Label-Free Four-Dimensional Visualization of Anaerobically Growing Electroactive Biofilms

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Abstract

Light sheet fluorescence microscopy (LSFM) allows non-destructive, label-free and in vivo imaging of large specimen, even at non-transparent surfaces. We show that LSFM can be applied for label-free analyses of prokaryotes on the example of electro-active biofilms. Biofilm growth is linked to the production of current serving as measure of metabolic activity in vivo by monitoring with high spatial and temporal resolution. After 35 h of exponential growth, a homogeneous biofilm with a thickness of 9 μm was formed. This was followed by a stratification of the biofilm including the formation of 3D structures over the next 100 h. Light reflection was sufficient to visualize the biofilm structure and development over time and the terminal morphology was confirmed using fluorescence staining. This proof of concept on using LSFM for investigation of biofilms opens the door for its application in the entire field of microbial ecology.

Key terms

light sheet fluorescence microscopy; Geobacter; live cell imaging; electroactive biofilm growth

Biofilms represent the most widespread living form of microorganisms in nature (1). Their prevalence ranges from geomicrobiology via microbial corrosion, possessing a high monetary significance, to health-related fields like disease and infection. Thus, mechanisms of biofilm formation and control are of highest relevance. However, the techniques for investigating natural biofilms in vivo are limited (2). Sampling destroys the spatial organization of biofilms and experimental set-ups like flow cells have to be adapted to serve the needs of analysis and hence do not fully resemble the natural habitat. Additionally, labeling (either by staining or by the use of genetic markers) as required for standard confocal laser scanning microscopy (CLSM) limits the applicability to a few model organisms or end-point samples. The shortcomings of established microscopy techniques for studying microbial biofilm growth resulted in an arsenal of methods, for instance, confocal Raman microscopy (CRM) (3), soft X-ray microscopy (4), biophotonic imaging (5), or optical coherence tomography (OTC) (6). All aforementioned methods possess a significant potential, but also inhere inevitable drawbacks like the need of suitable chemical markers (CRMs (3)), degradation at point of exposure (X-ray microscopy (4)), the need for bioluminescent bacteria (biophotonic imaging (5)) or limited resolution for very small cell aggregates (biophotonic imaging (5)), optical coherence tomography (6)).

Live cell imaging of eukaryotic organisms experienced a significant development during the last decade. Especially the advancement in light sheet fluorescence microscopy (LSFM), or selective plane illumination microscopy, provides a highly sensitive, high-resolution imaging platform for rapid three-dimensional imaging of large specimen. In contrast to confocal microscopy, the use of a thin light sheet to scan the object avoids photo damage (refer to Ref. (7) for a comprehensive introduction and comparison to confocal fluorescence microscopy). Due to this minimal illumination...
intensity, an optical analysis with improved temporal resolution leading to four-dimensional data sets becomes possible. Successful application examples of LSFM already cover zebrafish, fly embryos, Caenorhabditis elegans, mouse embryos and organs as well as plants (8, 9). First attempts for the visualization of microorganisms have also been made (10, 11), but natural biofilm development on surfaces has never been investigated. In contrast to most other methods, LSFM is not restricted to samples grown on transparent surfaces, for example, cover glass.

In this study, we show for the first time that LSFM can be used for label-free real-time imaging of natural bacterial biofilm development by continuously monitoring anaerobic biofilm growth and activity. So far, these electroactive biofilms have mainly been analyzed for their productivity (possible as continuous real-time measurement) or for their thickness and structure (destructive analysis for single time points), with very few exceptions including the utilization of CRM (12) and OTC (13). Therefore, detailed understanding of the correlation between growth, structure, and functionality of electroactive biofilms is missing, so far.

**Materials and Methods**

**Microorganisms and Cultivation in Standard Electrochemical Cells**

Growth of the electroactive biofilms was achieved using well-characterized natural wastewater derived electroactive biofilms dominated by *Geobacter* sp. as inoculum (14, 15). Refer Refs. (16, 17) for a general introduction on electroactive biofilms and microbial electrochemical technologies. The biofilms were grown under anaerobic conditions in standard 250 mL three-electrode electrochemical cells. The working electrode (WE) and counter electrode consisted of a stainless steel wired (d = 0.6 mm; Goodfellow GmbH, Germany) graphite rod (d = 10 mm, L = 40 mm, A = 13.4 cm², quality CP-2200; CP-Graphitprodukte GmbH, Germany). The WE served as anode (electron acceptor) at 0.4 V vs. SHE (standard hydrogen electrode). This potential was controlled using a potentiostat (MPG-2; Bio-Logic SAS, Clai, France) and a reference electrode (Ag/AgCl sat. KCl, +0.197 V vs. SHE, SE 11; Xylem Analytics, Germany; Sales GmbH & Co. KG Sensortechnik Meinsberg, Germany). An artificial wastewater medium based on phosphate buffer including acetate (10 mM) was used as only source of carbon and electrons for the microorganisms. Increased current production during biofilm growth was going along with the formation of a visible red biofilm on the working electrode being composed of *Geobacter* sp.

