Usage of microscopy techniques for the detection of biofilms among various microorganisms

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Biofilms are highly organized microbial structures which can live on abiotic and biotic surfaces such as medical implants and human tissue. Direct observation of the structure of biofilms is essential to understand their development and functions. The demonstration of biofilms on surfaces is technically challenging and has so far relied mostly on the use of scanning electron microscopy (SEM), transmission electron microscopy and confocal scanning electron microscopy (CLSM). Light and electron microscopy techniques require a dehydration process that reduces the total volume of the matrix and alters its architecture. For a detailed understanding of the formation and presence of microbial biofilms more advanced microscopy techniques should be used since the analysis have to include detection of the microorganims and the matrix. In various studies the analysis of the biofilm formation on different materials and surfaces has been carried out through SEM. However, SEM analysis requires biofilm fixation and dehydration, factors that could alter its characteristics. With the advent of CLSM, biofilm can be evaluated without the necessity of fixation and dehydration, allowing the biofilm to keep its original architecture. CLSM has been evaluated among various microorganisms like *Candida albicans* and nontuberculous mycobacteria. The studies revealed that CLSM gives detailed information about the ultrastructure of biofilms produced by these organisms. In this review article usage of novel microscopy techniques for the detection of biofilms among various microorganisms have been discussed.

Keywords: Microscopy, biofilm, microorganism

1. Introduction

Biofilms are communities of interacting microbes that are embedded in a matrix of extracellular polymers and protein. Mature biofilm infections have higher resistance to antimicrobials and the host immune defence than do their genetically-identical planktonic counterparts. The mechanical integrity of the biofilm matrix contributes to the difficulty of removal and harmful effects of the biofilm. Many harms done by biofilms arise from the mechanical integrity of the biofilm matrix [1]. In this brief review article usage of novel microscopy techniques for the detection of biofilms among various microorganisms have been discussed.

2. Scanning electron and confocal laser scanning microscopy

One of the biggest advantages of fluorescence microscopy is the possibility to study live samples in their natural hydrated state and the consequences that this brings with it. With scanning electron microscopy (SEM) and transmission electron microscopy (TEM), the samples require preparation involving dehydration, fixing and embedding, which may cause disruptive shrinkage and loss of the biofilm matrix that can affect 73 to 98% of the in vivo biofilm mass; furthermore, the time-consuming and tedious nature of the sectioning process for TEM constitutes a big drawback for this technique [2].

Imaging-based technologies are widely used to observe and quantify the transport of solutes through biofilms. Perhaps the most common and widely available optical technique is fluorescence-based microscopy. Fluorescence facilitates direct visualization of molecules in real time inside the biofilm. This enables investigators to calculate their effective diffusivity, determine interactions between the probe and the matrix, and to map voids, channels, and micro colony structures in biofilms. In comparison to microsensor technology, fluorescence imaging can typically cover a larger region of interest, ranging from hundreds of nanometers up to millimeters. In recent years, confocal and two-photon fluorescence microscopy has yielded three dimension information of fluorescence distribution through greater depths of biomass with less invasive methods relative to micro-electrode probing. Furthermore, fluorescence imaging can be used to visualize the biofilm matrix by directly labeling matrix components. This method has provided valuable information on the matrix structure, localization, and composition. Fluorescence imaging techniques provide high spatial resolution for identifying cell micro-clusters, densely packed films, and fluid-filled channels and voids. However, imaging is limited to fluorescence labeling of a molecular probe, and the introduction of the label can perhaps perturb the probe's behavior [6].

Scanning electron microscopy (SEM) is a powerful tool for structural analysis in a variety of fields such as physics, electronics, and chemistry. SEM is also widespread in biology, however its use to study live tissues and cells is hindered by the biologically incompatible requirements that sample visualization is done in vacuum, and for the sample to be conducting. To undergo SEM, biological samples must undergo a sequence of procedures that is time-consuming, labor-intensive, and chemically hazardous. Standard SEM protocols make routine use of highly toxic and corrosive

materials such as osmium tetroxide, cacodylic acid, tannic acid, guanidine hydrochloride which pose serious health hazards. Finally, the samples are sputtered with conducting materials such as gold or iridium. The procedures take many hours during which samples are passed through multiple substances with varying water content, inevitably undergoing changes in the process [3].

