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## TECHNICAL INNOVATION

## Quantitative tracking of the growth of individual algal cells in microdroplet compartments†

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In this paper we introduce a simple droplet-based microfluidic system consisting of two separate devices to encapsulate and culture microalgae, in contrast to cultivation in bulk liquid medium. This microdroplet technology has been used to monitor the growth of individual microalgal cells in a constant environment for extended periods of time. Single cells from three species of green microalgae, (two freshwater species *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, and one saline species *Dunaliella tertiolecta*), were encapsulated and incubated in microdroplet compartments of diameter of  $\sim 80\ \mu\text{m}$ , and their growth analysed over 10 days. In all cases, the doubling time of microalgae grown in microdroplets was similar to growth in bulk. The growth of *C. reinhardtii* in microdroplets of varying diameters and with different initial cell numbers per droplet was investigated, as well as the effect of varying medium conditions such as pH and nitrogen concentration. This methodology offers the opportunity to study characteristics over time of individual cells and colonies, as well as to screen large numbers of them.

## Introduction

Microalgae are simple photosynthetic eukaryotes which are responsible for approximately half of Earth's fixation of atmospheric carbon.<sup>1</sup> At present, microalgae are mainly grown for the production of high-value products, e.g.  $\beta$ -carotene from *Dunaliella salina*.<sup>2,3</sup> However, when grown

under certain specific conditions, such as nutrient limitation, some species can also produce lipids, which could be used as a feedstock for the production of biodiesel.<sup>4</sup> Biofuels, such as biodiesel from microalgae, have the potential to provide a low-carbon alternative to fossil-derived transport fuel, because growth of the feedstock uses photosynthesis to fix atmospheric carbon dioxide, which is then released on combustion. As a result, significant attention has recently been paid to maximising the production of lipids and other high-value chemicals from microalgae by optimising conditions of growth,<sup>4–7</sup> determining the most appropriate algal species<sup>8,9</sup> and developing photobioreactors. For this to be effective, we need to have a robust understanding of factors that limit algal growth both for an individual cell, and at scale. Much of our prior understanding comes from environmental studies that consider the growth of microalgae in natural ecosystems, in an effort to understand the drivers of eutrophication. Those studies have extensively assessed the roles of various nutrients,

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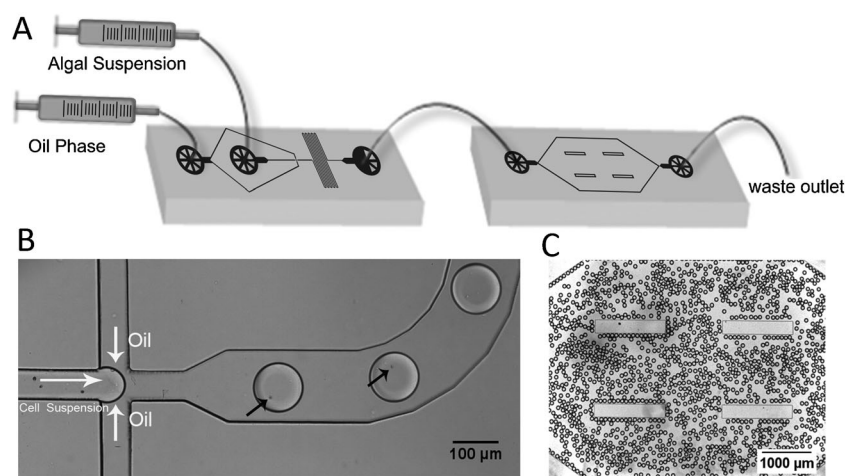
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## Insight, innovation, integration

Here, we introduce a simple droplet-based microfluidic system consisting of two separate devices to encapsulate and culture microalgae, in contrast to cultivation in bulk liquid medium. This system is used to monitor the dividing and growth of individual microalgal cells in a constant environment for many days. The methodology

enables the study of individual algal cells, rather than as populations, and can thus further our understanding of the behaviour of algae as living organisms. The approach holds the potential to build a high-throughput platform for screening both for algal mutants, as well as oil-rich algae.



**Fig. 1** Encapsulation of algal cells in microdroplets and their incubation in a separated reservoir. (A) Schematic diagram of droplet generation and incubation. Microdroplets containing algal cells were generated in the left flow-focusing microfluidic device, then transferred *via* PE tubing, and incubated in the reservoir (right) under continuous illumination of  $55 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . (B) A bright-field microscopy image showing algal cells (highlighted by black arrows) being encapsulated in microdroplets. The oil flow and cell suspension flow are illustrated by white arrows. (C) A bright-field microscopy image showing microdroplets containing algal cells stored in the reservoir. FC-40 with 2% Rainsance EA surfactant was used as the oil phase.

such as nitrate and phosphate, on stimulating algal growth. For example, Yun *et al.* (2003)<sup>10</sup> constructed a photosynthetic model for *Chlorella* that was based on the assumption that growth is limited by light. All cells were approximated to the “average cell” in the culture and growth was considered to be uniform across all cells. However, the model is reductionist, and assumes it is possible to scale up from one cell to a culture, without taking into account the likely heterogeneity of algal cultures. The problem with studies in bulk is that all data are averaged across the culture, so assessment of this heterogeneity is not possible. The ability to cultivate and monitor the growth of single cells in individual environments would be highly beneficial. Moreover, studies of single algal cells potentially facilitate the study of cell-to-cell interactions, the separation of mutants within a species, or of different species in mixed populations, and the stochastic behaviour of individual cells.

Recently, microfluidic devices have been introduced for the study of single cells (*e.g.* mammalian cells,<sup>11</sup> stem cells,<sup>12</sup> yeast cells<sup>13</sup> and bacteria<sup>14</sup>) in femtolitre to nanolitre aqueous droplets, suspended in oil (*e.g.* mineral or fluorinated oil). Different methods of trapping droplets in devices have been developed in order to monitor the growth of cells over time.<sup>13,15,16</sup> Such devices are generally made from poly-(dimethylsiloxane) (PDMS) or glass, with PDMS being advantageous as it is permeable to gases such as  $\text{O}_2$  and  $\text{CO}_2$ .<sup>17</sup> Using microfluidic devices, the chemical environment of the cell culture can be manipulated in ways not possible with conventional microwells.<sup>12</sup> Furthermore, high throughput screening of microdroplets at frequencies in excess of 1 kHz is possible using such systems.<sup>18–21</sup> These properties suggest that microfluidic technology could be a valuable tool for the study of microalgae.

