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Application of Fluorescence Spectroscopy for Microbial Detection to Enhance Clinical Investigations

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Abstract

Microbial biofilms are complex multi-layered communities of bacteria and fungi which cause a range of oral and other diseases. Efficient detection of biofilms is important for the clinical management of diseases they cause and for providing an endpoint to clinical treatments. For bacterial biofilms, bacterial metabolites such as porphyrins are important molecules for diagnostic purposes, since they fluoresce in the red and infrared regions of the spectrum. Fluorescence is a versatile and powerful diagnostic approach for detection of bacterial biofilms, particularly in dentistry. This chapter provides an overview of fluorescence spectroscopic methods for detection and analysis of biofilms and their derivatives such as deposits of dental calculus and how current technology can be extended using photon-counting detectors. Fluorescence can be used to help discriminate these from healthy tissues. The approaches described have broad applications to clinical and industrial situations where non-invasive detection of microbial biofilms is important.

Keywords: bacterial biofilms, clinical diagnosis, fluorescence spectroscopy, fluorophores, porphyrins

1. Introduction

The interactions of light with matter are heavily dependent on the wavelength of the light and the response of the target to that light. Major interactions include scattering, absorption and fluorescence. Various fluorescence spectroscopic methods have been used to analyse tissues and materials according to their fluorescence properties. In this chapter, the principles of fluorescence spectroscopy are discussed, with particular reference to the diagnostic values

of porphyrin derivatives for the detection of infected biological tissues and applications of photon counting.

2. Fluorescence phenomena

Luminescence is a general term for the emission of radiation, which incorporates both fluorescence (a short-lived process) and phosphorescence (a long-lived process), as well as other phenomena such as bioluminescence in living organisms in which chemical reactions generate light. In fluorescence, the absorption of light of a particular wavelength results in the emission of light of a longer wavelength. This emission of light occurs as fluorophores get de-excited from a higher energy level to a lower energy level [1, 2]. When light is absorbed, the fluorophore becomes electronically excited, but the lifetime in the excited state is very short, and there is a rapid decay to a lower energy level. Fluorescence occurs if the transition is between states of the same electron spin and phosphorescence if the transition occurs between states of different spins. Fluorescence and phosphorescence phenomena are illustrated in the Jablonski energy diagram shown in **Figure 1**. Many naturally occurring substances fluoresce, including

