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A microfluidic platform for long term monitoring of algae in a dynamic environment

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Abstract

Culturing cells in microfluidic “lab-on-a-chip” devices for time lapse microscopy has become a valuable tool for studying the dynamics of biological systems. Although microfluidic technology has been applied to culturing and monitoring a diverse range of bacterial and eukaryotic species, cyanobacteria and eukaryotic microalgae present several challenges that have made them difficult to culture in a microfluidic setting. Here, we present a customizable device for the long-term culturing and imaging of three well characterized strains of cyanobacteria and microalgae. This platform has several advantages over agarose pads and demonstrates great potential for obtaining high quality, single-cell gene expression data of cyanobacteria and algae in precisely controlled, dynamic environments over long time periods.

Keywords

cyanobacteria; microfluidics; dynamics; Synechocystis; Synechococcus; Chlorella

Introduction

The application of microfluidic technologies to biological research has increased significantly over the past decade (1). Microfluidics allows precise spatial and temporal control of the cellular environment and is an ideal tool for studying dynamic cellular processes such as natural and synthetic gene networks and intracellular signaling (2; 3; 4; 5). Time lapse imaging of cyanobacteria has traditionally been done by growing cells on agarose pads, but this is highly limiting in terms of image quality and experimental duration.

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Supplementary information

Supporting Information Available: Additional information on methods is available. This material is available free of charge via the Internet at http://pubs.acs.org/.

Conflicts of Interest

The authors declare no conflicts of interest.

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In recent years, several microfluidic devices have been developed for single cell imaging of cyanobacteria. One such device combines microfluidics and agarose with patterned linear tracks to enable the visualization of single lines of bacteria and cyanobacteria (5; 6), however this device is limited to species that can grow in linear rows. Another device uses PDMS and hydrogel cages on a glass slide to trap single cells and minimize physical stress on the cells caused by fluid exchange (7), but the trap was designed for relatively short experimental run times. Both of these devices utilized porous substrates that allow fluid exchange without adding a lot of mechanical stress, but they do not provide flexibility in terms of species morphology or allow for dynamic media profiles over long time periods.

There are several issues that have caused difficulty in culturing algae and cyanobacteria in microfluidic devices. Photosystem II (PSII) of photosynthetic organisms is susceptible to irreversible damage at all light intensities. A cell can repair the photodamage by synthesizing new proteins to replace the damaged ones. However, when the rate of photodamage exceeds the rate of repair, photoinhibition, or slow down in photosynthesis and growth, is observed (8). Reactive oxygen species (ROS) are byproducts of aerobic metabolism and are also generated by the photosynthetic electron transport chain in photosynthetic organisms. Excess ROS can cause oxidative stress that inhibits the repair of PSII (9). ROS can be generated from various types of environmental stresses such as CO_2 limitation, moderate heat (10), and also from excited fluorescent proteins (11), which poses a major challenge when imaging cyanobacteria in a microfluidic device.

Another concern for culturing these species in microfluidic devices is maintaining the balance of CO_2 and pH. CO_2 is needed for photosynthesis, but certain species such as *Synechocystis* and *Synechococcus elongatus* are sensitive to acidic pH, and pH is affected by the CO_2 concentration of the culture medium. As CO_2 gets consumed by cells during photosynthesis, the pH of the medium increases. We can supplement CO_2 to the medium to prevent depletion, but that has a side effect of decreasing the pH. This balance is difficult to control when dealing with the small volumes of medium presented by microfluidic devices.

In this study we address these and other design challenges by developing a flexible microfluidic platform that is suitable for the long term culture and imaging of cyanobacteria. We demonstrate the functionality of the device by performing experiments with two cyanobacterial strains *Synechocystis* sp. PCC6803, and *Synechococcus elongatus* PCC7942, and a microalgal strain *Chlorella sorokiniana*. These three species are commonly used as model organisms and are also considered as potential sources of renewable energy and specialty chemicals, as many scientists are trying to use synthetic biology and metabolic engineering to increase their biomass and productivity (12; 13; 14; 15; 16).

Due to its relatively simple circadian clock, *S. elongatus* serves as an important model cyanobacterium and has been the target of several single cell microscopy studies (4; 5; 17). While these studies have led to important discoveries about circadian networks, including the observation of stable circadian oscillations (17) and the coupling of circadian and cell division phases (4), the use of agarose pads limits the experimental duration due to rapid nutrient and water depletion and leads to lower signal due to agarose autofluorescence. Microfluidic devices can extend the experimental duration and can also provide a stronger

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signal and higher quality data. In addition, our microfluidic platform has a Dial-A-Wave (DAW) system that enables the precise temporal control of media exposure, which is novel to cyanobacterial research and is useful for characterizing cellular dynamics in more natural environments as well as for observing novel cellular functions derived from synthetic biology approaches.

