Continuous live cell imaging of cellulose attachment by microbes under anaerobic and thermophilic conditions using confocal microscopy

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Abstract

Live cell imaging methods provide important insights into the dynamics of cellular processes that cannot be derived easily from population-averaged datasets. In the bioenergy field, much research is focused on fermentation of cellulosic biomass by thermophilic microbes to produce biofuels; however, little effort is dedicated to the development of imaging tools to monitor this dynamic biological process. This is, in part, due to the experimental challenges of imaging cells under both anaerobic and thermophilic conditions. Here an imaging system is described that integrates confocal microscopy, a flow cell device, and a lipophilic dye to visualize cells. Solutions to technical obstacles regarding suitable fluorescent markers, photodamage during imaging, and maintenance of environmental conditions during imaging are presented. This system was utilized to observe cellulose colonization by *Clostridium thermocellum* under anaerobic conditions at 60°C. This method enables live cell imaging of bacterial growth under anaerobic and thermophilic conditions and should be widely applicable to visualizing different cell types or processes in real time.

Key words: thermophile; lipophilic dye; cellulose; biofuel; confocal laser scanning microscopy; biofilm

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Introduction

Cellulosic biomass has been identified as an attractive feedstock for producing biofuels and other high value-added bioproducts by microbial fermentation due to its abundance and renewability (Hromadko et al., 2010; Dale, 2011; Huang et al., 2011; Mazzoli et al., 2012). At present, however, no microbes have been identified that possess all of the metabolic capabilities to efficiently and cost-effectively produce biofuels directly from biomass in a single processing step, a process known as consolidated bioprocessing (CBP). As such, research efforts are focused on understanding the process of microbial cellulose degradation and utilization, and engineering these capabilities into microorganisms suitable for industrial-scale biofuel production via CBP (Lynd et al., 2008; Huang et al., 2011; Mazzoli et al., 2012). Many of the cellulose-utilizing microorganisms that are being engineered for

biofuel production, such as *Clostridium thermocellum* and *Caldicellulosiruptor* sp., are obligate anaerobes and thermophiles (Lynd et al., 2002; Hamilton-Brehm et al., 2010). These microorganisms attach to cellulosic biomass and form biofilms in the process of cellulosic degradation (Wang et al., 2011a, 2011b). In recent years, our understanding of microbial attachment to surfaces and biofilm formation has been revolutionized by the ability to visualize microbes under physiological conditions using imaging, particularly confocal laser scanning microscopy (CLSM) (Palmer and Sternberg, 1999). However, the dynamic process of cellulolytic biofilm formation in thermophiles has only been studied by examining samples sacrificed at various experimental stages (Wang et al., 2011a, 2011b; Dumitrache et al., 2013). This is due to the experimental challenges of providing both anaerobic and thermophilic environments for live cell imaging.

Live cell imaging techniques offer the opportunity to observe microbial behavior within the context of a living

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biofilm. Flow cells have become the tool of choice for *in vivo* investigation of biofilms since their development (Caldwell and Lawrence, 1986; Palmer and Sternberg, 1999). A flow cell is a chamber that facilitates the growth of microbial biofilms under controlled conditions on a surface that can be mounted onto a microscope stage for direct visualization in a non-destructive manner. By allowing the flow cell to remain mounted on the microscope stage, many images can be collected over time to analyze the temporal and spatial dynamics of cell attachment and biofilm formation. Although many flow cell designs are available (Zinn et al., 1999; An et al., 2001; Pamp et al., 2009), few have been reported to operate in both anaerobic and thermophilic conditions. For studies requiring anaerobic conditions, Hansen et al. (2000) enclosed a flow cell inside an inflatable glove chamber and placed the entire chamber in a 34°C room for temperature control. In order to image the cells, however, the flow cell was removed from the anaerobic chamber and exposed to air. Similarly, O'Sullivan et al. (2009) fabricated an airtight flow cell with recirculation of growth medium from an anaerobic vessel placed in a 37°C incubator, and Filoche et al. (2004) maintained a flow cell in anaerobic conditions by flowing nitrogen gas and controlling the temperature at 37°C with a water bath. But, these flow cells were disconnected and moved for microscopy and operated in mesophilic conditions. In one published study (Horn et al., 1999), both anaerobic and thermophilic conditions were maintained by sealing hyperthermophilic anaerobes inside glass capillaries placed on a heated stage of a light microscope. This microscope was enclosed inside a heated chamber to preserve temperature at 90 to 98°C and cell division was observed within this system. This was a closed system, however, that did not allow for the addition of fresh growth medium or other treatments.