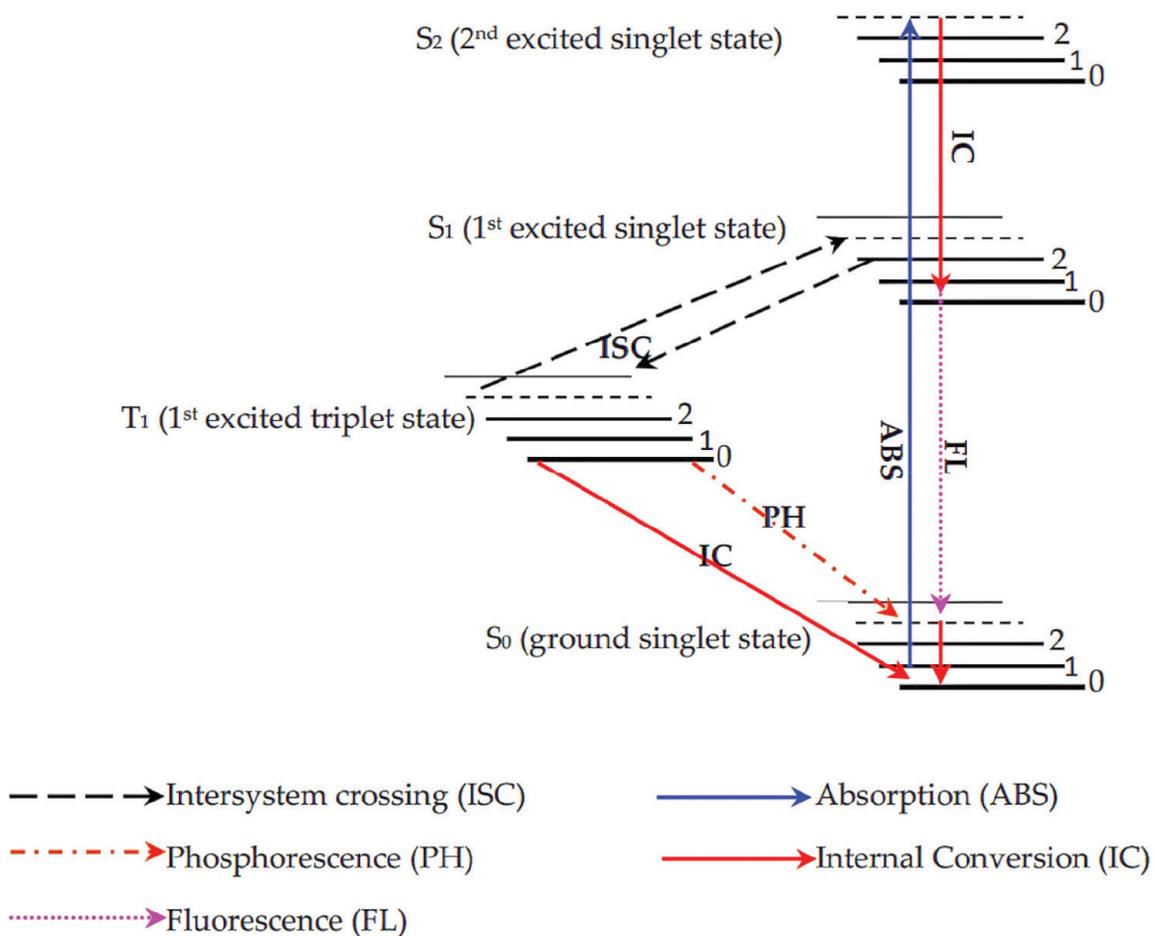


Figure 1. Jablonski energy diagram showing fluorescence and phosphorescence processes. Based on Ref. [3].

some minerals, fungi, bacteria, keratin, collagens and other components of body tissues. This is termed 'primary fluorescence' or 'autofluorescence'.

As the molecule absorbs energy, it transitions from the lower ground singlet state (S_0) to a vibrational level of an excited singlet state S_n ($n = 1, 2, \dots$). The excited molecule loses energy partly through internal conversion without photon emission, and then it spontaneously releases a lower energy photon as it returns back to the singlet ground state [1, 4]. Light emission occurs within one microsecond of light exposure. Molecular fluorescence emissions persist only as long as the incoming stimulating radiation is continued, unlike phosphorescence, where light is emitted as a persisting 'afterglow' long after the incoming exciting light is no longer present.

The light that is emitted by fluorescence is readily distinguishable from the excitation light because it has a longer wavelength. This relationship is known as Stokes law and is named after Sir George Stokes, who published the first major paper on fluorescence [5]. For example, when a molecule absorbs short wavelength ultraviolet (UVA) light in the region of 315–400 nm, the emissions may be in the visible spectrum, such as visible red, in the case of porphyrins. Likewise, when excited by visible light wavelengths, porphyrins emit light in the near-infrared range.

3. Fluorescence spectroscopy

In fluorescence spectroscopy, also known as fluorometry or spectrofluorometry, fluorescence emissions from a sample are elicited using a range of wavelengths, and the emissions measured. The sample is typically in solution in a cuvette, and it is excited by near monochromatic light or by monochromatic light from a laser. Nearly monochromatic light can be produced using a monochromator, where a broad spectrum lamp such as halogen lamp is used (**Figure 2**), and

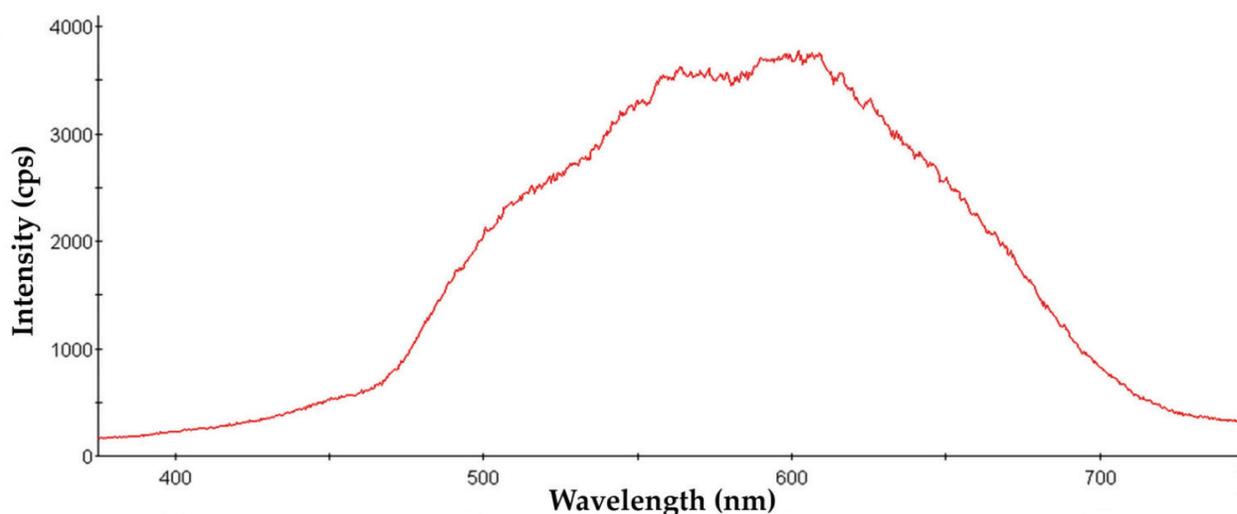


Figure 2. Emission spectrum from a halogen lamp.

the output is passed through a slit onto a diffraction grating, as shown in **Figure 3**. Even though fluorescence is emitted in all directions from the fluorophores within the sample, fluorescent emissions are detected normal to the incident beam path. This reduces the impact of stray light and the incident light wavelength. The detector (5) is usually a photomultiplier tube (PMT) or a photodiode array. These convert the intensity of the fluorescence emissions into an electrical output for subsequent analysis [1].

