Imaging Oral Biofilm and Plaque

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Abstract

Oral biofilm is a primary determinant of oral health, yet our ability to detect, map, and characterize it in vivo remains extremely limited. Moreover, there exists an as yet unmet but pressing need for characterizing its properties and response to prevention and intervention measures. Because clinical mapping of oral biofilm has been primarily restricted to macroscopic plaque staining techniques combined with naked eye visualization, additional means of assessing and quantifying oral biofilm in situ at high levels of resolution are currently under development. This chapter 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 this chapter, the principles behind optical approaches to imaging and characterizing oral biofilm, as well as their feasibility and applicability for imaging in situ are reviewed.

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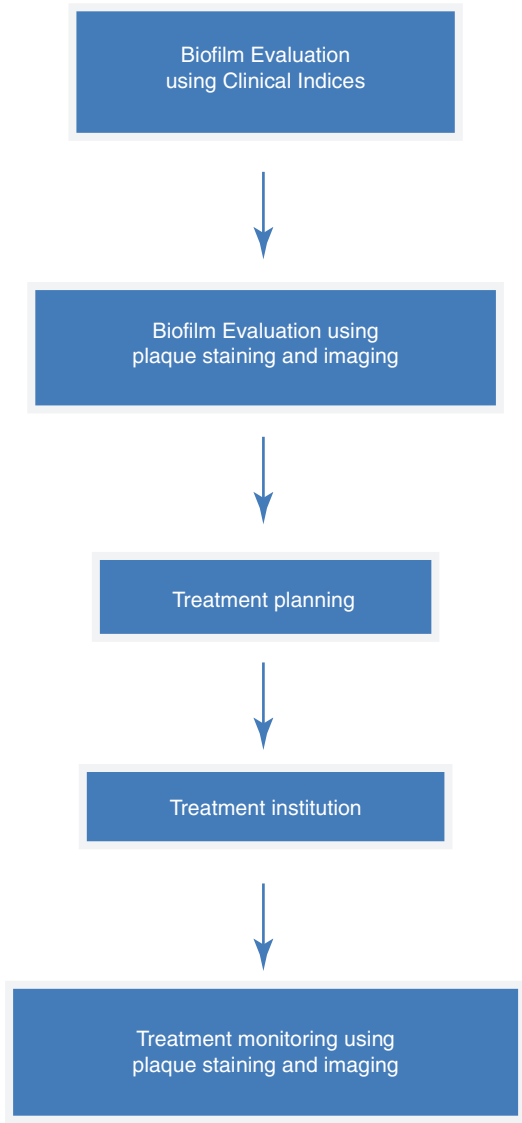
Background



Biofilm affects all aspects of our daily lives and is seminal in establishing, maintaining, and evaluating oral health [1]. Oral biofilm is typically referred to as dental plaque. It colonizes oral structures rapidly, and its thickness increases slowly with time. Although the literature reports a wide range of values, typically its thickness would approximate 20–30 μm after 3 days [2]. Initially, selective adsorption of salivary biopolymers on the enamel surface leads to the formation of the acquired salivary pellicle [3]. The adsorbed layer is a dynamic biofilm that can affect interactions at the interface between tooth surface and oral cavity [3–7]. Much research has been reported on the potential health hazards posed by biofilm [8], and microbial biofilms are implicated in the etiopathogenesis of many oral conditions including caries and periodontal disease [2]. Biofilms can have undesirable effects, for example, when they colonize medical and dental implants, but they can also be harnessed for beneficial purposes, such as when they are used for waste treatment [9]. Oral biofilm contains a multitude of factors important to oral microbial ecology and tissue surface properties. For example, the morphology of the oral biofilm layer appears to affect bacterial binding to the tooth surface [10, 11]. Because of this ambiguity and complexity of oral biofilm, understanding oral biofilm correctly is an essential process to reveal and prevent oral diseases (Table 1).

The first rudimentary characterization of dental plaque or biofilm was performed by van Leeuwenhoek with a microscope in 1683 [12]. However, despite enormous technological advances since that time, our ability to evaluate oral plaque accurately remains very limited due to the instability of the oral biofilm and the physical limitations on the use of microscopes within the oral cavity. It is only the recent development of miniaturized, multimodal high-resolution imaging technology that has begun to permit intra-oral analysis of oral biofilm (Table 2).

