In vivo fluorescence in clinical oncology: fundamental and practical issues

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In vivo fluorescence in clinical oncology : fundamental and practical issues

Non-invasive measurements of fluorescence in tissues have the potential to provide new tools in clinical oncology, for screening and diagnostics of early-stage malignancy, for defining tumour extent and spread to adjacent tissues and as a guide for optimizing localized treatments of solid tumours. Much of the impetus for this work has come from parallel developments in photodynamic therapy with fluorescent photosensitizers, but the fluorescence of endogenous molecules (tissue autofluorescence) may also play a role for some applications. In this paper, the fundamental principles of fluorescence measurements in tissue are discussed, the literature on tumour fluorescence is reviewed, and the major outstanding issues in the future development of clinical fluorescence techniques are examined.

Key words : Fluorescence — Oncology — Spectroscopy — Imaging — Haematoporphyrin derivative

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Abbreviations. *Hp :* haematoporphyrin ; *HpD :* haematoporphyrin derivative ; *NADH :* reduced nicotinamide dinucleotide ; *PDT :* photodynamic therapy

Introduction and principles

Fluorescence — based techniques are being used increasingly in biomedical science, as, for example, in fluorescence activated flow cytometry and cell sorting and fluorescence microscopy. These techniques commonly use exogenous fluorescent dyes as markers for specific cellular or tissue components. The use of in vivo tissue fluorescence as a clinical tool is much less developed [1], but includes studies in tissue metabolism, cardiovascular disease, ophthalmology and oncology. The purpose of this paper is to review the work which has been done in oncology, to identify some of the limitations of current approaches and to point to possible future developments. The discussion will be focussed primarily on tissue fluorescence measurements in situ.

In general, the potential applications of fluorescence measurements in clinical oncology can be classified as : cancer screening and diagnosis, tumour staging, tumour localization, including the defining of tumour margins and surgical guidance, and quantification or spectroscopy of endogenous or exogenous fluorophores. In screening, diagnosis and staging the current applications are very limited. Localization and quantification have been more widely used, particularly with photosensitizers as exogenous fluorophores, because of the parallel development of photodynamic therapy, PDT [2, 3].