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. SEM allows for collection of high resolution images useful in evaluation of bacterial interaction, extracellular polymeric substances organization and biofilm morphology, which assists in a greater understanding of formation and persistence. 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 absorbed by the surface molecules which excites the surface molecules and causes a low energy, secondary electron to be ejected or 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 [6].

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 [6].

Scanning electron microscopy has represented a breakthrough in the past 40 years. The sweeping of the sample surface with an electron beam and the detection of secondary or reflected electrons provides excellent resolutions of 10 nm or less. However, it requires the existence of a large vacuum and a metal coating and does not allow the examination of samples which produce water vapour when placed in a vacuum chamber, as this interferes with the electron beam. Therefore, biological samples require a long dehydration process which is very destructive and increases the risk of introducing artefacts and destroying the more delicate structures. The metal coating can also hide fine surface details in biological samples. Some authors have found that the external polysaccharide layer and much of the bacterial flora may disappear during the critical state. Environmental scanning electron microscopy is a substantial modification of the conventional technique that allows visualization of virtually any sample without dehydration or conductive dipping, as it allows the introduction of a gaseous atmosphere in the sample chamber (therefore also called “wet mode”). There are openings throughout the column that limit the pressure and help maintain vacuum gradients, so that there is an optimal vacuum in the electron beam while preserving a low vacuum in the sample chamber. Water vapour is the gas most commonly used in this technique and it acts as an amplifier of the secondary electrons signal released from the surface of the sample after the first scan by the electron beam. The positive ions resulting from this amplification help to compensate the negative charge generated, and thus there is no need for a conductive metal coating of the samples. A special, positively charged gas detector collects the signal amplified by secondary electrons [10].

The demonstration of biofilms in human tissue sections is technically challenging and has so far relied mostly on the use of SEM and transmission electron microscopy. Scanning electron microscopy imaging of the tonsil surface showed the presence of single and clustered bacteria attached to the surface and in the crypts. Bacterial cells present in a biofilm are usually encased in a hydrated (typically 95%-99%) matrix, which is prone to dehydration artifacts. When using SEM or transmission electron microscopy, only the remnants of the original hydrated structure can be visualized. In our study, SEM analysis was hampered by the presence of blood cells on the surface of the tissue, masking bacterial presence on these surfaces. Therefore, not all of the tissue specimens collected were suitable for SEM analysis [12].

Of the fluorescence microscopies, CLSM is the one that provides more advantages over conventional optical and electronic microscopies, and it represents an evolution of epifluorescence or incident-light fluorescence. The most important feature of CLSM is the capability of isolating and collecting a plane of focus from within a sample, which is achieved by spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus [2].

In many research areas, it is the scale of observation that determines what the observer considers to be reality, and biofilm research is no exception to this rule. Several authors have described biofilms in chronic sinusitis using gram staining and transmission electron microscopy, techniques that require a dehydration process, due to the cells present in a biofilm being usually encased in a hydrated (typically 95%-99%) matrix. Therefore, when using scanning electron microscopy (SEM) or transmission electron microscopy, only the remnants of the original hydrated structure can be visualized. In contrast to electron microscopy, preparation of tissue for CLSM does not require dehydration and thus the structure of a biofilm is better preserved [4].

The biofilm lifecycle and the matrix components have preferably been investigated by means of confocal laser scanning microscopy (CLSM). This method has provided valuable insight into the biofilm development; however, the information on the detailed ultrastructure of the biofilm is difficult to image by light microscopes. Electron microscopy

(EM) has the needed resolution and hence magnification to offer a more detailed insight into the ultrastructure of the biofilm as well as its environment. Several EM techniques have been used to investigate biofilms, with scanning electron microscopy (SEM) as the predominant choice Conventional SEM methods are far from optimal for investigation of water-containing specimens such as biofilms because the technique requires dehydration of the sample. In most cases, the choice of microscope is based on availability and not the suitability [5].

