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Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review

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Abstract

Biofilms are microbial communities attached to a surface and embedded in an extracellular polymeric substance which provides for the protection, stability and nutrients of the various bacterial species indwelling. These communities can build up in a variety of different environments from industrial equipment to medical devices resulting in damage, loss of productivity and disease. They also have great potential for economic and societal benefits as bioremediation agents and renewable energy sources. The great potential benefits and threats of biofilms has encouraged researchers across disciplines to study biofilm characteristics and antibiofilm strategies resulting in chemists, physicists, material scientists, and engineers, to develop beneficial biofilm applications and prevention methods. The ultimate outcome is a wealth of knowledge and innovative technology. However, without extensive formal training in microbes and biofilm research, these scientists find a daunting array of established techniques for growing, quantifying and characterizing biofilms while trying to design experiments and develop innovative laboratory protocols. This mini-review focuses on enriching interdisciplinary efforts and understanding by overviewing a variety of quantitative and qualitative biofilm characterization methods to assist the novice researcher in assay selection.

This review consists of four parts. Part 1 is a brief overview of biofilms and the unique properties that demand a highly interdisciplinary approach. Part 2 describes the classical quantification techniques including colony forming unit (CFU) counting and crystal violet staining, but also introduces some modern methods including ATP bioluminescence and quartz crystal microbalance. Part 3 focuses on the characterization of biofilm morphology and chemistry including scanning electron microscopy and spectroscopic methods. Finally, Part 4 illustrates the use of software, including ImageJ and predictive modeling platforms, for biofilm analysis. Each section highlights the most common methods, including literature references, to help novice biofilm researchers make choices which commensurate with their study goals, budget and available equipment.

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INTRODUCTION

A biofilm is a complex, three-dimensional microbial community that grows at an interface and interacts with the surrounding environment [1,2]. Biofilms have great potential to be exploited as a renewable aid in applications of waste, soil and water remediation through the sequestration and conversion of potentially toxic compounds [3–5]. Furthermore, biofilms have the potential to revolutionize energy and chemical production as a renewable source of biocatalysis and electrochemical cells [6,7]. Unfortunately, biofilms are also a major medical issue which cause of 60–80% of microbial infections and present a unique challenge in regards to disease diagnosis and treatment [8,9]. Biofilms also pose societal and industrial concern through equipment contamination leading to loss of productivity, product recall and potential epidemic [10,11]. It is these innovations and challenges which fuel the interdisciplinary study of biofilms and the need for greater understanding of best practices in biofilm research.

Biofilms are typically composed of multiple microbial species which exhibit complex community organization and cooperation leading to emergent properties that assists in organism survival in harsh conditions. Within biofilms, cells communicate with small molecules in order to coordinate activities contributing to the survival of the community which can influence the biofilm composition and structure. Biofilm architecture typically consists of live and dead bacterial cells, extracellular polymeric substances and other materials secreted by the cells [12]. Although the structure and spatial organization is primarily dictated by bacterial species and ratio of bacterial species, bacteria adapt the physical structures and material properties of the matrix based on changes in microbial populations and environmental conditions such as shear stresses, nutrient availability, and competing organisms as a survival measure. As a bonus to these adaptations, biofilms demonstrate increased hardiness against harsh chemical conditions, starvation, and antimicrobial agents. In fact, the adaptability of the biofilm matrix has been suggested to be a key component of biofilms persistence in harsh environments due to decreased ability for antimicrobial agents to diffuse through the structure thereby allowing only sub-lethal exposure of cells to these agents. Furthermore, the close proximity of cells allows increased potential for bacteria to spread antimicrobial resistance which limits the future materials and methods that can be used for anti-biofilm treatments. Finally, environmental elements incorporated into the matrix can be used as nutrients during starvation conditions [13]. Although, much progress has been made in understanding these complex features of biofilms much work remains. The complexities of present challenges are best met by a multidisciplinary approach capable of addressing not only the traditional biological properties of the films, but also their dynamic chemical, physical, and material properties.

Biofilm dynamics and complex architecture creates challenges for basic measurements regarding the number of viable cells, mass accumulation, biofilm morphology, and other critical properties. These challenges are not in the measurements themselves, but in the lack of standardized protocols for characterization and uniform training availability for individuals wanting to contribute to biofilm related projects without formal training in biofilm research to discern the optimal characterization method for their study. For example, biofilm accumulation measurements can focus on total dry mass, total organic carbon, number of live cells, or total number of cells (live and dead). Film morphology studies could involve two-dimensional surface structures illuminated through staining techniques and light microscopy or three-dimensional features revealed by confocal scanning laser microscopy (CSLM) [14,15]. Appropriate choice of techniques based on information required, equipment availability, cost and ease-of-use will be facilitated by this guide. In the following sections the most commonly used methods of biofilm characterization will be discussed in detail as a resource to aid in planning of biofilm characterization experiments.

QUANTITATIVE CHARACTERIZATION METHODS

One of the most basic and most commonly acquired types of bacterial measurements, whether in planktonic or biofilm cultures, is the determination of how much is present. A variety of direct and indirect methods have been used to quantify cells in biofilms. Direct counting methods permit enumeration of cells that can be cultured, including plate counts, microscopic cell counts, Coulter cell counting, flow cytometry, and fluorescence microscopy. Indirect measurement methods include the determination of dry mass, total organic carbon, microtiter plate assays, ATP bioluminescence, total protein, and quartz crystal microbalance. It should be noted that many methods, both direct and indirect, involve homogenization of the biofilm to disperse cells in a liquid medium prior to analysis via a commercially available homogenizer and vortex mixing [16–18].

Direct Quantification Methods

Direct methods for biofilm quantification are those that rely on direct observation for quantification of the desired parameter (number of cells, total biofilm volume, etc.). Imaging and automated cell counting are the most common methods of biofilm quantification. Furthermore, the use of stains or fluorescent markers, in order to more accurately identify cells of interest and distinguish from culture debris, allow for easier and increased accuracy of cell counting and data interpretation. Imaging methods, including light and confocal microscopy provide manual platforms to count cells and determine total biofilm volume. Instruments incorporating flow, such as automated cell counters and flow cytometers, provide mechanized methods. These different direct methods will be described in subsequent sections.

Determination of viable cell numbers by plate count (colony forming units/ml or CFUs)—Viable cell enumeration, aka CFU/ml assay or aerobic plate count, is a standard quantification method that is used to determine the number of viable cells [19–21]. The basic concept of this assay is to separate the individual cells on an agar plate and grow colonies from cells, therefore differentiating living from dead cells and quantifying the live cells

without the assistance of dyes or instrumentation. The procedure starts with a liquid planktonic culture or a mature biofilm which is suspended and homogenized in liquid medium via scraping, vortexing or sonicating. The plating method involves the aseptic removal of aliquots of the suspended biofilm, followed by serial dilution and plating onto nutrient containing agar. After incubation is complete (usually 24–72 hours), colonies are counted on the plates, and the number of cells per milliliter (cfu/mL) in the original culture are calculated using the mean colony counts, the volume of culture plated, and the dilution factor from the suspended biofilm to the plate. If the biofilm quantity is small, as might be collected from a 96-well culture plate, the number of cells may be insufficient to determine a significant difference in colony number using this method. In order to increase the number of cells for colony counting, the biofilm can be suspended from each sample into a specified volume of sterile liquid medium and grown at a suitable temperature with shaking (e.g., 37°C at 180 rpm). It is important to note the incubation time and keep it uniform to expand each culture by the same amount. It is advisable to have an experiment control which received no treatment when a culture expansion is undertaken as the final enumeration will be relative and may benefit from normalization of the final count. When working with a mixed culture, it is good to note that bacteria replicate at different rates. Therefore, the culture expansion may not be appropriate as it will disrupt the ratio of cells from the original biofilm. Furthermore, the consideration to the colony forming incubation time may need to be extended to accommodate for slow colony forming bacteria [20]. Enumeration is a particularly useful quantification method in pure cultures as optical density (OD) can be measured prior to plating to obtain a calibration curve used to correlate cell number and absorbance. Thereby in future experiments, absorbance of a sample of unknown cell number can then be measured to determine the cell concentration [22,23].

The CFU technique typically does not require highly specialized or advanced equipment and can be performed in most laboratory situations by trained individuals. Obtaining consistent results requires some practice with plating and media preparation. One important consideration for choosing this method is that only live cells, capable of forming a colony, will be counted. However, this technique may not be preferable in all situations because it is time and labor intensive, sometimes requiring days to perform enough replicates to obtain reproducible results [24]. Furthermore, since the biofilm requires suspension, errors can occur due to bacterial clumping and if antimicrobial treatment was used, carryover can occur. This technique is also vulnerable to counting error and user bias, especially when the given number of colonies is high and/or the count is done manually, but this error can be mitigated through the use of manual colony counters (such as ImageJ).

Flow-based cell counting—A more automated way to count cells is a pair of methods in which cells in liquid culture flow through narrow apertures and are measured as they pass. Coulter counting and flow cytometry both require the biofilm to be homogenized and suspended in liquid cultures. While Coulter counters are less expensive, flow cytometry potentially yields more information about cells during measurement.

The Coulter method involves passing charged particles in an electrolyte solution through an aperture that is part of an electrical circuit [25,26]. The presence of the particle alters the impedance of the circuit, and is registered as a change in voltage. The change in voltage is

correlated to particle size, enabling the technique to distinguish individual bacterial cells. The voltage pulses are then counted over a period of time and correlated with cell number. This method requires a Coulter Counter instrument, which tend to cost thousands of dollars. This technique is very simple but unfortunately cannot differentiate live and dead cells.

Another flow counting method utilizes a flow cytometer^[16,27]. In this technique, cells flow through a narrow opening, causing them to pass through single file. A laser is used to detect the cells as they pass via scattering, absorbance or intrinsic and extrinsic fluorescence measurements. The major advantages of flow cytometry are the speed, simplicity and accuracy associated with measurements. A great deal of additional information about the cells, including the cell dimensions as well as surface properties, metabolic activity and the differentiation state of the cells, may be simultaneously gathered using this method with additional cell staining or endogenous fluorescent tags (such as GFP)^[28]. The chief disadvantage of this method is the considerable initial expense of the instrument which is not commonly found in many labs and typically costs between \$50,000–100,000. It is also important to note that not all flow cytometers record volume but are focused-on number of events, therefore not all instruments can yield a cell count per unit volume.

