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Pulsed Laser-Triggered High-Speed Microfluidic Fluorescence-Activated Cell Sorter

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Pulsed Laser-Triggered High-Speed Microfluidic

Fluorescence-Activated Cell Sorter

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Mechanical Engineering

by

Yue Chen

2014
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ABSTRACT OF THE DISSERTATION

Pulsed Laser-Triggered High-Speed Microfluidic

Fluorescence-Activated Cell Sorter

by

Yue Chen

Doctor of Philosophy in Mechanical Engineering

University of California, Los Angeles, 2014

Professor Pei-Yu Chiou, Chair

Over the past decade, various microfluidic fluorescence-activated cell sorter (FACS) systems have been demonstrated aiming to provide a fully enclosed environment for sterile, contamination and infectious free sorting, and better downstream microfluidic integration for further analysis after sorting. The biggest challenge of μFACS systems, however, is the orders of magnitude lower sorting throughput and purity than commercial aerosol based FACS (90% purity at 70,000 cells sec⁻¹). To solve this problem, the key innovation is to find a faster switching mechanism. In this dissertation, the importance of the cell sorting in academic
research and clinical practice will be discussed at first. Among the commercial FACS machines, the state-of-the-art performance is achieving above 90% purity at 70 000 cells sec$^{-1}$. Other reported μFACS presents 1~2 order of magnitude lower in throughput.

In this context, we attempt to use the phenomena of the pulsed laser induced cavitation bubble to switch particles/cells in high speed. The first version simply excited the bubble in the microfluidic channel that is running parallel to the sample channel. The bubble expansion deformed the elastic PDMS channel wall and induced fluid swing in the sample channel which carried target particle/cell flowing into the collection channel. This first trial demonstrated the fast dynamics of this switching mechanism and the strong power the cavitation bubble can affect the flow. However, the deformation of the channel wall extended over hundreds of microns in length, resulting in low post-sort purity. To overcome this, the second version designed a connecting nozzle between the sample channel and the bubble channel. During the expansion of bubble, a liquid jet was created through the nozzle to the sample channel, which limited the disturbance range to tens of microns. With 2D hydrodynamic focusing, the pulsed laser activated cell sorter (PLACS) demonstrated performance with above 90% post-sort purity at 3 000 particles sec$^{-1}$. The drop of post-sort purity at higher throughput was due to the lack of the third dimension focusing which created large time variation in cell arrival time between the detection and switching zones. Then 3D hydrodynamic focusing that utilized multilayer PDMS
with vertical vias solved the synchronization issue but also allows efficient particle switching using a smaller bubble with a smaller perturbation volume, and a shorter on-off switching cycle. As a result, the 3D PLACS was able to sort at 23,000 cells sec\(^{-1}\) with ~90% purity or at 45,000 cells sec\(^{-1}\) with 45% purity within a single channel. However, the large amount of diluting sheath fluid needed for tight 3D focusing requires a high initial cell concentration in samples (>10\(^7\) cells ml\(^{-1}\)), which can result in cell clogging in channels, and high pressure pumps to drive fluid through microchannels at high speeds. Then inertial focusing was employed as a substitute to hydrodynamic focusing. The integrated system achieved sorting at 10,000 particles sec\(^{-1}\) with >90% sort purity or 6,000 cells sec\(^{-1}\) with >80% sort purity and used 10 times lower initial concentration cell samples than that in sheath-based PLACS.

The achieved PLACS not only outperformed other μFACS but also achieved performance at a level comparable to conventional aerosol-based FACS. It is anticipated that by integrating with upstream and downstream microfluidic cell analysis functions, the developed PLACS will greatly facilitate biomedical research and clinical diagnostics.
The dissertation of Yue Chen is approved.

Chih-Ming Ho

Tsu-Chin Tsao

Michael Alan Teitell

Pei-Yu Chiou, Committee Chair

University of California, Los Angeles

2014
To my dear parents
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PUBLICATIONS


PRESENTATIONS


Chapter 1. Introduction

1.1 Overview

Cell sorting is a widely used and standard tool for basic biology studies, drug development and clinical diagnosis in which enrichment of cells-of-interest from heterogeneous populations is a fundamental step prior to analysis [1, 2]. For example, many studies require identification and isolation of certain subpopulation cells from whole blood which consists 55% plasma, 44% erythrocytes (red blood cells), and less than 1% leukocytes (white blood cells) [3]. Mostly, isolation of subpopulation from diversely classified leukocytes is of interest. HIV/AIDS research needs isolation of CD4+ cells (one subpopulation of white blood cells) from whole blood efficiently [3]. Similarly, identification and separation of parasite-infected red blood cells is crucial for malaria diagnosis and treatment [4]. In non-invasive prenatal diagnosis, separation of erythroblast from mother’s peripheral blood is needed [3]. Another long-lasting interest is the isolation of circulating tumor cells (CTC) which is almost one in a few million unwanted cells [5, 6]. While there might be different constraints in individual application, a separation method that has high purity and high throughput is commonly desired by all these applications [4].

Cell separation techniques vary depending on the intrinsic properties of the different cell populations [7]. The distinguishing criteria can be divided into labelled and non-labelled.
Common physical characteristics including size, shape, density, adhesion, deformability, dielectric properties and surface affinity can be used as intrinsic biomarkers for non-labelled cell separations [5]. These separations can be achieved by sorting techniques such as membrane-based filtration [1, 3], centrifuge [8], hydrodynamic phenomena [9-13], dielectrophoresis [14-18], acoustophoresis [19-21], immune-affinity [22] and so on. However, they cannot distinguish cells with similar physical property but different biological characteristics. In order to more specifically distinguish subpopulation with different biological properties, labels such as fluorescence, magnetic labelling or special antigen/antibody should be applied. Correspondingly, techniques to sort labelled cells include fluorescence sorting, immunomagnetic sorting [23] and adhesion-based sorting [22]. Adhesion and magnetic beads based methods can only sort upon external membrane antigens and hardly sort based on multi-parameters [3]. Although it is possible to sort multi-parameters by magnetic force [24], high-purity and high-throughput sorting have not been reported yet.

On the other hand, fluorescence-activated cell sorting (FACS), following its invention in 1969, has become widely used in biomedical research laboratories and hospitals for clinical diagnostics [25, 26]. Not surprisingly, it becomes the “gold standard” [27] due to its mature engineering development, sensitivity and high throughput. Meanwhile, the versatile fluorescence labels, which are commonly available and acceptable, enable simultaneous
multiparametric analysis of the physical and chemical characteristics making it attractive to widespread applications [28]. Generally, immunolabelling is the dominant method, against both extracellular and intracellular targets. Besides, an expanding number of fluorescent probes for morphological and physiological cell measurements are now available, such as DNA binding dyes, viability dyes, cell tracking dyes and quantum dots [29].

1.2 Technical considerations in FACS

To separate target cells from initial mixture, the baseline is to preserve the viability of the cells, which sets some constrains on the choice of sorting methods and the working condition. The selectivity of an active sorting system means whether a target cell can be effectively detected and efficiently sorted out. In FACS, it depends on the accuracy of the detection and the efficiency of the sorting mechanism. The accurate detection indicates that the system will not miss a target (false negative) or send positive signal on a non-target (false positive). Upon sorting decision is given by detection system, an efficient sorting mechanism should be able to successfully move the target cell out from the mixture and not affect other cells. These two factors will affect post-sort purity in collection (positive) and waste (negative) outputs. Purity is defined as

\[ purity = \frac{n_{target}}{n_{total}} \times 100\% \]

where \( n_{target} \) is the number of target cells and \( n_{total} \) is the total cell number in the
considered cell sample. The higher of the post-sort purity in the collection output, the better. In contrast, the lower of the post-sort purity in the waste output, the better. However, they can be hardly achieved at the same time [4]. High post-sort purity in the collection output is more important in most applications because it reduces post-sort sample handling and eliminates the need for additional labels or tests on the cells [4].

Another important parameter related to purity is the enrichment ratio, which is defined as

$$enrichment\ ratio = \frac{purity_{post-sort}}{purity_{pre-sort}}$$

Sometimes it is ambiguous to use this parameter as the measurement of the performance as the definition varies in different studies [4]. High enrichment ratio cannot represent good performance by itself because it may start with extremely low pre-sort purity. As a result, it should be reported along with the post-sort purity.

The ratio of target cells to total cells, which is called pre-sort purity, is of great importance. In the field of rare cell sorting, which is the major application of FACS, high purity sorting from an initial mixture with target cell ratios ranging from $1:10^9$ to $1:10^4$ is challenging [3]. To get enough number of target cells to start with in the following steps, the sorting is expected to be as fast as possible in order to get rid of the abundant cells quickly. With the throughput of the sorting system and the target cell ratio together, the total initial sample quantity and processing time can be estimated. A suitable sorting system should have the capability to handle this
amount of cells in a reasonable time.

Concentration of the initial mixture cell sample (in the unit of cells per unit volume) must not be too low to require overly long separation time, nor too high to saturate the separation system. The relationship between cell spacing (in the unit of m), traveling speed (in the unit of m sec\(^{-1}\)) and the throughput (in the unit of cells sec\(^{-1}\)) is

\[
\text{Throughput} = \frac{\text{cell speed}}{\text{average distance between neighboring cells}}.
\]

And average distance between neighboring cells can be estimated as

\[
\text{average distance between neighboring cells} = \frac{\text{channel crosssection area}}{\text{cell concentration}}.
\]

Thus, high cell concentration is wanted for high throughput sorting. In contrast, high concentration samples will cause cell sentiment and aggregation during the separation procedure. In that case, external agitation might be needed. In FACS, cells are supposed to arrive one at one time. However, if the initial concentration is too high and not enough sheath flow is added, neighboring cells may not be spaced enough and arrive at the same time, potentially generating sorting errors. So there is always trade-off between throughput and cell concentration.

Dilution is an issue related to sheath flow that is used to focus cells in most cases. As a result, additional enrichment step will be needed. If a sheathless focusing scheme is employed, there is no such problem. However, to achieve high efficient sheathless focus is another
challenge, especially for rare cell sorting. For example, if the focusing efficiency is 99.9% which means that one cell is out of focus in 1000 cells. Assuming the initial target cell ratio is 1% and post-sort purity is 100%, the post-sort purity in collection output will decreased to 91%. If the focusing efficiency drops to 99%, then the amounts of the collected target cells and the unfocused cells are relatively equal resulting 50% post-sort purity. In order to achieve high post-sort purity, the ratio between target cells and unfocused cells in certain volume should be relatively large.

Most applications for FACS require fluorescence label on target cells and use positive selection to sort them out. However, labelling on target cells is not necessary in negative selection. In negative selection, the background cells are labelled instead of target cells. In rare cell sorting, it generates a lot more triggering which needs much more effort. But when minimal labelling and manipulation on target cells is wanted, negative selection will become a good candidate. Surely, there are other constrains arising from specific applications that should be considered case-by-case.

1.3 Conventional FACS

Figure 1.1 shows the working principle of a conventional FACS [30]. Basically, it focuses a cell suspension into a single stream with three-dimensional sheath flows through a small nozzle. Droplets leaving the nozzle are selectively and electrostatically charged based on the
fluorescence of an encapsulated cell. Then they are sorted by electrostatic deflection at a typical (not state-of-the-art) throughput 10,000 cells per sec [27]. This high-throughput facilitates both research and clinical applications, such as isolating sub-populations of immune cells for molecular analysis and sorting of circulating tumor cells (CTCs) from blood samples in a reasonable time [27].

