ADVANCED MICROFLUIDIC PLATFORM FOR EPITHELIAL AND ENDOTHELIAL CELL DIMORPHIC COCULTURE

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science
Graduate Department of Mechanical Engineering
University of Toronto

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Abstract

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Biological cells cultured in a 3D microenvironment are more realistic and exhibit characteristics found in vivo when compared to 2D cell cultures. However, culturing 3D cellular structures such as epithelial mammary spheroids and endothelial cells simultaneously is tedious using conventional cell cultureware. To address this issue, we have developed a CNC-milled microfluidic system that enables simultaneous culturing of 3D tumor spheroids and endothelial-lined lumens. We employed an accessible polishing techniques to make optical quality concave features in milled microfluidic platforms. This system can be used to potentially study the complex and dynamic cellular interactions of cancer development, angiogenesis and other malignancies involving the interactions between different cell types of different morphologies.
Dedication

To mummy and daddy, for everything.
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B.1 Dimorphic Chip (Top) SprutCAM model

B.2 Dimorphic Chip (Bottom) SprutCAM model
Chapter 1

Introduction

1.1 Motivation

Today, biologists continually rely on two experimental strategies: (1) flat two-dimensional (2D) in vitro tests in Petri dishes and well plates that are high-throughput, simple and low-cost but are not physiologically relevant, or (2) three-dimensional (3D) in vivo animal models that are more complex and represent conditions in the body, but time-consuming, intensely laborious and expensive. Studies have shown that cells cultured in a 3D microenvironment are more realistic and exhibit characteristics found in vivo as opposed to 2D cell cultures. In drug studies, cytotoxicity for cells in 3D is drastically different from that of their 2D counterparts thus suggesting that there are unique cell-cell and cell-ECM interactions as a result of the complex geometries of tissues and organs [26, 60]. Different geometric, mechanical and biochemical factors are known to steer different cell lines into a specific, more realistic phenotype. For example, a relatively simple way to achieve 3D cellular architecture is the creation of tumor spheroids, a collection of cells that aggregate under non-adherent culture conditions. Spheroids are good biological models because like cancer tumor cells they need ample vascularization in order to grow in size. There have been numerous studies showing multiple ways of creating spheroids in a high throughput, consistent manner using microfluidics. Also, biologists have been able to recapitulate the inner lining of blood vessels using endothelial cells in microfluidic devices. Together, the aforementioned suggest that culturing spheroids in the presence of endothelial cells should induce angiogenesis and a microfluidic approach holds much promise as a means of achieving this.

In the past decade, there has been much interest in microfluidics and its potential to revolutionize healthcare especially with the continued development of cheaper point-of-care diagnostic devices that
are more physiologically relevant, use minimum reagent and could potentially be operated by unskilled workers in developing countries. Researchers have shown the effective use of these technologies for various drug discovery applications [68]. Current research focuses on microfabrication using soft-lithography techniques and polydimethylsiloxane (PDMS) which is unsuitable for commercialization and presents issues with adsorption, elasticity and leaching. There has been limited studies on rapid prototyping techniques such as micro milling and the use of thermoplastics which is significant because the majority of biological cultureware are made using polystyrene. Thus, there is a need to develop innovative methods for fabricating microfluidic devices that can not only be used for research but easily translated into commercial products for mass adoption.

1.2 Thesis Overview

The goal of this research thesis is to design and fabricate a microfluidic device that advances the study of angiogenesis by enabling the culture of blood vessel models in close proximity to tumor spheroid models similar to *in vivo* conditions. This device will adapt features from proven PDMS-based angiogenenic assays to polystyrene and will possess the following characteristics:

- **Fabrication:** utilizing micromilling as a means to rapidly prototype and introduce complex design features onto microfluidic devices in a cost effective manner.

- **Polystyrene:** creating a device using a material similar to current tissue culture treated substrate

- **3D Culture:** using the geometry of a micromilled device and basement membrane additives to induce the formation of physiologically relevant *spheroid bodies* and *lumen structures*.

- **3D Coculture:** creating a microfluidic device to culture two different cell types simultaneously with provisions for intercellular interactions.

- **Dimorphic** 3D coculture: creating a microfluidic device to culture two different cell types with two different morphologies simultaneously with provisions for intercellular interactions.

The work presented in this thesis serves to introduce an added level of sophistication to the platforms used for current angiogenesis research, focusing on the culture of tumor and blood vessel models on a single chip. The microfluidic device serves to aid cancer research by providing insights into the process

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1The term ‘dimorphic’ is used here to highlight that this device provides an unprecedented level of control in the morphology of the cells when compared to a conventional coculture platform
of chip$^2$ fabrication, post-milling processing and what design features are necessary for the 3D coculture of two different cell types.

\footnote{‘chip’ is used to refer to a milled plastic part or an assembly of plastic parts bonded to form a microfluidic device}
Chapter 2

Background - Literature Review

2.1 Problem statement

Angiogenesis is one of the more studied biological phenomenon due to the ubiquity and utility of the vascular network in understanding biological systems and processes. In fact, sustained angiogenesis or the growth of new blood cells, has been identified as one of the underlying principles that enable the growth and proliferation of cancer cells [73]. As a result, cancer researchers have been interested in drug compounds that halt or slow down cell growth by preventing the recruitment of blood vessels. The problem is that complex diseases like cancer are nuanced and involve many physiological processes that are not well understood. Therefore, the current drug development process is costly, time-consuming and there is a high failure rate among drugs that make it to the final phase. To put this into perspective, consider that a few decades ago, getting a drug through research and development and into the market was $10\times$ cheaper and $3\times$ faster than it is now (Fig. 2.1).

The reason for this discrepancy is that current biological assays including Petri dishes and animal testing suffer from poor physiologic context, overly involved experimental procedures and a lack of specificity. More importantly, these tests are not sufficient to study complex malignancies that affect multiple organ systems such as cancer. \textit{In vitro} tests involving Petri dishes, albeit being easily accessible and commonplace, do not fully recapitulate the cellular level microenvironment. On the other hand, \textit{in vivo} animal testing procedures are very expensive, time-consuming and inefficient at predicting the efficacy of drugs. To complicate matters even further, given a specific drug molecule, patients respond differently to the same treatment regimen. Therefore, even after extensive preclinical testing, the best practitioners still have to resort to a very ineffective approach when treating a disease like cancer: they
Figure 2.1: R&D productivity for new drugs has seen a steady decline in recent decades. (a) Drugs take a longer time to get through the development process, (b) have a low probability of successful clinical trials, and (c) are increasingly expensive to test and validate [Boston Consulting Group, 2011].

serve a cocktail of drugs and hope that one of them works. This coarse ‘trial and error’ method calls for the development of a new model that offers better predictive power than those currently available.

Microfluidics, the manipulation of fluids in channels with dimensions less than a millimeter, has been identified as one plausible approach to accelerating the drug discovery process (Fig. 2.2). The concept of microfluidics has enabled significant advancements in the field of biomedicine with the rising influence of ‘life-on-a-chip’ referring collectively to ‘lab on a chip’, ‘organ on a chip’, ‘tissue on a chip’ technologies. Researchers have shown promising results from the use of these technologies for basic analytical applications including sample handling, reagent mixing, separation and detection [42, 54, 68] to more sophisticated biological inquiry such as the formation of spheroid bodies and angiogenesis. The emergent question becomes how to incorporate the growing body of knowledge and capabilities into the design of an advanced device that can potentially better predict drug efficacy.

Figure 2.2: Drug testing platforms. (a) 2D Petri dishes and (b) animal models are no longer sufficient to study the increasingly complex biological mechanisms of many malignancies. (c) Microfluidics provides a means to circumvent the drawbacks of these conventional methods.
The proposed solution to bridge the gap between cell-based assays and animal studies is the use of microfluidic three-dimensional (3D) cell cultures. 3D cell culture includes extracellular matrix (ECM), modified surfaces, scaffolds, hanging drops and bioreactors as opposed to two-dimensional (2D) culture on flat Petri dish-like surfaces. Microfluidic devices with different geometric and structural conformations have been known to steer different cell lines into forming a more realistic phenotype such as spheroids with 3D architecture [1,60,71]

Spheroids are good 3D biological models because like cancer tumor cells they need ample vascularization in order to grow in size. There have been numerous studies showing multiple ways of creating spheroids using microfluidics in a high throughput, consistent manner (Fig. 2.3A) for cell mono- and cocultures [26,39,60]. Also, researchers have been able to recapitulate the inner lining of blood vessels using endothelial cells in cuboidal (Fig. 2.3C) and tubular (Fig. 2.3D) microfluidic devices to show that a gradient in vascular endothelial growth factor (VEGF) induces angiogenesis [7,17]. This has given credence to studies interested in the amalgamation of cancer tumor and blood vessel models in a microfluidic platform as a tool for drug discovery.

The newest development in this field is the prevascularized tumor (PVT) spheroid model (Fig. 2.3B) [16] which involves the direct coculture of primary endothelial cells, tumor cells and fibroblasts in a

Figure 2.3: The next frontier in angiogenesis research involves combining tissue engineering and tumor biology in a microfluidic setting. (a) Tumor spheroids can be cultured in a hanging drop [60] and (b) prevascularized tumor cells [16] can be used to study solid tumor progression. (c-d) Angiogenesis can be induced by exposing (c) an endothelial monolayer [17] or (d) 3D microvessel to an exogenous VEGF gradient [7] (Scale bar represents 100µm).
fibrin matrix. This model exhibits robust angiogenesis and creates a contiguous vessel network that vascularizes the spheroid and extends into the surrounding matrix. Using the PVT platform, one can potentially probe basic research and drug development questions regarding the mechanisms of tumor initiation and development. We follow this approach and take it one step further by suggesting that ‘indirect’ coculture of tumor spheroids and endothelial cells would enable insights into tumor-induced angiogenesis and a microfluidic approach provides a plausible means of pursuing this hypothesis.

2.2 State of the Art

The process of engineering and validating a microsystem that models tumor angiogenesis requires a multidisciplinary approach involving knowledge of fluid dynamics, microfluidics, mechanical design, material selection as well as cell and tissue engineering. Fundamentally, it is a step towards providing a technology that could potentially eradicate some of the current inefficiencies in the pharmaceutical and medical industry. For economic and ethical reasons, systems that more accurately mimic the human tissue environment will need to be considered to optimize preclinical and pre-animal selection of the most promising molecules from the large and growing pool of drug candidates [47]. Due to the breadth of information published on angiogenesis, cancer, tumor angiogenesis, and drug discovery, this review will focus specifically on identifying and analyzing previous research results that inform the design and fabrication of an advanced microfluidic device that can be used to model tumor angiogenesis using the Michigan Cancer Foundation-7 (MCF-7) breast cancer cell line and Human Umbilical Vein Endothelial Cell (HUVEC) lines. Emphasis will be placed on techniques that are available and accessible at the Integrative Biology and Microengineered Technologies Laboratory (IBMT, University of Toronto) such as microfluidics, soft lithography, micromilling, cancer cell culture and lumen formation.

2.2.1 Microfluidics

Microfluidics is the technological field that involves the manipulation of small amounts of fluids in micrometer-sized channels to exploit the distinct characteristics of fluids in these microchannels such as laminar flow and diffusion-based transport phenomena [69]. The field of microfluidics came about as an adaptation of successful technologies in the semiconductor industry and really took off after the development of the soft lithography technique which primarily uses the poly(dimethylsiloxane) (PDMS) elastomer [44]. The choice of materials has since been expanded to include thermoplastics such as polystyrene (PS), poly(methylmethacrylate) (PMMA), cyclo-olefin copolymer (COC) and polycarbonate (PC) [75]. Consequently, microfluidic devices are fabricated using techniques adapted from the
semiconductor and plastics industries such as micromachining, photolithography, replica molding, embossing, and injection molding [19, 27, 75].

Microfluidics has been applied to make in vitro assays more realistic and adaptable to various applications by integrating various complex environmental factors, including those that facilitate 3D cell migration, proliferation and differentiation. The miniaturization of bioanalytical techniques through microfluidics presents several benefits including: the reduction of the size of equipment, fast analysis, short reaction times, parallel operation for multiple analyses, possibility of portable devices, low sample and reagent volumes, low waste levels and unique physical consequences of microscale fluid flow [19]. Assays based on microfluidics provide robust platforms for studying 3D cell migration and heterotypic cell-cell interactions in cancer invasion and for testing the efficacy of potential treatment of drugs. Features such as the dimensions of access channels and the means for seeding cells, changing media and regulating flow are chosen to be of a comparable size to their in vivo counterparts. By providing cells a well-controlled 3D environment, microscale systems can meet the demands of this increasing trend toward reproducing the true complexity of tissues and organs in vitro as models of pathophysiological processes [14].

2.2.2 Microfabrication methods

The current pace of research in the development and application of microscale systems for cell biology has been facilitated by an increasing number of vital collaborations between engineers and biologists. However, the tendency for biologists to choose conventional and proven methods rather than the novel and potentially more effective, albeit uncertain methods proposed by engineers, hinders communication between these two research cultures [5]. Therefore, for the engineer, it is imperative to establish as much evidence as possible in order to convince the skeptical biologists. In general, the choice of fabrication method in microfluidics is determined by several factors, such as available technologies, equipment, cost, speed, fabrication capabilities and the preferred material substrate [19]. Early microsystems relied heavily on silicon and micromachining techniques which were vestiges of the semiconductor industry. However, for the purpose of this report, the two most relevant methods of microfabrication in biomedical research are: 1) soft lithography using PDMS and 2) micromilling of thermoplastics.

