Manipulating the Mechanical Microenvironment: Microdevices for High-throughput Studies in Cellular Mechanobiology

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Determining how biological cells respond to external factors in the environment can aid in understanding disease progression, lead to rational design strategies for tissue engineering, and contribute to understanding fundamental mechanisms of cellular function. Dynamic mechanical forces exist in vivo and are known to alter cellular response to other stimuli. However, identifying the roles multiple external factors play in regulating cell fate and function is currently impractical, as experimental techniques to mechanically stimulate cells in culture are severely limited in throughput. Hence, determining cell response to combinations of mechanical and biological factors is technically limited. In this thesis, microfabricated systems were designed, implemented and characterized to screen for the effects of mechanical stimulation in a high-throughput manner. Realizing these systems required the development of a fabrication process for precisely-aligned multilayer microstructures, and the development of a method to integrate non-traditional and clinically-relevant biomaterials into the microfabrication process. Three microfabricated platforms were developed for this application. First, an array was designed for experiments with high mechanical throughput, in which cells cultured on a surface experience a range of cyclic, uniform, equibiaxial strains. Using this array, a novel time- and strain-dependent mechanism regulating nuclear β-catenin accumulation in valve interstitial cells was identified.
Second, a simpler system was designed to screen for the effects of combinatorially manipulated mechanobiological parameters on the pathological differentiation of valve interstitial cells. The results demonstrate functional heterogeneity between cells isolated from different regions of the heart valve leaflet. Last, a microfabricated platform was developed for high-throughput mechanical stimulation of cells encapsulated in a three-dimensional biomaterial, enabling the study of mechanical forces on cells in a more physiologically relevant microenvironment. Overall, these studies identified novel biological phenomena as a result of designing higher-throughput systems for the mechanical stimulation of cells.
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List of Abbreviations

2D……………Two-dimensional
3D……………Three-dimensional
AFM…………Atomic force microscopy
ANOVA……..Analysis of variance
BSA…………Bovine serum albumin
DMEM………Dulbecco’s modified eagle media
EBM…………Eagle’s basal media
ECM…………Extra-cellular matrix
FBS…………Fetal bovine serum
FEA…………Finite element analysis
FITC…………Fluorescein isothiocyanate
HTS…………High throughput screening
MSC…………Mesenchymal stem cell
MSL…………Multilayer soft lithography
PA……………Polyacrylamide
PAVIC………Porcine aortic valve interstitial cells
PBS…………Phosphate buffered saline
PDMS………Poly (dimethylsiloxane)
PEG…………Poly(ethylene) glycol
PPFC……….Parallel plate flow chamber
PU……………Polyurethane
ROI…………Region of interest
SMA…………Smooth muscle actin
TCP…………Tissue culture plastic
TGF-β1……..Transforming growth factor beta one
THF…………Tetrahydrofuran
UV……………Ultra violet
VIC…………..Valve interstitial cell
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For those who fail,
but refuse to stop trying.

And for those who keep them going.
Chapter 1

1 Introduction

1.1 Motivation

Mechanical forces play a critical role in regulating cellular function. As early as 1892, Julius Wolff described bone remodeling, in which bones change shape, density and stiffness when mechanical loading conditions are altered[1]. For this reason, astronauts experience severe bone loss after extended periods in microgravity[2]. Similarly, muscles develop as a result of exercise, and skin forms hard calluses under repeated wear. Only recently however, have these organ-level phenomena been traced to cellular function, and findings that cells are exquisitely sensitive to mechanical forces have prompted great interest in how mechanical forces broadly regulate tissue homeostasis, disease progression, and cell fate and function. Understanding fundamental mechanisms of how cells interact with the mechanical microenvironment could eventually result in predictive control of cell fate and function, which will enable rational design strategies for tissue engineering and identify avenues for cell-based regenerative therapies.

Currently, typical strategies to manipulate cell function for these purposes include presenting selected microenvironmental cues based on prior knowledge and informed reasoning. However, this approach has only met with limited success, and significant advances in predictive control will likely arise from an improved understanding of the integrated response of cells to multiple regulatory stimuli in the microenvironment[3]. As such, an emerging trend in regenerative medicine strategies is high-throughput screening using cell-based arrays[4]. By systematically manipulating biomaterials[5], matrix proteins[6], cell sources and soluble factors[7] in an array-based format, researchers are able to determine how cells respond to various parameters in the environment. In particular, such assays have emerged to accelerate drug discovery in the pharmaceutical industry, but this approach too has only met with limited success. One factor that could account for the low success rates of these approaches is the inability of current state-of-the-art high-throughput screening platforms to incorporate physiologically relevant mechanical forces in the screening parameters. Dynamic mechanical forces exist in vivo, and are known to play a pivotal role in driving cell fate and function[8]. In the case of load-bearing
cells, mechanical stimuli can supplant other factors[9]. Furthermore, mechanical stimuli can alter cellular response to other stimuli[10-12], and are hence critical features of the microenvironment. The inability of high-throughput screening systems to capture mechanical features of the \textit{in vivo} microenvironment may be the critical limiting factor in the discovery of positive ‘hits’ that are then translatable to therapeutic applications. Thus, there is a need to develop high-throughput screening technologies that incorporate dynamic mechanical stimulation to expedite scientific discovery and innovation.

Dynamic mechanical stimulation can be achieved by deformation of an elastic substrate, to which cells are attached. Currently, the most widely used systems for mechanical stimulation of cells through substrate deformation are a range of platforms produced by Flexcell International Corporation[13], which are based on a standard 6-well plate format. These systems tend to be relatively large in size due to limitations in manufacturing technologies, and are expensive, cumbersome, require large quantities of expensive reagents, and are low in mechanical throughput. These factors have limited the widespread use of mechanically dynamic bioreactors in screening applications. Despite the demonstrated importance of mechanical forces in regulating and modulating cell fate and function, no currently available system is capable of screening for mechanical stimulation in cell-based assays. This deficiency precludes systematic screening for multiple \textit{combinations} of mechanical, chemical and matrix parameters.

The past decade has seen the rapid emergence of microfabrication technologies for biological and biomedical applications. Microfabricated systems are designed and executed with precise control over feature sizes with micron-level resolution. Microsystems provide a number of advantages over conventional technologies including the ability to exploit favourable scaling laws at smaller length scales; increases in portability and throughput via system miniaturization; and the potential to integrate multiple components on a single chip. This “lab-on-a-chip” approach is quite promising for biomedical applications, and we have already seen the development and deployment of microfabricated point-of-care diagnostic tools and miniaturized high-throughput bioreactor systems[14]. Recent progress in such fabrication technologies could alleviate some of the manufacturing concerns in developing array-based platforms to precisely apply mechanically forces to cells in culture. In addition, the substantial decrease in equipment footprint can dramatically increase screening throughput, thereby offering the possibility of systematically screening multiple combinations of parameters in the cellular microenvironment.
*Combinatorially* probing cellular response to various types of stimuli using conventional techniques generates an unfeasibly large number of experimental conditions, and would require alternative approaches. Microfabricated systems also require greatly reduced quantities of reagents, can speed up experimental times and efficiency, can improve precision, and because they are batch fabricated, could eventually be inexpensively manufactured to produce disposable testing platforms. Hence, microfabrication technologies may have the ability to accelerate scientific discovery and innovation, as compared to more conventional macroscale techniques.

Although there are some notable exceptions, microfabricated systems are generally yet to be accepted tools in traditional biological research labs. The last decade has seen an explosion in microfabricated technologies for biomedical applications (including a few involving mechanical stimulation of cells) but most of these reports are largely focused on the proof-of-principle of device operation, and much work remains to be done in effectively translating these technologies to mainstream biomedical applications. Though such approaches to cell culture boasts great promise for advances in biomedical sciences, the technology is yet to deliver on its potential and be broadly used by non-specialists in the field. Although a number of reasons for resistance to these technological changes have been identified (including equipment complexity, ease of use, and reliability), the impetus to solve these challenges will primarily come from effective and meaningful demonstrations of the utility of these systems over conventional approaches. Hence, meaningful demonstrations and advances in biological knowledge by exploiting the specific advantages attainable through the use of microfabricated systems will be critical in translating these technologies to more mainstream use.

Thus, the overall goals of the work presented in this thesis are to first develop microfabricated systems to apply dynamic mechanical stimulation to cells in culture; and second, to experimentally demonstrate the utility of this approach by exploiting the advantages of microtechnologies to conduct biological experiments that would be very challenging to perform using more conventional systems.

### 1.2 Thesis Overview

This thesis is organized into nine chapters as follows. Chapter 2 reviews the literature on microfabricated technologies designed to study the interplay between mechanical forces and biological systems. Distinctions are drawn between cellular biomechanics and cellular
mechanobiology, and emphasis is placed on the specific demonstrated and promised advantages of each of the reviewed technologies, as well as critical bottlenecks and disadvantages of these approaches. Chapter 3 describes the aims and specific objectives of the work presented in this thesis. Chapter 4 presents a multilayer soft lithography microfabrication technique to produce well-aligned multilayer structures while eliminating shrinkage-induced registration errors of poly(dimethylsiloxane) (PDMS). This approach is used to fabricate an array of pneumatically actuated vertically-driven microposts. Chapter 5 describes the work done in modifying the array of microposts to simultaneously and cyclically apply a variety of uniform equibiaxial strains to cells cultured on a PDMS film. The array is characterized and utilized to create a high mechanical throughput culture system, to study the influence of a range of mechanical strain magnitudes on protein regulation within the cell. The study describes the use of the array to determine the effects of equibiaxial mechanical strain magnitude and duration on activation of the canonical Wnt/\(\beta\)-catenin signaling pathway in cardiac valve interstitial cells. Chapter 6 addresses one of the technological limitations of the devices described in Chapter 5: an inability to conduct long-term mechanical stimulation, due to loss of adhesion between the cells and the mechanically active PDMS substrate. In order to resolve this, a technique to integrate a clinically-relevant and customizable polyurethane material into the standard PDMS microfabrication process was developed and characterized; and shown to improve long-term cell culture over standard materials. Chapter 7 describes the utilization of this polyurethane-PDMS technology in engineering composite culture films to address this adhesion issue, and further addresses the usability limitations of the devices described in Chapter 5: the complex fabrication process limits the number of devices that can practically be produced. By using a simpler, but less precise platform, we demonstrate high-throughput combinatorial screening of multiple mechanobiological parameters in the culture environment, by systematically manipulating combinations of matrix proteins, chemical cues and mechanical stimulation. This platform is then applied to study the functional differences between interstitial cells from different layers of the aortic valve leaflet. Interesting findings reveal new exploratory research directions, and demonstrate layer-dependent heterogeneity in the behaviour of these primary cells. Chapter 8 describes the future direction of this work, in which cells are encapsulated in an array of mechanically active three-dimensional biomaterial microconstructs, to enable high-throughput screening in more physiologically relevant three-dimensional culture systems which better mimic the \textit{in vivo} environment for many load-bearing cell types. Lastly, Chapter 9 provides
conclusions from the engineering approaches and biological studies described, and contains recommendations for future work in this area.

1.3 Overview of contributions

A large number of people contributed to the work presented in this thesis, and I have been fortunate to work with each of them. In particular, all aspects of the described projects benefited from thorough discussion with my supervisors, Dr. Craig Simmons and Dr. Yu Sun. In this section I present a detailed record of contributions to the experiments and studies presented herein, both by myself and my colleagues.

The work presented in chapter 4 is anomalous in that it is entirely my own. I conceived the idea and conducted the described experiments and procedures. The manuscript for the work was written by myself with subsequent editing by my supervisors, and published in the *Journal of Micromechanics and Microengineering* in 2009.

The device and experimental components in Chapter 5 is work that is done largely by myself. I was responsible for device design, simulation, fabrication, testing, characterization, and the development of peripheral supporting equipment. Anthony Ho, an undergraduate summer research student under my supervision, replicated and tested additional peripheral components for device actuation. The biological experiments attempted on the platform prior to the successful experiments with translocation of β-catenin were done in collaboration with a number of people: Yan Michelle Zhao and Dr. Lidan You provided access, training and guidance in using their live-cell calcium imaging system; and Wen Li Kelly Chen provided assistance in culture and proliferation experiments with the mouse mesenchymal C3H10T1/2 cell line. The successful (published) experiments were designed in collaboration with Jan-Hung Chen, who also performed immunostaining, while I imaged, analyzed and interpreted the results. The manuscript for this work was mostly written by myself, with contributions from Jan, and subsequent editing by my supervisors. The work was published in *Lab on a Chip* in 2010.

The concept of integrating polyurethane culture substrates into PDMS microdevices (Chapter 6) was originally developed by myself after helpful discussions with Dr. Paul Santerre. Yoan Kagoma, an undergraduate student under my supervision, independently worked out the fabrication methodology for polyurethane films, and we worked together on integrating them
into the PDMS microfabrication process. Two other undergraduate students under my supervision worked on subsequent aspects of this project: Bogdan Beca characterized spreading on the different substrates; and Rachel Tonelli-Zasarsky conducted the microfluidic shear adhesion studies. I designed and directed the experiments and interpretation of results; and Rachel, Bogdan and I worked together on data analysis. Dr. Jian Wang from the department of Dentistry conducted SEM imaging of the films. The manuscript was written by myself with subsequent editing by my supervisors, and published in *Biomaterials* in 2009.

Development of the engineered composite diaphragms (in Chapter 7), and the bulging method to apply strains to cells was originally my idea, and begun by Bogdan Beca, under my supervision. Cameron Lam, also an undergraduate student, took on this project in 2009, and was entirely responsible for identifying and developing the analytical model to characterize surface strains, assisted in device design, and successfully tuned and mastered the fabrication process. All devices used in this study were prepared by Cam. At this stage, I was primarily responsible for device design and characterization. Dr. Morakot Likhitpanichkul, a postdoctoral fellow in the Simmons lab, originally developed the idea to apply mechanical forces to spatially distinct cell populations, and all biological experiments were performed in collaboration with her. Morakot was also responsible for the cell isolation, culture, and image analysis on the devices. We are presently working together on the manuscript for submission to a journal.

The three-dimensional mechanical stimulation systems described in Chapter 8 is work done primarily by myself and by Gong Hao (Billy) Wang, an undergraduate student I supervised. I designed, fabricated and characterized the culture system, and also conducted all the experiments and simulations presented. Billy optimized the biomaterial system and developed the polymerization protocol required to maintain cell viability in micropatterned poly(ethylene glycol) microconstructs. Wen Li Kelly Chen provided assistance and training for Billy in culture of the C3H10T1/2 mesenchymal stem cell line for viability studies. Some of the solutions developed in this project were the result of very helpful discussions with Dr. Morakot Likhitpanichkul (Simmons lab) and Dr. Mohamed Abdelgawad (Sun lab). The manuscript was written by myself, with subsequent editing by my supervisors, and published in *Biomaterials* in 2010. In separate experiments related to the use of this platform, but not included in this thesis, Sarah Kwon and Dr. William Stanford provided embryonic stem cells, antibodies and guidance
in some of the preliminary experiments performed using this system; and Zahra Mirzaei conducted the immunostaining procedures for these devices.

Lastly, work presented in Chapters 4, 5 and 8 garnered interest from the *Journal of Visualized Experiments*, which aims to produce video protocols for various experimental techniques. Working with their team of videographers and editors, we developed a script highlighting the critical and unusual parts of the fabrication process, and filmed the procedure to fabricate these devices. The video protocol is currently in press and will appear in 2010.
Chapter 2

“You must feel the force around you… everywhere, yes.” -- J.M.Y.

2 Background and Literature Review

2.1 Introduction

The mechanical behaviour of biological cells dynamically changes in response to chemical stimulation\cite{15}, mechanical forces\cite{16} and cell to cell contacts\cite{17}. The cytoskeleton provides mechanical support to the cell, reflects cell phenotype\cite{18-20}, has been shown to play a pivotal role in transducing mechanical stimuli in the environment\cite{21}, and may be a critical determinant of cell fate and function\cite{22, 23}. The cytoskeleton is instrumental in regulating processes including programmed cell death\cite{24}; differentiation\cite{9}; adhesion, polarity, contractility and migration\cite{25, 26}; and gene uptake, protein expression, secretion and metabolic activity\cite{27, 28}. Furthermore, disease progression can be dependent on alteration of the cytoskeleton: metastatic cancer cells become more compliant to facilitate motility\cite{29, 30}; red blood cells in patients infected with the malaria parasite become stiff and more adherent, preventing re-entry into the liver\cite{31}; and red blood cells in patients with sickle cell anemia have difficulty flowing in capillaries, causing circulatory problems\cite{32, 33}. Hence, the importance of recognizing the cell as being a mechanical entity, both in terms of its behaviour in the surrounding environment, and in being responsive to surrounding mechanical conditions is becoming more apparent in various biological studies.

This increasing recognition of the critical role played by mechanics in regulating biological form and function has given rise to two distinct areas of research: biomechanics and mechanobiology\cite{34, 35}. *Biomechanics* is “the application of the principles of mechanics to study living organisms and their components”\cite{34}. The application of biomechanics at the cellular scale continues to be ripe with challenges and opportunities. This includes both understanding the intrinsic mechanical properties of a cell, and determining how the cell mechanically interacts with the external microenvironment. Mechanical behaviour of the cell is quite complex, and cannot be isolated to a specific structural component, but rather arises from the collective mechanical interactions of multiple filamentous proteins\cite{22}. The mechanical
properties of cells reflect cell phenotype, and can be used as a marker for disease progression. Adhesion, migration and contractility of cells are mechanically-oriented processes through which cells manipulate and remodel the environment, and are hence of critical importance in wound healing, progression of certain diseases and developmental biology. Thus, cytoskeletal changes may indicate or even trigger pathogenic responses, and the ability to probe the mechanical nature of the cell could provide insight into how cells receive and integrate regulatory signals from the surrounding environment.

In contrast to biomechanics, mechanobiology is “the application or analysis of the role of mechanical forces in eliciting a molecular response, leading to a quantifiable change in form and/or function.” The importance and influence of environmental mechanical conditions on cell fate and function has been thoroughly established and is the subject of multiple reviews. Mechanobiology is a key component in pathobiology; development and morphogenesis; and in many specialized tissues such as skeletal bone, tendon, heart valves, intervertebral disc and cartilage. The ability to precisely manipulate the mechanical microenvironment to understand the mechanisms and processes by which mechanical forces regulate cell function will also require novel experimental platforms and techniques.

Microfabricated technologies can provide viable solutions to some of the problems associated with understanding both cell biomechanics and mechanobiology. Applications of these microtechnologies in this field have prompted a few recent reviews on the subject. In this chapter, we aim to provide a categorical summary of how emerging microfabricated technologies can be used to study various aspects of biomechanics and mechanobiology; and to critically appraise these technologies in terms of their advantages and disadvantages over more conventional macroscale techniques.

2.2 Advantages at the Microscale

Microfabricated systems yield a number of advantages that are applicable in working with cells. First and foremost, microfabricated features can span the range of sub-cellular (<1 μm) to tissue (>1 mm) dimensions, making them ideally suited to study a range of biological systems. The ability to fabricate on this length scale enables the development of arrayed structures, which can greatly increase experimental throughput. Given the inherent heterogeneity of biological
systems, the need for higher throughput experimental techniques becomes immediately apparent[55]. This is particularly true when studying single cells, where ensemble averaging of population response can wash out single cell dynamics[56]. Likewise for rare cell populations (such as primary stem cells), in which only a few cells from a mixed population are relevant to the study at hand. Furthermore, the synergistic response of cells to multiple environmental parameters necessitates high-throughput systems to screen for the effects of combinatorial stimuli. Hence, one of the critical advantages and strong attractions of microfabricated systems in biomechanics and mechanobiology is the ability to provide increased throughput to probe more samples under more experimental conditions.

Microfabricated technologies also allow the integration and consolidation of multiple functional components on a robust, single-piece chip. Actuation and sensing mechanisms can be integrated directly onto a single chip, and this concept of having a “lab-on-a-chip” can result in highly portable and versatile miniaturized systems. The reduction of pieces of equipment can substantially improve the usability of an experimental technique, and the ability to fabricate batches of arrays means that microfabricated systems will eventually be low-cost and disposable.

Control of displacements and features at the microscale allows highly accurate control over experimental parameters and conditions. For example, replacement of fluid in a microchannel requires only a few microliters, and laminar flow in the channels ensures that the resident liquid is expelled without contamination of the incoming fluid. This also allows for very rapid switching of experimental culture conditions, enabling the maintenance of a steady-state environment, and highly accurate control of mass transport, both in terms of time and space. The ability to reliably fabricate features at the microscale can also greatly simplify experimental setups and procedures, without relying on sensitive and expensive equipment to control environmental parameters over large areas or volumes. Often, this is a result of favourable physics that arise from operating at the microscale, which can be used to create conditions not possible at larger scales.

Lastly, materials used in microfabricated systems, such as the ubiquitous poly(dimethylsiloxane) (PDMS), are generally biocompatible, and easily integrated with most other techniques and equipment used in traditional wetlabs, including inverted microscopes, fluorescent labeling and
imaging techniques, and autoclave sterilizers, making these technologies relatively simple to incorporate into the standard infrastructure of a wet-lab.

2.3 Measurement and Biomechanics

Microfabricated techniques to measure the mechanical aspects of cell form and function can be broadly categorized as either passive measurement of the mechanical properties of a cell, or measurements of forces actively exerted by cells on their surrounding environments. Both passive and active measurements are strongly dependent on cell type and environment. In this section, we first survey the various microfabricated techniques used to characterize the passive mechanical properties of a cell. Second, we categorize the large number of micro-scale approaches to this problem based on the practical advantages gained by designing and using microtechnologies. We then survey microdevices designed to measure mechanical interactions with the surrounding environment, including adhesion strength and traction forces. In general, microfabricated approaches to these measurement problems can provide substantial improvements in sensing resolutions and throughput, as compared to more conventional macroscale systems.

2.3.1 Mechanical characterization

Biological cells are typically considered to have elastic moduli ranging in magnitude from 1 to 10,000 Pa, and are structurally complex enough that different measurement techniques produce disparate results on the same cell population[57]. The length scales of features fabricated using microtechnologies are particularly well-suited to characterizing single cells (~20 µm in diameter) and improving measurement throughput in a cell population. Further, the actuation and sensing technologies available are promising in terms of increasing sensitivity and resolution by precise application and measurement of forces and deformations.

When a biological cell is modeled as a linear elastic solid, the parameter typically used to describe its mechanical properties is the Young’s modulus, which relates applied stresses and cell deformation. Mechanical characterization given this assumption requires the application of a known force and measurement of a resulting displacement, or vice versa. However, linear elastic solid models are often unable to adequately describe the mechanical behaviour of cells, and so is often characterized using a dynamic frequency-dependent shear modulus. This
modulus is independent of frequency for normal solids, and is linearly proportional to frequency for liquids. The material behaviour of cells is between the characteristics of solids and liquids, displaying a non-linear, frequency-dependent response in shear modulus. This is due to the complicated deformation of various constituents of the mechanical structure supporting the cell. Mathematical models including the liquid drop, viscoelastic, and tensegrity based models developed for single cell biomechanics have been thoroughly reviewed by others [58, 59], but the issue is briefly mentioned here to elucidate some of the complexities that arise in designing systems to characterize these mechanical properties.

The spatial resolution of systems designed to probe cell biomechanics is a critical design parameter, as some techniques such as platen-based compressive measurement assume a biological cell to be a homogenous body and characterize the ‘whole-cell’ deformation in response to an applied force, while others are capable of spatially resolving mechanical properties in different regions of the cell.

2.3.1.1 Traditional tools to characterize cell mechanics

Standard techniques to probe the mechanics of single cells have been previously reviewed[58, 60, 61], and the most common are briefly recapitulated here (schematics in Figure 2-1). Lumped mechanical properties of single cells can be probed by several methods. Microplate compression consists of applying a known force to plates sandwiching an adherent cell, and observing the resulting deflection[62, 63]. Optical stretching[36, 64] involves using opposed laser beams to apply a stretching force to a cell in suspension, and the resulting deformation is observed through a microscope. In micropipette aspiration[65, 66] a controlled suction pressure is applied to a cell via a micropipette, and the resulting distention of the cell into the pipette can be used to calculate the mechanical properties of the cell, using analytical or computational models. Micropipette aspiration is a versatile technique in that it can be conducted on both adherent and suspended cells and spatial resolution can be controlled by changing the pipette diameter, enabling characterization of the whole cell or of sub-cellular cell segments. Magnetic twisting cytometry[67], gradients[68] and bead manipulation in an optical trap[69, 70] can be used to measure sub-cellular material properties, by applying precise forces to beads attached to receptors on the cell membrane, via a magnetic field or a laser-based optical tweezers system. Alternatively, atomic force microscopy[71] can be used to create a “stiffness map” of the cell, by
rastering a vibrating cantilever across the surface and observing the deflection of the cantilever resulting from the applied force.

Figure 2-1. Key experimental techniques in cell biomechanics. (A) Microplate compression; (B) Optical stretching; (C) Electrodeformation; (D) Micropipette aspiration; (E) Use of optical tweezers or magnetic force on a cell-attached bead; (F) Atomic force microscopy

The key limitation to each of these standard techniques is in equipment complexity. Each of the experimental setups can be challenging to operate, and requires skilled and trained operators. Peripheral equipment can be expensive and is rarely portable, making measurements outside a lab environment difficult. These techniques are also typically limited in throughput. Given the heterogeneity inherent in cell biology, and the number of environmental conditions that influence the cytoskeletal structure and the mechanical nature of the cell, high-throughput approaches are necessary to provide a better understanding of mechanical behaviour in various microenvironments. Though an ideal solution to probe single cell mechanics does not yet exist, microfabricated approaches have been designed to alleviate some of these issues.
2.3.1.2 Mechanical characterization of cells using microsystems

We categorize the surveyed microdevice systems by advantages over currently available techniques: increased system integration, reduction in experimental complexity, improved usability, and increases in throughput.

2.3.1.2.1 Component integration and miniaturization

Integrating actuation and sensing components into a single chip can reduce the number of peripheral and external components required to perform a characterization experiment. This could lead to more robust, compact, efficient and accurate systems. To illustrate this point, magnetic bead cytometry is an excellent case study. Functionalized magnetic microspheres are bound to integrins on the cell surface. External magnets can then be used to apply a well-characterized force to the integrins, and to the attached cytoskeleton. Magnetic actuation is particularly appealing because it is a non-contact method. The force at a distance created by a magnetic dipole is inversely proportional to the cube of the distance, and hence small positioning errors in systems in which the magnetic dipole is manually positioned could result in large errors in applied force. De Vries et al., successfully integrated three and four micro magnetic poles on a glass substrate, which conduct magnetic flux into a sharp tip, precisely patterned in relation to each other[72]. This scheme allows for 120 pN of force to be delivered to a magnetic microsphere, and by adding force vectors for each pole, a force can be applied along any vector in two dimensions. Though De Vries et al. did not use this system to conduct mechanical characterization studies, they did manipulate particles within live cells, demonstrating the ability to apply force to particles in a biological system. Hence, this system could conceivably be used to characterize the mechanical properties of cells using a technique similar to magnetic bead cytometry.

Conducting parallel experiments in such a magnetic scheme would be limited, as mechanical characterization would require continuous optical monitoring of the single cell and deforming bead. The requirement for optical observation is a significant barrier to parallelization in most experiments in cell mechanics. The integration of electrical displacement or strain sensors directly into the platform would resolve such issues, and one such approach is to design electrostatically interacting silicon surfaces, across which a small voltage is applied. Measured capacitance between the interlocking comb structures can then be used to infer separation of the
combs. Hence measurement of applied displacements can be automated, increasing throughput of such characterization systems. The interlocking structures can also be used as actuators, by applying a voltage between the combs. This actuation scheme has been employed in a silicon-based MEMS device capable of applying precisely controlled forces to separate two[73] and four[74] adjoining plates. A single cell cultured across both plates would resist the applied uniaxial and biaxial loads, and plate displacement can be tracked visually to characterize viscoelastic cell response (Figure 2-2). Capacitance-based force sensors can be incorporated into this fabrication process to automatically track deformations. A similarly themed device makes use of electrothermal deformation as the actuation strategy, in which electrical current causes heating in a resistive V-shaped suspended silicon structure, attached to a plunger. On heating, the V-shaped actuator bends, and the plunger compresses a cell in a dielectrophoretic trap[75].

Figure 2-2. Silicon-based devices for mechanical characterization of cells. (A) Separated plates across which a cell is cultured (Source: Serrell et al.,[76], with permission from Springer). (B) Biaxial stretching mechanism for a cell cultured in the center of the gap; and (C) the electrostatic comb drive required to actuate such a structure (Source: Scuor et al.,[74] with permission from Springer).

Measurements of cell mechanics using the electrostatic actuation technique were not reported, possibly because of 1) the difficulties in precisely positioning a single cell on a single point over a large area; or 2) the need to maintain cells under conductive liquid media, which leads to current-induced heating, charge shielding, and electrochemical reactions in the media[77]. More
recent finding that the use of high-frequency actuation voltages in electrostatic actuation eliminates electrochemical reactions does suggest that this problem is solvable[78]. However, in spite of demonstrated maintenance of cell viability, the impact of such electric fields on biological function is still to be determined. The thermal actuator developed in[75] was used to compress a cell, but the authors did not provide results for the mechanical characterization. Thermally actuated systems may not cause electrochemical problems in cell culture media, but do have a related disadvantage in terms of maintaining local temperatures, to which cells are exquisitely sensitive[79]. Furthermore, the size of the actuation structure in the electrostatic and electrothermal actuation systems requires a large device area, limiting the scalability and throughput of such systems. In addition, the challenging and expensive silicon fabrication process typically results in a low-device yield, further reducing possibilities for increased throughput and parallelization.

Directly applying electrostatic forces to cells in suspension may be more feasible for mechanical characterization, but analysis is limited to non-adherent cells. The applications of dielectrophoretic forces for biomedical applications such as cell sorting, identification and manipulation are covered in other reviews[77], but this idea was first applied to mechanical characterization in 1984, when Engelhardt et al. characterized the viscoelastic response of erythrocytes[80] placed in a high-frequency electric field. The original experimental setup consisted of two razor blade electrodes immersed in culture media, between which the cell was trapped by the dielectropheretic forces. More recent studies[81, 82] have used more robust approaches with electrodes micropatterned directly onto glass substrates to apply the requisite electric field. However, this method still requires the application of strong electric fields, which may influence cell biology, and which may be further exacerbated by the requirement for ionically conductive media. Additionally, analytical methods to relate raw data (voltage-cell deformation) to the mechanical properties of cells need to be developed.

