Interactive Droplet Manipulation for Microfluidic Single-cell Screening

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by

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A dissertation submitted to the faculty of KAIST in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Bio and Brain Engineering. The study was conducted in accordance with Code of Research Ethics¹.

2011. 12. 5.
Approved by

[Signature]
Professor Je-Kyun Park

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미세액적의 인터랙티브 조작

엄 유 진

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2011년 9월 27일

심사위원장 박 제 균
심사위원  남 윤 기
심사위원  정 기 훈
심사위원  이 승 구
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ABSTRACT

This thesis proposes a well-designed microfluidic system with hydrodynamic control of microdroplets can serve as a simple, feasible, and beneficial platform for biological assay. The selected experiment to assess practicability of pico to nanoliter microdroplet system is the assay for screening gene library inside *Escherichia coli* (*E.coli*). Cell-based screening is conventionally carried out in test tubes or well plates in which single cell isolation or reaction can be hardly achieved. The single-cell based assay platform requires the process of single-cell isolation, mixing with reagents for identifying certain gene expression, incubation with supplementary cell media, and recovery of the reacted cells for further analysis.

This work proposed that microdroplets generated in two-phase fluid are the most compatible tool for single-cell isolation, and the method of secondary breakup of droplets to encapsulate single cells inside microdroplets is first introduced. The secondary breakup of droplet which is obtained from the conventional microchannel for droplet generation, into smaller sizes by simple integration of micro-groove structures or a pinched flow. While conventional microfluidic tool achieves single-cell isolation efficiency of less than 30%, droplets obtained from the secondary breakup collects about 50% of single-cell containing droplets, and the proportion of multiple-cell containing droplets are kept less than 16%.

Mixing with reagents or supplementary cell media for droplet-based microfluidics requires merging events of droplets. Here we report the use of the abacus-groove structure for sequential addition of droplets. The abacus channel brings droplets together in a chamber with a control channel, which is analogous to biasing in electronics to change the pressure difference in the chamber and precisely determine the number of added droplets with no external forces integrated to the system other than syringe pumps. This method for droplet merging can be applied in addition of single type of droplets into various numbers, addition of two droplets at different ratio, and sequential merging and transport of droplets in multiple chambers serially connected with each other.

Finally, we created a platform for microdroplets which integrates multiple functions including easy trapping and consistent addition of droplets. We aligned a mesh-grid structure over the microwells which acts as a microchannel structure with an open access, and created a stable microdroplet array. The mesh-grid as a microchannel aids in guiding the trace of droplets into the microwells underneath for stable storage, and the open system enables integration of additional manipulation tools to each droplet. With the device, more than 80% of the wells are filled with single-cell droplets, and the following process of droplet-merging, incubation, and selective recovery of droplet was successfully demonstrated in one platform. Each droplet in the array forms a stable environment of pico-liter volume for cell-to-cell reactions and cell based screening application.
In this thesis, integration of each functioning tool of microdroplet system, which is a main bottleneck of the recent microfluidic technology, is realized to completely carry out the whole process of biological assay. This technique is expected to provide a microfluidic method not only for cell screening application but also in various applications which requires handling of small volume of materials. Furthermore, this work has created a novel environment to study interaction between the single cells in high throughput manner, and is expected to be applied to fast screening of microbial cells directly obtained from nature, of which more than 99% in existence cannot be cultured with conventional laboratory system.

**Keywords:** Microdroplet, Droplet Array, Droplet Coalescence, Droplet Manipulation, Single cell screening
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Nomenclature

**Alphabetic Letters**

d  Diameter of a droplet  
F  Viscous drag force  
H  Channel depth  
I₁  Inlet of the oil phase at the T-junction channel  
I₂  Inlet of the water phase at the T-junction channel  
I₃  Inlet of the oil phase at the pinched flow region  
L  Channel length  
n  the number of cells in a drop  
P₁  Pressure at the inlet of a chamber  
P₂  Pressure at the outlet of a channel  
ΔP  Pressure difference across the oil and the water phase  
Q  Flow rate  
Q₁  Flow rate from the inlet I₁  
Q₂  Flow rate from the inlet I₂  
Q₃  Flow rate of the the inlet I₃, the control flow  
Qₐ  Flow rate of dispersed phase  
Qₒ  Flow rate of oil phase  
R  Fluidic resistance between two points in a rectangular channel  
r₁  Radii of curvature at the front interface of a droplet  
r₂  Radii of curvature at the back interface of a droplet  
T₁  Time interval between the first droplets in each set of droplets for merging  
W  Width of a channel  
Wₜ  Width of expansion channel  
Wₚ  Width of the pinched flow region right before the expansion channel  
Wₚₐ  Width of main channel at the T-junction  
w  Normal velocity of the oil flow on the microwell array in direction towards the bottom of the wells
\( w_n \) Normalized velocity of \( w \)

**Greek Letters**
- \( \alpha \) Dimensionless parameter
- \( \gamma \) Interfacial tension between the oil and the water phase
- \( \eta \) Viscosity of the continuous phase
- \( \lambda \) Average number of cells per drop
- \( \nu \) Velocity of the continuous phase

**Abbreviations**
- Abil EM90 Cetyl Dimethicone copolyol
- Ca Capillary number
- *E.coli* *Escherichia coli*
- FACS Fluorescence-activated cell sorting
- LB Luria-Bertani
- PDMS Polydimethylsiloxane
- PLP Pyridoxal-5’- phosphate
- Span 80 Sorbitan Monooleate
- TEM Transmission Electron Microscopy
- TPL Tyrosine phenol-lyase
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CHAPTER 1.

Introduction

1.1. Conventional cell-based screening

Single-cell studies are explored to analyze cell population heterogeneity and cell-to-cell variations. Single-cell screening is executed by observing traits of each cell in large sample of either one type of cells or mixture of different kinds of cells to collect particular cells of interest. Many of the benchtop biological assays are run in test tubes or microwell plates with 96 or 384 wells per plate to carry out parallel analysis of reagents of a few hundreds of microliter in volume. The purpose for using microwell plates aims for high-throughput screening. However, the dimensions of the conventional multiwell plates are too large to be applicable to single-cell screening because a single cell usually has a volume of ~1 pL, a size of ~10 μm. For the analysis requiring lower volume with higher density, 1536 or 3456 well-plates are currently available but still the minimum well center-to-center spacing is around 2 mm. The area of each chamber of the wellplate is even larger than the area covered by the objective’s field of view in most of optical systems [1]. The microarray technology, which is developed by spotting the samples on solid substrates by pin printing or microstamping, provides smaller spots, each ranging from 50 to 300 μm in diameter. However, physical boundaries such as wall structures are still required when there is a need to isolate each content completely from each other for further analysis. Well-type structure is especially needed to treat individual cells for different treatments of liquid samples and to incubate cells for a long time providing cell culture media.
Moreover, in order to isolate single cell in each confinement repetitive pipetting of serial dilution is requires (Fig. 1.1). Although combining with robotic liquid manipulators will help in automation of the process and in handling of the samples as little as a few microliters, it is still an inefficient method using numbers of test tubes or microwells, The dilution method to load one cell in each chamber usually results in deposition of individual cells roughly one out of three wells. Large portions of the wells will either be empty or contain multiple numbers of cells. To induce proper cell behaviors with low numbers of cells, controlling the number of cells per unit confinement is important [1-2]. Therefore breakthrough towards more quantitative technology of single-cell confinement which will also allow higher throughput process is demanded.
1.2. Microfabrication and microfluidic tools for cell screening

Microfabrication and microfluidic tools can help facilitate the single-cell isolation process [1-3]. The development of microfabrication technologies such as wet and dry etching or soft lithography attracted many of the researches to create microwell array for cell-based experiments. Microfabricated well array allows a large number of wells per unit area and confine cells in a single-cell compatible dimension [4-6]. As shown in Table 1.1, microwells fabricated either by etching silicon materials [6] or glass [7] resulted in diameters of 10-20 μm. Micromolding of polydimethylsiloxane (PDMS) [5]or hydrogel materials [4]create microwells in tens to hundreds of diameter. The main bottleneck of the microfabricated well array is difficulty in addressing the wells of these small dimensions from bulk environment. For relatively large microwells, the scheme of wiping the sample dropped on the well array by hand with a cover glass is used. For microwells of tens of μm, the method of loading cells into the wells with the aid of microfluidics such as receding meniscus-induced docking was introduced. However, the expected ratio of wells filled with single cell is usually less than 30% of the whole array.

Table 1.1. Previous literatures on microfabricated wells of various materials for loading single cells.

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<td>Material</td>
<td>Glass</td>
<td>Silicon</td>
<td>PDMS</td>
<td>PEG</td>
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<tr>
<td>Well diameter</td>
<td>20 μm</td>
<td>10 μm</td>
<td>10 μm</td>
<td>200-450 μm</td>
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<tr>
<td>Cell handling method</td>
<td>Capillary force</td>
<td>Pipette Micromanipulator</td>
<td>Receding meniscus</td>
<td>Wiping</td>
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Figure 1.2 Fluorescent microscopic image of *E.coli* displaying green fluorescence.

In addition, once cells are loaded into the microwells, it is hard to access individual wells for different treatment and recovery of cells for further assay. There is also a challenge in handling of microbes such as *Escherichia coli* (*E. coli*) which has relatively small size and irregular shape compared to large mammalian cells with spherical shape (Fig. 1.2).

1.3. Microdroplet technology and droplet manipulation

The lab-on-a-chip technology which is developed with microfluidics grows as an alternative of the conventional benchtop tools by handling small fluid volumes in a chip of a few millimeters to centimeters in dimension. Microfluidic design provides a good solution to the problem of accessing microfabricated structure. In particular, two-phase fluidic system, bubbles or droplets, has found applications in serving as liquid microconfinement themselves for their abilities of compartmentalization. Microdroplets can play the role as sample transporter and microreactor for chemical or biochemical reactions, fabrication of tailored colloids and controlled synthesis of various materials. Especially for biological assay, microdroplets have great advantage in high-throughput encapsulation of biomolecules and live cells or organisms in pico- to nanoliter volumes, and the confinement is completely
separated by the surrounding oil phase (Fig. 1.3) [8-9].

Figure 1.3. Microdroplets generated from a microchannel to encapsulate various materials including biomolecules and microorganisms.

Other advantages of microdroplets over other microwells of microfluidic tools include shorter diffusion distance, fast mixing, a decrease in time for analysis, and low dispersion of samples. Microdroplets enables more active control of the assay procedures as a transporting system by timed control of generation and behaviors inside the carrier flow. Unlike solid wells, they can move or stop, split or merge with others, and select different paths for sorting, with timed intervals [10-12]. Although many attempts to manipulate the behavior or droplets rely on external power such as pneumatic valve [13] or electrical control [19], the innate nature of bubbles or liquid droplets in immiscible fluid is sometimes sufficient to perform
predicted behaviors in a well-designed microfluidic circuit. Possibilities are found in the
study of periodicity of bubble-oscillatory patterns in microfluidics [14], and reversibility of
initial inter-droplet time intervals which can encode and decode signals and represent them
as the binary number [15]. Path selection of droplets or bubbles in microchannels under the
influence of flow resistance and the presence of other bubbles or droplets is also used to
perform various logical operations [12, 16]. The behaviors of droplets which “think” [17] in
the act of flowing greatly simplifies the integration process of valve and switches usually
required in fluid-based experiments.