A spectrofluorimeter can show the range of fluorescence emissions at a particular constant excitation wavelength, or alternatively it can be used to record the excitation spectrum that gives rise to emission at a specific constant wavelength. The same principles as used in a spectrofluorimeter for sample analysis can be applied under field conditions or in clinical settings. The major challenges are in choosing the appropriate wavelength(s) of light to use, determining the most appropriate source of that light and ensuring that the detector system is sufficiently sensitive.

Fluorescence spectroscopy has become a useful analytical approach in many fields, including biochemistry, biophysics and biomaterial sciences. In recent years, molecular fluorescence analytical approaches have been developed to investigate fluorophores within biological samples [4].

A range of systems have been developed that give the user the choice of different excitation wavelengths, so that particular fluorophores of interest can be targeted. Using lasers as light sources, particularly semiconductor diode lasers, ensures that the emitted light is

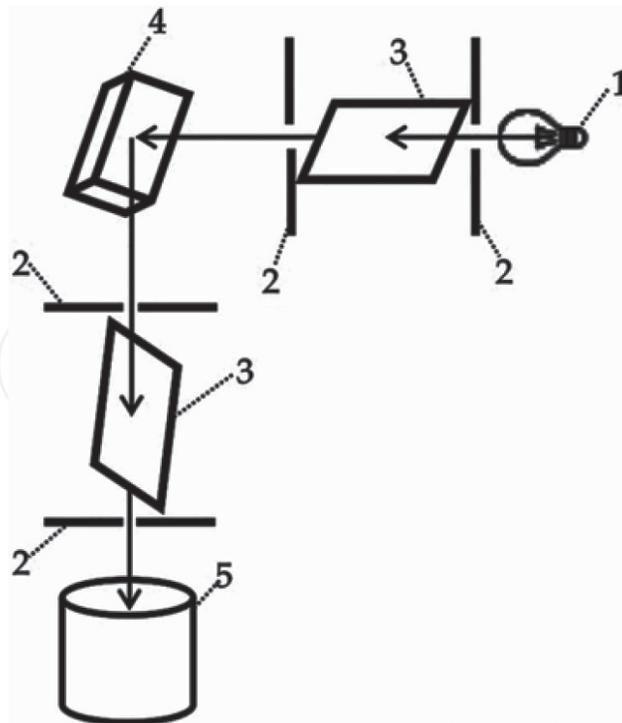


Figure 3. Schematic design of a spectrofluorimeter. 1 = excitation light source; 2 = slit; 3 = grating; 4 = sample cuvette; 5 = detector.

monochromatic, although there may be a drift in wavelength as the diode laser warms up from a cold start. The low power consumption and high electrical conversion efficiency of diode lasers make them well suited for use in portable systems. Diode lasers can be pulsed at a high frequency and can operate in chopped continuous waves as well as in superpulsed modes. By attenuating the intensity of the laser beam from a pulsed laser, the mean number of photons can be reduced dramatically and therefore it is possible to achieve single photon effects, if that is desired.

The detectors used in fluorescence diagnostic systems have included charge-coupled devices (CCDs), photodiodes and photon-counting detectors based on photomultipliers or avalanche diodes. Charge-coupled devices and photodiodes are well suited to fluorescence devices where the light source is running continuously or in long pulses, while photomultipliers and avalanche diodes are necessary to detect single photons.

4. Photomultiplier tube

A key component of a fluorescence system designed to work with faint laser light is a photomultiplier tube (PMT). This device amplifies the current generated by incident photons in the order of 100 million times, by using several dynode stages, enabling individual photons to be detected even when the incident light intensity is extremely low. Depending on the nature of the window through which the incident light enters the PMT, light in the ultraviolet, visible and near-infrared range may be detected, for photon-counting purposes or for high-sensitivity light detection techniques [6]. A simplified schematic design of a PMT is shown in **Figure 4** [7]. An electric field accelerates photoelectrons released from the photocathode, with increased numbers of secondary electrons released from successive dynodes as the incoming electrons collide with them, achieving amplification at each dynode through secondary emission. The electrons from the final dynode reach the anode, creating the signal current, which then gives final reading for light-induced fluorescence. A typical delay from an incoming photon striking the photocathode, causing electrons to be emitted through photoelectric actions, and a current pulse being measured from electrons reaching the anode, is in the order of 50

