

ULTRA HIGH-THROUGHPUT FLUORESCENCE DETECTION FOR SINGLE CELL
APPLICATIONS IN DROP MICROFLUIDICS

by

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of

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ABSTRACT

Conventional methods in microbiology can be limited by assay execution and analysis times, phenotypic dominance within bulk communities, reagent volumes, and single-use supply costs. These limitations can be overcome using drop-based microfluidics. In this discipline, pico-liter sized, water-in-oil emulsions serve as independent “test tubes,” allowing for the compartmentalization of community constituents and interrogation at the single cell level. Furthermore, two-phase, continuous flow microfluidic devices enable drop populations to be manipulated and analyzed at kilohertz rates according to experimental needs. In this research, a fluorescence-based method for drop analysis and sorting was developed and applied, in conjunction with other microfluidic techniques, to perform assays in microbiology. The applications explored include cell dormancy within *P. aeruginosa* subpopulations, microalgae lipid accumulation for the production of biofuels, optimization of microbially-induced calcite precipitation (MICP), and human norovirus infectivity. Results from each application include: 1. The hibernation promoting factor (Hpf) was found to play a key role in the maintenance of *P. aeruginosa* viability during planktonic starvation. 2. Progress was made on a Nile Red based, ultra high-throughput, single cell algal lipid detection platform. 3. MICP was demonstrated at the single cell level. 4. A drop based human norovirus infection platform was attempted using human B cells as the viral host.

CHAPTER ONE

DROP MICROFLUIDICS: INTRODUCTION AND BACKGROUND

Microfluidics Background

Microfluidics is regarded as both a science and a technology, but the term itself is defined as the manipulation of small volumes of fluid within micrometer scale channels [1]. As the field has grown, it has demonstrated promise in its ability to revolutionize techniques for chemical, biological, and biochemical analyses. Accelerated by soft lithography [2] and advances in low-cost photolithography, the technology presents a platform for sample processing that uses a fraction of the footprint, materials, and time when compared to existing approaches for analyses. Microfluidic devices boast the ability to precisely control flow through the use of integrated pumps [3], valves [3], and temperature controls, as well as the ability to chemically and optically analyze constituents of the flow.

Each of these characteristics have assisted in giving rise to the “Lab on a Chip” (LoC) concept that microfluidics strives to embody. Most simply, this concept attempts to automate and miniaturize all aspects of a biological or chemical assay on a device no larger than the palm of your hand. Similar to the way a computer chip may use binary inputs to output useable information, microfluidic chips accept raw materials, manipulate or analyze them, and produce a useable output. The LoC concept has recently gained traction in academia and industry. As academic groups strive to push the science into new frontiers, start-up teams are aiming to refine promising technologies for

commercialization. Companies such as Fluidigm and Dolomite have seen success in the research and device fabrication arenas, respectively.

The maturation of microfluidics has resulted in the rise of several relevant subcategories including glass capillary, paper [4], and drop based microfluidics. The focus of this review is drop microfluidics and its implications in microbiology.

Drop Microfluidics

Conventional methods in microbiology require high volumes and many single-use supplies such as petri dishes and test tubes. As a result, biological samples are prepared and analyzed in bulk communities allowing for phenotypic domination and extended turnaround times. To combat this, drop microfluidics uses picoliter size droplets as independent batch reactors for miniaturized biological assays. The cornerstone of this discipline is its ability to compartmentalize individual cells or desired groups of cells, and furthermore, its ability to manipulate and analyze these drop populations at kHz rates.

Drop microfluidics offers a significant advantage over conventional techniques in microbiology as it allows users to identify and isolate rare occurrences among cell populations. As an example, consider a simple growth assay. Conventional methods in microbiology include plating a known dilution of the original culture and counting colony forming units (CFUs) after a specified incubation period. While this will provide insight into the concentration of viable cells within the culture, it does not provide information about the fraction of the population that fails to grow; non-growing cells are not visible with the naked eye. Using available fluorescent technologies and current drop

interrogation methods, these non-growing populations can be detected and isolated for further analysis.

Drops range in size from approximately 10-100 μm in diameter, however, even at their largest volumes, drop microfluidics still yields more than one million independent reactors per mL of sample used. Using pump driven flow, drops are manipulated and analyzed using microfluidic devices or “chips.” Each chip is specially designed to carry out a specific function or set of functions. Current drop manipulation techniques include drop formation, fluid injection [5, 6], immobilization [7], splitting and merging, re-injection, and fluorescent detection and sorting[8-10].

To promote the understanding of the concepts, processes, and terms used throughout this review, a description of the drop construct as well as the relevant manipulation techniques is provided in the remainder of this introduction.

Drop Construct and Stability

A drop is an emulsion of aqueous solution in oil and is stabilized at the interface by a surfactant (Figure 1-1). In this work, the inner or dispersed phase consists of an aqueous cell culture while the outer or continuous phase consists of a fluorinated oil (HFE 7500) and dissolved fluorosurfactant [11, 12]. Fluorinated oils are preferentially used in drop microfluidics because of their increased capacity for dissolved oxygen, which is essential for the maintenance of aerobic conditions within such small volumes. Additionally, fluorocarbon oils are both hydrophobic and lipophobic making them ideal for inhibiting diffusion of biological components between drops.

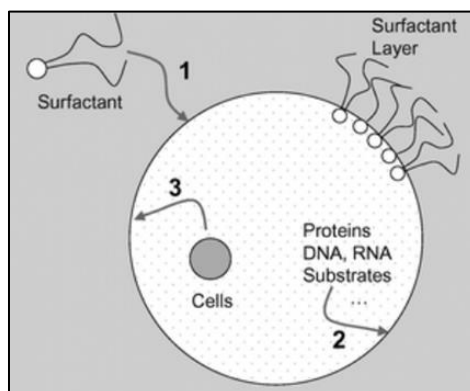


Figure 1-1. Dissolved surfactant diffuses through the continuous phase and assembles at the drop interface. Non-ionic hydrophilic moieties do not interact with charged biomolecules [11].

Stabilizing fluorosurfactants consist of hydrophilic heads and hydrophobic tails and for adequate performance must meet two requirements. The first requirement is that the hydrophilic portion of the molecule be biologically inert. Many surfactants rely on ionic interactions, however, using an ionic head group poses the risk of unwanted interaction between the surfactant and charged biomolecules within the drop. Such molecules include DNA, RNA, proteins, and various media constituents [13]. The second requirement is that the surfactant must prevent drop coalescence. Hydrophobic regions of the molecule must be compatible with fluorocarbon oils and must also possess long enough tails to ensure long term drop stability. Meeting these requirements is particularly difficult as the number of commercially available fluorosurfactants are limited [11, 12]. Companies that sell fluorosurfactants include Dolomite, Bio-Rad, and Ran Biotechnologies.

State of the art fluorosurfactants are equipped with perfluorinated (C_6 to C_{10}) polyether (PFPE) fluorocarbon chains and polyethylene glycol (PEG) heads [11]. These molecules have been found to be large enough to promote long term stability, small

enough to promote efficient diffusion throughout the continuous phase, and biologically inactive when in close proximity to drop constituents. With the appropriate surfactant, drop integrity is maintained for days at a time [11] allowing for downstream analysis or manipulation.

Dimensionless Numbers

Prior to discussing the physical properties associated with drop dynamics, it is useful to define some dimensionless numbers. The most important to consider in drop formation are the:

Reynolds number $Re = \text{Inertial Forces} / \text{Viscous Forces}$

$$Re = VD/\nu$$

Capillary number $Ca = \text{Viscous Forces} / \text{Surface Tension}$

$$Ca = \mu V / \gamma$$

Weber number $We = \text{Inertial Forces} / \text{Surface Tension}$

$$We = \rho V^2 L / \gamma$$

Where V is the fluid velocity, D is the hydraulic diameter, ν is the kinematic viscosity, μ is the dynamic viscosity, γ is the interfacial tension between the inner and outer phase, ρ is the fluid density, and L is the drop diameter.

Like all microfluidic systems, the characteristic length (hydraulic diameter, D) of the channel is very low, resulting in a Reynolds number <1 . This indicates that inertial forces are negligible and the overall flow can be considered laminar. The lack of turbulence makes convective mixing of multiple phases difficult within drops, however, there are device design features that promote this phenomenon [14, 15].

Drop stability relies heavily on the interfacial tension between the inner and outer phases. Without this stability the drop could not exist. The relationship between the interfacial tension and the viscous forces is represented by the Capillary number. In any scenario, $Ca < 1$ is desired. This indicates that the drop's interfacial tension is the dominant force. At $Ca > 1$ viscous forces begin to dominate and the drop becomes distorted. Similarly, the relationship between the interfacial tension and the inertial forces is represented by the Weber number. When $We < 1$ the result is a stable interface while $We > 1$ results in drop distortion. Analyzing each of these dimensionless numbers can assist in determining the appropriate flow rates for a given drop size.

Drop Formation

The geometries used for drop formation are the T-junction and the flow focusing junction (Figure 1-2). T-junctions are characterized by a single inner phase channel intersecting with a single outer phase channel to form a "T" shape. Alternatively, flow focusing devices are characterized by a single inner phase channel intersecting with two outer phase channels to form a "+" shape. In general, flow focusing is the more sophisticated method as it offers more design parameters. It will be the only method considered in this thesis.

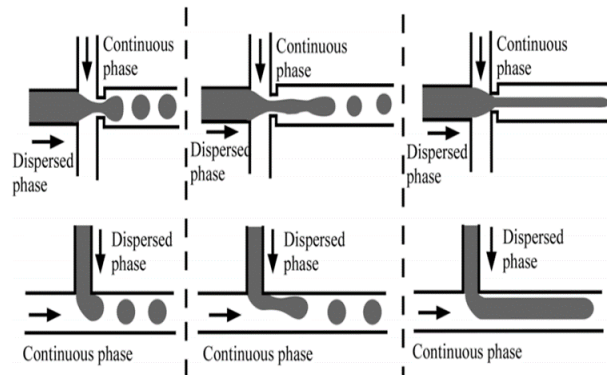


Figure 1-2. Dripping, jetting, and threading regimes for flow focusing and T-junction devices [16].

Flow focusing devices operate under two primary regimes: dripping and jetting. Each name is provided by the mechanism with which drops are formed and is characterized by the relative forces acting on the inner and outer phases. Dripping is characterized by low Ca and is achieved at lower fluid velocities as interfacial tension and viscosity are held constant for a given multiphase system (recall, $Ca = \mu V / \gamma$). To visualize, consider a dripping faucet and the correlation between the dripping mechanism and fluid velocity becomes intuitive [17]. Alternatively, jetting occurs as Ca approaches 1 and is associated with higher fluid velocities. Using the faucet analogy, it is easy to realize that even a steady stream of water would undergo surface instability and break into drops if given enough distance .

Pico-Injection

Pico-injection is a method for adding picoliter volumes of reagents to existing drops [6]. It is useful for carrying out multistep reactions or when the addition of a reagent at a later time period is necessary.

The mechanism for injection occurs at a junction where the injection stream meets the drop stream (Figure 1-3). As each drop passes through the junction, its interface is actively destabilized [18, 19] by an electric potential greater than 30V [6]. This destabilization occurs long enough for a portion of the injection fluid to coalesce with each passing drop. The amount of fluid injected is controlled by the pressure applied by the injection stream as well as the velocity of each passing drop. As the pressure increases or the drop velocity decreases, more fluid will be injected. This approach offers sub-picoliter precision and can be combined with additional injection streams for the injection of multiple reagents [6].

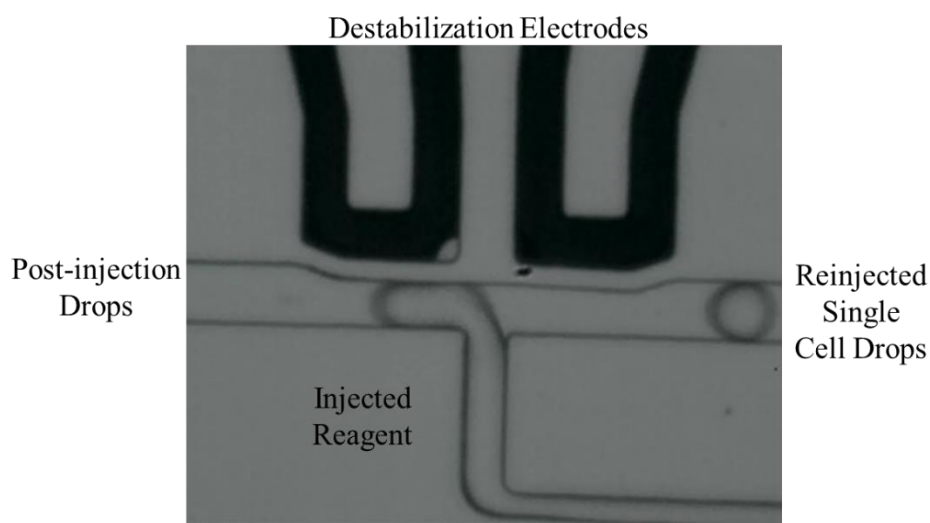


Figure 1-3. Microscopy image of the mechanism used during pico-injection. This image was taken in the Chang lab using an Edgertronic high speed camera.

Drop Immobilization

Although most of drop analysis is done using ultra high-throughput methods, there are instances when lower throughput analysis is beneficial. For example, tracking individual cells over the course of time or imaging subpopulations of drops requires such

analysis. In these instances it is necessary to immobilize the drops. This can be done using a dropspots device [7]. The dropspots device is a two dimensional array which serves as a well plate for individual or groups of drops. These devices are easily adapted for imaging via inverted microscopy and incubation for single cell growth monitoring. Furthermore, drops and their constituents can be recovered for additional analysis by flushing the device into the desired container [7].

Reinjection

During biological assays it is often necessary for a sample to undergo a desired reaction for a particular length of time. This duration of time can be spent in incubation, agitation, starvation, etc. In any case, it is necessary to encapsulate the sample, store the drops, and eventually reinject them into an appropriate analysis or manipulation chip. Reinjection is commonplace in drop microfluidics and is easily accomplished due to the density differences between the aqueous phase and the oil phase (HFE 7500 Specific Gravity=1.614).

Due to the need for reinjection, drops are typically stored in syringes as this allows the user to quickly interface a stored drop population with a device. During reinjection, the syringe is positioned vertically allowing the drop population to rise to the top and thus be reinjected at a high volume fraction. Once inside the chip, reinjected drops are typically reintroduced to an oil/surfactant stream. This stream provides the interface with additional surfactant and also helps to adequately space each drop.

Drop Detection and Sorting

As discussed, the benefit of drop microfluidics is two-fold. First, compartmentalization of cells eliminates phenotypic domination among cell populations. Second, analysis of each drop is done in an ultra high-throughput fashion. Current methods for ultra high-throughput drop interrogation are primarily fluorescence-based and emulate those used in flow cytometry [9, 20, 21]. During these assays, drop contents are engineered to produce an emission spectrum indicative of a particular result. As an example consider the growth assay previously discussed. Using green fluorescent protein, increased growth would be characterized by a more intense fluorescence. This emission signal is detected and evaluated against user defined parameters. Furthermore, evaluated subpopulations can be isolated using dielectrically driven sorting capabilities. This process is discussed in greater detail in chapter 3 of this thesis.

CHAPTER TWO

DEVICE FABRICATION

Introduction

Historically, microfluidic devices were constructed using photolithography techniques and rigid materials such as silicon and glass. However, the processes involved in constructing these devices required significant expertise and materials were expensive. Furthermore, they were not well suited for biological analysis as they were opaque and did not allow for sufficient gas diffusion. As a result, groups began to construct devices from plastics using soft lithography [2], a process pioneered by the Whitesides group at Harvard University.

The cornerstone of soft lithography is an elastomer block containing an imprint of the desired channel architecture. Of the materials used, polydimethylsiloxane (PDMS) is the most widely accepted among current microfluidic groups. PDMS is both clear and colorless allowing devices to interface well with microscopes and optical analysis tools. Its surface is biologically inert and can be functionalized to interact differently with different fluids. This is particularly useful for rendering surfaces hydrophilic or hydrophobic in drop microfluidics. Last, the elastomer is porous and provides excellent diffusion of gases making it ideal for the study of biological samples.

Finished PDMS devices are bonded to a solid substrate, typically glass, yielding a series of confined micro-channels. These devices are easily interfaced with fluidized chemical and biological agents. Through the use of accessible inlet and outlet holes,

appropriately sized polyethylene tubing is physically inserted into the device allowing for externally controlled fluid flow.

Fabrication via soft lithography requires five main steps: mask design, photolithography/master fabrication, soft lithography, bonding/surface treatment, and electrode fabrication. With the exception of mask printing, which is outsourced due to resolution requirements, all of these processes can be done in the laboratory. The materials and equipment for this process are listed in Table 1.

Equipment	Make/Model
Centrifuge	Champion F-33D
Hotplate	Any. 0-200°C Range
UV light source	OAI 150
Glass plates (exposure)	
Nitrogen assembly	Cleanroom Supply
SU-8	MicroChem
PGMEA	KMG Electronic Chemicals
Isopropyl alcohol	BDH Chemicals
Silicon wafer	Univesity Wafer: Test grade, 3”, SSP
Wafer forceps	Excelta
Pyrex glass dishes	
PDMS	Dow Corning Sylgard 184
Glass plates (bonding)	Corning, 2947-75x50
Plasma cleaner	Harrick Extended Plasma Cleaner
Vacuum pressure gauge	Edwards Pirani 501
Vacuum pump	Varian SH-100
Vacuum hose	Ideal Vacuum Products
Gas fittings and valves	Swagelok
Biopsy punch	Harris Uni-Core, 0.75mm
Razor blade	
Oxygen tank and hosing	
Oven	Barnstead Lab-line 3510

Table 2-1. A list of the materials used to assemble the in-lab photolithography and soft lithography station.

Mask Design

Devices are designed in Autocad or a similar drafting software. Governed by the desired drop size and final device footprint, an appropriate two-dimensional device blueprint can be created. Many of the functional components of a device already exist, making it easy to borrow design aspects from other devices. Lastly, due to the challenges involved in keeping devices clean, each design incorporates as much redundancy as the footprint will allow. Typically, 20 or more single devices are incorporated into a single chop. Figure 2-1 shows a .dwg file of a typical drop sorting device.

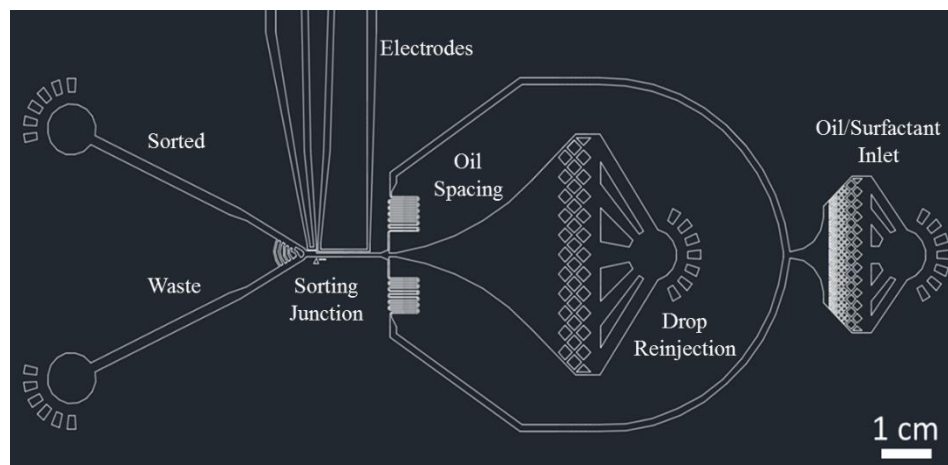


Figure 2-1. Example of a microfluidic device design drawn in AutoCAD. This particular design is for a 25 μm drop sorting device.

Once complete, the design is printed on a transparency and the product is referred to as the “mask” (Figure 2-2). Each mask is printed such that the space where flow will take place is transparent and the remainder is black. Mask printing is the only portion of the fabrication process that is outsourced. However, the process has quick turn-around and is usually completed within two to three business days. Popular printing companies

include FineLine Imaging or CAD/Art Services. Each of these groups guarantee adequate resolution for features above 10 μm . This is an important limitation to consider throughout the design process.

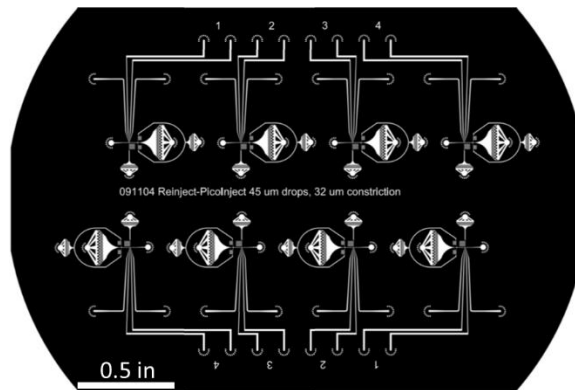


Figure 2-2. Prior to printing, Cad Art Services sends a PDF of the print-ready mask for feature confirmation. This example is a pico-injection design.

The ease with which new designs can be created and incorporated into a single mask allows for efficient prototyping of novel devices.

Photolithography/Master Fabrication

The process of fabricating microfluidic devices from PDMS is referred to as soft lithography, however there is still an aspect that requires photolithography. This portion is known as the “master”. The device master serves as the negative or the mold that will ultimately imprint the channels upon the PDMS. While this is a process most suitable for a cleanroom environment, the relatively low cost and high level of redundancy allows for in-lab fabrication in most cases. Adequate cleaning and attention to detail will often yield a high quality product.

Master fabrication is done entirely in-house, and can be completed in three steps: spin coating, exposure, and development. The photolithography station is both compact and inexpensive (Figure 2-3). The following sections discuss the custom equipment used for in-lab photolithography as well as the processes associated with each step.



Figure 2-3. The in-lab photolithography and soft lithography station is housed in a fume hood. It consists of a spin coater, a nitrogen source, a baking unit, a development unit, and various chemical supplies.

Spin Coating

Master fabrication begins by spin coating photoresist onto a silicon wafer. SU-8 3050 by MicroChem, which is designed for film thicknesses between 40-100 μm , is often used as a default. However, different products are available for devices requiring different channel thicknesses. Since the photoresist becomes the negative of a given device and the film thickness is directly related to the height of the channels, it is important to ensure that the film has the proper thickness and is uniform across the area

of the wafer. The manufacturer has published precise instructions for achieving desired film thicknesses from each fluid (http://www.microchem.com/Prod-SU8_KMPR.htm).

A typical spin coater has a center piece upon which the wafer is mounted and held in place by vacuum during the spinning process. However, these systems are expensive and unnecessary if budget does not allow. To bypass this piece of equipment, a centrifuge was modified with a custom “chuck” (Figure 2-4). 3D printed, the chuck is symmetrical to ensure centered spin, and serves as the wafer’s mounting surface. Additionally, the inside of the centrifuge is lined with aluminum foil to mitigate the mess associated with excess resist (Figure 2-4).



