

# New Technologies for Studying Biofilms

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**ABSTRACT** Bacteria have traditionally been studied as single-cell organisms. In laboratory settings, aerobic bacteria are usually cultured in aerated flasks, where the cells are considered essentially homogenous. However, in many natural environments, bacteria and other microorganisms grow in mixed communities, often associated with surfaces. Biofilms are comprised of surface-associated microorganisms, their extracellular matrix material, and environmental chemicals that have adsorbed to the bacteria or their matrix material. While this definition of a biofilm is fairly simple, biofilms are complex and dynamic. Our understanding of the activities of individual biofilm cells and whole biofilm systems has developed rapidly, due in part to advances in molecular, analytical, and imaging tools and the miniaturization of tools designed to characterize biofilms at the enzyme level, cellular level, and systems level.

## INTRODUCTION

The results of recent biofilm characterizations have helped reveal the complexities of these surface-associated communities of microorganisms. The activities of the cells and the structure of the extracellular matrix material demonstrate that biofilm bacteria engage in a variety of physiological behaviors that are distinct from planktonic cells (1–3). For example, bacteria in biofilms are adapted to growth on surfaces, and most produce adhesins and extracellular polymers that allow the cells to firmly adhere to the surfaces or to neighboring cells (4–6). The extracellular material of biofilms contains polysaccharides, proteins, and DNA that form a glue-like substance for adhesion to the surface and for the three-dimensional (3D) biofilm architecture (4). The matrix material, although produced by the individual

cells, forms structures that provide benefits for the entire community, including protection of the cells from various environmental stresses (7–9). Biofilm cells form a community and engage in intercellular signaling activities (10–19). Diffusible signaling molecules and metabolites provide cues for expression of genes that may benefit the entire community, such as genes for production of extracellular enzymes that allow the biofilm bacteria to utilize complex nutrient sources (18, 20–22). Biofilm cells are not static. Many microorganisms have adapted to surface-associated motility, such as twitching and swarming motility (23–28). Cellular activities, including matrix production, intercellular signaling, and surface-associated swarming motility suggest that biofilms engage in communal activities. As a result, biofilms have been compared to multicellular organs where cells differentiate with specialized functions (2, 29). However, bacteria do not always cooperate with each other. Biofilms are also sites of intense competition. The bacteria within biofilms compete for nutrients and space by producing toxic chemicals to inhibit or kill neighboring cells or inject toxins directly into neighboring

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cells through type VI secretion (30–33). Therefore, biofilm cells exhibit both communal and competitive activities.

The complexity of biofilms (and the complexity of the technologies required to study biofilm activities) is compounded by the fact that biofilms are inherently heterogeneous (34). In natural biofilm communities, the biofilm structure may be stratified as different organisms migrate to their optimal position for access to light, oxygen, nutrients, secondary metabolites, and signaling compounds (35–37). Even in biofilms composed of one species, subpopulations of cells show heterogeneous activities. In a recent review (34), three general factors that contribute to biofilm heterogeneity were described: (i) physiological heterogeneity, where the bacteria adapt to their local environmental conditions. As oxygen or nutrients diffuse into the biofilms from their sources and are utilized by the bacteria, chemical concentration gradients develop. The chemical gradients may intersect and overlap with gradients of waste products or signaling compounds, forming many unique microenvironments within biofilms that are not mimicked by growth of planktonic bacteria. The bacteria respond to their local environmental conditions, and therefore the physiology of individual cells may differ from other cells that are in close proximity (38–40). (ii) Genetic variability, where mutations may occur in initially clonal populations of cells. Cells within the community may develop mutations causing cellular differentiation (41, 42). This genetic variability may account for the identification of biofilm subpopulations that differ from the rest of the community, such as the rugos or mucoid strains that arise during biofilm growth (43–47). (iii) Stochastic gene expression events, where subsets of cells express the same genes at different levels, even when cells experience very similar environmental conditions (48, 49). Stochastic events promote division of labor, increasing the functional and morphological complexity of biofilms (50). Stochastic events may help account for the formation of some subpopulations of cells that differ from the rest of the community, such as the persister cells, which are cells that have enhanced tolerance to antibiotics compared to other cells in the community (51–54). Since chemical and physiological heterogeneities are tightly linked, variation within biofilm communities is an important factor to consider in designing experiments for biofilm studies.

Many discoveries concerning the physiology, genetics, and ecology of microorganisms growing in biofilms have been made since the first edition of this book. These discoveries have been made possible in part due

to the rapid technological advances that have occurred in biological research. Included in these advances is the adaptation of new molecular, analytical, and imaging strategies. Technological developments include the application of “omics” technologies in biofilm research. For example, high-throughput DNA sequencing technologies (next-generation sequencing) have been used for genomics and metagenomics studies and have provided insight into the genetic coding potential of biofilm organisms and into biofilm community structures (55–58). Transcriptomics approaches, including RNAseq, microarrays, and reverse transcription quantitative PCR (RT-qPCR), have advanced our understanding of global and localized gene expression processes that occur within biofilms (59–62). Mass spectrometry has also facilitated our understanding of biofilm proteomics and will be useful for metabolic profiling of biofilms (metabolomics) (63, 64).

Biofilm imaging has also provided a greater appreciation for the complexity and dynamics of biofilms. Advances in imaging technology have led to the ability to obtain 3D images of hydrated biofilms in real time. Fluorescent proteins, which are available in a variety of colors, allow imaging and differentiation of the bacterial cells (36, 65–68). When fused to promoter sequences or other proteins, the fluorescent proteins also enable imaging of localization in microbial gene expression (5, 69). Fluorescent staining combined with confocal scanning laser microscopy (CSLM) and high-speed computing provides information on cell localization and heterogeneity. A few fluorescent stains are now available for certain components of extracellular materials, providing 3D structural analysis of the biofilm extracellular material (70–73). Fluorescent *in situ* hybridization (FISH) has provided information on the community structure of biofilms (74–78). Other imaging approaches allow characterization of biofilm chemistry, including nuclear magnetic resonance imaging that provides information on water dynamics within biofilms (79, 80), while Fourier transform infrared spectroscopy (FT-IR) analysis and Raman imaging of biofilms allow characterization of cellular and extracellular compositions.

The use of new technology for the study of biofilms is dependent upon the scales of interest in characterizing biofilms. These scales can be at the systems level (using omics or imaging approaches), the cellular level (using approaches such as microfluidics and laser microdissection), or the gene/enzyme level (using mutagenesis, enzyme activity, and gene fusion approaches). By combining these different strategies, it may be possible to obtain a comprehensive, systems-level analysis of the

structure, function, and dynamics of microbial biofilms. In this article, we review some of these new technologies to study biofilms and provide information on some emerging technologies that will likely be applied to biofilm research.

## TRADITIONAL METHODS FOR BIOFILM GROWTH UNDER LABORATORY CONDITIONS

Biofilms are studied in their natural environment and in laboratory scale bioreactors, which are designed to provide models of natural biofilms. The bioreactors can be considered in two general formats: operating under static conditions or continuous flow conditions (Table 1). Using static conditions, biofilm bacteria may undergo growth phases, (lag, exponential, and stationary phases) as in typical planktonic laboratory cultures. Continuous flow reactors are usually operated under “wash-out” conditions. In this case, the residence time of the bioreactor chamber is shorter than the doubling time of the bacteria. This results in most of the planktonic cells being washed out of the reactor, while only those bacteria that are able to adhere to a surface remain within the reactor. The surfaces containing the biofilms may be coupons that can be removed from the reactor for analyses, or even the walls of the reactors, which can be imaged directly.

The static biofilms have been particularly useful for a number of biofilm studies (Fig. 1). In particular, O’Toole and coworkers utilized a relatively simple method to perform high-throughput screening of transposon mutants that were impaired in biofilm formation (26, 81). In this screening method, strains of bacteria, each with a random transposon insertion, are incubated in the wells of microtiter plates. Following incubation, the planktonic cells are removed by washing, and the biofilm cells that remain associated with the wells of the plate are stained with a colorimetric indicator such as crystal violet. The amount of crystal violet taken up by the cells is then assayed, providing a quantitative indicator of the cellular amount of the biofilm, and the mutant strains are compared to the wild-type strains. This approach has become a well-accepted model for biofilm formation and is often used as a first step in determining if a mutation in a particular gene affects the ability of the bacteria to adhere to a surface and develop into a biofilm. Once the putative biofilm-impairment mutation is identified by sequencing the site of transposon insertion, the mutant strains can be compared to the wild type using lower-throughput approaches (such as imaging or molecular

approaches). A modification of this microtiter plate biofilm assay uses the Calgary device (82). In the Calgary device, rather than assaying the biofilm that grows on the wells of a microtiter plate, the biofilms grow on pegs that are immersed in the wells of the microtiter plate. The advantage of this approach is that the bacteria must adhere to the pegs to be assayed. This reduces the interference of planktonic cells, which may remain at the bottom of the microtiter plate wells following washing but are not actually biofilm bacteria.