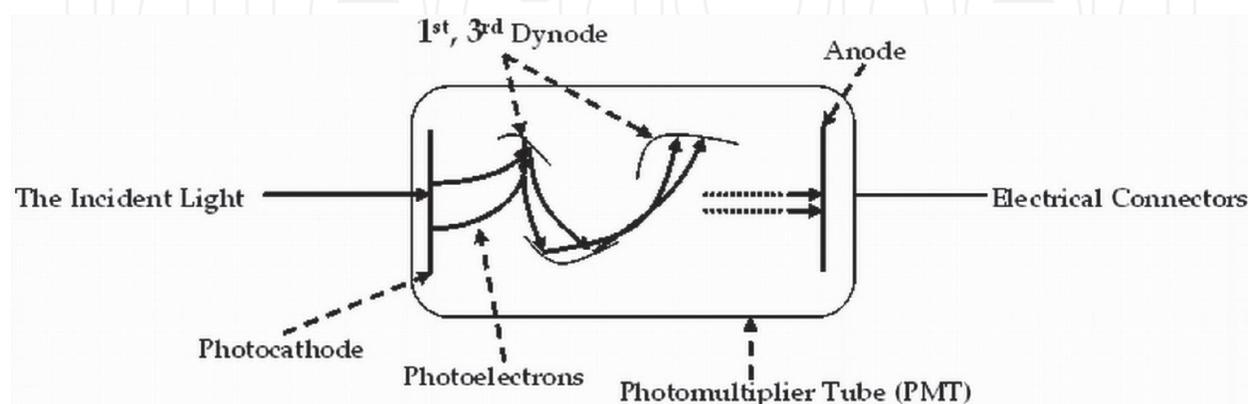


Figure 4. Schematic diagram of photon-counting photomultiplier tube. Based on Ref. [7].

nanoseconds. This brief time interval allows the distinction to be made between fluorescence and phosphorescence, since in the latter the emission of light continues for some time after the excitation pulse of light has ended.

Semiconductor devices such as avalanche photodiodes are now considered as alternatives to PMT for some applications. Their gain may however be less than that for PMTs. When very high gains are needed, single photon avalanche diodes can be used, applying voltages that are well above their breakdown voltage, for short intervals.

5. Fluorimeter equipment

The basis of many laboratory studies of fluorescence of biological materials is the spectrofluorimeter, which can determine the fluorescence characteristics of individual pure substances or mixtures of substances. A typical spectrofluorimeter is the FluoroMax-3 manufactured by JY-Horiba. This instrument is used widely to perform high-resolution fluorescence measurements. It has a standard configuration as shown in **Figure 3**, with a broad spectrum xenon arc lamp, excitation and emission monochromators before and after the sample and a photomultiplier tube for detection. Wavelength selection is achieved using the optical gratings of the monochromators. These diffract the incident beam, dispersing it into its constituent wavelengths. In addition, adjustable 'slits' are used at the entrance and exits of the grating, which can be used for resolving particular wavelengths. On the excitation monochromators, the slits control the bandpass (range) of light that is incident on the cuvette sample. On the other hand, the slits of the emission grating determine the intensity of the emitted fluorescence recorded by the PMT sensor. There is a reference photodiode which is used to correct for variations in the intensity of the emissions from the xenon lamp at different wavelengths.

The spectrofluorimeter interfaces with a computer, and a dedicated software package (DataMax) is used for data acquisition. A post-processing application manages the acquisition of emission and excitation profiles and allows the time course of fluorescence events to be followed. A constant wavelength analysis application allows multiple samples to be analysed at single wavelengths, similar to a microplate reader. A real-time display application allows individual hardware parameters such as slit width to be adjusted while immediately viewing the consequential changes in emission intensity.

6. Fluorescence analysis software

A typical example of software used for fluorescence spectroscopic analysis is MicroCal™ Origin. This software from OriginLab Corporation operates on a Windows® platform. The front end of the software has a spreadsheet design that is column oriented. The user has access to various templates to simplify workflow. The software can generate a range of 2D

and 3D graphs. Data analysis in Origin includes curve fitting and peak analysis. Curve fitting is achieved through a non-linear least squares approach. There are several platform-independent open-source programmes with similar functions, such as QtiPlot and SciDAVis.

7. Bacterial biofilms

Bacterial biofilms have a complex 3D architecture. They may be composed of a single bacterial species or of multiple species living within distinct microenvironments. Biofilms are ubiquitous in moist environments [8, 9], where they allow bacteria to resist environmental physical stresses such as shear stresses from fluid flow, as well as chemical stresses from adverse environmental pH or eH. The biofilm structure provides a physical barrier to the diffusion of most biocides [10]. Organisms located deep within in biofilms exist in a dormant or quiescent metabolic state. The low rate of proliferation makes them resistant to antibiotics that target bacterial replication [11] or the synthesis of new bacterial cell membranes [12].

In the industry, biofilms are a constant problem in the food processing industry [13–15], as well as in ventilation systems [16] and in water treatment [17]. Biofilms pose major problems in healthcare because they adhere to surgical implants of various types, as well as to most body surfaces, causing chronic infections when the opportunity arises [18]. Examples of diseases related to biofilms include periodontitis which causes loss of bone and connective tissue attachment of teeth [19–21] and chronic lung infections in individuals with cystic fibrosis [22]. Medical devices that suffer problems from biofilms include central venous catheters [23], endocardial pacemaker leads [24], prosthetic heart valves [25], orthopaedic devices [26] and urinary catheters [27].

