Published in final edited form as:

Microbiol Spectr. 2015 August ; 3(4): . doi:10.1128/microbiolspec.MB-0016-2014.

New Technologies for Studying Biofilms

MICHAEL J. FRANKLIN^{1,2}, CONNIE CHANG^{1,3}, TATSUYA AKIYAMA^{1,2}, and BRIAN BOTHNER^{1,4}

¹Center for Biofilm Engineering, Montana State University-Bozeman, Bozeman, MT 59717

²Department of Microbiology and Immunology, Montana State University-Bozeman, Bozeman, MT 59717

³Department of Chemical and Biological Engineering, Montana State University-Bozeman, Bozeman, MT 59717

⁴Department of Chemistry and Biochemistry, Montana State University-Bozeman, Bozeman, MT 59717

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

Correspondence: Michael J. Franklin, franklin@montana.edu.

Conflicts of interest: We disclose no conflicts.

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 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,

Author Manuscript

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).

Author Manuscript

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.

Author Manuscript

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

Author Manuscript

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 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 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 μm . 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 (μTAS)” 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 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 polydimethyl-siloxane (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 biofilms, 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 μm 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 bio-volume 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 fibrosis 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 meta-genomics 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 Kikirde (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 Kikirde (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 micro-arrays 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 micro-arrays 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 micro-array 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 comprise 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 posttranslational 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 posttranslational 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 un-targeted 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.

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, tetra-zolium 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 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 nondestructive 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 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 nondestructive 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.

Acknowledgments

This review is dedicated to the late Dr. David C. White, who spent much of his productive career developing innovative approaches designed to characterize microorganisms and their metabolic activities in natural environments. The authors thank Dr. Phil Stewart, Ms. Kerry Williamson, and Ms. Betsey Pitts for their helpful discussions of this review. This work was supported in part by NIH/NIAID award AI113330 (M.J.F.).

References

1. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel NC, Dosgupton M, Marine JJ. Bacterial biofilms in nature and disease. *Annu Rev Microbiol.* 1987; 41:435–461. [PubMed: 3318676]
2. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol.* 1995; 49:711–745. [PubMed: 8561477]
3. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science.* 1999; 284:1318–1322. [PubMed: 10334980]
4. Flemming HC, Wingender J. The biofilm matrix. *Nat Rev.* 2010; 8:623–633.
5. Hung C, Zhou Y, Pinkner JS, Dodson KW, Crowley JR, Heuser J, Chapman MR, Hadjifrangiskou M, Henderson JP, Hultgren SJ. *Escherichia coli* biofilms have an organized and complex extracellular matrix structure. *MBio.* 2013; 4:e00645–00613.10.1128/mBio.00645-13 [PubMed: 24023384]
6. Soto GE, Hultgren SJ. Bacterial adhesins: common themes and variations in architecture and assembly. *J Bacteriol.* 1999; 181:1059–1071. [PubMed: 9973330]
7. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol.* 2005; 13:34–40. [PubMed: 15639630]
8. Singh R, Ray P, Das A, Sharma M. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. *J Med Microbiol.* 2009; 58:1067–1073. [PubMed: 19528167]
9. van de Mortel M, Halverson LJ. Cell envelope components contributing to biofilm growth and survival of *Pseudomonas putida* in low-water-content habitats. *Mol Microbiol.* 2004; 52:735–750. [PubMed: 15101980]
10. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science.* 1998; 280:295–298. [PubMed: 9535661]
11. De Kievit TR, Iglewski BH. Quorum sensing, gene expression, and *Pseudomonas* biofilms. *Methods Enzymol.* 1999; 310:117–128. [PubMed: 10547786]
12. Fuqua WC, Winans SC, Greenberg EP. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol.* 1994; 176:269–275. [PubMed: 8288518]
13. Parsek MR, Greenberg EP. Quorum sensing signals in development of *Pseudomonas aeruginosa* biofilms. *Methods Enzymol.* 1999; 310:43–55. [PubMed: 10547781]
14. Parsek MR, Greenberg EP. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc Natl Acad Sci USA.* 2000; 97:8789–8793. [PubMed: 10922036]
15. Passador, L.; Iglewski, BH. Quorum sensing and virulence gene regulation in *Pseudomonas aeruginosa*. In: Roth, JA., et al., editors. *Virulence Mechanisms of Bacterial Pathogens*. American Society for Microbiology; Washington D.C: 1995. p. 65-78.
16. Pesci EC, Pearson JP, Seed PC, Iglewski BH. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol.* 1997; 179:3127–3132. [PubMed: 9150205]
17. Ueda A, Wood TK. Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog.* 2009; 5:e1000483.10.1371/journal.ppat.1000483 [PubMed: 19543378]
18. Whiteley M, Lee KM, Greenberg EP. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA.* 1999; 96:13904–13909. [PubMed: 10570171]
19. Zhu J, Mekalanos JJ. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell.* 2003; 5:647–656. [PubMed: 14536065]

20. Brint JM, Ohman DE. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J Bacteriol.* 1995; 177:7155–7163. [PubMed: 8522523]
21. Pearson JP, Pesci EC, Iglewski BH. Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol.* 1997; 179:5756–5767. [PubMed: 9294432]
22. Tielen P, Rosenau F, Wilhelm S, Jaeger KE, Flemming HC, Wingender J. Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. *Microbiology.* 2010; 156:2239–2252. [PubMed: 20360178]
23. Glessner A, Smith RS, Iglewski BH, Robinson JA. Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of twitching motility. *J Bacteriol.* 1999; 181:1623–1629. [PubMed: 10049396]
24. Heydorn A, Ersboll B, Kato J, Hentzer M, Parsek MR, Tolker-Nielsen T, Givskov M, Molin S. Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. *Appl Environ Microbiol.* 2002; 68:2008–2017. [PubMed: 11916724]
25. Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Camara M, Williams P, Haas D. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol.* 2004; 186:2936–2945. [PubMed: 15126453]
26. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol.* 1998; 30:295–304. [PubMed: 9791175]
27. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Mol Microbiol.* 2006; 62:1264–1277. [PubMed: 17059568]
28. Wang Q, Frye JG, McClelland M, Harshey RM. Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol.* 2004; 52:169–187. [PubMed: 15049819]
29. Aguilar C, Vlamakis H, Losick R, Kolter R. Thinking about *Bacillus subtilis* as a multicellular organism. *Curr Opin Microbiol.* 2007; 10:638–643. [PubMed: 17977783]
30. Basler M, Ho BT, Mekalanos JJ. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell.* 2013; 152:884–894. [PubMed: 23415234]
31. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature.* 2012; 483:182–186. [PubMed: 22367545]
32. Gibbs KA, Urbanowski ML, Greenberg EP. Genetic determinants of self identity and social recognition in bacteria. *Science.* 2008; 321:256–259. [PubMed: 18621670]
33. Moscoso JA, Mikkelsen H, Heeb S, Williams P, Filloux A. The *Pseudomonas aeruginosa* sensor RetS switches type III and type VI secretion via c-di-GMP signalling. *Environ Microbiol.* 2011; 13:3128–3138. [PubMed: 21955777]
34. Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. *Nat Rev.* 2008; 6:199–210.
35. Becraft ED, Cohan FM, Kuhl M, Jensen SI, Ward DM. Fine-scale distribution patterns of *Synechococcus* ecological diversity in microbial mats of Mushroom Spring, Yellowstone National Park. *Appl Environ Microbiol.* 2011; 77:7689–7697. [PubMed: 21890675]
36. Kim W, Racimo F, Schluter J, Levy SB, Foster KR. Importance of positioning for microbial evolution. *Proc Natl Acad Sci USA.* 2014; 111:E1639–E1647. [PubMed: 24715732]
37. Ramsing NB, Ferris MJ, Ward DM. Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. *Appl Environ Microbiol.* 2000; 66:1038–1049. [PubMed: 10698769]
38. Rani SA, Pitts B, Beyenal H, Veluchamy RA, Lewandowski Z, Davison WM, Buckingham-Meyer K, Stewart PS. Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J Bacteriol.* 2007; 189:4223–4233. [PubMed: 17337582]
39. Rasmussen K, Lewandowski Z. Microelectrode measurements of local mass transport rates in heterogeneous biofilms. *Biotechnol Bioeng.* 1998; 59:302–309. [PubMed: 10099341]

40. Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydorn A, Molin S, Pitts B, Stewart PS. Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol.* 2004; 70:6188–6196. [PubMed: 15466566]
41. Boles BR, Thoendel M, Singh PK. Self-generated diversity produces “insurance effects” in biofilm communities. *Proc Natl Acad Sci USA.* 2004; 101:16630–16635. [PubMed: 15546998]
42. Hansen SK, Rainey PB, Haagenzen JA, Molin S. Evolution of species interactions in a biofilm community. *Nature.* 2007; 445:533–536. [PubMed: 17268468]
43. Allegrucci M, Sauer K. Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *J Bacteriol.* 2007; 189:2030–2038. [PubMed: 17189375]
44. Hansen SK, Haagenzen JA, Gjermansen M, Jorgensen TM, Tolker-Nielsen T, Molin S. Characterization of a *Pseudomonas putida* rough variant evolved in a mixed-species biofilm with *Acinetobacter* sp. strain C6. *J Bacteriol.* 2007; 189:4932–4943. [PubMed: 17468252]
45. Kirisits MJ, Prost L, Starkey M, Parsek MR. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol.* 2005; 71:4809–4821. [PubMed: 16085879]
46. McEllistrem MC, Ransford JV, Khan SA. Characterization of *in vitro* biofilm-associated pneumococcal phase variants of a clinically relevant serotype 3 clone. *J Clin Microbiol.* 2007; 45:97–101. [PubMed: 17093036]
47. Valle J, Vergara-Irigaray M, Merino N, Penades JR, Lasa I. sigmaB regulates IS256-mediated *Staphylococcus aureus* biofilm phenotypic variation. *J Bacteriol.* 2007; 189:2886–2896. [PubMed: 17277051]
48. Baty AM 3rd, Eastburn CC, Diwu Z, Techkarnjanaruk S, Goodman AE, Geesey GG. Differentiation of chitinase-active and non-chitinase-active subpopulations of a marine bacterium during chitin degradation. *Appl Environ Microbiol.* 2000; 66:3566–3573. [PubMed: 10919822]
49. Baty AM 3rd, Eastburn CC, Techkarnjanaruk S, Goodman AE, Geesey GG. Spatial and temporal variations in chitinolytic gene expression and bacterial biomass production during chitin degradation. *Appl Environ Microbiol.* 2000; 66:3574–3585. [PubMed: 10919823]
50. Vlamakis H, Aguilar C, Losick R, Kolter R. Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev.* 2008; 22:945–953. [PubMed: 18381896]
51. Gelens L, Hill L, Vandervelde A, Danckaert J, Loris R. A general model for toxin-antitoxin module dynamics can explain persister cell formation in *E. coli*. *PLoS Comput Biol.* 2013; 9:e1003190.10.1371/journal.pcbi.1003190 [PubMed: 24009490]
52. Koh RS, Dunlop MJ. Modeling suggests that gene circuit architecture controls phenotypic variability in a bacterial persistence network. *BMC Syst Biol.* 2012; 6:47. [PubMed: 22607777]
53. Lewis K. Persister cells, dormancy and infectious disease. *Nat Rev.* 2007; 5:48–56.
54. Mulcahy LR, Burns JL, Lory S, Lewis K. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol.* 2010; 192:6191–6199. [PubMed: 20935098]
55. Deneff VJ, Mueller RS, Banfield JF. AMD biofilms: using model communities to study microbial evolution and ecological complexity in nature. *ISME J.* 2010; 4:599–610. [PubMed: 20164865]
56. Grzymalski JJ, Murray AE, Campbell BJ, Kaplarevic M, Gao GR, Lee C, Daniel R, Ghadiri A, Feldman RA, Cary SC. Metagenome analysis of an extreme microbial symbiosis reveals eurythermal adaptation and metabolic flexibility. *Proc Natl Acad Sci USA.* 2008; 105:17516–17521. [PubMed: 18987310]
57. Inskeep WP, Rusch DB, Jay ZJ, Herrgard MJ, Kozubal MA, Richardson TH, Macur RE, Hamamura N, Jennings R, Fouke BW, Reysenbach AL, Roberto F, Young M, Schwartz A, Boyd ES, Badger JH, Mathur EJ, Ortmann AC, Bateson M, Geesey G, Frazier M. Metagenomes from high-temperature chemotrophic systems reveal geochemical controls on microbial community structure and function. *PLoS One.* 2010; 5:e9773.10.1371/journal.pone.0009773 [PubMed: 20333304]
58. Xu P, Gunsolley J. Application of metagenomics in understanding oral health and disease. *Virulence.* 2014; 5:424–432. [PubMed: 24642489]

59. Ishii S, Suzuki S, Norden-Krichmar TM, Tenney A, Chain PS, Scholz MB, Nealson KH, Bretschger O. A novel metatranscriptomic approach to identify gene expression dynamics during extracellular electron transfer. *Nat Commun.* 2013; 4:1601. [PubMed: 23511466]
60. Folsom JP, Richards L, Pitts B, Roe F, Ehrlich GD, Parker A, Mazurie A, Stewart PS. Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. *BMC Microbiol.* 2010; 10:294. [PubMed: 21083928]
61. Lenz AP, Williamson KS, Pitts B, Stewart PS, Franklin MJ. Localized gene expression in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol.* 2008; 74:4463–4471. [PubMed: 18487401]
62. Williamson KS, Richards LA, Perez-Osorio AC, Pitts B, McInnerney K, Stewart PS, Franklin MJ. Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. *J Bacteriol.* 2012; 194:2062–2073. [PubMed: 22343293]
63. Beale DJ, Barratt R, Marlow DR, Dunn MS, Palombo EA, Morrison PD, Key C. Application of metabolomics to understanding biofilms in water distribution systems: a pilot study. *Biofouling.* 2013; 29:283–294. [PubMed: 23458161]
64. Secor PR, Jennings LK, James GA, Kirker KR, Pulcini ED, McInnerney K, Gerlach R, Livinghouse T, Hilmer JK, Bothner B, Fleckman P, Olerud JE, Stewart PS. Phevalin (aureusimine B) production by *Staphylococcus aureus* biofilm and impacts on human keratinocyte gene expression. *PLoS One.* 2013; 7:e40973.10.1371/journal.pone.0040973 [PubMed: 22808288]
65. Klayman BJ, Klapper I, Stewart PS, Camper AK. Measurements of accumulation and displacement at the single cell cluster level in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol.* 2008; 10:2344–2354. [PubMed: 18557771]
66. Lam AJ, St-Pierre F, Gong Y, Marshall JD, Cranfill PJ, Baird MA, McKeown MR, Wiedenmann J, Davidson MW, Schnitzer MJ, Tsien RY, Lin MZ. Improving FRET dynamic range with bright green and red fluorescent proteins. *Nat Methods.* 2012; 9:1005–1012. [PubMed: 22961245]
67. Shaner NC, Lin MZ, McKeown MR, Steinbach PA, Hazelwood KL, Davidson MW, Tsien RY. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat Methods.* 2008; 5:545–551. [PubMed: 18454154]
68. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Methods.* 2005; 2:905–909. [PubMed: 16299475]
69. McLoon AL, Kolodkin-Gal I, Rubinstein SM, Kolter R, Losick R. Spatial regulation of histidine kinases governing biofilm formation in *Bacillus subtilis*. *J Bacteriol.* 2011; 193:679–685. [PubMed: 21097618]
70. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol.* 2006; 59:1114–1128. [PubMed: 16430688]
71. Baird FJ, Wadsworth MP, Hill JE. Evaluation and optimization of multiple fluorophore analysis of a *Pseudomonas aeruginosa* biofilm. *J Microbiol Methods.* 2012; 90:192–196. [PubMed: 22587931]
72. Chen MY, Lee DJ, Tay JH, Show KY. Staining of extracellular polymeric substances and cells in bioaggregates. *Appl Microbiol Biotechnol.* 2007; 75:467–474. [PubMed: 17245574]
73. Gloag ES, Turnbull L, Huang A, Vallotton P, Wang H, Nolan LM, Mililli L, Hunt C, Lu J, Osvath SR, Monahan LG, Cavaliere R, Charles IG, Wand MP, Gee ML, Prabhakar R, Whitchurch CB. Self-organization of bacterial biofilms is facilitated by extracellular DNA. *Proc Natl Acad Sci USA.* 2013; 110:11541–11546. [PubMed: 23798445]
74. Amann R, Ludwig W. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol Rev.* 2000; 24:555–565. [PubMed: 11077149]
75. Briley KA, Camilleri LB, Fields MW. 3D-fluorescence *in situ* hybridization of intact, anaerobic biofilm. *Methods Mol Biol.* 2014; 1151:189–197. [PubMed: 24838887]
76. DeLong EF, Wickham GS, Pace NR. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science.* 1989; 243:1360–1363. [PubMed: 2466341]
77. Moller S, Pedersen AR, Poulsen LK, Arvin E, Molin S. Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative *in situ*

