MICROFLUIDIC CHIPS
FOR CELLULAR HETEROGENEITY STUDIES

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Abstract

In recent years several studies have found significant heterogeneity among even purified cells that were previously treated as if all cells were essentially identical. But since many measurements have been reported as averages over large numbers of cells, assuming that all cells are same when they might really be a mixture of different cell subtypes, this can lead to incorrect or at least imprecise and harder to replicate results. There is a macroscopic technique for single cell analysis known as dilution method: diluting and depositing cells in a conventional platform such as a 96 well plate. However, it is very labor-intensive, low-throughput, low single-cell loading efficiency, and poor reproducibility and thus is rarely performed.

We have designed a high-throughput microfluidic chip which solves these problems and is superior to existing devices in allowing heterogeneous cell studies at scales and accuracies not previously possible and with minimum labor and cost. This chip performs 1) single-cell capture and culture to generate their colonies, and 2) sorting multiple specific target cells. Hydrodynamic force and magnetic force were studied to position or to sort cells in pre-determined locations precisely. We designed a novel hydrodynamic guiding structure which can automatically capture and position single cells into each microwell with high capturing efficiency, using only gravity flow, with which we could capture 80% of cells at a single cell resolution from all the injected cells (~ 2 orders of magnitude improvement, comparing to the state-of-art and widely adopted
passive weir structures for single cell trapping). This hydrodynamic guiding scheme was applied to a high-throughput microfluidic array chip, which is the first chip capable of culturing single cells into their clonal colonies inside individual microwells and introducing test-reagents to their clones. To track cells carefully over time and ensure clonal outgrowth from single cells, we should prevent cell migration between neighbouring microwells, which can generally happen in other reported single cell culture chips. We integrated a surface patterning technique into a microwell, which effectively confines cells’ movement inside each microwell. In addition, we utilized a gravity flow caused by pressure difference between inlet and out reservoir, which allows us a simple and easy operation without the necessity of external equipments. Using this chip we can detect heterogeneity of cells identify subtypes of clones and monitor drug responsiveness. We observed three different subtypes grown from a prostate cancer cell line, PC3 cells. These distinctive subtypes have different morphologies and proliferation rate, as well as different drug responsiveness. This single cell clonal chip can be extended to a larger array as well as used for multiple reagents by integrating pneumatic valves.

An alternative method of cell sorting for heterogeneity studies has been explored where the objective cells have well-known identifiable surface markers. It is also desirable to collect cells at a specific target size especially for effective drug screening purpose. A macro magnetic sorter can separate target cells; however, the screened cells are not 100% pure and their sizes vary in a wide range. We devised a novel magnetic sorter which can separate multiple target cells into corresponding microwells. We have designed a magnetic sorter based on the phenomenon that magnetic particles move
towards the minima of field and flowing electrical current generates controllable magnetic field. The magnetic sorter could separate different-sized cells by generating a local magnetic field gradient with the integrated current-carrying lines. We successfully demonstrated three different sizes of magnetic beads can be sorted under the different electrical current through the embedded current-carrying lines in three successive sorting units. More unit stages can be added and the number of stages can be determined to meet the sorting purpose.
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Chapter 1.
Introduction

1.1 Heterogeneity of Cells

Most cell-based biological assays have traditionally emphasized a volume of data acquisition and analysis at a population level. Information on their proliferation, differentiation, how cells respond to their environment has been analyzed from the data averaged across large groups of cells, rather than from their distributions. Thus they could miss rare (but important) events and have been unable to analyze cells that are only produced in small numbers. In recent years several studies have reported that individual cells are not identical but different even in a purified cell line, showing their cellular heterogeneity [1-12]. It is not always possible to separate and assay them for heterogeneity study on the given cell types because all the sub-phenotypes do not have their own clear surface markers yet. But differences among cells exist and are often identifiable through their changes in progeny, differentiation potential, subtle but repeatable differences in proliferation and morphology, or physiological response to
various treatments. For example, bone marrow stromal stem cells form colonies of various sizes, growth rates and morphology ranging from fibroblast-like spindle-shaped cells to large flat cells [8]. Analyzing this heterogeneity and targeting specific cell subtypes such as cancer-stem cells has great potential for diagnostic and therapeutic treatments [9, 10].

Recently Li et al have revealed that there are three clonal subtypes represented by holoclones, meroclones, and paraclones in a cell line (PC3) for human prostate cancer [1]. Figure 1 shows a conceptual diagram of the PC3 cell line, which is a mixture of three different subclones. They could identify three types easily from their morphologies and their proliferation rates. As shown in Figure 2, holoclones consist of tightly packed small cells and paraclones of larger and fewer cells, whereas the meroclones were made of cells of intermediate sizes and numbers. They observed high level expression of three known stem cell markers (CD44, α2β1 integrin, and β-catenin) from only holoclones, whereas meroclones and paraclones showed markedly reduced expression, indicating that three clones have different characteristics and only holoclones contain self-renewing stem like property.

We have chosen the prostate carcinoma cell line, PC3 cells, as test cells to verify our devices because its heterogeneity is already well established with identified subtypes as described earlier. In addition, potentially different drug responsiveness from identified sub-phenotypes can provide insight and valuable data in order to develop chemotherapeutic agents for curing the second most malignant tumor, prostate cancer.
Figure 1.1 Conceptual diagram of the PC3 cell line spontaneously generating three different sub phenotypes (holo-, mero-, and paraclones) over time. Coloring and marked morphological differences are added for emphasis.

Figure 1.2 Photographs of three different subclones from PC3 cell line: holo-, mero-, and paraclones [1].
1.2 Conventional Methods for Heterogeneity Study

There have been two conventional methods useful for heterogeneity study. One is single cell method enabling to observe cellular behaviors from separate single cells and the other is sorting method, separating and purifying only target cells in a group, which share the same surface markers.

The single cell method is a basic and essential technique to reveal (otherwise hard to even recognize) cellular heterogeneity. Because studies based on this method are not subject to the averaging effects and one different from bulk-phase population-scale methods, they offer a level of discrete and closer observation to individual single cells, which is unavailable with traditional methods. Serial dilution method has been used to enable single cell analysis, which relies on high dilution and deposition of cells in conventional platforms such as 96 well plates. However, it is very labor-intensive and inefficient in single cell loading yield and reproducibility, thus it is seldom performed.

The alternative conventional method for heterogeneity study is a flow-cytometry, Fluorescent-Activated Cell Sorting (FACS), where cells labeled with fluorescent markers are suspended in solution, and injected with high speed to an observation area in a single file by sheath flows, then sorted with high accuracy (>99%) according to their fluorescence. However, it is useful only when the target cells have clear distinct surface markers to differentiate them from other cells; therefore, it limits the application only to exploring well-known cells.
On the other hand, recent development of the microfluidic devices allows us to manipulate cells even in a single cell resolution with higher efficiency and repeatability, facilitating cellular heterogeneity study, which will be discussed in the next section.

1.3 Microfluidic Chips for the Study of Heterogeneous Cells

As an alternative method which can replace conventional techniques, microfluidic devices have been developed by many research groups. There are advantages over the conventional methods due to its small scale: small necessary quantities of samples and reagents, low cost, short analysis time, and small form factors applicable to portable analytical devices [13]. Furthermore, introducing a soft lithography technique using polydimethylsiloxane (PDMS) as a microfluidic material facilitates even more extensive exploratory research [14]. A soft elastomer PDMS has advantages over silicon/glass such as: 1) easy-to-fabricate because all the microfluidic components can be replicated from mold structure by pouring liquid phase PDMS, 2) inexpensive since the mold can be used multiple times and low cost of material itself, 3) flexibility which enables to integrate pneumatic valves, 4) optical transparency which easily accommodate florescent assays, and 5) compatibility to biological studies (permeable to gases and non-toxic to cells). In addition, a lot of individual microfluidic components have been developed in the last decade, which can be easily integrated for multiple functions in a single chip.
example, pneumatic valves [15, 16] and peristaltic pumps [17] for selective control of reagents, mixers [18-20] and concentration generators [21, 22] for providing various reagent concentrations, cell capturing structures [23-26] for accurate cell positioning, etc.

Recently, single cell assay has drawn a high attention in microfluidic chip development [4, 23, 27-29]. This is due to the imminent need in cell assay research. The heterogeneity nature of cells requires single cell assay in a controlled microenvironment and the microfluidic chip can provide an ideal platform for this. Several microfluidic devices capable of single cell assay have been reported, which provides a enabling technology to further explore of cell behaviors in detail. They made the single cell assay possible by positioning individual single cells into pre-determined locations at a single cell resolution using either active or passive manipulation.

One of the active single-cell capture schemes using external forces to manipulate and control a position of cells inside microfluidic chips is dielectrophoresis [30, 31] or optical image driven dielectrophoresis [32]. Dielectrophoresis (DEP) is a phenomenon in which a force is exerted on a uncharged dielectric particle (which can be a cell in our biological applications) as a result of polarization induced by non-uniform electric field [33-35]. The DEP method provides accurate cell manipulation and selective cell capturing; however, it needs physiological media of high conductivity to generate dielectrophoresis, limiting its potential application and requires large external equipments as well as high expertise to control.
On the other hand, passive methods using mainly either hydrodynamic force to trap single cells such as hydrodynamic weir [24, 36, 37] or gravity force in a small microwell array, are attractive in terms of its simplicity and low-expertise requirement. However, their capturing efficiency (the ratio of the number of the total captured cells to the injected cells) is relatively poor (less than 1 %) and may not be adequate for handling rare cells such as stem cells or circulating tumor cells.

Table 1.1 Comparison of current available methods for heterogeneity study

<table>
<thead>
<tr>
<th></th>
<th>Conventional methods</th>
<th>Microfluidic methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency (single cell capture)</td>
<td>Fair</td>
<td>Best</td>
</tr>
<tr>
<td>Accuracy (manipulation)</td>
<td>N/A</td>
<td>Best</td>
</tr>
<tr>
<td>Labor intensive (operation)</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Expertise (necessary for operation)</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Cost (device/equipment)</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Space for isolated cells</td>
<td>Best</td>
<td>Good</td>
</tr>
<tr>
<td>Cell tracking (e.g. Migration blocking)</td>
<td>Best</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Most importantly, these previous chips only focused on single-cell capturing but did not provide the necessary conditions for clonal culture such as cell isolation, migration blocking, and space for isolated and traceable colony growth. Analogously, other microfluidic devices have developed effective migration-blocking schemes [39-49] but not in the context of single-cell capturing and clonal colony formation. Table 1 summarized the current techniques for heterogeneity cell study.