Biofilms may also be cultivated under static conditions on coupons placed in the wells of microtiter plates or as pellicles at the air-water interface (83). Depending on the organism cultivated (and the native environment of the isolate) certain cells may prefer growth under static conditions. For example, Chimileski et al. (84) showed that the *Archaeon Haloferax volcanii* forms thick biofilms when cultured under these static conditions but did not form substantial biofilms when cultured under continuous flow. This type of growth may reflect the preference for static conditions where these *Archaea* are commonly found in their natural environment.

Another type of static biofilm is the “colony biofilm” (85). Colony biofilms are cultivated on filters that are placed on the surface of agar petri plates. The filters are then transferred to fresh medium at regular intervals, giving the biofilms a semi-continuous supply of fresh nutrients. The greatest advantage of this approach is that these biofilms are easy to grow, using inexpensive laboratory material. The biofilms formed using this approach are generally thick and are therefore useful for a variety of experiments, such as cryoembedding and thin-sectioning to obtain vertical cross-sections of the biofilms (86). By using differential staining on colony biofilms, information on the heterogeneity of biofilms has been obtained (38). In addition, colony biofilms have been useful for determining gene expression heterogeneity within biofilms (61, 62). Colony biofilms have also been used to assay the rate of diffusion of antibiotics through model biofilms (87). Of course, the disadvantage of these biofilms is that since there is no continuous flow of medium, the bacteria are not forced to adhere to a surface or to the matrix material. Since there is no wash-out, planktonic cells may interfere with the biofilm assays.

Continuous-flow reactors have advanced biofilm studies by providing better mimics of natural biofilms. Reactors commonly used for continuous flow include the CDC reactor (88), the drip-flow reactor (89), and flow reactors designed for CSLM imaging (90–92) (Fig. 2). The CDC reactor is a fairly large-scale reactor

**TABLE 1** Methods for *in vitro* cultivation of biofilms

Format/technique	Experimental features	Applications and examples	References
<b>Static biofilms</b>	<ul style="list-style-type: none"> <li>• Low or no shear</li> <li>• No replacement of medium</li> <li>• No cell washout</li> </ul>		
Microtiter plate	<ul style="list-style-type: none"> <li>• High throughput</li> <li>• Limited biomass</li> </ul>	<ul style="list-style-type: none"> <li>• Phenotypic screening of mutant libraries</li> <li>• Attachment and early biofilm development studies</li> <li>• Biomass quantification with staining</li> </ul>	<a href="#">26</a> , <a href="#">81</a>
Calgary device (MBEC)	<ul style="list-style-type: none"> <li>• High throughput</li> <li>• Peg material may be modified</li> <li>• Biomass may be recovered from pegs</li> <li>• Limited amount of biomass</li> </ul>	<ul style="list-style-type: none"> <li>• Phenotypic screening of mutant libraries</li> <li>• Antibiotic susceptibility studies</li> <li>• Microscopy with fluorescent probes</li> <li>• Biomass quantification with staining</li> </ul>	<a href="#">82</a>
Colony biofilm	<ul style="list-style-type: none"> <li>• Large biomass in short amount of time</li> <li>• Inexpensive laboratory materials</li> <li>• Low throughput</li> </ul>	<ul style="list-style-type: none"> <li>• Antibiotic susceptibility and penetration studies</li> <li>• Chemical gradient measurements using microelectrodes</li> <li>• Heterogeneity studies using microscopy and fluorescent probes</li> <li>• Cryosectioning studies for gene expression heterogeneity</li> </ul>	<a href="#">38</a> , <a href="#">40</a> , <a href="#">61</a> , <a href="#">62</a> , <a href="#">85</a> , <a href="#">87</a>
<b>Continuous flow biofilms</b>	<ul style="list-style-type: none"> <li>• Continuous supply of fresh medium</li> <li>• Adjustable shear force</li> <li>• Low to medium throughput</li> </ul>		
CDC reactor	<ul style="list-style-type: none"> <li>• Special surface materials may be used</li> <li>• Multiple biofilms are formed simultaneously</li> <li>• Suitable for time-course study</li> <li>• May be used for anaerobic cultures</li> </ul>	<ul style="list-style-type: none"> <li>• Antibiotic susceptibility/viability studies</li> <li>• Microscopy studies with fluorescent probes</li> <li>• Applicable for omics studies</li> </ul>	<a href="#">88</a>
Drip flow reactor	<ul style="list-style-type: none"> <li>• Special surface materials may be used</li> <li>• High gas transfer</li> <li>• Heterogeneous biofilm</li> <li>• Large biomass in short time</li> </ul>	<ul style="list-style-type: none"> <li>• Antibiotic susceptibility/viability studies</li> <li>• Chemical gradient measurements using microelectrodes</li> <li>• Heterogeneity studies using microscopy fluorescent probes</li> <li>• Cryosection and laser capture microdissection followed by transcriptomic analysis</li> <li>• Biofilm-immune cell interaction</li> </ul>	<a href="#">38</a> , <a href="#">61</a> , <a href="#">89</a>
Imaging flow cells	<ul style="list-style-type: none"> <li>• Real-time detection</li> <li>• Surfaces can be modified</li> <li>• Appropriate for short-time experiments</li> </ul>	<ul style="list-style-type: none"> <li>• Real-time imaging</li> <li>• Monitoring attachment, development, and detachment phases</li> <li>• Microscopy with fluorescent tags</li> <li>• Attenuated total reflection Fourier transform infrared spectrometry</li> <li>• Hydrodynamics in biofilm by nuclear magnetic resonance</li> </ul>	<a href="#">40</a> , <a href="#">79</a> , <a href="#">80</a> , <a href="#">91</a>
<b>Microfluidics</b>	<ul style="list-style-type: none"> <li>• Miniaturization</li> <li>• Custom platform</li> <li>• Adjustable shear force</li> </ul>		
Microfluidics flow cells	<ul style="list-style-type: none"> <li>• Precise control of hydrodynamics</li> <li>• Low to medium throughput</li> </ul>	<ul style="list-style-type: none"> <li>• Microscopy with fluorescent tags</li> <li>• Spatial heterogeneity</li> <li>• Quorum sensing</li> <li>• Effect of hydrodynamics on biofilm growth and detachment</li> <li>• Formation of streamer phenotype</li> <li>• Measurements of adhesion forces</li> </ul>	<a href="#">123</a> , <a href="#">124</a> , <a href="#">125</a> , <a href="#">126</a> , <a href="#">127</a> , <a href="#">131</a> , <a href="#">133</a>

(continued)

**TABLE 1** Methods for *in vitro* cultivation of biofilms (continued)

Format/technique	Experimental design	Applications and examples	References
Micropatterned polymer stencils	<ul style="list-style-type: none"> <li>High throughput</li> <li>Both static and flow conditions</li> <li>Surface materials can be changed or coated</li> <li>Topographically patterned surfaces can be used</li> </ul>	<ul style="list-style-type: none"> <li>Microscopy with fluorescent tags</li> <li>Chemical heterogeneity and quorum sensing</li> <li>Attachment and early biofilm development</li> <li>Spatial heterogeneity</li> </ul>	<a href="#">103</a> , <a href="#">121</a>

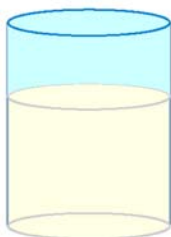
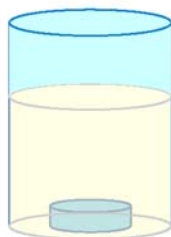
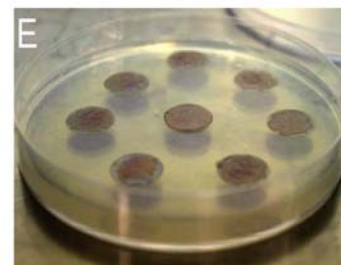
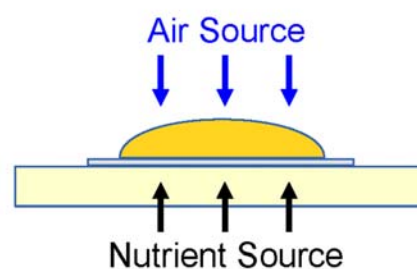
that provides medium inlet and outlet ports. The reactor chamber contains rods with removable coupons that can be made of a variety of different materials, such as glass, hydroxyapatite, or metals. The biofilms form on the surface of the coupons, which may then be removed aseptically over time. Experiments performed on coupon biofilms include direct imaging of the samples using CSLM or other imaging approaches, cell viability studies (colony forming unit and direct counts of bacteria), or omics studies. Since the CDC reactors are usually run under wash-out conditions with fluid shear, the biofilms formed on the coupons are often thick and mimic natural biofilms. The disadvantage of this approach is that the reactors often have large medium reservoirs, making this approach low-throughput.

