**In vivo oral biofilm analysis by confocal laser scanning microscopy: methodological approaches**

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The scientific community is showing an increasing interest in exploring the *in vivo* formation of the undisturbed human oral biofilm, because understanding the processes involved may open new avenues for interfering with the pathogenic properties of biofilms. The present chapter is focused on *in vivo* human models of undisturbed oral biofilm analysed by confocal laser scanning microscopy (CLSM). The different types of oral appliances and substrates used, the several microbiological and microscopic methods applied in combination with CLSM, as well as CLSM technical aspects are discussed. Finally, a new microscopic technique is described, the so called, confocal endomicroscopy, which will offer numerous exciting opportunities for the *in vivo* microscopic investigation in the field of Dentistry.

**Keywords:** dental plaque; dental biofilm; *in vivo* models; Confocal Laser Scanning Microscopy; Confocal Endomicroscopy.

1. Introduction

In nature, the immense majority of micro-organisms are adherent to the surfaces on which they grow, forming biofilms [1]. Of the various definitions of the concept of biofilm to be found in the literature, we would like to draw particular attention to the one proposed by Donlan and Costerton in 2002 [2]: a biofilm is “microbially-derived sessile community characterized by cells that are irreversible attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription”.

The oral biofilm and, specifically, dental plaque, is considered to be an example of a specialized microbial biofilm [3,4]. More than 6000 species-level phylotypes have been identified in dental plaque -determined by pyrosequencing analysis- [5]. The adherent biofilm that forms on the hard and soft tissues of the mouth constitutes the principal aetiologic agent in the origin of caries, gingivitis and periodontal disease [6-8]; these can therefore be considered as biofilm-mediated diseases.

Confocal laser scanning microscopy (CLSM) has been used in the biological sciences since 1961, when the concept of “optical sectioning” of a biological specimen was introduced. CLSM is a variation of white light microscopy in which specimens are visualized by stimulating light emission by a low power laser beam after application of fluorescent contrast agents. CLSM provides the capacity for direct, non-invasive, serial optical sectioning of intact, thick, live specimens with a minimum of sample preparation; in addition, it shows a marginal improvement in lateral resolution [9].

To date, more than 150 papers on oral biofilm analysis by CLSM have been published. There is now a specific interest in exploring the early stages of the *in vivo* formation of the oral biofilm on undisturbed samples as an understanding of the processes involved may open new avenues for acting on the pathogenic properties of biofilms [10-13]. This chapter provides a detailed description of the methodological characteristics of CLSM analysis of *in vivo* models of undisturbed human oral biofilm.

2. Limitations of the *in vitro* and destructured *in vivo* human oral biofilm models

The *in vitro* development of biofilm models led to significant advances in the study of oral biofilms [14-18]. However, *in vitro* oral biofilm models tend to involve limited numbers of species and, in addition, they are created under conditions that still cannot adequately reflect the physiological situation in the mouth [19-22]. Factors related to the oral cavity, such as the turnover rate of saliva, the ability of antibacterial substances to adhere to the pellicle of the tooth or the surface of soft tissues in order to achieve their effects, and the interaction with unculturable bacteria, cannot be modelled in *in vitro* experiments [12].

At the present time, the scientific community recognizes that *in vitro* models cannot guarantee the creation of oral biofilms whose composition and structure is comparable with those that form *in vivo* [20-22]. For this reason, some authors consider that the results obtained in *in vitro* studies should be interpreted with caution [12, 20, 23], and that there is a need to develop *in vivo* biofilm models that can subsequently be analysed intact *ex vivo* [20, 23-25].
Many authors have used microbiological plate culture techniques to study the composition of the in vivo dental plaque (DP) biofilm and the antimicrobial activity of different agents, such as chlorhexidine (CHX) [26-29]. However, the numerous disadvantages associated with the use of culture-dependent methods are well known [12, 30]. Fluorescence methods have also been used to analyse the oral composition and antibacterial effect of some antiseptics on the in vivo DP-biofilm [31-34].