2.2.2.1 Soft lithography - Poly(dimethylsiloxane) (PDMS)

Up until the late 1990s, fabrication of microdevices necessitated conventional micromachining of silicon/glass and involved etching and lithography techniques which required the use of cleanroom facilities and equipment [19]. This was until George Whitesides and his group were able to use a combination
of high-resolution printing and contact photolithography to generate a variety of microstructures with micrometer dimensions in a process known as soft lithography. In this technique, a computer aided design (CAD) file is printed on a flexible transparency using a commercial printer. This transparency is then used as a mask in contact photolithography. After development, the pattern of photoresist is used as the master to cast replicas of PDMS. This rapid prototyping offered several advantages because: 1) transparencies took less time and money to produce, 2) the development of photoresist to create a relief on silicon was easier, and 3) more flexible types of patterns could be produced using this method compared to the etching needed during micromachining of silicon (Fig. 2.4) [15].

In general, soft lithography uses organic and polymeric materials that are referred to as ‘soft’ matter by physicists, of which the most widely used for microfluidics is PDMS. The features of PDMS that make it attractive to work with are: 1) it is a moderately stiff elastomer which allows it to conform to a surface and achieve atomic-level contact useful in forming and sealing microfluidics systems, 2) it is non-toxic and readily available from commercial sources for acceptable prices, 3) it is optically transparent, 4) it is intrinsically hydrophobic but can be modified by brief exposure to an oxygen plasma to become hydrophilic, 5) It can adhere and seal reversibly or after oxidation, irreversibly to many different types of substrates [44]. These features make PDMS a common fixture in microfluidics engineering laboratories around the world. From the perspective of commercialization, however, PDMS becomes a major limiting factor because manufacturing costs and production volumes do not scale up favorably with soft lithography. Additionally, shipping, packaging, and storage of surface-treated PDMS

![Figure 2.4: Soft lithography procedures. Using photolithography, (a) SU-8 is spin-coated on a silicon wafer and, (b) a transparency mask is used to transfer channel patterns to (c) the layer of SU-8. (d) PDMS mixed solution is poured on the wafer and cured then (e) detached before (f) punching holes, autoclaving, plasma treatment and bonding [49].](image)
microdevices remains challenging given that hydrophobic recovery tends to revert surface treatments to their original state. Thus, when manufacturability is factored into design, it becomes necessary to explore other fabrication methods and materials.

2.2.2.2 Micromilling – Thermoplastics

Traditional microfabrication methods are limited in their choice of materials that can be patterned and the method of processing cannot be extrapolated to other materials for different applications. On the other hand, polymers such as polystyrene (PS), polycarbonate (PC) and cyclo olefin polymer (COP) are becoming increasingly relevant in the development of novel microdevices with the benefit of having a relatively cheap and high throughput fabrication process. There are two major classes of polymers: (1) thermoset polymers, which are heavily cross-linked and hard after curing, and (2) thermoplastic polymers which can be molded into any shape at their glass transition temperature and then cooled to a glassy state. Micromolding methods include soft lithography for elastomeric polymers (which could be either thermoplastic or thermoset), injection molding (thermoset and thermoplastic polymers), hot embossing (thermoplastic polymer) and micromilling.

Micromilling involves the use of small end mills (< 1mm diameter) to remove bulk material in a process analogous to macro-scale milling (Fig. 2.5A-C). With micromilling, features of varying height can all be made in the same process, instead of multiple steps required as in a lithography-based PDMS

Figure 2.5: Micromilling enables the rapid prototyping of thermoplastic devices that are suitable for cell culture. (A) Schematic showing the workflow of a basic milling operation from design to device. (B) A CNC micromill in operation. (C) A micromilled thermoplastic device. (D) Phase (left) and fluorescent(right) images of stromal cells at 4× (top) and 10× (bottom) magnification [23].
fabrication approach [12]. This method has the ability to rapidly create three-dimensional, high aspect ratio microscale features in thermoplastics for research purposes. However, machining a common thermoplastic material such as PS is non-trivial due to its low elastic modulus (3.2 GPa), low melting temperature (240°C), low glass transition temperature (100°C), and low thermal conductivity (0.18 W/(m·K)) [41]. The low thermal conductivity causes poor dissipation of the heat generated by deformation and friction in the cutting zone leading to poor surface finish, burr formation, and error in feature dimension of channels [72]. Friction and heat generation in the cutting zone is related to the amount of material each tooth removes per revolution in the tool travel direction (chip load). Finding the optimal cutting depth, spin speed and feed rate is critical to producing high quality microfeatures. Since cutting precision for micromachined chips are on the micron level, vibration and rate of travel have a large effect on the accuracy produced which is dependent on spindle run-out, actuator and motor resolution, and controller software, and the manufacturing tolerances [36]. Although milling induced surface roughness compromises image quality, it does not affect emergent phenotypical properties of cells in culture [23].

Thermoplastics have a distinctive edge over PDMS because manufacturing costs for plastics decrease significantly for high production volumes, and plastics do not degrade during long-term storage [5]. From the biologists perspective, the most commonly used material for in vitro cell-based research is polystyrene (PS), due to its commercial availability in tissue culture plasticware and unique qualities that set it apart from alternatives such as PDMS (Table. 2.1). PS is amenable to mass manufacturing processes that facilitate translation of microscale systems from simple laboratory tools to commercially marketable products, further expanding its potential impact in biological research [5]. Techniques such as embossing (patterning sheets of thermoplastics against a master (stamp) using pressure and heat), and injection molding are used in routine microfluidic designs for industrial applications [19].

2.2.2.3 Bonding

Bonding is critical for lab-on-a-chip devices as it confines solvents to a defined region, prevents uncontrolled spillage of reagents, reduces the risk of biological contamination and minimizes evaporation [55].

<table>
<thead>
<tr>
<th>PDMS</th>
<th>PS</th>
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<tbody>
<tr>
<td>Permeable, prone to bulk absorption</td>
<td>Impermeable</td>
</tr>
<tr>
<td>Excellent bonding to various substrates including glass</td>
<td>Acceptable self bonding</td>
</tr>
<tr>
<td>High feature fidelity after bonding</td>
<td>Prone to deformation after bonding</td>
</tr>
<tr>
<td>Highly compliant</td>
<td>Non-compliant after curing</td>
</tr>
<tr>
<td>Not suitable for mass manufacturing</td>
<td>Scalable manufacturing</td>
</tr>
<tr>
<td>Expensive to set up clean room infrastructure</td>
<td>Relatively cost effective</td>
</tr>
</tbody>
</table>

Table 2.1: Differences between PDMS and PS in a biological research setting
A major challenge is being able to achieve easy and consistent bonding in a laboratory setting. Bonding of PS has not been as well documented as other thermoplastics and this can be attributed to the fact that PS deforms easily beyond its glass transition temperature [5,38]. Two common methods for bonding PS are solvent and thermal bonding but they both have their drawbacks. Solvents such as acetone can produce a strong bond but also introduce deformations in channel dimensions. It also leaves traces of organic solvents in channels which might bias the response of cells to biological cues. On the other hand, thermal diffusion is carried out by applying heat and pressure slightly below the glass transition temperature of PS which causes the polymer chains to reorganize, diffuse and link to form workable bonds. The downside to this method is that it is difficult to maintain uniform interfacial contact across the area to be bonded. Also, it can be challenging to figure out the right pressure and temperature combination that forms strong bonds without deforming the plastic [59, 75]. Other methods exist to bond different thermoplastics. For example ethanol can be used to activate the surface of PMMA and a subsequent exposure to UV irradiation crosslinks the acrylate monomers to form a tight seal between two PMMA substrates [57]. Adhesives could also be employed but these require precision alignment of a stencil and also raises concerns over possible sample contamination.

2.2.3 Spheroid culture

One of the key benefits of microfluidics for basic biology is the ability to control parameters of the cell microenvironment at relevant length and time scales. The goal in cell culture is to recapitulate the cellular microenvironment while also maintaining enough simplicity so that experimental replicates can be performed to achieve statistically significant results. Biologists would prefer that experimental procedures remain accessible and manageable even when microfluidics is expected to add more sophistication to experimental design. Aside from the choice of material for device fabrication, design considerations of particular importance to microfluidic cell culture include the geometry and dimensions of the culture region and the method of pumping and controlling fluid flow [74]. Effective manipulation of these parameters should provide insights on how tissues and organs maintain homeostasis and how cells within organs lose or overcome these controls in cancer. 3D cell culture models particularly spheroids present an avenue to bridge the gap between cell-based assays and animal studies in the hope of reducing experimental uncertainties arising from monolayer cultures and hence the cost of subsequent drug screening processes. Spheroids are self-assembled spherical clusters of cell colonies cultured in environments where cell-cell interactions dominate over cell-substrate interactions. They naturally mimic avascular tumors with inherent metabolic (oxygen) and proliferative (nutrient) gradients resulting in three distinct and
Figure 2.6: The anatomy of a spheroid. Spherical cell cultures have a concentric arrangement of different cell populations. Cells at the core undergo apoptosis due to the diffusion limit of nutrients, oxygen, growth factors and an accumulation of waste. Cells in the middle quiescent zone stay dormant and while cells in the periphery proliferate.

physiologically relevant regions: necrotic core, quiescent zone and the proliferative zone (Fig. 2.6) [32]. Spheroids can serve as an improved assay format for testing drug efficacy because: 1) they model the 3D architecture of tissues including multicellular arrangement and extracellular matrix deposition found \textit{in vivo}, 2) they have diffusional limits to mass transport of drugs, nutrients and other factors, similar to \textit{in vivo} tissues, and 3) rare cells such as cancer stem cells or primary stem cells may be incorporated and maintained in spheroids, which can facilitate targeting these specific cells with drugs [35].

The general criteria for selecting a spheroid production method include: production efficiency, spheroid size uniformity, possible damage or influence on cellular physiology, convenience and suitability for subsequent applications. However, the fundamental requirement of spheroid generation is preventing cells from attaching to the cultureware substratum [25,39]. This occurs spontaneously in hanging drops and microchannel or membrane surfaces that are treated to be resistant to cell adhesion such that cell-cell interaction dominates over cell-substrate interactions (Fig. 2.7) [51, 60]. In addition, any method that concentrates suspended cells to high density such as low speed centrifugation can potentially facilitate spheroid formation particularly in poorly aggregating cells [25,26,29,61].
Figure 2.7: Various processes can induce spheroid formation. For example, (a) geometrical concentration in a semi-spherical hanging drop [60], (b) mechanical concentration by centrifugation in a concave-bottomed well-plate [29], and (c) convection-based concentration in a microfluidic chamber [26].

2.2.3.1 MCF-7 cells

A prime candidate for forming spheroid bodies as a cancer tumor model is the MCF-7 cell line. MCF-7 human breast cancer cells will only grow in spheroid cultures, if environmental conditions are optimized with regard to pH, CO$_2$ content and composition of the culture medium, serum batch and agitation of the medium [37]. For this cell line, the addition of Matrigel or other extracellular ECM proteins such as collagen and fibronectin have been shown to enhance spheroid assembly by providing cell-matrix support. The ECM is a critical part of the tumor-cell microenvironment that modulates cell homeostasis in vivo [67]. It binds a wide variety of soluble growth factors and other effector molecules including VEGF, which greatly slows their diffusion and therefore serves to fine-tune their local concentrations and gradients [22]. Experimental evidence also reveal that hormones like 17 beta-estradiol in the culture medium of MCF-7 cells might be required to sustain spheroids as functional tissue units [6,39].

2.2.3.2 Imaging

3D imaging is important to study the development of the necrotic core resulting from the inherent spheroid oxygen gradient. Because tumor spheroids are in liquid media and are not adhered to a substrate, they are difficult to image when floating as free spheroids. Image quality decreases significantly as a function of imaging depth. However, high-quality images of live cells within 3D tumor spheroids derived from established breast cancer cell lines were reportedly obtained using a thermo-reversible cell mountant [45]. Other imaging optimization that allowed significant increase in image quality were: 1) using culture chambers with thin borosilicate glass bottoms rather than 96 well PS plates, 2) the use of a water immersion rather than a standard dry imaging objective, and 3) increasing laser power or light output as a function of increase in imaging depth [46]. The most important recommendation for obtaining the best images with the least amount of laser damage is familiarity with the microscope.
hardware and software because experienced operators can more rapidly establish appropriate settings for an imaging experiment. This is crucial because the less the specimen is scanned prior to image collection, the longer the specimen will remain viable [40].

2.2.4 Angiogenesis

As part of tumor growth and proliferation, studies have demonstrated that in many spheroids there is an increase in the secretion of hypoxia-induced VEGF, a protein known to stimulate vasculogenesis and angiogenesis in tumor cells (Fig. 2.8) [7, 62]. Angiogenesis, the expansion or extension of existing vasculature is necessary to deliver oxygen and nutrients to ischemic or avascular regions in solid tumors. As tumors grow, they begin to produce a wider array of angiogenic molecules [10]. The recognition that endothelial cells control tumor mass is crucial for a more complete understanding of how oncogenes initiate tumor growth [20].

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 micrometers of blood vessels, the diffusion limit for oxygen. Without blood vessels, tumors cannot grow beyond a critical size. Similarly, without an efficient blood supply, it would be difficult to deliver anti-cancer drugs to all regions of a tumor in an efficient manner. Vessels in an embryo are assembled from endothelial precursors (vasculogenesis). Subsequently, this primitive network expands by

Figure 2.8: 3D endothelial lined lumens (ELL) form angiogenic sprouts in response to VEGF gradient [8]. A three-channel microfluidic device connected by hydrogel-filled diffusion ports is seeded with endothelial cells in the middle chamber. A VEGF gradient is created by perfusing VEGF through one of the neighbouring channels while the other acts as a control.
sprouting (angiogenesis) [10]. VEGF gradients and fluid forces cooperate to control endothelial sprouting and morphogenesis [50]. Because of the importance of angiogenesis in tumor growth, metastasis, and overall cancer progression, therapeutic strategies have been developed around the concept of inhibiting angiogenesis with drugs and other angiostatic agents to restrict blood supply to the tumor.

