

A Droplet Microfluidic Platform Used to Encapsulate Single Pre-formed Cancer Spheroids in Hydrogel Microenvironments

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Cancer has been a leading cause of death around the world for many years. Even with the emerging technologies seen in these times, there is still a lack of truly personalized treatments since cancer tumors are not fully understood, especially between different cancer patients. 3D models known as patient derived tumor organoids (PDTOs) have been gaining traction as *in vitro* models to mimic a patient's tumor outside of their body. Grown from the patients' cells into cell clusters (spheroids) or organoids (mini parts of the tumor tissue), these models can serve as precise avenues for personalized drug discovery or studying tumor complexity. However, there are still limitations to these 3D models due to conventional fabrication methods. In order to achieve personalized care, the models being tested on should be consistent through each batch, reliable, and affordable. Traditionally, organoid development from cells aggregating in hanging droplets or rotating in flasks for maintained cell-aggregate suspension, can cause batch-to-batch inconsistencies (lack of uniformity between samples), low throughput, and often require high volume of reagents.

To recreate a 3D suspension for cells in a more native environment, hydrogels have become popular biocompatible scaffolds capable of sustaining cell growth. Hydrogels themselves have been widely studied for ideal cell environments, yet common methods of cell encapsulation into these hydrogels (e.g. manually pipetting) do not address the mentioned limitations, but rather introduce inconsistencies.

Droplet microfluidics (DM), with the inclusion of hydrogels, has become a technology that has assisted in organoid fabrication, addressing conventional limitations. DM can create uniform aqueous droplets at high frequencies through controlled emulsion, using microchannels. These uniform droplets allow for controlled cell encapsulation with minimal reagent usage, thus addressing the drawbacks of traditional organoid formation and cell encapsulation techniques. Yet, the downside to using DM for cell encapsulation (whether it is multi- or single- cell encapsulation) is the high initial concentration of cells used at the inlet reservoir (millions), compared to the typical sample size obtained from patient biopsies (thousands).

To still leverage the advantages of DM, thoughts of encapsulating pre-formed cell clusters or spheroids (instead of forming them with the DM devices) can aim to lower the number of initial cells used, and aid in growing spheroids into organoids. Thus, the proposal of this thesis was to employ DM and defined hydrogels to encapsulate pre-formed spheroids into their own microenvironment, for the goal of supporting the growth of primary cancer patient cells into PDTOs in a robust and uniform

manner. This thesis intends to give a better understanding of the need of using DM for pre-formed spheroid encapsulation, and the ways to achieve a robust system for this purpose.

The first chapter of this thesis provides a deeper background of the motivation to the overarching goal and a further breakdown of the project tasks. Specifically, projects were designed around two aims: (1) optimizing portions of the system and (2) validating the system. In addition to this background information, more context is given through a literature review found in Chapter 2. This literature review further justifies the rationale of the work by providing more insight into each component.

After outlining protocols used in each project (Chapter 3), the optimization work began, starting with finding the best way to form spheroids that would enter the DM device (Chapter 4). It was important that the mechanism of forming these spheroids was also robust and uniform to maintain the advantage over conventional spheroid fabrication techniques. A well-established micropattern technology known as the AggreWell™ was implemented due to its capabilities of forming uniform spheroids in a high throughput manner with a low quantity of cells. Using PDMS replicas of the AggreWell™ (referred to as PDMS AWs) caused for adjustments to typical protocols followed when using the original AggreWell™. Parameters such as surface treatment, cell culture conditions, and collection methods, were studied to find the most appropriate outcome of spheroids for the intended application.

Continuing in the avenue of optimization, the other portion of Chapter 4 focused on exploring DM devices and fabrication techniques. After comparing parameters such as mold fabrication through 3D printing and soft lithography, chip design and geometry (T-junction versus double flow focusing (DFF)), and system tubing size, the selected chip design was a DFF junction, similar to one used in previous work of single cell encapsulation. The other factors ensured that uniform droplet formation and single pre-formed spheroid encapsulation could be achieved, in part of making the system robust.

Using the decisions made in the optimization work, validation of hydrogel selection, encapsulation efficiency, and spheroid viability were assessed in the main chapter (Chapter 5). This thesis work aimed to demonstrate the capabilities of the DFF DM device with the use of stratified flow. A hydrodynamic focusing stream of either Gelatin Methacrylate (GelMA) or sodium alginate hydrogel precursors helped to gather spheroids into their own droplets. Depending on the flow conditions, the width of the flow focusing stream varied, affecting the encapsulation efficiency. Along with focusing width, the concentration of spheroids at the inlet reservoir also affected the encapsulation efficiency. This study was able to show spheroid encapsulation with a range of spheroid quantities; from 1000 to 7000 total spheroids in 500 μ L of hydrogel precursor.

Lastly, the spheroid-laden hydrogel droplets were crosslinked and assessed for stability in cell culture conditions over time. With this, the spheroid viability was also tested to ensure the overall system was not too harsh on them. As most spheroids showed a high viability, this was enough to satisfy the proposed objective and conclude that this system has the potential to be further explored.

In conclusion, the overall system showed success in robustly encapsulating pre-formed spheroids, in hopes of being applied to patient derived samples for uniform growth into relevant 3D models. As this work is preliminary, Chapter 6 outlines recommendations and suggestions to future work, to guide this project towards the overarching goal.

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Table of Contents

Author's Declaration	ii
Abstract	iii
Acknowledgements	vi
List of Figures	x
List of Tables.....	xiv
List of Abbreviations.....	xv
Chapter 1 : Introduction.....	1
1.1 Background	1
1.2 Research Objectives	3
1.3 Thesis Outline.....	4
Chapter 2 : Literature Review	6
2.1 Introduction	6
2.2 Patient Derived Tumor Organoids.....	6
2.2.1 Spheroids vs organoids.....	6
2.2.2 Conventional organoid fabrication methods and their limitations	8
2.2.3 Recent technology for improved organoid fabrication.....	9
2.3 Hydrogels as 3D Matrices	10
2.3.1 Hydrogel crosslinking mechanisms.....	10
2.3.2 Hydrogels used for cell support and growth.....	12
2.4 Droplet Microfluidics	13
2.4.1 Types of droplet microfluidic devices	13
2.4.2 Hydrogels used in droplet microfluidics	15
2.4.3 Applications of droplet microfluidics for the formation of spheroids and organoids.....	16
2.4.4 Single bio-particle encapsulation using droplet microfluidics	17
2.5 Summary and Perspective	17
Chapter 3 : Experimental Setup and Methods.....	19
3.1 Introduction	19
3.2 Forming Spheroids Using PDMS Replicas of the AggreWell™ (PDMS AWs).....	19
3.2.1 PDMS AggreWell™ replica fabrication.....	19
3.2.2 Cell seeding into PDMS AggreWell™ replicas	20
3.2.3 Spheroid collection.....	21

3.3 Droplet Microfluidic (DM) Device Fabrication and Setup	21
3.3.1 PDMS DM chip fabrication.....	21
3.3.2 DM chip accessories and setup.....	22
3.4 Hydrogel Fabrication and Characterization.....	22
3.4.1 Gelatin Methacrylate (GelMA) fabrication	22
3.4.2 Sodium Alginate and Calcium Chloride fabrication	23
3.4.3 Compression testing	23
3.4.4 Viscosity measurements	24
3.5 Hydrogel Microparticle (HMP) Collection and Washing	24
3.5.1 Crosslinking HMPs	24
3.5.2 Washing HMPs.....	24
3.6 Cell Viability	24
Chapter 4 : Optimization of System Components	25
4.1 Introduction	25
4.2 Optimizing Spheroid Formation Using PDMS Replicas of the AggreWell™	25
4.2.1 PDMS AggreWell™ replica treatment effect on spheroid growth.....	25
4.2.2 Spheroid collection methods	31
4.2.3 Effect of spheroid size and cohesiveness based on culture time	32
4.3 Droplet Microfluidic (DM) Device Parameter Optimization	33
4.3.1 DM chip molds fabricated using 3D printing vs soft lithography	34
4.3.2 DM junction comparison.....	37
4.3.3 Inlet tubing inner diameter effect on spheroids entering the DM device	38
4.4 Summary of parameters.....	39
Chapter 5 : Studying a Droplet Microfluidic System for Single Pre-formed Cancer Spheroid Encapsulation in Hydrogel Microparticles	41
5.1 Introduction	41
5.2 Droplet Microfluidic Device and System Selection	42
5.3 Hydrogel Criteria in the Droplet Microfluidic System.....	44
5.4 Flow Focusing Width Variation and its Effect on Spheroid Encapsution.....	48
5.4.1 Narrow flow focusing width.....	49
5.4.2 Medium flow focusing width	50
5.4.3 Wide flow focusing width	52

5.5 Spheroid Concentration Variation	53
5.6 Hydrogel Microparticle (HMP) Droplet Collection	55
5.6.1 HMPs after crosslinking and washing	58
5.6.2 Compression testing on crosslinked HMPs	60
5.7 Spheroid Viability	62
5.7 Discussion	64
5.8 Summary	65
Chapter 6 : Concluding Remarks	67
6.1 Thesis Conclusions	67
6.2 Recommendations and Future Directions.....	69
6.2.1 Recommendations	69
6.2.2 Future Directions	70
Letters of Copyright Permission.....	73
References	74
Appendices	90
Appendix A: Spheroid Counting	90
Appendix B: LEXT Images of Different DM Molds	91
Appendix C: Classification of Flow Focusing Width	92
Appendix D: Representative Images of Live/Dead Assay on Spheroids Encapsulated in Alginate-GelMA HMPs.....	93
Appendix E: Experimental Setup and Methods Images	96
Appendix F: Droplet Content Graphs.....	97

List of Figures

Figure 1-1: Flow chart of thesis project tasks.....	3
Figure 2-1: Simplified illustration of a cell, a spheroid, and an organoid for comparison. [Created with BioRender.com]	7
Figure 2-2: Illustrations of different common techniques for fabricating organoids. From “Bhattacharya, A., Alam, K., Roy, N.S. et al. Exploring the interaction between extracellular matrix components in a 3D organoid disease model to replicate the pathophysiology of breast cancer. J Exp Clin Cancer Res 42, 343 (2023)” used under Creative Commons CC-BY-4.0 DEED, open access [67].	8
Figure 2-3: Droplet generation through passive and active techniques. From “P. Zhu and L. Wang, Lab Chip, 2017, 17, 34” used with permission from Royal Society of Chemistry [117].	14
Figure 2-4: Types of droplet microfluidic junctions. (A) T-junction (B) Flow-focusing (C) Coaxial. From “D. Liu, M. Sun, J. Zhang, R. Hu, W. Fu, T. Xuanyuan and W. Liu, Analyst, 2022, 147, 2294” rearranged under creative common CC-BY-NC 3.0 DEED, open access [118].	15
Figure 4-1: Cells seeded at different densities in untreated PDMS replicas of the AggreWell™. (a) L929 cells at t = 0 days (b) the collection of the L929 spheroids at t = 48 hrs; the top pictures were taken from the microscope eyepiece; the bottom pictures were taken from the lens view (scale bar = 100 µm). (c) Images of pyramid-microwells where L929 cells are attached to the PDMS surface (d) Images of MDA-MB-231 cells that experienced the same attachment to the PDMS.....	26
Figure 4-2: Different parameters implemented on the culture conditions of the PDMS AggreWells™. All PDMS AWs had a seeding density of 120k cell/PDMS AW (100 cells/spheroid) and were cultured for 24 hrs in cell culture media cocktail, unless otherwise specified. (a) Different concentrations of serum (FBS % v/v) in the cell culture media with L929 cells. (b) Effect of UV time on the PDMS during sterilization. MDA-MB-231 cells were cultured in the PDMS AWs afterwards with regular cell culture media. (c) Difference between using an old PDMS AW (made at least 2 weeks before cell seeding) and a new PDMS AW (less than 2 weeks old) for culturing MDA-MB-231 cells. (d) MDA-MB-231 cells in a new PDMS AW without UV treatment.	29
Figure 4-3: Cells seeded at different densities in Pluronic treated PDMS replicas of the AggreWell™. (a) L929 cells at t = 1 day (b) MDA-MB-231 cells at t = 3 days. The top pictures were in the PDMS AWs; the bottom pictures were the collection. Scale bars = 100 µm.	30
Figure 4-4: L929 spheroids reverse straining method implemented. (a) Schematic of the reverse straining process. (b) Images of collected L929 spheroids at different stages of the collection process:	

(i) not strained, (ii) strained sample (containing spheroids less than 70 μm and single cells), (iii) reverse strained, the desired solution of uniform spheroids ($> 70 \mu\text{m}$).	31
Figure 4-5: L929 spheroids at same initial concentration of 50 cells/spheroid (60 000 cells/PDMS AW). Spheroids were grown in the PDMS AggreWells TM and collected at different time points. Scale bars = 100 μm	33
Figure 4-6: Images of chips made from (a) 3D printed and sanded mold and (b) soft lithography silicon wafer mold. The channel walls are more defined and smoother when using soft lithography versus the bumpiness seen with 3D printing, even with gentle sanding.	35
Figure 4-7: A DM chip (running with polybeads) made from a 3D printed T-junction mold with channel dimensions of 100x100 μm . Instances of uneven droplet formation with this fabrication and geometry were not favored for the system. Green circles indicate uniform droplets, red circles show non-uniform droplets.	36
Figure 4-8: Encapsulation of polystyrene microbeads (90 μm) to demonstrate different geometries. (a) T-junction, 3D printed mold. (b) double flow focusing, silicon wafer mold. There were instances where the T-junction encapsulates more than one bead per droplet, where it looks like the DFF separated them, thus making it more favorable.....	38
Figure 4-9: Spheroids entering 3D printed T-junction chips with different inlet tubing diameters. Spheroid concentration at the inlet was $\sim 8000/\text{mL}$. (a) Inlet tubing with inner diameter of 0.030 in ($\sim 750 \mu\text{m}$). (b) Inlet tubing with inner diameter of 0.010 in ($\sim 250 \mu\text{m}$). A larger inlet tubing (750 μm) allowed for more spheroids (about 100 μm in diameter) to pile onto each other, not making it favorable when coming into the chip for single spheroid encapsulation.....	39
Figure 5-1: (a) Diagram of fluid flow through the DFF chip - 1) Continuous phase of mineral oil with 3% v/v Span 80; 2) dispersed phase 2 of a less viscous hydrogel precursor; 3) dispersed phase 1 of a more viscous hydrogel precursor mixed with spheroids; 4) outlet to droplet collection. (b) Schematic of chip geometry at the junctions for channel size and fluid flow division. Subscript definitions: dispersed phase 1 (D1), dispersed phase 2 (D2), continuous phase (C), junction (j), orifice (or), main channel (m). (c) PDMS chip with reference for size.	43
Figure 5-2: (a) Schematic of settling time of spheroids based on hydrogel precursor viscosity. Settling time is slower with increase of viscosity. (b) Approximately 4000 spheroids in 500 μL of 2% w/v sodium alginate. Spheroids showed significant settlement after about 20 minutes; pictures taken in 3-minute intervals.	46

Figure 5-3: Pseudo colorized images of each type of flow focusing width to help distinguish the streams: (a) Narrow, (b) Medium, and (c) Wide. Spheroid quantity was kept constant at 5000 spheroids, settled in 500 μL of 5% w/v GelMA ($\eta \approx 2$). Focusing width varied based on pressure change of the dispersed phase 1. Scale bars = 300 μm	49
Figure 5-4: Snapshots of successive time points for a narrow flow focusing width ($\lambda \approx 0.83$) with 5% GelMA, $\eta \approx 2$, and 5000 spheroids settled at the inlet. This case showed a low amount of encapsulation, about 27%. Yet, the narrow focus helped streamline the spheroids into their own droplet really well, considerable for targeting single spheroid encapsulation each time.	50
Figure 5-5: Snapshots of successive time points for a medium flow focusing width ($\lambda \approx 0.74$) with 5% GelMA, $\eta \approx 2$, and 5000 spheroids settled at the inlet. This case showed a decent amount of single spheroid encapsulations, about 48%.....	51
Figure 5-6: Snapshots of successive time points for a wide flow focusing width ($\lambda \approx 0.65$) with 5% GelMA, $\eta \approx 2$, and 5000 spheroids settled at the inlet. This case showed a high amount of single spheroid encapsulations, about 54%. However, it is coupled with many multi-encapsulations, not favorable for batch-to-batch consistency.....	53
Figure 5-7: Spheroid concertation variation with focusing width kept medium ($0.70 \leq \lambda \leq 0.75$). (a) Image of different spheroid quantities suspended in 500 μL of 2% w/v sodium alginate. (b) Varying spheroid quantities at the inlet reservoir depicted in (a). (c) Spheroid quantity variation settled in 500 μL of 5% w/v GelMA. Higher initial spheroid quantities allowed for more spheroids present for encapsulation into the hydrogel droplets. Scale bars = 300 μm	54
Figure 5-8: Collection baths for HMPs. (a) 2% w/v CaCl_2 solution. Droplets would collect on the side of the container and proceed to float to the top. (b) Mineral oil with 3% v/v Span 80. Droplets would fall directly to the bottom of the container. (c) 1% w/v CaCl_2 solution. Droplets exhibited similar behavior to the 2% w/v CaCl_2 bath. Arrows indicate movement of droplets. For aqueous solutions, droplets aggregated to the side and slowly floated up.....	58
Figure 5-9: HMPs after crosslinking and washing with media. The first two photos were droplets made from the alginate-GelMA combination, where the initial GelMA was dissolved in DPBS; (a) collected in cold 2% w/v CaCl_2 solution, (b) collected in 1% w/v CaCl_2 solution. (c) Droplets made from alginate-GelMA combination, where the initial GelMA was dissolved in PBS, and collected into cold mineral oil. (d) Droplets made from the GelMA-GelMA combination and collected into cold mineral oil. All HMPs were crosslinked with UV for 2 min.	59

Figure 5-10: Alginate-GelMA HMPs collected in 2% w/v CaCl_2 solution after being washed and culture in serum free media at 37°C. (a) Viewed after 24 hours. (b) Viewed after 5 days.	60
Figure 5-11: (a) Compression test stress-strain curves of different alginate-GelMA HMPs. The right-hand (blue) y-axis is meant for the 2% CaCl_2 curve. (b) Image of an HMP collected in mineral oil before compression ($t = 0$ sec) and at the maximum load ($t = 20$ sec) demonstrating the MicroTester.....	61
Figure 5-12: Ratio of green to red (live to dead) signal from Live/Dead assay at (a) 24 hours and (b) 5 days. Signals were normalized to spheroid area and averages of the spheroids in the representative photos were used as demonstration. Mineral oil at day 5 was not shown due to spheroid clusters overwhelming signals in coalesced HMPs.	62
Figure 5-13: HMPs cultured in serum media after 24 hours. Cells seemed to spread away from the spheroid, outside of the HMP. The spheroid circled in red shows the case where the cells (smaller dots) were moving away from the spheroid. The spheroid circled in green shows the case of a spheroid more intact.	63
Figure 6-1: Photo summary of the final system process	69

List of Tables

Table 2-1: Types of Crosslinking and Common Hydrogels Associated with Each Mechanism.....	11
Table 4-1: Potential Parameters Affecting Cell Attachment onto PDMS	27
Table 4-2: Descriptions of Various 3D Printed DM Molds	36
Table 4-3: Summary of Tested Parameters for Spheroid Formation Using PDMS AWs	39
Table 4-4: Summary of Tested Parameters DM Device Selection.....	40
Table 5-1: Inlet and Outlet Fluids Used for the Selected DFF Device.....	43
Table 5-2: Parameter Considerations for the DM System for Single Spheroid Encapsulation	44
Table 5-3: Hydrogel Preparation Information	47
Table 5-4: Classification of Flow Focusing Width Based on Viscosity Ratio of $\eta = 2$	48
Table 5-5: Comparison of Single Spheroid Encapsulation Efficiency for Varying Inlet Quantities ...	55
Table 5-6: Collection Methods and Parameters for Crosslinking HMPs after Droplet Formation	57
Table 5-7: Summary of Parameters for Overall System.....	66

List of Abbreviations

2D	Two Dimensional
3D	Three Dimensional
PDTO	Patient Derived Tumor Organoid
ECM	Extracellular Matrix
DM	Droplet Microfluidics
HMPs	Hydrogel Microparticles
MCSs	Multicellular Spheroids
GFs	Growth Factors
LCST	Low Critical Solution Temperature
UCST	Upper Critical Solution Temperature
GelMA	Gelatin Methacrylate
PDMS	Polydimethylsiloxane
PDMS AWs	PDMS AggreWells™
BSC	Biosafety Cabinet
DI	Deionized
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
DPBS	Dulbecco's Phosphate Buffered Saline
MA	Methacrylic Anhydride
CaCl₂	Calcium Chloride
SLA	Stereolithography
DFE	Double Flow Focusing
ID	Inner Diameter
FFW	Flow Focusing Width

Chapter 1: Introduction

1.1 Background

Cancer has been a prevalent disease that has affected millions over time. According to the Centers for Disease Control and Prevention (CDC), as of 2021 cancer is the second leading cause of death in the United States [1,2], with recent estimations of over six hundred thousand deaths in the year 2023 [2]. There is still a high cancer death toll despite significant developments in medicine, due to a lack of truly personalized treatment or the lack of understanding of tumor characteristics, especially between cancer types and patients [3–5]. Molecular based precision medicines, that target specific genes or biomarkers, are an example of recent medicines that have enhanced cancer prognosis [4–6]. However, limitations still rise with this approach as they are generalized for cancer types and do not account for tumor heterogeneity [4–8]. Additionally, typical models used for drug screenings and cancer studies, such as two-dimensional (2D) cell cultures or animal-derived models (patient-derived xenografts) have lacked physiological relevance compared to tumors from the human body, also making it difficult to treat the needs of specific cancer patients [9–11].

That's where the emerging model of cancer tumor organoids has become a new path for cancer research [3,9,11]. An organoid is a three-dimensional (3D), self-organizing tissue structure made to resemble an organ, or in the case of cancer organoids, it would resemble a tumor [11–13]. Ideally, a patient's primary tumor cells can be grown in a 3D microenvironment to mimic the inside of the body and develop into organoids outside of the body. These patient-derived tumor organoids (PDTOs) can be superior models to those currently used, and allow for fundamental or personalized medication studies, advancing the treatment and care of cancer patients [3]. Similarly, spheroids, which are cell clusters, have been used as simple 3D models for anti-cancer drug screening, as they can showcase cell interactions and properties of solid tumors, in a less complex manner [9,14].

That being said, the area of forming and screening these 3D models still has some limitations that need to be addressed. Most of these limitations stem from the method of production, where improvements are sought out in increasing throughput and reproducibility (batch-to-batch consistency), and lowering cost [15–17]. In traditional methods, 3D cell culture relies on aggregation in hanging droplets, air-liquid interfaces, or rotating flasks of cell culture media to avoid cell attachment to the bottom of a plate [16–18]. Alternatively, hydrogel scaffolds became a method of interest to encapsulate and suspend cells in an environment more native to them; the extracellular matrix (ECM). The ECM is an external substance that surrounds cells to provide them with structural and biochemical support

[19,20]. Hydrogels are natural, synthetic, or hybrid crosslinked polymers that form 3D networks capable of swelling to hold a sizable amount of water [21]. Hydrogels have piqued interest in replicating the ECM due to their favorable properties such as hydrophilicity, biocompatibility, controllable stiffness, and capability of suspending cells [20–24].

With well-defined hydrogels being developed to replace conventional ones that also decrease batch-to-batch consistency (Martigel) [20,25–28], there is still a limitation with conventional methods of encapsulating cells or larger bio-models inside the hydrogel scaffolds in a robust and controllable manner [17]. Conventional methods like manual pipetting, high-pressure emulsification, or nozzle dripping lack control and uniformity in droplet size, encapsulation content, repeatability, and large-scale production [29–32].

Droplet microfluidics (DM) is a technology that has been able to overcome the conventional droplet forming and cell encapsulation limitations because it can produce monodispersed droplets of hydrogels at high frequencies, and the parameters of the droplet size and content can be tuned and controlled more easily [33,34]. It is a technique that uses immiscible fluids to create discrete droplets. The flow is pressure driven and the regime is laminar, so this is safe for encapsulating biological entities such as cells, spheroids, and organoids [32,35,36].

DM has shown many instances of being a high-throughput system for forming spheroids and organoids through multi- and single-cell encapsulation [37–39]. Typically, the number of cells needed at the inlet reservoir is on the order of millions [40]. Yet, when considering sample quantity obtained from biopsies of cancer patients, that cell concentration is difficult to achieve as it's typical to acquire a few hundred thousands at most [41–43]. Also considering that traditional methods such as the hanging droplet, can use as low as thousands of cells [40,44], this should be factored into using DM as a tool for enhanced PDTO formation.

Thus, pre-formed spheroids can be considered as a means of encapsulating patient tumor cells while taking advantage of the DM technology for housing spheroids in their own microenvironment, for monitored growth into organoids, with controlled parameters. Since spheroids are much larger than single cells, a lower quantity and concentration can be used. In this case of needing to analyze larger bio-particles, or potentially jump-start the growth of organoids, there is a lack of studies that showcase the capabilities of droplet microfluidics for such encapsulation [45]. In this thesis, the aim is to demonstrate DM for large bio-particle encapsulation such as pre-formed spheroids.

1.2 Research Objectives

Due to the increase in demand of ideal 3D models for personalized cancer treatment, overcoming the limitations of current fabrication methods is the main motivation behind this project. Thus, the overall research will aim towards improving the growth and analyzation process of spheroids into organoids by implementing the high-throughput technology of droplet microfluidics for single-spheroid encapsulation, allowing for the controlled generation of numerous tunable microcapsules. Defined hydrogels will be leveraged to better house and support the growth of cancer spheroids, ultimately yielding more consistent lab results and aiding advancements in health research.

As there is a long-term goal of this system, it is ideal to begin with preliminary work to optimize and overcome any new challenges. This thesis work is the introductory portion to lead the overall project. The main objective of this thesis is to implement defined hydrogels into droplet microfluidic systems to encapsulate individual cancer spheroids and support their growth, creating a controlled microenvironment to grow organoids. To achieve this goal, two main aims were established to steer projects in a meaningful direction. Figure 1-1 displays a flow chart to visualize the breakdown of the tasks that led the project.

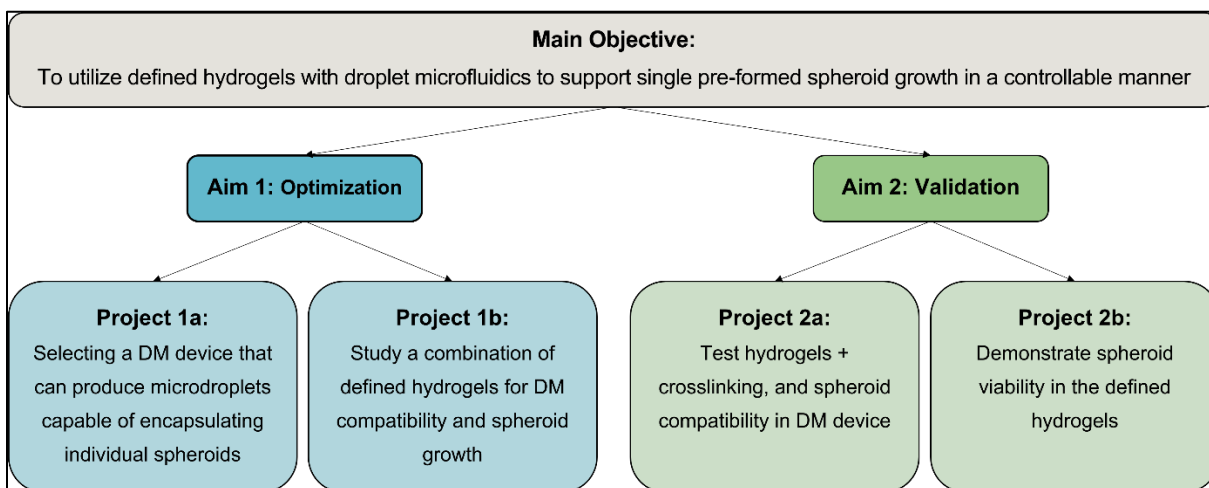


Figure 1-1: Flow chart of thesis project tasks

Since there are different components to this work, it was important to breakdown each to understand how to combine the contributing factors smoothly. The first aim was generalized to optimizing parts of the system. Project 1a was intended to seek the best droplet microfluidic device that could accommodate spheroids and produce the required microdroplet for single spheroid encapsulation with consistency.