The choice of an appropriate microscopy platform is dependent on the cellular process being observed. In this case, visualizing the attachment of microbes to cellulose is difficult using traditional light microscopy because cellulosic biomass is opaque and blocks light transmission. The use of fluorescence microscopy, on the other hand, provides greater contrast and the ability to simultaneously distinguish multiple cell types or processes depending on the selection of fluorescent dyes or fusion proteins. The availability of suitable fusion proteins or dyes for use with fluorescence microscopy is a significant issue with respect to live cell imaging under anaerobic and thermophilic conditions. The fluorescent dyes used in previous anaerobic flow cell studies, such as the nucleic acid dye SYTO 9, worked well for end-point sampling and visualization but are unsuitable for continuous live cell imaging due to its toxicity to cells (Hansen et al., 2000; Filoche et al., 2004; Dumitrache et al., 2013). The use of Green Fluorescent Protein (GFP) and its derivatives have greatly enabled the development of live cell imaging studies, but

GFP requires oxygen and is incompatible with imaging in anaerobic conditions (Shaner et al., 2005). An ideal flow cell design for cellulosic biofilm studies should overcome these challenges by maintaining anaerobic conditions and a thermophilic environment while being compatible with fluorescence microscopy. In this article, an imaging system is described that fulfills these conditions and can be used for continuous live cell imaging of microbial cellulose utilization by thermophilic cellulose-degrading microbes as well as other applications that require anaerobic conditions and high temperatures.

1 Materials and methods

1.1 Flow cell design

A commercially available flow cell (RC-31, Warner Instruments, Hamden, USA) was placed on the stage of a Zeiss LSM 710 confocal microscope (Jena, Germany) equipped with an environmental control chamber (Incubator XL LSM710 S, PeCon GmbH). Biomass samples were placed in a slot formed by a piece of gasket that was sandwiched between two coverslips and clamped by the upper and lower plates of the flow cell. Vacuum grease was applied on the edges of these coverslips to form an airtight seal. As illustrated in **Fig. 1**, an anaerobic medium bottle and an anaerobic waste bottle were connected to the inlet and outlet of the flow cell by Viton tubing (SCP Science, Champlain, USA). A small peristaltic pump (P720, INSTECH, Plymouth Meeting, USA) was used to pump medium through the flow cell into the waste bottle at a flow rate of 10 mL/hr and create a positive pressure in the system. Two gas bags (SKC, Eighty Four, USA), one filled with 70% N_2/30% CO_2 and the other with vacuum, were inserted into the medium and waste bottles, respectively, in order to equilibrate headspace pressures between the two. Three temperature probes, one placed on the in-line heater (SC-20, Warner Instruments, Hamden, USA), the second on the top of the flow cell, and the third on the bottom of the flow cell, provided feedback signals to a Dual Channel Bipolar Temperature Controller (CL-200A, Warner Instruments, Hamden, USA), which controlled the flow cell temperature at 60°C with a heating plate. An Objective Heater (Bioptechs, Butler, USA) was also installed on the CLSM lens and set at 60°C to help maintain temperature. The environmental control chamber was set at 35°C to further insulate the flow cell from temperature fluctuations. Approximately 10^7 cells were injected into the flow cell through an inoculation syringe connecting to the inlet tubing.