In the polyvinyl chloride (PVC) tubing used in much industrial and biomedical equipment, biofilms form readily when these are exposed to reticulated water. Surface colonisation is enhanced by calcium compounds (such as calcium carbonate) and adherent organic molecules present as contaminants in water [28]. Small diameter tubing gives laminar flow characteristics, with a high central flow rate and slow peripheral flow rates, with bacteria segregated near the walls of the tubing. The surface-to-volume ratio is high in small diameter tubing, since less than 100 mL of water may be spread over more than 1500 cm² of available surface. Tubing which has a diameter of 2 mm or less is particularly problematic in terms of the rapid formation of dense biofilms when connected to reticulated water or fluids which are not first rendered sterile.

Current management strategies that are used to control biofilms include flushing, purging with air or other gases, treatment with nitric or other acids and application of biocides including glutaraldehyde, sodium hypochlorite, hydrogen peroxide, ozone, silver ions or iodine [28, 29]. Such treatments are designed to reduce the problem of blockage of narrow tubing from biofilms. In addition to equipment failure, biofilms create health risks for patients through renal dialysis equipment [30]. They also form in the tubing in dental chairs [31], from where water may be aerosolized with pathogenic legionella or mycobacteria.

Because bacterial biofilms in pipes and tubing resist many chemical agents, treatments which have a physical removal action (such as scraping or agitation) are commonplace [12, 14]. Laser-generated cavitation can have powerful cleaning actions and can detach biofilms [32]. The same approach using lasers can be used to ablate biofilms, provided an appropriate laser wavelength and exposure parameters are used [33].

With regard to the detection of bacterial biofilms within pipes and tubing, levels of loosely attached bacteria can be quantified indirectly by using as a surrogate measurement the levels of bacteria in the fluid that exit the pipe in question. It is generally not practical to sample the tubing itself for the presence of bacteria, as this could be destructive. While the sampling of exit fluids for viable bacteria is used widely [34], this method is time-consuming since such samples require at least several days of incubation in the laboratory.

Real-time assessment of biofilm levels would be of great advantage in allowing precise control over the dosing of biocides and the timing of purging and other biofilm control measures. Ideally, such an assessment would be undertaken externally (i.e. through the tubing) [35, 36] in real time, without having to shut down or interrupt the system for testing and maintenance. A useful approach for determining the presence of bacterial biofilms in tubing made of PVC and similar transparent polymers may be light-induced fluorescence, applied externally (i.e. passing through the tubing walls) or applied internally using an optical fibre. This diagnostic approach has been used successfully within the narrow confines of the root canals of teeth [37]. By applying coherent (laser) or near-coherent light, fluorophores within the bacterial biofilm or the overlying fluid become excited. Not only could the levels of bacteria be assessed in a quantitative manner, but it should be possible to apply another laser to create cavitation and shock-waves inside the tubing to fragment and disrupt the biofilm. This concept of laser-generated internal shock waves has been applied successfully to debriding the root canals of teeth [38].

By selecting appropriate excitation sources and filters, fluorescence-based analysis systems can identify and quantify the target of interest in a tissue or on a surface. This selective fluorophore approach has been used for kidney stones, tumours, dental filling materials [39, 40], dental caries [41], dental plaque biofilms [2] and dental calculus [42]. The latter four sample types have been identified in diseased and healthy sites, employing optimal excitation wavelengths for fluorescence detection and then coupling this to a feedback-controlled second laser system for ablation. For biofilm detection within tubing, issues such as fluorescence from the liquid carried in the tubing and from the tubing itself need to be addressed. This is why it is essential to determine the excitation-emission ranges for various target materials using fluorescence spectroscopy under defined conditions in the laboratory using the type of spectrofluorimeter equipment and software described earlier.

A key objective is to disrupt and inactivate bacterial deposits without damaging the internal structure of the pipe or tubing. For effective disruption or ablation of biofilms, laser energy can be absorbed in both solid and fluid components. Key elements of bacterial biofilms from this perspective include water, calcific deposits and bacterial porphyrins. One can assume that at least 65% of bacterial biofilm volume is water because this is the typical water content of individual bacteria. The amount of energy absorbed by bacterial biofilms will vary according to the laser wavelength used, the concentration of the absorbing fluorophores and their

absorption coefficient [43]. The absorption of fluorophores in biofilms can be assessed across a range of possible excitation wavelengths via spectroscopy [44]. The absorption of light by the tubing is also assessed using the same approach.

A challenge in the use of fluorescence in very small confined environments where there are low levels of microorganisms is the detection limit of the system used to detect fluorescence emissions. This is where changing from a conventional photodiode to an avalanche photodiode can be considered. Moreover, the choice of semiconductor used will be affected by the wavelength range of interest. For detecting levels of planktonic bacteria of around 5 colony-forming units per mL in volumes of 20 μL , such as in the case of the root canal system of a molar tooth root, a system using conventional photodiodes to measure fluorescence from pulsed laser light is working at its limits. Moving to an alternative approach using faint laser emissions and thus single photon counting should increase the overall sensitivity of the system dramatically. Using such an approach, it should be possible to achieve detection of a single organism, provided it is in the range of the optical detection system. Specific improvements such as micro-patterned optical fibre tips have been developed to allow wide-angle detection of microorganisms in confined narrow canals.