Light and fluorescence microscopy—Cell counting and biofilm 3D characterization can be accomplished using several microscopy methods ranging from simple light microscopy of suspended biofilms to volume and morphology measurements of attached biofilms using confocal laser scanning microscopy (CLSM). In this subsection, we will describe various methods for quantifying biofilm, from cell counting to total biofilm volume, using microscopy. Furthermore, we include a brief guide of common tools for introducing fluorescence to samples for analysis.

Compound light and fluorescence microscopes—Structures, as small as bacterial cells, can be visualized by a compound light microscope. Resolution of typical bacterial cells, which are 2–8 μm in length, requires total magnification of 200x or greater. Contrast enhancement methods such as phase contrast or differential interference contrast (DIC) can improve total quality of the images and make cells more visible. The cost of compound light microscopes ranges from the hundreds to the tens of thousands of dollars. Fluorescence microscopy extends the optical capabilities of light microscopy to intrinsic or added fluorescent light emission, which greatly expands the information that can be collected from this method^[29]. Fluorescent microscopes are equipped with a high-intensity lamp to excite fluorescent molecules, and fluorescent filters which allow specific bands of excitation and emission light to reach the sample and the observer, respectively. The cost of conventional fluorescent microscopes is in the tens to hundreds of thousands of dollars depending on the sophistication of the model and additional features, such as attached camera and fluorescence filters, above the base available model. The cost of fluorescent stains and consumables are in the tens to hundreds of dollars range.

Cell counting using microscopy may be done in very immature biofilms in place or on homogenized/suspended biofilms with a chamber counting slide. This may be done with unstained cells or stained cells, and with light microscopy or fluorescence microscopy. Images of *Pseudomonas aeruginosa* biofilms stained with crystal violet at different

incubation times are shown in Figure 1. With immature biofilms (Figure 1A), individual cells can be distinguished and counted. This can be time consuming, require many images for reproducibility and be subject to user bias as mentioned with colony-counting. Furthermore, in mature biofilms (Figure 1D) a three dimensional structure is formed making counting via imaging even more complicated and difficult.

Once a biofilm grows past the early stage and takes on a third dimension, manual counting with a light microscope requires homogenization and suspension for counting with a Petroff-Hausser chamber counting slide. These are specialized glass microscope slides with precisely defined sample volume and an etched two-dimensional grid on the bottom which can be used to determine the cell density (cells/mL) of a suspended biofilm [30–32]. After homogenization/suspension, the cells are then visualized and manually counted in each grid section. The average count from several grids can be used to calculate the number of cells in the original suspension with the known volume of liquid over the grid. A possible complication in this technique is the potential for motile cells to cross into different grid sections during measurement. However this is easily overcome by taking an image of the grid and counting on the image. This technique can be limited by non-representative samples and its innate inability to distinguish live cells from dead. However, the use of various metabolic or selectively permeable stains can increase visibility of the cells and distinguish living from dead cells to make counting more accurate. This technique is simple, easy to implement, and inexpensive as it only requires a light microscope, a standard instrument of cell culture labs, and Petroff-Hausser slide which costs approximately \$800.

Furthermore once a mature film has formed, analysis of total biofilm volume and morphology can provide important information regarding biofilm construction and morphology without disrupting the physical structure of the film. Microscopy can be used to determine or estimate the total surface coverage and volume of a biofilm, including the extracellular polymeric matrix. For example, the total surface area coverage of the biofilm may be determined in (Figure 1D). In addition, by calibrating the microscope focal height, the depth of the biofilm layer may be found by determining the height of the top of the biofilm and the height of the surface on which the biofilm is attached [33,34]. Another example of using fluorescence microscopy to measure total biofilm is shown in Figure 2, in which the image of PA14 colonizing a *Arabidopsis thaliana* root is used to determine (Figure 2A) the thickness of the biofilm layer on the root.

Confocal scanning laser microscopy—Confocal laser scanning microscopy (CLSM) is a specialized form of microscopy that produces high-resolution, sharp images of biofilms in three dimensions [35–38]. 3-D imaging is made possible because the confocal optics can focus on a very small volume in the sample while excluding light from other locations. The area of focus is scanned across the sample to produce high-resolution 2-D “slices” at various heights that are assembled to produce a final 3D image (Figure 2A). Furthermore, confocal microscopy can utilize single or multiple excitation lasers to view multiple fluorescent markers sequentially or simultaneously [37]. The cost associated with confocal microscopes varies widely depending on the system configuration but typically starts at hundreds of thousands of dollars at start-up. These instruments also require experienced and highly trained users for accurate measurement and analysis. Furthermore, the cost associated with

purchase of fluorescent dyes as well as confocal compatible media and containers can be in the hundreds of dollars range.

Fluorescent dyes and proteins—Although intrinsic biomolecules, such as NADH and NAD(P)H or chlorophyll, which have fluorescent properties can be used in fluorescence microscopy, fluorescent dyes and proteins are very often used to introduce fluorescence into a sample to be analyzed. Fluorescent dyes are often fluorescent molecules, known as fluorophores, or biomolecules connected to fluorophores, which absorbs and emits light while incorporated in the biological structure. The emitted light is detected into for image generation to analyze biofilm features, such as spatial cellular viability, shape and function throughout the growth/treatment period. Another option for obtaining cell fluorescence is to genetically modify the organism to express a fluorescent protein. While these options increase preparation time either at the method level (producing a fluorescent cell) or the sample level (biofilm staining), the additional information is often useful for greater understanding of cellular growth and life within the biofilm [14,39]. Here we introduce some common classes of fluorescent dyes and proteins which are used for analysis of biofilms.

There are many commercially available fluorescent stains which are useful for any application including fluorescent microscopy, confocal microscopy, and flow cytometry. These include innately fluorescent molecules, fluorophores connected to biomolecules or molecules with a fluorescent derivative. The stains are available in a variety of emission colors (red, green, orange, and violet) which allow for the analysis of multiple dyes on a single sample. Localization of the stains within/upon the cell depends on the chemical structure or properties of the molecule to which the fluorophore is attached. Table 1 includes a summary of commonly used dyes with information regarding cellular localization, whether the dye indicates viability and references for experiment planning. For example, DAPI (4',6-Diamidino-2-phenylindole dilactate) is a dye highly selective for nucleic acids which will localize near the DNA whereas lipophilic dyes, such as FM 4–64, remains in the cell membrane. Many dyes provide information about viability depending on cell membrane permeability such as SYTO 9 and Propidium Iodide (PI) which both fluoresce in the presence of nucleic acids but PI is not cell membrane permeable, and will not stain living cells, while SYTO 9 freely enters living cells. A different mechanism for labeling live cells is that of calcein stains which are cell membrane permeable and nonfluorescent until converted to the fluorescent derivative via acetoxymethyl ester hydrolysis by intracellular esterases of the living cells [40]. This mechanism has advantages, as the hydrolysis-dependent fluorescence allows calcein to persist in the extracellular fluid of the biofilm without causing interference in the image/quantification process eliminating the need for extra wash steps and improving accuracy.

Another way of inducing cell fluorescence is to genetically engineer foreign DNA into the bacteria resulting in the production of fluorescent gene products. This is most often performed by introduction of a plasmid, a small section of foreign DNA, although incorporation of foreign DNA into the bacterial genome may be useful for tracking gene expression [41]. Green fluorescent protein (GFP) and variations of GFP such as enhanced green fluorescent protein (EGFP), when produced by the cell, causes the cell to fluoresce green, emission between 400 and 600 nm, when excited by UV light, between 350 and 450

nm in healthy cellular conditions [41,42]. The resulting emission can be used to count cells and track real-time biofilm accumulation [39]. Biofilms expressing GFP can be assessed for green alone or in conjunction with other fluorescent stains such as PI as shown in Figure 2A. In these images, taken two hours and six hours after flow cell inoculation, the *Pseudomonas aeruginosa* (PA) biofilm contains EGFP therefore living cells appear green while PI, from the culture media, accumulates in dead cells which appear red. Genetic modification can have many advantages compared to staining including relative stability against photobleaching and the ability to pass on the plasmid to daughter cultures thereby maintaining the modification into many cultures while stains must be reintroduced at each experiment. However, given the cost of vectors and labor associated with cloning the cells, creating the organism is mostly preferable if fluorescence analysis is used often. There are many advantages to GFP as it is a convenient fluorescent reporter for biofilm studies, and it does not appear to interfere with cell growth and function. A variety of colored fluorescent proteins are now available such as Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) which allows for separate labeling of cells in co-culture or multiple labeling in a single cell [42,43]. Biofilms with GFP can be visualized *in vitro* such as in a flow cell (Figure 2A) or *in situ* such as the PA on *Arabidopsis thaliana* roots (Figure 2B). A possible disadvantage in many but not all fluorescent dyes and proteins is the potential for interference in the cellular processes resulting in toxicity or changes in the cell which may limit the types of characterization possible. Customer Service of vendors and previous literature can provide helpful consultation in understanding whether the dye is appropriate for the desired data collection, and will allow for unhindered growth after use.

Advantages and Disadvantages of Microscopy

In general, microscopy has the advantage of producing fascinating images that can be used directly in publications or quantified using imaging software. Images often improve readability of publications and allow the reader to interpret the observations made by the microscopist. A major advantage of microscopy is the ability to quantitatively analyze biofilms without the need for harvesting and resuspension thereby allowing the natural structures to be maintained [35,36,44]. The use of dyes and fluorescence allows for increased information to be obtained about spatial and temporal cellular viability and function without destruction of the biofilm although introduction of fluorescence also increases preparation time either at the method level (producing a fluorescent cell) or the sample level (biofilm staining) [14,39]. Unfortunately, image selection is subject to bias, although measures can be taken to alleviate this fact. Random selection of images or consistent selections of image location between multiple samples are two commonly used techniques. Furthermore, in order to obtain statistical significance from analysis, a large library of images will be needed which can be time consuming. In the case of fluorescent images, care must be taken in experiment planning to assure that the cells are being imaged consistently. One must avoid fluorophore quenching or photo bleaching, which result from chemical and light exposure causing decreased or eliminated fluorescence from the fluorophore, which may lead to unrepresentative results. If data collection includes quantification of fluorescent intensity, care must also be taken to ensure that all settings are uniform across the library of images. Image collection and analysis with image analysis software, such as open source ImageJ, is a commonly used qualitative and quantitative characterization method [45,46].

