

Confocal Laser Microscopy on Biofilms: Successes and Limitations

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Introduction

Biofilms are communities of microbial cells and extracellular polymeric substances (EPS) attached to a surface. Bacteria exist preferentially in the biofilm state in nature and in many engineered systems. In this state, microbes tolerate antibiotics and disinfectants. Bacterial biofilms are now implicated in a wide range of human diseases and infections including cystic fibrosis, otitis media, chronic wounds, artificial joint and prosthesis infection, and many nosocomial infections. Biofilm-related infections and industrial fouling problems cost billions of dollars each year.

Imaging of bacterial biofilms with microscopes has been an essential and transformative method in biofilm research. Fluorescence microscopy can elucidate specific biofilm components and cellular activities that cannot be separated otherwise. In particular, confocal fluorescence microscopy extends that examination through the thickness of a fully hydrated, *in-situ* biofilm, affording the potential for 3D, non-invasive, time-lapse imaging.

This article discusses some striking examples of the insight provided by confocal fluorescence microscopy into biofilm structure, composition, and heterogeneity, and will enumerate some limitations of this imaging process.

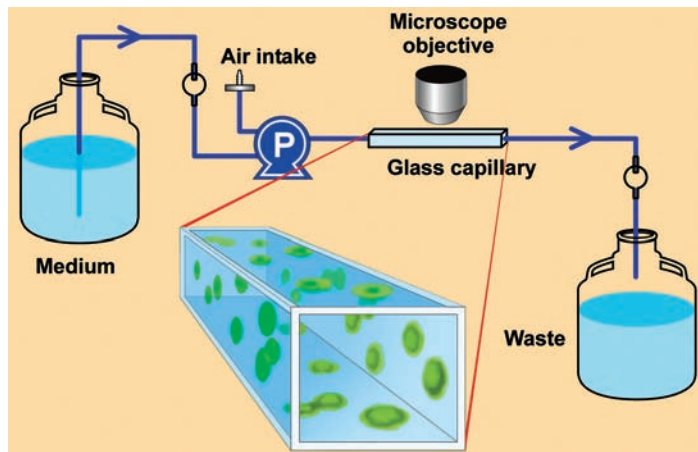


Figure 1 Illustration of a 1mm square glass capillary flow-cell. Biofilm microcolonies are imaged through the glass ceiling of the capillary. Drawing by J.Meyer, MSU-CBE.

Biofilm Growth Methods

Bacteria

Bacteria used in this work were *Staphylococcus epidermidis* ATCC 35984; *Pseudomonas aeruginosa* PA01, and a number of PA01 fluorescent protein constructs: pMF230-*gfp* (constitutively expressed green fluorescent protein), PA01 pMF230-*cfp*, or -*yfp* or PA01 pAB1 (inducible *gfp* expression) (Werner *et al.*, 2004); or a dental biofilm consortium: *Streptococcus oralis* ATCC 10557, *Streptococcus gordonii* ATCC 10558 and *Actinomyces naeslundii* ATCC 19039.

Growth Reactors

Biofilms were grown in either the CDC reactor (Goeres *et al.*, 2005) or a 1mm square glass capillary flow cell (Werner *et al.*, 2004).

In both reactor systems, a no-flow inoculation or attachment period was followed by the continuous flow biofilm growth period until the desired cell density was reached.

The CDC reactor consists of a stirred 1L beaker fitted with a stopper from which hang 8 rods. Each rod holds 3 glass biofilm growth surfaces or coupons, 1.27 cm² in area. The reactor is operated as a continuously stirred tank reactor, using parameters detailed in ASTM Method E2562-07 with the addition that, for the organisms used in these studies, the reactors were incubated at 37°C. After 18 hours of stirred inoculation and 48 hours of growth in continuous flow conditions, coupons were aseptically removed from the reactor, stained, rinsed, and imaged while submerged in water.

The capillary flow cell reactor uses a 1mm square glass tube (Friedrich and Dimmock), roughly 4 inches long, as the biofilm growth surface. The tubing walls are 0.17 mm +/- 0.1mm thick. The reactor is plumbed on either end so that nutrients are pumped in and effluent is pushed out and is operated under continuous flow conditions for 24 hours at 37°C. Capillary flow cell biofilms are usually imaged through the ceiling of the reactor, as shown in Fig. 1.