Figure 2-4. The 3D printed spin coater "chuck" and its assembly onto the centrifuge rotating shaft.

The mounted wafer is currently held in place by double-sided tape. While the quality of this adhesion at high RPM's is questionable, it can be maximized by ensuring clean surfaces and adequate pressure when attaching the wafer.

Spin coating procedure:

1. Clean the wafer: Given the scale of microfluidic features, even small imperfections can render a master useless. To promote successful master fabrication, it's important to begin with a clean wafer (Univesity Wafer: Test grade, 3", SSP). In this case, each wafer is best cleaned using a combination of isopropyl alcohol and pressurized nitrogen gas.
2. Attach double sided tape: Apply two pieces of double sided tape (approximately 2 cm x 1 cm each) to the unpolished side of the wafer (Figure 2-5). It is important to remember the location of each piece as it will assist in wafer removal. Removing the tape backing is easily accomplished with a razor.

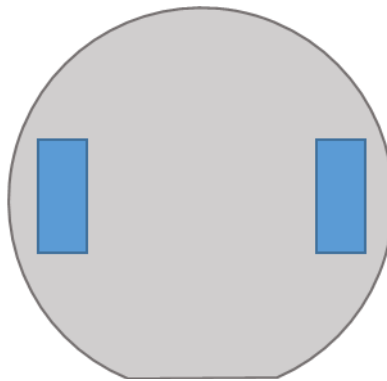


Figure 2-5. Taping scheme used for wafer adhesion during the spin coating process.

3. Attach the wafer to the spin coater chuck: Assuring a clean wafer, center the wafer on the chuck (tape side down). Using a gloved hand, press down gently to provide sufficient adhesion. Smudges and imperfections on the outer edges of the wafer are not detrimental as the device features do not extend to the wafer's edge.
4. Apply photoresist and spin coat as prescribed by MicroChem: MicroChem SU-8 has known mutagenic properties, and must be handled with gloves. Proper disposal of the liquid phase includes waste segregation. Once the fluid has

solidified it can be disposed of as regular waste. See the instructions prescribed by MicroChem for precise fluid volumes and revolution rates.

5. Remove the wafer from the chuck: Using wafer tongs, gently pry the taped portions of the wafer from the surface of the chuck.

A successfully coated wafer is first baked (pre-exposure bake, PEB) to remove the solvent within the photoresist (SU-8 3000: gamma butyrolactone; SU-8 2000: cyclopentanone). Baking is done via 95 °C hot plate and in close proximity to the spin coating centrifuge. It is essential to cover the wafer during baking as excess UV light can cause the resist to crosslink in undesired areas. In this instance, each wafer was covered with an aluminum foil lined Pyrex dish. Successfully baked wafers should be inspected for imperfections and prepared for the exposure process.

Exposure

The purpose of the exposure process is to crosslink the photoresist in the regions associated with the mask's transparent channels. This crosslinked portion of the wafer ultimately becomes the negative of the device and is used during soft lithography. This process is outlined in Figure 2-6.

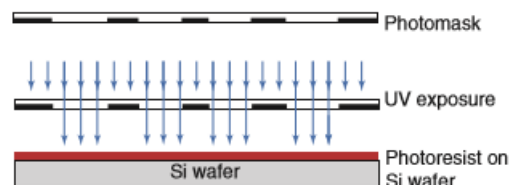


Figure 2-6. UV light is projected through the device mask and onto the spin coated photoresist [9].

Exposure procedure:

1. Power on UV light source: Once on, the OAI Model 30 UV light source must remain on for 30 minutes. Failure to do so will result in decreased mercury bulb life.
2. Remove the double sided tape from the wafer: Failure to remove tape increases the risk of breaking the wafer while removing it from surfaces throughout the exposure/development process.
3. Prepare the wafer for exposure: Place the wafer on a 4"x4" glass plate. Verifying correct orientation, place the mask on top of the wafer. Cover the wafer/mask with a second glass plate and clamp the glass plates together.
4. UV expose the wafer: Place the glass plate assembly in exposure chamber, set the exposure time, and press the yellow "Expose" button. Exposure times vary depending on the film thickness, but required intensities are outlined on the appropriate MicroChem data sheet.
5. Remove the wafer for baking: Carefully remove the mask from the surface of the wafer and the wafer from the glass plate using the wafer tongs. Place the wafer on the hot plate for the soft baking process. Cover the wafer to inhibit further exposure.
6. Store the mask in a safe place: Masks should be carefully stored as scratches can damage the device features.

Each successful exposure/soft bake yields a wafer with visible features. If the expected device features are not present, it is likely that the wafer was underexposed or

the baking temperature was too low (MicroChem). To resolve this issue, repeat the exposure process for an appropriate duration or re-calibrate the hotplate.

The UV light source purchased for this process was not functional upon arrival. It was missing several essential internal components including an aluminum heat sink, ceramic standoff, cooling fan, and mercury bulb. Each piece was easily identified and replaced with the assistance of OAI and ABM, two companies providing UV exposure services.

Development

The purpose of the development process is to remove the fraction of the photoresist that was not crosslinked during exposure. This is completed using the recommended developing solvent, propylene glycol monomethyl ether acetate (PGMEA). Ideally development is performed by submerging the wafer in a sonicated PGMEA bath. The ultrasonic vibrations allow the solvent to relieve the finer features of a microfluidic device. However, this piece of equipment is not necessary and can be bypassed using flat-bottomed Pyrex weighing dishes and manual agitation. This photolithography set-up uses two 4" flat-bottomed Pyrex weighing dishes. The first dish is intended for PGMEA only, and the second for a combination of PGMEA and isopropanol.

Development procedure:

1. Place the exposed wafer, feature side up, in the bottom of the "PGMEA ONLY" dish.
2. Cover the wafer with PGMEA and agitate: SU-8 is a solvent developable resist.

Although alternative developers are available, PGMEA is the manufacturer

recommended product. Approximate development times are provided on the photoresist data sheet; however, these times can vary depending on the level of agitation available.

3. Rinse the developer solvent: Remove the wafer from the “PGMEA ONLY” dish and rinse with isopropanol in the “PGMEA/ISOPROPANOL” dish. A completely developed wafer will rinse clear. If a cloudy solution forms, the wafer is underdeveloped. This issue can be resolved by re-rinsing the wafer with PGMEA to complete development and repeating the isopropanol rinse.
4. Hard bake: Completely developed wafers should be cured by hard baking via hot plate at 150-200°C. Typically, the temperature is increased gradually over the course of 20-30 minutes. The hard bake helps to further crosslink the deposited resist.

The hard baked wafer is complete and ready for soft lithography. For convenience, the wafer is stored in a disposable petri dish to protect against debris and to serve as a vessel for the PDMS mold.

Soft Lithography

The actual structure of the device is provided by the PDMS (Dow Corning Sylgard 184) mold [2]. Using the device master and soft lithography techniques, a PDMS block containing an imprint of the device channels is made.

Soft lithography procedure:

1. Mix the elastomer and curing agent in a 10:1 w/w ratio.

2. Pour the liquid PDMS over the master: The liquid PDMS is poured directly over the device master. The amount of PDMS added to the petri dish is determined by the desired device thickness. Thinner devices are useful under circumstances where the emulsion is prone to high evaporation rates as evaporation can be combated by saturating the PDMS with water. Thinner devices result in shorter saturation times.
3. Remove the gas bubbles from the device: Thorough mixing of the elastomer/curing agent mixture leads to the incorporation of large amounts of air into the mixture. These bubbles can be removed via house vacuum and desiccation chamber.
4. Thermally cure the PDMS mixture: Once the air bubbles have been removed, bake the device at 40-60°C until the PDMS has hardened. The baking process is not necessary as the PDMS will cure at room temperature; however, curing at room temperature requires more time.
5. Extract the PDMS slab from the master: Each newly cured PDMS slab is physically extracted from the petri dish. This is done by cutting through the PDMS along the perimeter of the underlying master with a razor blade. Once a successful perimeter is cut, the slab is gently peeled from the silicon surface.
6. Re-pour with new PDMS: To increase master longevity, each cut device should be immediately re-poured. This seal protects each master from dust and debris.

Each extracted device was prepared for bonding. Bonding preparation includes punching of inlet/outlet holes (Harris Uni-Core 0.75) and thorough cleaning. The holes

punched into the device have a diameter of 0.75 mm; however, the elastic properties of the PDMS easily accommodate the 1.09 mm outer diameter tubing used for sample injection. Additionally, PDMS molds are cleaned using scotch tape. In doing so, take care not to leave the tape on the mold for extended periods of time as this will leave an adhesive residue on the device. A schematic of the soft lithography process is provided in Figure 2-7.

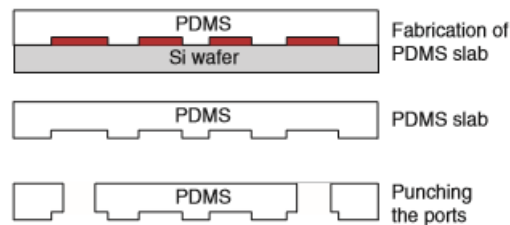


Figure 2-7. PDMS is poured over the master and cured. Once cured, the PDMS is cut from the master and prepared for bonding [9].

Bonding and Surface Treatment

The PDMS block provides the channels with height and a two dimensional footprint, however the device is ineffective without a substrate. This issue is most commonly resolved by bonding the block to a clean glass plate via plasma treatment. By subjecting the bottom surface of the PDMS to oxygen plasma, the exposed methyl side groups of the PDMS are replaced by hydroxyl groups. Likewise, the exposed glass surface is stripped of electrons. After treatment and upon contact of the two surfaces, the functionalized PDMS is covalently bonded to the glass surface. Successfully bonded devices can withstand pressures up to 75 psi [22].

Surface oxidation was achieved using a Harrick Plasma Cleaner and oxygen plasma. Oxygen plasmas were generated at very low pressures (approx. 300 mTorr) and under high power radio frequency (RF). Low pressures were achieved via a vacuum pump (Varian SH-100). In this case, a dry scroll pump was used to prevent combustion associated with pure O₂. An image of the bonding station is provided in Figure 2-8.

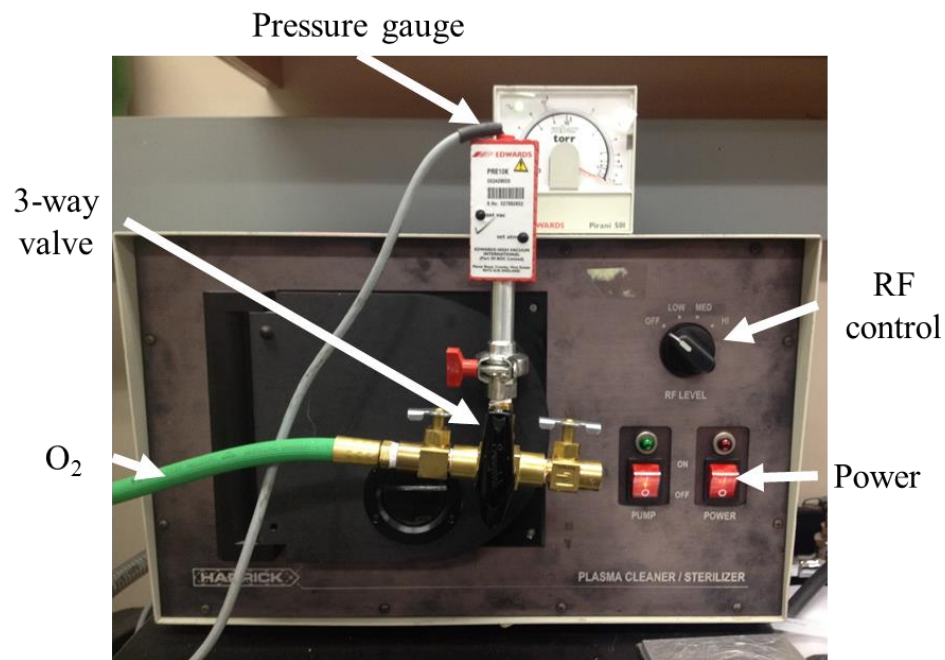


Figure 2-8. Plasma cleaner components.

Plasma treatment procedure:

1. Clean the plasma chamber: Impurities within the plasma chamber, can significantly impact the quality of the plasma. To minimize this effect, clean the inside of the plasma chamber and the chamber door with isopropanol and a cleanroom wipe.

2. Clean the PDMS and substrate: At such small scales the presence of dust and debris are detrimental to device operation. However, adequate cleaning can combat channel blockage. PDMS is cleaned using Scotch tape (3M) and the glass substrate is cleaned using isopropanol and nitrogen gas. Place cleaned PDMS (channel side up) and substrate on a cleaning platform (aluminum foil wrapped 4" glass plate) and inside the plasma chamber.
3. Plasma clean the PDMS and substrate: Turn the three-way valve to the vertical position, and decrease the pressure within the chamber by turning on the vacuum pump (Varian SH-100). Allow the pressure in the chamber to reach its lowest possible point.
4. Fill the vacuum chamber with O₂: Open the oxygen tank completely and adjust the regulator pressure to 10 psig. Slowly open the three-way valve towards the O₂ until the pressure reaches 300 mTorr. Once the pressure has stabilized at 300 mTorr, allow the 3-5 minutes for the vacuum chamber to fill with O₂. Note: There is significant room for improvement at this step. Installing high quality needle valves in series with the three-way valve would increase the ease and accuracy in reaching 300 mTorr.
5. Initiate the plasma: Turn on the plasma cleaner power and turn the RF dial to high. Verify the presence of the plasma and treat the device for 50 seconds. Successful plasmas are blue, pink, or purple depending on the impurities present in the chamber. At the end of 50 seconds turn off all components, close the O₂ tank, and open the three-way valve to the room air.

6. Bond the device: Once the chamber has filled with room air, open the chamber door and quickly remove the cleaning platform without touching the PDMS or substrate. Using a gloved hand and touching only the sides, attach the channel side of the PDMS block to the cleaned glass surface. Carefully press out any air bubbles. Test the integrity of the bond by gently lifting up on the corner of the device. Depending on the quality of the plasma used, the device may require a baking process to promote bonding. If this is the case, place the entire device in a 60°C oven for 15-30 min.
7. Surface Treatment: This process is adapted from the surface treatment procedure presented in Mazutis et al. To prevent hydrophilic wetting, the bonded device is treated with commercially available Aquapel. This product functionalizes the device's inner walls making them hydrophobic. To treat the inner surfaces, coat each channel with Aquapel, allow the product to sit for 10-30 seconds, and then flush the channel with air. Once each surface has been treated, bake the device at 60 °C for 10-15 minutes.

An outline of the entire bonding/surface treatment process is provided in Figure 2-9.

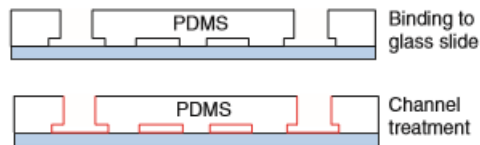


Figure 2-9. The PDMS and substrate surfaces are bonded via plasma treatment. Additionally, the channel surfaces are functionalized [9].

The vacuum used for this process is a dry-scroll vacuum and relies on clean tip seals for adequate function. Compromises in tip seal integrity can result in a vacuum chamber failure. Upon receipt, the Varian SH-100 struggled to reach the pressures necessary for plasma generation and, over time, began to exhibit audible differences while powered. After investigation, the tip seals and their housing were found to be compromised. Replacement tip seals are inexpensive and available through Capitol Vacuum Parts. Upon replacement, the tip seal housing was thoroughly cleaned of dust and debris, and the pump was reassembled.

To maintain sufficient pump/tip seal life, ensure that the inside of the plasma chamber is cleaned prior to powering the pump. In addition, the chamber door should remain closed when the plasma cleaner is not in use. This will prevent dust from entering the vacuum hose itself.

Electrode Fabrication

Devices such as pico-injectors or drop sorters require active manipulation through electric fields. To achieve this, these devices require electrodes and external leads. Using previously introduced equipment (plasma cleaner and hotplate), electrodes can be fabricated in one of two ways. In the first method electrode channels are filled with low melting temperature solder (Indium Corporation of America) [23, 24]. In the second, electrode channels are filled with a salt solution [5, 25]. The solder method was used for all applications discussed in this review.

Electrode fabrication procedure:

1. Heat the bonded device: The melting temperature of the solder is 60°C as indicated by Indium Corporation of America. Using a hot plate set to 80°C, heat the entire device.
2. Melt the solder into the electrode channels: Using tweezers, insert a segment of the solder wire directly into the device. Allow the inserted solder to melt until the entire channel is filled and a small bead of solder protrudes from the channel outlet. To ease future troubleshooting processes, consider the solder channel inlet to be where the external lead posts will be inserted. Complete this process for each solder channel.
3. Plasma clean the soldered device and posts: The surface of the solder is hydrophobic, a feature that does not promote a quality connection between the injected solder and the inserted post. To decrease the hydrophobicity of the solder, plasma treat the entire device and the solder post. Plasma treatment oxidizes all of the exposed surfaces leaving them hydrophilic and creating a more reliable solder-post connection. The plasma treatment process for this application is identical to device bonding, however, the treatment time is reduced to 30 seconds.
4. Insert the lead posts into the device: Remove the device from the plasma chamber and reheat using the 80°C hotplate. In an effort to keep all hardware as clean as possible, leave the lead posts in the chamber until the device is sufficiently heated. Once the device is heated, insert the posts into the solder channel inlets. This may require additional force depending on the quality of the punched inlet

holes; post installation is easier with straighter holes. Repeat for the other set of lead posts.

5. Test the connection integrity with an ohmmeter: Successfully fabricated electrodes will yield a small resistance indicating a congruency between each lead of the ohmmeter. To test the electrode attach one lead of the ohmmeter to the newly inserted post and the other end to its corresponding outlet bead. If there is no resistance, then the connection was not successful. Successful connections have a resistance of 2-5 Ω .
6. Backfill unsuccessful electrodes: Due to the small, round contact surface between the lead post and the injected solder, initial connections are often unsuccessful. This issue can be easily resolved by back filling the solder channel. To do so, re-heat the chip until the internal solder has melted. Remove the outlet bead using a small pair of wire cutters to promote a flat contact surface. Insert and inject a small piece of solder into the channel outlet. The amount of solder inserted into the channel requires some judgement. Too little and the incomplete connection is not resolved, however, too much and the electrode channels will begin to constrict the drop detection channel. Overfilled electrode channels are fixed using continued heat exposure. Prolonged heat will cause the pressure in the electrode channel to increase until the excess solder is ejected from the channel. Repeat this process until the connection in each electrode has been verified.

7. Seal the lead posts to the PDMS block using a UV curable epoxy (Loctite 352).

Curing times vary depending on the approximate bond thickness and are available on the product's technical data sheet.

CHAPTER THREE

FLUORESCENT DETECTION AND SORTING

Introduction

Drop detection is a fluorescence based process with a work flow similar to that of flow cytometry (FC) [20]. In each case, every particle or droplet is excited, produces an emission signal for detection, and is evaluated against a sorting threshold. Although FC is a mature technology providing benefits including increased throughput and color detection, it is very expensive and all constituents are analyzed as a part of a continuous stream. Drop detection allows for each of the same functional features, however the emulsion platform assures each microreactor is independent from the next. This difference allows users to identify and isolate reactions that would otherwise be undetectable.

During detection, a population of drops under specifically engineered conditions pass through laser light one at a time. At rates up to 4 kHz [9, 20, 26-29], the fluorophores within each drop are excited and produce emission signals proportional to their concentration within the drop (Note: frequencies of approximately 30 kHz have been reached using multilayer design features [10]). Each signal is independently detected by a photomultiplier tube (PMT). The detected light is converted to a voltage, which is subsequently received by custom LabVIEW software in real time. This voltage is displayed on a user interface as a local maximum. The operator can use these maxima to define an experiment specific threshold for detection.

Similarly, user defined thresholds can be used to physically sort for drops of interest. In this case, drops are detected in the same manner; however, when the output signal exceeds the defined sorting threshold an amplified voltage is sent to the sorting chip and the desired drop is pulled into a sorted channel. Drops are pulled via dielectrophoresis and due to their size, must be subjected to potentials exceeding 600 V [9]. Drops that remain below the threshold are not subjected to the electric field and are passively transported down a waste channel. This mechanism is discussed in greater detail throughout the remainder of this chapter.

Detection Stand Equipment and Assembly

The detection stand design under consideration is currently in use at a few institutions around the country including the School of Engineering and Applied Sciences at Harvard University and is outlined by Mazutis et al. Prior to assembly, a list of necessary equipment was compiled from colleagues affiliated with the Harvard SEAS. Table 3-1 contains the final list of equipment used to construct the first droplet detection stand.

Equipment	Make/Model	Qty.
Inverted microscope	Motic AE31	1
473 nm laser	DHOM-W473-50mW	1
Syringe pump	New Era NE-500 OEM syringe pump	3
Photomultiplier tube (PMT)	Hamamatsu H10723-20	2
High Voltage Amplifier	Trek 2220	1
Computer	Dell Optiplex 9020	1
Data acquisition card	National Instruments PCIe-7852R	1
LabVIEW software	National Instruments	1
PMT DC power supplies	HP 6216C/OTE HY3003	2
Breakout box	National Instruments CA-1000	1
Optics breadboard	Thorlabs	1
High speed camera	Edgertronic monochrome (IR filter removed)	1
68 pin cable	National Instruments SHC68-68-RMIO	1
Filters	Semrock	
▪ Dichroic beam splitters	Di01-R405/488/561/635	1
	FF776-Di01	1
	FF555-Di03	1
▪ Bandpass	FF03-525/50	1
	BLP01-568R	1
Optics hardware	Thorlabs	
▪ Ø25 mm 830nm longpass	FGL830	1
▪ SM1 lens tube	SM1L10 (1" thread depth)	2
▪ General purpose clamp	CL5	4
▪ Ø1" Aluminum mirror	PF10-03-G01	2
▪ Kinematic Mount	KM100 (for Ø1" optics)	2
▪ Threaded end cap	SM1CP2M	4
▪ Ø1/2" Post holder	PH1	1
▪ SM1 lens tube	SM1L30 (3" thread depth)	1
▪ Iris diaphragm	SM05D5 (Ø0.7 – Ø5 mm)	1

Table 3-1. A comprehensive list of the equipment used in detection stand construction.

Optical Train Assembly

Fluorescence is commonly used in microscopy and can be particularly useful when coupled with applications in microbiology. There are a plethora of commercially available dyes, beads, and biomolecules that are designed to aid in the understanding of these systems. Currently, the most widely used methods for ultra high-throughput drop detection are fluorescence based, however, these capabilities vary depending on the light source(s) and filter combinations used.

