공학석사학위논문

미세유체플랫폼 내 미세조류 배양 및 실시간 관찰 연구와 생체지질염색 방법

Microfluidic Platform for Real-time Observation and Vital Lipid Staining of Microalgae

2012년 8월

서울대학교 대학원

기계항공공학부

김 민 서

Abstract

Microfluidic Platform for Real-time Observation and Vital Lipid Staining of Microalgae

Kim, Minseo

School of Mechanical and Aerospace Engineering
The Graduate School
Seoul National University

In recent years, one of the most promising alternative energy resources is biomass, which can be converted to biofuel, such as biodiesel and bioethanol. Microalgae are the next generation of green-fuel since they can produce neutral lipids and hydrocarbons in large quantities; yet several challenges to the success of microalgal biofuel still exist; these include strain development, medium optimization, reactor design, metabolic engineering, and product purification.

The green microalgal capability of producing materials for biofuel is depending on many environmental factors of photosynthetic eukaryotes such as light exposure, CO_2, nutrient concentration, temperature and pH. Microfluidic device can serve the precise spatial and temporal control by taking advantages of the basic characteristics of laminar flow and is easy to observe single cellular behaviors. Considering these facts, we have studied microalgae to newly focus

on microfluidic platform. Among several meaningful microalgal species for biofuel, much of our current research is involved with *Chlamydomonas reinhardtii*, which is one of the model organisms.

In this paper, we introduce a microfluidic platform to cultivate and observe microalgae in real-time even after staining cells vitally. We have utilized microchannels of microfluidic platform to maintain location of cells and prevent cells from being lost. As a result of the process of photolithography, the height of the channel was 5 μm and it was reasonable to the average height of *C. reinhardtii* cells.

To estimate microalgal viability in a microfluidic platform, we observed cells after cell loading and imaging. On the conclusion, channels of microfluidic platform were effect to observe microalgal cell division or change in real-time even after vital lipid staining. We ultimately expect this microfluidic platform to bring out meaningful results from cells dedicating as microfluidic tool for the study of microalgae.

Keywords : Microfluidics, Microalgae, Biodiesel, Vital lipid staining, Real-time observation

Student Number : 2010-24061

Contents

List of Figures

1. Introduction

Microalgae are unicellular microscopic (2-200 μm), autotrophic organisms which grow by photosynthesis and are the eukaryotic representatives [1]. Microalgae can transform carbon dioxide from the air and light energy through photosynthesis to various forms of chemical energies such as polysaccharides, proteins, lipids and hydrocarbons [2].

There is increasing interest in using microalgae as a lipid feedstock for the production of biofuels. The great attraction of microalgae is that their potential biofuel production capacity far exceeds that from land-based crops [3]. Inputs for cropping area include irrigation, planting, fertilization, and harvesting can be greatly minimized with an aquatic crop for microalgae, with a well-designed system [2]. Microalgae also grow rapidly and can double its biomass as much as eight times in a day during exponential growth [2].

Microalgae possess large droplets of unbound triacylglycerols (TAGs) which are of prime interest as a biofuel source. Lipids used for these purposes are TAGs that can be converted to fatty acid methyl esters (biodiesel) [4]. Analyzing those microalgal neutral lipid droplets is the first step in the development of algal biofuels as an economically viable fuel source. However, technical challenges exist for the cultivation of high oil content microalgae. When microalgae are put into a stressful environment, such as nutrient starvation, carbon uptake is used for storing energy rather than reproduction, thus producing more lipids [2]. Nonetheless, conditions of lipid accumulation are currently optimized using slow, labor-intensive

screening processes [4].

Increasing the screening throughput would help reduce the development cost and time to commercial production [4]. Here, we demonstrated an initial step towards this goal in the development of a poly-di-methyl-siloxane (PDMS) microfluidic platform capable of real-time observation of microalgal culturing and stress conditions (Figure 1.1). Microfluidic platform contained microchannels that are suitable for average height of *Chlamydomonas reinhardtii*, which is one of the model organisms and important to genetic study for production of biodiesel [4].

Also, in order to develop feasible production processes for microalgal biodiesel, the isolation of high neutral lipid producing microalgae is crucial [5]. Since the established Nile Red method for detection of intracellular lipids has been successful only for non-vital staining conditions, more broadly applicable and vital detection methods would be desirable. High levels of DMSO (20-30% vol/vol) and elevated temperatures (40℃) have been used recently to accelerate the permeation of this dye into targeted cells [6]. Under these conditions, microalgae are incapable of surviving. Therefore, BODIPY 505/515 and BODIPY 500/510, lipophilic bright green fluorescent dyes were tested for detection of intracellular lipids in *C. reinhardtii*. Optimum BODIPY concentrations were determined for lipid staining in *C. reinhardtii*. Compared to Nile Red, BODIPY 505/515 and BODIPY 500/510 were more effective in staining *C. reinhardtii* and showed resistance to photobleaching, maintaining its fluorescence longer than 30 minute and showed less background fluorescence.

