Digital Microfluidic Lab-on-a-Chip Platform for the Culture and Analysis of Three-Dimensional Multicellular Spheroids

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering

By
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2014
ABSTRACT OF THE DISSERTATION

Digital Microfluidic Lab-on-a-Chip Platform for the Culture and Analysis of Three-Dimensional Multicellular Spheroids

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Doctor of Philosophy in Biomedical Engineering

University of California, Los Angeles, 2014

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Cell spheroids are compact, multicellular aggregates grown in vitro that mimic the three-dimensional morphology of in vivo tissues, thereby providing a more physiologically relevant tissue and disease model than cells cultured in two-dimensional monolayers. Despite the advantages of cell spheroids, their use in routine biomedical research has been limited, in part by the lack of automated, user-friendly, and flexible techniques for cell spheroid culture and analysis. Digital (droplet) microfluidics (DμF) enables the manipulation of discrete drops of liquid through the controlled application of electric fields. Because it allows automated and flexible liquid handling, DμF has the potential to address many of the shortcomings associated with current cell spheroid culture techniques. This dissertation describes the design, fabrication,
and operation of a DμF platform that enables the culture and analysis of three-dimensional, multicellular spheroids.

To enable spheroid culture on a DμF device, a novel device architecture was developed that incorporates through-holes, or ‘wells’, in the bottom plate of the device that allow for the formation of hanging drops of controlled volume and composition. With the ability to create and address hanging drops in situ, protocols for automated cell spheroid culture were developed. Using DμF liquid handling, spheroids can be initiated and maintained on the device for at least 96 hours, exhibiting good viability (>90%) and size uniformity (~8% CV intra-experiment, ~16% CV inter-experiment). Automated spheroid-based drug screening and migration assays were also performed, demonstrating the ability to create and assay spheroids with higher-order tissue properties as well as stimulate relevant physiological processes. An optimized DμF peptide mass fingerprinting (PMF) sample preparation technique that could be useful for the in situ preparation and analysis of spheroid protein secretions was also developed.

The platform described here advances the field of digital microfluidics by introducing a novel functionality, hanging drop formation, which enables the culture of large, three-dimensional micro-tissues on a DμF device. Improvements to the handling of biological solutions on a digital microfluidic device are also presented. This DμF platform also represents an advance in the field of tissue engineering by providing a novel means of automating the culture and analysis of individually addressable cell spheroids. A DμF platform that facilitates the culture and analysis of cell spheroids can potentially lower the barriers to adoption for the use of cell spheroids in routine biomedical research. Ultimately, broader use of spheroids in cell-based assays and
screens has the potential to improve pre-clinical drug development efficiency and provide more physiologically relevant insights into our basic understanding of tissues and diseases.
The dissertation of Andrew Peter Aijian is approved.

________________________________________
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2014
I dedicate this thesis to my inspiring and loving parents, my encouraging and uplifting siblings, and my patient and supportive wife.
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Acknowledgments

I express my sincere gratitude to my advisor, Professor Robin Garrell, for the support, mentorship, and advice she provided during my time at UCLA. One of the most valuable aspects of my graduate research experience was the freedom she offered me to explore my intellectual curiosity and the independence to pursue my own research interests. Her enthusiasm for science and higher education is contagious and, through her mentorship, I learned a great deal about microfluidics, professionalism, and leadership.

I extend a special thanks to all of my collaborators, whose expertise and resources were critical to the completion of this work. I am particularly appreciative of Prof. Joseph and Dr. Rachel Loo, Prof. Heather Maynard, Prof. Andrea Kasko, Prof. C. J. Kim, and Prof. Tatiana Segura, as well as the students in their labs, for providing access to their facilities and equipment.

I am also grateful for the assistance, insights, and friendship shared by current and former members of the Garrell lab, particularly Debalina Chatterjee, Alex Tucker-Schwartz, Robyn Hodgkins, and Brian Bender. I have also enjoyed the friendship and camaraderie of my fellow MCTP trainees, High School Nanoscience Program volunteers, and my BME classmates.

I am particularly grateful for the unfailing support of my family, especially my wife, who supported and encouraged me daily, and my parents, who instilled in me a curiosity about the world and a value for education; their generosity and encouragement has allowed me to pursue a higher education and for that I am forever grateful.
Chapter 4 of this thesis is adapted from the paper: “Digital Microfluidics for Automated Hanging Drop Cell Spheroid Culture,” which was submitted to the *Journal of Laboratory Automation* (SAGE Publications) on 07/02/2014 for publication in the special edition: Microengineered Cell- and Tissue-Based Assays for Drug Screening & Toxicology Applications. The content is reproduced here in accordance with the SAGE Publications’ Global Journal Author Reuse Policy. This paper was supported with funds from the NSF IGERT: Materials Creation Training Program (MCTP) – DGE-0654431, a 2013-2014 Dissertation Year Fellowship awarded to Andrew Aijian from the UCLA Graduate Division, and discretionary institutional funds from Prof. Robin Garrell (UCLA). Prof. Robin Garrell, Ph. D, provided oversight as the PI for this paper.

Chapter 5 is adapted from a manuscript that is in preparation for publication. This paper is co-authored by Brian Bender, who contributed to the fabrication of DμF devices used in this work and the development of protocols for assaying gel-encapsulated spheroids. Brian Bender also conducted spheroid-based migration assays on the DμF device and performed analysis of spheroid invasion using confocal laser scanning microscopy. Prof. Robin Garrell provided oversight as the PI for this paper. This research described in this paper was supported with funds from a 2013-2014 Dissertation Year Fellowship awarded to Andrew Aijian from the UCLA Graduate Division, the 2013-2014 Dr. Ursula Mandel and Malcom R. Stacy Endowed Fellowships awarded to Brian Bender, and discretionary institutional funds from Prof. Robin Garrell (UCLA).
Chapter 6 was adapted from the article, “Fluorinated liquid-enabled protein handling and surfactant-aided crystallization for fully in situ digital microfluidic MALDI-MS analysis,” A. P. Aijian, D. Chatterjee and R. L. Garrell, Lab on a Chip, 2012, 12, 2552. DOI: 10.1039/C2LC21135A. This paper is reproduced here in accordance with the rights retained by journal authors as outlined by the Royal Society of Chemistry. This paper was co-authored by Dr. Debalina Chatterjee, Ph.D., who developed the microfluidic device fabrication protocols used in this work. Prof. Robin Garrell provided oversight as the PI for this paper. This paper was supported with funds from the NSF IGERT: Materials Creation Training Program (MCTP) – DGE-0654431.
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- **Aijian, A. P.*, Bender, B. F.*, Garrell, R. L.** “Digital Microfluidics for Spheroid-Based Invasion Assays.” *(In preparation)*
- **Aijian, A. P., Garrell, R. L.** “Digital Microfluidics for Automated Hanging Drop Cell Spheroid Culture.” *Journal of Laboratory Automation. (accepted for publication)*

Presentations (* - presenting author)


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Chapter 1. Overview of Thesis

1.1 Background, motivation and objective

“Microfluidics” refers to tools and techniques for the precise manipulation of small volumes of liquid. The development of tools and techniques to facilitate the handling of small volumes of liquid arose from the adaptation of CMOS fabrication techniques to pattern silicon wafers with micro-scale three-dimensional features in the late 1970s. Bassous developed methods for the anisotropic etching of silicon to create nozzles for ink-jet printing, which became the first major commercial application of microfluidics.[1] Such etching techniques were subsequently used for the development of miniaturized sensors and actuators such as gas chromatograms,[2] ion-sensitive field effect transistors,[3] and capillary electrophoresis systems.[4] The concept of the “lab-on-a-chip” originated with Andreas Manz’s proposal of a “miniaturized total chemical analysis system (μ-TAS),” in which he envisioned “…the use of micromachining to fabricate chemical sensors, chemical analysis systems, or even laboratories on the scale of a silicon chip.”[5,6] Manz recognized that the ability to control liquid at the micro-scale would provide numerous benefits for the analysis of fluid samples such as reduced sample and reagent consumption, rapid and high-throughput operations, reduced costs, and automation capabilities. Since Manz’s demonstration of a micro-scale capillary electrophoresis system in 1992, microfluidic technologies have been widely implemented in various industries including healthcare, sensing and electronics, energy, and basic research.
Microfluidics has found widespread use within the field of biomedical science as tools for chemical and biomolecular liquid handling, sensing, and diagnostic applications. The scope of cell-based microfluidic applications, however, is much narrower, especially those pertaining to three-dimensional (3D) cell or tissue models. Human tissues have a hierarchical structure in which the properties of the tissues at the micro-scale impact the behavior and function of higher-ordered tissues and tissue systems. Thus, the ability to understand and model tissues at the micro-scale is fundamental to advancing biomedical science and technology. With the ability to enable precise control over liquids and materials at the micro-scale, microfluidic technologies have the potential to be excellent tools for modelling the tissue microenvironment. For this reason, researchers have recently begun to develop ‘body-on-a-chip’ technologies, which utilize microfluidics to create miniaturized, in vitro models of one or more physiological systems. The motivation for this work was to develop a body-on-a-chip platform that would leverage the advantages of digital (droplet-based) microfluidics to facilitate and enable micro-scale three-dimensional tissue engineering for basic research and applied biomedical applications.

### 1.2 Thesis outline

This dissertation provides background information pertaining to digital microfluidics and multicellular spheroids and describes the fabrication and operation of a DμF platform for cell spheroid culture and spheroid-based assays and screens. Chapter 2 reviews the theory and applications of digital microfluidics. This chapter covers the mechanisms of electrowetting and liquid dielectrophoresis, as well as the design and fabrication of DμF devices. Relevant DμF bioanalytical methods and biological liquid handling techniques are also reviewed.
Chapter 3 provides background on three-dimensional cell culture, with particular emphasis on multicellular spheroids. The differences between two-dimensional and three-dimensional in vitro cell culture are discussed. Various techniques for three-dimensional cell culture are also compared. This chapter also describes the advantages, limitations, and applications of three-dimensional cell spheroids as a tissue model.

Subsequent chapters consist of research articles that have been published or submitted for publication, describing the development and use of a DμF platform for cell spheroid culture and biomolecular analysis.

Chapter 4 is an article titled, “Digital Microfluidics for Automated Hanging Drop Cell Spheroid Culture,” which was submitted for publication in the Journal of Laboratory Automation in the Microengineered Cell- and Tissue-Based Assays for Drug Screening & Toxicology Applications special edition. This article describes the design, fabrication, testing, and validation of the DμF spheroid culture platform. The primary advancements described in this paper are: (i) the ability to form individually addressable hanging drops of reproducible volume on a DμF device, and (ii) the ability to maintain cells in culture within a hanging drop for multiple days (at least 96 h), allowing for the formation and assaying of cell spheroids. To demonstrate the utility of this platform, a proof-of-principle drug screen was performed, exploring the impact of insulin exposure on the chemosensitivty of colon adenocarcinoma spheroids in vitro.
Chapter 5 is an article draft entitled, “Digital Microfluidic Platform for Cell Spheroid-Based Invasion Assays,” which was in preparation at the time of writing. This paper describes the use of DμF to encapsulate spheroids in hanging drops of a collagen hydrogel to model cell invasion. It is shown that collagen gels up to 50 kPa can be formed on the DμF device and that medium can be efficiently added to and extracted from the collagen gels. The invasion of human fibroblasts from spheroids encapsulated within collagen is observed by confocal microscopy. The ability to either enhance or inhibit migration through the addition of exogenous migration modulating agents is also demonstrated. Using this platform, an in vitro model for cancer-associated fibroblasts (CAF) is developed by stimulating fibroblast migration through the exposure of fibroblast spheroids to secretions from human colon adenocarcinoma spheroids.

Chapter 6 is an article that was published in Lab on a Chip (Lab Chip, 2012, 12, 2552-2559, DOI: 10.1039/C2LC21135A) titled, “Fluorinated liquid-enabled protein handling and surfactant-aided crystallization for fully in situ digital microfluidic MALDI-MS analysis.” It describes a novel technique for handling protein solutions on a digital microfluidic device for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) sample preparation. This work demonstrates the benefits of using a fluorinated liquid ambient phase for the handling of protein solutions, as well as the use of fluorinated surfactants for enhancing matrix crystallization on a fluorinated surface. The protein sample preparation techniques described in this chapter could be useful for the in situ analysis of protein secretions from spheroids grown on a DμF device.
Chapter 7 summarizes and reviews the primary contributions of this research and the potential impact and future research directions enabled by this work. Chapter 8 contains the bibliographic information for all of the references cited in this dissertation.

Together, these chapters describe the development of a novel digital microfluidic functionality – hanging drop formation – and how this functionality can be used to automate the culture and analysis of three-dimensional micro-tissues, as well as engineer a physiologically relevant tissue microenvironment.
Chapter 2. Digital Microfluidics Theory & Applications

2.1 Electrowetting Theory

The effects of an electric field on the apparent wetting behavior of a liquid were first observed by Gabriel Lippmann in 1857 who developed a relation, known as Lippmann’s law (2.1), that describes the effective solid-liquid interfacial tension of a liquid in contact with an actuated electrode, $\gamma_{SL}^{eff}$, as a function of the non-actuated interfacial tension, $\gamma_{SL}$, the electric potential applied to the electrode, $V$, and the capacitance at the liquid-electrode interface, $C$.[9]

$$\gamma_{SL}^{eff}(V) = \gamma_{SL} - \frac{C}{2}V^2$$  \hspace{1cm} (2.1)

In 1993, Berge combined Lippmann’s law with Young’s Law (2.2)[10],

$$\gamma_{SG} = \gamma_{SL} + \gamma_{LG}\cos(\theta)$$  \hspace{1cm} (2.2)

which describes the balance of forces in a three-phase (solid-liquid-gas) system at equilibrium as a function of the interfacial tensions of the interfaces in the system ($\gamma_{SL}$, $\gamma_{SG}$, $\gamma_{LG}$) and the contact angle of the liquid on the solid ($\theta$), to arrive at the Lippmann-Young relation (2.3)[11]:

$$\cos(\theta) = \cos(\theta_0) + \frac{C}{2\gamma_{LG}}V^2$$  \hspace{1cm} (2.3)

The Lippmann-Young relation describes the contact angle of a liquid in contact with an electrode as a function of the initial contact angle of the liquid, the applied electric potential, and the liquid-gas interfacial tension. This change in the apparent contact angle of a liquid on a surface in response to an electric field is termed “electrowetting.” Because of the susceptibility of aqueous solutions to undergo electrolysis when exposed to an electric current, most electrowetting studies and applications utilize a dielectric and/or hydrophobic
coating on top of the electrode to prevent direct contact between the liquid and the electrode and to maximize the initial contact angle, so as to maximize the electrowetting effect. The addition of the dielectric layer increases the capacitance between the liquid and the electrode, thereby requiring higher voltages to achieve electrowetting. Electrowetting that occurs on a dielectric-coated electrode is referred to as ‘electrowetting-on-dielectric’ (EWOD). Figure 2.1 shows the change in contact angle for an aqueous drop on a dielectric-coated electrode as a function of applied voltage.[12]

![Figure 2.1](image)

**Figure 2.1. Contact angle as a function of applied voltage.** The contact angle of an aqueous drop of liquid on an electrode coated with 1 µm silicon dioxide and 20 nm Teflon-AF decreases in accordance with the Lippmann-Young relation until contact angle saturation occurs at 77V. (Figure adapted with permission from reference [12]. Copyright 2009 American Chemical Society)

As shown in Figure 2.1, the apparent contact angle of a liquid drop on a dielectric-coated surface will decrease with increasing applied potential until contact angle saturation is
reached. Contact angle saturation refers to the voltage regime in which the contact angle of a drop no longer decreases with increasing voltage. To date, the mechanisms for contact angle saturation are widely debated and unresolved.

The electrowetting effect is a result of an electrostatic attraction between the liquid and the electrode upon the application of the electric potential. As depicted in Figure 2.2, fringe fields near the edge of the droplet induce an electrostatic Maxwell stress that pulls on the contact line of the drop, causing the drop to spread, or wet, the surface. [13,14] This spreading causes a reduction in the apparent contact angle of drop ($\theta_s$ in Figure 2.2), as predicted by the Lippmann-Young relation. It is important to note, however, that on an insulated surface, the actual contact angle remains constant at the micro-scale with or without an applied potential. Thus, the electrowetting effect does not result from changes in the interfacial tensions in the system, but rather describes the change in the shape of a drop such that the Laplace pressure balances the electrostatic force on the contact line. Thus, the electrowetting effect is determined by a balance of forces near the three-phase line, which can be modeled by rearranging equation 2.3 to yield

$$\gamma_{LG} \cos(\theta) = \gamma_{LG} \cos(\theta_0) + \frac{C}{2} V^2$$

(2.4)

that shows that the apparent contact angle of the drop is determined by a combination of the capillary line force and the electrostatic pressure.
Figure 2.2. Mechanism of the electrowetting effect. Upon application of an electric potential to an electrode, the fringe fields near the three phase contact line induce a horizontal electrostatic force normal to the contact line and oriented outward. Note that while the macroscopic, apparent contact angle, $\theta_s$, changes in response to the electric field, the actual contact angle does not. (Figure reproduced with permission from reference [13]. Copyright Taylor & Francis 2012)

2.2 Liquid Dielectrophoresis

While the electrowetting effect is observed for conductive liquids, non-conductive liquids can also exhibit a physical response in the presence of an electric field. A dielectric object in a non-uniform electric field is subject to a ponderomotive force by virtue of its polarizability. This phenomenon, called dielectrophoresis (DEP), applies to both solid and liquid materials in the presence of a non-uniform electric field. Depending on the relative permittivities of the dielectric object ($\varepsilon_1$) and surrounding medium ($\varepsilon_2$), the dielectrophoretic force will drive the object towards the region of maximum field intensity ($\varepsilon_1 > \varepsilon_2$, “positive DEP”) or minimum field intensity ($\varepsilon_2 > \varepsilon_1$, “negative DEP”). Figure 2.3 illustrates the positive and negative DEP effect on a dielectric object in a non-uniform electric field. Non-conductive liquids, or
Conductive liquids at frequencies above a critical, cross-over frequency, behave as dielectrics and therefore can be manipulated through DEP. While the translational forces for the electrowetting effect are localized near the drop interfaces and contact lines, liquid DEP is a body force with the force density spread throughout the bulk of the drop.

**Figure 2.3. Dielectrophoresis of a dielectric particle in a non-uniform electric field.** In a non-uniform electric field, a dielectric body of permittivity $\varepsilon_1$ in an ambient medium of permittivity $\varepsilon_2$ will be polarized and drawn either towards the region of field maxima (if $\varepsilon_1 > \varepsilon_2$, “positive DEP”) or repelled from the region of field maxima (if $\varepsilon_2 > \varepsilon_1$, “negative DEP”) by dielectrophoresis.

Liquid DEP and the electrowetting effect have been utilized in a variety of applications, including the development of tunable liquid lenses [15], electrowetting displays [16], and digital microfluidic lab-on-a-chip (LOC) platforms [17].
2.3 Digital Microfluidics

2.3.1 Droplet Manipulation

In 1998, Washizu was the first to utilize the electrowetting effect to drive the movement of droplets.[18] The manipulation of discrete droplets of liquid using electric fields is referred to as ‘digital microfluidics’ (DμF). The manipulation of droplets in digital microfluidics is achieved by placing a drop of liquid on an array of closely spaced electrodes, and selectively applying an electric potential to an electrode adjacent to one side of the drop, as shown in Figure 2.4. When a potential is applied to an electrode that is sitting beneath just a portion of the drop footprint, the drop is drawn towards the actuated electrode through a combination of electrowetting and liquid dielectrophoretic forces. For conductive solutions at low frequencies, charges accumulate at the drop surface, shielding the interior of the drop from the electric field. Thus the electric field lines contour the drop surface, concentrating at the three-phase contact line nearest the applied electrode (Figure 2.5 (a), (b)). In this case, the electrowetting effect dominates droplet movement, and the three-phase contact line on the side of the drop nearest the active electrode is drawn towards the electrode due to Coulombic attraction, resulting in a change in the drop shape near the actuated electrode as the apparent contact angle changes, as described in section 2.1. The difference in the drop shape between the leading edge (the edge of the drop nearest the actuated electrode) and the trailing edge (the opposite edge of the drop) results in a gradient of Laplace pressure within the drop in which the pressure at the leading edge of the drop is lower than the pressure at the trailing edge of the drop. This pressure gradient as well as the electrostatic attraction of the contact line to the electric field generated by actuated electrode combine to drive the translation of
the droplet towards the actuated electrode. Figure 2.6 summarizes the different droplet translation forces in the case of electrowetting-dominated droplet movement.

**Figure 2.4. Schematic of a two-plate digital microfluidic device.** To manipulate drops of liquid on a DμF device, the electrode on one side of the drop is actuated while the other remains grounded. The droplet is drawn towards the actuated electrode until it is centered above the electrode. Activating individual electrodes sequentially along an array of electrodes allows the droplet to be transported across the array. (Figure reproduced with permission from reference [19]. Copyright Royal Society of Chemistry 2008)
Figure 2.5. Electric field distribution within a drop of water at various frequencies. At low frequencies, an electric field does not penetrate a conductive liquid, and thus concentrates at the three-phase contact line, driving electrowetting. At high-frequencies, a conducting liquid can behave as a dielectric, allowing the field to penetrate the drop, preventing the concentration of the field at the three-phase contact line, thereby reducing the electrowetting effect. As the dielectric nature of the liquid increases DEP forces become more dominant. (Figure reproduced permission from reference [20]. Copyright AIP Publishing LLC 2009)
Figure 2.6. Summary of DμF translation mechanisms. Cross sections of free-body diagrams for various mechanisms of DμF droplet translation. Boxes 1, 2, and 3 at the top correspond to Case (1), (2), and (3) beneath. The electrostatic force from the actuated electrode attracts charges accumulated at the three-phase contact line (Case 1), which disrupts the balance of the surface tension between the leading and trailing edges of the drop (Case 2), and also creates a gradient in Laplace pressure across the drop in which the pressure at the trailing edge is greater than the pressure at the leading edge. All these mechanisms contribute to the translation of drops in DμF. (Figure reproduced with permission from reference [13]. Copyright Taylor & Francis 2012)
For insulating solutions (conductive solutions at high frequencies can behave as insulating solutions), there is no charge accumulation at the surface of the drop so the electric field penetrates the drop, reducing the concentration of the field at the three-phase contact line, diminishing the lateral Coulombic force that drives electrowetting. In this case DEP forces dominate droplet movement.

