

Engineering Platforms for Advancing Plant Synthetic Biology

**A Dissertation Presented for the
Doctor of Philosophy
Degree**

The University of Tennessee, Knoxville

Tayler Marie Schimel McNeillie

December 2020

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DEDICATION

To my parents

Cindy and Brian

my sister

Kylee

and my partner

Brannon

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my advisor, Dr. Scott Lenaghan for his years of patient guidance and advice. Scott has been a great role model and mentor and he has positively influenced the way I design experiments, communicate scientific research, and how I manage research teams. I have learned a tremendous amount from Scott and will forever be grateful to him for encouraging my goals.

Dr. Andy Sarles has been instrumental in my work and deserves many thanks for his insights. I would like to thank Dr. Neal Stewart for his valuable input. I would also like to acknowledge the financial support offered through the Defense Advanced Research Projects Agency (DARPA) Advanced Plant Technologies (APT) program.

I will treasure my time in the Center for Agricultural Synthetic Biology for the colleagues I've had the good fortune to learn from and the lifelong friends I've made. I want to thank Mary-Anne Nguyen in particular, for the countless hours of conversation and deliberation, for always sharing snacks, and for her constant support and friendship.

ABSTRACT

This work describes research aimed at adapting advanced engineering systems for plant biotechnology. The droplet interface bilayer (DIB) is a robust and versatile platform for replicating model cell membranes, providing a bottom-up approach for synthetic cell- and tissue-like structures. In this work, a microfluidic device featuring five inlets, one for the continuous oil phase and four discrete aqueous channels for droplet generation was designed. Droplet production rates were controlled by adjusting the applied pressure of each inlet; and thus, altering the droplet sequence for capturing linear DIB networks in a downstream hydrodynamic trapping array. This microfluidic system provides a high-throughput method for generating DIB networks of complex droplet patterning for the purpose of studying higher-order combinatorial networks and signal cascades. Further, this work characterizes pressure-driven flow sensitivity to changes in microchannel resistance and differences in hydrodynamic trapping behavior compared to mechanically-driven flow.

In addition, this research work developed a custom laser system to image plant canopies expressing a wide variety of fluorescent protein genes in leaves for the purpose of phenotyping transgenic plants expressing multiple constitutive or inducible fluorescent proteins. The fluorescence-inducing laser projector (FILP) system is a critical instrument for accessing the efficacy of environmental phytosensors, which are plants engineered to detect and report environmental stimuli.

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CHAPTER I INTRODUCTION

1.1 Microfluidics in biotechnology

Microfluidics has been applied to numerous fields encompassing the fundamental sciences, with applications to cell and molecular biology of particular interest. Specifically, the application of microfluidics to plant biotechnology has significant potential for advancing our current understanding of plant cell biology, developing advanced tools for bioengineering, and even enabling on-chip organogenesis. Traditional methods for plant tissue culture and molecular screening rely on the use of bulk tissue, with limitations to the number of samples, available space, time, and costs associated with “high-throughput” screening. Typically, construct screening and phenomics in plant science use the plant or callus tissue as the primary unit cell. In these cases, populations of cells still serve as the minimal measurable unit, ignoring the variability of individual cells. By combining microfluidics and synthetic biology the speed at which constructs can be screened can be rapidly accelerated, along with enabling assessment at the individual cell level (Figure 1.1). Considering the pluripotency of plant cells, and the ability to regenerate entire plants from a single cell, the effect on a cell-by-cell basis is critical. In the near-term, the greatest advantage that microfluidics has to offer plant science is a platform for high-throughput single cell engineering and analysis. In order to prototype and validate microfluidic designs for use in plant biotechnology, droplet-based systems were first designed, built, and tested to discern guiding principles.

1.2 Droplet-based microfluidics

In numerous studies, droplets have been used as proxies for cells, to enable evaluation of microfluidic designs, and to assess fundamental aspects of microfluidics. In general, droplets are formed within a microfluidic device by three methods: cross-flow, co-flow, and flow focusing. In this proposal we will focus on cross-flow, more specifically a T-junction geometry. A T-junction is formed by the

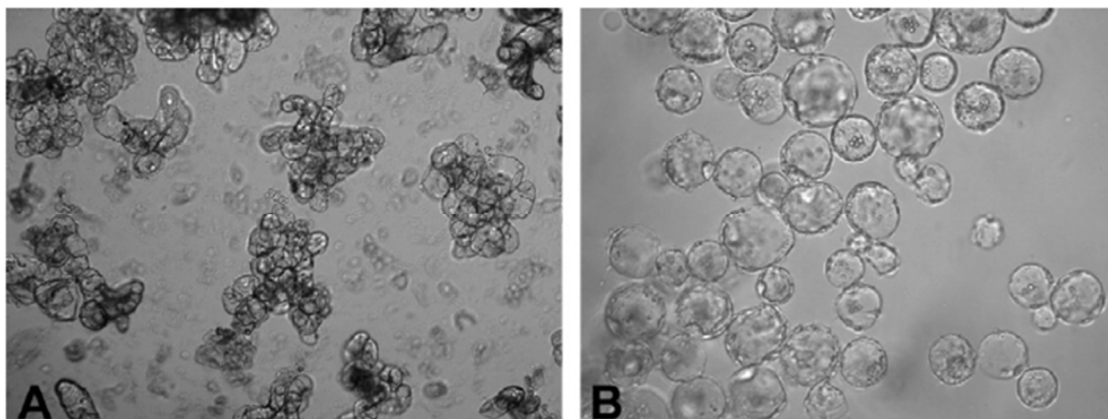


Figure 1.1 Image of switchgrass cell suspension culture (A) and isolated protoplasts (B).

intersection of two orthogonal channels, the main channel is the continuous phase carrier fluid and the side channel is the dispersed phase, which breaks into droplets. Droplets of differing composition can be produced by designing two opposing T-junctions, allowing for increased complexity of the system. Droplet size can be controlled by channel geometry, applied pressures or flow rates, capillary and Reynolds numbers, and the ratios of viscosity and density of the two phases. Once generated, droplets are carried downstream within the device often for sorting or fluorescence screening [9, 10]. This represents the simplest use of droplet-based microfluidics, and in the following section we will discuss more advanced manipulations that more closely mimic cells.

One of the simplest droplet configurations used to mimic cells are droplet interface bilayers (DIBs). In DIBs, droplets are placed into contact, without coalescence, due to lipid carriers, which naturally organize at the interface between droplets and the surrounding environment (most often oil). The interface between two droplets acts as a synthetic cell membrane that allows proteins to insert and diffusible molecules to cross. DIBs are commonly formed by manual pipetting of droplets forced together by mechanical manipulation; however, multiple droplets have been connected in complex compartmentalized networks [11-13]. Within the last several years, DIB network formation has been embraced by microfluidics, meeting the demand for greater precision and higher throughput [14-17]. For controlled microfluidic droplet generation and/or DIB formation, droplets can be trapped and forced together by hydrodynamic trapping or encapsulated within another droplet (double emulsion, multisomes) [17, 18]. Trapping is of particular interest for studying asymmetric DIBs (A/B droplet pairings) with bilayers functionalized with membrane proteins. Microfluidic droplet trapping arrays allow for the study of reactions and signaling over a much longer time course. Large droplet networks, formed manually, have been used to create light sensors and bio-batteries [19]. 3D droplet networks have also been created showing the communication pathways

formed by α -hemolysin pores [20]. By adapting microfluidic design to accommodate more complex droplet patterns that are trapped sequentially in large arrays, the study of real-world problems, such as biomedical devices for multiplexed diagnostics, high-throughput screening of chemical and biological processes, and detection of rare or stochastic events via compartmentalized samples, has been accomplished [21]. Further, several examples of the incorporation of cells with droplet-based microfluidics will be discussed below.

HEK (human) and 3T3 (mouse) cells were encapsulated, with uniquely barcoded primer beads, in droplets for single cell transcriptomics (Figure 1.2A) [22]. The microchip design joined two aqueous flows, one containing the cells and the other containing the barcoded primer beads in lysis buffer. Immediately following droplet formation by flow focusing, the two solutions were mixed in a mixing channel and the cells were lysed to release mRNAs which then hybridized to the primers beads. A destabilizing reagent was added to break the droplets and the contents were collected off-chip for bulk transcriptomics. This design allows for the library sequencing of 10,000 single cells in 12 hours. While this represents a sophisticated system for single cell analysis, a more common purpose for cell encapsulation is for sorting (Figure 1.2B) [2]. Protoplasts derived from *Marchantia polymorpha* were transformed to express the yellow fluorescent protein (YFP) mVenus and cell concentrations were adjusted so that no more than one protoplast was encapsulated per droplet for accurate quantitation of fluorescence intensity [2]. After droplet formation, the droplets were interrogated by a laser for the quantification of a reporter fluorescent protein. Fluorescent intensity at 633 nm was used to quantify chlorophyll autofluorescence indicating whether a droplet contained a protoplast or was empty. The same method was used to measure mVenus expression of the protoplasts. In both cases, droplets containing positive cells, as identified by the laser, were sorted from empty droplets and droplets containing non-fluorescent cells using a dielectrophoresis force. Another method

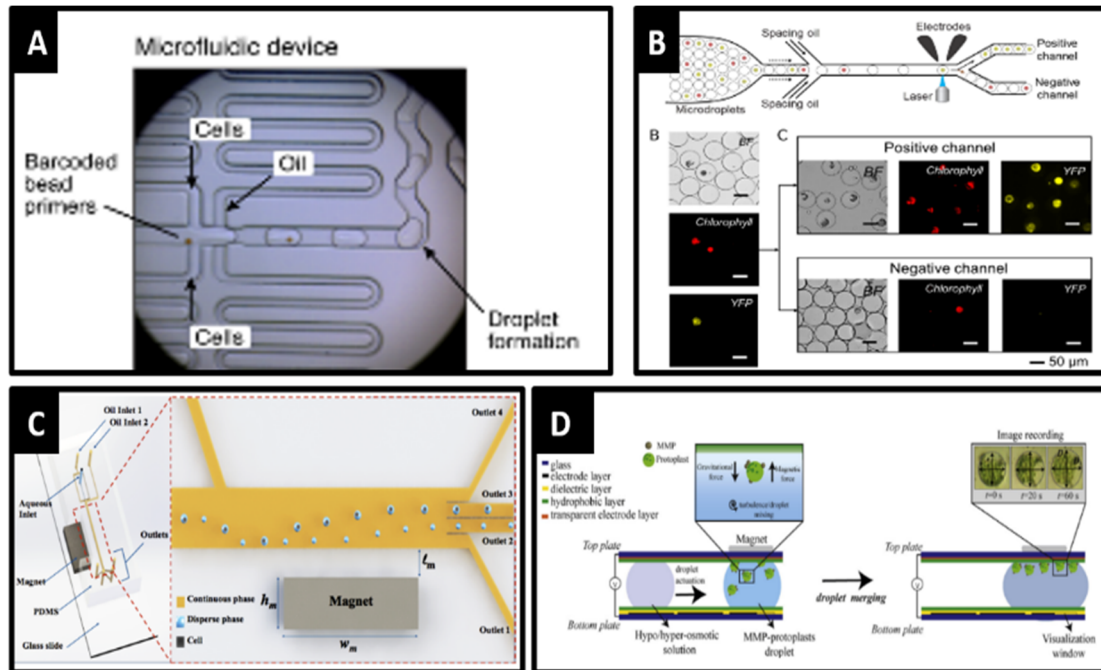


Figure 1.2 Microfluidic devices for encapsulating cells in droplets. (A) Encapsulation of HEK (human) and 3T3 (mouse) cells in droplets with DNA-barcoded beads for off-chip analysis of individual cells by RNA-sequencing [14]. (B) Encapsulation of *M. polymorpha* protoplasts for assessment of gene expression activity and droplet sorting [2]. (C) Device for magnetically sorting droplets encapsulating cells from empty droplets [4]. (D) Protoplasts encapsulated in droplets and merged with hypo/hyper-osmotic solution [6]. Cells were labeled with magnetic nanoparticles for immobilizing on the top plate for imaging physical response.

for cell sorting is shown in Figure 1.2C where a microfluidic device utilized cell encapsulation for cell sorting by magnetism [4]. The authors observed a size difference between droplets containing cells and empty droplets. Their diamagnetic size-based sorting of the two droplet types achieved ~84% purity. This sorting method is remarkable because it does not require cell labeling, auxiliary power supply, electrodes, or laser beams. In addition to cell sorting, droplet microfluidics are also used for studying individual cell response to a highly controlled environment. Droplet microfluidics further enables the measurement of water permeability of single isolated plant protoplasts. For example, Figure 1.2D shows a design for the proof-of-concept for a microchip to study water potential at the single cell level [6]. The device generates two droplets of asymmetric composition; the first droplet encapsulates plant protoplasts labeled with magnetic nanoparticles. The second droplet contains a hypo/hyper-osmotic solution. The magnetic nanoparticles immobilized the cells on the microchip surface for easier imaging and analysis. Upon merging of the two droplets, the response of the cells to the non-isotonic solution could be observed at the single cell level. This method is an easy solution for single cell physiological measurements. This study is similar to Goal 1 of the proposed work, but magnetic microparticle labeling of the protoplasts is overly complex and their system has little control of cell spacing and placement. These publications clearly illustrate a need for highly controlled, droplet manipulation.

1.3 Cell-based microfluidics

Single cell analysis has become increasingly popular because of the known heterogeneity that exists within any cell population [23, 24]. Single cells have been individually captured in U-shaped traps, optical traps, droplets and microwells depending on study requirements. Applications of cell-based microfluidics include studies on cell morphology, gene expression and protein production, intercellular communication, and cell differentiation. Despite decades of research on all of

these topics in mammalian and yeast cells, microfluidic devices have only recently been adopted by the field of plant biotechnology. Plant cell-based microfluidics has focused primarily on cell culture, protoplast fusion (e.g. electrofusion and chemically-induced fusion), and high-throughput analyses [1, 3, 5, 6, 8]. For these studies, cells are typically captured in large chambers or flown through wide channels. Although such techniques have been proven successful for cell culture and electrofusion, advancements can be made for single cell capture, analysis, transfection and gene expression. Many plant cell studies have been conducted with protoplasts, cells where the cell wall has been digested by enzymes but the plasma membrane is intact. Protoplasts can be isolated from most plant tissue (leaves, roots, shoots) and are more easily genetically modified by chemical and electrical methods due to their lack of a cell wall. The pluripotency of protoplasts means they can dedifferentiate, divide, and re-differentiate into various cell types based on controlled culture conditions [25]. This makes them ideal candidates for single cell studies, enabling real-time monitoring and perturbation during growth and differentiation. Due to the dearth of microfluidic studies using plant cells and protoplasts, a complete summary of research follows.

The first study to culture plant protoplasts within a microfluidic device was reported in 2006 (Figure 1.3A) [1]. The microchip design was a large channel with pillars at both ends for trapping the cells in the channel, but allowing for solution exchange. Tobacco protoplasts were first introduced into the device and then the culture media was injected at a low flow rate (50-100 $\mu\text{L/hr}$) for the remainder of the experiment. Cell division began after 2 days and formation of microcolonies occurred within 2 weeks, significantly faster than in the petri dish. The advantages to on-chip culture include a small cell population size, adjustable continuous cultures, and a more consistent environment that reduces stress on the cells.

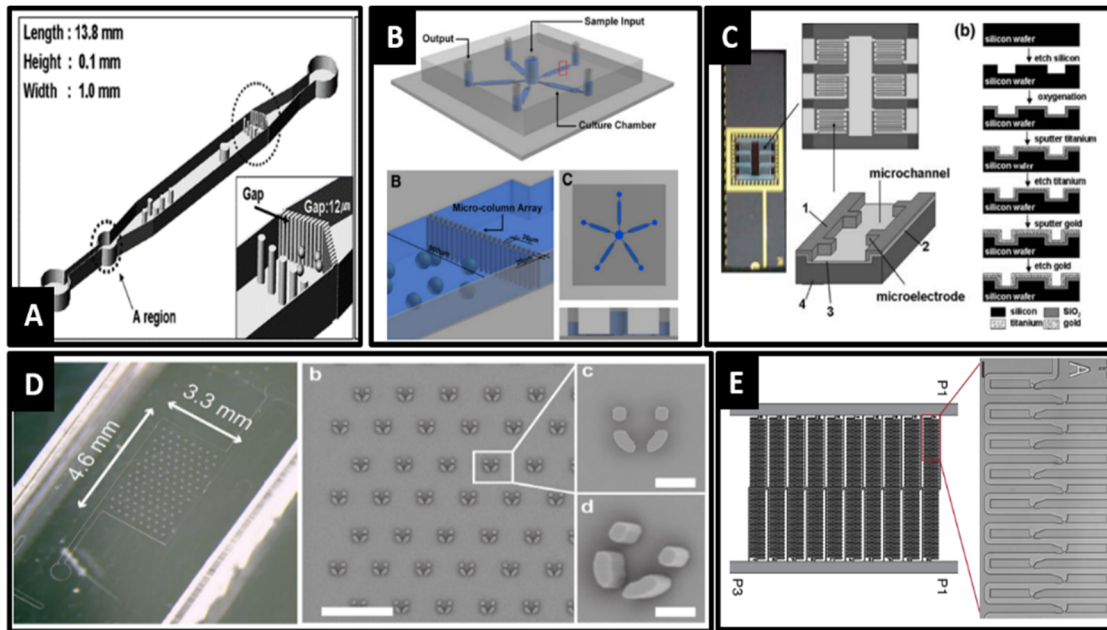


Figure 1.3 Microfluidic methods of trapping single cells. (A) Device for the continuous culture of *Nicotiana tabacum* protoplasts, trapped in device by pillars blocking the outlet [1]. (B) Microchip for the culture and chemical fusion of tobacco mesophyll protoplasts [3]. (C) Device for the trapping and electrofusion of N1H3T3 (mouse embryo fibroblast) cells [5]. (D) Flow-through array of U-shaped traps for capturing single protoplasts for real-time tracking of growth and development of tissues over time [7]. (E) Multi-generational lineage tracking of CD8⁺ (lymphocytic leukemia) cells to identify subtle signatures from cell cycle in various biological contexts [8].

A second study altered the previously described design by creating a pentagon structure from the same chamber layout, but went a step further by studying the chemical-induced fusion of tobacco mesophyll protoplasts (Figure 1.3B) [3]. The protoplasts were introduced to the device through a central inlet, which branched out to five separate chambers. 95% protoplast viability was achieved and the first division of cultured protoplasts occurred at 5 days. The microchambers were filled with cell masses after 12 days of culture. In addition to cell culture, the device was used for the chemical induction of protoplast fusion. Cell fusion has been a common method of genetic engineering in plant cells. Cells were seeded in the chambers and treated with 35% PEG for 10 minutes before being washed with the culture medium. A fusion rate of 28.8% was achieved.

Electrofusion is another method for cell fusion in microfluidics. One example of microfluidic cell electrofusion is shown in Figure 1.3C. The microchip contained 1,368 pairs of high aspect ratio silicon microelectrodes, covered with titanium and gold thin film, across six microchambers [5]. Cucumber mesophyll protoplasts were flown into the serpentine microchannels and subjected to three stages of electrical signals for cell alignment, electroporation, and cell fusion. Cell pairs were aligned in the wells lining the microchannel walls by applying a quasi-sine AC signal. The alignment signal was quickly switched to a pulse signal of 3-7 kV/cm for 600-1,000 ms. This method produced a cell fusion efficiency of 42% for 4 kV/cm, and 44% for 4.5 kV/cm electric pulse strength.

An interesting study of plant protoplasts in microfluidics involved the hydrodynamic trapping of single cells (Figure 1.3D). The authors captured single *P. patens* protoplasts in a flow-through array of 100 U-shaped traps measuring 45 μm x 60 μm [7]. The number of traps filled by at least one protoplast was $\sim 90\%$. After protoplast loading, the chamber was filled with culture media. The study went on to show proof-of-concept for the division steps and cell wall regeneration, the

growth of protonemata, and the induction of leafy buds when using the phytohormone cytokinin. The design ultimately enables the imaging of protoplast regeneration to plants at high resolution over long periods of time.

Another preliminary study upon which the research in this dissertation is based also utilized hydrodynamic trapping for single cell capture (Figure 1.3E) [8]. A hydrodynamic trapping array was design for the capture of single CD8+ T-cells (of a lymphocytic leukemia cell line). In contrast to the previously discussed hydrodynamic flow-through array, this microchip organized the hydrodynamic traps in a serial array, thereby enabling ordering for multiple generations. This novel study tracked the lineage of single cells by culturing two generations on-chip and then releasing the cells for off-chip sequencing. This allowed for the comparison of gene expression between sister, cousin, and unrelated cells. The importance of this study for the purposes of the proposed research is the trapping of individual cells for single cell analysis, under various flow conditions, over long time scales.

Hydrodynamic trapping is an advantageous method for measuring characteristics of individual cells, especially with the occurrence of solution exchange. Trapping cells in an array makes them easier to identify and track physical and behavioral changes over time [26]. Such a platform could be adapted for a number of common plant cell studies including temperature, chemical, and osmotic stress.

As such, the research in this dissertation takes aim at 2 main objectives that advance the current state of knowledge regarding microfluidics and plant biotechnology: 1) develop a method for automatically generating complex DIB networks and 2) develop a system for standoff detection of transgenic whole plants.

1.4 Gaps and objectives

1.4.1 Scientific gaps

We define the following scientific gaps:

Gap 1. Droplet interface bilayers provide a simplified mimic of a cell membrane with which synthetic biology and compound screening applications can be more easily investigated. Microfluidics is a platform with which DIBs can be formed robotically with higher throughput; however, there is a need for greater complexity than the existing A/B droplet ordering currently available. While large DIB networks have been formed manually by pipette and 3D printing, microfluidics can automate formation of larger networks of smaller droplets, while also being faster and more precise. The applications of DIBs in microfluidics are greatly increased when the possible number of solution inputs is increased. We designed a microchip containing four input channels for biological relevant solutions and controlling the pattern of droplet production in a way which allows for the downstream trapping of complex combinations of DIB networks. With these networks, more complex biological assays and signaling studies may be performed.

Gap 2. Reverse genetics and phenomics have revolutionized plant biology and bridged plant phenotypes with genes to transform agricultural fields. Genetically encoded fluorescent proteins have revolutionized plant biology paradigms in gene expression, protein trafficking, and plant physiology. While green fluorescent protein in plant canopies was first imaged over 25 years ago, modern phenomics has largely ignored fluorescence as a transgene expression device despite the expanding FP color palette available to plant biologists [27-30].

1.4.2 Research objectives

The following objectives attempt to address the scientific gaps mentioned above:

Objective 1 (addressing Gap 1). Design and fabricate a microfluidic device for generating droplets of four discrete compositions. The device needs to be able to capture four droplets in a trap array for generating complex droplet networks. Numerous droplet network patterns must be created in a controllable manner. In order to increase the complexity of DIB networks, this first goal is to develop a method for forming complex patterns of DIBs for biological assay applications.

Objective 2 (addressing Gap 2). The purpose was to conduct phenomic analysis of individual protoplasts to gain insight on how plants might respond to external stimuli, Objective 2 considers whole genetically modified plants. To address Gap 2, a laser system will be designed and built for imaging numerous fluorescent proteins in plant canopies from > 3 m standoff.

CHAPTER II
PRESSURE-DRIVEN GENERATION OF COMPLEX
MICROFLUIDIC DROLET NETWORKS

A version of this chapter has been submitted for publication by Tayler M. Schimel McNeillie:

Tayler M. Schimel, Mary-Anne Nguyen, Stephen A. Sarles, Scott C. Lenaghan. "Pressure-driven generation of complex microfluidic droplet networks." (2020).

TMS, AS, and SL conceived of the presented idea. TMS carried out all experiments and analysis. TMS wrote the paper with input from all authors.

2.1 Abstract

Droplet interface bilayers (DIBs) mimic the cell membrane and provide a model membrane platform for studying basic biophysical processes. This paper demonstrates a pressure-driven microfluidic system for the rapid and automated generation of alternating DIB networks, each comprised of four aqueous nanoliter droplets. The microfluidic device features five inlets, one for the continuous oil phase and four independent aqueous channels for droplet generation at T-junctions. Droplet production rates are controlled by adjusting the applied pressure of each inlet; therefore, inlet pressures control the sequence of droplets produced in the main channel and further stored in a downstream hydrodynamic trapping array. Each trap is designed to capture and hold in place one row of four adjacent droplets that promote the spontaneous formation of three interfacial lipid bilayers per network. The potential for more combinations of droplets in each network enables an increased complexity necessary for performing parallel multiplexed biological assays. We further examined flow behavior in response to changes in resistance of the microfluidic device when using a pressure driven source and demonstrated that droplet size and flow rate decreased significantly as droplets filled the traps and increased the total resistance. These findings give insight into the differences between pressure-driven and syringe pump-driven droplet

production and hydrodynamic trapping. This microfluidic system provides a high throughput method for generating DIB networks of complex droplet patterning.

2.2 Introduction

Due to the intrinsic complexities of biological membranes and difficulty in designing experiments to interrogate the membrane of single cells, droplet interface bilayers (DIBs) provide a simplified and effective tool to study a variety of multifaceted biological processes. DIBs are formed when two aqueous-in-oil lipid-coated droplets are in close enough proximity to allow opposing lipid monolayers to “zip” together and form a lipid bilayer. When three or more droplets are brought together in this way, a DIB network is formed, which provides a higher-order, compartmentalized test system for analyzing complex biosystems, such as cell-free bioreactors [31, 32], transmembrane proteins [33, 34], integral membrane ion channels [35, 36], and compound screening [37, 38]. While the most common method for forming DIBs is manual pipetting of droplets, this strategy is severely limited by relatively large droplet sizes and a lack of control over droplet placement, which complicates the formation of DIB networks. While large multi-compartment DIB networks have been formed manually by pipetting and 3D printing [13, 20, 39-41], microfluidics offer significant potential to reduce droplet volume and increase precision and automate the production of DIB networks.