BODIPY 505/515 is highly lipophilic dye used to label a wide

range of lipids such as fatty acids, phospholipids, cholesterol, cholesteryl esters, and ceramides [5]. When BODIPY 505/515 is added to culture of live microalgal cells, the dye stains intracellular oil-containing organelles, known as lipid bodies, within minutes [6]. The dye accumulates in these lipidic intracellular compartments by a diffusion-trap mechanism [6]. BODIPY 505/515 has a high oil/water partition coefficient, which allows it easily to cross cell membranes and organelle membranes [6]. Consequently, probes incorporating this fluorophore are more likely to mimic the properties of natural lipids [3]. The fluorescence quantum yield of the BODIPY dyes is not diminished in water. Also, BODIPY 500/510 is effective fluorescence resonance energy transfer donors to longer-wavelength BODIPY probes. Absorption of BODIPY 500/510 is well matched to argon-ion laser excitation at 488 nm, allowing analysis of cellular fatty acid uptake by confocal microscopy [2].

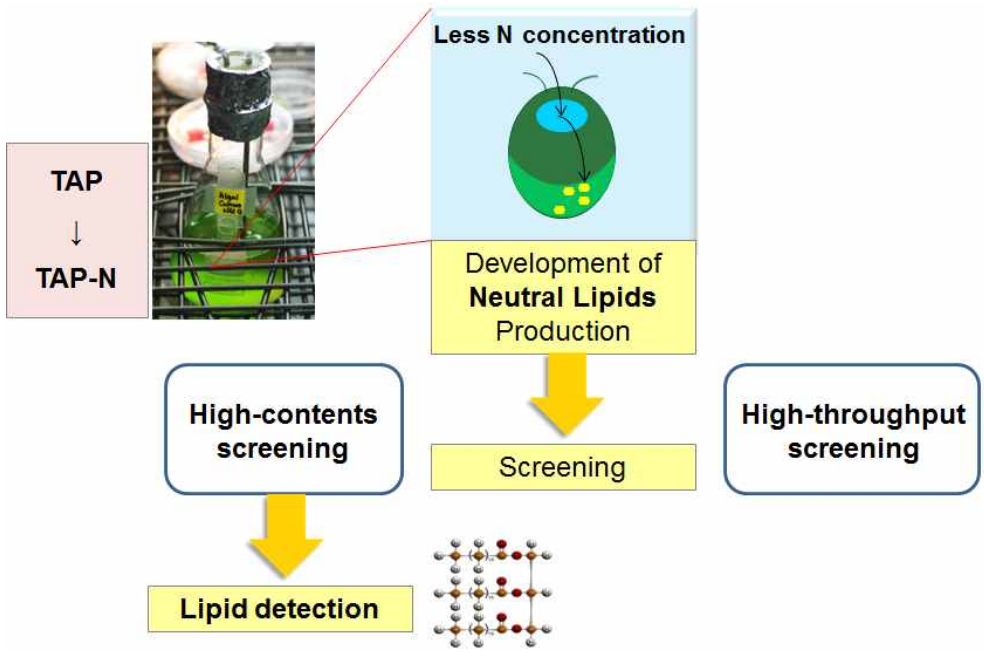


Figure 1.1 Schematic view for development of neutral lipids production of *C. reinhardtii* in Nitrogen starvation condition and screening for variation

2. Materials and Methods

2.1 Algal Cultures

Chlamydomonas reinhardtii (CC-503) was obtained from the University of Texas at Austin (UTEX). CC-503 is a wall-deficient strain and commonly used for preparation of the DNA used for genetic study.

2.1.1 Growth Conditions

C. reinhardtii have been grown previously in 100 ml or 200 ml Erlenmeyer flask in order to maintain the best microalga for synchronization. Cultures were maintained in TAP (Tris-Acetate-Phosphate) medium at 23℃. The cultures were kept illuminated with lamps at an irradiance level of 30±5 μmol m-2 s-1 photosynthetically active radiation (PAR) constant illumination on an orbital shaker with photoperiod 12:12 h (light/dark). An irradiance level was detected by the light meter (LI-250A; LI-COR Biosciences) and the quantum sensor (LI-190SA; LI-COR Biosciences). When cell concentration is from 5.0×10^6 to 1.0×10^7 cells/ml (late log phase), one milliliter of culture was centrifuged at 2,000 x g for 5 min at room temperature, the supernatant was removed and culture was resuspended at around 5.0×10^4 cells/ml in TAP medium.

2.1.2 TAP medium

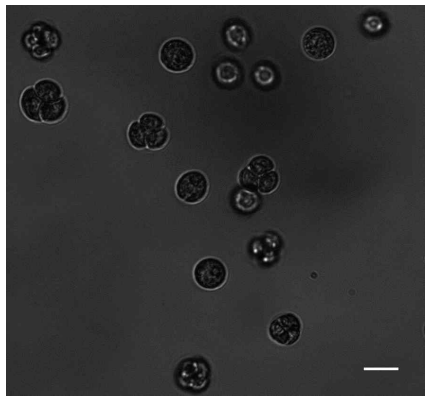
The cells were cultured in TAP medium, consisting of NH_4Cl (7.48 mM), $MgSO_4$ (406 μM), $CaCl_2$ (340 μM), K_2HPO_4 (540 μM), KH_2PO_4 (463 μM), 20 mM Tris, 17.4 mM acetate, H_3BO_3 (184 μM), $ZnSO_4$ (76.5 μM), $MnCl_2$ (25.5 μM), $FeSO_4$ (17.9 μM), $CoCl_2$ (6.77 μM), $(NH_4)_6MO_7O_{24}$ (0.88 μM), $CuSO_4$ (6.29 μM), and Na_2EDTA (148 μM).

