AUTOMATED, SPATIO-TEMPORALLY CONTROLLED CELL MICROPRINTING
_WITH POLYMERIC AQUEOUS BIPHASIC SYSTEMS

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AUTOMATED, SPATIO-TEMPORALLY CONTROLLED CELL MICROPRINTING
WITH POLYMERIC AQUEOUS BIPHASIC SYSTEMS

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Thesis

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ABSTRACT

Bottom-up tissue engineering aims to use building blocks of cells and biomaterials and assemble them into larger constructs that mimic microstructural features in living tissues. Cell printing is a promising bottom-up approach to spatially control placement of building blocks and create organized constructs. The following thesis presents the design, fabrication, and implementation of a three-dimensional bioprinter for cell printing. This automated cell printing microtechnology is based on the use of an aqueous two-phase system (ATPS) interfaced with a three-axis motorized system. Cells suspended in the denser aqueous dextran (DEX) phase are loaded into printing tips, which are placed onto the cartridge of the motorized system. Using a computer interface, tips are lowered in the vicinity of a biological surface maintained in the immersion, aqueous polyethylene glycol (PEG) phase to perform a horizontal motion and autonomously dispense their contents onto the surface. Then tips are retracted out of the PEG phase. The motorized ATPS technology allows precise spatial and temporal control of the printing process and supports printing fully viable cells. In this thesis, a systematic study was conducted on the resolution of ATPS-mediated cellular patterns. This resolution was shown to depend on several factors including the dimensions of the printing tips, lateral speed of tips during
horizontal motion, and the loaded volume of the DEX phase in the tips. The finest resolution of $322 \pm 64.5 \, \mu m$ was obtained with a tip diameter of $200 \, \mu m$ at a printing tip speed of $17 \, mm/s$. Higher speeds result in unstable DEX patterns that break into drops due to capillary instability, and thus are avoided. A number of printing substrates tested in addition to a cell monolayer found that decellularized matrices can serve as a substrate for cell printing with ATPS. Using the principles from the characterization studies, multicellular constructs were created to demonstrate the potential of this approach for spatio-temporally controlled cell placement. The ATPS printing microtechnology is a step forward toward developing well-organized, three-dimensional tissue constructs.
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CHAPTER I
INTRODUCTION

The past few decades have witnessed an emerging and growing field of study to deliver “manufactured” organs and tissue constructs that address present limitations associated with organ replacement [1]. The field known as tissue engineering seeks to deliver this promise. Tissue engineering uses engineering principles to create platforms that combine biological materials and living cells to form functional constructs. A major challenge is currently the lack of technologies to engineer spatially-defined, multi-cellular constructs that resemble the three-dimensional architecture of native tissues such as muscle, kidney, and liver and allow organized placement of different types of cells within the construct. Traditional tissue engineering methods employ a top-down approach, in which a porous and biodegradable polymer scaffold is seeded randomly with a cocktail of cells of the target tissue [2-4]. Despite advances in fabrication technologies, distributing cells with defined positioning within scaffolds remains impractical. To address this difficulty, a number of “bottom-up” methods have been developed to create constructs using cells and biomaterials as building blocks and assemble them in spatially-defined shapes [5]. Among these include a number of promising techniques that use passive or active methods to pattern cells in a 2-D plane [6-10]. Passive cell patterning
methods rely on selective adhesiveness of the surface to indirectly guide cell alignment and adhesion whereas active cell patterning methods use pins or dispensing tools to directly control placement of cells and biomaterials on a surface.

The following report uses a recently developed active cell patterning technology based on a polymeric aqueous two-phase system (ATPS) [11,12]. The ATPS consists of polyethylene glycol (PEG) and dextran (DEX) as phase-forming polymers. Cells suspended in the denser DEX phase are loaded in dispensing tools and directly positioned onto a biological surface maintained in the PEG immersion phase to form a cell-confining pattern and allow highly localized adhesion of cells to the underlying substrate. Unlike majority of active cell patterning techniques, this approach enabled patterning of cells with 100 % viability of printed cells, thus making it useful for tissue engineering applications [11]. Previous work using ATPS was performed using manual liquid handling tools that only allowed control over printing circular patterns of cells. Thus, the objective of this work was to construct an automated platform to facilitate control over ATPS-mediated printing and enable control over printing of user-defined patterns of cells.

**Specific Aims**

1. Construct, program, and calibrate a three-dimensional bioprinter to print cells in a spatially-controlled manner.

2. Perform a parametric investigation of ATPS-mediated cell printing using the bioprinter.
3. Demonstrate the feasibility of ATPS-mediated cell printing towards tissue engineering by creating heterocellular niches in multiple configurations, and user-defined cellular patterns.
2.1 Tissue Engineering

In their 1993 seminal article published in *Tissue Engineering*, Vacanti and Langer summarized the first formal definition of Tissue Engineering according to its development since the first NSF-sponsored workshop in 1988 as, “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue or organ function” [1]. Over the past two decades, tissue engineering has developed in much the same way as it did before this formalized definition, and has been used to successfully treat impaired tissues such as bone, skin, eye, and cartilage. However, engineering tissues of more complex architecture such as liver, pancreas, and muscle has not resulted in the same success. Earlier technologies were guided under the assumption that cells were “smart” and if only placed in close proximity to one another would know how to interact and develop into a functional construct. As the field has developed, tissue engineers have come to better understand and appreciate the signals exchanged between cells and their surroundings. Further progress relies on improved understanding of tissue formation, maintenance, and functionality in vivo [13], in order to develop technologies that can recreate these processes in
The development of many native tissues is orchestrated through a number of cellular and molecular processes [14] that result in specific interactions to drive morphogenetic events forming architecturally complex and functioning tissues [15-17]. For example, in cardiac tissue, communication between endothelial cells and surrounding smooth muscle cells shapes and defines blood vessels and capillaries [14]. In nerve tissue, neurons can only survive in close contact with Schwann cells [14]. The development of embryonic skin has been shown to closely rely on dermal fibroblast-keratinocyte interactions [14]. Accordingly, a major requirement for in vitro tissue engineering approaches to develop functional biological substitutes for complex, cell-dense tissues is to mimic morphogenetic patterns that facilitate close cell-cell interactions.

2.2 Tissue Engineering Approaches

Current approaches aimed at engineering tissue constructs can be divided into two main categories of top-down and bottom-up techniques that are briefly described below.

2.2.1 Top-down approaches

Classical tissue engineering methods utilize porous, biodegradable polymeric scaffolds made from synthetic or natural materials with tunable chemistry and and/or mechanical properties [18-20]. In addition, natural scaffolds have been derived by decellularizing animal-derived tissues and organs [21-22]. A cocktail of cells suspended in growth media is loaded into the scaffold to populate it. Interactions between cells and the scaffolding material provide physical support for cells and guide maturation of cells and formation of a
construct. To aid morphogenetic developments such as blood vessels and capillaries, constructs are placed within a bioreactor and furnished with necessary chemical stimuli such as growth factors [23, 24], and/or mechanical stimuli such as fluid force [25]. A common shortcoming with this approach is its lack of control to uniformly distribute and organize cells within prescribed geometries throughout the scaffold. A number fabrication techniques have shown some promise to address the lack of control when incorporating cells into scaffolds. Using photolithography, three-dimensional hydrogels can be created with spatially-controlled chemical and mechanical properties to facilitate selective cell adhesion [26-27]. Rapid prototyping via three-dimensional printing of hydrogel solutions can create complex-shaped scaffolds that contain well-organized network of pores to control distribution of cells [28]. Another fabrication process uses variation of high-pressure carbon dioxide to create a composite polymer scaffold with user control over porosity and distribution, and has been shown to induce spatio-temporal delivery of multiple growth factors [29]. Despite advances in scaffold patterning and design, scaffold fabrication techniques still face limitations to create structures of predefined geometries that resemble intricate microstructural features of native tissues. In addition, current scaffolding materials often do not fully degrade prior to implantation and may elicit inflammatory responses at the site of implantation [30].

2.2.2 Bottom-up approaches

To address limitations associated with the top-down techniques, a number of bottom-up approaches have been developed. Table 2.1 provides an overview
of these methods along with their advantages and disadvantages. Instead of incorporating cells in a 3D scaffold, bottom-up approaches use cells and biomaterials as building blocks and assemble them into three-dimensional constructs through passive or active methods. The ability to control the assembly process at a micro scale is a major advantage of bottom-up approaches and is supported by the fact that most tissues and organs consist of discrete modular components (building blocks), each containing microstructural features that work together to perform simple and complex functions. For example, the kidney is made up of tiny functional units called nephrons, the pancreas of islets, and the liver and breast of lobules.

Building blocks can be created in many different forms: cell aggregates in two dimensions (selective surface adhesiveness) or three dimensions (spheroids), cell-laden hydrogels, cell sheets, and user-defined cellular patterns by direct printing. A variety of techniques have been utilized to create and assemble these building blocks into more complex constructs.

(i) **Patternning using cell adhesiveness**

Patterned cell-adhesive substrates are used to indirectly guide cell adhesion and alignment in a two-dimensional plane. A number of microfabrication techniques have been developed to generate adhesive islands of defined shape and size, transferred on a substrate using a rubber polydimethyl siloxane (PDMS) stamp. Cells preferentially interact with and adhere to the regions of the surface decorated with the adhesive material [31-37]. For example, one microfabricated surface designed with
microgrooves showed significant improvements in endothelial cell retention and alignment compared to non-patterned surfaces [38]. Surfaces can also be fabricated that contain smaller morphological features such as capillaries [40]. A major advantage of this approach is the potential of single cell resolution [34]. A major disadvantage, however, is the need for physical contact of the stamp with the surface to facilitate patterning. This disadvantage causes cells to adhere in irregular shapes and makes it unfeasible to pattern cells onto an existing layer of cells or natural biological matrices that are necessary to develop multilayered cellular constructs.

(ii) Patterning using cell aggregates:

Cells can be constrained in different configurations to form three-dimensional objects such as compact spherical aggregates known as spheroids [41], or other geometries [42], which can then be assembled to form larger, more complex three-dimensional structures. To do this, a classical well-plate is coated with non-adhesive hydrogel material. Cells are then seeded onto the non-adhesive well plate. Because the well-plate is coated with a non-adhesive hydrogel solution, cells are forced to settle at the well bottom under gravity and form a three dimensional structure that eventually results in a microtissue. Replica molds can be created with custom-designed wells and used to generate larger constructs of different geometries. After removed from their molds, however, cell aggregates cannot retain their size but shrink with time in the absence of tension.
applied from honey comb-shaped molds [43-44]. In addition, cells remain confined within hydrogels inhibiting direct cell-cell interactions, which are important to tissue morphogenesis.