- hybridization and scanning confocal laser microscopy. *Appl Environ Microbiol.* 1996; 62:4632–4640. [PubMed: 8953734]
78. Schramm A, De Beer D, Wagner M, Amann R. Identification and activities *in situ* of *Nitrospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. *Appl Environ Microbiol.* 1998; 64:3480–3485. [PubMed: 9726900]
 79. Hornemann JA, Codd SL, Fell RJ, Stewart PS, Seymour JD. Secondary flow mixing due to biofilm growth in capillaries of varying dimensions. *Biotechnol Bioeng.* 2009; 103:353–360. [PubMed: 19191352]
 80. Hornemann JA, Lysova AA, Codd SL, Seymour JD, Busse SC, Stewart PS, Brown JR. Biopolymer and water dynamics in microbial biofilm extracellular polymeric substance. *Biomacromolecules.* 2008; 9:2322–2328. [PubMed: 18665639]
 81. O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol.* 1998; 28:449–461. [PubMed: 9632250]
 82. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol.* 1999; 37:1771–1776. [PubMed: 10325322]
 83. Branda SS, González-Pastor JE, Ben-Yehuda S, Losick R, Kolter R. Fruiting body formation by *Bacillus subtilis*. *Proc Natl Acad Sci USA.* 2001; 98:11621–11626. [PubMed: 11572999]
 84. Chimileski S, Franklin MJ, Papke RT. Biofilms formed by the archaeon *Haloferax volcanii* exhibit cellular differentiation and social motility, and facilitate horizontal gene transfer. *BMC Biol.* 2014; 12:65. [PubMed: 25124934]
 85. Wentland E, Stewart PS, Huang C-T, McFeters GA. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnol Prog.* 1996; 12:316–321. [PubMed: 8652119]
 86. Huang C, McFeters G, Stewart P. Evaluation of physiological staining, cryoembedding, and autofluorescence quenching techniques on fouling biofilms. *Biofouling.* 1996; 9:269–277.
 87. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother.* 2000; 44:1818–1824. [PubMed: 10858336]
 88. Goeres DM, Loetterle LR, Hamilton MA, Murga R, Kirby DW, Donlan RM. Statistical assessment of a laboratory method for growing biofilms. *Microbiology.* 2005; 151:757–762. [PubMed: 15758222]
 89. Goeres DM, Hamilton MA, Beck NA, Buckingham-Meyer K, Hilyard JD, Loetterle LR, Lorenz LA, Walker DK, Stewart PS. A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nat Protoc.* 2009; 4:783–788. [PubMed: 19528953]
 90. Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE. Optical sectioning of microbial biofilms. *J Bacteriol.* 1991; 173:6558–6567. [PubMed: 1917879]
 91. Nivens DE, Ohman DE, Williams J, Franklin MJ. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J Bacteriol.* 2001; 183:1047–1057. [PubMed: 11208804]
 92. Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, Molin S, Kharazmi A, Hoiby N, Mathee K. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. *J Med Microbiol.* 2004; 53:679–690. [PubMed: 15184541]
 93. Xu KD, Franklin MJ, Park CH, McFeters GA, Stewart PS. Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms. *FEMS Microbiol Lett.* 2001; 199:67–71. [PubMed: 11356569]
 94. Lewandowski Z, Beyenal H. Limiting-current-type micro-electrodes for quantifying mass transport dynamics in biofilms. *Methods Enzymol.* 2001; 337:339–359. [PubMed: 11398441]
 95. Dunsmore BC, Jacobsen A, Hall-Stoodley L, Bass CJ, Lappin-Scott HM, Stoodley P. The influence of fluid shear on the structure and material properties of sulphate-reducing bacterial biofilms. *J Ind Microbiol Biotechnol.* 2002; 29:347–353. [PubMed: 12483477]

96. Rani SA, Pitts B, Stewart PS. Rapid diffusion of fluorescent tracers into *Staphylococcus epidermidis* biofilms visualized by time lapse microscopy. *Antimicrob Agents Chemother*. 2005; 49:728–732. [PubMed: 15673757]
97. Xi C, Marks D, Schlachter S, Luo W, Boppart SA. High-resolution three-dimensional imaging of biofilm development using optical coherence tomography. *J Biomed Opt*. 2006; 11:34001. [PubMed: 16822051]
98. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature*. 2014; 507:181–189. [PubMed: 24622198]
99. Groisman A, Lobo C, Cho H, Campbell JK, Dufour YS, Stevens AM, Levchenko A. A microfluidic chemostat for experiments with bacterial and yeast cells. *Nature Methods*. 2005; 2:685–689. [PubMed: 16118639]
100. Leung K, Zahn H, Leaver T, Konwar KM, Hanson NW, Pagé AP, Lo C-C, Chain PS, Hallam SJ, Hansen CL. A programmable droplet-based microfluidic device applied to multiparameter analysis of single microbes and microbial communities. *Proc Natl Acad Sci USA*. 2012; 109:7665–7670. [PubMed: 22547789]
101. Locke JC, Elowitz MB. Using movies to analyse gene circuit dynamics in single cells. *Nat Rev Microbiol*. 2009; 7:383–392. [PubMed: 19369953]
102. Nichols D, Cahoon N, Trakhtenberg E, Pham L, Mehta A, Belanger A, Kanigan T, Lewis K, Epstein S. Use of ichip for high-throughput *in situ* cultivation of “uncultivable” microbial species. *Appl Environ Microbiol*. 2010; 76:2445–2450. [PubMed: 20173072]
103. Weibel DB, DiLuzio WR, Whitesides GM. Microfabrication meets microbiology. *Nat Rev Microbiol*. 2007; 5:209–218. [PubMed: 17304250]
104. Wessel AK, Hmelo L, Parsek MR, Whiteley M. Going local: technologies for exploring bacterial microenvironments. *Nat Rev Microbiol*. 2013; 11:337–348. [PubMed: 23588251]
105. Boedicker JQ, Li L, Kline TR, Ismagilov RF. Detecting bacteria and determining their susceptibility to antibiotics by stochastic confinement in nanoliter droplets using plug-based microfluidics. *Lab Chip*. 2008; 8:1265–1272. [PubMed: 18651067]
106. Zhang Q, Lambert G, Liao D, Kim H, Robin K, Tung C-K, Pourmand N, Austin RH. Acceleration of emergence of bacterial antibiotic resistance in connected microenvironments. *Science*. 2011; 333:1764–1767. [PubMed: 21940899]
107. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. *Science*. 2004; 305:1622–1625. [PubMed: 15308767]
108. Gefen O, Gabay C, Mumcuoglu M, Engel G, Balaban NQ. Single-cell protein induction dynamics reveals a period of vulnerability to antibiotics in persister bacteria. *Proc Natl Acad Sci USA*. 2008; 105:6145–6149. [PubMed: 18427112]
109. Boedicker JQ, Vincent ME, Ismagilov RF. Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. *Angew Chem Int Ed Engl*. 2009; 48:5908–5911. [PubMed: 19565587]
110. Balagaddé FK, You L, Hansen CL, Arnold FH, Quake SR. Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. *Science*. 2005; 309:137–140. [PubMed: 15994559]
111. Rowat AC, Bird JC, Agresti JJ, Rando OJ, Weitz DA. Tracking lineages of single cells in lines using a microfluidic device. *Proc Natl Acad Sci USA*. 2009; 106:18149–18154. [PubMed: 19826080]
112. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, Jun S. Robust growth of *Escherichia coli*. *Curr Biol*. 2010; 20:1099–1103. [PubMed: 20537537]
113. Ottesen EA, Hong JW, Quake SR, Leadbetter JR. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science*. 2006; 314:1464–1467. [PubMed: 17138901]
114. Baret J-C, Miller OJ, Taly V, Ryckelynck M, El-Harrak A, Frenz L, Rick C, Samuels ML, Hutchison JB, Agresti JJ. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. *Lab Chip*. 2009; 9:1850–1858. [PubMed: 19532959]