Imaging

A Leica TSC-SP2 AOBs confocal microscope was used for all imaging, with a laser excitation source of 488, 561, or 633 nm. In some cases, a Spectra Physics MaiTai 2-photon attachment was used instead of, or in addition to, those single photon excitation sources.

All biofilms were stained prior to imaging with stains specific to total bacterial cells, EPS and matrix components, or to a particular aspect of bacterial activity. Flow cell biofilms were stained *in situ*; that is, stain was pumped into the reactor to fill the volume, then the flow was stopped. The biofilm was stained statically for the desired time, then rinsed and immediately imaged. Flow cell biofilms were imaged with an N PLAN 20X 0.4NA dry objective or for very thick biofilms, a 40X W 0.8NA U-V-I 3.3mmWD dipping objective. CDC reactor coupons were removed from the reactor and placed in a 35mm diameter petri dish. 200µL of stain was gently pipetted onto the coupon surface, and allowed to remain for the necessary staining period. When staining was complete, filtered water was gently added to the 200 µL of liquid on the coupon surface to rinse until clear, then water was added to the dish to cover the coupon by roughly 2mm. Biofilm on the coupon surface was imaged immediately in this state with either a 40X W 0.8NA U-V-I 3.3mmWD or a 63X W 0.9 NA U-V-I 2.2mmWD dipping objective. Images were processed in either Imaris software for qualitative presentation (Bitplane Inc.) or MetaMorph software (Molecular Devices) for quantitative analysis.

