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 prosthetic 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.

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 Reactions
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 200μL 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 (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 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.

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.
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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.

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 pMF230cfp and PAO1 pMF230yfp 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.

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.

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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References

ASTM E2562-07 Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor


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