Microdroplet-Based Screening of Freshwater Green Algae

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담수 녹조류 선별을 위한
미세액적 기반의 스크리닝 기술 개발

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Advisor: Professor Je-Kyun Park

by

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Department of Bio and Brain Engineering
KAIST

A thesis 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 Ethics\footnote{Declaration of Ethical Conduct in Research: I, as a graduate student of KAIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.}

2013. 10. 24
Approved by

__________________________
Professor Je-Kyun Park
담수 녹조류 선별을 위한
미세액적 기반의 스크리닝 기술 개발

이 도 현

위 논문은 한국과학기술원 박사학위논문으로
학위논문심사위원회에서 심사 통과하였음.

2013 년 10 월 24 일

심사위원장 박 제 근 (인)
심사위원 남 윤 기 (인)
심사위원 정 기 훈 (인)
심사위원 김 필 남 (인)
심사위원 한 종 인 (인)
This thesis presents an integrated microfluidic platform for interactive single-cell screening of lipid-rich microalgae using droplet microfluidics. To achieve the efficient production of biofuels from algal biomass with economically viable process, selection and growing of highly productive lipid-rich algal strains should be preceded. Especially, the heterogeneity in the lipid accumulation among the individual cells in the same species should be monitored and investigated in a single cell level. Extraordinary advances in droplet microfluidics systems have been made on the basis of the development of droplet manipulation technology with encapsulation method in a single cell level. Monodispersed hydrogel microcapsules can be considered one of the microreactors which encapsulate a cell of interest by utilizing the droplet manipulation technology in a high-throughput manner, and are then transported downstream to allow the quantitative monitor the lipid accumulation of each alga. Also, microcapsules ensure the algal cell viability and capability of other analysis after determination. To guarantee the enhanced single-cell encapsulation efficiency, a new fluid introduction scheme with reduced particle adsorption was first proposed. This idea is based on a novel microparticle injection technique with lateral interconnection, which prevents particle loss, assisted by sample injection along the direction of fluid flow. Biological sample fluids, including fluorescent microparticles, and mammalian (U937) and green algae cells (*Chlorella vulgaris*), were injected directly via a through-hole drilled in the lateral direction, resulting in a significant reduction in microparticle attachment. It was confirmed that the proposed method accomplished a 100% enhancement of single-cell encapsulation compared to a Poisson distribution. At the high concentration (5×10^7 cells/mL) of *C. vulgaris*, the fraction of droplets containing single cells increased gradually from 28.67% to 40.00%. At a lower concentration (1×10^7 cells/mL), there was an increase in the percentage of droplets including single cells from 15.33% to 35.33%.

Secondly, a facile and robust microfluidic platform enabling uniform interval control of flowing droplets for the precise temporal synchronization and pairing of picoliter droplets with a reagent was presented. The microbridge structures interconnecting the droplet-carrying channel and the flow control
channel were associated to derive a fluidic pressure drop between two microchannels via the microbridge structures, reordering flowing droplets with a defined uniform interval. The droplet intervals were flexibly and precisely adjusted through the change of the control oil flow rate. With this mechanism of droplet spacing, the gelation of the alginate droplets as well as control of the droplet interval was simultaneously achieved by additional control oil flow including calcified oleic acid. In addition, controlled synchronization and pairing of two distinct droplets were demonstrated by parallel linking identical microfluidic modules with distinct sample inlet. As a new microfluidic interactive sorting technologies, a hybrid optoelectrofluidic platform which allows on-demand release of target microparticles from specific microwell with simple optical configuration was also suggested. The combination of an optoelectrofluidic device and the photopolymerizable hydrogel microwell arrays enables interactive and programmable releasing using optically induced virtual electrodes. The microparticles can be passively loaded to the separate microwells via sedimentation and the trapped particle was individually repelled and positioned close to the ground electrode by optically-induced negative dielectrophoretic forces when a dynamic image pattern was projected into an area of the specific microwell under the application of AC signal with 20 V bias at 100 kHz. I also demonstrated the selective and parallel release of distinct particles from each microwell.

Finally, all the above-mentioned technologies, including encapsulation, manipulation, and interactive selection, were contributed to develop a new biofuel screening platform based on the droplet-microfluidics. Three species of green microalgae, (Chlorella vulgaris, Chlamydomonas sp. and Botryococcus braunii) were encapsulated in the monodispersed hydrogel microcapsule, and the quantitative comparison of their lipid content of individual cells in situ by tagging with BODIPY dye was performed. Stochastic heterogeneity in the lipid content was verified under a highly viable physiological condition, implying that other analyses were possible after the determination of lipid content. Furthermore, the designed microwell arrays enabled us to distinguish the BODIPY fluorescence response of a single live alga within the microcapsules. The newly proposed microcapsule-based interactive platform for screening of lipid-rich microalgae in this dissertation is now technically ready-to-use for exploiting the alternative energy and will provide versatile tools for environmental engineering research.

**Keywords:** Hydrogel microcapsule, Lipid productivity, Microalgae, Microdroplet, Microfluidics, Optoelectrofluidics, Single-cell encapsulation
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# Nomenclature

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<thead>
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<th>Symbol</th>
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<tbody>
<tr>
<td>Ca</td>
<td>Capillary number</td>
</tr>
<tr>
<td>h</td>
<td>Channel height</td>
</tr>
<tr>
<td>k</td>
<td>Droplet containing number</td>
</tr>
<tr>
<td>Qc</td>
<td>Control oil flow rate</td>
</tr>
<tr>
<td>Qo</td>
<td>Oil flow rate</td>
</tr>
<tr>
<td>Qr</td>
<td>Aqueous flow rate</td>
</tr>
<tr>
<td>Qw</td>
<td>Water flow rate</td>
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<tr>
<td>(v_{\text{max}})</td>
<td>Maximum velocity</td>
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## Greek letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>(\gamma)</td>
<td>Surface tension at the water/oil interface</td>
</tr>
<tr>
<td>(\Delta p)</td>
<td>Pressure drop</td>
</tr>
<tr>
<td>(\eta)</td>
<td>Viscosity of fluid</td>
</tr>
<tr>
<td>(\lambda)</td>
<td>Average number of cells per droplet</td>
</tr>
<tr>
<td>(\mu_{\text{oil}})</td>
<td>Viscosity of continuous phase</td>
</tr>
</tbody>
</table>

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>Three-Dimensional</td>
</tr>
<tr>
<td>a-Si:H</td>
<td>Hydrogenated Amorphous Silicon</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating Current</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled Detector</td>
</tr>
<tr>
<td>CFD</td>
<td>Computational Fluid Dynamics</td>
</tr>
<tr>
<td>DEP</td>
<td>Dielectrophoresis</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorter</td>
</tr>
<tr>
<td>FADS</td>
<td>Fluorescence-Activated Droplet Sorter</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>LCB</td>
<td>Low Conductivity Buffer</td>
</tr>
<tr>
<td>LCD</td>
<td>Liquid Crystal Display</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-Activated Cell Sorter</td>
</tr>
<tr>
<td>nDEP</td>
<td>Negative Dielectrophoresis</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PECVD</td>
<td>Plasma Enhanced Chemical Vapor Deposition</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>Polyethylene Glycol Diacrylate</td>
</tr>
<tr>
<td>PR</td>
<td>Photoresist</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>RIE</td>
<td>Reactive Ion Etch</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-Assembled Monolayer</td>
</tr>
<tr>
<td>SiNx</td>
<td>Silicon Nitride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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1.1. Biofuels from microalgae

The energy crisis associated with the shortage of fossil fuels has grown all the more serious since the past few decades.[1,2] In response to the skyrocketing their prices and the sudden acceleration to global warming by the Greenhouse effect, substantial concerns has grown that the discovery and development of alternative energy sources. Biofuels, which includes biodiesel from oil crops, soybean, sunflower, corn and microalgae, have ignited interest and research efforts as a potential alternative of fossil energy due to their sustainable productivity. Most experts forecast that the global biofuels industry will increase more than double in the next decade. In the Republic of Korea, from 2006, the production of renewable energy has been dramatically increased, that portion of biodiesel production was 5.75% in 2010 (Figure 1.1). Among the many great feedstocks, microalgae are the best choice for the biofuel production due to their capability of high oil yields and fast growth-rate.[3,4] Furthermore, sustainable energy from microalgae would be competitive with other biofuel sources for providing the answer to the world food crisis. However, there have been several economic issues to realize the large-scale biodiesel production that the recovery of microalgal biomass is currently expensive.[5] Most strains of microalgae are typically very small with diameter and have thick cell wall, thus the lipid extraction by the dewatering mechanism is more complicated.[6] Also, the relatively low concentration of biomass in the individual algae cell limits its commercial applications due to the requirement of high energy of the centrifuge with the costliness. Therefore, selection and growing of highly productive lipid-rich algal strains should be preceded to achieve the efficient production of biofuels from algal biomass
with economically viable process.[7-9] Many environmental scientists have focused on the interspecies comparison to investigate the product of biomass productivity and lipid content under different environmental conditions.[10] On the basis of literature data, the fatty acid profiles have identified consistently with the recent experimental reports, but there was an individual difference among the algae cells of same species under equal environmental conditions. Hence, the isolation of lipid-rich microalgae in a single-cell level that can be applied to select and collect certain strains of microalgae for high-yield lipid production is required (Figure 1.2).
Figure 1.1. Yearly production of new and renewable energy from 1999 to 2010 in the Republic of Korea. Renewable energy includes solar, bio-gas, biodiesel, wood, wind, hydros, fuel cells, and waste gas. From 2006, the production of renewable energy has been dramatically increased. Percentage refers to the proportion of biodiesel production (Reference: Yearbook of Energy Statistics, Korea Institute of Energy Research (KIER), 2011).
Figure 1.2. Algae biofuels production process. The algal cell can be cultured and divided by photosynthesis, which is the process that converts CO₂ into organic compounds with high energy content. Lipids induction can be started under the environmental change, such as nutrient starvation, temperature shift, and change in salinity and pH. After production, the algae strain needs to be harvested and isolated and the lipids will be extracted with the remaining cell recovered. To isolate the best algae strain, a new selection strategy such as Lab-on-a-chip technique will be required to maximize the potential of microalgae for biofuel production.
1.2. Microdroplet technology for single-cell based screening

For understanding the intrinsic specificity and feature under physiological environments, it is essential to regulate the cellular function and measure the signatures of cells with single cell resolution. Most biological measurement tools such as fluorescence microscopy and flow cytometry have enabled the study of the individual cell measurements with numerous advances.

Microfluidics, which refers to the fluidic behavior and precise compartmentalization of fluids in microstructures,[11,12] has been a driving force for innovations in simplifying and enabling high-throughput and multiplexed biological experiments for single-cell analysis.[13-17] The application of microdroplet technologies to bioanalytical systems, which advance droplets as tiny-volume units of fluid that are enveloped by immiscible fluid such as oil, has resulted in a state-of-the-art method of isolation and encapsulation of individual cells for a variety of purposes.[18-20] Droplet-based microfluidics can achieve rapid production of highly monodispersed droplets in micro-sized diameter,[21,22] and thus droplets can be used to construct a cell-like environment which includes a single cell.[23] Contamination-free isolation of single cells can be achieved by stabilizing the droplet interface with surfactants,[24,25] which leads to facilitate the long-term biological experiments such as amplification of DNA by polymerase chain reaction (PCR),[26-29] directed molecular evolution,[30-33] and cell culture.[34,35]

Another fascinating example of the significant capability of droplet-based microfluidics is the synthesis and assembly of hydrogel microcapsules. Hydrogels are an exciting class of
scaffolding polymer in biomedical applications because of their high biocompatibility, "smart" responsibility of the local environment, and ease of crosslinking under mild reaction conditions which are acceptable to the encapsulated cells. Various material compositions can be utilized to fabricate hydrogels, including alginate, agarose, polyethylene glycol (PEG), polyvinyl alcohol and hyaluronic acid (HA). The advantageous points of droplet-based microfluidics would be able to fabricate highly monodispersed microcapsules containing individual cells with a uniform size in a high-throughput manner.[36-38] This cell-loaded hydrogel microcapsules allows for an effective delivery of the microcapsules into the targeted site and cell manipulation in the maintenance of high viability and cultivability.[39] Several microfluidic approaches were demonstrated for the encapsulation of single cells of yeast,[40] mammalian,[37,41,42] cartilage,[43] embryonic cells,[44] and multicellular organisms[42] in hydrogel microcapsules. Such hydrogel microcapsule-based encapsulation strategy will serve to further applications in cell-based diagnostics.

However, there is an intrinsic limitation for studies single-cells in droplets because of the stochastic cell loading dictated by Poisson statistics, which is given by the following the equation (1.1).[42,45] The probability of a droplet containing $k$ cells is:

$$f(k,\lambda) = \frac{\lambda^k e^{-\lambda}}{k!}$$

where $\lambda$ is the average number of cells per droplet. During droplet generation, variability in the number of cells per droplet makes a critical constraint which reduces the number of empty and multi-cell droplets. At the low concentration approximately $1\times10^6$ cells/mL, cells are not evenly distributed in the cell suspension, which leads to increase the majority of the
empty droplets with low efficiency of the single-cell encapsulation. Compact cell loading at higher concentration will also perturb the Poisson statistics due to the channel clogging and forming of multi-cell droplets. To enhance the efficiency of single-cell encapsulation into monodisperse droplets, several methods were introduced such as using hydrodynamic focusing and sorting,[21,46,47] a laser,[48] closed-packed[49] and deterministic ordering of cells.[50,51] However, these methods require the additional flow, specific dimensions of microchannel, high input flow rate and compact loading of the cells at higher concentration. In spite of the high yield of single-cell droplet encapsulation, it will be potential to damage to the cells due to high shear rate. Also, the high pressure would lead to fluid leakage at the fluid inlets due to the fluidic resistance.
1.3. Spatiotemporal control of droplets for microfluidic interactive manipulation

The characterization of droplet formation and dynamics was well-organized over a range of fluid viscosity, flow rate, and interfacial tension between the oil and water phase. In two-phase flow, the formation of plugs can be characterized by the dimensionless capillary number, $Ca$,

$$Ca = \frac{\eta v}{\gamma}$$

where $\eta$ (kg m$^{-1}$ s$^{-1}$) is the viscosity of fluid, $v$ (m s$^{-1}$) is the velocity of the flow and $\gamma$ (N m$^{-1}$) is the surface tension at the water/oil interface. The two classic methods to form a droplet train from a single solution or fluid are the generation of droplets in a T-junction and flow-focusing.

The outstanding characteristics of droplets that represent potential in the field of interactive droplet manipulation include the ability of synchronization and precise combination of droplet train containing distinct reagents.[52] Additionally, microfluidic merging of the synchronized droplets opens up opportunities to achieve the combinatorial pairing and coalescence for multiplexed assays and polymer encapsulation. Several technologies of spatiotemporal droplet manipulation for droplet merging in microfluidic networks have already been applied for various applications to puzzle out biological and chemical problems.

Spontaneous and combinatorial pairing of droplets requires precise synchronization of approaching droplets in time and space by adjusting the droplet interval. Numerous
considerations are involved in the design of microfluidic networks to prevent irregularities in the spacing control of droplets, such as integration with additional microfluidic components including electrode for applying electric field[53,54] or multi-layered chambers[55] for temporal stopping droplets. Especially, integrating valves with microfluidic network can produce the large number of droplets and control their size and composition by sequentially switching on and off the microvalves corresponding to each fluid.[56-58] Arbitrary pairing of droplet trains can also be generated in cartridges of many different reagents by on-demand aspiration and be used for a range of applications.[59,60] In addition, passive hydrodynamic coupling at two opposing nozzles to produce droplet pairs was demonstrated for flexible control of pairing aspects.[61,62] Using a ladder structure interconnected between two main channels, the pressure difference between two microchannels was balanced automatically by the cross flow of carrier oil through the ladder structures.[63] The railroad-like channel network facilitates the precise manipulation of pairwise droplets for droplet merging in a passive manner.[64]

To perform the consecutive study in droplet-based microfluidic devices, it is necessary to build a microfluidic platform to transport and immobilize droplets for convenient signal detection, and to retrieve selected droplets. A large number of microstructure arrays, stem from the microarray, maximize the strength of fast production of droplets to facilitate the high-throughput and multiplexed screening of droplets. Recent strategy in droplet-based microfluidic systems for trapping droplets have demonstrated using the hydrodynamic confinement of droplets within the static[65,66] and dynamic microarray.[67] mesh-
integrated microdroplet array,[68] hydrophilic-in-hydrophobic micropatterned surface,[69] and anchor structure for active guidance.[70,71] Also, the electric field was applied to trapping droplet in the microwell within the microchannel[72] and the integrated circuits.[73] In addition, the retrieval techniques of the entrapped individual droplet from microstructure are important to the droplet screening. Recent progress has introduced to develop the user-friendly sorting, however, they require complex fabrication and high-cost equipment such as laser,[65,74,75] electrode[76], and pneumatic actuation.[77]
1.4. Research objectives

This thesis, focused on developing and establishing a new biofuel screening platform based on the droplet-microfluidics, which is able to single-cell encapsulate the high biodiesel producing microalga into the monodispersed hydrogel microcapsules with a high-throughput manner. The picoliter-sized alginate hydrogel microcapsules, are obtained with high throughput and high uniformity, serve as a smart identification (ID) card which contains biometric signature such as their phenotype, lipid content and viability. By incorporating with the technique of interactive droplet manipulation, the identification and selection of the lipid-rich microalgae in a single cell level is performed in this thesis.