Results and discussion

Our goal was to design a microfluidic device that can culture many species of different shapes and division patterns over long time periods. We based the design of our device on a previously published yeast device (18) (Figure 1A). Media from ports 1 and 2 pass through the Dial-A-Wave (DAW) function generator and mixer, which combines the two inputs at a precise ratio defined by the user. The DAW system can deliver any desired waveform of biochemical inducer for dynamic stimulation of cells inside the culture chambers (18). Excess medium goes to port 3, and cell waste passes to ports 4 and 5. One design criterion of the microfluidic device is that the cell chamber height should be slightly lower than the smallest dimension of the cell to keep the cells inside the chamber constrained to a monolayer and also to allow slow media perfusion without perturbing the cells.

Three devices with different cell chamber heights were customized to each individual strain imaged for this study. All devices have a ring-shaped culture chamber of 1.4 mm diameter. *Synechocystis* has an average cell diameter of 1.75 μm, and its cell chamber was made to a height of 1.25 μm. *S. elongatus* has an average width of 1 μm and length of 3 μm, and its cell chamber has a height of 0.74 μm. A channel structure with an aspect ratio lower than 1:10 (height:width) is prone to collapse, so support posts must be added within the structure to prevent collapse. Circular posts of 30 μm diameter and rectangular posts of 12–45 μm × 15 μm were added radially around the cell chambers of the devices of *Synechocystis* and *S. elongatus*, respectively (Figure 1B, C). The distance between circular posts is 25–40 μm and the distance between rectangular posts is 20–30 μm. The cell chamber for *C. sorokiniana* has a 3.25 μm height, so support posts are not necessary. To prevent phototoxicity from image acquisition, we employed a highly sensitive Electron-Multiplying Charged-Coupled Device (EMCCD) camera and a white light laser with small focal area to limit superfluous light exposure. In addition, we found that a combination of supplementing the culture medium with sodium bicarbonate and infusing humidified 5% CO_2 into the culture chamber helped the balance of CO_2 and pH and was critical to cell growth.

To test the functionality of our platform, we grew *Synechocystis, S. elongatus*, and *C. sorokiniana* in the device and measured their growth rates and fluorescence. All cell types exhibited healthy morphology and fully colonized the glass surface of the culture chamber within a few days (Figure 2, rows A, B, and C respectively). We loaded a small number of cells per field of view to allow sufficient space for growth over the course of the experiment. The growth rates of photoautotrophic organisms are highly dependent on the light intensity, and the experimental conditions can be tailored to the organism of interest. All three types of cells had similar doubling times (mean +/− standard deviation) in microfluidic devices as in shaker flasks under the same light intensity (Table 1). In a microfluidic device, cells are spread out in a single layer and hence would experience an effective higher light intensity

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than counterparts growing in bulk culture that would experience shading effects from neighboring cells. Continuous perfusion at a constant flow rate ensured that the cells had sufficient access to nutrients.

We developed a tracking algorithm that is able to segment images, identify individual cells, and track their growth and fluorescence over time (Figure 3). The *Synechocystis* strain used for our experiments employs a lac repressible promoter P_{trc1O} expressing eYFP (19). Without lacI being expressed, eYFP was constitutively expressed and was imaged using a yellow filter. Chlorophyll emits red autofluorescence and was imaged using a red filter. We imaged the cells at 100×, using both fluorescence channels for up to 68 hours, and we were able to track cell area and fluorescence over the experiment duration. Automated single cell tracking was performed using a custom Matlab algorithm. A mask was created to identify and label individual cells (Figure 3A, B). Area and fluorescence of single cells were measured and tracked, where a significant drop in cell area indicates a cell division event (Figure 3C). This enabled us to calculate the time for each cell division event to give us statistics on the growth rate inside our device (Figure 3D).

Our device also presents the opportunity to track individual cellular behaviors over long time periods. As an example, we can track circadian rhythms of gene expression in the model species *S. elongatus*. We used a previously developed strain that expresses YFP-SsrA(LVA) under control of the rhythmic *kaiBC* promoter (4; 20). The *kaiBC* promoter drives the endogenous expression of the *kaiB* and *kaiC* genes, which in combination with KaiA make up the central oscillator of the cyanobacterial circadian clock. Previous work showed that increasing light intensity from ~25 to ~50 μE m^{-2} s^{-1} shortened the average cell cycle duration by an approximate factor of 2 while the average circadian period remained around 24 hours (4). To determine if this result is reproducible in our device, we altered the light intensity to ~100 μE m^{-2} s^{-1} and observed that the cell cycle was further shortened while the circadian period stayed around 24 hours (Figure 4B, C). We tracked 134 cells for 92 hours and demonstrated that our device allows robust growth in a monolayer in comparison to bulk culture.