An alternative approach has been to use an external precision stage to apply a known displacement to the biological sample and using electrostatic combs as capacitative sensors[83] or visually observing lateral cantilever deflection[76, 84-87], to measure the reaction force of the sample. Such systems were used to determine the viscoelastic response of a cell to an applied step displacement. Although this approach does reduce the degree of on-chip integration, the use
of the external motion system provides a strong actuation force over a long stroke length; and eliminates the issue of electrochemical reactions in the culture media.

One key issue that affects all the devices outlined in this section is that of cell positioning. In all the mentioned devices, a single cell has to be placed in a precise location in order to measure their mechanical properties. The use of robotic micromanipulation tools or micropatterning techniques[88-90] may alleviate this issue, but does make the experimental process more complicated and challenging.

2.3.1.2.2 Simplified device operation

Microfabricated structures can be used to either measure or apply biologically relevant forces and displacements, attaining high levels of precisions with a mechanism simpler than a commercial force transducer. Silicon and polymeric cantilevers are particularly useful as force sensors, as deformation can be visually tracked and related back to applied force using a simple analytical equation for cantilever bending.

Integrated sensors to measure post displacement via electrical resistance changes in an attached strain gauge have been developed[91, 92], and these could be utilized in high throughput applications. In current approaches to cell biomechanics, post deformation is more easily measured optically, and microfabricated cantilevers are able to provide the size and force resolution necessary for mechanical characterization of single cells. Though the atomic force microscope (AFM) is based on a similar concept, the ability to customize microfabricated posts has enabled parallelization and probing cell mechanics under various conditions. Sasoglu et al. used a micromanipulator to maneuver an array of PDMS sensing posts designed to measure the stiffness of an array of cells patterned on extracellular matrix-coated spots on a glass substrate. Though they successfully fabricated and characterized a device with suitable accuracy and resolution requirements[93], and have also developed computer methods for parallel analysis of the array[94], experiments probing cell mechanics have been proposed but not performed as yet.

Sun and co-workers leveraged the concept of force-sensing cantilevers to mechanically characterize oocytes during microinjection. A robotically-controlled micromanipulator was used to puncture the cell membrane under displacement control, and deflection of surrounding micropillars was monitored to assess forces required for injection (Figure 2-3). The ability to
produce large arrays of cantilevers enabled the authors to observe previously unknown differences in stiffness between young and old mouse oocytes[95].

![Figure 2-3](image-url)

**Figure 2-3.** System to measure force required to inject a mouse oocyte. (A) Schematic demonstration of cell surrounded by micropillars; Force measurement device from the (B) side) and (C) top view. Posts deflect as force is applied, and tracking post deflection enables the calculation of applied forces required for membrane penetration (Source: Liu et al., [96], with permission from the Institute of Physics Publishing).

### 2.3.1.2.3 Improved Usability and Throughput

Microfabricated systems can also improve the usability and applicability of more standard techniques, by using microstructures to precisely control cell position. For example, optical stretching requires suspended non-adherent cells to be positioned at the correct location between two focused beams of laser light. Guck and coworkers used microfluidics to improve the usability of their technology, and developed a capillary based microfluidic system which integrates fiber optic cables delivering the laser beams to cells positioned by microfluidic flow[97]. The integrated system can measure the viscoelastic signature of 50-100 cells per hour in a rapid, serial process. In this demonstration, the group was able to compare the distribution of deformability between a normal epithelial cell line and a cancerous one, and found significant differences between the two.

Similarly, AFM is commonly used to measure applied forces under load control across a cell surface, but is challenging to use with non-adherent cell populations. Rosenbluth et al. made use of microfabricated wells to physically trap non-adherent leukocytes, for rapid measurement with
The microwell system was able to trap cells in defined locations during the testing process, greatly speeding up measurements. The AFM applies a compressive load at a point, but alternatives exist to apply platen-like compressive loads to cells in culture. On the macroscale, microplate compression techniques are cumbersome and delicate, but microfluidic compressive testing systems require relatively fewer pieces of supporting equipment. These devices consist of a thin suspended layer of PDMS in a multilayer PDMS device, pressurized to come in contact with cells cultured on a surface. Viscoelasticity of the cells under compression can be determined by observing cell deformation under a microscope after a known pressure is applied. Kim et al., used such a device to demonstrate differences in viscoelasticity between cancerous and normal cells.

Micropipette aspiration is another technique which is inherently slow and requires extensive operator training. In order to eliminate the need for a skilled operator, Moraes et al. integrated the micropipette into a microfluidic channel, to allow cells to be localized close to the pipette tip, where a suction pressure draws them towards the pipette tip. The resulting experimental procedure was simple enough to be performed by untrained and minimally supervised undergraduate students as part of a teaching course. The same group has also investigated the use of parallel channels in PDMS for high-throughput micropipette aspiration of suspended cells.

2.3.2 Force Measurement

Microengineered systems to measure the mechanical interactions between cells and the substrate to which they are adhered can substantially improve force and spatial resolution over conventional techniques. In this section we review microfabricated systems to measure cell adhesion and cell-generated traction forces.

2.3.2.1 Adhesion

Traditional techniques used to measure cell adhesion include atomic force microscopy, in which a cantilever tip is used to ‘scrape’ a cell off a substrate, while observing the force required to do so, and an extended micropipette aspiration technique in which a second pipette is used to place a bead in contact with a cell, and pull them apart using suction at various pressures. Adhesion assays can also be conducted in parallel plate flow chamber (PPFC) systems.
which cells are allowed to attach onto a substrate, and a shear profile is applied to determine the fraction of cells remaining on the substrate at each shear level. The low throughput of these techniques limits the practical use of these tools in understanding the effects of multiple environmental parameters on cell adhesion.

Microfabricated approaches provide higher throughput by applying precisely controlled shear forces to detach cells from a substrate within a microfabricated channel. Work in the Griffiths and Jensen labs demonstrated a shear device that enables both short- and long-term culture of cells in PDMS microchannels, with separated fluid channels to perfuse nutrients and to apply shear stresses to the cells in culture. They determined the fraction of cells remaining adhered as a function of time and shear magnitude, and demonstrated differences in adhesion between cells cultured with and without epidermal growth factor. By manipulating the width of fluidic channels on the same chip, the group was able to use a single pressure differential to create a variety of shear magnitudes in different regions of the device[108]. Young et al., used a simpler device with uniform channels, but leveraged the ability to increase experimental throughput to conduct experiments involving eight culture conditions simultaneously on a single chip. They compared adhesion strengths of closely related but functionally distinctive vascular and valvular endothelial cells, on glass substrates coated with six different concentrations of the extracellular matrix proteins fibronectin and collagen, demonstrating matrix and cell-type dependent adhesion patterns[109].

Adhesion differences between cell types can also be maximized by manipulating the physical features of the substrate on which cells are grown. Kwon et al. demonstrated the use of nanotopographically patterned substrates integrated in microfluidic shear channels to selectively control adhesion of cancerous cells[110]. More detailed studies of detachment in microchannels have also been conducted, including measurement of cell-substrate contact area over time and under shear; fluorescence-based analysis of focal adhesion formation; and development of models to calculate the adhesive stresses exerted at each point of contact[111, 112].

The concept of using the fraction of cells remaining under shear as a defining feature for the subpopulation has also been exploited to enrich rare cell populations by depleting unwanted, adherent cells[113, 114]. Widening single channels designed to produce a linearly increasing shear stress profile along the channel length, clearly show the shear level at which cell
detachment occurs[115]. More complex devices operating on the same basic principles have been developed by Guiterrez and Groisman, who integrated valves into the microfluidic chip, enabling greater precision and automated control of applied adhesion assays[116].

Figure 2-4. Microfluidic devices to test adhesion of cells to various substrates. (A) Varying channel dimensions enables multiple shear forces to be applied simultaneously on a single chip (Source: Lu et al.,[108] with permission from American Chemical Society). (B) Use of multiple flow channels enables higher-throughput testing of adhesion on multiple matrix protein coatings (Source: Young et al., [109] – reproduced by permission of The Royal Society of Chemistry). (C) Channels with varying shear stress profiles along each channel (Source: Gutierrez et al., [116], with permission from the American Chemical Society); and (D) logarithmic design used to apply a linearly increasing shear stress along the microchannel length (Source: Plouffe et al., [115], with permission from the American Chemical Society).
2.3.2.2 Traction Forces

Traction forces are typically measured by examining deformations of the surrounding microenvironment. The wrinkling of thin, compliant films in response to cells exerting traction forces at the surface can be used to determine cell traction force fields[117]. Automated image tracking procedures can be used to track deformation fields for textured materials[118] or to image the displacement of embedded fiduciary markers in the underlying substrate[119], which can then be deconvolved to calculate traction forces.

Deconvolving displacement vector fields to calculate applied forces is a computationally intensive process. More critically however, each of these techniques are limited to relatively soft systems, where it is difficult to isolate the effects of substrate stiffness (and hence cell spreading) on cell behaviour. Microfabricated silicon cantilevers were able to partially address these issues. Cells cultured on separated adhesive pads exert mechanical forces as they migrate, which bends the silicon cantilever along one direction. Tracking cantilever deflection allowed the use of simple analytical models to calculate traction forces, and altering cantilever size provided force sensors of different stiffness[120, 121]. This approach was extended to a two-dimensional substrate by Chen and coworkers, who developed a bed of polymer microneedles, on top of which cells were cultured. Microneedle deflection in response to cell-generated traction forces were monitored, and a simple analytical model (section 3.1.2 of this chapter) can be used to determine the forces required for those deformations, providing a computationally simple and spatially-defined ‘map’ of force generation[122] (Figure 2-5). Matrix proteins patterned on the micropost tips can also be used to test cell traction forces under various matrix protein stimuli, limit cell adhesion to specific areas, and study traction forces arising from these decoupled parameters. To improve the usability of this system, image processing techniques to improve the accuracy and speed of microneedle displacement measurements have been developed[123]. Several novel biological mechanisms have been identified using this system of force measurement, and have been extensively covered in recent reviews[52].

The microfabricated polymer cantilever approach has been recently extended to cells cultured in a three-dimensional culture system. Legant et al. developed a modified micropost system, embedded within a polymerizable cell-laden hydrogel biomaterial. As cells in the hydrogel exert contractile forces and the matrix undergoes deformation, the force with which the hydrogel
contracts can be measured by deflection of the posts[124](Figure 2-5). Interestingly, the group found that the force of contraction increased with stiffness of the post. This suggests that cells ‘feel’ the increased stiffness of the posts by transmission of mechanical stiffness through the gel, and undergo increased contractility as a result of the change of stiffness.

Figure 2-5. Microfabricated devices to measure cell-generated traction forces. (A) Cells cultured on an array of polymeric microneedles deflect the needles. Deformation is tracked to create a ‘map’ of cellular traction forces (Source: adapted from Tan et al., 2003 [122], with permission from the National Academy of Sciences). (B) Cells cultured in microfabricated collagen gels generates tugging forces which can then be monitored via post deflection (Source: Legant et al., 2009 [124], with permission from the National Academy of Sciences).
This behaviour suggests that the line drawn between cell biomechanics and cell mechanobiology is not quite clear. There exists a complicated and exquisitely sensitive feedback mechanism between internal forces generated by the cell, and forces applied to the cell[125]. Hence, although the experiments described in this section are designed to merely measure passive cytoskeletal mechanics and the active forces generated, the forces required to probe the system could change the results, further emphasizing the need for multiple measurements in a high-throughput format.

2.4 Manipulating the Mechanical Microenvironment

In this section, we survey microengineered approaches (schematics in Figure 2-6) to manipulating mechanical cues in the cellular environment, in order to: 1) control the microenvironment in ways that are not possible with conventional technologies; and 2) use high-throughput systems to study the effects of parametric or combinatorial mechanobiological stimuli on cell function. Surveyed technologies are categorized based on the engineering approach taken to manipulating the mechanical cues. These include spatially-defined surface features; systems to apply stimulation to cells by direct manipulation of liquids; and systems that interact with cells using forces applied through a solid structure. Where relevant, macroscale systems that produce similar mechanical environments are briefly reviewed, to emphasize the benefits and drawbacks of using microfabricated approaches for these specific purposes.
Figure 2-6. Key experimental techniques in cell mechanobiology: (A) adhesion patterning; (B) surface topography; (C) Fluid shear; (D) Hydrostatic compression; (E) Substrate deformation.

2.4.1 Engineering surfaces

Critical mechanobiological cues can be provided by designing the interface between culture materials and the cell itself. Surfaces can be engineered with natural or synthetic matrix proteins to control cell adhesion with sub-cellular resolution in length, enabling fundamental studies of the relationships between mechanical cell spreading area, matrix composition and cell fate and function. The surface topography is also a critical component, and both micro- and nano-patterned surfaces play a key role in modulating cell behaviour. The development of model systems for these studies would not be possible without microfabricated approaches.

2.4.1.1 Spatial Control of Adhesion

A variety of techniques are available to pattern two-dimensional culture surfaces, restricting cell attachment to specific regions (reviewed elsewhere[88, 126]). Briefly, adhesive proteins can be spatially patterned on a substrate using a number of different techniques, and the remaining areas are rendered non-adhesive to cell attachment using a suitable chemical or physical method. One of the most commonly used techniques to precisely pattern protein features on a surface is microcontact printing[127, 128], in which a PDMS stamp with microfabricated features is used to transfer patterns of proteins onto the desired substrate, much like a carved-potato stamp.
Alternatively, a PDMS stencil can be fabricated with through-holes at the regions to be patterned. Adhesive proteins deposited on top of the stencil come in contact with the underlying substrate only at specific regions. Removal of the stencil results in the formation of a pattern of adhesive proteins or cells[129]. Alternatively, this method can be used to selectively activate the surface by plasma treatment, before subsequent deposition of the matrix proteins and blocking agents [130, 131]. Removable microfluidic channels can also be used to deliver adhesive molecules to specific regions on a substrate[132]. These techniques enable the study of cells that are mechanically confined to specific regions or allowed to spread; cells that are shaped such that they generate different cytoskeletal tension fields in different parts of a pattern. These techniques can also be coupled with other fabrication paradigms to create more complex microenvironments, including substrate-bound protein gradients[133] and designing temporally-manipulated matrix environments[134, 135] with electrically programmable adhesive surfaces[136]. Such approaches are promising in their ability to determine the temporal aspect of mechanobiology, but have not yet been used to directly manipulate the mechanical microenvironment.

Controlling available cell spreading area results in the mechanical structure of the cell being altered. Cells patterned on sub-cellular adhesive regions remained balled up on the substrate, while cells patterned on larger adhesive regions spread out over the available area. This geometric control of cell shape was found to regulate life and death: a large percentage of cells that were not allowed to spread underwent apoptosis, while spread cells maintained healthy phenotypes[137]. Regulation of cell spreading area was found to play a critical role in cell differentiation. Constrained mesenchymal stem cells (MSCs) differentiate to adipocytes, while MSCs that were allowed to spread underwent osteogenesis[9](Figure 2-7).
Figure 2-7. Control of cell adhesion area directs stem cell differentiation (Source: adapted from McBeath et al., 2004 [9], with permission from Elsevier). (A) Cells cultured on patterns of increasing size cause (B) increases in osteogenic or adipogenic differentiation, independent of chemical stimulation.

Pattern shape has also been shown to have a substantial impact on how cells function. Cells patterned on square islands have been shown to develop increased traction forces, as opposed to cells patterned on round islands[138], demonstrating oriented control of cytoskeletal tension. Cell orientation also direct lamellipodia extension[139] and migration[140], as cells migrate towards the blunt end of a tear-drop shaped pattern[141]. Differentiation of stem cells is also related to cytoskeletal tension, as demonstrated in a study by Ruiz et al., in which MSCs at the edge of multicellular islands undergo osteogenic differentiation, while those in the center become adipocytes[142]. More recently, Kilian et al. have demonstrated this phenomenon in single cells: high-aspect ratio patterns increased acto-myosin contractility in the cell, and promoted osteogenic MSC differentiation, whereas more rounded patterns form fewer stress fibers and differentiate towards an adipocyte lineage[143]. Three-dimensional control of cell adhesion and spreading within a homogenous material remains to be attained, but shaped microwells have been successfully used to manipulate cell geometries[144].

The development of sub-cellular micropatterning techniques has enabled precise control over mechanical constraints applied to cells, and has resulted in a substantially better understanding of
how cells behave in various environments. Hence, controlling adhesion on two-dimensional substrates is an indispensable tool in cell mechanobiology.

2.4.1.2 Physical Topography

Cells are able to sense physical topography at a number of different scales: curvatures in the underlying substrate[145], micro-scaled ridges and grooves[146], nanoscale topographies[147], and anisotropic gradients in topography[148]. The effects of physical topography have been shown to be more influential on cell alignment and function than patterned chemical cues[149], and have been shown to better recapitulate in vivo cell behaviour[150, 151]. These substantial mechanobiological effects on cell adhesion, alignment and migration[152] have been well-established since the 1990s[146], particularly on substrates fabricated with micropatterned topographical grooves of varying heights and widths. As such, the standard techniques and methods are not reviewed in detail here, but interested readers are referred to a number of relevant reviews on the subject[52, 153, 154]. Recent novel contributions to this existing field include the use of a PDMS platform to produce a substrate with reconfigurable microtopographies. Compression of a PDMS substrate results in 550-800nm high features, spaced ~6µm apart, and cells repeatably switched orientations in response to the applied topographical cues[155]. Other recent studies have shown that nanoscale topographies have a profound influence on cell function. MSCs differentiate to osteoblasts under the influence of nanopatterned substrates, without osteogenic components in the nutrient media[156]. Cell geometry, action potential conduction velocity and cell-to-cell coupling in nanopatterned cardiac tissue constructs are extraordinarily sensitive to the underlying patterns[157]. Likewise, neurons are able to sense nanometer-scale roughness[158]. Thus, both micro- and nano-topographies can play important roles in cellular response to the mechanical microenvironment, and can be eventually used to manipulate migration and matrix production in tissue engineering applications.

2.4.2 Controlling fluids

Fluid flow is a defining component of the in vivo mechanical environment, and is particularly relevant to endothelial cells[159], which are exposed to both continuous and pulsatile shear stresses in vivo. Fluid shear stress plays a critical role in development and differentiation, and a large number of temporal and spatial shear stress patterns and magnitudes exist in vivo[45, 160].
Hydrostatic pressures are also known to play a role in cell biology, particularly in cartilage[8] and ocular tissues[43]. Macroscale equipment used to apply fluid shear stress, such as parallel plate flow chambers and cone-and-plate systems are reviewed elsewhere, as are systems to apply hydrostatic forces to cells in culture[13]. These macroscale setups tend to bulky, consume large volumes of reagents and are relatively low in throughput. Miniaturizing these systems allows for greater throughput in creating multiple experimental conditions, device usability and portability, and precise control and uniformity in applied stimulation stresses.

2.4.2.1 Shear Stress

The use of artificial microfabricated channels is a suitable approach to mimic many in vivo environments as the reduction in scale enables well-controlled laminar flow in the channels. Simple PDMS channels can be used in combination with passive pumping[161], pressure-driven flows or syringe pumps to apply shear to cultured cells. For example, Higgins et al. used a single microfluidic channel to study the behaviour of sickle-type red blood cells in a physiologically relevant environment[33]. Pressure (gravity)-driven flow was used to drive the defective red blood cells through a channel, to study the effects of geometric, physical, and biological factors in vascular occlusion and rescue.

Various design considerations need to be factored into scaling such simple channel systems up for higher-throughput studies, and one of the key criterion is the method for driving fluid flow through the system. External connections to various devices can often hinder scalability, and passive pumping is one technique which does not require these external connectors. In passive pumping, surface tension differences between droplets of different sizes at either end of a microfluidic channel drive fluid flow[161]. Although passive pumping has not yet been used to apply physiologically relevant shear stresses to cells, the authors suggest that this is one possible application of the technique[162]. Beebe and coworkers have used this principle to demonstrate an automated high-throughput microfluidic system, in which a robotic system deposits and removes droplets across an array of microfluidic channels[162]. Syringe pump and pressure-driven flows are harder to implement in high-throughput systems, but serve adequately for devices designed for lower-throughput experiments. Careful design of the microfluidic channels can be used to maintain increased throughput while minimizing the world-to-chip interface connection issues that arise: connections are typical sources of device failure. Channels with
varying widths connected to a single fluid delivery source can be used to generate a range of fluid velocities, and hence applied shear stresses across a single device[108]. Channel bends and curves can also be used to apply spatially distinctive shear stresses[163]. Carefully designed channels of increasing width can also be used to apply linearly increasing shear stresses across a single channel[164].

More complicated technologies have also been developed to further miniaturize such systems. The development of the ‘Quake valve’[99] enabled the large-scale integration of multiplexed microfluidic valves on a single chip[165]. Using a multilayered PDMS microfluidic system, the valves consist of a pressure control channel which deforms thin PDMS films to block flow in fluidic channels – much like stepping on a garden hose. The valves can be used to direct fluid flow or drive it by operating as a peristaltic pump. Using these valves, an automated, high-throughput microfluidic cell culture system was developed, in which 96 culture chambers could be individually addressed[7]. Such systems have not as yet been used to explicitly explore the effects of shear stress on cultured cells, but can do so in principle.

Use of the Quake system of valves still requires an undesirably large number of world-to-chip interfaces. Technology developed in the Takayama lab may address this concern: commercially available Braille displays are used to deform the base of a flexible microfluidic channel[166]. Pin actuation can be independently and automatically controlled to manipulate fluid within the microchannels. Although it is not as scalable, it is simpler to implement and has been used to apply shear stress to endothelial cells in culture[167].

Microengineered technologies for fluid shear can also improve functionality in a variety of ways. In situ measurements of shear stresses can be made using MEMS-based ‘hair’ sensors, incorporated directly into the shear channels[92]. Direct readouts can also be integrated into the microfluidic devices – for example, Tolan et al. have developed an integrated luminescence detection system in which fluorescent reagents react with erythrocyte lysates to simultaneously monitor various biochemicals produced by erythrocytes under shear in underlying channels[168].

### 2.4.2.2 Hydrostatic Pressure

Classification of hydrostatic pressure as “mechanical” stimulation is somewhat contentious: increases in external pressure cause increases in internal pressure, presumably resulting in no net
cell deformation. Changes in cell function may instead be due to differences in gas solubility at different pressures, if gas concentrations are not controlled independently of the applied pressure. The only presently reported microfabricated system designed to apply hydrostatic pressures to cells was developed by Sim et al., who used a single pressure source to create a range of deflections in suspended PDMS membranes of various diameters. The differing deflections cause different pressures in isolated culture chambers, enabling the high-throughput evaluation of MSC response to a range of hydrostatic pressures[169].

2.4.3 Solid deformation

Mechanical cues presented to cells by way of deformations of the external environment are critical factors in cell regulation. Depending on the stiffness of the surrounding matrix, cell-generated traction forces generate large or small deflections, which are then sensed and dictate cell fate and function[170]. Similarly, cells sense deformations in their surroundings caused by externally applied deformations, and respond accordingly[8, 13].

2.4.3.1 Substrate stiffness

Substrate (two-dimensional) and matrix (three-dimensional) stiffness is generally manipulated by means of differentially crosslinked polymer substrates. Polyacrylamide (PA) systems are perhaps the best established biomaterial substrate for this purpose, and was first used in the 1990s to study cell locomotion and focal adhesion formation as a function of substrate stiffness[171]. Engler et al. more recently used PA gels to show that substrate stiffness directs stem cell lineage differentiation. MSCs differentially displayed neurogenic, myogenic and osteogenic differentiation on substrates with modulus increasing from 0.1 to 40 kPa[172]. More recently, poly(ethylene) glycol (PEG) has shown promise as a “blank slate” fully customizable matrix material with a considerable range of formulation-dependent mechanical modulus values[173]. PEG hydrogels are particularly interesting in terms of microfabrication, as they have been integrated into microfluidic devices using molding[174] and photopatterning[175-177] techniques. Direct molding of PEG hydrogels has resulted in a high-throughput combinatorial screening system for stiffness, adhesive ligands and chemical cues[178, 179].

In addition to the possible increases in throughput due to miniaturization, two further advantages are gained in microfabricated environments to study substrate stiffness. First, micropatterned
mechanical gradients can be defined in both two-[180-182] and three-dimensional[183] culture systems to study the effects of non-uniform substrate stiffness. Photopolymerizable hydrogels with varying concentrations of crosslinker are flushed into a microfluidic channel, and a standard gradient generator channel scheme and UV lamp are used to create polymerizable hydrogel structures with a stiffness gradient[153, 180], in order to study adhesion, spreading and migration in complex environments.

Second, when working on the length scale of tens of microns, spacing between hydrogel surfaces and adhesive structures can be used to modulate effective stiffness experienced by cells. Though not ostentatiously a “microfabricated device”, Yip et al. report a culture technique by which cells cultured on thick (~1mm) collagen gels experience lower stiffness than thin (~10 μm) gels firmly attached to a glass coverslip[184]. As is the case with the fictional princess who is able to feel a hard pea beneath several mattresses[185], cells ‘feel’ the stiffer substrate through the thin, compliant hydrogel. This effect is also seen in the microfabricated three-dimensional culture platform developed by Legant et al. (previously reviewed in 2.3.2.2), in which PDMS posts of different dimensions anchor a cell-laden collagen hydrogel. Cell contractility is influenced by posts of different dimensions, as cells experience the differing stiffness through the matrix material[124]. This approach to manipulating mechanical stiffness avoids complicating factors in changing concentrations of crosslinking agents, such as an increase in adhesion sites with increasing crosslink density.

2.4.3.2 Externally Applied Strains

Although in vivo mechanical strain modes are quite complex, the effects and underlying mechanisms can be studied using simplified in vitro models. Strains can be applied to cells cultured on substrates by uniaxial, biaxial, equibiaxial, compressive and tensile loading, in both two- and three-dimensional materials[186]. Cells are sensitive to strain magnitude[187], applied strain field[188] and stimulation frequency[189]; and response to these mechanical parameters are modulated by the other features of the microenvironment. Current platforms for the mechanical stimulation of cells[13] are generally limited in throughput, and are hence unable to probe combinations of mechanobiological parameters. The most commonly used commercially available systems for such stimulation are produced by the FlexCell Corporation, in which 6-well plates with deformable, elastomeric bases are positioned over a loading post. Vacuum is applied
beneath the substrates, and the cultured cells are stretched over the loading post. The design is quite versatile in that different strain fields can be applied by changing the shape of the loading post, and the size of the wells enables stimulation of a large amount of biological material. This allows the use of a wide variety of biological techniques to assay for cell response to strain, but makes these systems impractical for studying rare or limited cell populations. The systems is also limited to 24 samples, each undergoing the same mechanical strains; and hence screening for the effects of a range of mechanical forces is impractical. Yet, the Flexcell systems remain the most popular commercial platforms with which to apply mechanical strains to cultured cells.

A few microfabricated systems have been developed for these applications. Kurpinski et al. [190] and Wang et al. [191] aligned MSCs and fibroblasts along topographically patterned stretchable substrates, before applying uniaxial strains in a macroscale bioreactor. They demonstrated that mechanical stimulation influences gene expression, protein expression and proliferation differentially, dependent on the direction of strain to the aligned patterns. Tan et al. used a similar topographical patterning approach to align cells along specific orientations, but applied a pressure differential across circular patterned diaphragms[192]. The pressure differential caused the diaphragm to bulge, creating non-uniform anisotropic biaxial strains in different regions of the device. Gopalan et al. followed a similar approach, except the diaphragms were distended by a loading post in a manually actuated screw-type system[193]. Leduc and coworkers extended this idea and created a system in which thin films containing embedded microfluidic channels are strained by bulging, enabling the culture of cells in microfluidically controlled mechanically dynamic environments[194, 195].

Each of these systems is relatively limited in throughput. To address this, Takayama and coworkers used a commercial Braille display system to apply non-uniform substrate deformations to cells cultured on thin films. The films were distended by the hemispherical-headed pin, applying non-uniform strains. Independent control over individual pins across the array enabled simultaneous screening of cyclic loading frequencies ranging from 0.2 to 5 Hz, and differences were found in degree of alignment of various cell types in response to frequency and stimulation duration[189].

However, no system currently exists to apply well-characterized uniform strains to cultured cells, in a high-throughput manner. Similarly, no system is capable of screening for a variety of
culture parameters in combination with mechanical stimulation. Lastly, no system exists in which mechanical forces can be applied to cells cultured in a three-dimensional environment in a high-throughput manner. Addressing these limitations is the focus of this thesis, and required simultaneous development in fabrication techniques, materials design and device design.

Figure 2-8. Microfabricated systems for mechanical stimulation of cells by substrate deformation. (A) A microtextured substrate is used to align cells, and then apply strains to oriented cells using a macroscale bioreactor system (Source: Kurpinski et al., 2006 [190], with permission from the National Academy of Sciences). (B) A Braille display integrated into microfabricated well system, in which the displacement pin creates biaxial strains on the cell surface (Source: Kamotani et al., 2008 [189], with permission from Elsevier).

2.5 Discussion and Conclusions

The development and use of microfabricated tools for experiments in biomechanics and mechanobiology can have a profound impact on understanding the relationship between mechanics and cellular form and function. Increases in experimental throughput and
Experimental simplicity can substantially improve our understanding of rare cell populations, and how these cells respond to varied parameters. Microfabrication also allows designers to combine multiple stimulation and measurement techniques. Sniadecki et al. linked cell biomechanics and mechanobiology in developing a system designed to measure traction forces in response to an externally applied point deformation[196]. Combinatorial stimulation with a variety of mechanical cues is also possible. To date, this has included combining topography with shear[197] and with substrate strain[190, 192, 193, 198]. Work in evaluating combinatorial sensitivity of cells to other forms of mechanical cues is ongoing.

However, recent findings have suggested that the use of certain microfabricated systems in studying biological cells may have under-appreciated and substantial side-effects. Beebe and coworkers have determined that PDMS, used ubiquitously in microfabricated devices, sequesters small bioactive molecules and releases small chain polymers into the surrounding media, which is then incorporated into the cell membrane[199, 200]. This suggests that alternative techniques such as hot embossing, to create microfluidic channels using generally accepted materials for cell culture, such as polystyrene would be a more acceptable approach. Alternatively, others have investigated using coating films of polyurethane on PDMS materials, to provide cell adhesion sites and improve biological compatibility[201]. In general, better characterization of the effects of microdevice materials and cell culture techniques on biological function is needed, before such techniques can be broadly adopted into mainstream wetlabs.