In two-phase flow, hydrodynamic pressure is strongly influenced both by the size of the
droplets flowing in the channels and the parameters of the fluid phases. One of the
dimensionless capillary number,

\[
Ca = \frac{\eta v}{\gamma}
\]  

(1.1)

where \( \eta \) is the viscosity of the continuous phase, \( v \) is the velocity of the continuous phase,
and \( \gamma \) is the interfacial tension between the oil and the water phase, plays a key role in
determining droplet dynamics such as fission or droplet break off. Also, the curvature of the
interface produces a pressure difference between the two phase called Laplace pressure that
can be defined by the Young-Laplace equation,

\[
\Delta P = \gamma \left( \frac{1}{r_1} + \frac{1}{r_2} \right)
\]  

(1.2)

where \( \Delta P \) is the pressure difference across the oil and the water phase, and \( r_1 \) and \( r_2 \) are the
two principal radii of curvature of the interface. In addition, the number of drops
simultaneously flowing inside the channels influences the velocity of the flow. The size and

-6-
shape of the droplets, drag force acting on them in the carrier flow, the interfacial tension and the surface energy all provides the potential parameters of controlling the behaviors of droplets for designed usages. On the other hand, because a droplet has a high sensitivity to small perturbations, either in fabrication or operation, predicting the fate of droplets in microfluidic networks becomes very difficult, which calls for a sophisticated design considering various parameters including pressure, flow rate, frequency, and size of incoming droplets.

1.4 Research objectives

A well-designed microfluidic system with hydrodynamic control of microdroplets can serve as a simple, feasible, and beneficial platform for biological assay. Simplicity comes from the control only by hydrodynamic means of flow rates and the channel geometry without the need of external forces and complex fabrication. Feasibility is the capacity of droplets which can move or stop, break, and merge and mix with one another, enabling various performances of the containers with precise control of both time and space. By achieving the hydrodynamic control of microdroplets of predicted behaviors, the system can be useful and provide a better tool for biological assay than conventionally used equipments.

In this thesis, the hydrodynamic control of microdroplets will be designed specifically for the purpose of single-cell encapsulation, droplet merging and mixing, droplet incubation, and droplet recovery. The selected experiment to assess practicability of hydrodynamic microdroplet system is the assay for screening gene library inside *E.coli* which was conventionally carried out in test tubes or well plates. The detecting material of the specific enzyme-expressing gene from the library is another genetically engineered *E.coli* provided
kindly by researchers from Bio-chemistry & Energy Research Center of KRIIBB (Korea Research Institute of Bioscience & Biotechnology) which will specifically react in the presence of product from enzyme activity of the target cells and emit green fluorescence (Fig. 1.4a).

Figure 1.4 Schematic description of cell screening based on the detection of enzyme activity using cell-to-cell interaction. (a) Detecting the product of the enzyme activity from mixture of various cells obtained from host cells of metagenomic library or directly from environment. (b) of gene screening of the host cells (either from natural environment or from metagenomic library) by reporter cells throughout the cell-to-cell interaction.
Therefore, this model assay requires the process of single-cell isolation of the cells containing gene library and detection cells and merging two droplets containing each cell as shown in Fig. 1.4b. Incubation of droplets for enough time with supplementary cell media, and collection of the reacted droplets for cell recovery and further analysis are also the functions system has to achieve. The droplet-based platform is expected to be helpful especially in single-cell isolation and single-cell based reaction, which were difficult to be performed quantitatively with the conventional benchtop equipments, and also to reduce the whole reaction time. As described in table 1.2, our objective focuses on simple supplement and mixing of additional reagents which required complex method in previously developed microfabricated devices and applicability even to the microbial cells of various shapes [18]. Also the device should have advantages over other droplet-based system with simple design and ability to provide open access to individual droplets.[19-22]

Table 1.2. Microfluidic trapping method of droplets or cells for single-cell based analysis.

<table>
<thead>
<tr>
<th>Type</th>
<th>Microwell array</th>
<th>Micropost-trap</th>
<th>Droplets in wells</th>
<th>Stabilized droplets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>He et al., Anal Chem, 2005</td>
<td>Bai et al., Lab Chip, 2010</td>
<td>Shim et al., JACS, 2009</td>
<td>Brouzes et al., PNAS, 2009</td>
</tr>
<tr>
<td>Number of arrays</td>
<td>18,000</td>
<td>1,500 for drops Up to 6,000</td>
<td>4,000</td>
<td>900</td>
</tr>
<tr>
<td>Single cell trap (Cell type)</td>
<td>&gt;80% Mammalian cells</td>
<td>70-90% Mammalian cells</td>
<td>27% Microbial cells</td>
<td>~30% Mammalian cells</td>
</tr>
<tr>
<td>Addition of the Reagents</td>
<td>Limited</td>
<td>Pairing only &gt;50%</td>
<td>Impossible</td>
<td>Limited Electrical coalescence</td>
</tr>
<tr>
<td>Selection</td>
<td>Possible</td>
<td>Impossible</td>
<td>Impossible</td>
<td>Fluorescence detection</td>
</tr>
<tr>
<td>Device complexity</td>
<td>Simple</td>
<td>Moderate</td>
<td>Complex</td>
<td>Complex</td>
</tr>
</tbody>
</table>
1.5. Chapter outlines

This dissertation is articulated into the following sections:

Chapter 1 gives a brief introduction about traditional biological methods in cell-based screening, microfluidic technology for cell-based applications, and development of microdroplet technology. Subsequently, the aims and objectives for development of new microdroplet-based array for screening of single cells are presented.

Chapter 2 deals with the efficient method for single-cell encapsulation in microdroplets. In this chapter, design of the secondary breakup of microdroplets to enhance the proportion of single-cell droplets, and various breakup and sorting method of droplets were demonstrated.

Chapter 3 describes the novel droplet-merging device developed in this dissertation: the hydrodynamic control of the number of droplets to be merged including combinatorial control of ratio of two different droplets for mixing. This chapter also introduces another method to merge droplets in practical application where surfactant, which hinders demulsification of droplets are removed by addition of chemicals in alcohol group.

Chapter 4 introduces the fabrication of droplet-array with microgrid-integrated microwell array. The design was investigated with droplet trap efficiency and the possibility of droplet-merging. Integration with other droplet-modules to the microgrid-array, and procedures to trap and merge droplets in the array-form are described.
Chapter 5 presents the newly developed microdroplet-array and its demonstrations to the application of single-cell based screening. Stable trapping, merging with other reagents, stable incubation, and droplet recovery were demonstrated in the mesh-integrated microwell array. Through the cell-reaction demonstration, actual usage into single-cell based screening is discussed.

Chapter 6, lastly, presents discussion and conclusions of the newly developed methods to trap, merge, incubate, and recover microdroplets via microgrid-integrated well array for single-cell based screening application.
Chapter 2.
Single-cell encapsulation in microdroplets

2.1. Introduction

To generate cell-containing droplets using microfluidics, cells are first diluted in the aqueous phase and injected through a T-junction [23-24], or a focusing channel [25] along with the immiscible oil phase. Controlling the number of loaded cells per droplet has been a barrier for droplet-based single-cell analysis, due to the stochastic limitations of single-cell loading. The number of cells encapsulated in droplets follows Poisson statistics at the best, showing the portions of the single-cell droplet to be less than 40% depending on the initial cell concentration in the aqueous phase [26-29]. When the fraction of single-cell droplets reaches near 40%, the number of multiple-cell droplets also increases to be more than 30%, and reducing the multiple-cell droplets as low as 4% will result in only 22% of single-cell droplets and the majority of empty droplets [27, 29]. Poisson distribution (Fig. 2.1) is given by the following equation (2.1)

\[
f(n; \lambda) = \frac{\lambda^n e^{-\lambda}}{n!}
\]

where \( \lambda \) is the average number of cells per drop, and \( n \) is the number of cells in the drop.

Since the empty droplets are often preferred rather than overloading droplets with several cells, single-cell encapsulation efficiency is usually kept less than 30% as with the limited dilution in general arraying techniques [5, 8, 22, 27, 30]. This number is not advantageous to achieve one of the research goal, realization of droplet-merging to bring two different cells in one droplet for reaction, because chances a droplet will contain each cell after merging
will be even lower (~10%).

Figure 2.1 A Poisson distribution of which most of single-cell isolation method follows at different \( \lambda \) (average number of cells per drop).

There have been several efforts to enhance the efficiency of single-cell encapsulation into droplets up to 100%, including hydrodynamic self-organizing [31] and close-packed ordering of cells [32], which controls the periodicity of the drop formation with the periodicity of cell-discharging at the point of droplet generation. Another approach, hydrodynamic self-sorting, separates cell-containing droplets from the smaller empty droplets by volume-dependent drifting in the outlet [33]. However, these methods were demonstrated in the limited flow rate conditions only with spherical mammalian cells, which are relatively larger than most of the microbial cells. The innate problems of cell clustering could not be solved. There was a case of single-cell isolation of microbes by repeatedly splitting droplets until some of them resulted in having one cell inside, but this scheme is not developed for high-throughput analysis [34].
2.2. Principle of design

2.2.1 Problems to overcome

Microbial cells, *E. coli* in this case, have rod-shapes of 2-5 μm in length and isolation to single cells has depended solely on the control of the concentration. However, during microfluidic droplet generation, the value of \( \lambda \) does not guarantee to be 1 only by controlling the concentration of cell-media and the size of droplets. At the low concentration, as \(~1 \times 10^6\) cells/mL, theoretically spherical droplets of appropriate diameter is \(~120\) μm, but cells are not evenly distributed in the media at the point of droplet-generating inlet (Fig. 2.2a), which will result in higher percentage of empty or multi-cell droplets (Fig. 2.2b and Fig. 2.3). Compact loading of the cells at higher concentration will reduce irregular distribution in the media, and when the concentration is in the range of \(5-20 \times 10^7\) cells/mL, the volume a single cell takes up is \(5-20\) pL, which is \(21-34\) μm in diameters for droplets (Table 2.1). However, the dimension of the channel needs to be reduced to create small droplets compatible for single-cell isolation, and when cells flow through the channel they may easily form clusters of multiple cells, also perturbing the Poisson statistics. Therefore, the single-cell containing efficiency of droplets generated from the typical T-channel is usually worse than the value expected from the Poisson distribution.

<table>
<thead>
<tr>
<th>( \lambda )</th>
<th>Cell concentration</th>
<th>Droplet diameter</th>
<th>Droplet volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5x10^7 cells/mL</td>
<td>34 μm</td>
<td>20.6 pL</td>
</tr>
<tr>
<td></td>
<td>10x10^7 cells/mL</td>
<td>27 μm</td>
<td>10.3 pL</td>
</tr>
<tr>
<td></td>
<td>20x10^7 cells/mL</td>
<td>22 μm</td>
<td>5.57 pL</td>
</tr>
</tbody>
</table>
Figure 2.2 (a) A microscopic image of microfluidic T-channel where droplets encapsulating *E.coli* from the inlet are generated. (b) Distribution of number of cells per droplet from the T-channel.
Figure 2.3 Microscopic images of droplets loaded with cells generated from a typical T-channel with diameter controlled to be (a) 27 μm and (b) 50 μm.
In this work, we introduce a scheme of secondary breakup of a large droplet with high concentrations of cells into smaller sizes compatible to containing one microbial cells (Fig. 2.4a). By collecting the droplets of the size which are most likely to contain one cell with the aid of the hydrodynamic separation [35-36], we can increase the proportions of single-cell droplets in the outlet (Fig. 2.4b). Another advantage is the internal flow induced in large droplets flowing inside a microchannel before the secondary breakup which will help in distribution of cells.