Project 1b was centered around choosing appropriate hydrogels that would be able to run through the DM device and had biocompatibility for cellular growth. Although these projects separate portions of the system, they would be expected to be conducted simultaneously as they do affect each other.

Aim 2 was led towards validating the system. Project 2a was proposed to test how the alternative hydrogels, with single spheroid encapsulation, could be crosslinked compatibly with the droplet microfluidic device. To finish the system, Project 2b would demonstrate spheroid viability to check that the spheroids survive the overall process.

The approach to microfluidic chip optimization focused on using previous work towards single cell encapsulation and applying the channel geometries, droplet size, and hydrogel viscosity to be accommodating for pre-formed spheroids. The proposed selection of hydrogels was influenced by previous work and literature review while considering material properties such as polymer structures, stiffness, and crosslinking methods.

1.3 Thesis Outline

The expected layout of this thesis is explained in this section to wrap up Chapter 1 that was meant to introduce readers to the motivation behind this work and layout the aims and approach towards resolving the proposed need.

Chapter 2 follows with a thorough literature review to understand each main component of the entire work. First, establishing the difference between spheroids and organoids is outlined followed by limitations to their common formation techniques and the requirements for growth *in vitro* are explored. Next, hydrogels and their crosslinking mechanisms are touched upon. Work with cells and hydrogels is used to select options for implementing in this thesis work. Droplet microfluidics is then explained to gain an understanding of how the technology works, its application with hydrogels and 3D cell culture, and work done towards single particle encapsulation (to compare to single spheroid encapsulation). To wrap up the chapter, a brief summary and perspective is outlined to tie together the key take away points from the literature review and apply them to working on the goal at hand.

Chapter 3 is the methods and materials section used to explain the procedures and tools used for the main portion of this work. It starts by explaining how the spheroids were fabricated before entering the DM device. Then, the fabrication of the DM device is briefly outlined along with the required tools for system setup. Hydrogel fabrication is explained afterwards, also explaining the implementation of the spheroids into the hydrogels before entering the DM chip and crosslinking. Additionally, hydrogel characterization techniques are explained for compression testing and viscosity measurements. Briefly

after, methods used for the collection of hydrogel microparticles (HMPs) created by the DM device are described. Ending the chapter is the protocol used for cell viability screening.

Chapter 4 leads the work by discussing parameters used in optimizing the system. It is broken down into two main parts: spheroid fabrication using replicas of the AggreWell™, and droplet microfluidic device selection. This chapter explains why certain methods and processes were chosen for spheroid fabrication and the DM system, after facing obstacles in different trials.

Chapter 5 is the main chapter that showcases and validates the entire system together. It starts by explaining more details about the selected DM device and the parameters that were studied for the achievement of single spheroid encapsulation. The selected hydrogels were justified in this section as well, to accommodate the spheroids while matching criteria of the selected DM chip. The results of these studies then follow, showcasing the successful use of the device. To validate the system, the collection process of the spheroid laden HMPs is briefed followed by a live/dead assay to show the survival of the spheroids through the system.

To bring the thesis to an end, Chapter 6 reviews the conclusions and take away points of the work done. Additionally, a short discussion on recommendations and future considerations for this project are mentioned to bring it to the next level and attain the intended long-term goal of achieving a robust, high-throughput system to enhance PDTs for cancer studies.

Chapter 2: Literature Review

2.1 Introduction

Seeking to improve methods of growing and monitoring spheroids into organoids for cancer research applications, it is important to review the work that has already been done towards similar goals. In this section, a literature review was done in search for understanding the difference between spheroids and organoids and how they are typically grown, the hydrogels used for 3D encapsulation and suspension, and the droplet microfluidic device proposed for the enhanced encapsulation and droplet formation. Additionally, it was important to review any similar work to either build on or improve the approach and compare methodologies towards improving the overall system. To wrap up this chapter, a summary and perspective is given to gather the thoughts behind using this information for the bulk work.

2.2 Patient Derived Tumor Organoids

With the ongoing need of finding the most effective way to treat individual cancer patients, patient derived tumor organoids (PDTOs) have become favorable, physiologically relevant *in vitro* models capable of being used to study personalized medication, tumor heterogeneity, anti-cancer drug screening, and more [3]. It is important to understand what these models are comprised of and the necessary tools to sustain their growth that mimic the *in vivo* conditions (inside the human body) while maintaining consistency through each batch, for accurate research and testing. This section is meant to give a better understanding of the difference between spheroids and organoids, and the limitations of common fabrication methods. In brief, the recent methods targeting fabrication improvements are mentioned to segway into the other sections of this review chapter.

2.2.1 Spheroids vs organoids

In general, spheroids and organoids are 3D cellular structures that have been useful tools for biological and health research. Due to this similarity the terms are sometimes used comparably [9], however, there are a few key differences that set them apart, making it useful to know the distinction for the best application. Figure 2-1 shows simplified illustrations of the architecture of a spheroid versus an organoid, compared to a single cell.

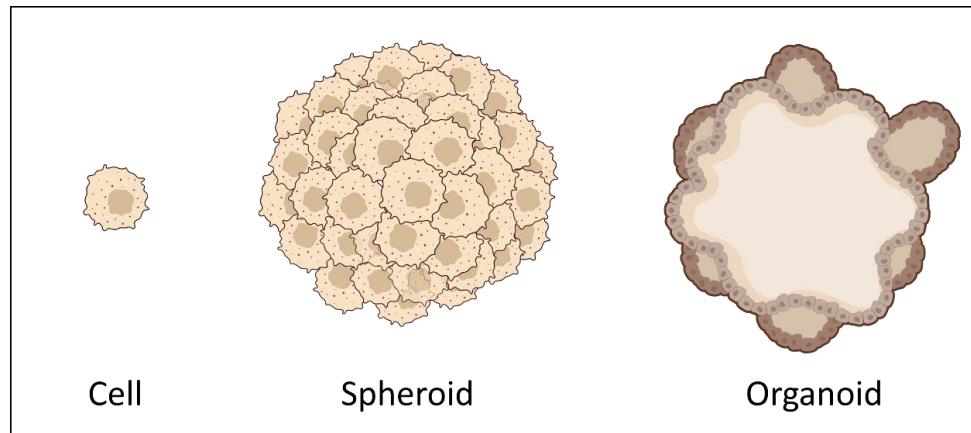


Figure 2-1: Simplified illustration of a cell, a spheroid, and an organoid for comparison.
[Created with BioRender.com]

Spheroids are typically classed as simple cell aggregates that can form without the presence of a scaffold and are not adhered to a substrate [46]. They show great cell-cell and cell-matrix interactions and thus can replicate to some degree the functions of the 3D cellular state that happens *in vivo*, after being cultured for a short time [9,46]. Their low complexity makes them easier to work with while still overcoming the limitations that 2D and animal models entail [9,14,47]. Spheroids can be unicellular or multicellular (MCSs) [46]. MCSs may be favorable to further recapitulate the tumor microenvironment that is composed of several members, especially since the main cells used are typically cancer cell lines instead of tissue, primary cancer or stem cells [9,48]. Due to this, further developed spheroids larger than 500 μm in diameter can replicate functions of avascular solid tumors due to their architecture but are not suited for long-term culture [14,48–50]. In the context of this thesis, the term spheroids will refer to cell clusters formed by scaffold-free environments reliant on cell interactions.

On the other hand, organoids are more complex 3D structures that can self-organize and -renew to truly mimic the properties of the primary organ tissue [11,12]. Their growth is better supported in a basement membrane or 3D matrix to assist with simulating the native microenvironment [51,52]. Typically, stem cells are used due to their ability to differentiate into different cell types that would exist in the original tissue structure [51]. In the case of tumor organoids, primary cells from a patient's tumor can also be grown into physiologically and functionally mimetic tissue [52,53]. From this ability, their growth can be monitored for long periods of culture for use in tumor studies [54–56], drug screening [57,58], biobanking [59,60] and more [3,11,61]. It is important to note that some cancer organoid structures may resemble properties of spheroids, and may not always resemble Figure 2-1.

To summarize, spheroids and organoids are both meaningful 3D cellular models that can progress research positively. Since they are models growing outside of their natural state, they need certain tools and supplements to ensure growth is successful. That is why the fabrication techniques are important for the behavior of the cells' growth into more complex, mimetic structures [51,52].

2.2.2 Conventional organoid fabrication methods and their limitations

The history of 3D cell culture can be traced back to the early 1900s, where the hanging droplet method was administered [62–64]. Expectedly, as technology has progressed, so have the techniques for improving 3D cell culture. There are several excellent papers that have detailed the fabrication of spheroids and organoids in traditional methods [9,18,65–67]. Figure 2-2 by Bhattacharya et al. [67] nicely illustrates those common techniques.

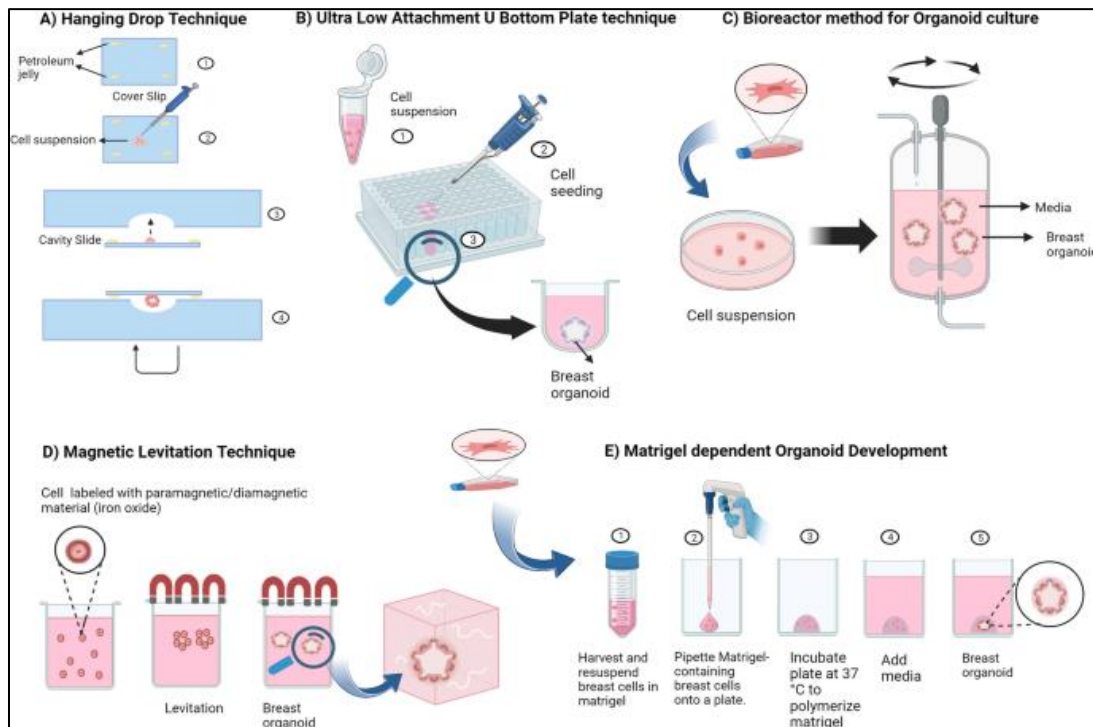


Figure 2-2: Illustrations of different common techniques for fabricating organoids. From “Bhattacharya, A., Alam, K., Roy, N.S. et al. Exploring the interaction between extracellular matrix components in a 3D organoid disease model to replicate the pathophysiology of breast cancer. *J Exp Clin Cancer Res* 42, 343 (2023)” used under Creative Commons CC-BY-4.0 DEED, open access [67].

Although these developed methods have overcome preceding limitations, they still have flaws that can be overcome with newer technologies. A common drawback amongst the methods mentioned in Figure 2-2 is not being able to efficiently supply each organoid with its own microenvironment in a high throughput and controllable manner [17]. A key factor to establish for improving these models is to ensure that repeatability can be achieved, so that there is confidence in working with the same models throughout each batch [37]. Ideally, each organoid would be supplied with the same amount of nutrients and growth factors and be suspended in a physical matrix with consistent mechanical properties [68]. Ensuring that the microenvironment is the same in each batch helps steer organoid growth in the same direction each time.

2.2.3 Recent technology for improved organoid fabrication

From the drawbacks mentioned in the section above, further development in improving organoid fabrication has been ongoing. Micropatterning and microfluidics have been recent favorable methods to enhance fabrication, thus there has been a rise in using these technologies [17].

Micropatterning refers to typically stamping small patterns onto solid substrates coated with ECM proteins, techniques and examples well reviewed by Velasco et al. [17]. In similar ways, patterning substrates themselves can achieve the same goal [69,70]. The AggreWell™ by STEMCELL Technologies is similar to micropatterning in the sense that it is a well with a series of inverted pyramid-like microwells, capable of catching cells to form them into spheroids [71]. It has been used for the formation of spheroids and embryoid bodies, using an initial low cell count, after 24 hours of culture, depending on the cell type [72–76]. This technology can be beneficial for forming cell clusters, however it may not be suitable for long term culture, due to limited microwell size, and chance of spheroids merging as they are not completely confined to their own microenvironment. After the spheroids form, they can be transferred into a hydrogel scaffold, also enabling a faster organoid growth process [77].

Microfluidics has become more popular in incorporating 3D cell culture due to its low waste and cost, and high throughput capabilities [78–81]. In the realm of microfluidics, droplet microfluidics has been utilized more because of volume control. Its combinations with hydrogels have been favorable for forming 3D scaffolds with cell encapsulation, typically for growth into spheroids [24,38,78,82]. However, not much work has been done in encapsulating pre-formed spheroids with DM, likely due to the size difference between single cells (1-20 μm) and spheroids (100-500 μm).

2.3 Hydrogels as 3D Matrices

There has been plenty of work around studying and developing hydrogels for cell encapsulation applications, as outlined in several review papers [20–23,83]. When considering supporting organoid growth *in vitro*, an ECM replica is important to not only help provide the 3D environment that naturally occurs, but to also provide the necessary biochemical cues to enhance the growth of the organoids. Natural hydrogels tend to resemble the ECM structure more than synthetic due to the naturally occurring binding sites and growth factors (GFs) [20,26]. However, synthetic hydrogels can be chemically modified to house the appropriate proteins, enzymes, and GFs that cells would need to thrive [25]. There can also be hybrid or semi-synthetic hydrogels where natural polymers are modified to be more controllable [84].

This section is meant to help understand these hydrogels and their properties for cell growth. By identifying what previous studies have done, a reliable hydrogel selection can be implemented with the droplet microfluidic system, compatible with the desired criteria. Briefly mentioned are the mechanisms used to crosslink hydrogels. Following this, a small exploration of hydrogels was done through searching commonly used gels for cell culture in hydrogel microenvironments.

2.3.1 Hydrogel crosslinking mechanisms

An important component of hydrogels is how they are crosslinked as this can affect the mechanical and physical properties of the gels [85,86]. Crosslinking is a means of reactions between polymer chains to form stable networks, leading to a jelly-like material [85]. Common types of crosslinking methods are broken down in Table 2-1 which also showcases typical hydrogels associated with the different crosslinking mechanisms [21,86]. Depending on the application of the hydrogel, each crosslinking mechanism may be more favorable than the others. Crosslinking via non-covalent interactions such as ionic forces and thermally induced reactions, are seen as less harsh thus are often coined as more biocompatible than other mechanisms [85–87]. Other mechanisms, such as photopolymerization and click chemistry, utilize forming networks via covalent bonds [86]. These types can be favorable due to their strength and durability compared to the non-covalent mechanisms which can be reversible [88,89]. It is important to have these properties in mind when considering implementing hydrogels with droplet microfluidics to sustain the microenvironment for organoids.

Table 2-1: Types of Crosslinking and Common Hydrogels Associated with Each Mechanism

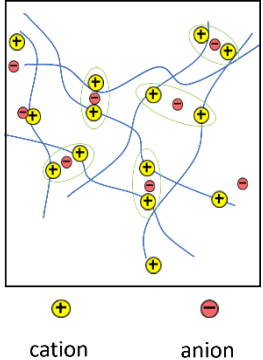
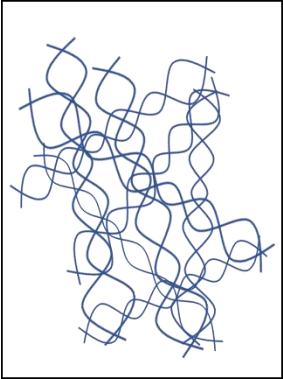
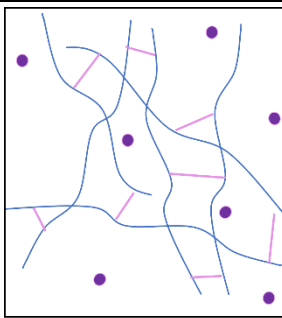
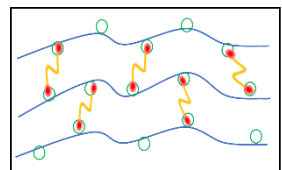
Crosslinking Mechanism	Description	Hydrogel	Schematic
Ionic Forces	- Polymers interact with oppositely charged molecules or polyions [87].	- Alginate is a polysaccharide, polyanion crosslinked with divalent ions, typically Ca^{2+} or Ba^{2+} which are mild and non-toxic [90–92]. - Chitosan is a natural polymer forming complexes with polyanions or anion molecules such as sulphates, pectin, and tripolyphosphate [22,87,93,94].	
Thermally Induced Reactions	- Hydrogels have a sol-gel transition at a critical temperature that causes polymer coils to form clustered helices attracting hydrophobic interactions, hydrogen bonding, or van der Waals forces [86,95,96] - Low critical solution temperature (LCST) hydrogels gel at higher temperatures. Upper critical solution temperature (UCST) hydrogels gel at cooler temperatures [86,97]	- Gelatin and agarose are common natural polymers that exhibit gelation below the UCST whereas Matrigel gels above the LCST [96] - ‘N’ substituted poly(acrylamide), such as Poly(<i>N</i>-isopropylacrylamide), are synthetic polymers capable of gelation above the LCST [86,98]	

Photo-polymerization	- Involves the use of a photoinitiator that can cause the reaction of free radical polymerization upon a certain wavelength [99]. The most common range of light energy is 200-400 nm (UV light).	- Common UV crosslinked hydrogels are gelatin methacryloyl (GelMA), polyethylene glycol diacrylate (PEGDA), and methacrylated hyaluronic acid (MeHA) [99]	 ● photoinitiator
Click Chemistry (Michael Addition)	- This reaction occurs with a nucleophilic donor and electrophilic acceptor [86]	- Common to use thiol-based donors and acrylate or vinyl sulfone acceptors [100] - e.g., PEGDA and thiolated natural polymers (chitosan, gelatin, hyaluronic acid) [100,101]	 donor acceptor

2.3.2 Hydrogels used for cell support and growth

In the search for a favorable hydrogel scaffold to support cell growth in a controllable manner, it was best to view what others have studied, along with reviews about hydrogel properties [102–104]. Gelatin Methacrylate (GelMA) came up as a commonly used hydrogels for cell applications due to its hybrid composition, containing biochemical cues while adopting easily tunable mechanical properties [84,105,106]. It has been used in cell encapsulation with droplet microfluidics [107,108], micro/nano patterning [105,109,110], and bioprinting [111]. As illustrated in Table 2-1, GelMA fits under photopolymerization due to the methacrylation of the gelatin. But this polymer can also be thermally crosslinked at cooler temperatures because of the properties of gelatin, making it cable of a hybrid crosslinking techniques, called GelMA+ [110].

Another very common hydrogel is alginate. Sodium alginate is a natural polymer that can form a gel-like structure when exposed to divalent ions, such as Ca^{2+} , creating the alginate hydrogel [89,91,112]. Due to the mild crosslinking technique, it has been widely used with cells for 3D encapsulation [113–115]. Additionally, its application in droplet microfluidics is very common, as it does not exhibit significant changes from temperature. Zhang et al. [92] made a very thorough review

on the many ways researchers have implemented alginate in DM and techniques used for crosslinking the hydrogel.

It is believed that the combination of GelMA and alginate has not been done together in the context of DM cellular encapsulation.

2.4 Droplet Microfluidics

Droplet microfluidics is means of producing microdroplets from flow instabilities with control, minimal waste, low cost, and high-throughput [29,116]. It involves a chip, often made from polydimethylsiloxane (PDMS) or glass, with microchannels that can vary in geometry and size [29]. This section is meant to explain DM devices and parameters, and its use in the application of hydrogels and cell encapsulation for forming spheroids and organoids. Due to the desire of achieving single pre-formed spheroids encapsulation, work around single particle encapsulation is briefly touched upon.

2.4.1 Types of droplet microfluidic devices

Due to its massive progression over the last few decades, droplet microfluidic devices have been growing. The main categories of DM devices are known as passive and active [117]. Figure 2-3 from Zhu et al. [117] summarizes droplet formation using both methods. Passive droplet microfluidics uses the principles of emulsion to form droplets with immiscible fluids, while active DM typically uses external energy. This review focuses on passive DM due to its operation simplicity, minimized cross-contamination, and reduced risk when implementing bio-particles [78].

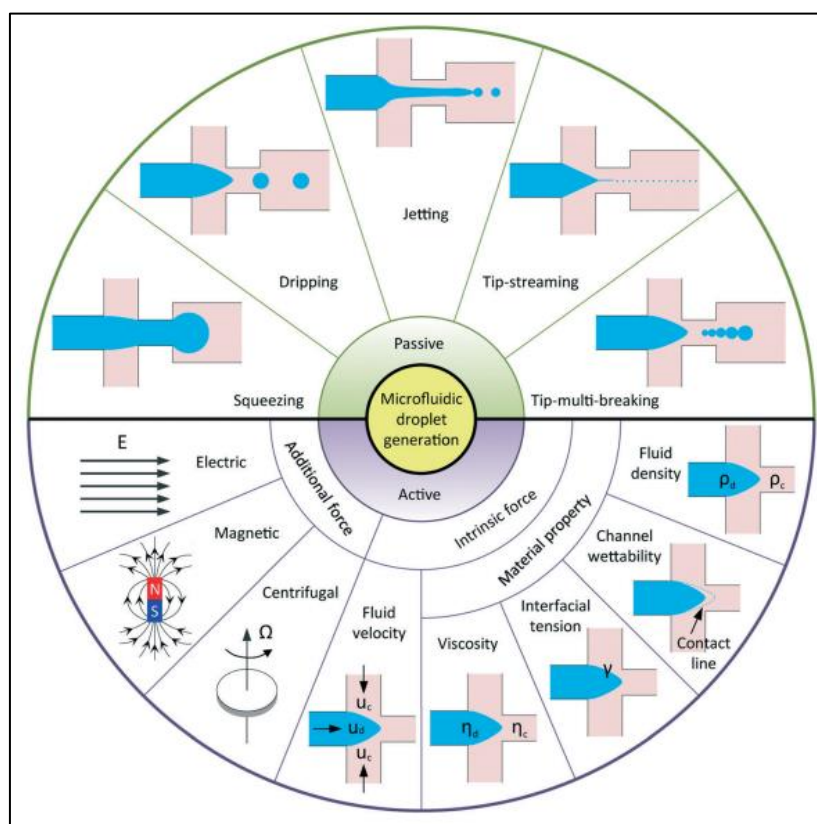


Figure 2-3: Droplet generation through passive and active techniques. From “P. Zhu and L. Wang, Lab Chip, 2017, 17, 34” used with permission from Royal Society of Chemistry [117].

Passive DM can also be broken down based on the type of geometry used. Figure 2-4 displays the different types of junctions a DM device can have; cross-flow (T-junction), flow-focusing, and coaxial flow (co-flow) [118,119]. The T-junction geometry is most often implemented in DM designs due to its simplicity in fabrication and use, but may be limited to a minimum droplet size of 10 μm because of design constraints [119]. Coaxial junctions have similar droplet size constraints as the droplets produced with this geometry are larger than the tip diameter [119,120]. Meanwhile, flow-focusing junctions are capable of much smaller droplet sizes, on the hundred-nanometer scale, thus can be more favorable for nano-applications [119]. In the simplest manner, regardless of the channel geometry, DM devices contain two immiscible phases: the continuous and dispersed phase [121]. The continuous phase acts as the droplet generator and carrier fluid, whereas the dispersed phase is the contents of the droplet.

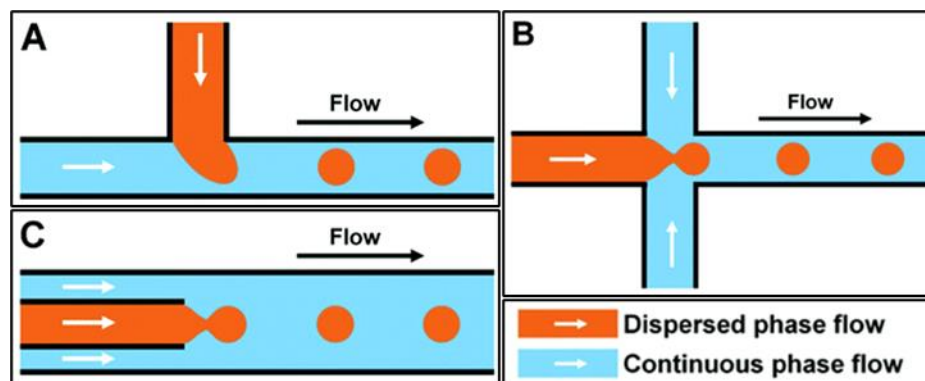


Figure 2-4: Types of droplet microfluidic junctions. (A) T-junction (B) Flow-focusing (C) Coaxial. From “D. Liu, M. Sun, J. Zhang, R. Hu, W. Fu, T. Xuanyuan and W. Liu, *Analyst*, 2022, 147, 2294” rearranged under creative common CC-BY-NC 3.0 DEED, open access [118].

2.4.2 Hydrogels used in droplet microfluidics

Droplet microfluidics is favorable for the large number of microdroplets that can be obtained in a short amount of time with high quality [33]. Hydrogel droplets formed by DM will be referred to as hydrogel microparticles (HMPs). As with any device, there are parameters to consider with DM when it comes to integrating hydrogels for the successful production of HMPs [121,122]. DM is beneficial for gaining control over droplet properties, but the device and fluid properties are what can influence the resulting HMP. Channel size and geometry play a main role in droplet size; however, altering the viscosities and/or flow rates of each phase can also affect the outcome of the droplet produced [121,123].

In the case of producing HMPs, the simplest type of emulsion is known as water-in-oil (W/O) [119]. With the hydrogel precursor (non-crosslinked hydrogel) acting as the ‘water’ or aqueous phase, this means the DM channels should be hydrophobic to ensure good wettability and proper droplet formation. In like of wanting good droplet formation, the immiscibility between the dispersed and continuous phase is also important, otherwise there is no emulsion [119,120]. This justifies that in the case of W/O emulsion, the continuous phase must be hydrophobic to contrast with the aqueous dispersed phase and maintain formed droplets. The use of a surfactant can also help stabilize the droplet interface so that the emulsion is steady until broken to retrieve the droplets [124].

More particularly, HMPs, which are essentially small hydrogel droplets, have been used as 3D networks for cell and bacteria encapsulation and culture [125–127], mechanisms for drug delivery

[128,129], and a means for screening [130,131]. In the interest of this thesis, focusing on DM for cell encapsulation will help identify techniques currently used.

2.4.3 Applications of droplet microfluidics for the formation of spheroids and organoids

Derived from the limitations of traditional organoid forming techniques, droplet microfluidics became a popular platform for gathering the cells in a 3D matrix for aggregation and growth into spheroids or organoids, owing to its mass production and trend towards repeatability [78]. There have been many great review papers on using droplet microfluidics to fabricate spheroids and organoids, in cases of using cancer cells or other types of cells [17,24,39,40,78].

Typically, cells are suspended in a hydrogel precursor and are encapsulated by droplet formation with an oil phase. Depending on the type of hydrogel and crosslinking methods the researcher would like to achieve, chip geometries and collection techniques may vary [37,82,132,133]. A commonality between the various different spheroid forming DM systems is the initial cell concentration at the inlet reservoir, usually on the order of millions [107,134–136]. This can be a disadvantage when trying to apply the system to patient derived primary cells, as biopsy amount and culture of primary cells is limited [43,137].