2.2 Microfluidic platform

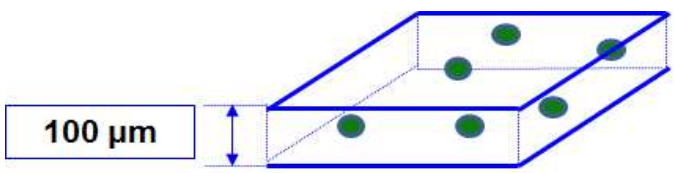
2.2.1 Device design

A microfluidic platform is motivated by imaging limit for microalgal cells in glasses as they are at different height in 0.1 mm height of glasses (Figure 2.1 (a) and (b)). Microalgae can float easily and even move around because cells are motile when they are vegetative state. To accomplish the aims of this study, a microfluidic device containing microchannels was required for isolating microalgae on real-time observation (Figure 2.1 (c)).

(a)



(b)



(c)

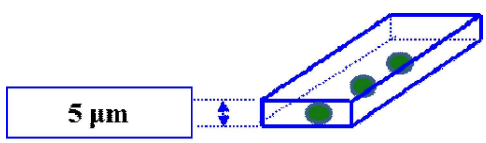


Figure 2.1 Imaging problem between glasses and idea for device design (a) Microalgal DIC image on glasses (IX 81 (40x); scale bar = 5 μm) (b) Scheme of microalgal cells between glasses and (c) in a microchannel of microfluidic platform

2.2.2 Photolithography

To make microfluidic platform, film photomask was drawn with commercial design program (AutoCAD, DE). Photolithography technique was processed to fabricate replicable molds (Figure 2.2). (1) For photolithography process, prepared wafer (test grade, US) was cleaned with plasma etcher (FEMTO Science, Korea). (2) Layer on a clean wafer was spin-coated with SU-8 5 (Microchem, US) for 5 μm height of microchannels (3000 rpm, 30 sec). The wafer was then soft-baked on a hot plate, 1 minute on 65℃ and 3 minute on 95℃ continuously. (3) Film photo mask (Hanaltech, KR) was then tightly contact to the surface of a wafer. UV radiation with 365 nm wavelength was followed for the next step using conventional 500 W mercury lamp (Osram, DE) for 4 seconds (30 mW/sec, Seongwoo MST, Kr). After the expose, the wafer was post-baked on a 95℃ hotplate for 1 minute. The wafer was developed (AZ1500 thinner, KR) leaving exquisite patterns on the wafer.

2.2.3 Monolithic bonding

To obtain complete mold piece from replicable molds fabricated through photolithography, we used polydimethylsiloxane (PDMS, Sylgard 184, Dow chemical, US) which is biocompatible, non-flammable, optically transparent, gas permeable and very flexible. PDMS was degassed with vacuum to get clear polymer before use.

The patterned wafer was prepared and coated with fresh

mixed PDMS to make 5 μm microchannels over the patterns. After then, the wafer with PDMS was cured on a 95℃ hotplate for 1 hour (Figure 2.2 (4)). Replica molds were cut out into proper pieces and holes were punched out to get a complete piece. The bonding side of the complete piece was cleaned with sticky tape and the sterilized glass cover slip was dusted. Both of the surfaces were oxygen air plasma treated and bonded.

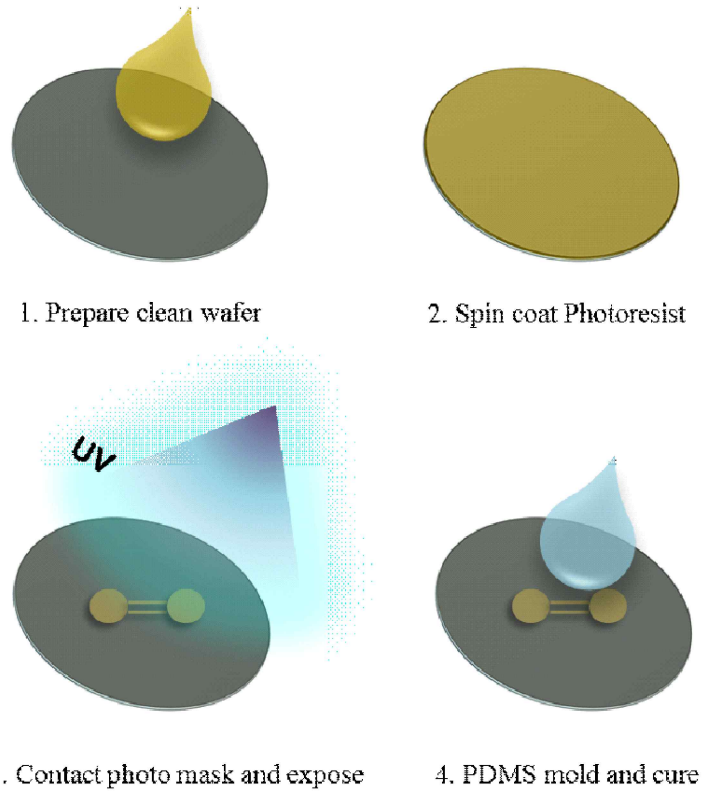


Figure 2.2 Fabrication process of microfluidic platform

2.3 Real-time observation

2.3.1 Morphological variation and reproduction

When *C. reinhardtii* cultures were in exponential phase, cells were prepared for an experiment on real-time observation. The aim of this work was dependent on successful cell loading on a microfluidic device. For this purpose, cell concentration was from 2.0×10^6 to 2.5×10^6 cells/ml.