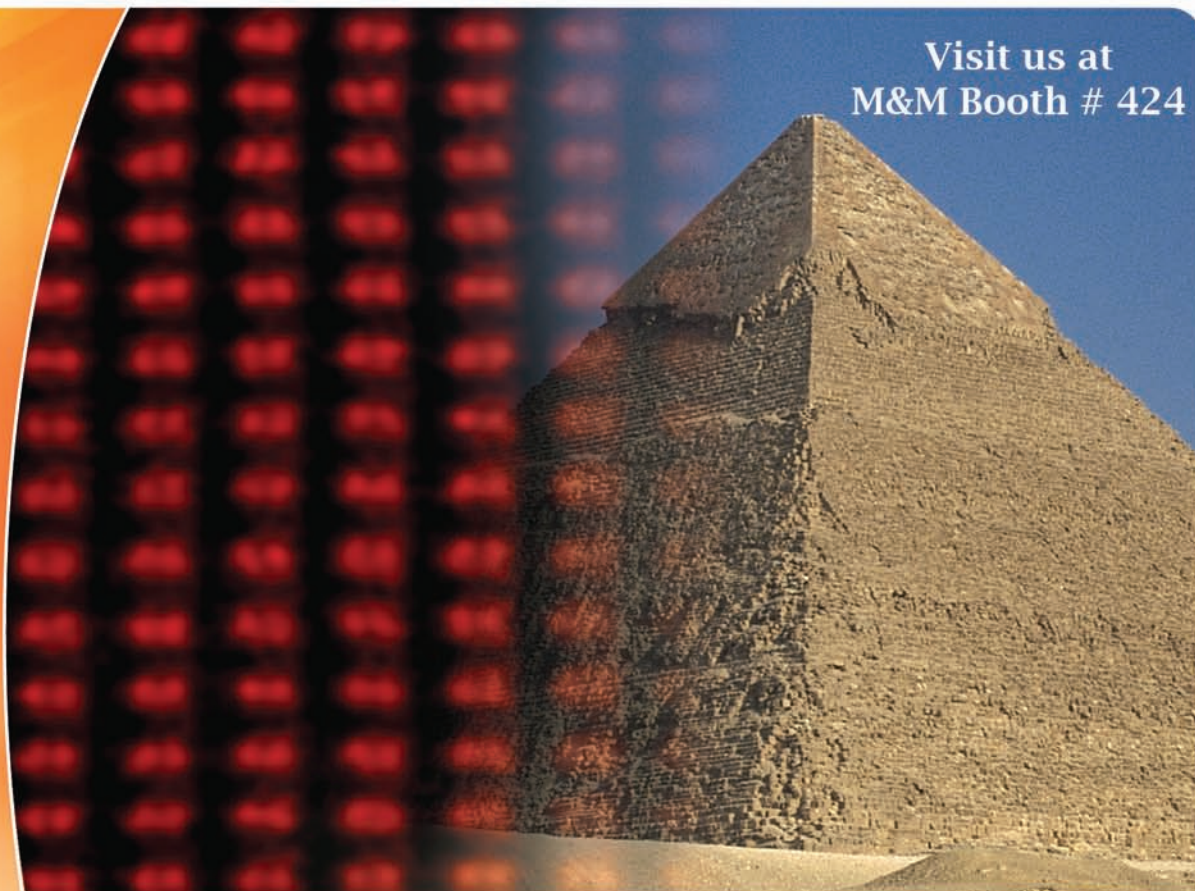
Imaging Results

Strengths of confocal scanning laser microscopy for imaging microbial aggregates include:

3D structural imaging with the potential to discriminate individual cells

A single fluorescent stain for either total or active cells applied to an untreated biofilm can produce the 3D images shown in Figures 2a and 2b respectively. Figure 2a is a shadow projection of a confocal stack of images taken of a *P. aeruginosa* (ATCC 15442) CDC biofilm stained with FM[®]1-43 (Molecular Probes/Invitrogen). FM[®]1-43 has not been used extensively to stain biofilms, so it is not yet well understood as a biofilm probe. It is a lipophilic styryl dye, and it appears to bind only to cell membranes. Our data indicate that FM[®]1-43 binds to all bacterial cells in a biofilm—dead or live—with very high specificity.

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About the image: Sub-Angstrom image of rows of dumbbell-shaped Si atoms. The specimen was prepared with the Fischione Model 1010 Ion Mill. Information is present down to 0.6 Angstrom. Image courtesy of the Oak Ridge National Laboratory.

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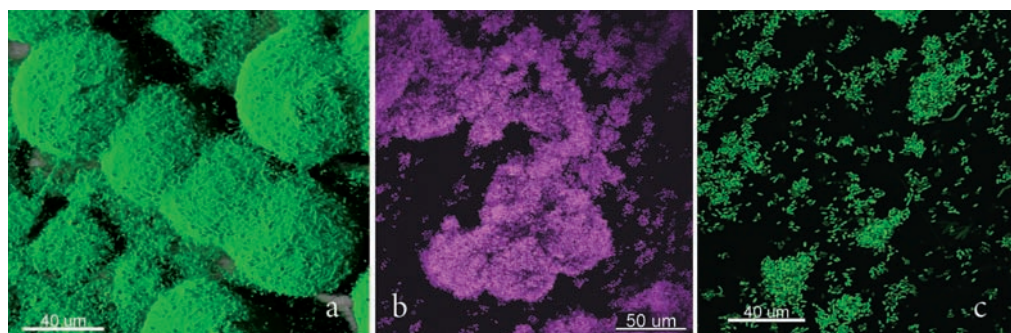


Figure 2 3-D structural imaging with the potential to discriminate individual cells. a) *P. aeruginosa* biofilm stained with FM*1-43; b) *S. epidermidis* biofilm stained with Calcein violet AM; c) individual cells of an FM*1-43-stained *P. aeruginosa* biofilm.

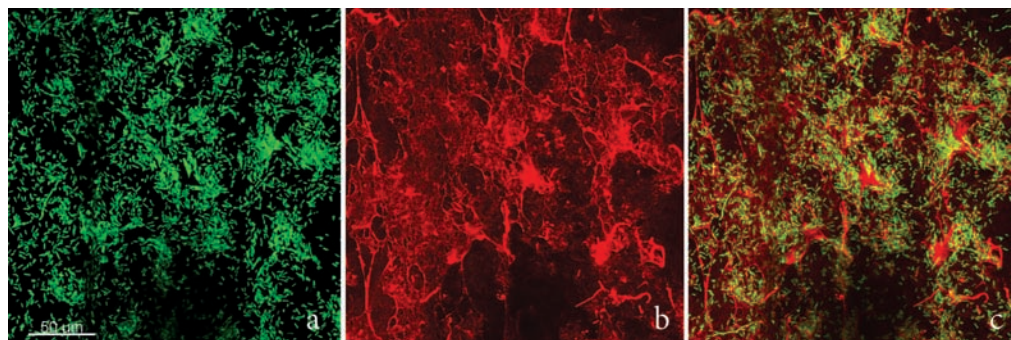


Figure 3 Discrimination of multiple specific constituents or activities. a) *gfp*-expressing *P. aeruginosa* cells; b) the biofilm matrix stained with Bodipy*630/650-X, SE; c) the overlay of cell and matrix images.

