Microfluidic Device Technology for Cell and Droplet Sorting, Encapsulation, Storage, and Lysis

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Microfluidic Device Technology for Cell and Droplet Sorting, Encapsulation, Storage, and Lysis

A dissertation presented
by
Kiryakos S. Mutafopulos
to
The John A. Paulson School of Engineering and Applied Sciences
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Applied Physics
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Microfluidic Device Technology for Cell and Droplet Sorting, Encapsulation, Storage, and Lysis

Abstract

Microfluidics enables researchers and clinicians to manipulate fluids, particles, and cells at the micron scale, providing a vast array of potential methods for studying cell populations at the single-cell level. In this thesis, I present techniques and methods that improve the use of microfluidics for particle and fluid manipulation by incorporating surface acoustic waves and passive flow techniques for cell sorting, droplet generation, sample encapsulation, cell lysis, and droplet storage. I provide a general overview of surface acoustic waves (SAW) based microfluidic mechanisms for particle manipulation, and present two microfluidic devices using these mechanisms for rapid fluorescence activated cell sorting (FACS). I demonstrate a new technique for droplet generation using SAW and step-emulsification channel geometries. Using this technique, I present a variety of applications such as: the ability to selectively encapsulate samples into droplets upon fluorescent detection, thus eliminating or minimizing the production of empty droplets, or the need to sort droplets based on their contents; the ability to lyse and encapsulate cells, and the ability to inject small volumes of fluids into individual droplets. I also present a microfluidic device that generates and passively stores up to hundreds of water-in-oil droplets, preserving the order in which they are produced. By drawing an analogy between fluid flow and electrical resistance networks, I present a model to determine the geometry of devices that can store a controllable number of droplets. The device may assist microfluidic applications incorporating droplet incubation and long-term observation.
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Citations to Previously Published Work and Work Submitted for Review

Chapter 2 has been published as:


Chapter 3 has been submitted to and is under review by Lab on a Chip as:


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Chapter 5 presents a device that has been patented as:

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Dedication

For my grandfather, Kiryakos Mutasfopoulos. I want to thank you for being an outstanding role model and someone to look up to.
Chapter 1: Surface Acoustic Wave (SAW) Microfluidics
Introduction

Microfluidics deals with the flow of fluids and suspensions in devices (commonly referred to as chips), comprised of sub-millimeter sized channels and has emerged as an important platform in chemistry, biology and medicine.\(^1\)\(^-\)\(^3\) In biology and medicine for example, the ability to operate on this scale has enabled researchers to handle populations of cells at the single-cell level to study cell heterogeneity. With the aim to improve the functionality, versatility, and performance of these cell-handling microfluidic devices, researchers have integrated other technologies into this platform. One powerful and enabling integration is surface acoustic wave (SAW) technologies, which have traditionally been used in the telecommunication industry for signal processing and filtering.\(^4\) This novel combination has received significant attention in the microfluidics community for its ability to precisely and accurately manipulate fluids, and individual cells. These SAW-based microfluidic devices offer an assortment of beneficial and unique combination of features such as compact size, and easy integration with other on-chip components since SAW-based components are widespread in cellular phones.\(^4\) The acoustic power intensity and frequency of SAW are in a range of similar scale to cells and can be adjusted to be either safe or harmful to cells.\(^5\)\(^-\)\(^7\) High-frequency acoustic waves provides fast fluidic actuation and large forces that can be used to introduce chaotic advection to a microfluidic channel, thereby enabling rapid, effective manipulations of fluids and particles.\(^8\)\(^,\)\(^9\) SAWs manipulate particles and cells by means of the primary acoustic radiation force applied by the surrounding fluid: manipulating fluid by means of acoustic waves leaked into the fluid.\(^9\) This contact-free manipulation eliminates the potential for sample contamination, and enables precise control of particles by tuning the frequency of the SAW.
SAWs are generally produced by applying an appropriate electric field to a piezoelectric material. The piezoelectric material, in turn, generates a propagating mechanical stress. A typical SAW device uses at least one set of metallic interdigital transducers (IDTs) fabricated on the surface of a piezoelectric substrate. An IDT consists of a set of connected metallic electrodes, or ‘fingers’ on the nanometer to micrometer scale that generate a SAW when an alternating current electrical signal in the radiofrequency range (RF) is applied across the two sets of connected fingers. The waves propagate from each finger along the surface of the substrate and interfere constructively as a result of the IDT introducing an electric field on the substrate. This in turn generates a SAW displacement amplitude on the order of nanometers to micrometers.

In this chapter, I present the basic principles and mechanisms of SAW-based microfluidics with an emphasis on particle and cell manipulations. I report three different interdigital transducer (IDT) designs, and quantify their ability to manipulate particles and cells in a microchannel.
**General Overview of SAW-based Microfluidics**

*Interdigital Transducer (IDT) for Surface Acoustic Wave (SAW) Generation*

The IDT is patterned on the surface of a piezoelectric substrate such as quartz, lithium tantalate, or lithium niobate. It is responsible for generating a SAW when radio frequency (RF) signal is applied and the structure of the IDT determines the bandwidth and directivity of the generated SAW. An IDT consists of an array of metallic finger pairs whose geometry determines the characteristics of the resulting SAW. By changing the number (np), spacing, and aperture (overlapping length) of the fingers, one can adjust the size, position, and direction of the SAW. For a single-pair IDT, the pitch (p) defined as the spacing between opposing fingers can determine the center-frequency wavelength ($\lambda_0$) of the SAW and is generally calculated as shown in equation 1:

$$\lambda_0 = 2p \quad (EQ \ 1)$$

In general, the IDT is designed to have an array of single-pair fingers of width (a) and pitch (p) and setting the metallization ratio\textsuperscript{10} to one-half as shown in equation 2:

$$\frac{a}{p} = \frac{1}{2} \quad (EQ \ 2)$$

By setting the metallization ratio to one-half, the coupling problem between periodic fingers due to the propagation of the SAW is typically reduced and is proportional to the capacitance of the IDT.\textsuperscript{11} The aperture (overlapping length) of the IDT determines how the SAW is distributed and its size as it propagates across the fingers. A general overview of the IDT structure is shown in Fig 1 and an illustration of the IDT aperture is shown in Fig 2.
IDT structures can be designed in a variety of ways, with the most common structure being the straight single-pair IDT, where single opposing electrodes are repeated uniformly forming a comb-like structure, as shown in Fig. 1. This design is commonly used in the microfluidics community for the production of either standing surface acoustic waves (SSAW) or traveling surface acoustic waves (TSAW),\textsuperscript{12-20} however, many other designs exist. For example, a focused IDT (F-IDT) consists of pairs of annular electrodes that focus SAW energy to a spatially small focal point.\textsuperscript{17, 21} A chirped IDT has a gradient of the finger width directed along the SAW propagation direction, enabling SAW generation over a wide range of frequencies.\textsuperscript{22} Alternative to the chirped IDT design is the tapered or slanted finger IDT, which has a gradient of electrode finer width directed perpendicular to SAW propagation direction enabling it to generate narrow SAW beams of varying frequency along its finger length.\textsuperscript{23-25} Each of these designs have their own set of advantages and disadvantages and the choice of IDT design for use in microfluidics depends on the chip requirements.

We pattern our IDTs onto 128\textdegree{} Y-cut lithium niobate using standard photolithography methods as described in chapters 2 and 3, as shown in Fig 3. This crystal represents a rotated Y-cut, where the surface normal makes an angle of 128\textdegree{} with the Y-axis of the crystal. The surface wave propagates in the X-direction of the crystal. The material distinguishes itself with excellent electro-optical properties, a higher piezoelectric coupling to the wave, and gives lower bulk-wave generation than other materials.\textsuperscript{10, 26} However, lithium niobate is very sensitive to temperature changes, making the transducer fabrication more challenging.
SAW Generation in Microfluidic Channels

When a SAW contacts a liquid, the liquid’s increased viscosity, relative to the substrate, causes part of the SAW to refract into the liquid as a longitudinal wave. Because of this acoustic refraction, the mode of SAW changes to a form called ‘leaky SAW’,\(^9\) as illustrated in Fig 4. The SAW propagates from left to right along the piezoelectric substrate surface within the depth of a single wavelength.\(^{27}\) The refracted wave moves along the direction given by the refraction angle known as the Rayleigh angle:

\[
\theta_R = \arcsin\left(\frac{C_1}{C_s}\right) \quad (EQ \ 3)
\]

where \(C_1\) and \(C_s\) denote the acoustic wave velocities of the fluid and piezoelectric substrate, respectively. For SAW propagating on 128° Y-cut lithium niobate at room temperature, the SAW velocity is approximately 3980 m/s,\(^{10}\) and the speed of sound in water is 1490 m/s result in a Rayleigh angle of approximately 22°. The refracted longitudinal waves generate a force in their propagation direction and induce flow within the confined fluid. The boundaries of the fluid reflect the actuated liquid and lead to internal streaming, commonly referred to as SAW-induced acoustic streaming.\(^9, 28-31\) The streaming can be visualized by introducing small particles or dyes into the effected liquid.\(^{28, 32}\) The SAW-induced streaming pattern varies dramatically with the shape of the confined liquid, as well as the incident position, operating frequency of the IDT, and angle of the SAW.\(^{33}\)
Figure 1: General IDT Structure Overview

Illustration of a single-pair straight IDT. The width (a), pitch (p), and center-frequency wavelength ($\lambda_0$) of the electrodes are highlighted with a dotted line. The aperture (overlap width) of the electrodes is also shown. We adjust each of these features to control the wavelength and size of the SAW.
Figure 2: IDT aperture (overlapping length) overview

The aperture or overlap length influences the amplitude distribution and effects the amount of diffraction created and the size of the beam that enters the channel. As the overlength decreases the beam width decreases, and diffraction at the edges of the beam increases.\textsuperscript{28}
**Figure 3**: Single-pair straight IDT Patterned onto 128° Y-cut Lithium niobate

The width \( (a) \) of the fingers and pitch \( (p) \) are 12.5 \( \mu \text{m} \) and 25 \( \mu \text{m} \), respectively. The center-frequency wavelength is approximately 50 \( \mu \text{m} \).
SAW is excited by means of an IDT on the surface of a piezoelectric substrate. Underneath a liquid, the SAW is converted to a leaky SAW, radiating pressure waves at the Rayleigh angle ($\theta_R$) into the fluid. The result is an emission of a sharp beam of longitudinal sound waves into the liquid.
Acoustic Stream Flow (ASF) and Acoustic Radiation Force (ARF)

Many SAW-based microfluidic devices use only a single IDT to generate a TSAW to efficiently actuate (mix, pump, jet) fluid in a microfluidic channel and/or manipulate particles and cells within a microchannel.\textsuperscript{18, 19} The actuation of fluids via TSAWs is referred to as acoustic streaming flow (ASF) and is generated by the dissipation of acoustic waves in the fluid, whereas the manipulation of particles is a result of the acoustic radiation force (ARF) and depends on the TSAW’s frequency, particle diameter, and relative densities of the fluid and particles.\textsuperscript{18} A simplified a model has been proposed to determine whether or not a particle or cell is subject to ARF.\textsuperscript{18} The model draws on work dating back to 1934 regarding the analysis of incompressible particles in acoustic fields\textsuperscript{34} and states a $k$ factor defined as:

$$k = \pi d \frac{f}{c_f} \quad (\text{EQ 4})$$

where $d$ is the particle diameter, $f$ is the TSAW’s frequency, and $c_f$ is the speed of sound in the fluid.\textsuperscript{18} Fig 5 shows the relationship between SAW frequency, particle diameter and the $k$ factor. For $k > 1$, the ARF on the particle or cell dominates the drag force induced to the particles via ASF.\textsuperscript{18} The ARF can derive the suspended particle or cell of adequate diameters in the direction of the acoustic wave propagation. When $k < 1$, the particles or cells are not affected by the ARF and the effect of ASF dominates. Smaller particles or cells will typically move with the ASF vortices as the ARF is unable to drive them along the acoustic wave. The ASF typically induces a drag force on these small particles:

$$F_D = 3\pi \mu d \nu \quad (\text{EQ 5})$$

where $d$ is the diameter of the particle, $\mu$ is the dynamic viscosity of the fluid, and $\nu$ is the particle velocity. For applications that require cells or particles of a given diameter to be
deflected across a channel, the frequency of the IDT is set to ensure that $k > 1$. The force acting on the particle or cell obeys the relationship:

$$< F_{ARF} > = Y_T \pi d^2 \frac{<E>}{4}$$ (EQ 6)

where $d$ is the diameter of the particle or cell, $<E>$ is the time-averaged acoustic energy density, and $Y_T$ is the acoustic radiation factor that is a function of the particle’s mechanical properties and size.\textsuperscript{35-39}
**Figure 5:** $k$ Factor modeling for four different IDT frequencies on 128° Y-cut lithium niobate

The $k$ factor model vs. particle diameter for four different frequencies is shown. As the frequency of the SAW increases smaller particles are dominated by acoustic radiation forces (ARF) rather than acoustic stream flow (ASF).
Comparison of IDT Designs for Microfluidic Devices

To characterize and quantify SAW behavior in a microfluidic channel, we designed three different single-pair IDT structures: straight, apodized, and tapered. The straight IDT design comprises an array of opposing fingers that are uniform in length, width, and spacing, as shown previously in Fig 1. The apodized IDT design comprises an array of opposing fingers that are uniform in width and spacing, but the length of one side of the fingers varies, as shown in Fig 6. This results in an IDT with a varying overlap length and provides a suitable comparison to the straight IDT design when determining how to control the SAW distribution in the channel. The tapered IDT design comprises fingers whose pitch varies laterally across the aperture. The pitch decreases linearly with position between a larger pitch on one side and a smaller pitch on the opposite side; therefore, the change in pitch enables the frequency to vary with the position along the IDT (Fig 7, Fig 8, and Fig 9). Due to the tapering of the electrodes the aperture does not equal the overlength lap of the finger pairs and is instead calculated by:

\[ W_{\text{Eff}} = \frac{f_0}{N_p(f_H-f_L)} W \] (EQ 7)

where \( f_0 \) is the center-frequency of the IDT, \( N_p \) refers to the number of finger pairs, \( f_H \) and \( f_L \) are the highest and lowest operating frequencies of the IDT, and \( W \) is the total aperture (overlap length) of the fingers. For both the straight and apodized IDTs, we fabricate and test two: 50 \( \mu \)m and 23 \( \mu \)m wavelength, and for the tapered IDT, only a 23 \( \mu \)m wavelength. For all IDT designs we test particle deflection and acoustic streaming in microfluidic channel with a cross-section that is 200 \( \mu \)m wide and 30 \( \mu \)m deep channel.
**Figure 6:** Apodized IDT Patterned onto 128° Y-cut Lithium Niobate

The width (a) of the fingers and pitch (p) are 12.5 µm and 25 µm, respectively. The center-frequency wavelength is approximately 50 µm.
Figure 7: Schematic of a Tapered IDT

Due to the tapering of the fingers, the IDT can effectively generate SAW across a range of frequencies. $W$ is the overlap width (aperture) and both bus bars have different widths ($a$), pitches ($p$), and wavelengths ($\lambda$), with $(a_1, p_1, \lambda_1) > (a_2, p_2, \lambda_2)$. The period varies linearly along the IDT ($y$-axis). The slope of the tapered fingers determines the aperture by limiting the resonant frequency.
**Figure 8:** Tapered IDT Patterned onto 128° Y-cut Lithium Niobate

This particular IDT shown, has a frequency range of 160-180 MHz. The total overlap length is 0.5 mm.
Figure 9: Tapered IDT Actuated at Different Frequencies

Overlay images of 10 µm beads flowing through a straight microfluidic channel with a cross section of 200 µm by 30 µm and a single inlet and outlet adjacent to a tapered IDT actuated at different frequencies is shown. As the frequency increases the position of the SAW moves across the total aperture (W) from the top to the bottom of the IDT.
Particle and Cell Deflection

For all three IDT designs we measure the amount of deflection or displacement of K562 cells (~15 µm), 4 µm and 10 µm polystyrene beads experience upon acoustic wave interaction at different RF power levels sent to the IDT. We plasma-bond the IDTs to a PDMS micro-molded channel containing a sample inlet, two sheath flow inlets to adjust the position of particles and cells in the channel to ensure they flow past the region where the SAW is generated, and one outlet channel, as shown in Fig. 10. For all deflection measurement experiments, we flow the sample inlet at 1.5 mL/hour, and the left and right sheath fluid at 4 mL/hour and 9 mL/hour, respectively. The IDT is positioned in an air pocket separated from the fluidic channel by a 50 µm PDMS wall. All cells and particles are fluorescently labelled and excited with a 488 nm laser. Upon excitation, the cells or particles are detected by a photomultiplier tube that measures the fluorescence from the particles or cells, generating a voltage proportional to the intensity of the incident light. This voltage is digitized by a data acquisition card and analyzed in real time using the card’s field programmable gate array to detect and analyze peaks in the fluorescence signal. When peaks corresponding to desired cells are detected, an RF signal is applied to the IDT for 25 µs at set power level. We apply four different power levels (34 dBm, 36 dBm, 38 dBm, and 40 dBm) to quantify relationship between power level and deflection. We record high-speed videos of particles or cells being deflected by the IDTs and track the position of each particle or cell over time using particle tracking software, as shown in Fig 11. We then plot the average particle trajectory for each power level in the x- and y-component. Fig. 12 shows an example a plot from the tracking data for a 50 µm wavelength apodized IDT.
The chip comprises a sample inlet, and two sheath inlets to flow focus particles and cells in a straight line as they flow past the region where the IDT is positioned (blue box). A laser is positioned upstream of the IDT position to detect cells and particles. Upon detection the IDT is actuated and we record high-speed videos in the blue box region for deflection measurements. The sample inlet is followed by a spiral microchannel to inertially flow focus cells and particles into a nearly ordered line.