![Principle of conventional cell sorter](image)

**Figure 1.1 Principle of conventional cell sorter [30].**

The speed of the sorting is limited by the droplet formation speed which is subject to the law of fluidics. Studies have already shown that the maximum droplet generation rate is proportional to the jet velocity, which in turn is proportional to the square root of the jet pressure. The relationship can be expressed by [30]

\[
  f_{optimal} = \frac{\sqrt{2P/\rho}}{4.54D_{jet}}
\]

where P is the pressure inside the droplet across the nozzle orifice, \( \rho \) is the density of the liquid,
\(D_{\text{jet}}\) is the diameter of the droplet. Current state-of-the-art devices can sort at a maximum rate of 70,000 cells sec\(^{-1}\).

Table 1.1 Summary of current commercial FACS from different companies

<table>
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<tr>
<th>Name/ Company</th>
<th>Maximum Throughput</th>
<th>Maximum Sorting Rate</th>
<th>Analyzed Parameter</th>
<th>Sorting Purity</th>
<th>Yield</th>
<th>Other Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influx/ BD</td>
<td>200,000 events/sec</td>
<td>70,000</td>
<td>24 (7 lasers)</td>
<td>98%</td>
<td>&gt;80%</td>
<td>6-way sorting</td>
</tr>
<tr>
<td>MoFlo Astrios/ BECKMAN COULTER</td>
<td>&gt;100,000 particles/sec</td>
<td>&gt;70,000 sort decisions/sec</td>
<td>18 (7 lasers)</td>
<td>&gt;99%</td>
<td>&gt;90%</td>
<td>6-way sorting</td>
</tr>
<tr>
<td>iCyt/ Reflection</td>
<td>4×150,000 events/sec</td>
<td>4×70,000 events/sec</td>
<td>18</td>
<td>&gt;99%</td>
<td>--</td>
<td>4 parallel modules</td>
</tr>
<tr>
<td>Gigasort/ Cytonome</td>
<td>--</td>
<td>144×2,000 cells/sec</td>
<td>9</td>
<td>--</td>
<td>90%</td>
<td>Microfluidic based, 144 sorters in parallel</td>
</tr>
</tbody>
</table>

1.4 Microfluidic FACS

However, current aerosol based FACS suffers several drawbacks. Aerosols accompanying high-speed droplet generation and sorting in conventional FACS are always concerns for both sample contamination and operating personnel safety when sorting infectious samples [31]. Perhaps more importantly, aerosol-based FACS does not provide a convenient interface for integration with upstream pre-sort or downstream post-sort sample preparation or analysis.
modules, especially with many of the novel microfluidic modules developed over the past decade.

To address this problem, various closed-form microfluidic FACS systems [2-4, 27, 32-34] have been developed over the past decade to provide sterile (contamination and infectious agent-free) sorting and improved downstream device integration for additional molecular analysis following sorting. For example, integration with an upstream label-free “rough” sorting module, such as based on inertial or dielectrophoretic forces can increase the throughput for rare cell sorting [10, 35]. Similarly, upstream integration with lysis and solution exchange sample preparation is possible creating a complete diagnostic solution – from body fluid to result [2]. Also, integration with downstream modules facilitates single cell analysis by keeping small numbers of sorted cells on the same chip, which reduces the risk of losing rare cells [36]. Besides solving the aerosolization issue and offering upstream and downstream integration capabilities, microfluidic FACS systems also has strong advantages in handling structures or flows at a scale commensurate with that of single cells. This offers greater control over single cell analysis in realizing true point-of-care (POC) lab-on-a-chip (LOC) systems [3, 37, 38]. Furthermore, microfluidic FACS can handle a small number of cells while consuming less reagents and producing less chemical waste [32].

In academic research field, many efforts have been putting on developing an on-chip
microfluidic based cell sorting technology. For example, live E. coli can be electro-osmotically switched for sorting at a throughput of 20 cells sec$^{-1}$ and enriched by 30-fold on a microfluidic chip [39]. Dielectrophoresis force has been reported to switch microparticles at a throughput of 300 particles sec$^{-1}$ [40, 41]. But because cells cannot survive in the low conductive buffer it used, it can be hardly applied to cell sorting. Employing a polydimethylsiloxane (PDMS)-based pneumatic valve, a sorter has achieved a throughput of 44 cells sec$^{-1}$ with 40% yield and 83-fold enrichment [42]. In this device, the slow rate of pneumatic control valve actuation blocks further increases in switching speed. Solenoid valve can also be used to switch droplets containing various number of target cells at a throughput of 30 droplets sec$^{-1}$ [43]. The slow response of the solenoid valve also limits the throughput. Optical force is another mechanism used in microfluidic switching [44-47]. High after-sort purity of > 90% has been demonstrated with a throughput of ~100 cells sec$^{-1}$ using HeLa human cancer cells [44]. Light can also heat up sol-gel to block the pathway in order to switch cells into collection channel at a frequency of 5Hz [29, 48, 49]. However, to increase the throughput, the light intensity should be increased which will damage the cells [50]. A sorter utilizing a piezoelectric actuator with a PDMS valve provided an enrichment of ~230-fold and after-sort purity of ~65% at 1,000 cells sec$^{-1}$ [51]. Overall, the major challenge of μFACS systems to date is the low sorting throughput and purity, compared to conventional aerosol-based FACS that yield >90% purity at 70,000
cells sec\(^{-1}\) [52]. In some fields such as oncology, stem cell research, or infectious disease biology, high purity sorting for rare target cells at high-throughput is essential. For example, the separation of human T-lymphocytes (CD4+) from the whole blood with high accuracy [27], the selection of circulating tumor cells (CTCs) from blood samples at high-throughput (7.5 ml in a few hours) [3], the isolation of fetal erythroblasts, lymphocytes, and stem cells from maternal blood at high purity (1 fetal red blood cell per 10\(^5\) to 10\(^7\) maternal red blood cells) [53] are all challenging applications. The following table summarizes the characteristics of above mentioned microfluidic cell sorting methods.

Table 1.2 Summary of microfluidic cell sorting methods

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Throughput</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electro-osmosis</td>
<td>20 cells sec(^{-1})</td>
<td>Easy fabrication, simple control</td>
<td>High voltage, low cell viability, low throughput</td>
</tr>
<tr>
<td>Dielectrophoresis</td>
<td>300 particles sec(^{-1})</td>
<td>Simple control</td>
<td>Small force, low throughput, complex fabrication, buffer incompatibility</td>
</tr>
<tr>
<td>Pneumatic valve</td>
<td>44 cells sec(^{-1})</td>
<td>Compatible with PDMS microfluidic devices, high purity</td>
<td>Complex fabrication, large perturbation volume, low throughput</td>
</tr>
<tr>
<td>Optical force</td>
<td>100 cells sec(^{-1})</td>
<td>Easy fabrication, contact-free manipulation, high purity</td>
<td>Small force, low throughput, strong light intensity may damage cells</td>
</tr>
<tr>
<td>Sol-gel transition</td>
<td>5 cells sec(^{-1})</td>
<td>Easy fabrication, contact-free manipulation, multi-channel sorting</td>
<td>Need special media, slow response, low throughput</td>
</tr>
<tr>
<td></td>
<td>1 000 cells sec(^{-1})</td>
<td>Simple control, strong force</td>
<td>Large perturbation volume, low throughput</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------</td>
<td>------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td><strong>PZT actuator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pulsed laser triggered</strong></td>
<td>45 000 cells sec(^{-1})</td>
<td>High purity, high throughput, contact-free manipulation, fast response, strong force, easy fabrication</td>
<td></td>
</tr>
</tbody>
</table>
1.5 Pulsed laser induced cavitation bubble

When a laser pulse with energy surpassing a threshold is focused in a liquid medium (e.g. water), the intense optical field generates heated plasma within the focal volume. The sharp temperature increase and thermoelastic stress built-up induces a cavitation bubble at the laser focus within nanoseconds [54]. The bubble lifetime and bubble volume are determined by the laser wavelength, laser fluence at the focal spot, and pulse duration [55]. Multiple bubbles can be generated at the same time through digital hologram [56]. Pulsed laser induced cavitation has recently been demonstrated as an effective mechanism for high speed microfluid actuation [57-63]. The high vapor pressure inside the cavitation bubble provides mechanical force to induce high speed fluid flow, which has been applied for creating transient cell membrane permeability for molecular delivery [64] and gene transfection [65], lysing cells near the bubble [66], high-speed droplet generation [67] and enhancing fluid mixing [68].

Pulse laser induced cavitation bubbles can produce large pressure and forces. For example, the pressure inside a cavitation bubble with a size of few hundred micrometers at its initial stage is higher than a few GPa due to the ultrahigh temperature water vapor induced by laser breakdown and hot plasma [54]. A bubble expands faster than collapse [69]. During the bubble expansion process, the bubble undergoes an adiabatic process in which the expansion of bubble results in the decrease of the vapor temperature and the saturated vapor pressure (PV is
constant). When the bubble expands to a size of tens of micrometers in diameter, the bubble pressure is still in the range of 1 ~10 MPa (10~100 atm). During the collapse process of a cavitation bubble, the water vapor gets re-absorbed back to water after cooled down. The two-dimensional bubble radius during collapse can be modeled as [70]

\[
(R\ddot{R} + \dot{R}^2)\log \frac{R}{R_\infty} + \frac{1}{2} \dot{R}^2 = \frac{P_\infty}{\rho}
\]

where \( R \) is the time dependant radius, \( R_\infty \) is the distance at which fluid velocity is zero, \( P_\infty \) is the pressure far from the bubble and \( \rho \) is the liquid density. The maximum radius indicates the energy that absorbed to generate the bubble and the relationship can be expressed as [70]

\[
E_B = \frac{2}{3} \pi (P_\infty - P_V)R_{max}^3
\]

where \( P_\infty \) is the static pressure of the surrounding liquid (101 325 Pa) and \( P_V \) is the vapor pressure of the liquid (2640 Pa at 22°C). For a bubble with radius of 100 μm, the bubble energy can be 0.2 μJ. If this amount of energy is used to heat up local volume, the temperature rise is estimated around 0.05°C which can be negligible.

In the following chapters, I will introduce the pulsed laser triggered high speed switch (see Chapter 2), 2D pulsed laser activated cell sorter (PLACS) (see Chapter 3), 3D PLACS (see Chapter 4) and PLACS integrated with inertial focusing (see Chapter 5).
Chapter 2. Pulsed Laser Triggered Microfluidic Switch

2.1 Overview

Pulse laser induced cavitation bubbles can produce large pressure and forces. Pulsed laser induced cavitation has recently been demonstrated as an effective mechanism for high speed microfluid actuation. In our proposed device, the bubble expansion is used to deform the elastic microchannel wall, serving as the mechanical switch to alter the flow pattern in the adjacent particle channel. Because of the rapid bubble dynamics, a fast switching time on the order of microseconds can be achieved by controlling the bubble size and the pulsed channel dimensions.