For the first experiment, a WE with a well-grown 36-days-old biofilm was visualized with the LSFM. Therefore, the complete WE was removed from the electrochemical cell and introduced into the measurement chamber of the microscope which was then filled with 20 mL phosphate buffer (1.8 g L⁻¹ Na₂HPO₄, 0.223 g L⁻¹ NaH₂PO₄, 8.5 g L⁻¹ NaCl, pH 7.2).

**Cultivation in LSFM Chamber**

An identical biofilm as described above was used as inoculum for starting the growth experiment in the LSFM setup. In this case, the three electrodes (WE: cuboid graphite rod 20 × 2 × 1 mm, counter electrode: graphite rod 5 × 25 mm and RE, see Supporting Information S1 for details of materials) were assembled in the microscope measurement chamber using a tailor-made set-up (Figs. 2A and S1).

The WE for biofilm growth was placed in a transparent tubing within the imaging chamber. This compartment was connected on the top end to a reservoir made of a 10 mL syringe into which both the counter electrode and the RE were placed and that was continuously deoxygenated with nitrogen gas. All electrodes were in contact with the medium. Buffer exchange was accomplished by a connected tubing at the bottom end to another 10 mL syringe used to carefully replace the solution at the WE in a regular manner. An optically transparent FEP tube (ElringKlinger Kunststofftechnik GmbH, Fluorinated Ethylene Propylene, 3 mm diameter (18)) shielded the working electrode from the solution in the measurement chamber (distilled water, continuously flushed with nitrogen). An ultra-low current module allowing measurements down to 1 pA was used with the potentiostat (SP-200; Bio-Logic SAS, Clai, France) and connected to the electrodes. A constant potential of 0.4 V versus SHE was applied at the WE. Current production was continuously measured and recorded every 30 s. The measurement chamber was kept at 30°C for the whole experiment by the integrated temperature control.

**Microscopy and Microscopic Data Analysis**

A standard ZEISS Lightsheet Z.1 with incubation equipment was used for all experiments (Carl Zeiss Microscopy GmbH, Jena/Germany). The setup was equipped with dual-side illumination and for evaluation the site was used that gave the best signal-to-noise ratio (SNR). Imaging was done at 30°C. Illumination was by 405 nm or 488 nm laser excitation (both 50 mW). Detection was with W Plan-Apochromat 20×/1.0 DIC objective. The signal was detected with a pco.edge 5.5 sCMOS camera. Typical exposure times varied between 100 and 200 ms. For detection of reflected light, an emission filter transmittant for the laser line was employed. For fluorescence detection, the emission band excluded the excitation line.

All 3D renderings and analysis was performed in the arvis Vision 4D software (version 3.1.3). For quantitation, the 3D Denoising, Intensity Threshold and Merge Operation
Figure 1. Visualization of growth substratum without any biofilm (A, scale bar 50 µm) compared to a three-dimensional adult biofilm (B) based on light reflection (here excitation with 405 nm). The same biofilm sample was reanalyzed after LIVE/DEAD nucleic acid fluorescent staining (C) confirming the organization of the individual cells and the three-dimensional organization of the biofilm as seen in reflection. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 2. (A) Schematic of experimental setup combining electrophysiological equipment with the LSFM system. (B) The current production (blue) as a measure of electrochemical and hence metabolic activity and the reflected light mean intensities in gray values of 16 bit images (green) were measured during the exponential growth phase. (C) The pictures (scale bar 50 µm) show top views (maximum intensity projection) on the biofilm at increasing time points (t1-t8, see B) after inoculation based on light reflection (excitation with 405 nm). [Color figure can be viewed at wileyonlinelibrary.com]
of the arivis Vision 4D Analysis Pipeline was used (arivis AG, Munich, Germany).

**Staining Procedure**

The biofilm used for the first experiment was stained using the nucleic acid staining kit LIVE/DEAD® BacLight™ Bacterial Viability Kit (Life Technologies) containing SYTO®9 and propidium iodide (PI) (excitation/emission: 480/500 nm for SYTO®9 and 490/635 nm for PI). The electrode with the biofilm was incubated in 10 mL medium containing 0.3 mL of each staining solution. The biofilms were incubated for 10 min. Afterwards, the medium with the staining solution was exchanged for fresh medium and the biofilm was immediately analyzed. Sample analysis was performed with 488 nm excitation, beam splitter SBS LP 490 nm and emission filter BP 505–545 nm for SYTO®9 and beam splitter SBS LP 560 and emission filter LP 585 nm for PI.