The fluorescence from endogenous fluorophores (autofluorescence) or

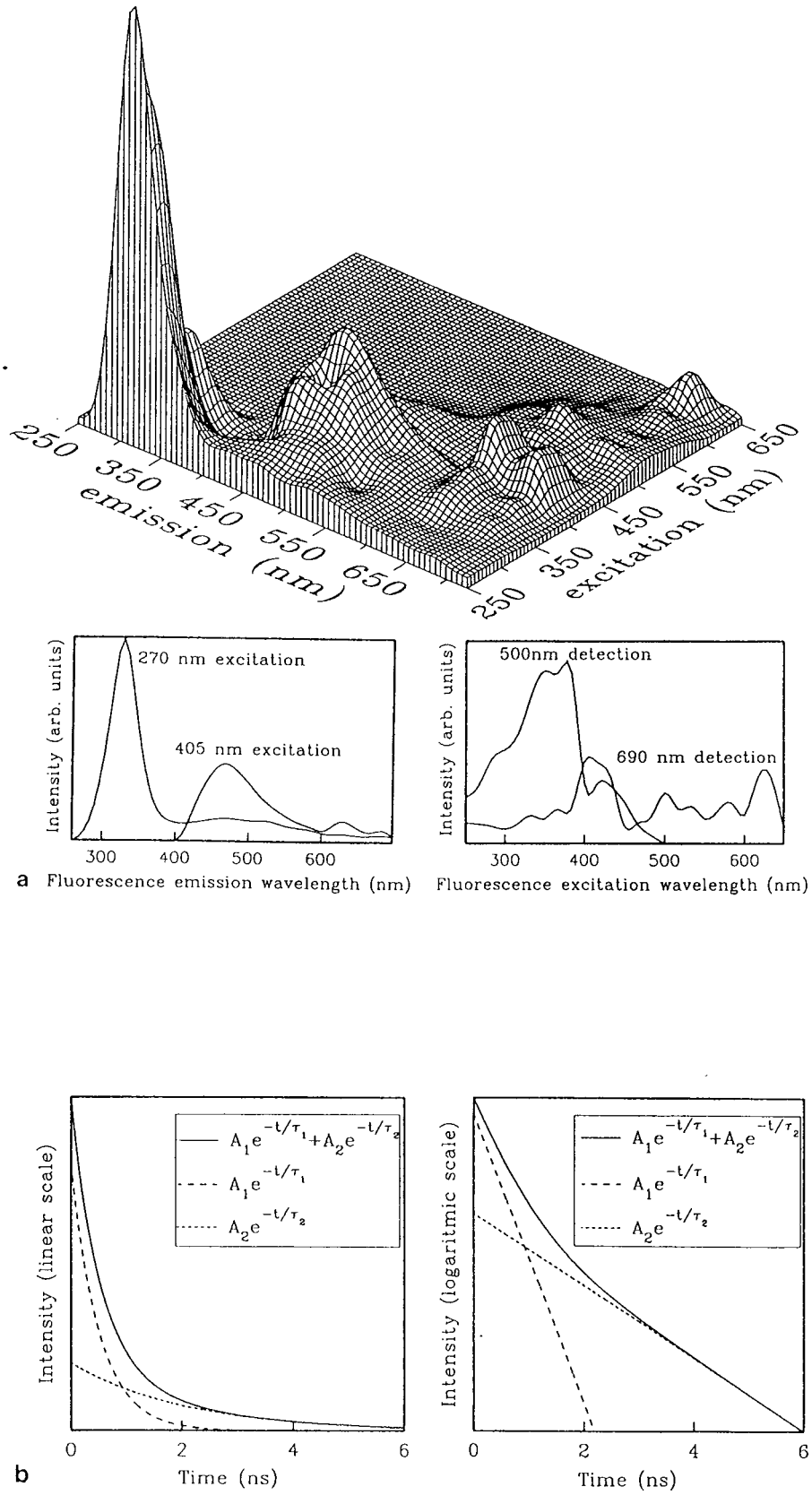

Fig. 1.a, b. Schematic illustration of **a** a fluorescence excitation-emission matrix (EEM) for excitation wavelengths from 250-650 nm and emission wavelengths from 250-700 nm, and excitation and emission spectral properties at selected wavelengths. Spectra are time-integrated. **b** A fluorescence multicomponent decay curve, $S = A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2}$ $A_1 = 80$, $A_2 = 20$, $\tau_1 = 1$ ns, $\tau_2 = 4$ ns

from exogenous fluorophores administered systemically or locally can be used in several ways. For screening or diagnosis by autofluorescence, distinguishing malignant from benign disease or normal tissue relies on differences in the concentration of specific molecules due to the disease process or in the tissue microenvironment, causing changes in the autofluorescence characteristics. With exogenous fluorophores, the differentiation is based primarily on differences in the uptake or retention of the fluorophore, or of its carrier molecule.

As illustrated in Fig. 1, any fluorophore in a particular environment has characteristic excitation and emission spectra. In the simplest case, fluorescence detection can be performed at a single excitation wavelength, λex, and a single emission wavelength, λem. However, extracting the maximum information may require measurements at more than one wavelength and, in the limit, full 2-D spectra may be used. Measurements of the fluorescence decay lifetime, τ, may provide complementary information [4, 5]. The fluorescence decay may be multicomponent, where more than one fluorophore contributes to the signal at the particular (λex, λem) values used. Potentially, other parameters in fluorescence recordings, such as polarization, could also be utilized, but the diagnostic value of these has not been demonstrated.

In principle, the measurement for any combination (λex, λem, τ) may be performed either as a point-by-point measurement, for example, using an optical-fibre fluorescence probe placed on or in the tissue, or as a fluorescence image, viewing the tissue surface (Fig. 2). Both techniques may be used either directly or through a fibroptic endoscope. Clearly, the use of imaging involves some trade-off in the spectral/temporal content of the measurement as compared with a point detection system, because of the complexity and cost of instrumentation and increased measurement time. Both approaches usually involve post-processing of the fluorescence signals, for example, to form spectral ratios $S(\lambda ex_1, \lambda em_1, \tau_1)/S(\lambda ex_2, \lambda em_2, \tau_2)$ or to subtract background fluorescence.

In vivo fluorescence measurements may be either « relative » or « absolute ». For most applications to date, the absolute magnitude of the fluorescence signal is ignored, but to quantify the tissue concentration of known fluorophores, the absolute fluorescence signal is required. This depends of the intensity and irradiance distribution of the exciting light, the optical attenuation of the tissue at both λex and λem the fluorescence yield in situ, and the efficiency and sensitivity of the light collection optics and detector at λem.

Review of studies to date

This review section is divided into four subsections : A) the biochemical origin of the fluorescence, B) light transport in tissue and its effect on fluorescence signals, C) fluorescence

