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TREATMENT

Imaging of oral biofilms

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Abstract

Oral biofilm is a primary determinant of oral health. Because clinical mapping of oral biofilm has been primarily restricted to macroscopic plaque staining techniques, additional means of assessing and quantifying oral biofilm in site at high levels of resolution are currently under development. This study addresses emerging optical imaging modalities for evaluating in vivo oral biofilm noninvasively. Desirable attributes include informing on variables that translate into clinical decision-making guidance to improve diagnosis, better treatment planning and outcomes, ease and speed of use, appropriate cost for the indicated setting, patient-friendly probes, and reliability. In addition, the principles behind optical approaches to imaging and characterizing oral biofilm, as well as their feasibility and applicability for imaging in situ are reviewed. The results include optical images of salivary pellicle and dental plaque including their inner structures. Ultimately, the results of this study can be used to develop clinical methods for preventing and intercepting oral diseases.

Keywords Oral biofilm · Plaque · Noninvasive · Probe · Optical approach · Oral disease

Quick Reference/ Description

Oral biofilm, typically known as dental plaque, is a major factor in the evaluation, establishment and maintenance of oral health. The current clinical methods for examination have limited ability to detect, map and characterize oral biofilms. Therefore, adjunctive imaging techniques for minimally invasive in vivo

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evaluation and quantification of oral biofilms at higher resolution are required for clinical mapping and characterization of the biofilms.

Overview

Imaging modalities	Indications
Confocal laser scanning microscopy (CLSM)	- High-resolution imaging of a biofilm for intra-oral analysis
Two photon and multiphoton microscopy (MPM)	- To image and reveal the microstructure of oral biofilms and calculus - To overcome some of the major disadvantages of conventional CLSM
Atomic force microscopy (AFM)	- To provide microstructural information of the biofilm - Analysis of the electronic properties of a specimen surface at an atomic resolution level - Elucidate the nanoscale morphology of bacteria within biofilms - Association of nano-indentation techniques and AFM can facilitate the detection and characterization of salivary pellicle - To image unstained macromolecular structures in fixed and living cells
Light sheet fluorescence microscopy (LSFM)	- For imaging deep within transparent structures like biofilm or entire organisms
Optical coherence tomography (OCT)	- In vivo non-invasive imaging of the macroscopic features of surface and subsurface tissues - In vivo imaging of the salivary pellicle and the oral biofilm - To overcome the limitations of conventional plaque imaging tools
Optical coherence microscopy (OCM)	- Higher resolution in vivo OCT imaging - To overcome the limitations of conventional plaque imaging tools

Materials/instruments

- Conventional optical microscope
- Intra-oral imaging probe holder
- Extra-oral imaging probe holder
- Optical coherence tomography
- Multiphoton fluorescent microscopy

Procedure

Oral biofilm, also called as dental plaque, is a principal determinant in the assessment and maintenance of oral health. However, clinical mapping and quantification of oral biofilms in situ still remain a challenge. Naked eye visualization and macroscopic plaque staining methods are the primarily available means for clinical mapping of oral biofilm. Therefore, additional imaging approaches for in vivo assessment and quantification of oral biofilms at higher resolution in a minimally invasive manner are required for mapping and characterization of these biofilms.

Taking into consideration this need for adjunctive imaging techniques, several optical imaging modalities have emerged over recent years for non-invasive in vivo assessment of oral biofilm (Table 1). The desirable features of these novel imaging techniques are

- Information on variables for providing guidance during clinical decision-making to improve diagnosis
- Improved treatment planning and outcomes
- Reliability
- Patient-friendly probes
- Ease and speed of use
- Reasonable cost for the indicated setting

Oral biofilm and its clinical implications

Oral biofilms affect almost all aspects of our daily lives. It shows rapid colonization over oral structures with a slow increase in thickness over time. The thickness usually approximates 20–30 μm after 3 days. Initially, the acquired salivary pellicle is formed that can influence the interactions occurring at the tooth surface and oral cavity interface.

Oral biofilms are seen as potential health hazards and are commonly implicated in the etiopathogenesis of various oral diseases like dental caries and periodontal disease. They also consist of numerous elements that are vital for the oral ecology and tissue surface characteristics. Thorough understanding of this entity is necessary for identification and prevention of several oral conditions due to the complex nature of oral biofilms.