Table 1 Overview of chapter content: techniques for oral diagnosis

Existing techniques	Available minimally invasive methods	New imaging methods
Clinical examination; Clinical indices; Plaque staining	Plaque staining combined with image analysis techniques; Confocal laser scanning microscopy; Atomic force microscopy	Optical coherence tomography; Optical coherence microscopy; Multiphoton microscopy; Light sheet fluorescence microscopy

Table 2 Conventional imaging approaches for oral biofilm

Technology	Advantages	Disadvantages
Confocal laser scanning microscopy (CLSM)	Very high resolution (1 μm)	Limited imaging depth (100 μm) Photo damage to sample
Multiphoton fluorescence microscopy (MPM)	Greater imaging depth than CLSM (>100 μm)	Photo damage over threshold
Atomic force microscopy (AFM)	Ultra-high atomic level resolution	Surface imaging only
Light sheet fluorescence microscopy (LSFM)	Optical sectioning and high-resolution imaging Imaging depth to 1 cm	Samples must be mounted

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) have been used widely for high-resolution imaging of biofilm, and in recent years in vivo imaging has become possible [13–17]. This technology is based on a conventional optical microscope but instead of a lamp, a laser beam is focused onto the sample. CLSM offers several advantages over conventional wide-field optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane, and the capability to collect serial thin optical sections (0.5–1.5 μm) from thick specimens (ranging up to 50 μm or more) [18]. But CLSM has limitations, such as the limited number of excitation wavelengths available with common lasers which occur over very narrow bands and are expensive to produce [19], limited speed due to point-by-point imaging, the harmful nature of high-intensity laser irradiation to living cells and tissues that can cause photo damage [20], and the high cost of operation [21]. Its effectiveness can be expanded using staining techniques including fluorescence in situ hybridization (FISH), some of which can be used in vivo. For example, FISH techniques combined with CLSM have been used to image natural heterogeneous biofilm on fixed orthodontic appliances [22]. Indeed, CLSM has been used in various medical fields including dentistry, otorhinolaryngology, and obstetrics [23] to evaluate biofilm, and many attempts including spinning disc confocal laser scanning microscopy have been made to overcome the identified disadvantages [24]. Despite these numerous efforts, natural oral biofilm in the oral cavity has not successfully been imaged using CLSM without staining of some sort to enhance contrast. Typically, oral biofilm studies using CLSM have been performed in vitro or using a variety of biofilm growth media including discs in the oral cavity [25–27]. In vitro oral biofilm models tend to involve limited numbers of species, and they are created under artificial conditions that still cannot adequately reflect the physiological situation in the mouth [28–30].

Two Photon and Multiphoton Microscopy

First described in 1990 by Winfried Denk and James Strickler [31, 32], multiphoton (two or three photon) microscopy was developed to overcome some of the seminal disadvantages of conventional CLSM. This fluorescence imaging technique uses near-infrared excitation light to elicit fluorescence in selective tissue components or materials. It can also excite fluorescent dyes in tissue explants and in tissue or animal models [33]. Multiphoton fluorescence microscopy (MPM) also has been used to image oral biofilm and calculus [34, 35], whose microstructure it reveals very effectively (Figs. 1 and 2). However, similar to CSLM, although to a somewhat lesser degree, above certain intensities MPM can lead to impaired cellular reproduction, formation of giant cells, oxidative stress, and apoptosis-like cell death [36, 37].

Atomic Force Microscopy

Also known as scanning probe microscopy (SPM), atomic force microscopy was introduced by Binnig et al. in 1986 [38]. Using this technol-

