Photodynamic tumor therapy and on-line fluorescence spectroscopy after ALA administration using 633-nm light as therapeutic and fluorescence excitation radiation

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Abstract. Photodynamic therapy (PDT) and on-line fluorescence spectroscopy were carried out on human tumors after 5-aminolevulinic acid (ALA) administration using 633-nm light of a dye laser as therapeutic radiation and as fluorescence excitation radiation. This has the advantages of (1) enabling use of one laser for PDT and fluorescence diagnosis only, (2) enabling the possibility of on-line fluorescence measurements, and (3) exciting protoporphyrin molecules in deep tissue layers. Monte Carlo calculations were carried out to determine excitation and fluorescence photon distribution in case of red and violet excitation radiation. The results show the possibility of depth-resolved measurements on the fluorophore distribution by variation of excitation wavelength. The high penetration depth of 633-nm radiation results in a higher ratio of the 700-nm protoporphyrin fluorescence of the xenotransplanted tumor I_t to the skin I_s compared with 407-nm excitation. No values greater than 1 for the ratio I_t/I_s were found in the case of intravenous ALA injection even for red excitation. Therefore, a large amount of ALA will be metabolized in the skin and can cause photosensitivity of the patient when applied systematically. In contrast, protoporphyrin fluorescence limited to the pretreated skin area was detected in case of topically applied ALA to patients with mycosis funcoides and erythroplasy of Queyrat. The influence of remitted excitation light and of the spontaneous radiation from the laser as well as the possible excitation of food-based degradation products of chlorophyll has to be considered in high-sensitivity fluorescence measurements.

Subject terms: PDT; ALA; fluorescence detection; Monte-Carlo simulation; tumor therapy; tumor diagnostics.

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1 Introduction

ALA (5-aminolevulinic acid) is a precursor in the biosynthesis of the metalloporphyrins heme, hemin, and chlorophyll. Each nucleated mammalian cell is able to synthesize heme-containing enzymes. The schematic pathway of heme synthesis is depicted in Fig. 1. ALA-synthetase catalyzed condensation of glycine with succinyl-CoA leads to the formation of ALA and in further catalyzed reactions to protoporphyrin IX (PP IX). Ferrochelatase-catalyzed iron insertion results finally in heme. Heme provides negative feedback on the activity of the enzyme ALA synthetase and therefore limits the concentration of intracellular ALA and PP IX. However, external ALA administration bypasses normal heme regulation. This results in highly efficient PP IX biosynthesis.[1]

PP IX is a well-known fluorescent photosensitizer. Light excitation results in the population of the first singlet state and in its conversion into a triplet state by intersystem crossing. PP IX in this long-lived triplet state is able to transfer energy to molecular oxygen. This results in the formation of singlet oxygen, which causes cytotoxic reactions. These photodynamically induced reactions can be used for photodynamic therapy (PDT) of cancer and other diseases.[2] Kennedy and Pottier[3] have used ALA-induced formation of PP IX in cancer treatment. They applied topical ALA to various skin cancers and obtained selective accumulation in tumor tissues. This selectivity can be explained by the highly efficient penetration through defective stratum corneum in the case of skin cancer and the absence of ALA diffusion into epidermis in the case of the surrounding skin with the intact barrier of stratum corneum.[4]

Localization, efficacy, and pharmacokinetics of ALA-induced biosynthesis of PP IX can be measured using fluorescence detection. The fluorescence excitation spectrum of

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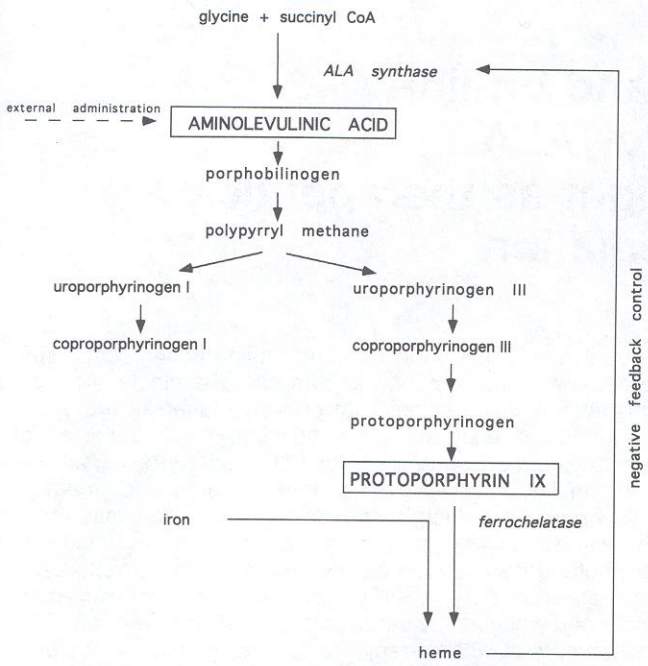

Fig. 1 Schematic pathway of heme biosynthesis.

aqueous protoporphyrin (PP) solution consists of an intense band in the violet spectral region (Soret band) and four smaller Q bands at longer wavelengths. Therefore, fluorescence excitation around 400 nm should be optimal. However, biological tissue contains a large amount of endogenous chromophores, which absorb short-wavelength radiation, like hemoglobin with the main absorption band around 415 nm. This leads to a short penetration depth of the excitation radiation compared with red light excitation.