A common methodological characteristic of all these papers is that evaluation of the supragingival bacterial plaque was performed on material previously removed from the surface of the tooth [31-34], whereas the subgingival bacterial plaque was obtained by paper point sampling or by mechanical debridement [28, 35], which is likely to disturb the delicate three-dimensional architecture and organization of the DP-biofilm [10, 13, 19, 36]. Another disadvantage of this type of studies is that the level of penetration of an antimicrobial agent into the plaque samples cannot be evaluated as the samples are dispersed for analysis [33]. This methodology therefore does not provide an optimal analysis of the architecture and organization of in vivo DP-biofilms, as well as of the action of antimicrobial agents on its structure [11, 21].

3. Undisturbed in vivo oral biofilm analysis by confocal laser scanning microscopy

In the early 1990s, Watson et al [37, 38] suggested the possible application of CLSM in vivo clinical dental research, and Netuschil et al, in 1998, [39] were the first investigators to use this non-invasive method to analyse the thickness and bacterial viability of undisturbed human DP-biofilm. Later, some authors designed special removable oral appliances that held a number of discs on which growth of the DP-biofilm took place [11, 22, 40]. This undisturbed DP-biofilm was then analysed using CLSM [11, 22, 40].

At present, the scientific community considers that this methodological design is the most suitable approach for studying the in vivo architecture and physiology of DP-biofilm formation on dental materials, as well as the antibacterial effect of antimicrobials on this microbial structure.

3.1. Removable appliances and substrates to collect dental plaque biofilm

It has been demonstrated that oral biofilms on mucosal surfaces are different from those on hard tissues, and that the composition of supragingival dental plaque differs from that of subgingival plaque [25]. As a result, these factors need to be taken into consideration when designing models of in vivo DP-biofilm.

Probably due to the marked inter-individual variability detected in the characteristics of DP-biofilm [10, 11, 20, 41, 42], in the majority of the studies the number of volunteers who had worn the removable appliances ranged from 3 to 10 [11, 42-46].

With regard to the type of removable appliance used to collect the supragingival dental plaque, Wood et al [10, 47], Watson et al [21], and Robinson et al [48] used the “Leeds in situ device”, composed of a nylon ring holding an enamel substrate, as previously described by Robinson et al [49], on which the DP-biofilm grew. These devices were bonded to free buccal surfaces of the first or second upper molars by means of a composite resin, providing a stagnation site for the formation of DP-biofilm [49]. The research group led by Kolenbrander [42, 50-52] designed two bilateral mandibular stents (spanning the posterior buccal surfaces from the first premolar to first molar), each of which contained several discs. A number of other authors have used different types of individualised acrylic splints for growing DP-biofilm [11, 12, 20, 22, 40, 53]. Recently the authors of the present chapter designed individualised splints of the lower arch for each volunteer in the study being performed. Figure 1 shows this splint formed of two vinyl sheets, an internal sheet with a thickness of 1 mm to which 6 discs were attached, and an external sheet with a thickness of 0.5 mm that was fenestrated to permit contact of the vestibular surface of the discs with the saliva whilst protecting them from the action of the cheeks and tongue; similar to previous designs [11, 20, 22], several discs were positioned on each hemiarch and inserted towards the interdental area between two adjacent teeth in order to imitate an approximal DP-biofilm which is only minimally influenced by the shear forces of the oral soft tissues. This particular design ensured that the biofilm was not touched or disturbed during removal or repositioning of the appliance.
A number of solid substrates of different characteristics have been used in the published studies on DP-biofilm, including human enamel [10, 39, 40, 50, 51], bovine enamel [12, 46, 53], bovine dentine [41, 46], hydroxyapatite [54], and polished glass [11, 13, 20, 22, 39]. Zaura-Arite et al [41] used bovine dentine discs, where three parallel grooves were cut perpendicular to the surface (each 200 µm wide and approximately 500 µm deep) in order to imitate fissured dental plaque. Other authors have described DP-biofilm formation on dental restorative materials, using various substrates: amalgam, gold, ceramic, resin composite, compomer, glass-ionomer cement, and polymethyl methacrylate [43, 55, 56].

