A microfluidic perfusion platform for cultivation and screening study of motile microalgal cells

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Systematic screening of algal cells is getting huge interest due to their capability of producing lipid-based biodiesel. Here, we introduce a new microfluidic platform composed of an array of perfusion chambers designed for long-term cultivation and preliminary screening of motile microalgal cells through loading and releasing of cells to and from the chambers. The chemical environment in each perfusion chamber was independently controlled for 5 days. The effect of nitrogen-depletion on the lipid production, phototaxis behavior in the absence of Ca^{2+} , and cytotoxic effect of herbicide on microalgal cells was successfully monitored and compared with simultaneous control experiments on the platform. The present methodology could be extended to effective screening of algal cells and various cell lines for the production of biodiesel and other useful chemicals. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4871522>]

I. INTRODUCTION

Microalgae, photoautotrophic organisms, and the simplest and most primitive plants are generally more efficient converters of solar energy than terrestrial plants. The microalgae produce high energy through photosynthesis by combining only sunlight, H_2O , and CO_2 . Recently, global warming and expected depletion of fossil fuels make it a target of much research and speculation as an affordable and sustainable feedstock for energy and chemicals.¹

Lipids, one of the biochemical fractions, can be converted into biodiesel by transesterification process. However, the production of biofuel from microalgae is too expensive to be commercially competitive with petroleum-based fuels. Therefore, a number of technological advances have to be made to bring down the costs of making the biofuel to the level of petroleum. These include controlling the metabolism of algae to increase lipid content by genetic engineering, selecting strains with high natural lipid content, developing simpler and cheaper methods of transesterification, and optimizing the design of bioreactors to achieve maximum biomass yield. Furthermore, the lipid accumulation in most microalgal species is dependent on environmental stress and culturing conditions such as light intensity, nutrient limitation, salinity, temperature, pH, and growth phase.² Multitude of these growth factors demands enormous time and efforts for screening cells and optimizing growth conditions. For commercial production to be viable, therefore, an efficient approach is required to reduce the development cost and time.

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Microfluidics has been used as an alternative platform of cell study, ranging from single cell to three-dimensional tissue.³ For example, a variety of mammalian cells, bacteria, and yeast were grown in a microfluidic channel, droplet, and chamber where tiny amounts of volume and medium are required. It is surprising, however, to find that the study of microalgae in microfluidics is largely unexplored compared to other cells except for encapsulation of microalgal cell into different droplets or microstructure.^{4–9} The encapsulation approach, while useful, has many limitations when it is confronted with cell capture, cell recovery after culture, change of medium, and inhibition of evaporating water,^{7,9} in particular, for motile cells such as microalgal cells. Cavity structures were used to capture motile cells.^{4,5,8} However, the entrance is freely accessible to cell allowing its escape. Although Holcomb *et al.* suggested a very simple microfluidic system for microalgal culturing and environmental studies,⁷ they used a static fluidic system and it is impossible to change the medium without losing cells. Therefore, new microfluidic platform is required to address these problems.

We present in this study, a microfluidic perfusion platform composed of multiple microchambers equipped with pneumatic valves that can resolve the limitations of the current microfluidic approaches including encapsulation methods. The platform is equipped with 2 μm -height pillar structures as lattice to prevent cell from escaping. It allows for loading of cells to the cage, effective exchange of nutrients, and their recovery after experiment for ordinary imaging and other purpose. We demonstrate its use in long-term cultivation with no drying problem of 5–6 μm -size *Chlamydomonas* cell, a model organism of microalgae and the production of lipid.¹⁰ We also demonstrate its versatility by studies on the effect of chemical treatment on the phototaxis and cytotoxicity. Although not shown here, the microfluidic perfusion chip can be expanded to large format for high throughput screening.

II. EXPERIMENTAL

A. Chip design

As shown in Fig. 1(a), operation of 2×4 array of perfusion chambers was controlled by a fluidic line-up consisting of 16 pneumatic valves, 11 inlets, and 5 waste ports. The 5 inlet ports (i0, iu1-2, and id1-2) were switched by combination of 4 valve lines (vu1-3 and vd1-2) to regulate the medium flow at the flow controller (Fig. 1(b)). In order to conduct experiments at desired conditions, two different mediums could be supplied to two rows of 4 chambers each through upper and lower inlets (iu3-4 and id3-4), while one identical medium could be injected to both rows of chambers through a flow controller (Fig. 1(c)).

