

In-vivo fluorescence detection and imaging of porphyrin-producing bacteria in the human skin and in the oral cavity for diagnosis of acne vulgaris, caries, and squamous cell carcinoma

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ABSTRACT

Certain bacteria are able to synthesize metal-free fluorescent porphyrins and can therefore be detected by sensitive autofluorescence measurements in the red spectral region. The porphyrin-producing bacterium *Propionibacterium acnes*, which is involved in the pathogenesis of acne vulgaris, was localized in human skin. Spectrally-resolved fluorescence images of bacteria distribution in the face were obtained by a slow-scan CCD camera combined with a tunable liquid crystal filter. The structured autofluorescence of dental caries and dental plaque in the red is caused by oral bacteria, like *Bacteroides* or *Actinomyces odontolyticus*. "Caries images" were created by time-gated imaging in the ns-region after ultrashort laser excitation. Time-gated measurements allow the suppression of backscattered light and non-porphyrin autofluorescence. Biopsies of oral squamous cell carcinoma exhibited red autofluorescence in necrotic regions and high concentrations of the porphyrin-producing bacterium *Pseudomonas aeruginosa*.

These studies suggest that the temporal and spectral characteristics of bacterial autofluorescence can be used in the diagnosis and treatment of a variety of diseases.

1. INTRODUCTION

The naturally occurring autofluorescence of cells and tissues is based on biomolecules containing intrinsic fluorophores, such as the amino acids tryptophan and tyrosine, the coenzymes NAD(P)H and flavins, and porphyrins. Porphyrins are derivatives of the tetrapyrrole porphyrin. They are the intermediate products in the synthesis of the metalloporphyrins heme, hemin, and chlorophyll. Normally, the concentration of metal-free porphyrins in cells and tissue is extremely low. However, abnormalities in heme synthesis due to enzyme defects (e.g. porphyrias) can cause abnormal porphyrin accumulation¹.

In addition to enzyme defects, various microorganisms can produce high levels of endogenous porphyrins. For example, certain species of the genus *Bacteroides* produce protoporphyrin IX (PP). These anaerobic bacteria belong to the normal flora of the oral cavity and intestine². Also *Propionibacteria* synthesize porphyrins³ as do some strains of *Clostridium* or *Actinomyces*⁴. The significance and optimal conditions for bacterial porphyrin synthesis are not well studied.

The normal flora of human skin contains large amounts of the Gram positive bacterium *Propionibacterium acnes* (*P. acnes*). *P. acnes* is involved in the pathogenesis of the widespread skin disease acne vulgaris. From in-vitro studies with bacterial cultures, it is known that this bacterium synthesizes coproporphyrin (CP) and protoporphyrin (PP)^{3,5,6}.

Porphyrins absorb mainly around 400 nm and emit in the red spectral region. It should be therefore possible to detect porphyrin-producing bacteria by their autofluorescence.

Indeed, as early as 1927, Bommer⁷ investigated his patients with a Wood lamp and reported orange and red fluorescence from skin and teeth.

Tumor tissue may also exhibit red autofluorescence, but the precise nature and source of the fluorophores (e.g. mutagenic cells with enzyme defects or bacteria) are not well characterized⁸⁻¹⁷.

The goal of this study was to detect and image various pathological bacteria in human skin and mouth by laser-induced autofluorescence.

2. MATERIALS AND METHODS

2.1. In-vivo fluorescence spectrometer

The spectrometer consists of a krypton ion laser at 407 nm as excitation source, a fiber optical sensor, a polychromator and optical multichannel analyzer as detection unit⁶.

2.2. Steady-state imaging

Images of the 407 nm excited fluorescence were obtained by means of a color CCD camera combined with a dichroic filter (cut-off wavelength: 580 nm) or with a slow scan, cooled CCD camera combined with a birefringent tunable liquid crystal filter for spectrally-resolved imaging (VariSpec, Cambridge Research and Instrumentation), Fig. 1.

2.3. Time-gated imaging

Time-gated imaging in the ns-region was performed with a highly sensitive CCD camera with a fast shutter system (minimal time gate: 5 ns)¹⁸. Fluorescence excitation was provided by a frequency-doubled, Q-switched Nd:YAG laser at 532 nm (pulse duration: 2 ns), Fig. 2.