Owing to the ability of microfluidic channels to generate large numbers of consistent picoliter sized droplets [15, 17, 18, 42] and the laminar flow environment, microfluidics is well suited for the generation of DIBs and DIB networks. As such, several microfluidic techniques have been developed for creating DIBs within microfluidic devices. Multisomes, water droplets encapsulated by a larger oil-in-water droplet, have been formed by generating alternating aqueous droplets by two opposing T-junctions and then encapsulating asymmetric droplet pairs in an oil droplet using a flow-focusing channel geometry [17]. Thus

multisomes form a DIB between the internal droplet pair, as well as between the inner droplets and the external aqueous phase. Multisomes have been validated as model bioreactors with one encapsulated droplet containing a membrane impermeable compound and the other a membrane permeable primary amine with a reaction inducing a fluorescence response. Another strategy used a pressure pump system to generate droplets with a double T-junction joined at a Y-junction to enable alternating droplets [15]. The alternating droplets were captured by pillars located within the main channel. Further, passive molecular permeation and ion-channel mediated permeation of molecules and ions across the bilayer was confirmed using fluorescent assays. A third approach generated droplets by flow-focusing for creating different sized droplets (300-1500 pL), which were then sorted according to size by deep grooves in the channel and held in place under constant flow by micropillars that outlined the grooves [42]. The DIB network size was dictated by the length of the grooves and networks as large as 20 droplets, all of a single droplet type, were assembled. Mass transfer across a 5-droplet network was demonstrated by an enzymatic reaction resulting in a fluorescent response. In another study, two opposing T-junctions were used to create a stream of alternating droplets that were captured in a hydrodynamic trapping array to form DIBs from two droplets of asymmetric composition [18]. Thin film electrodes were deposited at trap locations to obtain *in situ* current measurements corresponding to bilayer growth. Simultaneous electrical interrogation of 8 DIBs was used to monitor the insertion of alamethicin peptides into the bilayer. Thus, a few studies have demonstrated microfluidic formation and assaying of 2-droplet DIBs from droplets with differing compositions. However, advancing the capabilities of microfluidic DIB networks for multivariate systems would benefit from a combinatorial assembly of DIB arrays in which multi-droplet DIB networks are built from a larger number of possible droplet compositions combined in varying combinations.

While microfluidics has demonstrated effective formation of DIBs and DIB networks, there is currently a limitation in the complexity of droplet arrays that can be formed from one or two inlets. Relatively simple A/B droplet ordering has been achieved with two inlets [15, 17, 18, 43-47]; however, to study more complex interactions, such as signal transduction or combinatorial screening, droplet arrays and DIB networks with greater diversity in contents and droplet-droplet pairing are needed. To generate droplets in a microfluidic device, fluids can be driven by a syringe pump or by applied pressure. Syringe pumps have the advantage of a fixed volumetric flow rate; however, they also have a slow response time (seconds to hours) and periodic pulsations. The slow response time make syringe pumps unsuited for advanced droplet patterning because the flow rates and resulting droplet production rates cannot be quickly altered. Alternatively, pressure-drive flow response time is as fast as 40 ms and flow is pulseless, but with the potential of backflow. Two ways in which the complexity of droplet networks can be improved is by increasing the number of droplet-generating channels and by adjusting the droplet pattern. Pressure control systems are able to individually control the flow of numerous fluids simultaneously, allowing for multiple droplet types to be generated independently. Droplet patterns can thus be easily manipulated by driving flow with a pressure-driven pump. The controllability of these systems make it possible to apply varying sinusoidal pressure waves to multiple independent inlets, resulting in changes in droplet production rates. Different DIB patterns may even be possible by applying different pressure waveforms across opposing T-junction input pressures. Increasing the number of possible droplet types and allowing for advanced patterning of droplets with a pressure-driven flow system, enables the design of microfluidic studies for compound chemical screening, electrical and chemical signaling, and altering chemical or protein concentrations [11, 12, 48].

Herein, we report a multi-channel, pressure-driven microfluidic droplet system for the automated generation of arrays of complex DIB networks, a term that we use to describe linear droplet quartets of controllable droplet ordering. The method utilized T-junction channel geometry and a hydrodynamic trapping array to generate and capture up to 35 possible droplet combinations paired from four discrete aqueous compositions in a continuous oil phase. A multi-channel, pressure-driven pump system was used to vary the droplet production rates and thus the patterns of droplets generated at four inlets. The effects of dynamic input pressures on the droplet production rates, droplet sizes, and droplet production sequences were examined. Droplets were captured in a downstream hydrodynamic quad-trap array, forming four-droplet networks. Further, a circuit-based model was employed to model the change in flow response as droplets were trapped in the device. This work furthers understanding of pressure-driven droplet production and sequential hydrodynamic trapping for complex DIB networks, which provides a tunable platform for assembling and studying combinatorial multicomponent biosystems.

2.3 Results and discussion

2.3.1 Microfluidic design

We designed a microfluidic device, comprised of a continuous phase channel, which splits into two channels at the inlet, and two pairs of opposing T-junctions positioned in parallel for generating droplets from four independent inputs (Figure 2.1). The main channel width was 125 μm and the channel height was 125 μm across the entire device. Aqueous inlets tapered to 40 μm for better control over droplet size [49]. Post droplet generation, the split continuous phase channels rejoin by a Y-junction where droplet streams were combined to form the droplet patterns. A by-pass channel (shunt), followed the Y-junction and allowed droplets

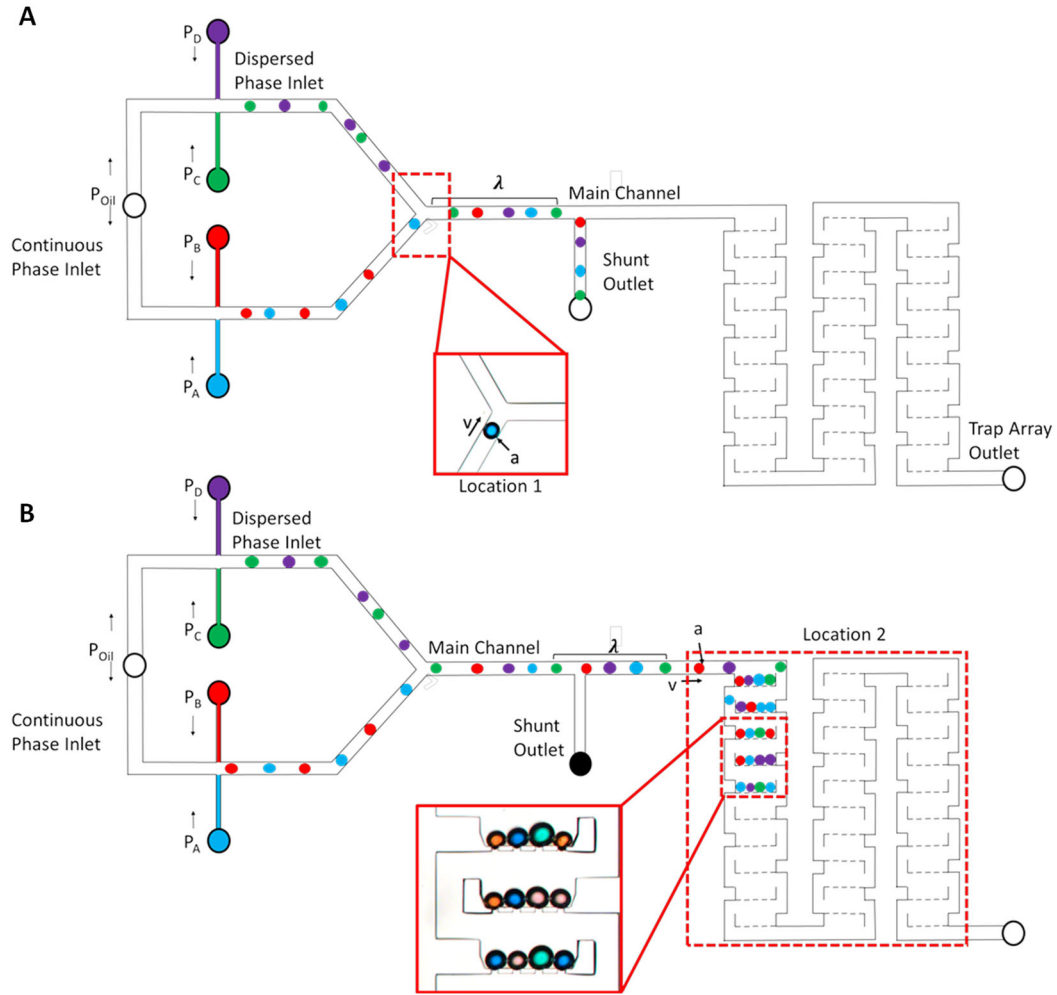


Figure 2.1 Schematic of the quad T-junction device generating complex droplet networks under pressure-driven control. (A) While a desired pattern is being established, droplets exit the device through the open shunt channel (left). (B) Once a pattern has been stabilized, the bypass channel is closed and droplets proceed down the main channel to the droplet trapping array. (---) indicates fields of view for shunt open pattern stabilization and shunt closed trapping imaging.

to exit the device when open. The shunt was left open prior to trapping to allow internal flow and droplet production to stabilize. Once a consistent pattern of droplets at the inlets was established, the bypass channel was closed to route droplets to the trapping array.

The trapping array was a serpentine channel of droplet traps placed in series to form DIB networks for biological studies (Figure 2.2, A.1), as previously described [18]. Each trap, designed to capture a row of four droplets, was made up of rectangular compartments 440 μm wide with four narrow exit channels 35 μm x 20 μm x 125 μm referred to as bleed valves. Droplets that enter the trap and block the bleed valves increase the equivalent hydraulic resistance of the trap section. Once a 4-droplet trap filled, the increased resistance caused the following droplets to bypass the filled trap and continue down the main channel to the next unfilled trap. Based on the electrical circuit model (Figure A.2), where the resistance of a trap is less than the resistance of the main channel, sequential filling of the trap array was predicted.

2.3.2 Droplet production rate

All fluid flow was generated by controlling the input pressure using an Elveflow OB1 pressure controller, which enabled individual control of the oil and four separate aqueous solutions. The ratio of aqueous pressure to oil pressure was 1.2-1.9. First, appropriate DC input pressures were found by manually adjusting the applied pressure for each aqueous input until an equal number of droplets at the same frequency was generated. A pressure sine wave was then applied to each input with an amplitude of 0.5-1 mbar above the constant pressure to create fluctuating droplet production rates, higher amplitudes resulted in jetting of the aqueous phases and lower amplitudes yielded fewer types of droplet combinations. A period of 10 minutes was chosen because that was approximately the amount of time required to fill 30 traps. Phase shifts of 90°, 120°, 150°, and

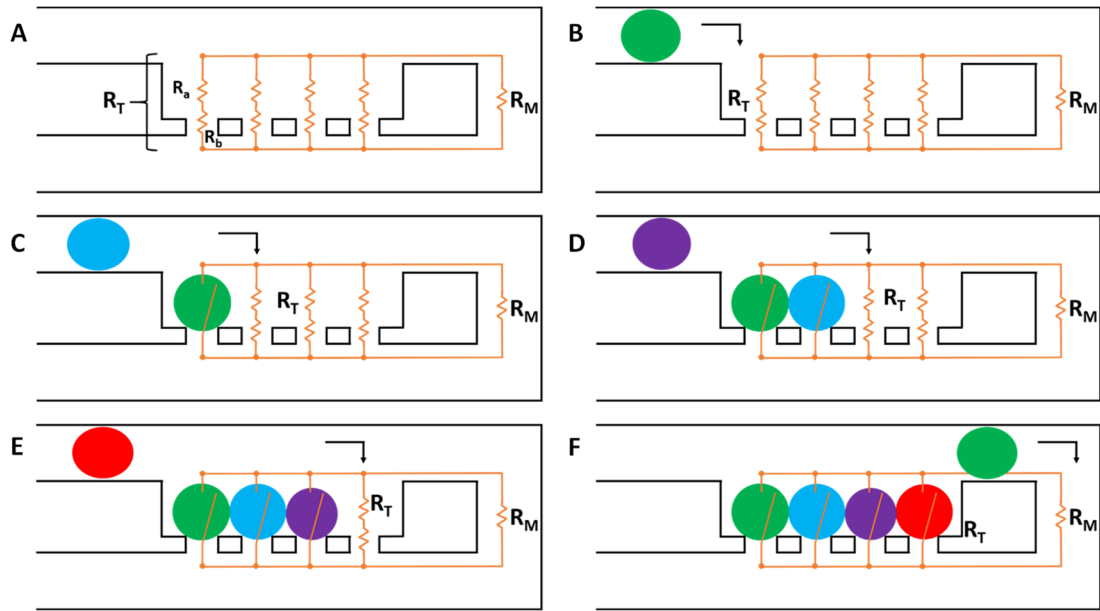


Figure 2.2 Schematic of hydrodynamic flow resistances in a 4-droplet trap. (A) Diagram of unfilled trap, where each lane of the unfilled trap has a resistance of $R_T = R_a + R_b$. (B) Droplet 1 enters the trap when R_T is less than the main channel resistance R_M . (C) Droplet 2 enters the open lane of the trap if $R_T < R_M$. (D) Droplet 3 enters the third lane of the trap if $R_T < R_M$. (E) Droplet 4 enters the fourth and last lane of the trap if $R_T < R_M$. (F) Once all four lanes in the trap are filled, R_T becomes greater than R_M and all following droplets in the droplet sequence bypass the filled trap and continue through the main channel.

180° were applied to each input of opposing T-junction pairs and thus the droplet sequence varied. Droplet sequences were evaluated downstream at the Y-junction, (Figure 2.1, Location 1), and the cross-sectional area and velocity of each droplet was calculated. Cross-flow at the Y-junction pinched off large aqueous droplets (>150 µm in diameter), occasionally generated in experiments with the shunt open, into multiple droplets, therefore droplet sizes were measured before the junction to avoid altering the droplet sequence prediction. Droplet velocities were measured downstream of the Y-junction, since this was the velocity of the droplets as they entered the trap array. Once a stable pattern was established, the shunt was sealed with tape, routing droplets to the trapping array.

Droplet production rates, λ , were measured at both Locations 1 and 2 by:

$$\lambda = \frac{1}{\Delta t_d} \quad (5)$$

where Δt_d was the time between two droplets of the same type. Production rates were measured for every droplet during the time of the experiment. Figure 2.3 shows the production rates of each droplet type (i.e. A, B, C, D) versus time for when a 120° phase shift in sinusoidal pressure is applied between inlets A and B and C and D; additional droplet production rates for 90°, 150°, and 180° phase shifts are included in Figure A.3.

Droplet production rate is a function of the water-to-oil applied pressure ratio [50]. In agreement, the data show that an increase in the pressure applied to the aqueous phase correlates to an increase in the rate of droplet production, up to the point of jetting. Large pressure differences between aqueous inputs, up to 2 mbar, in a pair of opposing T-junctions can cause one droplet type to dominate its opposing droplet type and cause the sequences of a single droplet type. Spikes in

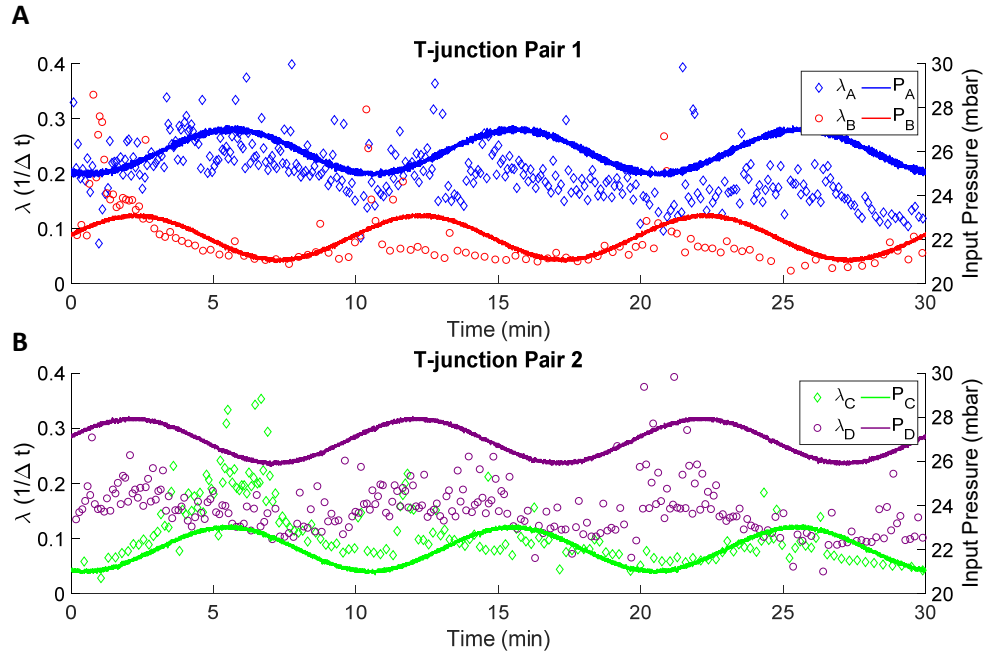


Figure 2.3 Droplet sequences are generated by applying sinusoidal pressure waves and thereby altering the droplet production rates. Peak points of droplet productions rates ($\lambda > 0.3 \text{ s}^{-1}$) occur by the rapid generation of two droplets of the same type; similarly, low points in droplet production rates ($\lambda < 0.02 \text{ s}^{-1}$) occur when there are long pauses between droplet generation. (A) Input pressure waves and corresponding droplet production rates for droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding droplet production rates for droplets formed at the second pair of opposing T-junctions.

droplet production rate indicate rapid generation of two droplets of the same type, causing patterns that were less likely to be repeated. Droplet production rates for the 120° phase shift data averaged between 0.07 and 0.17 s⁻¹ for the four droplet types, with a minimum range of 0.02-0.1 and production rates peaked at 0.25-0.38 (excluding spikes in droplet production).

2.2.3 Droplet size

For both syringe pump and pressure-driven flow, droplet size is predominately controlled by channel geometry. Flow rates and pressure ratio are a second-order determinant of droplet size, although there is a stronger effect with pressure systems [51]. In the current design, and in agreement with other studies with a constant oil pressure, the acceptable pressure range of the aqueous input for consistent droplet production was very narrow, on the order of 1-2 mbar [50, 52]. When dispersed phase pressure (ΔP_{A-D}) increased in relation to the continuous phase, droplet size increased, and when ΔP_{A-D} was too high unbroken, water flow occurred; if ΔP_{A-D} was too low, droplet production ceased. For trapping purposes, droplet size was an important factor. The traps in this study were designed to capture droplets that were 125 μm in diameter. Droplets smaller than 45 μm in diameter squeezed through the 35 μm wide bleed valve. Larger droplets (diameter > 125 μm) protruded out of traps, partially blocking the main channel and increasing the pressure drop along the main channel which prevents direct trapping of droplets in successive traps. Droplet cross-sectional area was measured and the equivalent circle diameter was calculated (Figure A.6). Due to refraction around the perimeter of the droplets, a fraction of the actual droplet size was measured. With the microchip's fixed channel geometries, droplet sizes ranged from 40-225 μm and 40-150 μm in equivalent circle diameter when the shunt was open and closed respectively, by varying the relative oil and water applied pressures in mbar. Figure 2.4 shows the change in droplet diameters in response to the variance of applied water pressure, where the minimum droplet

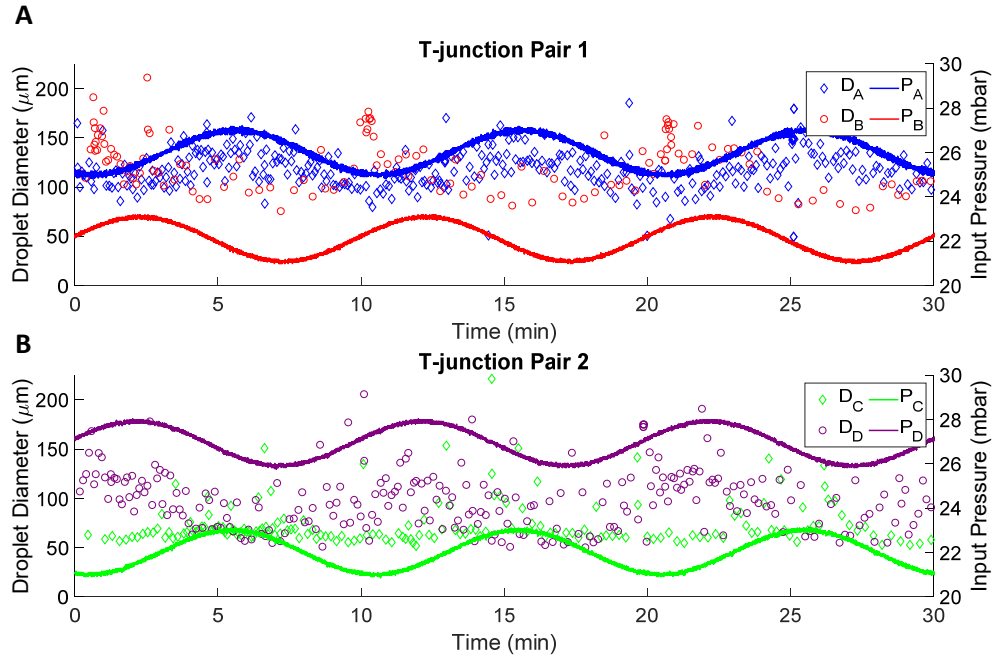


Figure 2.4 Droplet size change with applied pressure input. (A) Input pressure waves and corresponding diameters of droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding diameters of droplets formed at the second pair of opposing T-junctions.

size range for the four aqueous phases is 44-66 μm and maximum of 210-222 μm , with a mean of 70-120 μm . Data for additional pressure phase shifts is given in Figure A.7-9. Droplet diameters above 150 μm indicate aqueous slugs, which were broken into multiple droplets by crossflow at the Y-junction, which prevented large droplets from entering and disrupting DIB networks in the downstream trap array.

2.2.4 Droplet patterns

By manipulating the aqueous input pressure, and consequently the droplet production rates, the generated droplet sequences were controlled for the purpose of creating complex DIB networks. Droplet sequences were first assessed at viewing Location 1 (Figure 2.1) when the shunt was open as a way to preview the patterns that would be captured upon closing the shunt for trapping. Droplet sequences were analyzed by two distinct methods: (1) every droplet in the sequence was considered to be the first of a set of four droplets to create a pattern and (2) the droplet sequence was first split into sets of four droplets and then a pattern was identified for each quartet (Figure 2.5). Possible droplet combinations are given in Table 2.1. The first method, referred to as “all viewed combinations,” accounts for the possibility of irregularities in droplet generation or indirect trapping that could shift the start of a series of four droplets arriving in a given trap when attempting to predict droplet patterns. For example, if one droplet in the sequence bypassed an open trap and the next four droplets in the sequence were correctly trapped, that pattern would exist in the “all viewed combinations” but not in the “ideal” patterns. The second method is used for identifying the “ideal” and “trapped” patterns. Ideal patterns are based on the first 4 droplets landing in trap 1 and the second four droplets in trap 2 etc., with no missed droplets or traps. For all viewed combinations in the shunt open case (SO-AV), droplet sequence was monitored for thirty minutes, the time required for three sine wave phases, to allow for a baseline standardization of potential patterns (Figure 2.6A).

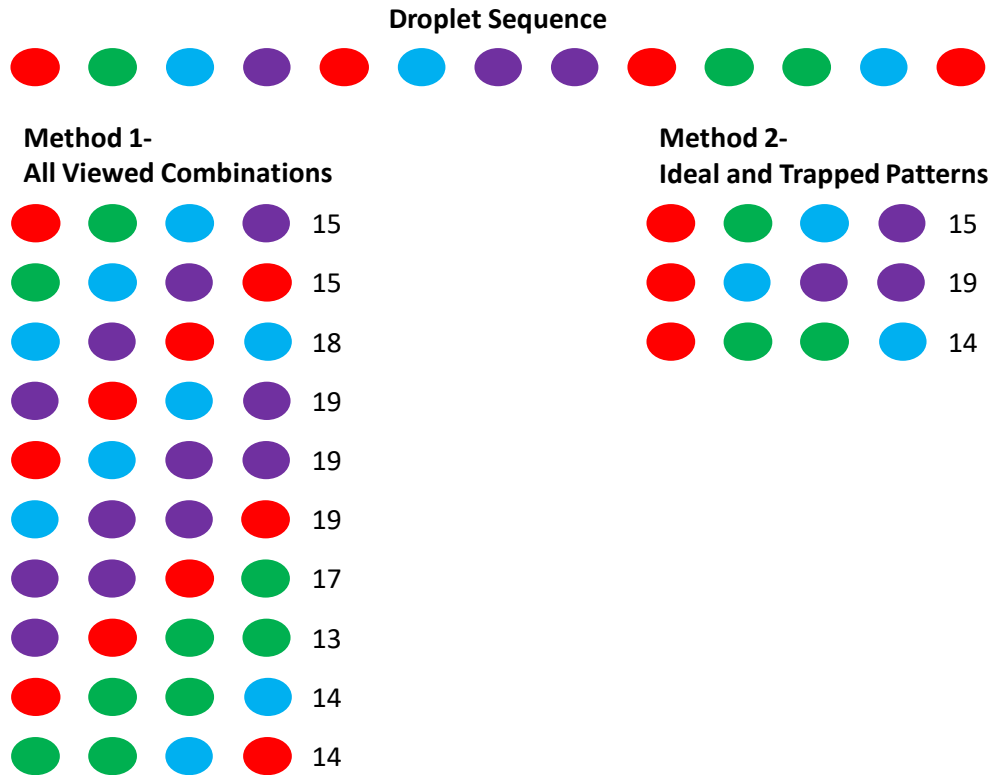


Figure 2.5 An example of identifying droplet combinations and patterns from generated droplet sequence. Given a random 13-droplet sequence, droplet patterns were identified by two methods: 1) all view combinations – every droplet in the sequence is taken to be the first in a droplet quartet, resulting in this scenario with 10 droplet combinations, 2) the droplet sequence is first separated into sets of 4 and then patterns are identified from the droplet quartets, in this example the result was 3 droplet patterns. Numbers to the right of each droplet quartet are the combination identification number for each grouping.

Table 2.1 Numbers were assigned to each possible combination of 4 droplets to simplify identification.