Normally, radiation in the red spectral region of a dye laser is used for PDT with porphyrins, whereas a krypton ion laser at 407 nm serves as excitation source for the fluorescence diagnosis of superficial tumors.[2]

In this paper, we report on the use of a dye laser at 633 nm for both ALA-PDT and fluorescence measurements. In addition, this procedure allows on-line detection of fluorescence modifications during the treatment. These changes are the result of photodynamically induced porphyrin destruction.[5]

2 Materials and Methods

2.1 *Experimental Setup*

The radiation of tunable dye laser (Kiton Red 620 = Sulforhodamine B, 633 nm, 40 mW) was coupled into the central fiber of a fiber bundle. Eight peripheral fibers transmitted the fluorescence via polychromator into a multichannel analyzer. A dichroic filter was placed in front of the entrance slit of the polychromator to reject parts of the remitted excitation radiation. In addition, an interference filter (633 ± 3 nm) was fixed between resonator and excitation fiber to separate background radiation due to spontaneous emission (see Figs. 2 and 3).

A modified fluorescence spectrometer was used for the measurement of fluorescence excitation spectra.

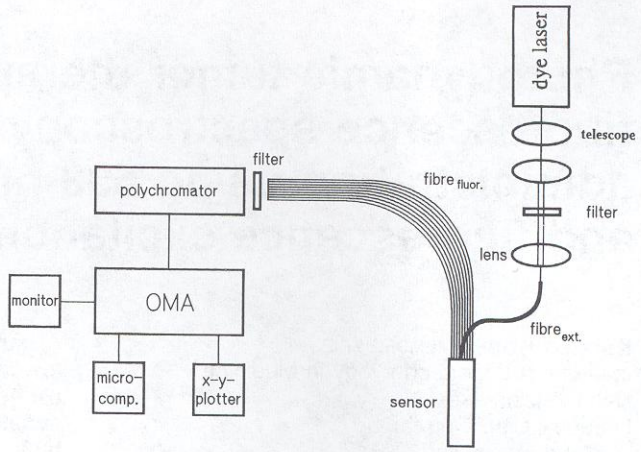

Fig. 2 Experimental setup for PDT and on-line fluorescence measurements.

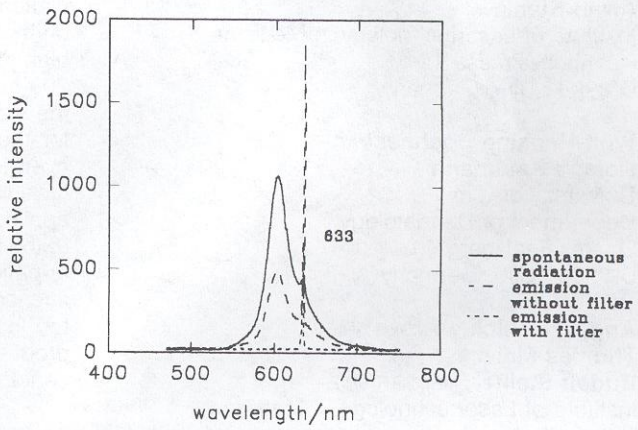

Fig. 3 Spontaneous and stimulated emission of the dye laser with and without an external interference filter at 633 nm.

2.2 *Objects of Investigation*

Human G3 colon tumors were transplanted in female athymic nude mice subcutaneously (NMRI nu-nu, 25 g); each mouse had two tumors. One tumor was irradiated, the other served as control. The skin covering the tumor and surrounding tissue was carefully opened for PDT and fluorescence measurements. ALA-hydrochloride (Sigma, Germany) was dissolved in NaOH and PBS (final pH = 6.8) and given intravenously (vein of the tail) in a concentration of 280 mg/kg body weight.

Written consent was obtained from two patients with mycosis fungoides and erythroplasia of Queyrat who volunteered in participating in fluorescence recordings during topical PDT. Six hours prior to therapy, an ointment containing 20% ALA was applied topically to the lesions to be treated. In the patient with mycosis fungoides in the eczematous stage, lesions at the right forearm were selected for treatment. The patient with erythroplasia of Queyrat showed large areas of erosion covering about 50% of the glans penis.

3 Theory

The usual mathematical approach to describe the light propagation in tissue is the radiative transfer theory.[6,7] The ra-

diative transfer equation can be solved using the Monte Carlo method.[8] This method is often used to calculate absorption, remission, and transmission. However, only few simulations concerning the fluorescence light are reported in the literature.[9] We developed a fluorescence Monte Carlo program, which in contrast to Keijzer et al.,[9] directly simulates the photon propagation through the tissue including the possibility that the incident photons may undergo a fluorescence process.