1.2 Bacterial growth

Clostridium thermocellum (ATCC 27405) was used as a model anaerobic and thermophilic bacterium in this flow cell study. *C. thermocellum* was cultivated in
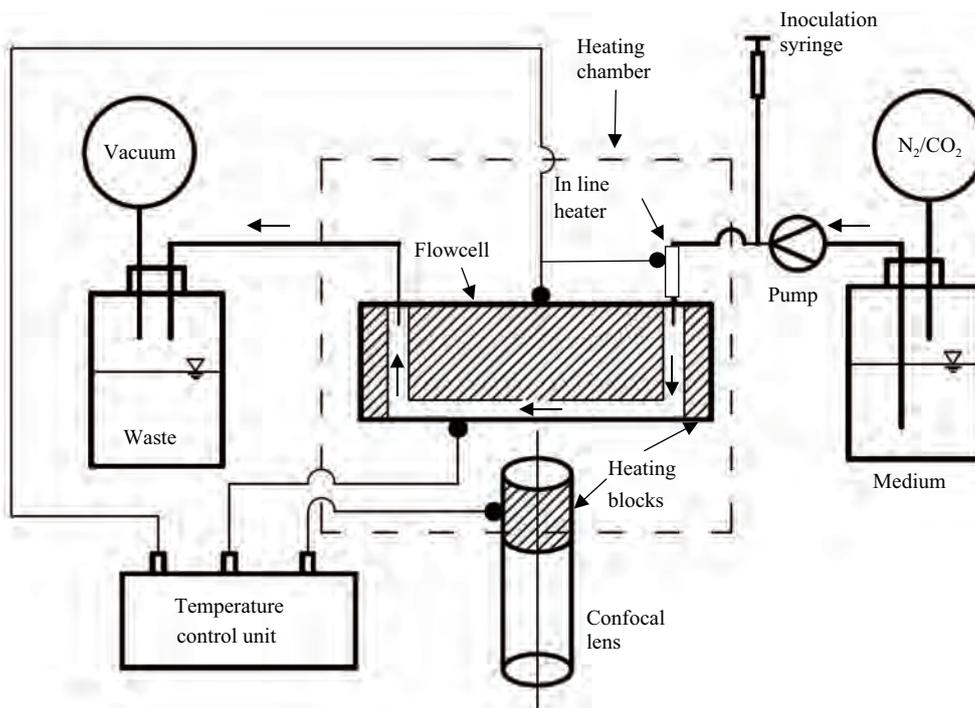
Fig. 1 Schematic illustration of the flow cell system. Details are given in the Methods section.

the following medium: 4.5 mmol/L KCl, 4.7 mmol/L NH$_4$Cl, 4.0 mmol/L MgSO$_4$·7H$_2$O, 10 mmol/L K$_2$HPO$_4$, 23 mmol/L MOPS, 1 mmol/L CaCl$_2$·H$_2$O, 6 mmol/L Na$_3$Citrate·2H$_2$O, 10 mmol/L urea, 1X Wolf's vitamins, 1X Wolf's minerals, 0.5 mg/L resazurin, 0.5 g/L yeast extract, 5.6 mmol/L cysteine HCl, and 6 mmol/L NaHCO$_3$. The headspaces of both medium and waste bottles were purged with a 70% N$_2$/30% CO$_2$ gas mixture to create an anaerobic environment. Resazurin was added as an oxygen indicator and cysteine was added as a reductant. Cellulosic biomass including regenerated cellulose membranes (Whatman RC58, Maidstone, Kent, UK), pulp nanofiber (softwood kraft pulp) with hemicelluloses (22.3 wt.%) and lignin (10.3 wt.%), which was disk-milled, or cotton fibers collected from Kendall Q-Tips Cotton Tip Applicators, were used as substrates in the flow cell.

