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EMERGENT BEHAVIOR OF CELLS ON MICROFABRICATED SOFT POLYMERIC SUBSTRATES

BY

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DISSERTATION

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Abstract

In recent years, cell based bio-actuators like cardiomyocytes and skeletal muscle cells have emerged as popular choices for powering biological machines consisting of soft polymeric scaffolds at the micro and macro scales. This is owing to their unique ability to generate spontaneous, synchronous contractions either autonomously or under externally applied fields. Most of the biological machine designs reported in literature use single cells or cell clusters conjugated with biocompatible soft polymers like polydimethylsiloxane (PDMS) and hydrogels to produce some form of locomotion by converting chemical energy of the cells to mechanical energy. The mode of locomotion may vary, but the fundamental mechanism that these biological machines exploit to achieve locomotion stems from cell substrate interactions leading to large deformations of the substrates (relative to the cell size). However, the effect of such large scale, dynamic deformation of the substrates on the cellular and cluster level organization of the cells remains elusive. This dissertation tries to explore the emergent behavior of cells on different types of micro-scale deformable, soft polymeric substrates.

In the first part of the dissertation, contractile dynamics of primary cardiomyocyte clusters is studied by culturing them on deformable thin polymeric films. The cell clusters beat and generate sufficient forces to deform the substrates out of plane. Over time, the clusters reorient their force dipoles along the direction of maximum compliance. This suggests that the cells are capable of sensing substrate deformations through a mechanosensitive feedback mechanism and dynamically reorganizing themselves. Results are further validated through finite element analysis.
The development, characterization and quantification of a novel 1D/2D like polymeric platform for cell culture is presented in the second part. The platform consists of a 2D surface anchoring a long (few millimeters) narrow filament (1D) with a single cell scale (micro scale) cross section. We plate C2C12 cells on the platform and characterize their migration, proliferation, and differentiation patterns in contrast to 2D culture. We find that the cells land on the 2D surface, and then migrate to the filament only when the 2D surface has become nearly confluent. Individual and isolated cells randomly approaching the filament always retract away towards the 2D surface. Once on the filament, their differentiation to myotubes is expedited compared to that on 2D substrate. The myotubes generate periodic twitching forces that deform the filament producing more than 17 μm displacement at the tip. Such flagellar motion can be used to develop autonomous micro scale bio-bots.

Finally, the design and fabrication of a polymeric micro-pillar based force sensor capable of measuring cellular focal-adhesion forces under externally applied stretch is discussed. The force sensor consists of arrays of uniformly spaced PDMS micro-pillars of 1-2 μm diameter and 2-3 μm spacing on a macroscale PDMS substrate. The tips of the micro-pillars are selectively patterned with fluorescently labeled ECM proteins using micro-contact printing to promote cell adhesion while simultaneously acting as markers for strain measurements. Cells adhere and spread on top of the pillars causing them to deform. When stretched, the cells reorganize their internal structure and modulate their traction forces in response to the applied stretch. The dynamically varying cellular forces in response to the stretch are computed by measuring the cell induced displacements estimated by isolating the displacements caused by the applied stretch from the net displacements of the tips.
To Dad and Mom.
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5.1 Different types of interacting cells and clusters of cells in tandem with soft engineered scaffolds are expected feature in advanced biological machine designs. Reproduced from: www.ebics.net

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xv
# List of Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MEMS</td>
<td>Microelectromechanical Systems.</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix.</td>
</tr>
<tr>
<td>FA</td>
<td>Focal Adhesion.</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane.</td>
</tr>
<tr>
<td>FEA</td>
<td>Finite Element Analysis.</td>
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<tr>
<td>FEM</td>
<td>Finite Element Method.</td>
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<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma.</td>
</tr>
<tr>
<td>DRIE</td>
<td>Deep Reactive Ion Etching.</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy.</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene.</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein.</td>
</tr>
<tr>
<td>RIE</td>
<td>Reactive Ion Etching.</td>
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<tr>
<td>MKF</td>
<td>Monkey Kidney Fibroblast.</td>
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<td>DIC</td>
<td>Digital Image Correlation.</td>
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Chapter 1

Introduction

1.1 Summary

There is vast experimental evidence to suggest that cellular behavior can be significantly influenced by cell-cell and cell-substrate interplay. Several biological functions have been found to be directly linked to cellular interactions with their micro-environments. For example, embryonic stem cells were found to differentiate into different lineages when cultured on substrates of varying elastic modulus [10]. This suggests that cells can sense changes in their surroundings through various mechanisms and in turn regulate their bio-physical and bio-chemical status accordingly. In recent years, researchers have exploited cell-substrate interactions to engineer several novel biological machines capable of performing useful functions. These biological machines are expected to play a big role in biomedical applications in the near future. This chapter examines the current state of understanding in cell-substrate interactions, various engineered tools and methods employed in studying the same and finally their applicability in the design of biological machines. A brief overview of the organization of this dissertation is provided at the end of the chapter.

1.2 Emergent behavior of cells

Cells seldom perform their functions individually. Instead, they rely upon other cells to form clusters and organize themselves in temporally evolving three-dimensional structures. This kind of time evolution in their structural morphologies and the dime dependent reor-
ganization of their internal dynamics depend on several factors such as their interactions with other cell clusters, substrate, extracellular matrix and their microenvironment. Their behavior is further guided by several bio-chemical and bio-mechanical cues. However, despite all the extensive literature available on cellular interactions and their effects on cellular functions, the fundamental process guiding the emergence of cellular behavior as a result of such interactions is not well understood. The following sections provide some background on cell-substrate interactions and various engineered tools and mechanisms developed to study them.

1.3 Mechanism of cell-substrate interaction

Cells interact with their underlying extracellular matrix (ECM) through integrin receptors. These receptors are about 10 nm wide and are present throughout the cell membrane. Their primary function is to link the cytoskeleton to the ECM [11]. Integrins are freely diffusive within the cell until they find ligand sites on the ECM. Once, they are attached to the ECM, the integrins change their conformation and start recruiting proteins that connect them to the cytoskeleton. Depending on the nature of the adhesion site, the integrins may dynamically change their clustering and may recruit more proteins to improve the adhesion strength and biochemical signaling activity [12, 13]. Hence, it is now well established that integrins act as sensors of biochemical and biomechanical changes in the microenvironment, cytoskeletal regulators and centers of signal transduction [12,13]. Furthermore, it is widely believed that the mechanism through which focal adhesion (FA) sites are assembled and disassembled are regulated through mechanical force as there is a very strong linkage between the FAs and the acto-myosin networks of the cytoskeleton (see Fig. 1.1). The cells constantly remodel the ECM through actin-myosin generated forces by constantly tugging and pulling the ECM fibrils [14–16]. Such remodeling is essential to accommodate newly synthesized ECM to regulate the structure of new tissues or for wound healing [11]. Since, there is such a
strong correlation between mechanical forces and the arrangement of FAs and cytoskeleton, it’s obvious that their arrangement depends on the mechanical rigidity of the underlying substrate. This leads to the conclusion that cells actively probe their microenvironments and reorganize according to the forces sensed by them [17]. Such forces play an important role in the final morphological evolution of the tissues [18,19].

1.4 Micro and nano-engineered tools for studying cell-substrate interactions

Micro and nanotechnologies have now become integral to understanding the regulatory and sensory behavior of cells and their interactions with substrates. Various engineered tools and methods have been used over the years to understand cellular mechanics as a function of substrate geometry, topology, and surface chemistry and for measuring cellular forces at the micro and nano scale. Some of these tools include new-generation biocompatible and protein-conjugated materials like gels and biopolymers, microfabricated soft polymeric structures of various designs, biological microelectromechanical systems (bioMEMS). Some of these tools and techniques are discussed briefly in the following sections.

1.4.1 ECM printing techniques

One of the most popular techniques to study the effect of spatially varying ECM distribution on cell function is called microcontact printing [20–22]. In this technique geometrically controlled patches of ECM are printed on soft substrates and the consequent cell behavior is studied. In this technique poly(dimethylsiloxane) (PDMS) is molded into predetermined shapes to generate a stamp using conventional microfabrication techniques. The stamps are then conjugated with ECM proteins and printed onto the surfaces of soft substrates such as gels and PDMS (Fig. 1.2 (a-c)). The regions where cell adhesion is not desirable may
be printed with self assembled monolayers (SAMs) like pluronic F127. Direct printing of ECM proteins also leads to satisfactory transfer of ECM to desired regions of the substrate [14,23,24]. When cells are seeded on such patterned substrates, they spread and conform to the shapes and sizes of the printed proteins. Based on these techniques of cell culture it has been found that cell shape is a potent regulator of cellular function [25–29]. For example, when liver cells were cultured on relatively small areas versus large areas, they seemed to enhance their liver specific functions as measured by their albumin secretion rates [29]. Recent literature has shown that cell shape regulates many other behaviors of cells, like their differentiation [30], organization of their migratory components [31,32] and even stem cell differentiation [28]. Other technologies to print ECM on soft substrates include dip-pen lithography [33–35] where ECM can be patterned with nanoscale precision (Fig. 1.2 (d-f)).

1.4.2 Surface topology manipulation

The effect of surface topology on cell behavior was first investigated by Curtis and Vale in the 1960s [36]. They cultured chicken embryo heart explants on silica fibers of 8 μm and 40 μm and found that the fibroblasts migrated out of the explants onto the fibers and formed sheets between them (Fig. 1.3 (a)). When cultured on carbon nanofibers mimicking the topology of ECM proteins, osteoblasts exhibited increased proliferation compared to glass surfaces (Fig. 1.3 (b)). Alkaline phosphatase activity also improved on these substrates. In another study, cells were seeded on substrates developed using electrospinning of a polymer solution of polyamide onto glass [37]. When breast epithelial cells were seeded on these substrates, they formed multicellular spheroids (Fig. 1.3 (c)). Other techniques to manipulate surface topology include, etching micro and nanoscale grooves on different substrates. Cells seeded on nanogrooves (Fig. 1.3 (d-e)) elongated themselves and aligned in the direction of the groves [38–40]. Such morphological changes of cells and cell clusters has been found useful in vivo applications for implantable scaffolds [41,42].
1.4.3 Measurement of cellular forces

Since, mechanical forces play such an integral part in determining cellular behavior and functions, it is essential to be able to measure such forces exerted by the cells on their substrates. A technique to measure cellular forces was first developed in the 1980s [43]. The forces were measured by using thin films of silicone that deformed due to the applied forces from the cells (Fig. 1.4 (a)). A thin film of silicone rubber was polymerized by exposing a small amount on a glass coverslip to a flame [43–46]. When cells were cultured on these films, they would deform the films and subsequently their traction forces would be measured by measuring the wrinkling of the films. Many of the modern day techniques to measure cellular forces depend on microfabrication and the use of micro and nanoscale materials to measure forces. One of the most popular techniques used for measuring cellular forces is called traction force microscopy where non-wrinkling substrates such as hydrogels and other soft polymers are used to measure the cell induced deformations [47–49]. Fluorescent micro/nano-particles are embedded in these polymeric substrates and cells are allowed to attach and deform them (Fig. 1.4 (b-d)). The deformations are accurately measured by tracking the displacements of the fluorescent particles and the forces are back-calculated using the mechanical properties of the polymer substrates used. Based on several traction force experiments over the years it has been found that the cellular forces depend greatly on factors such as the substrate stiffness, cell spread area, ligand density etc. [50–55]. Local cellular forces directly at the focal adhesion sites are measured using microfabricated pillar like substrates [2, 56–59]. Briefly, in this technique cantilever structures such as pillars are made of either silicon or other polymeric materials such as PDMS using microfabrication techniques (Fig. 1.4 (e)). The diameters of the pillars are on the order of the focal adhesion diameters to ensure that there is only one focal adhesion site per pillar. The mechanical properties of the pillars are tuned by varying the dimensions of the pillars and also the material properties of the substrate. When cells are seeded on these structures, they deform
the pillars and the forces are calculated by measuring the pillar deformations.