2.4. Objects of investigation

Bacteria in human skin and the oral cavity were detected by *in vivo* measurements on volunteers. In addition, biopsies of squamous cell carcinoma (1 hour after operation) were used.

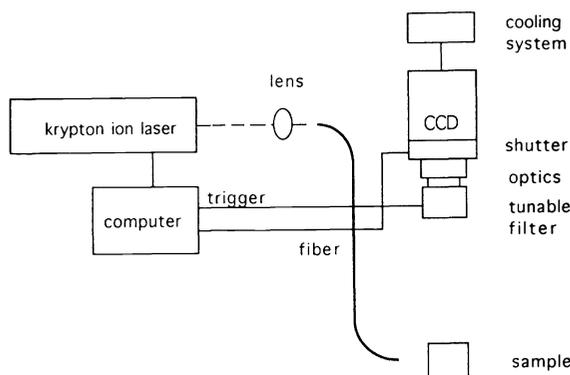


Fig.1 Set-up for spectrally-resolved autofluorescence imaging

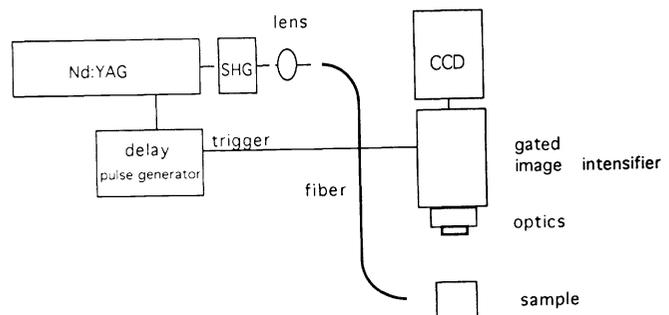


Fig. 2 Set-up for time-gated autofluorescence imaging



Fig. 3 Spectrally-resolved images of skin autofluorescence

3. RESULTS AND DISCUSSION

3.1 In-vivo detection of bacteria in human skin

Fluorescence imaging and spectral analysis of different skin areas (face, back) was carried out on 30 persons with and without acne vulgaris. All persons excited with the 407 nm radiation of the krypton ion laser showed strong fluorescent spots in the skin, mainly in the nasal region and in pimples of acne patients. These spots correspond to sebaceous follicles which contain large amounts of *P. acnes*. They differ in color (yellow or red).

The average spectral fluorescence characteristics, as well as the fluorescence of single fluorescent follicles were obtained by spectrally-resolved fluorescence imaging and image analysis. Fig. 3 documents 13 fluorescent images of the nasal region at different wavelengths in the region 590 nm - 720 nm obtained with the tunable filter. The determination of spatially-resolved exact spectra is complicated because of the wavelength-dependent bandwidth and wavelength-dependent transmission of the filter. However, main fluorescence bands in the region 580 nm - 600 nm and around 635 nm could be determined. The first maxima is typical of fluorescent metallo-porphyrins, like Zn-PP and Zn-CP, whereas PP in hydrophobic environment emits around 635 nm. Fig. 4 shows processed color images at 590 nm and 630 nm where spatial differences are clearly observed.

Extracted sebaceous follicles showed spectra similar to the in-vivo skin spectrum. We isolated *P. acnes* from these follicles. The spectral behavior of the bacteria grown on agar was different, Fig. 5. We found bacterial colonies with either a 635 nm fluorescence band (PP), a 620 nm band (CP), and both 635 nm and 620 nm bands. No metalloporphyrin peak was found. The incorporation of zinc may therefore occur in the skin by the uptake of dermal zinc. We varied the pH-value of the agar medium in order to study the pH influence on the spectral behavior but found no dependence of the ratio of the fluorescence intensities at 635 nm to 620 nm.