In this paper, we present a simple microfluidic system consisting of two separate devices to generate droplets containing one or more algal cells, and incubate them for up to 10 days.

To show the general utility of the approach, single cells from three species of green microalgae (two freshwater species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, and one saline species, *Dunaliella tertiolecta*) were encapsulated and incubated in the devices. By varying the diameter of the droplets and the initial number of cells per droplet, different growth conditions of *C. reinhardtii* cells were analysed and compared.

## Results

### The encapsulation of algal cells in microdroplets and long-term stability of the microdroplets

In this paper, three quite different species of green microalgae were investigated: *C. reinhardtii*, a flagellated freshwater species of diameter  $\sim 10 \mu\text{m}$ , which is often used as a model species in biological experiments; *C. vulgaris*, a non-motile freshwater species with a smaller diameter ( $\sim 2 \mu\text{m}$ ); and *D. tertiolecta*,  $\sim 10$ – $12 \mu\text{m}$  in diameter, a saline species, which is able to grow in conditions of varying pH and salinity. Cells were initially grown in bulk using conditions recommended by the CCAP (Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, UK). Once the cells reached stationary phase, they were diluted with fresh medium to a concentration of  $\sim 3.5 \times 10^6 \text{ cells ml}^{-1}$ , before being introduced into the microdroplet device that generated the suspension of droplets in oil. This concentration of cells was used to ensure a large number of the generated droplets initially contained a single algal cell. Continuous illumination of  $55 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  was used.

The microdroplet system used was modular, consisting of two separate devices, to generate and incubate droplets respectively (Fig. 1A). Droplets were generated in the first device, shown on the left in Fig. 1A, using flow-focusing geometry,<sup>22</sup> where a stream of the algal cell suspension meets

two streams of fluorinated oil at the 'generating nozzle' (Fig. 1B; Supplementary Movie S1). The diameter of the droplets and frequency of generation were controlled by the diameter of the generating nozzle, as well as the ratio of the flow rate of the oil phase ( $F_{\text{oil}}$ ) to that of the algal cell suspension ( $F_{\text{aqu}}$ ). Relatively high flow rates were used ( $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 200 \mu\text{l h}^{-1}$ ) to prevent the suspension of algal cells settling in the inlet channel. All the algal cells going into the inlet stream were encapsulated into separate droplets. Using these flow rates and a generating nozzle of dimensions  $50 \mu\text{m} \times 75 \mu\text{m}$  (width  $\times$  depth), monodisperse droplets of diameter  $\sim 80 \mu\text{m}$  (volume  $\sim 268 \text{ pl}$ ) were formed. The microdroplets were stable and did not fuse with each other whilst being transferred from the droplet-generation device (Supplementary Movie S2) into the reservoir device. The reservoir could store approximately 1500 droplets (diameter  $80 \mu\text{m}$ ) (Fig. 1C), allowing many experiments to be carried out in parallel. The modular nature of the microdroplet system makes it possible to add features for increasingly complex operations, e.g. to sort cells after incubation.

The shrinkage of droplets due to the diffusion of water into the PDMS matrix<sup>16,23</sup> has previously limited the time microdroplets can be stored. To avoid this problem, the PDMS matrix was maintained in a saturated environment by storing the reservoir in a Petri dish containing de-ionised water to balance the diffusion of water from the droplets into the matrix (Supplementary Fig. S1). Droplets stored in this reservoir were stable for at least 20 days (Supplementary Fig. S2). The ability to maintain droplets for this time allowed the observation of cells over the life span of the different microalgal species.

### Culturing *Chlamydomonas reinhardtii* in microdroplets

Fig. 2A shows the distribution of the number of *C. reinhardtii* cells encapsulated per droplet, when the initial cell density was  $\sim 3.5 \times 10^6 \text{ cells/ml}$ . As expected, this follows a Poisson distribution, as has been reported previously in the literature for both *E. Coli*<sup>11</sup> and mammalian cells.<sup>14</sup> Fig. 2C (i–v) shows how the cells in the microdroplets (diameter  $80 \mu\text{m}$ , volume  $268 \text{ pl}$ ) divided over 6 days after encapsulation in droplets. After 2 days the mother cell had divided into a number of daughter cells, and this division process continued until the cells reached stationary phase,  $\sim 4$  days after inoculation. This can also be seen in Fig. 2B, which demonstrates that the cells were in the log-growth phase between 1 and 4 days after encapsulation, and reached a maximum cell concentration of  $\sim 25$  cells per droplet after 5 days (equivalent to a concentration of  $9.3 \times 10^7 \text{ cells ml}^{-1}$  in a droplet of volume  $268 \text{ pl}$ ). The results in Fig. 2B were obtained by direct observation of the cells in the microdroplets under the bright-field microscope. Fig. 2D(i–ii) are higher magnification images of the cells encapsulated in microdroplets. These images clearly show the division process, where the mother cell splits into 2–8 daughter cells confined within the cell wall, before the newly-formed cells separate from each other. The algal cells exhibited a growth cycle typical of *C. reinhardtii*, and the motility of the cells did not appear to be affected by encapsulation in the microdroplets (Supplementary Movie S3). During the whole

experiment, no significant effect due to the neighbouring droplets (empty or full) was observed.