1.5 Application to biological machines

Biological machines are fast emerging as promising candidates for several important biomedical applications such as targeted drug delivery, microsurgery, biomolecular sensing etc. These machines consist of at least one biological component made up of either single living cells or cell clusters or even subunits of cells, like molecular motors. Biological machines may vary in their size scales, ranging from a few nanometers to several millimeters depending on the applications they are designed for. In recent years, biological machine designs consisting of single cells or cell clusters in conjunction with biocompatible soft polymeric scaffolds have received much attention. This is primarily to exploit the mechanical properties of the scaffolds to provide some kind of predesigned shape or form to the machine in addition to their elastic and visco-elastic properties. In most of the bio-machine designs reported in recent years the eventual functional goal of the machine is achieved by the cells deforming the soft scaffolds in some specific predetermined way in a quasi-static or periodic fashion. The popular choices of cells in these studies are either primary cardiomyocytes extracted from neonatal rats or skeletal muscle cell derived from primary tissues or commercially available cell lines. This is due the fact that these cells are capable of generating contractile mechanical actuations either spontaneously or under the influence of external fields (electric, magnetic etc.) large enough to deform the soft polymeric scaffolds they are cultured on. Polydimethylsiloxane (PDMS) and hydrogel materials like polyethylene glycol (PEG) and polyacrylamide (PA) are amongst most extensively utilized scaffold materials for bio-machine fabrication. In addition to their excellent mechanical, optical and bio-compatible properties, these scaffold materials can be used to conjugate several different extracellular matrix (ECM) proteins which in turn allow for culturing different cell types. A few representative examples of different biological machines at different size scales are discussed in the following sections.
1.5.1 An artificial jellyfish from rat cardiomyocytes

Bio-hybrid devices were constructed by reverse engineering a jellyfish to replicate its three-dimensional swimming motion in-vitro using rat cardiomyocytes conjugated with PDMS as scaffold material [3]. The artificial design was developed by studying the structural configuration, stroke kinematics and fluid-solid interactions of a scyphozoan jellyfish Aurelia aurita. The jellyfish like constructs, termed as medusoids consisted of a bilayer of contracting cardiomyocytes and PDMS arranged radially around a central disk. The contracting cardiomyocytes deformed the lobes in a periodic fashion to generate propulsion (see Fig. 1.5). The contraction of the cardiomyocytes was not spontaneous, instead it was achieved by externally applied pulsating electric fields to periodically deform the medusoids in alternating power and recovery strokes where the construct deformed into a quasi-closed bell shape and the natural open lobe form respectively. The engineered constructs were able to replicate the locomotion of jellyfish in terms of their propulsion dynamics and velocity profiles to a great extent. However, it was found that the swimming behavior of the artificial jellyfish constructs were limited to just one stereotypic mode of propulsion as there was no means to control the dynamics of muscle actuation locally for achieving maneuverability and steering control.

1.5.2 3-D printed walking machines

Biological machines termed as bio-bots capable of walking and crawling were developed by Chan et al [4]. The bio-bots consisted of a biological bimorph structure composed of primary rat cardiomyocytes cultured on 3D printed PEG hydrogel scaffolds. A base structure was included in the cantilever to provide asymmetry to the design and enable it to walk in one direction. The cantilever bimorph structure acted as the actuator to power the bio-bot. This was achieved by seeding sheets of rat cardiomyocytes cells on the PEG cantilever structure and allowing them to deform the cantilever structure to first produce a curvature due to the
quasi-static forces exerted by the cells and then produced a periodic deformation to propel
the bio-bot in the desired direction. The magnitude of the curvature and the displacement
depended on the interplay between the cantilever thickness and the cell generated forces (see
Fig. 1.6). The maximum velocity, average displacement power per stroke and the average
contraction frequency were $236 \mu\text{m s}^{-1}$, $354 \mu\text{m}$ and $1.5 \text{Hz}$ respectively. The physical and
chemical cues for the cells were controlled steriolithographically using a 3D printer.

Another type of walking bio-bot was developed using a different asymmetric design and
powered by engineered skeletal muscle strips contracting under an electric field [5]. The bio-
bot was capable of generating a walking/crawling motion with maximum velocity of around
$156 \mu\text{m s}^{-1}$, which was estimated to be over $1.5$ body lengths per min. The geometric design
and the mechanical and bio-chemical properties of the PEG hydrogels was controlled using
steriolithographic 3D printing. Furthermore, the effect of collagen I, fibrin extracellular
matrix proteins and insulin like growth factors on the contraction dynamics of the skeletal
muscle strips was characterized (see Fig. 1.7). The mechanism of locomotion was further
characterized by modeling and simulation.

1.5.3 An artificial microswimmer at low Reynold’s number

Microorganisms such as bacteria and some eukaryotes like spermatozoa are natural micro-
wimmers. They propel themselves using thin filamentous structures called flagella. The
shape of the flagella and the mechanism of swimming may vary between organisms. For
example, some bacteria have helical flagella that rotate and produce propulsion while most
spermatozoa swim by producing an oscillatory wave like motion of their flagella. It has
been proven that this mode of swimming is highly efficient for micrometer size scales where
the viscous effects dominate the inertial effects. However, a self propelled, synthetic flag-
ellar swimmer operating at low Reynolds number hasn’t been demonstrated until recently.
Our group developed a microscale, biohybrid swimmer enabled by a unique fabrication pro-
cess [6]. The micro-swimmers termed as bio-bots were fabricated by culturing neonatal rat cardiomyocytes on microfabricated polydimethylsiloxane (PDMS) functionalized with extracellular matrix proteins. The PDMS device consisted of a rigid head and a long slender tail. The beating of the cardiomyocytes caused periodic oscillation of the tail. This oscillatory motion of the tail generated propulsive forces to overcome the viscous drag resistance of the medium resulting in a net forward motion of the bio-bot (see Fig. 1.8). The swimming speed was estimated to be between 5-10 $\mu\text{ms}^{-1}$. A two-tailed swimmer swimming at 81 $\mu\text{ms}^{-1}$ was also demonstrated. A predictive hydrodynamics based model was developed to provide design cues for the swimmer and predict the final propulsion velocities and forces. Such devices are expected to be useful in a variety of applications such as targeted drug delivery by swimming through blood streams and bio-chemical sensing of toxins and pheromones. Furthermore, this small-scale, elementary biohybrid swimmer could also serve as functional units in more complex biological machines.

1.6 Overview

This chapter provides a brief introduction to the field of cellular mechanics from the perspective of cell substrate interactions and their applications for developing biological machines. Several important attributes of cells pertaining to their regulation of behavior based on their interactions with their microenvironments was discussed. Some methods to measure cellular forces were also highlighted. The remainder of this dissertation focuses primarily on the biomechanical interactions of cells with microfabricated thin, soft structures and consequent changes in their bio-chemical and biophysical behavior as a result of such interactions.

In chapter 2, the contraction dynamics of cardiomyocyte clusters is studied by culturing them on novel one-dimensional soft polymeric structures. In chapter 3, the development, characterization and quantification of a novel 1D/2D like polymeric platform for cell culture
is presented. The platform consists of a 2D surface anchoring a long narrow filament (1D) with a single cell scale (micro scale) cross section. We plate C2C12 cells on the platform and characterize their migration, proliferation, and differentiation patterns in contrast to 2D culture. Chapter 4, describes the development of a novel microfabricated substrate for measuring cellular forces in response to externally applied strains with pico to nano-newton resolutions. Finally, chapter 5 concludes the dissertation and provides some directions for future work.
Figure 1.1: Schematics showing organization of the force sensing elements of the cells. The cells connect to the ECM at focal adhesion sites using integrins which are connected to the cytoskeleton. Cells respond to changes in the substrate rigidity by increasing their spread area on harder substrates and by migrating towards them from softer regions. Adapted from [1].
Figure 1.2: Various ECM printing techniques on substrates for cell culture. (a-c) Process flow for microcontact printing ECM of various shapes and sizes on soft substrates and live cell spreading on printed areas. (d-e) Show immunofluorescent images of vinculin and actin distributions in the cells cultured on microcontact printed substrates. (f) Dip pen lithography technique used for directly printing ECM on substrates. Adapted from [2].
Figure 1.3: Manipulating surface topologies using micro-nano fabrication techniques. (a) Fibroblast sheets between silica fibers. (b) SEM image of the corneal epithelial basement membrane of a Macaque monkey. (c) SEM of carbon nanofibers used for cell culture. (d-e) Cells spreading on nanoscale grooves. Adapted from [2].
Figure 1.4: Various cell force measurement techniques. (a) Force measurement using wrinkling silicone thin films. (b-d) Traction force microscopy techniques using soft polymeric substrates embedded with fluorescent micro/nano beads. (e) Microfabricated pillar substrates for cell force measurement. Adapted from [2].
Figure 1.5: A jellyfish like reverse engineered construct was developed by culturing primary cardiomyocytes on deformable lobes around a central disk. The jellyfish construct is capable of generating propulsion due to cardiomyocyte contractions aided by an externally applied electric field. Adapted from [3].
Figure 1.6: Walking bio-bots were developed by culturing primary rat cardiomyocytes on 3D printed PEG hydrogels. The cantilever bimorph structure is capable of walking in fluids due to the asymmetric design and cardiomyocytes contractions. Precise control of cell placement and mechanical properties of the cantilever structure is possible using steriolithographic 3D printing technologies. Adapted from [4]
Figure 1.7: Walking biobots were developed from 3D printed soft polymeric materials. Propulsion was achieved using skeletal muscle strips capable of generating forces when paced with an electric field. Precise geometric and spatial control is possible using 3D printing technologies. Adapted from [5].
Figure 1.8: Artificial swimmers capable of swimming at low Reynolds numbers were developed. The swimmers consist of a PDMS based flagellar structure and are driven by primary rat cardiomyocytes. A hydrodynamics based model predicts the propulsion velocities and forces for a given design. Adapted from [6].
Chapter 2

Emergent dynamics of cardiomyocyte clusters on deformable polymeric substrates

2.1 Summary

Contractile dynamics of primary cardiomyocyte clusters is studied by culturing them on deformable thin polymeric films. The cell clusters beat and generate sufficient forces to deform the substrates out of plane. Over time, the clusters reorient their force dipoles along the direction of maximum compliance. This suggests that the cells are capable of sensing substrate deformations through a mechanosensitive feedback mechanism and dynamically reorganizing themselves.

2.2 Motivation

In recent years, cardiomyocytes have emerged as the most popular choice of bioactuators for powering biological machines consisting of soft polymeric scaffolds at the micro and macro scales [3–6, 60–62]. This is owing to their unique ability to generate spontaneous, synchronous contractions consuming only glucose as their primary energy source [63]. Most of the biological machine designs reported in literature use cardiomyocytes conjugated with biocompatible soft polymers like polydimethylsioxane (PDMS) and hydrogels to produce some form of locomotion by converting the chemical energy of the cardiomyocytes to mechanical energy. The mode of locomotion may differ in each case but the fundamental mechanism that these biological machines exploit to achieve locomotion stems from cell substrate interactions leading to large deformations of the substrates (relative to the cell size).
However, to date none of the studies have tried to understand the effect of such large scale, dynamic deformation of the substrates on the cellular and cluster level reorganization of the cardiomyocytes.

