FLUORESCENT ACTIVATED ACOUSTIC CELL SORTING STRATEGIES

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ABSTRACT

The cell sorter has received more attentions in recent years. The successful cell sorting can benefit biological or biomedical engineering field a lot. Biomedical experiments need exact controlling of the cells or other biological factors use. Researchers always need to extract or separate specific cell type from a group of mixtures. The traditional flow cytometry has been taken those jobs for decades. However, the traditional cell sorters are so expensive and may cause the damage of subjects. Acoustofluidic, a newly developing sorting method, has been brought onto the table with the advantage of high biocompatibility, cheap components, and portable size. In my senior project, I have been worked closely with Liqiang Ren on acoustic wave cell sorter in the acoustofluidics group of Dr. Huang in Pennsylvania State University. We have been working on improving the throughput rate of fluorescent acoustic wave cell sorter. Using the focused interdigital transducers and fluorescent recognizing system, I have fabricated the acoustic wave cell sorting device with the throughput rate as high as 720 events per second and increased the purity of the living cells from 20 percent to 78 percent.
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Chapter 1

Literature Review

As the science development, scientists keep pushing the size limit of the subjects studying to extremely small and trying to understand how everything is “built”. Chemistry scientists mostly study molecules; physicists always work with atoms, electrons, or photons; biologists deal with cells all the time; material science engineers design all kinds of cool material with small units as small as Nano-level. The technologies to manipulate those small particles or subjects are badly needed. The background and essential the principles using by fluorescent activated cell sorters are illustrated in this chapter.

1.1 Introduction

For biology and biomedical engineering field, recognizing cells or biological factors is always a big topic to be taken care of. There are over 200 types of cells and countless types of proteins in human body. The sorting process directly affect whether scientists can extract correct subject to study. Bioengineers need to grow the tissues with the absolutely right cells and other necessary small units, like growth factors or biomaterials. They also need to separate species of biological units from the samples for analyzing purposes. The sorting process, in this occasion, is one of the most essential parts. Over the past 40 years, this job has been taken by flow cytometry \[1\], which mostly use electromagnetic method to separate particles especially cells (see Figure 1). The samples are prepared with dyes so that different types of particles will have different recognizable features. We put dyed samples into the machine with sheath flow which can squeeze the particles to the middle of the channel to form ordered single-line of particles \[2\]. The laser will activate the fluorescent marks and the detectors can read the type and size information carried by the fluorescent of the particles. At the end of the channel, droplets of the fluid will form due to the vibrating
nozzle. One droplet will contain the negative charge and only one particle. The computer controlled electromagnets are operated based on the information gathered by the detector and apply electric forces with varies strength and direction to guide droplets to different groups to achieve the sorting.

Figure 1-1 Principles of traditional flow cytometry (a) particles with no fluorescent dye (b) particles with fluorescent dye. The picture is cited from literature 2
However, the revolutions are happening because of the weaknesses of the traditional flow cytometry. First, charges and the static electrical forces in the electromagnetic operations may damage the cells integrity and reduce the cells viability so that the sorted sample are not reusable. Secondly, the traditional flow cytometry machines are usually very big and are difficult to carry around (See Figure 2). Finally, the existing commercial flow cytometry machines can cost more than 100 thousand dollars with enough functions that a normal bio-lab needs. A new generation of flow cytometry with excellent biocompatibility, small size and low cost is an irresistible developing trend.

Figure 1-2 A normal Flow Cytometry Machine. It is a lot bigger than a desktop computer

In the recent decade, the microfluidics has emerging as a new promising field. According to George M. Whitesides in his work, “It is the science and technology of systems that process or manipulate small (10⁻⁹ to 10⁻¹⁸ liters) amounts of fluids, using channels with dimensions of tens to hundreds of micro-meters.”
There are many methods of manipulating cells by using microfluidics. Coupling with microfluidic channels, techniques like dielectrophoresis \(^4,5\), hydrodynamic flows \(^7\), optical tweezers \(^8,9\) and etc. are developing.