We have designed a high-throughput microfluidic chips which solve these problems and are superior to the existing devices, allowing heterogeneous cell studies at the proper scales and accuracies and with minimum labor and cost. The proposed device can culture single cells to generate their colonies from single cells within individual microwells inhibiting cell migration. Moreover, we have also developed a device capable of sorting multiple specific target cells using a controllable local magnetic field through current-carrying wires.

1.4 Thesis Organization

Chapter 2 and 3 will be dedicated to explain the design and development of single cell clonal culture array chip using hydrodynamic force as a cell manipulation method. Chapter 2 will focus on a novel hydrodynamic guiding structure which can capture single cells automatically into pre-determined positions with a high efficiency over 80%. Its
operation principle, analysis, experimental results and high-throughput applications will be discussed in detail. In chapter 3, single cell array chip for clonal culture based upon the hydrodynamic single cell capture structure will be presented. Unique features of the device enabling clonal culture and the biological experimental results will be mainly explained. Chapter 4 will present an alternative method for heterogeneity study, magnetic manipulation for sorting different sized cells using controllable local magnetic field. Operational principle, analysis, fabrication process, and experimental results will be included.

A comprehensive summary and conclusions of the performed researches are given in chapter 5. Also potential directions for future researches will be explored.
Chapter 2.
Highly Efficient Single Cell Capture Using
Hydrodynamic Guiding Structures

We present a novel single cell capture scheme using hydrodynamic guiding structures in a microwell, with which we could load cells into microfluidic array chips automatically and precisely with a high capturing efficiency. It splits a fluid flow into two paths: a short path at the center and long de-touring flow paths in the side. The hydrodynamic resistance of the flow paths is different from each other. Due to the resistance difference, majority of the injected fluid as well as cells will take the low resist central path and accordingly cells will be captured in the capture sites located in the central path at single cell resolution. Its feasibility was successfully demonstrated by capturing microbeads (15μm in diameter which is similar to mammalian cells), showing that more than 80% of all the injected microbeads were captured in the designated capture sites. Using this highly efficient capture scheme, the required number of cells for single cell assay can be dramatically reduced, which is beneficial to the applications with scarce supply of cells such as circulating tumor cells.
2.1 Introduction

Typically all the information about cells such as proliferation, differentiation, response to external stimuli etc. was derived by averaging over relevant individuals assuming that purified cell samples are uniform; however, cells are known to be often not identical but heterogeneous [3-9, 11, 50-52]. Therefore, single-cell analysis enables a more precise understanding of differences between individual cells, which would lead to, for example, better understanding of diseases such as cancer. There is a macroscopic technique for single cell analysis, namely, a dilution method: diluting cell samples down to a concentration of a single cell per hundreds of microliter and deposit single cells in a conventional platform such as a 96 well plate. However, it is very labor-intensive, low-throughput, low single-cell loading efficiency, and poor reproducibility and is thus rarely performed. On the other hand, microfluidic devices enable high-throughput cell manipulation with high controllability even in a single cell level. A few groups have reported microfluidic chips which are providing platforms to analyze single cells by incorporating active or passive single-cell capturing schemes. Active single-cell capture scheme such as dielectrophoresis [30, 31] and optical image driven dielectrophoresis [32] provides accurate cell manipulation and selective cell capturing; however, it needs physiological media of high conductivity to generate dielectrophoresis, limiting its potential application and requires large external equipments as well as high expertise to control. On the other hand, passive methods such as hydrodynamic weir [24, 36, 37, 53] is attractive in terms of its simplicity and low-expertise requirement, but their capturing
efficiency (the ratio of the total captured cells to the injected cells) is relatively poor (less than 1%) and may not be adequate for handling rare cells such as stem cells or circulating tumor cells. Here, we present a hydrodynamic capture scheme which has high efficiency of cell capturing as well as the advantages of the passive method. This scheme can be easily applied and expanded to high-throughput microfluidic array and we can load single cells into each microwell automatically.

2.2 Principle of Operation

Figure 2.1 shows the schematic view for the proposed microwell array chip. As seen in a unit microwell, we designed a hydrodynamic guiding structure which divides the injected flow into two streams: path A through the center and path B along the sides (path B consists of two symmetric ones) around a single cell capture site in a microwell. Path A pass through the center of the microwell and it has a shorter length than path B which is along the border of the microwell. Path A has a smaller flow resistance than path B. Due to the flow resistance difference, most introduced flow (as well as injected cells), will take path A rather than path B. In addition, path A was designed to have a smaller width than the cell diameter, which enables to capture only a single cell precisely into its capture site while it attempts to pass through. Once the cell is captured, path A becomes blocked and accordingly its flow resistance becomes much larger than path B. As a result, all the remaining cells will pass through path B and will be captured in the following
microwells. This hydrodynamic passive capture structure can significantly increase cell capture efficiency and confine only single cells into each capture sites.

Figure 2.1 Schematic diagram of a unit microwell with a high single-cell capture efficiency. Due to the flow resistance between path A and path B, injected beads/cells will take path A and be captured because the width of path A is smaller than the diameter of the beads/cells. The rest of the incoming beads/cells will pass through path B (the width of path B is larger than the diameter of the beads/cells)
2.3 Design and Fabrication

The capturing efficiency depends on the flow resistance difference between path A and path B which is inversely proportional to the volumetric flow rate. We analyzed the volumetric flow rate ratio of path A to path B using a simplified equation given by[54]:

\[
\frac{Q_A}{Q_B} = \left( \frac{C_B(\alpha_B)}{C_A(\alpha_A)} \right) \left( \frac{L_B}{L_A} \right) \left( \frac{W_B + H_B}{W_A + H_A} \right)^2 \left( \frac{W_A \cdot H_A}{W_B \cdot H_B} \right)^3
\]

(1)

where \( Q \) is the volumetric flow rate, \( C \) is the product of Darcy friction factor \( (f) \) and aspect ratio \( (\alpha) \), the aspect ratio is defined as either height/width or width/height such that \( 0 \leq \alpha \leq 1 \), \( L \) is the channel length, \( W \) is the channel width, \( H \) is the channel height, and the subscripts \( A \) and \( B \) denote path A and path B. To simplify the equation, a resistance of the wide flow channel next to the path A is ignored, which is 10 times less than the flow resistance of path A. The probability with which an injected cell goes through path A, namely the probability that an injected cell can be captured in a capture site, can be defined as capturing efficiency. Assuming the initial position of injected cells across the channel width is uniformly distributed, the probability can be obtained as the ratio of the flow amount through path A to the total injected flow amount \( (Q_A / (Q_A + Q_B)) \). From the equation (1), there are two main design-parameters determining the capturing efficiency: one is a ratio of channel lengths \( (L_B/L_A) \) and the other is a ratio of channel width \( (W_B/W_A) \). Larger channel length ratio and smaller channel width ratio are desired in order to get higher capturing efficiency. However, there are limitations to determine these dimension,
depending upon the size of targeting cells. First, the width of path A ($W_A$) should be designed to be smaller than the diameter of the targeting cells because path A works as a flow passageway as well as a capturing structure. Second, the width of the path B ($W_A$), and the channel height ($H_A$ and $H_B$) should be larger than the diameter of the cells to make cells flow fluently without any possible clogging problems. Considering these limitations (a diameter of our targeting cells, a prostate cancer cell line (PC3), ranges from 12µm to 22µm), we have determined the dimension of the proposed hydrodynamic guiding structure. We maintained the width ratio as small as possible to get a higher capturing efficiency. On the other hand, we test the four different length ratios (type I ~ IV: ratios are 1:20, 1:40, 1:80, and 1:160, respectively) to study the dependence of the capturing efficiency on the length ratio, as listed in table 2.1.

**Table 2.1 Dimension of path A and path B (unit is µm).**

<table>
<thead>
<tr>
<th></th>
<th>Length</th>
<th>Width</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path A</td>
<td>10</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Path B</td>
<td>200 (I), 400 (II), 800 (III), 1600 (IV)</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure 2.2 shows the fabrication process. The 25 µm thick-mold was patterned on a bare silicon substrate using epoxy-based negative photoresist, SU8-10 (Microchem, MA). Mold fabrication procedure was straightforward. In order to promote the adhesion
of SU-8 on silicon bare surface, the entire native oxide layer was removed by dipping the silicon bare wafer into 10% Hydrofluoric acid solution for 3 seconds, since the adhesion of SU-8 on oxide layer is poor. After removing the native oxide layer, SU8-10 was spun at 1200 rpm for 30 seconds (with a ramping speed of 100 rpm/second), then soft-baked at 65 °C and 95 °C on hotplate for 2 minutes and 5 minutes, respectively. Using MABA6 aligner (Karl Zeiss, Germany), the soft-baked SU8 layer was exposed for 10 seconds, followed by post-exposure-baked at 65 °C and 95 °C on hotplate for 1 minute and 2
minutes, respectively. Then, they were developed with SU-8 developer for 1 minute. PDMS (Sylgard 184, Dow Corning, MI) was casted on the SU-8 patterned mold and cured on a 90°C hotplate for 3 hours. Then, the cured PDMS was cut into pieces and detached. Prior to this process, we coated Trichlorosilane monolayer (Sigma Aldrich, MO) on the mold so as to detach the cured PDMS easily. Holes for inlets and outlets were formed by a custom-made punch (2mm in diameter). After treating the bonding surface of PDMS pieces and a glass piece with O₂ plasma (600 mTorr, O₂ 100 sccm, 100 W, 10 second), the PDMS was bonded to the glass slide on a 65 °C hotplate for 2 hours. The microwell size is 400 μm in diameter and the flow channel is 150 μm in width and 25 μm in height. The fabricated device was shown in Figure 2.3(a). The four difference length ratio was realized as shown in Figure 2.3(b).

Figure 2.3 (a) Photograph of the fabricated microwell array chip, (b) Tested unit microwell structures; 4 different length ratios of path A and path B
2.4 Experiment

To verify the hydrodynamic capturing scheme, we performed a test using microbeads (15 μm in diameter, micromer-M, Micromod, Germany) to imitate movement of PC3 cells inside the microwell array chip. A syringe pump (KDS101, KD scientific, MA) was connected through tygon tubes into an inlet port of the microwell array chip to generate continuous and constant fluid flow. The movement of the injected microbeads was monitored under a stereotype microscope (SMA1500, Nikon, Japan). To prevent the injected microbeads from being stuck on the bottom surface during operation, we suspended the microbeads in 22% sucrose solution which has the same density as the microbeads. We also added a surfactant, 1% Triton X-100 (Sigma-Aldrich, MO) to inhibit microbeads’ unspecific binding to the inner microchannel walls. Suspended microbeads with a concentration of $1.8 \times 10^5$ beads/mL were injected from the inlet at a constant flow rate of 1.5 μL/min. It took less than 1 minute for the microbeads to fill all the capturing sites with single microbeads. As shown Figure 2.4, the microbead moves toward the central path A rather than the de-touring path B due to the flow resistance difference: After a single microbead is trapped in the capture site, the following microbeads go through path B and are trapped in the next microwells (the capturing movie clip can be found in [55]). A capturing efficiency was obtained by counting all the injected microbeads through one column (8 successive microwells) until all the capture sites were occupied; the capturing efficiency was calculated as 41% (type I), 58% (type II), 75% (type III), and 82% (type IV), respectively and is in agreement with the analysis.
based upon the equation (1) as shown in Figure 2.5. This is a significant improvement (two orders of magnitude) compared to other passive hydrodynamic weir single-cell capturing schemes, which were inferred to have less than 1% in capturing efficiency based upon their reported loading condition and result [36, 53]. As shown in Figure 2.5, we can expect to increase the capturing efficiency even more by employing a higher length ratio between path A and B, though it may require larger area for an individual microwell.