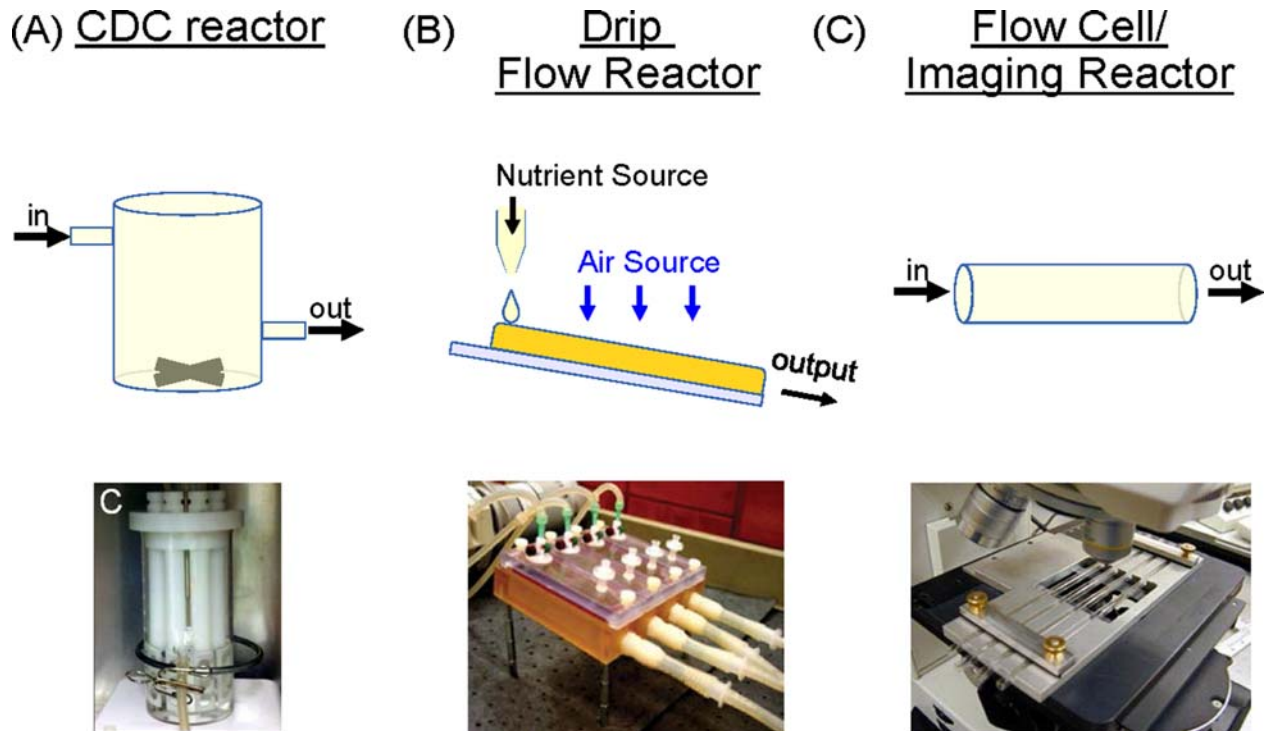
A similar type of continuous flow reactor is the drip-flow reactor ([89](#)). In these reactors, medium is dripped

onto the surface of a coupon, and the medium flows across the coupon. Biofilms cultured using this format are often thick and provide a good mimic for some natural biofilms, where there is fluid flow across a surface with an air interface. Biofilms cultured using the drip flow format often show heterogeneity across the slide from the initial site of the medium input to the outlet. In some cases the biofilms develop streamers, as observed in many environments such as streams with rapid fluid flow. Biofilms cultured under drip-flow conditions are amenable to cryoembedding and thin-sectioning, allowing studies of biofilm vertical heterogeneity ([61](#), [93](#)). Drip-flow cultured biofilms have also been used in combination with microelectrode sensors to study chemical gradients within biofilms ([39](#), [94](#)).

Biofilms may also be cultured under continuous flow conditions using imaging flow cells ([90–92](#)). These flow

**FIGURE 1** Examples of methods for biofilm cultivation under static conditions. (A) Biofilm cultured at the air-water interface, forming a pellicle. Published with permission from reference [83](#). (B) Biofilm cultured on a glass coupon under static conditions. Published with permission from reference [84](#). (C) Example of biofilm growth as a colony biofilm. Published with permission from reference [84](#). [doi:10.1128/microbiolspec.MB-0016-2014.f1](https://doi.org/10.1128/microbiolspec.MB-0016-2014.f1)

**(A) Static/Batch****(B) Static/Coupon****(C) Colony Biofilm**



**FIGURE 2** Examples of continuous-flow reactors for biofilm cultivation. (A) CDC reactor with medium inlet and outlet ports. Biofilms form on coupons arranged on removable Teflon rods. Published with permission from reference 88. (B) Drip-flow reactor with medium inlet and outlet ports and air exchange ports. Biofilms form on removable slides. Published with permission from reference 89. (C) Capillary flow cell for imaging biofilms. Published with permission from [http://centerforgenomicsciences.org/research/biofilm\\_flow.html](http://centerforgenomicsciences.org/research/biofilm_flow.html). doi:10.1128/microbiolspec.MB-0016-2014.f2

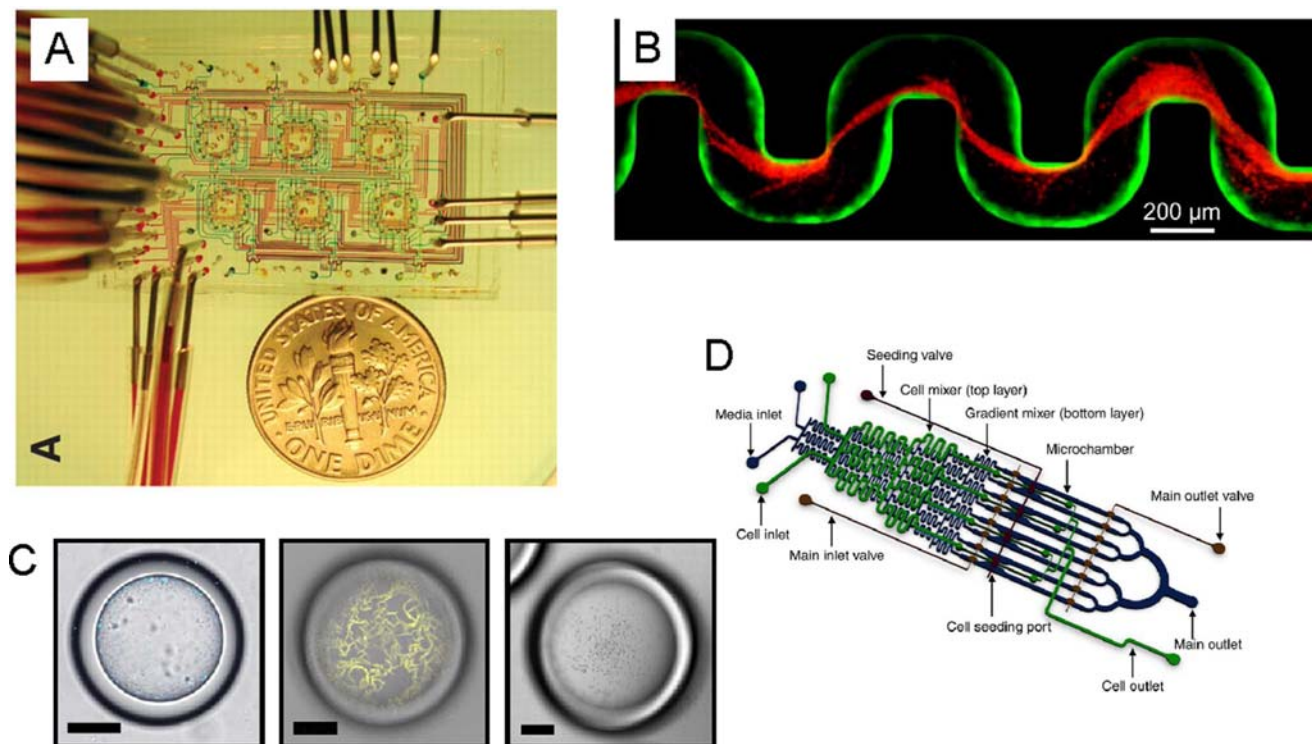
cells usually contain a window, composed of a microscope cover slip, that allows observation of the biofilms by microscopy. Imaging of these biofilms by CSLM provides information on the 3D architecture of the biofilms. A modification of this approach is the capillary flow cell biofilm (95–97), in which biofilms are cultured in small square capillary tubes. Biofilms that form on the walls of the capillary tubes can then be directly imaged. A disadvantage of this approach is that because the tubes are so small, they clog easily and therefore are most appropriate for short-term experiments.

