APPLICATION OF ACOUSTOFUIDIC TECHNOLOGIES IN CELL BIOLOGY

A Dissertation in
Molecular, Cellular and Integrative Biosciences

by

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ABSTRACT

The advances in cell biology have benefited from the invention of new technologies. Over the past two decades, microfluidics has emerged as a powerful platform for cell analyses and studies. In particular, acoustofluidic technologies offer some attractive features and have great application potential in cell biology and its related fields. This dissertation presents the development of acoustofluidic or microfluidic platforms that tackle some current challenges faced in cell biology. First of all, an acoustofluidic-based multichannel droplet sorter is demonstrated that can effectively sort picoliter droplets into multiple outputs. The future development of a fluorescence-activated droplet sorter (FADS) can realize the screening and sorting of cells based on secreted biomarkers. Secondly, an acoustofluidic-based, automatic, and continuous-flow cell washing device is developed and its application in the transfer of inflammatory cells from human sputum samples is reported. This acoustofluidic device preserves the viability and integrity of transferred inflammatory cells and effectively removes residual dithiothreitol (DTT) utilized during sputum liquefaction to facilitate more accurate identification of inflammatory lung cells from human sputum samples. Thirdly, a cell co-culture platform is developed that can form organized cell co-culture for studying heterotypic cell-cell interactions. The study of cancer cell mobility within co-culture demonstrates its utilization as an in vitro platform for the evaluation of the invasiveness of cancer cells and anti-tumor drug screenings. At last, a single-cell virology platform is developed that enables high-throughput, real-time, and automated observation of single-cell viral infection dynamics. The model studies of poliovirus have revealed enormous stochastic behavior that likely plays out in vivo that contributes to post-infection outcomes. The single-cell level investigations of viral infections unravel the delayed replication of an attenuated poliovirus mutant and provide insight into the evolutionary dynamics of viruses under selection pressure of antiviral drugs.
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Chapter 1

Introduction

The introduction of micro/nano-technologies into biology can often revolutionize the way biological research is conducted. For example, the invention of DNA microarray technology has greatly stimulated the study of gene expression profiling. Over the past two decades, the advance in microfluidic technologies has been creating powerful tools for cell biologists to realize automatic processing of cell samples; precise control of cellular microenvironment; reconstruction of multicellular organization; and high-throughput analysis of single cells. Solving these challenges that are encountered in conventional methods can potentially pave the way to new discoveries in cell biology and its related fields. Recently, acoustofluidic (i.e., fusion of acoustics and microfluidics) technologies have opened a new frontier in microfluidics with great application potential in cell biology. Acoustofluidic technologies use non-invasive, non-contact acoustic forces to achieve precise, tunable, and automatic manipulation of cells. These attractive features make acoustofluidic technologies a good candidate in fulfilling many unmet needs in cell biology. In this chapter, I will first review some current technological challenges in cell biology that have motivated my dissertation work. An introduction to acoustofluidic technologies will then be given followed by an overview of the organization of my dissertation.

1.1 Motivation

Discoveries in biology are driven by our curiosity about the mysterious and fascinating biological world. However, our endeavors in biological research are sometimes limited by the technologies available. This situation spurs the contributions from other fields like mathematics,
physics, chemistry, and engineering to developing novel biological tools, which push forward biological research and can have revolutionary impact on the field. For instance, the invention of flow cytometry enabled the high-throughput characterization of single cells and had tremendous impact on cell biology and clinical diagnostics.

During the past two decades, microfluidics — the manipulation of small amounts of fluids in micrometer-scale channels — has emerged as an important platform for chemical, biological, and medical research. Additionally, microfluidic systems have dimensions comparable to the sizes of cells, which makes them especially useful tools in cell biology. Numerous microfluidic platforms have been developed for automatic processing of cell samples (e.g., separation, sorting, purification, and lysis), precise control of cellular microenvironment (e.g., chemical, mechanical, and temperature), on-chip cell culture/co-culture, and single-cell analysis. Despite the attractive capabilities these platforms offer over conventional approaches, their application in biological laboratories has been relatively lacking. This limited application of microfluidic platforms in biology is partially attributed to the gap between engineering and biology and the disconnection between platform development and utilization by end-users. As a Ph.D. student receiving multi-disciplinary training in both a microfluidics lab and a biology lab, I see this as an opportunity for me to push the application of microfluidic platforms in cell biology and its related fields.

My dissertation work is motivated by the following technological challenges faced in cell biology and its related fields:

**Automatic processing of cell samples:** Processing of cell samples is an indispensable step in cellular research. In many cases, the cell samples are heterogeneous and require separation or sorting steps to isolate the cells of interest. Fluorescence-activated cell sorter (FACS) has been widely used in cell biology and clinical diagnostics to isolate target cells based on surface biomarkers. However, this dependency on surface biomarkers hinders the utilization of FACS in
isolating cells based on secreted biomarkers (e.g., screening of B cells based on antibody production). This issue can be solved by encapsulating single cells into individual droplets so that the secreted biomarkers can be fluorescently labeled and detected. The subsequent selection of droplets containing the cells of interest from the pool requires effective droplet sorting technique, which has motivated my work described in Chapter 2. Cell washing or transfer is another routine sample preparation procedure used in various cell biology studies. Conventionally, cell washing or transfer is realized through centrifugation, which has several drawbacks such as high shear stress, bulky instrument, manual sample handling, and discontinuous fluid flow. These limitations have motivated my work described in Chapters 3 and 4 to develop biocompatible, miniaturized, automated, and continuous-flow cell washing or transfer platforms.

**Investigation of heterotypic cell-cell interactions:** Cell culture is the basic technique in cell biology. Cells residing in their *in vivo* niches can interact with other cell types and *in vitro* co-culture of different cell populations is desired to reconstruct such heterotypic cell-cell interactions. Traditionally, different cell populations are randomly mixed and grown together on cell culture plates to form cell co-cultures. However, the organization of cell co-culture arising from random mixture is governed by spontaneous rearrangement of cells with little control on the final cell pattern. Precise reconstruction of heterotypic cell-cell interactions *in vitro* requires the co-culture of different cell types in a highly controlled manner. This demand has motivated my work described in Chapter 5.

**Investigation of heterogeneous virus-cell interactions:** Within a population of genetically identical cells, the phenotype of each cell varies due to differential gene expressions. Given the impact of cell cycle on gene expression, even greater complexity is created when a population of cells is growing asynchronously. On the other hand, virus population, especially RNA viruses, consists of genetically distinct variants due to mutations during viral replication. As a result, the pathogenesis of viral infection is determined by heterogeneous responses from single cells.
However, such heterogeneous virus-cell interactions are obscured when studied at the population level — *i.e.*, by monitoring the response of a population of cells to a challenge by a population of viruses. Therefore, new technologies to study viral infection dynamics at the single-cell level are of great interest, which has motivated my work described in Chapter 6.

To solve the challenges listed above, I have focused on developing different microfluidic platforms during my Ph.D. study. In recent years, standing surface acoustic wave (SSAW)-based acoustofluidic technologies have received significant attention in the microfluidic community with great application potential in cell biology. The SSAW technique uses non-invasive, non-contact acoustic forces for cell manipulation and has the following appealing features:

**High biocompatibility**: The SSAW technique uses non-invasive acoustic forces for cell manipulation. The input power and frequency of the acoustic wave are in a range similar to those used in ultrasonic imaging during pregnancy, which has been proven to be extremely safe. Therefore, the acoustofluidic platforms developed during my Ph.D. study are safe and preserve the viability and integrity of the cells and the ability of the cells to proliferate.

**Automatic and continuous-flow operation**: The SSAW technique can manipulate cells in continuous-flow and the operation of the device can be automatically controlled by syringe pumps and function generators. These features make it possible for the acoustofluidic platforms to be incorporated with other microfluidic platforms to realize fully integrated and automatic systems for cell analyses and studies.

**Non-contact and label-free cell manipulation**: The SSAW technique uses non-contact acoustic forces for cell manipulation. This non-contact cell manipulation is advantageous over contact-based approaches (*e.g.*, micropipette aspiration of cells) to avoid potential impact on the cell membrane. The SSAW-based cell manipulation does not rely on pre-labelling of cells (*e.g.*, magnetic labelling) but instead makes use of cells’ intrinsic physical properties (*e.g.*, size,
density, and compressibility). This label-free feature simplifies sample preparation procedure and allows the cell to be processed and studied on its native form.

**Good tunability:** In the SSAW technique, the formed SSAW field can be finely tuned by changing the frequency or phase of the two surface acoustic waves (SAWs)\(^7,8\). This gives the SSAW technique very good tunability in cell manipulation.

These recognized features of the SSAW technique give it great application potential in cell biology and its related fields, which will be elaborated in the following chapters of my dissertation.

1.2 Introduction to the SSAW-based acoustofluidic technologies

1.2.1 Generation of SAWs

The acoustofluidic technologies used in my dissertation work rely on the generation of SAWs (Fig. 1-1). A typical SAW device is fabricated by depositing interdigital transducers (IDTs) on the surface of a piezoelectric substrate, which can generate electric potential in response to mechanical stress, or vice versa. When a radio frequency (RF) signal of certain frequency is applied to the IDTs, the piezoelectric substrate vibrates and generates propagating mechanical stress. This mechanical vibration results in the propagation of acoustic waves along the surface of the piezoelectric substrate. The wavelength of the generated SAWs is determined by the period of the IDTs (\(i.e., \) the distance between two repeating electrodes) while the amplitude of the generated SAWs can be changed by tuning the input power of the RF signal.
Figure 1-1. Generation of SAWs. SAWs propagating along the surface of the piezoelectric substrate in both directions are generated when an RF signal is applied to the IDTs.

### 1.2.2 Formation of the SSAW field

Acoustofluidic manipulation of cells is realized through the formation of the SSAW field (Fig. 1-2). When a pair of IDTs is aligned in parallel on a piezoelectric substrate, two identical SAWs propagating in opposite directions can be generated when an RF signal is applied to both IDTs. When the travelling SAWs encounter the fluid inside the microchannel, they generate longitudinal-mode leakage waves. The interference between them forms a SSAW field inside the microchannel, with a periodic distribution of pressure nodes (with minimum pressure amplitude) and pressure antinodes (with maximum pressure amplitude). Cells present in the SSAW field experience an acoustic radiation force \( F_r \) that can be expressed as

\[
F_r = -\left( \frac{\pi p_0 v_c \beta_f}{2 \lambda} \right) \phi(\beta, \rho) \sin(2kx),
\]

\[
\phi(\beta, \rho) = \frac{5\rho_c - 2\rho_f}{2\rho_c + \rho_f} - \frac{\beta_c}{\beta_f}.
\]
where $p_0$, $V_c$, $\lambda$, $k$, $x$, $\rho_c$, $\beta_c$, and $\beta_f$ are acoustic pressure, volume of the cell, SAW wavelength, wave vector, distance from a pressure node, density of the cell, density of the fluid, compressibility of the cell, and compressibility of the fluid, respectively. Eq (2) describes the acoustic contrast factor, $\phi$, which determines whether the cells move to pressure nodes or antinodes in the SSAW field: the cells will aggregate at pressure nodes if $\phi$ is positive and antinodes if $\phi$ is negative. Cells suspended in normal culture medium or buffer solution have positive acoustic contract factors and will be pushed towards the pressure nodes in the established SSAW field. This is the basic mechanism of cell manipulation under static condition.

Figure 1-2. Cell patterning in the SSAW field. A cross-view schematic of the formation of the SSAW field inside a microchannel is shown. Cells are pushed to the pressure nodes (PN) in the established SSAW field.

When there is flow inside the microchannel, cells also experience the Stokes drag force ($F_d$) which can be expressed as

$$F_d = -6\pi \eta R_c u_r,$$

where $\eta$, $R_c$, and $u_r$ are viscosity of the fluid, radius of the cell, and relative velocity of the cell, respectively. In this flowing condition, the movement of cells in the SSAW field is governed by the effect of the acoustic radiation force and the Stokes drag force and will be further discussed in Chapter 3.
1.3 Organization of the dissertation

In this dissertation, our research efforts on developing different microfluidic platforms to solve current technological challenges in cell biology and its related fields are discussed. Chapters 2-6 are expanded versions of published, submitted or soon to be submitted work. Each chapter begins with a short introduction section to specify the objectives of that particular chapter and a brief literature review to distinguish our work from existing work.

In Chapters 2-4, we describe acoustofluidic platforms related to automatic processing of cell samples. An acoustofluidic device capable of sorting picoliter water-in-oil droplets into multiple outputs is described in Chapter 2. An automatic, continuous-flow cell washing device and its application in the transfer of inflammatory cells from human sputum samples are described in Chapters 3 and 4.

In Chapter 5, we present a cell co-culture platform for the investigation of heterotypic cell-cell interactions.

In Chapter 6, we present a single-cell virology platform for the investigation of heterogeneous virus-cell interactions.

Finally in Chapter 7, a summary of my dissertation work is given and potential directions for future research are discussed.
Chapter 2

Acoustofluidic-based multichannel droplet sorting

The encapsulation of single cells in isolated droplets has been a useful tool for studying small molecules or proteins secreted from single cells. The screening of encapsulated cells requires effective on-chip handling and sorting of droplets of interest. In this chapter, we report an acoustofluidic device that is capable of sorting picoliter water-in-oil droplets into multiple outputs using standing surface acoustic wave (SSAW). This device integrates a single-layer microfluidic channel with interdigital transducers (IDTs) to achieve on-chip droplet generation and sorting. Within the SSAW field, water-in-oil droplets experience an acoustic radiation force and are pushed towards the acoustic pressure node. As a result, by tuning the frequency of the SSAW excitation, the position of the pressure nodes can be changed and droplets can be sorted to different outlets at rates up to 222 droplets s\(^{-1}\). With its advantages in simplicity, controllability, versatility, non-invasiveness, and capability to be integrated with other on-chip components such as droplet manipulation and optical detection units, the technique presented here could be valuable for the development of droplet-based cell screening platforms.

