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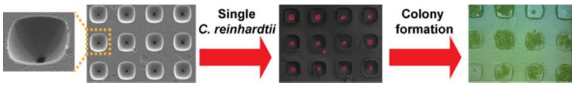

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Single microalgae capture, culture, and colonies retrieval using hemispherical perforated microwell structure

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A hemispherical perforated microwell structure was presented for efficient capture of *Chlamydomonas reinhardtii* (*C. reinhardtii)* at single cell level, cultivation to form colonies, and retrieval of the colonies to serve as a seed for large-scale cultivation. This solution-phase colony formation and recovery could overcome the tedious and time-consuming process of solid-phase agar plate based colony selection. The proposed microdevice was composed of three layers: from top to bottom, a cell solution layer for injection and recovery of a microalgal solution, a hemispherical perforated microwell array, and a solution manipulating layer through the control of hydrodynamic force.

The rapidly moving microalgal cells (wild type and hygromycin B-resistance mutant) loaded in the top layer moved into the microwell holes and captured at the single cell level by applying a withdraw mode in the bottom layer with high efficiency (>90%) within 1 min. Then, subsequently the single cell based cultivation in a hygromycin B containing medium was performed to generate colonies in the hemispherical microwell structure. While the wild type suffered from cell death, hygromycin B-resistance mutant survived well and grew into a colony within 2 days. The produced colonies in the microwells were recovered by applying a release mode in the bottom layer, so that the hydrodynamic force was exerted vertically to push out the colonies to the outlet in 10 sec. The recovered cells were cultured in a large scale medium in a flask. The recovered *C. reinhardtii* was confirmed as a hygromycin B-resistance mutant by identifying the hygromycin gene in the polymerase chain reaction (PCR). The here presented microdevice could perform solution phase based single cell capture, colony formation, and retrieval of colonies for further large scale cultivation, which could replace tedious and time-consuming solid phase agar plate processes with 7-fold time reduction.

Keywords: *Chlamydomonas reinhardtii*, single cell capture, culture, colony, retrieval, hemispherical perforated microwells

Introduction

Alternative energies to compensate for fossil energy has been eagerly dedicated, and among them, bioenergy which uses biological sources such as biomass for producing fuel has been considered promising.[1] *C. reinhardtii*, one of eukaryotic green alga, has been extensively investigated, and is the most widely used strain as a source of bioenergy such as hydrogen and biodiesel. The ideal strain of *C. reinhardtii* should show high photosynthetic efficiency,[2] rapid growth rate, and high lipid content. To obtain such superlative microalgae, many biologists prepared genetically engineered *C. reinhardtii* libraries, and screened them to isolate a promising strain.[3] However, the conventional process for genetic modification including electroporation or a bead beating method suffers from low efficiency,[4] and the strain selection based on the solid phase agar plating needs a prolonged time.[5] In particular, the agar plated colony formation requires more than 2 months depending on the microalgal strain, which could be a bottleneck for development of biomass based energy production. Routine procedure for colony formation and recovery consists of dispersion of cells on an agar plate at single cell level, cultivation to produce a colony, and picking-up a colony for large scale culture in a flask. Since the whole process is carried out on the solid phase agar plate, the diffusion rate of nutrient

and antibiotics to the cells was very limited, resulting in low efficiency for isolating genetically modified *C. reinhardtii*.

Current advancement of microfluidics or lab-on-a-chip technology has demonstrated its excellent performance in chemical and biological analysis with low sample consumption, high speed, automation, and portability.[6-10] A variety of cellular analysis microdevices for studying high-throughput cell culture, single cell analysis, and cell to cell interaction have been presented.[11-18]

To substitute the conventional process of agar plating colony formation and retrieval, the microdevice which can handle single cells should be required. Since the microfluidic device can be fabricated with a similar scale to that of cells, the precise manipulation of cell capture and isolation is possible. One of the widely used microdevices for single cell analysis was a microwell array type. Rettig *et al.* reported a PDMS microwell device for trapping cells with high efficiency by controlling the well diameter.[19] Liu *et al.* fabricated a rounded microwell structure which was imprinted from well-ordered polystyrene beads and enzyme kinetic analysis was carried out at the single cell level.[20] Lee *et al.* developed a microfluidic main channel with small side channels on each side wall, which enables selective cell trapping at lateral microfluidic junctions by fluid suction and they monitored direct cell-cell communication.[21] Although the previous reports demonstrated the high performance for single cell manipulation, the used microdevices were limited in the single cell based culture and recovery.