115. Gomez-Sjoberg R, Morissette DT, Bashir R. Impedance microbiology-on-a-chip: microfluidic bioprocessor for rapid detection of bacterial metabolism. *J Microelectromechanics Syst.* 2005; 14:829–838.
116. Mach AJ, Di Carlo D. Continuous scalable blood filtration device using inertial microfluidics. *Biotechnol Bioeng.* 2010; 107:302–311. [PubMed: 20589838]
117. Wu Z, Willing B, Bjerketorp J, Jansson JK, Hjort K. Soft inertial microfluidics for high throughput separation of bacteria from human blood cells. *Lab Chip.* 2009; 9:1193–1199. [PubMed: 19370236]
118. Mao H, Cremer PS, Manson MD. A sensitive, versatile microfluidic assay for bacterial chemotaxis. *Proc Natl Acad Sci USA.* 2003; 100:5449–5454. [PubMed: 12704234]
119. Stocker R, Seymour JR, Samadani A, Hunt DE, Polz MF. Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches. *Proc Natl Acad Sci USA.* 2008; 105:4209–4214. [PubMed: 18337491]
120. Connell JL, Wessel AK, Parsek MR, Ellington AD, Whiteley M, Shear JB. Probing prokaryotic social behaviors with bacterial “lobster traps. *MBio.* 2010; 1:e00202–00210.10.1128/mBio.00202-10 [PubMed: 21060734]
121. Eun Y-J, Weibel DB. Fabrication of microbial biofilm arrays by geometric control of cell adhesion. *Langmuir.* 2009; 25:4643–4654. [PubMed: 19215108]
122. Kim KP, Kim Y-G, Choi C-H, Kim H-E, Lee S-H, Chang W-S, Lee C-S. *In situ* monitoring of antibiotic susceptibility of bacterial biofilms in a microfluidic device. *Lab Chip.* 2010; 10:3296–3299. [PubMed: 20938507]
123. Kim J, Hegde M, Kim SH, Wood TK, Jayaraman A. A microfluidic device for high throughput bacterial biofilm studies. *Lab Chip.* 2012; 12:1157–1163. [PubMed: 22318368]
124. Skolimowski M, Nielsen MW, Emnéus J, Molin S, Taboryski R, Sternberg C, Dufva M, Geschke O. Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies. *Lab Chip.* 2010; 10:2162–2169. [PubMed: 20571689]
125. Lee J-H, Kaplan J, Lee W. Microfluidic devices for studying growth and detachment of *Staphylococcus epidermidis* biofilms. *Biomed Microdevices.* 2008; 10:489–498. [PubMed: 18204904]
126. Rusconi R, Lecuyer S, Guglielmini L, Stone HA. Laminar flow around corners triggers the formation of biofilm streamers. *J R Soc Interface.* 2010; 7:1293–1299. [PubMed: 20356880]
127. Drescher K, Shen Y, Bassler BL, Stone HA. Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. *Proc Natl Acad Sci USA.* 2013; 110:4345–4350. [PubMed: 23401501]
128. Seminara A, Angelini TE, Wilking JN, Vlamakis H, Ebrahim S, Kolter R, Weitz DA, Brenner MP. Osmotic spreading of *Bacillus subtilis* biofilms driven by an extracellular matrix. *Proc Natl Acad Sci USA.* 2011; 109:1116–1121. [PubMed: 22232655]
129. Wilking JN, Angelini TE, Seminara A, Brenner MP, Weitz DA. Biofilms as complex fluids. *MRS Bull.* 2011; 36:385–391.
130. Hohne DN, Younger JG, Solomon MJ. Flexible microfluidic device for mechanical property characterization of soft viscoelastic solids such as bacterial biofilms. *Langmuir.* 2009; 25:7743–7751. [PubMed: 19219968]
131. De La Fuente L, Montanes E, Meng Y, Li Y, Burr TJ, Hoch H, Wu M. Assessing adhesion forces of type I and type IV pili of *Xylella fastidiosa* bacteria by use of a microfluidic flow chamber. *Appl Environ Microbiol.* 2007; 73:2690–2696. [PubMed: 17293518]
132. Nadell CD, Bassler BL. A fitness trade-off between local competition and dispersal in *Vibrio cholerae* biofilms. *Proc Natl Acad Sci USA.* 2011; 108:14181–14185. [PubMed: 21825170]
133. Hong SH, Hegde M, Kim J, Wang X, Jayaraman A, Wood TK. Synthetic quorum-sensing circuit to control consortial biofilm formation and dispersal in a microfluidic device. *Nat Commun.* 2012; 3:613. [PubMed: 22215088]
134. Metzker ML. Sequencing technologies: the next generation. *Nat Rev Genet.* 2010; 11:31–46. [PubMed: 19997069]

135. Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW. Direct detection of DNA methylation during single-molecule, real-time sequencing. *Nat Methods*. 2010; 7:461–465. [PubMed: 20453866]
136. Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, Fligner CL, Singh PK. Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. *Proc Natl Acad Sci USA*. 2012; 109:13769–13774. [PubMed: 22872870]
137. Chivian D, Brodie EL, Alm EJ, Culley DE, Dehal PS, DeSantis TZ, Gihring TM, Lapidus A, Lin LH, Lowry SR, Moser DP, Richardson PM, Southam G, Wanger G, Pratt LM, Andersen GL, Hazen TC, Brockman FJ, Arkin AP, Onstott TC. Environmental genomics reveals a single-species ecosystem deep within Earth. *Science*. 2008; 322:275–278. [PubMed: 18845759]
138. Dick GJ, Andersson AF, Baker BJ, Simmons SL, Thomas BC, Yelton AP, Banfield JF. Community-wide analysis of microbial genome sequence signatures. *Genome Biol*. 2009; 10:R85. [PubMed: 19698104]
139. Goltsman DS, Dasari M, Thomas BC, Shah MB, VerBerkmoes NC, Hettich RL, Banfield JF. New group in the *Leptospirillum* clade: cultivation-independent community genomics, proteomics, and tran-scriptomics of the new species “*Leptospirillum* group IV UBA BS. *Appl Environ Microbiol*. 2013; 79:5384–5393. [PubMed: 23645189]
140. Yelton AP, Comolli LR, Justice NB, Castelle C, Denef VJ, Thomas BC, Banfield JF. Comparative genomics in acid mine drainage biofilm communities reveals metabolic and structural differentiation of co-occurring archaea. *BMC Genomics*. 2013; 14:485. [PubMed: 23865623]
141. Inskeep WP, Jay ZJ, Herrgard MJ, Kozubal MA, Rusch DB, Tringe SG, Macur RE, Jennings R, Boyd ES, Spear JR, Roberto FF. Phylogenetic and functional analysis of metagenome sequence from high-temperature archaeal habitats demonstrate linkages between metabolic potential and geochemistry. *Front Microbiol*. 2013; 4:95. [PubMed: 23720654]
142. Klatt CG, Wood JM, Rusch DB, Bateson MM, Hamamura N, Heidelberg JF, Grossman AR, Bhaya D, Cohan FM, Kuhl M, Bryant DA, Ward DM. Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential. *ISME J*. 2011; 5:1262–1278. [PubMed: 21697961]
143. Klatt CG, Inskeep WP, Herrgard MJ, Jay ZJ, Rusch DB, Tringe SG, Niki Parenteau M, Ward DM, Boomer SM, Bryant DA, Miller SR. Community structure and function of high-temperature chlorophototrophic microbial mats inhabiting diverse geothermal environments. *Front Microbiol*. 2013; 4:106. [PubMed: 23761787]
144. Hasan NA, Young BA, Minard-Smith AT, Saeed K, Li H, Heizer EM, McMillan NJ, Isom R, Abdullah AS, Bornman DM, Faith SA, Choi SY, Dickens ML, Cebula TA, Colwell RR. Microbial community profiling of human saliva using shotgun metagenomic sequencing. *PLoS One*. 2014; 9:e97699.10.1371/journal.pone.0097699 [PubMed: 24846174]
145. Wang J, Qi J, Zhao H, He S, Zhang Y, Wei S, Zhao F. Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease. *Sci Rep*. 2013; 3:1843. [PubMed: 23673380]
146. Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, Vazquez-Baeza Y, Jansson JK, Gordon JI, Knight R. Meta-analyses of studies of the human microbiota. *Genome Res*. 2013; 23:1704–1714. [PubMed: 23861384]
147. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature*. 2007; 449:804–810. [PubMed: 17943116]
148. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. *Nature*. 2009; 457:480–484. [PubMed: 19043404]
149. Fullwood MJ, Wei CL, Liu ET, Ruan Y. Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. *Genome Res*. 2009; 19:521–532. [PubMed: 19339662]
150. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prijbelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol*. 2012; 19:455–477. [PubMed: 22506599]

151. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. *Genome Res.* 2007; 17:377–386. [PubMed: 17255551]
152. Huson DH, Richter DC, Mitra S, Auch AF, Schuster SC. Methods for comparative metagenomics. *BMC Bioinformatics.* 2009; 10(Suppl 1):S12. [PubMed: 19208111]
153. Namiki T, Hachiya T, Tanaka H, Sakakibara Y. MetaVelvet: an extension of Velvet assembler to *de novo* metagenome assembly from short sequence reads. *Nucleic Acids Res.* 2012; 40:e155. [PubMed: 22821567]
154. Zerbino DR, Birney E. Velvet: algorithms for *de novo* short read assembly using de Bruijn graphs. *Genome Res.* 2008; 18:821–829. [PubMed: 18349386]
155. McLean RJ, Kakirde KS. Enhancing metagenomics investigations of microbial interactions with biofilm technology. *Int J Mol Sci.* 2013; 14:22246–22257. [PubMed: 24284397]
156. Blainey PC. The future is now: single-cell genomics of bacteria and archaea. *FEMS Microbiol Rev.* 2013; 37:407–427. [PubMed: 23298390]
157. Landry ZC, Giovanonni SJ, Quake SR, Blainey PC. Optofluidic cell selection from complex microbial communities for single-genome analysis. *Methods Enzymol.* 2013; 531:61–90. [PubMed: 24060116]
158. McLean JS, Lombardo MJ, Badger JH, Edlund A, Novotny M, Yee-Greenbaum J, Vyahhi N, Hall AP, Yang Y, Dupont CL, Ziegler MG, Chitsaz H, Allen AE, Yooseph S, Tesler G, Pevzner PA, Friedman RM, Neelson KH, Venter JC, Lasken RS. Candidate phylum TM6 genome recovered from a hospital sink biofilm provides genomic insights into this uncultivated phylum. *Proc Natl Acad Sci USA.* 2013; 110:E2390–E2399. [PubMed: 23754396]
159. Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS. Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci USA.* 2002; 99:5261–5266. [PubMed: 11959976]
160. Hentzer M, Eberl L, Givskov M. Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. *Biofilms.* 2005; 2:37–62.
161. Mikkelsen H, Duck Z, Lilley KS, Welch M. Interrelationships between colonies, biofilms, and planktonic cells of *Pseudomonas aeruginosa*. *J Bacteriol.* 2007; 189:2411–2416. [PubMed: 17220232]
162. Schoolnik GK, Voskuil MI, Schnappinger D, Yildiz FH, Meibom K, Dolganov NA, Wilson MA, Chong KH. Whole genome DNA microarray expression analysis of biofilm development by *Vibrio cholerae* O1 E1 Tor. *Methods Enzymol.* 2001; 336:3–18. [PubMed: 11398407]
163. Waite RD, Paccanaro A, Papakonstantinou A, Hurst JM, Saqi M, Littler E, Curtis MA. Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. *BMC Genomics.* 2006; 7:162. [PubMed: 16800888]
164. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature.* 2001; 413:860–864. [PubMed: 11677611]
165. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, Soboleva A. NCBI GEO: archive for functional genomics data sets: update. *Nucleic Acids Res.* 2013; 41:D991–D995. [PubMed: 23193258]
166. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 2002; 30:207–210. [PubMed: 11752295]
167. Stead MB, Marshburn S, Mohanty BK, Mitra J, Pena Castillo L, Ray D, van Bakel H, Hughes TR, Kushner SR. Analysis of *Escherichia coli* RNase E and RNase III activity *in vivo* using tiling microarrays. *Nucleic Acids Res.* 2011; 39:3188–3203. [PubMed: 21149258]
168. Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, Loh E, Gripenland J, Tiensuu T, Vaitkevicius K, Barthélemy M, Vergassola M, Nahori MA, Soubigou G, Regnault B, Coppee JY, Lecuit M, Johansson J, Cossart P. The *Listeria* transcriptional landscape from saprophytism to virulence. *Nature.* 2009; 459:950–956. [PubMed: 19448609]

169. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet.* 2009; 10:57–63. [PubMed: 19015660]
170. Gifford S, Satinsky B, Moran MA. Quantitative microbial metatranscriptomics. *Methods Mol Biol.* 2014; 1096:213–229. [PubMed: 24515372]
171. Moran MA, Satinsky B, Gifford SM, Luo H, Rivers A, Chan LK, Meng J, Durham BP, Shen C, Varaljay VA, Smith CB, Yager PL, Hopkinson BM. Sizing up metatranscriptomics. *ISME J.* 2013; 7:237–243. [PubMed: 22931831]
172. Jorth P, Turner KH, Gumus P, Nizam N, Buduneli N, Whiteley M. Metatranscriptomics of the human oral microbiome during health and disease. *MBio.* 2014; 5:e01012–01014.10.1128/mBio.01012-14 [PubMed: 24692635]
173. Klatt CG, Liu Z, Ludwig M, Kuhl M, Jensen SI, Bryant DA, Ward DM. Temporal metatranscriptomic patterning in phototrophic *Chloroflexi* inhabiting a microbial mat in a geothermal spring. *ISME J.* 2013; 7:1775–1789. [PubMed: 23575369]
174. Liu Z, Klatt CG, Wood JM, Rusch DB, Ludwig M, Wittekindt N, Tomsho LP, Schuster SC, Ward DM, Bryant DA. Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. *ISME J.* 2011; 5:1279–1290. [PubMed: 21697962]
175. Cattoir V, Narasimhan G, Skurnik D, Aschard H, Roux D, Ramphal R, Jyot J, Lory S. Transcriptional response of mucoid *Pseudomonas aeruginosa* to human respiratory mucus. *MBio.* 2013; 3:e00410–00412.10.1128/mBio.00410-12 [PubMed: 23143799]
176. Dugar G, Herbig A, Forstner KU, Heidrich N, Reinhardt R, Nieselt K, Sharma CM. High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet.* 2013; 9:e1003495.10.1371/journal.pgen.1003495 [PubMed: 23696746]
177. Mandlik A, Livny J, Robins WP, Ritchie JM, Mekalanos JJ, Waldor MK. RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. *Cell Host Microbe.* 2011; 10:165–174. [PubMed: 21843873]
178. Taveirne ME, Theriot CM, Livny J, DiRita VJ. The complete *Campylobacter jejuni* transcriptome during colonization of a natural host determined by RNAseq. *PLoS One.* 2013; 8:e73586.10.1371/journal.pone.0073586 [PubMed: 23991199]
179. Pérez-Orsorio AC, Williamson KS, Franklin MJ. Heterogeneous *tpoS* and *rhIR* mRNA levels and 16S rRNA/rDNA (rRNA gene) ratios within *Pseudomonas aeruginosa* biofilms, sampled by laser capture microdissection. *J Bacteriol.* 2010; 192:2991–3000. [PubMed: 20348255]
180. Van Oudenhoove L, Devreese B. A review on recent developments in mass spectrometry instrumentation and quantitative tools advancing bacterial proteomics. *Appl Microbiol Biotechnol.* 2013; 97:4749–4762. [PubMed: 23624659]
181. Vaudel M, Sickmann A, Martens L. Introduction to opportunities and pitfalls in functional mass spectrometry based proteomics. *Biochim Biophys Acta.* 2013; 1844:12–20. [PubMed: 23845992]
182. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol.* 2002; 184:1140–1154. [PubMed: 11807075]
183. Boes N, Schreiber K, Hartig E, Jaensch L, Schobert M. The *Pseudomonas aeruginosa* universal stress protein PA4352 is essential for surviving anaerobic energy stress. *J Bacteriol.* 2006; 188:6529–6538. [PubMed: 16952944]
184. Fong JC, Karplus K, Schoolnik GK, Yildiz FH. Identification and characterization of RbmA, a novel protein required for the development of rugose colony morphology and biofilm structure in *Vibrio cholerae*. *J Bacteriol.* 2006; 188:1049–1059. [PubMed: 16428409]
185. Kalmokoff M, Lanthier P, Tremblay TL, Foss M, Lau PC, Sanders G, Austin J, Kelly J, Szymanski CM. Proteomic analysis of *Campylobacter jejuni* 11168 biofilms reveals a role for the motility complex in biofilm formation. *J Bacteriol.* 2006; 188:4312–4320. [PubMed: 16740937]
186. Patrauchan MA, Sarkisova SA, Franklin MJ. Strain-specific proteome responses of *Pseudomonas aeruginosa* to biofilm-associated growth and to calcium. *Microbiology.* 2007; 153:3838–3851. [PubMed: 17975093]

187. Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *J Bacteriol.* 2005; 187:4327–4337. [PubMed: 15968041]
188. Rao J, Damron FH, Basler M, Digiandomenico A, Sherman NE, Fox JW, Mekalanos JJ, Goldberg JB. Comparisons of two proteomic analyses of non-mucoid and mucoid *Pseudomonas aeruginosa* clinical isolates from a cystic fibrosis patient. *Front Microbiol.* 2011; 2:162. [PubMed: 21863142]
189. Sauer K, Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol.* 2001; 183:6579–6589. [PubMed: 11673428]
190. Schreiber K, Boes N, Eschbach M, Jaensch L, Wehland J, Bjarnsholt T, Givskov M, Hentzer M, Schobert M. Anaerobic survival of *Pseudomonas aeruginosa* by pyruvate fermentation requires an Usp-type stress protein. *J Bacteriol.* 2006; 188:659–668. [PubMed: 16385055]
191. Wen ZT, Baker HV, Burne RA. Influence of BrpA on critical virulence attributes of *Streptococcus mutans*. *J Bacteriol.* 2006; 188:2983–2992. [PubMed: 16585759]
192. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis.* 1997; 18:2071–2077. [PubMed: 9420172]
193. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics.* 2001; 1:377–396. [PubMed: 11680884]
194. Marouga R, David S, Hawkins E. The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal Bioanal Chem.* 2005; 382:669–678. [PubMed: 15900442]
195. Maaty WS, Wiedenheft B, Tarlykov P, Schaff N, Heinemann J, Robison-Cox J, Valenzuela J, Dougherty A, Blum P, Lawrence CM, Douglas T, Young MJ, Bothner B. Something old, something new, something borrowed; how the thermoacidophilic archaeon *Sulfolobus solfataricus* responds to oxidative stress. *PLoS One.* 2009; 4:e6964.10.1371/journal.pone.0006964 [PubMed: 19759909]
196. Petrova OE, Sauer K. Dispersion by *Pseudomonas aeruginosa* requires an unusual posttranslational modification of BdlA. *Proc Natl Acad Sci USA.* 2012; 109:16690–16695. [PubMed: 23012421]
197. Denef VJ, Shah MB, Verberkmoes NC, Hettich RL, Banfield JF. Implications of strain- and species-level sequence divergence for community and isolate shotgun proteomic analysis. *J Proteome Res.* 2007; 6:3152–3161. [PubMed: 17602579]
198. Jiao Y, D'Haeseleer P, Dill BD, Shah M, Verberkmoes NC, Hettich RL, Banfield JF, Thelen MP. Identification of biofilm matrix-associated proteins from an acid mine drainage microbial community. *Appl Environ Microbiol.* 2011; 77:5230–5237. [PubMed: 21685158]
199. Pessi G, Braunwalder R, Grunau A, Omasits U, Ahrens CH, Eberl L. Response of *Burkholderia cenocepacia* H111 to micro-oxia. *PLoS One.* 2013; 8:e72939.10.1371/journal.pone.0072939 [PubMed: 24023794]
200. Santi L, Beys-da-Silva WO, Berger M, Calzolari D, Guimaraes JA, Moresco JJ, Yates JR 3rd. Proteomic profile of *Cryptococcus neoformans* biofilm reveals changes in metabolic processes. *J Proteome Res.* 2014; 13:1545–1559. [PubMed: 24467693]
201. Schmid N, Pessi G, Deng Y, Aguilar C, Carlier AL, Grunau A, Omasits U, Zhang LH, Ahrens CH, Eberl L. The AHL- and BDSF-dependent quorum sensing systems control specific and overlapping sets of genes in *Burkholderia cenocepacia* H111. *PLoS One.* 2012; 7:e49966.10.1371/journal.pone.0049966 [PubMed: 23185499]
202. Wu WL, Liao JH, Lin GH, Lin MH, Chang YC, Liang SY, Yang FL, Khoo KH, Wu SH. Phosphoproteomic analysis reveals the effects of PilF phosphorylation on type IV pilus and biofilm formation in *Thermus thermophilus* HB27. *Mol Cell Proteomics.* 2013; 12:2701–2713. [PubMed: 23828892]
203. Vera M, Krok B, Bellenberg S, Sand W, Poetsch A. Shotgun proteomics study of early biofilm formation process of *Acidithiobacillus ferrooxidans* ATCC 23270 on pyrite. *Proteomics.* 2013; 13:1133–1144. [PubMed: 23319327]

204. Wolters DA, Washburn MP, Yates JR 3rd. An automated multidimensional protein identification technology for shotgun proteomics. *Anal Chem.* 2001; 73:5683–5690. [PubMed: 11774908]
205. Cabral MP, Soares NC, Aranda J, Parreira JR, Rumbo C, Poza M, Valle J, Calamia V, Lasa I, Bou G. Proteomic and functional analyses reveal a unique lifestyle for *Acinetobacter baumannii* biofilms and a key role for histidine metabolism. *J Proteome Res.* 2011; 10:3399–3417. [PubMed: 21612302]
206. Chopra S, Ramkissoon K, Anderson DC. A systematic quantitative proteomic examination of multidrug resistance in *Acinetobacter baumannii*. *J Proteomics.* 2013; 84:17–39. [PubMed: 23542354]
207. Evans C, Noirel J, Ow SY, Salim M, Pereira-Medrano AG, Couto N, Pandhal J, Smith D, Pham TK, Karunakaran E, Zou X, Biggs CA, Wright PC. An insight into iTRAQ: where do we stand now? *Anal Bioanal Chem.* 2012; 404:1011–1027. [PubMed: 22451173]
208. Huynh TT, McDougald D, Klebensberger J, Al Qarni B, Barraud N, Rice SA, Kjelleberg S, Schleheck D. Glucose starvation-induced dispersal of *Pseudomonas aeruginosa* biofilms is cAMP and energy dependent. *PLoS One.* 2012; 7:e42874.10.1371/journal.pone.0042874 [PubMed: 22905180]
209. Pham TK, Roy S, Noirel J, Douglas I, Wright PC, Stafford GP. A quantitative proteomic analysis of biofilm adaptation by the periodontal pathogen *Tannerella forsythia*. *Proteomics.* 2010; 10:3130–3141. [PubMed: 20806225]
210. Solis N, Parker BL, Kwong SM, Robinson G, Firth N, Cordwell SJ. *Staphylococcus aureus* surface proteins involved in adaptation to oxacillin identified using a novel cell shaving approach. *J Proteome Res.* 2014; 13:2954–2972. [PubMed: 24708102]
211. Booth SC, Workentine ML, Wen J, Shaykhutdinov R, Vogel HJ, Ceri H, Turner RJ, Weljie AM. Differences in metabolism between the biofilm and planktonic response to metal stress. *J Proteome Res.* 2011; 10:3190–3199. [PubMed: 21561166]
212. Kouremenos KA, Beale DJ, Antti H, Palombo EA. Liquid chromatography time of flight mass spectrometry based environmental metabolomics for the analysis of *Pseudomonas putida* bacteria in potable water. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2014; 966:179–86.
213. Martinez P, Galvez S, Ohtsuka N, Budinich M, Cortes MP, Serpell C, Nakahigashi K, Hirayama A, Tomita M, Soga T, Martinez S, Maass A, Parada P. Metabolomic study of Chilean biominer bacteria *Acidithiobacillus ferrooxidans* strain Wenelen and *Acidithiobacillus thiooxidans* strain Licanantay. *Metabolomics.* 2013; 9:247–257. [PubMed: 23335869]
214. Mosier AC, Justice NB, Bowen BP, Baran R, Thomas BC, Northen TR, Banfield JF. Metabolites associated with adaptation of micro-organisms to an acidophilic, metal-rich environment identified by stable-isotope-enabled metabolomics. *MBio.* 2013; 4:e00484–00412.10.1128/mBio.00484-12 [PubMed: 23481603]
215. White AP, Weljie AM, Apel D, Zhang P, Shaykhutdinov R, Vogel HJ, Surette MG. A global metabolic shift is linked to *Salmonella* multicellular development. *PLoS One.* 2010; 5:e11814.10.1371/journal.pone.0011814 [PubMed: 20676398]
216. Workentine ML, Harrison JJ, Weljie AM, Tran VA, Stenroos PU, Tremaroli V, Vogel HJ, Ceri H, Turner RJ. Phenotypic and metabolic profiling of colony morphology variants evolved from *Pseudomonas fluorescens* biofilms. *Environ Microbiol.* 2010; 12:1565–1577. [PubMed: 20236162]
217. Want EJ, O'Maille G, Smith CA, Brandon TR, Uritboonthai W, Qin C, Trauger SA, Siuzdak G. Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass spectrometry. *Anal Chem.* 2006; 78:743–752. [PubMed: 16448047]
218. Want EJ, Cravatt BF, Siuzdak G. The expanding role of mass spectrometry in metabolite profiling and characterization. *Chembiochem.* 2005; 6:1941–1951. [PubMed: 16206229]
219. Tautenhahn R, Patti GJ, Kalisiak E, Miyamoto T, Schmidt M, Lo FY, McBee J, Baliga NS, Siuzdak G. metaXCMS: second-order analysis of untargeted metabolomics data. *Anal Chem.* 2010; 83:696–700. [PubMed: 21174458]
220. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G. METLIN: a metabolite mass spectral database. *Ther Drug Monit.* 2005; 27:747–751. [PubMed: 16404815]