### MINIATURIZATION APPROACHES TO BIOFILM CULTIVATION AND CHARACTERIZATION

In many cases, high-throughput genetic and microbial physiology studies have relied on miniaturization of bio-reactors (Fig. 3). An important development in the study of biofilms at the cellular level has been the use of microfluidics. Microfluidics is the precise manipulation and control of fluids in microscale channels that are typically

less than 100  $\mu\text{m}$ . The channel dimensions define the volumes that are handled, which range from nanoliters to picoliters. Microfluidics is a field that has grown since its inception in the 1990s, following the advent of technology from the semiconductor industry and the field of microelectromechanical systems. Microfluidic devices, commonly called “lab-on-a-chip” or “miniaturized total analysis system ( $\mu\text{TAS}$ )” devices, allow rapid sample assaying by a reduction in sample volumes (98).

The field of microfluidics is characterized by the study of fluid flow at microscale lengths, which greatly differs from macroscale flow. At this length scale, the flow of fluids is laminar and is described by low Reynolds numbers ( $Re$ ).  $Re$  is a dimensionless quantity defined by the ratio of inertial to viscous forces in a fluid. In microfluidic systems,  $Re$  lies in the laminar regime, where mixing due to turbulent flow is nonexistent and molecular transport across streamlines occurs through diffusion, instead of convective mixing. The modeling and computational analysis of fluid flow in microfluidic channels is simplified under these conditions. At submillimeter-length scales, forces that become significant include



**FIGURE 3** Examples of microfluidics approaches applied to biofilm research. (A) Example of a microfluidics device for precise control of fluids. Published with permission from reference [110](#). (B) Biofilm streamers forming within a microfluidics flow channel. Published with permission from reference [127](#). Microdroplet biofilm reactor showing phenotypic switching of cells and simultaneous change in expression from cyan fluorescent protein to the yellow fluorescent protein. Published with permission from reference [259](#). (D) Schematic representation of microfluidics flow cell. Published with permission from reference [133](#). [doi:10.1128/microbiolspec.MB-0016-2014.f3](https://doi.org/10.1128/microbiolspec.MB-0016-2014.f3)

interfacial tension and capillary forces. These can be tuned to manipulate the wetting properties, and therefore flow or movement, of fluids in microfluidic devices. Due to the tunability of the surrounding fluid flow and chemical composition, microfluidics allows a remarkable degree of control over the physical and chemical environment of microorganisms, whose sizes are within the same order of magnitude as that of microfluidic channels. Since these small microenvironments can be easily manipulated using microfluidics, this technique is useful for studying samples from individual cells to small populations. In addition, microfluidic devices are often made from materials that are compatible with light microscopy, such as optically transparent polydimethylsiloxane (PDMS) bonded to glass, which is optimal for viewing single cells and fluorescent particles or dyes.

There has been a wealth of information obtained from the study of single bacterial cells and small populations of cells in microfluidic devices and in other microfabricated environments ([99–104](#)). Microfluidics has been used as a new approach for cultivating bio-

films, for studying the dynamics of biofilm formation and detachment, for high-throughput assays, and for biofilm sensing. These studies may be important in understanding the early stages of biofilm growth. Examples include the effect of antibiotics upon individually isolated bacterium ([105](#)), the evolution of bacterial antibiotic resistance in connected microenvironments ([106](#)), cell variability in bacterial persistence ([107, 108](#)), quorum sensing in small numbers and aggregates of bacteria ([109](#)), and growth rates and lineages of single cells or small populations in microscale channels or chemostats ([99, 110–112](#)). Microfluidics has also been applied to multigene analysis of single cells using microfluidic digital polymerase chain reaction ([113](#)), the sorting of individual cells in droplet microfluidics ([114](#)), isolation of bacteria in fluids ([115–117](#)), and bacterial chemotaxis ([118, 119](#)). There are alternative methods for studying small numbers of bacteria in confined spaces that are not microfluidics-based but offer the same advantages as microfluidics, including controlled microenvironments and microscopically viewable substrates ([104, 120](#)).

Since small volumes are easily handled using microfluidics, large-parameter spaces can be systematically explored at fast rates, making high-throughput sampling possible. Thus, these lab-on-a-chip technologies become complementary to traditional experimental methods in the laboratory. In one study, arrays of biofilms were created using micropatterned polymer stencils (121). Various bacterial and fungal biofilms were grown upon hundreds of circular stencil arrays that were 100  $\mu\text{m}$  in diameter. These high-throughput arrays of biofilm substrates were interfaced within a microfluidic device to study biofilm growth in both static and flow conditions. In another study, biofilms were exposed to eight different concentrations of signals in a device containing eight microchambers with a single gradient diffusive mixer to perform high-throughput testing of chemical compounds (122, 123). Another chip was able to generate gradients of dissolved oxygen (124).

Microfluidics has been used to study the effect of hydrodynamics on the growth and detachment of biofilms. By changing the flow velocity and fluid shear in locations within the devices, biofilms can be grown to various thicknesses within the channels. For example, *Staphylococcus epidermidis* was found to grow as a monolayer in regions of high flow velocity and multilayered in regions of low flow velocity (125). Other studies have examined suspended filamentous biofilms or streamers of *Pseudomonas aeruginosa* that are formed in curved microfluidic channels (126). Those studies showed that secondary flows within the device cause the formation of streamers. These biofilm streamers are a major component of biofilms in natural, medical, and industrial environments (127).

Biofilms are comprised of rigid bacteria, soft viscoelastic extracellular matrix, and small molecules that may provide signaling or generate forces for spreading and survival (128, 129). The physical properties of biofilms may be characterized using microfluidics and can provide a fundamental understanding of the physical properties of biofilms at the microscale. For example, a combination of CSLM and a flexible microfluidic device was used to characterize the viscoelastic properties of *S. epidermidis* and *Klebsiella pneumoniae* biofilms (130). In addition, precise control of fluid forces by microfluidics has been used to measure adhesion forces of pili in cellular attachment to surfaces (131).

Since biofilms frequently exist as a heterogeneous community of cells (34), microfluidic techniques have been used to organize the spatial and temporal location of cells and to simplify the complexity of these heterogeneous populations. One study used a combination of molecular genetics, microfluidics, and microscopy to

show a fitness trade-off between two strains of *Vibrio cholerae* biofilms, in which one strain that was able to produce extracellular polymeric substance (EPS) competed with another that was deficient in its ability to produce EPS (132). That study showed that a single phenotype, extracellular polymeric substance secretion, governs a fitness tradeoff between colonization of biovolume and dispersal to new locations. In another study, a quorum sensing circuit was used to create a dual-species biofilm where a microfluidic device was used to control the consortia of bacteria (133).

## OMICS APPROACHES TO BIOFILM STUDIES

### Genomics and Metagenomics Approaches for Biofilm Studies

The advent of high-throughput sequencing technologies (such as Illumina, Roche 454, SOLiD, and Pacific Biosciences [PacBio]) (134) has allowed investigators to obtain genome-scale information on biofilms. Some of these technologies also provide information on genome modification (epigenetics) (135), which may be one mechanism that affects gene expression and physiology of biofilm cells. These new sequencing technologies have been used to obtain complete genome sequences of thousands of microorganisms, and the cost of obtaining genome sequences continues to drop, making complete genome sequences of many more strains feasible. The next-generation sequencing technologies combined with functional genomics studies (such as transposon mutant libraries and transcriptomics studies) have provided information on cellular activities within biofilms, the role of essential genes in biofilm formation, as well as the community structure of natural biofilms.

Next-generation sequencing technologies can provide billions of nucleotides of sequence for an individual sample. Therefore, this technology can be used to obtain sequence information on entire microbial communities (metagenomes). Analyzing data of conserved genes from the community (such as 16S ribosomal RNA and multi-locus sequence typing) allows the characterization of the microbial community structure and, to some extent, the abundance of individual microorganisms. Since metagenome data are useful for obtaining information on all genes (not just conserved genes), this information can be used to obtain the genetic potential of microbial communities.

Metagenome analysis has been applied to low-diversity biofilms, including the microbial community associated with the later stages of lung degeneration of cystic fibro-

sis patients, which is composed of very few species, particularly *P. aeruginosa* and *Burkholderia cepacia* (136). Another low-diversity environment analyzed by metagenomics is a deep subsurface sample, where the complete genome of an essentially monospecies community was assembled (137). Biofilms may have an intermediate level of diversity, particularly when environmental conditions impinge on a community, allowing growth of only certain types of microorganisms that are adapted for that environment. An example of biofilm communities that have been analyzed by metagenomics is the biofilms associated with acid mine drainage (138–140). Biofilms associated with thermal features of hot-spring communities have also been characterized by metagenome analysis (141–143). Next-generation sequencing technology is amenable to very complex microbial communities and has been used to characterize community structures of the human microbiome, including the oral biofilm microbiome (58, 144, 145) and the gut microbiome (146–148).