In this study, we propose a hemispherical perforated microwell structure which has a large hole on the top and a small hole at the bottom. Our unique microwell array design enables us to capture single cells by hydrodynamic force with ease and high efficiency. In addition, the hemispherical shape serves as a culture chamber, so the single cell based colony generation could be conducted. The perforated microwell structure also allowed us to perform the retrieval of the produced colonies by applying a back-pressure from the bottom. Thus, our methodology can replace the whole process of an agar plate in a solution phase. We used two types of *C. reinhardtii* (wild type and hygromycin B-resistance mutant) to demonstrate our rationale of high throughput screening of genetically modified *C. reinhardtii* with high rapidity, accuracy and reliability.

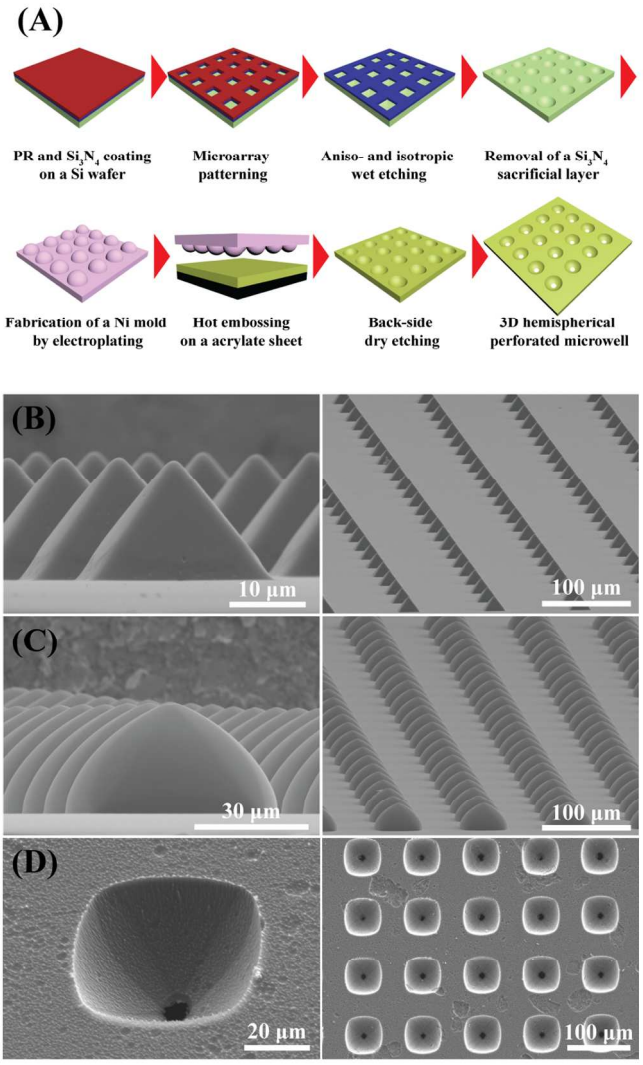

Fig. 1 (A) Fabrication process of hemispherical perforated microwell structure. SEM images of a replicated PDMS from a Si wafer (B) after anisotropic wet etching and (C) aniso- and isotropic wet etching. (D) SEM image of a large-scale hemispherical perforated microwell structure.

Experimental

Chemicals and materials

A positive photoresist (PR) (S1818) and an MF™-CD-26 developer were purchased from Rohm and Haas Electronic Materials Limited Liability Company (USA). A <100> Si wafer was obtained from iTASCO (Korea). An isotropic wet etching solution was prepared from a mixture of hydrofluoric acid (DC chemical, 50%), nitric acid (Junsei, 70%), and acetic acid (SAMCHUN, 99.5%). An aqueous KOH solution (Sigma Aldrich 85%) was used as an anisotropic wet etchant. 3-Aminopropyltriethoxysilane (APTES), 3-glycidoxypropyl trimethoxysilane (GPTMS) and hygromycin B were purchased from Sigma Aldrich (Korea). A PDMS prepolymer and a curing agent (a Sylgard 184 elastomer kit) were purchased from Dow Corning Corporation (USA). An acryl sheet with 30 μm thickness was ordered from Sejin T. S. Co., Ltd. (Korea). Primers were made from Integrated DNA Technologies (USA). Vistex 111-50 was ordered from FSI Coating technologies (USA). *Chlamydomonas reinhardtii* strain CC-125 (*mt+ nit1 nit2*) was obtained from Chlamydomonas Resource Center (University of Minnesota, USA), and an i-genomic BYF DNA extraction mini kit was purchased from iNtRON Biotechnology (Korea). TaKaRa LA Taq with GC buffer was purchased from Takara Korea Biomedical Inc. (Korea). The fluorescence image of *C. reinhardtii* strain was visualized under a confocal laser microscope (Nikon, ECLIPSE, C1si, Japan).