There is also a substantial divide to cross in terms of expertise: device design, fabrication and validation require specific and detailed skill sets, and it is difficult for experts in microdevice design to thoroughly understand the relevant biology, and vice versa. Devices can often be complicated to operate in practice, and are frequently unable to provide a reliable platform to study biological systems. Simplifying device designs may aid in solving these issues of usability, but more generally, bridging the gap between microdevice engineers and cell biologists will require truly interdisciplinary collaborations, or integrative thinkers in both areas developing tools to understand and answer specific biological questions. However, the promise of such techniques is powerful, and successful research programs integrating these disciplines will result in new insights and advances in both.
Chapter 3

3 Thesis Objectives

There exists great potential in using microfabricated systems to study the influence of mechanical forces on cell fate and function. The ability to conduct massively parallel, combinatorial manipulation of mechanobiological factors in the culture environment can provide insight into how cells integrate the regulatory stimuli presented to them, and identify exploratory avenues for research in cell biology. However, aside from some notable exceptions, the use of microfabricated devices is largely limited to those with expertise in this field. Hence, there exists a divide between technology development and practical applications in scientific discovery and innovation. A strong impetus for translation of these approaches from the engineers’ domain to mainstream use in a cell biology lab will be demonstrations of practical use of these platforms in identifying novel biological findings that would be extremely challenging to observe using conventional technologies.

Specifically in the domain of applying mechanical forces to cells in culture, this thesis focuses on (1) developing microfabricated platforms to systematically apply dynamic mechanical stimulation to cells in culture in a higher-throughput manner than is currently possible; and (2) using these platforms to obtain novel biological insight into the influence of microenvironmental mechanobiological parameters on cell function. In order to attain these goals, a number of technological advances are required, including the ability to fabricate precisely-aligned arrays of multilayer structures; and the ability to design microfabrication-compatible materials to maintain cell adhesion under dynamic mechanical load. Hence, the objectives of this thesis are as follows:

I. To develop techniques to fabricate large arrays of multilayer structures
   a. Demonstrate the advantages of such techniques in making large, dense arrays of well-aligned microstructures.
   b. Test these fabrication techniques in developing a platform for mechanical stimulation of cells in two- or three-dimensional culture.
II. To study the influence of strain magnitude in regulating cell function

a. Design, test and characterize a microfabricated system to simultaneously apply a range of uniform equibiaxial strains to cells cultured on an array.

b. Utilize the system to investigate the influence of mechanical strain magnitude in regulating β-catenin translocation into the nucleus of valve interstitial cells (VICs).

c. Identify critical improvements that need to be made before moving towards a useful array-based combinatorial screening platform.

III. To investigate the feasibility of incorporating alternative materials into the conventional soft lithography process to improve cell adhesion

a. Develop techniques to integrate polyurethane into the soft lithography fabrication process.

b. Compare primary cell adhesion-related properties of these hybrid material devices.

IV. To study the influence of combinatorial mechanobiological parameters in regulating cell function

a. Design, test and characterize composite material culture films intended to improve cell adhesion and maintain mechanical properties of microfabricated PDMS films under cyclic load in long-term culture.

b. Develop a practical, microfabricated screening platform capable of systematically manipulating mechanical, chemical and matrix cues presented to adherent cells.

c. Utilize this technology to study the influence of these parameters on myofibroblast differentiation of cells isolated from different layers of the aortic heart valve leaflet.
V. To study cells under mechanical stimulation in environments that better mimic in vivo situations.

a. Design, test and characterize a microfabricated platform to apply mechanical stimulation to cells cultured in three-dimensional microenvironments.

b. Investigate the relationship between biomaterial deformation and cell deformation in the biomaterial constructs.

Throughout the thesis, the advantages and disadvantages of using such microfabricated approaches in probing the outlined problems will be emphasized, in the hopes of making a relevant case for the future use of these systems in mainstream biological studies.
Chapter 4

“… in which we realize that a good squeeze might be all that we desire.”

4 Developing fabrication capabilities

Development of complex three-dimensional structures envisioned for the precise application of mechanical forces to cells cultured on a substrate (as in Chapter 5), requires specialized fabrication techniques. The need to culture cells on a deforming substrate immediately suggests the use of poly(dimethylsiloxane) or PDMS, a curable, transparent and biocompatible elastomer used ubiquitously in microfabricated systems. However, shrinkage of PDMS complicates alignment registration between layers during multilayer fabrication. This often hinders development of large-scale microfabricated arrayed devices. Here, a rapid method to construct large-area, multilayered devices with stringent alignment requirements is reported. This technique, which exploits a previously unrecognized aspect of sandwich molding, or ‘squeeze’ fabrication, improves device yield, enables accurate alignment over large areas of multilayered devices, and does not require strict regulation of fabrication conditions or extensive calibration processes. To demonstrate this technique, a microfabricated base platform for the mechanical stimulation of cells was developed and characterized. High device yield and accurate alignment within 15 µm were achieved over three layers for an array of 108 device units spread over a 6.5 cm² area, demonstrating fabrication of well-aligned devices with greater ease and efficiency than previously possible.

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1 Work presented in this chapter was published in the following journals:


4.1 Introduction

Multilayer soft lithography (MSL) is a fabrication paradigm in which layers of patterned elastomeric polymers are aligned, stacked and bonded to create complex three-dimensional monolithic structures. The ability to fabricate three-dimensional microstructures enabled the fabrication of complex microfluidic control components [99, 204-210], which in turn have enabled the consolidation of various experimental techniques in biology, chemistry and materials synthesis onto a single microfabricated platform [7, 211-214].

The most commonly used structural material in developing these microfabricated systems is polydimethylsiloxane (PDMS), due to a number of suitable properties of this polymer[215]. However, PDMS undergoes a small degree of shrinkage during curing, and this shrinkage can greatly impact process flow in rapid prototyping of array-based multilayer systems. The shrinkage-induced alignment registration problem arises when layers containing dense device arrays are fabricated by multiple methods and stacked, as is often the case in MSL. The most frequent example occurs when cast PDMS is peeled from a mold, in which features on the bulk slab no longer align with those fabricated by spin coating onto a master.

Several approaches to this problem have been utilized, including modifying the PDMS material[216], curing samples at room temperature[217], and designing devices with high tolerances to misalignment, or which have a small footprint, to minimize the total displacement of features due to shrinkage. Unfortunately, PDMS is not easy to chemically modify, takes prohibitively long to completely cure at room temperature, and can undergo a small degree of shrinkage in spite of these measures. Designing systems with large alignment tolerances limits the potential for miniaturization, and may not be desirable in many applications. Designing devices over a small footprint severely limits the ability to prototype high-throughput systems.

The most common solution to this fabrication problem has been to characterize the degree of shrinkage of PDMS, and scale the master size accordingly[211]. This approach has been successfully used to create functional large-area microdevices[218]. However, the devices must be designed to tolerate large misalignments, and using the iterative characterization technique to fabricate arrayed structures with exacting alignment requirements remains challenging. Furthermore, the degree of shrinkage of PDMS is not constant but dependent on several factors
including cure temperature and time, PDMS component ratios, and layer thickness[219]. These parameters introduce further issues with the characterize-and-scale approach to the shrinkage-induced alignment registration problem. Firstly, curing temperatures and environmental parameters must be strictly controlled, in order to achieve a consistent degree of shrinkage. In a non-cleanroom, multi-disciplinary, multi-user environment, maintaining tight control of these curing parameters can be difficult. As demonstrated by Lee et al.[219], small variations in temperature can cause a change in shrinkage, which, depending on microdevice size, will cause a misalignment between layers. This variability leads to low yield in fabricating multilayer devices. Second, because the amount of shrinkage is dependent on layer thickness, a single calibration result cannot be used in the fabrication of multiple prototype designs. Thus, individual calibration experiments need to be performed for each design. The same applies to using various PDMS component ratios, as is often done to improve adhesion in multilayer devices[99]. Third, shrinkage calibration techniques will never provide a perfectly accurate scaling factor. While small errors may be acceptable for low-throughput, proof-of-concept devices, this technique cannot be scaled up to larger-area devices with high feature densities, without extensive time-consuming and iterative calibration studies.

This fabrication issue lengthens the concept-to-prototype turnaround time for MSL devices. It also effectively limits the massive parallelization theoretically possible in such microsystems. Furthermore, it limits miniaturization and causes difficulties in implementing designs that have more stringent alignment requirements in fabrication. In order to address these concerns and provide a scalable fabrication solution, we made use of a technique termed ‘sandwich mold fabrication’ or ‘squeeze fabrication’ first described by Jo et al.[220]. This exclusion molding method has been used for a variety of purposes[221, 222], but has remained unrecognized as an effective solution in solving the shrinkage-induced PDMS alignment registration issue. In this paper, we rapidly microfabricate a precisely aligned multilayer, densely-packed, large-area, array of vertically actuated posts to illustrate the advantages of this technique in overcoming the alignment complications caused by PDMS shrinkage.
4.2 Materials & Methods

4.2.1 Fabrication of Mold Masters

SU-8 photoresist (Microchem, Newton, MA, USA) was patterned onto 3” × 2” glass slides (Fisher Scientific, Ottawa, ON, Canada), using parameters outlined by the manufacturers. Single and multi-layer masters were fabricated with heights ranging from 40 to 350 µm. After standard processing, the masters were hard baked at 80°C for three days, and treated with the silanization agent (Tridecafluoro-1,1,2,2-tetra-hydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA, USA), under vacuum conditions[223], to facilitate mold-release of the patterned PDMS layers.

4.2.2 Materials

The sandwich mold fabrication process requires a 3” C-clamp and two 3.5” × 2.5” metal plates. Transparencies for inkjet printers (Grand & Toy, Toronto, ON, Canada) were cut to an appropriate size and used as the plastic backing film. Foam pads (Silver Dollar, Toronto, ON, Canada) were purchased from a craft supply store, and cut to size. Sylgard 184 PDMS (Dow Corning, purchased through A.E.Blake Sales Ltd., Toronto, ON, Canada) was mixed in a standard 10:1 curing ratio and poured onto an SU-8 master. The uncured PDMS was then spread by tilting the master and degassed in a vacuum chamber. As per Figure 4-1, the master was then placed on a foam pad on a metal plate. The plastic film was carefully placed on top of the PDMS so as to avoid trapping air bubbles. A silanized glass slide was then placed on top of the sandwich, followed by a second foam pad, and a second metal plate. It was necessary to silanize this glass slide to enable easily removal from any PDMS that was squeezed out of the sandwich. The sandwich was then compressed in a C-clamp and cured at 80°C for at least four hours. Following curing, the sandwich stack was removed from the oven, disassembled, and the transparency was carefully peeled from the master. The cured PDMS adheres preferentially to the transparency and can be trimmed to size. This procedure was repeated for each layer.
Figure 4-1. Schematic of the sandwich mold fabrication process[220]. (A) Liquid PDMS is sandwiched between the microfabricated mold and a plastic film. (B) The sandwich is then placed in a multilayer stack of rigid metal plates, foam pads and a glass slide. The stack is clamped, and cured at elevated temperatures. (C) The stack is then disassembled and the plastic film and patterned PDMS layer is peeled away from the microfabricated mold.

4.2.3 PDMS Bonding

PDMS surface modification was achieved using a corona discharge treater (Electro-Technic Products, Chicago, IL, USA)[224]. For the base layer, a cleaned glass substrate and the PDMS
layer were plasma treated, placed in conformal contact, and baked on a hot-plate at 80°C for 20 minutes. Once cooled, the plastic backing film was peeled from the bonded structure, an easy process due to the permanent bond between the PDMS layer and the underlying substrate. The strength of this bond also prevented any warping or deformation of layers due to thermal stresses generated during fabrication. A similar process was followed when adding additional layers of PDMS: layers were aligned, plasma treated, and bonded, before peeling away the plastic backing film.

4.2.4 Alignment of Multiple Layers

Alignment between multiple layers was achieved using a home-made alignment system (Figure 4-2), in which a micromanipulator (Siskiyou, Mission Viejo, CA, USA) was fitted with an arm and a vacuum chuck, and was used to manually position a layer over the substrate. The substrates were mounted on a rotary platform (Newmark, Grants Pass, OR, USA) to correct rotational errors. Alignment between the two layers was monitored using a Navitar 12x zoom system (Navitar, Rochester, NY, USA), which provides a large depth-of-field at high magnifications. The PDMS layers were plasma treated, aligned manually, and then carefully brought into contact with each other. While methanol has been used as a surfactant between PDMS layers during alignment, it has also been shown to swell PDMS to a small extent[225]. This could interfere with the fine alignment accuracy, and hence was not used. The structure was then placed on a hot plate at 80°C for 20 minutes to complete the bonding process.
4.2.5 Demonstration Structure: Array of Cylinders

An array of two-layer cylinders (Figure 4-3) was produced using two fabrication methods: the conventional approach used in the Quake valve process[99], and squeeze fabrication. For both cases, the layers were aligned rotationally and at the bottom left corners of the arrays. In the conventional fabrication method, the two PDMS layers were prepared by casting and by spin coating. The two layers were cured in an oven at 80°C for four hours. The cast layer was peeled, aligned with the spin-coated layer and bonded. In the sandwich technique, two sandwich mold fabrication steps were used to produce two patterned layers. The layers were stacked, aligned and bonded as described. In each of the steps of this technique, the PDMS film is never released from a rigid substrate, thereby preventing shrinkage from occurring.
Figure 4-3. An array of two-layer structures fabricated by conventional and sandwich fabrication methods. The bottom left corner of both arrays was aligned, and the resulting registration error can then be observed across the array. No registration error was observed for the sandwich molding process.
4.2.6 Demonstration Structure: Micro-post Array

Fabrication of the micro-post array required three SU-8 mold masters. The process, illustrated in Figure 4-4, began by fabricating three sandwich mold films from the SU-8 masters. The first film was bonded to a glass substrate, and the backing film was peeled away. The second sandwich film acts as a mold for an additional layer of PDMS. To provide structural rigidity during fabrication, this mold was affixed to a glass slide using double-sided tape. The rigid mold was then silanized, and an 80 µm thick layer of uncured PDMS was spin-coated onto it. This layer was partially cured in an oven for 15 minutes at 80°C. After removing the glass slide from the mold, it was aligned and plasma-bonded with the first sandwich film. A second partial curing step of 15 minutes at 80°C strengthened the bond and the silanized PDMS mold was peeled away from the structure. The third sandwich mold layer was then aligned and plasma-bonded to complete the structure. Access ports were cored into the PDMS device, and Nanoport fittings (Upchurch Scientific, Oak Harbor, WA, USA) were used to connect the micro-post array to a Schwarzer rotary pump (Schwarzer Precision; Essen, Germany), capable of producing positive pressures up to 6 kPa.

Figure 4-4. Schematic outline of the fabrication process for the micro post array
4.2.7 Measurement of Post Displacements

To demonstrate functional actuation and consistent operation of an array of precisely aligned microstructures, the distance over which the micro-posts are displaced was measured on two 5 × 5 Braille display arrays, fabricated with varying geometries. A Wyko optical surface profilometer (Veeco Instruments Inc.; Woodbury, NY, USA) was used to determine heights of the pins at rest and when actuated. The results were grouped based on the geometry of each micro-post actuation unit, and presented as a mean displacement distance ± standard deviation for measurements of at least three Braille units (Figure 4-7; see Results & Discussion).

4.3 Results and Discussion

Cited advantages of the sandwich mold fabrication technique include the ability to reproducibly fabricate patterned layers of controlled thicknesses, and the ease of handling and transferring delicate films of PDMS. Not previously recognized however, is the fact that throughout the fabrication process, the PDMS is never released from a rigid substrate: either the plastic backing film, or the underlying substrate. Because PDMS is never given the opportunity to shrink, this method enables the fabrication of multilayered structures without any registration problems between layers.

4.3.1 Technique demonstrations

As a demonstration of this feature, the conventional and sandwich mold fabrication methods were used to produce identical multilayer structures: a rectangular array of 108 cylinders, fabricated over a 2 cm × 2.5 cm area (Figure 4-3). Both samples were aligned such that rotational errors were eliminated, and the bottom left corners of the array registered accurately.

Using the conventional fabrication process resulted in an increasing registration error across the array, culminating in a 210 µm misalignment at the top right corner. This corresponds to a 0.7% shrinkage of the cast layer of PDMS, which is within the range of accepted values of PDMS shrinkage, but not for similarly reported curing conditions[219]. This demonstrates the variability in shrinkage seen when working in a multi-user environment without tightly controlled fabrication conditions. In terms of comparing this with conventional fabrication methodologies, the demonstration of poor registration shown here is admittedly biased, because
no attempt was made to optimize the master size to account for shrinkage, as is often the case when using this technique. However, with relatively lax control over processing conditions, and without any time-consuming optimization or calibration procedures, the sandwich mold fabrication produced a device with no measurable alignment error across the array.

To demonstrate a functional application of this technique, we developed a miniaturized post array. Commercially-available refreshable Braille displays have been used as actuators in microfluidic devices as a simple, quick, robust and inexpensive alternative to on-chip valve components. The Braille display has been used to actuate valves, pump fluid[166], and to apply mechanical stimuli to adherent cells[189]. The platform developed in this study further miniaturizes the Braille display system and can be used as a modular base for various end-user defined applications in complex microfluidic control.

Figure 4-5. (A, B) Schematic quarter-section views of each micro Braille actuator, at rest and while actuated. (C, D) Demonstrated functionality of the micro Braille actuator.

The working principle of the micro-post unit is demonstrated in Figure 4-5. The three layer structure consists of a lower actuation cavity beneath a microfabricated post. The post is raised by applying a positive pressure to the actuation cavity (Figure 4-5). A third structural layer provides a mounting platform to support microfluidic channels or membranes. Because of the
small spacing between the pin edge and this structural support, each micro-post structure requires alignment accuracies of better than 15 µm to operate. Figure 4-6 displays a top-down view of the structure and demonstrates accurate layer alignment and the stringent tolerances required. For this application, the size of each actuation cavity was increased across the array, to produce micro-posts with varying actuation characteristics. Figure 4-7 demonstrates the changing actuation heights of the posts as a result of the change in device dimensions across the array, for an applied pressure of 6 kPa. The results indicate the ability to create vertical displacements ranging from 2 µm to 84 µm using a single pressure source, demonstrating functional actuation of an array of microstructures which require a high degree of alignment accuracy to operate. To further demonstrate the scalability of the sandwich mold fabrication technique, we increased the number of devices per chip and successfully fabricated an aligned array of 108 Braille units over a 1” × 2” area (Figure 4-8).

Figure 4-6. Top-down view of a section of the micro Braille display, demonstrating stringent alignment requirements of the array. The small misalignments observable away from the center of the image are due to perspective distortion artifacts inherent in using a single imaging path on a dissecting stereoscope.
Figure 4-7. Actuation heights of the micro Braille pins for a pressure of 60 mbar, for cavity radii varying from 250 to 700 µm (mean ± standard deviation, n = 3).

Figure 4-8. A 9 × 12 array of micro post actuators, across a 6.5 cm² area.
With no observable registration errors across the large array, we believe that the scalability of this technique is limited only by the size of masters that can be fabricated. The technique is robust to variations in fabrication conditions, a situation common in most multi-disciplinary labs. Furthermore, besides solving the shrinkage issue, the plastic backing films act as support structures for the PDMS layers during alignment, eliminating handling problems caused by the deformability and thermal expansion of PDMS.

### 4.3.2 Limitations

There are a few limitations to this technique. First, although the sandwich mold fabrication process effectively eliminates shrinkage across a large area, significantly reduces calibration time for each designed device, and improves functional device yield, it does require additional fabrication steps. Given the high degree of alignment accuracy and the high device yield that results from using this technique, we feel that this is an acceptable compromise during the conceptual prototyping phase of device development. However, it may be a concern during large scale production of such devices. Second, the sandwich mold fabrication technique occasionally fails to completely clear PDMS from the master features, resulting in a thin film of PDMS, rather than a clear through-hole. This happens more often with SU-8 masters greater than 100 µm in thickness, due to variations in height across the master. Often, this variability can be incorporated into the device design, such that a thin film of residual PDMS does not impact device function, as in valve structures. However, if a clear via is essential, it may be necessary to use a needle to manually remove any remaining PDMS. This is a labour intensive process, and not suited for large devices. An alternative solution would be to modify the perforated membrane process outlined by Luo et al.[226], or to use a photopolymerizable PDMS as demonstrated by Carlborg et al.[227].

### 4.4 Conclusions

This chapter reports the use of a method to overcome the shrinkage-induced alignment registration problem in prototyping multilayer PDMS microsystems. The sandwich fabrication method originally reported by Jo et al.[220] enables rapid fabrication of dense, large-area, well-aligned structures in MSL, without strict regulation of fabrication conditions or extensive calibration processes. Because PDMS layers are never released from a rigid substrate throughout
the procedure, polymer shrinkage does not occur, and hence the registration error between layers is eliminated. This aspect of the technique can significantly reduce the turnaround time in prototype development of well-aligned, high-throughput, multi-layered devices, and has been shown to be successful in rapidly fabricating a functional array of microstructures with stringent alignment requirements.
Chapter 5

“… in which time and stress take their toll.”

5 Uniform substrate strains

Presently, no technique exists to simultaneously and systematically screen for the effects of a range of uniform mechanical forces on cellular function. In this chapter, a novel microfabricated array capable of simultaneously applying cyclic equibiaxial substrate strains ranging in magnitude from 2 to 15% to small populations of adherent cells is developed and characterized. The array is versatile, and capable of simultaneously generating a range of substrate strain fields and magnitudes. The design can be extended to combinatorially manipulate other mechanobiological culture parameters in the cellular microenvironment. To demonstrate this technology, the array was used to determine the effects of equibiaxial mechanical strain on activation of the canonical Wnt/β-catenin signaling pathway in cardiac valve interstitial cells. This high-throughput approach to mechanobiological screening enabled the identification of a possible co-dependence between strain magnitude and duration of stimulation in controlling β-catenin nuclear accumulation. More generally, this versatile platform has broad applicability in the fields of mechanobiology, tissue engineering and pathobiology.

5.1 Introduction

The large number of factors in the cellular milieu that can impact biological function has necessitated the development of high-throughput approaches to study cell behavior in vitro. Microtechnologies have enabled rapid screening of cellular response to biomaterials[5], extracellular matrix proteins[6], cell-cell interactions[228, 229], substrate stiffness[180], soluble

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2 Work presented in this chapter was published in:


factors[7] and chemical gradients[230]. More recently, the development of multimodal high-throughput stimulation platforms[7, 231] has demonstrated interesting combinatorial effects between stimulation types. Such platforms are of particular relevance to research in tissue engineering and drug discovery, as they show that cellular response is dependent on multiple factors in the cellular microenvironment.

Mechanical forces play an important role in driving critical cellular processes, such as apoptosis, matrix deposition, gene and protein expression[8, 232] and stem cell differentiation[9]. Mechanical factors have also been shown to modulate cellular response to other stimuli, including matrix proteins[10], chemical cues[11], and genetic therapies[12]. In some cases, mechanical factors can supplant other stimuli in driving cellular response[9]. Thus, mechanical cues are important considerations when defining the cellular microenvironment and investigating its effect on cell fate and function. However, despite the demonstrated importance of mechanical forces in regulating and modulating cellular function, high-throughput screening for cellular response is typically conducted in mechanically static conditions.

The in vivo mechanical environment is complex and combines a number of loading modalities. Fluid-related stimulation has been successfully recreated on microfabricated platforms, in which microfluidic channels have been used to apply hydrostatic pressures[169] and shear stresses[109, 167] to cultured cells. Progress has also been made in developing substrate deformation-based stimulation platforms in which cells are “stretched”. The Braille display-actuated system developed by Kamotani et al.[189] can screen for the effects of stimulation frequency, but is unable to generate uniform strains or probe cellular response to a range of strain magnitudes. Microdevices developed by Tan et al.[192] are capable of screening for cellular response to multiple substrate deformations, but a single experiment is limited to three anisotropic biaxial strains at a fixed magnitude, and cells have been shown to differentially respond to distinct strain fields and magnitudes[233]. Furthermore, the system cannot be readily expanded to screen for the integrated cellular response to multiple mechanobiological parameters.

In this chapter, an array-based microfabricated platform designed to simultaneously apply a range of cyclic uniform substrate strains to single cells or small colonies of cells is described. In contrast to existing mechanical stimulation systems, the microfabricated array can currently conduct high-throughput screens for the effects of strain magnitude and has been specifically
designed to integrate with other microtechnologies that enable individual control over the extracellular matrix, fluid shear stresses, and chemical stimulation applied to isolated cell colonies, thereby enabling massively parallel combinatorial screening for mechanobiological culture parameters.

As a first demonstration of this technology, the platform was used to probe the temporal and strain-magnitude dependent accumulation of β-catenin in the nuclei of a primary interstitial cell population, with a high frequency of mesenchymal progenitor cells[234]. β-catenin is a critical protein in the canonical Wnt signaling pathway (Figure 5-1) that regulates the proliferation and differentiation of mesenchymal progenitor cells[235-238]. In the absence of signals from the canonical Wnt pathway, cytoplasmic levels of β-catenin are maintained through the action of a β-catenin destruction complex. In the presence of Wnt signaling molecules, this destruction complex is inactivated, resulting in hypophosphorylation of β-catenin and subsequent translocation into the cell nucleus[236]. In the nucleus, β-catenin participates in the differential transcriptional regulation of target genes in a dose-dependent manner[235-238].

Recent studies have shown that mechanical stimuli alone can induce nuclear translocation of β-catenin[239-241]. Capitalizing on the high-throughput capabilities of the developed platform, our results suggest that levels of nuclear accumulation of β-catenin in mesenchymal progenitor cells is both time- and strain magnitude-dependent. The identification of this relationship would have required a significantly larger time-investment, using conventional low-throughput techniques. Because of the demonstrated link between levels of β-catenin present in the nucleus and stem cell differentiation[235-238], these results may have implications for processes involving mechanoregulation of cellular differentiation, including tissue engineering, development and the pathobiology of load-bearing tissues.
Figure 5-1. Schematic representation of the canonical Wnt/β-catenin signaling pathway.  
(A) In the absence of Wnt molecules, cytoplasmic β-catenin is phosphorylated by the Axin/APC/GSK3 destruction complex, and is then targeted for degradation.  
(B) Wnt binding to surface receptors inhibits the Axin/APC/GSK3 destruction complex, leading to accumulation of β-catenin and subsequent translocation into the nucleus where it activates target genes. Figure reproduced from [238], with permission from the Journal of Biological Chemistry.

5.2 Materials & Methods

Unless otherwise stated, all chemicals and reagents for cell culture were purchased from Sigma-Aldrich (Oakville, ON, Canada); fluorescent dyes from Invitrogen (Burlington, ON, Canada); and all other equipment and materials from Fisher Scientific (Ottawa, ON, Canada).
5.2.1 Device design and simulation

The design of each element within the microdevice array has similarities to the macroscale system described by Schaffer et al. [242]. Each unit on the microfabricated array consists of a loading post suspended over an actuation cavity. Positive pressure applied to this cavity distends the loading post upwards, which deforms a flexible cell culture substrate (Figure 5-2). A lubricant between the loading post and the culture membrane prevents stiction between the two materials. Simultaneous application of a range of substrate strains across the array was achieved by changing the size of the actuation cavity, while keeping the size of the loading posts constant. For a single applied pressure, an increase in actuation cavity size leads to an increase in vertical displacement of the loading post, which in turn creates a larger strain on the cell culture substrate. Because the loading posts have identical dimensions, the sizes of the mechanically stimulated areas remain constant across the array. Finite element simulations to assess the feasibility of this actuation scheme were conducted in ANSYS (Figure 5-3; ANSYS Inc.; Canonsburg, PA, USA).

5.2.2 Device fabrication

The device consists of multiple layers of patterned poly(dimethylsiloxane) (PDMS; Dow Corning, purchased through A.E. Blake Sales Ltd, Toronto, ON, Canada), fabricated using multilayer soft lithography [99]. To fabricate the three-layer PDMS base structure (Figure 5-4), three master molds were fabricated on 3”×2” glass slides, with SU-8 negative photoresist (Microchem; Newton, MA, USA). Sandwich mold fabrication [220] was used to produce three patterned layers of PDMS on transparencies (Grand & Toy; Toronto, ON, Canada), which were transferred sequentially to a rigid glass slide. This fabrication technique prevents shrinkage between device layers [202], enabling us to realize large, dense arrays of precisely-aligned microstructures (Figure 5-2F). Connectors were then attached to the air pressure and lubrication channels. Connectors with a large dead-space volume were used to trap air bubbles from the lubricant before injection into the lubrication channels. For experimental simplicity, all subsequent experiments were conducted on a smaller 6×5 array.
Figure 5-2. (A) Schematic cross-sectional view of a single mechanically active unit on the array. (B, C) Schematic of a unit in its (B) resting and (C) actuated positions. (D,E) Stereoscopic images of the device (D) at rest and (E) when actuated. (F) 9 × 12 array of mechanically active culture units within a 6.5 cm² area.
Figure 5-3. Finite element simulation model for device actuation at increasing pressures (A-F).
Figure 5-4. Fabrication process for mechanically active culture arrays.
Following fabrication of the base platform, an ‘actuated’ fabrication process was used to complete device construction. In these steps (Figure 5-4) the device was operated during continued fabrication. A 15 μm thick ‘culture film’ of PDMS was spin-coated onto a transparency, and cured in an oven for 4 hours at 80°C. A 65 kPa vacuum (Barnant Air Cadet single-head pump) was then applied to the actuation cavities on the base structure, to lower the posts and prevent them adhering to the culture film. The PDMS base structure and culture film surfaces were oxygen plasma treated with a corona discharge unit (Electrotechnic Products; Chicago, IL, USA), placed in contact with each other and heated on a hotplate at 90°C for five minutes to create a permanent bond[224]. A lubricating solution of 90% glycerol in deionized water was carefully injected into the lubrication channels to fill the spaces between the lowered posts and the culture film. Trapped air pockets were found to shrink over time (as air diffused through the PDMS walls), and it was found that keeping the device at 40°C removed any trapped air within ten minutes. The full procedure was completed within 20 minutes, which prevented the PDMS surfaces from returning to their hydrophobic states. The loading posts were then released, and the transparency peeled away from the device, leaving the culture film suspended over the lubricated loading posts.

For those devices that were used for cellular studies, a single PDMS gasket was plasma bonded around the device to hold culture media over the cells grown on the array. The devices were then sterilized by soaking in 70% ethanol, air-dried, and exposed to germicidal UV light for 30 minutes. The device surface was plasma activated and incubated with 100 µg/mL Type I collagen (Becton Dickinson; Mississauga, ON, Canada) in 0.02 N acetic acid overnight at 4°C.

5.2.3 Strain Characterization

Characterization of surface strains on the device was conducted by tracking displacements of fluorescent beads. Fluorescent polystyrene beads (Bangs Laboratories; Fishers, IN, USA) of 1 μm diameter were suspended in methanol and vortexed for one minute to break up bead aggregates. The bead solution was immediately pipetted onto the device surface, and the methanol was allowed to evaporate. The devices were imaged on a stage heated to 37°C with a 40× objective (air-immersion; 0.6 NA) under a fluorescent microscope (Olympus IX71; Olympus Microscopes; Markham, ON, Canada) when strained and when at rest. Actuation
pressures (30 kPa) were applied to the actuation cavities using an eccentric diaphragm pump (SP 500 EC-LC; Schwarzer Precision; Germany).