A DμF device can be modeled as an RC circuit, providing a convenient method to calculate the electromechanical forces acting on a drop as a function of the material properties of the device. For example, the model DμF system in Figure 2.7, which represents a commonly used DμF device design, can be broken down into six circuit elements from which the total energy in the system, $U$, can be calculated from the energy stored in each element, $U_i$, from the equation $U = \sum_{i=1}^{6} C_i V_i^2$ where $C_i$ and $V_i$ are the capacitance and voltage drop, respectively, across the $i$th circuit element. The force can be calculated from $F = \frac{\partial U}{\partial x}$ to give:

$$F = \frac{\partial U}{\partial x} = \varepsilon_0 \frac{\varepsilon}{2} y \left[ k_t \frac{(V-V_0)^2-(V-V_2)^2}{d} + \frac{k_d (V_0-V_1)^2-(V_2-V_3)^2}{D} + \frac{k_p k_t (V_1^2-V_3^2)}{k_p d' + k_t d} \right]$$

(2.5)

Where $\varepsilon_0$ is the permittivity of free space, $x$ is the length of the liquid-filled region, $y$ is the width of the electrode, $k_t$, $k_d$, and $k_p$ are the dielectric constants of the Teflon-AF, liquid, and parylene layers, respectively, $d$, $d'$, and $D$ are the thicknesses of the parylene, Teflon, and inter-plate spacing, respectively, and $V_i$ is the voltage drop across the individual circuit components, as labeled in Figure 2.7. The combination of electrowetting and DEP forces allows a wide variety of conducting and non-conducting liquids to be manipulated on a DμF device (Figure 2.8).
A common DμF device design includes an electrode array coated with the dielectric polymer parylene-c and the hydrophobic fluoropolymer Teflon-AF on the bottom plate, and a continuous electrode coated with Teflon-AF on the top plate. Each layer, including the liquid and ambient medium, can be modeled as a component in a circuit, from which the total energy and electromechanical force can be derived. (Figure reproduced with permission from reference [21]. Copyright Royal Society of Chemistry 2009)
Figure 2.8. Liquids moveable on a DµF device. A variety of polar and non-polar liquids can be manipulated using a combination of electrowetting and DEP forces. Higher frequencies and reduced inter-plate gap heights increase the strength of the DEP force, facilitating the movement of non-polar, insulating liquids. Liquids with very low complex permittivities (<10^{-10}) are difficult or impossible to move on a DµF device. (Figure reproduced with permission from reference [22]. Copyright Royal Society of Chemistry 2006)

2.3.2 DµF Device Design and Materials

Digital microfluidic devices can operate in either one- or two-plate configurations. In a one-plate (“open”) system, droplets are transported across a single substrate that contains both the actuating and ground electrodes. In a two-plate device, the droplet is sandwiched between two plates with the actuating and ground electrodes patterned on one or both of the plates. Because the force acting on a drop is greater in a two-plate configuration with small inter-plate spacing, and because a two-plate configuration minimizes the air-liquid interface, thus mitigating droplet evaporation, most DµF devices are closed systems.
By varying the size of the electrodes or the inter-plate gap height, droplets of a wide range of volumes can be manipulated using DμF. Droplets of volumes as low as single digit pL can be generated through the serial splitting of parent and daughter drops, or through the use of very small gap heights.[23,24] Alternatively, droplets with volumes as large as 1 mL have been manipulated by increasing the inter-plate spacing as well as the size of the electrodes within the array.[25] Song et al. developed a DμF device scaling model for droplet actuation, splitting, dispensing, and mixing, which is summarized in Figure 2.9.[23] From Figure 2.9 it is evident that the voltage required to achieve these liquid handling functions is a function of four parameters: thickness of the dielectric layer (t), the dielectric constant of the dielectric material (ε_r), the inter-plate spacing (d) and the characteristic length of the electrode (L).[23] According to Song et al., splitting a droplet requires the highest voltage of any liquid handling function and, in an ambient medium of air, can only be achieved for 

\[ \left( \frac{T}{\varepsilon_r} \left( \frac{d}{L} \right) \right)^{\frac{1}{2}} \leq 0.4, \]

else the voltage required for splitting is greater than the saturation voltage and splitting cannot occur.[23] From equation 2.5, the force on the droplet increases as the thicknesses of the dielectric layer, hydrophobic layers, and inter-plate spacing decrease. Thus, using the minimal dielectric thickness that will enable reliable droplet handling while still minimizing the chance of dielectric breakdown (i.e. ensuring that the breakdown voltage, V_{BD}, for both the hydrophobic and dielectric layers is greater than the saturation voltage, V_{Sat}, for liquid handling) is desired for optimal device design and performance. This condition can be achieved using 1 μm of parylene-c as the dielectric and 0.2 μm of Cytop® as the hydrophobic coating, which corresponds \( \frac{T}{\varepsilon_r} = 0.42 \), and thus a \( \left( \frac{d}{L} \right) \) value of 0.38 is the maximum aspect ratio (inter-plate spacing to electrode length) allowable for droplet splitting to occur in air.
Thus, splitting droplets on the order of \( pL \) to \( \mu L \), requires electrode lengths and inter-plate spacings ranging from \( L = 14 \, \mu m \), \( d = 5.2 \, \mu m \) to \( L = 1.4 \, mm \), \( d = 0.52 \, mm \), respectively.

Figure 2.9. DuF Device Scaling Limits. (A) The minimum voltage required for droplet movement (\( V_T \)) as well as dispensing and splitting a drop across three electrodes as a function of dielectric thickness and relative permittivity (\( t, \varepsilon_r \)), the inter-plate spacing (\( d \)), and the characteristic length of the electrode (\( L \)). (B) The breakdown voltage of either a Cytop or Teflon-AF hydrophobic layer on 1 \( \mu \)m of parylene-C. Under these conditions, only Cytop is capable of sustaining the electric field for voltages up to and exceeding the saturation voltage. (Figure adapted from ref. [23])
Table 2.1 lists the range of published design and operating parameters for DμF devices.

Table 2.1. Typical materials and operating conditions for a DμF device.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operating voltage</td>
<td>$10^0 - 10^2$ V</td>
</tr>
<tr>
<td>Operating frequency</td>
<td>DC-20 kHz (EWOD), $&gt;10^5$ Hz (DEP)</td>
</tr>
<tr>
<td>Inter-plate spacing</td>
<td>$10^0 - 10^3$ μm</td>
</tr>
<tr>
<td>Electrode dimensions</td>
<td>$10^1 - 10^3$ μm</td>
</tr>
<tr>
<td>Drop volumes</td>
<td>$10^0$ pL – $10^1$ mL</td>
</tr>
<tr>
<td>Dielectric materials</td>
<td>Parylene-C, Parylene-N, Parylene-HT, PDMS, SU-8, Polyimide, Si$_3$N$_4$, Ta$_2$O$_5$, SiN, SiO$_2$, Al$_2$O$_3$, BST, BZN, Saran® wrap (polyethylene)</td>
</tr>
<tr>
<td>Hydrophobic materials</td>
<td>Teflon-AF®, Cytop, FluoroPel, BYK® Silclean® 3700, Rain-X®</td>
</tr>
<tr>
<td>Electrode materials</td>
<td>Gold, ITO, Aluminum, Chromium, Silver, Silver Ink, Graphene,</td>
</tr>
<tr>
<td>Substrate materials</td>
<td>Silicon, Glass, PCB, Paper</td>
</tr>
<tr>
<td>Dielectric layer thickness</td>
<td>$10^{-1} - 10^1$ μm</td>
</tr>
<tr>
<td>Hydrophobic layer thickness</td>
<td>$10^1 - 10^3$ nm</td>
</tr>
</tbody>
</table>

2.3.3 DμF Capabilities and Applications

Using electrowetting and DEP-driven liquid handling, droplets of liquid can be dispensed from on-chip reservoirs, translated across an electrode array, merged, mixed, or split. Because droplet movement is controlled through the application of an electric potential to a particular electrode or sequence of electrodes, the manipulation of droplets can be automated through the use of computer software that controls the relaying of an electric potential to the individual electrodes within the array. Thus a sequence of electrode actuation steps can be programmed allowing for fully automated liquid handling. Liquid handling operations can also be conducted either in series or in parallel, enabling the automation of independent tasks for multiple droplets simultaneously. Consequently, DμF enables significantly higher throughput liquid handling than is possible using manual liquid handling techniques, attaining
some of the advantages of robotic liquid handling and other technologies used in array-based assays.

Because of the high degree of control one has over the design and operation of a DμF device, DμF provides precise control over liquid handling. Utilizing droplet volume metering techniques, such as capacitance or impedance feedback control, or optimized electrode geometries, droplets can be dispensed from on-chip reservoirs with a volume variation of ±1%.\[26,27\] Droplets can be transported along an electrode array at speeds up to ~25 cm/s in air and ~0.5 cm/s in oil.\[28\] DμF also enables rapid mixing, allowing mixing of µL-scale drops in <3 seconds under optimal mixing conditions.\[29\] Additionally, droplets of immiscible liquids can be merged to form either oil-in-water or water-in-oil droplet emulsion systems on the device.\[30,31\]

Leveraging the robust liquid handling capabilities of the DμF platform, researchers have used DμF to automate the liquid handling for many chemical and biological workflows. For instance, a number of researchers have utilized DμF to automate the complex and tedious sample handling protocols associated with protein sample preparation for analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), using DμF to perform protein precipitation, denaturation, disulfide reduction, thiol alkylation, trypsinization, and matrix co-crystallization completely in situ.\[31-33\] Integrating resistive heating elements into the device to allow precise temperature control, enabling nucleic acid assays and protocols such as PCR, qPCR, gene transformation, single-cell genomics (SCG), hybridization assays, and gene sequencing.\[34-39\] Magnetic and dielectrophoretic particle
manipulation methods have been integrated into DμF platforms, allowing cell and particle sorting, as well as enabling heterogeneous reactions and immunoassays.[40,41] Various electrochemical analytical methods have also been developed for the DμF platform, including impedance spectroscopy, capacitance sensing, or cyclic voltammetry.[42-44] A number of optical analytical techniques, such as optical microscopy, absorbance spectroscopy, fluorescence imaging and spectroscopy, photodetection, and surface plasmon resonance imaging have also been demonstrated on the DμF platform.[36,45-48]

DμF has been shown to enable cell culture protocols and cell-based assays. By removing the hydrophobic layer in select locations on the top or bottom plate of the device, or by depositing extracellular matrix (ECM) materials on the device surface, cells can be made to form adherent monolayers on the device.[49,50] Digital microfluidic liquid handling can then be used to deliver all the reagents necessary for maintaining cell culture and performing in situ subculture. Au et al. have determined optimal surfactant types and concentrations for enabling movement of complex proteinaceous solutions while maintaining maximal cell viability.[51] Au also showed that below a frequency of ~10 kHz, the electric fields used to drive droplet movement have a negligible impact on cell viability or gene expression. Droplets of sol-gel solutions can also be manipulated on a DμF device for the encapsulation of cells within hydrogels.[52,53] Figure 2.10 shows a sampling of various bioassays or protocols performed on a DμF device.
Figure 2.10. DμF bio-analytical capabilities and functionalities. Digital microfluidics has been used to enable various *in situ* analytical capabilities and biological sample handling techniques, including: adherent monolayer cell culture [49], peptide mass fingerprinting-MALDI MS [32], PCR [34], magnetic bead-based heterogeneous immunoassays [54], encapsulation of cell suspensions within hydrogel discs [52], quantitative PCR [47], fluorescence microscopy [55], surface plasmon resonance imaging [36], dielectrophoretic particle and cell sorting [56], single cell genomics [39], capacitance sensing [26], impedance spectroscopy [43], and chemiluminescence detection [47]. Images reproduced with permission from Royal Society of Chemistry, Macmillan Publishers Ltd, American Chemical Society, and Elsevier.

Through microfluidic automation of genomic, proteomic, and cellular assays and screens, DμF can reduce required sample and reagent volumes, eliminate tedious manual sample handling, reduce user-error and variability, reduce assay times, and increase assay throughput, making DμF a powerful tool for advancing biomedical research.
Chapter 3. Cell Spheroids

3.1 Tissue structure and physiology

3.1.1 Cellular Organization

Tissues are collections of cells and biomolecules that are organized, interact, and communicate in a specific fashion to serve a particular function. The dimensions of human tissues span five orders of magnitude: from individual cells ($10^1 \, \mu m$) to the complex, interconnected network of organs, vasculature, muscles, bones, and neurological pathways comprising the human body ($10^0 \, m$). Figure 3.1 shows this hierarchy.

Figure 3.1. Hierarchy of human tissues. The human body is comprised of a collection of different cell types that combine to form tissues. Various tissues organize to form organs, which function together as a system to support the human body. (Image adapted from reference [57])
The primary unit of tissue is the cell. All cells within the body are derived from pluripotent embryonic stem cells, which can differentiate along one of three pathways: the ectoderm, mesoderm, or endoderm lineages, to become any type of somatic cell found in the human body (excluding extraembryonic tissues). Adult stem cells, which are multi-potent cells found within various tissues, are similar to embryonic stem cells in possessing self-renewal and differentiation capabilities, but are more committed to a single lineage. The stem cell lineage is depicted in Figure 3.2.

Figure 3.2. Embryonic stem cell (ESC) differentiation lineage. ESCs from the inner cell mass of the blastocyst can differentiate into any tissue in the body. (Image adapted from ref. [58])
The interactions among cells and between cells and the extracellular matrix (ECM) dictate the behavior and function of tissues. In an epithelial state, cells interact with each other at their lateral faces, are in contact with an extracellular matrix (ECM) at one end (basal surface) and are exposed to a lumen at the other end (apical surface). Epithelial cells are characterized by their tight intercellular junctions and their basal-apical polarization. Cell-cell adhesions are controlled by adherens junctions (primarily cadherin interactions), tight junctions, and desmosomes. In a mesenchymal state, individual cells adhere to and migrate through a three-dimensional ECM. Mesenchymal cells do not form well-defined intercellular interactions and possess a front-end/back-end polarity rather than an apical-basal orientation. Figure 3.3 illustrates the transition of cells from an epithelial to mesenchymal state.

**Figure 3.3. Epithelial-to-mesenchymal transition (EMT).** Cells within the epithelial state are tightly-packed and possess a distinct apical-basal polarity. Epithelial cells, however, can transitional into a mesenchymal state in which cells disrupt their intercellular interactions, adhere strongly to the ECM, and migrate through the basement membrane. (Figure adapted with permission from ref. [59]. Copyright Douglas S. Micalizzi 2010.)

### 3.1.2 Extracellular Matrix

The extracellular matrix is a network of fixed and soluble proteins and polymeric sugars that provide both structural support and biochemical signaling to cells and tissues (Figure 3.4).
The composition and structure of the ECM is dynamic and varies widely among different tissue types, developmental stages, or injury/disease states. Table 3.1 lists various components of the ECM as well as some of their biological functions.

**Figure 3.4. Functions of the ECM.** The ECM functions as a structural support for cells and is involved in chemical and mechanical cell signaling. The dynamic ECM microenvironment is critically involved in tissue homeostasis. (Figure reproduced from ref. [60] with permission from Elsevier. Copyright 2010)
<table>
<thead>
<tr>
<th>ECM component</th>
<th>Functions</th>
</tr>
</thead>
</table>
| Collagen: fibrillar (I, II, III, V, XI, XXIV, XXVII) | • Structural scaffold  
• Controls stiffness, resists tension  
• Binds adhesion factors (for example, fibronectin)  
• Binds some growth factors (for example, BMP2)  
• Porous: allows amoeboid migration strategies |
| Collagen: nonfibrillar (I-XXVII, except fibrillar types) | • Broadly serve many ECM and cell-adhesion functions, including: binding other ECM proteins and proteoglycans to aid ECM organization and stability; aiding fibrillar collagen formation; forming networks as barriers for solute transport, including basement membrane (VI); modulating cell migration and proliferation |
| Fibrin                                             | • Structural matrix in wound healing  
• Controls stiffness, resists tension  
• Binds adhesion molecules  
• Easily degraded and remodeled |
| Fibronectin                                        | • Structural matrix in tissue repair  
• Promotes the assembly of an insoluble, fibrillar ECM network  
• Exists in both soluble and insoluble forms  
• Binds integrin receptors  
• Links integrin receptors to other ECM molecules |
| Elastin                                            | • Provides elastic recoil |
| Laminin                                            | • Structural component of basement membrane  
• Binds integrin receptors  
• Anchors cells to the basal lamina  
• Contributes to cell differentiation, shape, and movement |
| Proteoglycans (Perlecan, Agrin, Decorin, Biglycan, Fibromodulin) | • Resist compression  
• Hinder water transport  
• Hinder macromolecular transport  
• Bind growth factors and chemokines  
• Electrokinetic effects |
| Matricellular proteins                             | • Intermediate, weak adhesion |
| Matrix Metalloproteinases                          | • Enzymatically cleave various ECM molecules  
• Remodel ECM  
• Modulate activity of ECM signaling molecules |
| Tissue Inhibitors of Metalloproteinases            | • Inhibit the activity of matrix metalloproteinases |
Cells respond to both the mechanical and chemical characteristics of the ECM. The stimulation of a biochemical cellular response by mechanical forces is called mechanotransduction, and has been shown to influence cell processes such as proliferation, migration, differentiation, and apoptosis.[63,64] The ECM, in addition to providing structural support and mechanical signaling, can harbor growth factors, cytokines, matrix proteases, and other non-structural proteins that result in signal transduction through the binding of soluble or immobilized ligands or chemical moieties.[65,66] Figure 3.5 shows an example of ECM-stimulated mechanical and chemical signaling.
Figure 3.5. Mechanical and chemical stimulation of cardiomyocytes. Cardiomyocyte function is directly related to both the mechanical and chemical properties of the extracellular matrix. Integrins and stretch-activated ion channels respond to mechanical stimuli, such as stiffness, or mechanical load, while growth-factor receptors and G-protein coupled receptors respond to soluble chemical stimuli in the ECM. (Figure reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology ref. [63]. Copyright 2009)

Cells interact with the extracellular environment through a variety of transmembrane receptor proteins. The integrin family of proteins, consisting of 24 different heterodimer transmembrane receptors, binds a wide variety of ECM components with varying degrees of
specificity. Over 50 other proteins, including proteoglycans, receptor tyrosine kinases, and cluster of differentiation complexes (CD) are also involved in cell-ECM interactions.[67] The communication between the cell and the ECM has been described as a “dynamic reciprocity” in which a cell-ECM binding event alters the cytoplasmic domain of the transmembrane receptor, initiating a signaling cascade that ultimately induces a change in gene expression, leading to the expression and secretion of proteins that alter the ECM.[68] The balance of this cell-ECM signaling cycle is key to the transition of cells from an epithelial state to a mesenchymal state, and vice-versa, and is integral to processes such as tissue homeostasis, wound healing, and tumor invasion.[64]

The restructuring and reorganization of cells in response to extracellular cues is the foundation of tissue morphogenesis, in which cells organize in a particular fashion so as to achieve a certain function or optimal configuration. A variety of extracellular conditions, including molecular composition, chemical gradients, shear stresses, and mechanical gradients, contribute to tissue morphogenesis. For example, it is hypothesized that local anisotropies in the distribution of tension, as well as the presence or absence of particular ECM proteins determine branching points in the epithelium, giving rise to tissue buds, tubes, or bifurcations that ultimately lead to the formation of branched organs such as the salivary glands, mammary glands, lungs, pancreas, and kidneys.[60,69] Similarly, angiogenesis and vessel sprouting result from endothelial cell alignment and tube formation along on collagen I fibrils, which also stimulate increased expression of membrane-type matrix metalloproteases (MT-MMPs) that break down the basement membrane, further facilitating the sprouting of vessels.[70,71] Thus, the local composition and mechanical properties of the ECM
microenvironment direct both the inter-cellular interactions at the single-cell level, and the organization and architecture of cell clusters at the multicellular level, ultimately leading to the formation of higher-order, functional tissues.

3.2 *In vitro* tissue models

*In vitro* cell culture dates back to the works of Leo Loeb, who, ca. 1897, studied the growth of tissues in agar and other “...environments that differ from those found in the body under natural conditions," and Ross Harrison, who in 1910 developed the hanging-drop culture technique, in which fragments of tissues were maintained in a pendant drop of cell culture medium for days at time.[72] Since then, many techniques and methods have been developed for the study of cells and tissues in *vitro*. *In vitro* cell culturing techniques can be categorized by their products: two-dimensional, three-dimensional, or suspension cultures. Two-dimensional (2D) cell culture refers to the culture of cells on a flat substrate, on which cells form adherent monolayers. Three-dimensional (3D) culture refers to the culture of cells in any manner that allows for the cells to move or migrate in three-dimensions. Suspension culture is a cell culture technique in which individual cells are maintained and proliferate as a suspension in culture media. While suspension culture can be used to grow three-dimensional tissues under certain conditions, it is used primarily for the culture of hematopoietic cells or other cells adapted for protein production, and is not used routinely for the study of solid tissues. This chapter focuses predominantly on two- and three-dimensional cell culture techniques.
3.2.1 Two-dimensional cell culture

In two-dimensional cell culture, cells are grown in adherent monolayers submerged in growth medium. Two-dimensional cell culture has been the standard *in vitro* culture technique used since its development in 1916, primarily due to the fact that most mammalian cells can be expanded in adherent culture, as well as the relative ease of maintaining cells in culture dishes, the low cost of materials necessary for adherent cell culture, and the vast selection of commercial products specifically designed for the maintenance and assaying of cells in two-dimensional monolayers. [73,74]

The microenvironment for cells cultured in two-dimensions is depicted in Figure 3.6. In 2D cell culture, cells adhere to the surface of the tissue culture vessel (typically polystyrene – PS) or to proteins adsorbed to the vessel. Cells adhere to the substrate through the formation of focal adhesion complexes, which are large, dynamic protein complexes that bind intracellular actin bundles to the ECM and transmit signals regarding ECM properties. Focal adhesions initiate at the edge of the cell and undergo a dynamic assembly/disassembly process as the cell migrates and spreads onto the surface. Because the cell is only able to form adhesions in the two-dimensional plane of the culture dish, many cells assume a flat, spread-out morphology in 2D cell culture. Additionally, because of contact inhibition, cells grown in 2D dishes will form monolayers, and exhibit minimal intercellular interaction. Typically cells grown in a 2D culture dish are exposed to uniform culture conditions, both on the substrate and medium faces of the cell.
3.6 Cell adhesion and migration in 2D cell culture. Cells cultured on two-dimensional substrates in vitro form focal adhesion complexes that utilize integrins to form a link between the cellular cytoskeleton and the ECM on the substrate. As cells migrate, new adhesion complexes can assemble and existing ones can disassemble. Filopodia and lamellipodia form as the cell encounters new adhesion sites at the leading edges of the cell. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology ref. [75]. Copyright 2002)

3.2.2 Three-dimensional cell culture

Numerous methods for three-dimensional cell culture exist; they can generally be categorized as either scaffold-based or scaffold-free. Scaffold-based methods utilize an extracellular, structural scaffold, such as hydrogels of ECM proteins, reconstituted basement membrane, or synthetic polymers to support the suspension of single cells or cellular aggregates within a three-dimensional space. The use of scaffolds is thus advantageous for modeling three-dimensional systems in which cell-matrix interactions dominate. Scaffolds can be engineered to contain various chemical functionalities or mechanical properties that mimic the natural extracellular matrix or stimulate particular cellular behaviors, such as migration or differentiation.[76] Scaffolds can also be designed with specific geometries to guide the
formation of desired cellular or tissue structures.[77-79] Table 3.2 lists various fabrication methods, materials and applications for 3D scaffold-based culture.