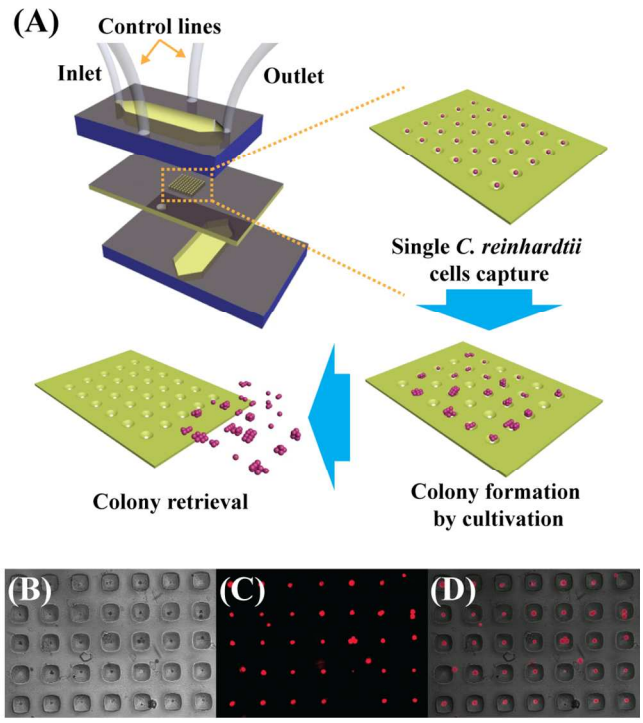

Fig. 2 (A) The entire process for the high-throughput single cell capture, single cell based culture to form colonies, and retrieval of the produced colonies. (B) The bright field, (C) the fluorescence and (D) the merged images showing uniform single *C. reinhardtii* capture in the hemispherical perforated microwell structure.

Fabrication of a hemispherical microstructure

Fig. 1A shows the fabrication scheme for a large-scale single cell manipulating microstructure in an acryl sheet. A Si wafer was coated with a 300 nm-thick Si_3N_4 layer and a positive PR, and then a square dot array with 20 μm dimension was patterned through a conventional photolithography process. The exposed Si_3N_4 layer was removed by reactive ion etching (RIE) with CF_4 plasma, and the background positive PR was cleaned with acetone. An anisotropic wet etching of a Si wafer was performed in a 5 M KOH aqueous solution for 30 min and then, an isotropic etching was followed for 5 min in a HF, HNO_3 and CH_3COOH mixture solution (the volume of HF, HNO_3 and CH_3COOH was 20, 35 and 55 mL) to form concave microwells in a Si wafer. After vigorous washing with distilled water and drying, the remaining Si_3N_4 layer was removed by RIE with CF_4 plasma. The resultant Si wafer which contained concave hemispherical patterns was used as a template for making a hard Ni mold. Through the electroplating process against the Si wafer, the convex micropattened Ni mold was fabricated. To transfer the hemispherical micropattern onto the polymer matrix, the acryl sheet was hot-pressed by a Ni mold with 25 Mpa at 100 °C for 15 min. As a result, the concave micropatterned acrylate sheet was generated. To make the bottom holes on the hemispherical micropattern, the backside of the acryl sheet was etched by RIE in O_2 plasma. The produced acryl sheet retains a large-area hemispherical perforated microwell structure which has a large hole on the top and a small hole at the bottom.