2.2 Principle

Figure 2.1 shows the schematic of the pulsed laser triggered microfluidic switch. The device consists of a Y-shaped microchannel with two outlets, collection and waste. Using hydrodynamic flow focusing, the input particles are focused slightly off the centerline of the main channel and go into the waste channel after the Y junction. Separated by a thin wall, another channel runs in parallel with the waste channel. This channel is positioned above the objective lens, which focuses the laser pulse to induce optical breakdown of the liquid medium and the subsequent cavitation bubble. As the bubble expands, the channel wall, being elastic, is
deformed and squeezes the waste channel on the opposite side. This transient deformation of
the channel wall alters the flow pattern in the main channel. A particle approaching the fork of
the Y junction is thus switched to flow in the collection channel. Rapid switching based on
deforming and restoring of the channel wall takes place on the time scale of few microseconds
to hundreds of microseconds, depending on the bubble size, pulsed channel dimensions, the
channel wall width, and material elasticity. On the other hand, the switched particles are
shielded from the direct impact of the explosive expansion and collapse of the bubble. This
way minimum stress is exerted on the particle during switching, and a high level of sample
integrity and viability can be maintained.

Figure 2.1 Schematic of the pulsed laser triggered microfluidic switch. (a) Before switching,
input particles are hydrodynamically focused into the waste channel. (b) During switching, a
cavitation bubble is induced in the pulsed channel by focusing a pulsed laser beam. The bubble
expansion deforms the thin PDMS wall and alters the flow in the sample channel. (c) After
switching, the switched particle goes into the collection channel.
2.3 Device fabrication and experimental setup

In our experiments, fabrication of the microchannels employed the conventional casting method. A master mold was made by patterning SU-8 photoresist on a silicon wafer. PDMS was poured onto the master mold to replicate the channel structures. After curing, the PDMS layer was peeled off from the master mold and bonded to a glass cover slide after oxygen plasma treatment. The physical dimensions of the channels are shown in Figure 2.2. All channel flows were controlled by syringe pumps (KD Scientific). Two other converging channels at the beginning of the main channel provided the sheath flows to focus the sample in the main channel. A light-absorbing dye was added to the pulsed channel flow to reduce the optical breakdown threshold and offer a wider tuning range of the induced bubble size. The laser source was a Q-switched, frequency-doubled neodymium-doped yttrium aluminum garnet laser (Continuum, Minilite I), with a pulsewidth of 6 nsec and 532 nm in wavelength. The laser beam was directed into the fluorescence port of an inverted microscope (Zeiss, AxioObserver) and focused through an objective lens (Zeiss, Epiplan-Neofluar 50×, 0.8 numerical aperature). The pulse energy used was 400 μJ, and the focus was positioned at 50 μm above the glass coverslip bottom and 50 μm away from the deformable wall. A time-resolved imaging system was constructed to capture the cavitation bubble and the PDMS wall deformation (Figure 2.2). In this system, an intensified charge-coupled device camera
(Princeton Instrument, PIMAX-II) recorded frames with an exposure time as short as 500 psec. The fluorescent dye cell and the flashlamp provided illumination pulses at variable delay times in synchronization with the camera exposure. This system enables us to capture the full dynamics of the switching event from few nanoseconds to hundreds of microseconds after the pulsed laser trigger.

![Figure 2.2 Experimental setup. The pulsed laser beam is focused in the microchannel through the objective lens to trigger the switch. To image instances from 20 to 400 nsec after the pulsed laser trigger, a fluorescence pulse generated by exciting the dye cell provides the illumination during the camera exposure. Time delay is controlled by the optical fiber length. For imaging instances from 2 μsec, the flashlamp is used instead.](image-url)
2.4 Results

Figures 2.3 (a)-(d) show the transient flow pattern change in the microchannel due to the bubble induced by a single nanosecond laser pulse. Dye solution was focused slightly off the centerline of the main channel and went into the waste channel on the left after the Y junction (Figure 2.3 (a)). A cavitation bubble induced by the focused laser pulse deformed the PDMS channel wall, resulting in higher resistance on the flow into the waste channel (Figure 2.3 (b)). In Figure 2.3 (c), 6 μsec after the laser pulse, part of the dye flow was switched across the centerline and went into the collection channel on the right. By 15 μsec after the laser pulse, the PDMS wall as well as the dye flow pattern resumed to the original state as before switching (Figure 2.3 (d)). Particle switching was also demonstrated on our device. Polystyrene microspheres of 10 μm in diameter were hydrodynamically focused in the main channel with an estimated speed of 0.05 m sec\(^{-1}\). Before switching, all particles flew into the waste channel on the left (Figure 2.3 (e)). To allow sufficient time for the switched particle to travel downstream after passing the fork of the Y junction, we imaged the particle flow at 1 msec after switching. Figure 2.3 (f) shows that a particle was switched to the collection channel on the right. To characterize the switching time of our device, we measured the deformation of the PDMS wall at various delay times from the triggering laser pulse (Figure 2.3 (g)). The deformation reached a maximum of 20.6 μm at 2 μsec delay. At 10 μsec after the laser pulse, the
channel wall had returned to about 10% of the maximum deformation. By 15 μsec, the wall deformation was indiscernible.

Figure 2.3 (a)-(d) Dye switching in a Y-shaped microchannel. (e) and (f) Switching of 10 μm polystyrene microspheres. (g) Maximum deformation of the PDMS channel wall from 400 nsec to 15 μsec after the pulsed laser trigger. Bar=50 μm.
As an initial test to evaluate the reliability of our switch, we repeatedly excited the cavitation bubble and deformed the PDMS wall for 1000 times. No noticeable difference was observed in the wall deformation pattern, and leaking of the channels due to weakening of the wall bonding to the glass substrate did not occur (Figure 2.4 (a)-(d)).

![Figure 2.4 (a)-(d) Reliability test of the fabricated microchannels. 1000 times actuation was tested without observing degradation effects. Bar=50 μm.](image)

2.5 Conclusion

Conventional FACS has been a standard tool in biological and medical analyses, capable of a sorting speed as high as 70 000 cells sec\(^{-1}\) [3]. For μFACS to reach a speed comparable to that of the conventional ones, a fast switching mechanism with a switching time on the order of
microseconds is required. In this study, a microfluidic switch with a switching time of 10 μsec triggered by a pulsed laser beam is demonstrated. Microspheres of 10 μm in diameter, size of most mammalian cells, have been switched in a Y-shaped microchannel. In the initial test, our proposed switch could be reliably actuated 1000 times without observing degradation effects. This device holds the potential for high-speed and high-efficiency cell sorting on a microfluidic chip.
Chapter 3. 2D Pulsed Laser Activated Cell Sorter (2D PLACS)

3.1 Overview

In Chapter 2, we demonstrated that a laser-induced cavitation bubble can deform an elastic membrane for switching an adjacent fluid or particle flow. In that prior case, the membrane deformation extended over hundreds of microns in length, which perturbed a large fluid volume and affected sample flows upstream of the switching region, yielding lower sort purity. Here we demonstrate a microfluidic based, high-speed and high-purity pulsed laser activated cell sorter termed PLACS which utilizes small volume liquid jets induced by pulsed laser triggered cavitation bubbles for sample switching. A complete switch cycle in PLACS, as characterized by the bubble lifetime, is 30 μsec. Bubble expansion and collapse transiently switches fluid flow inside the microchannels. The actuated fluid volume and the actuation location can be precisely controlled by the deposited laser pulse energy and laser focus position. By using all optical switching and eliminating any on-chip active components, the microfluidic chip is made of a single-layer PDMS channel bonded to a glass cover slide, which can be low cost and disposable. Furthermore, PLACS is compatible with the vast repertoire of PDMS-based microfluidic structures for performing additional upstream or downstream functions and analyses.
3.2 Principle

The design of PLACS is depicted in Figure 3.1 (a). The microfluidic chip consists of a main channel with two outlets, collection and waste. The pulsed channel runs in parallel with the main channel and is connected to the main channel through a straight narrow nozzle at the tip of the Y junction. Using hydrodynamic focusing, the input samples are focused to a narrow stream and flow into the left waste outlet. As a desired object flows through the fluorescence detection region upstream of the Y junction, a laser pulse is triggered and focused through a high NA objective lens into the pulsed channel after an optimal delay time. This induces an explosive cavitation bubble which expands and displaces the surrounding fluid creating a high speed liquid jet through the nozzle into the main channel. This liquid jet deflects the desired object into the collection channel. A rapid switching cycle of 30 μsec is demonstrated based on the expansion and collapse rate of a cavitation bubble (Figure 3.2). Using a nozzle to focus the deflecting liquid jet narrows the switching region in the sample stream and minimizes the disturbance range on neighboring objects to 60 μm (Figure 3.3). This is essential for achieving high throughput and high sorting purity. In this work, the volume of liquid jet delivered into the sample channel as captured by the time resolved image is ~90 pL (Figure 3.2 at 15 μsec). Combined with the capability of operating at high sample flow speeds of ~1 m sec⁻¹, PLACS can achieve mammalian cell sorting at high speeds with high purities.
Figure 3.1 PLACS operation. (a) Schematic of the cell sorter. The sample flow is hydrodynamically focused into the waste channel. As the desired object flows through the fluorescence detection region upstream form the Y junction, the laser pulse is triggered after an optimum delay and induces a cavitation bubble in the adjacent pulsed channel. The bubble expansion produces a high-speed liquid jet that deflects the desired sample towards the collection channel for sorting. (b) Time-resolved images of the cavitation bubble generated by the focused pulsed laser beam in the microfluidic cell sorter. (c) Fluorescent particle switching
in PLACS. Without switching, the particle flows towards the left waste outlet. With fluorescence activated switching, a laser pulse was triggered and the particle was sorted into the collection outlet on the right.

Figure 3.2 A cavitation bubble was generated within nanoseconds after laser pulse arrival. The bubble grew to a maximum diameter of 230 μm in the major axis and 205 μm in the minor axis within 3 μsec and began to collapse. During bubble expansion, the surrounding fluid was displaced at the speed of the bubble front and a high-speed liquid jet was induced in the connecting nozzle into the main sample channel, deflecting the targeted object towards the collection channel. The liquid injected into the sample channel is 140 μm (liquid jet length) × 20 μm (nozzle width) × 30 μm (channel height) ~ 90 pL (see 15 μsec). The bubble collapsed completely by 30 μsec after the laser pulse. Allura Red dye (concentration 67 mg ml⁻¹, Sigma-Aldrich) was added in the pulsed channel flow. Laser pulse energy was 31 μJ.
Figure 3.3 Switching window and optimum object switching delay. Particle switching efficiency was measured at different laser pulse delays ranging from 5 to 75 μsec after detection. The highest switching efficiency of 96% was obtained at a delay of 35 μsec and decreased to 0% at 75 μs. The perturbation range of neighboring objects is ~60 μm, estimated by the particle speed (0.8 m sec⁻¹) and the 75 μsec window. The distance between the detection region and Y junction was 50 μm. The sorting efficiency was obtained by analysing particle traces from 50 switching events for each delay condition.

3.3 Device fabrication and experimental setup

The device was fabricated using a conventional replica molding technique. Microchannel features were photolithographically defined in SU-8 (MicroChem 2025) photoresist molds on a silicon wafer and then replicated onto a PDMS (Sylgard 184) layer. The PDMS replica was bonded to a glass cover slide substrate after oxygen plasma treatment. The measured microchannel height was 30 μm. Sheath flow channels were 80 μm wide. The sample channel
width was 80 μm, which separated into 40 μm wide collection and waste outlet channels at the Y junction. The pulsed channel width was 300 μm and the straight nozzle connecting the sample and pulsed channel had a channel width of 20 μm and length of 100 μm.