This detection stand uses a 473 nm laser. This wavelength was chosen because of its ability to excite both green and red fluorophores, which are commonly used in microbiology. For example, green fluorescent protein is a common method for illuminating bacteria through the process of genetic modification. Also, live/dead stains typically use a combination of green and red emitting dyes to indicate cell viability.

Despite this, the stand is not limited to excitation by blue light only. The first filter in the optical train is a dichroic mirror (Di01-R405/488/561/635). This mirror is designed to reflect wavelengths well suited for fluorescent excitation and allows future operators to increase the versatility of the system by installing additional laser lines. A list of potential wavelengths and compatible fluorophores can be found in table 3-2.

Wavelength Range	Compatible Fluorophore
370-410	BFP
473-491	GFP, FITC, YFP
559-568	RFP, mCherry, TRITC
632-647	Cy5, Alexa 633

Table 3-2. Usable excitation wavelengths and their compatible fluorophores.

The microscope's internal filter cube is housed under the objective turret and holds a second dichroic (FF776-Di01). This mirror is designed to facilitate the reflection of both excitation and emission spectra (<776 nm) to and from the focal plane. Also, when used in conjunction with a long pass (> 830 nm) filter at the bright field light source, this dichroic protects the PMTs from unwanted light pollution.

Lastly, a single PMT is not capable of differentiating between detected wavelengths without the help of external filtration. As a result two PMTs were used, one for the detection of 500-550 nm (green, PMT 1) light and the other of light greater than 555 nm (red, PMT 2). This was accomplished through the use of a band-pass filter, a long-pass filter, and a third dichroic. The dichroic (FF555-Di03) was used to reflect light below 555 nm towards PMT 1 and light above 555 nm toward PMT 2. Furthermore, light entering PMTs 1 and 2 was filtered by a green band-pass filter (FF03-525/50) and a red long-pass (BLP01-568R), respectively. A schematic of the optical train assembly is provided in Figure 3-1.

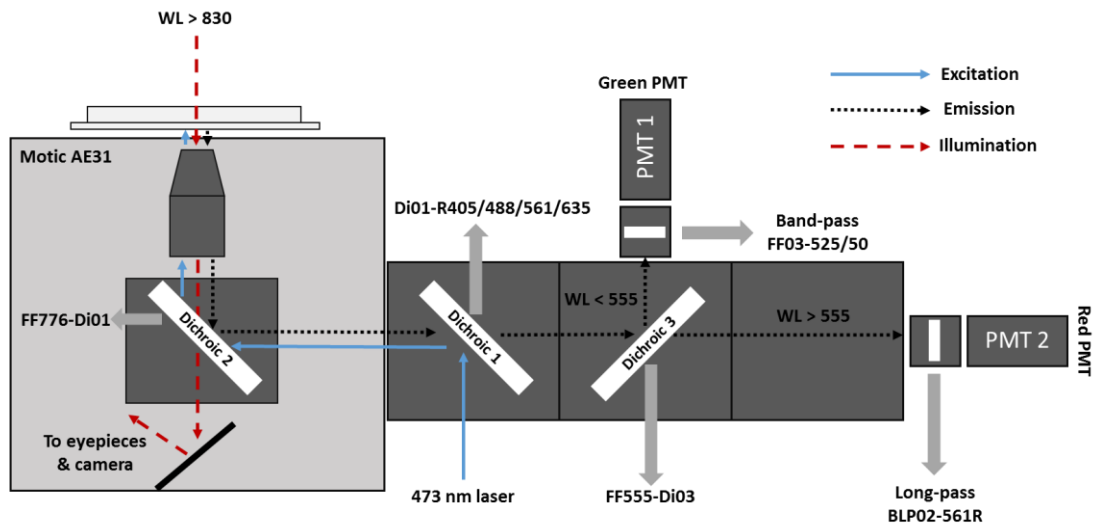


Figure 3-1. Schematic of the rear optical train and microscope hardware set-up.

Microscope and Laser Assembly

The centerpiece of the detection stand is an inverted microscope with fluorescence illumination and camera mounting capabilities. While make and model are not essential, this set-up employed a Motic AE31 with a rear fluorescence port and top mount camera port. Since the microscope must be easily accessed by the operator, optical train, syringe pump network, and electrical hardware, it was important to leave ample space on all sides. A schematic of this device's footprint is seen in Figures 3-2 and 3-3. In this case, the microscope was mounted on the left-hand side of an optics breadboard. Although orientation of the microscope was not a prescribed detail, limitations in available space, cord lengths, and hardware accessibility provided no other option.

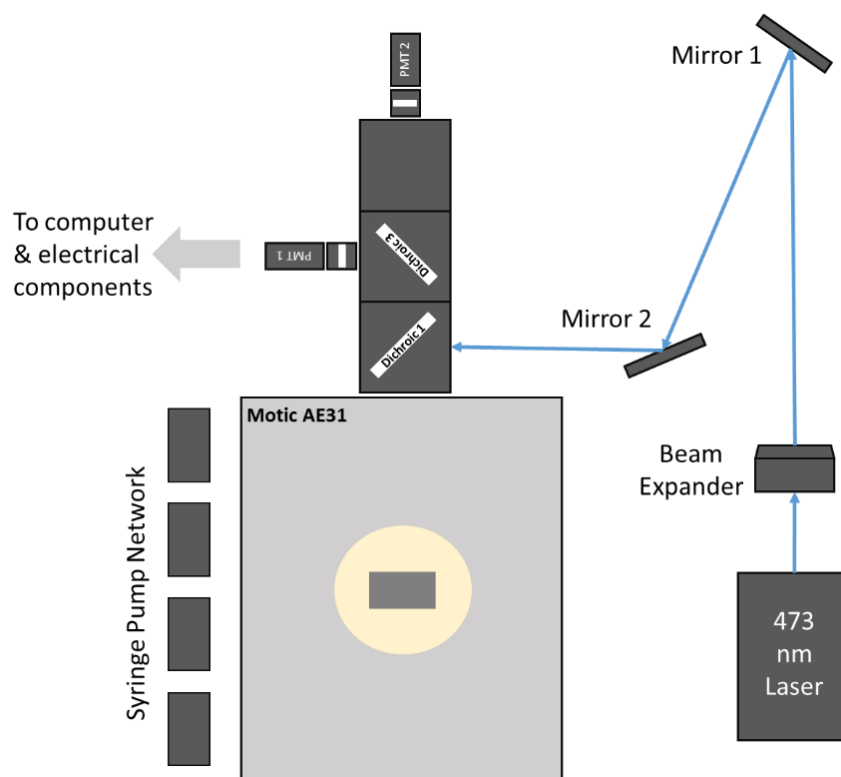


Figure 3-2. Schematic of the detection stand footprint and hardware orientation.

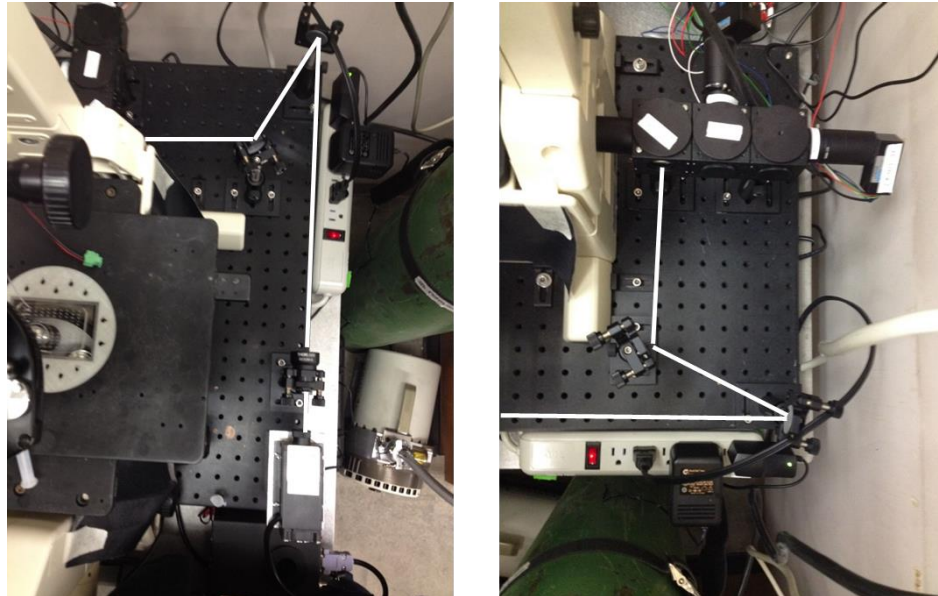


Figure 3-3. Images of the modified detection stand footprint and the excitation beam path.

Using optical hardware positioned to the right of the microscope, 473 nm light was transmitted from a laser source to the back aperture of the microscope objective. To begin, a custom aluminum mount was machined to house the laser source. The mount provided a means of reaching incident beam height requirements, while the aluminum assisted in unit temperature control. From the source, the beam passed through a beam expander, which ensured that the light reaching the objective would fill its aperture. Prior to entering the rear optical train, the beam was afforded two degrees of freedom. These mirrors were used to control the light's lateral and vertical tilt so that it could be centered in the imaging plane.

Once inside the optical train, the beam was reflected off of the first dichroic mirror (Di01-R405/488/561/635) and into the internal filter cube. Here it was reflected off of a second dichroic mirror (FF776-Di01) and into the back aperture of the objective

where it was finally projected onto the imaging plane. Due to limitations in high speed imaging resolution, precise laser alignment was imperative. To do so, each piece of optics equipment was set by order of incidence, beginning with the laser source and ending with the microscope's internal filter cube.

Alignment procedure:

1. Level the optical platform: Minor unwanted tilt could be compensated for with the pitch and roll features on each mirror mount, however, larger grades were difficult to overcome. Using a 360° level and its adjustable feet, the breadboard was leveled to minimize tilt.
2. Set the initial height of the beam: To set the beam's source height, the source post was adjusted until the incident beam hit the center of the microscope's internal filter cube or until the beam was reflected through the center of the cube's upper opening. Subsequent mirror heights were set using incident light from the laser source.
3. Choose a general location and orientation for all optical equipment: The position and orientation of the laser source was chosen such that the layout left a maximum amount of space for adjusting mirror locations during system maintenance. Furthermore, the risk of stray light was minimized. In this case, the laser was directed towards the back of the detection stand.
4. Position subsequent mirrors: Downstream mirrors allow for additional degrees of freedom in beam alignment. In this case, two subsequent mirrors were positioned

such that light could be directed into the opening at the side of the rear optical train.

5. Set mirror reflection angles: Mirror angles were determined by first reflecting light directly back to the laser source. Once achieved, the angle of the mirror was adjusted until the beam direction fit system needs. Reflecting the beam back to the source ensured appropriate tilt angles at each reflection point. Approximate mirror positions and angles can be seen in Figures 3-2 and 3-3.
6. Center the incident beam in the focal plane: To verify that the beam was centered in the imaging plane, a custom laser target was created in AutoCAD using a series of concentric circles intersected by crosshairs. However, due to the limitations in camera resolution, adequate alignment verification required viewing the beam through the mounted camera. Once aligned, all components were secured with bolts.
7. As an alternative to the target presented in the previous step, beam alignment can be achieved using the beam's reflection off of a surface. In this case, the solder channels from a sorting device were used as the reflective surface. When viewed through the eyepiece, the focal plane contains two sources of light: a point and a "halo". A properly aligned beam has the point centered within the halo. Figure 3-4 illustrates both a successful and an unsuccessful alignment.

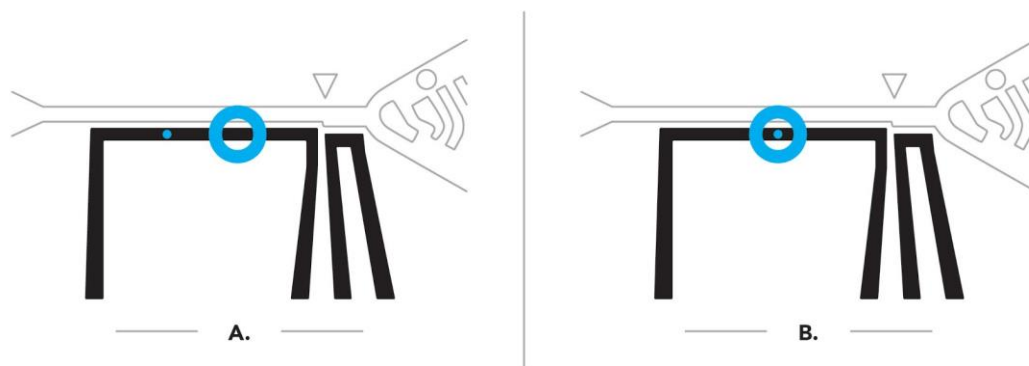


Figure 3-4. Illustration of the final alignment process. A. The incident laser and its reflection are not in the same location indicating that the laser is not perfectly aligned. B. The incident beam is within the reflection. This is the ideal scenario.

The stand employs long working distance objectives. Although this is not as important at lower magnifications, objectives with higher magnifications may otherwise not work given the thickness of the device's glass slide. Further, it is important to have some freedom to adjust the focal plane at different locations along the Z-axis to allow for broader excitation. Adjusting the focal plane just above or just below the center of the detection channel will allow for a larger area of incident light, thus more evenly exciting the entire drop. This is particularly important when analyzing single cells as they may not always be centered in the drop.

The completed detection stand was covered with black curtains hanging from a custom PVC frame to protect from light pollution.

Detection, Data Acquisition, and User Interface Integration

During drop detection, emitted light is detected by a PMT. Based on its intensity, the light signal is translated to a voltage which is subsequently interpreted by a custom

LabVIEW interface. However, before this process can be realized, there are several pieces of hardware that must be electrically integrated. To best communicate how this integration was achieved, this section follows the flow of information from detection to the computer interface.

PMT Integration

The PMT is the detection stand's mode of detection. It is electrically powered and has 6 leads: 2 low voltage inputs, a ground, a reference voltage output, a control voltage input, and a signal output. The 2 low voltage inputs provide power to the device and are connected to their respective positive and negative 5V sources. The ground is connected to the power supply ground. The reference voltage is insulated and left unconnected. The control voltage is connected to the breakout box and interfaces with the computer to adjust sensitivity. Lastly, the signal output is connected to the breakout box which interfaces with the computer to display drop signals.

To produce positive and negative 5V signals, two DC power supplies were used. By connecting the positive terminal of one to the negative terminal of the other and setting both to 5V, the positive and negative signals were generated. The connection between the two served as the ground. The leads for each PMT interfaced easily with the combined power supplies allowing both PMTs to be powered. It is important to note that the ground terminals on each power supply served no actual purpose in this application.

The signal output and the control voltage leads were connected to a breakout box which housed a 68 terminal connector block. In order to best interface each of these

leads, a banana clip was attached to the control voltage and a BNC connector was attached to the coaxial lead of the signal output.

Breakout Box and DAQ Card Integration

The breakout box houses a connector block that integrates the data acquisition card (DAQ card) with each of the PMTs and the high voltage amplifier used for sorting. On its face there are labeled female connection ports for the control voltage and output leads of each PMT as well as the high voltage input. Each of these ports contains an internal wire that is terminated at one of the connector block's 68 screw terminals. A map of the connector block is seen in Figure 3-5.

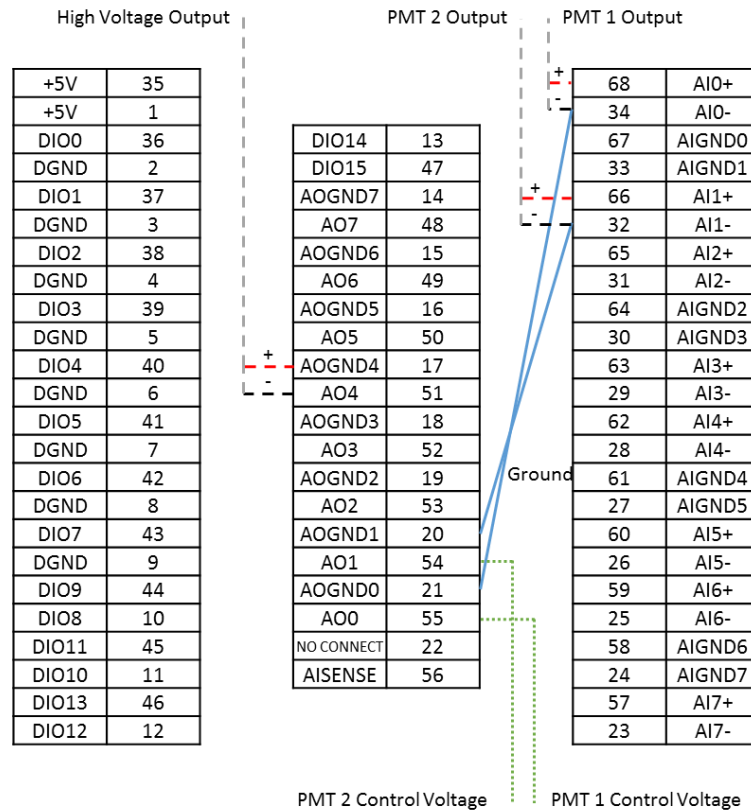


Figure 3-5. Schematic of the PMT/FPGA and FPGA/High voltage integration.

This pinout has the capacity to interface with both analog (denoted by “A”) and digital (denoted by “D”) signals, however, only the analog channels were used in this application. Additionally, this particular DAQ card has the capacity to handle 8 different analog channels (denoted by the numbers 0-7). For this detection stand, channels 0-3 were reserved for potential PMTs, but because only 2 were installed, channels 2 and 3 were left empty. Channel 4 was designated for the high voltage amplifier input. Figure 3-5 illustrates the wiring scheme used to install each PMT.

The control voltage for each PMT was connected to an output channel on the connector block indicating that the control voltage was an input with respect to the PMT. This signal was determined by the user through LabVIEW. Additionally, the PMT output for each PMT was a coaxial signal and thus contained positive and negative leads. The positive lead was connected to a positive input channel, while the negative lead was connected to the negative input for the same channel. Finally, each of the negative inputs were grounded with an output ground terminal (i.e. AOGND0). Grounding the negative leads allowed for only positive signals to be acquired by LabVIEW.

LabVIEW Detection Software Installation and Set-up

LabVIEW is a graphical coding language for that provides virtual interfaces (VI) for laboratory systems. In this application the detection and sorting program, which was written by colleagues at Harvard University, provided a user interface for the detection and sorting stand. The program communicates with the DAQ card to provide a visual output of the detected drops. As received, the sorting program was configured to interact with a different DAQ card (National Instruments PCIe-7842R) than was purchased

(National Instruments PCIe-7852R). The two are equipped with comparable capabilities, but have different input/output configurations. As a result, the LabVIEW programs were unable to communicate with the new card as written, and a new LabVIEW project was created.

FPGA Project Procedure:

1. In LabVIEW, FPGA projects must reference a “target,” which is the appropriate DAQ card (National Instruments PCIe-7852R). To begin an FPGA project, use the “File” menu and select “New.”
2. The following window offers a list of project templates. Select “LabVIEW FPGA Project.”

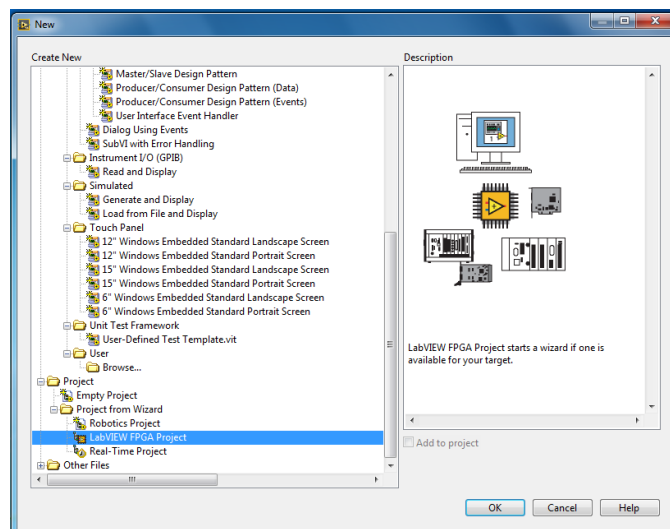


Figure 3-6. A screenshot of the new LabVIEW project window.

3. Next, the wizard prompts the user to choose the desired FPGA target style and location. For the installed National Instruments PCIe-7852R, “R Series Multifunction RIO on My Computer” was chosen.

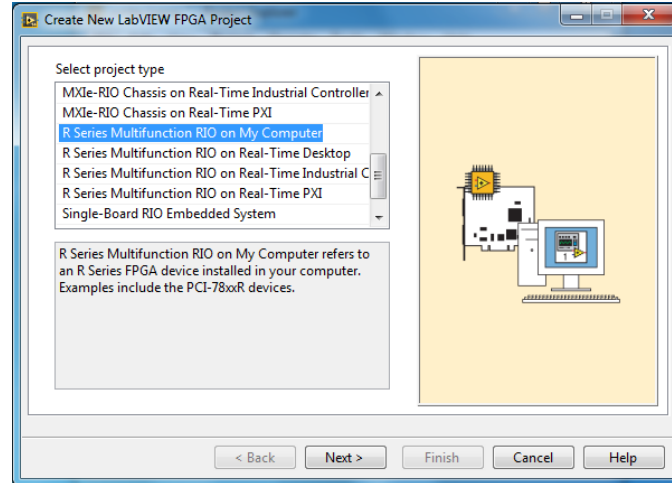


Figure 3-7. A screenshot of the FPGA target style window.

4. The wizard will locate and itemize any devices matching this description. Select the appropriate device.

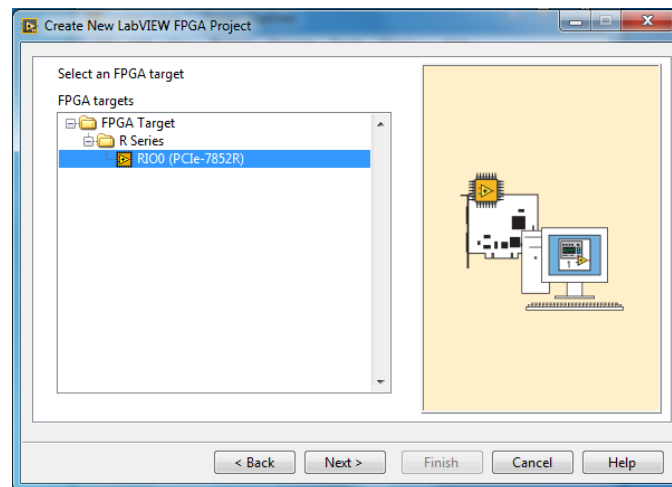


Figure 3-8. A screenshot of the available FPGA targets window.