In our model, the excitation beam with a uniform profile is directed perpendicular to the tissue surface. At the interaction points the photons are absorbed with the probability given by

$$[\mu_{a1}+(1-q)\mu_{a2}]/\mu_t \ ,$$

or converted into a fluorescence photon with the possibility of $q\mu_{a2}/\mu_t$, otherwise it is scattered. The quantity μ_{a2} stands for the absorption coefficient of the chromophore, which emits the fluorescence light with the fluorescence quantum efficiency q, μ_{a1} is the absorption coefficient of all the other chromophores, the total absorption coefficient μ_a is the sum of μ_{a1} and μ_{a2}, and the attenuation coefficient μ_t is the sum of μ_a and the scattering coefficient μ_s. We do not use the principle of weighting, which is normally used in Monte Carlo simulations to reduce computation time. If the photon is absorbed, a new incident photon is launched into the tissue.

The direction of a photon that is transformed into a fluorescence photon is given by an isotropic angular distribution. The photon then is characterized by the new optical coefficients of the fluorescence wavelength μ_{af}, μ_{sf}, and the mean cosine of the scattering angle g_f. At the interaction points the fluorescence photon has the probability μ_{af}/μ_{tf} to be absorbed and μ_{sf}/μ_{tf} to be scattered.

To reduce the computation time, each absorption event was considered to be a fluorescence excitation process ($q=1$). Therefore, some simulation results have to be corrected by a linear factor. In particular, the calculated value of remitted fluorescence light has to be divided by $\mu_a/q\mu_{a2}$.

To test the program we derived an equation for the root mean square of the penetration depth D of the photons, which follows from a statistical calculation of the random walk of the photons through the random medium. Here D is the distance between the incident point of the photons and the point of absorption of the fluorescence light. This equation is exact in the sense of the transport equation.

$$[\langle D^2\rangle]^{1/2}=\left\{\frac{2}{\mu_a[\mu_a+\mu_s(1-g)]}+\frac{2}{\mu_{af}[\mu_{af}+\mu_{sf}(1-g_f)]}\right\}^{1/2} ,$$

with the assumption that medium is infinity and the source is located inside. If the second root term is set to zero, the equation gives the root mean square of the penetration depth of the excitation photons. It was found that the simulation results correspond exactly with the values obtained from the upper formula.

The fluorescence Monte Carlo program was used to determine the remission and the distribution of excitation and fluorescence photons in tissue for 405-nm excitation ($\lambda_f=630$ nm) and for 630-nm excitation ($\lambda_f=700$ nm). The four physical quantities: μ_{a1}, μ_s, g, and the refractive index n, needed for the simulation, were chosen according to data from the literature, e.g., Cheong et al.,[10] as shown in Table 1. The value of μ_{a2} was estimated to μ_{a2} (405 nm) $=0.3\ \text{mm}^{-1}$ and μ_{a2} (630 nm) $=0.07\ \text{mm}^{-1}$, according to the formula $\mu_a=\varepsilon c\ \ln 10$, were ε stands for the extinction coefficient and c for the dye concentration in tissue. The value of q was assumed to be 0.2.

Figure 4 demonstrates the calculated lateral distribution of remitted excitation and fluorescence photons in the case of an excitation beam with 2.5-mm radius. Note that the 405-nm photons are remitted in a relatively small area around the incident excitation beam. In contrast, the points of remitted 630-nm and fluorescence photons can be located some millimeters away. Differences occur also in the distribution of remitted fluorescence photons of 630 and 700 nm.

The dependence of remitted fluorescence on the tissue depth where the fluorescence photons are generated is depicted in Fig. 5. In the case of 630-nm excitation deep lying chromophores can be excited. In contrast, nearly no fluorescence photon will be remitted from a depth of 2 mm with 405-nm excitation.

The mean fluorescence excitation depth in which the remitted fluorescence photons are excited is calculated in dependence on the lateral distribution on the skin surface (Fig. 6). The ratio of the depth for 630-nm excitation to the depth for 405-nm excitation is 2.7 inside the incident beam and becomes greater than 3 for lateral distances greater than 3 mm.

4 Studies on Autofluorescence before ALA Administration

In vivo fluorescence measurements on female nude mice before ALA incubation indicated autofluorescence of the skin with an intense peak around 677 nm for both 407 and 633-nm excitation. However, differences occurred in the spatial dependence of the fluorescence intensity. A pronounced emission in skin regions covering the stomach and intestine areas was found in case of red light excitation in contrast to violet excitation. Thus, a clear localization of the intestine organs by noninvasive *in vivo* measurements was possible with 633-nm radiation (Fig. 7). The strong autofluorescence of these organs and the weaker skin fluorescence (low accumulation of the fluorophores) are caused by fluorescent degradation products of chlorophyll, which is a main content of the mouse food pellets. We investigated the raw materials of the pellets and found that the emission spectra of cereal corns (maize, soya, wheat, oats) correspond with the *in vivo* skin spectrum (Fig. 8). The fluorescence excitation spectrum ($\lambda_f=677$ nm, detection range; 350 to 630 nm) consists of bands at 410, 505, 533, 558, and 613 nm.

Table 1 Four physical quantities needed for the simulation.