### Culturing *Chlorella vulgaris* cells in microdroplets

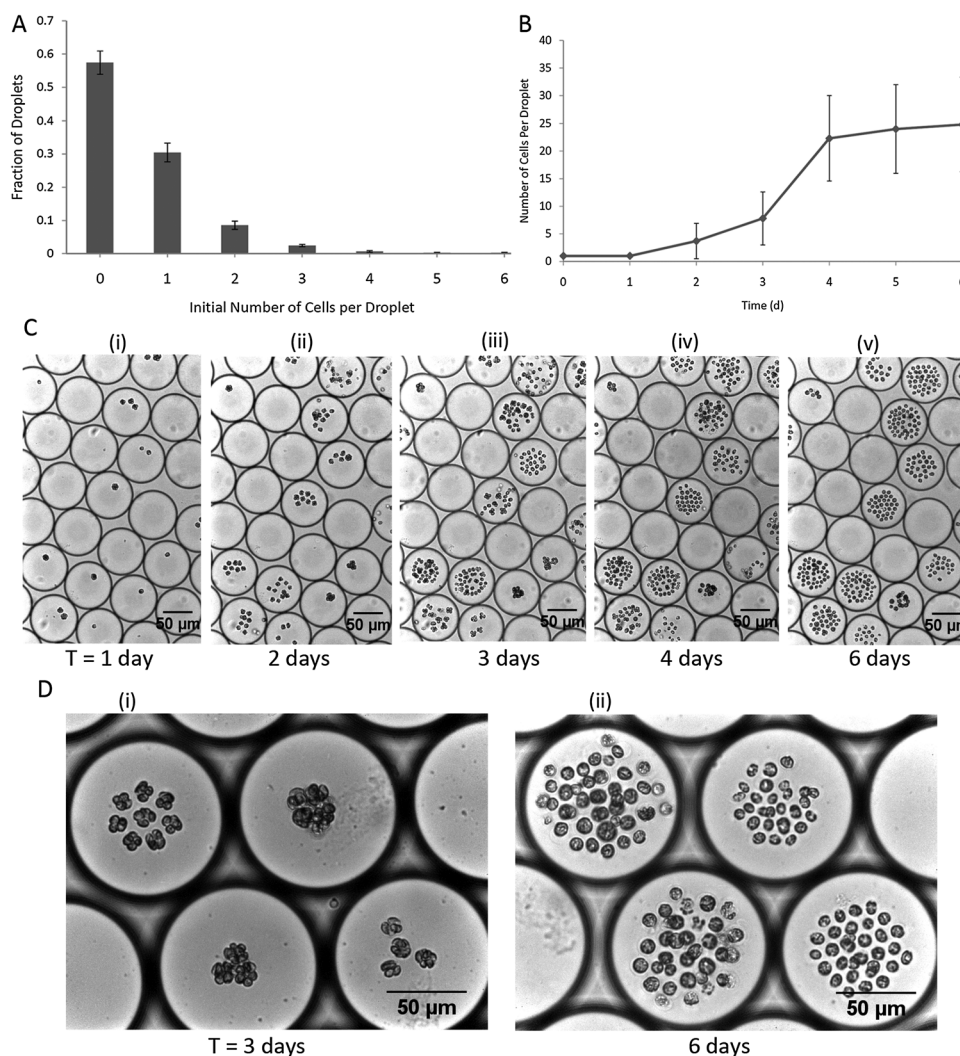
As previously mentioned, *C. vulgaris* cells are  $\sim 2 \mu\text{m}$  in diameter, much smaller than *C. reinhardtii* cells, and unlike *C. reinhardtii*, are non-motile. Fig. 3A shows the distribution of the numbers of cells of *C. vulgaris* encapsulated per droplet, when the initial cell density was  $\sim 3.5 \times 10^6 \text{ cells/ml}$ . By comparing Fig. 2A and 3A, it can be seen that when *C. vulgaris* cells were encapsulated, there was a greater proportion of microdroplets containing more than 2 cells than was the case with *C. reinhardtii*. This is a consequence of both the smaller size of the *C. vulgaris* cells and their lack of motility, making them more prone to aggregate. Another consequence of the size of the cells was that they were hard to distinguish from water droplets generated by condensation using bright field imaging (Fig. 3B). Accordingly, the cells were imaged by fluorescence microscopy to detect chlorophyll fluorescence (excitation filter 460–500 nm, emission filter  $> 600 \text{ nm}$ ) (Fig. 3C). Nonetheless, the growth of *C. vulgaris* in microdroplets followed a similar profile to that of *C. reinhardtii* (Fig. 3D). In microdroplets (diameter  $\sim 80 \mu\text{m}$ , volume  $\sim 268 \text{ pl}$ ) *C. vulgaris* entered log phase after  $\sim 2$  days and reaching a final concentration of  $\sim 120$  cells per droplet ( $\sim 4.5 \times 10^8 \text{ cells ml}^{-1}$ ) after 8 days.

The ability to look at very small cultures derived from a single algal cell has the potential to provide new insight into the effect of external factors that are known to affect bulk cultures. For example, the effect of altering the concentration of nitrogen (in the form of nitrate) in the medium has been investigated extensively.<sup>4,6,24</sup> Nitrogen depletion is a common procedure to increase lipid content of cells, but can have a detrimental effect on growth of cultures. A corresponding observation was made in microdroplets. The growth curves of *C. vulgaris* grown under different initial conditions of nitrate are shown in Fig. 3D. It can be seen that the growth of *C. vulgaris* in medium where the concentrations of nitrate are 8.8 mM and 14.0 mM show a similar profile, with cells entering log phase after  $\sim 2$  days and reaching a final concentration of  $\sim 120$  cells per droplet ( $\sim 4.5 \times 10^8 \text{ cells ml}^{-1}$ ) after 8 days. However, algal cells grown under nitrogen limitation (initial nitrate concentration of 1.8 mM) displayed a quite different growth curve. In this case, the log phase of growth was shortened and a maximum concentration of cells of only 60 per droplet ( $\sim 2.25 \times 10^8 \text{ cells ml}^{-1}$ ) was reached after 5 days. This suggests that these cells exhausted the available nitrogen earlier, and therefore were unable to divide further.

