



# Micro-compartmentalized cultivation of cyanobacteria for mutant screening using glass slides with highly water-repellent mark



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## ABSTRACT

Photosynthetic microorganisms such as cyanobacteria have attracted attention for their potential to produce biofuels and biochemicals directly from CO<sub>2</sub>. Cell isolation by colony has conventionally been used for selecting target cells. Colony isolation methods require a significant amount of time for cultivation, and the colony-forming ratio is potentially low for cyanobacteria. Here, we overcome such limitations by encapsulating and culturing cells in droplets with an overlay of dodecane using glass slides printed with highly water-repellent mark. In the compartmentalized culture, the oil phase protects the small volume of culture medium from drying and increases the CO<sub>2</sub> supply. Since a difference in cell growth was observed with and without the addition of antibiotics, this compartmentalized culture method could be a powerful tool for mutant selection.

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## 1. Introduction

Photosynthetic microorganisms, including cyanobacteria and microalgae, have attracted a growing interest in biofuel production. These organisms are efficient at converting solar energy and recycling CO<sub>2</sub>, and thus, biofuel production does not compete with agriculture for water, fertilizer, and arable land. Estimates suggest that nearly 50% of the global net primary fixation of carbon by photosynthesis occurs in ocean waters dominated by phytoplankton. For these reasons, there is an increasing interest in utilizing photosynthetic microorganisms to fix CO<sub>2</sub> and produce biofuels. Photosynthesis-driven conversion of carbon dioxide to biofuels and biochemicals using genetically modified cyanobacteria has previously been investigated [1–5]. For example, ethanol, 1-butanol, and isobutyraldehyde (a precursor to isobutyl alcohol) have been produced directly from CO<sub>2</sub> [3–5]. Cyanobacteria are attractive candidates for biofuel production, since genome characterization has facilitated genetic engineering of host cells [6].

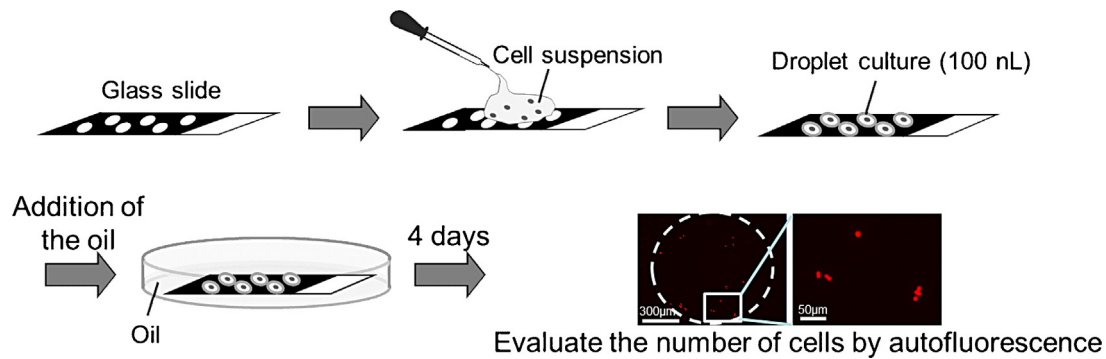
To improve biofuel productivity, it is important to develop an effective screening method for the selection of useful mutants. The general approach for mutant screening involves cell isolation following colony formation in agar nutrient media, followed by the

identification of target mutants by evaluating their activity after culturing in liquid media. For a long time, “toothpicks and logic” were considered sufficient for screening [7]. However, cell isolation on agar plates cannot be carried out efficiently for organisms with low growth rates and/or low colony-forming ratios. In cyanobacteria, the doubling time for *Synechococcus elongatus* PCC7942 is more than 10 h (with 5% CO<sub>2</sub> bubbling), and the number of colonies formed in a solid medium is less than 10% of the number of cells before plating. A significant amount of time is required for culturing single cells into colonies that are large enough to visualize and select from agar plates. This inherently limits the throughput of mutant screening. To address this problem, some have proposed methods for encapsulating single cells in aqueous droplets [8–10] and agarose microparticles [11].