1.3 Anaerobic fluorescent proteins

Escherichia coli TOP10 cells (Invitrogen, Carlsbad, USA) were used for cloning and grown at 37°C in LB medium supplemented with 50 μg/mL kanamycin. *Clostridium cellulolyticum* H10 was cultured at 34°C anaerobically in a modified vitamin medium (Higashide et al., 2011) with cellobiose as the carbon source. For agar plates, 1.0% Bacto agar was added to the medium. The modified vitamin medium was prepared under anaerobic conditions and supplemented with 15 μg/mL erythromycin as appropriate. The *E. coli–C. cellulolyticum* shuttle vector used in this study was pWH199, which was modified from plasmid pAT187 as described previously (Higashide et al., 2011). To insert the anaerobic fluorescent protein

Evoglow (*afp*) (Drepper et al., 2007) into pWH199 downstream of a *Clostridium pasteurianum* ferredoxin (Fd) promoter, a DNA fragment containing the *afp* was amplified from pGLOW-Pp1-stop (The evoglowR basic kit, Evocatal) by PCR using forward primer (5′-CAAAAGGATCCAATGATCAACGCAAAACTCCTGCA ACTGATG-3′) and reverse primer (5′-CAAAAACGCGTTCAGTGCTTGGCCTGGC CCTGCTGCCGGCG-3′). The PCR product was digested with BamHI and MluI enzymes and then ligated into pWH199 treated with the same enzymes, resulting in plasmid pCCFanaerobic. The correct construction of pCCFanaerobic was verified by sequencing. The plasmids were transformed into wild-type *C. cellulolyticum* by electroporation as described by Higashide et al. (2011). The transformants were spread on vitamin agar plates supplemented with 15 μg/mL erythromycin. The plates were incubated at 34°C anaerobically in BD GasPak plastic bags for 5 to 7 days until single colonies appeared.

1.4 Microscopy

A Zeiss LSM 710 confocal microscope (Jena, Germany) was used. The planktonic cell count was determined using a Thoma cell counting chamber (Blaubrand, Wertheim, Germany) and an Axioskop2 Plus microscope (Zeiss, Thornwood, USA) with phase contrast illumination. For live cell imaging of *C. thermocellum*, we tested each of the long-chain dialkylcarbocyanines in the Lipophilic Tracer Sampler Kit (L7781, Invitrogen, Eugene, Oregon, USA) at a concentration of 2.0 μmol/L in anaerobic conditions at 60°C to determine which dye performed best under these

conditions. Based on these results, we used SP-DiOC$_{18}$(3) (EX/EM: 497/513 nm) in this flow cell study. SYTO9 (Invitrogen, Carlsbad, CA) was used to stain harvested cells or end-point samples.

2 Results and discussion

2.1 Fluorescent protein assessment

GFP is commonly used as a fluorescent reporter for live cell imaging studies but it requires oxygen for fluorophore development; thus, it is not suitable for visualizing cells grown under anaerobic conditions. Recently, the fluorescent protein Evoglow (Drepper et al., 2007) was developed which is compatible with both aerobic and anaerobic conditions. To determine whether Evoglow was a suitable alternative for our system, Evoglow was expressed from a plasmid in the genetically tractable mesophilic anaerobe *Clostridium cellulolyticum*. Following growth at 34°C, images were collected demonstrating that Evoglow could be expressed and detected in these cells (**Fig. 2a**). It was noted, however, that cells expressing Evoglow photobleached quickly under the imaging conditions tested. Moreover, although Evoglow was expressed in *Clostridia* strains grown under mesophilic conditions, *Clostridium thermocellum* has an optimal growth temperature of 60°C and molecular genetic methods for *C. thermocellum* (ATCC 27405) are not yet routinely performed. Since Evoglow is limited to use with genetically tractable organisms and the thermostability of Evoglow was unknown, a different approach was adopted to identify fluorescent dyes that were compatible with live cell imaging under anaerobic conditions at high temperatures.

2.2 Fluorescent dyes

Fluorescent dyes are broadly employed for visualizing cells or cellular components for fluorescence microscopy applications. For microbial studies, the cell permeable, nucleic acid dye SYTO9 has proven very useful as it penetrates into nearly all cells and can be used to localize and quantify microbes in sacrificed samples (Hansen et al., 2000; Filoche et al., 2004; Dumitrache et al., 2013). Because SYTO9 binds nucleic acids, however, it can be toxic to cells and unsuitable for long term imaging studies (Tebaldi et al., 2010). The long-chain lipophilic carbocyanines, on the other hand, have been widely used as tracers for live cells as they do not appear to affect cellular viability, development, or basic physiology (Honig and Hume, 1986, 1989). In this study, nine different lipophilic carbocyanines, DilC$_{18}$(3), DiIC$_{18}$(3)-DS, SP-DilC$_{18}$(3), 5,5'-Ph$_2$-DiIC$_{18}$(3), DiOC$_{18}$(3), SP-DiOC$_{18}$(3), DiI$_{18}$(5), DiIC$_{18}$(7), and 4-Di-16-ASP, were tested for fluorescence intensity and toxicity in *C. thermocellum*. Each dye was added to the growth medium at a concentration of 2.0 μmol/L and cell growth and fluorescence was monitored after 24 hr at 60°C. Two of the dyes, SP-DilC$_{18}$(3) and