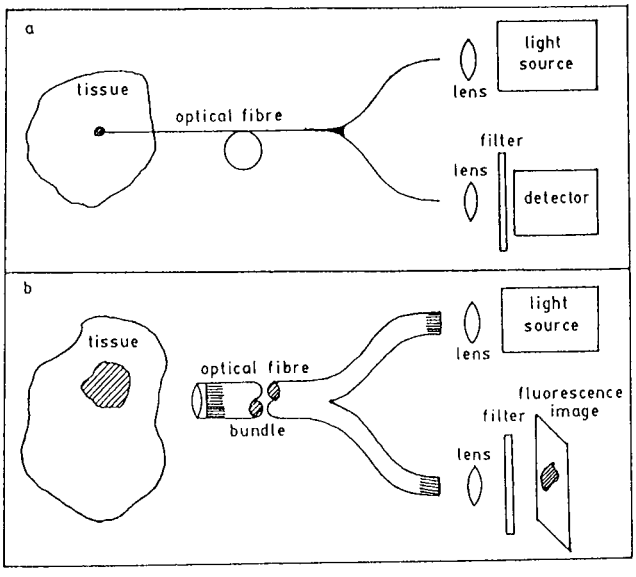

Fig. 2a, b. Illustration of in vivo fluorescence measurement techniques. **a** Point measurement with fibreoptic probe. **b** Fluorescence imaging via a fibreoptic endoscope onto a 2-D detector array

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(A more detailed bibliography is available from the authors on request)

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detection strategies and clinical instrumentation, and D) clinical applications and results.

Fluorophores

Various fluorophores contribute to tissue autofluorescence in the near UV and visible region. Although the in solu fluorescence properties of most fluorescent aromatic molecules present in tissue are well known [6, 7], the detailed interpretation of fluorescence signals from tissue is difficult due to the composite, generally unstructured, spectra and dependence on the in vivo microenvironment. The measured spectra may also be influenced by wavelength-dependent optical reabsorption by tissue chromophores such as haemoglobin [8] : for example, Fig. 3 illustrates the effect of melanin absorption on fluorescence from skin. 2-D excitation-emission spectra [9] and time-resolved spectra [10] have been used to identify the tissue fluorophores contributing to total tissue fluorescence by comparing these with the characteristics of the isolated fluorophores in solu. Identification of tissue fluorophores can also be aided by fluorescence microscopy or microspectrofluorimetry to determine their intra- or extra-cellular location and tissue distribution [11, 12]. Major tissue fluorophores which may be relevant to oncologic applications are tryptophan [13], collagen and elastin

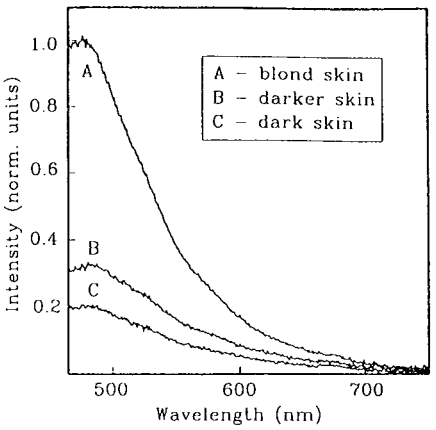

Fig. 3. In vivo autofluorescence spectra from three different skin types (337 nm excitation)

[9, 10], reduced nicotinamide adenine dinucleotide, NADH, and its phosphate, NADPH [14, 15], flavins and flavoproteins [16, 17], beta-carotene [10], and porphyrins [18] (see Table 1).

The development of exogeneous fluorophores as tumour markers is closely associated with that of photodynamic therapy (PDT), i.e the use of light-activated photosensitizers to destroy solid malignant tumours [2]. Experiments with fluorescence substances to photosensitize tissue were first performed as early as 1899/1900. In the 1940's it was found that injected porphyrins, particularly hematoporphyrin, Hp, accumulated in many

Table 1. Fluorescence data for some endogenoux tissue chromophores for potential oncologic applications measured in solution or as a powder

Fluorophore/ substance	Main excitation peaks (nm)	Main émission peaks (nm)	Fluorescence lifetimes (ns)	Refs
Trytophan	275	350	2.8, 1.5	10,18
Collagen	340 270 285	395 395 310	9.9, 5.0, 0.8	10,18
Elastin	460 360 425 260	520 410 490 410	6.7, 1.4 7.8, 2.6, 0.5	10,18
NADH	350	460	0.6, 0.2	10,18
FMN	440	520	4.7	112
Beta-carotene		520	9.6, 2.0, 0.3	10
Endogenous porphyrins	400	610 675		17

NADH : nicotinamide adenine dinucleotide, reduced form ; FMN : flavin mononucleotide (riboflavin-5'-phosphate)

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malignant tissues, embryonic tissue, lymph nodes, traumatized tissue, and to rapidly growing tissues in general [19]. Quantitative fluorescence studies in a range of human tumours using Hp were carried out during the next decade [20, 21]. Subsequent investigations using other fluorophores such as eosin, fluorescein, tetracycline, acridine orange and phenoxazines were encouraging but not extensive enough to draw firm conclusions [22].