It is known that cardiomyocytes are mechanosensitive to their local environment and are capable of sensing the substrate elasticity among other mechanical factors to remodel and reorganize their internal structures. For example, it has been reported that cardiomyocytes generate the highest force when cultured on hydrogel substrates mimicking the elasticity of the heart tissue at 10 kPa. The sarcomere alignment was also found to be different on different stiffness substrates [64, 65]. The contraction frequencies as well as the fraction of isolated cardiomyocytes are also heavily correlated to the natural elasticity of the heart tissue [66, 67]. When cultured on polymeric micropillar substrates, the cells generated higher contraction forces on stiffer pillars at the expense of contraction velocities. The sarcomere lengths and z-band widths also seemed to increase at higher stiffness [68].

Mechanical microenvironment also plays an important role in the modulation of the adhesion structures of cardiomyocytes. The size and frequency of the adhesion structures were shown to increase in response to externally applied stretch and cell contractility [69–71]. Traction force microscopy studies performed assuming applied forces to be localized on focal adhesion sites revealed the magnitude of the applied forces and force dipoles. Applied cell force was estimated to be about 15 nN per focal adhesion and the force dipoles were in the 6 pN-m range. When normalized to a full cardiomyocyte, the force scales to about 200-500 nN range [72, 73]. Furthermore, when cultivated on soft, flexible silicone membranes, the cells generated pleat like wrinkles with a spacing of 1.9 μm. The observed pleats seemed to be aligned with the z-disks in the cells [74].

All the aforementioned observations of cardiomyocyte mechanosensitivity to their local
microenvironment lead us to believe that the large deformations of the biological machines must induce some cellular reorganization and change in the contraction dynamics of the cardiomyocyte clusters. This is suspicion is further validated by the fact that in most of the cases the cells are not even spatially patterned to generate contractions in the desired direction. The cells seem to voluntarily contract in the direction of maximum deformation of the substrates in most cases. The objective of this study is identify the fundamental reason for this specific behavior of cardiomyocytes on soft substrates under large deformations and probe ways to tune the contraction dynamics of the cells by exploiting the cell-substrate interactions.

2.3 Materials and methods

2.3.1 Thin film substrate preparation

One-dimensional polydimethylsiloxane (PDMS) cantilever beams (henceforth referred to as filaments) were developed using a technique previously described by the authors [6,60,75]. The filaments were incubated in fibronectin (BD Bioscience, 25 µg/mL) extracellular matrix (ECM) protein for about 3 hours and rinsed in PBS 2-3 times. The ECM functionalized filaments were transferred to a custom prepared substrate which was previously incubated in Pluronic F127 (1% in PBS, Sigma-Aldrich) for 20 mins to prevent cell attachment. The substrate consisted of two square silicon chips of 4mmX4mm edge and 500 µm thickness separated by a distance equal to the length of the filaments glued to a polystyrene petri-dish. The large reservoir regions of the filaments were attached to the silicon chips through stiction, ensuring reasonable tautness in the filaments.
2.3.2 Substrate configuration and cell plating

Primary cardiomyocyte cells extracted from neonatal rats were then plated on the filaments. Several cardiomyocytes congregated and formed clusters on different locations of the filaments within 12 hrs of cell plating. The cluster sizes typically varied between 70-80 μm in diameter in the limiting case. A schematic of the filament configuration and cell plating methodology is shown in Figs. 2.1(a-c). Filaments of two different widths were chosen for the study. The ‘narrow’ filaments were between 30-40 μm in width and 7-10 μm in thickness while the ‘wide’ filaments were between 90-100 μm in width and 10-15 μm in thickness (Note: the thickness was only changed to keep the mechanical stiffness of the two filaments same). The two widths were so chosen to isolate the effect of filament geometry on the contraction dynamics of the cardiomyocytes. As the cluster sizes ranged from 70-80 μm, the wider beams were expected to contain the clusters completely within their boundaries without any of the cells sensing the edges while cells on the narrower beams were able to sense the edges of the filaments. Fig. 2.1(d) shows cardiomyocyte clusters on narrow beams. Figs. 2.1(e-f) show the clusters on wider beams. As can be seen the clusters on the wider beams are fully contained within the filaments and do not feel the edges. Fig. 2.2 shows the phase contrast images of the cell clusters on various configurations of the filaments.

2.3.3 Experimental protocol and imaging

The cardiomyocyte clusters start to contract spontaneously within 24 hrs of cell plating. At that time the filaments were cut 500 μm away from a contracting cluster near the fixed end of the filament in such a way that the 500 μm region away from the cluster would be free to deform due to the contraction forces applied by the cluster (cantilever configuration with the cells contracting at the base of the cantilever). This was done in order to create a feedback mechanism for the cells to sense the deformation of the filament. The hypothesis was that the cells would sense the deformation in the longitudinal direction and reorient
their contraction force dipoles in that direction (along the length of the filament). In the control case the filaments were not released but instead were left fixed such that there would be no feedback and thus no mechanical cues for the cells to change their force dipoles one way or the other. Contracting clusters on both the free cantilever structures and the fixed control filaments were observed and recorded 24 hrs and 48 hrs after releasing the filaments (48 hrs and 72 hrs after cell plating respectively) using phase contrast microscopy.

2.3.4 Analytical methodology

A statistical analysis of the contraction dynamics of the clusters was performed by tracking the positions of 30 randomly chosen points spread all around the clusters using a custom built MATLAB based particle tracking program incorporating a cross correlation algorithm. A force dipole orientation angle was defined as the angle made by the longitudinal axis of the filaments with an imaginary line passing through the points of maximum displacement calculated using the tracking program. In other words the dipole orientation angle indicated the direction of maximum contractions of the clusters. By this definition, smaller angles (closer to 0°) meant that the clusters were contracting along the length of the filaments and larger angles (closer to 90°) meant that they were contracting orthogonal to the longitudinal axis of the filaments.

2.4 Results and discussions

2.4.1 Cluster dipole orientation

Fig. 2.3 shows histogram distributions of the dipole orientation angles for clusters on various different beams. Four clusters on four different free cantilever filaments were analyzed in both the narrow and wide cases while three clusters on three different fixed control filaments were analyzed in each of the narrow and wide cases. The dipole angles in both the narrow and
wide free cantilever filaments were observed to be much smaller compared to their respective control cases. The dipole orientation angles for clusters on the control filaments were up to three times higher than the free cantilever case. This indicated that the cell clusters were indeed mechanosensitive to substrate deformations and suitably reoriented their dipoles based on the substrate deformation feedback. Furthermore, it was also observed that the dipole orientation angles for clusters on the free cantilever filaments further reduced over time indicating that the dipole orientation was dynamic and changed with time. As was expected, no specific trend was observed in the control filaments. Since, the effect was observed on both the narrow and wide cantilever filaments, it may be concluded that the reorientation of the dipoles was not due to geometric cues alone but was a result of cellular mechanosensitivity to substrate deformations.

2.4.2 Orientation of instantaneous angles

A cardiomyocyte cluster is composed of several cardiomyocyte cells and the cluster contracts when individual cells contract in unison in a synchronized manner. Therefore, analyzing the orientation dipole angles of the clusters alone does not present a complete picture of the cellular dynamics and time dependent reorganization taking place in the cluster. This may be better understood by analyzing the instantaneous angles of contractions of individual points in the cluster. The dynamics of the individual points may be approximated to the dynamics of single cells within the cluster. Fig. 2.4 shows the histogram distributions of instantaneous angles made by individual points (30 per cluster) with respect to the longitudinal axis of the filaments. Figs. 2.4(a-b) are histograms for two different clusters on narrow cantilever beams while Figs. 2.4(c-d) represent histograms for two different clusters on fixed narrow filaments. As can be seen, most of the points on the narrow free cantilever filaments had instantaneous angles ranging from 0-20° while those on the fixed control cases had instantaneous angles ranging from 20-40°. This indicates that the cells within the clusters on the narrow free cantilever filaments reoriented themselves along the longitudinal axis of the filaments as
a result of the feedback from substrate deformations. Furthermore, in the control cases there were points with instantaneous angles beyond 60° while in the free cantilever case the limiting angles were in the range of 40-50°, again indicating that cellular reorganization indeed took place.

It may be argued that the cellular reorganization observed in Fig. 2.4 was purely due to geometric effects as the cells were able to sense the edges of the narrow beams and may have reorganized based on geometric cues alone. In order to further validate the hypothesis that substrate deformation and not geometric cues were responsible for cellular reorganization, similar analysis as in Fig. 2.4 was performed for clusters on wide filaments. The results are as shown in Fig. 2.5. As can be seen in this case too, the free cantilever filaments had instantaneous angles ranging from 0-20° while those on the fixed control cases instantaneous angles were between 20-40°. This proves conclusively that the feedback from substrate deformation alone can cause the cells to reorganize and reorient their contraction dynamics.

2.4.3 Instantaneous angle as a function of time

Instantaneous angles of points on the cardiomyocyte clusters also changed with time. In fact it was observed that for the free cantilever cases the angles actually reduced over time. Fig. 2.6 shows a polar plot representation of the instantaneous angles computed for two different clusters on two different wide beams for both free cantilever and fixed control represented together (i.e., 60 points per plot) for different time points. Fig. 2.6(a) and Fig. 2.6(b) represent instantaneous angle data from 24 hrs after release (48 hrs after plating) for free cantilever and fixed control cases respectively. Fig. 2.6(c) and Fig. 2.6(d) represent instantaneous angle data from 48 hrs after release (72 hrs after plating) for free cantilever and fixed control cases respectively. It is evident that in the free cantilever case, the number of points between the 0-20° range after 48hrs was higher than those at 24hrs. This indicates that the cells reorganize themselves over time as a result of substrate feedback. No such
reduction in the instantaneous angles was observed in the fixed filament control case. In fact no specific trend in the change in instantaneous angles was observed.

