Abstract

High-Throughput Microfluidic Platform for Real-time Investigation of Lipid Droplet Accumulation in Microalgae

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Microalgae offer great promise to contribute a significant portion of the renewable fuels that is required by the Renewable Fuels Standard. Algal biofuels is based mainly on the high lipid content of the algal cells and thus would be an ideal feedstock for high energy density transportation fuels, such as biodiesel, green diesel, green jet fuel and green gasoline. With high lipid productivity of dominant, fast-growing algae is a major prerequisite for commercial production of microalgae oil-derived biodiesel. However, under optimal growth conditions, large amounts of algal biomass are produced, but with relatively low lipid contents. Meanwhile, species with high lipid contents are typically slow growing. Currently, the single cells observation and quantification of lipid accumulation after the stationary growth phase under various stress conditions is still a challenge. To solve this issue, we have

conducted the microfluidic platform to investigate the development of lipid droplet in individual microalgae, *Chlamydomonas reinhardtii* by immobilizing monolayer cells on the glass surface coated with gelatin. In addition, our novel platform able to eliminate the absorption of BODIPY fluorescence into the polydimethylsiloxane (PDMS) microchannel and also the media can be changed easily. In the end, the lipid droplet accumulation was observed in-real-time at the single cell resolution under different conditions of light and nutrient, allowing the correlations among lipid trigger conditions and lipid production, as evidenced with BODIPY 505/515 fluorescence lipid staining.

Keywords: Microalgae, Microfluidic, Single cell immobilization, Lipids accumulation, Real–time observation, Glass-PDMS sandwich device

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1. Introduction

1.1. Microfluidics and cell biology

Microfluidic technology is creating powerful tools for cell biologists to control the complete cellular microenvironment, leading to new questions and new discoveries. Microfluidics is a well understood physic domain and now can be used to develop tools for cell biology [1]. By simply miniaturizing macroscopic systems and taking advantage of the possibility of massive parallel processing, microfluidic has advantages in reducing sample and reagent consumption as well as in producing high-throughput biological experiments. Specific effects of laminar flow at the micron-scale also enable spatial control of liquid composition at subcellular resolution, fast media and temperature changes, and single cell handling and analysis. As the result, microfluidic can reduce the experiment cost and time consuming with excellent result performances.

There has been a wide range of studies carried out to identify and develop efficient lipid induction techniques in microalgae by controlling the extracellular environment such as nutrients stress, osmotic stress, radiation, pH, temperature, heavy metals and other chemicals. In addition, several genetic strategies to increase triacylglycerides production are currently being developed. Recently, as the new trend, microfluidic device came out as the best choice for microalgae research, opening the new window for biodiesel production as well as alternative energy for human being.

1.2. Current applications of microfluidic in microalgae research

Because of the depleting supplies of fossil fuels and the accumulation of carbon dioxide in the environment, substantial concerns have prompted the discovery and development of alternative energy sources. Therefore, microalgae appear to be the only source of renewable biodiesel that is promising alternative to fossil energy and capable of meeting the global demand for transport fuels with high yield, sustainable nature as well as improving the quality of environment. However, many issues remain to be solved, mainly regarding the economic viability of biofuels, before realizing commercial production and the recovery of microalgae biomass. There are hundred thousand algae strains with very different growth rate and lipid contents [2, 3]. So, the isolation of high-performance strains; e.g., lipid-rich and yet fast-growing cells (or cells with superb lipid productivity), is one such challenging issue. Currently, the process is to identify of suitable algal strains that possess high constituent amounts of total lipids of cells based on some conventional “one sample at a time” method such as gravimetric and gas chromatography or flow cytometer combining with extraction procedures. However, a major drawback of the conventional method is the time- and labor-intensive, making it difficult to screen large numbers of algae. Therefore, we are required to establish the more simple and effective technique for rapid screening. By that way, microfluidics system offers a powerful tool to accomplish labor-intensive and thus could benefit from a more streamlined, integrated approach. Microfluidic technology can bring the great chances to accelerate the algae research to get the economic target as well as time-consuming.

Microalgae culturing on the microfluidic device is a good ideal due to precise manipulation of fluids at small length scales (micrometers to millimeters) [4] and high through-put manner [5]. Recently, microdroplet monodisperse semi-permeable microcapsules [6] composed of an alginate-poly-L-lysine (PLL) membrane for the observation of encapsulated cells to track the single cells under supplying the nutrients as well as allowing the wastes pass through semi-permeable membrane. However, this fabrication process and manipulation are so complex.

Moreover, microfluidic droplet carried out the key solution to deal with the problem related to single cells cultivation at the high-throughput manner [6, 7]. With this technique, several groups succeed to trap the single microalgae in microdroplet with nanoliter –scale of media [8]. They quantified the lipid content of individual microalgal cells by fluorescence dye, but they only measured the lipid at time points because the fluorescence dye encapsulated with cells will be bleaching at time go by. Another issue of the microdroplet culture is the difficulty to supply the nutrient continuously. Thus, the droplet volumes need to be calculated with enough nutrients for cells viability during the culture time. The CO_2 concentration of the media culture also can be dropped and caused the pH changing effect on the cells viability for long time culturing.

Other group using the laser freezer for single cells trapping and observation individual cells [9] with high resolution and the capability of three-dimensional manipulation, but they require complex optical systems and high optical energy. Besides, another group optimized the light intensity for maximize the lipid content by integrating the 96 wells plate and 96 wells plate reader. With the optical microplate system consists of the 8 x 12 matrix of LEDs at the bottom of the standard 96 wells plate, the light illumination of

each of well can be controlled individually. Therefore, they can investigate the light-dependent growth rates and photosynthetic efficiency [10].

The conventional method for quantifying cellular lipids to determine viability as a biofuel resource is based on solvent extraction and weighing. Additional analytical techniques such as thin-layer chromatography, high-performance liquid chromatography or gas chromatography are required for complete characterization of the cellular lipids [11]. These techniques tend to be time-consuming, labor intensive and require large sample volumes. Therefore, they are not feasible for species screening towards economic production. The development of efficient rapid assessment tools and advance techniques for the screening of biofuel-directed microalgae strains needs to be established [12, 13]

1.3. Motivation and objective

To date, the ability to track and isolate single microalgae, particularly in a rapid and high-throughput manner, becomes a real-challenge in screening mutants with high lipid productivity. It is a current practice that the lipid productivity of species is compared in a batch and lump sum manner. In this way, however, any differences at the single-cell level even among the same species and batch, which in fact exist, cannot be considered. Lipid quantification in microalgae is firstly introduced in microfluidic device but they still have some disadvantages [5, 8]. The drawbacks of this platform are not able for tracking the lipid accumulation continuously at real time with individual cells and also difficulty to control the culture conditions. Besides, successful biodiesel production depends on the selection of fast-growing, lipid-rich microalgae from a large number of microalgae species. Therefore, the lipid content of individual algae should be determined because of the lipid accumulation in each single cell is so different at the same culture condition. Thus, the single-cell cultivation method is a key solution for recording changes in the lipid body of individual cells as well as the duration on their cell cycle phase without the need for synchronous cell cultivation.