In the case of Ding et al. [132], they were able to incorporate a low initial cell count into their DM device when encapsulating colorectal cancer cells for growth into organoids. The lowest amount of cells in the droplets was 20 cells. A low number of cells per droplet may take longer to grow due to the stiffer environment of the hydrogel, and not all cells may clump together given the gaps between each other in the droplet.

Another concern with using a low number of cells is the amount of empty droplets that can arise, thus taking away from the efficiency of this particular method of forming spheroids. Sun et al. [38] documented droplet encapsulation efficiency when using 500k cells for creating multicell tumor spheroids. This inlet condition had 90% empty droplets, compared to when they used 10 million cells which had no empty droplets. For implementing single cells from the beginning of the process, efficiency is important to gain the production of spheroids in mass-produced way. If looking to continue the growth of pre-formed spheroids, efficiency is less important compared to the other benefits DM can supply such as minimal reagents, control, manipulation and less cross contamination, but of course still a factor in achieving a robust overall system.

In terms of encapsulating larger bio-particles, Mesquita et al. [45] showed a glass capillary DM device for the encapsulation of human stem cell spheroids in alginate hydrogels. However, this chip

may not be able to employ the further exploration of hydrogels or ways towards enhancing the spheroids' growth. Additionally, the initial cell concentration used was 2 million to form the embryoid bodies that entered their DM device.

2.4.4 Single bio-particle encapsulation using droplet microfluidics

For cases of non-uniformities with multi-cell encapsulation (lack of cell number control) or in the interest of wanting to grow or analyze individual cells, single cell encapsulation became a branch of cell-laden droplets researchers were exploring [138,139]. The work for single particle encapsulation has been heavily done to seek the most efficient or meaningful system [140–142]. In a typical passive network, suspended particles are employed into the DM chip at high flow rates and follow the Poisson distribution, so the efficiency or number of single particles in one droplet can vary or be low [143].

In previous work, this distribution was overcome. Nguyen (2019, UW Space) [144] demonstrated the use of stratified flow and contrasting viscosity fluids for the encapsulation of single embryonic stem cells in their own hydrogel droplets. Using a double flow focusing geometry, the center hydrodynamic focusing stream of contrasting aqueous fluids allowed the cells to align, even if they came in groups from the off-chip inlet reservoir. To leverage this work, scaling up the geometry to fit the needs of pre-formed spheroids (which would be about 10 times as large in diameter to a single cell) can be a way of exploring more robust encapsulation of spheroids or larger bio-particles. The focusing stream can become a key player in aiding with single spheroid encapsulation, especially with the different behavior of the spheroids in the inlet reservoirs.

2.5 Summary and Perspective

After gaining more insight into the multiple components needed for improving the growth of patient derived tumor organoids, it is shown that there is always room for improvement in new systems. By understanding what an organoid is and needs for supported growth *in vitro*, leveraging well defined and compatible hydrogels with droplet microfluidics is a great combination towards a system that is robust and controllable.

Due to the limited number of cells that can be extracted from a biopsy, aggregating the cells in a gentle manner before implementing a high throughput technology to encapsulate them in a controlled microenvironment for enhanced growth can be favorable. Additionally, jump starting the organoid growth process by starting with pre-formed cell clusters would be favorable for a system to be robust

and deliverable. Spheroids in the early stage may be more fragile compared to single cells in the sense that the cells are loosely packed together. So when considering encapsulating them, the method should be minimally harsh and allow for sizable amount of room for spheroid passage into their own microenvironment. Since spheroids are much larger and denser than single cells, different inlet conditions need to be made when implementing them into the DM device.

Chapter 3: Experimental Setup and Methods

3.1 Introduction

This chapter describes the main materials and methods used for the overlying setup of the project. The purpose is to outline the procedures followed when running each step of the process, so that methods can be reproduced or critiqued.

3.2 Forming Spheroids Using PDMS Replicas of the AggreWell™ (PDMS AWs)

3.2.1 PDMS AggreWell™ replica fabrication

The use of polydimethylsiloxane (PDMS) replicas of the AggreWell™, referred to as PDMS AWs, helped form spheroids in a robust and uniform manner. The PDMS AWs were fabricated using a 2-step replication process. The original AggreWell™ 400 (STEMCELL Technologies) itself comes as a 24-well plate with each well (surface area of 2 cm²) containing 1200 smaller microwells. Each microwell is an upside-down pyramid-like shape (pyramid-microwells) 400 µm in size (400 µm width, length, and depth), allowing the cells to gather at the bottom to form spheroids. To make the most of the original pattern, PDMS replicas of the 2 cm² AggreWell™ were made and used as molds (a positive pattern) to create several PDMS AggreWells™ (that would contain the original microwell pattern) that would fit into a 12-well plate. Figure E-1 in 0 shows images of the replication process.

PDMS molds of the original 24-well AggreWell™ 400

The process began with mixing PDMS (Sylgard 184 Elastomer, Dow Corning) base and curing agent at a ratio of 10:1 (base to curing agent). After thoroughly mixing for at least 5 minutes, the mixture was degassed for 30 minutes until all air bubbles were gone. The degassed PDMS was poured into several wells of the original 24-well plate AggreWell™ 400, about two-thirds full. This was cured for 4-6 hours at 60 °C before demolding. Once the replicas were removed from the plate, they were silanized – the molds were placed in a vacuum desiccator with ~20 µL of Trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (Sigma-Aldrich) and vacuumed for 30 minutes and left over night. The treated molds were replicated 3 times with PDMS before use for cell work.

PDMS AW replicas

Using the silanized 2 cm² AggreWell™ molds, the molds were placed in a 12-well plate (VWR) with the pattern side facing up and PDMS was poured on top almost filling the well. This was cured for 4-6 hours at 60 °C before demolding. Once the PDMS AWs were completely demolded, they were cleaned with 100% ethanol and blow dried. In a biosafety cabinet (BSC), the PDMS AWs were cleaned and soaked in 70% ethanol for 10-15 minutes, and the remaining ethanol was aspirated afterwards. The sterile PDMS AWs were placed into a new 12-well plate, using 70% ethanol to lubricate the sides. Once the PDMS AWs were secure in the well plate, they were treated with 700 µL of sterile 5% w/v Pluronic F-127 (Sigma-Aldrich) for 30 minutes [77]. The Pluronic was made by dissolving the powder into deionized (DI) water at 60°C, and sterile filtered once fully dissolved, using a 0.2 µm syringe filter (Pall Corporation). 700 µL of the 5% Pluronic was added into each PDMS AW and centrifuged at 4000 RPM for 5 minutes to remove air bubbles, ensuring the Pluronic reached the bottom of each pyramid-microwell. After 30 minutes, the Pluronic was removed and the PDMS AWs were washed twice with sterile DI water and once with Dulbecco's Modified Eagle Medium (DMEM -/+/: without sodium pyruvate / with 4.5 g/L glucose / with L-glutamine, Wisent). Lastly, 700 µL of cell culture media was added to each of the PDMS AWs and centrifuged at 4000 RPM to remove air bubbles. The PDMS AWs would then be ready for cell seeding.

3.2.2 Cell seeding into PDMS AggreWell™ replicas

For this project, an immortalized mouse fibrosarcoma cell line known as L929 (ATCC) was used to create the cancer spheroids used for DM encapsulation into HMPs. In preliminary tests an immortalized human breast cancer cell line, MDA-MB-231 (ATCC), was also used and cultured in the same manner as the L929.

Cell culture

L929 cells were cultured in a T25 flask with a cell culture media cocktail of DMEM (-/+/: Wisent) + 10% Fetal Bovine Serum (FBS, Wisent) + 1% Penicillin-Streptomycin (Pen Strep 10 000 U/mL, Gibco). Cell culture media was changed every other day and cells were passaged when ~90% confluent. To passage the cells, 0.05% trypsin was made by diluting 0.25% Trypsin/EDTA (0.25%/2.21mM, Multicell) with phosphate buffered saline (PBS). 3 mL of 0.05% trypsin was incubated with the cells at 37°C for 3-5 minutes, then neutralized with double the amount of cell culture media. Live cells were counted using Trypan Blue Solution (4% w/v, Corning) to stain any dead cells. After centrifugation of

the cell suspension, the supernatant was aspirated, and the remaining cell pellet was resuspended in 1 mL of fresh cell culture media. The desired cell number was taken from this suspension.

Cell seeding

After cells were passaged, the appropriate quantity of cell suspension to obtain 60 000 cells was added to each PDMS AW. This would result in each pyramid-microwell containing 50 cells to start forming the spheroids. The plate was centrifuged at 100xg (~ 800 RPM) for 5 minutes to evenly distribute the cells. The plate was left to culture in a 5% CO₂ incubator at 37°C, for 4 days.

3.2.3 Spheroid collection

After the spheroid formation, they were collected through gentle agitation and pipetting. The suspension in the PDMS AW was collected through a 70 µm strainer (Nylon mesh, Fisher brand) that was flipped upside down. Once all spheroids were strained, the strainer was slightly submerged into a 35 mm culture plate (Thermo scientific) with ~2 mL of cell culture media. A gently swishing action was done to detach the spheroids from the bottom of the strainer. The solution with spheroids was collected into a 15 mL falcon tube (VWR), and 100 µL was taken for counting. After the spheroids settled at the bottom of the tube, they were aspirated and mixed with hydrogel precursors.

3.3 Droplet Microfluidic (DM) Device Fabrication and Setup

3.3.1 PDMS DM chip fabrication

The master for making PDMS chip replicas is made on a silicon wafer using common soft-lithography techniques, which are well established previously [145]. The specific device mold chosen was made by a previous lab member, Nguyen (2019, UW Space) [144], a similar design in which is discussed in their thesis. PDMS was mixed in the same fashion as used for AggreWell™ PDMS replica fabrication. The mixed PDMS was poured onto the silicon wafer mold that was encased in an aluminum bowl, then cured for 2 hours at 90°C. The PDMS replica was then cut to fit onto a standard glass microscope slide (75x25x1 mm, VWR VistaVision) and holes were punctured at the inlets and outlet. To form the chip itself, the PDMS replica was oxygen plasma bonded (Harrick Plasma) onto a glass microscope slide. The substrates were cleaned with 100% isopropyl alcohol (IPA) and dried with nitrogen. Once cleaned, they were placed in a vacuum chamber until 200mTorr where oxygen was flooded into the chamber for 16 seconds. At 500mTorr, plasma at 29.7 W was turned on for 14 seconds.

Once the vacuum was released, the PDMS and the glass were pushed together, creating a permanent bond. After plasma bonding, the chip was baked at 95°C for 10 minutes, followed by 170°C for 2 days to regain hydrophobicity. Cut tubing used for chip setup was temporarily placed in the holes of the chip to retain the proper hole diameter as the PDMS continued to cure in the long bake.

3.3.2 DM chip accessories and setup

This device was run by pressure-driven flow using a pressure pump (Fluigent MFCS-8C), and imaging was done using an inverted microscope (Nikon Ti-E Inverted Microscope) and NIS-elements software. To begin the setup, the pump and microscope were turned on, followed by opening the software. HPFA tubing (1/16x0.010x50ft, IDEX Health and Science) was used to connect the chip to the pump reservoirs. First, the chip was primed with light mineral oil (neat, Sigma) containing 3% v/v Span 80 (Sigma-Aldrich) for 30 minutes to swell the PDMS. The hydrogels and pre-formed spheroids were then able to be implemented into the chip. Figure E-2 in 0 shows images of the system setup.

3.4 Hydrogel Fabrication and Characterization

3.4.1 Gelatin Methacrylate (GelMA) fabrication

Synthesis of the GelMA polymer

The process began by dissolving gelatin (Type A, Sigma-Aldrich) into PBS at 10% w/v at 60°C for 1 hour, or until the gelatin was completely dissolved. The round bottom flask was submerged in a 60°C water bath to help with heat distribution so the gelatin would not solidify during the process. While stirring the solution, Methacrylic Anhydride (MA) (Sigma-Aldrich) was slowly added (about 1 drop/2 seconds). The amount of MA added was 20% w/v to achieve 80-90% methacrylation. Once all the MA was added, the solution was mixed for an additional 1 hour. After this reaction hour, pre-warmed 40°C PBS was added to the solution to achieve a 2X dilution (double the volume of the current batch) to stop the reaction. The mixture was centrifuged at 5000 RPM for 5 minutes, at the supernatant was collected for dialysis. The dialysis was done in 12-14kDa MWCO dialysis tubes (Spectra/Por) tightly closed and submerged in a DI water bath maintained at ~40°C with a fish tank heater. The water was changed every 2 hours the first 2 days, then once a day for a total of 7 days. After the dialysis, the solution was transferred into 50 mL falcon tubes (VWR) and frozen at -80°C overnight. The frozen GelMA was then lyophilized over a 3 day period, creating the GelMA powder. The powder was kept at -80°C until needed. The GelMA powder was pre-weighed into 100 mg aliquots and then autoclaved to sterilize.

Fabrication of GelMA hydrogel precursor

To make the GelMA prepolymer solution, first the photo-initiator solution was made by dissolving Irgacure 2959 (Sigma-Aldrich) into either PBS or Dulbecco's Phosphate Buffered Saline (DPBS, with Ca and Mg, Corning) at 0.5% w/v and left at 60°C until fully dissolved. After the Irgacure was dissolved, the solution was sterile filtered in a BSC using a 0.2 µm syringe filter (Pall Corporation). The sterile photo-initiator was then mixed with the autoclaved GelMA powder (dissolved at 60°C overnight) to achieve a 10% w/v GelMA hydrogel precursor that acted as a base solution for further dilution. The 10% w/v precursor was diluted to either 5% w/v or 2.5% w/v with cell culture media, or 5% w/v diluted with the spheroid suspension in media.

3.4.2 Sodium Alginate and Calcium Chloride fabrication

Fabrication of sodium alginate hydrogel precursor

To make the sodium alginate precursor solution, sodium alginate powder (from brown algae, Sigma-Aldrich) was dissolved in PBS at 4% w/v at 60°C. The 4% w/v solution was then sterilized by autoclave, and kept at 4°C until needed. The sterile 4% w/v solution was diluted to 2% w/v with either cell culture media or the spheroid suspension in media.

Fabrication of calcium chloride solution

To make the solution that would act as a collection bath for crosslinking the sodium alginate, calcium chloride (CaCl₂) (anhydrous 4-20 mesh, Fisher Scientific) was dissolved in DI water at 2%, 1%, and 0.5% w/v at 60°C. The solutions were sterile filtered in a BSC with a 0.2 µm syringe filter, after fully dissolved.

3.4.3 Compression testing

Compression tests were done on hydrogel microparticles using the MicroTester G2 by CellScale. A platen size of 2 mm was glued onto a beam size of 0.5588 inches. Once assembled, the holder container was filled with DPBS and an anvil was placed at the front of the container. In the system, the camera was set to 3X zoom, the displacement was 75% of the diameter of the HMP, and the data was recorded as a ramp at 5 Hz. When everything was setup, the sample was loaded, the beam was adjusted above the HMP and the system was started. Force versus displacement graphs were obtained from the run and analyzed into stress-strain curves using Excel.

3.4.4 Viscosity measurements

Viscosity measurements were taken on hydrogel precursors using the Brookfield Digital Rheometer (MODEL DV-III+). The disk used was the CPA 52Z (sample size = 500 μ L). Loaded samples were exposed to increasing RPMs and viscosity measurements were recorded to obtain an average viscosity.

3.5 Hydrogel Microparticle (HMP) Collection and Washing

3.5.1 Crosslinking HMPs

After forming the hydrogel precursor droplets in the DM chip, the droplets were collected in either 4°C mineral oil with 3% v/v Span 80, or various concentrations of CaCl₂ at 4°C, that were kept in 2 mL microcentrifuge tubes (Globe Scientific). The cold baths were meant to induce thermal gelation of the GelMA to reduce risks of coalescence before photo-crosslinking for a permanent gel structure that is the HMP. Collected hydrogel droplets were kept at 4°C for 5-10 minutes before beginning transferred to a 24-well plate and UV crosslinked with a 395 nm UV-A lamp (MAXIMA™ 3500 Series, Spectro-UV), for 2 minutes at a distance of about 13 cm.

3.5.2 Washing HMPs

After HMPs were crosslinked, they were carefully washed with DMEM (-/+/-) + 1% Pen Strep. Samples were washed about 3 times, and then kept in either DMEM (-/+/-) + 1% Pen strep or cell culture media and incubated at 37°C, 5% CO₂ for 24 hours up to 5 days.

3.6 Cell Viability

HMP samples containing spheroids were washed twice with DPBS to remove as much media as possible. A separate solution of DPSB was used to mix the Live/Dead™ assay (Viability/Cytotoxicity Kit, Invitrogen) at a ratio of 1:4 (live:dead). Calcein AM was used as the green-fluorescent dye to detect live cells, and ethidium homodimer-1 was used as the red-fluorescent dye to detect the dead cells. 200 μ L of the assay solution was added to each batch of samples in a 24-well plate. The solution was incubated for 1 hour at 37°C before imaging. Fluorescence images were taken using an inverted fluorescence microscope (Observer Z.1, Zeiss). Images were analyzed using Image J macro, where intensity of each signal was obtained, normalized to the spheroid's area.

Chapter 4: Optimization of System Components

4.1 Introduction

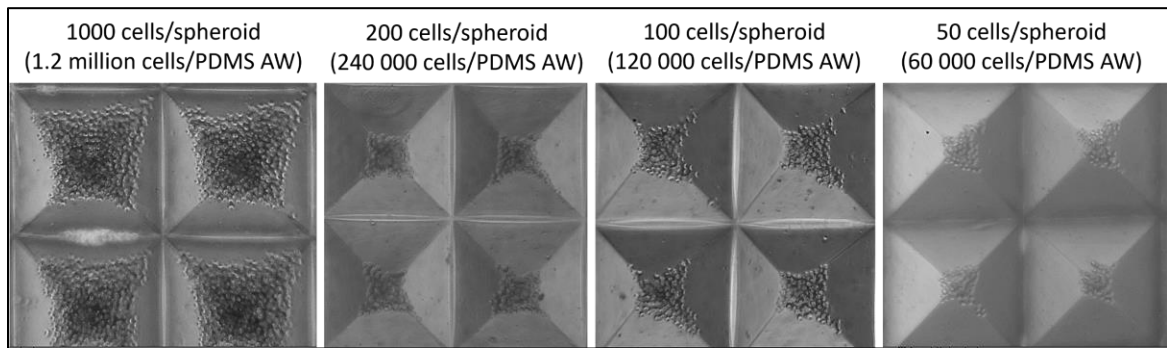
There are challenges to overcome in order to develop a robust system that can produce uniform droplets for the encapsulation of pre-formed spheroids. This chapter helps to depict the different approaches for running the system before some of the final decisions were made. Many factors affected the formation of the cancer spheroids or the droplet generation using droplet microfluidics. In the case of forming the spheroids, the written protocol was made after addressing issues during initial fabrication, which are elaborated on in section 4.2. For selecting the DM device, a couple of fabrication techniques were compared for exploring and prototyping potential geometries, and system set up, discussed in section 4.3.

4.2 Optimizing Spheroid Formation Using PDMS Replicas of the AggreWell™

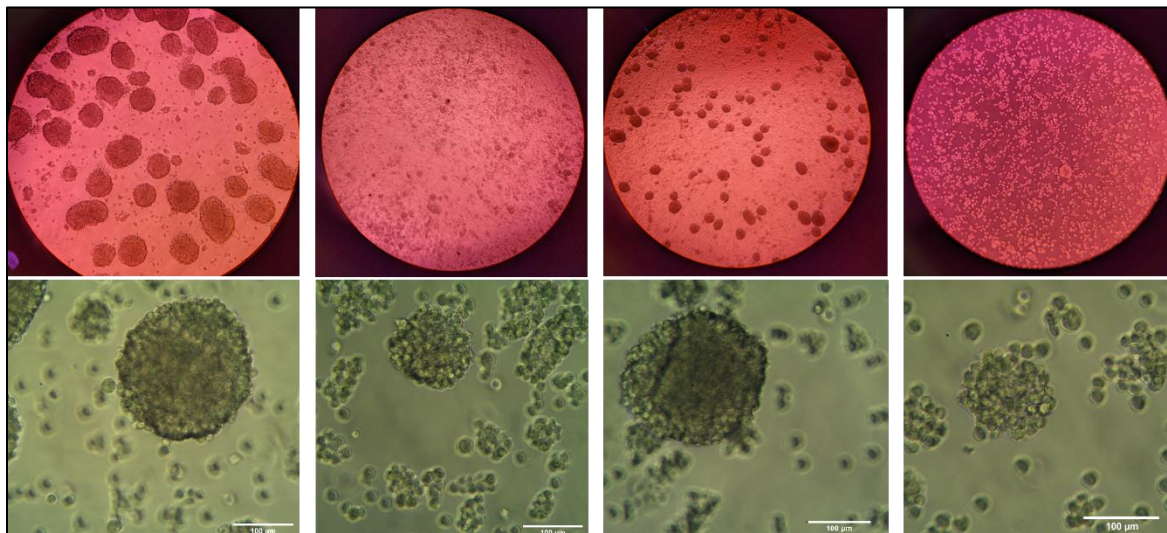
To initiate the spheroid making processes, protocols from past work or from the company of the AggreWell™ (STEMCELL Technologies) were followed. A 2-stage replica forming process was done to make PDMS replicas of the AggreWell™ (PDMS AWs). However, issues appeared when simply trying to seed L929 cells in the PDMS AWs directly, following older protocols. Parameters affecting spheroids formation were addressed which also influenced the final collection method. Additionally, there was an effect on how well growth or spheroid formation was based on initial cell density or culture time in the PDMS AWs. This section of Chapter 4 is meant to showcase how the final protocol for forming spheroids came about based on the challenges faced in the beginning.

4.2.1 PDMS AggreWell™ replica treatment effect on spheroid growth

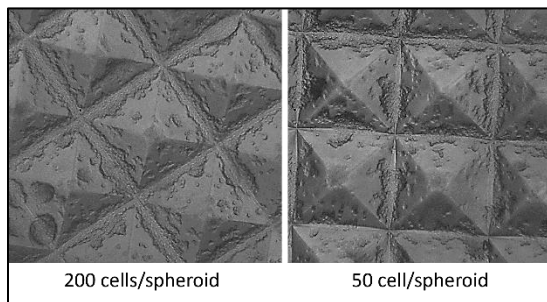
From the starting protocol, PDMS AWs were sterilized with 70% ethanol and sat under UV light in a BSC for at least 30 minutes. Once the ethanol was removed, the PDMS AWs were washed and cell culture media was added, air bubbles were removed by centrifuging, and cells were seeded after. It was commonly observed that after 24 hours, the cells would start to attach to the PDMS instead of sticking with each other. Figure 4-1 shows a couple examples of this instance and the spheroid collection results. With a higher seeding density, the spheroids did initiate more, but still there were many areas of the PDMS AWs that the cells spread onto (Figure 4-1 (c)). This was also observed with another cancer cell line, MDA-MB-231, shown in Figure 4-1 (d), indicating that the original PDMS AW method was not adaptable to all cell types.



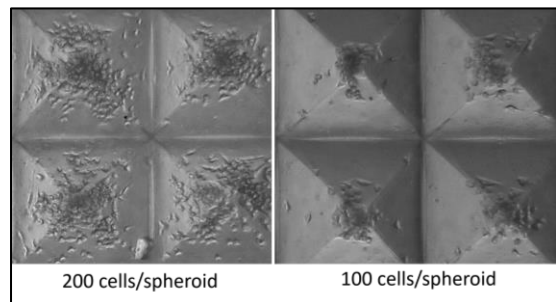
(a)



(b)



(c)



(d)

Figure 4-1: Cells seeded at different densities in untreated PDMS replicas of the AggreWell™. (a) L929 cells at $t = 0$ days (b) the collection of the L929 spheroids at $t = 48$ hrs; the top pictures were taken from the microscope eyepiece; the bottom pictures were taken from the lens view (scale bar = 100 μm). (c) Images of pyramid-microwells where L929 cells are attached to the

PDMS surface (d) Images of MDA-MB-231 cells that experienced the same attachment to the PDMS.

The occurrence of non-uniform clustering or cell spreading made the whole system lack a sense of repeatability as it was not certain how spheroids would form. Thus, it was important to discover what in the original protocol may have been influencing the cells, affecting their attachment to the PDMS. Table 4-1, coupled with Figure 4-2, outline some parameters that were potentially affecting the cells' behaviors on the PDMS AWs and the rationale for testing them. These parameters influenced how the trials were run and ultimately how to move on with the spheroid making process for the cancer cells.

Table 4-1: Potential Parameters Affecting Cell Attachment onto PDMS

Parameter	Effect on Cell Attachment + Rationale
(a) Serum concentration in cell culture media	- FBS could aid in cell attachment - Concentrations were varied to test results of 0, 3, 5, and 10 % v/v serum to see if less serum could prevent cell attachment while still helping with growth
(b) UV exposure time	- UV exposure to PDMS could be similar to plasma treatments in becoming ideally hydrophilic - Testing time points of UV exposure during the sterilization stage was done to see if a reduced amount would help prevent cell attachment
(c) PDMS AW age	- Age of PDMS post curing may change (stability or stiffness) - Testing older made PDMS AWs versus freshly made ones would help depict if the different self-life was encouraging more cell adhesion

Serum (FBS) is an important supplement in cell culture, and due to its composition, it encourages cells to adhere to their substrate [146]. That is why varying the concentration from 0% to the typical 10% was done to see if there was a major effect on spheroid formation with the PDMS. As shown in Figure 4-2 (a), serum concentration did not seem to heavily influence the cells' attachment in favor of spheroid formation. Due to this observation, the cell culture media with 10% FBS was kept to ensure optimal cell growth for future processes.

The UV during sterilization may have also been affecting the surface energy of the PDMS after long term exposure [147]. Cells tend to have an ideal range of surface wettability for adhesion [148], so treating the surface towards this range, similar to the effects of plasma treatment on PDMS, would not

be favorable. However, altering the time of UV exposure did not seem to influence different cell behavior (Figure 4-2 (b)), suggesting UV exposure may be eliminated overall, since the 70% ethanol was the key component in the sterilization process anyway.

Lastly, the stiffness of the substrate can play a role in cell adhesion, where some cells may prefer a stiffer substrate for adhesion [149]. As the PDMS sits overtime (over years), it continues to cure slightly, thus making it stiffer [150] and it may also become more unstable. Although selected PDMS AWs were not expected to sit longer than a month if used, it was still worth checking to see if there were any noticeable changes from the PDMS age. In Figure 4-2 (c), comparing the use of an old PDMS AW (made at least more than two weeks prior to cell seeding) and a freshly made PDMS AW showed more promising results, even being exposed to 30 minutes of UV for sterilization. Since the UV and PDMS age seemed to be the major contributors to the cell attachment based on the tested parameters, it was decided that a new PDMS AW with no UV treatment be used as the substrates to grow the spheroids (Figure 4-2 (d)).