### Culturing *Dunaliella tertiolecta* cells in microdroplets

*D. tertiolecta* is a motile and unicellular saline species with the cell size of 10–12  $\mu\text{m}$ . The intracellular composition of *D. tertiolecta* is highly dependent on the salinity of its environment. For example, Takagi *et al.* (2006)<sup>5</sup> reported that the cultivation of *Dunaliella* cells with high initial concentrations of NaCl of  $\sim 1.0 \text{ M}$ , and the addition of NaCl during culture increased significantly the total lipid content to  $\sim 70 \text{ wt\%}$  of the dry cell mass. The intracellular





**Fig. 2** Culturing *C. reinhardtii* cells in microdroplets (diameter  $\sim 80\ \mu\text{m}$ , volume  $\sim 268\ \text{pl}$ ) at  $25 \pm 2\ ^\circ\text{C}$  under continuous illumination of  $55\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ . (A) Distribution of *C. reinhardtii* cells in droplets when  $F_{\text{oil}} = 1000\ \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 200\ \mu\text{l h}^{-1}$ . (B) Growth curve of *C. reinhardtii* cells in microdroplets. (C) i–v Bright-field images showing the increase in the number of cells per droplet, over the 6 days after inoculation. The same microdroplets are shown in each image. (D) i–ii *C. reinhardtii* cells dividing in microdroplets. Again, the same microdroplets are shown in both images.

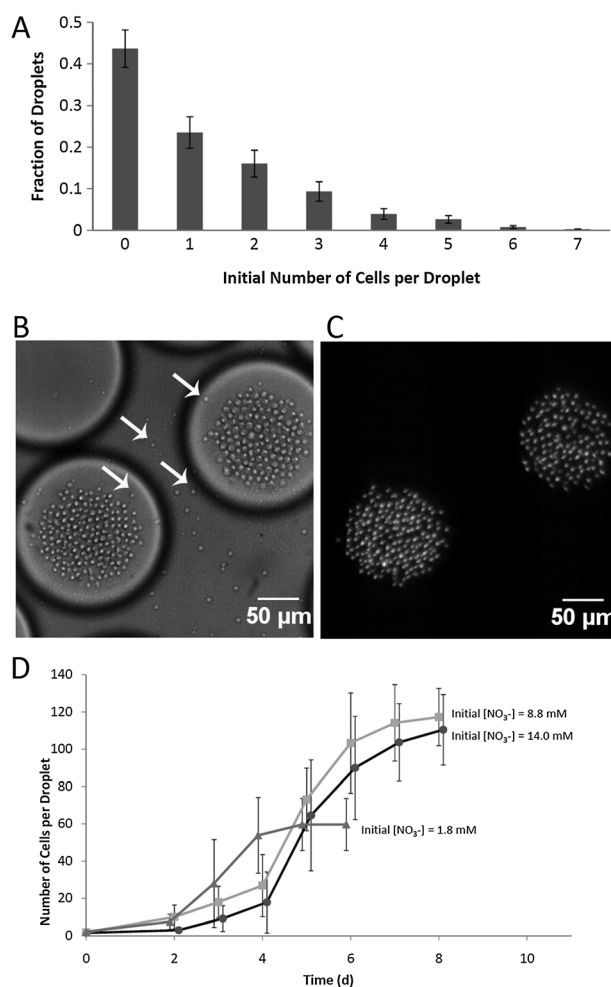
concentration of glycerol has also been reported to be dependent on the extracellular salt concentration in the medium.<sup>25</sup> This species therefore offers an opportunity to test whether the microdroplet system could be used to investigate altered growth conditions. Algal growth in medium with initial concentrations of NaCl of 0.5 M, 1.5 M and 4.0 M were defined as growth under low, recommended (standard medium) and high salinity, respectively. Owing to their similar size and motility, the distribution of the number of *D. tertiolecta* cells encapsulated per droplet, from an initial cell density of  $\sim 3.5 \times 10^6\ \text{cells ml}^{-1}$ , was observed to be similar to that with cells of *C. reinhardtii* (Supplementary Fig. S3). The growth curves are shown in Fig. 4A. It can be seen that the cells grew fastest and reached the highest final cell concentration of  $\sim 40$  cells per droplet ( $1.5 \times 10^8\ \text{cells ml}^{-1}$ ) when grown at the recommended salinity (Fig. 4B).

The ability of *D. tertiolecta* to grow in microdroplets at different pH values between pH 6.1 and 9.1 was also

investigated (Fig. 4C). At pH values 7.5 and 8.4, similar growth curves were observed, where the cells entered stationary phase after about 9 days and reached a final concentration of cells of  $\sim 40$  cells per droplet ( $\sim 1.5 \times 10^8\ \text{cells ml}^{-1}$ ). At lower and higher pH values the final concentration of cells only reached  $\sim 28$  cells per droplet ( $\sim 1.0 \times 10^8\ \text{cells ml}^{-1}$ ). These results support the conclusion that *D. tertiolecta* prefers to grow under neutral or slightly basic environments, and their growth is inhibited under more acidic or basic conditions.

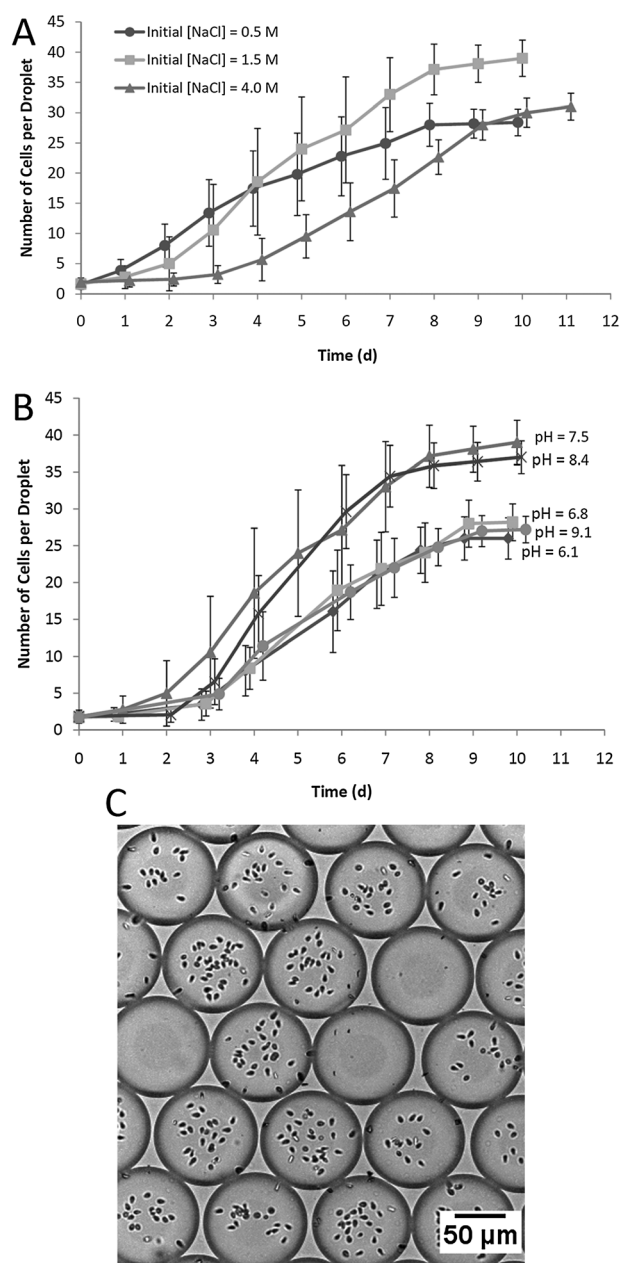
#### More detailed analysis of algal growth in microdroplets