Figure 2.4 Capturing sequence of the injected microbeads: (a) injected microbeads move towards path A and is captured in the center of microwell, (b) the following microbeads take the detouring path B and can be captured following microwells. Red arrows indicate the direction of microbeads. Scaling bar represents 100 µm.
We also captured and cultured live PC3 cells. Prior to cell loading, the chip was prepared by flowing 0.01% collagen for more than 1 hour in 37 °C incubator so as to promote cell adhesion on the inner surface. Suspension of PC3 cells (~ 5×10^4 cells/mL) in cell culture media (RPMI (culture media: acronym of Roswell Park Memorial Institute, Sigma Aldrich, MO) + 10% FBS (Fetal Bovine Serum, Sigma Aldrich, MO)) was

Figure 2.5 Capturing efficiency as a function of ratio of channel length (L_B/L_A) (for calculation, we used W_A=10 µm, L_A=10 µm, and H_A= H_B= 25 µm). Triangular dots represent the average of the capturing efficiency in the experiments with 15 µm microbeads in 8 cascading microwells (error bar indicates a standard deviation, N=8).
Figure 2.6 Photograph of captured single green fluorescence activated PC3 cells in microwell array. Only one PC3 cell was captured at each capturing site in the microwell. In the image, 39 microwells are occupied with single cells out of 42 microwells, only three microwells were not occupied.

prepared and injected through the inlet. Less than 3 μL of cell suspension media was enough to load single cells to the array. The capturing result was shown in Figure 2.6. Single green dot in each microwell represent a PC3 cell (for clear optical observation, we used green fluorescence activated PC3 cells). Over 90% of microwells were occupied only one cell. After capturing cells, we cultured them for 3 days by keeping the microwell array chip in an incubator (37 ºC, 5% CO₂, and 90 % humidity). In order to supply nutrients to the cells, we replaced the old culture media with new fresh one once a day. Figure 2.7 shows the 3 days culture result, where we can see that a captured single PC3 cell grew and became 3 cells.
We also demonstrate that the hydrodynamic guiding structure can be modified to capture a specific number of cells in a microwell as well as control an initial distance between the captured cells. As shown in Figure 2.8, instead of having one capture site in the path A, we implemented three of them and tested their feasibility with myoblast stem cells as shown in Figure 2.8. With this modification, we could successfully capture three cells and have demonstrated that the number of captured cell numbers in each microwell can be controlled as well as the initial distance between the captured cells.

![Figure 2.7 Single cell clonal outgrowth; photographs were taken in an identical microwell at day 0 and day 3.](image)

![Figure 2.8 Photograph of three captured myoblast cells in a microwell. The number of cells and distance between the captured cells can be controlled by optimizing the hydrodynamic guiding structure.](image)
2.5 Hydrodynamic Guiding Structure for High-Throughput Assay Applications

2.5.1 Operation Principle

We explored the expandability of the hydrodynamic guiding structure for high-throughput application in order to load single cells automatically with a high capturing efficiency as well as introduce multiple reagents simultaneously to obtain a huge volume.

Figure 2.9 Schematic diagram of a microwell array. Cells are loaded vertically in columns and reagents are introduced to the microwells in parallel along row channels by selectively open/close valves. In addition, each microwells can be completely isolated from the rest of microwells by closing all the valves.
of experimental data. Figure 2.9 shows the overall schematic view of the proposed microfluidic chip. We integrated valves in-between every microwell, which can open and close the fluidic channels selectively. We load cells vertically in column direction, inject reagents horizontally in row direction, and isolate cells in each microwell by closing all the integrated microvalves.

2.5.2 Pneumatic Valve Design

We adapted pneumatic valves to the high-throughput microwell array chip. We reviewed the current available pneumatic valves for optimal design in our microwell chips [15, 56-58]. Figure 2.10 shows two types of most commonly used pneumatic valves [58]: one is a push-down valve and the other is a push-up valve. These pneumatic valves consist of two flexible PDMS layers on a rigid glass substrate, forming fluidic and actuation channels. In both valves, when high pneumatic pressure is applied to the actuation channel, its volume expands and the membrane between the actuation and fluidic channels is deflected and blocks the fluidic channel. To make a complete sealing of the valves, the fluidic channels should be designed to be in a rounded shape as shown in Figure 2.10. In a push-down valve, when the membrane pushes down to seal off the lower fluidic channel, the thickness of the membrane varies from the edge of the channel to the middle so that it requires higher pressure to make enough deflection around the edge region of the fluidic channel for complete sealing. Despite the requirement of high
pressure actuation, the push down valve scheme is most widely adapted because it allows to integrate other components on the bottom of the fluidic channel, such as electrodes, to incorporate other useful functions. On the other hand, in a push-up valve, where the membrane deflects upwards to seal off the upper fluid channel, the thickness of the membrane is uniform; therefore, it relatively requires less actuation pressure. However, it is hard to make patterns on the bottom surface of the fluidic channel. Since our scheme does not require any pattern on the bottom surface, we have chosen a push-up valve in our application for lower pneumatic actuation pressure.

![Figure 2.10 Two types of pneumatic valves: (a) Push-down valve and (b) push-up valve. High pneumatic pressure deflects a membrane to seal off the fluidic channels.](image)

### 2.5.3 Fabrication of High-Throughput Array Chip

The first prototype, a 8×8 microwell array chip, was designed and fabricated by integrating pneumatic valves, which is capable of 1) loading single cells with high
efficiency using the hydrodynamic guiding structure, 2) injecting four different reagents simultaneously to microwells in every tow rows without cross contamination by selectively closing the integrated pneumatic valves, and 3) isolating each microwell by closing all the pneumatic valves. Figure 2.11 shows fabrication procedure, which is composed of largely three steps. The first step is to make the microfluidic channels, the second step to fabricate the actuation channels and the final step to bond the fluidic and actuation layers on a glass substrate.

Figure 2.11 Fabrication process procedure: two separate PDMS parts (fluidic layer and control layers) are prepared in each separate mold. Then, these two parts are bonded to a glass substrate.
2.5.3.1 Mold Fabrication of Fluidic Channels

Conventional photoresist-based photolithographic techniques are used to produce the mold on which the PDMS layers are casted. This fluidic channel layer includes all the microchannels, microwells and valves. Only the valve parts were patterned and reflowed with positive photoresist (AZ9260) to generate a rounded shape. All other microchannels and microwells (including hydrodynamic guiding structure in the microwells) were patterned with an epoxy-type negative photoresist, SU8-10, which is represented by rectangular shape in Figure 2.11.

Fabrication process for the flow channels starts by making by removing the native oxide from a 4-inch silicon wafer by dipping it into hydrofluoric acid (10% in D.I. water) and treating the wafer with hexamethyldisilazane (HMDS; Sigma Aldrich) vapor for 1 min at room temperature to promote the adhesion of the photoresist. Then, AZ9260 positive photoresist is spin-coated on the wafer at 2000rpm for 2sec, to make a final thickness of ~ 32 µm. The coated AZ9260 is rehydrated for more than 3 hours at room temperature and baked on a hot plate at 85 °C for 15 minutes. The AZ photoresist is exposed to a UV light (100 mW, 180 seconds, 365 nm center wavelength, on a Karl Suss MA6 contact aligner) through a mask containing the design of the flow channels. The mask is transferred to a chrome photomask from a high resolution transparency mask printed at 20,000 dpi (CAD Art Services, Inc. OR) from an AutoCAD (AutoDesk Inc., CA) design file. After exposure, the photoresist is developed in diluted a AZ400K developer (1 AZ400K : 3.5 D.I water; Microchemicals Inc., Germany) for 7 ~ 10 minutes.
The patterned AZ photoresist is reflowed and hardbaked on a hot plate by ramping the temperature from initial 65 °C to 180°C at 10 °C/5 minutes, to obtain a rounded pattern, with a peak height of 38 ~ 42 µm. After refloowing and hardbaking, the wafer is exposed to O₂ plasma (600 mTorr, O₂ 100 sccm, 100 W) for 5 minutes to remove the photoresist residues and the native oxide layer is removed by dipping it into 10% HF acid (in D.I water) for 5 seconds to promote adhesion of the following SU8 photoresist process. Subsequently, a 38 ~ 42 µm thick layer of SU8-50 negative photoresist is spin-coated at 3000rpm for 30seconds on the wafer and baked on a hot plate at 65 °C for 5 minutes and 95 °C for 20 minutes. The SU8 is exposed to a UV light (100 mW, 30 seconds, same aligner as before, using the mask for fluidic channels), post-exposure-baked on a hot plate at 65 °C for 1 minute and 95 °C for 10 minutes, and developed in a SU8 developer (Microchem) for 2 minutes. This process generates all the fluidic structures including microchannels, microwells, valves, and hydrodynamic guiding structures in each microwell. Figure 2.12 shows the photographs taken at each steps in the process of AZ9260 patterning: refloowing, and final mold generation.

2.5.3.2 Mold Fabrication for Actuation Channels

Actuation channels are formed by patterning all the valve parts and their connections using only one layer of SU8. This mold is made by removing the native oxide of a starting silicon wafer by dipping it in 10% HF (in D.I. water) to promote the adhesion of SU8. Prior to making a mold structure for the actuation channels, a blanket
layer by SU8 was formed on the entire surface because it can provide uniform wetting properties to the PDMS, which ensures a uniform PDMS spin-coating thickness. For this blanket layer, SU8-2 was spin-coated at 2000 rpm for 30 seconds (ramping speed: 100 rpm/second) targeting the final thickness of 3 µm and soft-baked at 65 °C for 1 minute and 95 °C for 3 minutes. The coated SU8 was exposed by a UV light (100 mW, 10 seconds) without a mask (blank exposure) and then post-exposure-baked at 65 °C for 1 minute and 95 °C for 1 minute, respectively. On the top of the blanket layer, SU8-10 was

Figure 2.12 Mold fabrication process for a fluidic layer: (a) AZ9260 pattern, (b) after reflow process, and (c) SU8 pattern on the reflowed AZ9260.
spin-coated at 1000rpm for 30 seconds (ramping speed: 100 rpm/second) targeting 30 µm in thickness and soft-baked at 65 °C for 2 minute and 95 °C for 7 minutes, respectively. The coated SU8 was exposed by a UV light (100 mW, 15 seconds) with a mask for the actuation channels and then post-exposure-baked at 65 °C for 1 minute and 95 °C for 4 minutes, respectively. Finally, the SU8 was developed by a SU8 developer for 2 minutes. This process generates all the actuation channels including valves and their connection channels.