2.4 Vital Lipid staining

Cultures were grown to late log phase in nitrogen-replete TAP medium and were resuspended at 2.0×10^6 to 2.5×10^6 cells/ml in nitrogen-depleted TAP(TAP-N) medium, in which NH_4Cl was omitted and KCl was used. After TAP-N culture, *C. reinhardtii* cells were stained with fluorophore with cell concentration from 2.0×10^6 to 2.5×10^6 cells/ml.

2.4.1 BODIPY 505/515

We have used BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Invitrogen Molecular Probes, Carlsbad, CA) to visualize lipid distribution in *C. reinhardtii*. A 1 mg/ml stock solution of BODIPY 505/515 was made by dissolving the dye in anhydrous dimethylsulfoxide(DMSO) and stored in dark. To investigate lipid droplet formation after acclimation to nitrogen deprivation, cells were visually assayed for nonpolar lipid accumulation using laser scanning confocal microscopy after 15 minute incubation in dark with BODIPY 505/515. There were reported that BODIPY 505/515 labeling concentration of 1-10 μM with DMSO vehicle concentrations of 0.02-2% were suitable for staining some microalgae [6]. To determine the optimal BODIPY 505/515 and vehicle concentration for *C. reinhardtii*, we demonstrated tests for BODIPY 505/515 staining with concentrations from 0.3 to 1.0 μg/ml BODIPY and from 0.03 to 0.1% DMSO (Figure 3.3). Microalgal cells were stained with BODIPY 505/515 and this mixture was incubated for 15 min at room temperature in darkness. BODIPY 505/515 fluorescence was determined using a confocal microscope equipped with a 488-nm argon laser and 633-nm laser. Upon excitation by a 488-nm argon laser, BODIPY 505/515 detected neutral lipids of microalgal cells. Also, excitation by a 633-nm laser was used as an autofluorescence of microalgae for detection of background fluorescence.

Furthermore, we did cell viability assay for sets of BODIPY and DMSO concentration. Cultures in TAP medium at early exponential phase were incubated with sets of dye and control in 4 days. Cells were cultured in 96 well plate and optical density of cells

at 680 nm was detected by the microplate spectrometer (Multiskan GO; Thermo Scientific).

2.4.2 BODIPY 500/510

Similarly, we have used BODIPY 500/510 (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid; Invitrogen Molecular Probes, Carlsbad, CA) to visualize lipid distribution in *C. reinhardtii*. A 1 mg/ml stock solution of BODIPY 500/510 was made by dissolving the dye in anhydrous dimethylsulfoxide(DMSO) and stored in dark. To investigate lipid droplet formation after acclimation to nitrogen deprivation, cells were imaged using epifluorescence microscopy after 30 minute incubation in darkness with BODIPY 500/510 and washing.

3. Results and Discussion

3.1 Microfluidic platform

It was found that a microfluidic device containing microchannels of 5 μm height (Figure 3.1) was well suited for this task as the height of 5 μm was reasonable for the average height of *C. reinhardtii* cells. Furthermore, *C. reinhardtii* of vegetative state could not move around on average.

3.2 Real-time observation

3.2.1 Real-time observation of *C. reinhardtii* in microchannels

After cell loading in a microfluidic device, we observed and took DIC images with IX 81 microscope in real-time in dark photoperiod of *C. reinhardtii*. Exposer time for imaging was 30 ms and light intensity for expose was 0.82 μmol m-2 s-1. Images were taken with duration of 10 min. After imaging in dark photoperiod, we incubated the microfluidic device for light period. We could observe same *C. reinhardtii* cells of the microfluidic device until 60 hour after cell loading. As shown in Figure 3.2, we could observe morphological variation and reproduction of *C. reinhardtii*. Division time of individual cells was different but cells were divided in dark

photoperiod on average. One cell was divided to 2 cells and after then, divided to 4 cells. Also, size of each cell was getting smaller in dark photoperiod representably. It is matched within formation from transient decreasing standard curve with OD_{680} in dark photoperiod.