Combination ID	Droplet Pattern	Combination ID	Droplet Pattern	Combination ID	Droplet Pattern
1		13		25	
2		14		26	
3		15		27	
4		16		28	
5		17		29	
6		18		30	
7		19		31	
8		20		32	
9		21		33	
10		22		34	
11		23		35	
12		24			

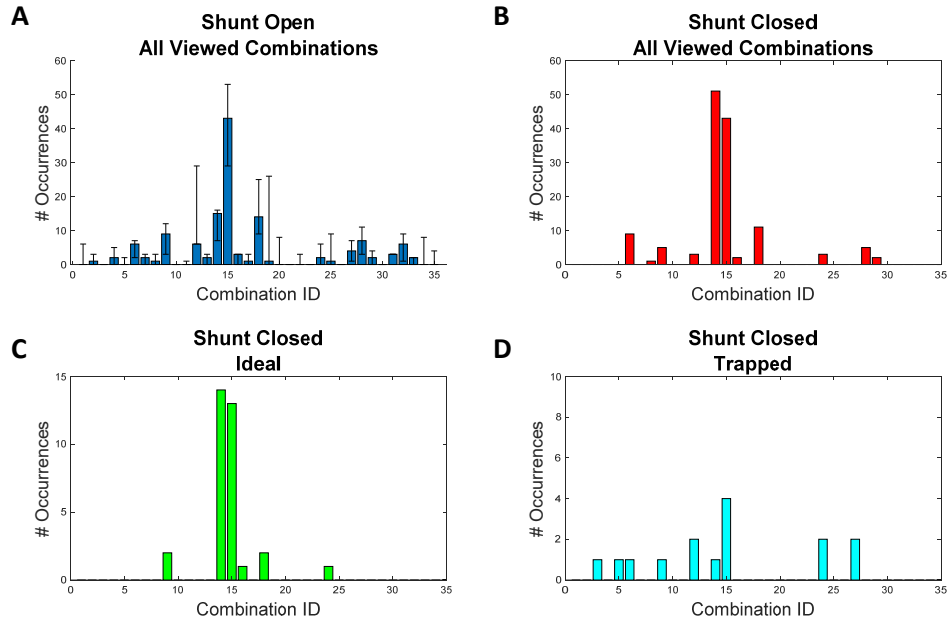


Figure 2.6 Generated droplet patterns for 120° pressure shift in the cases of (A) open shunt- all viewed combinations, (B) shunt closed- all viewed combinations, (C) shunt closed ideal pattern, and (D) trapped patterns. All viewed combinations are comprised of four droplets, identified starting with every droplet in the droplet sequence and regardless of order. Ideal patterns are comprised of four droplets, identified by every 4th droplet in the droplet sequence, starting with the first droplet observed after the shunt is closed. These patterns are the droplet combinations that are predicted to be trapped if errorless trapping were to occur in the device. Trapped patterns are droplet combinations that are observed once all traps in the array are filled with four droplets.

After consistent droplet patterns were established, the shunt was closed and droplets were analyzed at viewing Location 2 (Figure 2.1B) for the length of time required to fill the whole trap array (~10 minutes) (Figure 2.6B). Fewer droplet quartets were available for analysis in all other cases relative to SO-AV due to the reduced observation time which, therefore, accounts for much of the decrease in pattern distribution. If the first droplet in the dataset was the first to be trapped and perfect sequential trapping were to occur, the observed pattern was given by the shunt closed ideal pattern (SC-Ideal) histogram (Figure 2.6C). “Trapped” patterns were the observed droplet patterns in the array of traps at the completion of the experiment (Figure 2.6D). It should be noted that the most prevalent pattern in the trapped sample data is Combination ID #15, which is the pattern most frequent in the original SO-AV data. Combination ID #15 is a DIB network containing one droplet of each of the four droplet types and was the most common pattern to occur in all of the studied phase shifts. This was primarily due to aqueous pressures being balanced prior to each sine wave application, resulting in comparable droplet production rates among the four aqueous inputs [43].

These experiments were carried out for input pressure phase shifts of 90°, 120°, 150°, and 180° applied between inlets A and B and C and D (Figure A.10-12). Images of the array of trapped droplet networks are shown in Figure A.13. Lower phase shifts in input pressure were anticipated to be more consistent because of the reduced pressure imbalance, therefore the 90° shift experiment was conducted three times to demonstrate the repeatability of this method (Figure A.14). Values in Table 2.2 represent the number of unique patterns observed, the number of droplet quartets, and the efficiency of predictive patterns shows the percentages of observed trapped patterns that match the predictive droplet combination patterns in SC-Ideal, SC-AV, and SO-AV. Only droplet networks containing four droplets at the time point that all traps were filled were counted. The efficiency of predictive patterns percentages illustrates the accuracy of the predictive pattern's

Table 2.2 The number of different patterns observed and the number of droplet quartets that occurred for each experiment. Efficiency of predictive patterns is the percentage of observed trapped patterns that matched the predictive shunt closed-ideal (SC-Ideal), shunt closed all viewed combinations (SC-AV), and shunt open all viewed combinations (SO-AV). Percentages were calculated by dividing the number of the trapped patterns that correspond to the comparative identified pattern by the total number of trapped patterns.

Phase shift	SC-Ideal			SC-AV			SO-AV		
	Number of observed patterns	Number of droplet quartets	Predictive score	Number of observed patterns	Number of droplet quartets	Predictive score	Number of observed patterns	Number of droplet quartets	Predictive score
90°	7, 9, 8	25, 25, 11	62.4 ± 6.4%	10, 13, 11	100, 114, 42	81.1 ± 5.7%	19, 22, 25	504, 879, 637	92.3± 0.2%
120°	6	33	53.3%	11	135	73.3%	29	471	93.7%
150°	8	25	47.6%	9	97	52.3%	18	303	85.7%
180°	15	24	40.0%	18	94	73.3%	30	661	80.0%

ability to anticipate which droplet patterns will be trapped. Fluctuations in the size and frequency of droplet production caused indirect trapping and thus errors in pattern prediction [53]. As expected, trapped patterns more closely match the SC-Ideal patterns for the lower phase shifts and there was less accuracy in trapped pattern predictions when the phase shift between input pressures was greater. In this study, the 180° phase shift data exhibited the most droplet combinations with 30 and 15 unique droplet patterns in SO-AV and SC-Ideal experiments, respectively. In comparison, the 90° phase shift experiments only 12 and 7 patterns were observed for SO-AV and SC-Ideal, respectively. Depending on the requirements of future experiments, this system may be altered to either produce a broader range of distinct DIB patterns with fewer repetitions in the trap array or to produce fewer patterns with more replicates. For the former, a greater number of patterns may be achieved by increasing the number of traps, encouraging indirect trapping by altering channel geometry, or selecting a surfactant that enable a higher droplet production rate and decreasing the period of the input pressure wave to promote variability in droplet pairing. For the later, options to reduce the variety of patterns may be realized by reducing indirect trapping or by decreasing the amplitude of the pressure wave. For this paper, aqueous input pressures were set to generate droplets 1:1 at opposing T-junction pairs before applying a sine wave: thereby favoring droplet quartets containing one of each of the four droplet types and neighboring patterns containing three droplet types with one type being repeated. Additionally, it is possible to set the initial pressures to generate droplets at a rate of 2:1 or even 3:1 before applying a pressure wave and thus more extreme patterns, such as quartets of only one or two droplet types, would be created. For many potential studies, improving the predictive patterns' efficiency to anticipate the droplet patterns (predictive score) that would be trapped is a necessity, which can be realized by improving the direct trapping of droplets. Direct trapping and trapping efficiency can be improved by increasing the length of the main channel between traps; however, doing so would increase the resistance of trap array.

2.2.4 Resistance sensitivity

Shunt channels have been used extensively for droplet sorting with syringe pump systems. The designed shunt was 3 mm in length, giving it a resistance of $4.4 \times 10^4 \text{ Pa s } \mu\text{L}^{-1}$. With the trapping array resistance of $7.6 \times 10^4 \text{ Pa s } \mu\text{L}^{-1}$, droplets preferentially exit the microchip through an open shunt channel. With the syringe pump droplet production system, the shunt may be closed with little effect on droplet production [54]. In comparison, pressure-driven droplet production results in decreased droplet velocity within the main channel decreasing by 35%, 37%, 57%, and 13% for the 90°, 120°, 150°, and 180° conditions, respectively (Figure 2.7); droplet velocity also became more uniform after shunt closure (Table 2.3). The size of droplets was also shown to significantly decrease and become more uniform upon shunt closure dropping from an average equivalent circle diameter of 92.7 μm to 30.4 μm (Figure 2.9, Figure A.15-17). The higher resistance of the trap array in comparison to the resistance of the shunt channel caused a decrease in droplet production rate, size, and velocity in response to closing the shunt when using a pressure pump source. This finding was confirmed by the results of the electric circuit model (Figure A.18, Table A.1) comparison of flow behavior between syringe pump system and pressure-driven system. Similarly, as the traps were filled, the velocity and size of droplets entering the trap array continued to decrease. The array of captured droplets shown in Figure A.13 of the 120° phase shift data depicts the overall reduction in droplet size, where droplets in the first, second, and third column have an average diameter of 107.6, 97.43, and 72.4 μm respectively. A consequence of this change in size is that traps at the beginning of the array are most likely to capture fewer than the design-intended 4-droplet network and traps farther along in the array are likely to contain more than four droplets due to incomplete blockage of the bleed valve. This effect can easily be corrected by connecting a flow rate sensor to each fluid input; allowing for flow rate control via pressure. Alternatively, a less expensive solution is to lower the oil

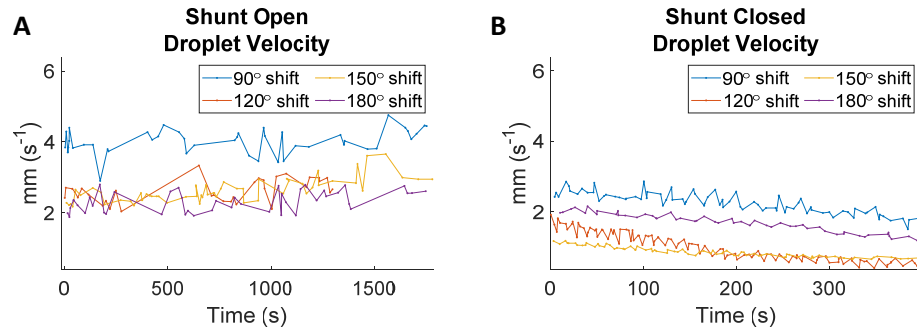


Figure 2.7 Plot of individual droplet velocities, (A) Droplet velocities when the shunt is open and droplets are freely allowed to exit the device. (B) Droplet velocities when the shunt is closed and droplets are being trapped. Droplet velocity decreased by 13-57% upon shunt closure and slowed further as traps filled.

Table 2.3 LineFit equations and R² values corresponding to droplet velocity graphs in Figure 2.7.

° Phase Shift	Shunt Open		Shunt Closed	
	LineFit	R ²	LineFit	R ²
90°	$y=1.8E4x+3.9$	0.089	$y=-2.1E3x+2.7$	0.789
120°	$y=3.1E4x+2.4$	0.19	$y=-2.9E3x+1.6$	0.868
150°	$y=5E4x+2.3$	0.538	$y=-1.1E3x+1.1$	0.86
180°	$y=2.2E4x+2.2$	0.168	$y=-3E3x+2.3$	0.894

pressure as needed while traps are filled: thereby increasing the aqueous to oil pressures ratio and thus the droplet size.

2.3 Methods

2.3.1 Materials

Squalene was purchased from Sigma-Aldrich (UK). 1,2 Diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabama, US). Aqueous solutions were prepared by mixing food color with DI water. DPhPc and DOPC were dissolved in squalene at concentrations of 2 mg mL⁻¹ for initial droplet sequence experiments. DPhPc was dissolved in squalene at a concentration of 6 mg mL⁻¹ for trapping experiments.

2.3.2 Device design and fabrication

Two pairs of opposing T-junctions set in parallel were used for droplet production of four aqueous types. The two incoming droplet streams were joined at a Y-junction, routed through microchannels, and captured in an array of hydrodynamic traps. Each trap was designed to capture and hold in place four droplets in a linear chain. Microchannel geometry was optimized for 125 µm diameter droplets.

Microfluidic devices were fabricated using polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, US) and standard soft lithography techniques. Silicone master wafers were produced using NFR photoresist on a silicon wafer, spun to produce a resist thickness of 2 µm. The resist was exposed to CD-26 and baked at 120°C before Bosch etching. To prevent PDMS adhesion to the silicon master, the wafer was silanized by vapor deposition by chlorotrimethylsilane for 24 hours. PDMS at a 10:1 ration of base to curing agent, was then poured over the silicon master, degassed in a vacuum desiccator chamber and cured for at least 2 hours at 80 °C. The PDMS devices were cut and then peeled off of the master and holes were

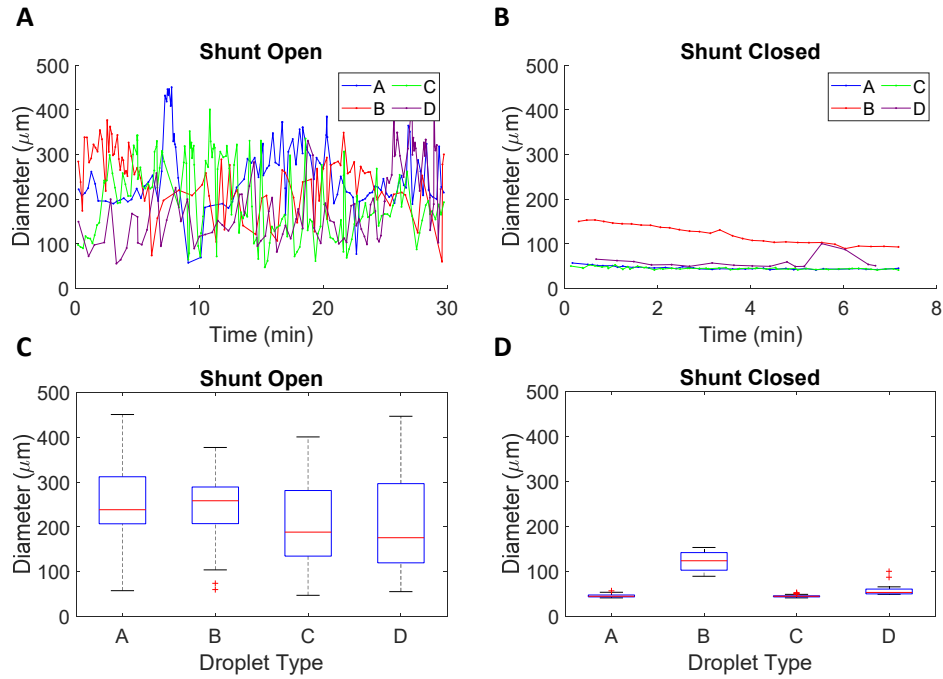


Figure 2.8 Droplet cross-sectional area for a 120° shift shunt open and shunt closed cases. (A) Droplet size measurements when shunt is open. Droplet size fluctuations are evident. (B) Droplet size measurements when shunt is closed. Droplet size decreases slowly as traps are filled. (C) Droplet size when shunt is open. Droplet size varies greatly and slugs are often produced. (D) Droplet size when shunt is closed. Droplet size is decreased and more consistent, when the shunt is closed.

punched at the inlet and outlet ports. PDMS devices were then cleaned and bonded by plasma oxidation to PDMS coated microscope slides. The bonded microchips were heated again at 80 °C for 2 hours. It is common for microdroplets to shrink over time due to fluid absorption into PDMS walls. To minimize this effect, microchips were soaked in water for at least an hour before being used for experiments.

2.3.3 Microfluidic device operation and modeling

Two Elveflow OB1 pressure controllers (Elveflow, France) were used to regulate the applied pressure to the oil and all aqueous phases. All pressures were independently applied in the range of 20-60 mbar. Sampling rates were every 0.05 seconds for every channel. The system allowed for on-demand generation of droplets at each of the four T-junctions by adjusting the pressure of each phase simultaneously by Elveflow, a computer controlled software. PTFE tubing and 23 gage blunt stainless steel needles were used to connect the aqueous solution containers to the inlet ports of the microfluidic device.

A confocal microscope (Olympus IX83) was used for all experiments. Images and videos were acquired (up to 58 frames/s) using an Olympus DP74 color camera and CellSens Dimension software. An objective lens of 4x was used. CellSens Dimension's Count and Measure software and MATLAB were used to analyze and process recorded videos and images. Matlab Simscape software was used to model the microfluidic device using the electrical circuit analogy. Syringe-pump-driven flow input was modeled as DC current and pressure-driven flow input was modeled as DC voltage.

2.4 Conclusion

In this work, we developed and validated a pressure-driven microfluidic system for the generation of complex alternating DIB networks. By varying the pressure waves applied to each of the aqueous inputs it was possible to alter droplet

sequences in a controllable manner, with the potential to capture varied combinations of droplets. Combined with the validated quad-trapping arrays, three serially connected DIBs could be formed, providing a platform for communication between droplets. In this study, a 90° phase shift applied between opposing T-junctions for droplet production yielded the most accurate droplet sequence predictions for which droplet patterns would be trapped, with 92.3% of trapped patterns matching the SO-AV sequence and an average of 8 unique patterns being trapped. While the 180° phase shift data only predicted 80% of the patterns that were trapped, it exhibited the widest range of patterns with 15 unique patterns trapped. Prediction efficiency can be improved by improving direct trapping of droplets and the number of observed patterns can be increased by widening the phase shift between input or increasing the amplitude of the input pressure wave. Further, we characterized pressure-driven flow behavior in response to small changes in resistance. In the current design, the velocity at which droplets travel down the main decreased by 13-57% when the shunt was closed; a characteristic that does not match syringe pump-driven flow where flow rate is constant. Droplet velocities continued to decrease as traps in the array were filled and droplet sizes became smaller, indicating that pressure-driven flow was exceptionally sensitive to any increase in resistance. Based on this finding, flow rate sensors may be necessarily coupled with each pressure inputs to counteract this occurrence. This work has furthered the understanding of pressure-driven microfluidic droplet generation and hydrodynamic trapping to facilitate the creation of complex DIB networks for experiments analyzing complex biosystems.

APPENDIX A

Visual bilayer confirmation

Figure A.1 shows the visual confirmation of bilayer formation between droplets trapped in a microfluidic device, using a concentration of 6 mg ml⁻¹ DPhPc in squalene oil.

Microfluidic Circuit Model

Microfluidic channel design can be guided by modeling the system as an equivalent electric circuit [55, 56]. In the equivalent model, steady-state pumping sources which control flow rate, such as a syringe pump, are treated as DC current supply [57, 58]. Similarly, DC pressure pumping sources, which control input pressures, are treated as a DC voltage source. A resistive flow model, based on the electrical circuit analogy, was used to determine microfluidic channel dimensions required for droplet sorting and trapping in a multi-trap array. In an electrical circuit, Ohm's law relates the voltage (V , volt) to the current (I , amp) and the electric resistance (R_e , Ω_e ohm):

$$V = IR_e \quad (1)$$

In microfluidics, the Hagen-Poiseuille law similarly relates the pressure difference across a microfluidic channel (ΔP , mbar) to the flow rate Q ($\mu\text{L min}^{-1}$) through the channel's hydraulic resistance (R_h , Ω_h):

$$\Delta P = QR_h \quad (2)$$

Thus, the pressure difference and flow rate are comparable to the voltage and current, respectively. For Poiseuille flow in a rectangular channel, the

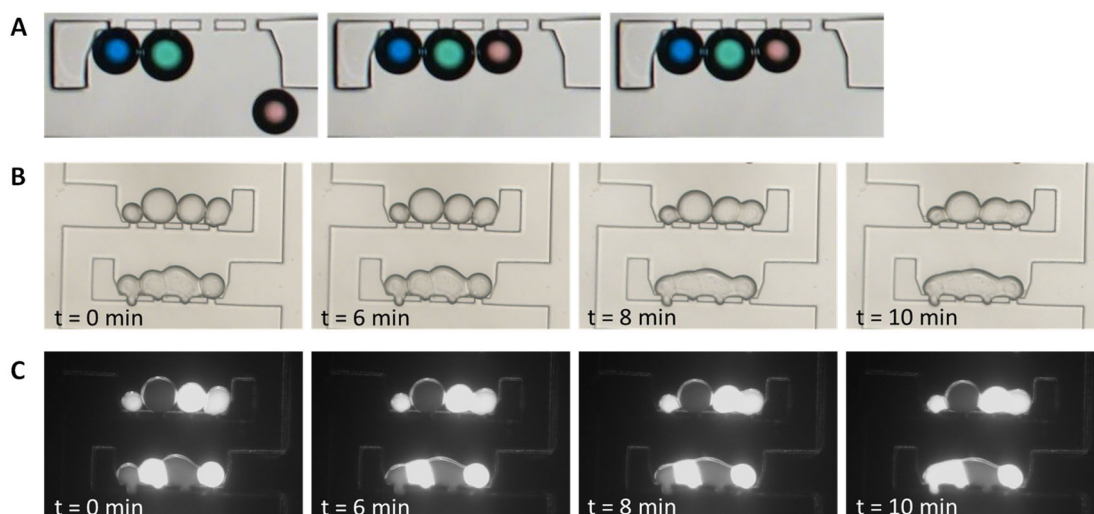


Figure A.1 Visual confirmation of DIB formation 1. A) Water droplets dyed with food coloring exhibit relatively small bilayer area. B) Brightfield images of water droplets containing calcein or α -hemolysin showing clear bilayer formation in the droplet network. Bilayer area is larger without the presence of food color dye. C) Fluorescence image of water droplets containing calcein (bright droplets) or α -hemolysin (dark droplets) showing that the bilayer is stable and droplets have not coalesced.

hydrodynamic resistance is given by:

$$R_h = \frac{12\mu L}{h^3 w} \left[1 - \frac{192}{\pi^5} \times \frac{h}{w} \times \tanh\left(\frac{\pi w}{2h}\right) \right]^{-1} \quad (3)$$

where μ ($\text{kg } \mu\text{m}^{-1} \text{ s}^{-1}$) is the viscosity of the continuous phase (i.e. oil for a water droplet in oil system) and L , w , and h are the microchannel section length, width, and height (μm) respectively. This is an approximation as droplets in the main channel alter the resistance slightly [59-61].

Previously, it was shown that multiple hydrodynamic trap arrays, located parallel to the main channel, can be used to sequentially trap droplets to enable DIB formation [18]. Each trap has a narrow exit channel, referred to as a bleed valve, at the opposite side of the trap from the main channel. Droplets enter traps when the resistance of the vacant trap (R_T) is less than the resistance of the main channel (R_M). Droplets will remain in place as long as the resulting pressure drop across a filled trap is also less than the Laplace pressure. The Laplace pressure, P_L , of a droplet blocking a bleed valve is given by:

$$P_L = 2\gamma \left(\frac{1}{w_b} - \frac{1}{w_t} \right) \quad (4)$$

where γ (mN m^{-1}) is the interfacial tension at the liquid interface and w_b and w_t are the widths of the bleed valves and trap in microns, respectively [51, 62]. Once a trap is filled by a droplet, the resistance in the trap increases greatly (modeled using an open circuit), such that all subsequent droplets bypass the filled trap and continue through the main channel to the next empty trap.

Droplet production rates

Figures A.3-5 show the droplet production rates of 90° , 150° , and 180° phase shifts between sine wave pressure inputs. Droplets are generated by two sets of

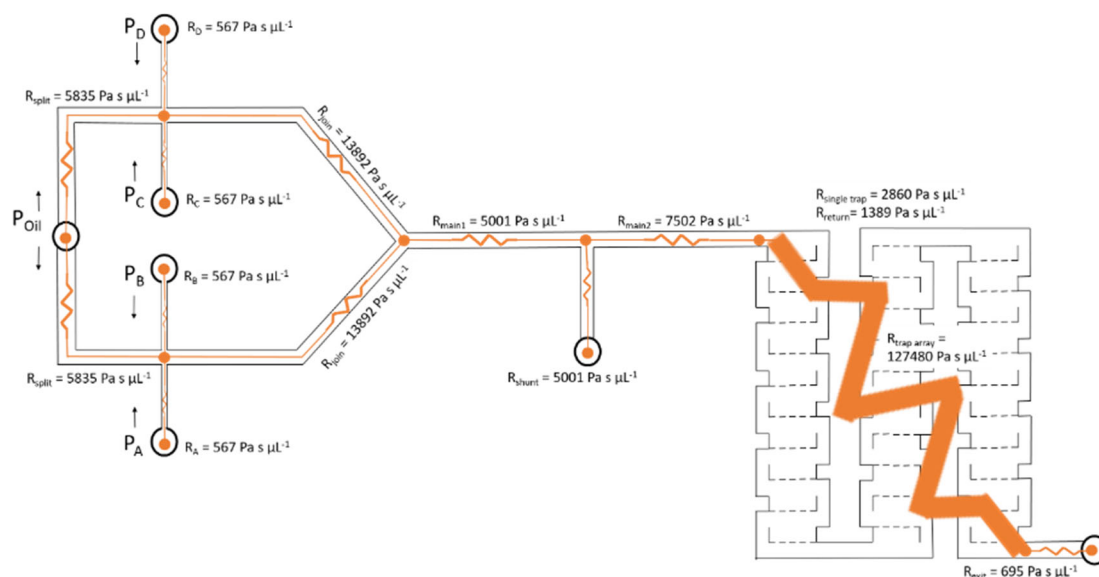


Figure A.2 Schematic of the designed circuit model with the calculated resistance of channel sections.

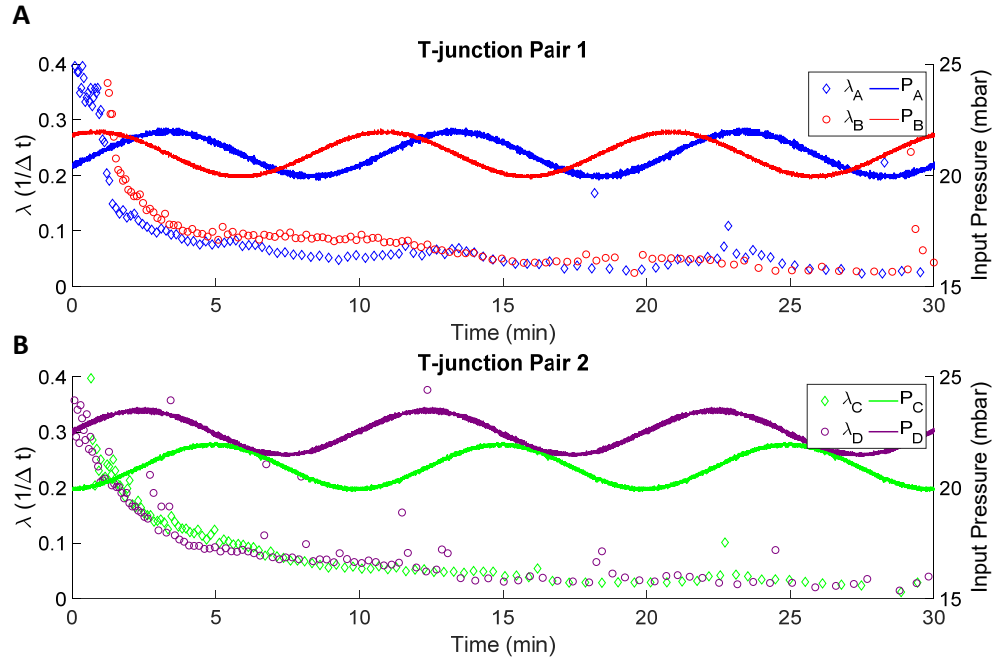


Figure A.3 90° phase shift. Patterns are generated by applying sinusoidal pressure waves and thereby altering the droplet production rates and the resulting droplet sequence. (A) Input pressure waves and corresponding droplet production rates for droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding droplet production rates for droplets formed at the second pair of opposing T-junctions.