Scaffold-free methods for 3D in vitro cell culture promote the aggregation of cells by minimizing cell-substrate interaction and maximizing cell-cell interactions. Scaffold-free systems are thus excellent models for tissues with a high degree of cell-cell interactions. Scaffold-free 3D culture can be achieved by growing cells on non-adhesive surfaces, such as nano-patterned materials or at liquid-liquid interfaces, or by inducing constant motion of cell suspensions such that cells are unable to adhere to any substrate and can only form cell-cell adhesions. The most common techniques for forming scaffold-free cellular aggregates include the hanging-drop technique, the use of non-adhesive micro-molds or well-plates and the use of rotary vessels. The hanging drop technique involves creating pendant drops of a cell suspension, which allows cells to aggregate into compact, spherical aggregates, called 'spheroids,' at the curved air-liquid or liquid-liquid interface at the bottom of the drop.[80] Alternatively, non-adhesive materials, such as agarose gels, can be used to coat well-plates or can be patterned with micro-wells that induce cell aggregation by inhibiting cell-substrate interaction.[81] Nano-patterned surfaces of certain materials exhibit minimal cell adhesion, and so can cause cells to adhere preferentially to other cells compared to the substrate, resulting in the formation of spheroid-like aggregates.[82] Rotary vessels, originally developed by NASA to study tissue growth in microgravity conditions, utilize a slowly rotating vessel containing a cell suspension to create microgravity conditions in which cells are in minimal contact with the vessel walls and thus adhere primarily to other cells, forming
three-dimensional, spheroidal aggregates.[83] Table 3.3 compares various scaffold-based and scaffold free 3D culture techniques.
Table 3.2. Materials and applications for scaffold-based 3D cell culture (Adapted from [84])

<table>
<thead>
<tr>
<th>Method</th>
<th>Polymers</th>
<th>Unique factors</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biodegradable porous scaffold fabrication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent casting/salt leaching method</td>
<td>Absorbable polymer (PLLA, PLGA, collagen, etc.)</td>
<td>Biodegradable controlled porous scaffolds</td>
<td>Bone and cartilage tissue engineering</td>
</tr>
<tr>
<td>Ice particle leaching method</td>
<td>PLLA &amp; PLGA</td>
<td>Control of pore structure and production of thicker scaffolds</td>
<td>Porous 3D scaffolds for bone tissue engineering</td>
</tr>
<tr>
<td>Gas foaming/salt leaching method</td>
<td>PLLA, PLGA &amp; PDLLA</td>
<td>Controlled porosity and pore structure sponge</td>
<td>Drug delivery and tissue engineering</td>
</tr>
<tr>
<td><strong>Microsphere fabrication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent evaporation technique</td>
<td>PLGA, PLAGA</td>
<td>High-density cell culture, due to the extended surface area</td>
<td>Bone repair</td>
</tr>
<tr>
<td>Particle aggregated scaffold</td>
<td>Chitosan, HAP</td>
<td>High mechanical stability</td>
<td>Bone, cartilage, or osteochondral tissue engineering</td>
</tr>
<tr>
<td>Freeze drying method</td>
<td>PLGA, PLLA, PGA, PLGA/PPF, Collagen, and Chitosan</td>
<td>3D porous sponge structure, durable and flexible</td>
<td>Tissue engineering scaffolds</td>
</tr>
<tr>
<td>Thermally induced phase separation</td>
<td>PEG, PLLA</td>
<td>Highly porous scaffold for cellular transplantation</td>
<td>Complicated shapes for tissue engineering applications</td>
</tr>
<tr>
<td><strong>Injectable gel scaffold fabrication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceramic-based injectable scaffolds</td>
<td>CP ceramics, HAp, TCP, BCP, and BG</td>
<td>Porosity and bioreabsorbability</td>
<td>Cartilage tissue engineering</td>
</tr>
<tr>
<td>Hydrogel-based injectable scaffolds</td>
<td>Hydrophilic/hydrophobic diblock and triblock copolymer combinations of PLA, PGA, PLGA, and PEG. Copolymers of PEO and PPO and polyoxamer. Alginates, collagen, chitosan, HA, and fibroin</td>
<td>Biomimetically, exhibit biocompatibility and cause minimal inflammatory responses, thrombosis, and tissue damage</td>
<td>Cartilage, bone tissue engineering, and drug delivery</td>
</tr>
<tr>
<td><strong>Hydrogel scaffold fabrication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micromolding</td>
<td>Alginate, PMMA, HA, PEG</td>
<td>Microgels, biologically degradable, mechanical and physical complexity</td>
<td>Insulin delivery, gene therapy, bioreactor, and immunosolation</td>
</tr>
<tr>
<td>Photolithography</td>
<td>Chitosan, fibronectin, HA, PEG, PNIAAm, PAA, PMMA, PAam, and PDMAEM</td>
<td>Microwells, microarrays, controlled size and shape</td>
<td>Microdevices, biosensors, growth factors, matrix components, forces, and cell-cell interactions</td>
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<tr>
<td>Microfluidics</td>
<td>PGS, PEG, calcium alginate, silicon and PDMS</td>
<td>Microbeads, microrods, valves, and pumps</td>
<td>Sensing, cell separation, cell-based microreactors, and controlled microreactors,</td>
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<tr>
<td>Emulsification</td>
<td>Gelatin, HA, and collagen</td>
<td>Microgels, microsensors, cell-based diagnostics</td>
<td>Sustainable and controllable drug delivery therapies</td>
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<td><strong>Acellular scaffold fabrication</strong></td>
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<tr>
<td>Decellularization process</td>
<td>Biological tissues</td>
<td>Retain anatomical structure, native ECM, and similar biomechanical properties</td>
<td>Tissue engineering</td>
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*Table 3.2 continued on next page*
<table>
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<tr>
<th>Keratin scaffold fabrication</th>
<th>Fibrous scaffold fabrication</th>
<th>Functional scaffold fabrication</th>
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<td><strong>Self-assembled process</strong></td>
<td><strong>Nanofiber electrospinning process</strong></td>
<td><strong>Growth factor’s release process</strong></td>
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<tr>
<td>Keratin</td>
<td>PVA, PLA, PLGA, PCL</td>
<td>Collagen, gelatin, alginate, chitosan, fibrin, PLGA, PLA, and PEG</td>
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<tr>
<td>Biocompatibility</td>
<td>High surface area, biomechanical, and biocompatibility</td>
<td>Membranes, hydrogels, foams, microsphere, and particles</td>
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<td></td>
<td>Drug delivery, wound healing, soft tissue augmentation, synthetic skin, coatings for implants, and scaffolds for tissue engineering</td>
<td>Angiogenesis, bone regeneration, and wound healing</td>
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<td><strong>Sponge replication method</strong></td>
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<td>PU sponge, PVA, TCP, BCP or calcium sulfate</td>
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<td></td>
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<td>Interconnected porous ceramic scaffolds</td>
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<td>Bone tissue engineering</td>
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<td><strong>Simple calcium phosphate coating method</strong></td>
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<tr>
<td></td>
<td></td>
<td>Coating on: metals, glasses, inorganic ceramics and organic polymers (PLGA, PS, PP, silicone, and PTFE), collagens, fibres of silk, and hairs</td>
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<td></td>
<td></td>
<td>Improve biocompatibility or enhance the bioreactivity</td>
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<td>Orthopedic application</td>
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<td><strong>Inkjet printing process</strong></td>
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<td></td>
<td>Sodium alginate</td>
<td>To build complex tissues composed of multiple cell types (Hydrogel scaffold)</td>
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<td>Biosensor development, microdeposition of active proteins on cellulose, biochips and acellular polymeric scaffolds</td>
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<td><strong>Melt-based rapid prototyping process</strong></td>
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<td>Biodegradable polymers or blends</td>
<td>Complex 3D solid object, good mechanical strength</td>
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<td>Honey comb structure scaffold, hard-tissue scaffolds</td>
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<td><strong>Computer-aided design (CAD) data manipulation techniques</strong></td>
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<td>Design and fabrication of patient-specific scaffolds and automated scaffold assembly algorithm</td>
<td>Develop a program algorithm that can be used to design scaffold internal architectures</td>
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<td><strong>Ceramic scaffold fabrication</strong></td>
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<td><strong>Automation and direct organ fabrication</strong></td>
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<tr>
<td>Method</td>
<td>Advantages</td>
<td>Disadvantages</td>
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<tr>
<td>-----------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
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</table>
| Liquid Overlay (Nonadhesive surfaces)    | - Inexpensive  
- Simple  
- Easy to scale | - Variation in size, cell number and shape  
- Not individually addressable |
| Hanging Drop                            | - Inexpensive  
- Simple  
- Good size control  
- Rapid Formation  
- Compatible with co-culture  
- Individually addressable aggregates  
- Easy to trace spheroid assembly | - Labor intensive  
- Difficult to scale |
| Rotary Vessel                           | - Simple  
- Massive production possible  
- Long-term culture  
- Dynamic control of conditions  
- Co-culture possible | - Specialized equipment required  
- Variation in size, cell number, shape  
- Not individually addressable |
| Spinner Flask                           | - Simple  
- Massive production possible  
- Long-term culture  
- Dynamic control of conditions  
- Co-culture possible | - Specialized equipment required  
- Variation in size, cell number, shape  
- High shear force  
- Not individually addressable |
| Scaffold/ Hydrogel                      | - Provides 3D extracellular support  
- Provides biochemical signaling cues | - Lot-lot variability  
- Additional equipment/steps required  
- Not individually addressable  
- Limited control over size/shape |
| Pellet culture                          | - Simple  
- Rapid aggregation of cells  
- Individually addressable aggregates | - High shear force  
- Massive production difficult |
| Micromold                               | - Well controlled spheroid size  
- Well controlled spheroid shape  
- Co-culture possible  
- Individually addressable aggregates possible | - Additional equipment/steps required  
- Individual addressability difficult for high density spheroids |
| External force (electric, magnetic acoustic) aggregation | - Rapid cell aggregation  
- Individually addressable aggregates possible | - Potentially undefined effects to cells  
- Requires specialized equipment and culture conditions |
In both scaffold-based and scaffold-free 3D cell culture, cells are able to migrate and proliferate in three-dimensions and are exposed to other cells or an extracellular scaffold in all dimensions. Compared to two-dimensional cell culture, cells cultured in three-dimensions exhibit a significantly higher degree of intercellular interactions, possess a more three-dimensional morphology, and exhibit a higher degree of cellular heterogeneity. Importantly, because cells grown in three-dimensions can form intercellular interactions, they can assemble into epithelial-like cell arrangements similar to physiological tissues with basal, lateral, and apical surfaces. Because 3D cell structures experience both cell-cell and cell-ECM interactions, these cells will form a variety of epithelial junction complexes, such as adherens junctions, desmosomes, and tight junctions, in addition to the focal adhesion complexes that mediate cell-ECM interactions. Additionally, in contrast to 2D cell culture, cells cultured within a three-dimensional aggregate experience different microenvironments because of the nutrient and metabolic mass transport gradients that arise within a 3D aggregate. This cellular heterogeneity results in differential gene and protein expression patterns. Figure 3.7 illustrates some of the spatial gradients that arise in a three-dimensional cell culture model.
Figure 3.7. **Nutrient and metabolic gradients within a 3D cell spheroid.** Due to the high cell density of multicellular spheroids, nutrients and metabolic products such as oxygen and glucose experience diffusion-limited mass transport. Spatial nutrient gradients give rise to cellular heterogeneity within the aggregate as can be seen by inhomogeneous lactate and ATP distribution profiles. Additionally, cells > ~250 μm from the spheroid surface experience hypoxic conditions leading to the formation of necrotic regions within the interior of the aggregate. (Figure reproduced from ref. [86] with permission from Elsevier. Copyright 2010.)

### 3.2.3 Advantages and disadvantages of 2D vs. 3D cell culture

There are advantages and disadvantages to both 2D and 3D cell culture. The primary advantages of 2D cell culture are the relative ease and standardization of 2D cell culture protocols. Also, because many mammalian cell types can be expanded in 2D cell culture, and
because of the relatively low cost of cell culture supplies, 2D cell culture is used widely for routine *in vitro* tissue culture. As the standard method for *in vitro* tissue culture, a large number of assays and reagents, as well as automation equipment and protocols, have been developed specifically for 2D cell culture, enabling high-throughput and high-content cell-based assays. Two-dimensional tissue culture fails, however, to recapitulate many physiologically relevant properties of *in vivo* tissues. For instance, cells grown in two-dimensions do not establish intercellular contacts which are fundamental to tissue function and behavior. Many cells grown in flat dishes lose their cuboidal shape and adopt a flattened morphology, resulting in an unnatural mechanical state. Additionally, cells in monolayers are exposed to uniform environmental conditions, which is not the case for in tissues *in vivo*. Various studies indicate that the gene and protein expression profiles for cells grown in two-dimensional adherent monolayers differ significantly than what is observed from the same cells *in vivo*. [87-91]

The primary advantage of 3D tissue culture is that it can generate more physiologically-relevant tissue models than 2D culture. For instance, the intercellular interactions established in 3D cell culture enable cell signaling and communication mechanisms that cannot be achieved in two-dimensional cell culture. Cells in certain 3D culture environments can adopt the cuboidal shape and the basal-apical polarization observed *in vivo*. This physiologically relevant structural and mechanical environment translates into gene and protein expression profiles that more closely correlate with *in vivo* conditions than cells grown in monolayers. Additionally, cells grown in three dimensions can exhibit higher order tissue structures and morphologies such as the formation of hypoxic cores in dense tissue aggregates [92], the
formation of lumen or crypt structures similar to those found in various tissues *in vivo* [93], and the formation of endothelial tube-like structures.[94]

While more physiologically relevant than monolayer cells, 3D tissue culture entails practical challenges, and despite the variety of techniques available, no single method has been adopted as a standardized technique. Consequently, few commercial assay kits or reagents have been designed for three-dimensional cell-based assays. This lack of standardization makes research comparisons difficult, prompting many researchers to eschew 3D cell culture in favor of more standard 2D tissue culture. Furthermore, 3D tissue culture protocols are typically more labor intensive, often requiring extra equipment and handling steps compared to 2D cell culture. For example, the hanging drop spheroid technique typically requires numerous manual pipetting steps and very careful handling of the culture vessel so as not to disturb the hanging drops, thus limiting the throughput and scale. Once created, the hanging drops usually cannot be assayed or interrogated unless manually transferred to a separate vessel. Other 3D culture methods, such as the use of rotary vessels, spinner flasks, or non-adhesive flat-bottom plates, provide limited control over the size and shape of the spheroids and do not allow spheroids to be interrogated individually, making these techniques poor tools for cell-based assays. Scaffold-based methods require extra work to prepare the scaffolds, and are susceptible to lot-to-lot variability. Similarly, micro-mold techniques require additional reagents and preparation steps and, in the case of high-density micro-wells, do not allow individual addressability. In sum, while 3D tissue culture methods provide superior physiological relevance, technical barriers have limited their utility and adoption.
3.2.4 Applications of three-dimensional tissue models

Three-dimensional in vitro tissue culture has applications in both basic and applied biomedical science. The primary application of 3D cell culture is the development of clinical tissue engineered products for regenerative medicine. With an aging global population, there is a substantial need for products and technologies that can regenerate or restore function to aging or damaged tissues. Estimates for the global market for clinical tissue engineered products range from ~$4-15B in 2014, with musculoskeletal, skin, cardiology, and dental tissue engineered products comprising the bulk of the market.[95,96] The most successful commercial tissue engineering products to-date are a class of synthetic bone-graft products marketed by various medical device manufacturers such as Medtronic (Infise®), Baxter (Actifuse®), and DePuy (α-BSM) for the regeneration of bone tissue following surgeries such as anterior lumbar interbody fusions. These synthetic bone graft products consist of an extracellular matrix scaffold material, such as collagen, silicate, or calcium phosphate to provide structural support for the growth and migration of cells and may also include growth factors, such as bone morphogenic protein 2 for biochemical stimulation of bone growth. Other tissue engineered products utilize a combination of cells and ECM to create a 3D, metabolically-active synthetic tissue, such as Organogenesis Inc.’s Dermagraft®, a human fibroblast-loaded ECM mesh material for the treatment of diabetic foot ulcers. The combination of cells, scaffold materials, and growth factors into three-dimensional environments that mimic and encourage the growth of human tissues has led to the development of a wide variety of clinically successful tissue engineered products for regenerative medicine.
Three-dimensional tissues can also serve as excellent models for basic and pre-clinical research. As more sophisticated tissue and disease models than 2D *in vitro* cultures, 3D cultures can yield insights into basic biological processes such as embryogenesis, morphogenesis, differentiation, and tumorogenesis.\[97,98\] Because gene and protein expression patterns differ significantly depending on the cellular microenvironment, tissue models that more accurately mimic *in vivo* conditions are essential to advancing our understanding of biology at both the molecular, cellular, and systemic levels.

One particular area that stands to benefit from the use of enhanced *in vitro* tissue and disease models is pre-clinical drug development. Drug development cost estimates range from ~$1-5 billion for a single drug [99,100], with the (capitalized) costs split evenly between pre-clinical and clinical testing. Thus, tools that can provide more accurate disease models *in vitro* and improve drug candidate selection prior to the initiation of expensive clinical trials are needed. As tissue and disease models of intermediate complexity, three-dimensional micro-tissues fill a gap in the drug-screening paradigm, bridging 2D cell culture and animal models (Figure 3.8). Many researchers have noted significant differences in drug efficacy *in vitro* between 2D and 3D tissue models.\[81,101-103\] Such pre-clinical insights have the potential to reduce drug failure rates of drug candidates later during clinical trials.
Drug development involves testing pharmaceutical candidates against a progression of increasingly complex and expensive disease models. Three dimensional cell culture provides disease models of intermediate complexity that bridge the gap between 2D cell culture and animal models.

**Figure 3.8. Drug screening paradigm.**
The adoption of 3D cell culture in routine assays and screens remains limited due to the inefficiencies and non-standardization of 3D cell culture techniques. It is estimated that less than 30% of cancer and molecular biologists utilize 3D cell culture in their research and that less than 20% of drug leads generated by the pharmaceutical industry are developed using cell-based phenotypic assays.\[104,105\] While recent advances have been made to automate the traditionally cumbersome hanging drop technique to enable high-throughput formation and screening of individual spheroids of well-defined sizes and shapes \[80\], such automated methods require the use of robotic liquid handling instruments, which, though accessible to large research organizations, can be prohibitively expensive or superfluous for smaller, academic research labs, where high-throughput experiments may not be necessary. Various other techniques utilizing 96-well plates with specialized coatings also require robotic instrumentation to benefit from automation.\[81,106\] Furthermore, the accurate dispensing and handling of sub-microliter volumes, sol-gel solutions, or particle suspension can be difficult using some types of robotic liquid handling systems.\[107\] Thus, automated, flexible, and accessible tools and technologies that facilitate the culture and assaying of three-dimensional tissues for cell-based assays and screens have great potential to promote the adoption of 3D cell culture in routine biomedical research.
Chapter 4. Digital Microfluidics for Automated Hanging Drop Cell Spheroid Culture

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Manuscript submitted for publication in Journal of Laboratory Automation and Screening, Special Issue: Microengineered Cell- and Tissue-Based Assays for Drug Screening & Toxicology Applications
4.1 Abstract

Cell spheroids are multicellular aggregates, grown in vitro, that mimic the three-dimensional morphology of physiological tissues. While there are numerous benefits to using spheroids in cell-based assays, the adoption of spheroids in routine biomedical research has been limited, in part, by the tedious workflow associated with spheroid formation and analysis. Here we describe a digital microfluidic platform that has been developed to automate liquid handling protocols for the formation, maintenance, and analysis of multicellular spheroids in hanging drop culture. We show that droplets of liquid can be added to and extracted from through-holes, or ‘wells,’ fabricated in the bottom plate of a digital microfluidic device, enabling the formation and assaying of hanging drops. Using this digital microfluidic platform, spheroids of mouse mesenchymal stem cells were formed and maintained in situ for 72 h, exhibiting good viability (>90%) and size uniformity (%CV <10%, intra-experiment, <20% inter-experiment). A proof-of-principle drug-screen was performed on human colorectal adenocarcinoma spheroids to demonstrate the ability to recapitulate physiologically relevant phenomena such as insulin-induced drug resistance. With automatable and flexible liquid handling, and a wide range of in situ sample preparation and analysis capabilities, the digital microfluidic platform provides a viable tool for automating cell spheroid culture and analysis.
4.2 Introduction

Cell spheroids are multicellular compact aggregates, grown in vitro, that have a three-dimensional (3D), spherical morphology. Unlike cells grown in two-dimensional adherent monolayers, cells grown in three dimensions possess a high degree of intercellular interactions and exhibit relatively complex nutrient and metabolic mass transport gradients. These lead to cellular heterogeneity within the 3D aggregate and to gene and protein expression patterns that more closely mimic in vivo tissues.[91,108-112] The differential expression profiles result in significant differences in cellular behaviors such as drug sensitivity, differentiation capacity, malignancy, function, and viability. For example, hepatocellular carcinoma cells grown as spheroids exhibit more physiologically relevant levels of cytochrome P450 activity and albumin secretion compared to cells grown in monolayers.[113] Another example is mammary epithelial cells, which exhibit basement membrane-induced apoptosis resistance when grown in three dimensions, but are susceptible to apoptosis in monolayer culture.[114] Because of their enhanced physiological relevance compared to monolayer cell cultures, cell spheroids can provide more accurate models for cell-based assays and screens. Improved tissue and disease models not only enhance basic research, but can be extremely valuable in commercial research, particularly in the pharmaceutical industry, where the failure rates for drug candidates entering clinical trials are typically >80%.[115-119]

Despite the known advantages of 3D cell cultures, their use in cell-based assays and screens has been limited. It is estimated that <30% of cancer and molecular biologists utilize 3D cell culture and that <20% of drug leads generated by the pharmaceutical industry are developed through cell-based phenotypic assays.[104,105] A major reason for the relatively low adoption of 3D
cell models is the limited number of user-friendly, flexible, and automated methods for performing spheroid culture and analysis.[120,121] While a variety of technologies and methods are available for culturing 3D micro-tissues, each approach has limitations that make it unsuitable for routine assays and screens.[85] Non-automated spheroid culture methods, such as the manual hanging-drop technique or the use of micro-molds, are inexpensive and relatively simple, but interrogating individual spheroids requires manual transfer to a separate vessel. Additionally, the non-automated methods often require a significant amount of manual sample handling, which can be tedious, time-consuming, and prone to variability and error.

A number of microfluidic techniques for spheroid culture have been developed that can enable high-throughput and massively parallel spheroid formation, but which do not support the interrogation of spheroids individually on the same device.[122-125] Rotary vessels and spinner flasks can also be used to generate a large number of spheroids, but provide limited control over spheroid size and also do not allow for in situ assaying of individual spheroids. Alternatively, specially engineered well plates, such as those capable of supporting hanging drop culture [80] or those with non-adhesive surfaces designed to induce cell aggregation,[106] are compatible with robotic liquid handling equipment, enabling automation and high-throughput processing. However, the ability to automate spheroid culture and analysis using these methods requires access to robotic liquid handling equipment, which can be prohibitively expensive to acquire, operate, and maintain for many research labs, particularly those in academic settings where the emphasis may not be on high-throughput experiments. Additionally, functionalities necessary for spheroid culture and analysis, such as in situ microscopy, mixing, and temperature control, require additional, often expensive, hardware to be added to the liquid handling instrument.

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Thus, there is a need for a spheroid culture and analysis technology that provides some of the advantages of automation, in a platform that is more accessible than current automated methods. We propose that digital microfluidics (DµF), a flexible and precise microfluidic liquid handling technology, can be used to automate cell spheroid culture and analysis as well as provide some unique benefits over existing automated techniques.

Digital microfluidics is a type of microfluidic platform that enables the manipulation of discrete droplets of liquid in either an air or liquid ambient medium through the spatially and temporally-controlled application of electric fields.[13,126,127] The application of an electric potential across the solid-liquid contact line generates a combination of electrostatic and/or dielectrophoretic forces, depending on the frequency of the applied field and the relative permittivities of the liquid and ambient phases. Sequentially applying an electric potential to an array of planar electrodes can enable the translation of droplets across the array,[128] and can also be used to split, merge and mix droplets.

Here we present a digital microfluidic device that enables the formation of hanging drops to allow in situ cell spheroid culture. With the ability to automate liquid handling, and with a wide range of in situ bioanalytical techniques developed for the DµF platform, DµF can ultimately provide a powerful tool for automation of spheroid-based assays and screens.

4.3 Materials and Methods

Bone marrow-derived mouse mesenchymal stem cells (ATCC® CRL-12424™) were generously donated by Prof. Tatiana Segura (UCLA). HT-29, human colorectal adenocarcinoma cells (ATCC® HTB-38™) and BJ, human foreskin fibroblasts (ATCC® CRL-2522™) were purchased
from ATCC. Leibovitz L-15 cell culture medium, Dulbecco’s Modified Eagle Medium (DMEM), penicillin-streptomycin (P/S) solution (10,000 U Pen., 10 mg Strep./mL), L-glutamine, fetal bovine serum (FBS), and the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells were obtained from Life Technologies (Carlsbad, CA). Pluronic® F-68 was purchased from Sigma (St. Louis, MO). Cytop® (CTL-809M) and CYSOLV-180 were purchased from Bellex International Corporation (Wilmington, DE). Human recombinant insulin was purchased from R&D Systems, Inc. (Minneapolis, MN) and Irinotecan HCL was purchased from BIOTANG, Inc. (Lexington, MA).

Device fabrication was conducted in the CNSI Integrated System Nanofabrication Cleanroom at UCLA. Briefly, water white glass substrates (LabScientific, Inc. CAT# 7787) were coated with 1100 Å indium tin oxide (ITO) via sputtering and were patterned with electrodes via photolithography and reactive ion etching. For this work, the substrate with the patterned electrode array was used as the top-plate and an un-patterned ITO-coated slide was used as the bottom-plate. Prior to coating with the dielectric, through-holes were manually drilled into specific locations on the bottom-plate using a benchtop drill press and diamond-coated drill bits. Through-holes were also drilled into the footprint of the reservoir electrodes in the top-plate to provide a world-to-chip interface. The top-plates were then coated with 3–4 μm of dielectric polymer parylene-C (Specialty Coating Systems) by vapor deposition. The top and bottom-plates were rendered hydrophobic by spin coating ~300–400 nm of Cytop on each. Prior to use, the walls of the wells in the bottom-plate were gently scraped with a diamond-coated drill bit to remove the Cytop coating and expose the hydrophilic glass surface. A schematic of a DμF device assembly is shown in Figure 4.1.
Figure 4.1. Device schematic and dimensions. Through-holes in the top plate allow for the addition of solutions to on-chip reservoirs, while through-holes, or ‘wells,’ in the bottom plate allow for the formation of hanging drops. Drops that are delivered to a well are drawn into the well spontaneously upon contact with the hydrophilic well wall. Addition of multiple drops to a well allows for the formation of a hanging drop with a curved air-liquid interface. Cells suspended in the drop can aggregate at this interface, forming a single spheroid within the drop.