2.1 Introduction

In recent years, droplet-based microfluidic platforms\(^{10,11}\) have demonstrated tremendous potential in many chemical and biological assays due to their advantages of low sample consumption, high throughput, short mixing time, elimination of cross contamination, and capacity to be integrated with other lab-on-a-chip devices\(^{12-17}\). In particular, the high throughput inherent in droplet microfluidics makes it extremely promising for various chemical or biological
screenings.\textsuperscript{18−24} Droplet-based screening largely relies on precise isolation of individual droplets of interests from other droplets; thus it is essential to develop effective, on-chip droplet sorting techniques. Passive sorting methods such as hydrodynamic self-sorting\textsuperscript{25} and deterministic lateral displacement-based sorting\textsuperscript{26} make use of the size difference between droplets, but they are unable to sort droplets with similar sizes. Meanwhile, many active droplet sorting techniques have been developed in recent years.\textsuperscript{27−42} Among the existing micro-object manipulation techniques, acoustic-based approaches offer significant advantages in compactness, versatility, and non-invasiveness. Recently, low-power acoustic waves have been extensively utilized in micro-object (\textit{e.g.}, bubble, particle, cell, organism, and droplet) manipulation due to their harmless nature to biological objects.\textsuperscript{41−68} In our previous studies, we have used standing surface acoustic waves (SSAW) to achieve focusing, separation, sorting, and patterning of microparticles and cells in microfluidics.\textsuperscript{69−75}

In this chapter, we report a SSAW-based, on-chip, multichannel droplet sorting technique that takes advantages of the tunability and control\textsuperscript{74,75} offered by chirped interdigital transducers (IDTs). Our SSAW-based approach is fundamentally different from the recently demonstrated traveling surface acoustic wave (TSAW)-based droplet-sorting technique\textsuperscript{42}: our SSAW-based droplet sorting method manipulates the objects (\textit{i.e.}, droplets) directly by the acoustic radiation force, while the TSAW-based method actuates the fluid (\textit{i.e.,} oil) that surrounds the objects through the acoustic streaming effect. Our method has excellent controllability and large range of translation, which renders it capable of precisely sorting cells into a great number (\textit{e.g.}, five) of outlet channels in a single step. This is a major advantage over most existing droplet-sorting methods, which typically only sort droplets into two outlet channels. Our approach is simple, versatile, and compact. We expect that it can be valuable in many chemical or biological analytical processes to facilitate the development of micro total analysis systems (\textmu TAS).\textsuperscript{76−83}
2.2 Working mechanism

The integrated droplet-sorting device consists of a flow-focusing, droplet-generation component\textsuperscript{11} and a SSAW-based, droplet-sorting component (Fig. 2-1a). The channel geometry was formed via standard soft lithography.\textsuperscript{84} In all of our devices, the height of the polydimethylsiloxane (PDMS) microchannel was 40 µm. The widths of the water-phase inlet, two oil-phase inlets and two sheath-flow inlets were 50 µm, 50 µm and 100 µm, respectively. The widths of the main channel and the five outlet channels were 200 µm and 45 µm, respectively. The two oil sheath flows were used to control the inter-droplet spacing. The PDMS microchannel and a pair of narrow chirped IDTs (with period of 360–400 µm and aperture of 300 µm or 500 µm) were aligned in parallel. Chirped IDTs are a variation of regular interdigital transducers (IDTs) which contain a linear gradient in their finger period.\textsuperscript{74,75} As a result, the wavelength of the excited surface acoustic waves (SAWs) is dependent upon the input frequency of the RF signal, while the amplitude of the SAWs is controlled by input voltage. The use of chirped IDTs rather than regular IDTs in our experiment is due to their capability to change the pressure node distribution of the generated SSAW field by tuning the input frequency. This is the basis for sorting droplets into multiple outputs.

The mechanism of multichannel droplet sorting is illustrated in Fig. 2-1c. When the chirped IDTs are excited at two different frequencies ($f_1$ and $f_4$), the SSAW with wavelengths of $\lambda_1/2$ and $\lambda_4/2$ ($\lambda = cf$, where $c$ is the SAW propagation velocity on the LiNbO$_3$ substrate) are formed, respectively. Since the microchannel is intentionally positioned closer to one chirped IDTs than the other, the pressure node located inside the microchannel is the $n^{th}$ node from the centerline of the two IDTs. And the absolute distance between the node and the centerline at these two excitation frequencies are $n(\lambda_1/2)$ and $n(\lambda_4/2)$, respectively. Therefore, the translation of the droplet ($\Delta x$) at the pressure node can be described as $\Delta x = n (\lambda_1 - \lambda_4)/2 = n (c/f_1 - c/f_4)/2$ for a
frequency change from $f_1$ to $f_4$. As a result, droplets exit through different outlets (Fig. 2-1a). Therefore, our SSAW-based droplet sorter is capable of precisely and controllably sorting droplets to different outputs through the control of excitation frequencies.

![Diagram](image)

Figure 2-1. Working mechanism of droplet sorting. (a) A schematic of the SSAW-based multichannel droplet sorter. The shaded area indicates the SSAW field. (b) An optical image of our SSAW-based multichannel droplet sorter. (c) A cross-section view showing the SSAW-based, multichannel droplet-sorting mechanism.

### 2.3 Methods

#### 2.3.1 Device fabrication

The fabrication procedure for the SSAW-based droplet-sorting device (Fig. 2-1b) includes: (1) the fabrication of a SAW substrate; (2) the fabrication of a PDMS microchannel; (3) the bonding of the PDMS microchannel to the SAW substrate (Fig. 2-2). To fabricate the SAW substrate, a double layer of chrome and gold (Cr/Au, 50 Å/500 Å) was deposited on a photoresist-
patterned 128° Y-cut lithium niobate (LiNbO₃) wafer (500 µm thick, double-side polished) using an e-beam evaporator (RC0021, Semicore, USA), followed by a lift-off technique to form the pair of chirped IDTs. The chirped IDTs in our experiment have 27 pairs of electrodes with the width of electrode and spacing gap increasing linearly from 90 to 100 µm by an increment of 1.25 µm, each width repeating three pairs. The PDMS microchannel was fabricated by standard soft-lithography using SU-8 photoresist. After holes were drilled on the PDMS microchannel for inlets and outlets with Harris Uni-Core 1.0 mm punch, the PDMS microchannel was treated with oxygen plasma in a plasma cleaner (PDC001, Harrick Plasma, USA) for 3 min with the SAW substrate. The PDMS microchannel was then aligned and bonded on the SAW substrate between the chirped IDTs after which the whole device was cured at 65 °C for at least three days before experiments to ensure that the surfaces of the microchannel were hydrophobic.

Figure 2-2. Device fabrication procedure: (1) the fabrication of a SAW substrate; (2) the fabrication of a PDMS microchannel; (3) the bonding of the PDMS microchannel to the SAW substrate.
2.3.2 Experimental Setup

All of the experiments were conducted on the stage of an inverted microscope (Eclipse Ti-U, Nikon, Japan). In order to eliminate the virtual image introduced by using a double-side polished LiNbO$_3$ substrate, we placed a polarizer in the light path and adjusted it to a certain angle. For droplet generation, Fluorinert$^\text{®}$ FC-40 (Sigma, USA) with 3 wt% DuPont Krytox 157 FS (ChemPoint, USA) was used as continuous oil phase while deionized water was used as dispersed water phase. Fluid prepared in 1 ml plastic syringes (Becton Dickinson, USA) was injected through polyethylene tubing (Becton Dickinson, USA) into the microchannel using syringe pumps (neMESYS, cetoni GmbH, Germany). An RF signal function generator (E4422B, Agilent, USA) was used to generate RF signals at desired frequencies, which were applied to the chirped IDTs after amplification by a power amplifier (100A250A, Amplifier Research, USA) to generate the SSAW field.

2.3.3 Characterization and data analysis

A fast camera (SA4, Photron, Japan) was connected to the microscope for data acquisition at a speed of 2000 frames per second (fps) during the experiment. Recorded data was analyzed with Image J (NIH, Bethesda, MD, USA). The droplet position was traced by analyzing the gray value of the stacked image of 500 frames and plotted in MATLAB. The droplet generation was analyzed from the captured video with the “Plot Z-axis Profile” function and plotted in MATLAB. The input pulsed signal after amplification was recorded with a digital phosphor oscilloscope (load set at 1 MΩ) (DPO4104, Tektronix, USA) and plotted in MATLAB.
2.4 Results and discussion

2.4.1 SSAW-based droplet switching

We first tested the device’s ability to switch droplet streams to each of the five outlet channels. In this experiment, droplets with diameter of ~50 µm were generated. At the beginning of the experiment, we tuned the input SSAW frequency to identify five working frequencies ($f_1 = 10.02$ MHz, $f_2 = 10.20$ MHz, $f_3 = 10.35$ MHz, $f_4 = 10.46$ MHz, and $f_5 = 10.60$ MHz) under which droplets could be directed into five outlet channels when the SSAW was excited. Then, by applying an RF signal periodically sweeping among these five working frequencies with a mean peak-to-peak voltage of around 48.0 V to the chirped IDTs, we were able to switch droplet flows among the five outlets (Fig. 2-3).

Figure 2-3. SSAW-based droplet switching. (a─e) show the directed droplet flows into five outlet channels under five different SSAW frequencies. The blue arrow indices the target outlet and the dashed lines indicate the aperture position of the chirped IDTs relative to the microchannel.
The effect of input voltage on the lateral displacement of droplets across the microchannel was investigated for two different SSAW frequencies (10.02 MHz and 10.20 MHz) using the same device under the same flow conditions. The RF signal was applied to the chirped IDTs with four different mean peak-to-peak voltages (30.0 V, 38.4 V, 48.0 V and 60.0 V), and the position of droplets within the last 2400 µm of the main channel at each voltage was recorded and plotted (Fig. 2-4). When the chirped IDTs were excited, higher input voltage generated stronger SSAW field with larger acoustic radiation force, which pushed droplets faster in the y-direction. This indicated that the velocity of droplet in the y-direction increased with the input voltage. Under the excitation of the 10.02 MHz RF signal, because the pressure node (the node nearest to the droplet stream) was located far from the centerline of the microchannel, the droplets never reached the pressure node and were still migrating in the y-direction as they left the SSAW field. Because the flow rates were constant, each droplet traveled through the SSAW field in the x-direction for the same amount of time regardless of the voltage. Therefore, the lateral displacement of droplets increased with the voltage (Fig. 2-4a). As a result, the sorting outcome was also influenced: droplets exited through the uppermost outlet at 60.0 V and 48.0 V while through the second uppermost outlet at 38.0 V and 30.4 V (data not shown). In contrast, under the excitation of the 10.20 MHz RF signal, the pressure node was shifted nearer to the centerline of the microchannel. Hence, droplets would have reached the pressure node before leaving the SSAW field above certain voltage. The lateral displacement of droplets did not increase after \( X = 1870 \) µm at 60.0V because droplets had already reached the pressure node (Fig. 2-4b, red curve). In this circumstance, droplets could never flow into the uppermost outlet regardless of the input voltage.
2.4.2 SSAW-based single droplet sorting

In order to develop a fluorescence-activated droplet sorter (FADS), the ability to sort a single droplet out of a stream is desirable. Therefore, we tested the feasibility of single droplet sorting by applying a pulsed RF signal to the narrow chirped IDTs. The diversion of single droplets from the main stream into four different target outlets was realized in our device (Fig. 2-5). When the SSAW was off, all the droplets flowed into the center outlet channel by default (Fig. 2-5). When a pulsed signal of desired frequency with certain pulse width was applied, one could deflect a single droplet into the desired target outlet channel. By applying a 15 ms wide pulsed...
signal of 10.20 MHz or 10.45 MHz with mean peak-to-peak voltage of 48.0 V, we successfully diverted a single droplet either upward or downward to the neighboring target outlet (Fig. 2-5b and 2-5c). The flow rates of the water-phase inlet, two oil-phase inlets and two sheath-flow inlets were 0.2, 8.0 and 1.7 µl/min, respectively. In comparison, the sorting of a single droplet into the uppermost or lowermost target outlet is more difficult, since a larger lateral displacement of the droplet is required. To realize this, a longer pulse width and higher input voltage could help based on our discussion above. The flow rates of the water-phase inlet, two oil-phase inlets and two sheath-flow inlets were changed to 0.1, 8.0 and 3.0 µl/min, respectively. Under this condition, we applied a 35 ms wide pulsed signal of 10.05 MHz or 10.60 MHz with mean peak-to-peak voltage of 54.0 V to sort a single droplet into the uppermost or lowermost target outlet (Fig. 2-5a and 2-5d).

Figure 2-5. SSAW-based single droplet sorting. (a─d) The sorting of single droplets into four different target outlets under pulsed RF signal. The dashed lines indicate the aperture position of the chirped IDTs relative to the microchannel.
At last, we evaluated the throughput of our SSAW-based droplet sorter by using the chirped IDTs with smaller aperture (300 µm), which gave us a better chance to sort a single droplet from its neighboring droplets (Fig. 2-6). In this experiment, droplets (diameter ~ 40 µm) were generated roughly every 4.5 ms, corresponding to a droplet generation rate of 222 droplets s⁻¹ (Fig. 2-6a). In order to sort single droplets out of the stream, we applied a 10.20 MHz periodically oscillating pulsed signal with a pulse width of 4.5 ms and pulse period of 18 ms to the chirped IDTs (duty cycle is 25%) (Fig. 2-6b). As a consequence, a single droplet was sorted into the target outlet about every 18 ms (Fig. 2-6c–g). The sorting performance of our SSAW-based approach is promising since whenever the SSAW was on during the pulse of 4.5 ms, one droplet was sorted out. Therefore, we have good reasons to expect that with the integration of an optical-detection unit to detect the target droplet and a trigger-control unit to control the SSAW generation, we can better match the SSAW generation with the target droplet to achieve single droplet sorting with high accuracy. To further increase the throughput in our future work, there are several possible approaches. The first approach is to fabricate chirped IDTs with an even smaller aperture (e.g., 200 µm) and more pairs of electrodes. This modified design can help to generate a narrower SSAW field while reducing the effect of acoustic dispersion at the edge. Thus, it can help to sort single droplets with closer inter-droplet distances at higher throughput. The second approach is to apply a pulsed signal with a higher voltage. Under higher voltages, the acoustic radiation force is larger, which pushes the droplet at a higher velocity in the y-direction. Therefore, a shorter time or pulse width (and thus less translational distance of the droplet in the x-direction) will be needed to sort the single droplet into a certain target outlet. This shorter time can also lead to higher throughput single droplet sorting. The third approach to increase the sorting throughput is to decrease the distance between the two IDTs and bond the microchannel closer to the IDTs. This closer distance between the two IDTs can also reduce the effect of acoustic dispersion to increase the sorting throughput.
2.5 Conclusions

In this chapter, we report an acoustofluidic-based multichannel droplet sorter. Our device integrates on-chip, water-in-oil droplet formation with SSAW-based, multichannel droplet sorting. Our device takes advantages of the excellent controllability and tunability offered by chirped IDTs and can sort picoliter droplets into five (or more) outputs at rates up to 222 droplets s$^{-1}$ without external labeling. With further optimization, this device can be integrated with an optical-detection module and other on-chip functions to enable a SSAW-based, micro fluorescence-activated droplet sorter (µFADS) for screening of small molecules or proteins secreted from single cells.
Chapter 3

Acoustofluidic-based cell washing

Cell washing is an indispensable sample preparation procedure used in various cell biology studies. In this chapter, we report an acoustofluidic-based microfluidic device for cell washing in a continuous flow. In our approach, the acoustic radiation force generated in a SSAW field is utilized to actively extract cells from their original medium. A unique configuration of tilted-angle standing surface acoustic wave (taSSAW) is employed in our device, enabling us to achieve >98% recovery rate and >97% washing efficiency. We also demonstrate the functionality of our device by preparing high-purity (>97%) white blood cells from lysed blood samples through cell washing. Our acoustofluidic-based cell washing device has the advantages of label-free manipulation, simple device, high biocompatibility, high recovery rate, and high washing efficiency. The technique presented here is an ideal continuous-flow cell washing unit and can be useful for future development of automated cell analysis systems.

3.1 Introduction

Cell and bead washing are important experimental procedures widely employed in biological studies and biomedical research. Taking fluorescent cell labeling as an example, a typical protocol requires the labeled cells to be washed after incubation with a fluorochrome. This cell-washing step is necessary to remove the unreacted fluorochrome and to optimize the staining result. In transplantation of cryopreserved and thawed hematopoietic stem cells (HSCs), washing of HSCs is required for dimethyl sulfoxide (DMSO) removal to decrease the adverse effects associated with DMSO infusion. Besides cell washing, bead washing has also been
routinely used in molecular biology and immunology. For instance, multiple bead-washing steps are needed to change reagents when affinity-based DNA purification is performed using QIAEX® beads.