An assembled microdevice for single *C. reinhardtii* cell manipulation

An integrated microdevice, whose size was 3 cm × 2 cm, for manipulating single *C. reinhardtii* cells consisted of three layers as shown in Fig. 2A. The top layer has a PDMS microfluidic channel (width × length × height = 600 × 21000 × 250 μm) in which *C. reinhardtii* cells were injected and recovered. The middle layer was the hemispherical microstructure patterned acryl layer. The bottom one has a PDMS microchannel (width × length × height = 2800 × 9000 × 250 μm), which was connected with control lines to change the flow direction of a cell solution in the top layer. The top and bottom PDMS microchannel was fabricated by a conventional soft lithography procedure and aligned perpendicularly to each other. To bond the acryl sheet with the PDMS layers, each layer was exposed to O_2 plasma for 2 min to generate hydroxyl groups and oxygen radicals on the surface, and then the PDMS and acryl sheet were immediately immersed in a 2% (v/v) APTES solution and a 2% (v/v) GPTMS solution, respectively, at 70 °C for 60 min. This process produced the terminal amino-functional groups on the PDMS surface and the epoxide groups on the acryl surface. After drying with N_2 gas, the functionalized PDMS layers (top and bottom) were permanently bonded with an acryl sheet (middle) at 70 °C for 24 h through the amine-epoxide reaction. Then, the inlet and outlet of the top PDMS layer, and the two control lines of the bottom PDMS layer were connected with syringe pumps.

Transformation of *C. reinhardtii* by electroporation

For nuclear transformation to produce hygromycin B-resistance mutant, the *C. reinhardtii* cells were grown in a Tris Acetate Phosphate (TAP) medium under continuous light (50 μmol photons m^{-2} s^{-1}) at 23 °C in a shaking incubator with an agitation rate of 120 rpm until cell density reached 1×10^6 cells mL^{-1}. Cell wall was removed by treatment with autolysin in a TAP medium. Cells were collected by centrifugation and resuspended in a TAP medium containing 40 mM sucrose to make a final cell density of 1×10^8 cells mL^{-1}. The transforming DNA fragment including hygromycin B-resistance gene *aph7"* was amplified by PCR from the pHyg3 plasmid using HgF (5'-CAAGCTTCTTTCTTGCGCTATGA-3') and HgR (5'-AAGCTTCCATGGGATGACGGG-3') primers under the following conditions: 94 °C for 1 min, 30 cycles of (94 °C for 30 sec, 56 °C for 30 sec, and 68 °C for 2 min), and 68 °C for 5 min.[22] For electroporation, 300 ng of DNA was added to 40 μL of a cell suspension and the mixture was transferred to an electroporation cuvette with 2 mm gap. An electric pulse of 250 V was applied to the sample for 8 ms using an ECM 830 electroporator (Harvard Apparatus). After electroporation, cells were resuspended in 10 mL of a TAP medium supplemented with 40 mM sucrose and incubated

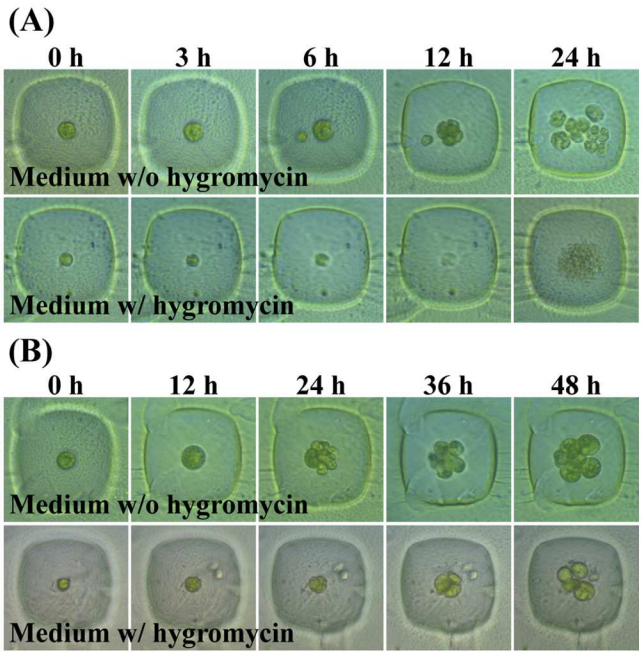

Fig. 3 (A) Time-lapse images of the cultivation using a wild type single *C. reinhardtii* cell without (top panel) and with (bottom panel) hygromycin B. (B) Time-lapse images of the cultivation using a mutant single *C. reinhardtii* cell without (top panel) and with (bottom panel) hygromycin B.

under dim light for 16 h. Transformants were selected on a TAP agar plates containing 15 μg mL^{-1} hygromycin B.