The initial primary characterization of dental plaque was done by van Leeuwenhoek in 1683 using a microscope. In spite of the technological advances over time, the ability of accurately evaluating dental plaque is limited because of its dynamic nature and the physical limitations on intraoral utilization of microscopes. Recent development of miniaturized, multimodal high-resolution imaging technology has now permitted intraoral analysis of oral biofilms.

Table 1 Techniques for the evaluation of oral biofilms

Existing techniques	Currently available minimally invasive imaging techniques	Newer imaging techniques
Clinical examination	Plaque staining combined with image analysis techniques	Optical coherence tomography
Clinical indices	Confocal laser scanning microscopy	Optical coherence microscopy
Plaque staining approaches	Atomic force microscopy	Multiphoton fluorescence microscopy
		Light sheet fluorescence microscopy

Practical considerations for imaging modalities

The formation of an oral biofilm needs firm attachment of the salivary pellicle to the tooth surface. The pellicle begins to form immediately after tooth brushing and provides a foundation for the development of dental plaque. Hence, the ability to effectively image oral biofilms is critical for examining the effects of the preventive and therapeutic measures employed for biofilm control. Bacteria in oral biofilms operate as an organized, coordinated and metabolically integrated microbial community. The features of this microbial community differ considerably from the sum of its components requiring multimodality approaches to map the complex features of the biofilm.

The multimodality approaches usually combine traditional techniques like bioassays with the capabilities of innovative imaging. The ability of a device to consecutively image the exact same location in the oral cavity over time (image colocalization) is also an important factor for relevant intra-oral imaging. Image colocalization can be achieved using imaging jigs or probe holders that are customized for a specific site and use (Fig. 1).

The image colocalization devices can be used for the quantification of the oral biofilms at specific time points and locations. These tools can also be used to assess the effects of certain preventive or treatment measures that are appropriate for biofilm-related diseases such as caries, periodontal diseases and peri-implantitis. Nanoparticles and advanced dyes can also be used to improve the selectivity and resolution of in vivo imaging.

Novel imaging modalities of oral biofilms

Confocal laser scanning microscopy

The use of confocal laser scanning microscopy (CLSM) for high-resolution imaging of biofilms is prevalent. In vivo imaging with CLSM has become possible in recent years. CLSM is based on the use of a conventional optical microscope along with a laser beam that is focused onto the specimen. It is useful for the identification of

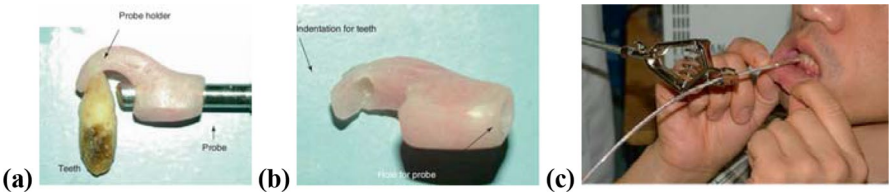

Fig. 1 Prototype intra-oral imaging probe holder. **a** The probe location is accurately controlled by its placement through a slot within the probe body. **b** The custom fabricated probe body is reproducibly fixated through a custom groove fitting onto an adjacent tooth. **c** Prototype extra-oral imaging probe holder. To ensure reproducible re-imaging at exactly the same location during multiple imaging events and to minimize movement artifacts, a multi-joint imaging probe holder for OCT imaging was fabricated. (Figure: courtesy of J.H.B.)

specific proteins after being labelled with markers or antibodies. CLSM offers various benefits but also has several drawbacks (Table 2).

Adjunctive staining techniques like fluorescence in situ hybridization (FISH) can be implemented along with CLSM to enhance its effectiveness. A few of these staining techniques can also be performed in vivo. The FISH techniques have been utilized in combination with CLSM for imaging natural heterogeneous biofilms present on fixed orthodontic appliances. The implementation of CLSM is prevalent in various medical fields for biofilm analysis.

To overcome the drawbacks of CLSM, several alternative approaches like spinning disc confocal laser scanning microscopy were attempted. In spite of these attempts, the use of CLSM for intraoral imagining of a natural oral biofilm has not been successful without additional staining to enhance contrast.

Two photon and multiphoton microscopy

Multiphoton microscopy (MPM) was first introduced by Winfried Denk and James Strickler in 1990 to overcome some of the major pitfalls of conventional CLSM. It is a fluorescence imaging technique that utilizes near-infrared excitation light to generate fluorescence in selective materials or tissue components. Multiphoton fluorescence microscopy can also excite fluorescent dyes in tissue models and tissue explants.