Figure 2.4. (a) Schematic representation of secondary breakup of droplets which break large droplets with multiple number of cells into smaller sizes containing single cell in each. (b) Selection of droplet-size which has the highest probability to contain one cell inside. (c) Internal flow is induced in large droplets flowing inside a microchannel which will help in distribution of cells.
2.2.2 Design of the secondary breakup of microdroplets

The micro-groove design for random breakup of droplets as shown in Fig. 2.5a, was to create small droplets different in size between single-cell containing droplets and the ones with multiple cells or no cell. The system has to work under the broad range of flow rates and initial cell-concentration conditions. The primary droplet generated before the random breakup induced the internal flow [37] and this mixing effect helped in reducing the problem of clustering of cells in the inlet. Then the groove structures induce deformation of droplets and shear forces to break them into various sizes. The repeated expansion and contraction of cross-sectional area along the groove-structures yields disruption of droplets by elongating and splitting them. The whole device consists of three main parts: T-junction for droplet generation, groove structures, and hydrodynamic sorting parts (Fig. 2.5b). By making the grooves slightly slanted to 30°, the pressure drop along the length of the groove moves droplets especially large ones containing multiple cells from the bottom side of wall to the other, towards the top outlet. The main purpose of the design was for collecting high percentage of single-cell droplets and about 50% of droplet portion was discarded to the top outlet when cell recovery was not the main issue.
Figure 2.5 Schematic figure of the device for secondary breakup of droplets using microgrooves for single-cell encapsulation. (a) Enlarged image of the microgrooves which act as to break droplets into small sizes. (b) The device consists of three parts including (1) T-junction, (2) microgrooves for droplet breakup, and (3) pinched-flow section to sort out single-cell droplets.
Although there are several methods to break droplets with channel-geometries including the T-shaped bifurcating channel [38] and the poly(dimethylsiloxane) (PDMS) pneumatic valve [39], the size of resulting drops is hard to predict with such designs due to the hydrodynamic resistance change of the channel during the operation and complex design of the channel. By integrating micro-groove structures on top and bottom walls of the channel, and creating the terrace structure, the dispersed phase passing through the narrow groove inflates into an elongated shape, and the elongated dispersed phase tries to return to the spherical droplets due to interfacial tension at the terrace. This spontaneous droplet formation is said to lead to very low energy input during the process, and the interfacial tension is dominant compared with the inertial force and the drag force of the continuous phase. This dominant interfacial tension leads to fairly monodispersed droplet formation at relatively low flow rate conditions. We designed the height of the upper channel to be 20 $\mu$m and the lower channel structure 10 $\mu$m. The distance between the grooves was 30 $\mu$m. Therefore, most of the droplets will have diameters around $10^{-30}$ $\mu$m falling at the terrace from the groove structure, along with satellite droplets, because droplets of diameter less than 30 $\mu$m will restore their spherical shape and will not be deformed due to high surface tension.

We applied the pinched-flow sorting module [35] as shown in Fig. 2.6, which is effective in eliminating the empty satellite droplets resulted from the droplet breakup process at the micro-grooves. The outlet of the collection channel after the size-based separation was designed in accordance with the following equation,

$$L = W_B \cdot \frac{d}{2} \times \frac{W_B}{W_P}$$

(2.2)

where $d$ is the droplet diameter, $W_B$, width of expansion channel, $W_P$, width of the pinched
flow region right before the expansion channel, and L is the channel length from the side wall of the expansion channel. For droplets around 20 μm in diameter, L is estimated to be 660 μm. We designed the position of the selection outlet and the waste outlet accordingly to collect droplets around 25-30 μm, which will most likely to contain single cells.

Figure 2.6 Schematic drawing of the pinched-flow separation of different sized droplets.

With design of the channel with groove structures, creation of some large droplets containing multiple cells are expected to result. Also, separation by pinched-flow design cannot handle large number of droplets at one time because droplets have to pass through the pinched-flow region in a line for the best results. Therefore we designed the additional top outlet to reduce the number of droplets. Although the resulting droplets collected in the selected outlet may have high percentage of single-cell droplets, there are many cell-containing droplets to be discarded at the other outlets as well. To reduce the cell loss, we designed another type of channel for the secondary breakup.
Figure 2.7 Schematic figure of the newly designed device for secondary breakup of droplets using the pinched flow section to break and collect single-cell containing droplets.

Fig. 2.7 is the newly designed schematic representation of the secondary breakup of droplets with the enlarged image of the pinched-flow section. The main channel from T-junction which has width $W_m=120 \ \mu m$ tapers to $W_p=20 \ \mu m$. We can estimate the diameter of droplets, $d$, produced from the pinched-flow section with equation (2.3)[40]

$$d = W_p\left(1 + \alpha \frac{Q_d}{Q_o}\right)$$

(2.3)

With $\alpha \sim 1$, the flow rate of the oil flow $Q_o$ was set to be 40 $\mu$L/h and $Q_d$, the flow rate from T-junction 20 $\mu$L/h, to break droplets into diameter 30 $\mu$m. The satellite droplets produced during the second breakup of droplets flow out to the upper waste by separation.
2.3. Experimental

The channel was fabricated with two layers of PDMS slabs engraved with channel structures as shown in Fig. 2.8, molded from standard soft lithography of negative photoresist SU-8 2010. Polyethylene tubing connects the channels to the glass syringes, which are controlled by syringe pumps. To verify the single-cell encapsulation, we used enhanced green fluorescence protein expressing *E.coli* DH5α. The continuous oil phase used was mineral oil with 1 wt.% Span80 as surfactant, and the aqueous phase containing cells was Luria-Bertani broth (LB medium). First, the larger monodisperse droplets with high concentrations of cells (5–20 × 10^7 cells/mL) were created at a T-junction.

Figure 2.8 Schematic image showing bonding of two layers of PDMS slabs each engraved with patterns for a droplet-breakup channel.
2.4. Results and discussion

2.4.1 The breakup and sorting of droplets

The breakup of droplets as shown in Fig. 2.9a and 2.9b is followed by the pinched flow region where they are sorted by size following different streamlines as shown in Figure 2.9c. Satellite droplets flow out to the uppermost outlet, and larger droplets to the next branch of the outlet. We can select the outlet branch which will contain most of the single-cell droplets. Too many droplets at the separation region may block the streamline of others and decrease the separation efficiency. We divided the outlet into two next to the groove structure in order to filter out some relatively large droplets to the top outlet.

To demonstrate the device works with various sizes of initial droplets, size distributions of droplets before and after the pinched flow sorting were measured with the primary droplets of 220 pL and 400 pL created at the T-junction. For generation of 220 pL droplets, the flow rate of the oil phase injected to the inlet I$_1$ was 12 μL/h, and the cell media to I$_2$ was 3 μL/h. For the droplet of 400 pL, the flow rate of the oil phase was decreased to 9 μL/h. The rate of the focusing flow via inlet I$_3$ for the pinched-flow separation was kept at 35 μL/h. Fig. 2.10a and 2.10b are the volume distributions of droplets measured in the reservoir A and the outlet A, which had initial volume of 220 pL and 400 pL, respectively. In the reservoir A, droplets of 220 pL resulted in more satellite droplets, but most of the resulting droplets fall into the volume range less than 9 pL for both cases, which have diameter of approximately 26 μm in spherical shape. More than 85% of the resulting droplets at the outlet A had volumes in the range of 2–5 pL, 15.5–22 μm in diameter of spheres regardless of the initial droplet volumes. Therefore we could obtain fairly monodispersed droplets after random breakup even when the initial size of the primary droplet and the flow rate conditions were
Alternative design of the groove structure to reduce the cell loss is shown in Fig. 2.11. The groove structures are changed to be straight instead of being slanted, and the width of the channel along the groove structure is increased to be 280 μm to dramatically elongate droplets and lower capillary number for more complete breakup and reduce the production of large droplets during breakup. This design reduced the cell loss by removing the top outlet but the number of secondary droplets generated was too much for the pinched-flow separation to precise.

Figure 2.9 (a) Microscopic image of the microgroove structures with (b) the enlarged view showing the deformation (necking indicated with arrows) of droplets when passing through the groove structures. Scale bars are 100 μm. (c) Droplets separating in the outlet as passing the pinched-flow section.
Figure 2.10 Volume distributions of droplets in the reservoir A after the secondary breakup and in the outlet A after pinched-flow sorting of droplets, when initial volumes of the large droplets were (a) 220 pL and (b) 400 pL.

Figure 2.11 Alternative design of the groove structure to completely break droplets into small sizes and reduce the cell loss, (a) the groove structures and (b) sorted droplets after pinched-flow separation.
The results from newly designed channel of secondary breakup are shown in Fig. 2.12. Here the first large droplets break for the second time at the pinched flow channel with satellite droplets containing no cells separated to the outlet at the same time (Fig. 12a). The initial size of the first droplets did not affect the size of the secondary droplets considerably as long as the overall flow rate of the channel did not have a dramatic change (Fig. 12.b)

Figure 2.12 (A) Microscopic image of the secondary droplet breakup at the pinched flow section. (Scale bar is 200 μm) (b) Comparison of the size of droplets from the secondary breakup with different initial droplet-sizes from the first breakup. (Scale bars are 100 μm.)
Figure 2.13 Comparison of the volume distribution of droplets from the device with microgroove structure and the pinched flow breakup, measured at the outlet after the secondary breakup and sorting process.

Fig. 2.13 shows the comparison of size distribution of resulting droplets generated from the previous design of groove structure with the new design of the pinched flow breakup. For droplet handling and trapping which will be discussed in Chapter 4 in this thesis, larger droplets were more convenient. Therefore we designed the new channel to generate droplets slightly larger (30 μm in diameter) than the groove-structure channel, which is still a compatible size to encapsulate single cells inside.
2.4.2 The collection of single-cell droplets

Fig. 2.14a and 2.14b are fluorescence microscopic images taken at the reservoir A and the outlet A, respectively. The initial loading concentration of cells through the inlet I₂ was $20 \times 10^7$ cells/mL, and the volume of the primary droplet was 315 pL. The reservoir A includes satellite droplets containing no cell and some larger droplets having multiple cells inside. In the outlet A, high proportions of single-cell containing droplets could be observed.

To verify the device works at various cell concentration, we prepared cell media of three different concentrations, 5, 10, and $20 \times 10^7$ cells/mL. Using the scheme of random breakup and sorting, the fraction of single-cell containing droplets reached more than 50% (Fig. 2.15). For high concentrations of $20 \times 10^7$ cells/mL, droplets of diameter around $20 \mu m$, which were smaller than the average sizes of droplets from the outlet A, were more suitable, so the selected outlet was the upper branch of outlet A. Even at lower concentrations $5 \times 10^7$ cells/mL, having the estimated value of $\lambda=0.4$ when $25 \mu m$ droplets are formed, satellite droplets removal from the initial droplet resulted in the concentration increase by 1.4 which is equivalent to 5.2% enhancement of the value of one-cell droplet according to Poisson distribution. For each case, 400 droplets were analyzed with three devices. In the case of T-junction channel, the ratio of numbers of multiple-cell droplet to single-cell droplet was 1.1, 2.2, and 3.7 when the initial cell-loading concentration was 5, 10, and $20 \times 10^7$ cells/mL, respectively. In the secondary breakup device using groove structure, the ratio was reduced to 0.1, 0.3, and 0.3 for each concentration condition.

The results of new design using the pinched-flow secondary breakup are shown in Fig. 2.16. The large droplets first generated at the T-junction (Fig.2.16a) completely break at the pinched-flow section (Fig. 2.16b) without cell loss. The single-cell encapsulation efficiency
also maintained to be about 50% (Fig. 2.16c).

Figure 2.14 Fluorescence microscopic image showing droplets containing GFP-expressing *E.coli* (a) in the reservoir A right after the secondary breakup (scale bar, 50 μm) and (b) in the outlet A after passing through the pinched-flow separation part. Scale bars are 50 μm.
Fig. 2.15 The change in distribution of the number of cells per droplet collected in the outlet from the conventional T-channel device and the device with microgrooves.
Figure 2.16 The result images from the new design of the secondary breakup at the pinched flow channel showing (a) the first large droplets containing high concentration of *E.coli* at the T-channel (scale bar, 100 μm) and (b) the secondary breakup at the pinched flow section (scale bar, 200 μm). (c) The resulting distribution of the number of cells per droplet at the target initial loading concentration of cells.