λ/nm	405	630	670
n	1.4	1.4	1.4
g	0.7	0.8	0.85
μ_{a1}/mm^{-1}	0.8	0.2	0.15
μ_{s1}/mm^{-1}	30	14	10

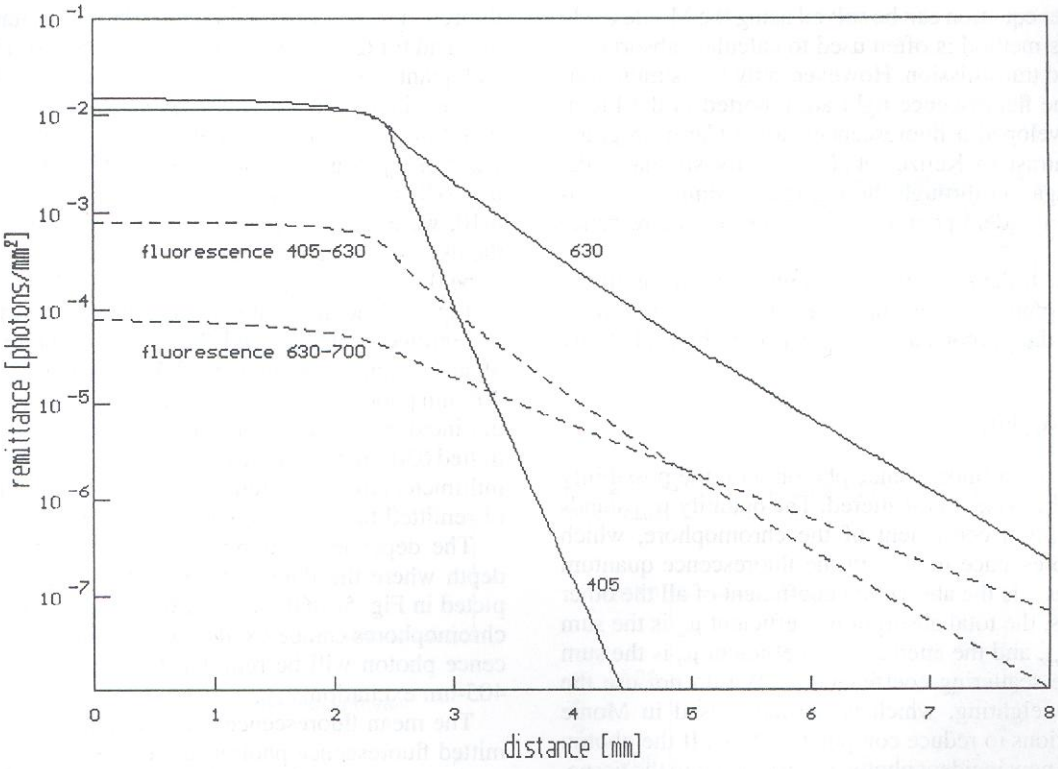

Fig. 4 Remission of incident and fluorescence photons in dependence on the lateral distance from the incident beam (2.5 mm). The points of remitted incident photons at 630 nm and of fluorescence photons can be located some millimeters away in contrast to the remitted 405-nm photons.

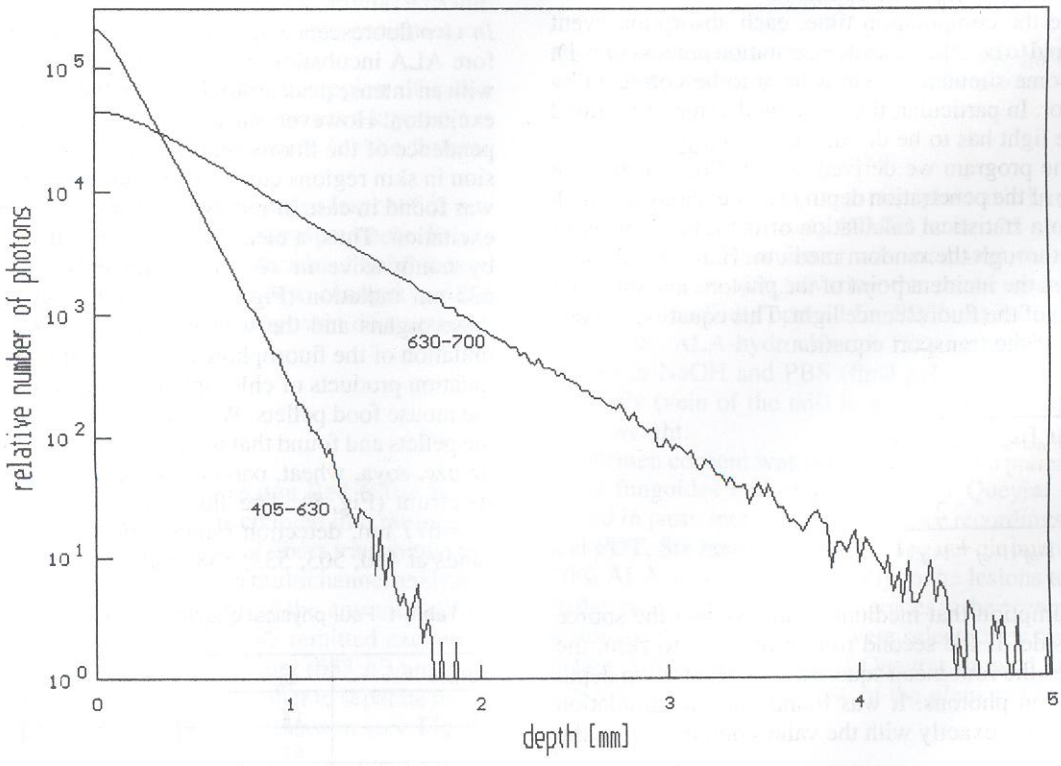

Fig. 5 Depth-resolved remittance of fluorescence photons. Deep lying chromophores can be excited in case of 630-nm excitation.