In this study, encapsulation of cyanobacteria in a droplet culture was investigated for cell screening without colony formation on agar plates. Using glass slides printed with highly water-repellent mark, we conducted micro-compartmentalized cultivation from single cyanobacteria cells by covering micro-droplets in an oil phase. This oil phase can protect small volumes of culture medium from drying and increase the transfer of CO<sub>2</sub> from the air to cells, since, it has a higher absorption constant than water. This micro-compartmentalized culture method offers promise for the screening of useful cyanobacteria mutants, such as high growth strains and strains resistant to specific metabolic products, and for single colony isolation for many kinds of microalgae that can fix CO<sub>2</sub>.

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**Fig. 1.** Overview of the droplet culture method.

Droplets were prepared by laying the cell suspension on a glass slide printed with highly water-repellent mark. The volume of one droplet is approximately 100 nL. Cyanobacteria were cultured for four days after the glass slide was covered with oil. After culture, growth was evaluated using cell autofluorescence.

## 2. Materials and methods

### 2.1. Culture conditions

*S. elongatus* PCC7942 was cultured at 30 °C under a light irradiance of 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The strain was grown on BG11 medium (1.5 g/L  $\text{KNO}_3$ , 0.4955 g/L  $(\text{NH}_4)_3\text{SO}_4$ , 0.006 g/L citric acid anhydride, 0.006 g/L ferric citrate, 0.001 g/L  $\text{Na}_2\text{EDTA}$ , 1.03 g/L  $\text{NaCl}$ , 0.039 g/L  $\text{K}_2\text{HPO}_4$ , 0.0739 g/L  $\text{MgSO}_4$ , 0.038 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.020 g/L  $\text{Na}_2\text{CO}_3$ , 1000 $\times$  trace minerals [2.86 g/L  $\text{H}_3\text{BO}_3$ , 1.81 g/L  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.222 g/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.39 g/L  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.079 g/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.0404 g/L  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) [12].

### 2.2. Selection of oil

The stationary phase culture fluid (5 mL) was mixed with 5 mL of oil (dodecane, mineral oil, oleyl alcohol, and oleic acid) in a test tube and cultured under light. After three days, cells were stained with YO-PRO<sup>®</sup>-1 iodide (Abs, 491 nm; Em, 509 nm; Y3603, Invitrogen, Life Technologies, Carlsbad, CA, USA) and the number of live and dead cells were counted by tallying red and green colors, respectively, using fluorescence microscopy (Model IX70, Olympus Co., Ltd., Tokyo, Japan) [13].

To confirm cell growth with overlaid oil, cyanobacteria were cultured with oil in 5%  $\text{CO}_2$  for four days and the growth was monitored by measuring absorbance at 730 nm ( $\text{OD}_{730}$ ) using a digital colorimeter (miniphoto518R, Taitec, Saitama, Japan) and an ultraviolet and visible spectrophotometer (V-630 BIO, JASCO Corporation, Tokyo, Japan).

### 2.3. Micro-compartmentalized cultivation

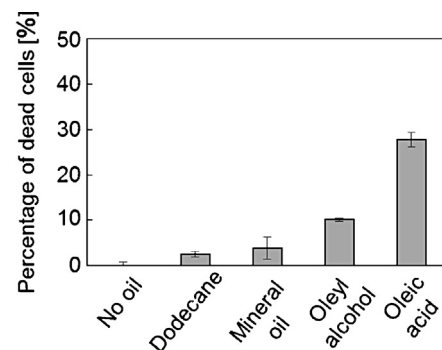
*S. elongatus* was cultured in test tubes under 5%  $\text{CO}_2$  until  $\text{OD}_{730} = 0.8$ . To make the 5%  $\text{CO}_2$  environment, Anaero Pack- $\text{CO}_2$  (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) was used. The culture was diluted in BG11 at 1 cell per 100 nL ( $10^4$  cells/mL). Droplets were prepared by laying 1 mL cell suspension on a glass slide printed with highly water-repellent mark (high-density amino group introduction coat, 570 holes of 1 mm in diameter, 480  $\mu\text{m}$  spaces between holes; Matsunami Glass, Osaka, Japan). Due to the patterning of the hydrophobic area (spacing between holes) and hydrophilic area (holes), droplets were formed. Based on the number of cells in a droplet and the cell concentration of the suspension, the volume of one droplet was approximately 100 nL. After the glass slide was covered with oil, the cells were cultured in micro-compartmentalized droplets for four days (Fig. 1). The oil phase was equilibrated with BG11 medium beforehand by mixing dodecane and BG-11 medium at a ratio of 1:1 by volume, followed