Indirect Quantification Methods

Biofilm growth can often be determined indirectly using a proxy marker which infers the biofilm quantity. Examples of these markers include dry mass, total protein content, DNA, RNA, polysaccharides, or metabolites. Indirect quantification methods all involve the basic assumption that the substance or property to be quantified correlates to the number of cells, or that the amount of protein/DNA/mass is consistent from cell to cell. This assumption has been validated for biofilms making these methods extremely useful [47]. It is best practice to verify indirect methods with direct methods since they are only proxy quantification based on metabolic function and biomolecule production which can be dependent on organism, culture conditions and age.

Dry mass—Dry mass, usually expressed as mass per unit area, or biofilm density is a widely used marker that can lead to quick growth quantification. To find the dry mass, the biofilm with growth substrate is placed in an oven at a constant temperature until the water is removed and a constant weight is achieved [48,49]. Alternatively, if the substrate is heat sensitive, the biofilm can be scraped from the surface, suspended in physiological saline, precipitated with cold ethanol, and the precipitate collected for analysis [17]. The drying temperature is dependent on researcher preference and substrate heat tolerance. For example, while some researchers have employed 60°C others have used 100–105°C, both temperatures with their respective drying time achieve full drying of the sample but were chosen based on researcher preference based on specific aspects of the experiment at hand [47,48]. The main objective is to utilize a constant temperature and corresponding time to achieve a completely dry sample with minimal disturbance of the biofilm or substrate. After drying, the sample is weighed, the biomass is scraped from the substrate and the substrate is weighed. Dry biomass is the difference in weight between biomass on the substrate and the substrate with no biomass. The dry biomass is normalized to the growth area of the wet biofilm for the calculation of biomass per unit area of film or to the wet biofilm volume for biofilm density [48,49].

The disadvantage of dry mass measurements is that they do not differentiate cell mass from different film components such as the extracellular matrix. The use of this method is also dependent on the growth substrate as it must be heat resistant at the drying temperature or easily separated from the biofilm so it is not included in the biomass calculation. Another disadvantage of this method is that the sample cannot be used for any other characterization methods after drying. The main advantages of this method are the relative ease and cost effectiveness since it requires relatively “low-tech” lab equipment, such as a drying oven and a balance, which are standard laboratory equipment.

Total organic carbon—Total organic carbon (TOC) is an indirect measurement of the amount of carbon in a sample associated with organic compounds or carbon compounds derived from living things (proteins, lipids, urea, etc.). This is opposed to elemental carbon (EC), such as graphite or coal, and inorganic carbon (IC), consisting of simple compounds including simple carbon oxides (CO and CO₂), carbonates, carbides, and cyanides. The three carbon sources can be distinguished due to differences in conditions required for degradation into CO₂ of the various carbon compounds [50,51]. TOC measurements are often used to

determine environmental water quality and for testing of instrument cleanliness in the pharmaceutical industry, as well as quantification of biofilm accumulation [52–54].

The TOC quantification of biofilms is usually performed as a two-step process in which total carbon (TC) and IC are measured and used to determine TOC [55,56]. The biofilm is broken down, and the IC is converted into CO₂, typically via heated acidification and detected by infrared spectroscopy. Next, all carbon in the sample is converted into CO₂, usually via heated oxidation, and the TC is measured. The TOC is then inferred by the difference between these two values (TOC = TC – IC) [55]. The exact method of sample preparation and quantification is determined using instruments such as the Oceanic International Carbon Analyzer, Analytik Jena Multi N/C 2100S, or a UIC incorporated Model CM5012 CO₂ coulometer. However, the final calculation for TOC is universal and not instrument dependent [54–56]. Therefore, the major expense and drawback of this method is the cost of a specialized TOC dedicated instrument, which costs around \$20K. Another drawback is the lack of specificity in quantification as TOC measures the carbon content of the entire biofilm including the bacteria and the extracellular polymeric substances (EPS). Estimation protocols exist for differentiating carbon from cells and carbon from EPS thereby alleviating this drawback [55].

Although TOC provides a marker to quantify the amount of biofilm present this value must be correlated using a direct analysis method (CFUs, cell count, etc.) to generate a method-independent value. Also, it has been shown that the amount of carbon in a given volume of cells is dependent on the health status of the bacteria [53]. Thus, additional correlations must be made under each set of conditions, and a new standardization protocol, or control, has to be performed each time the experiment is performed.

Crystal violet assay—Gram staining is one of the most used and well optimized methods in microbiology for identification and visualization of bacteria [57]. The primary component and commonly used dye for gram staining is crystal violet, a basic trianiline dye which is cell membrane permeable in gram positive and negative cells [58]. Traditionally, in the gram staining process, a mordant, typically an iodine-iodide mixture, is added which complexes the crystal violet inside the cell cytoplasm. This complex is membrane impermeable in gram positive cells, due to the greater cell's membrane thickness holding the complex in, but breaks through the thin membrane of gram negative cells. This leaves gram positive cells purple in color after a de-colorization with an ethanol solution allowing for differentiation between gram positive and negative bacteria via microscopy [58]. However, if differentiation between gram types is not the goal, the mordant can be omitted, both gram positive and negative cells will take up the crystal violet and the dye will freely pass from the cell during the de-decolorization step allowing for the quantification of crystal violet via spectroscopy. This quantification has proven extremely useful as a cell estimate for biofilm growth [23,59–61].

The schematic in Figure 3 explains a basic biofilm accumulation assay performed in a multi-welled plate. The growth media and planktonic cells are removed from the plate and washed with deionized (DI) water leaving only attached biofilm (Figure 3A). A 1% solution of crystal violet in DI water is added and the biofilm incubated with the dye at room

temperature for a period of time, typically 5 to 30 minutes. After incubation, the dye solution is removed, and the biofilm washed several times with DI water to remove free dye (Figure 3B). The decoloring solution can then be added, to a volume greater than or equal to the original culture media volume, and incubated with the biofilm for 10–30 minutes. The decoloring solution typically consists of a 90–95% ethanol solution but other decoloring solutions such as pure ethanol or ethanol with acetone or acetic acid can also be used as the objective is to solubilize the CV [23,61,62]. Finally, the CV infused decoloring solution is transferred to a clean 96 well plate with appropriate blanks of decoloring solution to be assessed for absorbance at 530–600 nm, depending on the instrument's filter availability, with a multi-well plate UV-Vis spectrometer [23,60,61].

While the crystal violet Microtiter plate assay consists of several steps, it is relatively easy to perform, reproducible, and allows researchers to rapidly analyze multiple samples simultaneously. It is relatively inexpensive as it does not require the purchase of specialized equipment, and the dye is inexpensive with a shelf life of years if protected from contamination. Furthermore, the crystal violet assay can be modified for biofilms grown in a variety of reactors. Figure 4 shows a schematic for measurement of optical density (OD) over-time data for the growth of a *Pseudomonas aeruginosa* (PA) biofilm in a Center for Disease Control (CDC) reactor at the start of the exponential growth (“log”) phase. The main disadvantage of this assay is the nonspecific nature in that it does not distinguish between live and dead cells. Another disadvantage of this assay is the many variables (incubation times, incubation temperatures, decoloring stain, etc.) which can introduce batch variability into the assay results [62]. However, the adoption of a standardized protocol available in literature and the employment of a control for normalization can eliminate method variability.

Tetrazolium salt—Tetrazolium salts are one of the most widely used tools in biology for monitoring metabolism *in vitro* [63]. A variety of salts, summarized in Table 2, successfully utilized for biofilm evaluation have been developed which allow for quantification and visualization of cellular viability and metabolism via UV-Vis and fluorescence spectroscopy. While the exact mechanisms of reduction are still under scrutiny and vary between organism and salt type, the overall concept can be generalized in the following manner.

The tetrazolium salt of choice is diluted into a physiologically relevant solution, such as media or saline, and the biofilm is allowed to incubate for 1–3 hours at culture temperature or room temperature. During this time the colorless salt is reduced by cellular cofactors and enzymes from cellular metabolism, indicative of and proportional to cellular viability, into the corresponding formazan molecule which is detectable by visual or fluorescent spectrometers or microscopes [63,64]. The reduction can result in water soluble or water insoluble formazan dictating end work-up and analytical steps. Water soluble formazans solubilize in the treatment buffer and therefore can be immediately detected via spectrometric analysis [21,31,65–67]. These are often used for real-time evaluation of cellular viability and metabolism. Water-insoluble formazan crystallizes and becomes trapped within the cell membrane during the reduction process. Therefore, the crystals can be evaluated via flow cytometry and microscopy, on a per cell basis, within the cell or dissolved in a solvent, such as DMSO or alcohol with 0.1 N HCl, for overall quantification [68–70]. Figure 5

demonstrates visualization of a PA01 biofilm grown in a Drip Flow Reactor stained with insoluble formazan CTC using fluorescent microscopy. The formazan crystal has fluorescent properties allowing for visibility with fluorescent microscope as shown in Figures 5B and 5C.