As one of the major advantages of microfluidic technology is the precise and dynamic control of the cellular microenvironment, we tested the ability to dynamically stimulate the cells in the culture chambers using the Dial-A-Wave functionality. To mimic nitrogen fluctuations in nature, we cultured *C. sorokiniana* in BG11 medium without nitrate and subjected the cells to pulsing of 100 ppm ammonia at different periods. We monitored the cell proliferation and chlorophyll autofluorescence, which can be viewed as a measure of the efficiency of photochemistry. That is, when autofluorescence is higher, less light is being converted to energy indicating a slow down in photosynthesis. As verification of this hypothesis, we observed that chlorophyll autofluorescence (Figure 5, green line) decreased when ammonia was introduced (red line), providing a nitrogen source for cellular proliferation. These types of time course induction experiments can provide an opportunity to study dynamic phenomena that could not be replicated on an agarose-based platform and can provide great insight into the behavior of cells in more natural environments.

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Conclusions

Previous implementations of microfluidics for cyanobacteria have employed hydrogel or agarose due to the concern of mechanical stress. In this study, we demonstrated the use of a new PDMS device for growing and imaging cyanobacteria and microalgae in a monolayer. We found that minimizing the exposure settings but staying above the detection threshold can permit normal cell growth while still capturing gene expression dynamics. The addition of sodium bicarbonate to the medium in conjunction with CO_2 infusion is critical for pH and CO_2 balance. In addition, the Dial-a-Wave functionality enables the probing of cyanobacteria and algae in a dynamically changing environment, opening the door for experiments that can elucidate native behaviors or can facilitate the search for optimal conditions for the production of biofuel precursors or other specialty chemicals.

Materials and methods

Design and fabrication

The devices were fabricated by first creating a multi-layer master-mold using photolithography. Negative photoresists (Microchem Corporation) were used to create the master-mold according to established methods (18). Specifically, SU-8 2001.5 was spun at 2700 rpm to generate a 1.25 μm trap layer for *Synechocystis*. SU-8 2000.5 was spun at 565 rpm to generate a 0.74 μm trap layer for *S. elongatus*. SU-8 2003 was spun at 1600 rpm to generate a 3.25 μm trap layer for *C. sorokiniana*. The other layers for all three devices were the same. SU-8 2005 was spun at 660 rpm and 1200 rpm to generate the main channel and chaotic mixers, respectively. The devices were then made by pouring and curing polydimethyl-siloxane (PDMS) onto the master-mold. PDMS was prepared by mixing Sylgard 184 Elastomer curing agent and base (DOW Corning) in 1:10 ratio. PDMS was poured onto the master-mold, degassesd for 30 min, cured for 1 hour at 80 degrees C, and then carefully peeled off the mold. Holes for the five ports were punched using a 0.5 mm Harris Uni-Core hole puncher (Ted Pella, Inc) and the devices were subsequently bonded to glass coverslips (Corning) via oxygen plasma exposure (Jelight UVO cleaner Model no. 42, 0.6 scfm O_2, 3 min).

Cell culture and device loading

Synechocystis, S. elongatus, and *C. sorokiniana* were maintained at 30 degrees C, 5% CO_2, and 35 μE m^{-2} s^{-1} white light in a standard incubator (Percival). Cells were cultured in BG11 medium (Sigma), with pH adjusted to 7.8–8.0 with NaOH after 50× dilution. Cells were grown to OD_{730} of 0.8 prior to device loading. For the circadian experiments with *S. elongatus*, cells were entrained to 12 hour light and 12 hour dark cycle for 3 days prior to imaging. The device was first wetted by gently pushing sterile water plus 0.075% tween 20 (G Biosciences) through the ports and channels with a syringe to remove all air bubbles. Syringes containing media were connected to ports 1 and 2, and syringes containing sterile water were connected to ports 3 and 4. For *C. sorokiniana* experiments that used two different media, 0.5 μg/mL Sulforhodamine 101 (Sigma), a red fluorescent dye, was added to the BG11 with 100ppm ammonia to help visualize medium switching in the mCherry fluorescence channel. 100mM sodium bicarbonate was supplemented to the BG11 as a pH

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buffer against CO_2 fluctuations. Finally, a syringe containing the cell suspension was connected to port 5. Cells were loaded into the chamber by flicking the line connecting the cell syringe to port 5 to create pressure waves. Cells in the main channel that did not enter the chamber were washed away by reversing the flow direction without perturbing cells inside the chamber. During the microscopy experiment, the microfluidic device was contained within an environmental chamber maintained at 30 degrees C with humidified 5% CO_2 and fluid flow delivered using hydrostatic pressure. 0.022 inch inner diameter PTFE tubing (Cole-Parmer) and 23 gauge stainless steel luer stub (Becton Dickinson) pins were used to connect the ports of the device to syringes.