The first quantitative in vivo study with exogenous fluorophores was performed in 1960 [23] and in the same year a derivative of haematoporphyrin (HpD) as a fluorescent tumour marker and photosensitizer was reported, which was shown subsequently to be a better tumour localizer than Hp itself [24, 25]. HpD was first tested clinically in patients with bronchial, oesophageal, cervical or vaginal tumors. All bronchial and oesophageal tumours and 94 % of the cervical/vaginal lesions showed positive reddish fluorescence from the HpD [26]. Several other clinical studies [e.g. ref. 27, 28] with HpD during the period 1964-76 also showed high sensitivity and specificity for malignant tissue in lung, oesophagus, head and neck, cervix and urinary bladder.

By the end of the 1970's the use of HpD as a tumour marker for fluorescence diagnostics was growing rapidly, largely due to the breakthrough in the use of HpD as a photosensitizer for photodynamic therapy [29]. Many questions arose regarding the composition of HpD [30], which markedly affects the tissue uptake, retention and microlocalization of the fluorescent and photodynamically active components. HpD is not simply a mixture of porphyrin moieties, but rather is an unstable mixture of several monomeric, dimeric and aggregated forms of porphyrins. Equilibria between the different monomeric fractions and for dimerization and/or aggregation of monomeric fractions [31], and the resultant fluorescence characteristics, are sensitive to micro-environmental factors such as pH, ionic strength, temperature and concentration of the molecules [32]. About two-thirds of HpD are monomeric porphyrins, which are too hydrophilic to penetrate cell membranes efficiently, while porphyrin dimers and higher aggregates are more hydrophobic and form the main tumour-localizing fractions. The photophysical properties of HpD differ for the monomeric fractions and the dimers, so that these can be distinguished spectroscopically. Hp itself has a strong absorption band around 280 nm, another major absorption band at about 400 nm, (the Soret band) and four minor absorption bands in the visible region above 500 nm. The fluorescence emission shows a double peak in the red around 610 and 670 nm [33]. The

Table 2. Fluorescence data for some examples of exogenous fluorophores for oncologic applications measured in solution

Fluorophore	Main excitation peaks (nm)	Main émission peaks (nm)	Fluorescence yield %	Fluorescence lifetimes (ns)	Optimal times for Fluorescence *in vivo* (h)	Phototoxicity	Refs
HP	405	610	9	13.5	2-4	Yes	46,48
BpD-MA	400	690		5.5	2-4	Yes	49,50
HpD	400	610	2-7	15.5, 2.5	12-72	Yes	33,34
MACE	410	664		3.7		Yes	51,52
$Sn.NT2H_2$	410	645	16	0.65		Yes	53
AlSPc	350	675	4	5.3	24-48	Yes	54
NBA	620	660				No	55

[a] Not necessarily clinically significant phototoxic at doses for fluorescence diagnostics
Hp : haematoporphyrin ; BpD-MA : benzoporphyrin monoacetate ; HpD : haematoporphyrin derivative ; MACE : mono-L-aspartyl chlorin e6 ; Sn.NT2H2 : reduced tin metallo purpurin ; AlSPc : tetrasulphonated aluminium phthalocyanine ; NBA Nile blue A

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higher aggregates with high uptake/retention in tumour, and hence photodynamic effect, are almost non-fluorescent.

In vivo and ex vivo fluorescence have been used to study the dynamics of HpD uptake and retention in tissue [35-37]. After i.v. injection the fluorescence intensity in all tissues reaches a maximum at about 2 h. A weaker fluorescence signal is seen after 4 h, followed by a second phase of increased signal. The total porphyrin concentration (determined by radiolabelling) shows a single broad peak at around 24 h. Malignant tumours generally retain porphyrins longer than other tissue types, but the uptake varies considerably between different tumour types and even within the same tumour. The maximum HpD concentration ratio between malignant tumour and surrounding tissue is typically between 2:1 and 5:1, except in the brain (> 10:1), where the intact blood brain barrier prevents HpD reaching the normal tissue [38]. The mechanism(s) of HpD retention in tumour are not well understood. No marked differences in HpD uptake can be found between malignant and normal cells in vitro. The main retention mechanism(s) must, therefore, lie in the structure or physiology of the tumour [39].

Transformations between monomeric, dimeric and higher aggregated states in the various fractions taking place after i.v. injection may be very important for tumour detection [30, 40]. The highly fluorescent monomers injected are not retained in tissue and the dimers and aggregates are only faintly fluorescent. Transformation to the monomeric state takes place in situ, as supported by the observation that the fluorescence excitation and emission spectra of HpD in vivo are very similar to those in preparations containing serum or surfactants [41, 42]. It likely also accounts for the second peak in the early fluorescence-time curve in vivo. Time-resolved fluorescence spectroscopy is of particular value to study these transformations, since the monomers and dimers have very different lifetimes (~ 14.5 and 2.5 ns, resp.) [5, 33, 34, 43]. The polarization characteristics of monomers and dimers are also different [44], and this may be used for microscopic imaging of the distribution of the various fractions.