In this study, we aim to develop the surface immobilization technique on the glass surface coated by gelatin for screening individual cells in the microfluidic system. Furthermore, we proposed to make the glass-PDMS sandwich microfluidic device for monitoring the lipid accumulation in-real-time with high magnification of lipid droplet stained with BODIPY fluorescence.

2. Methods and Materials

3.1. Device design and simulation

A schematic of the microfluidic device used in the following experiments is shown in Figure 2.1A. Our platform was modified from the gradient device [14] and was composed of three main parts which were two pairs of inlets, a cell culture chamber, and an outlet. The central chamber is separated with two side channel by micro posts in order to reduce the effect of shear stress on cells during the continuous flow experiment (Figure 2.3A). Comsol Multiphysic 4.3 was used for 3D model simulation with laminar flow solving condition. The 3D model was drawn by using commercial Autodesk Inventor 2012 (USA, CA) and then was imported into the Comsol simulation.

2.2. Photolithography

Standard photo-lithography techniques were used to fabricate the PDMS portion of the microchip. To make the device, two individual film photo-mask sets were drawn with commercial design program (AutoCAD, DE) to make the two layers of the device (Figure 2.1). Film photo-mask was made by Hanall Tech (Korea). Photo-lithography technique was processed to fabricate replicable molds (Figure 2.1B). For photolithography process, prepared wafer (Test grade, US) was cleaned with plasma etcher 5 min at 50 W (FEMTO science, Korea). Then, the wafer was put on the hot plate in 25 min at 120 ^{o}C to evaporate the water on the surface. Surface of a clean wafer was spin-coated with SU-8 50 (Microchem, US) for 25 μm height of thickness as microchannels (3000 rpm, 30 seconds). After spin-coating process, the wafer was soft-baked on a hot plate, at 65 ^{o}C for 2 min and at 95 ^{o}C for 10 min

continuously. The first photo mask film was then tightly contact to the surface of the coated -wafer. The mask was exposed with UV radiation at 365 nm wavelength for the next step using conventional 500 W mercury lamp for 20 seconds (30 mW/sec). After the exposure, the wafer was post-baked at 65 ^{o}C for 1 min and at 95 ^{o}C for 3 min.

In the immobilization experiments, we did not need required to sandwich the glass in the PDMS device, so one step photolithography was used to make the device for these experiments. In case of device for screening the lipid production, the master mold was fabricated with double layers as shown in Figure 2.1 C. The second step photolithography was conducted by using another wafer as same as one step photolithography.

2.3. Chemicals and materials

Chlamydomonas reinhardtii (C-503) were purchased from *Chlamydomonas* Resource Center at University of Minnesota. Gelatin from bovine skin type B was purchased from Sigma Aldrich, USA. Polydimethyl Siloxane (PDMS) was purchased from Dow Corning Corp. (Midland MI, USA). Photoresist SU-8 series were from MicroChem Corp. (Newton MA, USA). 4" Silicon wafer was from Uni Sill Tech. (Korea). Other chemical and reagent used in this study were provided by Sigma-Aldrich Co. (St. Louis MO, USA) unless otherwise mentioned.

2.4. *Chlamydomonas reinhardtii* culture

Chlamydomonas reinhardtii is a ~10 μm, unicellular, green alga with multiple mitochondria, two anterior flagella for motility and mating, and a chloroplast that houses the photosynthetic apparatus and critical metabolic pathway [15]. C-503 is commonly used strain for preparation of the DNA for genetic study. *C. reinhardtii* have been grown previously in 100 ml or 200 ml Erlenmeyer flask in order to maintain the best microalgae for synchronization. Cultures were maintained in TAP (Tris-Acetate-Phosphate) medium at 23 ^{o}C. The cultures were kept illuminated with lamps at an irradiance level of 40 $\mu W/cm^2$ photosynthetically active radiation (PAR) constant illumination on an orbital shaker at 125 rpm with light circle 12:12 h (light/dark). An irradiance level was detected by using the light meter (LI-250A; LI-COR Biosciences) and the quantum sensor (LI-190SA; LI-COR Biosciences). When the cell concentration was within 5.0 x 10^6 to 1.0 x 10^7 cells/ml (late log phase), one milliliter of culture was centrifuged at 2,000 x g for 5 min at room temperature. After that, the supernatant was removed and cells were re-suspended to get 5.0 x 10^4 cells/ml in TAP medium.

TAP medium was optimized for *C. reinhardtii* culture. The TAP medium was consisted of phosphate buffer, nutrient stock, and Hunter's Trace metals. Phosphate buffer (K_2HPO_4 54 g, KH_2PO_4 25 g were dissolved in 500 ml of dH_2O), nutrient stock (NH_4Cl 20 g, $MgSO_4$. $7H_2O$ 5 g, $CaCl_2$. $2H_2O$ 2.5 g were dissolved in 500 ml of dH_2O. Hunter's trace metals (H_3BO_3 11.40 g, $ZnSO_4.7H_2O$ 22.00 g, $MnCl_2$. $4H_2O$ 5.06 g, $FeSO_4.7H_2O$ 4.99 g, $CoCl_2$. $6H_2O$ 1.61 g, $CuSO_4.5H_2O$ 1.57 g, Mo_7O_{24} (NH_4). $4H_2O$ 1.10 g were dissolved in 250 ml of dH_2O). The three solutions were sterilized and stored at 40^{o}C. One liter of TAP media was made up from 20 ml 1M Tris/Cl Buffer, 1 ml

Phosphate buffer, 1 ml Hunter's Trace Metals, 10 ml Nutrient stock and the final media was adjusted to pH 7 by adding 1 ml Glacial Acetic Acid.

2.5. Surface immobilization of *C. reinhardtii*

Gelatin derived from Bovine type B is combined from 3 types of amino acid which are Glycine, Proline and Hydroxyproline with very rich hydrogen group (Figure 2.4 B). Gelatin was dissolved with PBS 1X to make the working solution. After dissolved with PBS 1X, the solution was kept in water bath at 37 ^{o}C for 5 min to get the absolute dissolve. Figure 2.4A shows the whole process of microalgae surface immobilization on glass surface that was coated with gelatin. pH of solution, gelatin concentration, coating time, immobilization time, and cells concentration were optimized. Firstly, the glass coverslip was treated with oxygen plasma etcher for 1 min with 50 w to generate hydrophilic surface with abundant hydroxyl group on the surface. After the plasma treatment, the PDMS was immediately placed on the pre-treated glass coverslip to enclose the microchannels. Then, the gelatin solution was loaded and was incubated at

37 ^{o}C to allow the gelatin deposited and bond with the hydrophilic glass surface. Then glass surface was washed with TAP media for three times. After this step, the thin layer of gelatin was coated on the glass substrate. Then, the *C. reinhardtii* were introduced and allowed to settle and attach on the gelatin by hydrogen bonding networks. pH of gelatin solution was evaluated at pH 6, 7, and 8. Gelatin concentrations range was from 1 mg/ml to 10 mg/ml. Coating time of 1, 3, 6, and 12 h were evaluated according to a previous procedure as Figure 2.4A. Immobilization time range was from 0 to 6 h and was optimized as described previously. Cell concentration was evaluated at 10^{6}, 10^{7}, and 10^{8} cells/ml.