Single *C. reinhardtii* cell capture, culture and retrieval

Prior to injecting the *C. reinhardtii* cells (wild type and hygromycin B-resistance mutant) in the top layer, all the microchannels were filled with a Vistex 111-50 solution and incubated at 70 ^{o}C for 1 h to make the microchannel surface hydrophilic. In order to capture single *C. reinhardtii* cells in the hemispherical perforated microwell array, a withdraw mode was set from the control lines of the bottom PDMS layer with a flow rate of 200~400 μL/h, while the sample fluid in the top PDMS layer was pulled out to the outlet with a flow rate of 100~200 μL/h. The injected cell concentration was ~1000 cells/mL. Hydrodynamic force was exerted from top to bottom in the perforated microwells, so single cells were stuck in each well. Once the single *C. reinhardtii* cells were captured, a TAP medium containing hygromycin B was injected for cultivation. The flow rate of the top and bottom layer was reduced below 50 μL/h and the proliferation from the captured single cell was monitored by a confocal laser microscope. Once the colonies of *C. reinhardtii* were formed in the hemispherical microwells, it was released to the outlet of the top layer by applying an infuse mode from the bottom layer with a flow rate of 10 mL/min.

Gel electrophoresis for identification of hygromycin B-resistance gene

Collected *C. reinhardtii* colonies from the microwells were cultured in a T25 flask for more than 2 weeks to obtain sufficient population of cells. The prepared cells were lysed by an i-genomic BYF DNA extraction mini kit to obtain genomic templates. The PCR cocktail was prepared by using a TaKaRa LA Taq with a GC Buffer, and the primers were designed for amplification of hygromycin B-resistance gene. A 25 μL PCR reaction consisted of 12.5 μL of 2X GC buffer, 4 μL of dNTP mixture, 2.25 μL of deionized distilled water, 1 μL of a template solution (1 ng/mL), 0.25 μL of a TaKaRa LA Taq and 2.5 μL of a forward primer (100 pmol/μL, 5'-ATGACACAA-GAATCCCTGTTACTT-3') and 2.5 μL of a reverse primer (100 pmol/μL, 5'-AGAGGAACTGCGCCAGTTCC-3'). PCR thermal cycling was carried out by an initial activation step at 94 °C for 1 min, 30 amplification cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 120 sec, followed by a final extension step at 72 °C for 5 min. The entire PCR was finished within 2 h. To confirm the PCR products, gel electrophoresis was conducted on a 1% agarose gel in a 0.5X TBE buffer at 135V voltage for 60 min. After separating the PCR products, the gel was incubated with an ethidium bromide solution for staining for 30 min and then the product bands were observed using a UV transilluminator.

Results and discussion

Fig. 1A describes the overall scheme for preparing the hemispherical perforated microwell array. The hemispherical perforated microwell structure with open holes would be adequate not only for stabilizing the cells in position during the bioassay process, but also for manipulating the capture and release of single cells as demonstrated below. To achieve such a unique micropattern array, the concave micropatterned Si wafer was fabricated through the combination of aniso- and isotropic wet etching. As a first step, the Si wafer was anisotropically etched in an aqueous KOH solution to produce concave pyramidal structure, which was confirmed by the replication of PDMS from the etched Si wafer (Fig. 1B). Subsequently, the Si wafer was isotropically etched in a HF, HNO_3 and CH_3COOH mixture with an etching rate of 2~3 μm/min. The concave pyramidal microstructure on the Si wafer was changed to the concave Taj Mahal style as shown in the replicated PDMS (Fig. 1C). A Ni mold was fabricated by an electroplating process against the concave Taj Mahal shaped Si wafer.

The corresponding convex micropattern of a Ni mold could be transferred to the acryl polymer sheet by hot embossing. We chose the acryl sheet as a substrate because of facile bonding with PDMS by surface modification and relatively uniform dry etching rate with O_2 plasma. After hot embossing with the convex Ni mold, the backside of the acrylate sheet was etched out with the RIE in the presence of O_2 plasma. The diameter of the bottom hole could be controlled by etching time, and 5 μm diameter was patterned with 45 min etching time (120W, 100 sccm), whose size is smaller than that of single *C. reinhardtii* cell. Fig. 1D shows an enlarged and top view of the large-scale