Porphyrins have been shown to photobleach rapidly in tissue [45], so that very sensitive detection systems are required for HpD fluorescence diagnostics in order to minimize the excitation irradiance required. An example of published excitation and emission spectra of HpD in vivo is shown in Fig. 4.

The main disadvantages of HpD as an exogenous fluorophore for tumour diagnostics are that it is not a well characterized single stable chemical compound, has a relatively low selectivity for malignant tissue, causes skin photosensitivity, has a relatively low fluorescence yield, and about 24 h delay is required after injection for optimal contrasts. It is of interest, therefore, to investigate other fluorophores and alternative delivery vehicles. Although much of his work has been « spin-off » from the better PDT agents, search for the ideal characteristics of a photosensitizer for PDT are only partly overlapping with those for fluorescence diagnostics [37, 46, 47]. Characteristics of a few potential candidates are summarized in Table II. Among other interesting fluorescent dyes suggested as tumour markers are metalloporphyrins with near-infrared fluorescence emission around 900 nm [56].

Various alternative drug delivery

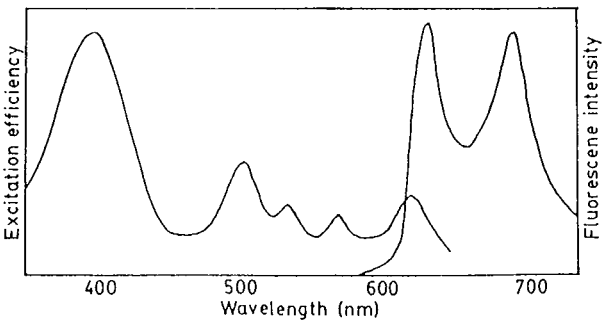

Fig. 4. In vivo fluorescence excitation and emission spectra of HpD 15 h after an i.p. injection of 20 mg/kg body weight in RUC-2 tumour bearing rats. (Adapted from ref. [42])

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systems have also been proposed, both for fluorescence diagnostics and for PDT. One approach is to encapsulate the molecules in liposomes, for i.v. injection [57]. This allows the use of fluorophores that are otherwise insoluble in aqueous media. The in vivo fluorescence from zinc phthalocyanine in dipalmitoyl phosphatidylcholine (DPPC) liposomes have been demonstrated in tumours, and the tissue distribution and kinetics determined by fluorescence spectroscopy [58]. In a second method the fluorophore is coupled to a monoclonal antibody, using the selectivity in the antibody uptake to target the tumour for diagnosis [47, 59] or PDT [60, 61]. The advantage is that any nontoxic dye of high fluorescent yield that can be anti-body coupled can be used, regardless of its intrinsic tumour-localizing properties. Two recent studies [47, 59] using fluoroscein coupled to anti-carcinoembryonic antigen (anti-CEA) monoclonal antibodies showed fluorescence signals in tumour about 8-fold greater than that obtained with a standard dose of HpD [47]. The use of topical administration of fluorescent tumour markers (or photosensitizers) to avoid systemic toxicity is also attractive for certain applications, but the results to date have been mixed [62, 63].

Effects of light attenuation in tissue

Considering for simplicity only time integrated fluorescence, the signal, S (λex, λem), measured by any given instrument depends on several factors : i) the spatial distribution of the excitation light at wavelength λex, which depends on the tissue absorption and scattering properties at λex, ii) the fluorescence quantum yield, Q (λex, λem), in the tissue and spatial distribution of the fluorophore within the detection volume, iii) the « escape function » which determines the probability that fluorescence photons emitted at some point in the tissue will propagate to the detector, which depends on the optical absorption and scattering of the tissue at λem,