Bead positions were analyzed using a semi-automated customized algorithm in ImageJ (NIH). Briefly, the images were converted to binary images, and bead locations were tracked between image sets using a modified particle tracking algorithm. A visual representation of particles and displacement vectors was used to manually identify false traces, which were removed from the dataset. The displacement vector sets were then analyzed using a custom-written Matlab program (Mathworks; Natick, MA, USA), to best fit a point from which radial bead displacement originated. This central point was used to calculate changes in radial bead location. An arbitrary reference line was used to calculate changes in circumferential bead location, and hence, radial and circumferential strain parameters were extracted for each bead. Standard plane fitting algorithms were then used to calculate nominal strain values for each unit in the array. In order to characterize the strains created across a device array, this procedure was repeated for at least three units of each geometry across the array. Graphical results for radial and circumferential strains are reported as means ± standard error (n ≥ 3). This procedure was repeated for a second array, with similar results.

To determine the effects of material fatigue on the culture film, the device was placed in cell culture conditions (37°C, 5% CO2), and cyclic pressure was applied using a solenoid valve (Pneumadyne; Plymouth, MN, USA), driven at 1 Hz for $10^3$, $10^4$, and $10^5$ cycles. At each of these time points, the strain fields for 15 randomly selected units were characterized using the above-mentioned procedure. Radial strains at each time point were normalized to their initial values, and the results reported as percentage means ± standard deviation.

### 5.2.4 Cell Culture and Immunostaining

Primary porcine aortic valvular interstitial cells (VICs) were isolated by enzymatic digestion as previously described[184] and used between passages two and three for all experiments. VICs contain a large population of mesenchymal progenitor cells with multilineage differentiation potential, similar to that of mesenchymal stem cells[234].

Collagen-coated devices were washed three times with phosphate-buffered saline and preconditioned for two hours at 37°C with Dulbecco’s Modified Eagle Medium supplemented
with 10% fetal bovine serum. VICs were then seeded on the device surface at 20,000 cells/cm² and maintained in supplemented DMEM for 24 hours (37°C, 5% CO₂) to allow cell spreading and surface attachment. The cell culture arrays were then cyclically stimulated at 1 Hz for three and six hours before fixing the cells in 10% neutral buffered formalin (NBF) overnight at 4°C.

To detect β-catenin expression, a standard immunocytochemistry procedure was followed. Briefly, NBF-fixed cells were permeabilized and blocked with 3% bovine serum albumin (BSA). Cells were then incubated with rabbit polyclonal β-catenin antibody (Abcam) followed by AlexaFluor 568 goat anti-rabbit IgG. The cell nuclei were counterstained with Hoechst 33258. Since the devices were maintained in a humidified incubator, condensation of droplets beneath the loading post can hamper imaging using a standard fluorescent microscope. In order to obtain clear images, actuation cavities and the channel network were backfilled with water in a vacuum chamber prior to imaging. Images were then collected using a 40× objective (0.6 NA) on a fluorescent microscope (Olympus IX71; Olympus Microscopes; Markham, ON, Canada) with a CCD camera (QImaging Retiga 2000R; QImaging; Surrey, BC, Canada).

5.2.5 Cell Image Analysis

Nuclear β-catenin levels were measured by applying a threshold binary function to the nuclear images and manually counting the number of cells in each field of view. The binary images were used to mask the cytoplasmic β-catenin images, and the integrated fluorescent density across all the nuclei was determined. The average β-catenin levels per nucleus were calculated for each image. Nuclear β-catenin levels measured by fluorescent analysis have been shown to correlate directly with those measured by Western blotting[239]. Similarly, cytoplasmic β-catenin levels were measured by subtracting the integrated nuclear fluorescent intensity from the integrated cellular fluorescent intensity. The results were reported as means ± standard deviation (n = 3). Data were analyzed using one-way ANOVA tests for each time point. Post-hoc pairwise comparisons were conducted using the Student-Newman-Keuls method. All statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA).
5.3 Results

5.3.1 Computational Simulations

Finite element simulations were conducted to test the actuation principle of the device and to determine expected strain field profiles. As a first demonstration of these experiments, circular loading post geometries were used, which theoretically should provide strains equal in magnitude in the radial and circumferential directions (equibiaxial) along the culture film surface[243]. Radial and circumferential strains obtained in the culture region over the 400 µm diameter loading posts (Figure 5-5) demonstrated increasing strains with an increase in actuation cavity size, for a single applied pressure of 2.5 kPa. Within a 200 µm diameter region, radial and circumferential strains were relatively uniform, varying by a maximum of 0.22% across this area of interest. The strain field was also isotropic and equibiaxial, with no more than a 0.17% difference between radial and circumferential strains. It was also found that the area of uniform strain decreased with an increase in membrane thickness (data not shown). Consequently, the microdevices were realized with a 15 µm thick culture film, which should significantly improve strain uniformity as compared to the simulation results, which assumed a 20 µm thick film.
Figure 5-5. Finite element simulations demonstrating strain field profiles and device feasibility. (A) Axisymmetric finite element geometry used to obtain a range of (B) radial and (C) circumferential strains across the culture film using a single applied pressure for a range of increasing actuation cavity sizes. Increasing strains are found for increasing sizes of actuation cavity geometry. A uniformity of 0.0022 is obtained within a 100 µm diameter radius, and equality between radial and circumferential strains is within 0.0017.
5.3.2 Device Characterization

A typical plot of radial and circumferential strains for a unit on the array is shown in Figure 5-6B, with an accompanying line of best fit from linear regression. Measurements of radial strain were found to show a slightly larger spread closer to the center of the loading post, due to limitations in measurement resolution for small bead displacements. Use of this technique to measure circumferential strains was found to be less robust, especially close to the arbitrary reference line. This is due to the measurement resolution limitation and heightened sensitivity of measured circumferential strains to the location of the center of the loading post (the origin of radial deformation), which cannot be precisely determined. However, the ensemble measurement is reasonably accurate and repeatable when using a large number of tracking beads.

A single strain value at the center of each loading post was calculated based on these regression results in order to compare strain magnitudes across an array actuated at 30 kPa (Figure 5-6C). The results demonstrate that the array functioned as intended, providing relatively equal radial and circumferential strains, which increase in magnitude across the array. To simplify data presentation, each actuation cavity size was assigned a nominal strain value based on these results: 2, 3, 5, 8 and 15% for cavity sizes ranging from 800 to 1400 µm in diameter. These strains are within the range of those most often used in macroscale mechanobiological experiments[8].

Long-term testing of the device was found not to significantly impact the strains produced (Figure 5-6D) on any of the units in the array. There were no significant differences in substrate strains after the device operated for 1,000, 10,000 or 100,000 cycles (p = 0.967). These results demonstrating insignificant levels of material fatigue are supported by long-term fatigue studies of PDMS valves, which have a similar structure and were tested for over four million cycles[99].

5.3.3 Mechanosensitivity of β-catenin

Levels of cytoplasmic β-catenin were not significantly impacted by stimulation time or strain magnitude. However, accumulation of β-catenin in the nuclei of the valve mesenchymal progenitor cells was found to be significantly affected by strain levels and stimulation time (Figure 5-7; Figure 5-8). After three hours of stimulation, there was a general increase in nuclear β-catenin with increasing strain levels. There was significantly greater β-catenin translocation in
cells stimulated at 15% strain than in mechanically static controls (p = 0.063). After six hours of stimulation, there was elevated nuclear β-catenin accumulation in cells stimulated at 3% (p = 0.093), 5% (p = 0.074), and 8% (p = 0.005) strain. However, in cells strained at 15% for six hours, nuclear β-catenin returned to levels comparable to static controls (p = 0.827).

Figure 5-6. Characterization of strain on device surface. (A) Fluorescent bead displacement for a single unit on the array, upon device actuation (red: original bead location; green: deformed bead location). (B) Radial and circumferential strains across a single unit on the array, plotted as a function of radial distance and angle from an arbitrary reference line. (C) Radial and circumferential strains (mean ± standard error, n > 3) achieved across a 5 × 5 array (inset). (D) Strain values normalized to their initial values after cyclic deformation (mean ± standard deviation, n = 15).
Figure 5-7. The influence of mechanical substrate strain on \(\beta\)-catenin accumulation in the nucleus. Representative fluorescent images of Hoechst-stained nuclei (left, blue channel), \(\beta\)-catenin (center, red channel), and the merged channels (right). VICs shown are fixed and stained after 3 h of cyclic mechanical stimulation at 0%, 3%, 8% and 15% strain magnitudes. Nuclear \(\beta\)-catenin accumulation (white arrows) increased with applied strain.
5.4 Discussion

High-throughput approaches to studying biological systems have significantly increased the ability to better understand and manipulate cells for tissue engineering, drug discovery and fundamental cell biology studies. Though mechanically active environments have been shown to significantly impact cellular response to other stimuli, most high-throughput screening techniques are limited to mechanically static environments. To address this issue, we have developed a microfabricated platform that can rapidly screen for the effects of multiple cyclic substrate strains. More broadly, the platform can be used in combination with currently available high-throughput screening techniques for other factors that impact cellular function, enabling a massively parallel approach to screening for combinatorial mechanobiological factors in the cellular microenvironment.

The selected device design is versatile in terms of the strain fields that can be applied to individual cell populations. The strain profile on each array unit is dependent on the shape of the loading post[244]. Modifying a single mask in the fabrication process can produce an array with many loading post shapes. Rectangular and elliptical posts with changing aspect ratios can create a range of anisotropic biaxial strains. In these initial experiments, the system was limited to circular loading posts, which generate uniform equibiaxial strain fields on the culture films. The
strain fields produced are similar to those of the Flexcell systems (Flexcell International Corporation, Hillsborough, NC, USA), a popular commercial platform for macroscale cell stretching[13]. Though the maximum strains achievable in the Flexcell systems are as high as 25%, the range of strains actually used in the equibiaxial stimulation of cells most often falls between 3 and 10%[8], which are well within the operating parameters of the designed device. In contrast to existing macro- and microscale mechanical stimulation systems, our device generates multiple uniform strain levels on a single platform and when scaled up can provide a 256-fold increase in experimental throughput over commercially-available systems. Thus, the versatility and increased throughput of the microfabricated platform provide advantages that can speed discovery and improve our understanding of mechanoregulation of cell function.

5.4.1 Specific design limitations

There are three design limitations to the device as presented here. First, the PDMS gasket used to contain the culture media in the present work potentially allows paracrine communication between cell populations across the array. Though the platform has been designed such that the distance between mechanically active units on the array is greater than the diffusion distance of common paracrine signaling molecules[13], convective transport and mechanical agitation caused by the moving loading posts remains a concern. In order to address this issue, the device was designed to allow cells to be cultured on a large, flat, unobstructed surface. Hence, simple PDMS wells can be integrated on the flat culture surface of the device to segregate soluble paracrine cues on different portions of the array. The pitch between elements in the array was designed based on the standard 1536-well plate format to allow commercial robotic liquid handling equipment to automatically control chemical stimulation factors across such an array of wells. Alternatively, microfluidic networks can also be used to control chemical stimulation or apply shear stresses to specific areas of the array. Hence, this platform design can be extended to enable screening for the effects of combinations of mechanical and chemical cues.

Second, a common problem associated with loading-post based deformation systems is that cells experience heterogeneous strains away from the center of the loading post. The ability to spatially control cellular adhesion patterns across the array can address this issue. The flat device surface allows adhesive extracellular matrix proteins to be readily printed on the surface using well-established techniques, such as a commercially-available protein plotters[6], a PDMS
stamp for microcontact printing\cite{245}, or an elastomeric stencil to apply matrix protein solutions to specific areas of the device\cite{221}. Furthermore, such an approach would extend the capabilities of the system to manipulate extracellular matrix protein type and concentrations in combination with cyclic mechanical substrate strain.

Third, this platform is not well-suited to real-time imaging of cells under mechanical stimulus using simple microscopy, as the culture membrane is displaced out of the focal plane. Though this can be achieved using auto-focusing microscopy systems or post-processing image manipulation algorithms, these are non-trivial solutions. Furthermore, the primary advantage to this research platform is in experimental throughput; assessing real-time cellular response to multiple mechanical stimuli would require simultaneous imaging of all the units in the array, which would require specifically designed imaging systems. Hence, the system has been designed for and is best suited to determine end-point biological response to a range of mechanical substrate strain conditions.

5.4.2 Advantages of microfabrication

Moving to a microfabricated format when designing mechanically active culture systems yields a number of generic advantages: smaller quantities of expensive cell culture and immunostaining reagents are used; the experimental equipment has a significantly reduced footprint; a single fabrication step can be used to generate a large number of experimental conditions without manual intervention; the length scale is ideally suited for single cell studies; cells can be visualized directly on-chip using bright field or fluorescent microscopy; and the potential for completely automated experimental techniques can be more easily realized. Additionally, unlike macroscale systems\cite{246}, applied strain levels in the microscale system did not change at high cycle numbers. Furthermore, microfabrication of mechanically active cell culture platforms also brings a specific advantage that is not possible on similar macroscale instruments. Mechanically straining cells on flexible substrates generates reactive normal fluid stresses caused by the associated movement of fluid over the deforming substrate, which can unintentionally influence cell function\cite{247}. Because cells must be cultured under liquid media, these two forms of mechanical stimulation cannot be decoupled. On the microscale however, the total perturbation to the system required to produce a specific strain is significantly reduced. For example, creating a 15% substrate strain in a 3.6 cm diameter well plate requires the substrate to be stretched by
540 mm. Creating a similar substrate strain in a 500 µm diameter film requires the substrate to be stretched by 75 µm, reducing the distance through which cells are displaced by nearly 4 orders of magnitude. Though the issue of unintentional loading stresses is not completely eliminated, this platform reduces reactive normal forces and their confounding effects on the response of cells to substrate stretch.

5.4.3 Critical limitations and disadvantages

Designing mechanically active culture experiments on the microscale also has limitations, specifically: (1) reduction in numbers of cells; (2) increase in stiction forces; and (3) material limitations of microfabrication. First, because of the reduced number of cells available for analyses, the utility of the mechanically active array is limited to high-level screens for fluorescently-tagged proteins or morphological assays. The reduction in the number of mechanically stimulated cells makes techniques such as RT-PCR, ELISA and Western blotting challenging with current technologies. Hence, as is the case with most high-throughput screening systems, this array is best suited for narrowing research focus to certain specific microenvironmental parameters of interest.

Reducing the size of system components increases the problem of stiction between the loading post and the cell culture film. In this microdevice, solid PDMS microstructures are required to interact with a low coefficient of friction. Hence, selection of an appropriate lubricant was critical for successful device operation. Standard lubricants and oils were unsuitable because they migrated through the PDMS, caused swelling, and in some cases were toxic to cultured cells. Glycerol is biocompatible, does not swell PDMS[225], and has been shown to have a low coefficient of friction between hydrophilic PDMS surfaces[248]. Because oxygen plasma-activated PDMS surfaces maintain their hydrophilic nature under water[223], the fabrication process was designed to rapidly cover both contact surfaces with a glycerol/water lubricant, shortly after plasma treatment. In this way, the culture film can slip over the post and deform reliably. Experimental characterization showed little difference in generated strain fields when using 30% or 90% glycerol (data not shown), in spite of significant differences in viscosity of the fluids. The fluid viscosity could affect the deformation rate, but has little influence on end-point strains. Visual observations confirmed that maximum deformation was reached well within the applied pressure cycle of 0.5 seconds when using a 90% glycerol lubricant.
Microfabrication also limits the materials that can be used as a cell culture substrate on these devices. Substrate material has been shown to significantly impact cellular function[249, 250], and though PDMS provides a convenient culture substrate for short term studies such as those reported here, it does not support long-term culture[201] and is not a common biomaterial. Either modifying the PDMS[251] or incorporating alternative biomaterials into the microfabrication process[201] would enable longer-term experiments and potentially improve the translatability of these devices to screen biomaterials for tissue engineering applications.

More broadly, this is a complicated device to fabricate. Due to the time required to fabricate working devices, it is challenging to conduct combinatorial manipulation of multiple mechanobiological factors in the environment, especially when working with heterogeneous populations of primary cells.

5.4.4 Significance of biological findings

As an initial demonstration of this technology, the effects of mechanical strain on β-catenin nuclear accumulation were studied in primary mesenchymal progenitor cells derived from the aortic valve interstitium. The aortic valve contains a strikingly large subpopulation of mesenchymal progenitors[234], which are under constant mechanical strain in vivo[252]. Wnt signaling pathway components, including β-catenin, are elevated in valvular interstitial cells in diseased aortic valves[253], with calcified lesions often occurring in regions subjected to high mechanical stress[254]. Hence, an understanding of how mechanical strain modulates β-catenin nuclear accumulation in valve-derived mesenchymal progenitors may help in understanding the pathobiological basis of aortic valve sclerosis. β-catenin plays a critical role in regulating mesenchymal progenitor cell proliferation and differentiation and is normally kept at a relatively low level in the cytoplasm by active degradation[235-237]. Upon activation of the canonical Wnt signaling pathway, β-catenin degradation is inhibited and β-catenin is translocated into the nucleus, where it can accumulate and regulate gene transcription[238]. Though mechanical stimuli have been shown to induce nuclear translocation of β-catenin[239-241], the effects of cyclic strain magnitude and duration on β-catenin regulation are not defined. Our results (Figure 5-7; Figure 5-8) suggest that though cytoplasmic levels of β-catenin are not substantially affected by applied substrate strains, nuclear accumulation of β-catenin is both time- and strain magnitude-dependent, where large strains accelerate the cycle of nuclear translocation and
subsequent degradation. Smaller strains require longer times for protein translocation but achieve similar levels of accumulation. These results identify two hypotheses for further study. First, different magnitudes of substrate strains either differentially affect translocation into the nucleus, or differentially affect the degradation or removal of \( \beta \)-catenin from the nucleus. Either possibility results in the accumulation of nuclear \( \beta \)-catenin, which has a demonstrated effect on cellular function. Second, there is a relationship between substrate strain magnitude and the temporal dynamics of \( \beta \)-catenin nuclear accumulation. Taken together, these initial findings suggest a novel time- and strain-dependent mechanotransduction mechanism by which different strain magnitudes may elicit distinct temporal responses in mesenchymal progenitors. As is the case with most high-throughput screening platforms, these results serve to identify a starting point for further quantitative studies detailing the impact of nuclear \( \beta \)-catenin levels and residence time on cellular function. The current experiments, in which only two variables (strain magnitude and duration) were tested, would have been significantly more time consuming if using existing macroscale equipment, demonstrating the need for high-throughput techniques to study cellular response to multiple mechanobiological stimuli.

5.5 Conclusions

A microfabricated array was designed and developed to simultaneously test cellular response to a range of cyclic equibiaxial substrate strains. The device generates uniform strains from 2 to 15% across the array, a range most often used in macroscale experiments. As a first demonstration of this technology, the effects of mechanical stimulation on the differential accumulation of \( \beta \)-catenin in the nuclei of mesenchymal progenitor cells were investigated. The experiments revealed a complex relationship between stimulation time, strain magnitude, and nuclear accumulation of the protein, an effect that may not have been observed without a high-throughput approach to these studies. More generally, this platform can be used to create a variety of strain fields and can be extended to systematically and combinatorially manipulate multiple mechanobiological parameters, including extracellular matrix, chemical cues, and mechanical stimulation, for high-throughput screening research.
Chapter 6

“… in which mere surface changes are shown to significantly improve long-term compatibility.”

6 Integrating alternative materials

A critical limitation in the utility of the device described in Chapter 5 is the inability to conduct long-term stimulation experiments: VICs lose adhesion to the PDMS substrates when subjected to cyclic strain over extended periods, preventing the study of longer-term, functional cell commitment and transition in response to mechanical strain. More generally, our results suggest that the use of PDMS as a culture substrate precludes long-term culture and may significantly impact cell response. The experiments described in this chapter address the broader possibility of incorporating polyurethanes into PDMS devices. Here, a method to integrate polyurethane (PU), a well-studied and clinically relevant biomaterial, into the PDMS multilayer microfabrication process is described, thereby enabling the exploration of long-term cellular response on alternative substrates in microdevices. To demonstrate the utility of these hybrid microdevices for cell culture, initial cell adhesion, cell spreading, and maintenance of protein patterns on PU and PDMS substrates are compared. The results demonstrate that the use of a generic polyurethane substrate in microdevices enables cell culture comparable to that on tissue culture plastic in longer-term studies. More generally, this technique can improve the impact and applicability of microdevice-based research by facilitating the use of alternate, relevant biomaterials while maintaining the advantages of using PDMS for microdevice fabrication.

6.1 Introduction

Cellular function is regulated by several features of the external microenvironment, including substrate, chemical, and mechanical factors. Rapid progress is being made in developing high-throughput experimental approaches to study cellular response to various stimuli: growth

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3 The work presented in this chapter was published in:

factors, chemical cues and culture parameters[7], mechanical forces[109, 189, 192, 255],
extracellular matrix proteins[6], gradients in chemical stimuli[210], and co-culture
conditions[256]. These approaches are building towards techniques to determine the integrated
cellular response to combinations of stimuli, thereby improving understanding of fundamental
cell-microenvironment interactions and guiding developments in biomaterial design and tissue
engineering.

Advances in many high-throughput methods can be attributed to the development of
microfabricated technologies. Specifically, soft lithography fabrication of polydimethylsiloxane
(PDMS) microdevices shows great promise in developing miniaturized systems to study cell
biology. PDMS is an excellent material for microfabrication: it is elastomeric, transparent, non-
toxic, gas permeable, inexpensive, chemically stable, can be cured at low temperatures, and can
retain micropatterned features during processing[215]. The availability of this material has been
an important factor in driving microdevice development.

Multilayer soft lithography[99] has been used to form more complex PDMS structures, and has
led to fully integrated devices capable of systematically, combinatorially and rapidly probing
biological systems. However, many multilayer soft lithography systems are limited to culturing
cells directly on a PDMS substrate[7, 152, 189, 192, 194, 255, 257-263]. PDMS is poorly suited
for and rarely used in standard cell culture experiments, particularly those requiring extended
culture periods of several days. The substrates on which cells are grown can have a substantial
impact on cellular function[249, 250], and using PDMS for this purpose is one factor that
hampers the adoption of microdevices as a mainstream technology for biomedical research.
Chemical modification of the PDMS surface to improve cell culture is possible[195, 251, 263,
264], but challenging and not easily accessible to many research groups with expertise in
microfabrication.

The inability to use more common biomaterial substrates in novel microdevices limits their
utility for applications in cell biology, biomaterials, and tissue engineering research. For
example, PDMS is a poor material for tissue engineering scaffolds because it is highly resistant
to degradation, has non-physiological mechanical properties, and is difficult to fabricate into
porous structures. Biological results obtained using PDMS microdevice research platforms may
not be applicable to the systems for which the studies are intended, because the substrate
materials will, by necessity, be different. Hence, there is a need to conduct these microdevice studies on relevant culture substrates and biomaterials. The use of alternative materials to create hybrid devices[265], or to completely replace PDMS with conventional biomaterials as the primary structural material in microdevices[266-268] are potential approaches; however, material properties and processing requirements of these alternative materials can make complex multilayer microfabrication difficult.

To improve the relevance of biological results obtained using such PDMS microfabricated platforms, a simple, robust and easily accessible method to integrate polyurethane (PU) substrates into the multilayer PDMS soft lithography process is presented. The polyurethane class of polymers was selected based on the ease with which it can be chemically tailored for a specific application, as well as its widespread use in implantable medical devices[269, 270] and tissue engineering scaffolds[271-274]. Thus, our approach retains the advantages of using PDMS in microfabrication while enabling studies with a well-established, clinically-relevant biomaterial. In this paper, we describe and characterize the process to integrate Tecoflex®, a generic formulation of polyurethane, into multilayer PDMS microdevices. In addition to the general advantages of being able to conduct cellular experiments on substrates relevant to clinical applications, we have quantitatively demonstrated specific improvements in long-term cell culture conditions when using polyurethane over PDMS as a culture substrate in microdevices.

6.2 Materials and Methods

Unless otherwise stated, all chemicals and reagents for cell culture were purchased from Sigma-Aldrich (Oakville, ON, Canada); fluorescent dyes from Invitrogen (Burlington, ON, Canada); and all other materials from Fisher Scientific Canada (Ottawa, ON, Canada).

6.2.1 Polyurethane film preparation

Polyurethane (PU) films were formed by dip-coating handling slabs into a solubilized polymer. Tecoflex® aliphatic polyurethane pellets (SG-80A; Lubrizol Corporation; Wickliffe, OH, USA) were dissolved in tetrahydrofuran (THF) at concentrations ranging from 2-10 wt%, in preparation for dip-coating. PDMS was used to make the handling slabs, as it is flexible, and the PU films were easily released from this silicone material. Sylgard 184 PDMS (Dow Corning,
purchased through A.E. Blake Sales Ltd., Toronto, ON, Canada) was mixed in a standard 10:1 curing ratio and poured into an Omniwell tray (Nunc; Rochester, NY, USA). The PDMS was then degassed in a vacuum chamber to remove air bubbles, before curing overnight at 80°C. The PDMS was then peeled from the tray and cut to size. This handling slab was then dip-coated in the PU solution. THF causes the PDMS to swell, so the immersion time was minimized to less than two seconds. After dip-coating, samples were quickly transferred to a beaker, stored vertically, covered and left to dry for at least two hours at room temperature. Drying the samples in a container was critical, as excessive airflow was shown to cause rough, opaque surfaces. A Wyko optical profilometer (Veeco Instruments Inc., Woodbury, NY, USA) was used to characterize the dip-coated PU films. A section of the film was cut and peeled away, and the step-height and surface roughness were measured for each of the solution concentrations used in the dip coating procedure (n=3). A concentration of 5% PU in THF was used in all subsequent experiments in this chapter.

6.2.2 Integrating PU films into PDMS microfabrication

Integration of a PU film into microfabricated PDMS devices was achieved using a ‘mortar layer’ of partially cured PDMS as an intermediary layer[275] between a fully cured PDMS sheet and the PU coated on the handling slab. Briefly, the process (schematic outlined in Figure 6-1A) involved preparation of PDMS in the standard 10:1 ratio, and spin-coating a 15 μm film of PDMS on a plastic surface (Ink-jet transparency, Grand & Toy; Toronto, ON, Canada). This thin PDMS film was partially cured for 10 minutes at 80°C. The PDMS device layer and the partially cured PDMS film were treated with oxygen plasma (corona discharge treatment, Electro-Technic Products; Chicago, IL, USA), and placed in contact with each other. The sandwich was cured for an additional 10 minutes at 80°C, and the transparency was peeled away, leaving a partially cured ‘mortar layer’ of PDMS adhering to the fully-cured PDMS device layer. This mortar PDMS layer and the PU film (mounted on the handling slab) were then treated with oxygen plasma and placed in contact with each other, for a further 10 minutes at 80°C. The handling slab was then peeled from the PU-PDMS structure, which was then fully cured for 4 hours at 80°C.
Figure 6-1. Fabrication process flow. (A) Bonding a PU film to a PDMS slab. (i) The PDMS layer is placed in contact with a partially cured ‘mortar layer’ of PDMS. (ii) The PDMS is peeled away, retaining the partially cured PDMS in the regions of contact. (iii) The PDMS is then brought in contact with a dip-coated PU film, (iv) which is subsequently peeled away from the handling slab. (B and C): Schematic cross-sections of multilayer PDMS microchannels with (B) a PDMS culture surface and (C) a PU culture surface. (D): Fabricated microchannel with integrated PU culture surface. The channel was filled with a red dye to allow easy visualization.
6.2.3 Multilayer microfabrication of PU-PDMS structures

A simple microchannel device, which mimics the fabrication process for a complex multilayer PDMS system while maintaining experimental simplicity, was formed from two layers of PDMS (Figure 6-1B-D). In this study, the channels were fabricated with widths of 1500 µm and lengths of 35 mm. An upper layer with a rectangular channel relief pattern was formed by replica molding on microfabricated SU-8 masters. Briefly, the channel patterns were drawn in AutoCAD, and printed on a transparent film using a high-resolution laser plotter (City Graphics; Toronto, ON, Canada). SU-8 25 photoresist (Microchem; Newton, MA, USA) was spin-coated at 1000 RPM on pre-cleaned 3” × 2” glass slides. The slides were pre-baked, exposed to UV light through the printed film, post-baked and developed, using parameters outlined by the resist manufacturers. The resulting SU-8 structures were optically profiled and determined to be 50 µm high. PDMS was then mixed, poured, degassed, cured, and peeled from the SU-8 master.
Fully cured sheets of unpatterned PDMS were also prepared for the base layer of the microchannel, and both layers were then cut to size. For devices without PU films (Figure 6-1), the PDMS channel and base layers were bonded to each other and then to a clean glass slide, by treating the surfaces with oxygen plasma. For microchannels with PU culture substrates (Figure 6-1C), a PU film was bonded to the PDMS base layer using a mortar layer as described in Section 6.2.2. The process was then repeated with the upper channel layer: a partially cured PDMS film was transferred to the upper channel layer, plasma-treated, and placed in contact with the PU-PDMS structure before completing the curing process.

To test the adhesion strength between polyurethane and PDMS using this bonding method, a test structure was fabricated, consisting of a PU membrane suspended over a hole cored into a PDMS slab (Figure 6-2A-B). The base was formed from a fully cured PDMS slab with holes cored in it using a 5 mm disposable biopsy punch (Sklar Instruments; West Chester, PA, USA). The PU-PDMS structure was formed by first applying a partially cured PDMS mortar film (on a plastic inkjet transparency) to the PDMS base. Because the PDMS mortar film had not fully solidified, the mortar did not form a suspended membrane over the cored PDMS slab and remained on the transparency surface in these regions. The PU film was then bonded to the PDMS base. The PDMS base was then plasma-treated and sealed to a glass slide. To visualize the resulting structures, the suspended membranes were coated with 15 nm of platinum and visualized in a scanning electron microscope (Hitachi). To test the adhesion strength between the polyurethane and PDMS, increasing pressure was applied across the polyurethane membrane by connecting the PDMS cavity to a tank of compressed nitrogen via a pressure regulator, and visually observing membrane distention at various pressures.

### 6.2.4 Evaluation of cell response to PU versus PDMS substrates

In addition to the general advantages of being able to integrate a clinically-relevant biomaterial into the microfabrication process, the demonstrated incorporation of the Tecoflex® material into PDMS devices may yield specific technical advantages in improving device utility and biological relevance, particularly for long-term culture. To test this hypothesis, we compared cell adhesion, spreading, and initial attachment strength to native PDMS and PU substrates. Because cell adhesion to PDMS substrates is often promoted by adsorption of extracellular matrix proteins prior to cell seeding, we also compared cell adhesion to plasma-treated PDMS coated with type I
collagen (PDMS+ECM) and plasma-treated PU coated with type I collagen (PU+ECM). Tissue culture-treated polystyrene (TCP) was used as a positive control substrate in some experiments.