(iii) **Cell-laden microgels:**

Three dimensional blocks made of hydrogels or other biomaterials in various shapes can serve as a natural support matrix for cells while imparting control over their spatial arrangements [44, 45]. Hydrogel building blocks are linked together by emulsification, photolithography, microfluidic techniques, or micromolding [45]. Each of these assembly methods has its particular advantages for creating constructs of defined shapes. Emulsification features a two-phase mixture of aqueous droplets containing cells and hydrogel precursors within an organic phase [45]. Once the droplets containing cells and hydrogel precursors gel, they are assembled by a centripetal force that randomly pools the blocks together into a larger unified pattern [45]. Microfluidic platforms can provide greater control over assembling hydrogel blocks by driving two immiscible liquids through separate microscale channels that converge at a third channel to shape hydrogels of prescribed shape and size [45]. Photolithography-based techniques bypass the need to use potentially harmful organic solvents, by directly passing UV light through transparent regions of a film onto cell-laden microgels positioned on a photosensitive polymer [45]. Micromolding can also synthesize cell-encased hydrogels by using a PDMS stamp to first mold a liquid-phase hydrogel solution containing cell
suspension and then, gel it using UV radiation [45]. Although these methods for assembling cell-laden microgels can potentially generate structures with controlled spatial distribution of multiple cell types, current methods employ cell-damaging solvents, physical contact from the PDMS stamp, and mutation-inducing radiations that compromise cell viability and functionality.

(iv) **Cell sheet patterning:**

Two dimensional sheets of cells have been used as building blocks of fairly thick structures and through manual stacking of cell sheets in layers. [46-47]. Cells are first grown to a confluent monolayer on a temperature-responsive polymer; the monolayer is then released from the polymer surface at a certain temperature, removed using a gelatin-coated plunger and layered on a second monolayer. This technique has been used to successfully reconstruct the ocular surface of the eye in four patients using the patients’ own oral mucosal epithelial cells [46]. Another version of this technique separately cultured sheets of fibroblasts and smooth muscle cells until cells deposit sufficient ECM. The sheets were then rolled around a cylindrical mandrel and cultured for at least 8 weeks [46-47]. Afterwards endothelial cells were seeded onto the construct producing a vessel-like structure. A corporation, Cytograft, now employs this technique towards developing grafts for patients requiring severe dialysis. However, this approach still suffers from layer-to-layer binding
between sheets and is limited in terms of patterning tissues of specific geometries.

(v) Direct cell patterning and printing:

Direct patterning methods use pins or dispensing tools to position biomaterials and/or cells with precise spatial control. These methods have certain advantages over passive patterning methods: they can be automated to allow for scalable and reproducible production of tissue-like constructs; they can actively manipulate multiple cell populations without relying on physical contact of the surface; and they can achieve fine resolution of patterns. In direct contact patterning, solid pins are used to position cells onto gels [48-49] although physical contact from the pin can damage and/or contaminate cells. To address this problem, a number of non-contact approaches have been developed that utilize mechanical, thermal, and/or piezoelectric actuating forces to dispense cells and biomaterials through an orifice towards a defined position on the substrate [52-59]. Exertion of external forces on cells and the need for special additives and/or concentrated buffer solutions to shrink cells in order to eject them from the nozzle are major concerns [59]. In addition, in thermal or piezoelectric inkjet printing methods, cells are ejected on substantially dry surfaces to avoid their dispersion and the loss of pattern shape; this and the need for thermal or electrical forces in the ejection process may compromise the viability and functionality of cells [58-59]. Laser-assisted printing is an orifice-free, non-contact approach that utilizes local
evaporation of a laser-absorbing layer to generate a high pressure and propel small volumes of cell suspension located subjacent to this layer onto a substrate [61-63]. This method avoids the use of additives during preparation, and accommodates more than one cell type. The use of a laser, however, remains a concern due to a possibility of inducing mutations in cells. A recently developed microfluidic platform that uses dielectrophoresis demonstrated the ability to directly pattern/manipulate cells in a configuration similar to units of liver tissue (hepatocytes) through two controllable electrodes [9]. A viability/cytotoxicity assay revealed that cells remain 95% viable after patterning and maintained their physiological behaviors such as cell migration and differentiation. Electrode-dependent guidance, however, limits patterning to only surfaces decorated with arrays of electrodes and not on biological surfaces such as a cell monolayer or extracellular matrix surfaces, making multi-layer assemblies unfeasible.

2.3.1 Aqueous two-phase system (ATPS) mediated cell microprinting

Recently, a non-contact method based on the use of aqueous two-phase systems has been adopted to autonomously dispense a suspension of cells without any actuation forces. This approach, developed by Tavana et al., served as the fundamental technology for this report [11-12]. Previous work showed the capability of this approach to print bioreagents and cells in arbitrary shapes; however because printing was performed with manual liquid handling tools, it could only control droplet-shaped patterns. ATPS microprinting has been
adopted towards printing genetic materials such as lentiviral vectors and nucleic acids on a monolayer of cells in arbitrary shapes [11]. Results from these studies demonstrate that genetic materials remained confined to the DEX phase droplets during transfection. In addition, stem cells have been directly printed in circular patterns onto a layer of support stromal cells and show that homotypic and heterotypic intercellular interactions play a major role on fate determination of stem cells and that such interactions can be harnessed for tissue engineering applications using stem cells [12, 70]. Altogether, these studies provided evidence that ATPS microprinting is a promising technology for spatially defined printing of biomaterials and/or cells on cell and ECM support surfaces.

2.3.2 Aqueous two-phase systems and cell partition

An aqueous two-phase system forms when aqueous solutions of two polymers above certain concentrations are combined. The resulting mixture gives rise to two liquid layers, both of which are highly aqueous (as high as 95%). The top phase is enriched with the polymer with lower density while the bottom phase is enriched with the denser polymer. Phase separation is influenced by a number of physical and chemical properties of the polymers and the aqueous solvent used. Particular properties of significance include the molecular weight of phase-forming polymers and overall charge distribution of polymer chains, temperature of the solutions, and pH and ionic composition of the aqueous solvent. Most significantly, separation is determined by the concentration of each polymer in the aqueous solvent and often occurs at low polymer concentrations,
making ATPS suitable for a wide-range of biological applications targeted at the segregation of biomolecules.

The specific polymer concentrations giving rise to phase separation are described with a phase diagram. A phase diagram uniquely characterizes an ATPS, delineates the range of polymer concentrations above a binodal curve that result in phase separation [64-65], and provides the composition of each phase (Fig. 2.1). A phase diagram can experimentally be constructed by creating two separate aqueous solutions, each enriched with a high concentration of a different polymer [66]. A wide range of dilutions from each solution is prepared. Dilutions from one polymeric solution are mixed with those of the second polymeric solutions in conical tubes and allowed to equilibrate and form two separate phases with a sharp interface. The weight of each conical is measured and recorded. ATPS in each tube is titrated drop-wise with water until a one-phase system forms. The final weight of the conical containing the one-phase system is recorded and used to calculate the weight of diluent added just prior to one-phase formation. A binodal curve is determined through plotting the final composition of each system. Figure 2.1 shows the phase diagram of an ATPS consisting of Polyethylene glycol (PEG) with Mw: 35K and dextran (DEX) with Mw: 500K.

Due to their high-water content, ATPS have widely been used for separation and purification of biological materials including cells, proteins, bacteria, cell organelles, nucleic acids, and other species in macro- [67] and micro-scales [68-69]. Molecules or particles that do not contribute to phase
separation are mixed with the ATPS. Based on the surface properties of particles and several physicochemical factors including polymer molecular weight, ionic content of the aqueous solvent, electrical potential differences between the two phases, temperature of the solutions, and the interfacial tension between the top and bottom phases, particles often will favor to reside in one of three phases: bottom phase, top phase, or the interface.

This report uses ATPS of microliter volumes that consists of aqueous DEX and PEG phases, to print cells on various support surfaces. These two polymers are selected due to their ability to quickly dissolve in aqueous media and form ATPS at relatively small concentrations [64, 66]. In this report, aqueous solutions of DEX and PEG are prepared in complete growth media. Owing to its larger density, the aqueous DEX phase always forms the bottom phase and hence, is used as the printing phase that contains cells. Accordingly, the surface to print on (cell monolayer or biomaterial surface) is maintained in the PEG phase. To achieve efficient cell printing using ATPS, cells must favor and remain confined to the bottom DEX phase. Cell partition in ATPSs has previously been shown to be mainly a function of the interfacial tension between the two aqueous phases, \( \gamma_{12} \), i.e.

\[
- \log K \propto \alpha \gamma_{12}, \tag{1}
\]

where \( K \) denotes the partition coefficient, i.e., the ratio of the number of cells in the bottom phase to the total number of cells in the top and bottom phases and interface between them, and can vary between 0 and 1 [68]. The variable \( \alpha \) is an empirical constant. Thus, cell partition to the bottom phase can be easily
manipulated by minimizing the PEG-DEX interfacial tension, $\gamma_{12}$. The interfacial tension between two phases can be determined either through direct measurements or from a known phase diagram using the following equation:

$$\log \gamma_{12} = A + B \log(TLL),$$  \hspace{1cm} [2]

where $A$ and $B$ are experimental constants specific to each individual two-phase system and TLL denotes the tie-line length, shown as $CB$ in Figure 2.1. The tie-line shows the concentration of each polymer in the top and bottom phases. The lower the polymer concentration of the system, the smaller the tie-line length, and from equation 2, the smaller the two-phase interfacial tension is. This principle was previously tested [12] using two ATPS pairs and showed high sensitivity of cell partition to interfacial tension. The first pair consisted of 5.0 % (w/w) PEG (Mw: 25K), 6.4% (w/w) DEX (Mw: 500K), and the second pair comprised of 8.0 % (w/w) PEG (Mw: 8K), 10.0 % (w/w) DEX (Mw: 500K). The experiments measured interfacial tension and partition coefficient with both pairs. Due to lower concentrations of the phase-forming polymers in ATPS pair 1, which fall very close to the binodal curve, it resulted in a lower interfacial tension (12 $\mu$N/m), and hence a higher partition coefficient of $K = 0.78$. It was also confirmed that this pair generated high-fidelity, uniform patterns of fully viable cells. Thus, the ATPS pair 1 was selected for all cell printing experiments in this report.

2.4 Summary

Tissue engineering is an emerging field that aims to create biological substitutes for diseased or damaged tissues. A major challenge in the field of tissue engineering is creating two- and three-dimensional multi-cellular
constructs with defined spatial organization of cells, and is a fundamental need to mimic the architecture of living tissues, where homotypic and heterotypic cellular interactions are key to tissue formation and function. Current tissue engineering approaches focus on creating multi-cellular constructs from either the top-down or bottom-up. Both approaches seek to provide a supportive niche for cells to guide their proliferation, differentiation, and maturation and generate functional constructs. Although both classes show some promise, bottom-up approaches (Table 2.1) can potentially overcome limitations in cell placement and alignment that are difficult to achieve in most top-down approaches. To achieve the goal of creating functional, three-dimensional tissue constructs with spatial organization of cells, the bottom-up approaches should satisfy the following criteria: (i) organized cell placement and spatial assembly of multiple cell types in a reproducible manner (ii) direct and non-contact assembly of multiple cell types and/or cell layers without exerting damaging forces, (iii) retention of cell viability and functionality during assembly steps and long periods of culture, (iv) minimal use of harmful and toxic chemicals such as profuse amounts of mineral oil and buffer reagent, (v) avoiding the use of complicated surface treatments that prevents scale-up, (vi) avoiding mutation-inducing radiations such as UV used to photocrosslink hydrogels, and (vii) efficient layer-to-layer assembly of cell layers within the three-dimensional construct. This thesis will investigate the ability for ATPS biphasic microprinting to satisfy criteria (i, ii, iv, and vi), and its potential to satisfy criteria (iii, v, vii).
### Table 2.1. Existing bottom-up tissue engineering approaches