The acoustophoresis recently has joined the list for its advantages of contactless manipulation, low cost, high controllability and high biocompatibility \(^10\). The most outstanding advantage of acoustophoresis techniques in the microfluid environment is the biocompatibility. The frequency of the acoustic wave for manipulate cells are normally smaller than 50 MHz, which is within the safety frequency range of medical ultrasonic devices\(^12\). The energy in the acoustic wave is safe enough for the cells in the samples. The acoustic particle sorter devices can easily align, separate or sort particles or cells without any techniques directly contacting the fluids. Furthermore, all we need to have are function generators and amplifiers to generate the acoustic wave. The device has a great potential to be designed into small portable size. The electronic components have already been well developed so that the device can be much cheaper than those devices using other sorting techniques. If the acoustic micro-fluidics cell sorts are fully developed, the devices be labeled with clean, safe, convenient and economic.

The drawbacks of the acoustic wave cell sorters do exist. The limited throughput rate is a major problem that needs to be solve. In 2009, Johansson et al. have designed the first bulk acoustic waves based fluorescent activation cell sorters and achieved 27 cells per second \(^13\). The record was broken with 60 events per second and then 150 events per second in the following years \(^14,15\). However, the stunning fact is that the commercial flow cytometry cell sorters can reach over 10 thousand events per second throughput now. The acoustic wave cell sorter needs a lot more improvements. In my senior project, I have worked closely with Liqiang Ren. We are working on the method to improve the throughput of acoustic wave cell sorters. The mainly considered important factors are the shapes of the interdigital transducer, the focus width of the acoustic wave effective area, the activation time of the transducer and the speed of the fluid inside the channel. With carefully chosen of the parameters, I have fabricated an acoustic wave cell sorter with throughput of 715 events/s and increase the purity of the living cells from 20 percent to 78 percent.
1.2 Principles of Acoustic Wave Cell Sorters

This subsection includes the essential principles of fluorescent activated acoustic cell sorters we are working on. The fluorescent activated acoustic cell sorter is based on the micro-fluid environment, acoustic wave generation and the fluorescent activation mechanisms.

1.2.1 Micro-fluid Environment

The fluid has two main types of flows: laminar flow and turbulent flow. The laminar flow has smooth stream line and the particles in it have highly ordered motion. The turbulent flow, on the contrary, has fluctuate velocities and highly disordered motion. There is actually another type of flow named transition flow, but since it is a just a transition state between laminar flow and turbulent flow, we are not interested here. The magic of the microfluidics is based on its viscosity property. The viscosity dominates over inertia in the microfluidics environment 16. The predictable and regular laminar flow is the main consideration in our study, while the turbulent flow can be neglected. The laminar flow is far more easily to work with than the turbulent flow. During the sorting process, we need the particles to follow the order and be as predictable as possible. The laminar environment of micro-fluidics provides us a good sorting condition.

The acoustic wave used in the acoustic cell sorters have two types: Bulk Acoustic Waves (BAW) and Surface Acoustic Wave (SAW). The bulk acoustic waves are like the acoustic wave in the earthquake. It propagates in the bulk entity. As shown in Figure 1.3 (a), the bulk acoustic wave penetrates the PDMS channel walls and apply pressure on the fluid inside the channel. The surface acoustic waves propagate on the surface of the piezoelectric material (See Figure 1.3 (b)). The interdigital transducer (IDT) will be connected to the function generator. The piezoelectric layer will sense the voltage patterns and generate acoustic waves along the surface of the layer. The acoustic wave can leak into the fluid, which the piezoelectric material is in direct contact with, and then generate pressure field inside the fluid.
As the energy is confined in the substrate and does not go through other materials, the loss of energy can be minimized. In addition, the SAW can be generated with higher frequencies so that the particles can be more easily controlled. The advantage of higher frequency and less energy dissipation drive us using the surface acoustic wave instead of bulk acoustic wave.