5. A preview of the project is provided. This preview shows all of the FPGA channels and outlines the project.

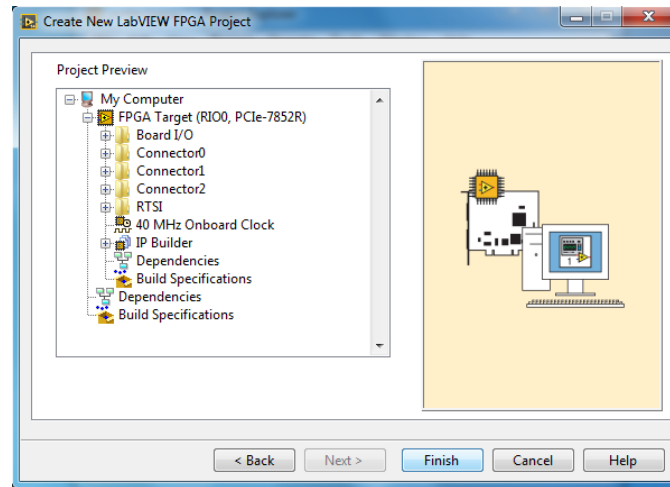


Figure 3-9. A screenshot of the new FPGA project outline window.

6. Once the new project has been created, copy the folders “subVis,” “fpgaVis,” and “mainVis” into the project. Distribute the contents of each folder accordingly.

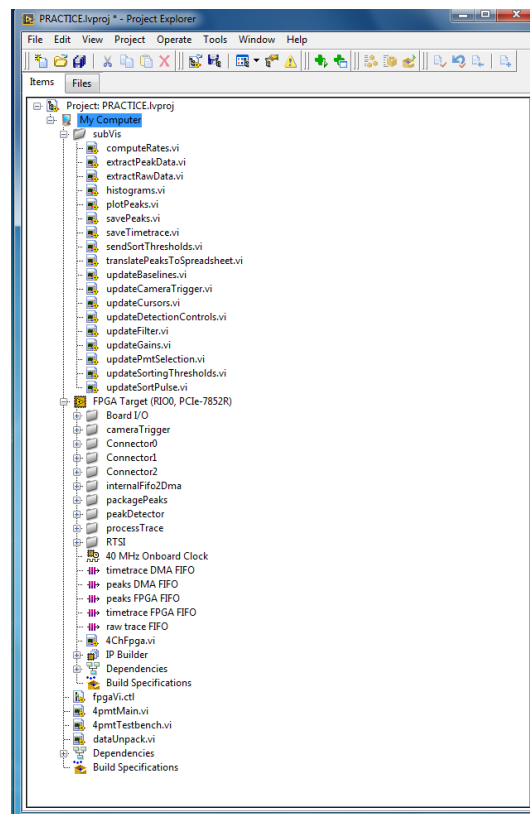


Figure 3-10. A screenshot of the "subVi," "fpgaVi," and "mainVi" distribution window.

7. Before the detection and sorting interface can be executed, the “4ChFpga.vi” file must be configured to accommodate the changes in I/O nomenclature.

Additionally, the project must be compiled from this VI. To reassign the I/O nodes of the “4ChFpga.vi,” independently open the VI and navigate to the block diagram, which acts as the program’s code. Broken pathways are indicated by red X’s and can be restored by selecting an appropriate I/O channel. To change from the 7842R to the 7852R, each node was reassigned to its equivalent analog or digital I/O. For example, where the 7842R node read “AI0,” the equivalent 7852 node read “Connector 0, AI0.” Save the VI when each node has been resolved.

8. To compile the project, right-click the “Build Specifications” icon within the “FPGA Target (RIO0, PCIe-7852R)” and select “New” → “Compilation.”
9. The project compilation is built from a top level VI. In this case, that VI is the recently altered “4ChFpga.vi.”

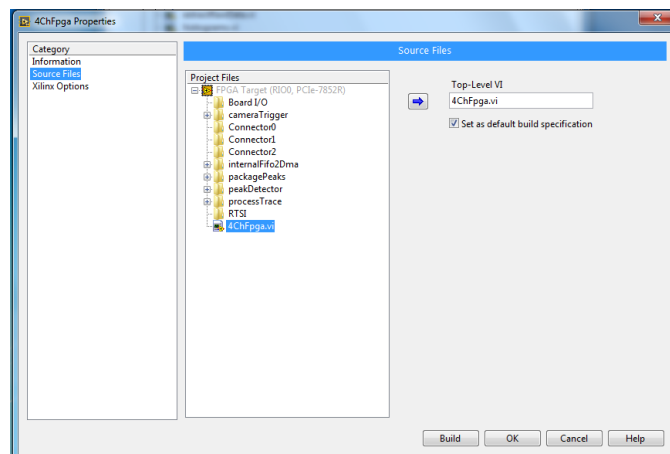


Figure 3-11. A screenshot of the project compilation window.

10. Select the appropriate VI and choose “Build.”

11. Once the program is successfully built, the “4pmtMain.vi” VI can be used to execute the detection and sorting interface.

Syringe Pump Installation

Pressure driven flow used to drive fluids in each application was provided by syringe pumps (New Era NE-500 OEM syringe pump). The combination of their compact size, LabVIEW interface, and networking capabilities made for efficient integration of 4 New Era pumps into the existing system.

Similar to the detection and sorting stand, the pump network is operated by a LabVIEW user interface. However, prior to user communication, the pump network must be recognized by the computer and the LabVIEW program “Pumps.” Detailed instructions for network setup and pump operation are provided on the detection stand computer.

High Speed Camera Integration

Visualizing on-chip operations is useful for determining the efficacy of a particular manipulation. However, the high frequencies and small length scales associated with microfluidics make this impossible with the naked eye. This limitation was mitigated by the incorporation of a high speed camera. The Edgertronic Monochrome camera was an affordable and powerful option for this detection stand. While the resolution and speeds are not quite comparable to some of the more expensive options (i.e. Phantom, Photron), the interface and usability minimize added complexity to the system. Additionally, these limitations can be alleviated by precise laser alignment as the

speeds drastically increase at lower resolutions. Precise alignment is particularly important when recording the sorting process because adjustments to the field of view for visualization purposes will displace the laser from the appropriate detection location. Error by distances as low as 100 μm can make recording the sorting process difficult.

Detection Stand Modifications

Microscope Upgrade

Upon completion, several of the apparatus' shortcomings were illuminated. The first was the difference in focal distance between the eyepiece and the camera. The system relies on an objective to deliver emitted light to each PMT. As a result, the eyepiece focal plane must be positioned at a location along the z-axis such that the maximum amount of emission light can be captured for detection. Further, high frequencies make it desirable to view flows via high speed camera. The difference in focal distance did not allow the user to record when the eyepieces were in focus, but also did not allow adequate light capture when the camera was in focus. Thus, recording the sorting process was impossible.

The second shortcoming was the lack of precision in X and Y manipulation. The Motic AE31 microscope was not equipped with an XY stage or manipulator, and those available were not outfitted with adaptors capable of holding the glass slides used for each microfluidic device. In an attempt to overcome this, a manipulator was purchased and a fiberglass stage, roughly cut to the size of the device, was fastened to it. While this

provided some relief in XY manipulation precision, it still allowed for approximately 1-2 mm of error in either direction due to cutting imperfections.

Third, the microscope's internal filter cube was salvaged and allowed for freedom in the angle of the dichroic mirror within the filter cube (FF776-Di01). This made it difficult to ensure that the incident laser light was being reflected straight up into the objective.

Each of these issues was addressed by replacing the Motic AE31 with a more capable Nikon Diaphot 300. The Edgertronic fast camera is manufactured with a Nikon F-mount and is most compatible with Nikon lenses and devices. The difference in focal distance between the eyepiece and the camera was negligible in the Diaphot. However, due to limitations in the camera's resolution at high speeds, it is still necessary to make sure the excitation beam is centered as best as possible. This is most essential when attempting to record the sorting junction.

Further, the Nikon microscope was equipped with a significantly larger stage and more precise manipulator. Using a 3D printer, a platform was designed to fit the device footprint. The amount of error associated with X and Y movement was negligible.

Last, the Nikon's internal filter cube housing was much more accessible and a new filter cube was purchased. The new filter cube ensured that the angle at which the dichroic mirror was set would allow the incident light to be reflected at a 90 degree angle. In addition, the exposed housing made laser alignment and establishing a beam height much simpler.

Despite the Nikon's increased performance, this model has been discontinued and replacement/missing components are difficult to find. The slider for the internal filter cube is missing and the proper cube location is denoted by two pieces of tape. While this is effective, bumping the equipment or laser table can cause this piece to move and the laser to become misaligned.

Detection Stand Location and Accessibility

The original detection stand was positioned in an area of the lab providing limited operator access. This made system maintenance a challenge and daily operation inefficient. To resolve this, the laser table supporting the platform was moved providing the operator with more work space and exposing the optical hardware. This exposure allowed for easier maintenance and allowed spectators to observe the detection and sorting processes. This is important for teaching purposes. Due to the complexity of the system, it is beneficial to make quality observations and receive hands on training before operating the apparatus. Figure 3-12 displays the differences in overall system orientation, available space, and accessibility.

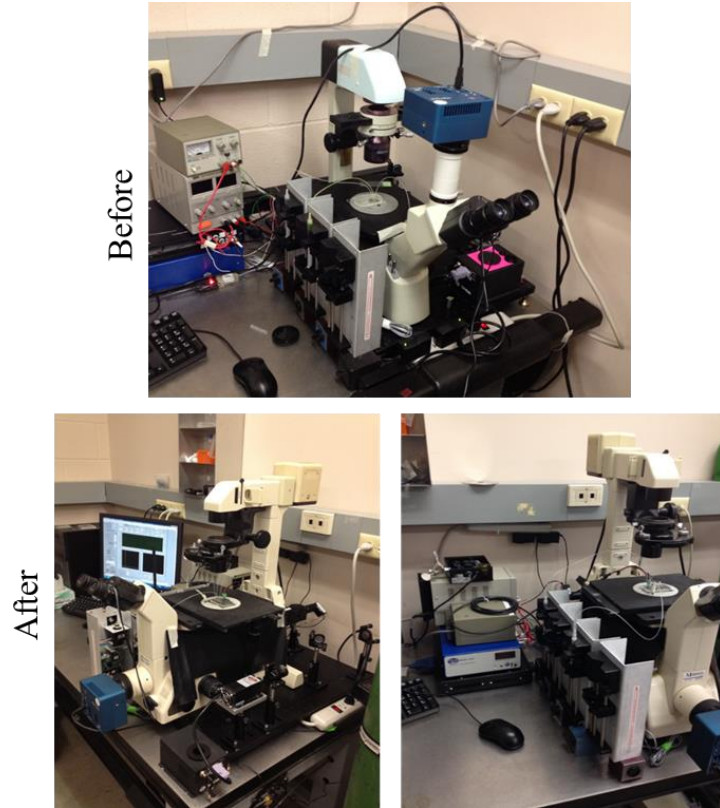


Figure 3-12. Comparison of system orientation, available space, and accessibility before and after modifications.

Expanding Fluorescent Detection Capabilities

In an effort to increase system versatility, a 405 nm laser and a third PMT (PMT 3, 425-465 nm) were added to the system. Because of the system's small footprint, integration of these components required relocation and removal of some optical hardware as well as the addition of other hardware. A schematic of the final optical train, system footprint, and breakout box configuration can be seen in Figures 3-13, 3-14, and 3-15. The added components have been colored orange.

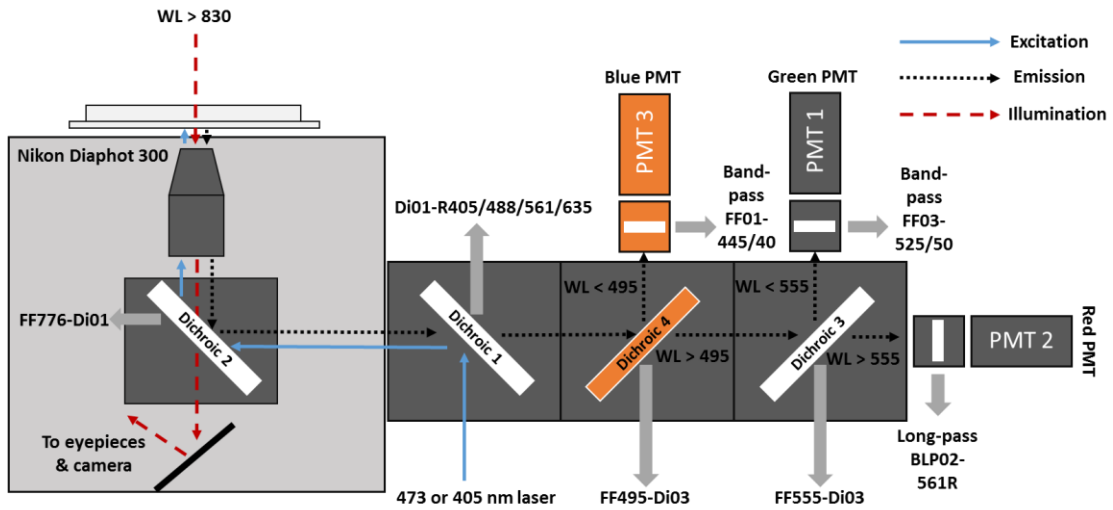


Figure 3-13. Schematic of the modified optical train.

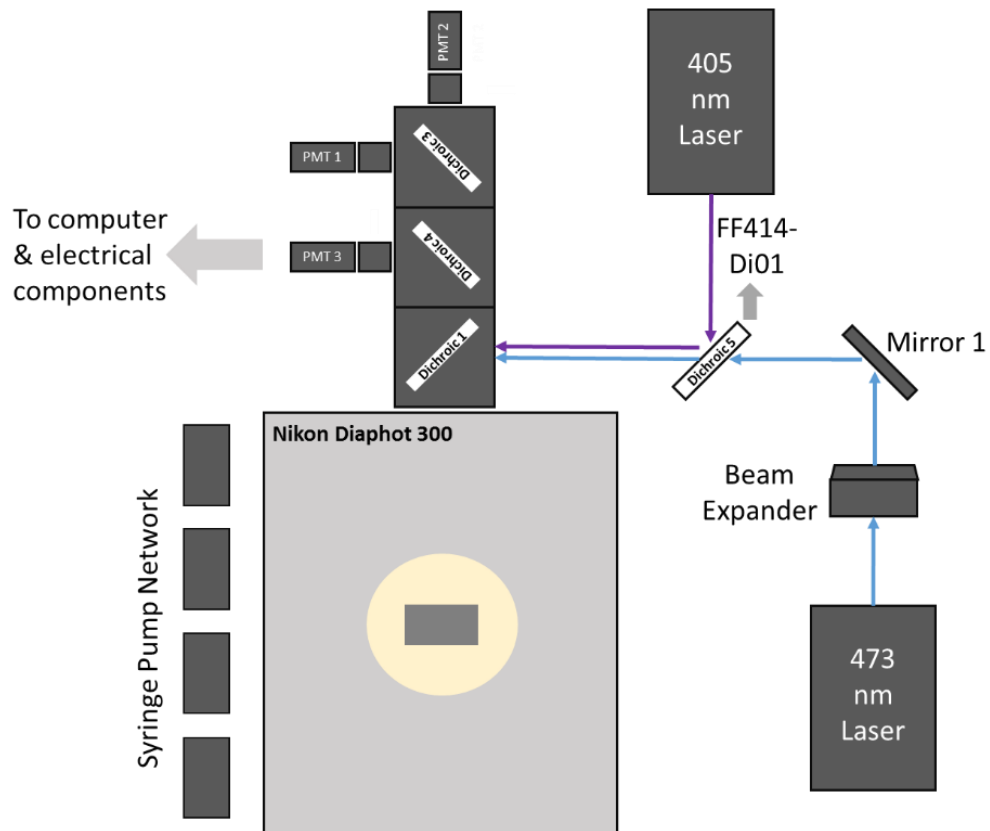


Figure 3-14. Schematic of the modified system footprint.

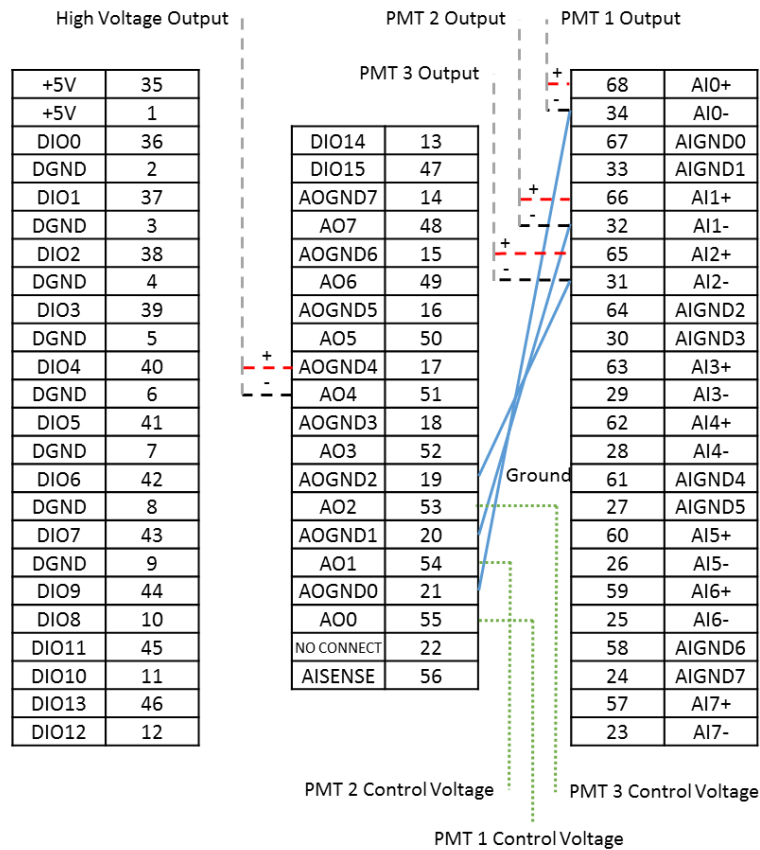


Figure 3-15. Schematic of the modified PMT/FPGA configuration.

Integration of a second laser requires precise alignment of both lines as it may be necessary to excite with both wavelengths at the same time. Although most fluorescent materials are excited by UV light, there are some instances when it is useful to quickly change excitation colors. For example, UV light is often good for inducing autofluorescence in media, however, it is also harmful to cell viability. An easy exchange between the two lines allows the operator to determine characteristics such as drop frequency using the UV line, but quickly change to a safer mode of interrogation using the blue (473 nm) laser.

Detection and Sorting Stand Operation

Detection stand operation requires full system integration. To simplify, the workflow will be discussed in the context of drop sorting because drop detection is the foundation of this process. To begin, the desired drop sample, an oil/surfactant syringe, and outlets are attached to the sorting chip via polyethylene tubing and the device is positioned over the microscope's objective turret. Taking care to choose a high quality device with effective electrodes, the high voltage leads are attached to the sorting chip's electrode posts. It is best to attach all of the leads prior to aligning the laser with the detection channel as slight movements will easily compromise the laser alignment. Laser alignment is easily accomplished using the microscope stage manipulator. An image of the fully integrated device is provided in Figure 3-16.

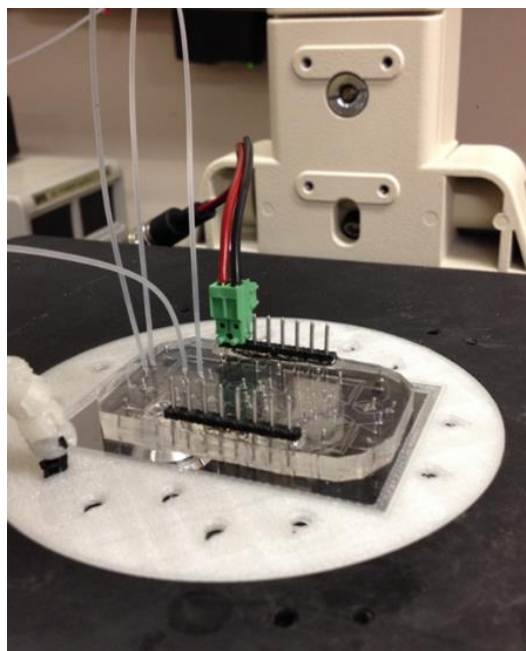


Figure 3-16. Complete optical, electrical, and fluidic device integration.

Drop Reinjection

Reinjected drops flow through a detection channel in single file fashion where they are subjected to excitation via 473 nm laser light (Figure 3-17). Subsequent emission signals are transmitted through the objective and into the PMT via the rear optical train. This pathway is illustrated in Figure 3-13.

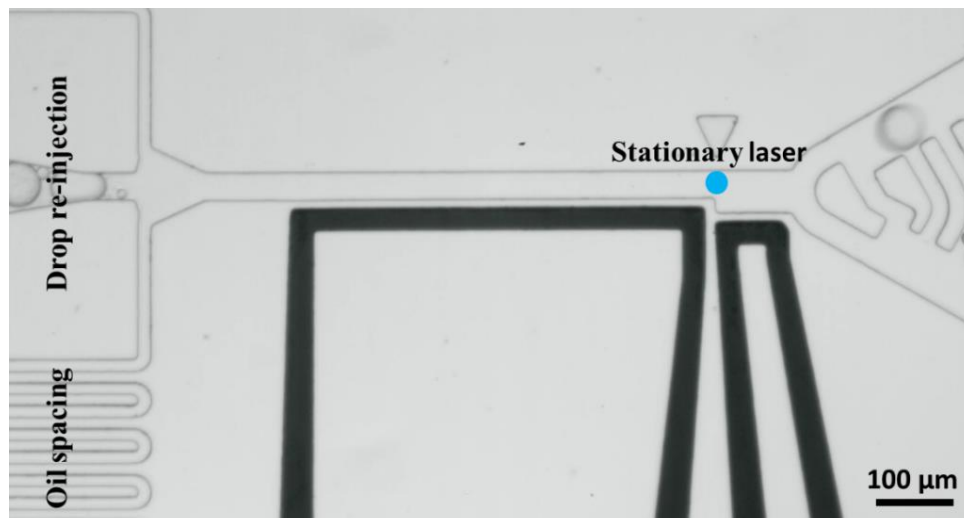


Figure 3-17. A microscopic image of the architecture and work flow used for drop detection via sorting device.

Operating LabVIEW

Detected emission signals are displayed in LabVIEW in real time. The LabVIEW interface (Figure 3-18) provides a number of control parameters and information including PMT sensitivity, detection parameters, sorting parameters, acquisition progress, and sorting pulse. The interface also provides several data monitoring and exporting features. In an effort to provide a thorough understanding of the LabVIEW interface, we will go through each area in detail.



Figure 3-18. A screenshot of the LabVIEW interface during drop detection. Local peaks indicate the fluorescence within a drop is being detected. The magnitude of the peak is determined by the emission intensity.

PMT Sensitivity. Sensitivity is controlled independently for each PMT using the gain function presented in Figure 3-19. This feature allows the user to adjust the amplitude of the PMT output signals by increasing or decreasing the PMT control voltage. It is important to monitor the signal output when adjusting this parameter. Output signals exceeding 4 volts can be detrimental to the PMT. Typical gains used are in the range of 0.4-0.6 depending on the intensity of the fluorophore within the drops.