### 2.5. Conclusions

Although we used green fluorescence expressing *E.coli* to verify the result, this scheme will obtain consistent results without labeling the cells. Since the structure does not need much space, it can be easily incorporated with other devices for further usage in microfluidic applications. The high proportions of single-cell encapsulated droplets collected in the outlet...
will result in enhancement of data from single-cell reaction, with high speed and less interference from other neighboring cells. The fraction of single-cell containing droplets and ratio to droplets with multiple numbers of cells are compared in Table 2.2 with conventional droplet-generation method. If supported by more efficient hydrodynamic sorting method, the efficiency and monodispersity of single-cell droplet encapsulation can be further increased.

Table 2.2 Comparison of the single-cell encapsulation efficiency from the secondary breakup of droplets with Poisson distributions and conventional T-channel.

<table>
<thead>
<tr>
<th>A (cell concentration)</th>
<th>Poisson distributions</th>
<th>Conventional T-junction</th>
<th>Random Breakup &amp; Sorting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5       1  2</td>
<td>0.5       1  2</td>
<td>0.5      1  2</td>
</tr>
<tr>
<td>Empty droplets</td>
<td>61%       37% 14%</td>
<td>39%       10% 5%</td>
<td>59%      36% 31%</td>
</tr>
<tr>
<td>Single-cell droplets</td>
<td>30%       37% 27%</td>
<td>29%       28% 20%</td>
<td>36%      50% 53%</td>
</tr>
<tr>
<td>Multi-cell droplets</td>
<td>9%        26% 59%</td>
<td>32%       62% 75%</td>
<td>5%       14% 16%</td>
</tr>
<tr>
<td>Multi-cell to single-cell droplet ratio</td>
<td>0.3 0.7 2.2</td>
<td>1.1 2.2 3.7</td>
<td>0.1 0.3 0.3</td>
</tr>
</tbody>
</table>
Since the model assay for this droplet-breakup scheme is to be applied is cell screening of gene library, it is also important not to have too many loss of cells. The new design of the pinched-flow channel which results in the secondary breakup of droplets and separation of satellite droplets at the same time, is compared with the first design of the slanted groove structures (Fig. 2.17). The number of large droplets with higher probability of containing multiple number of cells can be significantly reduced using this design and eliminated the extra outlet for discarding large droplets and cell loss.

Figure 2.17 Comparison of the structure of two different droplet-breakup channel for single-cell encapsulation. (a) A design with multiple groove structures and the pinched-flow separation of droplets. (b) The use of pinched-flow solely for breakup and separation of droplets at the same time. Scale bars are 100 μm.
Chapter 3.
Controlled merging of microdroplets

3.1. Introduction

One of many characteristics of droplets that show potential in the field of fluidic computation include the ability of a droplet to merge with other droplets to form bigger ones or to split into smaller droplets. Also, the fact that velocity of the dispersed phase can be set to be different with the continuous phase flow carrying them can be the building block of more complex circuit. Although splitting of a droplet is relatively easy with modification of the channel geometry that deforms and extends the shape of a sphere [38], merging of droplets is hard to achieve because both the time and the space have to be manipulated for synchronization of approaching droplets. Instead of merging droplet to droplet, we can bring multiple components to one inlet of the T-junction right before generating droplets, but diffusional contamination occurs even before droplet generation and exact amount of each component stream is hard to estimate (Fig. 3.1). If droplets have different sizes, they flow at different velocities in one channels and are able to merge in the downstream [41]. Creating velocity gradient using a tapered chamber allows droplet approaching by slowing down the flow rate and slight increase in the distance between adjacent droplets when leaving the chamber facilitates the event of merging [42-43]. Fluid rectifying channel also removes the continuous phase between droplets and allows merging of multiple droplets [44]. Electric forces [45-46] or optical forces have been implemented to enhance the merge rate [47]. However, previous results have been insufficient to quantitatively estimate the merging ratio of droplet numbers. When merging is accomplished by changing the flow rates of the
continuous phase or dispersed phase, other factors such as the size of droplets or the pattern of droplet-generating states are immediately affected [24, 44, 48]. Also, the location of droplet merging in the channel is hard to predict in the stream of continuous flow. The systems have to overcome the limited range of flow rates and the size of droplets on the events of droplet addition. Temporal trapping or retention of droplet in a designated place will be suitable for efficient merging and temporal storage of information in microfluidic circuit, although permanent storage of droplets have been accomplished with surface tension-guided docking sites [49]. There are also methods of chemical merging for droplets which will be introduced in chapter 4 and chapter 5.

![Figure 3.1](image)

Figure 3.1 Loading multiple components into droplets at the point of droplet-generation at the T-channel.

### 3.2. Principle of design

In this work, we integrated the functions of temporal stopping, merging, and splitting of droplets into one device to design a controllable droplet adder. The microchannel structure mimics an ancient counting tool, abacus, still used in Asian countries today. The method of
counting is very intuitive. Counting beads slide in grooves or along wires one by one. Our device adopts the approach of assembling liquid droplets in the groove structure instead of the solid beads, and when certain amounts of droplets reach the stopping position, they are autonomously evacuated from the microfluidic chamber rather than by using fingers. Fig. 3.2a shows the design of the droplet adding chamber with the cross-section of A-A'. We implemented another inlet for a control flow as bias, which sets the number of droplets to be added when droplet-generating flow rates are constant. Exclusively changing the control flow without integration of valves or external forces, demonstrations of a single-droplet addition, a multi-column addition with a carry, and merging of different droplets in the microfluidic abacus channel is illustrated.

Figure 3.2 (a) A schematic image of the microchamber for droplet merging. (b) Cross-section A-A’ of the droplet-merging chamber.
A control channel which is analogous to biasing in electronics can also be used to precisely determine the number of added droplets, when all other experimental conditions are fixed including the size of the droplets and the frequency of droplet-generation. Overall, a droplet is first confined in the corner of the groove because of the surface tension and the sudden change in the velocity in that region. While trapped, a number of incoming droplets from the droplet generating channel are sequentially added there and merge as one big droplet. As droplets enter the chamber the resistance of the channel will increase. The fluidic resistance $R$ between two points in a rectangular channel can be expressed as

$$R = \frac{\Delta P}{Q} = \frac{\alpha \eta L}{WH^2},$$

where $\alpha$ is dimensionless parameter, $\eta$ fluid viscosity, $L$ channel length, $W$ channel width, and $H$ channel depth, and shown schematically in Fig. 3.3. Setting the flow rate $Q$ constant during the process, the increase in $R$ will result in the increase of $\Delta P = P_1 - P_2$. This pressure increase will push the droplet out of the groove as they overcome the surface tension forces in the droplet. The added number can be manipulated by changing the control flow rate $Q$ through the additional control channel which will also increase $\Delta P$, and we can manipulate when the droplet will be evacuated from the addition chamber before certain numbers of droplets are reached when all other parameters are fixed. As droplets are added and become larger, the viscous drag force ($F \propto d \eta \nu$) of oil flowing upon the droplet will also become larger, where $d$ is diameter of the droplet assumed to be in spherical shape, $\eta$ is the viscosity, and $\nu$ is the velocity of the oil phase. When certain diameter is reached at the stopping position, droplets gain enough velocity to flow out of the expanding chamber. Increasing the flow rate of $Q_3$ will also increase the drag force and droplets flow out quickly with less
number of droplets merged.

Figure 3.3 Top view of the droplet-merging chamber showing streamlines of fluid flow and a simplified model of a channel network represented with register elements of electric circuit.
3.3. Experimental

The device is consisted of three parts: the droplet generating channel, the droplet adding chamber, and the outlet. First, droplets are produced in the droplet generating channel which can be either a T-junction [24, 50-51] or a focusing channel [52-53] in geometry. Then droplets are transported to the droplet-adding chamber which is an expanding channel with an abacus groove. The width of the expanding channel broadens to slow down the speed of droplets. Abacus groove is engraved in another layer which forms the bottom of the channel, and acts as a guidance of droplet flow in the chamber. The groove structure in this report is designed to have bent-shape with rectangular corners in which a droplet bumps into the wall and temporarily stops, providing a fixed location of droplet-stopping events. The control flow (bias flow) is introduced to the expanding channel through this groove layer and changes the velocity of flow in the chamber. The main flow of liquid which comes from the droplet-generation region veers towards the side depending on the shape of the groove, with the control flow entering the region through the control channel. The outlet channel from the adding chamber is narrow in the width to restore the flow rate and the resulting droplets are quickly evacuated from the adding chamber.

The device requires two slabs of PDMS aligned and sealed together (Fig. 3.4). The upper layer has the droplet-generating T-junction with two inlets for the continuous phase and the dispersed phase, and the expanding channel of the droplet-adding chamber. The bottom layer has an inlet for the control flow which is linked to the abacus groove. All channels in each of the slab were created by first transferring the pattern of a shadow mask to a negative photoresist film spun upon a silicon wafer. Then we poured a mixture of PDMS and curing agent (10:1 ratio) upon the resulting silicon master, and removed it after
curing. The depth of the channels in the upper layer was designed to be 40 μm, and the lower layer to be the half, 20 μm.

Mineral oil (Sigma-Aldrich Co., St Louis, MO) was used as the continuous phase including the control flow without no surfactant for efficient merging of droplets, and solutions of red and green food dye (Kemide Co., Jeonju, Korea) were used to create the dispersed phase. All fluids were injected via the syringe pumps (Pump 11 Pico Plus; Harvard Apparatus, Inc., Holliston, MA).

Figure 3.4. A schematic figure showing top and bottom layers of the PDMS slabs, each comprised of an expanding channel and the abacus groove. The complete channel is obtained by sealing two slabs of the channel layers.
3.4. Results and Discussion

3.4.1 Single-droplet addition

Fig. 3.5 is the schematic figure of the whole layout of the droplet-adding system. $Q_1$ is the inlet flow for the continuous phase (mineral oil) and $Q_2$ is the inlet flow for the dispersed phase (red dye) to generate droplets at the T-junction (I). $Q_3$ is another inlet flow of oil which provides the control flow connected to the lower layer of the device. It is branched off from the main flow of $Q_3$ to show that more branches can be added to a single control channel for further applications including multiple chamber control not shown in this report.

We performed experiments at three different conditions of constant $Q_1$ and $Q_2$ (Table 3.1). In each trial, only the flow rate of control flow ($Q_3$) was changed. First, the size and frequency of droplets were tested to examine the effect of the control flow. The length and the time intervals of the droplets were measured when they flow through the channel with the width of 60 μm and the height of 40 μm, before entering the adding chamber. The results indicate that droplets maintained the size and the generation frequency in the range of standard deviation as show in the Table 3.1, regardless of the change in control flow rates ($Q_3$) in each trial. Under the microscope, we could observe that a droplet first introduced to the expanding channel was temporally trapped at the stopping position and sequential addition of two, three, four or more droplets occurred at different $Q_3$. After certain numbers are reached they form large droplets and are evacuated to the outlet.

In all three trials, the number of merging droplets decreased with the increase in the flow rate of the control channel (Fig. 3.6). The experiments were conducted until the flow rate of $Q_3$ allowed no more droplet addition. At the maximum flow rate of $Q_3$, a single droplet temporally stopped at the stopping position and soon slipped out of the chamber before the
second droplet arrived (corresponding to the added number of droplets “1” in the graph). The faster velocity of the control flow applied larger forces to the droplet that it was quickly evacuated from the abacus channel with less time to merge with other droplets (Fig. 3.7a). The time intervals between the first droplets arriving at the stopping position of the abacus groove ($T_1$) in each set of addition correspond to the number of droplets merged (Fig. 3.7b). The faster the flow rate of $Q_3$, the shorter the renewal intervals of each addition set, and the less the number of the droplets was added. According to the change in the flow rate of the control flow ($Q_3$) when all other experimental conditions follow trial 3 in Table 3.1., were achieved.