With the aim of collecting subgingival dental plaque as predominantly undisturbed biofilm, Wecke et al [19] designed carriers consisting of gold foil or polytetrafluoroethylene membranes that were inserted into the periodontal pockets of patients suffering rapidly progressive periodontitis whereas, Schaudinn et al [57] studied in vivo bacterial biofilm in the endodontic root canals of teeth extracted after failed endodontic treatment.

In the majority of the studies on DP-biofilm, the time the appliance remained in the oral cavity varied between 4 hours [42, 51, 52, 55] and 7 days [12, 21, 48], depending on the type of DP-biofilm to be analysed. Auchill et al [20] demonstrated that the mean thickness of 48-hour biofilm -with a range from 14 to 150 µm- was not affected by the position of the removable device within the oral cavity (maxillary buccal region vs mandibular buccal region) or by the position of the disc (distal vs mesial; right vs left). In addition, Arweiler et al [11] observed that disc location in the oral cavity affected neither the mean bacterial viability values -with a range from 64% to 77%- nor the bacterial viability pattern in the 48-hour biofilm. Although the roughness of the surface of the substrate and its free energy are considered to be important factors for in vivo growth of the DP-biofilm [20], Netuschil et al [39] found no major differences in the thickness of 48-hour biofilm on using enamel or glass discs; some authors recommend using glass to avoid any optical disturbance due to the known autofluorescence of enamel [39, 44]. Jung et al [46] detected a significantly higher number of bacteria adherent to bovine dentine discs than to bovine enamel discs, and that the number of bacteria on the dentine increased with increasing oral exposure time (at 2 hours and 6 hours), whereas it remained relatively constant on the enamel.

Interestingly, Wood et al [10] observed that the DP-biofilm generated over a 4-day period varied within each disc: the biofilm was thicker at the enamel disc/ring junction (depth of 75 to 220 µm) and thinner towards the centre (depth of 35 to 215 µm).

### 3.2. Microbiological techniques in combination with confocal laser scanning microscopy

It has been shown that the use of a combination of different microbiological techniques is the only means to achieve a realistic representation of the bacterial spatial distribution and dynamics of DP-biofilm and to be able to examine the role of distinct species as coadhesive microbial agents in dental plaque [12, 46].

- **Fluorescence-labelled antibodies**

   In some studies of the undisturbed in vivo DP-biofilm of healthy subjects, fluorescence-labelled polyclonal antibodies have been used in combination with CLSM to look at the initial stages of biofilm development (at 4, 6, and 8 hours), and at the specific interactions between early colonizers of the tooth surface (such as *Streptococcus* spp., *Actinomyces* spp., and *Veillonella* spp.) [50-52]. Gu et al [54] explored the possibility of using 3 well-characterized monoclonal antibodies against *Streptococcus mutans*, *Actinomyces naeslundii*, and *Lactobacillus casei*, to identify these important members of the oral microbial community in the DP-biofilm, while Chalmers et al [50] used antibodies...
against *Streptococcus mutans.* It has been observed that this methodological approach does not disturb the biofilm structure and allows simultaneous assessment of the relative proportions and the spatial arrangement of several pathogenic genera (or species) within DP-biofilm grown *in vivo*, together with real-time monitoring of the dynamic changes involved in biofilm formation [54].

However, it has been recognized that the characteristics of the *in vivo* DP-biofilm can potentially influence the specificity of the antibodies, as well as the conjugation of the respective fluorescent labels to the antibodies could block the antigen recognition sites of the antibodies [54]. In consequence, Gu et al [54] stated that each newly raised fluorescently labelled antibody should be analyzed regarding their detection abilities and behaviour under various biofilm conditions.