Conventionally, cells or beads are washed using centrifugation methods. To ensure adequate cell washing, a typical cell-washing protocol involves centrifuging the cells at $1000 \times g$ for 10 min and for multiple rounds, which can be labor-intensive. In addition, cells experience high shear stress during high-speed, long-duration centrifugation processes, which may cause cell damage. Research data show that the percent hemolysis of red blood cells (RBCs) washed by centrifugation methods is significantly higher (~ 0.74%) compared with unwashed RBCs (~ 0.22%). In addition to low biocompatibility, another limitation for the centrifugation approach is its difficulty for in-line integration, which is necessary to realize automatic, micro total analysis systems (µTAS). Thus, the development of a simple, biocompatible, and continuous-flow microfluidic cell/bead washing device will address many unmet needs in cell biology and analytical chemistry.

To overcome the limitations of centrifugation-based cell-washing methods, researchers have been developing microfluidic techniques to wash cells and beads in a continuous flow. Several passive approaches have been demonstrated based on deterministic lateral displacement, microstructure-guided railing, hydrodynamic filtration, pinched flow fractionation, or inertial microfluidics. In these devices, cells or beads passively migrate from the original medium to the wash solution in the microchannels. However, these approaches offer limited control of cell/bead movement since the passive migration is predetermined by the geometry of the microchannel, size of the cell/bead, and/or flow conditions. In order to have better control of the cell/bead movement during the washing process, researchers have made efforts to use external forces to manipulate cells/beads, leading to the development of several active cell-washing techniques. Cells/beads flowing in these devices are subject to
external forces such as magnetic forces,\textsuperscript{108} dielectrophoretic (DEP) forces,\textsuperscript{109–111} or acoustic forces.\textsuperscript{59,112–116} As a result, beads or cells can be actively extracted from their original medium stream and placed into a wash solution. Among these cell/bead manipulation technologies, acoustic methods offer significant advantages in terms of label-free manipulation, biocompatibility, and versatility.

Recently, our group has utilized standing surface acoustic waves (SSAWs) to accomplish label-free manipulation of various micro-objects (e.g., beads, cells, droplets, and microorganisms).\textsuperscript{70,71,74,75,117} Particularly, a unique configuration of tilted-angle standing surface acoustic wave (taSSAW) was introduced in our recent paper in which the interdigital transducers (IDTs) were inclined at a specific angle to the flow direction.\textsuperscript{118} Compared with traditional SSAW approaches where the IDTs are aligned in parallel with the flow direction,\textsuperscript{55,56,119} this taSSAW approach offers a significantly larger lateral displacement for cells/beads. As a result, cells/beads can be separated based on differences in size, density and compressibility with high efficiency.

We explore the potential application of this taSSAW approach in the development of a SSAW-based cell/bead washing device. We first tested washing 10 µm beads and characterized the performance of our SSAW device for bead washing. By optimizing the input voltage, we were able to wash beads with high recovery rate (>98%) and high washing efficiency (>97%). For the demonstration of cell washing, we investigated the purification of white blood cells (WBCs) from lysed blood samples. Preparation of WBCs from whole blood through RBC lysis is widely employed in immunology and clinical diagnosis.\textsuperscript{120,121} After RBC lysis, WBC washing is necessary to remove the lysis buffer which can be detrimental to WBCs during long-term exposure. In addition, cell debris produced during RBC lysis also needs to be removed to reduce interference and background noise during further WBC analysis. For the optimization of WBC washing, we considered the differences between WBCs and debris in terms of size, density and compressibility and conducted numerical simulations to evaluate washing performances under
different input voltages. After numerical simulations, we demonstrated that our SSAW device was able to isolate WBCs from lysed blood samples with high-purity (>97%) while preserving cell integrity. The technique described here is expected to be an ideal continuous-flow cell/bead washing module and has great potential in the development of various micro total analysis systems (μTAS). 82,122

3.2 Working mechanism

Our SSAW-based cell/bead washing device was fabricated by bonding a polydimethylsiloxane (PDMS) microchannel to a piezoelectric substrate (Fig. 3-1). The microchannel is placed between a pair of IDTs and inclined at a specific angle (15°) relative to the IDTs. When a radio frequency (RF) signal is applied to the pair of IDTs, surface acoustic waves (SAWs) are generated from both IDTs. The SAWs propagate in opposite directions on the substrate surface and leak into the liquid inside the microchannel. The interference between them forms a SSAW field and causes pressure fluctuations in the liquid. As a result, a periodic distribution of pressure nodes (regions of minimum pressure amplitude) and pressure antinodes (regions of maximum pressure amplitude) is formed inside the microchannel (Fig. 3-1a). Particles flowing into the SSAW field experience the acoustic radiation force \( F_r \) and Stokes drag force \( F_d \) that can expressed as Eq (1) to Eq (3) described in Chapter 1. Particle movement in a SSAW field is governed by these two forces.

Since the microchannel is inclined at a specific angle to the IDTs, cells or beads flowing into the SSAW field will deviate from their original medium stream due to the competition of the acoustic radiation force and Stokes drag force (Fig. 3-1c). As a result, cells or beads can be washed out from the original medium and collected through the upper outlet.
3.3 Materials and methods

3.3.1 Device fabrication

To fabricate our SSAW-based cell/bead washing device (Fig. 3-1b), we first deposited a double layer of chrome and gold (Cr/Au, 50 Å /500 Å) on a photoresist-patterned lithium niobate (LiNbO₃) wafer (128° Y-cut, 500 µm thick, and double-side polished) using an e-beam evaporator (RC0021, Semicore, USA). Then we used a lift-off technique to expose the pair of IDTs with a period of 200 µm and width of 8 mm. The PDMS microchannel (1 mm wide in the main channel) with three inlets (650, 300, and 150 µm wide, respectively) and two outlets (300 µm wide, respectively).
µm wide) was fabricated by standard soft-lithography using SU-8 photoresist (channel height is 75 µm). A Harris Uni-Core 0.75 mm punch was used to drill holes for inlets and outlets. For device bonding, we first placed the PDMS microchannel and the LiNbO₃ substrate in a plasma cleaner (PDC001, Harrick Plasma, USA) for 3 min. Immediately after plasma treatment, we aligned the PDMS microchannel with markers on the LiNbO₃ substrate in between the IDTs and bonded with a 15° tilt angle between the microchannel and the IDTs. After bonding, we cured the whole device at 65 °C overnight before use.

### 3.3.2 Sample preparation

Both bead and cell samples were used in our experiments. For illustrating bead deviation in the SSAW field, 1 µl of 9.77 µm polystyrene microparticles (Dragon Green, Bangs Laboratory, USA) was diluted in 500 µl of 100 µM Rhodamine B (Sigma, USA) solution with 0.1% sodium dodecyl sulfate (SDS, Sigma, USA) added (final bead concentration was around 1.5 × 10⁴/ml). For the demonstration of SSAW-based bead washing, 2 µl of 9.77 µm (Dragon Green) and 0.87 µm (Rhodamine WT) polystyrene microparticles (Bangs Laboratory, USA) were mixed in 500 µl of 0.1% SDS solution (final bead concentrations were around 3 × 10⁴/ml and 4 × 10⁷/ml, respectively). To characterize the recovery rate and washing efficiency, we mixed 10 µl of 10 µm polystyrene microparticles (Sigma, USA) with 2 µl of 0.87 µm polystyrene microparticles (Rhodamine WT, Bangs Laboratory, USA) in 500 µl of 0.1% SDS solution (final bead concentrations were around 4 × 10⁶/ml and 4 × 10⁷/ml, respectively).

Human whole blood was purchased from Zen-Bio, Inc. To prepare the cell sample, 1 ml of blood was mixed with 10 ml of 1× RBC Lysis Buffer (eBioscience, USA) for 3 min to lyse most of the red blood cells (RBCs). Then the prepared cell sample was centrifuged at 400 × g for 5 min, resuspended in 1 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) solution.
(Santa Cruz Biotechnology, USA), and fixed at room temperature for 30 min. After fixation, the cell sample was centrifuged again and resuspended in 1 ml of PBS (Gibco, Life Technologies, USA) for use in experiments (final concentration of WBCs was around $5 \times 10^6$/ml).

### 3.3.3 Experimental setup

All of the experiments were conducted on the stage of an inverted microscope (Eclipse Ti-U, Nikon, Japan). To eliminate the double images caused by using a double-side polished LiNbO$_3$ substrate, we placed a polarizer in the light path and adjusted it to a certain angle during the experiments. To generate the SSAW, we applied amplified RF signals at desired frequencies and input voltages to the IDTs using an RF signal function generator (E4422B, Agilent, USA) and a power amplifier (100A250A, Amplifier Research, USA). A digital phosphor oscilloscope (load set at 1 M$\Omega$) (DPO4104, Tektronix, U.S.A) was used to measure the input voltages. In all of our experiments, the frequencies of the applied RF signals were in the range of 19.40 to 19.60 MHz and the input voltages were in the range of 20 to 40 V$_{pp}$, unless specified otherwise. Bead or cell samples prepared in 1-ml plastic syringes (Becton Dickinson, USA) were injected into the center inlet through polyethylene tubing (Becton Dickinson, USA) using syringe pumps (neMESYS, cetoni GmbH, Germany). Sheath flows (0.1% SDS solution for bead experiment and PBS for cell experiment) were introduced through two side inlets. Sheath flow introduced through the upper inlet also acted as a wash solution for collecting washed cells/beads.

### 3.3.4 Data acquisition and analysis

A fast camera (SA4, Photron, Japan) and a CCD camera (CoolSNAP HQ2, Photometrics, USA) were connected to the microscope for data acquisition. To calculate recovery rate and
washing efficiency, a hemocytometer was used to measure the concentrations of 10 µm and 0.87 µm beads in collected samples. For the characterization of WBC washing, flow cytometry results were collected using a Beckman Coulter FC500 Flow Cytometer and analyzed using FlowJo software (Tree Star, USA). Image J (NIH, Bethesda, MD, USA) was used for video processing.

3.4 Results and discussion

3.4.1 Demonstration and optimization of SSAW-based bead washing

In order to evaluate the taSSAW approach for continuous-flow cell/bead washing, we first demonstrated SSAW-based bead washing. We mixed 9.77 µm green fluorescent microparticles with 0.87 µm red fluorescent microparticles and introduced them into the center inlet. In this experiment, the 9.77 µm beads represented the target beads we would like to wash out and the 0.87 µm beads represented the original medium. Fluorescence images of the outlet region were taken when the SSAW was off and on (Fig. 3-2). When the SSAW was off, the 9.77 µm beads flowed within the original medium stream and exited through the lower outlet; while when the SSAW was on, the 9.77 µm beads were washed out and exited through the upper outlet in the wash solution.

During this experiment, we found that the input voltage clearly affected bead-washing results. If the input voltage was too low, the generated acoustic radiation force was too weak to deflect all the target beads from the original medium stream. As a result, only a portion of target beads were washed out from the upper outlet. In contrast, when the input voltage was too high, the acoustic streaming effect became obvious, and mixing between the wash solution and original medium occurred. Under this circumstance, some of the small beads were also extracted and
exited through the upper outlet together with the target beads, reducing bead-washing performance.

Figure 3-2. SSAW-based bead washing. (a) When the SSAW was off, the 9.77 µm beads exited in original medium. (b) When the SSAW was on, the 9.77 µm beads were washed out and exited in wash solution. Green: stacked images of the 9.77 µm beads. Red: fluorescence images of the 0.87 µm beads indicating the original medium.

In order to optimize the input voltage for bead washing, we conducted an experiment to study the impact of input voltage on the bead-washing performance. In this experiment, we mixed 10 µm beads and 0.87 µm beads in 0.1% SDS solution. The flow rates of the upper inlet, center inlet, and lower inlet were 5, 1, and 2 µl/min, respectively. The flow rates of the two outlets were controlled equally with the syringe pump in withdrawal mode. To evaluate the device performance, we examined both recovery rate and washing efficiency. Recovery rate is defined as the percentage of 10 µm beads that are washed out and collected through the upper outlet and
washing efficiency is defined as the percentage of 0.87 µm beads exiting through the lower outlet. Before the SSAW was applied, all of the beads flowed out through the lower outlet and the concentrations of 10 µm and 0.87 µm beads from the lower outlet were measured as references. Then, we applied 19.58 MHz RF signals with five different input voltages (16.8, 21.2, 26.8, 34.0, and 42.4 V\textsubscript{pp}) to the IDTs, collected samples from the upper outlet, and measured the concentrations of 10 µm and 0.87 µm beads. All sample collections and corresponding measurements were repeated three times for each input voltage.

Figure 3-3. Optimization of the input voltage for bead washing. (a) Influence of input voltage on recovery rate and washing efficiency for bead washing. The error bars represent the standard deviation (n=3). (b) Stacked image showing outlet region of the SSAW-based bead-washing experiment at the optimized condition.

The calculated recovery rate and washing efficiency at each input voltage were plotted (Fig. 3-3a). When we increased the input voltage from 16.8 to 26.8 V\textsubscript{pp}, the recovery rate increased, indicating that more 10 µm beads were washed out. At 26.8 V\textsubscript{pp}, the acoustic radiation
force was already strong enough to wash out almost all of the 10 µm beads so that the recovery rate did not change when we further increased the input voltage. The influence of input voltage on washing efficiency is the opposite. At low voltages (16.8 and 21.2 V_{pp}), the washing efficiency was almost 100%. From 26.8 to 42.4 V_{pp}, the washing efficiency decreased dramatically because 0.87 µm beads started to be extracted and collected through the upper outlet as well. Considering the balance between recovery rate and washing efficiency, the optimized input voltage at this frequency and flow condition was 26.8 V_{pp} for bead washing, where we achieved around 98.7% recovery rate and 97.2% washing efficiency.

From the stacked image of bead washing at this optimized condition (Fig. 3-3b), we saw that all of the 10 µm beads exited through the upper outlet and almost all of the 0.87 µm beads exited through the lower outlet. This result agreed with our characterization of recovery rate and washing efficiency, and illustrates the excellent performance of our SSAW-based bead washing device.

### 3.4.2 SSAW-based WBC washing

After optimizing the input voltage for bead washing, we conducted experiments to purify WBCs from lysed blood samples using our SSAW-based cell washing device. The flow conditions and excitation frequency were kept the same as in the optimized bead-washing experiment while an input voltage of 34.0 instead of 26.8 V_{pp} was used. We used higher input voltage because cells have a smaller acoustic contrast factor than beads, thus experiencing smaller acoustic radiation forces. Under the microscope, WBCs appeared as big, dark spheres and were easy to focus. Apart from WBCs, we also found a considerable number of small objects with various sizes and lighter color, which were debris produced during sample preparation. When there was no SSAW applied, all of the WBCs exited the microchannel through the lower outlet,
mixed with debris (Fig. 3-4a). When the SSAW was applied at the input voltage of 34.0 \text{ V}_{\text{pp}}, almost all of the WBCs got washed out and collected through the upper outlet, while the debris remained in original flow stream and exited through the lower outlet (Fig. 3-4b).

Figure 3-4. SSAW-based WBC washing. (a) Influence of input voltage on recovery rate and washing efficiency for bead washing. The error bars represent the standard deviation (n=3). (b) Stacked image showing outlet region of the SSAW-based bead-washing experiment at the optimized condition.

