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REVIEW ARTICLE

FLUORESCENCE IMAGING AND POINT MEASUREMENTS OF TISSUE: APPLICATIONS TO THE DEMARCATION OF MALIGNANT TUMORS AND ATHEROSCLEROTIC LESIONS FROM NORMAL TISSUE

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Abstract—The possibilities of using laser-induced fluorescence for tissue diagnostics are discussed. The tissue types investigated are malignant tumors and atherosclerotic lesions. Studies with natural autofluorescence as well as with fluorescent tumor markers are included in this paper. Fluorescence emission and decay data are presented for some tissue chromophores contributing to tissue autofluorescence. Optical spectroscopic characteristics of fluorescent malignant tumor markers are analyzed and instrumental designs for clinical applications are discussed. Images recorded with a multicolor fluorescence imaging system developed in Lund are presented.

INTRODUCTION

Laser-induced fluorescence (LIF)† has been used for many years in chemical and photobiological analysis. During recent years it has attracted interest in medicine, especially in tissue diagnostics. The possibility of demarcating malignant tumors, as well as atherosclerotic lesions from surrounding normal tissue has been in the focus for LIF applications. Quantitative measurements of any chromophore in tissue are very difficult due to the many chromophores in tissue with broad and partly overlapping absorption and fluorescence spectra, and also due to the fact that light propagation in tissue is strongly influenced by scattering. Furthermore, the penetration depth of light in biological tissue is less than a few mm, which means that only superficial regions can be studied using fluorescence. Therefore, a useful application of LIF is in tissue surface diagnostics, for which the exact interpretation of the fluorescence signal is not necessary. The objective is instead to find statistically significant differences between diseased and normal tissue. Such differences are caused by variations in the concentration and distribution of a few chromophores between the diseased and normal tissue. Discussions regarding the statistic analysis of tissue fluorescence diagnostics data are presented by Balchum *et al.* (1982), Profio *et al.* (1983), Alfano *et al.* (1989), O'Brien *et al.* (1989) and Andersson-Engels *et al.* (1990a).

TISSUE FLUORESCENCE CHARACTERISTICS

Several characteristics of tissue fluorescence can be utilized in tissue diagnostic measurements. The fluorescence intensity at a fixed wavelength for various excitation wavelengths (excitation spectra) and the fluroescence intensities at several wavelengths for a fixed excitation wavelength (emission spectra) have been used in measurements. A third property that can be utilized is the rate at which the fluorescence decays following pulsed excitation (fluorescence lifetimes). In Fig. 1 fluorescence decay curves at 380 nm after 337 nm excitation are shown for an atherosclerotic lesion and a non-diseased artery wall. Because several chromophores in biological tissue contribute to the fluorescence, no simple exponential decay can be expected. The curves are fitted to three exponential decays. Furthermore, fluorescence anisotropy, i.e. the dependence of the fluorescence on the polarization angle between the excitation and emission light, might be useful for fluorescence diagnostics in tissue. These characteristics differ for the various chromophores in tissue. From a technical point of view, the simplest and most straightforward way of distinguishing between the contributions from the various tissue chromophores is to study the fluorescence emission spectrum. Using multichannel detection, simultaneous measurements of all emission wavelengths can be performed, eliminating the risk of data dis-

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†*Abbreviations*: BPD-MA, benzoporphyrin derivative monoacid; Hp, hematoporphyrin; HpD, hematoporphyrin derivative; LIF, laser-induced fluorescence; PDT, photodynamic therapy; PHE, poly hematoporphyrin ester; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotaminamide adenine dinucleotide, reduced form; TSPc, tetrasulfonated phthalocyanine.

