
Single-cell resolution imaging of bacterial biofilms

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Status

Biofilms are ubiquitous surface-attached communities of bacteria embedded in an extracellular matrix [1]. Biofilms represent a predominant bacterial lifestyle in nature and in man-made environments – ranging from the ordinary, e.g., sewage systems, to the exotic, e.g., Yellowstone hot springs. However, biofilms can be problematic in clinical and industrial settings: they can cause chronic infections such as in cystic fibrosis patients and they can damage materials in industry.

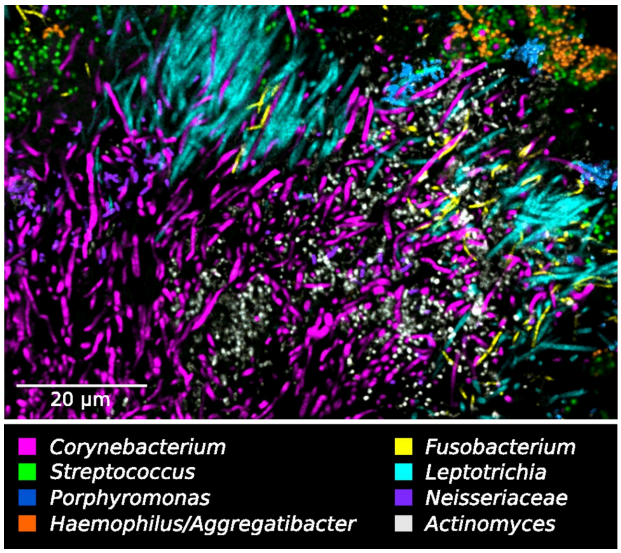

Fig. 1. Oral biofilms imaged with CLASI-FISH. Different colors correspond to different bacterial genera. Image adapted from Ref. [2].

Imaging is increasingly playing a central role in biofilm analyses. Indeed, high resolution imaging of biofilm internal structures has revolutionized our understanding of how cells are organized in biofilms, how extracellular matrix components are distributed, and how biofilm structures respond to environmental challenges including shear flow, starvation, and osmotic stress.

Fixation of biofilm samples treated with DNA stains enabled bulk visualization of biofilm cells and the general contours of the biofilms. Recently, techniques including fluorescence *in situ* hybridization (FISH) of 16S rRNA were combined with fixation to define biogeographies inside polymicrobial biofilms. A seminal example (Fig. 1 and Ref. [2]) shows an oral biofilm in which Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH) was combined with metagenomic sequence analysis to reveal the spatial organization of the different bacterial genera. Specifically, it was shown that the oral bacterial consortium consisted of a radially arranged, nine-taxa structure organized around a core of filamentous cyanobacteria. A step-by-step accession model was proposed to explain the observed pattern, taking into account the metabolic and adhesive properties of the different bacterial species.

In addition to revealing the spatial distributions of bacteria in mixed-species biofilms, biofilm fixation procedures allowed high resolution imaging and segmentation of biofilms into individual cells. The ability to identify positions and orientations of cells in biofilms allowed researchers to use concepts and tools from colloidal physics to rationalize the observed cellular packing. The first work to exploit single-cell imaging in fixed biofilms used line scanning confocal microscopy to study *Staphylococcus epidermidis* biofilms [3]. By tracking the centers of the spherical cells and analyzing the radial distribution function, biofilm compactness parameters were quantified. Surprisingly, the

packing characteristics of cells in biofilms were found to vary dramatically even within a single sample, ranging from a disordered liquid phase to an open, fractal-like structure. More recently, using images of fixed samples obtained at different times during biofilm maturation, the architectural transitions undergone by cells in *Vibrio cholerae* biofilms were revealed [4]. Specifically, in a mature biofilm cluster, vertical cells reside at the biofilm center and radially orientated cells are present at the periphery.

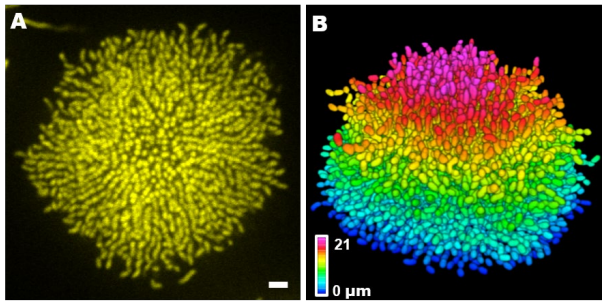

Figure 2. Single-cell live imaging of biofilms. (A) Cross-sectional image of the bottom cell layer of a growing *V. cholerae* biofilm cluster at 18 h and (B) the corresponding segmented image with color-coding according to *z* position. Scale bar: 3 µm. Images adapted from Ref. [5].