Figure 2b is a shadow projection of an *S. epidermidis* CDC biofilm stained with Calcein violet AM (Molecular Probes/Invitrogen). CAM violet is a fluorogenic esterase substrate that passively diffuses into bacterial cells, is hydrolyzed by cellular esterase present in live cells, and is trapped in its fluorescent form inside the cell. The CAMs (available in green, red, violet or blue form) are often used as bacterial activity probes. CAM violet was imaged using the 2-photon confocal, with excitation at 785nm. This biofilm was not treated with an antimicrobial, so one would expect the majority of cells in the biofilm to be “live,” as the staining indicates. Comparison of Figures 2a and b shows clear differences in morphology of pseudomonas biofilms compared to staph. The pseudomonas biofilm has a “haystack” cluster morphology characteristic of that strain, whereas the staph biofilm has a much denser structure, composed of tightly packed cells.

Figure 2c is a close-up of an FM* 1-43 stained *P. aeruginosa* biofilm showing individual cells. These images illustrate the range in visual scale possible with confocal microscopy of biofilms. Images 2a and b are on the scale of community assessment, where larger-scale effects such as disinfection and removal or the effect of fluid shear on biofilm structure can be monitored. In contrast, the ability to visualize single cells allows us the opportunity to monitor the state of individuals in the community. We can observe protein expression in individual cells or watch cells toggle between two physiological states as indicated by different fluorescence emission colors (Vlamakis et al, 2008).

Discrimination of multiple specific constituents or activities

For imaging purposes, biofilm biomass is often divided into three simplified categories: total bacterial cells, active cells, and EPS or matrix. A major challenge of research-relevant biofilm microscopy is to find fluorescent stains that can bind specifically to one of the three biofilm biomass components and also be used in concert with other specifically binding stains.

Two examples of dual staining are shown in Figures 3 and 4. Figure 3 is a confocal image of a *P. aeruginosa* PAO1 pMF230 capil-

lary flow cell biofilm. The green and red channels are shown separately in panels 3a and 3b, and the overlay is shown in panel 3c. The *gfp*-expressing bacteria are green fluorescent, and the biofilm has been stained with red fluorescent Bodipy*630/650-X SE, which has bound to the matrix. Bodipy*630/650-X SE is an amine-reactive dye that may adhere to the matrix or to the lipopolysaccharide capsule of different bacteria depending upon the state, the age of the biofilm, the species, or growth conditions. The biofilm matrix is notoriously difficult to stain. Because of this, panel a of Figure 3 might be considered a typical, straightforward image of a biofilm, and it is what we see in transmitted light images. It is the addition of the previously invisible structural components shown in panel b that hints at the complexity of biofilms.

Figure 4 is also a flow cell biofilm, but this time the bacterial strain is the inducible *gfp* pseudomonas construct PAO1 pAB1. In this experiment, the biofilm was treated with 20 mg/L chlorine for 1 hour, before inducer was added. The green fluorescence reveals biofilm bacteria that were

still able to produce *gfp* after the chlorine treatment. Finally, the biofilm was counterstained with rhodamine B (red fluorescent), which should stain all cells, regardless of state. The layer of red fluorescence shows that cells on the periphery of the biofilm cluster were unable to produce *gfp* after chlorine exposure, or were dead, whereas the cells in the more protected center of the cluster were still able to produce protein, and would be considered live on the basis of this activity.