All microfluidic liquid handling was performed using a custom LabView application to control electrode actuation. Liquid handling was performed at 100–115 V\textsubscript{pp} AC and at a frequency of 18.5 kHz. Analysis of hanging droplet liquid exchange was performed by measuring the absorption of a standardized solution of brilliant blue dye prepared in water before and after liquid exchange cycles using a Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer. The liquid exchange process is described in more detail below.

For the preparation of cell solutions for use on the DµF device, cells were thawed and seeded in polystyrene dishes in growth medium (DMEM, 4 mM L-glutamine, 10% FBS, 100 U/mL P/S solution). Cells were grown to ~80% confluency, trypsinized, and re-suspended in spheroid growth medium (Leibovitz L-15 medium, 4 mM L-glutamine, 7.5% FBS, 100 U/mL P/S, 0.04% Pluronic® F-68) at cell densities ranging from ~7.5e5 – 1e6 cells/mL for culture on the device.
Detailed schematics of the experimental setup, as used in this work, are shown in Figure 4.51 in the Supplemental Information. Prior to use, the devices were sterilized by dipping them in a 70% aqueous ethanol solution and gently drying with compressed air. For device operation, the bottom-plate was placed on an aluminum holding plate that contained a milled recess below the location of the wells to allow hanging drops to form beneath the device. The bottom-plate of the device was sealed to the aluminium plate using silicone grease (Dow Corning High Vacuum Grease). The bottom of the recess was enclosed with a glass slide to prevent exposure of the hanging drops to the laboratory environment during drop formation. To minimize evaporation, 1.5 µL of 10 cst silicone oil was pre-loaded into each well prior to the formation of hanging drops. Additionally, a small amount of water was placed in the enclosed recess to create a humidified environment. The top-plate was secured to another aluminum plate and was interfaced with the bottom-plate such that particular electrodes in the top-plate aligned with the location of the wells in the bottom-plate. The two plates were separated by a custom designed adhesive silicone spacer (Grace Biolabs, Bend, OR) to create a gap height of 300 µm and were secured using binder clips. Drops of cell-suspension were added to the reservoir electrodes via through-holes drilled into the top-plate.

Hanging drop and spheroid formation were achieved by dispensing droplets of cell suspension from the reservoir and moving the droplets to the location of a well. Upon contact with the hydrophilic wall of the well, droplets were pulled into the well via capillary forces. Addition of multiple droplets to a well resulted in the formation of a hanging drop. Exchange of the medium within the hanging drop was achieved by performing the following sequence of steps one or more times per well: (1) delivering a drop of fresh medium to a well, (2) using electrowetting
actuation to repeatedly pull out and release a liquid finger from the well to facilitate mixing of the liquid in the well, (3) extracting a drop from the well of twice the volume of the amount initially delivered, and (4) adding another drop of fresh medium to the well. Devices were kept in an incubator at 37 °C and 95% relative humidity at all times except during liquid handling.

For confocal imaging, spheroids were stained with fluorescent markers by incubation in imaging medium for 2 h at 4 °C followed by 30 min at 37 °C to ensure enhanced staining of the interior of the spheroid.[129] The imaging medium consisted of 2 μM calcein-am and 4 μM ethidium homodimer-1 (Life Technologies, LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells) in Hank’s Balanced Salt Solution (HBSS, Life Technologies) supplemented with 1 mg/mL ascorbic acid, 25 mM HEPES buffer solution – pH 7, 100 U/mL P/S, 100 μM non-essential amino acids, and 4 mM L-glutamine; 1N NaOH was used to adjust the pH to 7.2. Following staining with imaging medium, the spheroids were washed with HBSS containing 1 mg/mL ascorbic acid. A custom PDMS imaging chamber was secured beneath the bottom plate such that flooding the wells with the HBSS solution caused the hanging drops to detach from the device into individual wells within the PDMS chamber, enabling the spheroids to be imaged directly from the device. Figure 4.S1 in the Supplemental Information shows a schematic of the device interfaced with the PDMS imaging chamber.

Confocal imaging was performed using a Leica TCS SP2 confocal microscope. Spheroid images were constructed by creating a maximum projection of multiple z-plane sections spaced 3–7 μm apart. The proportion of living cells within a spheroid was estimated by counting the number of live (green) and dead (red) cells in five different, equally spaced z-planes throughout the
spheroid. ImageJ was used for all image analysis, which included hanging drop volume, as well as spheroid viability, diameter, and aspect ratio measurements.

4.4 Results and Discussion

4.4.1 Device design and operation

To enable hanging drop formation, through-holes, or ‘wells,’ were fabricated into strategic locations in the bottom-plate of the device. The schematic in Figure 4.1 shows the basic principle of DμF hanging drop formation along with typical device dimensions. Hanging drops are formed when droplets of liquid are delivered to the location of a well and, upon making contact with the hydrophilic walls of the well, get pulled into the well spontaneously via capillary forces (Figure 4.2a). Adding multiple drops to a well results in the formation of a curved liquid-air interface that protrudes beneath the bottom-plate, similar to a hanging drop (Figure 4.2b). To ensure the formation of a hanging drop, the wells were designed such that the Bond number (Bo, a dimensionless parameter describing the ratio of gravitational to surface tension forces) of the system is greater than \(~0.3\), which is within the range where gravitational forces begin to influence the shape of the meniscus.[130-132] A Bo\(\geq 0.3\) requires a well diameter of \(\geq 2.4\) mm.
Figure 4.2. Hanging drop formation on a digital microfluidic device. (a) A series of images showing a top-down view of the insertion of drops of cell media (dyed blue for enhanced visualization, ~1.2 µL) into a well on the device. (b) A series of images showing a side-view of a well after the addition of multiple drops to the well. The drops insert spontaneously into the well and, after a sufficient volume has been added, form a hanging drop with the curved interface necessary to induce cell aggregation.

To simplify device fabrication protocols, the top plate contained the actuating electrodes and the bottom-plate contained the ground electrode. While either the actuating or ground plate can be modified with through-holes and used as the bottom-plate to support hanging drop formation, we found that incorporating the wells into the plate containing the actuating electrodes was more difficult because the holes needed to be drilled precisely within the footprint of an electrode, which occasionally resulted in damaging the electrode. Additionally, decoupling the wells and
actuating electrodes allows for the actuating top-plate to be removed and replaced in the case of dielectric breakdown, without disrupting the hanging drops in the wells in the bottom-plate.

Dielectric breakdown occurs when the electric field across the dielectric layer exceeds the dielectric strength of the material, resulting in localized, physical destruction of the dielectric layer. Dielectric breakdown typically occurs on the actuating plate, where charges within the drop and electric field lines outside the drop concentrate near the droplet edge closest to the actuated electrode, creating a region of locally elevated electric field strength.\[12,20,133\] If the degree of dielectric breakdown is minor, droplets can still be transported normally across the location of the breakdown. Significant dielectric breakdown can cause electrolysis of aqueous solutions as a result of current flow into the drop, and can also damage critical electronic connections on the device, thereby impeding droplet movement (“pinning”). For the top-plates used in this work, the entire spheroid culture process, which required ~800-1200 total electrode actuation steps for the culture of 6-8 spheroids, could typically be achieved without the occurrence of dielectric breakdown. Approximately one out of every four devices showed evidence of dielectric breakdown at some point during the culture protocol, typically during the 48 h medium exchange process, i.e., after the hanging drops had been formed and all the cell handling had been completed. Because the spheroids were maintained in hanging drops beneath the bottom plate and were relatively far from either of the interior surfaces of the top- and bottom-plates, dielectric breakdown did not disrupt or affect the spheroids within the hanging drops.
To allow visualization of droplet handling, the actuating electrodes in the top plate were made from a transparent conductive material, indium tin oxide (ITO). Videos of liquid handling and hanging drop formation are provided in the Electronic Supplemental Information.

The wells in the bottom plate contain a tapered opening on the top side to aid in the insertion of drops into the well. Droplets that reach the edge of a well can experience canthotaxis, or pinning at the intersection of two interfaces, due to both the change in geometry at the well edge and the difference in surface properties between the hydrophobic surface of the bottom plate and the hydrophilic interior of the well walls. By tapering the inside walls of the well to form an acute angle with the surface of the bottom plate, as opposed to a right angle formed by a cylindrical through-hole, the pinning effect on a drop of liquid at the edge of the well is reduced, facilitating droplet insertion into the well.

The volume of a hanging drop is determined by the number of drops dispensed from a reservoir and added to a well. Thus, the volume and reproducibility of droplet dispensing from the reservoirs are critical to the volume and reproducibility of the hanging drops. To determine the variation in dispensed drop volumes, we used image analysis to measure the volumes of 144 drops of an aqueous surfactant solution (0.04% Pluronic® F-68) dispensed from different reservoirs across three different devices using a programmed dispensing sequence. An aqueous surfactant solution was used so that the surface tension of the liquid and, consequently, the volume of the dispensed drops, would be similar to that of the growth medium solution subsequently used in the cell culture experiments, which also contained 0.04% Pluronic® F-68. The dispensed droplet volume was determined by measuring the area of the drop in contact with
the top-plate using device features of known dimensions as a scale, and multiplying by the known distance of the inter-plate gap. While in actuality the sidewalls of the droplet are curved, to simplify the volume measurements we used the straight-wall, cylindrical approximation to calculate droplet volumes, which is a reasonable approximation considering the relatively small droplet aspect ratio on our devices (h/w ~0.15) and the contact angle of approximately 100°.[134] The distribution of droplet volume measurements is shown in Figure 4.3. The average volume of a single dispensed drop was 1.75 ± 0.13 µL (7.7% CV; %CV = coefficient of variation). This degree of droplet volume variation is consistent with reproducibility values from other electrowetting devices that do not utilize capacitance metering to control dispensing volumes. Droplet dispensing reproducibility can be improved to low single-digit %CV by employing capacitance metering methods or by optimizing reservoir and dispensing electrode design.[135-137] The variation in the volumes of hanging drops was also determined (Figure 4.4). Hanging drops formed from four, five, and six dispensed drops had average volumes of 7.4 ± 0.5 µL, 8.8 ± 0.8 µL, and 10.2 ± 0.5 µL, respectively, corresponding to %CV range of 5–9%. This volume range was chosen because, for the devices used in this work, at least four drops are required to fill a well and form the curved surface necessary for cell aggregation.
Figure 4.3. Distribution of dispensed drop volumes. 144 drops were dispensed from different reservoirs across 3 different devices using a pre-programmed droplet dispensing sequence. The average drop volume was 1.75 μL and the %CV of the volume of all drops dispensed was ~8%.

Figure 4.4. Size and variation of hanging drop volumes. Hanging drops comprised of four, five, and six dispensed drops had volumes of 7.4 ± 0.5 μL, 8.8 ± 0.8 μL, and 10.2 ± 0.5 μL, respectively (N = 8 hanging drops formed for each condition). For each condition, the variation in hanging drop volume was <10%. Hanging drops of volumes up to ~55 μL can be formed on the devices used in this work, however, only 7-10 μL is needed to form cell spheroids via the hanging drop technique.
It should be noted that the volume and reproducibility data shown here represent results from a particular dispensing sequence and device arrangement (i.e., gap height = 300 µm). Various droplet volumes can be dispensed on a DµF device by simply altering the gap height and/or changing the dispensing sequence. The devices used in this work support the formation of hanging drops up to ~55 µL before the drops detach from the well due to their weight. We observed that hanging drops of larger volumes can be supported by varying the thickness of the bottom plate, the well geometry, or the surface tension of the liquid comprising the drop (determined experimentally, data not shown).

Conventional spheroid culture protocols call for ~50% medium exchange every 48 h for optimal growth.[81,138] Thus, to enable long term hanging drop spheroid culture, protocols for in situ medium exchange using digital microfluidic liquid handling were developed. Medium exchange requires extracting the spent medium from a hanging drop and replacing the spent medium with fresh medium. Liquid can be extracted from a hanging drop by using the electrodes adjacent to a well to pull out a drop of liquid. Repeating the process of extracting and adding drops of medium to a well, as described in the Materials and Methods section, results in the exchange of the medium within the well. Assuming the hanging drop initially contains the volume of four dispensed drops, the medium exchange protocol theoretically allows for exchange of 40% and 64% of the initial drop volume after one and two exchange cycles, respectively (according to the dilution rate for this particular exchange protocol: C = 0.6^n, where C = the concentration of spent medium in the drop, and n = the number of exchange cycles). The video “Hanging Drop Liquid Exchange” in the Electronic Supplemental Information shows two cycles of the liquid exchange protocol. Using a hanging drop of a standardized brilliant blue dye solution to mimic spent
medium and DI water to represent fresh medium, we assessed the degree of exchange by measuring the change in dye concentration of the hanging drop after successive exchange cycles by visible spectrophotometry. Figure 4.5 shows that the dye concentration calculated from UV-Vis absorption are consistent with the theoretical predictions, indicating that >50% medium exchange can be achieved with one or more exchange cycles. These data also indicate that DµF can provide precise control over the composition of the hanging drop, which is critical for performing cell-based assays and screens.

![Liquid in Well:](image)

**Figure 4.5. Extent of liquid exchange, predicted and experimental results.** The extent of liquid exchange after one and two exchange cycles was monitored by measuring the change in absorbance of the dyed hanging drop solution and calculating the concentration from a standard curve. The dilution of a hanging drop after each cycle can be seen in the images above the plot. The agreement between the measured concentrations and the predicted values indicates that thorough mixing of the hanging drop is achieved during each exchange cycle and that DµF provides good control over the composition of the hanging drop. Error bars indicate the standard deviation of measurements from three different experiments.
4.4.2 Cell Spheroid Culture

After establishing the ability to form a hanging drop and conduct medium exchange, a complete cell spheroid culture protocol was performed to demonstrate proof-of-principle for fully automated DμF cell spheroid culture. Droplets of mouse mesenchymal stem cell (MSC) suspension in growth medium were delivered to wells to form hanging drops of ~7-10 μL (~5250-7500 cells/drop). Pluronic® F-68 was included in the growth medium to minimize the adsorption of proteins to the hydrophobic surface of the device, which can impede the movement of proteinaceous solutions.[139] At 0.04%, Pluronic® F-68 is known to be non-cytotoxic.[140] Leibovitz L-15 medium was used for spheroid culture because it is buffered by phosphates and free-base amino acids instead of sodium bicarbonate. This medium allows cell growth in the absence of a controlled CO₂ atmosphere; our current digital microfluidic setup is operated outside of an incubator at ambient atmospheric conditions. During liquid handling, the microfluidic apparatus was kept at ~37 °C by placing a thin-film polyimide heater in contact with the aluminium device holder. After liquid handling, devices were transferred to an incubator at 37 °C and relative humidity of 95%. To prevent fluctuations in atmospheric conditions between the liquid-handling and incubation periods, the incubator was also maintained at ambient atmosphere (i.e., without CO₂ control).

Medium exchange was performed once daily. During culture, the spheroid sits at the bottom of the hanging drop, which is ~1.8 mm below the top opening of the well (assuming a 7.4 μL drop in a 2.5-mm diameter well). Because liquid from the drop is extracted from the top opening of the well and medium exchange never requires extraction of more than 25% of the initial hanging
drop volume, the spheroid remains settled within the hanging drop throughout the medium exchange protocol and does not get extracted from the well.

Figure 4.6a shows confocal micrographs of typical spheroids of mouse mesenchymal stem cells cultured on the DµF device over the course of 72 h using automated sample handling protocols. The spheroids were stained with calcein-AM and ethidium homodimer-1 to indicate living (green) and dead (red) cells, respectively. Counting the number of living and dead cells at various z-planes within the spheroid indicated that the spheroids exhibited >90% cell viability. The spheroid diameter was measured at 24, 48 and 72 h (Figure 4.6b) following hanging drop formation using a USB-microscope (Dino-Light AD4013TL). A seeding density of 7.5x10^5 cells/mL produced spheroids of up to ~400 µm after 72 h in culture. The size and viability of the spheroids generated on the DµF platform are consistent with those obtained through other hanging drop techniques over the same timeframe using similar cell number conditions.[80] Intra-device spheroid diameter variation was ~8%; this is comparable to other hanging drop techniques, which exhibit a %CV range of ~3% (for robotic liquid handlers) to 15% (for manual methods), and is superior to spheroid generation on non-adhesive flat-bottom well plates, which show spheroid diameter variation of up to 40–60%.[141,142] Because the cell density is the same for each hanging drop, the intra-experiment spheroid diameter variation is attributable to the variation in the volumes of the hanging drops. The inter-device variation in spheroid diameter (i.e., for spheroids grown on different devices) was 14%, 18%, and 18% for spheroids at 24, 48, and 72 h, respectively (Figure 4.6b). The relatively larger inter-experiment variation compared to the intra-experiment results is likely due to variations in cell densities between the different experiments. For this work, cell suspensions of ~7.5x10^5 cells/mL were prepared based
on hemocytometer measurements, which can exhibit variability of 10-40% depending on cell concentration.[143,144] More precise cell-density measurement techniques, which can achieve a %CV of <3%,[145] would reduce the inter-experiment spheroid diameter variability. Figure 4.6c shows the distribution in spheroid aspect ratio (ratio of spheroid major axis to minor axis) for 77 spheroids of various cell numbers and types. The average aspect ratio for the spheroids cultured on the DµF platform was 1.15 ± 0.09, corresponding to a CV of ~8%. The spheroid aspect ratio was measured after at least 48 h in culture to allow spheroid compaction to occur. Table 4.1 summarizes performance characteristics for spheroid culture conducted on a DµF device.
Figure 4.6. Cell spheroids formed by DµF liquid handling  (a) Representative images of spheroids of mouse mesenchymal stem cells (mMSC) grown on a digital microfluidic device after 24, 48, and 72h of in situ incubation. Each image is of a different spheroid. Spheroids exhibit >90% viability during this time-frame as determined by staining with calcein-AM/ethidium homodimer-1 to visualize living (green) and dead (red) cells.  (b) The spheroids of mMSC formed from cell suspensions of ~7.5e5 cells/mL had diameters of 249±34 µm after 24 h (N=12 spheroids), 327±58 µm after 48 h (N=10 spheroids), and 425±75 (N=8 spheroids) after 72 h in culture. The data here represent average spheroid sizes at each time point from 6 separate experiments.  (c) The distribution and average aspect ratio of spheroids grown on the DµF platform (N=77 spheroids). The data represent measurements from HT-29 colorectal carcinoma spheroids, BJ fibroblast spheroids, and mouse MSC spheroids with diameters ranging from 100 100 h (N=12 solid line indicates the average aspect ratio value (1.15), the dashed lines indicate +/- one standard deviation (0.09).
Table 4.1. Performance characteristics of DµF devices used in this work

<table>
<thead>
<tr>
<th>Performance Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanging drop volume reproducibility</td>
<td>~8%</td>
</tr>
<tr>
<td>Maximum hanging drop volume</td>
<td>~55 µL</td>
</tr>
<tr>
<td>Intra-experiment spheroid diameter variation</td>
<td>~8%</td>
</tr>
<tr>
<td>Inter-experiment spheroid diameter variation</td>
<td>~16%</td>
</tr>
<tr>
<td>Spheroid size (diameter) range</td>
<td>50-700+ µm</td>
</tr>
<tr>
<td>Average spheroid aspect ratio</td>
<td>1.15 ± 0.09</td>
</tr>
</tbody>
</table>

a. Average %CV of droplet volume for hanging drops.
b. Volume range specific to devices used for this work. Max volume = volume above which droplets detach from the device; determined experimentally.
c. Average % CV of spheroids formed on the same chip. N = 30 total spheroids across eight different experiments.
d. Average % CV of spheroids formed on different chips. N = 30 total spheroids across 8 different experiments.
e. Values represent the smallest and largest spheroids obtained over a period of 24-72 h on the DµF devices. These values do not represent minimum or maximum possible sizes.
f. Aspect ratio = ratio of spheroid’s major axis to minor axis. N = 77 spheroids of different cell types.

With the ability to initiate and maintain viable spheroids in culture as well as freely add, mix, and extract liquid from a hanging drop, the DµF platform enables automation of spheroid-based assays and screens. To demonstrate this capability, we performed a proof-of-principle spheroid-based drug screen, using DµF to examine the impact of insulin exposure on the chemosensitivity of colon cancer cells to treatment with the chemotherapeutic agent irinotecan. Insulin has been shown to cause resistance to chemotherapy in certain colon cancer cell lines via activation of the PI3K/Akt pathway.[146,147] For the drug screening assay, hanging drops of HT-29 human colon adenocarcinoma cells were initiated and maintained on a DµF device for 48 h to allow for the formation of compact spheroids. After 48 h of culture, the medium for some spheroids was exchanged for medium containing 500 nM insulin, while the remaining spheroids received normal growth medium. The insulin-induced drug resistance effect has been observed in HT-29 cells in vitro at insulin doses of 100-1000 nM.[147,148] Spheroids were allowed to incubate in
their respective medium for 24 h after which the medium was exchanged for medium containing 100 μM irinotecan, or, for controls, normal growth medium. Previous studies had shown that HT-29 spheroids exhibit ~20-50% cell death upon exposure to 100 μM irinotecan.[81,101,149] The DµF drug screening assay workflow is depicted in Figure 4.7a. To evaluate drug toxicity, the diameter of each spheroid was measured at 48, 72, and 96 h. Figure 4.7b shows the average normalized diameter (the ratio of spheroid diameter at 96 h to the diameter at 48 h) of the spheroids for the different assay conditions. Spheroids that received just the drug treatment exhibited a ~20% decrease in diameter, while those that were exposed to insulin prior to drug treatment did not exhibit any decrease in size. These results are consistent with the insulin-induced drug resistance effect observed in HT-29 cells in vitro and, to our knowledge, represent the first time this effect has been demonstrated using a three-dimensional HT-29 colon cancer model.
Figure 4.7. Spheroid-based drug screening protocols and results. (a) Diagram illustrating the workflow for a spheroid-based drug screen performed on human colorectal adenocarcinoma cells (ATCC HT-29). After 48 h of compaction, spheroids received either normal or insulin-containing medium. After another 24 h, the spheroids received either normal or drug-containing (irinotecan) medium, and were incubated for another 24 h. (b) Comparison of the normalized spheroid diameter ($D_{96h}/D_{48h}$) for spheroids exposed to the different drug screen assay conditions. The error bars indicate +/- one standard deviation from the average for each condition (medium + medium: N = 4, insulin + medium: N = 4, medium + drug: N = 6, insulin + drug: N = 8).

Another interesting result of the colon cancer spheroid-based drug screen was the formation of ‘colonospheres’: spherical structures composed of several colonic mucosal epithelial cells that appear as rounded-off epithelial cysts.[150] The colonosphere morphology signifies a reorganization from a spherical aggregate into one that more closely mimics the morphology of the colon epithelium, which contains numerous glandular and crypt structures. Figure 4.8 shows examples of colon cancer spheroids exhibiting the colonosphere morphology. This phenotype is
of particular interest in cancer research, as literature suggests that colonospheres exhibit a relatively high proportion of cells with a cancer-stem-cell phenotype, which is critical to tumor formation and growth.[151]

![Colonosphere morphologies](image)

**Figure 4.8. Colonosphere morphologies.** HT-29 colon adenocarcinoma spheroids exhibited ‘colonosphere’ morphologies after 96 hours of in-vitro hanging drop culture on a DµF device. Images (a)-(c) are confocal projections of spheroids showing the development of glandular or crypt-like folds, similar to the morphology of the colon epithelium. Image (d) is a confocal cross-section image taken at a z-plane ~75 µm into the spheroid interior. The image shows the large lumen/crypt structure that is indicative of colonosphere morphology. The spheroids in these images are stained with calcein-AM/ethidium homodimer-1 to visualize living (green) and dead (red) cells. Scale bars correspond to 200 µm.