2.5.3.3 PDMS Replication from Molds

A microwell array chip consists of two PDMS layers: one with the microchannels where the liquid flows (fluidic layer) and the other with the dead-ended channels which is used to control the valve operation (actuation layer). As discussed earlier, the push-up valve is adapted in this design, so that the fluidic channel corresponds to the upper layer and the actuation channel to the lower layer.

Before replicating the pattern to PDMS, the mold was prepared by coating trichlorosilane monolayer on the mold surfaces to facilitate the PDMS detachment process by preventing the bonding between silicon surface and PDMS. The fabricated mold of the fluidic channel was replicated by pouring 30 g of uncured PDMS (20 elastomer : 1 cross-linker) aiming for 3 mm thickness. For the membrane, uncured PDMS (5 elastomer : 1 cross-linker) was spin-coated at 1700 rpm for 30 seconds, aiming for 16 µm of the PDMS membrane thickness, which serves as the membrane between actuation
and fluidic channels. Since its thickness determines the pressure for closing the valves, we should accurately control the PDMS thickness by adjusting the spin-coating speed. After casting the uncured PDMS on the both molds, we cured them on a leveled surface for more than 3 days at room temperature, which prevents the shrinkage of PDMS, which generally happens during curing procedure.

After curing, the thick fluidic layer was gently peeled off from its mold. The bottom surface of the thick fluidic part and the top surface of the thin actuation part, which is still on the mold, were treated by O2 plasma (600 mTorr, O2 100 sccm, 100 W) for 10 seconds. Then, these two parts were aligned by MJB3 aligner (Karl Suss, Germany), and bonded and cured on a hot plate at 60 °C for 2 hours to ensure the irreversible bonding. After bonding process of two PDMS parts, it was peeled off from the actuation mold and the holes for inlets / outlets are formed by a custom-made punch (inner diameter: 1mm). These holes permit the connection of 1/16” Tygon tubing to the chip, which can fit tightly into the ports on the chips. The bottom surface of the PDMS layers and the top surface of a substrate were treated by O2 plasma (same condition as above) and they were irreversibly bonded by curing it on a hot plate at 60°C for 2 hours.

2.5.4 High-Throughput Assay Chip

Figure 2.13 shows the fabricated microchip. The microwell size is 400 μm in diameter and the flow channel is 150 μm in width and 40 μm in height. Cell suspension and reagents are injected using syringe pumps and its fluid stream is controlled by the
pneumatic valves integrated on the chip. Pneumatic pressure is applied from a highly compressed external nitrogen tank. Because PDMS is gas permeable, the nitrogen in the control channels can penetrate the PDMS membrane and generate air bubbles in the fluidic channels. In order to avoid this problem, we filled the control channels with deionized water prior to the valve operation. Thus, we could maintain the pneumatic pressure without leakage and bubble-generation. Figure 2.14 shows the picture taken when four different color dyes are injected along every two rows while each rows was isolated from each other by closing the horizontal valves at 15 psi. The result shows there is no cross-contamination and it could be maintained more than 24 hours by continuously supplying 15 psi of pressure. Figure 2.14(B) shows the photograph for microwell isolation test.

Figure 2.13 Photograph of the fabricated 8×8 microwell array for single cell assay capable of injecting four different reagents simultaneously, enabled with pneumatic valve actuation
We also extended the microwell array size even larger up to 1024 (32×32) for massively parallel application, in which we can inject 8 different reagents simultaneously to every 4 rows by selectively closing the valves as shown in Figure 2.15.

Figure 2.14 (a) Photograph of the drug injection test. Four different food dyes are injected in every two rows and each row was isolated from the rest by closing the valves to prevent cross-contamination. (b) photograph of microwell isolation test. All the valves are closed during isolation operation.
2.6 Conclusion

We have developed a hydrodynamic single cell capture scheme and successfully demonstrated that single cell capturing efficiency significantly increased over previous devices by more than two orders of magnitude. We can obtain a high capturing efficiency of 80% by adjusting the dimension of each channel. We demonstrated the massively-parallel loading of cells at single cell resolution in a short period of time (~ 2 minutes) and succeeded in culturing the captured cells for three days. This high efficient capturing scheme can be easily expanded to a larger array platform for high-throughput assays and adapted for various single cell analysis biochips. We showed the expandability of the array size up to 1024 (32 × 32) microwells and integrated pneumatic valves to control the selective introduction of reagent flows for given applications.
Chapter 3.

Single Cell Clonal Culture Array Chip

3.1 Introduction

Conventional assays generally assume that purified cell samples are uniform and that average measurements are sufficient. However, recent studies have shown that this is often not true in metabolic, proliferative and differentiation potentials and response to drug treatments [2-7, 59]. Unfortunately only some of these between-cell differences have clear markers that would allow FACS to be useful for identifying different cell subtypes. The rest are undetectable and it is almost impossible to independently separate and track not just those cells but their individual progeny, and to observe differences in proliferation rate, morphology and physiological response. Macroscopic techniques for separation and isolation do exist (essentially diluting cell samples down to a single cell per plate/well) but are very labor-intensive, low-throughput, less physiologically accurate,
and are thus rarely performed.

Recently several groups have reported microfluidic devices pursuing single cell analysis to overcome the demerits of the dilution method [23-25, 28, 32, 54, 60-62]. They successfully demonstrated that microfluidic chips can easily load single cells into their predetermined positions in a high-throughput manner; however, their schemes do not allow the captured cells to grow over time due to 1) lack of space for their growth and proliferation, 2) inability to isolate and track cells from the original mother cells, and 3) low capturing efficiency at single cell resolution (less than 1%).

We have demonstrated a working prototype capable of automatically capturing and isolating single cells into an 8×8 array of microchambers. Our prototype allowed us to track each single-cell progeny for differences in proliferation and morphology over several days, and was able to successfully identify three cell subtypes in a well-known cell line (PC3) which were recently discovered [1]. We successfully measured differences in drug responsiveness and correlated them to cell subtypes, showing clear and repeatable differences in drug response among them. We have also demonstrated our ability to selectively harvest cells from specific microchambers to perform further probing a cell subtype of interest.

### 3.2 Design and Fabrication

We designed and fabricated a novel single cell clonal culture chip which
overcome the limitations of conventional single cell methods (e.g. serial dilution method [1]) and current microfluidic chips developed for single cell analysis [24, 25, 30-32, 36, 63] by employing 1) a hydrodynamic guiding structure to capture single cells very effectively and efficiently, 2) a migration blocking structure for effective isolating individual clones and tracking the descendents from the captured single cells, and 3) a simple scheme for loading cells or media/drugs by perfusion using a gravity flow to realize ease-to-use for point-of-care applications. We aimed to develop a low-cost and portable chip and it was realized such that its size is less than one inch square at a cost of a few dollars in materials to produce, and require no external equipments except for microscopes and incubators.

### 3.2.1 Single Cell Capture

We developed a novel hydrodynamic guiding structure incorporated in a microwell array for highly efficient cell capturing. We can capture more than 60% cells from all the injected cells and fill more than 80% of microwells at single cell resolution. This scheme was explained in detail in chapter 2.

Here we have modified the structure for the optimal capturing of prostate cancer cells (PC3) by avoiding 1) cell clogging inside the microchannels and 2) multiple cells capturing. Because a size of target (PC3) cells is not uniform but distributed with large a deviation from its average (12 ~ 30 µm in a diameter; different from uniformly sized
microbeads which were used to evaluate our hydrodynamic guiding structure in chapter 2), we need to increase the channel height more than 30 µm in order to prohibit larger cells from unspecific trapping or clogging in undesired locations (our test chip for capturing 15 µm microbeads has 25 µm of channel height). Cell clogging can lower the cell capturing efficiency as well as block the fluid stream and supply of culture media/reagents. Thus, we increase the microchannel height to 40 µm so that the injected cells can flow freely without any clogging; however, the increased channel height can

Figure 3.1 Modified single cell capturing structures: (a) previous design for capturing uniformly-sized microbeads (15 µm in a diameter), (b) optimized capturing structure which can capture PC3 cells without cell clogging and multiple cell loading.
cause multiple cells captured in a capture site because the captured cells cannot effectively block the fluid flow. In order to address this multiple cell loading issue, we modified our capturing site as shown in Figure 3.1. By using two SU8 mold layers, we could restrict the height of the capture sites to 20 µm, adequate for single PC3 cells, while keeping the rest of microchannels and microchamber height to be 40 µm. With this modified structure, we could capture single cells without any clogging at the cost of lowering the capturing efficiency around 60% from 80%.

### 3.2.2 Migration Blocking

In order to study cellular heterogeneity, we need to monitor behaviors of single cells over time. Because during the culture cells tend to proliferate and move around, it becomes very difficult to keep tracking and confining them inside the microwells that the cells are initially loaded. Figure 3.2 shows the cell migration in a microwell observed from our test experiment. As shown in the figure, the number of cells increased and the cells migrated into the next microwells, so that eventually it becomes very hard to tell which cells are clonally cultured from the original single cell. Therefore, it is required to block the captured cells from being migrated to the neighboring microwells. Selective surface modification has been developed to confine the cells inside a predefined area. By incorporating this method in our hydrodynamic guiding structure, we could successfully culture the clones from the captured single cell only within the microwell without cross-
contamination. Using this scheme, we could easily track and monitor cells over their progeny.

![Cell migration](image)

**Figure 3.2** Observation of cellular migration and proliferation from a captured single PC3 cell in an identical microwell over 5 days. Their active migration makes impossible to confine the cells in the same microwell that they were initially captured.