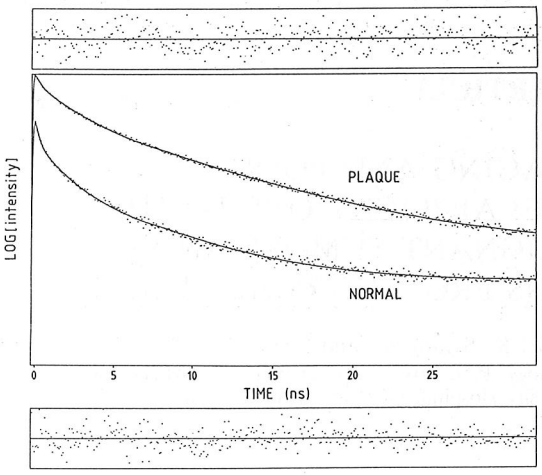


Figure 1. Fluorescence decay curves for human artery samples *in vitro*; calcified plaque (upper) and normal vessel wall (lower). The laser spot was approximately 0.5 mm in diameter. The laser exposure time was 3 min. The samples were kept humid during the investigation. The decays were fitted to $\tau_1 = 7.6$ ns ($A_1 = 22\%$), $\tau_2 = 2.5$ ns ($A_2 = 39\%$) and $\tau_3 = 0.37$ ns ($A_3 = 39\%$) for plaque and $\tau_1 = 6.1$ ns ($A_1 = 9.3\%$), $\tau_2 = 1.8$ ns ($A_2 = 29\%$) and $\tau_3 = 0.22$ ns ($A_3 = 62\%$) for non-diseased vessel wall. The weighted residuals are given for plaque (above) and normal (below). The excitation wavelength was 337 nm and the emission wavelength 380 nm.

tortion due to movements of the investigated tissue region. By using a combination of the different measurement procedures, it may be possible to better distinguish between the contributions to tissue fluorescence from various tissue chromophores. Fluorescence microscopy investigations as described by Blankenborn and Braunstein (1958) and chemical extraction used by e.g. Banga and Bihari-Varga (1974) are other methods useful in defining the chromophores responsible for tissue fluorescence.

In order to gain a better undertanding of the structure and the variations in the tissue fluorescence specta, it would be useful to be able to separate the contributions from different chromophores in the tissue. The major molecules in biological tissue which contribute to the fluorescence signal under near-UV light excitation, have been identified by Baraga *et al.* (1989), Chance *et al.* (1962), Chance and Schoener (1966) and Kozikowski *et al.* (1984) as tryptophan (350 nm emission), chromophores in elastin and collagen (380 nm), nicotinamide adenine dinucleotide (NADH) (470 nm), flavins (520 nm) and melanine (540 nm). Experiments carried out in our laboratory support these findings. Fluorescence decay curves as well as fluorescence emission spectra were recorded for some of these substances and the results are presented in Table 1. Fluorescence from endogenous porphyrins may also be present in the red spectral region, as obtained by Auler and Banzer (1942) and used by Yuanlong Yang *et al.* (1987) for tumor localization. Furthermore, non-fluorescent chromophores, such as hemoglobin, also influence the resulting tissue fluorescence signal by reabsorption of the fluorescent light. The superposition of these contributions to the fluorescence signal results in a smooth, largely unstructured tissue spectrum. Since the absorption and fluorescence characteristics of the various tissue chromophores are known, it should be possible to estimate their concentrations by using a simple theoretical model for the interaction of light with tissue and fitting a model curve to the recorded spectrum. Such theoretical modeling has been described by Profio *et al.* (1984), Richards-Kortum *et al.* (1989) and Andersson-Engels (1989). The major substances contributing to the fluorescence in the near-UV region from an artery wall have been identified by Baraga *et al.* (1989) to be collagen and elastin. Studies of the fluorescence emission and decay in our laboratory suggest that the contributions to the tissue fluorescence from these substances are larger in atherosclerotic lesion than in normal artery wall, leading to a difference between the spectrum from the superficial part of an atherosclerotic lesion and that from a normal artery wall excited at around 340 nm. For malignant tumors the accumulation of HpD gives the main contribution to the difference between malignant tumor and normal surrounding tissue. Furthermore, the intrinsic blue-green tissue autofluorescence can be useful in tumor localization. A decrease in the overall fluorescence intensity in malignant tissue when excited in the near-UV region has been reported by Montán *et al.* (1985). Recently the same observation was reported by Lohmann *et al.* (1989). This decrease in the fluorescence intensity may be due to a change in the equilibrium between the weakly fluorescent NAD^+ and the strongly flourescent NADH. This phenomenon has been observed in a variety of malignant tumors both in human malignancies investigated *in vivo* and in experimental tumors in rats.