by three periods of centrifugation at 5000  $\times$  g. Cell growth in each micro-compartmentalized droplet was evaluated by detecting cell autofluorescence (chlorophyll a and phycocyanin) using fluorescence microscopy. To detect autofluorescence, an excitation filter (520–550 nm), a dichroic mirror (565 nm) and an emission filter (580 nm) were used. The analysis of acquired images was performed using an EMCCD camera (Luca 658  $\times$  496 pix, Andor Technology Ltd., Belfast, U.K.) and image analysis software (Andor IQ, Andor Technology Ltd.). The fluorescence images were taken under the condition that no signal was detected in a droplet lacking cells. We assessed the red points, which were supposed to indicate cells in the fluorescence images. After that, the cells in phase difference images were counted. The specific growth rate of droplet cultures was compared with that of normal liquid cultures without dodecane in 18 mm test tubes.

## 3. Results and discussion

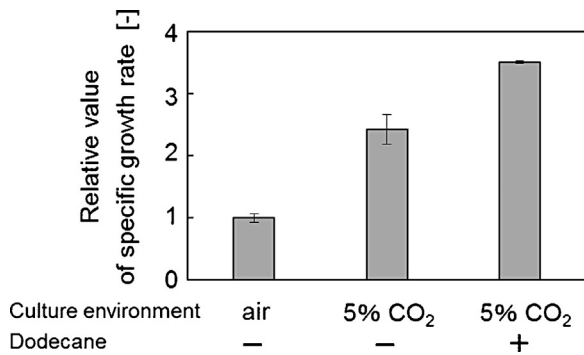
### 3.1. Selection of oil

For the selection of an oil phase for micro-compartmentalized cultivation, *S. elongatus* in stationary phase were incubated for three days with an overlay of oil. The cell death rate of *S. elongatus* increased consecutively according to treatments with dodecane, mineral oil, oleyl alcohol, and oleic acid; in the case of oleic acid, dead cells comprised 28% of the sample (Fig. 2). Little toxicity was observed with an overlay of dodecane, with only 2% dead cells. Although both dodecane and mineral oil had low toxicity,