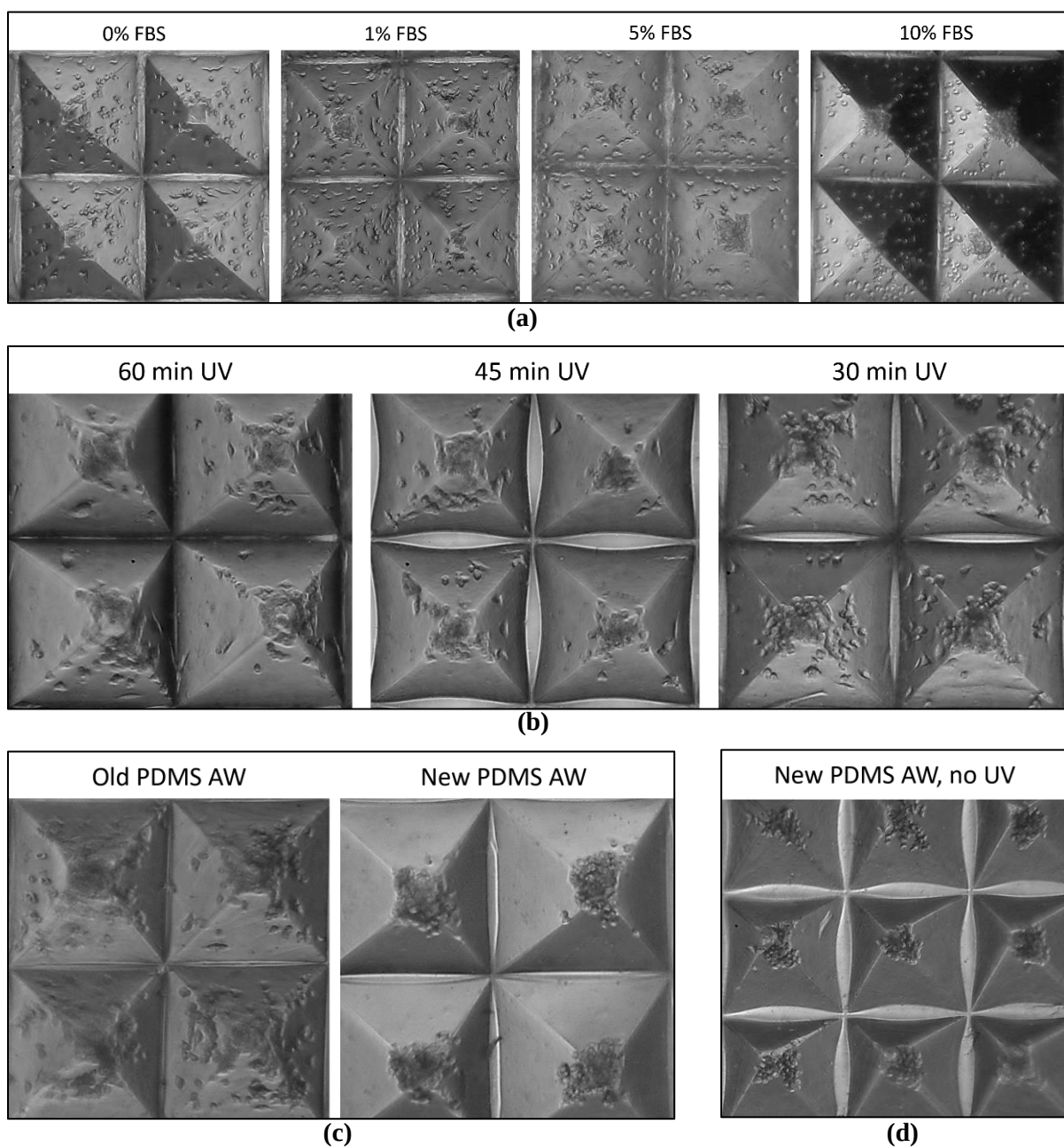


Figure 4-2: Different parameters implemented on the culture conditions of the PDMS AggreWells™. All PDMS AWs had a seeding density of 120k cell/PDMS AW (100 cells/spheroid) and were cultured for 24 hrs in cell culture media cocktail, unless otherwise specified. (a) Different concentrations of serum (FBS % v/v) in the cell culture media with L929 cells. (b) Effect of UV time on the PDMS during sterilization. MDA-MB-231 cells were cultured in the PDMS AWs afterwards with regular cell culture media. (c) Difference between using an old PDMS AW

(made at least 2 weeks before cell seeding) and a new PDMS AW (less than 2 weeks old) for culturing MDA-MB-231 cells. (d) MDA-MB-231 cells in a new PDMS AW without UV treatment.

After recognizing the best case of the above parameters, which was to use PDMS AWs made less than two weeks prior to cell seeding and avoiding UV sterilization (only using 70% ethanol), the results were still not very consistent. This is what encouraged the search for a solution to treat the PDMS to make the surface less favorable for the cells and cause them to stick together. In searching, Pluronic came up as a solution to treating the PDMS [77,151], as it binds to PDMS's hydrophobic sites and exposes hydrophilic tails, prohibiting the attachment of cells and proteins thus allowing cells to form clusters instead of spreading out [152–154].

Figure 4-3 shows PDMS AWs treated with 5% w/v Pluronic-F127, culturing both L929 and MDA-MB-231. The results were more promising in that they showed the cells having better success of sticking together and forming spheroids. Due to the way in which the L929 cells formed more cohesive spheroids in a shorter time compared to the MDA-MB-231, this cell line was chosen to moving forward.

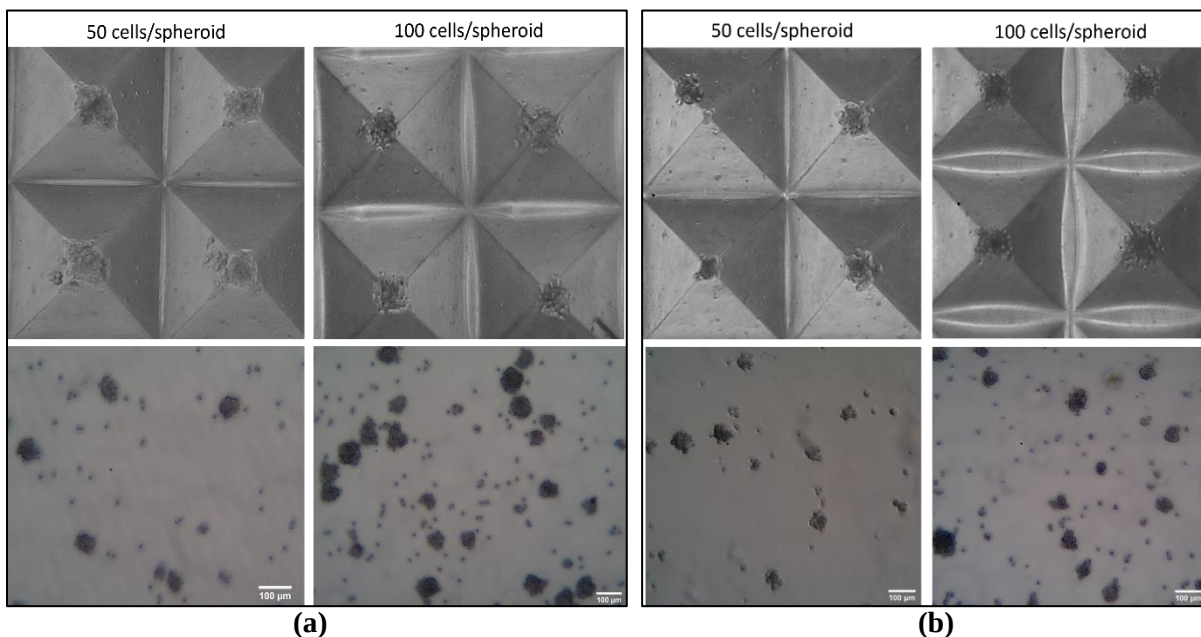


Figure 4-3: Cells seeded at different densities in Pluronic treated PDMS replicas of the AggreWell™. (a) L929 cells at t = 1 day (b) MDA-MB-231 cells at t = 3 days. The top pictures were in the PDMS AWs; the bottom pictures were the collection. Scale bars = 100 μm.

4.2.2 Spheroid collection methods

Although the Pluronic was a great improvement to the PDMS AWs, after collection of the spheroids, there still remained single cells or smaller spheroids that either did not grow well, or were detached during the collection process. This was what inspired reverse-straining the collected solution. Reverse straining refers to straining the solution, and obtaining what was collected in the strainer mesh, then rinsing this into a separate solution. Figure 4-4 (a) illustrates the method of reverse straining used with a common cell strainer (70 μm). The results of the different collection/straining steps are shown in Figure 4-4 (b), where it is clear how the reverse straining really helped pick out uniform spheroids to have a uniform solution to work with the DM device for spheroid encapsulation.

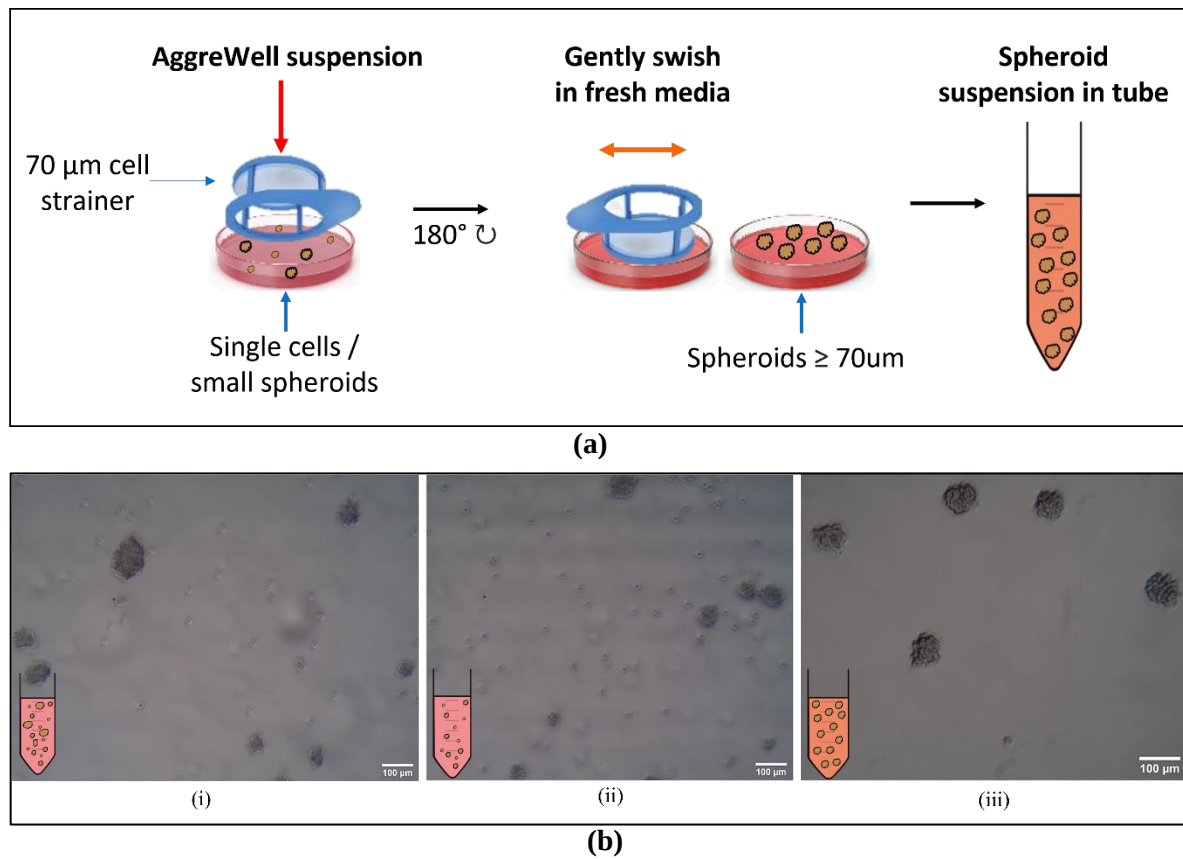


Figure 4-4: L929 spheroids reverse straining method implemented. (a) Schematic of the reverse straining process. (b) Images of collected L929 spheroids at different stages of the collection process: (i) not strained, (ii) strained sample (containing spheroids less than 70 μm and single cells), (iii) reverse strained, the desired solution of uniform spheroids ($> 70 \mu\text{m}$).

4.2.3 Effect of spheroid size and cohesiveness based on culture time

With an updated protocol for culturing the spheroids more uniformly and constantly, it was also important to check how the results of the spheroids would be after different times in the PDMS AWs. Due to some influence of wanting to target a lower initial cell density, lower amounts of initial cell seeding were continued with, using 60 000 cells/PDMS AW, equivalent to 50 cells/spheroid. Due to the smaller number of seeded cells, the initial size of spheroids after 24 hours would be small and more fragile (with respect to the cell lines used). As seen in Figure 4-1, even though there was no treatment, spheroids at higher density of seeded cells were much more compact, due to more cell-cell interaction.

So, to test the results of how the spheroids would turn out at the same seeding density and fabrication process at different collection time points, L929 spheroids were collected after 1, 3, 4, and 5 days of culture in the PDMS AWs. Figure 4-5 shows the results of collection at different days. The longer culture times resulted in spheroids that were larger, more compact and slightly less uniform, with a higher total spheroid count. 0 outlines the protocol used for counting the spheroids.

It was important to consider an appropriate culture period that could allow the spheroids to survive through the droplet microfluidic device, while also maintaining uniformity. A 4 day culture was selected as it resulted in a high number of spheroids, of decent size and uniformity, with confidence that they would successfully run through the DM device with minimal damage.

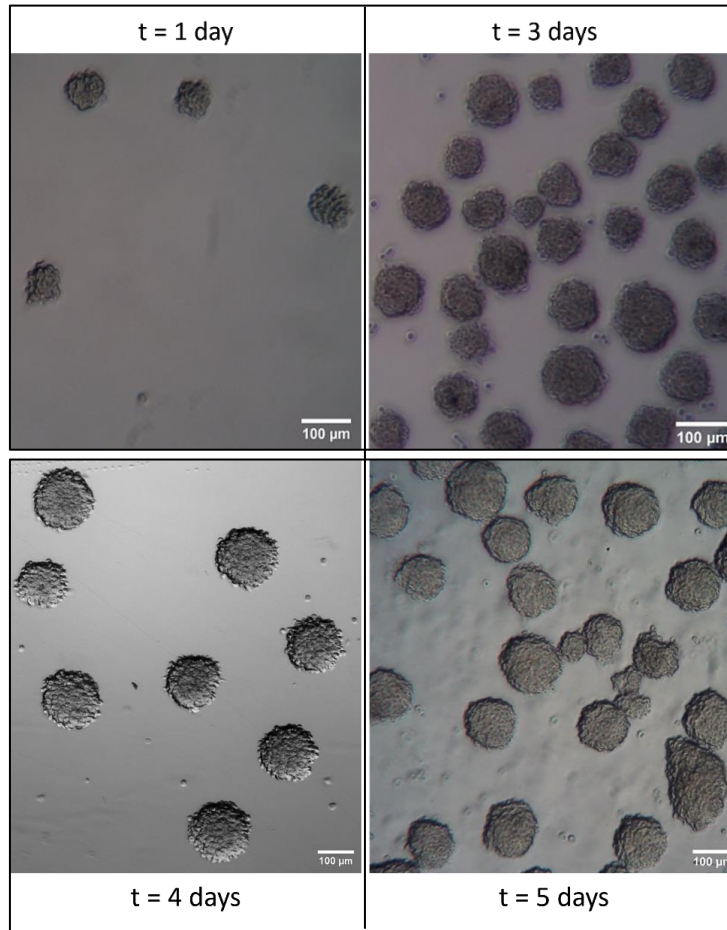


Figure 4-5: L929 spheroids at same initial concentration of 50 cells/spheroid (60 000 cells/PDMS AW). Spheroids were grown in the PDMS AggreWells™ and collected at different time points. Scale bars = 100 μm

4.3 Droplet Microfluidic (DM) Device Parameter Optimization

To move forward with a complete system, a DM device that was suitable for pre-formed spheroid encapsulation and uniform droplet formation needed to be chosen. There have been many DM chip fabrication techniques with the rise of microfluidic technologies. In this study, the traditional method of soft lithography, and 3D printing were considered to compare fabrication techniques. Alongside fabrication methods, factors such as chip geometry and system setup (tubing) also played a role in selecting the final setup.

It is important to note that in some instances, 90 μm polystyrene microbeads (polybeads, Poly Science) were used to mimic the spheroids to eliminate cell culture time, to test some parameters more quickly. However, there were some cases where the polybeads would either stick to the reservoir, tubing or PDMS chip, potentially due to the washing technique causing an electrostatic charge, attracting them to the different materials. Due to this occurrence, polybead concentration was not heavily relied on, and thus the beads were preferred for demonstration when showcasing different geometries.

4.3.1 DM chip molds fabricated using 3D printing vs soft lithography

Considering fabrication methods of DM molds, the two that were explored were soft lithography with a silicon wafer or stereolithography (SLA, 3D printing) using the Form 3 (Formlabs). Soft lithography is a common way of fabricating microfluidic chips due to its high resolution. 3D printing is a convenient way for rapid prototyping, that can assist in making decisions on what type of geometries to use. It is also an easier way of making larger channel heights, a challenge that may arise with soft lithography.

When trying different molds made by soft lithography, premade molds by previous lab members were chosen to save on time and expenses that soft lithography fabrication can entail. The main mold focused on in later trials was a double flow focusing (DFF) design, developed by Nguyen (2019, UW Space) [144]. Its channel height (180 μm) was comfortable for spheroids (~100 μm in diameter). Other silicon wafer molds usually had heights of 50-60 μm , thus were used for practice or demonstration.

Decisions for 3D printing molds initially came with testing simpler designs (e.g. T-junction) and viewing the capabilities of SLA for smaller features (microchannels). Due to limitations on the resolution of 3D printing, chip molds came out with a rough surface. This roughness was not only visually poor, but it could also interfere with stable droplet formation (likely at smaller channel dimensions). Figure 4-6 shows the comparison of a T-junction chip made from a 3D printed mold that was slightly sanded (a) versus a mold made by soft lithography (b). Zoomed in parts of channel walls help to depict the main differences: the surface and wall roughness, and channel linearity.

Interestingly enough, the way in which the molds were printed in the 3D printer and their location on the print bed also affected the roughness or finish of the mold. Some of these patterns were noticed during trials, however further 3D printing optimization was not continued due to time constraints and the scope of the project. But to briefly address some of the roughness issues, post-process treatments like sanding or coating were tested. A coating technique, developed by Ramasamy et al. [155], was promising for reduced roughness. However, due to the timing of using this method (in its development

stage) there were uncertainties of its toxicity to cells and its cause of slight channel dimension modifications, thus it was not considered moving forward.

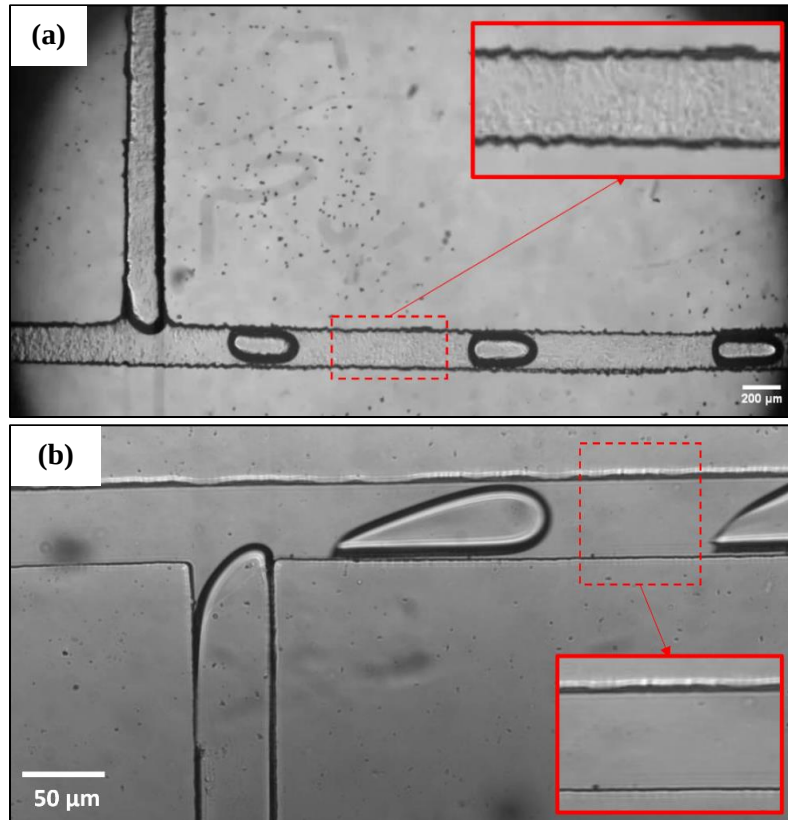


Figure 4-6: Images of chips made from (a) 3D printed and sanded mold and (b) soft lithography silicon wafer mold. The channel walls are more defined and smoother when using soft lithography versus the bumpiness seen with 3D printing, even with gentle sanding.

Table 4-2 summarizes some of the different 3D printed molds made to compare fabrication technique, geometry, and roughness (surface finish). This table is meant to serve as a documentation of different ways the chips were printed, in case of future use. 0 displays some images taken on the LEXT confocal microscope (Olympus), to show roughness of molds more visually.

Table 4-2: Descriptions of Various 3D Printed DM Molds

Chip Geometry (w/h, μm)	Resin Type	Print Angle	Post-Processing Treatment	Roughness Description
T-junction short channel, 300/300	Tough 2000	45°	None	Bumpy / visible layers
			Sanded	Channel tops much smoother, walls still rough
			Coated	Smoother all around, coating piled on the sides of the walls
T-junction long main channel, 200/200	Clear	Flat	Sanded	Decent smoothness, main roughness on the walls of the channel (hard to sand)
T-junction channels, 100/100	Clear	Flat	None	Pretty rough, causing unstable droplet formation
DFF, 150/150	Clear	Flat	None	Very rough walls

In considering a simple system with the T-junction geometry, shrinking the channel width to 100 μm was tested with the idea that the spheroids would be linearly sorted before droplet encapsulation. However, after trying this mold, the effect of the roughness was visible by unstable droplet formation, Figure 4-7.

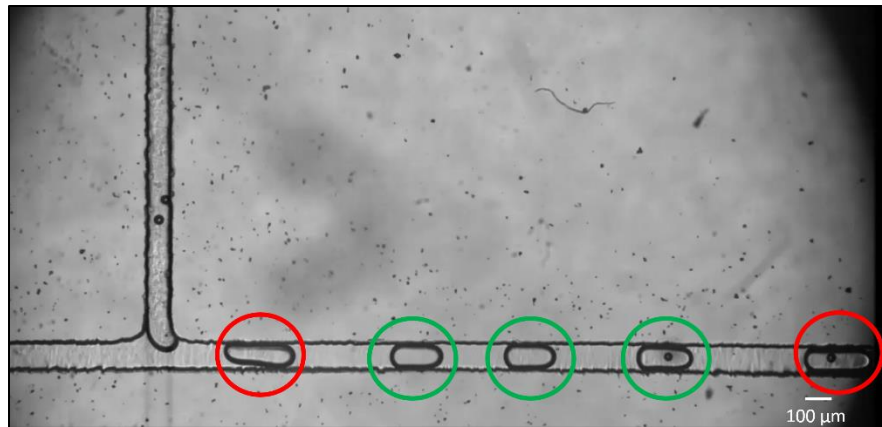


Figure 4-7: A DM chip (running with polybeads) made from a 3D printed T-junction mold with channel dimensions of 100x100 μm . Instances of uneven droplet formation with this fabrication

and geometry were not favored for the system. Green circles indicate uniform droplets, red circles show non-uniform droplets.

After working with the different 3D printed molds, it seemed that the 200 μm molds printed with clear resin were the best form of fabrication and might have been the limits of SLA 3D printing for this application. Due to the limitations of 3D printing, soft lithography was the preferred route. The decision was also influenced by having an established silicon wafer mold that could accommodate the spheroids, thus this geometry and mold was chosen moving forward. Yet, 3D printing was still a useful tool that was used to gain an idea of any geometry modifications in a quick manner.

4.3.2 DM junction comparison

Based on literature and practice, the T-junction type channel gave rise to a simple system. Thoughts of this junction were tried using mainly the 3D printing to achieve a comfortable channel height for the spheroids. On the other hand, from previous work, the double flow focusing chip seemed promising to implement with spheroids as its work with single cell encapsulation was successful.

To compare the performance of the two geometries, chips were tested with polybeads in Figure 4-8. In many instances using the T-junction, there were portions of the run where polybeads would be encapsulated together instead of individually (a), where the DFF could separate them into their own droplets (b). It was decided that having a slightly more complex configuration be worth it to ensure spheroids would get their own droplet, with decent efficiency. Thus, the DFF device was favored more than the T-junction.

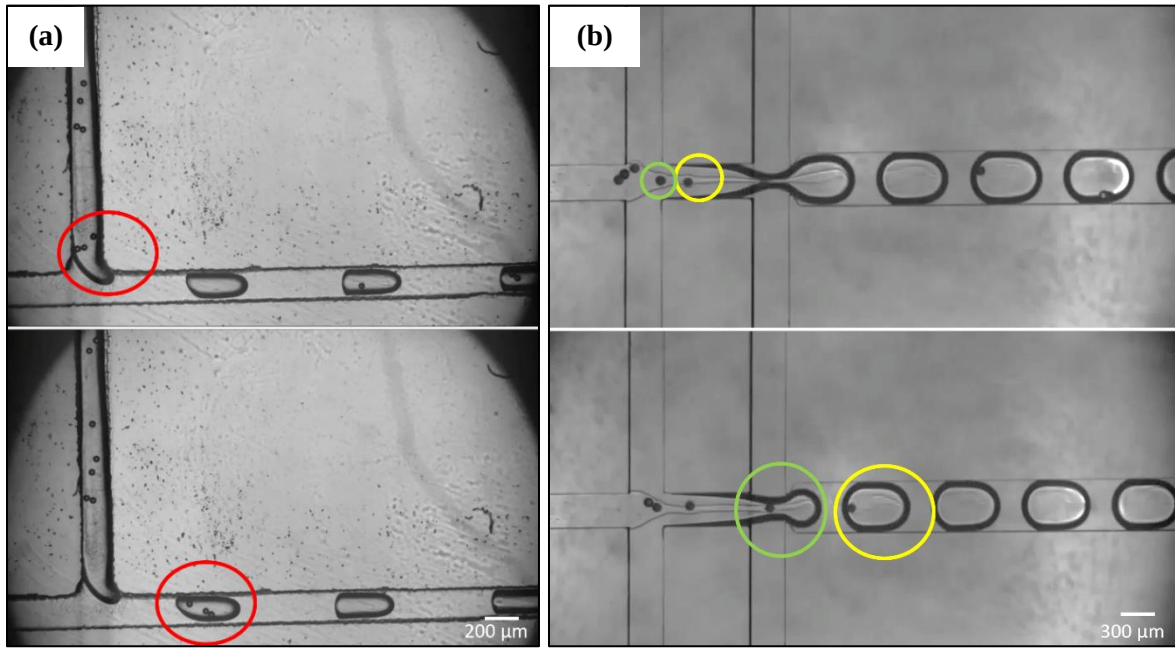


Figure 4-8: Encapsulation of polystyrene microbeads (90 μm) to demonstrate different geometries. (a) T-junction, 3D printed mold. (b) double flow focusing, silicon wafer mold. There were instances where the T-junction encapsulates more than one bead per droplet, where it looks like the DFF separated them, thus making it more favorable.

4.3.3 Inlet tubing inner diameter effect on spheroids entering the DM device

In addition to the chip geometry, the inlet tubing diameter played a role in how the spheroids came onto the chip. Figure 4-9 shows this by comparing tubing with 2 different inner diameters (ID) on a T-junction chip for spheroid encapsulation. In this case, spheroids were used instead of polybeads due to similar issues mentioned previously. As seen in Figure 4-9 (a), an ID much larger than the spheroids brought them onto the chip in larger groups. Even in the case of using the DFF, this would be difficult to separate spheroids into their own droplets. Of course, the starting inlet concentration of the spheroids can affect the variation of spheroids onto the chip, but a smaller, comfortable size tubing would still be better to implement. It also made sense to maintain similar channel widths compared to the tubing for a more uniform system and reduced fluctuation in pressure drop. However, it is worth noting that having too small of an ID would reduce the flow of the spheroids, and risk getting clogged easily.