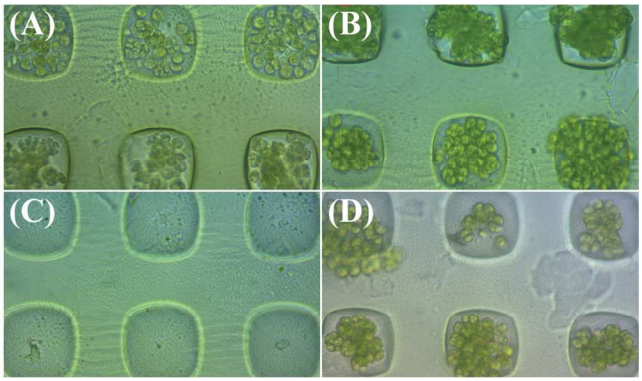

Fig. 4 Colony formation from single *C. reinhardtii* cell in each microwell. (A) Formation of wild type *C. reinhardtii* colonies in 24 h without hygromycin B. (B) Formation of mutant *C. reinhardtii* colonies in 48 h without hygromycin B. (C) Cell death of wild type *C. reinhardtii* with hygromycin B. (D) Formation of mutant *C. reinhardtii* colonies in 48 h with hygromycin B.

hemispherical single cell capture microarrays that retain a large hole on the top and a small one at the bottom. The funnel shape enables the flow control vertically by hydrodynamic force, so that the capture of a single cell per well and the release of the colony could be tuned. In addition, the Taj Mahal shape could be fitted to the cellular morphology, minimizing the physical deformation of the captured cell, and could serve as a culture chamber. While the sole anisotropic etching produced pyramidal micropatterns which are not suitable for securing captured cells, the sole isotropic etching generated perfectly rounded micropatterns which make it difficult to control the size of bottom holes by RIE. On the other hand, Taj Mahal patterns which were generated by combination of aniso- and isotropic wet etching contained a sharp tip at the bottom, so the tuning of a bottom hole to be smaller than a single *C. reinhardtii* cell was feasible.

Fig. 2A describes the entire process for the high-throughput on-chip single cell capture, colony formation, and retrieval. The hemispherical microarray patterned acryl layer was bonded with the top and bottom PDMS layers. In the top PDMS layer, the cell solution was loaded in the inlet and recovered in the outlet. In the bottom PDMS layer, two control lines were connected to the microfluidic channel which function as a withdraw or infuse mode for cell manipulation. The top PDMS microfluidic channel was positioned orthogonal to the bottom PDMS channel. If the flow of the bottom layer was withdrawn by a syringe pump with a flow rate of 200~400 μL/h, the hydrodynamic force was exerted from the top layer to the bottom layer through the hemispherical holes, leading the single cell directed toward the microhole arrays. On contrary to the conventional microwell based cell capture which relies on gravity force for cellular settlement, our methodology forced individual cells to move into each pocket with high speed (within 1 min) and to be fixed in the bottom hole by hydrodynamic force. Without such a driving force, it is almost impossible to localize the rapidly drifting *C. reinhardtii* at the