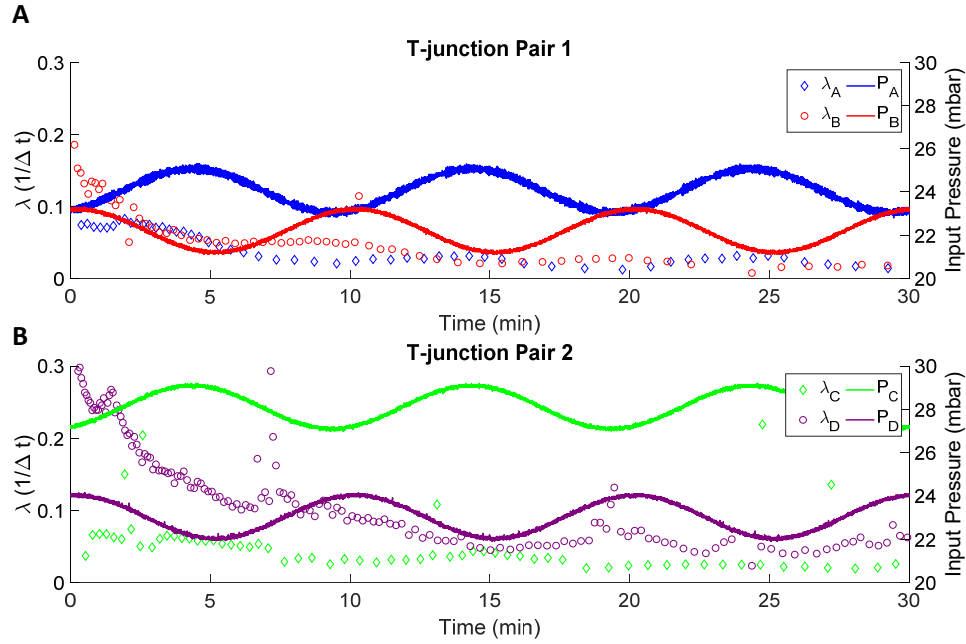


Figure A.4 150° phase shift. Patterns are generated by applying sinusoidal pressure waves and thereby altering the droplet production rates and the resulting droplet sequence. (A) Input pressure waves and corresponding droplet production rates for droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding droplet production rates for droplets formed at the second pair of opposing T-junctions.

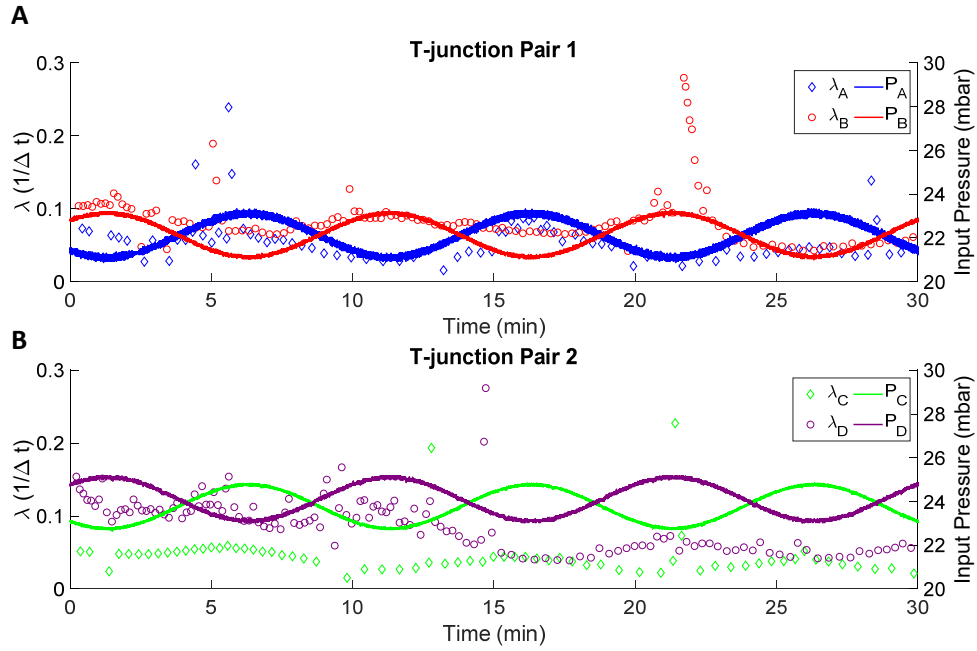


Figure A.5 180° phase shift. Patterns are generated by applying sinusoidal pressure waves and thereby altering the droplet production rates and the resulting droplet sequence. (A) Input pressure waves and corresponding droplet production rates for droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding droplet production rates for droplets formed at the second pair of opposing T-junctions.

opposing T-junctions set in parallel. A phase shift is applied to the input pressures between each set of T-junctions, while the continuous phase pressure is held constant. Individual droplet production rates trend with the corresponding input pressure. Droplet production rates are highest at pressure peaks.

Measuring Droplet Size

CellSens Count and Measure software uses threshold color detection to identify objects and measure the droplet cross-sectional areas (Figure A.6). Threshold detection identified droplet types based on color. However, due to refraction at the curvature points, this method excluded droplet edges. To correct for the fractional size measurement, 5 droplets of each color type were also measured by hand in order to estimate a coefficient for size correction: 1.547 ± 0.233 , 1.133 ± 0.133 , 1.277 ± 0.056 , and 1.507 ± 0.068 for blue, red, green, and purple droplets respectively. Droplet diameters measured by software were then multiplied by their respective corrective coefficients.

Figures A.7-9 show the adjusted droplet equivalent circle diameters of 90°, 150°, and 180° phase shifts between sine wave pressure inputs, respectively. Droplet diameter increases as the pressure ratio of disperse phase to continuous phase increases.

Droplet patterns

Droplet patterns are generated for the purpose of capturing droplet networks in a hydrodynamic trap array. Each trap is designed to capture and hold in place four droplets. By analyzing droplet patterns before closing the shunt, droplet patterns can be predicted for trapping. Figure A.10-12 show histograms comparing the droplet patterns for shunt open all viewed (SO-AV), shunt closed all viewed (SC-AV), shunt closed ideal (SC-Ideal) and actual trapped patterns.

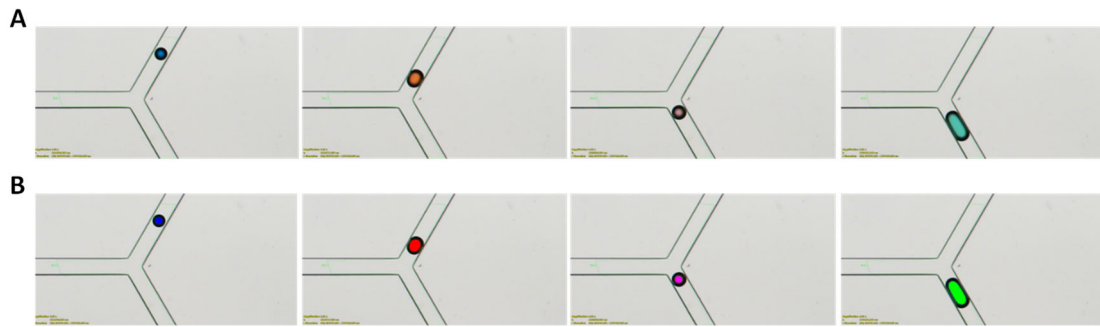


Figure A.6 Droplet cross-sectional area was measured by CellSens Count and Measure software. A) Droplets with blue, red, purple, and green food dye before measurements were taken. B) Threshold detection of droplets measuring cross-sectional area. Droplet edges are excluded from the measurements due to refraction, leading to measured sizes being less than the actual droplet size.

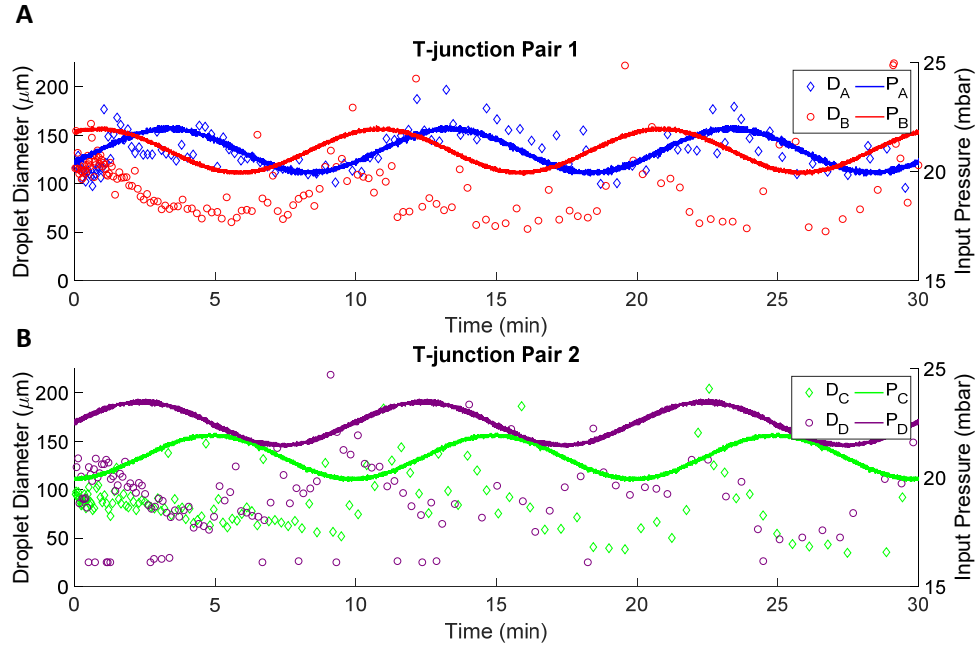


Figure A.7 90° shift. Droplet size change with applied pressure input. (A) Input pressure waves and corresponding diameters of droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding diameters of droplets formed at the second pair of opposing T-junctions.

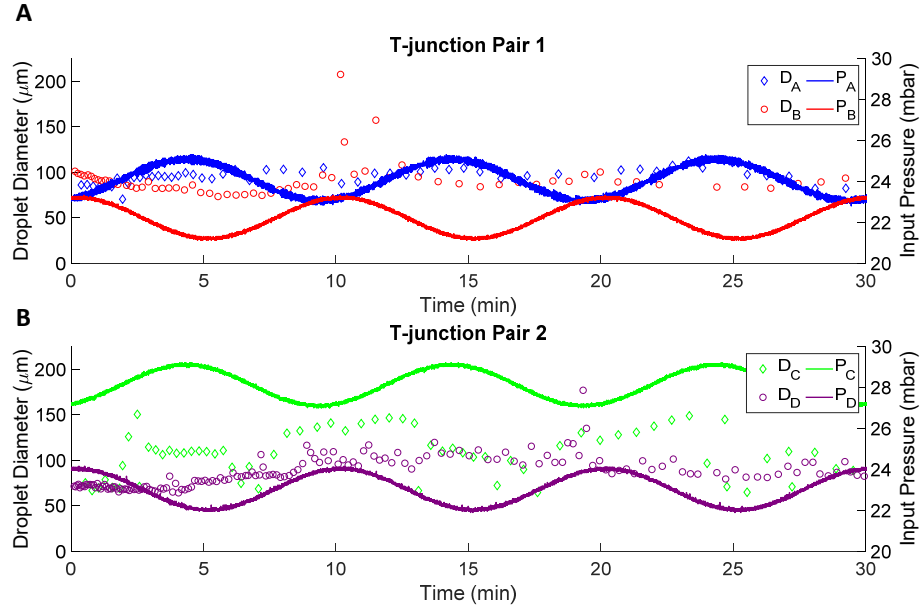


Figure A.8 150° shift. Droplet size change with applied pressure input. (A) Input pressure waves and corresponding diameters of droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding diameters of droplets formed at the second pair of opposing T-junctions.

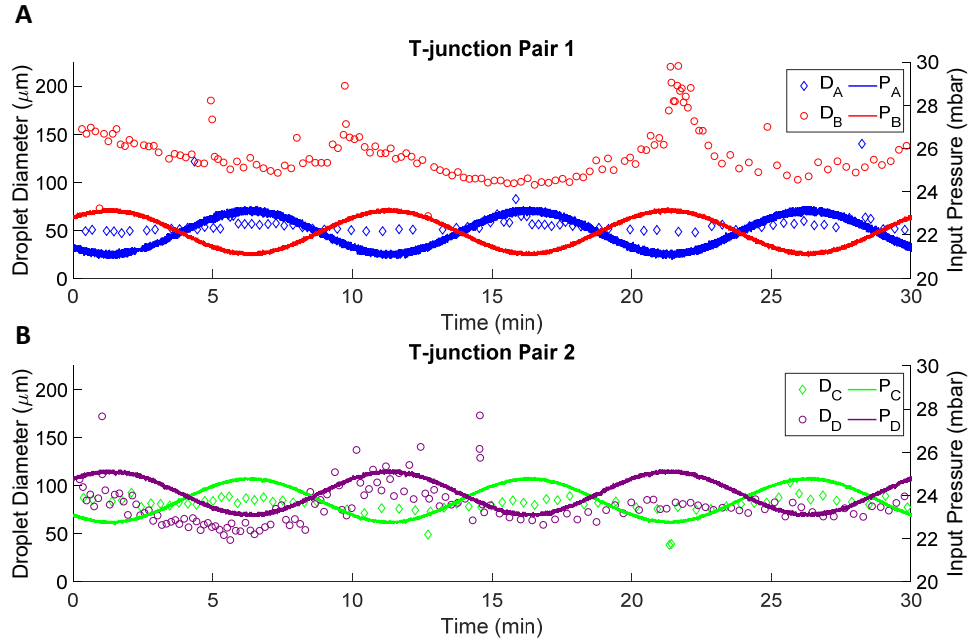


Figure A.9 180° shift. Droplet size change with applied pressure input. (A) Input pressure waves and corresponding diameters of droplets formed at the first pair of opposing T-junctions. (B) Input pressure waves and corresponding diameters of droplets formed at the second pair of opposing T-junctions.

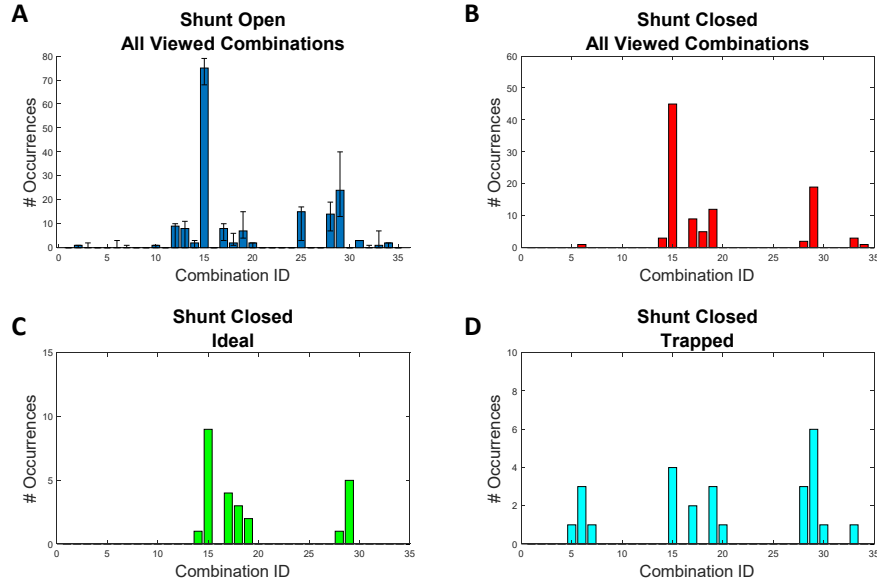


Figure A.10 Generated droplet patterns for 90° pressure shift in the cases of (A) open shunt- all viewed combinations, (B) shunt closed- all viewed combinations, (C) shunt closed ideal pattern, and (D) trapped patterns. All viewed combinations are comprised of four droplets, identified starting with every droplet in the droplet train and regardless of order. Ideal patterns are comprised of four droplets, identified by every 4th droplet in the droplet train, starting with the first droplet observed after the shunt is closed. These patterns are the droplet combinations that are predicted to be trapped if errorless trapping were to occur in the device. Trapped patterns are droplet combination networks that are observed once all traps in the array are filled with four droplets.

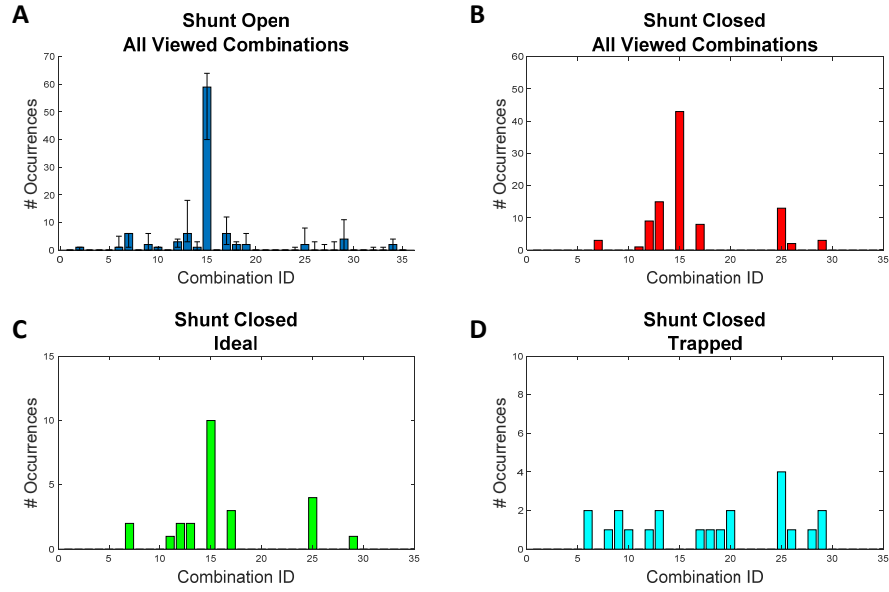


Figure A.11 Generated droplet patterns for 150° pressure shift in the cases of (A) open shunt- all viewed combinations, (B) shunt closed- all viewed combinations, (C) shunt closed ideal pattern, and (D) trapped patterns. All viewed combinations are comprised of four droplets, identified starting with every droplet in the droplet train and regardless of order. Ideal patterns are comprised of four droplets, identified by every 4th droplet in the droplet train, starting with the first droplet observed after the shunt is closed. These patterns are the droplet combinations that are predicted to be trapped if errorless trapping were to occur in the device. Trapped patterns are droplet combination networks that are observed once all traps in the array are filled with four droplets.

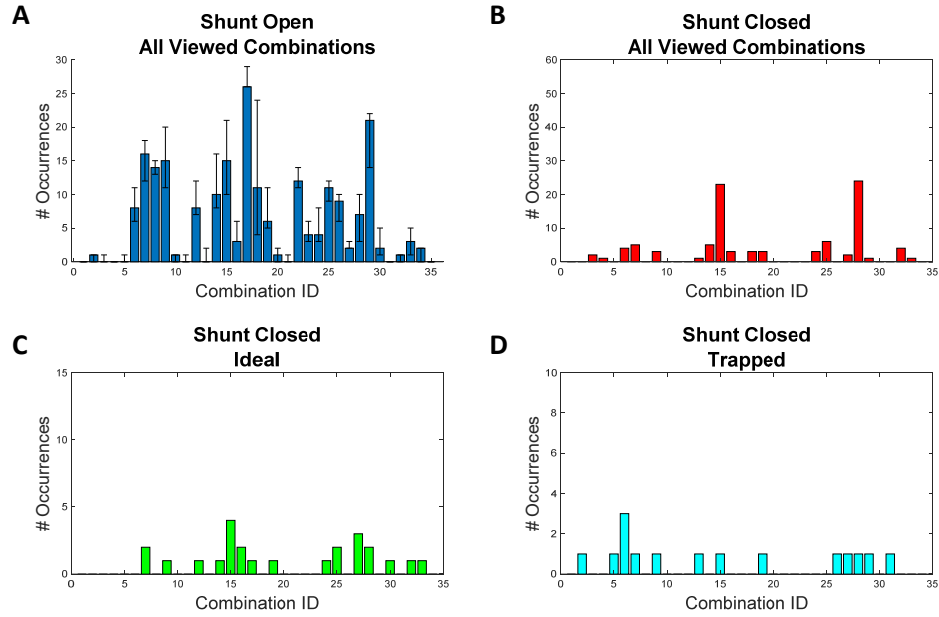


Figure A.12 Generated droplet patterns for 180° pressure shift in the cases of 9A) open shunt- all viewed combinations, (B) shunt closed- all viewed combinations, (C) shunt closed ideal pattern, and (D) trapped patterns. All viewed combinations are comprised of four droplets, identified starting with every droplet in the droplet train and regardless of order. Ideal patterns are comprised of four droplets, identified by every 4th droplet in the droplet train, starting with the first droplet observed after the shunt is closed. These patterns are the droplet combinations that are predicted to be trapped if errorless trapping were to occur in the device. Trapped patterns are droplet combination networks that are observed once all traps in the array are filled with four droplets.

Trapped droplet patterns

Figure A.13 shows the final results of trapped droplet patterns. Trapped patterns discussed in main article were determined at these time points, when all traps were filled. Traps that remain empty at this point in time indicate that the pillars between bleed valves were damaged. Droplets enter the trap array from the main channel visible in the top right of each image and the direction of flow is initially from right to left and then winding through the trap array. The top right trap is taken as the first trap in the array and the bottom left is the 30th. Droplets become noticeably smaller in the left most column of traps, giving the only occurrences of 5 droplets being captured. The majority of occurrences where only 3 droplets were captured in a trap is also seen to happen in the right most column, thus verifying that droplet size decreased as the traps were filled. Trapped droplet networks of 3 or 5 droplets were not counted in trapped pattern results, this accounts for some, but not all, of the errors in pattern results. Videos of droplets being trapped show occasional indirect trapping, likely due to pressure fluctuations in the system.

Pattern Repeatability

Pattern replicates were done for the 90° shift experiment (Figure A.14); each trial was run on a separate microchip. Combination #15 was the most common and consistent across all trials, while there was more variability among all other patterns. The variability in pattern results is primarily due to the inconsistencies between microchips and the consequentially different pressure inputs for and equilibrium of droplet production rates. Patterns should therefore be established for each new microchip independently.

Comparison of droplet equivalent circle diameter for shunt open vs. shunt closed

Droplet cross-sectional areas were measured by CellSens software and converted to equivalent circle diameter. Droplet diameter decreased significantly upon shunt closure and became more uniform (Figure A.15-17).

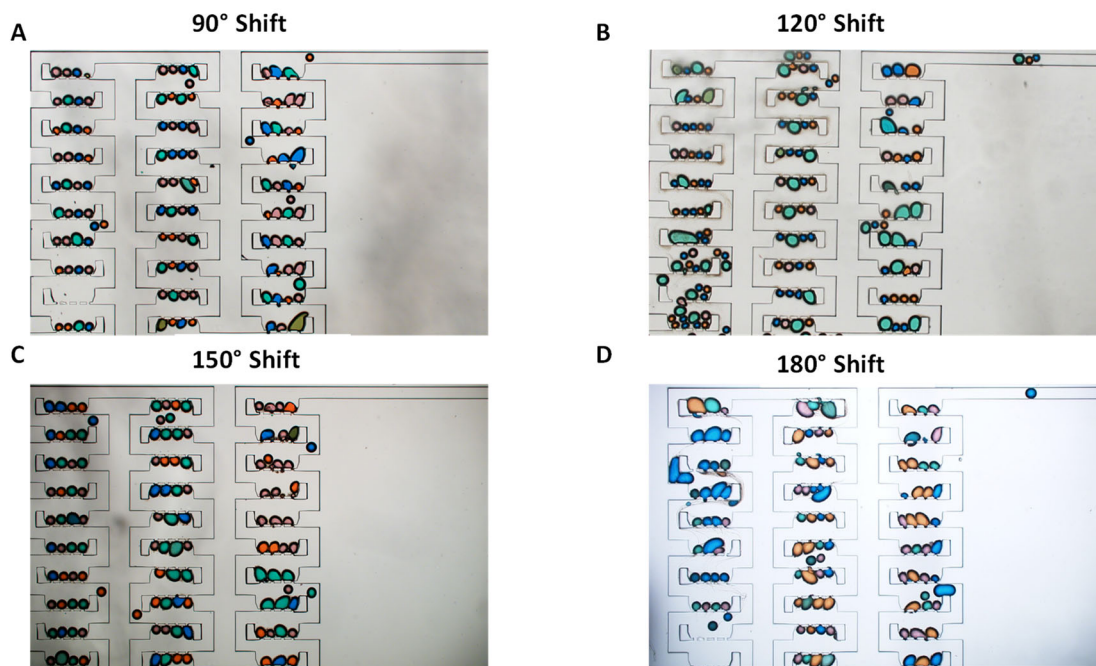


Figure A. 13 Images of final trapped droplet network for 90°, 120°, 150°, and 180° phase shift data sets.