In the Figure 2.5 shows the immobilization process from initial empty chamber until the abundant of cells were settled and were immobilized on the glass surface through gelatin matrix. To calculate the efficiency of immobilization, we counted the cells number in the area of 125 μm x 125 μm with different position in the cells chamber. Each experiment was reproduced 5 times at the same condition.

2.6. Viability Assay

C. reinhardtii were plated and immobilized on glass bottom type 96-well plates. After the immobilization and incubation process, cells were treated with 10 ul of Ez-Cytox solution to each well and were incubated for 30 min in incubator before the measurement with 96 well-plate reader. Cell viability was monitored by measuring the solution absorbance at 450 nm. The control value was done without gelatin in each well-plate with the same procedure.

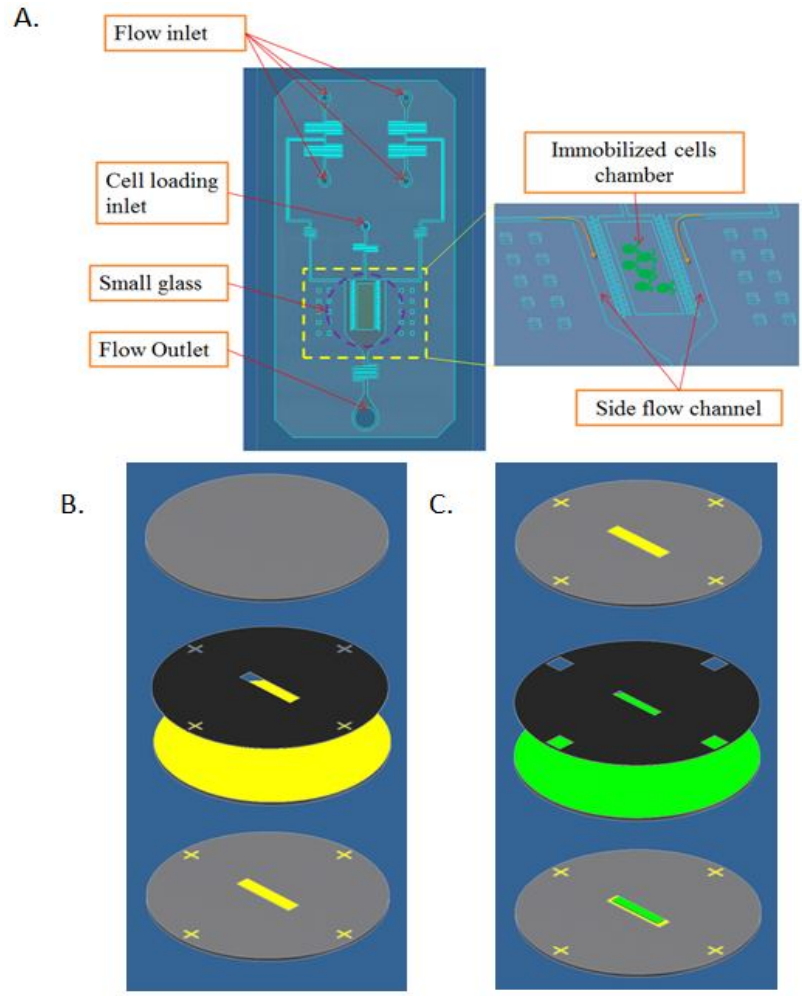

Figure 2.1 Microfluidic device and two step Photolithography

(A) Schematic of the device. (B) One step photolithography to make the 25 µm microchannel height. (C) Two step of photolithograpy to make 2 layers of glass-PDMS sandwich device for lipid droplets observation experiment.

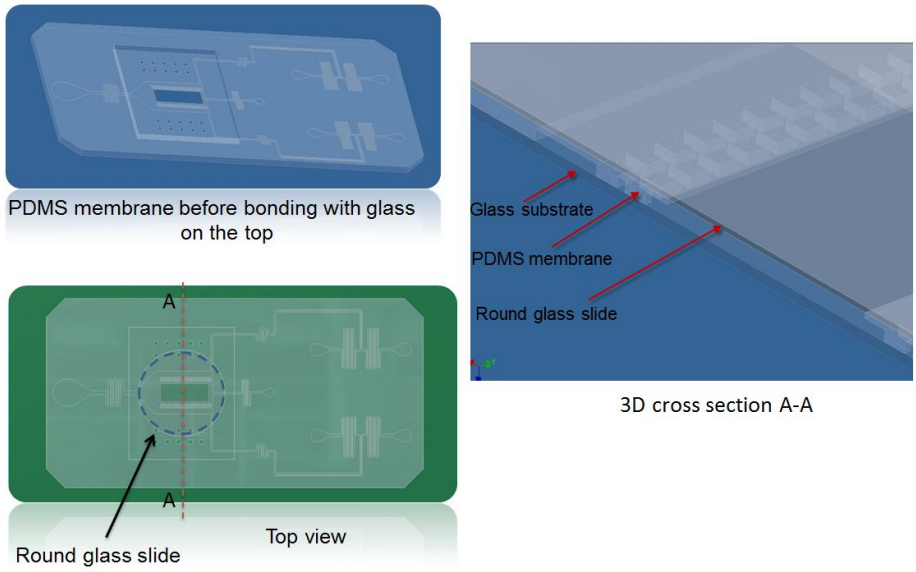

Figure 2.2 Illustration of the microfluidic device for high-throughput screening of microalgae lipid accumulation at single cells level

(A) 3D membrane device with opening at middle channel. (B) Membrane device with 50 μm height was bonded with ground cover glass (12 mm of diameter). (C) Cross section of the device

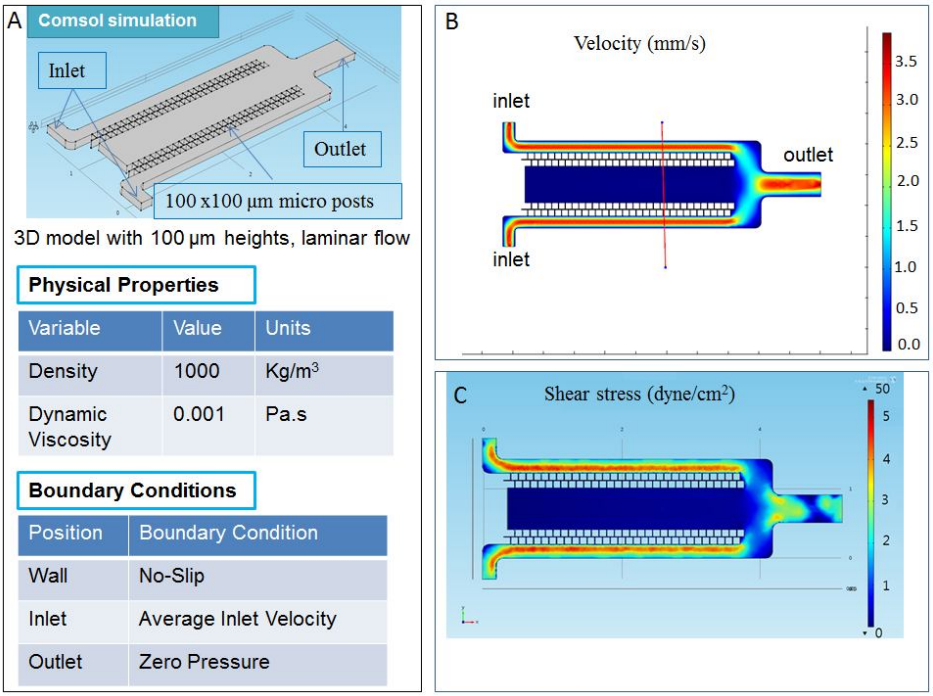

Figure 2.3 Simulation models by Comsol multiphysic

(A) 3D model of the device with physical properties and boundary conditions. (B) Velocity simulation result with 5 mm/s at inlet flow. (C) Shear stress simulation result indicated the shear stress in the cells chamber below 1 dyne/cm^2