DiIC$_{18}$(3)-DS, stained the cell membranes of *C. thermocellum* and remained stably fluorescent under thermophilic growth conditions (**Fig. 2b, c**). Of these dyes, SP-DilC$_{18}$(3) showed a brighter fluorescent signal than DiIC$_{18}$(3)-DS (**Fig. 2b**). Hence, SP-DilC$_{18}$(3) was chosen as a fluorescent marker for *C. thermocellum* in this study. Since the dyes were initially tested when *C. thermocellum* was growing on a soluble substrate (cellobiose), SP-DilC$_{18}$(3) was added to a culture of *C. thermocellum* growing in the presence of a solid substrate to determine whether the dye affected the ability of *C. thermocellum* to attach to cellulose. The cell density on cellulose chads for *C. thermocellum* cells treated with SP-DilC$_{18}$(3) was similar to untreated cells stained after harvest with SYTO9 (**Fig. 3a, b**), suggesting that SP-DilC$_{18}$(3) does not interfere with cell attachment.

The effects of SP-DilC$_{18}$(3) concentration on cell growth was also evaluated to determine the optimum concentration for live cell studies. For this study, SP-DilC$_{18}$(3) was added in a range of 0 to 2.5 μmol/L into the medium of *C. thermocellum* containing 0.4% cellobiose as a substrate and growth was measured for 24 hr. These results indicate that higher concentrations of SP-DilC$_{18}$(3) inhibit growth (**Fig. 3c**). Conversely, cellular fluorescence increases with SP-DilC$_{18}$(3) concentration (results not shown). From these data, a concentration of 0.1 μmol/L SP-DilC$_{18}$(3) was chosen for live cell studies since the fluorescent signal was acceptable and the growth rate of *C. thermocellum* was comparable to untreated controls.