Figure 3.5. The layout of two layers of the channel for the single-droplet addition. Dashed circles indicate three main parts of the system: the droplet generating channel, the droplet adding chamber, and the outlet.
Table 3.1 Experimental conditions and the length of the droplets from the droplet generating channel, passing through the droplet-merging chamber.

<table>
<thead>
<tr>
<th>Trial</th>
<th>$Q_1$ ($\mu$L/h)</th>
<th>$Q_2$ ($\mu$L/h)</th>
<th>$Q_3$ ($\mu$L/h)</th>
<th>Length (µm) (std*)</th>
<th>Time intervals (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>0-100</td>
<td>198.9 (3.6)</td>
<td>0.8</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>2</td>
<td>0-60</td>
<td>306.7 (18.0)</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>6</td>
<td>0-180</td>
<td>163.5 (0.6)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Figure 3.6 Microscopic images of the droplet-merging chamber showing two to five droplet-additions. Scale bars are 100 µm. The upper images show the sequential arrivals of the droplets and they are merged before leaving the chamber as shown in the lower images.
Figure 3.7 (a) The graph plotting the number of droplets added in each set of conditions. As the flow rate of control channel ($Q_3$) increases, the number of additions decreases. (b) The time intervals between the first droplets in each set of addition. The time was measured when the first droplet arrives at the stopping position.
3.4.2 Multi-column addition

Depending on the materials inside droplets, one droplet can convey versatile information. Along with on-off or binary calculation, manipulations of multiple numbers of droplets are necessary for broad lab-on-a-chip applications. The serial linking of the droplet adding chambers can work as a more complex counter. The layout of the system with three columns of the abacus channels is shown in Fig. 3.8. After certain numbers of droplets are merged in the first column (the rightmost), the resulting droplet is transported to the next column as a carry and it repeats the adding procedure again. The point in this design is to have all three columns to add the same number of droplets. The control flow is again introduced to the first adding chamber for this purpose. Since the resulting merged droplets are large in sizes, we splitted the added droplet with a T-junction, in the course of the outlet of the first and the second column, before it is transported to the next column.

The dimension of the chamber and the abacus groove is optimized to hold two to four droplets of diameters around 70-100 μm. In the experimental trial, we first set the flow rate of $Q_1$ and $Q_2$ to the range in which each three columns can add similar numbers of droplets. However, making all of them to add the exact same number is a difficult procedure, so we adjust the condition with the control flow (Table 3.2). Increasing the flow rate of $Q_3$ decreased the number of added droplets in the first column, and increased overall speed of the flow in all three chambers. On the other hand, the number of droplets to be added tends to increase in the second and third column, because the resultant droplet from the previous column is smaller in diameters and arrives at faster rates. On the contrary, decreasing the flow rate of $Q_3$ increased the number of droplets in the first column and decreased the number in the next two columns. However, the change of the two columns according to the
flow rate of $Q_3$ is relatively slow compared to the immediate effect on the first column, and we could adjust the desired number based on the observation from the first column. As shown in the result of Trial 2, all three columns could hold up to three droplets. After three droplets are merged in the first column, it moves to the second column and waits until two more droplets arrive resulting from consecutive six more droplets in the first column. Then the resulting droplet (original 9 droplets) again moves to the third column, waits again to fulfill the three resulting droplets (original 27 droplets) and evacuates to the outlet (Fig. 3.9). The lower images of the Figure 3.9 show the split of the droplet at the T-junction before it is transported to the next column. Less than a half of the droplet volume is transported autonomously to the next column and the leftover is evacuated to the outlet because the flow resistance is smaller towards the outlet than toward the next columns. Fig. 3.10 shows the time intervals ($T_1$) of the first droplets arrived in each column in each set of addition. The horizontal axis of the droplet number indicates the order of the first droplets arrived in the first column. The average time interval ($T_1$) of the first column is 1.45 s, the second column, 4.32 s, and the third, 13.28 s. $T_1$ ratio of the second column to the first column is 2.99, and the third column to the second column is 3.07. The resulting droplet from the third column can represent the unit of 32, the second column 31, the first column 30 in decimal numbers, both with the added number of droplets and the time intervals.
Figure 3.8 The layout of two layers of the channel for the three-column addition.

Table 3.2 Experimental conditions to adjust the number of droplets in three-column addition.

<table>
<thead>
<tr>
<th>Trial</th>
<th>(Q_1) (µL/h)</th>
<th>(Q_2) (µL/h)</th>
<th>(Q_3) (µL/h)</th>
<th>Added number of droplets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1st column</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>6</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>6</td>
<td>180</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 3.9 Microscopic images of three columns-addition. Each column accepts three droplets and after they are merged, they evacuate to the next column and to the outlet. The lower images shows the sequential droplet splitting at the T-junction before it moves to the next column.
Figure 3.10 The time intervals between the first droplets in each set of addition. The time was measured when the first droplet arrives at the stopping position. The droplet number is in the order of the incoming droplets.
3.4.3 Addition of two different droplets

Additions of two inputs of droplets were performed in the channel layout as shown in Fig. 3.11. Unlike the previous channels with a single droplet inlet, the adding chamber has two droplet inputs coming from the side of the expanding channel. The control channel is designed to enter the chamber through the center, linked to the abacus groove. Experimental Trial 1 and 2 only changed the control flow to change the number of droplets added, when all other conditions are fixed. Under the condition of Trial 3, we could acquire alternate addition of two different combinations of droplets (Table 3.3). Figure 3.12a is the microscopic images of 1:1 addition of the red and the green droplet. When the control flow rate is reduced, the number of addition is increased to total four droplets (2 greens and 2 reds) (Figure 3.12b). In this device, merging of different combinations of droplets was possible under the same condition with the aid of the control flow for precise adjustment. In Trial 3, a red droplet was added to the every other sets of 3 green droplets, resulting in the alternate output of green droplets and the mixed ones (Figure 3.12c).
Figure 3.11 The layout of two layers of the channel for two different droplet-addition.

Table 3.3 Experimental conditions when for two different droplet-addition.

<table>
<thead>
<tr>
<th>Trial</th>
<th>$Q_1$ (μL/h)</th>
<th>$Q_2$ (μL/h)</th>
<th>$Q_3$ (μL/h)</th>
<th>$Q_4$ (μL/h)</th>
<th>$Q_5$ (μL/h)</th>
<th>Added number of droplets (G: green; R: red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>0.05</td>
<td>26</td>
<td>0.05</td>
<td>35</td>
<td>1(G) + 1(R)</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.05</td>
<td>15</td>
<td>0.05</td>
<td>35</td>
<td>2(G) + 2(R)</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>4</td>
<td>10</td>
<td>1</td>
<td>25</td>
<td>3(G); 3(G) + 1(R)</td>
</tr>
</tbody>
</table>
Figure 3.12 (a) One green droplet is added to one red droplet when the control flow rate is 26 μL/hr. (b) Two green droplets are added with two red droplets when the control flow rate is decreased to 15 μL/hr. (c) Three green droplets are merged, and one red droplet is added to the alternative green droplets (Trial 3 of table 3.3). After the first green droplets (yellow circle) are evacuated, a red droplet is added to the next green droplets (red circles).
3.5. Conclusions

In this work, quantitative control of the number and the location of the droplet-based addition was demonstrated by temporally stopping the droplets with a sudden change of velocity in the microfluidic abacus channel. In most of the microfluidic droplet platforms, the function of the continuous phase fluid has been confined in the generation of droplets. This report introduces another approach of the flow to manipulate dispersed fluids more accurately in two-phase microfluidics, and new scheme of fluidic calculation analogous to the abacus structure with biasing function. The working speed of the system shown here is not very different with other microfluidic computation devices, which is in the order of 1 s down to 1 ms, and is not comparable with today’s silicon-based chips [54]. However, when applications are found in the field of microfluidics and lab-on-a-chips, the advantages include minimizing of the integration, interfacing, and conversion process of input or output signals with external forces or electronic signals, by directly performing the calculations of fluidic operations. Controllability of the number and periodicity of droplets provides the major advantages using this platform in microfluidic chemical or biological reactions which require time and volume-dependent reactions. Further developments to aid in the cell screening system will include optimization of channel dimensions suitable for single-cell containing droplets. Also, this system is designed for serial addition of droplets, each merging occurring one at a time, but development of this controlled merging scheme into a parallel merging system for high-throughput applications requiring the same amount of reaction time for each micro-container is demanded.

In this work very low concentration of surfactant was used to help merging of droplets. However, in most applications oil phase contains high concentration of surfactant to stabilize
the droplets, which will hinder the events of merging. Therefore design of microchannel structure which brings droplets into one place at the same is not enough to induce high-throughput merging of droplets [12]. Instead of physically inducing droplet coalescence, there is a chemical demulsification method which utilizes addition of other chemicals into the oil phase to induce phase separation. This method is utilized in later chapters of the thesis.
Chapter 4.

Microdroplet array with microgrid integration

4.1. Introduction

In previous two chapters, the work of single-cell encapsulation and merging chamber of microdroplets was investigated. In order to achieve the final goal of developing a droplet-based single-cell screening system, establishment of stable droplet array which can culture cells in droplets for long hours and secure droplets in place in the process of droplet merging and reaction is necessary.

Limitations in previous microdroplet-based array fabricated by either chemical patterning [55] or reservoir inside a microchannel [56] include instability of the droplets for long term incubation and lack of accessibility to the individual droplets for further reaction process or recovery. Usually trapping droplets is more difficult than trapping cells or particles because of droplets do not sediment quickly in the flow of oil and will not be trapped inside a small wells where their shapes have to be deformed and requires high surface energy. There has been a method to transfer droplets with the help of surfactant from each functional module[57] but without physical boundary for trap, it is hard to keep droplets in a fixed position for long time for tracking and inspection. With physical boundary, a sophisticated design is necessary to predict the behaviour of droplets which are highly sensitive to small perturbation including pressure, flow rate, the number of droplets which will hinder combination of various functioning modules of droplet manipulation. With help of microfluidics, microchannel array to store droplets stably has been developed[21, 58-60], but technique for repetitive addition of droplets or selection of specific droplets in a closed
channel by inspection is yet to be accomplished.

Although each method of droplet manipulation has been successfully demonstrated, integration into whole system has been a problem of droplet technology in carrying out a complete biological assay. The dynamic handling of microdroplets inside a microchannel requires a very complex system of stacking multi-layers of channels or integration of electrode which results in very bulky system. Usually relatively simple microfluidic array for droplets can perform only one kind of functional operations. However, repetitive procedures and multiple functions usually necessary in biological experiments cannot be achieved. Practically, capturing small diameter of droplets in tens of micrometer targeting single cells is especially difficult with their surface tension being high, and removal of unwanted droplets trapped in the array is also problematic.

4.2. Principle of design

We developed a simple method of creating picoliter-droplet array by integrating microgrid aligned on the microwell array. The aim of the design is to create a stable droplet incubation system which allows trapping of droplets with high efficiency, supplement of additional droplets or oil, and open access for easy recovery of the contents and integration with other microfluidic modules. We utilized a flat metal grid which allows free flow of oil to draw and guide droplets into the wells aligned beneath, for more stable storage and addition of other reagent-containing droplets(Fig. 4.1). The spacer is placed under the grid to hold oil phase right above the microwell array and also permit the easy flow of oil.
Figure 4.1 Micro-mesh grid aligned with microwell array to efficiently trap droplets for single-cell based screening.