ATP bioluminescence—ATP bioluminescence testing is a well-established microbial test in food and biomedical communities for the presence of microbial contamination on surfaces [71,72]. Adenosine triphosphate (ATP) is a nucleoside triphosphate which acts as the primary energy source in all organisms and thereby a prime marker for viability. Bioluminescence refers to the process by which organisms convert chemical energy to light. The most common ATP bioluminescence assay utilizes the enzyme luciferase, responsible for light production in fireflies. At low ATP concentrations luciferase is a reaction which produces light with a linear correlation to the amount of ATP present in solution [73]. Therefore, the amount of light can be used to infer biofilm viability and biomass [71,73]. The basic reaction proceeds in two steps; the first being the complexing of Luciferase, Luciferin, and ATP to create luciferyl adenylate complex. The second step is the oxidation of luciferyl adenylate with oxygen into oxyluciferin which results in the emission of a photon detected at approximately 550 to 570 nm [71,74].

The protocol for quantification of bioluminescence is relatively simple and can be performed on suspended or attached cells. First, culture media is removed and the biofilm is washed with water or buffer to remove extracellular ATP. Second, the biofilm is suspended and cells are lysed to release intracellular ATP making it available to the luciferin-luciferase. In the third step, the intracellular ATP released is added, or vice versa, to the reaction reagent consisting of luciferin, luciferase, magnesium ions, buffer for pH maintenance, etc. in a luminometer-appropriate cuvette or multi-well plate. The half-life of the luminescent complex is approximately 30 minutes. Therefore, the light emitted should be detected as quickly as possible following the addition of biological sample/reagent [23]. The best practice is to quantify the emitted light every 10–30 seconds over a limited time frame so that the readings can be averaged for a total ATP estimation. This average is compared to an ATP standard quantified by the same protocol in the same conditions in which the quantity of ATP added in step 3 is known. The procedure outlined is the step-by-step sequence of the assay. However, commercially available kits can be purchased which include an optimized ratio of lysis detergent, luciferase, luciferin, buffer, and ions required sold as lyophilized powder only requiring the addition of deionized water reducing the protocol to the addition of one reagent to the biological sample, incubation and quantification of emitted light via a luminometer [75,76].

This assay is very reliable, can be performed quickly, and only requires a luminometer for analysis. Basic luminometers, which read a single cuvette at a time, cost around \$1000 while higher tech instruments that are capable of reading 96 well plates and/or have automatic reagent addition, can cost \$10K or more. The assay is highly accurate at low ATP levels. However, many variables such as poor ATP extraction, fluctuations in temperature and pH, insufficient ratio of luciferin to luciferase can lead to data variance. Therefore, it is typically recommended that a kit and not a homemade reagent is used for the assay [74,77]. Commercial assays cost a few hundreds of dollars and include reagent and standards, which

can be used to perform 200–1000 assays. In addition to the relatively high cost of the assays and instrumentation, this method has the distinct limitation that the instrument must be regularly calibrated to confirm accuracy [23].

Alternatively, some researchers have striven to make a non-destructive bioluminescence assay for biofilm observation over time, high throughput screening or targeting of specific bacteria in a diverse biofilm [78,79]. This method requires the production of recombinant bacteria, through the introduction of a plasmid similar to that discussed previously for GFP modification, for the endogenous production of luciferase and luminescence quantified via a luminometer [78–81]. If ATP bioluminescence assays are frequently used for high throughput experiments, the cost and effort of creating a recombinant bacteria may be warranted. Otherwise, the previously outlined commercially available assay is sufficient for general use.

Total protein determination—One widely accepted surrogate for total biofilm growth is total protein content. Assuming that protein content is approximately similar between cells, protein content has been found to correlate with the number of cells in biofilms in biofilms of wetland microcosms [54]. However, variability of protein production across species, age and culture conditions may result in deviation from direct correlation with cell number making this a method to be used in conjunction with strict experiment controls and verified with more direct quantification methods [82,83]. To assess this, the biofilms are removed from their substrate and homogenized in a liquid suspension. The cells are lysed in a manner consistent with the protein determination method to be used. For instance, some protocols require incubation in the presence of a strong base at 55°C or a solution with detergent and protein precipitation with trichloroacetic acid (TCA). This lysis buffer should be made protease free as the presence of proteases, enzymes that break down proteins, would decrease the sample quality. After lysis, the protein content can then be measured by color change resulting from the dye-protein interaction via a UV-Vis spectrometer. The change in absorbance of the colored species at a particular wavelength, dependent on dye-protein interaction product, is proportional to the concentration of protein by the Beers-Lambert law.

There are many established methods for total protein content determination. Among the most commonly used are the Bradford, Lowry, and bicinchoninic acid (BCA) methods. The Bradford method is simple, consisting of the addition of a known volume of protein sample to an acidic Bradford reagent containing Coomassie Brilliant Blue G-250 dye [84]. The lysed sample or standard protein is added to the Bradford reagent and incubated for a short time period, i.e., 10–30 minutes, at room temperature or 37°C (to decrease the required reaction time). During this incubation, the protein binds to the dye resulting in a spectral shift from brown (absorbance at approximately 465 nm) to blue (optimal absorbance at approximately 595 nm) [85,86]. The protein binding is dependent on the presence of positively charged amino acids in the protein structure interacting with the net negatively charged dye via Van der Waals, ionic and hydrophobic interactions [87]. Therefore, the change in absorbance at 595 nm is measured and converted to concentration of total protein via a BSA standard curve. A second common method is the Lowry assay. The original Lowry protein assay, or its more modern modification, is based on oxidation-reduction chemistry in two steps [88–91]. First, the protein sample reacts with cupric sulfate and tartrate with a ten minute incubation time at room temperature to form a tetradentate copper complex from four

peptide bonds and one copper atom. In the second step, a Folin phenol reagent is added, and the light blue color of the tetradentate copper complex is intensified by the transfer of electrons to a phosphomolybdic/phosphotungstic acid complex in the Folin phenol reagent during incubation at room temperature of 30 minutes or greater, and the final color absorbs optimally at approximately 750 nm. The exact mechanism has been investigated, but has not been completely clarified to date. The protein suspension buffer is a critical consideration because the Lowry assay is sensitive to detergents, potassium ions, most surfactants, chelating agents (i.e., Ethylenediaminetetraacetic acid, EDTA), some sugars routinely present in culture media and reducing agents interfere with the assay and can result in erroneous color changes [84]. The third commonly used protein quantification method is the BCA assay. The chemical mechanism of the BCA protein assay is very similar to the Lowry assay utilizing the reduction of copper ions by proteins resulting in a spectral shift. However, the Folin reagent of the Lowry method is replaced with bicinchoninic acid (BCA) which can be performed in one step rather than two [54,92]. The protein sample and BCA kit reagents, a carbonate buffer containing BCA reagent and a cupric sulfate solution, are incubated for 30 minutes at room temperature and the absorbance is optimally analyzed at 562 nm. The main advantage of the BCA protein assay is the compatibility with most surfactants making it appropriate for use with most common cell lysis reagents although it is still vulnerable to chelating agents. The BCA assay is widely used, and the method is simple because it is commonly noted in literature as being performed by “manufacturer’s instructions” which commonly includes the combining of Reagents A and B and the addition of the sample diluted with lysis buffer to bring the sample concentrations within the standard curve [93,94]. In addition to these traditional methods, a variety of other colorimetric and fluorescent protein assays have been described, including specialty assays for histidine tags, antibodies, etc. and many are commercially available as assay kits [95–97].

Protein quantification is a quick, commonly available assay which allows for a relative assessment of biofilm growth. Assay kits usually cost \$100–300 depending on kit size. These typically include assay reagents for 100–1000 samples, in the range of 1–2000 ul protein per ml sample depending on the sensitivity of the assay chosen, and vials of BSA standard. When assaying very small amounts of protein it is advisable to run a standard, typically BSA, with every plate or assay set due to non-systematic variation in pipetting (i.e., poor/inexperienced technique), variations in room temperature or incubation time, etc. Although several of the kits are sensitive to numerous interfering agents, it is possible to plan lysis buffers and choose kits to avoid any interference. In the case that interference is unavoidable, minor cases can be accounted for by running a background blank of just the suspension buffer. Otherwise, samples can be treated via gel filtration, dialysis or protein precipitation to remove interfering substances [54,90,95]. The major grievance with this assay is the inability for differentiating between cellular and extracellular protein which may cause error in cross species biofilm comparisons and treatments which decreases EPS protein without harming the cells. This can be avoided via methods of cellular extraction where the extracellular protein is removed from the cells, or utilizing protein quantification in parallel with a cell viability assay may be advisable.

Quartz crystal microbalance—Quartz crystal microbalances (QCMs) allow for the nondestructive measurement of biofilm accumulation as a function of time [98]. The instrument consists of a small disc of Astatine (AT)-cut single crystal quartz (Figure 6D), which is a piezoelectric material that is driven at the resonant frequency of the disc by an applied oscillating potential difference. The disc may be coated, e.g., by Gold (Au) or Silicon Oxide (SiO₂), and serves as the growth substrate. This disc resides in the flow channel of the bioreactor so that the biofilm is formed on the disk surface. The resonant frequency is a function of the system's mass, so microgram changes in mass are proportional to the shift in resonant frequency, thereby allowing measurement of biofilm accumulation as it is forming [99]. Tam et al. illustrates effective use of QCM technology to show the effect of environmental conditions and genetic manipulations on the growth rate of *Streptococcus mutans* biofilms [100]. In this study, a direct correlation between wet mass of the film and QCM frequency shift is shown, giving a quantitative measure of mass from the QCM device.

Additional information about the viscoelastic properties can be obtained when the applied potential is turned off so exponential decay of the oscillation can be monitored. This type of measurement is called quartz crystal microbalance with dissipation monitoring (QCM-D). The dissipation factor measured in this technique is sensitive to the surface mass density of the film and the mechanical coupling of the film to the crystal surface and to the surrounding medium [101]. QCM-D allows for dynamic measurements in a liquid environment and is a non-destructive technique. Thus, the dependence of biofilm quality and formation kinetics on environmental conditions, such as pH or additive concentrations, can be considered [102–106]. QCM-D requires models to interpret the data and gives estimates for film thickness, shear stress, and viscosity of biofilms [101,107–109]. These parameters are of particular interest for efforts to mechanically remove biofilms. QCM-D remains an underutilized technique for biofilm characterization primarily used by physicists and engineers but microbiologists have not extensively explored this option as an interdisciplinary method.

