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Time-gated microscopic imaging and spectroscopy in medical diagnosis and photobiology

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Abstract. An experimental setup was developed for time-gated (nanosecond) fluorescence spectroscopy and imaging of microscopic samples. This makes it possible to depict individual components of complex fluorophores and to measure specific metabolites on the basis of their decay times. The field of applications includes the selective detection of intrinsic fluorophores and of photosensitizers in single cells, skin, and teeth, as well as of photosynthetic pigments in plants under various stress conditions.

Subject terms: time-resolved fluorescence imaging; microspectrofluorometry; photosensitizers; photosynthesis.

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1 Introduction

As has been reported previously,[1,2] various components of complex fluorophores in cells and tissues can be differentiated on the basis of their decay times. This fact was used in fluorescence diagnosis, in particular to discriminate the emission of tumor-specific markers or photosensitizers against the autofluorescence of the tissue.[2–4] After pulsed laser excitation the fluorescence was measured in a certain time gate that was adapted to the lifetime of the relevant fluorophore and allowed for suppression of shorter lived or longer lived components. For a broader field of applications a microscopic setup was developed, where the detection of fluorescence spectra and images is combined. These applications include the detection of individual components of complex photosensitizers in cells and tissues, first steps of diagnosis of skin and teeth (caries) on the basis of specific fluorophores, and measurements of defects to the photosynthetic apparatus of plants.

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2 Materials and Methods

2.1 *Materials*

Photosan 3 (PS 3)—a complex and highly aggregated porphyrin photosensitizer with some tumor-localizing properties[1] (obtained from Seehof Laboratorium, Wesselburener Koog, Germany)—was studied in an aqueous and methanol solution (100 to 300 μg/ml), as well as in single RR 1022 epithelial cells after 3 or 24 h of incubation (5 μg/ml culture medium). In addition, PS 3 and protoporphyrin—as produced endogenously from aminolaevulinic acid (ALA)—were measured in the chick chorioallantoic membrane (CAM)[5] and in human skin after laser ablation of the stratum corneum. ALA-induced photosensitization has been reported for the epidermis, as well as for basal cell and squamous cell carcinoma, which originate from malignant transformation of epidermal cells.[6] Therefore, topical application

lived monomers are located in the plasma membrane, whose contribution to the fluorescence signal at 0 to 5 ns is comparatively low. The nuclear membrane, mitochondria, and (in some cases) the cell nucleus were identified as further sites of monomer accumulation. Since monomeric porphyrins were reported to have the best photosensitizing properties,[13] these sites, as well as the plasma membrane, are supposed to be the main intracellular targets of photosensitization.

The method of time-gated photodiagnosis has so far been applied to the skin and teeth of individual patients. In the first case an ALA-containing cream was applied to part of an arm, where within small spots the stratum corneum had been ablated by an Er:YAG laser, such that ALA could be taken up by the epidermal cells. This may reflect a situation similar to a skin tumor (basal cell or squamous cell carcinoma) that selectively takes up ALA after topical application. When detecting fluorescence from the ablated spots and their surroundings, the emission peak of ALA-induced protoporphyrin (around 635 to 640 nm) can be clearly distinguished from the autofluorescence of the tissue. The ALA-induced peak, however, becomes most prominent if a "late" time gate (e.g., 15 to 20 ns after the laser pulse) is selected (Fig. 5). Figure 6 shows the time-integrated and the time-gated (20 to 45 ns) fluorescence images detected at $\lambda \geq 590$ nm. Whereas Fig. 6(a) shows the autofluorescence from the entire illuminated part, in Fig. 6(b) only the ALA-induced fluorescence from the ablated spot is obtained. This, again, proves the advantage of the time-gated detection method.

Figure 7 shows the fluorescence decay kinetics from a carious and a noncarious region of a human tooth. The integral emission from a healthy part of a tooth is often stronger than that of a carious part. Caries fluorescence, however, shows characteristic decay times of porphyrins (see earlier: PS 3) and may be due to porphyrin-producing bacteria. Diagnostics of caries may become possible if a large time delay (about 25 ns or more) between the exciting laser pulse and fluorescence detection is selected. In Fig. 8 the time-integrated and the time-gated (30 to 55 ns) images of the incisors of a human patient with carious regions at the bottom are demonstrated. Only in Fig. 8(b) does the caries become evident.