2.2.4.1 Lumen models

The ability to study angiogenesis and investigate the effects of various factors on angiogenic responses is critical for furthering our understanding of the mechanisms of cancer development, as well as for the development of new and effective therapies [7]. Studies of engineered microvasculature models with physiologically relevant geometries such as bifurcations and junctions within a matrix that is permissive of remodeling and 3D cell culture indicate the usefulness of microvessels for the study of angiogenesis in healthy and pathological scenarios [76]. Current angiogenesis assays and lumen formation processes are time-consuming, labor-intensive, expensive, require significant skill in surgery, are challenging to do repeatedly, and are consistently difficult to scale up (Fig. 2.9) [2,13,18].
2.2.4.2 Viscous patterning

The technique that is most amenable to plastic fabrication is viscous fingering (Fig. 2.10), wherein a less viscous fluid displaces a more viscous fluid resulting in finger-like projections of the less viscous fluid in a rectangular channel [8]. The underlying concept is fairly simple: at the microscale, the less viscous liquid develops a projection of fingers as it contacts the more viscous liquid. After initial instability, a single finger dominates the flow. The reason for this can be understood simply in terms of ‘shielding’. Since the tendency is for fingers of mobile fluid to grow in the direction of the pressure gradient in the more viscous fluid, a finger slightly ahead of its neighbors quickly outruns them and shields them from further growth [53]. Viscous fingering enables the creation of endothelial-lined lumen monolayers that can be used to mimic blood vessels either automatically using robots or manually by passive pumping. Passive pumping is based on the principle that if two drops are of different sizes, the smaller drop has a higher internal pressure than the larger one. It is a consequence of the Young-Laplace equation and remains the easiest way to pump fluids through a channel using differential pressure. Using passive pumping, it is possible to induce flow at a nearly constant rate as long as the small drop placed on the inlet is being replaced rapidly before it loses half its volume. Pumping rate can be modulated by controlling the width of the channel and the radii of the drops [66]. Passive pumping is a form of differential pressure flow. Other ways of inducing flow in a microchannel include electroosmotic and electrokinetic flow [15,68].

Figure 2.10: Viscous finger patterning. Lumens can be patterned in rectangular channels by the passive pumping of media through a more viscous liquid (hydrogel).
2.3 Discussion

2.3.1 Soft lithography vs. Micromilling

A core challenge facing biological microfluidic research is the issue of fabrication and what material and fabrication methods to be used. The two major contenders, for the purposes of this report, are soft lithography and micromilling. Soft lithography is the current gold standard and has been ubiquitously used in microfluidic chips to develop complex biological assays. It is mostly carried out in a clean room and requires relatively expensive equipment compared to micromilling. One issue that favors soft lithography against micromilling is consistency (i.e. low variability). With soft lithography, once the master has been fabricated, one can be certain that every derivative PDMS device will be identical in form and dimensions. However, there are more chances of batch variability in a micromilling operation. This is because the process of setting up the bulk plastic for machining requires skill and any error such as an uneven surface can result in stress from the milling bit causing design features to budge and flex during the cutting process. These concerns pose a challenge to the inexperienced user similar to the significant loss of time that accompanies mistakes by new users of the clean room.

For a tumorigenic model of angiogenesis which relies on the stable gradient formed by the release of angiogenic chemokines in the cellular media, the porosity of PDMS is an extremely significant barrier to adoption. PS which is very familiar to biologists is an attractive alternative fabrication material. Although PS is relatively novel in the microfluidic realm, it does not permit the diffusion of small molecules. It is also more amenable to high volume manufacturing and when coupled with micromilling can support quick iterations between designs. For soft lithography, it takes 3-5 days to go from drawing a new design, to ordering the mask, to creating a new master and finally making a PDMS mold that gets bonded to a flat substrate [75]. With micromilling of PS, one can go from conception to working prototype in a matter of hours. Additionally, micromilling also presents the opportunity to form spheroids-on-a-chip by using ball-nosed bits to mill out rounded bottom mini-wells which could be further treated to induce spheroid formation. This operation is extremely difficult to do using lithographic techniques.

2.3.2 Challenges of plastic microfabrication

Some of the broad challenges related to microfabrication with plastics in a research setting include bonding, bonding alignment, tool breakage, and surface roughness. A fundamental part of the fabrication process is bonding of the featured part to a substrate. Bonding methods must be adapted and optimized for the demanding task of enclosing micron-scale, or even sub-micron scale, fluidic channels without
excessive deformation of the channel cross-sections [58]. With PDMS, this can be irreversibly done by simply plasma-treating the surfaces to be bonded (normally PDMS and glass) to induce hydrophilicity, and establishing contact with slight pressure. However, bonding processes related to PS are suboptimal due to much less research published in this area as compared to PMMA and PC [38, 75]. It is expected that as more microfabrication research is done using PS, bonding techniques will be optimized. The same effect of optimization by widespread adoption is projected to rectify the all too common issue of tool breakage. Optimal material-specific tool spin speed and feed rates for micro endmill bits will become established in coming years. Regarding the rough topography of milled channels that the cell experiences, it is hypothesized that rough channel walls will be ‘smoothened’ by the viscous fingering process which basically coats the channel walls with gel. The effective result of resolving the aforementioned problems and fabricating microfluidic devices out of PS is that it will provide added credibility to engineers as they converse with biologist to adopt new innovative tools for probing cells.

2.3.3 Organs on a chip

There is a desperate need in the pharmaceutical industry for tools that predict the behavior of potential new drugs in humans. The purpose of developing improved experimental models through organ-on-chip technologies is to accelerate progress in fundamental research, to increase efficiency in drug discovery, and to advance the translation of new knowledge into clinical outcomes [73]. These assays must package microsystems in a highly reproducible and easily manipulated format that integrates all the miniaturized components onto a single chip and could be used routinely by technicians [69]. Consequently, access channels employed for seeding cells, changing media and regulating flow are designed to be easily manipulated and of a comparable size to small blood vessels, and large enough to seed cells but small enough to retain significant concentrations of soluble factors released into media [14]. This tradeoff between realism and simplicity is a constant dilemma in the development of new in vitro models. In vitro models have the distinct advantage of being relatively robust, predictable and repeatable. In vitro tissue models are also relatively fast, high-throughput, simple, can be thoroughly analyzed and can be set up with a healthy, modified or diseased human tissue. The main advantage of in vitro model systems is also their main disadvantage: they are simple and this reductionist approach means that they fail to mimic key aspects of the human body. This failure to reproduce physiologically relevant factors can cause skewed results, misinterpretations and false conclusions [21]. An evident scenario where in vitro constructs will not replicate human physiology and drug response is with the idea of interconnected organ-on-chip (OoCs) constructs without correct relative size. Presently, there is not yet a full understanding of how
biological scaling laws apply to multiple, coupled OoCs systems [70]. Therefore it is very important to validate microfluidic chip models thoroughly by comparing with the *in vivo* tissues of interest [63].

### 2.3.4 Mass Adoption

The general trend in microfluidics is developing devices that perform one specific function, but in order to achieve their maximum potential, these devices may have to be integrated in some manner. One way to achieve this is a modular architecture consisting of a microfluidic breadboard with active electromechanical control structures and a passive fluidic component consisting of channels and reaction chambers [48]. Ideal components of a microfluidic breadboard will include valves, pumps, reagent mixers, cell counters, media and waste reservoirs. The importance of such integration and the creation of standards will help drive user adoption and prevent unnecessary duplication of efforts among researchers.

Although theoretically sound, the proper implementation of this idea will require a detailed assessment of all the research done in the lab-on-a-chip space to determine what type, number and configuration of components ends up in breadboard. A likely scenario is that multiple breadboards will be developed for specific microfluidic niches. For example, a researcher investigating angiogenesis or neovascularization might require a breadboard with more pumps and valves to simulate shear stress while a cancer biologist might need one with more sophisticated detection capabilities to monitor the production and consumption of chemokines and other secreted factors in culture media. Furthermore, finding ways to automate or semi-automate procedures such as passive pumping will play a huge role in making microdevices more robust [66]. If a task can be carried out by simply designing a series of channels in a chip and letting the capillary effect and other microfluidic effects work without direct control, then that task becomes a cheap, portable, and disposable package that can be used anywhere without a need for expensive lab equipment [64]. The onus is on the engineer to thoroughly simplify how the end-user interfaces with microdevices. Here, microfluidic devices can borrow a leaf from their predecessor: the semiconductor industry. Most internet users are not aware of the inner workings of microchips yet are ardent users of electronic devices embedded with silicon chips.

The successful miniaturization and commercialization of fully integrated microfluidic systems relies on the availability of reliable microfluidic components as well as detection and quantification schemes. For a tumor angiogenesis chip where shear stress and chemokine concentrations play a vital role in the results of experiments, it is necessary to have tools that can accurately monitor these parameters. A possible avenue for further research is a time-wise characterization of the properties of the ECM in a microfluidic chip. It has been posited that a feedback mechanism exists by which cells that sense mechanical stress
respond by altering their protein expression pattern and thus remodelling the ECM to meet changing mechanical requirements [11]. What signals are triggered and how does it affect angiogenesis in vivo? How does the concentration of matrix proteins used in spheroid formation affect the recruitment of blood vessels towards tumor cells? Does the cell-cell adhesion that the ECM promotes influence the motility of cells thus negating the hypoxic effect on angiogenesis? Obviously, these are big questions that will take years to answer but ultimately inform the accurate recapitulation of in vitro conditions on any device involving ECM so that optimal concentrations and conditions of the basement membrane and the response of cells and tissues in vivo are properly defined [4, 28]. Tackling these questions will produce insights that will be useful for creating a ‘body-on-a-chip’ device consisting of multiple organ models (Fig. 2.11).

2.4 Conclusion

The development and commercialization of sophisticated microsystems such as a “tumor-on-a-chip” device requires a multi-step design process. Currently, there is huge demand and promise for this type of technology in the rugged terrains of the developing world where the lack of trained personnel demands cheap and easy-to-use devices that can carry out complex tasks [3]. However there are also lucrative opportunities in industrialized countries to utilize the many advantages that microfluidics bring. Due to the novel and dynamic nature of design in the microfluidics industry, it is necessary to efficiently and effectively assess different testing parameters, and employ application specific optimization. This
implies that at every step during fabrication, design decisions should be made that acknowledge and leverage existing infrastructure wherever possible. For example, new chips should be designed to take advantage of existing platforms (Fig. 2.12). From an engineering perspective, the emphasis should be on tackling the outstanding technical challenges that surround the transfer of microfluidic technologies from research to commercialization.

Figure 2.12: Bio-rad cell counter composed of an analytical instrument and microfluidic disposable chips. A standardized universal platform like this that can run multiple microfluidic devices(‘apps’) will accelerate mass adoption of thermoplastic microfluidics technology.
Chapter 3

Thesis Objectives

Microfluidics presents a new medium for studying the complex nuances of cancer cell biology with increased throughput and reduced reagent consumption. Presently, microfluidic devices are predominantly used in academia and industry for research purposes. Although commercial biomedical applications of microfluidics is growing, it is still far from being ubiquitous. In view of this divide between the myriad of research work being done in this field and the number of commercialized products, a key aim of this thesis is to demonstrate the utility and simplicity of micromilling for fabricating plastic microfluidic devices that could be used for studying tumor and endothelial cell biology, and to provide evidence that illustrate why these technologies would be more easily translatable to a marketable product if fabricated.

Figure 3.1: Design of a dimorphic co-culture chip. (a) Tumor model compartment composed of uniform spherical pits in a circular arrangement, (b) blood vessel model compartment, and (c) a hydrogel-filled diffusion port (or ‘interaction zone’) that links both compartments.
in plastic.

To achieve this goal, we focused on developing a suite of protocols for streamlined thermoplastic microfabrication. Polystyrene was the main substrate material of interest because of its ubiquity in cell culture studies. Two cell types were studied: (1) a mammary epithelial cancer cell line (MCF-7) and (2) human umbilical vein endothelial cells (HUVECs). Studying two different cell types allowed direct manipulation of phenotypic differences to create a microfluidic platform with advanced capabilities. The platform shown in Figure 3.1 requires two separate compartments for culturing the two different cell types and a zone for intercellular interactions. In order to validate this hypothesis, we designed three chips: (1) a spheroid chip for creating MCF-7 tumor models, (2) a lumen chip for modelling blood vessels using HUVECs, and (3) an angiogenesis chip that combines the features of both (1) and (2) with an interaction zone where cytokines from both compartment can form a diffusion gradient and potentially trigger a physiologically relevant response (Fig. 3.1).

The list of objectives reflects the need to first develop reliable techniques for fabricating micromilled thermoplastic devices prior to examining specific applications. The objectives and specific aims of this thesis are:

1. To design and fabricate three separate microchips for: (i) spheroid only, (ii) blood vessel only, and (iii) spheroid plus blood vessel coculture (Chapter 3)
   (a) Determine what milling parameters are necessary to form concave features.
   (b) Develop milling strategies that minimize imprecision due to user error.

2. To optimize micromilling parameters to achieve smooth plastic surfaces on all chip geometries (Chapter 4)
   (a) Determine the appropriate protocol for post-milling processing

3. To perform macroscale spheroid cultures and optimize culture conditions (Chapter 5)
   (a) Determine what parameters are necessary to form spheroids
   (b) Measure and compare spheroid quality from different reagent groups

4. To perform spheroid culture on chip (Design #1) (Chapter 5)

5. To perform vessel culture on chip (Design #2) (Chapter 6)

6. To combine both spheroid and vessel cultures on single chip (Design #3) (Chapter 6)
(a) Design and fabricate a multi-compartmental microfluidic device suitable for dimorphic co-culture.

Throughout this thesis, emphasis will be placed on scalable engineering and significant biological applications will be used to illustrate the impact of thermoplastic research in the mass manufacturing of microfluidic devices. Subtle nuances of thermoplastic microfabrication in a research setting will be discussed and possible improvements will be provided.
Chapter 4

Microfabrication - Micromilling

Micromilling is a subtractive manufacturing process that uses a cutting tool rotating at high speeds to remove unwanted material from a stockpiece. The precise controls needed for this operation is done using computer numerical control (CNC) which improves repeatability, minimizes human error and facilitate the conversion from computer-aided design (CAD) models to finished parts. It mitigates the prevailing issue with popular fabrication methods which is that they are multi-step processes and therefore not amenable to low volume prototype development as is required in a research environment.