The major advantage of this technique is the monitoring of mass accumulation to ng/cm² accuracy in real-time without sacrificing the sample which has assisted in a greater understanding of biofilm attachment and allows for the investigation with multiple analytic techniques, such as assays for quantifying viability and gene/protein expression, on a single sample. A major disadvantage of this method is the cost of specialized equipment, electronics, software and consumables which can range from a simple, single channel device, such as that available through openQCM®, for \$600 to fully automated, high-throughput devices from Q-Sense for thousands of dollars. Figure 6 shows an example of the openQCM® device that has the QCM crystal mounted in a small flow chamber. The electronics are based on the micro Arduino microcontroller board. All hardware and software are open source, so they can be adapted easily to a user's needs. Another disadvantage of this system is that resonant frequency is highly sensitive to changes in temperature and pressure making the maintenance or accounting for fluctuations of those variables during data collection important.

Alternative Quantitative Characterization Methods

Literature accounts show that biofilms can also be analyzed using DNA, RNA, and polysaccharide quantification [91,110–117]. Although these are often used as direct techniques with the assumption that each cell will have similar DNA, RNA and polysaccharide quantities per cell, it is advisable to provide these quantifications in tandem with more direct methods, such as CFU or cell counting as the EPS matrix contains DNA, RNA and polysaccharide components from previously lysed cells giving these techniques the similar grievance as total protein with the inability to discern cellular from EPS components [12]. Furthermore, particular RNA and polysaccharide expression quantities have been noted to change by organism or growth environments [118,119]. Therefore, literature precedence for quantification of these compounds in a particular organism is highly suggested.

Fluorescence Spectroscopy is an explored but not commonly utilized method of indirect quantification. This method assumes similar fluorescence emission intensity is exuded by each cell upon excitation, therefore utilizing intensity as a surrogate quantification value for cell number and can be performed on suspended biofilm [120]. This method can take advantage of autofluorescence of metabolic molecules (NAD/NADH, FAD, tryptophan, etc.), and cells can be transfected to produce fluorescent proteins as previously discussed with GFP, or cells can be dyed with a fluorescent stain [69,120–122]. The presence of metabolic molecules and fluorescent intensity of proteins are influenced by extra- and intra-cellular conditions. Therefore experiments should be run with a negative control, a sample with the same culture conditions without treatment agent [122]. Although not a quantification method, 2D fluorescence spectroscopy can be used as a non-destructive, time-resolvable method to elicit information on the physiological state of the biofilm [122]. This method requires some specialized equipment but when combined with other technology can provide information on physical interactions between the biofilm and surrounding medium. Wolf et al. used this technique in combination with Artificial Neural Networks to study biofilm formation *in situ* and analyze biofilm growth with respect to process parameters [123].

Radioactive-labeling has also been used for the quantification of biofilms by attaching an isotope to a biological molecule, such as thymidine or glucose, and observation via microscopy, scintillation/gamma-ray counter [124–126]. Specialized equipment and training is required due to the potentially harmful effects of excessive radiation exposure.

QUALITATIVE CHARACTERIZATION METHODS

Quantitative methods of biofilm characterization are often accompanied and assisted by representation with qualitative methods such as imaging the physiological biofilm surface, structure evaluation of surface roughness, morphology, spatial organization, and interaction of the biofilm with the environment. Previously we have discussed light and fluorescent microscopic methods which are increasingly being used for quantitative and surface structure analysis due to the ease of use and the ability to visualize living biofilms. In this section, we describe Scanning Electron Microscopy (SEM) because it is the most commonly used method for structural analysis through high resolution imaging.

Scanning Electron Microscopy

SEM can be used to develop a high resolution, magnified image of surface topography. Overall magnification can range from about 10–500,000 times, making this technique invaluable in the analysis of microscopic structures, including those of biofilms [19,127]. SEM allows for collection of high resolution images useful in evaluation of bacterial interaction, EPS organization and biofilm morphology, which assists in a greater understanding of formation and persistence [128–130].

SEM operates in a manner similar to conventional fluorescent microscopes. However, instead of using a beam of photons to observe a sample, SEM utilizes a concentrated beam of electrons. After passing through a number of electromagnetic lenses, the electron beam strikes the sample and two major scenarios occur: the electron is (1) absorbed by the surface molecules which excites the surface molecules and causes a low energy, secondary electron to be ejected or (2) scattered off the surface, i.e., a high energy, backscattered electron. The former is picked up by the secondary electron sensor and converted into a digital image, similar in concept to photons detected in fluorescent microscopy. Due to the low energy of secondary electrons, these images tend to only display the surface of the sample [19,127,131].

SEM imaging techniques fall into one of two categories depending on the origins of the detected electrons: secondary electron or backscattered electron. While secondary electron analysis is the primary SEM imaging technique, most SEM instruments are capable of reading back scattered electrons. Back scattered electrons are caused by high energy electrons from the incident electron beam being scattered on the surface. These back scattered electrons can be used to generate a low resolution image which indicates locations of chemical variance. The frequency of such a scattering event scales with the atomic weight of the probed atoms thereby measuring differences in chemical composition.

An advantage of electron microscopy is the easy availability of tandem spectroscopic techniques for quantitative elemental analysis. Energy-dispersive x-ray spectroscopy (EDX) generates a spectrum that is indicative of the ratios of elemental surface composition of a sample by detecting the X-rays emitted from atoms when the incident electron causes a surface atom to lose a core-shell electron, the secondary electron, leaving the atom in an excited state. The surface atom subsequently returns to its ground state by releasing energy in the form of an X-ray, which is then detected.

A major disadvantage of SEM analysis is that it cannot be performed on living samples, as testing is done under high vacuum, and extensive preparation is required prior to the analysis of biological samples. Sample preparation includes fixation, removal of all moisture, and coating the sample with a thin layer of a conductive metal. Sample fixation is typically achieved via an aldehyde solution, which covalently bonds proteins to preserve the secondary and tertiary protein structure [132]. Samples are then dehydrated via a series of graded alcohol treatments. As moisture in the sample can interfere with the ability of the SEM to achieve sufficient vacuum conditions total dehydration of the sample is vastly important for optimal resolution of imaging. Since biofilms consist of approximately 97% water, the total dehydration for imaging can result in unavoidable distortions of size and structure in the sample [133,134]. Finally, the sample must also be conductive to allow for

dissipation of static charges which can result in ‘artifacts’ or structures which obstruct the image. This is usually achieved by sputter coating with a metal, such as gold or platinum, requiring the purchase of additional equipment and chemicals. Alternatively, biological samples can be impregnated with osmium, via the osmium-thiocarbohydrazide-osmium (OTO) staining method which incorporates the heavy metal salt into the lipid membrane effectively eliminating specimen charging [135–137]. An emerging technology with great potential to significantly decrease biological sample preparation time is the use of Ionic Liquids. These molten salts which remain liquid at room temperature are resistant to vaporization, even under high vacuum, and provide conductive coating without the need for sample fixation and drying. Furthermore, they have been successfully used on a variety of biological samples including biofilms [133,138].

To overcome difficulties of sample preparation, the method of Environmental Scanning Electron Microscopy (ESEM) allows for untreated samples to be imaged without the need for complete dehydration or a vacuum [129,139,140]. ESEM uses all image generation techniques of SEM (i.e., background scattering, secondary electron, transmission, etc.). However, the technique has limitations due to the distance the electron beam travels through gas molecules which compromises resolution. Furthermore, although the samples can remain wet it is still not advisable to use viable samples because the electron beam can harm the sample. Finally, the most notable disadvantage of ESEM is that the instrument is still in development, and a commercial version is not available for purchase yet. Therefore, use of this technique is done by modifying currently available SEMs requiring researchers to frequently shift between modes or the designation of an instrument for ESEM.

Despite some shortcomings of SEM use, this technique is highly advantageous as the high resolution of the surface images can reveal details about biofilm structure and topography that are unmatched by many other microscopy techniques [19,127].

Alternative Qualitative Characterization Methods

The topological structure and chemical properties of biofilm surfaces can be assessed using scanning electrochemical microscopy (SECM) [141,142]. This technique employs a microelectrode, on a micrometer scale, in the presence of an appropriate redox reagent to scan a surface and induce a redox reaction when potential is applied between the tip and the surface [143]. This versatile technique can provide an extra dimension to 3D models of biofilms based on the distribution of reactive groups used to determine how extracellular polymeric substance (EPS) components are distributed at the biofilm surface. SECM requires specialized equipment which makes instrument availability and access a limiting factor of use.

Although not commonly utilized currently, literature precedence exists to analyze biofilms with atomic force microscopy (AFM). AFM can characterize the components on the underlying substratum as well as the substratum interactions [144]. AFM would be useful in understanding biofilm characteristics such as roughness, topography, and stiffness but, similar to other techniques, requires specialized equipment costing more than \$100K and trained operators.

Spectroscopic analyses of biofilm are becoming increasingly recognized for usefulness as a non-destructive method for greater understanding of biofilm aggregation, adhesion and EPS composition. Infrared (IR) and Raman spectroscopic characterization utilizes the absorption (IR) and inelastic scattering (Raman) of light to identify chemical signatures via probe free, *in situ* analysis. Infrared spectroscopy provides the vibrational information through the use of IR light, whereas Raman typically uses more energetic light, usually supplied by a near IR, visible, or ultraviolet laser, to provide similar information. As a biofilm is a 3D structure, the IR and Raman spectroscopy is limited to surface spatial-chemical changes in the biofilm thereby providing greater understanding of biofilm structure and intercellular communication. IR signals are typically much stronger but produce a significantly worse signal-to-noise ratio due to the overwhelming water signal. Raman signals while weaker are not clouded by water and can be detected with cheaper detectors. Surface Enhanced Raman Spectroscopy (SERS) utilizes surface properties of metal surfaces to enhance the weak Raman signals by factors in range of $10^6 - 10^8$. While SERS enhancement opens up new possibilities the heat generation from the power of the laser and the antimicrobial properties of the metal surfaces present experimental problems for biofilm studies. Despite some difficulties, IR and Raman are good methods to use in conjunction with one another, with confocal scanning light microscopy (CSLM), or with specialized IR compatible surfaces [145–148].