An elegant example of a widely deployed fluorescence device is the DIAGNOdent [45, 46]. This was developed for detecting dental caries (tooth decay) [47] and uses a pulse 655-nm diode laser as the light source and a photodiode detector to collect near-infrared light that is filtered through a long pass filter. The detection system is gated so that only fluorescence emissions that correspond with laser pulses are assessed, to thus remove the effects of ambient light and background noise. Work in our laboratory showed that it can also be used to detect infections within the root canals of teeth [48]. The fluorescent yield of a healthy surface decreases much more than the infected region, as excitation wavelength increases in the red spectral region [49, 50]. For removing biofilms *from the outside* of teeth, a pulsed Er:YAG laser has been combined with the DIAGNOdent system and linked to a feedback control system [51]. More recently, this approach has also been used for addressing biofilms and planktonic bacteria *inside* teeth [37], which is technically much more challenging because of issues of access.

The current level of technology deployed in dental practice for fluorescence diagnostics includes systems with LED illumination and charge-coupled device (CCD) or complementary metal oxide semiconductors (CMOS) sensors, such as in intra-oral cameras as well as diode laser-based systems such as the DIAGNOdent Classic, DIAGNOdent Pen and KEY-3 laser (all from KaVo, Biberach, Germany) [52]. The intra-oral cameras use continuous wave emissions from multiple LEDs as the light source. A challenge for using CMOS sensors with such devices is the so-called 'rolling shutter' effect seen when the handpiece is being moved, due to the refresh rate used. CCD image sensors are considered to have better sensitivity for light detection than CMOS but are more expensive. They can operate well for detecting light emissions in the near infrared, which is useful for detection of bacteria [41, 52, 53], provided that a long-pass filter is used to remove reflected excitation light as well as ambient daylight and work-place lighting. In the case of the DIAGNOdent, only light wavelengths above 680 nm are measured [45–47].

8. Porphyrins and biofilm fluorescence

The literature identifies porphyrin derivatives as a potential fluorophore in bacterial by-products, based on the peak fluorescence of bacterial biofilms compared to the known emissions of porphyrins at a particular wavelength range [54, 55]. Porphyrins are derivatives of haemoglobin-related molecules known as tetrapyrrole porphyrins. These are involved in the biosynthesis of metalloporphyrin heme [ferroprotoporphyrin (Fe^{2+})], hemin [ferriprotoporphyrin (Fe^{3+})] and chlorophyll [54]. Aerobic cells can synthesise heme proteins (**Figure 2**). The main porphyrins in biosynthesis are protoporphyrin IX (PP IX), coproporphyrin III (CP III), uroporphyrin III (UP III) and hematoporphyrin IX (HP IX). These are linked through heme biosynthesis, as shown in **Figure 5**.

Solutions of porphyrin derivatives show fluorescence upon red excitation, particularly PP IX, which has strong near-infrared fluorescence around 825 nm when irradiated at 655 nm. The fluorescence yield increases linearly with the concentration of PP IX [56]. Testing various fractions derived from high-performance liquid chromatography (HPLC) of carious dentine