Although milling has been used in woodwork and metal fabrication for years, it has only recently been investigated as a means of creating microfluidic devices in plastic. The benefits of exploring this novel fabrication method is two-fold; biomedical engineers need to know the limitations and capabilities of micromilling and harness best practices from machinists that could be leveraged to accelerate adoption in laboratories. In addition to being an economical means of rapid prototyping in a research setting, micromilling enables the fabrication of complex concave structures that are otherwise tedious using conventional methods. With the advent of microtools and precision CNC machines, endmills of different sizes and shapes measuring hundreds of microns can be readily purchased and used to fabricate high quality devices in various materials ranging from steel to glass. For biological applications, plastics such as polystyrene, cyclin olefin copolymer (COC) and poly methyl methacrylate (PMMA) remain favorable options compared to PDMS because they are low cost and easily amenable to high volume manufacturing processes. This is important for mass production and for reducing the chasm between research and commercialization.

A major drawback micromilling as a means of rapidly prototyping microfluidic devices is poor surface finish. Micromilling thermoplastics, especially polystyrene, results in a dull and translucent surface
finish due to the machine marks made by the milling bit as it makes discrete steps during the cutting process. This roughness, which could vary up to the micrometer range, could be caused by scallops, burrs and sub-optimal drilling conditions. Microfluidic devices that are used for biological experiments require sufficiently smooth surfaces to enhance cell proliferation and most importantly enable bright field imaging.

In this chapter, we introduce a process for polishing milled polystyrene to give optical quality surface finish (Fig. 4.1). The review by Guckenberger et al. [23] served as a major reference and resource for the development of the devices presented here.

Figure 4.1: The thermoplastic microfabrication process. A sheet of plastic undergoes (a) micromilling, (b) polishing, and (c) bonding before it is ready for use.

### 4.1 Materials and Methods

#### 4.1.1 Premilling

##### 4.1.1.1 SolidWorks

All micromilled devices were designed using Solidworks (Dassault Systemes, Velizy-Villacoublay, France). Certain design conventions were held across all chips: (1) all features were designed on the front plane to minimize orientation manipulation on SprutCAM (Nabervzhnye Chelny, Russia), (2) chips measured either 25×30 mm or 25×75 mm to fit in the microscope slide holder, and (3) where two chips were to be bonded, 1.12 mm diameter through-holes were added to the corners of each chip. The holes were equidistant (2mm or 4mm) from the nearest edge (Fig. 4.2) for bonding alignment. The files were then saved in a .IGES format. To fabricate multiple chips at a time, a SolidWorks part could be added to an assembly file before saving.
Figure 4.2: Design convention for alignment ports. Through holes were milled at two diagonal corners of the chip to enable accurate bonding for multilayered devices.

4.1.1.2 SprutCAM

The commercial software SprutCAM was used to convert 3D SolidWorks files into machine language (G code). Endmills and milling parameters were selected on the software and a .TAP file was generated. Using SprutCAM, one could also approximate the milling times for each design and get a computerized visualization of design fidelity.

4.1.1.3 Workpiece preparation

Polystyrene raw materials were purchased in three stock thickness: 1.2mm, 2mm and 3mm (#ST313120, #ST313200 and #ST313300, Goodfellow Cambridge Ltd., Huntingdon, England). Stock sheets were cut into 85mm by 60mm blocks using a razor blade, cutter or scissors, and taped to a 6” × 12” × 2” Grade AA granite block (Standridge Granite Corporation, Santa Fe Springs, CA, USA) which was secured to the mill worktable using clamps (#32580, Tormach) at each of the four corners (Fig. 4.3). All cuts were performed on the front face of the plastic to enable accurate x and y orientation. After cutting, the sheets were either milled as is or bonded together to form thicker pieces.

4.1.2 Milling

To begin milling, the granite block was wiped down using acetone to remove any trace of coolant from a previous machining cycle. Double-sided tape was then laid atop the granite block followed by a clean piece of transparency that is enough to protect the workpiece. Next, tape was laid on the transparency preferably perpendicular to the orientation of the tape on the granite block. After creating a TAP file using SprutCAM (Appendix B), the machine is zeroed for orientation and immediately milled using a Tormach CNC Machine (Tormach, Waunakee, Wisconsin, USA). Each well was milled using a 1/8” ball
Figure 4.3: Milling setup. The stock piece is taped to a granite block which provides a flat surface for milling. The block is held by four clamps at four corners and provides a perfectly flat surface for milling end bit. Wells 1 & 2 underwent a roughing waterline followed by a finishing plane operation with a step size that was 20% and 10% of the bit diameter respectively, while wells 3 & 4 were milled using roughing waterline, finishing plane and finishing waterline operations with a step size of 5% and 2% of the bit diameter respectively. After milling, wells 1&2 were polished.

4.1.3 Post Milling

After milling, the chip was slowly removed either by hand or with the aid of a spatula. The spatula was particularly important for thin chips to provide an even force and prevent the chip from breaking during the removal process. Once off the mill, the chip was washed with soap and water to remove coolant residue. Residual tape was peeled off and the chip was washed again and then dried with nitrogen gas. Furthermore, depending on the application, the chip was vapor polished, plasma treated or both.

4.1.3.1 Vapor Polishing

The two large faces of the chip to be polished were covered in a layer of transparent DUCK tape (Duck EZ Start® Packaging Tape Clear #299002) and the edges were trimmed so that the four unmasked sides are properly exposed. Using a needle, holes were poked through the tape right above the rough features to be polished. The hot plate was set to 40°C and while the temperature was equilibrating, the chip was placed on the perforated plate of the vacuum chamber with 6ml of acetone. The vacuum pump was run for 30 seconds to seal the chamber and the whole setup was placed on the hot plate and held for 5-10 minutes depending on the design. Once the time elapses, atmospheric pressure is restored in
the chamber by releasing the valve, and the chip is allowed to cool for 5-10 minutes before it is removed and placed in a laminar flow hood overnight.

Figure 4.4: Vapor polishing setup. The chip is taped to the bottom side of the middle plate in the vacuum chamber above a pool of solvent. The solvent vaporizes and comes in contact with the plastic surface melting a thin layer and smoothing any surface roughness.

4.1.3.2 Surface Roughness Analysis

Surface roughness plot was measured with a KLA Tencor profilometer stylus of angstrom level sensitivity that was used to trace a cross-section of the well at 20\(\mu\)m/s collecting data at the rate of 200 Hz using 3mg of force. For round-bottom wells, the roughness average (Ra) i.e. average deviation of profile from mean line, was calculated by fitting a semi-circle into a graph of the well topography using an iterative excel model.

\[
X_a = \frac{1}{n} \sum_{i=1}^{n} |y_i|
\]

4.2 Results

Since surface roughness is dependent on various milling parameters, a simulation was run to see how milling time varied with various endmills, depth of cut and step size. To illustrate the effect of feed rate on milling time and how this influences device production rate, different feed rates were compared between three endmills. It was observed that in all cases as expected, the smaller endmill took longer for each feed rate (data not shown). The milling time for a given endmill showed a power law relationship
to the feed rate. Therefore as feed rate goes down, the milling time spikes upwards at an increasing rate. The data also showed that increases in the depth of cut and step size also resulted in a reduction of milling times.

### 4.2.1 Vapor polishing reduces surface roughness

Fig. 4.5 represents the surface profile data from polished and unpolished micromilled semi-spherical wells with a 1/8” (3.179 mm) diameter. A major issue with the stylus instrument is that it only provided a two-dimensional representation of the surface. 3D visualizations using an optical profilometer was impractical because of the transparency of polystyrene. Even after the surface was coated in gold, there was a lot of scattering of light such that it was impossible to record any useful data.

It was observed that rough surfaces from milling are a function of step size, mill diameter, spin speed,

![Image of micromilled polystyrene slide](image)

**Figure 4.5:** (a) Photograph of a micromilled polystyrene(PS) slide. Two wells on the left were polished by exposing the surface to acetone for 90 seconds giving a lens like finish. The two wells on the right were not polished and remained translucent. (b) Surface profile of a polished and an unpolished well was obtained from a stylus profilometer. RMSE of polished well = 0.1065µm, RMSE of unpolished well = 1.276µm
feed rate and substrate. For ball end bits, the step size is proportional to scallop height (the part of the stock left over in between tool offsets). However, reducing the step size to minimize scallop height could significantly increase milling time. The optimal step size for a given operation is determined by what can be compromised: milling time, surface finish or tool life. It should be noted that for a given step size, a larger tool will always create smaller scallops (Fig. 4.6).

4.2.1.1 Polystyrene solvent processing

The rough surfaces resulting from milling were polished using various solvents. Among these, acetone gave the best results when compared to acetonitrile (Fig. 4.7) and water. Each of these solvents were tested at 100% concentration and where acetonitrile was very aggressive, water vapor had no visible effect on the surface roughness. The ineffectiveness of water as a polishing solvent eliminated the possibility that heat was responsible for the smoothening effects of vapor polishing.

The use of acetone for polishing introduced internal stresses in the plastic which resulted in minor cracks (crazing) within the substrate and also significant warping. Crazing is normally caused by the combined presence of residual stress in a thermoplastic sheet and an aggressive solvent or liquid. We hypothesized that there is some milling-induced stress and the difference in shrinkage rate of the plastic surface post-polishing results in crazing. Warping was especially noticeable for chips were the residual plastic after milling was < 500µm. To counteract this, a targeted polishing approach was implemented which involved taping the top and bottom surfaces of the chip and then poking holes right above features that needed to be polished (fig. 4.8). After trying different types of tape, it was noticed that paper-based tapes tended to absorb acetone which ruined the surface of the chip and scotch tape tended to rip instead of puncture thus limiting the extent of polishing control. However, EZ Start® packaging tape (Duck brand) worked well to protect the chip surface and could be easily punctured to provide
vapor accessible holes. Proper storage of the chip in a clean dust-free environment such as the laminar
flow hood also helped in preventing crazing. Another issue with vapor polishing is that a dark band
appears on the periphery of concave surfaces after polishing. This affects proper imaging, but the bands
are minimal with the addition of media and most other liquid reagents.

4.3 Discussion

4.3.1 Feature fidelity

The most important part of any milling operation is the mill itself. Choosing the right CNC mill system
is necessary for ensuring high quality end products. Most mills are defined by their work envelope, feed
rate, spindle speed, power, automatic tool changer, precision and accuracy. High end industrial milling
machines have advanced features such as high maximum speed and automatic tool change and these
enhance the accuracy and general capabilities of the mill. In micromilling, surface roughness depends
on multiple factors: spindle speed, vibration of spindle, temperature of room and parts, tool backlash
during acceleration motion. Less expensive machines such as the Tormach have linearization tolerances
that limit the milling step size, and this constraint is heightened by the use of SprutCAM which produces
low-resolution code. To minimize the surface roughness given these conditions, best practices have to
be employed. One of these is to mill the center, the floor and the sides of channels respectively when
pocketing channels (fig. 4.9)

The selection of milling parameters such as speed and feedrate was informed by previous publications,
online forums and web-based speed/feed calculators. This information was used to minimize surface

Figure 4.7: Brightfield microscope images of polished micromilled surfaces using different solvents. (a)
Vapor polishing with acetone results in optical quality surfaces while (b) acetonitrile is more aggressive
and produces surfaces with a lot of crazing (Scale bar= 2mm).
roughness or milling time. The biggest endmill was always used where possible to minimize cutting twice while depth of cut and step size were set at 5-100% of tool diameter depending on the operation to minimize roughness or milling time.

To compensate for substrate unevenness and zeroing errors where necessary, the bottom level of the workpiece was set to be slightly deeper than the substrate thickness when milling out 2D contours or through holes. A higher occurrence of zeroing error was observed when milling multiple chips as a slight unevenness on one side of the workpiece gets magnified along the length of the chip.

### 4.3.2 Mechanism of Vapor polishing

The process of vapor polishing involves bringing the plastic in contact with the vapor of an applicable solvent. It works by melting a thin layer at the surface of the plastic which then spreads due to surface tension thus smoothening out parts for an optical finish. It is quick, easy, and requires equipment and chemicals that are easily accessible in the lab. The vapor is capable of accessing small crevices which makes it suitable for smoothening the internal features of micro devices. The solvent used typically have a low boiling point and rapidly evaporates thus leaving no chemical contaminant which can jeopardize biological research. Other means of polishing plastic include flame polishing and mechanical plastic polishing. However, these are either more dangerous, less effective or require a lot more skill than vapor
polishing. Additionally vapor polishing can occur in batches, that is, multiple chips can be polished at once for a more streamlined production process. During vapor processing, it was observed that taping the bottom side of the chip to the bottom of the middle plate in the vacuum chamber facilitated the polishing step. Vapor polishing may also be used as a means of polishing milled thermoplastic surfaces that would later be used as negative molds for epoxies in thermal embossing. Further work has to be done to characterize vapor polishing for different thermoplastics, and how it relates to solvent concentration. It is hypothesized that high solvent concentrations will be more aggressive and result in the deformation of microscale features such as channels. Vapor polishing of PS using acetone works presumably because of their solubility parameter relationship. It would be important to quantify the maximum milled surface roughness that can be polished with minimum deformation of channels so as to minimize milling time.

4.3.3 Optical Clarity

It was noticed that devices with concave surfaces such as channels with a circular cross-section have optical properties that compromise experimental observation and analysis. While they accurately mimic lumens in the body, the rounded cross-section introduces complexity in the path of light as it travels from the plastic-air-plastic or plastic-media-plastic interface. For the case of air, the parallel incidence light would reflect and refract at the two interfaces between PS and the air (Fig. 4.10). There are 3 regions of light: Region I contains light travelling through the middle cross-section of the channel which gets refracted twice and still falls within the acceptable range of the objective lens; Region II contains light travelling through the middle cross-section which gets refracted twice but falls outside the range of the objective; and Region III contains light which gets refracted once and falls outside the range of the objective lens.