The work presented here advances on previous DµF cell culture studies that established the ability to seed and maintain cells in adherent monolayer culture on a DµF device over an extended period of time.[49,152-154] Those studies confirmed that the electric fields used to drive droplet movement have negligible detrimental impact on cell viability, and developed protocols for the manipulation of complex biological solutions. Other work has demonstrated
the ability to encapsulate a suspension of cells within hydrogel posts between the plates of a DµF device.[52,53,155] The encapsulation of cells within hydrogel posts provides a useful tool for modelling cell-matrix interactions, which are key to understanding the cellular microenvironment and important physiological processes such as the epithelial-mesenchymal transition.[156] However, while the use of hydrogel posts enables cell growth in three dimensions, there are certain limitations associated with these techniques. When using inter-plate gel posts, the thickness of the cell aggregates within the gel is limited to the thickness of the gap between the plates of the digital microfluidic device (typically ≤ ~300 µm). Additionally, when cells suspensions are encapsulated in gel-posts, the cells are randomly distributed throughout the gel, providing little control over the size and morphology of the aggregates that form. Lastly, these methods require the use of a scaffold or matrix to support 3D cell culture; in some cases, this can be disadvantageous, because the scaffold materials may require extra sample preparation steps, can be expensive, are susceptible to lot-to-lot variability, may consist of non-physiological materials, can complicate sample recovery/analysis, can restrict the movement of cells or nutrient transportation, can interfere with screening compounds, and may not allow recapitulation of processes that rely on a high degree of cell-cell interactions such as embryogenesis, morphogenesis, or tumorigenesis.[97,157-160] The platform described here allows scaffold-free three-dimensional cell culture. That said, because solutions can be freely added to or extracted from a hanging drop, cell suspensions or compact spheroids within a hanging drop could be encapsulated in a scaffold material if desired. This platform also enables the growth of spheroids that exceed the thickness of the inter-plate gap of a DµF device, allowing for the formation of spheroids that exhibit physiologically relevant morphologies specific to large aggregates, such as the development of a necrotic, hypoxic core that can occur
within spheroids >400-500 µm in diameter.[85,121,161,162] Because not all cell types form spheroids, and because the behavior of individual cells or small cell clusters encapsulated within an extracellular matrix can provide interesting physiological insights, the hanging-drop and gel-post techniques for three-dimensional cell culture on a DµF device are complementary.

The platform described here also provides a number of unique advantages compared to existing spheroid culture techniques. The primary advantages of the DµF system are automation and the flexibility of the liquid handling protocols. By automating liquid handling, digital microfluidics can enable increased throughput and minimize hands-on time compared to manual spheroid culture methods, potentially reducing variability and human-error in spheroid culture and assay protocols. Digital microfluidics also allows droplets to be manipulated either sequentially or simultaneously and droplet handling can be pre-programmed for complete automation, or can be controlled in real-time allowing for assay flexibility and reconfigurability. Because DµF provides temporal and spatial control over the handling of discrete drops of liquid, any type of solution can be added to or extracted from any particular well at will. Thus, spheroids can be exposed to a wide variety of stimuli such as drug candidates, different cell types, differentiation factors, genetic modulators, and cell secretions in a highly controlled fashion. Additionally, because liquid movement on a DµF platform is not confined to channels, liquid can be freely exchanged from one hanging drop to another, allowing controlled communication between different spheroids on a device. The ability to extract solution from a well allows for in situ or ex situ analysis of secretions or extracellular conditions from distinct spheroids at any point throughout the spheroid culture. This precise control over the composition and analysis of the spheroid microenvironment is difficult or impossible to achieve using other spheroid culture
techniques. For example, while flow-based microfluidic techniques are advantageous for massively parallel and/or high throughput spheroid culture protocols, such methods are non-ideal for assays that require flexible or reconfigurable liquid handling or precise and selective control over the microenvironment of individual spheroids. Likewise, microarray or micro-well techniques, in which cells passively aggregate in defined locations on a patterned substrate, allow for high-throughput and uniform spheroid formation, but do not allow for compartmentalization or interrogation of individual spheroids.

While robotic liquid handling systems do allow for automation of spheroid culture and analysis, digital microfluidics enables unique liquid handling capabilities that are difficult or impossible to achieve using robotic liquid handling. For example, digital microfluidics allows for the interrogation of hanging drops either individually or in parallel, enables handling of very small volumes of liquid (pL–µL),[24,163] allows for magnetic or dielectrophoretic sorting of cells or beads,[40,41,56,164] enables programmable and spatially controlled heating of individual or multiple locations,[32] supports rapidly sequential delivery of reagents to single or multiple locations,[165] allows for in situ electrochemical detections,[36,43,166] and allows for the formation of hydrogels with controllable geometry and orientation.[52,167,168] Additionally, a wide range of bioanalytical capabilities including mass spectrometry sample preparation,[31] PCR,[169] qPCR,[47] immunoassays,[54] surface plasmon resonance imaging,[170] and fluorescence imaging,[55] have been developed for the DµF platform, providing in situ analytical and multiplexing functionalities that could be challenging to incorporate into a robotic liquid handling spheroid-culture workflow.
The DµF platform described here does have certain limitations compared to other automated spheroid culture techniques. The primary limitation of the system is the relatively low throughput compared to robotic liquid handling systems. Because all of the liquid handling in digital microfluidics is performed in the same two-dimensional plane, the device must accommodate both the wells and the transportation electrodes, which limits the number of wells that can be placed on a device. By contrast, the liquid handling path for robotic liquid handling systems usually occurs on a different plane than the well-plate, allowing the wells to be packed closer together. Thus digital microfluidics cannot achieve the same well density that is possible using hanging-drop well-plates, and is best suited for research environments in which medium-throughput processing is sufficient. While the prototype devices used in this work are limited to 46 actuating electrodes, which enables the formation of up to eight hanging drops, a more advanced DµF setup, such as the DropBot,[171] an open source DµF hardware and software system which allows for hundreds of individually addressable electrodes, would enable >50 spheroids to be maintained and addressed on a single device. Although this well density is considerably lower than commercially available 96- or 384 hanging drop well plates (3DBiomatrix Inc., InSphero), operating multiple DµF devices simultaneously would increase the throughput. Digital microfluidics also operates at lower working volumes than other automated spheroid culture methods (7–12 µL hanging drops on this DµF platform compared to 20–30 µL for a 384 well hanging drop plate[80]), which, while advantageous in some respects, can also present challenges. Specifically, smaller drops are more susceptible to evaporation, which can alter the composition of the hanging drop. Smaller hanging drops also require a higher cell density than larger hanging drops to achieve a spheroid of the same size. Working with higher cell densities necessitates more precise liquid handling as spheroid size is related
directly to hanging drop volume and cell density. Additionally, hanging drops with higher cell density require more frequent medium exchanges. These challenges, however, can be mitigated by employing humidity controls to minimize droplet evaporation, droplet dispensing control techniques to provide precise control over hanging drop volumes, and programmed liquid handling sequences to automate medium exchange. Lastly, DμF devices are susceptible to dielectric breakdown during prolonged operation, which can interfere with assay procedures. However, dielectric and hydrophobic material selection and deposition techniques are active areas of research in digital microfluidics and many design parameters, such as the materials, thicknesses, and organization of the dielectric and hydrophobic layers, as well as the ambient medium (i.e. air vs. oil) and operating voltage and frequency can be optimized to minimize the chance of dielectric breakdown.[172-175] The performance of optimized devices can support at least 25,000 droplet actuation steps without dielectric breakdown, which is sufficiently reliable for commercial applications.[48]

The work presented here demonstrates that digital microfluidics, with highly flexible and automated liquid handling capabilities, and compatibility with a variety of in situ analytical techniques, has the potential to serve as a powerful tool for automated cell spheroid culture. Ultimately, a digital microfluidic platform that facilitates cell spheroid culture and analysis may help increase adoption of three-dimensional cell-based assays and screens in routine biomedical research.

4.5 Acknowledgements

The authors thank Professor Heather Maynard (UCLA, Dept. of Chemistry and Biochemistry) for providing access to cell culture facilities and equipment. All confocal laser scanning
microscopy was performed at the CNSI Advanced Light Microscopy/Spectroscopy (ALMS) Shared Resource Facility at UCLA, supported with funding from NIH-NCRR shared resources grant (CJX1-443835-WS-29646) and NSF Major Research Instrumentation grant (CHE-0722519). The authors also thank Dr. Matthew J. Schibler (ALMS Shared Resource Facility) for assistance with the confocal microscopy performed for this work. This work was supported by NSF-DGE UCLA IGERT Materials Creation Training Program (MCTP) [0654431], institutional funds, and a Dissertation Year Fellowship (to APA).

4.6 Supplemental Information

Figure 4.S1. Cross-section schematic of the digital microfluidic setup used in this work. The schematic shows how the devices were assembled to enable hanging drop formation and culture. Silicone oil is pre-seeded into each well to engulf the hanging drops and mitigate evaporation during incubation. A small amount of aqueous buffer is placed in the enclosed recess below the hanging drops to provide a humidified environment, which also helps minimize evaporation. The schematic illustrates the device with the PDMS imaging chamber secured below the bottom-plate and sealed with a glass coverslip. For confocal imaging, the wells were flooded with imaging medium so that the hanging drops would detach from the wells and fall into their respective compartments within the PDMS imaging chamber. Spheroids could then be imaged directly from the aluminum plate. The schematic is not drawn to scale. Dimensions are provided for reference.
Figure 4.S2. Top-down schematic of DμF device. Schematic showing a top-down perspective of the electrode array layout for the devices used in this work. The devices enabled formation of up to 8 individually addressable hanging drops. The schematic illustrates hanging drops containing spheroids within the wells on the device.
Chapter 5. Digital Microfluidics for Spheroid-Based Invasion Assays

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5.1 Abstract

Cell invasion is a key process in tissue growth, wound healing, and tumor progression. Most invasion assays examine cells cultured in adherent monolayers, which fail to recapitulate the three-dimensional nuances of the tissue microenvironment. Multicellular cell spheroids have a three-dimensional morphology and mimic the intercellular interactions found in tissues in vivo, thus providing a more physiologically relevant model for studying the tissue microenvironment and processes such as cell invasion. Spheroid-based invasion assays often require tedious, manually intensive handling protocols, or the use of robotic liquid handling systems, which can be expensive to acquire, operate, and maintain. Here we describe a digital microfluidic (DµF) platform that enables formation of spheroids by the hanging drop method, encapsulation of the spheroids in collagen, and the exposure of spheroids to migration-modulating agents. Collagen sol-gel solutions up to 4 mg/mL, which form gels with elastic moduli up to ~50 kPa, can be manipulated on the device. In situ spheroid migration assays show that cells from human fibroblast spheroids exhibit invasion into collagen gels, which can be either enhanced or inhibited by the delivery of exogenous migration modulating agents. Exposing fibroblast spheroids to spheroid secretions from colon cancer spheroids resulted in a >100% increase in fibroblast invasion into the collagen gel, consistent with the cancer-associated fibroblast phenotype. These data show that DµF can be used to automate the liquid handling protocols for spheroid-based invasion assays and create a cell invasion model that more closely mimics the tissue microenvironment than traditional, two-dimensional techniques. Ultimately, a DµF platform that facilitates the creation and assaying of three-dimensional, in vitro tissue models could make automated 3D cell-based assays more accessible to life sciences researchers.
5.2 Introduction

The invasion of cells into their surrounding environment is an essential process in wound healing, tissue growth, and tumor metastasis.[176,177] Cell migration and invasion is dictated by the complex communication and signalling pathways between cells and the extracellular matrix (ECM) of the tissue microenvironment. The nature and extent of this invasion are controlled by multiple parameters, including cell type, matrix type, matrix stiffness, and soluble extracellular signalling cues.[177-179] In vitro assays that can model the complex, three-dimensional cellular microenvironment can yield valuable insights into cellular migration and invasion, and facilitate the discovery and development of treatments and therapeutics for many types of injury and disease.

Most cell migration assays are currently performed using systems that rely on the motility of single or adherent cells. For example, in transwell assays, adherent cells migrate through pores in a membrane in response to a stimulus gradient.[180,181] Another common migration assay known as a “scratch test” measures the degree to which adherent, monolayer cells migrate into a void created on a substrate by the physical removal of a region of cells, or by otherwise preventing growth in the void region.[176] While these systems provide valuable insight into cell migration, they do not take into account the complex conditions found in three-dimensional tissues in vivo, such as cell signalling associated with intercellular interactions, the epithelial-mesenchymal transition, the cellular heterogeneity that exists within 3D aggregates, or migration mechanisms specific to a 3D microenvironment.[98,176,182-185]
Spheroid-based cell migration and invasion assays have been developed to provide more physiologically relevant models.[176,181,186] These assays typically use 3D cell spheroids encapsulated within a hydrogel matrix. Cell invasion can be studied by introducing chemical or physical stimuli to modulate cell motility, which can be quantified in situ by microscopy. These assays typically require multiple manual pipetting steps,[180] rendering them labor intensive, prone to variability and human error, and difficult to scale. Complex and laborious assay protocols are major factors limiting the adoption of more advanced invasion assays.[180]

Here we describe a digital microfluidic (DμF) platform that is capable of automating all the liquid handling steps necessary for performing spheroid-based invasion assays. Our previous work developed the capability to perform automated hanging drop cell spheroid culture on a DμF device.[187] In this work, we advance upon that technique by developing protocols for the encapsulation and maintenance of spheroids within a collagen hydrogel to study cell migration and invasion.

Digital microfluidics is a liquid handling technique that enables the manipulation (dispensing, translating, splitting, and mixing) of discrete pico- to microliter droplets of liquid on a planar array of electrodes through the controlled application of electric fields.[188-190] Because digital microfluidics enables automation of complex assay workflows, DμF systems have been used for a variety of biological applications [191] such as on-chip cell culture,[192] hydrogel formation,[52,53,193,194] and cell spheroid culture and analysis.[187,195] By automating liquid handling, DμF can reduce or eliminate the tedium associated with manual assay protocols. Additionally, DμF can provide lower sample volume requirements, reduced costs, and superior
liquid handling flexibility compared to other automated techniques, such as robotic liquid handling.[180]

To demonstrate the ability of DµF to enable spheroid-based invasion assays, we used DµF to encapsulate spheroids of human fibroblast cells in hanging-drops of collagen gel. As a key component of the human stroma, fibroblasts play a central role in wound healing and tumor progression and are commonly used to model cell migration and invasion. Through the delivery of migration modulating agents to individual spheroids, we show that gel-encapsulated spheroids can be interrogated and assayed in situ. To demonstrate a physiologically relevant invasion model, we show that DµF can be used to simulate paracrine signalling in the tumor microenvironment, providing a convenient method to model tumor-stromal communication and the cancer-associated-fibroblast (CAFs) phenotype in vitro. Cancer-associated-fibroblasts are stromal fibroblasts that are ‘activated’ by chemical and mechanical signalling in the tumor milieu. These activated fibroblasts are crucial to establishing the structural and biochemical properties of the tumor microenvironment, and have been implicated in promoting tumor invasion and metastasis.[196,197] Thus, an in vitro model for studying the properties and behaviors of CAFs would be a valuable tool in cancer research.

By automating sample handling and reducing the requirement for manual intervention, the DµF platform has the potential to lower the barriers to adoption for more advanced, spheroid-based migration assays. Ultimately, greater adoption of more physiologically relevant, three-dimensional cell-based assays can yield new insights into homeostasis and morphogenesis for both healthy and diseased tissues.
5.3 Materials and Methods

5.3.1 Materials and Reagents

HT-29 human colorectal adenocarcinoma cells (ATCC® HTB-38™) and BJ human foreskin fibroblasts (ATCC® CRL-2522™) were purchased from ATCC. Leibovitz L-15 cell culture medium, Dulbecco’s Modified Eagle Medium (DMEM), penicillin-streptomycin (P/S) solution (10,000 U/mL Pen., 10 mg/mL Strep.), L-glutamine, fetal bovine serum (FBS), and the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells were obtained from Life Technologies (Carlsbad, CA). Bovine collagen I solutions were purchased from Trevigen, Inc. (Gaithersburg, MD; Cat. #: 3442-005-01) and Corning Life Sciences (Tewksbury, MA; Prod. #: 354231). Hoechst 33342 solution was purchased from Fisher Scientific (Pittsburgh, PA). Acti-stain 488 phalloidin was purchased from Cytoskeleton Inc. (Denver, CO). Human bone morphogenetic protein 2 (BMP-2) and Pluronic® F-68 were purchased from Sigma (St. Louis, MO). Prostaglandin E2 (PGE2) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cytop® (CTL-809M) and CT-SOLV 180 were purchased from Bellex International Corporation (Wilmington, DE).

5.3.2 Device Fabrication and Operation

The DμF devices used in this work consisted of two separate plates: a top plate that contained an array of driving electrodes, and a bottom plate that contained a continuous ground electrode and the through-holes necessary for spheroid formation. The devices were fabricated according to previously described protocols in the California NanoScience Institute (CNSI) Integrated System Nanofabrication Cleanroom at UCLA.[187] Briefly, the top-plate substrates (75 x 50 x 1 mm water white glass slides - LabScientific, Inc., Cat# 7787) were sputter-coated with 110 nm of
indium-tin-oxide (ITO) to provide a conductive surface. The ITO was then patterned with the array of driving electrodes using standard photolithography techniques. Through-holes were manually drilled into the footprints of the reservoir electrodes on the top-plates to provide a world-to-chip interface that allowed for the addition of liquid to the devices. The top-plates were subsequently coated with 4-6 μm of Parylene-C via vapor deposition and ~400 nm of Cytop via spin-coating.

The bottom plate of the DµF device consisted of a 1.7 mm thick soda lime glass slide with through-holes, or ‘wells,’ drilled into specific locations within the plate. The bottom-plate was similarly coated with a layer of ITO and Cytop to produce a hydrophobic ground electrode. The Cytop was removed from the well walls by physical abrasion in order to expose the underlying glass, rendering the wells hydrophilic.

A schematic of the DµF device setup used in this work is shown in Figure 5.1. For device operation, the bottom plate was placed on an aluminum holding plate that contained a milled window below the wells to allow hanging drops to form. A glass slide was placed underneath to create an enclosure that would prevent exposure of the hanging drops to the laboratory environment during drop formation and spheroid culture. A small amount of aqueous buffer was placed in the enclosed recess beneath the wells to create a humidified environment. To minimize evaporation, 1.2 μL of 10-cst silicone oil were pre-loaded into each well prior to the formation of hanging drops. The top-plate was secured to another aluminum plate and was interfaced with the bottom-plate such that particular electrodes in the top-plate aligned with the location of the wells in the bottom-plate. The two plates were separated by a custom designed silicone spacers (Grace
Biolabs, Bend, OR) to create a gap height of 300 μm and were secured using binder clips. Droplets of cell suspension were added to the reservoir electrodes via through-holes drilled into the top-plate. Droplet actuation was achieved using an AC potential of 100-120Vpp at 18 kHz.

Figure 5.1. Digital microfluidic device for spheroid culture. To enable spheroid culture, through-holes, or wells, were fabricated into the bottom plate of the DµF device. Droplets of cell-suspension were dispensed and delivered to the location of the wells where they were pulled into the wells spontaneously via capillary forces. Adding multiple drops to a well resulted in the formation of a pendant drop, allowing the cells to settle into an individual, compact spheroid at the bottom of the drop. Aqueous buffer was placed beneath the hanging drops to create a humidified environment for long-term hanging drop culture.

5.3.3 In Situ Spheroid Culture

Cell solutions of either HT-29 human colorectal adenocarcinoma cells or BJ human fibroblasts were used to form cell spheroids. To prepare the cell suspensions, cryopreserved cell stocks were thawed and seeded in polystyrene dishes in growth medium (DMEM, 4 mM L-glutamine, 10% FBS, 1% P/S solution). Cells were grown to ~80% confluency, trypsinized, and re-suspended in spheroid growth medium (Leibovitz L-15, 4 mM L-glutamine, 7.5% FBS, 1% P/S, 0.04% Pluronic® F-68) at ~1e6 cells/mL for culture on the device. Prior to use, the devices were
sterilized by rinsing them with a 70% aqueous ethanol solution and gently drying with compressed air.

All liquid handling steps were performed using a custom LabView program that enabled the application of the electric potential to any individual or combination of electrodes on the device.

To initiate hanging drop spheroid culture, droplets of cell suspension were dispensed from on-chip reservoirs and delivered to the location of a well, where they were pulled into the well spontaneously via capillary forces. Approximately 5 to 6 dispensed drops of ~1.75 µL each were needed to form a hanging drop with a curved surface that protruded beneath the bottom plate; the curvature at the bottom of the drop is necessary for the aggregation of cells into a spheroid. A thin film polyimide heater was placed in contact with the bottom aluminium holding plate to maintain the device at 37 °C during the handling of cell solutions. At all other times, the devices were kept in an incubator at 95% relative humidity and 37 °C.

For all spheroid assays, medium exchange was performed every 24 h following hanging drop formation by adding fresh medium and extracting spent medium from the hanging drop according to the following sequence: (1) deliver a drop of fresh medium to the hanging drop; (2) mix the liquid in the drop by repeatedly pulling out and releasing a liquid finger from the well; (3) extract two drops of liquid from the hanging drop; (4) deliver another drop of fresh medium to the hanging drop.
5.3.4 Migration Assays

For migration assays, hanging drops were created from a suspension of human foreskin fibroblasts at ~1e6 cells/mL. Cells were allowed to aggregate for 24 h into compact spheroids prior to encapsulation in collagen gel. The encapsulation process involved extracting the medium from the hanging drop and replacing it with a bovine collagen I solution (0.5-4 mg/mL bovine collagen I, 1% P/S, 4 mM L-glutamine, 0.04% Pluronics F-68, in Leibovitz L-15 medium, PH = 7). The DμF device was then placed in the incubator to allow the collagen drop to gel at 37 °C. To demonstrate the ability to assay spheroids with exogenous migration modulating agents, some spheroids were encapsulated in collagen solutions containing either BMP-2 (100 ng/mL) or PGE2 (5 μg/mL).

Cell migration was quantified 24 h after spheroid encapsulation by staining the hanging drops, per the manufacturer’s protocols, with Hoechst 33342 for imaging nuclear DNA and Acti-stain 488 phalloidin for visualizing actin filaments. The stained spheroids were imaged by confocal microscopy. Cell migration was quantified by measuring the radial fluorescence distribution and the total fluorescence intensity for each spheroid using ImageJ. For image analysis, the confocal images were converted into binary images to normalize the fluorescence intensity across multiple experiments and samples. The Radial Profile Plot plugin for Image J was used to measure the radial fluorescence intensity in the region outside the perimeter of the spheroid.[198] Total migration was measured by measuring the integrated fluorescence density of the stained actin everywhere outside the perimeter of the spheroid.
The viability of collagen-encapsulated spheroids was measured using the Life Technologies LIVE/DEAD® Viability/Cytotoxicity Kit according to the manufacturer’s protocols. The percentage of viable cells was quantified by counting the number of living and dead cells in multiple z-planes throughout each spheroid.

The physical properties of the collagen gels formed in situ were analyzed using atomic force microscopy (AFM) and scanning electron microscopy (SEM). AFM was used to obtain the Young’s modulus of the collagen sample by probing the hydrated gel and calculating the stiffness based on cantilever deflection. Bruker DNP-D and Bruker MSCT-B probes were used in a fluid environment. The spring constants of the probes were calibrated by measuring the thermal noise and fitting the response of a simple harmonic oscillator in fluid. The sample stiffness was measured by fitting probe deflection curves according to the Sneddon model and eliminating close range sample-probe interactions.[199,200] Scanning electron microscopy was performed in order to visualize and characterize the collagen matrix morphology and cell adhesion. For SEM imaging, gel samples were first fixed in a 10% buffered formalin solution for 2 h. A step-wise dehydration in methanol was then performed by subsequently submerging the samples in 25, 50, 75, 90, and 100% methanol solutions for at least 10 min each. The samples were super-critically dried and sputter coated with gold before SEM imaging.