While obtaining genomic and metagenomic data is now relatively inexpensive and fast, analyzing the sequence information can be complicated and time-consuming. With most next-generation sequencing technologies, the sequence reads are short, and therefore the data must be assembled or else the reads must be binned onto the sequence of known microorganisms. New sequencing methods include long reads with PacBio sequencing and paired-end reads (149). Bioinformatics tools are being developed that allow the assembly and analysis of genomic and metagenomic DNA sequence data (e.g., 150–154). While these and other bioinformatics approaches have provided vast amounts of information on the structure and coding potential of a variety of different natural biofilms, complete assembly of genomes is still difficult. Repetitive and similar sequences in metagenome data are difficult for computer programs to handle and can lead to errors such as chimeric sequences and gaps in genome sequences.

Metagenome assemblies are complicated by the diversity of the biofilm community, which in natural environments can range from low diversity to very complex community structures. Several approaches have been developed to generate metagenome information from short reads of next-generation sequence information from biofilm samples. McLean and Kakirde (155) described two general approaches. One approach uses the isolation of DNA directly from the environment, fragmenting the DNA and performing direct sequencing using next-generation sequencing. This approach has the advantage of providing high coverage of DNA sequence

from the environmental sample. One disadvantage of this approach lies in the difficulty in determining the source of the DNA (for example, whether the DNA is derived from cellular or extracellular material or if it is from live or dead cells). Assembling the reads into genome information is also complicated, since assembly may generate chimeric sequences or incomplete data sets. Binning the data based on known marker genes or genome sequences is useful for analyzing the number of reads associated with a particular organism. Binning is dependent on whether genome sequence information is available for the organisms isolated from that particular environment. McLean and Kakirde (155) also discussed the use of metagenomic sequencing for biofilms by first using large DNA fragment libraries cloned into fosmid, cosmid, or bacterial artificial chromosomes (BACs). The advantage of this approach is that the linkage of genes can be obtained, since the sequence reads are obtained from adjacent large DNA fragments. This helps reduce misassembly and chimeric assembly. A disadvantage of this approach is that an additional DNA cloning step is required, which may discriminate against certain DNA sequences and therefore reduce the diversity of DNA sequences available in the metagenome library.

An emerging approach for obtaining DNA sequences directly from environmental samples without first cultivating the bacteria is the use of single-cell genomics (156, 157). This approach was recently applied to a biofilm from a hospital sink, where a new phylum of uncultivated bacteria was identified (158). With this approach, single cells are first isolated by fluorescent activated cell sorting, laser tweezers, or microfluidics. The genomic DNA of the isolated cells is then amplified by using multiple strand displacement amplification to obtain enough DNA for sequencing (159). The advantage of this approach is that chimeric sequences are reduced, since the sequence information is known to be derived from individual cells. Therefore, this approach provides not only information on the type of organism in the environment, but also information on the organisms' metabolic capabilities, as was shown for the novel hospital sink isolate. With the decrease in cost and time for sequencing as well as improvement in sequencing approaches, single-cell genomics has the potential to become a major technique for metagenomic analysis of environmental biofilms.

### Transcriptomics Approaches for Biofilm Studies

For many years investigators have asked the question “What makes biofilm cells different from planktonic

cells?” Questions about the physiology of biofilm bacteria include: Why do infectious biofilms have enhanced resistance to antibiotics compared to planktonic cells? Why do they show enhanced resistance to host defensive processes? How do environmental biofilm cells differ in metabolic activities compared to the same strains growing planktonically? One approach to address these questions is to analyze the global expression profile of the bacteria when cultured under biofilm conditions and under planktonic conditions. The RNA expressed under the different conditions is isolated, and all RNA transcripts are quantified. These transcriptomics studies have been applied to a variety of biofilms and have provided important information on the gene expression patterns that are unique to biofilm cells (e.g., 60, 160–164). Until recently, transcriptomics studies have relied on microarrays and RT-qPCR. With the advent of next-generation sequencing technologies, it is now possible to analyze the entire transcriptome of biofilms by RNAseq, the sequencing of cDNA libraries generated from pools of RNA. With this RNAseq approach, the number of RNA transcripts for each gene can be quantified for cells growing under different conditions such as in biofilm and in planktonic cells.

For microarray studies, the total RNA from cells cultured under the different conditions is isolated. The RNA is reverse transcribed to cDNA using reverse transcriptase and hybridized to oligonucleotide probes arrayed on a surface. The fluorescently labeled cDNA is then quantified, using either one-color arrays (Affymetrix-style arrays) or two-color technology, where the cDNAs from the different samples are labeled with different fluorescent dyes. The array data are normalized so that a direct comparison between samples may be made. The data from these biofilm studies as well as other transcriptome studies are deposited in the Gene Expression Omnibus database and are freely available for other investigators to examine for new patterns in gene expression (165, 166). While microarrays have been very informative for transcriptome studies, standard microarrays are made to detect known and predicted genes and thus cannot detect small RNAs located at intergenic regions. Several studies used tiling array, a subtype of microarray with customized chips carrying partially or nonpartially overlapping oligonucleotide probes to cover the whole-genome sequence. With tiling array, an abundance of small RNAs as well as posttranscriptionally modified RNAs can be detected (167, 168).

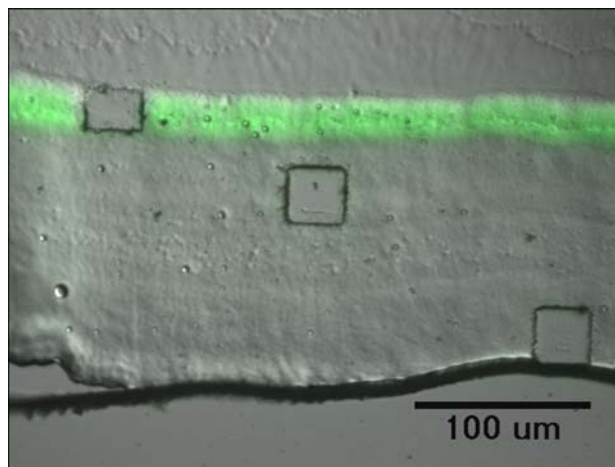
With the advent of next-generation sequencing, it is now possible to obtain a quantitative description of the bacterial transcriptome using sequencing (RNAseq)

(169). RNAseq has several advantages over microarray studies for transcriptome analysis of biofilms. The greatest advantage of RNAseq is that an array platform does not need to be available for every organism of interest, making this approach amenable to any species of *Bacteria*, *Archaea*, or *Eukarya*. RNAseq is amenable to metatranscriptomics studies, since the genome sequences of the organisms of interest do not need to be known *a priori* (170, 171). It has been applied to several environmental biofilm samples, including characterizing the transcriptome of the human oral microbiome (172) and hot-spring microbial mats (173, 174). Another advantage is that RNAseq provides information that may not be available with the microarray approach, such as the presence of small noncoding RNAs expressed from intergenic regions and information on the sites of promoter sequences and operon structures (175–178).

While the global transcriptomics approaches provide an average value for gene expression over the entire biofilm population, they do not provide information on local heterogeneity of the cells (34). Several approaches, including microfluidics, have been developed to address the question of localized transcriptional processes within biofilm. Another approach to studying localized biofilm processes is the combination of laser capture microdissection and transcriptomics (61, 62) (Fig. 4). Using these approaches, subpopulations of bacteria from different regions of biofilms are isolated and captured using laser capture microdissection. RNA and DNA are extracted from the captured cells and analyzed directly by using RT-qPCR and qPCR for individual gene analyses (61, 179). Alternatively, the RNA or DNA may be amplified using multiple strand displacement for transcriptomics analysis (62). This approach has the advantage of excellent sensitivity and large dynamic range and therefore can be performed for quantitative analysis of gene expression for a few cells from defined regions within the biofilms.

### Proteomics and Metabolomics Approaches for Biofilm Studies

The technological advances and a general realization of the power of omics approaches to the study of complex biological systems have advanced the application of these studies to biofilm biology. Two rapidly expanding approaches within this field are proteomics and metabolomics (180, 181). From an information standpoint, proteomics and metabolomics are a step closer to biofilm physiology than genomics and transcriptomics, since the proteins and metabolites are biomolecules that com-



**FIGURE 4** Example of a biofilm vertical transect, showing GFP-gene expression heterogeneity. Areas were cut from different biofilm strata and captured for transcriptomics analyses. Published with permission from reference 61. doi:10.1128/microbiolspec.MB-0016-2014.f4

prise most of the enzymatic activity of the cells and the metabolic substrates and products of those enzymes. However, neither proteomics nor metabolomics provide data sets that are as complete as methods focused on nucleic acids (180, 181). It is now routine to obtain deep coverage of transcribed regions of a genome using transcriptomics approaches, but there has yet to be a truly comprehensive proteomics or metabolomics investigation. This is largely due to a combination of technical and practical challenges, one of which is that amplification of the target is not possible in proteomics. This puts a technical limit on detection in that protein concentration can span five orders of magnitude in a single cell. This obstacle can be overcome through fractionation, but this has practical limitations based on time, cost, and value. Fortunately, the balance between value-added data and expense is now strongly in favor of collecting proteomics and metabolomics data from biological samples.