In order to find out the most appropriate cell migration blocking method for PC3 cells, we tested several previously proven methods for other cells [39-43, 64-68] including 1) multiple groove patterns on a glass surface with 2, 4, 8 µm line and space patterns in a trench depth of 2 and 4 µm, 2) physical walls using SU8 on a glass surface with a height of 3.5 µm and 10 µm and 3) selective surface patterns using chemicals (Pluronic copolymer coating on SU8 walls). The first two methods (physical grooves and walls) could not prohibit PC3 cells from migrating over the patterns (Figure 3.3), but the selective surface modification with Pluronic copolymer, F108, could effectively stop their migration on the edges of patterns (Figure 3.4).
Figure 3.3 Test results for migration blocking with groove patterns (with line/space of 2µm/2µm, 4µm/4µm, 8µm/8µm and SU8 walls. Neither of them could prohibit cells from being migrated over the patterns
We chose the selective costing of F108 for cell migration blocking. This has adequate properties such as bio-compatibility and antifouling for our applications [39, 40, 68]. The triblock copolymers are composed of hydrophobic poly propylene oxide (PPO) domain at the center and two flexible hydrophilic poly ethylene oxide (PEO) chains, which are attached at each end of the PPO domain as shown in Figure 3.5. Antifouling properties of Pluronic copolymers come from the fact that the Pluronic copolymer’s
hydrophobic PPO binds strongly to hydrophobic surface via hydrophobic-hydrophobic interactions, while leaving the flexible hydrophilic PEO chains free in solution to repel proteins and other adsorbents from the surface by steric repulsion [69]. Therefore, when the surface of substrate is hydrophobic, Pluronic copolymer can be coated and the surface becomes protein-repelling and cells would not sit on. On the other hand, when a surface is hydrophilic, Pluronic copolymer cannot be strongly bonded to the surface and it remains as protein-adsorbing, which cells prefer to adhere to.

![Chemical structure of pluronic triblock copolymer, F108](image)

**Figure 3.5 Chemical structure of pluronic triblock copolymer, F108**

We designed our device to utilize this property by selectively generating a hydrophobic region with SU8 on a hydrophilic glass substrate. Figure 3.6 shows our unit microwell structure, where the hydrodynamic guiding structure for efficient cell capturing was incorporated with the cell migration blocking structure of SU8 patterns on a glass substrate along the boundary of microwell.

We successfully demonstrated that the selectively coated Pluronic copolymer works as an effective cell migration barrier by culturing PC3 cells in the unit microwells as shown in Figure 3.7.
Figure 3.6 Schematic diagram of unit microwell which includes 1) cell migration blocking patterns and 2) hydrodynamic single cell capture structures.

Figure 3.7 Photograph of cultured PC3 cells in a microwell for 4 days. Cell migration was effectively blocked by selective coating of triblock Pluronic copolymer on the SU8 wall.
3.2.3 Gravity Flow

One of the advantages of microfluidic devices over conventional methods is that it needs relatively very small amount of fluid (cell culture media, reagents, etc.). But due to the low volume, the stagnant fluid in microwells can become easily deprived of ingredients or full of cells’ waste, thus periodical or continuous replacement of the fluid is required to maintain the constant concentration of all the ingredients such as growth factors to maintain the cell viability over time. Otherwise, the altered concentration may lead to incorrect, imprecise and harder to replicate results.

Typically this fluid replacement was performed by using 1) syringe pumps or 2) peristaltic pumps using three successively integrated pneumatic valves [17, 56] or placing Braille actuators underneath the microfluidic devices [70]. Syringe pump injection, the most common method, is very beneficial since it can supply a continuous fluid flow at a controllable flow rate by pressurizing constantly the syringes filled with reagents. But it needs forking tubes tightly to microfluidic chips in order to flow the fluid from syringe pumps to the chips. This causes several disadvantages such as air-trapping in the tubes, and debris generation while connecting tubes in PDMS-based devices. On the other hand, peristaltic pumps use the actuation of more than three successive valves pneumatically or with external Braille actuators. It can control flow very well by adjusting the frequency of valve operations. But high pressure can cause leakage problem and physical actuation can wear the actuating membranes. Active fluid delivery methods are good at controlling the precise flow rate but they should be connected to either externally actuators (syringe
pumps or Braille actuators) or high pressure air, which limits the portability of the microfluidic devices.

We utilized the simplest and easiest way to generate a flow using gravity. Instead of connecting to external actuators, we make two big holes (reservoirs) for inlet and outlet and fill the liquid levels in the two reservoirs different to generate a continuous gravity flow from the reservoir with a higher liquid level to the one with a lower level. Its flow rate is also controllable by adjusting our liquid level or hydrodynamic resistance of the microchannels. Most of all, gravity flow makes our microfluidic devices “portable” because it does not require any external components for actuation. It is very important for microfluidic devices to be portable especially for cell assay applications because we need to move them frequently from an incubator to a microscope and bio-benches back and forth.

3.2.4 Fabrication

Figure 3.8 shows a fabrication procedure for the single cell clonal culture chip. It is composed of 3 major steps: 1) PDMS microchannel fabrication, 2) glass substrate fabrication, and 3) bonding between PDMS and glass substrate.

As explained in Chapter 3.2.1, the single cell capture site has a half height (20 µm) of other main microchannel and microwells (40 µm). In order to realize this structure, we used two layers of SU8 mold. The first one has 20 µm in thickness and defines the
capture site. The second layer has 40 µm in thickness and is composed of all other microfluidic parts. As the first step, the silicon wafer was prepared by removing native oxide on silicon, which was performed by dipping the wafer in HF solution (diluted 1:1 in deionized water) for 5 seconds and rinsed completely with deionized water for more than 3 minutes. The possible moisture on the silicon wafer was evaporated on a hotplate of 120 °C for 2 minutes. On the prepared silicon wafer, SU8-10 was spin-coated at 2000 rpm for 30sec targeting 20 µm in thickness (ramping rate of spinning speed was 500 rpm / second) and soft-baked in two steps, at 65 °C for 1 minute and at 95 °C for 3 minutes respectively. After cooling down the baked photoresist at room temperature for more than 1 minute, we exposed it with MABA6 aligner. The photoresist was post-exposure-baked in two steps, at 65 °C for 1 minute and at 95 °C for 2 minutes consecutively. Then it was developed in a SU8 developer for 1.5 minutes and rinsed with isopropyl alcohol. On the top of the single cell capture mold, we generated the pattern for microchannels and microwells. We spin-coated SU8-25 at 2000 rpm for 30 seconds (targeting thickness is 40 µm and the ramping rate was 500 rpm / second). After spin-coating, we planarized it by placing the wafer on the top of flat surface for more than 30 minutes. Solvent in the coated SU8 was evaporated by two steps of soft-baking, at 65 °C for 2 minute and at 95 °C for 6 minutes. By exposing a UV light for 12 sec at 20 mW, we could activate photosensitizer in SU8 and the exposed photoresist was cross-linked during the two steps of post-exposure-baking, at 65 °C for 1 minute and at 95 °C for 3 minutes. Then, the unexposed area was developed by SU8 developer for around 2 minutes and rinsed with isopropyl alcohol. The fabricated SU8 mold was replicated with PDMS. PDMS was
prepared by mixing the component A and B with 10:1 ratio and removing all the air bubbles which were trapped during the mixing process by evacuating in a desiccator for 2 hours. As a separate process, we coated SAM (Self-assembled monolayer) of trichlorosilane (1H-1H-2H-2H) on the SU8 mold, which was performed by evaporating trichlorosilane in a small chamber to the mold on a hotplate at 140°C for more than 1 hour. We poured the prepared PDMS to the SAM-coated mold and cured it on a hotplate at 95°C for 3 hours. After curing, we cut it into pieces using a razor blade and made holes for reservoirs using a punch for biopsy (with an inner diameter of 6 mm).

Figure 3.8 Fabrication procedures for single cell clonal culture chip comprising of 3 main steps: 1) PDMS microchannels and microwell array, 2) substrate preparation for F108 selective coating by SU8 patterning and 3) bonding between prepared PDMS and glass substrate.
The substrate was prepared by a hydrophobic SU8 patterns on hydrophilic glass substrate. We used the glass wafer in order to observe cells under an inverted microscope as well as to provide hydrophilic surface for selective coating of Pluronic copolymer F108. Because the adhesion of SU8 on glass substrate is very weak, we treated the glass wafer in O₂ plasma (250 mTorr, 80 W) for 5 minutes to make rough glass surface and also removed all the possible moisture by putting it on a 150°C hotplate for more than 30 minutes. On the prepared glass wafer, we spin-coated SU8-5 at 5000 rpm for 30 seconds targeting 3.5 µm and it was soft-baked at 65°C for 1 minute and at 95°C for 2 minutes. Then it was exposed for 3 seconds at 20 mW using a MABA6 aligner. After post-exposure-bake, at 65°C for 1 minute and at 95°C for 1 minute, we developed it using SU8-developer and rinsed with isopropyl alcohol. The patterned wafer was cut into pieces using a dicing saw machine.

Figure 3.9 Fabricated chip for single cell clonal culture.
Two prepared pieces, the PDMS microchannel part and the SU8 patterned glass part, were bonded together. We treated O$_2$ plasma (250 mTorr, 80 W) only on the PDMS pieces for 60 seconds but did not treat the glass pieces because it may alter the hydrophobic surface of SU8 to hydrophilic. The two pieces were aligned and bonded using a MJB-3 aligner. The bonding was reinforced by baking on a hotplate at 80 °C for 1 hour. Figure 3.9 shows the fabricated device.

3.3 Biological Experiment

In order to show feasibility of our fabricated single cell clonal culture chip for cellular heterogeneity study, we loaded and cultured a well-known cell line (PC3 cells; human prostate cancer cells) which is recently discovered to have three different cell subtypes [1, 71]. Our chip allowed us to track each single-cell progeny for differences in proliferation and morphology over several days, which other current state-of-the-art microfluidic chips cannot provide. Using this chip, we could identify three different subtypes of PC3 cells, which are holo-, mero-, and paraclones, and observe differences in drug responsiveness. We also successfully demonstrated our capability to selectively harvest cells by using a detachable PDMS lid and local trypsinization.
3.3.1 Clonal Culture from Single Cells

We added 100 µL of PC3 cell suspension made of $5 \times 10^4$ cells/mL into the inlet of the conditioned chip (The preparation steps are described in detail in section 3.3.1.2 in this chapter). Gravity flow from the pressure difference between the inlet and outlet makes the cell suspended solution flow through the microfluidic channels. While they flow through the microchannels, hydrodynamic guiding patterns integrated in each

![Figure 3.10 Three different types of subclone outgrowth from PC3. The left sides are the photographs from Petri-dish [1] and right two columns are cells cultured in the fabricated microchip](image)

Figure 3.10 Three different types of subclone outgrowth from PC3. The left sides are the photographs from Petri-dish [1] and right two columns are cells cultured in the fabricated microchip
microwell enable automatic single cell capturing with a high capturing efficiency (~60%). After capturing single cells, we emptied the inlet and refilled it with fresh culture media, then cultured them by putting it in a 37 °C incubator. Culture media from the inlet could generate a continuous gravity flow for one day; therefore, we replaced the media in the inlet once a day.