2.4.4 Finite element analysis of substrate compliance

In all the aforementioned analyses it was observed that the instantaneous angles of cardiomyocyte clusters on the fixed filaments were curiously biased towards 20-40°. This observation led us to believe that the preferred orientation could purely be a function of non uniform substrate stiffness arising from geometric and edge boundary constraint effects. To probe this phenomena further a finite element methods (FEM) analysis was performed using a commercially available FEM software called ANSYS. The geometry, dimensions of the cantilever and fixed filaments and boundary conditions were maintained so as to simulate the experimental conditions. Point forces and dipole forces (45 µN separation corresponding a typical cluster diameter) of unit magnitudes of 1 µN were applied along various directions near the fixed boundary of the filaments under different configurations. Fig. 2.8 shows the results of the FEM analysis. A unit force of 1uN applied at a point close to the fixed end of the cantilever filament produced a maximum displacement along the longitudinal axis of the beam as shown in Fig. 2.8(a). Similarly, a dipole force applied along different directions near the fixed edge of the cantilever too revealed that the maximum displacement occurred along the longitudinal axis as shown in Fig. 2.8(b). Based on the observations it may be inferred that the apparent stiffness sensed by the cells is the least along the longitudinal axis. A similar analyses performed on fixed filaments revealed that the maximum displacement was along a direction oriented at 40° from the longitudinal axis as shown in Figs. 2.8(c-d), indicating that the apparent stiffness sensed by the cells is the least along that direction. The results of the FEM simulations seem to be consistent with the experimental observations.
2.5 Overview

In conclusion, a one-dimensional soft polymeric platform to study the contraction dynamics of neonatal rat cardiomyocytes has been developed. The contraction dynamics of the cells and cell clusters have been found to be sensitive to the substrate deformations caused by the contraction itself. This indicates the presence of a feedback mechanism through which the cells sense the substrate deformations and in turn self reorganize and orient their contraction dipoles along the direction of substrate deformation. The instantaneous angles made by points on cell clusters also change with time indicating that the cellular reorganization is in fact a dynamic process.
Figure 2.1: Filament configuration and cardiomyocyte cluster formation. (a) shows the initial configuration of the fibronectin functionalized filament on the substrate before cell plating. (b) primary cardiomyocyte cells from neonatal rats are plated on the filaments. (c) the filament is cut 500 μm away from a contracting cardiomyocyte cluster at the base of the cantilever after 48hrs of cell plating. (d) cardiomyocyte clusters on a narrow filament (scale bar: 25 μm). (e-f) cell clusters on wide beams (scale bar: 100 μm). The highlighted regions in yellow indicate clusters confined within the filament.
Figure 2.2: Phase contrast images of cell clusters on the different types of filaments analyzed. (a) Shows a cluster of cells on a narrow fixed beam. The cluster is ellipsoidal in shape and tends to grow beyond the edges of the beam. (b) A cluster of cells on a narrow cantilever beam. (c) Cluster of cells on a wide fixed beam. The cells assume a more circular morphology and remain within the edges of the beam. (d) Shows a cluster of cells on a wide cantilever beam.
Figure 2.3: Histograms of cardiomyocyte contraction force dipoles. (a) Histogram of contraction force dipoles for clusters on four different narrow cantilever beams (free to deform). (b) Histogram of dipoles on three different fixed filaments (control case, no filament deformation allowed). (c) Histogram of force dipoles on wide cantilever beams. (d) Histogram of force dipoles on fixed wide control filament.
Figure 2.4: Histograms of instantaneous angles of points tracked on different cardiomyocyte clusters on narrow filaments. (a-b) histograms of instantaneous angles of points on clusters contracting on narrow and free cantilever beams. A Gaussian fit indicates that most of the points have instantaneous angles between 0-20°. (c-d) histograms of instantaneous angles of points on clusters contracting on narrow and fixed control filaments. A Gaussian fit indicates that most of the points have instantaneous angles between 21-40°.
Figure 2.5: Histograms of instantaneous angles of points tracked on different cardiomyocyte clusters on wide filaments. (a-b) histograms of instantaneous angles of points on clusters contracting on wide and free cantilever beams. A Gaussian fit indicates that most of the points have instantaneous angles between 0-20°. (c-d) histograms of instantaneous angles of points on clusters contracting on wide and fixed control filaments. A Gaussian fit indicates that most of the points have instantaneous angles between 21-40°.
Figure 2.6: Polar plots comparing instantaneous angles of points on cardiomyocyte clusters on wide filaments over time. (a) instantaneous angles of points on clusters contracting on wide cantilever beams at day 2 of cell plating (24 hrs after releasing). (b) instantaneous angles on fixed wide control filaments at day 2 of cell plating. (c) instantaneous angles on wide cantilever beams at day 3 of cell plating (48 hrs after releasing). (d) instantaneous angles on fixed wide control filaments at day 3 of cell plating.
Figure 2.7: Polar plots comparing instantaneous angles of points on cardiomyocyte clusters on narrow filaments over time. (a) instantaneous angles of points on clusters contracting on narrow cantilever beams at day 2 of cell plating (24 hrs after releasing). (b) instantaneous angles on fixed narrow control filaments at day 2 of cell plating. (c) instantaneous angles on narrow cantilever beams at day 3 of cell plating (48 hrs after releasing). (d) instantaneous angles on fixed narrow control filaments at day 3 of cell plating.
Figure 2.8: Results from finite elements analyses of fixed and cantilever filaments. (a) displacements produced by a unit force of 1 μN applied at a point close to the fixed support of the cantilever along different directions. (b) displacements produced by a unit dipole force applied along various directions of the cantilever. (c) displacements produced by a unit force of 1 μN applied at a point close to the fixed support of the fixed filament along different directions. (d) displacements produced by a unit dipole force applied along various directions of the fixed filament. The brown arrows indicate the softest direction (corresponding to maximum displacement).
Chapter 3

Cell culture on one-dimensional polymeric structures

3.1 Summary

In this chapter, the development, characterization and quantification of a novel 1D/2D like polymeric platform for cell culture is presented. The platform consists of a 2D surface anchoring a long (few millimeters) narrow filament (1D) with a single cell scale (micro scale) cross section. We plate C2C12 cells on the platform and characterize their migration, proliferation, and differentiation patterns in contrast to 2D culture. We find that the cells land on the 2D surface, and then migrate to the filament only when the 2D surface has become nearly confluent. Individual and isolated cells randomly approaching the filament always retract away towards the 2D surface. Once on the filament, their differentiation to myotubes is expedited compared to that on 2D substrate. The myotubes generate periodic twitching forces that deform the filament producing more than 17 μm displacement at the tip. Such flagellar motion can be used to develop autonomous micro scale bio-bots.

3.2 Motivation

Bio-hybrid structures developed by combining mammalian cells with soft polymeric scaffolds have received much attention recently [61,62]. Such structures, often termed as bio-actuators or bio-machines hold tremendous potential in various applications ranging from development

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of synthetic organs to drug screening, drug delivery, micro-surgery, sensing and actuation, autonomous or controlled directed motion (walking/swimming/crawling), transportation of cargo, information processing and other similar in-vivo and in vitro applications that require unconventional techniques and devices [4–6].

In all of the aforementioned structures, the choice of the polymeric scaffold and the associated cell types determine their mechanical and bio-chemical properties and thus their functionalities. Polydimethylsiloxane (PDMS) is fast emerging as the polymer of choice for developing such bio-hybrid structures owing to its several unique properties - its excellent biocompatibility, transparency for a broad range of light spectra, robust mechanical properties, easy conjugation with ECM matrix proteins, negligible degradation in cell culture media at elevated temperatures for extended periods of time [76–78].

Recent studies have successfully developed and demonstrated bio-actuators and biomachines capable of performing some of the aforementioned tasks using clusters of mammalian cells in conjunction with soft polymeric scaffolds like PDMS and hydrogels. A 1-dimensional, microscale, bio-bot capable of swimming in fluids at low Reynolds number was developed using PDMS filaments [6]. Neonatal rat cardiomyocyte clusters cultured on PDMS filaments were used as actuators to achieve a propulsion speed of up to 10 μm/s with a single filament. Propulsion speeds as high as 80 μm/s were observed in swimmers with two filaments. Other studies have utilized cardiomiocytes beating in clusters and conjugated with PEG hydrogels to achieve walking motion. Chan et al. developed a walking bio-bot powered by cardiomyocytes on 3D printed hydrogels [4]. Their design consisted of a 'biological bimorph' cantilever structure scaffold where cardiomyocytes act as actuators to drive the bio-bot. They were able to achieve walking speeds of up to 236 μm/s in fluidic environments. Tanaka et al. developed micropumps by utilizing the contractions from a sheet of cardiomyocytes on PDMS scaffolds uniquely designed to pump fluids [79]. They were able to make fluid flow measurements using 1 μm polystyrene beads suspended in the fluid. The fluid oscillating frequency was observed to be 0.7 Hz and a linear displacement of
upto 150 μm was observed. The fluid flow rate was measured to be 2 nL/min. They went on to further improve their design by wrapping a sheet of cardiomyocytes around spherical PDMS scaffold [63]. Swimming jellyfish like structures have been realized by culturing cardiomyocytes on thin elastomers arranged in freely moving lobes around a central disc [3].

There are several limitations to using primary cardiomyocytes for bio-bot applications, namely- cells are relatively large in size leading to space limitations on structures, scaling of force generated is not feasible as the cells do not multiply and proliferate, not very useful for co-culture with other cell types like neurons, primary cells are not feasible for long term experiments because they cannot be cryopreserved and require sacrificing of rats for each experiment. Skeletal muscle cells are viable alternatives to cardiomyocytes as functional components of futuristic bio-bot designs. This is due to some of the intrinsic advantages they present in terms of control and guidance, force scalability, robustness and most importantly the ability to co-culture with other cell types. However, only a few studies have been reported that take advantage of skeletal muscle cells for bio-actuator and bio-bot applications. Skeletal muscles have been primarily utilized to study the active tension in Myotubes in isolation or in clusters on silicon, PDMS and collagen [80–82]. Other applications include the development of engineered skeletal muscle tissues in 3D. Engineered skeletal muscle was developed using myotubes and synthetic tendons [83–85]. Other methods to develop skeletal muscle tissues include culturing myotubes in pre-fabricated scaffolds and suspending between PDMS pillars [86].

In this chapter, the development of a novel 1D polymeric platform and culture technique to form C2C12 derived myotube filaments is presented. Patterns of cell migration and mitosis on the 1D substrate are reported. Parameters like nuclei orientation, spread area, deformation and cell density on the 1D substrate are quantified and compared with those on flat PDMS substrates. Techniques for precise control of cell placement on the 1D structure are outlined. Finally, methods for myotube formation and eventual bio-actuator realization are described. The novel techniques presented here will be useful in improving
the performance and functionalities of existing micro-swimmers and future biological machines by integrating subunits like neurons and vascular networks for control and guidance of biobots and for other higher order functions.

3.3 Materials and methods

3.3.1 Silicon mold and device fabrication

The 1D PDMS structures were fabricated using a previously developed process [75] by the authors. Briefly, a silicon wafer was patterned and etched using standard microfabrication techniques as shown in Fig. 3.1(a-b). Photoresist was spun coated on a 4'' Si wafer and patterned using standard photolithography techniques. The wafer was then etched using a dry etching process in an inductively coupled plasma reactive ion etching (ICP-DRIE) machine. The etch depth and thus the depth of the final PDMS structure was determined by the etch time. A thin layer of polytetrafluoroethylene (PTFE) was deposited on the wafer after the completion of the etching process to lower the adhesion of PDMS to the Si wafer and to prevent delamination of the structures during peeling off from the molds. The device design was such that there were 4-5 microchannels of width 7-10 μm and 2-3 mm in length connected by squares of 3 mm edges on either sides. These squares are referred to as reservoirs where PDMS (Sylgard 184, 4:1 base to crosslinker ratio) was poured as shown in Fig. 3.1(c). Liquid PDMS filled the microchannels in about 2 minutes due to capillary draw [75] as shown in Fig. 3.1(d). PDMS in the molds was then cured at 60° C for 12 hours. The molds with the cured PDMS structures were submerged in ethanol for about 15 mins to allow PDMS to swell, which facilitated the peeling process. The structures were then released manually and transferred to polystyrene petri dishes and thoroughly rinsed in PBS before extracellular matrix functionalization. A schematic diagram of the finally peeled PDMS structure is shown in Fig. 3.1(e). The scanning electron micrographs of the final structures are shown in Figs. 3.1(f-g).
3.3.2 1D substrate preparation and extracellular matrix functionalization

The structures were air dried after rinsing at room temperature for 2-3 hours in a sterile environment. Fibronectin (BD Bioscience, 25 µg/mL) was the preferred extracellular matrix for cell attachment on the structures. They were incubated in fibronectin for about 3 hours after which they were again rinsed in PBS 2-3 times. Following ECM functionalization, the structures were transferred onto two different types of substrates depending on the experiments to be performed. For the purpose of high resolution immunofluorescence imaging the structures were transferred onto a glass bottom petri-dish. For the purposes of time lapse imaging and bio-actuator experiments they were transferred to another substrate which was custom prepared to minimize cell adhesion. These substrates were prepared by gluing two square silicon chips of 4mmX4mm edge and 500 µm thickness separated by a distance equal to the length of the 1D structure (filaments) and incubating the entire substrate in Pluronic F127 (1% in PBS, Sigma-Aldrich) for 20 mins to ensure negligible cell adhesion on regions without ECM. The ECM coated PDMS structures were then mounted on the Si chips. The Si chips acted as suspenders which prevented any kind of interaction between the cells on the filaments and those on the petri-dish. The final configuration of the PDMS structure mounted on Si chips in a petri-dish is shown in Fig. 3.2(a). Cells of required density were plated on the structures as shown in Fig. 3.2(b). The cells landing on the PDMS surface adhered, while the others remained rounded and were washed away during subsequent media change procedures. The attached cells elongated and eventually started to proliferate and move towards the 1D filaments as shown in Fig. 3.2(c).