2.3 Live cell imaging of *C. thermocellum* growth on cellulosic biomass

Two types of cellulosic biomass, pulp nanofiber chad and cotton fibers, were used as substrates for *C. thermocellum* in separate flow cell experiments. The presence of biomass was detected by microscopy based on its autofluorescence in the red region of the spectrum while the *C. themocellum* cells stained with SP-DilC$_{18}$(3) fluoresced green. *C. thermocellum* growth and attachment were visualized in three dimensions by collecting optical sections at a spacing of 0.5 μm. These Z-stacks are projected as maximum intensity projections in **Fig. 4**. These images demonstrate that *C. thermocellum* attached and formed colonies on the surface of pulp nanofiber chad after 24 hr of incubation (**Fig. 4a**), based on the comparison with the blank images taken at the beginning of the experiments (data not shown). Similarly, biofilms were also formed by *C. thermocellum* on a cotton fiber (**Fig. 4b**). These data indicate that the growth conditions maintained in the flow cell provided a suitable environment for *C. thermocellum* growth and attachment on cellulosic biomass.
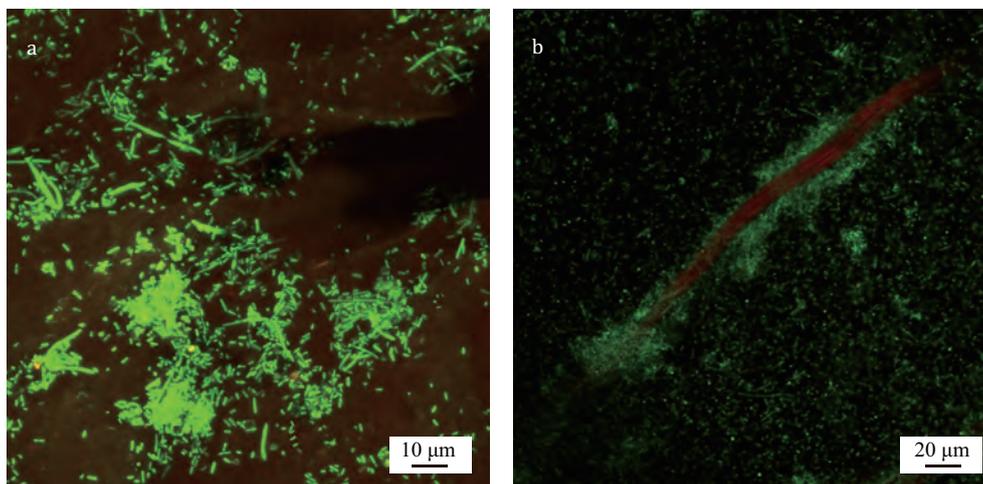
C. thermocellum cells that were visualized during growth, however, seemed to grow more slowly than cells that were not exposed to scanning lasers (data not shown), which is likely the result of photodamage. Proposed mech-
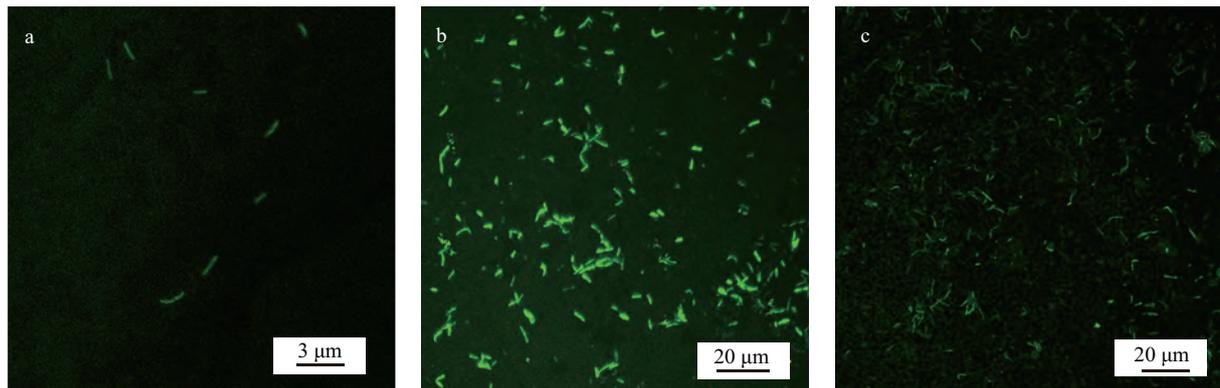
Fig. 2 Evaluation of fluorescent dyes suitable for imaging *C. thermocellum*. (a) *C. cellulyticum* cells grown at 34°C expressing Evoglow from a plasmid; (b) *C. thermocellum* cells grown at 60°C for 24 hr and stained with 2.0 μmol/L SP-DilC$_{18}$(3) or (c) 2.0 μmol/L DilC$_{18}$(3)-DS.
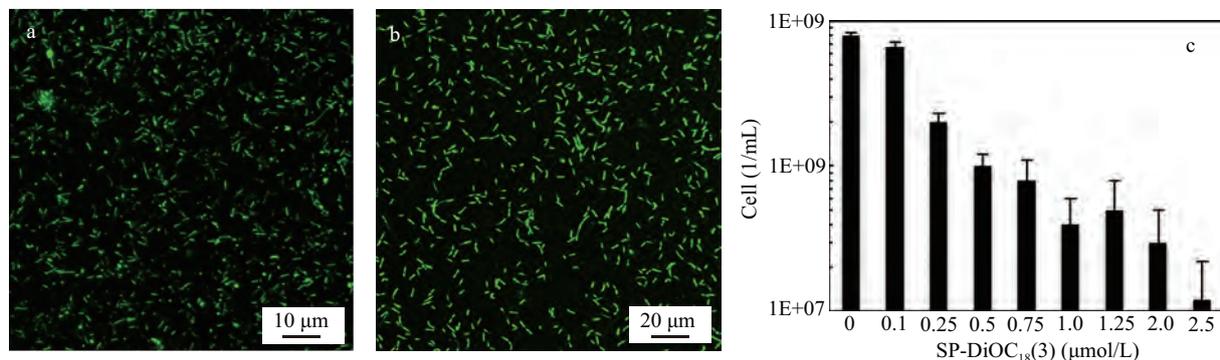
Fig. 3 Growth of *C. thermocellum* in the presence of SP-DilC$_{18}$(3). The attachment of *C. thermocellum* cells to cellulose chads was measured in (a) presence or (b) absence of SP-DilC$_{18}$(3). For the control (b), *C. thermocellum* cells were detected using SYTO9. (c) *C. thermocellum* cells were grown for 24 hr in the presence of different concentrations of SP-DilC$_{18}$(3) to determine an optimal concentration for growth and imaging, each error bar represents the standard deviation of triplicate samples.