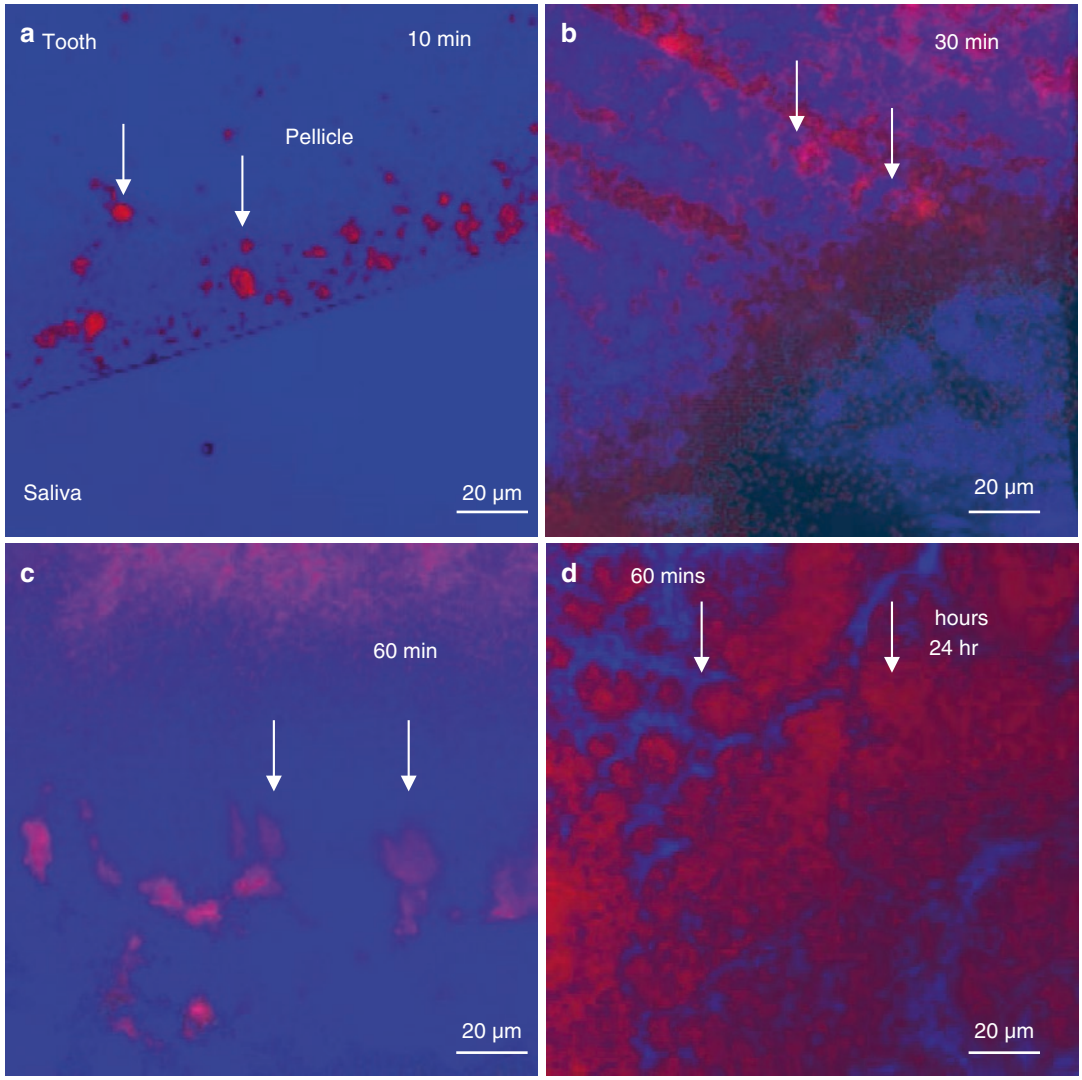


Fig. 1 Progressive growth and development of pellicle (white arrows) on the same tooth sample. Top view of 3D-reconstructed MPM images at progressive saliva incubation time points. (**a**) 10-min incubation. (**b**) 30-min incubation. (**c**) 60-min incubation. Blue signal originates from tooth and saliva, 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 covers the pellicle. (Figure Courtesy P.W.S)

ogy, researchers were able to establish that the dental pellicle is a stiff, viscoelastic solid with a dense undulating morphology [39]. In addition to providing microstructural information, AFM can also analyze the electronic properties of a sample surface at an atomic resolution level [40]. Using this technology, the nanoscale morphology of bacteria within biofilms can be elucidated [41]. For example, S. mutans *within a biofilm* was characterized at a nanoscale level of resolution [42]. Moreover, associated nano-indentation techniques uniquely permit the detection and characterization of salivary pellicle [43]. While CLSM is useful for identifying specific proteins subsequent to labelling with markers or antibodies, AFM can be used to image unstained macromolecular structures in fixed and living cells [44]. However, because AFM images are obtained by