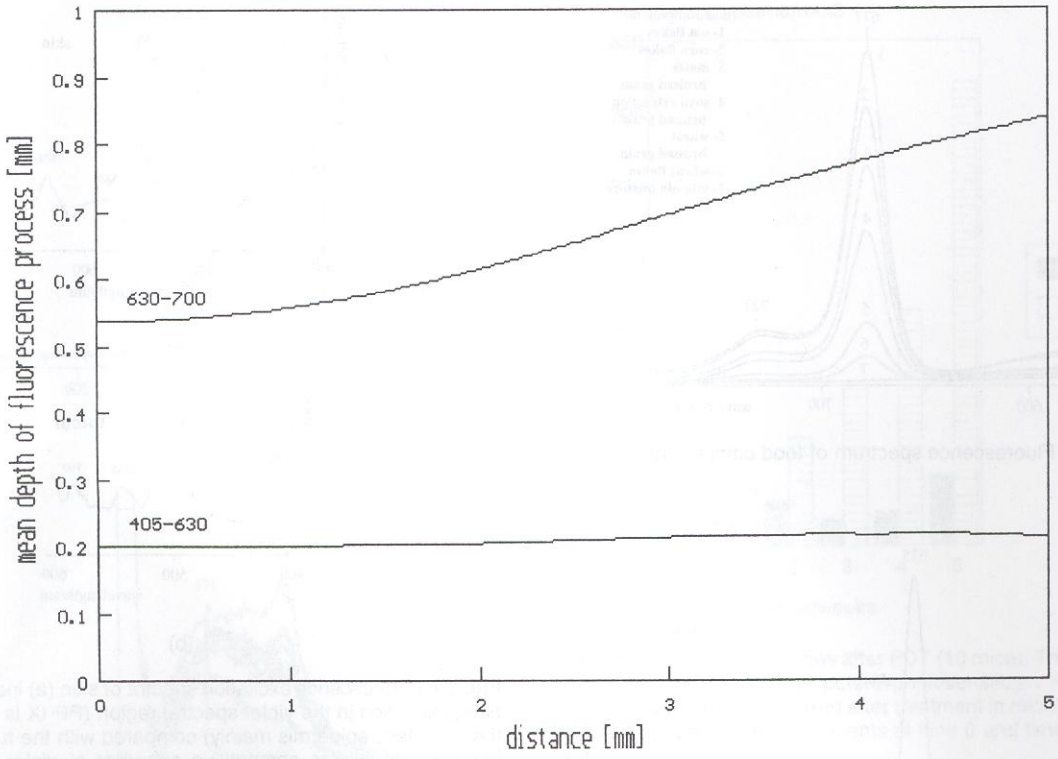

Fig. 6 Mean depth of fluorescence process versus lateral remittance distance. Fluorescence photons, which are remitted far away from the incident 630-nm beam, were generated in deeper tissue layers than remitted photons in short distance.

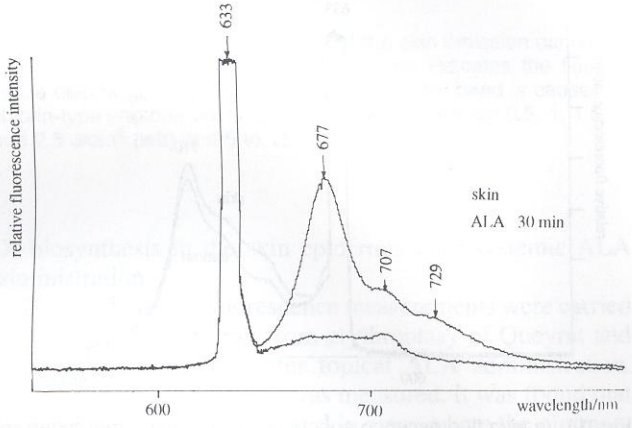

Fig. 7 *In vivo* fluorescence of different skin regions. Upper curve: on skin above the stomach and lower curve: 1 cm away. The peaks at 677 and 727 nm can be attributed to the accumulation of food-based fluorescent degradation products of chlorophyll, whereas the 707-nm peak indicates the beginning of PP biosynthesis after ALA injection. The peak dominates 4 h later.

These results of food fluorescence confirm the findings of Weagle et al.[11] They suppose pheophytins or pheophorbides as responsible chromophores, which can be formed by a photoinduced demetallation process and by phytol chain removal.[12]

The stomach fluorescence was simulated in a simple model consisting of a nonfluorescent tissue layer of 2 mm and a deeper lying fluorescent layer ($\lambda_f = 700$ nm, $q = 1$). The calculation gives a fraction of remitted fluorescence photons compared with the number of incident photons of 0.25% in case of 630-nm radiation and values lower than 0.01% for 405-nm excitation.