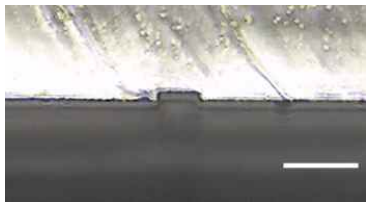
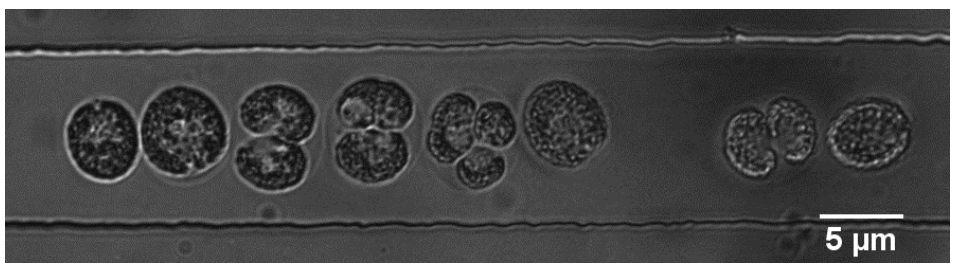


Figure 3.1 5 μm height of microfluidic platform for real-time observation (scale bar = 50 μm)

(a)



(b)

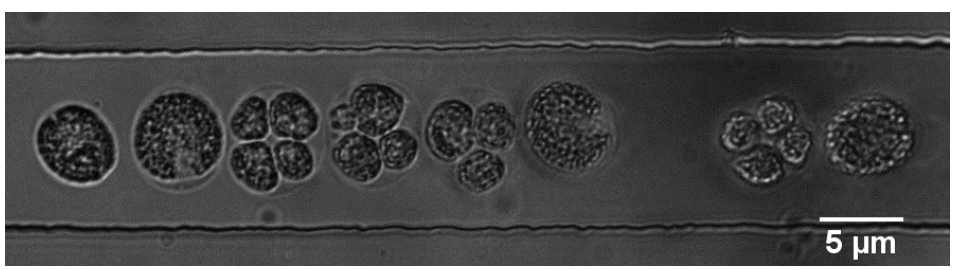


Figure 3.2 Real-time observation of *C. reinhardtii* in a microchannel (a) *C. reinhardtii* after 24 h culture in a microchannel (b) morphological variation and reproduction of *C. reinhardtii* after 26.5 h culture in a microchannel (IX 81 (40x); scale bar = 5 μm)

3.3 Vital lipid staining

3.3.1 BODIPY 505/515

After 96 hour nitrogen-deplete TAP-N culture, microalgal cells were stained with concentrations from 0.3 to 1.0 μg/ml BODIPY 505/515 with form 0.03 to 0.1% DMSO (Figure 3.3). Cells after 96 hour TAP-N culture were used because their fully lipidic state would be helpful to detect neutral lipid with BODIPY staining.

Neutral lipid images of the confocal microscope were taken using 488 nm excitation and a 500- to 600-nm band-pass emission filter. The green fluorescence of images (Figure 3.3) was stained lipid bodies. As shown in Figure 3.3 and 3.4, working concentrations of BODIPY 505/515 and DMSO were 0.5 μg/ml BODIPY with 0.03% DMSO and 1.0 μg/ml BODIPY with 0.03-0.05% DMSO.

Also, the aim that was to test the effectiveness, specificity and the repeatability of BODIPY 505/515 as lipid staining in *C. reinhardtii* was accomplished even in low level of BODIPY 505/515 and DMSO concentration.

Furthermore, we did cell viability assay of BODIPY 505/515 with TAP medium culture. All cultivation sets of BODIPY 505/515 dye concentration for optimization experiments were good viability in 4 days. Especially, standard curves from culture with 0.5 μg/ml BODIPY 505/515 (0.05% DMSO) and 1.0 μg/ml BODIPY 505/515 (0.05% DMSO) were similar to that from TAP culture (Figure 3.5). On the conclusion, 1.0 μg/ml BODIPY 505/515 (0.05% DMSO) was best for vital lipid staining because it was good result from both of

cell viability assay and imaging.

3.3.2 BODIPY 500/510

Neutral lipid images of epifluorescence microscope (Nikon Ti-e; 60 x) were taken using 480 nm excitation and a 500- to 530-nm band-pass emission filter. BODIPY 500/510 was also effective to stain lipids of *C. reinhardtii* clearly even after 24 hour TAP-N culture (Figure 3.6).