Three sides of the microchamber were surrounded by medium supply channel with a shallow bottom gap (2 μm height and 10 μm interval) of pillars (30 $\mu\text{m} \times 30 \mu\text{m}$, square) so that perfusion of the medium into the chamber is allowed across the interval space from the medium supply channel, but the escape of the cells out of the chamber is prevented. The remaining wall side of the microchamber is equipped with a pneumatic valve (vu8 and vd8) and it could be reversibly connected into a cell loading/harvesting microchannel (iu5 and id5) for loading or releasing cells to/from the chamber. The cells in the microchamber could be cultivated and treated with various compositions of media or/and chemicals supplied through separately controlled two rows of winding microchannel with right angle.

B. Chip fabrication

The microfluidic perfusion chip was fabricated by micromolding method of polydimethylsiloxane (PDMS) using a multi-layer soft lithography.¹¹ Two types of molds were produced for microfluidic chambers/channels and pneumatic valves. The mold for microfluidic chamber/channel was prepared by three photolithographic process (see supplementary material, Fig. S1).¹² A 3-in. silicon wafer was coated with a photoresist (SU-8 2, MicroChem, Newton, USA) to form 2 μm thick pillar structure. After exposure to UV light through a film photomask for the pillar structure, the post-baked wafer was developed using 1-methoxy 2-propyl acetate. For the second photolithographic process for medium channels, a diluted photoresist (SU-8 50,

FIG. 1. Design of a microfluidic perfusion platform for cultivation and study of microalgal cells. (a) Overall fluidic line-up for 16 pneumatic valves (black, vu1–vd8), 12 medium channels (red, i0/w0, iu1–id4, and wu2/wd2), and 4 cell loading/harvesting channels (blue, iu5/id5 and wu1/wd1). (b) Close-up design for flow controller to supply the medium into culture chamber. (c) Close-up design and optical image for 2×4 array of perfusion chambers. The chamber (green) is connected to the cell loading/harvesting channel (blue) through a concave channel (cyan) over which a valve (gray) passes. Perfusion solution comes into the chamber through a shallow gap (yellow). (d) Schematic drawing (left) and SEM image (right) of a perfusion chamber shown from bottom.

MicroChem, Newton, USA) with 1-methoxy 2-propyl acetate in the ratio of 90:10 (wt. %) was over-coated on the wafer with mold for pillar structure to form $30\ \mu\text{m}$ thickness and was then fabricated using the same process for pillar structure as mentioned above. The last photolithographic step for microfluidic channel with valve was performed with a positive photoresist (AZ 4620, AZ Electronic Materials, Seoul, Korea) by multilayering on the patterned wafer. The $15\ \mu\text{m}$ thick photoresist coating on the wafer was patterned for the fluidic channel having valve functions, including the connecting channel between the cell loading/harvesting channels and the chambers, then developed with AZ 400 K (1:4) (AZ Electronic Materials, Seoul, Korea). The triple-layer patterned wafer was put on a hot plate at 135°C for 30 s for re-shaping the AZ 4620 pattern into rounded channels to seal it completely, so that there will be no leaking during valve operation.

Another mold for pneumatic valves was fabricated using the diluted photoresist (SU-8 50) to render $15\ \mu\text{m}$ thickness by the photolithographic process on a silicon wafer (see supplementary material, Fig. S2).¹² Both molds were treated with chlorotrimethylsilane at 70°C for 30 min for easy release of a cured PDMS chip from the mold. Two types of PDMS mixtures with 10:1 and 20:1 ratios of base and curing part (Sylgard 184, Dow Corning, Seoul, Korea) were made. The 10:1 PDMS mixture was poured on the microfluidic mold to form 5 mm thickness. The mold for the valve was coated with the 20:1 PDMS mixture by spinning at 2000 rpm for 90 s to obtain $50\ \mu\text{m}$ of thickness. Both PDMS prepolymers were partially cured at 70°C for 30 min. The PDMS chip for the fluidic channel was peeled off from the molds and was punched with a 19 gauge blunt needle to make the inlet and outlet ports. Then the fluidic layer was aligned over the valve layer under a microscope (SZH, Olympus, Tokyo, Japan), and baked for 1 h to seal the two PDMS layers. Finally, the prepared PDMS perfusion chip was punched for valve ports and mounted on a glass slide with a partially cured 20:1 PDMS layer, and post-treated by keeping the whole chip in an oven at 70°C for 1 day. Finally, the PDMS based microfluidic perfusion chip was fabricated as shown in Fig. S3 of the supplementary material.¹²

C. Device operation

The inlet of the microfluidic perfusion chip was connected to one end of a tubing through a needle (21 gauge). The other end of the tubing was joined to a syringe to perfuse media or to load cells into the chamber using a syringe pump (Legato 180, KD Scientific, Holliston, USA). The inlet of the pneumatic valve was connected through the tubing to a three-way solenoid valve (LHDA1223111H, The Lee Co., Westbrook, USA) on a manifold that was connected to an air-pressure regulator (2100-2c-L, CKD, Aichi, Japan). The air-pressure was monitored with a pressure gauge (G49D, CKD, Aichi, Japan). The microfluidic valve and one end of the tubing near the chip were filled with water for its operation without the production of air bubble in the microfluidic channel. The opening and closing of the microfluidic valve was controlled by applying 12 V of electricity to the solenoid valve using a manual switch.