Figure 5.2 illustrates the tumor secretion fibroblast stimulation assay protocol. To model tumor-stromal communication and invasion, fibroblast spheroids and HT-29 human colon adenocarcinoma spheroids were formed on the same device. After 24 h of aggregation, the medium from HT-29 spheroid hanging drops was replaced with serum free medium (SFM) to
allow for the enrichment of spheroid secretions. The fibroblast spheroids were maintained in normal growth medium. After an additional 24 h, medium from the fibroblast spheroids was extracted and replaced with drops of medium extracted from the HT-29 spheroids to expose the fibroblasts to the HT-29 spheroid secretions. Drops of 1 mg/mL collagen solution were then added to the wells to create hanging drops with a final collagen concentration of ~0.5 mg/mL. For controls, fibroblast spheroids that were not exposed to HT-29 spheroid secretions were also encapsulated in collagen. After 24 h of encapsulation within collagen, the hanging drops were stained for actin filaments and imaged by confocal microscopy. Migration was quantified by measuring the radial distribution and integrated fluorescence density of stained actin everywhere outside of the body of the spheroid.
Figure 5.2. Tumor secretion assay protocol. 24 h after the imitation of hanging drops, both HT-29 and fibroblast cells formed compact spheroids within their respective drops. At the 24 h point, spent medium was extracted from the HT-29 and fibroblast spheroids (steps 1,2) and replaced with serum free medium and normal growth medium, respectively (steps 3,4). After 24 more hours of incubation, medium was extracted from the fibroblast spheroid (step 5) and replaced with medium extracted from the HT-29 spheroid (step 6). Collagen was then added to the fibroblast spheroid (step 7) and the spheroids were allowed to incubate for another 24 h prior to imaging.

5.4 Results

To create a model for three-dimensional cell invasion on the DµF platform, spheroids of human fibroblasts were grown in hanging drops and encapsulated within a collagen hydrogel. Fibroblasts were used because they are the most abundant cell type in connective tissues and fibroblast motility plays a critical role in both wound healing and cancer invasion.[201] We used collagen gels because collagen is a naturally abundant extracellular matrix protein and is widely
used as a matrix in migration and invasion assays.[176,181] Bovine collagen I was used because the slower gelation rate allowed the collagen solutions to be manipulated longer on the device compared to more rapidly gelling rat tail collagen I solutions. Collagen solutions of up to 4 mg/mL could be actuated (dispensed, translated back and forth across a four electrode path four times, and inserted into a well) on our DμF devices. Solutions of higher concentrations of collagen moved too slowly for practical assay protocols. Solutions of up to 4 mg/mL bovine collagen I could be manipulated on the device for ~10 minutes at room temperature before movement became sluggish due to the onset of gelation of the collagen. Figure 5.3 shows representative SEM images and the mechanical properties of collagen gels of varying concentrations formed on a DμF device. The SEM images of the gels reveal a highly interconnected network of collagen fibrils. AFM measurements indicate that the Young’s Modulus (YM) increases linearly from 1-4 mg/mL up to an average of ~50 kPa. The YM deviates from linearity at concentrations < 1mg/mL. The Young’s moduli measured for the various collagen concentrations used in this work were consistent with values reported in literature.[202-205] This modulus range spans the elastic moduli of a wide variety of tissue types including lung, breast tumor, kidney, liver, brain, cardiac muscle, skeletal muscle, spinal cord and lymph node tissues.[206]
Figure 5.3. Collagen gel characterization. Collagen solutions up to 4.0 mg/mL could be handled on our DµF devices. (a) SEM images of hanging drop gels formed from 0.5, 1.0, 2.0, and 4.0 mg/mL collagen solutions. Each image was acquired at 10,000x magnification (Scale bar = 2 μm). (b) Young’s moduli measurements of collagen gels formed on the DµF platform as determined by AFM. Error bars correspond to the standard deviation from at least 10 separate measurements.

Extended (>24 h) spheroid culture assays require the ability to perform medium exchange. To determine if medium could be effectively exchanged from a collagen hydrogel, a standard solution of dyed collagen was used to form hanging drops. The change in the concentration of the dye in the collagen gel after successive liquid exchange cycles, as described in the Materials and Methods, was measured by UV-vis absorption spectroscopy. Figure 5.4 depicts the degree of medium exchange achieved after multiple exchange cycles. The data show that two exchange
cycles result in the exchange of >50% of the liquid in a hanging-drop gel, which is sufficient for performing cell spheroid culture. According to the medium exchange protocol used in this work, and assuming an initial hanging drop volume of five dispensed drops, the liquid in the well dilutes at a theoretical rate of $C = C_0(2/3)^n$, where $C$ = the concentration of dye in the hanging drop, $C_0$ = the initial concentration of dye in the hanging drop, and $n$ = the number of exchange cycles. The measured concentrations after each exchange cycle agree closely with the predicted values, demonstrating the ability to precisely control the composition of the hanging drop.

![Figure 5.4. Liquid exchange from hanging collagen gel drops.](image)

To demonstrate the ability to assay spheroids with migration modulating agents, bone morphogenic protein 2 (BMP-2), a growth factor known to stimulate migration in a variety of cell types, and prostaglandin E2 (PGE2), which is known to inhibit fibroblast migration, were added to the spheroids to induce and inhibit migration, respectively.
Untreated spheroids, which were encapsulated in collagen without any exposure to exogenous migration modulating agents, were used as a control.

Figure 5.5 compares the degree of migration for BMP-2-treated, PGE2-treated, and untreated fibroblast spheroids in collagen. Consistent with both 2D and 3D migration assays reported in literature, fibroblast spheroids encapsulated within collagen hanging drops on the DµF device exhibited cell invasion within 24 h.[181] Confocal imaging of fluorescently labelled actin filaments clearly shows the invasion of cells from the periphery of the spheroid into the surrounding gel. As expected, the addition of BMP-2 to the gel stimulated enhanced migration of cells from the spheroid while PGE2-treated spheroids exhibited reduced migration compared to the untreated controls. Spheroids exposed to 100 ng/mL BMP-2 showed an average ~85% increase in invasion compared to spheroids maintained in standard growth medium while PGE2 treated spheroids exhibited a ~33% decrease in invasion compared to untreated controls. The data illustrate that PGE2-treated spheroids exhibit migration out to ~50 µm from the spheroid perimeter after 24 h, compared to ~170 µm for untreated and BMP-2-treated spheroids (Figure 5.5c). Compared to the untreated controls, the BMP-2-treated spheroids, exhibit greater overall fluorescence at each radial distance, indicating a greater number of cells migrating from the spheroid.
**Figure 5.5. 24 h migration assays.** (a) Representative images of actin stain spheroids encapsulated within collagen gels and exposed to either standard medium or medium supplemented with either PGE2 or BMP-2. Scale bars = 300 µm. (b) A comparison of the total fluorescence measured outside of the spheroid perimeter for untreated, PGE2-treated, and BMP-2-treated spheroids. The data has been normalized to the average value of the untreated controls, which was set = 1. The dashed lines indicate the average change in migration compared to the untreated controls (solid line). The * indicates values that differ at the 99% CI as determined by a Student’s t-test. (c) The average radial fluorescence distribution of untreated, PGE2-treated, and BMP-2-treated spheroids (data normalized to max fluorescence).

Consistent with literature, we also observed that fibroblast migration varied with gel stiffness.[209-211] In this work, fibroblast migration increased with increasing collagen gel concentration. On average, the 4 mg/mL collagen gels exhibited a 46% increase in total migration, while the 0.5 mg/mL gels exhibited an average 40% decrease in migration compared
to the 1 mg/mL gels. These data are consistent with other reports of a positive correlation between fibroblast motility and collagen gel stiffness \textit{in vitro}.\textsuperscript{[212]} Cell migration in three-dimensions is regulated by both the formation of stable focal adhesion complexes, which anchor the cell to the matrix and serve as traction sites, and the activity of matrix proteolytic enzymes, which provide a migratory path through the localized degradation of the dense extracellular matrix.\textsuperscript{[212,213]} Research has shown that focal adhesions formed on soft gels (<1 kPa) are transient and less stable than those formed on stiffer gels, thus limiting cell migration in soft gels.\textsuperscript{[214,215]} Additionally, it has been shown that the expression of matrix proteolytic enzymes in fibroblasts correlates positively with matrix stiffness, resulting in increased fibroblast migration in stiffer gels.\textsuperscript{[216,217]}

do not support the formation of stable focal adhesion complexes, thereby limiting cell motility. Additionally, the expression and activity of matrix proteolytic enzymes

Viability staining indicated that encapsulating the fibroblast spheroids in collagen does not decrease cell viability. The gel-encapsulated fibroblast spheroids exhibited viability >90\% after 96 h in culture (48 h in solution and 48 h in collagen), which is comparable to spheroids cultured in suspension for the same duration.\textsuperscript{[187]}

To demonstrate the ability to model a physiologically relevant cell invasion process, we used the D\textalpha{}F platform to explore tumor-stromal communication \textit{in vitro}. Paracrine secretions from tumors are known to induce an activated phenotype in adjacent fibroblasts, characterized by the production of \(\alpha\)-smooth muscle actin and enhanced invasiveness.\textsuperscript{[218,219]} To mimic paracrine
signaling in the tumor microenvironment, secretions from human colon carcinoma spheroids cultured in hanging drops were delivered to fibroblast spheroids immediately prior to encapsulation in collagen. A comparison of the migration from tumor-stimulated and non-stimulated fibroblast spheroids is shown in Figure 5.6.

Figure 5.6. Comparison of HT-29 secretion-stimulated and untreated fibroblast spheroids. (a) Representative images of actin-stained, collagen-encapsulated fibroblast spheroids. Red bars correspond to 100 µm. (b) The average total fluorescence intensity of stained actin outside of the spheroid perimeter for both untreated and HT-29 treated spheroids. Data normalized to the fluorescence intensity of the untreated spheroids. (N = 7 spheroid for each condition) (c) Average radial distribution of the fluorescence intensity of stained actin outside of the spheroid perimeter. Data normalized to fluorescence intensity value at the spheroid perimeter. (N = 7 spheroid for each condition)

The results, shown in Figure 5.6, reveal enhanced migration for tumor secretion-stimulated fibroblast spheroids, supporting evidence that paracrine signaling from tumor spheroids induces
fibroblast activation in the tumor microenvironment.[183,220,221] The HT-29 treated spheroids exhibited a >130% increase in actin fluorescence outside of the spheroid perimeter compared to untreated controls. On average, cells from HT-29 treated spheroids exhibited migration out to ~270 µm from the spheroid perimeter compared to ~90 µm for untreated spheroids. Another interesting observation from these experiments is the evidence of multiple modes of fibroblast migration occurring simultaneously. In three dimensions, cells can exhibit different migration mechanisms depending on a variety of factors including matrix stiffness, density, and orientation, as well as the activity of matrix proteases.[211,222,223] The HT-29-treated spheroids in Figure 6 exhibit characteristics of: (a) mesenchymal-type migration, characterized by cellular polarization and the formation of invadopodia; (b) amoeboid-type migration, characterized by individual, non-adherent cells that have migrated outside of the spheroid perimeter, and (c) collective-type cell migration, characterized by migration of multicellular streams or aggregates from individual spheroids. Non-treated spheroids also appear to exhibit multiple modes of migration, however, migration was lower overall compared to the HT-29 stimulated spheroids.

5.5 Discussion

This work builds upon the cell culture and assaying capabilities established for the DµF platform. Previous works have developed the ability to culture cells in adherent monolayers, encapsulate cell suspensions within hydrogels, and culture cell spheroids in solution on a DµF device.[49,53,187] While cell spheroids in a hanging drop solution provide excellent in vitro tissue models, the addition of an extracellular scaffold allows for cell-ECM signaling, which is paramount to tissue homeostasis and morphogenesis. By enabling the encapsulation of three-dimensional spheroids in an extracellular matrix, this platform allows the modeling of processes
that are highly dependent on cell-ECM interactions, such as the epithelial-mesenchymal transition, which plays a key role in important physiological processes such as embryogenesis, wound-healing, and tumor progression, and which cannot be modeled effectively using cell monolayers or encapsulated cell suspensions. Additionally, the methods described here can be extended to any type of spheroid-forming cell type and thermoresponsive sol-gel formulation. While not explored in this work, other gelation techniques, such as photopolymerization, could also be employed to encapsulate cell spheroids.

The DμF platform described here provides various advantages over existing spheroid-based invasion assays. Primarily, we have shown that DμF can effectively automate all of the liquid handling steps necessary for spheroid culture and encapsulation in collagen. Advanced DμF systems, such as the DropBot system or commercially developed systems that have integrated droplet volume metering, temperature control, and hundreds of addressable electrodes would enable fully automated, ‘walk-away,’ operation and medium-throughput (dozens of wells per chip) assay capabilities.[40,47,171] These systems can also have integrated sample treatment and analytical capabilities such as localized heating, magnetic bead separation, electrochemical detection, fluorescence microscopy, and absorption spectroscopy, providing a level of in situ analysis that is difficult or impossible to achieve using robotic liquid handling systems. Such a platform would enable more sophisticated yet user-friendly assay capabilities than is possible with current spheroid-based invasion assays.

The DμF platform described here does have certain limitations. The primary limitation of DμF is the relatively lower throughput compared to robotic liquid handling systems. Migration assays
using well insert membranes, for example, are compatible with robotic liquid handling equipment, which can process up to 96 wells simultaneously. Unlike robotic liquid handling, DμF liquid handling occurs on the same plane as the wells, which limits the well density on the chip. Thus, in the case of cell-based assays, the DμF platform is best suited for research environments in which medium-throughput sample processing is sufficient. Another limitation of the platform described here is the incompatibility with certain multi-component gel type systems that form crosslinks upon mixing (i.e. alginate). If the gelation kinetics of a multi-component system are faster than the mixing kinetics on the DμF device, then the addition of one component to the other results in the formation of a gel barrier at the interface of the drops or a gel with inhomogeneous crosslinking density throughout the drops. Despite these limitations, the automation, flexible liquid handling, and in situ analytical capabilities of the DμF platform make it a powerful tool for cell-based assays and screens.

5.6 Conclusions
We have developed protocols for the encapsulation of cell spheroids in hanging-drops of collagen gel using DμF. The encapsulation of spheroids within a three-dimensional, ECM scaffold allows for the creation of a tissue microenvironment that mimics tissues in vivo. With the ability to automate spheroid formation, gel encapsulation, and the addition of exogenous agents to spheroids, the DμF platform described here would reduce the tedium associated with manual 3D invasion assays and provide an alternative to robotic liquid handling instruments for assay automation. Making three-dimensional cell-based invasion assays easier to perform and more accessible to life sciences researchers could ultimately lead to new insights into tissue homeostasis and morphogenesis, as well as the development of novel treatments and therapies for a variety of injuries and diseases.
5.6.1 Acknowledgements

The authors thank Prof. Heather Maynard (UCLA) for providing access to cell culture facilities and equipment. Confocal laser scanning microscopy was performed at the CNSI Advanced Light Microscopy/Spectroscopy Shared Resource Facility (ALMS-SRF) at UCLA, supported with funding from NIH-NCRR shared resources grant (CJX1-443835-WS-29646) and NSF Major Research Instrumentation grant (CHE-0722519). The authors also acknowledge Dr. Matthew Schibler (UCLA ALMS-SRF) for assistance with confocal microscopy.
Chapter 6. Fluorinated liquid-enabled protein handling and surfactant-aided crystallization for fully in situ digital microfluidic MALDI-MS analysis

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Published in \textit{Lab on a Chip}, RSC Publishing, Royal Society of Chemistry

Received 21st November 2011, Accepted 26th March 2012

First published on the web 28th March 2012
6.1 Abstract

A droplet (digital) microfluidic device has been developed that enables complete protein sample preparation for MALDI-MS analysis. Protein solution dispensing, disulfide bond reduction and alkylation, tryptic digestion, sample crystallization, and mass spectrometric analysis are all performed on a single device without the need for any ex situ sample purification. Fluroinated solvents are used as an alternative to surfactants to facilitate droplet movement and limit protein adsorption onto the device surface. The fluorinated solvent is removed by evaporation, and so does not interfere with the MALDI-MS analysis. Adding a small amount of perfluorooctanoic acid to the MALDI matrix solution improves the yield, quality and consistency of the protein-matrix co-crystals, reducing the need for extensive ‘sweet spot’ searching and improving the spectral signal-to-noise ratio. These innovations are demonstrated in the complete processing and MALDI-MS analysis of lysozyme and cytochrome c. Because all of the sample processing steps and analysis can be performed on a single digital microfluidic device without the need for ex situ sample handling, higher throughput can be obtained in proteomics applications. More generally, the results presented here suggest that fluorinated liquids could be used to minimize protein adsorption and improve crystallization in other types of lab-on-a-chip devices and applications as well.

6.2 Introduction

Droplet or ‘digital’ microfluidics has emerged as an effective method for performing various types of chemical reactions and biological assays in a rapid and programmable manner. [34,36,54,224-226] Using electrowetting and liquid dielectrophoresis, discrete droplets of liquid can be dispensed, moved, merged, and split across patterned, dielectric covered electrodes for
lab-on-a-chip functions. [227-229] Sample preparation for high throughput proteomics applications on a droplet microfluidic platform has been explored by various research groups. Peptide mass fingerprinting (PMF) is a technique used to identify proteins from their proteolytic peptides. While a powerful tool in proteomics, PMF requires multiple sample handling steps (protein denaturation, disulfide bond reduction, alkylation of free thiols, enzymatic digestion, sample crystallization) that can be tedious and time consuming if performed manually. [230,231]

Thus, technologies that enable the automated preparation of protein samples for analysis by mass spectrometry could significantly facilitate proteomics research. Because of the ability to dispense, move, and mix small volumes of liquid on a digital microfluidic device in a controlled fashion, droplet microfluidics offers the potential to address some of the sample handling bottlenecks of peptide mass fingerprinting. We and others have shown that the processing steps for peptide mass fingerprinting can be performed on a digital microfluidic device, providing a rapid means of preparing samples for MALDI MS analysis.[32,33,224,232,233]

Several aspects of the sample preparation process, however, have limited the implementation of digital microfluidics in high throughput proteomics. Protein solutions are difficult to dispense and move on a hydrophobic surface, due to non-specific adsorption. When a protein encounters a hydrophobic surface, adsorption occurs if the protein undergoes a conformational change to maximize interactions between the hydrophobic peptides within the protein and the hydrophobic surface. This is an energetically favorable process as the hydrophobic interactions result in an increase in the conformational entropy of the polymer and an increase in the translational entropy of the solvent molecules that are displaced from the surface. [234,235] In digital microfluidics, proteins can also adsorb to the surface as a result of electrostatic attraction of charged proteins to
the surface when the actuating voltage is applied. [236] Irreversible adsorption of proteins to the hydrophobic surface of a digital microfluidic device results in the formation of hydrophilic areas on the device, causing the aqueous droplets to ‘stick’ to those areas, rendering droplet movement difficult or impossible.

In our previous MALDI sample preparation work, we found that protein solutions with concentrations as low as 5 μM were sometimes unmovable on our devices. [232] Reducing the protein concentration, or adding surfactants to the sample, enabled droplet movement, however; both approaches resulted in poor MALDI spectral S/N due either to the reduced amount of protein analyte or to spectral interference from the surfactant. To make the digital microfluidic platform an effective tool for MALDI sample preparation, a method for enabling droplet movement without sacrificing spectral quality is needed.

The use of low viscosity oils have been shown to facilitate droplet movement by encapsulating droplets, forming a barrier between the droplet and the device surface, limiting contact and thus protein adsorption. [48,237] With silicone or mineral oils there is a risk that the solvent and analyte will partition into the ambient oil phase. Also, the presence of a non-volatile ambient phase disallows certain functions, such as in situ drying of the sample droplet. Furthermore, the silicone oils that are typically used are non-volatile and cannot be completely removed, potentially resulting in mass spectral interference. The build-up of non-conductive oils on lens elements can lead to surface charging, impairing mass spectrometer operation.
Fan et al. recently demonstrated the ability to remove a silicone oil shell from an encapsulated droplet using an on-chip reservoir of hexane to dissolve the oil. [238] While effective, this approach is limited by the miscibility of various organic solvents with hexane, which could result in an undesirable change in droplet composition. Also, multiple hexane reservoirs would be required to remove the silicone oil from different drops in order to avoid cross contamination. This could be burdensome, given the limited space available on a digital microfluidic device.

Luk et al. have shown that the movement of protein solutions can be greatly facilitated by adding small amounts of Pluronics, PPO/PEO/PPO triblock copolymers that prevent protein adsorption. [139] A comprehensive analysis demonstrated that using Pluronics is an effective and biocompatible approach to facilitating the movement of proteinaceous solutions on a digital microfluidic device. [51] Surfactants can significantly interfere with the MALDI analysis of low level peptides, however, so they must be removed from the sample prior to analysis. [33,239,240] This adds processing steps, limiting the overall throughput.

A further challenge is that while a hydrophobic surface facilitates the movement of droplets on the device, we have found that MALDI matrix co-crystallization is relatively poor on fluoropolymer surfaces, requiring crystallization to be performed manually off-device, or the incorporation of hydrophilic patches on the device surface, where crystallization can be enhanced. [232]

To address these issues, we have adapted the sample preparation process to be conducted in a fluorinated liquid ambient environment. Like other types of oils used to assist droplet movement
in digital microfluidics, fluorocarbon liquids can facilitate droplet movement. The use of fluorininated liquids is advantageous because they have relatively low boiling points and are solvophobic, making them immiscible with aqueous and organic solvents and poor solvents for protein analytes. [241-243] Consequently, a fluorinated liquid can be used to assist droplet movement on a digital microfluidic device with minimal sample loss to the ambient phase. It can then be quickly and passively removed by evaporation at room temperature, allowing protein samples to crystallize with MALDI matrix directly on the device.

Lastly, we address the issue of poor MALDI crystal formation on fluoropolymer surfaces by adding to the matrix solution a fluorinated surfactant that has been shown to enhance desorption/ionization on silicon mass spectrometry (DIOS-MS) of peptides from fluoropolymer surfaces. [244] These methods for improving droplet movement and crystallization enable the peptide mass fingerprinting sample processing to be performed entirely in situ, without the need for manual sample purification or extra fabrication steps to create a crystallization compatible surface. Together, these strategies significantly improve digital microfluidic sample processing of proteomic samples.

6.3 Experimental

Lysozyme from chicken egg white, cytochrome c from horse heart, equine myoglobin, fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA), ProteoMass™ P14R MALDI-MS standard, ammonium bicarbonate, 2,5 dihydroxybenzoic acid (DHB), Pluronics® F-68 and F-127 and Octyl-β-D-glucoside (OBG) were purchased from Sigma (St. Louis, MO). Autolysis resistant, TPCK treated, mass spectrometry grade trypsin was purchased from G Biosciences (Maryland Heights, MO). Cytop® was purchased from AGC Chemicals Americas,
Inc. (Exton, PA) and Teflon-AF® was purchased from DuPont (Wilmington, DE). Acetonitrile and trifluoroacetic acid were purchased from EMD Chemicals (Gibbstown, NJ). Fluorinert® FC-40 and FC-75 were purchased from Acros Organics (New Jersey, USA). Novec® Engineered Fluid HFE-7500 and HFE-7100 were purchased from 3M (St. Paul, MN). Whole cell *E. coli* and pentadecafluorooctanoic acid (PFOA) from TCI America (Portland, OR), was generously provided by Prof. Joseph Loo (UCLA).

Stock solutions of ammonium bicarbonate (50 mM), tris(2-carboxyethyl)phosphine (TCEP, 350 mM), *N*-ethymaleimide (NEM, 100 mM), OBG (17 mM), lysozyme (500 μM), myoglobin (295 μM), and cytochrome *c* (240 μM) were prepared in deionized water. Stock solutions of trypsin (8.6 μM) were prepared according to the manufacturer’s protocol. Stock solutions were kept frozen and working solutions used within one day of preparation. Working solutions of TCEP (5 mM) and NEM (5 mM) were prepared by diluting the stock solutions with deionized (DI) H₂O, and combined in a 1:1 mixture when performing sample processing. Working solutions of lysozyme (5 μM), myoglobin (6 μM), cytochrome *c* (8 μM), whole cell *E. coli* (2.5 mg/ml), and trypsin (0.5 μM) were prepared by diluting stock solutions with 50 mM ammonium bicarbonate. All protein working solutions had a pH of 8.6. Solutions of MALDI matrix 2,5-dihydroxybenzoic acid (15 mg/mL in 20% acetonitrile, 0.1% TFA) were prepared immediately prior to use in DI H₂O.