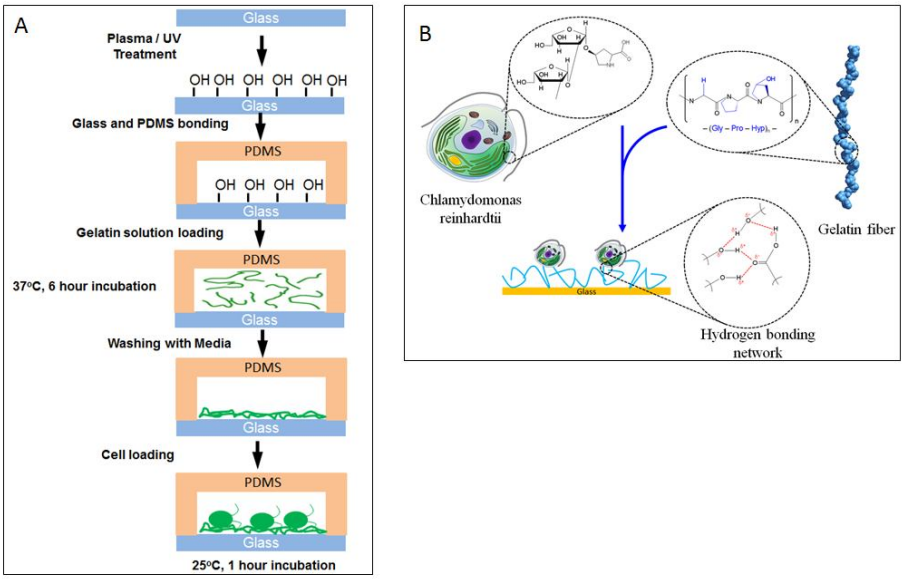

Figure 2.4 Microalgae surface immobilization through gelatin

(A) Procedure for gelatin coating on glass surface in the microfluidic channel. (B) Surface immobilization mechanisms: molecular structure of algae cell membrane and gelatin fiber, and hydrogen bonding networks between outer *C. reinhardtii* and gelatin fibers.

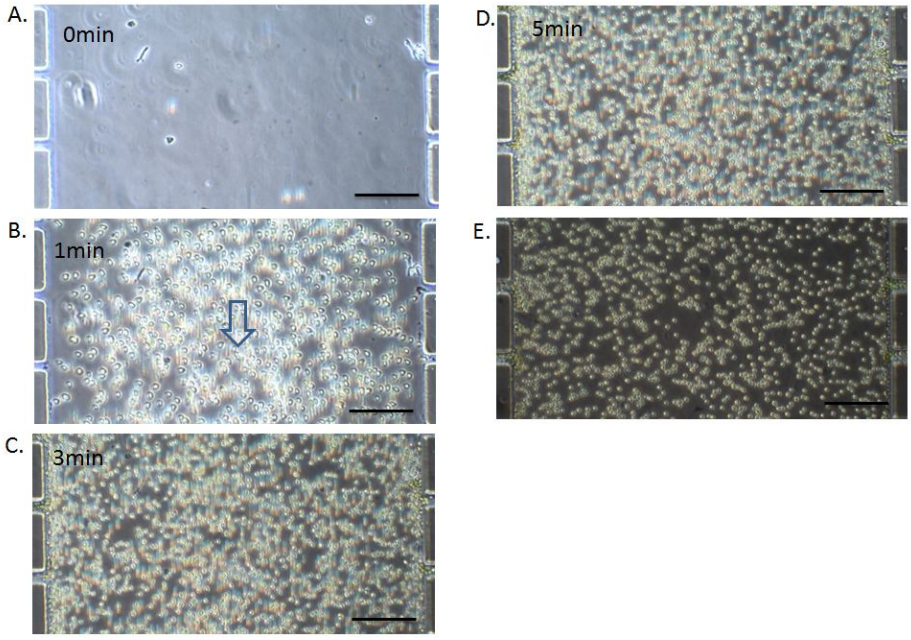

Figure 2.5 Cell immobilization process

(A) Initial stage without cells in the chamber. (B) Cell loading by gravity flow: 100 µl cells in micro-tip after 1 min of loading. (C) Cell loading after 3 min. (D) Cell loading after 5 min. (E) Immobilized cells after washing away non-immobilized cells. Scale bar 100 µm

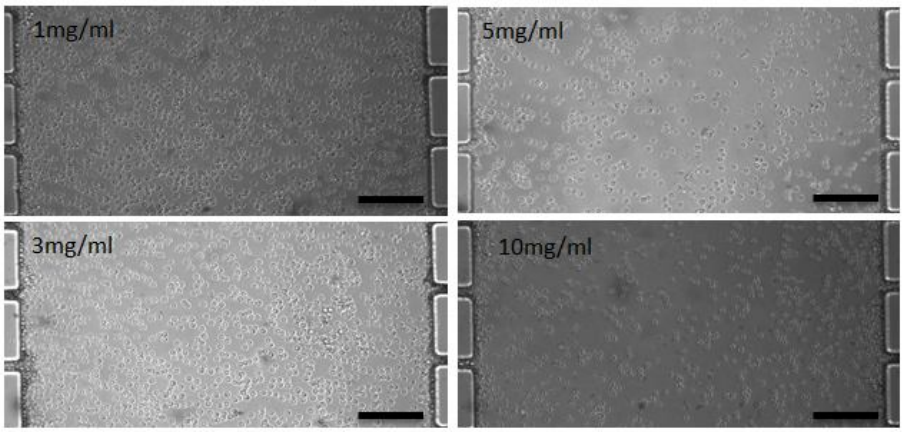

Figure 2.6 Microalgae were immobilized with various gelatin concentrations

The number of cells immobilized on the glass surface coated gelatin decreased when the gelatin concentration was increased from 1 mg/ml to 10 mg/ml. Scale bar 50 μm