6.2.4.1 PAVIC isolation and cell culture

Primary porcine aortic valve interstitial cells (PAVICs; a fibroblast-like cell) were used as model primary cells. Cells were isolated by enzymatic digestion as described[276] and used between passage two to six for all experiments.

6.2.4.2 Adhesion and spreading measurements

Cell adhesion and spreading were measured on pieces of PDMS and PU-PDMS cut to size and placed in 12-well plates. The substrates were soaked in 70% ethanol, air-dried, and exposed to UV light for 30 minutes to sterilize them. Samples to be coated with matrix proteins were exposed to oxygen plasma and incubated with 50 µg/mL type I rat-tail collagen (Becton Dickinson, Mississauga, ON, Canada) overnight at 4°C. The substrates were then washed three times in phosphate-buffered saline (PBS) before seeding PAVICs at 3,000 and 10,000 cells/cm² for spreading and adhesion experiments respectively, and incubating at 37°C, 5% CO₂. To assess initial adhesion, the samples were washed with PBS 12 hours after seeding and fixed in 10% neutral-buffered formalin. They were then stained with Hoechst 33342 nuclear dye and imaged with a 10× objective under a fluorescent microscope (Olympus IX71; Olympus Microscopes; Markham, ON, Canada) with a CCD camera (Retiga 2000R; QImaging; Surrey, BC, Canada). The number of adhering cells were counted manually in five fields of view for each condition and repeated at least three times.

To determine spread area, samples were cultured for three and six days, washed in PBS, fixed and stained for 10 minutes in 1% (w/v) crystal violet stain solubilized in 100% ethanol. They were then washed thoroughly in water and imaged using bright-field microscopy. Cell spread area was measured using ImageJ (NIH) and determined in at least three fields of view, for four samples per condition.

6.2.4.3 Microfluidic adhesion strength assay

The strength of cell adhesion to the various substrates was measured in microchannels using a shear assay, as described by Young et al.[109]. Microchannels with and without PU layers were
fabricated as described, mounted to glass slides, and assembled with polyethylene tubing as inlet ports for fluid injection and withdrawal. Channels were sterilized by flushing them with 70% ethanol for 10 minutes and with sterile PBS for an additional 10 minutes. Sterile air was flushed through the device to evacuate the channels. Channels were dried by placing them on a hotplate at 50°C for 20 minutes. For those channels to be treated with oxygen plasma before collagen coating, the tip of a corona discharge unit was inserted into the inlet port of the microchannel. The plasma was observed to traverse the channel, and this treatment was maintained for 30 seconds. To coat the channels with an ECM protein, type I collagen was flushed into the channels at a concentration of 50 μg/mL and incubated at room temperature for 30 minutes. Sterile PBS was used to displace the collagen, and the channels were rinsed three times.

PAVICs were fluorescently labeled with a vital nuclear dye prior to loading them into the microchannels by incubating with Hoechst 33342 (2 μg/mL in fully supplemented DMEM) for 30 minutes at 37°C, 5% CO₂. They were then washed with PBS and maintained in fully supplemented media for at least 30 minutes. Cells were trypsinized from the culture flasks and re-suspended in supplemented media at a concentration of 10×10⁶ cells/mL. Using a syringe, 200 μL of the cell suspension was injected into each of the microchannels, and incubated for 2 hours at 37°C, 5% CO₂ to allow initial cell adhesion and spreading on the microchannel substrate.

A multi-channel syringe pump (Cole-Parmer, Montreal, QC, Canada) was used to clear the microfluidic channels of non-adhered cells by flushing them with supplemented DMEM at a rate of 3 mL/hr for 2 minutes. To investigate initial adhesion strength, cells were subjected to shear treatments for 2 minutes each at rates of 30 mL/hr and 60 mL/hr. These flow rates translate into shear levels of 98 and 195 dynes/cm², respectively, based on equations used by Young et al. [109] for similar experiments.

\[
\tau_w = \frac{2 \mu Q}{wh^2} \left( \frac{m+1}{m} \right) (n+1)
\]

where \(\tau_w\) is the applied shear, \(Q\) is the volumetric flow rate, width \(w = 1500\mu m\), height \(h = 50\mu m\), viscosity of the culture media \(\mu = 0.72 \times 10^{-3} \text{ kg/ms at 37°C}\). \(m\) and \(n\) are calculated based on the aspect ratio of the channels, and were found to be 60.17 and 2 respectively.
The number of cells in each channel was determined before and after shear treatments by imaging the nuclei at five marked locations along the channel length, using a fluorescent microscope (Leica; Wetzlar, Germany) and camera (Hamamatsu Photonics; Hamamatsu, Japan). These experiments were repeated for two to four channels per condition.

### 6.2.5 Assessment of protein pattern maintenance in long-term culture

Maintaining the ECM protein microenvironment in cell culture conditions is an important requirement for long-term cell culture. In order to assess the maintenance of ECM protein patterns on PU and PDMS substrates, protein pattern fidelity was monitored over time. PDMS samples were prepared by bonding a thin PDMS sheet to a glass substrate. PU samples were prepared by dip-coating a silane-coated glass slide (Sigma) in 5% PU solution. An elastomeric stencil[129, 221] was used to pattern proteins into an array of 300 µm diameter circles. The stencil was constructed by exclusion molding[220] to create a thin film of PDMS with through-holes which was then bonded to a thick PDMS gasket. The sample and stencil were then sterilized by exposure to UV exposure light for two hours. The stencil was carefully placed on the substrate where it formed a conformal contact. The sample was then plasma-treated through the stencil, and 50 µg/mL of FITC-labelled bovine type I collagen (Exalpha Biologicals Inc.; Watertown, MA) was incubated in the gasket for two hours at room temperature. The protein was aspirated, and the sample washed with sterile PBS, before peeling away the stencil. The sample was then washed with 3% bovine serum albumin (BSA) in PBS to block the non-patterned regions on the substrate.

The patterned substrates were incubated with DMEM supplemented with 10% FBS, and fluorescently imaged at several time points over the course of four weeks. At each time point, the media was aspirated and samples were washed with sterile deionized water. A consistent exposure time of 500 ms with a gain of 10 was used to ensure an appropriate intensity comparison between samples. To quantify protein pattern fidelity, the total fluorescent intensity was measured inside and outside the patterned circles (n = 20), and the results scaled to the ratio of the measured areas. The ratio between the fluorescing pattern and the background was recorded and normalized to the ratio for that pattern at day zero.
6.2.6 Statistical Analyses

Data were analyzed using one and two-way ANOVA, as appropriate. Post-hoc pairwise comparisons were conducted using the Student-Newman-Keuls method. All statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA). Graphical results are plotted as means ± one standard deviation.

6.3 Experimental Results

6.3.1 Polyurethane film characterization

PU film thickness was found to increase for increasing concentrations of solubilized polymer (Figure 6-3A). For concentrations ranging from 2 to 10 wt%, film thickness increased from 4.6 ± 1.2 µm to 40.4 ± 3.6 µm. The increase was not linear over the range of concentrations tested, but increased sharply at concentrations greater than 6 wt%. Surface roughness was found to increase from 56 ± 18 nm to 202 ± 91 nm with higher concentrations of PU in the solvent (Figure 6-3B). The variation in roughness across each sample increased dramatically at concentrations higher than 6 wt%. The surface of the PU film was smooth and free of defects. Visual inspection confirmed the thickness of the films determined via surface profilometry (Figure 6-2A, B). Qualitatively, we found that films less than 6-7 µm thick were more prone to tears during handling. The surface wettability of native and plasma-activated PU were determined by water contact angle measurements (Table 6-1) and compared to the wettabilities of PDMS. The results indicated that plasma-activated PU and PDMS are more hydrophilic than native PU and PDMS substrates.
Figure 6-3. Characterization results for dip-coated polyurethane on a PDMS handling substrate. (A) Polyurethane film thickness and (B) surface roughness, measured as a function of the concentration of solubilized polyurethane (PU) used in dip-coating.
Table 6-1. Contact angle measurements for native and oxygen plasma treated polyurethane (PU) and polydimethylsiloxane (PDMS) surfaces. Measurements are based on the contact angles of drops of deionized water (5 µL) pipetted onto the material surface (n = 5).

<table>
<thead>
<tr>
<th></th>
<th>Native Substrate</th>
<th>O₂ plasma treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU</td>
<td>61.6 ± 1.8°</td>
<td>38.0 ± 3.6°</td>
</tr>
<tr>
<td>PDMS</td>
<td>104.4 ± 6.7°</td>
<td>3.1 ± 2.8°</td>
</tr>
</tbody>
</table>

6.3.2 Integrated PU films in microfabrication

PU culture surfaces were successfully integrated into multilayer microchannels (Figure 6-1A-D) using the mortar layer method, and were tested at flow rates of up to 7 mL/min, at which they maintained structural integrity and did not leak. On testing the adhesion strength between the PU and PDMS films in the suspended membrane structure (Figure 6-2A, B), the PU membranes underwent elastic deformation up to pressures of ~35 kPa without peeling away from the PDMS. At ~70 kPa, the membrane integrity was compromised before exceeding the adhesion strength at the interface of the two materials. The PU-PDMS structures were stored under PBS in a humidified incubator for three days, and this did not affect the bond strength.

6.3.3 Initial cell adhesion

Significant differences were found in the number of cells initially adhered to each of the substrates (p < 0.001; Figure 6-4). The number of cells that adhered initially to the PU, PU+ECM and PDMS substrates was significantly lower than that of the TCP control (p < 0.01). No statistically significant difference was found between initial adhesion to TCP and PDMS+ECM. Plasma treatment and collagen coating of PDMS improved initial cell adhesion significantly over native PDMS (p<0.01). No differences were found between the PU, PU+ECM and PDMS+ECM substrates; or between the PU, PU+ECM and PDMS substrates.
6.3.4 Initial cell adhesion strength

To determine the strength of initial cellular adhesion to various substrates and treatments, cells were introduced into the microfluidic channels (Figure 6-1D) and sheared by fluid flow. The proportion of cells remaining after two levels of shear (Figure 6-5A) provided a quantitative measure of adhesion strength for the various substrates and treatments.

The number of cells remaining after shear depended significantly on the substrate material (p < 0.001) and the shear level (p = 0.022) but there was no interaction between these factors (p = 0.901), by two-way ANOVA (Figure 6-5B). The PDMS substrate retained fewer cells than any other substrate (p < 0.001), and there were no significant differences in the number of cells remaining after shear on the PU, PU+ECM or PDMS+ECM substrates.
Figure 6-5. Results of the microfluidic shear assay probing initial cellular adhesion strength to the substrates. (A) Representative images of Hoechst nuclear-stained cells remaining in microchannels after increasing shear treatments (scale bar = 500 μm). (B) Comparison of cells remaining on each substrate following two shear treatments, normalized to the number of cells adhered in the static condition (*p < 0.001 compared to other substrates).
Figure 6-6. A comparison of cell spreading areas on different substrates, six days after seeding. (A-E): Representative pictures of cells under these conditions. (F, G): comparison of cell areas across the samples at day 3 and 6 (* p < 0.05; ** p < 0.01)
6.3.5 Cell morphology and spreading area

The morphology of PAVICs seeded on TCP is typically well-spread with long processes. For the first 12 hours after seeding, there was no significant difference between cell areas on each of these substrates. However, after three days in culture, cells were significantly less spread on PDMS than any of the other substrates (p < 0.01; Figure 6-6F). Furthermore, cells seeded on the PDMS+ECM substrate exhibited a significantly smaller spread area than those on the TCP control (p = 0.023). Cells on the PU, PU+ECM and TCP control samples spread similarly (Figure 6-6F). By day six (Figure 6-6G), cell spreading areas remained similar on TCP, PU and PU+ECM, and each case exhibited significantly better spreading than on the PDMS and PDMS+ECM substrates (p = 0.036).

6.3.6 Protein pattern maintenance

The maintenance of extracellular matrix protein (type I collagen) on plasma-treated PDMS and PU substrates was compared in culture conditions (Figure 6-7). The protein patterns degraded quickly on PDMS substrates, such that by day six in culture, the fluorescently labeled patterns on PDMS were undetectable. In contrast, protein patterns were maintained for at least 26 days on PU substrates (after an initial drop to 30% of the original level over the first 12 days). The normalized contrasts were statistically different based on both substrate material (p < 0.001) and culture time (p < 0.001), and showed a strong interaction between these two factors (p < 0.001). The pattern fidelity was significantly reduced on the PDMS substrate as compared to the PU substrate for every time point after the first 24 hours (p < 0.001). After day 8, there were no significant reductions in pattern fidelity on the PU substrate.
Figure 6-7. Assessment of protein pattern fidelity over time in culture conditions. (A): Evolution of a single protein spot patterned on PDMS and PU substrates. (B): Normalized contrast of the patterns ($n = 20$ for each time point) over 26 days.
6.4 Discussion

The ability to answer many complex questions in cellular biology is significantly facilitated by the utility and availability of microfabricated devices for cell culture applications. These microdevice-based approaches can have a substantial impact on fields such as tissue engineering, drug discovery, and fundamental cell biology. However, in many multilayer microfabricated systems, cells must be cultured on PDMS substrates, which can significantly impact their function. To address this issue, we have developed a technique to integrate polyurethane, a well-established, clinically-relevant class of biomaterials, into the PDMS microfabrication process, and have demonstrated this using a generic formulation of polyurethane as a model biomaterial. Thus, we maintained the advantages of using PDMS as a structural material in microfabrication while (1) improving long-term cell culture potential of soft microdevices and (2) enabling the use of alternative biomaterials in microfabricated systems, thereby improving the applicability of microdevices in cell biology and biomaterials science.

Polyurethane elastomers are easily customizable and can be designed for specific biomedical applications[269],[277]. Tecoflex® solution-grade polyurethane has excellent physical properties as a cell adhesion substrate[278], and can be processed by a variety of techniques. For this application, dip-coating was the most suitable for several reasons. Without expensive equipment or materials, dip coating rapidly and reproducibly forms PU films with thicknesses and optical properties consistent with those typically used in microfabrication. Surface roughness can be easily minimized by isolating the sample from airflow during drying. Dip-coating also allows flexibility in the type of handling slabs used, which can be particularly important in the microfabrication process. In this case, a flexible PDMS handling slab was used to facilitate peeling away from the rigid glass-PDMS-PU device layer. If the device layer is flexible, a glass slide can be used as the handling slab, and produces more uniform PU films. However, the adhesion between bare glass and PU is stronger than that of dip-coated PDMS to PU. Hence, this requires greater experimental skill to complete the film transfer, and was not used in this study. When transferred to the PDMS substrate, the PU film is complete and free of wrinkling, and the bond strength between the two materials is sufficient for most microfabricated devices, as demonstrated by bulge testing and the operation of hybrid PU-PDMS microfluidic channels.
Consistent with the findings of other researchers[279], we found that native PDMS is a relatively poor substrate for culture of adherent cells, as it was significantly worse than the control TCP, matrix-coated PDMS, bare PU and matrix-coated PU in terms of initial cell adhesion, adhesion strength, and cell-spreading area. This is likely due to the strong surface hydrophobicity, which has a demonstrated negative impact on cellular adhesion and function[280]. In microdevices in which cells are cultured on PDMS, the substrate is often coated with an ECM protein to improve cellular adhesion. Several methods to coat PDMS with matrix protein exist[130, 281, 282]. Wipff et al.[282] compared several techniques of coating PDMS with ECM and found significantly improved cellular response for their multi-step PDMS preparation process over other more common techniques. However, they did not compare their method with protein adsorption to plasma-activated PDMS surfaces, which can potentially impact the activity of the adsorbed matrix protein and is commonly used when conducting biological experiments on microfabricated PDMS platforms[122]. Though it has been demonstrated that greater quantities of collagen adhere to hydrophobic surfaces[283] such as native PDMS, there are concerns that matrix proteins adsorb to these surfaces in different conformations, depending on surface wettability[284-286]. These conformational differences can affect cellular adhesion and function. In the case of fibronectin and vitronectin, adsorption to a hydrophobic surface has been shown to significantly reduce the bioactivity of the molecule[284-286]. Whether this applies to collagen specifically has not been addressed. For the purposes of this study, we elected to deposit proteins on plasma-activated substrates, based on lower cell spreading areas and adhesion strengths observed on matrix-coated hydrophobic PDMS, as compared to matrix-coated hydrophilic PDMS (data not shown). Furthermore, others have demonstrated that protein adhesion to plasma-activated polyurethane is covalent[287], which was supported by our observation that protein patterns survived for close to four weeks in culture conditions on plasma-activated polyurethane substrates. Characterization of surface wettability by contact angle measurements indicated that plasma-activated PU is more hydrophilic than native PU (Table 6-1). Since covalent ECM binding on PU is associated with plasma-activated, hydrophilic surfaces, we made the PDMS surfaces hydrophilic as well, in order to minimize experimental differences caused by conformational changes in collagen, and to accurately compare cellular response based on substrate material properties alone.
In terms of initial cell adhesion and adhesion strength, there were no significant differences between collagen-coated PDMS, polyurethane and collagen-coated polyurethane, suggesting that these substrates are interchangeable in terms of suitability for short-term cell culture. It should be noted however, that some polyurethanes have been designed or modified specifically for cell adhesion. For example, Dennes et al. reported a method to modify the Tecoflex® polyurethane with RGD peptide, achieving a several-fold increase in cellular adhesion[288]. Techniques have been reported to improve and optimize initial cell adhesion to generic polyurethanes by modifying the surface with oxygen plasma prior to seeding[289]. For this study, polyurethane was neither designed nor optimized for cell adhesion, and these modifications can only further improve this polymer’s desirability as a culture substrate over PDMS.

For longer-term cell culture, there are notable differences in cell response for the PDMS and PU materials. Cell spreading area is a measure of cellular response to a substrate, and has a demonstrated impact on cellular function[9]. After three days in culture, cells grown on the PDMS and PDMS+ECM substrates were significantly less spread, while cell spreading area on the polyurethane substrates was comparable to those on TCP. After six days in culture, cells on the polyurethane substrates remain similar in spreading area to those on TCP, and cell spreading area in this group was significantly larger than on the PDMS substrates (Figure 6-6). This could be due to the dimethylsiloxane chains in PDMS migrating to reduce the surface energy, thereby renewing the surface hydrophobicity[290], or to a loss of adsorbed ECM protein from the surface. The loss of protein pattern fidelity on PDMS substrates (Figure 6-7) supports this hypothesis, although it is unclear as to whether those proteins are being removed, or are being replaced in competition with proteins in the supplemented media. The poor cell spreading area observed on PDMS as early as three days after seeding suggests that the proteins are not being replaced by adhesion proteins in the serum-supplemented media (e.g., vitronectin), and the resulting functional differences could have a significant impact on cell function. The inability of PDMS to maintain adhesion to matrix proteins under culture conditions, even in the absence of cells, could explain the unsuitability of PDMS for mechanically dynamic, long-term cell culture studies.

The Tecoflex® polyurethane substrates performed just as well as PDMS in short-term culture conditions and showed significant improvement over PDMS for longer experiments. More generally, polyurethanes have a well-established chemistry and are already in widespread use for
implanted devices, tissue engineering scaffolds, and cell biology experiments. Hence, the integration of polyurethane into PDMS microdevices not only extends culture time, but also enables the incorporation and control of clinically-relevant culture substrate conditions in multilayer microfabricated research platforms.

### 6.5 Conclusions

Polyurethane was successfully integrated into the PDMS multilayer soft lithography fabrication process, so as to improve the long-term cell culture capabilities of microdevices. While initial cell adhesion, spreading and adhesion strength on PU and matrix-coated PDMS were similar, the PDMS surface was not able to maintain matrix proteins and cell spreading in long-term culture. Hence, integrating PU into PDMS devices maintains the advantages of using PDMS in soft lithography, while improving the long-term cell culture capabilities of these devices, thereby increasing the overall impact, accessibility and applicability of microfabricated devices in biomedical research.
7 High-throughput combinatorial screening

Biomaterial advances in integrating polyurethane with PDMS microsystems described in the previous chapter are utilized in this chapter to fabricate composite culture films, which maintain the ease of microfabrication and suitable mechanical properties of PDMS, while improving adhesion of cells to the mechanically deforming substrates. In order to address the issues of device complexity and limited production capabilities encountered in Chapter 5, this chapter focuses on the development and utilization of a substantially simpler microfabricated system, in which a composite film is distended by the application of pressure across the film. Although this approach sacrifices the uniformity and precision of applied strain fields, the simplified process enables medium-scale production of such devices, in order to conduct combinatorial studies in mechanobiology. In order to demonstrate the utility of this simplified technology, we use this platform to study heterogeneity within a limited quantity of VICs isolated from different regions of the heart valve, in terms of their functional transition to a pathological phenotype in response to combinatorially manipulated mechanical, chemical and matrix cues.

7.1 Introduction

Aortic valve sclerosis is a common disease affecting 20-30% of the population over the age of 65, and is associated with a 50% increased risk of other cardiovascular disorders[291]. Pathological changes in the aortic valve include increased cellularity, lipid accumulation, and deposition of disorganized matrix, leading to fibrotic thickening and the formation of focal subendothelial lesions, leaflet stiffening, imperfect coaptation, and disturbed hemodynamic flow[292]. Complete valve replacement is the only currently available medical therapy for this condition.

Valve interstitial cells (VICs) are the primary mediators of tissue homeostasis, matrix remodeling, and disease progression[293]. *In vivo* differentiation of VICs to the myofibroblast phenotype is strongly correlated with the formation of lesions, matrix disarray, and fibrosis[294-297], implicating myofibroblasts as having key roles in disease progression, likely through their
increased contractility and secretion of matrix proteins[298]. The incorporation of α-smooth muscle actin (SMA) into stress fibers is considered the defining characteristic of differentiated myofibroblasts[299], and has been shown to increase force generation[300] by the cell.

The influence of multiple microenvironmental conditions on the differentiation of VICs to the pathological myofibroblast state is poorly understood. VICs exist in a complex environment, and myofibroblast differentiation is known to be influenced by matrix stiffness[184], mechanical stimulation[301], chemical factors[302-304] and matrix proteins[303], which potentially act in combination to regulate cell function[8, 305]. Furthermore, although the valve is only a few hundred microns thick, valve composition is highly structured and organized, consisting of three distinct layers: the fibrosa on the aortic side; the spongiosa; and the ventricularis on the ventricular side. We suggest that VICs isolated from these spatially distinct regions exhibit differing myofibroblast differentiation potential in response to microenvironmental stimuli. This hypothesis is supported by the observations that layers exhibit differing matrix compositions in vivo[306], that layers experience distinct mechanical conditioning stimuli as the leaflet bends[307], and that focal lesions preferentially form in the fibrosa, whereas the ventricularis is relatively disease protected[296]. Endothelial cells on either side of the valve leaflet are known to exhibit distinct genotypes[308] and responses to shear stress[309], but spatial heterogeneity in the myofibroblast differentiation potential of VICs remains undefined.

In order to rapidly screen for the combinatorial effects of matrix proteins, chemical cues and mechanical stimulation in multiple spatially isolated cell types, we developed a microfabricated in vitro culture system designed to apply two distinct cyclic substrate strains to cultured VICs, in combination with other mechanobiological parameters. Using this platform, we demonstrate that mechanically induced myofibroblast differentiation of side-specific VICs is differentially modulated by matrix and soluble cues, and that these results have implications in understanding disease pathobiology and heart valve tissue engineering.

### 7.2 Materials & Methods

Unless otherwise stated, all chemicals and reagents for cell culture were purchased from Sigma–Aldrich (Oakville, ON, Canada); fluorescent dyes from Invitrogen (Burlington, ON, Canada); and all other equipment and materials from Fisher Scientific Canada (Ottawa, ON, Canada).
7.2.1 Device overview

The microfabricated device consists of an array of suspended circular films. Cells cultured on the films experience radial and circumferential strains when the films are distended by the application of pressure, via a network of underlying PDMS microchannels. Groups of films are segregated using PDMS gaskets, enabling multiple matrix proteins, chemical cues and cell types to be probed in parallel (Figure 7-1). In order to improve long-term adhesion between cells and the substrate, polyurethane (PU) culture films were integrated into the PDMS microfabrication process, as previously described[201]. Polyurethane can covalently bind matrix proteins[287], enabling long-term culture, and presumably improve cell adhesion under cyclic load. However, free-standing films of PU were observed to exhibit creep over early cyclic loading, and hence are not suitable for this application. To address this issue, diaphragms consisting of a thick (45-100 µm) film of PDMS were layered with a thin (<1 µm) film of PU, to create a composite material structure, maintaining the advantages of PDMS in microfabrication and in material properties, while improving adhesion under cyclic mechanical load.

7.2.2 Fabrication process

The complete fabrication process is illustrated in Figure 7-2, and consists of preparation of a PDMS base layer and a composite PDMS-PU culture film, and bonding the two together. The PDMS base layers of the devices are fabricated by sandwich molding[220] against a multilayer master mold. Masters were fabricated on 3” × 2” glass slides, with SU-8 negative photoresist (Microchem; Newton, MA, USA), following standard parameters outlined by the manufacturer. Two-layer devices were produced using subsequent alignment and exposure steps, creating masters with a distribution channel network 150 µm thick, connecting pillars 300 µm thick (heights verified by optical surface profilometry). SU-8 masters were silanized and sandwich molding was used as previously described[202] to produce a negative replica film of the mold master on the transparency, where the SU-8 pillars form vertical through holes. This patterned PDMS layer is then bonded and transferred onto a clean glass slide using an oxygen plasma-generating corona discharge unit (BD-20A; Electrotechnic Products; Chicago, IL, USA).

PU films were formed by dip-coating an untreated PDMS handling slab into a 0.5 wt% solution of SG-80A Tecoflex® polyurethane (Lubrizol Corporation; Wickliffe, OH, USA) in tetrahydrofuran, and allowing it to dry overnight in a covered beaker[201]. The resulting film is
less than one micron in thickness, and does not adhere tightly to the PDMS slab. The exposed PU surface was treated with oxygen plasma, and uncured PDMS was spin-coated onto the slab to form a film of PDMS, 45-100 μm thick. The PDMS was partially cured in an oven at 80 °C for 20 minutes, until the PDMS layer remains sticky, but does not deform permanently. The fabricated device and PU-PDMS composite layers were then plasma treated, placed in conformal contact, and baked on a hot plate for at least 10 minutes at 80 °C to permanently bond the substrates. The handling slab was then carefully peeled away, leaving the composite PDMS-PU film tightly adhered to the device.

Figure 7-1. Device overview. (A, B) Composite films consisting of a thick layer of PDMS and a thin layer of PU are (A) suspended over an actuation cavity; and (B) distended by the application of a pressure waveform. (C) High-throughput system consisting of 12 wells, each containing 9 actuatatable culture films; fabricated on a 3” x 2” glass slide.
Holes were then cut into a thick PDMS gasket, which was plasma bonded to the device surface to segregate groups of culture films. PDMS connectors (previously described in [187]) were then used to connect the array of wells to a regulated pressure source.

### 7.2.3 Operation parameters

Each device consists of an array of 108 circular suspended films, separated by the PDMS gasket into twelve groups of nine units each. Circular films were each two millimeters in diameter, and two film thicknesses were selected to produce two distinct strain fields under differing pressures, generated using miniature rotary vane pumps (SP-135 FZ and SP-135 FZ-LC; Schwarzer Precision; Germany), and controlled using solenoid valves (Pneumadyne; Plymouth, MN, USA) driven at 1 Hz. Relatively low strains were produced by applying a pressure of 6 kPa across a 100 μm thick film, and high strains were produced using an 11 kPa pressure source across a 45 μm thick diaphragm (Table 7-1).

Figure 7-2. Fabrication process for the composite culture films and simplified mechanical stimulation system.
7.2.4 Analytical model of surface strain

Analytical models characterizing deformation of a clamped circular plate have been previously developed [310], and the following equations were used to relate peak vertical deflection with radial and circumferential strains generated across the surface:

\[ \varepsilon_r(r) = \frac{1}{E} (\sigma_r - \nu \sigma_\theta) \]  
Equation 7-1

\[ \varepsilon_\theta(r) = \frac{1}{E} (\sigma_\theta - \nu \sigma_r) \]  
Equation 7-2

where \( \varepsilon_r \) and \( \varepsilon_\theta \) are the radial and circumferential strains respectively, and are functions of the radial distance \( r \). \( E \) is the Young’s modulus and \( \nu \) is the poisson ratio of the deforming material. Further:

\[ \sigma_r = -\frac{6D}{h^2} \left( \frac{d^2 w}{dr^2} + \nu \frac{dw}{dr} \right) + \frac{1}{r} \frac{d\phi}{dr} \]  
Equation 7-3

\[ \sigma_\theta = -\frac{6D}{h^2} \left( \frac{1}{r} \frac{dw}{dr} + \nu \frac{d^2 w}{dr^2} \right) + \frac{d^2 \phi}{dr^2} \]  
Equation 7-4

where \( D \) is the flexural rigidity of the circular film and is defined by

\[ D = \frac{Eh^3}{12(1-\nu^2)} \]  
Equation 7-5

Here \( h \) is the thickness of the diaphragm and \( w(r) \) and \( \Phi(r) \) are defined as:

\[ w(r) = f \left[ 1 - \left( \frac{r}{a} \right)^2 \right]^2 \]  
Equation 7-6

\[ \Phi(r) = \frac{f^2 E}{12} \left[ \left( \frac{5 - 3\nu}{1-\nu} \right) \left( \frac{r}{a} \right)^2 - \frac{1}{4} \left( \frac{r}{a} \right)^4 + \frac{1}{9} \left( \frac{r}{a} \right)^6 - \frac{1}{48} \left( \frac{r}{a} \right)^8 \right] \]  
Equation 7-7

and \( f \) and \( a \) are the measured deflection and radius of the film, respectively.
7.2.5 Strain characterization

Characterization of surface strains was conducted by tracking the vertical displacement of fluorescent beads. Fluorescent polystyrene beads (Bangs Laboratories; Fishers, IN, USA) of 1 μm diameter were suspended in methanol and vortexed for 1 min to break up bead clusters. The solution was then pipetted onto the device surface and allowed to dry at ambient temperature. Confocal microscopy (Fluoview 300, Olympus Microscopes; Markham, ON, Canada) of the fluorescent beads was used to track diaphragm displacement at rest, and when actuated. Side-reconstruction of the confocal images (Figure 7-3 A,B) was used to determine vertical diaphragm displacement. These results were then used in the previously described analytical model to determine the applied radial and circumferential surface strains (Figure 7-3C). This imaging and analysis procedure was repeated after $10^0$, $10^5$ and $10^6$ actuation cycles to determine mechanical stability of the diaphragms under cyclic load. Video microscopy was used to determine the time constants required for actuation and relaxation of the bulging diaphragm. Peak deflection was tracked for each video frame, and time constants were calculated as the average time required for 95% actuation or relaxation of the diaphragm.