**Figure 10:** Particle and Cell Deflection Chip Schematic
Figure 11: 50 µm wavelength apodized IDT actuated at 36 dBm for 25 µs

Overlay images from a high-speed camera recording of a fluorescent labeled 10 µm bead deflected by an apodized IDT. Beads flow through a chip (as described in Fig 9.), and upon detection are deflected by a surface acoustic wave. Videos generated by the high-speed camera are then uploaded to particle tracking software (Tracker) and the particles position over time is collected to measure the particle velocity and deflection by an IDT.
Figure 12: Particle Deflection Plot from Tracker for 10 µm Polystyrene Bead Deflected by an Apodized IDT

2-D particle position of a 10 µm bead deflected by an apodized IDT at four different power levels. As the power level increases the particle is deflected further away. The error bars correspond to the standard deviation of the x and y position of the particle.
For all IDTs and power levels, we find that the 10 µm beads deflect further away than the K562 cells, despite the cells being 5 µm larger on average. We attribute this behavior to the variation of ARF according to the material properties of the sample. Since cells and polystyrene beads have entirely different mechanical properties that substantially impact their acoustic radiation factor (Y_T) and for cells it is estimated to be an order of magnitude smaller than polystyrene beads,\textsuperscript{39, 41} the force experienced by the cells is smaller, as shown in Fig 13. Furthermore, we observed poor deflection of 4 µm beads for both 23 µm and 50 µm wavelength (~168 MHz and 78 MHz, respectively) IDTs. For the 50 µm wavelength IDTs, we attribute this to the k factor being less than one for this condition. The 23 µm wavelength condition did deflect the 4 µm beads further but still significantly lower than the 10 µm beads and K562 cells, as shown in Fig. 14. We assume this to be a result the k factor being much larger for the 10 µm beads and K562 cells compared to the 4 µm beads. For the straight, apodized, and tapered IDT with a wavelength of 23 µm, we apply a frequency of approximately 165 MHz to the IDT. We observed that this wavelength deflected K562 cells further than the 50 µm wavelength condition. A summary of the deflection for all conditions is shown in Table 1.
**Figure 13**: Overlay images from high-speed camera recordings. Images shows K562 cells and 10um beads deflected by an apodized IDT at four different power level settings. Sorting pulse for all applied signal generator power levels is 25us.
**Figure 14:** 4 µm Bead Deflected by a Tapered IDT

Fluorescent labeled 4 µm bead deflected by a tapered IDT. The power level and pulse length of the IDT is 40 dBm and 25 µs, respectively.
A table summarizing the average maximum deflection a particle or cell experienced for each condition. Ten measurements are made and averaged for each condition.

### Table 1: Summary of Deflection Results

<table>
<thead>
<tr>
<th>IDT Design</th>
<th>Sample</th>
<th>Wavelength (µm)</th>
<th>Max Deflection at 40 dBm (µm)</th>
<th>Max Deflection at 38 dBm (µm)</th>
<th>Max Deflection at 36 dBm (µm)</th>
<th>Max Deflection at 34 dBm (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straight</td>
<td>10 µm Bead</td>
<td>50</td>
<td>98±5</td>
<td>98±12</td>
<td>81±14</td>
<td>50±4</td>
</tr>
<tr>
<td>Straight</td>
<td>4 µm Bead</td>
<td>50</td>
<td>21±9</td>
<td>17±21</td>
<td>7±11</td>
<td>0</td>
</tr>
<tr>
<td>Straight</td>
<td>K562 Cell</td>
<td>50</td>
<td>78±3</td>
<td>47±11</td>
<td>36±6</td>
<td>15±7</td>
</tr>
<tr>
<td>Straight</td>
<td>10 µm Bead</td>
<td>23</td>
<td>97±5</td>
<td>71±8</td>
<td>54±11</td>
<td>40±3</td>
</tr>
<tr>
<td>Straight</td>
<td>4 µm Bead</td>
<td>23</td>
<td>45±15</td>
<td>32±15</td>
<td>18±18</td>
<td>5±8</td>
</tr>
<tr>
<td>Straight</td>
<td>K562 Cell</td>
<td>23</td>
<td>70±12</td>
<td>50±9</td>
<td>46±3</td>
<td>21±16</td>
</tr>
<tr>
<td>Apodized</td>
<td>10 µm Bead</td>
<td>50</td>
<td>85±4</td>
<td>76±16</td>
<td>65±7</td>
<td>32±5</td>
</tr>
<tr>
<td>Apodized</td>
<td>4 µm Bead</td>
<td>50</td>
<td>18±7</td>
<td>8±24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Apodized</td>
<td>K562 Cell</td>
<td>50</td>
<td>30±13</td>
<td>22±12</td>
<td>12±9</td>
<td>0</td>
</tr>
<tr>
<td>Apodized</td>
<td>10 µm Bead</td>
<td>23</td>
<td>108±5</td>
<td>96±7</td>
<td>79±5</td>
<td>70±8</td>
</tr>
<tr>
<td>Apodized</td>
<td>4 µm Bead</td>
<td>23</td>
<td>39±14</td>
<td>27±8</td>
<td>22±6</td>
<td>8±6</td>
</tr>
<tr>
<td>Apodized</td>
<td>K562 Cell</td>
<td>23</td>
<td>79±6</td>
<td>66±7</td>
<td>38±16</td>
<td>22±3</td>
</tr>
<tr>
<td>Tapered</td>
<td>10 µm Bead</td>
<td>23</td>
<td>101±7</td>
<td>94±6</td>
<td>74±13</td>
<td>55±11</td>
</tr>
<tr>
<td>Tapered</td>
<td>4 µm Bead</td>
<td>23</td>
<td>34±22</td>
<td>26±9</td>
<td>20±5</td>
<td>0</td>
</tr>
<tr>
<td>Tapered</td>
<td>K562 Cell</td>
<td>23</td>
<td>87±14</td>
<td>70±14</td>
<td>25±7</td>
<td>12±6</td>
</tr>
</tbody>
</table>
Summary and Conclusion

The results provide insight for microfluidic sorting applications that require rapid deflection of particles or cells into a separate channel. By recording high-speed videos of particles or cells being deflected by SAW, the measured deflection for a given IDT can be obtained and used to design channel geometries and IDTs that enable deflected particles to be sent into a separate channel upon fluorescent detection solely using acoustic radiation forces (ARF), rather than acoustic streaming flow (ASF). The deflection data demonstrates the ability to deflect cells and particles travelling at an average velocity of approximately 650 mm per second as far as 80 µm or more with a SAW pulse of 25 µs. The advantage of selectively deflecting particles using ARF will permit sorting pulses at least one order of magnitude shorter than using ASF, since ASF based microfluidic cell sorters typically operate on the order of a few hundred microseconds.42
Chapter 2: Enhanced Surface Acoustic Wave Cell Sorting by 3D Microfluidic
Chip Design
**Introduction**

Fluorescence-activated cell sorting (FACS) is a method for extracting desired cells based on their biological characteristics. These characteristics are distinguished using fluorescence-based assays. A sheath flow focuses cells to a narrow stream, accelerating cells to high velocity and separating them along the direction of flow. This stream must be aligned with the optics of the instrument to make accurate measurements. To sort cells, the fluid stream is broken into droplets that contain single cells. Droplets containing target cells are selectively charged and subsequently deflected in an electric field. The entire process occurs rapidly enough for FACS instruments to operate at rates as high as tens of kHz, while still retaining high purity. However, FACS also suffers from several limitations of the technique: the large volumes of sheath fluid required to reach high velocities make it difficult to sort small numbers of cells; moreover, the droplet aerosol produced during sorting poses a potential biohazard when using infectious cells. One way to overcome these limitations is to use microfluidic devices composed of micro-scale flow channels that can handle minute volumes of fluid in a closed device without producing drops. The small volume of each microfluidic device reduces the dead volume of the system and minimizes the loss of sample. Alignment of microfluidic devices is simplified because the flow channels are embedded reproducibly within each device; moreover, cross contamination between different samples can be eliminated by replacing the device, rather than cleaning it. Furthermore, microfluidic devices can sort cells without producing aerosols, alleviating the potential risks associated with sorting hazardous samples. Microfluidic cell sorters implement a variety of actuation mechanisms, such as piezoelectric actuation, dielectrophoresis of droplets, optical manipulation
such as pulsed laser induced cavitation,\textsuperscript{48, 53} and surface acoustic wave (SAW) deflection.\textsuperscript{42, 54, 55} In spite of the benefits of microfluidic devices for cell sorting, they are not widely used because microfluidic devices cannot match the speed of FACS instruments; to become widely used, microfluidic devices must be able to sort at higher rates.\textsuperscript{5} Surface acoustic waves have the potential to reach high rates, offering a robust and contactless microfluidic method for sorting cells by sound using high speed electronics.\textsuperscript{8} Devices exciting standing acoustic waves have been used to dynamically pattern the acoustic field within the microchannel to achieve fine control over each cell’s spatial position,\textsuperscript{13, 23, 54-56} but they have yet to reach rates comparable to FACS, when used to perform the sorting. In SAW devices using standing waves the acoustic wavelength determines the pressure node spacing that is used to separate the cells and that is therefore fixed for a desired node spacing. Traveling SAW devices can be operated at different frequencies without changing the device design, enabling the use of increasing frequencies to increase the acoustic force exploited for sorting without changing the device design,\textsuperscript{5} and therefore are promising candidates for high speed microfluidic sorting even though yet they do not reach FACS rates. However, if these devices could be improved by developing a more efficient way to exploit the sound pulse for deflection, traveling SAW devices would be more widely adopted for cell sorting applications.

Here, we demonstrate a microfluidic cell sorter based on traveling SAW actuation that screens and sorts cells at rates approaching those of commercial FACS instruments. The device contains multi-layer features that enhance the capabilities of a SAW sorter by harnessing the component of the acoustic wave oriented normal to the plane of the substrate. The multi-layer features consist of a
three-dimensional flow-focusing nozzle and a slanted ceiling groove, which guides cells to the retention outlet following SAW deflection. We find operating conditions which yield efficient sorting in this device, and sort fluorescently-labelled cells from mixed samples. The device achieves sorting at a rate of 9,000 events/s with 60% purity and yields of 92% purity, while operating at 1,000 events/s; this level of performance approaches that of a FACS instrument operating in its high-purity mode.

Results and Discussion

A SAW that impinge on the interface of a fluid in a microfluidic device, refracts and establishes longitudinal acoustic waves in the fluid, as shown in Fig 15.\(^9\) The angle of refraction for SAWs is known as the Rayleigh angle, \(\theta_R\), and depends on the speed of sound in the liquid, \(v_l\), and the speed of the SAW on the substrate, \(v_s\), according to Snell’s law, \(\sin \theta_R = v_l/v_s\).\(^9,57\) The refracted acoustic wave exerts forces aligned with the direction of wave propagation on cells flowing through the microfluidic device.\(^3,5,8,16,57\) Because the SAW travels along the lithium niobate surface several times faster than the acoustic wave in the liquid,\(^10\) the refracted wave is largely aligned with the substrate’s surface normal.\(^58\) The device is oriented such that the refracted wave pushes cells mainly upward in the vertical, or \(+z\), direction. We present a device that sorts cells based on their vertical deflection actuated by the refracted acoustic wave. We could not observe the formation of standing acoustic waves caused by the acoustic reflection at channel walls as has been reported elsewhere,\(^15,59,60\) probably of the small impedance mismatch of the fluid and PDMS material and the channel dimensions. We also did not observe any
near field effect such as streaming roll as has been reported in Devendran et al.,\textsuperscript{61} or acoustic interference patterns\textsuperscript{62} because of the short pulse length and the comparatively large size of the cells. The proposed design enhances sorting performance, because it harnesses a larger proportion of the power carried by the SAW. We achieve this by a 3-dimensional design of the microfluidic channel.