D. Maintenance of microalgal culture

Experiments were conducted using three types of microalgal cells, *Chlamydomonas reinhardtii*, C-124 (mt- [137c], wild type), CC-400 (cw-15 mt+, cell wall-less and nonmotile), and CC-4333 (cw15 arg7-7 sta6-1::ARG7 mt+ [Ball Bafj5], starchless).¹³ The strains, CC-124 and CC-400, were obtained from KRIIBB (South Korea). The other strain was obtained from the *Chlamydomonas* Resource Center (USA). The cells were maintained on agar plates or in liquid culture of tris-acetate-phosphate (TAP) medium¹⁴ under 2000 lux of white light from fluorescence tubes at 25 °C. The *Chlamydomonas* cells for experiment were taken from an exponentially growing liquid TAP culture.

E. Cultivation of *Chlamydomonas* cells in microfluidic perfusion chamber

The microfluidic chip was sterilized by rinsing with 70% of ethanol and washing with TAP. *Chlamydomonas* cells (CC-4333) were loaded into two rows of microfluidic perfusion chambers through the cell loading/harvesting channel (iu5 and id5 of Fig. 1(a)) in a PDMS chip. And the microchambers were disconnected from the cell loading/harvesting channel by blowing air into the pneumatic channel to close the valves (vu8 and vd8 of Fig. 1(a)), then perfused with TAP (iu3 of Fig. 1(a)) or nitrate-depleted TAP (id3 of Fig. 1(a)) media at 0.5 μ /min of flow rate up to 5 days. The microfluidic chip was placed on the stage of an inverted microscope (ECLIPSE Ti-U, Nikon, Tokyo, Japan) to monitor the cell growth by imaging with a color CCD camera (FOculus F1, NET GmbH, Finning, Germany) or a monochrome camera (Rolera EM-C², QImaging, Surrey, Canada). The image was analyzed using an image processing software, ImageJ¹⁵ to investigate the relative growth of cells. The cell was illuminated with the continuous light from a halogen lamp of the microscope. The part or all of the cells in the microfluidic perfusion chamber were enabled to release through the cell loading/harvesting channel (wu1 and wu2 of Fig. 1(a)) by opening the pneumatic valve (vu8 and vd8 of Fig. 1(a)) and injecting medium through the perfusion channels. After 5 days of cultivation, the lipid contents in the released cell sample were stained with 0.5 mg/ml of Nile Red and imaged at 540 nm with 480 nm of excitation. Image of chlorophyll fluorescence was also captured at 680 nm with 480 nm of excitation.

F. Phototaxis of *Chlamydomonas* cells in microfluidic perfusion chamber

The microfluidic chip was perfused with 4% of Pluronic F-68 for 1 h and washed with TAP. *Chlamydomonas* cells (CC-124) were harvested from a fresh culture and suspended in TAP with 0.2% Pluronic F-68.¹⁶ The media were supplemented with 0.2% of Pluronic F-68 to reduce the adhesion of cells to the chamber wall of microfluidic chip. The operation of the chip was performed in a similar way to the cultivation of cells. The cells were loaded into the microperfusion chambers. The upper and lower 4 chambers in two rows were perfused with complete TAP and Ca²⁺-depleted TAPs, respectively. The cells were kept in dark for 30 min and illuminated with blue LED (peak wavelength, 480 nm, 245 mcd (3.9 V)) supplied with 3 V

power supply. The movement of cell was recorded by taking images, and the longitudinal distribution of cells in the microchamber was analyzed by using a software, ImageJ.

G. Effect of herbicide on *Chlamydomonas* cells in microfluidic perfusion chamber

Chlamydomonas cells (CC-400) were loaded into the microfluidic perfusion chambers. The chip was perfused with TAP medium using a common channel (i0 of Fig. 1(a)) at the normal condition. For cytotoxicity test, the flow controller of the chip was switched to perfuse one row of chambers with TAP containing 1 μ M of methylviologen as a herbicide (id3 of Fig. 1(a)). The other row of chambers was perfused with normal TAP (iu3 of Fig. 1(a)). The cells were illuminated with light of 3000 lux from a halogen lamp (Osram, Berlin, Germany) to induce cytotoxic effect of methylviologen. Images of bright field and chlorophyll fluorescence were captured for 1 h. TAP medium with 10 μ M of SYTOX (Invitrogen, Carlsbad, USA) as a staining dye was perfused through one inlet (iu1 of Fig. 1(a)) to check their viability by staining the dead cells in the chamber. Images of the green fluorescence of dead cells were captured at 580 nm with 480 nm of excitation.