Fig. 4.2a shows the side view of the mesh-integrated array system. The grid structure allows free flow of oil to pass through, and draws and guides droplets into the wells aligned beneath. Comparison of the microwell with and without microgrid integrated on the microwell array in the velocity towards the bottom of microwell is shown in Fig. 4.2b. The velocity \( w \) which facilitates the trap of droplets is much higher because of the microchannel structure formed right above the microwells. Vertical velocity (see \( w \) in Fig 2b) towards the wells is almost zero near the surface opening of the wells, and air will start to fill the wells as oil starts to evaporate. Therefore, as shown in Fig. 4.2c, droplets placed on the microwell array without the grid do not flow into the wells. Without the grid, droplets in oil will follow bulk flow of oil outside the microwells and will not flow into the narrow wells. However, the mesh-grid above the microwells acts like a microchannel structure to hold thin film of oil.
in the space between a grid and the PDMS array instead of bulk flow. When droplets fall through the grid following the streamline of the oil, they will move directly into the wells because the space between the PDMS array and the microgrid is set to be smaller than the size of droplets. They tend to stay in the wells where their shape deformation can be minimized, thus reducing surface energy. Once droplets are settled in the wells, residual droplets can be flushed out with the additional oil flow (Fig. 4.2d).
Figure 4.2 Comparison of the microwell with and without microgrid integrated on the microwell array. (a) Side view of the mesh-integrated microwell array. (b) Comparison of velocity in the direction towards the microwell which facilitates the trap of droplets. (c) Droplets placed on the microwell array without the grid do not flow into the wells. (d) Droplets placed on the microgrid first flow through the grid structure and to the wells because their surface energy will be lower in the microwells instead of the narrow space between the grid and the PDMS well.
Because each well must be occupied by one droplet with a single cell, we used the following criteria: (a) the microwell must be wide enough for a droplet, but not too wide to fit multiple droplets; and (b) the microwell must be deep enough to protect the droplet from being washed out by flow in the rinse steps, but not so deep that more than one droplet may be trapped on top of another. Because droplets for single-cell encapsulation are 25-35 μm in diameter, the mesh grid with 43 μm of the hole-width with 20 μm of the wire dimension was chosen to freely pass the droplets and the oil. The array was aligned beneath the microgrid with 25 μm space to let oil and the untrapped droplets out of the system. The microwell array was designed to the same pitch with the microgrid (63 μm) by having 40 μm in width and 23 μm apart from each other. The dimensions are summarized in Table 4.2 and the schematic image of the mesh grid is shown in Fig. 4.3. The force attracting droplets to the well ($F_γ$) is large enough to hold one droplet in a spherical shape to reduce its surface energy. As long as $F_γ$ is larger than the driving force of the oil flow, droplets will settle inside the microwells. Therefore, the space between the PDMS array and the microgrid is set to be slightly smaller than droplets so first droplets experience larger $F_γ$ due to low surface energy, and next droplets come into the place move to the next well or out with the oil flow.

Table 4.2 Commercially available microgrid, showing dimension of the 400-mesh grid chosen for single-cell droplet trap application.

<table>
<thead>
<tr>
<th>Microgrid</th>
<th>Hole</th>
<th>43 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS well</td>
<td>Wire</td>
<td>20 μm</td>
</tr>
<tr>
<td></td>
<td>Well width</td>
<td>40 μm</td>
</tr>
<tr>
<td></td>
<td>Well depth</td>
<td>30 μm</td>
</tr>
<tr>
<td></td>
<td>Spacer</td>
<td>s</td>
</tr>
</tbody>
</table>
Figure 4.3 Commercially available microgrid, showing dimension of the 400-mesh grid chosen for single-cell droplet trap application.

Because the mesh grid provides an open structure to the microwells, merging events of additional droplets could be easily performed with the chemical demulsification method, instead of integrating complex hydrodynamic merging channel or active components such as electrode. The presence of chemical additive such as butanol is known to shift equilibrium of the two phase system resulting in merging droplets in the confinement [61]. The open structure of the mesh grid also allows selective recovery of droplets from the array with a glass capillary tube.
4.3. Experimental

The structure of the array consists of a flat metal grid aligned above the PDMS microwells with spacers between them. The microgrids are commercially available in the electron microscopy application as TEM (Transmission Electron Microscope) support films. The materials are usually made of copper, nickel, or gold. Since the target diameter of droplets for single-cell applications are in the range of 25-35 μm, the 400-mesh grid with the dimension of the hole, 43 μm and the bar, 20 μm was purchased from Electron Microscopy Science (PA, USA). The PDMS microwell was fabricated by conventional photolithography and PDMS molding procedures. The spacer of 25 μm in height was fabricated by spincoating PDMS at 2500 rpm for 60 seconds, resulting in thin film of PDMS. The film was bonded with the PDMS slab containing the microwells and the resulting PDMS array was aligned with the microgrid under a microscope (Fig. 4.4). Dimension of the grid and microwell should be designed compatible with droplets for trap. Droplets too large will not pass through the grid and to the well. Droplets of small diameters will tend to be trapped more than one per one microwell. Also, they will easily flow out through the space between the grid and the array, resulting in more empty wells.

Fig. 4.5 describes the procedures of trapping microdroplets through the microgrid-integrated array and also merging droplets. Droplets are transferred to the grid from other droplet-generation modules by temporarily storing them in microcapillary. The excessive oil or droplet removal is facilitated by placing absorbing material such as absorption paper or cotton materials beside the grid. Droplet-merging after second droplets are placed in the array was facilitated by dropping octanol-containing mineral oil on the array, which will be discussed in the next section on merging of droplets. In this work, we used 40% (v/v)
octanol in mineral oil for its insolubility in water. Well-known material for demulsification of two-phase system is butanol. We used octanol (40% (v/v) in mineral oil) instead because octanol is insoluble in water and has less effect on droplets of water phase. The whole course of droplet trap, oil change, and addition of other droplets can be repeated many times for each purpose. Unlike other conventional well-structure which is blocked by surrounding walls, microgrid structure placed above the well array with spacer between them allows free flow of oil, therefore easily drawing the droplets into the mesh as oil phase flow out, and effectively removing air bubbles which may hinder the positioning of droplets.

Figure 4.4 Procedure for the assembly of the grid-integrated microwell array. (a) The PDMS microwell-array was fabricated by the conventional photolithography and PDMS molding. (b) The PDMS spacer which is fabricated by spin-coating of uncured PDMS to make the membrane was bonded on two sides of the slab of microwell array. (c-d) The micro-mesh grid was aligned on the microwell-array. (e) Microscopic image of the aligned mesh-grid on transparent PDMS wells. (f) Wetting of the microwell array with a drop of oil.
Figure 4.5 Schematic diagram for trapping and merging of droplets in the mesh-integrated microwell array. Each process of droplet trap, oil change, and addition of other droplets can be repeated several times.
4.4. Results and Discussion

4.4.1. Droplet trapping

Microdroplets occupy about 900 wells when placed in the PDMS well array under the metal grid (Fig. 4.6a). The number of array is limited only by the size of the microgrid commercially available and can be increased if the microgrid with larger area is fabricated. Fig. 4.6b is the enlarged image of the droplets trapped under the micromesh aligned on the microwells. The result shows 95% of the squares of the well array containing one droplet (Figure 4.6c) if droplets of proper sizes are selected. The graph shows the fraction of the wells containing single droplets is the hinges when droplets are around 40 μm in diameter. Droplets too large will not be able to pass through the grid and to the well efficiently. Droplets of small diameters tend to be placed more than one per one microwell, and also easily flow out through the space between the grid and the array, resulting in more empty wells. Droplets of 40 μm in diameter filled about 95% of the microwell array, and droplets of 30 μm in diameter resulted in about 80% of single-droplet occupancy slightly less than 40 μm droplets.
Figure 4.6 (a) Whole image of the metal grid placed on the PDMS microwell array with droplets trapped. (b) The enlarged image of the droplets trapped under the micromesh aligned on the microwells. (c) The graph showing the fraction of the wells according to the number of droplets trapped inside the microwells.
4.4.2. Droplet merging and recovery

In chapter 3, we created an environment for droplet merging to occur by synchronizing the time and space of droplets. The design becomes complex when more droplets are needed to take the parts in fusion. In addition, droplets are stabilized by high percentage of surfactant present in the oil phase, which will hinder droplet-merging in most of the practical application. Coincidence of the time and place of droplets alone is not enough. In this microdroplet-array, we utilized the chemical demulsification method of microdroplets instead of designing for complex merging channel. Butanol is known to shift the equilibrium of the two phase system of emulsion to that of separate oil and water phases. The demulsification process acts well in the mixture of mineral oil and Span 80 surfactant. Although butanol has very low solubility in water, to keep it away from cell-media droplets, we used octanol instead which is insoluble in water. Demulsification process is much slower with octanol in mineral oil compared with butanol, but with high concentration (40% (v/v) in mineral oil with Span 80, and 100% octanol for mineral oil with Abil EM 90) droplet merging occurred. 1:1 merging of droplets occurred in 83% of the wells (Fig. 4.7). With butanol, the merging event is much faster even in concentration as low as 5% (v/v) in mineral oil.

Glass capillary tube of 30 μm in diameter used to take out droplets from the microwells. It is also used to transfer droplets to the mesh-integrated array for trapping (Fig. 4.8).
Figure 4.7 The microscopic images showing merging of microdroplets trapped inside the microwells underneath the grid. (a) Trap of the first droplets in the microwells. (b) Addition of red droplets (the second droplets) to the initial droplet array previously formed with the first droplets. Scale bars are 100 μm. (c-d) Sequential images of before and after drop of octanol-containing oil is added to remove the surfactant present inside the oil phase, for droplet merging.
Figure 4.8 Recovery of selected droplets from the mesh-integrated microwell array with a microcapillary.
4.5. Conclusion

In our work, we utilized a flat metal grid which allows free flow of oil to draw and guide droplets into the wells aligned beneath, for more stable storage and addition of other droplets. The microdroplet array will help integration of single-cell encapsulation and droplet-merging modules by stably storing droplets inside the membrane through-holes. Optimizing the size of droplets for real experimental purposes, setting the optimal dimension for the effective trap, and designing a method for further recovery of droplets were investigated. When dimensions are compatible, trapping of droplets with high efficiency resulted in more than 90% of microwells filled with single droplets. Realizing supplement of additional droplets or oil, and open access for oil phase change or easy recovery of the contents showed the possibility to be utilized in cell-based screening application.
Chapter 5.  
Single-cell based screening with microdroplets

5.1. Introduction

We can detect the presence of specific-enzyme secreting cell by co-culturing with reporter cell which displays fluorescence signal by the product of the enzyme activity. The average response of the cells, commonly interpreted as the response of all cells in the sample does not guarantee the reactions of single cells. Additionally, the effect of cell number (signaling, interactions, etc.) on cell behavior is still often neglected due to difficulties in monitoring such detailed phenomena (Fig. 5.1). Common aims of the device for single-cell analysis are time-efficiency, high-throughput, instrument compatibility, transparency (for imaging and optical properties), cell accessibility (for further manipulation and treatment), stability and robustness (cell tracking), sensitivity, mimicking of in vivo conditions, and user-friendliness.

Figure 5.1. The model reaction of the enzyme-secreting cells and the detection cells inside a conventional test tubes.
Co-culturing of two different cells can be performed with coencapsulation of the enzyme-expressing cell and the reporter cell by merging of single-cell containing droplets. If we cannot utilize the single-cell encapsulation channel elaborated in Chapter 2 into the droplet trapping and analysis system, the efficiency of single-cell trap and creating environment for cell-to-cell interaction is to be kept very low as described in Fig. 5.2a.

