Acoustofluidic Innovations for Cellular Processing On-Chip

by

Korine Allison Ohiri

Department of Mechanical Engineering and Materials Science
Duke University

Date: _________________________

Approved:

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Benjamin Yellen, Supervisor

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Ashutosh Chilkoti

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Kris Wood

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Mechanical Engineering and Materials Science in the Graduate School of Duke University

2018
ABSTRACT

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Abstract

The advent of increasingly proficient cell handling tools has led to a drastic maturation of our understanding of life on the microscale. Thus far, impressive strides have been made towards creating efficient and compact systems for manipulating cells on-chip. Multiple exciting cell handling methodologies have been explored and incorporated into cell separation and analysis tools ranging from passive hydrodynamic based systems to active magnetic and optoelectronic systems. Of these tools, acoustofluidic technologies, which employ the use of sound waves to manipulate cells in microfluidic environments, show great promise. These technologies offer a myriad of benefits including labeled or unlabeled cell manipulation, gentle handling of cells, long range manipulation of cells, and simplified fabrication compared to other active systems (e.g. magnetics, optoelectronics). Accordingly, in this dissertation I develop novel acoustofluidic tools that can combine with existing technologies and expand upon the array of systems available to scientists in biology and medicine for cell handling.

In my first experimental chapter, I characterize and develop elastomeric magnetic microparticles that can be used in future applications for multi-target cell separation from complex mixtures. These particles are comprised of a varying loading of magnetic nanoparticles evenly and stably distributed throughout a silicone matrix. Consequently, these particles uniquely exhibit a “dual contrast”, whereby they undergo
positive magnetophoresis and negative acoustophoresis in water. Further, I show that these particles can be functionalized with biomolecules via chemical modification by linking a biotin group to the surface-accessible amine groups on the particles using carbodiimide chemistry. These functionalized particles can then non-specifically or non-covalently bind to streptavidin molecules. Additionally, I characterize both the magnetic and acoustic properties of these particles by quantifying the magnetic susceptibilities and extent of acoustic focusing after 3 seconds for each particle formulation (i.e. 3 wt. % magnetite, 6 wt. % magnetite, 12 wt. % magnetite, 24 wt. % magnetite, and 48 wt. % magnetite in solids), respectively. Finally, I demonstrate a simple ternary separation of my 12 wt. % formulation from unlabeled human umbilical vein cells (HUVEC, non-magnetic) and magnetic beads that exhibit a positive acoustic contrast using magnetic and acoustic-based separations to highlight the unique properties of the mNACPs.

In my second experimental chapter I characterize and develop a novel acoustofluidic chip that employs the use of a trap and transfer approach to organize a high-density array of single cells in spacious compartments. My approach is based on exploiting a combination of microfluidic weirs and acoustic streaming vortices to first trap single cells in specific locations of a microfluidic device, and then transfer the cells into adjacent low shear compartments with an acoustic switch. This highly adaptable, compact system allows for imaging with standard bright field and fluorescence microscopes, and can array more than 3,000 individual cells on a chip the size of a
standard glass slide. I optimize the hydrodynamic resistance ratios through the primary
trap site, the bypass channel, and the adjacent compartment region such that particles
first enter the trap, subsequent particles enter the bypass, and particles enter the
compartment regions of a clean acoustofluidic chip upon acoustic excitation. Further, I
optimize the acoustic switching parameters (e.g. frequency and voltage), and prove that
acoustic switching occurs due to the generation of steady streaming vortices using
particle tracking methods. Uniquely, my system demonstrates for the first time the
manipulation of single cells with an array of streaming vortices in a highly parallel
format to compartmentalize cells and generate a single cell array.

Finally, for my third experimental chapter, I demonstrate the biological relevance
of the acoustofluidic chip I designed in my third chapter. First, I determine the trapping
and arraying efficiencies of cells in my acoustofluidic chip to be 80 and 67 %
respectively. Here, the arraying efficiency represents the percentage of single cells in the
compartment regions and is dependent on both the trapping efficiency and the acoustic
switching efficiency (which is roughly 84 %). Additionally, I observe the adhesion,
division, and escape of single PC9 cells from the compartment regions of my
acoustofluidic chip at 8 hour increments over 24 hours and identify potential obstacles
for quantitative analysis of cell behavior for motile populations. In these studies, I found
that it is possible to incubate arrayed single cells on-chip. Finally, I demonstrate that
single cells can be stained on chip in a rapid and facile manner with ~ 100 % efficiency either before or after adhesion to the surface of the microfluidic chip.

While the studies described herein address but a small fraction of the wider need for next-generation cellular manipulation and analysis tools, I present meaningful knowledge that can expand our understanding of the utility of acoustofluidic devices. Importantly, I (i) characterize for the first time a new type of particle that exhibits both negative acoustic contrast and positive magnetic contrast and (ii) develop a novel acoustofluidic chip that exploits the use of steady acoustic streaming vortices to generate a single cell array.
Dedication

To my family, Erica Wilson, Kerianne Richards, Kamar Duval, Constance Lawrence, my late father Tony Duval, and my husband Ugonna Ohiri, who have been my rock throughout this process.
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1. Introduction

The continued development and improvement of cellular capture, isolation, and analysis tools is vital in addressing a wide variety of biomedical challenges facing the scientific community. Accordingly, there exists the monumental task of inventing increasingly efficient methods to process complex biological samples (e.g. tissue samples, whole blood, sputum) such that individual populations of interest can be de-bulked and discretized into a single-cell array for longitudinal studies. Microfluidic tools have shown great promise introducing this next-generation of cell processing technologies, but there is still much room for growth and improvement in this young field.

1.1 Whole Blood De-Bulking

Whole blood is a complex mixture comprised of red blood cells (RBCs), white blood cells (WBCs; lymphocytes, monocytes, and granulocytes), platelets, plasma (contains proteins, sugars, and fats), and rare circulating species (e.g. circulating tumor cells, circulating fetal cells, circulating stem cells, etc.). Each of these populations contain properties of interest (e.g. immune response of WBCs, antigens in plasma) that warrant further study, thus necessitating the development of whole blood de-bulking technologies. Interest in the identification and isolation of rare circulating cells or rare cell states (e.g. dormant HIV infected T Cells) has rapidly grown ever since the emergence of ‘liquid biopsies’, where screening, diagnosis, and prognosis of diseases is
conducted using biological fluidic samples (i.e. urine, whole blood, cerebral spinal fluid). Consequently, a vast array of labeled and label free techniques for identifying and isolating cell populations have been developed.

Labeled cell manipulation techniques require the spontaneous uptake of synthetic beads\textsuperscript{2-4}, binding of intracellular receptors\textsuperscript{5}, or- more frequently- binding to the surface of the cell.\textsuperscript{6-8} The target binding site and labeling efficiency also varies wildly depending on the cell population (or sub population) of interest and whether negative selection (i.e. labeling and de-bulking all non-target cells) or positive selection (i.e. labeling and de-bulking target cells) is desired. Negative selection is comparatively more expensive and less effective, as it requires a wider variety of labeling beads and higher labeling efficiencies to successfully de-bulk all non-target cells.\textsuperscript{9} Additionally, negative selection is a poor option when target cells must be de-bulked from a rare or poorly characterized solution (e.g. de-bulking tumor cells from WBCs). However, this method is preferred to positive selection when labeling target cells can potentially disrupt downstream analysis (e.g. excess magnetic beads in a de-bulked cell culture).

The most common labeled separation techniques capable of both positive and negative selection are fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS).\textsuperscript{10,11} When employing the former method, cells are first fluorescently labeled then drawn into the FACS machine where they are aligned in a single flow stream and individually interrogated for their fluorescent signal, size, and morphology.
The number of lasers involved in interrogation is dependent on the level of sophistication of the machine, the maximum of which is 18 florescent channels, a forward scatter channel (size), and side scatter channel (morphology or granularity). After interrogation, cells are given a charge depending on their fluorescent signature and sorted into multiple outlets (Figure 1.a).13

Figure 1: Common cell sorting methodologies. (a) Schematic of fluorescent activated cell sorting13 (b) Magnetic activated cell sorting process where magnetic species (black) remain trapped while non-magnetic species (white) are depleted.14
In contrast, MACS involves magnetically labeling target cells (or non-target cells) and injecting them into a high gradient magnetic field. MACS devices are comprised of a permanent magnetic clamp that fits a specially designed syringe with paramagnetic columns leading to the outlet. Magnetically labeled objects become trapped along magnetic columns in the syringe when mounted onto the permanent magnet, while label free cells freely flow through. After this first separation, the syringe is removed from the magnetic column, thus releasing the trapped particles which are then ejected out of the syringe (Figure 1.b).^{14} In addition to FACS and MACS there are numerous new technologies and research efforts that manipulate labeled cells by miniaturizing key elements from both of these machines into microfluidic devices using alternative fields or using passive sorting methods.

Both labeled and label free cell separation can be categorized into active or passive methods. Active separation involves the use of an applied external field (e.g. magnetic, acoustic, optical) to sort cells while passive separation techniques do not require an external field or power input (e.g. affinity based assays, deterministic lateral displacement). Active separation can be further categorized into type one, where an external field is used but there is no power input, and type two, where both an external field and power input is required. Typical active separation fields include magnetic, acoustic, electrokinetic (e.g. dielectrophoresis, electroosmosis, electrophoresis), optical, and mechanical forces.^{15} Typical passive separation techniques include inertial focusing,
pinched flow fractionation, deterministic lateral displacement, and cellular immobilization techniques.\textsuperscript{15}

Aside from mechanical forces, all the active forces are dependent on the applied field and material properties of the target cells and suspending fluid. Accordingly, magnetic forces are dependent on the magnitude of the gradient of the magnetic field and magnetic susceptibilities of the suspension; while conversely, acoustic forces are dependent on the applied pressure field, densities, and compressibilities of the suspension. Additionally, electrokinetic forces are dependent on the applied electric field and corresponding dielectric properties or charges associated with the suspension while optical forces are dependent on the applied optical beam and associated refractive indices of the suspension. Contrastingly, mechanical forces enable sorting by incorporating flow switches and structural valves into bioseparation devices. Analogous to mechanical forces, passive separation techniques are dependent on the structure of the microfluidic channel as well as the size of the target cells in suspension.

Inertial focusing causes the migration of target cells to stable positions along the microfluidic device due to lift forces in long, serpentine channels. Additionally, pinched flow fractionation separates cells in solution by size due to lift forces associated with the broadening of the microfluidic channel. Deterministic lateral displacement also separates cells in solution by size due to the prescribed arrangement of columns in a microfluidic channel that causes smaller cells to travel along a separate preferential
direction from larger cells. Finally, immobilization techniques rely on the binding (either specifically or non-specifically) of target cells to capture sites (e.g. pillars, wells) in the microfluidic channel while unbound cells are depleted from solution. Each of these separation mechanisms provide the following key advantages: inexpensive, low impact on cell viability, capable of immunospecific capture, or capable of dynamic manipulation as highlighted in Table 1.

Table 1: Key advantages of various sorting mechanisms.*

<table>
<thead>
<tr>
<th>Type</th>
<th>Sorting Mechanism</th>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inexpensive</td>
</tr>
<tr>
<td>Active</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acoustic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electrokinetic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optical</td>
<td></td>
</tr>
<tr>
<td>Passive</td>
<td>Inertial focusing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pinched flow fractionation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deterministic lateral displacement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immobilization</td>
<td></td>
</tr>
</tbody>
</table>

* Dark regions signify sorting mechanism belongs to group

Due to these unique benefits, many of these mechanisms have been (or are in the process of being, since 2015) successfully incorporated into commercial cell sorting devices, as detailed in Table 2 below. Please note that CTC is an initialism for “circulating tumor cells”.
Table 2: Microfluidic cell sorting technologies.

<table>
<thead>
<tr>
<th>Type of Device</th>
<th>Sorting Product</th>
<th>Sorting Mechanism(s)</th>
<th>Company/Collaborative Entities (Acquired by)</th>
<th>Key Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passive</td>
<td>ClearCell® FX System</td>
<td>Geometric features to trap cells by size and stiffness (^{17,18})</td>
<td>Clearbridge Biomedics, Pte Ltd.</td>
<td>Automated and label-free isolation of CTCs from 8 mL of blood within 1 hr</td>
</tr>
<tr>
<td></td>
<td>Rare Cell Isolation Chip</td>
<td>Deterministic lateral displacement (^{19}) and affinity capture (^{20})</td>
<td>GPB Scientific, LLC</td>
<td>Separation of rare cells (other than CTCs) by size and immunospecific immobilization</td>
</tr>
<tr>
<td>Active (Type 1)</td>
<td>CTC-iChip</td>
<td>Deterministic lateral displacement and magnetophoresis (^{21})</td>
<td>Janssen Diagnostics, LCC / Massachusetts General Hospital</td>
<td>Isolation of CTCs from whole blood with capabilities for positive and negative selection</td>
</tr>
<tr>
<td></td>
<td>LiquidBiopsy®</td>
<td>Magnetophoresis (^{22,23})</td>
<td>Cynvenio Biosystems, Inc. (Distribution: Thermo Fisher Scientific, Inc.)</td>
<td>Automated isolation of CTCs from whole blood at 5 mL/hr and enables molecular analysis</td>
</tr>
<tr>
<td>Active (Type 2)</td>
<td>Free Flow Acoustophoresis Chip</td>
<td>Free Flow Acoustophoresis (^{24})</td>
<td>AcouSort AB</td>
<td>Label-free approach to acoustically sort cells by size in a continuous flow microchip device</td>
</tr>
<tr>
<td></td>
<td>DEPArray™ System</td>
<td>Electrokinesis (^{25})</td>
<td>Silicon Biosystems, Inc. (Menarini Group, Co.)</td>
<td>Individually traps cells in dielectrophoretic cages for micromanipulation</td>
</tr>
<tr>
<td><strong>GigaSort™</strong></td>
<td>Pneumatic chambers to hydrodynamically displace fluid</td>
<td>Cytonome, Inc.</td>
<td>Parallel microchip for cell identification and sorting up to 48,000 cells/sec</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
<td>----------------</td>
<td>----------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Jet Flow Sorter</strong></td>
<td>Bubble-jet flow generated from Joule heating</td>
<td>JSR, Co. / IMEC vzw</td>
<td>On-chip imaging and cell identification to sort 2,000 cells/sec for each channel</td>
<td></td>
</tr>
<tr>
<td><strong>MACSQuant® Tyto</strong></td>
<td>Fluorescence detection and electro-mechanical switching</td>
<td>Owl Biomedical, Inc. (Miltenyi Biotec GmBH)</td>
<td>Disposable cartridge with a high frequency switch for sorting up to 30,000 cells/sec</td>
<td></td>
</tr>
<tr>
<td><strong>Sorting Chip for Cell Sorter SH800</strong></td>
<td>Focuses and detects cells for post-chip sorting via FACS technology</td>
<td>Sony Biotechnology, Inc.</td>
<td>Disposable cartridge that aligns automatically in a permanent workstation for cell identification</td>
<td></td>
</tr>
<tr>
<td><strong>Wolf™ Cell Sorter</strong></td>
<td>Fluorescence detection and acoustophoretic displacement</td>
<td>NanoCellect Biomedical, Inc.</td>
<td>Sorts over 330 cells/sec while removing contaminants such as cell-free DNA</td>
<td></td>
</tr>
</tbody>
</table>

### 1.2 Single Cell Analysis

The earliest form of cellular analysis came with the invention of the simple light microscope in the 16th century. While crude, this pioneering method gave scientists such as Robert Hooke insight into the underlying structure of individual cells. Decades later, technology has drastically improved and applications for cellular analysis range from improving our fundamental knowledge of cellular functions and interactions (e.g.
studying signaling pathways of stem cells, understanding the intricacies of
tumorigenesis)\textsuperscript{12,34,35} to aiding in the screening, diagnosis, and prognosis of diseases,\textsuperscript{36} to
drug development, toxicity, and efficacy studies.\textsuperscript{37-39} Accordingly, an array of single-cell
analysis techniques have been developed (Table 3). These techniques encompass a
combination of genomics (i.e. gene level interrogation), transcriptomics (i.e. mRNA level
interrogation), proteomics (i.e. protein level interrogation), metabolomics (i.e. metabolite
level interrogation) and various labeling (e.g. antibody) and imaging (e.g. fluorescent,
nuclear magnetic resonance, optical) techniques.

**Table 3: Single Cell Analysis Techniques.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Information Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell labeling and staining\textsuperscript{40,41}</td>
<td>Detection of membrane or intracellular proteins using fluorescent or optical imaging</td>
</tr>
<tr>
<td>Antibody staining of proteins\textsuperscript{12}</td>
<td>Measurement of gene expression products</td>
</tr>
<tr>
<td>Fluorescent imaging of RNA in situ\textsuperscript{42}</td>
<td>Quantification of RNA molecules in fixed cells</td>
</tr>
<tr>
<td>Fluorescent imaging of fusion proteins\textsuperscript{43}</td>
<td>Measurement of protein dynamics in live cells</td>
</tr>
<tr>
<td>Patch-clamp technique\textsuperscript{44}</td>
<td>Measurement of electrophysiological signals from live neurons</td>
</tr>
<tr>
<td>FACS\textsuperscript{12,13}</td>
<td>Sorting of fluorescently labeled single cells based on up to 18 membrane proteins</td>
</tr>
<tr>
<td>Electrospray Ionization Mass Spectrometry\textsuperscript{45,46}</td>
<td>Provides information on structure and molecular mass of analytes</td>
</tr>
<tr>
<td>DNA sequencing\textsuperscript{47}</td>
<td>Enables DNA profiling</td>
</tr>
<tr>
<td>Nuclear magnetic resonance imaging\textsuperscript{48,49}</td>
<td>Images the anatomy and active processes of a cell</td>
</tr>
</tbody>
</table>

Traditional systems biology approaches to cellular analysis\textsuperscript{34,50} employ bulk
measurements of cellular signals that compensate for noise\textsuperscript{51,52} amongst homogenous
populations by providing an ensemble averaged response. Contrastingly, single-cell analysis allows for the dynamic discovery and investigation of rare cellular events\textsuperscript{53} (e.g. digital response of a population to a stimulus) or populations\textsuperscript{54-56} that are often lost during bulk measurement. Due to the length scale of single cells, microfluidic systems offer the most practical environment to optimize the throughput, automation, and cost effectiveness of single-cell studies.

1.3 Microfluidic Devices

Microfluidic systems are engineered to process small pockets or streams of fluid (10\textsuperscript{-9} to 10\textsuperscript{-18} liters) in microscale (10\textsuperscript{-4} to 10\textsuperscript{-7}) channels.\textsuperscript{57} These systems have proven to be capable of performing a variety of cellular analysis tasks such as DNA sequencing\textsuperscript{58}, cell transfection\textsuperscript{59}, and polymerase chain reaction (PCR) DNA amplification.\textsuperscript{60} This technology offers an assortment of additional benefits such as reduced reagent consumption, increased automation (i.e. reduction of skilled labor), shorter analysis times, disposability, decreased footprint, and increased temporal resolution and sensitivity, while reproducing much of the same analytical information as traditional bulk instrumentation (e.g. FACS, MACS) with a comparable quality.\textsuperscript{15} Accordingly, a vast array of microfluidic devices have been constructed using varying fabrication techniques suited to the application of the device such as micro-machining, replica-molding, embossing, injection molding, in situ construction injection, laser ablation, photolithography, and 3D printing.\textsuperscript{61,62} Of these methods, replica molding,
micromachining, and photolithography techniques are the most common. Here, each common technique is categorized into: (i) poly(dimethylsiloxane) (PDMS) based, (ii) silicon based, and (iii) SU-8 based and are described below.