3.3.3 Cell culture and imaging

C2C12 myoblast cells (ATCC, Manassas, VA) were cultured in growth media (GM) composed of DMEM (Life Technologies, Grand Island, NY, Cat. No. 41965-039) supplemented
with 10% fetal bovine serum (ATCC, Manassas, VA, Cat. No. 30-2020) and 1% Penicillin-Streptomycin (ATCC, Manassas, VA, Cat. No. 30-2300). The C2C12 myotubes in GM were seeded on the ECM functionalized structures at a cell density of about 200,000 cells/cm² and incubated at 37°C and 5% CO₂ for 1 hour before any experiments were performed. The process of differentiation to myotube was initiated by replacing GM with fusion media (FM) comprising of 2% horse serum (ATCC, Manassas, VA, Cat. No. 30-2040) in place of the fetal bovine serum in GM. One hour after plating the cells on the suspended structures, time lapse imaging in phase contrast mode was carried out using an Olympus IX81 microscope with an incubation chamber. The filaments were observed at 10X magnification in an environmental chamber under biological conditions. Images were captured automatically at 2 minute intervals for 40 hours.

3.3.4 Immunohistochemistry and fluorescence imaging

The cells were first washed three times with PBS and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Next, they were permeabilized with 0.3% Triton-X100 for 3 min and blocked in 5% Bovine Serum Albumin (BSA) for 10 min at room temperature. The cells were then immunostained with monoclonal sarcomeric myosin antibody MF-20 (Developmental Studies Hybridoma Bank, Iowa City, IA) for 1h at room temperature and rinsed 3 times with PBS. F-actin staining was done using tetramethylrhodamine (TRITC) conjugated phalloidin (Sigma-Aldrich, MO) at 50 μg/mL concentration for 1h at room temperature followed by a PBS rinse. Finally, nuclei were stained using DAPI (1:1000) for 20 min at room temperature. The samples were incubated in PBS overnight and imaged using a Zeiss LSM 700 confocal scanning laser microscope.
3.4 Results and discussion

3.4.1 Unique cellular migration and mitosis

Time lapse imaging of the C2C12 myoblasts for extended periods of time revealed their migration patterns and cellular mitosis on 1D substrates. Figs. 3.3(a-j) show a set of phase contrast images obtained over 40h after the cells were plated. Note that the geometry of the 1D substrate contains irregularities in the form of notches and groves. The plated cells preferentially landed on the reservoir pads with large flat surface area instead of the filaments. Almost no cell landed on the filament due to its narrow width (<10 µm). Fig. 3.3(a) shows the filament along with the groove 1h after cell plating when time lapse imaging started. Over time the cells started to migrate towards the filaments from both the reservoirs. Part of a cell was first spotted 8h 20 min after the beginning of the time lapse as represented by the arrow in Fig. 3.3(b). The single cell, without the influence of any other cells in its vicinity, moved towards the groves from right to left of the image (Fig. 3.3(c)). The cell constantly sensed its microenvironment by extending its filopodia. The moment the cell sensed the presence of the groves (Fig. 3.3(d)) it returned towards the reservoir following the same path that it had taken to reach the groove (Fig. 3.3(e)), and completely disappeared from the field of view (Fig. 3.3(f)) as it moved further towards the right of the image towards the reservoir. As time progressed, cells began to divide and proliferate in and around the reservoirs and the free space available for the cells to migrate and expand starts to shrink. As a result, the cells were forced to move towards the groove due to ‘peer pressure’ (Fig. 3.3(g)). More and more cells started to appear in the field of view as they were forced to make their way towards the filaments from either side, bridging the groves (Fig. 3.3(h)). After about 29h the cells were completely confluent on the filament. During mitosis the cells partially detached themselves from the 1D substrate, assumed a spherical morphology and appeared to be dangling from the structure as shown in Fig. 3.3(h,i). The daughter cells merged with the rest of the cells and adhered to the 1D substrate. In the course of the time lapse many cells divided in the
same way and migrated and merged with other cells. At the end of the time lapse around
the 40h time mark (Fig. 3.3(j)), several cells could be observed in spherical morphologies
and the entire structure was confluent with at least one layer of cells. The velocities of the
cells under different circumstances are quantified in the next section.

3.4.2 Migration velocities

Migration velocities of the cells at various different stages of the time lapse observation
were calculated using a simple particle tracking cross-correlation algorithm in MATLAB.
Fig. 3.4(a) shows the displacement of the first observable cell (Fig. 3.3(b-e)) as a function
of time. The migration velocity of the cell on the smooth part of the 1D surface under
no influence from other cells seemed to be fairly constant (at about 1 \( \mu m/min \)). The cell
suddenly stopped when it encountered the grooves indicated by the flat region in the curve.
Interestingly, the cell did not stay at rest for too long- instead it reversed its direction and
moved at a much faster rate (almost twice the forwards velocity) to return towards the
reservoir. Fig. 3.4(b) represents the instantaneous forward and return velocities of single
cells under various circumstances. The 'single' case indicates cell moving in isolation without
the influence of other cells. The forward and return velocities in this case were 1.05 \( \mu m/min \)
and 1.94 \( \mu m/min \) respectively. Multiple 'case' indicates a single cell under the influence of
multiple cells. The velocities in this case were 2.26 \( \mu m/min \) and 4.79 \( \mu m/min \). Finally 'new'
indicates the velocities of newly formed cells immediately after cell division migrating with
a forward and return velocity of 1.71 \( \mu m/min \) and 3.6 \( \mu m/min \) respectively. In all the cases
it was observed that the return velocity was almost twice the forward velocity indicating
biophysical bias towards quick change in directions. It may also be noted that the cells
under the influence of other cells tended to move faster in either directions when compared
to isolated cells. This indicates that the cell migration on 1D substrates is dependent on and
affected by the phenomena of 'crowding' and 'peer pressure'. Actin and nuclei distribution
of the cells are characterized and quantified in the next few sections.
3.4.3 Actin and nuclei distribution

The actin and nuclei distribution of the C2C12 myoblasts on the 1D filaments and plain PDMS are as shown in Figs. 3.5(a-i). These images were obtained after fixing and staining the myoblasts for 24h after plating them on the structures. The cells were still in the growth medium (GM) and hadn’t started to fuse into myotubes. Figs. 3.5(a-c) show the actin and nuclei distributions of the cells at a relatively lower magnification of 10X in order to visualize the intersection between the filaments and the reservoir (left of the images). Dense clustering of actin was observed at the intersection. The cells seemed to be uniformly distributed forming a confluent layer over the filament. The nuclei seemed to be fairly well aligned along the length of the filament (Figs. 3.5(d-f)). The actin distribution seemed to be fairly uniform and dense over the entire structure. Furthermore, the nuclei seemed to be elongated and stretched assuming an ellipsoid morphology. We believe this happens due to the geometric confinement of the cells on the surface of the filaments. Similar morphologies of cells were observed in the literature when plated on patterned 1D shapes like lines on soft substrates like PA gels [87, 88]. Figs. 3.5(g-i) show the actin and nuclei distribution of the cells plated on flat, large area, PDMS substrates. It may be noticed that the nuclei orientation and actin distribution have no specific pattern of organization. Instead, they seem to be more random when compared to the filament case as there is no geometric confinement of the cells.

3.4.4 Three dimensional characterization

Three dimensional fluorescence images were recreated from the confocal z-stacks of the cells on the filaments. The Z resolution of the stacks was about 0.7 μm. Cells completely wrapped around the filaments from all four sides as opposed to just attaching on the sides with the highest surface areas (Figs. 3.6(a-c)). One would have expected the cells to migrate from the thinner sides to the wider sides in order to spread out. But it appears that the cells
occupied all the surfaces evenly. However, it must be noted that the morphologies of the
nuclei and actin distribution are different on the top surface and the edges than on the sides.
The nuclei on the sides and edges seemed to be more deformed as compared to those on the
wider top surface. The actin density was higher along the edges. The nuclei seemed to be
flattened due to the actin network passing over them and exerting downward forces. It also
appeared that the cells had accumulated at the bottom of the filaments too although at a
lower density as compared to the top side.

3.4.5 Nuclei orientation

The orientation angle of the nuclei (about 40 cells) of C2C12 myoblasts on the filaments
and flat PDMS is measured using ImageJ. The data is represented in a polar plot (Fig. 3.7(a-b)).
On the filament structure most of the nuclei were oriented between 160 – 180°
(around 22 cells) indicating that the cells aligned themselves along the length of the filaments.
This is due to the geometric confinement of the cells on the filaments as discussed in the
previous section. The nuclei on flat PDMS substrates did not show orientation bias towards
any direction as can be noted from Fig. 3.7(b). The orientation angles were spread over
0 – 180°. This indicates that given the freedom to grow, proliferate, migrate and expand
on flat PDMS substrates without any microscopic geometric constraints, the cells will orient
themselves randomly.

3.4.6 Nuclei deformation and spreading

Nuclei spread area of the C2C12 myoblasts on the filaments and flat PDMS was calculated
using ImageJ. Box plots of nuclei spread area shown in Fig. 3.7(d). The circular markers at
the center of the shaded boxes represent the mean nuclei spread area. The horizontal lines
through the shaded boxes indicate the median nuclei spread area and the lower and upper
edges represent the 25th and 75th percentile, respectively. The whiskers extending vertically
from each box represent the standard deviation. The median nuclei spread area of the cells on the filament was about 90 μm² whereas in the case of flat PDMS substrate the nuclei spread area was much higher at 130 μm². This indicates that the narrow geometry of the structures tend to compact the cells thus leading to a smaller median spread area. On the other hand the cells can spread freely on flat PDMS substrates as there are no geometric constraints. A direct consequence of the compaction effect produced by the filaments was the increased cell density as shown in the histograms in Fig. 3.7(c). There were more than 40 cells per 10000 μm² area of the structure as compared to about 35 cells per 10000 μm² on flat PDMS substrates. The higher density of nuclei is expected to produce thick myotubes in relatively shorter time frame compared to PDMS substrates. The lateral deformation of the nuclei is quantified using a parameter called the circularity index of the nuclei using ImageJ. Circularity index (CI) was calculated using the relation, CI = 4 × π × [Area]/[perimeter]^2. Hence, a CI = 0 indicates an infinitely elongated polygon and a CI = 1 indicates a perfect circle. CI of nuclei on the filament was estimated to be about 0.6 while that of the nuclei on flat PDMS is estimated to be about 0.8 as shown in Fig. 3.7(e).