Figure 1-3 The demonstration of two different acoustic waves generated in acoustic cell sorter. (a) the bulk acoustic wave (b) the surface acoustic wave

If we apply two signals in opposite direction to the IDTs, the two opposite surface acoustic wave can interference with each other and, with the right frequency, we can generate standing surface acoustic waves. The SSAW can apply constant pressure gradient to the fluid. At the nodes of the waves, the pressure is at minimum level so the particles will be pushed toward those nodes. With this property, we can apply forces to particles to push them to the predicted locations. According to the literature, the primary acoustic radiation force acting on any micro-particles in a SSAW field can be expressed as

\[ F_r = -\left(\frac{\pi P_0 V_c \beta w}{2 \lambda}\right) \varphi(\beta, \rho) \sin(2kx) \]
\[ \varphi(\beta, \rho) = \frac{5\rho_c - 2\rho_w}{2\rho_c - \rho_w} \frac{\beta_c}{\beta_w} \]

where \( p_0, \lambda, V_c, \rho_c, \rho_w, \beta_c \) and \( \beta_w \) are acoustic pressure, acoustic wavelength, volume of the particle, density of the particle, density of the fluid, compressibility of the particle and compressibility of the fluid. As we can see from this equation, the force is proportional to the frequency of acoustic wave and basically has a shape of Sine wave.

### 1.2.2 Acoustic Wave Generation

The acoustic waves are generated by coupling the electrodes (IDTs) and piezoelectric material. Our goal is to convert the electric energy into mechanical energy. The piezoelectric materials provide us the media to achieve that purpose. The AC signals are generated by the function generator and connected to the interdigital transducers. The piezoelectric material will vibrate with the alternative voltage signal and propagate the vibration along the surface (see figure 1.4).

![Diagram](image)

**Figure 1-4** Demonstration of Piezoelectric substrate. (a) the piezoelectric material reactions to voltage change. (b) The acoustic pressure propagation on the surface of the substrate
There are two main types of IDTs for different conditions. Like rails, the IDTs contain a lot of "fingers". The geometry of the fingers can be different. For type one IDT, the fingers are all straight and parallel to each other. It is normally called Standard Interdigital Transducer (SIDT, see Figure 1.5 (a)). In this case, the generated acoustic wave is parallel and have a rectangular-shape working zone. This type of IDT can also be made into tilted IDT by tilting the IDT and having a specific angle between the straight fingers and the longitude of the channel (see Figure 2.4 b). Separation of cells is one of the main applications for tilted IDT. SIDTs are often used when the subjects have large difference in size and density.

Figure 1-5 Different types of IDTs (a) Standard IDT. (b) Tilted IDT. (c) Focused IDT.
The other type of IDT is called focused interdigital transducer (FIDT, see Figure 1.5 (C)). The fingers are all curved and are arranged in the geometry of a part of a circle\(^ {18}\). In this way, the effective acoustic area can be focused with much shorter width than the SIDT. The energy is also focused in this condition so that the pressure on the particles will be larger and the effect of the acoustic wave will be more effective. FIDTs are needed when we need smaller effective area and shorter interaction time between particle and the acoustic wave. Channel

1.2.3 Fluorescent Activated Acoustic Cell Sorter

One of the main parts of the fluorescent activation cell sorter is the fluorescent recognition (Refers to Figure 2.6). After we centered the particles in the channel with sheath flow, we need to recognize the particle with a fluorescent activation fiber and a fluorescent detector. The fluorescent activation fiber constantly emits an optical beam and as the subject particle goes through, the beam will activate the fluorescent elements on the particle. If the particle’s fluorescent is activated, the particle will release specific fluorescent and be detected by the detector immediately. When the recognized particles pass through the acoustic force effective area, the trigger system controlled by computer program will turn on the acoustic wave and push the particle out of the line of normal particles. Finally, the particle will be collected from target outlet because it deviates from the other particles that do not have fluorescent.

![Figure 1-6 Fluorescent Acoustic Wave Cell Sorter Schematic](attachment:image.png)
The performance of the fluorescent acoustic cell sorter is determined by throughput and sorting rate. The throughput represents how many particles or cells, including both types of the particles, pass through the device per second. Assume the width of the effective acoustic wave area is $W$ and the average velocity of all of the particles is $V$, the time need for the particle to pass the effective area is:

$$T = \frac{W}{V}$$

Since we can only push one particle at a time to avoid errors, the time for the particle to pass through the acoustic force field must be larger or equal to $T$.

$$T_{real} \geq T$$

The throughput, $R$, is how many particles can pass through the sorter per second, so

$$R = \frac{1s}{T_{real}} \leq \frac{1s}{T} = \frac{V}{W}$$

As we can see from the equation, the throughput rate depends on the velocity of particles and the width of the acoustic wave force field. We can always increase $V$, but the error would be much larger because the SSAW may accidentally push some unwanted particles or cells away from their original path. So we need to focus on decreasing $W$ to get higher $R$.