Key to both proteomics and metabolomics are advances in chromatography and mass analysis. Liquid chromatography (LC) coupled with electrospray ionization and a high resolution mass analyzer (LCMS) has evolved into a straightforward and very sensitive method for the analysis of a wide array of biomolecules. Quantitative information can be compiled from thousands of proteins from bacterial systems, and post-translational modifications can readily be tracked.

There are two primary approaches used in proteomics: two-dimensional differential gel electrophoresis (2D DIGE) and shotgun proteomics analysis. Variants

of each approach exist. 2D DIGE was developed first and has been used in several studies of biofilm proteomes. For example, Sauer et al. (182) used 2D DIGE to characterize the changes in proteome patterns of *P. aeruginosa* over time, demonstrating that biofilm formation of this organism is a developmental process. 2D DIGE has also been used to characterize strain variations in biofilm proteomes and the effect of environmental conditions on biofilm intercellular and extracellular proteomes (183–191). With 2D DIGE, proteins are identified by first cutting the protein spots from the gel. The protein in the dissected piece of gel is analyzed using in-gel proteolysis, which amounts to adding trypsin and then analyzing the peptides that are released using liquid chromatography/mass spectrometry. The peptide mixture is separated over a reverse-phase column, and then the mass of the intact peptide and the fragmentation pattern are collected. Peptide fragmentation is highly predictable, allowing sequence tags to be generated. The experimental data is compared with an *in silico* pattern generated from the predicted protein coding regions of the organism of interest. This process can be automated, and femtomolar sensitivity is now routine.

Traditionally, the 2D DIGE approach has required staining of the proteins with stains such as silver or coomassie blue. The proteomes of cells cultured under separate conditions are run on separate gels, and then the intensities of individual protein spots are compared using image analysis software. A more recent advance allows relative quantification between samples by labeling each protein sample with different colors of fluorescent tags (192, 193). Samples are then mixed together and separated by charge (pI) and molecular weight. After the gel has been run, the intensity of protein spots from each sample is determined using high-resolution imaging. Over a thousand protein spots can be visualized on a single 24-cm gel, and the specific sample of origin for a given spot can be determined by which color tag it has. Often an internal standard is added to the gel to facilitate analysis across gels and experiments. Sophisticated image analysis software that can warp images and adjust for variations in background and protein loading has been developed (194). 2D DIGE is well suited for rapid screening of samples for global changes. In addition, because post-translational modifications of proteins usually lead to changes in protein pI or molecular weight, 2D DIGE is extremely sensitive to global and specific changes in proteins such as phosphorylation (195, 196). The capability to investigate post-translational modification in an untargeted way is a strength of 2D DIGE.

Shotgun proteomics has now been applied to biofilm samples for metaproteomics studies of cellular and extracellular proteins (197, 198) and for characterizing the effects of biofilm growth, environmental conditions, and intercellular signaling of individual strains of biofilm organisms (e.g., 199–203). Shotgun proteomics uses direct digestion of a complex protein sample with trypsin and LCMS analysis of the ensuing peptide mixture (204). This step produces a highly complex mixture of peptides, which from a bacterial cell may contain >50,000 distinct peptides. To handle this complex solution, multidimensional chromatography is normally used. The standard approach separates peptides into fractions using strong cation exchange, with each fraction then being analyzed using reverse-phase LCMS as above. The advantage of the shotgun approach is that 2,000 to 4,000 proteins can routinely be identified, providing a deeper view into the proteome. The method can also be entirely automated, allowing for high-throughput work. Recent advances using long reverse-phase gradients allow direct analysis of total proteome digests with 5,000 to 10,000 protein IDs from a sample. Comparisons between biofilm samples can be made directly using label-free approaches or through the addition of isobaric tags for relative and absolute quantitation (iTRAQ) (205–210). While iTRAQ adds additional steps to sample preparation, it facilitates multiplexing of up to eight samples in a single LCMS run, dramatically reducing the amount of instrument time that is required.

Metabolomics is the most recent addition to the omics quiver that has been applied to biofilms (211–216). Metabolomics has a distinct advantage over other omics approaches, such as transcriptomics and proteomics, since the latter two only report on biological potential and not the activity, whereas the presence and changes in abundance of metabolites are direct readouts of biological activity. As with proteomics, the sensitivity and speed of leading-edge LCMS instruments facilitate detection of thousands of compounds in cellular extracts and extracellular solutions.

Untargeted metabolomics primarily relies on LCMS because it enables the analysis of the widest variety of compounds with excellent quantification, reproducibility, and sensitivity (217, 218). In addition, the high accuracy of modern mass analyzers allows chemical formulas to be generated for a majority of the detected molecular features. The downside to using LCMS is that chemical formulas are highly redundant, and fragmentation patterns cannot be readily predicted as with peptides. Fortunately, data analysis tools such as

XCMS, metaXCMS, and MZmine are freely available (219). These tools link to databases such as METLIN (220, 221) and the Human Metabolome (222), which contain >80,000 compounds. More specific analyses, such as those focusing on central carbon metabolism and lipids, are well served by gas chromatography–mass spectrometry, for which spectral libraries based on fragmentation patterns are available, facilitating direct compound identification. The specific technique to be used again depends on the question being asked. Metabolomics experiments can be designed to be untargeted surveys, which are a powerful approach for the identification of novel compounds and unbiased analysis of microbial states (64). On the other hand, highly targeted approaches can make quantitative analyses of hundreds of compounds in under ten minutes if large numbers of samples must be processed or high time resolutions of a biological response are needed.

## BIOFILM IMAGING

One of the most important advances in the study of biofilm structure, function, and dynamics has been the ability to visualize hydrated living biofilms in three dimensions, over time, using CSLM. The first 3D images of biofilms were obtained by CSLM approximately 25 years ago (90). Now, CSLM is available in many labs and core facilities, and therefore this technology is now used in almost all biofilm studies (Table 2). Important advances in CSLM for the analysis of microbial biofilms have been the optics and lasers, image analysis software, and high-speed computing power which allows image analysis of large data files and also allows time-lapse imaging of biofilm developmental processes. Chemical and molecular biological advances have also improved the ability to image biofilms in three dimensions. In particular, the use of fluorescent proteins and fluorescent probes allows imaging by multiple fluorochromes for assays of the individual biofilm components simultaneously. Another new development in imaging technology is super-resolution microscopy such as photoactivated localization microscopy (PLAM), fluorescence photoactivation localization microscopy (FPLAM), and stochastic optical reconstruction microscopy (STORM) (223–225), imaging techniques that give resolution down to tens of nanometers, below the diffraction limit of light. These techniques use photoactivatable fluorescent proteins or probes to perform imaging with much greater resolution than traditional light or CSLM microscopy. It is likely that high-resolution approaches will soon find an application in biofilm research.