3. Other fluorescence scanning microscopy techniques

Both fluorescence recovery after photobleaching and fluorescence correlation spectroscopy are powerful quantitative tools for measuring local diffusion coefficients for multiple locations within a biofilm beyond initial diffusive rates. These techniques provide a greater spatial resolution to resolve transport properties within a biofilm. Importantly, these methods allow for separation of free diffusion from anomalous diffusion, facilitating the discrimination of solutes that interact with matrix components from those that are nonreactive. Furthermore, these techniques can be applied after the fluorescent probe has reached equilibrium within the biofilm matrix to monitor the reactivity of the solute on extended timescales, which can be on the order of days [7]. Scanning electrochemical microscopy is a scanning probe technique and electrochemical tool that is widely used to probe surfaces and surface reactions [8]. 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. 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. The resulting emission can be used to count cells and track real-time biofilm accumulation. Biofilms expressing GFP can be assessed for green alone or in conjunction with other fluorescent stains. *Pseudomonas aeruginosa* 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 which allows for separate labeling of cells in co-culture or multiple labeling in a single cell. Biofilms with GFP can be visualized *in vitro* such as in a flow cell [6].

Light sheet fluorescence microscopy provides an alternative approach to three-dimensional fluorescence imaging that surmounts these limitations. Here, fluorescence excitation is shaped into a thin sheet, emission from which is imaged onto a camera by a perpendicular lens. In essence, this is simple widefield imaging, but with the thin sheet providing optical sectioning to illuminate a single plane. Constructing a three-dimensional image only requires scanning in the one perpendicular dimen- sion. Notably, the illuminated volume is roughly equal to the detected volume, minimizing photodamage [9].

Conventional electron microscopy (EM) has been widely used to image biofilms at sub-nanometre resolution. However, the sample is under vacuum, making pretreatments that might affect the soft and hydrophilic structure of biofilms (e.g., dehydration) basically unavoidable. Recently, atmospheric scanning electron microscopy (ASEM) was developed to observe biological samples at atmospheric pressure. ASEM allows an inverted SEM to observe a wet sample from below while an OM simultaneously observes it from above. Cells can be cultured, fixed and imaged in the specialized sample dish (ASEM dish) at atmospheric pressure. The dish has an electron transparent, 100 nm-thick silicon nitride film window in its base to allow SEM, and holds a few millilitres of solution, enabling direct cell culture under stable conditions. Importantly, at 30 kV acceleration voltage ASEM observes the 2–3 μm-thick specimen regions directly above the film, allowing structures near the film to be visualised at 8 nm resolution. In addition, the time-consuming sample preparation generally required for immuno-EM is not required for immuno-ASEM and the effort involved is comparable to that for immuno-OM [13].

4. Microorganisms and biofilm microscopy techniques

Microbial biofilms are ubiquitous in nature and of importance for a number of environmental processes. However, biofilms are also the underlying cause of persistent infections in various parts of the human body, including the teeth, urinary tract, heart valves, cystic fibrosis lungs, middle ears, nasal passages and sinus cavities, bones, prostate, and chronic wounds. In addition, biofilm formation on implants and catheters gives rise to problematic infections in connection with the use of medical devices including urinary catheters, central venous catheters, fracture fixation

devices, dental implants, joint prostheses, vascular grafts, cardiac pacemakers, breast implants, mechanical heart valves, penile implants, and heart assist devices [14].

Studies using confocal laser scanning microscopy of biofilms formed in laboratory experimental systems by fluorescence-tagged bacteria have provided detailed knowledge about structural biofilm development. Biofilm formation can initiate when bacteria attach to a surface or to each other and form aggregates. The biofilm developmental cycle is believed to include the processes: i) transport of solitary microbes to a surface or each other, ii) initial attachment of the microbes to the surface or each other, iii) formation of microcolonies, iv) maturation of the biofilm, and v) dispersal of the biofilm. In many bacteria, the initiation of biofilm formation and the pro- duction of biofilm matrix components occur in response to high intrabacterial levels of the second messenger molecule c-di-GMP, whereas dispersal of the biofilm occurs in response to low levels of c- di-GMP [14]