**Fig. 2.** The percentage of dead cells in the presence of oil.

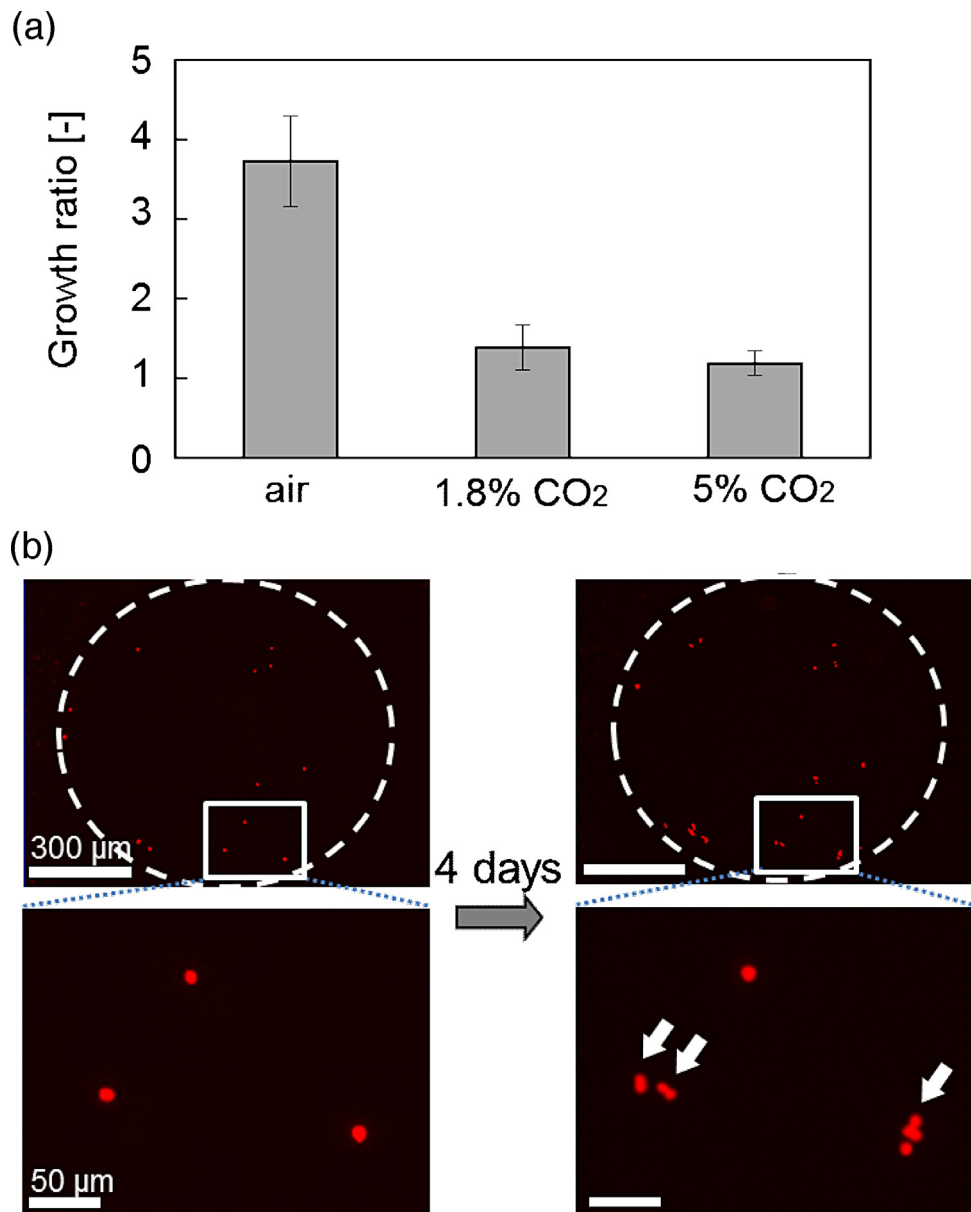
Cyanobacteria in the stationary phase were incubated with an overlay of dodecane, mineral oil, oleyl alcohol, or oleic acid in the presence of light (50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). After three days, cells were stained with YO-PRO and the numbers of live and dead cells were estimated by tallying counts of red and green cells, respectively, using fluorescence microscopy.



**Fig. 3.** The effect of 5% CO<sub>2</sub> supply and a dodecane layer on growth in test tubes. *S. elongatus* was cultured by exposing the test tube to air or 5% CO<sub>2</sub>. Furthermore, the strain was cultured with dodecane overlaid in 5% CO<sub>2</sub>. The y-axis shows the relative value of the specific growth rate. Each value was normalized by the specific growth rate without dodecane in air.

dodecane has high CO<sub>2</sub> absorption. The abilities of dodecane and mineral oil to absorb CO<sub>2</sub> are approximately 1.7 and 1.1 times higher than that of water, respectively [14,15]. Dodecane, the oil with the lowest recorded cytotoxicity and high CO<sub>2</sub> absorption, was subsequently used for micro-compartmentalized culture.

To examine the influence of dodecane on cell growth in test tubes, *S. elongatus* was cultured with overlaid dodecane supplied with 5% CO<sub>2</sub>. When *S. elongatus* was cultivated under 5% CO<sub>2</sub>, the specific growth rate increased 2.4-fold compared to that in normal air conditions. The specific growth rate increased a further 3.5-fold when cultivated under 5% CO<sub>2</sub> with an overlay of dodecane (Fig. 3). We assume that the CO<sub>2</sub> supply into the culture medium was enhanced in conditions with an overlay of dodecane. Consequently, an increase in cell growth was observed in cultures grown under 5% CO<sub>2</sub> with overlaid dodecane.