PDMS is by far one of the most popular materials in the scientific community for fabricating microfluidic devices due to its unique material properties and low cost. Advantages of PDMS based microfluidic devices include flexibility (i.e. the ability to incorporate pneumatic valves for flow switching), optical transparency (i.e. transparent down to 230 nm), biocompatibility (i.e. waterproof, non-toxic, gas permeable), rapid water-tight sealing with glass, simple fabrication of multi-layered devices, and rapid prototyping. PDMS based devices are most often fabricated using replica molding, a technique originated in Prof. George Whitesides’ group in the late 1990s. In this technique, first a master mold is fabricated using standard photolithography (Figure 2.a). Next, PDMS prepolymer (base and cure) are poured on to the master and left to cure (Figure 2.b-c). Finally, the replica PDMS device can be removed, oxidized, and sealed on a glass slide (Figure 2.d-e). As the master mold can be easily fabricated and used multiple times (> 30), this methodology is preferred during design optimization when multiple iterations of a device are considered. However, disadvantages of this method include poor compatibility with long term cell culture, analyte adsorption due to hydrophobicity, and diffusion of small molecules due to the permeability of PDMS.
Figure 2: Replica molding technique for fabricating PDMS based microfluidic devices.\textsuperscript{64}

The next most common material for microfluidic device fabrication is silicon. Benefits of these systems include precise fabrication of high-aspect ratio channels, high acoustic reflectance in water, high temperature tolerances, impermeability to liquids and solutes, biocompatibility (i.e. non-toxic, chemically inert), and industrial scalability.\textsuperscript{68,69} Disadvantages of silicon based devices include complex fabrication and high cost. Silicon based microfluidic devices are commonly fabricated using bulk micromachining
techniques, which consist of isotropic (rounded) or anisotropic (straight) chemical (liquid or vapor, typically isotropic) or plasma etching (typically anisotropic).\textsuperscript{68,70} Generally, chemical vapor etching is conducted by creating volatile silicon compounds from a reaction of the silicon substrate with highly reactive halogen molecules. Additionally, wet etches are conducted using highly reactive acids, bases, or non-aqueous compounds that aggressively oxidize the silicon. Typical chemical etchants for silicon include fluorine based compounds (xenon difluoride (XeF\textsubscript{2})), sulfur hexafluoride (SF\textsubscript{6}), potassium hydroxide (KOH), tetramethyl ammonium hydroxide (TMAH), and ethyleneadimine pyrocatechol (EDP).\textsuperscript{71} Chemical etches can be combined with passivation layers and plasma etches, in which focused ion beams bombard unprotected surfaces of the silicon, in order to fabricate straight, well-defined, microchannels. The most common of these hybrid methods capable of fabricating microfluidic channels (~10\textsuperscript{-6} to ~10\textsuperscript{-4} deep) is deep reactive ion etching (DRIE), which most often uses the ‘Bosch process’. There are two main steps in this process, first the surface of exposed silicon (i.e. not protected by photoresist or metal layers) is bombarded with a beam of ions and a chemical etchant, typically SF\textsubscript{6}. Next, a passivation layer, typically C\textsubscript{4}F\textsubscript{8}, is deposited to protect the side walls of the microchannel. This process is highly selective (≈50-100:1 photoresist selectivity and ≈120-200:1 silicon dioxide selectivity) and cycles until the prescribed depth is reached.\textsuperscript{70}
A third useful material in microfluidic device fabrication is SU-8, an epoxy-based negative photoresist. SU-8 is comprised of an acid-labile oligomer (Bisphenol A Novolak epoxy oligomer) and a photoacid generator (triarylsulfonium hexafluoroantimonate salt). Upon irradiation, the photoacid generator decomposes and protonates the oligomer, which then cross-links during heating. This leads to a highly mechanically, thermally, and chemically stable structure that can withstand temperatures up to ~200°C and maximum stresses of ~34 MPa. Consequently, SU-8 based microfluidic system offer a variety of benefits such as biocompatibility, high compatibility with integrated circuit processes, potential optical transparency (above 360 nm for the SU-8 3000 series), high aspect ratio structures, and chemical inertness. Importantly, unlike PDMS and silicon based chips, SU-8 based devices are more easily integrated into devices with multiple metallic layers (e.g. for the incorporation of underlying integrated circuitry) due to the increased ability to align and pattern microchannels on top of deposited layers without extra equipment (e.g. PDMS aligner) or complicated processes (e.g. depositing gold patterns across the top of silicon surfaces into etched trenches). As such, SU-8 offers an interesting alternative to these devices but still maintains key disadvantages relative to each material such as low mechanical strength and difficulty creating a high-strength water-tight seal.

Ultimately, the appropriate material and fabrication method for a microfluidic device is constrained by its application and operating principals. While a silicon-based
device is ideal for driving mechanisms that require a high acoustic reflectance (e.g. bulk acoustophoretic manipulation) or operating temperature (e.g. thermocapillarity), it is a poor choice for driving mechanisms that require multi-layer devices (e.g. the deposition of magnetophoretic tracks or gold wires across microchannels) as patterns will be distorted by the gap between the top and bottom of the etched silicon trench. Here, SU-8 or PDMS based devices would be more appropriate for multi-layer designs since the walls can be erected as the last step after depositions. Along with engineering constraints, market considerations such as stage of development, cost, end-user, and disruptive technologies (e.g. 3D printing) inform the development of microfluidic devices.

1.4 Commercial Systems for Single-Cell Analysis

With the improvements of microfabrication and system automation tools, trapping and arraying tens of thousands of cells in a compact microfluidic system has become increasingly feasible. Thus, three major technologies have risen to dominate the microfluidic single cell analysis space: the DEPArray by Silicon Biosystems, the Beacon Platform by Berkeley Lights, and the C1 system by Fluidigm. Akin to its name, the DEPArray traps and manipulates single cells using positive dielectrophoretic forces (Figure 3.a) such that electrodes can be serially activated to transport a cell of interest (green) along a prescribed trajectory for extraction and downstream analysis. The Beacon Platform works similarly to the DEPArray, except this unique system allows for
the transport of cells into “pin” compartment regions using “virtual electrodes”. These electrodes can be generated on demand, having dynamic and flexible addressability using phototransistor based optoelectronic tweezers.\textsuperscript{75} Contrastingly, Fluidigm’s C1 system serially traps cells using purely passive hydrodynamic forces and a complex valving system (Figure 3.b). Here, the microfluidic path is designed such that a single cell (yellow) will be trapped at the entrance of an isolated compartment. Once loaded, cells can be injected into compartments, processed, and analyzed on-chip.

![Figure 3: Schematic of commercialized single-cell analysis devices. (a) DEP array with cells freely suspended in solution while off (top) and trapped in DEP cages when on (bottom). (b) Single cell hydrodynamic trapping scheme of the C1 system. Images for the DEPArray (https://www.youtube.com/watch?v=ANZKlsBpK_c) and C1 System (https://www.youtube.com/watch?v=Yg8yEeKoB2Q) found online.](image)

Despite the disparate operating principles for these systems, they both offer unique benefits and shortcomings in their design concerning power consumption, throughput, complexity, and analysis capabilities. While the DEPArray is capable of
manipulating a large array of cells (~10,000~10,000), it requires a continuous input of power as each trapping site on the array consumes energy during use. This exposes a severe limitation in the design of the DEPArray, as power consumption for this device increases with throughput. Furthermore, as cellular transport is also controlled by dielectrophoretic traps, throughput is additionally constrained by the ability to both trap and transport cells onto available cages (30,000 in all). The Beacon Platform overcomes some of these limitations with virtual electrodes, however this complex system requires advanced image recognition and a fully integrated light modulator. Contrastingly, while the C1 system requires a relatively nominal power input, the maximum throughput of the system (as of 2015) is limited to 800 cells per experiment due to the extensive complexity (e.g. due to multiple fluidic and pneumatic lines or complex flow trajectories) of the multi-layered PDMS structure.

Additionally, it is important to note that the DEPArray has limited processing and analysis capabilities. Its main purpose is to serve as an identification and retrieval tool for fluorescently labeled targets of interest. Along with this, the Beacon Platform has an ultra non-fouling surface coating that may interfere with adherent cell culture, along with lacking the ability to dynamically exchange fluids due to the unbalanced fluidic resistance in the chip. Contrastingly, the C1 system is capable of performing multiple operations on-chip including real time PCR analysis (i.e. extraction, amplification, and harvesting of DNA) from each individual cell (Figure 4).
Figure 4: Operation of C1 system. (a) Single cells captured in hydrodynamic trap. (b) Cells are lysed. (c) Cell DNA released. (d) DNA amplification and thermal cycling conducted while mixing with peristaltic pumps. (e) Amplified DNA harvested.

While this *on-chip* analysis capability firmly establishes the C1 system as the most advanced microfluidic single-cell analysis technology, it still lacks the ability to perform longitudinal studies. Unlike the Beacon Platform, both the C1 system and DEPArray are poorly suited to culture cells for extended periods of time (e.g. > 1 week), capture the progeny of cells, or perform time-lapse images over the lifetime of a cell.\textsuperscript{76} Further, though the Beacon Platform overcomes many of these limitations, this new promising system is still in its infancy (i.e. launched late 2016) and intimately complex to fabricate and operate. Consequently, there is still much room for growth and competition in the research and development of single-cell microfluidic technologies.

1.5 Pre-Translational Systems for Single-Cell Analysis

There has been a burgeoning research effort to produce efficient microfluidic single-cell analysis systems that improve upon existing technologies but have yet to be commercially translated. This pre-translational space is largely populated with droplet microfluidic systems and trap-based systems (e.g. hydrodynamic and well plates).
Similar to the C1 system, most of these technologies isolate individual (or a small collection of) cells into trap sites for further analysis *on-chip*. These applications range from *on-chip* PCR, to multiplex measurements of secreted molecules, to cell sorting based antibody secretion, to lineage tracking of single cells. Though there is a wealth of new applications offered by these pre-translational systems, the operating principals of the system dictates its versatility.

Microwell plates are the least dynamic pre-translational system but are the most compatible with existing technologies and easily scaled. Specifically, more than 85,000 individual 50 μm PDMS based microwells and over 200,000 10 x 12 x 30 μm polystyrene based microwells can be arrayed on a simple glass slide (25 x 75 mm²). Briefly, PDMS based microwells can be fabricated using silicon masters and replica molding (see 1.3 Microfluidic Devices) while polystyrene based chips are made using the lithographie galvanostatic ennoblement (LIGA) process. Similar to replica molding, the LIGA process involves lithography and molding steps but additionally requires an electroplating step to create the mold. Once fabricated, microwells can be sterilized, loaded with cells, and temporarily sealed using glass pre-treated with capture species (e.g. secondary antibodies). During incubation, proteins secreted from cells are captured on the glass surface and subsequently detected with fluorescent species (e.g. labeled antigens) (Figure 5). These systems are compatible with commercial microarray scanners and also capable of tracking the lineage and mapping the gene expression of
single cells (Figure 5). Despite these advantages, microwells suffer from poor single-cell capture efficiency of polydisperse populations and complex retrieval mechanisms such as by micromanipulator or manually.

![Figure 5: Capture and analysis of single cells (or cell pairs) on microwell plates.](image)

Simple hydrodynamic traps are comparably more complex than microwell plates as they require careful consideration of the fluidic resistance in the chip and precise fabrication to direct the flow into trap sites. Despite these added constraints, hydrodynamic trapping systems offer benefits similar to the microwell assembly including, but not limited to, scalability, cell-pair studies, and lineage tracking of single cells. Along with these benefits, simple hydrodynamic traps are capable of capturing individual cells with high resolution and performing PCR (or other common analysis methods) on-chip with the incorporation of pneumatic valves (as seen in Fluidigm’s C1 system). These systems are typically made using replica molding, but can work with any water proof material as long as there is not significant distortion (i.e. swelling of walls) between the original geometry and final product. The basic operating principal of this
chip relies on designing flow junctions with specified fluidic resistances such that trap sites have a lower resistance than the bypass channels (Figure 6).

![Figure 6: Typical hydrodynamic trap configuration for cell-pair studies. (a) Fluidic circuit overlaid on schematic of microfluidic element. (b) Zoom in of fluidic junction. $Q_1$ is greater than $Q_2$ due to the lower fluidic resistance through the trapping stream.](image)

Once trap sites are filled the resistance through the trap increases, thus making the bypass channel the preferential path. Simple hydrodynamic traps can be grouped into two categories: linked and confined. Linked configurations are simple traps that do not have an attached, isolated chamber. While this system is ideal for cell lineage tracking or simple cell-pair studies, they are at risk of cross contamination. Alternatively, confined configurations are akin to the C1 system, where cells are first hydrodynamically trapped, then transported to an isolated chamber. These systems are ideal for confined reactions such as PCR and detection of eluted species but are typically designed for a single function and are not made to be dynamic.

Finally, the newest and most complex group of pre-translational technologies are droplet-based systems. These systems employ the use of two phase “oil-in-water”
platforms to encapsulate cells, reagents, and bio-sensing beads in individual droplets. Cells are suspended in media (i.e. aqueous or water phase) and co-flowed into a fluorinated carrier oil. The choice of carrier oil due to its impact on the gas levels in the droplet (e.g. by dissolving ~20 times oxygen than water) and the quantity of organic molecules in the droplet (e.g. by being a poor solvent) and thus strongly impacts the viability of cells and detectability of secreted molecules. As droplet-based systems operate under continuous flow, they are capable of performing a multitude of functions on encapsulated cells (Figure 7). These functions include but are not limited to sequential isolation, detection (i.e. via co-encapsulation with bio-sensing beads), sorting at a rate of ~200Hz using external forces (e.g. dielectrophoresis, acoustophoresis), and recovery by bursting the droplets. As such, droplet microfluidic systems can be used in a variety of bioanalytical applications such as drug screening, DNA sequencing, and single-cell analysis.

Figure 7: Cell encapsulation using emulsion microfluidics. Potential applications include (a) single copy amplification, (b) whole genome amplification, and (c) barcoding.
Despite the numerous benefits of droplet microfluidics, these systems have the lowest single cell capture efficiency due to the random nature of encapsulation. As such, the probability (P(x)) of encapsulating x cells in an individual droplet is predicted by the following Poisson distribution:

\[ P(x) = e^{-\lambda} \frac{\lambda^x}{x!} \]  

[Eq. 1]

Here, \( \lambda \) represents the mean number of objects to be encapsulated (e.g. cells or beads) per droplet. Accordingly, the probability of encapsulating a single cell is dependent on the concentration of the original cell solution. Encapsulation efficiencies in droplet microfluidic systems further decreases when attempting to co-encapsulate one cell with one bio-sensing bead or a cell pair of interest. This results in longer encapsulation times\(^7\) (~ 1 h) and reagent waste.

In summary, each commercialized and pre-translational technique has drawbacks that can be categorized into the following flaws:

1) **Lacks separate reaction chamber for individual cells**  
   - DEPArray, Hydrodynamic trapping systems
2) **Low throughput** (i.e. < 1000 cells can be captured and analyzed at a time)  
   - C1 System, Hydrodynamic trapping systems
3) **Poor single cell capture efficiency**  
   - Droplet based systems, Microwell plates
4) **Complex operation** (i.e. multiple parameters must be manipulated simultaneously or sequentially for proper functioning of the device)  
   - DEPArray, C1 System, Droplet based systems, Beacon Platform
5) **Lacks ability to dynamically exchange fluids** (e.g. drug treatments, deliver fresh media)  
   - DEPArray, Microwell plates, Beacon Platform
Accordingly, while each of these methodologies enable some form of single-cell organization and analysis, there is a clear need for a hybrid system that retains the benefits of existing technologies while simplifying and streamlining the overall design of microfluidic single-cell templating systems.

1.6 Summary of Experimental Studies

The typical process for isolating, organizing, and analyzing individual cells requires first collecting the sample, then separating cell populations using positive (i.e. labeled target) or negative (i.e. un-labeled target) sorting mechanisms, next organizing the target population on a cellular array, and finally performing on-chip genotypic or phenotypic analysis of each individual cell or subpopulation of interest (Figure 8).

As described earlier in this chapter (1.3 Microfluidic Devices), miniaturizing and automating this work flow using an integrated microfluidic system allows for profound benefits (e.g. reduced reagent consumption, and increased precision) as compared to its bulky counterparts. While individual processes in this work flow have already been adapted into commercial microfluidic platforms (1.1 Whole Blood De-Bulking, 1.4 Commercial Systems for Single-Cell Analysis), most of these technologies rely on a single well-established mechanism to manipulate cells (e.g. magnetophoretic trapping and hydrodynamic trapping) and few of these systems incorporate technologies still in
the nascent stages of development (e.g. acoustofluidic chips). Accordingly, I introduce hybrid acoustofluidic technologies that increase the functionality and feasibility of a holistic microfluidic tool for cellular processing.

I first describe a novel class of dual contrast particles that enables multi-target separation using both magnetic and acoustic forces (Study I, Figure 9.a). Next, I describe a hybrid array in which cells are serially trapped using hydrodynamic course alignment then transported to individual apartment sites using acoustically actuated fine alignment (Study II, Figure 9.b). Finally, I validate our hybrid cellular array by performing basic cellular analyses (e.g. viability studies, drug response studies, cell-pair generation) (Study III, Figure 9.c).

Figure 9: Proposed acoustofluidic innovations. (a) Study I details a tunable class of dual contrast particles, (b) Study II details systems and methods to organize cells in a hybrid acoustofluidic array and, (c) Study III details basic analysis capabilities of the hybrid array.
2. Characterization and Development of Dual Contrast Bio-Sensing Particles

Separation with biofunctional magnetic beads is the current gold standard for the capture, isolation, and downstream manipulation of many target analytes due to their ease-of-use and prevalence. This well-understood approach has had far-reaching implications in a variety of research efforts, including positive and negative selection strategies for debulking cellular suspensions,\textsuperscript{20,97-99} establishing a platform for fundamental \textit{in vitro} studies,\textsuperscript{100} and the organization of single cells on magnetophoretic arrays.\textsuperscript{101-104} Despite significant benefits such as low cost, ease of use, and broad applicability of magnetic manipulation, traditional magnetic separation relies on a single force which generally fails to robustly sort non-labeled or weakly diamagnetic materials. This causes difficulty separating magnetic targets from each other (e.g. multiplex targeting, removal of unbound magnetic beads prior to cell culture) or from concentrated non-magnetic solutions (e.g. whole blood separation).\textsuperscript{105} Additionally, magnetic separation can lead to the entrainment of contaminating species into collection ports or a decrease in throughput of the separation mechanism. Acoustic separation, however, overcomes these limitations by applying a differential force on particles or cells in suspension based on their inherent mechanical properties, or acoustic contrast factor. Consequently, I have engineered a new class of magnetic, negative acoustic contrast particles (mNACPs), Figure 10.a) that undergo positive magnetophoresis
(Figure 10.b) and negative acoustophoresis\textsuperscript{106,107} (i.e., by migrating to the pressure antinodes of a standing wave) in water (Figure 10.c). These particles enable new combinations of acoustic and magnetic manipulation for microfluidic separations, assays, and other applications such as colloidal assembly.