For the characterization of WBC washing, we collected WBC samples before and after applying SSAW, and analyzed the samples using flow cytometry (Fig. 3-4c-d). After SSAW-based cell washing, the percentage of debris present in the WBC sample decreased from 22.6\% to 2.16\%. Therefore, we were able to collect WBCs in clean PBS solution with more than 97\% purity. To evaluate the biocompatibility of our SSAW-based cell washing device, we also
measured the viability of WBCs collected after cell washing. Three different samples were collected after cell washing: original lysed blood sample; washed WBCs; and microchannel control (WBCs collected through the lower outlet when no SSAW was applied). The cell viabilities of the three samples were all around 80% and showed no significant difference, indicating that the viability of WBCs was not compromised after cell washing (Fig. 3-5). Collectively, these results demonstrate that our SSAW-based cell washing device can indeed take advantages of the difference in size and physical property between WBCs and debris to successfully purify WBCs from lysed blood samples with good performance and high biocompatibility.

![Figure 3-5. Viability of WBCs in original sample, washed WBCs, and microchannel control.](image)

### 3.5 Conclusions

In this chapter, we have applied our recently introduced taSSAW approach to develop and test an acoustofluidic-based cell washing platform. The successful application of our device is demonstrated by high-purity (>97%) WBC preparation. With the advantages of label-free manipulation, simple device, high biocompatibility, high recovery rate, high washing efficiency, and compatibility with other on-chip components, our acoustofluidic-based cell-washing
technique offers an excellent option for on-chip, continuous-flow cell washing and can be used for in-line integration of various μTAS.123–126
Chapter 4

Acoustofluidic transfer of inflammatory cells from human sputum samples

For sputum analysis, the transfer of inflammatory cells from liquefied sputum samples to a culture medium or buffer solution is critical because it removes the inflammatory cells from the presence of residual dithiothreitol (DTT), a reagent that reduces cell viability and interferes with further sputum analyses. We report an acoustofluidic platform for transferring inflammatory cells using standing surface acoustic wave (SSAW). In particular, we exploit the acoustic radiation force generated from a SSAW field to actively transfer inflammatory cells from a solution containing residual DTT to a buffer solution. The viability and integrity of the inflammatory cells are maintained during the acoustofluidic-based cell transfer process. Our acoustofluidic technique removes residual DTT generated in sputum liquefaction and facilitates immunophenotyping of major inflammatory cells from sputum samples. It enables cell transfer in a continuous flow, which aids the development of an automated, integrated device for on-chip sputum processing and analysis.

4.1 Introduction

The analysis of human sputum samples is critical for diagnosis of pulmonary diseases such as tuberculosis and asthma.\textsuperscript{127–129} Patients with severe asthma may be stratified into distinct immunological phenotypes per analysis of cell populations in induced sputum samples.\textsuperscript{130,131} Standard procedure for sputum liquefaction calls for the mixture of sputum samples with dithiothreitol (DTT), a strong reducing agent which decreases cell viability and interferes with further sputum analyses.\textsuperscript{132–134} Therefore, the inflammatory cells have to be
transferred to a culture medium or buffer solution in order to avoid the negative effects of DTT on downstream sputum analysis.

Conventionally this cell transfer step is realized through centrifugation, which has several drawbacks. Centrifuges are bulky and sometimes expensive. They are often not suitable for resource-limited regions, where point-of-care diagnostics of pulmonary diseases are needed. In addition, in these centrifugation-based conventional sputum processing/analysis assays, sputum samples need to be handled manually by the operator, often in open air, and run through several instruments. This presents significant biosafety concerns to the operator. This also hinders the integration with continuous-flow components toward a fully automated sputum processing/analysis system. Moreover, the manual transfer of fluid upon centrifugation is prone to inter-operator variability. With these drawbacks, there is a pressing demand for a biosafe, biocompatible, low-cost, automated, point-of-care platform to transfer the inflammatory cells from liquefied sputum samples to a culture medium or buffer solution in continuous flow.

With the advantages of low cost, miniaturization, and automation, microfluidics is suited to fulfill this unmet demand. Several microfluidic platforms for sputum liquefaction and analysis have already been demonstrated. However, the microfluidic transfer of inflammatory cells from liquefied sputum samples has not yet been demonstrated. Although various microfluidic techniques have been developed for the transfer of cells across flow streams, none of these techniques has been validated in transferring inflammatory cells from human sputum samples.

In this chapter, we demonstrate the first microfluidic platform for the transfer of inflammatory cells from liquefied human sputum samples. This cell transfer device is built upon our acoustofluidic (i.e., the fusion of acoustics and microfluidics) based cell manipulation platform. It features a tilted alignment between a microchannel and interdigital transducers (IDTs), which generate a standing surface acoustic wave (SSAW) field.
Inflammatory cells present in liquefied sputum samples are thereby transferred from the residual DTT solution to the buffer solution when SSAW is applied. This acoustofluidic cell transfer process decreases the concentration of DTT in sputum samples by 93% and thereby avoids the adverse effects of DTT on sputum analysis. Results from live/dead and histological staining show that our acoustofluidic device preserves the viability and integrity of transferred inflammatory cells, demonstrating the biocompatibility of our acoustofluidic-based inflammatory cell transfer platform. Moreover, flow cytometry analysis shows that the removal of residual DTT from liquefied sputum samples by our acoustofluidic approach yields better antibody labeling and accurate identification of major inflammatory cells. With the advantages of high biocompatibility, high biosafety, low cost, miniaturization, automation, and ease of in-line integration, our SSAW-based acoustofluidic platform is an excellent candidate for the development of an automated and fully integrated sputum processing/analysis system.

4.2 Working mechanism

Our acoustofluidic device (Fig. 4-1, inset) was fabricated by bonding a polydimethylsiloxane (PDMS) microchannel with a pair of IDTs on a piezoelectric substrate. The microchannel is aligned with the IDTs at a tilt angle of 15°. When the IDTs are excited by radio frequency (RF) signals, two identical surface acoustic waves (SAWs) are generated and propagate in opposite directions. The interference between the waves establishes a SSAW field inside the microchannel. Because of the tilted alignment, periodically distributed pressure nodes and antinodes are formed at an angle relative to the flow direction. When the liquefied sputum sample flows into the SSAW field, inflammatory cells present in the sample are subjected to an acoustic radiation force ($F_r$) and the Stokes drag force ($F_d$) that can be expressed as Eq (1) to Eq (3) described in Chapter 1.
Inflammatory cells present in the liquefied sputum sample have a positive acoustic contrast factor, so they are forced towards pressure nodes in the SSAW field. Because of the angle between the pressure nodes and the flow direction, inflammatory cells which pass through the SSAW field deviate from the flow due to the combination of the acoustic radiation force and the Stokes drag force. As a result, inflammatory cells are successfully transferred from the liquefied sputum to the buffer solution and are collected at the upper outlet.

Figure 4-1. Schematic of our acoustofluidic device for transferring inflammatory cells from liquefied sputum samples to a buffer solution using SSAW. Inset: a photograph of our acoustofluidic device.

4.3 Materials and methods

4.3.1 Sample preparation

Human sputum samples were collected at the National Institutes of Health (NIH) from an asthmatic patient who provided informed consent to participate in the Institutional Review Board (protocol 99-H-0076) of the National Heart, Lung, and Blood Institute. After collection, sputum
samples were transported on ice to The Pennsylvania State University. Upon arrival, the sputum sample was mixed with 0.1% DTT (Sputolysin® reagent, Cat# 560000, EMD Millipore, USA) at a 1:4 ratio, vortexed for 30 seconds, and incubated at room temperature for 15 minutes. After incubation, the sample was filtered through a sterile cell strainer of mesh size 100 µm (Cat# 22363549, Fisher Scientific, USA). The filtered sample was centrifuged at 800 rpm for 5 minutes. After centrifugation, the pelleted cells with residual DTT were re-suspended for the cell transfer experiment in 1 ml of phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), 0.5% Pluronic F-68 (PF-68), and 2.5 mM EDTA.

4.3.2 Experimental setup

The acoustofluidic cell transfer experiment was conducted on the stage of an inverted microscope (Eclipse Ti-U, Nikon, Japan). The IDTs were excited using an RF signal function generator (E4422B, Agilent, USA) and a power amplifier (100A250A, Amplifier Research, USA). The liquefied sputum sample and a PBS buffer (1% BSA, 0.5% PF-68, 2.5 mM EDTA) were injected into the center inlet and two sheath flow inlets using 1-ml syringes (MFR# 102-ST1C, McKesson, USA) and polyethylene tubing (Cat# 598322, Harvard Apparatus, USA). To transfer inflammatory cells for flow cytometry analysis, PBS supplemented with 1% mouse serum was used as a sheath flow. Flow rates were controlled by syringe pumps (neMESYS, cetonI GmbH, Germany). A fast camera (SA4, Photron, Japan) was connected to the microscope for video acquisition.
4.3.3 Cell count and DTT quantification

Cell concentrations of sputum samples before and after acoustofluidic-based inflammatory cell transfer were counted by hemocytometer. Cell recovery rate was calculated as the percentage of cells successfully transferred and collected through the upper outlet. DTT concentrations of sputum samples before and after acoustofluidic-based inflammatory cell transfer were quantified using a commercial DTT assay kit (Cat# DTT200, ProFoldin, USA): (1) 100 µl of DTT solutions from 100 µM to 0 µM were prepared in the wells of a 96-well plate (Cat# 07-200-565, Corning, USA) through two-fold serial dilution; (2) 100 µl of 100x diluted sputum samples were added into the wells; (3) 25 µl of Dye C55 was added into each well; (4) After incubation at room temperature for 10 minutes, the fluorescence intensity at 535 nm (excitation at 485 nm) of each well was measured using a plate reader (Infinite® 200 PRO, Tecan, Switzerland); (5) A linear standard curve was generated, and the original concentrations of DTT within sputum samples were interpolated.

4.3.4 Cell viability

Cell viability was assessed using live/dead staining. Samples were stained with Calcein-AM (Cat# C3100MP, Life Technologies, USA) to identify live cells, and propidium iodide (Cat# P4864, Sigma-Aldrich, USA) to identify dead cells. After staining, samples were examined by fluorescence microscopy to calculate the percentage of live cells.

4.3.5 Modified Wright–Giemsa staining

Sputum samples were added to the two chambers of a Shandon™ Double Cytofunnel™ (Cat# 5991039, Thermo Scientific, USA) assembled on Shandon™ Double Cytoslides™ (Cat#
5991055, Thermo Scientific, USA) and were centrifuged at 600 rpm for 10 minutes in a cytocentrifuge (Cytospin™ 3, Thermo Scientific, USA). After centrifugation, the slide was air-dried and stained with a Kwik-Diff™ staining kit (Cat# 9990700, Thermo Scientific, USA): (1) 10 dips in reagent 1 (fixative, green); (2) 5 minutes in reagent 2 (eosin, red); (3) 2 dips in reagent 3 (methylene blue, blue); (4) rinsed with deionized water. After staining, the slide was examined under an inverted microscope (Eclipse Ti-U, Nikon, Japan). Staining results were captured using a digital single-lens reflex (DSLR) camera (D3S, Nikon, Japan) connected to the microscope. A differential cell count was performed (200 to 250 cells in total) to calculate the percentage of major inflammatory cells (eosinophils and neutrophils) in the sputum sample.

4.3.6 Flow cytometry

To label the inflammatory cells, 5 µl of Alexa Fluor® 488 anti-human CD45 antibody (Cat# 304019, BioLegend, USA) and 5 µl PE anti-human CD15 (SSEA-1) antibody (Cat# 301905, BioLegend, USA) were added to 100 µl of sputum sample before and after cell transfer, and incubated at room temperature for 30 minutes. Another 100 µl of liquefied sputum sample was also incubated without antibodies and used as a non-stained control. After incubation, the three samples were washed with 900 µl of PBS, fixed with 4% paraformaldehyde (Cat# sc-281692, Santa Cruz Biotechnology, USA), re-suspended in 500 µl of PBS, and examined using a commercial flow cytometer (FC500, Beckman Coulter, USA). The flow cytometry results were analyzed using commercial software (FlowJo, Tree Star, USA).
4.3.7 Statistical analysis

Data were presented as group means ± standard deviations (SD). Welch two-sample t-tests were conducted using R. A p-value of less than 0.05 was considered statistically significant.

4.4 Results and discussion

4.4.1 Transfer of inflammatory cells using SSAW

We first validated the successful transfer of inflammatory cells from liquefied sputum samples using our acoustofluidic device. Each sample was injected into the center inlet at a flow rate of 1 µl/min, and PBS buffer was injected into the upper and lower inlets at flow rates of 5 and 4 µl/min, respectively. When the SSAW was not applied, all of the inflammatory cells along with the residual DTT solution exited through the lower outlet (Fig. 4-2a). In order to transfer inflammatory cells from residual DTT solution to PBS buffer, we applied RF signals (19.54MHz, 34.0 V_{pp}) to the IDTs. Most of the inflammatory cells were transferred to the PBS buffer and collected through the upper outlet when the SSAW was applied (Fig. 4-2b). We observed that a small fraction of cells was difficult to transfer, even when we increased the input voltage. This fraction could be oral squamous cells produced during collection of the sputum sample, or cell debris produced during sample processing. We collected three samples from the upper outlet after cell transfer and calculated the cell recovery rate to be 83.9 ± 5.1%.

To characterize the removal of residual DTT through acoustofluidic cell transfer, we quantified DTT concentrations of sputum samples before and after cell transfer. The DTT concentration before acoustofluidic cell transfer was 573.1 ± 37.1 µM and decreased by 93% to 40.0 ± 14.9 µM after acoustofluidic cell transfer (Fig. 4-2c, p-value = 0.0004, two-sample t-test). These results demonstrate that our acoustofluidic-based inflammatory cell transfer device
removes residual DTT used for sputum liquefaction, which may affect cell viability and further cellular analysis.\textsuperscript{132-134}.

Figure 4-2. Transfer of inflammatory cells using SSAW. (a–b) Stacked micrographs showing the transfer of inflammatory cells using SSAW: (a) When the SSAW was off, inflammatory cells exited through the lower outlet in residual DTT solution; (b) When the SSAW was on, most of the inflammatory cells were transferred to the PBS buffer and collected through the upper outlet. Scale bar = 200 µm. (c) DTT concentrations before and after the cell transfer (n=3). ***: p-value < 0.001.

4.4.2 Cell viability and morphology

After demonstrating the successful transfer of inflammatory cells using our acoustofluidic device, we evaluated the effect of cell transfer on cell viability. We first checked the liquefied sputum sample and found the cell viability to be 41.5%. The relatively low cell viability could stem from prolonged storage of sputum samples after collection due to transportation delay (> 4 hours). Four samples of inflammatory cells were collected through the upper outlet after cell transfer. As a positive control, four samples of inflammatory cells were collected through the lower outlet when the SSAW was not applied. The cell viability of each sample was quantified and normalized to that of the original liquefied sputum sample. Normalized cell viabilities of positive control samples and samples after acoustofluidic cell transfer were 92.1 ± 12.9% and 87.1 ± 8.9%, respectively (Fig. 4-3, n = 4, p-value = 0.5498, two-sample t-test). Therefore, exposure to SSAW during cell transfer did not affect the viability of inflammatory cells in sputum samples.
Figure 4-3. Comparison of normalized cell viabilities between the positive control samples and the sputum samples after acoustofluidic cell transfer (n=4). N.S.: not significant (p-value > 0.05).