III. RESULTS AND DISCUSSION

A. Chip design and operation

Simple perfusion systems were proposed for the cultivation of adherent liver and HeLa cells.^{17–19} We developed here a perfusion system for the cultivation of free floating cells by incorporating pneumatic microvalves. Two pieces of the patterned PDMS for fluidic channels/chambers and pneumatic valves were overlaid with precise alignment to prepare a PDMS perfusion chip with a 2×4 array of chambers and mounted on a glass slide coated with PDMS adhesive layer. The array of microchambers is constructed for it to be suitable for real time observation of cell behavior under microscope. It was designed to accommodate changes in growth environment by regulating the medium using a fluidic line-up consisting of 16 inlet/waste ports and 16 pneumatic valves as shown in Fig. 1. Different media could be supplied to each row of 4 chambers using two separate inlets (iu3/4 for upper row and id3/4 for lower row) with the help of valves. Fig. 2(a) and supplementary material Fig. S4¹² provide visualization of the flow of fluorescent dye solution into the bottom row, contrasting the flow of water with no staining into the upper row. Alternatively, both rows of microfluidic perfusion chambers could be supplied with identical medium through bifurcated central fluidic channel by opening one or combined medium channels (i0, iu1, iu2, id1, and id2 of Fig. 1), depending on the medium composition, and closing medium A and B channels (iu3/4 and id3/4 of Fig. 1), as shown in Fig. 2(a) and supplementary material Fig. S4.¹² These operations demonstrated the capability to provide various environmental conditions for living organisms in the chambers, which can be utilized to study the cell viability and subsequent *in-situ* imaging analysis with staining dyes. Comparison with control experiment can also be carried out simultaneously. In addition, the four chambers of a row under identical condition produce enough data for a statistical evaluation.

The channel dimension surrounding the chamber played an important role for quick perfusion of the prepared composition of medium into chambers within a certain time. When the medium channels at both inlet and outlet were symmetrically designed with 90 μ m of width, the perfusion influx of the medium into the chamber was too slow to reach the equilibrium in concentration between the chamber and the fresh medium (see supplementary material, Fig. S5).¹² However, when the perfusion channel width was unsymmetrically designed with 30 μ m inlet part and 90 μ m outlet part, a quick equilibrium was achieved due to build-up of the influx flow pressure that accelerated the perfusion of fresh medium through small gap into the chamber.

To investigate the perfusion kinetics in the microfluidic perfusion chip, the progression of fluorescence from the channel inlet to the 4 chambers in a row was observed. The fluorescence intensities measured at 5 locations (inlet of medium channel and 4 microchambers) with time generally increased in a sigmoidal manner and reached steady state within 75 s (Fig. 2(b)). The

FIG. 2. Fluidic properties of the designed microfluidic perfusion system for cultivation of microalgae. (a) Two rows of microchambers are supplied with identical fluorescent dye media (left) or with water and fluorescent dye media (right). (b) Perfusion kinetics in the medium channel and 4 chambers. The fluorescent intensities are measured with time at 5 sites indicated with stars in (a). The flow rate was $0.1 \mu\text{l}/\text{min}$. The intensities were normalized relative to the final values at steady state. The slope of fluorescent intensity curve corresponding to the speed of change in solute concentration was calculated from the graph by numerical differentiation. Shown is the representative data of two independent experiments.

differential curves of fluorescence, obtained by numerically differentiating the fluorescence curves to represent the flow rate (Fig. 2(b)), indicate that the flow rate progressively decreased with extended flow from the inlet to and across the chambers. However, the linear flow speed in the channel was consistently maintained in the range of $560\text{--}600 \mu\text{m}/\text{s}$ throughout. The flow speed in the chambers decreased to two thirds of that in the medium channel, presumably due to the presence of the micropillars between channel and chamber. It is advantageous that the reduced flow rate in the chamber lowered the shear stress to *Chlamydomonas* cells. These analyses confirmed that the prepared medium could be delivered reliably into the chambers with nearly no delay in time and no change in composition, which is important for cell cultivation and cell screening study. Only $\sim 25 \text{ s}$ was taken to exchange the medium on demand in culture chambers.