In our design, the microfluidic device is bonded directly onto a lithium niobate substrate adjacent to an interdigital transducer (IDT). When a RF signal is applied, the IDT generates travelling SAWs. Acoustic waves are excited in the channel adjacent to the IDT, in what is referred to as the sorting region of the device. Cells enter the sorting region of the device through the vertical flow-focusing nozzle. A slanted groove extends above the sorting region and enhances the deflection of cells by acoustic waves. Immediately after the sorting region, the device’s main channel bifurcates: each cell either flows directly into the default outlet or acoustic waves actuate the cell into the sorting outlet. The positions of the distinct features of the sorting device with respect to the IDT are shown in Fig 16.
Figure 15: A Multi-Layer Design for Cell Sorting with Surface Acoustic Waves

A cross-section of the device is shown to illustrate the refraction of surface acoustic waves. The interdigital transducer (gold) generates a surface acoustic wave (purple). The surface acoustic wave travels along the substrate surface (white rectangle) in the +y direction. The surface acoustic wave refracts, upon contact with the fluid (light blue) within the microfluidic device, forming a longitudinal acoustic wave (red arrows) in the liquid. Refraction occurs at a small Rayleigh angle, $\theta_R$. The refracted acoustic wave exerts an acoustic radiation pressure on the cells (white circle) as well as driving fluid flow by acoustic streaming (blue arrow).
Figure 16: Slanted Groove Device Overview

The design developed for cell sorting using surface acoustic waves is illustrated. The flow channel of the microfluidic device (blue) is positioned next to the interdigital transducer (IDT). The flow channel has a cell inlet and two sheath inlets through which the sample and sheath flows for flow focusing are injected respectively. The cell phase flow and sheath flows meet at the vertical flow-focusing nozzle; cells flow through the vertical flow-focusing nozzle into the sorting region of the device (red rectangle). Cells are probed by the optical system and a sorting pulse is applied, when a target cell is detected. Desired cells are sorted using acoustic waves and are deflected to leave the sorting region through the sorting outlet, while the rest of the cells pass through the sorting region unperturbed via the default outlet.
The design uses multi-layer features to create flows with vertical components. The vertical flow-focusing nozzle is a multi-layer feature formed at the intersection of the cell phase inlet with the channels containing the sheath flow. The cell inlet channel has a vertical constriction just prior to where it converges with the sheath channels, so the sheath flows focus the cell sample phase laterally and downward into a narrow thread at the bottom of the channel. This ensures that all cells flow along the bottom of the channel and are confined into a small region initially. If cells manage to reach the top of the channel by acoustic deflection, they interact with a different multi-layer feature, the slanted ceiling groove. The slanted groove channels fluid along the groove, setting up a flow that carries cells laterally across the sorting region of the device. The magnitude of the lateral flow decreases with distance from the groove, and it is negligible at the bottom of the channel. The flows created by the multi-layer features of the device are illustrated in Fig 17. Cells can thus be sorted based on their vertical position, because the flow in the groove directs cells at different heights into different outlets.
Figure 17: Particle Interaction with Slanted Groove

(a) After the flow focusing nozzle, sheath flows (blue arrows) confine the flow from the cell inlet (green dashed line) into a narrow thread at the bottom of the channel. At the top of the channel, the flow is pulled across the channel by the slanted groove (red arrows). The fluid within the groove flows along the groove’s long axis, but the flow of liquid at the bottom of the channel is largely unperturbed. (b) Cells lacking the desired characteristics (white circles) are not sorted and exit the device through the waste outlet without interacting with the flow within the groove. (c) If a target cell (green circles) is detected, surface acoustic waves are applied (purple arrows). They refract into the device and deflect the cell into the groove, where it is carried by the flow within the groove across the channel and out of the device through the retention outlet.
The vertical position of cells can be set by triggered acoustic wave actuation. In the absence of acoustic waves, cells transit directly through the sorting region of the device and leave through the default outlet without interacting with the slanted groove, as shown in Fig. 17b. However, an acoustic wave pulse can deflect target cells, using the refracted wave to selectively push cells vertically to the top of the sorting channel. At the top of the channel, the flow within the slanted groove guides these cells laterally across the sorting channel and into the sorting outlet, as shown in Fig. 17c. The experimentally observed cell tracks captured during device operation verify that cells follow these expected trajectories with and without acoustic wave actuation, as illustrated in Fig. 18a and 18b respectively. Although cells are still deflected without a slanted groove, the lateral displacement is much smaller as compared to deflection with the groove, as depicted in the cell track shown in 17a. Thus, the slanted groove only interacts with cells deflected by the refracted wave converting their vertical motion into lateral motion that can be used to segregate cells.
**Figure 18:** The Slanted Groove Enhances Cell Deflection Using Surface Acoustic Waves.

(a) When no pulse is applied, the cell follows straight along the same trajectory as the bulk of the cell phase fluid. The cell passes through the sorting region and underneath the slanted groove without deflection and exits the device through the default outlet (waste outlet). (b) When a cell is detected, a radio frequency signal of 38.26 dBm is applied for 100 μs to generate a surface acoustic wave pulse. In the device with a slanted groove, the refracted acoustic wave deflects the cell into the slanted groove, where it is carried across the sorting region of the channel by the flow of sheath fluid within the groove. The sorted cell moves laterally more than 150 μm and exits the device through the sorting outlet. (c) For devices without a slanted groove, the sorted cell is still deflected by the acoustic wave, but the cell is only displaced about 50 μm laterally under the same acoustic conditions. The scale bars correspond to 50 μm.
To quantify the sorting success and to optimize devices with slanted grooves, we measure the sorting performance using the tracks of moving cells. When a pulse of acoustic waves is applied to a cell, we use a high-speed camera to record the corresponding cell track; we combine the results from several cell tracks to determine a sorting success rate. If we increase the power carried by the acoustic wave, we can increase the sorting success rate. For every condition we test, we measure how much power is required to exceed a 90% success rate and define this as the threshold power. Moreover, if we operate in a regime where the power required is below the threshold power, we expect better sorting performance. We observe how cell velocity and acoustic wave pulse length affect the sorting performance to determine the screening rate these devices can achieve. We also measure how well different types of cells can be sorted. In addition, we want to understand how the geometry of the groove impacts sorting performance. For these devices, the parameters are coupled, so we vary one parameter at a time to understand the overall behavior of the device. The threshold power was tested against flow rate, pulse length, groove width, and groove height as shown in Fig. 19 to determine the ideal threshold power. We aim to find settings that can yield sorting at high event rates by choosing a high cell velocity and a low pulse length; however, we keep the sorting success high using groove dimensions that enable sorting at low power levels and exceeding the threshold power.
**Dependence on flow velocity**

The velocity at which cells transit through the sorting region of the device limits the screening rate of the device because it effects the exposure time of the cell to the acoustics. Moreover, if a cell is still in the sorting region of the device when the next cell enters the region of deflection, the two cells cannot be sorted independently. To prevent this, cells must transit quickly through the sorting rates, there appears to be a direct correlation between threshold power and applied flow rate, as the cell deflection becomes limited by its exposure to the acoustic wave pulse. The relationship between the threshold power of the SAW pulse and the overall device flow rate is depicted in Figure 19a. The results show that cells can be deflected consistently even at high flow rates of 60ml/h.

**Dependence on pulse length**

The minimum pulse length with which cells can be deflected is particularly important, because the shorter the pulse length is, the higher the event rate can be. We determine the threshold length of the SAW pulse for a range of radio frequency (RF) power levels. As the RF power increases, pulses with shorter durations provide enough energy to deflect cells. Cells can be efficiently actuated provided the SAW pulses are at least 20 µs long, as shown in Figure 19b. Because the device can successfully deflect cells with short bursts of acoustic waves, the length of the pulse does not limit the instrument’s performance at high event rates.
Different Cell Types

Our sorting device also needs to be able to sort a variety of cell types. We use the same range of power levels and pulse lengths to test whether the device can sort adherent and non-adherent cell types. For adherent cell type, we chose MDCK cells which are approximately 8 µm in diameter. For non-adherent cell type, we chose K562 cells which are approximately 15 µm in diameter. Both classes of cells can be reproducibly deflected into the groove with similar SAW pulse parameters, also shown in Figure 19b. However, the threshold power slightly differs between the cell types, due to small differences in the average size or acoustic contrast of the cells. Nevertheless, the slanted groove device can actuate both adherent and non-adherent cells with the short pulse lengths necessary to attain high screening rates.

Dependence on Groove Dimensions

The shape and orientation of the groove may also be tuned to improve sorting performance. We examine the impact of groove geometry on cell actuation in our design by varying the groove width, height, and angle independently. As the groove is widened, less power is necessary to cause the cell to interact with the flow within the groove, as demonstrated in Figure 19c. While it is possible to sort cells without any groove or with a very shallow groove, the best sorting results are obtained with grooves fabricated at a height of 25 µm, as shown in Figure 19d. This corresponds to the deepest groove that gives reliable results. Different groove angles show no significant effect on the threshold power required for sorting. While there may be slight variations due to changes in the effective aperture of the groove or in the flow speed along the groove, these effects are within the range of measurement error, and appear to have very little effect on the threshold power. Our results demonstrate
that both the depth and the width of the groove provide geometrical tuning parameters, which can influence the interaction of cells with the groove when SAWs are applied.

Operating Limitation

Because the threshold power for each parameter is within the operating range of the device, we should be able to achieve sorting at high event rates; however, there are additional device limits to consider. In terms of the acoustic wave power, the SAW cannot push cells high enough to interact with the groove for power levels below 3 W. Increasing the power increases the success rate of sorting, but only until about 10 W, when we start to see IDT damage in the form of chip cracking. To increase the rate, we would also like to increase the total flow rate, but, in our device, for flow rates exceeding 60 ml/h, the cells are not effectively confined to a narrow thread, which prevents us from testing SAW actuation with higher cell velocities. There are also limits on the groove geometry. When the height and width of the groove are increased to 50 µm or 160 µm respectively, cells begin to enter the groove without any applied SAW, which is detrimental to the purity of the recovered cells. Having these limitations in mind the optimal condition of sorting and prevention of false positive in the sorting channel is a groove width in the range of 120 µm – 160 µm and a groove height of 25 µm – 50 µm. In all subsequent experiment we therefore choose a width of 120 µm and height of 25 µm to stay away from the boundaries where false positive occur. Despite these limitations, there are a wide range of conditions for which the slanted groove device provides stable and reliable operation.
Figure 19: Cell Sorting Performance of Groove-Enhanced Devices

The symbols on each plot are centered on the mean of the three independent threshold values, while the error bars depict the full range of threshold values. For points without visible error bars, the marker size exceeds the extent of the error bars. We used a groove width of 120 µm, a height of 25 µm, flow rate of 45.5 ml/h and a pulse length of 50 µs. (a) As the flow rate is varied, the threshold power required for sorting increases, except at the lower range of flow rates, where it appears there is a minimum amount of power necessary for sorting. (b) As the applied radio frequency power is increased, the length of the pulse necessary to deflect a given cell type decreases. The device actuates both, adherent Madin-Darby canine kidney cells (MDCK; open symbols, ○) and non-adherent chronic myelogenous leukemia cells (K-562; filled symbols, ●) with performance levels sufficient to achieve high speed cell sorting. (c) The threshold power decreases linearly as the groove is widened. (d) The threshold power changes non-monotonically as the groove height is increased, but sorting with the lowest required power for deepest tested grooves.
**Sorter Performance**

The extent to which we can achieve reliable operation with our sorting device can be measured by applying the sorting conditions we determined here to cell samples. For a given cell phase flow rate, a high cell density is required to reach a high event rate. However, this increases the chance of coincidence events. To measure the sorter’s performance at different cell densities, we prepare a reference sample of K-562 cells in which the cell density and the fraction of fluorescent cells are known. The sorter extracts the fluorescent cells. The purified sample is collected and the recovered cells are imaged using a confocal microscope to obtain an independent measurement of cell purity. To elucidate how the purity of the sorted fraction depends on event rate, we increase the density of cells in the mixed sample, while keeping the cell phase flow rate constant. We also operate the device with two different sheath flow rates and two different groove widths, to measure how these parameters affect sorter performance. These experiments provide a realistic picture of how devices with slanted grooves will perform.

**Purity**

The sorter achieves high purity at low event rates, but the purity decreases, as the concentration of cells increases with a trend that appears linear, as shown in Figure 20a. The data fits to a line, which intersects the purity axis at 93% and whose slope represents a loss in purity of 4.3% each time the event rate increases by 1,000 events/s. This y-intercept represents the theoretical maximum purity for the set of devices we tested. Although this fit describes the average performance of these sorting devices, we examine the distributions of the residuals for each parameter in more detail, to see if the operating flow rate or the slanted groove width influence
device performance. For different sheath flow rates, there is no clear difference between the purity of the recovered samples. However, when we test different groove widths, we observe that the purity of samples isolated using devices with a 40 µm groove is higher than for devices with an 80 µm groove. This difference was statistically significant with a probability of only 0.28% indicating that these sets of residuals are drawn from the same distribution. Box plots showing the distributions of residuals grouped by applied flow rate and groove width are plotted in Figure 20b and c respectively. Because the width of the slanted groove has a clear effect on device performance, we fit the data from different groove widths independently. The purity of samples recovered using the device with the 40 µm groove decreases 4.0% per 1,000 events/s with a theoretical maximum at 95.7%, while the purity of samples recovered from the device with the 80 µm groove decreases 4.5% per 1,000 events/s and the fit intersects the purity axis at 91.1%, as shown in Figure 20d. These results indicate that decreasing the width of the slanted groove in our sorting design provides a means of improving the purity of the sorted sample, for the whole range of event rates.

The observation that the device with a narrow groove yields higher purity suggests that the groove plays another role in the sorting process. Because cells that enter the groove are carried across the sorting channel to the sorting outlet, it must be more difficult for non-target cells to enter the narrow groove. We propose that the groove acts as a spatial filter; cells can only enter the groove, if they are aligned with the groove when the acoustic wave is applied. This effect offers a unique advantage compared to previous SAW sorting designs, in which the sorting purity can only be increased by changing the design of the SAW transducer or the operating flow rates.
With the 40 µm groove, our design can achieve on average 92% purity at 1,000 events/s; moreover, the device succeeds to enrich cells at event rates of nearly 10,000 events/s.

Although the purity appears low, our characterization experiments show that the slanted groove is capable to operate at high rates. In conventional FACS instruments, high levels of purity require detection and elimination of coincidence events. Our instrument could be improved by incorporating the hardware and software designed for FACS instruments. In addition, the nozzle used for vertical flow focusing is a relatively simple design. While it serves to illustrate the principle of operation of the device, it could be further optimized to increase the spacing between cells and to minimize the dispersion of cell velocities. Moreover, after sorting with the slanted groove device, the viability of the sorted fraction of cells remains high, greater than 96% based on membrane integrity. As a result, we believe that cell sorters based on traveling SAWs are already promising and will benefit from the fast pace of development in cell sorting using microfluidics and will soon be able to compete with FACS instruments.
Figure 20: Sorting Performance of Sorting Devices with Slanted Grooves

The purity of each recovered sample is plotted relative to the event rate at which the sample is sorted. (a) All the data points follow the same general trend. The entire data set fits to a line whose slope indicates a decrease in purity of 4.3% each time the event rate increases by 1,000 events/s and whose intercept indicates that the theoretical maximum purity of the sorter is 93% ($R^2 = 0.817$). We determine the residuals of the data with respect to this fit, and group them based on the total sheath flow rate that was applied in (b) and the slanted groove width in (c) for each experiment. The distributions of the residuals are plotted as box and whisker plots for each group. The horizontal lines contained within each of the boxes indicate the median values; the upper and lower edges of the boxes indicate the upper and lower quartiles respectively; and the whiskers show the full range of the data. Outliers are indicated using stars. When the data are grouped according to the total sheath flow rate, the difference between the two populations is not significant ($p = 0.90$).
However, when the data are binned according to the width of the slanted groove, the devices with the narrower groove produce samples that are about 5% more pure than the devices with the larger groove, and there is a statistically significant difference ($p = 0.0029$) between the distributions for the 40 µm and 80 µm grooves. We further measure the effect of groove width on performance by fitting the data from the different groove widths independently. The data and the fits for the different groove widths are shown in (d). Filled circles (●) are used for samples sorted with a 40 µm groove device, while empty circles (○) represent samples sorted with the 80 µm groove. The device with the 40 µm groove has a slope of -4.0% per 1,000 events/s and an intercept at 95.7% ($R^2 = 0.849$), while the device with the 80 µm groove has a slope of -4.5% per 1,000 events/s and an intercept at 91.1% ($R^2 = 0.879$).
Conclusions

Cell sorters with slanted grooves use traveling SAWs to sort cells rapidly to high levels of purity. The design features guide the vertically translated cells to isolate desired cells. The sorter operates at high rates, approaching those of commercial FACS instruments, and features a high purity mode for recovery of enriched samples. Like other microfluidic cell sorters, the fluid handling region is enclosed and aerosols are not produced by the acoustic waves in the system; therefore, the sorter could find application particularly in screening bio-hazardous samples without the need for additional containment measures. Moreover, the same SAW device platform is compatible with both cells and droplets, so a single instrument could provide users with both FACS and droplet sorting capabilities. The slanted groove devices demonstrated here could be further enhanced by integrating numerous recent advances in flow focusing, inertial microfluidics, and SAW microfluidics. As a result, the SAW-actuated cell sorter with a slanted groove offers a promising alternative to both traditional FACS instruments and other microfluidic methods of sorting that could see widespread use.
Materials and Methods

Device Layout

The device consists of a PDMS replica containing the device’s flow channels bonded to a SAW substrate next to an IDT. Drawings of the IDT design and the microfluidic device are created using AutoCAD (Autodesk, Inc., San Rafael, CA). The structures defined in the drawings are transferred photomasks for lithography. These photomasks are used to fabricate molds for the PDMS replicas and to pattern metal onto each SAW substrate to form IDTs.