CRITERIA FOR FLUORESCENCE DIAGNOSTICS

Due to the unstructured nature of fluorescence spectra from tissue molecules, low-resolution spectroscopy is adequate for tissue diagnostics. In fact, for most cases it is satisfactory to record only a few fluorescence intensities to extract enough information for tissue diagnostics. An automatic evaluation of these signals would be easy. Such an evaluation should preferably compare a few suitable fluorescence intensities by forming a dimensionless function, independent of experimental parameters that remain largely unknown. Such parameters are excitation energy, detection efficiency and geometrical position of the studied tissue with respect to the detection system. A dimensionless quantity can also be formed in such a way that it is insensitive to chromophores in the tissue which have exactly the same influence on the two fluorescence intensi-

Table 1. Fluorescence peak emission wavelengths, decay constants and amplitudes for fluorescent substances excited at 337 nm

Substance	Peak emission wavelength (nm)	Evaluated wavelength (nm)	A_1 (%)	A_2 (%)	A_3 (%)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)
NADH	470	450	21.4	78.6		0.64	0.27	
NAD+	380	430	100			1.21		
Tryptophan	390	380	27.7	72.3	—	2.78	1.47	—
Collagen	390	380	29.7	45.5	24.8	9.94	4.99	0.78
β-Carothene	520	550	6.6	27.8	65.5	9.65	1.96	0.35
Elastin	410	380	11.7	35.8	52.5	6.60	2.11	0.38

The fluorescence decay $I = \Sigma A_i \exp(-t/t_i)$ of each substance was evaluated as consisting of 1, 2 or 3 superimposed exponential decays, according to the best fit.

ties from which a dimensionless ratio is formed. All these effects are cancelled by forming a ratio, since both fluorescence intensities are equally influenced.

Since the fluorescence yields of tissue chromophores are relatively low and the tolerable excitation energy is limited by unwanted side-effects of tissue illumination, such as photobleaching of the fluorescent chromophores and photodynamic effects, the tissue fluorescence signal is weak. For this reason a sensitive detection system must be employed and special care must be taken to suppress any background light. Normally, a photomultiplier or an image intensifier followed by a linear photodiode array or a CCD camera is used as a detector. There are two ways of avoiding disturbances in the fluorescence signal by background light. One is to ensure that no illumination of the tissue, other than the excitation light, is present during the fluorescence recording, the other is to modulate the fluorescence signal in time to make it possible to suppress background light by temporal filtration. In endoscopical applications no background light is present, apart from that from the endoscope lamp. By blocking this during fluorescence recording, the effect on the fluorescence signal due to background light can be eliminated. This idea has been utilized in several diagnostic fluorescence systems, e.g. by Kinsey *et al.* (1978), Profio *et al.* (1979, 1984b), Hirano *et al.* (1989), Baumgartner *et al.* (1987), and Andersson *et al.* (1987a). Another technique has to be applied in situations when the background light cannot be completely blocked. One useful technique is to use a pulsed excitation source and to gate the detection system with very short gating times. In this way the effective active detection time can be reduced by six orders of magnitude, enough to suppress very bright continuous-wave (CW) background light to an undetectable level. This technique is used in many laser spectroscopic applications and has been implemented in a few fluorescence diagnostic systems, e.g. those reported by Andersson-Engels *et al.* (1990a,b).

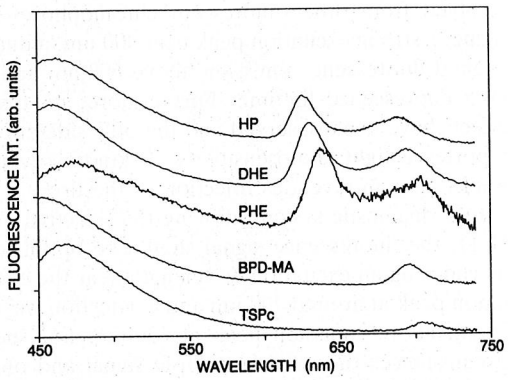


Figure 2. Fluorescence spectra from colon adenocarcinoma (DMH-W49) inoculated subcutaneously on the hind leg of a Wistar/Furth rat. The rats were injected with; HP, fluorescence recorded 2 h post injection, DHE (48 h), PHE (48 h), BPD-MA (3 h) and TSPc (24 h). The drug dose was 15 mg/kg body wt (HP, DHE, PHE and TSPc) and 2.4 mg/kg body wt (BPD-MA). Excitation wavelength was 337 nm except for PHE where 405 nm was used due to the low fluorescence yield. The drug retention times were chosen for optimized tumor selectivity.