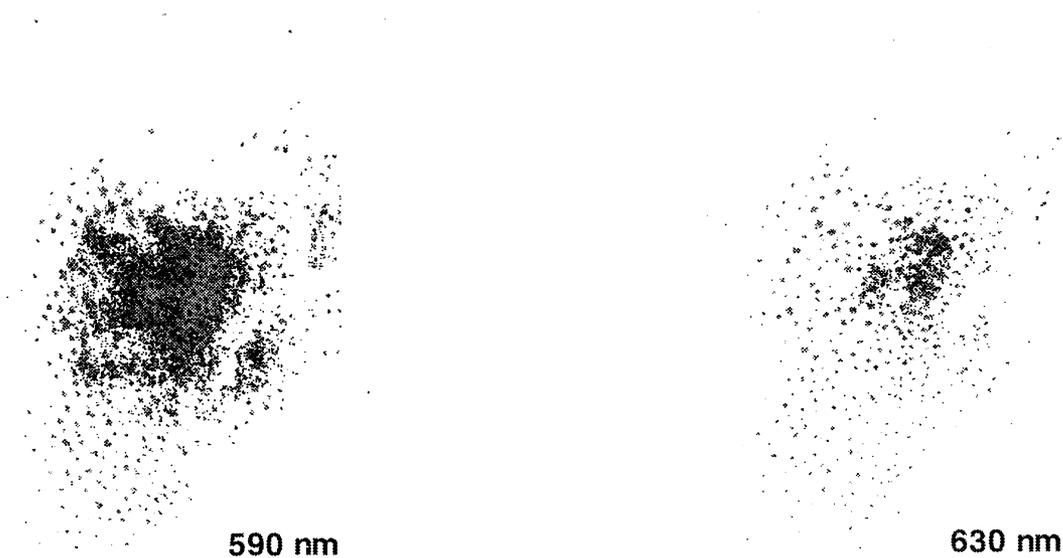


Fig. 4 Autofluorescence images of human skin in the nasal region at 590 nm and 630 nm. The spots indicate the presence of porphyrin-producing *Propionibacterium acnes*.

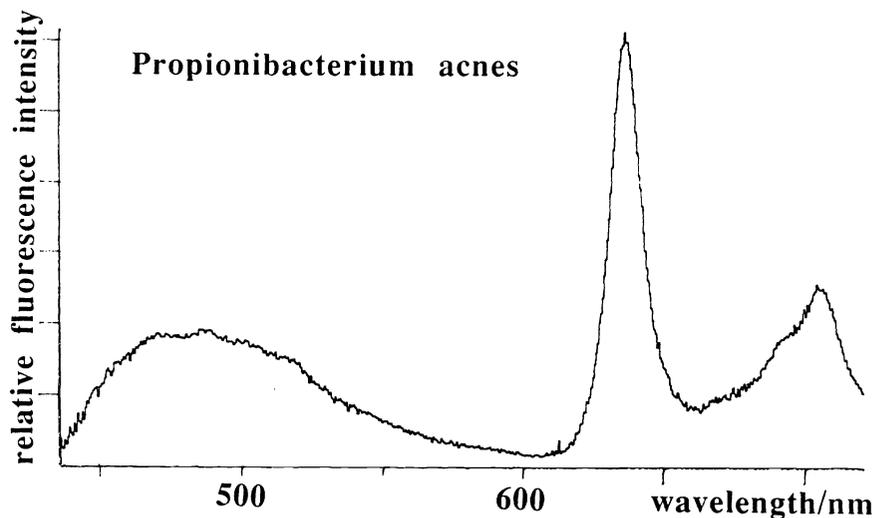


Fig. 5 Autofluorescence spectrum of *Propionibacterium acnes* grown on agar

3.2. Detection of caries- and dental plaque-involved bacteria

We investigated the autofluorescence of about 100 freshly extracted human teeth using 407 nm excitation, the optimal excitation source for porphyrins. It was found, that healthy dental tissue (enamel, dentin) showed a broad emission band in the short wavelength part of the visible spectrum. In contrast, the fluorescence spectrum of all carious lesions consisted of maxima in the red spectral region with a main band at 635 nm. A clear differentiation between healthy and carious tissue was possible. Less than 10% of the teeth showed an additional band around 590 nm and shoulders at 620 nm, Fig. 6. In vivo measurements on patients, using a single detection fiber, showed similar spectra in regions of dental caries.