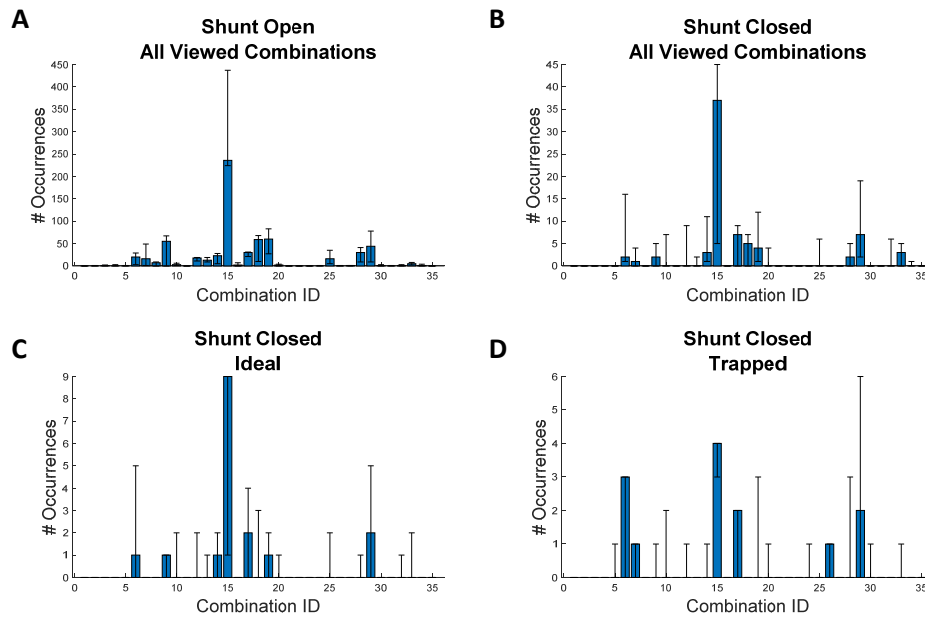


Figure A.14 The results of three replicates of the 90° shift experiments. Blue bars indicate the median number of occurrences of a specific pattern with error bars indicating the minimum and maximum number of occurrences observed.

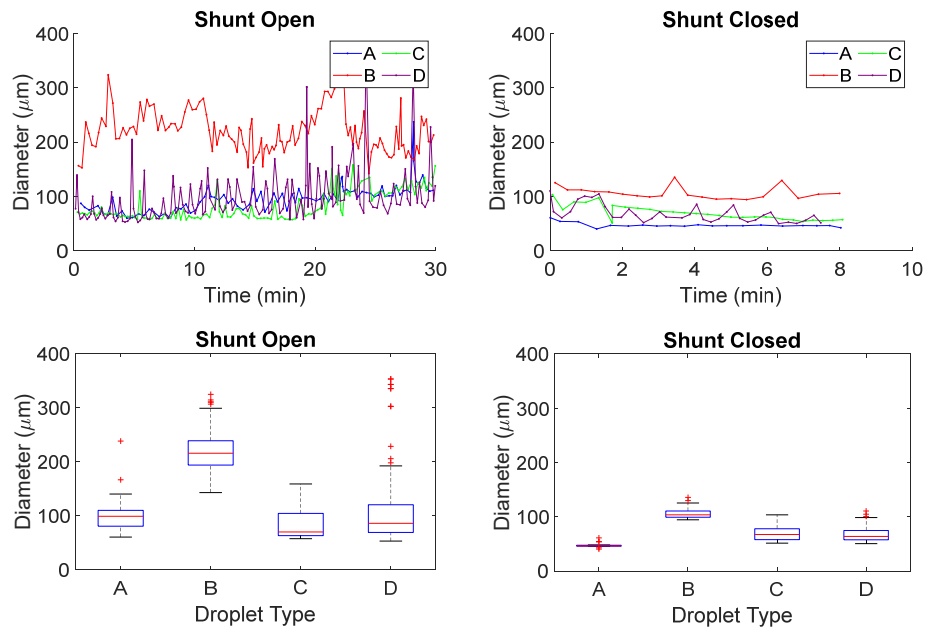


Figure A.15 Droplet cross-sectional area for a 90° shift shunt open and shunt closed cases. (A) Scatter plot of droplet size measurements when shunt is open. Droplet size fluctuations are evident. (B) Scatter plot of droplet size measurements when shunt is closed. Droplet size decreases slowly as traps are filled. (C) Boxplot of droplet size when shunt is open. Droplet size varies greatly and slugs are often produced. (D) Boxplot of droplet size when shunt is closed. Droplet size is decreased and more consistent, when the shunt is closed.

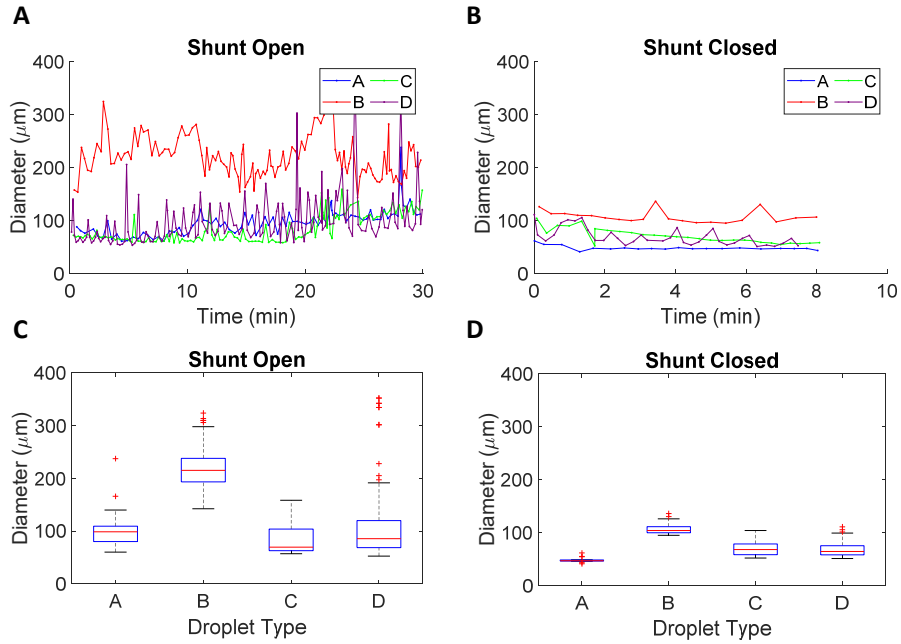


Figure A.16 Droplet cross-sectional area for a 150° shift shunt open and shunt closed cases. A) Scatter plot of droplet size measurements when shunt is open. Droplet size fluctuations are evident. B) Scatter plot of droplet size measurements when shunt is closed. Droplet size decreases slowly as traps are filled. C) Boxplot of droplet size when shunt is open. Droplet size varies greatly and slugs are often produced. D) Boxplot of droplet size when shunt is closed. Droplet size is decreased and more consistent, when the shunt is closed.

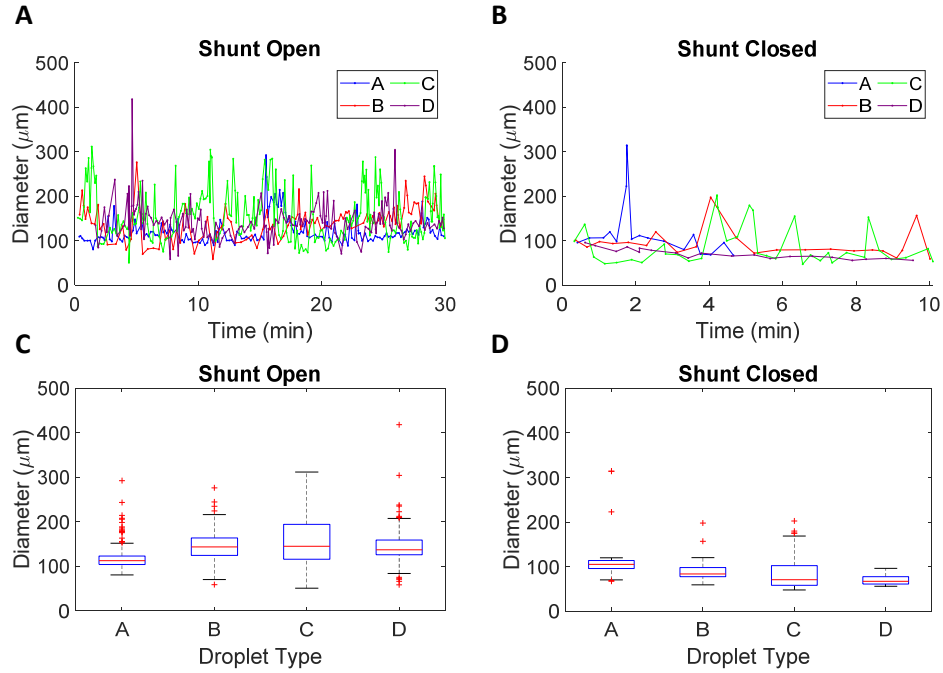


Figure A.17 Droplet cross-sectional area for a 180° shift shunt open and shunt closed cases. A) Scatter plot of droplet size measurements when shunt is open. Droplet size fluctuations are evident. B) Scatter plot of droplet size measurements when shunt is closed. Droplet size decreases slowly as traps are filled. C) Boxplot of droplet size when shunt is open. Droplet size varies greatly and slugs are often produced. D) Boxplot of droplet size when shunt is closed. Droplet size is decreased and more consistent, when the shunt is closed.

Simulink resistive circuit model

Microfluidic channel design can be guided by modeling the system as an equivalent electric circuit [55, 56]. An electrical circuit model of the microfluidic design was developed using MATLAB Simscape software for both syringe pump and pressure-drive flow for the purpose of illustrating the differences between the two pump systems (Figure A.18). In the electrical circuit analogy, a syringe pump's applied flow rate can be modeled as DC current and applied pressure is modeled as DC voltage [57, 58]. For the syringe pump model, input currents were set as 16.7 pA and 8.3 pA for the continuous phase and four aqueous phase channels respectively, comparable to flow rates of $1 \mu\text{L min}^{-1}$ and $0.5 \mu\text{L min}^{-1}$ (where $1 \text{ A} \approx 1 \text{ m}^3 \text{ s}^{-1}$ and $1 \text{ m}^3 \text{ s}^{-1} \approx 6 \times 10^7 \mu\text{L min}^{-1}$) [56]. For modeling pressure-driven flow, voltages of 3000 V and 4000 V were applied to the continuous and aqueous phases respectively, comparable to 30 mbar and 40 mbar (where $1 \text{ V} = 1 \text{ Pa}$ and $1 \text{ Pa} = 0.01 \text{ mbar}$). For each system, the current and voltage at the Y-junction and at the array outlet were calculated at five stages of device operation: (1) the shunt was open, allowing droplets to exit the device, (2) immediately after the shunt was closed, before any droplets are trapped, (3) when 10 of the traps are filled, (4) when 20 traps were filled, and (5) when all 30 traps are filled with droplets. Results of this effort are shown in Table A.1. The syringe-pump-driven model gives a constant current of 4.99 pA at the Y-junction and array outlet and the voltage remains constant at $1.01 \times 10^{-7} \text{ V}$ while the shunt is closed and the traps are filling. Therefore, in syringe-pump driven flow, the flow rate and pressure are not expected to change as droplets fill the system. Conversely, for the pressure-driven model, the current and voltage calculated at the array outlet are shown to decline as the traps are filled: indicating a decrease in flow rate within the device. This finding confirms the previously discussed experimental results of droplet velocity decreasing as traps are filled. Additionally, the drop in pressure at the array outlet was confirmed by connecting a pressure sensor to the exit channel, where there

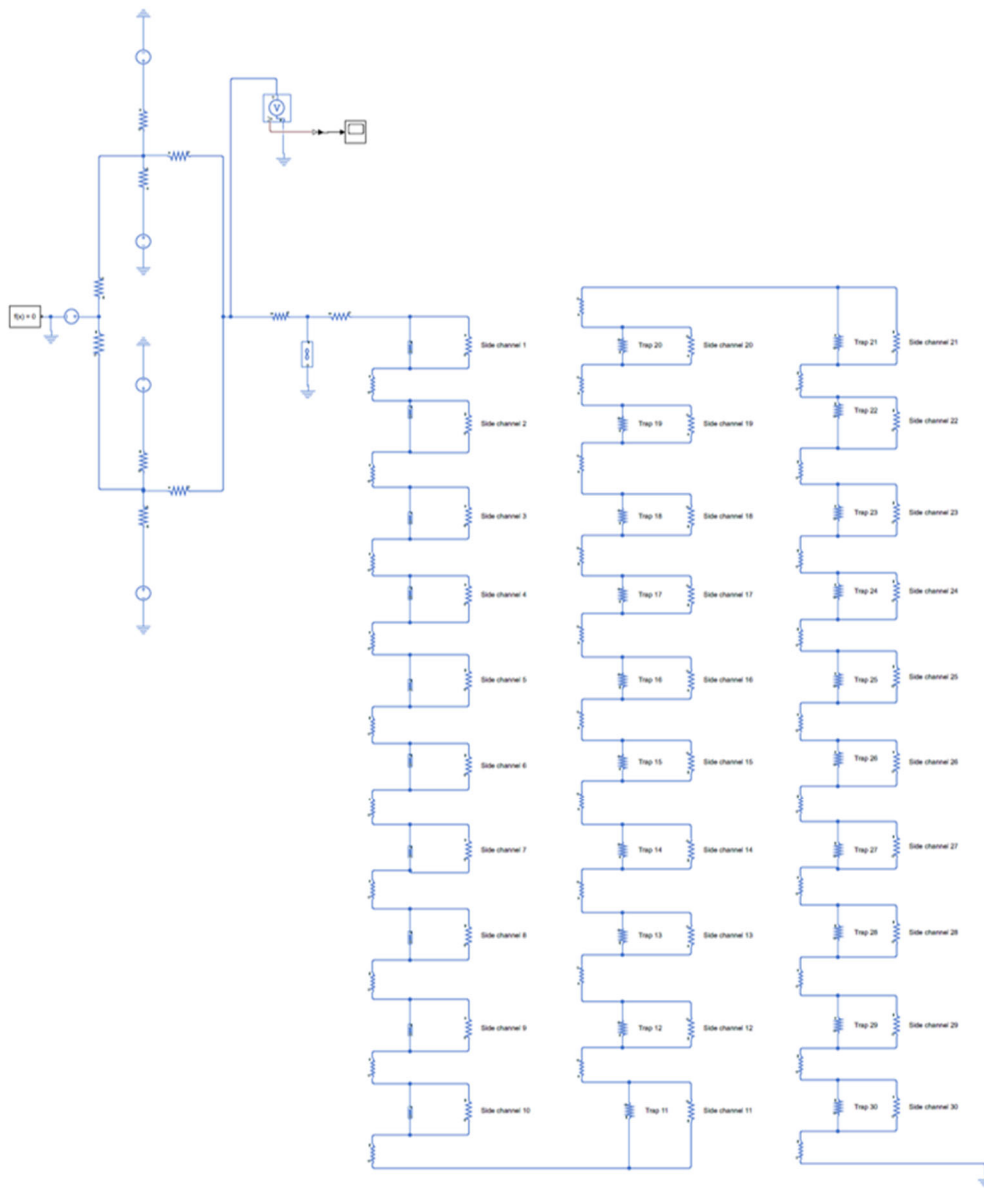


Figure A.18 Resistive circuit model of a microfluidic device for generating, sorting and trapping complex droplet networks.

Table A.1 Comparison of flow behavior between syringe pump system (current input) and pressure-driven system (voltage input).

Voltage and Pressure Measured at Array Outlet				Current at Array Outlet			
	Experimental Pressure (mbar)	Theoretical Input Constant Voltage (V)	Theoretical Input Constant Current (V)	Experimental Droplet Velocity ($\mu\text{m s}^{-1}$)	Flow Rate ($\mu\text{L min}^{-1}$)	Theoretical Input Constant Voltage (A)	Theoretical Input Constant Current (A)
Shunt open	-	0	3.08E+05	-	-	0	4.34
Shunt closed; empty	993.7	69	6.70E+18	2427	2.27	0.05	69.3
Shunt closed; 10 full	934.8	63	1.03E+19	2341	2.19	0.046	69.3
Shunt closed; 20 full	568.1	57.9	2.12E+19	1997	1.87	0.043	69.3
Shunt closed; 30 full	437.6	53.6	9.63E+04	1511	1.42	0.039	69.3
Voltage and Pressure Measured at Y-junction				Current at Y-junction			
	Experimental Pressure (mbar)	Theoretical Input Constant Voltage (V)	Theoretical Input Constant Current (V)	Experimental Droplet Velocity ($\mu\text{m s}^{-1}$)	Flow Rate ($\mu\text{L min}^{-1}$)	Theoretical Input Constant Voltage (A)	Theoretical Input Constant Current (A)
Shunt open	1409.7	2244.8	6.55E+05	4013	3.76	0.5617	69.3
Shunt closed; empty	-	3625.5	6.70E+18	-	-	0.5617	69.3
Shunt closed; 10 full	-	3656.25	1.03E+19	-	-	0.5617	69.3
Shunt closed; 20 full	-	3682	2.12E+19	-	-	0.5617	69.3
Shunt closed; 30 full	-	3704	6.64E+06	-	-	0.5617	69.3

is a significant change in pressure after 20 traps are filled (Figure A.19). The alteration in flow behavior when using pressure-driven flow, as opposed to syringe-pump-driven flow, is not ideal for this hydrodynamic trap design. However, this effect can easily be corrected by connecting a flow rate sensor to each fluid input; allowing for flow rate control via pressure. Alternatively, the continuous phase oil pressure can be made to decrease as traps fill with droplets.

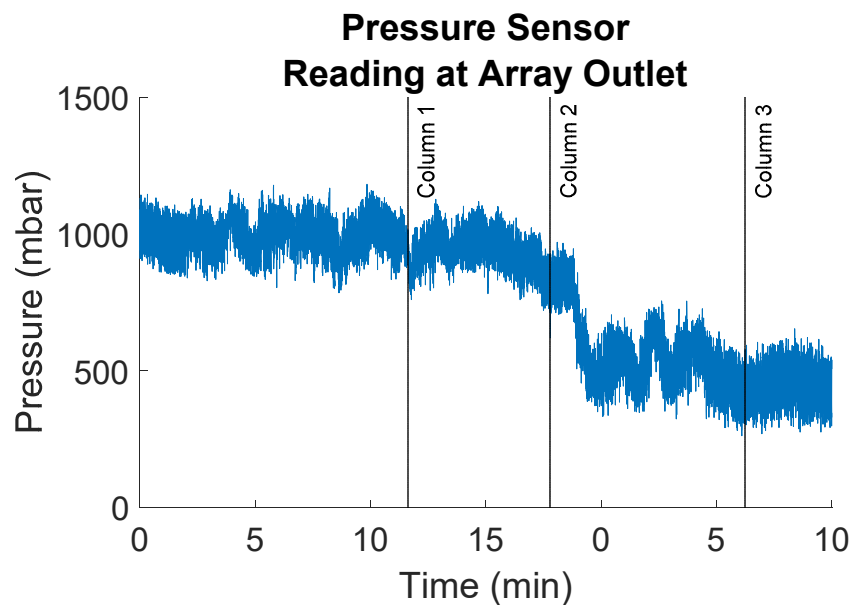


Figure A.19 Pressure sensor output recorded by Elveflow pressure sensor placed at trap array outlet. Lines indicate times at which the first ten traps are filled, twenty traps are filled, and all thirty traps are filled, $t_{10}=4.65$ min, $t_{20}=7.12$, and $t_{30}=10.5$ min respectively. Pressure drops significantly as traps fill.

CHAPTER III
FLUORESCENCE-BASED WHOLE PLANT IMAGING AND
PHENOMICS

A version of this chapter was originally submitted by Stephen B. Rigoulot:

Stephen B. Rigoulot, Tayler M. Schimel, Jun Hyung Lee, Robert G. Sears, Holly Brabazon, Jessica S. Layton, Li Li, Kerry A. Meier, Magen R. Poindexter, Manuel J. Schmid, Erin M. Seaberry, Jared W. Brabazon, Jonathan A. Madajian, Michael J. Finander, John DiBenedetto, Alessandro Occhialini, Scott C. Lenaghan, C. Neal Stewart, Jr. "Imaging of multiple fluorescent proteins in canopies enables synthetic biology in plants." *Plant Biotechnology Journal* (2020). <https://doi.org/10.1111/pbi.13510>

For the work reported in this chapter, TMS travelled to the Special Technologies Lab in Santa Barbara, CA for a one-week training session on class 4b laser operation for fluorescent plant imaging. TMS played a major role in the designing and building of the FILP instrument and designed and built the laser enclosure. TMS communicated with the Laser Safety Office at the University of Tennessee to ensure that FILP within safety guidelines and regulations. TMS optimized the operation of FILP for multiple projects, collected and analyzed data generated by FILP, trained fellow researchers, and continues to repair and maintain the system. TMS also identified a simplified method for analyzing plant fluorescence, adapted that method to meet the needs of multiple projects.

CNS, JDB, SCL, TMS, and SBR conceived of the research. JDB, JAM, MJF, SCL, TMS, and CNS played roles in designing and building the FILP instrument. SBR, TMS, JHL, SCL, and CNS wrote and prepared the manuscript. SBR, KAM, HB and JHL were responsible for the organization of figures. SBR, TMS, JHL, HB, MJS, KAM, MRP, JSL, MJS, AO, SCL and EMS were responsible for the design and construction of all constructs tested, plant care, potato cell culture, protoplast assays, agroinfiltration experiments, confocal microscopy, and FILP imaging and spectroscopy. KAM, RGS and SBR designed and executed the Y2H and BiFC

experiments. AO and LL transformed and characterized stable GFP expressing potato. MJS and SBR developed the agroinfiltration apparatus and produced the Supplementary video. HB and JWB were responsible for the conception and writing of the Fluorologger software. SBR and KAM designed the custom plant stand.

This research was developed with funding from the Defense Advanced Research Projects Agency (DARPA) Award No. HR0011-18-2-0049 and Department of Energy (DOE) Grant No. DE-SC0018347. The views, opinions and/or findings expressed are those of the authors and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government. (Approved for Public Release, Distribution Unlimited).

3.1 Abstract

Reverse genetics approaches have revolutionized plant biology and agriculture. Phenomics has the prospect of bridging plant phenotypes with genes, including transgenes, to transform agricultural fields. Genetically-encoded fluorescent proteins (FPs) have revolutionized plant biology paradigms in gene expression, protein trafficking, and plant physiology. While the first instance of plant canopy imaging of green fluorescent protein (GFP) was performed over 25 years ago, modern phenomics has largely ignored fluorescence as a transgene expression device despite the burgeoning FP color palette available to plant biologists. Here we show a new platform for standoff imaging of plant canopies expressing a wide variety of FP genes. The platform—the fluorescence-inducing laser projector (FILP)—uses an ultra-low-noise camera to image a scene illuminated by compact diode lasers of various colors, coupled with emission filters to resolve individual FPs, to phenotype transgenic plants expressing FP genes. Each of the 20 FPs screened in plants were imaged at > 3 m using FILP in a laboratory-based laser range. We also show that pairs of co-expressed fluorescence proteins can be

imaged in canopies. The FILP system enabled a rapid synthetic promoter screen: starting from 2000 synthetic promoters transfected into protoplasts to FILP-imaged agroinfiltrated *Nicotiana benthamiana* plants in a matter of weeks, which was useful to characterize a water-stress-inducible synthetic promoter. FILP canopy imaging was also accomplished for stably-transformed GFP potato and in a split-GFP assay, which illustrates the flexibility of the instrument for analyzing fluorescence signals in plant canopies.

3.2 Introduction

Phenomics seeks to tightly connect genotype to phenotype across various environmental conditions [63, 64], which would enable translation of lab-based research to agricultural production and sustainability [65]. The various scales of phenotyping currently cover ranges from sub-micron/microscopic to satellite-based imaging of > 2000 km, with tremendous disconnect between these scales. We posit that the ‘sweet spot’ to connect genes to phenotypes as well as genomes to phenomes—for both reductionistic-mechanistic levels and ecological levels—lies at the scope of the plant canopy (meters); currently there is a technological void at this range. At the microscopic level where most basic research takes place, studies assess cellular-to-subcellular activities using state-of-the-art microscopes and molecular probes, for which innovations are numerous [27, 66].

At the whole-plant-to-field level of assessment, there is tremendous potential for detecting environmental stresses on crops. The chief problem with “small scale” laboratory studies is that they are confined to tightly-controlled artificial conditions. Field experiments and radiometric models of vegetation remote sensing, enabling phenome-to-genome associations, have several key problems: 1) ‘real-life’ systems generate data that is extremely noisy due to optical artifacts (incomplete illumination, subpixel mixing, bidirectional effects, etc.); and 2) in complex

environments connecting robustly-measured phenomes to genes and genomes is tenuous [63, 64, 67].

In plant biology and agriculture, the most useful optical signals would be those that are unambiguous and occupy distinct spectral wavelengths from endogenous plant molecules. Leaf-produced compounds such as alkaloids, terpenoids, and chlorophyll produce sizable spectral ‘noise’ in plants in the form of autofluorescence [68]. In addition to avoiding spectral noise, heterologous signals should be directly tied to traits and genes. Indeed, a collection of these “ideal” spectral signatures could be stacked for multispectral signaling to expand the diversity of applications. FPs fit these criteria and can be universally imaged in plant organs. Certainly, canopy-level FP imaging is facile for UV-excitable FPs such as (near) wildtype GFP [69] and recently-characterized GFP variants, such as those expressed in ornamental plants [70]. UV-excitable GFP can be easily imaged at the sub-meter level, e.g., seedlings and small canopies, because emission filters are not required and GFP fluorescence may be seen in the dark [30]. Previously, researchers have developed an inexpensive imaging system using blue LED arrays to excite GFP engineered into *Arabidopsis* [71], in which dichroic filter cubes were coupled with an inexpensive camera, which could image cm-scale seedling ‘canopies.’ At the other end of the cost spectrum a portable laser-induced fluorescence imaging (LIFI) system containing a tripled Nd:YAG laser (355 nm) has been used to excite UV-excitable GFP in plants at a standoff (3 m), but this instrument was very expensive [72]. In order to move to higher efficiency light sources and multiple wavelengths, non-imaging techniques were explored to frequency modulate 405 nm laser diodes and a fluorescence spectrometer was used to detect signals at distances greater than 2 km in field experiments [73, 74]. All current remote FP-imaging systems lack flexibility with regards to imaging a variety of FPs and cannot simultaneously image multiple FPs in multiple plants at the canopy level. Here we show the performance of a relatively

inexpensive custom device (< \$50,000 USD), FILP, that images plant canopies expressing various FPs at > 3 m in a laboratory setting in both constitutive and induced modalities. In addition, we show how FILP enables advanced plant biotechnology and synthetic biology by imaging fluorescence signatures in plant canopies that are the product of an abiotic stress-induced synthetic promoter driving GFP.