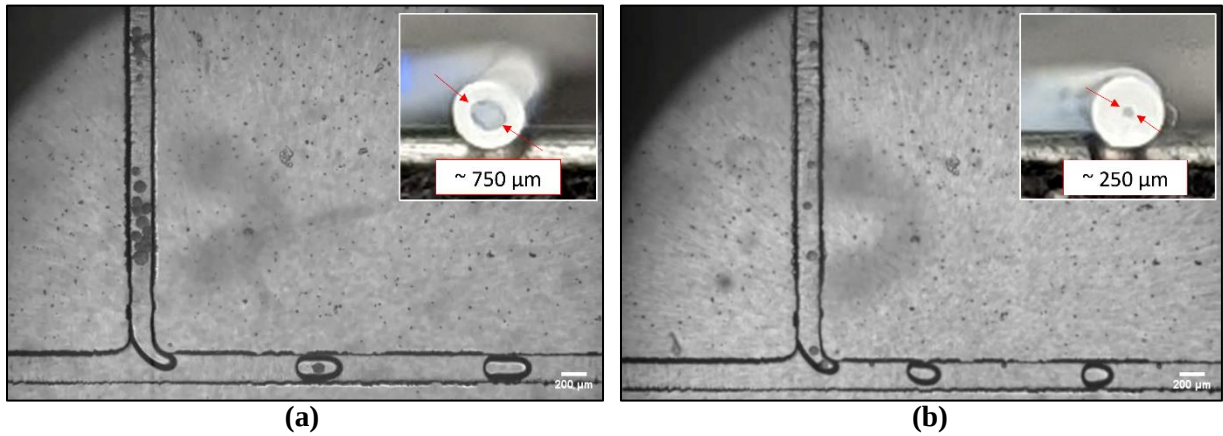


Figure 4-9: Spheroids entering 3D printed T-junction chips with different inlet tubing diameters. Spheroid concentration at the inlet was $\sim 8000/\text{mL}$. (a) Inlet tubing with inner diameter of 0.030 in ($\sim 750\text{ }\mu\text{m}$). (b) Inlet tubing with inner diameter of 0.010 in ($\sim 250\text{ }\mu\text{m}$). A larger inlet tubing ($750\text{ }\mu\text{m}$) allowed for more spheroids (about $100\text{ }\mu\text{m}$ in diameter) to pile onto each other, not making it favorable when coming into the chip for single spheroid encapsulation.

4.4 Summary of parameters

Overall, studying different approaches to the spheroid and droplet formation helped to conclude the parameters used moving forward. Table 4-3 and Table 4-4 summarize the parameters that were faced during optimization, the rationale behind testing them, and the overall decisions/conclusions drawn from experimental results. Each table focuses on each main component, that is the spheroid formation and DM device selection, respectively. In short, the final system would be comprised of the DFF geometry, with $250\text{ }\mu\text{m}$ ID tubing, using spheroids that were initially fabricated with 50 cells each after 4 days of culture in cell culture media with 10% FBS.

Table 4-3: Summary of Tested Parameters for Spheroid Formation Using PDMS AWs

Parameter	Rationale	Conclusions
Cell culture media serum concentration	Serum in media could help cells adhere to the substrate	- No significant change in cell behavior with less serum 10% FBS (standard) was chosen for culture

PDMS Surface	• UV sterilization could potentially mimic plasma treatment, encouraging cell attachment • Aged PDMS AW could be favored for cell attachment	• UV sterilization was eliminated (only 70% ethanol was used) • New PDMS AWs were used • PDMS was treated with 5% Pluronic F-127
Collection Method	• See if straining could help with spheroid suspension uniformity while yielding a decent number of spheroids	• Reverse straining (using 70 μm cell strainer) was applied to obtain a uniform spheroid collection
Spheroid Culture Time	• View effect of time on spheroid cohesiveness for DM use, without growing too large or non-uniformly	• Culture time effected spheroid cohesiveness and size • A 4 day culture time was selected for implementing into DM

Table 4-4: Summary of Tested Parameters DM Device Selection

Parameter	Rationale	Conclusions
Fabrication method of molds (3D printing vs soft lithography)	• Tested to see capabilities of newer technology (3D printing) versus tradition fabrication methods (soft lithography) • 3D printing could offer rapid prototyping for testing different geometries quickly	• 3D printed molds resulted in rough surfaces effecting droplet formation • Soft lithography was chosen overall
Channel Geometry (T-junction vs DFF)	• T-junction could offer a simple system • DFF has been established for single particle encapsulation	• DFF performed better for single particle encapsulation, thus was chosen overall
Inlet Tubing ID (750 vs 250 μ m)	• Tubing ID could affect the amount of spheroids flowing onto the chip	• 250 μm ID was chosen as it showed less clusters of spheroids coming onto the chip

Chapter 5: Studying a Droplet Microfluidic System for Single Pre-formed Cancer Spheroid Encapsulation in Hydrogel Microparticles

5.1 Introduction

Droplet microfluidics (DM) has become an emerging technology to achieve high throughput and controllability for spheroid formation [36,135]. However, most works look at forming spheroids from individual cells, whether with single or multi- cell encapsulation [38,139]. There has been a lack of work around encapsulating larger bio-particles, including pre-formed spheroids. In applications like forming patient derived tumor organoids (PDTOs), usually cell count from biopsy samples are low [43], so it would be impractical to apply them to current DM operations that use millions of cells at the initial reservoir. Pre-forming cells into cell clusters (spheroids) before bringing them on chip and encapsulating them in hydrogel microparticles (HMPs) brings rise to a new way of growing and analyzing a low quantity of cells in a meaningful manner, while jump starting organoid growth.

In this chapter, the capabilities of DM for pre-formed cancer spheroid encapsulation in a couple of biocompatible hydrogels is demonstrated with varying systems parameters. Using an optimized fabrication method to form cancer spheroids, these were implemented in a double flow focusing (DFF) droplet microfluidic device, a similar design of which was used in previous work for single cell encapsulation [144]. Due to the differences between individual cells and spheroids, adjustments had to be made, mainly at the inlet reservoir and the geometry of the chip, to account for spheroid density and fragility. Similarly, the efficiency would be expected to reduce due to the significant number of less spheroids than cells. Typically, in droplet microfluidic cell encapsulation, the starting concentration of cells is on the order of millions. In the case of using pre-formed spheroids, the total amount is on the order of thousands. To account for efficiency, different flow conditions, spheroid concentrations, and hydrogel precursors were shown.

Involved in completing the system was collecting the spheroid laden HMPs. Based on the chosen hydrogels, different collection methods were approached for successful crosslinking of the hydrogels. To validate the entire system, the spheroids were cultured over time and tested for cell viability to ensure cell survival afterwards.

5.2 Droplet Microfluidic Device and System Selection

The ideal DM geometry selected would aid in single spheroid encapsulation, while maintaining a decent efficiency. In previous work, Nguyen (2019, UW Space)[144] demonstrated that single cell encapsulation be possible up to 70% efficiency using a double flow focusing (DFF) geometry. Importantly with this geometry, the use of contrasting aqueous fluids was leverage for stratified flow, creating a hydrodynamic flow focusing stream that allowed for cells to be streamlined into their own droplets. Using this geometry, the dimensions of the chip were increased to fit the needs of the spheroids, which can be about 10 times as large in diameter as a single cell. The focusing stream became a key player in aiding with single spheroid encapsulation, especially with the challenges of inlet reservoir conditions (later discussed). Figure 5-1 demonstrates the selected chip by showing the geometry and PDMS chip. D1, D2, and C refer to the dispersed phase of the middle aqueous stream (red, inlet 3), dispersed phase of the outer aqueous stream (blue, inlet 2), and the continuous non-aqueous phase (yellow, inlet 1), respectively. L_j is the junction length, W_{or} is the orifice width, and W_m is the main channel width. The fluids used in each channel are also expressed in this figure, correlating to Table 5-1 for the two main cases used in this study. Along with these parameter, two main ratios were focused on: viscosity ratio (η) and flow rate ratio (λ) between the two dispersed phases, where μ represents the viscosities of the fluids and Q is the volumetric flow rate:

$$\eta = \frac{\mu_{D1}}{\mu_{D2}} \quad (1)$$

$$\lambda = \frac{Q_{D2}}{Q_{D1} + Q_{D2}} \quad (2)$$

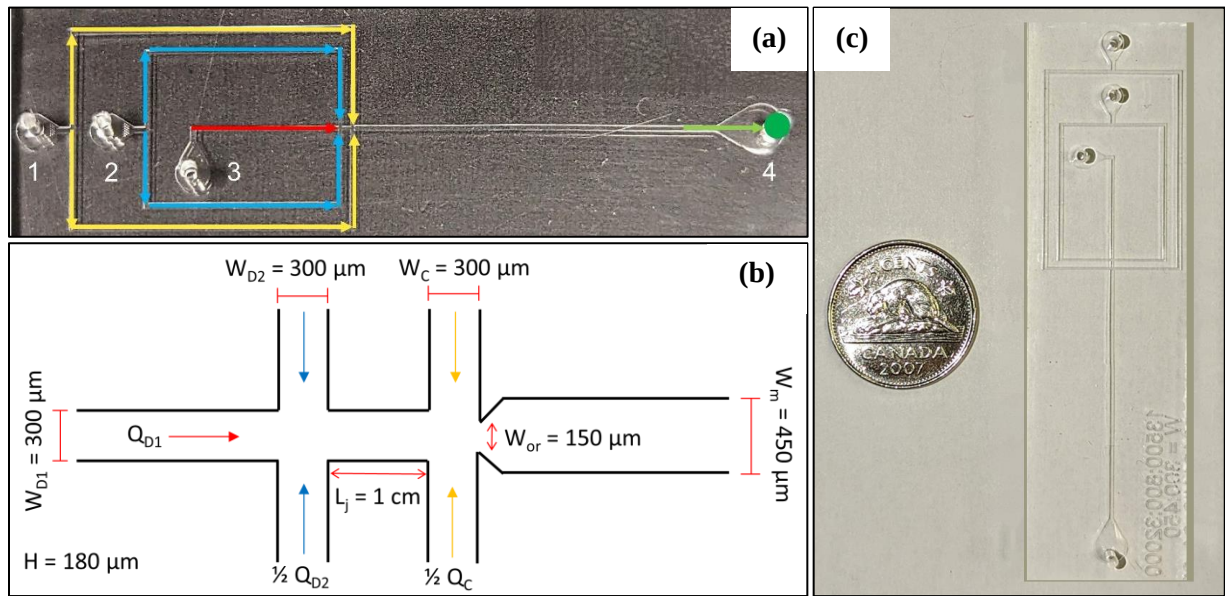


Figure 5-1: (a) Diagram of fluid flow through the DFF chip - 1) Continuous phase of mineral oil with 3% v/v Span 80; 2) dispersed phase 2 of a less viscous hydrogel precursor; 3) dispersed phase 1 of a more viscous hydrogel precursor mixed with spheroids; 4) outlet to droplet collection. (b) Schematic of chip geometry at the junctions for channel size and fluid flow division. Subscript definitions: dispersed phase 1 (D1), dispersed phase 2 (D2), continuous phase (C), junction (j), orifice (or), main channel (m). (c) PDMS chip with reference for size.

Table 5-1: Inlet and Outlet Fluids Used for the Selected DFF Device

Case	Inlet 1	Inlet 2	Inlet 3	Outlet
1	Mineral oil + 3% v/v Span 80	5% w/v GelMA	2% w/v sodium alginate	Cold CaCl_2 solution or cold mineral oil + Span 80
2		2.5% w/v GelMA	5% w/v GelMA	Cold mineral oil + Span 80

Before moving forward with this design, it was important to also understand the parameters that could affect the overall performance. Table 5-2 (and Table 5-1) summarizes the main parameters investigated and the rationale behind them for selecting the best case for the overall system. In later sections, each parameter will be elaborated on further.

Table 5-2: Parameter Considerations for the DM System for Single Spheroid Encapsulation

Parameter	Rationale for Studying/Varying
Hydrogel Precursor Concentration	- Concentration would affect viscosity which would effect the focusing stream as well as droplet formation - Hydrogel precursor viscosity would affect the spheroid settling time in the inlet reservoir, changing the way in which spheroids would enter the chip
Flow Focusing Width (FFW)	- Studying the changes in FFW would show how the single spheroid encapsulation efficiency would vary - A range of 3 types of widths were established to find the ideal condition
Spheroid Inlet Concentration	- Varying this condition would help find an ideal spheroid concentration for a decent encapsulation efficiency - It would also be beneficial to observe lower concentrations to view the system's ability to handle a small amount of sample, when considering the ideal application of PDTs
Crosslinking Techniques	- Proper crosslinking at certain stages would affect the uniformity of the HMPs after collection - Varied concentrations of CaCl_2 solution were used to find the optimal solution that would successfully crosslink the alginate, and try to separate the droplets from the oil phase. - Temperature and UV exposure were also factors for considering crosslinking GelMA

5.3 Hydrogel Criteria in the Droplet Microfluidic System

Following the criteria Nguyen proposed for optimal single particle encapsulation, the viscosity ratio between the two dispersed phase aqueous fluids was important to consider. For stable flow, D1 should be more viscous than D2, where the viscosity ratio is between 2 to 20 [144]. While this ratio was important, it was necessary to be mindful of the viscosities going into the chip itself. Fluids that were too viscous or exhibited non-Newtonian behavior would have challenges in droplet formation or channel clogging [156,157].

Gelatin Methacrylate (GelMA) was first considered being used based on previous work done, and its great biocompatibility. It was often seen that GelMA less than 10% w/v was used in DM devices to reduce risks of clogging, due higher viscosity and faster thermal gelation time at room temperature with higher w/v percentages [32]. The GelMA also needed to be stiff enough to support the spheroids, and not risk breaking or degrading quickly after permanent (UV) crosslinking. That is where the decision to use 5% w/v GelMA ($\mu \approx 5\text{-}7 \text{ mPa.s}$) came from. To accompany the 5% GelMA as the middle aqueous stream, 2.5% w/v GelMA ($\mu \approx 2.5 \text{ mPa.s}$) was selected as the outer aqueous stream for the first case, giving a viscosity ratio of $\eta \approx 2$.

In addition to the considerations for the hydrodynamic focusing stream and droplet formation, the viscosity of the hydrogel precursor also affected the conditions of the spheroids at the inlet reservoir. Due to the spheroids being more dense than single cells, their suspension time in fluids would differ, where the spheroid settling time would be quicker than that of single cells and vary based on fluid viscosity.

Figure 5-2 (a) illustrates the instance of viscosity effecting settling time of the spheroids. When 5% w/v GelMA was first used with the spheroids, it was observed that they would settle in less than 2 minutes. The settling was occurring as the reservoir was being attached to the pump system, so by the time the system was ready to go, the spheroids sat at the bottom of the inlet reservoir. In this case, the inlet tubing was targeted at the bottom of the reservoir. Comparing to Nguyen's observation with single cells, the cell suspension was able to last about an hour before noticeable changes to the behavior of cells coming onto the chip (settling and aggregation) [144].

After considering another common biocompatible hydrogel, sodium alginate, similar parameters were kept in mind. It was found that using 2% w/v sodium alginate ($\mu \approx 51 \text{ mPa.s}$), was the highest concentration without being too viscous for the DM device. It was also observed that the spheroids stayed suspended for a longer duration of time in the 2% alginate, due to its higher viscosity. Figure 5-2 (b) shows the real time settling of the spheroids in 2% sodium alginate, where images are taken in 3-minute intervals, showing significant settling after about 20 minutes. In this case, the inlet tubing was kept roughly in the middle of the fluid suspension.

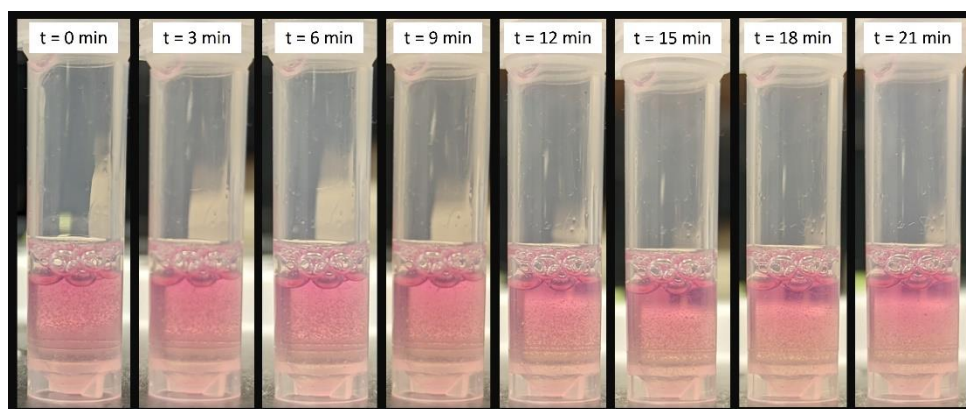
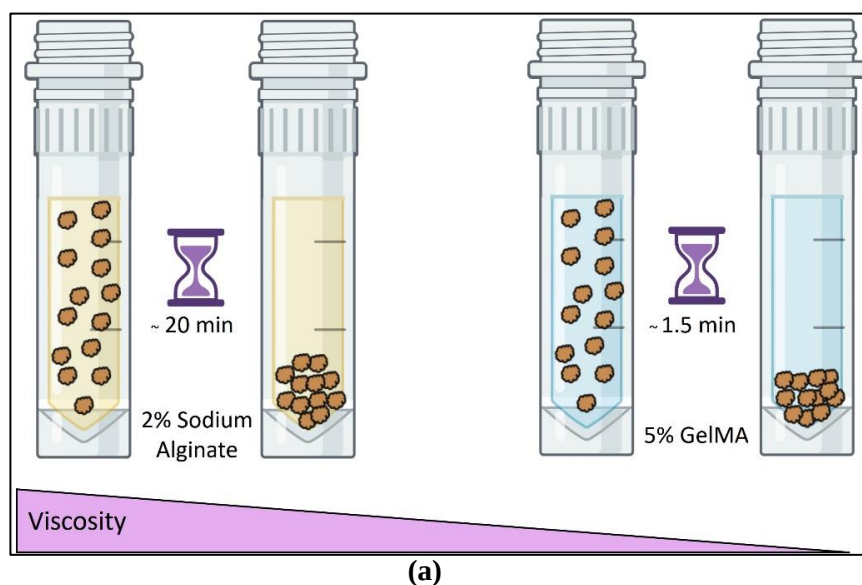


Figure 5-2: (a) Schematic of settling time of spheroids based on hydrogel precursor viscosity. Settling time is slower with increase of viscosity. (b) Approximately 4000 spheroids in 500 μ L of 2% w/v sodium alginate. Spheroids showed significant settlement after about 20 minutes; pictures taken in 3-minute intervals.

The difference of the spheroids lasting in the suspension while running the chip, versus collecting them from the bottom of the reservoir, would affect the way the spheroids come onto the chip. If spheroids were sat too long at the bottom of the reservoir, they would start to clump together, making it hard to separate them into individual droplets on the chip. It was preferred to use 2% w/v sodium alginate to suspend the spheroids and gain a more even distribution of spheroids flowing onto the chip. However, using 5% w/v GelMA was still successful with single spheroid encapsulation when the

number of spheroids was not too high, due to the hydrodynamic focusing stream. This can be favorable for the case of a low amount of sample size where a smaller number of spheroids may be obtained.

These observations, as well as the coupled criteria for this DFF geometry, were the main motivations behind the 2 selected hydrogel combinations that were outlined in Table 5-1.

It was also beneficial to understand how the hydrogels were made, specifically what they were dissolved in. Table 5-3 outlines the fabrication of the selected hydrogels, and what was considered for later crosslinking.

Table 5-3: Hydrogel Preparation Information

Hydrogel	Fabrication	Considerations
2% w/v sodium alginate	4% w/v sterile solution (dissolved in PBS) was diluted with cell culture media and spheroids to obtain 2% w/v	DMEM contains calcium. It is possible a small degree of crosslinking was occurring when the media was mixing with the alginate
5% w/v GelMA	- A 10% w/v solution (dissolved in PBS) was diluted with cell culture media and spheroids to obtain 5% w/v OR - A 10% w/v solution (dissolved in DPBS) was diluted with cell culture media to obtain 5% w/v	With considering PBS vs DPBS, DPBS can contain calcium and magnesium (both capable of crosslinking alginate). Introducing these ions briefly in the chip can initiate alginate crosslinking
2.5% w/v GelMA	A 10% w/v solution (dissolved in PBS) was diluted with cell culture media to obtain 2.5% w/v	Implementing cell culture media in the hydrogel would help sustain the cells through the journey until later incubated with media at the end

5.4 Flow Focusing Width Variation and its Effect on Spheroid Encapsulation

The key player in the DFF chip is the hydrodynamic flow focusing width. Nguyen (2019, UW Space)[144] demonstrated that the focusing width varies with mainly the flow rate ratio (λ) and viscosity ratio (η) between the two dispersed phases. To demonstrate the effect of the FFW, 5% GelMA was used as D1 and 2.5% GelMA was used at D2. Since in this case the viscosity ratio remained the same at $\eta \approx 2$ ($2.4 \leq \eta \leq 2.8$), the pressure adjustments driving D1 flow from the inlet reservoirs affected the focusing width. The determining factor for varying focusing widths was compared to Nguyen's case of using a viscosity ratio of $\eta = 2$. For initial judgment while running the chip, 3 main stages were used to determine the difference in flow conditions. Table 5-4 describes the type of focusing width with the classification used for the initial judgment. It also compares the expected flow rate ratios of the system based on Nguyen's study. Percentages were determined by finding the ratio of the width of the focusing stream relative to the channel width, using Image J and the given scale bar. The figure and description used is outlined in 0.

Table 5-4: Classification of Flow Focusing Width Based on Viscosity Ratio of $\eta = 2$

Classification of FFW	Ratio to channel width (percentage)	Expected Total Dispersed Phase Flow Rate Ratio (λ)
Narrow	~ 20 - 25%	0.85 - 0.8
Medium	~ 29 - 33%	0.75 - 0.70
Wide	~ 40%	0.65

To illustrate the focusing widths with this geometry, Figure 5-3 displays the three different thicknesses of FFW in the same inlet condition, colorized to depict where the focus stream is (pink). The different FFWs are important in demonstrating how single spheroid encapsulation efficiency can differ. Efficiency was estimated over a short time span, similar for all conditions, counting the number of encapsulations to the total number of droplets (around 20 droplets analyzed for each case). Videos chosen were the best-case scenarios for each condition, to give a brief understanding of how the different FFWs affect the spheroid encapsulation. It is important to note that over longer time observations, efficiency would decrease. The three cases are further outlined in the following subsections, each kept with condition of about 5000 spheroids settled in 500 μ L of 5% GelMA as D1

and 2.5% GelMA as the D2. Figure F-1 in 0 also graphically compares the ratios of different droplet contents for each FFW case, for another type of visual representations.

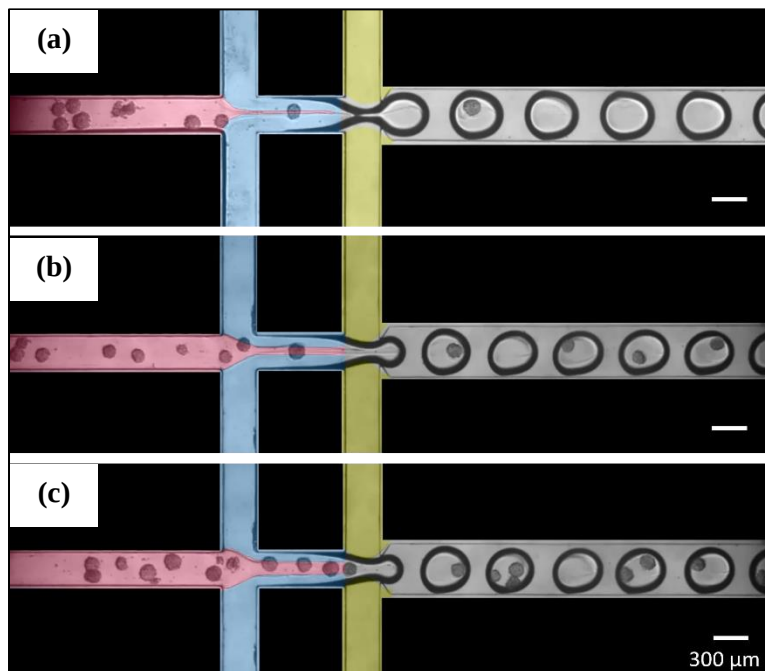


Figure 5-3: Pseudo colorized images of each type of flow focusing width to help distinguish the streams: (a) Narrow, (b) Medium, and (c) Wide. Spheroid quantity was kept constant at 5000 spheroids, settled in 500 μL of 5% w/v GelMA ($\eta \approx 2$). Focusing width varied based on pressure change of the dispersed phase 1. Scale bars = 300 μm .

5.4.1 Narrow flow focusing width

Having a narrow focusing stream meant the spheroids would enter the chip at a slower rate, so it was expected that there would be less spheroid encapsulations compared to other cases. Figure 5-4 shows snapshots at different time frames to visualize the encapsulation process, with the system running with a narrow focusing width ($\lambda \approx 0.83$). The narrow focusing width may be more favorable for ensuring single spheroid encapsulation, but would overall lack in general encapsulation efficiency, due to the high number of empty droplets. The results of this parameter estimated an efficiency of about 27%.

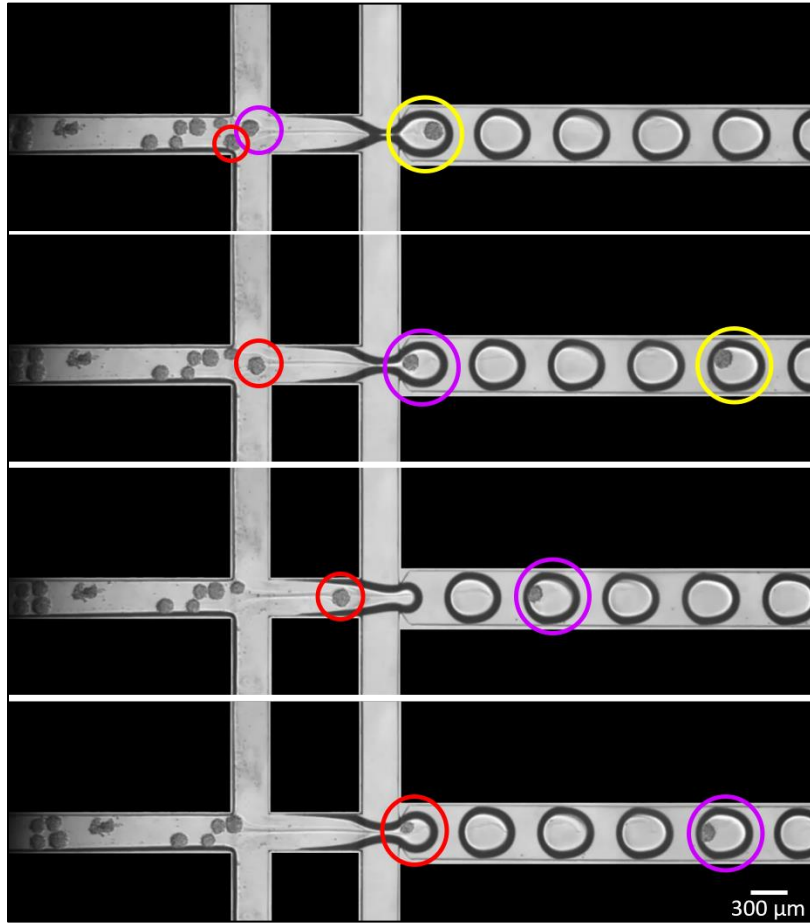


Figure 5-4: Snapshots of successive time points for a narrow flow focusing width ($\lambda \approx 0.83$) with 5% GelMA, $\eta \approx 2$, and 5000 spheroids settled at the inlet. This case showed a low amount of encapsulation, about 27%. Yet, the narrow focus helped streamline the spheroids into their own droplet really well, considerable for targeting single spheroid encapsulation each time.

5.4.2 Medium flow focusing width

Setting the flow conditions to create a medium size focusing width would aim towards a moderate inflow of spheroids onto the chip. The focusing stream would still showcase its ability to help spheroids flow into their own droplets with a higher intake to increase encapsulation efficiency. Figure 5-5 shows snapshots at different time frames to visualize the encapsulation process, with the system running with a medium focusing width ($\lambda \approx 0.74$). The medium focusing width overall showed an encapsulation efficiency of about 48%, a good increase compared to the narrow width. In the snapshots used, there were no instances of multi-encapsulation, but this was observed from time to time over longer runs.

The medium width was successful at breaking apart spheroid groups that would enter the chip together, showcasing the importance of the focusing width. This condition of a medium FFW showed promising results moving forward in the system.

To compare this case with Nguyen, who used a medium focusing width ($\lambda = 0.7$) in their application of single cell encapsulation, their efficiency was up to 70%. Although, considering the conditions at the inlet, where Nguyen used 5×10^6 cell/mL versus the 5000 spheroids/500 μL for this study, the results are still comparable. To make 5000 spheroids, only about 240 000 cell are required initially. To note again, cells are about 10 time smaller in diameter than spheroids, so a cell's progression onto the chip would behave differently to that of a spheroid.