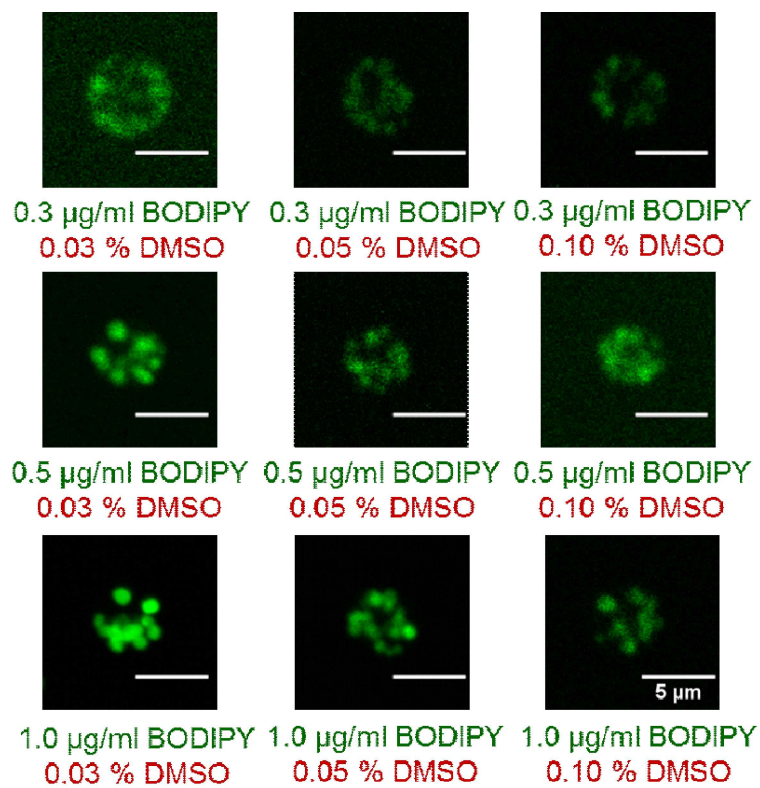


Figure 3.3 Optimization of BODIPY 505/515 for staining of *C. reinhardtii* after 96 h TAP-N culture (Confocal microscope (40x); scale bar = 5 μm)

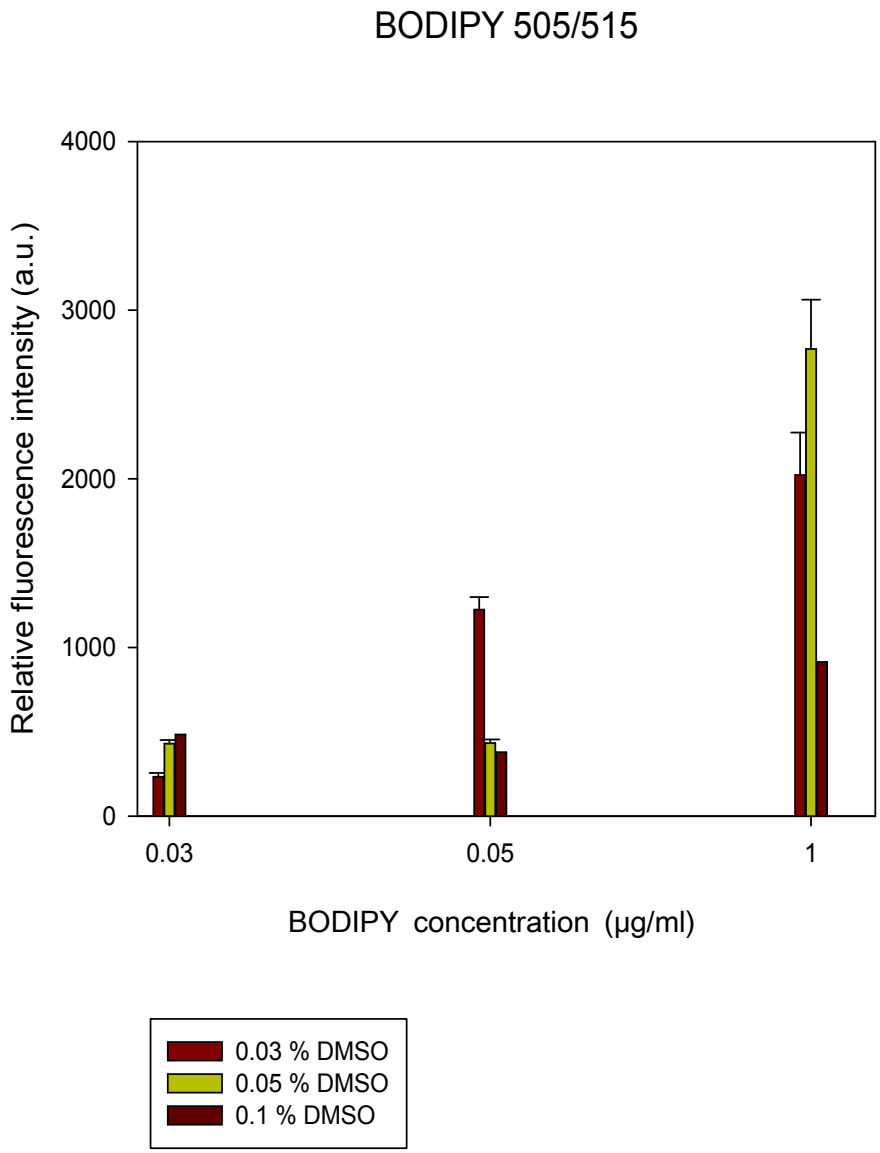


Figure 3.4 Relative fluorescence intensity of BODIPY 505/515 staining of *C. reinhardtii* after 96 h TAP-N culture