The refracted light captured by the objective lens is dependent on the lens aperture angle ($\alpha$) governed
Figure 4.10: An illustration of the light path in a concave microfeature. Light rays passing the blue path (Region I) is captured by the microscope. Light from the orange path (Region II) is refracted away from the visible region of the observer. Meanwhile, light in the purple path (Region III) is reflected into the plastic at the polysytrene-air interface

by the equation for the objective lens numerical aperture,

\[ \frac{\alpha}{2} = \arcsin \left( \frac{NA}{n} \right) \]

where NA is the numerical aperture of the objective, \( n \) is the refractive index of the lens immersion material. By this calculation, an incident light bigger than \( \frac{\alpha}{2} \) will not be captured. Therefore, lenses with a higher NA or materials of similar refractive indices are necessary to enhance visibility

### 4.3.4 Solvent-Induced Deformation

Overexposure of channels to acetone can result in deformation (rounding) of channels and for shallow features, it could result in channel collapse (Fig. 4.11). This suggests that there are optimal parameters in terms of exposure time and acetone concentration that create an optical quality device with minimum channel deformation. This rounding of channel corners as a result of polishing might be useful for cell culture applications where sharp corners are not physiologically relevant such as blood vessel modelling.
4.3.5 Alignment

To the authors knowledge, there has been no published papers on the accuracy of different alignment methods for microfluidic devices. Further work includes more research into alignment precision for microfluidic devices with multiple parts, i.e., where chips to be bonded contain microchannel features. The two possible approaches which are suitable for further studies can be broadly classified into internal and external aligners (Fig. 4.12). External aligners require no change to the chip and could be in the form of a jig or external plastic frames whose contours are identical to the corners of the chip. For any of these alignment methods to work, the aligners should extend from the bottommost to the topmost chip but not protrude higher than the last chip.

Figure 4.12: Two methods for aligning chips: (a) Pegs pass through both chip parts at two aligned holes or (b) Alignment jig placed on the periphery of chips keep them aligned. The alignment holders are diagonally separated to ensure a stable setup.
4.3.5.0.1 Lego-block model: The alignment method which we investigated involved one peg (male) and one hole (female) chip to be bonded. The pins were incorporated into the chip design such that the male chip snaps into the female chip. Experimental evidence suggest that the Lego-design combined with vapor polishing enables a press-fit mechanism for bonding simple devices without the need for high temperature and pressure equipment. This peg-hole approach suggests the possibility of fabricating injection molded chips with complimentary alignment pegs and holes such that multiple stacks can be created in a scalable interchangeable manner similar to lego blocks (Fig. 4.13). This will have to be investigated further as alignment is a critical part of bonding multipart microfluidic devices.

Figure 4.13: Lego-block model. Illustration of the (a) three chip styles consisting of a double-sided male chip, a double-sided female chip and a single-sided male chip. (b) A sample 3-chip assembly consisting of two one-sided male chips and a female chip. (c) A sample 5-chip assembly consisting of two one-sided male chips, two female chips and one double-sided male chip.
Chapter 5

Multicellular Tumor Spheroids (MCTS)

There is a growing trend towards the use of spheroids as models for the study of basic cell mechanisms including proliferation, metabolism, differentiation, angiogenesis and anti-cancer therapies in drug development assays [31, 39, 52, 56, 60, 65]. Spheroids are defined as ‘spherically symmetric aggregates of cells analogous to tissues, with no artificial substrate for cell attachment’ [24]. These mimic the in vivo biological properties of tumors by expressing oxygen, nutrient and pH gradients within the spheroid resulting in a heterogeneous population consisting of a proliferating rim, quiescent intermediate region and necrotic core.

Although the advantages of 3D cultures are well known, it is difficult to ascertain the best technique for creating spheroids that exhibit a specific physiological response. Various parameters have been shown to promote single-tumor spheroid formation including centrifugation, cell density, estrogen, basement membrane, concave and non-adherent surface [29,39]. Each of these have either a physical or biochemical mechanism for promoting cell-cell adhesion over cell-substrate adhesion. However, it is not clear how they affect the structural integrity of a given spheroid or how to measure this parameter.

To assess the functional relevance of generated spheroids, previous reports have measured cell growth, cell viability, growth factor secretion and the cytotoxic effects of drugs on 3D versus 2D cultures [51,60]. However, these published methods reveal no easily accessible method for evaluating and classifying ‘cell aggregates’ and ‘single-tumor’ despite the common usage of these terms. Given that a clear distinction between these two descriptors is neccessary, and it is not always possible to visually analyze these 3D structures via existing imaging methods, it is beneficial to investigate other means of classifying...
spheroids. In this chapter, the effects of various combinations of additives known to induce spheroid formation was investigated. The study measured the circularity, solidity and cell viability of spheroids formed in commercial well plates. This was used to determine optimal conditions to achieve a streamlined high throughput method for generating spheroids in a micromilled plastic device. The motivation is clear from a practical perspective: combine the biologically significant attributes of microfluidics, plastics and spheroids to create a scalable drug testing platform.

5.1 Materials and Methods

Five different substrates were used for spheroid experiments: 1) Flat bottom 96 well plate (#353072 Falcon Corning Incorporated, NY), 2) Round-bottomed 96 well plate (Falcon, Becton Dickinson, NJ), 3) ULA Round-bottomed 96 well plates (#89089-826 Corning Incorporated, Corning, NY), 4) Micromilled single spheroid well plates, 5) Micromilled multi-spheroid well plates.

5.1.1 Device Design and Fabrication

Two micromilled devices were primarily used in this study: 1) a U-bottomed device with similar dimensions to the conventional 96-well plate (Fig. 5.1A) and 2) a large well with smaller pits inside (Fig. 5.1B). The devices were formed from polystyrene using micromilling and vapor polishing techniques described in Chapter 3. Briefly, well patterns were designed in SolidWorks, converted to IGS format and transferred to SprutCAM to be converted to machinable .TAP file format. To create a 4mm thick polystyrene slab, two 2mm thick PS sheets measuring 65mm × 85mm each were cut and bonded using a Carver Press (Carver Inc., Wabash, IN, USA) set at 500PSI, 90°C (both platens) and held together for 20 minutes. This was followed by using water through a chiller (McMaster Carr Supply Company, Elmhurst, IL, USA) to cool the platens before removing the bonded chip.

5.1.1.1 U-bottom well plate analogs

For the 96-well plate analog, wells with a radius of curvature of 3.175mm and an additional height of 0.625mm were milled into a 4mm thick PS sheet using a 1/8" ball end bit. The milling procedure involved a roughing waterline followed by finishing waterline with same bit and finishing plane operation. The outer perimeter of the well was selected as the job zone. A 2D contouring step with a 1/8" cylindrical bit was used to dissociate the chip from the workpiece with the perimeter of the chip selected as the job zone (Table B.1).
Figure 5.1: Two micromilled devices for the formation of spheroid bodies: (A) A 12-well micromilled plate with dimensions similar to a conventional 96 well plate was milled using a 1/8" ball endmill and (B) a simple modified chip with multiple spherical pits per well to increase the spheroid throughput.

5.1.1.2 Multiple Spheroid Chip

In the multiple spheroid chip (MSC), 5 wells each containing 7 spherical pits were milled into a 3mm sheet of PS. A 1/8" square endmill was used to mill out the reservoir section of the chip via a roughing plane followed by a finishing waterline operation. The job zone was specified using the inside perimeter of the reservoir and the outside perimeter of the well. A 1/8" ball endmill was used to chip away at the well in a rough waterline operation. A 1/16" ball endmill was used to mill the pits via roughing plane, finishing waterline and finishing plane steps. A 0.02" square endmill was used in a roughing waterline operation to mill the diffusion ports that connect the wells to the reservoir. The perimeter of the chip was milled with a 2D contour operation using a 1/16" square endmill (Table B.2).

After milling, the chips were vapor polished (6ml acetone, 5 mins) for optical clarity and stored in the laminar flow cabinet (AirScience USA LLC, Fort Myers, Fl., USA) overnight. This was followed by attaching the chip to an Omnitray (Thermo Fisher Scientific Inc., Waltham, MA, USA) using double-sided tape (Scotch Brand, St. Paul, MN, USA) and exposing it to UV-light in the biosafety cabinet (Labconco Model No. 3440809) for at least one hour. Subsequently, Design A was coated with 6% BSA for 20 mins while no further surface modification was done to Design B.

5.1.2 Cell Culture

Prior to performing any of the spheroid experiments, MCF-7 cells (ATCC® HTB-22™) were maintained in Dulbeccos Modified Eagles Medium (DMEM) (#11995-065 Gibco by Life Technologies) supplemented
with 10% v/v fetal bovine serum (FBS) (#12483-020 Gibco by Life Technologies), 1% v/v penicillin-streptomycin (#15140-122 Gibco by Life Technologies) and 1% v/v HEPES (#15630-080 Gibco by Life Technologies) in regular tissue culture flasks. The cells were cultured in a humidified incubator (37°C, 5% CO₂). Cell suspensions for the spheroid seeding were made by aspirating the media and rinsing with 1X DPBS (#14190-250, Life Technologies Corporation, Grand Island, NY, USA) dissociating cells with 0.25% w/v trypsin EDTA (Gibco 25200, Invitrogen Co.), centrifuging dissociated cells at 130×g for 7 mins at room temperature, and resuspending the cells in growth media. Cell density was estimated using a hemocytometer and a bright field microscope. After cell counting, cells were subcultivated at a 1:3-1:6 ratio with media renewal 2-3 times per week.

5.1.3 Experimental Preparation

Experiments involving Matrigel (BD Biosciences), estradiol (Cayman Chemical Company, Ann Arbor, Michigan), insulin (#19278 Sigma Life Sciences) and pluronic (BASF, Florham Park, NJ) were carried out using a 96-well plates. There were no variations in the procedure for flat-bottomed, round-bottomed or ULA round-bottomed plates. Prior to beginning cell culture, media was mixed with the appropriate supplement in a 600µl, 1.5ml or 2ml Eppendorf tubes and this was stored in the fridge. The appropriate number of cells were dispensed into each vial after the cell count, depending on the desired cell density and 50-200µl of this cell-media solution was then added to each well. A wide range of cell seeding densities, from 500 cells/well to 50,000 cells/well, was tested based on previously published work. Media was replaced every 2-4 days based on seeding density. BD Matrigel™ basement Membrane Matrix Growth Factor Reduced (BD Biosciences) aliquots were prepared and stored in the freezer in 40µl or 200µl batches. For each experiment, an appropriate number of aliquots was thawed overnight. To prevent premature polymerization, Matrigel was placed in ice at all times and all cultureware or media coming in contact with the gel were pre-chilled by either placing in the freezer at least 1 hour prior to cell culture or overnight in the fridge. Prior to beginning experiments, vials were made with the correct Matrigel-media mix and stored in the fridge until cells were counted and ready to be seeded into wells. In cases where phenol red was not desired, phenol-free media was used. Estradiol was used at 10⁻⁸M. Insulin was used at 1µM. Pluronic F68 was used at a 0.1% v/v concentration. Depending on the parameters to be tested, the cells were centrifuged at 1000g force and 21°C (or 0°C when Matrigel is added) for 10 mins with minimum acceleration/deceleration to facilitate aggregation at the bottom of the plate. Plates were sealed with parafilm for easy handling inside the centrifuge. However it is essential to take off the parafilm before placing the plates in the incubator as this could possibly interfere with
the diffusion of gases into the plate.

5.1.4 Imaging

The 2D morphology of spheroids was observed and recorded using an inverted EVOS Microscope (Life Technologies Inc., Carlsbad, CA, USA) 4X objective (NA: 0.13, WD: 16.9 ) with brightfield and fluorescence imaging functions.

5.1.5 Characterization of spheroid size and shape

Spheroids were formed using ULA 96-well round-bottomed plates and imaged every day after seeding using a brightfield EVOS microscope. From these images, the size, circularity and solidity of the spheroid was determined using the java-based image analysis program ImageJ (http://rsb.info.nih.gov/ij/). All values are presented as mean standard deviation (independent samples n=3). Single-factor analysis of variance (ANOVA) test was performed using Microsoft Excel for comparison among the different reagent treatments (Fig. 5.1 ), and Tukeys test was used for post-hoc pair-wise comparisons. Differences were considered significant for $p < 0.05$.

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Table 5.1: 9 different groups were tested for their spheroid forming potential. Each group contained a combination of parameters; Phenol-red (P), Round-bottomed well (R), Ultra-low adherence surface (U), Centrifugation (C), Estradiol (E), Insulin (I), Matrigel (M) and Pluronic (Plu).

5.2 Results

5.2.1 Well Plate Spheroids

To assess the different reagents and experimental conditions that induce spheroid formation, the effects of phenol red (P), round-bottomed (R), ultra-low adherent bottom (U), concave surface (C), estradiol (E), insulin (I), Matrigel (M) and pluronic (Plu) were tested. Concavity was tested using regular off-the-shelf 96 well flat and round-bottomed plates. Regular culture plates were coated with 6% BSA to inhibit cell adhesion. It was observed that cells did not aggregate in the flat-bottomed plates or uncoated
Figure 5.2: Brightfield microscope images of MCF-7 cells cultured on flat and round-bottomed 96-well plates with different treatments. (a) Cells in a regular round-bottomed well plate with no coating did not form spheroids. (b-c) Cells in a flat-bottom well plate did not aggregate to form spheroids both (b) with and (c) without low-adherence substrate coating. The cells in the round-bottomed well showed more aggregation than those in the flat-bottom.

In total, nine cases were tested and these were separated into 3 groups: no phenol red, phenol red and an alternative additive pluronic (table 5.1). Phenol red free media was investigated because phenol has a structural resemblance to nonsteroidal estrogens and stimulates the proliferation of estrogen receptor positive MCF-7 breast cancer cells. Additionally, pluronic was tested to explore a new mechanism for creating spheroids in microfluidic chips.