It is known that long-term cell culture in the gas permeable PDMS²⁰ based chip and the encapsulated droplet needs humid space to suppress the water evaporation in media.^{7,9,21} Severe water absorption causes a problem of significantly reducing the transmission of light into microalgal cells grown under photoautotrophic condition. In contrast, the developed microfluidic perfusion system caused no drying problem even during long-time cultivation in outdoor environment. Therefore, it is convenient to observe the cell behavior in real time by installing the transparent chip system on the stage of microscope that simply reduced the working distance from an objective lens.

B. Cultivation of *Chlamydomonas* cells in microfluidic perfusion chamber

The microfluidic perfusion chamber was first used for the cultivation of microalgal cells at different conditions. Free floating microalgal *Chlamydomonas* cells (starchless strain CC-4333) were loaded into the 4 chambers of each row through cell loading/releasing channel equipped

with pneumatic valves. The cells were captured in the rectangular cage of chamber when the valve was closed, and fresh medium was continuously supplied through the shallow gap in the bottom of the wall that acted as a barrier between the media channel and the chamber.

As the cells in each row of 4 chambers were cultured under identical conditions, the two rows of chambers could be used to culture the cells under two different media conditions. The flow rate for the media was maintained in the range of 0.1–0.5 $\mu\text{l}/\text{min}$ because a higher flow rate pushed the non-adherent cell into the corner near to the efflux of the perfusion chamber. The cell reproduction was comparatively observed up to 5 days under nitrogen replete and deplete conditions (Fig. 3).

FIG. 3. Long-term cultivation of *C. reinhardtii*, CC-4333 strain, in a microfluidic perfusion chamber. The cells loaded into each row of 4 chambers were fed with complete (TAP) or N-deplete TAP (TAP-N) through perfusion channel. (a) Optical images of cells grown in complete TAP for 0, 1, 2, 3, 3.5, and 3.8 days of cultivation. The 5 days cultured cells were reduced by opening the valve (0h) and resume to cultivate (3h). (b) Relative growth rate curves of cells in TAP and TAP-N media, normalized to the maximum growth rate. The inset shows two cell chambers after 4 days of cultivation. Shown is the representative growth curve of three independent experiments. (c) Comparative microscopic images of bright field, autofluorescence from chlorophyll (red), staining with Nile Red dye (yellow) of cells grown in TAP and TAP-N media.

In the early growing stage of cultivation, the growth of cells was very slow, even though there was still division of cells (Figs. 3(a) and 3(b)). In the TAP medium, the growth rate became faster, and exponentially increased on the second day of cultivation, the perfusion chamber being nearly full with cells on the third day. Note that the growth rate was plotted from the captured images of cells inside the 4 chambers, after taking average value of transparency. The doubling time was 8–9 h, which was consistent with the previous studies in bulk and droplet cultivation of *Chlamydomonas* wild type 12.²¹ During the exponential phase, the growth rate in the microfluidic perfusion chamber seemed to be faster compared to those in the bulk phase cultivation as shown by the slopes of cell growth (supplementary material, Fig. S6)¹² and the droplet system.^{9,21} The microchambers with 30 μm height are ideal photobioreactors that can expose enough light even to full density of cell, unlike in bulk photoreactor with severe light blocking problem by self-shading. It was also reported that the viability of *Chlamydomonas* cells in the droplet compartments or the closed chamber depended on cell density, droplet size, and the size of inoculum.²¹ Possible depletion of essential nutrients and accumulation of waste products in the closed system would affect the growth of cells. However, the microfluidic perfusion chamber (volume of 1 nL) could be appropriately refreshed with a flow rate of 2–8 nL/s, and the wastes from cells were diffused out of the chamber, resulting in a better viability and productivity. In addition, the cell population could be reduced by opening the valve on the loading/releasing channel when needed. After 4 days of culture, the fully crowded cells were released from the chamber (0 h of Fig. 3(a)), but the cell population in the chamber increased again after 3 h of the second cultivation (3 h of Fig. 3(a)), demonstrating the viability of cells even in the crowded dense condition. Therefore, this platform can be used as a tool for keeping living cells over a long term with intermittent control of the cell population.