The IDT has a tapered or fanned design. In the tapered design, the pitch of the IDT fingers varies laterally across the transducer. The pitch increases linearly with position between a smaller pitch on one side of the transducer and a larger pitch on the opposite side; therefore, the resonant frequency also varies with position along the transducer. In our IDT, the resonant frequency range is between 161 and 171 MHz. We have chosen this comparatively high frequency since the effect of acoustic streaming increases with the square of the frequency. The width of the resonance region is determined by the full width of the IDT and the frequency difference between the two edges; it is approximately 100 μm in our design. The metallization ratio is the fraction of the IDT in each finger repeat that is covered by metal; this ratio is kept constant at 0.5. Bus bars on either side of the IDT connect to square pads with 1.5 mm side length, through which external voltages are applied to all the IDT fingers with minimal resistance. Additional markings delimit each transducer so that the IDTs can be cut from the wafer into individual squares with 17.4 mm side length. The design is etched into a chrome mask (Photo-Sciences Inc.,
Torrance, CA) to ensure that the actual finger widths closely match the designed values.

The microfluidic device has three layers, each fabricated using a separate photomask. The first layer contains only the shallow channel that serves as the vertical flow-focusing nozzle. The pattern for the nozzle extends underneath both the cell inlet region and the sorting channel to ensure that the nozzle is not sensitive to the alignment of subsequent layers. The constriction in the nozzle is designed to be nominally 40 µm long, reducing the chance that cells will clog the nozzle. The sheath channels form a Y-shape with the sorting channel, so that no stagnation points are formed, as the flow emerges from the nozzle. The nozzle is offset from the center of the channel, so that variations in flow rate or other unwanted perturbations will not cause cells to enter the retention channel spuriously.

The bulk of the device’s features are on the second layer, including the sheath and cell inlets, the main flow channel, and the device outlets. In addition, the fingers of the IDT are situated beneath an air gap, to prevent the acoustic waves from leaking into the PDMS device. The thickness of the PDMS separating the air gap from the liquid in the channel is also minimized to reduce power loss. This air gap does also exist in the second device layer. The third layer only contains the slanted groove, which is patterned on top of the sorting region of the channel. The groove is drawn 230 µm wide, slightly less than the full sorting channel width, ensuring that even if the groove is slightly misaligned from the sorting channel, the wall of the channel where the acoustic wave encounters the liquid will not be distorted. Distortions of the channel wall cause the acoustic wave to refract at unexpected angles. Each layer
contains at least two sets of alignment marks consisting of an asymmetrical pattern of crosses,\(^6\) enabling different layers to be aligned precisely on top of each other. The masks for the individual microfluidic device layers are ordered from CAD/Art Services, Inc. (Bandon, OR) and imaged with a resolution of 25,400 dpi.

*Interdigital Transducer (IDT) Fabrication*

Interdigital transducers are fabricated using the lift-off process described in the protocol from the Center for Nanoscale Systems at Harvard University.\(^7\) The substrates are 4 inch wafers of black lithium niobate (Precision Micro-Optics, LLC, Woburn, MA). Black lithium niobate is effective in SAW applications and exhibits less pyroelectric effect than undoped lithium niobate, making it convenient for lithographic processes that require baking at high temperature.\(^4\) We choose 128° Y-X lithium niobate, because it has high SAW velocity and strong coupling.\(^10\)

Each wafer is cleaned by spinning it at 4000 rpm, spraying it with acetone and isopropanol, and letting it continue to spin until dry. Residual moisture is removed with a dehydration bake at 180°C for 1 minute. We ease the rate of temperature change by placing the wafer on a hot plate at 115°C for 1 minute just prior to and immediately following baking at 180°C. Resist is dispensed onto the wafer using a disposable dropper. A layer of LOR3A resist (MicroChem, Westborough, MA) is added to the wafer surface; then, the wafer is spun at 4000 rpm to create a layer 300 nm thick. We bake the resist at 180°C for 4 minutes, using the same temperature ramping method as for the dehydration bake. We then add a layer of Shipley 1805 (MicroChem) and spin that at 4000 rpm. This layer is baked for 1 minute at 115°C. The photoresist layers are exposed to UV light through the chrome
mask with the IDT pattern on a contact mask aligner (MJB4, Karl Süss, Garching, Germany). We develop the pattern by immersing the wafer in CD-26 developer (Microposit, Austin, TX) for 75 s to form a shadow mask for E-beam deposition. We rinse the wafer clean with water and blow it dry with nitrogen. We clean the exposed surface of the wafer using an oxygen plasma cleaner (SCE106, Anatech, Union City, CA) with 75 W of RF power and an oxygen gas flow rate of 40 sccm for 20 s. We deposit 10 nm of titanium as an adhesion layer, followed by 50 nm of gold using electron beam physical vapor deposition (Denton Explorer 14, Denton Vacuum LLC, Moorestown, NJ) to form electrodes on the wafer. The photoresist is then lifted off by soaking the wafer in Remover-PG (MicroChem) at 80°C until all the excess resist is removed, about 60 minutes. We add a layer of Shipley 1813, and bake it at 115°C for 1 minute to form a protective layer. We use a dicing saw (DAD321, DISCO Corp., Tokyo, JPN) to score lines 250 µm into the lithium niobate. The wafer breaks cleanly along the scored lines, yielding up to 21 devices per wafer. The IDTs are cleaned with acetone to remove the protective layer, prior to use.

**Soft Lithography**

We perform multi-layer lithography to create molds for PDMS replicas. The layers are processed following the method recommended in the manufacturer’s data sheet for SU-8 3025 photoresist (MicroChem). For each layer, we dispense a small amount of resist onto the wafer. We spin the wafer at 3000 rpm to create a layer that is 25 µm thick. We pre-bake each layer for a total of 12 minutes at 95°C, rotating the wafer 180° on the hot plate after half the bake time has elapsed. The photomask for a particular layer is aligned with any underlying features and the layers of photoresist are patterned with UV light on a contact mask aligner (ABM, Scotts...
Valley, CA). The resist is then post exposure baked for 1 minute at 65°C and 5 minutes at 95°C. At this point, additional layers can be added on top of any existing layers by following the same procedure. Once all the layers are post exposure baked, we develop the features by immersing the wafer in polyethylene glycol monomethyl ether acetate (484431, Sigma-Aldrich Co. LLC, St. Louis, MO) for 5 minutes using an orbital shaker (Roto Mix 8x8, Thermo Fisher, Waltham, MA) for mixing. After development, we rinse the wafer with isopropanol and blow it dry with nitrogen. We place the wafer in a 100mm plastic petri dish. The wafer is now ready to serve as a mold for creating replicas in PDMS.

We mix PDMS (Sylgard 184, Dow-Corning, Midland, MI) base and cross-linker in a 10:1 weight ratio using a Thinky mixer (AR-100, Thinky Corp., Tokyo, Japan). The mixer runs in mixing mode for 30 s and degassing mode for another 30 s. We pour the uncured PDMS on top of the mold. We degas the PDMS for 10 minutes, then place the mold in the oven at 65°C overnight. Once the PDMS is cured, we cut around the edge of the wafer using a scalpel and lift the PDMS replica out of the mold. Each PDMS replica contains 16 independent devices; the replica is cut into individual devices prior to use. Interface holes are created with a biopsy punch (Uni-Core, GE Healthcare Life Sciences, Pittsburgh, PA). We use 0.75 mm diameter holes for the inlets and 1.5mm diameter holes for the outlets. We clean the replicas by sonication in isopropanol for 5 minutes, then blow them dry. At this point, individual PDMS devices can be bonded onto a substrate with the IDT pattern in the instrument’s sample holder for sorting experiments.
Sorter Apparatus

The sorting apparatus is similar to that detailed in previous publications, except that the microscope is custom built using modular optomechanics (Thorlabs Inc., Newton, NJ) instead of using a commercially available microscope. A 473 nm laser with 100 mW of output power (LRS-0473, Laserglow Technologies, Toronto, ON) excites fluorescence in the sample. The laser beam is expanded (BE-05-10-A, Thorlabs Inc.), and steered into the microscope. A cylindrical achromat (ACY254-200-A, Thorlabs Inc.) and a microscope objective (Nikon CFI Plan Apochromat Lambda, 10X/0.45NA, Micro Video Instruments, Inc., Avon, MA) focus the laser beam into a line in the microscope’s focal plane. The objective also collects any fluorescence emitted by the sample. While excitation light gets reflected by the excitation dichroic (FF495-Di03-25x36, Semrock, Inc., Buffalo, NY) and up through the objective, the emitted fluorescence passes through the excitation dichroic. The fluorescence reflects off the fluorescence dichroic (FF605-Di01-25x36, Semrock, Inc.) towards the photocathode of a photomultiplier tube (H10723-20, Hamamatsu Photonics K.K., Hamamatsu, Japan). A colored glass longpass filter (FGL495, Thorlabs Inc.) and a dielectric bandpass filter (FF01-520/44-25, Semrock, Inc.) are placed between the fluorescence dichroic and the photomultiplier tube (PMT) to attenuate noise sources of light, so that the PMT provides an accurate measurement of fluorescence. The microscope’s field of view is illuminated using an infrared light emitting diode (M780L2-C1, Thorlabs Inc.). The infrared light passes through both microscope’s dichroic filters, and gets reflected by a turning mirror (CM1-P01, Thorlabs Inc.). The infrared image is focused onto the sensor of a fast camera (HiSpec1, Fastec Imaging, San Diego, CA) by a tube lens (AC254-100-B-ML,
Thorlabs Inc.). The fast camera enables the system to record high framerate videos of the sorting process. A manual stage (Leica) provides fine adjustment of the sample position with respect to the optical system.

The photomultiplier tube module measures the fluorescence from the sample, generating a voltage proportional to the intensity of the incident light. This voltage is digitized by the data acquisition card (PCIe-7842R, National Instruments Corp., Austin, TX) and analyzed in real time using the card’s field programmable gate array to detect and analyze peaks in the fluorescence signal. When peaks corresponding to desired cells are detected, a sorting pulse is generated. The sorting pulse is a +3.3V signal, which controls the output of a RF waveform generator (SMB100A, Rohde & Schwarz, Munich, Germany) through its pulse modulation input. The output of the waveform generator is intensified using a high gain RF amplifier (LZY-22+, Mini-Circuits, Brooklyn, NY). When the amplified RF signal is applied to the IDT, the IDT generates SAWs in response. An associated PC can be used to set threshold values for peak detection and sorting and to monitor system performance. Using this system, the fluorescence from cells passing through the sorting region of the device is analyzed in real time, and pulses of SAW are applied to sort desired cells with minimal latency.

A custom-made sample holder supports the microfluidic device. The base plate of the sample holder holds the IDT securely in a milled slot. The center of the baseplate is cut away to enable light to transmit through the sample and to permit the microscope to focus on the device. A glass slide is cut to size and placed under the IDT to provide mechanical support. A clear piece of lithium niobate is fastened
underneath the glass slide in an orientation chosen to cancel the effects of substrate birefringence. A printed circuit board (PCB) routes signals from the amplifier to the IDT. The amplifier and the PCB are connected using standard RF adaptors (SMA to MMCX male), and electrical connections from the PCB to the IDT are created, when pogo pins mounted on the board are pressed into contact with the metal pads. The PCB is held in place by fixing it to the base plate using M3 screws. An acrylic spacer ensures that the pins exert enough force to hold the IDT in place and make consistent electrical contact, but not so much force that the substrate cracks under the stress. The spacer is milled to 3.7 mm and laser cut to accommodate the mounting screws, the shape of the PCB, and any electrical components on the lower side of the PCB. Each PDMS device is bonded to the substrate using mechanical force. The PDMS device forms three sides of the device’s flow channel, while the lithium niobate substrate serves as the bottom of the flow channel. A clamp is fashioned from a 6mm thick sheet of acrylic; it is laser cut to permit fluid connections to pass through to the PDMS device. The clamp presses the PDMS device onto the substrate using M2 screws fastened to the baseplate. Once assembled, the entire sample holder fits into the microscope stage.

_Device Characterization Experiments_

Madin Darby canine kidney (MDCK) and human chronic myelogenous leukemia (K-562, ATCC, Manassas, VA) cells are harvested prior to each day’s experiments. The MDCKs have fluorescent nuclei, following stable transfection with green fluorescent protein fused to a nuclear localization sequence, while the K-562 cells are stained by adding calcein AM (Life Technologies, Grand Island, NY) to the
cell suspension at a concentration of 1 µM and incubating the suspension at 37°C for 20 minutes. The cells are re-suspended into injection buffer at between 5 and 10 million cells per ml. Injection buffer consists of 18% Optiprep (D1556, Sigma-Aldrich Co. LLC) by volume, 6 U/ml DNAse I (New England Biolabs Inc., Ipswich, MA), and 3 mM magnesium chloride in 1X phosphate buffered saline (PBS, P3813, Sigma-Aldrich Co. LLC, St. Louis, MO).

Unless otherwise specified, we use a PDMS device with a vertical flow-focusing nozzle that is 50 µm wide, 40 µm long and 25 µm tall; a sorting channel that is 250 µm wide and 50 µm tall; and a slanted groove that is 120 µm wide, 25 µm tall, and whose long axis is tilted 60° from the overall direction of flow. The flow rate of the cell phase is 0.5 ml/h, while the sheath fluid has a flow rate of 45 ml/h. The sheath fluid is 1X PBS. One quarter of the sheath flow comes from the inlet nearest the waste outlet and three quarters of the flow from the inlet on the retention side of the device.

The frequency of the RF pulse used to generate SAWs is kept constant at 163.1 MHz, except when the groove width is changed, then we tune the frequency to ensure that the SAW aligns with the groove. For each distinct condition, we run control experiments to ensure that cells are not sorted, when the instrument is triggered, but the RF source is off. Unless noted, no cells enter the retention channel unexpectedly for the conditions we tested. Fast movies of individual sorting events are analyzed to determine whether the cell is successfully deflected into the retention outlet or not.
**Sorting Experiments**

As detailed for the characterization experiments, K-562 cells are harvested from culture just prior to conducting the experiment. To create reference libraries of cells, we mix the sample of cells carefully with a pipette and collect 10% of the cell suspension by volume. This fraction of the cells is stained with calcein AM at 1 μM for 20 minutes at 37°C, while the remaining cells are kept unstained. The two fractions are then combined and the cells are re-suspended in injection buffer at the target cell density.