The fabrication of digital microfluidic devices was similar to previously described protocols [32] and was conducted in the Nanoelectronics Research Facility and the CNSI Integrated System Nanofabrication Cleanroom at UCLA. Briefly, 100 Å chromium and 1000 Å gold were
deposited onto glass substrates by electron beam evaporation and patterned into electrodes via photolithography. The dielectric layer consisted of parylene-C (Specialty Coating Systems, ~2 μm) and was applied via vapor deposition. A fluoropolymer coating was then applied by spin coating either Cytop or Teflon-AF onto the substrates at 2000 rpm for 60 seconds and post-baking on a hot plate at 110 °C for 5 min and 180 °C for 15 min. The top plate, an ITO-coated glass slide, was coated with fluoropolymer as well.

MALDI-MS sample processing experiments were carried out by dispensing solutions from on-chip reservoirs. For experiments in which a fluorinated liquid was used to assist droplet movement, 2 μL of the fluorinated liquid was first pipetted onto each reservoir electrode. Droplets of TCEP/NEM, lysozyme, trypsin, and DHB, 1.4 μL each, were then pipetted onto reservoir electrodes and sandwiched between the top and bottom plates with gap heights ranging from 200 to 350 μm. Droplet actuation was achieved by applying an AC potential (120-150 V, 18 kHz) to an electrode on the bottom plate adjacent to the edge of the droplet. First, lysozyme and TCEP/NEM droplets were dispensed, merged, and allowed to mix for 5 min. A droplet of trypsin solution was then dispensed and merged with the reduced and alkylated lysozyme droplet and allowed to mix for 15 min. Mixing was enhanced by moving the droplet back and forth across three electrodes. Following tryptic digestion, two droplets of DHB were dispensed and mixed with the protein digest solution. Immediately after the addition of the DHB, the top plate was removed and the device was moved into a fume hood where the droplets were allowed to crystallize. A schematic of the microfluidic device and the sample preparation steps is shown in Figure 6.1. All sample processing steps were performed at room temperature. For comparison of microfluidic and manual sample preparation methods, protein solutions were processed
manually by performing reduction/alkylation as well as tryptic digestion steps for > 1 hr at 37 °C. Digested samples were then crystallized on a stainless steel MALDI plate using a matrix solution of 15 mg/mL DHB in 20% (v/v) acetonitrile and 0.1% (w/v) TFA.

**Figure 6.1. Device layout and sample processing steps.** The gold electrodes represent the electrode array used to perform the protein digestion steps. The black lines indicate wires connecting the electrodes to contact pads elsewhere on the device. Prior to adding sample processing droplets to the device, drops of fluorinated liquid were pipetted onto the reservoirs (large electrodes). Sample processing drops (protein, TCEP/NEM, Trypsin, DHB) were then added to the reservoirs and became encapsulated in a fluorinated liquid shell. The top plate was then put in place to begin sample processing. Step 1: Droplets of the protein solution and the TCEP/NEM solution are dispensed from their reservoirs, mixed, and allowed to incubate on device for 5 min. Step 2: A droplet of trypsin solution is dispensed and merged with the reduced and alkylated protein droplet and is allowed to mix for 15 min on the device. Step 3: Following tryptic digestion, a droplet of DHB matrix solution is dispensed from it’s reservoir and is merged with the digested protein droplet. After merging the droplet of matrix solution with the digested protein, the top plate of the device is removed and the bottom plate is placed in a fume hood where any remaining fluorinated liquid is allowed to evaporate and the sample is allowed to crystallize.
Protein adsorption to the fluoropolymer surface was examined using contact angle measurements and fluorescent imaging of residual protein from a droplet sitting on the surface. For the contact angle measurements, a 3-μL droplet was placed on a Cytop surface either in air or submerged in FC-40, and monitored by video camera for 20 min. Contact angles were measured by capturing still images from the video at various time points using a FTA4000 contact angle goniometer (First Ten Angstroms, Portsmouth, VA). Experiments were performed at least three times for each solution to obtain an average value. For residual protein analysis, 2-μL droplets of 1 mg/mL solutions of FITC-BSA were pipetted onto a Cytop-coated coverslide and allowed to sit either in air or fluorinated liquid for 20 min in a humidified chamber (Petri dish partially filled with water). After 20 min, the droplets were wicked away with a Kimwipe® and non-adsorbed protein was washed away by submerging the coverplate in DI H₂O for 10 min. The droplet contact areas were then imaged using a Zeiss Axiovert 200M fluorescence microscope. The fluorescence intensity of the adsorbed protein was quantified by using the Zeiss AxioVision image processing software to calculate the densiometric mean value of fluorescence from each image.

The effect of adding PFOA to the matrix solution was examined by crystallizing samples containing a MALDI standard peptide in matrix solutions both with and without PFOA. After testing various concentrations of PFOA, we found that incorporating 0.005 to 0.01% (m/v) PFOA into the standard matrix solution did not adversely affect droplet movement and yielded high quality MALDI mass spectra. The resulting samples were analyzed by acquiring mass spectra from five different regions from each crystallized sample spot (center, upper left, upper right, lower left, lower right) to account for possible ‘sweet spots’. For each matrix condition, 75
total spectra were acquired from 15 different samples with at least 150 laser shots for each spectrum. Solutions of tryptic digest of myoglobin containing 0.06% Pluronic F-68 were also crystallized on fluoropolymer surfaces, using matrix solution with and without 0.0075% PFOA, and analyzed by mass spectrometry.

MALDI mass spectra were acquired by inserting devices containing dry crystals into a specially milled MALDI plate that enabled desorption/ionization directly from the surface of the device. All mass spectra were obtained using an Applied Biosciences Voyager DE-STR mass spectrometer in positive ion, reflector mode using a delayed extraction time of 170 ns and accelerating voltage of 20 kV.

6.4 Results and Discussion

6.4.1 Surfactant-free movement of protein solution

The fluorinated liquids Fluorinert® FC-40 and FC-75 and Novec™ Engineering Fluid HFE-7500, HFE-7100 were tested for their ability to facilitate protein droplet movement. Fluorinert® FC-40 (a mixture of perfluorotri-n-butylamine and perfluoro-n-dibutylmethylamine) and FC-75 (perfluoro-2-butyltetrahydrofuran) are perfluorinated compounds. Novec™ HFE-7500 (3-ethoxy-1,1,2,3,4,4,5,5,6,6,6-dodecafluoro-2-trifluoromethyl-hexane) and HFE-7100 (a mixture of methyl nonafluorobutyl ether and methyl nonafluoroisobutyl ether) are semifluorinated compounds. Movability was defined as the ability to move a droplet back and forth three times across eight electrodes. These fluorocarbon liquids were selected because they are readily available; they do not necessarily represent the best options for facilitating droplet movement, and other perfluorinated liquids might perform as well. All of the fluorinated liquids tested
resulted in enhanced movement of the protein solutions compared to movement in air. Movement of lysozyme solutions at concentrations up to 500 μM (7.15 mg/mL), without any added surfactants, could be achieved when a fluorinated liquid was used as an ambient fluid. Engineering Fluid HFE-7500 and Fluorinert® FC-40 facilitated movement of protein solutions for an extended period of time (>30 min) on the device. Fluorinert FC-75 and HFE-7100 initially enhanced the movement of protein solutions, but movement ceased upon evaporation of the solvent. Re-addition of solvent to the droplet restored movability.

The concentrations of protein solutions that are movable using fluorinated liquids are lower than the maximum movable protein concentrations (10 to 50 mg/mL) reported by Luk et. al., who used surfactants to prevent adsorption,[139] but are still >100 times more concentrated than what is movable without any additives or ambient liquid. Because the presence of surfactants in the protein sample can interfere with the analysis of peptides, fluorinated liquids represent an alternative to Pluronic additives when the protein concentration is low enough that the MALDI analysis might be adversely affected by the presence of surfactants.

Figure 6.2 shows a sequence of droplet dispensing for 2.5 mg/mL whole cell E. coli. Without any added surfactants or fluorinated liquid, this complex biological solution cannot be moved. The image sequence shows that with the fluorinated liquid, the solution can be moved. Movement of protein solutions required only enough fluorinated liquid to engulf the drop of solution. Upon dispensing from on-chip reservoirs, a thin fluorinated oil ‘shell’ encapsulated each droplet, facilitating the movement of the protein solution. We observed effects similar to those reported by Brassard et al. and Fan et al., who described reduced threshold voltages, lower
voltage droplet splitting and dispensing, and faster movement for water-silicone oil core-shell droplet actuation compared to droplet actuation in air. [245,246] The subsequent evaporation of fluorinated liquid allows analysis of the sample without interference from the ambient liquid. The rate of droplet evaporation can be tuned by varying the amount or type of fluorinated liquid added to the droplet.

Figure 6.2. Dispensing of aqueous droplets engulfed in fluorinated liquid. Sequence of images depicting the dispensing of a droplet of 2.5 mg/mL whole cell E. coli in an ambient droplet of HFE-7500 and the subsequent evaporation of the fluorinated liquid over time. The solid arrow in the t = 0 picture indicates the outer edge of the fluorinated liquid, while the dashed arrow indicates the outer edge of the cell solution droplet.
Because proteins are amphiphilic molecules, they can assemble at an interface causing a reduction of the interfacial tension. In the case of protein adsorption at a solid-liquid interface, this change in interfacial tension can be monitored by contact angle goniometry. [247] The adsorption of proteins to a hydrophobic surface can thus be detected by a decrease in the contact angle of a sessile drop on the surface. While contact angle change is protein species, concentration, and time dependent, nanomolar - micromolar concentrations of protein are generally sufficient to induce a measurable change (up to ~25%) in contact angle of a droplet on a hydrophobic surface. [248,249] Figure 6.3 compares the the time-dependent contact angles of a sessile droplet of protein solution on the Cytop surface in air to those of an identical droplet engulfed in FC-40. A reduction in contact angle can result from evaporation if the droplet is in a ‘pinned’ state, or from protein adsorption on the device surface. The data show that adding the fluorosolvent prevents a decrease in droplet contact angle from evaporation and non-specific adsorption, both of which are common problems when manipulating protein solutions in air. For the contact angle analysis, the aqueous droplets do not float on the denser fluorinated liquid because the aqueous-fluorocarbon interface is energetically more favorable than the aqueous-air interface, so the droplets remain completely surrounded by fluorinated liquid. This observation is supported by our previous work examining the spontaneous insertion of an aqueous droplet into a droplet of an immiscible, hydrophobic liquid.[250-252]
Figure 6.3. Contact angle change in fluorinated liquid and ambient environments. Relative change in contact angle over time for 1 mg/mL lysozyme (circles) and cytochrome c (triangles) in 50 mM ammonium bicarbonate buffer in an FC-40 environment and in air. The change in contact angle of buffer solution in air is due to evaporation (squares).

We also monitored protein adsorption by examining the residual protein left on the device top plate after allowing drops to sit on the surface for 20 min. Figure 6.4 shows a comparison of FITC-BSA adsorption to a Cytop-coated top plate from a droplet in air (a) and engulfed in FC-40 (b). The reduction in fluorescence from the surface of the device upon the addition of FC-40 indicates a decrease in protein adsorption. Au et. al. have recently shown that Pluronic additives at concentrations as low as 0.02% can facilitate movement of complex protein solutions for extended periods of time (>40 min). [140] From Figure 6.4(c), it is clear that encapsulating protein droplets in select fluorinated liquids can reduce surface fouling as effectively as adding...
Pluronics. A sensitivity analysis of the fluorescence microscopy technique used for analyzing protein adsorption suggests that less than 20 fmol of protein adsorbs to the surface of the device from droplets engulfed in fluorinated liquid. (See supplemental information Figure 6.S8). Thus, the use of a fluorinated solvent is an improved technique for facilitating electrowetting driven droplet movement when sample drying and minimal surfactant interference are required.

![Fluorescent images](image)

**Figure 6.4. Protein adsorption in fluorinated liquid and ambient environments.** Fluorescent images of FITC-BSA adsorbed to a Cytop surface after 20 min from droplets in (a) air and (b) FC-40. (c) A comparison of the relative intensity of fluorescence from adsorbed proteins from droplets containing 1mg/mL FITC-BSA to Cytop surfaces in various environments: [1] in air. [2] with 0.02% Pluronic F-127 added. [3] with 0.02% Pluronic F-68 added. [4] engulfed in FC-40. [5] engulfed in FC-75, and [6] engulfed in HFE-7500. Error bars indicate the standard deviation of relative intensities from at least five different drops.
While fluorinated liquids enhance the movement of protein solutions on a digital microfluidic device, adsorption of enzymes to the aqueous/fluorous liquid interface can result in denaturation of the enzymes. [253] To preserve enzyme activity, small amounts of surfactant can be added to the protein solution to prevent protein denaturation at the liquid/liquid interface. Figure 6.5 compares MALDI spectra from digests performed without (a) and with (b) 0.005% (w/v) Octyl-β-D-glucoside (OBG) in the trypsin solution. The complete disappearance of the lysozyme molecular ion peak at ~14.3 kDa demonstrates that incorporating the surfactant maintained enzyme activity. At such a low concentration, the OBG does not interfere with MALDI spectra.

![Figure 6.5](image.png)

**Figure 6.5. Effect of surfactants on trypsin activity in fluorinated liquid-engulfed drops.** MALDI mass spectra of lysozyme digest performed in FC-40 without (upper) and with (lower) 0.005% (w/v) OBG in the enzyme solutions. An OBG concentration of 0.005% is enough to restore enzyme activity without causing MALDI interference. Plots are offset to show comparison, (*) indicates the intact lysozyme (undigested) peak.

Fluorinated solvents can plasticize the thin fluoropolymer film on the device surface.[254] Plasticization was evident in our experiments by the appearance of visible rings in the film as the
solvent evaporates, and by a change in the contact angle for plasticized and non-plasticized films ($\theta_c$ acetonitrile = 78.2 ± 1.6° on Teflon, 73.5 ± 2.5° on plasticized Teflon). A small degree of plasticization did not significantly inhibit droplet movement, but, movement was optimal when plasticization was minimized. Plasticization or solvent intercalation into minute pores of the film can also be beneficial in the present application, as they render the films less permeable to small molecule solutes and solvents compared to non-plasticized films, due to the occupation of free volume in the film by the fluorinated molecules.[243,255] In one study, films of Teflon-AF doped with up to 15 wt% Fluorinert FC-70 exhibited increased density as well as decreased permeability, diffusion, and partition coefficients for various organic solutes, enhancing the fluorous nature of the film.[256] This decrease in film permeability may contribute to the enhanced movability of solutions in fluorinated liquids and prevent contamination of the surface.

6.4.2 PFOA enhanced MALDI mass spectra from fluoropolymer surfaces

We have previously reported difficulty in obtaining quality MALDI spectra from fluoropolymer surfaces due to poor crystal distribution.[232] The addition of fluorinated surfactants to samples has been shown to enhance spectral quality for DIOS MS from fluorinated surfaces[244] and was examined here for its effect on MALDI spectra. We observed that samples containing PFOA crystallized rapidly (< 5 min) and consistently formed crystals with similar morphology on fluoropolymer surfaces, while samples without PFOA crystallized slowly (>10 min) and exhibited inconsistent sample to sample morphology (Figure 6.6).

To compare the quality of spectra acquired from samples with and without 0.0075% PFOA in the matrix, we crystallized 30 samples of peptide standard solution, 15 with and 15 without PFOA,
on Cytop surfaces. We acquired five spectra from various regions within each sample spot in order to account for possible ‘sweet spots.’ The peptide standard peak could be detected with a S/N >3 in 88% of all spectra acquired from samples containing PFOA, compared to just 44% from samples without PFOA, demonstrating the efficacy of PFOA in enhancing MALDI spectra from fluorinated surfaces. All samples containing PFOA formed numerous crystal needles, the typical DHB crystal morphology, while samples without PFOA showed various crystal morphologies and occasionally did not crystallize at all on the fluoropolymer surfaces. As shown in Figure 6, the addition of PFOA to the matrix allows samples to crystallize on a hydrophobic fluoropolymer surface (a) with a similar, needle-like morphology as samples crystallized on a standard MALDI plate (c). Because MALDI signal reproducibility is extremely sensitive to crystal homogeneity,[257] it is essential that the matrix composition reliably yields high quality crystals. Although the crystal area was larger when PFOA was added to the matrix (because the lower surface tension leads to a larger droplet footprint), samples crystallized with PFOA required less ‘sweet spot’ searching than samples without it, thereby reducing the analysis time. Increased reproducibility of quality spectra and reduction of acquisition time are essential for enhancing the throughput of any automated MALDI process.

We found that PFOA enhanced crystallization only on the fluoropolymer surfaces of our devices and not on a stainless steel MALDI plate. We suspect that the fluorous nature of the PFOA molecule allows it to interact closely with the fluoropolymer surface, while presenting hydrophilic domains to the aqueous droplet that can serve as nucleation sites for crystallization on the otherwise hydrophobic surface. Other work has shown increased spectral reproducibility from 2,5-DHB crystallized samples by sandblasting stainless steel MALDI plates to roughen the
surface, resulting in more crystal nucleation sites and smaller, more evenly distributed crystals.[258] From Figure 6.6, it is clear that the addition of PFOA to the matrix solution enhances nucleation on fluoropolymer surfaces, leading to the formation of more evenly distributed crystals compared to the standard matrix preparation (15 mg/mL DHB, 30% aq. ACN, 0.1% TFA).

![Figure 6.6. Fluorinated surfactant-enhanced matrix co-crystallization.](image)

**Figure 6.6.** Fluorinated surfactant-enhanced matrix co-crystallization. A comparison of typical crystal morphology for digested FITC-BSA samples crystallized on Cytop with (a) and without (b) PFOA in the matrix solution. (c) FITC-BSA samples crystallized on a stainless steel MALDI plate using a standard matrix preparation (15 mg/mL DHB, 30% acetonitrile, 0.1% TFA).

Figure 6.7 shows that the addition of fluorinated surfactant to the matrix solution can enhance spectral quality even from samples containing Pluronics that are crystallized on a fluoropolymer surface. The addition of PFOA to samples containing Pluronics yielded crystals with a long, needle-like structure, similar to samples crystallized without Pluronics (crystal images in
supplemental information). It appears that promoting sample crystallization on the fluoropolymer surface partially negates the detrimental effects of the detergents. This observation is consistent with those of Zhang and Li, who found that various sample crystallization conditions can reduce the degree to which a surfactant additive interferes with the mass spectra.[239] Comparable results were obtained for matrix crystallization on Cytop and Teflon-AF surfaces.

Figure 6.7. Fluorinated surfactant-enhanced MALDI spectra. Comparison of typical MALDI spectra of tryptic digests of myoglobin containing 0.06% Pluronic F-68 crystallized on Teflon-AF with matrix solutions either with (top) or without (bottom) 0.0075% PFOA. Plots are offset to facilitate comparison. The (*) indicates peaks identified as myoglobin tryptic peptides in MASCOT.

6.4.3 Peptide mass fingerprint

Using the optimized conditions for protein movement and sample crystallization, mass spectra of lysozyme and cytochrome c tryptic digests were obtained from samples fully processed on-chip. The sample processing steps: disulfide bond reduction, free thiol capping, and tryptic digestion for lysozyme and tryptic digestion for cytochrome c, were accomplished in 30 min at room
temperature on the device. With fluorinated liquid enhanced droplet movement, four lysozyme samples could be processed simultaneously on a single device, limited simply by the design of the electrode array and the number of electrodes that can be addressed in our automated set-up. An analogous system could easily be designed to allow high throughput sample processing. Mass spectra obtained from complete on-chip sample processing of protein solutions in fluorinated liquid are shown in the upper spectra of Figure 6.8 (a) and (b). The spectra show high ionization efficiency of peptides, high S/N, and negligible interference from the small amounts of surfactants added to maintain enzyme activity. There is no evidence of contamination from residual fluorinated solvent. The lower spectra in Figure 6.8 show typical mass spectra obtained from protein solutions containing 0.06% Pluronic F-68 that were processed manually at optimal temperature, digested for over 2 h, and crystallized on a fluoropolymer surface without added fluorinated surfactant. A detailed description of how the MALDI spectra were analyzed is presented in the Supplemental Information. It is evident that even under the optimal conditions for sample preparation, interference from Pluronics is substantial. Alternatively, when using a fluorinated liquid to enhance droplet movement and/or PFOA in the matrix solution, high quality MALDI spectra can be obtained directly from the device surface without the need for additional, ex situ sample purification steps.
**Figure 6.8. In situ DμF peptide mass fingerprinting mass spectra.** Representative spectra of lysozyme (a) and cytochrome c (b) tryptic digests. The spectra are offset for comparison. The upper spectra in each plot were acquired from samples processed on a digital microfluidic device in a fluorinated liquid environment with 0.005% OBG in the protein drops and 0.0075% PFOA in the matrix drop. The lower spectra in (a) and (b) correspond to samples containing 0.06% Pluronic F-68 that were processed manually and crystallized on a fluoropolymer surface. The insets show a close-up of the lower spectra. Tryptic peptides from the top spectra identified by MASCOT exhibit sequence coverages of 45% and 64% for lysozyme and cytochrome c, respectively. Spectra in (c) and (d) correspond to lysozyme and cytochrome c tryptic digests, respectively, that were processed manually using extended digestion times (> 1 hr), optimal digestion temperature (37 °C), and were crystallized on a stainless steel MALDI plate. The spectra are normalized to the maximum signal intensity of the corresponding spectra in (a) and (b).

As seen in Figure 6.8, spectra obtained from on-device sample processing (top spectra in a and b) are of comparable quality to spectra obtained from samples processed manually under optimal conditions (c and d). While the samples processed manually exhibited higher signal intensity, the number of peptides identified and sequence coverage of the digested proteins were similar for the microfluidic and manual sample processing techniques. For both the manual and the digital microfluidic preparation of lysozyme, the MALDI spectra exhibited 7 peptides identified and a
sequence coverage of 45%. For the cytochrome c analysis, the digital microfluidic approach resulted in the identification of 13 peptides for a sequence coverage of 64%, compared to 15 identified peptides and a sequence coverage of 79% for the manual sample preparation approach. A complete peptide mass fingerprinting protocol was performed in less than a quarter of the time of the manual approach and required no manual pipetting steps apart from the initial sample loading. The reduced time, elimination of manual sample handling, and reduced reagent volumes compared to manual preparations are important advantages of performing assays at the microscale, and demonstrate the potential for the digital microfluidic platform to enhance proteomics through automated sample processing. To our knowledge, this is the first time in which the complete protein preparation process, from droplet dispensing to mass spectra acquisition, has been performed entirely in situ.

6.5 Conclusions

We have demonstrated the ability to manipulate protein solutions on a digital microfluidic device without the need for ex situ surfactant removal, allowing us to perform MALDI sample processing steps and crystallization completely in situ. While the use of Pluronics remains essential for the manipulation of concentrated protein solutions, fluorinated liquids provide an alternate means of facilitating droplet movement when surfactants can potentially interfere with the analysis of dilute protein solutions. The benefits of using a fluorinated liquid to enable movement of protein solutions may be extended to other digital microfluidic processes in which surfactant additives are undesirable, such as cell based assays in which surfactants may impact cell function.[259] We have also shown that the addition of perfluorooctanoic acid to the MALDI matrix solution can significantly enhance MALDI mass spectra obtained from fluoropolymer surfaces by reliably forming evenly distributed matrix crystals on the hydrophobic
surface. These enhancements make the digital microfluidic approach to MALDI sample preparation a viable option for high-throughput proteomic analyses. More generally, these strategies can be used to minimize protein adsorption and improve crystallization in other types of lab-on-a-chip devices.