Figure 10: Overview of dual contrast particle technology. (a) Conceptual schematic (not to scale) of a functionalized particle in a magnetic field and an acoustic standing wave (white crosses represent streptavidin molecules and black boxes represents biotin). (b) Bright field image of particles disordered (left) and aligned due to imposition of a magnetic field (right). (c) Fluorescent image of particles focused in acoustic standing wave (particles are focused along the walls of the microchannel, corresponding to the pressure antinodes). (d) Images of suspensions of particles containing 0, 3, 6, 12, 24, and 48. wt.% Fe\textsubscript{2}O\textsubscript{3} (from left to right). (e) Bright field image of particle synthesis using an oil-in-water capillary microfluidic system. Scale bars represent 200 μm.

The sorting of particles due to magnetic forces depends on their magnetic susceptibility ($\chi$), while acoustophoresis in standing waves depends on their acoustic contrast factor ($\varphi$):\textsuperscript{108}

$$\varphi = \frac{5\rho_p - 2\rho_o}{2\rho_p + \rho_o - \frac{\beta_p}{\beta_o}}$$  \hspace{1cm} \text{[Eq. 2]}
which can be either positive or negative depending on the relative density, $\rho_p$ and $\rho_o$, and bulk compressibility, $\beta_p$ and $\beta_o$, of the particle with respect to the suspension media. Most biological materials, such as cells and biopolymers, exhibit a positive acoustic contrast factor. Materials that exhibit a negative acoustic contrast are thus strongly preferred for separation systems.

Towards this end, I have developed a new class of particles that exhibits both positive magnetic contrast and negative acoustic contrast, which allows these particles to be separated from native biological tissues or cells and commercial magnetic beads.$^{109,110}$

**Contributions:**

For the results discussed in this chapter (2. Characterization and Development of Dual Contrast Bio-Sensing Particles) I worked in collaboration with researchers within Duke University and at Elon University. Professor Benjamin Evans synthesized the precursor FFPDMS formulations and trained me on how to precipitate iron nanoparticles and incorporate them in to the amine functional, PDMS-based matrix to independently make more FFPDMS if need be. Dr. Nick Carrol and I worked on optimizing the droplet microfluidic platform for FFPDMS to form monodisperse microparticles. I independently fabricated particles using bulk homogenization, optimized the particle functionalization routine, and quantified the acoustic response of each particle formulation using particle tracking methodologies. Professor Benjamin Evans additionally measured the magnetic susceptibility of each particle and provided
useful discussions on the analysis for the magnetics section. Finally, Mr. Robert Gutiérrez and I quantified the magneto-acoustic ternary separation together.

**2.1 Synthesis of mNACPs**

I have demonstrated the ability to adjust the acoustic and magnetic contrast factors by preparing mNACPs with controlled ratios of magnetic nanoparticles embedded in an elastomeric, silicone matrix (i.e., 6-7% aminopropylmethylsiloxane-dimethylsiloxane copolymer; Gelest, Inc.; Figure 10.d), a composite material referred to as ferrofluid polydimethylsiloxane (FFPDMS). This combination of materials yields an overall low bulk modulus that allows the particles to be magnetically responsive while exhibiting a negative acoustic contrast in water.

These particles can be synthesized rapidly using homogenization techniques, or they can be made using emulsion (oil-in-water) microfluidics systems to enable their synthesis in a near-monodisperse form (e.g., with a 9% coefficient of variance; Figure 10.e). Precursor ferrofluid was synthesized using previously described methods, where 100% nanoparticles were produced using co-precipitation techniques then stabilized in an elastomer. Briefly, the ferrofluid was comprised of magnetic nanoparticles uniformly stabilized in 6-7% aminopropylmethylsiloxane-dimethylsiloxane copolymer. During bulk homogenization, this ferrofluid was added to a 0.5 wt.% solution of CTAB in deionized water and was subsequently cross-linked with 37 wt.% formaldehyde in
water. In addition to bulk homogenization, magnetoelastomeric particles were also synthesized using a capillary microfluidic oil-in-water emulsion device (Figure 11).

![Figure 11: Capillary oil in water microfluidic device](image)

I prepared an injection capillary to flow the ferrofluid oil droplet phase by tapering a cylindrical glass capillary and subsequently inserting the injection capillary into a square capillary. Next, I inserted a cylindrical collection capillary into the square capillary from the other end, as pictured in Figure 1e, to confine fluid flow near the injection tip. To form ferrofluid drops, ferrofluid was co-flowed (500 μL/hr) with an aqueous continuous fluid comprising 0.5 wt.% poly(vinyl alcohol) with 1.85 wt.% formaldehyde (30,000 μL/hr) in deionized water. The resultant drops were collected in a glass vial and sat overnight.

### 2.2 Functionalization of mNACPs

Following this, I show that the surfaces of the particles can be functionalized with biomolecules via chemical modification by linking a biotin group to the surface-accessible amine groups using carbodiimide chemistry. I measured the adsorption of fluorescently labeled streptavidin to the biotinylated particles under two conditions. For
the “biotin-blocked” samples, we pre-blocked the streptavidin by exposure to excess biotin to probe the level of non-specific adsorption; for the “unblocked” samples, streptavidin remained unmodified such that the binding pockets of the protein were free to interact with surface-bound biotin.

To functionalize particles, a 0.5 wt.% solution of sulfo-NHS (N-hydroxysulfoxuccinimide) biotin in 1xPBS was added to magnetoelastomeric particles suspended in 0.5 wt.% CTAB in deionized water and mixed for 30 min. After centrifuging at 2000xG for 4 min and resuspending in 0.5 wt.% CTAB in deionized water three times, Alexa Fluor® 488 fluorescent streptavidin (33.3 μM) was added to the suspension and incubated overnight. Afterwards, the particles were centrifuged at 2000xG for 4 min and resuspended in 0.5 wt.% CTAB in deionized water three times. Once functionalized, the relative loading of particles with fluorescent streptavidin can be estimated using standard flow cytometry techniques (Figure 12). The fluorescent loading of a sample is directly related to its fluorescence intensity \( I_F \) as determined by flow cytometry. This in turn is converted to a relative channel number \( RCN \) and molecules of equivalent streptavidin \( MESA \) according to the following equations:

\[
RCN = 256 \cdot \frac{\log_{10}(10I_F)}{\log_{10} bin} \tag{Eq. 3}
\]

\[
MESA = \frac{10^{m(RCN)+b}}{n} \tag{Eq. 4}
\]

where \( m \) and \( b \) are determined using calibration particles (SPHERO™ Rainbow Calibration Particles) and a calibration fit provided by the manufacturer, \( bin \) is the total
bin number of the flow cytometer, and $n$ is the number of fluorophores per streptavidin molecule ($n = 4$ for the aliquots of streptavidin used in our study).

![Diagram](image)

**Figure 12**: Fluorescence histograms and the corresponding molecules of equivalent of streptavidin (MESA) data (see text for details). Data sets include biotinylated “control” particles without exposure to fluorescent streptavidin, “biotin-blocked” particles adsorbed with streptavidin exposed to biotin, and “unblocked” particles chemically modified with streptavidin (biotin-blocked).

The particles from the unblocked samples exhibited the highest average fluorescence (i.e., by two orders of magnitude above the control), followed by the biotin-blocked samples. I also measured “control” particles that were not exposed to fluorescent streptavidin. These results suggest that while nonspecific adsorption can lead to surface-decoration of adsorbed proteins, biotin-based coupling is comparatively more effective.

### 2.3 Characterization of mNACPs

To characterize the magnetic and acoustic properties of the mNACPs, my collaborator Professor Benjamin Evans (Elon University) and I measured the magnetic susceptibility and acoustic focusing profiles of particles with different weight loadings.
of magnetic nanoparticles. I also calculated the approximate acoustic contrast factor for a chosen particle formulation.

2.3.1 Magnetic Characterization

I first synthesized different formulations of mNACPs (i.e., 3, 6, 12, 24, and 48 wt.% Fe₂O₃ nanoparticles) using bulk homogenization. Subsequently, each population of particles was rinsed in deionized water, weighed, and redispersed in 0.5 mL of a solution containing 1 wt.% gelatin and 0.5 wt.% hexadecyltrimethylammonium bromide (CTAB) in deionized water. An aliquot of each solution was transferred to a sample tube and chilled for 2 min at 4°C to set gelatin. The elastic network of the gelatin prevented the sedimentation of mNACPs during the measurement process. Also, the low concentration of particles in the gelatin allowed us to characterize mostly non-interacting particles. Magnetic susceptibility measurements were then conducted at room temperature on an MK 1 magnetic susceptibility balance (Sherwood Scientific, Ltd.) at a field of 3500 G. Susceptibilities per unit mass of microsphere are listed in Table 4, Column 3. A sample of 100% magnetite nanoparticles yielded a susceptibility of 2.41x10⁻⁴ m³/kg on the MK 1 balance. By comparing the susceptibility of each microsphere formulation with this value, Professor Benjamin Evans and I were able to calculate the wt.% nanoparticles in each of our formulations (Table 4, Column 4), which is in agreement with our expected values (Table 4, Column 1). The results indicate that
the magnetic susceptibility of the particles is linear ($R^2=0.996$) with respect to weight percent loading of Fe$_3$O$_5$.

### Table 4: Magnetic Loading and susceptibility measurements

<table>
<thead>
<tr>
<th>Nominal FFPDMS Formulation (wt. %)</th>
<th>Microspheres in Gelatin Solution (wt. %)</th>
<th>Microsphere Volumetric Susceptibility at 3500 G ($\times 10^5$ m$^3$/kg)</th>
<th>Microsphere Volumetric Susceptibility at 3500 G</th>
<th>Calculated FFPDMS Formulation (wt. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.51 ± 0.02</td>
<td>0.65 ± 0.03</td>
<td>0.006 ± 0.000</td>
<td>2.70 ± 0.11</td>
</tr>
<tr>
<td>6</td>
<td>0.48 ± 0.02</td>
<td>1.37 ± 0.05</td>
<td>0.013 ± 0.001</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>0.57 ± 0.03</td>
<td>3.07 ± 0.17</td>
<td>0.033 ± 0.002</td>
<td>12.7 ± 0.7</td>
</tr>
<tr>
<td>24</td>
<td>0.57 ± 0.03</td>
<td>6.0 ± 0.3</td>
<td>0.077 ± 0.005</td>
<td>24.9 ± 1.3</td>
</tr>
<tr>
<td>48</td>
<td>0.65 ± 0.03</td>
<td>11.0 ± 0.5</td>
<td>0.188 ± 0.013</td>
<td>45.4 ± 2.1</td>
</tr>
</tbody>
</table>

### 2.3.2 Acoustic Characterization

After magnetic characterization, we stained mNACPs ($2.3 \times 10^6$ particles/mL) with Nile red dye and injected them into a microfabricated, silicon-based acoustofluidic chip (note, we describe the methods for fabrication elsewhere) at a rate of 100 μL/min to characterize their acoustic properties. After generating an acoustic standing wave at the fundamental harmonic (2.43 MHz, 40 V$_{pp}$) across the 300 μm microchannel for 3.0 sec, I recorded fluorescence microscopy images of particles as they flowed down the channel through the inspection region and obtained fluorescence intensity profiles across the channels using ImageJ (NIH) (Figure 13). As shown in Figure 13, the relative slope of each focusing profile diminished with increasing magnetic loadings, indicating a general decrease in the concentration of mNACPs focused at the pressure antinodes. As the time required for acoustic focusing is directly related to the magnitude of the acoustic...
I deduced that, qualitatively, the magnitude of the acoustic contrast factor for the particles in water decreases with increasing magnetic loadings, thus suggesting that we can effectively tune the acoustic contrast factor of the mNACPs.

Figure 13: Acoustic characterization of particles. (i) Fluorescent images of 3 wt.% (top) and 48 wt.% (bottom) Fe$_2$O$_3$ samples (2.3x10$^6$ particles/mL) after focusing in an acoustic standing wave for three seconds (flow rate = 100 μL/min). Scale bar represents 100 μm. (ii) Relative fluorescence intensity profiles across the width of a half wavelength acoustic standing wave for each particle composition.

To demonstrate a proof-of-concept multi-target separation with these microparticles, I selected the mNACP formulation with 12 wt.% Fe$_2$O$_3$. I first sought to estimate the acoustic contrast factor of this formulation by video analysis of particle trajectories as they focus to the antinodes of a standing pressure wave. Standing pressure waves contain distinct regions of acoustic potential energy minima that depend on the acoustic contrast factor of a particle relative to the suspending fluid ($\varphi$).$^{108,115}$ The primary acoustic radiation force ($F_p$) exerted on particles suspended in a medium subjected to a standing pressure wave is described by:$^{116}$

$$F_p = V_p k E_{ac} \cdot \sin(2ky) \cdot \varphi$$  

[Eq. 5]
where the pressure nodes and anti-nodes are assumed to exist at the locations where $2k_y$ are multiples of $\pi$. The strength of the trapping force is determined by the volume of the particle ($V_p$), the corresponding acoustic energy density ($E_{ac}$), and the corresponding wavenumber of the standing pressure wave ($k$). When the particle is near the centerline of the microfluidic channel, the expression for force can be simplified with the small angle approximation, which allows for the acoustophoretic focusing velocity of a particle ($u_y$) to be expressed through a force balance as:

$$u_y(y) = \frac{2a^2kE_{ac}}{3\eta} \cdot 2ky \cdot \varphi = Cy$$  \hspace{1cm} \text{[Eq. 6]}

where ($a$) is the radius of the particle, ($\eta$) is the viscosity of the fluid, and ($y$) is the distance of the particle from the centerline of the channel. It is important to note that the density of these particles ($1,066 \text{ kg/m}^3$) led to prolonged suspension in solution and thus friction forces due to contact with the lid or trench of the microfluidic device are neglected. Consequently, the radii, velocities, and corresponding positions of the particles in suspension as they focus in a standing pressure wave were determined using epifluorescence microscopy and ImageJ analysis software (NIH) to quantify their acoustic response.

Acoustofluidic characterization was conducted using a microfabricated silicon chip for particle tracking experiments and glass capillary resonator for ternary sorting experiments (Figure 14) (details for fabricating such devices are described elsewhere).
Particle positions were evaluated at a frame rate of 8 frames per second using ImageJ analysis software (NIH) and injected at a rate of 1 $\mu$L/mn. The linear approximation for acoustic force was considered to be valid in the range of $-50 \leq y \leq 50$ (Figure 15, red line). Our calibration procedure involved first determining the acoustic energy density based on the focusing capabilities of polystyrene beads which are known to exhibit an acoustic contrast factor of $\approx 0.328$. The fit between theory and experiment for polystyrene beads is shown in (Figure 15.a).

![Figure 15: Particle velocities in water of (a) polystyrene calibration beads and (b) mNACPs with a 12 wt.% Fe$_2$O$_3$ formulation at different locations across the channel. Polystyrene (PS) beads, which exhibit a positive acoustic contrast factor in water, moved toward the center of the channel (corresponding to the pressure node; $y = 0$), while the mNACPs moved away.](image)
Subsequently, mNACPs with a 12 wt.% $\text{Fe}_2\text{O}_3$ formulation were injected into the same inspection region at a rate of $1\,\mu\text{l}/\text{mn}$ to track their focusing behavior and comparatively determine their acoustic contrast factor (Figure 15.b). With this approach, the resulting acoustic contrast factor of the particles was estimated to be $\approx -3$, indicating that the bulk compressibility of this formulation is roughly $2 \times 10^{-9}\,\text{Pa}^{-1}$. Notably, these particles also exhibit a uniform magnetic response ($\chi = 0.033 \pm 0.002$), thus suggesting that this particle formulation may be useful for separations and bioassays requiring dual contrast.

### 2.4 Magneto-Acoustic Ternary Separation Study

Using this 12 wt.% $\text{Fe}_2\text{O}_3$ particle composition, my undergraduate mentee, Robert Gutiérrez, and I performed a ternary separation from human umbilical vein endothelial cells (HUVEC, non-magnetic) and magnetic beads with positive acoustic contrast using magnetic and acoustic-based separations to highlight the unique properties of the mNACPs (Figure 16). To determine the separation purities, Mr. Gutiérrez and I quantified the number of particles and cells before and after each separation step using optical microscopy and a standard laboratory grade hemocytometer (Hausser Scientific Co.). First, magnetic separation of mNACPs (2.2x$10^5$ particles/mL) and magnetic, positive acoustic contrast particles (mPACPs, 9.5x$10^4$ particles/mL) from unlabeled HUVEC cells (3x$10^5$ cells/mL) was performed using a standard column (MACS® MS Column; Miltenyi Biotec) with the OctoMACSTM magnet. As expected, all of the cells
(i.e., 100±0%) were effectively removed from the solution during this step, thus leaving only two target populations (mPACPs and mNACPs) (Figure 16, middle). Next, we acoustically separated mNACPs from mPACPs by flowing the solution into an acoustofluidic glass capillary\textsuperscript{118} while the attached piezoelectric transducer was excited (2.43 MHz, 40 V\textsubscript{pp}). The acoustofluidic device was comprised of a lead zirconate titanate (PZT) piezoelectric transducer (841, APC International; 2.46 MHz resonance frequency) attached to a cylindrical borosilicate capillary (300 μm ID; VitroCom, Inc.) and inlet and outlet tubing connections.\textsuperscript{117} The capillary rested on polydimethylsiloxane (Sylgard 184; Dow Corning Corp.) slabs to reduce damping effects on wave propagation within the device (Figure 14). The generation of an acoustic standing wave caused the mNACPs to migrate to the pressure antinodes and remain trapped against the walls of the capillary, while the remainder flowed into the outlet at the center of the channel.
Figure 16: Two-step magnetic and acoustic sorting process. (a) Initial mixture comprised of mNACPs, mPACPs, and mammalian cells. (b) Magnetic separation is shown to remove cells from mNACPs and mPACPs. (c) Acoustic separation is shown to separate mNACPs from mPACPs (n = 5).

I found that this simple acoustofluidic device can separate mPACPs from mNACPs with purities of $96 \pm 6\%$ and $85 \pm 6\%$ respectively (Figure 16, bottom). Representative fluorescent images of the serial separation are shown in Figure 17.
Figure 17: Representative fluorescent images corresponding to separation study with blue: HUVEC cells; green: mPACP; red: mNACP. (a) Initial mixture (before MACS), magnetic separation with (b) field on and (c) field off, acoustic separation with (d) field on and (e) field off. Scale bars represent 100 μm.

Additionally, we compared our sorting performance to previously published work in which magnetic particles were separated from each other (Table 5).\textsuperscript{119-121}

**Table 5:** Summary of throughput and separation efficiency of various microfluidic methods for separation of magnetic particles.