The cells are sorted using a slanted groove sorting device. We use the standard nozzle geometry and an RF pulse with 38.26 dBm of instantaneous power and 100 μs duration at 164.1 MHz. The flow rate of the cell suspension is kept constant at 0.5 ml/h. The device is operated with a range of cell densities to test different event rates. We measure the purity from devices operating at two different sheath flow rates, 45 ml/h and 60 ml/h, and using two different groove widths, 40 μm and 80 μm. The remaining control parameters are kept are held constant. Here, the sheath flow is 1X PBS. The actual rate of fluorescent events is measured by the sorting instrument and the projected total event rate is obtained by dividing this by the measured purity of the initial reference library. We set the thresholds for sorting to ensure that pulses are only applied when we expect only a single fluorescent cell to be present in the channel, by ignoring the lower and upper extremes of fluorescence. In addition, when the sorting rate is high, we further limit the sorting thresholds to set the sorting rate below 500 events/s, reducing the chance that the IDT will be irreversibly damaged. The fluorescence of the cells recovered from the
retention outlet is measured using a confocal microscope (SP5, Leica Microsystems Inc., Buffalo Grove, IL). In addition to using calcein to measure the proportion of labelled cells in the recovered sample, DRAQ5 (Life Technologies, Grand Island, NY) is added at a final concentration of 500 nM to label the DNA of all cells present in each sample. To measure cell viability after sorting, we add ethidium homodimer (Life Technologies, Grand Island, NY) to 2 µM final concentration and incubate the cells with the dye for 20 minutes at 37°C. The images are analyzed using a custom Matlab (The Mathworks, Inc., Natick, MA) script to detect fluorescence in the three separate fluorescence channels. The purity of the sorted fraction is the ratio of cells labeled with calcein to the total number of cells, and the viability is the difference between unity and the ratio of dead cells to total cells.
Chapter 3: Traveling Surface Acoustic Wave (TSAW) Microfluidic

Fluorescence Activated Cell Sorter (μFACS)
**Introduction**

Conventional fluorescence activated cell-sorting (FACS) is a valuable and widely-used tool in molecular and cellular biology, which optically screens each cell and encapsulates it into an aerosolized droplet that is charged so that it can be electrostatically deflected to be sorted. However, despite its utility and wide-spread applicability, FACS has some unavoidable drawbacks that can be eliminated by sorting with a microfluidic device. As a bulk technique with millimeter sized components, FACS requires orders of magnitude larger sample volumes\(^{43}\) than microfluidic devices with micron sized channels, to achieve similar yields.\(^{20, 45, 72-75}\) Microfluidic fluorescent activated cell sorters (µFACS) eliminate the use of an aerosol nozzle that can damage cells or create safety concerns when handling infectious cells.\(^{76}\) Furthermore, microfluidic devices are much easier to align optically and are disposable, eliminating potentially biohazardous clean-up steps that a FACS machine requires after each use.\(^{45, 47, 72, 73}\) Lastly, the channel geometries of microfluidic devices can be curved to utilize the inertial effects of fluid flow to control cell position, which facilitates high-throughput analysis.\(^{77, 78}\) Several cell-sorting mechanisms have been used in microfluidic devices, including piezoelectric actuation, surface acoustic waves (SAW), and pulsed laser-activated cell sorting (PLACS). Each of these techniques sort at rates that are comparable to FACS, offering an attractive alternative;\(^{17, 20, 39, 48, 53, 72-75, 79}\) for example, PLACS can achieve 90% purity at 1,000-3,000 events per second,\(^{53, 74}\) but creates cavitation bubbles in the channel that potentially harm cells. Moreover, standing surface acoustic waves (SSAW) have demonstrated the ability to focus and sort 2,500 cells per second while achieving 90% purity.\(^{75}\) However, the SSAW wavelength is fundamentally
determined by the device geometry, constraining the range of its application and precluding adjustment after device fabrication.

By contrast, traveling surface acoustic waves (TSAW) deflect cells into a separate channel with no wavelength constraint,\textsuperscript{20, 73} thus facilitating TSAW integration with a wide variety of channel geometries without modification.\textsuperscript{17, 20, 73, 80} Despite this advantage, TSAW has not thus far demonstrated microfluidic cell sorting under high-speed conditions with switch cycles fast enough to achieve sorting rates comparable to FACS; the ability to sort cells rapidly using a microfluidic device has the potential to replace conventional FACS machines, providing users with smaller scale devices that are disposable and can handle small volumes.

In this chapter, we report a \(\mu\)FACS device that combines a spiral channel for inertial flow focusing with a tapered interdigital transducer (IDT) that generates a 25 \(\mu\)s TSAW pulse to rapidly deflect cells into a separate channel upon fluorescence detection. The device sorts cells at rates up to 5,000 events per second while maintaining cell viability in excess of 90%; moreover, at rates up to 2,000 events per second, the device maintains sorting purity above 90%, comparable to FACS. The device, which has a theoretical maximum sorting rate of 40 kHz, demonstrates the high sorting performance capabilities of TSAW-based FACS and provides an attractive alternative to conventional sorting methods.
Results and Discussion

The cell-sorting device is composed of polydimethylsiloxane (PDMS) molded microchannels bonded to a lithium niobate substrate containing a tapered IDT. We pattern the IDT onto 128° Y-X lithium niobate, which serves as the piezo-electric material to create TSAWs, as well as the substrate to seal the PDMS device. To optimize TSAW sorting, the cells are spatially ordered in a straight line and positioned close to the surface of the lithium niobate substrate side of the channel to guarantee interaction with the pulsed acoustic wave in the sorting region. To accomplish this, cells entering the device first flow through a spiral channel, which inertially flow focuses them into a single ordered line, as shown in Fig. 21a. We use a spiral channel, since fluid flowing through this channel geometry experiences centrifugal acceleration, creating two counter-rotating Dean vortices in the top and bottom halves of the channel.\(^{77, 78, 81-86}\) These vortices position cells in the fluid into a nearly ordered single file.\(^{78, 81-84, 87}\) To ensure the spiral channel can inertially focus cells that are 10 to 15 \(\mu\)m in diameter, we set the hydraulic radius of the channel, defined as \(D_h = \frac{2hw}{h+w}\) where \(h\) and \(w\) are the height and width of the channel cross section, respectively, to 51 \(\mu\)m, to satisfy the particle confinement ratio inequality, \(\lambda > 0.07\), where \(\lambda\) is the ratio between particle diameter and hydraulic radius.\(^{77, 84}\) Furthermore, we set the length of the spiral channel to be 61.5 mm, the flow rate in the channel to 1.5 mL per hour, in accordance to design rules for inertial focusing.\(^{78}\)
Figure 21: Overview of the Sorting Device

(a) Cells are flowed through a spiral microchannel to focus and align the cells into a single file. The spiral channel causes cells to experience both inertial migration and influences from Dean vortices, minimizing the number of positions a cell can occupy in the channel. By minimizing the number of positions, the likelihood that cells will deviate from the desired flow path, an event known as misfocusing, is minimized.\textsuperscript{13}

(b) The outlet of the spiral channel connects to a vertical flow focusing nozzle flanked by two sheath flow channels at higher flow rates than the spiral channel to accelerate, further align, and space cells upon entering the sorting region and to further minimize misfocusing events. (c) Cells are sequentially interrogated by a laser and detector in the sorting region. Cells that are fluorescently labeled are detected and deflected into a separate channel (keep) by the IDT.
At the exit of the spiral, our cell-sorting device contains a vertical flow-focusing nozzle, a multi-layer feature which introduces a vertical constriction at the intersection with the two sheath-flow channels, as shown in Fig. 21b. The vertical constriction focuses the cells into a narrow sample core stream towards the bottom of the channel, maximizing the interaction of cells with the acoustic wave. Additionally, the sheath flow further separates the cells and positions them within the sorting region, as shown in Fig. 21b. Upon entering the sorting region, the cells are illuminated by a 473 nm laser to excite fluorescence in labeled cells; the fluorescent light is detected by a photomultiplier tube (PMT) and triggers a signal generator to activate the IDT. The IDT induces a 25 µs TSAW pulse that deflects the fluorescent cells into a separate keep channel, as shown schematically in Figure 1c. When the IDT is not activated, cells flow unaffected through the sorting region and into the waste outlet channel. A microscope image of the sorting region, with the IDT and outlet channels is shown in Figure 22. The combination of inertial and vertical flow focusing features together guide cells to enter the sorting region one at a time for localized and precise cell-acoustic wave interaction for reproducible cell deflection.
Figure 22: Individual frames from a high-speed camera video recording (10,000 fps) of a fluorescently labeled Mycl-9E10 cell being sorted by the traveling acoustic wave (flow direction is from left to right).

(a) Fluorescently labeled cell, circled in red, enters the sorting region and is detected. A non-fluorescently labeled cell, circled in blue, is shown entering the waste outlet channel as the fluorescently labeled cell enters the sorting region. (b) When a cell is detected, 12.5 W of power is applied to the IDT to generate a traveling surface acoustic wave for 25 µs into the sorting region. (c) Deflected cell entering the keep outlet channel after interacting with the applied TSAW. Scale bar is 100 µm
A radio frequency (RF) signal of 162 to 164 MHz is applied to the IDT, generating a TSAW that is refracted into the sorting region of the fluid channel adjacent to the IDT, leading to a deflection of the detected cell into the keep channel, as shown in Fig 21c. We taper the electrode pairs of the IDT to allow the adjustment of the TSAW position along the direction of flow in the channel (x-direction in Fig. 1c) by tuning the RF of the signal generator, ensuring optimal cell deflection and compensation for slight variations in IDT alignment from fabrication. The IDT is placed beneath an air pocket separate from the fluid in the sorting region, to prevent acoustic waves from leaking into the PDMS device. The thickness of the PDMS separating the air gap from the liquid in the sorting region is minimized to reduce power loss. When the TSAW impinges on the interface of a fluid it refracts and establishes longitudinal acoustic waves in the fluid; these waves deflect cells by acoustic radiation forces into a separate outlet channel.

For all experiments the flow rates are held constant to maintain a uniform flow velocity. The sample flow rate is 1.5 ml per hour, while the left and right sheath flow rates are 4 and 8.5 ml per hour respectively. The right sheath fluid operates at a higher flow rate to direct cells entering the sorting region into the waste outlet channel when the IDT is off. We compare three different IDT power levels to determine the amount of power needed to deflect cells successfully at high speeds. We repeat each power-setting experiment five times across four separate chips to quantify reproducibility, and test against three different cell lines: K562 (ECACC 89121407), Mycl-9E10 (ECACC 85102202), and 357-101-4 (ECACC 92030603) cells. We record high-speed videos of cells flowing through the sorting region; we track individual cell positions using open-source tracking software (Tracker, Open Source
Physics, physlets.org/tracker), quantifying cell velocity and deflection. In each of the experiments, we reconstruct the trajectories of an average of twenty cells through the sorting region.

*Cell Velocity and Deflection*

To demonstrate the high-speed capabilities of our sorting device, we record high-speed videos of cells entering and exiting the sorting region at 10,000 FPS and use the tracking software to measure the average velocity of sorted and non-sorted cells in the direction of flow (x-component) in the sorting region as shown in Figure 23 a, b. We find the average x-component velocity for non-sorted cells of all three cell lines is close to 1.5 m/s. We do not observe a large variation in velocity for non-sorted cells since the cells are run under the same flow conditions and are of similar size; using a commercial cell counter (Countess FL II Automated Cell Counter, ThermoFisher, Waltham, MA) we find that the average cell diameters of the K562, Myel-9E10, and 357-101-4 cells are approximately 15 µm, 10 µm, and 10 µm, respectively. For sorted cells of these types, we find the average x-component velocity to be approximately 1.3 m/s; this slight decrease in velocity is a result of the sorted cells being deflected vertically and laterally across the sorting region away from their mean flow path. The average x-component velocity measurements are shown in Figure 23c.

We actuate the IDT at three different power levels to determine the amount of power required to successfully deflect a target cell away from the mean flow path and into the keep channel. We observe that increasing the power applied to the IDT
increases the amount of deflection a cell experiences in the sorting region. A supply of 5 W of power or less to the IDT does not deflect a cell into the keep channel, while a supply of 8 W or 12.5 W to the IDT does successfully deflect cells. For each of the IDT power settings, we normalize, and plot for each cell line the 2-D position of sorted and non-sorted cells in the sorting region as shown in Figure 23 d,e,f. We observe that at 12.5 W, the Mycl-9E10 cells are deflected slightly further in the Y-direction than at 8 W; therefore, we actuate the IDT at 12.5 W for all velocity measurements, purity performance experiments, and cell viability measurements. These observations in cell deflection correlate with the widely discussed coherence between particle deflection and the IDT input power.17, 92-94
Figure 23: High-Speed Camera Analysis of Cells for Velocity and Position Measurements

(a) Multiple frames from a sort event are superimposed to create an image depicting the trajectory of a sorted and non-sorted cell (cells in image are Mycl-9E10) (b) Multiple frames from (a) after Tracker analysis super imposed to create in image depicting Tracker analysis. (c) Average velocity measurements for tested cell lines in the sorting region. Individual cell velocity measurements are obtained from high-speed camera videos analyzed and averaged using Tracker. Both target and non-target cells are measured for comparison. Sorted cells experienced a lower velocity on average due to deflection into the keep outlet channel. (d-f) Normalized deflection plots for all three cell lines under the same flow conditions. (Flow direction is from left to right). (d) K562 normalized X and Y position in the sorting junction for non-target and target cells deflected at 5 W, 8 W, and 12.5 W. (e) Mycl-9E10 normalized X and Y position in the sorting junction for non-target and target cells deflected at 5 W, 8 W, and 12.5 W. (f) 357-101-4 normalized X and Y position in the sorting junction for non-target and target cells deflected at 5 W, 8 W, and 12.5 W. Error bars represent confidence intervals.
**Sorting Purity**

For purity measurements, we label 10% of the total number of cells with calcein AM fluorescent dye and sort them from non-labeled cells for all event rates. We count cells obtained from the keep channel using the commercial cell counter to determine the percentage of stained cells present. We repeat purity experiments ten times at each event rate condition for all three cell lines, and use a different microfluidic chip for each experiment to determine reproducibility.

We adjust the cell event rate by changing the concentration of cells processed through the sorting device while maintaining the same flow conditions. We define the event rate here as the projected number of cells entering the sorting region per second, which we estimate based on the total number of cells and sample flow rate. At low event rates the sorting device accomplishes high purity, but the purity declines as the event rate is increased; we observe that all three cell lines follow a similar decrease in purity as the event rate increases, as shown in Fig. 24. We observe that as the cell concentration rises, the probability of more than one cell being present during a sorting event also increases; in these coincident events, multiple cells are deflected into the keep channel at the same time, decreasing the purity. In commercial FACS machines, higher levels of purity are obtained by the detection and elimination of such coincident events; introducing coincident event detection software, as commonly found in conventional FACS instruments, would likely maintain sorting purity above 90% at all tested event rates.
Figure 24: Purity performance of the sorting device

A starting population of 10% target cells for all event rates. The average keep purity of each recovered sample is plotted relative to the cell line and event rate. Each data point represents the average from 10 sorting runs and the error bars represent confidence intervals. Cells obtained from the keep outlet are measured on the Countess FL to determine the fraction of fluorescent cells present. Event rate is controlled by adjusting cell concentration of the sample. Decrease in purity is assumed to result from a lack of coincident event logic software and increasing sample concentration. (Insert) Countess FL images of 357-101-4 cells before and after sorting at 2000 events per second. White circle indicates fluorescent labeled cells, black circle non-labeled cells.
Viability

To assess cell viability, we stain cells with 0.4% trypan blue (Thermo Fisher Scientific, USA) and quantify the percentage of live and dead cells using the commercial cell counter, sorting with the same power and pulse settings. We collect four cell viability measurements during eight sorting runs for each cell line to determine if cell viability is affected by the acoustic wave, the sorting device, or both. We measure cell viability prior to loading the cell suspension into the sorting apparatus. During a sorting run, we extract fractions of the keep and waste for viability measurements. We then obtain a fourth viability measurement from a fraction of the same cell solution used for the sorting experiment that is never loaded into the sorting apparatus as a control. We observe a small decrease in viability of a few percent for the keep cells in comparison to the input, waste, and control viability measurements, as summarized in Table 2. We attribute this small decrease due the fact that cells collected from the keep collection tubes are centrifuged and resuspended to provide a higher concentration of cells for accurate measurements using the commercial cell counter. It is also possible that this small decrease is due to the amount of power used to deflect fluorescent labeled cells. Further studies would be required to determine if increased acoustic power would decrease cell viability.
Table 2: Average cell viability measurements for each cell line