TUMOR DETECTION AND LOCALIZATION

Tissue diagnostics for the localization of malignant tumors can be performed by studying the tissue autofluorescence, as suggested by Alfano *et al.* (1984) and Andersson *et al.* (1987b) and has been tested clinically by Yuanlong Yang *et al.* (1987) and Andersson-Engels *et al.* (1990a). The properties utilized were the fact that endogenous porphyrins which fluoresce in the red tend to accumulate in malignant tissue, as discussed by Auler *et al.* (1942), and a change in blue-green fluorescence as discussed above.

Most studies have, however, utilized the dual-peaked fluorescence emission in the red spectral region of the tumor-localizing drug hematoporphyrin derivative (HpD) after excitation in the near-UV or blue region, see Fig. 2. Porphyrins were found by Auler *et al.* (1942) and Figge *et al.* (1948)

to accumulate in tumor tissue. Intravenously injected HpD has been used extensively during the past 10 years for photodynamic treatment of malignant tumors (see e.g. Dougherty (1984) and Manyak *et al.* (1988)), but it can also be utilized as a tumor marker in fluorescence diagnostics of tissue, as reviewed by Profio (1988) and Andersson-Engels *ete al.* (1989a). The main properties of a tumor marker for fluorescence diagnostics are a selective accumulation in malignant tissue and a high fluorescence yield with a characteristic feature that makes it possible to resolve it from tissue autofluorescence. HpD is a good tumor marker in these respects. It exhibits a differential accumulation 24–72 h after injection; the ratio for malignant and normal tissue is about 4:1 (this factor is weakly dependent on the tumor type). HpD has fluorescence characteristics which makes it easy to be resolved from other fluorescent chromophores in tissue: a strong excitation peak near 400 nm, a dual-peaked fluorescence emission above 600 nm and a long fluorescence lifetime. Furthermore, no toxic effects have been observed and the only side-effect reported is light photosensitivity of skin for several weeks after intravenous injection of the drug.

In a diagnostic system utilizing the selectivity of HpD, the fluorescence signal should be optimized by choosing an excitation wavelength near the excitation peak at around 400 nm and a detection wavelength at the emission peak at 630 nm. For such circumstances the maximum HpD signal and only weak fluorescence from other chromophores in tissue will result, since most tissue chromophores have their excitation maximum in the UV region and exhibit only a small amount of absorption in the violet and blue regions. Much clinical experience of such examinations has been gained by several groups, especially Balchum *et al.* (1987) and Hayata *et al.* (1982). Furthermore, the tissue autofluorescence signal should be subtracted to increase the sensitivity of the HpD fluorescence. This can be achieved by using differential excitation or emission spectroscopy near the maximum in the excitation or emission spectrum of HpD, since tissue autofluorescence excitation and emission spectra are both relatively constant in these regions.

Montán *et al.* (1985) suggested that by utilizing the differences in both autofluorescence and HpD fluorescence between malignant and normal tissue, the diagnostics could be improved. It is not at all clear which wavelengths should be chosen to achieve this. Shifting the excitation wavelength further to the UV causes the tissue autofluorescence to increase. The optimal excitation wavelength for fluoresence emission spectroscopy depends on which wavelengths are to be evaluated, but 370 nm was found to be appropriate if the autofluorescence was evaluated at 470 nm and the HpD signal at 630 nm (Andersson-Engels *et al.*, 1988). By forming the ratio between the HpD signal at 630 nm and the tissue autofluorescence at 470 nm, we have previously shown that the discrimination between a malignant tumor and surrounding normal tissue increased by about a factor of 1.5 compared with the pure HpD signal (Andersson-Engels *et al.*, 1989b). Such an evaluation also has the advantage of using a dimensionless quantity.