As mentioned, the maxima at 635, 620 and 590 nm correspond to the emission peaks of PP, CP and Zn-PP in hydrophobic environments. The question arises regarding the origin of these endogenous porphyrins in dental caries. We investigated various cell cultures which can be found in caries and dental plaque. No porphyrin fluorescence in the red spectral region was measured for the bacterial strains *Streptococcus mutans* and various *Lactobacteria* grown on agar plates. However, a strong fluorescence with maxima at 635 nm and 700 nm was detected in the bacterial strains *Actinomyces odontolyticus*, *Bacteroides intermedius* (strongest fluorescence, Fig. 7) and *Pseudomonas aeruginosa*. The microorganism *Candida albicans* and the *Corynebacterium* emitted around 620 nm (CP). These results demonstrate that the red autofluorescence characteristic of caries and dental plaque is based on porphyrin synthesizing oral bacteria found in dental lesions^{19,20}.

High-contrast in-situ video-images of the porphyrin-producing bacteria in the teeth of patients can be obtained by time-gated fluorescence measurements using an appropriate ultrashort time interval of detection. The idea is to consider the different fluorescence decay kinetics of the various endogenous fluorophores and to choose an appropriate time window which isolates the compound of interest. In addition, scattered excitation light can be excluded with sufficient time-delay between ultrashort laser excitation and detection. A preferential application of this method is the detection of metal-free porphyrin monomers. These fluorophores have fluorescence decay times greater than 10 ns. Other endogenous chromophores possess shorter fluorescence lifetimes.