221. Tautenhahn R, Cho K, Uritboonthai W, Zhu Z, Patti GJ, Siuzdak G. An accelerated workflow for untargeted metabolomics using the METLIN database. *Nat Biotechnol.* 2012; 30:826–828. [PubMed: 22965049]
222. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazzyrova A, Shaykhtudinov R, Li L, Vogel HJ, Forsythe I. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res.* 2009; 37:D603–D610. [PubMed: 18953024]
223. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. Imaging intracellular fluorescent proteins at nanometer resolution. *Science.* 2006; 313:1642–1645. [PubMed: 16902090]
224. Hess ST, Girirajan TP, Mason MD. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys J.* 2006; 91:4258–4272. [PubMed: 16980368]
225. Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods.* 2006; 3:793–795. [PubMed: 16896339]
226. Neu TR, Lawrence JR. Investigation of microbial biofilm structure by laser scanning microscopy. *Adv Biochem Eng Biotechnol.* 2014; 146:1–51. [PubMed: 24840778]
227. Jackson KD, Starkey M, Kremer S, Parsek MR, Wozniak DJ. Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J Bacteriol.* 2004; 186:4466–4475. [PubMed: 15231778]
228. Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, Givskov M, Whitchurch CB, Engel JN, Tolker-Nielsen T. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol.* 2008; 10:2331–2343. [PubMed: 18485000]
229. Christen B, Fero MJ, Hillson NJ, Bowman G, Hong SH, Shapiro L, McAdams HH. High-throughput identification of protein localization dependency networks. *Proc Natl Acad Sci USA.* 2010; 107:4681–4686. [PubMed: 20176934]
230. Lew MD, Lee SF, Ptacin JL, Lee MK, Twieg RJ, Shapiro L, Moerner WE. Three-dimensional superresolution colocalization of intracellular protein superstructures and the cell surface in live *Caulobacter crescentus*. *Proc Natl Acad Sci USA.* 2011; 108:E1102–E1110. [PubMed: 22031697]
231. Li G, Young KD. Isolation and identification of new inner membrane-associated proteins that localize to cell poles in *Escherichia coli*. *Mol Microbiol.* 2012; 84:276–295. [PubMed: 22380631]
232. Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proc Natl Acad Sci USA.* 2008; 105:3076–3081. [PubMed: 18287048]
233. Chai Y, Chu F, Kolter R, Losick R. Bistability and biofilm formation in *Bacillus subtilis*. *Mol Microbiol.* 2008; 67:254–263. [PubMed: 18047568]
234. Lydmark P, Lind M, Sørensen F, Hermansson M. Vertical distribution of nitrifying populations in bacterial biofilms from a full-scale nitrifying trickling filter. *Environ Microbiol.* 2006; 8:2036–2049. [PubMed: 17014502]
235. Kofoed MV, Nielsen DA, Revsbech NP, Schramm A. Fluorescence *in situ* hybridization (FISH) detection of nitrite reductase transcripts (*nirS* mRNA) in *Pseudomonas stutzeri* biofilms relative to a microscale oxygen gradient. *Syst Appl Microbiol.* 2012; 35:513–517. [PubMed: 22300865]
236. Rosenthal AZ, Zhang X, Lucey KS, Ottesen EA, Trivedi V, Choi HM, Pierce NA, Leadbetter JR. Localizing transcripts to single cells suggests an important role of uncultured deltaproteobacteria in the termite gut hydrogen economy. *Proc Natl Acad Sci USA.* 2013; 110:16163–16168. [PubMed: 24043823]
237. Behnam F, Vilcinskis A, Wagner M, Stoecker K. A straightforward DOPE (double labeling of oligonucleotide probes)-FISH (fluorescence *in situ* hybridization) method for simultaneous multicolor detection of six microbial populations. *Appl Environ Microbiol.* 2012; 78:5138–5142. [PubMed: 22582069]

238. Valm AM, Mark Welch JL, Rieken CW, Hasegawa Y, Sogin ML, Oldenbourg R, Dewhirst FE, Borisy GG. Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. *Proc Natl Acad Sci USA*. 2011; 108:4152–4157. [PubMed: 21325608]
239. Valm AM, Mark Welch JL, Borisy GG. CLASI-FISH: principles of combinatorial labeling and spectral imaging. *Syst Appl Microbiol*. 2012; 35:496–502. [PubMed: 22521573]
240. Schneider D, Fuhrmann E, Scholz I, Hess WR, Graumann PL. Fluorescence staining of live cyanobacterial cells suggest non-stringent chromosome segregation and absence of a connection between cytoplasmic and thylakoid membranes. *BMC Cell Biol*. 2007; 8:39. [PubMed: 17767716]
241. Créach V, Baudoux AC, Bertru G, Rouzic BL. Direct estimate of active bacteria: CTC use and limitations. *J Microbiol Methods*. 2003; 52:19–28. [PubMed: 12401223]
242. Nielsen JL, Aquino de Muro M, Nielsen PH. Evaluation of the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride for activity studies by simultaneous use of microautoradiography and fluorescence *in situ* hybridization. *Appl Environ Microbiol*. 2003; 69:641–643. [PubMed: 12514052]
243. Breinbauer R, Kohn M. Azide-alkyne coupling: a powerful reaction for bioconjugate chemistry. *Chembiochem*. 2003; 4:1147–1149. [PubMed: 14613105]
244. Liechti GW, Kuru E, Hall E, Kalinda A, Brun YV, VanNieuwenhze M, Maurelli AT. A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. *Nature*. 2014; 506:507–510. [PubMed: 24336210]
245. Siegrist MS, Whiteside S, Jewett JC, Aditham A, Cava F, Bertozzi CR. (D)-amino acid chemical reporters reveal peptidoglycan dynamics of an intracellular pathogen. *ACS Chem Biol*. 2013; 8:500–505. [PubMed: 23240806]
246. Spiers AJ, Bohannon J, Gehrig SM, Rainey PB. Biofilm formation at the air-liquid interface by the *Pseudomonas fluorescens* SBW25 wrinkly spreader requires an acetylated form of cellulose. *Mol Microbiol*. 2003; 50:15–27. [PubMed: 14507360]
247. Elzinga EJ, Huang JH, Chorover J, Kretzschmar R. ATR-FTIR spectroscopy study of the influence of pH and contact time on the adhesion of *Shewanella putrefaciens* bacterial cells to the surface of hematite. *Environ Sci Technol*. 2012; 46:12848–12855. [PubMed: 23136883]
248. Quilès F, Humbert F. On the production of glycogen by *Pseudomonas fluorescens* during biofilm development: an *in situ* study by attenuated total reflection-infrared with chemometrics. *Biofouling*. 2014; 30:709–718. [PubMed: 24835847]
249. Suci PA, Geesey GG, Tyler BJ. Integration of Raman microscopy, differential interference contrast microscopy, and attenuated total reflection Fourier transform infrared spectroscopy to investigate chlorhexidine spatial and temporal distribution in *Candida albicans* biofilms. *J Microbiol Methods*. 2001; 46:193–208. [PubMed: 11438184]
250. Holman HY, Miles R, Hao Z, Wozel E, Anderson LM, Yang H. Real-time chemical imaging of bacterial activity in biofilms using open-channel microfluidics and synchrotron FTIR spectromicroscopy. *Anal Chem*. 2009; 81:8564–8570. [PubMed: 19775125]
251. Pätzold R, Keuntje M, Theophile K, Muller J, Mielcarek E, Ngezahayo A, Anders-von Ahlften A. *In situ* mapping of nitrifiers and anammox bacteria in microbial aggregates by means of confocal resonance Raman microscopy. *J Microbiol Methods*. 2008; 72:241–248. [PubMed: 18255179]
252. Pätzold R, Keuntje M, Anders-von Ahlften A. A new approach to non-destructive analysis of biofilms by confocal Raman microscopy. *Anal Bioanal Chem*. 2006; 386:286–292. [PubMed: 16868726]
253. Sandt C, Smith-Palmer T, Pink J, Brennan L, Pink D. Confocal Raman microspectroscopy as a tool for studying the chemical heterogeneities of biofilms *in situ*. *J Appl Microbiol*. 2007; 103:1808–1820. [PubMed: 17953591]
254. Sandt C, Smith-Palmer T, Comeau J, Pink D. Quantification of water and biomass in small colony variant PAO1 biofilms by confocal Raman microspectroscopy. *Appl Microbiol Biotechnol*. 2009; 83:1171–1182. [PubMed: 19529929]