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<td>Assembly is random and occurs in cell-damaging solvents.</td>
</tr>
<tr>
<td></td>
<td>Photolithography [45]</td>
<td>Direct assembly of hydrogel blocks without the use of cell-damaging solvents</td>
<td>Assembly require mutation-inducing radiations to gel hydrogel blocks.</td>
</tr>
<tr>
<td></td>
<td>Micromolding [44,45]</td>
<td>Direct and easy assembly of hydrogel blocks.</td>
<td>Assembly requires physical contact from a stamp and mutation-inducing radiations to gel hydrogel blocks.</td>
</tr>
<tr>
<td><strong>Indirect microcontact patterning</strong></td>
<td>Photolithography [34]</td>
<td>Precise resolution of feature size. Capable of patterning more than one cell type.</td>
<td>Patterning relies on contact from PDMS stamp</td>
</tr>
<tr>
<td></td>
<td>Other surface treatments (i.e., surface properties) [31-33, 35-37]</td>
<td>Patterning allows for analysis of a wide array of physiological functions</td>
<td>Cannot accommodate patterning of more than one cell type.</td>
</tr>
<tr>
<td><strong>Micromold templates to create spheroids</strong></td>
<td>Hydrogel-coated microwells [41-43]</td>
<td>Capable of creating micro-size structures of specific shape and geometry</td>
<td>Does not accommodate all cell types.</td>
</tr>
<tr>
<td><strong>Cell sheet technology</strong></td>
<td>Nanoengineered surface [46-49]</td>
<td>Structures exhibit robust mechanical properties and cell-cell junctions</td>
<td>Limited control of positioning and resolution</td>
</tr>
<tr>
<td><strong>Direct contact patterning</strong></td>
<td>Pins [50-51]</td>
<td>High-throughput patterning</td>
<td>Physical contact involved may damage and/or contaminate cells. No continuous patterning.</td>
</tr>
<tr>
<td></td>
<td>Thermal and/or piezoelectric forces [52-53, 57, 59]</td>
<td>High-throughput and continuous patterning</td>
<td>Cannot accommodate multiple cell types and requires special additive and buffer concentrated solutions harmful to cells</td>
</tr>
<tr>
<td></td>
<td>Laser-guided [61-63]</td>
<td>Orifice-free actuation accommodates placement of multiple cell types</td>
<td>Surface requires complicated and potentially cell-damaging pretreatments and does not allow continuous patterning</td>
</tr>
<tr>
<td></td>
<td>ATPS-mediated [11-12]</td>
<td>Dispensing does not require cell-damaging ejection forces and allows for continuous printing. Accommodates multiple cell types</td>
<td>Current technology cannot control patterning of non-circular shapes</td>
</tr>
<tr>
<td></td>
<td>Microfluidics/electrode-mediated [9]</td>
<td>Accommodates patterning of multiple cell types with spatial control similar to native environments</td>
<td>Cannot accommodate patterning of multiple layers</td>
</tr>
</tbody>
</table>
Figure 2.1. Phase diagram of an aqueous two-phase system comprising polyethylene glycol (PEG) and dextran (DEX) as phase forming polymers. Point A represents the initial concentration of each polymer in the entire solution and points C and B represent the compositions of top and bottom phases equilibrium, respectively. The line connecting all three points is called a tie line and is a unique property of the given two-phase system.
CHAPTER III

DESIGN AND IMPLEMENTATION OF A 3D BIOPRINTER

3.1.1 Design and Fabrication

To print cells in a spatially and temporally controlled manner with aqueous two-phase system (ATPS)-based printing technology, the designed system should meet the following specifications: (i) three-axis motion control to precisely register coordinates on the printing surface, (ii) sufficient travel range in a horizontal plane to cover surface area of commercially-available culture plates (i.e., 60-mm Petri dish), (iii) sufficient load-capacity to accommodate additional components such as printing tools, (iv) computer-interface capability, (v) software program compatible with Microsoft operating system, and (vi) small footprint to be housed inside a standard sterile culture hood. A set of three linear axis programmable stepping motors, which met these criteria, and two unipolar motor-controllers that featured an easy-to-use graphical user interface for programming were purchased from Velmex, Inc. Two linear slides coupled to the y-direction and z-direction motors had travel distances of four inches (101.6 mm). To accommodate for a greater range of horizontal motion across the culture dish, the slide on the x-direction motor was specified to have a travel distance of six inches (152.4 mm). In addition, the Z-axis linear slide had sufficient load capacity (10 lbs) to carry the added printing tools. Two stepping motor-
controllers with linked controls (bus cable) provided the capabilities of manual and automated positioning of all three motors. Computer-programming of motor-driven linear assemblies was realized through a user-friendly Computer Optimized Stepping Motor Operating System (COSMOS) compatible with Windows operating system.

The linear slides were assembled in an XYZ configuration using the provided mounting cleats as shown in Fig. 3.1A and 3.1B. To enable 3D motion capability without tilting of the system, the slide in the x-direction was cleated to a large aluminum plate (Fig. 3.1D). Cable wires, attached to the motor plate on each linear slide, were connected to matching cables on the motor-controllers (Fig. 3.1A and 3.1B). Since each motor-controller can control a maximum of two stepping motors, the third motor was connected to a second controller. Both motor controllers were linked by a bus cable (Fig. 3.1C), and could be used to provide simultaneous movement of all three motors. Interfacing the motor-controllers with a PC was realized using a RS-232 converter.

After proper selection and assembly of the three-dimensional motorized system, a number of sub-assemblies were needed to facilitate ATPS printing on biological materials. A holder for a 60-mm polystyrene dish (Fig. 3.2B) and a cartridge for housing printing tips (Fig. 3.2A) were designed in SolidWorks and fabricated using high-powered machining tools. The holder was made of a rectangular piece of Plexiglas containing a negative relief of a 60-mm polystyrene petri dish (Fig. 3.2B) and was mounted onto a supporting stage designed to house different sizes of culture plates. In order for the Z-axis linear slide to reach the dish surface and accommodate different sizes of culture plates, the
supporting stage consisted of two stacked breadboards. The bottom breadboard was secured to the aluminum plate supporting the linear slides, and bolted to the top breadboard (Fig. 3.1D and 3.1E). For printing multiple patterns at once, a cartridge made of PTFE was designed to mount up to six pipette tips. Each tip slot within the cartridge was designed with several tapered sections matching the architecture of standard gel-loading pipette tips, to prevent wiggling of the tip during mounting and printing, and inconsistent tip placement upon subsequent rounds of printing. To support motion across the printing stage, an arm consisting of two separate pieces of Plexiglas was designed and fabricated. The first arm piece (Fig. 3.2C) was designed with a cut-out in the shape of the cartridge to hold it in place. To ensure easy and secure attachment of the cartridge to this piece of the arm, a magnet was placed at the rear of the cartridge. To avoid interference of this piece with the supporting stage during motion in the Z-direction, a second piece was added to the arm (Fig. 3.2D). This piece also extended the cartridge to move over the entire printing stage. Fig. 3.1D shows the entire assembly.

The last subcomponent, a rectangular piece of aluminum containing a roller ball bearing mounted to the end plate of the Y-axis linear slide, was used to provide extra support to the system and enable smooth horizontal positioning of the Y-axis linear slide (Fig. 3.1D). In summary, the 3D automated machine is comprised of three motorized linear slides positioned in horizontal, vertical, and orthogonal directions. A rectangular piece of Plexiglas containing a negative relief of a 60-mm polystyrene dish mounted onto supporting stage is used to accommodate the printing surface. A maximum of six printing tips equally
spaced can be loaded into a homemade rectangular PTFE cartridge. The motorized linear slides are connected to a programmable controller, providing the user with an interface and a wide-range of control over movement of the printing tips. The final system was repainted and placed within a culture hood (Fig. 3.1A) to demonstrate its utility for printing cells and biomolecules onto different biological surfaces in a sterile environment.

3.2.1 Programmed motion of the 3D bioprinter

The capability of the ATPS cell microprinting technology for spatial control over cell placement was previously demonstrated using manual liquid handling tools. The automated ATPS printing system now features temporal control and enhanced spatial control of positioning cells by use of a Computer Optimized Stepping Motor Operating System (COSMOS) software package that communicates to the motor-controllers via a serial port. COSMOS employs a command language made of ASCII (text) characters. Although it features drivers for various programming languages such as LabVIEW and Matlab, the work performed in this project used the built-in language. Since each linear slide consisted of a stepping motor that moves in stepwise increments, the motor-controllers read in commands in “step” units. Each revolution of a stepping motor shaft consisted of 400 steps and equaled 1 inch. Commands are passed to each motor indicating exact speed, forward or reverse direction, and travel distance. A command for speed was entered as,

$$SmMx,$$ [3]

where $S$ signals a speed index to the motor-controller, $m$ represents the motor-axis, and $M$ signals to the motor an $x$, speed in steps/sec. The motor-controllers
were limited to a speed range of 1-6000 steps/sec, or 0-15 in/sec (0-38.1 mm/sec). Similarly, a command for the travel distance is entered as,

\[ \text{ImMx or ImM-}x, \]

where I signals a distance index to the motor-controller, in either the clockwise or counterclockwise direction as indicated by the presence or absence of a hyphen between the M and x. The resolution of motion on each linear slide is 6.35 µm. Complex motion profiles are programmed using a Continuous Index Mode to enable smooth transitioning between motors. Having two motor-controllers also enables coordinated motion of multiple motors to produce angles, arcs, and circles. Multiple commands are compiled into a script and stored as a program for future use.

The computer-programming capabilities of the reported ATPS microprinting system provided the motivation for the remainder of the report. This setup was used to perform a set of parametric characterization studies to understand the capabilities and limitations of the ATPS microprinting and enable a number of proof-of-concept experiments for spatio-temporal control over cell printing important to bottom-up tissue engineering applications. Characterization studies consisted of printing linear and user-defined patterns. The program scripts for printing linear patterns and user-defined patterns such as the letters “UA” are shown in appendix B.

3.3.1 Calibration of the bioprinter

To use the reported motorized system for ATPS microprinting, a series of measurements were performed prior to each experiment: (i) the printer head
(cartridge containing printing tips) was calibrated to the printing surface to ensure that when the tips are lowered into the plate, the tips will be within a few hundred micrometers vicinity of the plate surface without touching it. To calibrate the printer head to the dish, Z-axis motor was manually jogged toward the dish until the printing tool touched a sample dish floor at which it was then programmed to ascend to a programmed height plus <78 steps, or <500 um. Secondly, the plate holder was calibrated to the plate to ensure that the plate is an exact, pre-defined orientation with respect to the holder. A matching line was etched on the dish holder and Petri dish using a fine scalpel and ruler, for duplex cell printing experiments of chapter 5. Lastly, the tips were calibrated to the cartridge to ensure that they are all at the same level.