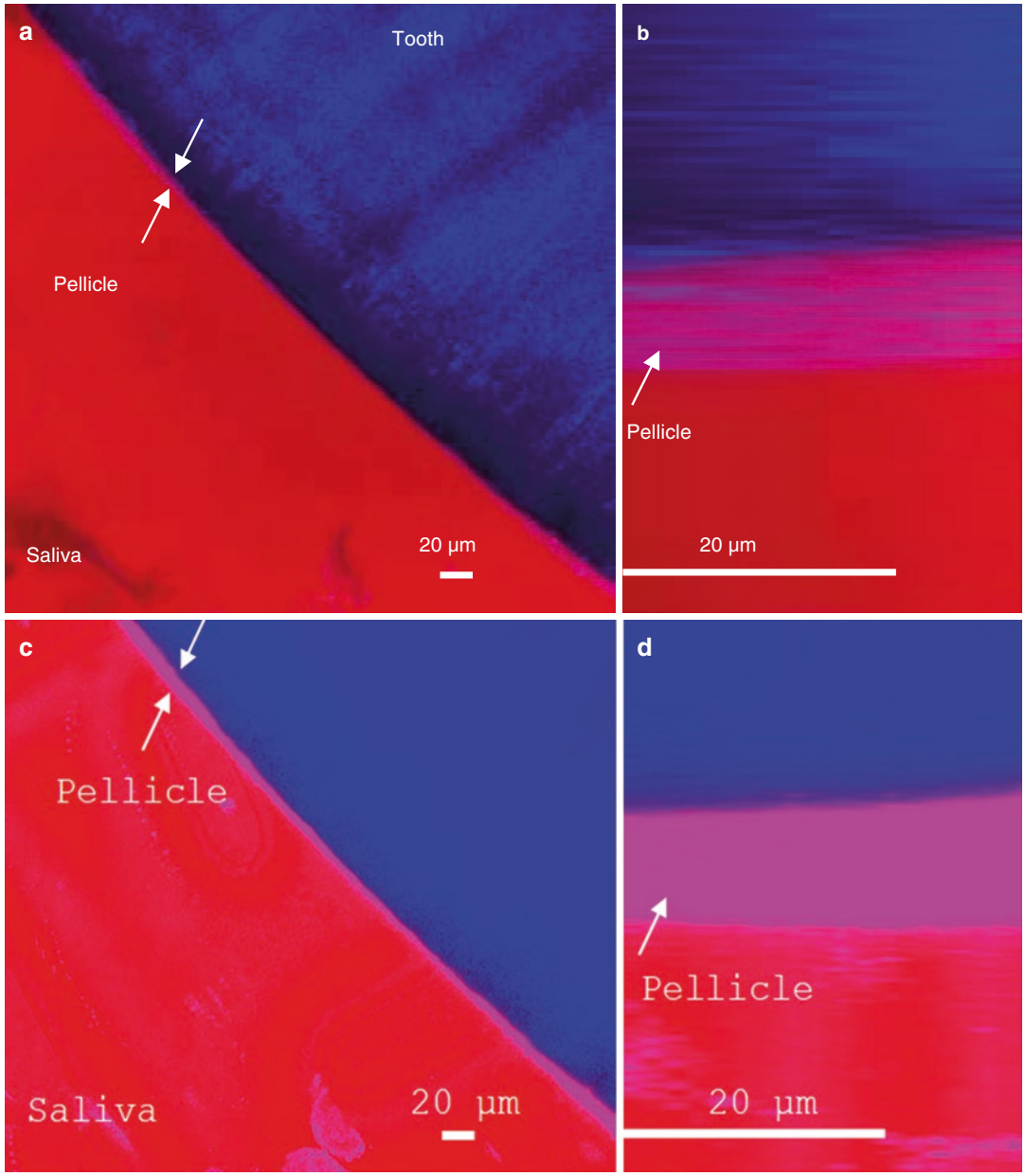


Fig. 2 MPM images showing pellicle growth and development over time (white arrow). (**a**) Tooth incubated in saliva for 30 min. Top view of 3D-reconstructed images. (**b**) Optically sectioned lateral view of (**a**). Coarse layers and voids in the pellicle layer are clearly visible. (**c**) Same tooth incubated in saliva for 120 min. Increased thickness of pellicle layer is visible. (**d**) Inner structure of pellicle layer is more dense and compact than at earlier time point. (Figure Courtesy P.W.S)

measuring forces on a sharp tip that are created by its proximity to the sample surface [38, 45], this technology can only image the cell membrane surfaces of biological samples and cannot directly visualize the interior of the cell [38, 45].

Light Sheet Fluorescence Microscopy

Light sheet fluorescence microscopy (LSFM) also called selective plane illumination micros-

copy (SPIM) or ultramicroscopy is a fluorescent light microscope imaging technique which is differentiated from CLSM in that it does not require a spatial pinhole to eliminate out of focus light. It was first described by Henry Siedentopf and Richard Adolf Zsigmondy in 1903, who were awarded the Nobel Prize for this work in 1925 [46]. LSFM functions as a combined non-destructive microtome and microscope that uses a plane of light to optically section and view samples with subcellular resolution. This technique is well suited for imaging deep within transparent structures such as biofilm or entire organisms, and because samples are exposed to only a thin plane of light, specimen photobleaching and phototoxicity are minimized compared to wide-field fluorescence, confocal, or multi-photon microscopy. Three-dimensional imaging is possible using LSFM [47]. Compared with confocal and two-photon microscopy, LSFM is able to image samples up to a thickness of 1 cm [37]. Despite its many advantages, LSFM applications to oral biofilm imaging and the oral cavity in general are still lacking, primarily because samples must be mounted prior to imaging. A common method of LSFM sample preparation is sample embedding in an agarose cylinder [47], which is clearly not suitable for biofilm imaging.

Need for New Technology

The greatest disadvantage of many of the aforementioned imaging devices is that they cannot be applied directly in the oral cavity. Biofilm within its oral environment is complex and dynamic. From the moment that it is extracted from the oral cavity, its properties change. Even within the oral biofilm itself, the bacteria are not uniformly distributed. Microcolonies aggregate in various shapes and size. It is for this reason that researchers have turned to techniques such as liquid chromatography-mass spectrometry to analyze the proteome of salivary pellicle proteins for mapping bacterial presence and properties [1]. This allows the identification of various organisms such as *Actinomyces naeslundii, Steprococcus oralis, Streptococcus mutans, Fusobacterium nucleatum, Veillonella dispar, Candida albicans* in the salivary pellicle [48]. The bacterial species diversity in the oral cavity indicate is estimated at approximately 500 species [49] (Table 3).