### 3.4.7 Myotube formation

The C2C12 myoblasts were incubated in fusion media (FM) after 24h of cell plating. Fully differentiated myotubes were obtained on the filaments after 6 days in the FM (Fig. 3.8). Myotubes extended all the way from the reservoir to the filaments as shown in the phase contrast image in Fig. 3.8(a). Furthermore, we were able to control the placement and length of myotubes on the PDMS structure through spatial patterning of ECM and pluronic F127 (Fig. 3.8(b)). One side of the structure was made to adhere to the petri-dish surface through stiction, and ECM was carefully placed over the reservoir and 300-400 μm of the exposed PDMS structure. Once, the ECM was conjugated to the exposed surface, the entire structure was released and incubated in pluronic F127 leading to a very specific region of interest where myotubes could grow (see Fig. 3.8(b)). The nuclei, actin and myosin
The myosin organization in these images was of particular interest as it showed the formation of thick myotubes around the filament in a very short span of 6 days. It usually takes longer to fuse myotubes on PDMS substrates (between 10-14 days) [7]. The reason for the myotube fusion in such short duration may be due to the alignment and compaction of the C2C12 myoblasts achieved through geometric confinement on the filaments. It is reported in the literature that myoblasts cultured on line patterns on 2D substrates fuse faster than those cultured on flat substrates [7,89]. Such functionality is highly desirable in bio-actuators and micro-swimmers.

### 3.4.8 Bio-actuator characterization

Fully differentiated myotubes are known to twitch and contract [90,91]. We made use of this property of the myotubes to develop periodically contracting bio-actuators. We cultured the myoblasts on suspended filaments and differentiated the cells to form myotubes. Once the myotubes were formed after about six days of culture in fusion media, one end of the filament was manually cut off such that it resembled a cantilever beam. Fig. 3.9 shows such a structure with Myotubes cultured all along the length of the filament. The filament doesn’t look straight anymore because the regions with higher density of Myotubes are deformed due to the higher traction stresses applied by the Myotubes. A contracting myotube was observed close to the base of the cantilever as shown in Fig. 3.9. This Myotube produced enough periodic contractile force to actuate the entire beam. We tracked the displacements of three distinct points separated by few hundred micro meters along the length of the beam using simple particle tracking cross-correlation algorithm in MATLAB. The view graphs on the right show the horizontal and vertical displacement profiles of the three points labeled in the phase contrast image. As can be seen, the peaks of the three curves on both the plots align perfectly (although with varying magnitudes) indicating that there is just one source of actuation on the beam. The maximum peak displacement was observed to be as high...
as 17 μm for point 3. Such deformations may be utilized to develop autonomous biobots. Note that here the bio-actuator contracts purely based on spontaneous twitching of the myotubes. The contraction of the bio-actuator due to spontaneous twitching of myotubes implies that swimming bio-bots similar to those demonstrated before [6] but with much higher control and improved functionalities may be obtained using the described methods. Furthermore, it may be possible to enhance the contraction magnitude and frequencies using electrical and optogenetic means. Work is already underway to develop electrically and optogenetically actuated micro-swimmers using C2C12 derived myotubes by the authors and shall be reported in future publications.

3.5 Overview

A novel 1D polymeric platform and a cell culture methodology have been developed, characterized and quantified for robust bio-actuator and bio-bot development using C2C12 cells. The developed techniques are expected to improve the performance and functionalities of existing micro-swimmers and future biological machines by allowing the capability to integrate subunits like neurons and vascular networks for control and guidance and other higher order functions. The unique geometry of the 1D filaments enhances cell density and increases cellular compaction, thus leading to the formation of mature and thick myotubes in shorter time frames with autonomous contraction capability. Cellular migration and mitosis pattern on the 1D substrate has been characterized by observing migrating cells on the filaments under various geometric conditions. Precise control of Myotube placement on the filaments has been demonstrated through spatial patterning of ECM proteins and cell repellent molecules. An example bio-actuator design capable of autonomous contraction has been demonstrated. The developed platforms and techniques will be used to realize electrically and optogenetically controlled bio-bots in the near future.
Figure 3.1: One dimensional PDMS structure fabrication methodology. Si micro-molds are prepared using photolithography and dry etching of Si wafers. (a) Photoresist is patterned on a Si wafer using standard photolithography techniques. The wafer is then etched in an inductively coupled plasma- deep reactive ion etching (ICP-DRIE) machine to the desired depth. (b) The etched wafer is then coated with polytetrafluoroethylene (PTFE) covering the side walls and the bottom surfaces of the micro channels. PDMS (4:1 base to cross-linker ratio) droplets are poured on the reservoirs of the micro-channels. (c) The PDMS fills the micro-channels through capillary draw. (d) Solid 1D PDMS structures connected by the reservoirs may be peeled off after curing at 60° C for 12 hours. (e) Scanning electron micrograph of the cured 1D PDMS structures showing a smooth and continues topography (f) Zoomed SEM image showing a single device with a broad region closer to the reservoir transitioning smoothly to a thinner structure.
Figure 3.2: Final PDMS structure configuration and cell plating. (a) Shows the final configuration of the PDMS structure on the Si chips in a petri dish just before cell plating. The PDMS structure is coated with ECM while all the other surfaces are coated with pluronic F127. (b) Schematic of rounded cells on all the 2D surfaces immediately after cell plating. Note that the cells do not land on the 1D regions due to limited surface area available to adhere. (c) Elongated cells after several hours of plating. Cells divide and proliferate after few hours of plating. Once, the reservoirs are confluent, the cells start moving towards the 1D structure and eventually cover the whole structure.
Figure 3.3: Time lapse imaging of C2C12 myoblasts over 40 hour. (a) Free standing device coated with fibronectin after one hour of cell plating. (b) A single cell starts to appear on the right side of the image as indicated by the arrow. (c) The cell is now entirely in view and it is moving towards the groves along the side of the filament. (d) The filopodia of the cell extend and sense the presence of the groove as an irregularity in the topology. (e) The cell doesn’t proceed forward anymore and returns. (f) The cell completely exits the field of view moving further towards the reservoir from where it came. (g) A cell reappears along with several other cells moving in a group as the free space in and near the reservoir region is starting to shrink as a result of proliferating and migrating cells crowding the area. (h) The cells temporarily detach from the device to form spherical structures just before cell division. (i) The newly formed cells from the dividing spheres are shown by the arrows. (j) At the end of the time lapse the entire structure is confluent with cells. Several spherical structures may be observed indicating that many cells are at the stage of division at this point. (k-l) Zoomed in images of the highlighted regions in (i) and (j) respectively showing a confluent layer of cells and newly formed cells on the structure. (Scale bar: 100 μm)
Figure 3.4: Displacement tracking and velocity data from the time lapse images. (a) Shows the horizontal tracking data of a single point on a cell moving in isolation without the influence of any other cells during the initial phase of the timelapse. (b) Instantaneous forward and return velocities of single cells under various different circumstances.
Figure 3.5: Actin and nuclei organization of C2C12 myoblasts on the 1D PDMS devices and plain PDMS substrates. (a-c) show the actin and nuclei organization of the cells on the PDMS devices at 20X magnification (Scale bar: 100 μm). (d-f) show the actin and nuclei organization of the cells on the PDMS devices at 40X magnification. The nuclei seem to be elongated and the actin fibers show a directional preference along the length of the device. (g-i) show the actin and nuclei organization of the cells on plain PDMS. (Scale bar: 25 μm).
Figure 3.6: Three dimensional visualization of the PDMS device confluent with C2C12 myoblast cells on all sides. (a-c) show the three dimensional nuclei and actin organization of the C2C12 cells on the top and side surfaces of the PDMS structure. It may be observed that the nuclei on the edges and sides are more deformed than the ones on the top surface. (d-f) show the cross sectional view of the nuclei and actin distribution on the PDMS device. It may be observed that the cells wrap around the device completely but in a non-uniform manner. The device dimensions in the image are 200X30X10 μm. (Scale bar: 50 μm)
Figure 3.7: Quantitative comparisons of the nuclei orientation, deformation and cell spreading on the 1D devices and flat PDMS substrates. (a-b) show polar plots representing the nuclei orientation angles of the C2C12 cells on 1D PDMS structures and flat PDMS substrates respectively. Angles closer to 0° and 180° imply nuclear orientations along the neutral axis of the 1D PDMS beam. It may be observed that the nuclei on the 1D device tend to be oriented along the beam axis while those on the flat PDMS substrates have no particular orientation bias. (c) shows a bar plot of the cell density on 1D device and flat PDMS substrate. The cell density is calculated by counting the number of nuclei within a given area. (d-e) show box plots of nuclei spread area on 1D device and flat PDMS substrate. The circular marker in the center of the shaded box represents the mean nuclei spread area. The horizontal line through the shaded box indicates the median nuclei spread area and the lower and upper edges represent the 25th and 75th percentile, respectively. The whiskers extending vertically from each box represent the standard deviation. (f-g) show box plots of nuclei circularity index on 1D structure and flat PDMS substrate. Circularity is calculated using the relation: circularity = $4 \times \pi \times [\text{Area}] / [\text{perimeter}]^2$. Hence, 0 indicates an infinitely elongated polygon and 1 indicates a perfect circle.
Figure 3.8: Characterization of fully differentiated C2C12 derived myotubes on the 1D PDMS substrates. (a) shows a phase contrast image of fully matured myotubes after day 6 of cell plating. (b) shows a phase contrast image of Myotube formation on only one side of the PDMS device through selective patterning of fibronectin (scale bar: 50 μm). (c-f) show nuclei, actin and myosin organization in the differentiated myotubes after day 6 of cell plating. The myotube formation observed through myosin staining indicates total maturation around the 1D device and extending along the length of the device.
Figure 3.9: Demonstration of Myotube powered bio-actuator. The phase contrast image shows fully differentiated myotubes formed along the length of the 1D structure which is released at one end to behave like a soft cantilever beam. The spontaneous periodic twitching of the myotubes closer to the base of the cantilever (indicated by the dashed circle) deforms the entire beam in a cyclic fashion. Other dense myotubes (denoted by arrows) exert enough traction forces to bend and deform the beam. The view graphs on the right show the horizontal and vertical displacements of three different points (denoted as points 1-3) on the beam as the myotube close to the base contracts periodically. (Scale bar: 100 μm)
Chapter 4

Measuring cellular traction forces under applied stretch

4.1 Summary

It has been well established that cells respond to externally applied stretch. For example, C2C12 skeletal muscle cells differentiate faster under an applied cyclic loading; passive cardiomyocytes begin to contract when stretched; vesicles in axons of neurons alter their dynamics due to applied stretch. In most cases, cells respond to stretch by reorganizing their cytoskeletal machinery to optimally respond to whatever stretch is applied on them. This usually leads to a force build up or relaxation in the stress fibers of the cells. Consequently, the cells start to polymerize more and more actin fibers in the cytoskeleton to build more force. However, to date no method has been reported to measure cellular forces under applied stretch. This chapter describes the development a novel device and techniques to measure traction forces applied by cells and cell clusters under applied uniaxial loading.

4.2 Materials and methods

4.2.1 Fabrication of Si micromolds

Silicon micromolds for the micropillar array were fabricated by modifying a previously developed process [60]. Briefly, a silicon wafer was patterned and etched using microfabrication techniques as shown in Fig. 4.1(a-c). A positive tone photoresist (MEGAPOSIT SPR220) was spun coated on a 4” Si wafer and patterned with a photomask using standard
photolithography techniques. The wafer was then etched using a dry etching process in an inductively coupled plasma deep reactive ion etching (ICP-DRIE) machine. The etch depth and thus the length of the final PDMS structure was determined by the etch time. A thin layer of polytetrafluoroethylene (PTFE) was deposited on the wafer after the completion of the etching process (see Fig. 4.1(d)) to lower the adhesion of PDMS to the Si wafer and to prevent delamination of the pillar structures during peeling off from the molds. The micro-mold design was such that there were 12 square chips of 15 mm edge on a 4” diameter and 0.5 mm thick Si wafer. Each of these square chips contained a 3 mm square region at the center where circular pits of 2 μm diameter and 5 μm center to center spacing were etched using the described microfabrication technique.