Figure 3.10 shows the photographs taken one hour and 4 days after cell loading. We could observe three different clones cultured out of a PC3 cell line, holo-, mero-, and paraclones, based upon their morphology and proliferation rate. Cells in holoclones were small and proliferated fast. On the other hand, cells in the paraclones occupied a larger area and seldom proliferated, and cells in meroclones showed medium size and

![Cell population increase over days in each type of PC3 clones](image)

*Figure 3.11 Cell population increase over days in each type of PC3 clones*
intermediate proliferation rate, which is exactly matched with single culture result from a conventional dilution method [1]. Cell population increase over time in each phenotype was plotted in Figure 3.11. The average doubling time was 1.7 days (a time for the number of cells to be doubled) in the microfluidic chip which is comparable to the result of 1.5 days from conventional Petri-dish culture. This indicates our in microfluidic chips can provide good micro-environment for cell culturing.

3.3.1.1 Chip Preparation Procedure for Cell Loading

Prior to cell loading, we prepared the chip to an optimal condition for cell culturing. As a first step, we sterilized our chips by exposing UV light for 5 minutes in a biobench. Then we evacuated the chips in a dessicator for 30 minutes to remove air which may be trapped in pores in PDMS. Immediately after ventilation, we added 100 µL of 1 w% F108 (in deionized water, Pluronic copolymer for migration blocking) to the inlet and aspirate from the outlet using a pipette bulb to initiate the gravity flow. Since the pores in PDMS were evacuated, the solution can be introduced without generating any air bubbles inside the microchannels (Air bubble trapping is a typical problem in PDMS-based microfluidic devices). After coating the hydrophobic surfaces of SU8 on the bottom surface and the PDMS microchannel walls with F108 for 10 minutes, we washed it by flowing deionized water for 5 minutes (removed all the liquid from inlet and outlet, then added 100 µL of deionized water into the inlet). Then, the glass surface in the
microchannels was coated by 0.01% collagen to promote the adhesion of cells only on the glass surface by adding 100 µL of the collagen solution instead of deionized water. Collagen can be selectively coated only on to the glass substrate due to the anti-fouling property of F108 (which was discussed in Chapter 3.2.2). After coating the collagen for 1 hour in 37 °C incubator, we washed out and replaced it with cell culture media (RPMI + 10% FBS) and placed the chip in 37 °C incubator overnight, which will soak the PDMS with the cell culture media.

3.3.1.2 PC3 Cell Preparation for Loading

We also prepared PC3 cell suspension to load cells into the prepared chips. First, we detached the cells cultured in a 35mm Petri-dish. All the culture media from the Petri-dish was aspirated and 2 mL of trypsin was added, then it was incubated in 37 °C for 5 minutes for enabling trypsin to detach the cells. After detaching, we centrifuged the cell suspension to separate the cells from trypsin, and then we replaced the trypsin with cell culture media. Lumped cells owing to the centrifuge process were dissociated into single cells by repeating pipetting around several tens of times. The cell concentration from the suspension was checked by a hemocytometer and was adjusted to around 5×10³ cells/mL by adding the culture media.
3.3.2 Chemodrug Responsiveness

*In vitro* chemotherapy test is very important to predict the effectiveness of the drug and extensive studies have been performed to discover new drugs and search for the most appropriate therapy. Because they are based upon the data averaged across the different groups of cells, rather than from their distributions, it may lead to incorrect or at least imprecise conclusions. For example, although a newly developed drug can kill 90% of prostate cancer cells, we cannot judge its effectiveness because the rest 10% may have stem-like property and they can relapse by renewing themselves. We hypothesized that each sub-phenotype can have different drug responsiveness to a given chemotherapy and it can provide a clue to discover a novel drug or develop a very effective chemotherapy.

We utilized our clonal culture chips for testing chemotherapy. First, we captured PC3 single cells in the fabricated microwell array and cultured them into their clones for several (4 ~ 6) days till identifying subclones. Then, we introduced a drug and its effectiveness was checked by monitoring viability (a ratio of the live cells out of all the cells) from each sub-phenotype. We tested a Docetaxel, one of the representative drugs for prostate cancer. We injected 40 nM/mL of Docetaxel after 5 days of culture and observed their responsiveness 1 day after the treatment (Figure 3.12). Dead or dying cells changed their morphology to a rounded shape and detached from the bottom surface. But live cells kept adhered and spread to channel surface. Based on the morphological change of the cells, we could notice the three separate subtypes of cells showed different resistance to the drug. The drug killed most of holoclones (more than 80%). On the other
hand, it could not kill the paraclones as effectively as the holoclones (less than 30%) and the meroclones shows the intermediate result (around 50%) between holo- and paraclones (Figure 3.13). This particular result might be explained by the different proliferation rate of the three subtypes because Docetaxel is effective during cell division.

Figure 3.12 Different responsiveness of three sub-phenotypes to Docetaxel. Dead/dying cells become rounded
To demonstrate that our prototype can test other drugs than Doxetaxel, we have chosen, Etoposide, which also works during the cell division process. We introduced 10 \( \mu \text{M/mL} \) of Etoposide at day 6 (after identifying subclones) and observed the effectiveness of the drug two days later. In addition, we used a well-established staining method to indicate live and dead cells; 1) live cells with Calcein AM (Invitrogen, CA), which makes them green fluorescent and 2) dead/dying cells with Annexin X (invitrogen, CA), which makes the membrane surface partially red. Figure 3.14 shows the result images. Although
interpretation was subjective since cells undergoing apoptosis can be stained by both of Calcein AM and Annexin X, the result was similar to the ones with Docetaxel; most of holoclones became dead, paraclones were not affected much, and meroclones were in between, as expected. This clearly demonstrates the ability of our device to investigate the existence of subpopulations within cancer cells and test for different response to cancer treatments.

Figure 3.14 Different responsiveness of three sub-phenotypes to Etoposide. Calcein AM and Annexin X staining methods were utilized for identifying live/dead cells.
3.3.3 Selective Harvest

The single cell clonal culture chip can identify three different subclones from PC3 cell line and isolate each clone to its initial microwell. It would be very beneficial to collect each clone out of the microfluidic chip because this selective harvest can facilitate heterogeneity study by acquiring genetic fingerprints on each separate clone. However, unfortunately there have been no techniques succeeded reported to successfully collect clones out of microfluidic chips up to date.

We demonstrated to show the feasibility of the selective harvest from our single cell clonal culture chip by a local trypsinization method. Our group has already presented a selective harvest scheme based upon microfluidic logic circuit using integrated pneumatic valves [72]. However, this scheme requires external high pressure air through complicated tube connections. Instead, we simply detach a PDMS lid and collect the desired cells by locally trypsinizing them. To enable the detachment of the PDMS lid, we did not make a permanent bonding between the PDMS part and a glass substrate. Without O₂ plasma treatment, we simply placed the PDMS piece on the glass substrate and

![Figure 3.15 Photographs of PC3 cells during 5 days culture in PDMS detachable chip](image)

**Figure 3.15 Photographs of PC3 cells during 5 days culture in PDMS detachable chip**
evacuate for 30 minutes in order to make the PDMS surface contact to the glass surface without a gap. This temporal bonding could maintain the fluid flowing through the microchannels without any leakage. We fabricated the lid-detachable chip and loaded cells.

As shown in Figure 3.15, cell grew well for 5 days without migrating into the interface between PDMS and glass. On 5th day, we detached the lid. We immersed the chip inside the cell culture media and lifted it up very gently to minimize the possible cell damage. To check the cell viability after the detachment, we kept monitoring cells for 1 day in an incubator. Figure 3.15 shows the images taken right after and 1 day after the detachment, which indicate that cells survived well after the lid removal.

Figure 3.16 Photographs of PC3 cells right before, after, and 1 day after the PDMS lid removal (tracked from an identical microwell).
We also successfully showed the feasibility of local trypsinization. Figure 3.17 shows the experimental setup. Small piece of glass was prepared by culturing cells very densely on the top of the glass. We placed a PDMS piece which has a circular hole which was generated by 2mm biopsy punch. Then, we add a trypsin drop into the hole to detach the cells exposed only in the hole. After warming it up in a 37 °C incubator, we collected the detached cell from the hole using a 10 uL pipette. The detached cells were deposited and cultured in a 96 well to see if the cells could survive after detachment. Figure 3.18 shows the experimental results. There is no cell remained in a circular area which was defined by the hole of the PDMS piece and the deposited cells into a 96 well plate survived and elongated on the surface. These two successive experiments confirmed that we can collect cells selectively using detachable chips and local trypsinization techniques. Smaller holes (in PDMS) with fine pipetting will enable the selective harvest of cells from microfluidic devices and this method is applicable to other microfluidic devices.
3.4 Conclusion

We have successfully demonstrated that our prototype could be used for single cell clonal culture and drug screening purpose. Our chip enabled 1) to capture single cells automatically and efficiently by employing hydrodynamic guiding structure and 2) to monitor and track cells over their progenies by isolating cells inside individual microwells with Pluronic copolymer selective patterning technique. In addition, we utilized a gravity flow generated by pressure difference between the inlet and outlet to supply cell culture media and reagents gently without the necessity of external equipments. We fabricated the $8 \times 8$ microwell array and tested it by loading human prostate cancer cell line (PC3 cells) which was reported to have three difference sub-phenotypes. After 4 days of culturing, we could observe the generation of the three different phenotypes:
holo-, mero-, paraclones identified by their morphology and proliferation rate. After identifying PC3’s heterogeneity, we investigated their drug responsiveness by injecting Docetaxel to correlate them to cell subtypes, which showed clear and repeatable differences in drug responses among them. We have also demonstrated our ability to selectively harvest cells from specific microwells to perform further probing on a cell subtype.
Chapter 4.
Magnetic Cell Manipulation

In this chapter, we present a novel programmable magnetic cell sorter which separates cells with different sizes by adjusting local magnetic fields using electric current. The cell sorter consists of a series of separation units comprising ferromagnetic nickel lines and current-carrying gold lines. The nickel lines magnetized by external permanent magnets and the current passing through the gold lines generate a local magnetic field gradient and magnetic force which will attract cells coated with superparamagnetic nanoparticles. By adjusting the magnetic force in each unit, we can sort the target cells by sizes selectively. The nickel lines placed in the front part of each unit align the incoming cells to the center of the channel to make the initial condition of cells uniform before sorting. We successfully demonstrated this scheme using commercial polymer beads coated with magnetic particles (6, 10, and 15μm in diameter).
4.1 Introduction

Specimens obtained from patients contain target cells as well as other cells. It is desirable to collect only the target cells of a specific size with small variation especially for drug screening purpose. There are two conventional macro methods which can separate the target cells: fluorescence activated cell sorter (FACS) and magnetic activated cell sorter (MACS). FACS can separate multiple target cells from a collection of cells with more than 99% accuracy by tagging target cells with various fluorescence markers, however, it requires experienced staff with high-expertise to operate, and is not affordable for every single laboratory or clinic to own and maintain it. On the other hand, MACS can separate cells at a reasonable cost. But its separation accuracy is less satisfactory compared to FACS. Therefore, there have been many efforts to improve MACS performance by adopting microfluidic techniques. Microfluidic magnetic sorters could improve their separation accuracy by confining all the samples in a narrow microchannel to expose cells to a local high-gradient magnetic field.