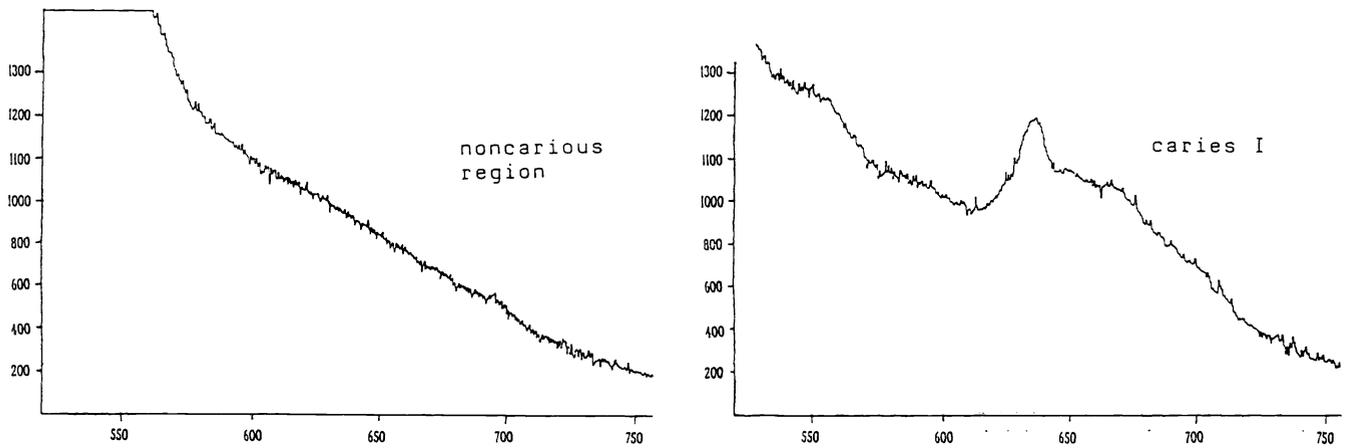


Fig. 6 Autofluorescence spectrum of human teeth with carious lesions

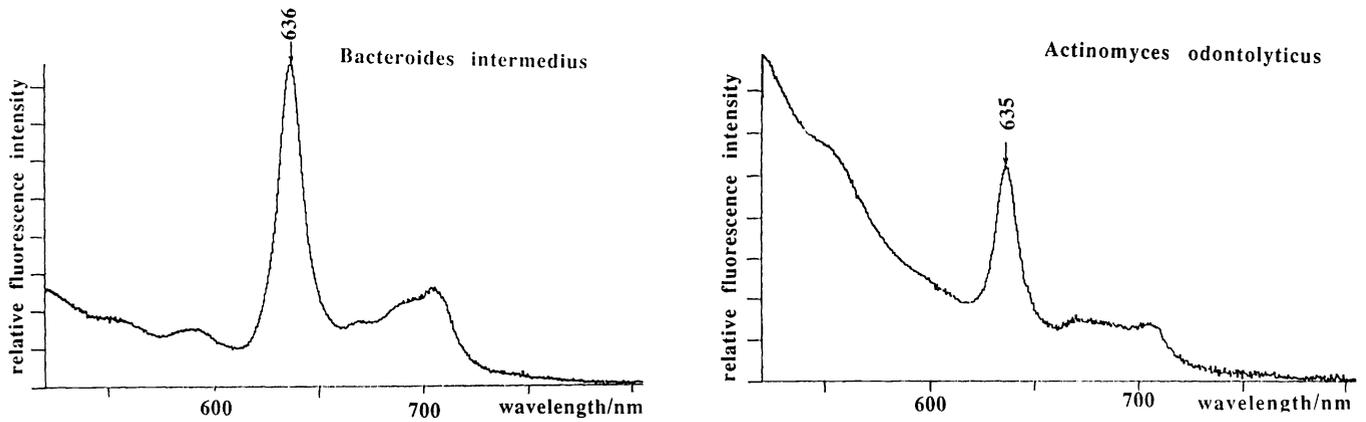


Fig. 7 Autofluorescence of *Bacteroides intermedius* (left) and *Actinomyces odontolyticus* (right)

At first, we took an time-integrated image (cw, detection range: 590-800 nm) of the teeth of a patient with multiple caries- and dental plaque regions. The picture shows nearly homogeneous fluorescence over the entire tooth. This image is mainly determined by autofluorescence with a maximum in the short-wavelength spectral region (Fig. 8, left). Next, we used the time-gated fluorescence technique. Interestingly, with an appropriate time-delay of more than 10 ns (here: detection time: 30-55 ns) only caries and plaque regions become obvious (Fig. 8, right) due to the high concentration of PP-producing bacteria¹⁸.