In order to investigate further the growth of algal cells in microdroplets, the effect of the initial number of cells per droplet and the volume of the droplets on cell growth was investigated using *C. reinhardtii* as the test species. Fig. 5 shows the growth curves of cells in droplets of diameter  $80\ \mu\text{m}$  (268 pl) (A) or  $130\ \mu\text{m}$  (1150 pl) (B), when the initial



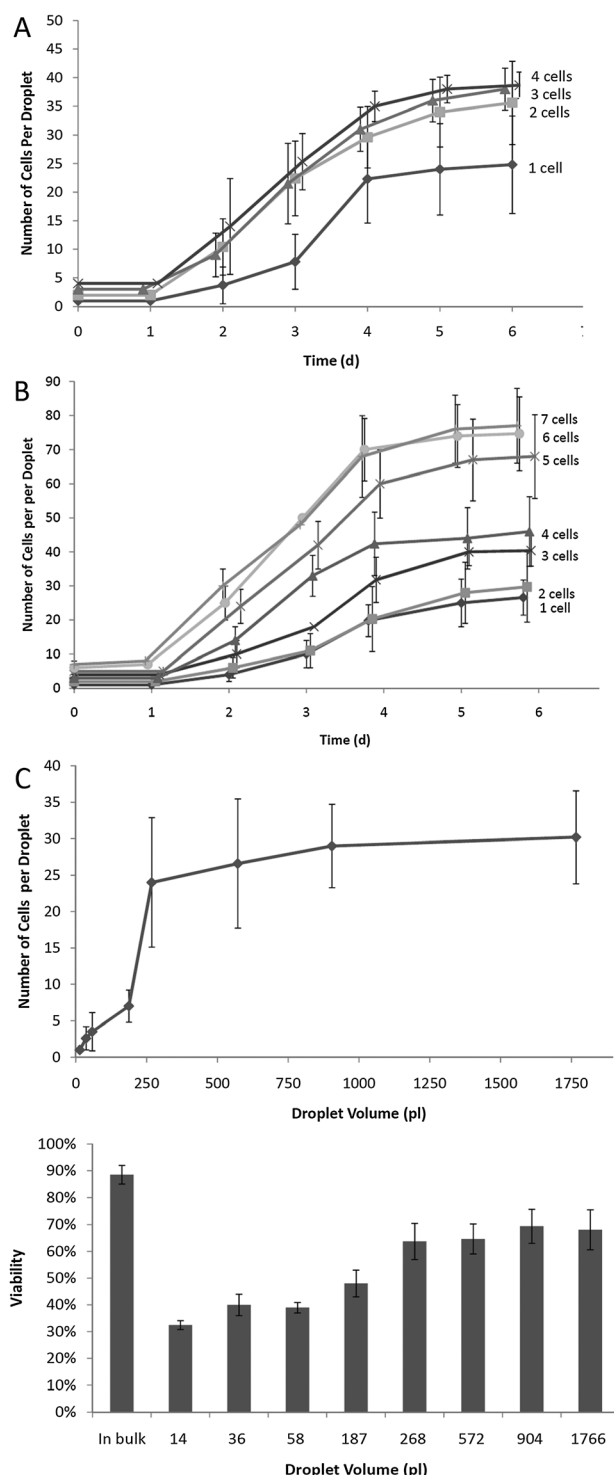
**Fig. 3** Culturing *C. vulgaris* cells in microdroplets (diameter  $\sim 80\ \mu\text{m}$ , volume  $\sim 268\ \text{pl}$ ) with varying levels of nitrate at  $25 \pm 2\ ^\circ\text{C}$  under continuous illumination of  $55\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ . (A) Distribution of cells in droplets when  $F_{\text{oil}} = 1000\ \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 200\ \mu\text{l h}^{-1}$ . (B) Bright-field images of *C. vulgaris* cells in microdroplets after 8 days of growth under concentration of nitrate of 8.8 mM. Some of the water droplets of similar size to *C. vulgaris* cells generated by condensation of evaporated DI water are indicated by white arrows. (C) Fluorescent image of *C. vulgaris* cells (excitation filter 460–500 nm, emission filter  $> 600\ \text{nm}$ ) in microdroplets after 8 days of growth under concentration of nitrate of 8.8 mM. (D) Growth curve of *C. vulgaris* in microdroplets at different concentrations of nitrate in the medium.

number of cells per droplet was varied. For the  $80\ \mu\text{m}$  droplets, similar growth curves were observed when the droplets initially contained 2 or more cells, with the maximum number of cells per droplet,  $35\text{--}40$  cells ( $\sim 1.4 \times 10^8\ \text{cells ml}^{-1}$ ) being reached after 5 days (Fig. 5A). However, when the droplets initially contained only one cell, the maximum number of cells per droplet reached only  $\sim 25$  ( $\sim 1.1 \times 10^8\ \text{cells ml}^{-1}$ ). In the larger droplets, the maximum number of cells per droplet ( $\sim 70\text{--}80$ ) (equivalent to a concentration of  $8.8 \times 10^7\ \text{cells ml}^{-1}$  in a droplet of volume  $904\ \text{pl}$ ) was achieved when the droplets contained over 6 cells immediately after encapsulation (Fig. 5B). When the droplets initially contained fewer than 6 cells, the final number of cells reached was lower and was found to increase with the increasing initial cell number.