First, I introduce a new microfluidic interconnection for microparticle injection, which utilizes lateral flow in the PDMS-based microfluidic device. The proposed device utilizes the lateral movement of the microparticles and cells resulted in a significant reduction in nonspecific binding to microchannel walls with no surface modification. In this regime, single-cell encapsulation into monodispersed microdroplets was successfully demonstrated and yielded an efficiency enhancement due to the reduced number of empty droplets.

Second, I propose a platform for spatiotemporal control of individual droplets. I present a facile and robust microfluidic platform enabling uniform interval control of flowing droplets for the precise temporal synchronization and pairing of picoliter droplets with a reagent. With this mechanism of droplet spacing, the gelation of the alginate droplets as well as control of the droplet interval was simultaneously achieved by additional control oil flow including calcified oleic acid.
Third, I demonstrate an optoelectrofluidic device integrated with photopolymerizable hydrogel microwell arrays for facile selection of individual microcapsules. This combination enables interactive and programmable releasing using optically induced virtual electrodes. I also demonstrate the selective and parallel release of distinct capsules from each microwell.

With this new manipulation method, I first demonstrate the encapsulation of high-level biodiesel-producing microalgae into an alginate hydrogel microcapsule based on droplet-based microfluidics, as well as the in situ analysis of lipid content at the single-cell level. Finally, I achieve the interactive selection of the microcapsules containing lipid-rich microalgae by the quantitative comparison of their fluorescence signal.
Figure 1.3. Research objectives of this thesis. Using a new microfluidic interconnection for microparticle injection, which utilizes a lateral flow in the PDMS-based microfluidic device, I present a facile and robust microfluidic platform enabling uniform interval control of flowing droplets for the precise temporal synchronization and pairing of picoliter droplets with a reagent. With this new manipulation method, I first demonstrate the encapsulation of high biodiesel producing microalgae into an alginate hydrogel microcapsule for quantitative estimation of the lipid content of individual microalgae.
Figure 1.4. Through this dissertation: (i) Single-cell encapsulation of green microalgae with enhanced efficiency was demonstrated. (ii) The gelation of the hydrogel alginate droplets containing microalgae as well as control of the droplet interval was simultaneously achieved. (iii) The feasibility of the entrapment of the microalgae-laden hydrogel microcapsule and the selection of the microcapsule containing the lipid-rich microalgae were proposed and examined.
1.5. Chapter outlines

This thesis is articulated into the following sections:

**Chapter II** first describes the enhanced single-cell encapsulation based on a new fluid introduction scheme with reduced particle adsorption. A 100% enhancement of single-cell encapsulation compared to a Poisson distribution under the proposed regime was confirmed.

**Chapter III** deals with a facile and robust microfluidic platform enabling uniform interval control of flowing droplets for the precise temporal synchronization and pairing of picoliter droplets. In this chapter, the achievement of the gelation of the alginate droplets as well as control of the droplet interval was presented.

**Chapter IV** presents a new microfluidic interactive sorting technologies by using the hybrid optoelectrofluidic platform which allows on-demand release of target microparticles from specific microwell.

**Chapter V** describes a new biofuel screening platform based on the droplet-microfluidics, which is able to single-cell encapsulate the high biodiesel producing microalga into the monodispersed hydrogel microcapsules with a high-throughput manner. Three species of green microalgae, (*C. vulgaris*, *Chlamydomonas* sp. and *B. braunii*) were encapsulated in the monodispersed hydrogel microcapsule, and the quantitative comparison of their lipid content of individual cells by tagging with BODIPY dye was performed.

**Chapter VI**, lastly, presents discussion and conclusions of the newly developed methods for microcapsule-based interactive platform for screening of lipid-rich microalgae.
CHAPTER II: Microfluidic single-cell encapsulation

with lateral interconnections

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2.1. Introduction

In response to the growing demand for miniaturized analytical systems, the microfluidic systems have been widely used in the fields of biology, chemistry, and nanotechnology.\cite{78}

In particular, a technique for generation of cell-containing nanoliter droplets improves manipulation and screening capabilities to facilitate high-throughput analysis.\cite{41,50,68,79,80}

Most droplet-based microfluidic devices have been fabricated from a hydrophobic polymer, poly(dimethylsiloxane) (PDMS),\cite{81,82} by a rapid prototyping method, which is widely used due to its high biocompatibility, good optical transparency, and compatibility with lab-on-a-chip techniques. The strong antipathy of water to the hydrophobic wall is an attractive attribute and facilitates formation of stable droplets. However, there are significant opportunities for the adsorption of organic solvents, small molecules, and particles around the channel inlet due to the innate hydrophobic nature of PDMS microchannels.\cite{83}

Nonspecific interactions, such as hydrophobic and Van der Waal’s interactions, between particles and the microchannel wall are major causes of reduced reliability and functionality in PDMS-based microfluidic devices.

To address the above issue, various strategies for prevention and elimination of biofouling have been introduced (Figure 2.1). In particular, \textit{in situ} surface modification of PDMS microchannels is commonly carried out by chemical treatments using self-assembled monolayer (SAM) coatings.\cite{84-86} However, the main shortcoming of these methods is that there is no guarantee of uniform coating and durability. For example, their use may result in undesirable reactions when surfactants (e.g., Tween 20 and Pluronic solution) react with the
oil phase. The unwanted debris generated by the reaction causes clogging, which interrupts particle movement and decreases cell encapsulation efficiency. The rapid recovery of the original hydrophobicity within several minutes to several hours is also a critical drawback for long-term microfluidic studies. Interestingly, the electrohydrodynamic buoyancy can be used for removal of adherent particles. Kim et al.[87] proposed a wall loss reduction technique operated by an AC electric potential from interdigitated electrodes integrated at the bottom of the microchannel. Kim et al.[88] reported a method for nanofabricating polyethylene glycol (PEG) hydrogels on the microchannel surface to improve surface hydrophobicity. Although they showed impressive results, complicated electrode structures and intensive fabrication processes were required, which limits application in integrated microsystems.
Figure 2.1. Various strategies for prevention and elimination of biofouling. It is important to reduce the settling force around the microfluidic inlet for reduction of microparticle accumulation. *In situ* surface modification of PDMS microchannels is commonly carried out by chemical treatments using self-assembled monolayer (SAM) coatings. The high input flow rate passing through the well-defined interconnections is also required.
Since encapsulation into droplets follows a Poisson distribution,[42] surface fouling results in a decrease in particle encapsulation efficiency due to the higher rate of empty droplets relative to positive droplets. The occasional clogging by adsorption at the narrow constriction also provokes inefficient particle loading in drops. This inefficiency has been improved by deterministic single-cell encapsulation within droplets using inertial ordering with a high flow rate.[50,89] Despite the high yield of single-cell encapsulation, there is an increased possibility of cell damage due to the high shear rate. In addition, the high pressure would result in fluid leakage at the inlets due to fluidic resistance.

In the former approach, a through-hole type interconnection for particle injection, the plastic tubing was penetrated and connected through inlet holes that were perpendicular to the flow direction.[90-92] As the flow was injected, the settling force around the inlet would be increased, and thus particle adhesion would be aggravated by particle sedimentation [Figure 2.2(a)]. Introduction of sample fluid, including microparticles and cells, with a high flow rate may contribute to prevention of particle adhesion, but it is difficult to tune the low droplet frequency and control the droplet size accurately at such high flow rates. Solvas et al.[93] utilized a mini-magnetic stirrer to stir the cell suspensions within a commercial syringe that contained a tiny magnetic stir bar. However, this method cannot guarantee prevention of particle adhesion around the microfluidic inlet. As a remedy for the adsorption problem, I propose a novel interconnection approach assisted by lateral injection of the sample fluid via a direct side interconnection [Figure 2.2(b)]. The inlet holes connected to a syringe tube were easily punched through the side of the PDMS microfluidic device in the
same direction as the fluid flow. Several other methods have already been described for delivering fluid laterally into silicon-based microfluidic channels using complex fabrication methods [94,95] or into PDMS-based microfluidic channels through a glass capillary [96], but no attempts have been made to realize the well-defined interconnection through the side of the PDMS microfluidic device with a simple fabrication process to connect the plastic tube. This simple punching method contributes to the well-defined interconnection through the side of the PDMS microfluidic device. In addition, the defined interconnection holes were fabricated quickly and robustly irrespective of the complexity of the microfluidic network. By connecting the injection tube to the defined holes directly, I demonstrate smooth introduction of microparticles and cells with reduced particle adsorption. In addition, I present two significant applications to emphasize the advantageous points of the digital microfluidics: (i) multilayer 3D microdroplet circuits, and (ii) encapsulation of single cells in microdroplets. This microparticle injection method reduces the number of empty droplets while increasing the single-cell encapsulation efficiency.
Figure 2.2. A schematic cross-sectional drawing of (a) the conventional PDMS-based microfluidic device and (b) the proposed microfluidic device for microparticle injection. (a) Conventionally, the plastic tubing containing sample fluid penetrated and was connected through the inlet holes, which were perpendicular to the flow direction. As fluid was injected, the settling force components acting in a negative z direction would be increased around the inlet, and thus particle adhesion would be aggravated by particle sedimentation. (b) In the proposed device, the plastic tubing was connected to the well-defined interconnection through the side of the PDMS microfluidic device in the same direction as the fluid flow. In this regime, the laterally injected microparticles and cells are directed into the middle of the fluid stream, which is free of both the settling force and nonspecific surface–particle interactions.
2.2. Experimental

2.2.1. Design and fabrication

Figure 2.3 shows a schematic diagram of the overall process for fabrication of the microfluidic device with lateral interconnections. A microfluidic device was fabricated using a conventional PDMS (Sylgard 184; Dow Corning, Midland, MI) molding process. The mold for the PDMS replica was fabricated by SU-8 patterning on a silicon wafer. For microparticle applications, the channels were 100 μm in width and 35 μm in thickness. The outlets for fluid collection were punched out using a punch with a diameter of 1.5 mm (Harris Uni-Core Punch; Ted Pella Inc., Redding, CA). A PDMS layer with microchannels and another PDMS layer reversibly faced each other. Then, the inlet hole was punched at the side of the faced device in a lateral direction using a punch with a diameter of 5.0 mm. Direct insertion of the needle into the side of the microfluidic device may give rise to inaccurate penetration and damage the PDMS around the punched site. This structural mismatch would lead to unexpected leakage around the inlet. In addition, to embed the ferrule successfully, the thickness of the whole PDMS device should be greater than 6 mm.

The residual PDMS fragment was removed using a pair of tweezers. To align and permanently bond the PDMS replicas, each layer was treated with oxygen plasma. To form a perfect seal against the side of the microfluidic device and Tygon tubing (0.06" outer diameter, Tygon R-AAQ04103; Saint-Gobain Performance Plastics Corp., Akron, OH), I used a commercially available microfluidic ferrule (flangeless fittings for 1/16" OD tubing; Upchurch, Oak Harbor, WA). The ferrule, which had an outer diameter of 0.46 cm and a
length of 0.56 cm, was assembled into the punched inlet port (diameter, 5.0 mm). After casting uncured PDMS around the ferrule and inlet port, the microfluidic device was stored at 70 °C for 1 h. This curing process supports robust fixation of the ferrule without fluid leakage. To avoid wetting of the PDMS microchannel by the aqueous phase, I flushed the microchannel with oil phase before introduction of the aqueous phase.
Figure 2.3. A schematic diagram of the overall process for fabrication of the microfluidic device with lateral interconnections. The defined interconnect holes were robustly fabricated in a lateral direction using a biopsy punch with a diameter of 5.0 mm. After removal of the PDMS fragment, the microfluidic ferrule was assembled into the punched inlet port to improve the alignment and achieve perfect sealing.
2.2.2. Materials

Fluorescein isothiocyanate (FITC) solution (Sigma-Aldrich, St. Louis, MO) with a concentration of 50 μg/mL was prepared. Red fluorescent polystyrene beads with a diameter of 15 μm were purchased from Invitrogen Corporation (Carlsbad, CA). The beads were prepared in 6% Pluronic F68 solution (Sigma-Aldrich) at a concentration of approximately 5×10^5 /mL. For droplet-generation experiments, the continuous oil phase used was mineral oil (Sigma-Aldrich) without surfactants. Solutions of yellow and green food dye (Kemide Co., Jeonju, Korea) were used to produce the dispersed phase.

The human histolytic lymphoma monocyte (U937) cell line was cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 100 units/mL penicillin G, and 100 μg/mL streptomycin. Cell cultures were maintained in a humidified atmosphere containing 5% CO2. Then, cells were centrifuged at 1000 rpm for 3 min to remove the supernatant and stained with 10 μM CellTracker Green CMFDA (Molecular Probes Inc., Eugene, OR). *Chlorella vulgaris* cells were precultivated for 3 days at 20 °C in non-saline BG 11 medium under constant shaking and continuous illumination with a 3000 lux lamp. Suspensions of ~1×10^5 and 5×10^5 cells/mL were used in the experiments.
2.2.3. Experimental setup

All microparticle-containing fluids were introduced using a syringe pump (KDS200; KD Scientific Inc., Holliston, MA). The trajectories of the fluorescent microparticles were recorded with a charge-coupled device (DS-U1; Nikon Instruments Inc., Melville, NY). The movements of droplets, including single cells, were recorded with a computer-controlled high-speed camera (Hotshot 512sc; NAC Image Technology, Simi Valley, CA) mounted onto an inverted optical microscope (TS100; Nikon Co., Tokyo, Japan).
2.3. Results and discussion

A ferrule-coupled inlet port for facile connection of the device to a commercial syringe was constructed successfully. Figure 2.4(a) shows an image of the fabricated microfluidic device laterally connected to a Tygon tube. The device consists of two layers: one for the microchannel (height, 40 μm) and the other for the non-patterned slab. To characterize the performance of the proposed interconnection, I conducted a leakage test as sample fluid was continuously injected into the linear microchannel [Figures. 2.4(b) and 2.4(c)]. Red dye and FITC solution were placed in a syringe. There was no apparent leakage around the connectors when the fluids were infused directly by pushing the syringe piston using finger force. At the same time, the injected fluid was collected continuously at the outlet. Also, the commercial microfluidic ferrule was recyclable and endurable for repetitive manipulations such as insertion or removal of tubings. I confirmed that there was no leakage around the microfluidic ferrule when the tubing was inserted repeatedly more than 30 times.

For further investigation of applicability in droplet microfluidics, generation and addition of two droplet inputs were performed (Figure 2.5). Unlike the structure presented in Figure 2.4, the microchannel for droplet generation and transport was formed on each PDMS slab. Two inlets for application of the liquid phase were constructed laterally using the proposed fabrication method. Solutions of yellow and green food dye were successfully introduced into each inlet without leakage or fluctuation. By adjusting the flow rate of the aqueous and oil phases, I could generate liquid droplets and control their size at the T-shaped channel. One green droplet generated at the T-junction of the top microchannel was transported to the
adding chamber (cross-section b–b′) via the microchannel (cross-section a–a′) and merged with another yellow droplet that originated from the bottom microchannel. The merged droplets were successfully collected at the outlet. The oil phase was injected into the oil inlet at a flow rate of 20 μL/h. The water flow rate was maintained at 4 μL/h. Thus the proposed microfluidic system, which uses multilayer 3D microdroplet circuits, could be applied to advanced combinatorial reactions, such as nanoparticle synthesis and hydrogel microcapsule encapsulation. In addition, the high integrity of the lateral interconnection to the droplet-based microfluidic device means that this novel approach can contribute to advanced applications of cell-laden droplet manipulation, such as individual isolation and monitoring of single cells.
Figure 2.4. (a) Photograph of the fabricated device (a) without and (b) with the microfluidic ferrule and plastic tubing. The device consists of two layers: one for the microchannel (height, 40 μm) and the other for the non-patterned slab. The microchannels were filled with dye solution to facilitate visualization. Results of the leakage test. The yellow dye (c) and fluorescein isothiocyanate (FITC) solution (d) were continuously injected into the linear microchannel.
Figure 2.5. Multilayer three-dimensional microdroplet circuits. Microchannels for generation and transportation of droplets were formed on each PDMS slab. Two inlets for application of the liquid phase were constructed laterally using the proposed fabrication method. Microscopy image showing generation, transport, and merging of green and red microdroplets. One green droplet generated at the T-junction of the top microchannel was transported to the adding chamber (cross-section b–b’) via the microchannel (cross-section a–a’), and merged with another yellow droplet that originated from the bottom microchannel. The merged droplets were successfully collected at the outlet.
I verified the reduction in adsorption when microparticles were injected within the microchannel. The 15-μm red fluorescent beads that were completely attached to the microchannel surface were no longer transported to the outlet via the linear microchannel. By enumerating immovable beads 10 min after fluid introduction, I could evaluate the probability of particle adsorption in the conventional and the proposed devices. Because the areas for particle/cell counting were different in the two devices, the number of adsorbed particles/cells was normalized to a certain area (1 mm²) of inlet part. As shown in Figure 2.6, the number of attached particles per mm² of surface area was varied according to the flow rate (25–200 μL/h). The number of attached particles according to flow rate (25–200 μL/h) is shown in Figure 2.6. The number of attached particles was significantly decreased using the proposed microparticle injection method. Despite the relatively low input flow rate, I was able to manipulate most of the introduced microparticles freely. However, at lower flow rates, the adsorption of particles around the inlet was problematic using the conventional approach. These results suggest that most particles settled immediately due to the sedimentation force under low-flow-rate conditions. Microparticles cannot be manipulated once they are attached to the PDMS surface. This is critical for loading of single particles or cells into the droplets.