Fixed biofilm imaging strategies transformed the understanding of which cells are present and where each cell resides. However, fixed biofilms could not be used to study temporal changes in biofilm formation, preventing understanding of the full dynamical process of biofilm development from a single cell to a three-dimensional (3D) community. Recent improvements in confocal microscope design, availability of fluorescent proteins possessing increased photostability and quantum efficiency, and development of new computer algorithms that are particularly useful for resolving small objects (i.e., bacterial cells) made imaging of living biofilms with single-cell resolution possible. First, *V. cholerae* biofilm clusters were imaged as they grew from the founder cell to 10,000 cells [5]. The biofilm clusters transition from a two-dimensional (2D) branched morphology to a dense 3D cluster with a nematically ordered core (Fig. 2). Combining single-cell live imaging, mutagenesis, and agent-based computer simulations revealed the cellular ordering inside the biofilm to be the physical consequence of a competition between biofilm expansion and cell surface adhesion [6]. Specifically, during the initial 2D expansion phase, friction with the surface due to surface adhesion proteins impedes the expansion of the biofilm cluster. As a result, cells at the center of the cluster are under compressive force and transition from lying parallel to the surface to re-orienting perpendicular to the surface. Once verticalized, these cells send their progeny further into the third dimension, thereby creating a dome-shaped 3D biofilm cluster.

Current and Future Challenges

The ability to image individual live cells in 3D bacterial biofilms now makes possible the study of their behaviors. By measuring the levels of expression of specific genes using fluorescent reporters, questions that can now be addressed include whether cells in different regions of a biofilm produce distinct subsets or levels of quorum-sensing autoinducers, whether cells in close proximity to one another coordinate to build particular portions of the biofilm architecture, and whether persister cells that survive transient antibiotic exposure arise in specific locations in the biofilm. Moreover, single-cell imaging can be extended to polymicrobial biofilms to reveal the rich dynamics underpinning how different species compete or cooperate during biofilm development.

Several challenges need to be overcome to generate the next wave of information regarding spatiotemporal development of biofilms. For example, can we follow cell lineages inside biofilms? Can we resolve the shapes of individual biofilm-dwelling cells? Can we image biofilms in complex 3D environments similar to those found in nature? Here, we highlight some approaches that are being pursued to address these challenges.

1) The time resolution of imaging will need to be increased to follow cell lineages in 3D biofilms. Lineage tracing has revolutionized our understanding of eukaryotic development. Indeed, the fate of each individual cell has been mapped in model organisms such as nematodes and zebrafish using lineage tracking [7]. Achieving lineage tracing for 3D biofilms is more challenging due to issues arising from the small sizes of bacterial cells along with phototoxicity and photobleaching, which currently

limit the time between consecutive imaging frames to > 10 minutes. Ideally, one would need at least 5-10 time-steps between each bacterial division event, which imposes an upper limit of 3-5 minutes between image acquisitions. Light sheet microscopy is poised to overcome this challenge due to the dramatically reduced phototoxicity and photobleaching of the technology [7]. The Dual-View Inverted Selective Plane Illumination microscopy (diSPIM) setup is particularly well suited for visualizing bacterial biofilm geometry [8, 11]. In addition to hardware improvements, new software developments are required to trace lineages in 3D biofilms, including improving segmentation accuracy and incorporating tracking algorithms similar to those developed for 2D bacterial colonies.

2) Image analysis procedures need to be extended to bacteria with more complicated shapes than rods and spheres, for example, filamentous bacteria and spirochetes [9]. In the case of *V. cholerae*, which has been the focus of many of these single-cell analyses, the bacterium is a curved rod. However, current confocal optical resolution does not allow quantitative assessment of individual cell curvature inside of biofilms. Having algorithms that can extract detailed shape information for individual cells will allow researchers to ask questions such as: do bacteria change their shapes during biofilm maturation? What is the correlation between individual bacterial shape and the overall biofilm architecture? How does heterogeneity in bacterial shape affect cellular packing inside a biofilm?

3) The current imaging setup (i.e., unconstrained biofilms on flat glass) is far from the environments in which biofilm-forming bacteria reside in nature or during infection. The geometries, stiffnesses, surface topographies, and surface chemistries of substrates all influence biofilm development. Thus, a key challenge will be to adapt imaging procedures such that complex surfaces with non-ideal optical properties are made suitable for high-resolution imaging. As an example, bacteria such as *Bacillus subtilis* form biofilms in the 3D soil environment. Optically transparent particles mimicking soils (such as irregularly shaped glass beads) need to be developed and coupled with new imaging protocols that can handle the non-flat geometry of the growth substrate. Moving away from solid substrates, many clinically relevant biofilms, such as those made by *Pseudomonas aeruginosa,* form while embedded in the mucus layer in the lungs of cystic fibrosis patients [10]. Mucus is complex with respect to thickness, chemical composition, and stiffness, and furthermore, these properties vary from patient to patient. Understanding how the heterogeneous mucus substrate as well as other in-host or in-environment milieu affect biofilm development will be required if the promise of new therapeutic or industrial approaches to chronic biofilm infections/biofouling/clogging is to be met.

Concluding Remarks

The ability to visualize the location, orientation, shape, and progeny of individual cells in 3D biofilms has begun to define the key biophysical steps driving biofilm formation. Ultimately, we envision that single-cell imaging technology will become routine for biofilms. Together with genetic and biochemical perturbations, and the use of non-uniform substrates, we will gain a comprehensive understanding of how bacteria build their communities cell by cell.

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