**Fig. 4** Culturing *D. tertiolecta* cells in microdroplets (diameter  $\sim 80\ \mu\text{m}$ , volume  $\sim 268\ \text{pl}$ ) with varying levels of salinity and pH at  $25 \pm 2\ ^\circ\text{C}$  under continuous illumination of  $55\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ . (A) Growth curve of *D. tertiolecta* in microdroplets when cultured in medium with low (0.5 M), recommended (1.5 M) and high (4.0 M) concentrations of NaCl. (B) Growth curve of *D. tertiolecta* in microdroplets at various pH values in medium with 1.5 M NaCl. (C) Bright-field image showing *D. tertiolecta* cells in microdroplets 10 days after encapsulation in medium of pH 7.5 with 1.5 M NaCl.

Fig. 5C shows how the final number of cells per droplet changed with the droplet volume after 6 days of culturing, when one cell was initially encapsulated per droplet. It can be seen that with droplets of diameter greater than  $80\ \mu\text{m}$  ( $>268\ \text{pl}$ ), similar final cell numbers of  $\sim 25\text{--}30$  ( $\sim 1.1 \times 10^8\ \text{cells ml}^{-1}$ ) were observed. Growth curves for droplets of these volumes show similar profiles over 7 days (Supplementary Fig. S4). However, with droplets of smaller volumes, algal



**Fig. 5** Investigating the effect of droplet volume and initial number of cells per droplet on the growth of *C. reinhardtii* at  $25 \pm 2^\circ\text{C}$  under continuous illumination of  $55 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . (A) Growth curves of *C. reinhardtii* in droplets of diameter  $80 \mu\text{m}$  (268 pL) with different initial cell number (1, 2, 3, or 4). (B) Growth curves of *C. reinhardtii* in droplets of diameter  $130 \mu\text{m}$  (1150 pL) with different initial cell numbers (1, 2, 3, 4, 5, 6, or 7). (C) The change in the final number of cells per droplet and the final cell density with varying droplet volume, after 6 days of culturing. (D) Comparison of *C. reinhardtii* cell viability after 6 days of culturing in bulk (100 mL conical flasks) and in droplets of different volumes.

growth was restricted and lower final numbers of cells were reached. For droplets of diameter  $\sim 30 \mu\text{m}$  (14 pL), the cells did not grow at all (Supplementary Fig. S5). Fig. 5D shows the results of a viability assessment after 6 days of culturing in microdroplets, using the fluorescein diacetate (FDA) staining method. It can be seen that the viability of the algal cells increased with the droplet volume, from  $\sim 30\%$  alive in the smallest droplets to  $\sim 70\%$  in the largest. However, in all cases, the viability of algal cells cultivated in microdroplets was lower than those cultured in bulk ( $\sim 90\%$  alive).

## Discussion

Two key parameters required to compare the growth of microalgae in microdroplets and in bulk for the 3 species of microalgae investigated in this paper are shown in Table 1. Growth in bulk took place in shake flasks of volume 100 mL, rotating at 150 rpm under continuous illumination of  $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  light (45% greater intensity than the light source used for cultivation in microdroplets). It can be seen that for all 3 species, similar doubling times were achieved when cultivating in bulk and in microdroplets. However, despite the lower intensity of light used for cultivation in microdroplets, higher final concentrations of cells were achieved when the three species were grown in microdroplets rather than in bulk. This is demonstrated for the growth of *C. reinhardtii*. The final maximum cell concentration of  $\sim 1.1 \times 10^8 \text{ cells mL}^{-1}$ , initially cultured from one cell in droplets of  $80 \mu\text{m}$  ( $\sim 4.4 \times 10^6 \text{ cells mL}^{-1}$ ), is higher than the maximum concentration of  $1.2 \times 10^7 \text{ cells mL}^{-1}$  in bulk culture system, derived from the same initial concentration of cells. This may be due to 'self-shading' in the flasks, where cells deeper in the bulk culture receive significantly less light than those on the top, reducing the available light per cell. When cultivated in microdroplets, each cell is exposed to the same intensity of light and therefore this effect is not observed.

Another advantage of growing microalgae in microdroplets rather than in bulk is that individual cells can be tracked over time by microscopy. This is illustrated in Fig. 2C (i–v), which shows that inoculation of one *C. reinhardtii* cell per droplet results in cell division, but the rates of division of individual cells differed significantly. Indeed, in this experiment the final number of cells per droplet after 6 days of cultivation varied between 8 and 40. This stochastic heterogeneity provides new insights into algal culture that are opaque to bulk studies. Detailed mechanisms for explaining the cellular heterogeneity in algal cultures are desirable, and microdroplet studies could clearly facilitate them. *C. reinhardtii* cells are easily visible in bright field, but even with the much smaller *C. vulgaris* cells, it is possible to track them individually by detecting chlorophyll fluorescence (Fig. 3C).

Our results also show that algal growth in microdroplets is dependent on the initial number of cells per droplet and the droplet volume. For a specific droplet volume, the final cell concentration was found to increase with the increasing initial cell number per droplet. However, the final cell concentration stopped increasing and remained constant when the initial cell number increased beyond a certain number, which depended on the droplet volume. The most probable explanation of this



**Table 1** Comparison of key parameters of algal cell growth in microdroplets and bulk

	<i>C. reinhardtii</i>		<i>C. vulgaris</i>		<i>D. tertiolecta</i>	
	Bulk culture	Droplet culture	Bulk culture	Droplet culture	Bulk culture	Droplet culture
Doubling time (h)	8	8	12	12	24	24
Maximum cell density (cells ml <sup>-1</sup> )	$1.2 \times 10^7$	$1.1 \times 10^8$	$2.5 \times 10^8$	$4.5 \times 10^8$	$9.0 \times 10^6$	$1.5 \times 10^8$

is depletion of nutrients in the droplets. After the nutrients are consumed, the cell number would not increase. Bigger droplets contained more nutrients which can support the growth of more cells. These parameters can therefore be varied to achieve a desired outcome. For example, algal cells may be prevented from growing, whilst keeping them in a controlled environment, by reducing the volume of the microdroplets. However, the long term culture of algal cells in microdroplets is likely to result in decreased cell viability, in part due to the nutrition depletion in such small volumes (pL). Boedicker *et al.*<sup>26</sup> showed that the confinement of bacteria in microdroplets can induce high-density behaviour in small populations and may be used as a general system in which secreted signal molecules regulate some fundamental biological functions. Microdroplet technology could be used to determine if similar phenomena are observed when culturing microalgae in such environments.