I also confirmed injection of human histolytic lymphoma monocytes (U937) according to the initial cell loading concentration using both the conventional and proposed methods (Figure 2.7). To quantify cell attachment, the number of adsorbed U937 cells per mm² of surface area was considered for each of the two methods. The input flow rate was maintained at 25 μL/h. Using the conventional method, the numbers of adsorbed cells were 347 ± 7, 930
± 76, and 2208 ± 148 for injected cell concentrations of 5×10^6, 1×10^7, and 5×10^7 cells/mL, respectively. In contrast, using the proposed method, the numbers of adsorbed cells for the same injected cell concentrations were 164 ± 28, 490 ± 99, and 1705 ± 358, respectively. Thus, the number of attached cells was markedly decreased using the proposed method, and the difference in cell attachment between the two methods decreased with increasing cell loading concentration. This may have been because cell–cell and cell–surface interactions would be enhanced with increasing cell concentrations. This result suggests that introduction of cells with minimal cell loss could be achieved using a lower concentration of cells than with the conventional injection method. This novel approach may thus be useful for continuous separation and isolation of rare cell types. For further evaluation of time-lapse cell attachment within the proposed device, the conventional [Figure 2.8(a)] and proposed methods [Figure 2.8(b)] were compared with regard to continuous cell loading in a straight microchannel with an input flow rate of 200 μL/h. The green microalga, C. vulgaris, which is of great interest for second-generation biofuels,[7] was tested. While most of the adsorbed cells accumulated around the plastic tube in the conventional microfluidic device, few cells were adsorbed in the proposed microfluidic device. Previously attached cells could exacerbate cell accumulation due to cell–cell interactions, which may result in channel clogging upon long-term introduction of fluid. However, in the proposed microfluidic device, channel clogging due to cell accumulation did not occur around the inlet until 35 min after fluid introduction, indicating that the proposed injection method is more robust for long-term microfluidic applications. Unexpectedly, entrapped air bubbles were observed around the
inlet, but did not affect device performance (Fig. 7). Therefore, I believe that my system is useful as an experimental platform for adsorption-free cell injection irrespective of cell size for several cell manipulation applications.
Figure 2.6. Plot of attached particle number per mm$^2$ of surface area according to the input flow rate in the conventional and proposed microfluidic device. In the proposed microfluidic device, I was able to manipulate freely most of the introduced microparticles without attachment around the inlet, despite the relatively low input flow rate.
Figure 2.7. Reduction of mammalian cell attachment by lateral injection. Fluorescence images of attachment of U937 cells around the inlet of the (a) conventional and (b) proposed microfluidic device upon loading of $5 \times 10^7$ cells/mL. (c) Plot of the attached cell number per mm$^2$ of surface area according to initial cell loading concentration. The number of attached cells was markedly decreased by use of the proposed method, and the difference in cell attachment between the two methods decreased with increasing cell loading concentration.
Figure 2.8. Continuous loading of *C. vulgaris* cells into the linear microchannel with injection times of 10–35 min using the (a) conventional and (b) proposed methods. While most of the adsorbed cells were accumulated around the plastic tube in the conventional microfluidic device, few cells were adsorbed in the proposed microfluidic device.
For practical purposes, this microfluidic approach was used to encapsulate single cells into monodispersed droplets with improved efficiency. In particular, screening of lipid-abundant microalgae, such as *C. vulgaris*, requires appropriate cell culture techniques and single-cell encapsulation strategies. Various droplet-based microfluidic platforms have been used to enhance single-cell encapsulation efficiency, such as hydrodynamic self-sorting,[46] close-packed,[49] and deterministic ordering of cells.[50,89] However, these methods require an additional flow, specific microchannel dimensions, a high input flow rate, and compact loading of the cells at higher concentrations. Thus, the intrinsic problem of cell attachment, which leads to cell loss, could not be solved. The reduction in the number of attached cells that resulted from use of the proposed injection method is critical for determining the efficiency of single-cell encapsulation into droplets. I compared the percentage of droplets containing single cells under two different initial cell loading conditions (1×10⁷ and 5×10⁷ cells/mL) collected at the outlet of the conventional and proposed microfluidic devices. For generation of droplets ~60 μm in diameter, the oil phase and cell culture medium flow rates were 200 and 25 μL/h, respectively [Figure 2.9(a)]. Using the conventional method, the distribution of encapsulation efficiency was similar to the Poisson distribution, which yields a higher percentage of empty droplets than those containing multiple cells. The ratio of single-cell droplets did not exceed 30% regardless of the cell concentration used, and the percentage of cell-containing droplets decreased as the initial cell loading conditions decreased. In contrast, my cell injection method was effective in reducing the percentage of empty droplets, which was due to the reduction of cell attachment, and also resulted in an increase in the
percentage of single-cell-containing droplets. Figure 2.9(b) shows an enlarged view of a microscopy image of single-cell encapsulation. As shown in Figure 2.9(c), at the high concentration of $5 \times 10^7$ cells/mL, the fraction of droplets containing single cells increased gradually from $28.67 \pm 4.62\%$ to $40.00 \pm 3.46\%$ ($n = 3$). At a lower concentration ($1 \times 10^7$ cells/mL), there was a noticeable decrease in the percentage of empty droplets, leading to an increase in the percentage of those containing single cells from $15.33 \pm 1.15\%$ to $35.33 \pm 11.02\%$ ($n = 3$) [Figure 2.9(d)].

To enhance the single-cell encapsulation efficiency compared with the conventional method based on Poisson statistics, I significantly reduced cell adhesion by weakening the vertical settling force using the proposed method. The ratio of cell-encapsulating droplets (including single- and multiple-cell droplets) increased from $17\%$ to $36\%$ at a lower concentration ($1 \times 10^7$ cells/mL), and $44\%$ to $54\%$ at a higher concentration ($5 \times 10^7$ cells/mL). Also, the ratio of empty droplets was markedly decreased at the lower concentration of $1 \times 10^7$ cells/mL. However, an increase in the initial cell concentration resulted in two negative effects on single-cell encapsulation into droplets. First, as mentioned previously, if the initial loading concentration was high, the effects of cell–cell and cell–surface interactions were dominant. The cells already attached to the microchannel surface trigger cell–cell adhesion and form cell aggregates, thus hindering the smooth introduction of cells around the microfluidic inlet. This implies that the increase in the cell-encapsulating droplet ratio was relatively low at the higher concentration of $5 \times 10^7$ cells/mL. Second, according to the Poisson distribution, the number of multiple-cell droplets also increased with increasing
initial cell loading concentration. The same phenomenon occurred in the proposed device due to the large number of cells loaded. Further efforts should focus on reducing the fraction of multiple-cell droplets by integrating microfluidic droplet separation components.
Figure 2.9. Single-cell encapsulation into monodispersed picoliter droplets. (a) The adsorption-free introduction of *C. vulgaris* cells in the direction of flow led to the generation of single-cell droplets by hydrodynamic focusing. The initial cell loading concentration was 5×10^7 /mL. (b) Enlargement of the microscopy image of single-cell encapsulation. Gray arrows indicate droplets encapsulating single cells. Scale bars: 200 μm. (c,d) Percentage of droplets containing single cells according to initial cell loading concentration, (c) 1×10^7 and (d) 5×10^7 cells/mL, in the conventional and proposed microfluidic devices.
Compared with other single-cell droplet encapsulation platforms and conventional methods based on Poisson statistics, this approach has the following remarkable advantages. First, this novel interconnection approach does not limit the design of the microchannel, for example, the aspect ratio and complexity. The use of high integrity, low cost, and reusable interconnections results in broad applicability in microfluidic biochemical reactions that require manipulation of individual cells without cell damage. Second, the proposed encapsulation approach could be employed for various cell concentrations, including relatively low concentrations. Since sorting, detecting, and isolating rare cells remains challenging,[98] this method could be used to encapsulate rare cell types, such as low-abundance primary cells and circulating tumor cells, into droplets for droplet-based single-cell analysis.

Several modular approaches to building integrated modular microfluidic systems have been developed recently.[99] For fastening and bonding of the microfluidic modules with each other, the up-and-down connection approach was utilized as a breadboard. This approach suffers from an increased duration of sample passage, which leads to both extra time being required for sample transportation and the existence of a dead volume. However, using the proposed interconnection, leakage-free interconnections together with various microfluidic modules may facilitate miniaturization of the interconnection components by reducing the space required. Furthermore, the proposed interconnection could be coupled with other microfluidic cell sorting systems, such as a fluorescence-activated cell sorter (FACS),[100] magnetic-activated cell sorter (MACS),[101] or fluorescence-activated droplet
sorter (FADS),[102] to enhance the purity and recovery rate by reducing sample loss.
2.4. Conclusions

The nonspecific interactions between microparticles and PDMS microchannels results in disadvantages, such as channel clogging due to adsorption of microparticles and cells and reduced single-cell encapsulation efficiency. Here, I demonstrated a new microfluidic interconnection for microparticle injection, which utilizes lateral flow in the PDMS-based microfluidic device. Inlet holes, which are capable of leakage-free fluid introduction, were robustly and accurately punched into the side of a double-layer PDMS microfluidic device. The lateral movement of the microparticles and cells resulted in a significant reduction in nonspecific binding to microchannel walls with no surface modification. Consequently, single-cell encapsulation into monodispersed microdroplets was successfully demonstrated and yielded a twofold enhancement of efficiency due to the reduced percentage of empty droplets. This inexpensive, easy, reusable, and high-integrity interconnection technology is compatible with various microfluidic systems and appropriate for a number of biomedical applications, such as microfluidic cell culture, isolation of rare cell types, and single-cell encapsulation within microdroplets.
CHAPTER III: Microfluidic interactive platform for

spatiotemporal control of droplets

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3.1. Introduction

Precise temporal control of microfluidic droplets such as synchronization and combinatorial pairing of droplets is required to achieve a variety range of chemical and biochemical reactions inside microfluidic networks. To prevent inconsistency in the spatiotemporal control of droplets, several efforts for microfluidic integration were reported, which introduced in Chapter 1.3, but there are limitations in the requirement of further incorporation of other microfluidic components.

To address the above needs, I suggest a facile and robust microfluidic control module which is integrated with simple microbridge structures[103] connected to additional inlets for adjusting the interval between two approaching microdroplets. Temporal control of droplets is achieved by the flow rate in a control channel, without complicated control of droplet-carrying flow rates, any additional microfluidic components and external forces. By adjusting the flow rate of control oil flow as bias, the droplet interval is dynamically altered when droplet-carrying flow rates are constant (Figure 3.1). I also demonstrate the robustness of the temporal spacing method for practical applications of alginate droplets by introducing the oil phase including calcium chloride as a control flow. Based on the difference of control oil flow rate, alginate droplets containing live cells are rapidly solidified and efficiently collected at the outlet with suitable droplet spacing. Moreover, controlled pairing of droplets is demonstrated by temporal synchronization of generated droplet pairs.
Figure 3.1. A schematic of the microfluidic device integrated with microbridge structures interconnecting droplet-carrying and control channels. Two inlets of the droplet-carrying channel (inlets 1 and 2) are for introduction of reagent ($Q_r$) and oil flow ($Q_o$), and the inlet of the control channel (inlet 3) is for introduction of control oil flow ($Q_c$). A fluidic pressure drop between two channels via 45 microbridges can be used to adjust the interval at the droplet-carrying channel, enabling the flexible and precise temporal control of droplets.
3.2. Materials and method

3.2.1. Materials

Sodium alginate (A0682-100G, Sigma) was dissolved in distilled water by 1% (w/w) and filtered with a 0.22-μm syringe filter (Millex-GV, Millipore) to remove any clumps of alginate. Solutions of orange food dye (Kemide Co., Jeonju, Korea) were mixed with alginate solution to help visualize droplets. Oleic acid without any surfactant was introduced as a continuous phase and the alginate was gelated by the oleic acid (Sigma) with calcium. 0.6 g of calcium chloride (C7902-500G, Sigma) was dissolved in 25 mL of oleic acid via ultrasonication. Due to the low solubility of calcium chloride and oleic acid, calcium chloride was dissolved in 25 mL of 2-methyl-1-propanol (J.T. Baker, Deventer, The Netherlands) via ultrasonication. After mixing of the calcium chloride and oleic acid at a ratio of 50% (v/v), the 2-methyl-1-propanol was distilled in a convection oven at 65 °C for a day.

Human leukemic monocyte lymphoma U937 cell line was cultured in RPMI 1640 medium (Invitrogen, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 100 units/mL penicillin G and 100 μg/mL streptomycin. The cell cultures were maintained in a humidified atmosphere containing 5% CO₂. Then cells were centrifuged at 1000 rpm for 3 min for removing supernatant and stained with 10 μm CellTracker Green CMFDA (Molecular Probes Inc., Eugene, OR).
3.2.2. Design and fabrication of a microfluidic device

A microfluidic device was fabricated by using a conventional poly(dimethylsiloxane) (PDMS) (Sylgard 184; Dow Corning, Midland, MI) molding process (Figure 3.2). The elastomer was mixed with a curing agent in a ratio of 10:1 (w/w). The negative photoresist SU-8 2025 (MicroChem Corp., Newton, MA) on a silicon wafer was used for a PDMS mold. PDMS was cast on the mold and cured for 3 h in a convection oven at 65 °C for complete cross-linking. The PDMS channel was irreversibly sealed with a glass slide after exposure to oxygen plasma for 30 s. Because the hydrophobic PDMS microchannel became hydrophilic during plasma treatment, the device was stored in a convection oven at 65 °C for 2 days to recover hydrophobic nature of the microchannel. After the fabrication process, the microfluidic devices were sterilized with 6% (w/v) Pluronic F127 in distilled water prior to cell loading without mammalian cell attachment. Subsequently, the microchannels were flushed with an oleic acid.

I prepared liquid droplets in mineral oil mixed with 0.5% (w/w) Span 80 (Sorbitan monooleate, Sigma–Aldrich Co.) by hydrodynamic flow focusing at the T-junction where each stream of oil and liquid is came from two different injection channels. The entire dimension of the device as shown in Figure 3.3 was about 33 mm × 6 mm. The widths of the aqueous and oil channels were 40 and 60 μm, respectively. The width and length of the microbridge were 15 and 300 μm, respectively. The width of outlet detection region for measuring the spacing of gelated alginate droplets was 300 μm. The height of the channel was 35 μm. The diverging microchannel of detection zone nearby the outlet plays a role in
the velocity reduction of the gelated alginate capsules and thus the U937 cells inside the capsules would be easily observed in the microscope.