To understand the position of each axis-motor with respect to the dish holder, a coordinate system was devised. To do this each motor was jogged to four boundary points on the dish, and the coordinates registered on COSMOS (Fig. 3.3). The origin (Point 1, Fig. 3.3) indicates that the X-axis and Z-axis were 6.03 and 28.9 mm from the printing surface, respectively. Point 3 in Fig. 3.3 indicates the X-axis motor could move 38.1 mm in the horizontal direction and the Z-axis motor, 33.7 mm in the forward direction before the printing tool reached the dish wall.
Figure 3.1. Overview of 3D ATPS Printer. (a) Entire System, (b) Three-Axis Linear Sliding Motors, (c) Motor-Controllers, (d) 3-axis motorized linear slides stabilized onto aluminum platform, (e) Close-up view of printer stage and cartridge with mounted tips.
Figure 3.2. (a) Teflon cartridge designed to house six pipette tips, (b) Holder for 60-mm petri dish, (c) First piece of arm used to house the printing cartridge, and (d) Extension piece of arm used to provide cartridge motion over the entire printing stage.
Figure 3.3. A coordinate system showing travel distances of each axis-motor at four different locations on the printing surface. Pt 1; (6.03, 28.9, 31.8), Pt. 2; (44.1, 28.9, 31.8), Pt. 3; (44.1, 62.5, 31.8); Pt. 4; (6.03, 62.5, 31.8). Units are in mm.
CHAPTER IV
MATERIALS AND METHODS

4.1. Overview

This thesis designed and fabricated an automated system to print cells with spatial and temporal control using of a polymeric ATPS. Prior to adopting this technique towards tissue engineering applications, a series of studies were conducted to characterize the effect of several factors on the printing process discussed in Chapter 5. The experimental work involved preparation of aqueous PEG and DEX stock solutions, preparation of bio-ink (printing phase, Fig. 4.1a) and bio-paper (printing surfaces, Fig. 4.1b), calibration and adjustment of the three-axis motorized system (Fig. 4.1c), design and fabrication of printing tips, printing linear and parallel cell patterns in co-culture, and image analysis and measurements.

4.2. Preparation of aqueous PEG and DEX stock solutions

For all printing experiments, an ATPS with 5.0% (w/w) polyethylene glycol (PEG, Mw: 35k) and 6.4 % (w/w) dextran (DEX, Mw: 500k), was used as phase-forming polymers. PEG and DEX were obtained from Sigma and Pharmacosmos, respectively. Each phase was individually prepared with these concentrations in growth medium. The DEX phase was prepared twice the final concentration to account for mixing with the PEG and cell suspension at a 1:1
ratio (Fig. 4.1a). Centrifuge tubes containing the solutions were allowed to rest in a vertical position at room temperature for ~2 hours for the polymer to fully dissolve. The stock solutions were then stored at 4 °C.

4.3. Preparation of printing surface and printing phase

For each experiment, the printing surface was maintained in 5 % (w/w) PEG (Fig. 4.1b). Five different types of surfaces were prepared or provided to assess the stability of printed patterns of the DEX phase, including decellularized matrices, cell monolayer replica, live cell monolayers of different confluence, molecularly smooth films of poly(D,L-lactic acid) (PDLA), and PDLA with fabricated striped microgrooves of 10 and 20 µm pitch. Preparation of each surface is briefly discussed:

(i) Decellularized matrices of MDA-MB-231 breast cancer cells and C2C12 mouse myoblast cells were prepared by treating a confluent monolayer with a 0.25% (v/v) Triton X-100 (Sigma) solution in PBS for 15 minutes [71]. The monolayer was then washed three times with PBS to ensure the complete removal of all cell constituents except for the protein matrix laid down by cells on the surface. When prepared at least a day before printing, samples were maintained in PBS and stored at 4 °C until use.

(ii) To create cell monolayer mold replicas with negative and positive indentations, cells were first fixed in a -20 °C methanol solution for 5 min and washed with PBS three times. A 10:1 mixture of polydimethyl siloxane (PDMS) and a curing agent (Sylgard 184, Dow Corning) was prepared, degassed under a vacuum desiccator until all bubbles disappeared, poured on fixed cells, degassed
again, and baked in a 65 °C oven overnight. The cured PDMS was cut out with a surgical blade and transferred to an empty 60-mm petri dish to serve as the mold with negative indentations. To create a mold with positive indentations (i.e., same morphology as the cell monolayer), the PDMS mold containing negative indentations was placed in a vacuum chamber and treated with silane for ~2 hours, then covered with a layer of PDMS, degassed, and baked overnight at 65 °C. Then the two PDMS slabs were carefully separated. The top layer, possessing positive indentations was used as the substrate for printing. To render it hydrophilic, the surface was exposed to oxygen plasma at 200 mTorr for 30 sec.

(iii) Cell monolayers of various confluence were prepared by adjusting seeding cell density to achieve 50, 75, and 100 % confluence for printing.

(iv) Films of PDLA were prepared in three conditions; plain surface, and striped microgrooves of 10 and 20 µm pitch. Using the method reported in [72], first a 10:1 mixture of cured PDMS was prepared, oxidized for 15 minutes, and covalently bonded to a glass slide. The PDMS sheet was then separated from the glass slide using tweezers and weight (i.e., a known peel force). The amount of weight applied when peeling determined the pitch of the strips.

The printing phase for all characterization studies consisted of a 6.4 % (w/w) DEX solution containing small traces of diluted fluorescein isothyiocyanate-dextran 500k conjugate (FITC-DEX). The FITC-DEX dilution was prepared in de-ionized water (1.25 mg/ml), until its concentration was reduced to 5 % of the original stock. The resulting solution was then mixed with the non-fluorescent...
DEX phase to facilitate fluorescent imaging. All printing tips were loaded with 8 µl of the printing phase and then programmed to descend into the PEG-covered surface for studies conducted to investigate the printing tip speed, tip inner diameter, and surface type. To study the effect of loaded volume in the tips, the amount of loaded DEX above the capillary column of the tips was imaged using a light microscope and measured. For cell printing experiments, the printing phase consisted of a 1:1 (v/v) ratio of 12.8 % (w/w) DEX (non-fluorescent) and cell suspension. Cells were grown until 90-100 % confluent, washed with PBS, trypsinized, centrifuged down, and counted on a hemacytometer. For imaging purposes, cells were first stained with a 2 µM calcein-AM solution for 30 min prior to printing. To generate linear patterns containing a dense population of cells, the cell suspension was centrifuged a second time and cells resuspended in an appropriate volume of media to result in a density of 12×10⁶ cells/ml after mixing 1:1 with the 12.8% DEX phase solution. This suspension was thoroughly mixed prior to each successive round of printing to prevent cells from accumulating at the bottom of the centrifuge tube.

4.4 Calibrations of the three-axis motorized system

The three-axis motorized system was calibrated at the beginning of each experiment. The printer head containing the cartridge and printing tips was programmed to descend into an empty dish until the tip touched the dish surface. The printer head was then programmed to ascend ~500 µm (78 steps) to prevent tips from scraping the printed surface during printing. Another script of commands was developed to print individual patterns for characterization.
studies. The printing tips were programmed to descend into the dish (Fig. 4.2C), perform a linear forward motion, ascend out of the dish, and then move an offset distance to prepare for the next print. The offset distances between each printed line for co-culture cell printing experiments were determined based on measurements of line width from characterization studies with FITC-DEX printing.

4.5 Design and fabrication of printing tips

To study the effect of cross-sectional diameter of printing tips on printed patterns resolution, tips with four inner diameters of 200, 300, 575, and 750 µm were used. Tips of 200 µm were fabricated using a crude extrusion method. The new tips were designed to match the contour with the exact dimensions as gel-loading tips in other studies in this project that diameters of 300 µm. To make sure the tips have with the same geometry and dimensions as the gel-loading pipette tips, designs were created after carefully inspecting and accurately measuring dimensions of gel-loading tips under a microscope (Fig. 4.3a, b). Designs were submitted to a stereolithography rapid prototyping center for fabrication. Due to limitations in precision with the stereolithography tool, only two of the six designed tips could be fabricated according to our specifications. Both of these tips had larger diameters than gel-loading tips, i.e., 575 µm and 750 µm. To use tips with diameters smaller than 300 µm, a gel-loading tip was placed over a hot plate to soften and forceps used to pull the elongated end. The extruded tips were then placed onto a cutting board, carefully sliced above the original tip end point in the extruded region, and imaged under a microscope for
inner diameter measurements. This extrusion process yielded tips with a diameter of 213.7 µm (rounded to 200 µm for this study). All sample tips were used to print linear patterns of FITC-DEX at speeds of 5-22 mm/s. Figure 4.2 shows a computational design of a printing tip.

4.6 Creating heterocellular niches in multiple configurations

To create heterocellular niches or duplex prints in multiple configurations, 300 µm tips were loaded with 8.0 µl of the DEX phase containing green fluorescing cells and placed in the cartridge. Linear patterns of green cells were individually printed at a speed of 22 mm/s on the surface covered with the PEG phase. After each individual pattern was printed, registered coordinates of the x-axis stepping motor were manually recorded to know the coordinates for printing subsequent patterns of a different color. Once all green-cell patterns were printed, the printer head was programmed to return to its origin point of coordinates and the printed surface was placed in a cell culture incubator for 1 hr to allow cells to attach. Then ATPS media was replaced with complete growth media and cells were returned to the incubator for another 3 hrs to spread. The culture dish was loaded with the PEG phase again and placed back on the printing stage for printing red cells. Next, printing tips were loaded with red fluorescing cells and placed in the cartridge. The printer head was offset twice the distance (400 µm) of previously measured pattern widths at 22 mm/s from characterization experiments and linear patterns of the red cells were printed.

For duplex cell printing in orthogonal and acute geometries, the dish was rotated
at a forty-five degree and a ninety degree angle with respect to the stage prior to the second printing step.

4.7 Creating user-defined shapes

Patterns in the shapes, “UA,” (standing for the University of Akron) and, “NEOMED,” (standing for Northeast Ohio Medical University) were printed by continuous dispensing of C2C12 cells suspended in the DEX phase from an extruded pipette tip (inner diameter ~ 200 µm). The pattern, “UA,” was printed in three separate steps (Fig. 5.7d). First, the letter U was printed with a loaded volume of 10 µl at a speed of 3.2 mm/s. Second, the x-axis motor was programmed to offset 1 mm to the right of the U, and the y-axis motor forward by the programmed side-length of the U, 7.4 mm. Then, the same tip was emptied of its contents and reloaded with 10 µl of the same printing phase, and a backward U was printed. For printing the middle line feature in the, the y-axis motor was programmed to move backwards half the height of the backward U, the tip reloaded with a volume of 5.25 µl, and a line connecting both ends of the backward U was printed to result in the shape, “A”.

Similarly, the pattern, “NEOMED,” was printed in eight separate steps, where only the letter, “E,” required more than one step. Letters N, O, M, and D were printed with a loaded volume of 8 µl at a speed of 6.3 mm/s. The continuous index feature on the computer program enabled smooth motor-transition to print the diagonal sections in the shapes, N and M. Letter E was printed in two separate steps. First, the three outside line features were printed in one continuous stroke with a loaded volume of 8 µl at a speed of 6.3 mm/s.
The middle line feature was then printed with a loaded volume of 5.25 µl at a speed of 6.3 mm/s. After all shapes were printed, the dish was incubated for 1 hour, after which the two-phase media was replaced with regular culture media, and the dish was returned to the incubator for 3 hrs. Then, printed patterns were imaged under a microscope to measure pattern resolution.