- **Fluorescence *in situ* hybridization**

It has recently been shown that fluorescence *in situ* hybridization (FISH), a technique that employs fluorescence-labelled species-specific DNA probes, is an useful method for the detection of bacteria without disruption of their natural environment [12, 13, 19, 42, 44-46]. Several authors have used the combination of FISH with CLSM to obtain images of three-dimensional reconstructions of the natural microbiological environments of *in vivo* DP-biofilm, evaluating the levels of up to 4 different micro-organisms in the biofilm formation after different periods of time [12, 13, 19, 42, 44-46]. The most important oral micro-organisms studied using the FISH/CLSM techniques have been the genera *Streptococcus* (the most widely studied), *Actinomyces*, *Veillonella*, *Prevotella*, and *Treponema*, as well as species such as *Actinomyces naeslundii* and *Fusobacterium nucleatum* [12, 13, 19, 42, 44-46].

Al-Almad et al [12] considered that the advantages of using FISH to identify micro-organisms in DP-biofilm include the possibility of detecting unculturable bacteria and the faster development of new probes compared to the production and characterization of antibodies. Besides, a good correlation between FISH and the results after staining with 4′-6-diamino-2-phenylindole dihydrochloride (DAPI) has been detected [58]. On the other hand, one of the limitations of FISH is that it uses ribosomal (r) RNA-targeted probes, and its sensitivity is therefore limited by the metabolic state of the bacteria within the DP-biofilm, as metabolically inactive micro-organisms are generally considered to have a lower cellular ribosomal content (relative “inaccessibility” to FISH probes) [59]. Dongari-Bagtzoglou [59] stated that to detect metabolically active cells more specifically, it is possible to synthesise FISH oligonucleotide probes that bind to intergenic spacer regions in rRNA genes, which are quickly degraded during ribosome maturation in bacteria. These probes would therefore only detect bacteria that are producing new rRNA at the time of sampling [60]. Also, FISH requires specimen fixation including dehydration, potentially leading to similar problems to those observed with transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and it cannot be utilized to study the dynamic changes occurring in live biofilms [54, 59].

- **Molecular biology techniques**

When studying clinical isolates of *Veillonella* spp. obtained from undisturbed *in vivo* DP-biofilm, Palmer et al [52] not only used fluorescence-labelled antibodies/CLSM but also simultaneously applied molecular techniques including phylogenetic characterization of clinical isolates by 16S rRNA gene sequencing. Those authors then analyzed the relationship between the isolates at strain level using enterobacterial repetitive intergenic consensus (ERIC) PCR fingerprinting, a technique that provides an assessment of diversity within a species (microdiversity) [52].

- **Conventional culture-dependent techniques**

In some studies on *in vivo* DP-biofilm by CLSM, the number of colony forming units obtained from the biofilm samples was quantified using conventional microbiological techniques [46, 52, 61]. In the series by Jung et al [46] and von Ohle et al [61], the cultural viability was examined by plating the serially diluted biofilm samples on different types of non-selective media for aerobic and anaerobic bacteria. On the contrary, in the series by Palmer et al [52] a selective-medium such as *Veillonella* enrichment agar plates was used.

- **Other techniques**

Von Ohle et al [61] demonstrated the utility of microelectrodes to provide a non-destructive, real-time measurement of the effects of sucrose and CHX on the physiology of DP-biofilm. The microelectrode data were correlated with microscopic examination by CLSM of the biofilm structure, thus allowing a relationship to be made between biofilm physiology and structure [61].

### 3.3. Fluorescence techniques in combination with confocal laser scanning microscopy

Since Netuschil first used fluorescence techniques to investigate the DP-biofilm in 1983 [62], many authors have employed a variety of fluorochromes, including acridine orange [63], fluorescein [10], DAPI [46], fluorescein diacetate plus ethidium bromide [22, 39, 40, 41, 53], and SYTO 9 plus propidium iodide (PI) [33]. Both fluorescein plus ethidium
bromide and SYTO 9 plus PI offer the possibility of selectively staining live and dead bacteria; these fluorochromes have therefore been widely used to analyse bacterial viability in the majority of studies on undisturbed in vivo DP-biofilm (mainly fluorescein plus ethidium bromide) [22, 33, 39, 40, 41, 53].