3.2.3. Experimental setup

The droplet movements were recorded by a charge-coupled detector (CCD) camera (DS-U1; Nikon Instruments Inc., Melville, NY). A commercial image analyzing program, i-Solution (IMT i-solution Inc., Korea) was used to measure the interval of droplets. All fluids were injected and motivated via the syringe pumps (Pump 11 Pico Plus; Harvard Apparatus, Inc., Holliston, MA) at a range of volumetric flow rates.
**Figure 3.2.** Fabrication process. A microfluidic device was fabricated by using a conventional PDMS molding process. The negative photoresist SU-8 2025 on a silicon wafer was used for a PDMS mold. PDMS was cast on the mold and cured for 3 h in a convection oven at 65 °C for complete cross-linking. The PDMS channel was irreversibly sealed with a glass slide after exposure to oxygen plasma.
Figure 3.3. Channel design of the microfluidic device and enlarged view of fabricated microbridge structures. All fluidic channel and microbridge structures were fabricated from poly(dimethylsiloxane) (PDMS) using standard soft lithographic methods. The overall thickness of the channel was designed to be 35 μm.
3.3. Results and discussion

Figure 3.1 illustrates a schematic diagram of the droplet interval control module integrated with microbridge structures interconnecting droplet-carrying and control channels. By incorporating microbridge structures bridging two microchannels, a pressure drop through 45 microbridges can be used to adjust the interval at the droplet-carrying channel. First, droplets are generated at the T-junction[104] in the droplet generating channel (see inlets 1 and 2), and then conveyed to the temporal control region which is connected to the microbridge structures. The control oil flow is introduced to the control channel with additional inlet (see inlet 3) and changes the velocity of the droplet-carrying flow via the microbridge structures. The long and narrow microbridge structures play a role in maintaining the shape and size of droplets, and changing the interval of neighboring droplets quickly.

To figure out the flow characteristics along the microbridge by the control oil flow, I calculated the concentration changes by two flows for different flow rate in the control channel of 20 and 80 μL/h with a fixed flow rate of 10 μL/h in the droplet-carrying channel using a commercial CFD solver (CFD-ACE+; ESI Group, Huntsville, AL) [Figure 3.4(a)]. The insets are the enlarged views of the concentration distribution by the change in the control oil flow rate (Q_c). The augmented migration of additional flow passing through each microbridge from the control channel appeared as the control oil flow rate increased. Also, I plotted the calculated concentration distributions across the lateral position of microchannel at the outlet of droplet-carrying channel as the control oil flow rate increases [Figure 3.4(b)].
The relatively high input pressure field from the control oil flow generates pressure difference between two flows across the microbridge and thus gives it a chance to increase the droplet interval. The simulated tendency of pressure drop between two microchannels at the first microbridge structure for different flow rate in the droplet-carrying channel of 10 and 20 μL/h was plotted as the control oil flow rate increases [Figure 3.4(c)]. I also calculated and plotted the normalized velocity distribution through the microbridges according to the microbridge number [Figure 3.4(d)]. The $y$-axial velocity at the center of each microbridge decreases more steeply under the higher flow rate condition of the control channel. This means that the number of microbridge structures is enough for shifting of the control oil flow toward the droplet-carrying channel at the various flow rate conditions. Modulating the number of microbridge structures would be necessary to achieve the temporal spacing at the wide or narrow range of the control flow rate.
Figure 3.4 (a) Simulation results of flow characteristics with an increase of the additional flow from the control channel. The cross-sectional images acquired nearby outlet show the concentration distributions of two different flows at a control oil flow rate ($Q_c$) of 20 and 80 μL/h, respectively, maintaining a constant oil flow rate ($Q_o$) of 10 μL/h. An additional oil flow field causes the migration of control oil flow passing through each microbridge due to a fluidic pressure drop between two channels, thereby enhancing the velocity of the droplet-carrying flow and inducing the temporal spacing of droplets. (b) Plot of the concentration distributions along the line from A to B in panel a as the flow rate in the control channel ($Q_c$) increases from 2.5 to 80 μL/h. (c) Plot of the calculated pressure drop between two channels according to the control oil flow rate ($Q_c$) at two different oil flow rate ($Q_o$). (d) Plot of the calculated normalized velocity distribution through the microbridges according to the microbridge number. Each data point represents the $y$-axial velocity at the center of each microbridge at the two different control oil flow rate ($Q_c$).
I could predict that the spacing between droplets would be regarded as a function of the control oil flow rate. The negative pressure drop would decelerate a train of droplets. Therefore, the droplet interval in the droplet-carrying channel was flexibly and precisely tuned by controlling the flow rate in the control channel at a fixed flow rate in the droplet-carrying channel.

Figures 3.5(a)–(c) shows the microscopic images of varying droplet interval within droplet-carrying channel with respect to the flow rate in the control channel (Q_c) from 5 to 80 μL/h. The fixed flow rates of aqueous (Q_r) and oil phase (Q_o) in each trial were 1 and 10 μL/h [Figure 3.5(a)]; 2 and 10 μL/h [Figure 3.5(b)]; 4 and 20 μL/h [Figure 3.5(c)], respectively. The ‘initial’ interval of droplets, which is the same with droplet generating pattern at the T-junction without any temporal control, increased as the flow rate of aqueous phase decreased. The results indicate that droplets introduced to the droplet-carrying channel were temporally controlled by the additional control oil flow along the microbridges. Therefore, the interval between two approaching droplets could be adjusted by simply changing the flow rates in the control channel without any additional operation of droplet-carrying flow. I measured the droplet interval with respect to the control oil flow rate (Q_c) at the condition of droplet generation with an oil flow rate (Q_o) of 10 and 20 μL/h [Figures 3.5(d) and 3.5(e)]. The droplet interval was defined as the distance between two neighboring droplets which were passed through the temporal control region and located near the outlet. When the flow rate in the control channel (Q_c) was adjusted from 5 to 80 μL/h, the droplet interval was changed from 114.94 ± 4.98 to 1336 ± 14.93 μm, at the fixed condition of droplet generation with a
reagent flow rate \( (Q_r) \) of 4 \( \mu L/h \) and oil flow rate \( (Q_o) \) of 20 \( \mu L/h \) \( (n = 3) \). The zero value of the droplet interval indicates unpredictable merging of adjacent droplets due to insufficient spacing. As shown in Figures 3.5(d) and 3.5(e), the change of droplet interval against the control oil flow rate was relatively greater as the reagent flow rate \( (Q_r) \) became lower than 10 \( \mu L/h \). In addition, the droplet interval increased as the flow rate in the control channel increased, without any effect on the droplet size or generating pattern. These droplet movements can be described based on momentum balance at the pressure-driven flow through a narrow rectangular channel of height \( h \) and length \( L \).[105] The maximum velocity is then found from an overall momentum balance, \( v_{\text{max}} = \frac{\Delta p h^2}{8 \mu_{\text{oil}} L} \), where \( \Delta p \) is the pressure drop; \( \mu_{\text{oil}} \) is the viscosity of continuous phase. Thus, the droplet velocities can be changed by adjusting pressure difference between two channels which depends on the control oil flow rate \( (Q_c) \). The linear tendency of the predicted pressure difference as shown in Figure 3.4(c) is similar to the experimental ones.

For complete temporal control of droplets, it is important to maintain a stable flow throughout the microchannel. The relatively low control oil flow rate \( (Q_c \leq 20 \mu L/h) \) at the low oil flow rate \( (Q_o = 10 \mu L/h) \) may be insufficient in temporal controlling of droplets due to the small variations in flow fluctuation from the pumps. Also, insufficient spacing between adjacent droplets owing to low volumetric flow rates may influence the resistive hydrodynamic coupling effects with several droplets.[106] As shown in Figure 3.5(a) and 3.5(b), the interval control data showed some irregular variations, resulting from an unstable microflow at the flow condition as mentioned above. This handicap at low flow rate levels
can be overcome by reducing the length and increasing the number of microbridge structures for achieving sufficient droplet spacing.
Figure 3.5. Microscopic images of the gelated hydrogel capsules taken at the channel outlet with respect to the control flow rate ($Q_c$) of (a) 40, (b) 60, and (c) 80 μL/h. (d) Plot of the droplet interval measured at the detection zone with respect to the control oil flow rate at the condition of alginate droplet generation with an oil flow rate ($Q_o$) of 10 μL/h, while the reagent flow ($Q_r$) varied from 1 to 10 μL/h. The results indicate that alginate droplets introduced to the droplet-carrying channel were temporally controlled and polymerized by the additional flow including calcified oleic acid.
For practical purpose, this microfluidic module was used for on-chip polymerization and recovery of hydrogel capsules. The additional control oil flow including calcified oleic acid leads to adjust droplet interval and gelation of the alginate droplet simultaneously through the microstructures. The demonstrations of the interval control of alginate droplets were performed within microchannels integrated with microbridges. Figures 3.6(a)–(c) shows the enlarged images of the alginate capsules gelated with calcified oleic acid taken at the channel outlet injected with different flow rates (40, 60, and 80 μL/h) in the control channel. The diameter of the alginate capsule was approximately 69.6 μm. Figure 3.6(d) shows the plot of the measured droplet interval with respect to the control oil flow rate (Q_c). When the flow rate in the control channel was adjusted from 20 to 60 μL/h, the droplet interval was changed from 710 ± 17.32 to 2930 ± 88.88 μm, at the fixed condition of droplet generation with an reagent flow rate (Q_r) of 1 μL/h and oil flow rate (Q_o) of 10 μL/h (n = 3). The results indicate that alginate droplets introduced to the droplet-carrying channel were also temporally controlled and polymerized by the additional control oil flow along the microbridges. Figure 3.7 shows the enlarged images of the U937 cell-laden alginate capsules gelated with calcified oleic acid taken at the channel outlet injected with different flow rates (40, 60, and 80 μL/h) in the control channel. The diameter of the cell-laden alginate capsule was approximately 99.8 μm. The fixed flow rates of alginate solution (Q_r) and oleic acid (Q_o) were 1 and 10 μL/h, respectively. When the flow rate of calcified oleic acid was high, the shape of polymerized alginate capsules was not spherical and uniform because the calcium passage through the microbridges was increased. Also, the polymerization would be accelerated when
the concentration of calcium chloride was high. The insufficient calcium passage from the control channel due to the lower control oil flow rate (lower than $Q_c = 20 \mu L/h$) would lead to unexpected droplet merging in the collecting chamber because of weak gelation (data not shown). The encapsulated cells were located on the droplet surface due to the circulation flow inside the relatively large droplet, which is not good for the viability. With suitable droplet spacing and improved single-cell encapsulation efficiency by incorporating a microfluidic flow-focusing device for reduction of droplet size and maintenance of cell viability, the device can be further applied to single cell-based analysis systems.
Figure 3.6. Microscopic images of the gelated hydrogel capsules taken at the channel outlet with respect to the control flow rate ($Q_c$) of (a) 40, (b) 60, and (c) 80 μL/h. (d) Plot of the droplet interval measured at the detection zone with respect to the control oil flow rate at the condition of alginate droplet generation with an oil flow rate ($Q_o$) of 10 μL/h, while the reagent flow ($Q_r$) varied from 1 to 10 μL/h. The results indicate that alginate droplets introduced to the droplet-carrying channel were temporally controlled and polymerized by the additional flow including calcified oleic acid.
Figure 3.7. Microscopic images of the cell-laden alginate capsules with varying interval taken at the channel outlet with respect to the control oil flow rate \((Q_c)\) of (a) 40, (b) 60, and (c) 80 \(\mu\)L/h. Dashed circles indicate the U937 cell encapsulated in the alginate capsule. The fixed flow rates of alginate solution \((Q_r)\) and oleic acid \((Q_o)\) were 1 and 10 \(\mu\)L/h, respectively.
On the basis of this droplet interval control, I also demonstrated a dynamic temporal control of two distinct droplets using an additional inlet for another sample. As shown in Figure 3.8(a), non-dyed (blue) and dyed (orange) droplets were generated at 1st and 2nd T-junctions, respectively. The diameter of each droplet was approximately 59.4 µm. For synchronization of droplets, each droplet was temporally controlled by each control oil flow (e.g., $Q_{c1}$ and $Q_{c2}$) along the microbridges and brought together in the confluence channel nearby outlet. Due to the difference in the droplet interval of the two distinct droplets by changing control oil flow rate, the ratio in droplet pairs could be efficiently adjusted. The ratio of droplet pairing (non-dyed: dyed) could be observed in the confluence channel which was dynamically adjusted as 1:2 ($Q_{c1} = 80 \mu$L/h and $Q_{c2} = 10 \mu$L/h), 1:1 ($Q_{c1} = 20 \mu$L/h and $Q_{c2} = 20 \mu$L/h), and 2:1 ($Q_{c1} = 20 \mu$L/h and $Q_{c2} = 60 \mu$L/h), as shown in Figures. 3.8(b)–(d). The fixed flow rates of each sample ($Q_{r1}$ and $Q_{r2}$) and each oil phase ($Q_{o1}$ and $Q_{o2}$) were 4 and 20 µL/h, respectively. Droplet pairing with various ratios of two distinct droplets would be reproducibly produced by changing the flow rates in each control channel. Also, the entire length of each produced droplet pair would be controlled as a function of flow rate difference between two control oil flows. By adjusting the number of additional inlets including various samples and control channels, the component and sequence of droplet pairs would be controlled. Compared with other droplet pairing methods, the remarkable advantage of this approach is that the interval and pairing ratio of particular droplets can be flexibly and precisely controlled by the control oil flow without any extra operation of droplet-generating flow and integration issues for external fields. The utility of periodic control of droplets
provides broad applicability in microfluidic biochemical reactions which require time and volume-dependent reactions. Furthermore, other microfluidic components such as pillar microstructure[107] and built-in electrode[108] can be applied to enhanced coalescence of droplets for achieving advanced combinatorial reactions in a large scale screening applications. Particularly, current platform can be adapted to carry out on-chip serial dilution for measuring rapid enzymatic kinetics, studying protein–protein interactions and performing biological dilution assays. It is anticipated that the proposed on-chip polymerization method combined with a droplet pairing technique will be applicable for paired capsule-based analysis[67].
Figure 3.8. Production of combinatorial droplet pairs by droplet synchronization in the microfluidic channel. (a) The layout of the parallel-linked channel for the dynamic control of droplets and the confluence channel for the production and observation of droplet pairing. Non-dyed (blue) and dyed (orange) aqueous phase was introduced to each inlet. Non-dyed and dyed droplets emerged from each T-junction were temporally controlled by changing the flow rate of each control flow ($Q_{c1}$ and $Q_{c2}$). Microscopic images of the dynamic control of droplet interval at the microbridge and adjusted droplet pairing ratio (non-dyed: dyed) of (b) 1:2, (c) 1:1, and (d) 2:1 at the confluence channel when (b) $Q_{c1} = 80 \ \mu$L/h and $Q_{c2} = 10 \ \mu$L/h, (c) $Q_{c1} = 20 \ \mu$L/h and $Q_{c2} = 20 \ \mu$L/h, and (d) $Q_{c1} = 20 \ \mu$L/h and $Q_{c2} = 60 \ \mu$L/h, respectively.
Each inset in (b)–(d) shows a schematic of pairing sequence in droplet pairs. White and orange arrows in each droplet pairing images indicate non-dyed and dyed droplets, respectively. All conditions of droplet generating flow were fixed as $Q_{r1} = Q_{r2} = 4 \, \mu\text{L/h}$ and $Q_o = 20 \, \mu\text{L/h}$.
3.4. Conclusions

In this paper, I have designed and characterized a microfluidic device integrated with simple microbridge structures for the temporal control of aqueous droplets. As the control oil flow rate increased, the droplet interval was increased without any effect on the droplet size or generating pattern. This temporal control of droplets will give an opportunity to achieve precise droplet synchronization. I also validated that the additional control oil flow including calcified oleic acid promoted rapid gelation of alginate capsules and collection with sufficient spacing at the outlet. The main drawback of using oleic acid as a continuous phase is that the prolonged exposure to the oleic acid will cause damage to cells due to its acidity and low gas permeability. However, these questions relating to biocompatibility would be settled by including the flushing-out process of oleic acid after the gelation of alginate capsules to prevent the viability declining.[109] In this respect, introducing of the pure oil instead of oleic acid as a control flow can be further used to preserve cell viability during the gelling process. The proposed microbridge structures will also play a critical role in performing the on-chip rapid oil exchange. Parallel control of two distinct picoliter droplets for synchronization and production of combinatorial droplet pairs was also demonstrated. It is anticipated that this microfluidic module will support and develop the advanced droplet-based experiments for a wide range of biochemical applications, including biological assay, combinatorial synthesis, and high-throughput screening.
CHAPTER IV: Optoelectrofluidic device for interactive retrieval of microparticles

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4.1. Introduction

To understand the intrinsic property and measure the signatures of single cells with elimination cell-to-cell interaction from neighbouring cells under physiological environments, it is essential to isolate an individual cell in a tiny volume.[110] Emulsion droplets including microgel polymer and a single cell play a role as templates for “tailored” hydrogel microcapsule synthesis based on droplet-based microfluidics.[111] A monodisperse hydrogel microcapsule that contains a living cell serves for a variety of applications in capsule-based assay, which covers receptor/ligand studies, enzyme assays, and single-cell screens.[36-39] Especially, the inherent advantages of hydrogel microcapsules compared to liquid emulsions are that they allows for a continuous supply of nutrients and oxygen due to their high permeability, which prevent death of the encapsulated cells and ensure to the long-term manipulation without droplet evaporation. Also, the microcapsule-microcapsule interaction could be observed by the molecular diffusion between paired microcapsules.[67]

Microcapsules can be transported, arranged, and retrieved in two-dimensional arrays to facilitate storage, time-lapse imaging, high-throughput analysis, and selection.[17,65] Well-established microfabricated well arrays make droplets and microcapsules be loaded passively or actively.[68] However, unlike entrapment methods, there were not many reported techniques for selective retrieval of individual microcapsules from the high-density indexed arrays to recollect microcapsules which contain a requested cell. Although several methods of microcapsule ejection have been successfully demonstrated by applying hydrodynamic force,[112] laser,[113] pneumatic valving,[77] and optical scattering force,[75] difficulty in
integrating complicated microfluidic components into whole platform has limited the applicability of microfluidic screening technology.