4.8 Image analysis and measurements

After patterns were printed and confirmed to be stable in ~5 min, the Petri dish was placed under a light microscope for imaging. To determine the width of the printed patterns, fluorescent images of FITC-DEX patterns were captured along several sections of the pattern at a magnification of 1.63X and analyzed using AxioVision software (Zeiss). Because printed lines of the DEX-containing aqueous phase exhibit jagged edges along their column due to hinging of the DEX phase on the cells of the surface, the area of the pattern was measured in AxioVision. An average line width was then calculated by dividing the calculated area by the vertical length of the image, as provided by the image scale.
Figure 4.1. A) Preparation of bioink includes mixing the DEX phase with cell suspension. The bioink is then loaded into the printing tips. B) Preparation of biopaper includes immersing a monolayer of cells or decellularized matrix in the PEG phase. C) Side view of printing process. Printing tips are loaded into the cartridge. The cartridge is then programmed to descend into PEG solution resulting in autonomous dispensing of the bioink.
Figure 4.2. Sample image of custom-designed printing tips used to study the effect of tip dimensions on pattern resolution. (a) Overall view of entire printing tip, (b) Close-up of the capillary column, (c) Cross-sectional view of top face in (a). The tip diameter is 300 µm.
CHAPTER V
PARAMETRIC INVESTIGATION OF ATPS-MEDIATED CELL PRINTING USING THE THREE-DIMENSIONAL BIOPRINTER

5.1 Introduction

The developed bioprinter provided an automated platform to employ ATPS-mediated printing to (i) achieve greater spatial and temporal control over cell positioning, (ii) investigate pattern stability and fidelity, (iii) create heterocellular niches in multiple configurations, and (iv) generate user-defined cellular patterns. Unlike few existing cell printing approaches that use external mechanical, thermal, electrical, or fluidic forces to eject cells from a nozzle, printing with ATPS is autonomous without a need for forces that compromise viability of printed cells. Dispensing of the printing DEX phase within the immersion PEG phase takes place because an extremely small interfacial tension between PEG and DEX phases (12 µN/m) cannot hold the DEX phase at the tip of the printing tip against the force of gravity, causing the DEX phase to autonomously dispense independent of any cell-damaging forces. This small interfacial tension was critical to maintaining full cell viability and enables printing user-defined non-circular cellular patterns.

A major interest is to determine and parametrically understand the resolution of ATPS cell microprinting, i.e., how small a contact area it can
generate and conditions of stability of printed patterns. Intrinsically, the resolution depends upon the interplay of gravity that tends to flatten printed patterns and net force of the three interfacial tensions at the three-phase contact point of the PEG-DEX-substrate discussed in Fig. 5.2. Experimental work has shown that the resolution of printed patterns can also be controlled by several tunable design parameters including printing tip speed, loaded volume of printing phase in the tip, and printing tip dimensions. To capture the effect of each of these parameters, systematic tests were conducted varying one parameter while keeping others fixed. In addition, the pattern stability was investigated at different printing tip speeds and with different printing substrates. Results are presented below.

5.2 Characterization Studies

5.2.1 Effect of printing speed

A system has been designed that provides the user with an interface to program tunable speed for the printing tips in X, Y, and Z directions. To perform printing of a simple linear pattern, tips containing cell suspension in the DEX phase were brought over the dish using the Y-axis and X-axis motors, lowered into the dish containing the PEG phase using the Z-axis motor, moved laterally with a pre-defined travel distance across the dish using the Y-axis motor, and then moved out of dish back to a next position to prepare for the subsequent printing step. The width of each linear pattern was found to depend on the speed of the Y-axis motor, and hence the speed of the printing tip, as it swept across the dish. The Z-axis motor, responsible for lowering the tips, was programmed to
descend with a low speed (1.6 mm/s). A slow insertion speed of the tip is necessary since each tip is tapered with a long and thin capillary column at its end (Fig. 4.2b) resulting in a slight delay of about ~1 second before dispensing. The Y-axis motor was programmed to sweep the dispensing tips across the dish at a user-defined speed. It is this speed that effected the resolution, or width, of printed patterns.

To capture the effect of lateral speed of dispensing tips on print resolution, individual linear patterns of FITC-DEX were printed through a single printing stroke, across a wide range of speeds of 3-22 mm/s. Experiments at each speed included a minimum of five replicates. All experiments were conducted with tips of 300 µm inner diameter, which were loaded with a volume of 8 µl. The results from these experiments are shown in Fig. 5.1a and can be divided into three regimes. In regime I, the lateral dimensions of the printed patterns directly depended on the DEX volume dispensed over a unit area of the surface swept by printing tips ($R^2 = 0.98$). As the tip speed increased within 3-12 mm/s, the size of patterns decreased laterally due to less spreading on the surface. The smallest pattern size is 487.5 ± 86.2 µm. Since cells are always maintained in the DEX phase, this relationship between speed and pattern width was also observed in cell patterns (Fig. 5.1b), and therefore this principle can be applied towards cell printing. In regime II, increasing the tip speed up to 22 mm/s did not produce narrower lines anymore; here the width of printed lines remained constant at 487.9 ± 50.0 µm. Further increase in the printing tip speed beyond those tested in regime II resulted in unstable lines that showed necking along the patterns and
broke into droplets (regime III). In this regime, the printed lines were initially 408.1±16.8 µm, but immediately pinched off at several locations.

In all three regimes, the balance of forces at the point of contact of the three phases of PEG-DEX-cell monolayer (Fig. 5.2a) determined the final contact area, or pattern resolution. Potential energy acting on the dispensing DEX phase, i.e., mgh, pulled it down to flatten it on the surface. Upon contacting the surface, a net force resulting from the balance between the three interfacial tensions, \( \gamma_{PD}, \gamma_{PC} \) and \( \gamma_{DC} \), was generated to resist and limit the spreading of the pattern to a completely thin film. To confirm this explanation, the potential energy due to the weight of the liquid column in the printing tip was calculated:

\[
\rho: \text{density} \approx 1000 \frac{kg}{m^3} \\
v: \text{volume} = 6 \mu l = 6 \times 10^{-8} m^3 \\
g = 9.8 \frac{m}{s^2} \\
h = 26 + 1.3 \ mm = 0.02713 \ m \\
mgh = \rho \times v \times g \times h = 1.59 \times 10^{-6} J
\]

To compare this value with \( \gamma_{PD} \) (0.012 mJ/m²), the gravitational energy per unit area was calculated:

\[
A_{\text{tip orifice}} = \frac{\pi}{4} (300 \times 10^{-6})^2 = 7.068 \times 10^{-8} m^2 \\
\frac{mgh}{A_{\text{tip orifice}}} = 22569.9 \frac{mJ}{m^2}
\]
The potential energy value was significantly greater than $\gamma_{PD}$ allowing gravity to pull the DEX phase out of the dispensing tip to continuously dispense.

To determine pattern thickness of experimental prints, a cell monolayer was cultured on a thin glass slide. The slide was then placed on the bottom of a cuvette and immersed in the PEG phase. A sessile drop of the DEX phase was formed on a monolayer of C2C12 cells immersed in the PEG phase. Sideview images were taken (Fig. 5.2b) and the drop thickness and contact angle were measured. Measurements showed a 39.2 µm thickness at the drop apex with a contact angle of 25° (Fig. 5.2b). The small thickness and the low contact angle was due to an ultralow interfacial tension $\gamma_{PD} = 12 \mu N/m$, and indicated why patterns less than a few hundred microns in width may not be achievable with ATPS microprinting.

In regime II, the pattern width was independent of the tip speed at higher speeds despite smaller dispensed volumes. At higher speeds, the interfacial tension $\gamma_{PD}$ (through its horizontal component), would tend to reduce the pattern width to compensate for smaller dispensed volumes. However this force was not large enough to overcome the net force $|\gamma_{PC} - \gamma_{DC}|$ that acts to pin down the pattern on the cell layer. As a result, patterns in this regime of speeds are referred to as energetically metastable for the fact they are not in their lowest energy state and can break if sufficient energy is provided, e.g., through vibrations. Finally in regime III (above 22 mm/s of printing tip speed), the dispensed volume was too small to counteract local pressure gradients along the three-phase interface of patterns that arise due to large local curvatures. As a
result, patterns became hydrodynamically unstable, pinched off at several
locations, and resulted in individual drops.

The breakage is initiated by curvatures of different radii along the interface of the
PEG-DEX-cell layer and therefore differential capillary pressures. The printed
phase (DEX) is a liquid inside the body of another liquid (the PEG immersion
phase) and tends to be in a minimal energy state. Thus, when the net force
$|\gamma_{PC} - \gamma_{DC}|$, could no longer resist the capillary pressure imposed by the
differential curvatures along the DEX phase pattern, liquid drained from pinched
areas of higher pressure (larger curvature) to bulging areas of lower pressure
(smaller curvature) along the pattern column and created local necking that
resulted in the breaking of the pattern into droplets (Fig. 5.3). This phenomena,
which resembles Plateau-Rayleigh instability [73], limited patterns to lateral sizes
of $405.1 \pm 7.4 \mu m$, and is theoretically applicable to other aqueous biphasic
systems having an interfacial tension of $12 \mu N/m$.

5.2.2 Pattern instability at high printing speeds

To explain pattern instability, an experiment was conducted to print
patterns at high speeds of $>22 \text{ mm/s}$ on a confluent cell layer. To rule out any
possibility that external forces from vibrations in the PEG phase induce
breakage, an LED lamp was shined on patterns immediately after printing.

Patterns were observed to immediately begin changing shape after dispensing.
Time-lapse images of an unstable pattern were captured to analyze the dynamic
changes in the curvatures along the pattern. A total of sixteen images were
captured and six were selected for quantitative analysis. Using the image
processing toolbox in MATLAB, edges on the two sides along the pattern were extracted, and radii of curvatures on both ends were measured (Fig. 5.3a). Laplace pressures at the site of necking were calculated from the Laplace equation of capillarity to determine the evolution of capillary pressure in the course of necking until breakage:

$$\Delta P = \gamma_{PD} \left( \frac{1}{R_1} + \frac{1}{R_2} \right), \tag{5}$$

where $\gamma_{12}$ represents the interfacial tension between the PEG and DEX phases, and $R_1$ and $R_2$, are the measured radii of curvature values at the site of necking (Fig. 5.3b). From Fig. 5.3b, radii of curvature shrunk with respect to time and breakage occurred as capillary pressures increased until liquid drains from the pinched area with a Laplace pressure of 130 mPa and radii of curvature of 220 µm to a bulging area with a radii of curvature of 150 µm.

5.2.3 Loaded volume

In addition to increasing the speed of the printer head carrying the tips, experimental studies showed the amount of volume of printing phase loaded into printing tips also affected pattern width. To systematically capture this effect, tips were loaded with 6-8 µl of FITC-DEX at increments of 0.5 µl and linear patterns printed a single programmed speed of 1200 steps/s (7.6 mm/s) onto a confluent monolayer of C2C12 cells. A minimum volume of 5 µl was needed to fill the tip just above the capillary portion and initiate autonomous dispensing within seconds of tip insertion in the PEG phase. Tips loaded with smaller volumes can autonomously dispense from the tip but often requires the tip to remain stationary in the PEG phase for over a minute to allow enough DEX mass from inside the
tip to overcome the interfacial tension, $\gamma_{PD}$, at the dispensing end of the tip. In addition, printing with loaded volumes below 6 µl resulted in patterns that broke into droplets similar to those in regime III of fig. 5.1a.