Figure 5.3: Sample brightfield microscopy images of representative spheroids for each tested reagent group. The label notation E.#.D.#.W.# refers to the experiment number, day the image was captured and well number respectively. Images were taken at 4x magnification.
5.2.1.1 Circularity and Solidity

There was a morphological difference in the types of spheroids formed as some reagents induced a more compact and circular aggregate comparable to previously published reports. In order to compare the quality of spheroids formed, the circularity and solidity of 2 day old spheroids were calculated. To calculate circularity, the perimeter and cross-sectional area of each cell aggregate was acquired using Image J. Using the raw microscope image (figure 5.4A), a high contrast black and white image (figure 5.4B) was generated and a radius of 10,000 pixels was used to remove surrounding small clumps of aggregated cells (figure 5.4C). This filtered image formed the basis of a high resolution outline of the cell (figure 5.4D) which was used to measure the cross-sectional area (A) and perimeter (P). Circularity (C) of individual cell aggregates was calculated using the formula,

\[ C = 4 \cdot \pi \cdot \frac{A}{P^2} \]

To calculate solidity on Image J, the cross-sectional area calculated above was divided by the spheroid convex area, as determined by the convex hull.

Circularity (Fig. 5.5) was used as a proxy for the 3D roundedness of each spheroid and solidity (Fig. 5.6) was used in the same way to evaluate compactness of the cells in the spheroid. Cell compactness

Figure 5.4: Spheroid image analysis. The (a) brightfield microscopy image is converted into a (b) high-contrast image, (c) filtered to remove extraneous signal for solidity measurements and, (d) filtered again to extract just the outline for circularity measurements.
Figure 5.5: Circularity measurements for several groups of spheroids. Images of samples (n=3) from each group were acquired daily and after Day 2, the circularity index was analyzed. Final score represents the average of 3 spheroids cultured at $5 \times 10^3$, $7.5 \times 10^3$ and $1 \times 10^4$ cells per well. Data is expressed as the mean ± s.d.

Figure 5.6: Solidity measurements for several groups of spheroids. Images of samples (n=3) from each group were acquired daily and after Day 2, the solidity index was analyzed. Final score represents the average of 3 spheroids cultured at $5 \times 10^3$, $7.5 \times 10^3$ and $1 \times 10^4$ cells per well. Data is expressed as the mean ± s.d.
and roundedness is critical to spheroid evaluation because it ensures that a diffusion gradient is formed between the core and periphery of the spheroid, facilitates physiologically accurate spheroid density and, enables the formation of reproducible uniform spheroids.

5.2.1.2 Determining spheroid quality

Single-factor ANOVA showed that statistical differences were present for both circularity \( (p < 10^{-7}) \) and solidity \( (p < 10^{-4}) \). When the circularity and solidity graphs were sorted in order of increasing indices, distinct clusters emerged (Fig. 5.7). Furthermore, Tukey’s pairwise comparison was performed for both circularity and solidity, leading to the identification of statistically different groups.

First, in terms of circularity, F-PRUCM was statistically different from all other conditions, and showed highest circularity values. Groups A, C, and G were similar in circularity, and all statistically different from the groups B, D, and E. Finally, H is not statistically different from any condition except for F, due to its high sample variability (Fig. 5.8A).

Group I is an anomaly because although statistically different from A and G, it is similar to C in circularity. However, solidity data shows that it is statistically different from A, C, F and G (Fig. 5.8B). Thus we can divide the groups into distinct spheroid categories:
1. F- which produces the most homogeneous spheroids

2. A, C, G- all similar with medium spheroid homogeneity

3. B, D, E, I- result in heterogeneous spheroids or aggregates

4. H- is too variable to make any scientific conclusions

Figure 5.8: Sorted circularity and solidity data. Quantitative analysis show a) a larger range in circularity data when compared to b) solidity data.

5.2.1.3 Cell viability & reproducibility

The formation of spheroids occur after 24-48h of culture and there is increased compactness over time first 1-3 days (Fig. 5.9). The differences in area seen in the growth curve is attributed to the difference in compaction for each tested case although variations in seeding density might have also played a role in the discrepancy.

Given the results above, a **low adherent substrate, concave surface with an appropriate cell seeding density greater than 5000cells/mL** are necessary but not sufficient requirements for the formation of spheroids(diameter > 300μm) using MCF-7 cells.
Chapter 5. Multicellular Tumor Spheroids (MCTS)

5.2.2 Micromilled chips

Insight from section 6.2.1. was used to create uniform spheroids in both conical and U-bottom micromilled well plates. Conical wells formed more compact spheroids than U-bottom wells (data not shown) however imaging in conical wells is significantly more difficult due to the high angle of incidence caused by the increased curvature of the compartment thus spherical wells were used for the rest of the experiments.

Figure 5.9: Spheroid growth over time. Size is determined by the area of MCF-7 spheroids acquired using ImageJ analysis software over a 7-day culture. \( n=3 \) for each spheroid group seeded at \( 5 \times 10^3 \), \( 7.5 \times 10^3 \) and \( 1 \times 10^4 \) cells per well. Data is expressed as the mean.

Figure 5.10: Multiple uniform spheroids formed in a 50\( \mu \)l micromilled well with 7 spherical pits. a) Cells were seeded at a high concentration (1.05 \( \times \) 10\(^6 \) cells/ml) to facilitate rapid aggregation. b) The addition of pluronic into the cell suspension prevents cell-substrate adherence.
Centrifugation, Matrigel and all the other non-essential additives were omitted in search of a solution that induced cell-substrate non-adherence which facilitated spheroid formation. This was realized with the addition of 0.002-0.1% plurones F68 which was sufficient to prevent cell adhesion in the MSC chips but has been proven to not affect cell viability. The gravity-driven formation of semi-aggregated cellular mass during the seeding process facilitate subsequent spheroid formation (Fig. 5.10).

5.3 Discussion

The multiple spheroid microfluidic design uses multiple recesses with concave bottoms to rapidly capture settling cells in a uniform manner and funnel them to promote cell-cell interactions. Based on theoretical calculations (Fig. 5.11) and data from other published papers, wells were seeded between 5-10k to generate $350 - 400 \mu$m spheroids where the diffusion limits of oxygen and nutrients result in a necrotic core.

Cells were initially centrifuged after seeding in order to force immediate formation of a compact circular pellet and thus avoid the generation of multiple aggregates from a single well. Interestingly this was not a necessary step as non-adherent concave surfaces caused cells to aggregate under the influence of gravity. The only exception was in experiments that involved Matrigel where the lack of centrifugation resulted in either multiple medium sized spheroids or one major spheroid with satellite spheroids. One possible explanation is that Matrigel increases the viscosity of the cell media which extends the settling time of cells. Experiments suggests that a new batch of Matrigel polymerizes slower than old batches and are therefore more suitable for spheroid formation. Matrigel-induced spheroids tend to have the
Figure 5.12: Batch variability of Matrigel, gel polymerization and improper handling can lead to inconsistent results. (a) Brightfield image at 10x magnification of a spheroid seeded at $2 \times 10^4$ cells/well with 10% Matrigel additive. Premature polymerization results in the formation of spheroid bodies of various sizes. (b) Seeding cells at high density ($2 \times 10^4$ cells/well) without Matrigel nor centrifugation but supplemented with insulin and estradiol can produce homogeneous spheroids.

best morphology but there is a degree of variability between experiments (Fig. 5.12). The inconsistency in Matrigel composition might account for the varied results.

5.3.1 Centrifugation and cell seeding density

Cell-cell proximity is a significant factor in spheroid formation and a high cell density or increases the likelihood that each cell will have a neighbor to attach to. In a regular round-bottomed 96 well plate without centrifugation, cell aggregation can be challenging below a $5 \times 10^3$ cells/ml seeding density per well. The propensity for cells to aggregate is also determined by the radius of curvature of the well.

Figure 5.13: Higher cell density produces 3D structures that are less spheroidal and more flattened, resembling a ‘pancake’. (a) Flourescence image at 10x magnification of a spheroid seeded at $2 \times 10^4$ cells per well. Calcein(Green) and Ethidium(Red) was used to stain for live and dead cells respectively. (b) Brightfield microscopy image of the same spheroid at 4x magnification.
A large radius of curvature would increase the available settling area and decrease cell density at the bottom of the plate. When cells are distant from their neighbors, there is a higher likelihood of multiple little spheroids per well. This effect is magnified in the presence of viscous media (Matrigel-media). However, above 10k seeding density per well, spheroids appeared more flattened approaching a pancake geometry. This is evident as cells seeded at a 20k/well density formed 800\(\mu\text{m}\) spheroids after Day 2 where theoretical calculations predict 600\(\mu\text{m}\) (Fig. 5.13). Since this discrepancy is too large to be attributed to cell proliferation or incorrect model parameters, the z-axis of the spheroid has to be 50% of the x and y axis. Based on this hypothesis, it is reasonable for there not to be a necrotic ‘core’ but a necrotic ‘plane’.

5.3.2 Non-adherent surface

This was determined to be one of the most important parameters in spheroid formation. The hanging drop plate was abandoned early in the project because the method was relatively tedious and provided no additional benefit over the 96 well round-bottomed ULA plates. Spheroids were formed in the 96 well round-bottomed ULA plates in all conditions making it the most reliable control tested. It is posited that an adherent biodegradable microsphere that is denser than media might enhance the circularity and solidity of spheroids.

5.3.3 Estrogen & Phenol red & Insulin

The effect of estradiol on spheroid formation is unclear. In the absence of phenol-red, it seemed to have a detrimental effect on spheroid formation based on the circularity of groups B, D, E & I. Moreover, where phenol-red and estradiol were both present or both absent (Group A, C, & G), medium quality spheroids were produced. Since estradiol is an important hormone in MCF-7 growth, and phenol-red binds to the same receptors, a possible explanation for this phenomenon is that there is an optimal amount of estrogen hormone required for spheroid formation. On the other hand, phenol-red appears to be responsible for driving mitogenic activity based on the growth curve of groups F, G & H (Fig. 5.9). Insulin also facilitated spheroid growth but had no apparent effect on the circularity or solidity of spheroids.

5.3.4 Pluronic

Surface engineering can be used to tailor surface properties such as adhesion, adsorption, corrosion, wettability and lubrication. These artificial treatments are can generate surfaces that are more stable
against heat and pH treatment via easily controllable composition and surface coverage. To fight against non-specific protein physiosorption, exposing the surface to a surfactant (such as Pluronic F68) reduces the surface tension of the solution and prevents physiosorption on hydrophobic surfaces (D. Irimia 2003, Development of a cell patterning technique using poly(ethylene glycol) disilane). Pluronic F68 was tested in different concentrations; 0%, 0.002%, 0.01%, 0.1% and 1%. Interestingly a 0.002% pluronic concentration induced spheroid formation in non-ULA plates where 0.1% concentration had been reported to have no adverse effect on cell behavior (Barbulovic-Nad, 2008). The effects of pluronics could also result from several layers forming a phase boundary between aqueous media and the substrate (Luk 2008). Unfortunately the physiochemical interactions governing protein adsorption is not fully understood.

### 5.3.5 Micromilled chips

The work presented in this research show that micromilling can enable the production of uniform, reproducible spheroids in a fast efficient manner (Fig. 5.14). The robustness of this approach makes it amenable to both 96-well and microdevice formats. Customizable well concavity enable researchers to tailor their device to a specific cell type or intended spheroid size. Using common flow techniques, spheroid media changes can be easily automated and spheroids could be retrieved and counted by turning the chip upside down and adopting a flow cytometry based technology. Furthermore, multiple spheroids in a well can enable 3D angiogenesis experiments or act as a tumor model for a human-on-a-chip device.

In micromilled chips, cells appear to respond differently to various reagents similar to their behavior in commercial 96 well plates. However they also appear smaller but this is most likely due to the lens-like effect as light travels from the outer flat bottom to the inner curved surface of the chip. Incomprehensibly, there was significant variability in aggregate formation efficacy between experiments employing the same cell line and number of cells resulting in coherent aggregate formation in one case, and then failing to

![Day 0 and Day 2 images of micromilled chips](image)

Figure 5.14: A device for customized production of spheroids by controlling the number of pits in a micromilled well. This platform is useful for engineering multiple spheroids per well.
Figure 5.15: The 6 different spheroid morphologies

do so in subsequent attempt. There were six reoccuring morphologies that might provide insight into the mechanism of spheroid formation (Fig. 5.15).

After investigating potential causative factors such as passage number, passage frequency, temperature of media and serum concentration, it might be that variations in specific protein expression between MCF-7 cultures may explain this problem. A similar problem in the formation of embryoid bodies was solved by introducing a Rho-associated protein kinase (ROCK) inhibitor [61]. The peptide sequence tripeptide arginine-glycine-aspartic acid (RGD) in ECM which is responsible for cell attachment may be playing a role here. Additionally Matrigel degradation post-thawing might be contributing to the inconsistency in experimental data. That being said, there appears to be a relationship between the concavity of a surface, cell seeding density and the spheroid formation probability. For a given spheroid size, there is an optimal cell density and surface concavity for a spheroid to form in the absence of Matrigel.
Chapter 6

3D Thermoplastic Platform for Coculture Model

The microvasculature is an extensive organ that mediates organ-tissue interactions and plays a role in common pathologies such as cardiovascular diseases and cancer. Angiogenesis, the physiological process through which new blood vessels form from preexisting vessels, is a hallmark of cancer as it permits microscopic tumors to grow beyond the oxygen diffusion limit. It is a fundamental step in the transition of tumors from a benign state to a malignant one leading to the use of angiogenesis inhibitors in the treatment of cancer. The presence of blood vessels where there should be none affects the mechanical properties of a tissue leading to higher chances of failure. Conversely, the absence of blood vessels in a repairing tissue may inhibit essential functions leading to coronary abnormalities. To mimic the in vivo structure and function of tissues with tubule structures such as blood vessels or mammary ducts, it is important to generate in vivo lumen structures. This is based on the underlying hypothesis that the structure and function of biological entities are inextricably linked and thus recapitulating an in vivo function in vitro would involve replicating its structure. For example, comparing lumen-based models to traditional cell culture methods have shown that in vitro culture conditions affect cell shape, mechanical stress and secreted factors for both endothelial and mammary epithelial cells [9].