The sorting rate represents the maximum number of particles the device can sort per second. The acoustic wave sorting process can be seen as a process of generating a pulse. The pulse has a width and we assume the width is $T_{pulse}$. The pulse can be turned on $\frac{1}{T_{pulse}}$ times per second. So the sorting rate is equal to $\frac{1}{T_{pulse}}$. However, as far as we concern, the $T_{pulse}$ is so small that $R$ is much smaller than the sorting rate. Therefore, improving $R$ is the priority of the research.
2.1 Device Fabrication

Before the fabrication, we need to design the IDT patterns in the computer software (see Figure 2.1). In my senior project, I used FIDT patterns with 500 µm arc radius and 20° arc degrees \(^{18}\). The design should also include the channel so that we can bond the channel with IDTs together seamlessly. The size of the channel in my project have to be 60 µm height and 120 µm wide. The patterns are sent to mask producers to fabricate the UV impenetrable masks.

The substrate of the device is made of 128° Y-cut, X-propagating lithium niobate (LiNbO\(_3\)) which is a piezoelectric material. We will apply standard photolithography process to it. The LiNbO\(_3\) wafer is spun-coated with SU8 photoresist. The SU8 photoresist is a negative photoresist so that the UV exposed part are insoluble to the photoresist developer. Then we cover the designed mask on our substrate and expose them together to UV light. The substrates are developed with photoresist developer to wash the soluble photoresist off. With an e-beam evaporation process, we can deposit two thin metal layers onto the photoresist residue which have been exposed to UV. The metal layers contain Chromium and Gold (the
thickness of the layers are 5nm and 50 nm, respectively). After a lift-off process, the FIDTs can be observed. The substrate is then finished.

The master mold for channel building can be produced with similar process as piezoelectric substrates. With the help of SU8 photoresist, the IDT and channel patterns can be built on the master mold (silicon wafer). We bake the mold until the structures are stable. This time we do not use e-beam evaporation to deposit metal layers. We use PDMS mixtures which are made with PDMS base and cross-linker with a ratio of 10:1. We stir the mixture until the two conjugate parts are mixed uniformly and then pour the PDMS mixture onto the mold in a wafer holder. The PDMS mixture is full of air bubble now, so we need to put them into the vacuum chamber to get all of the air bubbles out (See Figure 2.2 (a)). Finally, the holder with the PDMS mixture and the master mold is put into the oven (See Figure 2.2 (b)) to cure the PDMS at 65°C until the PDMS mixture is firm enough. The PDMS channels can be peeled off after the curing is done.

![Figure 2-2 Equipment demonstration for channel fabrication (a) The vacuum chamber. (b) The baking oven.](image)

We put both the substrate and PDMS channel and the substrate into the plasma vacuum chamber to finish plasma treatment (See Figure 2.3). The ions in the plasma chamber can break the bonds on the surface of the material to increase the surface energy. When the plasma treatment is done, we can easily bond the channel and the substrate together because they can form bonds on the contacting surfaces in order
to reduce the surface energy. The channel needs to be perfectly aligned with the substrate to prevent leakage of the fluid in the experiment.

In my project, I have used 10 µm polystyrene particles diluted with 0.5% sodium dodecyl sulfate (SDS) water solution as test subject one for parameter calibration. Then Hela cells need to be prepared with fluorescent elements. The Hela cells originally have concentration of 10⁶/mL. Half of the cells are dyed with Calcein green AM, a permanent cell dye. Those cells were mixed with 1 uL Calcein green AM in 100 uL volum and incubated in dark room for 15 min. Then we centrifuged the solution with 800 rpm for 5 mins and removed the upper level clear solution. The cells at the bottom are re-suspended with PBS solutions to adjust to the desired concentration. Finally, we mixed the Calcein green AM labeled cells with unlabeled cells at random ratio for further sorting experiment.