Table 3 The required elements for imaging oral biofilm in situ

Direct application to the oral cavity
No physical or chemical preparation of the biofilm required
None or minimal effects on bacteria and cells
Standardized images available for purposes of quantification and comparison

Optical Coherence Tomography

Optical coherence tomography (OCT) is a high-resolution optical technique that permits minimally invasive imaging of near-surface abnormalities in complex tissue. OCT combines principles similar to those of ultrasonic imaging. Whereas ultrasound produces images from back-scattered sound "echoes," OCT uses infrared light waves that reflect off the internal microstructure within the biological tissues. Cross-sectional images of tissues are constructed in real time, at near-histologic resolution. This permits in vivo noninvasive imaging of the macroscopic characteristics of surface and subsurface tissues. Two-dimensional images may be combined to generate 3D images that can be sectional and manipulated in many ways. In vivo OCT images are acquired in seconds or less using a handheld probe; therefore, they can be used in the clinical setting [26]. Higher resolution in vivo OCT imaging is possible by using optical coherence microscopy (OCM) [50, 51].

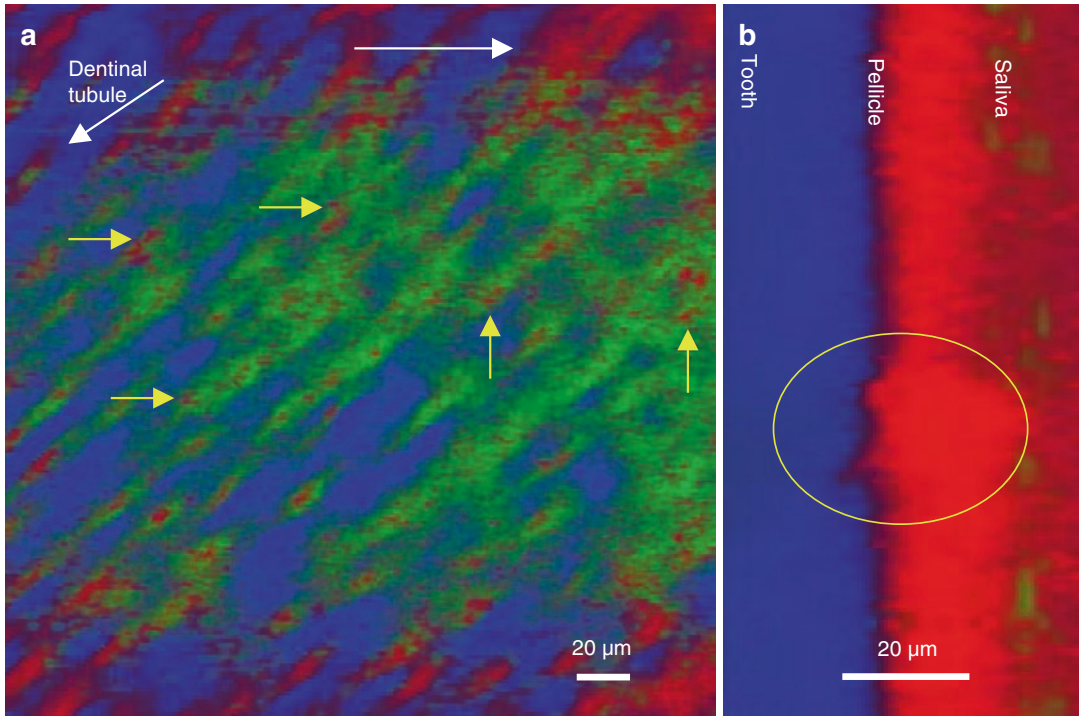


Fig. 3 Low- and high-resolution OCM images using fluorescein stain showing the pellicle after 120-min incubation in saliva. (**a**) Top view of 3D-reconstructed image. The fluorescein is seen as a pink stain at the saliva/tooth interface (arrows). (**b**) Optically sectioned lateral view of 3D-reconstructed 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 P.W.S)

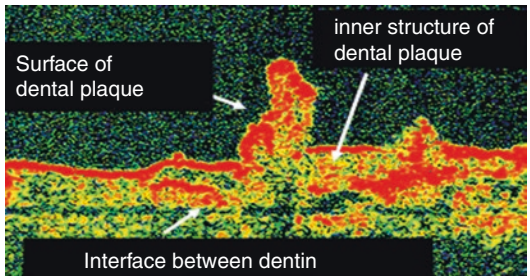


Fig. 4 High-resolution in vivo OCT image showing a vertical optical section of human subgingival dental plaque on the dentin surface of the tooth root. As well as mapping high-resolution image of outer surface of dental plaque, the inner structure, and interface between dentin and dental plaque (the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

OCT or OCM can be combined with in vivo multiphoton microscopy (MPM), generating high-resolution imaging of specific tissue components and fluorescence using many wavelengths of light [52]. Using combined OCT and OCM, noninvasive imaging of physiological, pathological, and preventive processes becomes possible (Fig. 3). Thus, OCT is well suited for in vivo oral biofilm imaging, overcoming many of the limitations of conventional plaque imaging tools. Figure 4 shows an OCT image of undisturbed dental plaque on an extracted human molar (Fig. 4). The salivary pellicle can also be imaged effectively (Fig. 5).