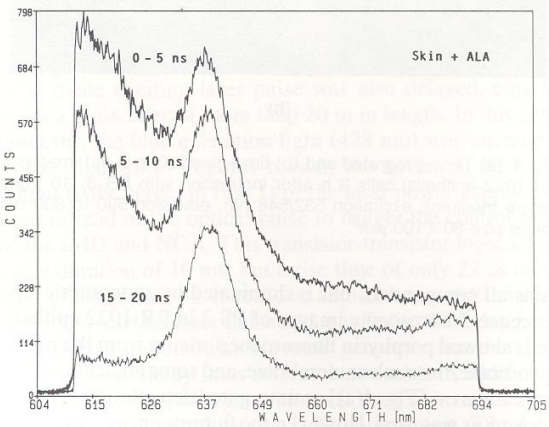
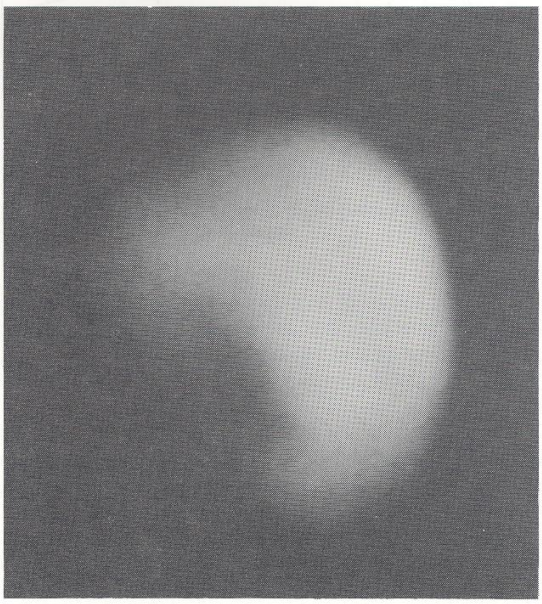


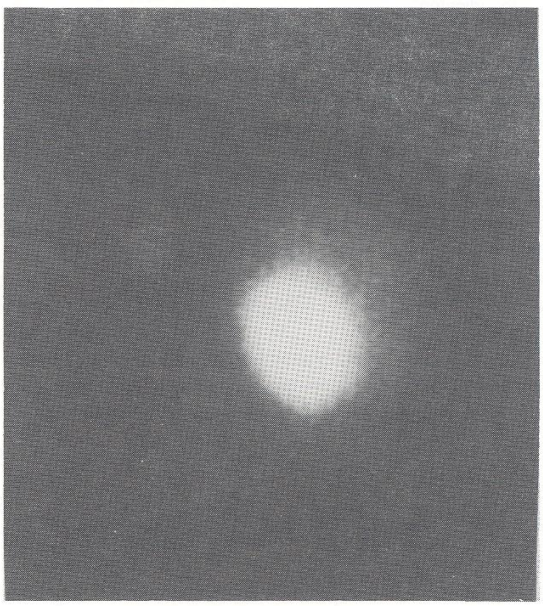
Fig. 5 Fluorescence spectra of human skin within different time gates after topical application of ALA; excitation wavelength 532 nm.

3.2 *Photosynthesis*

As demonstrated recently,[14] defects to the photosynthetic apparatus of plants can be correlated with a "long-lived" component of chlorophyll fluorescence (≥3 ns as compared with 100 to 600 ps for the intact photosystems). The relative



(a)



(b)

Fig. 6 *In vivo* fluorescence of human skin after local ablation of the stratum corneum: (a) time-integrated detection and (b) time-gated detection at 20 to 45 ns; excitation wavelength 532/546 nm, emission measured at 590 to 800 nm, image size 2.5×3 mm^2.