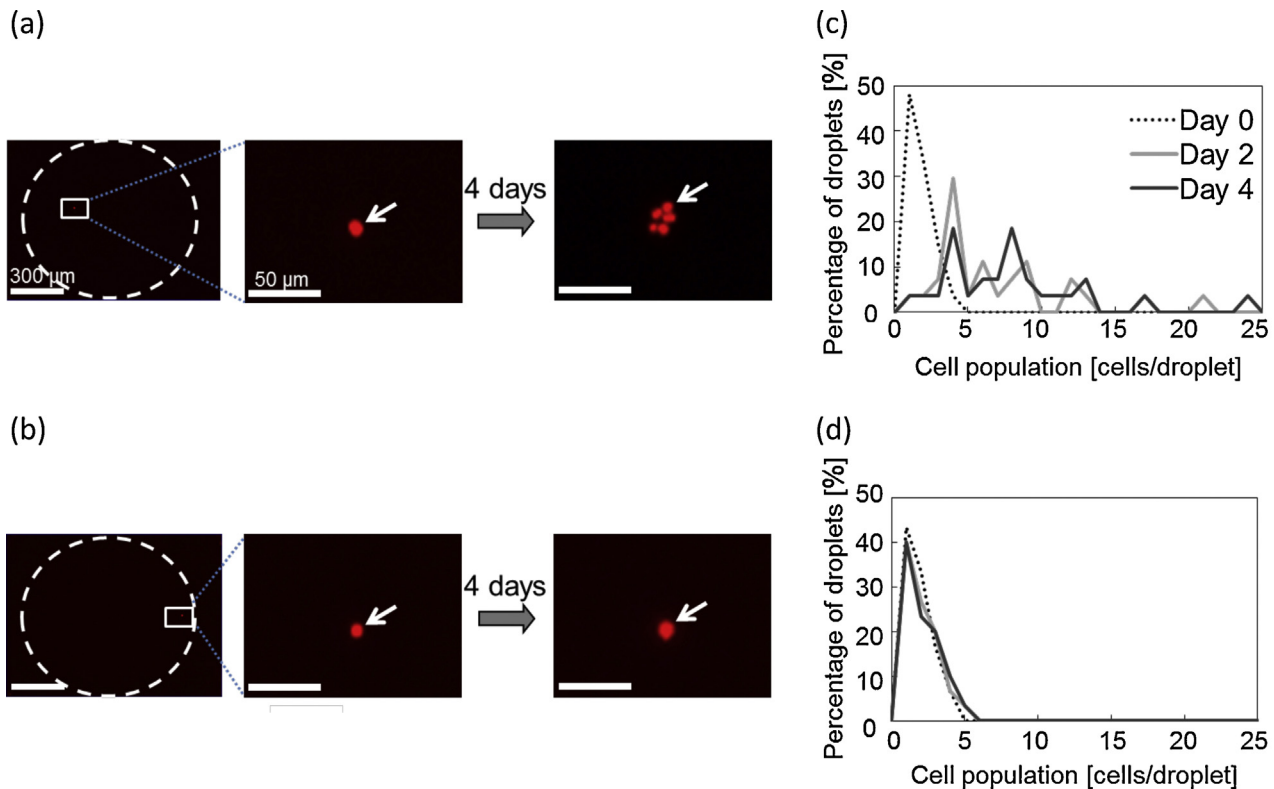


**Fig. 4.** The growth of *S. elongatus* at different CO<sub>2</sub> conditions in droplet culture with an overlay of dodecane.

(a) The growth ratio of droplet cultures in the air, 1.8% CO<sub>2</sub> and 5% CO<sub>2</sub>.

Growth ratios were calculated by dividing number of cells at day 4 by the number of cells at day 0. Approximately 15 cells were in each droplet at the beginning of the experiment.

(b) Fluorescence images of droplet cultures in the air.



**Fig. 5.** The growth of *S. elongatus* in the presence or absence of chloramphenicol.

(a) Fluorescence images of droplet cultures using normal BG11.

(b) Fluorescence images of droplet cultures using BG11 with 15 µg/mL chloramphenicol.

(c) Changes in the cell populations of each droplet cultured using normal BG11.

The cells in each of 27 droplets were counted.

(d) Changes in the cell populations of each droplet cultured using BG11 with 15 µg/mL chloramphenicol. The cells in each of 30 droplets were counted.