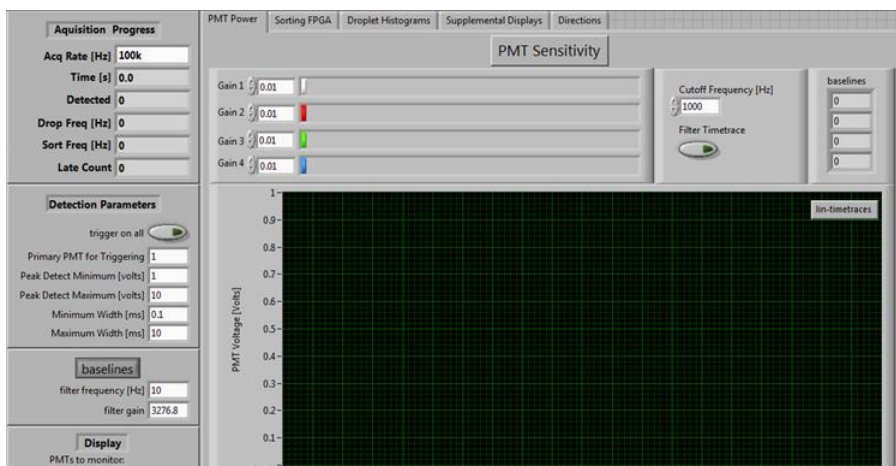


Figure 3-19. A screenshot of the interface's PMT power page. In this section the user can define aspects of detection including PMT sensitivity and detection thresholds.

Detection Criteria. Drop detection is a means of evaluating drops against user defined thresholds to quantify populations of interest. Detection can be accomplished through a single PMT signal, in this case blue, green, or red, or all PMT signals (blue, green, and red). In any case, each triggering signal must meet the defined requirement. Populations of interest are defined by signal peak and width (“time inside”). The peak is useful for differentiating between drops containing different fluorophore concentrations or intensities, while the width is useful for determining drops outside of a desired size range.

Acquisition Progress. Acquisition progress displays key information about the progress of a detection session including the amount of time the program has been running, the number of drops detected, and the frequency with which drops are being detected. However, the displayed detection frequency can be misleading as it does not account for empty drops. The acquisition rate is a user defined parameter used to control the rate at which the FPGA acquires samples from the PMTs. The NI PCIe 7852R FPGA has a maximum sampling rate of 750 kS/s (kilosamples/second), however, the acquisition rate is limited to approximately 200 kHz as the PMT output cannot exceed this frequency.

Sorting Criteria. Sorting thresholds operate similarly to detection thresholds, but with slightly different controls. “X select” and “Y select” correspond to axes on a data distribution plot found at the bottom of the “sorting FPGA” tab. These axes can be set to a specific PMT or to a signal duration. Furthermore, each of the X and Y controls offers

the operator the freedom to choose maximum and minimum values. This allows for sorting of drops within a precise region of interest and is particularly useful when incorporating fluorescent barcodes. When a specific drop satisfies the defined sorting threshold, a user defined electric potential is initiated, amplified, and transferred to the chip's electrodes. A large yellow signal appears next to the drop emission peak when a drop has exceeded the sorting threshold (Figure 3-20).

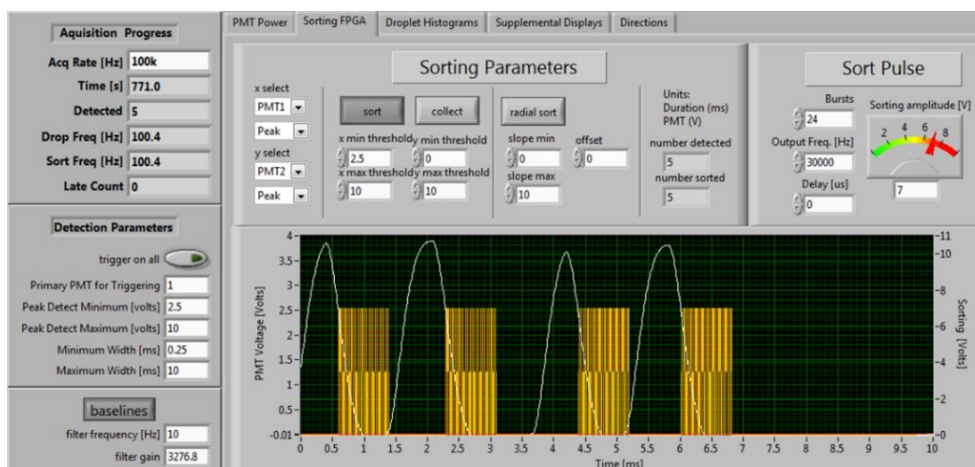


Figure 3-20. A screenshot of sorting interface. The yellow rectangles indicate that a given peak has met the user-defined threshold and an electric field has been initiated.

Sorting Pulse. When a drop meets the sorting criteria, a sorting potential between 0.6-1.0 kV is initiated [9]. This pulse is defined by the user and its parameters are set according to the size, speed, and frequency of the drops being sorted. The yellow signal shown in Figure 3-20 indicates that a drop has met the sorting criteria and has been recognized in LabVIEW. The height of this yellow signal is indicative of the sorting threshold (volts) and the width is indicative of the pulse duration. Furthermore, any delay defined by the user is also visible on the interface. These displays help the operator ensure that the chosen sorting pulse is not interfering with subsequent drops.

The voltage defined by the sorting amplitude is slightly misleading. Due to limitations of the analog output terminals, only half of the voltage is output by the FPGA and only half of the defined voltage is received by the high voltage amplifier. The amplifier is rated to amplify at 200 V/V and corresponding amplified signals are available in table 3-3. Additionally, the amplifier produces a -2.7 V signal without any input. This inherent signal appears to compensate the amplified voltage at low inputs (i.e. 0.1-0.4 V), however, at higher voltages, compensated signals are due to decreased amplification efficiencies. Using a voltmeter, relevant input and output voltages were measured inside the breakout box and at the sorting leads, respectively. These voltages are displayed in table 3-3.

LabVIEW Output (V)	Amplifier Output (V)	LabVIEW Output (V)	Amplifier Output (V)
0	-2.7	3	295
0.1	7.2	4	387
0.2	17.2	5	475
0.3	27.1	6	560
0.4	37.1	7	633
0.5	47	8	703
1	97	9	767
2	196	10	825

Table 3-3. Relevant LabVIEW voltage inputs and high-voltage amplifier outputs.

Sorting Mechanism. The sorting device contains two outlets and is designed such that only drops subjected to the electric potential are pulled into the ‘sorted’ channel (Figure 3-21). This preferential flow is achieved by a 1.5x [9] increase in hydraulic resistance within the sorted channel. Specifically, the wall of the sorting junction bumper extends further into the sorted channel than the waste channel. This difference in flow

resistance is enough to passively transport drops into the waste channel as long as the drops are adequately spaced. Inadequate drop spacing results in increased resistance to flow within the waste channel and can cause unwanted drops to passively travel into the sorted channel. The recommended amount of inter-drop spacing is ten times [9] the drop diameter.

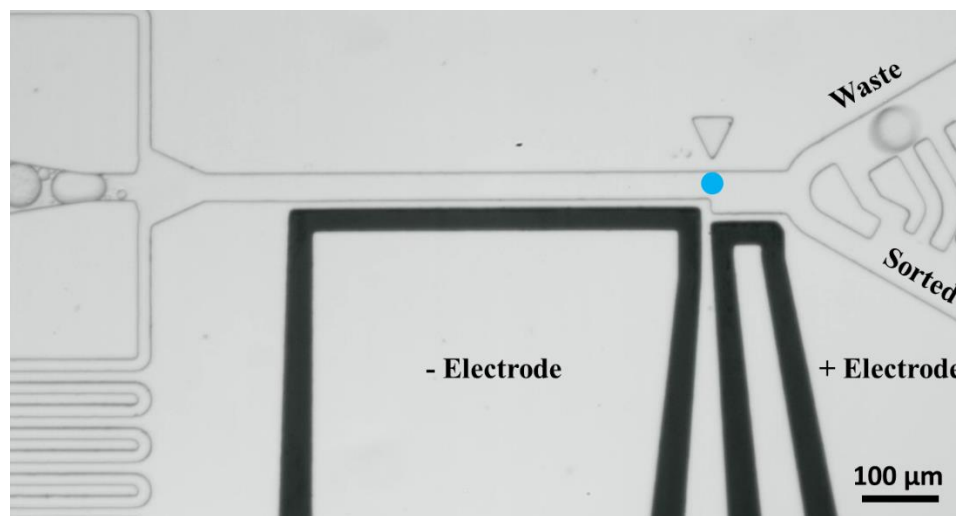


Figure 3-21. A microscopic image of the workflow used for sorting.

Data Logging. The LabVIEW interface is set-up to acquire data, write it to a text file, and save the file in a designated folder. There are two types of data the program can record, peak data and timetrace data. The peak data is typically the most useful as it can be recorded for extended periods of time and is easily analyzed using common data analysis software (i.e. Excel, Minitab, R). Timetrace data requires custom MATLAB code to analyze and the files can quickly become too large to work with.

Prior to saving data, the user must set a destination folder. Use the folder icon to activate the browsing function (Figure 3-22). Once inside navigate to the desired folder and choose “Current Folder.” Next, create a unique filename addon and select “peaks” to

record peak data. A file ID number will automatically be added for multiple files containing the same filename addon. Recording timetrace data requires the same process, however there is separate folder icon for selecting the base folder.

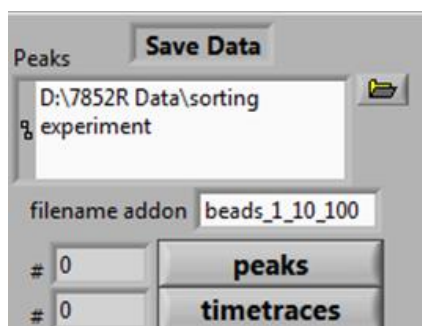


Figure 3-22. A screenshot of the widget used for saving peak data.

CHAPTER FOUR

USING DROP MICROFLUIDICS TO QUANTIFY THE INFLUENCE OF HPF ON
RIBOSOME HIBERNATION AND THE MAINTENANCE OF *P. AERUGINOSA*
VIABILITY DURING STARVATIONIntroduction

Drop microfluidics uses micro-scale water-in-oil emulsions as batch bioreactors for miniaturized biological assays. Similar to the way conventional techniques use petri dishes and liter-scale volumes of media to perform growth assays on bulk communities, a drop can be used to perform the same assay on a single cell. When used in conjunction with available fluorescence capabilities, the drop-based growth assay provides the added advantage of allowing users to visualize, detect, and quantify both growing and non-growing constituents at the single cell level. Additionally, ultra high-throughput drop analysis and sorting methods make it possible to automate subpopulation quantification and isolation.

Pseudomonas aeruginosa is an opportunistic pathogen responsible for chronic wounds and lung infections in cystic fibrosis patients. Because these infections often exist as biofilms, they are particularly resistant to antimicrobial treatments. Antimicrobial resistance in biofilms can occur through several different mechanisms, however, the mechanism under investigation is resistance among subpopulations via cell dormancy [30].

In a previous study, Williamson et al. investigated an antimicrobial resistant, dormant subpopulation at the bottom of *P. aeruginosa* biofilms where oxygen and nutrient were scarce [31]. Their study indicated that there was a decrease in overall mRNA, but an abundance of rRNA and ribosome hibernation factor mRNA at the bottom of the biofilm [31]. Thus, antimicrobial resistance was thought to be linked to two ribosome associated proteins, the ribosome modulation factor (Rmf) and the hibernation promoting factor (Hpf). Although their relative importance can vary from species to species, these proteins are known to play a role in maintaining cell viability during dormancy. These proteins dimerize 70S ribosomes to form translationally inactive 100S ribosomes during stress [32, 33]. This process has been termed ribosome hibernation. A colony forming unit (CFU) assay done in conjunction with the current microfluidic research confirmed the importance of Hpf over Rmf in *P. aeruginosa* ribosome preservation.

In this study, drop microfluidics was used to interrogate green fluorescent protein (GFP) expressing *P. aeruginosa* wildtype (PAO1:pMF230) and *hpf* mutant (Δhpf) cultures at the single cell level. Drop compartmentalization and cultivation of single cells offered a significant advantage over traditional plating techniques as they allowed for the visualization and quantification of non-growing populations of starved PAO1:pMF230 and Δhpf planktonic cultures. Results indicated a reduction in Δhpf cell resuscitation when compared to wildtype suggesting that the presence of Hpf, not Rmf, was directly related to the resuscitation of cells exposed to prolonged starvation.

Methods and Materials

Inducing Cell Dormancy

The two *P. aeruginosa* strains under investigation were the wildtype (PAO1:pMF230) and the *hpf* mutant (Δhpf), each containing Gfp. Cell dormancy observed at the bottom of the biofilm was imitated through starvation of planktonic cultures for 0-5 days using phosphate buffered saline solution (PBS). Each day 1 mL samples of each strain were prepared by re-suspending 100 μ L of the starved culture in 900 μ L tryptic soy broth (TSB) yielding a cell density of approximately 6×10^6 cells/mL on day 0. Samples were collected from 3 sequentially run biological replicates, each requiring 7 days from start to finish.

Cell Encapsulation and In-Drop Incubation

Using flow focusing techniques, each sample was encapsulated into 15 μ m diameter drops. Drops were produced for 1 hour using flow rates of 100 μ L/hr and 375 μ L/hr for aqueous and oil phases, respectively. This yielded approximately 56×10^6 individual drops. Encapsulation rates varied depending on the strain and the number of days starved. Figure 4-1 demonstrates the difference in encapsulation rates for each culture and each starvation time point. The initial increase in encapsulation rate (day 0-1) was due to continued cell division after starvation was initiated. Continued cell division during starvation influences single cell encapsulation rates during subsequent days and as a result can influence the number of drops exhibiting growth. Cell encapsulation follows a Poisson distribution [34] and can be modeled using the following equation:

$$P(X = x) = e^{-\lambda}[\lambda^x/x!]$$

Where X is the probability of encapsulating x number of cells, λ is the mean number of cells encapsulated, and x is number of cells. According to this distribution and the mean encapsulation rate, it is more likely to observe an initial encapsulation of 2 or more cells within the Δhpf culture. The probability of a drop having a single cell during any given day is outlined in Table 4-1.

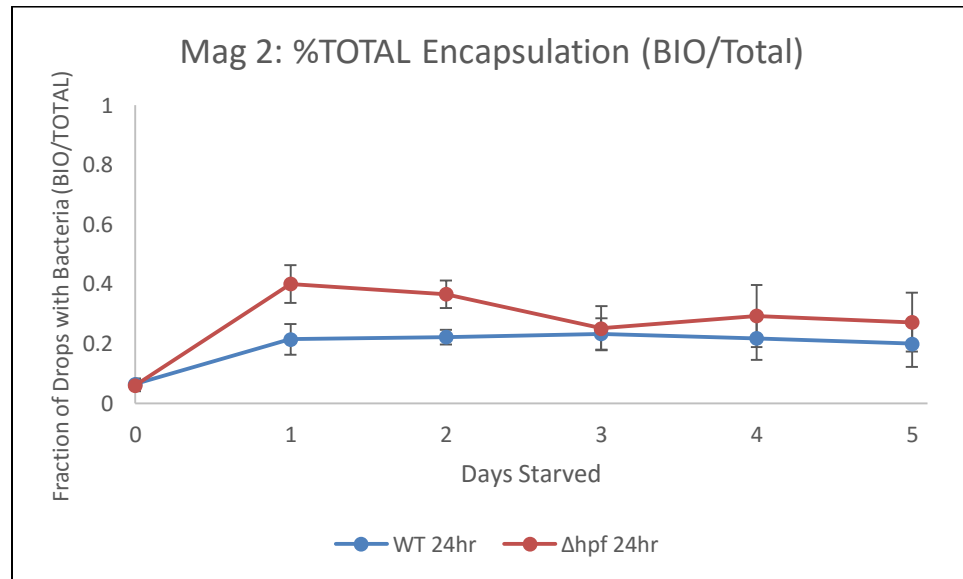


Figure 4-1. The fraction of total drops containing biological material at each time point.

Days Starved	WT			Δhpf		
	1 Cell	2 Cells	3 Cells	1 Cell	2 Cells	3 Cells
0	93.79%	0.19%	0.06%	94.14%	2.84%	0.06%
1	80.69%	8.66%	0.62%	66.99%	13.42%	1.79%
2	80.10%	8.89%	0.66%	69.36%	12.69%	1.55%
3	79.25%	9.21%	0.71%	77.77%	9.78%	0.82%
4	80.41%	8.76%	0.64%	74.61%	10.93%	1.07%
5	81.89%	8.18%	0.55%	76.19%	10.36%	0.94%

Table 4-1. The probability of a biological drop containing 1, 2, or 3 cells at each day according to the Poisson distribution.

After formation, the drops were captured in a 3 mL plastic syringe and were incubated for 24 and 48 hours at 37°C. The emulsions were agitated using a Hula mixer set to 30°C and 1 RPM. Emulsion agitation was a means of promoting homogeneous gas exchange and recirculating surfactant throughout all parts of the emulsion.

Drop Imaging via Dropspots Device

At 24 and 48 hours, a portion of each drop population was re-injected into a dropspots [7] device for immobilization and imaged using confocal microscopy (Leica TCS SP5 II) (Figure 4-2). Z-stack images were taken (20x objective, 1.5 μm Z-resolution, at 3 different magnifications for a total of 9-11 images per sample. Magnification 1 captured 250-400 drops, magnification 2 captured 125-200 drops, and magnification 3 captured 15-25 drops. In addition to transmission imaging, Gfp signals were captured using 476 nm laser excitation and 500-550 nm emission detection. These parameters were carefully chosen as they closely matched the capabilities of high throughput drop detection (473 nm laser, 500-550 nm emission detection).

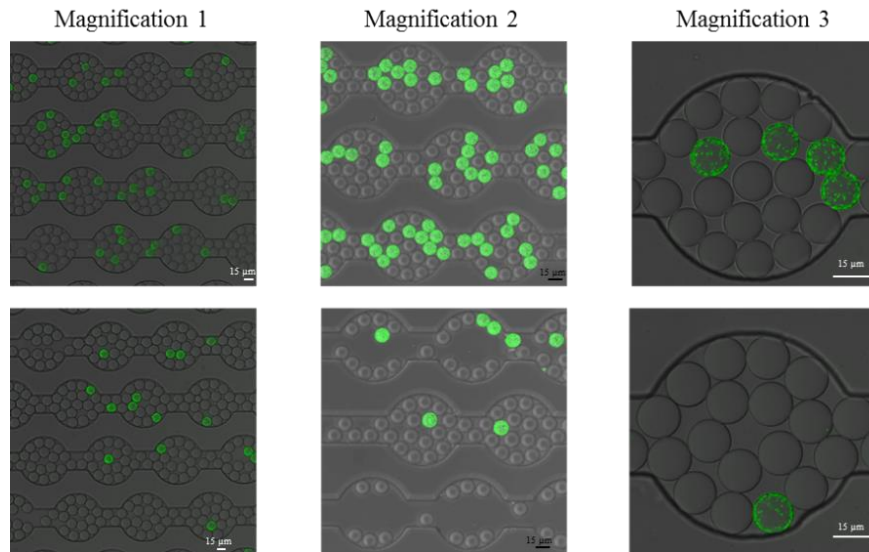


Figure 4. Sample images indicating approximate sample for each magnification. Top row: WT. Bottom row: Δhpf .

Image Analysis

Image analysis and manual drop counts were completed using Metamorph image analysis software. Important metrics under consideration were drop size (drops outside of the desired area threshold were not considered), total drop count (total number of drops under consideration), positive drop count (number of drops exhibiting full growth), single cell drop count (number of drops containing single cells), intermediate growth drop count (number of drops containing partial growth), and positive non-fluorescing drop count (number of drops containing growth, but not expressing Gfp). Examples of each drop population are provided in Figure 4-3. When possible, counts were taken from maximum projections, which is a Z-stack reconstruction transposing the maximum intensity of all layers onto one image. A slice by slice approach was taken when drop movement and artifact fluorescence made this ineffective. Single cells were differentiated from artifacts based on motility.

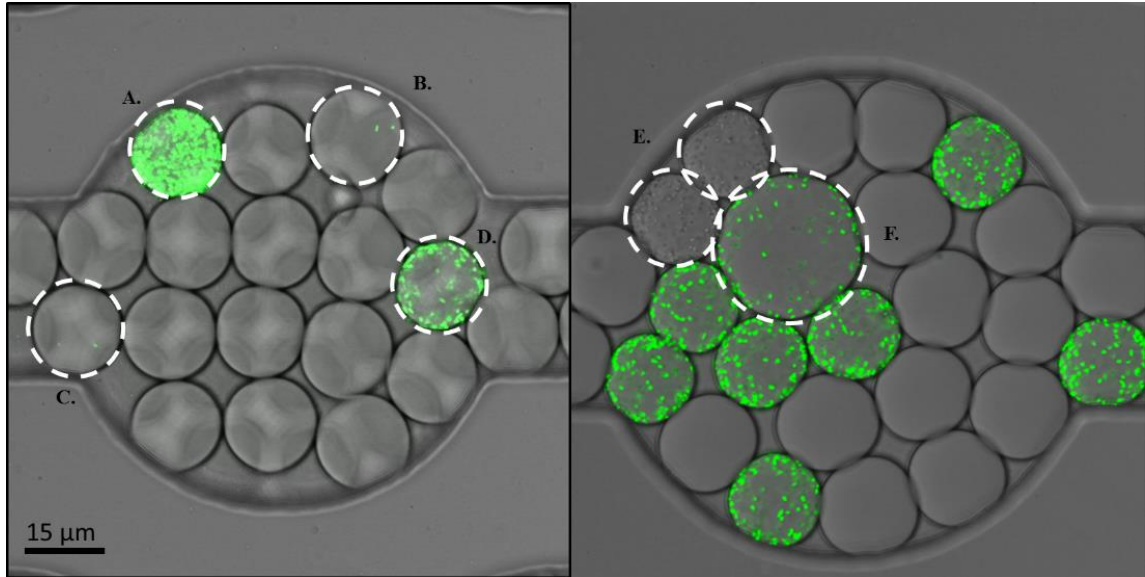


Figure 4-3. Samples of each drop population. A. Positive drop. B-C. Single cell drop. D. Intermediate drop. E. Positive, non-fluorescing drop. F. Coalesced, positive drop.