In recent years, microfluidics has emerged as a powerful tool for mimicking physiologically relevant three-dimensional (3D) structures. To bridge the gap between in vivo and in vitro assays, rectangular microchannels of varying geometries and surface properties have been routinely fabricated on PDMS. Although these can be useful for the study of single cells, cell suspensions and biochemical reactions, they do not replicate the circular cross section of structures such as blood vessels. The study and
manipulation of blood vessels is extremely important in understanding biological processes because abnormalities in the vascular system can lead to a host of human pathologies such as cardiovascular diseases and cancer which have a high morbidity in the developed world. Modeling an artificial capillary vessel requires a physiologically relevant tubular geometry. Analysis of flow profiles in microchannels show that channel geometry could contribute to the biophysical behavior of blood flow [34]. Price et al. studied vessel stability as a function of pressure and shear stress showing that low flow produced short-lived leaky vessels. It was found that a circular cross section ensures a uniform velocity and diffusion profile with no stagnation at the corner edges as in a rectangular channel [43]. PDMS techniques to create rounded channels have been reported in recent literature: Bischel et al. devised a method for patterning lumens through ECM hydrogels via viscous finger patterning [8], Fiddes et al. exploited the polymerization of a liquid silicone oligomer around a gas stream as both are coaxially introduced into a cuboidal microchannel [18], YueFei et al. used smooth and highly flexible microwires as PDMS molds to create microchannels of different topological shapes [30] and, Lee et al. fabricated round channels through the meniscus formed around a solid-liquid PDMS sidewall due to surface tension [33]. However, the fabrication and experimental limitations of PDMS necessitates the developments of more accessible, high-throughput methods for creating round microchannel geometries which is not dependent on the stringent demands of soft lithography techniques. In this chapter, we demonstrate a simple method for creating channels with a circular cross section using micromilling. We seek to add to the growing list of physiologically relevant lumen structures used as in vitro models to mimic vessels and ducts. Whereas cellular self-assembly as well as biologically derived and synthetic materials have been used to generate random endothelialized microtubules, our method introduces an unprecedented level of control of microvessel geometry. The method relies on the ease of creating complex three-dimensional designs by micromilling and proven techniques for the simultaneous culture of multiple cell types in a microfluidic chip [49].

6.1 Materials and Methods

6.1.1 Design: Dimorphic coculture chip

This chip contains two identical microsystems consisting of a straight cylindrical channel connected to a multi-spheroid well compartment via a diffusion port (Fig. 6.1). For the bottom chip, the cylindrical channel had a diameter of 300µm and was 8.28mm long (fig 6.2B). The diffusion port had a rectangular cross section of 520µm by 100µm and was 970µm long. The inlet and outlet ports were 1mm and 2mm
in diameter respectively. The seven spherical pits start as 570\(\mu\)m long cylinders and end as a rounded bottom with an 800\(\mu\)m radius of curvature. The top side of the chip had an identical semicylindrical feature but there was no diffusion port (Fig. 6.2A), the inlet and outlet ports were through-holes, the semi-spherical well was replaced by a large access port, and there was a retention groove around the access port to keep the media contained.

Figure 6.1: The microfluidic dimorphic coculture chip. (a) Two microsystems on a 25×30 mm chip. (b) Each microsystem contains a cylindrical lumen, with an inlet and outlet port, connected to a well compartment via a diffusion port (interaction zone). Retention grooves surround the well compartment to contain media and prevent cross-experimental contamination.

6.1.2 Milling
6.1.2.1 Top chip

The semispherical well was first milled out using a roughing plane, finishing waterline, finishing plane, and a finishing combine operations with a 1/16” ball endmill. This was followed by a finishing plane and finishing waterline operation with a 1/32” ball endmill. The cylindrical channel was milled using three finishing plane operations with a 1/32” ball end bit. The inlet/outlet/alignment ports were milled using hole machining and waterline operation with a 1/32” cylindrical bit. When milling through holes, a hole machining step was first done using a chip removing strategy followed by a waterline. This is important to prevent plastic from melting and tool breakage. The perimeter of the chip was milled using a 2D contouring operations with a 1/32” cylindrical endmill. To achieve accurate milling results scallop was set to 0.001”, ‘check workpiece’ was selected so only residual thicknesses above 0.001” are machined, roll type was set to ‘surfaces’, for the step-over type ‘retract-approach’ was selected and hole machining was done with a chip removing drilling type in 5 steps. (See appendix b for milling details)
6.1.2.2 Bottom chip

The perimeter of the chip was milled first using a 2D contouring operations with a the 1/32” cylindrical bit. The inlet, outlet, and alignment ports were milled using hole machining and waterline operations with a 1/32” cylindrical bit. The diffusion port and media retention groove was milled using a roughing waterline with a 0.02” cylindrical endmill. The cylindrical section was milled using three finishing plane operations with a 0.01” ball endmill (See appendix b for milling details).

Figure 6.2: Dimorphic Coculture Chip. Image of (a) the top chip showing through holes (retention grooves, inlet, outlet, and spheroid access ports), and (b) the bottom chip showing the base of the spherical pits. (c) Photograph of the top and bottom chips bonded to make a complete device.

6.1.3 Post-milling processing

After milling the chip was vapor polished for 5 minutes (see chapter 3), plasma treated for 3 minutes, bonded and UV sterilized for >1hr prior to cell culture.

6.1.4 Cell culture

MCF-7 cells (ATCC®HTB-22™) were maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) 1% v/v penicillin-streptomycin and 1% v/v HEPES in regular tissue culture flask. The cells were cultured in a humidified incubator (37°C, 5% CO₂).

Cell suspensions for the spheroid and monolayer seeding were made by dissociating cells with 0.25% w/v trypsin EDTA (Gibco 25200, Invitrogen Co.), centrifugation of dissociated cells at 130×g for 7 mins at room temperature, and resuspended in growth media. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD, USA) and maintained with endothelial growth medium (EGM-2) with Bullet Kit (EGM-2MV; Lonza, Walkersville, MD, USA) on regular tissue culture flasks. The cells were cultured in a humidified incubator (37°C, 5% CO₂). Cell suspensions for the monolayer seeding were made by dissociating cells with 0.25% w/v trypsin EDTA (Gibco 25200, Invitrogen Co.),
centrifugation of dissociated cells at $300 \times g$ for 7 mins at room temperature, and resuspended in growth media. Cell density was estimated using a hemocytometer and a bright field microscope.

Cell culture in the dimorphic chip is a serial process involving multiple steps (Fig. 6.3): (1) the channel was filled with a hydrogel solution (Matrigel: thawed overnight) which pinned at the outer edge of the diffusion channels due to surface tension, (2) the solution was then aspirated from the main channel leaving the solution behind only in the diffusion channels. This was incubated for 20 minutes to polymerize the hydrogel, (3) the wells were then coated with 6% bovine serum albumin (BSA) for 20 minutes to inhibit cell-substrate attachment, (4) 100ng/ml fibronectin is used to coat the cylindrical channel to facilitate cell-substrate adhesion, (5) spheroids were created by seeding 5000 cells into the wells, (6) external force in the form of centrifugation was used to induce cell aggregation, and (7) to form the mammary duct model, cells were seeding into the cylindrical channels at a density of
10,000 cells/µl and the whole assembly was rotated by 90 degrees at regular 20 minute intervals for 2 cycles. Alternatively, the dishes containing the chips are attached to a motor which is spun at 2RPM for 30 minutes inside of an incubator at 37°C.

### 6.1.5 Cell staining and Image Acquisition

To image MCF-7 and HUVEC-lined lumens, cells were cultured for 1-2 days, rinsed with a 1X DPBS solution and stained with the membrane-permeable dye calcein-AM at a 1:1000 dye to DPBS dilution. This was used to determine cell attachment and viability on the milled surface. To test for monolayer formation, HUVEC cells were grown on fibronectin-coated (100 µg/µl) rectangular channels for 1-2 days, fixed with 4% paraformaldehyde (PFA) (Fisher Scientific, Hampton, NH, USA) and stained for CD31. Cells were permeabilized with 0.1% triton X, blocked with 3% BSA and 0.1% Tween 20, immunolabeled with mouse anti-human CD-31 primary antibody, and fluorescently labelled with secondary antibody AlexaFlour 488 (1:100 dilution, Molecular Probes, Eugene, OR, USA). Hoechst 33342 (1:1000 dilution, Molecular probes) was used to stain the nuclei. To enhance image quality and mitigate against fluorescent aggregate spots, the secondary antibody was vortexed prior to mixing, mixed with blocking buffer and then vortexed again. The solution was then centrifuged at 14000RPM for 5 mins. Fluorescently labeled samples were imaged using a Nikon confocal microscope (Nikon Instruments, Tokyo, Japan) and both 2D and volume-rendered images were acquired using NIS Elements Advanced software (Nikon Instruments, Tokyo, Japan). Brightfield images were collected with an EVOS inverted microscope.

### 6.1.6 Circularity measurement

The circularity of the cylindrical channel was measured by tracing a multi-side polygon around the cross-section of a 2D brightfield image on ImageJ. Each channel was measured three times and a total of five channels were measured for statistical significance.

### 6.2 Result

#### 6.2.1 Circular cross-section microchannel array

A simple method for creating 3D cell-lined lumens using micromilling was demonstrated. The circular cross-section of these lumens are practically significant for mimicking the human vasculature in vitro. To analyze the circularity and fidelity of the milled lumens, two semi-circular channels (800 µm diameter) were bonded together and the circularity of the cross-section of the resultant cylinder was measured. The
results show a significant increase in circularity and uniformity from the conventional cuboidal channel geometry (Fig. 6.4).

### 6.2.2 Cylindrical profile

The lumens in the truncated structures were lined with HUVECs and stained with calcein to visualize the 3D lumen geometry. Imaging deep channels resulted in signal attenuation as light could not penetrate through the entire channel. However, 3D volume-rendered images of calcein stained endothelial cells (Fig. 6.5) showed that there is indeed a curved profile. To verify that a monolayer was formed, a CD31 stain was carried out (Fig. 6.6). The image for the top plane was acquired by turning the chip upside down and imaging.

Figure 6.4: Uniform lumens formed using micromilling. (a) The cross-section of micromilled cylindrical channels was measured to calculate circularity and uniformity. (b-c) The circularity was higher than that of square channels with low variability between samples. Perimeter: 5.61mm ± 0.08mm, Area: 2.42mm² ± 0.03mm² and Circularity: 0.97 ± 0.02. Data expressed as mean ± s.d.
6.2.3 Coculture of tumor spheroids and blood vessel model

By combining the multiple spheroid chip (Chapter 4) with the blood vessel models, we were able to create a chip that allowed for the dimorphic coculture of cells separated by a hydrogel-filled diffusion port. Both cell types were cultured for 24 hours and then imaged on an EVOS microscope (Fig. 6.7).

6.3 Discussion

6.3.1 Imaging concave surfaces

Imaging the contents of the dimorphic coculture chip presented challenging problems that have not been completely resolved yet. First, due to different protocols for staining HUVECs and spheroids, only HUVECs were stained in the chip since the spheroids could be seen under a brightfield microscope. The result of this was that under fluorescence microscopy, only the HUVECs in the cylindrical channel were stained.
Figure 6.7: Dimorphic coculture of epithelial and endothelial cells. The brightfield image of spheroid bodies was overlayed with a fluorescence image of HUVECs in the cylindrical channels. The varying depth between spheroids and HUVECs(1mm) results in the spheroids being out of focus and a loss of image quality. MCF-7s were seeded at $7.5 \times 10^3$ cells/spherical pit and the HUVECs were also seeded at $7.5 \times 10^3$ cells/µl. Scale bar is 1mm

visible and with brightfield imaging, only the spheroids were visible. Second, to view both images, the fluorescence and brightfield images had to be overlayed necessitating the use of an EVOS microscope which produces lower quality images when compared to the Nikon confocal. Third, the staining of HUVECs was done in non-sterile conditions and involved the introduction of reagents that harm the integrity of spheroids. Although staining the spheroids at the same time as the HUVECs could resolve the issues mentioned above, four other outstanding challenges remain: 1) the scan area for the spheroids and HUVECs is large (10mm$^2$), 2) the spheroids and HUVECs focus at different depths, 3) refraction due to the concavity of the spherical pits and cylindrical channels distorts the light path (Chapter 4.3.3), and 4) polystyrene has been known to exhibit autofluorescence after thermal bonding. Although these challenges do not prevent further work on the device, being able to capture a representative image of dimorphic coculture would serve as a credible validation moving forward.

### 6.3.2 Time-dependent gel contraction

Over time, the gel appears to contract which results in cells leaking from one section to another through the bottom face of the diffusion port. This occurs as early as during the first HUVEC seeding step. Plasma treating surfaces that come in contact with hydrogel helps to promote hydrogel-substrate adhesion. However, it is possible that imprecise alignment might be offsetting the effects of surface tension in pinning gel at the interface where the diffusion port opens up to the spheroid compartment. Preliminary experiments suggest that offsetting negative pressure by sealing the spheroid compartment after gel polymerization is an effective way to prevent HUVECs from breaking through the gel.
Figure 6.8: Gel contraction over time ruins the structural integrity of the monolayer at the gel interface. (a-d) The images represent 15 minute intervals during which endothelial cells are seeded on each side of the channel starting from the bottom face and proceeding in a clockwise direction ending in the face with the diffusion port.