Non-invasive time-lapse imaging

Using time-lapse confocal imaging, the action of an antimicrobial against a biofilm can be visualized in real time and then analyzed quantitatively. CAM green is a fluorogenic esterase substrate, like CAM violet discussed above, that is excited with a 488 nm laser and is viewed with a typical FITC emission bandwidth. CAM is trapped inside intact bacterial cells once it is hydrolyzed. If, however, the cell membrane is compromised by an antimicrobial agent such as chlorine or a quaternary ammonium compound, the fluorescent CAM will leak out. In this context, CAM becomes a probe for intact cells, or a means of separating live (green) and compromised or dead (not green) cells. We can record this entire process using time lapse imaging on the confocal: cell loading with CAM, application of the antimicrobial and loss of fluorescence.

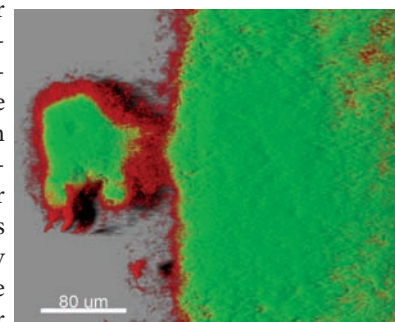


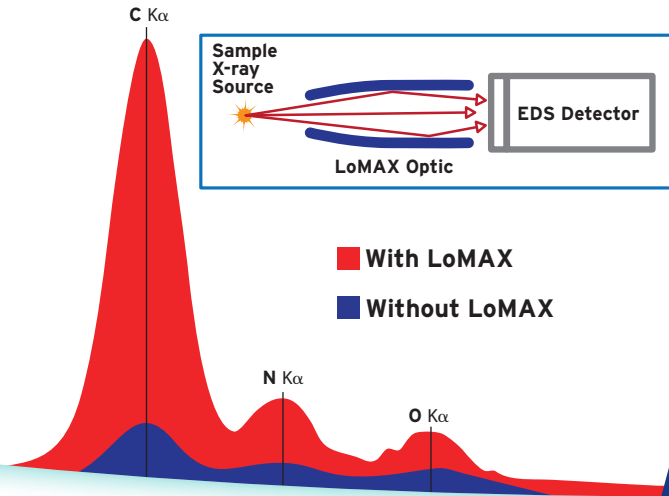
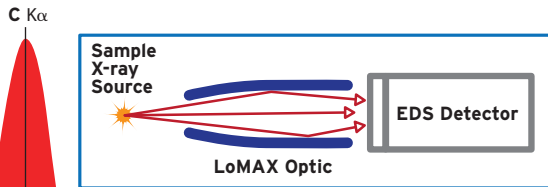
Figure 4 Imaging of antimicrobial efficacy. A *P. aeruginosa* biofilm, imaged after chlorine treatment, *gfp* induction, and counterstaining with rhodamine B (red). Green cells are still able to produce *gfp*, while the red cells are not.

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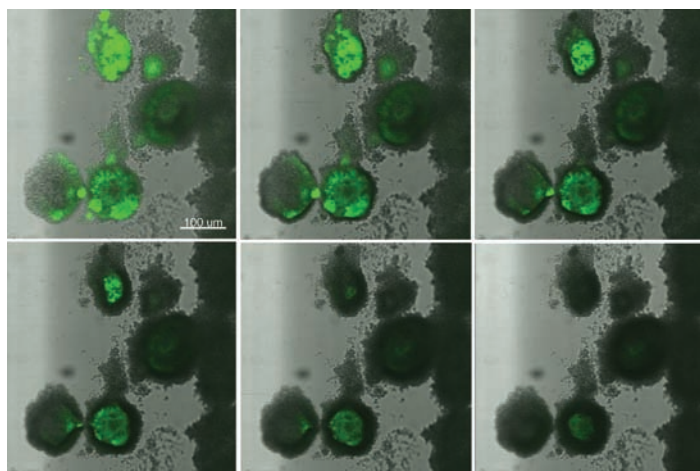


Figure 5 Non-invasive time-lapse imaging. The images in this panel are a series of overlays of transmission and fluorescence channels, beginning at the upper left, and ending at lower right. Biofilm clusters are stained with green fluorescent CAM, which leaks out of live cells during a 15 minute treatment with the mouthwash additive CPC.