The passive methods use ferromagnetic guiding structures integrated on a substrate [73-77]. From an the externally applied magnetic field generated by permanent magnets, the ferromagnetic structures (coplanar stripes [73-76] or multiple pillars/posts [77]) are magnetized and combine high-gradient magnetic field inside a microchannel as shown in Figure 4.1[73]. As a result, when magnetic beads/cells are introduced in a continuous fluid flow, they are attracted to the maxima of magnetic field: on the top of guiding lines or on the wall of ferromagnetic posts. From this, magnetic and non-
magnetic beads/cells can be separated. The active method uses magnetic field generated by the electrical current through conducting lines. It was utilized not only to separate cells as shown in Figure 4.2 [78] but also to manipulate magnetic beads by changing the applied current [79-81]. Though these microfluidic magnetic sorters can increase the separation accuracy, they can only separate magnetically labelled cells from non-labelled cells.

Figure 4.1 Example of passive cell separation methods [67-68]: (a) Illustration of magnetic field distribution inside a microchannel by an integrated ferromagnetic pattern, (b) movement of cells which are coated with magnetic nanoparticles under fluid flow and magnetic field, and (c) experimental results of separation of magnetic beads (dots in the left side) from non-magnetic beads (dots in the right side).
In this work, we developed a microfluidic magnetic cell sorter which is able to separate target cells by magnetic labeling as well as sort them by their size. A programmable magnetic cell sorting scheme has been designed and realized combining ferromagnetic lines and current-carrying metal lines to control local magnetic fields.

Figure 4.2 Example of active cell separation methods [75]; (a) A serpentine conductor pattern with semi-encapsulated permalloy. It can generate a magnetic field by flowing electrical current through the conductor. The field is reinforced by the embedded permalloy. Photographs (b) before and (c) after the bead injection. Only the area where current excited magnetic field holds the beads.
4.2 Device Concept and Principle of Operation

Figure 4.3 shows the schematic of the proposed microfluidic magnetic sorter. Each unit stage consists of two guiding elements: ferromagnetic lines and current-carrying lines. The ferromagnetic lines induce a high magnetic field gradient by concentrating external magnetic field from permanent magnets. Current flow through the current-carrying lines also generate local magnetic fields, which can be easily controlled by adjusting the current. These local magnetic fields trap the cells along to the ferromagnetic/current-carrying lines and the trapped cells move along the lines by hydrodynamic force from fluid flow. (These local magnetic fields attract the flowing cells and alter their movement only when the cells are coated with sufficient magnetic nanoparticles.)

The operation principle of each unit stage is as follows (Figure 4.3(a)). As the injected cells approach the ferromagnetic lines, they are attracted down to the bottom and move toward the center of the main channel (A → B) by a combination of magnetic and hydrodynamic forces. Once the cells reach to the center, the laminar flow in the microchannel maintains the cells in the center stream (B → C). When the cells flow over the current-carrying lines, the local magnetic field attracts the cells along the lines and loads them into the microwell (C → D). Cascading the unit stages multiple times allows sorting cells in various sizes by programming the current differently in each stage. The size of the attracted cells depends on the amount of current; the larger the current the larger the attracted cells are. For example as shown in Figure 4.3(b), three unit stages...
connected in series can sort three different size cells by applying different current in each unit stage in an increasing order. Smaller cells are captured at the first stage and larger cells at the last stage. More stages can be added for sorting other different sizes of cells.

Figure 4.3 Operation principle of magnetic cell sorting: (a) Unit stage and (b) Sorting the magnetic nanoparticle-coated cells of three different sizes by cascading three stages in series.
4.3 Design and Analysis

The device was designed to separate different sizes of magnetically labeled cells. The microchannel has a height of 30 µm and width of 600 µm for the main flow path and contains the two set of magnetic gradient generating lines: ferromagnetic lines and current-carrying lines. Shallow microchannels may cause a cell clogging problem and deep microchannels require stronger magnetic force to attract the flowing cells because the magnetic force exerted on the cells becomes weaker as they are far from the magnetic lines [73]. We chose 30 µm for the main channel height considering the variation of target cell size (typical average size of mammalian cells is around 10 ~ 20 µm). Narrow drain channels (5 µm in width) were connected to the microwell in order to make the injected fluid to flow through the microwell but to hold the cells. Ferromagnetic lines were designed to effectively guide the injected cells to flow along the center stream in the main microchannel by disconnecting the lines at the center area. The lines incorporate ten nickel patterns which are 40 µm in wide and 2000 Å in thickness with a pitch of 80 µm in a 26° angle from x-axis. The current-carrying lines were designed to guide cells to the microwell. The lines are composed of ten gold patterns which are 40 µm in width and 5000 Å in thickness with a pitch of 80 µm in a 26° angle from x-axis. The dimension of the guiding lines was determined based on our test experiments.

We analyzed our device with numerical simulations based upon 2-dimensional approximation to calculate the corresponding electrical currents for sequential sorting. Magnetically labeled cells were assumed as a polymer microbead coated with magnetic
nanoparticles for simplicity in calculation. We considered two forces dominantly acting on a magnetic microbead. One is the magnetic force, $F_M$, induced by the magnetic field gradient and the other is the strokes drag force, $F_D$, due to the viscous drag exerted by the suspending medium on a moving microbead.

Figure 4.4 Illustrated cross-sectional view of a microchannel with the embedded ferromagnetic and current-carrying lines. The area of force calculation for sorting is marked with red dash lines.

Figure 4.4 illustrates the cross-sectional view of the microchannels. All the injected microbeads passing through the microchannels experience magnetic force induced from the magnetized ferromagnetic lines and electrical current from wires. While flowing over the ferromagnetic lines, they are attracted and guided to the center of the channel. After that, they receive the magnetic force formed by electrical current. When the magnetic force exceeds the drag force that pulls the bead away from the wires (i.e. $F_M > F_D \sin(\theta)$), the magnetic microbead deflects and follows the current-carrying lines as
We focused our analysis on how the different sized microbeads can be selectively sorted out by controllable electrical current. The parameters utilized in both the calculation and experiments are summarized in Table 4.1. For the calculation, we assumed that 1) all the injected magnetic beads flow on a substrate surface in the microchannels due to the ferromagnetic lines, 2) the flow velocity profile has a parabolic shape distribution in channel height direction (fully developed flow), 3) magnetic beads are not saturated under a weak magnetic field condition (< 0.5 T) [82], and 4) magnetic beads (polymer beads coated with magnetic nanoparticles) can be considered as if all the
magnetic nanoparticles are concentrated at the center of the beads.

Table 4.1 Parameters used for force calculation

<table>
<thead>
<tr>
<th>For drag force calculation</th>
<th>For magnetic force calculation</th>
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<tbody>
<tr>
<td>Channel height</td>
<td>30 µm</td>
</tr>
<tr>
<td>Average fluid velocity in a main channel</td>
<td>100, 300, 500 µm/s</td>
</tr>
</tbody>
</table>

The Stroke drag force is given by equation (1):

\[
F_D = 6\pi \eta \nu R
\]  

(1)

where \( \eta \) is fluid viscosity, \( \nu \) is a fluid a velocity, and \( R \) is a radius of a magnetic bead. Since the fluid velocity has a parabolic profile in a microfluidic channel [83], the drag force varies along the position of beads. Thus, drag force depends not only on a bead radius but also on the vertical position of a bead under a given fluid velocity. Based upon equation (1), we calculated the drag force on different sized microbeads in three different fluid velocities (100, 300, and 500 µm/s for average velocity), assuming beads are flowing on the bottom surface of a microchannel due to the attraction caused by
ferromagnetic lines in the previous stage. The calculated drag force is shown in Figure 4.6.

![Figure 4.6: Calculated drag forces on different sized beads under three different fluid velocities. Fluid was assumed to contain 22% sucrose (in deionized water), with a viscosity of 1.941.](image)

We simulated the magnetic force generated by the electrical current flowing through multiple coplanar rectangular parallelepiped gold lines integrated on a substrate, based on a modeling from Furlani et al [84, 85]. We also considered the applied external magnetic field from the permanent magnets and assumed it is uniform in the region of analysis as 0.065 T. Figure 4.7 shows the profile of the calculated magnetic force in x-
direction (same as the fluid flow direction) on a 10 µm bead for 33 mA in the current-carrying wires. The magnitude of the force (absolute value) reaches its maxima at the edges of the wires, and the minima at the center of the wires, and the mid points in the space between the wires.

Figure 4.7 Calculated magnetic forces applied to a 10 µm bead when it passes through the current-carrying wires flowing 33 mA.
In addition, we calculated the maximum magnetic force according to the applied current (Figure 4.8), which clearly shows the dependence of the force on the current amount. Based upon the calculation on the two forces, we could obtain the required current to generate enough magnetic force to overcome the drag force and attract the magnetic beads to the corresponding microwells (Figure 4.9). For example, in order to sort out 6, 10 and 15 µm beads successively in the flow with a velocity of 300 µm/s, we need to apply the current 20, 33 and 50 mA, respectively.

Figure 4.8 Calculated magnetic forces on different size beads with varying electric current.
4.4 Verification of the Model

4.4.1 Simplified Modeling of Microbeads

In order to simplify the simulations for magnetic force calculation, we have assumed a magnetic bead (polymer bead coated with magnetic nanoparticles) as if all the magnetic nanoparticles are concentrated at the center of the beads. To verify the validity

Figure 4.9 Calculated minimum required current to overcome the drag force to guide the magnetic microbeads.
of this assumption, we calculated the magnetic force when 50 and 500 magnetic nanoparticles are coated around the bead surfaces. Figure 4.10 shows the calculated results compared with our simplified model. The profiles of the calculated magnetic force are in x-direction for a 10 \( \mu \text{m} \) bead in the case that the current over the current-carrying lines is 33 mA. As shown in Figure 4.10, the differences are negligible; less than 0.01%; this verifies our assumption that the microbeads coated with magnetic nanoparticles can be treated as a point quantity.