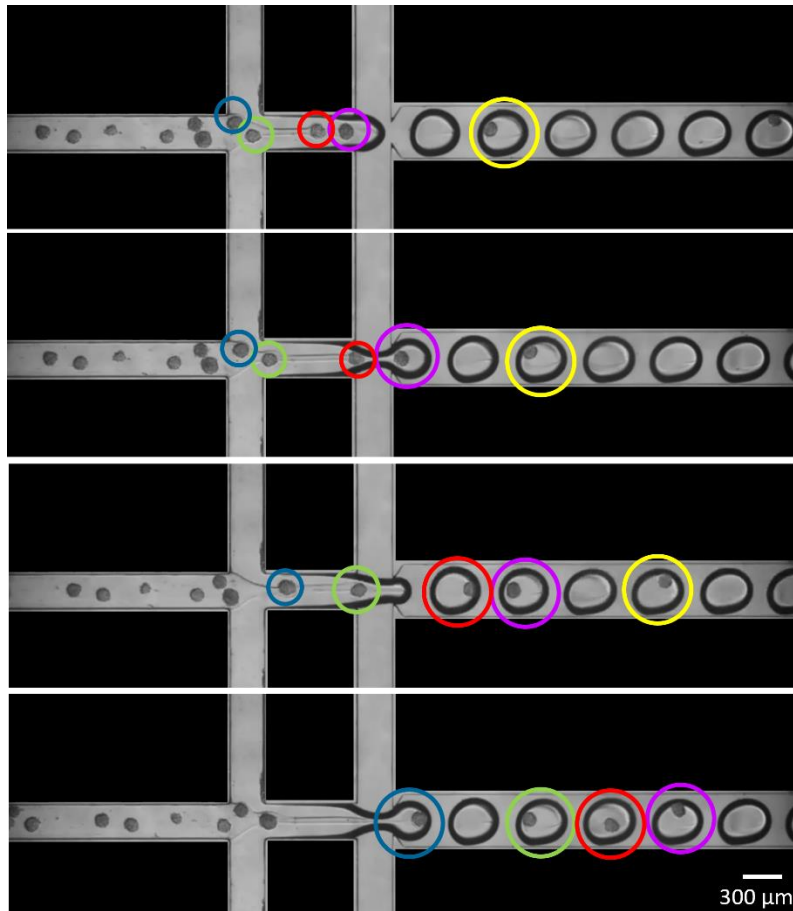


Figure 5-5: Snapshots of successive time points for a medium flow focusing width ($\lambda \approx 0.74$) with 5% GelMA, $\eta \approx 2$, and 5000 spheroids settled at the inlet. This case showed a decent amount of single spheroid encapsulations, about 48%.

5.4.3 Wide flow focusing width

The use of a wide focusing width came with the risks of more multi-encapsulations (droplets with more than one spheroid inside). The flow of spheroids would be higher than the other cases and the focusing stream may be too wide that it would not do its intended job. Figure 5-6 shows snapshots at different time frames to visualize the encapsulation process, with the system running with a wide focusing width ($\lambda \approx 0.65$). The wide focusing width encapsulation efficiency was looked at in two ways. For single spheroid encapsulation, it produced roughly 54%, and when considering total encapsulations (including multi) it was 75%. Although about 70% of the total encapsulations were of single spheroids, this condition may not be worth the tradeoff of the highest encapsulation efficiency. With multi-encapsulated spheroids, there is a decrease in uniformity when considering growing these spheroids into organoids. To be consistent between batches, one spheroid per droplet is favored, especially with passive DM techniques and lack of sorting droplets.

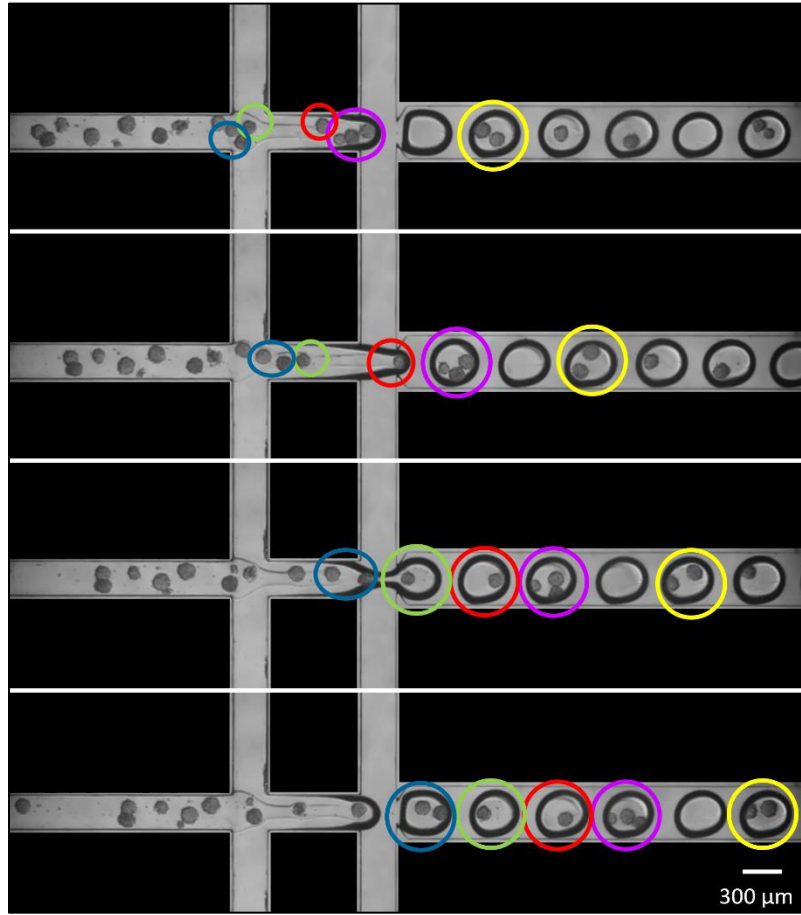


Figure 5-6: Snapshots of successive time points for a wide flow focusing width ($\lambda \approx 0.65$) with 5% GelMA, $\eta \approx 2$, and 5000 spheroids settled at the inlet. This case showed a high amount of single spheroid encapsulations, about 54%. However, it is coupled with many multi-encapsulations, not favorable for batch-to-batch consistency.

5.5 Spheroid Concentration Variation

Similar to how the flow focusing width affected the efficiency of single spheroid encapsulation, the amount of starting spheroids also had an effect on this. Figure 5-7 demonstrates this effect by showing snapshots of different inlet quantities of spheroids either suspended in 500 μL of 2% w/v sodium alginate, (a) and (b), or settled in 500 μL of 5% w/v GelMA (c).

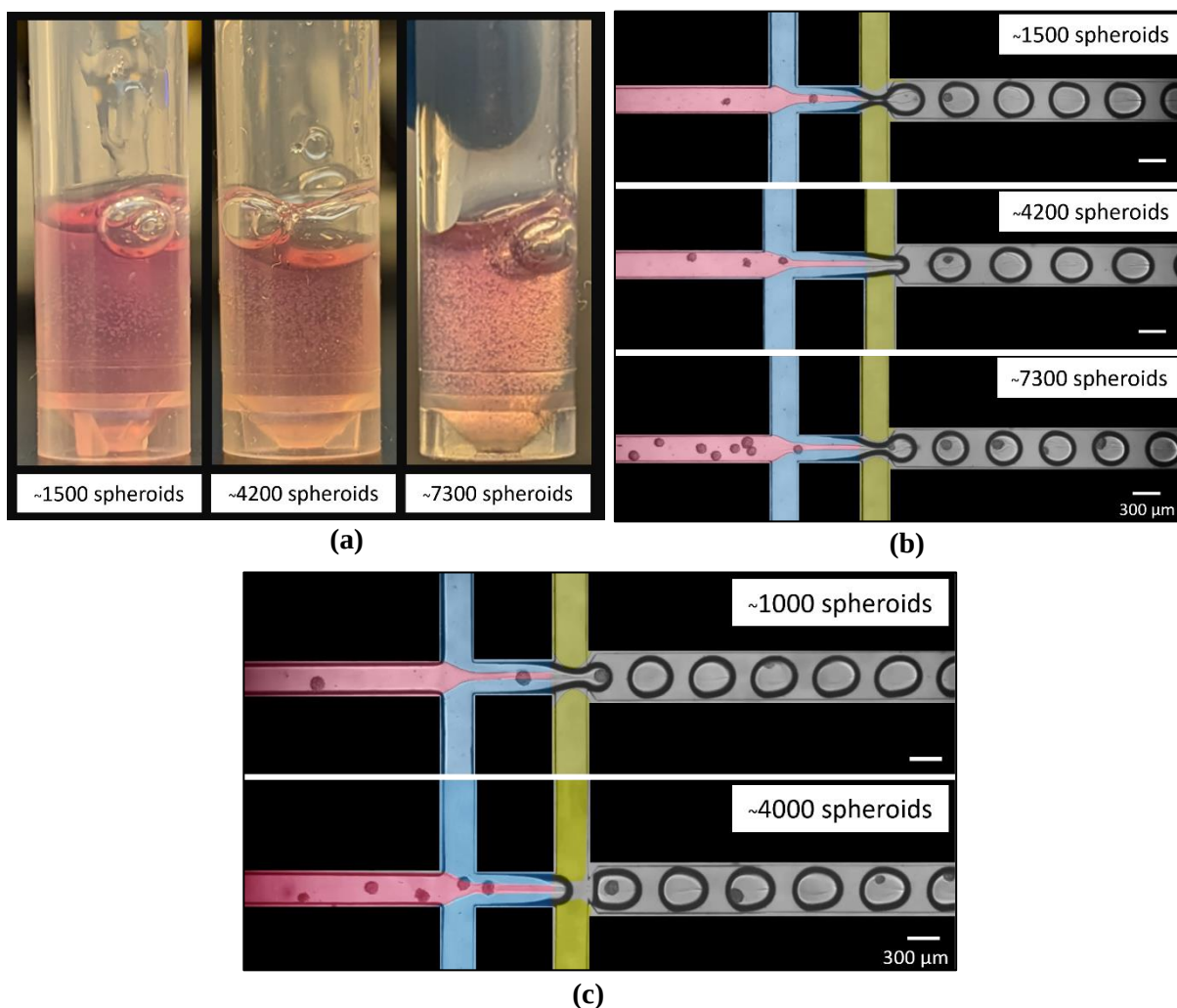


Figure 5-7: Spheroid concentration variation with focusing width kept medium ($0.70 \leq \lambda \leq 0.75$). (a) Image of different spheroid quantities suspended in 500 μL of 2% w/v sodium alginate. (b) Varying spheroid quantities at the inlet reservoir depicted in (a). (c) Spheroid quantity variation settled in 500 μL of 5% w/v GelMA. Higher initial spheroid quantities allowed for more spheroids present for encapsulation into the hydrogel droplets. Scale bars = 300 μm

In the same way the efficiency was estimated for the varying FFW, this was done for varying spheroid concentrations at the inlet reservoir, with both kinds of hydrogel combinations. The best instances for each case were analyzed by counting the number of droplets with single spheroids to the total number of droplets generated in the selected time frame. Table 5-5 summarizes the values for direct comparison. Figure F-2 in Appendix F also graphically compares the ratios of different droplet

contents for each case of spheroid concentration and inlet condition, as another type of visual representation.

Table 5-5: Comparison of Single Spheroid Encapsulation Efficiency for Varying Inlet Quantities

Hydrogel Condition	Quantity of Spheroids at the Inlet	Single Spheroid Encapsulation Efficiency
Case 1, spheroids suspended in 500 μ L of 2% w/v sodium alginate	~ 1500	19%
	~ 4200	20%
	~ 7300	45%
Case 2, spheroids settled in 500 μ L of 5% w/v GelMA	~ 1000	30%
	~ 4800	44%

Overall, as expected, the higher number of spheroids in the inlet generated higher encapsulation efficiency. However in those cases, there were more chances of multi-encapsulations, a similar trend noticed by Tang et al.[139] in their study of single bead encapsulation using their unique system. This case of multi-encapsulations is not as high as when using the wide FFW. For example, the case of 7300 spheroids in 2% sodium alginate resulted in about 18% of the encapsulation being multi-encapsulations. Having a combination of high concentration with higher spheroid flow rate would be the unideal case of plenty of multi-encapsulations.

So, when considering the initial spheroid quantity at the inlet, the most favorable are the higher quantities in each hydrogel case. Yet, considering that when the 1000 spheroids settled at the bottom of the reservoir showed successful encapsulations, this would be favorable for a low sample size because to make 1000 spheroids, only 60 000 cells would be needed in the initial seeding in the AggreWell™.

5.6 Hydrogel Microparticle (HMP) Droplet Collection

Once the spheroid laden HMPs were formed within the DM chip, they needed to be collected off-chip to crosslink the hydrogels to form gelled HMPs. Due to the use of 2 different types of hydrogels, different parameters were investigated when considering the crosslinking for GelMA versus sodium alginate.

GelMA is a thermal sensitive hydrogel in that it can thermally gel at cooler temperatures, and remain crosslinked at maintained low temperatures. However, once exposed to UV light, it will permanently gel by covalent crosslinking, allowing it to be a stable gel at warmer temperatures such as 37°C. The gel formed by sequential crosslinking through the act of cooling the GelMA first and then photo-crosslinking is known as GelMA+ [110]. The idea to do this with both cases of hydrogels was to minimize droplet coalesce during collection, as direct UV light during collection was limited based on the setup and equipment. Although there was a surfactant (Span 80) present in the oil, often the droplets would merge off-chip if not kept cool.

Since GelMA was present in both cases of hydrogel combinations on the outer portion of the HMP, all collection baths were cooled to 4°C and kept on ice during collection at the microscope to achieve thermal crosslinking. For the case of only GelMA combinations, cold mineral oil with Span 80 was used as the collection bath.

When implementing sodium alginate, the ionic crosslinking mechanism was used. Alginate hydrogel forms after mixing with divalent ions, such as Ca^{2+} . It was commonly found that researchers use CaCl_2 solution as the crosslinker solution due to its mild effects for biocompatibility [92]. In the case of the alginate-GelMA combination, 2 main baths were considered: CaCl_2 solution (in DI water) and mineral oil.

Table 5-6 outlines the different collection baths used for considerations on crosslinking. They were all cooled to 4°C to thermally crosslink the outer GelMA to reduce risk in coalescence before UV crosslinking.

Table 5-6: Collection Methods and Parameters for Crosslinking HMPs after Droplet Formation

HMP Composition (outer/inner)	Collection Bath	Rationale
Case 1: 5% GelMA / 2% sodium alginate	4°C 2% w/v CaCl ₂ solution (0.18 M)	• Common concentration for crosslinking sodium alginate with calcium chloride solution
	4°C 1% w/v CaCl ₂ solution (0.09 M)	• Tried to combat droplets floating in the collection bath by selecting concentrations of CaCl ₂ that were less dense than 2% sodium alginate
	4°C 0.5% w/v CaCl ₂ solution (0.045 M)	
	4°C Mineral oil + 3% Span 80	• Oil helped keep droplets segregated
Case 2: 2.5% GelMA / 5% GelMA	4°C Mineral oil + 3% Span 80	• No chemical crosslinkers needed • Oil helped keep droplets segregated

When collecting alginate-GelMA HMPs in oil, there was no additional calcium present to fully crosslink the alginate, so this type of combination was expected to be different. When trying to decide on the concentration of the CaCl₂, the most common was 2% w/v. That is where the initial bath was used. However, during collection, it was found that the droplets would float to the interface of the oil instead of sinking as expected. Figure 5-8 (a) and (b) shows an example of how the collection looked like when using an aqueous solution (CaCl₂) versus the oil solution (mineral oil + Span 80).

A potential speculation for this occurrence was due to the density between sodium alginate and the CaCl₂ solution. After comparing these parameters, lower CaCl₂ solution concentrations were used (1% and 0.5%) to achieve densities less than that of 2% sodium alginate [158,159]. Figure 5-8 (c) shows HMPs collected in 1% CaCl₂ solution. This change did not reduce the floating, suggesting the oil around the droplets was influencing its behavior. Yet, the droplets were still able to crosslink when they sat in the solution for some time, eventually breaking from the oil after collection for UV crosslinking.

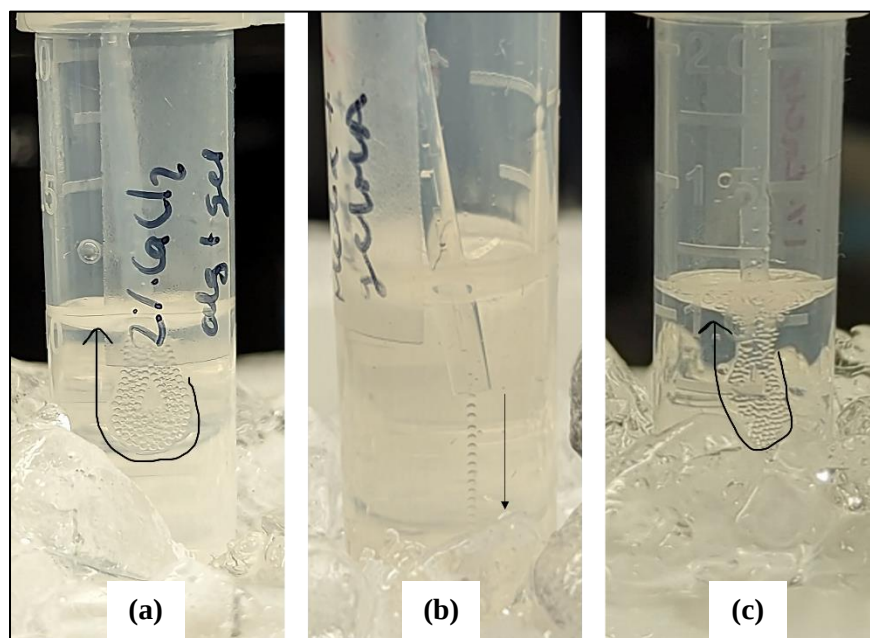


Figure 5-8: Collection baths for HMPs. (a) 2% w/v CaCl₂ solution. Droplets would collect on the side of the container and proceed to float to the top. (b) Mineral oil with 3% v/v Span 80. Droplets would fall directly to the bottom of the container. (c) 1% w/v CaCl₂ solution. Droplets exhibited similar behavior to the 2% w/v CaCl₂ bath. Arrows indicate movement of droplets. For aqueous solutions, droplets aggregated to the side and slowly floated up.

After collection, the HMPs were exposed to UV for 2 minutes. The UV was meant to permanently crosslink the GelMA for stable HMPs for further use at warmer temperatures. From previous trials and literature, 2 minutes was selected as the optimal time for crosslinking the GelMA.

5.6.1 HMPs after crosslinking and washing

When HMPs were fully crosslinked, they needed to be washed from either the oil or extra CaCl₂ solution and replaced with fresh media. Figure 5-9 shows some collection examples from different droplet hydrogel combinations and collection baths. In general, HMPs had uniform structure and similar sizes between their own batch. Often times, alginate-GelMA HMPs collected in the oil bath came out the most uniform, potentially due to no interference from alginate crosslinking. The GelMA-GelMA HMPs were not very consist in collection, where troubles of droplet coalescence remained a challenge. That is why the alginate-GelMA combination was more favored.

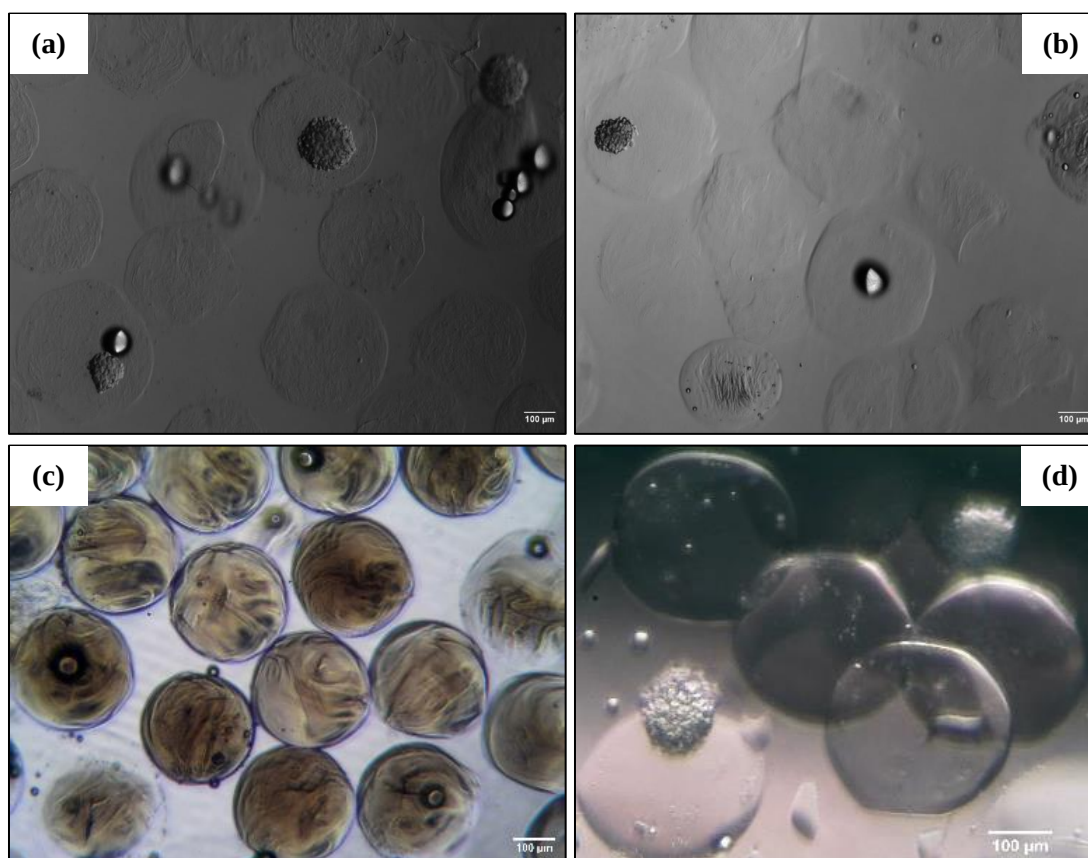


Figure 5-9: HMPs after crosslinking and washing with media. The first two photos were droplets made from the alginate-GelMA combination, where the initial GelMA was dissolved in DPBS; (a) collected in cold 2% w/v CaCl_2 solution, (b) collected in 1% w/v CaCl_2 solution. (c) Droplets made from alginate-GelMA combination, where the initial GelMA was dissolved in PBS, and collected into cold mineral oil. (d) Droplets made from the GelMA-GelMA combination and collected into cold mineral oil. All HMPs were crosslinked with UV for 2 min.

An important part of the HMP's role was not only to encapsulate the spheroids, but to maintain its structure for the time of the spheroids' growth at 37°C. Thus, viewing the HMPs' ability to last longer than at least a few days was a good sign it could house the spheroids long-term. Figure 5-10 shows HMPs after 24 hours and 5 days for the case of alginate-GelMA collected in 2% w/v CaCl_2 solution. In general, HMPs lasted at least 5 days. If there were any instances of bursting, it was visible right after the collection wash. The bursting was a common issue which seemed like a random effect. Due to the large number of variables that made up the system, it was difficult to pinpoint exactly what may have

caused bursting. However, potential leading factors may have been the timing between crosslinking the alginate and GelMA, or osmotic pressure when washing the HMPs in a different solution (media).

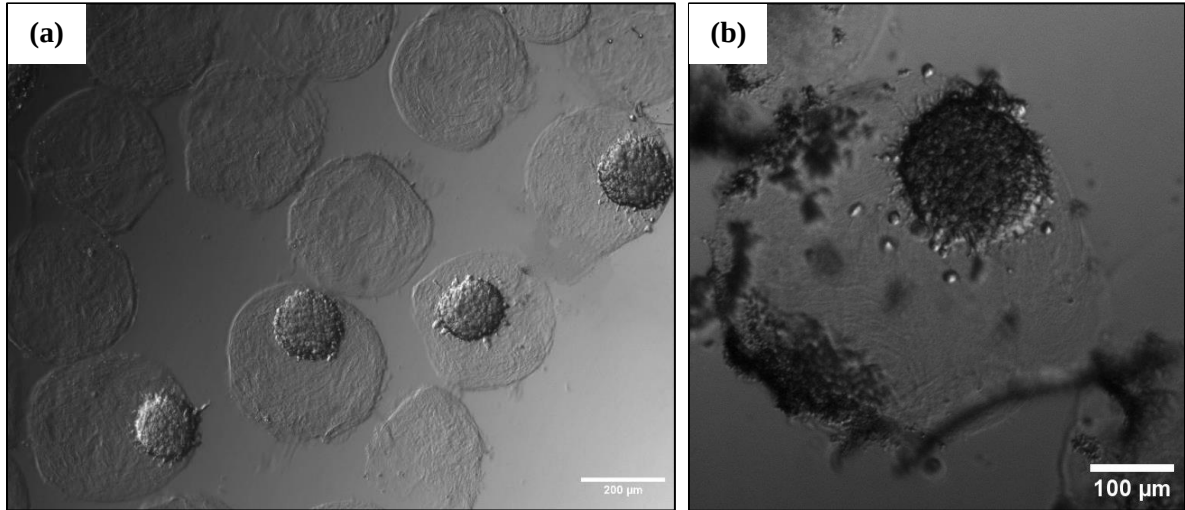


Figure 5-10: Alginate-GelMA HMPs collected in 2% w/v CaCl_2 solution after being washed and culture in serum free media at 37°C. (a) Viewed after 24 hours. (b) Viewed after 5 days.

5.6.2 Compression testing on crosslinked HMPs

As an extra characterization measure, the HMPs that were made in the DM device and crosslinked in specific manners were tested for elasticity using the MicroTester (CellScale). Due to time limitations and learning curves, only a few types of HMPs were tested, so these results act as preliminary to gain an idea of the stiffness of the gels based on crosslinking method. Figure 5-11 shows the results of tests on HMPs collected in 2% and 1% w/v CaCl_2 (0.18 M and 0.09 M respectively) solution and mineral oil. Since the data were a bit rough with many fluctuations, it was hard to fully calculate Young's modulus. However, the overall linear region of the stress-strain curve (0-20% strain) was used to estimate a value, method inspired by Wang et al. [160]. The estimated Young's modulus (kPa) for HMPs collected in 2% CaCl_2 solution, 1% CaCl_2 solution, and mineral oil were 64.43, 1.46, and 4.87, respectively. From the graph and this data, it appears that the crosslinked alginate with 2% CaCl_2 was stiffer compared to when it was 1% CaCl_2 or just GelMA and sodium alginate present. This would be understandable as increasing crosslinking density tends to have an increased effect on stiffness. These values can also be comparable to those in literature for sodium alginate and GelMA individually. Modulus for 2% sodium alginate crosslinked in 0.5 M CaCl_2 for 10 minutes was found to be about 14

kPa [161]. The modulus for 5% GelMA, crosslinked for 60 seconds under 6.9 mW/cm² UV light was about 5 kPa [162]. Further tests on GelMA combinations will be useful to compare the stiffness of when no alginate is present.