The observation that algae growing in microdroplets responded to changes in environmental conditions including changes in pH and salinity, in the same way as would be expected for bulk samples demonstrates the relevance of the system for exploring the effect of altering environmental parameters such as light intensity, carbon dioxide levels *etc.* in order to optimise the growth of algae. Similarly the response to nitrogen deprivation means that microdroplets can be used to explore lipid production in algae, by direct detection of lipids in encapsulated microalgae. There are many screening programmes around the world to identify fast-growing species that accumulate lipid. The use of microdroplets is particularly suited for this process because single cells can be introduced into droplets, the droplets can be sorted from one another, and the desired cells then used to inoculate further cultures (*i.e.* the testing is not destructive). The single cells could be derived from heterogeneous environmental samples, although it would be necessary to establish protocols for harvesting and preparing the samples for microdroplet encapsulation. The concept could be extended further to screen mutagenised libraries to identify mutants with specific beneficial features. One desirable trait for large scale cultivation is to reduce the antenna size to minimise self-shading. Cells with this characteristic would have reduced chlorophyll fluorescence, and we have demonstrated that our system can be adapted to follow this parameter. Once mutant lines had been identified, they could be maintained in the droplets and their growth characteristics studied: depending on the cause of the reduced antennae, there may be detrimental effects on growth, so this could be an additional parameter for the screen.

In conclusion, it is evident that the use of microdroplet technology provides a powerful tool for analysing the growth of algal cells. In this paper, we have demonstrated that 3 different algal species can be grown successfully in

microdroplets over a time period of  $\sim 10$  days, whilst the environment can be easily manipulated to show growth information under varying conditions. This methodology can be extended to the culture of mutagenised algal libraries and other cell lines by altering their environmental parameters. As well as the biotechnological applications described above, the system provides a unique approach to study a range of physiological or behavioural features of microalgae at the level of an individual cell, including motility, gametogenesis, and cell division. In this context a recent paper has illustrated the use of a lab-on-a-chip approach to study the cell cycle in *C. reinhardtii* by cultivation in a fixed microchamber.<sup>27</sup>

## Experimental

### Fabrication of microfluidic devices

All the microfluidic devices were fabricated from poly-(dimethylsiloxane) (PDMS) using conventional soft lithographic techniques.<sup>28,29</sup> The microchannel architectures were first designed with AutoCAD software and printed onto high-resolution photomasks. Next, the negative photoresist, SU-8 2025 (MicroChem), was spin-coated onto silicon wafers of diameter 76 mm (Compart Technology). The pattern of the photomask was imprinted on the silicon wafer using a MJB4 mask aligner (Süss MicroTec) under a UV light source of wavelength 365 nm, before the silicon wafer was developed by submerging in 1-methoxy-2-propyl acetate (Sigma-Aldrich) for several minutes. The pre-polymer Sylgard 184 (Dow Corning) was mixed with a cross-linker in a ratio of 10:1 w/w and then poured onto the patterned silicon wafer and degassed in a dessicator. After curing at 80 °C for 6 h, the PDMS layer was cut and peeled off the wafer. Inlets and outlets were punched using a biopsy punch (Kai Industries) with an outer diameter of 1 mm. The PDMS device was bonded to a glass slide after plasma treatment in a Femto plasma cleaner (Diener electronic). The microchannels were then rendered fluorophilic by flushing with Aquapel (Duxback), then fluorinated oil. The devices were baked at 100 °C for at least 4 h and cooled to room temperature for use.

### Preparation of algal cells

*C. reinhardtii* wild type 12, *D. tertiolecta* strain CCAP 19/6B and *C. vulgaris*, strain 211/11B were obtained from the Culture Collection of Algae and Protozoa at Dunstaffnage Marine Laboratory, Oban, Scotland. Each was routinely cultured in 100 ml conical flasks at  $25 \pm 2$  °C under continuous illumination of 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and shaken at 150 rpm. Five percent CO<sub>2</sub> in air (v/v) was bubbled into the cultures of *C. vulgaris* and *D. tertiolecta*. Once the cells had reached stationary phase, 1 ml of cell suspension was centrifuged,

washed with fresh medium and diluted to a concentration of  $\sim 3.5 \times 10^6$  cells  $\text{ml}^{-1}$  in more fresh medium. The concentration of cells was determined by direct counting using a bright-line haemocytometer (Sigma-Aldrich). The diluted cell suspension was then used to generate microdroplets. The media used for each strain are listed below:

TAP medium<sup>30</sup> was used for culturing *C. reinhardtii*, and contained (per litre): 2.42 g Tris, 25 ml TAP salts, 0.375 ml phosphate solution, 1 ml Hutner's trace element and 1 ml glacial acetic acid. The TAP salts consisted of (per litre): 16 g  $\text{NH}_4\text{Cl}$ , 4 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 2 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , whilst the phosphate solution contained (per litre) 288 g  $\text{K}_2\text{HPO}_4$  and 144 g  $\text{KH}_2\text{PO}_4$ .

Bold's Basal Medium with 3-fold nitrogen and vitamins (3N-BBM + V) was used to culture *C. vulgaris*, and contained (per litre): 0.75 g  $\text{NaNO}_3$ , 0.075 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.225 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.225 g  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 0.525 g  $\text{KH}_2\text{PO}_4$  and 0.075 g  $\text{NaCl}$ . To this, 6 ml of trace element stock solution was added per litre of medium which contained (per litre) 0.75 g  $\text{Na}_2\text{EDTA}$ , 97 mg  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 41 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 5 mg  $\text{ZnCl}_2$ , 2 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  and 4 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ . After autoclaving, 1 ml of  $1.2 \text{ g l}^{-1}$  vitamin  $\text{B}_1$  and 1 ml of  $1 \text{ g l}^{-1}$  vitamin  $\text{B}_{12}$  were added to each litre of medium.