<table>
<thead>
<tr>
<th>Study</th>
<th>Separation Method</th>
<th>Throughput ((\frac{\text{beads}}{\text{min}}))</th>
<th>Separation Efficiency Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ohiri et al., 2016</td>
<td>Acoustic</td>
<td>(3.15 \times 10^4)</td>
<td>(\approx 85 – 96%)</td>
</tr>
<tr>
<td>Krishnan et al., 2007</td>
<td>Dielectrophoretic/ Magnetic/ Size\textsuperscript{119}</td>
<td>(2.5 – 3)</td>
<td>(\approx 80 – 94%)</td>
</tr>
<tr>
<td>Adams et al., 2008</td>
<td>Magnetic/ Size\textsuperscript{120}</td>
<td>(1.67 \times 10^7)</td>
<td>(\approx 91 – 100%)</td>
</tr>
<tr>
<td>Afshar et al., 2011</td>
<td>Magnetic/ Size\textsuperscript{121}</td>
<td>(3 \times 10^3 – 6 \times 10^5)</td>
<td>(70 – 80%)</td>
</tr>
</tbody>
</table>
It is important to note that the separation performance of these studies heavily relies on complex microfluidic devices, labeling density, magnetic loading, and size of the labeling bead while our new approach relies simply on the opposite sign of the acoustic contrast factor of two types of biofunctional particles. Additionally, the throughput of our method may be improved if necessary by adding additional capillaries in parallel.\textsuperscript{122} While there remains ample opportunity for optimization, we note that even our non-optimized system compares favorably against previously reported studies involving separating magnetic particles from each other.

This separation data suggests the mNACPs can be efficiently separated from other magnetic species (e.g., magnetically labeled cells, magnetic polystyrene beads) in a facile manner using acoustic separation.

\textbf{2.5 Conclusions}

In conclusion, this work introduces a new class of biofunctional particles that combines the benefits of well-established magnetic labeling technologies with the unique capabilities of negative acoustic contrast particles for efficient separation of a wide range of potential analytes (e.g., small molecules, macromolecules, viruses, and cells). The effective acoustic force exerted on a cell-mNACP complex can be tuned by adjusting the size of the mNACP, labeling density, and acoustic contrast coefficient of the mNACP\textsuperscript{112}, whereas the magnetic force can be tuned with the volume loading of the
particle. With this insight, I envision future studies in which mNACP-cell complexes are engineered to migrate to the antinode of an acoustic standing wave for rapid and discriminatory separation from unlabeled blood cells.\textsuperscript{106,123} As these separated complexes retain their magnetic response, they can be seamlessly integrated with magnetic manipulation techniques for downstream analysis.\textsuperscript{101-104} The characterization of these dually responsive mNACPs establishes a necessary understanding of the particles that enables a number of fundamental and bioanalytical studies.
3. Characterization and Development of a Hybrid Acoustophoretic Trap and Transfer Approach

Single cell analysis enables new insights into the heterogeneity within a cell population that were previously concealed using traditional bulk ensemble measurement techniques.\textsuperscript{12,51,52} The field is currently receiving significant attention\textsuperscript{124-127} and is expected to open up a plethora of applications in basic and clinical research for fields ranging from oncology, to immunology, neuroscience, and beyond.\textsuperscript{53,128-131} Accordingly, there has been great interest in innovating new techniques that can organize single cells into discrete chambers and monitor their behavior over time.

Typically, single cell arraying technologies involve wholly passive or active manipulation methodologies (1.4 Commercial Systems for Single-Cell Analysis). While passive mechanisms have proven useful for trapping single cells, active mechanism enable more flexibility and control during transfer. Previously, research in the group has focused on actively manipulating magnetically labeled cells after separation.\textsuperscript{104,132} However, despite significant advantages of this technology such as fine spatiotemporal cells and active path switching capabilities, these two-dimensional systems suffered from slow coarse alignment, poor longitudinal viability on-chip, and complex fabrication. Thus, in my work, we sought to retain the flow switching capabilities of active systems while incorporating benefits from three-dimensional passive systems such as rapid course alignment, low power consumption, simple fabrication, and isolated cellular
compartments. Accordingly, I combine traditional hydrodynamic cell trapping mechanisms for use as “coarse alignment” with active acoustic forces for use as unlabeled “fine alignment” to create a hybrid device and reduce the overall complexity of microfluidic bioanalytical devices while increasing the associated efficiency, speed, and throughput.

**Contributions:**

For the results shown in this chapter (3. Characterization and Development of a Hybrid Acoustophoretic Trap and Transfer Approach) I worked in collaboration with researchers within Duke University in Professor Kris Wood’s Lab from the Pharmacology and Cancer Biology Department, gained invaluable assistance from my undergraduate mentee, Mr. Sean Kelly, and had useful discussions and aid from my lab mate Mr. Jeffrey Motschman. I independently measured the hydrodynamic resistance through the microfluidic chip using MATLAB and analytical solutions for Poiseuille flow and modeled the hydrodynamic flow in COMSOL Multiphysics. In conjunction with my advisor, Professor Benjamin Yellen, I prototyped and developed multiple designs for the basic acoustofluidic element as well as performed preliminary investigations into the acoustic response of each prototype. To expedite our design process, I independently modeled the acoustic radiation force and steady acoustic streaming using COMSOL Multiphysics. Mr. Sean Kelly was instrumental in building off my previous work and procuring the final particle tracing graphs by automating the
particle tracking process with a MATLAB code and repeating experiments with a high-speed camera. In collaboration with Mr. Jeffrey Motschman, Mr. Sean Kelly additionally performed useful auxiliary studies (Figure 28) regarding the acoustophoretic motion of particles and cells in the compartment region of the acoustofluidic element as well as programed an automated routine to quantify the number of fluorescent particles/cells in suspension in the compartment and trap regions. Mr. Jeffrey Motschman performed experiments for optimizing the cell trapping mechanism on chip and I quantified them manually. Mr. Kevin Lin provided PC9 and AML cells along with useful discussions relevant to cell culture. Finally, I independently performed experiments for optimizing and quantifying the acoustic transfer mechanism.

### 3.1 Optimization of Hydrodynamic Resistance through the Chip

There is a fine balance needed to form a high density, hybrid array. As the packing density of the individual elements on the chip increase, the features of the chip must decrease. However, decreasing these features leads to challenges arraying larger cells and an increasingly larger fluidic resistance through the chip which poses significant challenges during priming (i.e. filling the microfluidic cavity with a physiological buffer). Thus, my first task involved optimizing the hydrodynamic resistance through the chip.
3.1.1 Theory on Hydrodynamic Resistance

The Navier-Stokes equation describes fluid flow in a velocity field. This fundamental relation describes that the rate of change in the momentum is equivalent to the sum of convention forces, pressure forces, and viscous forces within the fluidic system. Neglecting external forces, this equation reduces to:

\[ \rho \left( \frac{\partial \mathbf{u}}{\partial t} \right) = -\rho (\mathbf{u} \cdot \nabla) \mathbf{u} - \eta \nabla^2 \mathbf{u} - \nabla P \]  \hspace{1cm} [Eq. 7]

where \( \rho \) is the density of the fluid, \( \mathbf{u} \) is the fluid velocity field, \( \eta \) is the dynamic viscosity of the fluid and \( P \) is the pressure field. In the case of Poiseuille Flow, the flow field is assumed to be steady, viscous, and incompressible. Accordingly, the Navier-Stokes equation reduces to:

\[ \nabla P = \eta \nabla^2 \mathbf{u} \]  \hspace{1cm} [Eq. 8]

This equation can be analytically solved to determine the velocity field and maximum velocity \( (u_{\text{max}}) \), where

\[ u = \frac{R^2-r^2}{4\eta} \left( -\frac{dp}{dx} \right) = u_{\text{max}} \left( 1 - \frac{r^2}{R^2} \right) \]  \hspace{1cm} [Eq. 9]

\[ u_{\text{max}} = \frac{R^2}{4\eta} \left( -\frac{dp}{dx} \right); r = 0 \]  \hspace{1cm} [Eq. 10]

Here, \( R \) is the radius of the channel cross section and \( r \) is the radial position along the cross section. In this case the expression for mean linear velocity, \( \bar{u} \), is

\[ \bar{u} = -\frac{1}{8\eta} \left( \frac{dp}{dx} \right) R^2 \]  \hspace{1cm} [Eq. 11]

where \( \mu \) is the dynamic viscosity, \( \frac{dp}{dx} \) is the pressure gradient, and \( R \) is the radius of the channel. Accordingly, the linear velocity can be converted to volumetric flow rate:
\[ \bar{u} = \frac{\bar{Q}}{A_c} \]  

[Eq. 12]

The equation for mean linear velocity can be re-written as the Hagen-Poiseuille equation in a form analogous to Ohm’s law where volumetric flow rate is directly related to the pressure drop:

\[ \Delta P = \frac{dp}{dx} = R_h \bar{Q} \]  

[Eq. 13]

where \( \Delta P \) is the pressure drop, \( R_h \) is the hydraulic resistance, and \( Q \) is the volumetric flow rate. This resistance is dependent on the dynamic viscosity of the fluid and the geometry of the microfluidic channels. Particularly, hydraulic resistance is:

\[ R_{circ} = \frac{8\eta L}{\pi R^4} \]  

[Eq. 14]

for circular channels,

\[ R_{sq} \approx 28.47 \left( \frac{\mu L}{w^2 h^2} \right), w = h \]  

[Eq. 15]

for square cross sections and,

\[ R_{rec} = \frac{12\eta L}{wh^3 \left( 1 - \frac{h}{w} \right) \tanh \left( \frac{n\pi w}{2h} \right)} \]  

[Eq. 16]

for rectangular cross sections, which is approximately

\[ R_{rec1} \approx \frac{12\eta L}{(wh^3) - 0.63w^3}, w > h \]  

[Eq. 17]

\[ R_{rec2} \approx \frac{12\eta L}{(hw^3) - 0.63w^3}, h > w \]  

[Eq. 18]

where \( l \) is the length of the channel, \( w \) is the width of the channel, \( h \) is the height of the channel assuming, \( A \) is the cross-sectional area of the channel, and \( P \) is the wetted
perimeter. Knowing the hydraulic resistances, the ratio of the volumetric flow rate through a junction can be determined using (Eq. 13).

3.1.2 Design of the Arraying Elements

I optimized the hydrodynamic trapping step in each of my four prototypes by tuning the three volumetric flow rates \((Q_1, Q_2, \text{ and } Q_3)\), in which the hydrodynamic resistance of each branch is carefully controlled. Here, I assume a cell suspension flows steadily into a trifurcation in a microfluidic device with resistances \(R_1, R_2, \text{ and } R_3\) where region 1 is cell loading site (e.g. weir) for a 15 \(\mu\)m cell, region 2 is the bypass channel, and region 3 is the inlet to the compartment region. When a cell is not present in the loading site \(R_1\) should exist at a low resistance state, but once a cell has been loaded \(R_1\) should exist at a high resistance state and thus redirect cells to the bypass channel. Thus, for proper functioning of the device I require:

\[
R_{1lo} \ll R_2 \ll R_3, R_{1hi}
\]

[Eq. 19]

without having a constraint on the relationship between \(R_3\) and \(R_{1hi}\). Furthermore, all channels in the microfluidic chamber have the same initial height \(h\) but different widths \(w_1, w_2, \text{ and } w_3\).

The geometry of the weirs is designed such that an unoccupied weir has the lowest fluidic resistance, whereas an occupied weir has higher fluidic resistance than the bypass channel. This design ensures that after a weir traps a single cell, subsequent cells are diverted towards the bypass channel until one of them gets trapped in the next
unoccupied weir. This process allows the weirs across the entire chip to be loaded within minutes. Since my devices were fabricated through single-level Silicon etch, we tuned the fluid resistances by adjusting the lengths and widths of each channel section. The bypass channel was designed to have a width commensurate to several cell diameters (in our case 35 μm), which helped to reduce clogging but required long serpentine bypass channels to match the desired resistance ratios. The weirs have widths of 6 μm and lengths of 4 μm, from which we derive a condition that the length of the bypass channel must be at least ~1mm long to maintain the condition R2/R1>2, which ensures that most fluid flow goes through the trap as compared to the bypass segment, thus increasing the probability of capturing cells in the weirs.

To avoid unintentionally moving cells into the compartments prematurely, we included physical constrictions in the compartment region to raise the fluidic resistance. This section was designed to achieve a resistance ratio R3/R2>2 with a similar purpose of biasing most fluid flow to go through the bypass segment compared to the compartment. To visualize the flow patterns, I show the flow patterns from COMSOL calculations for the case when the weir is occupied as (Figure 18.a) or is empty (Figure 18.b). As expected, flow is highest through the unoccupied weir, followed by the bypass region, and finally lowest through the compartment region and occupied weir (Figure 18.c).
As expected, the highest flow rate is through the trap, followed by flow through the bypass, with flow through the apartment being the smallest. This trend holds true for
four all prototypes that will be explored in this chapter. As one piece of evidence, in
Figure 19 below, it can be clearly seen that the highest flow rate is through the trapping
region, followed by the bypass region and apartment as expected for one of my earlier
prototypes.

![Fluorescent image of 0.2 µm polystyrene microparticles flowing through multiple fluidic elements in prototype 1. Scale bar is 100 µm.]

As another piece of evidence, it can be seen in Figure 20 below that during priming (e.g.
filling the microfluidic chip with an aqueous phase) compartment regions are the last to
fill in two different prototypes.

Further, to confirm these flow predictions in later prototypes, I injected 15 µm
polystyrene beads (50,000 beads/mL; Sigma Aldrich Corp.) into the device and flowed
them through the chip at a flow rate of 50 µL/min. I note here that I used large beads for
the purpose of completely occluding the weir, which causes the flow profile through the
region to more closely match the expected behavior of deformable cells entering the
trap. As expected, beads first populate the weir, thus decreasing flow through this region and causing subsequent beads to travel through the bypass (Figure 18.d). In this way, cells and beads can be hydrodynamically loaded into weirs, then intentionally transferred into the compartment region when an acoustic force is activated.

Figure 20: Priming in (a) prototype 2 and (b) a variation of prototype 3 using the pressure driven Elveflow System.

During exploration of the behavior of cells and particles in my acoustofluidic array, I encountered various issues related to the hydrodynamic resistance that informed my decision to modify the design of the chip. Though I explored many alternative configurations prior to adopting the hydrodynamic trifurcation trapping design, they
were ultimately ineffective and thus I will forgo discussion of these earlier prototypes. Instead, in this chapter of my document as previously stated I focus on four key configurations for the acoustofluidic array (Figure 21).

![Figure 21: Basic configurations for acoustofluidic chips with decreasing fluidic resistance. Here, (a) prototype 1 has the longest fluidic paths (b) prototype 2 has more paths in parallel than series and (c) prototype 3 has larger channels and fewer connecting lines are shown and (d) prototype 4 shows a variation of the third prototype separated into 8 different conditions.](image)

The first configuration (prototype one) was my earliest design that effectively trapped single cells (Figure 21.a). While this design fit the most array elements compared to the other two (> 7,000) it also had the highest fluidic resistance ($\approx 1 \times 10^{15} \text{ Pa} \cdot \text{s/m}^3$). This substantial fluidic resistance lead to numerous issues during
the preparation and operation of the chip, including insufficient priming downstream (Figure 22), long priming times (> 4 hrs at 5 $\mu$L $mn$), and the large amount pressure necessary to fill the cavity with fluid on a time scale of less than 6 hours ($\gg$ 2000 mbar). It is important to note that at higher flow rates (e.g. priming by hand) and thus higher pressures, this design had the highest probability of failing due to leaking at the inlet.

![Image](image1.png)

**Figure 22:** Issues priming first prototype of acoustofluidic single cell array with high fluidic resistance. (a) Templating elements upstream are fully primed while (b) templating elements downstream are filled with air bubbles after priming overnight.

To address these issues, I modified the design of the acoustofluidic array (prototype two) to reduce the number of elements in series, effectively dropping the total resistance of the chip to $\approx 2 \times 10^{14} Pa \cdot s/m^3$ (Figure 21.b). While this improvement drastically decreased the amount of time necessary to fully prime the acoustic chip (< 2 hrs at 25 $\mu$L $mn$) and the amount of pressure necessary to prime the chip on a time scale of less than 6 hours (< 1500 mbar), the lengthy inlet and outlet fluidic lines still introduced a significant amount of unnecessary resistance into the design. Additionally, the 20 $\mu$m
opening to the apartment was too small to allow for PC9 cells ($d \geq 15 \mu m$) to be transported into the apartments, unlike the smaller OCI-AML2 cell line ($d \leq 12 \mu m$). To address this, for prototype 3 I increased the channel width to 35 $\mu m$ and converted the inlets and outlets to a bus configuration (Figure 21.c). In this new configuration, priming can be accomplished manually and rapidly (<5 minutes) and the fluidic resistance dropped to $\approx 1 \times 10^{14} Pa \cdot s/m^3$. Finally, for the fourth and final prototype, the third design is separated into 8 separate sections with corresponding inlet/outlet regions and returning to a branched configuration. While this change held the fluidic resistance roughly constant, being $\approx 1 \times 10^{14} Pa \cdot s/m^3$ (Figure 21.d), this updated configuration additionally allows for multiple experiments to be run on one chip. These high-level design changes led to the development of four different prototypes for my acoustofluidic chip.

To confirm this analytical prediction for the resistance through prototype four, I fully primed a section of the microfluidic chip with water to remove all air bubbles and tracked the amount of time it took to fill 100 $\mu$L reservoirs attached to the outlet of the microfluidic chip to determine the associated flow rate through a section at each pressure (Figure 23). As expected, the experimental data matched a linear trend, indicating that the resistance remains constant through each experimental data point (indicated by the red ‘x’) and thus no major flow disturbances occurred during measurement.
Figure 23: Relationship between pressure and volumetric flow rate of a single section from a prototype 4 chip. Red x’s indicate measured data while the blue dot indicates the threshold pressure ($P_{th} = 25$ mbar) to prevent cellular escape.

Additionally, using this data I calculated the measured resistance through a single section connected to tubing and a bubble bypass (see Appendix) to be approximately $\approx 2.27 \times 10^{14} \ Pa \cdot s/\ m^3$. Here, we see that experimental results are roughly consistent with analytical results. While the results are the same order of magnitude, as expected, variations between these numbers can be due to a variety of sources of error including inaccuracies in the applied pressure, fluctuations from the pressure source, inaccuracies of the analytical solution, non-uniformity of the etch depth in the microfluidic chip, and added resistance due to additional tubing and connections.
3.1.3 Design of the Acoustofluidic Chip

For use in single cell analysis, this technology requires the following fundamental components: (i) a sterile microfluidic channel and apartment (ii) hydrodynamic single cell traps for the “coarse alignment” step of our templating process (iii) an acoustophoretic switch to template the cells into the apartments, and finally (iv) a biocompatible, functionalized surface to prevent the fouling of cells in the device. I use standard cleanroom techniques to fabricate variations of sterile silicon-glass based microfluidic chips. Each chip is comprised of inlet regions, outlet regions, and array sites (Figure 24).

![Figure 24: Image of the third prototype. Silicon region is 75 x 25 mm and contains >3,000 templating elements. Inlet region is indicated with a right facing arrow.]

These array sites include 3,000-8,000 individual elements depending on the chip configuration (four prototypes total) and contain a cell capture site, cell bypass, and cell apartment. The third prototype has been heavily tested, and consists of 96 parallel microfluidic channels, each having 40 compartments in series at an areal density of approximately 4 compartments per mm² (cmpts/mm²). I have also tested a fourth prototype, that is comprised of smaller devices with the same basic design that have a footprint of 15 mm x 25 mm consisting of 16 parallel channels with 31 compartments in
series with a total of 496 compartments. To demonstrate the feasibility of higher density designs, we also tested earlier designs (prototype 1 and 2) that have higher densities < 12 cmpts/mm², allowing for ≈5,000-7,000 cells to be organized in a device the size of a glass slide (Figure 25).