We have some experience in the use of the SYTO 9 plus PI dual stain, which enables bacteria with intact cytoplasmic membranes (emitting green fluorescence) to be differentiated from bacteria with damaged membranes (emitting red fluorescence) [30], as showed in Figure 2. This technique has therefore been considered particularly useful for analysis of the spatial distribution of bacterial viability within biofilms and of the antimicrobial activity of some antiseptics, such as CHX [33, 64, 65]. We have also suggested that the SYTO 9 plus PI fluorescence assay could be very useful for the simultaneous analysis of the effect on different oral ecosystems (e.g., saliva and DP-biofilm) of antimicrobials that affect the integrity of the cytoplasmic membrane [30]. Nevertheless, taking into account the limitations associated with SYTO 9 plus PI fluorescence assay [30], the results obtained with this technique should be considered to be semi-quantitative with respect to viability [61].

In some papers on undisturbed in vivo DP-biofilm using fluorescence-labelled antibodies/CLSM or FISH/CLSM, the antibodies and the different oligonucleotide probes were conjugated or labelled with different fluorochromes (e.g., Alexa Fluor dyes, ATTO dyes, Cy dyes, SYTO dyes, DAPI, and calcifluor) in order to identify a specific bacterial genus or species, all bacteria, or even bacterial components such as extracellular polysaccharide [12, 13, 19, 42-46, 50-52]. In this type of studies (antibodies/CLSM and FISH/CLSM), an obstacle with CLSM is the limited availability of fluorescent probe colours that have non-overlapping emission signals, meaning that only 2 to 4 distinct microbial taxa (depending on the microscope) can be differentiated in any one image [44].

A further methodological factor to be considered with regard to fluorescence is the problem of correct penetration of the fluorescent dyes. Paramonova et al [46] showed that poor penetration of the fluorescent dyes into biofilms thicker than 120 µm leads to a noticeable underestimation of biofilm thickness by CLSM. An additional problem with thick biofilms is the risk of underestimating bacterial numbers due to the degree of fading of the fluorophore when multiple optical sections are performed [44, 66]. Other disadvantages that may be associated with the fluorescence staining include: the existence of a percentage of people (8-12% of males and almost 1% of females), who are green/red colour blind from extracting any meaningful information from the images [67], the influence of the type of bacteria (e.g. Actinomyces spp. often results in an irregular distribution of fluorescent signals, possibly because of insufficient permeability of the bacterial cell walls) and of its physiological state (exponential-growth phase vs stationary phase) about the staining properties [45, 68], some fluorochromes showed a significant non-specific binding to the matrix and great background fluorescence [69] or the detection of intermediate colors of “unknown” interpretation [68].

Recently, Chalmers et al [50] applied quantum dot (QD) based primary immunofluorescence for Veillonella spp. and for receptor polysaccharide (RPS) bearing Streptococcus spp. to achieve high, single-cell, resolution and to analyse spatio-temporal relationships between these bacteria in 6-hour and 8-hour biofilm communities. The results supported the use of QD-based immunofluorescence as a tool for the study of undisturbed in vivo DP-biofilm, as it detects multispecies communities similar to those revealed in previous studies using antibody-Alexa Fluor conjugates published by the same research group [51, 52]. Furthermore, it has been stated that QD conjugates offer significant advantages over traditional fluorophore fluorescence: QD conjugates have a high specificity and orders of magnitude higher sensitivity than can be achieved with traditional fluorophores [70]; QD luminescence is photostable and size-tunable, with narrow, symmetric emission spectra and broad continuous excitation, allowing excitation of multiple QD with a single wavelength [50, 71]. Chalmers et al [50] concluded that one application that benefits greatly from the photostability of QD conjugates is micromanipulation of viable, spatially resolved multispecies communities from the surface of the substrate.