Nitrogen is an essential element for a living organism as a major component of nucleic acid and protein. The growth of *Chlamydomonas* cells in the N-depleted TAP medium was observed in the other row of 4 chambers in the identical microfluidic chip. When compared to those in the N-replete TAP medium, we observed similar green growth of cells up to 1.5 days and maintained the slow growth rate with very low exponential increase (Fig. 3(b)). N-depletion is known to induce lipid accumulation in microalgal cell. The lipid accumulation in the N-depleted cells was visualized by selectively staining intracellular lipid with Nile Red as a fluorescence dye.²² Fig. 3(c) shows a stronger fluorescent intensity from several lipid droplets of significantly larger size (1.6 ± 0.2 (SD) μm , $n=4$) in the N-depleted cells grown for 5 days ($P < 0.01$ in t test), in contrast to the weak fluorescence due to small size of lipid droplets (0.8 ± 0.1 (SD) μm , $n=4$) in the cells grown in complete medium. It is apparent that the promoted accumulation of cellular carbon product without cell division resulted in larger size ($5.9 \pm 0.4 \mu\text{m}$) of the N-depleted cell than the control ($4.7 \pm 0.7 \mu\text{m}$) as observed by the bright field image and the autofluorescence from chlorophyll, which is consistent with the reported work.¹² The demonstration illustrates that the microfluidic device can serve as a very useful platform for developing optimum cultivation conditions. In addition, this microfluidic chip equipped with pneumatic valves facilitates cell loading and releasing manipulation of non-adherent or/and motile cell. Actually, the cells were collected from chambers to stain the lipid with hydrophobic organic dye (Nile Red) by conventional process because strong adsorption of the hydrophobic dye on PDMS surface inhibited cell staining in the chamber as reported.⁷

Holcomb *et al.* shows staining of lipid droplet in microalgal cells with BODIY 493/503 only after removal of PDMS piece from glass bottom.⁷ However, the present microfluidic perfusion chamber conducted harvesting and staining of lipid-containing cells without destruction of the chip. These performances imply that the microfluidic perfusion chamber can be a valuable working module in multiple arrayed format when the chamber is equipped with individual addressing mode.

Gas-permeable PDMS caused a problem of solution evaporation from the space inside the PDMS chip in long term-cultivation of microalgae. This was resolved with maintaining the chip in a humidifying chamber,^{7,9,22} which complicated the system and observation. The perfusion method supplies fresh media and water to a target, making the setting simpler and provides easy accession of microfluidic chip to microscope. The continuous supply of fresh medium

resulted in very high-density growth of cells, similar to feed-batch culture. This would give a clue for real application in the field of biomass.

C. Phototaxis of cell in a microfluidic perfusion chamber

One of the most striking behaviors of *Chlamydomonas* is swimming with a velocity of about 100–200 $\mu\text{m/s}$.²³ It is known that the cell adjusts its swimming path with respect to incident light in a finely tuned manner.^{23,24} The loaded cells, wild strain CC-124, were illuminated with blue LED. The cells started to be biased with 1–2 s of time delay after turning on the light, resulting in a shift of the cells to opposite side of the chamber that was demonstrated in the microfluidic chamber (Fig. 4(a)). The cell avoided strong light, showing negative phototaxis as reported.²⁵ To confirm the role of calcium ion, it was removed from the medium with a chelating agent, 1 mM of ethylene glycol tetra-acetic acid. In the absence of calcium ion, the cell did not respond to the strong light. It is reported that the calcium ion flow into the flagella via voltage-dependent Ca^{2+} channels activated by photoreceptor-induced depolarization of the

FIG. 4. Phototaxis of *C. reinhardtii*, CC-124 cells in a microfluidic perfusion chamber. Cells are loaded in the microfluidic chamber and supplied with TAP or Ca^{2+} -deplete TAP (TAP- Ca^{2+}) through perfusion channel. (a) Micrographs showing negative phototaxis of cells in TAP and no response in TAP- Ca^{2+} media at 0 and 40 s after illumination. The arrows indicate the direction of the blue light inducing phototaxis. (b) Cell distribution as a function of vertical position along the illuminating direction in the chamber. Light was turned on at 2 s. The vertical bars are standard deviations. * means $P < 0.01$, when compared with time 0 s. Shown is the representative data of two independent experiments.

plasma membrane.²⁶ The depletion of calcium ions shuts down the signal, inhibiting the phototaxis. Moreover, the response of phototaxis was quantified with cell distribution as a function of vertical position along the illuminating direction in the chamber, of which the average and standard deviation were determined by ImageJ (Fig. 4(b)). In the absence of directional light, the random and homogeneous cell distribution rendered the center of the microfluidic perfusion chamber as a mean location with large standard deviation. The continuous illumination decreased the deviation along with cell accumulation, showing a sigmoidal curve with $t_{1/2} \sim 9$ s (the time to take for the mean location of cells at the middle of the chamber) and

FIG. 5. Effect of methyl viologen on *Chlamydomonas* cells in a microfluidic perfusion chamber. Cells are incubated in the microfluidic chamber under light and supplied with TAP (control) or methyl viologen-containing TAP (MV) through perfusion channel. (a) Cell survival is determined with SYTOX staining and chlorophyll fluorescence. (b) Representative cells from control and methyl viologen-treated samples at high magnification for chlorophyll and SYTOX fluorescence. (c) Effect of methyl viologen on the chlorophyll fluorescence of cell. The chlorophyll fluorescence of each cell were measured using ImageJ and averaged (*, $P < 0.01$ in t test). Shown is the representative growth curve of two independent experiments.

nearly complete shift of cells within 1 min. The difference in distribution could be detected within 10 s with statistical significance (Fig. 4(b)).