A sub-region of the device surface, a 600 μm diameter circle concentric with the circular film, was selected as the analysis region of interest (labeled ‘ROI’ in Figure 7-3C), so as to minimize radial and circumferential strain variation across the device surface, while maintaining a suitably large culture area for cells to adhere (Table 7-1). Each of the following analyses for cell experiments was limited to this region of the deforming substrate.

Table 7-1. Summary of device parameters and characterization results.

<table>
<thead>
<tr>
<th>Radius</th>
<th>Thickness</th>
<th>Pressure</th>
<th>Strain (within a 600 μm diameter ROI)</th>
<th>Nominal strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mm</td>
<td>100 μm</td>
<td>6 kPa</td>
<td>Radial: 3.4 ± 0.3 %</td>
<td>Low (~3%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Circumferential: 3.4 ± 0.3 %</td>
<td></td>
</tr>
<tr>
<td>1 mm</td>
<td>45 μm</td>
<td>11 kPa</td>
<td>Radial: 12.4 ± 0.2%</td>
<td>High (~12%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Circumferential: 11.3 ± 1.7%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7-3. Strain characterization across device surface. (A, B) Fluorescent beads were used to track culture film displacement (green = bead locations at rest; red = bed locations when actuated). (A) Top view of bead displacement; and (B) confocal image of side view were used in conjunction with an analytical model to (C) calculate radial and circumferential strains across the device surface. A selected region of interest (ROI) was used to minimize strain variations across the surface, while maintaining a suitable culture area for cells.
7.2.6 Layer specific isolation of VICs

Hearts were obtained from 8-month-old pigs from a local abattoir (Quality Meat Packers, Toronto, ON). Aortic valve leaflets were excised and rinsed in phosphate buffered saline (PBS) containing 1% penicillin and streptomycin and 0.5% amphotericin B. Rinsed leaflets were incubated in collagenase (type I, 150 Units/mL) in N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer with 0.36 mmol/L calcium chloride (pH = 7.4) for 20 minutes at 37 °C to digest the leaflet matrix. Leaflets were vortexed and lightly scraped on both surfaces to remove endothelial cells. Each leaflet was then pinned and secured (ventricular surface up) to a dissecting dish filled with black wax, using four needles (25 gauge) passing through only the fibrosa. A micro-knife (Fine Science Tools Inc; North Vancouver, BC, Canada) was used to gently cut into the spongiosa layer to remove the fibrous connections between the ventricularis and the fibrosa, while the ventricularis was gently grasped and pulled upward with fine dissecting forceps. Only the area below the nodulus of Arantius, where the tissue structure is distinctly trilayered, was separated and taken for cell isolation. Separated fibrosa and ventricularis sides of the selected region were incubated in hyaluronidase (type I-S, 2 mg/mL) in PBS for 1 hour at 37 °C. This step cleanly digested the spongiosa layer partially attached on both fibrosa and ventricularis sides (histological data shown in Appendix E). Cells from the spongiosa were released into the hyaluronidase, and subsequently discarded. The cleaned fibrosa and ventricularis layers were then separately minced and incubated in collagenase (type I, 150 Units/mL, Sigma) in TES buffer for a second digestion step on a rocker at 37 °C for 2 hours. Undigested tissues were removed using a cell strainer (pore size 70 μm), and volumes of supplemented medium (Dulbecco’s modified Eagle’s medium; DMEM, with 10% fetal bovine serum and 1% penicillin and streptomycin) equal to the separate cell suspension volumes were added to each, and centrifuged at 284 × g. Supernatant was removed and the cells were re-suspended in supplemented medium and plated on tissue culture-treated polystyrene.

7.2.7 Device preparation and cell culture

Devices were sterilized by immersion in 70% ethanol for 5 minutes, and subsequent exposure to a UV germicide lamp for 45 minutes in a biological safety cabinet. Device surfaces were exposed to oxygen plasma for 30 seconds each, using the hand-held corona discharge unit, and incubated with either collagen (100 μg/mL in 0.02N acetic acid), or fibronectin (100 μg/mL in
PBS) overnight at 4 °C. Immediately prior to seeding, devices were washed three times in PBS, and preconditioned with supplemented DMEM for 2 hours at 37 °C.

For adhesion studies, collagen-coated devices with and without the polyurethane layer were prepared, and a mixed population of cells (between P2 and P5) were seeded and allowed to spread on the device for 24 hours, and mechanically stimulated at ‘high’ strains at 1 Hz for an additional 24 hours. For the side-specific studies, the freshly isolated cells from the fibrosa and ventricularis were allowed to grow in supplemented medium until they reached approximately 90% confluence. Cell populations were then trypsinized using standard cell culture protocols, and seeded on the device surface at 15,000 cells / cm² in supplemented DMEM, and with 5 ng/mL of transforming growth factor (TGF)-β1. Cells were allowed to adhere and spread for 24 hours (37 °C, 5% CO₂) before the application of low and high levels of mechanical stimulation (at 1 Hz) for 24 hours. Cells were then fixed in 10% neutral buffered formalin overnight at 4 °C, and permeabilized with 0.1% Triton X-100.

7.2.8 Image collection and analysis

Devices maintained in a humidified environment tend to form condensation droplets beneath the composite diaphragms, which can hamper image collection. To obtain clear images, the underlying channel network was backfilled with deionized water in a vacuum chamber to eliminate optical distortion caused by water droplet condensation. Images were then collected using a 10× objective on a fluorescent microscope (Olympus 1X71, Olympus) with a CCD camera (QImaging Retiga 2000R, QImaging; Surrey, BC, Canada).

Cell adhesion was assessed by counting the number of cells remaining on the nine mechanically active PDMS and PU-PDMS culture films in each well, and expressed as a percentage of the number of adherent cells on control (mechanically static) devices. After the stimulation period, cells were fixed and stained with a Hoechst 33258 nuclear counter stain. Results for cell counts on the mechanically active substrates are expressed as mean ± standard error percentages of the number of cells on the mechanically static samples (n = 6).

To detect α-SMA expression, a standard immunocytochemistry procedure was followed. Briefly, fixed cells were permeabilized and blocked with 3% bovine serum albumin and incubated with monoclonal mouse anti-alpha SMA antibody (Clone 1A4, Sigma) followed by
AlexaFluor 568 goat anti-mouse IgG (Invitrogen). Cell nuclei were counterstained with Hoechst 33258. Myofibroblast differentiation was determined by counting the number of cells incorporating α-SMA into cytoskeletal stress fibers [311]. Cells containing three or more α-SMA positive stress fibers were considered as myofibroblasts, and expressed as a percentage of the total number of cells in the defined region of interest. For each condition 8-10 regions were assessed and averaged, and expressed as means ± standard deviations. Data are analyzed using multiple comparison ANOVA tests in SigmaStat 3.5 (Systat Software Inc.; San Jose, CA, USA). Post-hoc comparisons were conducted using the Tukey method.

7.3 Results

7.3.1 Device characterization

Measured deformation of the device surface enables the calculation of strains applied to cells on the culture film. The radial and circumferential strains obtained for the two types of devices manufactured are shown in Figure 7-3C. Limiting analysis area to a 600 μm diameter region of interest (ROI) was based on maximizing the number of cells available for analysis, while simultaneously providing distinctly different strain conditions in which the range of high strains is at least twice that of the range of low strains. Typical cell counts for this ROI range from 110 to 150 cells after 24 hours of cyclic stimulation at 1 Hz. The 600 μm diameter ROI produces a comparatively low strain field of 3.4 ± 0.3% in the radial and circumferential directions; and a comparatively high strain field of 12.4 ± 0.2% and 11.3 ± 1.7% in the radial and circumferential directions (Table 7-1). Nominal titular values of 3% and 12% are used for brevity in this discussion.

7.3.2 Composite diaphragm performance

Although free-standing PU films show greatly improved adhesion over PDMS [201], the material undergoes creep, as evidenced by sagging in the culture films after cyclic actuation. Cyclic loading of the composite PU-PDMS films showed no discernible changes in strain over 100,000 actuation cycles (Figure 7-4A), a result previously demonstrated for microfabricated PDMS films by us and others [99, 187]. Hence, inclusion of the PU coating does not appear to alter the mechanical stability of the PDMS films. The actuation and relaxation time constants of the culture film were defined as the time required to undergo 95% of the final deformation in
response to a step application of pressure, and were found to be 0.21 and 0.42 seconds respectively (Figure 7-4B). Complete actuation and relaxation of the bulging films were observed when using a square pressure waveform of 1 Hz frequency.

The use of the PU coating layer significantly improved adhesion (p < 0.001 by one-way ANOVA) over the uncoated PDMS films, on which 20% of the primary cells remained adhered after 24 hours of cyclic stimulation at 12% strain. By contrast, more than 75% of the cells cultured on the composite films remained adhered and well-spread under similar loading conditions (Figure 7-5).

![Figure 7-4](image.png)

**Figure 7-4.** Characterization of the mechanical properties of composite culture films over multiple actuation cycles. (A) No changes in strain observed over 100,000 loading cycles; and (B) video microscopy demonstrated the dynamic response times of the diaphragm in response to a square pressure waveform.
7.3.3 Side-dependent mechanosensitivity of VICs

Myofibroblast differentiation levels under each of the tested experimental conditions are reported in Figure 7-6A (substrates coated with type I collagen) and Figure 7-6B (substrates coated with fibronectin). In terms of individual culture parameters, cell type most strongly influenced myofibroblast differentiation across repeated experiments. Consistently increased levels of myofibroblast differentiation were observed in cells from the ventricularis over cells from the fibrosa for all culture conditions (p < 0.001). The presence of exogenous TGF-β1 also significantly increased myofibroblast differentiation in all conditions (p < 0.05).

Interaction effects between culture parameters were also significant. On collagen, increased differentiation of cells from the fibrosa occurred only at high strain levels (p < 0.001), but the presence of TGF-β1 induced similar increases at both low and high strains, as compared to the static case (p < 0.005). Differentiation of ventricularis cells increased for both strain levels (p < 0.001), with or without TGF-β1. On fibronectin, differentiation of both cell populations was increased as compared to on collagen, but the effects of mechanical stimulation on the fibrosa cells were not statistically significant.

Figure 7-5. Adhesion differences between the collagen-coated PDMS substrates and the collagen-coated PDMS+PU composite substrates after 24 hours of mechanical stimulation (1 Hz) at ~12% strain. Results are normalized to static control samples (* p < 0.001).
Figure 7-6. Myofibroblast differentiation of side-specific VICs cultured on (A) Type I collagen; and (B) fibronectin-coated surfaces; with selected statistical comparisons highlighted (* p < 0.01, n = 7-8, experiments repeated three times).
7.4 Discussion

7.4.1 Technical advantages over previous work

Our previous work (Chapter 5) has demonstrated the development and use of a microfabricated multilayer piston-based deformation system, which provides uniform equibiaxial stimulation to small colonies of cells across an array. However, four key drawbacks were identified in the design and operation of those devices. First, the fabrication process is quite challenging, and producing enough devices to adequately address the heterogeneous responses in biological systems can be difficult. Second, although the system provides high mechanical throughput, the ability to study the combinatorial effect of multiple mechanobiological cues is limited. This is due to the rapid increase in the number of experimental conditions required when screening multiple parameters, and the practical limitations in the production rate of the described devices. Third, uniformly stimulated cells are limited to a culture area about 300 μm in diameter, which limits the number of cells available for analysis. Scaling this area up is challenging without switching to macroscale fabrication technologies. Lastly, the materials used in that study limit experiments with primary cells to six hours, as poor adhesion between the PDMS and the cells cause cell loss during mechanical stimulation.

The present device addresses these issues. It is relatively simple to fabricate. Individual units on the array can be scaled up to mechanically stimulate larger cell populations. The number of units per device can easily be scaled to create individually segregated groups of mechanically stimulated populations, to study the effects of combinatorial mechanical stimuli on cell function. Last, the development and use of the composite PU-PDMS films enables substantially improved adhesion after 24 hours of cyclic stretch. PU can be precisely designed and tailored for specific applications, and hence, even though this demonstration shows approximately 75% of the cells remaining after 24 hours of cyclic stretch (Figure 7-5), adhesion can be further strengthened using customized chemical modification and synthesis techniques developed by others[288]. Furthermore, polyurethane formulations can be customized and tailored for clinical applications, and polyurethane is commonly used as a scaffold material in tissue engineering[312]. Hence, use of this material can improve the clinical relevance of these studies and translational capacity of these technologies.
Applying well-characterized non-uniform deformation fields to study the mechanoresponse of cells in *in vitro* culture is an established approach[313]. Although the primary criticism of distending macroscale culture diaphragms by the application of a pressure differential is the non-uniformity in strains created[13], careful characterization and selection of the analysis region of interest can mitigate these issues. For the low strain case, strains spanned a range of 0.6% across the selected 600 μm diameter ROI, which is equivalent in magnitude to strain ranges in more complex ‘uniform’ systems[187]. This applies to radial strain in the high strain case as well. Circumferential strain in the high strain case was more variable, spanning 3.4% across the ROI, but the low and high strain cases remain mechanically distinct (Table 7-1; Figure 7-3).

Bolstering the poor fatigue resistance of PU with the thicker PDMS films enabled repeatable mechanical stimulation produced over 100,000 cycles. Observed differences were inconsistent between 10,000 and 100,000 actuation cycles, strongly suggesting that these are a result of errors in measurement of vertical displacement (Figure 7-4). This advantage in creating reproducible mechanical strains represents a significant advantage over current macroscale technologies, which have been shown to undergo cyclic strain-related fatigue as early as 1000 cycles[246].

Other key advantages over macroscale technologies are the ability to produce a large number of mechanobiological culture conditions within a small footprint, with minimal external infrastructure. Such devices are particularly important when the number of available cells is limited. In the particular biological phenomena explored here, the VIC primary cells cannot be expanded indefinitely, as extended culture of these cell types on tissue culture plastic has been shown to induce the myofibroblast phenotype[294]. More broadly, a portion of this cell population has been shown to consist of mesenchymal progenitor cells, and the frequency of these progenitors has been shown to decrease after multiple population doublings[234], thereby changing the characteristic behaviour of this cell type. Hence, given the limited quantity of cells available, and the large number of environmental parameters that influence myofibroblast differentiation, a microfabricated high-throughput approach is desirable to adequately explore this system.
7.4.2 Significance of biological results

The influence of matrix proteins, mechanical stimulation and chemical cues on myofibroblast differentiation has been generally established for fibroblasts[298, 299]. The effects of TGF-β1[303] and collagen and fibronectin matrix proteins[314] on myofibroblast differentiation specific to valvular interstitial cells has also been explored. These experiments, for the first time, examine the relative influence of each of these parameters combinatorially on spatially segregated sub-populations of VICs which had been previously considered as the same cell type. Combinatorial effects observed include the interactional effect between the presence of TGF-β1 and differing levels of mechanical stimulation: on the collagen-coated surfaces, fibrosa cells exhibit an increase in myofibroblast differentiation only at high strain levels, unless in the presence of TGF-β1, in which case increased levels are observed at low strains. This ‘mechanical dose’ dependency is also observed with cells from the ventricularis, in which higher strains increase differentiation.

The signaling mechanism relating these stimuli is currently unknown, but a number of potential avenues have been identified. Since mechanical stress alone is not sufficient to increase myofibroblast differentiation in the absence of TGF-β1[300, 315], it is reasonable to assume that TGF-β1 sourced through the fetal bovine serum supplement or produced by the cells in a latent form plays a role in the observed combinatorial mechanosensitivity. Hence, a likely mechanism for this combinatorial effect is mechanical modulation of the bioactivity of TGF-β1[316-318]. However, it is uncertain whether this mechanism applies to exogenous TGF-β1, which is not introduced in a latent complex with other molecules. Potentially, exogenous TGF-β1 could bind with cell-produced latent TGFβ1 binding protein-1, creating mechanically activated latent reservoirs[298]. More complex relationships could also explain this combinatorial sensitivity, including the mechanically-induced incorporation of α-SMA into the cell stress fibers, which may subsequently play a role in defining cell response to exogenous chemical stimulation; or the production of additional interacting molecules, such as the ED-A splice variant of fibronectin, known to influence myofibroblast response to TGF-β1[299].

The influence of matrix proteins on regulating response to mechanical stimuli is also clear. Cells from the fibrosa demonstrate increased myofibroblast differentiation on fibronectin coated substrates, as compared to collagen-coated substrates. The effects of mechanical stimulation on
fibronectin-stimulated cells from the fibrosa were not significant, while cells from the ventricularis continued to show significant responses to mechanical stimulation. This suggests that either fibronectin causes a saturation of α-SMA incorporation in fibrosa stress fibers, thereby ‘washing out’ any mechanical effects, or that fibronectin inhibits the mechanical response of cells from the fibrosa. Since the addition of TGF-β1 significantly increases myofibroblast differentiation of the fibrosa cells cultured on fibronectin, this indicates that myofibroblast differentiation is not saturated, although there may be differing saturation levels for various stimuli. Regardless of mechanism, these results demonstrate significant matrix- and mechanically-dependent differences in behaviour from cells on either side of the valve leaflet. Speculatively, these differences in phenotype may be due to environmental regulators, such as differing mechanical conditioning of the interstitial cells in different layers[307], or through spatially distinct endothelial cell-mediated signaling on the aortic and ventricular sides of the valve[308, 309]. Alternatively, cells in the fibrosa and ventricularis layers of the valve may be inherently or developmentally different in their response to environmental stimuli. Although further investigations into the reasons for these differences are required, taken together this data shows distinct functional characteristics of cells from either side of the valve, and demonstrates the need to separately consider these cell types in understanding valve pathobiology and mechanobiology.

7.5 Conclusions

By addressing the issues that arose in operating the microfabricated platforms described in Chapter 5, we have developed a system which allows for high-throughput, long-term, combinatorial manipulation of mechanobiological culture parameters in the cellular microenvironment. Rather than providing a proof of concept device, this work demonstrates the potential for microdevices in addressing problems that would be challenging to resolve with conventional macroscale technologies. Observing differential responses in the limited number of cells available from either side of the valve in response to this range of combinatorially manipulated parameters would require a substantial investment of time, reagents, and equipment. The use of microtechnologies as a screening platform has resulted in the discovery of new directions for future research into the reasons for this spatial heterogeneity within the valve. The specific results obtained in this study may have implications in valve tissue engineering and in better understanding valve pathobiology.
Chapter 8

“… now in 3D! ”

8 Three-dimensional mechanically active culture

High-throughput screening techniques for cellular response are often unable to account for several factors present in the in vivo environment, many of which have been shown to modulate cellular response to the screened parameter. Culture in three-dimensional biomaterials and active mechanical stimulation are two such factors. In this chapter, we integrate these microenvironmental parameters into a versatile microfabricated device, capable of simultaneously applying a range of cyclic, compressive mechanical forces to cells encapsulated in an array of micropatterned biomaterials. The fabrication techniques developed here are broadly applicable to the integration of three-dimensional culture systems in complex multilayered polymeric microdevices. Compressive strains ranging from 6% to 26% were achieved simultaneously across the biomaterial array. As a first demonstration of this technology, nuclear and cellular deformation in response to applied compression was assessed in C3H10T1/2 mouse mesenchymal stem cells encapsulated within poly(ethylene glycol) hydrogels. Biomaterial, cellular, and nuclear deformations were non-linearly related. Parametric finite element simulations suggested that this phenomenon was due to the relative stiffness differences between the hydrogel matrix and that of the encapsulated cell and nucleus, and to strain stiffening of the matrix with increasing compression. This complex mechanical interaction between cells and biomaterials further emphasizes the need for high-throughput approaches to conduct mechanically active experiments in three-dimensional culture.

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4 Work presented in this chapter was published in:


8.1 Introduction

High-throughput screening (HTS) has been a critical technology in driving drug discovery, tissue engineering and fundamental cell biology research[319]. However, these techniques are hindered by the inability of standard HTS platforms to account for a number of factors present in the in vivo cellular microenvironment[320]. Technological progress in microfabricated systems has enabled the simulation of complex cellular environments, while maintaining the arrayed format suitable for systematic HTS. Currently, factors such as chemical stimulation[7], the extracellular matrix[6, 180, 231] and cell-biomaterial interactions[5] can be precisely manipulated. However, HTS techniques for drug discovery, biomaterial development, and tissue engineering require the inclusion and manipulation of other parameters to better recreate a more realistic in vivo environment.

One such parameter is the dimensionality of the culture system. Three-dimensional cell culture has been shown to significantly impact cell function[321, 322], and is a core approach in tissue engineering to produce functional tissue replacements[323, 324]. Three dimensional culture has also been shown to modulate cellular response to chemical factors in the microenvironment[325-327], suggesting that biomolecular screens must incorporate this aspect of dimensionality to produce clinically relevant results[328]. Advances in biomaterial technologies have enabled spatial definition of three-dimensional structures for tissue engineering[329-331], and these approaches have only recently enabled high-throughput screening in three-dimensional culture systems[178, 179].

Dynamic mechanical forces also play a role in driving cell function in vivo[308, 332]. Many cells are exquisitely sensitive to applied mechanical stimulation in both two- and three-dimensional environments[8, 333]. Mechanical forces modulate cellular response to other stimuli[9-11], and are often used to encourage appropriate cell growth and function in engineered tissues[334]. For example, dynamic compression has been shown to regulate chondrocyte apoptosis in cartilage explants[335]; chondrocyte matrix biosynthesis[336]; intervertebral disc cell gene and protein expression[337]; bone homeostasis[338]; and stem cell chondrogenesis[339], osteogenesis[340] and adipogenesis[341]. Compression has also been shown to modulate cellular response to other stimuli[342], and thus has potential in making HTS assays more suitable to clinically-relevant applications.
Creating a range of mechanically dynamic compressive microenvironments in three-dimensional culture is challenging with conventional equipment and techniques. To realize mechanically active HTS arrays, this chapter reports on the incorporation of an aligned photopolymerized biomaterial array, stably micropatterned within a multilayer PDMS microfabricated platform. This integration of biomaterial and microfabrication technologies results in a microdevice capable of simultaneously and systematically varying compressive strain levels across an array of three-dimensional, cell-laden biomaterial microconstructs.

To demonstrate the functionality of this system, an array of poly(ethylene glycol) (PEG) biomaterial microconstructs containing mouse mesenchymal stem cells were simultaneously compressed to varying degrees. Cellular deformation resulting from the applied mechanical stimulation was measured via fluorescent confocal microscopy, in order to rapidly assess the minimum levels of strain required to cause detectable cellular deformation. A parametric finite element model was used to further probe and provide a potential explanation for the observed nonlinearity in cellular deformation.

8.2 Materials & Methods

Unless otherwise stated, all chemicals and reagents for cell culture were purchased from Sigma-Aldrich (Oakville, ON, Canada); fluorescent dyes from Invitrogen (Burlington, ON, Canada); and all other equipment and materials from Fisher Scientific Canada (Ottawa, ON, Canada).

8.2.1 Hydrogel Chemistry

Hydrogel arrays were produced by mask-based photolithography of a cell suspension in a PEG precursor[343]. Poly (ethylene glycol) diacrylate (PEGDA) with a molecular weight of 3.4 kDa was purchased from Laysan Bio (Arab, AL, USA). A hydrogel precursor solution consisting of 10% w/v PEGDA and 10% w/v PEG (8 kDa) in unsupplemented Eagle’s Buffered Media (EBM) was prepared. Irgacure 2959 (Ciba Specialty Chemicals; Tarrytown, NY, USA) was dissolved in 1-vinyl-2-pyrrolidone (100 mg/mL) to form a photoinitiator stock solution. This stock solution was then added to the hydrogel precursor solution to a concentration of 0.4% w/v of photoinitiator. The precursor solution was vortexed thoroughly and passed through a 0.45 μm syringe filter, before being mixed in a 1:1 ratio with either unsupplemented EBM or a suspension of C3H10T1/2 mouse mesenchymal stem cells (ATCC, Manassas, VA; 8×10⁶ cells/mL for
viability studies and $2 \times 10^6 \text{ cells/mL}$ for compression experiments), depending on the experimental conditions required.

### 8.2.2 Device Fabrication

The PDMS device consists of 25 vertically actuated loading posts, arranged in a $5 \times 5$ array to produce five replicates of five distinct mechanical conditions (Figure 8-1). (A) Microfabricated device a $5 \times 5$ array of mechanically active three-dimensional culture sites (green dye in the pressurized actuation channels). (B) Increasing actuation cavity size across the array enables a range of mechanical conditions to be created simultaneously. (C) Cylindrical hydrogel polymerized on a loading post in the mechanically active culture array.). The device pitch is based on a 1536-well plate. The array of loading posts is suspended over actuation cavities of varying diameters, and posts are raised by applying pressure beneath the suspended membranes via an integrated network of channels in the PDMS device (Figure 8-2). A solenoid valve (Pneumadyne; Plymouth, MN, USA) was used to apply a step pressure of 55 kPa generated by an eccentric diaphragm pump (SP 500 EC-LC; Schwarzer Precision; Germany). By varying the diameters of the actuation cavities, a single pressure source can be used to create a range of vertical displacements across the array.

Devices were fabricated using Sylgard 184 PDMS kits (Dow Corning, purchased through A.E. Blake Sales Ltd., Toronto, ON, Canada) using sandwich mold fabrication to prevent alignment registration errors between multiple device layers. Briefly, a layer of PDMS was cured while compressed between an SU-8 patterned glass master and a transparency film (Grand & Toy; Toronto, ON, Canada). The patterned PDMS layers preferentially adhere to the transparency, from where they can be bonded and transferred to a 3"×2" glass slide, or another patterned PDMS layer. A series of cross-shaped marks were fabricated directly into the PDMS device to facilitate alignment with corresponding marks on the photopolymerization mask. To reduce the gas permeability of the PDMS device, a layer of Parylene-C was deposited using a PDS 2010 Labcoter® 2 system (Specialty Coating Systems; Indianapolis, IN, USA). Clear tape was used to mask the device around the post array, before a $1 \mu\text{m}$ thick conformal layer of Parylene-C was deposited over the surface. Peeling the tape away removed the Parylene film from the device periphery.
A 200 μm thick slab of PDMS was cast in a petri dish, and small sections of this slab were bonded as spacers between the multilayer device and a glass coverslip (Figure 8-2), creating a thin polymerization chamber above the loading posts. The coverslip was functionalized by treatment with a 2% v/v solution of 3-(trimethoxysilyl) propyl methacrylate[176] to provide binding sites for the polymerized hydrogels.

A connector was then attached to the actuation cavity network. Imaging the encapsulated cells was found to be severely hampered by the formation of condensate droplets in the actuation cavities of the device during the course of the experiment. In order to prevent this, the actuation cavities were backfilled with deionized water, prior to further fabrication steps.

### 8.2.3 Hydrogel lithography

A fluorescent microscope light source (X-Cite 120, EXFO Life Sciences; Mississauga, ON, Canada) was modified to provide UV illumination. The fiber optic guide and microscope adapter were removed from the fluorescent microscope, and set up in a custom-made vertical polymerization system. The broad-spectrum source was passed through a 365 nm narrow band filter (removed from a BlakRay illumination lamp; UVP; Ottawa, ON, Canada) and a manual shutter system, to illuminate a removable lift stage. The stage was raised towards the light source, such that the incident UV dose to the device was 17.5 mW/cm² (measured using a 365 nm intensity meter), with negligible variation in intensity over a 2 cm × 2 cm area.

The cell suspension and hydrogel precursor mixture were injected into the polymerization chamber using a long 25-gauge needle (Figure 8-2B). Care was taken to ensure that no air bubbles were trapped within the chamber. Enough fluid was injected to ensure that the edges of the polymerized array were at least 1 cm away from the air-fluid boundary.

A printed transparent film (CAD/Art Services; Bandon, OR, USA) was used to selectively photopolymerize the hydrogel. Arrayed circular patterns (500 μm in diameter) were used for all experiments. The mask was placed on the device surface. Alignment was conducted manually with the aid of a Navitar 12× zoom vision system (Navitar; Rochester, NY, USA). The stage was then illuminated with UV light, selectively polymerizing the hydrogel.
The arrays were washed three times by injecting sterile EBM into the device chamber to displace the unpolymerized precursor solution. For those experiments with encapsulated cells, devices were incubated for at least 1 hour before fluorescent staining and mechanical compression.

Figure 8-1. (A) Microfabricated device a 5 × 5 array of mechanically active three-dimensional culture sites (green dye in the pressurized actuation channels). (B) Increasing actuation cavity size across the array enables a range of mechanical conditions to be created simultaneously. (C) Cylindrical hydrogel polymerized on a loading post in the mechanically active culture array.
Figure 8-2. Fabrication process for mechanically active three-dimensional cell culture arrays.
8.2.4 Characterization of PEG polymerization

In order to investigate the effects of microdevice materials on hydrogel microconstruct formation, the photopolymerization procedure was conducted on multiple substrates. As with device fabrication, a PDMS spacer was bonded between methacrylated glass coverslips and three substrate materials: bare glass, PDMS and Parylene-coated PDMS, to create polymerization chambers of different materials. An additional oxygen-depleted PDMS substrate was created by placing a PDMS-based chamber under vacuum (Savant VLP80 rotary vacuum pump) for 20 minutes immediately prior to polymerization. Photopolymerized arrays were imaged under a stereoscope (Olympus; Markham, ON, Canada). A standard binary threshold level was applied in ImageJ (NIH), and automated ellipse fitting algorithms were used to measure the dimensions of the polymerized hydrogels across the area of interest.

8.2.5 Fluorescent staining and microscopy

Microconstruct deformation was assessed by mixing 1 μm diameter FITC fluorescent beads (Bangs Laboratories; Fishers, IN, USA) into the hydrogel solution before polymerization. Confocal microscopy (Fluoview 300, Olympus) was used to collect three-dimensional images of each hydrogel cylinder when at rest and when the device was actuated, at a magnification of 10× (n=3).

Cell viability in the polymerized arrays was assessed using a calcein AM and ethidium homodimer LIVE/DEAD cytotoxicity assay. To measure cytoplasmic and nuclear deformation in response to mechanical stimulation, cells were stained with calcein AM and DRAQ5 nuclear stain. The use of calcein AM served the dual purpose of staining the cellular cytoplasm and ensuring that the particular cell under study was viable. Confocal images were collected at 40× magnification for each of the five mechanical conditions across the array, when the hydrogels were at rest and under compression.