Image Data Statistical Analysis

Statistical analysis was completed using binary logistical regression and the statistical analysis software, R. The data set contained high sample size (n) variability making it difficult to assume a Gaussian distribution in some cases. In addition, the variance of the counted positive fractions was much lower at the earlier time points (i.e. most points indicated 100% positive drops for days 0, 1, and 2 of starvation) and those that did vary were skewed in one direction only (many were 100%, but one or two may have been lower). Binary regression uses odds to assign a weight to each data point according to n . In this case there were two possible drop outcomes, positive or single cell and each was assigned a binary marker, 0 or 1, respectively. The model analyzes the odds that a desired outcome will occur regardless of sample size or distribution.

Ultra High-Throughput Drop Detection

Detection of incubated drop populations was completed using a custom fluorescent based drop detection stand [9]. Data retrieved from this platform include the signal peak (V) and signal duration (time inside, ms) for each detected drop. During preliminary detection, day 4 starvation drops were individually interrogated for the presence of Gfp using a 473 nm laser after 24 hour incubation. Emission signals were further conditioned by a green bandpass filter (Semrock FF03-525/50) before being detected by a designated green PMT. Preliminary detection data was collected for 30 seconds at a time yielding approximately 1000-2000 analyzed biological drops. All detection was completed using the 10x Motic objective and the modified Nikon detection stand.

Due to successful preliminary data, a 6 day, 3 biological replicate detection experiment was performed. Cell culture preparation and drop formation were identical to the image analysis experiment and fluorescence detection analysis was completed after 24 hour incubation only.

Results and Discussion

Due to differences in the number of total drops per image (Magnification 1: 250-400 drops, magnification 2: 125-200 drops, magnification 3: 15-25 drops), results were best represented as a percent of the total drop sample (%TOTAL) or a percent of the drops containing bacteria (%BIO). In either case, drops containing growth (intermediate or complete) were regarded as 'positive' and drops containing single cells were regarded

as ‘single cell.’ Additionally, the highest magnification images were omitted from data analysis due to bias during the imaging process (these regions were not selected randomly).

In-Starvation Cell Division

Using %TOTAL information we observe that each strain demonstrated continued cell division upon being transferred to the PBS starvation media. This is indicated by the increase in positive drops between day 0 and day 1 of starvation (Figure 4-4). The continued growth ceases after day 1 of starvation indicating that imitated cell dormancy does not occur until after this time point. Interestingly, the continued growth is consistently 15-20% higher among the Δhpf cultures than the PAO1:pMF230 suggesting a reduction in induced ribosome hibernation and cell dormancy. Furthermore, similarities in the average positive drop fraction at day 0 suggests that this is not a result of a higher initial cell density, but the influence of Hpf. After day 2, a significant reduction in Δhpf positive drops was observed suggesting significant ribosome degradation. It was unclear whether non-replicating cells were still viable.

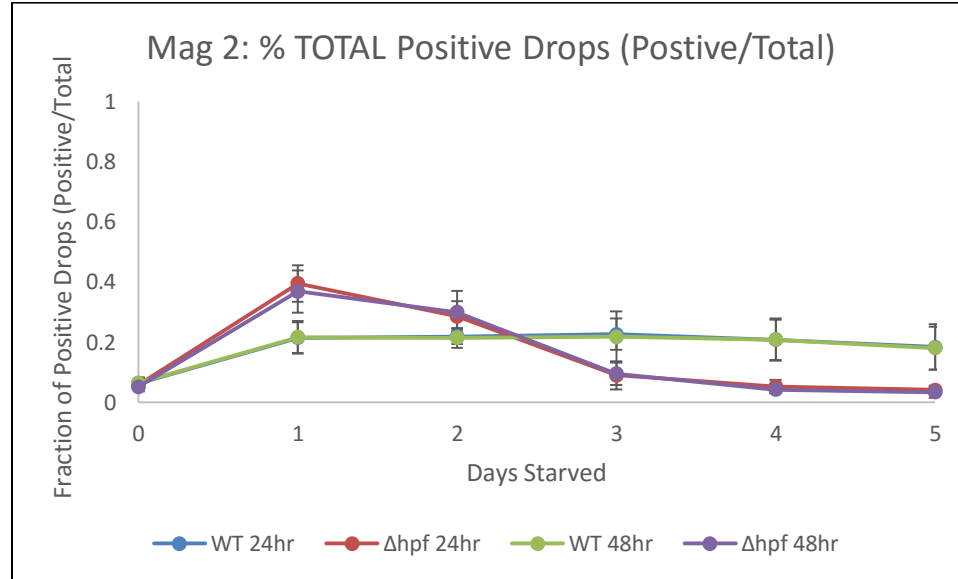


Figure 4-4. The fraction of total drops displaying positive fluorescence at each time point.

Cell Resuscitation: Image Analysis Experiment

A %BIO analysis was used to quantify the relationship between relative positive and single cell drop fractions (Figure 4-5). During each biological replicate, both cultures exhibited a high relative fraction of positive drops on days 0 and 1. This indicated that ribosome preservation and Hpf were not yet necessary for the maintenance of cell viability. However, a significant reduction in the relative fraction of Δhpf positive drops was observed during days 2-5 indicating a lack of ribosome preservation. Interestingly, a reduction in the fraction of wild type positive drops was also observed during days 2-5. Although this reduction was significantly less than what was observed in the mutant culture, it suggests that prolonged starvation can have an adverse effect on cell resuscitation even when Hpf is present. Despite this observation, this analysis could not conclude the viability of the wild type single cell drops. It was unclear whether these cells were still in the induced stationary phase, had experienced significant levels of ribosome

degradation and were awaiting resuscitation, or were no longer viable. Additionally, it is important to note that the increase in multiple cell encapsulation probability did not appear to influence the prevalence of single Δhpf drops at later starvation points.

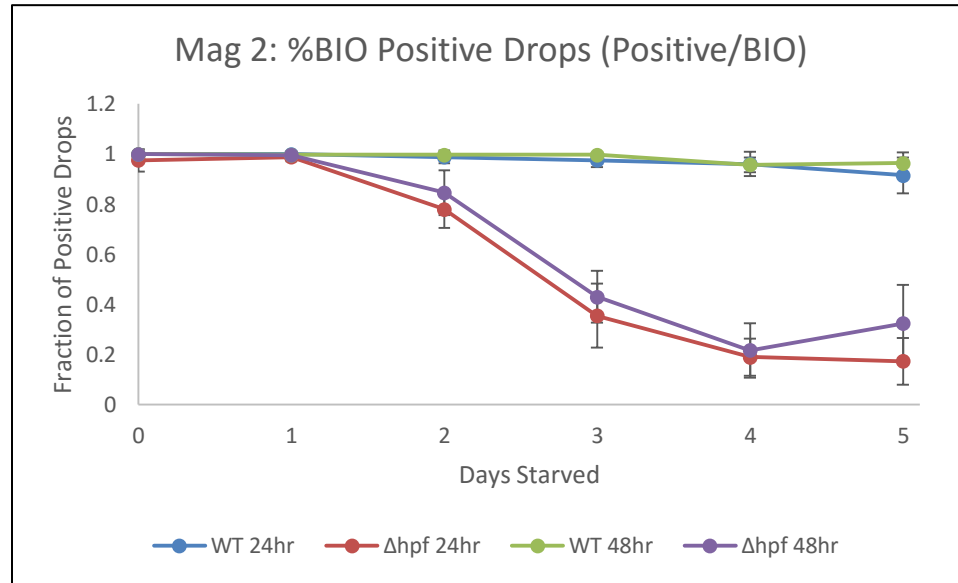


Figure 4-5. %BIO positive drops. Each curve is an average of the 3 biological replicates.

Using binary regression and considering all interactions between culture strain, days starved, and incubation time, the influence of each of the variables was found to be statistically significant. This means, for example, that there is a statistically significant difference between the WT and Δhpf %BIO positive drops when data points from each day and incubation time are averaged.

Of the two way interactions, only two indicated potential significance by producing F-values slightly greater than the critical F value, 3. These included the interaction between culture strain and days starved, and culture strain and incubation time. The interaction between days starved and incubation time, and the three-way interaction were not considered significant.

Of these interactions, the most interesting was the interaction between culture strain and incubation time, specifically the interaction between Δhpf and incubation time. A correlation between resuscitation and incubation time supports the notion of ribosome degradation during starvation and a threshold ribosome concentration for regrowth. To analyze this interaction, the data set was split so that only Δhpf and days 2-5 were considered (Figure 4-6). Days 0 and 1 were removed from this analysis because there was no indication of compromised regrowth at these time points. Using binary regression, the effect of incubation time was found to be statistically significant when averaged over days 2-5 of starvation. The associated P-value for this test was 9e-05. However, upon analyzing each day independently, it was found that the difference in resuscitation between incubation times was not statistically significant at day 4. This finding was visually supported by comparing the day 4 Δhpf points in Figure 4-6.

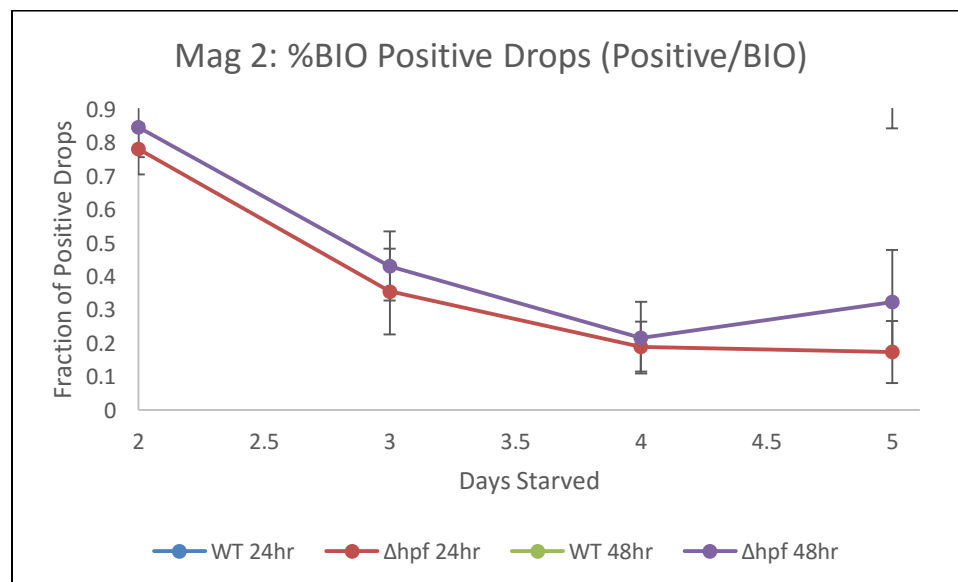


Figure 4-6. 24 and 48 hour comparison of Δhpf at days 2-5 of starvation.

Preliminary Detection: Day 4 Starvation

To determine the capability of ultra high-throughput drop detection for this application, a day 4 starvation, 24 hour incubation sample was run. The preliminary detection data is presented in histogram form. In this case, the x-axis is representative of 500-550 nm emission intensity.

TSB media, which exhibited negligible auto-fluorescence during preliminary detection (Figure 4-7), was used as a baseline. Detected signals were scarce and likely due to artifacts within the media. Notice that the scale on the y-axis has a maximum of 9, while the scales on the WT and Δhpf histograms are much larger.

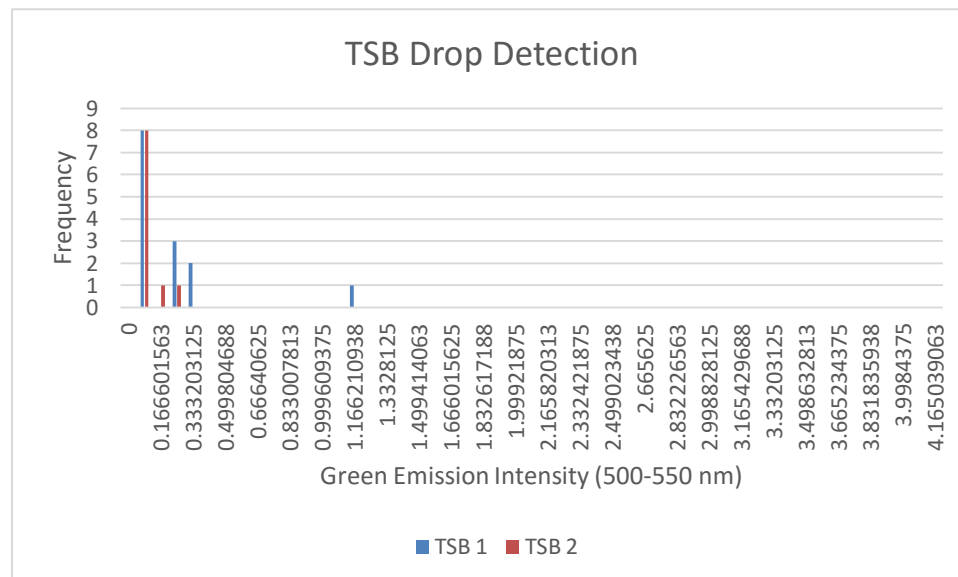


Figure 4-7. TSB baseline during preliminary drop detection.

Although exact quantification of positive and single cell drops requires further system calibration, the relationship between the day 4 starvation, 24 hour incubation PAO1:pMF230 and Δhpf cultures was consistent with what was found through image analysis. The wildtype exhibited a high fraction of positively fluorescing drops (Figure 4-

8), while the mutant did not. Conversely, the mutant exhibited a very high frequency of drops in bins 1-3 when compared to the wildtype.

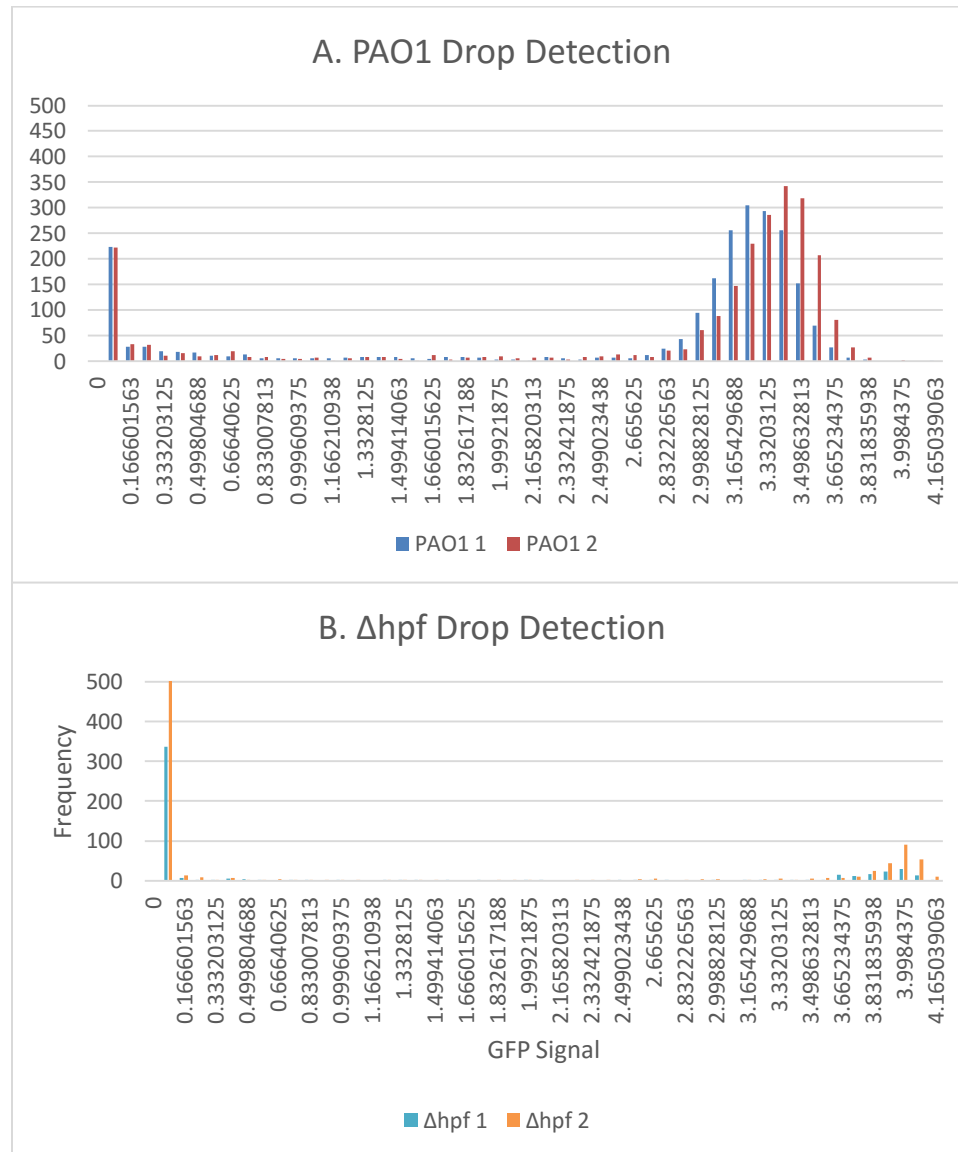


Figure 4-8. Day 4 starvation, 24 hour incubation (A.) wildtype and (B.) Δhpf preliminary detection data.

Due to the consistencies seen between the preliminary detection data and the image analysis data, a 6 day, 3 biological replicate detection experiment was performed.

Detection Experiment Data Filtration

During incubation, a fraction of the drops coalesce due to evaporation and dry surface interactions. These drops were visually excluded during image analysis; however, size discrimination during detection is more involved. Initially peak data is examined as a function of signal duration. Due to the finite volume of a given drop, there exists a maximum amount of time during which the fluorescent signal can exceed the defined threshold. This value is referred to as the “time inside.” Although points can be excluded based on this value within LabVIEW, they are often included to provide a more complete data set. Because the throughput associated with drop detection is so high, large data sets make it easy to determine the maximum time inside associated with the desired drop size, and to filter the data accordingly. Figure 4-9 demonstrates how this filtration process can be completed using time inside vs drop number and peak vs time inside plots. In Figure 4-9A, data points above the filter line are characterized by long time inside values. This indicates that the bacteria are dispersed within a larger than normal volume. These are drops that have coalesced during incubation. Similarly, points to the right of the filter line in Figure 4-9C represent the same phenomenon.

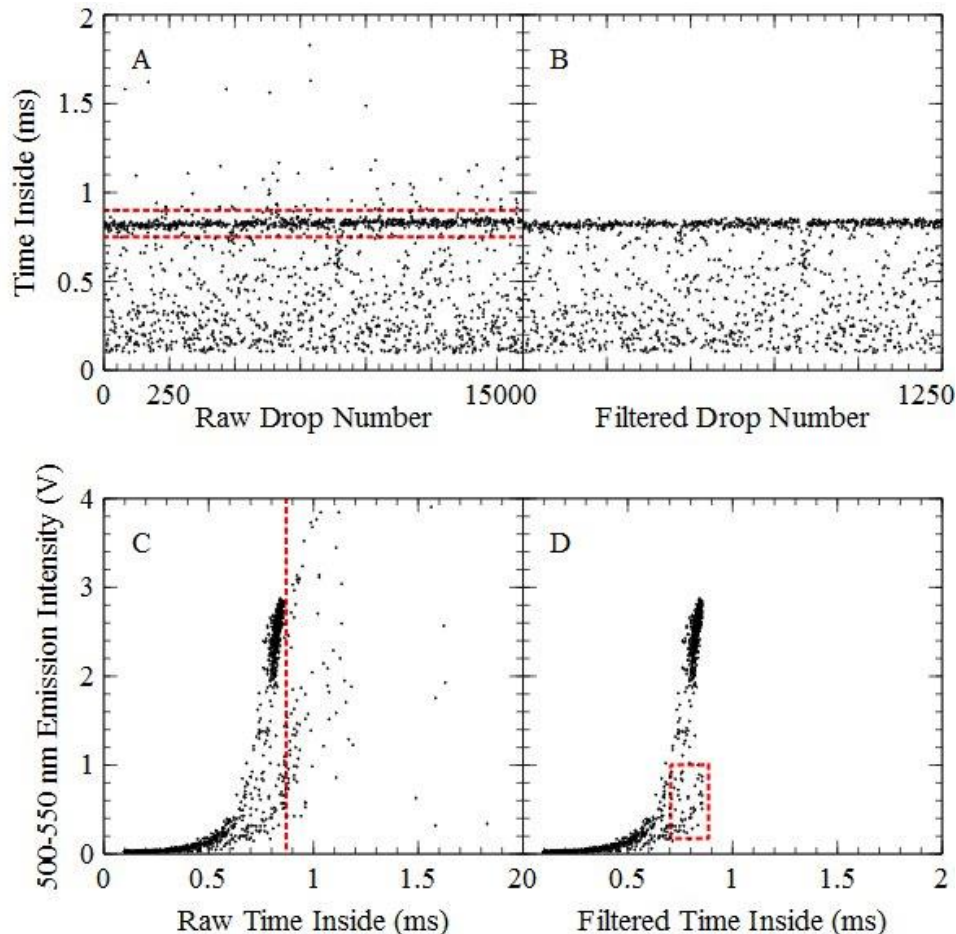


Figure 4-9. Detection data filtration process. A. The time inside vs. raw drop number plot displays each drop's signal duration in chronological order. The dark black band indicates positive 15 μm drops. B. The time inside vs. filtered drop number plot shows the removal of points greater than the maximum. C. Raw Peak vs. time inside. D. Filtered Peak vs. time inside. The dotted box indicates a region where data points may represent coalesced, low intensity drops.

While this approach is ideal for excluding high intensity, coalesced drops, it does not address low intensity, coalesced drops. Filtration of drops within the marked region of Figure 4-9D can be accomplished through in depth chronological drop analysis or by evaluating the data set against a user defined exponential fit. This type of filtration requires constant and consistent flows and is often easier to visualize on a log-normal plot

where the relationship between emission intensity and time inside appears linear for a constant drop size. Exponential fit filtration was not used for this portion of the study.

The fluorescent intensity is produced by the bacteria within the drop, and as a result not every drop produces the same intensity for the same amount of time. Assuming a constant flow rate, the filtered peak vs time inside data set takes on an exponential trend. Drops represented by the points at the bottom left of the figure contain single cells or low growth. The low intensity suggests that there is little Gfp present within the drop, and the low duration suggests that the bacteria do not take up the entire drop volume. As the number of bacteria within the drop increase, the intensity also increases, but more importantly, the duration reaches a maximum. At this point, an increase in bacteria concentration only results in an increase in the signal's peak. Plotting the data in this manner illuminates the portion of the population referred to as the "intermediates." This is the portion that are growing, but have exhibited delayed resuscitation. Although this could have been achieved via image analysis, the process is laborious and was not completed in this study. Despite this, the analyzed detection results are presented in bimodal fashion for ease of comparison with the previous experiment.