P. aeruginosa is an opportunistic human pathogen that is by far the best-characterized, most widely studied model organism for biofilm formation. The widespread use of *P. aeruginosa* as a model organism for biofilm formation has two principle causes. First, *P. aeruginosa* readily forms biofilms on a large variety of surfaces (and multicellular, biofilm-like aggregates in liquid suspension) and under a wide range of growth conditions, including on contact lenses, in water treatment facilities and pipes, and on oil spills. Second, *P. aeruginosa* biofilm infections are an important factor that detrimentally affect the outcome for patients with implants or medical devices, chronic obstructive pulmonary disease, chronic wounds, and cystic fibrosis. Thus, near-ubiquity and high practical impact together have led to the selection of *P. aeruginosa* as a model organism in the majority of biofilm related research. Intriguingly, Professor Howard Stone's group at Princeton has shown that shear stress applied by fluid flow increases the residence time of surface-adhered *P. aeruginosa* cells. The mechanical interaction between an individual bacterium and a surface can be probed using atomic force microscopy (AFM). AFM uses the deflection of a cantilever to image the height features of a surface and to measure forces of interaction between a surface and the cantilever. AFM is a powerful tool for studying planktonic cells and how small numbers of cells interact with surfaces [1].

The early *P. aeruginosa* biofilm generally consists of a subpopulation of non-motile cells forming small microcolonies, and a subpopulation of motile cells, which move on the surface between the small microcolonies. After formation of the initial microcolonies structural biofilm development by *P. aeruginosa* depends on the prevailing conditions. For example, flat biofilms are formed in flow-chambers irrigated with citrate minimal medium, while heterogeneous biofilms containing mushroom-shaped multicellular structures are formed in flow-chambers irrigated with glucose minimal medium. Formation of the flat *P. aeruginosa* biofilm in flow-cham- bers irrigated with citrate medium was shown to occur via ex- pansive surface-migration of cells from the initial microcolonies. CLSM time-lapse microscopy indicated that a shift from non-motile to migrating cells occurred when the initial microcolonies reached a certain size, suggesting that the shift may by induced by a limitation arising in the initial microcolonies [15].

Candida albicans biofilms originate from cells that adhere to a surface, and generally consist of highly entangled hyphae (filaments of elongated cells), clusters of pseudo-hyphal and yeast-form cells, voids, and extracellular matrix. After 24–48 h growth on a surface submerged in liquid culture medium, dense biofilms range in thick- ness from 100 to 600 mm. Under 5–10 magnification, a biofilm has the appearance of very fine 'wet felt'. Long, non-entangled apical hyphae may extend an additional 500 mm into the medium, while basal hyphae may invade the substratum. These features add greatly to the thickness and complexity of the biofilm. Fluorescence imaging of intact specimens requires optical sectioning microscopy, most commonly confocal scanning, 2-photon scanning, or selective-plane illumination [16].

One of the most important traits for assessing *Candida* biofilm development is architecture. SEM and CLSM have been used to assess cellular phenotype, extracellular matrix, relative growth, and architecture. However, both methods have limitations and artifacts and can lead to variability, depending upon how they are performed. There have been two procedures used for SEM analysis of *Candida albicans*. If biofilms are fixed with glutaraldehyde in cacodylate buffer, flash frozen, and then freeze-dried, one preserves the collapsed ECM but loses resolution of the hyphal and yeast morphologies. If biofilms are fixed and dehydrated, hyphae and yeast cells are imaged, but the ECM is lost. As noted by Little et al. for bacterial biofilms, solvent replacement of water from a biofilm removes matrix components. In addition, whether the SEM preparation is frozen or dehydrated after fixation, native architecture is lost. CLSM can provide information on internal archi- tecture, cellular phenotype, and biofilm thickness. In contrast to the SEM procedure, there is no dehydration step after fixation with paraformaldehyde or other fixatives, and thus more of the native architecture is preserved. CLSM involves staining rather than surface shadowing, and the collection of hundreds of optical sections of a biofilm through the *z* axis provides information about different depths. Staining artifacts, however, can arise in CLSM. For instance, when a 48-h fixed biofilm is overlaid with a solution of calcofluor, which targets cellulose and chitin, staining is most intense in the upper portion of the biofilm, presumably because of disproportionate binding of the dye to hyphae at the top of the biofilm during dye penetration. CLSM is, however, effective at comparing the thicknesses of biofilms, and with advanced processing software, it can be used to image the architecture of intact hyphae in side views of projection images. An additional problem arises when CLSM is used to view the ECM with dyes such as calcofluor, which binds to chitin and cellulose. These ECM polysaccharides are also localized in the hyphal wall. In the case of calcofluor, hyphae stain far more intensely than the diffuse ECM. One must therefore lower the laser intensity to view hyphae by CLSM, at the expense of viewing the ECM. If intensity is increased, the ECM can be visualized at the

expense of resolving distinct hyphae, because hyphal walls stain so much more intensely. Light attenuation is less problematic when multiphoton CLSM is applied [17]. The authors emphasized that, preparing cells for imaging, such as for SEM, can collapse a biofilm, destroy the architecture, or extract the ECM and CLSM preserves architecture but does not provide high resolution images of cellular phenotype and can include staining artifacts [17].