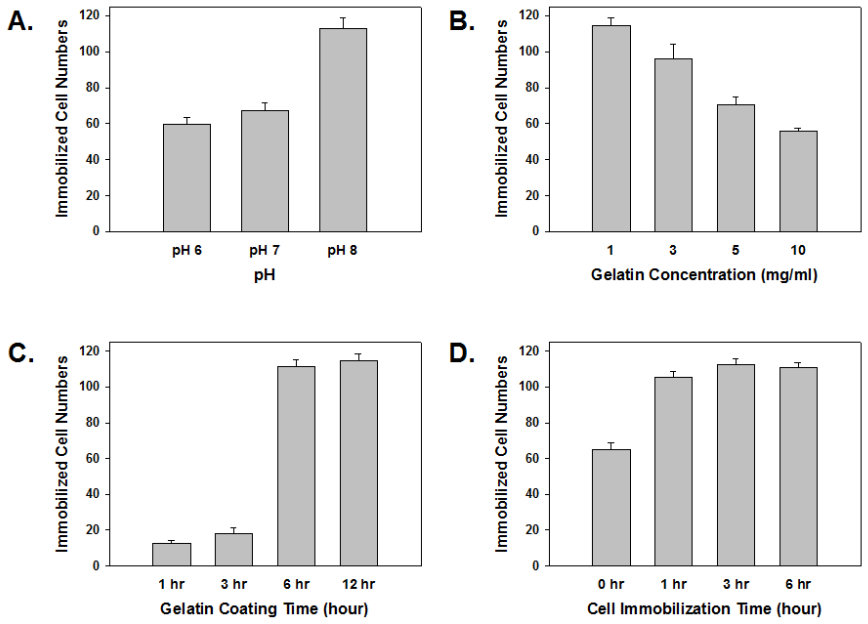

Figure 2.7 Optimal conditions for high efficiency of cells immobilization

(A) pH of PBS used for making the gelatin solution (B) 1 mg/ml, 3 mg/ml, 5 mg/ml, 10 mg/ml of gelatin concentration (C) Gelatin coating time at 1 h, 3 h, 6 h, 12 h (D) Cell immobilization time at 0 h, 1 h, 3 h, 6 hr.

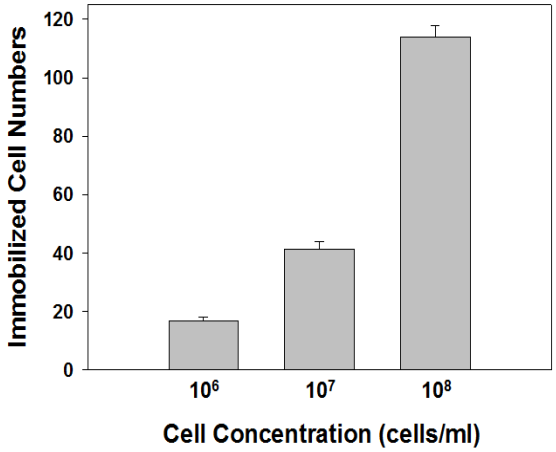

Figure 2.8 Effect of cell loading concentration on efficiency of cells immobilization.

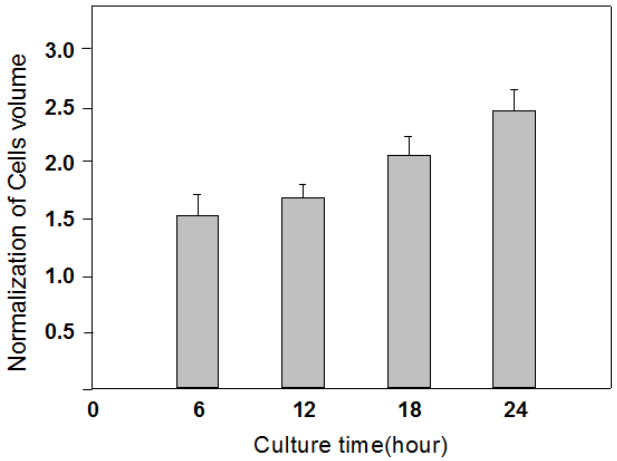

Figure 2.9 The cells size increased for 1 day cell culture with TAP media

The individual cells were imaged at every 6 h. The later cell size was normalized with the initial cells by measuring the area of the cell.

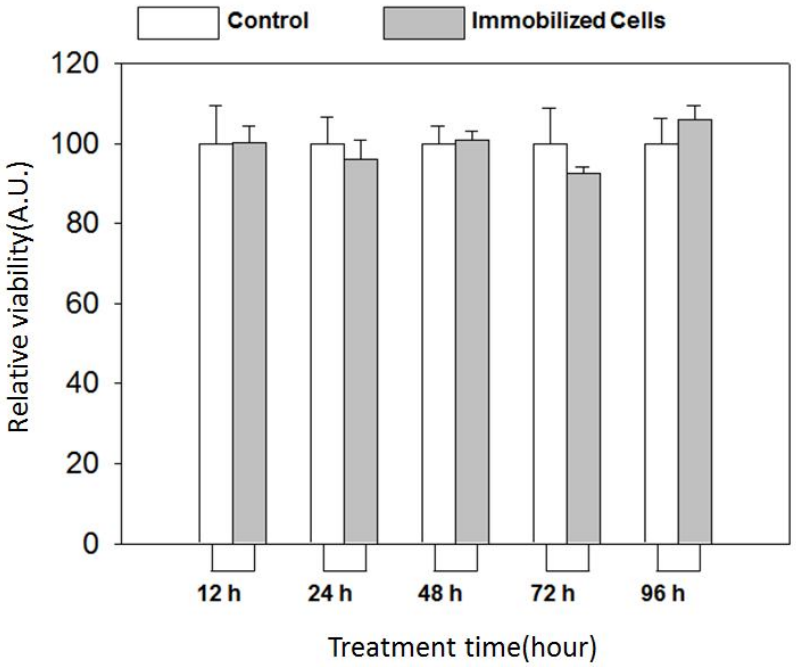

Figure 2.10 Viability of immobilized and non-immobilized cells

3. Microalgae dynamic assay for lipid accumulation study

To visualize and quantify the lipid content at the single cells manner in the microfluidic device is a real challenge because of the motility characteristic of *C.reinhardtii*. Furthermore, there other issue is the strongly absorption of lipophilic fluorescent dye such as BODIPY into the PDMS, resulting the high background noise compare to the signal from the cells body, as shown in Figure 3.1A. To solve this problem, we have developed the novel platform for observation of the lipid droplets accumulation in single microalgae with strong fluorescent signal from BODIPY 505/515 lipid staining dye, as presented in Figure 3.1B.

3.1. Glass –PDMS Sandwich device fabrication

The whole fabrication process is showed in detail in Figure 3.2 with 6 simple steps. The master device with two step photolithography was fabricated by the process in Figure 2.1B, C. First, the silicon wafer with SU-8 microstructure was poured with the PDMS prepolymer (ratio 10:1). Then, the PS sheet (15 mm x 15 mm) was slightly aligned on top of the mold and mounted the heavy weight to create the high pressure between the PS sheet and SU-8 surface (Figure 3.2 B). After baking for 1h on hot plate at 95 ^{o}C, PS sheet was peeled off gently and the PDMS membrane was remained (Figure 3.2C). The membrane device was cut off and was put on the transparent film as a support substrate. The upper surface of membrane was bonded with small glass slide (12 mm of diameter) by using the oxygen plasma treatment 50 W for 1 min. After that, the PDMS prepolymer was poured on the top of device to make the thick 5 mm PDMS layer (Figure 3.2.E). After 1h baking on hot plate at 95 ^{o}C

and peeling off the transparent film sheet gently, the inlet and outlet were punched to make holes. The device was irreversibly sealed with a glass slide after exposure to oxygen plasma for 1 min. The glass-PDMS sandwich device was used for the surface immobilization right after sealing.