3.3 Results

3.3.1. Fluorescence-inducing laser projector (FILP) imaging of plant canopies

The flexibility of FILP lies in the ability to select diode lasers for FP excitation and custom filters for filtering emission. In the initial build, violet (400 nm), blue (465 nm) and green lasers (524 nm) were installed in the system. These excitation wavelengths were chosen because they can excite virtually any FP variant in the current color palette (Figure 3.1). The diode laser beam was homogenized to produce an illumination pattern that was highly uniform (flat and smooth), which maximized spatial resolution across the area of illumination, which was approximately 400 cm². Emission filters, housed in an automated filter wheel, were specifically chosen to prevent crosstalk between multiple FPs. The final major component of the system was a laboratory-grade digital camera that enabled the capture of high-resolution whole plant images. As designed, the system allows modular substitution of both diode lasers and emission filters to customize imaging of fluorescent signatures in plants, which can be accomplished at the end-user level. The goal of our study was to conduct near-simultaneous imaging of multiple, spectrally-distinct FPs at the whole canopy level in plants as a new modality of plant phenomics, and to then apply FILP imaging to enable synthetic biology.

To determine the flexibility of the FILP system, 20 FPs were characterized in agroinfiltrated *Nicotiana benthamiana*. The inherent excitation peaks for each FP

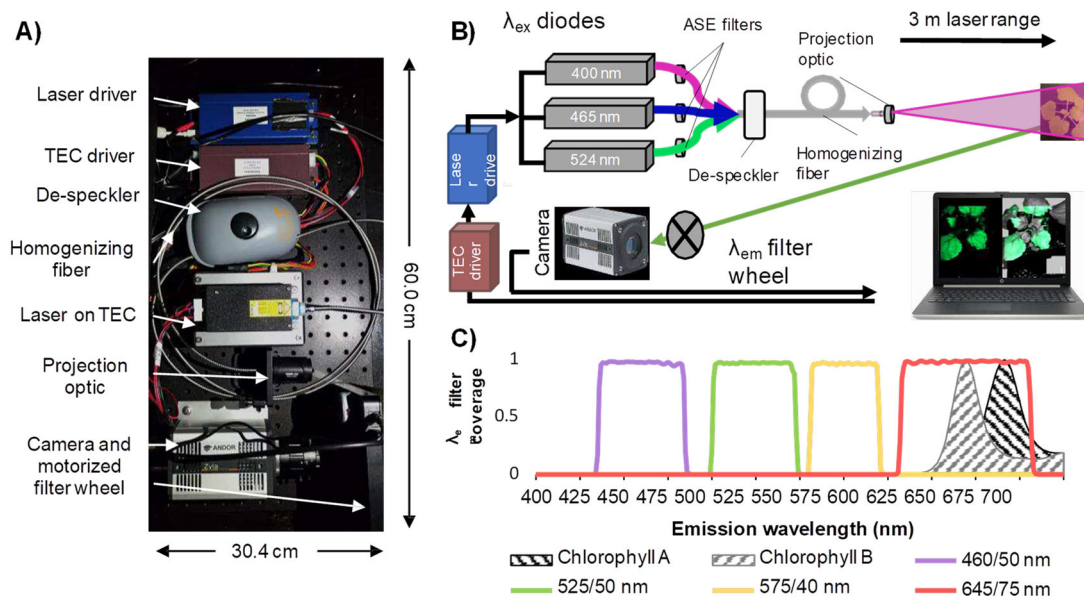


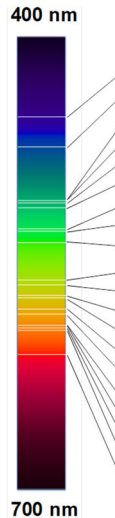
Figure 3.1 Fluorescence-inducing laser projector (FILP) components. (a) Photograph of FILP system breadboard. (b) Schematic illustrating the setup of components. Abbreviations: Thermo-electric cooler (TEC); amplified spontaneous emission (ASE) (c) Combined line and area plot. Line plots show the wavelengths covered by the 460/50 nm, 525/50 nm, 575/40 nm and 645/75 nm notch filters, in which the first numeral is the center point and the second numeral is the breadth of the notch filter. The line plot also includes area plots which indicate the auto-fluorescence emission by chlorophylls A (excitation 614 nm) and B (excitation 435 nm) in diethyl ether.

variant ranged from UV (395 nm) to orange (578 nm) with emission peaks from blue (454 nm) to red (611 nm) (Table 3.1). The FPs also had a wide range of extinction coefficients, quantum yields, and oligomerization states to allow for a plethora of multi-spectral imaging schemes in plants. Each of these FP genes was placed under the control of a constitutive doubled 35S promoter in a common vector and expressed in *N. benthamiana* using either whole plant or whole leaf vacuum-agroinfiltration [75]. Given the range of FP characteristics, many of which were suboptimal for the initial laser diodes and emission filters chosen for FILP, we were surprised that all FPs could be imaged in the plant canopies (Figure 3.2). Owing to these suboptimal excitation and emission matches (FPs vs. FILP), in some cases, coupled with differences in relative brightness of FPs, there was a four-fold difference between power requirements for imaging among FPs in plants and the respective imaging channels (Table B.1). Fluorescence imaging was complemented by on-the-plant fluorescence spectroscopy measurements that modeled the three laser excitation frequencies (Table B.1). The heatmap from fluorescence spectroscopy data as well as signal-to-noise ratios of each FP emission peak, relative to a buffer infiltrated control excited at the same wavelength, was congruent with imaging results (Table B.1). Therefore, we conclude FILP imaging is an apt indicator of FP expression in transgenic plants.

3.3.2. Multiplexed fluorescence imaging in plant canopies

Of the 20 FPs initially screened in plants, four of these were selected as top performers with regards to stacking and/or imaging together in single plants or plant canopies: mTagBFP2, mEmerald, TurboRFP and mScarlet-I (Figure 3.3). The ‘top FP’ emissions were in four distinct color bands and were consistently brighter than others in those color bands. Compared with other color band emissions, blue light-emitting FP candidates were few. When designing FILP components, we purposefully matched the laser and emission filter to the optimal blue fluorescence protein spectra of mTagBFP2 (Figure 3.1 and Table 3.1).

Table 3.1 Fluorescent proteins engineered into and imaged in plants using the FILP laser range. Abbreviations: excitation λ (Ex), emission λ (Em); extinction coefficient (EC); quantum yield (QY); relative brightness (RB). * indicates that the EC, QY and RB data was taken from avGFP, the wild-type GFP.



Fluorescent protein	Donor organism	Oligomerization	Ex (nm)	Em (nm)	EC ($M^{-1}cm^{-1}$)	QY	RB	Primary reference
mTagBFP2	<i>Entacmaea quadricolor</i>	Monomer	399	454	50,600	0.6	32.4	Subachet et al., 2011
mTurquoise	<i>Aequorea victoria</i>	Monomer	434	474	30,000	0.8	25.2	Goedhart et al., 2010
mGFP5ER*	<i>Aequorea victoria</i>	Monomer	395/473	509	25,000	0.8	19.8	Harper et al., 1999
mEmerald	<i>Aequorea victoria</i>	Monomer	487	509	57,500	0.7	39.1	Cubitt et al., 1998
mT-sapphire	<i>Aequorea victoria</i>	Monomer	399	511	44,000	0.6	26.4	Ai et al., 2008
Clover	<i>Aequorea victoria</i>	Dimer	505	515	111,000	0.8	84.4	Lam et al. 2012
mVenus	<i>Aequorea victoria</i>	Monomer	515	527	104,000	0.6	66.6	Kremers et al., 2006
YPet	<i>Aequorea victoria</i>	Dimer	517	530	104,000	0.8	80.0	Nguyen and Daugherty, 2005
PhiYFP	<i>Phialidium sp.</i>	Dimer	525	537	115,000	0.6	69.0	Shagin et al., 2004
mOrange-ER	<i>Discosoma sp.</i>	Monomer	548	562	71,000	0.7	49.0	Shaner et al., 2004
mKO2	<i>Verrillifungia concinna</i>	Monomer	551	565	63,800	0.6	39.6	Sakaue-Sawano et al., 2008
LSS-mOrange	<i>Discosoma sp.</i>	Monomer	437	572	52,000	0.5	23.4	Shcherbakova et al., 2012
TurboRFP	<i>Entacmaea quadricolor</i>	Dimer	553	574	92,000	0.7	61.6	Merzlyak et al., 2007
tdTomatoER	<i>Discosoma sp.</i>	Tandem Dimer	554	581	138,000	0.7	95.2	Shaner et al., 2004
TagRFP	<i>Entacmaea quadricolor</i>	Dimer	555	584	100,000	0.5	48.0	Merzlyak et al., 2007
mScarlet-H	Synthetic construct	Monomer	551	592	74,000	0.2	14.8	Bindels et al., 2017
mRuby3	<i>Entacmaea quadricolor</i>	Monomer	558	592	128,000	0.5	57.6	Bajar et al., 2016
mScarlet-I	Synthetic construct	Monomer	569	593	104,000	0.5	56.2	Bindels et al., 2017
pporRFP	<i>Porites porites</i>	Tetramer	578	595	54,000	1.0	55.0	Alieva et al., 2008
mBeRFP	<i>Entacmaea quadricolor</i>	Monomer	446	611	65,000	0.3	17.6	Yang et al., 2013

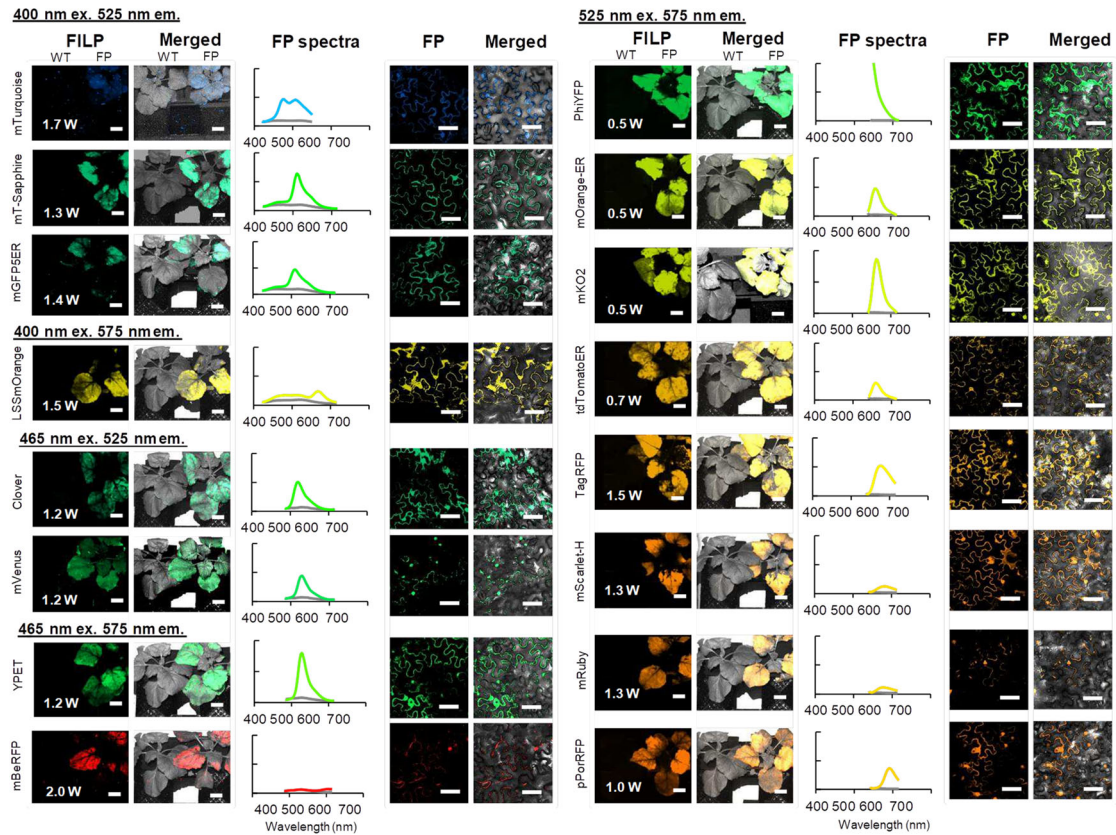


Figure 3.2 Images of plants expressing a wide variety of fluorescent protein genes taken using the FILP instrument at a 3 m standoff. Fluorescence spectroscopy measurements and confocal micrographs included for comparisons. Exposure times used in FILP-based imaging are found in Table S1. Y-axis for all plots is scaled to 5×10^5 CPS except for PhiYFP, which is scaled to 1.0×10^6 CPS. Scale bars for FILP images represent 2.5 cm at a detection distance of 3 m while scale bars for confocal images represent $50 \mu\text{m}$.

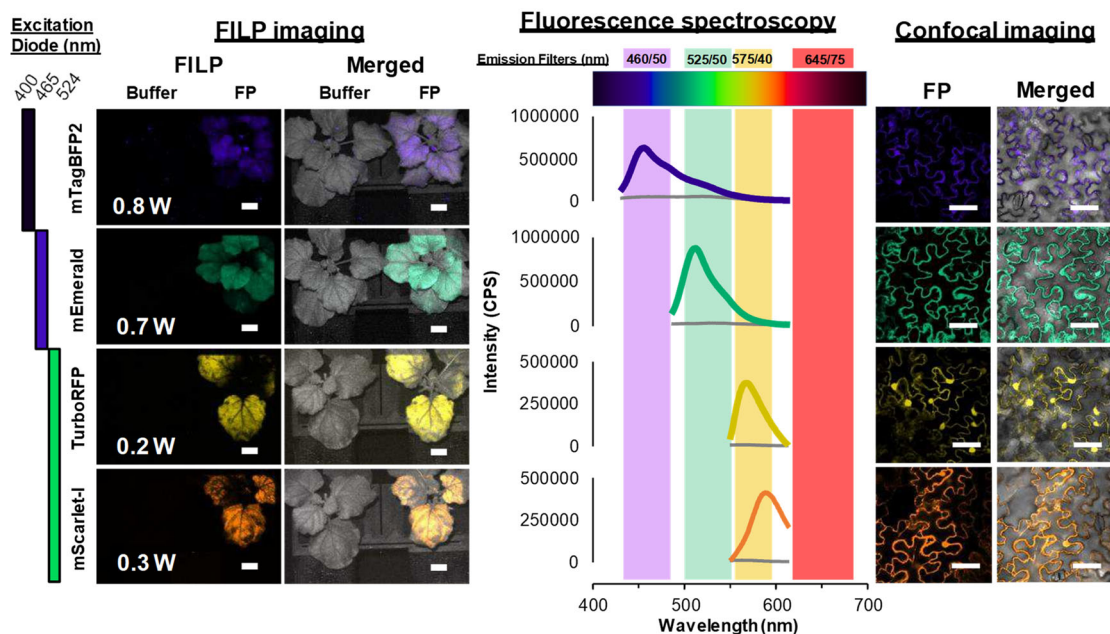


Figure 3.3 Top-performing fluorescent proteins for standoff detection in plants by the FILP device. Bars along the left side of the figure indicate which laser was used for excitation of each fluorescent protein, whereas the emission filter corresponds to the emission peak taken from the fluorescence spectroscopy data. The laser power (W) used is displayed in the bottom left of the images of plants. Exposure time for FILP images was 150 ms. Scale bars for FILP images represent 2.5 cm at a detection distance of 3 m. Scale bars for confocal images represent 50 μ m.

Nonetheless, both mTagBFP2 and mTurquoise could be imaged in plants using the same laser/filter combination (Figure 3.2 and Figure 3.3). Other potential top performing FPs included both yPet and PhiYFP (Figure 3.2), which would be very good choices for single FP reporters, but their emission spectra overlapped with both green and orange emitters in the 525/50 nm and 575/40 nm emission filters, which prohibited their use for simultaneous co-expression with other FPs. Top FPs were also selected based on their emission peaks which were aptly spaced across the visible spectrum to facilitate robust combinatorial detection pairs. mTagBFP2 or mEmerald paired with TurboRFP or mScarlet-I were easily differentiated by FILP (Figure 3.4). One potential concern in imaging multiplexed FPs in the same plant cells was Förster (or fluorescence) resonance energy transfer (FRET) [76]. FRET occurs when one FP emits at the same spectrum that excites a second FP, which could potentially make dual FP detection ambiguous and confounding within the same plant. Therefore, we tested for evidence of FRET prior to selection of the four color band ‘winners’ by fluorescence spectroscopy. We observed no detectable second emission peak in the spectrophotometric measurements taken on any two co-expressed FP combinations; i.e., no FRET was observed in our agroinfiltrated samples (Figure 3.4). The initial FILP components were selected to allow for capture of optimal FP emission spectra. However, optimization of the laser light source has been facile to excite most the FPs tested using a small fraction of the total laser power available in the system. Selection of alternative emission filters may facilitate simultaneously observing more than two FPs in the same plant.

Given the high irradiance potential of lasers, it is reasonable to question whether prolonged exposure to any of the lasers might potentially injure plants. Upon exposing transgenic plants to the highest available laser power at each wavelength for up to 60 min, no apparent leaf damage was observed, nor were off-type phenotypes seen after long-term laser exposure (Figure B.1).

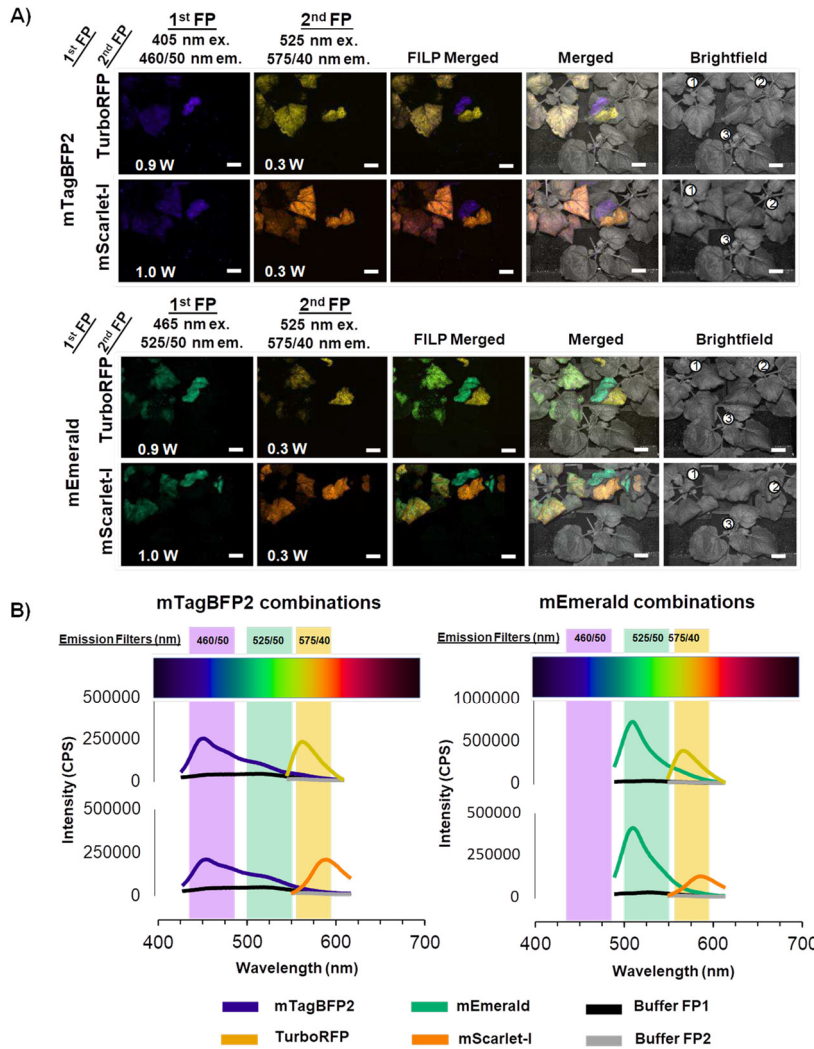


Figure 3.4 FILP and fluorescence spectroscopy measurements of co-expressed fluorescent protein genes in plants. (A) FILP images of four pairwise combinations of fluorescent proteins synthesized in plants. Brightfield images for each of the combinations indicates the placement of the three plants with a circled number: 1) vacuum co-infiltrated, 2) syringe infiltrated individual FPs and 3) buffer control. The buffer control is common among combinations. The FILP images were acquired sequentially at 150 ms exposures. (B) Line plots correspond to fluorescence emission measurements taken on agroinfiltrated whole plants for dual fluorescent proteins. Black lines designate the buffer control plant reading for the 1st FP and the buffer for the 2nd FP is in grey. Scale bars for FILP images represent 2.5 cm at a detection distance of 3 m.

3.3.3. Imaging stable transgenic GFP potato plants

To demonstrate the potential for FILP usage as a phenomics tool in the high-throughput selection of stable transgenic plants, a 2x35S::mEmerald GFP construct was engineered into the potato (*Solanum tuberosum* cv. 'Desiree') nuclear genome. Among 14 transgenic events, FILP imaging of young potato plants indicated that Events 1, 2, and 3 were most highly fluorescent; on that basis we inferred these were the highest GFP expressers (Figure 3.5). Conversely, Events 9 and 12 appeared similar to WT plants with regards to GFP fluorescence (Figure 3.5). These observations correspond with qRT-PCR-transcript abundance for each event. Thus we conclude that FILP imaging is useful as a screening tool for both agroinfiltrated plants as well as stable transgenic plants to gauge the relative level of gene expression between events, using an FP gene as a marker.

3.3.4 Synthetic biology enablements: synthetic promoter screen-to-fluorescent plants, and protein-protein interactions via BiFC observed in plant canopies

In this study, a 2000-member synthetic promoter library was screened in protoplasts under osmotic stress. Among six putative hits from the protoplast screen of the synthetic promoter library, one candidate synthetic promoter (referred to here as JL1; Figures B.4 and B.5) was selected from protoplast transfection experiments and further characterized in agroinfiltrated *N. benthamiana* canopies (Figure 3.6A and 3.6B). Using FILP, within three-to-five days-post osmotic stress treatment, strong inducible GFP expression could be detected in *N. benthamiana* leaves relative to control treatments. The FILP-imaged green fluorescence corresponded with the fluorescent spectroscopy data that was taken from the time-course post-osmotic-stress treatment (Figure 3.6A and 3.6B).

In addition to imaging single and dual FPs in plants, FILP was useful for canopy-level fluorescence phenotyping for other FP assays that have typically been

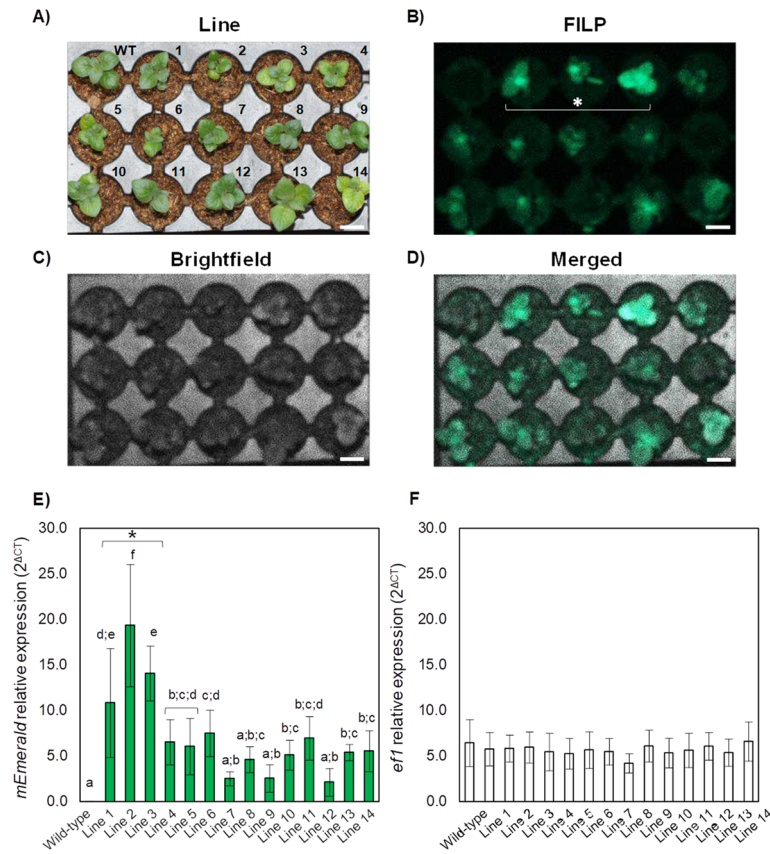


Figure 3.5 Comparison between FILP-images of stably-transformed potato plants expressing an mEmerald GFP gene and the relative GFP transcription of each independent transgenic line. (a) The line numbers correspond to the 14 independent lines expressing GFP. A non-transgenic potato (WT) was included. (b) FILP images of transgenic potato plants synthesizing various levels of GFP. (c) Bright-field and (d) merged images are shown. The FILP images were acquired using the 465 nm ex. 525/50 em. at 0.70 W with 150 ms exposure. Scale bars represent 1 cm for all images. (e) Relative qRT-PCR transcription levels of GFP compared to those of the *L8* reference gene (y axis: $2^{\Delta CT}$) among transgenic potato lines (x axis: transgenic lines 1-14 and wild-type controls) are shown. (f) Relative expression of the endogenous gene *ef1* comparing to the same *L8* reference gene. Data are expressed as means $\pm$ standard deviations of 3 biological replicates and 3 technical replicates per biological replicate. Data were analyzed using ANOVA and Tukey HSD mean separations ($p < 0.05$). Statistical significance among lines is indicated by letters (a-f). High mEmerald-expressing lines (line 1-3) are indicated with asterisk (*).