Monfredini et al, demonstrated by using two different assays (crystal violet and confocal microscopy) that *C. albicans/C. dubliniensis* isolates obtained from patients with persistent candidemia produced substantially more biofilm mass when compared to a control group of isolates obtained from patients with single episodes of fungemia [18]. The authors highlighted that, from a clinical perspective, *Candida* biofilms are associated with negative effects, as they provide a safe environment for cells, acting as reservoirs for persistent sources of infections and potentially adversely affecting the function of implanted devices. The discrepancies in results related to the clinical impact of biofilm in the mortality rates of candidemia may be secondary to the fact that mortality in these patients is related not only to fungal sepsis but also to the multiple risk factors and severe underlying diseases documented in this population and the usage of biofilm detection detection methods are important much in demand [18].

Confocal scanning laser microscopy shows that phagocytes enhance the ability of *C. albicans* to form biofilms and that these phagocytes appear as unstimulated rounded cells, inducing significantly less damage to biofilms than their planktonic counterparts. *C. neoformans*, an encapsulated fungus that can cause meningoencephalitis in immunocompromised patients, shows resistance to oxidative stress, but it becomes considerably more susceptible to cationic antimicrobial peptides when switching to the biofilm mode of growth. Defensins with higher net positive charges (β-defensin-1, β-defensin-3) interact strongly with negatively charged biofilm surfaces, while their hydrophobic characteristics enable them to enter the cells' membranes and create temporary pores through which cell contents leak, leading to cell death. In contrast, defensins with lower net positive charges (α-defensin-3 and magainin-1) are less efficient against biofilms, implying that the greater affinity to biofilms results in increased biofilm susceptibility [11].

The ultrastructure of mycobacterial biofilms has also been studied with different methodologies. CLSM combined with two fluorescent dyes, Nile Red (Sigma-Aldrich Co., St. Louis, MO, USA) and LIVE/DEAD BacLight (Invitrogen, USA), has been used to analyze the phenotypic characteristics of biofilms formed by some RGM (growth rate, percentage of covered surface, percentage of live/dead bacteria, and autofluorescence), showing differences between species. In this study, Muñoz- Egea et al. showed that maximum thickness for *Mycobacterium fortuitum* and *Mycobacterium chelonae* biofilm was detected at 72 h, but other non-pigmented RGM reach maximum thickness at 96h. *M. chelonae* covered a smaller surface area than *Mycobacterium abscessus*, but a greater area than *M. fortuitum* and *Mycobacterium mageritense*. Interestingly, autofluorescence, which has been found among different mycobacterial species can be detected not only in sessile bacteria but also in the extracellular matrix. It was speculated that coenzyme F420 could be involved in this phenomenon. This molecule may be secreted by the bacterial components of the biofilm, and is thus detectable in extracellular matrix using CLSM [19].

5. 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. 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 (biofilm staining) level. 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 [6].

Advantages of SEM include its wide-array of applications, the detailed three-dimensional and topographical imaging and the versatile information garnered from different detectors. SEMs are also easy to operate with the proper training and advances in computer technology and associated software make operation user-friendly. In addition, the technological advances in modern SEMs allow for the generation of data in digital form. Although all samples must be prepared before placed in the vacuum chamber, most SEM samples require minimal preparation actions [20].