The basic setup for the third prototype is shown in Figure 26(a-b). This chip has dimensions consistent with a glass slide (i.e. 25 x 75 mm) and fits 3,840 individual compartments.

Figure 25: Image of the first prototype. Silicon region is 75 x 25 mm and contains >7,000 templating elements. Inlet region is indicated with a right facing arrow.

In this way, cells only enter the apartment region of a clean device when the acoustic force is activated. Hence, there is no need for a continuous input of power. The basic switching junction is a trifurcation, consisting of the weir (i.e. primary trap site), a bypass channel, and a cellular compartment ( Figure 26.c).
Figure 26: Acoustofluidic single cell array. (a) Image of chip in the aluminum manifold. (b) Schematic of entire set-up indicating the location of the chip, inlet, outlet, and piezoelectric transducers. (c) Image of individual acoustofluidic element with characteristic length \( R_L \) and comprised of a weir (1), cellular bypass (2), and compartment region (3). Scale bar indicates 100 \( \mu \text{m} \).

As can be seen, using results from 3.1.1 Theory on Hydrodynamic Resistance and 3.1.2 Design of the Arraying Elements, the hydrodynamic flow profile was finely tuned to reliably capture single cells at the weirs, without unintentionally transferring the cells into the compartments, except when desired. While optimizing the hydrodynamic resistance through the chip informed most of my design changes, I also surveyed the acoustic response of various configurations for the individual elements to optimize the acoustic switching efficiency.
3.2 Optimization of the Acoustic Switch

Acoustic approaches have demonstrated the ability to control the positions and orientations of single cells in a label-free manner, and they can additionally be operated directly in cell culture media. “Bulk acoustic wave” (BAW) devices can be built from single layer silicon or glass microfluidic channels and designed to resonate at well-characterized frequencies that correspond to the device geometry.\textsuperscript{133-137} Moreover, these systems can be operated with a simple piezoelectric transducer mounted underneath the device; however, BAW devices have limited ability to change the position of the focusing nodes, and thus have mainly been used in bulk continuous flow sorting applications. As I uniquely seek to exploit BAW for single cell analysis, in this section of my dissertation I explore the response of particles to bulk acoustic excitation in this novel, complex geometry.

Acoustic control of particles in complex geometries (e.g. trifurcations, bended channels) is largely unexplored. While most BAW based devices involve simple, long rectangular or circular channels, tortuous paths such as those explored herein can lead to various poorly understood structural resonances. Thus, approaching the unknown, I started with the assumption that I could form a standing acoustic wave in the entrance channel of my acoustofluidic element, and use acoustophoresis to transport particles and cells across streamlines to the node of the standing wave. However, it quickly became clear that other positions were also attracting particles through a mechanism that did not have its origin in prescribed pressure nodes and seemed to be from acoustic streaming. Thus, a large part of my effort was devoted to resolving which force dominates and
where. This required a combination of numerical simulation, and experimental particle tracking with different types of transducers, voltages, and frequencies. Thus, in this section I discuss the theory behind the COMSOL simulations I used to predict the acoustophoretic motion of particles in the chip and experimentally investigate various designs that I produced while optimizing the hydrodynamic resistance through the chip.

### 3.2.1 Theory for COMSOL Simulations of Steady Acoustic Streaming and Radiation Forces in Acoustofluidic Element

To begin to understand the underlying mechanisms behind this focusing behavior I first simulated steady acoustic streaming and the acoustic radiation force in an individual acoustic element using COMSOL Multiphysics. I note that in this section, I focus my simulations on the acoustofluidic element used in prototype four and that the larger purpose of these simulation was to allow me to iterate through the design space rapidly. Further, this simulation routine was adapted from an open source code from COMSOL and work by Muller et. al. where they developed a comprehensive process for modeling the acoustophoretic motion of particles in a simple rectangular cavity.\(^{138}\)

The behavior of complex acoustic waves in a quiescent fluid can be approximated using a perturbation expansion. Before excitation, I assume that the temperature \((T)\), pressure \((p)\), and velocity \((\nu)\) are at a steady state (subscript 0). Acoustic perturbations can then be modeled as frequency dependent first and second order effects (subscript 1 and 2 respectively) such that:

\[
T = T_0 + T_1 + T_2
\]  
[Eq. 20]
\[ p = p_0 + p_1 + p_2 \]  \[ \text{[Eq. 21]} \]
\[ \vec{\nu} = \vec{\nu}_0 + \vec{\nu}_1 + \vec{\nu}_2 \]  \[ \text{[Eq. 22]} \]

These higher order terms can be solved using the thermodynamic heat transfer equation, the kinematic continuity equation, and the dynamic Navier-Stokes equation.\(^{139}\)

Accordingly, governing equations for ultrasonic fields to the first order are:

\[
\partial_t T_1 = D_{th} \nabla^2 T_1 + \frac{\alpha T_0}{\rho_0 C_p} \partial_t p_1
\]  \[ \text{[Eq. 23]} \]
\[
\partial_t p_1 = \frac{1}{\gamma \kappa} [\alpha \partial_t T_1 - \nabla \cdot \vec{v}_1]
\]  \[ \text{[Eq. 24]} \]
\[
\rho_0 \partial_t \vec{v}_1 = -\nabla p_1 + \eta \nabla^2 \vec{v}_1 + \beta \eta \nabla (\nabla \cdot \vec{v}_1)
\]  \[ \text{[Eq. 25]} \]

where \(D_{th}\) is the thermal diffusivity, \(\alpha\) is the isobaric thermal expansion coefficient, \(C_p\) is the specific heat capacity, \(\gamma\) is the specific heat capacity ratio, and \(\kappa\) is the isentropic compressibility. These first order fields are coded into the *Thermoviscous Acoustics* module of COMSOL Multiphysics and can be used to plot the acoustic pressure distribution in the acoustofluidic element (Figure 27).
Figure 27: COMSOL simulations of acoustic standing pressure wave in acoustofluidic element excited at 1.4 MHz.

While this simulation has limitations such as two-dimensional analysis, inaccurate actuation mode, and no study of higher order resonances, I note that my results are consistent with experimental studies by Kelly et al.\textsuperscript{140} where the predicted location of the acoustic node is below the trifurcation point (Figure 28).
Consequently, traditional acoustic focusing of cells to the node of the generated pressure standing wave is insufficient for directly transferring cells into the entrance channel of the compartment region. However, acoustic transfer can still occur due to vibration of the sharp edge of the entrance corner to the compartment region. Thus, the following computational routine outlined by Muller *et. al.* can still prove useful for predicting the generation of acoustic microvortecies and nodes in this region of the element.

Solutions to the first order acoustic fields can be used as source terms to solve for the non-linear second order fields using the *Laminar Flow* module in COMSOL Multiphysics. Assuming effects from the second order temperature field are negligible,
Due to the harmonic time dependence of these acoustic perturbations, first order fields average to zero over a complete cycle while second order fields remain and give rise to acoustic radiation forces and steady acoustic streaming. Using (Eqs. 26-27), the behavior of acoustic fields in each element of the microfluidic single cell array can be approximated with a pre-constructed thermoacoustics simulation from COMSOL Multiphysics software.

Summarily, first order fields were solved using the built-in *Thermoviscous Acoustics* module. Solutions from this module were used as source terms for the built-in *Laminar Flow* module to solve for second order pressure and velocity fields. This, in turn, is used to model the steady acoustic streaming that occurs as a result of acoustic excitation (Figure 29).
Figure 29: COMSOL simulations of steady acoustic streaming in acoustofluidic element excited at 1.4 MHz.

As can be seen, this simulation predicts the formation of significant streaming vortices at the sharp edges of the entrance channel to the compartment region.

Finally, the general form of the acoustic radiation force is:

$$F_{rad} = -\frac{4\pi a^3}{3} \left[ \frac{1}{2} k_0 Re [f_1 p_1 \nabla p_1] - \frac{3}{4} \rho_0 Re [f_2 v_1 \cdot \nabla v_1] \right]$$  \[Eq. 28\]

$$f_1 = 1 - \frac{k_p}{k_0}$$  \[Eq. 29\]

$$f_2 = \frac{2(1-\Gamma)(\rho_p-\rho_0)}{2\rho_p+\rho_0(1-3\Gamma)}$$  \[Eq. 30\]

$$\Gamma = -\frac{3}{2} [1 + i \left(1 + \frac{\delta}{a}\right)] \frac{\delta}{a}$$  \[Eq. 31\]

$$\delta = \frac{2\eta}{\rho_0 \omega}$$  \[Eq. 32\]

Here, $\delta$ is the viscous penetration depth which indicates the length scale over which the acoustic wave develops into its bulk value. It is important to note that this general form simplifies to the primary acoustic radiation force equation used in chapter 2, $F_{rad} = f(V_p, k, E_{ac}, \varphi)$ in special cases where simple rectangular chambers are excited by a one-dimensional pressure standing wave. Using the built-in Particle Tracing module and Equations 28-32, I can solve for the acoustic radiation force (Figure 30).
Figure 30: COMSOL simulations of acoustic radiation force in acoustofluidic element excited at 1.4 MHz. (a) Simulations for a particle that exhibits a positive acoustic contrast factor and (b) particle that exhibits a negative acoustic contrast factor. Dashed boxes indicate predicted acoustic focusing locations along the entrance channel of the compartment region.

Here, we see that the predicted location of acoustic focusing for PACPs aligns with my pressure simulation, while the predicted location of acoustic focusing for NACPs aligns with experimental results from Kelly et. al. Interestingly, however, there are multiple locations at sharp edges along the entrance channel of the compartment region that are predicted to lead to acoustic focusing for both PACPs and NACPs as well.
For each simulation, I chose a mesh such that the boundaries are well resolved according to previously published results.\textsuperscript{139} Additionally, following methods from Muller et al.,\textsuperscript{141} I assume an input velocity excitation of $\mathbf{v} = v_{bc} e^{-i\omega t}$ on all boundaries where $v_{bc} = \omega d$. Here, I similarly choose $d = 0.1$ as this value has been previously shown to be consistent with experimental results.\textsuperscript{142} With these specifications, I simulated the first order pressure field, acoustic streaming field (assuming $\mathbf{v}_0 = 0$), and acoustic radiation force field to inform my experimental studies.

As can be seen, acoustic excitation in microfluidic devices leads to two potential mechanisms of acoustic streaming due to the contact of viscous fluid with sharp vibrating boundaries; the first describes steady rotational fluid flow near the boundaries of the channel and the second describes oscillatory flow propagating outside of the viscous boundary layer.\textsuperscript{143} While acoustic tweezing studies have primarily focused on cellular manipulation using oscillatory flow (e.g. acoustic standing waves in a wide chamber),\textsuperscript{96,144,145} steady acoustic streaming patterns within the viscous boundary layer can also lead to predictable manipulation of objects in suspension via either pumping\textsuperscript{146} or trapping in vortecies.\textsuperscript{147,148} These studies, however, have been traditionally conducted in large open cavity systems with little consideration for the complex geometries necessary for my single cell array (e.g. trifurcations, bends, and wall tapering).

Additionally, as my system seeks to maximize packing density, the characteristic length scale of my channels is significantly smaller (i.e. by an order of magnitude, $\sim 10 \, \mu m$ vs 69
~100 μm) than those in traditional acoustofluidic chips and thus boundary effects will have a larger influence on particle manipulation. As such, I sought to determine whether acoustic radiation forces, or steady acoustic streaming could be exploited to manipulate single cells on-chip in a highly parallel format.

### 3.2.2 Experimental Investigation of the Acoustic Switching Behavior in Earlier Prototypes

Beginning this study, I predicted that there are three main parameters that influence how effectively particles and cells switch into the apartment region upon acoustic excitation:

1) Excitation frequency (i.e. establishment of acoustic standing waves in the device)
2) Excitation voltage (i.e. strength of acoustic force in the device)
3) Injection velocity (i.e. balance between acoustic trapping at corners and drag from fluid)

To gain a deeper understanding of the acoustic response of particles in my device, I explored various designs at frequencies on (or near) resonance with the entrance of the apartment region. For these experiments, I began with the second hydrodynamic configuration (Figure 21.b). Despite the promising responses particles had to acoustic excitation in this configuration at 700-850 kHz, I found that while cells indeed exhibited an acoustic switching response they oftentimes switched down to the bypass as opposed to up into the apartment at frequencies within this range (Figure 31).
To investigate potential reasons for this behavior I (i) performed an experimental sweep to find an alternative frequency regime where cells more often switched up into the apartment as opposed to down and (ii) modeled the acoustic pressure and primary radiation force at key frequencies where cells had high switching rates into the compartment. From the cursory sweep, I identified 2-3 MHz as an additional frequency range of interest. Interestingly, this range includes 2.55 MHz, which is the on-resonance excitation frequency of the apartment entrance in this configuration (290 μm long). From
In this cursory experimental sweep, I qualitatively observed that 2.8 MHz driven at 16 \( V_{pp} \) proved to be particularly effective in the first and second prototype (Figure 32).

![Figure 32: Trajectory of cell in single cell array (configuration 2) with acoustics on at 2.9 MHz and 16 \( V_{pp} \). Red circle indicates final position of cell. Scale bar is 50 \( \mu \)m.](image)

I additionally observed individual cells within the compartment region drifting with the residual pressure gradient in the fluid and subsequently rapidly focusing to two regions of interest in the compartment region upon acoustic excitation at 2.9 MHz (Figure 33).
The behaviors shown above led me to postulate that acoustic focusing due to acoustic radiation forces could be achieved (i) in this complex element, consistent with findings by Kelly et. al.,\textsuperscript{140} and (ii) at frequencies slightly off resonant from the entrance region to the apartment. Accordingly, using COMSOL Multiphysics I simulated the 2D acoustic radiation force in my basic acoustofluidic element for these early prototypes at 840 kHz and 2.9 MHz (Figure 34). As can be seen, while there is no apparent standing wave above the trifurcation point of the acoustofluidic element excited at 840 kHz, there are two apparent focusing points at the mouth and entrance in the acoustofluidic element excited at 2.9 MHz. Importantly, this simulation is roughly consistent with results observed from my preliminary experiments (Figure 33).
Figure 34: COMSOL simulations of acoustic force in individual acoustofluidic element at (a) 840 kHz and (b) 2.9 MHz.

Additionally, 2D models of the first order acoustic pressure further confirm the conclusion that a standing wave with defined nodes is generated in the apartment region of the acoustofluidic elements (Figure 35).

Figure 35: COMSOL simulations of first order acoustic pressure in individual acoustofluidic element at 2.9 MHz (a) zoomed out and (b) zoomed in.
Thus, with these preliminary experiments on my first prototypes I have shown that (i)
acoustic focusing due to acoustic radiation forces indeed can exist within the
acoustofluidic element, consistent with results from Kelly et. al.\textsuperscript{140} and that (ii)
cells/particles can be switched at the trifurcation junction upon acoustic excitation. While
these results are promising, the question remains whether switching occurs due to
acoustic radiation forces or steady acoustic streaming.

Further, while the first and second prototype allows for the robust and repeatable
switching of single cells into the compartment region, it is unsuitable for organizing
larger cells (e.g. \( d \geq 15 \mu m \)) due to the relatively small 20 \( \mu m \) width of the entrance
region. Accordingly, I additionally tested a variation of prototype 3, in which the width
of entrance channel to the compartment region varies from 40-120 \( \mu m \). Before fabricating
the physical chip, I simulated the expected acoustic radiation force at 1.34 MHz, which is
on-resonance with the new wide channel design (Figure 36). The following key
characteristics can be observed from these simulations:

1) As the apartment entrance widens, the predicted focusing region near the center of
the entrance becomes more defined.
2) The predicted focusing region near the center of the entrance curves downwards
towards the corners of the walls.
3) There is a predicted acoustic force focused towards all corners of the
compartment region including the entrance, intermediary bend, trap site, and
mouth of the compartment.
Figure 36: COMSOL simulations of acoustic force in individual acoustofluidic element (prototype 3) at 2.9 MHz. The width of the entrance to the apartment region varies between (a) 40 μm (b) 60 μm (c) 80 μm and (d) 100 μm.

In addition to modeling the acoustic force for varying apartment widths, I also modeled the acoustic radiation force (on resonance) when the apartment width was 120 μm and open or included a shelf (Figure 37).
Interestingly, while the acoustic element without a shelf followed the same general trend seen in Figure 37 above, the acoustic element with a shelf shows a relatively flat node which is consistent with current literature.\textsuperscript{139,141} Also, it is noteworthy that there is a slight kink in the acoustic focusing region seen in Figure 37.b. This could potentially be due to the presence of the thin bypass entrance directly underneath the apartment that may cause a slight attenuation of the incident wave.

Following simulations, I fabricated a series of acoustofluidic chips (prototype 3) and tested the sorting mechanism and efficiency in this new design. I first tested the templating behavior of particles into apartments with wide entrances (\(\sim 120 \mu m\)). For these studies, particles were first hydrodynamically trapped, then slowly withdrawn to roughly the center of the apartment entrance, after which acoustics was turned on. Two
key behaviors were observed during acoustic excitation near resonance. In some instances, particles were attracted up into the apartment region (Figure 38.a), but in other instances particles were attracted to sharp edges/turns of the acoustofluidic element (Figure 38.b). This behavior is consistent with simulations, which showed acoustic forces pointing upwards towards a node and sideways towards the corners. It is important to note that there was a slight residual flow field towards the inlet of the microfluidic chip.

Figure 38: Particle response to acoustic excitation at 1.41 MHz and 16 Vpp in prototype 4. Particles (a) migrated upwards into the acoustofluidic apartment or (b) migrated towards sharp corners of the acoustofluidic element. Red circles indicate final position of particles. Scale bar is 200 µm.

Interestingly, once acoustics were turned off and particles were withdrawn back to the proceeding trap to reset the study, they would latch onto the loading dock of the proceeding acoustofluidic element much like the behavior seen in 3.1.1 Theory on Hydrodynamic Resistance during acoustic excitation at lower frequencies. Furthermore,
though acoustics were still off, these particles that had been trapped at the loading dock exits would enter the apartment during injection (Figure 39). Particles could be released from the loading docks and captured in the hydrodynamic trap after repetitive injection and withdrawal motions.

![Figure 39: Particle flowing into the apartment region after capture on the loading dock of the proceeding acoustofluidic element with no acoustics on. Red circle indicates final position of particle. Scale bar is 100 µm.](image)

Next, I tested the sixth variation of prototype four that included a shelf to study the impact of the location of the node in the apartment entrance on the acoustic response of the particles. While there was slight particle migration when the acoustofluidic chip was excited at 2.21 MHz, this motion was significantly slower than other acoustic responses seen at similar excitation voltages (e.g. 16 Vpp). Consequently, I performed a cursory sweep of the input frequency and found particles exhibited a rapid and substantial response to excitation between 1.3 MHz and 1.5 MHz. Interestingly, particles in the same row roughly aligned along the same focusing location in the
channel (Figure 40.a). To investigate this behavior further, I modeled the acoustic response of a single element excited at the same frequency, 1.41 MHz, (Figure 40.b) and confirmed that the predicted location of the node in simulations is roughly consistent with the focusing location of the particles (Figure 40.c).