Each experimental volume was tested with a minimum of six replicates. To consider small volume inconsistencies due to manual loading of printing tips, tips were placed under a microscope before printing for imaging, and the reagent height above the capillary portion was measured (Fig. 5.4a). To eliminate the effect of printing tip speed on patterns breaking into droplets, individual patterns of FITC-DEX were printed at one arbitrarily selected speed that generated a pattern width within the stable regime (7.6 mm/s). Fig. 5.4b shows that, within the range of volumes studied, a consistent increase in the pattern width corresponded to an increase in the loaded volume at a constant lateral speed of the printing tip. A larger loaded volume translates into a larger height of the DEX phase in the tip and therefore, a larger potential energy (hydrostatic pressure). The potential-kinetic energy balance resulted in a dispensing speed of the DEX phase from the tip as

$$v = \sqrt{2gh},$$

where $h$ denotes the height of the DEX phase in the printing tip and $g$ is the gravitational acceleration. When the DEX phase is loaded to a greater height, a greater potential energy (hydrostatic pressure) translates into a larger kinetic energy that results in a faster dispensing speed, i.e., larger flow rate. Thus, at a constant printing tip speed, the dispensed DEX phase will cover a greater contact area with the surface at larger loaded volumes. The outcome was similar to
printing with lower tip speeds at a constant volume (Fig. 5.1a). In conclusion, the
loaded volume of the printing phase provided another tunable means to change
the lateral dimensions of printed patterns and spatially control placement of
reagents and cells.

The results, however, cannot be applied to printing tips of different
geometries. The particular tip under study was a standard gel-loading tip that
featured a narrow, elongated end connected to a small triangularly-shaped
“reservoir” above it (Fig. 4.2a, b; Fig. 5.4a). The range of volumes studied in this
section only spanned the triangularly-shaped reservoir. Loaded volumes greater
than 8 µl filled the tip above this reservoir region; however pattern width, did not
change appreciably and remained constant at 934.2 ± 99 µm (data not shown in
Fig. 5.4). Because the interest was to generate narrow patterns only, volumes
larger than 8 µl were not considered further. Also, larger loaded volumes
dispensed larger amounts during insertion of the tip into the PEG phase,
resulting in an blob at the beginning of patterns that made measurements of
pattern width inconsistent for comparison.

5.2.5 Printing tip diameter

The rate of the dispensing phase (i.e., the volumetric flow rate) was also
dependent on the cross-sectional area of the printing tip through the following
relation:

\[ Q = vA, \]

Because ATPS printing is autonomous, varying the inner diameter can passively
control the rate at which liquid dispenses and consequently how wide patterns
spread. Tips used in studies on the printing tip speed, loaded volume, and surface type (discussed below) included standard gel-loading pipette tips with a measured capillary inner diameter of 312.9 µm (rounded to 300 µm). To systematically study the effect of other tip dimensions on patterns width, new tips with different capillary diameters were designed in SolidWorks (Section 4.5). For each tip diameter, the pattern width decreased at higher speeds. This trend was more pronounced for 575 µm and 300 µm tips, where the patterns continuously decreased in size with increase in the printing tip speed. With the largest diameter tips (750 µm), the DEX phase tended to prematurely dispense upon insertion into the PEG phase solution before printing (horizontal motion of the tip) had started. To avoid this problem, the insertion speed of the printing tip was increased (through the z-axis motor) to 63.5 mm/s from 3.2 mm/s used with smaller diameter tips, but still yielded inconsistent dispensing at the onset of printing causing large variations in pattern width between samples. The inconsistent dispensing explained less significant decreases in patterns width with tips of diameter 750 µm. Among the tips studied, extruded tips with a measured 213.7 µm diameter (rounded to 200 µm), were the least likely to prematurely dispense at the onset of printing. Hence, patterns width exhibited less variations at each tested speed.

At a given speed, tips with a smaller inner diameter of 200 µm and 300 µm resulted in significantly finer patterns (p<0.05). The thinnest stable pattern was 322 ± 64.5 µm obtained using an extruded tip of 200 µm at 17 mm/s. Further increase in the speed with the 200 µm tip resulted in unstable patterns. The fact
that patterns printed with a 200 µm tip diameter were statistically smaller than patterns using a 300 µm tip diameter suggests that finer resolutions may be achievable by using tips of smaller diameters. However, the change in slope of pattern width printed with 200 µm at different speeds is much smaller than that for larger diameter tips. This implies that even if narrower patterns are obtained with smaller tip diameters, increasing the speed of the tip will not appreciably reduce it. Future works for printing smaller printed features should utilize tips with a finer cross-sectional area than those used in this study.

5.2.4 The effect of surface type on printed patterns

All characterization studies presented to this point were conducted on a confluent monolayer of cells as the printing surface (bio-paper) based on previous works that suggested pattern stability is partly due to interactions between the printing phase and the surface of living cells [11-12]. While the capability of direct, non-contact printing on cells is a major advantage of the ATPS microtechnology, printing on other surfaces could broaden the utility of this approach. This capability would be important for tissue engineering applications that require the use of bioactive matrices for cell support and growth. Direct observations of the PEG-DEX interface revealed certain points where the interface became anchored to the cell monolayer. This observation prompted the question whether morphology of cell monolayer and its micrometer-scale roughness pins down and stabilizes printed patterns, or interactions between DEX phase and biological molecules on cells facilitate it. To address this question, several types of substrates were selected including molecularly smooth
poly(D,L-lactic acid) (PDLA) films with a roughness of 1.9 nm [74], PDLA surfaces decorated with ordered microgrooves of 10 µm and 20 µm pitch (Fig. 5.6a), low cell-density surfaces (Fig. 5.6b), hydrophilic and hydrophobic polydimethyl siloxane (PDMS) replica of a monolayer of fixed cells (Fig. 5.6c), and a decellularized matrix with sub-micrometer roughness (Fig. 5.6e,f). The molecularly smooth PDLA surface, ordered microgrooved surface, and hydrophobic and hydrophilic cell layer replica surfaces were used to help elucidate the effect of surface roughness whereas the decellularized matrix could explain the contribution of biomolecules.

Linear patterns of FITC-DEX were printed on these substrates under similar conditions for the tip diameter (300 µm), loaded volume (8 µl), and printing tip speed (7.6 mm/s). To ensure that the tip speed did not affect pattern stability as in previous characterization studies, only a speed within the stable regime were used. All patterns printed onto the smooth PDLA, microgrooved PDLA, low cell-density and cell layer replica quickly became unstable and resolved into droplets (Fig. 5.6d). Contrarily, FITC-DEX patterns printed onto the decellularized matrix surface remained stable and intact over a period of 3 hrs. In addition, patterns on the decellularized matrix surface showed smoother edges than on a cell monolayer (Fig. 5.6g). In principle, the absence of curvatures can facilitate stable patterns of finer resolution. Decellularized matrices also supported linear cell patterns confirming the long-term stability of patterns (Fig. 5.6h). Altogether, this set of experiments indicated that the contribution of nanoscale and microscale surface roughness, ordered or random, is inadequate
to stabilize linear patterns of DEX and suggests that biomolecules play a key role for maintaining DEX patterns stable for long time periods. Our finding made by studies on different surface types is supported by a recent report that showed arbitrary-shaped DEX phase patterns remained stable on non-cellular surfaces containing molecularly rough heterogeneous patches [75]. It was shown that pattern stability is driven by the affinity of the DEX molecules in the printed phase to select functional groups on the surface. The difference in the affinity of DEX molecules in the printed phase for different functional groups on the surface gave rise to surface energy barriers that pin the contact line of the printed DEX phase.

Decellularized matrices are biologically active surfaces that promote cell adhesion and growth and thus provide an appealing alternative to cell monolayer for printing with ATPS. Similar to a cell monolayer, only a high-density matrix laid down by a 90-100% confluent layers of cells generate stable patterns of high-fidelity. This finding is similar to the discovery made in [75], that the gap between patchy patterns is also crucial in retaining stable line patterns.

5.3 Summary on printing resolution

Characterization studies revealed that the resolution of non-circular patterns are limited to 322 ± 65 µm. Attempts made in characterization studies to further increase the resolution resulted in the instability and breakage of printed patterns. The instability of patterns was caused by curvatures along the interface of patterns with the PEG phase and cell layer, giving rise to pressure gradients and resembling the Plateau-Rayleigh instability. Previous studies have found that curvatures can be minimized and potentially eliminated by using different
PEG and DEX concentrations with increased interfacial tension ($\gamma_{PD}$), printing on smoother surfaces such as a decellularized matrix. To test this principle, a preliminary study conducted using 4 % PEG 8K, 5 % DEX 500K ($\gamma_{PD} = 14 \mu J/m^2$) as phase-forming concentrations and generated patterns with lateral dimensions of 258 ± 13 µm before breaking into droplets. A disadvantage, however, was that the higher interfacial tension compromised the fidelity of cellular patterns, as cells were unable to effectively partition to the printing DEX phase (section 2.3.2). Decellularized matrices are most likely smoother surfaces [76] than cell layers and have given patterns with smoother edges (Fig. 5.6e). As a result, future attempts to increase pattern resolution should be focused on printing with narrow dispensing tips on decellularized surfaces.

5.4 Creating heterocellular niches in multiple configurations

Native tissues consist of multiple cell types organized into well-ordered microstructures where heterotypic cellular interactions play a crucial role in regulating tissue formation and function [13, 15-16]. Generating such well-organized constructs in vitro and replicating these interactions require a bottom-up approach, such as the ATPS microprinting, to enable controlled spatial arrangements of multiple cells in defined geometries. Thus, experimental studies were designed to demonstrate the capability of our automated ATPS microtechnology for printing high-fidelity patterns of cells with spatial and temporal control over cell placement. Duplex co-cultures of green and red fluorescently labeled mouse myoblast C2C12 cells were created in parallel, orthogonal, and acute geometries on cell layers and decellularized matrices.
Co-cultures featured side-by-side linear patterns of two cell colors with defined interspacing. The process resulted in uniform linear patterns of one cell type sandwiched within linear patterns of a second cell type (Fig. 5.7a). Lateral dimension measurements revealed that patterns could be printed with a consistent size down to 348 ± 10 µm without interspacing. For duplex cell printing in orthogonal and acute geometries, interspacing measurements between linear cell patterns in red showed that spatial control is consistent down to ± 19 µm. Altogether, duplex prints in multiple geometries successfully demonstrated the capability of ATPS microtechnology to print well-organized cellular arrangements with high reproducibility and microscale spatial control.

Results from proof-of-concept experiments demonstrated the capability to both spatially and temporally control cell printing. Spatial control was realized through the computer interface that allowed printing cell patterns at computer-registered coordinates on the printing surface. Temporal control was, in this context, demonstrated by the ability to print cells of different colors at predefined time points: cell patterns in green were first printed and precisely four hours later, cell patterns in red were printed. Pressure-induced printing utilizing a mechanical valve has shown temporal control by monitoring how fast and frequent the valve opened to eject cells but printing of all cell types is done in one uninterrupted step [51]. A distinguishing advantage of automated ATPS cell microprinting is therefore, the capability to control both the location of cell patterns and the time cell patterns are printed.
5.5 Creating user-defined shapes

Bottom-up engineering of some tissues (e.g., liver) require controlled positioning of cells in complex geometries. Current cell printing technologies can accommodate multiple cell types but are limited in terms of creating user-defined viable cell patterns [11]. Therefore, an experiment was designed to print user-defined patterns and show improvements in pattern generation based on the new automated platform. The narrow dispensing tip allowed greater control over dispensing to generate patterns of consistent size. A large loaded volume of 10 \( \mu l \) and slow speed of 3.2 mm/s allowed printing the shape “UA,” to generate features that had a consistent size with droplets generated upon insertion and retraction of the tip. The arc in shape “U”: could be programmed by carefully tuning the speed commands and incrementing small distances of the y-axis and x-axis motors. Programming such precise features like the arc were, however, not necessary because small droplets generated at the end of each line merged connecting lines in the shape of a curve. For printing the middle line feature in the A, since the length was half the distance of side line features and required the tip to insert at its beginning and retract at its end, small loaded volumes were used to prevent forming large droplets that would pull apart both ends from the line. In addition, since all three line features in the “A” were printed in less than one minute, droplets of 1 mm diameter in middle line feature could spread enough and disturb the fidelity of side lines.