Obviously, it would be desirable to find a more suitable tumor marker for fluorescence diagnostics than HpD. Higher selectivity for malignant tissue more rapidly after injection, higher fluorescence yield, preferably with very narrow excitation and/or emission peaks and no light photosensitization of the skin, are properties which would improve the possibility of early malignant tumor detection. Alternative drugs to HpD as photosensitizers for fluorescence diagnostics as well as PDT are under development. Fluorescence emission spectra from tumors inoculated in rats injected with different photosensitizers are shown in Fig. 2. For a discussion of the properties of these substances as tumor markers in fluorescence diagnostics, see Andersson-Engels *et al.* (1989b) and references cited therein. Some of the requirements of a good photosensitizer for PDT and a tumor marker for fluorescence diagnostics are the same. Such characteristics are a high selectivity for malignant tissue and a short retention time in tissue. It would also be advantageous if one drug could be used for tumor detection as well as treatment. However, a photosensitizer should have a strong absorption peak in the far red region, where light penetration in tissue is optimal and energy transfer reactions with oxygen are efficient. This means that the lowest excited singlet state would have an excess energy matching light of this wavelength (650–750 nm). A tumor marker, on the other hand, should have the first excited energy level at an energy matching light of a wavelength near 600 nm. In this region light penetration in tissue is much less, which means that light scattering in tissue will not blur a fluorescence image as much as is the case with longer emission wavelengths. The spatial resolution is therefore much higher in a fluorescence image of tissue at this shorter wavelength. Furthermore, photocathode-based detectors are more sensitive to this lower wavelength than to the far red region. Tumor markers with fluorescence at even shorter wavelengths should be avoided, since the tissue autofluorescence background will then be higher, as seen in Fig. 2. Much research work remains before a fluorescent tumor marker better than HpD can be introduced clinically.

PLAQUE IDENTIFICATION

Kittrell *et al.* (1985) indicated that LIF could be utilized as a tool for guiding optical fibers during laser angioplasty. In *in vitro* studies using UV light excitation it has been found by Deckelbaum *et al.*

(1987), Andersson *et al.* (1987c) and Andersson-Engels *et al.* (1989c) that by utilizing only the intrinsic tissue autofluorescence it is possible to distinguish between atherosclerotic lesion and non-diseased surrounding vessel wall. Various diagnostic criteria for atherosclerotic plaque and normal artery vessel wall have been proposed by Bergeron *et al.* (1988), O'Brien *et al.* (1989) and Andersson-Engels *ete al.* (1989c). The main difficulty with this method was identified by Oraevsky *et al.* (1987, 1988), and Richards-Kortum *et al.* (1987) to be interference effects in the fluorescence signal by the heavily absorbing hemoglobin in the blood. Andersson-Engels *et al.* (1990c) showed that by using a diagnostic criterion in the form of a ratio of two fluorescence intensities, equally attenuated by hemoglobin, the diagnosis was much less dependent on the presence of blood than any blood-dependent criteria. *In vivo* investigations by Bonner and Leon (private communication) confirm this observation.

In order to make it possible to use this tool to guide laser angioplasty, it is a necessary fact that the signal changes upon going through the plaque lesion to deeper layers in the artery wall, or the ablation laser will perforate the vessel wall. Since UV light has poor penetration in tissue, only the superficial layers are probed by fluorescence diagnostics. Gmitro *et al.* (1988) showed that only a 150 μm thick tissue layer contributed to the fluorescence signal under 325 nm light excitation. Cutruzzola *et al.* (1989) and Andersson-Engels *et al.* (1989c) showed that the fluorescence spectra of deeper vessel wall layers underneath a plaque lesion were similar to that of a normal artery wall. Some questions remain to be answered before LIF can be used to guide laser angioplasty. One is whether plaque has the same fluorescence characteristics at all depths or if the typical plaque signal only originates from the plaque surface. Another question is whether the laser ablation itself will change the fluorescence characteristics of tissue. Leon *et al.* (1988) and Papazoglou *et al.* (1989) reported a decrease in fluorescence intensity after laser ablation, while the spectral shape seemed to remain relatively unaltered. Lastly, even if the method seems to work in principle—and diagnostic guidance certainly would improve laser angioplasty—one question that must be raised is whether LIF would provide a practical method for the guidance of the optical fiber in thin coronary vessels filled with blood.