Optoelectrofluidics, which combines the advantages of optical and electrical manipulation, allows the programmable manipulation of particles or fluids based on optically induced electrokinetics induced by light.[114-116] The image-based manipulation of polymer microspheres,[117-119] microdroplets,[120-122] and hydrogel microcapsules[43] using a conventional display device such as a liquid crystal display (LCD) with simple optical configuration and low power energy.

Here, an interactive manipulation platform for selective retrieval of cell-laden hydrogel microcapsules using a light-activated particle manipulation technology (Figure 4.1) is demonstrated. A high-scalable and free-standing microwell array is consisted of UV-curable hydrogel, polyethylene glycol-diacylate (PEG-DA), offers loading and selective ejection of the microcapsule in a disposable and a reusable format. By applying an optoelectrofluidic device, the microcapsules of interest could be individually repelled and positioned to a collection-flow field from the PEG hydrogel microwells by negative DEP forces using a programmed image from a LCD module. Furthermore, I combined the present selective retrieval method with fluorescent microscopy to monitor the interspecies difference of lipid contents in the green microalgae, *Chlorella vulgaris*, *Chlamydomonas* sp., and *Botryococcus braunii*. It is important that the exhaustive selection of microalgae that possess high lipid content is challenging for powerful production of biodiesel.[8] The quantity of lipid content in each microalga within the microcapsule was investigated using lipophilic green
fluorescent dye. I acquired the massive fluorescent cell images by microscope and retrieved selectively the microcapsules which emit high fluorescence intensity using the optoelectrofluidic device. Using this platform, I was able to monitor and separate the microcapsule which contains lipid-rich microalgae at single-cell resolution.
Figure 4.1. Overview of an optoelectrofluidic device for selective retrieval of individual microcapsules. A schematic figure of the optoelectrofluidic device integrated with photopolymerizable hydrogel microwell arrays. A polyethylene glycol diacrylate-based microwell array (PEG-DA) was sandwiched between the top indium tin oxide (ITO) glass and the bottom photoconductive layer. The released microcapsule can follow the collection stream and come out of the device.
4.2. Working principles

To load the microbeads into the microwell arrays passively, the sample droplet contains microbeads, whose total volume is 500 nL, was placed in a 120 μm-height liquid chamber of the optoelectrofluidic device. After I placed the upper ITO layer with the space, the microbeads sank into the microwell array after 10 min. When I focused a programmed LCD image to the specific microwell, containing microbeads of interest, in the application of the AC signal, the microbeads were levitated as a result of the optically-induced negative dielectrophoretic (DEP) force. The collection fluid flow provided the transportation of target microbeads into the outlet reservoir. Thus, I could perform the interactive and selective retrieval of the particular microbeads by exposure of a programmed LCD image [Figure. 4.2(a)]. For predicting the microbead behaviour by the optically-induced nDEP force, I simulated the electric field distribution around a hydrogel microwell using a commercial CFD solver (CFD-ACE+, ESI group, Huntsville, AL) [Figure. 4.2(b)]. I assumed the AC voltage of 20 V at 100 kHz was applied across the illuminated area of the photoconductive layer where was at the bottom of the specific microwell and the ground electrode. As shown in the simulation results, the strongest electric field was attained at the edges of a microwell floor and the field strength decreased along the z axis. When the electric field is applied with an illumination, microbeads following the nDEP would float toward to the ground layer.
Figure 4.2. Device operation. (a) Microalgae-laden alginate hydrogel microbeads sediment into the hydrogel microwell array. I focus an image pattern from an LCD onto target microbeads, levitating the microbeads into the collection flow field with the optically-induced negative dielectrophoretic (DEP) force. (b) By applying an externally applied AC bias, this causes the formation of nonuniform electric field gradients around the microwell, which result in an nDEP response on microbeads.
4.3. Experimental

4.3.1. Fabrication of optoelectrofluidic device

The whole optoelectrofluidic device consists of a photoconductive electrode, free-standing hydrogel microcell arrays, and a bare ITO electrode. The double-stick tapes were used as gap spacers for the liquid chamber, of which the height is high enough to manipulate the microbeads. The photoconductive layer was comprised of four layers: 1) a 180 nm-thick ITO layer, 2) a 50-nm-thick n+ doped hydrogenated amorphous silicon (n+ a-Si:H) layer, 3) a 1 μm-thick intrinsic hydrogenated amorphous silicon layer (intrinsic a-Si:H), and 4) a 20 nm-thick silicon nitride (SiNx) layer. The ITO-coated glass substrates (Samsung-Corning Precision Glass, Asan, Korea) were prepared and a triple layer of n+ a-Si : H, intrinsic a-Si : H and SiNx was consecutively deposited by plasma enhanced chemical vapor deposition (PECVD) onto the substrate. Afterward, some regions were etched by reactive ion etch (RIE) for electric connections. A wire was connected after dicing the fabricated device into 37.5 mm × 25.0 mm sections.
4.3.2. Fabrication of hydrogel microwell array

The fabrication of the free-standing hydrogel microwell arrays was based on the injection micromolding (Figure 4.3). A PDMS template mold was place over a glass slide, and then a few microliters of the hydrogel precursor solution was introduced through the mold via capillary action. After the direct exposure of UV light to the precursor solution for 2 s, the PDMS mold was removed and the remaining unpolymerized precursor was removed by rinsing with distilled water. The fabricated hydrogel microwell array was mechanically stable and flexible thus it could be comfortably transferred and embedded into the optoelectrofluidic device without morphological distortion.
Figure 4.3. Fabrication process for hydrogel microwell array and hybrid optoelectrofluidic device.
4.3.3. Materials

Polystyrene microspheres with diameter of 75 μm were purchased from PolyScience, Inc. (Warrington, PA). Polyethylene glycol diacrylate (PEG-DA, MW575) was obtained from the Aldrich Chemical Co.. Low conductivity buffer (LCB)[123] was prepared by 10 mM HEPES, 0.1 mM CaCl$_2$, 59 mM D-glucose and 236 mM sucrose, pH 7.35.

4.3.4. Experimental setup

Experimental setup for the optoelectrofluidic selective retrieval is shown in Figure 4.4. A conventional upright fluorescence microscope (BA400T; Martin Microscope Company, SC) was applied to construct the optoelectrofluidic interactive manipulation system. A monochromatic LCD module (CP-S225; Hitachi, Japan) for the image generation was located on the illumination of the upright microscope. The programmed LCD image was projected onto the photoconductive layer of the optoelectrofluidic device on the sample stage. The condenser lens which is integrated in the microscope condenses and focuses of the LCD image.[124] An AC voltage of 20 V$_{pp}$ produced from a function generator (AFG310; Tektronix, OR) and applied across the two electrodes. The optoelectrofluidic motions of microparticles were captured with a CCD camera (DS-U1; Nikon Instruments Inc., NY) and analysed with commercialized image processing software (NIS-Elements; Nikon Instruments Inc., NY). To perform the interactive and programmable manipulation of LCD images, I used conventional software such as Microsoft PowerPoint™.
Figure 4.4. An experimental setup for optoelectrofluidic interactive retrieval system. A conventional upright fluorescence microscope was applied to construct the optoelectrofluidic interactive manipulation system. The programmed LCD image which was transmitted to the condenser lens was projected onto the photoconductive layer of the optoelectrofluidic device on the sample stage.
4.4. Results and discussion

To capture and analyse single microbead, I fabricated a patterned PEG hydrogel microwell array, in which the bottom of the microwell was punched, with regard to the several shapes (hexagonal, circle and square), and pitches (60, 120, and 240 μm). An overview of the microwell array is shown in Figure 4.5. The whole microwell dimension was 20 mm length, and 12 mm width. The microwell dimension was 120 μm diameter and 75 μm in height.

Figure 4.6(a) shows the fabricated whole optoelectrofluidic device consists of a photoconductive electrode, free-standing hydrogel microwell arrays, and a bare ITO electrode. A 20 × 12 mm-sized, PEG-DA-based 111 × 66 array of traps was integrated between the top ITO glass and the bottom photoconductive layer to capture and release of microparticles. The height of the spacers between the ITO glass and the photoconductive layer was 120 μm. To passively load the microbeads, a small amount of the microbead solution (~500 nL) was dropped onto the microwell arrays. The solution plug was successfully migrated towards the entire microwell by carefully covering the top ITO glass on the hydrogel microwell array. After microbeads were settled in the wells, residual microbeads were flushed out with additional flow. As a result, the single microbeads were successfully loaded in the 60 μm pitch, separated square-shaped microwell [Figure 4.6(b)]. By using the orbital shaker at least 2 h, I would enhance the efficiency of single-microbead loading.[125]
**Figure 4.5.** Bright field images of various PEG hydrogel microwells according to their shape (hexagonal, circle and square), and pitch (60, 120, and 240 μm). All of the diameters of microwell are same as 120 μm. Scale bar: 100 μm.
Figure 4.6. An overview of the optoelectrofluidic device integrated with hydrogel microwell arrays. A $20 \times 12$ mm-sized, PEG-DA-based $111 \times 66$ array of traps was integrated between the top ITO glass and the bottom photoconductive layer.
To figure out the $z$ axis manipulation of the hydrogel microbeads, I observed the selective levitation of single microbeads from microwells. Conventional optical configuration based on optical tweezers requires the high energy and the provision of space for a clear optical path, which hinders automatic image-based selection of microbeads of interest. I demonstrated the feasibility of selective floating a single hydrogel microbead without cell from a hydrogel microwell based on optoelectrofluidics with simple optical configuration. I checked the optoelectrofluidic on/off response whether the microbead was focused out for confirmation of the microbead levitation by optically-induced negative DEP force. First, I exposed a programmed LCD-image directly to the lower right-hand corner of the specific microwell in the application of the AC signal of 20 V and 1 MHz (Figure 4.7). When the light exposed with the AC voltage, an isotropic DEP force is induced from the light-activated virtual electrodes. As a result, I observed the diagonal movement of the target microbead and the levitation from the microwell, resulting that the microbead was defocused. After the voltage was turned off, the microbead was fallen back into the bottom of the microwell by the gravity force, and I could obtain an in-focus image of the microbead again. Therefore, I could perform the reversible and rapid manipulation of the hydrogel microbeads in $z$ direction by switching the AC voltages.

I also performed the directional movement of individual microparticles in a certain direction by using optoelectrofluidics (Figure 4.8). The key strategy was illuminating a light to a partial area of specific microwell under the application of AC voltage. Due to the optically-induced negative DEP force, the trapped microbead was individually repelled and
positioned close to the ground electrode in an opposite direction to the exposed light. Two schematic and video clip show the directional movement of a microbead with $-x$ [Figure 4.8(a)] and $+x$ [Figure 4.8(b)] direction. At present, the process of releasing a microbead takes about 15 s, with scanning an array site, aligning the light to the well, and floating and releasing a microbead taking about 5, 7, and 3 s, respectively. Relatively short selection period would be useful to facilitate the high-throughput screening, to minimize the cell damage with short exposure of light and low optical energy. Also, I could manipulate the microbeads in $-y$ and $+y$ direction (data not shown) in the same regime. Optoelectrofluidic directional movement of target microbeads would give an opportunity to enhance the sorting selectivity and efficiency.

The selective and parallel release of distinct particles from each microwell was finally demonstrated (Figure 4.9). When the light pattern is selectively positioned and then electric field is applied, the particle could be ejected from the microwell. This allows the user to select a position on the computer screen and pick out the corresponding particle from the particular microwell.
Figure 4.7. Optoelectrofluidic on/off response. I checked whether the microbead was focused out for confirmation of the microbead levitation by optically-induced negative DEP force. The diagonal movement of the target microbead was observed under the application of AC voltage of 20 V at 100 kHz. After the voltage was turned off, the microbead was fallen back into the bottom of the microwell by the gravity force.
Figure 4.8. Programmable microbead release in different directions, (a) –x and (b) +x direction. Sequence of bright-field and schematic images and showing levitation and release of a target microbead illustrate the operation of the optoelectrofluidic interactive retrieval. (i) A target microbead was docked on the bottom of the microwell without application of AC voltage. (ii) A focused light projecting on half of the well accompanied by application of the AC voltage of 20 V at 100 kHz triggers to levitate the microbead along the diagonal direction by the optically-induced negative DEP force. (iii) The microbead was completely released from the microwell. The collection fluid flow will remove a single targeted microbead to the collection outlet.
Figure 4.9. Optoelectrofluidic selective retrieval. The interactive selection of the distinct microbeads was demonstrated.
The selective release and recovery of the target microbeads from the microwell was performed (Figure 4.10). As shown in Figure 4.10(a), the microfluidic inlet and outlet were drilled through the ITO-coated film to introduce the continuous flow for recovery of the target microbeads. The film-based electrode is cheap, thin, and flexible, allowing us to easily handle, modify, and punch holes for fluidic operation. The PDMS slab including inlet and outlet holes was attached on the top ITO film to assist the tight interconnection between the plastic tubing and the microfluidic inlet. To release and recovery of the target microbeads selectively, first, the trapped target microparticles were selectively positioned close to the ground ITO electrode in an opposite direction to the exposed light. Subsequently, the fluid flow was introduced along $+x$ direction, recovering the target microbeads at the microfluidic outlet. Figure 4.10(b) shows an image of the fabricated optoelectrofluidic device. As shown in Figure 4.10(c), an individual microbead trapped in the microwell was interactively floated up and transported along the flow direction within 2.50 s in the application of voltage of 20 $V_{pp}$ at 100 kHz.
Figure 4.10. Recovery of the target microbeads. (a) The PDMS slab including inlet and outlet holes was attached on the top ITO film to introduce the continuous flow. First, the trapped target microparticles were selectively positioned close to the ground ITO electrode in an opposite direction to the exposed light. Subsequently, the fluid flow was introduced along $+x$ direction, recovering the target microbeads at the microfluidic outlet. (b) Photograph of the fabricated device. ITO-coated film was integrated instead of the ITO glass to facilitate the drilling holes for fluidic operation. The PDMS slab assists the tight interconnection between the plastic tubing and the microfluidic inlet. (c) Captured movie for interactive recovery of the target microbead using a light pattern in the application of voltage of 20 V$_{pp}$ at 100 kHz.
4.5. Conclusions

In conclusion, I have presented an interactive, programmable, selective retrieval platform for optoelectrofluidic selection of single hydrogel microbeads. I proposed the hybrid optoelectrofluidic device integrated with free-standing hydrogel microwell array, which is capable of loading the individual hydrogel microbeads separately. PEG-DA-based free-standing hydrogel microarray is simple to fabricate, mechanically stable without morphological distortion, and easy to transfer and embed. Optoelectrofluidic-based interactive selection from the microwell array provides the simplicity, immediacy, and accuracy. I performed the massive handling and intuitive and interactive manipulation of 75 μm microbeads by selective levitation of target microbeads from the specific microwell. Since the optoelectrofluidics allows us to manipulate more microparticles in larger area than the optical tweezers, the storage efficiency of single particles should be enhanced by integrating mesh-grid of other fluidic components.[68] This automated and interactive methodology would promise a powerful technique in high-throughput single-cell screening.
CHAPTER V: Microcapsule-based quantitative estimation of the lipid productivity of single algae cells

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5.1. Introduction

Microfluidics has driven innovations in biological research, exploiting several advantages of the unique fluidic behavior through sophisticated control within precisely fabricated microstructures. Even at the single-cell level, multiplexed biological experiments have been demonstrated using high-throughput microfluidic analysis.[13,14] Recently, some interesting microalgae research has been reported, including their use as miniaturized sensing devices for environmental pollution,[126-129] photobioreactors for biofuel production,[130,131] and cell culture devices for microalgae screening.[35,132-134] In fact, microfluidic devices make it possible to accomplish numerous algae-based studies that are challenging or even impossible to achieve using their macro-sized counterparts. One such task is the selection and/or screening of potent microalgae; e.g., cells with high lipid productivity, which is presented here.