Figure 40: Particle response to acoustic excitation at 1.41 MHz and 16 Vpp. (a) Alignment of particles in a row of elements along roughly the same focusing line. (b) COMSOL® Multiphysics simulation of acoustic radiation force in single element. (c) Zoom in view of second element from (a). Red circles indicates final positions of particles. Scale bar is 100 μm.

This response leads me to postulate that traditional acoustic focusing (e.g. generating a standing wave in the open cavity of the compartment entrance) can be used to switch particles and cells when the entrance channel to the compartment region is sufficiently wide. However, as these channels becoming increasingly thinner, particle are much
more likely to be acoustically attracted to the entrance corner of the compartment region due to either steady acoustic streaming or acoustic radiation forces. Despite these promising switching behaviors, I chose to eliminate the wide channel design from consideration due to the erroneous switching of particles into the apartment region when acoustics are off (Figure 39). Thus, to reduce the effects of this hydrodynamic switching behavior, I focused the bulk of my attentions on my final design, which has a compartment entrance channel width of 40 μm, much smaller than the shelf design but significantly larger than prototypes one and two.

### 3.2.3 Experimental Investigation of the Acoustic Switching Behavior in the Final Prototype

From early experiments, I found the most repeatable behavior was attraction to the corner in my final design. Thus, the purpose of this section is to better quantify the switching mechanism and associated parameters, then determine whether the attraction to the corner is due to steady acoustic streaming or acoustic radiation forces. After hydrodynamic trapping (Figure 41.a), the beads or cells can be transferred into the compartment regions by reversing the fluid flow to release them from their weirs and then establishing a slow forward flow to move them into the adjacent compartments under acoustic excitation. As can be seen, acoustic excitation of the chip caused beads to be strongly attracted to the leading corner of the compartment region, which is defined here as the transfer point depicted as the end point of the dashed line trajectory in Figure 41.b. Thereafter, the acoustic transducer was turned off and forward pressure was used
to push the beads into the compartment regions (Figure 41.c) to the final loading sites (Figure 41.d). Thus, the purpose of the acoustic switch is to move the beads into the streamlines that pass through the compartment. It is important to note that during this acoustic switching step, we kept the flow at a low speed (e.g. < 50 µm/s) to allow the acoustic force to dominate fluid convection.

Figure 41: Image sequence detailing the acoustic switching mechanism. (a) Beads are coarsely aligned in weirs. (b) Beads are acoustically confined to the leading corner of the compartment region. (c) Beads are flowed into the compartment region. (d) Beads are loaded in the compartment region. Scale bar indicates 200 µm.

To find the optimal parameters for acoustic attraction to the corners, I injected 8.5 µm polystyrene beads (50,000 beads/mL; Sigma Aldrich Corp.) into the chip and tracked the bead motion towards the corner at frequencies ranging from 1.1 to 1.6 MHz and applied voltages ranging from 2 to 5 Vpp (as read by the oscilloscope following
amplification). My method involved first applying backward pressure to remove the beads from the weirs and away from the switching junction, and next applying slow forward pressure to move towards the switching junction when the acoustic field was turned on. A successful switching event is one in which the bead was captured by the corner within 5 seconds. I used a 10X objective to visualize many switching processes simultaneously in a large field of view, which allowed us to obtain at least 6 measurements for each voltage/frequency pair, and the results are provided in Figure 42. As a visual aid, the data points are color-coded, in which red depicts 100% capture on the corner and blue depicts 0% capture. As a guide to the eye, we also provide a contour plot to show the conditions where trapping was most efficient, which was in the range of 1.35 – 1.42 MHz and at higher voltages.

Figure 42: Color plot of switching efficiencies of polystyrene beads onto the leading corners of the compartment region upon acoustic excitation (n=6
compartments). Shaded regions indicate average switching efficiencies over the specified range.

To confirm that this optimal frequency was caused by the channel dimensions and not by a characteristic resonance of the PZT actuator, I additionally tested this effect with PZT transducers having different resonant frequencies (705 kHz, 1.35 MHz, 2.9 Mhz). In all cases, the devices showed peak performance around 1.40 MHz, similar to the results shown in Figure 42. It is clear from Figure 42 that the particle switching effect has strong frequency dependence, which would imply that the acoustic fields are amplified due to certain structural features of the microfluidic channel; however, these measurements alone were not sufficient to conclude whether the effect was due to the acoustic radiation force in a standing acoustic wave or whether the particles are following the flow patterns produced by streaming vortices near the sharp corner.

Next, to better understand the acoustically excited flow patterns, I performed a series of experiments with my undergraduate mentee, Sean Kelly. First, 200 nm red fluorescent tracer particles were injected (0.1 wt % in CTAB; Sigma Aldrich Corp) into the fluid and used long exposures to enable visualization of the streaming patterns near the trifurcation. In the absence of an acoustic field, the flow patterns are random as expected. When the transducer was actuated at 1.4 MHz and 5 Vpp, I observed very clear streaming vortices, which spanned the length of the channels and were present with minor variations across all the junctions in each field of view (Figure 43.a). The high consistency of the streaming vortices across the entire chip indicated that it is
caused by the specific structural features of the compartment rather than the specific position of the PZT transducer, overall size of the chip, or other macroscale features.

Figure 43: Relationship between particle trajectory and streaming behavior. (a) Red fluorescent image of nanoparticles under acoustic excitation at 1.4 MHz and 5 V_{pp}. (b) Normalized velocity magnitudes of 8.5 µm polystyrene beads approaching the entrance corner of the compartment region. Scale bar indicates 100 µm.

Further evidence that the transfer effect is due to the presence of streaming vortices was obtained by tracking the trajectories of individual beads in the vicinity of each corner. Using the same 8.5 µm beads described above, we tracked the trajectories of ≈44 individual particles upon acoustic excitation at 1.4 MHz and 5 V_{pp} and overlaid them on the same plot (Figure 43.b). As a visual aid, the data points are colored according to their instantaneous velocity. The overall shapes of the trajectories and their good match with the shapes of the streaming vortices, as well as the strong spatial dependence of the bead velocity, all provide strong evidence that the trapping force is due to acoustic streaming. This finding is consistent with previous studies in which
large particles and cells were trapped in steady acoustic streaming patterns around oscillating edges.\textsuperscript{148-150}

Following this, to better characterize the strength and driving mechanism of the acoustic switching effect, we also measured the peak velocity of the beads as a function of the applied voltage. These measurements were taken with a high-speed camera at 240 frames per second, which could quantify trajectories of up to \(~1\) mm/s. The highest velocities were recorded close to the sharp corner, which allowed us to approximately measure the holding force based on extrapolation from the fluid drag on a sphere (Figure 44.a). The results indicate that the peak holding force is linearly related to the magnitude of the applied voltage, similar to the linear relationships observed by others at high acoustic excitations.\textsuperscript{151} We estimate that at the strongest acoustic excitations, the contact force is less than 100 pN, and should thus be gentle on the cells. From Figure 44.b it is clear that the peak holding force scales linearly with voltage, which contrasts with the acoustic radiation force that should roughly scale quadratically with the excitation voltage.\textsuperscript{133}
Figure 44: Relationship between contact force and excitation voltage. (a) Trajectories of 8.5 µm polystyrene beads approaching the entrance corner of the compartment region. (b) Plot of maximum force before contact versus voltage. $R^2 = 0.9656$.

Further, as a final piece of evidence, I note that both highly elastic PDMS-based microparticles and stiff polystyrene beads are attracted to the same position with
comparable velocities. Since it is well known that PDMS particles suspended in aqueous fluids exhibit an effective negative acoustic contrast factor, whereas polystyrene beads exhibit a positive acoustic contrast, these two materials should not be attracted to the same points in an acoustic energy landscape.\textsuperscript{152,153}

Due to contributions from the monopole and dipole coefficient (30-32), the relative material properties of the particle or cell in suspension dictate their preferred position in the acoustic energy landscape. While a positive monopole coefficient or negative dipole coefficient can cause particles in suspension to migrate to the pressure node, a negative monopole coefficient or positive dipole coefficient can cause particles to migrate to the pressure antinode. Typically, in one-dimensional systems these two coefficients are combined and referred to as the acoustic contrast factor.\textsuperscript{152,153} Although this acoustic contrast factor is an over-simplification of the two-dimensional migration observed in these studies, the basic concept remains that particles with negative (positive) acoustic contrast factor should move to the time averaged pressure maxima (minima) respectively.

For my purposes, both the observed engineered PDMS-based microparticles (NACPs) and cells are nearly neutrally buoyant with the surrounding fluid, having densities ranging from approximately 0.9 to 1.1.\textsuperscript{153,154} Thus, contributions from the monopole coefficient dominate. NACPs are more compressible than the suspending fluid and exhibit a negative monopole coefficient, whereas cells are less compressible
than cell media and thus exhibit a positive monopole coefficient. Thus, if migration were
due to the acoustic radiation force, we would expect that NACPs and cells would be
driven to different locations in the energy landscape. However, in my device both
the NACPs and PC9 cells focus to the same location (Figure 45), indicating that
switching is not due to an acoustic radiation force, and instead caused by steady acoustic
streaming.

![Image](image.png)

**Figure 45: Acoustic switching of NACPs and Cells.** (a) Capture of a NACP on
the leading corner of the compartment region. (b) Capture of a PC9 cell in the same
location on the leading corner of the compartment region. (c) Zoom-in of streaming
patterns in the trifurcation region for 300 nm red PS particles upon acoustic excitation
at 1.4 MHz and 16 V<sub>pp</sub>. Scale bar indicates 50 μm.

Importantly, the combination of these measurements thus allows me to reasonably
conclude that the acoustic switching mechanism is based on acoustic streaming rather
than an acoustic radiation force.
3.3 Conclusions

In conclusion, I have developed a trap and transfer process for organizing a high-density array of single cells. This approach relies on a combination of hydrodynamic capture of cells in weirs and then transfer of the cells into more spacious compartment chambers using an array of acoustic streaming vortices as local switches. To the best of my knowledge, this is the first demonstration of the consistent generation of an array of streaming vortices for use in massively parallel acoustic control of single cells. Importantly, due to the versatile nature of acoustic forces, my technique does not require a cell-labeling step, and allows for the microfluidic channels and acoustic switching functionality to be integrated into a single device layer, which improves manufacturability, allows for the facile exchange of fluids, provides a good substrate for optical imaging, and has high chemical compatibility for patterning different biomolecules, which can be used for incubating cells and probing their molecular processes in future studies.
4. Demonstration of the Biocompatibility and Biological Relevance of the Acoustophoretic Array

Microfluidic cell handling devices that enable the high throughput isolation and analysis of single cells are powerful emerging tools. These technologies are useful because they allow for the identification of rare cellular events and heterogeneous responses of cell populations to external stimuli that are often concealed in conventional ensemble averaging approaches.\textsuperscript{12,51,52} Thus, knowledge gathered from these microscale experiments have been used to illuminate insights into both cell biology and medicine.\textsuperscript{53,128} The remarkable genetic and phenotypic heterogeneity within patient tumors, for example, is believed to drive resistance to chemotherapy,\textsuperscript{156,157} and has motivated the development of new tools to assess the drug sensitivity of single cells.\textsuperscript{158,159} Likewise, the diversity in single cell immune responses play an important role in the maintenance of chronic infections, preventing the cure of diseases such as HIV.\textsuperscript{160}

Accordingly, the development of new single cell analysis systems, particularly those which can study rare cellular events (i.e., those that occur at frequencies of 1 in 10,000 or less), can make a major impact in basic research and drug discovery in fields ranging from cancer, to immunology, neuroscience, and others.\textsuperscript{129-131,161}

Thus, for my third aim, I demonstrate a proof of principle that the acoustofluidic device I have developed (see 3. Characterization and Development of a Hybrid
Acoustophoretic Trap and Transfer Approach) has biological relevance. Though I do not provide exhaustive volumes of data, I show that my device can be used to track single cells growing on chip and how they interact with their environment. I also show a proof of principle that it is possible to stain the cells and track them in time. Specifically, I (i) quantify the efficiencies related to generating a single cell array, (ii) study cell escape, adhesion, and growth on-chip, and (iii) demonstrate cell staining and individual interrogation on-chip. To accomplish this, I determined the trapping/ arraying efficiencies for cells on my chip and devised procedures to reliably and robustly deliver controlled volumes of biologically relevant fluids (e.g. primed media, cell stains) to the cells.

**Contributions:**

For the results shown in this chapter (4. Demonstration of the Biocompatibility and Biological Relevance of the Acoustophoretic Array) I largely designed and performed all experiments independently. However, Mr. Jeffrey Motschman performed experiments aiding my quantification of single cell trapping efficiencies on-chip and provided useful discussions for cell growth on-chip. Additionally, Dr. Ying Li provided cellular dyes and useful discussions for my staining protocol while Mr. Kevin Lin provided the PC9 cell line explored herein.
4.1 Cell Capture and Arraying Efficiencies On-Chip

PC9 cells used in these studies have roughly the same size as the polystyrene beads used in 3.2 Optimization of the Acoustic Switch (i.e. ~15 µm). However, cells are comparatively more deformable and can undergo lysis in surfactant solutions such as those used for bead experiments (see Appendix). Thus, the loading pressure and fouling of the chip surface pose greater concerns in cell experiments as cells may squeeze through the trap sites at high pressures (e.g. > 35 mbar) and may adhere to the chip surface in absence of any surface treatment or surfactant phase. To address this, I functionalize the surface of the microfluidic chip with BSA, incorporate the use of a biocompatible surfactant Pluronic® F-68, and limit the forward loading pressure to -20 mbar. In depth details on the loading routine can be found in the Device Loading section of the Appendix. Further, as described in 3.1.2 Design of the Arraying Elements, as the gap width of the trap increases, the volumetric flow rate through the trap increases. While this geometric effect causes more cells to be directed towards these primary trap sites, the combined increase in size and width of the trap leads to significantly larger cellular escape due to squeezing. Accordingly, due to a high incidence of escape observed with 7 µm gaps, I limited my trap size to 6 µm. I note here also that, as discussed in previous chapters, narrower traps (e.g. ≥ 5 µm) led to a lower volumetric flow rate through the trap region and thus lower trapping efficiencies.
With this in mind, after optimizing the acoustic transfer step in 3.2 Optimization of the Acoustic Switch and exploring expected variations due to cell studies, I next sought to demonstrate the feasibility of this trap and transfer approach to organize a single cell array in a highly parallel manner. Towards this end, in collaboration with Jeffrey Motschman, I first quantified the ability to fluidically trap PC9 cancer cells (400,000 cells/mL) in the trap sites. This high initial concentration reduced the time for cells to occupy every trap site compared to lower concentrations (e.g. < 100,000 cells/mL) in the microfluidic chip due to the higher density of cells introduced into the chip at once. However, as expected, as this value increases the incidence of cells clogging the bypass and various sections of the chip also increase. Thus, while 400,000 cells/mL is used for these experiments for the sake of repeatability, I advise that no more that 600,000 cells/mL be introduced into the chip at a time. With this constraint, I demonstrated the consistent ability to achieve 80 ± 5% single cell trapping efficiency (Figure 46.a-e, n = 3 chips). Blue, cyan, yellow, and red indicate 0, 1, 2, and 3 trapped cells, respectively. Information on data collection methods can be found in the Appendix.
Figure 46: Trapping efficiency for PC9 cells in the weirs of the trifurcation. (a) Representative image of trapped cells. Number of cells captured in individual trap sites for trial (b) one, (c) two, and (d) three. (e) Distribution of cells in n = 3 acoustofluidic chips. Scale bar indicates 200 µm.

In brief, however, data can be analyzed manually by visually inspecting each individual compartment to quantify the cell occupancy or data can be collected automatically using a MATLAB based script. For automated data collection, cells must be stained with a fluorescent dye. The efficiency can be improved by reducing the number of cell doublets entering the chip, and limiting cellular debris, which leads to clogging along the fluidic path (two blockages are clearly shown in Figure 46.c where multiple traps in series are empty, and doublets are colored in yellow). Next, consistent with methods described in 3.2 Optimization of the Acoustic Switch, I transferred the cells into their corresponding adjacent compartments using an acoustic sweep from 1.35 to 1.42 MHz, with a sweep rate of 1 Hz for over one second at an excitation of 16 V_{pp} (Figure 47.a).
Figure 47: Arraying efficiency for PC9 cells in the compartment region of the trifurcation. (a) Representative image of arrayed cells. Number of cells captured in individual array sites for trial (b) one, (c) two, and (d) three. (e) Distribution of cells in \( n = 3 \) acoustofluidic chips. Scale bar indicates 200 \( \mu \text{m} \).

As can be seen, after the second step of this process, we were able to consistently obtain a single cell array with an efficiency of 67 ± 4 % (Figure 47.b-e, \( n = 3 \) chips). It is important to mention that this number represents the total fraction of single cells present in the compartment regions of the acoustofluidic chip. Thus, this value is strongly dependent on the number of single cells originally trapped and the acoustic switching efficiency for each compartment site. Accordingly, with a trapping efficiency of roughly 80 %, this data indicates that the acoustic switching efficiency is approximately 83 %. As multiple factors such as retarded motion of cells during transfer due to fouling on the chip surface or surface roughness, cells sticking in trap sites, or pressure fluctuations due to a trapped bubble along the fluidic line or instability of the pressure source can
lead to a failed acoustic transfer to compartment regions, this number is reasonable.

While many of these factors are difficult to control, switching efficiencies can be improved by functionalizing the chip with a non-fouling brush, stabilizing the pressure source, and replacing manual transfer (see Appendix) with a LabVIEW based automated routine.

I additionally tested a high density acoustofluidic array (i.e. \(\approx12\) cmpts/mm\(^2\), see 3.1.3 Design of the Acoustofluidic Chip) with a footprint of 25 x 75 mm consisting of 8 sections of 20 compact acoustofluidic elements in series and 32 elements in parallel (e.g. > 5000 compartments total, Figure 48). Here, the vertical length from the top of the compartment region to the first bend in the bypass channel (Figure 48, solid line) is 255 \(\mu\)m while the length to the bottom of the bypass is 470 \(\mu\)m (Figure 48, dashed line). For this chip, I observed sufficient acoustic transfer across a broad range of frequencies, ranging from 2.7 to 2.9 MHz.
Figure 48: Single cell array generated in high density chip excited at 2.9 MHz and 16 V_{pp}. Solid line indicates distance from top of compartment region to the first bend of the bypass channel (i.e. 255 µm) while the dashed line indicates the distance from the compartment region to the bottom of the bypass (i.e. 470 µm). Scale bar is 200 µm.

As can be seen, though some of these cells proved to be too large for the compartment entrance, I was able to fill a simple array of my second prototype with single cells using a similar methodology as that developed for Figure 47. However, this slender geometry led to significant sticking of these large cells (e.g. ≥15 µm, seen in the first compartment of the first row in Figure 48) and clogging of the microfluidic chip due to their large size.
compared to the slender width of the microfluidic channels (i.e. 20 µm). Further, as cells are attracted to multiple regions in the trifurcation aside from the entrance corner of the compartment region (see 3.2.2 Experimental Investigation of the Acoustic Switching Behavior in Earlier Prototypes), at times these large cells would be attracted back towards the trap site or down towards the bypass during acoustic excitation due to the reduced distance from these other attraction points (e.g. < 5 µm from the cell surface to other corners of the 20 µm wide channels in microfluidic chip). Thus, multiple trials were required to successfully switch cells into the compartment region.