These results demonstrate the utility of the microfluidic perfusion chamber for tracking the phototile behavior of single cell using imaging techniques in a time- and chemical-saving manner. The phototaxis of *Chlamydomonas* and other motile cells in the microfluidic perfusion chamber can be used for screening of drug targeting ion channel or down stream signal transducer and effector. For high throughput screening with tiny amount of chemicals, microfluidics will be the best choice and this perfusion method makes the cells re-usable.

D. Cytotoxicity of cells in a microfluidic perfusion chamber

Discharge of agricultural chemicals into aquatic systems through the surface runoff poses a serious pollution problem.²⁷ We utilize here, the microfluidic system for assaying the extent of contamination by a herbicide. For the purpose, the acute effect of a reactive oxygen-producing herbicide, methyl viologen, on *Chlamydomonas* cells (nonmotile strain CC-400) was studied in the microfluidic perfusion chamber. The cells in one row were subjected to photo-oxidative stress by perfusing with methyl viologen-containing TAP medium under light for 1 h, while the cells in the other row were fed with normal medium as a control. The cells treated with methyl viologen exhibited decreased fluorescence of chlorophyll and they are stained with SYTOX green in the microfluidic perfusion chamber (Fig. 5(a)). It was reported that the reactive oxygen species injured the membrane, rendering the dye accessible to the nuclear DNA and also induced chlorophyll bleaching in microalgae.²⁸ When examined in detail under high magnification (Fig. 5(b)), the control cell was round and donut-shaped with smooth cell surface, exhibiting nearly homogeneous chlorophyll fluorescence with intact cytoplasmic membrane, showing negative staining with SYTOX green. However, the methyl viologen-treated cell is deformed into irregularly wrinkled surface, resulting from the loss of turgor pressure and dark dense aggregates in the cytoplasm. The intensity of chlorophyll fluorescence that is proportional to the amount of chlorophyll was decreased to ~70% of control (Fig. 5(c)).

The microfluidic perfusion chamber needed only ~100 s to exchange the medium on demand in culture chambers, as demonstrated by sequential change of medium from TAP, MV-TAP, and SYTOX for cytotoxic study. Morimoto *et al.* used monodisperse semi-permeable microcapsules to capture *Chlamydomonas* cells.⁶ But they needed flow-focusing device to produce the capsule and microfluidic channel with trapping holes. Ai *et al.* also demonstrated the use of microfluidic chip for monitoring of flagella in living *Chlamydomonas* cells.⁵ But they were not suitable for long term study and less reliable on cell capturing due to a chance of escaping cells from the microwell. Zheng *et al.* suggested an integrated microfluidic device with a polycarbonate membrane whose function is similar to the pillar structure of our microfluidic perfusion chip.²⁹ The membrane is not transparent and hinders optical cell observation. They used the chip for toxicity screening application that requires cell capture, incubation, and staining of cells for viability measurement. On the contrary, the platform presented in this paper is transparent, monolithic, and easy to change perfusion solutions, enabling reliable and convenient observation of cells.

IV. CONCLUSIONS

We have developed a microfluidic perfusion chamber that provides cages for cultivation of free floating *Chlamydomonas* microalgal cells. Such effective medium supplying enabled the developed chip to become a potential platform for studying free-floating *Chlamydomonas* cells at various conditions. The microfluidic chamber allows observation of cell activities on a single cellular or multicellular level with high and low magnification, respectively.

The fabricated system was used to culture non-adherent microalgal cells for 5 days with two types of media to investigate the effect of nutrition on lipid production, phototaxis, and cytotoxicity. The versatility of the microfluidic perfusion chamber would be well suited for microalgal lipid screening with culturing and analysis functions that could be integrated onto a single platform. The multiplex analyses in a high-density microfluidic format would make it possible

to perform high-throughput screening, which is a powerful platform for the study of motile organisms with applications in diverse fields such as biotechnology and pharmaceuticals.