As shown in Figure 3.4, the pulsed laser system was a Q-switched Nd:YVO₄ laser (EKSPLA, Jazz 20) operating at 532 nm wavelength, 8 nsec pulsewidth, and a repetition rate up to 100 kHz. The pulsed laser beam was expanded and focused by an objective lens (100×, NA 0.9) through the glass substrate into the pulsed channel. The laser focus was positioned 100 μm away from the connection nozzle to the main channel. The laser pulse energy deposited into the channel was adjusted using a half-wave plate followed by a polarizing beam splitter and was set at 31 μJ per pulse for switching. For sample fluorescence excitation, a 10 mW, 488 nm solid state laser (CyrstaLaser, DL-488-010) was reflected by a dichroic mirror (Chroma, z488rdc) and lightly focused into the main channel through a 25×, NA 0.4 objective lens from the PDMS side of the microfluidic chip. The emitted sample fluorescence was collected by the same objective lens and detected using a photomultiplier tube (Sens-Tech, P30CWAD5-01) after passing through a bandpass filter (Chroma, HQ510/20m) matching the fluorescence emission spectrum. An aperture preceded the PMT and was placed at the sample conjugate image plane. The aperture opening defined the fluorescence detection area to be ~30×80 μm covering the entire width of the sample channel and blocked scattered light from the laser pulse.
and plasma emission. The PMT signal was integrated using a DAQ card (National Instruments, PCI 7831R) at 100 kHz. FPGA logic was programmed using LabView (National Instruments) to perform real-time detection, threshold comparisons, and timed triggering of the pulsed laser.

To image and characterize the fast dynamics of the cavitation bubble, a flashlamp (High-Speed Photo-Systeme, Nanolite KL-M) with 11 nsec flash duration was used as the illumination for time-resolved photography. Images were taken by a CCD camera (Zeiss, AxioCam MRm) and the flash delay from the laser pulse was controlled by LabView FPGA.

Figure 3.4 PLACS experimental setup. For cavitation bubble induction, we used a Q-switched, 8nsec pulsewidth Nd:YVO₄ laser. The pulsed laser beam was focused by an objective lens (100×, NA 0.9) into the pulsed channel. Sample fluorescence was detected by a photomultiplier tube and sampled using a DAQ card and integrated every 10 μsec. FPGA logic was programmed using LabView to perform real-time detection, threshold comparison and timed triggering of the pulsed laser.
For particle sorting experiments, 10 μm green fluorescent beads (Thermo Fisher Scientific, G1000) and 10 μm non-fluorescent beads (Polysciences, 17136-5) were suspended in deionized water with 3% w/v Tween 80 (Sigma-Aldrich) to the desired concentrations. Sheath flows contained deionized water with 3% w/v Tween 80. All channel flows were driven by syringe pumps (Harvard Apparatus, PHD2000). Allura Red dye (concentration 67 mg ml$^{-1}$, Sigma-Aldrich) was added in the pulsed channel flow to reduce the laser energy threshold for bubble generation. The sample flow rate was 1.2 ml h$^{-1}$ and the average sample speed was 0.8 m sec$^{-1}$ in the main channel. For cell sorting, human Nalm-6 pre-B cells or B lymphoma Ramos cells cultured in RPMI 160 culture media with usual supplements were washed and re-suspended in phosphate buffered saline (1x PBS, pH 7.4) with 2% w/v bovine serum albumin (BSA) to the desired concentration. Fluorescent cell samples were obtained by staining Nalm-6 or Ramos cells with Calcein AM (Invitrogen). After sorting, the collected cells were incubated at 37°C, 5% CO$_2$ for 30 min in PBS before addition of propidium iodide (Invitrogen) for viability evaluation. The sample flow rate and flow speed settings were the same as the particle sorting experiments.

Sorted samples purities were analyzed by a flow cytometer (BD, FACSCantoII). Positive and negative control samples were used to configure the fluorescence gating conditions. For each sort condition, the average sort purity was obtained from measurements of 3 experiments.
In each measurement, the total number particles analyzed were ~5000. Cell viability based on propidium iodide exclusion was measured on the same flow cytometer with a sample size of ~5000 cells for each sorting experiment.

Human Nalm-6 pre-B cells were suspended in sorting buffer (1x PBS, pH 7.4, with 2% w/v BSA) to a concentration of ~1×10^6 ml⁻¹. The mixture ratio of Calcein AM stained cells versus unstained cells was 1 : 1. Cells were sorted through PLACS at room temperature and sorted cells were retrieved from both the collection and waste outlets (~1×10^6 cells in each sample). Unsorted cells were incubated in sorting buffer at room temperature for the same duration as the sorted cells. RNA was extracted from retrieved cells immediately after sorting using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocols. Real-time qPCR was performed according to manufacturer’s protocol on the Roche LightCycler 480 Real-Time PCR System with SYBER Green I Master mix (Roche). For base level gene expression evaluation, cells were incubated in culture media at 37°C, 5% CO₂ before mRNA extraction. For positive controls, Nalm-6 cells were incubated in media at 42°C for one hour for HSPA6 evaluation, whereas for FOS evaluation, cells were treated with 10 μg ml⁻¹ cycloheximide (Sigma-Aldrich) for two hours. Primers for gene expression were generated using Roche’s Universal Probe Library Assay Design Center online.

FOS (c-fos) Fwd: (CTACCACCTCACCGCAGACT)
Rev: (AGGTCCGTGCAGAAGTCCT).

HSPA6 Fwd: (TCATGAAGCCGAGCAGTACA)

Rev: (GTTTTTGGCAGCCACTCTGT).

GAPDH Fwd: (GCTCTCTGCTCCTCCTGTTC)

Rev: (ACGACCAAATCCGTTGACTC).

3.4 Results

In PLACS, once a desired object was detected, a nanosecond laser (532 nm wavelength, 8 nsec pulsewidth) induced a bubble that expanded to a maximum diameter (major axis) of 230 μm in 3 μsec after laser pulse arrival (Figure 3.1 (b)). Figure 3.1 (c) shows a fluorescent particle trace that was successfully switched into the collection outlet on the right by a pulsed laser triggered high speed bubble flow. Perturbation volume was estimated by measuring the maximum liquid jet volume injected into the main sample channel. As captured by time-resolved imaging, the liquid jet flow reached the center of the main sample channel while the bubble front reached the boundary of the pulsed channel (Figure 3.2 15 μsec). Estimated liquid jet volume is 140 μm (liquid jet length) × 20 μm (nozzle width) × 30 μm (channel height) ~ 90 pL.

The switching window or perturbation range was determined by measuring particle switching efficiency at different time delays after detection. Particles were detected at a distance of 50 μm upstream of the Y junction. Switching efficiency was measured for particles
at different locations with respect to the nozzle (translating to different time delays after detection) as the laser pulse was triggered. The highest efficiency was 96% obtained at a delay of 35 μsec and the efficiency decreased to 0% at a delay of 75 μsec and <40% at 5 μsec (Figure 3.3). Particles outside of this region (75 μsec × particle speed 0.8 m sec⁻¹ = 60 μm) were not switched and followed the original flow focusing streamline into the waste channel. The switching window and optimal delay time between triggering laser pulsing for switching and object detection depend on the sample flow speed and the detection region distance to the Y junction. Variable delay time between object detection by the PMT and laser triggering was controlled by FPGA programmed in LabView (see Experimental setup section). For different delay times ranging from 5 to 75 μsec, fluorescent particle traces were recorded and analyzed. Switching efficiency was obtained by measuring the percentage of successful switching events (particle trace going into the collection channel) in a total of 50 sampled fluorescent particle images.

We characterized the sorter performance at different object throughputs and initial mix ratios. Samples were prepared by mixing 10 μm green fluorescent beads with non-fluorescent polystyrene beads. Throughputs ranging from 3000 to 10 000 particles sec⁻¹ were obtained by keeping the green bead concentration at 5.7×10⁴ particle ml⁻¹ while increasing the non-fluorescent bead concentration accordingly. High purities (>90%) of the sorted beads were
obtained at a sorting throughput of 3000 particles sec\(^{-1}\) by analyzing the collection sample using a commercial flow cytometer (Figure 3.5 (a)-(b)). The fluorescent beads were enriched from an initial mix ratio of 0.0087 to a final ratio of 9.61, corresponding to an 1105-fold enrichment. At higher throughputs the sort purity decreases as a result of the narrowing of the average distance between adjacent particles (Figure 3.5 (c)). At a throughput of 10 000 particles sec\(^{-1}\) the collection purity was 45%, equivalent to a 426-fold enrichment. To evaluate sorter performance in switching individual particles at high frequencies compared to rare event sorting, bead samples with initial mix ratios ranging from 0.01 to 0.27 (unsorted purity 1% to 21%) were sorted at a throughput of 3000 particles sec\(^{-1}\). High collection object purity (94%) with low target object waste (1%) was maintained even at the highest initial mix ratio tested.
Figure 3.5 PLACS sorting results. (a) Mixed 10 µm green fluorescent and non-fluorescent polystyrene microspheres before sorting. Initial mix ratio is 0.0087. (b) Collected sample after sorting with a final mix ratio of 9.61. Fluorescent microsphere concentration was enriched by a factor of 1105. (c) Sort purity at different sorting speeds ranging from 3000 to 10 000 microspheres sec$^{-1}$. High purities (90 ± 3% (mean ± s.d.)) of the sorted particles were obtained at a sorting throughput of 3000 particles sec$^{-1}$. The collection purity measured at the highest speed tested (10 000 particles sec$^{-1}$) was 45 ± 15%. The sort purity decreased at higher
throughputs as the average distance between adjacent particles shortened. In these experiments, green fluorescent particle concentrations were kept constant ($5.7 \times 10^4$ particles ml$^{-1}$) while non-fluorescent particle concentrations were increased accordingly to obtain the target throughput. (d) Sort purity at different initial mix ratios ranging from 0.001 to 0.27 (unsorted purity 1% to 21%). At the initial mix ratio of 0.27, measured collection target particle purity was $94 \pm 3\%$ and waste target particle purity was $1 \pm 0\%$. Sorting speed was kept at 3000 particles sec$^{-1}$.

To evaluate mammalian cell sorting, human pre-B Nalm-6 or B lymphoma Ramos cells were stained with Calcein AM (green fluorescence) and mixed with untreated cells at the desired ratios. Results showed at sorting speeds of 560 and 1500 cells sec$^{-1}$, >90% purity with ~90% cell viability were obtained for sorting fluorescent Nalm-6 cells (Table 3.1), a performance comparable to similar experiments performed on a high-end commercial FACS. For high-speed cell enrichment, Ramos cells were sorted at 10 000 cells sec$^{-1}$ and 20 000 cells sec$^{-1}$. The obtained sort purities were 48.6% and 37.4%, corresponding to enrichment factors of 473 and 298.5 respectively (Table 3.1). Under these sorting conditions, cells sorted into the collection or waste channels exhibited the same stress levels seen in unsorted cells, as measured by HSPA6 and FOS stress-response gene expression (Figure 3.6). These results showed that hydrodynamic cell focusing and cavitation bubble triggered liquid jet switching
did not exert additional stress on the cells being sorted.