3.2. System installation

Pressure pump (Onix, CellASIC^{TM}, USA) can be controlled very straightforward since it was manipulated through the ONIX^{TM} FG software. Constant or pulsed type of pressure was generated with the range of 0 psi to 10 psi. Pressure range can be controlled precisely with 0.005 psi unit and the time lag can be controlled in a millisecond order. Pressure was indirectly delivered to the device from 10 ml cylinder reservoir. The device was installed firmly on the stage of microscope and maintained the pressure for 7 days of cells culture.

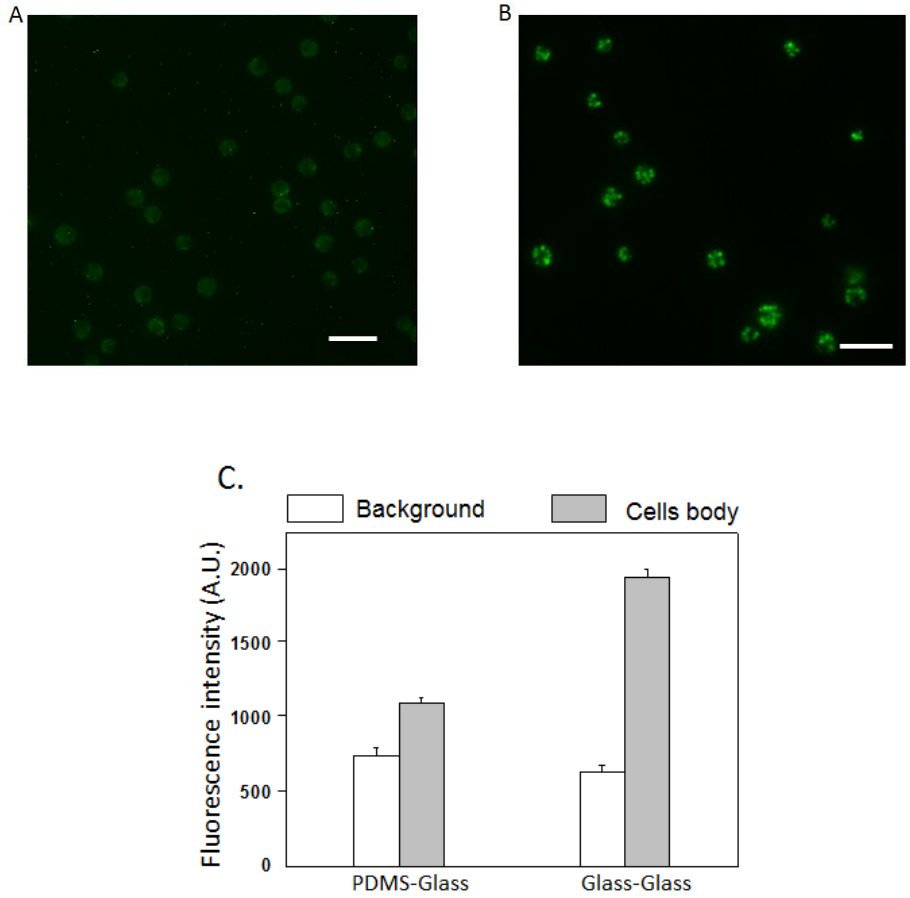

Figure 3.1 Lipid bodies were visualized in microfluidic device. The microalgae were stained with 1 µ M/ml BODIPY 505/515

(A) Cells were stained with 1 µM BODIPY in PDMS device, and the channel was bonded directly with glass substrate. The lipid droplets inside the cells cannot be visualized because of high background noise. (B) Cells were stained with 1 µM BODIPY in the PDMS device, with a small cover glass on the top to prevent the BODIPY absorption into PDMS surface. The lipid droplets can be visualized clearly inside the cell body. (C) The fluorescent intensity indicated lipid body and background in case A, B

3.3. Lipid body accumulation

Lipid droplets (LDs) are subcellular organelles containing neutral lipids such as triacylglycerols or cholesteryl esters surrounded by a phospholipid monolayer. When microalgae are stressed by nutrient limiting conditions, which still can permit carbon fixation by photosynthesis, the lipid biosynthesis pattern changes and neutral lipid accumulates to form lipid droplets localized in the cytoplasm, mainly in the form of triacylglycerols (TAG). There are many researches focus on the optimal cell life conditions for maximum the lipid accumulation as well as apply the gene mutants for create the super microalgae stain with high lipid production [16]. However, they are not only desired to get the high growth rate but also simultaneously considered the lipid accumulation [17]. In this research, the *C. reinhardtii* was stressed by eliminating the Nitrogen sources from TAP media and called TAP-N. The lipid droplets begin to accumulate in their cells body after 1 day cells stressed. With the glass-PDMS sandwich device, we can count the number of lipid droplets as well as monitor the increasing of lipid size.

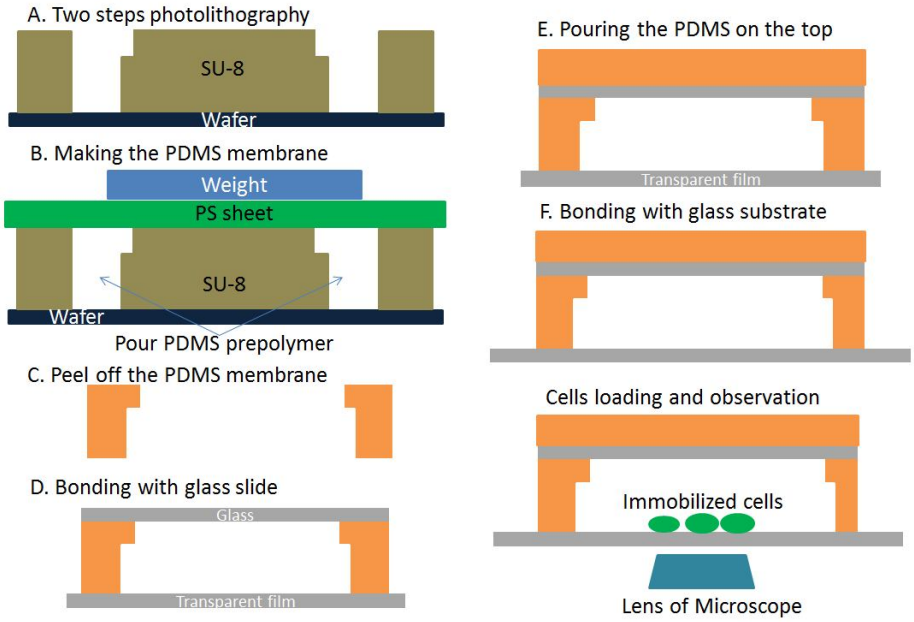

Figure 3.2 the procedure to make the glass-PDMS sandwich device

(A) Two step photolithography master mold with 25 μm for each layer (B) Pouring the PDMS pre-polymer and gentle align the polystyrene (PS) sheet and then put the weight on top of PS sheet to make the pressure (C) PDMS membrane device. (D) PDMS membrane device was put on the transparent film for easy to manipulate and bonded with small glass cover (12 mm of diameter) on the top. (E) Making the top PDMS layer. (F) Punching inlet and out let hole and bonding with the glass slide.