Small angle x-ray scattering (SAXS) can be used to study EPS components, structure and potentially molecular interaction. Although traditionally used to analyze proteins in crystals or suspension, the use of x-ray scattering probes shows promise for use of SAXS for studying interactions within a specimen [149–151]. Although underestimated due to “low resolution” SAXS has great potential to provide valuable insight into biological structures and molecular composition of biofilms [152].

Surface Plasmon Resonance imaging (SPRi) and Electrochemical Surface Plasmon Resonance (EC-SPR) are emerging techniques used to study bacterial physiology and electrochemical activity in real-time without labels [153,154]. Similar to the spectroscopic methods, this analytical method suffers from the need for specialized equipment and substrate coated with a conductive material typically gold.

Biofilm formation and virulence can be detected by colorimetric means using the Congo red agar method, in which microorganisms are cultured on dye infused agar. The outcome of this method is colony color change which can be used to determine whether microorganisms are biofilm producing (black) or not (red). This method is commonly used with polysaccharide rich, slime producing gram positive or gram negative bacteria [82,155].

INFORMATION ENHANCEMENT TOOLS AND PREDICTIVE MODELS

Mathematical models and computer programs (COMSTAT, ImageJ, etc.) can be used to analyze images and enhance new perspective on existing data that can be utilized in the development of descriptive/predictive models and biofilm quantification.

ImageJ

ImageJ has been applied to biofilm analysis in laboratory situations such as the automatic count of colonies from images [156]. This is in part due to the open framework of the program, which allows plugins and macros to be written and shared for specific applications. The collaborative nature of this system is what makes it so useful in research in general, and in biofilm analysis specifically [157,158].

ImageJ, originally called NIH Image, is a free, open source, Java-based imaging program that can be used on Windows, Mac, or Linux operating systems. It is capable of reading many image formats (JPEG, TIFF, GIF, DICOM, FITS, and BPM) and manipulating, analyzing, or processing the images in a number of different ways. For example, colony particles can be photographed or scanned with a desktop scanner and the bacterial clusters can be automatically counted. Figure 7 demonstrates a typical example obtained in our labs that show how ImageJ can achieve a colony particle count.

Perhaps the most useful feature of ImageJ in biofilm research is the ability to generate image stacks. Stacking not only allows for images with different fluorescent stains to be overlaid, but can create three dimensional images (z stacks) using data from techniques such as confocal microscopy. These three dimensional images are capable of not only revealing details of biofilm structures, but can be combined with fluorescent staining techniques to show distributions of different bacteria, proteins, or ion concentrations within the biofilms as a whole [159,160].

Mathematical Models to Quantify Biofilm Accumulation

Mathematical models of biofilm systems allow us to merge information from the many measurement techniques discussed previously into a coherent and unified picture. Information about form and function of the microorganisms in the film can be related quantitatively to biochemical factors such as growth kinetic parameters and to physical factors such as transport mechanisms, shear forces, and viscoelastic properties of the film [161].

Biofilms are very complex due to the various forms of attachment, detachment, growth, and transport of nutrients from the surface to the deepest layers, as well as the inter-bacterial forces that are in place when biofilms accumulate. Thus, biofilm structures should be treated using mathematical models of biofilm accumulation and activity, so that relationships among biofilm structure, rate of biofilm accumulation, and microbial activity in biofilms can be quantified within the same framework [162]. Much work has been done using computer programs (COMSTAT) to calculate bio-volume, surface area coverage, biofilm thickness distribution, mean biofilm thickness, microcolony volume, fractal dimension, roughness coefficient, average and maximum distance, and surface-to-volume ratio from three-dimensional stacks of biofilm images [39,116,163,164].

Mechanistic mathematical models associated with computational systems biology enhance the basic understanding of biological systems with complex physical parameters, such as biofilms [55,165]. Two modeling techniques are generally used: a dynamical systems approach where the dynamics of cell and metabolite concentrations are modeled with

differential equations and an individual-based simulation approach, which is particularly effective in combining multiscale dynamics, from molecular events to three-dimensional cellular organization [166,167].

Dynamical systems models of biofilm reactors now play a major role in designing wastewater treatment facilities since they allow for realistic predictions of mass transport and substrate conversion rates in these systems [161].

Individual-based simulation models treat each bacterial cell individually interacting with its local environment of metabolites, other cells, EPS, and fluids according to its own metabolic behavior. The time evolution of the system can be simulated starting from a few attached cells to fully developed three-dimensional film structures that can undergo detachment [167]. This type of model allows for addressing questions such as how the biofilm geometric structure depends on substrate concentrations [168–172].

CONCLUSION

The characterization of biofilms involves many techniques ranging from older established methods such as counting of bacterial colonies to more modern techniques such as fluorescent labeling of biofilms in conjunction with mathematical predictive modeling such as COMSTAT (Table 3). Today's federal funding climate is very competitive and successful proposals rely more and more on collaborative interdisciplinary work. Consequently, it is important for investigators to expand their knowledge in order to better critique and plan interdisciplinary projects. This review provided a quick overview geared towards new researchers on biofilm characterization method.

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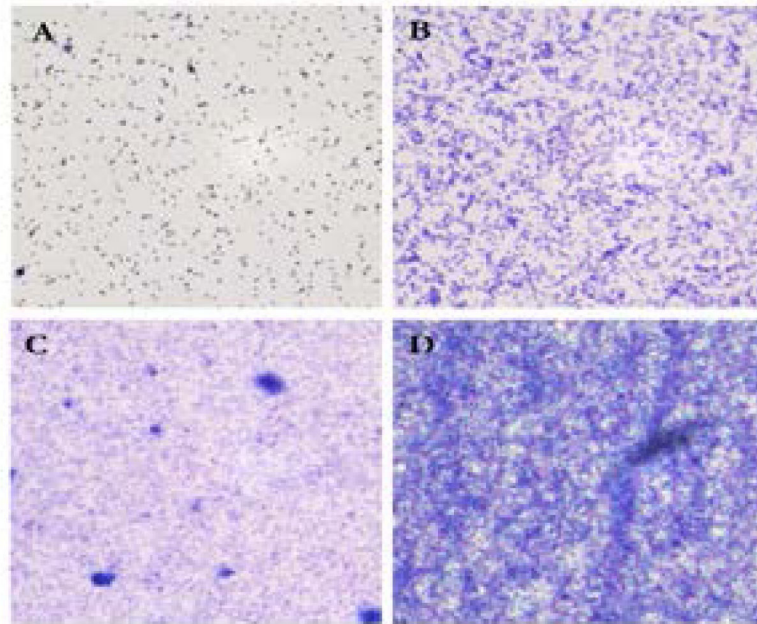


Figure 1.

Light microscopy of biofilms. Microscopy images of *Pseudomonas aeruginosa* (PA01) biofilm growth over time. Light microscopy images show the morphology of PA01 biofilm growth in a tissue culture plate with complete FAB media stained with crystal violet at 1 (A), 3 (B), 6 (C) and 24 (D) hrs after seeding in a static culture. Although light microscopy allows for the visualization of biofilm at all growth stages it has limitations to counting ability as the 3D structure of the film begins to form in later stages as can be observed in these images. Unpublished images by Christina Wilson at Doane University 2016.

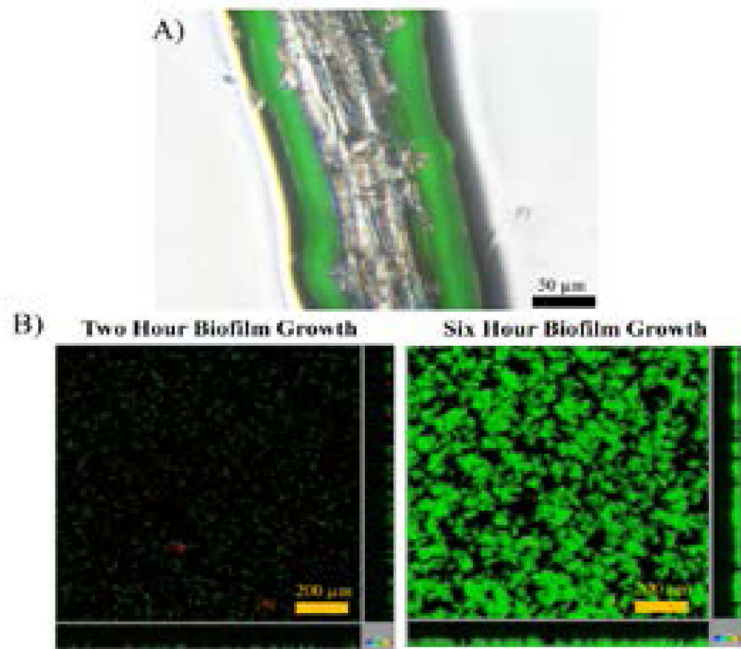


Figure 2.

Fluorescent microscopy of biofilms. Fluorescent images of biofilms. (A) Merged phase and fluorescent image of *Arabidopsis thaliana* roots colonized with EGFP expressing PA14. Overlays of fluorescence (green) and phase contrast (gray) images of *A. thaliana* roots infected with PA14 are shown. Unpublished Image by Cat Foster at Doane University 2014. (B) Confocal microscopy image of *Pseudomonas aeruginosa* (PA14) biofilm. PA14 growth *in vitro* two and six hours after inoculation under flow conditions. Viable cells are green (EGFP) and dead cells are red (stained with propidium iodide). Confocal allows for the monitor of x and y plane (central panel) and z axis (bottom and right panel) to characterize biofilm growth. Unpublished Image by Barbara Clement at the Helmholtz Institute for Infectious Disease Research, Susanne Häussler Lab 2014.