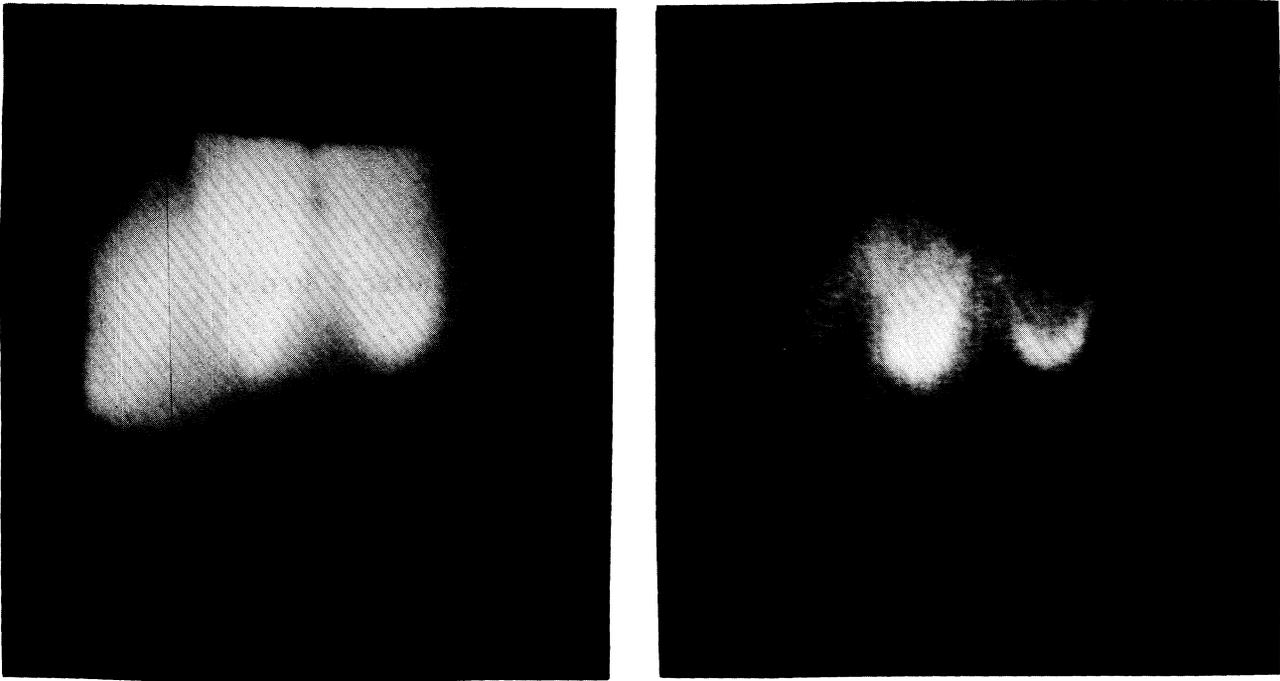


Fig. 8 In vivo bacteria detection in carious regions and dental plaque of human teeth by time-integrated imaging (left) and time-gated imaging (right)

3.3. Bacteria detection in necrotic tumor regions

Biopsies of 30 oral human squamous cell carcinomas were measured 1 hour after removal. The biopsies were stored in 0.9% NaCl. Only 20% of the biopsies showed a weak autofluorescence in the red spectral region (Fig. 9). These samples showed necrotic regions. No fluorescence was found in biopsies from surrounding healthy tissue. The autofluorescence spectrum of the carcinoma consisted of a major peak at 636 nm, typical of PP. Five biopsies showed an additional peak in the 580-600 nm region (Fig. 10, left) where Zn-PP emits. All fluorescent biopsies were stored in glass vials at room temperature and showed a significant increase in fluorescence over the following two weeks (Fig. 10, right). The band at 580 nm became the new spectral maxima, probably due to the insertion of zinc into endogenous PP. Metallation was also observed for free porphyrins stored in glass vials (glassware contains zinc)^{19,20}.

In another study we divided a freshly-removed, weakly-fluorescent biopsy into 3 parts. One was stored in NaCl solution, the second in formalin, and the third in NaCl solution incubated with antibiotics (Polymexin E). Only the first developed strong autofluorescence in the red spectral range.

The increase in fluorescence for NaCl-stored biopsies can be explained by microbial synthesis of PP. When samples were assayed for bacteria, the fluorescent *Pseudomonas aeruginosa* was isolated, Fig. 11.

Our results confirm that the red autofluorescence which can be found in some oral squamous cell carcinomas originates from fluorescent bacteria and not from enzymatic defects in heme synthesis. High concentrations of these bacteria can be found in necrotic and ulcerated tumor tissues.