Sophisticated tools for single-cell analysis based on a wide range of new microtechnologies have emerged in the last decade.[135] Optical[136] and magnetic tweezers[137] have unique strengths of high resolution and the capability of three-dimensional manipulation, but they require complex optical systems and high optical energy. Microwell arrays, miniaturized replicas of 96-well plates, allow cells to be localized and monitored at the single-cell level.[138] This approach, however, has not been applied to microalgae, primarily due to the small cell size. A feasible alternative would be microdroplet-based technologies, which exploit droplets as tiny-volume units of fluid that are enveloped by immiscible fluid, such as oil, and have resulted in a state-of-the-art method of isolation and
encapsulation of individual cells for a variety of purposes.\[19,35,132,139\] Highly monodispersed droplets of micro-sized diameter can be produced rapidly;\[21\] thus, a single cell can be encapsulated within a well-controlled microenvironment inside the droplet.

The applicability of droplet-based microfluidics can be improved substantially by the synthesis and assembly of hydrogel microcapsules.\[39\] Hydrogels are an exciting class of scaffolding polymer that have great potential in biomedical applications due to their high biocompatibility, smart-response nature to the local environment, and ease of crosslinking under mild reaction conditions. Hydrogel microcapsules, when loaded with cells, are transported to the target site in an effective way and allow manipulation of cells with high viability and cultivability. Thus far, various microfluidic approaches have been demonstrated for the purpose of encapsulating various cell types.\[36\] An attempt was made to encapsulate a type of algae into hydrogel microcapsules to enable continuous tracking of their motility,\[140\] although no cell screening on the basis of lipid productivity has yet been reported.

Herein, I demonstrate the encapsulation of high-level biodiesel-producing microalgae into an alginate hydrogel microcapsule based on droplet-based microfluidics, as well as the in situ analysis of lipid content at the single-cell level. The picoliter-sized alginate hydrogel microcapsules, which were generated with high throughput and high uniformity, serve as a smart identification card that contains a biometric signature, which comprises parameters including phenotype, lipid content, and viability. The proposed technique provides the following advantages over conventional liquid droplets: (i) microcapsules containing single
cells can be manipulated for long periods of time with little evaporation of medium, (ii) the microcapsules can be utilized with microcapsule-based assay systems for further cell research, and (iii) the trapped individual cell, when necessary, can be recovered easily from the alginate microcapsule by dissolving it in biocompatible buffer. To detect intercellular lipids and compare them among microalgal strains, I utilized a lipophilic bright green fluorescent dye, BODIPY 505/515. BODIPY staining is a nondestructive means of lipid determination in live algal cells. Because the stained cells are viable, further analyses are also possible.[141] In this study, I encapsulated single cells of three green microalgae species (*Chlorella vulgaris*, *Chlamydomonas* sp. and *Botryococcus braunii*) in monodispersed microcapsules and conducted an in situ comparison and analysis of heterogeneity in the lipid contents of individual cells within the microcapsules by staining with BODIPY dye (Figure 5.1). In addition, I designed the high-density microwell arrays for inserting cell-encapsulating microcapsules and successfully observed the BODIPY fluorescence responses of the different single live algae.
Figure 5.1. Design and operating principles for the rapid preparation of monodispersed alginate hydrogel microcapsules encapsulating algal cells. Schematic diagram of the microfluidic device with integrated microbridge structures for the generation of alginate hydrogel microcapsules containing algal cells with sufficient spacing.
5.2. Experimental

5.2.1. Design and fabrication of microchannels

A microcapsule generator with integrated microbridge structures (Figure 5.1) was fabricated in poly(dimethylsiloxane) (PDMS) by soft lithography. This device consisted of two channels for Na-alginate and oil (20 and 40 μm wide, respectively), the microbridge (15 μm wide and 300 μm long), the main channel (100 μm wide) and a diverging microchannel near the outlet (200 μm wide). A PDMS mold was obtained by patterning SU-8 photoresist (Microchem Corp., St. Newton, MA) on a silicon wafer using standard lithography. Liquid PDMS mixed with a curing agent (ratio of 7:1) was cast on the mold and cured for 3 h in a convection oven at 65 °C for complete crosslinking. The PDMS channel was irreversibly sealed with a glass slide after exposure to oxygen plasma for 30 s. As the hydrophobic PDMS surface of the microchannel became hydrophilic during plasma treatment, the microfluidic device was stored in a convection oven at 65 °C for 1 day to allow recovery of the hydrophobic nature of the microchannel. After the fabrication process, the microfluidic devices were sterilized with 1% (w/v) Pluronic® F127 in distilled water prior to cell loading without algal cell attachment. Subsequently, the microchannels were flushed with an oil phase.

5.2.2. BODIPY-stained algal cell preparation

*C. vulgaris* was cultivated at 20 °C in non-saline BG 11 medium. *Chlamydomonas* sp. (KMMCC-1681) and *B. braunii* (KMMCC-868) were obtained from the algal culture
collection at the Korean Marine Microalgae Culture Center (Busan, Korea). *Chlamydomonas* sp. and *B. braunii* were grown in JM medium prepared in sterilized natural freshwater. All cultures were incubated under constant shaking with an agitation speed of 120 rpm and continuous illumination with a 3000-lux intensity lamp. BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Invitrogen Molecular Probes, Carlsbad, CA) was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution of 100 mg/L. Incubation for 30 min in darkness at room temperature was used for all staining. BODIPY 505/515-stained cells were examined microscopically using an inverted epifluorescence microscope (IX51; Olympus, Tokyo, Japan).

### 5.2.3. Preparation of monodisperse alginate hydrogel microcapsules encapsulating algal cells

Sodium alginate (1% w/w; A0682-100G, Sigma) in JM media was prepared and filtered with a 0.22-μm syringe filter (Millex-GV, Millipore) to remove any clumps of alginate. Oleic acid (Sigma) with Abil EM 90 surfactant (4% w/w) was introduced as a continuous phase and the Na-alginate was crosslinked by the calcified oleic acid (24 mg/mL). The calcified oleic acid was prepared by dissolving 0.6 g of calcium chloride (C7902-500G, Sigma) in 25 mL of oleic acid via ultrasonication. Calcium chloride was dissolved in 25 mL of 2-methyl-1-propanol (J.T. Baker, Deventer, The Netherlands) via ultrasonication. After mixing calcium chloride and oleic acid at a ratio of 50% (v/v), the 2-methyl-1-propanol was distilled in a convection oven at 65 °C for a day. All fluids were injected and transported via syringe
pumps (Pump 11 Pico Plus; Harvard Apparatus, Inc., Holliston, MA) at a range of volumetric flow rates.

5.2.4. Cell viability assay

As a general staining method to determine cell viability, dual-fluorescence viability assays using nucleic-acid-binding fluorescent probes, such as SYTO and propidium iodide, have been used widely. Due to the autofluorescence of chlorophyll pigments, however, these assays could not be used in this study. Therefore, SYTOX® Green, which is used predominantly for staining phytoplankton species and can penetrate damaged cell membranes, was used. Because SYTOX Green fluorescence does not overlap with the red autofluorescence, the dead microalgae—which exhibit bright green fluorescence—could be distinguished easily.
5.3. Results

5.3.1. High-throughput production of alginate hydrogel microcapsules encapsulating microalgal cells

To fabricate the algae cell capsules, I used a previously developed microfluidic device with integrated microbridge structures (Figure 5.1). Briefly, the microbridge provides two advantages in microcapsule preparation: (i) the aggregation of surrounding microcapsules can be prevented by suitable microfluidic spacing, and (ii) the size of droplets can be maintained irrespective of the control flow rate. I dispersed microdroplets of Na-alginate in media containing an alga in a continuous phase of oleic acid with Abil EM 90 surfactant (4% w/w) using a flow-focusing method at a cross-junction [Figure 5.2(a)]. To generate spherical hydrogel microcapsules, I exploited the enlargement in channel height from 35 to 100 μm, which facilitated recovery of the droplet shape from a plug to a sphere. Further downstream, the additional control oil flow (Qc), which includes calcified oleic acid (24 mg/mL) with a surfactant, changes the velocity of the main flow via the microbridge structures. I could visualize the migration of control flow by means of the algal autofluorescence passing through each microbridge [Figure 5.2(b)]. The control flow occupied more space in the microchannel when Qc increased [Figure 5.2(c,d)], showing good agreement with the simulation, as reported previously.[97] As a result, the droplet interval can be adjusted actively within the main channel and near the outlet, when Qc is 150 μL/h [Figure 5.2(c)] or 900 μL/h [Figure 5.2(d)]. Figure 5.2(e) shows that the droplet interval increased significantly as Qc increased without any effect on the droplet-generating pattern. When Qc was adjusted
from 150 to 900 μL/h, the droplet interval changed from 34.40 ± 12.14 to 99.52 ± 20.48 μm, at a fixed droplet generation condition with a Na-alginate flow rate (Q_w) of 20 μL/h and oil flow rate (Q_o) of 50 μL/h.
Figure 5.2. (a) Bright-field micrograph showing cell encapsulation into a Na-alginate droplet at the cross-junction. (b) Fluorescence micrograph illustrating the migration of control flow passing through each microbridge. The additional control oil flow ($Q_c$), including calcified oleic acid, changes the velocity of the main flow via the microbridge structures. (c, d) Micrographs showing variation in droplet interval within the main channel (left panels) and near the outlet (right panels) at $Q_c$ of (c) 150 $\mu$L/h or (d) 900 $\mu$L/h. Scale bars: 50 $\mu$m. (e) Diameter distribution of alginate droplets shown under flow conditions of $Q_w = 30$ $\mu$L/h and $Q_o = 50$ $\mu$L/h. The generated alginate droplets were monodispersed with a mean diameter of 26.34 $\mu$m.
Figure 5.3(a) presents enlarged images of the fabricated cell-laden spherical microcapsule with different spacing under $Q_e$ values of 150 and 900 $\mu$L/h, respectively. Undesirable coalescence of the hydrogel microcapsule was observed at the relatively low control flow rate. As a result, I confirmed that suitable spacing between two approaching microdroplets guaranteed the stable formation of alginate hydrogel microcapsules. This unwanted coalescence and aggregation of hydrogel microcapsules due to insufficient spacing reduces both monodispersity and single-cell encapsulation efficiency.

I produced monodispersed sodium alginate droplets within the microchannel. Figure 5.3(b) illustrates the variability in droplet size with a flow rate ratio ($Q_w/Q_o$) of 0.4 when generating alginate droplets containing *C. vulgaris*. I utilized oleic acid with the Abil EM 90 surfactant (4% w/w) as the oil phase, and cell medium (BG 11 for *C. vulgaris*, and JM for *Chlamydomonas* sp. and *B. braunii*) containing Na-alginate (1% w/w) as the liquid phase. The microcapsules showed high monodispersity irrespective of the presence of a cell. The oil flow rate was fixed at 50 $\mu$L/h. I could adjust the microcapsule diameter from 19 to 36 $\mu$m. As the flow rate ratio increased, the droplet diameter decreased (data not shown). As depicted in Figure 2d, I generated monodispersed algal-cell-encapsulating alginate droplets with a mean diameter of 26.34 $\mu$m ($n = 170$). I confirmed that the percentage of single-cell-containing droplets at initial cell loading concentrations of $1 \times 10^7$ and $5 \times 10^7$ cells/mL was similar to the theoretical value of the Poisson distribution. Using the proposed device, up to 35% of droplets generated contained single algal cells [Figure 5.3(c)]. The efficiency of
single-cell encapsulation could be enhanced by incorporating the interconnection technique or secondary microfluidic networks.[46]
Figure 5.3. (a) Enlarged images of a fabricated cell-laden spherical microcapsule with different spacing under control flow rates ($Q_c$) of (I) 150 and (II) 900 μL/h. Aggregation of hydrogel microcapsules occurred at relatively low $Q_c$. (b) Diameter distribution of alginate droplets shown under flow conditions of $Q_w = 30$ μL/h and $Q_o = 50$ μL/h. The generated alginate droplets were monodispersed with a mean diameter of 26.34 μm. (c) Percentage of droplets containing single *C. vulgaris* cells at initial cell loading concentrations of $1 \times 10^7$ and $5 \times 10^7$ cells/mL. Up to 35% of droplets contained single algal cells.
5.3.2. Microcapsule-based interspecies comparison of the intracellular lipids in microalgae

Lipid productivity, which is calculated based on both the growth and lipid content of oleaginous microbes such as microalgae, is advantageous for microalgae-derived biodiesel production.[8] Obtaining microalgal species with such properties is critical; however, doing so is challenging, particularly in a high-throughput manner. To investigate the lipid content of a cell encapsulated within an alginate hydrogel microcapsule, the BODIPY fluorescent dye was employed and three microalgal species were investigated (Figure 5.4). Nile Red, which stains lipids within animal cells and microorganisms, was not suitable for this study, as it is unable to penetrate the thick walls of microalgal cells and both the dye itself and/or a carrier solvent are detrimental to cell viability; further, its emission spectrum overlaps with that of chlorophyll, so it interferes with accurate estimation of intercellular lipids. After staining with BODIPY, the lipid bodies in all three algal cells exhibited bright green fluorescence, although the intensity varied markedly [Figure 5.4(b), (d), and (f)].

Bright-field microscopic images of microcapsules containing single cells of three species were obtained in the microfluidic device [Figure 5.4(g), (i), and (k)]. The positions of cells inside the microcapsules varied due to the circulation flow within the droplet before gelation. To confirm and compare lipid accumulation of a single cell of among three species, I visualized the green fluorescence within the microcapsules by fluorescence microscopy [Figure 5.4(h), (j), and (l)]. The results suggest that the intracellular lipid levels were distinguishable, although the cell was encapsulated in the microcapsule. Figure 5.4(m) shows
the fluorescence intensities of the three microalgal species within microcapsules, which is indicative of their average lipid contents. *B. braunii* exhibited a higher lipid content than *C. vulgaris* or *Chlamydomonas* sp.
Figure 5.4. (a, b) Enlarged bright-field and fluorescence images of microcapsules containing single (a, b, g, h) *C. vulgaris*, (c, d, i, j) *Chlamydomonas* sp., and (e, f, k, l) *B. braunii* cells, respectively. Scale bars: 20 μm. (m) Fluorescence intensity profiles of each of the three microalgae within microcapsules (*n* = 60).
5.3.3. Microcapsule-based lipid profiling of individual algal cells

The conventional method of estimating the lipid content of microalgae represents only the average value of a population. Therefore, any heterogeneity in lipid accumulation is not reflected. Traditionally, the lipid content of microalgae is estimated by measuring the dry biomass via a gravimetric method, which requires at least 10–15-mg wet weight of cells and a day-long drying process. Furthermore, because post-extraction algae cannot be subjected to any additional analyses, fluorescence measurement using Nile red[142] and BODIPY[143] staining is more appropriate for single-cell lipid screening. A comparison between the lipid contents measured by Nile red or BODIPY and the lipid content showed a linear relationship, which has advantages of in situ measurement of single microalga within a microcapsule in a high-throughput manner.

I first confirmed the heterogeneity in the lipid accumulation of single cells from three different species in bulk. Figure 5.5 shows the bright-field and fluorescence images of the algal species, including *C. vulgaris* [Figure 5.5(a, b)], *Chlamydomonas* sp. [Figure 5.5(c, d)], (e, f) *B. braunii* [Figure 5.5(e, f)], respectively, to investigate the difference in lipid accumulation among individual cells. From the bright-field images, I could realize that there was diversity in cellular size, meaning of the heterogeneity in the growth rate, and in degree in green color, representing the spatial occupation of the chloroplast within the cell. When I stained cells using BODIPY, I also observed the heterogeneity in the green fluorescence despite the same species. Figure 5.5(g) represents the distribution of green fluorescence among individual cells in three species of algae. All three species showed a deviation of the
green fluorescence due to their heterogeneity in lipid content, which was observed in Figure 5.5b, d, and f.
Figure 5.5. Bright-field and fluorescence images of (a,b) C. vulgaris, (c, d) Chlamydomonas sp., (e, f) B. braunii, stained with BODIPY in bulk scale to show the heterogeneity in the lipid accumulation among the individual cells. Scale bars = 20 μm. (g) Histograms showing the distribution of green fluorescence among individual cells in three species of algae.
To identify heterogeneity among individual cells in a population, I isolated single-cell-containing hydrogel microcapsules and examined their lipid contents by BODIPY staining. The lipid contents of 10 randomly selected single *C. vulgaris*, *Chlamydomonas* sp., and *B. braunii* cells are shown in Figure 5.6(a), (b), and (c). I successfully generated monodispersed hydrogel microcapsules with a mean diameter of 26 μm, each of which contained a single microalgal cell. Accumulated lipid bodies, which are evident in these images, exhibited highly variable fluorescence signals among cells within each microcapsule. The normalized fluorescence intensity of individual cells within microcapsules is indicated at the bottom of Figure 5.6(a), (b), and (c). The fluorescence intensity was normalized to the maximum intensity of each species. Figure 5.7 shows the results of profiling of whole microcapsules and the fluorescence intensity distribution of individual cells of the three algal species tested.