Viability was determined by trypan blue and measurements were made on the Countess FL II. Input is defined as cell viability prior to sorting. The keep and waste are viability measurements of sorted and non-sorted cells respectively. Control is defined as a fraction of cells from the input placed on ice and never introduced into the device but measured after the sorting run.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>K562</th>
<th>Mycl-9E10</th>
<th>357-101-4</th>
</tr>
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<td>96.8 ±1.0</td>
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<tr>
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<td>91.8 ±2.9</td>
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<tr>
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<tr>
<td>Control</td>
<td>95.0 ±1.7</td>
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</table>
Conclusions

We demonstrate a microfluidic cell sorter that integrates inertial and hydrodynamic flow focusing with a TSAW to sort cells at throughputs comparable to conventional FACS. The device sorts cells and obtains sort purities in excess of 90% for event rates up to 2,000 events per second using a 25 µs acoustic wave pulse. We measure cell viability for three different live cell lines to demonstrate the gentleness of acoustic sorting. Our sorting device keeps all liquids enclosed and generates no aerosols, enabling applications that involve biohazardous samples. The sorting device could be improved by implementing coincident event logic software to discard cells that are too close together to be sorted individually, potentially maintaining purity above 90% at sorting rates exceeding 3,000 events per second. Microfluidic devices that use TSAW for cell sorting may have a broad spectrum of research and industrial applications such as cancer research, reproductive technologies, and rare cell enrichment.
Materials and Methods

Soft Lithography

The polydimethylsiloxane (PDMS) molded microfluidic channels of the device are comprised of two layers, each fabricated using a separate photomask. The first layer comprises of the vertical flow-focusing nozzle. The nozzle is designed to be nominally 190 µm long and extends underneath both the sample inlet and the sorting region. The second layer contains the sample and sheath inlet channels, the sorting region, the device outlets, and the IDT air pocket. The sample inlet consists of a 70 µm wide by 61.5 mm long spiral channel with five consecutive turns that leads to the sorting region. The sheath channels form a Y-shape with the sorting region to prevent stagnation points as flow emerges from the nozzle. The nozzle and sample inlet are offset from the center of the sorting region to ensure cells will not enter the keep outlet channel unless deflected by the pulsed acoustic wave. The air pocket is a rectangular shaped area that prevents acoustic waves from leaking into the PDMS device away from the intended sort region. The thickness of the PDMS separating the air pocket from the liquid in the sorting region is minimized while maintaining fluidic sealing to 50 µm to reduce power loss. Each layer contains two sets of alignment marks consisting of an asymmetrical pattern of crosses, enabling the two layers to be aligned precisely. The masks for the individual layers were ordered from CAD/Art Services, Inc. (Bandon, OR) and imaged with a resolution of 15,400 dpi.

We perform multi-layer lithography to create molds for PDMS replicas. We process the layers by following the method recommended in the manufacturer’s data sheet for SU-8 3000 series photoresists (MicroChem Corp., Westborough, MA). For each layer, we dispense a small amount of SU-8 3025 photoresist (MicroChem) onto
the silicon wafer. We spin the wafer at 4000 rpm to create a layer that is 20 µm thick. We pre-bake each layer for a total of 12 minutes at 95°C on the hot plate. We use a contact mask aligner (ABM, Scotts Valley, CA) to align and pattern with UV light any underlying features to the photomask (CAD/Art Services Inc., Bandon, OR). We then post-exposure bake the resist for 1 minute at 65°C and 5 minutes at 95°C, followed by immersing the wafer in polyethylene glycol monomethyl ether acetate (484431, Sigma-Aldrich Co. LLC, St. Louis, MO) for 6 minutes using an orbital shaker (Roto Mix 8x8, Thermo Fisher, Waltham, MA) for mixing. After development, we rinse the wafer with isopropanol and blow dry it with compressed nitrogen. We repeat these steps for each subsequent layer. After the layers have been developed, the wafer is now ready to serve as a mold for creating PDMS replicas.

We mix PDMS (Sylgard 184, Dow-Corning, Midland, MI) base and cross-linker in a 10:1 weight ratio using a Thinky mixer (AR-100, Thinky Corp., Tokyo, Japan). We de-gas the PDMS for 20 minutes and cure the mold in the oven at 65°C overnight to create a replica. We cut the PDMS replica into individual devices prior to use. We create inlet- and outlets holes with a 1.2 mm diameter biopsy punch (Uni-Core, GE Healthcare Life Sciences, Pittsburgh, PA). Next, we bond individual devices to the lithium niobate substrate using an oxygen plasma stripper (PE-50, Plasma Etch, Carson City, NV). During the bonding procedure, we align the PDMS device to the IDT so that the electrodes are situated beneath the air pocket, to prevent the acoustic waves from leaking into the PDMS device prematurely. The PDMS device forms three sides of the device’s flow channel, while the lithium niobate substrate serves as the bottom of the flow channel.
Interdigital Transducer (IDT)

The device uses an IDT with a tapered-finger design. The design is characterized by a continuously changing pitch of the IDT fingers from one side to the other; effectively varying the position of the resonant frequency laterally along the transducer. Our IDT resonant frequency ranges from 160 to 172 MHz. The excited SAW beam of the resonance region can be approximated by the electrode aperture and the frequency difference between each IDT pole. This dimension is approximately 30 µm in our design. The metallization ratio, a/p, which is the fraction of the electrode width, a, and pitch, p, is 0.5 throughout the transducer. Electrodes on either pole are interconnected by trapezoidal bus bars that merge into square contact pads to apply external voltages. The trapezoidal bus shape prevents the IDT from obstructing the flow channels of the PDMS slab.

We fabricate the IDTs in a lift-off process using a modified protocol from the Center for Nanoscale Systems at Harvard University. We use double polished 4” diameter black 128° Y-X cut lithium niobate that is 500 µm thick as the piezoelectric substrate, because it offers adequate optical transparency, strong electro-mechanical coupling with low bulk wave generation and high SAW velocity. In addition, the black, chemically reduced lithium niobate helps facilitating fabrication steps that involve baking on heat plates by effectively eliminating the pyroelectric effect. We clean each wafer with acetone and isopropanol. We remove any residual moisture on the wafer with a dehydration bake on a heat plate at 180°C for minimum 3 minutes. For every step involving baking, we hold the wafer 5 to 10 mm above the heat plate surface for about 20 seconds before placing it down, to ease temperature changes of the substrate and reduce the risk of cracking. Consequently, we hold hot wafers in
air for about 20-30 seconds to gently cool down the substrate to room temperature before proceeding with any next steps. We create a 300 nm thick sacrificial layer by spin coating LOR 3A (MicroChem Corp., Westborough, MA) at 3000 rpm on the cleaned wafer surface, followed by baking the layer at 180°C for 7 minutes. Subsequently, we spinShipley S1805 (MicroChem Corp., Westborough, MA) at 4000 rpm to form a 500 nm layer of photoresist on top of the sacrificial layer and baked for 1 minute at 115°C.

We expose the coated wafers to a UV dosage of 40 mJ/cm² and a wavelength of 405 nm using a mask-less alignment tool (MLA150, Heidelberg Instruments, Germany) to transfer the designed IDT patterns to the substrate. We develop the exposed patterns in CD-26 developer (Microposit MF, Dow Electronic Materials, Marlborough, MA) during a 75-second-long immersion, followed by a rinse with deionized water and drying the wafer with nitrogen. Prior to metal deposition, we clean wafers with oxygen plasma for 3 minutes at 150 W and 40 sccm gas flow (Anatech SCE-106 plasma barrel etcher, Anatech USA, Union City, CA) to remove organic residues from the substrate surface that could impair metal adhesion. We use electron beam physical vapor deposition (Denton Explorer 14, Denton Vacuum LLC, Moorestown, NJ) to create a 10 nm thick titanium adhesion layer, followed by 50 nm of gold to form IDTs on the wafer. To obtain the IDTs, the deposited wafers are soaked in a Remover-PG bath (MicroChem Corp, Westborough, MA) at 80°C for about 3 hours to lift-off the sacrificial layer and cleaned with isopropanol.
Sorter Apparatus

The sorting apparatus is similar to what has been described in previous works,\textsuperscript{51, 71, 73} and uses a custom-built microscope using modular optomechanical components (Thorlabs Inc., Newton, NJ). We expand and steer (BE-05-10-A, Thorlabs Inc.) a 473 nm laser with 100 mW of output power (LRS-0473, Laserglow Technologies, Toronto ON), into the microscope to excite fluorescently labeled cells. A cylindrical achromat (ACY254-200-A, Thorlabs Inc.) and a microscope objective (Nikon CFI Plan Apochromat Lambda, 10X/0.45NA, Micro Video Instruments, Inc., Avon, MA) focus the laser beam into a line in the microscope’s focal plane. Any fluorescence emitted by the cell is collected by the objective and the excitation light gets reflected by the excitation dichroic (FF495-Di03-25x36, Semrock, Inc., Buffalo, NY) and up through the objective, and the emitted fluorescence passes through the excitation dichroic. The fluorescence reflects off the fluorescence dichroic (FF757-DiO1-25x36, Semrock, Inc.) towards the photocathode of a photomultiplier tube (PMT) (H10723-20, Hamamatsu Photonics K.K., Hamamatsu Japan). We place a colored glass longpass filter (FGL495, Thorlabs Inc.) and a dielectric bandpass filter (FF01-520/44-25, Semrock, Inc.) between the fluorescence dichroic and the PMT to reduce noise sources of light to provide accurate measurements of fluorescence. To illuminate the microscope’s field, we use a 850 nm light emitting diode (LED) (48T1419, LZ1-30R400, Newark Element14, Chicago IL). The infrared light passes through both dichroic filters and is reflected by a steering mirror (CM1-P01, Thorlabs Inc.). The infrared image is focused onto the sensor of a high-speed camera (HiSpec1, Fastec Imaging, San Diego, CA) by a tube lens (AC254-100-B-ML, Thorlabs Inc.) The high-speed camera enables the system to record high framerate
videos of the sorting process. A manual stage (Leica) provides adjustment of the sample position with respect to the optical system.

The PMT measures the fluorescence from the sample, generating a voltage proportional to the intensity of the measured light. The voltage is digitized by a data acquisition card (PCIE-7842R, National Instruments Corp., Austin TX) and analyzed in real time using the card's field programmable gate array to detect and analyze peaks in the fluorescence signal. When peaks corresponding to desired cells are detected, a 25 µs sorting pulse is generated. The sorting pulse a 3 V signal, which controls the output of a RF waveform generator (SMB100A, Rhode & Schwarz, Munich, Germany) through its pulse modulation input. The output is amplified using a high gain RF amplifier (LZY-22+, Mini-Circuits, Brooklyn, NY). When the amplified signal applied to the IDT, the IDT produces SAWs in response. We use a PC to set threshold values for peak detection and sorting, and to monitor system performance. Using this system, the fluorescence from cells passing through the sorting region of the device are analyzed in real time, and pulses of SAW are applied to sort desired cells with minimal latency. The microfluidic device is supported by a custom-made sample holder that fits into the microscope stage, and the base plate of the sample holder holds the lithium niobate containing the IDT of the chip securely. We cut away the center of the baseplate to permit light to transmit through the sample and to focus the microscope into the channels of the device. A glass slide is cut to size and placed under the IDT to provide mechanical support. A clear piece of lithium niobate is taped underneath the glass slide in an orientation chosen to cancel the effects of the birefringence. A printed circuit board (PCB) routes signals from the amplifier to the IDT. The amplifier and the PCB are connected using standard RF
adaptors (SMA to MMCX male), and electrical connections from the PCB to the IDT are created when pogo pins mounted on the board are pressed into contact with the metal pads. The PCB is held in place by fixing it to the base plate using M3 screws. An acrylic spacer plate ensures that the pins exert enough contact force to hold the IDT in place and make consistent electrical contact. The spacer is milled to 3.7 mm and laser cut to accommodate the mounting screws, the shape of the PCB, and electrical components on the lower side of the PCB.

**Deflection, Purity, and Viability Characterization Experiments**

We harvest either K562 (ECACC 89121407), Mycl-9E10 (ECACC 85102202), or 357-101-4 (ECACC 92030603) cells prior to sorting experiments. We remove a fraction of the cell suspension and stain it by adding calcein AM (Life technologies, Grand Island, NY) to the cell suspension at a concentration of 1 µM and incubating the suspension at 37°C for 20 minutes. Stained cells are re-suspended into injection buffer at or between 3 and 12 million cells per ml depending on event rate desired. Injection buffer consists of 1% Optiprep (D1556, Sigma-Aldrich Co. LLC) by volume, 6 U/ml DNase I (New England Biolabs Inc., Ipwich, MA), 3 mM magnesium chloride, 10% fetal bovine serum (FBS) by volume in Dulbecco’s Modified Eagle’s Medium (DMEM, 10-013-CV, Corning). Mycl-9E10 and 357-101-4 cells are prepared the same with the exception that DMEM is replaced with Roswell Park Memorial Institute Medium (RPMI, R8758, Sigma-Aldrich). The flow rate of the cell phase is 1.5 ml/h, while the sheath fluid has a total flow rate of 12.5 ml/h. The sheath fluid is either DMEM with 10% FBS or RPMI with 10% FBS depending on the cell line used. The left sheath fluid comes from the inlet nearest the waste outlet at a flow rate of 4 ml/h, while the right sheath fluid comes from the inlet nearest the keep outlet at a
flow rate of 8.5 ml/h. The right sheath fluid operates at a higher flow rate to flow cells into the waste channel when the IDT is inactive.

The frequency of the RF pulse used to produce TSAWs is tuned at or between 162 and 164 MHz depending on IDT alignment to the sorting junction during device fabrication. This ensures that the acoustic wave is produced at the approximately the same position in the sorting region for each deflection and purity experiment. Unless noted, no cells enter the keep channel unexpectedly for the conditions tested. High speed videos of individual sorting events are analyzed to determine whether a cell is successfully deflected into the keep outlet or not. We centrifuge and resuspend cells collected from the keep outlet channel to obtain higher a concentration for accurate purity counts using the Countess FL II.

We measure cell viability by mixing cells with 0.4% trypan blue stain (Thermo Fisher Scientific, USA) in a volume ratio of 9 to 1. Each sample incubates at room temperature for one minute prior to injection into a disposable cell count board. We then load the injected cell count board into the Countess FL II and record the percentage of unstained cells to the total cell number.
Chapter 4: SAW Driven Droplet Generation, Encapsulation, Lysis, and Pico-Injection
**Introduction**

The ability to generate emulsions/droplets within a microfluidic channel is of significant importance for a variety of applications such as the generation of cell assays at the single-cell or single molecule level, and encapsulation of reagents at micro-, pico- or femtoliter volumes for reactions at the micron scale. This technique, known as droplet microfluidics, has provided an exceptional way to analyze cells independently by encapsulating them within aqueous droplets surrounded by an immiscible liquid, so that the reagents, cells, and reaction products are contained within a controlled microenvironment or ‘reaction-flask’. For the use of droplet microfluidics for single-cell encapsulation, the majority of the work performed in the field relies on the use of passive methods, whereby a population of cells or particles suspended in an aqueous solution (dispersed phase) are flowed into an immiscible fluid (continuous phase) that segregates the aqueous fluid into discrete droplets surrounded by the immiscible fluid. When a cell is surrounded by a portion of fluid that is segmented from the bulk fluid, the cell is encapsulated into a discrete droplet. This method is ideal because it is controlled by flowing the two fluids through channels or nozzles, such that the respective surface energies of the fluid and the channel geometries of the two phases, rapidly generate uniform droplets. This passive method provides a simple way to rapidly produce droplets without incorporating complex external and internal equipment. Nevertheless, this method has limitations that may be unfavorable to the user, such as the number of cells encapsulated per droplet is dictated by Poisson statistics, reducing the proportion of droplets that contain the desired number of cells and thus the effective rate at which single cells can be encapsulated. This method produces many empty droplets, and on occasion droplets that may have more than one cell, particle, or
molecule. Active methods for droplet production have the added advantage that the same forcing mechanism used to displace the aqueous phase into a non-aqueous phase has the potential to act on a solid-liquid interface such as a cell surrounded by fluid.