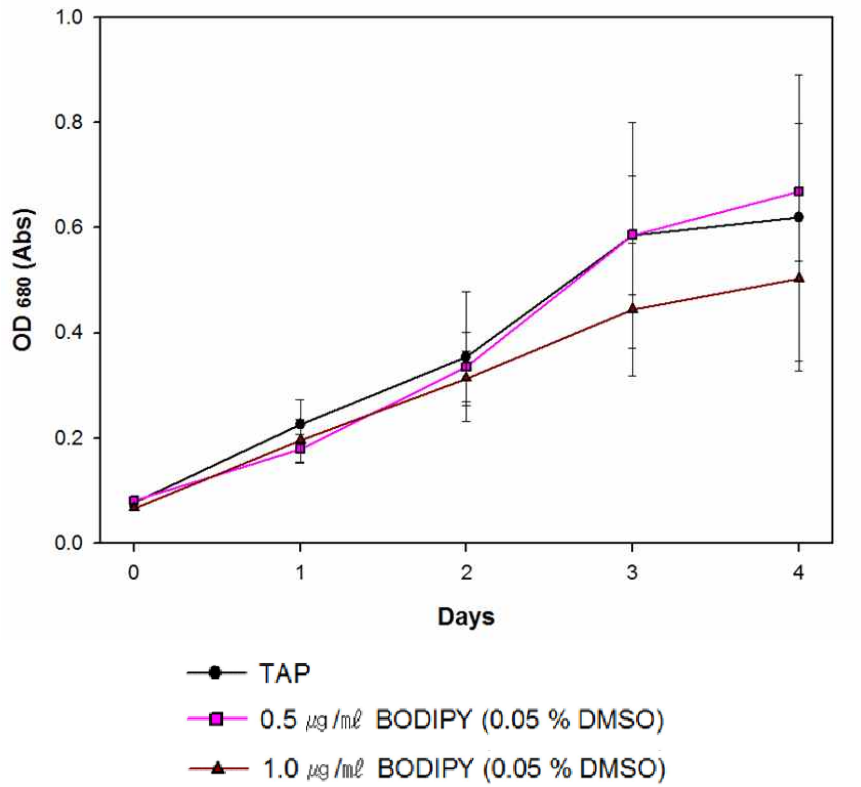


Figure 3.5 Cell viability assay of BODIPY 505/515 for *C. reinhardtii*

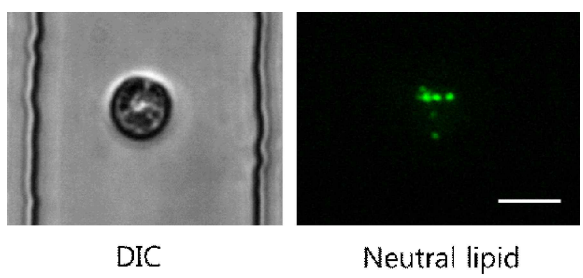


Figure 3.6 *C. reinhardtii* in a microchannel, after 24 h TAP-N culture (Nikon Ti-e (60x); scale bar = 5 μm)

3.3.3 Real-time observation

As shown in Figure 3.7, real-time observation of lipid variation was demonstrated with BODIPY 500/510 staining and epifluorescence microscopy (Nikon Ti-e). The cells after 72 hour TAP-N culture were used because their less fully lipidic state would be helpful to detect variation of neutral lipids with BODIPY staining. With this idea, we could observe lipid variation in real-time with photostable BODIPY successfully until 210 minute.

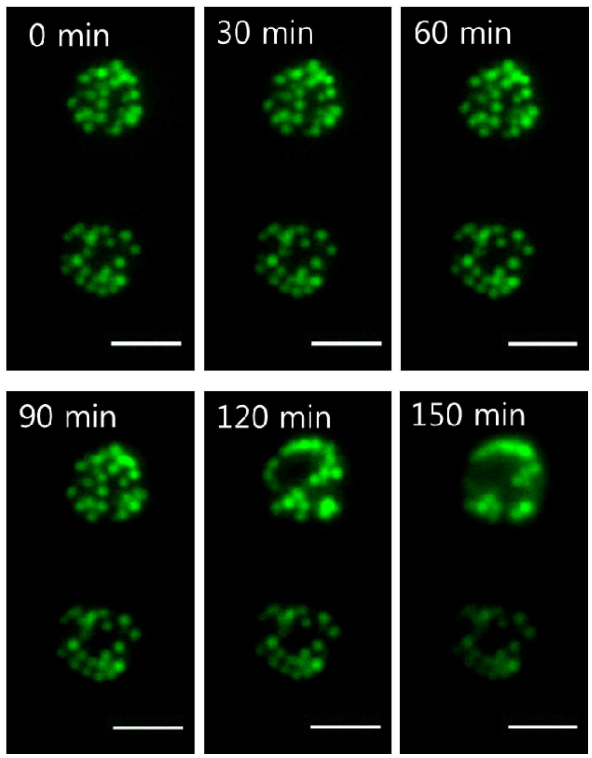


Figure 3.7 Lipid variation on real-time observation after 72 h TAP-N culture and lipid staining with BODIPY 500/510 (Nikon Ti-e (60 x); scale bar = 5 μm)

4. Conclusion

We focused on real-time observation of *C. reinhardtii* which is one of the model organisms because it is important to research of biodiesel and microalgae.

On the conclusion, microfluidic culture could be correlated with flask culture of microalgae. It was shown that real-time observation of microalgae which are motile and floated cells is easily demonstrated in microchannels of microfluidic platform.