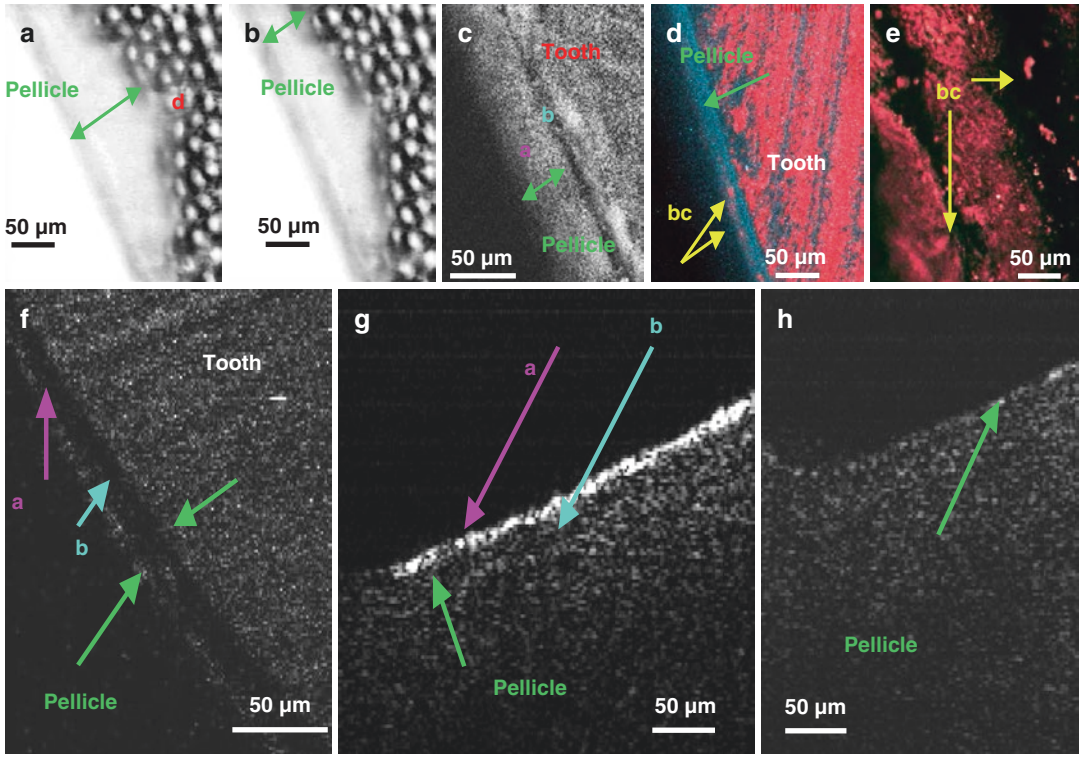


Fig. 5 (**a**, **b**) OCT images of dentinal surface and the overlying tooth pellicle and cross-sectional images of dentinal tubules (d). (**c**) MPM gray scale image showing outer (**a**) and inner (**b**) pellicle layers; (**d**, **e**) MPM fluorescence images showing pellicle and bacterial clusters (bc); (**f**) MPM gray scale image of tooth after rinsing with a commercial 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 (**h**). (Figure Courtesy J.H.B)

Practical Considerations

Biofilm formation requires the firm attachment of salivary glycoproteins or salivary pellicle to the tooth surface. This salivary pellicle forms immediately after tooth brushing [53], and it provides the basis for subsequent development of dental plaque. The ability to image oral biofilm is crucial for analyzing the effects of various preventive and interventional approaches to biofilm control [54]. However, quantitative biofilm imaging is challenging due to the complex, heterogeneous, dynamic properties of biofilms [55]. Oral bacteria in plaque do not exist as independent entities but function as a coordinated, spatially organized and fully metabolically integrated microbial community, the properties of which differ considerably from the sum of the component species [56]. Thus, multimodality techniques that combine traditional approaches (such as bioassays) with innovative imaging capabilities can provide the multi-factorial information to map out the complex properties of biofilm structures [57].

Another important factor for ensuring the relevance of intra-oral imaging is image co-localization, or the ability to image consecutively at exactly the same location in the mouth over time. This can be accomplished through various imaging jigs or probe holders tailored to specific site and use (Figs. 6 and 7). Using such devices, biofilm can be quantified at specific time points, locations, or to evaluate specific preventive or interventional approaches relevant to biofilm-related conditions including dental caries, periodontal diseases, and peri-implantitis (Fig. 8). Figure 9 shows the ability of standardized,