Each fluorescence intensity frequency corresponds to the green fluorescence of individual cells within a single microcapsule. Based on these findings, I propose classification of individual algal cells according to their lipid content; e.g., lipid contents on a scale of 0 to 1. Particularly, due to its exceptionally high lipid content, the botryoid-shaped microalgal species *B. braunii* has been considered the feedstock of choice for biodiesel production. Further, *B. braunii can* form blooms; these individual pyriform-shaped cells stand together and contain long hydrocarbon chains. To visualize the lipid contents of individual cells, I disintegrated the clump (or colony) by mild sonication. This physical disruption resulted in BODIPY being localized in lipid globules, which appeared to be round, without other cytoplasmic compartments [Figure 5.6(c)]. Thus, lipid bodies could be distinguished from
other parts of the cell as bright green structures, even though the individual microalga was encapsulated in the hydrogel microcapsule. The greater green fluorescence intensity, which indicates higher lipid content, indicates that *B. braunii* is a potent lipid-producer.

I constructed a microwell array with single microalga-containing microcapsules [Figure 5.6(d)]. Each microwell had a width, depth, and center-to-center distance of 35, 17, and 50 μm, respectively. In total, ~110,000 wells were present on a 15 × 15 mm substrate. Microcapsules filled ~60% of the microwell array. Use of a microstructure for more efficient insertion of microcapsules into microwells could allow ~95% of the microwell array to be filled.[68] Figures 5.6(e) and (f) show the portion of a microcapsule array which represents the magnified bright-field and fluorescence images of the entrapped microcapsules, respectively. Microcapsules encapsulating *B. braunii* cells were readily distinguishable from empty microcapsules by fluorescence microscopy. The differences in lipid content among the three microalgae in the microwells were estimated by their fluorescence [Figure 5.6(g)].
Figure 5.6. Microcapsule-based in situ profiling of the lipid contents of three microalgae species within hydrogel microcapsules at the single-cell level. Enlarged bright-field and fluorescence images of microcapsules containing single cells stained with BODIPY: (a) C. vulgaris, (b) Chlamydomonas sp., and (c) B. braunii, respectively. The normalized fluorescence intensities noted at bottom indicate the heterogeneity in lipid content. (d) Micrograph of the hydrogel microcapsule array. Hydrogel microcapsules containing single B. braunii cells were distributed randomly within the high-density microwell array. Magnified (e) bright-field and (f) fluorescence images of a trapped microcapsule. Microcapsules containing single cells were readily distinguishable from empty microcapsules by fluorescence microscopy. (g) Three different fluorescence responses within microcapsules in each of the microwells shown in panel f. Scale bars: 20 μm.
Figure 5.7. Enlarged bright-field and fluorescence micrographs of microcapsules containing single BODIPY-stained (a) *C. vulgaris*, (c) *Chlamydomonas* sp., and (e) *B. braunii* cells, respectively. In situ quantitative comparison of the normalized fluorescence intensity of (b) *C. vulgaris* (*n* = 60), (d) *Chlamydomonas* sp. (*n* = 60), and (f) *B. braunii* (*n* = 50) cells within microcapsules. The stochastic heterogeneity in algal lipid content was evaluated based on the fluorescence signals of single microalgae. Scale bars: 20 μm.
5.3.4. Lipid screening in single microalgae using hydrogel microcapsule arrays

Here, the successful results of hydrogel microcapsule trapping in a microwell array are presented. Microcapsules that contained a single *B. braunii* were sequentially introduced to the microwell array by gentle pipetting. The microcapsule introduction was repeated on the same microwell array as many as three times. Microcapsules filled approximately 90% of the microwell array [Figure 5.8(a)]. Figures 5.8(b) and 5.8(c) show the bright-field and fluorescence microscopic images, respectively, of the trapped microcapsules transferred from the microfluidic device to the microwell array, filling the microwells with single cells. The green fluorescent cells are *B. braunii* expressing BODIPY response.
Figure 5.8. (a) Microscopic images of the hydrogel microcapsule array showing trapping of single microcapsules. More than 90% of the wells contained one microcapsule containing a single *B. braunii* when the microwell size was comparable to the microcapsule diameter. (b) Bright field and (c) fluorescence images of the trapped microcapsules. Each green fluorescent cell is a *B. braunii* expressing BODIPY response. Scale bars: 200 μm.
The differences in lipid content among the *B. braunii* in the microwells were estimated using hydrogel microcapsule arrays. Figures 5.9(a) and 5.9(b) show a portion of a microcapsule array, representing the magnified bright-field and fluorescence images of the entrapped microcapsules containing single *B. braunii* cells, respectively. The field of view through the 10× objective lens is sufficient to capture the microcapsule entrapment within 165 microwells. Figure 5.9(c) shows the first (A) row of the microwell array. The trapped microcapsules within the microwell are noticeable against the empty well. Furthermore, the diversity of lipid heterogeneity among individual cells was examined by their BODIPY responses. However, autofluorescence of the THB photoresist was detected [gray line in Figure 5.9(d)]; thus, the relative fluorescence intensity of the BODIPY response from a single microalga was obtained after subtracting the autofluorescence of the microwell [black line in Figure 5.9(d)]. The single cells within the second and eighth microwell have higher fluorescence intensities than the other wells. In the same way, the lipid content of the remaining 154 microcapsules was examined in Figure 5.10. The red line in Figure 5.10 indicates the autofluorescence of THB photoresist.
Figure 5.9. Hydrogel microwell array with cell-containing hydrogel microcapsules. (a) Bright-field and (b) fluorescence images of a trapped microcapsule containing single *B. braunii* cells. (c) Magnified images of the first row of the microwell array. (d) Different fluorescence responses of single cells within microcapsules in each of the microwells based on the intensity profile on the line a-b in panel (b) (black lines). Gray lines indicate the autofluorescence of THB photoresist.
Figure 5.10. Fluorescence intensity profile of the hydrogel microwell array of Figure 5.9(b) (black line). Red lines indicate the autofluorescence of THB photoresist.
Quantitative scoring is one of the main issues in estimating the stochastic heterogeneity in algal lipid content, and in determining a critical selection criterion for a specific microalga with high lipid content. By analyzing the fluorescence profile obtained via image analysis, the lipid contents of single microalgae were scored (Figure 5.11). The values of the sorting threshold were set at 7.5, 15.0, or 22.5 a.u. to define the level of lipid accumulation (each red dotted line in Figure 5.11(b)). The score levels are expressed with colors based on their fluorescence intensity [Figure 5.11(c)]. For example, we translated (–) for \( \leq 7.5 \) a.u., (+) for \( 7.5 < x \leq 15.0 \) a.u., (2+) for \( 15.0 < x \leq 22.5 \) a.u., and (3+) for \( > 22.5 \) a.u.. While a single microalga existed in the microcapsules, some microcapsules showed a (–) score, reflecting the heterogeneity in lipid content among individual cells of the same species. This result also indicates that the platform is applicable to the identification and selection of individual hydrogel microcapsules containing lipid-rich microalgae.
Figure 5.11. (a) Snapshot of a fluorescence image of a trapped microcapsule containing single *B. braunii* cells. (b) Fluorescence intensity profile of the hydrogel microwell array of panel (a). The autofluorescence from photoresist of the wells was subtracted. The red dotted lines indicate the three different threshold values of 22.5, 15.0 and 7.5 a.u. (c) Results of quantitative scoring for the case in panel (b). We translated (–) for $\leq 7.5$ a.u., (+) for $7.5 < x \leq 15.0$ a.u., (2+) for $15.0 < x \leq 22.5$ a.u., and (3+) for $> 22.5$ a.u.. The fluorescence intensity and their score levels are expressed with colors (bright green: 3+, dark green: 2+, white: +, red: –).
**Figure 5.12.** Results of quantitative scoring for another eight cases.
5.3.5. Viability assessment

A cell viability assay was performed to see if the algal cell is actually live in the microcapsule. Figures 5.13(a) and (b) present bright-field and fluorescence microscopic images, respectively, of encapsulated C. vulgaris cells stained with SYTOX Green. Chlorella was readily visualized by its red fluorescence. Images of single-alga-containing microcapsules showed that live [Figure 5.13(c), (e)] and dead cells [Figure 5.13(d), (f)] were distinguishable by their red and bright green colors, respectively. Two distinct fluorescence signals after SYTOX staining of the other species (Chlamydomonas sp. and B. braunii) were also observed (data not shown). I also quantified microalgae viability [Figure 5.13(g)] both in bulk and within microcapsules. The viabilities of free C. vulgaris, Chlamydomonas sp., and B. braunii cells were 88.88%, 85.56%, and 75.56%, respectively, based on three replicates. Despite the acidity and low gas permeability of oleic acid, the percentage viabilities of C. vulgaris, Chlamydomonas sp., and B. braunii within the microcapsule were stable at ~70%. This was because oleic acid was exchanged for mineral oil at the microfluidic collection chamber, resulting in maintenance of cell viability within the alginate microcapsules. The viability of cells within alginate hydrogel microcapsules could be further improved by introduction of pure oil, such as mineral oil or hexadecane, as a control flow through the additional oil exchange microchannel.[97] A microfluidic device that enables continuous-flow extraction of microcapsules from an oil phase into aqueous phase might also increase the viability.
Figure 5.13. Viability of the three microalgae species. (a) Bright-field and (b) fluorescence micrographs of encapsulated *C. vulgaris* cells stained with SYTOX green. Magnified bright-field images of microcapsules containing single (c) live and (d) dead microalgae, which emit (e) red and (f) bright green fluorescence due to SYTOX Green staining. Scale bars: 20 μm. (g) Percentage of live cells in bulk samples (*n* = 30) and within microcapsules (*n* = 15) of the three species.
5.4. Discussion

Alginate is an excellent material that is preferred over other hydrogel precursors for the entrapment of a single microalga due to its biodegradability, rapid solidification using calcium ions, and high permeability to nutrients. Several groups have exploited microfluidics for the encapsulation of cells in spherical alginate microcapsules. Sugiura et al. presented a microfabricated device enabling to prepare size-controlled alginate microcapsule less than 300 μm. The device includes a thin and short silicon nozzle which utilizes the droplet formation by viscous drag force of the oil flow. However, the difficulty in complex silicon fabrication is still facing significant obstacles due to high production costs and poor efficiency.[144] Morimoto et al. reported an internal gelation process with CaCO₃ nanoparticles,[140] however, there was a drawback in terms of reduced viability due to gradual diffusion of acetic acid. Also, despite their monodispersity and spherical shape, the fabricated microcapsules were relatively large (~100 μm), making them inappropriate for encapsulation of a single small microalga. To date, the bulk immobilization of green microalgae has been demonstrated for only in situ monitoring of metal ion adsorption[145] and the assessment of eutrophication in flowing waters.[146] In the current study, I fabricated relatively small (~35 μm) microcapsules containing a single algal cell in a microfluidic device with an integrated microbridge structure. However, despite the presence of a surfactant layer, aggregation of the microcapsules near the outlet was observed frequently. Through temporal control by adjusting the spacing between two microcapsules, I
successfully fabricated spherical alginate microcapsules containing a single algal cell with no aggregation (Figure 5.14).
Figure 5.14. A comparison of fabrication technology for single-cell laden microcapsules.

<table>
<thead>
<tr>
<th><strong>Biomaterials 2005</strong></th>
<th><strong>Advanced Materials 2007</strong></th>
<th><strong>Biomicrofluidics 2011</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-nozzle array</td>
<td>Axisymmetric flow-focusing</td>
<td>Microbridge</td>
</tr>
<tr>
<td>Complex Si fabrication (Deep reactive ion-etching)</td>
<td>Complex fabrication (Stereolithography)</td>
<td>Simple fabrication (PDMS soft lithography)</td>
</tr>
<tr>
<td>Multi-cell encapsulation</td>
<td>Multi-cell encapsulation</td>
<td>Single-cell encapsulation</td>
</tr>
<tr>
<td>~160 micron diameter</td>
<td>~100 micron diameter</td>
<td>~30 micron diameter</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>CaCO₃</td>
<td>CaCl₂</td>
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</table>

Note: CaCO₃ (would be harmful due to the low acidity)
I determined the lipid content of single microalgal cells within alginate hydrogel microcapsules. Many microalgae accumulate lipids under environmental stresses, including nitrogen repletion, UV exposure, low light, temperature, salt stress, and nutrient deficiency. In particular, nutrient deficiency has a marked effect on algal lipid content. Thus, several studies of the effects of nutrient deficiency have been reported; however, the results reflected only the average lipid content of algal communities in response to an external stress. The advantage of microcapsule-based determination of the lipid content of a single cell rather than in bulk is that the effect of environmental stress on individual microalgal cells can be determined. The immediate response of a single live alga to a shift in environmental conditions could be monitored in real time by fluorescence microscopy. Furthermore, the integration of microfluidic components for microcapsule storage in the microwell array (Figure 5.6) and mounting of a microchannel to supply various chemical reactants to the individual microcapsules may provide a multiplexed screening platform for studies of individual algal cells. This approach will facilitate the discovery of cellular heterogeneity in algal cultures, which has been investigated only in bulk systems to date.

The heterogeneity in lipid content shown in Figure 4 is derived from the variability in cell status according to growth rate. Light is important for growth of algae, which ensures high lipid productivity via photosynthesis. However, bulk cultivation in 250-mL conical shaking flasks, rotating at 120 rpm under continuous illumination, suffers from insufficient light provision due to the high cell density. Cells deeper in the shaking flask receive attenuated light, less than the average light intensity per cell.[147] This ‘self-shading’ effect would be
greater in larger culture flasks as the cell density increases. This effect results in differences in growth rate and lipid accumulation among cells according to their position in the flask. This in turn leads to the difference in BODIPY fluorescence intensity.

Successful biodiesel production depends on the selection of fast-growing, lipid-rich microalgae from a large number of microalgal species. Thus the lipid content of an individual alga should be determined. Lipid-rich microalgae are generally identified using fluorescence-activated cell sorting (FACS), which can distinguish lipid-stained from unstained cells in a high-throughput manner. The combination of BODIPY staining with green fluorescence and FACS allows effective cell separation because the emission spectrum of BODIPY is spectrally separated from the red algal autofluorescence. However, use of FACS has some limitations. First, monitoring and measuring the behavior of individual cells is problematic. Also, typical FACS systems are expensive to purchase and maintain, and they require highly trained operators, making it problematic for environmental engineers to operate the complicated apparatus. The most important problem is that FACS does not ensure the viability of recovered cells. Post-FACS cells have a higher probability of damage and contamination, referred to as ‘cross-contamination’. On the other hand, using droplet-based microfluidics, a single alga can be encapsulated into a picoliter droplet. In particular, the proposed platform provides an appropriate environment for live microalgae within the hydrogel microcapsule with a continuous supply of culture medium, while avoiding cross-contamination from other microorganisms. Many suitable droplet-based microfluidics approaches for continuous droplet sorting based on cellular fluorescence signals have been
reported; these facilitate recovery and inoculation of the cells of interest. The stochastic heterogeneity in algal lipid content, which was estimated quantitatively in my system, is a critical selection criterion for a specific microalga with high lipid content. Furthermore, automated or interactive selection of a microcapsule containing the desired alga with no need for complex apparatus is required in various environmental technologies. An optoelectrofluidic interactive separation technique[115,116,118,124] would be appropriate for this purpose.
5.5. Conclusions

In the current study, I successfully encapsulated microalgal cells within alginate hydrogel-based microcapsules in a microfluidic device that incorporated a microbridge structure. This was confirmed using the microalgae *C. vulgaris*, *Chlamydomonas sp.* and *B. braunii*. To my knowledge, this is the first report that live algae cells could be encapsulated and their lipid contents determined in an on-line, real-time manner. By utilizing the rapid migration of the additional control flow, which includes a calcified oil phase, via a microbridge structure, I could fabricate stable alginate hydrogel microcapsules with suitable spacing; moreover, no aggregation of microcapsules was identified. The fabricated microcapsules showed characteristics of monodispersity, sufficient robustness in handling, stability for long-term manipulation, and maintenance of cell viability.

This unique combination of microcapsules and nondestructive fluorescence staining with BODIPY will facilitate follow-up biological analyses. Similar to previous reports, the fluorescence intensity differed significantly among the three species due to differences in lipid contents. The heterogeneity of lipid accumulation among individual cells of the same species was investigated by in situ profiling of the intercellular lipid at the single-cell level. I also combined the cell-encapsulation methodology with the microwell array technology to realize a lipid screening platform. Maintenance of cell viability within the microcapsules was confirmed by SYTOX staining. Therefore, microcapsule encapsulation protected the cells from damage during lipid screening procedures. The proposed microcapsule-based platform
for screening of lipid-rich microalgae is now ready for use in alternative energy development, and represents a versatile tool for environmental engineering research.
CHAPTER VI: Conclusions

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6.1. Summary

The ultimate goal of biofuel production is to enable the sustainable production of renewable energy as a warning for the increasing combustion of fossil carbon. Microalgae have much higher lipid contents than those of food crops and oil seeds, but they suffer from several constraints to the recovery of microalgal biomass due to the high expenditure. Therefore, the specific microalga containing high-yield lipid contents should be investigated and selected for efficient production of biofuels from algal biomass. In the conventional method, many researchers have focused on the interspecies comparison to examine the intracellular lipid content under different environmental conditions in a bulk scale. However, the difference of lipid content between the individual algae cells in the same species has not been considered until now.