These results demonstrate the capability of obtaining information on deep lying chromophores in case of long-wavelength excitation radiation. The ''background'' fluorescence caused by naturally occurring fluorophores and by fluorophores applied by food has to be considered in the fluorescence diagnosis.

Fortunately, the skin regions of the patients investigated before topical ALA administration showed no significant autofluorescence in the red spectral region.

5 Studies on ALA-Induced Fluorescence

The intravenous (I.V.) injection of ALA (280 mg/kg body weight) resulted in a strong fluorescence of the skin of tumor-bearing mice with intense peaks at 635 and 710 nm when excited with 407-nm radiation and emission intensities some orders of magnitude higher than the food-dependent autofluorescence. Figure 9 demonstrates the *in vivo* fluorescence of skin, tumor, and peritumoral muscle tissue, and Fig. 10 the fluorescence excitation spectrum 4 h after ALA application. The position of the bands are typical for endogenous PP.

ALA is metabolized into PP mainly in the upper parts of the skin (epidermis). The blood vessels are mostly located in the deeper-lying dermis. Therefore, the optimal excitation will be with violet light [see Fig. 10(a)]. In contrast, PP will be synthesized in all vital cells of the tumor as shown by confocal laser scanning microscopy on tumor spheroids[13] and hemoglobin will act as competitive absorber of the 407-nm radiation [Fig. 10(b)]. Therefore, ratios of emis-

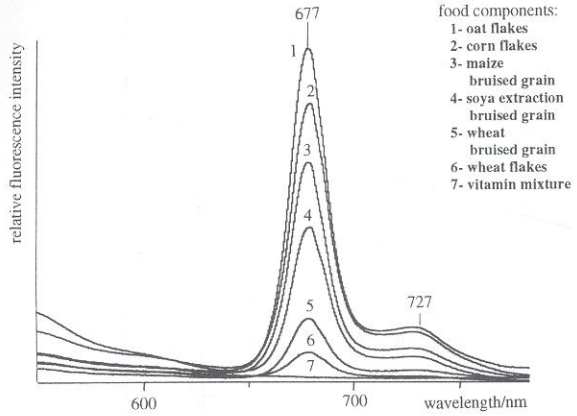

Fig. 8 Fluorescence spectrum of food components.

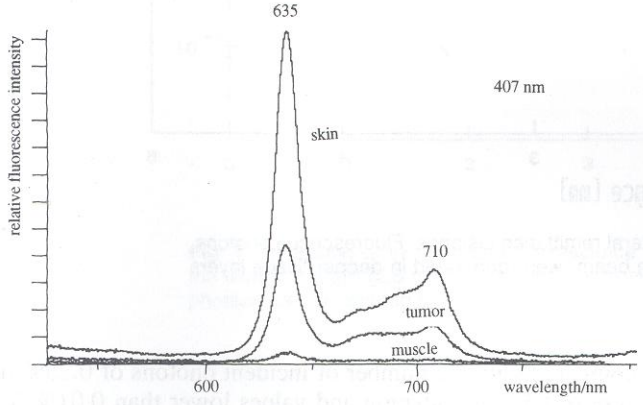

Fig. 9 *In vivo* fluorescence spectra of skin, tumor, and surrounding muscle tissue of a mouse 4 h after intravenous ALA injection. The mean ratio (five mice, four different tissue regions) of the 710-nm fluorescence of tumor to skin I_t/I_s was about 0.6 for 407-nm excitation.

sion signals from different tissues will be dependent on the excitation wavelength. The mean ratio I_t/I_s (five mice, four different tissue regions) of the fluorescence intensities (700 nm) of tumor I_t to skin I_s was around 0.6 when excited with 407 nm and 0.9 to 1.0 in the case of 630-nm excitation (Fig. 11). These findings document the high efficient biosynthesis of PP also in the skin which has to be considered in the case of systemic ALA administration.

The tumor and some areas of skin were irradiated for 30 min. Simultaneously, *in vivo* fluorescence was detected (in real-time modus). Figure 12 demonstrates the photoinduced modifications of the skin emission during this photodynamic treatment. The tumor emission showed a similar behavior. A decrease of the fluorescence intensity at 710 nm was found because of a process of photobleaching. The emission around 670 nm showed no decrease for radiant exposures lower than 5 J/cm^2 and then a slow bleaching effect with a lower rate than for the 710-nm peak. The 671-nm band became the new fluorescence maximum. This emission is different from the ''food peak'' and can be explained by the formation of fluorescent photoproducts. Recent studies[5] have shown that protoporphyrin is transformed into chlorin-type compounds,