Surface acoustic wave (SAW) microfluidics, provides a highly versatile mechanism for actively displacing fluids and particles simultaneously; presenting itself as an ideal candidate for droplet generation, encapsulation, and pico-injection. SAW has demonstrated the ability to control the ejection of particles in a single phase and to concentrate particles in solution at a water-oil interface, subsequently creating a droplet of those particles.\(^{103, 104}\) Furthermore, SAW microfluidics is easily integrated into fluorescent activated mechanisms, such as those used in fluorescent activated cell sorting (FACS).\(^{5, 16, 17, 73}\) This combination provides a means for selective and on-demand droplet generation, thus eliminating empty droplets and ensuring droplets only containing the sample of interest are produced.

In this chapter, I present a highly versatile microfluidic device that combines step-emulsification channel geometries and an IDT to generate pulsed SAWs to encapsulate particles or cells on-demand. In addition to cell and particle encapsulation, the device can simultaneously lyse and encapsulate cells by increasing the power and pulse length of the IDT to no longer displace cells without harm. Furthermore, by changing the power level and pulse length of the radio frequency (RF) signal sent to the IDT, I can control the size of a droplet produced by the SAW. Finally, I modify the channel geometries of the device to sequentially inject fluid into droplets.
**Results and Discussion**

The device comprises polymethylsiloxane (PDMS) molded channels with two inlets and two outlets to form two separate fluidic channels plasma bonded to a lithium niobate substrate patterned with a tapered IDT. We use the same tapered IDT designs as described in chapters 1 and 3 to control the position of the SAW. To enhance the bonding between the PDMS and lithium niobate, we coat the lithium niobate with 50 nm layer of silicon dioxide (SiO$_2$). We design the fluidic channels to be parallel to one another and separated by a PDMS wall, except for the region where the IDT is positioned. In this region, we place the IDT in an air gap separated from the fluidic channels by a 50 µm PDMS wall and leave a gap in the fluidic channels for the two fluids to interact when the IDT is actuated, as shown schematically in Fig 25. We flow an aqueous phase (dispersed phase) in the channel closest to the IDT and a non-aqueous phase (continuous phase) into the other adjacent channel. This channel arrangement enables us to take advantage of localized micro-vortices created by the SAW to direct the aqueous phase into the non-aqueous phase to produce droplets only when the IDT produces a SAW into the fluidic channel. The SAW generates acoustic streaming flow and micro-vortices that flow the aqueous phase into the non-aqueous phase. To break the aqueous phase into droplets upon entering the non-aqueous phase, we make the channel of the non-aqueous phase two times the height of the aqueous phase channel to form a step, as illustrated in Fig 26. The abrupt transition permits a droplet to form through a gradient in the Laplace pressure between the two fluids, a technique which has been used previously for step-emulsification devices.$^{105-108}$ More importantly, when there is no step present the device is unable to produce droplets, as shown in Fig 27. The combination of SAW and a step-emulsification channel geometry facilitates on-
demand droplet production. We balance the flow rates of both phases such that droplets are not created unless the IDT is actuated.

To characterize the performance of our device, we actuate the IDT at different power levels and pulse lengths to determine how these parameters control droplet size. In addition to droplet size, we demonstrate a technique to selectively sort and encapsulate samples upon detection using 10 µm beads and K562 cells. This technique demonstrates the utility of our device for selective and on-demand single cell or single-molecule encapsulation. Furthermore, we demonstrate by adjusting the power level of the IDT we can simultaneously lyse and encapsulate cells. Finally, we modify the dimensions of the device to demonstrate the ability to inject fluid into droplets for applications that require the need to add additional reagents to already formed droplets.
Figure 25: Channel Geometry for Droplet Production Applications

(1) Interdigital Transducer (IDT) for surface acoustic wave generation positioned in an air pocket (2) Fluidic channel of an aqueous phase (dispersed phase) (3) Fluidic channel of the non-aqueous phase (continuous phase). The channel is deeper than channel 2 to generate emulsions when the IDT produces a surface acoustic wave (SAW). The dashed line indicates the gap in the channel wall for the emulsions to form.
Figure 26: Overview of Droplet Generation via Surface Acoustic Wave (SAW)

Flow direction is in the x-direction towards the reader. (1) When the IDT is not actuated no droplets are generated and no aqueous liquid flows into the adjoining non-aqueous (in this case oil) channel. (2) The IDT is actuated and generates a SAW into the channel on the aqueous flow side. Upon entering the channel, the leaky SAW forces the aqueous liquid into a taller channel containing oil. (3) The transition from a shallow channel to a taller channel in the form of a step, allows for the aqueous phase to disperse into a droplet surrounded by oil.
Figure 27: Device with No Step

Individual frames from a high-speed video of the droplet generation channel with no step, to demonstrate the need for having a step. IDT is set to 41 dBm with a pulse length of 33 µs. Without the step, droplets are not generated when a SAW is produced in the channel.
On-Demand Droplet Generation via SAW

As described in the previous chapter, when a SAW transitions from air to liquid, the waves refract into the liquid in the form of leaky SAW. This leaky SAW produces longitudinal waves that attenuates within a few micrometers depending on the liquid density and piezoelectric substrate, which in turn establishes an effective body force acting into the fluid medium. The longitudinal waves propagate into the fluid at the Rayleigh angle ($\theta_R$), as shown in Fig 26. The generated body force can create significant acoustic streaming in the liquid and can facilitate pumping and ejection when the acoustic wave is pulsed into the liquid. In our device, the aqueous phase is pumped into a non-aqueous phase across a step to generate droplets, as illustrated in Fig 26.

We set the depth of the aqueous phase channel to 30 $\mu$m and the depth of the non-aqueous phase channel to 60 $\mu$m. Both channels are 70 $\mu$m wide, and the two phases interact across a 65 $\mu$m gap. We use 1X PBS for the aqueous phase and HFE-7500 for the non-aqueous phase, and flow the fluids at 500 $\mu$L/hour and 5 mL/hour, respectively. We determined the flow rates empirically, and found that these flow rates ensure that the aqueous phase does not passively generate droplets or leak into the non-aqueous phase, unless the IDT is actuated. Furthermore, for our device, we find that a flow rate ratio of 1:10 (aqueous:non-aqueous) prevents the aqueous phase from flowing into the non-aqueous phase, unless a SAW is generated into the channel.
Controlling Droplet Size

To determine how to create droplets of different sizes, we actuate the IDT at four different power levels, and test five different pulse lengths for each power. By varying the power level and pulse length we can increase or decrease the size of the droplet produced by the surface acoustic wave, as shown in Fig 28. We find that as both the power level and pulse length increase, the droplet size increases, as shown in Fig 29. As the power level increases the acoustic force generated by the IDT increases, thus displacing more fluid. To flow more fluid across the step and into the adjacent channel, we increase the pulse length of the SAW facilitating more time for acoustic streaming to take place, thereby causing a larger droplet to form. The combined increase of both parameters enables a wide range of droplet sizes to be generated (Fig 29).
**Figure 28:** Controlling Droplet Size

Individual frames exported from a high-speed camera (Hi-Spec) of SAW activated droplet production. (A) IDT set to 37 dBm and a 50 µs pulse length (B) IDT set to 35 dBm and a 67 µs pulse length.
Figure 29: Droplet Diameter (µm) as a function of power (dBm) and pulse length (µs)

As the power sent to the IDT increases the droplet diameter increases. By increasing the pulse length, more aqueous fluid is dispersed into the non-aqueous fluid causing larger drops to form.
Selective On-Demand Droplet Generation for Encapsulation of Samples

The ability to selectively encapsulate single-cells, particles, and molecules into discrete droplets overcomes the random distribution that occurs when passive encapsulation methods are used. These active mechanisms circumvent the limitations posed by Poisson statistics. By combining SAW-based fluorescent activated cell sorting (FACS) techniques as described in the previous chapters, with the channel geometry described in the previous sections, the device can selectively encapsulate samples on demand, ensuring each droplet contains a single-cell or particle, eliminating the need to sort droplets post sample encapsulation.

We flow either a mixture of K562 cells and 10 µm yellow-green fluorescent beads (Spherotech), or a mixture of calcein AM labeled and unlabeled K562 cells suspended in 1X PBS or DMEM, respectively with 10% (v/v) opti-prep at 500 µL/hour through the aqueous phase channel, to demonstrate selective on-demand droplet generation for encapsulation of samples. The cells and fluorescent beads are interrogated by a 488 nm laser, and when a fluorescent bead is excited by the laser, the light emitted by the bead is detected by a photomultiplier tube that then generates a voltage proportional to the intensity of the light emitted by the bead. The voltage is then digitized by a data acquisition card (PCiE-7842R, National Instruments) and analyzed in real time using the card’s field programmable gate array. When peaks corresponding to the beads are detected, a RF pulse is sent to the IDT by a RF waveform generator (SMB100A, Rhode & Schwarz). Before reaching the IDT, we amplify the pulse using a high gain RF amplifier (LZY-22+, Mini-Circuits). The amplified RF pulse delivered to the IDT generates a SAW, which then deflects and encapsulates the fluorescently labeled cell or bead into a water-in-oil droplet, as
illustrated in Fig. 6. For the non-aqueous phase, we flow HFE·7500 at 5 mL/hour through the non-aqueous channel. A schematic of the device used for these experiments is shown in Fig. 31.

To characterize the performance of our device for selective and on-demand encapsulation, we flow K562 cells and yellow-green fluorescent 10 µm beads at a total sample concentration of approximately 1x10^6 particles/mL, to detect and encapsulate only the fluorescent beads. To ensure that beads are being encapsulated into water-in-oil droplets upon detection we record high-speed videos in real time to make adjustments to the delay time between detection and IDT actuation as well as the power level and pulse length of the IDT, as shown in Fig 32. We find that the minimum power level and pulse length of the IDT to successfully deflect and encapsulate the beads to be 39 dBm and 50 µs, respectively. We collect the droplets generated by the device and measure the diameter and find the droplet size to be approximately 35 µm on average, as shown in Fig. 33. We also take images and count the concentration of K562 cells and 10 µm beads for each run that were not encapsulated into droplets, as shown in Fig 34.
Figure 30: Overview of Selective or On-Demand Encapsulation

(1) Cell flows through aqueous channel. (2) Fluorescent labeled cell is excited and detected by a laser. (3 and 4) Upon detection the IDT is actuated to produce a SAW to simultaneously deflect the cell and stream the aqueous liquid surrounding the cell into the adjoining oil channel. (5) When the IDT is turned off the aqueous liquid surrounding the cell is pinched off to form a droplet. (6) The encapsulated cell flow out of the device through the oil channel and the next flows past the laser.
Figure 31: Schematic of Microfluidic Device Used for Selective and On-Demand Generation and Encapsulation of Samples
**Figure 32:** 10 µm Bead Deflected and Encapsulated by SAW

Individual frames exported from a high-speed video of a 10 µm bead being deflected and encapsulated upon detection. (1) 10 µm bead (highlighted by blue circle) flows past a 488 nm laser (not visible) and is detected. (2) The IDT is actuated and produces a surface acoustic wave (SAW) into the channel to deflect and encapsulate the bead (yellow box). (3) A droplet is created containing the detected bead (blue circle) and an incoming K562 cell enters the channel (red circle). (4 and 5) Droplet containing the bead (blue circle) flows out of the device through the non-aqueous channel and the K562 cell (red circle) continues to flow past the IDT and is not encapsulated (Not shown).
Figure 33: 10 µm Fluorescent Beads Encapsulated in Droplets

Microscope images of beads sorted and encapsulated using SAW. The droplet diameters are approximately 35 µm. The droplets are collected from the device and imaged on a microscope.
**Figure 34:** Microscope Image of Sample Collected from the Waste Outlet

Microscope image of sample collected from the waste outlet. K562 cells (black circle) are not labelled and treated as a non-target or undesired sample. The fluorescent beads (green) are present in the waste indicating that not all beads were properly deflected and encapsulated.
To assess the sorting and encapsulation purity performance of our device, we compare two sample conditions, one where the percentage of beads in the total sample is 10% and the other is 5%. We count the percentage of droplets containing the 10 µm beads from the total population of droplets containing beads and cells. We also quantify the depletion performance of the device by measuring the percentage difference of beads in the original sample from the percentage of beads in the waste divided by the percentage of beads in the original sample. We find the purity for the 10% and 5% initial target sample population to be 83% ± 3.5% and 88% ± 4.5%, as shown in Fig 35. Furthermore, we find the depletion for 10% and 5% initial target sample population to be 86% ± 4% and 95% ± 5%, as shown in Fig 36.
Figure 35: Purity Performance of Selective Encapsulation

Total sample concentration is approximately $1 \times 10^6$ particles per mL. Target and non-target population consists of yellow-green fluorescent 10 µm beads and K562 cells, respectively.
**Figure 36:** Depletion Performance of Selective Encapsulation

Total sample concentration is approximately $1 \times 10^6$ particles per mL. Target and non-target population consists of yellow-green fluorescent 10 µm beads and K562 cells, respectively.
Lysis and Encapsulation of Cells

The ability to study cells at the molecular level is important for the investigation of genome sequencing to understand diseases and develop drugs. A common method to lyse cells involves the incorporation of chemicals that break down the cell membrane and or cell wall to release the components and can be incorporated with droplet microfluidics. While chemical based lysis methods are quite successful, they may interact with the sample in a way that interferes with molecular targets, such as DNA, RNA, or proteins that are to be analyzed or sequenced. Therefore, there is a need to develop a chemical-free method to lyse cells based purely on mechanical methods. Furthermore, the ability to lyse and encapsulate one cell at a time will enable researchers to study cell heterogeneity, understand antibiotic resistant bacteria, sequence cancer cells, and more. In this work, for the first time ever reported, I exploit the mechanical action of the surface acoustic wave (SAW) to lyse cells in a chemical-free manner while simultaneously encapsulating the lysed cell.

To achieve this, I use the same device described in this chapter but I adjust the power level and pulse length of the IDT to not only deflect the cell but to break open its membrane as it is encapsulated, as shown in figure 37. To verify cell lysis, we compare two different power levels of the IDT, and image the contents of the droplets, as shown in figure 38. We find that increasing the power level can determine whether or not a cell is lysed or not during the SAW actuated droplet encapsulation mechanism as discussed in earlier in this chapter. For all lysis experiments we use the tapered IDT design and find power levels under 42 dBm do not damage or lyse cell, while power levels above 42 dBm cause lysis.
**Figure 37:** Single-Cell Lysis and Encapsulation

Individual frames from high-speed video of calcein AM stained K562 cell lysed and encapsulated into a water-in-oil droplet. (1) Fluorescent labeled K562 cell (red circle) is detected (2) Upon detection, the IDT is actuated generating a surface acoustic wave (teal box) lysing the cell and generating a droplet. (3) The lysed cell is encapsulated as the droplet (blue circle) is formed. (4-6) Droplet containing lysed (blue circle) exits the device. Fragments of the cell can be seen in the droplet as it flows out down the channel.
Figure 38: (Left) Lysed K562 Cell in a Droplet (Right) K562 cell in a droplet

(Left) K562 cell lysed and encapsulated when the tapered IDT generates a SAW with a power level of 42 dBm. Fragments of the cell are visible and the entire droplet is fluorescent as a result. (Right) K562 cell encapsulated in a droplet when the IDT power level is kept below 42 dBm.
SAW Driven Pico-Injection of Droplets

One of the most important techniques used in droplet microfluidics is the ability to controllably add reagents to individual droplets also known as pico-injection. A popular and robust technique for pico-injection is the use of electric fields which can destabilize the water-oil interface of a water-in-oil droplet, allowing a reagent to enter the droplet.\textsuperscript{115} The interface ruptures due to an electrically induced thin-film instability which then causes a pressure difference across the droplet and the reagents to be injected.\textsuperscript{115-117} While this method of pico-injection is popular for its ability to inject controllable volumes of reagents into droplets at kilohertz speeds, there are no alternative methods that can achieve these controllable injection at such rates. Using similar channel geometries and our surface acoustic wave (SAW) techniques for droplet generation and sample encapsulation, we provide a SAW based method for injecting reagents into water-in-oil droplets. Here, we use the same general device design as described previously in this chapter, but eliminate the step geometry, and flow droplets through non-aqueous channel, and fluorescent dye through the sample channel.