Also, channels of microfluidic platform were effect to observe microalgal cell division or change in real-time even after vital lipid staining. We found effective and vital staining conditions with BODIPY 505/515 and BODIPY 500/510 for *C. reinhardtii*. We ultimately expect this microfluidic platform to bring out meaningful results from cells dedicating as microfluidic tool for the study of microalgae. Furthermore, microalgal analysis of lipid body growth or change on real-time observation would potentially be performed in mutants and transgenic algal strains to help elucidate genetic pathways involved in intracellular oil accumulation.

5. References

1. T. Mutanda et al., *Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production, Bioresource Technology*, 2011, 102:57-70

2. R. Sakthivel et al., *Microalgae lipid research, past, present : A critical review for biodiesel production, in the future, Journal of Experiment al Sciences*, 2011, 2(10):29-49

3. T. Skye R et al., *Analysis of lipid accumulation in microalgae*, *Plant Biology*, 2009

4. R. E. Holycomb et al., *Culturing and investigation of stress-induced lipid accumulation in microalgae using a microfluidic device*, *Anal Bioanal Chem*, 2011, 400:245-253

5. Govender T et al., *BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae*, *Bioresour Technol.*, 2012, 114:507-11

6. M. Cooper et al., *Visualizing "green oil" in live algal cells*, *Journal of Bioscience and Bioengineering*, 2010, 109(2):198-201

7. Li Y et al., *Inhibition of starch synthesis results in over production of lipids in Chlamydomonas reinhardtii*, *Biotechnol Bioeng.*, 2010, 107(2):258-268

8. V. H. Work et al., *Increased lipid accumulation in the Chlamydomonas reinhardtii st 7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains*, *Eukaryot Cell*, 2010, 9(8):1251-1261

요약

미세유체플랫폼 내 미세조류 배양 및 실시간 관찰 연구와 생체지질염색 방법

김 민 서

서울대학교 대학원
기계항공공학부

최근에 전망 있는 대체에너지 중 하나로 바이오디젤, 바이오에탄올과 같이 바이오연료로 전환 가능한 바이오매스가 각광받고 있다. 미세조류는 중성지질과 탄화수소를 대량으로 생산 가능한 차세대 그린연료이다. 그러나 여전히 미세조류 바이오연료의 성공을 위해서는 균주 개발, 배지 최적화, 반응기 설계, 대사 공학과 생산물 정제와 같은 여러 도전과제를 해결해야 한다.

바이오연료를 생산하는 미세조류의 능력은 광량, 이산화탄소 농도, 온도, pH와 같은 광합성 진핵생물의 많은 환경적 요인에 의존한다. 미세유체기기는 층류의 기본적 성질의 이점에 의한 정확한 시공간적 설계 및 수행이 가능하고 단일 세포 관찰이 용이하다. 이러한 요소들을 고려하여, 우리는 미세유체플랫폼에서의 미세조류 연구에 새롭게 초점을

맞추어 설계 및 수행을 했다. 이를 위해, 바이오연료를 위한 몇몇 의미 있는 미세조류 중에서 모델생물인 *Chlamydomonas reinhardtii*를 연구하였다.

이 논문에서 우리는 실시간 관찰과 생체지질염색을 위한 미세조류세포를 배양하고 연구하기 위한 미세유체플랫폼을 소개하였다. 특히 이 미세유체플랫폼에서는 실시간 세포 관찰이 가능하게 하는 미세채널을 보유하도록 제작되었다. 광식각 공정을 통하여, 5 μm 높이의 미세채널을 제작하였고, 이것은 *C. reinhardtii* 세포의 높이에 적합하고 세포 유동을 저지한다는 것을 확인할 수 있었다. 이후 미세유체플랫폼에서의 실시간 미세조류세포 관찰을 장시간 수행하였다.

또한 기존의 지질염색법은 *C. reinhardtii*의 세포 생장성을 저해하기 때문에, 실시간 세포 변화 및 지질 관찰에 한계가 있다. 따라서 *C. reinhardtii*의 중성지질 관찰을 위한 염색제의 선택 및 적정한 농도 선정과 생체지질염색 방법을 구축하였다.

주요어 : 미세유체역학, 미세조류, 바이오연료, 생체지질염색
실시간 관찰

학 번 : 2010-24061

감사의 글

대학원에 입학한지 벌써 2년 가까이 되어 석사 졸업을 하게 되었습니다. 존경하는 전누리 교수님, 친애하는 연구실 선배님들과 후배님들 덕분에 연구에 대해서, 그리고 연구 외적으로도 많은 것을 배우고 성장하여 사회에 나가게 되었다고 생각합니다. 돌아보면 짧은 2년 대학원 생활이어서 아쉽지만, 저의 지난 어떤 시간들보다 값지고 귀중한 시간이었고, 잊지 못할 것 같습니다. 늘 가까이에서 뵐 수 있던 교수님, 선배님들과 후배님들께 이제는 다음을 기약하는 인사를 드려야 한다는 것을 생각하니, 벌써 그리운 마음이 듭니다. 앞으로 멀티스케일 의기계공학 연구실의 졸업생으로서 감사하는 마음을 가지며 사회에 보탬이 되는 인재가 되도록 노력하고 또 노력하겠습니다. 그동안 진심으로 감사드립니다.

2012년 7월

김민서 드림