255. Chao Y, Zhang T. Surface-enhanced Raman scattering (SERS) revealing chemical variation during biofilm formation: from initial attachment to mature biofilm. *Anal Bioanal Chem.* 2012; 404:1465–1475. [PubMed: 22820905]
256. Ivleva NP, Wagner M, Horn H, Niessner R, Haisch C. *In situ* surface-enhanced Raman scattering analysis of biofilm. *Anal Chem.* 2008; 80:8538–8544. [PubMed: 18947197]
257. Ivleva NP, Wagner M, Szkola A, Horn H, Niessner R, Haisch C. Label-free *in situ* SERS imaging of biofilms. *J Phys Chem B.* 2010; 114:10184–10194. [PubMed: 20684642]
258. Wagner M, Ivleva NP, Haisch C, Niessner R, Horn H. Combined use of confocal laser scanning microscopy (CLSM) and Raman microscopy (RM): investigations on EPS-matrix. *Water Res.* 2009; 43:63–76. [PubMed: 19019406]
259. Chang CB, Wilking JN, Kim S-H, Shum HC, Weitz DA. Monodisperse emulsion drop microenvironments for bacterial biofilm growth. *Small.* 201510.1002/sml.201403125
260. Alonso C. Tips and tricks for high quality MAR-FISH preparations: focus on bacterioplankton analysis. *Syst Appl Microbiol.* 2012; 35:503–512. [PubMed: 22502862]
261. Tischer K, Zeder M, Klug R, Pernthaler J, Schattenhofer M, Harms H, Wendeberg A. Fluorescence in situ hybridization (CARD-FISH) of microorganisms in hydrocarbon contaminated aquifer sediment samples. *Syst Appl Microbiol.* 2012; 35:526–532. [PubMed: 22425347]
262. Majors PD, McLean JS, Pinchuk GE, Fredrickson JK, Gorby YA, Minard KR, Wind RA. NMR methods for in situ biofilm metabolism studies. *J Microbiol Methods.* 2005; 62:337–344. [PubMed: 15936835]
263. Renslow RS, Majors PD, McLean JS, Fredrickson JK, Ahmed B, Beyenal H. In situ effective diffusion coefficient profiles in live biofilms using pulsed-field gradient nuclear magnetic resonance. *Biotechnol Bioeng.* 2010; 106:928–937. [PubMed: 20589671]

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

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. doi: 10.1128/microbiolspec.MB-0016-2014.f2

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

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

FIGURE 5.

Gene expression heterogeneity, demonstrated by translational fusions of target proteins to the yellow fluorescent protein (YFP). (A) Translational fusion of the Ibpa protein to YFP, showing uniform distribution of Ibpa throughout the biofilm. Cells were counterstained with mCherry fluorescent protein (mChFP). (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 mChFP (M.J. Franklin, unpublished data). doi:10.1128/microbiolspec.MB-0016-2014.f5

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

TABLE 1

Methods for *in vitro* cultivation of biofilms

Format/technique	Experimental features	Applications and examples	References
Static biofilms	<ul style="list-style-type: none"> • Low or no shear • No replacement of medium • No cell washout 		
Microtiter plate	<ul style="list-style-type: none"> • High throughput • Limited biomass 	<ul style="list-style-type: none"> • Phenotypic screening of mutant libraries • Attachment and early biofilm development studies • Biomass quantification with staining 	26, 81
Calgary device (MBEC)	<ul style="list-style-type: none"> • High throughput • Peg material may be modified • Biomass may be recovered from pegs • Limited amount of biomass 	<ul style="list-style-type: none"> • Phenotypic screening of mutant libraries • Antibiotic susceptibility studies • Microscopy with fluorescent probes • Biomass quantification with staining 	82
Colony biofilm	<ul style="list-style-type: none"> • Large biomass in short amount of time • Inexpensive laboratory materials • Low throughput 	<ul style="list-style-type: none"> • Antibiotic susceptibility and penetration studies • Chemical gradient measurements using microelectrodes • Heterogeneity studies using microscopy and fluorescent probes • Cryosectioning studies for gene expression heterogeneity 	38, 40, 61, 62, 85, 87
Continuous flow biofilms	<ul style="list-style-type: none"> • Continuous supply of fresh medium • Adjustable shear force • Low to medium throughput 		
CDC reactor	<ul style="list-style-type: none"> • Special surface materials may be used • Multiple biofilms are formed simultaneously • Suitable for time-course study • May be used for anaerobic cultures 	<ul style="list-style-type: none"> • Antibiotic susceptibility/viability studies • Microscopy studies with fluorescent probes • Applicable for omics studies 	88
Drip flow reactor	<ul style="list-style-type: none"> • Special surface materials may be used • High gas transfer • Heterogeneous biofilm • Large biomass in short time 	<ul style="list-style-type: none"> • Antibiotic susceptibility/viability studies • Chemical gradient measurements using microelectrodes • Heterogeneity studies using microscopy fluorescent probes 	38, 61, 89

Format/technique	Experimental features	Applications and examples	References
Imaging flow cells	<ul style="list-style-type: none"> Real-time detection Surfaces can be modified Appropriate for short-time experiments 	<ul style="list-style-type: none"> Cryosection and laser capture microdissection followed by transcriptomic analysis Biofilm-immune cell interaction 	40, 79, 80, 91
		<ul style="list-style-type: none"> Real-time imaging Monitoring attachment, development, and detachment phases Microscopy with fluorescent tags Attenuated total reflection Fourier transform infrared spectrometry Hydrodynamics in biofilm by nuclear magnetic resonance 	
Microfluidics	<ul style="list-style-type: none"> Miniaturization Custom platform Adjustable shear force 		
Microfluidics flow cells	<ul style="list-style-type: none"> Precise control of hydrodynamics Low to medium throughput 	<ul style="list-style-type: none"> Microscopy with fluorescent tags Spatial heterogeneity Quorum sensing Effect of hydrodynamics on biofilm growth and detachment Formation of streamer phenotype Measurements of adhesion forces 	123, 124, 125, 126, 127, 131, 133
Micropatterned polymer stencils	<ul style="list-style-type: none"> High throughput Both static and flow conditions Surface materials can be changed or coated Topographically patterned surfaces can be used 	<ul style="list-style-type: none"> Microscopy with fluorescent tags Chemical heterogeneity and quorum sensing Attachment and early biofilm development Spatial heterogeneity 	103, 121

TABLE 2

Imaging strategies in biofilm studies

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

Technique	Experimental features	Types and modifications	Application and examples	References
			<ul style="list-style-type: none"> Quantification of biomass Spatial distribution of biomolecules 	
Nuclear magnetic resonance microscopy	<ul style="list-style-type: none"> Nondestructive Real-time detection 	<ul style="list-style-type: none"> Use of isotope-labeled substrates 	<ul style="list-style-type: none"> Water flow and water dynamics of EPS Dynamics of metabolites 	79, 80, 262, 263

Abbreviations: CLASI, combinatorial labeling and spectral imaging; ATR/FT-IR, attenuated total reflection Fourier transform infrared spectrometry; BrdU, bromo-deoxyuridine; BRET, bioluminescence resonance energy transfer; CARD-FISH, catalyzed reporter deposition-FISH; CTC, cyanoditolyl 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.