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Review

Confocal microscopy imaging of the biofilm matrix☆

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ABSTRACT

The extracellular matrix is an integral part of microbial biofilms and an important field of research. Confocal laser scanning microscopy is a valuable tool for the study of biofilms, and in particular of the biofilm matrix, as it allows real-time visualization of fully hydrated, living specimens. Confocal microscopes are held by many research groups, and a number of methods for qualitative and quantitative imaging of the matrix have emerged in recent years. This review provides an overview and a critical discussion of techniques used to visualize different matrix compounds, to determine the concentration of solutes and the diffusive properties of the biofilm matrix.

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Abbreviations: CBM, carbohydrate-binding modules; CLSM, confocal laser scanning microscopy; DDAO, 1,3-dichloro-7-hydroxy-9,9-dimethyl-2(9H)-acridinone; ECM, extracellular matrix; eDNA, extracellular DNA; EPS, extracellular polymeric substances; FLIM, fluorescence lifetime imaging; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; MALDI, matrix-assisted laser desorption ionization; PI, propidium iodide; SECM, scanning electrochemical microscopy; SIMS, secondary ion mass spectrometry; SPT, single particle tracking; STED, stimulated emission depletion microscopy; ThT, thioflavin T; WGA, wheat germ agglutinin.

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1. Introduction

The extracellular matrix of microbial biofilms is a highly complex scaffold, characterized by a multitude of structurally and chemically heterogeneous microenvironments. Its functions are manifold: It provides mechanical stability to the biofilm and protects the microorganisms from desiccation. It can act as a barrier against adverse chemical and biological influences, such as osmotic stress, acid/base challenges, oxygen, antibiotics and antiseptics, the host immune defense, and grazing protozoa. Moreover, it contributes to the sorption and storage of nutrients and trace elements, it is the location of numerous extracellular enzymatic reactions, and it keeps the microorganisms in tight contact to each other to facilitate genetic exchange and bacterial communication. If the biofilm is a microbial city, then the matrix is its infrastructure.

Polysaccharides were long believed to be the main macromolecular constituent of the extracellular matrix, and the abbreviation EPS, today used for extracellular polymeric substances, originally designated extracellular polysaccharides. Today it is well-known that a multitude of different biopolymers, including DNA, proteins, and lipids, i.e. in outer membrane vesicles, contribute to matrix structure and function.

For decades, the cellular components of biofilms held the center of research attention, as the microorganisms are the driving force behind both detrimental and beneficial effects of biofilms. The past ten years witnessed an increased focus on the matrix and its functional interplay with the microbiota. An integrated view on both compartments is necessary to attain in-depth understanding of biofilms and to develop target-oriented strategies for the control of biofilm-related problems.

Confocal laser scanning microscopy (CLSM) is a valuable tool for the study of biofilms, and in particular of the biofilm matrix, as it allows real-time visualization of fully hydrated, living specimens. The past years have brought about several new imaging technologies that improve the spatial resolution of light microscopy, and the Nobel Prize in Chemistry was in 2014 awarded to Eric Betzig, Stefan W Hell and William E. Moerner for their development of super-resolution optical microscopy. Confocal laser scanning microscopes are available in many research laboratories, and consequently, methods based on CLSM have evolved considerably in the past decade to retrieve information about the composition and the properties of the biofilm matrix. The aim of this review is to provide an overview and to discuss the opportunities and challenges of fluorescence labeling techniques that can be used to acquire either qualitative or quantitative information about the biofilm matrix.

2. Qualitative confocal microscopy imaging of matrix components

The functionality of bacterial biofilms is entwined with its microscale structure, as mass transport by diffusion and convection affects chemical gradients that dictate the limits of metabolic activity and the conditions in the microenvironment experienced by individual cells. The physical structure also affects the mechanical stability of the biofilm, and the protective properties of the matrix towards host immune cells and antimicrobial agents. There is currently no fluorescence labeling method available which visualizes the biofilm matrix in general, and this is due to the complex and highly variable composition of the matrix produced by different bacteria and under different environmental conditions. Each matrix component must therefore be stained individually (Table 1).

2.1. Polysaccharide staining

Polysaccharides are often an important part of the biofilm matrix where they contribute to cohesion, retention of water, sorption of organic and inorganic compounds, and protection against biocides and grazing protozoa (for recent reviews, see Arciola et al. (2015) or Flemming and Wingender (Flemming and Wingender, 2010)). Unfortunately a general stain for polysaccharides does not exist, as the chemical structure of matrix polysaccharides differs among different bacteria. Calcofluor white has been used for polysaccharide staining, but it binds only β-1,3 and β-1,4 glucans (Rasconi et al., 2009), which are found in cellulose and chitin but not in the more common matrix polysaccharide poly-β-1,6-N-acetyl glucosamine (Sadovskaya et al., 2005). A better approach is therefore the use of fluorescently labeled lectins, which was pioneered by Neu et al. (2001). Lectins typically recognize specific di- or tri-saccharides. Such oligosaccharides can be present both in the matrix and as glycoconjugates on the cell surface e.g. in the teichoic acids of Gram-positive bacteria and the lipopolysaccharides of Gram-negative bacteria. Glycoconjugates are also highly diverse in structure (Messner et al., 2013), and lectin staining therefore always starts with a large screening of commercial lectins to identify which are able to bind.

A new approach to carbohydrate staining was recently introduced by Nguyen et al. (2014), exploiting the high affinity of carbohydrate-binding modules (CBM): the non-catalytic carbohydrate-binding domain of polysaccharide-degrading enzymes. The authors constructed a green fluorescent (GFP) fusion protein with the carbohydrate-binding module 3 (GFP-CBM3) which has high affinity for cellulose. As a proof of concept, they showed that this new polysaccharide label did not bind planktonic cells, but only to *Escherichia coli* biofilms and flocs

Table 1
Overview of methods for visualizing the components of the biofilm matrix by confocal microscopy.

Target molecule	Staining principle	References
Polysaccharide adhesin	Calcofluor (for β-1,3 and β-1,4 glucans)	Rasconi et al., 2009
	Fluorescently labeled lectins (for various di- or tri-saccharides)	Reviewed by Neu et al., 2001
	GFP fusion protein with carbohydrate-binding modules from polysaccharide-degrading enzymes	Nguyen et al., 2014
Extracellular DNA	Cell impermeant DNA-binding fluorescent stains: TOTO®-1, TO-PRO® 3, PicoGreen®, DDAO, propidium iodide and SYTOX® stains	Specificity of stains evaluated by Okshevsky and Meyer, 2014
Extracellular proteins	Fluorescent stains that bind to all proteins: FilmTracer™ SyPro®	Lawrence et al., 2003, Frank and Patel, 2007
	Fluorescently labeled antibodies with specificity for individual proteins	Berk et al., 2012
	Dye with specificity for amyloid proteins	Kim et al., 2016
Extracellular amyloid	Thioflavin T staining	Ilanchelian and Ramaraj, 2004
	Fluorescently labeled antibodies with specificity for amyloid proteins	Larsen et al., 2007, Kim et al., 2016