Image capture

A Nikon Ti-Eclipse microscope fitted with a Photometrics QuantEM:512SC EMCCD Camera was used to collect time lapse images at 300× electron multiplying gain. 100× Phase brightfield images were taken every 5 minutes, and fluorescent images were taken every 30 minutes using programmable NIS-Elements control software. 50% intensity and 100 ms exposure (Lumencor SOLA light engine) was used for capturing yellow fluorescence. Cell autofluorescence was captured at 70% intensity and 5 ms exposure in mCherry channel. *C. sorokiniana* was imaged at 20× every 5 minutes, and its chlorophyll autofluorescence was captured by an Atto655 channel. Ammonia pulsing (switching between medium without nitrogen and medium with ammonia) was imaged in the mCherry channel. To provide light for photosynthesis, constant illumination of 20–100 $\mu E\ m^{-2}\ s^{-1}$ (Schott LLS LED) was used. The light source was programmed to turn off during imaging.

Single cell tracking and analysis

To obtain and analyze single cell data from time lapse images, we adopted a recently developed single cell tracking algorithm (21). To generate the mask and identify *S. elongatus* cells, we optimized the area and width parameters to minimize tracking errors. In the case of *Synechocystis*, we had to modify the algorithm to account for the more rounded shape of the cells to minimize tracking errors. We used the circularity of the cell, rather than the cell width, to filter out non-cell objects. Once single cell objects were identified, the tracking algorithm described previously (21) was applied to obtain single cell time course information about the area and mean fluorescence of the cells. By identifying large drops in single cell area trajectories we were able to calculate division times. Single cell tracking was not performed on *C. sorokiniana* because one cell divides into four new cells. The normalized total area occupied by cells was used to estimate the growth rate of *C. sorokiniana*.

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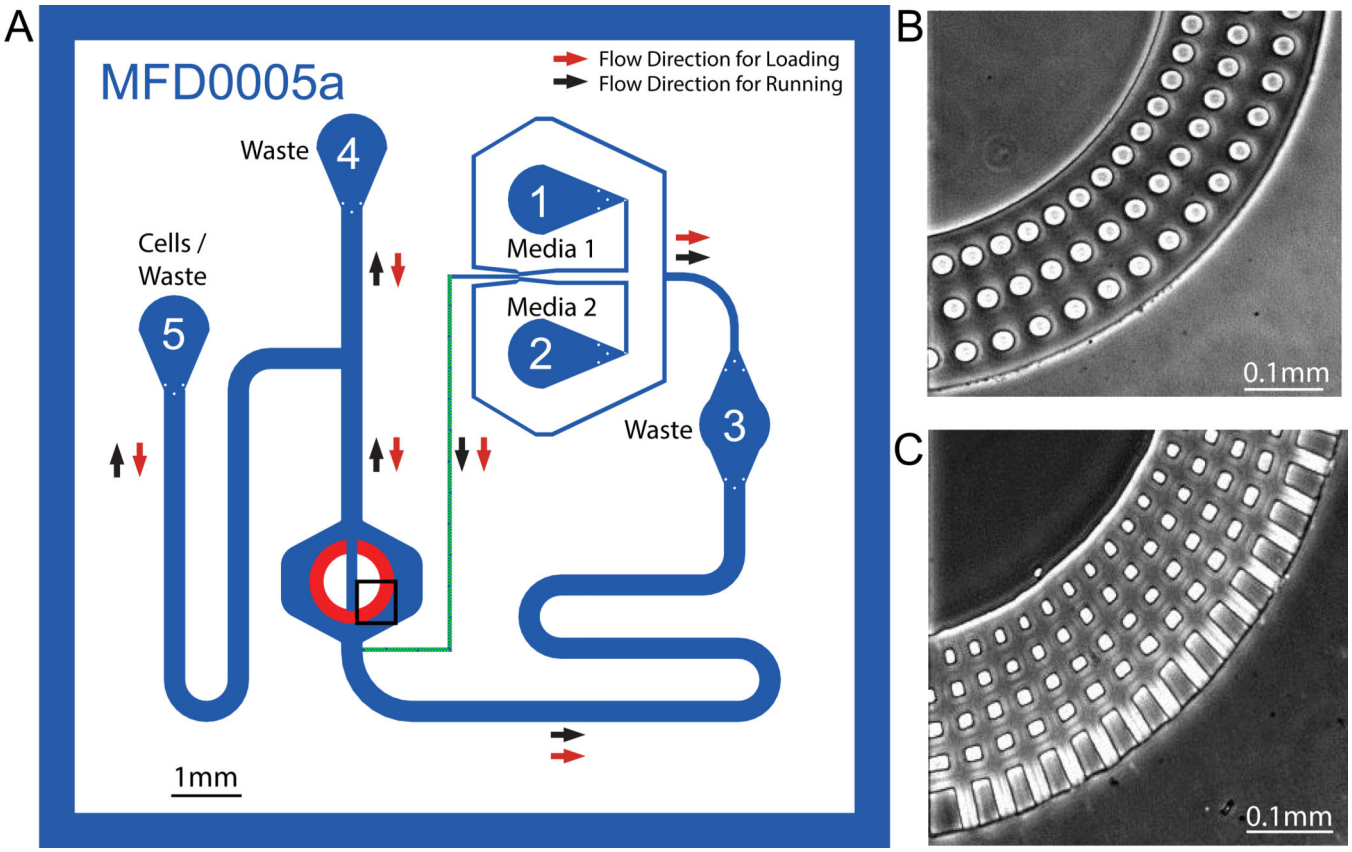