MPM can effectively demonstrate the microstructure of calculus and oral biofilms (Figs. 2, 3). It also has the ability to characterize and monitor oral biofilms within its natural environment. The main advantage of MPM is its greater imaging depth (> 100 μm). Photodamage and phototoxicity over a certain threshold is a major drawback of MPM.

Atomic force microscopy

Atomic force microscopy (AFM), also called as scanning probe microscopy, was described in 1986 by Binnig et al. The AFM can reveal microstructural information

Table 2 Advantages and disadvantages of confocal laser scanning microscopy

Advantages	Disadvantages
Ability to control the depth of field	Common lasers can produce a limited number of excitation wavelengths due to very narrow bands
Reduction or complete elimination of the unnecessary background data away from the focal plane	Generation of excitation wavelengths is expensive
Capability to obtain thin optical sections of 0.5–1.5 μm from thick samples	Limited speed because of point-to-point imaging
Very high resolution (1 μm)	Limited depth of imaging (100 μm)
	Photodamage to living cells and tissues due to toxic nature of high-intensity laser radiation
	High cost of device operation

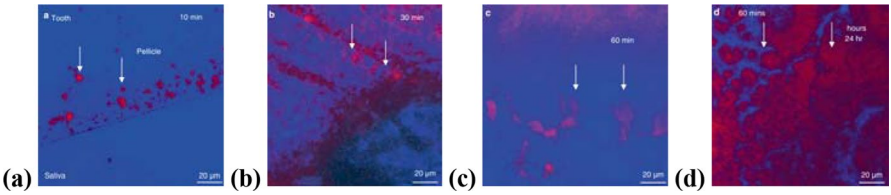

Fig. 2 Progressive growth and development of pellicle (white arrows) on the same tooth sample. Top view of 3D-reconstructed MPM images at progressive saliva incubation time points. **a** 10-min incubation. **b** 30-min incubation. **c** 60-min incubation. Blue signal originates from tooth and saliva, pink and red signals from salivary pellicle. Over time, the number and diameter of pellicle islands gradually increase. **d** 24-h incubation. Thick layer of biofilm over the pellicle. (Figure: courtesy of P.W.S)

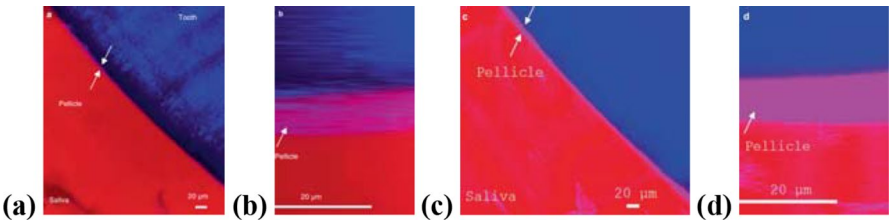

Fig. 3 MPM images showing pellicle growth and development over time (white arrow). **a** Tooth incubated in saliva for 30 min. Top view of 3D-reconstructed images. **b** Optically sectioned lateral view of tooth incubated in saliva. Coarse layers and voids in the pellicle layer are clearly visible. **c** Same tooth incubated in saliva for 120 min. Increased thickness of pellicle layer is visible. **d** Inner structure of pellicle layer is denser and more compact than at earlier time point. (Figure: courtesy of P.W.S)

and was used to show that the dental pellicle is a stiff and viscoelastic solid having a dense undulating morphology. It can analyze the electronic properties of the surface of a specimen at an atomic resolution level. Ultra-high atomic level of resolution is a major benefit of AFM.

The technology of AFM can help to interpret the nanoscale morphology of bacteria within oral biofilms. Additionally, detection and characterization of the salivary pellicle is facilitated by AFM in association with other nano-indentation techniques. AFM has the capacity to image unstained macromolecular structures in living and fixed cells. It can only be used to image the surfaces of cell membranes in biological test samples as the images from AFM are collected by measuring the forces on a sharp tip that are developed by its closeness to the sample surface. This surface imaging is the main limitation of AFM.

Light sheet fluorescence microscopy

Light sheet fluorescence microscopy (LSFM) is also known as selective plane illumination microscopy or ultramicroscopy. It is a fluorescent light microscope imaging approach that was first introduced by Richard Adolf Zsigmondy and Henry Siedentopf in 1903. LSFM is different from CLSM as it does not need a spatial pinhole for elimination of the out of focus light.