To address the above issues, droplet-based microfluidics was utilized to generate the highly monodispersed microcapsules encapsulating individual microalgae. Hydrogel microcapsules allow a high degree of permeability for continuous supplying of nutrients and maintenance of cell viability. In this dissertation, I first developed the microcapsule-based interactive platform for screening of high biodiesel producing microalgae compared to the related state-of-the-art studies. I performed the identification of the lipid content of the different species of microalgae encapsulated into the hydrogel microcapsule, and the selection of the lipid-rich microalgae by incorporating with the interactive microcapsule manipulation technology.
First, I suggested a novel interconnection microparticle injection technique, which prevents particle loss, assisted by sample injection along the direction of fluid flow. Sample fluids, including microparticles, and mammalian (U937) and green algae cells (C. vulgaris), were injected directly via a through-hole drilled in the lateral direction, resulting in a significant reduction in microparticle attachment. The proposed regime achieved a twofold enhancement of single-cell encapsulation compared to the conventional encapsulation rate, based on a Poisson distribution. At the high concentration (5×10^7 cells/mL), the fraction of droplets containing single cells increased gradually from 28.67% to 40.00% (n = 3). At a lower concentration (1×10^7 cells/mL), there was an increase in the percentage of droplets including single cells from 15.33% to 35.33%.

Second, I presented a facile and robust microfluidic platform enabling uniform interval control of flowing droplets for the precise temporal synchronization and pairing of picoliter droplets with a reagent. By incorporating microbridge structures interconnecting the droplet-carrying channel and the flow control channel, a fluidic pressure drop was derived between two fluidic channels via the microbridge structures, reordering flowing droplets with a defined uniform interval. Through the adjustment of the control oil flow rate, the droplet intervals were flexibly and precisely adjustable. With this mechanism of droplet spacing, the gelation of the alginate droplets as well as control of the droplet interval was simultaneously achieved by additional control oil flow including calcified oleic acid.

Third, I demonstrated an optoelectrofluidic device integrated with photopolymerizable hydrogel microwell arrays for facile selection of individual microparticles. A 20 × 12 mm-
sized, polyethylene glycol diacrylate-based 111 × 66 array of traps was integrated between the top ITO glass and the bottom photoconductive layer to capture and release of microparticles. The microparticles in liquid solution can be passively loaded to the separated microwells via sedimentation thus a real-time microscopic monitoring was allowed. Feasibility was demonstrated by capturing and releasing 75 µm-diameter plain polystyrene particles. When a dynamic image pattern is projected into an area of the specific microwell under the application of AC signal with 20 V bias at 100 kHz, the trapped particle was individually repelled and positioned close to the ground electrode by negative DEP forces. Thus I successfully achieved the ejection of specific particles from the microwells. I also demonstrated the selective and parallel release of distinct particles from each microwell.

At the last parts of this dissertation, I performed the microcapsule-based interactive screening of lipid-rich microalgae based on the quantitative estimation of the lipid content of individual microalgae. I successfully generated the alginate hydrogel microcapsules containing single cells from three species of green microalgae (C. vulgaris, Chlamydomonas sp. and B. braunii) in a high throughput manner. There was a significant difference of the fluorescence intensity, which indicates the intracellular lipid content, among three species of individual cells by incorporating the non-destructive fluorescence staining method with BODIPY. I also could observe the existence of the heterogeneity in the lipid accumulation among the individual cells in the same species.
6.2. Outlook, challenges, and future works

6.2.1 Outlook and challenges

This dissertation proposed technical innovations for the estimation of lipid content of single microalgae using droplet-based microfluidics. The single-cell based lipid screening is a very promising area of research in environmental engineering and cell biology, it currently faces several challenges. There are still lots of issues to be solved to be truly utilizable in environmental engineering field.

First, the procedure of BODIPY staining should be simplified. The additional inlet and fluid network for the introduction of BODIPY staining solution should be included in the microcapsule-generating microfluidic device. One droplet which isolates a single microalgae and fluorescence staining solution effectively will be generated by flow focusing method at a cross junction, and this leads to effective mixing. The fluidic networks could be helpful to save the amount of staining solution, to reduce the staining time, and to remove the labor-intensive process, which maximizes the advances of lab-on-a-chip technology. This will be a crucial development and contribution to the automated pretreatment system in the whole environmental engineering field.

Second, other microfluidic components should be integrated to perform the enhanced coalescence of spatiotemporally controlled droplets for achieving advanced combinatorial reactions in a large scale screening applications. I demonstrated a dynamic temporal control of two distinct droplets as the difference in the droplet interval of the two distinct droplets. The ratio in droplet pairs was adjusted rapidly by changing control oil flow rate. However,
the development of merging of the droplet pairs remains unfinished. Well-established microfluidic components for droplet coalescence have been demonstrated, which is capable to be easily integrated to the proposed microfluidic device. The coalescence of the droplet pairs with various ratios could be appropriate for generation of alginate hydrogel microcapsule with various compositions of hydrogel crosslinkers.

For effective construction of the microcapsules with a cell-friendly environment, biomaterial issues should be studied in the concrete. For specific type of microalgae, specialized 3D environments may be essential for appropriate lipid screening. The intrinsic properties of hydrogel materials should be investigated such as strength, Young’s modulus, permeability, and etc. There are lots of materials as a crosslinker for creation of microcapsules with tailored architectures.[111,149] I have demonstrated the continuous generation of hydrogel microcapsules using an alginate as a crosslinker, however many researchers have reported the fabrication of microcapsule consisted of various kind of crosslinkers besides sodium alginate.[38,150-153]

For quantitative estimation of lipid productivity of a particular microalga, more investigation of the method of increasing lipid content should be involved. As I represented in Figure 1.1, lipids accumulation can be begun under the environmental change including nutrient starvation, temperature shift, and change in salinity and pH. Above all, the most frequently reported method is nitrogen starvation, which is applied in this dissertation. Another lipid-accumulation method can be applied to my culture system, and the lipid-accumulated microalgae will be applied to the proposed lipid screening platform. It is
anticipated that the lipid accumulation of microalgae can be evaluated in a single cell level according to the several stress conditions.

It is important that a viable condition should be maintained to monitor of single microalga for estimation of lipid productivity. In this dissertation, in the Chapter 5, I used the BODIPY as a staining solution, which is not harmful to cells, to ensure their viability and capability of other biological experiments after lipid screening. However, the staining procedure requires a time-consumption and reduces its availability and ease of use. Alternatively, several label-free determination techniques have received much attention because they support a role as the basis of secondary assays. Several microfluidic platforms have collaborated on many label-free detection principles such as optics,[154,155] acoustic waves,[156] lensfree imaging,[157-159] etc. By integrating above techniques with the proposed microfluidic platform, I may expect to achieve the label-free lipid profiling of individual microalgae in a high resolution, with the maintenance of high viability of cells.

Ultimately, the proposed microfluidic platform can be extensively employed to identify the lipid content of microalgae whose species is not revealed. They are a large number of algal species in raceway ponds and sea, but only a few of them was revealed, even their lipid contents were not disclosed. The proposed microfluidic platform will be contributed to find the answer to the problem as I mentioned above. In addition, to prevent of predation by protozoa and contamination by other microbial organisms, biological contaminants should be separated from the sample fluids of microalgae. Microfluidic separation platform for subtle discrimination of unwanted contaminants can be easily integrated.
6.2.2 Future directions

Several studies of the effects of nutrient deficiency have been reported; however, the results reflected only the average lipid content of algal communities in response to an external stress. The advantage of the microcapsule-based determination of the lipid content of a single cell rather than in bulk is that the effects of environmental stress on individual microalgal cells can be determined. The immediate response of a single live alga to a shift in environmental conditions could be monitored in real time using fluorescence microscopy. This approach will facilitate the discovery of cellular heterogeneity in algal cultures, which has been investigated only in bulk systems to date (Figure 6.1).

Bacteria are motile and understanding their motile characteristics is important. By combining single-cell detection technology and droplet microfluidics, we can better understand the swimming mechanics of single bacteria. Droplet microfluidics can provide competition for nutrients among bacteria by caging them, like a birdcage (Figure 6.2).
Figure 6.1. Schematic figures for study of the effects of nutrient deficiency based on microfluidics.

Figure 6.2. Schematic figures for detection of single microalga movement within the microsphere. Droplet microfluidics can provide competition for nutrients among bacteria by caging them, like a birdcage.
All algae require sunlight, carbon dioxide, and nutrients to grow. Above all, sunlight is necessary for photosynthesis; thus, the uniform distribution of light in photobioreactors is a major limitation on biomass cultivation. If light is illuminated at a focused area a smaller photobioreactor, the photon usage efficiency of illumination will be enhanced versus the bulk situation. The unique combination of picoliter-sized cell-encapsulated microcapsules and optofludics for light guidance may provide a relevant design for a novel platform of a biofuel production reactor (Figure 6.3).

Large scale cultivation of microalgae under controlled, contamination-free conditions should be achieved to realize commercial production and the recovery of microalgal biomass. Thus, unwanted microbes and contaminants should be removed from the microalgae suspension. Based on differences in the electrical properties of contaminants and microalgae, we will be able to separate microalgae in a high-throughput manner. Additionally, if there is a linear relationship between the intrinsic electrical properties and the lipid content of microalgae, this approach will facilitate the high-throughput microalgae screening of potent microalgae, (e.g., cells with high lipid productivity), and in situ profiling of heterogeneity in lipid accumulation among individual cells in a population (Figure 6.4).
**Figure 6.3.** Schematic figures for picoliter-sized photobioreactor. The unique combination of picoliter-sized cell-encapsulated microcapsules and optofludics for light guidance may provide a relevant design for a novel platform of biofuel production reactors.

**Figure 6.4.** Schematic figures for high-throughput microalgae separation using isodieletric separation.
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광합성을 통해 자라는 미세조류는 바이오디젤 작물 중에서 생산성이 가장 우수하고 지속적인 햇빛과 이산화탄소를 제공하면 대량배양이 가능하기 때문에 미래의 대체 에너지 원으로 각광 받고 있다. 미세조류가 지질을 축적하는 방법은 광합성 등의 여러 가지가 있지만 그 중에서도 영양분의 공급을 제한하는 등의 외부 환경적인 스트레스를 가하게 되면 자가적으로 지질 함량을 폭발적으로 늘릴 수 있다. 이에 따라 수천 종에 달하는 조류 종에 대한 연구가 현재 진행 중이며, 중 간에 축적할 수 있는 지질 함량, 외부 스트레스에 의해 생기는 지질 함량의 차이 등 여러 가지 요인에 대한 학문적 고찰이 필요한 상황이다. 특히 미세조류로부터 지질 함량을 추출하기 위해서는 고비용을 수반하는 과정이 필요하기 때문에 효율적인 바이오 디젤 생산을 위해서는 지질 함량이 높은 종의 선별이 필수적이다. 현재 상황에서는 배치 타입에서의 지질 함량 정량화만 가능하다는 기술적인 한계가 존재한다.

본 연구에서는 단일 세포를 피코리터(10^{-12} 리터) 부피의 미세액배와 캡슐화하여 조작할 수 있는 미세액배기반의 단일세포 스케일인 기술을 도입하였다. 미세액배는 미세세포 내에서 서로 섞이지 않는 두 유체(물과 기름)를 동시에 주입하여 구조적인 제한을 주면 피코리터 부피의 미세액배를 초당 천 개 이상 생산할 수 있다. 기존의 일괄 세포처리법에 비해 미세액배 내에서 단일 세포의 세포 별 신호, 환경, 반응 등의 여러 정보를 동시에 받을 수 있는 장점이 있다. 특히, 조직공학이나 생체 모방 등에 쓰였던 수화젤 폴리머를 원료로 사용해 단일 세포가 포함된 미세액배를 생산하고 이를 교체시키면 수화젤 미세캡슐을 고속 생산할 수 있다. 수화젤 미세캡슐은 미세액배에 비해 얇게 용매에서의 조작이 가능하기 때문에 세포 배양액을 지속적으로 주입할 수 있으므로 세포 생장을 유지 및 장시간 배양이 가능하다.

본 학위과정에서는 연못이나 저수지 등에서 서식하는 *Chlorella vulgaris, Chlamydomonas sp., Botryococcus braunii* 등의 3 종의 납수 녹조류 종을 동일한 크기(평균 25 마이크로미터 지름)의 알 아크릴에 수화젤 미세캡슐에 단일 세포 단위로 캡슐화하고 이 중에서 지질 함량이 높은 단일 조류세포를 선별하기 위한 스크린 플랫폼을 개발하였다. 단일 조류세포 내의 지질 함량을 현장에서 즉시 측정하기 위하여 세포 내 지질을 선택적으로 염색할 수 있는 형광 염료인 BODIPY 를 세포에 염색하였다. 형광 현미경을 사용하여 종간 및 개체간의 지질 함량을 현장 측정한 후에 높은 지질 함량을 가지는 단일 조류세포가 캡슐화된 미세캡슐을 선택적으로 선별
하었다.

동일한 크기의 알지네이트 수화젤 미세캡슐을 고속 생산하기 위하여, 본 연구에서는 미세 다리(microbridge) 구조를 집적한 미세채널을 제작하였다. 기존에는 수화젤 미세캡슐이 고체화되기 이전에 이웃하는 미세액적의 간격이 충분하지 않으면 고체화되면서 응집되는 현상이 있었으나, 하나의 미세채널과 이를 연결하는 미세다리 구조를 집적하여 추가적인 오일 흐름을 유발하면서 미세액적의 간격을 늘렸다. 오일 유속이 증가함에 따라 미세액적의 간격 역시 증가하는 결과를 관찰하였다. 알지네이트 수화젤을 단시간 내에 고체화할 수 있는 경우 이를 오일에 혼합 및 주입하면서 미세액적의 간격을 증가시킴과 동시에 수화젤 미세캡슐로 고체화하였다.

특정 미세캡슐을 인터랙티브하게 선별하기 위하여 본 연구에서는 광전기유체소자에 미세 입자를 포함할 수 있는 마이크로웰을 집적하였다. 빛을 받는 부분만 가상 전극으로 전환할 수 있는 광전기유체소자의 장점을 활용하여, 광전기유체소자 내에 집적된 마이크로웰 중에서 특정 마이크로웰에만 빛 미세패턴을 조사하여 마이크로웰 내의 특정 미세입자에 음의 유전영동을 유발하도록 설계하였다. 20 Vp, 100 kHz의 AC 전압 하에서 빛 미세패턴에 조사된 특정 마이크로웰 입자는 z축 방향의 거동이 일어났을 관찰하였고, 광전기유체소자에 미세채널을 추가 집적하여 선별된 미세입자를 미세유체의 흐름에 의해 배출구에서 수득할 수 있도록 설계하였다.

끝으로, 알지네이트 수화젤 미세캡슐 내에서 BODIPY 에 염색된 3 종의 미세조류의 지질 함량을 분석하였다. 형광 반응을 관찰하여 앞서 발표된 문헌과 동일하게 수화젤 미세캡슐 내에서 Botryococcus braunii, Chlamydomonas sp., Chlorella vulgaris 순으로 지질 함량이 높음을 증명하였다. 중간 차이뿐만 아니라 동종 세포 중에서 개체 간의 지질 함량 차이 또한 확인할 수 있었으며 지질 함량이 높고 낮음에 따른 조류세포의 프로파일링을 수행하였다. 이는 기존의 일괄 세포처리법에서는 수행하기 어려운 결과이다. 또한 미세캡슐 내에서의 단일 세포의 생장률을 SYTOX 형광염료를 사용하여 측정한 결과 70% 이상의 생장률을 보임을 확인하였다.

본 연구에서 제안한 단수 녹조류의 개체 간 지질 함량의 프로파일링과 지질 함량이 높은 조류 중의 선별을 위한 미세액적 기반의 스크리닝 기술은 전 세계적으로 최초의 시도이다. 기존의 일괄 세포처리법에 비해 단일 세포 단위로 지질 함량을 평가할 수 있으며, 형광 유세포 분석기(FACS)에 비해 높은 생장률을 유지할 수 있다는 장점이 있다. 또한 미세액적 플랫폼을 활용하여 높은 처리율 하에서 고속 스크리닝이 가능하다.