8.2.6 Image analysis

Confocal images were processed using a semi-automated macro in ImageJ. For the fluorescent bead characterization studies, the confocal image stacks were resliced orthogonal to the principal axis of the hydrogel cylinders. A z-projection of the resliced stack was taken, the heights of the
cylinders under compression were measured, and the percentage changes in cylinder height were calculated.

In order to assess cellular and nuclear deformation under compression, a synchronized selection function was used to crop a separate image stack for each isolated cell in the photopolymerized cylinder, when at rest and when compressed. There were at least three cells in each image stack which were physically separate from other cells in the microconstruct. The stacks were then resliced orthogonally, and a z-projection of the stacks was applied to clearly define the outlines of the cytoplasm and nuclei. A threshold binary function was applied and ellipses were fitted to the cross-sectional view of the cells and nuclei when at rest and when under compression.

A mathematical descriptor for ‘nuclear shape’ and ‘cell shape’ was defined as the aspect ratio between the x- and y-axes of the fitted ellipse. ‘Cellular deformation’ and ‘nuclear deformation’ were defined as the ratios between the normal and compressed aspect ratios of the cells and nuclei. Deformation values for all the single cells within a hydrogel cylinder were averaged. These values were used to obtain a mean and standard deviation across three cylinders under similar mechanical conditions.

8.2.7 Statistical analysis

All statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc.; San Jose, CA, USA). One-way ANOVA tests were used to compare groups, and post-hoc comparisons were conducted using the Tukey method. Graphical results are reported as means ± standard deviation.

8.2.8 Finite element simulations

In order to assess the impact of the relative differences in stiffness between the hydrogel and the encapsulated cell on cellular deformation, finite element simulations were conducted in ANSYS (ANSYS Inc.; Canonsburg, PA, USA). An axisymmetric, large displacement model of a cell embedded within a hydrogel was developed using quadrilateral PLANE183 elements. The simulations were designed to be first-order approximations to this problem, and assumed isotropic linear elastic behaviour of the cell and the surrounding matrix. The matrix was defined as a 500 μm diameter cylinder, 100 μm in height, encapsulating a 20 μm diameter sphere, representing the cell. The cell was assumed to be fixed in the matrix, such that no slippage
occurs between the two interacting surfaces. The standard ANSYS mesh generator algorithm was used to create a mesh with 932 elements in the cell, and 6180 elements in the hydrogel microconstruct. The relative modulus between the two materials ($E_{\text{matrix}}^*$) was varied over seven orders of magnitude, and fixed deformation constraints were applied to the matrix to produce compressions of 1%, 5% and 10%. The resulting change in aspect ratio was determined for values of $E_{\text{matrix}}^*$ ranging from 0.001 to 1000.

8.3 Experimental results

8.3.1 Hydrogel integration and characterization

Polymerization chamber materials were found to play a significant role in PEG polymerization kinetics. The feature sizes of micropatterned hydrogel constructs were used to assess the degree of polymerization on various substrate materials commonly used in microfabricated devices. A significant reduction in microstructure diameters was observed on all PDMS substrates as compared to the glass substrate, even with a 2.5-fold increase in UV exposure time ($p < 0.05$; Figure 8-3). Depleting the oxygen levels in the PDMS substrate improved polymerization kinetics, but these results varied from experiment to experiment. Coating the PDMS surface with a thin conformal layer of Parylene-C consistently resulted in hydrogel features comparable to those produced on bare glass.

The viability of the encapsulated C3H10T1/2 cells was found to decrease significantly at polymerization times greater than 300 seconds (no viable cells found at 600 and 900 seconds of UV exposure). Since completed hydrogel polymerization on the PDMS device substrates was well in excess of this time, PDMS microdevices used in this study were coated with a 1 $\mu$m layer of Parylene-C to minimize exposure time and maintain cell viability. The critical parameters for accurate reproduction of PEG microstructures in the Parylene-coated PDMS microdevices were exposure time and intensity. For cylindrical hydrogel constructs 500 $\mu$m in diameter, an exposure time of 195 seconds at 17.5 mW/cm$^2$ was found to accurately replicate the mask pattern, and obtain an initial cell viability of 69.4 ± 3.4%, which is comparable to viabilities achieved in this biomaterial in other micropatterning studies[344]. Microconstruct diameters were similar across the 1 cm$^2$ area of the array for our polymerization system.
Figure 8-3. Micropatterning of hydrogel arrays on various materials used in microfabrication. The diameters across an array of hydrogel cylinders were measured on glass, PDMS, oxygen-depleted PDMS and Parylene-C coated substrates, for two UV exposure times. Polymerization on the Parylene and glass substrates for the same exposure time was not significantly different, whereas hydrogel features on all other conditions were undersized (* p < 0.05 as compared to polymerization on the glass substrate).

8.3.2 Microconstruct compression

Micropatterned PEG arrays were successfully integrated into the Parylene-coated PDMS devices, confirmed by direct visual observation (Figure 8-1C) and confocal microscopy (Figure 8-4A, B). Compressive deformation of the PEG microconstructs across the array was repeatable and ranged from 6.02 ± 0.80% to 25.54 ± 1.52%. (Figure 8-4C).
Figure 8-4. Characterization of hydrogel compression across the microfabricated array. (A) Orthogonally resliced confocal image of fluorescent bead markers within a single hydrogel cylinder over a unit on the array at rest and (B) when actuated at 55 kPa. (C) Nominal compression means achieved across a 5 × 5 array of hydrogel cylinders (mean ± standard deviation; n = 3).

8.3.3 Cell deformation

Cells encapsulated within the polymerized microconstructs remained rounded after 1 hour of incubation. They were evenly dispersed throughout the PEG matrix, but there was some indication of cell settling towards the bottom of the microconstructs during the alignment and polymerization procedure. Cellular deformation (as defined in section 8.2.7) was found to
increase non-linearly with increasing levels of compression (Figure 8-5F). The largest applied compression caused a significant cellular deformation \((p < 0.001)\), but all other deformations across the array were not different \((p > 0.269)\). Nuclear deformation (Figure 8-5G) in response to the applied compressive strains was not significantly different under any of the tested mechanical conditions \((p > 0.121)\).

Figure 8-5. Deformation of single cells within a compressed matrix. (A-E) Orthogonally resliced confocal images of single cells encapsulated in the PEG hydrogels under rest and active (compression) conditions.

### 8.3.4 Parametric finite element simulations

In order to gain a better understanding of the reasons for the observed non-linear relationship between matrix compression and cellular deformation, parametric finite element simulations were conducted. These simulation results (Figure 8-6) suggest that the ratio between the Young’s modulus of the cell and that of the surrounding matrix significantly impacts cellular deformation in a compressed matrix, and this effect is more dramatic at higher strain levels. Cellular deformations are small when a cell is encapsulated in a relatively soft hydrogel, but increase as the modulus of the matrix approaches that of the cell. Further increases in matrix
stiffness continue to affect cellular deformation, but the impact becomes noticeably smaller when the matrix is an order of magnitude stiffer than the cell.

Figure 8-6. Finite element simulations showing encapsulated cellular deformation in response to compression of the surrounding matrix. (B) Simulated cellular deformation for 1, 5 and 10% compression in the surrounding matrix for a range of matrix stiffness properties normalized to the cell stiffness ($E_{matrix}^*$)
8.4 Discussion

The ability to understand cellular response to factors in the microenvironment has been significantly improved by the availability of various technologies. Advances in HTS techniques have enabled rapid screening for the effects of a large number of chemical cues on cellular function, an approach critical to drug discovery, tissue engineering, and probing fundamental cell biology. However, most HTS assays are conducted on static tissue culture plastic, which does not reflect the complexity of in vivo systems. Mechanical stimulation and three-dimensional culture conditions are two factors that are known to modulate cellular response to other microenvironmental conditions, but remain unaccounted for in HTS techniques. Hence, there is a need to conduct high-throughput experiments in conditions that capture more of the complexities that define the cellular microenvironment. To address this issue, we have developed a technology that enables high-throughput mechanical compression of cells in a three-dimensional culture system.

Developing this system required the integration of aligned, micropatterned, cell-laden biomaterial arrays into multilayer PDMS devices. PEG was chosen as a model biomaterial to demonstrate this technology because of the established use of PEG as a photopatternable biomaterial for encapsulated cell studies[176, 345, 346]. The ability to comprehensively modify this biomaterial with adhesive ligands[347], establish chemical gradients[348], incorporate degradable crosslinking structures[349, 350], and control mechanical stiffness[173] makes PEG particularly suited to this application. Long-term culture of encapsulated cells has been demonstrated[351], and the use of chemical modifications to the hydrogel polymer has shown significantly improved cell behaviour in terms of viability and maintaining regular function[343].

Integrating micropatterned PEG microconstructs into these multilayer PDMS devices was challenging. Incorporation of three-dimensional biomaterials into microfabricated devices has been achieved by assembling the microdevice around the patterned material[174], and by designing ‘open-top’ microfluidics[14]. However, the physical constraints of the present compression system necessitated in situ patterned photopolymerization within a closed microfluidic system. Photopolymerization of immobile PEG structures is most often carried out in glass chambers[176, 343, 345], and though Liu et al. have reported encapsulation of cells within PDMS-glass microchannels, these have only been demonstrated for small hydrogel
microconstructs (< 50 μm diameter)[177]. In the polymerized arrays reported here, each hydrogel was 500 μm in diameter, in order to maximize the matrix volume unaffected by edge-related changes in local strain, and to obtain an adequate number of cells in each microconstruct for analysis.

Current techniques to integrate photopolymerized PEG into PDMS devices[177] do not scale well to 500 μm diameter features. We experimentally found that the polymerization time required to accurately define the hydrogel features in a PDMS-based polymerization chamber was in excess of the cytotoxicity threshold for C3H10T1/2 cells. This is because the polymerization process is quenched by oxygen, and the high oxygen-permeability of PDMS prevents polymerization, while maintaining an increased concentration of toxic photo-generated free radicals. Temporarily depleting the oxygen levels in the PDMS by vacuum treatment improved polymerization times. However, this method was inconsistent, as variations in the time required to align the photomask with the PDMS device created differently sized structures in different devices. To address this problem, the PDMS device was coated with a 1 μm thick layer of Parylene-C (Figure 8-3). Parylene-C is biocompatible[352] and the gas permeability of a PDMS-Parylene C composite is substantially lower than PDMS alone[353]. This approach is broadly applicable, and would enable the integration of cell-laden biomaterial arrays into most PDMS device designs.

There are a number of advantages in developing microfabricated platforms to study the effects of mechanical forces on systems. Some of the generic advantages are the minimized use of culture and immunostaining reagents; the ability to generate a large number of experimental conditions without manual intervention; the ability to work with small cell numbers; miniaturized equipment footprints; and reduced cost. Furthermore, the use of this system yields a specific advantage over current macroscale equipment. Compressive stimulation of cells is often accompanied by a transient increase in fluid pressure, which has been shown to impact cell function[169], as a ‘side effect’ of applying mechanical deformation[354]. Microfabricated constructs have a substantially higher surface to volume ratio than their macroscale counterparts, and hence the transient imbalance in fluid pressure reaches equilibrium more rapidly. Hence, these microfabricated arrays can be used to probe cellular response to mechanical compression while minimizing other confounding factors.
As a first demonstration of the mechanically active biomaterial array, cellular and nuclear deformation was assessed in response to matrix compressive strains ranging from 6 to 26%. These hydrogel strains were not translated linearly to encapsulated cells (Figure 8-5). Nuclear deformation was not significantly different regardless of applied mechanical strain, and cellular deformation only changed significantly at the highest strain levels. We hypothesize that the lack of cellular deformation at lower strain levels was due to the relatively low stiffness of the matrix, compared to the stiffness of the encapsulated cell. At higher strains, the pores in the PEG material collapse, increasing the effective modulus of the matrix[355, 356] and causing increased cell deformation. To further explore this hypothesis, first-order linear finite element simulations of cell deformation were conducted in which the relative differences in mechanical stiffness between the cell and the surrounding matrix were varied. The simulation results indicate that within a range of $E_{\text{matrix}}^*$, an increase in matrix stiffness can cause substantial changes in cell deformation for the same applied compression (Figure 8-6). This effect, similar to that reported by Guilak and Mow[354] for cartilage, supports the hypothesis that cellular deformation is impacted by the non-linear behaviour of the matrix and its stiffness relative to that of the cell.

Though an accurate determination of the relative stiffness difference between the cells and the matrix ($E_{\text{matrix}}^*$) is difficult, a rough estimate can be made. Compressing fibroblasts by micromanipulation suggests a stiffness modulus range from 0.1 to 10 kPa[243], and formulations of PEG hydrogels similar to those used in this work have a Young’s modulus of 0.1 to 1 kPa[356]. Hence, a rough estimate for $E_{\text{matrix}}^*$ at low compressive strains ranges from 0.01 to 1. The finite element simulations indicate that there is little change in cell deformation for all simulated hydrogel compressions in the lower end of this range of $E_{\text{matrix}}^*$. At the upper end of this range however, cell deformations become more substantial. Since PEG has been shown to stiffen at the higher compressive strains tested[356], it is plausible that strain stiffening at higher applied strains increase $E_{\text{matrix}}^*$ enough to cause a large increase in cellular deformation. Hence, this model of cell-biomaterial mechanical interaction may explain the non-linear cellular deformation results observed in the compression array. A similar argument applies to the low levels of nuclear deformation observed: the nucleus is three to four times stiffer than the cytoplasm[357], and hence is ‘shielded’ by the softer surrounding matrix. The observed complex interaction between cell and biomaterial further emphasizes the need for high-throughput approaches to experiments involving mechanical stimulation, particularly when
viewed in combination with the number of other microenvironmental parameters that can be varied.

Since the PDMS base platform of the microfabricated array is constructed using standard soft lithography techniques, simple modifications can further extend the utility of this platform technology. Hydrogels with several different chemistries can be formed on a single chip[358], or microfluidic gradient generators could be used with the PEG precursor chemicals, to create a polymerized array with a range of mechanical and chemical properties[359]. The ability to integrate PEG structures into microchannels also enables the delivery of chemical cues in a high-throughput, automated fashion using well-established techniques[7]. Hence, this technology promises rapid assessment of the integrated response of encapsulated cells to mechanical stimulation in combination with a multitude of other factors including hydrogel stiffness, matrix ligand density, hydrogel chemistry, and biochemical cues.

8.5 Conclusions

Mesenchymal stem cells encapsulated in an array of PEG microconstructs were successfully integrated into an active multilayer PDMS device. Using this system, mechanical compressive strains ranging from 6 to 26% can simultaneously be applied to sections of the array. As a first demonstration of the capabilities of this platform for mechanically active cell culture, nuclear and cellular deformation was assessed in response to various compressive strains. Finite element simulations suggest that the non-linear cellular and nuclear deformations arise from relative differences in the mechanical stiffness of the cell and the surrounding matrix. This information can be used to guide experimental study design in cellular response to compressive stimulation. More broadly, this platform enables HTS of cellular response in three-dimensional environments under combinatorially manipulated mechanobiological conditions. This approach may aid in the development of complex biomaterials and tissue engineering systems and in testing drugs in more physiologically-relevant environments.
Chapter 9

9 Conclusions and Recommendations

9.1 Conclusions

Microfabricated systems were designed, fabricated, tested, characterized and utilized to apply a range of dynamic mechanical strains to cells in two-dimensional culture systems, in a higher-throughput manner than is possible with conventional technologies. The influence of mechanical forces and mechanical forces in combination with other culture parameters were studied on VICs, and we (1) identified a potential time- and strain-magnitude dependent relationship between mechanical stimulation and accumulation of the \( \beta \)-catenin protein in the cell nucleus; (2) revealed previously unknown functional differences between cells isolated from spatially separated regions of the heart valve leaflet; and (3) identified parameters and combinations of parameters that influence myofibroblast differentiation in these VICs.

In order to conduct these biological experiments on microfabricated platforms, several advances in microdevice development and fabrication techniques were made. In order to create a structure to apply uniform equibiaxial strains to a culture surface, it was necessary to produce precisely-aligned multilayer microstructures; and for this to be useful as a screening tool, it was necessary to produce these structures in an array. We developed a fabrication methodology that is broadly applicable to constructing precisely aligned multilayer structures over large-area devices.

With this fabrication technology, we developed an array of mechanically active units, able to apply precisely controlled and uniform equibiaxial substrate strains to cells cultured on a device, and used this array to identify the relationship between \( \beta \)-catenin nuclear accumulation, stimulation time, and strain magnitude. Without a high-throughput system, these relationships would have been challenging to observe.

We then realized that although PDMS is widely used for cell culture in the microfabrication community, adhesion of primary cells to this substrate was poor under mechanically active
conditions. In order to maintain the mechanical properties and ease of microfabrication in using PDMS while improving the adhesion properties of the substrate, we developed a technique to integrate a clinically-relevant biomaterial, polyurethane, into the PDMS microfabrication process. This technique should be broadly applicable in improving device customizability and applicability for all open-top microfabricated devices for cell culture, as well as in some closed systems. We utilized this technology to develop microfabrication-compatible composite polyurethane-PDMS culture films for the purpose.

The second issue with the fabricated array is regarding usability: the devices are complex and challenging to fabricate, thereby limiting experimental throughput. To address this, we compromised on precision in application of mechanical strains and developed a microfabricated platform capable of combinatorially manipulating multiple stimuli in the cellular microenvironment; and we used this platform to study myofibroblast differentiation of the side-specific VIC populations in response to combinatorial microenvironmental stimuli. Given the limited cell population available for study, the number of culture parameters that could be manipulated, and the subtle combinatorial effects observed with these cell types, these studies would be time-consuming and impractical with conventional equipment, emphasizing the need for and utility of developing high-throughput alternatives.

We then developed a platform to study the influence of mechanical forces encapsulated within three-dimensional biomaterial constructs, a culture paradigm which is more physiologically relevant to many in vivo situations. Although mechanobiological effects have not as yet been demonstrated with this system, the non-linear relationship observed between cell deformation and biomaterial compression suggests that design of mechanically active three-dimensional systems must account for complex mechanical relationships between cell and matrix modulus, further emphasizing the need for high-throughput approaches to these studies.

9.2 Recommendations and future work

Given the current findings, there are a number of suggested directions that would be interesting and important to explore in future research.
9.2.1 Device technologies

One of the key limitations in using microfabricated devices for cell biology studies is the time and experimental skill required for device production. Given the inherent heterogeneity and inconsistencies in studying biological systems, such technologies are only practically useful if a large number of replicate conditions can be produced rapidly, and without years of training and experimental skill. Once the use of microdevices makes the transition from being ‘novel’ to being a ‘standard approach’, the use of this technology will begin making substantial contributions to biological discovery. However, in order to achieve this, a number of technological issues need to be addressed. First, devices should be designed to be as simple as possible. Second, connecting devices to peripheral systems are typically ‘weak points’ in device operation, prone to failure and leakage. For the devices presented in this thesis, use of a single pressure source for each device was a critical design parameter, to minimize the number of connections per device. Developing a cost-effective and robust connectivity solution for world-to-chip interfaces in microfabricated systems would greatly assist in alleviating some of the challenges in using these microfabricated platforms. Third, microfabrication processes are not amenable to mass or automated production. Particularly in the case of PDMS fabrication, translating prototyping techniques to large-volume production will require the development of new production and assembly techniques, in order to reduce the cost and increase the availability of these systems for biological study. Fourth, using microfabricated systems causes a reduction in the amount of biological material being tested, which hence limits biological analysis techniques. Microscopy (phase contrast or fluorescence) was used in this thesis for biological analyses, as there were typically too few cells available to use most biochemical and molecular assay techniques (e.g., Western blotting). While microscopy-based approaches are acceptable to initially select a small number of environmental conditions from a large parameter space for further study, in many cases additional biological insight requires the use of complementary quantitative analysis methods. Hence, in addition to the development of novel microfabricated technologies, parallel development of techniques to selectively isolate cells for analysis and assay with increased sensitivity and fewer cells would make these approaches more useful and relevant to biological studies. 
9.2.2 Technologies for mechanical stimulation

The technologies developed in this thesis yielded some interesting results which may be worth future exploration. In each of the culture film based mechanical stimulation platforms, no noticeable change in strains was observed over at least 100,000 cycles, which is a substantial improvement over conventional macroscale technologies. This raises the question: why did these materials behave so well? One hypothesis for this behaviour is that the film thickness prevents polymer chain reorganization, and thereby improves the material resistance to fatigue. Though this idea has been explored for rigid materials such as silicon, to our knowledge, the influence of dimensionality on soft polymeric materials has not been studied thoroughly, and could lead to interesting advances in materials technology.

The influence of scaling on the relevance of the substrate deformation systems to the applications for which the system is being used needs to be more thoroughly evaluated. For example, as discussed in Chapter 5, the distance through which a film is deformed to achieve similar strains on the microfabricated platform as opposed to conventional technologies is substantially reduced. This changes the normal reactive forces cells experienced as a result of fluid interactions. Hence, although substrate strain is being conserved as a result of the scaling, fluid-related stimulation is being altered, and this may have a profound impact on cell function. This raises the concern that discoveries made using these microfabricated platforms may not be translatable to large-scale equipment, which would be necessary for purposes such as tissue engineering. Hence, thorough characterization of the response of cells to these changing factors will be necessary to create translational research.

The three-dimensional stimulation systems have been characterized and demonstrated in this thesis, but can be improved to make a more useful contribution to the field of cell mechanobiology. Experimentally, one of the key limitations is the use of unmodified poly(ethylene glycol) as the encapsulating biomaterial. The material can be improved by chemically including adhesive ligands and by modifying the material to degrade with cell activity, all of which substantially improves cell viability and better mimics the in vivo microenvironment. Alternatively, the device can be modified to avoid the need for photopatternable biomaterials, by creating a well structure on top of each loading post. In this way, various natural or synthetic hydrogels could be polymerized in situ. This would improve
device applicability and cell viability and function. Furthermore, the number of cells in culture needs to be increased to meet requirements for more rigorous biological assay techniques.

Last, similar concerns regarding the influence of scaling laws apply to the three-dimensional platforms, as to the two-dimensional platforms. In the case of three-dimensional systems, microfabrication increases the surface area:volume ratio for the biomaterial constructs, and this affects transient hydrostatic pressures in the hydrogels as well as fluid shear on cells in the matrix as fluid equilibrium is reached. Hence, it is unclear whether findings on the microscale can be scaled up to macroscale culture systems without further characterization of cellular response to these forces.

9.2.3 β-catenin accumulation mechanisms

The time- and strain-magnitude dependent accumulation of β-catenin in the cell nucleus observed in Chapter 5 requires further study. First and foremost, this effect needs to be more rigorously and quantifiably studied using protein measurement techniques such as Western blotting. These techniques require a large number of cells, and hence, macroscale stimulation techniques will be required to confirm observations using our microfabricated screening platform. Second, the biological significance of these changes in nuclear accumulation levels of β-catenin need to be clarified. Although the measurements show significant differences in accumulation, and these results may be applicable to tissue engineering and in better understanding valve pathobiology, it is unclear if the observed differences in nuclear protein accumulation play a significant role in regulating cell function such as proliferation or differentiation. Third, it is unclear whether mechanical stimulation regulates β-catenin translocation into the nucleus; removal from the nucleus; or both. Determining this will be the first step in elucidating a signaling mechanism that regulates this mechanosensitive behaviour. Last, it is not immediately obvious what mechanotransduction mechanism would be sensitive to these differences in strain magnitude, and much work remains to be done in better understanding this phenomenon.

9.2.4 Side-specific myofibroblast differentiation

Similar questions are raised in examining the side-specific myofibroblast differentiation observed in response to multiple environmental parameters: while the developed technology can
be used to select combinations of microenvironmental cues for further study, the small number of cells available is a challenge for more comprehensive and mechanistic analyses. The key questions raised in observing this data is 1) why are these side-specific cells differentially responsive to environmental conditions; and 2) what mechanisms coordinate the integrated myofibroblast differentiation response of these cells to multiple culture conditions.
References


Appendix A
Microfabrication protocol: SU-8 master fabrication

A1. SU-8 Seed layer fabrication

**Purpose:** to create a thin, crack-free SU-8 layer to improve adhesion of thick SU-8 layers to glass.

**Equipment Required:**
Glass microscope slides soaked in acetone bath (min: 2 weeks)
SU-8 5 (Microchem)
Digital hotplate with temperature control*
Spin coater*
UV Exposure system (Karl Suss mask aligner)*
Phantom etcher for oxygen plasma etching*

* All fabrication performed with equipment in the ECTI Cleanroom (Bahen Center, Room 7178)

**Slide preparation**
1. Remove glass slide from acetone bath
2. Rinse with isopropanol
3. Blow dry with Nitrogen gas
4. Keep on hotplate at high temp (~200°) for as long as possible (minimum 5 mins)
5. Use standard oxygen plasma clean protocol in Phantom etcher (300W RIE, 5 minutes)
6. Continue dehydration on hotplate (~200°C) for another 20 minutes.

**Fabrication Process**

Make sure that all white lights are OFF before opening the SU-8
1. Use Pasteur transfer pipette, drop approx 1mL of SU-8 on glass slide
2. Tilt slide to spread the SU-8 over as much area as possible
3. Place slide in spin coater, with the following parameters:

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4. Bake on hotplate at 65° for 2 minutes
5. Bake on hotplate at 95° for 5 minutes
6. Flood Expose in UV aligner at 16.2 mW/cm² (365nm wavelength) and 30.5mW/cm² (405nm wavelength) for 6 seconds (cleanroom Karl Suss mask aligner)
7. Post-Exposure bake on hotplate at 65° for 2 minutes
8. Post-Exposure bake on hotplate at 95° for 5 minutes
9. Allow sample to cool to room temperature
10. Immerse in SU-8 developer, mild agitation for 2 minutes
11. Wash thoroughly in DI water
12. Blow dry with nitrogen gas
13. Bake at 175° for at least 20 minutes to harden the cross-linked SU-8

**A2. SU-8 feature layer fabrication**

**Purpose:** process flow to microfabricate SU-8 molds. Parameters for various thicknesses and SU-8 types are provided in the accompanying SU-8 Fabrication Parameters excel file

**Equipment Required:**
Cleanroom equipment (as in A1) – spin coater, SU-8, swabs, masks, exposure unit, etc.*
* This fabrication process tested and performed regularly in the ECTI cleanroom

**General fabrication process:**
Numerical values for all parameters are listed in the following page.

1. Use glass slides with seed layers of SU-8 only.
2. Dehydration bake – hotplate, ~180 degrees, ~20-30 minutes
3. Let samples cool on cleanroom wipe
4. Pour SU-8 across sample, being careful not to introduce bubbles
5. If bubbles are excessive, try remove them with a needle. Be careful not to scratch the surface
6. Tilt sample to spread SU-8 over as much of the surface as possible.
7. Re-tilt to get the bulk of SU-8 back to the center, and mount in the spin coater
8. Spin at the appropriate parameters
9. While the sample is in the spin coater under vacuum, perform Edge-bead removal (EBR):
   a. Use mechanical EBR on the top surface (scrape ~2-3mm of the edges with a swab),
   b. use chemical EBR on bottom surface – swab with acetone-soaked swab to remove SU-8 drip-offs
10. Use ONE hotplate only, initially set at 65 degrees.
    - NOTE: ECTI cleanroom hotplates should be set at 5 °C higher than stated values.
11. Level the hotplate using the bubble level (found in the top ‘cupboard’ of the storage cabinet next to the profilometer in the cleanroom). This is important to keep uniformity across the sample. Strips of cleanroom wipes are the most effective balancing platform. Do not move hotplate after.
12. Keep slide on hotplate at 65 degrees for the appropriate time.
13. When time is up, set the hotplate to 95 degrees. Start timing after the readout shows 95+.
14. When time is up, set the hotplate to <=65 degrees, and wait to cool. When the readout is less than 65 degrees, remove the sample and place on a cleanroom wipe on the bench. Allow more time to cool to RT
15. Set the hotplate to 65 degrees again.
16. Expose in the aligner for appropriate time and settings.
17. Note: if using soft or hard contact, use the blank glass slide as the hard-mask backing, and place your mask directly on top of your sample before loading, ink side down.
18. Place samples on hotplate for the appropriate time
19. When time is up, set the hotplate to 95 degrees. Start timing after the readout shows 95+.
21. When time is up, set the hotplate to <65 degrees, and wait to cool. For really thick samples with lots of SU-8 cross-linking, allow it to cool on hotplate all the way to room temperature. Up to ~150um structures, cool to 60 degrees, and then remove and allow to cool to RT on wipe on cleanroom bench.

22. Wait overnight before developing. This gives the SU-8 film time to relax and it won’t crack.

23. Develop for however long it takes – check at ~10 minute intervals for the first 30 minutes, and then 5 minute intervals after that. Replace developer every 10 minutes or so.

24. Hard-bake according to parameters.

25. If hard-bake temperature ramps are too steep, cracking in thick SU-8 has been observed. Be careful – use an oven at low temperature for a long time if necessary.
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Appendix B
Microfabrication protocol: PDMS fabrication

B1. Casting

Purpose: pour and cast a slab of PDMS on an SU-8 master

Procedure: the master first needs to be silanized to minimize adhesion between the master and the cured PDMS.

Master Silanization

1. The SU-8 master is silanized in order to minimize adhesion between the master and the cured PDMS.
2. Place the master in a glass vacuum dessicator.
3. Pipette 60-70 µL of the silanization agent (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA) into a petri dish in the vacuum dessicator.
4. Using the VLP80 pump with the gas-ballast switch set to 1, pump down the dessicator until the lid cannot be moved from the chamber.
5. Switch the gas-ballast setting to 0, and continue pumping down for 1 minute.
6. Switch the dessicator inlet port to maintain vacuum and shut down the pump.
7. Leave for at least 1-2 hours, if not overnight.
8. Release vacuum, place master into a casting dish (top of a Nunc omniwell plate works well).

PDMS mixing and pouring

1. Place a weigh boat on the digital scale and tare to zero.
2. Pour in the desired quantity of PDMS monomer. Record the mass.
3. Multiply the mass by 1.10, and use a syringe to add enough cross-linking agent to reach this value.
4. Mix thoroughly with the syringe. Pour mixing results in ‘sticky’ PDMS after curing.
5. Pour over the sample in the casting tray.
6. Place in vacuum chamber and degas until the bubbles disappear.
7. Place in an oven at 80 °C to cure for at least 4 hours, if not overnight.