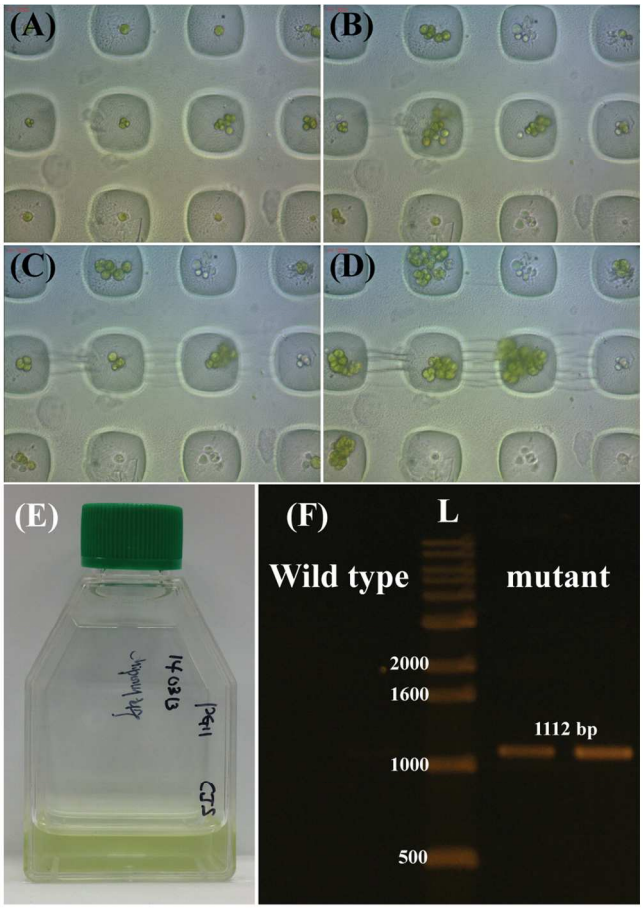

Fig. 5 Cultivation of both the wild type and the mutant from single cells. The mixture of the wild type and the transformed cells was loaded. Hygromycin resistance gene transferred mutant strain survived, while the wild type *C. reinhardtii* suffered from cell death. Cultivation time: (A) 0 h, (B) 12 h, (C) 24 h and (D) 48 h. (E) Digital image of a large scale culture using the recovered colonies as seeds. (F) Identification of hygromycin resistant gene from the recovered colonies by PCR and gel electrophoresis. L is the DNA ladder.

single cell level in a solution phase. Since we controlled the diameter of the top (60 μm) and the bottom hole (5 μm), only single *C. reinhardtii* (10~15 μm diameter) was be captured in each microwell and could not pass through the hole. Fig. 2B-2D shows the images of the captured single *C. reinhardtii* in the 3D hemispherical perforated microwell. The red fluorescence derived from chlorophyll of *C. reinhardtii* was monitored. The single cell capture was achieved with more than 90 % yield, and the cells were uniformly and stably positioned through the hydrodynamic fluidic control from the bottom layer. Thus, this stage would be equivalent to the single cell dispersion on an agar plate. After producing a single cell array, we successively performed the cultivation using two types of *C. reinhardtii* (wild type and hygromycin B-resistance mutant) with and without hygromycin B which is a well-known inhibitor for protein synthesis (Fig. 2A, middle).

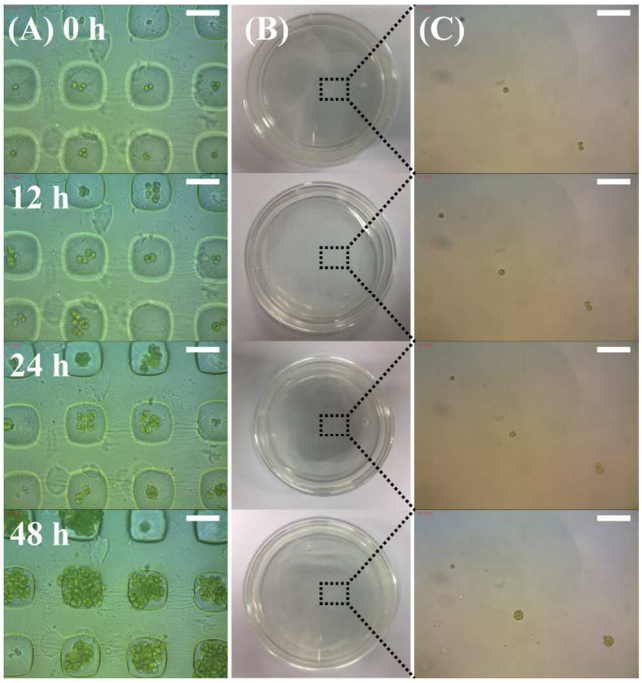

Fig. 6 Comparison of the colony formation time between (A) a hemispherical perforated microwell incorporated microdevice and (B) a conventional agar plating method. (C) Magnified images of (B). Scale bar: 50 μm.