Individual droplet acts as a separate well and can be stored in a large chamber with the use of amphipathic surfactant as in most of droplet-screening applications currently reported. The stability of droplets only depend on the quality of surfactant and throughput is relatively low. However their state becomes unstable as time passes because they will deviate from the initial position and tend to fuse with other droplets nearby. Identification and access of individual droplet is very limited. (Fig. 5.2b). Another form of droplet-array was created by designing the microstructures of reservoir inside a microchannel. Keeping the droplets inside a channel increases the stability, but has drawbacks that the design of the device becomes complex and it becomes difficult to combine with other devices of the droplet manipulation which will limit the utilization of various functions of droplets. The recovery of droplets after storage also becomes problematic.

We examined the applicability of the mesh-integrated array for single cell-based reaction by merging droplets containing specific enzyme TPL (tyrosine phenol-lyase)-expressing *E. coli* with the second droplets of reporter cells. The reporter cell is genetically modified *E. coli* which emits green fluorescence in the presence of the product (phenol) from the enzyme activity. To conduct biological assay, multiple steps of droplet manipulation have to be processed, including droplet generation with single cells, stable storage, and merging with additional droplets to activate reaction or supplement media (Fig.
The additional process such as changing the continuous oil phase of different constituent or recovery of droplets to recollect the selected cells for further analysis should be implemented.

The following function has to be actualized for the complete system. First, not only serial (sequential) operation of droplets but also parallel and simultaneous reactions has to be carried out to screen multiple cells at once. Second, each module such as the single-cell encapsulation into droplets and the supplementary delivery of cell media into each droplet has to be integrated. Third, stable storage of microdroplets inside the channel, and the change of the continuous phase (oil with different concentrations of surfactant) have to be easily performed. Lastly, the stored droplets for incubation also has to be recovered to recollect the gene library inside for further analysis.

![Diagrams](image)

Figure 5.2 (a) Schematic image showing the low probability of the chance that two droplets will meet, both containing single cells. (b) Droplets temporally stored in a microchamber stabilized with surfactant in oil.
Figure 5.3 Three functions required for droplet-based microfluidic platform for single-cell based screening application: (1) Single-cell isolation into droplets. (2) Stable storage of droplets. (3) Repetitive merging of droplets.

5.2. Experimental

We demonstrated the application of the microgrid-integrated microarray for an E.coli-based screening assay. Single-cell droplet-generating channel produced droplets having diameter of 30 μm [62] and they were captured in the mesh with high efficiency. Continuous events of merging of the cell-droplets with detector-droplets for cell-based screening and the media-droplets for long time incubation were accomplished.

The material change from the droplet-trapping assay in chapter 4 is in the use of surfactant Abil EM 90, instead of Span80. We used Abil EM 90 as surfactant instead of span 80 because Span80 has been reported to show high leakage of entrapped water-soluble molecules and not suitable for cell culture purposes [63]. Abil EM 90 in mineral oil also has demulsification effect [64]. The dimension of the microgrid and the PDMS microwell array used in this experiment is the same as in Chapter 4. The average cell concentration for experiments is set to be 1.2×10^8 cells/mL. Evaluation of the mesh-integrated microwell array
to test feasibility with single cells was performed with two different kinds of E.coli emitting green and red fluorescence for distinctive visualization.

First, the enzyme-expressing cells were encapsulated into single-cell droplets using the secondary breakup of droplet method [62], and they were transferred to the mesh-integrated microarray to be captured in the grid with high efficiency. Cofactor pyridoxal-5'-phosphate (PLP) to induce expression of enzyme TPL was also included inside the droplets. Continuous events of merging the additional droplets were performed with droplets containing reporter cells for detection of enzyme activity and droplets of LB media for long time incubation were accomplished, followed by the removal of the droplets to transfer the droplets to the agar plate for cell recovery. For encapsulating enzyme-expressing cells into droplets, we used 3%(w/w) Abil EM 90 as surfactant. All cell strains were modified and obtained from researchers of KRIBB. The initial loading concentration of cells into the microchannel for secondary breakup of droplets was $1.2 \times 10^8$ cells/mL for all experiments.

5.3. Results and Discussion

5.3.1. Trap of droplets containing single cells

Integration with the single-cell (E.coli) droplet-generating channel, which breaks the large droplets of high concentrations of well-dispersed cells into smaller sizes [62], was applied to the microgrid array. Single-cell containing droplets having diameter of 30 μm were captured in the array with high efficiency for the further process of reaction and incubation, and the cell-based screening assay. Fig. 5.4a and 5.4b shows the microscopic image of the single-cell (green fluorescence) containing droplets having diameter around 25–35 μm captured in the microgrid-integrated microwells with 48% efficiency (Fig. 5.4c).
Figure 5.4 Integration of the mesh-array with the single-cell droplet-generating channel. (a) The captured droplets on the micromesh contain single *E.coli* (green fluorescence) to be used for the further process of cell-based screening assay, and (b) the enlarged image. (c) Fraction of the wells filled with droplets according to the number of cells encapsulated.
5.3.2. Merging of droplets containing single cells

The integration with the microwell provides more stability that addition of more droplets into the previous ones become possible. The second droplets of red-fluorescence emitting cells were added to the first array of green-fluorescence cells. Although the efficiency of merging two different cells among droplets are 38% and does not seem to be high enough, it is higher than the number expected from Poisson distribution, and creation of microenvironment for cell-cell interaction of microbial cells in single-cell unit was first to be executed in microfluidic system no other previous method could achieve. Fig. 5.5 is the result of merging the second droplets with red fluorescent cells to the gree-fluorescence cell array, validating that the array can be used to co-encapsulate two different cells.

Figure 5.5 (a) Addition of secondary droplets containing red-fluorescence *E.coli* into the previously formed array of droplets with green fluorescent cells and (b) the enlarged image.
5.3.3. Droplet evaporation and transfer

The resulting 95% of the squares containing one droplet can make droplets stay in place even after the size shrinks due to evaporation. For long-term incubation the mesh can be covered with a large drop of mineral oil, and droplets are not affected for several hours. Droplets stay in place even after the size shrinks due to evaporation (Fig. 5.6a and 5.6b). Even with droplets (d=30 μm) of high concentration of red dye, which evaporated in a few tens of minutes, they stayed for more than an hour when covered with mineral oil on the array.

Figure 5.6 (a) The microscopic image of the mesh array showing 95% of the squares filled with single droplets. (b) The droplets fixed in the position after shrinkage due to evaporation. (c) Graph showing the shrinkage of droplet volume while trapped in the array.
For application to cell-droplets, the top of the droplet array was covered with mineral oil to prevent evaporation and supplementary media droplets were added to the array throughout the events at 1 hour intervals. By removing a droplet from the array and transferring it to solid agar plate, we could recover the target cell after culturing (Fig. 5.7).

Figure 5.7 A cell-droplet is removed from the mesh-integrated array (a) with a microcapillary with diameter in 20-30 μm and (b) transferred to the solid agar plate and cultured to show the recovery of the selected cells.

5.3.4. Cell-screening inside the array

Prior to experimenting with droplets, the reaction between the TPL-cells and the reporter cells was observed as shown in Fig. 5.8. The cells were co-cultured in a test tube by mixing two types of cell samples in total volume of 2mL. Fig. 5.8a shows the increase in the fluorescence from reporter cells start to appear even after an hour. The fraction of the cell numbers and the intensity also increase as time passes (Fig. 5.8b and 5.8c). However, since they all grow in the same media of the same container, the batch culture system cannot screen out the specific cells causing the results.
Figure 5.8 (a) The reporter cells with fluorescence signal when cultured with enzyme-expressing cells (TPL-cells) in conventional batch tube. (b) Comparison of the increasing population of the reacted reporter cells with time inside the test tube cultured with 10 μM phenol and TPL cells. (c) The increase in relative fluorescence intensity of the reporter cells in the test tube.
Next, the droplet-based cell screening experiments on the mesh-integrated microwell array was carried with three steps. First, the TPL-enzyme expressing cells and the reporter cells were separately cultured and prepared to have concentration of $1.2 \times 10^8$ cells/mL. Prior to the examination of single-cell droplets, droplets with multiple cells (5-10 cells per droplets) of host and the reporter cells were placed in the microwell array and studied. Cells did not grow well in presence of the surfactant Span80, so we changed the surfactant to Abil EM 90. Then we incorporated the single-cell droplet generator to the system. When two droplets merged, green fluorescence started to appear indicating the reaction. We compared the result of cell-interaction of single cell units with the results of multiple cells. Fig.5.9 shows the fluorescence images of the microwells when reporter cells start to emit fluorescence. Fig. 5.9a shows the result of fluorescence increases of reporter cells when cell concentration was about 5-10 cells per droplet and Fig. 5.9b shows the image when the proportion of single-cell droplet was controlled to be high with the secondary breakup channel for single-cell droplet generation. Figure 5.10a and 5.10b display graphs of population growth and increase in fluorescence activity comparing the multiple cells and the single-cell reactions. Since multiple cells will secret more enzyme and more products in the same confinement, the number of reacted reporter cells and fluorescence of overall system were higher than the results from the single cells. Less than 10% of the whole array showed reactions for single-cell droplet array and the results may be due to the low concentration of the product secretion from such low concentrations of cells and due to lack of cell viability in single-cell environment. We can estimate in this experiment that at single cell level, at least 3 h of incubation is required for sufficient accumulation of products and detection of reporter cells.
Figure 5.9 Microscopic images of the mesh-integrated microwell array trapping (a) the multiple numbers of the reporter cells cultured with enzyme-expressing cells in each droplet and (b) single-cell controlled droplets of reporter cells and enzyme-expressing cells.
5.4. Conclusions

We successfully achieved integration of the single-cell generation droplets resulting in more than 50% of single-cell encapsulated droplets to the microgrid-incorporated microwell array. The efficiency of single-cell encapsulation is the highest among any other microbial-cell isolation methods reported. This platform also carried out droplet merging events resulting in 37% of droplets showing cell-to-cell interactions. The evaporation of the cell-droplets was minimized by covering the wells with mineral oil, and addition of the media droplets for supplement. By creating the cell-friendly environment with the choice of materials including surfactant and octanol for droplet-merging, we could obtain result of
cell-cell interaction aimed for single-cell screening purposes. The host cells and the reporter cells are successfully coencapsulated in droplets, stored in the array for several hours, and the fluorescence signal from individual droplets was detected to increase with incubation time. With more investigation for more suitable environment of single-cell growth along with dimension of droplets, the microgrid-integrated microwell array will open a step towards high-throughput single-cell study.

In table 6.1, we compared the previous literature for single-cell analysis in microfluidics. The method developed in this thesis is comparable in the number of arrays, is excellent in the efficiency of single-cell trap especially with microbial cells, and has novelty in the functions of addition of other reagents and accessibility of droplets for recovery all in the simple design.

<table>
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<th>Microwell array</th>
<th>Micropost-trap</th>
<th>Droplets in wells</th>
<th>Stabilized droplets</th>
<th>Droplets in grid</th>
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<tr>
<td>He et al., Anal Chem, 2005</td>
<td>Bai et al., Lab Chip, 2010</td>
<td>Shim et al., JACS, 2009</td>
<td>Brouzes et al., PNAS, 2009</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>Number of arrays</td>
<td>18,000</td>
<td>1,500 for drops Up to 6,000</td>
<td>4,000</td>
<td>900</td>
<td>&gt;900</td>
</tr>
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<td>Single cell trap (Cell type)</td>
<td>&gt;80% (Mammalian cells)</td>
<td>70–90% (Mammalian cells)</td>
<td>27% (Microbial cells)</td>
<td>~30% (Mammalian cells)</td>
<td>~50% (Microbial cells)</td>
</tr>
<tr>
<td>Addition of the Reagents</td>
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<td>Pairing only &gt;50%</td>
<td>Impossible</td>
<td>Limited Electrical coalescence</td>
<td>Possible &gt;70%</td>
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<td>Impossible</td>
<td>Impossible</td>
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</tbody>
</table>
CHAPTER 6.
CONCLUSIONS

6.1. Summary

We have shown an interactive platform for manipulation of droplets by users can be simple, easy to use, and practical for real biological application in laboratory only with hydrodynamic control. Although there have been numbers of previous works focusing on a single functional manipulation of droplets, such as droplet merging or droplet trapping, realization of multiple functions in one platform has limited the use of the system in practical assay application or creation of novel methods. By integrating each droplet modules such as a single-cell droplet generator and chemical demulsification method of two phase fluid with the microgrid-integrated array, integration with the single-cell droplet generating channel, the mesh-integrated microarray allows immediate confinement of single cells and total isolation of each droplet-chamber throughout the whole process of droplet manipulation.