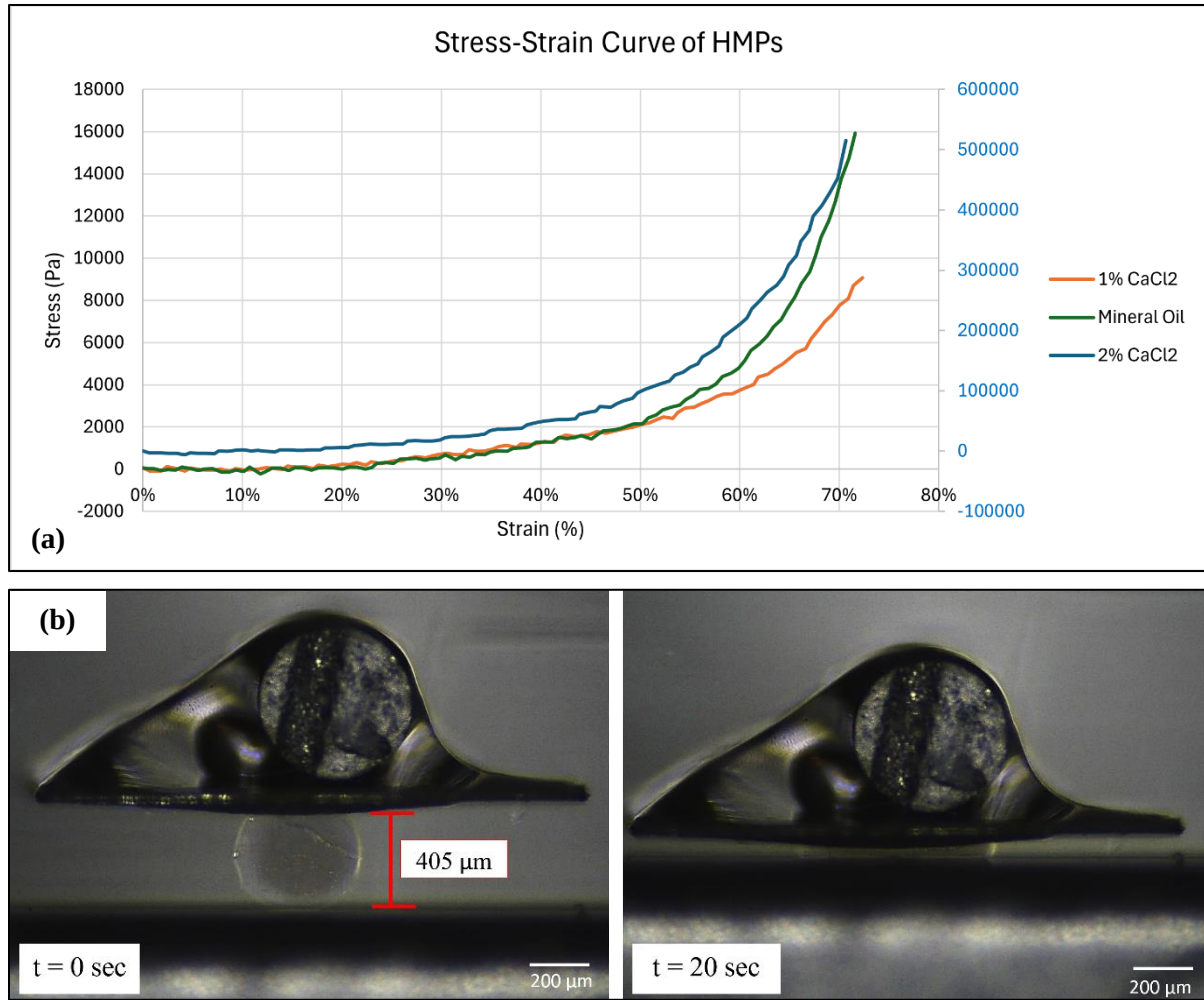


Figure 5-11: (a) Compression test stress-strain curves of different alginate-GelMA HMPs. The right-hand (blue) y-axis is meant for the 2% CaCl₂ curve. (b) Image of an HMP collected in mineral oil before compression (t = 0 sec) and at the maximum load (t = 20 sec) demonstrating the MicroTester.

5.7 Spheroid Viability

As a final measure to the entire system, the viability of the spheroids was important to test to ensure that this system was not only robust, but not harmful to the cells. As a preliminary test of viability, a Live/Dead assay was used to compare the amount of green versus red (live versus dead) signal coming from the spheroids: Figure 5-12 with representative images in 0, after 24 hours and 5 days. It is noted that in the 5 day test, the droplets collected in mineral oil were not included due to inconsistencies in encapsulation and droplet coalescence, causing larger groups of spheroids, not representative of the intended goal.

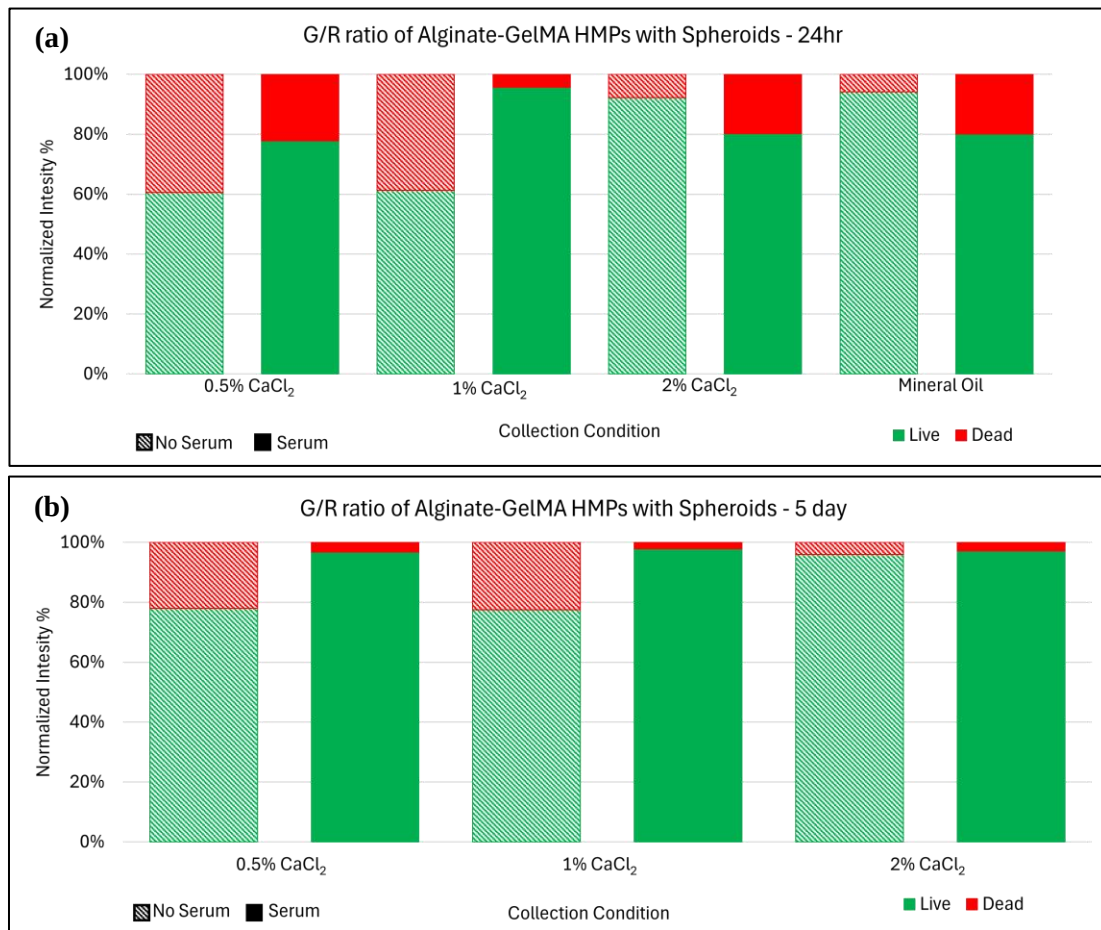


Figure 5-12: Ratio of green to red (live to dead) signal from Live/Dead assay at (a) 24 hours and (b) 5 days. Signals were normalized to spheroid area and averages of the spheroids in the representative photos were used as demonstration. Mineral oil at day 5 was not shown due to spheroid clusters overwhelming signals in coalesced HMPs.

Since there were various collection methods and post-collection conditions, it was also of interest to test the viability of the spheroids after being collected in different ways. All HMPs were formed with alginate-GelMA combination, where initial GelMA was dissolved in DPSB, with spheroids that were cultured for 4 days prior to being used in the DM device. Along with testing the crosslinking baths (outlined in Table 5-6), it was also of curiosity to see how cell behavior or viability would be when spheroids were cultured in media with and without serum (FBS). Overall, the results looked promising as the majority of the spheroids show above 80% green (live) signal, even after 5 days of culture. There is no conclusion to be drawn from this preliminary data on whether it is better to use serum in the media or not. However, there was an interesting occurrence after 24 hours of culture with serum media on some of the spheroids. The cells seemed to start spreading away from the HMP. Figure 5-13 shows this instance with an HMP collected in 2% CaCl_2 solution after 24 hours of culture in media with 10% FBS. As serum is a substance that encourages cell attachment, if the HMP was too soft, there is a chance that the cells were able to ‘leave’ and adhere to the tissue culture plate instead of staying in the HMP.

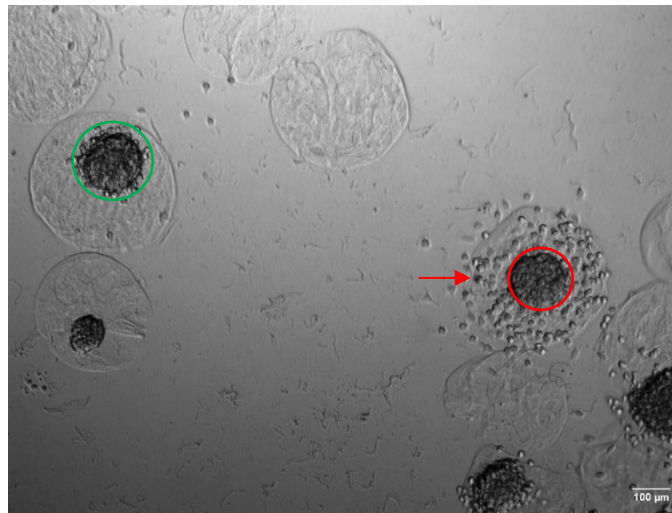


Figure 5-13: HMPs cultured in serum media after 24 hours. Cells seemed to spread away from the spheroid, outside of the HMP. The spheroid circled in red shows the case where the cells (smaller dots) were moving away from the spheroid. The spheroid circled in green shows the case of a spheroid more intact.

Since the spheroids are 3D, it was hard to be certain exactly how the spheroids were sat inside the HMP and that the entire image captured was representative of the entire spheroid. Using a fluorescent confocal microscope would have been more favorable for this type of quantification. However, due to

equipment limitations a standard inverted fluorescence microscope was used. Thus, this assay and imaging served more as a guideline to visually see if the majority of the spheroid remained alive after at least 24 hours and up to 5 days as a short-term time point. After acknowledging the outcome of this assay, other more meaningful viability tests, such as alamarBlue™ (Invitrogen), could be considered to quantify cell viability through proliferation measurements. Additionally, it will be good to replicate the system a few times to get significant results.

5.7 Discussion

After viewing the results of the system, key points can be taken to compare with Nguyen's system for single cell encapsulation and other systems that go beyond this for cell culture in the hydrogel microparticles. Beginning with efficiency, it was noted that the highest achieved efficiency of single pre-formed spheroid encapsulation was about 50%. Comparing this to the previous work using the DFF chip for single cell encapsulation, up to 70% efficiency was achieved [144]. A reduced efficiency was expected due to the differences in size and properties of single cells compared to spheroids.

Beyond considering efficiency, it was beneficial to compare the system to DM devices that used individual cells to form spheroids rather than encapsulating pre-formed spheroids. As mentioned previously, the number of cells used at the inlet reservoir is typically very high. With this high amount, of course a high number of spheroids can be produced, owing to the high throughput capabilities of DM. In cases like Sun et al.[38] who produced 200 spheroids/minute or Lee et al.[82] who produced 42 000 spheroids in 10 minutes, they both used a high range of initial cell concentration at 10 million/mL or 6 million/mL. This number of cells is not practical for applications in patient derived primary cells, due to the lack of sample obtained [43]. Comparing the pre-formed spheroid encapsulation, a typical droplet frequency was around 120 droplets/minute. If 50% efficiency is considered when using 5000 spheroids at the inlet reservoir, for this frequency, only about 60 spheroids would be encapsulated into hydrogel droplets. Of course, this is a much lower amount compared to those previously mentioned, however, only about 250 000 cells are needed to produce about 5000 spheroids using the AggreWell™.

Considerations of increased flow rate to increase droplet frequency may help with overall encapsulation quantity. Yet, the quantity of droplets with pre-formed spheroids encapsulated may not be as important as their growth in the hydrogel microenvironments. Encapsulating already formed spheroids, and not forming them inside of the hydrogel, may decrease the overall growth time for

organoid formation. Accelerating the growth time of organoids is also favored for improved PDTO formation, allowing for cancer analysis to begin more quickly.

In this work, gelatin methacrylate (GelMA) and sodium alginate were used, 2 common biocompatible hydrogels often used with cell culture. They have been separately used in droplet microfluidic devices for cell culture [32,38], but have not been seen together. Comparing this work's droplet stability and formation with other crosslinking techniques in other studies, it is noted that there is room for improvement for achieving reproducible and uniform droplets after the collection process. Using these studies can help guide the proper collection techniques, especially when considering a new combination of hydrogels. Yet the preliminary work done still showed decent results, mainly with alginate-GelMA hydrogels collected in 2% w/v CaCl_2 solution or mineral oil, and was partially validated with the cell viability assay.

Lastly, comparing the system with the one by Mesquita et al.[45] who also encapsulated stem cell spheroids, there are differences that allow each system to work for different cases. In their study, they used a glass capillary DM device with sodium alginate hydrogel, crosslinked as droplets were forming, to encapsulate stem cell spheroids. While this did show to produce decent uniform droplets, they did not report on the efficiency of the system. It was however mentioned that they used 2 million cells at initial seeding density to make 81 spheroids using a non-adherent plate. This thesis work was able to obtain thousands of spheroids using hundreds of thousands of cells by leveraging the high-throughput technology of the AggreWell™. In addition to this, the DFF geometry can offer a wider variety of hydrogels to be studied with the spheroids versus the co-flow geometry they used.

All-in-all, this system still has its differences to those published by other researchers but shows its potential for a new area of encapsulating larger bio-particles (like pre-formed spheroids) using lower amounts of reagents and materials.

5.8 Summary

Overall, a successful droplet microfluidic system was demonstrated for the purpose of encapsulating pre-formed spheroids into their own hydrogel microenvironments, in a robust manner. Using pre-formed spheroids made from a low number of cells, this system enables the path for use in forming PDTOs, in hopes to gather patient primary cell spheroids and support their growth uniformly.

The use of a double flow focusing device leveraged with contrasting aqueous fluids demonstrated a key role in effecting the efficiency of spheroid encapsulation. The medium focus width was more

favorable due to its consistency in single spheroid encapsulation and almost 50% encapsulation efficiency. Alongside this part of the system, the inlet conditions of the reservoirs influenced how spheroids entered the DM chip, thus the focus width showed more importance for cases of high spheroid concentration or spheroids settled in the reservoir. But the range of spheroid concentrations tested still proved to be reliable for the likely targeted amount of encapsulations desired.

Finishing off with droplet collection, there were many parameters that affected the outcome of the droplets, but the combination of alginate and GelMA collected in mineral oil or 2% w/v CaCl_2 solution seemed like promising hydrogels capable of culturing the spheroids. The collected encapsulated spheroids demonstrated decent viability, some cases higher than others, thus validating the system for use in aiding growth of pre-formed spheroids.

Table 5-7 summarizes criteria for the best system determined from the ones tested. These parameters can be followed to try to repeat the system and apply it to similar conditions, to obtain meaningful results to further improve the system for downstream use.

Table 5-7: Summary of Parameters for Overall System

Parameter	Description
Viscosity ratio of dispersed phases	$2 \leq \eta \leq 7$; $D1 > D2$ 2% sodium alginate (D1) with 5% GelMA (D2)
Flow rate ratio of dispersed phases	$0.70 \leq \lambda \leq 0.75$ (medium FFW) ; $D1 > D2$
Viscosity of D1	~ 50 mPa.s
Concentration of spheroids at inlet	$\sim 7000/500$ μL, suspended OR $\sim 5000/500$ μL, settled
Collection (outlet) bath	4°C 2% CaCl_2 OR 4°C Mineral oil with 3% Span 80

Chapter 6: Concluding Remarks

6.1 Thesis Conclusions

Towards finding unique and reliable models for cancer patients, the work demonstrated in this thesis showed a positive path towards this goal. The advantage of using this system in the application of PDOs can be to further enhance the growth of primary cancer cell clusters into more complex organoids structure in a controlled and high throughput manner. Models created from such system would be reliable for personalized medication or fundamental tumor studies, all in hopes to reduce the death toll seen through cancer.

To begin with the system, an understanding of what was needed was first gained through searching previous work and literature. This helped to shape the path for the scope of this project. Tackling the project from two main aims (that was optimization and validation), allowed for incremental growth of the overall process, to the final product showcased at the end.

Starting with the consideration of forming spheroids from a low number of cells, this mimics the case of biopsy sample that may be obtained in real life applications. The beginning of Chapter 4 showed the progress towards solidifying a protocol capable of forming these spheroids in a robust and repeatable manner, after facing different challenges using PDMS replicas of the AggreWell™.

Alongside spheroid optimization came droplet microfluidic device optimization. The exploration of 3D printing versus traditional fabrication methods gave rise to a new way of exploring different DM geometries in a cheap and quick fashion. Unfortunately, due to the limitations of 3D printing, this fabrication was not continued with. Instead, a mold made from soft lithography was chosen for its cleaner surface finish and established geometry. The double flow focus (DFF) geometry was validated when compared to a simple chip such as a T-junction geometry. Due to the goal of this work being targeted at single spheroid encapsulation, using the T-junction relied on how the system was setup more than the chip manipulating the spheroids into their own droplet, compared to the DFF chip. That is also why inlet tubing diameter was explored, to ensure there were less groups of spheroids coming onto the chip.

Through Chapter 4's optimization stage, it was shown what type of chip and setup would be used for the desired system, along with the type of spheroids that would be used to model the system's ability to handle large bio-particles.

Chapter 5 was the bulk of the work to wrap up towards the main objective. Beginning with selecting the biocompatible hydrogels used for the system, the main influence was on criteria from previous

work. The combination of either sodium alginate and Gelatin Methacrylate (GelMA) or different concentrations of GelMA were considered for different purposes. The GelMA-GelMA combination was mainly used to demonstrate the way in which the hydrodynamic focusing width of the DFF design could affect the single spheroid encapsulation efficiency. In doing so, the medium width was decided as the best one of the three due to its payoff in single spheroid encapsulation efficiency. At this flow condition, different spheroid quantities were tested, to demonstrate the range of the system. Both high and low numbers of spheroids were tested. For the application of PDTs and small sample sizes, a low number of spheroids would be favored. Yet, a high number increased efficiency, so this may be used in other cell applications where sample quantity is high thus spheroid formation can be higher.

As the target of this thesis was aiming for single spheroid encapsulation using passive DM, it was more important to focus on having more single spheroid encapsulations than multi-encapsulations. Having multiple spheroids in one droplet for the sake of spheroid growth would cause uncertainties when comparing to those with only one spheroid in the droplets. With this system, sorting was difficult at any stage, so the best thing was to rely on the flow of the spheroids and how they were encapsulated. Having plenty of empty droplets was not a large issue for this system, this would only decrease efficiency, and not interfere with the growth of the spheroids and their development. That is why with the current system, aiming for single encapsulations (versus multi) was the best option while still avoiding too many empty droplets (zero spheroids encapsulated).

For the system to be complete, validation through hydrogel stability and spheroid viability were shown. Although there were challenges with droplet collection and hydrogel crosslinking, overall, the process was deemed successful. Due to the high number of variables that may have affected each outcome, a better assessment of the materials can be done in future runs to ensure familiarity with the parameters, especially for repeatability demonstrations. But with the current condition showcased, the viability of the spheroids was mostly positive, concluding the potential this system has for pre-formed spheroid encapsulation.

To conclude, Figure 6-1 visually summarizes the intended process that each role of this project helped contribute to.

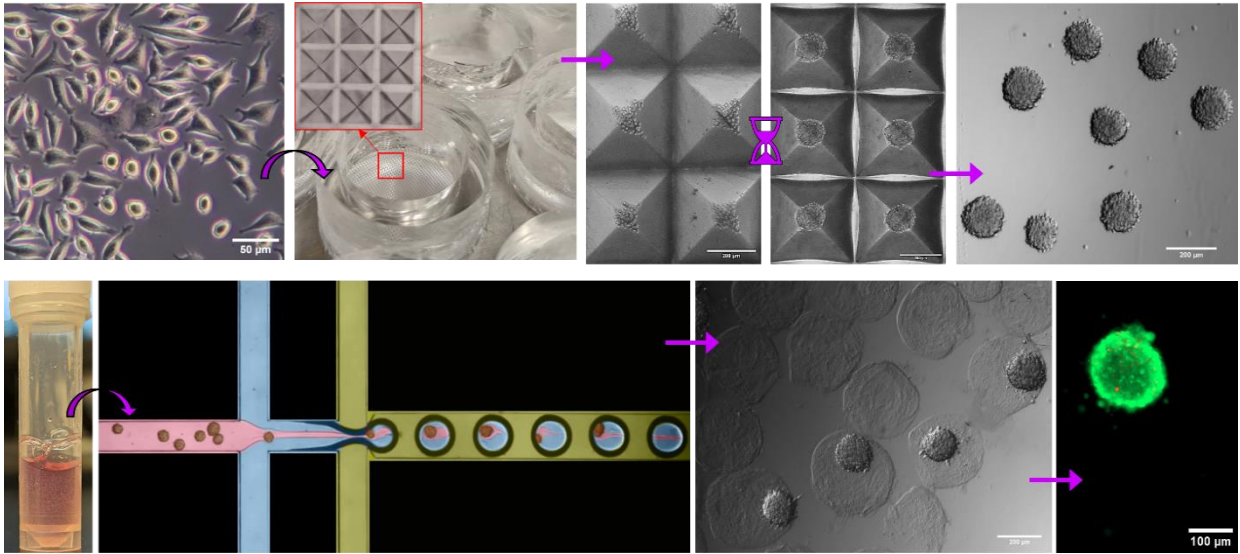


Figure 6-1: Photo summary of the final system process

6.2 Recommendations and Future Directions

6.2.1 Recommendations

With the project still showing imperfections in some areas, it would be helpful to outline recommendations for anyone considering implementing the work to other applications or continuing the progress potential of this system.

System consistency

An important factor to consider is the number of variables this system can produce. The combination of the droplet microfluidic system and the hydrogels compiled a wide number of factors that affected the outcome of the size, integrity, and stability of the hydrogel microparticles. For the side of droplet microfluidics, it was important to ensure the system parameters were as consistent as possible. Especially with tubing length, the variation could affect the resistance in the system, and with the use of a pressure pump, the system was more sensitive to change. Any change in resistance would affect the way the droplets were produced (due to changes in flow rate), so it would be important to maintain tubing consistency, or study the best tubing length for optimal droplet formation and collection.

To enhance the system for repeatability, it would be better to include the use of a flow sensor. This flow sensor could help measure the velocity at certain areas of the chip for a more accurate

representation of pressure flow. Having more quantitative data can allow the user to repeat the system more comfortably and make more reliable conclusions in terms of droplet uniformity and flow ratios.

Material variables

When looking into the hydrogel aspect of the system, any form of reagent combination and temperature changes should be considered systematically, so that if any issues arise, it would be easier to find where the problem may stem from. For example, the type of solution the hydrogel is made from (media versus water) can affect the behavior or crosslinking of the hydrogel in the chip or after collection. In this case of using sodium alginate with gelatin methacrylate, it was observed that bursting would occur sometimes after collection and washing. If chemical and system variables were better kept track of, it would have been easier to resolve this issue quickly, to achieve a more repeatable system.

Spheroid exposure time to external stimuli

Considering working with the spheroids with this system, their viability is important for continued growth into organoids. Due to the early stages of this project and equipment setup, device runs would be performed over long periods of time to obtain as many results as possible, before moving on to the stage of washing and culturing the spheroids in the hydrogel microparticles. That meant, some spheroids were sat in the collection baths longer than others, potentially affecting the viability or integrity of the hydrogel. It would be ideal to run the system as quickly as possible, collecting one case at a time so that each spheroid could have a fair chance to showcase if the system and hydrogel are working effectively, and factors such as equipment setup do not hinder any results.

6.2.2 Future Directions

As this project is rather preliminary to an overall system, there are some suggestions that can be made to further amplify the project in the right direction. However this project may progress, it seems that there are many avenues to build on to complete the project for the overlying goal of growing PDTs consistently and efficiently, to progress cancer treatment and research.

Studying optimal hydrogel matrix

Due to the sensitivity of cells, their environment can heavily influence the way in which they grow. To find the ideal matrix to grow them in, more work can be done surrounding the hydrogel fabrication

and composition. Using DM, this can be explored or the device itself can be enhanced to add other components to the microenvironment in a controlled and sizeable manner. When studying the material, it will be important to consider every little detail as this can affect the outcome of the hydrogel.

Especially when using hydrogels for the application of tumor organoid, their microenvironment is specific. It would be best to find hydrogels that can better mimic the tumor microenvironment to give cancer cells a similar environment to grow, mimicking *in vivo* conditions much more. A suggestion may be to look into more fibrous gels, such as collagen methacrylate, where the structure may resemble the tumor microenvironment better.

Another consideration with the hydrogels is to do more characterization on them. Having selections of different gels can be good, but more meaningful to characterize them in different stages to understand what properties are being used and what criteria works best for different cell types. For example, swelling was a property not studied in this work, but an important one that can also help understand porosity and effects on spheroids growth. Along with swelling, more mechanical testing can be done as well as degradation tests. Understanding degradation can help correlate the lasting time of the hydrogel integrity with the timing of spheroid growth, to calibrate the best combination depending on the growth rate of the spheroids/organoids.

Studying culture conditions of spheroids in the HMPs

An important project can stem from needing to observe the growth of the spheroids. The goal is to not only support the growth of the spheroids and show their viability after some time, but to show they can grow well in the HMPs, into the desired organoids, in a repeatable manner. This can be in line with studying the best HMP for the spheroids. It would be meaningful to also compare the growth of the spheroids in the different defined HMPs to droplets formed with the current gold standard, Matrigel, to show that the different hydrogels can replace Matrigel for the intended goal.

When going for this approach, it is expected to run into different challenges for organoid development. Retrieving the organoid after its growth time may be tricky with the current system as spheroid-laden droplets are scattered amongst empty droplets. When sorting can be implemented (discussed in *Studying spheroid behavior in DM*) then individual spheroid-laden droplets can be placed in their own culture wells to isolate them for the eventual retrieval after organoid development. Additionally, the growth itself may have complications if the growth factors or nutrients are not properly administered, or if they are not consistent between batches. In those cases, variability can still occur which goes against the intended goal. That is why studying this behavior would be ideal before

moving onto patient derived tumor cells so that the system shows repeatable with immortalized cell lines and can ideally be relied on for any other cell type.

Applying primary patient derived tumor cells

As a model for the system, an immortalized cancer cell line was used, but ideally the spheroids can stem from patient primary tumor cells, so their components can tend towards the growth into PDTOs. This would be an excellent way to further validate the system. This type of work may be considered after the overall system is better tweaked to ensure that samples are being used resourcefully. Additionally, since working with primary or stem cells is variable itself (as cells will differ from patient to patient), having a system that is repeatable in other contexts can help ensure that the growth of the PDTOs is reliable.

Studying spheroid behavior in DM

Another direction for this project would be to study the flow and behavior of the spheroids in the DM regime. Simulations can be established to study how the spheroids disrupt the flow, or how varying their shape and size can effect encapsulation rate.

It is important to note that the spheroids formed before entering the chip were not all perfectly the same. Due to this, observations could be made on how skewed sizes could affect the single encapsulation efficiency, as different size spheroids would have different local flow rates; some may catch up to others and be encapsulated together, lowering the single spheroid encapsulation. In addition to the variable spheroid sizes, there were observations made on its effect of flow focus disruption. This can be another study done to see if this disruption affects efficiency and droplet formation/contents.

In line with the analysis side, an active control system, using feedback pressure control, could be a compatible way to sort empty droplets or multi-encapsulations, increasing the efficiency much more and allowing for higher repeatability in the DM side of the system. An active control system would use controlled pressures to dictate quantity of droplets and where droplets should go depending on their contents (a form of sorting). Implementing this type of control would be much less harmful to the spheroids compared to other active DM systems or traditional sorting methods. With sorting implemented in the system, it can make it easier to analyze individual spheroids in their own microenvironment and make it easier to supply each spheroid with the same nutrients and growth factors, towards a repeatable system.