We design the microfluidic channels similar to the one shown in Fig 31, but include a T-junction droplet maker and make the non-aqueous channel the same depth as the sample channel, as shown schematically in Fig. 39. The width and depth of the channels are 70 µm and 30 µm, respectively. We flow 1X PBS into the droplet/sample inlet, HFE-7500 fluorinated oil into the oil inlet to generate droplets, and green fluorescent dye into the reagent/injection inlet at 125 µL/hour, 500 µL/hour, and 2mL/hour, respectively. Downstream of the inlets, we introduce a 55 µm gap in the channel walls that separate the reagent/injection channel from the
adjacent droplet channel. At this region we include an air-pocket adjacent to the reagent/injection channel separated by a 50 µm PDMS wall where the IDT is positioned. We then record high-speed videos and estimate the rate of droplets passing the region/gap in the channels where the green fluorescent dye is in contact with the droplets, and find the rate to be 125 droplets per second. Next, we set the IDT to generate a surface acoustic wave 125 times per second into the channel where the green fluorescent dye is in contact with the droplets to inject green fluorescent dye into each droplet that is made. For our chip design, we find that a power level and pulse length of 41.5 dBm and 33 µs, respectively, is sufficient to break the surface tension of the droplet and introduce fluorescent dye into the droplet, as shown in Fig 40. To verify this, we collect the droplets made and image with a confocal microscope, while exciting the droplets with a 488 nm to observe the number of droplets that contain fluorescent dye. We also keep the IDT off for a short duration to ensure that droplets containing no dye are produced to confirm that droplets only receive fluorescent dye upon interaction with the surface acoustic wave, as shown in Fig. 41 We find the droplets without dye to be approximately 75 µm and observe droplets with fluorescent dye to be approximately 86 µm in size. The increase in size is due to the addition of fluorescent dye into the droplet.
Figure 39: Schematic of SAW Pico-Injection Chip
**Figure 40:** Individual Frames from High-Speed Video of SAW Based Pico-Injection

High-speed videos are obtained of droplets being injected with green fluorescent dye. (1) Droplet flows through channel (C₂) and approaches a gap in the channel where fluorescent dye is present (C₁). (2) Droplet is now in the region where the fluids of Channels C₁ and C₂ are in contact with one another. (3) The IDT generates a SAW into the channel (red box) breaking the surface tension of the droplet and enabling green fluorescent dye to enter the droplet. (4-5) The SAW is turned off and the droplet continues to flow past the gap and begins to break off from the fluorescent dye. (6) The droplet breaks off and the next droplet begins to enter the frame.
**Figure 41:** Droplets Containing Green Fluorescent Dye from SAW Pico-Injection

Droplets collected from the device described in Figures 39 and 40. Images are taken using a confocal microscope with a 488 nm laser to excite and visualize the fluorescent dye.
Conclusion

The microfluidic device presented in this chapter demonstrates a variety of droplet-based microfluidics applications that SAW can be used for. We demonstrate the ability to selectively sort and encapsulate samples on-demand into droplets without producing empty droplets, thus eliminating the need to sort through droplets of interest after they are made. We adjust the power level of the IDT to individually lyse and encapsulate cells for applications that require molecular analysis, sequencing, etc. Furthermore, we modify the channel geometries of the device to inject reagents into droplets for applications that require but are not limited to: chemical and biological screening and genetic sequencing, addition of reagents for chemical processes, single-cell studies, and multistep reactions that require adding new reagents at different times. By taking advantage of the acoustic streaming and step-emulsification we can actively control droplet production, and simultaneously encapsulate samples into droplets.
Chapter 5: Microfluidic Device for In-Order Storage and Release of Multiple Droplets
Introduction

Droplet microfluidic devices generate and manipulate discrete micron-scale droplets with numerous applications in biology and chemistry \textsuperscript{115, 118, 119}. For example, droplet microfluidics have enabled biological and chemical assays to be compartmentalized into individual “reaction flasks” that can be transported, mixed, and analyzed, providing strong advantages such as the ability to screen genomics of single cells, study protein crystallization, and reduce reagent consumption \textsuperscript{51, 119-122}. Droplet microfluidics offer several major advantages: rapid detection of reagents down to the single-molecule level; monodispersity, that each droplet can have the same volume; and high throughput. The high frequency generation of similarly-sized droplets makes possible the production, processing, and analysis of large data sets that facilitate new laboratory and industrial uses \textsuperscript{102}. In particular, the ability to isolate and screen individual droplets within a single device is especially important for applications that require down-stream incubation steps, and optical measurements. One of the earliest on-chip methods for isolating droplets of interest is hydrodynamic confinement \textsuperscript{123}, which adjusts the hydraulic resistance of the microchannels to store droplets into individual compartments \textsuperscript{123, 124}. When one droplet enters a compartment, the hydraulic resistance of the channel changes such that the next droplet bypasses the now-occupied compartment and is directed to the next immediate compartment. This process repeats until every compartment is filled sequentially with a single droplet \textsuperscript{123}. These hydrodynamic confinement devices store one droplet per compartment, limiting the number of droplets that can be stored on a single device. To screen far larger numbers of droplets for high-throughput applications, the ability to store multiple droplets in each compartment is needed.
In this chapter, I demonstrate a microfluidic device that produces, stores, and can release up to hundreds of droplets in the order they were produced, in groups of up to several dozen in a series of identical storage compartments. To determine the geometric parameters to store a controllable number of droplets in each compartment, we present a method based on an analogy between the hydraulic resistance of fluid flowing in microfluidic channels and the electrical resistance of a circuit.

**Results and Discussion**

Existing devices that store single droplets include identical storage units, in which the flow is split into two channels: a bypass channel, and a storage channel that contains the storage compartment that drains through a pore, as shown in the schematic in Fig. 42A. Hydrodynamic confinement requires lower hydraulic resistance through the storage channel than through the bypass channel to direct a droplet into the storage compartment of the first empty storage unit it encounters. When a droplet enters the storage channel, it blocks the pore, obstructing the flow through this channel; the next droplet will flow through the bypass channel and into the storage channel of the next empty storage unit. In this single pore storage device, the flow ratio \( \frac{Q_S}{Q_B} \) is determined by:

\[
\frac{Q_S}{Q_B} < \frac{c_B(\alpha_B)}{c_S(\alpha_S)} \cdot \frac{L_B}{L_S} \cdot \left( \frac{H+W_B}{H+W_S} \right)^2 \cdot \left( \frac{W_S}{W_B} \right)^3
\]

EQ (8)

Where H, W, and L are the channel height, width, length, respectively, \( C(\alpha) \) is a function of the aspect ratio \( \alpha \), and subscripts S and B refer to the storage and bypass channels, respectively. When the \( Q_S > Q_B \), more flow is directed through the storage channel than through the bypass channel, allowing a droplet to be trapped.
The device contains multiple identical storage units, which each store multiple droplets. Here, we replace the single pore of the storage channel with a wall of pores that maintains $Q_S$ above $Q_B$, even as droplets accumulate until the final droplet blocks the last pore; subsequent droplets flow through the bypass channel and into the next storage unit, as shown in Fig. 42B. We control the number of droplets that fill each storage compartment by setting the number of pores and the volume of the storage channel.

To design the geometry of the porous wall, we draw an analogy between the hydraulic resistance of the flow of liquid through the channels of our microfluidic device and the electrical resistance of an electric circuit, which relates flow rate, pressure drop, and hydraulic resistance to current, voltage, and electrical resistance, respectively. In our device, the hydraulic resistance through the storage channel includes three components which add in series: the resistances of the storage compartment, the wall of pores, and the exit, as shown in Fig. 42C, which are each determined by:

$$R_H = \frac{12 \cdot \mu \cdot L}{W \cdot H^2 \left(1 - \frac{H}{W} \sum_{n=1,3,5}^{192} \tanh \left(\frac{n \cdot \pi \cdot W}{2 \cdot H}\right)\right)}$$

EQ (9)

where $n$ is the number of pores, which is set by convention to one for the bypass, storage, and exit channels. We demonstrate a device comprising multiple storage units of the type shown in Fig. 42B, which includes five pores in each storage channel, to store up to five 50 µm droplets per storage compartment. To prevent the droplets from easily flowing or squeezing through the porous wall, we set the width and length of the pores to 10 µm and 50 µm, respectively, and keep all channel depths at 50 µm. We find that keeping the pore five times smaller than the diameter of the droplet and setting the length of the pores equal to the droplet diameter prevents droplets from escaping. To operate the device, we use a
custom-built pressure driven flow system, and flow 1X PBS (PBS; Mediatech) and HFE-7500 (3M™ Novec™ Engineered fluid) with 3% (w/w) fluorinated surfactant (RAN Biotechnologies, Inc.) as the aqueous and continuous phase respectively. We generate water-in-oil (w-o) droplets with a flow-focusing junction\textsuperscript{125-127}, which flow through a channel into a series of storage units. When the first droplet enters the first storage unit, it flows into the storage channel because of lower hydraulic resistance, and blocks the pore furthest from the entrance. As subsequent droplets enter the storage unit, they also flow into the storage channel, blocking additional pores; the flow rate through the storage channel remains higher than that through the bypass channel until all of the pores are blocked by droplets. The next droplet then flows through the bypass channel, and into the next storage unit, where the process repeats, as shown in Fig. 43.
Figure 42: Overview of Passive Droplet Storage

(A) Single droplet storage: the hydraulic resistance of the storage channel remains lower than the bypass channel until a pore is blocked by a droplet. (B) Storage unit for multiple droplets, with a storage channel comprising a storage compartment, a porous wall, and an exit. (C) Circuit model for storage unit. Blue line refers to the hydraulic resistance of the bypass channel, the red line and green lines refer to hydraulic resistance of the storage compartment (SC) and exit (EX), respectively. Each of the purple lines refer to the hydraulic resistance of the pores that form the porous wall (PW).
Figure 43: Storage of four to five droplets in each storage unit, observed in bright-field with a fast camera on a microscope

At $t = 0$ ms, a droplet enters the first storage compartment of the device, which fills completely by $t = 500$ ms; at that time, the second compartment already contains one droplet. In between $t = 500$ ms and $t = 1000$ ms, the stage of the microscope was moved slightly to the right, so that only half of the first storage unit is still visible. At $t = 1000$ ms, the first two storage compartments are completely filled with droplets and the third storage compartment begins to fill with droplets. At $t = 1500$ ms, the stage was again moved slightly to the right, so that the second storage unit is the first visible unit: the third storage compartment is completely filled, while the fourth storage compartment loads.
To determine the scalability of our method, we vary the channel and pore dimensions in accordance with equation 2 to increase the number of droplets stored in each compartment to twenty-five and above, as shown in the device in Fig. 44. To prevent the last stored droplets from being pushed through the pores, we reduce the length of the first two pillars closest to the entrance of the storage channel, as shown in the figure. The inclusion of an additional pore to bypass the exit allows the controlled in-order release of droplets by changing the flow rates; after the aqueous phase is shut off, thereby generating no new droplets, increasing the flow rate of the continuous phase by a factor of two expels the stored droplets in the order they accumulated, as shown in Fig. 45. This additional pore does not otherwise affect droplet storage at normal flow rates.

Conclusion

I demonstrate a microfluidic device that can store up to dozens of droplets in each of many storage units, up to hundreds of droplets per device. Our storage and controllable release of all droplets maintain their order. The device may be useful for applications that require droplet samples to be stored, incubated and/or observed after production.
Figure 44: Microscope Image of a Larger Storage Unit

Storage of twenty-seven 50 µm droplets in a single storage unit. The length of the two pores closest to the storage channel entrance are truncated to prevent droplet escape.
We increase the flow rate of the continuous phase to push droplets out in the order they were stored. (A) Under normal operating flow rates, droplets are stored indefinitely. (B) Increasing the flow rate of the continuous phase forces droplets to deform through the horizontal pore. (C and D) Droplets escape through the pore into an outlet.

**Figure 45:** Dispensing Droplets Sequentially Through an Additional Pore
Materials and Methods

Soft Lithography

The polydimethylsiloxane (PDMS) molded microfluidic channels of the device comprise a single layer, fabricated using a photomask. We perform lithography to create molds for PDMS replicas. We process the layer of channels by following the method recommended in the manufacturer’s data sheet for SU-8 3000 series photoresists (MicroChem Corp., Westborough, MA). We dispense a small amount of SU-8 3050 photoresist (MicroChem) onto the silicon wafer. We spin the wafer at 3000 rpm to create a layer that is 50 μm thick. We pre-bake each layer on a hot plate at 95°C for a total of 20 minutes. We use a contact mask aligner (ABM, Scotts Valley, CA) to align and pattern with UV light any underlying features to the photomask (CAD/Art Services Inc., Bandon, OR). We then post-exposure bake the resist for 1 minute at 65°C followed by 5 minutes at 95°C, then immerse the wafer in polyethylene glycol monomethyl ether acetate (484431, Sigma-Aldrich Co. LLC, St. Louis, MO) for 6 minutes using an orbital shaker (Roto Mix 8x8, Thermo Fisher, Waltham, MA) for mixing. After development, we rinse the wafer with isopropanol and blow dry it with compressed nitrogen.

We mix PDMS (Slygard 184, Dow-Corning, Midland MI) base and cross-linker in a 10:1 weight ratio using a Thinky mixer (AR-100, Thinky Corp., Tokyo, Japan). We de-gas the PDMS for 20 minutes and cure the mold in the oven at 65°C overnight to create a replica. We cut the PDMS replica into individual devices prior to use. We create inlet- and outlet holes with a 1.2 mm diameter biopsy punch (Uni-Core, GE Healthcare Life Sciences, Pittsburgh, PA). Next, we bond the individual molds to glass slides using an oxygen plasma
stripper (PE-50, Plasma Etch, Carson City, NV). The PDMS mold forms three sides of the device’s flow channel, while the glass slide serves as the bottom of the flow channel. After bonding we treat the PDMS-glass channels with 2% (v/v) Trichloro (1H,1H,2H,2H-perfluorooctyl) silane (Sigma-Aldrich) in HFE-7500 to alter the surface wettability to hydrophobic, followed by flushing the channels with the continuous phase to remove excess silane.

Device Operation

For droplet generation we use HFE-7500 3M™ Novec™ Engineered fluid (HFE-7500: 3M) with 3% (w/w) surfactant (RAN Biotechnologies, Inc.) as the continuous phase and 1X solution of Phosphate-Buffered Saline (PBS; Mediatech) for the aqueous phase. We flow both phases into the microfluidic device using a custom pressure controlled driven system. In this system we fill two syringes that are separately connected to two separate gas regulators. We adjust the flow of the phases by adjusting the pressure in the syringes to produce 50 µm droplets, cease droplet production, or dispense droplets. We mount the device onto a microscope (Nikon Eclipse Ti2 Series) with a fast camera (Hi-Spec 1) and record time-lapse images of droplets storage and dispensing.
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