4.2.2 Fabrication of stretchable substrate with micropillar array

The 15 mm square silicon chips were then diced from the wafer and another square silicon chip of similar dimensions was glued to their bottom surface. This was done to increase the total depth of the chips from 0.5 mm to 1 mm (see Fig. 4.1(e)). This hybrid structure was then glued to the center of a 300 mm polystyrene petri dish with the etched circular pits facing upwards. Here, the silicon chips were meant to act as a positive mold for creating a 1mmX1mm well for holding cell culture media while the circular pits were meant to act as negative molds for fabricating the micropillar array. PDMS (Dow Corning, Sylgard 184) was thoroughly mixed in a 10:1 base to crosslinker ratio and degassed in a vacuum chamber for 30 mins to remove all the air bubbles. The degassed liquid PDMS was then poured into the hybrid Si and polystyrene molds (see Fig. 4.1(f)). The volume of the liquid PDMS used is carefully controlled during the casting process to obtain the desired thickness of PDMS on top of the Si mold. This thickness is very important from an imaging point of view as objective lenses of higher magnification operating under liquid medium like oil/water have small working distances on the order of 150-200 μm. Hence, we ensure that the volume of the liquid PDMS poured into our molds is such that the final film thickness does not
exceed 200 μm. The mixture is once again degassed in a vacuum chamber for 1 hr to get rid of all air bubbles. Even tiny bubbles are undesirable as they interfere with the strain field when stretched. Next, the PDMS is cured at 60°C for 12 hrs to complete the process of polymerization. After 12 hrs, the PDMS is allowed to cool at room temperature and then peeled using tweezers. The final stretching substrate is obtained by cutting rectangular sections of 80mmX30mm with the 15 mm square wells containing the micropillar arrays at their center (see Fig. 4.1(g)). PDMS micropillars of 1-2 μm diameter and 5 μm center to center spacing are obtained in the 3mmX3mm central region of the substrate as shown in the SEM micrographs in Fig. 4.2.

4.2.3 Microcontact printing of fluorescent fibronectin on pillar tips

Before, cell plating the PDMS pillars need to be functionalized with extracellular matrix proteins to promote cell adhesion. However, regular ECM adsorption techniques are not suitable for the pillar substrates as cell adhesion only on the top surface of the pillars is desirable for accurate force measurements. At the same time we also want to fluorescently label the pillar tips to later aid in tracking purposes for displacement measurements. We achieve this by microcontact printing green fluorescent protein (GFP) conjugated fibronectin on the top surface of the micropillars. A schematic diagram of the process is as shown in Fig. 4.3. First, we adsorb 50 μL of 25 μg/mL concentration, GFP conjugated fibronectin (Cytoskeleton Inc, cat. no. FNR02) on a flat PDMS stamp for 3 hrs. At the end of 3 hrs the stamp is rinsed three times in phosphate-buffered saline and blow dried using pressurized air. Five minutes before the fibronectin stamp is ready, the stretching substrate with the micropillar arrays is exposed to oxygen plasma of 50W power for 15 s in a reactive ion etching (RIE) machine. This is done to make the top surface of the pillars hydrophilic (cured PDMS is naturally hydrophobic) so as to improve its adhesion properties and enable
complete fibronectin transfer from the stamp to the pillar tips. Next, the stamp is carefully placed on top of the pillars using tweezers and allowed to rest for 30 mins. At the end of 30 mins, the stamp is carefully peeled off and the pillar tips are observed under a fluorescent microscope to ensure fibronectin transfer to the tips. The pillars may then be treated with Pluronic F127 (1% in PBS, Sigma-Aldrich) for 20 mins and rinsed off in PBS to ensure all the regions without fibronectin remain cell repellant.

4.2.4 Cell culture and imaging

Monkey kidney fibroblast (MKF) cells (from ATCC Inc., USA) were cultured in an incubator at 37°C and 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY, cat. no. 41965-039), supplemented with 10% fetal bovine serum (ATCC, Manassas, VA, cat. no. 30-2020), 2mM L-glutamine (ATCC, Manassas, VA, cat. no. 30-2214), 10mM HEPES (Life Technologies, cat. no. 15630080), and 1% penicillin/streptomycin (ATCC, Manassas, VA, cat. no. 30-2300). Prior to cell plating, PDMS substrates with micro-pillar arrays were incubated in cell growth medium at 37°C for 15 minutes for equilibration followed by sterilization under a germicidal UV lamp in a tissue culture hood for 20 minutes. Cells were plated on the central culture surface of the substrate with micropillars and incubated for 16 hours before imaging. The plating density was maintained at ~10,000 cells per substrate. Phase contrast imaging of the cells on the pillars and fluorescence imaging of the pillar tips were performed using an Olympus IX81 motorized inverted microscope.

4.2.5 Strain application using a stretching stage

A custom built stretching system [7] is used to apply known strains on the PDMS substrates. The substrate consists of a thin region (culture surface with micropillars) and thick regions (about 1.2 mm thick). The thick regions are used to handle the substrate and provide
structural support to the thin, 200 μm central region. The thin ends of the substrate are rigidly clamped to a stretching stage (Fig. 4.4). The linear stretching stage consists of an actuator (Newport Inc.) mounted on an aluminum base with adjustable clamps. Uniaxial stretch may be applied at one direction while the other being constrained, using the linear actuator. The applied strain was characterized to be uniform earlier.

4.2.6 Tracking pillar tips using digital image correlation (DIC) technique

2D displacement field produced by the cells was computed by digital image correlation method (DICM) using gray intensity of fluorescent pillar tips. The marker subset size, subset shape function, sub-pixel optimization algorithm, and sub-pixel intensity interpolation scheme were optimized. The theoretical resolution of DICM can reach 0.1 pixel size of the acquired images. Images were acquired by a 60X objective (each pixel 0.1 μm). Under these conditions, 1-2 μm pillar tips spanned more than 8 pixels on the image, with the brightest portion at center, giving the recognizable gray intensity features for correlation tracking. An open source MATLAB program based on the Sum of Squared Differences (SSD) correlation criterion was used to compute the gray intensity correlation of the fluorescent pillar tips. In the present analysis, the markers subset size was selected ranging from 5X5 pixel$^2$ to 25X25 pixel$^2$ through trial and error. A uniform grid matrix encompassing the entire target cell clusters’ contracting area was generated to obtain both transverse and longitudinal displacements of each grid node. During image processing, rigid body motions (translation and rotation, generally 2° to 3°) of the pillars were subtracted to obtain the net 2D displacement field ($u_x$ and $u_y$, which are sum of the stretch induced and cell-induced displacements of the pillars). The stretch induced components of the pillar displacements were subtracted from the net displacement by tracking the displacements of those pillars which are outside the cell area (and hence not affected by the cells). Since, all rows and all columns of the
pillar array have the same displacements in Y-axis and X-axis respectively, the cell induced
displacement of a pillar in X-direction and Y-directions are computed by subtracting the X
and Y components of the net displacement from the X component of another pillar in the
same column and the Y component of another pillar in the same row (outside the cell area)
respectively.

4.3 Results and discussion

4.3.1 Finite element analysis (FEA) and validation of the experimental methods

A finite element analysis (FEA) was performed to validate the experimental methods for
calculation of the cellular forces. A finite element model simulating the actual PDMS micro-
pillar array design was constructed and meshed. An array of 3X3 uniformly spaced pillars of 2 μm diameter and 5μm center to center spacing were considered on a square platform of 20 μm edge length. The elastic modulus of PDMS was chosen to be 2 MPa and the Poisson’s ratio was selected as 0.5. Known forces were applied on the tip (at the center) of each pillar (as shown in Fig. 4.5(a)). The substrate was constrained from one end as would be done during the experiments and a uniaxial stretch of 10% was applied from the other end as shown in Fig. 4.5(b). The model was solved under the aforementioned loading and boundary conditions. The tip displacement as a result of the applied loading and stretching were obtained from the model solution as shown in Fig. 4.5(c). Here, we are simulating the exact similar experimental scenario where the cells applying forces on the pillars would be stretched and the measured displacements would be used to compute their forces. Hence, when the model is solved using the computed displacements, it should return the original applied forces. This would indicate that the stretching didn’t cause any pseudo forces or displacements to appear. In the next iteration, the computed tip displacements were used
as the inputs to solve the model as shown in Fig. 4.5(d). The computed displacements were applied at the pillar tips and the substrate was again stretched. The model was solved and the forces at the pillar tips were computed. The computed forces are as shown in Fig. 4.5(e). They matched very closely with the applied loads conforming that indeed stretching of the substrate did not induce any pseudo forces or displacements in the system. Fig. 4.5(f) shows the displacements of the pillar under loads that are obtained from the difference of the applied and computed loads. As can be seen, the tip displacement and the displacement of the base are same due to the applied stretch indicating that there is no force acting at the tip. This once again, confirms that there are no induced pseudo forces due to the applied stretch.

4.3.2 Validating the measured strain fields

The stretching substrate is a rectangle of 80mmX30mm dimensions and consists of a central square pit of 15mmX15mm for holding cell culture media (see Fig. 4.6(a)). At the center of this square pit is the micropillar array spread over an area of 3mmX3mm. The thickness of the square pit is maintained at 20 μm while the rest of the substrate is about 1.2 mm thick. The substrate is stretched by constraining it at one end and applying a uniaxial stretch using a linear actuator from the other end as shown in Fig. 4.6(b). The application of uniaxial stretch elongates the length of the substrates while the width shrinks due to Poisson’s effect. Theoretically, the applied longitudinal strain $\varepsilon_x$ and the obtained lateral contraction are related as $\varepsilon_y = -\nu \varepsilon_x$. Hence, when the pillar tips are tracked we should observe a similar relationship in the X and Y displacements of the tips. Fig. 4.6(c) shows a phase contrast image of the tips of the pillars observed at 60X magnification before cell plating. This substrate was used for calibration of the strain fields without any interference from the cells. As can be seen, each of the pillars is well resolved under this magnification and they are uniformly spread out through the field of view. A uniaxial stretch of 8% is applied is applied at one end using the linear actuator while the other end is kept fixed.
This leads the pillars to move from the left to right of the screen. We bring the same pillars back into the field of view using a special microscope eyepiece attached with a reticle with calibrated cross-hair lines. We first align a feature on the substrate with the cross-hair of the eyepiece reticle before and after applying the stretch ensuring that we image the same set of pillars both times. An image of the stretched pillars can be seen in Fig. 4.6(d). The images from Figs. 4.6(c-d) are used to compute the displacements of the pillars using a digital image correlation software as describe in the methods section. The longitudinal displacement field of the pillars computed using DIC is as shown in Fig. 4.6(e). The distribution of the displacement field is along expected lines. The region closest to the applied stretch moves the most (right end) while the region closest to the fixed support moves the least (left end). The magnitude of the displacements depends on the magnitude of the applied stretch and the location tracked. Fig. 4.6(f) shows the transverse displacements of the pillars as a result of the Poisson shrinking. Again, the displacements seem to be along expected lines as the lower region moves upwards while the top edge is moving downwards, indicating that the substrate is under compression in the transverse direction. This is quite consistent with the expected Poisson’s effect.