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Exploring the interaction between extracellular matrix components in a 3D organoid disease model to replicate the pathophysiology of breast cancer

Author: Anamitra Bhattacharya et al

Publication: Journal of Experimental & Clinical Cancer Research

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Single-cell droplet microfluidics for biomedical applications

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Appendices

Appendix A: Spheroid Counting

To estimate the number of spheroids collected before running each batch, a counting technique inspired by the company of the AggreWell™ (StemCell Technologies) was used. After spheroids were reversed strained and collected into a 15 mL falcon tube, the solution was very carefully resuspended to distribute the spheroids evenly. After redistribution, a 100 μ L sample was taken from the suspension. A 48-well plate with a cross on the bottom was prepared, where the sample solution was put into the well. Spheroids were counted, and after final count, the number was multiplied by the total solution volume:

→ Spheroid count from 100 μ L sample = S_{100}

→ Total number of spheroids = $\frac{S_{100}}{100 \mu\text{L}} * \text{total volume of solution } [\mu\text{L}]$

The theoretical yield of spheroids would be 1200 per PDMS AW. It was expected that number be lower due to the PDMS AW fabrication and the reverse straining process.



Figure A-1: Microscope eyepiece view of spheroid counting

Appendix B: LEXT Images of Different DM Molds

The LEXT confocal microscope was a great tool at visualizing surface roughness of the 3D printed molds, and even effective for validating channel geometries. The following images are examples of some of the 3D printed molds and for comparison the DFF silicon wafer mold.

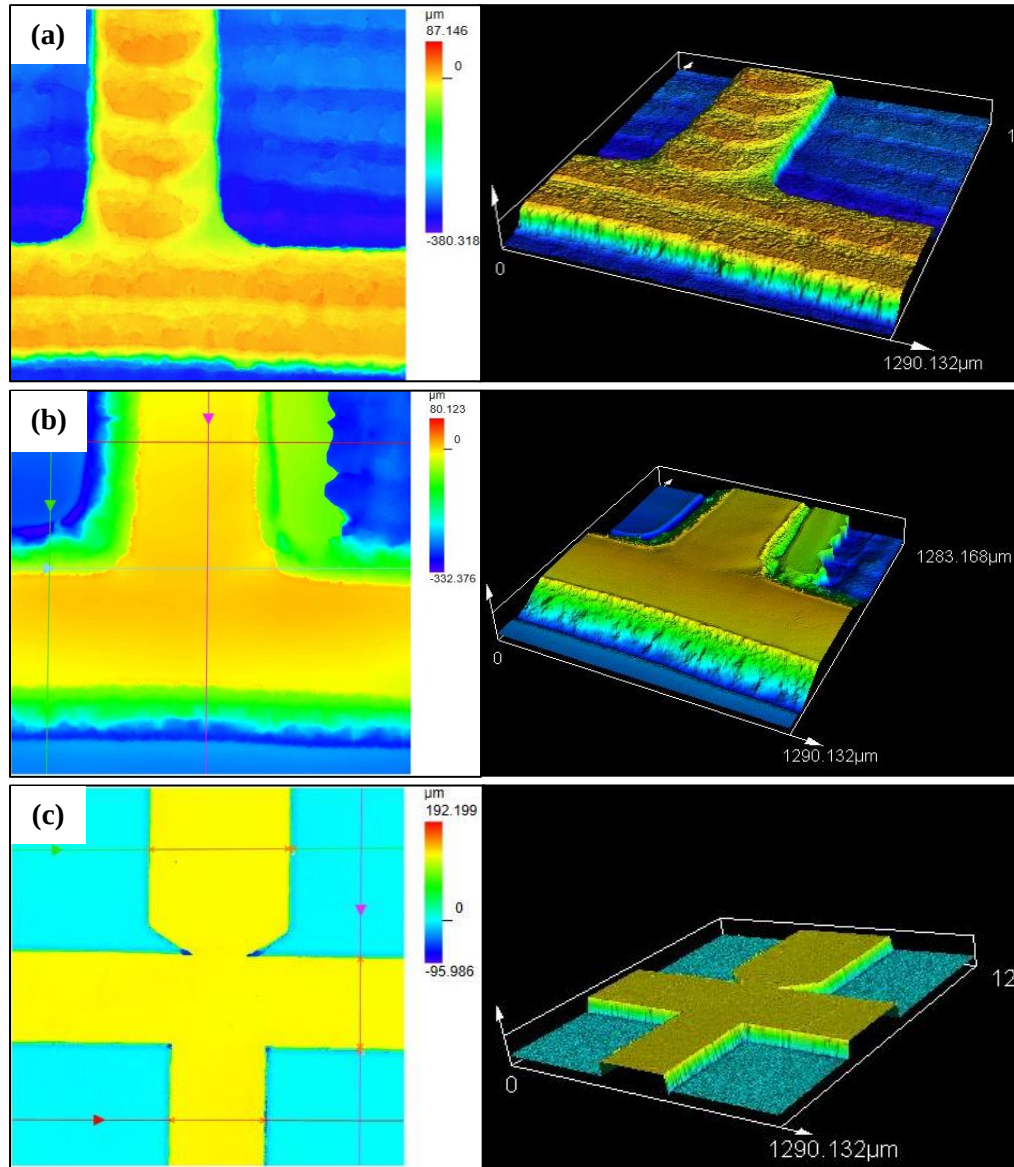


Figure B 1: LEXT images showing surface roughness of 3D printed vs silicon wafer molds. The first two show 3D printed T-junction (300x300) molds where (a) is not treated and (b) is sanded. (c) DFF silicon wafer mold.

Appendix C: Classification of Flow Focusing Width

To gain an idea of flow through the DFF device, previous work with this geometry was used as a guideline. From Nguyen (2019, UW Space) [144], there was a study on flow focusing width with respect to flow rate ratio between the two dispersed phases, at fixed viscosity ratio of 2. The representative image of this study is depicted below. Using the given scale bar (100 μm), the width of the focusing stream was measured and normalized to the width of the channel (100 μm). This ratio was then applied to the DFF device used for the spheroids to help approximate what regime the focusing width was in. Width classifications that were categorized as narrow, medium and wide, were based on visual judgments to give a rough category to the focusing widths studied below.

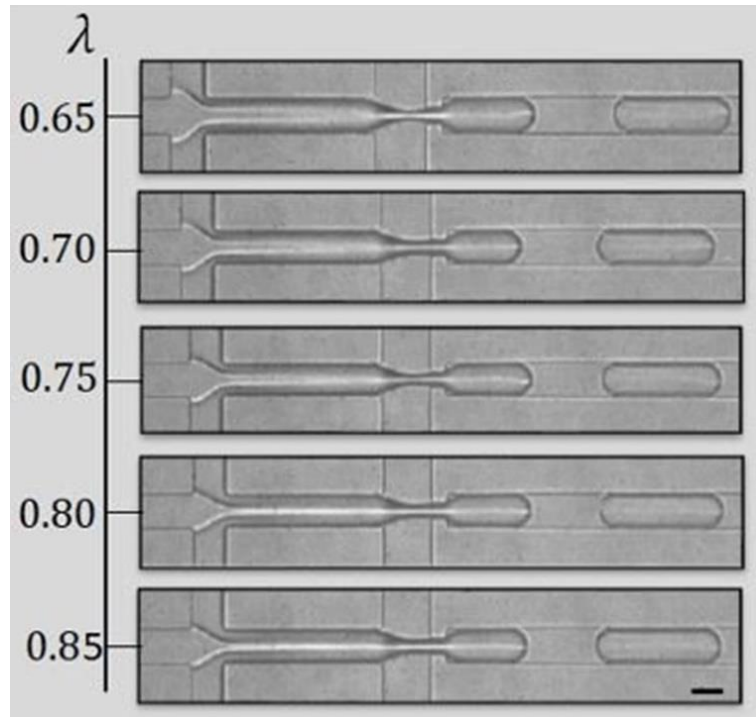
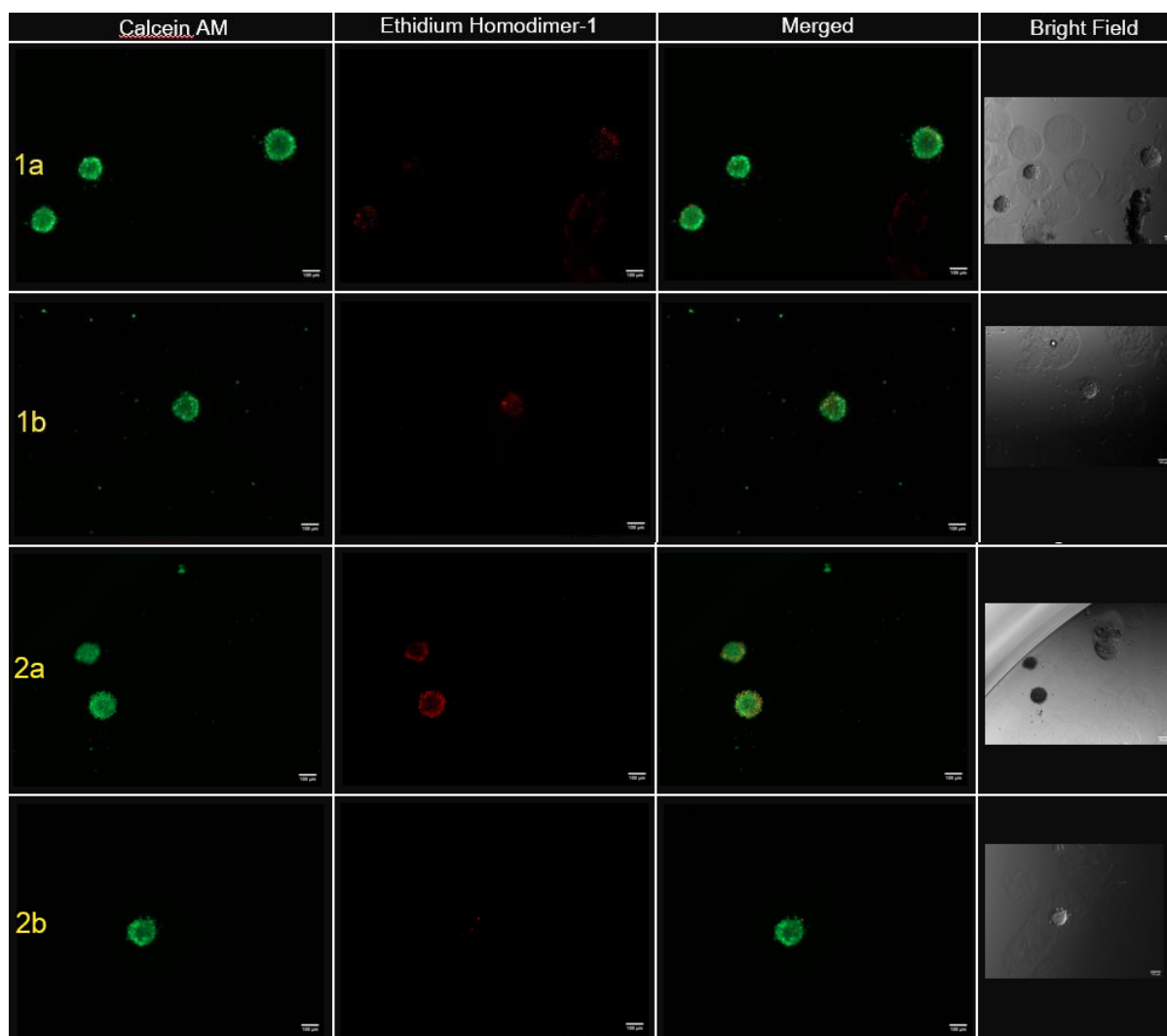


Figure C-1: Effect of flow rate ratio on flow focusing width. $\eta = 2$. (portion of image taken from Nguyen [144])

Appendix D: Representative Images of Live/Dead Assay on Spheroids Encapsulated in Alginate-GelMA HMPs

The following images correlate to the G/R ratio calculated in Section 5.7. To distinguish between the different collection baths, 1-4 was used for 2%, 1%, 0.5% w/v CaCl_2 solution and mineral oil, respectively. For the droplets cultured in media without and with serum, (a) and (b) will be denoted, respectively. Figure D-1 shows results for 24 hour culture and Figure D-2 shows results for 5 day culture.



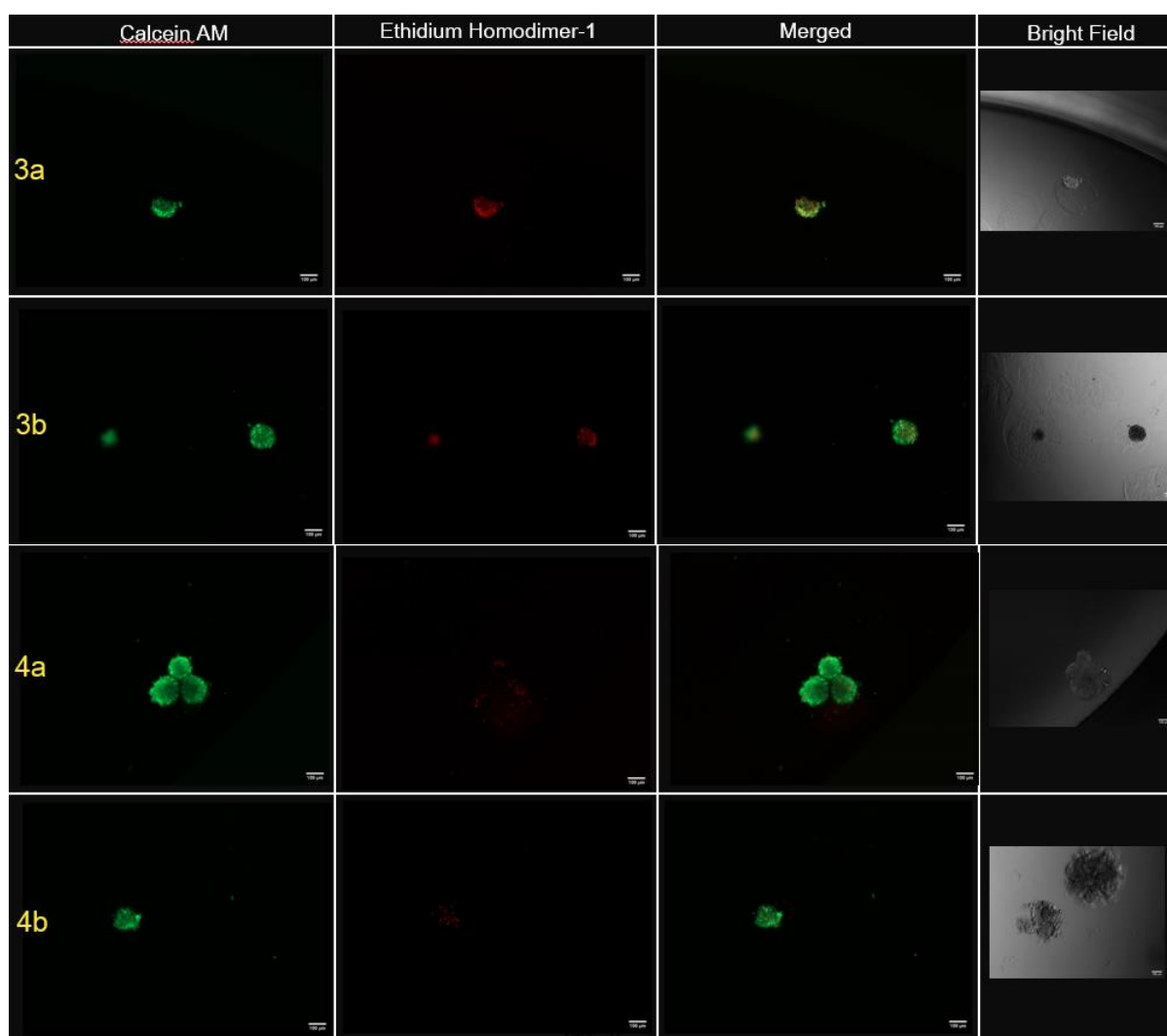


Figure D-1: Live/Dead Assay results of spheroids after 24 hours of culture in alginate-GelMA HMPs

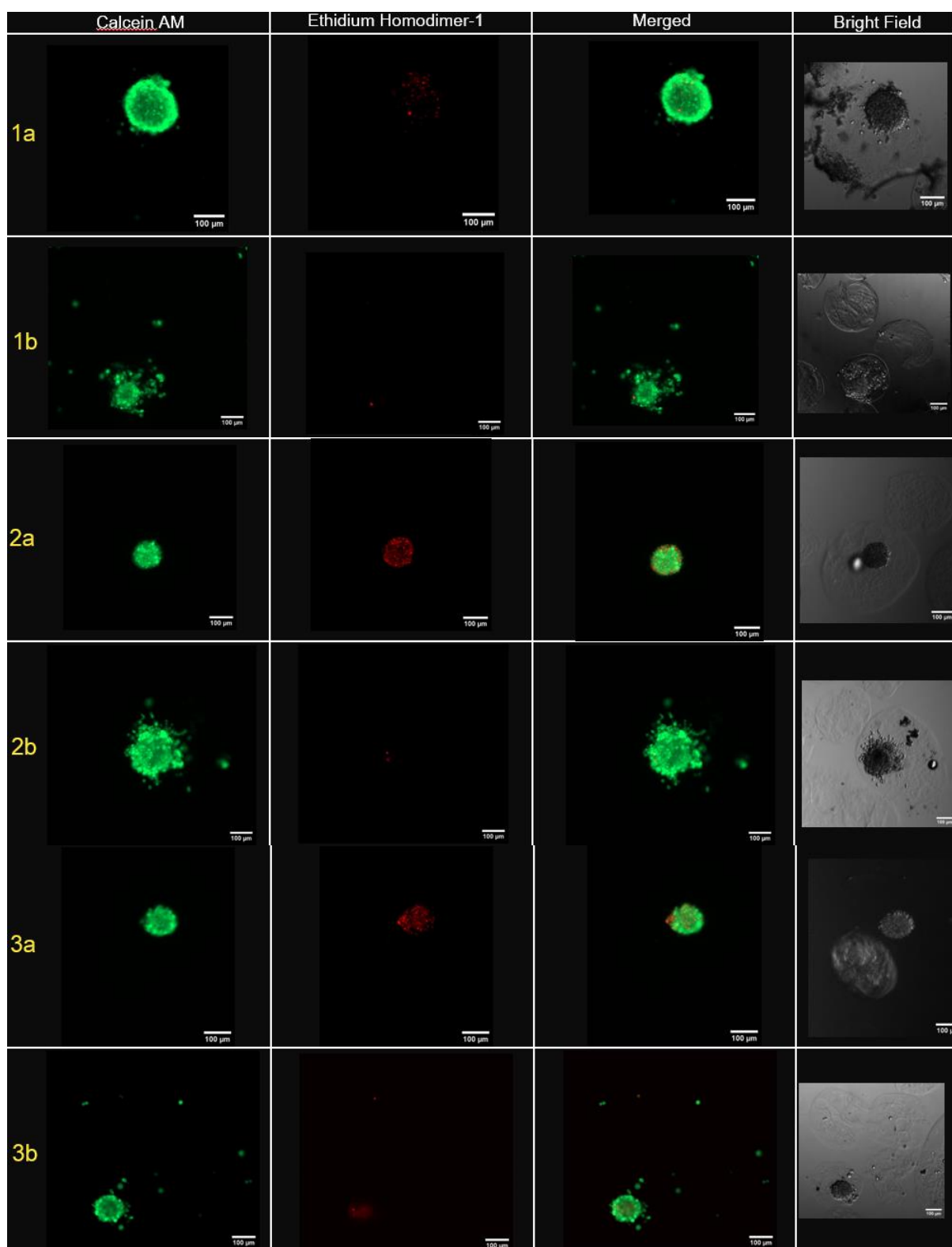


Figure D-2: Live/Dead assay results of spheroids after 5-day culture in Alginate-GelMA HMPs

Appendix E: Experimental Setup and Methods Images

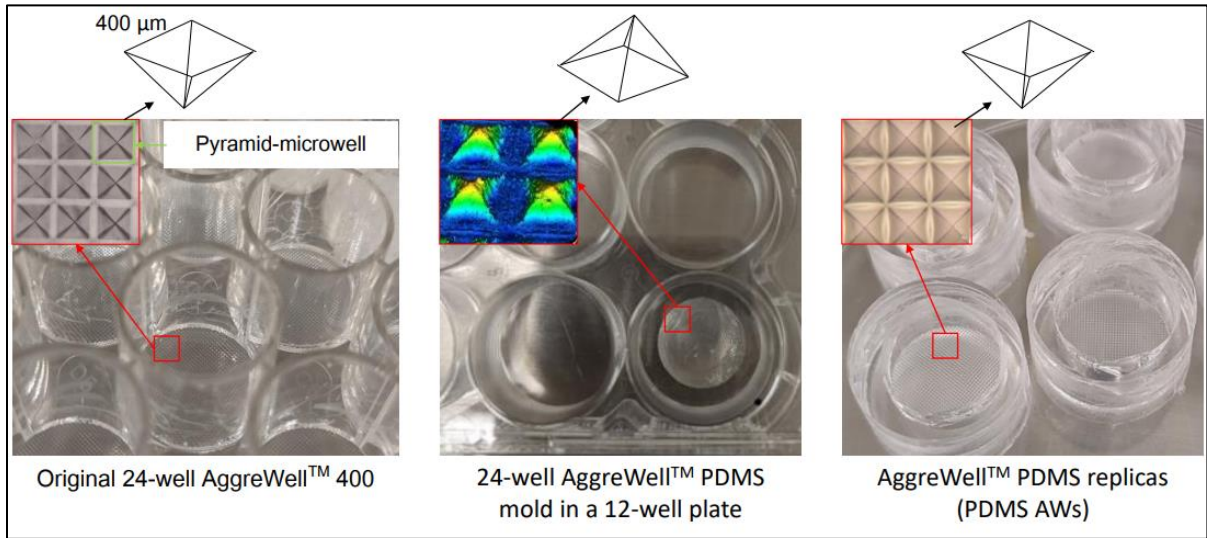


Figure E-1: PDMS AggreWell™ replication process

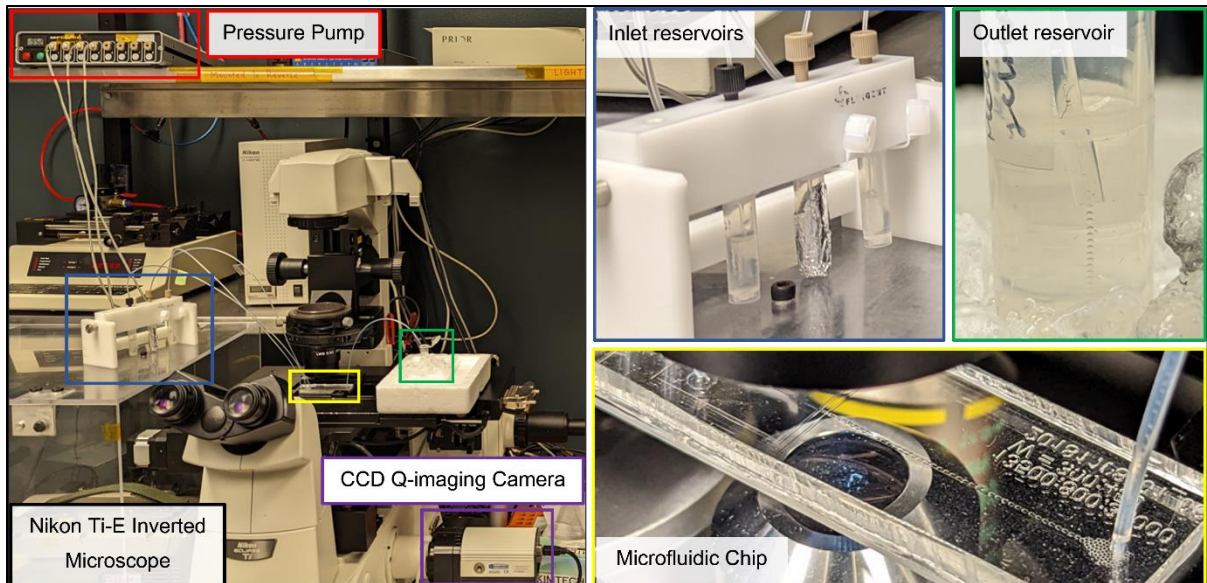


Figure E-2: System setup

Appendix F: Droplet Content Graphs

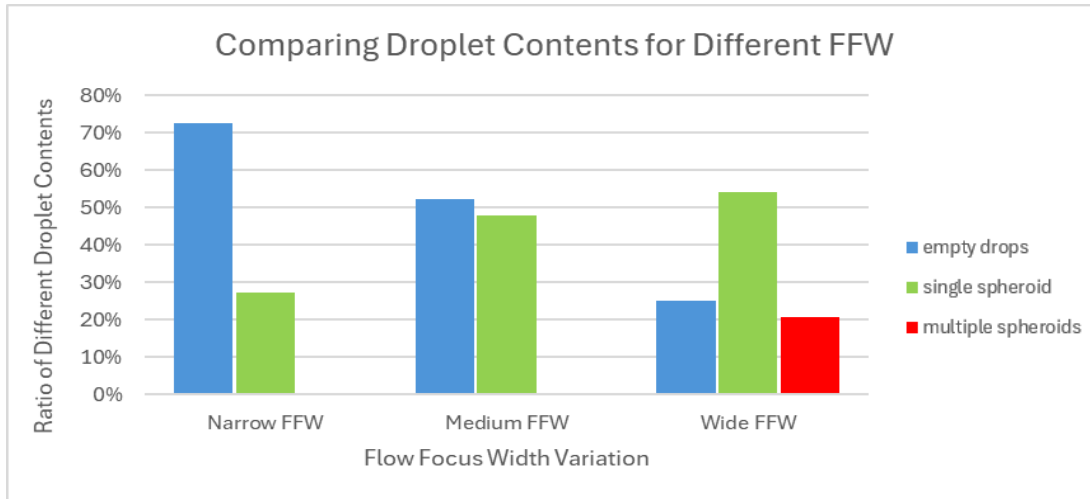


Figure F-1: Comparing droplet contents for different cases of flow focus width (FFW) – Narrow, medium, wide. Contents compared are empty droplets, droplets with single spheroids, and droplets with multiple spheroids. The droplet number was normalized to the total number of droplets analyzed. Condition is representative of efficiency calculated in section 5.4.

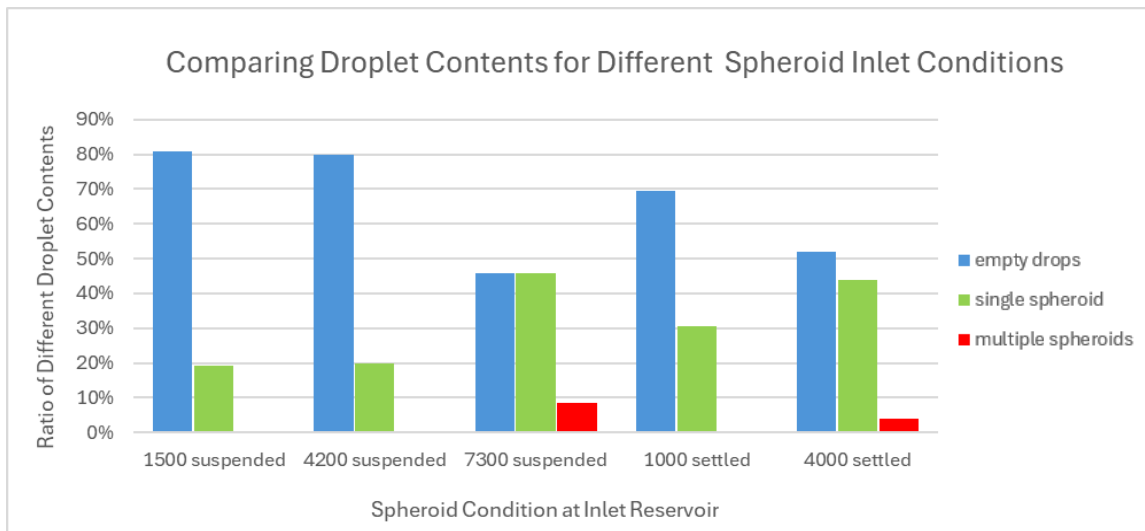


Figure F-2: Comparing droplet contents for different cases of spheroid concentrations and inlet conditions (suspended or settled). Contents compared are empty droplets, droplets with single spheroids, and droplets with multiple spheroids. Droplet number was normalized to the total number of droplets analyzed. Condition is representative of efficiency calculated in section 5.5.