Table 3.1 Sorting results of Nalm-6 human pre-B cells and B lymphoma Ramos cells at different throughputs (Col: Collection sample; W: Waste sample)

<table>
<thead>
<tr>
<th>Throughput (cells sec(^{-1}))</th>
<th>Cell type</th>
<th>Before sort</th>
<th>After sort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial green cell percentage (%)</td>
<td>Cell density (×10(^6) cells ml(^{-1}))</td>
</tr>
<tr>
<td>560</td>
<td>Nalm-6</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>1 500</td>
<td>Nalm-6</td>
<td>0.8</td>
<td>4.6</td>
</tr>
<tr>
<td>10 000</td>
<td>Ramos</td>
<td>0.2</td>
<td>30.6</td>
</tr>
<tr>
<td>20 000</td>
<td>Ramos</td>
<td>0.2</td>
<td>61.3</td>
</tr>
</tbody>
</table>
Figure 3.6 Evaluation of stress levels of Nalm-6 cells after PLACS sorting. The HSPA6 gene expression level indicates the cellular response to heat shock, and FOS gene expression reflects heat shock and fluidic shear stress. Gene expression levels were normalized to a GAPDH housekeeping gene for all cell samples under different conditions. Sorted cells retrieved from the collection and waste outlets showed expression levels that were statistically similar to the unsorted cells (incubated in sorting buffer at room temperature for the sort duration). A positive control for HSPA6 expression was provided by incubating Nalm-6 cells in culture media at 42 °C for one hour. For FOS expression, a positive control was provided by treating Nalm-6 cells with cycloheximide (CHX) for two hours.

The reproducibility of PLACS sorting process under repeated laser pulsing and bubble cycles was tested. We verified that the microchannels remained intact and exhibited no burning or leakage after 100 million (10^8) actuations (Figure 3.7). Cavitation bubbles after 100 million cycles showed no discernible changes in the bubble pattern, further ensuring reliable switching over long periods of device operation.
Figure 3.7 PLACS reliability evaluation. A laser-induced cavitation bubble (imaged at 3 μsec after laser pulse arrival) could be repeatedly produced >100 million times without showing significant bubble pattern alteration or microchannel degradation. The pulsed laser repetition rate was 10 kHz.

3.5 Conclusion

PLACS overcomes the limit of microfluidic-based fluorescence-activated cell sorting mechanisms in achieving simultaneously high speed, high purity, and high viability sorting. The bubble jet switching mechanism actuates a small and well-controlled fluidic volume for sample sorting which allows sorting of multiple cell types in any biological medium. Our current device operates with a bubble cycle time of 30 μsec. Shorter switching times are possible by utilizing a smaller bubble actuation volume and modified channel design. PLACS has the potential to bridge the sorting throughput gap between current microfluidic FACS and conventional electrostatic-droplet-based cell sorters.
Chapter 4. 3D Pulsed Laser Activated Cell Sorter  
(3D PLACS)  

4.1 Overview  

In the previous chapter, 2D PLACS achieved 90% sorting purity at 3,000 cells sec$^{-1}$ with high cell viability. However, the sorting purity dropped to 45% at 10,000 cells sec$^{-1}$ due to the lack of third dimension flow focusing in a device with only 2D sheath flows. Cells at different vertical positions in the fluid channel with a parabolic velocity profile reached the switching zone at different times after fluorescent detection. This created a major synchronization issue between detection and sample switching and decreased the switching efficiency, especially in high-speed flow situations where the switching window was small and actuation timing was therefore critically important. This synchronization issue also decreased the sort purity at high-throughput since a large perturbation volume was required to provide a larger switching zone to ensure that detected cells arriving at different times were sorted correctly. At high-throughput sorting speeds, the distance between neighbouring cells decreased. A large switching zone needed to capture all desired cells also increased the chance of capturing nearby unwanted cells which reduced the sort purity. If this synchronization problem is not solved, there remains a trade-off between switching efficiency and sort purity at high-throughput. There are many reported 3D focusing microfluidic devices but they all require special flow
In this chapter, we present a new 3D PLACS device that utilizes multilayer 3D PDMS channels with interlayer connection vias to achieve 3D sheath focusing. This approach not only solves the synchronization issue between detection and switching but also allows efficient particle switching using a smaller bubble with smaller perturbation volume to result in a more accurate and shorter switching cycle. 3D PLACS can achieve 90% purity sorting at a throughput of 23,000 cells sec\(^{-1}\). This is the first microfluidic FACS to perform single stage, single channel sorting at a throughput comparable to conventional aerosol based FACS on a fully enclosed microfluidic chip.

4.2 Principle

The principle of 3D PLACS is shown in Figures 4.1 and 4.2. The device consists of a main channel with two outlets, collection and waste. Utilizing thin film PDMS fabrication techniques, 3D hydrodynamic flow focusing can be realized. Samples are focused into the waste channel initially. When cells flow through the detection zone, their fluorescent signals are collected by a 25× N.A. 0.4 objective lens and detected by a PMT (photodetector module P30CWAD5-01 SENS-TECH). When a target cell is identified, a laser pulse (Q-switched Nd:YVO\(_4\), 8 nsec pulse width, 532 nm wavelength) is triggered to generate a cavitation bubble through a 100× N.A. 0.9 objective lens with a proper delay time. Figure 4.2
demonstrates a bubble with a diameter of 160 μm that creates a high-speed jet to deflect a fluorescent particle into the collection channel. At 5 μsec, the bubble expands to its largest size (Figure 4.2b) and induces a liquid jet through the nozzle. With this perturbation, detected fluorescent cells, originally flowing into the waste channel (Figure 4.2c), are deflected into the collection channel (Figure 4.2d). The bubble collapses and fully disappears within 20 μsec.
Figure 4.1 Schematic of a 3D PLACS. (a) 3D sheath flow focusing is achieved by multilayer PDMS structures with vertical vias connecting channels in different layers. Detected fluorescent particles are deflected into the collection channel by high-speed liquid jets induced by
by rapidly expanding laser cavitation bubbles that squeeze fluid across a micro nozzle. (b) Microfluidic structure of each layer.

Figure 4.2 Particle switching triggered by a focused laser pulse. (a) The main channel is 80 μm wide and is split into two 40 μm wide channels after the switching junction, one for collection and one for waste. The bubble excitation channel is 200 μm in width and increases to 450 μm at the bubble excitation location. The nozzle connecting bubble channel and the main channel is 50 μm in length and 20 μm in width; (b and c) Time-resolved images showing a jet created by a bubble. The bubble expands to its maximum diameter of 160 μm (major axis) in 5 μsec and
fully disappears by 20 µsec following the laser pulse; (c) fluorescent trace of a non-switched particle and (d) of a switched particle.

In the bubble excitation channel, red dye is added to reduce the laser threshold energy required to trigger a cavitation bubble. The cavitation bubble is excited at the mid-height of the channel where the highest flow speed occurs in a parabolic flow pattern. This high-speed flow not only helps remove residual bubbles that are not fully collapsed but also the heat generated at the laser excitation spot through fast convection flow, instead of through slow thermal diffusion. This prevents heat from accumulating at the same spot over time with high repetition rate excitation. Using these operating principles, PLACS enables reliable switching for >100 million bubble cycles without any thermal damage to surrounding low melting temperature PDMS structures.

4.3 Device fabrication and experimental setup

The microfluidic component of the 3D PLACS sorter consists of a bonded cover glass with three thin film PDMS layers containing through-layer vias and one bulk PDMS layer, as shown in Figure 4.1. This device consists of four inlets, one for sample introduction, one for vertical sheath focusing, one for lateral sheath focusing, and one for introducing fluid with red dye to the cavitation bubble channel. The through-layer vias solve the fluid routing issue for 3D hydrodynamic sheath focusing. The microfluidic device is fabricated using standard soft
lithography processes and a PDMS stamp. The detailed fabrication process is provided in references [73-75]. Briefly, a SU-8 mold was fabricated on a silicon wafer. A thin layer of Cytop (CTX-809A, AGC) was coated onto the mold. A thin layer of uncured PDMS mixed with curing catalyst platinum-divinyltetramethyldisiloxane complex is spin coated onto the mold. A flat PDMS stamp treated by trichloro (1H, 1H, 2H, 2H-perfluorooctyl) silane (Aldrich) is pressed onto the mold for 4 hours until the PDMS thin film is cured. The cured PDMS film is then peeled off from the Cytop treated mold. The elastic PDMS stamp insures complete removal of residual PDMS between the SU-8 mold and the stamp to create through-layer vias. The peeled off PDMS thin film with vias is bonded strongly to a glass substrate or other PDMS layers through oxygen plasma treatment. The stamp is detached from the thin film afterward. By repeating the PDMS thin film fabrication and transfer processes, multilayer PDMS channels with interlayer vias are built in this manner.

All channels in our microfluidic devices are 40 µm in height. The main channel is 80 µm wide and is split into two 40 µm wide channels after the switching junction, one for collection and one for waste. The bubble excitation channel is 200 µm in width and increases to 450 µm at the bubble excitation location to allow more space for bubble expansion.

The experimental setup is illustrated in Figure 4.3. A Q-switched Nd: YVO4 pulsed laser (EKSPLA, Jazz 20) is focused into the microfluidic channel through a 100× objective lens
The laser delivers 8 nsec pulses at 532 nm wavelength with a repetition rate up to 100 kHz. A polarizer and a beam splitter are added to continuously tune the laser pulse energy between 0~100 µJ to control the size of laser-induced cavitation bubbles. For cell fluorescence excitation, a CW laser (CrystalLaser, DL-488-010, 10mW) at 488 nm wavelength is reflected by a dichroic mirror (Chroma, z488rdc) and focused through a 25× objective lens (N.A. 0.4) into the microfluidic channel. The emitted fluorescence signal is collected by the same objective lens and then detected by a photomultiplier tube (Sens-Tech, P30CWAD501) after passing through a bandpass filter (Chroma, HQ510/20m) that matches with the emission spectrum of the fluorescence under detection. A CCD camera (Zeiss, AxioCam MRm) is positioned at the conjugate image plane for observation. A flashlamp (High-Speed Photo-Systems, NANOLITE, KL-M) that can emit 11 nsec duration flash light is used as an illumination light source to take time-resolved images for calibrating and characterizing the fast dynamics of cavitation bubbles and induced high-speed liquid jets. The PMT signal is sampled and integrated by a Labview DAQ card (National Instruments, PCI 7831R). FPGA logic is programmed to perform real-time detection, threshold comparisons, and timed triggering of the laser pulses and the flashlamp.
Figure 4.3 Experimental setup. A Q-switched Nd: YVO4 pulsed laser was focused into the middle of the bubble channel through a 100×/N.A. 0.9 objective lens. CW laser at 488 nm was used for fluorescence excitation through a 25×/N.A. 0.4 objective lens. The emission fluorescence was collected by the same objective lens and detected by a photomultiplier tube (PMT) which connected to a DAQ card for signal acquisition and processing. FPGA logic was programmed using Labview to perform real-time detection, threshold comparison, and timed triggering of the pulsed laser. To observe and characterize the bubble, a flash lamp was used to capture time-resolved images on a CCD camera.