To examine if exposure to SSAW during cell transfer compromised cell integrity, transferred cells were stained with modified Wright–Giemsa stain to inspect cell morphology and identify different cell types. Samples collected through the lower inlet without SSAW applied were used as a positive control. The appearance of stained cells after transfer appeared similar to that of the positive control group (Fig. 4-4). We identified major inflammatory cells common to sputum samples from asthmatic patients (e.g., eosinophils and neutrophils), as well as other cell types (e.g., macrophages and oral squamous cells). From a differential cell count, the percentages of major inflammatory cells (eosinophils and neutrophils) for the positive control and after acoustofluidic cell transfer were 27.6% and 29.5%, respectively. The modified Wright–Giemsa staining results indicate that our acoustofluidic-based inflammatory cell transfer device preserves cell integrity and is compatible with histological staining, which is commonly used in clinical diagnostics of pulmonary diseases.
4.3.3 Flow cytometry analysis

Since identification of major inflammatory cells using flow cytometry is a crucial step in immunophenotyping of sputum samples, we examined the effect of acoustofluidic-based cell transfer on flow cytometry analysis. Inflammatory cells in liquefied sputum samples were...
transferred using SSAW. Flow cytometry analysis was conducted on non-stained sputum sample, sputum sample before cell transfer, and sputum sample after cell transfer. The non-stained sputum sample was used to set the gates for CD45+ and CD15+ cells (Fig. 4-5a–b). Fluorescence signals of CD45 and CD15 in the sputum sample before acoustofluidic cell transfer (Fig. 4-5d–e) were weaker than after acoustofluidic cell transfer (Fig. 4-5g–h). This weaker fluorescence signal in the sputum sample before cell transfer is attributed to the inactivation of antibodies by residual DTT.\textsuperscript{148} The calculated percentages of CD45+/CD15+ cells (eosinophils and neutrophils) in sputum samples before and after cell transfer were 11.9% and 29.9%, respectively. Compared to the results obtained from a differential cell count (27.6% and 29.5%), the percentage of eosinophils and neutrophils (CD45+/CD15+ cells) in the sputum sample before cell transfer was underestimated. We quantified the percentage of co-population of eosinophils and neutrophils more accurately after cell transfer (Fig. 4-5i). Our flow cytometry results justify the necessity of the acoustofluidic cell transfer step after sputum liquefaction for identification of major inflammatory cells per flow cytometry.

\textbf{4.5 Conclusions}

In this chapter, we have demonstrated an acoustofluidic device which transfers inflammatory cells from liquefied sputum samples by SSAW. Our device preserves the viability and integrity of transferred inflammatory cells. In addition, by removing residual DTT utilized during sputum liquefaction, our acoustofluidic-based inflammatory cell transfer device yields more accurate immunophenotyping results per flow cytometry analysis. The increased accuracy is valuable for identifying inflammatory lung cells from human sputum samples. Compared with conventional centrifugation methods, our acoustofluidic-based approach offers advantages such as high biocompatibility, high biosafety, low cost, and miniaturization. Moreover, our
acoustofluidic device transfers inflammatory cells in a continuous flow. As such it is readily incorporated with other on-chip sputum processing or analysis units to realize an automated, fully integrated device for point-of-care diagnostics of asthma and other pulmonary diseases.

Figure 4-5. Flow cytometry results of (a–c) non-stained sputum sample; (d–f) sputum sample before acoustofluidic cell transfer; and (g–i) sputum sample after acoustofluidic cell transfer. The non-stained sputum sample was used to set the gates for CD45+ and CD15+ cells. The calculated percentages of CD45+/CD15+ cells (eosinophils and neutrophils) in sputum samples before and after cell transfer were 11.9% and 29.9%, respectively.
Chapter 5

Cell co-culture platform for studying heterotypic cell-cell interactions

Precise reconstruction of heterotypic cell-cell interactions in vitro requires the co-culture of different cell types in a highly controlled manner. In our approach, different types of cells were patterned sequentially in the standing surface acoustic wave (SSAW) field to form an organized cell co-culture. To validate our platform, we demonstrate a co-culture of epithelial cancer cells and endothelial cells. Real-time monitoring of cell migration dynamics reveals increased cancer cell mobility when cancer cells are co-cultured with endothelial cells. Our SSAW-based cell co-culture platform has the advantages of non-contact cell manipulation, high biocompatibility, high controllability, simplicity, and minimal interference of the cellular microenvironment. The SSAW technique demonstrated here can be a valuable analytical tool for various biological studies involving heterotypic cell-cell interactions.

5.1 Introduction

Cell culture techniques have greatly benefited various studies in biology, biochemistry, and biomedical engineering, and are used as in vitro platforms for drug screening in the pharmaceutical industry. However, cells residing in their in vivo niches experience a far more complex microenvironment than those maintained in conventional cell culture; they interact with neighboring cells, the extracellular matrix (ECM), and with soluble factors present in the microenvironment. In particular, interactions among different types of cells are crucial for the maintenance of normal cell function. For example, the interaction with nonparenchymal cells helps preserve the liver-specific functions of primary hepatocytes within co-culture. To study
heterotypic cell-cell interactions, co-cultures of different cell types are needed. Traditionally, different types of cells are randomly mixed and grown together on cell culture plates to form cell co-cultures. According to differential adhesion hypothesis (DAH), the differences in intercellular adhesiveness between different cell types can drive the movement and assortment of cells.\textsuperscript{155,156} However, the organization of cell co-culture arising from DAH is governed by spontaneous rearrangement of cells with little control on the final cell pattern. Therefore, engineering approaches that offer on-demand control of cell arrangement are desirable for the reconstruction of physiologically relevant \textit{in vivo} multicellular microenvironment.\textsuperscript{157–159}

To address the unmet needs of cell co-culture techniques with better controllability, researchers have developed many cell co-culture platforms using micropatterned surfaces,\textsuperscript{160–174} cell printing,\textsuperscript{175–177} detachable substrates,\textsuperscript{178,179} physical barriers,\textsuperscript{180–183} microfluidic traps,\textsuperscript{184–186} and dielectrophoresis (DEP).\textsuperscript{187–189} Among these, micropatterning techniques are the most widely used and generate organized cell co-cultures using an adhesion molecule- or microstructure-patterned substrate surfaces.\textsuperscript{160–174} However, cell adhesion to the micropatterned surfaces is a passive process with low controllability and cell behaviors within such co-cultures might be influenced by the artificially introduced substrate heterogeneity. In cell-printing methods, cells are actively deposited onto specific positions with relatively low patterning resolution.\textsuperscript{175–177} A recently developed detachable substrate method enables reconfiguration of the formed cell co-culture, but is not suitable for multicellular construction.\textsuperscript{178,179} Using solid microstructures as physical barriers or microfluidic traps, researchers can control the positions of a group of cells or single cells within co-culture in microfluidic channels.\textsuperscript{180–186} These platforms have fine resolution and good control of local cellular microenvironment,\textsuperscript{157} yet cells confined by these microstructures may experience unwanted mechanical stimuli potentially interfering with the study of heterotypic cell-cell interactions. The introduction of non-contact DEP forces for cell patterning can overcome this limitation.\textsuperscript{187–189} However, for cell manipulation, DEP methods
require a specially prepared culture medium with low ionic strength that is different from the cells’ *in vivo* microenvironment. Thus far, a cell co-culture technique with high biocompatibility, high controllability, and minimal interference of the cellular microenvironment has yet to be realized.

Previously our group has used standing surface acoustic waves (SSAWs) to precisely manipulate various micro/nano-objects (*e.g.*, beads, cells, droplets, nanowires, and microorganisms). In particular, in our early work we demonstrated that our SSAW platform, the so-called acoustic tweezers technology, could create a pattern of *suspended cells from a single cell type*. In this chapter, we introduce the phase-shift approach and microfluidic cell culture technique to our SSAW cell-manipulation platform and demonstrate SSAW-based cell co-culture (*i.e.*, patterning of *cultured cells from multiple cell types* with desired arrangement). Our SSAW-based cell co-culture technique can position multiple cell types using non-invasive, non-contact acoustic forces with high precision and high tunability. It also has the flexibility to operate in literally any medium and with different types of adherent cells. For the validation of our platform, we demonstrate a co-culture of epithelial cancer cells and endothelial cells and monitor the cell migration dynamics within the co-culture. In our platform, cell behaviors (*e.g.*, migration) are restricted by neither heterogeneous surface modifications nor physical barriers, which is advantageous over existing techniques (*e.g.*, micropatterning, detachable substrates) in studying cell-cell interactions. We expect that the SSAW-based cell co-culture platform described here will be a valuable tool for studying cell-cell interactions, tissue engineering, and drug screenings.
5.2 Working mechanism

The SSAW-based cell co-culture platform was made by bonding a polydimethylsiloxane (PDMS) microchannel between a pair of interdigital transducers (IDTs) fabricated on a lithium niobate (LiNbO₃) piezoelectric substrate (Fig. 5-1). The pair of IDTs are aligned in parallel. When a radio frequency (RF) signal is applied to both IDTs, two series of identical surface acoustic waves (SAWs) propagating in opposite directions are generated. The interference between these two series of SAWs forms a SSAW field, with a periodic distribution of pressure nodes (with minimum pressure amplitude) and pressure antinodes (with maximum pressure amplitude) on the piezoelectric substrate. When the resonating SSAW encounters the liquid medium inside the microchannel, it generates longitudinal-mode leakage waves, which cause pressure fluctuations in the medium.

Figure 5-1. Working mechanism of the cell co-culture platform. (a) Working mechanism of the patterned cell culture in our SSAW-based cell co-culture platform. (b) An optical image of our SSAW-based cell co-culture device.
When the RF signal is on, cells suspended in the culture medium inside the microchannel are aligned in parallel lines in the established SSAW field due to the acoustic radiation forces. When the RF signal is off, cells eventually settle down inside the microchannel and, in the absence of external flow, maintain their original pattern. Eventually, these cells attach to the surface of the piezoelectric substrate forming a patterned cell culture (Fig. 5-1a). To form an organized cell co-culture, different types of cells need to be patterned in different positions, which requires changing the distribution of pressure nodes and antinodes. In an established SSAW field, this can be achieved by tuning the relative phase between the RF signals applied to the pair of IDTs. With this phase-shift approach, different types of cells can be patterned sequentially in different positions to form an organized cell co-culture.

![Schematics of the SSAW-based cell co-culture procedure.](image)
For the formation of co-cultures of two different cell populations (Fig. 5-2), cells of type A are first seeded into the microchannel and patterned by SSAW (Fig. 5-2a-b). After type A cells attach to the bottom surface (Fig. 5-2c), cells of type B are introduced into the microchannel (Fig. 5-2d). To pattern type B cells in different positions from type A cells, we tune the relative phase between the RF signals applied to the pair of IDTs by 180°. Thus, type B cells are patterned in between type A cells with the same patterning period (Fig. 5-2e). As a result, two types of cells will grow in alternate lines after type B cells attach to form an organized cell co-culture (Fig. 5-2f).

5.3 Materials and methods

5.3.1 Device fabrication

To fabricate the SSAW-based cell co-culture device (Fig. 5-1b), a double layer of chrome and gold (Cr/Au, 50 Å /500 Å) was deposited on a photoresist-patterned 128° Y-cut LiNbO₃ wafer (500 μm thick, double-side polished) using an e-beam evaporator (RC0021, Semicore, USA), followed by a lift-off process to form a pair of IDTs with a period of 300 μm. A single-layer PDMS microchannel (12 mm long, 1 mm wide and 100 μm high) was fabricated by standard soft-lithography using SU-8 photoresist. After holes for an inlet and outlet were drilled into the PDMS microchannel with a Harris Uni-Core 1.00 mm punch, the PDMS microchannel was treated with oxygen plasma in a plasma cleaner (PDC001, Harrick Plasma, USA) for 3 min with the LiNbO₃ substrate. The PDMS microchannel was then aligned and bonded to the LiNbO₃ substrate between the IDTs. The entire device was then incubated overnight at 65 °C and sterilized under UV light for 30 min. The surface of the LiNbO₃ substrate inside the microchannel was then coated overnight with 100 μg/ml Collagen I Rat Tail (Gibco®, Life
Technologies, USA) in an ethanol solution before each experiment for better cell adhesion and growth.

5.3.2 Experimental setup and data acquisition

All of the experiments were conducted on the stage of an inverted microscope (TE2000-U, Nikon, Japan) with an installed microscope incubation system (Chamlide TC, Live Cell Instrument, South Korea). In order to eliminate the virtual image introduced by using a double-side polished LiNbO$_3$ substrate, we placed a polarizer in the light path adjusted at an angle. The SSAW is created by applying amplified RF signals to the IDTs using a function generator (AFG 3102C, Tektronix, USA) and power amplifier (25A250A, Amplifier Research, USA). A digital phosphor oscilloscope (load set at 1 MΩ) (DPO4104, Tektronix, USA) was used to determine the resonance frequency of the IDTs. A syringe pump (KDS210, KD Scientific, USA) was used to infuse fresh culture medium into the microchannel during long-term microfluidic cell culture. A charge-coupled device (CCD) camera (ORCA-Flash 2.8, Hamamatsu, Japan) was connected to the microscope for data acquisition. Cell movement trajectories were extracted from time-lapse images and analyzed using Nikon NIS-Elements Advanced Research (AR) software and plotted using R. Welch Two Sample t-test was conducted to compare cell movement data between two groups. All of the other image processes were conducted using Image J (NIH, Bethesda, MD, USA).

5.3.3 Cell culture and staining

HeLa cells (a cervical cancer cell line) were maintained in DMEM/F12 medium (Gibco®, Life Technologies, USA), supplemented with 10% fetal bovine serum (Gibco®, Life
Human Dermal Microvascular Endothelial (HMVEC-d) cells were purchased from ATCC and maintained in EndoGRO™-LS Complete Media (Millipore, USA). For co-culturing HeLa cells and HMVEC-d cells, EndoGRO™-LS Complete Media was always used as the culture medium. A CO₂ incubator (Nu-4750, NuAire, USA) was used to maintain a temperature of 37°C and a 5% CO₂ level during cell culture. CellTracker® Green CMFDA and CellTracker® Orange CMRA (Molecular Probes®, Life Technologies, USA) were used to label cells with green and red fluorescence following the manufacturer’s standard protocols. Cells grown to 80~90% confluency were trypsinized (Trypsin + 0.05% EDTA, Gibco®, Life Technologies, USA), washed with PBS, resuspended in fresh culture medium to desired cell concentrations, and seeded into the microchannel for the experiment. After the cells were treated and cultured in our SSAW-based devices, live/dead cell staining was conducted using Calcein AM and SYTOX® Orange (Molecular Probes®, Life Technologies, USA) to assess cell viability in our device after being treated by SSAW and cultured in microfluidic devices.