Fig. 4 Live cell imaging of *C. thermocellum* cells grown in the flow cell at 60°C under anaerobic conditions. Cells were grown in the presence of (a) pulp nanofiber chad; and (b) cotton fiber. Cells were stained with SP-DilC$_{18}$(3) and visualized after 24 hr growth.

anisms for cellular photodamage include transient local heating (Liu et al., 1996), two-photon absorption (Berns, 1976; Konig et al., 1995), and photochemical processes leading to the creation of reactive chemical species (Calmettes and Berns, 1983; Svoboda and Block, 1994). Although the exact mechanism behind the photodamage of *C. thermocellum* remains unclear, it is likely exacerbated

by frequent scanning (Mirsaidov et al., 2008). This issue is not unique to the flow cell study presented here, but rather is an issue faced for any live cell imaging application involving fluorescence microscopy.

To reduce the damaging effects of laser scanning while still producing useful data, the experimental design was altered to image the cells every 12 hr using only the green
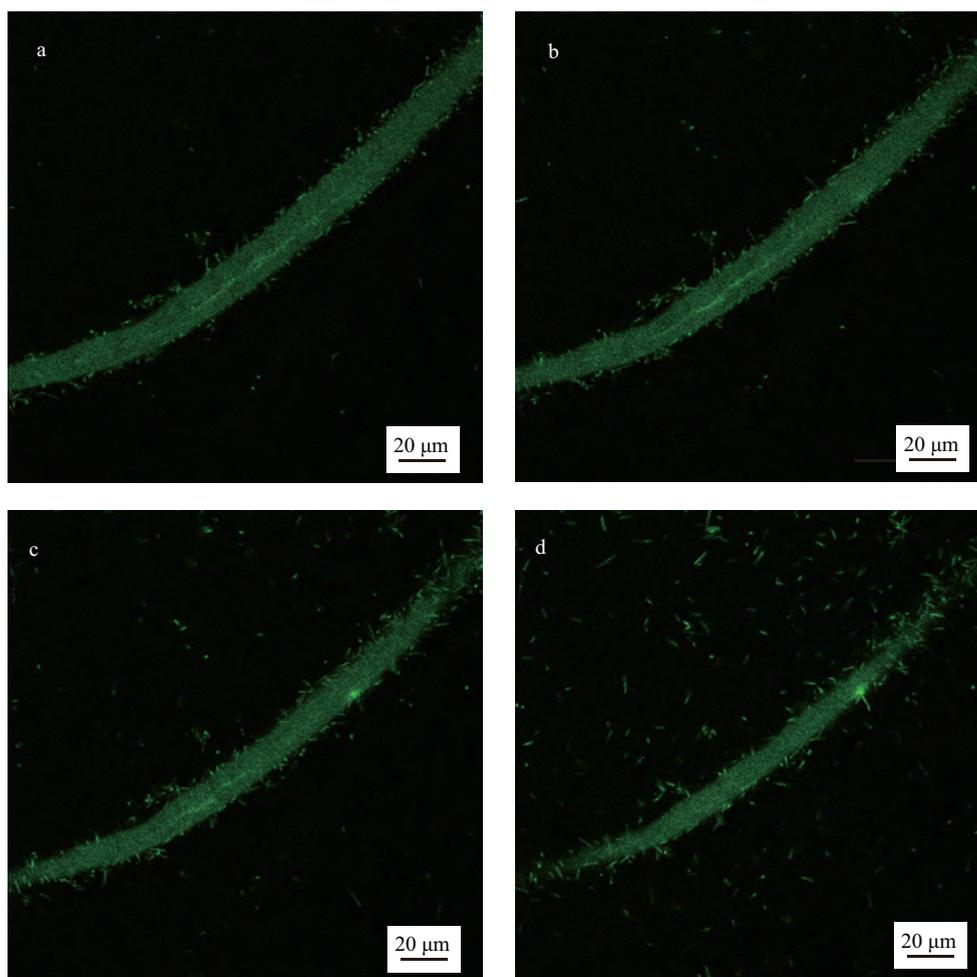
Fig. 5 Temporal dynamics of *C. thermocellum* colonization of a cotton fiber. Cells were stained with SP-DilC$_{18}$(3) and images were collected at (a) 12 hr, (b) 24 hr, (c) 36 hr, and (d) 48 hr after inoculation.