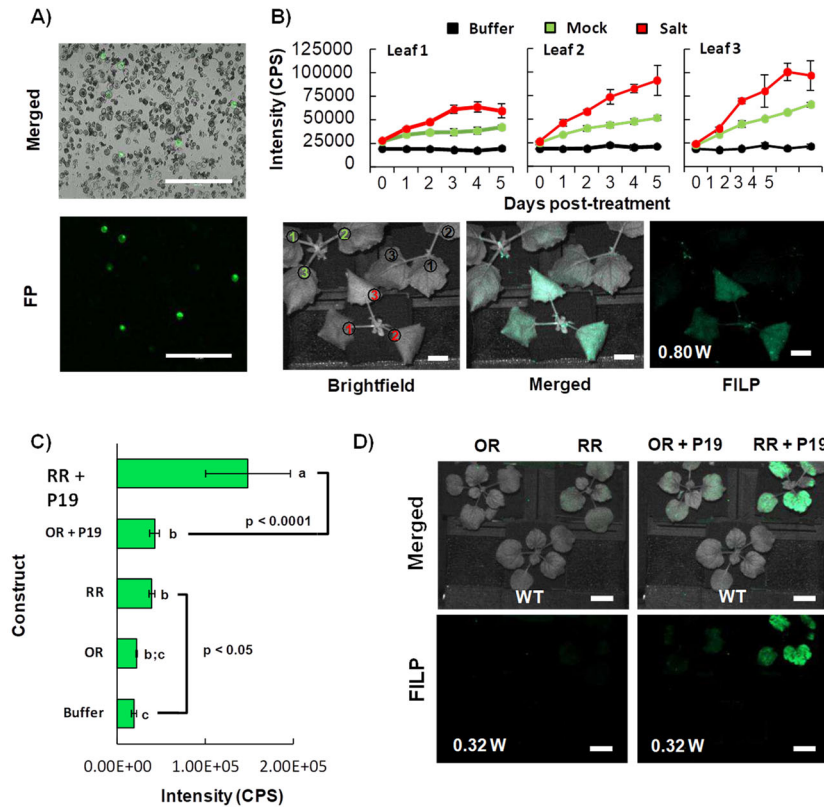


Figure 3.6 Synthetic inducible promoter and BiFC intensity data. (A) The ‘JL1’ synthetic promoter’ was induced by osmotica in a high-throughput screen, which was indicated by GFP production in transfected potato protoplasts (B) Timecourse of fluorescence measurements of *N. benthamiana* leaves synthesizing mEmerald GFP (509 nm em.) over 5 days following an osmotic-stress treatment. FILP images of the plants on day 5 after treatment. In the brightfield images, the identification of leaf numbers are indicated by representative numerals of different colors for treatments: buffer (black), mock (green) and salt (red), ¹Leaf 1, ²Leaf 2 and ³Leaf 3. FILP images for the inducible promoter construct ‘JL1’ were obtained using 400 nm ex., and 525/50 nm em. with a 150 ms exposure time. (C) Fluorescence intensity readings for BiFC constructs at the mVenus peak emission (527 nm em.) with and without the helper P19. (D) FILP images of all AtRAD51 BiFC constructs. RR, AtRAD51/AtRAD51, or, AtODB1/AtRAD51. FILP images for BiFC constructs were obtained 525 nm ex., and 575/40 nm em. using 150 ms exposure time. The scale bars for all FILP images represent 2.5 cm at a detection distance of 3 m.

restricted to cell-based characterization. One such application was bimolecular fluorescence complementation (BiFC), which is based on a split-GFP system which allows *in vivo* analysis of protein-protein interactions. To test for specific protein-protein interactions we used AtRAD51, which self-dimerizes in Y2H experiments (Figure B.2). AtODB1 was also cloned to test whether it participates in a protein interaction with AtRAD51 to mediate functions related to DNA damage repair, as has been demonstrated for homologs in humans [77]. However, in Y2H mating experiments this interaction does not appear to be conserved (Figure B.2A). For this reason, the combination was included in future BiFC experiments to serve as a negative control.

Gookin and Assmann (2014) designed a split-GFP system based on the mVenus FP variant, which was characterized in plant canopies in the present study (Figure IV.2). This mVenus design displays reduced background compared to other split-GFP systems by using an optimized split at amino acid 210 as well as including the P19 silencing suppressor [78]. Both combinations, AtRAD51:NVen210/AtRAD51:CVen210 and AtODB1:NVen210/AtRAD51:CVen210 were constitutively expressed in *N. benthamiana* agroinfiltration assays, with and without co-expression of P19 (Figure 3.6C and 3.6D). Fluorescence spectroscopy measurements shown in Figure 3.6C indicate that leaves expressing the AtRAD51 BiFC pair, without the help of the P19 silencing suppressor, were significantly brighter than leaves that were only infiltrated with buffer, but not different than the negative control (AtRAD51/AtODB1). By including the P19 silencing suppressor, the AtRAD51/AtRAD51 BiFC pair was approximately 3-fold brighter than the negative control that was also expressed with P19 (Figure 3.6C). These results were easily visible in FILP images of the infiltrated plant canopies (Figure 3.6D) and demonstrated how FILP can expand to any fluorescent protein derived technology.

3.4 Discussion

3.4.1 *Advanced plant canopy fluorescence phenotyping*

FILP is a relatively compact and inexpensive device that is versatile to image plant canopies for fluorescence signatures. Its interchangeable lasers and emission filters allows for unprecedented flexibility for fluorescence-based phenotyping in plants. As demonstrated, using 400 nm, 465 nm, and 525 nm diode lasers as well as bandpass emission filters at 460/50 nm, 525/50 nm and 575/40 nm, it can successfully detect 20 unique fluorescent proteins across a wide range of excitation and emission wavelengths, oligomerization states, and spectral properties (Table 3.1, Figure 3.2 and 3.3). Among these fluorescent proteins, we demonstrated that four very bright FPs could be co-imaged in pairs in canopies at > 3 m (Figure 3.4). All FPs were visible in whole leaves using far less laser power than the maximum (Table B.1). It is conceivable that more than 2 FPs in plants could be observed simultaneously after optimal bandpass filters are installed. Compared with the commercially-available FluorCam FC 800 (Photon Systems Instruments, Brno, Czech Republic) and the IVIS Lumina S5 imaging platforms (Perkin Elmer), FILP also allows for larger plant canopies e.g., 10-fold greater area, to be assayed in one view. Ideally, a FILP system could be employed as a fluorescence imaging station in an automated greenhouse setting. In such a system potted plants or mesocosms could be grown in the greenhouse and assayed at a FILP station configured for brief (millisecond-level) excitation. Dynamic temporal and spatial FP-tagged gene expression studies could be performed to connect both physiology and morphology with plant performance.

Of concern is the consequence of repeated- or long-term exposure of plants to relatively high-intensity light supplied by class 4 diode lasers. Especially of interest is exposure to the 400 nm laser, which is at the border of UV-A (320 nm-400 nm). Since DNA does not absorb UV-A—unlike UV-B or UV-C— the laser's mutagenic

effects are negligible. Experimentally, 10 J/cm² doses of UV-A have 300-fold less mutagenicity than UV-B on neonatal human fibroblasts. Doses of 5-to-10 J/cm² UV-A do not decrease yeast survival [79, 80]. In plants, low dose UV-A exposure may be beneficial with regards to growth, and photosynthesis, depending on species and/or varieties [81, 82]. Anecdotally, after performing thousands of separate FILP measurements on hundreds of plants, no off-target effects have been observed; nor has photobleaching been noted. Nonetheless, to provide a worst-case scenario, we exposed various FP-*N. benthamiana* combinations to continuous full-power from each of the lasers. Calculation of the radiation from an hour of continuous exposure shows that 15.7 J/cm² is the total dosage (Figure B.1A). Without beam expansion, the exposure from the laser would be considerably higher, however, by expanding the beam to 400 cm² for imaging, there are no adverse effects on the plants. We observed no morphological damage and no photo-bleaching of mTagBFP2, mEmerald, TurboRFP, and mScarlet-I produced in plants (Figure B.1B and B1C). It is also important to note that typical exposure times for FILP were < 500 ms, resulting in a maximum dose of 0.002 J/cm². Thus, we conclude that the FILP system does not damage plants during normal or exaggerated use.

3.4.2 Synthetic biology synergies for phytosensing applications

As a phenomics tool, FILP would provide unparalleled assessment of gene expression in transgenic plants, as shown by imaging a range of GFP in stably transformed potato (Figure 3.5). This capability predicates the enablement of detection of inducible gene expression in intact plants. Indeed, the authors produced the FILP system as a means to assess the efficacy of environmental phytosensors, which are plants engineered to detect and report environmental stimuli [72]. Phytosensors have been developed to monitor plant pathogenic bacteria in the field [83], but the production of clear and useful photonic signals remains challenging. In combination with advanced synthetic biology in plants [84],

especially in the area of synthetic promoters[85] and circuits[86], we are poised to enter the 'golden era' of gene-targeted phenomics. Arguably, abiotic stress detection, e.g., osmotic stress, at an early onset stage, has the potential to revolutionize agricultural productivity and sustainability [87].

Phytosensor design is being greatly improved by using stimulus-specific synthetic promoters to drive fluorescent proteins. Nonetheless, one vexing problem is performing high-throughput biology in plants. Herein, combining a high-throughput synthetic promoter screen with FILP phenotyping provided a rapid-genetics discovery platform in plants. In the example shown above, one postdoctoral scientist was able to screen up to 2000 synthetic promoters in protoplasts under an osmotic treatment and image the top candidate stress-inducible synthetic promoter in agroinfiltrated plant canopies in less than two months. The experiments were replicated in both space and time (Figure 3.6).

Beyond inducible promoter assays, FILP has also shown the ability to capture protein-protein interactions *in vivo* (Figure 3.6). Like the induction of the synthetic JL1 promoter in response to osmotic stress, such *in planta* detection of protein-protein interactions could lead to novel characterization of the timely association of protein complexes in the context of diverse activating stimuli within agricultural settings and beyond.

The utilization of FILP as a detection and phenomics tool is only limited by the feasible employment of fluorescent protein-based designs. Fortunately, fluorescent proteins have proven time and again as valuable characterization tools and have received much attention since the discovery of *Aequorea* GFP in 1962 [88]. For years, thought leaders in the burgeoning field of plant phenomics have identified bottlenecks for effective gene-to-phenotype identification in various modes of operation. Dhondt et al. noted that organ-level detection, increased

dimensionality, and sensor technologies are in need of a step change with regard to precision, as well as the need of direct links to plant physiology [89]. FILP directly addresses all of these needs through fluorescence imaging at the canopy level through expression of fluorescent protein genes.

3.5 Methods

3.5.1 Plant expression vectors for constitutive expression of fluorescent protein genes

The DNA coding sequences for the 20 fluorescent protein (FP) genes listed in Table 3.1 were mobilized into Invitrogen pENTR /D-TOPO cloning vectors (Invitrogen, Carlsbad, CA). Following colony PCR and validation by sequencing, the FP coding sequences were each recombined into the pMDC32 doubled 35S expression vector [75] via the LR Clonase reaction (Invitrogen, Carlsbad, CA). FPs were subsequently sequence-verified prior to transformation into *Agrobacterium tumefaciens* strain LBA4404.

Modular cloning was used for construction of the plant binary vector for mEmerald overexpression in potato. An expression cassette containing the double CaMV 35S, the full-length mEmerald CDS, along with the 35S terminator was assembled by modular cloning and integrated into the pAGM4723 destination vector containing an hygromycin cassette for selection [90-92].

3.5.2 Generation of transgenic lines

Stably-transformed potato (*Solanum tuberosum*) lines that overexpressed an mEmerald GFP gene under the control of the double CaMV 35S promoter were generated by *Agrobacterium*-mediated transformation and regenerated in selective media as previously described [93, 94]. Internode explants (~1 cm-length) were taken from one-month-old *in vitro*-grown potato plants; these were the target of transformation. Putative lines were assessed for transgene presence

and expression by real-time PCR and fluorography. Transgenic lines were grown *in vitro* in Magenta GA7 boxes containing selective propagation media (4.33 g/l Murashige and Skoog salt mixture; 170 mg/l sodium phosphate monobasic monohydrate; 100 mg/l inositol; 400 µg/l thiamine HCl; 30 g/l sucrose; 100 mg/L timentin; 20 mg/L hygromycin and 3 g/l phytigel; pH 5.6) as previously described [94, 95]. Plants were acclimated and then transferred to pots. Transgenic and non-transgenic potato was grown in a controlled environment at a temperature of 22-24°C and a cycle light/dark of 16 and 8 hours, respectively.

3.5.3 Total RNA extraction, cDNA synthesis and real-time PCR

For each plant, ~50 mg healthy leaf tissue was used for total RNA extraction using the Tri-Reagent method (Molecular Research Center, Inc, Cincinnati, OH, USA) according to manufacturer's protocol. RNA preparations were then subjected to DNase treatment and a step of cleaning using the RNA Clean & Concentrator Kit (Zymogen, Irvine, CA, USA). For each sample, 1 µg of clean total RNA was used for cDNA synthesis using the Super Script III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instruction. A NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was then used to measure cDNA concentration.

cDNA dilutions (1:3 v/v in nuclease-free H₂O) were used in real time PCR reactions. The reaction was assembled in a total volume of 15 µl in 1X PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), using 1 µl of diluted cDNA and 0.5 µM of each primer. The software Primer3 input v. 0.4.0 (Howard Hughes Medical Institute and by the National Institutes of Health) was used to design compatible pairs of primers with an annealing temperature of ~57°C and able to amplify a fragment of ~100 bp [96]. The pairs of primers 1Fw/1Rv were used to amplify *mEmerald*, whereas the pair of primers 2Fw/2Rv and 3Fw/3Rv were used to amplify the two internal control *ef1* (*Solanum*

tuberosum elongation factor 1- α ; XM_006343390.2) and *L8* (*Solanum tuberosum* 60S ribosomal protein L8: XM_006362853.2). In Table B2 the sequences of primers used for qRT-PCR are shown.

The QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform real-time PCR reactions using 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA), whereas amplification data were acquired using the QuantStudio™ Real-Time PCR Software v1.1 (Thermo Fisher Scientific, Waltham, MA, USA). The $2^{\Delta CT}$ method was used to represent gene expression data in transgenic lines along with wild-type controls. *L8* was used as reference gene to determine the expression levels of both *mEmerald* and the internal control *ef1* gene. Data are expressed as mean $\pm$ standard deviation (sd) of 3 biological replicates and 3 technical replicates per biological replicate per each transgenic line and wild-type control. The ANOVA post-hoc Tukey statistical analysis ($p < 0.05$) was performed using the IBM SPSS software to determine statistically significant difference among means.

3.5.4 Vacuum agroinfiltration of *N. benthamiana*

Agrobacterium was infiltrated according to Rigoulot et. al. (2019) with modification [97]. *Agrobacterium tumefaciens* strain LBA4404 was used for the infiltration of all fluorescent protein and BiFC constructs. Colony PCR was used to determine transformation of *Agrobacterium*. *Agrobacterium* was then grown from colonies overnight in 10 ml YEP media [10 g/L yeast extract, 10 g/L bacto peptone, 5 g/L NaCl, pH 7.0] with rifampicin (50 mg/L) and kanamycin (50 mg/L) selection at 28°C shaking (225 RPM). This culture was used as the seed culture for a 125 ml culture also grown overnight under the same conditions. *Agrobacterium* was resuspended in injection media (10 mM MES, 10 mM MgCl₂ and 100 μ M acetosyringone) to an OD600 value of 0.8 and this *Agrobacterium* solution was incubated for 3 hr at room temperature prior to infiltration. Four-week-old *N. benthamiana* plants grown under

long day conditions at 23°C were used for infiltration experiments. Vacuum infiltration of *N. benthamiana* plants were performed using a modified Nalgene vacuum desiccator (Cole Parmer, Vernon Hills, IL). The sidearm on the base was blocked using the PTFE cap (provided with purchase) and secured in place with parafilm. The stopcock on the desiccator lid was removed and PVC tubing was retro-fitted with a 1-5 ml pipette tip to connect the benchtop vacuum port with the vacuum desiccator. *N. benthamiana* plants were inverted and all aboveground tissues were submerged into a Magenta™ GA-7 boxes (Fisher Scientific, Catalog No. 50-255-176) filled with the *Agrobacterium* suspension. The Magenta box was housed inside of a Styrofoam support ring that was cut to the size of a large glass container to prevent the movement of the vessel during application and release of the vacuum. With the modified pipette tip end of the hose securely inserted into the vacuum desiccator lid, the vacuum was applied in 1 min intervals. This was repeated 3 times to achieve thorough infiltration of leaf tissue indicated by visible saturation of the leaf with the *Agrobacterium* solution. The vacuum provided by the benchtop vacuum port was measured to be -84 kpa or -12 psi. After infiltration, the plants were rinsed in a beaker of deionized water and then allowed to dry at room temperature. The plants were then returned to the growth room until fluorolog readings, FILP and confocal imaging were taken 72 hr post infiltration.

For co-infiltrated plants, *Agrobacterium* solution was adjusted for both FP gene constructs to an OD600 of 1.6, then FP constructs were mixed 1:1 and vacuum infiltration was conducted as previously described. Syringe infiltration of *N. benthamiana* leaves was conducted as described in Rigoulot *et al.* (2019) with *Agrobacterium* solution at an OD600 of 0.8.

3.5.5 Fluorescence spectroscopy of leaves

Prior to FILP measurements, the spectral characteristics of the youngest, fully-expanded leaf of each plant was quantified using scanning fluorescence

spectroscopy (Fluorolog®-3, Jobin Yvon and Glen Spectra, Edison, NJ, USA) using emission spectral acquisition by the FluorEssence Software (HORIBA Scientific, version 3.8.0.60). On-the-leaf fluorescence was measured using a fiber optic probe as described previously [98]. Excitation wavelengths matched Necsel laser diode wavelengths at 400 nm, 465 nm, and 523 nm with a slit width of 5 nm. Emission wavelengths were scanned from 415-615 nm, 480-615 nm, and 540-615 nm for the respective excitation wavelengths in increments of 1 nm. Leaves of the same developmental age were infiltrated with buffer as a negative control and measured for background fluorescence.

Fluorescence spectroscopy data was handled using custom software: the Fluorologger Shiny app, coded in R [99, 100]. The graphic user interface allows for the visualization and normalization of fluorolog data and the app is currently available on github at github.com/jaredbrabazon/Fluorologger. Output files from the Fluorolog (.dat format) were input into the application and with user input the data were normalized according to methods described in Millwood et al. (2003). A detailed user guide is available at the link provided.

The heatmap in Table B.1 was created by recording the fold change difference between an individual fluorescent proteins peak emission as described on FPBase.org and the background emission at this same point taken by the Fluorolog instrument (signal to noise).

3.5.6 Confocal microscopy

The same leaf tissue analyzed by fluorescence spectroscopy (Fluorolog) was imaged using an Olympus FV1200 confocal microscope (Olympus, Center Valley, PA, USA). Diode lasers (405 nm, 440 nm, 473 nm, 559 nm, and 635-nm) along with conventional Argon and HeNe (R) lasers were used to image plant tissues synthesizing fluorescent proteins with the appropriate excitation and emission

spectra (Table IV.1). The manufacturer's Olympus FV10-ASW Viewer software Ver.4.2a (Olympus, Center Valley, PA, USA) and the ImageJ analysis software (version 1.41o) were used to acquire and process confocal images, respectively [101].

3.5.7 Fluorescence-inducing laser projector phenotyping system

The fluorescence-inducing laser projector (FILP) is a custom-designed instrument primarily composed of components purchased from Necsel IP, Inc. (Milpitas, CA). The Necsel components include a custom NovaLum module with three independently-modulating laser diodes that emit at 400.3 nm, 464.9 nm, and 523.5 nm, a Thermal Platform Developer's Kit, an intelligent controller kit, and a homogenizing square core fiber (400 μm , 0.22 NA) used for flattening the Gaussian image produced by the lasers into a spatially-uniform flat-field, albeit with residual speckle from the laser coherence. This speckle was reduced by passing the fiber through a commercially-available aquarium pump to vibrate the fiber faster than the camera's exposure time, thereby smoothing and de-speckling the resulting image. Individual amplified spontaneous emission (ASE) filters were hand cut and mounted onto each laser diode to limit the transmitted wavelength. As such, a 405 ± 20 nm filter (ZET405/20X, Chroma Technology Corp., Bellow Falls, VT) was mounted onto the 400 nm diode, a 460 ± 36 nm filter (ET465/36 nm, Chroma Technology Corp.) was mounted onto the 465 nm diode, and a 524 ± 24 nm (FF01-524/24, IDEX Health & Science, LLC., Rochester, NY) was mounted onto the 523 nm diode. After assembly, the maximum power output of the complete Necsel system was 1.14 W @ 400 nm, 1.36 W @ 465 nm, and 1.45 W @ 523 nm. Further, control of the current (from the intelligent controller) allowed linear control over the laser power with R^2 values > 0.99 . To shape the beam for imaging whole plants, a projector lens (63-714, Edmund Optics, Barrington, NJ) was placed 6 mm in front of the homogenizing fiber to form a 400 cm^2 imaging square at a distance of 3 m.

Images of plants at ≥ 3 m was achieved using an Andor Zyla 5.5 sCMOS camera equipped with a 50 mm focal length lens (86-574, Edmund Optics). The camera was mounted to the same breadboard as the laser system, ensuring that the excitation and emission distances were identical. Control of the camera resolution, exposure time, and image acquisition was achieved using the free open-source software μ Manager. A five-position motorized filter wheel with USB control (84-889, Edmund Optics) was mounted between the camera lens and the sample to enable collection of images for specific wavelengths pertaining to the target fluorescent protein synthesized by plants. In the current version of FILP, three 50 mm emission filters were loaded onto the emission wheel: 460 ± 50 nm (ET460/50 nm), 525 ± 50 nm (ET525/50 nm), and 575 ± 40 nm (ET575/40 nm) (Chroma Technology Corp.).

To ensure user safety of the system and provide complete darkness for sampling, a custom laser range, 0.61 m x 0.91 m x 3.7 m, was assembled around the entire system using 25 mm construction rails and black hardboard (XE25 & TB4, ThorLabs, Newton, NJ). Plants were placed inside the enclosure through a door fabricated at the back of the enclosure using the same materials. Finally, a magnetic interlock was used to ensure that the class IV lasers could be operated as a class I system where the lasers were immediately and automatically turned-off if the door was opened.

A custom plant stand ("FILP's castle") to secure potted plants being imaged in the laser range was fabricated using 3D printing. The stand tilts potted plant samples 75 degrees forward to allow for maximum foliar exposure while using the laser system. The stand accommodates three square (7.6 cm) pots that are grown in an 18 cell flat (59-3080, Griffin Greenhouse Supplies, Inc., Tewksbury, MA). Designs for 3D printing are available upon request.

3.5.8 Image processing

Assembly of FILP and confocal microscope images was done using the ImageJ analysis software (version 1.41o) [101]. Color determination for each fluorescent protein was done using the Wolfram Demonstration Project (<https://demonstrations.wolfram.com/>), Colors of the Visible Spectrum plugin. Using the Adobe color space option, peak emission wavelengths were used to query for RGB values. These values, representing a percentage, were multiplied by the maximum value for the R, G or B decimal code (255). The resulting values were then used to establish look up tables (LUT) for the ImageJ software. Images are input into the ImageJ software independently. Adjustments to brightness and contrast were applied uniformly across images if necessary. Using the images to stack function, FILP or confocal fluorescent and brightfield (BF) images were overlaid. After using the composite image function and selecting the color option of the channels tool, pseudo coloring is applied to a selected image. Presets include the gray which can be used for the brightfield image as well as blue, green, red, etc. For more specific color palettes, a unique LUT was generated. By default, ImageJ applies different colors to the different channels (images) and these were changed using the channels tool color option. Images were exported as .tiff files for the construction of figures. We provide an example of the Wolfram player determination of R, G, B values, the conversion of these values to generate individual LUTs, the table of RGB decimal values for all FPs and a visual walkthrough of pseudo coloring (Figure B.3).

For the continuous laser exposure experiment of top performer FP infiltrated plants at full laser power, 16-bit black and white laser images were obtained every minute for a 60 minute time series using the multidimensional acquisition feature of μ Manager. These images were cropped using an automated action in Adobe Photoshop CC 2019 v.20.0.5 (Adobe Systems, Inc. San Jose, CA). Since the first 30 minutes show that the plants are adjusting to the orientation of the plant stand

and the alternative light environment, measurements were taken starting at the 30 min time point (T0) and continued until the end of the hour (T30). The cropped images were batch measured in ImageJ to record the mean pixel intensity for the same plant over time. The mean pixel intensity for each image was plotted relative to time and displayed in Figure B.1.

3.5.9 Potato cell suspension used for protoplast production

The preparation of potato cell suspension used for synthetic promoter screening of protoplasts was modified from a previously described method by Sajid and Aftab (2016). *Solanum tuberosum* cv. 'Desireé' was propagated by nodal explants into propagation media [4.33g/L MS salts (Phytotech M524, PhytoTech Labs, Lenexa, KS), 25 g/L sucrose, 100 mg/L myo-inositol, 0.17 g/L sodium phosphate monobasic, 0.44 g/L calcium chloride dihydrate, 0.4 mg/L thiamine HCL, 5 ml/L "complete vitamin stock" (for 100 ml; 40 mg glycine, 10 mg nicotinic acid, 10 mg pyridoxine HCL, 10 mg thiamine HCL), 3 g/L phytigel, pH 5.8, 1 ml/L MS (Phytotech M557) vitamins) in Magenta GA7 vessels under 16-h day, 8-h night fluorescent light conditions at room temperature (23°C). Sterile leaf explants, cut into 1-2 cm squares, were taken from propagates and callus was induced on callus induction (CI) media [4.33 g/L MS salts, 20 g/L sucrose, 2 g/L gelzan (solid media), pH 5.8, 1 ml/L MS vitamins, 4 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid)]. Callus was transferred to fresh CI plates every 2-3 weeks. After 4-5 weeks on CI media, approximately 2 g of green, friable callus was used to inoculate 20 ml of liquid CI media and grown on a platform shaker at 120-140 rpm for 7 d. The suspension was then filtered through a 425 µm sieve and transferred to a new 125 ml flask. After the filtered cells settled, 15 ml of liquid CI media was removed, replenished with fresh CI media and the suspension was grown again on a platform shaker at 120-140 rpm. After 7 d, an additional 30 ml of fresh liquid CI media was added to the flask and allowed to grow for another week. The cell suspension was maintained every 5-7 d by sub-culturing approximately 15 ml of the filtered

suspension culture into 30 ml of fresh media. Cells were periodically filtered through a 425 µm sieve to maintain consistency.