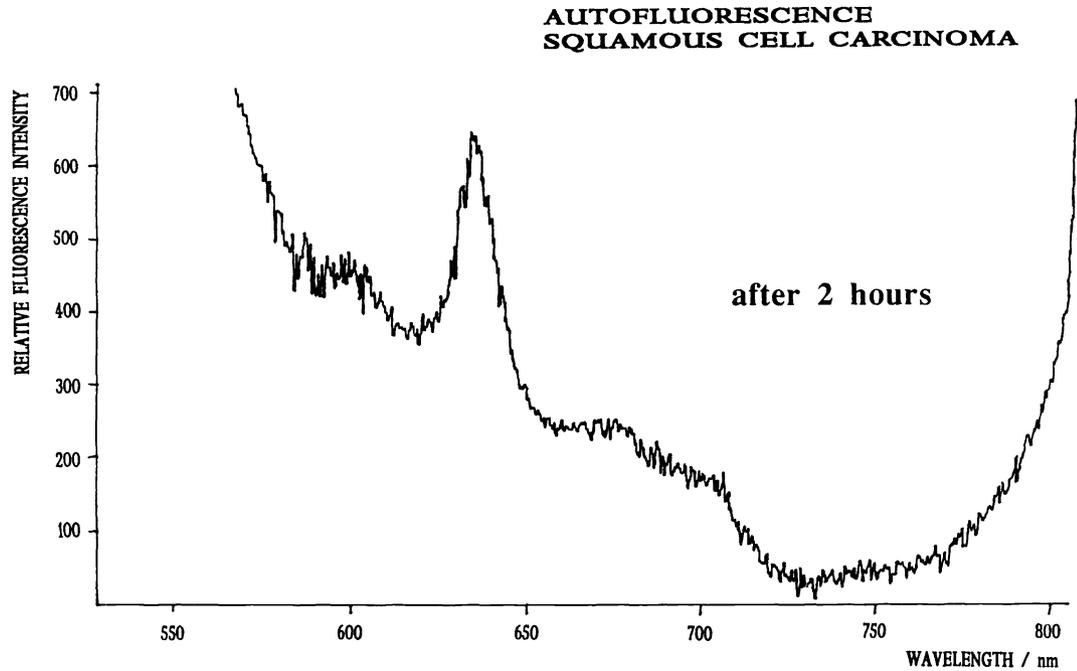


Fig. 9 Autofluorescence spectrum of squamous cell carcinoma

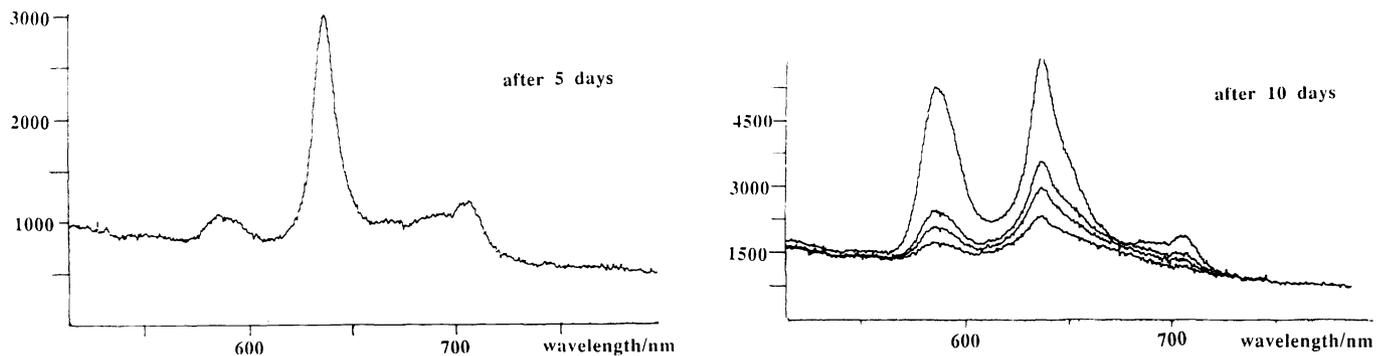


Fig. 10 Autofluorescence modifications of tumor biopsies stored in NaCl solution.
Right panel in presence of 407 nm irradiation with photobleaching

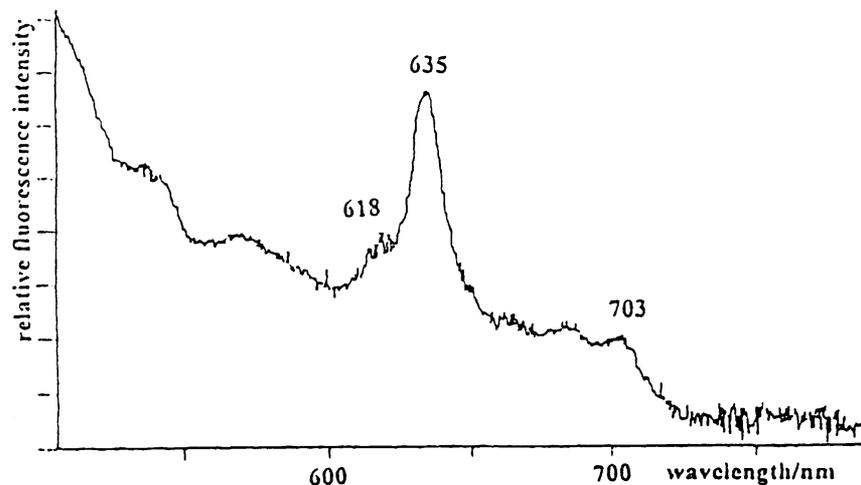


Fig. 11 Autofluorescence of *Pseudomonas aeruginosa*

4. Conclusion

In situ imaging and spectral analysis of pathogenic bacterial autofluorescence is a novel tool which can be used for the diagnosis of a variety of diseases. These techniques can provide information on the location, concentration, and the metabolism of microorganisms. High-contrast in situ "bacterial images" can be created and stored on video tape. In addition, fluorescence measurements can be used for feedback control during laser-based therapeutic procedures.

5. References

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