IMAGING INSTRUMENTATION

Fluorescence imaging systems for HpD-marked tumors have been developed by Profio *et al.* (1983) and others (for a review, see Profio, 1988). In one construction reported by Profio *et al.* (1986), the background can be intermittently subtracted by switching to a blue transmitting filter. To fully utilize

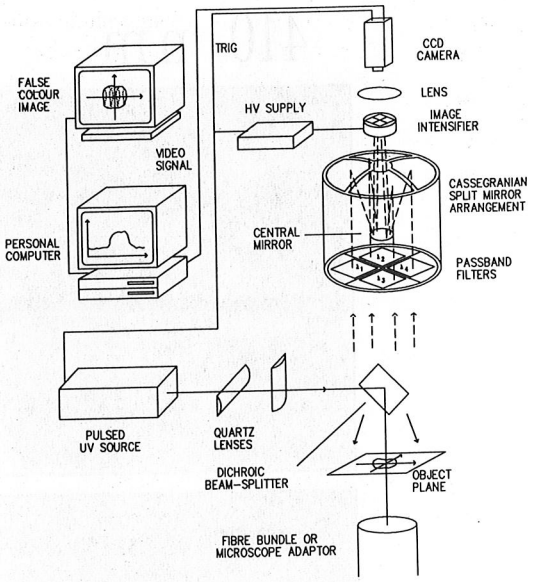
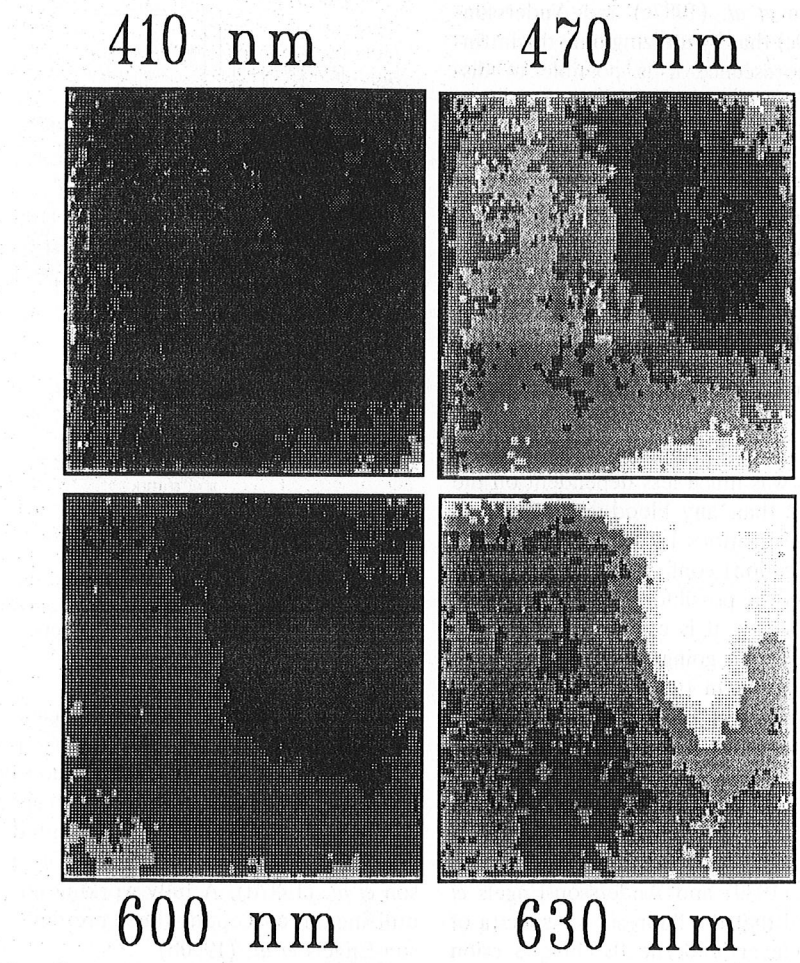


Figure 3. Experimental arrangement for the multicolor fluorescence imaging system developed in Lund.

the potential of the contrast enhancement concepts developed in connection with our point-monitoring studies, it is necessary to simultaneously image the fluorescence of the object in several spectral bands. A concept allowing this was presented by Montán *et al.* (1985) and was further developed by Andersson *et al.* (1987d). A fully working imaging system utilizing this concept has been presented by Andersson-Engels *et al.* (1990b).