B2. Bonding PDMS

Purpose: to irreversibly bond a slab of cured PDMS to a PDMS or glass substrate

Equipment Required:
Corona Discharge Treater (ElectroTechnic Products, BD-20A); Hotplate

Glass Slide Prep

1. Use fresh glass slide from acetone bath. Remove with tweezers and blow dry with Nitrogen spray.
2. Wipe slide with 70% Ethanol solution
3. Place glass slide on hotplate at ~100° C for ~10 minutes (dehydration step)
4. Replenish acetone bath with new glass slide

**PDMS Prep**
1. Cut and peel PDMS from master, check for dust.
2. If using old PDMS, or if dust is on sample, use clear scotch tape to lift off all particles

**Bonding Process**

**WARNING:** MAKE SURE THAT NO ELECTRONICS ARE WITHIN 5 FEET OF THE CORONA
1. Use the 3” Field Electrode (curved wire perpendicular to treater)
2. Adjust height of retort stand so that the lowermost point of the electrode is approximately ¼” above the sample height.
3. Turn on corona discharge unit, and play with the adjustment knob.
4. Try and set it for the softest, most uniform corona field possible
5. For a 1” x 3” Bonding area, approximately 20-25s of treatment is required, if using the field electrode. Adjust this number based on how much you have to move the treater over the surface.
6. Treat BOTH the Glass Slide AND the PDMS piece
7. Shut off the corona discharge treater
8. Be careful not to touch the surface – handle the PDMS and glass by the edges
9. Carefully put the samples in conformal contact with each other. No pressure required.
10. Place sample on hotplate at ~80° C for at least 20 minutes. If shorter time required, higher temperatures can be used.

Notes: increase in corona treatment time often means an increase in post-bonding bake time. Chip Tip from Beebe’s group indicates that for an excessive corona exposure, the sample needs to be left at <100° C overnight.

**B3. Spin coating PDMS**

Spin coating parameters listed here are characterized for the ECTI cleanroom spin coaters. Translating them to other coaters may be possible, but will result in minor variations in thicknesses.

PDMS is poured onto the substrate and degassed. The substrate is then placed on the spin coater chuck, and the vacuum is turned on. The spin coater is programmed as per the parameters in the following table. All accelerations are set at 88 RPM/s, (setting 001 for ACL on the coater). Each recipe uses four steps, with the RPM and times provided.
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Appendix C

Microfabrication protocol: Multilayer structures for mechanically dynamic cell culture^5

C1. Device description and operation

Devices are fabricated using multilayer soft lithography[99] in polydimethylsiloxane (PDMS), and are capable of simultaneously generating a range of mechanical conditions in individual cell culture locations across the microfabricated array. In this protocol, the steps to fabricate an array of pneumatically-actuated microposts are first described, followed by steps to modify the device to enable mechanically dynamic culture in both two-dimensional (2D) and three-dimensional (3D) culture paradigms. The outlined microfabricated approach increases throughput over existing macroscale systems, and is best suited for screening for the effects of a variety of mechanical conditions.

The operational principle for the device is based on an array of vertically actuated microposts. Microposts are fabricated on a freely-suspended diaphragm, and are raised and lowered by applying positive and negative pressures beneath the actuation diaphragm (Figure 1). A key feature of the array is that by varying the size of the actuation diaphragm, a single pressure source can be used to obtain a range of vertical displacements across the array. This principle is used to rapidly screen cellular response to a large number of mechanical stimulatory conditions across a single device.

Based on an analogous macroscale design by Schaffer et al.[242], our design includes a second suspended and lubricated cell culture film over the post, allowing cells to experience 2D substrate deformation as the culture film slips over the raised loading post. Alternatively, photopatterning an array of cell-laden hydrogels over the loading posts allows for compressive stimulation of cells in 3D culture. Detailed instructions in setting up these systems follow.

C2. Fabrication of the pneumatic microactuator array

Fabricating the pneumatic microactuator array requires stringent alignment in multilayer PDMS structures. This is challenging, due to shrinkage-induced alignment registration errors. To address this, we use a fabrication process termed 'sandwich mold fabrication'[220], shown to effectively eliminate this issue[202].

Materials Preparation

1. Single- or multi-level SU-8 masters for each of the various levels are fabricated in a cleanroom using standard procedures. For these devices, masters are 200-400 μm thick, depending on the application. Masters are hardbaked at 80 °C for three days prior to use.

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2. Clean glass slides and the SU-8 masters are silanized in a vacuum chamber, to prevent adhesion of curing PDMS to the materials. In a fume hood, 60 μL of the silanization agent (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA) is pipetted into a petri dish in the chamber. The bare glass and SU-8 masters are placed in the chamber and kept under vacuum overnight.

3. Two foam pads (available at craft supply stores) and an overhead inkjet transparency film are cut to sizes slightly larger than the SU-8 master used for sandwich mold fabrication.

Sandwich Mold Fabrication

1. Each layer of the multilayer device requires a PDMS layer molded using this method. A schematic of the sandwich mold fabrication process is provided in Figure 4-1.
2. PDMS monomer and crosslinker are thoroughly mixed in the standard 10:1 ratio. 3 mL of uncured PDMS mixture are deposited onto a previously fabricated SU-8 master, and degassed in a vacuum chamber.
3. The foam pad and SU-8 master are placed on a plexiglass base plate.
4. The transparency is carefully lowered onto the uncured PDMS, making sure to avoid trapping any air bubbles. Inkjet transparencies have a rough and a smooth side, and it is important that the smooth side contacts the uncured PDMS.
5. A silanized glass slide is placed on top of the transparency, beneath the second foam pad and a plexiglass top plate. The sandwich is then clamped together using a C-clamp.
6. The PDMS sandwich is cured in an oven at 80 °C for at least 4 hours.
7. The sandwich is removed from the oven and allowed to cool before removing the clamp.
8. The clamp is removed and the sandwich disassembled, and the foam pads are discarded.
9. These patterned PDMS layers and their handling transparency layers can then be stored in a dust-free environment until they are ready for use. We have successfully used PDMS layers up to two months old, with no noticeable deleterious effects.

Constructing the multilayer device

The schematic for fabrication of the microactuator array is provided in Figure 4-4, and operation of the actuators is shown in Figure 4-5. To fabricate the device:

1. The lowermost patterned PDMS layer is prepared by sandwich mold fabrication. For these experiments, circular patterns ranging from 600 to 1200μm in diameter were used.
2. The PDMS layer is then transferred to a clean glass slide. A corona discharge unit is used to treat the PDMS and glass surfaces with oxygen plasma[224], and the two surfaces are placed in contact with each other. The stack is then placed on a hotplate at 80 °C to complete the bonding process. The transparency can then be peeled away and discarded.
3. Depending on the success of the sandwich molding step, thin films of PDMS which have not been squeezed out from between the SU-8 features and the transparency may need to be removed. This can be done manually using a 25G needle under a stereoscope.
4. The second sandwich molded layer is formed as a negative replica mold of the desired diaphragm-and-post structure. This is because fabrication of large-area SU-8 masters is challenging, but the use of alternate fabrication processes could eliminate this extra step. In this demonstration, circular posts 500 μm in diameter were used. The negative replica
mold is silanized, and temporarily affixed to a glass slide using double-sided tape. Uncured PDMS is deposited, degassed and spin-coated onto this sandwich mold layer for 45 seconds at 1000 RPM, yielding an actuation diaphragm 60 μm thick. The spin-coated PDMS layer is partially-cured at 80 °C for ~20 minutes, or until the PDMS is sticky but does not deform permanently when touched.

5. The glass slide and double sided tape are removed from the partially-cured PDMS layer, which is then plasma-treated, inverted and placed in a custom-made aligner. The aligner consists of a vacuum chuck mounted to a micromanipulator (Siskiyou; Mission Viego, CA, USA). The first PDMS layer is then plasma-treated and placed on a rotary stage (Newmark, Grants Pass, OR, USA), beneath the vacuum chuck, and alignment between the two layers is observed using a Navitar 12x zoom machine vision system (Navitar; Rochester, NY, USA).

6. The layers are aligned using the manipulator, and carefully brought into contact. The sandwich is then removed from the aligner, and cured in an oven at 80 °C for a further 30 minutes. The transparency and silanized replica PDMS mold can then be peeled from the device and discarded. The device is then fully cured at 80 °C for at least four hours.

7. Depending on the type of mechanical stimulation experiment being conducted, additional PDMS layers can then bonded to this base device by repeating the outlined process. Details for the various types of mechanical stimulation experiments are provided in sections C and D.

Connectors
1. Once the device is completed, a needle can be used to clear the PDMS layer at the point of connection.
2. Commercial connectors such as the Nanoport (Upchurch Scientific; Oak Harbor, WA, USA) can then be used to interface with external tubing. To avoid the height requirements of commercial connectors, and thereby use standard petri dishes as device containers, we fabricate our own as follows.
3. Approximately 35 g of PDMS is cast and cured in an omni-well tray (Nunc; Rochester, NY, USA). The device is peeled, and cut into small gasket sections, from which a 1/8” hole is removed using a punch.
4. An 18G blunted needle is used to core out a section from the side of the gasket. A smaller 21G needle is used to remove the central core before withdrawing the 18G needle.
5. Approximately 15g of PDMS is cast and cured in an omni-well tray lid, peeled and cut into similarly sized section. This section is then cleaned with scotch tape, plasma-bonded to the top of the gasket, and the unit is plasma-bonded to the device, punched side down.
6. A second blunt 18G needle is then separated from the coloured plastic luer connector, inserted into the cored hole and sealed in place with a few drops of PDMS. The resulting connector is quite effective, economical and has a dead-space volume large enough to prevent air bubbles from entering the channels during liquid filling stages.

C3. Mechanically active 2D culture substrates
The array of actuated microposts can be used to create various strain profiles in a suspended polymer film, on which cells are cultured. Raising the post into a lubricated film causes the film to slip and deform around the post. Using a circular loading post results in an equibiaxial strain
distribution, but the design is versatile in that different post shapes can be used to create a variety of strain fields. To modify the actuator array for 2D culture experiments (schematic in Figure 5-4):

1. Bond a third PDMS layer to the device, as shown in Figure 5-4. The third PDMS layer consists of a two layer structure. The lower circle matches the size of the actuation cavity beneath the actuated microposts. The diameter of the upper circle is 100μm greater than the diameter of the loading posts. A 200 μm wide microchannel connects these features to a connection area, and will be used to lubricate the loading posts and culture film.
2. Fabricate and bond connectors to the device, as previously described.
3. Spin coat a 10-15 μm thick layer of PDMS onto the smooth side of a transparency (spin parameters: 4000 RPM, 120 seconds). Cure at 80 °C for at least 4 hours.
4. Apply a low-level vacuum (Barnant Air Cadet aspiration pump) to the actuators, lowering the array of posts.
5. Plasma treat and bond the spin coated PDMS film to the actuated microdevice. Place on a hotplate at 80 °C for 10 minutes. The posts and culture film will remain hydrophobic for a short time.
6. Fill the lubrication channels with a 90% glycerol in deionized water solution. In addition to lubrication, this formulation maintains the hydrophobicity of the PDMS indefinitely, reducing friction coefficients[248]. Air bubbles will remain trapped between the post and culture film. Place the device on a hotplate at 40 °C, and continue to inject lubricant into the channel, causing a small pressure increase. After a few minutes, the air bubbles will diffuse through the PDMS, and all surfaces will be lubricated.
7. Remove the negative pressure, releasing the posts.
8. Using a scalpel, cut around the thin PDMS film. Carefully peel the transparency backing away from the device.
9. Plasma bond a hand-cut PDMS gasket around the device culture area.
10. Sterilize the device by soaking in 70% ethanol for 5 minutes, and then under a germicidal UV light in a biological safety cabinet for 45 minutes.
11. Plasma treat the surface and incubate with 100 μg/mL collagen (or alternative ECM protein) overnight at 4 °C.
12. Wash the device with phosphate buffered saline (PBS), and seed cells at 10-15000 cells/cm², following standard cell culture protocols.

Detailed characterization of microdevice operation and biological experiments conducted on this platform have been published elsewhere[187].

C4. Mechanically active 3D hydrogels

Modifications to the device can also be used to enable compression of photopatterned, cell-laden, three-dimensional hydrogels in a high-throughput manner. In this example, we create 350 μm diameter polyethylene glycol (PEG) hydrogel cylinders, encapsulating C3H10T1/2 mouse mesenchymal stem cells, and apply compressive strains ranging from 5 to 25% across the array. This protocol can be used with more advanced hydrogel photopolymerization chemistries. To use the platform for experiments in three-dimensional mechanobiology (schematic in Figure 8-2):
**Device modifications:**

1. Coat the posts with a 1 μm thick layer of Parylene-C to reduce gas permeability and improve polymerization times in the PEG hydrogel system. This will reduce the cytotoxic effects of the free radical-based photopolymerization reaction. Pieces of scotch tape are used to mask the areas around the post, and the device is coated in a PDS 2010 LabCoter® 2 system (Specialty Coating Systems; Indianapolis, IN, USA).
2. The coated device is removed from the coating system, and the scotch tape peeled away, leaving a conformal layer of Parylene-C over the posts.
3. A glass coverslip is methacrylated[176] by immersion in a 2% v/v solution of 3-(trimethoxysilyl) propyl methacrylate in 95% ethanol for 2 minutes.
4. The coverslip is rinsed in 100% ethanol, and baked at 100 °C, leaving methacrylate groups on the surface.
5. A 200 μm thick PDMS spacer film is cast in a petri dish, and cut into small sections. Four of these small sections are plasma bonded around the edge of the methacrylated coverslip.
6. The spacers are then plasma bonded to the PDMS-areas of the device, covering the Parylene-C coated posts.
7. Device is sterilized by washing in 70% ethanol, and exposure to germicidal UV light in a biological safety cabinet for 45 minutes.

**In situ polymerization:**

Cell-laden PEG hydrogels were then photolithographically patterned into the devices[176, 345] as follows:

1. Prepare a 10% w/v PEGDA (3.4kDa, Laysan Bio; Arab, AL, USA) and 10% w/v PEG (8kDa, Sigma-Aldrich) solution in unsupplemented cell culture media.
2. Prepare a photoinitiator stock solution of 100 mg/mL of Irgacure 2959 (Ciba Specialty Chemicals; Tarrytown, NY, USA) in 1-vinyl-2-pyrrolidinone.
3. Thoroughly mix the two prepared solutions together to obtain a solution with a concentration of 0.4% w/v of photoinitiator. Filter the mixture through a 0.45 μm syringe filter to sterilize and remove particulates.
4. Trypsinize cell cultures and prepare a cell suspension in culture media, at twice the desired end-point cell concentration.
5. Mix the cell suspension and the filtered hydrogel precursor/photoinitiator solution in a 1:1 ratio.
6. Inject the solution into the microdevice using a long 25G needle. Carefully avoid trapping bubbles in the device.
7. Align a printed transparency mask to alignment marks on the device surface. Set up a UV illumination system to provide a UV dose of 17.5 mW/cm² at the device surface. Illuminate the hydrogel through the mask for 195 s. This time has been optimized for our system, and may need to be adjusted.
8. Wash away unpolymerized hydrogel precursor solution with PBS, and replace the PBS with culture media. Actuate the loading posts to compress the hydrogel constructs.

Detailed characterization of microdevice operation and biological experiments conducted on this platform have been published elsewhere[175].
C5. Peripheral equipment

Modified petri dishes are used to maintain sterility of cell cultures during actuation of the devices. A blunted and stripped 18G needle is epoxied into a hole drilled in the side of the petri dish. Tubing (Clay Adams Intramedic PE190; VWR International, Arlington Heights, IL, USA) is press-fitted to these connectors and connects to the controlled solenoid valves and pressure source.

Pressures to the devices are provided by external micropumps. For the 2D experiments, positive pressure values from 30-55 kPa were generated using a voltage-controlled eccentric diaphragm pump (SP 500 EC-LC 4.5VDC; Schwarzer Precision, Germany). A pulse-width modulation based voltage controller is used to vary the applied voltage to control pressure output. Solenoid valves and manifolds (S10MM-30-12-3 and MSV10-1; Pneumadyne, Plymouth, MN, USA) are used to create a cyclic pressure waveform. The valves are actuated with a 12 V signal, controlled by a square waveform function generator.

C6. Device characterization and use

Imaging on the device
A common issue with imaging cells or particles on the device is poor optical resolution due to the condensation of droplets beneath the PDMS film supporting the loading post. This condensation arises from temperature and humidity differences inside the incubator and after fixation or on a microscope stage. To resolve this problem:

1. Connect an open reservoir to the pressure inlet port, and fill it with deionized water. We use an open syringe with a luer lock connector for convenience.
2. Place the device and filled reservoir in a vacuum chamber, and pump down.
3. Wait 5 minutes, and bring the chamber to atmospheric pressure. Repeat two or three times. As air is displaced out of the channels of the device, it is replaced with water from the reservoir, eliminating the droplet condensates and providing a clear optical path for microscopy.
4. Image culture areas using bright field or fluorescence microscopy.

For live cell imaging, this procedure can be done before seeding the cells on the devices. However, channels must be designed large enough to prevent any viscous losses of pressure between units on the device.

Strain characterization by bead tracking
On the 2D culture systems:

1. Dilute 1 μm diameter fluorescent beads (Bangs Laboratories; Fisher, IN) in methanol to the desired concentration. Dilutions of 1:150 from the original concentrations (1% solids in bead buffer solution) were found to be suitable for our needs.
2. Plasma treat the device surface, and deposit 5-10 μL of bead suspension.
3. Wait for the methanol to evaporate. Repeat if bead concentration is too low.
4. Image the cell culture areas at rest, and under load.
5. Open-source automated particle tracking algorithms can then be used in ImageJ (NIH) to track bead displacements.
6. Displacement magnitude, direction and location parameters can then be used in a mathematical model to characterize strain fields on the device surface.

A similar procedure can be followed to characterize strains in 3D systems. Mix a quantity of fluorescent beads into the hydrogel precursor solution. In the case of the PEG hydrogel system, the pore sizes are much smaller than those of the 1 μm diameter beads. Beads are encapsulated in the hydrogel, and the system can be imaged using a confocal microscope. Bead displacements can then be tracked, analyzed and fitted to a deformation model.

C7. Discussion

Though conceptually simple, device fabrication does take some experimental skill and practice. Particularly in the case of 2D cell culture, alignment of the multiple layers in the device can be challenging, especially over a large-area array. Practically speaking, we can reliably achieve a 100% alignment success rate using devices with 50 μm of tolerance in spacing between adjacent features in multiple layers. We have also successfully demonstrated alignment with tolerances as low as 15 μm, but the alignment time required is substantially greater and is achieved at a lower success rate. Alignment of the mask to photopattern biomaterials onto the array is similarly challenging, but the posts can be designed to be quite large in comparison to the hydrogel cylinders, alleviating this constraint.

Other challenges involve cell culture on the materials described in this protocol. In the 2D stretching system, using PDMS as a culture film limits biological investigation to short-term experiments, as cell adhesion to the substrate begins to be substantially affected after 6 hours of stimulation. This can be rectified by covalently binding matrix proteins to the PDMS surface[263], or by replacing the PDMS cell culture substrate with a more clinically relevant polyurethane material, to improve cell adhesion and provide greater flexibility in conducted biological experiments[201]. In the described 3D system, the hydrogel chemistry is intended as an illustrative example only. Long-term culture of adherent cells requires the use of alternative natural or synthetic hydrogels, or the inclusion of adhesive ligands into the PEG matrix (which can be easily achieved using standard PEGylation chemistries)[360].
Appendix D
Microfabrication protocol: Composite diaphragm structures

This protocol was originally written by Cameron Lam.

**Purpose:** Fabrication of composite diaphragm structures (Chapter 7).

**Procedure:** multi-step process, as follows.

*Squeeze Fabrication (Silanized Glass/SU-8 Masters)*

Note: Epoxy masters can be used instead of glass/SU-8 and require a slight modification of the process described.

1. Place a layer of foam on top of an acrylic plate (Figure D-1).
2. Place the master on top of the foam with the features facing up
3. Pour 3 mL of PDMS on top of the glass master.
4. Spread the PDMS over the master by tilting to ensure that all features are covered.
5. Remove all air pockets in the PDMS by placing the masters in a vacuum chamber.
6. Press a transparency smooth side down on to the PDMS covered master to spread it.
   a. This should be done slowly such that no air pockets are formed between the PDMS and the transparency.
7. Place a silanized glass slide on top of the transparency directly above the master. This helps distribute the force evenly.
8. Put another layer of foam on top of the silanized glass slide and cover with another acrylic plate.
9. Use C-Clamps to press the acrylic plates together (make sure that the clamps are tight enough so that nothing moves, but doesn’t crack the glass inside).
10. Bake the entire set up at 80°C for 4 hours
11. Remove the set up from the oven and let cool. Once the set up has cooled to room temperature remove the foam sandwich from the acrylic plates.
   a. Remove the top layer of foam and the silanized glass master. The bottom layer of foam, the master, and the transparency should remain.
12. Remove the bottom layer of foam from the transparency – make sure the transparency is in a stable position so it cannot be pulled away from the master as this may peel off the PDMS squeeze fabrication.
13. Slowly remove the transparency from the master ensuring that no air pockets are formed during removal.
14. Remove a small portion of the edges of the PDMS squeeze fabrication using a scalpel to cut it away.
Figure D-1. Squeeze fabrication process

**Squeeze Fabrication (Silanized Epoxy Masters)**

Steps 1-8 using epoxy or glass masters are identical.

9. Put the entire acrylic plate set up on a level surface at room temperature.
10. Place weights on top of the set ups to apply force – do not use clamps.
    a. ~ 5 lbs per set up is effective
11. Let cure overnight.
12. Remove weights and cure at 80°C for about an hour. This will ensure that all PDMS has been cured.
13. Remove the set up from the oven and let cool at room temperature with a 5 lb weight on top of the acrylic plate. This ensures that the epoxy masters will return to their non-deformed shape properly.
14. Once the set up has cooled to room temperature remove the foam sandwich from the acrylic plates.
    a. Remove the top layer of foam and the silanized glass master. The bottom layer of foam, the master, and the transparency should remain.
15. Remove the bottom layer of foam from the transparency – make sure the transparency is in a stable position so it cannot be pulled away from the master as this may peel off the PDMS squeeze fabrication.
16. Slowly remove the transparency from the master ensuring that no air pockets are formed during removal.
17. Remove a small portion of the edges of the PDMS squeeze fabrication using a scalpel to cut it away.

**Polyurethane/PDMS Handling Slab**

1. Cast 28 grams of PDMS in an omni well tray lid and cure at 80°C for 4 hours.
2. Cut the PDMS to size – slightly larger than the devices will be.
3. Clean the PDMS slabs of debris by applying and removing tape repeatedly.
4. Prepare a beaker to dry the PDMS slabs in.
    a. Press PDMS squares to the bottom of the beaker to prevent the PDMS slab from slipping in the beaker.
5. Put 0.5 wt% PU in THF in a petri dish and soak the PDMS slab in the solution for 3 seconds.
6. Using tweezers pick up the PDMS, preferably at the corner, and place in the drying beaker standing up.
7. Cover beaker immediately with paper towel and an empty petri dish.
   a. If more than 1 slab will be drying in the same beaker wait 15 minutes before putting the second slab in to dry as this will cause streaking of the polyurethane.
8. Let dry overnight.

Gasket Fabrication

1. Cast 40 grams of PDMS in an omni well tray lid and cure at 80°C for 4 hours.
2. Cut the PDMS exactly to the size of the glass slide.
3. Using a scalpel cut out the wells from the PDMS.
   a. Alternatively a punch can be used to cut out the wells; however, the circular punches do not leave much room for alignment error.
4. Cut out the connector hole using a circular punch.

Chip Connector Fabrication

1. Cast a 60 gram PDMS slab in an omni well tray base and cure at 80°C for 4 hours.
2. Cut out a small square in slightly large enough to cover the inlet hole in the device base.
3. Punch out a circular hole from the top to the bottom of the PDMS square using a circular punch.
4. Using the sharpened 18 gage needle, punch a hole in the side of the PDMS square to the vertical through hole just created in the previous step. Remove all PDMS through the vertical through hole.
5. Cast a 10 gram PDMS slab in an omni well tray lid and cure at 80°C for 4 hours.
6. Cut out a square the same size or larger than the 60 gram piece.
7. Plasma treat the base 60 gram piece and the 10 gram piece and place together to cover one end of the vertical through hole.
8. Heat on a hot plate at 80°C for 10 minutes to make the bond permanent.

Device Fabrication

1. Clean a glass slide with ethanol and dry before proceeding.
2. Plasma treat the glass slide and it aside.
3. Plasma treat the PDMS squeeze fabrication and press it on top of the glass slide previously treated. Make sure no air is trapped between the two layers.
4. Heat the glass slide on a hot plate at 80°C for at least 10 minutes to make the bond permanent.
5. Remove the transparency from the glass slide slowly and the PDMS should remain on the glass.
6. Remove any excess PDMS from any through holes by using a blunted needle tip to scrape away thin layers of PDMS under a microscope.
7. Plasma treat the PDMS/PU handling slab (see Polyurethane/PDMS handling slab fabrication).
8. Pour 2 mL of PDMS (already de-gassed) on the handling slab and spread by tilting.
9. Spincoat handling slab to achieve desired PDMS thickness using the following recipes:

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<td>90</td>
<td>300:30</td>
<td>500:30</td>
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<td>0:60</td>
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<td>45</td>
<td>200:30</td>
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10. Bake the handling slab at 80°C for about 20 minutes. This timing is debatable depending on the thickness of the PDMS layer – just be sure that the thin PDMS layer is still sticky, but no longer deformable. Check the handling slabs at 15 minutes and continue to bake until PDMS is as described above.
11. Plasma treat the glass slide PDMS side up.
12. Plasma treat the PDMS/PU handling slab.
13. Starting at one end gently press the handling slab to the PDMS glass slide. Start at one end and gently roll a finger over the handling slab to ensure no air pockets are created.
14. Heat the pressed together pieces for at least 10 minutes on the hot plate at 80°C.
15. Cool to room temperature and very slowly remove the PDMS handling slab from the glass slide. The PDMS layer should come off of the handling slab with the polyurethane leave a thin membrane on the base.
16. Tape a transparency to a silanized glass slide using double sided tape.
17. Pour 2 mL of PDMS on to the transparency and de-gas.
18. Spin coat PDMS mortar layer as follows:

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19. Bake transparency at 80°C for 10 minutes until sticky, but no longer deformable.
20. Plasma treat transparency and gasket (see gasket fabrication).
21. Place gasket on to transparency and bake for and additional 5 minutes at 80°C.
22. Remove gasket from transparency, a thin layer of PDMS should come with it.
23. Plasma treat the PDMS side on the gasket and the base layer.
24. Align the gasket wells with the base layer membranes and press together slowly starting from one end to the other to ensure no air pockets get trapped.
25. Heat the device on a hot plate for at least 10 minutes at 80°C.
26. Plasma treat the gasket and the chip connector (see chip connector fabrication) around the inlet port.
27. Align the chip connector inlet port with inlet port on the gasket and press together. Heat on hot plate for 10 minutes at 80°C.
28. Press a blunted and bent 18 gauge needle into horizontal inlet hole of the chip connector.
29. Cover the chip connector/needle connection with PDMS and cure at 80°C for 4 hours.

Figure D-2 – Fabrication process for composite diaphragm structures
Appendix E
Histology data supporting layer-specific isolation of VICs from aortic valve leaflet

These histological stain images were prepared by Morakot Likhitpanichkul and Krista Sider. Morakot Likhitpanichkul developed, optimized and characterized the protocol for layer-specific isolation of VICs. The images included in this appendix demonstrate the clean enzymatic separation of the spongiosa from the fibrosa and ventricularis layers of the aortic valve. Movat’s pentachrome stain was used in each of the following images, in which cell nuclei and elastin are stained black, glycosaminoglycans are stained blue, and collagen is stained yellow. The histologically stained cross-sectional view of an aortic valve leaflet is shown in Figure E-1.

![Histology image](image1.png)

Figure E-1. Cross-sectional view of valve leaflet

Typical results for the physical separation of the fibrosa and ventricularis layers are shown in Figure E-2.

![Histology image](image2.png)

Figure E-2. Fibrosa (A) and ventricularis (B) layers after physical separation.
Enzymatic digestion in hyaluronidase removed the remaining spongiosa layer from both sides of the valve leaflet, as shown in Figure E-3.

Figure E-3. Fibrosa (A) and ventricularis (B) layers after physical separation and enzymatic digestion.

These histology sections demonstrate the clean separation of the fibrosa and ventricularis layers of the valve leaflet from the intermediary spongiosa. Tissues are then enzymatically-digested separately, yielding side-specific cell populations for the fibrosa and ventricularis.
Appendix F
3D confined compression systems

The device design and fabrication work presented in this appendix has not been published, but addresses one of the key failings of the unconfined compression system presented in Chapter 8. In order to position biomaterials in the devices presented in that Chapter, biomaterials were required to be photopolymerizable, and careful alignment was required to position the mask over the compressive microposts, which currently limits material compatibility and ease of use of the platform. In order to address this, we developed a confined compression system compatible with any polymerizable biomaterial.

Figure F-1. Working principles of device operation. (A-B): Schematic cross section of confined compression stimulation platform. (C) Biomaterials are polymerized into the system beneath a coverslip, and are confined to the region directly above the loading post.

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6 The work presented in this appendix was presented at the 2009 Micro Total Analysis systems conference:

Figure F-2: Device operation and calibration. (A, B): Collagen gels polymerized in device. (C) Resliced confocal images of polymerized biomaterial at rest and (D) when actuated. (E) Characterized strains across the array for a collagen-poloxamine biomaterial, under actuation pressures of 30 and 55 kPa (n = 3).
Cell-laden biomaterial prepolymer solutions are poured over the loading posts and polymerized in situ beneath a glass coverslip. Surface tension forces prevent the prepolymer from moving past the loading post (Figures F-1). We have successfully integrated both pH-polymerizable collagen gels and UV-polymerizable collagen-poloxamine gels into the microfabricated array. More generally, a wide range of other natural and synthetic polymeric biomaterials can be incorporated into this platform.

In the current device configuration, the cylindrical biomaterial constructs are 500 µm in diameter and 350 µm thick. Twenty-five of these constructs are formed within a 1 cm × 1 cm area, and the device area can be further scaled up to conduct massively parallel high-throughput biological experiments. In order to experimentally quantify the strains applied to the polymerized biomaterial, 1 µm diameter fluorescent beads were added to the collagen solution, and bead-laden collagen gels were polymerized on a smaller characterization array with actuation cavities of different sizes. The constructs were then imaged under a confocal microscope while at rest and when compressed with actuating pressures of 30 kPa and 55 kPa. Compressive strains ranging from 5% to 18% were obtained across the array (Figure F-2).

Although this system enables the use of most polymerizable biomaterials, the effect of confinement on the mechanical deformation of the gels is still to be determined. This includes the differing solid mechanical deformation in the confined loading case, as well as the theoretical increase in transient hydrostatic pressure of the construct. The influence of cyclic loading on the solid and fluid mechanical components may also play a role in ultimate construct deformation.
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