Approximating a Single Cell Intensity Range

To estimate an approximate intensity range for single cell drops, a single cell baseline was evaluated against a TSB baseline, which was not negligible due to adjustments in laser alignment. The results are presented in Figure 4-10, where the x-axis represents the 500-550 nm emission intensity in volts and the y-axis represents a relative frequency for that sample size. In this case, there is a marked increase in TSB associated

intensities from 0.02-0.035V. This increase is also present in the single cells samples because only 10-20% of the drops actually contained a cell. As the voltage increases from 0.04V and beyond, the proportion of detected single cell drops remains higher than that of the TSB drops. While definitive differentiation between TSB and single cell signals is difficult, the difference observed after 0.04V supports the detection of additional fluorescent material. For this study, an estimated range of 0.04-0.15V was used to define the presence of single cells.

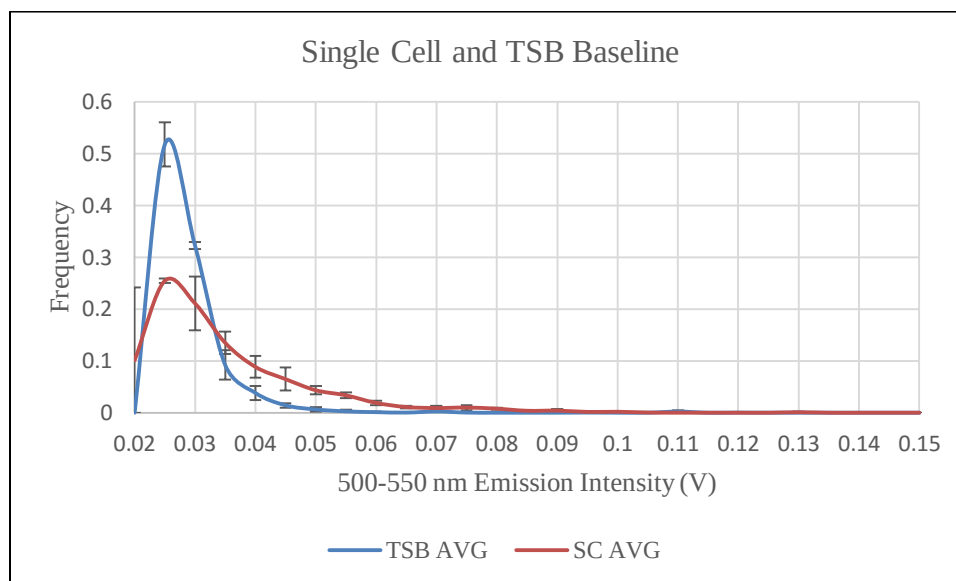


Figure 4-10. Selection of a fixed single cell intensity range was completed by comparing TSB and single cell drop baselines.

Cell Resuscitation: Detection Experiment

Due to the limitations in system calibration and single cell detection, an estimated %BIO analysis was used to quantify the relationship between detected single cell and positive drops. Although the *Δhpf* biological replicates used during detection exhibited a delayed reduction in resuscitation, the data obtained through detection also illustrate a

correlation between the presence of Hpf and cell viability maintenance during starvation (Figure 4-11). The delay in resuscitation reduction from day 2 to day 3 may be explained by an increased fitness level among the Δhpf community, a difference in culture preparation, or an increase in multiple cell encapsulation events. Additionally, the overall reduction was not as dramatic as it was in the image analysis replicates. The validity of this decreased reduction can be supported by the notion that the entire reduction have been shifted from day 2 to day 3.

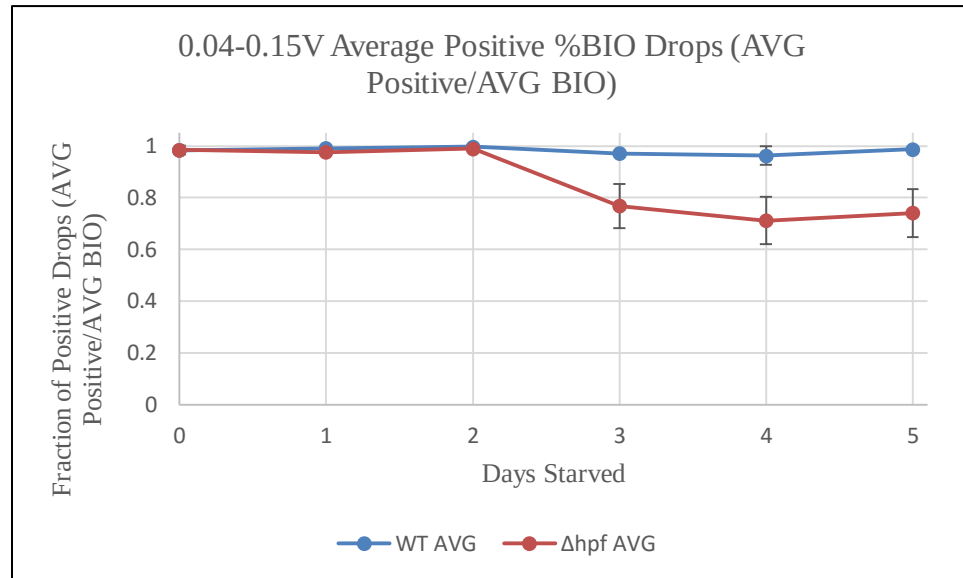


Figure 4-11. %BIO positive drops. Each curve is an average of the 3 biological replicates. Detection data was collected at 24 hours of incubation only.

One of the shortcomings associated with the detection results was the ineffective calibration method used. As a result, it is difficult to select a single cell intensity range as detection sensitivity can vary with the objective used, the focal plane, and slight movements in optical hardware. To mitigate these issues, the filtered data sets from each biological replicate were normalized against each sample's maximum observed intensity

(Figure 4-8). In this figure, each y-axis represents the proportion of the population characterized by a relative voltage range. Reduced Δhpf resuscitation during days 3-5 is represented by an increase in low voltage drop frequencies. Furthermore, this reduction appears to be more significant than what was reported using the fixed single cell range (0.04-0.15V).

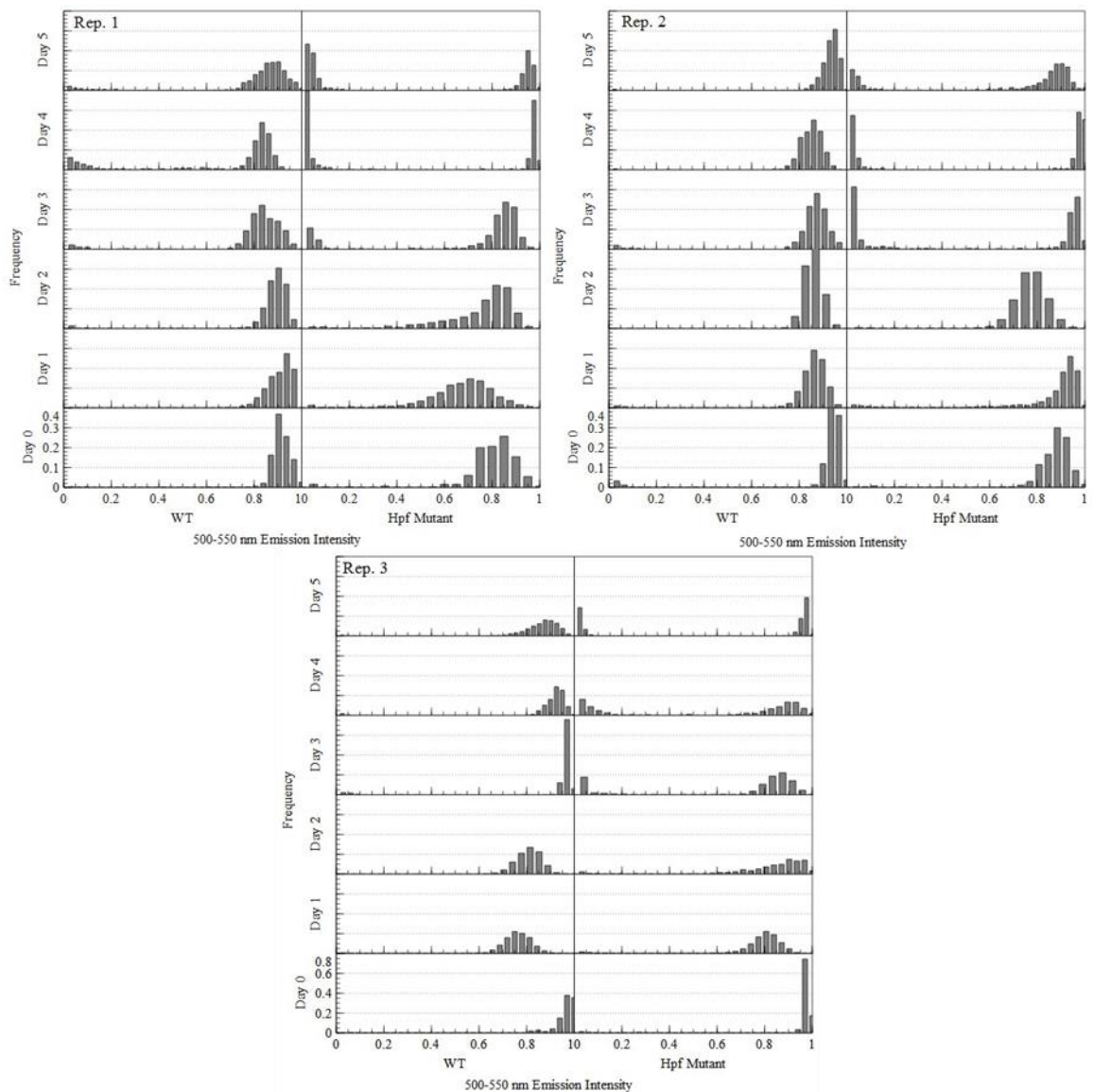


Figure 5. Drop intensity distributions. Each histogram contains only BIO signals and is normalized to the maximum intensity detected.

Conclusion

Single cell level resuscitation of starved cultures was not possible through conventional techniques as it was necessary to quantify individual non-replicating cells; however, this problem was resolved using drop microfluidics. Compartmentalization of single cells allowed each encapsulated cell the opportunity to propagate, while isolating each micro-reactor from the others. Image analysis demonstrated that an induced stationary phase was achieved in the wildtype culture, and that the lack of Hpf impacted the cells' ability to initiate this phase in the Δhpf culture. Furthermore, this study has also demonstrated a difference in the maintenance of cell viability between wildtype and *hpf* mutant cultures for starvation periods exceeding 1 day. Presumably, this difference is a direct result of ribosome degradation associated with the *hpf* mutation. This mechanism is supported by FISH and BioAnalyzer rRNA data gathered in conjunction with this experiment.

Similarly, ultra high-throughput fluorescent drop detection indicated a reduction in cell resuscitation among the Δhpf constituents. While the observed reduction was not as pronounced as what was observed through image analysis, the overall trend stood and the role of Hpf for ribosome preservation was supported.

The most important difference between these two means of data analysis was the amount of time required to gather and analyze the information. While extensive image analysis and visibly verified data are important for providing experimental proof of concept, extracting that information for 1000 total drops can take several hours. In comparison, drop detection has demonstrated the ability to effectively analyze thousands

of biologically relevant (not including empty) drops in a matter of minutes. In addition, drop detection does not require the aide of sophisticated microscopy equipment or image analysis software.

CHAPTER FIVE

ULTRA HIGH-THROUGHPUT MICROALGAE LIPID ACCUMULATION
ANALYSISIntroduction

Due to increasing atmospheric CO₂ levels, the effort to combat fossil fuels with sustainable fuel sources has accelerated in recent decades [35]. These efforts include a variety of plant derived ethanol and long chain hydrocarbon sources such as corn, soybean, canola, oil palm, and microalgae [36]. Among these, microalgae have shown the greatest promise in their ability to produce biodiesel in economically viable quantities. Some advantages microalgae provide are a high lipid to biomass ratio [37], low food source competition, efficient carbon fixation, short reproduction times, and the ability to be cultivated on low grade waters [38-42]. Figure 5-1 compares microalgae to other resources in terms of proposed land area required to meet 50% of the transportation fuel needs in the United States. In their study, Chisti et al. conservatively predict to accomplish this using a mere 2.5% of the country's existing cropping area [36]. While the potential for microalgae is exciting, many hurdles remain before it can become an economically viable resource. These include optimization of algal community constituents, growth, and lipid production, as well as dewatering and oil extraction processes, and scalable biodiesel production processes. This study focused on optimizing community constituents and species selection.

Crop	Oil yield (L/ha)	Land area needed (M ha) ^a	Percent of existing US cropping area ^a
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae ^b	136,900	2	1.1
Microalgae ^c	58,700	4.5	2.5

^a For meeting 50% of all transport fuel needs of the United States.

^b 70% oil (by wt) in biomass.

^c 30% oil (by wt) in biomass.

Figure 5-1. Estimation of biofuel land requirements [36].

Due to limitations in large scale cultivation capabilities, it's economically beneficial to grow algae on low grade waters and in mixed communities containing a consortia of algae, bacteria, and/or archaea. The interactions between these organisms has been a recent area of study and there is evidence to suggest a symbiotic relationship between certain algal and bacterial species [43-46]. Although the extent of these relationships is largely unknown, they are thought to involve potential metabolite exchange within the extra-polymeric region of the algal cell. This area is known as the “phycosphere” [47]. Separation and genetic identification of the high lipid producing algae would aide in the efforts to further understand these relationships and their potential roles in lipid production.

Although several methods exist for quantifying lipid production in bulk communities, identification and isolation of high lipid producing constituents has proven difficult. Using drop microfluidics and conventional lipid staining techniques, high lipid accumulating algae and their phycospheres show promise in their ability to be detected, quantified, selected for, and identified according to user defined thresholds. Furthermore, the drop platform leaves room for isolated regrowth and precise microenvironment

control allowing for characterization of environmental responses on a single cell level. This study aims to select for these organisms in two different communities. The first is a monoculture of green microalgae, and the second is a mixed culture cultivated on municipal waste water.

Algal Lipid Detection and Drop Sorting

The information presented in this section is largely the development of a repeatable and reliable drop based lipid detection and sorting method. As a result, the term “microalgae” will be used to encompass the previously mentioned mixed and monoculture populations.

Lipid Fluorescence and Detection/Sorting Scheme

Fluorescent quantification of algal lipid content has two potential modes. The first is through chlorophyll a and b fluorescence and the second is triglyceride staining Nile Red, which is currently the gold standard for algal lipid staining. Figure 5-2 displays both modes simultaneously. When excited by blue (473nm) laser light, the chlorophyll b within the green algae will emit a red signal, while the chlorophyll a emits a red signal when excited by 405 nm light [48]. This is useful as there is often an inverse relationship between accumulated lipid and cellular chlorophyll concentration. Furthermore, the emission spectra for both forms of chlorophyll begin around 600 nm and are detectable given the current equipment (PMT 2, >555 nm). Detection of Nile Red is more challenging. 473 nm light is approximately 57% of the maximum intensity excitation wavelength and the emission spectrum does not begin until 517 nm (ThermoFisher

Scientific). The detection bandwidth of PMT 1 ranges from 500-550 nm and the maximum relative intensity reached at that range is approximately 30%. Meanwhile, detection by PMT 2 begins at 555 nm resulting in a significant amount of Nile Red PMT 2 contributions. While this is not detrimental, a drastically reduced Nile Red signal can be expected due to the low excitation intensity and small detectable bandwidth. Additionally, falsely high chlorophyll intensities can be expected due to the crossover between each emission spectra.

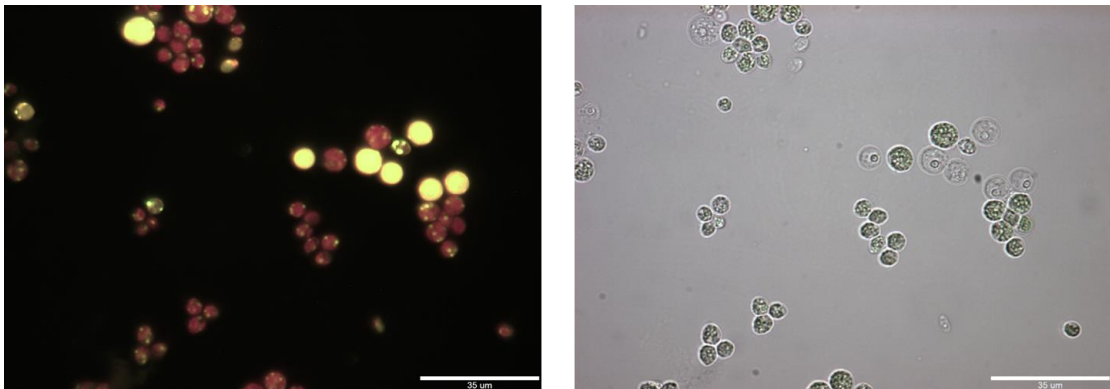


Figure 5-2. The left image displays the illumination of accumulated lipid within an algae cell via Nile Red. The right image displays the inverse relationship between chlorophyll and lipid concentrations. Cells that appear clear contain a high fraction of accumulated lipid (Image by Luisa Corredor Arias).

Logistically, detection via Nile Red is favored because, though the detectable signal is faint, it is not compromised by other sources. However, Nile Red also has poor stability over extended periods of time and its rapid bleaching is an obstacle for this mode of detection.

Ideally drop evaluation would be based off of both red and green emission signals. In doing so, three theoretical populations arise (Figure 5-3). The first are those emitting low green signals and high red signals. This population is representative of low

lipid producing cells. The second are those emitting high green signals and high red signals. This population is representative of drops likely receiving multiple cells during encapsulation. This population may be valuable for characterizing constituents closely associated with lipid production, but may not be valuable if culture enrichment is the final goal. Last, the third population are those emitting high green signals and low red signals. These drops are representative of cells containing high lipid and low chlorophyll content.

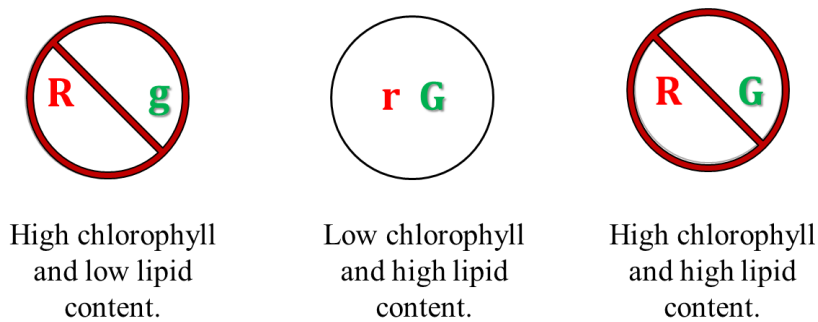


Figure 5-3. Illustration of the expected microalgae drop populations.

Due to crossover in emission spectra, the desired drop population has changed from a high PMT 1 and low PMT 2 signals to high signals for both PMT 1 and 2. As previously stated, the high PMT 2 signals indicate the presence of a cell and the high PMT 1 signals indicate a proportional Nile Red concentration. Quantification of chlorophyll contributions requires some form of calibration, which was not performed in this preliminary study.

Alternatively, a smaller red tail could also be achieved using more appropriate optical filters (Figure 5-4). Increasing the bandwidth of PMT 1 to 500-600 nm and

increasing the edge of PMT 2's long pass to >605 nm would remove the most intense portions of the Nile Red emission spectrum from the PMT 2 signals. Furthermore, these changes would also allow the most intense Nile Red emission wavelengths to be detected by PMT 1. In this case, it would still be best to use the PMT 2 signal strictly as a means of discriminating against empty drops.

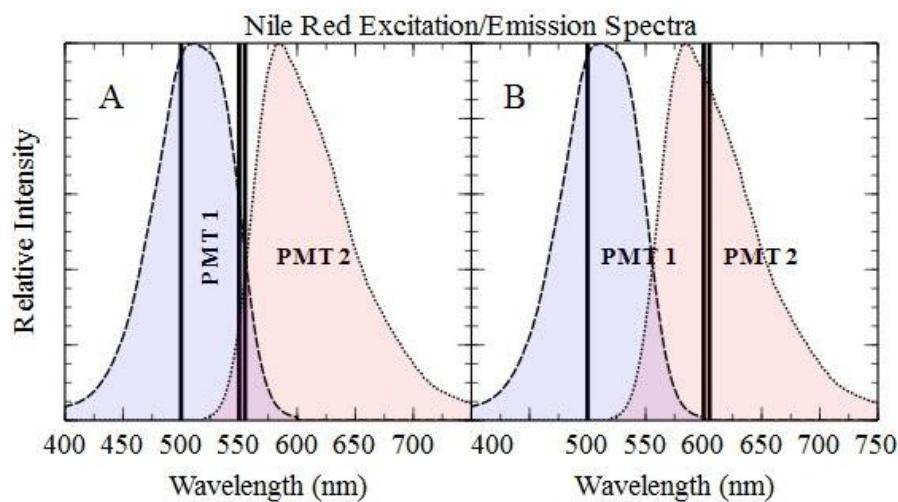


Figure 5-4. ThermoFisher Scientific. The excitation/emission spectrum for TAG staining Nile Red.

Proof of Concept: Nile Red Presence and Preservation

Prior to performing high-throughput fluorescent detection, a sample of stained algae cells were encapsulated and imaged via confocal microscopy. Imaging the drop contents helps to validate the peaks produced during detection. This is particularly important in this case as Nile Red has a short lifespan. These images indicated successful single-cell encapsulation as well as Nile Red presence and preservation during the drop making and storage periods.

Cell Encapsulation and Drop Detection Methods

Initially there were two concerns surrounding drop based algae interrogation. The first was the time scale for which Nile Red remains fluorescent and the second was the rate of lipid consumption within dark environments. Since the workflow for drop based experiments involves making, storing, and re-injecting drops, detection experiments can last for hours depending on the volumes required. To combat these concerns, two approaches to drop detection were proposed: two-step and single-step.

Two-Step Method. The first approach was a two-step approach. In this case, stained cells were encapsulated using a flow focusing device, temporarily stored, and detected using a drop sorting device. Syringes containing the stained cultures and drop populations were wrapped in aluminum foil and a non-transparent tubing was used to transport fluids to and from each microfluidic device. Since microalgae cells range in size from 10-15 μm in diameter, they were encapsulated in 50 μm drops. This size is typically used as a default as the drops are small enough to remain stable over time, but the associated channels are large enough to resist clogging.

Single-Step Method. The second approach was a single-step approach. In this case, stained cultures were encapsulated using the separation junction within the drop sorting device. This intersection is larger than what is seen in a typical drop making device, and the produced drops are also larger. However, formed drops are monodisperse, and the time between drop formation and detection/sorting is drastically reduced when compared to the two-step method. Additionally, the flow rates used for traditional drop

formation are ineffective in this situation as the spacing between drops is not large enough to prevent interference at the sorting junction. When using this method, the spacing flow rate was a minimum of 10 times that of the dispersed phase, and the rate of drop formation did not exceed 100 Hz. The actual flow rates used in a 50 μm system were based off of those presented by Mazutis et al.