3.4. Confocal laser scanning microscopy

Over the years, different microscopic techniques including light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have frequently been employed to visualise the microstructure of in vivo oral biofilms [72-75]. It can be difficult to achieve penetrative views of specimens using these techniques or the samples require preparation involving dehydration, fixing, and embedding, which may cause disruptive shrinkage and loss of biofilm matrix that can affect 73% to 98% of the in vivo biofilm mass [76]. TEM is prone to preparation artefact, mainly affecting delicate fluid-filled structures [10].

The advent of CLSM, though of lower resolution than TEM [44], has eliminated or considerably reduced these problems. With CLSM (using inverted or non-inverted microscopes), biofilms can be studied in their natural hydrated state (usually using a 63x water-immersion lens), with no requirement for dehydration, fixation, or staining [11, 20, 53]. In addition, the optical sectioning properties of CLSM mean that very thin optical sections in the horizontal plane (X-Y axes) can be taken at 0.5 to 2 µm intervals, at increasing depths through the biofilm (from the surface of the biofilm to its base), free from out-of-focus blurring [12, 13, 44, 53, 55].

Robinson et al [48] evaluated the 7-day DP-biofilm using CLSM without fluorescence solutions. The optical data were therefore recorded as a series of arbitrarily assigned grey levels numbered 1 to 20 according to the degree of reflectance observed. However, in the other studies published on undisturbed in vivo DP-biofilm, the authors used CLSM with fluorescent dyes to make elements visible [12, 13, 44, 53, 55]. The excitation value and emission range of
the fluorescence imaging were measured at different wavelengths depending on the fluorochrome used – e.g. the SYTO 9 and PI excitation values and emission range wavelengths are 488 nm (492–550 nm) and 561 nm (588–655 nm), respectively [30]. Fluorescence emission was analyzed in series of X-Y images in which each image corresponded to a single Z position (depth) [12, 13, 44, 53, 55], as showed in Figure 2.

DP-biofilm thickness is usually used as an initial quantitative measurement of biomass [10, 12, 22, 45]. DP-biofilm thickness has been defined as the distance between the substrate and the peaks of the highest cell clusters [77]. However, some authors observed that the substrate surface was never exactly horizontal with respect to the microscope stage, and the number of optical sections per specimen was not directly translatable to DP-biofilm thickness [44, 45, 51].

Bacterial quantification can be performed by manual counting, which requires high-magnification images to allow differentiation between single cells. Furthermore, to obtain representative estimates, a large number of consecutive images need to be recorded and counted, which is time-consuming [13]. Also it has been recognized that counting of cells in aggregates is complicated because densely packed cells cannot easily be separated during image segmentation [13, 75]. It is probably for these reasons that the majority of authors have employed automated (digital) image analysis tools that have been developed for the quantification of fluorescence signals [10-12, 20, 22, 39-42, 46, 53]. Figure 2 shows as in automated image analysis, the images of each fluorochrome are defined as channels (e.g., for the quantification of viability, SYTO 9 occupies the green channel and PI the red channel) and are subsequently converted to quantitative data. However, Dige et al [13] stated that the generation of quantitative data from images based on fluorescence signals is a complex process. A number of researchers therefore performed quantitative analyses based on the ratios of the area occupied by each micro-organism to the whole area (mainly in studies on biofilm viability) [10, 11, 20, 22, 39-41, 53]; others quantified the biomass of different targets (probes) in the DP-biofilm by setting the EUB338-corresponding fluorescence volume at 100%; they then calculated other targets as a percentage of the EUB338-fluorescing biomass (mainly in studies on biofilm topography) [12, 42, 46].

As a possible alternative, Dige et al [13, 45] recently suggested that the combined use of FISH/CLSM and stereological methods is an attractive tool for quantification of bacterial populations in undisturbed in vivo DP-biofilm. This technique provides an unbiased and reliable determination of the numerical contribution of specific species in mixed bacterial communities. Importantly, stereological methods avoid the inherent problems associated with varying intensity of the fluorophore, which gives rise to a possible bias in automated image analysis. However, the stereological methods have their own disadvantages: they are relatively time-consuming compared to automated digital image analysis, and they require prior knowledge of bacterial morphology [13].