The growth medium for *D. tertiolecta* was based on that used by Hejazi and Wijffels (2003)<sup>3</sup> and contained (per litre): 87.7 g  $\text{NaCl}$ , 0.51 g  $\text{KNO}_3$ , 1.23 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.075 g  $\text{KCl}$ , 0.044 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.84 g  $\text{NaHCO}_3$  and 12.1 g Tris. To this, 2.5 ml of trace element stock solution was added per litre of medium and contained (per litre): 1.00 g  $\text{H}_3\text{BO}_3$ , 1.80 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.2214 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.08 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.02 g  $\text{Na}_2\text{MoO}_4$ , and 1.52 g  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ . Iron-salting liquid stock was also added at 2.5 ml per ml medium and contained (per litre): 0.21 g  $\text{Na}_2\text{EDTA}$ , and 0.21 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . Finally, the pH value was adjusted to 7.5 with 1 M  $\text{HCl}$ , the solution was autoclaved and 1 ml of 0.1 M  $\text{NaH}_2\text{PO}_4$  was added to the medium.

### Generation and storage of microdroplets

Fluorinated oil FC-40 (Sigma-Aldrich) containing 2.0% w/w of EA surfactant (Raindance Technologies) was used as the immiscible oil phase, whilst the cell suspension with a concentration of  $\sim 3.5 \times 10^6$  cells  $\text{ml}^{-1}$  was used as the aqueous phase. These two fluids were loaded into 1 ml gas-tight syringes (SGE Europe Ltd.) with 25 G disposable needles and syringe pumps (Harvard Apparatus PhD 2000) were used to flow each phase into the flow-focusing microfluidic device (left device of Fig. 1B) through polyethylene (PE) tubing (ID = 0.38 mm, Becton Dickinson). When flow rates of  $1000 \mu\text{l h}^{-1}$  and  $200 \mu\text{l h}^{-1}$  were used for the oil and aqueous phase, respectively, and a generating nozzle of dimensions  $50 \mu\text{m} \times 75 \mu\text{m}$  (width  $\times$  depth) was employed, microdroplets of diameter  $\sim 80 \mu\text{m}$  were generated at a frequency of  $\sim 60 \text{ Hz}$ . These droplets were then directed through a winding channel to ensure the droplets did not fuse and stable droplets were being formed, before being transferred into the reservoir through polyethylene tubing. Once the reservoir had been filled, the inlet and outlet tubing of the reservoir was cut and sealed in order to capture the droplets. The reservoir was

stored in a petri dish containing de-ionised water, and covered with a lid containing holes for gas transfer (Fig. S1).

In order to generate droplets with varying volumes, generating nozzles of different dimensions were employed and different oil and cell suspension flow rates were used: for droplets of  $30 \mu\text{m}$ , a generating nozzle of  $20 \mu\text{m} \times 25 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 200 \mu\text{l h}^{-1}$ ; for droplets of  $41 \mu\text{m}$ , a generating nozzle of  $20 \mu\text{m} \times 25 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 800 \mu\text{l h}^{-1}$ ; for droplets of  $48 \mu\text{m}$ , a generating nozzle of  $40 \mu\text{m} \times 50 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 400 \mu\text{l h}^{-1}$ ; for droplets of  $71 \mu\text{m}$ , a generating nozzle of  $50 \mu\text{m} \times 75 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 2000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 500 \mu\text{l h}^{-1}$ ; for droplets of  $103 \mu\text{m}$ , a generating nozzle of  $100 \mu\text{m} \times 75 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 200 \mu\text{l h}^{-1}$ ; for droplets of  $120 \mu\text{m}$ , a generating nozzle of  $100 \mu\text{m} \times 75 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 800 \mu\text{l h}^{-1}$ ; for droplets of  $120 \mu\text{m}$ , a generating nozzle of  $200 \mu\text{m} \times 75 \mu\text{m}$  (width  $\times$  depth) and flow rates of  $F_{\text{oil}} = 1000 \mu\text{l h}^{-1}$  and  $F_{\text{aqu}} = 400 \mu\text{l h}^{-1}$ .

### Fluorescein diacetate (FDA) staining method<sup>31</sup>

A fluorescein diacetate (FDA) stock solution was prepared by dissolving 46 mg FDA (Invitrogen) in 10 ml acetone (11 mM). The emulsion of droplets in oil was collected from the reservoir into a Eppendorf tube of 0.5 ml. Two  $\mu\text{l}$  of 1*H*,1*H*,2*H*,2*H*-perfluorooctanol was then added to break the emulsion. The suspension was centrifuged at 3000 rpm for 5 min and the supernatant discarded. Ten  $\mu\text{l}$  TAP medium and 1  $\mu\text{l}$  FDA stock solution were added and the cell suspension was incubated for 20 min. Esterases in viable cells cleave FDA and form fluorescein, which fluoresces at  $\sim 520 \text{ nm}$  when excited with  $\sim 480 \text{ nm}$ , therefore the viability of the algae was taken as the percentage of the cells visible using a bright-field microscope which fluoresce using a fluorescence microscope at  $\sim 520 \text{ nm}$ .

### Image acquisition and analysis

The generation of microdroplets and the growth of cells in microdroplets over the time course of each experiment was imaged by a monochrome Phantom v7.2 camera (Vision Research) mounted to an IX 71 inverted microscope (Olympus). Bright-field images were taken and analysed via the Phantom software, ImageJ<sup>®</sup> and Labview<sup>®</sup> 8.2. The number of cells per droplet were counted directly from images and using ImageJ<sup>®</sup>.

Fluorescence images of the chlorophyll within the cells were obtained using an IX 71 inverted microscope (Olympus) operated in epifluorescence mode. The wide-field illumination from a mercury lamp (U-LH100HG, Olympus) was filtered and reflected using an appropriate dichroic mirror to obtain excitation light of wavelength 460–5 nm. The fluorescence emission was collected by an objective, filtered (600 nm long-pass edge filter), and finally captured with an EMCCD iXonEM + DU 897 camera (Andor Technology). By using a computer-controlled shutter, the sample was only excited during the acquisition, which greatly minimised photo-bleaching and other damage to the cells.



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