LSFM operates as a combination of a non-destructive microtome and a microscope that utilizes a plane of light for optical sectioning and viewing of specimens with subcellular resolution. This optical sectioning and high-resolution imaging are specific advantages of LSFM. It can be used to image deep within transparent structures like entire organisms or biofilms. In comparison to confocal, multiphoton or wide-field fluorescence microscopy, phototoxicity and photobleaching of samples are minimized as the specimens are subjected to only a thin plane of light.

LSFM can also be utilized for three-dimensional imaging. It has the ability to image specimens up to a thickness of 1 cm. Commonly, a LSFM sample is prepared by embedding the sample in an agarose cylinder. Therefore, in vivo application of LSFM for imaging oral biofilms and the oral cavity in general is lacking due to this requirement of mounting the samples before imaging.

The main limitation of most of the above-mentioned imaging modalities is the lack of direct application in the oral cavity. The nature of an oral biofilm is complex and dynamic within its natural environment. Its properties immediately begin to change when it is removed from the oral cavity. Uniform distribution of the bacteria within the oral biofilm is not observed. Bacterial microcolonies are present in various sizes and shapes. Therefore, methods like liquid chromatography–mass spectrometry have been used for analyzing the proteome of salivary pellicle proteins to map the presence and properties of bacteria.

The use of these adjunctive methods facilitates the identification of several organisms such as *Streptococcus mutans*, *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum* and *Candida albicans* in the salivary pellicle. The various elements required for ideal in vivo imaging of oral biofilms are

- Direct application in the oral cavity
- No requirement of physical or chemical preparation of the biofilm
- No to minimal effect on the cells, bacteria and tissues
- Availability of standardized images for quantification and comparison

Optical coherence tomography

Optical coherence tomography (OCT) facilitates the imaging of near-surface abnormalities of complex tissues in a minimally invasive manner. It is a high-resolution optical imaging method that follows principles similar to that of ultrasonic imaging. In OCT, infrared light waves are utilized that reflect off from the internal microstructure of biological tissues. This results in the construction of cross-sectional images of tissues in real time and at near-histologic resolution. OCT facilitates non-invasive in vivo imaging of the macroscopic features of the surface and subsurface tissues.

Three-dimensional images can be created by combining the two-dimensional images. Sectioning and manipulation of these images is possible in several ways. Hand-held probes are used to acquire in vivo OCT images; hence, making them useful in clinical settings. OCT overcomes several drawbacks of conventional plaque imaging tools and is best suited for in vivo imaging of oral biofilms (Fig. 4). It can also be used to effectively image the salivary pellicle and has the ability to characterize and monitor oral biofilms within their natural environment (Fig. 5).

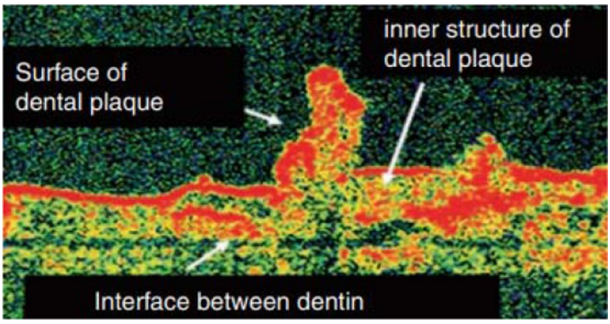

Fig. 4 High-resolution in vivo OCT image showing vertical optical section of human subgingival dental plaque on the dentin of the tooth root. High-resolution mapping image of outer surface of dental plaque, inner structure, and interface between dentin and the base of plaque are also distinguishable. (Figure: courtesy of J.H.B)

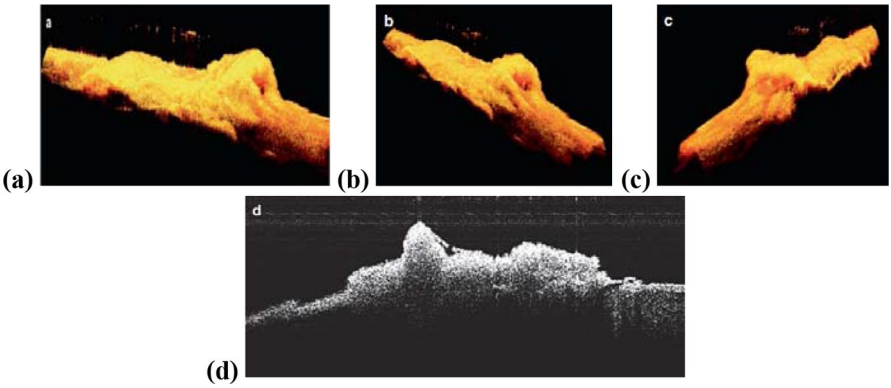

Fig. 5 In vivo, in situ OCT images of human dental calculus on the lingual surface of lower anterior incisors. **a**-**c** 3D-reconstructed OCT images. **d** 2-D raw image from optical sectioning of a 3D image (Figure: courtesy of J.H.B.)