3.5.10 Protoplast transfection

Potato protoplasts were isolated from cell suspension culture 3 d post-subculture. Five milliliters of packed cell volume was digested by a cell wall-digesting enzyme solution [0.4 M mannitol, 20 mM MES (pH 5.7), 20 mM KCl, 10 mM CaCl₂, 1% (w/v) bovine serum albumin (BSA), 5 mM β-mercaptoethanol, 4.4% (v/v) Rohament CL, 4% (v/v) Rohapect, 0.6% (v/v) Rohapect UF] in the dark at room temperature for 2 h with gentle shaking (88 RPM). After two washes with wash buffer [0.45 M mannitol, 10 mM CaCl₂] protoplasts were filtered through a 40 µm nylon mesh cell strainer (Fisher Scientific, Hampton, NH) and intact cells were purified on a 23% sucrose gradient. Protoplasts were then re-suspended in a MMg solution [0.4 M mannitol, 15 mM MgCl₂, 4 mM MES] to a concentration of 2 x 10⁵ protoplasts/ml.

For the protoplast transfection assay, PEG-mediated transfection was conducted according to Yoo et al. (2007) with modification [102]. Ten microliters of plasmid (1 µg/µl) was added to 100 µl of protoplast suspension. After adding 110 µl of 40% PEG solution [40% (w/v) PEG-4000 (Sigma), 0.2 M mannitol, 0.1 M CaCl₂], the mixture was incubated for 15 min at room temperature. The reaction was stopped by adding 440 µl of W5 solution [2 mM MES, 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl] and the protoplasts were centrifuged at 100 x g for 1 min, and then resuspended in 200 µl of WI solution [0.5 M mannitol, 4 mM MES, 20 mM KCl] for incubation in the dark for 48 h. Due to the osmolality of the MMg (449 mOsm), W5 (695 mOsm), and WI (544 mOsm) solutions used in this work, protoplasts received an osmotic stress during the transitions between these solutions.

Transformed protoplasts were observed for GFP expression using an EVOS M7000 imaging system (ThermoFisher Scientific) equipped with a GFP filter (excitation 470/22 nm, emission 510/42 nm).

3.5.11 Synthetic promoter screening

To identify candidate osmotic-stress inducible plant promoters, a library of synthetic promoters, ~2000 constructs (Stewart et al., unpublished data), was screened with a potato protoplast assay. Transformed protoplasts incubated under hypertonic conditions described above were observed for GFP expression after 48 h using an EVOS M7000 imaging system (ThermoFisher Scientific, Waltham, MA) equipped with a GFP filter (excitation: 470/22 nm, emission: 510/42 nm), and protoplasts were scored as positive or negative for induction based on green fluorescence. Promoters identified in the protoplast screen were then characterized in leaves by agroinfiltration assays in *N. benthamiana*. Of 8 putative hits, we characterized one of the more promising ones here: JL1 (Figure B.4 and B.5).

For the *N. benthamiana* canopy experiment, osmotic stress treatment was applied by watering each pot with 100 ml of NaCl solution (250 mM) 48 hr after agroinfiltration, followed by withholding water for 5 d to the partial wilt stage. The mock treatment consisted of 100 ml tap water applied every two days to each plant. Three biological replicates were used, and the experiments were repeated three times. Fluorescence spectroscopy measurements were taken immediately prior to NaCl treatment and then repeated every day for 5 d. FILP images were taken on the final day of the experiment. Leaves not previously measured by the fluorescence spectrometer were removed, including old leaves and new leaves produced since agroinfiltration. Plants were imaged using the 400 nm laser diode and observed using the 525/50 nm filter. Laser wattage was 0.8 W and the exposure time was 150 ms.

3.5.12 Yeast two-hybrid

The DNA coding sequences for AtRAD51(AT5G20850) and AtODB1 (AT1G71310) were cloned from Arabidopsis leaf cDNA and incorporated into Invitrogen pENTR /D-TOPO cloning vectors (Invitrogen, Carlsbad, CA). Following colony PCR and validation by sequencing, the coding sequences were each recombined into Gateway-compatible yeast expression plasmids via the LR Clonase reaction (Invitrogen, Carlsbad, CA). These plasmids, pGBKCg and pGADCg, feature c-terminal fusions for the GAL4 DNA binding domain and the GAL4 activation domain, respectively (Stellberger et al., 2010).

Both AtRAD51 and AtODB1 constructs, including empty vector negative controls were transformed into mating compatible *S. cerevisiae* strains AH109 and Y187. Transformation of yeast and mating experiments were conducted as previously described [97]. Tryptophan- and leucine-deficient media selected for yeast containing both pGBKCg and pGADCg constructs, respectively, while histidine and adenine deficient media selected for positive interaction.

3.5.13 Bi-molecular fluorescence complementation vector construction

Following the positive Y2H results for the AtRAD51 self-interaction, AtRAD51 was domesticated to remove BsaI/BbsI sites and re-cloned into the CDS1 no stop level 0 acceptor vector (pAGM1287) for use in Golden Gate cloning strategies [90-92]. Similarly, AtODB1 was cloned as a CDS1 no stop level 0 for use in negative controls. C-terminal BiFC fusion pieces were designed based on the Gookin and Assmann (2014) construct pDOE-01 CD3 1901 available from the Arabidopsis Biological Resource Center repository [78]. As with the production of the CDS1 no stop lvl 0 pieces, the N-terminal (NVen210) and the C-terminal pieces (CVen210) were domesticated and subsequently cloned into the C-terminal lvl 0 acceptor vector (pAGM1301) to generate modules for use with other Golden Gate compatible components. Remaining pieces were obtained from the Engler Golden

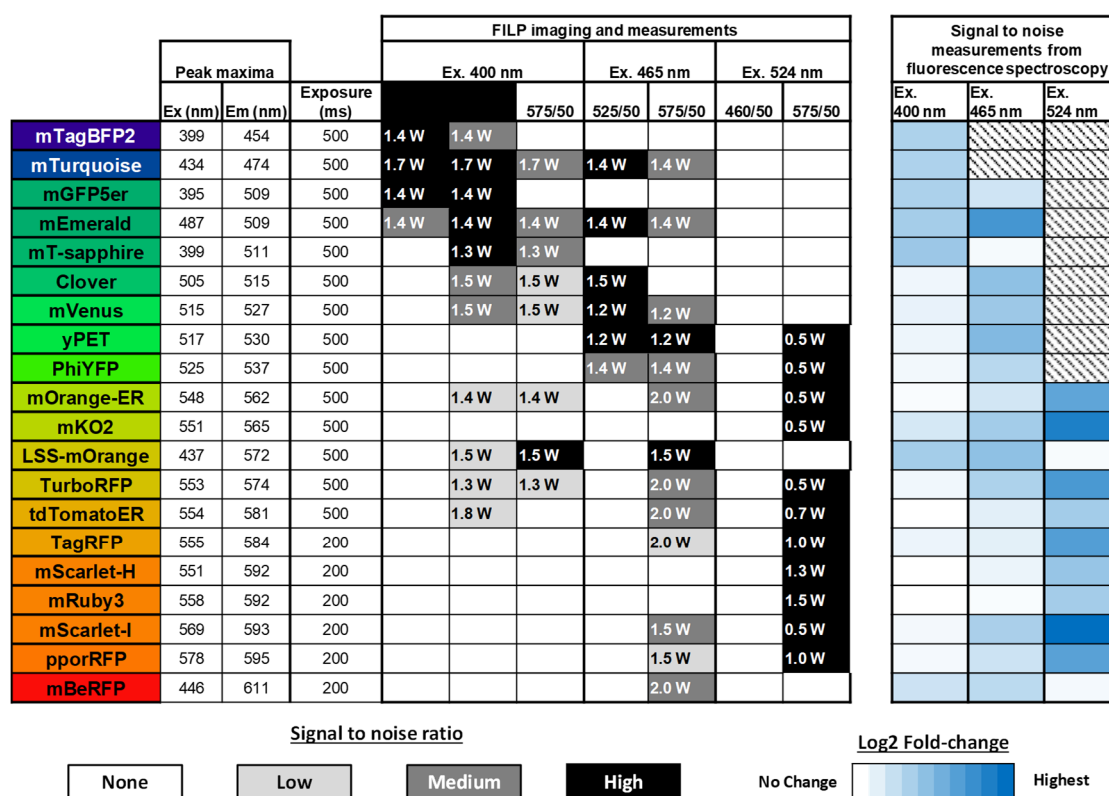
Gate Modular Cloning Toolbox for Plants [91]. All final expression constructs were assembled in the pAGM4723 level 2 vector. CVen210 fusion cassettes occupy position 1, NVen210 fusion cassettes occupy position 2 and if present the P19 helper cassette occupies position 3. All cassettes were produced in the reverse orientation. AtRAD51 and AtODB1 fusion cassettes were expressed using the 2 x 35s_5U-TMV promoter (pICH51288) and included the *A. tumefaciens* nos terminator (pICH41421). Helper plasmids for the + P19 multi-cassette vectors emulated the aforementioned pDOE-01 vector [78], using the *A. tumefaciens* mas promoter (pICH85281) and terminator (pICH77901) to regulate the Tomato Bushy Stunt Virus P19 silencing suppressor (pICH44022). Vectors at the level 0, level 1 and level 2 stages were validated using colony PCR, restriction digest and sequencing before conducting any further experimentation.

3.6 Conclusions

Our study represents the first demonstration of a ‘turn-key’ system of an osmotic stress phytosensor that can be detected optically at a stand-off. Moreover, the versatility of standoff detection using the suite of the FP color palette and the FILP diode laser-based phenotyping system represents an unprecedented application that clearly demonstrates the potential of FP-based phenomics in agriculture.

APPENDIX B

Table B.1 Heatmap of laser power required for the detection of fluorescent proteins. Laser diode power and emission filter combinations are scored from no signal (none) to the highest signal to noise ratio. Heatmap of fluorescence spectroscopy measurements shows fluorescence when compared to buffer infiltrated control at each fluorescent protein peak emission when excited by 400 nm, 465 nm, and 524 nm laser light. Diagonal line fill indicates that the excitation wavelengths exceed the reported peak emission value and therefore the fluorescence intensity for the peak emission cannot be calculated. Data acquired for this table and Figure 2 used an earlier version of μ Manager, which did not support the full capabilities of the Andor camera. As such, a higher laser voltage was required to collect this data, when compared to the data collected in the main text with a newer version of μ Manager.



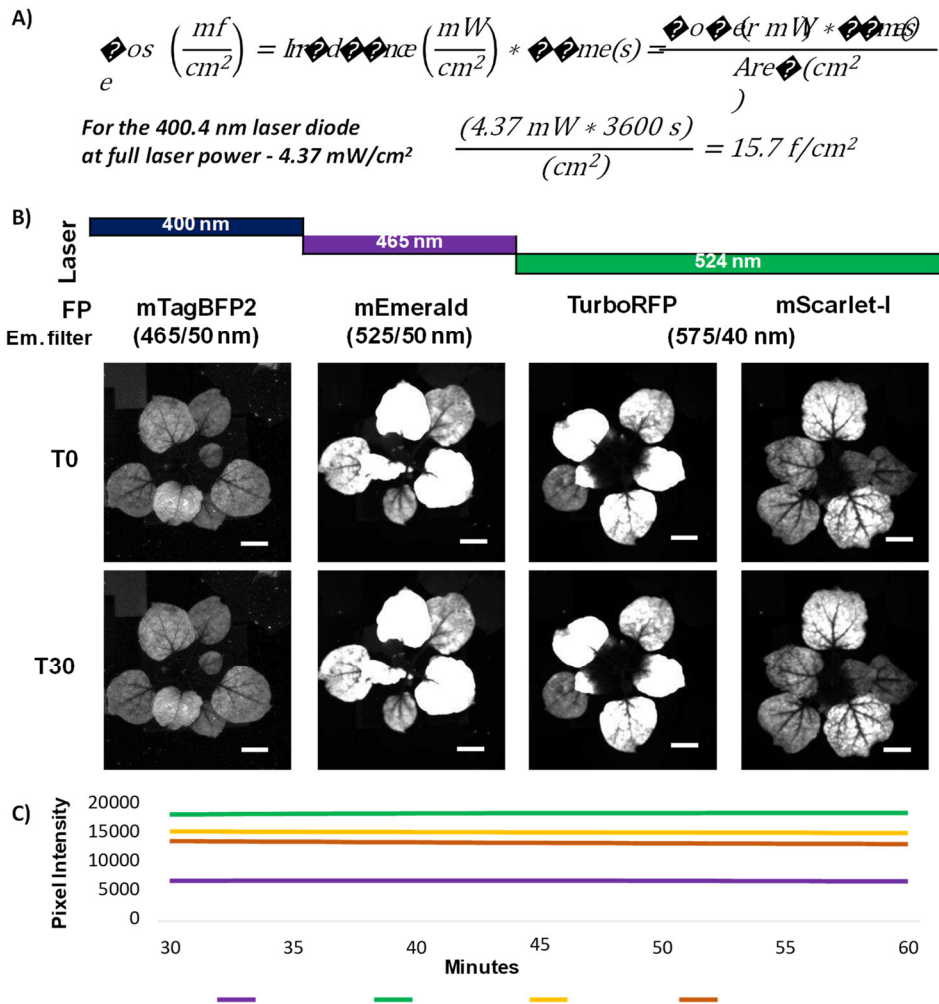


Figure B.1 Extended exposure of top performers to FILP lasers at full power. a) Equation to calculate laser dosage. Calculation of UVA radiation from 400 nm laser after 1 hour of exposure. b) Images of fluorescent protein gene-agroinfiltrated plants exposed to 60 min (T60) constant laser light exposure compared to the measurement taken at 30 min (T30) after each of the three different lasers excited four fluorescence proteins in plants. The bars indicate laser wavelength used. No false coloring was used to help facilitate comparisons. Due to orientation/movement of the leaves toward the light source over the first 30 minutes of exposure, only time points from 30-60 min could be used for analysis of photobleaching. c) Line plot showing a time series where average pixel intensity was measured over the last 30 minutes of an hour of continuous laser activity. Scale bars represent 2.5 cm.

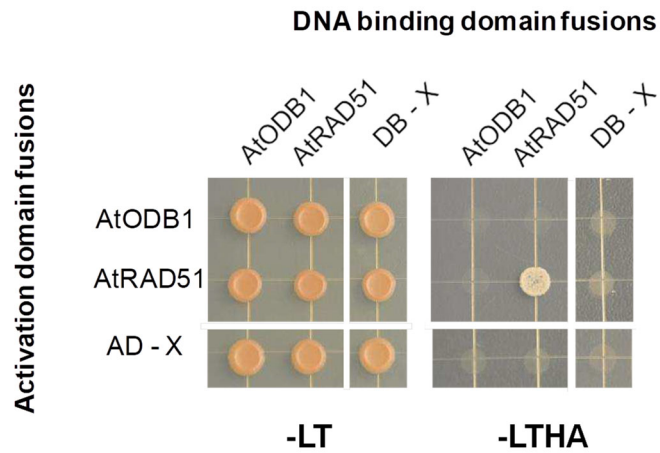


Figure B.2 Y2H for combinations of AtRAD51 – AtODB1. Y2H mating results for AtODB1 and AtRAD51. –LTHA panel shows the selection for interactions between DB and AD fusion pairs. AD-X and DB-X vectors were empty vector backbones and served as control.

Table B.2 Sequences of PCR primer used in this study. The primers are identified with different identification (id) and they are subdivided in forward (Fw) and reverse (Rv). For each primer the 5'-3' nucleotide sequence is indicated in the table.

Primers Forward		
id	name	sequence (5'-3')
1Fw	mEmerald-q2-FW	CTATATCACCGCCGACAAGC
2Fw	EF1-alpha-q2-FW	GATGGTCAGACACGTGAACA
3Fw	L8-q-FW	TGGTAATGTGTTGCCGCTTA
Primers Reverse		
id	name	sequence (5'-3')
1Rv	mEmerald-q2-RV	GGGTGTTCTGCTGGTAGTGG
2Rv	EF1-alpha-q2-RV	CCTTGGAGTACTTGGGGGTG
3Rv	L8-q-RV	GGCGTAATCACCAGAACACC

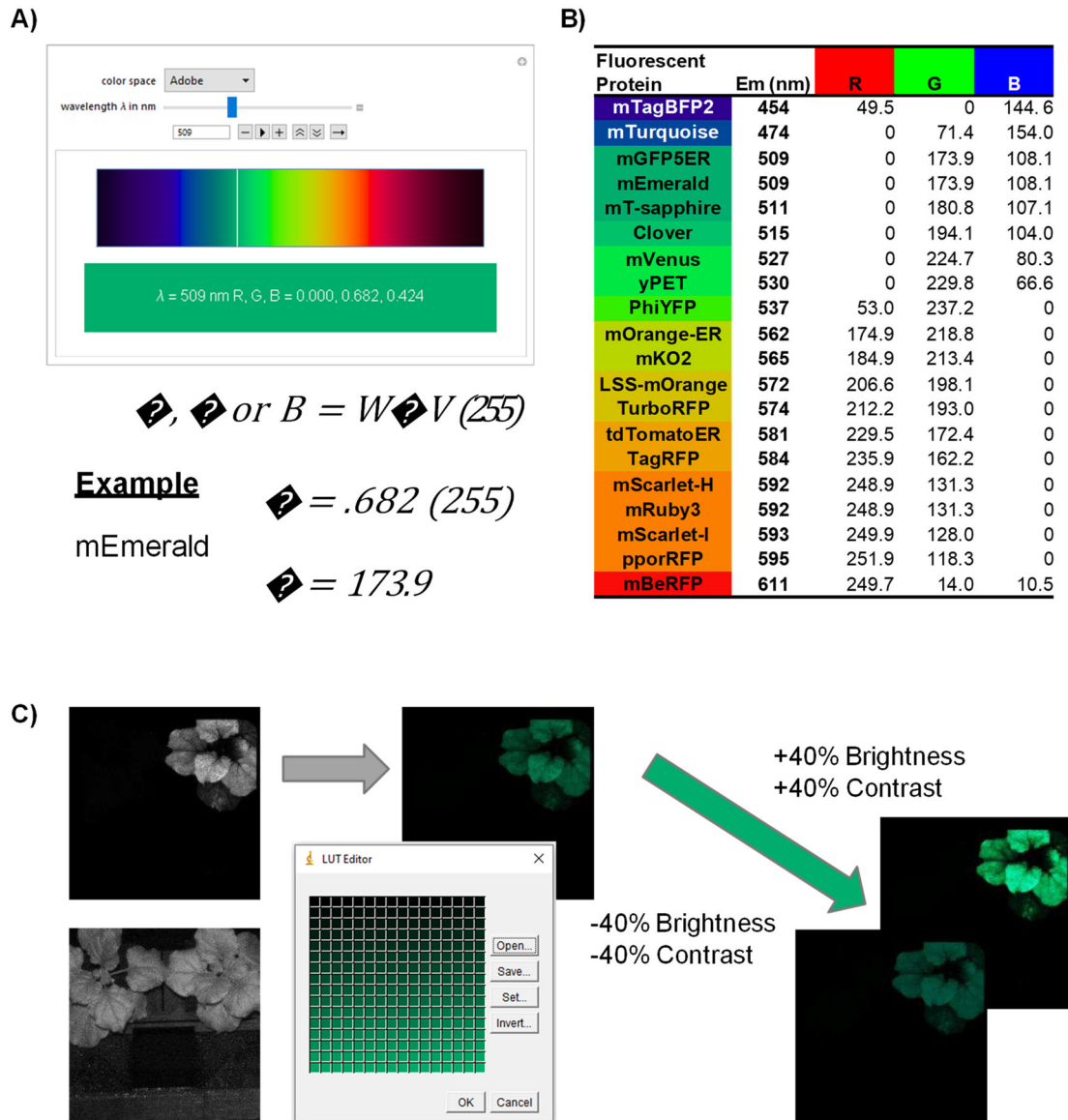


Figure B.3 Determination and application of image color. (a) Screenshot of the Wolfram Player Colors of the Visible Spectrum plugin for mEmerald peak emission (509 nm) and the formula for the conversion of the Wolfram Player Value (WPV) to red (R), green (G) or blue (B) intensity values. (b) Fluorescent protein table that contains peak emission values and the RGB intensity values used to establish the look-up tables for pseudo color application. (c) Example of image processing using the ImageJ software and the application of pseudo color to a FILP image includes a screenshot of the ImageJ LUT editor. The example shown is the mEmerald image used for the production of Figure 3.2.

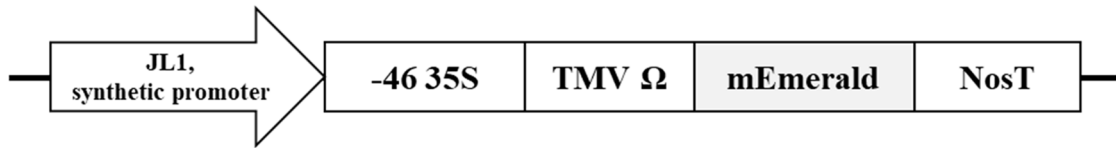


Figure B.4 Schematic diagram of the synthetic promoter-reporter gene construct. A synthetic promoter, JL1, was placed upstream of the cauliflower mosaic virus (CaMV) -46 35S (minimal) promoter followed by the tobacco mosaic virus (TMV) omega leader, driving an mEmerald GFP reporter gene.

5'GTTGAATGTTGAATGTTGAATGTTGAATGTTGAATGTTGAATGTTGAATCCTGCGC
 AAGACCCTTCCTCTATATAAGGAAGTTCATTTCAATTTGGAGAGGACTATTTTTACAAC
 AATTACCAACAACAACAACAACAACAACATTACAATTACTATTTACAATTACACCA
 TCGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAG
 CTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGG
 CGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCT
 GCCCGTGCCCTGGCCACCCCTCGTGACCACCTTGACCTACGGCGTGCAGTGCTT
 CGCCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCC
 GAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGA
 CCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTG
 AAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTAC
 AACTACAACAGCCACAAGGTCTATATCACCGCCGACAAGCAGAAGAACGGCATCA
 AGGTGAACTTCAAGACCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCG
 ACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACA
 ACCACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCG
 ATCACATGGTCCTGCTGGAGTTCGT3'GACCGCCGCCGGGATCACTCTCGGCATG
 GACGAGCTGTACAAGAGGTGAGTGGCGCGCCCGATCGTTCAAACATTTGGCAATA
 AAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTG
 TTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATG
 GGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATA
 TAGCGCGCAAACCTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATC-3'

Figure B.5 Nucleotide sequence of the 171 bp synthetic promoter and mEmerald GFP reporter gene (starting at the yellow highlighted ATG) fusion construct from the 5' to 3' direction.

CHAPTER IV SUMMARY AND CONCLUSION

4.1 Research overview

The overarching goal of this work was to adapt advanced engineering systems for plant biotechnology. To that end, Chapter 2 presents an automated high-throughput droplet generation and trapping platform for creating arrays of complex DIBs, for biological assay and signaling cascade applications. The microfluidic device featured five inlets, one for the continuous oil phase and four for separate aqueous solutions and droplet production rates were independently controlled by adjusting the applied pressure and thereby the droplet sequence. Sets of four droplets, of varying compositions, were captured in a hydrodynamic trapping array to create linear DIB networks containing three interfacial lipid bilayers per network. The potential for more combinations of droplets in each network enables an increased complexity necessary for performing parallel multiplexed biological assays. In Chapter 3, on the whole plant scale of plant biotechnology, a diode laser platform was designed for the goal of imaging and measuring fluorescent protein expression in the canopies of whole plants. The fluorescence-inducing laser projector (FILP) was used to image a wide variety of fluorescent protein genes in plant leaves for the purpose of phenotyping transgenic plants expressing multiple constitutive or inducible fluorescent proteins. This system was critical for evaluating the performance of environmental phytosensors, which were designed to sense and report environmental stimuli.

4.2 Conclusions

This section presents conclusions generated by each research objective and addresses the scientific gaps identified in Chapter 1 (objectives are restated here for reference).

4.2.1 Objective 1

Design and fabricate a microfluidic device for generating droplets of four discrete compositions. The device needs to be able to capture four droplets in a trap array

for generating complex droplet networks. Numerous droplet network patterns must be created in a controllable manner. In order to increase the complexity of DIB networks, this first goal is to develop a method for forming complex patterns of DIBs for biological assay applications.

The key contributions of this research include:

- Developed a microfluidic device for the generation of droplets of four discrete aqueous solutions, capable of altering droplet sequences in a controllable manner, for the purpose of trapping complex DIB networks.
- Analyzed droplet pattern results of applying various phase shift between sinusoidal pressure inputs. With this design, lower phase shifts in pressure yielded greater accuracy in pattern predictions, but fewer droplet combinations; larger shifts yielded decreased accuracy, but a wider range of droplet combinations. Prediction efficiency can be improved by improving direct trapping of droplets and the number of observed patterns can be increased by widening the phase shift between input or increasing the amplitude of the input pressure wave.
- Characterized pressure-driven flow behavior in response to small changes in resistance. As a hydrodynamic trap array fills with droplets, droplet velocity and size decrease, indicating that pressure-driven flow was exceptionally sensitive to any increase in resistance.

4.2.3 Objective 2

The purpose was to conduct phenomic analysis of individual protoplasts to gain insight on how plants might respond to external stimuli, Objective 2 considers whole genetically modified plants. To address Gap 2, a laser system will be designed and built for imaging numerous fluorescent proteins in plant canopies from > 3 m standoff.

The key contributions of this research include:

- Designed and built a fluorescence-inducing laser projector (FILP) to phenotype transgenic plants expressing fluorescent protein (FP) genes. Plants expressing one or more of 20 listed FPs were imaged at > 3 m in a laser enclosure. A heatmap from fluorescence spectroscopy data and signal-to-noise ratios of each FP emission peak concluded that FILP imaging is an apt indicator of FP expression in transgenic plants.

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VITA

Tayler M. Schimel grew up in Pendleton, Oregon with her younger sister Kylee. She received a B.A. in Physics at the University of Idaho in May 2013 and her M.S. in Biomedical Engineering from the University of Tennessee in August 2019. She is currently set to graduate in December 2020 with a Ph.D. in Biomedical Engineering at the University of Tennessee, Knoxville.

While at the University of Idaho, Tayler did her undergraduate research in the Physics department studying magnetic nanoparticles. As a graduate student, Tayler's work focused on combining plant synthetic biology and microfluidics. She taught Engineering Fundamentals 151, an intro to physics course, for 7 semesters before becoming a Project Integrator for her lab's DARPA-APT research project.

When Tayler is not in the lab, she enjoys spending her time at Rocky Top Crossfit or at one of Knoxville's many local coffee shops.