Accordingly, I next tested the acoustic transfer mechanism with a smaller suspension cell line OCI-AML2 cells (e.g. ≤ 12 µm), a human acute myeloid leukemia line (Figure 49). Though these cells are smaller compared to PC9 cells and led to less clogging in the chip, I still observed a high incidence of cells sticking in the trap regions of the microfluidic chip (see panel i-iii in compartment 05-16 in Figure 49).
Figure 49: Bright field image sequence of acoustophoretic templating of cells. Here, (i) acoustics are off and cells flow into trap region then (ii-iv) acoustics are turned on and cells flow into apartment. Scale bars represent 100 µm.
This, in turn, suggests that as I decrease the trap width, I increase the likelihood that cells may get stuck in the trap site. Further, I also observed more AML cells escaping from their trap sites after capture, likely due to their reduced size.

Thus, I have shown that a variety of cells with a range of sizes can be trapped and transferred using the technique developed in this dissertation. In both instances, the surface of the microfluidic device was modified via passive adsorption of proteins from the cell media to the surface and a BSA incubation step. While functionalization with an ultra non-fouling brush could improve my efficiencies, this ability to array and study cells without extensive surface modification (e.g. polymer functionalization) is promising because it reduces both the cost and preparation time for the device. After demonstrating the generation of single cell arrays in different chips that are optimized for both an adherent and suspension cell line, I next sought to show proof of principle that cells could be maintained and propagate inside chips over time.

4.2 Cell Behavior over Time on Chip

PC9 cells have both an adherent and suspension growth mode with morphologies ranging from spindle shaped cells to round shapes. Further, these cells are recognized as a robust model cell line for studying the effect of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) on non-small cell lung cancer (NSCLC) growth rates and subsequent development of drug resistance. As such this cell line, provided by Kevin Lin, my collaborator in Professor Kris Wood’s lab, is an ideal
candidate for proof-of-concept studies to demonstrate that the trap and acoustic switching methodology developed herein allows cells to survive long enough that it is possible to determine various phenotypic parameters, like cell motility, focal adhesions, nuclear position, etc., at multiple time points.

For each growth study, the microfluidic chip was treated with filtered (i.e. using 0.45 µm PTFE filters) 100 % BSA for 2.5 hours at 37°C to allow for passivation of the surface. I note here that for passivation and all remaining steps, while the microfluidic chip was on the 3D printed inverted microscope set-up (Figure 50), the heating element was turned on to maintain a roughly constant temperature of 37°C throughout the aluminum housing.

Figure 50: Total Assembled chip in 3D printed stage with reservoirs for withdrawal of cells into microfluidic chip.
Following passivation, I rinsed and replaced the warmed BSA with RPMI media doped with GlutaMAX\textsuperscript{TM}, 10 \% FBS, and 1 \% Pen/Strep that was previously primed in an incubator at an optimal gas concentration (i.e. 5 \% CO\textsubscript{2}) and temperature (i.e. 37°C) for > 1 hour. Next, following priming with cell media, a cell suspension (∼1 × 10\textsuperscript{6} cells/ mL) was added to the inlet ports and withdrawn into the microfluidic chip using an Elveflow pressure controller (i.e. -12 mBar, Figure 50). After arraying cells into the compartment regions using previously described methods (3.2.3 Experimental Investigation of the Acoustic Switching Behavior in the Final Prototype), a reservoir was added to the outlet port of the microfluidic chip and ∼100 µL of cell media was added to the inlet reservoir to allow steady gravity driven flow of fresh media through the chip.

I went on to image the behavior of single cells over time in the chips after t=0, t=8 hours, t=16 hours, and t=24 hours. In between imaging steps, the exterior of the aluminum housing was thoroughly cleaned with 70 \% ethanol and stored in an incubator. In these studies, cellular adhesion, escape, and division were tracked over time. These phenotyping classifications were chosen because they indicate important features for sustained cell viability on-chip such as viability after the trap and transfer process, cell motility over time, and cell growth over time respectively.

As I tracked the behavior of single cells in the compartment regions, I noticed some inconsistencies in the cell growth and adhesion rates on chip. Upon closer inspection, I found that the dynamic motion of cells once adhered to the surface of the
microfluidic chip caused random fluctuations in the population of adhered and divided cells across various, unpredictable compartment regions in the chip over time. This volatility in the data thus stunted my ability to draw firm quantitative conclusions on the adhesion and proliferation rates of individual cells in my chip. Accordingly, instead I will discuss the qualitative behavior of this cell line in the chip during my 24-hour observation period with the goal of informing future single cell studies on motile cell populations.

Unlike suspension cells that remain largely within the same confined region, I noticed that once adhered to the surface, PC9 cells uniquely could both escape (Figure 51) and enter (Figure 52) the compartment regions as shown below.
Figure 51: Single PC9 cell escaping from compartment region over time at (a) t=0 hours, (b) t=8 hours, (c) t=16 hours, and (d) t=24 hours. Scale bar is 100 µm.

In Figure 51.a-c, it is clear that a single cell started in suspension initially, then adheres to the surface after 16 hours. Subsequently, as shown in Figure 51.d, the cell then moves on to crawl out of the compartment region and escape through the loading dock by the 24-hour time point. Importantly, cell adhesion and subsequent movement indicates that the cell is alive on-chip, hours after being arrayed into the compartment region. I note I observed some cells escape from the compartment region by exiting up and out of the entrance channel as opposed to exiting down through the loading dock. However, most escape I observed was through the loading docks. This is likely caused by multiple cells having an initial position on or close to the loading docks directly following transfer into the compartments.

Additionally, I observed cells that were not originally arrayed in the compartment region entering through the bypass channel, as shown below in Figure 52. In this image, I observed the suspected original arrayed cell (indicated by a red circle) remain in the compartment region and appear to slightly adhere to the walls of this region. I additionally observed new cell, indicated by a green circle, enter the weir site of the acoustofluidic element from the bypass channel by t=8 hours, appear to squeeze through this constriction by t=16 hours, then return to its original location by t=24 hours. Finally, and most importantly, I observed a third cell (indicated by a blue circle) appear to travel from the bypass channel to the exit outlet of the loading dock of the
compartment by $t=16$ hours and within 8 hours crawl through the loading dock into the compartment region by $t=24$ hours.

Figure 52: Single PC9 cell entering the compartment region over time at (a) $t=0$ hours, (b) $t=8$ hours, (c) $t=16$ hours, and (d) $t=24$ hours. Scale bar is 100 µm.

In my initial attempts to analyze this data, I found that this escaping and entering effect significantly mutes any perceived trend in the adhesion or division of single cells on chip, even when removing any compartment region from the data set that appeared to be compromised by this stochastic behavior. However, in part due to the relatively large times between imaging (e.g. 8 hours), the challenge of determining which cells from the original population were in each compartment region proved to be insurmountable for my present studies in this chip configuration. This escaping and entering effect, however, may be reduced or eliminated by selectively increasing the
depth of compartment regions and thus increasing its surface area and providing cells more room to roam. A selective etch can be achieved by combining a hard mask of the entire chip (e.g. SiO2 based) onto the wafer surface with a photoresist-based mask that will be used to outline the compartment regions. Thus, the compartments can undergo a deeper, extended etch and after the photoresist layer is removed, the hard mask remains and can be used to define the shallow channel regions. Additionally, it may be possible to limit the motion of cells in the compartment region by selectively functionalizing their surrounding surface with a polymer coating, such as polyacrylamide,\textsuperscript{164} that is resistant to cell adhesion. This may be achieved by functionalizing the entire surface with a resistance polymer, and subsequently filling the chip with an aqueous hydrogel (or alternative solubilized matrix), rinsing the bypass channels with an oil to remove excess hydrogel from these regions, solidifying the matrix in the compartment regions, and finally replacing the oil with fresh media and steadily infusing this throughout the chip.

Along with observing cells adhering to the surface of the microfluidic chip and subsequently escaping from or entering compartment regions, I additionally noticed that some cells simply moved around their confined regions and did not escape within 24 hours, as shown in Figure 53 below.
Here, we see that the single cell adheres to the surface of the microfluidic chip by $t=16$ hours (Figure 53.c), similar to the cell shown in Figure 51. However, due to the spacious area provided in the compartment region, while the cell moves with flow towards the loading dock it remains within the compartment region at this final time point (Figure 53.d). Finally, I additionally observed some compartment regions that initially had a single cell not escape over the 24-hour period and divide once. A representative time series of this process is shown in Figure 54 below.
Figure 54: Single PC9 cell dividing over time at (a) t= 0 hours, (b) t= 8 hours, (c) t=16 hours, and (d) t=24 hours. Scale bar is 100 µm.

Here, it can be seen that initially the compartment region contains a single cell (Figure 54.a), which then swells after 8 hours and increases in size (Figure 54.b), to finally divide by 16 hours (Figure 54.c), and remain at the same position by the 24 hour time point (Figure 54.d).

From these observations, I anticipate that while I cannot perform definitive quantitative studies, there may be a discrepancy between cell growth on-chip and cell growth in a tissue culture dish due to the scarcity of nutrients in the compartment regions of the microfluidic chip or poor growth of single cells in isolation (i.e. minimum seeding densities). In this case, for future studies I can increase the pressure head at the inlet of the microfluidic chip, and thus the volumetric flow rate of nutrients though the
chip. Alternatively, I can mix conditioned media (i.e. cell secretome from a tissue culture) that may include important cytokines, growth factors, and hormones necessary for healthy growth\textsuperscript{165} with fresh media at the inlet reservoir. I note, however, that if taken directly from cell culture as opposed to purchasing an optimized supply from a vendor, this mixture may introduce toxins into the chip. Further, I believe there is a possibility that growth and adhesion rates \textit{on-chip} can be improved by exploring alternative surface functionalization routines.\textsuperscript{166}

Overall, the results from these experiments matched my expectation that cells can be incubated in the acoustofluidic chip and adhere and proliferate in this environment after arraying. Despite being unable to collect quantitative data due to the dynamic migration of adherent cells over time, this ability for cells to adhere and grow \textit{on-chip} allows for probing for changes in phenotypic characteristics over time.

\textbf{4.3 Cell Staining on Chip}

Physiological stains can play an important role in cell analysis \textit{on-chip} over time. The Hoechst stain, for example, has been shown to be useful for quantifying nuclear content in cell cycle staging and counting cells \textit{on-chip}. Hoechst 33342 is a cell-permeant nucleic acid stain that emits a blue fluorescence once it is bound to double stranded DNA. As such, Hoechst 33342 allows for the visualization of the cell nuclei. Accordingly, using this and similar stains (e.g. DAPI) scientist have garnered meaningful information \textit{on-chip} concerning the stage of individual cells.\textsuperscript{167,168} This in turn can be used in future
studies to identify cells that continued to grow past the G1 phase regardless of treatment with an EGFR inhibitor. Further, various other physiological stains for structural proteins such as tubulin and actin have been used to study variations in the cellular scaffold over time that could play an important role in cell division and motility. As a result, it is important to not only be able to culture cells on-chip, but additionally be able to stain single cells on-chip. Thus, in this section, I focus my efforts on characterizing and validated a method which enables cell staining and physiological characterization on-chip. Optimizing the balance between exchanging the suspending fluid in the system expeditiously and preventing cellular escape from the compartments during this exchange poses one of the largest problems in cell staining on chip because these two factors oppose each other. As the injection pressure increases, the flushing rate of the suspending fluid increases. However, increasing the injection pressure leads to a greater force on cells in the chip causing an increased incidence of escape through the loading docks. Consequently, I sought to quantify realistic flushing rates in my microfluidic chip.

I first calculated the expected time for exchanging media in the chip (i.e. entirely replacing the media in the chip with a new suspending fluid such as a staining solution) using the experimental resistance calculated in 3.1.2 Design of the Arraying Elements (i.e. $2.27 \times 10^{14}$ $Pa \cdot s/m^3$). As stated in 4.1 Cell Capture and Arraying Efficiencies On-Chip, high pressures lead to cells squeezing through thin features- such as the loading
dock or trap site of the acoustofluidic element. Thus, to avoid this, I set my threshold pressure to be 25 mbar to avoid loss of cells in the compartment region through the loading dock during fluid exchange. Using the experimental resistance, Equation 12, and setting the pressure to be 25 mbar I calculated the corresponding volumetric flow rate through one section to be ≈0.7 μL/min. Next, I estimated that the volume of the fluid in the microfluidic chip is roughly 3 μL (i.e. assuming that the chip is a rectangular bath). Knowing this volume, and assuming a volumetric flow rate of ≈0.7 μL/min, I predicted that it would take about 4 minutes to replace the suspending fluid in the chip if the new phase is suctioned from a reservoir. Thus, for subsequent staining experiments I introduce a new fluid phase into the microchip by suctioning the fluid from a reservoir at approximately – 25 mbar for 5 minutes.

After optimizing the infusion mechanism, I stained PC9 cells on-chip. Once cells were loaded into the compartment regions of the microfluidic chip, 100 μl of a Hoechst 33342 Dye (ThermoFisher; 0.05 μg/mL) solution was added to the inlets of the microfluidic chip and suctioned into the chip following the routine outlined above. After 5 minutes, the dye in the reservoir was replaced with media and the chip was rinsed at the same pressure for 10 minutes. Following rinsing, bright field and fluorescent images were captured across the microfluidic chip (Figure 55). As can be seen, the nuclei of single cells were stained on-chip.
Subsequently, composite images (i.e. bright field overlaid with fluorescent images) of individual compartments were visually inspected to confirm the presence or absence of the Hoechst stain in each cell. If the fluorescent signals of all cells in a compartment were clearly visible to the naked eye with no additional thresholding, that compartment was considered Hoechst 33342 +.

Figure 55: Representative (a) bright field and (b) fluorescent images of PC9 staining on-chip. Scale bar is 150 μm.

If a signal could not be seen, in all or one of the cells it was considered to have no stain, and if the compartment was empty it was not included in the quantification.
Conveniently, Hoechst 33342 is a rather robust stain that fluoresces brightly over any perceived background noise. As can be seen in Figure 56.a nearly 100 % of cells within compartments that contained cells (i.e. 99.8 %, n = 3 chips) were stained with the Hoechst 33342 dye. Additionally, it is clear from Figure 56.b that staining occurred in a largely uniform manner across the entire chip as only one location on-chip contained a cell without the Hoechst 33342 stain.

Figure 56: Staining efficiency on-chip. Shown here, (a) a pie chart of showing staining efficiency for n = 3 chips and (b) compartment regions from a representative trial that were empty, contained stained cells, or contained cells that were not stained
This lack of staining could be due to a variety of sources, such as a clog near that area of the chip that caused poor flow in the compartment, visual misidentification (e.g. spherical junk in the compartment), or DNA degradation/fragmentation due to cell death. Of these potential sources I believe that, due to the uniformity of cell staining around the unstained compartment and lack of clogs observed close to the region, most likely the rare staining failures are most likely a result late stage cell death or misidentification.

Finally, I next sought to combine cell incubation on-chip with cell staining on-chip using three separate structural stains (i.e. Hoechst 33342, TubulinTracker™, SiR-actin). For this experiment, the three-color staining solution was comprised of 0.05 µg/mL blue Hoechst Dye (ThermoFisher), 250 µM of green TubulinTracker™ (Invitrogen) with 0.2 wt. % Pluronic F-127, and 1 µM of a red SiR-actin Kit (SpiroChrome) with 10 µM verapamil (SpiroChrome; a broad-spectrum efflux pump inhibitor). For this staining solution, the Pluronic F-127 helps increase the solubility of the tubulin dye and the verapamil helps improve the staining of the actin dye. I additionally note that in order to effectively withdraw the staining solution from the inlet reservoir using negative pressure from the ElveFlow, liquid must completely fill the connecting tubing before connection such that there are no air bubbles along the fluidic path that may disrupt flow. Further, adhered cells cannot withstand the high shear from the from the prescribed -25 mbar for sustained periods of time (e.g. > 20 minutes) and eventually
delaminate (see Appendix). However, the three-color stain requires incubation for 45 minutes. Thus, after the chip is filled with the three-color staining solution (e.g. after 5 minutes at -25 mbar), the pressure is reduced to 5 mbar for the remaining 40 minutes.

Following the procedure outlined above, I successfully arrayed, incubated, and stained a single cell in the compartment region of my acoustofluidic chip (Figure 57).

Figure 57: Example of PC9 cell stained and incubated on-chip. Shown here, (a) the PC9 cell adhered to the surface after incubation overnight, (b) a fluorescent image of the tubulin stain (green), (c) a fluorescent image of the nuclear stain (blue), (d) a fluorescent image of the actin stain (red), (e) a composite fluorescent image of the nuclear and actin stain, (f) a composite fluorescent image of the tubulin and nuclear stain, and (g) a composite fluorescent image of the tubulin and actin stain. Scale bars are 50 µm.
Here, we see that with this new platform, single cells can be arrayed in compartment regions, incubated on chip using gravity driven flow (i.e. filling a reservoir with fresh media and allowing fluid to be pulled by gravity through the chip), and stained the next day. These results indicate that fluid can be dynamically exchanged on-chip in a facile and rapid manner. This additionally provides a proof of concept that cells can be both incubated and stained on chip to probe for various physiological changes over time.

4.4 Conclusions

In conclusion, I have demonstrated a proof of principle for my novel cellular array platform by testing single cell trapping and arraying, quantifying cell adhesion, escape, and division of time, and staining my cells with physiological markers at various time points. Specifically, in my first section I used a new trap and transfer approach to generate a single cell array with a trapping and arraying efficiency of ≈80% and ≈67 %, respectively, which allows me to array thousands of cells on a glass slide sized device. Further, in my second and third sections I demonstrated the ability to both grow and stain cells on-chip which allows for a powerful exploration of phenotypic characteristics of cells over time. Thus, the characterization and development of this platform technology enables longitudinal studies, where cells can be incubated and studied on-chip to study phenotypic responses of single cells to external stimuli in future studies over time.
5. Conclusions

Microscale acoustofluidic devices have proven to be powerful tools that enable the gentle and highly parallel manipulation of cells and particles in suspension. Bulk acoustic wave based (BAW) devices are particularly attractive due to their high chemical compatibility (i.e. inert to a variety of organics and caustic solutions such as sulfuric acid; unlike PDMS), high pressure threshold (i.e. $\gg 1000$ mbar), and inexpensive material requirements, and maintain the viability of cells during actuation. Further, these systems have been demonstrated to have a myriad of bioanalytical applications in the past ranging from continuous flow cellular de-bulking,$^{122,171}$ to characterization of the material properties of cells,$^{172}$ and medium switching.$^{173}$ While most of these applications have involved focusing objects in suspension to the nodes of a basic continuous flow rectangular design, I explored unconventional applications such as focusing to the anti-nodes of an acoustic standing wave and manipulating single cells in complex geometries. These unusual applications have steadily gained traction in over the past fifteen years due to key foundational papers by the López$^{106,123,153,174}$ and Laurell$^{175}$ groups and an array of important studies that explore acoustic streaming in basic geometries (e.g. square channels or near vibrating tips).$^{139,143,148,151}$ However, my studies on magnetic particles that exhibit a negative acoustic contrast factor and the generation of a single cell array using steady acoustic streaming vortices are the first of its kind. Thus, it is my
hope that this body of work will serve as another point of reference in our collective
effort to better understand and design impactful microfluidic tools.