Figure 1.

Schematic of our microfluidic device capable of imaging and subjecting different cell types to a dynamic environment. (a) The cell trapping chamber is colored red. Ports 1–3 make up the DAW dynamic stimulation generator, where 1 and 2 contain different media types for switching and Port 3 is a waste port. Port 5 is used to load cells and then becomes a waste port during during the imaging experiment. Port 4 is an additional waste outlet. Arrows indicate flow direction during experiment, where red represents the direction of flow during loading, and black represents the direction of flow during the imaging experiment. (b and c) Enlargement of the boxed area in (a) to show the cell chambers for (b) *Synechocystis* and (c) *S. elongatus*. PDMS posts (bright areas) are used to ensure that the low-height chamber doesn't collapse.

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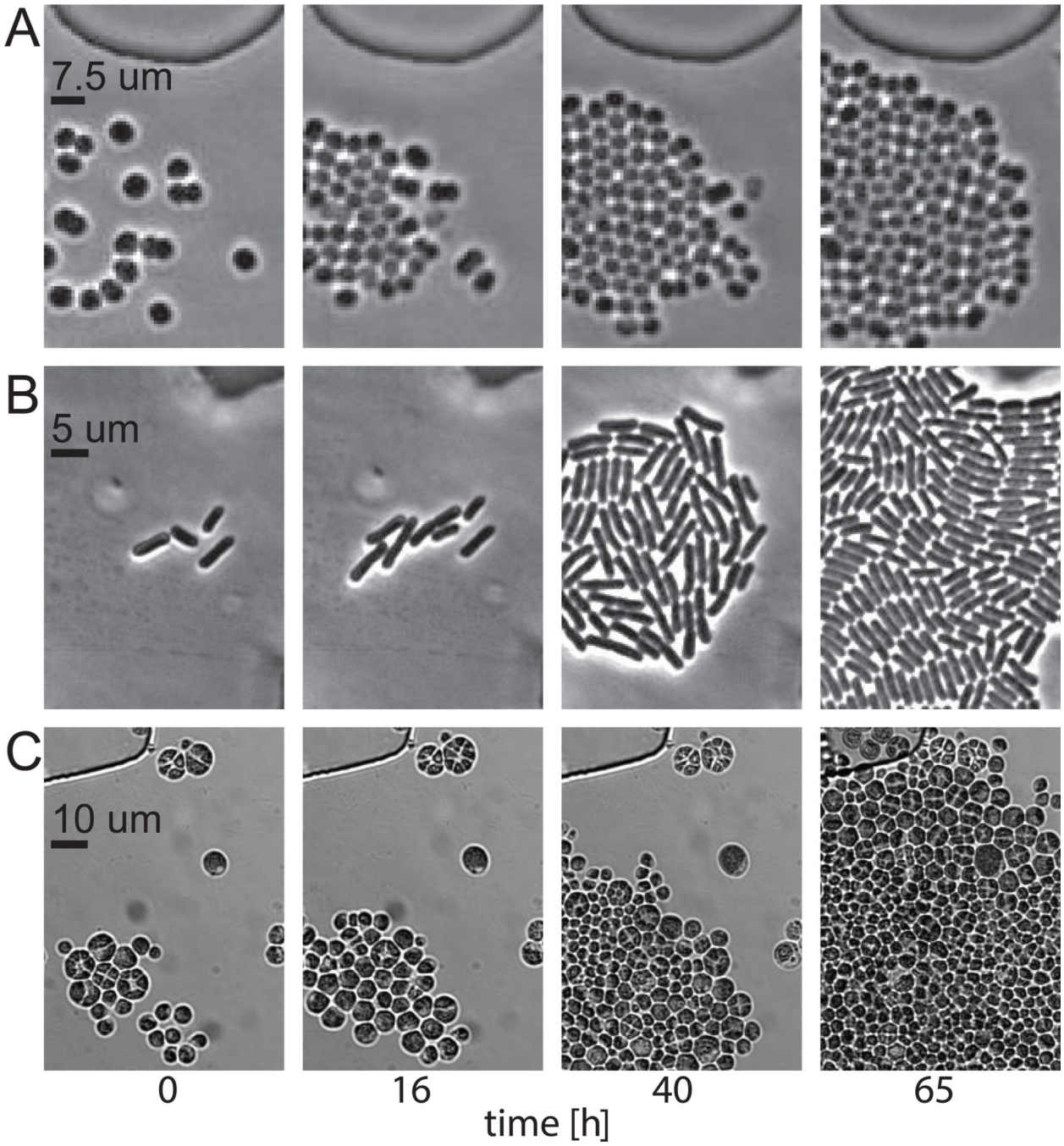