6.5.1 Acknowledgements

The authors acknowledge Drs. Joseph and Rachel Loo (UCLA) for assistance with MALDI analysis as well as Dr. Heather Maynard (UCLA) for support with fluorescent imaging. This work was supported by the NSF IGERT: Materials Creation Training Program (MCTP) – DGE-0654431, and the California NanoSystems Institute.
6.6 Supplemental Information

Figure 6.S1. Comparison of matrix co-crystallization conditions. A comparison of typical crystal morphology for digested FITC-BSA samples crystallized on Cytop with (a) and without (b) PDFOA in the matrix solution. Samples of digested FITC-BSA containing 0.02% Pluronic® F-68 crystallized on Cytop with (c) and without (d) PDFOA in the matrix. All samples crystallized with PDFOA exhibited the typical crystal structure of long, needle-like crystals originating from the outer rim of the spot. Samples crystallized without PDFOA exhibited unpredictable crystal structure. Samples crystallized without PDFOA but with 0.02% F-68 consistently formed compact crystal clusters.
All mass spectra were acquired from a Voyager DE-STR Mass Spectrometer from Applied Biosciences. The instrument settings for all spectra acquired were as follows:

Mode of operation: Reflector
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

Accelerating voltage: 20000V
Grid Voltage: 66%
Mirror voltage ratio: 1.12
Guide wire 0: 0%
Extraction delay time: 170 nsec

Laser intensity: 2716 – 2866
Laser Rep Rate: 20.0 Hz
Calibration matrix: 2,5-Dihydroxybenzoic acid
Timed ion selector: Off

TIS gate width: 30
TIS flight length: 1167

Prior to analysis by MASCOT, all spectra were modified in the Data Explorer™ (Applied Biosystems) according to the following modifications:

Gaussian Smooth: 7 points
Baseline Correction: yes
Peak insertion: when necessary

All spectra were internally calibrated using either a peptide standard, trypsin autolysis peak, or both.

Below are the mass spectra used in Figures 6.7 and 6.8. The peptides identified by MASCOT are listed along with the MOWSE score and protein identification.
Figure 6.S2. Peptides identified by MACOT for the spectrum in Figure 6.7 (with PDFOA). Spectrum calibrated to known peak at m/z = 1377.8344. * = identified peak.

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<td>R.LFTGHPETLK.F</td>
</tr>
<tr>
<td>65 - 78</td>
<td>1377.8344</td>
<td>1377.8344</td>
<td>1377.8344</td>
<td>0</td>
<td>0</td>
<td>K.HGTVVLTALGGILK.K</td>
</tr>
<tr>
<td>120 - 134</td>
<td>1501.5314</td>
<td>1501.5314</td>
<td>1501.6620</td>
<td>-87</td>
<td>0</td>
<td>K.HPGDFGADQQAMTK.A</td>
</tr>
<tr>
<td>147 - 154</td>
<td>940.8132</td>
<td>940.8132</td>
<td>940.4654</td>
<td>370</td>
<td>1</td>
<td>K.YKELGFQQ.</td>
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</table>

Match to: MYG_EQUBU Score: 110  Expect: 5.3e-06  
Myoglobin OS=Equus burchelli GN=MB PE=1 SV=2  
Nominal mass (Mr): 17072; Calculated pI value: 7.21  
NCBI BLAST search of MYG_EQUBU against nr  
Unformatted sequence string for pasting into other applications

Taxonomy: Equus burchelli  
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
Number of mass values searched: 6  
Number of mass values matched: 6  
Sequence Coverage: 51%
Figure 6.S3. Peptides identified by MASCOT for the spectrum in Figure 6.7 (without PDFOA). Spectrum calibrated to known peak at m/z = 1814.8959. * = identified peak.

<table>
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<th>Observed</th>
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<th>Mr(calc)</th>
<th>ppm</th>
<th>Miss</th>
<th>Sequence</th>
</tr>
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<tr>
<td>2 - 17</td>
<td>1814.8959</td>
<td>1814.8959</td>
<td>1814.8952</td>
<td>0</td>
<td>0</td>
<td>M.GLSDGEWQQVLNVWGK.V</td>
</tr>
<tr>
<td>18 - 32</td>
<td>1606.0096</td>
<td>1606.0096</td>
<td>1605.8475</td>
<td>101</td>
<td>0</td>
<td>K.VEADIAGHGQEVLIR.L</td>
</tr>
<tr>
<td>33 - 43</td>
<td>1270.9807</td>
<td>1270.9807</td>
<td>1270.6557</td>
<td>256</td>
<td>0</td>
<td>R.LFTGHPETLEK.F</td>
</tr>
</tbody>
</table>

Match to: MYG_EQUBU Score: 58 Expect: 0.83  
Myoglobin OS=Equus burchelli GN=MB PE=1 SV=2  
Nominal mass (Mr): 17072; Calculated pH value: 7.21
NCBI BLAST search of MYG_EQUBU against nr
Unformatted sequence string for pasting into other applications  

Taxonomy: Equus burchelli  
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
Number of mass values searched: 3  
Number of mass values matched: 3  
Sequence Coverage: 27%
Figure 6.S4. Peptides identified by MASCOT for the spectrum in Figure 6.8a (with PDFOA). * = identified peak. s = internal peptide standard.

<table>
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<th>Start - End</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>ppm</th>
<th>Miss</th>
<th>Sequence</th>
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<tr>
<td>33 - 39</td>
<td>874.4707</td>
<td>873.4635</td>
<td>873.4093</td>
<td>62</td>
<td>0</td>
<td>R.HGLDNYR.G</td>
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<tr>
<td>52 - 63</td>
<td>1428.5655</td>
<td>1427.5582</td>
<td>1427.6429</td>
<td>-59</td>
<td>0</td>
<td>K.FESNFNTQATNR.N</td>
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<tr>
<td>64 - 79</td>
<td>1753.6607</td>
<td>1752.6535</td>
<td>1752.8278</td>
<td>-99</td>
<td>0</td>
<td>R.NTDGSTDYGILQINSR.W</td>
</tr>
<tr>
<td>80 - 86</td>
<td>936.4212</td>
<td>935.4139</td>
<td>935.3708</td>
<td>46</td>
<td>0</td>
<td>R.WWCNDGR.T</td>
</tr>
<tr>
<td>115 - 130</td>
<td>1803.7220</td>
<td>1802.7148</td>
<td>1802.8886</td>
<td>-96</td>
<td>1</td>
<td>K.KIVSDGNGMNANWAVWR.N</td>
</tr>
<tr>
<td>116 - 130</td>
<td>1675.6655</td>
<td>1674.6582</td>
<td>1674.7937</td>
<td>-81</td>
<td>0</td>
<td>K.IVSDGNGMNANWAVWR.N</td>
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<tr>
<td>135 - 143</td>
<td>1045.5642</td>
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<td>1044.5352</td>
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Match to: LYSC_CHICK Score: 155 Expect: 1.7e-10
Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1
Nominal mass (Mr): 16228; Calculated pI value: 9.37
NCBI BLAST search of LYSC_CHICK against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Gallus gallus
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 7
Number of mass values matched: 7
Sequence Coverage: 45%
Figure 6.S5. Peptides identified by MASCOT for the spectrum in Figure 6.8b (with PDFOA). * = identified peak.

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<th>Start - End</th>
<th>Observed</th>
<th>Mr(expt)</th>
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<td>- MSDVEKGKKIFVQK.C</td>
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<td>24 - 39</td>
<td>1675.8798</td>
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<td>1674.9067</td>
<td>-20</td>
<td>2</td>
<td>K. GGKHKTGPNLHGLFGR.K</td>
</tr>
<tr>
<td>27 - 39</td>
<td>1433.7133</td>
<td>1432.7060</td>
<td>1432.7688</td>
<td>-44</td>
<td>1</td>
<td>K. HKTGPNLHGLFGR.K</td>
</tr>
<tr>
<td>27 - 40</td>
<td>1561.8344</td>
<td>1560.8271</td>
<td>1560.8637</td>
<td>-23</td>
<td>2</td>
<td>K. HKTGPNLHGLFGRK.T</td>
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<td>29 - 39</td>
<td>1168.5720</td>
<td>1167.5647</td>
<td>1167.6149</td>
<td>-43</td>
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<td>1296.6682</td>
<td>1295.6609</td>
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<td>1</td>
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<td>40 - 54</td>
<td>1598.7415</td>
<td>1597.7342</td>
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<td>R. KTGQAPGFTYTDANK.N</td>
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<td>40 - 56</td>
<td>1840.8555</td>
<td>1839.8482</td>
<td>1839.9115</td>
<td>-34</td>
<td>2</td>
<td>R. KTGQAPGFTYTDANKNK.G</td>
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<td>41 - 54</td>
<td>1470.6226</td>
<td>1469.6153</td>
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<td>41 - 56</td>
<td>1712.7707</td>
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<td>K. TQAPGFTYTDANKNK.G</td>
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<td>1734.9427</td>
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<td>-39</td>
<td>4</td>
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<tr>
<td>90 - 101</td>
<td>1478.7817</td>
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<td>1477.8140</td>
<td>-27</td>
<td>2</td>
<td>K. TEREDLIAYLKK.A</td>
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Match to: CYC_HORSE Score: 224 Expect: 2.1e-17
Cytochrome c OS=Equus caballus GN=CYCS PE=1 SV=2
Nominal mass (Mr): 11825; Calculated pI value: 9.59
NCBI BLAST search of CYC_HORSE against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Equus caballus
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 13
Number of mass values matched: 13
Sequence Coverage: 64%
Figure 6.8c. * = identified peak. s = internal peptide standard.

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<th>Mr (calc)</th>
<th>ppm</th>
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<td>874.5295</td>
<td>873.5222</td>
<td>873.4093</td>
<td>129</td>
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<tr>
<td>52 - 63</td>
<td>1428.6800</td>
<td>1427.6727</td>
<td>1427.6429</td>
<td>21</td>
<td>0</td>
<td>K.FESNFNTQATNR.N</td>
</tr>
<tr>
<td>64 - 79</td>
<td>1753.7850</td>
<td>1752.7777</td>
<td>1752.8278</td>
<td>-29</td>
<td>0</td>
<td>R.NTDGSTDYGILQINSR.W</td>
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<tr>
<td>80 - 86</td>
<td>936.5040</td>
<td>935.4967</td>
<td>935.3708</td>
<td>135</td>
<td>0</td>
<td>R.WWCDGDR.I</td>
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<tr>
<td>115 - 130</td>
<td>1803.8526</td>
<td>1802.8453</td>
<td>1802.8886</td>
<td>-24</td>
<td>1</td>
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<td>116 - 130</td>
<td>1675.7971</td>
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</table>

Match to: LYSC_CHICK Score: 151 Expect: 4.2e-10
Lysozyme C OS=Gallus gallus GN=LYZ PE=1 SV=1 Nominal mass (Mr): 16228; Calculated pI value: 9.37
NCBI BLAST search of LYSC_CHICK against nr

Taxonomy: Gallus gallus
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 7
Number of mass values matched: 7
Sequence Coverage: 45%
Figure 6.S7. Peptides identified by MASCOT for the spectrum in Figure 6.8d. * = identified peak. s = internal peptide standard.

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<th>Mr (calc)</th>
<th>ppm</th>
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<td>27 - 39</td>
<td>1433.8339</td>
<td>1432.8266</td>
<td>1432.7688</td>
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<td>1</td>
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<td>27 - 40</td>
<td>1561.8831</td>
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<td>1167.6149</td>
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<td>1598.7983</td>
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<td>1605.9209</td>
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<td>22</td>
<td>1</td>
<td>K. TEREDLIAYLK.K</td>
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</table>

**CYC_HORSE Score:** 251  
**Expect:** 4.2e-20  
**Nominal mass (Mr):** 11825  
**Calculated pI value:** 9.59  
**NCBI BLAST search of CYC_HORSE against nr**  
**Unformatted sequence string for pasting into other applications**  
**Taxonomy:** Equus caballus  

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P  
Number of mass values searched: 15  
Number of mass values matched: 15  
Sequence Coverage: 79%  
Matched peptides shown in Bold Red
Figure 6.S8. **Protein adsorption data.** (a – e) Fluorescence images showing adsorption of known amounts of protein to the hydrophobic surface of the device. To determine the sensitivity of fluorescence microscopy for detecting FITC-BSA adsorbed to the device surface 2 μL drops of various, known concentrations were deposited onto a Teflon® coated surface and allowed to dry. The dried protein spots were then imaged by fluorescence microscopy. (f) The sensitivity analysis reveals that as little as 20 fmol of protein is readily detected using fluorescence microscopy. (g) Figure 6.4 with the addition of fluorescence intensity data for 20 and 200 fmol of adsorbed FITC-BSA (data added as the rightmost columns). The addition of Pluronics®, or engulfing the droplets in fluorinated liquids, reduces the adsorption of proteins substantially compared to a droplet without surfactant additives sitting in air (leftmost column); protein adsorption is reduced to 20 fmol or less.
Chapter 7. Conclusions and Future Directions

7.1 Key findings and contributions

Chapters 4-6 represent the accumulation of five years of research towards the development of biomedical applications for the digital microfluidic platform. This work has contributed a number of advances to both digital microfluidics and three-dimensional, *in vitro* cell culture. The most significant contributions of this work are outlined below:

1) **Hanging drop functionality for DμF devices:** The primary advantage of incorporating hanging drop functionality into a DμF device is the ability to create or manipulate three-dimensional objects that exceed the thickness of the inter-plate spacing of the DμF device. While various DμF devices have been developed to manipulate or assay three-dimensional objects, each method limits the size of any object formed or handled on the device to the thickness of the inter-plate spacing.[25,52,53] As the inter-plate spacing is increased, larger electrode dimensions and higher voltages are required to enable the movement of droplets. The use of through holes to store liquid or objects outside of the plane of the inter-plate gap allows a much broader range of volumes to be handled and maintained on a DμF device while providing greater flexibility in the electrode size and operating voltage. Added benefits are that the through-holes can be used to transport liquids in three-dimensions; by stacking multiple devices vertically, droplets of liquid can be delivered to a through hole in either the top or bottom plate and be drawn through the hole from one level to another.
The ability to form stable hanging drops that are individually addressable represents a novel functionality for the DμF platform that enables a variety of new assays and techniques. In addition to enabling the formation of spheroids, hanging drops are used commonly in protein crystallization assays, oocyte/embryo culture, and bacterial motility assays [260-264]; with the ability to automate the formation and interrogation of hanging drops, DμF can be used to automate these assays or protocols. Any assay or technique that utilizes pendant drops can benefit from automated liquid handling using the DμF platform described here.

2) Automation of cell spheroid culture: With the ability to create and maintain hanging drops, the DμF platform described here enables automation of the formation, maintenance, and analysis of cell spheroids either in solution or in a hydrogel scaffold. Because liquid handling can be completely automated, the DμF platform requires minimal manual sample handling, thereby reducing the tedium associated with traditional hanging drop culture methods. To our knowledge, aside from robotic liquid handling instruments, the DμF platform described here is the only tool that can automate the formation and assaying of individual cell spheroids. While robotic liquid handling instruments support higher throughput than is possible using DμF, the costs of acquiring, operating, and maintaining robotic liquid handling instruments that are optimized for cell culture can be prohibitive for many research labs. The degree of throughput provided by these instruments can also be superfluous for academic research. Additionally, a wide range of in situ sample handling and analytical capabilities developed for the DμF platform, such as magnetic and electrophoretic bead separation, electrochemical detection, localized heating, and sol-gel handling, would be difficult or impossible to achieve using robotic liquid handling systems. Thus, an optimized
DμF platform would enable spheroid-based assays that are more sophisticated, yet easier to perform than is possible using current spheroid culture techniques.

The work presented here represents the first demonstration of using the DμF platform for the culture of discrete multicellular spheroids. Other DμF techniques have shown that encapsulating a suspension of cells in a gel-post that spans the inter-plate gap allows cells to form small cellular aggregates through clonal growth or interactions with neighboring cells.[52,53] Such aggregates, however, do not possess the compact, high-cell-density characteristics of multicellular spheroids. These methods also do not provide control over the number, size, or shape of the aggregates that form within the gel. Such techniques also require an extracellular scaffold to support cell-growth in three-dimensions. The platform described here allows the growth of large, compact, and individual spheroids of uniform size and shape, either in solution or in the presence of a scaffold. The size of the spheroids is not limited to the inter-plate spacing, as is the case for the gel-post techniques.

A unique advantage of DμF spheroid culture is the ability to generate biological samples in situ. Unlike most analytical microfluidic systems, in which a sample is introduced to the system and then processed or analyzed in a certain fashion, this DμF platform enables the formation and generation of samples in situ. For example, the aggregation of individual cells into a single spheroid results in the formation of a new sample that is functionally distinct from the individual cells that comprise it. Additionally, protein or genetic secretions from spheroids grown in situ can conceivably be harvested, processed, and analyzed on the same DμF device. Thus, DμF can automate entire bioassays, from sample generation through
sample analysis, entirely in situ, which allows for enhanced standardization, minimal manual intervention, and minimization of assay variability.

3) Alternative sample handling techniques for proteomics analysis: In situ handling and analysis of biological solutions is one of the main applications of digital microfluidics. One of the major challenges with the handling of biological solutions is the propensity for proteins to denature and adsorb to the hydrophobic surface of the DμF devices. The adsorption of proteins to the device surface reduces the hydrophobicity of the surface, making the movement of aqueous solutions difficult or impossible. Thus minimizing protein adsorption is critical to the handling of biological solutions. To this end, we developed protocols for handling biological solutions on a DμF device that do not rely on the presence of surfactants, which can interfere with certain analytical techniques such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). We showed that encapsulating proteinaceous drops in fluorinated liquids minimizes the adsorption of proteins to the device surface, facilitating the movement of drops of protein solution. Because fluorinated liquids are more volatile than silicone oils, which have also been used to facilitate DμF droplet movement, they can be passively removed via evaporation at room temperature, allowing sample analysis without interference from oil residues. Using these fluorinated liquids allows complete automation of peptide mass fingerprinting sample preparation on a DμF device without the need to purify the samples of surfactants ex situ prior to MALDI-MS analysis. We also discovered that the addition of small amounts (<0.01% w/v) of the fluorinated surfactant pentadecafluorooctanoic acid to the MALDI matrix solution dramatically improves co-crystallization of protein samples with the matrix on the
fluorinated surface of the device. Together, these developments enable fully *in situ* peptide mass fingerprinting analysis on a DμF device. A DμF platform that facilitates fully integrated proteomics analysis would be useful in both applied and basic life-science research.

Collectively, this research has advanced the DμF platform for automating the handling and analysis of biological solutions. This work contributes novel techniques for device design, fabrication, and operation, and introduces new applications and capabilities for digital microfluidics. By enabling liquid handling as well as sample formation, processing, and analysis on a single device, this work has made meaningful contributions towards the development of a true lab-on-a-chip platform for *in vitro* tissue engineering research.

### 7.2 Future Directions

The work presented here achieved the primary goal of establishing capabilities for three-dimensional cell culture and analysis on a DμF platform. There are, however, many areas where this work can be expanded upon to further advance microfluidics, tissue engineering, and drug screening.

From a device perspective, certain design and fabrication improvements would enhance the function and capabilities of the platform. For example, the use of Parylene-HT, a fluorinated parylene dielectric material, would provide both the dielectric protection and hydrophobic surface properties required for a DμF device.[265] Using this material would simplify and reduce the cost of device fabrication by eliminating the need to add the expensive Teflon® or Cytop® coating. Another design consideration would be the incorporation of micro-channels.
into the device to allow the continuous delivery or extraction of liquids from the hanging drops. While liquid exchange can be achieved successfully using solely electrowetting-based liquid handling, many tissues in vivo are exposed to a continuous perfusion of nutrients and metabolic products. Continuous perfusion capabilities would further increase the physiological relevance of the in vitro tissue microenvironment formed on the DμF device. The DμF platform described here would also be significantly improved by incorporating more advanced in situ analytical capabilities and increasing the well-density on the devices to support higher throughput spheroid culture. While the microfluidic control system used in this research was limited to 48 individually addressable electrodes, more advanced research and commercial-grade systems support hundreds of individually addressable electrodes.[40,48,171] Such systems would enable the formation of dozens of spheroids on a single chip. These systems also contain integrated volume metering and magnetic separation capabilities, which would improve the precision and reproducibility of the droplet and spheroid volumes and enable powerful in situ analytical capabilities. A more advanced microfluidic control system enabling integrated temperature, atmosphere, and humidity control is also necessary to realize the full potential of the DμF platform described here.

Various cell-culture and analytical processes and protocols remain to be validated on this platform. The spheroid-based culture and assay protocols developed in this work carried spheroid culture out to 96 h. Many assays of interest, such as the spheroid growth-delay drug toxicity assay require up to 14 days of hanging drop culture.[81] Although the hanging drop medium exchange protocols described in this work support sufficient medium exchange for long-term culture, the limits of spheroid growth, viability, and function in long-term culture need to be
elucidated for platform optimization. Additionally, culture protocols should be developed and validated for as many cell and tissue types as possible, including primary cells, stem cells, genetically modified cell lines, and even whole organisms such as zebrafish embryos or *C. elegans*. Exploring the culture of a wide variety of cell types for extended periods of time will further establish the capabilities and potential applications for the DµF platform. Additionally, in this work, we utilized primarily morphological analyses (spheroid diameter/shape, cell migration distance) to characterize spheroids. Future work would benefit from a more thorough molecular characterization of the spheroids to provide deeper insight into the molecular and cellular processes governing spheroid formation, growth, and response to stimuli. For example, immunohistochemical analysis of spheroids would allow better visualization of the spheroid interior and the cell organization and molecular distribution within the spheroid than confocal microscopy, which is limited in its ability to image deep into dense, three-dimensional tissues. Additionally, staining for molecular markers such as integrins, cadherins, and myosin proteins would provide a better understanding of the migration mechanisms responsible for cell invasion from encapsulated spheroids. Also, staining for hypoxia inducible factor and reactive oxygen species would allow the monitoring of cell response to hypoxic conditions within the interior of large spheroids. Such molecular analyses would yield valuable insights into the formation and behavior of three-dimensional tissues.

Optimization of the DµF device hardware as well as the cell-culture and analytical protocols for a variety of cell types would enable the development of more sophisticated three-dimensional tissue models and assays. For example, three-dimensional co-culture systems, consisting of more than one cell type, provide insights into intercellular interactions and communication, as
well as cellular organization and function, thereby offering a more advanced tissue model than single-cell-type systems. [266-268] Of particular interest is the potential to develop a human-microbial co-culture system which would provide an *in vitro* model of tissue-microbial interactions. Microbes are found throughout a variety of human tissues, but are especially crucial to gastrointestinal function and health.[269] The activity of the gut microbiota, for example, is known to impact the metabolism, toxicity, and efficacy of a wide variety of drugs, and is implicated in the progression of colorectal cancer.[270-272] Thus, *in vitro* tools that can effectively model human-microbial interactions in the tissue microenvironment would help enhance our understanding of the human microbiota and be useful in drug development.

Another application enabled by an optimized DμF spheroid culture system is the modeling of multi-organ systems. Because each spheroid can be addressed individually and cultured in a unique microenvironment, an optimized DμF spheroid culture platform could enable the formation of multiple different types of spheroids on a single device while allowing controlled communication between the spheroids. For example, a chip containing spheroids of various tissue types, such as esophagus, stomach, liver, kidney, pancreas, and colon tissues, could serve as an *in vitro* model of drug metabolism and accumulation as a drug is delivered from one organ to the next along a metabolic pathway. Alternatively, spheroids of a variety of healthy and diseased tissues could be cultured on a single device and exposed to drug candidates in order to assess the tissue-specific toxicity profile of the drug. A wide variety of *in vitro* body-on-a-chip systems could be developed using DμF for cell spheroid culture and analysis.
Combined with the advances in DμF droplet handling precision and throughput, *in situ* sample analysis, and hardware integration achieved in recent years, the novel features and protocols developed in this work establish a blueprint for a fully-integrated system for the culture and analysis of three-dimensional micro-tissues. The realization of an integrated system for automating three-dimensional cell culture would provide researchers with a tool with unmatched liquid handling and assay capabilities, facilitating greater adoption of three-dimensional tissue models in routine biomedical research.
Chapter 8. References


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