To minimize the size of droplets at the beginning and end of linear patterns, printing was done with volumes starting at 5 \( \mu l \), previously noted as the
minimum volume needed to initiate autonomous dispensing upon insertion of the
tip in the PEG phase. Loaded volumes below the selected 5.25 µl generated a
small enough droplet 900 µm in diameter at the beginning of the line feature, but
the lateral speed of the tip generated a line 335 µm in width. Due to the
difference in size between the droplet and line feature, pressure gradients from
local curvatures pulled apart the two features resulting in collapse of the line
pattern. To minimize these curvatures, the volume was increased in increments
of 0.5 µl until at 5.25 µl, a stable line could be printed. The resulting middle line
in the ‘A’ of fig. 5.7d shows droplets, 905 µm in diameter, connected to a linear
feature, 625 µm in width.

Measurements for pattern resolution on resulting images of “UA” and
“NEOMED” at the selected printing conditions were 660 ± 32 µm and 390 ± 31
µm, respectively. Using a smaller loaded volume and higher speed (8 µl and 6.3
mm/s), “NEOMED,” showed features of smaller sizes than “UA”, but
compromised their aesthetics as shown by the presence of blobs at the end of
line features in N, E, and M (Fig. 5.7e). The autonomous nature of dispensing
results in circular droplets close 1 mm in diameter, at the beginning and/or end of
each pattern depending on whether or not the tip retracted after printing the
feature. Several adjustments were made to generate a uniform width along each
letter of the pattern including maximum insertion and retraction speeds (63.5
mm/s), continuous transition between Y-axis and Z-axis motors, and using lower
tip speeds (6.3 mm/s). However, limitations of the current system still prevented
letters of finer resolution to be printed without leaving a large blob at the
beginning or end of printed patterns. As a result, conditions were set to print “UA” with features of similar sizes to circular droplets, and thereby generated patterns with relatively consistent lateral dimensions throughout.

5.6 Conclusions

The newly developed automated and programmable cell printing platform enabled spatial and temporal control over positioning of cells on biological surfaces. Characterization studies revealed that the resolution of non-circular patterns are limited to $322 \pm 64.5 \, \mu m$. Attempts to further increase the resolution resulted in the instability and breakage of printed patterns. To explain pattern instability, images of unstable patterns were analyzed at the point of breaking to show that breakage occurs due to the presence of different curvatures along the patterns that give rise to large capillary pressures. In addition, studies on different surface types suggested that interactions between the printed DEX phase and biomolecules on the surface are crucial to maintain stable patterns. This finding was based on observing stable patterns on decellularized matrices, but not on ordered and randomly rough substrates, and expands ATPS microprinting to include applications where cell-ECM interactions are important. To demonstrate the utility of this approach for spatially-temporally controlled cell printing, duplex patterns of cells were generated in multiple configurations. To show capabilities of printing complex geometries user-defined shapes ‘UA’ and ‘NEOMED’ were printed. Overall, the non-contact nature of this printing approach combined with spatial and temporal control from computer-registered
motors, can in principle enable layer-by-layer engineering of constructs with multiple cell types for tissue engineering applications.
Figure 5.1. (a) Variations of pattern width with the lateral speed of tips. Printed FITC-DEX patterns show a systematic decrease in width ($R^2 = 0.96$), a plateau at higher speeds, and instability at beyond 22 mm/s. All prints were done on a monolayer of C2C12 cells as the substrate. (b) Variations of cellular pattern width with the tip speed. Scale bar 500 µm in b.
Figure 5.2. (a) Schematic representation of balance between the three interfacial tension terms at the interface of PEG-DEX-cells. The schematic shows a cross-sectional view of a printed line. (b) Printed patterns have an average thickness of 25 µm.
Figure 5.3. a) Sequence of images showing image processing of an unstable linear pattern of FITC-DEX, representing Plateau-Rayleigh Instability. b) Capillary pressure calculations at the point of necking. Scale bar 300 µm in a and 500 µm in b.
Figure 5.4. (a) A printing tip of 300 µm inner diameter loaded with FITC-DEX; the arrow indicates the region above the capillary. (b) Printed patterns width changes linearly with the height of reagent above the capillary portion, within the volume range studied. Asterisks indicate data sets statistically different from each other (p<0.05).
Figure 5.5. The resolution of printed patterns varies with the inner diameter of the tip within the range of 200-750 µm. The asterisks indicate that data from different tip diameters at the same speed are statistically different ($p<0.05$).
Figure 5.6 (a) A PDLA surface decorated with 20 µm microgrooves, (b) 50% confluent cell monolayer. (c) PDMS mold replicate of fixed cells, and (e) a decellularized surface generated from a confluent MB-MDA 231 breast cancer cells. (f) Fluorescent staining of a decellularized matrix using a protein labeling dye. Scale bar: 200 µm in a, b, c, e, and f, 400 µm in d and g, and 300 µm in h.
Figure 5.7. Duplex printed co-cultures on a cell monolayer in (a) parallel, (b) orthogonal, and (c) acute angle configurations. User-defined spelling, “UA” in (d) and, “NEOMED” in (e). C2C12 cells were stained with green cell and red cell tracking dyes. Scale bar in a, b, and c is 500 µm, 300 µm in d and e.
Specific Aim 1: Construct, program, and calibrate a three-dimensional bioprinter to print cells in spatially-controlled manner.

This aim involved (i) configuring three motorized single axis linear slides according to pre-defined specifications, (ii) assembling them to enable 3D motion capability, (iii) design and fabrication of a (1) Teflon cartridge for mounting up to six pipette tips, (2) a rectangular piece of Plexiglas as a holder for a 60-mm polystyrene dish mounted onto a supporting stage, designed to house different sizes of culture plates, (3) two arms made of Plexiglas to house the cartridge and provide it motion over the entire printing stage, respectively, (iv) interfacing the assembly with a programmable controller to provide a wide-range of control over movement of the printer head, and (v) calibrating the movement of the printer head.

Specific Aim 2: Perform a parametric investigation of ATPS-mediated cell printing using the bioprinter.

To accomplish this, a series of characterization studies were performed to study the effect of three design parameters on cell printing with this bioprinter.
These were tip speed, loaded volume in pipette tips, and pipette tip inner diameter. In addition, a number of surface types were investigated to determine which surfaces enable stable patterns; a sub-confluent monolayer of cells, decellularized matrices, flat PDLA surface, PDLA surface with imprinted grooves, and hydrophobic and hydrophilic PDMS replica of a cell monolayer. Results from these studies showed that patterns of finer resolution can be achieved by (i) increasing the printer tip speed to 12 mm/s, (ii) decreasing loaded volumes in printing tip to 6 µl, and (iii) using finer tips of 200 µm diameter. Resolution of patterns was found to be limited to 322 ± 64.5 µm. Attempts to generate finer resolutions caused formation of curvatures along the pattern column that induce breakage of the pattern into droplets. Pattern stability was found to also depend on surface type. Only surfaces containing a high-density matrix laid down by a 90-100% confluent layers of cells and a confluent cell layer generated stable patterns of high-fidelity. This finding suggested that interactions between the DEX phase and biological molecules are crucial to retain stable patterns.

- **Specific Aim 3:** Demonstrate the feasibility of ATPS-mediated cell printing towards tissue engineering by creating heterocellular niches in multiple configurations, and user-defined cellular patterns.

  Demo experiments for creating heterocellular niches were performed by printing co-cultures of green and red fluorescently labeled mouse myoblast C2C12 cells in parallel, orthogonal, and acute geometries on cell layers and
decellularized matrices. To create user-defined cellular patterns, UA and NEOMED were printed on a monolayer of cells.
CHAPTER VII
FUTURE DIRECTIONS

This study demonstrated that ATPS-mediated cell printing is a promising approach towards creating two- and potentially three-dimensional multi-cellular constructs for tissue engineering applications. Future studies aimed towards this direction should first make improvements on the three-dimensional bioprinter to enable more systematic control of the printing tip during printing. A design for an improved cartridge that will hold fine printing tips (~200 µm) has already been completed and needs to be implemented. In addition, a feature needs to be added to the existing dish holder to lock the dish in place during printing.

Once these changes are implemented, two- and three-dimensional multi-cellular constructs can be easily created to study cell-cell and cell-ECM interactions. One study in progress involves printing endothelial cells in patterns of controlled size to assess the effects of both cell density and surface on the formation of capillary-like structures. Thus far, from experiments that have tested printing circular patterns with different densities of endothelial cells (ECs) on matrigel-coated plates a relationship between the two variables appears to exist, but no systematic studies have been performed to uncover this relationship. Future results from this study will provide a better understanding of conditions for ECs to form capillaries *in vitro.*
Future attempts towards creating three-dimensional multi-cellular constructs will need to address the following challenges, (i) define spatial placement of different cell types within different layers, (ii) identify a surface to harvest the construct after printing, (iii) utilize histological and immunohistochemical techniques (i.e., fixing, staining, and sectioning of construct) and analysis to determine the distribution of printed cells within the constructs, (iv) addressing limitations in vascularization of construct.

(i) **Define spatial placement of different cell types in different layers:**
Similar to what has been done with the 2D multi-cellular construct, a demo experiment should be designed and carried out to understand the capabilities of the current system for multi-layered printing in terms of positioning of cells within different layers. Experiments to date have only printed on a single confluent monolayer of cells, and therefore it was easy to determine how far to position the Z-axis motor containing the printing tips from the surface. Introducing multiple layers may require recalibration of the Z-axis motor to consider the added thickness, although the thickness of several micrometers is small enough compared to the distance between cell layer and the printing tips currently used.

(ii) **Identify a surface to harvest the construct after printing:**

Prior to beginning any multi-layering experiments, a surface should be selected for releasing printed cell layers from the surface. Some cell sheet technology approaches use a poly(N-isopropylacrylamide)-grafted (PIPAAm) culture dish that is temperature-responsive [46-47], to harvest and collect cell sheets. PIPAAm-grafted dishes are commercially available but require additional
tools and experience to collect the cell sheets after they have released from the surface.

(iii) **Fixing, staining, and sectioning of construct:**

To observe the cross-section of multi-layered constructs, they must be fixed with 4% paraformaldehyde and processed at certain sections. Each section is then stained with hematoxilin and eosin (H.E staining) along with certain antibodies to identify the presence of different cells and whether they have organized into microstructures. A number of standardized methods have been developed and can be consulted for performing these techniques [77]. The presence of microstructures can be detected with a confocal laser microscope.