Figure 2.

Time-lapse images of three species in microfluidic chambers at times t = 0, 16, 40, and 65 hours after inoculation to show growth and healthy morphology. (a) *Synechocystis*. (b) *S. elongatus*. (c) *C. sorokiniana*.

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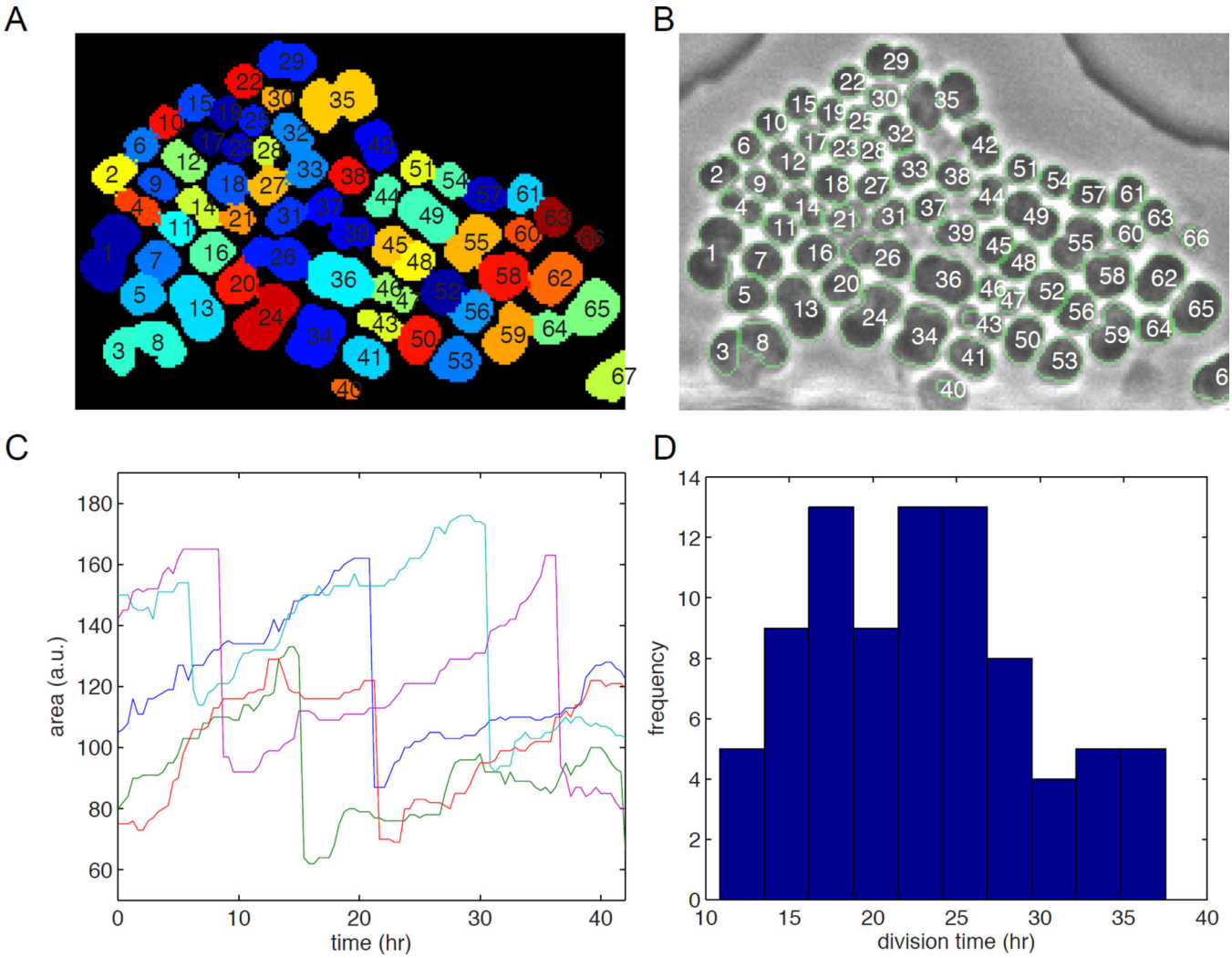

Figure 3.