Using image analysis we can measure the fluorescence intensity in a region in the biofilm throughout the time stack, calculate parameters such as penetration time and depth, and gain insight into differing modes of action for antimicrobial agents, as well as protective mechanisms exhibited by biofilms. A series of still images taken from such a time stack is shown in Figure 5a - f. Dental biofilm clusters attached to the ceiling of a capillary tube were imaged. The series shows an overlay of transmitted and fluorescence channels. It begins with the cells already loaded with CAM, and follows a 15 minute-long treatment under flow conditions with 0.05% cetylpyridinium chloride (CPC), a quaternary ammonium compound which is added to mouthwash and toothpaste. Over the 15 minute treatment time, CPC can be seen to work in through the outer layers of the biofilm, and a front of permeabilized, non-fluorescent cells moves in towards the center of the clusters. The region of more protected, still-fluorescent cells in the center of each cluster becomes progressively smaller as the CPC works its way in. It is important to note that this advancing edge of permeabilized cells is not a typical *spatio*-temporal pattern: not all treatments “chew” their way in to the center of a biofilm cluster. Some other antimicrobial agents tested affect cells throughout the entire cluster nearly simultaneously.

Evolution of 3D structures in time

The final example of a successful application of fluorescent confocal microscopy to biofilms is that of utilizing *xyz* scans over time. In this research, *P. aeruginosa* PAO1 pMF230*cfp* and PAO1 pMF230*yfp* were grown together in a capillary flow cell. The two-color system allowed microcolonies of the same strain to be identified and imaged early on, then followed through the development process to a mature biofilm. Figure 6 shows a series of *xyz* confocal stacks collected over a period of 72 hours, as a cyan cluster grows and expands in a predominantly yellow fluorescent field. This kind of imaging provides unique insight into community organization and development.

Limitations of confocal laser microscopy for imaging microbial biofilms

While confocal laser microscopy holds great potential for revealing the complexities of biofilm structure and behavior, there are also a number of limitations that we have encountered. These are listed briefly below.

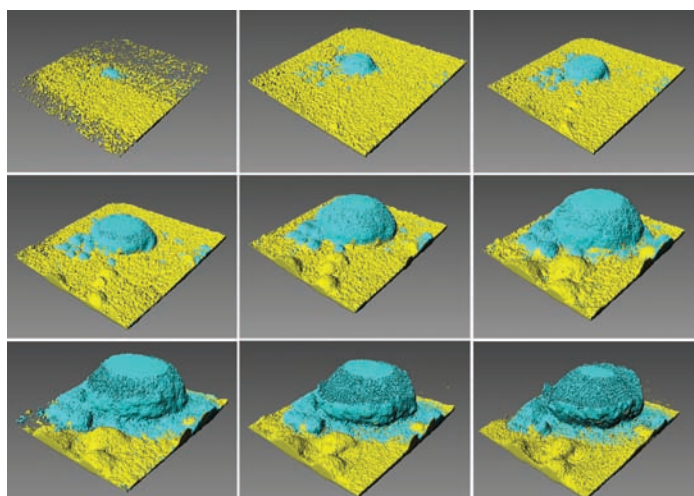


Figure 6 Evolution of 3-D structures in time. Time-lapse series showing *xyz* scans of a two-color *P. aeruginosa* biofilm (*cfp* and *yfp*) as it develops over 72 hours.

- Light is attenuated by the biofilm, limiting the depth of imaging to approximately 50 to 100 microns, depending upon the opacity of the biofilm.
- Individual cells are often only discernable at the surface of the biofilm or in areas where the biofilm is sparse. Within dense cell clusters, it usually becomes impossible to resolve individual cells.
- Tapping the potential to measure specific chemical and biological properties depends upon the availability of appropriate fluorescent probes (*i.e.*, component-specific probes) and the ability to cleanly separate emission signals.

Conclusions

Confocal imaging has become a critical aspect of biofilm research. Fluorescent stains, probes and reporter genes, used with a system designed for imaging provide the opportunity for creative and relevant research to elucidate biofilms. The information obtainable by these techniques has been valuable for discovering fundamental characteristics of biofilms, and has also provided useful insights for industry, such as visualizing in real time, and the action of specific antimicrobials. There is plenty of room and tremendous need for further exploration of fluorescent stains and combinations of stains that have not previously been applied to biofilm imaging. ■

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