3.4. Lipid droplet staining dye and solution preparation

BODIPY 505/515 (4,4-difluoro-1,3,5,7,-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Invitrogen Molecular Probes, Carlsbad, CA) is a green lipophilic fluorescent dye, serves as an excellent vital stain for the oil-containing lipid bodies of live algal cells. BODIPY 505/515 vital staining can be used in combination with fluorescent activated cell sorting to detect and isolate algal cells possessing high lipid content[18]. When BODIPY 505/515 is added to a culture of live algal cells, the dye stains intracellular oil-containing organelles, known as lipid bodies, within minutes. Especially, the intensity of BODIPY demonstrates the direct correlation with the lipid content in the cell, without affected by the cells viability, which make BODIPY as the best solution for rapid quantitative the lipid content in microfluidic device.

A stock solution of BODIPY 505/515 was prepared at 500 μM in 100 % DMSO and was stored at -20 ^{o}C shielded from ambient light when not use. To prepare the BODIPY working solution, 1μl stock solution was added with 499 μl TAP-N solution to create 500 ul BODIPY of 1 μM concentration.

3.5. BODIPY absorption into the PDMS

Many studies showed that hydrophobic fluorophor such as BODIPY able to absorb very easily and rapidly into the PDMS microchannel after loading [19], since both of BODIPY and PDMS have hydrophobic characteristic. This causes many problems especially during quantifying of the lipid content by using the light excitation intensity for lipid screening studies in PDMS microfluidic device. Some groups modified the surface of PDMS channel by using Paraffine wax and Parylene-coating in PDMS microfluidic channels to prevent the absorption of fluorescent dyes [20, 21]. However, the prevention from these techniques just maintained the characteristics of modifying surface less than one day. However, for microalgae screening, the cells need to be cultured up to 10 days. Therefore, these techniques cannot be used to the PDMS channel for long time cells culturing and BODIPY staining. Here, is the first time that glass-PDMS sandwich technique was proposed in order to quantify the lipid droplet in microfluidic device with the strong emitted signal of BODIPY. We developed the device to visualize the high signal from the lipid bodies stained with BODIPY without increasing the background noise. Figure 3.1 shows that 1 uM BODIPY 0.02 % DMSO in microfluidic chamber with PDMS on the top and also with glass sandwich PDMS. As the result, the lipid formation can be easily quantified at the individual cells manner and the lipid droplet can be clearly visualized with high magnification under fluorescent microscope. The number of lipid droplets can be straightforward counted to estimate the lipid accumulation at individual microalgae as the Figure 3.3A

3.6. Cell imaging and Data analysis

To validate the immobilization, live cell imaging was conducted. The whole images were acquired by inverted microscope (Olympus IX81, Japan) fitted with an environmentally controlled live cell chamber (Live Cell Instrument, Korea). The live cell chamber was set at 23 ^{o}C with 5 % CO_2. The PDMS device was illuminated by using external light source with 120 μmol photon $m^{-2}s^{-1}$ at the day circle. After culturing the cell for 3 h with TAP-N in flow, the TAP-N media was changed with TAP-N that contained 1 μM BODIPY for 5 min. After washing the remaining BODIPY in the chamber by reflowing with the TAP-N, the images were taken at 475 nm excitation and 515 nm emission wavelengths. The procedure was repeated for every 3 h of time interval for getting the time-lapse imaging. All of the acquired images were processed with Image J software (NIH, Bethesda, MD, USA) for lipid analysis.

4. Results and discussion

Figure 2.4B illustrates the cell wall composition of *C. reinhardtii*. The cell wall layer is composed of polymers which are rich with hydroxyproline (Hyp) that are connected to the carbohydrates, and known as glycoproteins. For the surface immobilization technique of microalgae in microfluidic device, gelatin is demonstrated as molecular-bridge through Hyp hydrogen bonding network between gelatin and outer cell wall of *C. reinhardtii*.

We examined the various physical and chemical treatments that were needed to be considered for immobilization. We measured immobilized cell numbers per 125 um by 125 um of glass substrate through acquired micrographs. Figure 2.7A shows the immobilized cell numbers with different pH of gelatin solutions evaluated at pH 6.0, 7.0, and 8.0. Immobilized cell numbers were increased with pH. Therefore, pH 8.0 was identified as the optimal pH value. As indicated in Figure 2.7B, immobilized cell number was influenced by the gelatin concentration. Immobilized cell numbers decreased with increasing with gelatin concentration. We concluded that optimal gelatin concentration for immobilization was 1 mg/ml. Our concluded optimal pH and concentration was relatively high and low value, respectively, we found the explanation for molecular phenomena of gelatin. Gelatin is commonly sensitive to degradation. The degree of degradation of gelatin is depended on physic-chemical parameters such as pH and concentration. The optimal pH and concentration result in a high degree of degradation. It seems that the result of degradation causes expose of Hyp, so it gives a chance to generate a hydrogen bonding networks between gelatin and *C. reinhardtii*.

Figure 2.7 C and D show the immobilized cell numbers as a function of coating time and treatment time, respectively. In Figure 2.7 C, between 3 hour and 6 hour, there was significant increase of immobilized cell numbers up to 6

hours. However, compared with the cell numbers of 6 h significant difference was not founded at 12 h. Therefore, we concluded that 6 h as the optimal gelatin coating time for immobilization. Same as the result from coating time, Figure 2.7 D indicates that optimal cell immobilization time was 1 hour incubation. We also validated the effect of cell loading number. As a result in Figure 2.8, high cell density shows high efficiency of immobilization.

Throughout the optimization, we concluded that the optimal condition for *C. reinhardtii* immobilization were 1 mg/ml (pH 8.0) of gelatin concentration and 6 h of incubation at 36 ^{o}C. After incubation, glass substrate was washed three times with TAP media. Then, cells are loaded and incubated at 25 ^{o}C for 1 h to settle down and to immobilize on the glass substrate (Figure 2.5E)

Based on the optimized conditions, we evaluated cytotoxic assays for validation of the microfluidic system. To determine the cell viability, the immobilized cells were evaluated with Ez-Cytox assay over 3 days. Figure 2.10 shows the comparison between non-immobilized cells as the control value and immobilized cells, immobilized cell showed no significant cytotoxic effect. This result indicated that *C. reinhardtii* immobilization was sufficiently non-cytotoxic and can be used to long time live cell manipulation on the microfluidic system.

After the immobilization, we can easily conduct any experiments related to real-time observation of the cell mitosis or the individual cells activities under TAP media culture. Figure 2.9 indicates that the cells volume increased before the cells going to the mitosis stage. The later cells volume was normalized with initial cells stage by measuring the area that cells occupy. The individual cells were tracked for their size for every 6 h, allowing the analysis of the increasing of each cell volume.

Furthermore, our novel platform was able to keep the single cells in position in the microfluidic channel for imaging, as well as for observation individual cells over 7 days. The device also can continuously supply the various nutrient environments by switching the inlet media without dynamic force effects on the immobilized *C. reinhardtii*. The single cells were cultured in continuous flow to make the constant extracellular conditions. After 4 days culturing with TAP-N, the lipid accumulation in cells increased dramatically with a larger size of lipid droplets as shown in Figure 3.3A. The size and the number of lipid droplets increased as the time go by. However, after a certain value, the number of lipid will stop, and the lipid size will be increasing.