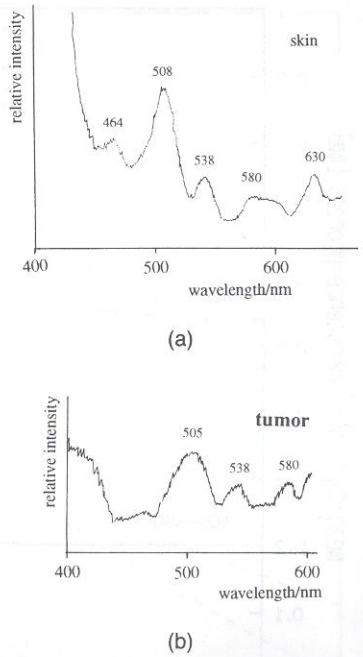

Fig. 10 Fluorescence excitation spectra of skin (a) indicating the optimal excitation in the violet spectral region (PP IX is synthesized in the bloodless epidermis mainly) compared with the tumor (b) where hemoglobin acts as competitive absorber of violet radiation. The bands correlate with the absorption maxima of PP IX in DMSO: 504, 540, 576, and 629 nm.

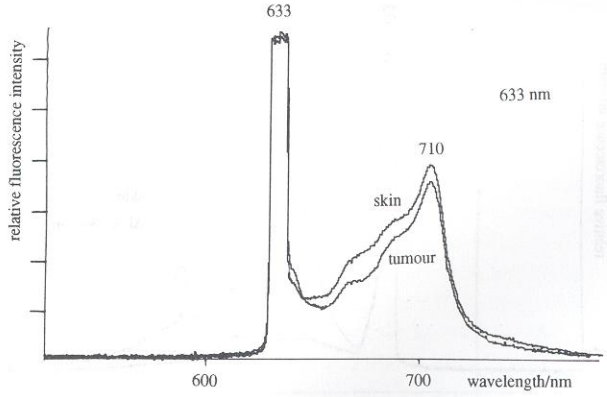

Fig. 11 *In vivo* fluorescence spectrum of skin and tumor when excited with 633-nm radiation. The main ratio I_t/I_s (five mice, four different tissue regions) of the 710-nm fluorescence of tumor to skin was nearly 1.

which are, in turn, also not photoresistant. The process of porphyrin photodestruction depends on singlet oxygen.

Tumor irradiation (two treatments) with a total radiant exposure of 100 J/cm^2 each session results in photodynamically induced tumor destruction. The tumor volumes of the treated animals were determined 0, 1, 2, 3, and 4 weeks after PDT. A significant reduction of the mean tumor volume was obtained compared with untreated control animals. However, a renewed tumor growth after PDT occurred in mice of no complete tumor destruction (Fig. 13).

The irradiation of the skin led to severe skin reactions (erythema, edema, scar formation) as a result of efficient PP

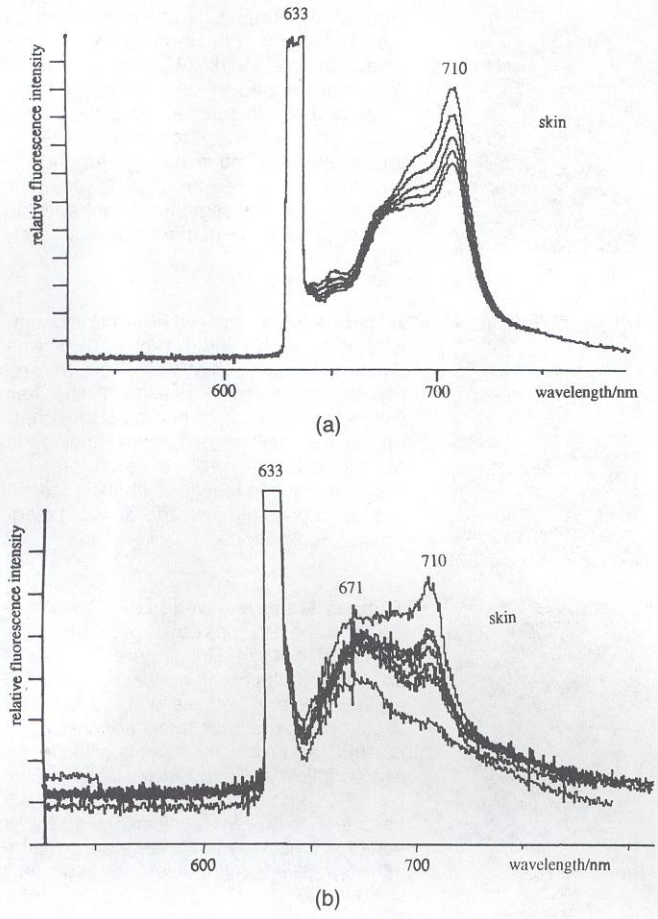

Fig. 12 Photoinduced modifications of the skin emission during the photodynamic treatment. The 710-nm band indicates the fluorescence of endogenous PP, whereas the 671–nm band is caused by chlorin-type photoproducts of PP. Radiant exposures: 0.5, 1, 1.5, 2, and 2.5 J/cm^2 (left) and 5 to 15 J/cm^2 right.

IX biosynthesis in the skin epidermis after systemic ALA administration.