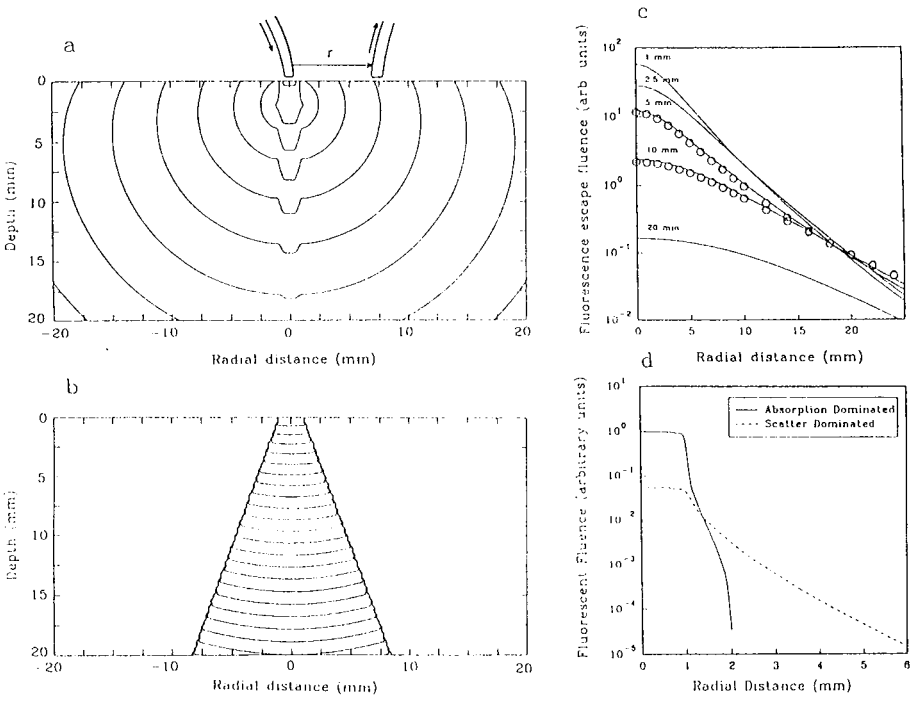

Fig. 5a-d. Light distribution and resulting fluorescence signal calculated for a semi-infinite tissue with uniform optical properties and fluorophore distribution. **a** Excitation light distribution calculated from diffusion theory using tissue-like optical properties at 630 nm ($(\mu_s' = 1\ mm^{-1}$, $\mu_a = 0.2\ mm^{-1})$; **b** excitation light distribution calculated from the Beer-Lamberts relation for tissue like optical properties at 337 nm ($\mu_a = 10\ mm^{-1}$) ; **c** fluorescence « escape » functions for various depths as a function of radial distance, r, in a medium with tissue like optical properties at 690 nm ($\mu_s' = 0.1\ mm^{-1}$, $\mu_a = 0.1\ mm^{-1}$) calculated from diffusion theory and measured in a tissue phantom and ; **d** resulting fluorescence signal versus radial distance, r, for the two excitation wavelengths, calculated as a volume integral of the light distribution and the fluorescence escape functions. Note the significant difference in the effective « sampling volume » of the fluorescence signal

iv) the collection geometry and photon sensitivity of the detector at λem, and v) the background contribution at (λex, λem) of other endogenous fluorophores.

The most general approach to obtain the fluorescence quantum yield or fluorophore concentration (or, more generally, the product of the two) is to determine, preferably non-invasively, the tissue absorption and scattering coefficients at both λex and λem by some fluorescence-independent technique [64], and then to apply an analytic or numerical model of photon transport in tissue to calculate the effects of light attenuation and system geometry. Spatially-resolved, steady-state or time-resolved diffuse reflectance spectrophotometry have recently shown promise for this purpose and are compatible with fluorescence instrumentation. A number of possible models to calculate the attenuation factors have been examined, including Monte Carlo computer simulation, which may be used for homogenous or heterogenous tissue optics and fluorophore distributions and for any definable source-tissue-detector geometries, or closed-from, analytic models of light propagation in tissue providing either exact or approximate solutions for more restriced cases. For example, studies have been performed using one-dimensional diffusion theory to model the sensitivity of the fluorescence signal to the presence of absorber at the fluorescence excitation or emission wavelengths [65, 55]. Other modelling and experimental studies of quantitative fluorescence in tissue [67, 69] have shown that the effects of light attenuation on the fluorescence signal are very dependent on excitation and emission wavelengths and on the measurement geometry and that, if possible, long wavelengths ($\geq$ 600 nm) should be used for both excitation and emission, to reduce the sensitivity to haemoglobin absorption and tissue optical heterogeneity.

It may also be speculated that 2-photon excitation of fluorescence in tissue would increase the effective sampling depth (and volume) through the use of high peak-power, low energy pulses of long wavelength light using short pulse laser sources. In addition, 2-photon excitation spectroscopy also opens energy levels which cannot be reached by 1-photon excitation. Fluorescence has been observed from HpD following 2-photon excitation in solution [70], but clearly extensive studies are needed to evaluate this approach, including possible tissue damage from multi-photon absorption in cellular structures, and technical issues involved in the laser sources and optical fibre delivery systems required.

Detection schemes and instrumentation

Different types of techniques and instruments for fluorescence diagnostics in vivo are summarized here. Although most of the instruments have been developed and designed for diagnostics based on HpD fluorescence, they could, with minor changes, be used with other exogenous tumour markers or with tissue autofluorescence. Reviews of existing fluorosensors in clinical oncology have been published recently [66, 71].