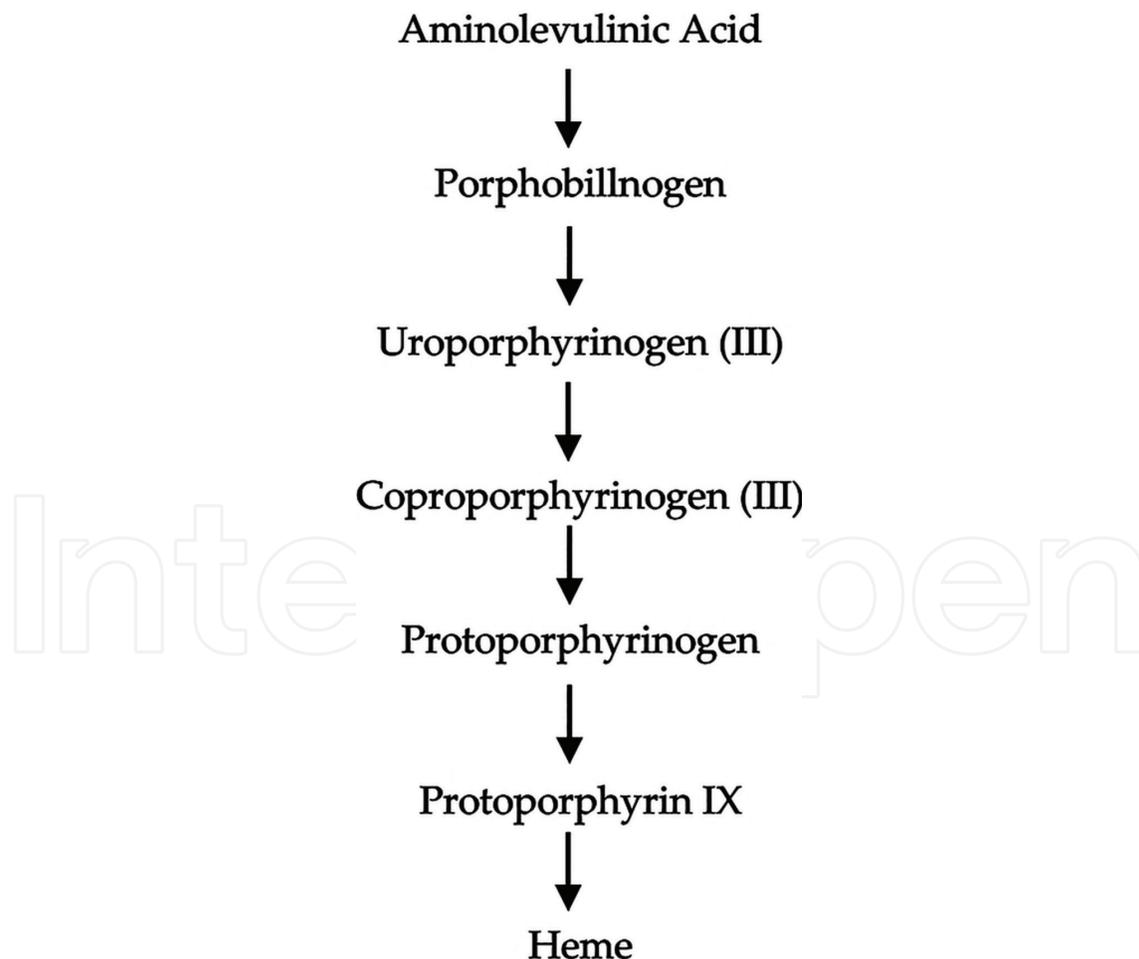


Figure 5. Schematic pathway of heme biosynthesis. Based on Ref. [54].

using 406 nm excitation has identified porphyrin, protoporphyrin, coproporphyrin and uroporphyrin as the main fluorescing compounds [54].

König had previously explored a similar concept while studying carious tooth tissue and had found emissions mainly in the visible red spectral region when samples were excited by a 407 nm (UVA) krypton ion laser [57]. Most carious teeth in König's experiments displayed a fluorescence maximum at 635 nm, and fewer than 10% of carious teeth examined showed additional maxima around 590 and 620 nm. These emission maxima correspond to the known emission peaks of protoporphyrin (633 nm), coproporphyrin (623 nm) and Zn protoporphyrin (593 nm) [57], as shown in **Figure 6**.

The fluorescence decay time (fluorescence lifetime) is the mean time during which the fluorophore remains in the excitation level before returning to the ground state. As shown in **Table 1**, the fluorescent decay kinetics of both protoporphyrin and carious tooth sample region are quite comparable, with a similar proportion of molecules having lifetimes of 3 and 17 ns [57].

In dental caries, the endogenous porphyrins are derivatives from bacteria [52]. As shown in **Table 2**, their presence is not a unique property of bacteria associated with dental caries. Bacterial strains such as *Bacteroides intermedius* and *Pseudomonas aeruginosa*, not associated with dental caries, when grown on agar plates and excited by 407 nm light, also display emission maxima at 635 and 700 nm [57]. Likewise, *Corynebacterium* species emit fluorescence around 620 nm, which corresponds to coproporphyrin fluorescence. Importantly, some key Gram-positive bacteria involved with dental caries, such as *Streptococcus mutans* and various *Lactobacilli* species, do not show strong porphyrin fluorescence in the red spectral region.

Table 3 presents summary details of the major and minor peak wavelengths for dental plaque biofilms growing on contaminated tooth surfaces. At 400–500 nm excitation wavelengths, the major and minor fluorescent peaks are mostly within the range of 610–614 nm, whereas