laser. This is a long interval but previous studies suggested that this time frame was suitable for capturing biomass attachment, colony formation, and biomass degradation (Wang et al., 2011b). Importantly, this frequency ensured that the cells had ample time to recover from exposures and continue growth. Using this experimental design, *C. thermocellum* colonization and degradation of a cotton fiber was visualized over the course of 48 hr (**Fig. 5**). Colonization of the cotton fiber by *C. thermocellum* was observed after 12 hr (**Fig. 5a**). At this point, few unattached cells were detected in the bulk solution. With longer incubation times, the attached *C. thermocellum* cells appear to have proliferated and the cotton fiber appears to have reduced size, presumably as a result of microbial hydrolysis (**Fig. 5b, c, d**). At these time points, the number of planktonic cells increased, which is likely due in part to cells detaching from the cotton fiber surface (Wang et al., 2011b).

The exact mechanisms for the photodamage of *C. thermocellum* remain unknown (Berns, 1976; Calmettes and Berns, 1983; Svoboda and Block, 1994; Konig et al., 1995; Liu et al., 1996). Images were collected using a confocal laser scanning microscope which is a popular choice for flow cell studies because it not only detects specific molecules with good signal-to-background ratio, but also restricts photodetection to light originating from the focal plane. However, even though signal is only collected from within the plane of focus, fluorescent molecules are also excited in the out-of-focus volume, which contributes to photobleaching and phototoxicity through absorption of excitation energy along the beam path, and by specimen scattering of both the excitation and emission photons. Alternative microscopy methods may reduce the photobleaching and phototoxicity issues if suitable exposures and time intervals cannot be identified for CLSM. Spinning-disk confocal microscopes, based on the parallel use of many pinholes, can reduce the excitation power needed to capture suitable images and reduce the effects of phototoxicity (O'Malley, 2008). Two-photon excitation is another technology suitable for live cell imaging. Two-photon excitation provides three-dimensional optical sectioning without absorption above and below the plane of focus. Consequently, this technique can be less phototoxic to live specimens, particularly when

used with infrared wavelengths which are less damaging to a variety of live cells (Benninger et al., 2008).

3 Conclusions

A CLSM-based flow cell can be assembled with commercially available equipment and chemicals for live cell imaging of microbes under anaerobic and thermophilic conditions. The lipophilic carbocyanines are economical fluorescent dyes with high thermostability and relatively low toxicity, which can be used for observing microorganisms under aerobic or anaerobic conditions. As with other live cell imaging applications, imaging conditions must be optimized to ensure minimal photobleaching and phototoxicity during acquisition. This experimental design represents one of the first reported methods for observing cell growth in real time under both thermophilic and anaerobic conditions.

Acknowledgments

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