4.4 Device Characterization

Three dimensional (3D) sheath flow focus

Synchronization between fluorescence detection and cell switching is critical for achieving high-purity, high-throughput sorting. In two-dimensional sheath flow focusing, samples are confined only in the lateral direction. Without third dimension confinement, cell positions in the vertical position are not uniform. In a microfluidic channel with a parabolic flow velocity...
profile, cells travel at different speeds at different vertical positions and arrive at the switching zone with different delay times. Accurate timing to trigger cavitation bubble formation is hard to predict in this configuration. This imprecision could result in failure to collect target cells or collecting non-target cells that reduces the sort purity. Third dimensional flow focusing is therefore crucial to solve this synchronization issue.

Figure 4.4a shows a side view of the 3D device. To provide vertical sheath flows without interfering with the paths of sample flow and lateral sheath flows, one via layer and two extra fluid routing layers are fabricated for the device.
Figure 4.4 Vertical hydrodynamic focusing. (a) Side view of a 3D device. The sample flow is sandwiched by upper and bottom sheaths. (b) Fluorescence image of vertical focusing at different sheath/sample flow ratios. The 100 nm red fluorescent particle solution is flowing in the sample channel at a flow rate of 1 ml h\(^{-1}\). The upper and bottom sheaths are DI water at flow rate of 0 ml h\(^{-1}\), 5 ml h\(^{-1}\) and 10 ml h\(^{-1}\), respectively. (c) Measurement of sample stream width.

The vertical focusing effect is tested using DI water to introduce vertical sheath flows to
focus a sample stream carrying 100 nm red fluorescent particles. The sample flow rate is set at 1 ml h$^{-1}$. Different sample-sheath flow rate ratios are measured by varying the vertical sheath flow rate from 0 ml h$^{-1}$, 5 ml h$^{-1}$ to 10 ml h$^{-1}$. Fluorescence images are taken in the main channel ~100 µm downstream of the merged junction of the sample and vertical sheath flows. At a 1:10 sample-sheath ratio, the width of the sample stream decreased from 40 µm to 10 µm (Figure 4.4b).

Lateral flow focusing is demonstrated in Figure 4.5a. A sample with red color dye flowing at a rate of 2 ml h$^{-1}$ is laterally focused by sheath flows at a total flow rate of 20 ml h$^{-1}$. At the collection and waste channel separation junction (Y junction), the focused sample stream is biased toward the waste channel (Figure 4.5b). The flow rate of the bubble channel is tuned such that the sample does not flow into the collection channel or flow through the nozzle into the bubble channel. In our studies, an optimal flow rate of 20 ml h$^{-1}$ is used in the bubble channel.
Figure 4.5 Lateral hydrodynamic focusing. (a) Sample (red color dye) flows at 2 ml h$^{-1}$ and the total lateral sheath flow rate is 20 ml h$^{-1}$. (b) The focused sample stream is biased towards the waste channel by the nozzle opening between the main channel and the bubble channel. The bubble channel flow rate is 20 ml h$^{-1}$.

**Switching window optimization and sort purity prediction**

Since bubble generation and destruction time is as short as 20 μsec, successful switching critically depends on controlling the time delay from detecting the cell to triggering a bubble for sorting while considering the traveling speed of a cell. As a fluorescent cell is detected, a cavitation bubble will be triggered at the optimal time delay as the cell travelling down to the sorting junction. Switching is the most effective only when the strongest liquid jet moment meets the cell passing by the Y junction. In our sorting experiments, the distance between the detection spot and the bubble trigger spot is 50 μm. The cell traveling speed is constant at 1.5 m sec$^{-1}$ due to 3D hydrodynamic focusing. We experimentally varied the delay time from detection to laser triggering and characterized the switching efficiency, which is defined as the probability of successful switching.

Laser pulse energy, nozzle design, and the timing of bubble triggering are critical parameters that have to be optimized to achieve high purity sorting in PLACS. To characterize the switching window, a sample solution containing 10 μm green fluorescent beads at a
concentration of $10^6$ beads ml$^{-1}$ is used. The calculated average distance between neighbouring beads is 2500 µm and the probability of more than one bead passing by the detection and switching zone at the same time is 0.8% by Poisson distribution estimation. When a fluorescent bead passes through the detection zone, a camera (Zeiss, AxioCam MRm) is triggered to take an image with an exposure time of 1 msec to capture the trace of this fast moving bead. By analyzing the images of particles’ fluorescent traces, the switching efficiency, the percentage of identified beads successfully switched into the collection channel, can be measured. Two parameters, laser trigger delay time and the pulse energy, are varied to characterize the switching efficiency. In the laser pulse energy test (Figure 4.6a), a device with a 10 µm wide and 100 µm long nozzle is used.
Figure 4.6 Switching window optimization. (a) Switching efficiency at different pulse energies and delay times. Larger pulse energies generate larger bubbles for higher switching efficiencies but also larger switching windows that could deflect non-target particles into collection channel. (b) Optimization of the nozzle design helps to increase the switching efficiency by narrowing the switching window to allow smaller bubbles for high purity sorting. (c) An optimized switching window is 25 µsec wide in time which equates to 40 µm in distance at a flow speed of 1.5 m sec\(^{-1}\). The highest switching efficiency of 97% occurs at a laser delay time of 18 µsec and decreases to 1% at 5 µsec and 30 µsec delays.

With a 100 µJ laser pulse energy, peak switching efficiency reaches 100% but the switching window is wide, which is not ideal for high purity sorting when the cell separation distance is small. As the laser pulse energy decreases, the switching window narrows but the peak switching efficiency also decreases. An ideal shape of a switching window profile should be a sharp peak to ensure high efficiency switching of target particles that excludes all non-target neighbouring particles. To generate a sharp peak switching profile, several nozzle designs with different lengths and widths have been tested (Figure 4.6b). The goal was to identify optimal nozzle dimensions that allow effective particle switching with minimum volume of liquid injected into the sample channel, a parameter affecting the width of switching window. Using a 20 µm wide and 50 µm long nozzle, and an excitation bubble of 160 µm in
diameter, an optimal switching profile is obtained with a peak switching efficiency at 97% and a switching window of 25 µsec, corresponding to a 40 µm switching range in the sample channel (Figure 4.6c) when the flow speed in the sample channel is 1.5 m sec$^{-1}$. The cut-off switching efficiency in defining our switching window is 1%, which means particles outside this switching window are almost impossible to get switched into the collection channel. The switching profile shown in Figure 4.6c is symmetric. This means non-target particles outside the ±20 µm range of the target particle will not be switched. However, if the non-target particle is located within 20 µm away from the target particle, the probability that it will be switched into the collection channel varies with its distance from the target particle.

4.5 Results

In practical sorting experiments, cells come into the detection and switch zone randomly. The separation distance between cells follows the Poisson distribution. For example, at a sorting throughput of 10,000 cells sec$^{-1}$, the average cell separation distance is 160 µm when cells flow at a speed of 1.5 m sec$^{-1}$ in the sample channel. According to the Poisson distribution, only 25% of cells will be within the ±20 µm range. For cells within this range, the probability for each cell to be switched is determined by the experimentally obtained switching profile. In theory, a sorting purity above 90% can be achieved at a throughput up to 11,000 cells sec$^{-1}$ when the flow speed in the sample stream is 1.5 m sec$^{-1}$ as shown in Figure 4.7b.
To validate the sorting capability for biological samples, Ramos human Burkitt lymphoma cells are stained with Vybrant® CFDA SE Cell Tracer (Invitrogen) and mixed with unstained cells at desired ratios. Cells cultured in RPMI 1640 with standard supplements were washed and re-suspended in phosphate buffered saline (1× PBS, pH 7.4). Staining was performed by incubating cells in PBS containing 1 µM carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) at 37°C for 15 min, followed by centrifugation and 3 washes with PBS. Following the third wash, cells were placed in 1 x PBS with 10% fetal bovine serum (FBS) and 1 mM ethylenediaminetetraacetic acid (EDTA) to prevent aggregation. The unstained cells were also transferred into the PBS/FBS/EDTA solution at the desired concentration, ranging from $10^7$ to $10^8$ cell ml$^{-1}$, which would correspond to different throughputs in the PLACS device. The stained cells were mixed with the unstained cells at a ratio of 0.001 (confirmed by conventional flow cytometry) after filtering out large cell clumps using a 40 µm pore-size cell strainer. Once the cell sample was prepared, it was transferred into a 3 ml syringe with two magnetic bars inside which agitate the sample solution to prevent sedimentation and aggregation during an experiment. The sheath fluid was PBS/FBS/EDTA and was filtered before pumping into the microchannels. The dye solution was prepared by mixing Allura Red dye (67 mg ml$^{-1}$, Sigma-Aldrich) into PBS and filtered. All the solutions were driven into the PLACS device by syringe pump (Harvard Apparatus, PHD2000). The
flow rates were fixed at 2 ml h\(^{-1}\) for the sample channel, 4 ml h\(^{-1}\) for the vertical sheaths, 20 ml h\(^{-1}\) for the lateral sheaths, and 20 ml h\(^{-1}\) for the dye channel. Under this flow rate setting, the cells were travelling at a speed of 1.5 m sec\(^{-1}\). After sorting, propidium iodide (Invitrogen) was added to the collected samples for viability testing.

After sorting, the collected samples were immediately analyzed by a conventional flow cytometer (BD, FACSCanto II). To obtain the proper fluorescent gating parameters, negative controls (unstained cells) and positive controls (green fluorescence stained cells and fixed dead cells with PI dye) were also analyzed. Moreover, forward and side scatter signals were also used to gate the proper cell population. From these analyses, purity and viability data were obtained for pre-sorted samples, collection channel samples, and waste channel samples. For each measurement, >1,500 cells were analyzed from collection channel samples and >10,000 cells for all other samples.

The bubble excitation location, bubble size (160 µm in diameter), and time delay between detection and laser excitation (18 µsec) are all fixed to ensure optimal switching performance.