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- ¹Q. Hu, M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, and A. Darzins, *Plant J.* **54**, 621 (2008).
- ²D. Pal, I. Khozin-Goldberg, Z. Cohen, and S. Boussiba, *Appl. Microbiol. Biotechnol.* **90**, 1429 (2011).
- ³G. B. Salieb-Beugelaar, G. Simone, A. Arora, A. Philippi, and A. Manz, *Anal. Chem.* **82**, 4848 (2010); S. Yazdi and A. Ardekani, *Biomicrofluidics* **6**, 044114 (2012).
- ⁴K. Matsumura, T. Yagi, A. Hattori, M. Soloviev, and K. Yasuda, *J. Nanobiotechnol.* **8**, 23 (2010).
- ⁵X. Ai, Q. Liang, M. Luo, K. Zhang, J. Pan, and G. Luo, *Lab Chip* **12**, 4516 (2012).
- ⁶Y. Morimoto, W. Tan, Y. Tsuda, and S. Takeuchi, *Lab Chip* **9**, 2217 (2009).
- ⁷R. E. Holcomb, L. J. Mason, K. F. Reardon, D. M. Crokep, and C. S. Henry, *Anal. Bioanal. Chem.* **400**, 245 (2011).
- ⁸Marcos and R. Stocker, *Limnol. Oceanogr.: Methods* **4**, 392 (2006).
- ⁹S. H. Au, S. C. C. Shih, and A. R. Wheeler, *Biomed. Microdevices* **13**, 41 (2011).
- ¹⁰A. R. Grossman, M. Groft, V. N. Gladyshev, S. S. Merchant, M. C. Posevitz, S. Prochnik, and M. H. Spalding, *Curr. Opin. Plant Biol.* **10**, 190 (2007).
- ¹¹M. A. Unger, H. P. Chou, T. Thorsen, A. Scherer, and S. R. Quake, *Science* **288**, 113 (2000).
- ¹²See supplementary material at <http://dx.doi.org/10.1063/1.4871522> for the three-step photolithographic process to fabricate the fluidic mold; the photomask of valve; the fabricated PDMS-based microfluidic perfusion chip; the operation of flow controller; the perfusion influx of solution into chamber with symmetric and asymmetric channel designs; and the slopes of cell growth in bulk and microfluidic perfusion chamber.
- ¹³V. H. Work, R. Radakovits, R. E. Jinkerson, J. E. Meuser, L. G. Elliot, D. J. Vinyard, L. M. Laurens, G. C. Dismukes, and M. C. Posewitz, *Eukaryot. Cell* **9**, 1251 (2010).
- ¹⁴*The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*, edited by E. H. Harris (Academic Press, 1989).
- ¹⁵M. D. Abramoff, P. J. Magalhaes, and S. J. Ram, *Biophotonics Int.* **11**, 36 (2004).
- ¹⁶T. M. Sobczuk, F. G. Camacho, E. M. Crima, and Y. Chisti, *Bioprocess Biosyst. Eng.* **28**, 243 (2006).
- ¹⁷P. J. Hung, P. J. Lee, P. Sabounchi, R. Lin, and L. P. Lee, *Biotechnol. Bioeng.* **89**, 1 (2005).
- ¹⁸P. J. Lee, P. J. Hung, and L. P. Lee, *Biotechnol. Bioeng.* **97**, 1340 (2007).
- ¹⁹M. Y. Zhang, P. J. Lee, P. J. Hung, T. Johnson, L. P. Lee, and M. R. K. Mofra, *Biomed. Microdevices* **10**, 117 (2008).
- ²⁰T. C. Merkel, V. I. Bondar, K. Nagai, B. D. Freeman, and I. Pinnau, *J. Polym. Sci., Part B: Polym. Phys.* **38**, 415 (2000).
- ²¹J. Pan, A. L. Stephenson, E. Kazamia, W. T. S. Huk, J. S. Dennis, A. G. Smith, and C. Abell, *Integr. Biol.* **3**, 1043 (2011).
- ²²P. Greenspan, *J. Cell Biol.* **100**, 965 (1985).
- ²³J. S. Guasto, K. A. Johnson, and J. P. Gollub, *Phys. Rev. Lett.* **105**, 168102 (2010).
- ²⁴Y. Hanada, K. Sugiyoka, H. Kawano, I. S. Ishikawa, A. Miyawaki, and K. Midorikawa, *Biomed. Microdevices* **10**, 403 (2008).
- ²⁵R. L. Stavis and R. Hirschberg, *J. Cell Biol.* **59**, 367 (1973).
- ²⁶R. Kamiya and G. B. Witman, *J. Cell Biol.* **98**, 97 (1984).
- ²⁷N. Warren, I. J. Allan, J. E. House, and A. Parker, *Appl. Geochem.* **18**, 159 (2003).
- ²⁸R. Prado, C. Rioboo, C. Herrero, and A. Cid, *Aquat. Toxicol.* **102**, 10 (2011).
- ²⁹G. Zheng, Y. Wang, Z. Wang, W. Zhong, H. Wang, and Y. Li, *Mar. Pollut. Bull.* **72**, 231 (2013).