The simplest technique to study tissue fluorescence is to irradiate the tissue externally or endoscopically and observe visible fluorescence with a dark-adapted eye. However, the sensitivity is low and the examination has to be performed in darkness, making a visual reflected white light examination impossible at the same time. Also, no signal processing can be performed, and the diagnosis is necessarily subjective. Electronic detection allows a more objective fluorescence measurement and both white-light and fluorescence studies can be performed in rapid sequence in real time. Further, multiple fluorescence signals S(λex, λem, τ), can be recorded and processed to subtract tissue autofluorescence and/or reduce geometric effects. The ratio between fluorescence and the diffusely reflected excitation light, can also be used [72], but care is needed to eliminate specularly reflected light. Pulsed or modulated light sources help suppress ambient background light [41, 42, 73].

With many fluorescence techniques it is easy to obtain a large amouts of data, which must be reduced to some simple functional values, F(S_1, S_2, ...), of the various measured signals, S_i (λex, λem, τ). Sophisticated, general-purpose equipment may have to be utilized to find the optimal functions. However, for routine clinical use, technical factors can restrict the options. Usually an imaging device is preferable, since it gives textural information, but imaging with spec-

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troscopic capabilities currently requires advanced technology. Determining the optimal functions, F, can be illustrated by HpD fluorescence. The in vivo excitation and emission spectra of HpD suggest that 405 nm excitation and 630 nm detection should be used. However, the autofluorescence and tissue optical properties must also be considered. For the best tumour-to-normal contrast, the HpD-to-autofluorescence ratio should as high as possible. For 405 nm excitation this is higher for the 690 nm peak of HpD than at 630 nm. Furthermore, the tissue autofluorescence is weaker if excited in the visible rather than the UV region. The superimposed autofluorescence can be measured, and hence subtracted, by i) using two excitation wavelengths at which the autofluorescence is similar, but the HpD excitation efficiencies differ, ii) using one excitation wavelength and two detection wavelengths, one corresponding to pure tissue autofluorescence and the other the sum of HpD and autofluorescence, or iii) exploiting the different time-characteristics of the tissue fluorophores and HpD. Measuring the autofluorescence also allows the calculation of a dimensionless ratio of fluorescence intensities, thus reducing the influence of the measurement geometry. Since light absorption by haemoglobin absorption markedly influences measured fluorescence signal levels and spectral shapes, wavelength regions with marked haemoglobin structures (e.g. around 415, 540 and 580 nm) should be avoided. Long wavelengths (λex, λem > 600 nm) give the largest tissue penetration.

The first fluorescence systems using electronic detection for tumour diagnostics were introduced in 1974 (in ophthalmology) and in 1978 (for endoscopy) [74, 75]. The former was a modified indirect ophthalmoscope, in which part of the fluorescence was split off to a photomultiplier tube (PMT), while the endoscopy instrument was designed for fluorescence bronchoscopy and utilized chopped light from a high pressure mercury arc lamp intermittently for white light illumination and fluorescence excitation (with a 405 nm bandpass filter). The light was delivered to the tissue by fibre optics. The imaging fibre bundle gave the reflected white light image, while a separate fibre guided the fluorescence through a bandpass filter (620-690 nm) to a PMT. The PMT signal was used to generate an audible tone. Similar systems for use in the bronchial tree and the bladder have been reported [76], and improved versions have also been subsequently developed [77], a current model of which can measure the fluorescence and diffuse reflectance intensities at up to six (λex, λem) values with ambient light subtraction and computer processing of the measured values.

Other lamp-based in vivo systems using scanning excitation and emission monochromators have been constructed [42]. Although the clinical use of such systems is limited by the long recording time, they may provide valuable data for the development of simpler clinical instruments. The use of a polychromator and photodiode array detector shortens recording times, makes in vivo measurements more feasible [78], and reduces movement artefacts. A limitation of lamp-based fluorosensors is the difficulty of suppressing scattered light at the detection wavelength. The monochromaticity and high brightness of lasers make them suitable as excitation sources, although this restricts the available excitation wavelengths. Spectrally-resolved piont measurements can be performed with laser excitation and multichannel detection [79, 80].

The simplest time-resolved point measurements can be performed using pulsed excitation and gated detection [14], with either a single emission wavelength, multiple discrete wavelengths or full spectra. More sophisticated techniques where full decay times are measured can be performed with pulsed excitation and a fast detector such as a streak camera or time-correlated single photon counter [81, 82]. An equivalent approach is to use frequency-domain fluorometry. No tissue fluorescence measurements using this method are, to our knowledge, published, but the high time resolution, relative technological simplicity and the possibility to extend this method to an imaging technique [83] make this attractive for future clinical applications.