Since the fluid flow rates are fixed, experiments at different throughput are achieved by varying the initial cell concentration. At low throughput, the average cell-cell separation distance is large and high purity (>95%) sorting can be achieved easily (Figure 4.7). For example, as shown in Table 4.1, a sorting purity of 96.2% is accomplished at a throughput of
6,000 cells sec\(^{-1}\) and a purity of 95.9% is also achieved at 11,000 cells sec\(^{-1}\). The average cell-cell separation distances at these two cell concentrations are 252 µm and 135 µm, respectively. When the throughput increased to 23,000 cells sec\(^{-1}\), the average distance between cells decreases to 64 µm, which is still larger than the switching window, but the sorting purity decreases to 89.6%. This means that the proportion of cells with separation distances smaller than 20 µm starts to become significant. The sorting purity, up to 23,000 cells sec\(^{-1}\), matches well with our prediction based upon a Poisson distribution. In fact, the experimentally obtained sorting purity is slightly higher than what we predicted since the Poisson distribution neglects the cell size effect, meaning, in real experiments, not that many cells will get into the effective switching zone as the theory predicts. At a throughput of 23,000 cells sec\(^{-1}\), the initial cell concentration was 4.2×10\(^7\) cells ml\(^{-1}\). By further increasing the cell concentration for higher throughput, the frequency of cell clogging at the switching zone increases substantially. This caused problems since aggregated cells could be lysed and stacked at the switching junction, blocking the channel and severely affecting the switching function. As a result, the collection purity dropped to 45.4%, much lower than what the theory predicted, at a throughput of 45,000 cells sec\(^{-1}\). During an sorting experiments, three methods were employed to avoid cell clogging: (1) adding 10% FBS (fetal bovine serum) and 1 mM EDTA (ethylenediaminetetraacetic acid) to the buffer medium; (2) pipetting the cell suspension and
filtering it with 40 μm pore size cell strainers to remove large clumps before introduction into microchannels and (3) stirring the cell suspension in a delivery syringe with magnetic stirring bars during sorting. From our experimental results, these methods effectively help prevent cell clogging for concentrations below 4.2×10^7 cells ml^{-1}. There are additional methods that can be considered as well, such as Teflon coating the microchannel [51] or designing a wider channel [76].
In our current 3D PLACS system, increasing the percentage of labeled cells at
high-throughput will not affect the sorting purity but the yield will drop with the increasing percentage of labeled cells. This means more labeled cells will not be captured by sorting. This limitation is due to laser energy instability at high pulse triggering rates. The nanosecond laser used in the current system requires a minimum delay of 200 μsec between two consecutive laser pulses when operating in the pulse-on-demand mode. If the second pulse is triggered within 200 μsec from the first laser pulse, the energy of the second pulse does not reach its maximum, which in turn affects the switching and leads to lower yields. Using a laser with a shorter pulse-on-demand time could solve this problem.

Cell viability testing is achieved by PI staining of sorted cells. No significant loss of viability is observed in the sorted sample, compared to unsorted cells.

4.6 Conclusion

3D PLACS overcomes the synchronization problem between cell detection and sorting, thus outperforming other microfluidic based cell sorters and achieving performance at a level comparable with conventional aerosol-based FACS. From the study of laser energy, nozzle shape, and bubble size, the switching window is optimized at 25 μsec, which equates to a 40 μm window at 1.5 m sec\(^{-1}\). Using this sharp window, the sort purity achieved 90% at 23,000 cells sec\(^{-1}\). Sort purity dropped to 45.4% at 45,000 cells sec\(^{-1}\) due to a cell clogging issue at the switch junction. 3D PLACS has the potential to substitute for conventional
electrostatic-droplet-based cell sorters. By integrating with downstream microfluidic cell analysis functions, 3D PLACS will greatly facilitate biomedical research and clinical diagnostics.
Chapter 5. Pulsed Laser Activated Cell Sorting with 3D Sheathless Inertial Focusing

5.1 Overview

3D PLACS in the previous chapter achieved a sorting performance of 90% sort purity at 23,000 cells sec$^{-1}$ and 45% sort purity at 45,000 cells sec$^{-1}$ using 3D sheath flows provided by multilayer PDMS structures [58]. However, the large amount of diluting sheath fluid needed for tight 3D focusing requires a high initial cell concentration in samples ($>10^7$ cells ml$^{-1}$), which can result in cell clogging in channels, and high pressure pumps to drive fluid through microchannels at high speeds. The need for 3D microfluidic channels also increases the complexity and the cost of device fabrication. In this context, sheathless 3D cell focusing provides a potential solution for this multi-faceted issue.

Several sheathless 3D cell-focusing mechanisms have been demonstrated so far. These include focusing using acoustic pressure [77, 78], optical [16, 47], magnetic [79], and dielectrophoretic forces [14, 16], hydrophoresis [80, 81] and inertial focusing [12, 82-84]. Among these, inertial focusing is a promising sheathless mechanism for integration with PLACS since both can operate at high speed ($>1$ m sec$^{-1}$). Other mechanisms, although providing a 3D cell focusing function, can only operate at relatively low throughput that are therefore not ideal for integration with PLACS. Furthermore, particle-particle interaction
phenomena [85, 86] in inertial microfluidics can be utilized to regulate the inter-particle separation between two closely positioned particles, or cells, to improve purity in high-throughput sorting.

In this chapter, we demonstrate an inertial focusing integrated sheathless PLACS for high-purity and high-throughput cell sorting in a single layer PDMS channel that can be easily and inexpensively fabricated. We also demonstrate an approach to enlarge the inter-particle distance of closely positioned particles, which may affect the sort purity at high throughput, by utilizing particle-induced hydrodynamic interactions in an expansion chamber.

5.2 Principle

As shown in Figure 5.1, the upstream channel design consists of a single low aspect ratio straight channel (80 μm in width and 40 μm in height) with a series of 30 vertical constrictions (20 μm in width, 18 μm in height, and 1 mm in periodicity), followed by a symmetric laterally expanded chamber structure (160 μm in width and 800 μm in length with an expanding slope of 2.86 degree). This main sample channel is geometrically engineered to enable 3D particle/cell focusing and inter-particle spacing control. The sample channel bifurcates into two outlets, collection and waste, after the switching junction. The train of focused particles/cells initially flows into the waste channel. Once fluorescence is detected by a photomultiplier tube, a cavitation bubble will be excited in another parallel channel, which is 150 μm in width and
expands to 300 μm at the bubble excitation location to allow enough space for bubble expansion, that is then connected with the main sample channel through a nozzle (20 μm in width and 50 μm in length) at the bifurcation. A rapidly expanding cavitation bubble triggered by the pulsed laser induced breakdown of water pushes high-speed fluid jets through the nozzle into the main channel to deflect the target particle or cell passing by into the collection channel.

Figure 5.1 Schematic of inertial PLACS. (i)-(ii) Randomly coming particles/cells are inertially focused into two positions in a 1:2 aspect ratio microchannel. (iii) A stepped microchannel induces secondary flows that lead to lateral migration of upper particles/cells in the channel. (iv) After passing through these stepped microstructures, the particles/cells are focused into a single stream. The y-z plane and x-z plan depict the particle/cell migration process. The focused particles enter into an expansion chamber structure whose function is to enlarge the separation distance between two closely positioned particles by particle-particle interaction in
inertial flows and reduce the coincident events. Particles passing through the stepped structure and the expansion chamber are then interrogated in the fluorescence detection zone. Once a target particle is identified, a laser pulse will be triggered to excite a cavitation bubble at a proper delay to induce a liquid jet through the nozzle to deflect the target particle/cell into the collection channel.

5.3 Device fabrication and experimental setup

SU-8 2010 and SU-8 2025 were patterned by standard photolithography on bare wafers for the first and second layers respectively. Then PDMS was poured on the mold and cured at 80 °C for 2 hr. After peeled off and cut into desired size, PDMS with structures was bonded to a piranha-cleaned thin cover glass (150 μm thick). Finally, the whole device stayed in the oven at 80 °C for 4 hr to enhance the bonding strength.

A Q-switched Nd:YVO₄ pulsed laser (EKSPLA, JAZZ 20, 532 nm wavelength, 8 nsec pulse width) was focused into the middle of the bubble channel through a 100×/N.A. 0.9 objective lens. CW laser (CrystalLaser, DL-488-010, 10 mW) at 488 nm was used for fluorescence excitation through a 25×/N.A. 0.4 objective lens. The emission fluorescence was collected by the same objective lens and detected by a photomultiplier tube (PMT) (Sens-Tech, P30CWAD501) which connected to a DAQ card (National Instruments, PCI 7831R) for signal acquisition and processing. FPGA logic was programmed using Labview to perform real-time
detection, threshold comparison, and timed triggering of the pulsed laser. To observe and characterize the bubble, a flash lamp (High-Speed Photo-Systems, NANOLITE, KL-M) was used to capture time-resolved images on a CCD camera (Zeiss, AxioCam MRm).

All the flows were driven by syringe pumps (Harvard Apparatus, MA, USA). High-speed camera (Vision Research, NJ, USA) mounted on a Nikon inverted microscope (Nikon, Japan) was used to take series of high-speed images at 4000 fps with 1 μsec shutter speed. The recorded images were processed by ImageJ and then analyzed using R.

5.4 Device characterization

Switching window optimization

Cavitation bubble dynamics are shown in the time-resolved images in Figure 5.2. A disk-shaped bubble expands to a maximum diameter of 160 μm within 3 μsec (Figure 5.2c) and fully collapses by 20 μsec (Figure 5.2d). Red dye (Allura, 67 mg ml⁻¹, Sigma-Aldrich) is added to reduce the threshold energy required to excite a cavitation bubble. With a liquid jet induced by a cavitation bubble through a nozzle, particles which are initially focused along a streamline that exits into the waste channel (Figure 5.2e) are pushed into the collection channel path (Figure 5.2f). The switching efficiency profile, which is defined as the probability of a successful switching event at various time delays, is experimentally constructed by counting successful switching events in 150 captured particle fluorescence images. The proper delay
time between detection and trigger of a laser pulse is set at the time that has the highest switching efficiency.

Figure 5.2 (a) Detection spot and pulsed laser spot location. (b) Flow rates of DI water in the main channel and fluid with red dye in the bubble channel were 400 μl min⁻¹ and 650 μl min⁻¹, respectively. The flow in the main channel was biased toward the bubble channel through the nozzle to minimize color dye following into the waste and collection channels. (c-d) time-resolved images showing cavitation bubble dynamics. A laser pulse triggered, disk-shape bubble with 160 μm in diameter expanded to its maximum in 3 μsec and fully collapsed at 20
μsec. (e) The fluorescence trace of a particle passing through the switching zone into the waste channel without triggering a bubble. (f) The fluorescence trace of a target particle that triggered a cavitation bubble and was deflected into the collection channel.

Here, a critical parameter to tune is the fluid flow rate in the bubble excitation channel. The peak switching efficiency is lower with a lower flow rate (for example 500 μl min⁻¹, as shown in Figure 5.3) in the bubble channel because the trace of the particles/cells is more biased away from the channel-separation tip, thus requiring a larger perturbation bubble to switch. By increasing the flow rate to 650 μl min⁻¹, the highest switching efficiency can be increased to 93% (Figure 5.3) while still keeping the focused particles/cells flowing into the waste channel. The optimized switching window for the sorter configuration here is 16 μsec in time or 32 μm in distance (16 μsec × 2 m sec⁻¹).
Figure 5.3 The tested switching efficiency profile (defined as the probability of a successful switching event at various time delays by counting successful switching events in 150 captured particle fluorescence images) with different dye flow rate settings. The proper delay time between detection and trigger of a laser pulse is set at the time that has the highest switching efficiency. By increasing the flow rate up to 650 μl min⁻¹, the highest switching efficiency is increased to 93%. The optimized switching window is 16 μsec in time or 32 μm in distance (16 μsec × 2 m sec⁻¹).

**Inertial focusing**

Fluid inertia is used to focus particles without sheath flows, using three separate channel regions: (i) a straight focusing region, (ii) a stepped region to bias a single stream, and (iii) an expansion region to maximize inter-particle spacing uniformity. With a particle/cell speed of 2 m sec⁻¹ and Reynolds number ($Re = \rho v D_h / \mu$, the ratio of inertial to viscous forces, $D_h$ is 53.33 μm calculated based on the dimension of the channel) of 107, particles/cells are focused by fluid inertia and equilibrate into two equilibrium positions in the initial low aspect ratio straight channel segment. This occurs due to a balance of two inertial lift forces originating from the shear gradient and the presence of the wall [85]. In the second region of the channel, vertical steps induce a pair of top-bottom asymmetric helical secondary flows, which cause the upper focusing position to become unstable and direct particles/cells to the other equilibrium position.
This results in 3D focusing of cells and particles into a single stream in the channel.