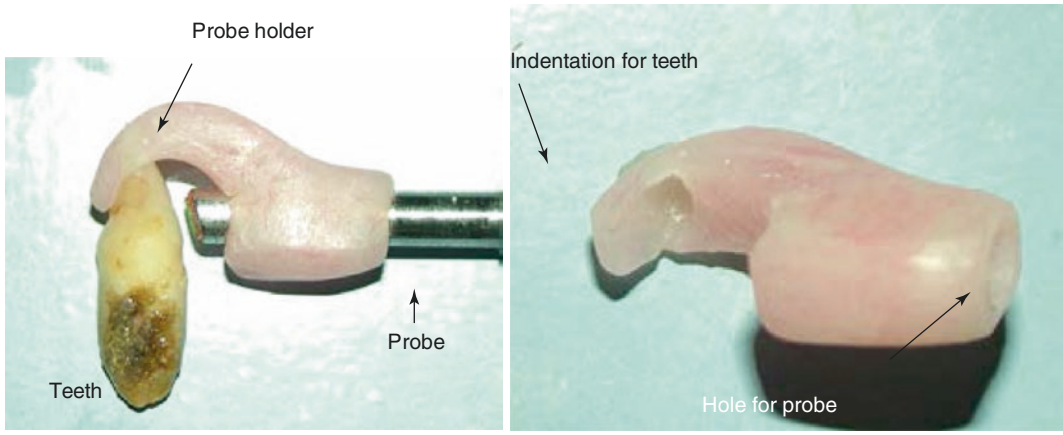


Fig. 6 Prototype intra-oral imaging probe holder. The probe location is accurately controlled by its placement through a slot within the probe body. The custom-fabricated probe body is reproducibly fixated through a custom groove fitting onto an adjacent tooth (Figure Courtesy J.H.B.)

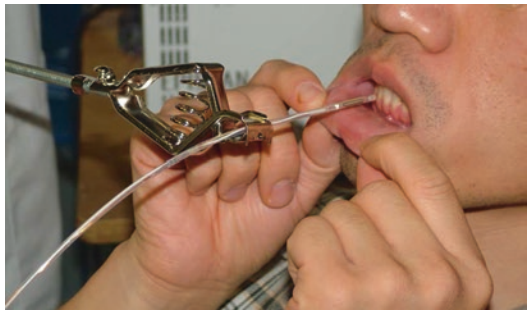


Fig. 7 Prototype extra-oral imaging probe holder. To ensure reproducible re-imaging at exactly the same location during multiple imaging events and to minimize movement artifacts, a multi-joint imaging probe holder for OCT imaging was fabricated. In the future, this multi-joints holder can be replaced with a robotic arm for automatic programmed imaging localization (Figure Courtesy J.H.B.)

co-localized OCM and MPM imaging techniques to map and quantify the effects of various anti-plaque agents [58, 59]. In vivo imaging resolution and selectivity can be further enhanced by the use of advanced dyes and nanoparticles. A preliminary study demonstrated the successful use of 15 nm and 18 nm diameter gold nanoparticles to visualize early pellicle development after tooth cleaning (Fig. 10).

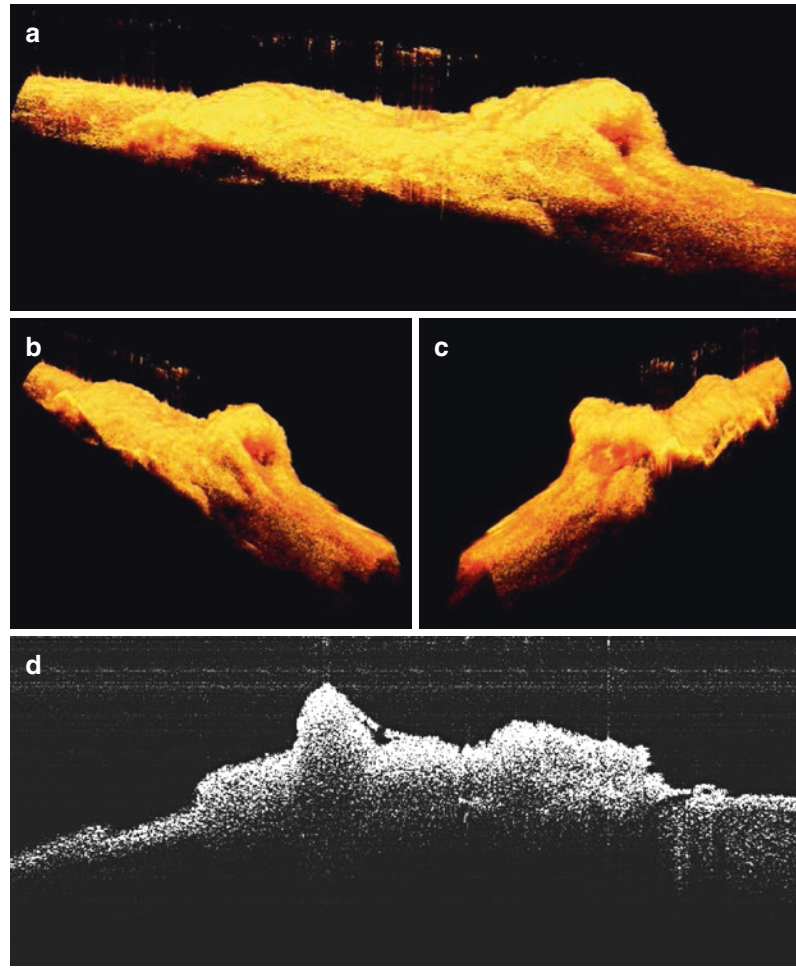


Fig. 8 In vivo, in situ OCT images of human dental calculus on the lingual surface of the lower anterior incisors. (**a**–**c**) 3D-reconstructed OCT images. (**d**) 2 D raw image prepared by optical sectioning of a 3D image (Figure Courtesy J.H.B.)