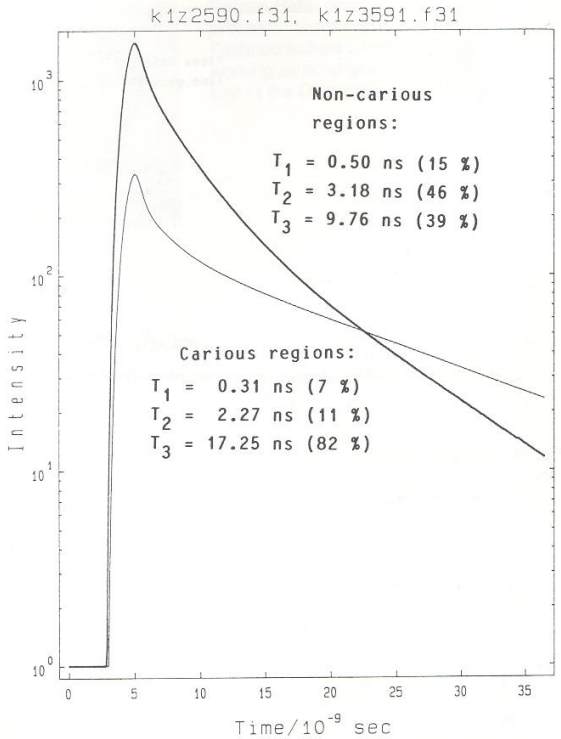
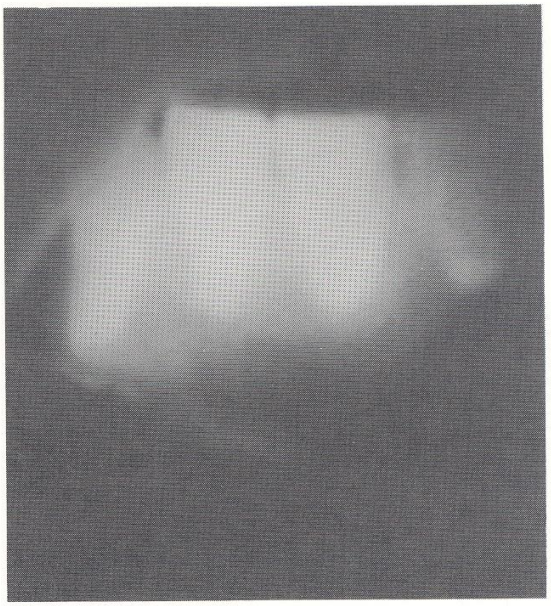


Fig. 7 Fluorescence decay kinetics of a carious and a noncarious region of a human tooth (picosecond excitation pulses at 390 nm; emission measured at 590 to 800 nm).

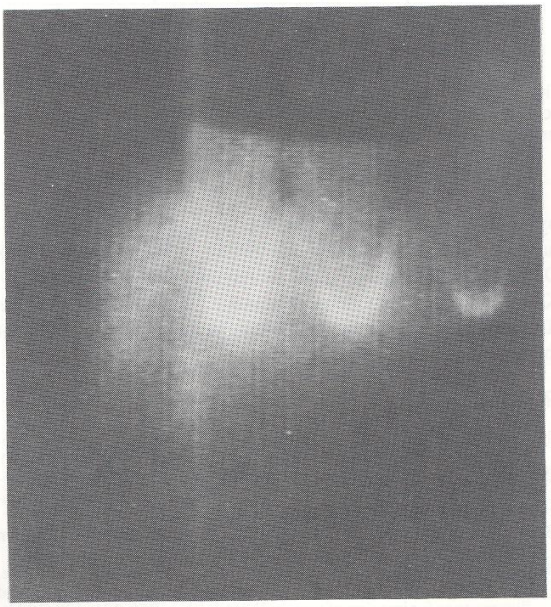
intensity of this long-lived component (I_3) in spruce needles increased with the damage class of the tree (Fig. 9) and was most pronounced in summer, when stress factors such as high light doses, drought, and increased ozone concentrations became prominent. Therefore, during one summer period in 1993, parts of a damaged spruce were exposed to reduced sunlight (about 20%, using a wire mesh), whereas other parts remained exposed to full sunlight. It became evident that (only) at reduced light conditions, I_3 of second-year needles decreased whereas the chlorophyll concentration increased during the summer season. The fluorescence spectra of the long-lived chlorophyll component was obtained within time gates of 10 to 15 ns or 15 to 20 ns (Fig. 10) after the exciting laser pulse. Only after exposure to full sunlight—but not to reduced light—a prominent peak at 685 nm was found, which can be attributed to photosystem II. Therefore, high light doses mainly affect the function of this photosystem.