5.4 Results and discussion

5.4.1 Culture of patterned cells

We validated the ability of our SSAW-based platform to create patterned cell culture. HeLa cells were patterned and cultured for up to 24 h (Fig. 5-3a-e). During this experiment, we applied a 12.78 MHz RF signal with a voltage of ~20 V_{pp} to pattern HeLa cells that were injected into the microchannel at a concentration of 4 × 10^6 cells/ml. The resulting pattern of parallel lines (Fig. 5-3a) had a period of approximately 150 µm, which matched well with the half wavelength of the applied SAW (λ = ~300 µm). After the cells were patterned in the SSAW field, we turned
off the RF signal and stopped the fluid to allow the cells to settle and adhere to the bottom surface of the microchannel. Although the cells were subject to movement of the residual flow before adhesion, this effect was minimal in the microfluidic environment. After 1 h of culture in the incubation system, the cells maintained the original pattern, with only slight cell movement (Fig. 5-3b). The cell movement during the adhesion process was due to cell expansion on the surface and did not affect cell patterns within 1 h of culture. We also found that all of the cells had attached within 1 h of culture; they were not flushed away by an external flow at this time. For long-term culture of the patterned cells, after 1 h we infused fresh culture medium into the microchannel with a syringe pump at a flow rate of 0.5 µl/min. Patterned HeLa cells were then cultured in the SSAW-based microfluidic device with this continuous medium infusion until the cells grew to full confluency at 24 h (Fig. 5-3c-e).

Figure 5-3. Patterned cell culture. (a-e) Micrographs showing culture of patterned HeLa cells in our SSAW-based microfluidic device for up to 24 h. (f) Live/dead staining results indicate that most of the HeLa cells remain viable at the end of 24 h culture (Green: live cells. Red: dead cells).
During this phase of the experiment, cells were exposed to the SSAW field only during the patterning process and for less than 10 s. No obvious cell damage associated with acoustic exposure was observed during the 24 h microfluidic cell culture. In addition, we conducted live/dead cell staining using Calcein AM and SYTOX® orange at the end of the 24 h cell culture (Fig. 5-3f). A count of the stained cells in three different SSAW-exposed areas showed that 99.26 ± 0.38% of the cells remained viable at the end of the 24 h culture, proving the non-invasive nature of our SSAW technique.

The IDTs used in our experiment were designed to have a fixed period of 300 µm with a resonance frequency of 12.78 MHz, which were used in all subsequent experiments to generate the best cell patterning result. IDTs with different designs can be used to pattern cells with different periods. Furthermore, it is also feasible to change the period of cell patterning in a single device using tunable SSAW generated from slanted-finger IDTs or chirped IDTs,73,75 which can give our SSAW-based cell co-culture platform an even greater degree of flexibility.

5.4.2 Co-culture with sequential cell patterning

After demonstrating a patterned culture of a single cell type, we examined the possibility of patterning and culturing multiple types of cells in one device. In our experiment, we employed the aforementioned phase-shift approach to form an organized co-culture of two different cell types (Fig. 5-4). In this experiment, green and red fluorescently labelled HeLa cells were used to represent two cell types. The green fluorescently labelled HeLa cells (type A cells) were first introduced into the microchannel at a concentration of 4 × 10⁶ cells/ml and patterned under an RF signal of 12.78 MHz, 20 Vpp, and 0° relative phase. After 2 h of culture with the SSAW off, the red fluorescently labelled HeLa cells (type B cells) were injected into the microchannel at the same concentration. They were patterned under an RF signal of 12.78 MHz, 20 Vpp, but with a
180° relative phase. After another 2 h of culture with the SSAW off, fluorescent images were taken. The change of the relative phase between the pair of IDTs from 0° to 180° will change the cell-patterning positions between the two rounds of cell seeding by switching between pressure nodes and antinodes (Fig. 5-4a). The fluorescent images in Fig. 5-4b-c represent the two groups of HeLa cells growing in patterned lines. From the merged image in Fig. 5-4d, we can see that the patterned lines for the two groups of HeLa cells are in an alternate manner, with a separation of less than 75 µm (1/4 of the applied SAW wavelength).

Figure 5-4. Co-culture with SSAW-based sequential cell patterning. (a) Mechanism of patterning two types of cells in different positions with the phase-shift approach. (b) Green fluorescent image showing firstly-seeded HeLa cells. (c) Red fluorescent image showing secondly-seeded HeLa cells. (d) Merged image showing green and red HeLa cells grown in alternate lines.

In addition to its advantages in high controllability, our SSAW-based cell co-culture platform requires only a small number of cells. During each round of cell patterning, usually only
2 µl of cell suspension (approximately $4 \times 10^6$ cells/ml) were injected into the microchannel using a pipette, which means that $< 1 \times 10^4$ cells were required for co-culture reconstruction for each cell type.

### 5.4.3 Investigation of cancer cell movement during co-culture

To further examine the functionality of our SSAW-based cell co-culture platform, we explored the interactions between epithelial cancer cells and endothelial cells in our system. For this study, we chose HeLa cells and Human Dermal Microvascular Endothelial (HMVEC-d) cells as our biological model. Prior to studying HeLa cell behaviors in co-culture, we first explored patterned mono-culture of HeLa cells. In the control experiment (Fig. 5-5a-b), two groups of HeLa cells were first labelled with green and red fluorescence, respectively. Then these two groups of HeLa cells were sequentially seeded and patterned in our device as described above. The cell concentration for each round of patterning was $3 \times 10^6$ cells/ml and the interval between two rounds of patterning was 1 h. Fluorescent images were taken at 2 h and 24 h time points. Green and red fluorescently labelled HeLa cells growing in alternate lines were formed at 2 h (Fig. 5-5a). When the HeLa cells grew to confluency at 24 h, this pattern of alternate lines was not disrupted, indicating that HeLa cell mobility in mono-culture is low (Fig. 5-5b). In our co-culture experiment, we introduced green fluorescently labelled HMVEC-d cells at the concentration of $3 \times 10^6$ cells/ml during the first round of cell patterning and red fluorescently labelled HeLa cells at the same concentration after 1 h. At 2 h time point of co-culture, we saw that HMVEC-d cells and HeLa cells grew in alternate line regions (Fig. 5-5c). However, at 24 h time point, the organized HeLa cell pattern was clearly disrupted indicating high HeLa cell mobility when co-cultured with HMVEC-d cells (Fig. 5-5d).
Figure 5-5. Fluorescent images of (a-b) HeLa cell mono-culture at 2 h and 24 h and (c-d) HeLa and HMVEC-d co-culture at 2 h and 24 h in our SSAW device.

In order to quantitatively investigate HeLa cell movement within mono-culture or co-culture, we cultured HeLa cells when HMVEC-d cells (in EndoGRO™-LS Complete Media) were present or absent in our SSAW device. As control experiments, off-chip mono-culture and co-culture of HeLa cells were also conducted in which cells were randomly seeded on a petri dish. For co-culture, HeLa cells were fluorescently labelled with Calcein AM for identification and seeded before the HMVEC-d cells. Time-lapse phase-contrast images were automatically taken every 20 min from 2 h to 12 h for all of the four groups (on-chip mono-culture, on-chip co-culture, off-chip mono-culture, and off-chip co-culture) to record cell positions. To analyze the cancer cell movements, we randomly picked 30 HeLa cells in each group and tracked their trajectories from 2 h to 12 h.
Figure 5-6. Quantitative analysis of HeLa cell movement in mono-culture and co-culture. (a-b) Three typical movement trajectories (in blue, green, and red lines) for HeLa cells in on-chip (a) mono-culture and (b) co-culture. (c) Average movement path lengths of the tracked HeLa cells plotted against culture time for the four groups. (d-e) Comparison of (d) average movement path length and (e) average distance from origin for the tracked HeLa cells at 12 h among four different groups. The error bars represent the standard deviation (n = 30 for each group, ns: P > 0.05, *: P ≤ 0.05, **: P ≤ 0.01, ***: P ≤ 0.001).

Three typical cell trajectories for on-chip mono-culture and co-culture (Fig. 5-6a-b) illustrated that HeLa cells co-cultured with HMVEC-d cells in our SSAW device had higher mobility than those cultured alone. This increased HeLa cell mobility was not caused by the higher cell density in on-chip co-culture because HeLa cells within confluent off-chip mono-
culture also showed low level of mobility. The average movement path lengths of the 30 tracked HeLa cells in each group were plotted (Fig. 5-6c). At 12 h, the average movement path lengths of the tracked HeLa cells in off-chip mono-culture, on-chip mono-culture, off-chip co-culture, and on-chip co-culture were 66.4 ± 11.2, 64.8 ± 19.5, 146.1 ± 21.3, and 174.0 ± 24.6 µm, respectively (Fig. 5-6d). From the comparison, we found that the mobility of HeLa cells in co-culture was much higher than that in mono-culture, either off-chip or on-chip. The average movement path length of the tracked HeLa cells in on-chip co-culture was only slightly larger than in off-chip co-culture, which indicated that the presence of endothelial cells increased the mobility of cancer cells either in a random co-culture or in an organized co-culture. However, when we compared HeLa cell trajectories between these two groups, we found that the movements of HeLa cells in the two groups were different. In the organized on-chip co-culture, HeLa cells tend to migrate away from their original positions while in the random off-chip co-culture, HeLa cells tend to wander around locally so that their final positions at 12 h were close to their original positions at 2 h. To illustrate this difference, we further plotted the average distances from the original positions for the four groups at 12 h (Fig. 5-6e). We saw that the average distance from the original positions for on-chip co-culture (48.7 ± 24.9 µm) was twice that in off-chip co-culture (23.1 ± 16.4 µm). One possible explanation is that compared with the random cell arrangement in off-chip co-culture, the organized cell arrangement in on-chip co-culture better facilitates gradient formation for the signaling molecules secreted from HMVEC-d cells, which can guide HeLa cells to migrate away from their original positions. These results indicate that our SSAW-based cell co-culture platform can be used for in vitro evaluation of the invasiveness of cancer cells and can be developed as an efficient tool for in vitro anti-tumor drug screenings.
5.5 Conclusions

We have developed a SSAW-based cell co-culture platform. Our platform takes advantage of the non-contact, non-invasive nature of acoustic forces, thus exerting minimal interference on the cellular microenvironment while preserving high cell integrity. The use of non-contact acoustic forces that pattern cells in a transient manner is valuable because cells are free of any major external stimulus (e.g., substrate heterogeneity, microstructure confinement, or mechanical stimulation) during culture. Thus, the interference of the cellular microenvironment by unwanted stimuli is minimized and the influence of heterotypic cell-cell interactions can be isolated for study. The SSAW-based cell co-culture platform described here provides a novel analytical tool for real-time, dynamic observation of cell behaviors within co-culture. The cellular-level resolution makes our SSAW-based cell co-culture platform an excellent candidate for reconstructing heterotypic cell-cell interactions — important for probing cell communication and multicellular tissue construction.
Chapter 6

Single-cell virology platform for studying heterogeneous virus-cell interactions

Heterogeneity of pathogen-host interactions poses a major obstacle to understanding complex physiological and pharmaceutical processes. Here, we report a single-cell virology platform that incorporates isolated culture of single cells, live-cell imaging, single-cell tracking and statistical pipeline for high-throughput analysis of viral infection dynamics at the single-cell level. We show that this platform separates individual infection dynamics from one another. In addition, our system allows a parallel study of multiple factors such as multiplicity of infection, cell cycle, genetic or drug perturbations. Our model studies of poliovirus have revealed enormous stochastic behavior that likely plays out in vivo that contributes to post-infection outcomes. Our investigations unraveled the delayed replication of an attenuated poliovirus mutant and provided insight into the evolutionary dynamics of viruses under selection pressure of antiviral drugs. This technology will open potential avenues to investigate otherwise inaccessible mechanisms of infectious diseases, and accelerate the discovery of more effective therapeutics.

6.1 Introduction

The stochastic responses of individual host cells to individual pathogens are often omitted by averaged measurements of large populations, which in turn makes it difficult to reveal the underlying mechanisms of complex physiological and pharmaceutical processes. These problems are further aggravated in the studies of virus-host interactions, the development of attenuated vaccine, and the screening of antiviral therapeutics with broad-spectrum efficacy, due
to the extremely high mutation rates and significant genetic diversity of viral population. Taking poliovirus (PV) as an example, one or two incorrect nucleotides are incorporated during each round of viral RNA replication, and the error frequency therefore creates a population of genetically distinct variants.\textsuperscript{204,205} Given the variation of host cells due to differential gene expressions and cell cycle, the complexity in virus-host interactions is further elevated. Consequently, the pathogenesis of viral infection is determined by outcomes in single cells. As such, new technologies to study viral infection dynamics at the single-cell level are of great interest to researchers in the field of virology.

Previously, researchers have demonstrated the variation in virus production from single infected cells\textsuperscript{206,207}. In these studies, viruses released from single cells were titered by plaque assay, which serves as a “gold standard” in conventional virology experiments and relies on the principle that single viruses will be amplified within a population of host cells to produce visible plaques of cell death. This assay is widely used to measure virus infectivity and to test antiviral drugs, but is limited by providing only the averaged information of populations, labor intensiveness, inconvenience of automation, low sensitivity, throughout and reproducibility, and high consumption of samples and reagents.

Recently, microfluidics has begun to overcome the difficulties in the traditional viral infection investigations. Microfluidic-based digital PCR\textsuperscript{208-210} or RNA interference\textsuperscript{211} enable virus-host interaction screening by encapsulating host cells and viruses in water-in-oil droplets or microfluidic chambers. Despite advances in economics of the scale, reproducibility, parallelization and automation, these approaches may miss the stochastic kinetics of individual infection events due to end-point analysis of fixed or lysed cells. The integration of a multi-layer, pneumatic-controlled, microfluidic cell culture system with live-cell imaging offers an excellent tool for dynamic cellular activity studies in improving the precision of single cell tracking, control over cell growth microenvironment, reagent consumption, and ability to work with small
number of cells.\textsuperscript{212,213} Although this system greatly enhanced our understandings in stem cell proliferation,\textsuperscript{214} cell signalling,\textsuperscript{215} and cell migration,\textsuperscript{216} applications to virus-host interactions have not been reported.

In order to apply such an integrated system to viral infections at the single-cell level, issues such as the crossing-infections from neighbouring infections and the limited number of single cells precluding any robust statistical analysis need to be addressed. Here, we describe a single-cell virology platform to (i) allow a high-throughput isolation of virus-infected single cells into individual wells, (ii) avoid cross-infections from neighbouring infection events by sealing each cell culture well via closing pneumatic valves, (iii) monitor the dynamics of single-cell viral infections using an automatic, time-lapse, live-cell imaging approach, (iv) premise a parallelized, multiple parametric or/and multiple conditional comparison, thereby addressing the technical limits of the conventional plague assay. Our studies of single-cell PV infection dynamics have revealed enormous stochastic behaviour that likely plays out \textit{in vivo} that contributes to post-infection outcomes. Our experimental data also unravelled the delayed replication of an attenuated poliovirus mutant and provided insight into the evolutionary dynamics of the virus under selection pressure of antiviral drugs. We expect that this single-cell virology technology promises to be as transformational to virology as single-molecule approaches have been for enzymology and cell biology.

\section*{6.2 Working mechanism}

An integrated microfluidic device that preforms 6400 viral infection events and executes isolation, culture and viral infection of single cells is described (Fig. 6-1). For precise comparison between different experimental conditions, our prototype device consists of four individual sample inlets, each connecting to 1600 wells. Here, HeLa S3 cells and GFP-modified poliovirus
(GFP-PV)\textsuperscript{217} were chosen as our biological model to investigate the single-cell viral infection. This GFP-PV has the GFP sequence inserted into the viral genome so that the GFP protein is expressed upon the replication of the viral genome. As a result, the dynamics of viral replication can be monitored by recording the GFP signal from a single infected cell at different points post-infection. After loading the cell/virus/drug solution into the device, single infected cells were trapped and sealed in individual cell culture wells by closing the integrated pneumatic valves. Dynamics of viral replication was automatically monitored using time-lapse fluorescence microscopy every half an hour in a high-throughput manner. Recorded time-lapse images (Fig. 6-2) were then processed using a customized MATLAB code to extract the fluorescence intensity of single infected cells and background fluorescence intensity of the well. The time-dependent GFP intensity curve of a single cell can be used to describe a single-cell viral infection dynamic pattern (Fig. 6-1b).