The disadvantages of a SEM start with the size and cost. SEMs are expensive, large and must be housed in an area free of any possible electric, magnetic or vibration interference. Maintenance involves keeping a steady voltage,

currents to electromagnetic coils and circulation of cool water. Special training is required to operate an SEM as well as prepare samples. The preparation of samples can result in artifacts. The negative impact can be minimized with knowledgeable experience researchers being able to identify artifacts from actual data as well as preparation skill. There is no absolute way to eliminate or identify all potential artifacts. In addition, SEMs are limited to solid, inorganic samples small enough to fit inside the vacuum chamber that can handle moderate vacuum pressure. Finally, SEMs carry a small risk of radiation exposure associated with the electrons that scatter from beneath the sample surface. The sample chamber is designed to prevent any electrical and magnetic interference, which should eliminate the chance of radiation escaping the chamber. Even though the risk is minimal, SEM operators and researchers are advised to observe safety precautions [20].

CLSM is a specialized form of microscopy that produces high-resolution, sharp images of biofilms in three dimensions. Three dimension 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 two dimension "slices" at various heights that are assembled to produce a final three dimension image. Furthermore, confocal microscopy can utilize single or multiple excitation lasers to view multiple fluorescent markers sequentially or simultaneously. 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 [6].

Biofilm structure can be evaluated using different microscopy techniques. However, some of these techniques, such as scanning electron microscopy, do not allow careful evaluation of the entire structural architecture of a biofilm. A more efficient alternative for this type of analysis is atomic force microscopy (AFM). AFM requires pre-treatment during sample preparation, but AFM is able to capture images of liquid samples in their physiological state. This microscopy technique is based on the variation of the force exerted between the tip of the probe and the surface of the sample [21].

There is evidence derived from AFM that the development of microbial biofilms also involves protein-based matrices. Fibronectin binding proteins (FnBPA) from *Staphylococcus aureus* were shown to promote intercellular adhesion via low-affinity, zinc-dependent homophilic bonds between FnBPA domains on neighbouring cells. Unlike the very strong and stable 'dock lock and latch' (DLL) bonds, homophilic bonds showed moderate strength and fast dissociation, which could be important for biofilm dissemination. For another *S. aureus* matrix surface protein G (SasG), AFM demonstrated zinc-dependent homophilic interactions between proteins on opposing bacteria, and unravelled the molecular elasticity of individual SasG molecules [22]. According to the researchers, the full potential of nanoscopy will be achieved when combining optical and force modalities and establishing these correlated platforms in microbiology should allow the identification and tracking of specific cellular components, while probing their biophysical properties (adhesion, elasticity) simultaneously on the same single cell, thus contributing to the important connection between their structures and functions. Toward this goal, correlated AFM-fluorescence imaging has been exploited to track cell surface dynamics during cellular morphogenesis [22].

Table 1 Advantages and limitations of microscopy techniques in the study of biofilms (modified from reference [23]).

Microscopy techniques	Advantages	Limitations
Light Microscopy	-Simple sample preparation -Cheap and easy to perform Imaging of larger parts of a sample compared to electron microscopy	-Limited magnification and resolution -Sample staining necessary -Morphotypic differentiation relatively gross -Lacking discriminatory detail
Confocal laser scanning microscopy	-Resolution compatible with single cell visualization -Reconstruction of 3-D images of a sample -No need for extensive computer processing	-Use of fluorophores is required -Limited number of reporter molecules (e.g. no universal matrix probes exist) -Interference of local properties of the biofilm with the fluorescence probes -Natural auto-fluorescence may hide signal of interest
Scanning electron microscopy	-Resolution higher than other imaging techniques (resolves surface details) -Good depth of field ability to image complex shapes -Wide range of magnifications	-Time-consuming sample preparation -Lacks vertical resolution -Preparation processes (fixation, dehydration, and coating with a conductive material) can destroy sample structure or cause artifacts
Atomic force microscopy	-Works under ambient conditions, minimizes pretreatments and artifacts even on liquid surfaces (enables in situ imaging). -Three dimension reconstruction Qualitative and quantitative assessment of living biofilms under physiological-like conditions	-Inability to obtain a large area survey scan -Sample damage or artifacts caused by tip shape and size (although generally considered negligible)

6. Conclusions

Microscopy techniques provide valuable and complementary information about different aspects of the biofilm structure of various microorganisms. A combination of methods is needed to obtain a more detailed and true biofilm detection. If a direct quantification of biomass is required microscopy techniques are crucial, but the nature of the analysis and some other factors like the expenditure, limit its comprehensive application.

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