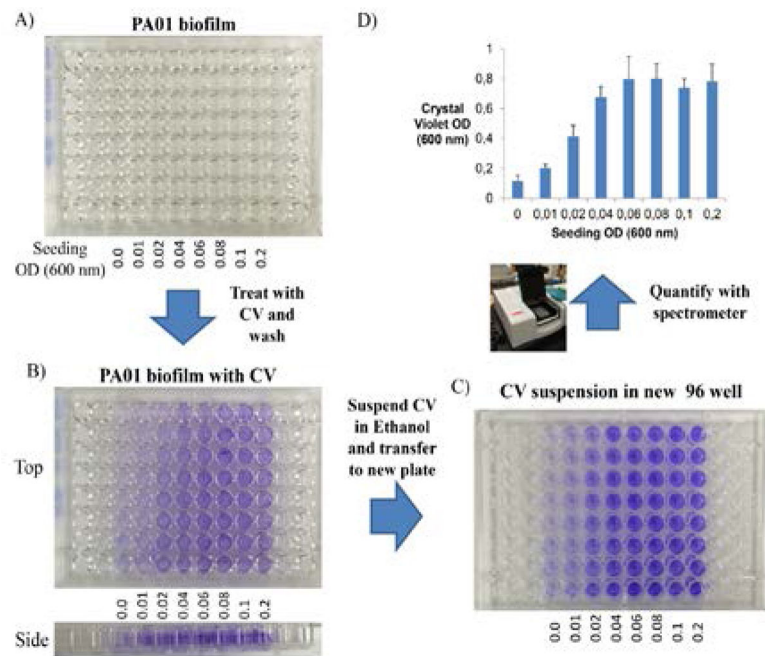


Figure 3. Schematic crystal violet assay on biofilms in a microtiter plate. Schematic of crystal violet assay on PA01 biofilm in a microtiter plate at 5 hr after inoculation. Biofilm formulation is difficult to distinguish with the naked eye (A) However CV is an unspecific dye which colocalizes with bacteria making it visible (B). An especially dense region of the biofilm will be formed on the outside edge of each well where the plate, media, and air intersect. This can be seen by a thin dark purple ring (side plate view). The crystal violet absorbed by the bacteria is proportional to the number of cells in the biofilm. Therefore when removed from the biofilm by ethanol and transferred to a clean 96 well plate (C) can be quantified by a UV-Vis plate reader (D). Unpublished data obtained by Christina Wilson at Doane University 2016.

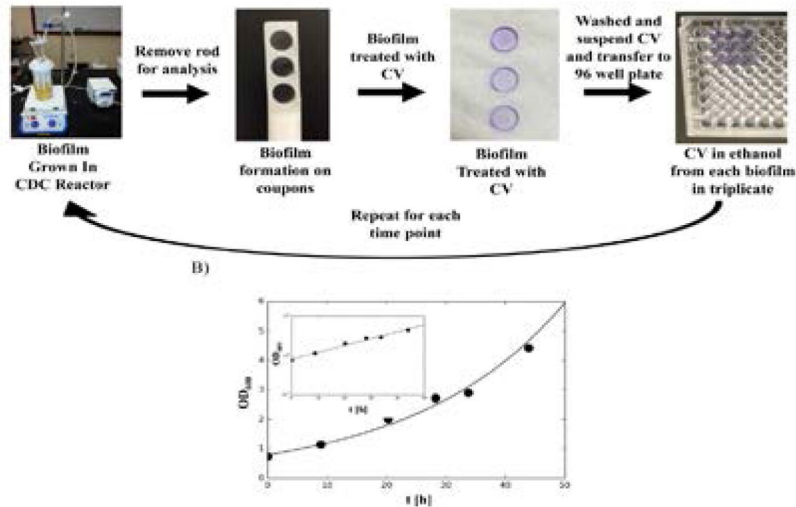


Figure 4. Schematic crystal violet assay of biofilms in a CDC reactor. Crystal Violet (CV) Assay of PA01 biofilm in a CDC Reactor. (A) Schematic of the assay repeated at each time point for development of growth curve. First, the biofilm is grown on coupons in the CDC reactor. After removal, the biofilm is treated with CV which clings to the bacteria surface until washed with ethanol. The absorbance (optical density) of the ethanol wash is measured at 600 nm as a surrogate for biofilm growth. Unpublished images obtained by Christina Wilson and Helena Valquier-Flynn at Doane University 2016. (B) Optical density (OD)-time data for the growth of a biofilm cell culture of PA01 on a glass surface. The cells are in the exponential growth (“log”) phase. Uncertainty in the OD measurements is less than or equal to the circle size. The solid line is a fit to an exponential function. The inset shows the same data and fit using a semi-log plot demonstrating how the exponential growth curve becomes linear when the log of optical density is plotted against time. The *Pseudomonas aeruginosa* (PA) biofilm was grown in 0.25% glucose (GL) and minimal media (MM). Unpublished data obtained by Chris Wentworth and Jeniffer Caballero at Doane University 2015.

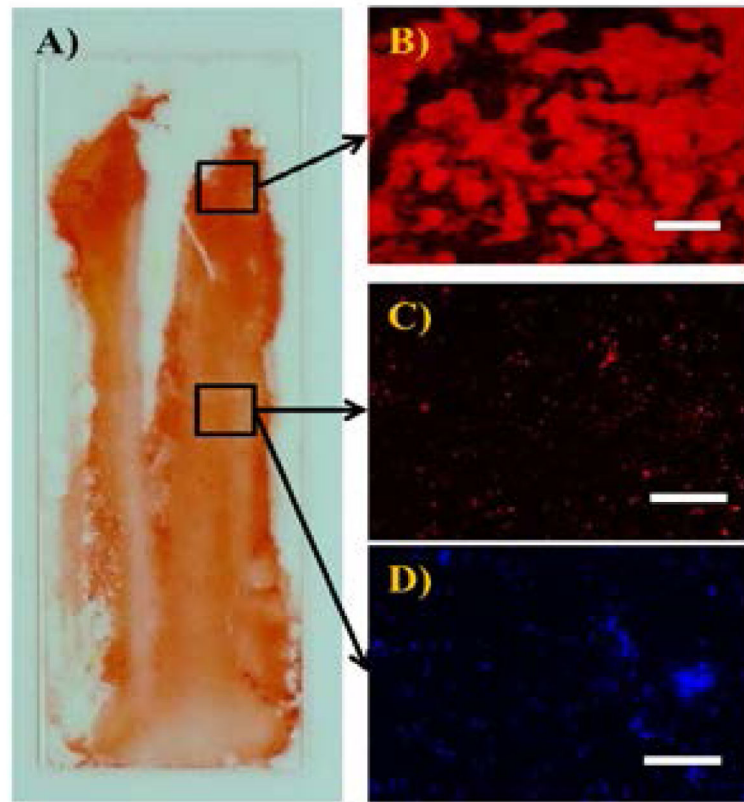


Figure 5. Images of biofilm stained with tetrazolium salt. Representative images of biofilm growth visualized with insoluble formazan derived from tetrazolium salt, CTC. Growth of biofilm on glass slide (A) has visible to the naked eye biofilm formation after 48 hrs growth in the drip flow biofilm reactor and staining with CTC (red) and DAPI (yellow). The biofilm can further be observed with fluorescent microscopy to qualitatively characterize biofilm shape and quantify area of coverage on glass slide (B) at low magnification and viability quantification with CTC derived fluorescent formazan only staining live cells (C) and DAPI staining all cells (D) at high magnification. Scale Bar C and D=200 μ m. Unpublished images obtained by Jasmin Sandoval at Doane University 2016.

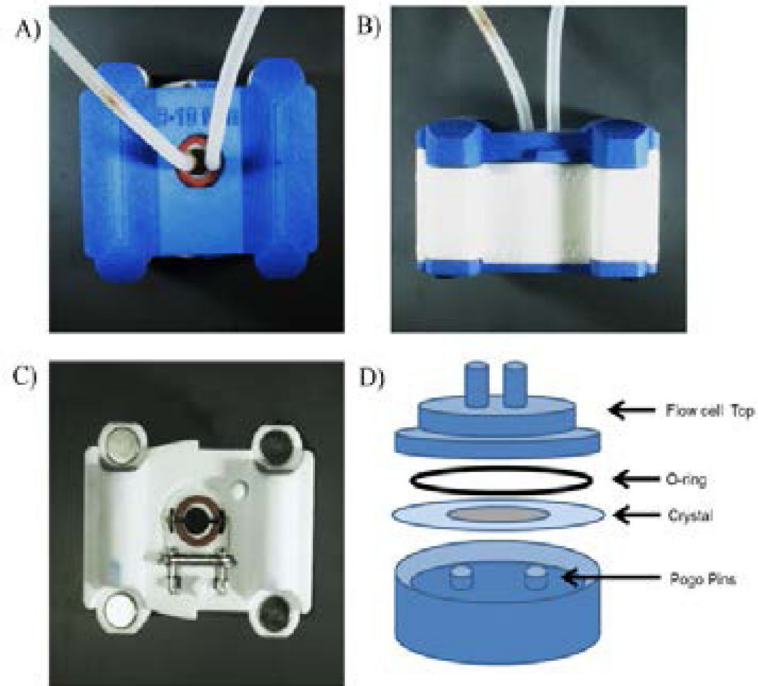


Figure 6. Quartz crystal microbalance biofilm reactor. The openQCM[®] test chamber with attached inflow and outflow tubing for media. Pictures of the (A) top, (B) front view and (C) inside the flow chamber including the quartz crystal and holder of the openQCM[®] test chamber. The chamber contains the required electronics in its base. (D) A cartoon of the flow cell configuration where biofilm would deposit on the crystal for biofilm quantification [100].