Figure 6-1. Single-cell virology platform. (a) Micrographs of an integrated microfluidic device with image and schematic structure of wells as insets. This device contains 6400 wells (green) connected with four separated sample inlets, and pneumatic control lines (red) to seal cell and virus in a well. Scale bar: 100 μm. (b) Illustration of single-cell viral infection dynamics in an isolated microfluidic well. The time-dependent GFP intensity curve indicates the viral infection dynamics of a single cell.
Figure 6-2. Experimental data. (a) Stitched bright-field image (3 hpi) showing wells connected to one inlet. (b) Three corresponding fluorescence images (6, 12 and 18 hpi) showing GFP signals of infected cells (white dots). Scale bar: 1 mm. (c) Enlarged images of the rectangular regions in (a) and (b). Four single infected cells can be identified. Scale bar: 200 µm. (d) Plots of the normalized GFP intensity versus hpi for the four single infected cells in (c).
6.3 Results

6.3.1 Isolated single-cell viral infection

Despite the fact that viral infection of single cells within a cell population has been investigated, the unpredicted and uncontrollable virus release and spread in the cell population may obscure the virus-host interaction dynamics. To manifest this situation, we conducted an experiment to compare the interactions between viruses and single cells within a cell population and a single-cell environment. In this experiment, HeLa S3 cells infected with GFP-PVs at a low MOI (~ 50 genomes/cell or 0.05 pfu/cell) were loaded into either a conventional cell culture plate or an integrated microfluidic device, and cultured in a microscope incubation system. Here, the MOI of 0.05 plaque-forming unit (pfu)/cell indicated that each cell was infected with less than 1 pfu on average and the percentage of infected cells should be around 5%. Bright-field and fluorescence images were automatically recorded every 30 minutes from 3 hours post-infection (hpi) to 36 hpi. The GFP signal of the infected cell was undetectable before 3 hpi. In the cell culture plate, only a few infected cells within a population expressed obvious GFP signals until 12 hpi and the intensities decreased after 18 hpi, while most of cells showed GFP signals at 30 hpi (Fig. 6-3a). In the isolated wells, only a small portion of single infected cells trapped in sealed single-cell environment showed heterogeneous GFP dynamics (Fig. 6-3b), while other single cells did not show obvious GFP expression during the whole process. The time-dependent GFP intensity curves of single infected cells within a cell population (Fig. 6-3c) and isolated wells (Fig. 6-3d) were obtained from the experimental data. For a typical infection event, the GFP signal increased around 3 to 4 hpi, raised dramatically until it reached a plateau around 8 to 12 hpi as a life cycle for PV replication. In the cell population condition, several GFP signals increased after 18 to 20 hpi, and reached their plateaus around 26 hpi (such as green, orange, and
brown curves in Fig. 6-3c). These late infection events were probably due to multiple-around infections by the viruses released from the initial infected cells (two bright cells at 12 hpi in Fig. 6-3b corresponding to blue and red curves in Fig. 6-3c). In contrast to this cell population approach, the isolated wells can compartmentalize individual single-cell viral infections and avoid the potential influence of virus release and spread. The heterogeneous patterns of the isolated single-cell viral infection dynamics obtained from first-around infections were shown in Fig. 6-3d. We have demonstrated the capability of our single-cell virology platform in studying the pathogen-host interactions without any crosstalk with other events.

Figure 6-3. Comparison of single-cell viral infection dynamics within a cell population and a single-cell environment. (a,b) Time-lapse automated imaging of GFP-expressing HeLa S3 cells in a cell population (a) and an isolated single cell environment (b). Cells in the plate or single cell in the sealed wells were imaged every 30 minutes. The well format isolates each individual infection event, while the plate format shows multiple-time infections. Scale bars: 100 μm. (c,d) Time-dependent normalized GFP intensity of single cells in the conventional culture plate (c) and the isolated microfluidic wells (d). Each single curve indicates the infection dynamics of a single cell.
6.3.2 Heterogeneity of single-cell viral infection dynamics

The heterogeneity of virus-host interactions is an intriguing feature of virology. We believe that the elucidation of this heterogeneity will reveal some inaccessible facts in conventional population-averaged analyses. Using our single-cell virology platform, we examined this heterogeneity with GFP-PV infected HeLa S3 cells. Single infected cells displayed astonishing heterogeneity in the kinetics and magnitude of GFP fluorescence (Fig. 6-4a). Importantly, an average time-dependent curve of all the normalized GFP intensity curves (Fig. 6-4b) represented a similar trend toward a typical one-step virus growth curve obtained using traditional methods (Fig. 6-6a).

In order to evaluate the substantial differences in single-cell viral infection dynamics, an analysis pipeline was developed to quantitatively and statistically characterize each time-dependent normalized GFP intensity curve. The single-cell viral infection dynamics were categorized into either an infection model as a sigmoidal curve (Fig. 6-4c) or an infection and lysis model as a double-sigmoidal curve (Fig. 6-4d). Three independent phenomenological parameters including maximum, midpoint, and slope were given to represent the maximum normalized GFP intensity reached during the time course, the time at which the intensity has risen to half of its maximum (the midpoint of the rise phase), and the slope of the normalized GFP intensity curve at the midpoint. From viral replication point of view, these three parameters may indicate the saturation level, kinetics, and speed of genome replication inside the infected cells. These parameters are expected to characterize the effect of different conditions such as MOI, genetic variation, and drug treatment.

Using this analysis model, we investigated the impact of MOI on the heterogeneity of single-cell viral infection dynamics. In our experiment, the responses of single HeLa S3 cells to GFP-PV infections at different MOIs were parallel monitored and compared on one device. We
analysed 92, 126, and 202 single infected cells at the MOIs of 50, 500, and 5000 genomes/cell respectively, which supports a meaningful statistical analysis (Table 6-1). The distributions of maximum appeared similar among MOI = 50, 500, and 5000 genomes/cell (Fig. 6-4e). Importantly, when the MOI increased from 50 to 5000 genomes/cell, the distributions of slope showed a right shift (Fig. 6-4f), while the distributions of midpoint showed a left shift (Fig. 6-4g), both suggesting a fast progression of viral infection at high MOI. In addition, the percentage of infections (infected single cells to all single cells) rose from 6.3% to 86.5% as MOI increased from 50 to 5000 genomes/cell (Fig. 6-4h). Our results indicate that the larger MOI causes more rapid progression of viral infection, both increasing the replication speed and reducing the generation time.

Figure 6-4. Heterogeneity of single-cell viral infection dynamics. (a,b) Individual normalized GFP intensity curves (a) and average normalized GFP intensity curve (b) based on time-lapse imaging of single infected cells. (c,d) Statistical models including infection model (c) and infection and lysis model (d). (e) The distributions of maximum over different MOIs showed insignificant difference. (f) The distributions of slope showed fast infections with a high MOI. (g) The distributions of midpoint confirmed a fast replication of viral genomes with a high MOI. (h) The percentage of infected cells increased from 6.3% to 86.5% by increasing MOI from 50 to 5000 genomes/cell.
Table 6-1. Two-sample t-test results of the MOI experiment. A Benjamini–Hochberg correction was performed to control the false discovery rate introduced by multiple testing. Adjusted P-values below 0.000005 rounded to 0.

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6.3.3 Cell cycle perturbation

It is well known that within a population, cells grow asynchronously and are at different growth stages due to cell cycle. We evaluated the influence of cell cycle on the heterogeneity of virus-host interactions that we have observed at the single-cell level. HeLa S3 cells were first labelled with a cell membrane-permeant, DNA-binding dye and sorted into G0/G1 and G2/M groups using a fluorescence-activated cell sorter (FACS) (Fig. 6-5a). Cells run through the FACS and collected without sorting were used as an unsorted control. Then these three groups of cells
were infected with GFP-PV at MOI = 500 genomes/cell and studied in our single-cell virology platform.

The average curves of single-cell viral infection dynamics using unsorted cells, G₀/G₁ cells, and G₂/M cells appeared overlapping (Fig 6-5b). Further analysis showed similar distributions of maximum, slope, and midpoint among the three groups (Fig. 6-5c-e). Results of t-tests confirmed that the difference in the distributions of maximum, slope or midpoint was not significant (P-values > 0.05) (Table 6-2). These results indicate that the contribution of cell cycle to the heterogeneity of single-cell viral infection dynamics that we have observed is insignificant.

Figure 6-5. Cell cycle experiment. (a) Sorting of HeLa S3 cells into G₀/G₁ and G₂/M groups based on cell cycle. Cells run through the FACS and collected without sorting were used as unsorted control. (b) Averaged curves of single-cell viral infection dynamics using unsorted cells, G₀/G₁ cells, and G₂/M cells. Data are presented as means ± standard errors of the mean (SEM). (c–e) Distributions of maximum, slope, and midpoint illustrated an insignificant contribution of cell cycle to the heterogeneity of viral infection dynamics at the single-cell level.
Table 6-2. Two-sample t-test results of the cell cycle experiment.

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6.3.4 Virus mutant perturbation

Like other RNA viruses, PV replicates with high mutation rates, thus exhibiting significant genetic diversity within the viral population.\textsuperscript{204,205} This genetic diversity serves as a reservoir for novel mutants that could potentially evade vaccination and cause polio outbreaks.\textsuperscript{220} Evolutionary theory predicts that the mutation rate of PV has been optimized by natural selection and that further increasing it will compromise viral fitness.\textsuperscript{221,222} Recently, we have discovered a novel PV mutant strain H273R, which exhibits reduced fidelity of the viral RNA-dependent RNA polymerase (RdRp) and increased error rate in viral replication.\textsuperscript{223} The previous studies in mouse model showed that H273R is attenuated \textit{in vivo} compared to WT, confirming the decreased viral
fitness due to the increased mutation rate. However, one-step-growth curve of H273R in HeLa cells at the population level revealed no significant difference relative to WT (Fig. 6-6a). This discrepancy motivated us to investigate the viral infection dynamics of H273R at the single-cell level.

Figure 6-6. Comparison of single-cell viral infections between WT and H273R. (a) One-step-growth curves of WT and H273R PV. Viral titer (pfu/ml) was plotted against hours post-infection. Data are presented as means ± SEM (n=3). (b) The distributions of maximum for WT and H273R appeared similar, without significant difference. (c) The distributions of slope for H273R showed a right shift with respect to WT, suggesting an increased replication speed for H273R. (d) The distributions of midpoint illustrated delayed viral replication of H273R compared to WT. (e) The distributions of start point contributed to the difference we have observed in the distributions of midpoint. (f) At high MOI (5000 genome copies/cell), the percentages of infected cells appeared similar between WT and H273R (86.5% and 87.6%, respectively). At low MOI (50 genome copies/cell), the percentage of infected cells for H273R (2.4%) was lower than WT (4.4%).

We infected HeLa cells with GFP-PV WT or GFP-PV H273R viruses at MOI = 50 or 5000 genomes/cell and studied the viral replication of single infected cells. Quantifications of the viruses based on pfu or genomes were determined (Table 6-3). The distributions of maximum for WT and H273R appeared similar at MOI = 50 or 5000, without significant difference (Fig. 6-6b). Interestingly, the distributions of slope for H273R (Fig. 6-6c, blue) showed a right shift with respect to WT (Fig. 6-6c, red), suggesting an increased replication speed for H273R. Recent
studies of another PV mutant strain K359R showed increased RdRp fidelity but reduced speed of genome replication. Together, these observations indicate an intrinsic antagonism between the fidelity and speed of viral replication. The distributions of midpoint (Fig. 6-6d) illustrated delayed viral replication of H273R compared to WT. This effect of elongated generation time of H273R relative to WT could be amplified over virus passaging, leading to a large difference in the accumulation and spread of virus. We also noticed that the difference in the distributions of midpoint between WT and H273R is largely attributed to the difference in the distributions of start point (Fig. 6-6e), which is the point at which a straight line representing the slope intersects the line of zero intensity. This observation implies some difference between WT and H273R at early points post-infection. The percentages of infected cells appeared similar between WT and H273R (86.5% and 87.6%, respectively) at high MOI (5000). At low MOI (50), the percentage of infected cells for H273R (2.4%) was lower than WT (4.4%) (Fig. 6-6f). This result is consistent with the decreased infectivity of H273R relative to WT, as indicated by the values of genomes/pfu (Table 6-3).

Table 6-3. Quantifications of the GFP-PV WT and H273R viruses based on pfu or genomes.

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<th>genomes/ml</th>
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<td>GFP-PV H273R</td>
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Table 6-4. Two-sample t-test results of the virus mutant experiment.

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6.3.5 Antiviral drug perturbation

Despite extensive efforts in developing antiviral drugs, the intrinsic genetic diversity and high mutation rates of RNA viruses allows for rapid acquisition of viral resistance to antiviral drugs. The existence of genetic diversity predicts the heterogeneous responses of the viral population to drug treatment. The dissection of this heterogeneity may shape our view of the underlying mechanism of drug resistance in RNA viruses, and calls for our investigation on cellular responses to the antiviral drug at the single-cell level. The antiviral drug used in our experiment was 2'-C-methyladenosine (2'-C-meA), a chain-terminating nucleotide analog that can be incorporated into the synthetic RNA and block the addition of the next correct nucleotide.

Prior to on-chip experiment, we first performed an off-chip screening experiment to determine the half maximal inhibitory concentration (IC\textsubscript{50}) of 2'-C-meA. We infected HeLa cells with GFP-PV WT at MOI = 500 genomes/cell under the treatments of 0 (DMSO control), 25, 50, and 100 µM 2'-C-meA. The percentages of infected cells at each drug concentration were 37.7%, 22.7%, 18.5%, and 0.5%, respectively. Therefore, the IC\textsubscript{50} of 2'-C-meA under this condition is around 50 µM.

For on-chip experiment, we infected HeLa cells with GFP-PV WT at MOI = 500 genomes/cell with 0 (DMSO control) and 50 µM 2'-C-meA and monitored the viral replication of single infected cells. The percentages of infected cells without and with drug treatment were 35.4% and 18.2%, respectively (Fig. 6-7a), consistent with our off-chip screening results. It is obvious that drug treatment decreased the maximum with respect to the control, which can be explained by the termination of viral replication due to 2'-C-meA incorporation (Fig. 6-7b). Surprisingly, we noticed that drug treatment also affected the slope distribution significantly (Fig. 6-7c). It is not likely that 2'-C-meA directly reduced the speed of viral replication considering its inhibition mechanism of chain termination. Thus, the influence of drug treatment on the slope
distribution may suggest that infections with larger replication speed have a higher chance of 2’-C-meA incorporation and are thereby more drug-sensitive. As a result, this subset of infections was inhibited by drug treatment, leading to the altered slope distribution. The distribution of midpoint was not changed significantly by drug treatment (Fig. 6-7d) (Table 6-5).

Figure 6-7. 2’-C-meA drug treatment results at the single-cell level. (a) The treatment with 50 µM 2’-C-meA (IC50 concentration) decreased the percentage of infected cells from 35.4% (0 µM, DMSO control) to 18.2%. (b) The distribution of maximum shifted to the left after drug treatment. (c) The distribution of slope also shifted to the left after drug treatment. (d) Kinetics of genome replication seemed not changed by drug treatment, indicated by similar distribution curves of midpoint. (e) 2D density plot of maximum versus midpoint showed that these two parameters were not correlated. (f) 2D density plot of maximum versus slope showed that these two parameters were correlated. After drug treatment, the subset of infections with high slope and high maximum was eliminated.

