Microscopy Methods for Biofilm Imaging

Subjects: Others
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Several imaging methodologies have been used in biofilm studies, contributing to deepening the knowledge on their structure.

Keywords: scanning electron microscopy ; variable pressure scanning electron microscopy

1. Introduction

Surface-attached microbial agglomerations were for the first time named as a “biofilm” by William J. Costerton in 1978 [1]. In the following years, he perfected this definition by also considering the host role and the three-dimensional (3-D) architecture. The definition of biofilm was thus implemented, expanding the concept toward a complex community of microorganisms living attached to a surface or interface, being enclosed in an exopolysaccharide matrix (Eps) of microbial and host origin arranged in a three-dimensional (3-D) architecture [2]. Bacterial species in biofilms exhibit cooperation [3], behaving as complex multi-cellular organisms [4]. Eps composition is complex and it may contain polysaccharides, proteins, nucleic acid, lipids, and metals. [5]. The complex array of chemically and functionally diverse biomolecules in the Eps has been termed the matrixome [6], which contributes to the peculiar characteristics of biofilm behavior. According to the National Institutes of Health (NIH), bacterial biofilms are responsible for up to 75% of infectious diseases in humans, as implant-related infections and/or tissue-associated infections [7]. In the European Union and European Economic Area, 8.9 million healthcare-associated infection episodes per year are due to biofilms [8]. These infections are often recurrent and resistant to antibiotic treatments [9][10] due to the particular characteristics of Eps that protect the resident microorganisms from the effects of host immunity or administered antimicrobial drugs [11]. It is of crucial importance nowadays to design or screen anti-biofilm molecules that can effectively minimize and eradicate biofilm-related infections. In this kind of investigation, the use of different microscopy techniques is required to better understand the intimate details of the biofilms’ ultrastructure, their 3-D organization, cell population behavior, and reactions after drug treatments [12]. The development of novel morphological investigation approaches is therefore crucial.

2. Microscopy Techniques Applied to Biofilm Imaging

2.1. Light Microscopy (LM)

Light microscopy (LM) is a basic imaging technique that is useful for providing the visual identification of biofilm presence and also has significant prognostic value [13]. It can be used for quantitative assessment of biofilm biomass, being easy and low cost to perform [14][15]. However, light microscopy has limited magnification and resolution, so it cannot be applied to describe the finest details of biofilm cell morphology or Eps architecture, but it can be coupled with Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) in correlative studies as in [16]. In this study on teeth microflora, light microscopy observation of semi-thin sections from demineralized teeth provided the best overall perspective of the root canal, enabling larger areas to be observed at low magnification. Samples observed with SEM did not show bacteria in dentine tubules, in contrast, when the same samples were demineralized and included in resin, their semi-thin section LM images revealed the presence of bacteria, then TEM images confirmed the LM findings [16].

2.2. Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) allows for the quantitative evaluation of structural parameters as biovolume (cells overall volume in the observation field), thickness, and roughness. Sample 3-D architecture representation and its time-dependent variation (real-time 4-D) can also be achieved [17]. CLSM was used in combination with a fluorescent stain and was successfully applied on different biofilms species [18][19][20][21]. The CLSM resolution level is singe cell dimension and using pathogen-specific probes labeled with different fluorescent dyes (FISH followed by CLSM) as described in [22], identification of a single species in multispecies samples is allowed. With the same approach, interspecies competition assessment as well as interference in-between species were analyzed [23]. In studies assessing drug antimicrobial effects,

CLSM was used, together with specific fluorophores, to discriminate between live or dead bacterial cells, localizing also their spatial distribution [24][25][26][27][28]. CLSM is a method of choice for biofilm visualization and quantification. Unfortunately, CLSM biofilm analysis has limitations due to the use of fluorophores, the existence of a limited number of reporter molecules, and the signal of interest might be hidden by the interference of intrinsic biofilm fluorescence with that of the probe.

2.3. Atomic Force Microscopy (AFM)

Bacteria respond to different mechanical signals [29] like adhesion forces originating during adhesion processes. During these events, bacterial surfaces deform [30], modifying the intra-bilayer pressure profile [31], which, in turn, changes bacterial gene expressions, transforming a planktonically growing cell into a biofilm growing one. Atomic force microscopy (AFM) allows for the quantification of adhesion forces existing among living cells, and between cells and surfaces [32][33]. The knowledge of how adhesion and viscoelasticity can modulate biofilm development may be important in the design of biofilm control strategies. Viscoelastic properties of biofilms influence antimicrobial penetration and removal of biofilm from surfaces and therefore performs a role in their protection against mechanical and chemical challenges [34]. This approach was recently used to demonstrate how amyloid protein production dramatically increases the stiffness of Pseudomonas biofilms [35]. AFM has been applied to obtain insights into biofilm 3-D developmental patterns [36][37][38][39][40][41]. Atomic force microscopy (AFM) allows for the quantification of biofilm biomass in terms of thickness and Eps amount based on height and roughness analyses from AFM images [42][43][44][45][46][47][48][49]. Vantages, disadvantages and application fields of non-electron microscopic techniques are summarized in Table 1.

Table 1. Most widely used non-electron microscopic techniques for biofilm study.

	Light Microscopy	**CLSM**	**AFM**
Pros	Simple protocols Cheap and easy to perform Large investigation area	Allows single cell visualization and 3-D imaging	Nondestructive technique that works under physiological-like conditions, allowing living biofilms qualitative and quantitative assessment with few treatments, sample 3-D structure reconstruction at nanometer scale.
Cons	Low resolution and magnification power, need for sample staining, gross morphological differentiation, finest details not visible	Use of fluorophores, limited number of reporter molecules, intrinsic biofilm fluorescence can interfere with probes fluorescence	Small scan area (max 150 × 150 µm), no image of bacterial cells sidewalls, possible surface damage during imaging due to tip interactions.
Applications	Visualization of biofilm formation and quantitative assessment of its biomass	Assessment of biofilm structural parameters, Biofilm 3D structure, identification and localization of living and death cells	Quantitative biofilm analysis, determination of adhesion forces, biofilm topography, in situ imaging.

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