Review

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Innovative techniques, sensors, and approaches for imaging biofilms at different scales

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Confocal laser scanning microscopy has become a standard technique for the investigation of hydrated interfacial microbial communities at the microscale. Multiphoton and spinning-disk microscopes provide new options for *in situ* imaging. Progress has been made in imaging structural aspects as well as interactions and processes. Advanced fluorescence techniques such as lifetime imaging and correlation spectroscopy are also available. Newly developed target-specific probes allow investigation of new aspects of microbial communities. Several new laser-based techniques are available including nanoscopy and mesoscale techniques. Nanoscopy techniques offer access to unprecedented resolution of hydrated microbiological samples at the scale of fluorescent gene products and macromolecules. Mesoscale approaches are important to address larger features and statistical issues of microbiological samples. This review presents the state of the art *in situ* biofilm imaging and assesses the pros and cons of laser-based imaging techniques in combination with a variety of sensor types at different scales.

Microbial biofilms

The default mode of growth for microorganisms in nature is that of association with interfaces of any type. Although it is now accepted that microorganisms usually have a life cycle which includes dispersal of differentiated cells [1], microbial communities are frequently found at interfaces as stationary biofilms. They may also occur as mobile bioaggregates, flocs, or granules. Other appearances in nature may be in the form of microbial mats as well as sediment biofilms, aquifer, soil biofilms, or symbiontic plant root biofilms. In industrial systems biofilms may be present as sludges and granules or as biofouling layers. In medicine biofilms are an important issue on tissues as well as on biomaterials including invasive devices and implants. All these biofilm systems are characterized by a diversity of microorganisms and a highly-complex matrix composed of extracellular polymeric substances (EPS). Owing to its complex makeup EPS remain the 'dark matter' of biofilm systems [2]. Although some matrix constituents such as polysaccharides and proteins have been known for decades, the composition and functionality of EPS are recognized as increasingly diverse. Today it is acknowledged that EPS include various types of polysaccharides, a range of different proteins, extracellular nucleic acids, amphiphilic compounds, and extracellular membrane vesicles as well as bacterial refractory compounds [2,3]. The potential role of matrix constituents has been discussed in a still-evolving concept of biofilm matrix functionality [3]. Recognized biofilm matrix functions include: structure, absorption, adhesiveness, repellence, cohesion, activity, surface activity, information, competition, nutrition, locomotion, redox regulation, and conductivity.

It is also accepted that the architecture of biofilm systems may be divided into intracellular and extracellular domains, although for particular features a gradient from the cell surface towards the external space may be present. Further, bacterial cells are not only bags filled with biochemical compounds but are highly organized, containing well-defined internal locales and intracellular organelles [4]. By contrast, the degree of extracellular organization, as random arrangements or specifically linked constituents, remains to be established. To resolve the intracellular and extracellular domains of biofilms, high-resolution microscopy techniques applied under *in situ* conditions are required. In addition, the structure and performance of biofilms at the mesoscale are of interest in natural as well as engineered systems. In this review we discuss biofilm imaging at the micro-, nano-, and meso-scale using laser-based techniques. For this purpose the various types of sensors are considered. Finally, digital image analysis is briefly touched upon.

Microscale: laser scanning microscopy (LSM)

Confocal laser scanning microscopy (CLSM) has revolutionized the structural investigation of hydrated microbiological samples since the early 1990s. Today CLSM represents a standard technique in many laboratories, and some research groups have begun to explore advanced applications (Table 1). The core instrument of any CLSM is a conventional upright or inverted epi-fluorescence microscope which is used for visual examination of the sample. LSM may be divided into confocal or one-photon LSM, and

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Table 1. Major advantages and limitations of laser-based imaging techniques