(iv) **Address limitations in vascularization of construct:**

Due to diffusion limitations that prevent the delivery of nutrients to and the removal of cellular waste from multilayered constructs thicker than ~200 µm, a well-organized network of capillaries is essential to support the viability of cells within the construct [78]. A current set of experiments focuses on the need to understand the environmental needs of endothelial cells which form the interior of capillaries and larger vessels. The specific parameters in view were: endothelial cell density and type of feeder gels and/or cells with growth factors needed to best facilitate blood vessel formation. The range of endothelial cell densities that have been tested are 15-22,000 cells/cm². The types of support surfaces tested are: matrigel, smooth muscle cells, and a combination of the two. Matrigel alone has been shown inadequate to stabilize blood vessels upon implantation [79]. Another study has also shown smooth muscle cells to be an inadequate to
induce formation of capillary-like structures without presence of 2-4 ng of vascular endothelial growth factor (VEGF) per/10^6 cells for each day [80]. As a result, combinations of matrigel containing VEGF and SMCs should be examined as a surface type to induce formation of capillary-like structures. In addition, future studies will need to develop a method to quantify the formation of capillary networks and then using this criterion determine optimal cell density and/or surface to facilitate vascularization of the construct using the ATPS microprinting approach.
BIBLIOGRAPHY


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APPENDICES
APPENDIX A
SAMPLE STATISTICAL TESTS

```
data:
  Input innerd linewidth;
  datalines;
  750  1013
  750  1314
  750  1308
  750  760
  750  854
  750  919
  750  1344
  750  1011
  750  1049
  575  664
  575  740
  575  529
  575  599
  575  509
  300  470
  300  446
  300  400
  300  410
  300  305
  300  282
;
proc glm;
  class innerd;
  model linewidth = innerd;
  means innerd/tukey cldiff;
title 'Compare Linewidth across Inner Diameters';
proc qplot;
  plot linewidth*innerd;
proc boxplot;
  plot linewidth*innerd;
  title 'anova results';
run;
```
The GLM Procedure
Tukey's Studentized Range (HSD) Test for Linewidth

NOTE: This test controls the Type I experimentwise error rate.

Alpha 0.05
Error Degrees of Freedom 17
Error Mean Square 27336.62
Critical Value of Studentized Range 3.62736

Comparisons significant at the 0.05 level are indicated by **.

<table>
<thead>
<tr>
<th>Difference</th>
<th>Comparison</th>
<th>Between Means</th>
<th>Simultaneous 95% Confidence Limits</th>
</tr>
</thead>
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<tr>
<td>750 - 575</td>
<td>401.31</td>
<td>165.33</td>
<td>638.49</td>
</tr>
<tr>
<td>750 - 300</td>
<td>679.28</td>
<td>455.73</td>
<td>902.82</td>
</tr>
<tr>
<td>575 - 750</td>
<td>-401.31</td>
<td>-638.49</td>
<td>-185.33</td>
</tr>
<tr>
<td>575 - 300</td>
<td>277.37</td>
<td>20.53</td>
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</tr>
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<td>300 - 750</td>
<td>-679.28</td>
<td>-902.82</td>
<td>-455.73</td>
</tr>
<tr>
<td>300 - 575</td>
<td>-277.37</td>
<td>-534.20</td>
<td>-20.53</td>
</tr>
</tbody>
</table>

Compare Linewidth across Inner Diameters
%% This program signals the Z-axis motor to lower inside the dish, Z-axis motor to perform a linear motion across the petri dish, the Z-axis motor to retract out of the dish, and the x-axis motor to offset in preparation for the next set of linear patterns.

F; Enable On-line mode
C; Clear all commands from current program
S2M500, ; Set Motor 2 (Z-axis) speed to 500 steps/sec (3.175 mm/s)
I2M3000, ; Motor 2 (Z-axis) descends 3000 steps into PEG solution
S3M1200, ; Set Motor 3 (Z-axis) speed to 1200 steps/sec (7.6 mm/s)
I3M5000, ; Motor 3 (Z-axis) moves 5000 steps in the forward direction
S2M2000, ; Set Motor 2 (Z-axis) speed to 2000 steps/sec (12.7 mm/s)
I2M-3000, ; Motor 2 (Z-axis) retracts 3000 steps to initial height
S3M2000, ; Set Motor 3 (Z-axis) speed to 2000 steps/sec (12.7 mm/s)
I3M-5000, ; Motor 3 (Z-axis) moves backward 5000 steps to starting point
I1M350, ; Motor 1 (x-axis) moves left 350 steps (2.22 mm)
R; Run current program

Sample script to print a linear pattern for characterization studies.
%% This program signals the Z-axis motor to lower inside the dish, coordinated motion between Z-axis and x-axis motors in a "U"-shaped pattern, and the Z-axis motor to retract out of the dish.

F, ;Enable On-line mode
C, ;Clear all commands from previous programs
S2M700, ;Set Motor 2 (Z-axis) speed to 700 steps/sec (4.44 mm/s)
I2M3000, ;Motor 2 (Z-axis) descends to 3000 steps into PEG solution
S3M1000, ;Set Motor 3 (Z-axis) speed to 1000 steps/s (6.35 mm/s)
I3M1170, ;Motor 3 (Z-axis) moves 1170 steps (7.43 mm) in the forward direction
S1M1000, ;Set Motor 1 (x-axis) speed to 1000 steps/s (6.35 mm/s)
I1M660, ;Motor 1 (x-axis) moves to the right 660 steps (4.2 mm)
S3M1000, ;Set Motor 3 (Z-axis) speed to 1000 steps/s (6.35 mm/s)
S2M6000, ;Set Motor 2 (Z-axis) to maximum speed of 6000 steps/s (38.1 mm/s)
I3M-1170, ;Move Motor 3 (Z-axis) in the backward direction 1170 steps (7.43 mm)
U77, ;Start Continuous Index
I3M1170; ;Motor 3 (Z-axis) moves 1170 steps (7.43 mm) in the backward direction
I2M-3000, ;Motor 2 (Z-axis) retracts 3000 steps to starting point
U99, ;End Continuous Index
R, ;Run current program

%% This program signals the Z-axis motor to lower inside the dish, coordinated motion between Z-axis and x-axis motors in a backward U-shaped pattern, and the Z-axis motor to retract out of the dish.

F, ;Enable On-line mode
C, ;Clear all commands from previous programs
I1M200, ;Motor 1 (x-axis) moves to the right 200 steps (1.2 mm)
I3M1170, ;Motor 3 (Z-axis) moves 1170 steps (7.43 mm) in the forward direction
S2M700, ;Set Motor 2 (Z-axis) speed to 700 steps/sec (4.44 mm/s)
I2M3000, ;Motor 2 (Z-axis) descends 3000 steps into PEG solution
S3M1000, ;Set Motor 3 (Z-axis) speed to 1000 steps/s (6.35 mm/s)
I3M-1170, ;Move Motor 3 (Z-axis) in the backward direction 1170 steps (7.43 mm)
U77, ;Start Continuous Index
I3M1170; ;Motor 3 (Z-axis) moves 1170 steps (7.43 mm) in the backward direction
I2M-3000, ;Motor 2 (Z-axis) retracts 3000 steps to starting point
U99, ;End Continuous Index
R, ;Run current program

%% This program signals the Z-axis motor to lower inside the dish, and the x-axis motor to complete the "A"-shaped pattern started with the previous program.

F, ;Enable On-line mode
C, ;Clear all commands from previous programs
I3M-585, ;Move Motor 3 (Z-axis) 585 steps (3.71 mm) in the backward direction
S2M1000, ;Set Motor 2 speed to 1000 steps/sec (6.35 mm/s)
I2M1000, ;Motor 2 (Z-axis) descends 1000 steps to starting point
S1M800, ;Set Motor 1 speed to 800 steps/sec (5.08 mm/s)
S2M1000, ;Set Motor 2 speed to 1000 steps/s (6.35 mm/s)
U77; ;Start Continuous Index
I3M1170, ;Motor 3 (Z-axis) moves 1170 steps (7.43 mm) in the forward direction 1170 steps (7.43 mm)
I1M660, ;Motor 1 (x-axis) moves 660 steps to the left
I2M-1000, ;Motor 2 (Z-axis) retracts 1000 steps
U99, ;End Continuous Index
R, ;Run current program

Sample script to print user-defined shapes (i.e., UA).
%CIRCFIT  Fits a circle in x,y plane
% function  [xc, yc, R, a] = circfit(x,y)
% Result is center point (yc,xc) and radius R.  A is an optional
% output describing the circle's equation:
%  x^2+y^2+a(1)*x+a(2)*y+a(3)=0

% by Bucher izhak 25/oct/1991

n=length(x);  xx=x.*x; yy=y.*y; xy=x.*y;
A=[sum(x) sum(y) n;sum(xy) sum(yy) sum(y);sum(xx) sum(xy) sum(x)];
B=[-sum(xx+yy) ; -sum(xx.*y+yy.*y) ; -sum(xx.*x+xy.*y)];
a=A\B;
xc = -.5*a(1);
yc = -.5*a(2);
R  =  sqrt((a(1)^2+a(2)^2)/4-a(3));

Make your lines verticle
%Have the circle fit and images with this file in the same directory
clc

I=imread('C:\Users\ADSA\Desktop\David\old\1-50_1.63X.tif');
J=imread('C:\Users\ADSA\Desktop\6_R.tif');
J=rgb2gray(I);
J=imadjust(J);
J=I;
thresh=100;
sigma =2;
thresh_C=[0.15,0.3];
BW = edge(J,'canny',thresh_C,sigma);
figure
imshow(BW)
[obj,n]=bwlabel(BW,8);
object = zeros(1,n);
for k=1:n
    [Y,X]=find(obj==k);
    object(k) = length(Y);
    if object(k)<thresh
        BW(Y,X)=0;
    end
end
figure
imshow(BW)
impixelinfo

ymin=input('Please enter minimum Y: ')
ymax=input('Please enter maximum Y: ')
Scale=input('Please enter scale of imaging (Unit/Pixel) : ')
[r,c]=size(BW);
CW=BW(ymin:ymax,5:c-floor(c/100)-2);
figure
imshow(CW)
[obj,n]=bwlabel(CW,8);
% [Rtrim,Ctrim]=find(obj1>0);
% [rl,cl]=size(CW1);
% CW=zeros(rl,max(Ctrim)-min(Ctrim)+20);
% CW=CW1(1:end,min(Ctrim)-10:max(Ctrim)+10);
if n>2
    disp('error!')
    break;
else
    for i=1:n
        Y2=[];X2=[];X3=[];
        [Y2,X2]=find(obj==i);
        X3=X2-min(X2);
        [xc, yc, R, a] = circfit(X3,Y2);
        disp(R*Scale)
    end
end
I_initial=imread('C:\Users\ADSA\Desktop\P2.tif');
I=imread('C:\Users\ADSA\Desktop\P3.tif');
%J=rgb2gray(I);
%K=imadjust(J);
J=I;
thresh=100;
sigma =2;
thresh_C=[0.15,0.3];
BW = edge(J,'canny',thresh_C,sigma);
figure
imshow(BW)
[obj,n]=bwlabel(BW,8);
object = zeros(1,n);
for k=1:n
    [Y,X]=find(obj==k);
    object(k) = length(Y);
    if object(k)<thresh
        BW(Y,X)=0;
    end
end
figure
imshow(BW)
impixelinfo
I_initial(find(BW==1))=255;
figure
imshow(I_initial)