Figure 2-3 Plasma Vacuum Chamber. The samples can be put inside for plasma treatment.
2.2 Acoustic Cell Sorting Preparation and Settings

After the channel and the substrate are well bounded, we need to connect the device with wires, plug in the inlets and outlets and pump the BSA&PBS solution through the whole channel. The BSA&PBS solution can help smooth the inner surface of the channel and lower the surface energy. The cells or particles are extremely easy to get stuck on the walls inside channels and sabotage the device.

The IDT wires are cross linked. In other words, the positive wire from the IDT on one side are connected to the negative wire from the IDT on the other side. The other two wires are dealt with the same manner. In this way, we can have the two IDTs generate acoustic waves in completely opposite directions. As I have mentions in the chapter 1.2.1, the acoustic wave applied to the channel will become a SSAW. The FIDTs are designed to generate 100 µm and 38.8 MHz acoustic waves. We generally adjust the function generator to the preferred value. The amplitude can be adjusted on the amplifier in the experiment for the preferred effect (See Figure 2.4).

![Figure 2-4 Function generator (a) and amplifier (b)](image)

Three syringes are fixed on the syringe pump (See Figure 2.5) with the sample in the middle and the sheath flow (PBS solution) at two sides. The speed of the pump can be adjusted on the computer.
The fluorescent activation fiber is put at one side of the IDT and slightly before the focus point. And the fluorescent detector is put at the other side of the IDT in tilted position (See Figure 3.2). If the detector is vertical to the channel and is exactly symmetry to the activation fiber, the high intensity laser light will cause strong background noise for detection and result in low detection sensitivity.

Figure 2-5 Syringe pump

Figure 2-6 Fluorescent recognition system in the acoustic cell sorter. (a) Microscopic view of the FIDT and fluorescent fibers. (b) The schematic of acoustic cell sorting process.
2.3 Experimental Procedure

With the device fabricated, the overall process is as following. The sample is centered with the selected sheath flow. Then the fluid is scanned by the fluorescent detection system; if the subject is labeled with fluorescent, the signal will be detected by the detector and the computer will activate a trigger to open the acoustic wave. The trajectory of the particle or cell will be slightly changed and eventually flows toward the target outlet. If the subject is not labeled with any fluorescent, the computer will not trigger the acoustic wave. The particle’s flowing trajectory will not be affected and flow toward the waste outlet.

We first examined the device with polystyrene particle solution and adjust the parameters to achieve functional sorting. Then we clean the channel with ethanol and change the sample to the Hela cells solution. The cell sample is sorted with the same parameters and gather the two type of cells into two different test tubes. The sorting process can be observed with the microscope and fast camera (See Figure 2.7). The sorting results will be analysis by a commercial flow cytometer (FC500, Beckman Coulter, USA).

Figure 2-7 Microscope and fast camera
Chapter 3

Results

3.1 Polystyrene Particles Sorting

With insufficient input power, we will not have enough force to deflect the polystyrene particles. In figure 4.1, the particle 1 is activated by the 488nm laser fiber and can be recognizable. However, when it reaches the acoustic force field, it was no deflected and flowed directly into the waste outlet.

![Figure 3-1 The particle sorting in low power condition](image)

We let the acoustic wave generator on and adjust the power input. We increase the input power to find a just enough level. The power input cannot be too big because too much power can generate heat in the flow and cause the damage to both subjects and channels. After adjustment, we have enough force to
deflect the particles. See in Figure 4.2, the particle is deflected upward and flow into the target outlet when the surface acoustic wave is on. When we turn the surface acoustic wave off, the particles were not deflected anymore and flew into the exit outlet.