4 Conclusion

The applications of time-gated fluorescence spectroscopy and imaging allow for (1) an *in vivo* differentiation of various components of complex photosensitizers; (2) the detection of ALA-induced protoporphyrin in human skin; (3) caries detection from the autofluorescence of teeth; and (4) the localization of defects in the photosynthetic apparatus of plants under high (environmental) light exposure. The method appears to be rather universal and may also be applied to quite different topics such as time-resolved transillumination or surface analysis in material science.



(a)



(b)

Fig. 8 *In vivo* fluorescence of human teeth using (a) time-integrated and (b) time-gated (30 to 55 ns) detection at 590 to 800 nm; excitation wavelength 532 nm.

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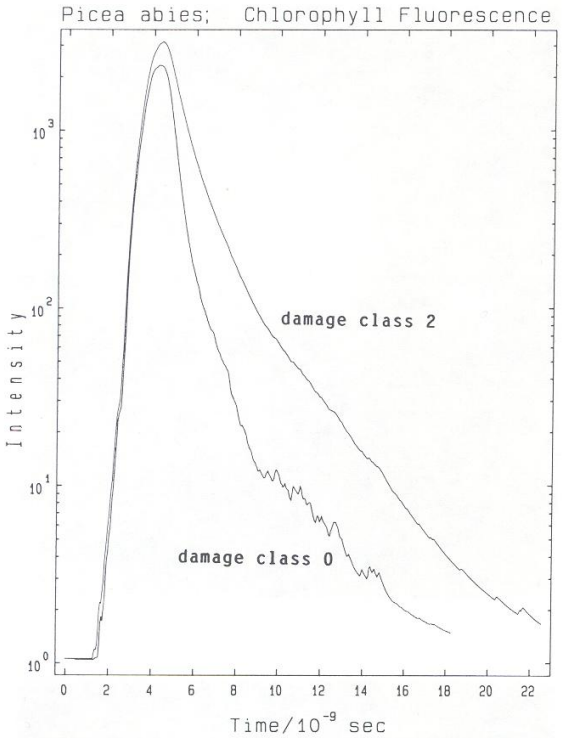

Fig. 9 Chlorophyll fluorescence of about 10 needles of a healthy (lower curve) and a declined (upper curve) spruce; excitation wavelength 668 nm, emission measured at 690 to 800 nm.

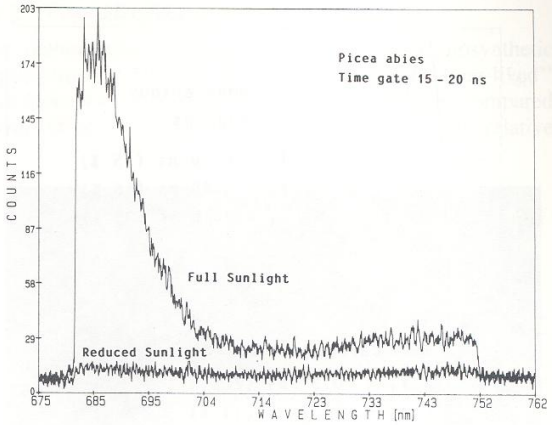

Fig. 10 Emission spectra of the long-lived component of chlorophyll fluorescence as measured in a time gate of 15 to 20 ns for needles with different light exposures (excitation wavelength 428 nm).

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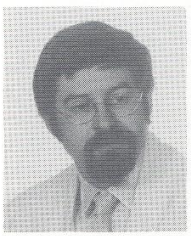

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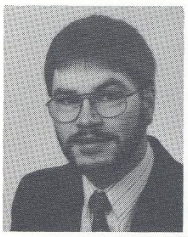

Karsten König received his PhD from the Friedrich Schiller University of Jena in the fields of optical diagnosis, photothermal spectroscopy, and time-resolved fluorescence spectroscopy. He has been working as a scientist at the Institute of Laser Technologies in Medicine at the University of Ulm and is currently profiting from a DFG fellowship at the Beckman Laser Institute in Irvine, California. His work is concentrated on studies of light-induced processes of photosensitizers and biomedical laser applications.

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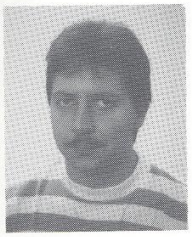

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