We developed algorithm to track the growth of *Synechocystis* to demonstrate the utility of the device. (a) Segmentation of phase images enables the identification of individual cells. A portion of the mask is shown to demonstrate the ability of the algorithm to identify single cells. (b) Segmentation allows us to outline the cells in the original image and measure their area (i.e. number of pixels). (c) A single cell tracking algorithm allows us to track individual cells over time and observe division events, highlighted by sharp drops in area between successive frames. (d) We measured 84 division events and found the average doubling time to be about 23 hours with a relatively wide distribution.

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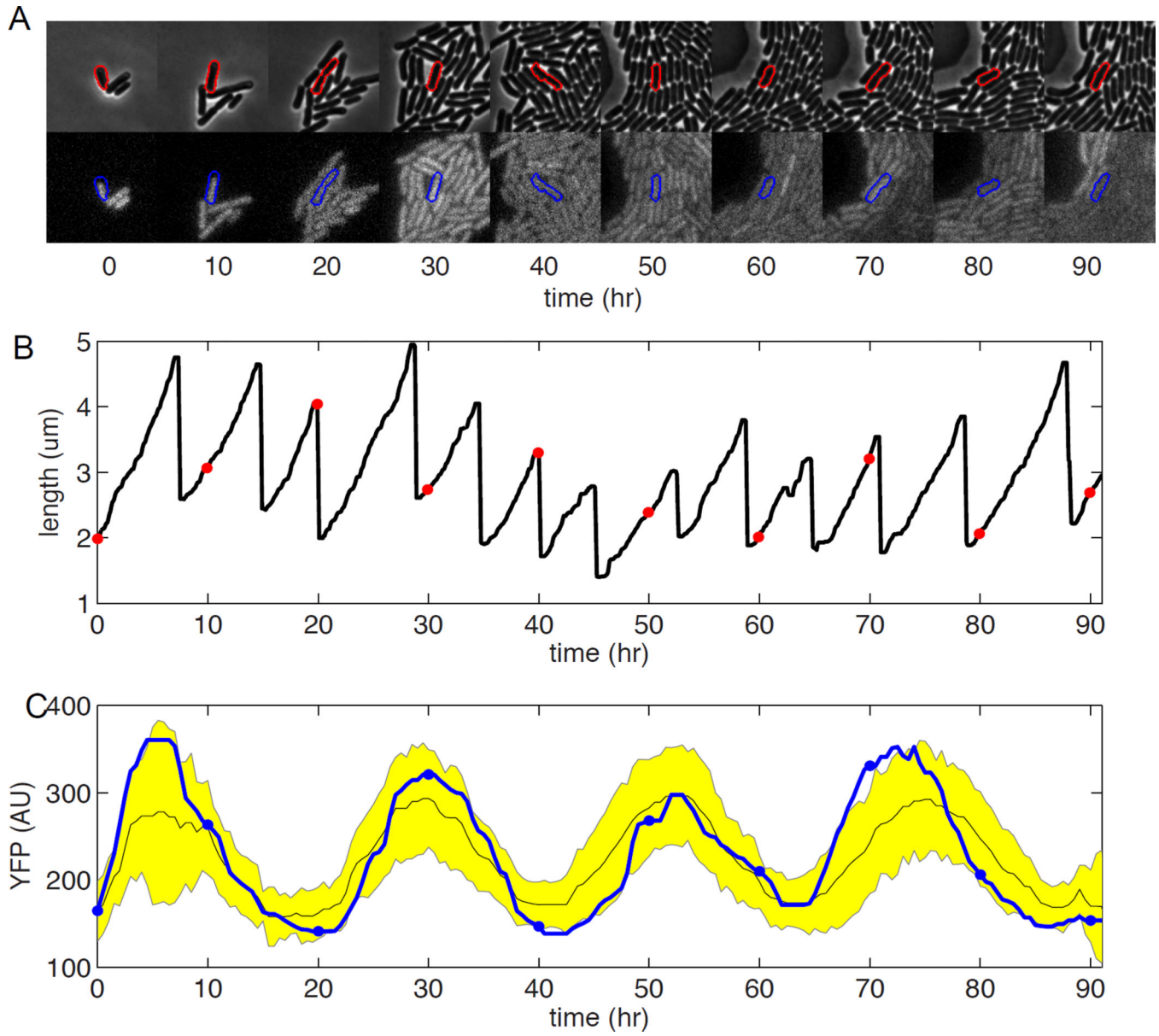

Figure 4.