Culture Mixing. In each case, the algae culture must be adequately mixed during the injection process. To accomplish this, a small magnetic stir bar was placed inside the syringe containing the algae culture. Using a small electronic motor (Frigelli Automation), the stir bar maintains some level of homogeneity within the culture reservoir. Successful stirring requires a minimum of 0.5 mL of solution at all times. Below this, the stir bar does not have enough space to spin freely.

Algal Clumping and Device Clogging. Clumping among algal communities has become the largest barrier to successful drop detection and sorting. Currently this issue is being combated through the use of low cell densities. However, at the cell concentrations necessary for adequate encapsulation rates, large aggregates of cells are blocked by the device's filters and in most cases restrict flow completely. This issue could potentially be resolved by removing the filters, however the likelihood of encapsulating more than one cell is increased. Furthermore, removing the filters requires additional device design and fabrication.

Mixed Community Preliminary Results

Preliminary mixed community detection work requires further analysis. Peak data provided by PMT 2 can only be used as a binary means of cell detection until the system is calibrated to accommodate Nile Red contributions. Current PMT 2 peak data contain an undecipherable difference between drops containing high chlorophyll and drops containing high Nile Red. Because >555 nm peaks are likely indicative of cell presence, simultaneous low magnitude 500-550 nm Nile Red signals may be used to determine lipid accumulation in the drop.

Example results outlined by Figure 5-5 were taken from mixed community samples and indicate sample chlorophyll and Nile Red raw emission intensity distributions triggered by both PMT 1 and PMT 2 thresholds. In this case, the desired low chlorophyll and high Nile Red population was not present in the raw data, as expected. However, the upper right quadrant represents cells producing high 500-550 nm fluorescence, and may represent high lipid accumulation.

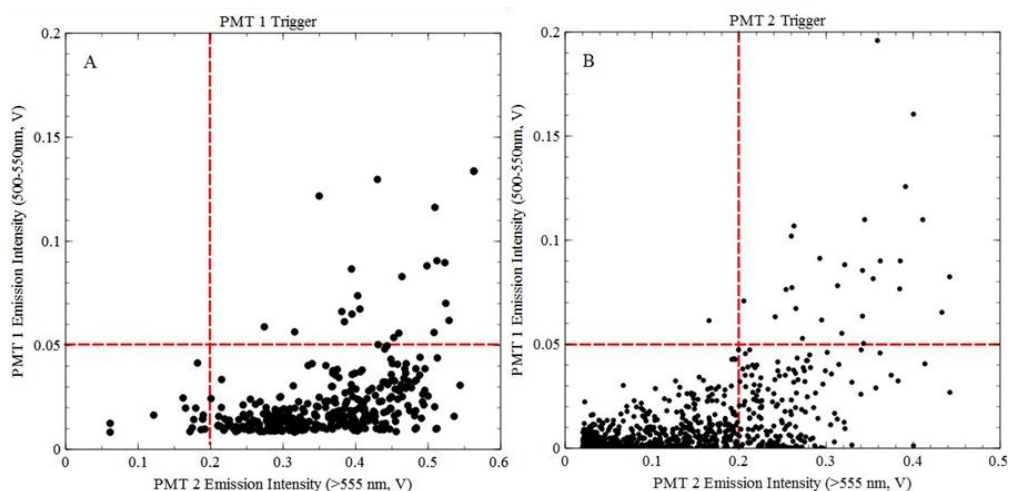


Figure 5-5. PMT 1 vs PMT 2 Triggered Preliminary Algae Detection Results.

In Figure 5-5A detection was triggered off of a 0.05 ms 0.02 V PMT 1 signal intensity. PMT 2 peaks present at this time were simultaneously recorded. Similarly, Figure 5-5B represents detection triggered off of a 0.1 ms and 0.008 V minimum PMT 2 intensity. PMT 1 peaks present during this time were simultaneously recorded. For future experiments of this nature triggering off of PMT 2 signals is recommended as this signal consistently has higher magnitudes and will provide the maximum amount of Nile Red information.

Future Work

Future work will involve continued development and optimization of the detection process including system calibration and optical hardware improvements. It will also include efforts to resolve algal clumping and device clogging. Finally, limitations associated with sorted fraction volumes will be addressed to aide in the ability to conduct enrichment and constituent characterization studies.

CHAPTER SIX

SINGLE CELL MICROBIALLY INDUCED CALCITE PRECIPITATION

Introduction

Microbially induced calcite precipitation (MICP) leverages the ureolytic activity found among microbial species' for the production of calcium carbonate [49]. This phenomenon can be used to aid in environmental remediation efforts [50-54] where conventional techniques are ineffective. Examples include repairs associated with subsurface fractures in the oil and gas industry [55], CO₂ sequestration [56, 57], and subsurface barriers. In addition, MICP can also be used for enhancing and repairing construction materials [58].

Although the process has proven effective at industrially relevant scales, there is ample room for improvements in efficiency and optimization at the micrometer scale. Using current drop manipulation techniques, drop microfluidics offers a platform for optimizing the MICP process at the single cell level. A functional system would allow researchers to quantify relationships between the organisms and mineral precipitation potentially reducing operation costs and increasing reaction efficiency.

MICP Process

The bacteria used for this study was *Escherichia coli* MJK2. This strain of *E. coli* was developed by Connolly et al. to express Gfp and ureolytic properties [59]. The calcite precipitation process, as it applies to this organism, is outlined in Figure 6-1 and begins

when the culture is exposed to urea [60]. Facilitated by the enzyme urease, urea hydrolysis causes an increase in system pH and the subsequent addition of calcium results in calcium carbonate precipitation.

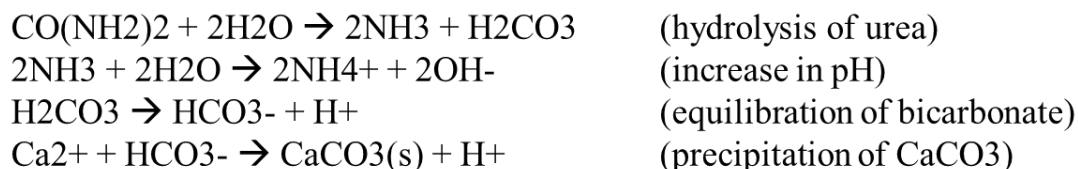


Figure 6-1. Annotated MICP reaction [60].

Methods and Preliminary Results

Single Cell Encapsulation and Reagent Injection

To begin, cell cultures were encapsulated in 50 μm drops such that there was no more than one cell per drop. This culture concentration was verified using confocal microscopy (Figure 6-2). The presence of Gfp makes bacteria makes bacteria quantification easy even at the single cell level, and in the image below, single cells are present in approximately 60% of the drops. Ensuring single cell distribution was particularly important for this study as there was speculation surrounding the ability to observe precipitation from single cells.

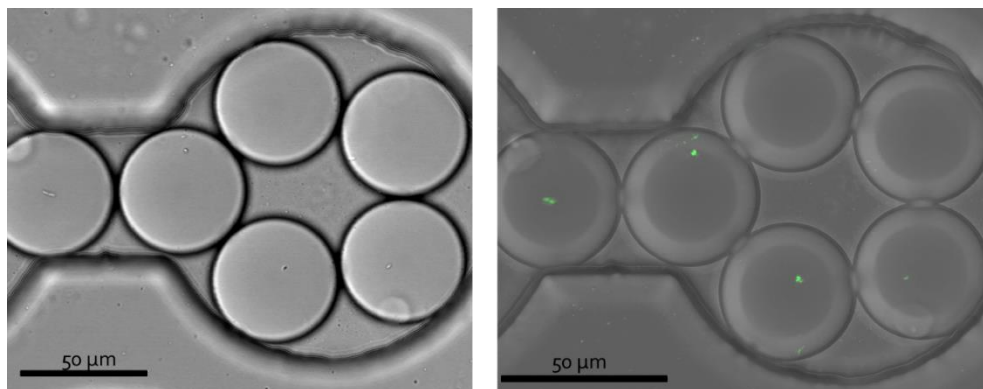


Figure 6-2. Single cell *E. coli* MJK2 encapsulation in 50 µm drops.

Next, the MICP process was tested by combining the cell culture and calcium/urea solutions in drops. Initially, the cell culture and reagent mixture were co-flowed in a flow focusing device (Figure 6-3). In this workflow, the drop maker contains two intersections. At the first intersection the cell culture is mixed with the calcium/urea mixture and the relative proportions are controlled by each fluid's respective flow rate. At the second intersection the culture/reagent mixture is encapsulated, forming monodisperse drops. However, due to excessive precipitation rates and the associated micrometer scale flows, calcite precipitation occurred prior to encapsulation blocking the first intersection.

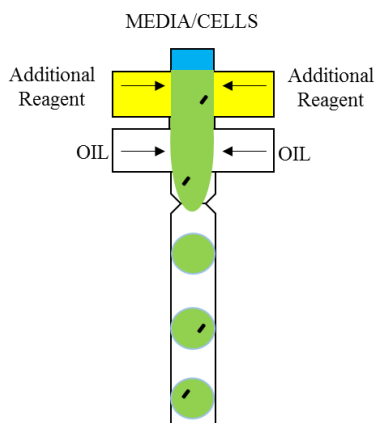


Figure 6-3. Illustration of the co-flow, flow focusing process.

This issue was resolved via pico-injection (Figure 6-4). This is a two-step process in which the bacteria were first encapsulated using a 50 μm flow focusing device and stored off-chip until drop production was complete. Following production, the drop population was reinjected into a pico-injection chip where the reagent mixture is actively added to existing drops. Although there is also a risk for precipitation outside of the drop with this approach, it was not observed during the trials performed. As a result, pico-injection is the preferred method for this type of precipitation study.

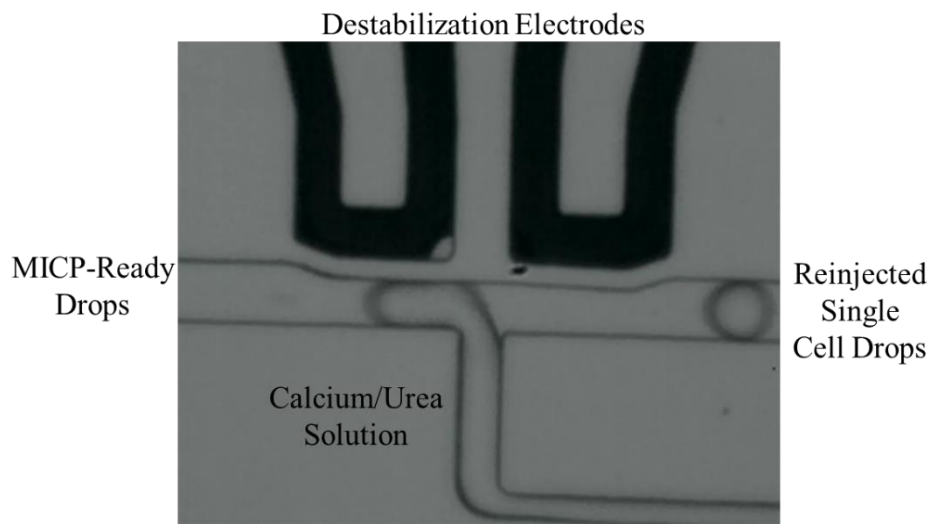


Figure 6-4. Microscopy image outlining the MICP pico-injection workflow.

Drop Imaging and Calcite Identification

MICP-ready drops were immobilized and imaged at a later time point. The drops displayed in Figure 6-5 were imaged after 3 days of incubation. The resulting structures suggested both bacterial growth, as indicated by the increase in Gfp, and the presence of a mineral precipitate, as indicated by the matrix-like scaffold.

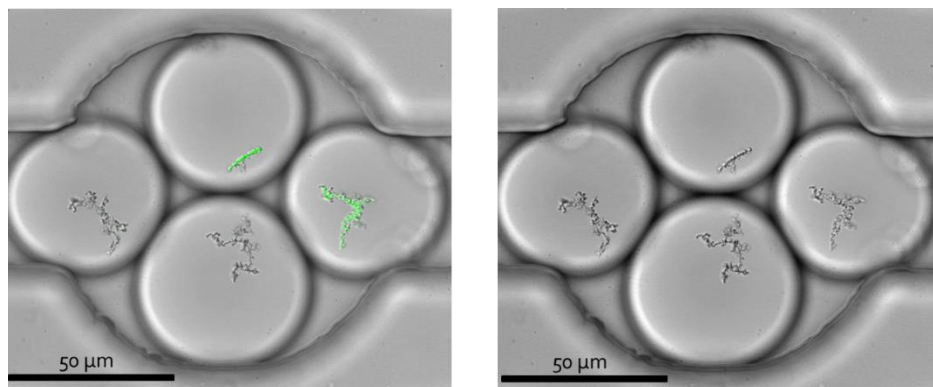


Figure 6. MICP progress after 3 days of incubation.

Precipitate formation was verified using confocal microscopy. Within the Leica confocal software suite is a reflection setting that can be used to capture light reflected off of solid surfaces (i.e. the mineral precipitate). The images taken during this analysis can be found in Figure 6-6. The left image was taken using reflection mode. In this case the white regions represent light that was reflected off of the mineral's surface. The right image is provided as confirmation that similar structures were observed.

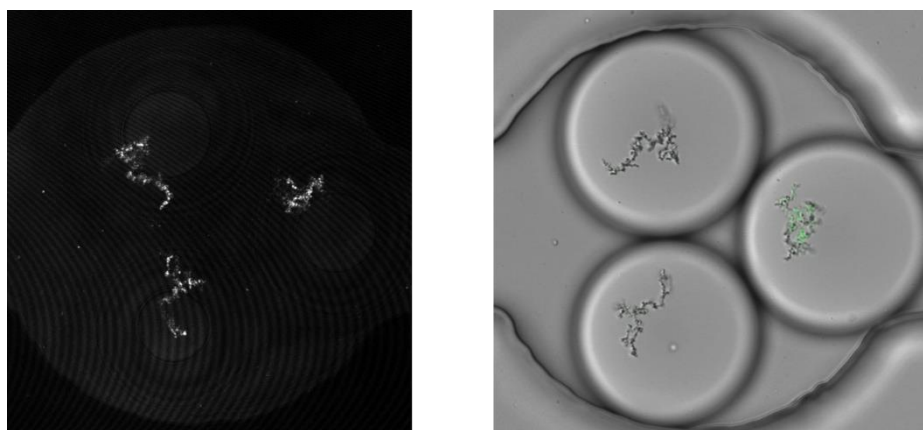


Figure 7. Confocal microscopy reflection mode images indicating the presence of mineral precipitate.

Despite these findings, it is unclear whether the precipitate formed was calcite, another form of calcium carbonate, or another mineral entirely. To verify the presence of calcite, x-ray diffraction analysis is required. This analysis is currently underway.

Future Work

Future work with this system will likely involve similar methods, however will seek to determine biological and precipitation responses to variations in reagent concentrations, reagent volumes, and bacteria concentrations. Furthermore, future studies will likely examine the composition of the precipitate using XRD or similar technology.

CHAPTER SEVEN

USING DROP MICROFLUIDICS TO QUANTIFY HUMAN NOROVIRUS
INFECTIVITYIntroduction

Rapid evolution and cellular tropism have made in vitro cultivation of noroviruses a challenge among research groups [61-66]. However, a recent study has developed a platform for in vitro human norovirus (HuNoV) cultivation in B cells [67]. In their study, Jones et al. realized the effect of enteric bacteria, particularly those expressing histo-blood group antigens [68-72] on B cell infection rates and leveraged the presence of these bacteria to increase HuNoV titers.

The current study sought to adapt the platform presented by Jones et al. for drop based single cell infectivity studies. Not only does drop compartmentalization allow for precise control over single cell environments, it also provides a means of performing directed evolution in virus populations [27]. These types of studies have the potential to better characterize and control the HuNoV cellular tropism that has long eluded the research community.

Methods and MaterialsBJAB Cell Culturing and Maintenance

The host cell used for drop based experiments was the B cell, BJAB. Culturing of this cell line was completed using instructions provided by collaborators in the Wobus

Lab at the University of Michigan. In addition, frozen BJAB stocks were also provided by the Wobus lab. Detailed passaging instructions are outlined below.

Media. There are two preparations of RPMI media used for BJAB culturing. The first is RPMI (50%), which contains RPMI (Fisher Scientific cat# MT10040CM) (use RPMI with L-glutamine. Add only FBS and Pen/Strep), 50% FBS, and 1x Pen/strep. The second is RPMI (10%), which contains RPMI, 10% FBS, and 1x Pen/strep. The Wobus Lab also recommended not adding BME to the either media.

Frozen Stock. Remove a vial of cells from LN2 freezer and thaw in a 37°C water bath until cells are just thawed. Add cells to 9 ml of RPMI (10%) and centrifuge at 1100rpm for 10 min. Aspirate medium and resuspend the pellet in 1 ml of RPMI (50%). Remove 10 µl to determine cell concentration. Seed the cells into two wells of a 6-well plate at a density of $5 - 7 \times 10^5$ cells per well. Add RPMI (50%) to bring the final volume to 2 ml. Incubate the plate at 37°C.

First Passage. Begin checking the culture 3 days after initial plating. A growing, healthy culture will begin to form clumps of cells. Initially, smaller clumps (10-20 cells) will be seen, but after 1-2 days of additional incubation at 37°C, larger clumps (40+ cells) should begin to form. When the culture concentration reaches $1 - 2 \times 10^6$ cells/ml, it can be split. Seed 5×10^5 cells per well of a 6-well plate. Add additional RPMI (10%) to bring the final volume to 2 ml. Incubate the plate at 37°C. At this point, begin expanding the culture into as many wells as possible.

Subsequent Passaging. It generally takes 3-4 days for a culture to reach $1-2 \times 10^6$ cells/ml. These cells do not do well being passaged too frequently and it is best to wait at least 4 days to split. BJABs are also sensitive to both high and low cell densities. They should not be seeded at densities less than 1×10^5 in 6 or 12 well plates and should not be kept at densities higher than 4×10^6 for extended periods of time.

BJAB Infection

BJAB infection was completed in bulk using an MOI of 100. To begin, a 6 ml BJAB culture was prepared containing a cell density of approximately $0.6-0.8 \times 10^6$ cells/ml. Using a 24 well plate, each of 6 wells were seeded with 165 μ l of the BJAB sample. A schematic of the sample distribution is provided in Table 7-1. Wells A-D were dedicated to drop based examination, while wells E-H were used as a bulk comparison.

	24 hours	72 hours	120 hours	Mock
Drops	A	B	C	D
Bulk	E	F	G	H

Table 7-1. Well plate labeling scheme. Wells A-D were used for drop studies, while wells E-H were used for bulk comparisons.

The virus sample was prepared by adding 3 unfiltered 20 μ l HuNoV samples to 940 μ l of 10% RPMI media. The HuNoV samples were provided by the Wobus Lab and contained an estimated 6×10^7 copies/ml. Once prepared, each well was inoculated with 165 μ l of virus solution (MOI 100). The mock wells were inoculated with 165 μ l of 10%

RPMI media. The inoculated well plate was incubated at 37 °C for 2 hours during infection.

Cell Encapsulation and Well Plate Preparation

After infection incubation, wells A-H were transferred into respective 1.7 ml microcentrifuge tubes. Using a table-top microcentrifuge, each solution was centrifuged in the biosafety cabinet until a pellet was formed. The supernatant was removed and each pellet was resuspended in 300 µl of 10% RPMI media. Once completed, the resuspended solutions from wells A-C were combined and set aside for encapsulation. The solution from well D was also set aside for encapsulation. The solutions from wells E-H were reseeded in a 24 well plate and brought to 2 ml by adding 1700 µl of 10% RPMI media. The regrowth conditions used for the bulk study are the same as those used for cell culturing. Reseeded bulk samples were incubated at 37 °C.

The needle/tubing assembly was prepared by cutting the tip of the plastic needle cover, inserting the needle into the tubing, and re-stringing the needle cover onto the needle/tubing assembly. Once complete, the encapsulation sample syringe (HSW 3 mL) was prepared by attaching the needle assembly to the sample syringe. The described assembly protects users against accidental needle punctures; however, it is important that this portion is completed prior to introducing the cell/virus solution. This process was repeated anytime a syringe was used.

The 900 µl A-C sample combination was pulled into the sample syringe and the end of the tubing was disinfected with 70% ethanol. The cell culture and HFE/surfactant mixture were interfaced with a 100 µm drop making device. Drops were produced using

flow rates of 2000 $\mu\text{l/hr}$ and 500 $\mu\text{l/hr}$ for the oil and culture phases, respectively. After flow equilibration, drop production was performed for 30 minutes/sample yielding approximately 250 μl of drops and 1000 μl of oil in each tube labeled A-C. Due to the available MOI and subsequent cell concentration used, it was estimated that approximately 17% of the drops contained 1 cell. Using a separate syringe, a similar process was completed for the sample from well D. Each of the prepared drops samples were incubated at 37 °C.

Post-incubation Sample Preparation

Incubated drop and bulk samples were removed at 24 (A and E), 72 (B and F) and 120 hours (C, D, G, and H). After incubation, the drop populations were broken by adding 10% perfluorooctanol by total volume and centrifuging until the two phases had completely separated. The supernatant was removed and transferred to a separate microcentrifuge tube. Because cells are more likely to exist at the oil/water interface, a small amount of oil was also included in the transferred sample. Additionally, 150 μl of bulk sample were transferred into a separated microcentrifuge tube. The collected samples were frozen at -80 °C until shipping.

Disinfection

HuNoV is highly contagious and causes acute gastroenteritis [73-77]. As a result, proper disinfection is important. In this case, a combination of UV, bleach, and 70% ethanol were used as disinfectants as recommended by our collaborators in the Wobus Lab. Following the removal of biological material, the biosafety cabinet was illuminated

with UV light for 10-15 minutes, after which each piece of equipment and the biosafety hood were wiped with a Clorox healthcare germicidal bleach wipes (VWR, 102877-772). To preserve the stainless steel within the biosafety cabinet, it is recommended that any and all bleach not be left to dry. All bleach was finally wiped clean using 70% ethanol.

Results and Discussion

Sample analysis for experiment requires RT-PCR completed by the Wobus Lab. This analysis was not completed prior to this review and the results will not be included. However, there was one key shortcoming in the experimental preparation and procedure. This was a lack of virus samples. MOI is the governing variable for each of the concentrations and volumes used throughout this experiment, and MOI is directly related to the number of virus copies initially available. The MOI in this experiment (100) did not allow a culture volume sufficient enough to accommodate mixing by stir bar, which may have resulted in a decreased encapsulation rate for later time points. Increasing the MOI for this experiment would allow for more cells and higher culture volumes during encapsulation.

Future Work

In their study, Jones et al. concluded that the presence of histo-blood group antigens promoted HuNoV infection of B cells. Although the samples used in this experiment were unfiltered stool samples, it may be beneficial to inoculate the infection

wells with a known concentration of *Enterobacter cloacae* [67] as an adequate concentration may not exist in the stool samples provided.

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