**TABLE 2** Imaging strategies in biofilm studies

Technique	Experimental features	Types and modifications	Application and examples	References
<b>Fluorescent imaging</b>				
Fluorescent protein	<ul style="list-style-type: none"> <li>• Wide range of colors</li> <li>• Multiple functions</li> <li>• Genetically encoded</li> <li>• No need for fixation</li> <li>• Real-time detection</li> <li>• Molecular techniques need to be developed for organisms</li> <li>• Cell physiology may be altered</li> </ul>	<ul style="list-style-type: none"> <li>• Photoactivatable</li> <li>• Photoconvertible</li> <li>• Photoswitchable</li> <li>• FP timer</li> <li>• FRET/BRET</li> <li>• Oxygen-independent FP</li> <li>• Unstable GFP</li> </ul>	<ul style="list-style-type: none"> <li>• Cell differentiation</li> <li>• Cell tracking</li> <li>• Expression profile</li> <li>• Protein localization</li> <li>• Activity indicator</li> <li>• High-resolution imaging</li> </ul>	<a href="#">36</a> , <a href="#">40</a> , <a href="#">48</a> , <a href="#">62</a> , <a href="#">65</a> , <a href="#">68</a> , <a href="#">91</a> , <a href="#">132</a> , <a href="#">227</a> , <a href="#">228</a> , <a href="#">229</a> , <a href="#">231</a>
Fluorescent <i>in situ</i> hybridization (FISH)	<ul style="list-style-type: none"> <li>• Wide range of colors</li> <li>• High target specificity</li> <li>• No genetic modification necessary</li> <li>• Exogenous addition</li> <li>• Fixation of sample is necessary</li> </ul>	<ul style="list-style-type: none"> <li>• Oligonucleotide probe</li> <li>• Peptide nucleic acid probe</li> <li>• CLASI-FISH</li> <li>• CARD-FISH</li> <li>• MAR-FISH</li> </ul>	<ul style="list-style-type: none"> <li>• Differentiation of multiple organisms</li> <li>• Cell localization</li> <li>• mRNA expression profile</li> <li>• High-resolution imaging</li> </ul>	<a href="#">78</a> , <a href="#">234</a> , <a href="#">235</a> , <a href="#">236</a> , <a href="#">237</a> , <a href="#">238</a> , <a href="#">239</a> , <a href="#">242</a> , <a href="#">260</a> , <a href="#">261</a>
Fluorescent stain	<ul style="list-style-type: none"> <li>• No genetic modification necessary</li> <li>• Exogenous addition</li> <li>• Can be specific for biomolecules and cellular components</li> </ul>	<ul style="list-style-type: none"> <li>• SYBR-green</li> <li>• FM1-43</li> <li>• CTC</li> <li>• BrdU-fluorescent antibody</li> <li>• Calcofluor</li> <li>• TOTO-1</li> </ul>	<ul style="list-style-type: none"> <li>• Matrix material and cellular component visualization</li> <li>• Activity indicator</li> <li>• Viability indicator</li> </ul>	<a href="#">38</a> , <a href="#">73</a> , <a href="#">226</a> , <a href="#">240</a> , <a href="#">241</a> , <a href="#">242</a> , <a href="#">246</a>
<b>Analytical imaging</b>				
Fourier transform infrared spectrometry (FT-IR)	<ul style="list-style-type: none"> <li>• Nondestructive</li> <li>• Real-time detection</li> </ul>	<ul style="list-style-type: none"> <li>• ATR/FT-IR</li> <li>• SR-FTIR</li> </ul>	<ul style="list-style-type: none"> <li>• Chemical composition of EPS</li> <li>• Quantification of biomass</li> <li>• Spatial distribution of biomolecules</li> </ul>	<a href="#">91</a> , <a href="#">247</a> , <a href="#">248</a> , <a href="#">249</a> , <a href="#">250</a>
Raman spectrometry	<ul style="list-style-type: none"> <li>• Nondestructive</li> <li>• Real-time detection</li> </ul>	<ul style="list-style-type: none"> <li>• Raman microscopy</li> <li>• SERS</li> </ul>	<ul style="list-style-type: none"> <li>• Chemical composition of EPS</li> <li>• Quantification of biomass</li> <li>• Spatial distribution of biomolecules</li> </ul>	<a href="#">249</a> , <a href="#">251</a> , <a href="#">252</a> , <a href="#">253</a> , <a href="#">254</a> , <a href="#">255</a> , <a href="#">256</a> , <a href="#">257</a> , <a href="#">258</a>
Nuclear magnetic resonance microscopy	<ul style="list-style-type: none"> <li>• Nondestructive</li> <li>• Real-time detection</li> </ul>	<ul style="list-style-type: none"> <li>• Use of isotope-labeled substrates</li> </ul>	<ul style="list-style-type: none"> <li>• Water flow and water dynamics of EPS</li> <li>• Dynamics of metabolites</li> </ul>	<a href="#">79</a> , <a href="#">80</a> , <a href="#">262</a> , <a href="#">263</a>

Abbreviations: CLASI, combinatorial labeling and spectral imaging; ATR/FT-IR, attenuated total reflection Fourier transform infrared spectrometry; BrdU, bromodeoxyuridine; BRET, bioluminescence resonance energy transfer; CARD-FISH, catalyzed reporter deposition-FISH; CTC, cyanoditoly tetrazolium chloride; FP, fluorescent protein; FRET, Förster resonance energy transfer; MAR-FISH, microautoradiography-FISH; SR-FTIR, synchrotron-radiation-based FTIR; SERS, surface-enhanced Raman scattering.

CSLM uses a combination of laser excitation of fluorescent molecules and fluorescence emission through a pinhole to filter out light that is not in the optimal focal plane. As a result, with CSLM there is no out-of-focus haze, which is typical for 3D specimens imaged using conventional light or epifluorescence microscopy. With CSLM, a series of in-focus 2D images in the X and Y planes are obtained at submicrometer intervals in the Z-direction. The 2D stacks are converted into a 3D

image using image analysis software. CSLM is ideal for imaging biofilms, since most biofilms have extensive 3D structure and can be stained with fluorescent probes or fluorescent proteins. Neu and Lawrence (226) published a comprehensive review of CSLM applied to biofilm research, including many published examples of CSLM techniques and applications. Here, we briefly describe a few of the approaches that are most commonly used to obtain 3D images of microbial biofilms.

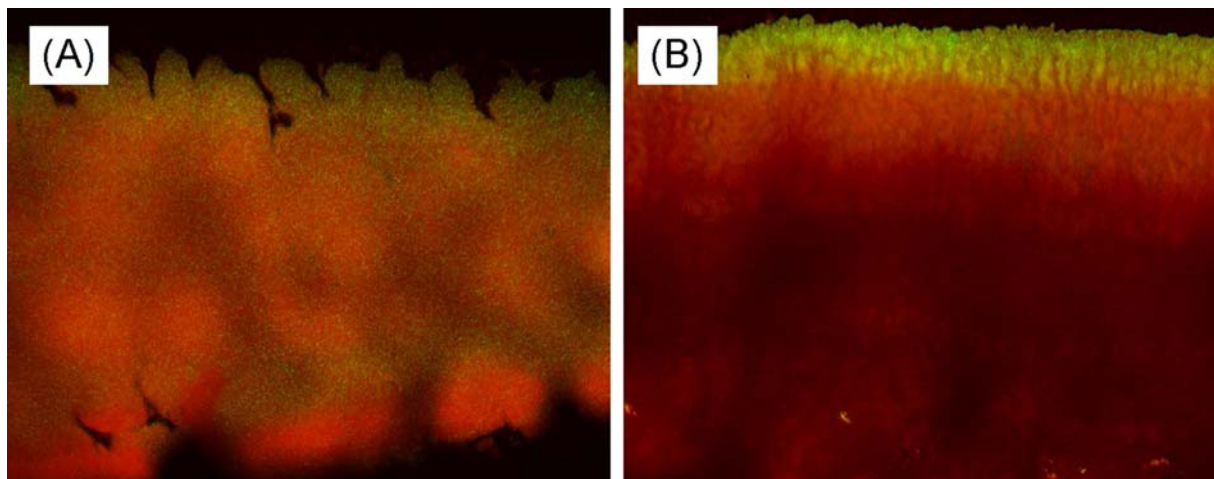
The most widely used approach of CSLM imaging in biofilm research is to culture biofilms of bacteria that contain fluorescent proteins (such as the green fluorescent protein [GFP]) in flow cells and to image the biofilms that form on the walls of the flow cells. The greatest advantage of using GFP-labeled cells is that the 3D architecture of the biofilm cells can be visualized during biofilm development, without the need to add external fluorescent dyes. Generally, the GFP is expressed from a constitutive promoter, allowing comparison of the biofilm formation properties of a wild-type strain and a mutant derivative that is impaired in biofilm formation (e.g., 91, 227). Since fluorescent proteins are now available in many colors (68), it is possible to differentially label multiple strains, species, or mutants with different colors of fluorescent proteins. These strains can then be cultured separately or in multispecies biofilms and imaged by CSLM (36, 65). These experiments may be used to assay the competitive advantage of a particular strain or the effect of a gene mutation on the competitive index of an organism (132, 228). While GFP-labeling of cells has provided excellent images of a variety of biofilms, there are some disadvantages to this approach. First, a genetic system must be available for the organism of interest to introduce and express the *gfp* gene in the cell. Second, the *gfp* expression may affect the growth of the cells, since energy is required to express this nonnative gene, often from a multi-copy number of plasmids. Third, although the GFP-labeling provides excellent images of the cellular material in the biofilms, it does not provide information on the extracellular component of the biofilm matrix material, which must be stained separately to provide a comprehensive view of the biofilm architecture.

A modification of the fluorescent protein labeling approach is to use the GFP (or another fluorescent protein) as a reporter gene (Fig. 5). Fluorescent reporter genes are used to assay the expression of a particular gene within the biofilms. Reporter gene fusions can act as transcriptional reporters (if the *gfp* is fused to a gene's promoter region) or translation reporters (if the *gfp* is fused in frame with another gene, generating a chimeric protein). Fluorescent reporter gene fusions combined with CSLM have allowed investigators to examine gene expression and protein production in biofilms and the effect of environmental conditions on gene expression. They have also been used to serve as a measure of metabolic activity within regions of the biofilms. Translational reporter fusions may be used to determine the localization of tagged proteins within cells (229–232). Combined with CSLM, fluorescent reporter genes are

particularly useful for examining gene expression heterogeneity in biofilms and for studying stochastic gene expression events (48, 233).