Fig. 3 shows the time-lapse images of the proliferation process starting from single *C. reinhardtii* in the microwell. The wild type *C. reinhardtii* initiated cell division in 12 h in the absence of hygromycin B, and total population rapidly increased from 12 h to 24 h (Fig. 3A, top panel). However, with the hygromycin B containing medium, the wild type cell showed no division and suffered from cellular disruption. All green pigmentation of *C. reinhardtii* cells disappeared after 12 h (Fig. S1). The cell debris was passed through the bottom hole (Fig. 3A, bottom panel). In case of the hygromycin B-resistance mutant *C. reinhardtii*, they proliferated even with the hygromycin B-containing medium (Fig. 3B). Single cell division of the mutant strain started in 24 h without hygromycin B (Fig. 3B, top panel), while 36 h was necessary for cell division with hygromycin B (Fig. 3B, bottom panel). These results showed that the growth rate of the mutant was much slower than that of the wild type, and the antibiotics could affect the growth rate of single mutant strain. Interestingly, as the mutant strain underwent cell division, the cells formed cluster-like morphology rather than complete individuals. Thus, the single hygromycin B-resistance mutant changed to a single colony in 36 h in the presence of hygromycin B. The resultant colony images of the wild type after 24 h and the mutant after 48 h are shown in Fig. 4A and Fig. 4B, respectively. Without hygromycin B, both the wild type and the mutant fully occupied the microwells. If hygromycin B was added, all the wild type single *C. reinhardtii* cells died, but the mutant survived and produced a colony (Fig. 4C and 4D). To mimic the real situations of the conventional agar plating, we loaded the mixture of the wild type and mutant, and cultured them from a single cell array. During cultivation with hygromycin B, the wild type *C. reinhardtii* suffered from cell death within 12 h, but the hygromycin resistant mutant was tolerated and successfully cultured (Fig. 5A-5D). The produced colonies were recovered in 60 sec by applying the infuse mode in the bottom microfluidic channel (Fig. 2A, right). The collected colonies were resuspended in a large scale flask, and the cultivation was scaled up (Fig. 5E). We lysed the cultured *C. reinhardtii* cells, and confirmed the existence of the hygromycin resistance gene (1112 bp) by colony PCR and gel electrophoresis (Fig. 5F). The target gene was clearly verified, suggesting that the collected *C. reinhardtii* was hygromycin B-resistance mutant.

Our unique platform allows us to monitor the whole process of the patterning of single *C. reinhardtii* cells, single cell based cultivation to form a colony, antibiotic effect on the cellular growth rate, and the retrieval of the colonies for further large scale cultivation. Thus, the conventional agar plating based colony formation and recovery could be replaced by the proposed microdevice. The solution phase process in our microdevice would be superior to the solid phase agar plate based method in terms of analysis time and simplicity.

To demonstrate these advantages of our methodology, we compared the time to generate colonies in the proposed microfluidic device with that of the conventional agar plating platform. Fig. 6 shows the time-lapse images of cultivation in a microfluidic device (Fig. 6A) versus an agar plate (Fig. 6B) for 48 h. Initially, the dispersed single cells could be seen in both cases. In case of the microfluidic cultivation, a TAP medium was continuously injected, so *C. reinhardtii* grew much faster than those of the agar plate due to sufficient nutrient supply. The diffusion rate of the nutrient can be estimated by the Stokes-Einstein equation.

$$D = k_B T / 6\pi\eta R$$

where k_B is the Boltzmann's constant, T is the temperature in Kevin, η is the solvent viscosity, and R is the radius of the molecules that diffuse. Since the difference of the viscosity value of the solution phase (a TAP medium) and a solid phase (an agar gel) is huge, the nutrient supply to the cells in the microfluidic device would be more efficient than that of the agar plate. Spatial confinement in the solid agar plate also leads to mitotic delay, resulting in a long cell division duration.[18] After 48 h, most of the single cells were changed to colonies in the microdevice, while single *C. reinhardtii* cells in the agar plate still showed a slow growth rate. The solution phase colony formation and retrieval in the microdevice could be complete in 48 hr, but the solid phase colony formation in an agar plate and manual colony picking took at least 2~3 weeks, demonstrating at least 7-fold time reduction using our proposed microdevice.[5, 23]

Conclusions

We successfully demonstrated the single cell capture, the colony generation, and the retrieval of colonies for large scale cultivation in an integrated microdevice which incorporated a hemispherical perforated microwell structure. By hydrodynamic fluidic control, the rapidly drifting *C. reinhardtii* cells could be uniformly and stably localized in each microwell at the single cell level with high efficiency, and subsequently cultured to form a colony in a short time. In addition, the produced colonies were securely recovered for the downstream large scale culture. As a model, hygromycin B-resistance mutant could be isolated from the wild type through the solution phase colony formation and retrieval process. Compared with the tedious and time-consuming conventional solid phase agar plating method, our methodology provides more rapid, reliable, and simple tools to select the genetically modified *C. reinhardtii* cells. Since it is feasible that the hemispherical microarray format could be expanded in parallel, high throughput screening would be possible regardless of cell types, enabling the screening process for searching for superior microalgae to be accelerated.

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Notes and references

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