PDT and on-line fluorescence measurements were carried out on patients suffering from erythroplasy of Queyrat and mycosis fungoides 6 h after topical ALA administration. High fluorescence intensity was measured. It was found that fluorescence occurred only in skin areas where the ointment was applied and lesions were present (Fig. 14). No ALA-induced emission was obtained in unaffected tissue areas indicating fast conversion into PP IX compared with diffusion times. Therefore, selective PP IX accumulation can be achieved by topical administration and photosensitivity of the patient and the risk of photodamage in healthy skin areas can be excluded. Dose-dependent photobleaching and photoproduct formation was observed during PDT. Therapy was tolerated without local anesthesia and caused only minor pains described as ''heat-like,'' although skin temperature did not rise more than 2°, as documented by thermorecording during the irradiation.

Acknowledgment

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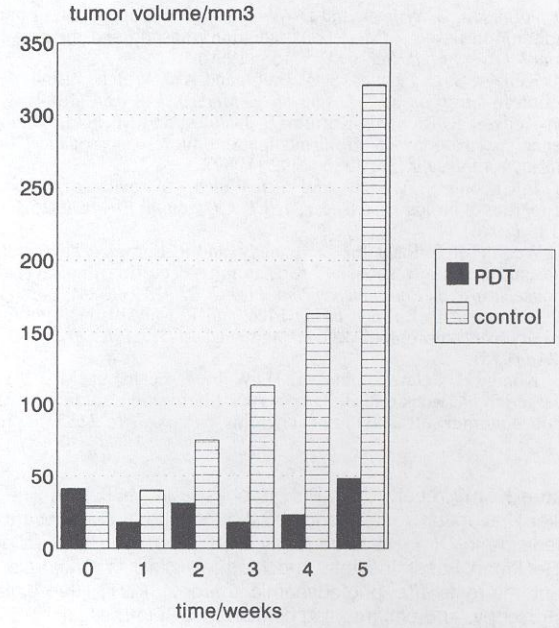

Fig. 13 Mean tumor volumes after PDT (10 mice). The ALA-induced protoporphyrin IX acts as potent photosensitizer. However, a renewed tumor growth occurred after treatment in mice of no complete tumor destruction. Two treatments at time 0 and time 2; 150 J/cm^2; 4 h.

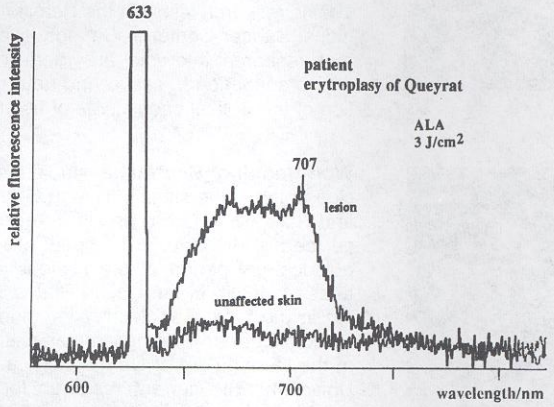

Fig. 14 Fluorescence spectrum of a patient with erythroplasy of Querat 6 h after topical administration of an ointment containing 20% ALA. Fluorescence occurred only in the region where the ointment was applied.

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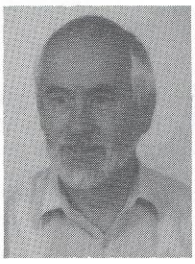
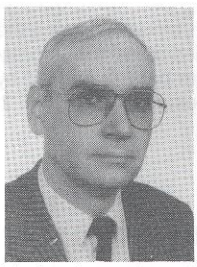
Roland Kaufmann studied medicine at the University of Berne, Switzerland, where he received his MD degree in 1979. After specializing in dermatology he became a senior lecturer at the University of Ulm. His clinical work focused on dermatologic surgery and oncology. Among his topics of recent research are laser applications in dermatologic treatment and the topical application of photodynamic therapy.

Angelika Rück received her PhD in chemistry at the University of Ulm in 1986. She did her thesis on high-resolution IR and MW spectroscopy. Since 1987 she has worked in the field of photodynamic therapy at the Institute of Lasertechnology in Medicine. Her current research interests are light-induced reactions of fluorescence probes in oncology and the possible diagnostics applications.



Thomas Meier received his PhD in physics from the Philipps-University, Marburg, Germany, in 1974. His doctoral thesis was on high-resolution spectroscopy. During his postdoctoral time he worked on laser spectrocopic methods in hfs spectroscopy. In 1982 and 1983 he was a visiting research fellow at Texas A&M, College Station, TX. Since he joined the Institute for Lasertechnology in Medicine in 1986 as senior scientist his activities have concentrated on basic problems of the applications of lasers in medicine.



Rudolf Steiner studied physics at the Technical University (TU) Munich. He worked as research scientist at C.N.R.S., Montpellier, France, and received his PhD in 1972 from the TU Munich. His doctoral thesis was in biophysics. As a North Atlantic Treaty Organization (NATO) research fellow he spent 1972 to 1973 in Montpellier, France, and afterward moved to the University of Düsseldorf, Medical School, where he built his own research group in biophysics and medical laser applications. He received his habilitation for biophysics in 1979. Since 1986 he has been professor at the University of Ulm and directs the Institute of Lasertechnologies in Medicine.