### 3.2. Micro-compartmentalized droplet cultivation

Droplet cultures of *S. elongatus* were investigated using glass slides printed with highly water-repellent marks measuring 1 mm in diameter. To examine the CO<sub>2</sub> concentration of dodecane-overlaid cultures, approximately 15 cells/droplet of *S. elongatus* were introduced in air (0.04% CO<sub>2</sub>), 1.8% CO<sub>2</sub>, or 5% CO<sub>2</sub> conditions. Although little increase in cell growth was observed under the 1.8 and 5% CO<sub>2</sub> conditions, cell growth was confirmed when cultured in air (Fig. 4a). Cell growth could be observed using fluorescence microscopy. Holes containing divided cells were detected as an enhanced fluorescence signal (Fig. 4b). Cell growth increased under 5% CO<sub>2</sub> in test tube cultures. The difference in suitable CO<sub>2</sub> conditions for cultures might be associated with differences in the specific surface area (the ratio of the interfacial area with dodecane to the volume of medium) in the droplet culture and test tube culture. An arrest of cell growth in the droplet culture whose specific surface area was large was considered to be due to a decrease in the pH of the medium following excessive adsorption of CO<sub>2</sub>. When phenol red was added to droplets with an overlay of dodecane, the color of the medium changed from red to yellow (indicating a decrease in the pH below 6.8) in 5% CO<sub>2</sub> conditions. We observed that cell growth in droplet culture with overlaid dodecane did not require CO<sub>2</sub> enrichment in the gas phase.

When *S. elongatus* was cultured in air, the specific growth rate of droplet cultures (0.336 day<sup>-1</sup>) was approximately 1.4 times higher than that of normal liquid cultures without dodecane in 18 mm test tubes (0.240 day<sup>-1</sup>). In other words, the doubling time of droplet cultures and test tube cultures was 50 and 69 h, respectively,

without shaking under air conditions. Another conventional method is the use of a solid culture with agar medium, which takes 2 weeks to form colonies. In contrast, the droplet culture requires less than 1 week because temporal observations are possible for evaluating cell growth. In addition to growth improvement, the number of colonies formed in droplet culture was approximately 70% whereas that in solid culture was less than 10% of the number of cells before culture. Therefore, we concluded that micro-compartmentalized droplet cultivation of *S. elongatus* was successfully conducted using dodecane as the organic solvent phase.

### 3.3. Single-cell culture of *S. elongatus* with addition of antibiotics

Cell growth was evaluated for cyanobacteria cultured under conditions of 1 cell/droplet using the droplet culture method. *S. elongatus* was cultured in the presence or absence of chloramphenicol. A concentration of 15 µg/mL chloramphenicol was used; this concentration is sufficient for arresting cell growth in test tube cultures. Fig. 5 shows the population of compartmentalized cells within each droplet. Approximately 30% of droplets contained single cells. The percentage of droplets containing zero, two, or three cells was 8, 23, and 18%, respectively. After culturing droplets for two and four days, cell growth was evaluated using fluorescence microscopy. In cultures without chloramphenicol, we could confirm growth from single cells. We observed changes in the cell population for each droplet. After two days of culturing, 48% of droplets contained five or more cells. After four days of culturing, this number further increased and approximately 72% of droplets

contained more than five cells. On the other hand, little growth was observed for cultures grown with chloramphenicol. Following the addition of antibiotics, changes in the cell population for each droplet indicated that the droplet cultivation method could be applied to mutant screening after transformation. Furthermore, daughter cells were observed to divide near parent cells (Figs. 4b and 5a,b). Therefore, even if all droplets did not contain single cell, cell growth could be continuously observed under the microscope

#### 4. Conclusions

In this study, droplet cultures were constructed using dodecane as an oil phase with little observed cytotoxicity. The oil phase resulted in an increased CO<sub>2</sub> supply to the droplet medium, and specific growth rates were higher compared to those observed for liquid cultures grown under normal air conditions. We anticipate that droplet culture can be applied to high-throughput screening for the acquisition of useful mutants, such as high-growth strains and strains resistant to specific metabolic products. In addition to these applications, we hope this method can be applied to single colony isolation for other microalgae that are able to fix CO<sub>2</sub> and are difficult to grow on agar plates due to drying.

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