In addition to the distributions of single parameter, we also studied the parameter correlations (e.g., maximum versus midpoint and maximum versus slope). The 2D density plot of maximum versus midpoint showed that these two parameters were not correlated and that drug treatment reduced the maximum without affecting the midpoint distribution significantly (Fig. 6-7e). The 2D density plot of maximum versus slope showed that these two parameters were correlated (Fig. 6-7f). Interestingly, we noticed that the subset of infections with high slope and high maximum was eliminated after drug treatment. From evolutionary point of view, this
observation may indicate that the development of drug resistance to 2’-C-meA stems from viruses with slow replication speed rather than those with fast replication speed, although the fitness of fast-replicating ones is higher when there is no selection pressure.

Table 6-5. Two-sample t-test results of the antiviral drug treatment experiment.

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6.4 Methods

6.4.1 Cell and virus

HeLa S3 cells and enhanced green fluorescent protein-modified poliovirus type 1 Mahoney (GFP-PV) were used in the experiment. HeLa S3 cells were obtained from American Type Culture Collection (ATCC) and cultured at 37°C in DMEM/F12 medium (Cat# 11320, Life Technologies, USA) supplemented with 10% fetal bovine serum (Cat# S11050H, Atlanta Biologicals, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Cat# 30-002-CI, Corning,
USA). For suspension cultures, HeLa S3 cells were grown in 125-ml flasks (Cat# 355117, Becton Dickinson, USA) shaken at 80 rpm (Mini Shaker 15, VWR, USA). Viruses were made from pMo-GFP-WT or pMo-GFP-H273R plasmids via plasmid linearization, purification of linear plasmid template, T7 transcription, and transfection of HeLa cells through electroporation. After harvest, the virus was purified via PEG precipitation and filtration. Quantifications of the virus based on pfu or genomes were conducted through plaque assay or real-time (RT) quantitative polymerase chain reaction (qPCR).

### 6.4.2 Experiment

Infection of cells was conducted off-chip by thoroughly mixing HeLa cells with GFP-PV in PBS at desired MOI for 20 minutes in suspension. After infection, HeLa cells were washed with PBS, centrifuged, resuspended in culture medium, and loaded into the microfluidic device. The microfluidic device was then placed inside a microscope incubation system (INUBTFP-WSKM-GM2000A, Prior Scientific, USA) assembled on the stage of an inverted microscope (Eclipse Ti, Nikon, Japan), which was equipped with a motorized stage (ProScan III, Prior Scientific, USA). The microfluidic device was connected to a customized pneumatic valve system that sealed single infected cells into each individual wells and prevented the spread of virus into the neighboring wells by applying pressurized nitrogen (30 psi). The cells were isolated in individual wells and cultured in a 37 °C humid environment with 5% CO₂ level. This experimental setup enabled automatic monitoring of single-cell viral infection dynamics through time-lapse, multipoint, and live-cell imaging.
6.4.3 Data acquisition and processing

The time-lapse images (including bright-field and fluorescence) of multiple positions of the microfluidic device were automatically acquired with a 10× objective and a charge-coupled device (CCD) camera (CoolSNAP HQ2, Photometrics, USA) controlled by software (NIS-Elements AR, Nikon, Japan) every 30 minutes for 21 hours or 33 hours starting at 3 hpi. After data acquisition, the time-lapse images were processed with a customized MATLAB script to extract fluorescence intensity of single infected cells and background fluorescence intensity of the well. After image processing, we manually examined the experimental data to exclude wells showing auto-fluorescence, dried wells, and wells showing out-of-focus GFP signals. Wells containing multiple infected cells were excluded by the MATLAB script during image processing. Empty wells and wells with no infection were excluded by the R code during model fitting.

6.4.4 Data modeling and analysis

The extracted time courses of normalized GFP intensity (cell intensity subtracted by background intensity and then divided by background intensity) were fitted with three different models: a linear model, a sigmoidal model, and a double-sigmoidal model and classified into four categories “no signal”, “infection”, and “infection & lysis”, and “ambiguous” based on the fitting results. For time courses showing “infection” and “infection & lysis”, parameters of biological relevance such as maximum, midpoint, and slope were extracted and used to characterize the heterogeneity of viral infection dynamics at the single-cell level. Two-sample t-tests with Benjamini–Hochberg correction were performed to compare the distributions of parameters among different groups and control the false discovery rate introduced by multiple testing.
6.5 Discussion

Our single-cell virology platform presented here enabled high-throughput, real-time, and automated observation of single virus-infected cells in an isolated micro-environment. Compared to population methods such as plaque assay, our platform studies viral infections at the single-cell level and has the ability to unravel the complexity of virus-host interactions. Our experimental data have revealed unexpected heterogeneity in the kinetics and magnitude of viral replication at the single-cell level. This single-cell analysis of viral infections helps to illustrate the delayed replication of an attenuated PV mutant, which is indistinguishable from WT when characterized using plaque assay. In addition, our single-cell virology platform offers a novel tool for screening the heterogeneous responses of the viral population to antiviral drug treatment and sheds light on the development of drug resistance. Currently, cells loaded into the microfluidic chips follow a Poisson distribution and the percentage of wells containing a single cell is ~ 20% at optimized condition, which could be further improved with microfluidic single-cell trapping design.227

In our current system, the dynamic process of viral replication was monitored by incorporating the GFP sequence into the viral genome. Real-time monitoring of other stages of the virus life cycle (e.g., viral entry or virion release) can also be realized via the fusion of a fluorescent protein to the structural protein of the virus.228,229 In addition, to further adapt our platform to the virology community, the genetically encoded reporter could be replaced with certain external reporter system so that the virus can be studied on its native form. Fluorophore-conjugated molecular probes targeting viral genomes or proteins, such as the recently demonstrated spherical nucleic acid (SNA) gold nanoparticle conjugate targeting the viral RNA, would be ideally suited for our platform.230 Recently, the CRISPR231 (clustered regularly interspaced short palindromic repeats)/Cas system has been demonstrated as a powerful gene editing tool that allows the modification of the organism’s genome at any desired location. This
makes it possible to fuse a fluorescent protein to a key component of the host signaling pathways (e.g., interferons) in response to a specific pathogen. The creation of such reporter cell lines can expand the application of our platform for elucidating various pathogen-host interactions.

In our combat against infectious diseases, one big threat is the dwindling arsenal due to the rapid emergence of drug-resistant pathogens. For instance, the emergence of “super bacteria” resistant to multiple antibiotics is becoming a severe threat to public health. This places a pressing need for both uncovering the underlying mechanism of drug resistance and discovering novel drugs. Our platform can facilitate the identification of drug-resistant variants of the pathogen by exploring the dynamic pathogen-host interactions at the single-cell level. Further studies of such variants can provide insight into the evolutionary dynamics of the pathogen and help decipher how drug resistance is acquired. In addition, the high-throughput and multiplex merits of our system also make it a good candidate for the future development of a single-cell drug-screening platform, in which the heterogeneous responses of the pathogen population to drug treatment will be evaluated. This single-cell level drug screening may facilitate the discovery of more effective therapeutics for infectious diseases caused by a myriad of pathogens such as viruses, bacteria, and fungi.
Chapter 7

Summary and prospects

7.1 Research summary

We are in an era in which inter-disciplinary research is embraced to tackle scientific challenges. The advances in biological and biomedical research have benefited from the collaborative efforts from many disciplines to develop novel technologies. The past two decades have witnessed the rapid growth of microfluidics and its application in biological and biomedical research. Recently, acoustofluidic technologies have demonstrated attractive advantages such as high biocompatibility, automatic and continuous-flow operation, non-contact and label-free cell manipulation, and good tunability; thus providing a powerful platform for cell analyses and studies. This dissertation has centered on developing acoustofluidic or microfluidic platforms to solve some technological challenges faced in cell biology and its related fields: automatic processing of cell samples; investigation of heterotypic cell-cell interactions; and investigation of heterogeneous virus-cell interactions.

The acoustofluidic-based multichannel droplet sorter I have developed takes advantages of the excellent controllability and tunability offered by the SSAW technique and can sort picoliter droplets into five (or more) outputs at rates up to 222 droplets s\(^{-1}\) without external labeling. It offers an effective droplet sorting unit for the future development of fluorescence-activated droplet sorter (FADS) through integration with an optical-detection module and feedback control module. Such FADS system can be useful in screening and sorting cells based on secreted biomarkers (e.g., small molecules or proteins secreted from single cells).
The acoustofluidic-based cell washing/transfer platform I have developed takes advantages of the large lateral displacement offered by the taSSAW approach and can wash beads or cells with high recovery rate (>98%) and high washing efficiency (>97%). It is the first validated microfluidic platform that transfers cells from human sputum samples. This acoustofluidic device preserves the viability and integrity of the transferred cells. In addition, the removal of residual DTT utilized during sputum liquefaction through acoustofluidic cell transfer yields more accurate immunophenotyping results per flow cytometry analysis, which is valuable for identifying inflammatory lung cells from human sputum samples. Moreover, because of automatic and continuous-flow operation, the acoustofluidic-based cell transfer device can be readily incorporated with other on-chip sputum processing or analysis units to realize an automated, fully integrated device for point-of-care diagnostics of asthma and other pulmonary diseases.

The cell co-culture platform demonstrated in my dissertation can form organized cell co-culture for studying heterotypic cell-cell interactions. It takes advantage of the non-contact, non-invasive nature of acoustic forces, thus exerting minimal interference on the cellular microenvironment while preserving high cell integrity. It provides a novel tool for real-time, dynamic observation of cell behaviors within co-culture and on-chip multicellular reconstruction. The demonstrated study of cancer cell mobility within co-culture suggests its utilization as an *in vitro* platform for the evaluation of the invasiveness of cancer cells and anti-tumor drug screenings.

The single-cell virology platform demonstrated in my dissertation enables high-throughput, real-time, and automated observation of single-cell viral infection dynamics in an isolated micro-environment. Compared to population methods such as plaque assay, it studies viral infections at the single-cell level and has the ability to unravel the complexity of virus-host interactions. Our experimental data have revealed astonishing heterogeneity in the kinetics and
magnitude of viral replication at the single-cell level, which likely plays out in vivo that contributes to post-infection outcomes. This single-cell analysis of viral infections helps to illustrate the delayed replication of an attenuated poliovirus mutant, which is indistinguishable from WT when characterized using plaque assay. In addition, our single-cell virology platform offers a novel tool for screening the heterogeneous responses of the viral population to antiviral drug treatment and sheds light on the evolutionary dynamics of viruses under selection pressure of antiviral drugs.

7.2 Prospects

Despite its rapid development over the past two decades, microfluidics has not yet lived up to the expectation to be a widely used tool in biological research and clinical diagnostics. The acoustofluidic or microfluidic platforms developed during the course of my dissertation work serve as examples how microfluidics can help solve some technological challenges in cell biology and its related fields. However, for these platforms to be exploited in routine biological or biomedical research, further platform improvement and application demonstration are needed.

The acoustofluidic droplet sorter developed in my dissertation work provides a controllable and tunable microfluidic droplet sorting module. The current throughput of our device is 222 droplets s⁻¹, which can be further improved by using focused IDTs. One major application potential of our acoustofluidic droplet sorter is the further integration with an optical-detection module and a triggering-control module to realize a miniature fluorescence-activated droplet sorter (FADS). Some colleagues in the Huang Lab have recently developed an acoustofluidic FACS system, which shares the same scheme and can facilitate the future development of the FADS system. Once realized, the FADS system can help select target droplets containing the cells of interest from the pool based on fluorescence signals and overcome
the limitation of current FACS systems to screen and sort cells based on secreted biomarkers instead of surface biomarkers.

For microfluidic platforms to be adopted by biological or biomedical laboratories, significant efforts are needed to integrate different functional units into complete systems. Such integrated systems should target at specific tasks and realize “sample-in, answer-out” function. One critical step in asthma diagnosis is immunological phenotyping of induced sputum samples to identify lung inflammatory cells. The current sputum processing and analysis techniques involve several labor-intensive, operator-dependent procedures such as sputum liquefaction, cell transfer, and flow cytometry analysis. In my dissertation work, I have developed an acoustofluidic cell washing device that can successfully transfer inflammatory cells from liquefied human sputum samples. Recently, my colleagues in the Huang Lab have also demonstrated an acoustofluidic sputum liquefier and an acoustofluidic flow cytometry chip. One potential future direction is to develop an integrated acoustofluidic point-of-care system that would allow on-chip processing of induced sputum samples and analysis of the percentages of different lung inflammatory cells in induced sputum samples from asthmatic patients. The development of such a system requires further engineering efforts to integrate separate functional units as well as external components (e.g., function generators, amplifiers, optical components, and syringe pumps) into a single prototype device. Once developed, the performance of the prototype device also needs to be validated with real clinical samples. This integrated prototype device is expected to provide a point-of-care asthmatic diagnostic tool and increase the availability of personalized treatment strategies to asthmatic patients in both research and community settings.

In addition to system integration, the adaptation of microfluidic platforms to some widely accepted standards in biological or biomedical research is also very important. The cell co-culture platform demonstrated in my dissertation provides a novel tool for probing heterotypic cell-cell
interactions. However, the lithium niobate/PDMS-based microchannel device may not appear appealing to cell biologists, who are used to plastic-based cell culture plates. To overcome this limitation, we can further develop an acoustofluidic-based cell co-culture platform that is compatible with conventional cell culture using petri dish or well plate. One possible strategy is to couple the acoustic waves into the culture medium inside the petri dish or well plate to pattern cells and form organized cell co-culture. The realization of such a coupling platform will promote the utilization of our acoustofluidic-based cell co-culture platform by cell biologists. In our current cell co-culture device, different cell populations were patterned in culture medium and grown on the substrate surface. Another future direction is to replace the culture medium with extracellular matrix (e.g., Matrigel™) so that 3D cell co-culture can be formed and investigated. Such improvement will even better mimic the in vivo cellular microenvironment and provide a useful tool for tissue engineering.

Although platform improvement plays an important role in pushing forward the application of our platforms, it is worth noting the solving real scientific problems lies at the heart of our research. During our model studies of poliovirus using the single-cell virology platform, we observed astonishing heterogeneity in virus-host interactions at the single-cell level. Elucidation of the underlying molecular mechanism in either the virus or host is the key to uncovering the mystery of viral pathogenesis. However, in order to correlate the phenotypic heterogeneity we observe with variances from the virus or host side, subsequent investigations of cellular transcriptome and viral progeny are highly desirable and will be the major focus of our future studies. To achieve this goal, we will integrate a micromanipulator connected with a micropipette into our system to achieve selective recovery of materials from wells of interest post-infection. Transcriptomic analysis of recovered single cells can be conducted to study whether variation in innate immunity can influence viral infection. In addition, phenotypic and genetic analyses of the released viral progeny can be conducted to probe the variance from the
virus side. The infectivity of viral population from the wells of interest can be compared by repeating the experiment to determine whether the observed phenotype is preserved. Circular sequencing (CirSeq) can be used to uncover the mutational landscape of viral progeny in each well. The combination of our single-cell virology platform with these existing tools may help us decipher the complexity of virus-host interactions.


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