**RESULTS AND DISCUSSION**

In a first experiment, the setup was evaluated for the label-free three-dimensional visualization of the biofilm on its natural surface for growth being in this case an anode, that is, an electrode that serves as terminal electron acceptor. Therefore, a mature electroactive biofilm, grown on a graphite electrode in a separate setup, was introduced into the LSFM measurement chamber. The light reflection (both at 405 nm and 488 nm) of the biofilm surface was sufficient to visualize its three-dimensional structure and to distinguish the biofilm volume from the surface (Fig. 1A,B; Supporting Information Fig. S12A and video S1). Established fluorescent staining (LIVE/DEAD nucleic acid staining) resembled the identical organization of the individual cells and the three-dimensional structures (Fig. 1C; Supporting Information video S2). Further continuous illumination was confirmed of being nondestructive. These results demonstrate that LSFM is suitable for label-free three-dimensional biofilm imaging.

In a next step, the instrumental setup was further adapted to house a blank cuboid graphite electrode (geometric surface area of 0.52 cm²) serving as terminal electron acceptor and surface for growth of an anaerobic electroactive biofilm (see Fig. 2A). The LSFM setup was supplied anaerobically with a buffer solution containing acetate as only electron donor and carbon source. After inoculation from an actively growing electroactive biofilm dominated by Geobacter sp. the formation of an identical biofilm started at the electrode in the LSFM-chamber (14, 15). As the metabolism of electroactive microorganisms is directly wired the external current, it can serve as measure of activity. The exponential growth phase of 35 h (Fig. 2B) was followed by stratification of the biofilm including the formation of 3D structures over the next 100 h.

During the exponential growth phase, a continuous increase in current production (Fig. 2B) and the formation of a homogeneous biofilm with a thickness of approximately 9 μm was observed. Thereby, the maximum current density of 19.1 μA cm⁻² after 35 h is comparable to standard experiments, for example, Refs. (19, 20). The increase in current concomitant with biofilm growth was also supported by the images. We noticed an increase of the mean intensity from 3,200 to 20,150 gray value levels (at 16 bit) after 26 h for a representative position (Fig. 2B). This increase in mean intensity represents an increase in the volume during biofilm formation. At 35 h, intensity levels dropped reflecting either that saturation in growth was reached due to insufficient nutrient supply or, being even more likely, that the biofilm starts to show compaction causing less penetration of the excitation light into the layer. At this point, a further increase...
in the biofilm volume cannot be exactly determined and only the change of the surface structure monitored.

3D denoising for a more quantitative measure of biofilm growth and expansion (Fig. 3) shows that the volume increased in the first 26 h of the experiment from $33 \mu m^3$ at t1 to about $638,321 \mu m^3$ at t5 in the observed region. There was a near linear correlation between produced current and bacterial growth during the initial stages of 22 h (t1 to t5). The nominal decrease to $324,806 \mu m^3$ at t8 is not due to a decrease in biofilm volume, due to the aforementioned change of the biofilm structure.

The formation of 3D structures was first observed after 48 h (Supporting Information Fig. S12B). The observed change in biofilm morphology is highly reasonable and has been described before (21, 22). Initially, the bacteria grow in closest vicinity to the electrode in order to minimize the electron transfer resistance. In this growth phase, the diffusion of acetate to the bacteria and the transport of charge balancing ions (like protons or sodium ions) to the bulk liquid are not limiting. This leads to the formation of a relatively homogenous, dense biofilm (showing already an increased volume over the first 26 h). This changes, when the biofilm grows thicker. From a certain biofilm thickness on (here about 9 µm), the counter ion transport and the acetate diffusion can become rate limiting for the metabolic activity of the single cell, while the overall activity increases (23). This needs further investigation, for example, on the change of the diffusion coefficients, the means of ion transport or the effect of shear stress.

For continuous monitoring of the biofilm growth during the exponential growth phase, regular stacks of 143 images were recorded (x,y step size 0.228 µm resulting in an area of 319 µm × 319 µm, stable z position).

The flexibility of the setup in combination with label-free, continuous, high-resolution, in vivo imaging underlines the extraordinary potential of LSFM for biofilm research and microbial ecology and health in general (11). For electroactive microorganisms, LSFM allows the monitoring of the growth and maturation of the biofilms that is the “structure,” while the current serves as online measure of metabolic activity that is the “function.” Thus, using LSFM, a clear link between structure and function can be established. This structure-function relationship needs to be further investigated not only for anodic, current producing, biofilms, but especially for microbial electrosynthesis (16, 24).

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