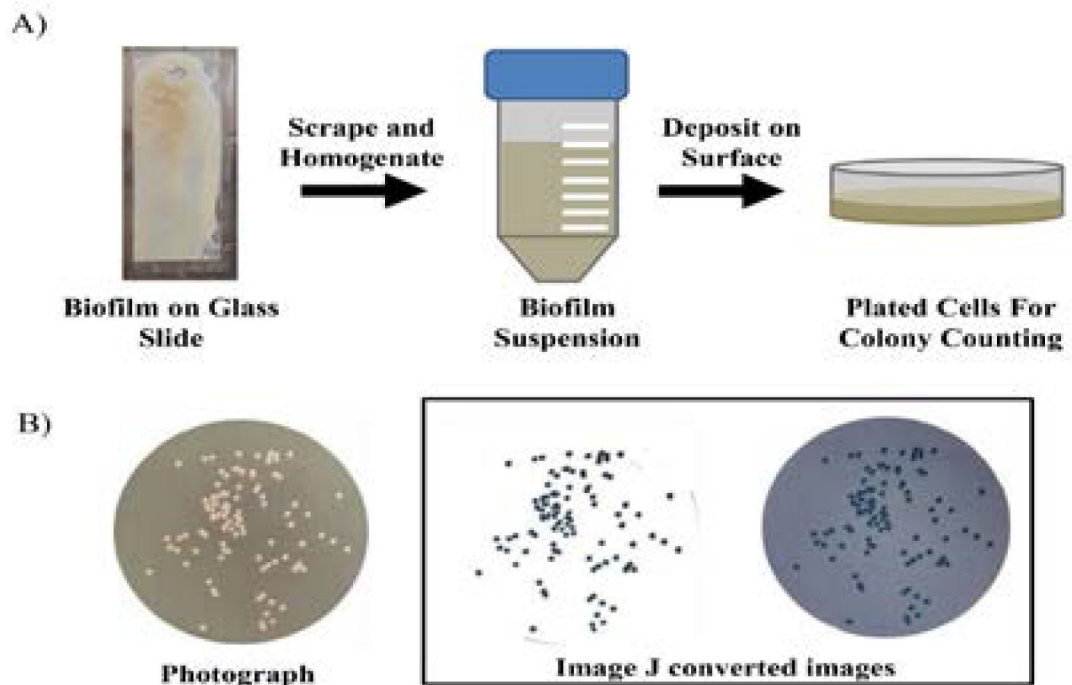


Figure 7.

Image J quantification of bacterial colonies from a biofilm (A) Schematic of biofilm collection from a drip flow reactor on a glass slide suspended in media and plated for colony counting. (B) Photograph of an agar plate with bacterial colonies. The photograph was analyzed with ImageJ so that colonies are black and the background is white in order to achieve maximum contrast between background and colony (left). In this case the program determined that the average size of a single colony was 108.16 pixels. The largest single colony was 135 pixels. If clusters were divided by average size of a colony, 91 colonies were counted. ImageJ was set up so that colonies are black and the background is grey in order to achieve minimal reflectance and uniformity between the background and the colony (right). In this case, if colony clusters were divided by the average size of a colony, 93 colonies were counted.

Table 1

Summary table of common fluorescent stains used for biofilm staining.

Name	Cellular Location	Membrane Permeability	Viability (Live/Dead/Both)	Reference
DAPI (4',6-Diamidino-2-phenylindole dilactate)	Nucleic Acids	Yes	Both	[14]
FM dyes	Cell Membrane Lipids	Yes	Both	[170]
SYPRO Ruby Biofilm Matrix Stain	Matrix Proteins	No	Both	[171]
Propidium Iodide	Nucleic Acids	No	Dead	[27,171,172]
SYTO	Nucleic Acids	Yes	Live	[27,116,172]
Calcein	Intracellular Space	Yes	Live	[140,172]

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Table 2

Summary table of commonly used tetrazolium salts for study of biofilms *in vitro* with water solubility and detection wavelength.

Name	Water solubility	Detection wavelength (nm)
MTT 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide	Insoluble	Abs: 550–570
CTC 5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride	Insoluble	Ex:540 Em:630
INT 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride	Insoluble	Abs:470
TTC 2,3,5-TriphenylTetrazolium Chloride	Soluble	Abs:480
XTT(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)	Soluble	Abs: 490

Table 3

Summary of major direct and indirect methods for characterization of biofilms.

	Time to Complete	Specialized Equipment Required	Biofilm Preparation	Notes	References
Plate Count, Viable cell enumeration	1–3 days	<i>Incubator- Consumables; disposable petri plates, culture flasks, suitable agar and medium</i>	Cells are removed from the substrate, homogenized, resuspended in liquid medium, diluted, and aliquots are plated, incubated and counted.	Most readily adaptable to liquid/planktonic cultures. This method only quantifies live cells, and an assumption is made that each colony derives from one original cell. The differing metabolic states of living cells in the biofilm may complicate determination of accurate number of cells in the biofilm. Must be confirmed by cell mass or surface area.	[21,23,25]
Light Microscopy	Minutes	<i>Compound, brightfield microscope</i>	Biofilm can be grown directly on a transparent substrate, such as a slide or coverslip, stained and observed directly.	Counting or observing mature biofilms is limited, as accumulation of extensive biofilm mass prevents observation of individual cells. Can be used in conjunction with dry mass measurements to acquire biofilm thickness and quantifying specific visual characteristics of the biofilm.	[19,52]
Manual Cell Counting Using a Microscope	Minutes to hours	<i>Hemocytometer; Brightfield or Fluorescent Microscope, Cell Stains</i>	Biofilm must be removed from the substrate and homogenized.	Relatively inexpensive, and easy to learn, but tedious. It does not distinguish live and dead cells, and motile cells are	[16,27,28]

	Time to Complete	Specialized Equipment Required	Biofilm Preparation	Notes	References
Automated Cell Counting, Coulter Counter	One to a Few Hours	<i>Coulter counter</i>	Biofilm must be removed from the substrate and homogenized	Coulter counter quantifies cells and particles and can distinguish entities by size. Very small cells may be difficult to count accurately.	[31,32]
Automated Cell Counting; Flow Cytometry	A few hours – more labeling and separation may take longer.	<i>Flow cytometer; Antibodies or fluorescent stains</i>	Biofilm must be removed from the substrate and homogenized. Flow cytometry requires cell populations to be labeled with a separate fluorophore for each cell type to be isolated. May not be efficient if you are only trying to count cells.	Flow cytometry can distinguish different cell types. This can be expensive, and requires technical expertise.	[33,34]
Fluorescent Microscopy and Staining	20 – 30 minutes.	<i>Brightfield/ Fluorescent Microscope; Fluorescent stains, antibodies or endogenous fluorescent proteins</i>	Biofilm can be grown directly on a slide or coverslip, stained, and observed <i>in situ</i> . Biofilm is stained with view of the desired outcome.	Some stains are potential mutagens. Stain should be chosen carefully-- not all stains penetrate the cell membrane, and not all are compatible with maintaining a living biofilm.	[14,27,37,140,140]
Confocal Fluorescent Microscopy	One to a few hours.	<i>Confocal Fluorescent Microscope; Fluorescent stains, antibodies or endogenous fluorescent proteins</i>	Can image in place biofilm (on a coverslip, e.g.). Cells must be labeled. Fluorophores can be selected according to a variety of purposes, such as distinguishing live and dead cells, staining nuclei/DNA, etc.	Biovolume can be calculated with appropriate software and computing capability. Usually requires a dedicated technician to run and maintain the instrument. Can image any cell or particle that has a fluorescent label that can be detected by the microscope. It is better used for structures and 3D architecture than counting cells. Can image within the thickness of the	[20,35,38,41,44,126,170]

	Time to Complete	Specialized Equipment Required	Biofilm Preparation	Notes	References
Determination of Dry Mass	Three – four hours.	<i>Analytical Balance: Lab oven capable of reaching 100 °C</i>	Film on substrate is dried, massed, then cleaned. Substrate is massed again.	Film area should be measured; thickness can be measured to give dry mass per unit of wet volume.	[42,43,48,63,88]
Total Organic Carbon	15 minutes per sample.	<i>TOC instrument</i>	Homogenization and resuspension.	A standard protocol can discriminate between carbon in EPS and cellular carbon.	[17,24,54]
Crystal Violet Assay	Two – four hours over two days.	<i>Plate reader or UV/VIS spectrophotometer: Gram Stain</i>	Indirect measure of biofilm growth. Cells are stained with crystal violet, washed, and the absorption of CV measured. Higher absorption relates to more biofilm mass.	Individual wells are somewhat variable, so controls, standards, and replicates are important. A 96-well plate adaptor is required. Also requires standard 96-well plates with flat bottoms.	[25,58,59,66,88]
Tetrazolium Salt Assay	Two – four hours.	<i>Tetrazolium Salt: Evaluation method: Microscopy, Flow Cytometry, Spectroscopy, etc.</i>	Direct or Indirect measure of biofilm growth. Cells are incubated with tetrazolium salt that is metabolically converted to formazan derivative. Evaluated via spectroscopy, microscopy or flow cytometry depending on the solubility of the formazan.	Insoluble formazan salts will be trapped in the cell membrane allowing for direct individual cell analysis. Soluble formazan may be collected from the media and quantified for an indirect quantification. Only living cells will convert the salt to formazan providing a measure of viability.	[23,27,63,65–68]
ATP Bioluminescence	A few hours.	<i>Assay Kit: Luminometer</i>	Incubation of biofilm on soy broth for up to 5 days.	The reagent is stable for one day at 15 to 25 °C or for one week when stored at 0 to 4 °C.	[73,75,77,78]

	Time to Complete	Specialized Equipment Required	Biofilm Preparation	Notes	References
Total Protein	A few hours	<i>Assay Kit: UV-Vis spectrophotometer</i>	Biofilms are scraped from their substrates and homogenized in a liquid suspension, often using a commercial homogenizer.	Protein determination methods are subject to interference from other substances, potentially present, such as certain ions, detergents, reducing agents, or other species.	[88,90,91,94]
QCM & QCMD	Minutes	<i>Quartz crystal microbalance Bioreactor</i>	For accumulation measurement, a calibration between frequency and cell number must be done.	Material property information from QCMD requires an appropriate theoretical model.	[96,99–101,104,106]
SEM	Hours to Days	<i>Scanning electron microscope: Sputtering Coater</i>	Biofilm can be grown on a coverslip (or other substrate) and directly imaged on the microscope. Samples must be fixed, dried, and coated with metal (Pt-Pd).	Toxic chemicals may be involved in some fixation techniques. Usually requires a maintenance contract and special housing conditions.	[21,39,88, 124–126,131,135,137]