In order to simultaneously obtain spatial and spectral resolution, the fluorescence light is divided with a multi-mirror arrangement as shown in Fig. 3. The sample is excited with a PRA Model LN250 nitrogen laser. The individually adjustable segments in the multi-mirror configuration allow four identical images to be arranged as quadrants on an intensified CCD matrix detector (Delli Delti Model CPI/NS2, modified). An interference filter arrangement in front of the split mirror system is used to filter out a fluorescence band for each of the four images. A dimensionless contrast function is calculated for each spatial location using the values in corresponding pixels in the four images. In this way an artificial image is formed. If proper filters are chosen and a proper dimensionless function is used, an image with high contrast for malignant tumors can be displayed using false-color coding. As an illustration of the capability of this system an image of an experimental malignant tumor is shown in Fig. 4. The tumor was a colon adenocarcinoma inoculated on the hind leg of a Wistar/Furth rat. Twenty-four hours prior to the investigation the animal was intravenously injected with 5 mg/kg bodyweight of Photofrin II, a commercially available hematoporphyrin derivative. At the time of the investigation the tumor size was 2 × 3 mm. Figure 4



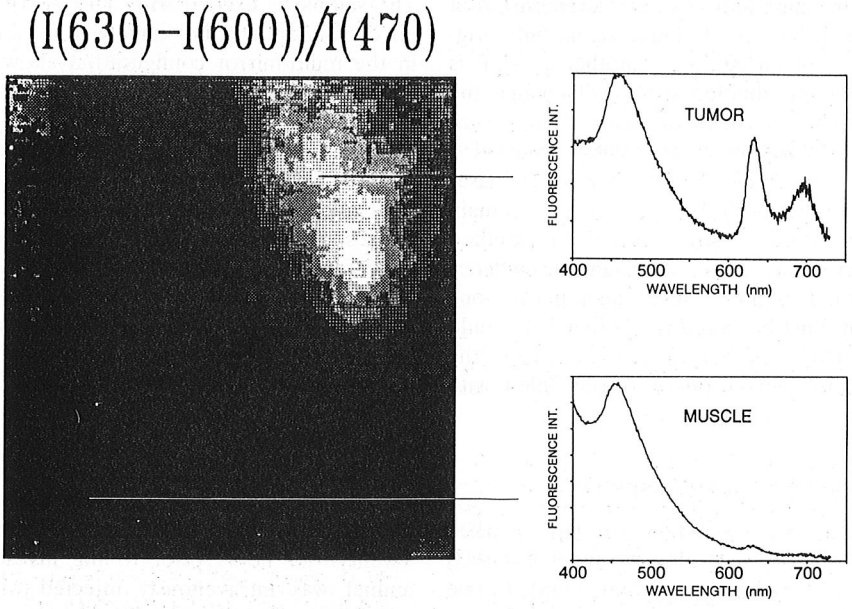


Figure 4. (top) Fluorescence images from a colon adenocarcinoma (DMH-W49) *in situ* inoculated subcutaneously on the hind leg of a Wister/Furth rat. The rat was injected with 5 mg/kg body wt DHE (Photofrin II) 24 h prior to the examination. The excitation wavelength was 337 nm. (bottom) Function image from the four subimages and corresponding spectra.

shows the four images as obtained from the camera, corrected for uneven illumination and the detection efficiency of the detector system. Using the function $F = (I(630\text{ nm}) - I(600\text{ nm}))/ I(470\text{ nm})$ a dimensionless image results, which is shown in the lower part of Fig. 4, together with fluorescence spectra from the tumor and the surrounding muscle. Apart from the advantage of being dimensionless, a better contrast between the tumor and the surrounding muscle results than in any of the sub-images. This is due to the fact that not only the HpD signal but also the tissue autofluorescence contributes to the demarcation of the tumor.

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