Finally, the concentration will directly affect the results of sorting. See in Figure 4.3, when the concentration of the sample is too large, the particles will flow with an extremely close distance. And when the SSAW is on, all of the particle near the fluorescent particle will be pushed upward together and the result of sorting will be highly imprecise. We have captured the moment when the non-fluorescent particles are pushed to the target outlet together with fluorescent particles (See Figure 3.3).
3.2 Hela Cell Sample Sorting

With all of the parameters tentatively set up, we switch the particles to the Hela cells. There are some moments of the sorting. In Figure 3.4, particle A and B are all live cells and they were detected by the fluorescent detector. Compared with particle C, A and B were all pushed out of the original trajectory and flow into the target outlet while particle C received zero force and flow into the waste outlet. After the sorting process was finished, we took the sample from target outlet to the commercial flow cytometer for qualitative analyzation. The flow cytometer examined the fluorescent intensity of the cells in the sample. Results are shown in Figure 3.5. The type of cells can be differentiated by the fluorescent intensity.
Because the cells were dyed with green fluorescent before doing any experiment, the labeled cells will have higher fluorescent intensity and the unlabeled cells will have lower intensity. As shown in Figure

Figure 3-4 Cell Sorting Captures

Figure 3-5 The Analysis of the Sample Before and After Sorting. (a) before the sorting. (b) after the sorting
3.5, the sample before sorting contain 80.9% unlabeled cells and 19.1% labeled cells. After the sorting, the percentage of the labeled cells was increased to 77.9.

Chapter 4
Discussion and Conclusion

4.1 Discussion

The throughput was calculated from observations. From the video recorded by the fast camera, we have count 18 events happened in 507 frames. Each frame is 49.3 micro-seconds.

\[
R \approx \frac{N}{T} = \frac{18 \text{ events}}{507 \times 49.3 \times 10^{-6} \text{s}} \approx 720 \frac{\text{events}}{s}
\]

where N is the events happened during the period and T is the time of the period. It turns out that we can reach as high as 720 events per second in the sorting process.

The purity of the labeled cells has been increased from 19.1% to 77.9%. There are still some unlabeled cells flow through the target outlet, although from our selective observation all of the sorting was correctly. The errors in the sorting process may have several sources. 1. The cells were not ideally uniform. As we can see from figure 3.3, if the concentration of cells is high locally, the dead cells may be sorted together with the live cells. 2. The overall concentration of the sample is high for this device. 3. The velocity of the fluid is too fast. The high velocity may cause the fluid slightly turbulent so that the acoustic force is not the only factor that can affect the trajectory of the cells. 4. The effects of acoustic force on the cells are more complicated than the normal particles. Cells do not have uniform density so that the force can cause other effects, like spins, rather than what we predicted.

Combined the throughput and the purity together, we found that there is a tradeoff. We can increase the fluid velocity and the concentration to increase the throughput, but the purity increase after sorting will
decrease. We have observed the throughput of $720 \frac{\text{events}}{s}$ with low rate of errors. The throughput of the overall process can be much higher. Theoretically, with FIDTs, the fluorescent acoustic cell sorter can have a throughput as high as 13800 events per second $^{18}$. However, that is just theoretically prediction. The purity of target samples will be much lower if we reach that throughput. There are more unpredicted restrictions for improving acoustic cell sorter throughput.

4.2 Summary

The sorting process was performed on both particles and Hela Cells. The input powers, sample concentration and fluid velocities can all affect the throughput rate and the sorting purity. If input power are not optimized, the cell sorter may or push the subjects too hard or not affect the subjects at all. If the concentration is higher than the tolerance range, the “innocent” cells may be pushed with fluorescent cells. With deliberately calibration of the parameters, we have achieved 720 event per second throughput and about 60 percent increase in purity of the live Hela cells. The purity cannot be easily increased to 100 percent without limiting the throughput rate.

Future study should consider the concentration uniformity. Although the overall concentration is not high, the local concentration may cause the mistakes in sorting process. More acoustic sorting mechanisms are to be explored. The sheath flow can be substitute with a pair of standard IDTs. By constantly applying acoustic forces, the cells can be aligned in the center of the channel to avoid unnecessary effect of sheath flow like turbulence effect. Furthermore, we can start to explore more recognition mechanism. Instead of having fluorescent detectors, we can use some protein or antibody conjugates. As we all know, the proteins usually have large structures and the strength of acoustophoresis forces also depends on the density of subjects. If we can have specific proteins bind to the cells and apply constant acoustic force to the group, the cells can be differentiated with higher throughput and purity.
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11. Cell Analysis at the Bench: Benchtop Flow Cytometers


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