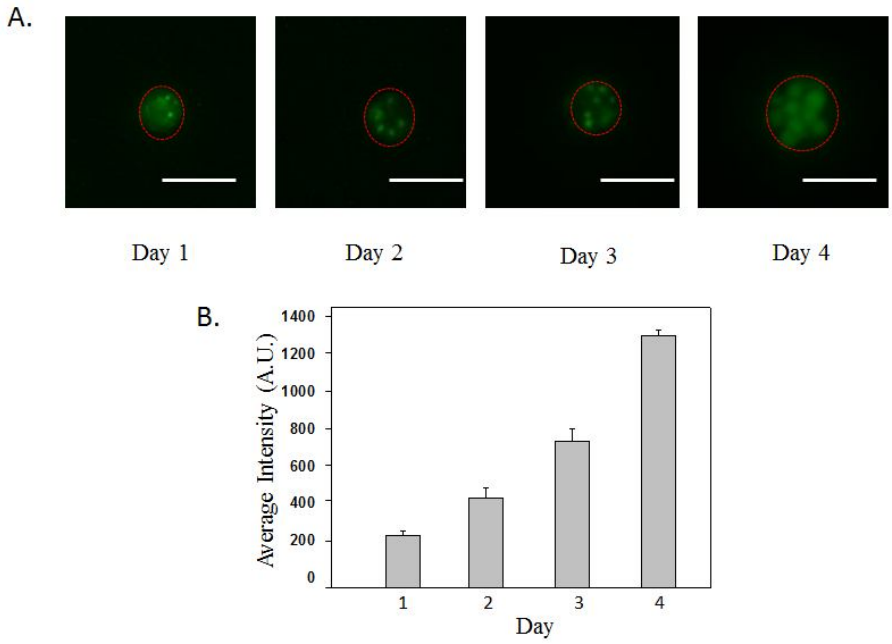

Figure 3.3 Lipid droplet accumulations in individual cells under stress condition by TAP-N for 4 days culture.

(A) The neutral lipid was visualized by BODIPY staining; the red circle indicates the cell wall boundary with many lipid droplets inside the cell body. (B) The graph illustrates the fluorescent intensity of lipid body for 4 days and cells were stressed with TAP-N. Scale bar 10 μm

5. Conclusion

In this research, we developed the novel method of immobilization the *chlamydomoas reinhardtii* through gelatin coated on the glass surface in microfluidic device. The hydrogen bonding networks between outer cells wall and gelatin fibers able to keep the cell in position for long time culturing as well as for observation on microscope. By using this method, we able to make the monolayer of cells immobilized in the microfluidic chamber. In addition, we optimized the gelatin coating conditions such as pH, concentration, and time for getting cell immobilization with high efficiency. Besides, cells viability assay was conducted to demonstrate that the immobilization process did not effect on the live microgalgae. We also successfully monitored the lipid droplet accumulation of the individual cells by using the glass-PDMS sandwich device. Based on our knowledge, this is the first report on live microalgae cells that lipid droplet could be monitored in real-time manner under long culturing time with controlled extracellular environment. Combination of this novel platform with non-destructive fluorescence staining BODIPY will propose an ideal method for screening the lipid content among individual cells.

From a broader perspective, this novel microfluidic system can be meaningful for studying the properties and dynamics of lipid droplet of *C.reinhardtii*

strain. This is also useful method for the screening of lipid-rich microalgae strain with low cost and rapidly manner.

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요약

미세조류 내 실시간 지질 형성 관찰을 위한 미세유체 고속탐색 플랫폼 개발

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미세조류는 현재 거론되는 화석 연료 에너지 고갈 및 그의 사용에 따라 발생하는 환경 문제 등에 대해, 재생 가능하고 친환경적인 대체 에너지 자원 중 하나로서 대두되고 있다. 미세조류를 이용한 바이오 연료는 기본적으로 미세조류 내에 존재하는 고함량의 지질의 이용을 바탕으로 한다. 미세조류의 빠른 성장과 빠른 체내 지질 생성률을 달성하는 것은 이를 이용한 바이오 연료의 산업화 및 상업화의 중요한 기반 기술이다. 그러나 일반적으로 알려진 최적의 배양 조건에서는 많은 개체수의 생산이 가능하나 상대적으로 적은 지질의 생성이 이루어 지며, 고함량의 지질을 가진 몇몇 종에서는 느린 생장속도를 보인다. 이 같은 한계점을 극복하기 위하여 단일 세포 수준의 관찰과 지질 축적의 정량 분석을 다양한 조건에서 고속으로 탐색할 수 있는 플랫폼 개발이 요구된다. 이에 대해 본 논문에서는 미세유체를 이용한 고속탐색 플랫폼으로서, 젤라틴을 이용하여 단일 세포 수준의 고정 및 지질 형성의 관찰이 가능한 디바이스를 개발하였다. 추가로 본 플랫폼에서는 BODIPY 지질 염색약의 Polydimethylsiloxane(PDMS) 미세 채널 사이로의 흡수를 막고, 빠른

배지의 교체가 가능하며, 실시간으로 단일세포 수준의 지질 축적을 빛과 배지 조성을 포함한 다양한 조건에서 관찰이 가능하다. 이는 지질 형성 조건 및 그 결과의 상관관계 분석을 통해 최적의 미세조류 지질 생산의 요건 탐색에 이용 가능할 것으로 기대한다.

Keywords: 미세조류, 미세유체, 단일 세포 고정, 지질 축적, 실시간 관찰, 유리-PDMS 샌드위치 디바이스

학번: 2011-24053

Acknowledgement

First and foremost, I would like to thank my advisor, Professor Jeon Noo Li. I am extremely grateful for the opportunity that he offered me to pursue the Master course at Department of Mechanical and Aerospace Engineering, Seoul National University. I am also thankful Professor Jeon Noo Li for giving me so much brilliant ideas to explore my research. Thank you very much for your kindness, honest advices, and patience in training me, and also for many other supports that have significantly changed my mind.

Secondly, I am grateful to all laboratory members who supported me very much during 2 years in SNU. Especially, I would like to appreciate many helps from Byun Jae Hwan, Park Jae Woo, Kim Su Dong, Jay M, Oh Soo Jung, Na Sang Cheol, Chung Min Hwan, Kang Myeong Woo, Hana, and Trung. With their helps, I learned many new things in our laboratory. I also had the great moments during MT trips as well as the warm dinner nights together. I will never forget these wonderful moments with them.

I sincerely would like to express my gratitude to all the Professors and staff members in Department of Mechanical Engineering, Seoul National University, for their great and interesting lectures, for their valuable advices, helps and kind instructions during my study.

I would like to thank my family and my Vietnamese friends in SNU. Having the assistance and support from them, I have overcome many obstacles to get the master degree at SNU.

Most sincerely,

Nguyen Thanh Qua

Master's Thesis

High-Throughput Microfluidic Platform for Real-time Investigation of Lipid Droplet Accumulation in Microalgae

미세조류 내 실시간 지질 형성 관찰을 위한 미세유체 고속탐색 플랫폼 개발

February 2014

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