6.3.3 Cylindrical lumen biological relevance

The lumens created via micromilling have highly reproducible dimensions which can be easily modified to fit the natural variation of microvessels in vivo. Also, introducing Matrigel into the diffusion port serves a second function of coating the lumen with ECM such that the endothelial cells reside on a biologically relevant and porous matrix. This was confirmed by mixing hydrogels with fluorophores, introducing the mix into a channel and then aspirating out. Image analysis showed that a small density of residual fluorophores was deposited on the channel walls. To be fully validated, these endothelialized tubes have to be tested for barrier function as preliminary data shows that HUVECs grown on a fibronectin-coated microchannel show a better monolayer than those grown on a hydrogel. It was observed that HUVECs formed more confluent monolayers in channels gel-free channels. Therefore, a design that introduces the hydrogel through a designated ‘middle’ channel might be necessary for certain applications. Experiments will be carried out to test that the endothelial monolayer on the hydrogel face is the only area of interest.
6.3.4 Dimorphic coculture chip

Micromilling could be utilized to form different configurations of 3D microchannels on a planar substrate. This technique makes it possible to design model systems to study intercellular communication mediated by the diffusion of soluble factors across a porous hydrogel. The dimorphic chip illustrates a simple but robust microfluidic assay combining 3D cancer tumor spheroids as well as a tubular lining of epithelial cells. This device takes advantage of the ease of micromilling concave geometries which enables intricate chip designs that allow for novel manipulation of cell behavior in a microfluidic platform. It consists of a hydrogel-filled diffusion port separating the two flanking cell types. The diffusion port is necessary to induce a 3D angiogenic response by facilitating the creation of a gradient of growth factors secreted by the tumor cells. It also provides the ability to control many aspects of the local cellular microenvironment and track changes through high resolution and real time images.

6.3.5 Plastic as PDMS molds

For research purposes, micromilling can be used to fabricate PDMS molds on plastic which would enable PDMS designs with concave surfaces. This will potentially eliminate issues related to crazing, imaging and the current inability to compare between experiments done in polystyrene versus PDMS.
Chapter 7

Conclusions and recommendations

Various techniques were developed to facilitate the design and rapid prototyping of thermoplastic microfluidic devices for investigations involving mammary epithelial and endothelial cells. Of particular interest was the formation of uniform spheroid bodies and endothelial-lined lumens. Using simple accessible techniques, we (1) introduced a processing technique for fabricating optical quality concave features in microfluidic devices, (2) demonstrated its application for forming multiple spheroids on a chip, and (3) added a vessel feature that enable dimorphic coculture on a chip. Spheroids were of interest in this study therefore it was imperative to first understand the conditions necessary for their formation. Different reagents and substrates were tested and optimized for efficiency and viability. Ultimately, a non-adherent concave substrate and basement membrane additive proved to be the reliable method for consistently producing spheroids. Using our micromilling competencies, we studied different milling parameters and how it affected the fidelity of microfeatures. We found that minimizing scalloping by using an appropriate end mill with small step sizes significantly increases the effectiveness of polishing. However, the optimal parameter values for milling and polishing was device dependent. The surface roughness of polished features was further analyzed using a stylus profilometer and it showed remarkable optical clarity in contrast to poor brightfield microscopy images resulting from milling marks. The difference in surface roughness is attributed to the tendency of the solvent to dissolve a thin layer of the milled surface effectively smoothening rough edges. Invariably, this process potentially deforms device features but we speculate that an optimal temperature and solvent concentration exists to optimize polishing while minimizing deformation. More research is necessary to test this hypothesis. Finally, a microfluidic device was designed for its potential use in the study of angiogenesis by culturing a tumor next to a blood vessel model. Using micromilling, we demonstrated the ability to form any arbitrary number of
spheroids on a device. We also showed a method for fabricating lumen features next to the spheroid compartment separated by a hydrogel diffusion port. More experiments will be needed to fix problems associated with gel contraction which compromises the diffusion barrier necessary to study interactions between cells in the two compartments. In conclusion, the utility of micromilling for microfabrication was demonstrated through a series of experiments designed to reveal easily accessible techniques for researchers to take advantage of microfluidics. The devices designed were simple and prove that when microfluidic systems are designed with high throughput manufacturing in mind, they offer a promising platform for the study of complex biological phenomena.

Given this platform, much work is required to expand the work presented in this thesis. The next steps involve:

1. Microfabrication
   (a) Creating a robust alignment jig for bonding different plastic thicknesses
   (b) Characterizing vapor polishing for PS and other thermoplastics

2. Spheroid growth
   (a) Increasing spheroid size and viability on micromilled devices
   (b) Comparing gene expression profiles to determine exactly how spheroid model mimics avascular tumors

3. Role of VEGF
   (a) Measuring the amount of VEGF and other chemokines produced as a function of spheroid size.
   (b) Investigating the minimum amount of VEGF required to induce angiogenesis.
   (c) Inhibiting the activity of VEGF while monitoring other secreted factors will shed light on other significant angiogenic factors in a more realistic fashion.

4. Technique for angiogenesis exploration
   (a) Investigating ways by which a tumors can be vascularized in vivo by co-culturing MCF-7 spheroids with endothelial cells in a hydrogel.
Appendix A

Best Practices

A.1 IGS files

1. If a concave feature is not represented properly on SPRUTCAM, change the options when saving the IGES file from Trimmed Surface (Type 144) to Bounded Surface (Type 143).

A.2 SprutCAM

2. When milling two operations using the same bit, the second bit should be selected from the ‘Used’ tab so there is no pause in the machining process.

3. On the first operation, set ‘tool change position’ to X0 Y0 Z50 so that the bit always returns to the origin after milling.

4. To prevent a finishing step from going through already milled features, go to the operation parameters → Trimming → set Check Workpiece to [0.1]. ‘Check part’ should also be selected.

5. The Z-cleanup parameter leaves a small piece of stock at the end of a waterline operation to create a smooth finish in the final pass on the z-axis. The Finish-level does the same for the side walls.

6. To minimize time and ensure feature stability, pre-cut workpiece close to desired size and shape, minimize the number of tool required and avoid very long and thin features.

7. When milling through-holes and 2-D contours, set the bottom level 50-100µm deeper to ensure that the bit goes completely through the substrate. This is a non-trivial time-save if there are zeroing errors or more importantly for unlevelled plastic sheets.
8. Carefully watch the simulation to identify and prevent the omission of features or milling of unwanted areas.

9. **Simulation Errors**
   
   (a) Contact plane on rapid feed! Increase the safe height to ‘2’ or reduce outer deviation in ‘parameters’ to the minimum.
   
   (b) Gouge of part! Select an appropriate ‘top level’. See if some feature is being selected that shouldn’t be.
   
   (c) Collision detected! The stem of the selected bit is too short.

A.3 **CNC Mill**

10. The tape used to secure the sacrificial layer should be perpendicular to the tape used to secure the work piece. This ensures minimum room for flexing of the plastic. They should also both extend beyond the material they are securing as the edge of the tape might uneven.

11. A straight line on the granite block will help in aligning your workpiece before milling.

12. The leftover portion of the substrate should be at least 0.5mm thick to prevent the collapsing of channels during pressure-thermal bonding.
# Appendix B

## Milling parameters for each device

### Table B.1: SprutCAM Milling parameters for 96 well plate analog

<table>
<thead>
<tr>
<th>Operation</th>
<th>Size of End Mill (mm, inches)</th>
<th>Type of end mill</th>
<th>Spin Speed</th>
<th>Feedrate</th>
<th>Depth of Cut (%)</th>
<th>Step Size (%)</th>
<th>Safe Distance (%)</th>
<th>Safe Level</th>
<th>Angle</th>
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<tbody>
<tr>
<td>Roughing waterline</td>
<td>3.175, 1/8</td>
<td>Ball</td>
<td>6000</td>
<td>600.8</td>
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<td>50</td>
<td>1</td>
<td>150</td>
<td>0</td>
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<tr>
<td>Finishing waterline</td>
<td>3.175, 1/8</td>
<td>Ball</td>
<td>6000</td>
<td>600.8</td>
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### Table B.2: SprutCAM Milling parameters for MSC

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<th>Feedrate</th>
<th>Depth of Cut (%)</th>
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### Table B.3: SprutCAM Milling parameters for Dimorphic Coculture Chip

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<th>Feedrate</th>
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Appendix B. Milling parameters for each device

Figure B.1: Dimorphic Chip (Top) SprutCAM model

Figure B.2: Dimorphic Chip (Bottom) SprutCAM model
### Appendix C

#### Spheroid Data Analysis

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<tr>
<th>Case</th>
<th>Experiment label</th>
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<th>Solidity</th>
<th>PRUCEIM</th>
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### post-hoc Tukey HSD Test Calculator results:

$k = 9$ treatments
degrees of freedom for the error term $\nu = 18$
Critical values of the Studentized Range $Q$ statistic:

\[
Q_{\text{critical}}^{\alpha=0.05, k=9, \nu=18} = 4.9552
\]

---

### Anova: Single Factor

#### CIRCULARITY

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Appendix D

Dimorphic Coculture Protocol

This protocol is a detailed work-in-progress outlining the most up to date method as of August 2015 for seeding two cell types in a microfluidic device such that they both attain different morphologies.

D.1 Chip Fabrication

D.1.1 Software

1. SolidWorks
2. SprutCAM

D.1.2 Milling

1. Tormach CNC Mill
2. Granite block
3. Polystyrene sheets (2mm, 1.2mm)
4. Square end mill
5. Ball end mills
6. Transparency
7. Double-sided tape
8. Acetone
D.1.3 Processing

1. Hot plate
2. Plasma treater
3. Vacuum chamber
4. Laminar flow hood
5. Nitrogen gas
6. Transparent tape

D.2 Cell culture

D.2.1 Reagents and Media

1. Dulbecco’s Modified Eagle Medium
2. Phosphate-buffered saline (PBS)
3. Penicillin-Streptomycin
4. HEPES
5. Fetal Bovine Serum
6. Trypsin
7. Bovine Serum Albumin
8. Matrigel

D.2.2 Glassware and Dishes

1. Falcon tubes (15ml)
2. Omnitray
3. Serological pipettes
4. 1 canister sterile Pasteur pipettes
5. Gloves
6. Test tube rack

7. Timer

8. Cell counter

**D.3 Procedure**

1. Create design on solidworks
   
   (a) Use 1.12mm alignment ports
   
   (b) Bottom chip is 2mm thick
   
   (c) Top chip is 1.27mm thick
   
   (d) Leave at least 0.5mm between bottom of well and bottom of chip
   
   (e) Round out corners of chip (aesthetics)

2. Convert to IGS

3. Convert to IGS

   (a) Hole machining should be done with chip removing drilling type and 5 steps.

   (b) 2D contouring should be done in 3 passes.

   (c) Select ‘check workpiece’

   (d) Select surface only for roll over type

   (e) Make sure tool change position for first operation is set to ‘X0 Y0 Z50’

   (f) For any errors, check safe level and length of stem.

   (g) If operation isn’t working, revise outer deviation.

4. Mill out chip

   (a) Place double-sided adhesive(DSA), transparency, DSA, substrate in that particular order. The adhesive should extend beyond the length of the substrate.
D.3.1 Post-processing

5. Wipe down chip and get adhesive off then wash with soap and water.

6. Dry off with nitrogen gas and cover the backside with EZ packing tape.

7. Vapor polishing the chip

   (a) Using DSA, tape the backside of the chip to the bottom of the middle plate in the vacuum chamber.

   (b) Pour 6ml of acetone and induce vacuum in the chamber for 30 seconds.

   (c) Lock the vacuum valve and put the setup on a 40 degrees Celsius hotplate for 5 minutes.

   (d) When the time is up release the vacuum.

8. Immediately place the middle plate in a laminar flow hood for 24 hours.

   **For Epithelial(MSC) culture, continue to Step 9**

   **For Epithelial-Endothelial culture, continue to Step 21**

D.3.2 For MSC Preparation only

9. UV sterilize for at least 1hr.

10. Coat channels with BSA for 20 minutes.

11. Rinse off with DPBS

D.3.2.1 Pre-cell culture

12. Create cell suspension at 7500cells/pit and 50ul/well i.e. 150k/ml and store in fridge

   (a) For four MSC microsystems with 7 pits each,

      i. Cell= 16.9µl

      ii. 5% Gel = 10µl

      iii. Media = 173µl
D.3.2.2 Cell culture

13. Start culturing MCF 7 cells

14. Aspirate BSA

15. Rinse wells with DPBS and aspirate from each individual recess as liquid tends to be trapped

16. Seed cells by adding 50ul of media to each well.

17. Let the droplet sit in incubator for 20mins so that cells settle.

18. Add 50µl of media.

19. After 8 hours, add 25µl of media at a 5:1000 with 5µM insulin (5:1000, insulin(1MG): media)


D.3.3 Epithelial-Endothelial Dimorphic culture

21. Put tape over the MSC compartment (without covering the bottom face of the diffusion port) and plasma treat the top and bottom chip for 3 mins.

22. Bond both chips at 1000lbs for 20mins

   (a) Make sure not to put any rubber on the chip with the MSC compartment.

   (b) Use two slabs of rubber (one below and the other above the alignment pin)

23. UV sterilize for at least 1hr.

24. Coat channels with fibronectin 4hours or overnight.

25. Aspirate fibronectin with aspirator.

26. Hydrogel: Mix 78µl collagen Type IV, 1.56µl NAOH and 20µl PBS (5X) and let sit for 5-10 mins for nucleation.

   (a) Add 25µl matrigel

   (b) Pin in diffusion port for 20 minutes. Look to see that gel goes in diffusion port.

27. Cover the MSC compartment with a negative PDMS mold or tape.

28. Fill the channel with fibronectin. (Pause Point)
D.3.3.1 HUVEC Culture

29. Start cell passaging

30. Aspirate fibronectin

31. Seed HUVECS at 7500/µl for 15 mins on all 4 sides. Do this twice to ensure even seeding.

32. After 4 hours replace HUVEC media

33. Culture HUVECs for 24 hours

D.3.3.2 MCF-7 Culture

34. Prepare MCF-7 media (0.1% pluronic + 5% gel)

35. Start culturing MCF-7 cells

36. Seed at 7500cells/crater
Bibliography