5.1 Summary

In this dissertation, I have developed two key acoustofluidic tools for cellular
processing on-chip (i) a class of dual-contrast bio-sensing particles and (ii) an
acoustofluidic single cell array. In both systems, cells and particles are manipulated in
suspension using a BAW based device, where acoustic perturbations are generated by
an attached piezoelectric transducer that in turn vibrates the highly reflective glass and
silicon substrates. In my first system, my main mode of acoustic manipulation is the
acoustic radiation force, while my second system uses steady acoustic streaming vortices
to manipulate particles and cells. The findings from both studies are relevant to the
development of a comprehensive acoustofluidic tool where target cells are first de-
bulked from suspension, then isolated on-chip for longitudinal studies.

In 2. Characterization and Development of Dual Contrast Bio-Sensing Particles,
I introduced a class of bio-sensing particles that focus to the anti-nodes of an acoustic
standing wave and undergo positive magnetophoresis. This unique capability has
important implications for biological separations due to the wide adaptation of magnetic
activated cell sorting (MACS) devices and the ability to rapidly acoustically separate
particles that exhibit a negative acoustic contrast factor (NACPs) from cells in a facile
manner. I show that these amine-functional microparticles can be coated with
streptavidin molecules for future binding assays, quantify the magnetic and acoustic properties of each particle, and demonstrate a ternary separation using a MACS device and a simple acoustic glass capillary (see Figure 14). As such, these functionalized microparticles can be easily integrated with existing MACS technologies and act as a secondary bio-sensing bead that can be used to isolate two magnetically labeled cell populations from each other using acoustic forces.

In 3. Characterization and Development of a Hybrid Acoustophoretic Trap and Transfer Approach, I optimize the trap and transfer process for my acoustofluidic single cell array using microparticles. Using analytical approximations and simulations, I optimized the resistance through the trap, bypass, and compartment region such that the resistance through the compartment region is at least four times higher than that through the trap and double that through the bypass. Further, using simulations and experiments, I found that acoustic streaming and acoustic radiation forces exists simultaneously in my device and that there are many regions that cells and particles are attracted to throughout the acoustofluidic element. Of these various regions, I found that attraction to the entrance corner of the compartment region and subsequent contact with the wall caused particles to switch from flow lines entering the trap to flow lines entering the compartment over a specified voltage and frequency range. To determine whether this attraction was due to acoustic streaming or acoustic radiation forces, along with my undergraduate mentee, I performed a series of particle tracking experiments
and found that (i) particle trajectories speed up towards the epicenter of the streaming vortex formed at the entrance corner of the compartment region and (ii) maximum particle velocities (immediately preceding contact) scale linearly with applied voltage. These two findings, along with evidence that NACPs and polystyrene microparticles that exhibit a positive acoustic contrast factor focus to the same location, led me to conclude that switching occurs due to the generation of steady acoustic streaming vortices. Further, it is important to note that the contributions of my work stem beyond ideating a new type of cellular array to contributing new knowledge about poorly understood fundamental acoustofluidic phenomena in a complex geometry.

In 4. Demonstration of the Biocompatibility and Biological Relevance of the Acoustophoretic Array, I show a proof of principle for my novel cellular array platform by (i) quantifying my single cell trapping and arraying efficiencies on-chip, (ii) demonstrating cell adhesion and growth on-chip, and (iii) demonstrating dynamic cell staining on-chip. I found that while my trapping and arraying efficiencies suffered due to fouling of the chip surface and blockages in the chip, the trap and transfer process is highly parallel and capable of arraying up to 67 % of single cells in a high throughput manner. Further, I found that I can incubate single cells in the compartment regions of these chips by steadily feeding fresh media into the chip using gravity driven flow and an incubator. A portion of these single cells can then adhere to the surface of the array or divide on-chip over 24 hours with no surface modification aside from passive adsorption.
of BSA. Further or alternative modification schemes (e.g. polymer functionalization, FBS adsorption, collagen or gel matrices) may lead to improved adhesion and division rates. Finally, I was able to stain cells on-chip after incubation overnight with a cocktail of physiological dyes comprised of Hoechst 33342, TubulinTracker™, and a SiR-actin kit. Thus, the characterization and development of the platform technology I introduced in my third chapter enables longitudinal studies, where cells can be incubated and studied on-chip to observe phenotypic changes of single cells over time and space.

5.2 Future Directions

The work described herein explores the preliminary stages of what could be a broader, comprehensive system. As of yet, there still exists a pressing need for an integrated microfluidic platform that enables the isolation of a cell population from a complex mixture (e.g. whole blood, tissue sample), the organization of this population on-chip, and the ability to incubate these single cells on-chip and study their response to a variety of environmental stimuli (e.g. drug treatments, other cells, or signaling proteins) over time. This could prove invaluable in a variety of applications, ranging from personalized care, to tissue engineering, and fundamental biology. While my work begins to answer this need, there are still many open questions remaining to accomplish this goal. In this section, I will suggest potential directions for future work for those who may follow in my steps, as well as highlight problems that I did not have the opportunity to solve.
The characterization and development of the class of dual contrast particles discussed in Chapter 2 serves as a necessary first step towards the ultimate goal of creating a tunable cell capture and isolation bead. The natural next step involves optimizing the binding affinity of the microparticles to a cell type of interest. Previous work that myself and collaborators from the López Lab have performed indicates that binding of elastomeric particles to cells can be challenging and lack repeatability. This could be due to a variety of sources, ranging from steric hindrance due to the large particle sizes (i.e. > 10 µm) to poor surface coverage of functionalized groups. These issues can potentially be overcome by exploring alternative functionalization routines (e.g. with antibodies or longer spacers between the particles and cells), tuning the size and concentration of the particles, and optimizing the reaction routine (e.g. mixing mechanism and reaction time). Following binding and separation of cells, the positive magnetic contrast of the cells can be leveraged to array these bound complexes on magnetographic or magnetophoretic arrays.

For the system related sections of my third and fourth chapter, I found that my trapping and arraying efficiencies are most heavily impacted by the formation of blockages in the chip, sticking of cells to the trap regions, or retarded motion of cells during arraying due to fouling on the chip surface or pressure fluctuations in the chip. These deleterious effects may be partially suppressed by functionalizing the chip surface with a non-fouling polymer brush and stabilizing the pressure source. Further, a stable
pressure source and uniform velocity of cells would enable the use of an automated LabVIEW based routine for the trap and transfer process. Along with this, further optimizing the acoustic coupling between the piezoelectric transducer and the acoustofluidic chip could enable acoustic transfer of cells at a lower actuation voltage.

Additionally, there are multiple biological considerations that can be explored and expand upon studies from my fourth chapter. Surface optimization, for example, may additionally increase cell adhesion and growth rates on-chip. Thus, a natural next step would be to explore alternative functionalization routines and microenvironments for the cells, such as POEGMA, collagen matrices, or various synthetic agarose gels. It is also important to note that different cells may grow at different rates and respond in a variety of manners to different microenvironment or surfaces. Along with optimizing the surface and microenvironment for PC9 cells, it would additionally be interesting to perform an array of studies that optimize for the ideal conditions for alternative set of cells of interest (e.g. t cells, stem cells, etc.). Outside of cell growth, this platform can be used for many practical future studies. In particular, it has been shown that the efficacy of combinatorial treatment approaches can be strongly impacted if drugs are taken sequentially rather than concurrently. This is an important consideration for PC9 cells, as EGFR-TKIs have been shown to potentially induce a cell cycle arrest in the G1 phase, and thus may interfere with alternative chemotherapeutic drugs that cause cell-cycle specific cytotoxicity in later phases (e.g. S, G2/M). As a result, a timely and
important follow on study for this work would be performing cell cycle staging for arrayed single cells \textit{on-chip} using the Hoechst Dye and exposing the cells to combinatorial treatments over time.

These suggestions represent a mere sample of the wide variety of follow-on studies enabled by this work. The expansive nature of these potential studies demonstrates the powerful impact of developing microfluidic systems for cellular manipulation. By optimizing the engineering properties of these systems and demonstrating a proof of concept for their application, I have introduced a new potential path for addressing the multifaceted problem of cellular processing \textit{on-chip}. 
Appendix

Materials

Acoustically Active Particles

6-7% Aminopropylmethylsiloxane)-dimethylsiloxane copolymer (AMS-162) was purchased from Gelest, Inc. Hexadecyltrimethylammonium bromide (CTAB, ≥98 %), 37 wt.% formaldehyde, poly(vinyl alcohol), and bovine serum albumin (BSA, heat shock fraction, pH 7, ≥98 %), were purchased from Sigma-Aldrich Co. EZ-Link™ sulfo-NHS (N-hydroxysulfoxuccinimide) biotin and Alexa Fluor® 488 fluorescent streptavidin were purchased from ThermoFisher Scientific, Inc. Gelatin was purchased from E.D. Smith® Foods, Ltd. SPHEROTM rainbow calibration particles and SPHEROTM carboxyl fluorescent magnetic particles were purchased from Spherotech, Inc. Human umbilical vein cells (HUVEC) and endothelial growth medium SingleQuotsTM kit was purchased from Lonza AG. Blue calcein AM dye live/dead cell viability kit was purchased from Invitrogen Corp. MACS® MS Columns and OctoMACSTM Magnet were purchased from Miltenyi Biotec GmbH. Silicon wafers were purchased from University Wafer, Inc. Borofloat® glass was purchased from Schott AG. Piezoelectric transducers were purchased from APC International, Ltd. Glass capillaries were purchased from VitroCom, Inc. Poly(dimethylsiloxane) (PDMS) was purchased from Dow Corning Corp. A standard laboratory grade hemocytometer was purchased from Hausser Scientific Co.

Acoustic Chip
The acoustofluidic chip was housed in a three-part aluminum manifold. The top component of the manifold uses standard ¼-28 threaded fittings (Idex Corp.) to make high pressure (i.e. > 1000 mbar) world-to-chip connection to the acoustofluidic device with pressure tight fittings (Figure 58).

Figure 58: Fluidic connections for acoustofluidic chip

The middle manifold had a recess along the top to hold the chip and another along the bottom to mount the zirconate titanate (PZT) transducers (1 1/8” x 1 1/16”, resonant frequency, $f_0 = 1.35$ MHz; APC International, Ltd.), which were bonded to the manifold with cyanoacrylate glue (Loctite® 495; Loctite Corp.). We improved the acoustic transmission into the chip by spreading a layer of electrode gel (Spectra® 360; Parker Laboratories, Inc.) between the chip and manifold. Finally, the bottom component encloses the transducer in the manifold to enable a closed chamber for a temperature
probe. For the backside configuration, access ports were included in the middle component (Figure 59).

**Methods**

**Acoustically Active Particles**

**Cell Preparation**

Human umbilical vein cells (HUVEC) were cultured in Endothelial Growth Medium SingleQuots™ Kit, which is doped with growth factors, cytokines, and supplements. Prior to separation, cells were stained with blue calcein AM dye from a live/dead cell viability kit (Invitrogen) and incubated for 30 min. Subsequently, cell
solutions were centrifuged at 200xG for 4 min and resuspended a fresh solution of 1.0 wt.% bovine serum albumin in deionized water two times.

**Acoustofluidic Chip**

*Device Fabrication*

Three acoustofluidic arrays were tested in this study. Two had topside access ports in which holes were drilled through the glass prior to bonding. The other was fabricated by through-wafer etching of inlet/outlet ports and then bonding to unpatterned glass to enable backside fluidic access ports. Photopatterning was achieved by spin coating Shipley S1838 photoresist (MicroChemicals, GmbH) onto 6” silicon wafer (University Wafer, Inc.) at a spin speed of 3000 rpm, baking them a 115°C for 60 seconds, then exposing them to 126 mJ of 365 nm UV radiation with a mask aligner (MA6/BA6, Karl Süss). These patterns served as a polymer mask for etching the microfluidic channels to a depth of approximately 18 µm using deep reactive ion etching (Pegasus deep silicon etcher; SPTS Technologies, Ltd.). We diced individual chips from the wafer when using the devices with topside access ports. For backside access ports, we used a second lithography step, in which the dice lines and inlets/outlet ports were patterned on the backside of the wafer in AZ9260 photoresist (MicroChemicals, GmbH), which was spin coated at 1800 rpm for 60 s, baked at 110°C for 3 min, then exposed to 3600 mJ of irradiation in the mask aligner. Subsequently, the wafer was bonded to a
carrier and a through-silicon etch was performed using deep reactive ion etching. For both configurations, individual chips were cleaned in piranha and anodically bonded to precut glass cover slips (Borofloat® Glass; Schott AG) to form a hermetic seal.

**Cell Preparation**

The PC9 cell line was cultured in RPMI 1640 with 10 % fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C in 5 % CO2. All cell lines were purchased from the Duke University Cell Culture Facility (CCF). Expression of ectopic luciferase was enforced through lentiviral transduction of cells, enabling stable expression of luciferase off of a PGK promoter (Addgene plasmid #64649). Lentivirus particles were produced through transient transfection of 293T cells using a three-plasmid system: expression clone + VSVG + δVPR as previously described. Cells were spininfected with lentivirus at 1200xg for 1 hour at 37C in the presence of 8µg/mL polybrene. 24 hours after infection, transduced cells were selected in 2µg/mL puromycin for at least 48 hours before experimental use.

**Device Loading**

For microparticle studies, the chip was primed by sequentially rising with 190 proof ethanol (Sigma-Aldrich Corp.) and deionized water. After the chip was primed, 8.5 µm polystyrene beads (50,000 beads/mL, Sigma Aldrich Corp.) suspended in 0.5 wt. % hexadecyltrimethylammonium bromide (CTAB) in deionized water were injected into the device. For cell studies, the chip was primed by sequentially rising with
190 proof ethanol (Sigma-Aldrich Corp.), 1X PBS buffer, and cell media (see cell preparation above). PC9 cells (400cells/μL) were mixed with a biocompatible surfactant (Pluronic® F-68; Life Technologies, 0.1 % v/v) and loaded into weirs under an oscillatory pressure profile (i.e. switching between pulses of -20 mbar for 8 seconds and 60 mbar for 2 seconds) from a reservoir at the outlet of the microfluidic chip using a pressure-controlled system (OB1 Pressure Controller; Elveflow). The oscillatory pressure profile was used to prevent cell adhesion or the formation of cell clusters on the back-side of weirs. After loading in the weirs, cells were transported to compartment sites using a three-step process. First, cells were withdrawn from weirs with negative pressure driven flow (i.e. -30 mbar) for 3 seconds then subsequently propelled towards the compartment region at various pressures for 10 seconds. Next, when the particle reached the corner, the acoustic generator was switched ON, exciting the attached piezoelectric transducer with an acoustic sweep from 1.35 to 1.42 MHz with a sweep rate of 1 Hz and at over one second at an excitation of 16 V_{pp}. Finally, after visually confirming that the particles in each field of view were captured at the corner, acoustics were turned off and cells were transported into compartment regions with positive pressure driven flow. We applied a positive pressure of ~30 mbar for relatively long durations of ~10 seconds, because this section had higher fluidic resistance and thus lower flow rates. Since there is a net positive pressure bias for each transfer cycle, cells that move into the compartments remain trapped there permanently. Meanwhile, it was possible to repeat this approach
for cells that were missed during a previous cycle and still trapped in the weirs. After repeating this process 4-5 times, we were able to transfer a large percentage of the cells that were trapped in the first step.

**Data Acquisition**

Switching efficiencies were determined by visually inspecting the particle trajectories when the acoustic field was turned on. Particle tracking data was extracted using a custom MATLAB program, which cropped a region of interest, stabilized the video, and extracted particle positions with circle tracking and thresholding. To quantify the frequency dependence of the trapping process, we used acoustic excitations at varying voltages and frequencies, and visually inspected the number of particles in the field of view (n=6) that were captured on the corners. An efficiency map was generated using a custom MATLAB script, with dark red circles indicating 100% capture and blue circles indicating 0% capture.

Additionally, to quantify the arraying efficiency across the chip acoustofluidic chips, we developed a custom Metamorph program (Molecular Devices, Inc.), which controlled the DMI-6000B microscope, XY automated stage (MS-2000; Applied Scientific Instrumentation) and camera to enable high-throughput imaging of each compartment in the array. Briefly, we first calculated the focal plane along the chip surface using fiducial alignment marks, and next bright field and fluorescent images were acquired in series along a prescribed XY path to map the entire array. The files were saved using a
custom naming format, and then manually inspected to quantify the occupancy in the trap and compartment regions of the acoustofluidic element. Heat maps were created by recording the number of cells in each compartment in a table using a custom MATLAB script to generate a color-coded grid indicating the occupancy of each compartment, with blue representing no cells and deep red representing three or more cells.

**Three Color Staining**

Cells eventually delaminate when exposed to high pressures (i.e. ≥ 25 mbar) for an extended period of time (Figure 60). Thus, it is paramount that the withdrawal (or injection) pressure is reduced once the new fluid phase fills the microfluidic cavity.

Figure 60: Cell delamination after 30 minutes of withdrawal at –25 mbar. Here, (a) shows the single cell at t=0, (b) shows the adhered cell after incubation overnight and (c) shows the delaminated cell after exposure to -25 mbar for 30 minutes. Scale bar is 50 µm.
References

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Biography

Korine Ohiri was born in Washington D.C. on July 29, 1991. She lived the first eight years of her life in Forestville, MD and was raised in Burtonsville, MD for the remainder of her formative years where she graduated from Paint Branch High School in 2009. That fall, Korine matriculated in the University of Maryland- Baltimore County (UMBC) where she was a Meyerhoff Scholar, MARC U-STAR Scholar, member of Tau Beta Pi, and mechanical engineering major. While she pursued her bachelor’s degree, Korine worked as an undergraduate researcher at Johns Hopkins University, Yale University, and Northwestern University over the summers. During the academic year, starting from her junior year, Korine worked on various projects in Prof. Liang Zhu’s Bioheat Transfer Laboratory. Korine also served on the student boards of the UMBC chapters of the American Society of Mechanical Engineers (ASME) and the National Society of Black Engineers (NSBE). She graduated in 2013 with a Bachelor of Science degree in Mechanical Engineering and honors.

Korine joined the research group of Prof. Gabriel López in the fall of 2013 where she focused on developing biofunctional microparticles with unique acoustic properties for applications in microfluidic bioseparations. In the spring of 2015 she transitioned to Prof. Benjamin Yellen’s group where she shifted focus to developing an acoustofluidic
chip for microfluidic single cell handling and analysis. During her time at Duke, she received the Dean’s Graduate Fellowship, National Science Foundation Graduate Research Fellowship, and the Research Triangle Materials Research Science and Engineering Center Fellowship. She won the Langmuir Graduate Student Award at the 90th ACS Colloid & Surface Science Symposium, mentored two undergraduate students, published her work in ACS Applied Materials & Interfaces, and is a co-author on four additional scientific publications. Korine also served on the Mechanical Engineering and Materials Science (MEMS) Committee, and was the Vice President of the Engineering Graduate Student Council (EGSC) at Duke. Korine’s lifelong goal is to innovate cutting edge technologies, continue her community service and outreach efforts, and encourage diversity in the sciences.