Single cell tracking of the growth and fluorescence of *S. elongatus*. (a)Phase contrast (upper panel) and YFP fluorescence (lower panel) images of cells tracked over a 92-hour period. The same cell is outlined in both panels with corresponding trajectories shown in panels (b) and (c) (red, phase; blue, YFP). (b) The length of the cell outlined in (a) during growth and division with red points corresponding to the select frames in (a). (c) The mean YFP of 134 cells (black line) with variability (+/− STD) indicated by the yellow shaded region. The blue trace corresponds to the mean YFP trace of the cell outlined in (a) with the blue dots matching select frames.

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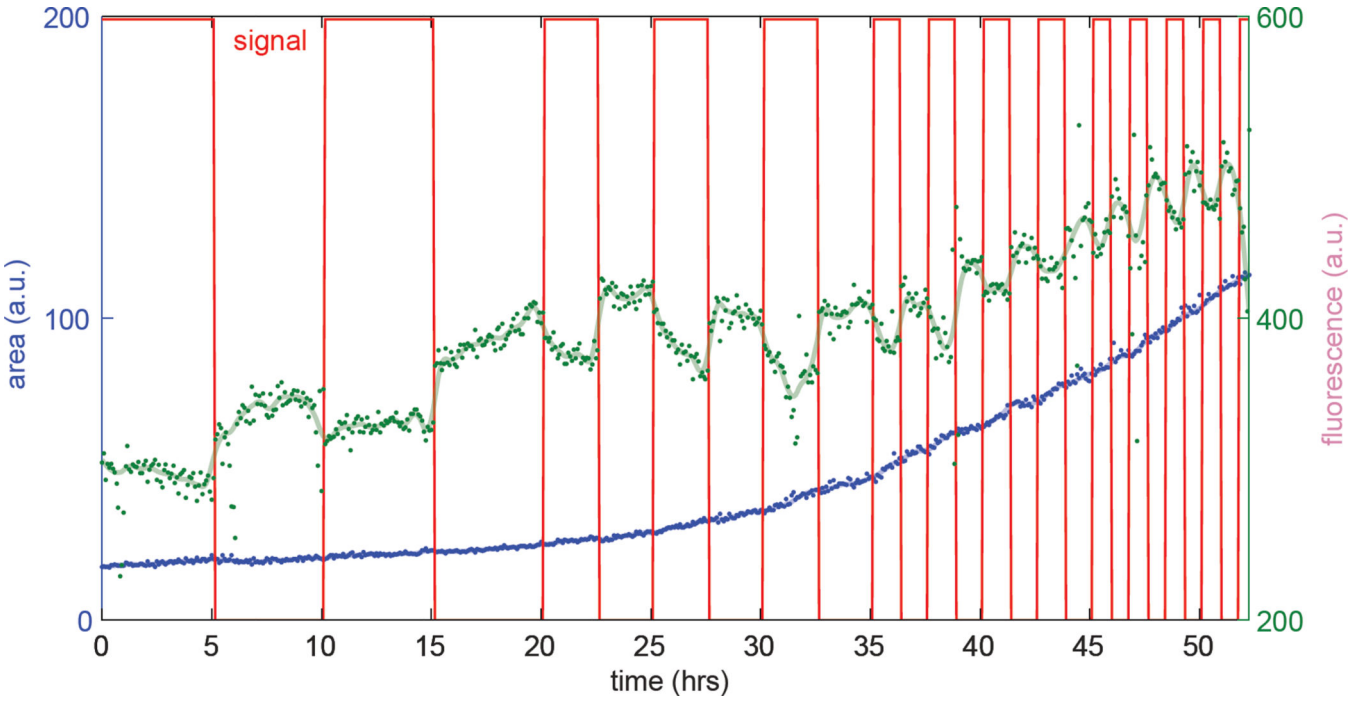

Figure 5.

To demonstrate the use of the DAWfunctionality, we monitored Chlorophyll fluorescence of *C. sorokiniana* under dynamic stimulation with pulses of ammonia. Chlorophyll fluorescence (green line) decreased when ammonia was introduced (red line). The total cell area (blue line) increased over time.

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Table 1

Comparison of microfluidic and bulk culture characteristics

Species	Culture format	Light intensity (μE m^{-2} s^{-1})	Doubling time (hr)
Synechocystis	Shaker flask	20	26.7 ± 0.5
Synechocystis	Device	20	22.8 ± 6.6
S. elongatus	Shaker flask	50	12.8 ± 4.0
S. elongatus	Device	50	7.3 ± 3.2
S. elongatus	Shaker flask	100	6.4 ± 0.8
S. elongatus	Device	100	5.7 ± 1.5
C. sorokiniana	Shaker flask	100	12.3 ± 3.1
C. sorokiniana	Device	100	9.5 ± 1.0