Optical coherence microscopy

The use of optical coherence microscopy (OCM) is beneficial for higher resolution in vivo OCT imaging (Fig. 6). In vivo multiphoton microscopy can be combined with OCM or OCT to develop fluorescence with several wavelengths of light and achieve high-resolution imaging of specific tissue components (Fig. 7). Non-invasive imaging of physiological, pathological and preventive processes is possible with combined OCT and OCM.

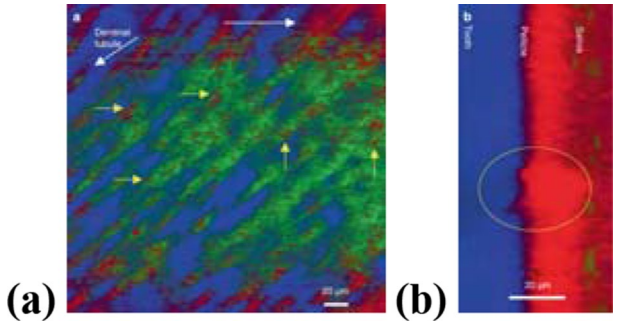

Fig. 6 Low- and high-resolution OCM images using fluorescein stain showing the pellicle after 120-min incubation in saliva. **a** Top view of 3D-reconstructed image. The fluorescein is seen as a pink stain at the saliva/tooth interface (arrows). **b** Optically sectioned lateral view of 3D-reconstructed image showing pellicle presence and structure in pink. The white circle indicates an area where the attachment between the pellicle and the underlying tooth is evident. (Figure: courtesy of P.W.S)

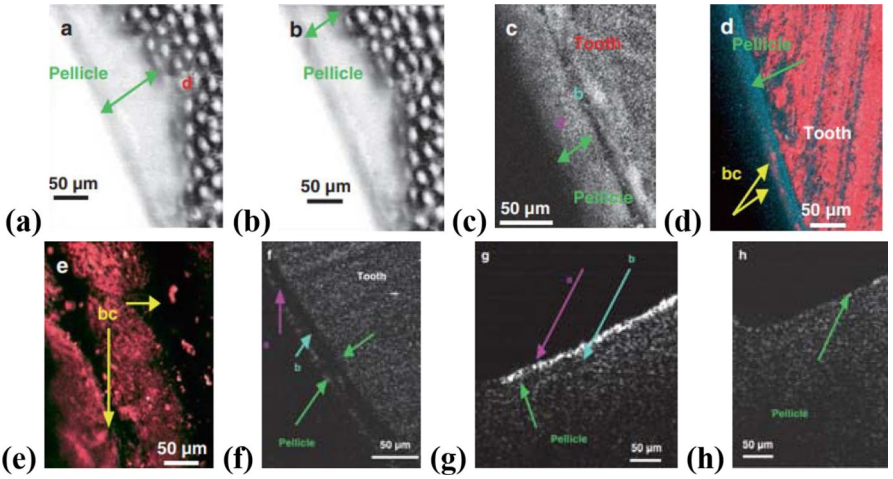

Fig. 7 **a**, **b** OCT images of dentinal surface and the overlying tooth pellicle and cross-sectional images of dentinal tubules (d). **c** MPM gray scale image showing outer (a) and inner (b) pellicle layers. **d**, **e** MPM fluorescence images showing pellicle and bacterial clusters (bc). **f** MPM gray scale image of tooth after rinsing with a mouth rinse containing 21.6% alcohol. The pellicle remains unchanged. **g**, **h** OCT images before (g) and after (h) wiping the tooth with 99% isopropyl rubbing alcohol for 1 min. Very little pellicle remains afterwards. (Figure: courtesy of J.H.B)

Pitfalls and complications

- Successful imaging of natural oral biofilms in the oral cavity using CLSM without additional staining to enhance contrast is still lacking.
- Multiphoton microscopy can cause impaired cellular reproduction, oxidative stress, giant cell formation and apoptosis-like cell death.
- Direct visualization of the cell interior is not possible with AFM.

- LSFM requires mounting of the samples before imaging.

Further reading

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