![Figure 4.10 Comparison of magnetic force where \( n \) represents the number of nanoparticles around a microbead. Except for \( n=5 \), the calculated forces are almost the same as our simplified model, verifying that our assumption are valid.](image)
4.4.2 Temperature Effects from Current Flowing

We analyzed how much the applied current may increase the temperature of fluid using Coventorware™. Temperature higher than 37 °C may harm the cells flowing though the microchannel. We simulated with all the same dimensions as our device design and assumed that heat was 1) generated by Joule heating in the current-carrying lines, 2) conducted to all the surrounding materials, and 3) dissipated through the top surface of PDMS and the bottom surface of silicon wafer by air convection. We also assume that the surrounding temperature is 300 °K. In order to analyze the worst case, we assume that fluid inside microchannel is not flowing but stagnant. Also, natural convection condition was assumed (a convection coefficient of 10 W/m²K in air).

Figure 4.11 shows the simulation results. The expected maximum temperature was simulated less than 307.5 °K, when we applied 100 mA, which is much larger than the calculated current to sort 15 μm beads. Moreover, the temperature increase in real experiment would be much less than our calculation since our calculation is based on stagnant fluid. In addition, we also considered a case that our device operates on the top of a cooler to keep the temperature of the substrate constant at room temperature. Figure 4.12 shows results. In this case, temperature increase was reduced below 1 °C. Therefore, our device can be utilized to sort real cells without any harmful effect from temperature increase.
Figure 4.11 Simulated temperature increase when heat is dissipated by a natural convection. (a) Temperature profile along the $x$-axis, and (b) Temperature distribution around the current-carrying lines.
Figure 4.12 Reduced temperature increase when heat is dissipated through a cooler underneath the device. (a) Temperature profile along the x-axis, and (b) Temperature distribution around the current-carrying lines.
4.5 Experiments

4.5.1 Fabrication

Figure 4.13 Fabrication process consisting of three main steps including 1) a substrate preparation for magnetic guiding structures, 2) PDMS lid for microchannels and microwells, and 3) bonding PDMS piece to the substrate.
To demonstrate the feasibility of the idea, we have fabricated a prototype device consisting of three unit stages. The fabrication process procedure was illustrated in Figure 4.13. We have three main fabrication procedures: 1) substrate preparation for magnetic guiding structures, 2) PDMS preparation for microchannels and microwells, and 3) bonding the PDMS piece to the substrate. At first, we prepared the magnetic guiding lines on a substrate. We deposited 1000 Å silicon nitride on a bare silicon wafer as an insulation layer, using low pressure chemical vapor deposition (LPCVD). Then we did lift-off process to generate a pattern of multiple parallel ferromagnetic lines, which were sputtered 2000 Å nickel layer. After removing all the possible photoresist residues from the lift-off process, by treating the wafer with O₂ plasma (600 mTorr, O₂ 100 sccm, 100 W) for 5 minutes, we patterned a E-beam evaporated 5000 Å gold layer by lift-off process again for the current-carrying lines. The patterned wafer was cleaned again in O₂ plasma for 5 minutes. Then the substrate preparation was finalized by passivating the entire surfaces with silicon nitride film using plasma enhanced chemical vapor deposition (PECVD) and dicing into pieces. In a separate silicon wafer, we made a mold structure for microchannels and microwells, using two steps of photolithograph process with epoxy-based negative photoresist, SU8 (Microchem, MA). A thin 5µm layer using SU8-5 was patterned for drain channels to supply continuous fluid flow through the microwells and then thick 30 µm SU8-10 was patterned for main microchannels and microwells. This SU8 mold structure was replicated by pouring liquid-phase polydimethylsiloxane (PDMS). The poured PDMS was cured at room temperature for 3 days to prohibit any shrinkage. Then, holes for inlet and outlet were generated using a custom-made punch
(1.5 mm in a diameter) and cut into pieces by a razor. Bonding surfaces of the separately prepared substrate and PDMS pieces were cleaned out by acetone, methanol, and isopropyl alcohol and then treated with O₂ plasma (600 mTorr, O₂ 100 sccm, 100 W) for 10 seconds to make strong bonding between the two pieces. Then, the two pieces were aligned under an aligner MJB-3 (Karlsuss, Germany). Then they were put on a hotplate at 60 °C for 2 hours to ensure the bonding. The fabricated device was shown in Figure 4.14.

Figure 4.14 Fabricated prototype device for sorting different sized cells. It consists of three unit stages. Three different sized magnetic cells/beads can be sorted into the corresponding microwells. Non-magnetically labeled cells or beads will be corrected in the outlet. Magnified view shows a fabricated unit stage comprising of ferromagnetic and current-carrying lines.
4.5.2 Experiments

We tested our fabricated prototype sorter by flowing a mixture of three different-size magnetic beads (6, 10, 15 μm in diameter, polymer microbeads coated with magnetite to emulate the cells, Micromod, Germany), which were suspended in a concentration of $4 \times 10^5$ beads/mL. To prevent the introduced microbeads from settling down by gravity (without magnetic attraction) during the operation, we suspended the microbeads in a 22% sucrose solution, instead of de-ionized water, which has the same density as the microbeads. We also added surfactant, 1% Triton X-100 (Sigma-Aldrich, MO), to inhibit microbeads’ unspecific binding to the inner microchannel wall. The suspended magnetic beads were injected using a syringe pump (KDS 200, KD Scientific, MA) at a constant flow rate of 0.16 μl/min and their movement was monitored under a stereotype microscope (SMA1500, Nikon, Japan) and recorded into a movie clip by a digital camera (coolpix S4, Nikon, Japan). Two permanent magnets were placed in parallel to the microchannel in order to magnetize the ferromagnetic lines inside the microchannel and guide the magnetic beads to flow along the center bottom region of the main microchannel. To adjust the external magnetic field, we designed a jig for two permanent magnets which can horizontally control the distance from the magnets to the device. We placed our device on a micro-stage to control vertically ($z$-directional position). Three separate power supplies were connected to each stage to control and supply the different electrical currents at the same time. Figure 4.14 shows the experimental setup.
From the mixture of three different size magnetic beads, we successfully collected 6 μm, 10 μm, and 15 μm beads in microwell #1, microwell #2, and microwell #3, respectively by applying the current of 21 mA/line, 37 mA/line, and 68 mA/line in the corresponding unit stage respectively, as shown in Figure 4.16. Our experimental current values were in a good agreement of our calculation. We applied the current in an increasing order in the cascaded stages. For example, at the first stage we sorted the smallest beads with the smallest current, and the next stage sorted the larger ones with larger currents. If the order is reversed, the first stage may sort the larger ones as well as the smallest one. Around 90 % of all the captured beads were collected in the
corresponding microwells. The errors were mainly due to the unstable flow generated by a syringe pump.

Figure 4.16 Experimental sorting results: (A) overall structure of the sorting chip, (B) 15 µm beads captured in the 3rd microwell, (C) a 10 µm bead captured in the 2nd microwell and (D) 6 µm beads captured in the 1st microwell.

4.6 Conclusion

We designed and fabricated a programmable cell separator to sort different size cells with the integrated current-carrying lines which generate a controllable local magnetic field. From a prototype consisting of three unit sorting stages, we successfully demonstrate that three different size magnetic beads (6, 10, 15µm in a diameter) can be
sorted into three separate microwells by controlling electrical current at each stage. More stages can be added and the number of stages can be determined to meet the sorting purpose.
Chapter 5.
Conclusion and Future Studies

5.1 Conclusion

In this study, we have designed and fabricated novel high-throughput microfluidic chips allowing heterogeneous cell assays by capturing single cells and isolating their clones to observe behaviors over their progenies.

We developed a hydrodynamic guiding structure which can automatically capture and position a single cell into each microwell with very high capturing efficiency by utilizing hydrodynamic resistance differences between the two flow paths. We have fabricated a prototype and tested it by flowing microbeads. More than 80 % of microwells were occupied with single microbeads (occupancy rate) and more than 80 % out of all the injected beads were captured at trapping sites in microwells (capturing efficiency). This is a significant increase compared with previous passive single cell
capturing devices reported up to date by more than two orders of magnitude. We also demonstrated the massively-parallel loading of cells in a short period of time (less than 2 minutes) and succeeded in culturing the captured cells. This hydrodynamic guiding structure can be easily adapted to high-throughput assay applications and we verified its feasibility thoroughly by extending it to a $32 \times 32$ (1024) microwell array chip integrated with pneumatic valves allowing 8 different reagents injection simultaneously.

We have developed a novel single cell clonal culture array chip, which is the first chip capable of culturing single cells into their clonal colonies inside individual microwells and introducing test-reagents to their clones. This chip can capture the injected cells automatically and efficiently into microwells at a single cell resolution by employing a hydrodynamic guiding structure. It also allows to track each single-cell progeny to monitor differences in cell proliferation and morphology change over several days. Every microwell contains a homogeneous phenotype of clones originated from an initially captured single cell. For supplying cell culture media or test-reagents, we utilized a gravity flow generated by pressure difference between inlet and out reservoirs. This allows us a simple and easy operation without the necessity of external equipments or apparatus. We have fabricated an $8 \times 8$ array prototype and tested it by loading human prostate cancer cell line (PC3 cells). It could identify three cell subtypes from PC3 cells and it measures the differences in drug responsiveness in each subtypes. We have also tested initial feasibility to selectively harvest cells from specific microchambers to perform further testing on a cell subtype.
An alternative method for heterogeneity study can be achieved by sorting cells with identifiable surface markers. It is desirable to collect target cells of a specific size with a small variation especially for effective drug screening purpose. We devised a novel magnetic sorter which can separate multiple target cells by their sizes, using the integrated current-carrying lines which generate local magnetic fields. We fabricated a prototype comprised of three unit stages and successfully demonstrate that three different size magnetic beads (6, 10, 15 µm in diameter) can be sorted into separate microwells by controlling electrical current in each stage. More stages can be added and the number of stages can be determined to meet the sorting purpose.

5.2 Future Studies

We have successfully demonstrated that our prototype can culture identifiable clones in individual microwells. This prototype can be improved by integrating other functions such as micropumps and microvalves to control microenvironment spatially and temporally, and adding multiple reagent inlets for high-throughput applications.

Another interesting research topic to improve the prototype is a selective harvest, one of the most demanding techniques especially for single cell assay chips, because the extracted cells from one sub-phenotype can be used to further biological probing such as gene expression analysis. The selective harvest scheme can be improved by integrating more sophisticated functions: 1) using aqueous two-phase systems that allow the
formation and localization of small droplets over a surface while preventing spreading or diffusion, 2) using a microfluidic logic system which can selectively provide trypsin and retrieve the corresponding cells out of selected microwells.
Bibliography