It is challenging to simulate particle and fluids interaction. Instead, we simulated fluids part to demonstrate the cross-sections of the flow deformation as fluids passed a series of stepped channels with the same flow condition used in the experiment (Figure 5.4). In the figure, color shows the normalized concentration: blue represents zero and red represents 1. With the initial condition being concentration of 1 on the two sides of the channel and 0 in the middle, the cross-sections after each step illustrate the whole process of helical secondary flow formation. Among the particles that are inertial focused at the equilibrium positions, the ones at the top and side positions will be driven to the bottom center position by this secondary flow.

Figure 5.4. Finite element analysis of helical secondary flow at Reynolds number of 107. Images show cross-section of normalized concentration at the beginning and after each steps
(total 30 steps) in the channel. The development of helical secondary flow is demonstrated with the initial condition of concentration of 1 on the sides of the channel and 0 in the middle. Among the particles that are inertial focused at the equilibrium positions, the ones at the top and side positions will be driven to the bottom center position by this secondary flow.

Figure 5.5a shows the focusing result of 10 μm polystyrene microspheres at a concentration of 0.1% w/w, which corresponds to a throughput of 10,000 particles sec\(^{-1}\) at a flow rate of 400 μl min\(^{-1}\). A standard deviation plot from 8,000 high-speed images (Figure 5.5a) shows that all particles are focused tightly into the waste channel. Previous studies found that the inertially focused particles/cells, due to the particle-particle interaction effects, tend to form trains with certain inter-particle separation distances, which for our channel dimensions and particle sizes is 22 μm (Figure 5.5b), a value smaller than the PLACS switching window measured in our experiments (Figure 5.3). Figure 5.5b shows a high-speed image of particles focused at the bottom inertial equilibrium position after the stepped structure but before entering the expansion chamber. To increase this inter-particle spacing we have implemented a third region in the channel – an expansion chamber which acts to slow down the particles and bring them closer into a hydrodynamic repulsive interaction regime, in which the viscous disturbance flows act to repel neighboring particles and push them apart [86]. After passing
through the expansion chamber, inertial lift forces again act to focus particles and maintain them at enlarged separation distances, as shown in Figure 5.5c. This process is possible because the train of particles is segmented, which means there are still large intervening regions with no particles. After slight widening of inter-particle separation distances, the particles are spread more uniformly while still satisfying conservation of mass.
Figure 5.5 Inertial focusing performance. (a) An image without flowing particles and an image showing the particle trace by stacking 8000 high-speed images of particles focused into a single stream and flowing into the waste channel. (b) Without an expansion chamber structure, interparticle separation distances have a distribution with a peak at 22 μm. There are 41% of particles whose separation distances with neighboring particles smaller than the 32 μm switching window. (c) After passing through a chamber structure, the distribution of interparticle separation distance shows a two-peak pattern with the major peak at 80μm and another peak at 22 μm. The percentage of the particles with interparticle distance smaller than the switching window reduces to 13%.

Using an expansion chamber increases the sorting purity at high sort rates by making the spacing between particles more uniform (less dependent on Poisson arrival statistics). Without the expansion region, we observe that 41% of the particles have an inter-particle separation distance smaller than 32 μm, which is the switching window. This means that non-target neighboring particles have a high probability to be switched into the collection channel. As a result, the sort purity in the device without an expansion chamber for regulating inter-particle separation distances drops to as low as 15% at a throughput of 10,000 particles sec\(^{-1}\). In contrast, by passing 3D focused particles through an expansion chamber, the percentage of particles with a separation distance smaller than the switching window is reduced.
to 13%.

5.5 Results

Particle sorting results

Sorting performance is assessed by sorting 10 μm (CV 2.4%) polystyrene microsphere mixtures at various concentrations corresponding to different throughputs. The sample flow rate is fixed at 400 μl min\(^{-1}\) while the bubble channel flow rate is 650 μl min\(^{-1}\). Both the initial mixtures and the post-sort samples are analyzed by a commercial flow cytometer (BD FACSCanto II). The sorting results with throughputs ranging from 6,000 particles sec\(^{-1}\) to 30,000 particles sec\(^{-1}\) are shown in Figure 5.6. The sorter can achieve > 90% sort purity at throughputs lower than 10,000 particles sec\(^{-1}\). The obtained post-sort purities were 91.3 ± 1.2% (mean ± s.d.) and 92.3 ± 1.4% for throughputs of 6,000 particles sec\(^{-1}\) and 10,000 particles sec\(^{-1}\). The post-sort purity drops at higher throughputs because of higher particle concentrations and smaller inter-particle separation distances. At throughputs 20,000 particles sec\(^{-1}\) and 30,000 particles sec\(^{-1}\), the obtained post-sort purities were 73.6 ± 2.2% and 45.9 ± 5.4% respectively.
Figure 5.6 Particle sorting results at different throughput. Initial mixture contains non-fluorescent 10 μm polystyrene particles with low percentage of fluorescent particles. After sorting, above 90% sort purity (fluorescent particle percentage) is achieved for throughputs below 10 000 particles sec\(^{-1}\). The obtained post-sort purities were 91.3 ± 1.2% (mean ± s.d.) and 92.3 ± 1.4% for throughputs of 6 000 particles sec\(^{-1}\) and 10 000 particles sec\(^{-1}\). At throughputs 20 000 particles sec\(^{-1}\) and 30 000 particles sec\(^{-1}\), the obtained post-sort purities were 73.6 ± 2.2% and 45.9 ± 5.4% respectively. All the purity data are confirmed by a conventional flow cytometer. For higher throughputs, the sort purity decreases because of decreased interparticle separation distance due to higher initial particle concentration.
Cell sorting results

Mammalian cell sorting is also demonstrated using Ramos human Burkitt lymphoma cells which are 11.29 μm in diameter but have a larger size variation (CV 9.3%) than particles (CV 2.4%). Stained cells by Vybrant® CFDA SE Cell Tracer (Invitrogen) are mixed with unstained cells at desired ratios. After tuning the density of the cell suspension, which is on the level of $10^6$ cells ml$^{-1}$, the mixture is introduced into the microchannel. After sorting, the collected cells are stained with PI dye to test the viability and analyzed by a commercial flow cytometer (Table 5.1). A sort purity of 80.1% is achieved at a throughput of 6,000 cells sec$^{-1}$. At higher throughputs, more unfocused cells stack at the bifurcation tip in a short time and cause the shifting of the bifurcation point. This not only changes the switching timing, decreases switching efficiency, but also causes device clogging. These unfocused cells partly come from the beginning of sample introduction before a stabilized fluid flow is established. After the fluid flow becomes stable, there is still a small percentage of unfocused cells (less than 1%). Decreasing the number of unfocused cells or increasing focusing efficiency by adding more steps at the inertial focusing stepped region may help reduce clogging probability. Widening the channel can also prevent device clogging [76]. The reasons for lower sort purity for cells than that for particles at the same throughput arise from both mis-focusing and mis-sorting. (1) The focusing efficiency for cells is lower compared to particles given the same experimental
conditions because cells are more deformable and have larger variations in size. Non-target cells can flow into the collection channel due to mis-focusing of variably sized cells. (2) The chamber structure is less effective in enlarging the distance between cells than it is for particles, partly because of size differences which lead to variations in cell velocities and inter-particle distances. Thus, there are more cells, compared to particles, with inter-cell distances smaller than the switching window, causing more non-target cells to be sorted into the collection channel.

Table 5.1 Results of sorting mammalian cells at different throughputs

<table>
<thead>
<tr>
<th>Throughput (cells per sec)</th>
<th>3000</th>
<th>6000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell sample density (×10^6 cells per ml)</td>
<td>0.45</td>
<td>0.9</td>
</tr>
<tr>
<td>Initial purity (viability)</td>
<td>6.4% (93.9%)</td>
<td>4.3% (81.1%)</td>
</tr>
<tr>
<td>Collection purity (viability)</td>
<td>85.6% (90.7%)</td>
<td>80.1% (78.5%)</td>
</tr>
<tr>
<td>Waste purity (viability)</td>
<td>1.5% (95.5%)</td>
<td>2.4% (71.9%)</td>
</tr>
</tbody>
</table>

5.6 Conclusion

In conclusion, we presented a 3D inertial focusing integrated PLACS for high throughput, sheathless, fluorescence activated cell sorting. Inertial focusing, which replaces 3D sheath flow focusing, uses 10 times lower initial concentration cell samples than that in sheath-based PLACS in order to avoid severe dilution effects from high volume sheath flows at the same
throughput. The unique particle-particle interactions in inertial flow also allow more uniformly distributed particles/cells to increase sort purity during high-throughput sorting. With an optimized switching window (peak switching efficiency of 93% and width of 16 μsec), sort purity >90% has been achieved at a throughput of 10,000 particles sec\(^{-1}\). Sorting mammalian cells with >80% sort purity at a throughput of 6,000 cells sec\(^{-1}\) is also demonstrated. To further improve the sorting performance, throughput and purity, a superior channel design is required to further reduce the chance of cell clogging and enlarge the inter-cell separation distances.
Chapter 6. Summary

In this dissertation, a brief review of the cell sorting technologies and their application in academic research and clinical practice has been summarized at the beginning. Because the specific characteristics of the fluorescence activated cell sorter (FACS), such as multi-parameter analysis, high purity and high throughput, it has widely applied in the past decades. Compared to aerosol-based conventional FACS, microfluidic FACS (μFACS) can offer a closed sterile environment and strong potential to integrate with upstream and downstream cell analysis microfluidic modules. However, the big challenge in μFACS is the speed, which is 1-2 orders of magnitude slower than conventional FACS. In order to overcome this problem, a novel switching mechanism has been introduced, which employs a pulsed laser triggered cavitation bubble to induce a liquid jet and deflect the passing by target particle/cell. The second chapter is the preliminary attempt to utilize the cavitation bubble to switch particle/cell. The strong force from the expansion of the bubble deformed the channel wall and successfully switched the passing by particle/cell into the collection channel. However, because the perturbation volume is big, it hardly achieved high purity sorting. The third chapter introduced a new channel design that can limit the perturbation volume to ~90 pL. With 2D hydrodynamic focusing, high purity sorting (90% purity) has been achieved at a throughput of 15 000 cell sec^{-1} or 3 000 cell sec^{-1}. The purity drop at high throughput is due to the lack of the
third dimensional focusing. Then in the fourth chapter, a 3D PLACS has been developed using PDMS thin membrane fabrication technique. It has achieved same post-sort purity (90%) at a throughput of 20 000 cells sec$^{-1}$, which is about 10 times higher than that in 2D PLACS. The potential issue with 3D hydrodynamic focusing is the large dilution of the sample by sheath. A potential solution is to use a sheathless focusing scheme to avoid sheath. In the fifth chapter, inertial focusing is integrated with PLACS because they both operate at high speed regime. After adding an expansion chamber to enlarge the particle-particle separation distance, it achieved 90% post-sort purity at 10 000 particle sec$^{-1}$ and 80% post-sort purity at 6 000 cell sec$^{-1}$. 
References