Laser techniques	Advantages	Limitations	Refs
Confocal laser scanning microscopy (CLSM) – point scanner	Many options for sample mounting (upright/inverted), high-resolution, optical sectioning, multichannel imaging, sequential/simultaneous	Low axial resolution, limitation in laser penetration; fast imaging only with special scanner	See references in Table 1 of [10]; [83]
CLSM – disk scanner	Ideal for motile objects and fast processes; options for other light sources	Low axial resolution, lower light transmission, fixed pinhole, slightly lower resolution	[84]
Two/multiphoton laser scanning microscopy (2PLSM)	Imaging in deep locations; excitation of UV fluorochromes and organic substances	Low axial resolution, limited availability, sometimes two-photon effects	[85]
4Pi microscopy	Nearly-equal resolution in all three dimensions	No routine application; no longer currently manufactured	[86]
Structured illumination microscopy (SIM)	Double resolution compared to CLSM; all fluorochromes can be used	Depth resolution ≈30 μm	[43,44]
Stimulated emission depletion (STED) microscopy	Resolution in the range of 70 nm; scanning instrument similar to CLSM	Depth resolution ≈30 μm; specific fluorochromes only with two-photon STED, more with continuous wave STED, many options with gated STED	[62,87]
Localization/blink microscopy (PALM, STORM, dSTORM, GSDIM)	Resolution under ideal conditions in the range of 40 nm	3D imaging is limited (TIRF-mode, plus few μm with particular approaches); fluorochrome/cocktail combinations to be evaluated for dSTORM and GSDIM	[45–49,64,88,89]
Optical coherence tomography (OCT)	Large area (mm^2) imaging without staining	Signal recorded is a reflection; possible shading effects	[76,90–93]
Ultrasound imaging	Large area (mm^2) imaging, no staining needed	Potential biofilm damage	[77]
Selective plane illumination microscopy (SPIM)	High-resolution imaging of deep locations in cell biology	Need for embedding of sample in gel; so far only a conference poster on biofilms	[80,94]
Photoacoustic tomography (PAT)	Imaging of deep locations in cell biology	Not applied in biofilms	[78]
Scanning laser optical tomography (SLOTy)	Imaging of transmission, reflection, and fluorescence	Chemical clearance required, not applied with biofilms	[79]
Mesolens	High-resolution large-area (mm^2) imaging	Prototype only, not used with biofilms	[82]

multi- or two-photon LSM. Confocal LSM represents the standard technique using laser sources in the visible or UV range. Two-photon LSM is a technique for special applications such as imaging in deep locations without bleaching out-of-focus areas and as an option for excitation of UV fluorochromes. There are also point-scanning laser microscopes for high resolution, and disc-scanning microscopes for imaging motility or fast processes. The laser options for excitation include continuous wave lasers (e.g., gas lasers, laser diodes, and solid-state lasers) as well as pulsed lasers which are tunable [e.g., multiphoton lasers (infrared lasers) and super-continuum light sources (white lasers)]. The LSM can be set up for reflection and fluorescence mode. Emission signals are detected with conventional photomultipliers or new detector types having higher quantum efficiency. Signals are intensities encoded in grey levels from 0–255 (8 bit) or 0–4095 (12 bit). One reason why the LSM (upright) configuration is advantageous for assessment of biological samples is the freedom of mounting the specimen. This comprises a range of devices such as cover well chambers, coverslip chambers, flow cells, as well as ordinary Petri dishes in combination with so-called water immersion or dipping lenses. A relatively new option for mounting and imaging are microfluidic devices which have been employed to study bacterial cells in well-defined microenvironments [5]. Whatever the configuration, the LSM design allows optical sectioning of biological objects resulting in either a 2D highly resolved single section or, in most applications, a series of highly resolved 2D sections at a defined step-size. The many advantages of confocal laser scanning microscopy and also the limitations have been discussed in detail elsewhere (see references in Table 1) [6]. An example of a complex microbial biofilm examined by CLSM is provided to illustrate the potential of the technique (Figure 1).

Resolution in LSM is given by the famous formula of Ernst Abbe which is roughly half the wavelength of light used. Nevertheless, with conventional LSM sub-diffraction resolution can be achieved by using triexciton imaging. Triexciton is a three-photon absorption process of single quantum dots resulting in a reduced point-spread function and thus an improved resolution. Triexciton imaging can be carried out with any LSM equipped with an argon laser source at 488 nm and specific quantum dots conjugated to a probe. This approach was tested with a 655 nm Q-dot–lectin conjugate for imaging bacterial cell envelopes (Figure 2A). Triexciton results in a 1.7-fold improved resolution in *xy* and *z* directions, which is remarkable compared to the so-called nanoscopy techniques described below [7]. It is puzzling why this approach has not found more applications. A completely different approach for improved resolution was suggested on the detector side of the