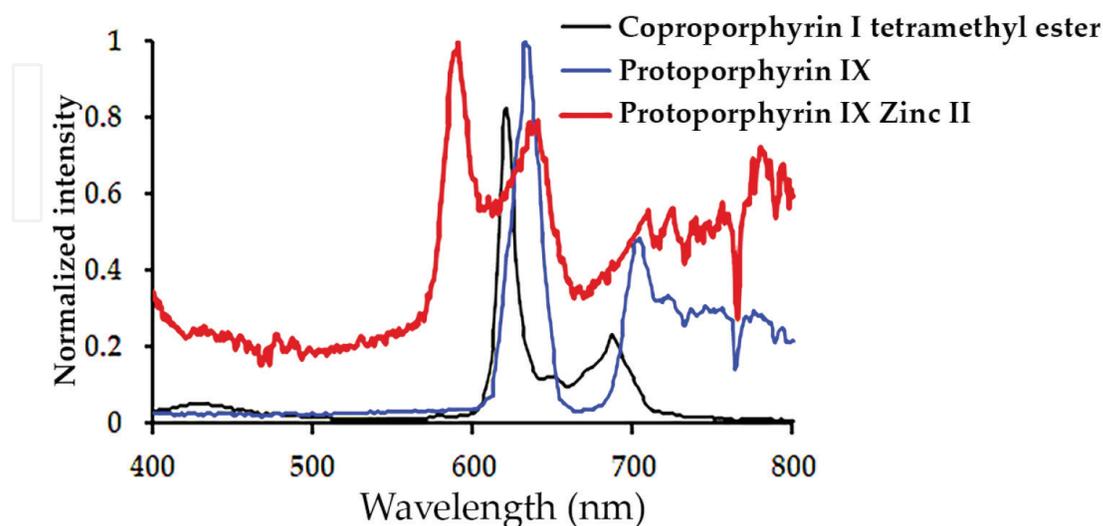


Figure 6. The normalized fluorescence spectra of various porphyrin derivative solutions at 350 nm excitation in the solvent dimethyl sulphoxide.

Sample type	Fluorescence lifetime (ns)	Fluorescent decay time (%)
Coproporphyrin	20	100
Protoporphyrin	3	11
	17	89
Zn-protoporphyrin	13	8
	2	92
Non-cariou region	0.5	15
	9.8	39
	3.2	46
Cariou region	0.31	7
	2.3	11
	17.3	62

Based on Ref. [57].

Table 1. Fluorescence decay kinetics of different porphyrins and samples from carious and non-cariou regions of teeth, and their percentage of occurrence.

Bacteria	Peak fluorescence
<i>Actinomyces odontolyticus</i>	635 nm
<i>Bacteroides intermedius</i>	636, 708 nm
<i>Pseudomonas aeruginosa</i>	636, 618, 703 nm
<i>Streptococcus mutans</i>	Non-fluorescent
<i>Streptococcus faecalis</i>	Non-fluorescent
<i>Lactobacterium casei</i>	Non-fluorescent
<i>Lactobacterium acidophilus</i>	Non-fluorescent
<i>Candida albicans</i>	620 nm
<i>Corynebacterium</i>	620 nm

Based on Ref. [57].

Table 2. The peak fluorescence of different oral microorganisms at 407 nm excitation.

with excitation at wavelengths above 500 nm, the major and minor peaks are now mainly in the near-infrared spectrum, particularly around 825 nm. The fluorescence profiles for dental plaque or biofilms on the tooth surfaces are less intense in the visible red spectrum than those for dental caries and dental calculus [53].

Excitation wavelengths	Major peaks	Minor peaks
400 nm	—	—
425 nm	—	610 nm.
450 nm	614 nm	625, 704 nm
475 nm	610 nm	708 nm
500 nm	610 nm	625, 740, 800, 822 nm
525 nm	—	706, 733, 768, 798, 800, 822 nm
550 nm	—	708, 748, 759, 783, 824 nm
575 nm	—	708, 742, 765, 779, 822, 849 nm
600 nm	—	757, 766, 794, 828 nm
625 nm	825 nm	762 nm
650 nm	825 nm	761, 794 nm

Table 3. Major and minor fluorescence emission peaks for dental plaque biofilm on teeth.

9. Conclusions

Fluorescence spectroscopy has a significant value for laboratory assessment of complex materials and mixtures, including biofilms. The principles can be applied directly to clinical devices that use fluorescence principles for improved diagnosis and clinical care [47, 58] in detection and diagnosis of bacterial biofilms from target biological samples. Fluorescence has particular applications for detecting bacteria because of their porphyrin derivatives, both within planktonic bacteria and within bacterial biofilms, and there already is good support for the presence of porphyrins within target tissue samples [59–61]. These porphyrin derivatives generate visible red emissions from bacterially contaminated sites, whereas healthy tissue sites that are free from bacteria lack such fluorescence. A key direction for further work is to move towards more sensitive methods for analysis, such as using faint laser emissions as an excitation source and either avalanche diodes or PMTs as detectors. This should allow detection thresholds to move down to the level of single bacteria.

Fluorescence spectroscopy can be used to extend the use and application of optical methods [62] and particularly light-induced fluorescence devices [63] in clinical practice. Fluorescence can be used to identify infected target surfaces and to guide clinicians by providing feedback during ablation. This allows infected sites to be detected and ablated using an autopilot approach with maximum accuracy [51, 64].

Past studies have shown that visible red (655 nm) laser-induced fluorescence has clinical applications for guiding bacterial removal, on the basis that near-infrared emissions are likely to be

from porphyrins of bacterial origin [21, 65]. Overall, visible light has applicability for eliciting fluorescence from porphyrins for detecting bacteria and their products present in infected tissues, with emissions in the visible red region. This highlights the value of fluorescence as a non-invasive adjunct to conventional clinical examination in detection and diagnosis of infected surfaces.

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