Irrespective of the quantification strategy, the dense packing of bacteria in DP-biofilms sometimes makes analysis difficult because of interference by fluorescence signals from adjacent, out-of-focus planes [44]. Other problems have also been detected: fading of the fluorophore following optical sectioning; differences in fluorescence intensities depending on the dyes used for probe labelling and in cell-specific uptakes of fluorescence stains; and varying thresholds [13, 45]. Sekar et al [78] therefore recommended that CLSM of the biofilm area/volume should include size standards such as microbeads during image acquisition in order to minimize the inherent error in quantitative measurements.

Among the possible drawbacks of CLSM, is the use of microscopy as the basis of the technique which makes the selection of regions of dental plaque for analysis highly observer-dependent [33]. The high density of DP-biofilms grown in vivo can limit the laser penetration depth of CLSM (conventional one-photon excitation) to the outer 40 to 100 µm of the biofilm cell clusters; this could mean that deeper areas are not reliably visualised [33, 61]. Kawaguchi and Decho [79] demonstrated that two-photon excitation CLSM could achieve deeper penetration of tissue biofilms and enhance resolution.

Schaudinn et al [57] proposed a combined method using more than one microscopic technique to provide unique information about a structure that is difficult or even impossible to obtain with a single imaging regimen. However, the application of combined microscopy techniques for the characterisation of undisturbed in vivo DP-biofilm has been underexploited [80], as few authors have used CLSM in combination with other methods of microscopy, such as SEM, TEM, or epifluorescence microscopy [10, 46, 56, 57].
4. Future perspectives

Recent technological advances in miniaturization have meant that CLSM can be integrated into a conventional flexible endoscope, or into transendoscopic probes, a technique known as confocal endomicroscopy (CEM) or confocal laser endomicroscopy (CLE). This newly developed technology has enabled endoscopists to collect real-time, in vivo histological images or “virtual biopsies” of the gastrointestinal tract. Clinical applications include not only the study of precancerous and cancerous lesions but also the identification of Helicobacter pylori infection and diagnosis of disorders such as coeliac disease and ulcerative colitis [81, 82]. CEM has also been used for the in vivo examination of human skin [83], cervix [84] and recently for microscopic imaging of the proximal and distal respiratory tracts [85].

Taking advantage of optical fibres, the miniaturization of optics, and improvements in beam scanning mechanisms, confocal images have been successfully obtained from the oropharynx [86]. CEM has been found to be suitable for non-invasive examination of the oral mucosa, enamel, and dental materials in vivo [87]. Whilst it is hard to predict the scope of its future applications, most recent papers have been focused on the clinical evaluation and diagnosis of oral cancer and precancerous lesions [88, 89]. Following this line, the potential for CEM applications for the in vivo analysis of undisturbed human DP-biofilm could be enormous.

Apart from morphologic visualisation, CEM offers an unique possibility to study pathophysiological events in their natural environment (functional imaging). The application of CEM to molecular imaging in basic and clinical sciences will enable us to increase our understanding of the cellular basis of oral physiology and pathophysiology. In consequence, future applications of CEM in Dentistry should include, among others, caries research, soft and hard tissue responses to biomaterials (e.g., dental implants), wound healing, and monitoring the effect of periodontal treatment protocols. This new technique also offers numerous exciting opportunities for the microscopic investigation

Figure 2. Bacterial viability quantification, analysing fluorescence emission in X-Y axes sections corresponding to different Z positions (depth). A) Green channel (shows intact cytoplasmic membranes); B) Red channel (shows damaged cytoplasmic membranes); C) Superimposed green and red channels; D) Fluorogram and segmentation; E) Quantification automatic data of a single section (e.g. bacterial viability= 28.61%)
of many clinical operative procedures in vivo, allowing the response of the oral tissues to be non-destructively monitored over time at high resolution.

References