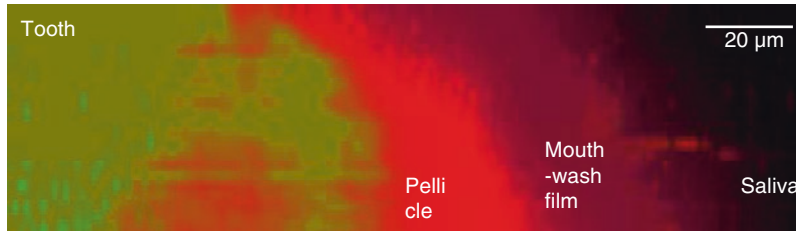


Fig. 9 Lateral 3D OCM image with superimposed MPM image of a tooth after rinsing with mouthwash (Listerine®). The tooth surface (green), overlying pellicle (red), film of mouthwash (purple), and saliva ((dark) brown) are all visible (Figure Courtesy J.H.B.)

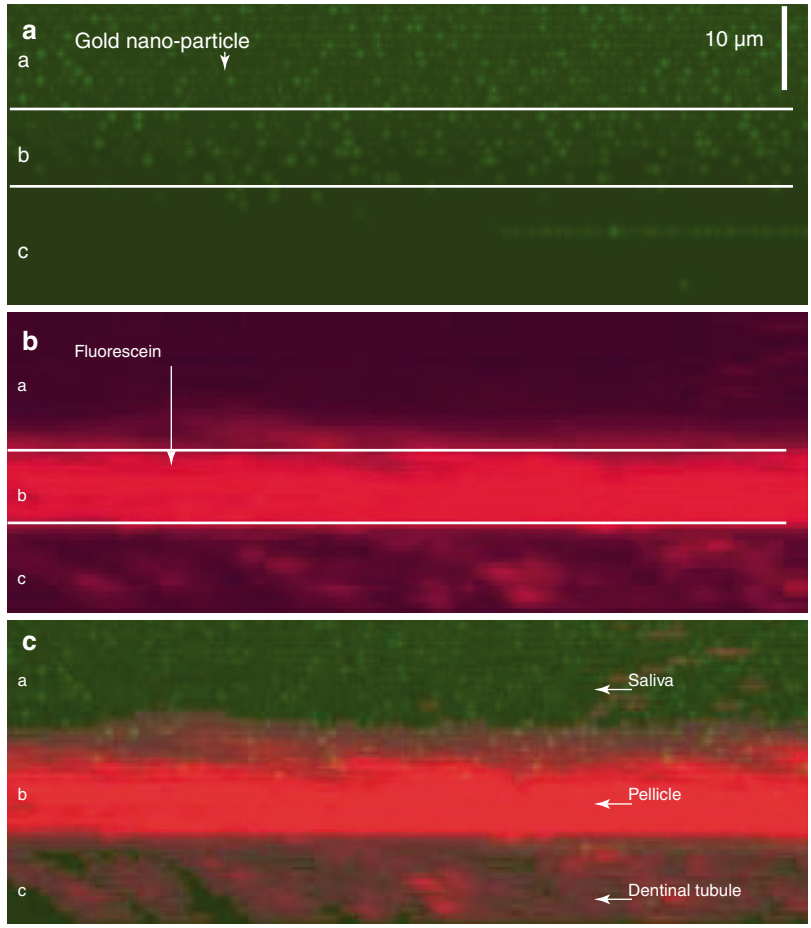


Fig. 10 Multimodality images showing use of gold nanoparticles for imaging the salivary pellicle. An extracted tooth was incubated in saliva mixed with 15 nm diameter gold nanoparticles and fluorescein for 120 min. (**a**) OCM image; (**b**) MPM image; (**c**) Combined OCM and MPM image. Zone a; Saliva. Gold nanoparticle (arrow) and fluorescein are present in this zone. Zone b; Pellicle. Fewer gold nanoparticles and strong fluorescein signal (arrow) are visible. Zone c; Outer dentin surface. Gold nanoparticles are unable to penetrate, but there is a strong fluorescein signal that permits visual differentiation between saliva, pellicle, and dentin (Figure Courtesy J.H.B.)

Conclusion

Mapping and quantifying oral biofilm in its natural environment remains challenging. Recent advances in imaging technologies such as OCT and MPM including multiple reference optical coherence tomography (MR-OCT) [59] are rapidly expanding our abilities to characterize and monitor oral biofilm within its natural environment. Applying cutting edge artificial intelligence machine learning techniques to such imaging data will enhance its value and relevance to improving oral health and understanding the complex role that oral biofilm plays in it.

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