4.3.3 Computing traction forces at focal adhesion sites of a cell cluster

After having validated the experimental technique and the measured strain fields, we move on to compute the actual cellular forces from a cluster of cells. Cells are cultured on the pillars as described in the methods section. Imaging is done after 16 hrs of cell plating in both phase contrast and fluorescent modes to observe the cells and the pillars respectively. Fig. 4.7(a) shows a cluster of cells after 16 hrs of cell plating under phase contrast at 60X magnification. The cell seems healthy and seems to have spread out well over the pillar surfaces. The cells spread naturally by applying forces on the substrates underneath them.
In this case too, the cell cluster applies traction forces on the tips of the pillars, deforming them. Fig. 4.7(b) shows the tips of the pillars underneath the cell cluster under fluorescent mode. Since, the pillar tips were functionalized with GFP conjugated fibronectin, they light up under green light. This pillar distribution is a consequence of the deformation caused due to the forces applied by the cells. The displacement field for the pillars may be obtained by comparing this image of the pillars with another reference fluorescent image of the pillars without any cells on them. A displacement field map thus obtained using a DIC software is shown in Fig. 4.7(c). From, the displacement map it may be observed that the maximum displacement is caused around the periphery of the cell cluster while the pillars around the central region of the cluster move relatively less. A maximum displacement of about 1.8 μm was observed close to the south east corner of the cluster. The force field computed from the displacement field is as shown in Fig. 4.7(d). A maximum focal adhesion force of about 8 nN was observed around the south-east periphery of the cell cluster. This force field is consistent with that reported in literature [92].

4.4 Overview

In this chapter a novel technique to measure cellular traction forces under an applied stretch was described. A new fabrication technique was developed by integrating micro-scale features (micropillars) with a macro-scale substrate for applying stretch while simultaneously maintaining the integrity of cultured cells. Computational validation of the developed experimental methods was performed using finite element analysis (FEA) and all the methods were validated successfully. The experimentally observed strain fields on a reference substrate were characterized and calibrated. Finally, cellular traction forces exerted by an island of cells were computed by tracking the tip displacements of the pillar substrates. The observed results were found to be consistent with those observed in literature.
Figure 4.1: Process flow for fabricating the stretching substrate with micropillar arrays.
(a) A positive tone photoresist spun coated on a 4'' Si wafer is exposed through a chrome photomask to UV light of 365 nm wavelength using an I-line tool. (b) The exposed regions are developed using AZ-400K base developer. (c) The developed regions are etched in an ICP-DRIE machine using Bosch process. (d) A thin layer (about 100 nm) of PTFE deposited after striping of the resist using oxygen plasma. (e) A silicon chip of similar dimensions as the etched chip is glued to the bottom surface. (f) PDMS in 10:1 base to crosslinker ratio is poured in a polystyrene petri-dish with the Si structures at the center. (g) Finally, the stretchable substrate is peeled into cut into required dimensions. The micropillar array is at the center of a square well used for cell culture and to hold culture media.
Figure 4.2: SEM micrographs of the PDMS pillars. (a) An array of 4X4 pillars on the stretchable substrate. (b) A zoomed in view of a single pillar. (Scale bar: 2 μm)
Figure 4.3: Microcontact printing of fluorescent fibronectin on pillar surfaces. (a) A flat PDMS stamp of 5mmX5mm dimensions. (b) Green fluorescent protein (GFP) conjugated fibronectin is adsorbed on the surface of the stamp for 3 hrs. (c) The stamp is carefully placed on the top surface of the oxygen plasma treated pillar surfaces. (d) The stamp is allowed to rest on top of the pillars for 30 mins. (e) At the end of 30 mins, the stamps carefully peeled off to reveal the pillar tips functionalized with the GFP-fibronectin.
Figure 4.4: The stretching system consists of a flexible PDMS substrate mounted on a linear stage with an actuator. Adjustable aluminum plates are used to clamp the substrate. The system is about 10" long. Reproduced from [7]
Figure 4.5: Finite element validation of the experimental procedures to compute cellular forces under applied stretch. (a) Known forces are applied at the tips of a meshed model of an array of nine pillars on a substrate of 5 um thickness. The pillars are 7 um long and 2 um in diameter. As in the experiment the substrate is constrained in one direction and a uniaxial stretch of 10% is applied at the other end. (b) 3D meshed model of the PDMS pillars. (c) Computed tip displacements due to the applied forces and the applied stretch. (d) The displacements obtained in the previous step are used as inputs in this step to back calculate the forces. (e) The computed forces at the pillar tips match the applied fields with great accuracy. (f) Displacement caused by the difference of the applied and computed forces resulted in zero net displacements of the pillar tips, indicating that no pseudo displacements or forces were induced due to stretching.
Figure 4.6: Validation of the experimentally obtained strain fields. (a) Initial state of the stretchable substrate before the application of stretch. (b) After the application stretch the length increases but the width decreases due to Poisson’s contraction. Theoretically, the applied longitudinal strain $\varepsilon_x$ and the obtained lateral contraction are related as $\varepsilon_y = -\nu \varepsilon_x$. (c) Phase contrast image of the original pillar positions at 60X magnification. (d) Pillar positions after the application of 5% stretch at the end of the PDMS substrate. (e) Computed X-displacement field using a DIC software from the displaced and original tip positions. (f) Computed Y-displacement field.
Figure 4.7: Measurement of cellular displacement and force fields. (a) A well spread out cell cluster on top of the micropillar array viewed under phase contrast microscopy. (b) Fluorescent pillar tips underneath the cell cluster under a fluorescent microscope. The pillars displaced due to the cell applying forces on them. (Scale bars: 20 μm). (d) Computed displacement field produced by the cell obtained by comparing reference image with the displaced pillar tip images using a DIC software. (e) Computed force field applied by the cell. The force is obtained by taking the product of a given pillar displacement with its stiffness.
Figure 4.8: Evolution of cellular forces as a function of applied stretch. (a) Force field measured immediately after stretching the cell cluster by 6%. (b) Force field after 20 mins of constant 6% stretch. (c) Force field measured immediately after stretching the cell cluster by 12%. (d) Force field after 20 mins of constant 12% stretch.
Figure 4.9: Magnitude of cellular forces as a function of stretch. (a) Maximum force applied by the cluster increases from about 7.9 nN at 6% stretch to about 18.8 nN at 12% stretch. Force builds up over time. (b) Root mean square force increases as a function of stretch, indicating that the total magnitude of the force applied by the cells increases with stretch and time.
Chapter 5

Future work and conclusions

5.1 Future directions

5.1.1 Designing higher order biological machines

This dissertation lays the foundation for understanding the emergent behavior of various cell types through the lens of their interactions with novel engineered soft substrates. This research is expected to be particularly useful in developing design principles for the realization of futuristic biological machines consisting of multiple cellular and engineered components acting in harmony to perform a useful task. An example of such a scenario is presented in Fig. 5.1, where multiple cell types are expected to come together and work in tandem with an engineered substrate to perform the functions of actuation, sensing and transport. The realization of such advanced biological machines would require a thorough of the emergent properties of the various cell types involved in intercellular and cell-substrate interactions. This dissertation is restricted in its scope to mechanically actuating cell types like cardiomyocytes, skeletal muscle, endothelial cells etc., but similar studies must be extended to other cell types like neurons and stem cells to predict their behavior in dynamic scenarios such as those presented in this dissertation.

5.1.2 Estimating trigger forces in cells

Several biological phenomena observed in cells like, differentiation, proliferation, mitosis etc. are force mediated. There is increasing evidence to suggest that cellular behavior changes
drastically based on the force they sense and generate often times by sensing matrix or substrate rigidity. However, the exact force levels at which the cells switch from exhibiting one behavior to another is not very clear. This is primarily because, the tools required to perform such measurements weren’t available thus far. This dissertation has presented (in chapter 4) a novel new device and techniques to measure such dynamically varying trigger (forces triggering a change in the usual behavior of a cell). For example, it is now understood that cells on stiffer substrates require nuclear transcriptional regulator YAP (Yes associated protein) localization in the cell nucleus and are tightly coupled to larger traction force generation [8,9]. However, the same studies also report that the cells are capable dynamically localizing YAP in the cytoskeleton or in the nuclei based on their microenvironment (see Figs. 5.2 and 5.3). With the force measurement techniques presented in this dissertation it will now be possible to measure with great accuracy the exact force levels at which such transitions of YAP happens in different cell types.

5.2 Conclusions

This dissertation focused primarily on the bio-mechanical interactions of cells with micro-fabricated thin, soft structures and consequent changes in their bio-chemical and biophysical behavior as a result of such interactions leading to unique emergent behaviors. A brief summary of the work presented in the different chapters of this dissertation is provided next.

In chapter 1, a brief introduction to the field of cellular mechanics from the perspective of cell substrate interactions and their applications for developing biological machines was provided. Several important attributes of cells pertaining to their regulation of behavior based on their interactions with their microenvironments was discussed. Some methods to measure cellular forces were also highlighted.
In chapter 2, the dynamic cross talk between contractile neonatal rat cardiomyocyte cluster and a deformable substrate with anisotropic compliance was explored. The cell clusters were found to be sensitive to the anisotropy. The clusters gradually oriented their contractile dipole direction along the most deformable direction of the substrate. The emergence of this orientation occurred over many cycles of contractile beating. This implies a dynamic feedback mechanism through which the cells sense the substrate deformations they produce by beating, and in turn optimize their contraction. The most deformable direction appeared to be the most optimum for the substrates studied in this chapter.

A novel 1D polymeric platform and a cell culture methodology were developed, characterized and quantified in chapter 3, for robust bio-actuator and bio-bot development using C2C12 cells. The developed techniques are expected to improve the performance and functionalities of existing micro-swimmers and future biological machines by allowing the capability to integrate subunits like neurons and vascular networks for control and guidance and other higher order functions. The unique geometry of the 1D filaments enhanced cell density and increased cellular compaction and lead to the formation of mature and thick myotubes in shorter time frames with autonomous contraction capability. Cellular migration and mitosis pattern on the 1D substrate was characterized by observing migrating cells on the filaments under various geometric conditions. Precise control of Myotube placement on the filaments was demonstrated through spatial pattering of ECM proteins and cell repellent molecules. An example bio-actuator design capable of autonomous contraction was also demonstrated. The developed platforms and techniques will be used to realize electrically and optogenetically controlled bio-bots in the near future.

In chapter 4, a novel technique to measure cellular traction forces under an applied stretch was described. A new fabrication technique was developed by integrating micro-scale features (micropillars) with a macro-scale substrate for applying stretch while simultaneously
maintaining the integrity of cultured cells. Computational validation of the developed experimental methods was performed using finite element analysis (FEA) and all the methods were validated successfully. The experimentally observed strain fields on a reference substrate were characterized and calibrated. Finally, cellular traction forces exerted by an island of cells were computed by tracking the tip displacements of the pillar substrates. The observed results were found to be consistent with those observed in literature.
Figure 5.1: Different types of interacting cells and clusters of cells in tandem with soft engineered scaffolds are expected feature in advanced biological machine designs. Reproduced from: www.ebics.net
Figure 5.2: YAP/TAZ localization in cell nucleus and cytoskeleton are greatly influenced by matrix rigidity and cell shape. By varying substrate rigidity and by patterning cells into certain shapes, YAP/TAZ can be made to transition from one location to another in the cell. Adapted from [8].
Figure 5.3: Intracellular YAP localization is tightly coupled to the cellular force generation. YAP is localized in the cytoplasmic regions on soft collagen gels while it is localized in the nuclear region in cells on hard glass substrates. Adapted from [9].
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