Biofilms may also be imaged by labeling the cells with fluorescent oligonucleotide probes. Fluorescent *in situ* hybridization (FISH) has traditionally been used to detect the abundance of multiple bacteria species in environmental samples (74) based on hybridization to the 16S rRNA. FISH has been used on a variety of biofilm samples. For example, FISH was used to determine spatial distribution of ammonia-oxidizing and nitrite-oxidizing bacteria in nitrifying aggregates or biofilms (78, 234) and the methanogen and sulfate-reducer components of anaerobic biofilms (75). Traditional and modified FISH have also been used to detect localized abundance of specific mRNAs in biofilm or termite gut microflora (235, 236). The microbial community in natural biofilm can be highly diverse. Recent development of combinatorial labeling and spectral imaging FISH (CLASI-FISH) has the potential to detect hundreds of microbial taxa in single microscopy imaging (237–239). The disadvantage of FISH approaches is that the samples have to be fixed with chemicals prior to the probe hybridization, which may disturb the structure of the biofilm and makes time-course studies difficult.

Fluorescent stains are also used in combination with CSLM for imaging biofilm cellular and extracellular matrix material. Neu and Lawrence (226) provided tables with comprehensive lists of fluorescent stains commonly used in biofilm research. The fluorescent stains are generally designed to bind a specific cellular component, such as DNA (e.g., propidium iodide, SYBR-green, and ToTo-1) or protein (e.g., Sypro-Ruby). The FM1-43 stain is interesting in that it intercalates into the cell membrane and provides definition of the bacterial cell wall when imaged using CSLM (240). In addition to staining cellular materials, fluorescent stains are available for studying metabolic activities within biofilms. For example, tetrazolium salts precipitate when reduced by the biofilm cells, forming a zone of fluorescence around the active cells (241, 242). In another example, bromodeoxyuridine (BrdU) is incorporated into the DNA of actively growing cells. Following incorporation, the active cells can be identified by staining the BrdU with fluorescent anti-BrdU antibodies. This approach was used to study spatial heterogeneity in *S. epidermidis* biofilms (38). A third approach which will likely find an application in biofilm research is the “click-chemistry” technology (243–245). In this approach, amino acid analogs are incorporated into the protein (or peptidoglycan) of actively growing cells. The analogs are then labeled with a fluorescent



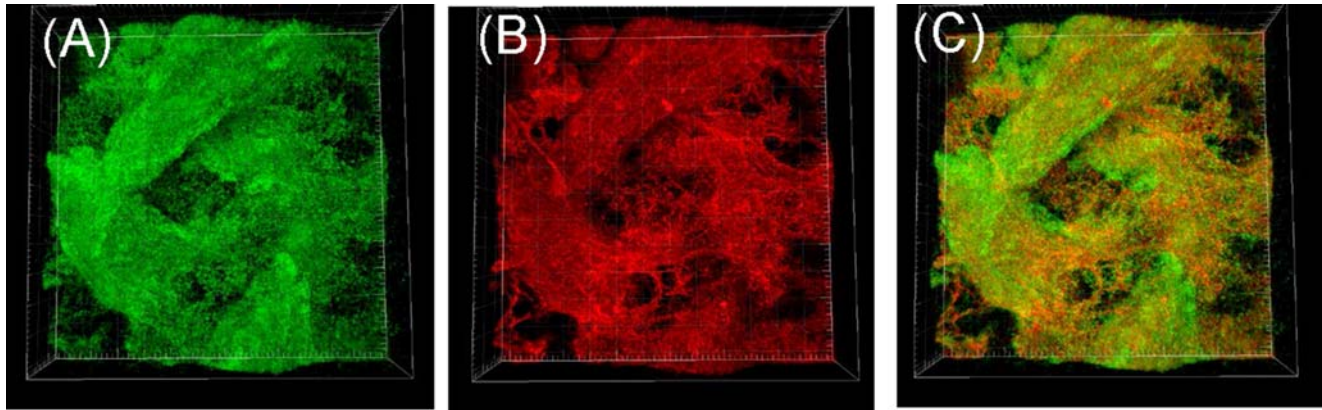
**FIGURE 5** Gene expression heterogeneity, demonstrated by translational fusions of target proteins to the yellow fluorescent protein (YFP). (A) Translational fusion of the lbpA protein to YFP, showing uniform distribution of lbpA throughout the biofilm. Cells were counterstained with mCherry fluorescent protein (mCFP). (B) Translational fusion of Rmf protein to YFP, showing that most Rmf production occurs in cells at the top of the biofilm. Cells counterstained with mCFP (M.J. Franklin, unpublished data). [doi:10.1128/microbiolspec.MB-0016-2014.f5](https://doi.org/10.1128/microbiolspec.MB-0016-2014.f5)

probe to identify the cells that are actively involved in protein or cell wall biosynthesis.

Most fluorescent dyes used in biofilm studies stain cellular components. Characterizing the extracellular matrix material by CSLM has been more challenging. In fact, the extracellular matrix of biofilms has been referred to as the “dark matter of biofilms” since it is difficult to image, having approximately the same refractive index as water (4). The biofilm matrix material is difficult to stain because it is complex. The matrix material is composed of a combination of polysaccharides, proteins, and extracellular DNA. The composition, particularly the polysaccharide components, and the relative amounts of the components vary for different species, and even for different strains, of bacteria. Therefore, it is unlikely that there will ever be a universally effective biofilm matrix stain. A few fluorescent stains are becoming available for CSLM studies of biofilm matrix components (Fig. 6). In particular, fluorescent lectins bind to specific sugars and can be used to stain certain extracellular polysaccharides. Since lectins are large molecules, they do not penetrate biofilms well. Ideally, small molecule stains will become available for staining the polysaccharide component of biofilms, such as Calcofluor, which is used to stain biofilms of strains that produce cellulose (246). Another component of the biofilm extracellular matrix material is extracellular DNA. Various stains are available that bind DNA, with the TOTO-1 iodide stain providing excellent contrast

between the biofilm eDNA component and the biofilm cells (73).

While the extracellular matrix material of biofilms is still difficult to image, it can be characterized by spectroscopic techniques. Attenuated total reflection Fourier transform infrared spectrometry (ATR/FT-IR) is a non-destructive technique that has been used to determine the chemical composition of biofilms by analysis of absorbance of infrared light by particular chemical groups (91, 247–249). For example, ATR/FT-IR was used to quantify changes in the biomass and chemical composition of extracellular matrix material in *P. aeruginosa* and alginate O acetylation–deficient mutants of *P. aeruginosa* (91). Synchrotron-radiation-based FT-IR with a microfluidic system was used to determine the responses of biofilm to antibiotic exposure and assaying uptake of antibiotics, cell lysis, and stability of biofilm by detecting changes in chemical composition (250). Raman spectrometry has also been used to define the chemistry of matrix materials in biofilms (251, 252). Raman spectrometry is based on the light scattering patterns detected after irradiation of a sample with monochromatic light. The frequency of scattered light differs among substrates and can be used to study the chemical composition of biofilm. Raman spectrometry with CSLM was used to determine spatial distribution biomass and water as well as chemical composition of wild-type and small colony variant *P. aeruginosa* biofilms (253, 254). Modified Raman spectroscopy–based analysis such as



**FIGURE 6** (A) Images of *P. aeruginosa* biofilms, where the cells constitutively express the GFP. (B) Extracellular matrix material stained with Bodipy 630/650-X NHS from Life Technologies. (C) Combined image showing GFP-fluorescent bacteria and Bodipy-stained matrix (M.J. Franklin, unpublished data). [doi:10.1128/microbiolspec.MB-0016-2014.f6](https://doi.org/10.1128/microbiolspec.MB-0016-2014.f6)

surface-enhanced Raman scattering has increased sensitivity and may detect components in the EPS that are not detectable by Raman microscopy (255–258). Finally, nuclear magnetic resonance imaging has been used to characterize water dynamics within biofilms, as well as molecular dynamics and diffusion of biomolecules in biofilms (79, 80). These analytical techniques are non-destructive methods for real-time detection of spatial heterogeneity of the chemical environment within biofilms.

## SUMMARY

Most microbial life grows in association with surfaces. Microorganisms that adapt to growth on surfaces adopt a variety of complex behaviors that include adhesion to the surface, expression of genes, and enzymatic activities that allow adaptation to a sessile lifestyle. The activities of microorganisms in biofilms are not uniform across the entire biofilm, and cells adapt to their local environments. Many complementary technologies are required to study the metabolic activities and the physical and chemical environments of biofilm-associated communities. These technologies include molecular approaches, such as gene expression and metabolomics studies to characterize the activity of the microbial cells, analytical approaches to understand the chemistry of biofilm organisms and their matrix materials, imaging approaches to characterize structure-function relationships of the microbial cells, and miniaturization studies to characterize the physical and heterogeneous properties of biofilms. By combining these and other new techniques, it is now possible to gain insight into these complex microbial communities from the gene to the systems level.

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