Prior to creating a droplet array, the device for single-cell droplets generation was developed. With conventional droplet-generating method, the proportion of single-cell containing droplets is usually kept less than 30%. Chances two different cells will meet in one droplet for reaction by droplet-merging will be even lower. We have presented the secondary breakup method of initial droplets into smaller droplets in the range of certain dimensions with simple hydrodynamics using the channel geometry or shear flow. The large droplets first generated from T-channel are compactly loaded with high concentration of cells to reduce the problem of cell adhesion, and they break for the second time at the
pinched flow channel into smaller sizes of average 30 μm in diameter which is compatible to containing one microbial cell (initially in media of $1 - 2 \times 10^8$ cells/mL). By loading high concentrations of cells into large droplets first, the problem of cell adhesion and uneven distribution of cells in a microchannel is greatly reduced. With this design around 50% of droplets collected in the outlet are expected to contain single *E. coli* inside. With the aid of hydrodynamic sorting module by sizes, this structure could be applied to collect single-cell droplet with percentage as high as 50%, in the various experimental conditions such as initial droplet volume, flow rate conditions, and the cell-loading concentration.

Second, a droplet-adding system utilizing the change in the pressure difference and the channel resistance with no other external forces other than syringe pumps was developed. The number of added droplets could be controlled without changing the size of the initial droplets and the device could be used to merge and mix different droplets at various ratios in a serial manner. Although this design is advantageous in bringing droplets into the same place at the same time, which are the key criteria for merging events, the methods need to be simpler to develop a ready-to-use platform in biological experiments performed in a laboratory.

For more practical application to cell-based screening system of microdroplets, we developed a novel and simple method of creating a femto-to-picoliter droplet array by utilizing microgrid structures. The micro-lattice formed by thin wires captures a droplet and guide them to the well-array aligned beneath by permitting relatively free flow of the oil phase. The trapped droplet inside the mesh stays in the position and this high stability enables long-term incubation, supplement of additional droplets, and recovery of the specific droplet. The array designed for the purpose of cell-based screening assay, and single-cell
droplets which will require the long-term incubation and open access to the droplets for the addition of reagents and specific selection. We could create 900 droplet-array only limited in numbers because of the size commercially available with grid structure. Since the array structure is separated from the droplet-generation channels, it could be integrated with the single-cell droplet generator by secondary breakup, and about 50% of the wells could be filled with single-cell containing droplets. The microgrid enabled repetitive process of droplet merging, and evaporation of droplets could be prevented by supplying the cell-droplets with media droplets. Any addition of droplets with chemical reagents or other cells could be obtained and about 37% of droplets could capture two different droplets with different cells, which will be only 13% achievable at most with previous method of dilution for single-cell capture. This research is also the first to merge droplets by chemical method of adding octanol in the oil. The oil change was also possible because the system is an open system. By integrating all functions listed above, we could screen cell reactions by cell-to-cell interaction of host and the reporter cells in single cell units.

6.2. Outlook and perspectives

In this research of interactive manipulation of microdroplets, simplicity comes from the control only by hydrodynamic means of flow rates and the channel geometry without the need of external forces and complex fabrication. Feasibility is tested by achieving the capacity of platforms to move, stop, break, and merge droplets, enabling various performances of the containers with precise control of both time and space. The system proved to be useful in real application by showing the applicability for single-cell based screening which cannot be performed with conventionally used equipments. Further
investigation of the optimal design of the mesh-integrated microarray platform in regards to the size, volume of droplets, the working flow rate, applications in various cell types and stable storage of droplets are the next research step.

With further development of the technique enabling the detection of single cells and low amounts of molecules secreted from single cells, the platform will advance the area of single cell studies and high-throughput screening. The ability of picoliter droplets which can immediately break and encapsulate various materials in liquid in a high throughput manner shows the most perspective outlook in the area of studying cells in natural environment, where there are millions of unknown microorganisms exist with no culturing method and screening tool in conventional laboratory equipment. In our work, the first step is to generate droplets from different samples of microorganisms which can be stocked in laboratory and create droplet array with the mesh-integrated microwell array. One target sample is *citobacter freundii* which also secrete TPL enzyme, at much lower concentration than the gene-modified TPL-*E.coli*. Once the system is validated in detecting the presence of *c. freundii* in arrays of droplets and selecting out the target cells from the mixture of different microorganism, this system will provide to be a valuable tool for discovery of new microorganisms and screen of novel gene sequences.

The openness of the system allows easy access to individual droplets and enables the cell recovery for further analysis. The possibility of cell recovery in microfluidics enables complete analysis of the biological assay which was hindered in most of the closed channel system. In this work, we carried out droplet handling processes including droplet transfer and recovery with microcapillary by manual approach, but automation process will be required for high-throughput applications. One option will be the use of micromanipulator,
but the equipment is expensive and also time-consuming when the number of array becomes larger. Since the device is an open system, we can integrate another microfluidic tools or channels. With careful design of microchannel with the ability of reversible bonding and accessibility to each of the well array, faster, simultaneous manipulation of droplets can be realized in the array.

Since the device is especially designed for applications in cell-to-cell interaction and single-cell based screening, creation of single-cell friendly environment is important. We removed all other external forces such as electric field which may affect the viability of cells. One parameter requiring further research will be the selection of materials both for droplets and the microwell structure. In this work, we used conventional cell media, but the surrounding fluid phase is oil with high concentrations of surfactant. Unexpected problems can include the lack of oxygen, osmotic pressure change due to quick evaporation of the media droplet, and bio-incompatibility of the surfactant. Finding a biocompatible surfactant which also enables merging of droplets in the presence of chemical demulsifier is very challenging. Materials of the aqueous phase and control of external environment of the system such as humidity management are also crucial for long term incubation.

The droplet-array integrated with microgrid structure is expected to provide a novel environment for complex two-phase microfluidics as well as new applications for the method of analysis in lab-on-a-chip devices. More options include not only in cell applications but there are lots of materials to be utilized in droplet technology including hydrogel, polymer, ionic liquid, liquid crystal, nanoparticles, etc. The stability of droplets placed in position inside the wells will greatly implement in many other applications from material science to chemical engineering which usually requires stable fixation of droplets
and long incubation time.
References


요 약 문

미세유체기반 단일 세포 스크리닝을 위한
미세액적의 인터랙티브 조작

본 학위논문은 미세액적의 유체역학적 제어를 위해 설계된 미세유체기반 시스템은 생물 기반 분석의 수행을 위한 간단하고, 적합하며, 유익한 플랫폼을 제공할 수 있음을 논한다. 피코에서 나노리터 크기의 미세액적의 실용성을 보일 수 있는 적용분야로는 Escherichia coli와 같은 미 생물 내의 유전자 라이브러리 스크리닝 실험이 있다. 세포 기반의 스크리닝 실험은 기존의 시험관이나 마이크로웰 플레이트에서 행해지고 있으나 단일 세포 단위의 세포 분리나 반응을 수행하는 어렵다. 단일 세포 기반 실험을 위한 플랫폼은 단일 세포의 분리, 특정 형질의 발현을 유도 또는 감지하기 위한 약물의 처리, 세포 배지의 공급 및 장시간 동안의 배양, 추가 분석을 위한 반응 세포의 수거 등의 조작이 가능하여야 한다.

먼저 이 연구에서는 이상 유체에서 생성되는 미세액적이 단일 세포 단위의 분리를 위해 가장 적합한 수단이 될 수 있을음을 제안하고 액적의 이차 분쇄 방법을 소개하였다. 이차 분쇄 방법은 기존의 미세액적 생성을 위한 미세유체 채널 내의 미세홈 구조들을 연결하거나 핀치형 유동을 도입하여 미세액적을 한 번 더 작은 크기로 분쇄하는 방법이다. 기존의 미세공정 또는 미세유체조작에 의한 방법에서는 단일 세포로의 분리 효율이 30%가 채 되지 않으나, 액적의 이차 분쇄 방법으로는 단일 세포를 포함한 액적을 50% 정도 수거할 수 있었으며, 다수의 세포를 포함하는 액적의 비율을 16% 이하로 낮추었다.

액적 기반 미세유체 시스템에서 반응물 또는 세포 배지의 공급을 수행하기 위해서는 액적이 합쳐질 수 있어야 한다. 이 연구에서는 주판형 홈 구조를 이용하여 미세액적이 모여 합쳐질 수 있도록 하였고, 전기회로의 바이어스와 같은 역할을 할 수 있는 컨트롤 채널을 통해 채널 내의 압력차를 변화시켜 추가적인 외부형의 작용 없이도 합쳐지는 액적의 개수를 조절하였다. 이 주판 형 구조는 한가지 액적을 다른 개수로 합치거나, 다른 종류의 액적을 다른 비율로 합칠 수 있으며, 연결된 여러개의 채널 공간 안에서 순차적으로 액적을 합치고 이동시킬 수도 있다.

마지막으로 본 연구에서는 미세액적을 손쉽게 모아 가두고 지속적으로 다른 액적과 합치기 위한 플랫폼의 통합 기술을 개발하였다. 미세 액적을 보관하기 위한 마이크로웰 어레이를 그룹화 형태의 격자구조물을 정립하여 열린 형태의 미세채널 구조가 결합된 미세액적 보관 어레이를 제작하였다. 이 격자구조물은 미세액적의 이동 경로를 보조해주어 미세액적이 마이크로웰에 안정적으로 가둬질 수 있도록 하는 동시에, 열린 구조이기 때문에 다른 추가적인 미세액적의 조작 및 다른 디바이스와의 연계가 가능하다는 장점을 가진다. 이 격자구조물이 정립된 플
랫폼을 이용하여 80% 이상의 마이크로웰에 단일 세포가 포함되어 있는 어레이를 형성할 수 있었습니다. 미세액적 간의 혼합, 장시간 배양 및 선택적인 액적의 수거 등의 기능이 한 디바이스에서 구현되도록 하였습니다. 어레이 내 피코리터 부피의 액적 각각은 단일 세포 단위에서의 세포간 상호작용이 일어나거나 세포 기반 스크린으로의 적용에 적합한 안정된 환경을 구축하였습니다.

본 학위논문에서는 최근 미세유체기반 기술이 극복해야 할 과제로 여겨지는 응용분야로의 적용을 위한 다양한 기능들의 통합 문제를 미세액적 기반 기술의 개발을 통해 해결하고자 하였습니다. 먼저 미세액적을 단일 세포 스크린에 필요한 여가기 기능을 구현함으로써 미세유체기반 플랫폼이 실제로 다양한 응용분야 쓰일 수 있는 가능성을 보였습니다. 더 나아가서는 단일 세포 단위의 상호 작용 반응을 대량으로 관찰할 수 있는 새로운 미세 환경을 만들었다는 의미를 가지며, 기존 실험실에서는 배양이 불가능한 현존하는 99% 이상의 미생물을 바로 캡처하여 고속으로 스크린 하기 위한 기술의 토대가 될 것으로 기대됩니다.

핵심어: 미세액적, 액적 어레이, 액적 합침, 액적 조작, 단일 세포 스크린ning