For clinical studies it is clearly an advantage to visualize the fluorescence from the entire area under investigation. In early fluorescence diagnostic instruments, image intensifiers were used to visualize the weak fluorescence

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signal. The first clinical fluorescence bronchoscope was presented in 1979 [84]. One problem with this early system was that normal white light bronchoscopy could not be performed at the same time as the fluorescence examination. Rapid switching between white light and fluorescence was, therefore, developed, together with video recording [85]. Other, more recent one-colour imaging devices have been reported. CCD cameras have recently become competitive in sensitivity to image-intensified cameras, and their use in single-wavelength imaging fluorosensors has been demonstrated [86]. A gated image intensifier together with pulsed excitation is required to reject the ambient light. An alternative is to use a rapidly-scanned laser beam and a point detector to construct the fluorescence image [87].

In general, the restriction to a single wavelength seriously limits the reliability of fluorescence diagnosis [85]. One solution is to combine point spectroscopy with imaging instruments, so that any suspicious region can be investigated spectroscopically [88]. A second, general approach is to perform sequential imaging at several excitation or emission wavelengths [89, 90]. Systems for simultaneous multicolor imaging have also been developed, for example utilizing specially designed optics to obtain images of the same tissue area at 4 different wavelengths side-by-side on one camera [91]. The four images can then be computer processed for viewing of the optimized contrast function image. Another system uses two separate intensified cameras with different bandpass filters to perform subtraction and/or ratio-ing [92]. A final approach is to perform time-gated imaging to reduce the influence of autofluorescence [91, 93].

Clinical applications and results

Fluorescence diagnostics based on HpD and tissue autofluorescence will be discussed separately here. In recent studies with direct viewing of HpD fluorescence, the main interest has been visualization of tumours in conjunction with PDT. For example, a study in 409 patients with various malignancies was carried out using an Argon-ion (488-514 nm) or He-Cd (442 nm) laser or a Black lamp (365 nm) for excitation. The patients received 5 mg HpD per kg body weight 48-72 h before the fluorescence examination, and sites investigated included lung, oesophagus, stomach, rectum, urinary bladder, other urinary or genital organs, oral cavity, eye, breast, skin and brain. Of the 405 total lesions, 344 revealed red fluorescence, 32 gave equivocal response and no fluorescence was observed from the remainder. Mild photosensitization of the skin resulted in 20 patients.

Several clinical studies of bronchial and bladder tumors have utilized a filter-wheel point fluorosensor system [75, 95]. Tumour edges could be determined and bronchial carcinoma in situ (CIS) could be detected in sputum cytology positive, chest X-ray negative patients and in patients where disease was not visible by normal white-light. Bladder CIS could also be detected using 2.5 mg/kg HpD at 2-48 h [96], the best lesion discrimination being at 2 h after injection.

The results from the first fluorescence bronchoscopies using an image intensifier with HpD were presented in 1979 using a filtered mercury are lamp (405 nm) and subsequently using Kr-ion laser excitation (406.7, 413.1 and 415.4 nm). Fluorescence bronchoscopy was equally good in detecting the tumours as standard white light bronchoscopy and better delineated the tumour extent. The method was sensitive enough to detect small tumours (1-1.5 mm thick) and generally showed a high sensitivity, but relatively low specificity. In a review of clinical studies in 78 patients, 75 tumours showed positive fluorescence [97].

A ratio-ing fluorometer using Kr-ion laser excitation was used in a strudy with 72 patients (0-2 mg/kg HpD at 4, 48 or 72 h) with visible bronchogenic tumours ranging from small to obstructing [98]. The fluorescence intensities at 690 ± 40 nm (red) and 562 ± 30 nm (green) were detected, and a red-to-green ratio was calculated, and compared for tumour and normal tissue in each patient. Tumour ratios 1.9-14.3 times those in normal tissue were obtained in HpD-injected patients, while values between 1.1-2.2 were found in non-injected patients. In a study of low dose HpD, 36 patients were examined before HpD injection, 24 patients at 24 h after a 0.25 mg/kg injection, and 7 patients after a 2 mg/kg injection of HpD. The lower HpD dose resulted in about two times higher red fluores-

cence than with no HpD. The red/green ratios from tumour tissue were generally lower than with high dose, while those for normal tissue were comparable. All CIS and dysplastic lesions had higher red/green ratios than normal tissue.

A dual-wavelength excitation fluorometer (λex = 611, 632 nm, λem = 690 nm) was used clinically on seven patients with local recurrent breast carcinoma on the chest wall [100], scanned by a point probe 24 and 48 h after. 0.5 mg/kg HpD. In four patients, histopathologically-verified metastases were found. In two patients no metastases were found by either fluorescence or random biopsies.

An instrument with multiwavelength detection and Kr-ion laser excitation has been reported in a small number of patients with malignant tumours in various organs [10], while multichannel detection instrument using HeCd laser excitation (442 nm) has also been used successfully in animals for intraoperative detection of metastases [102]. A dual-wavelength imaging system, using two excitation wavelengths to subtract autofluorescence, has been used recently in patients, with encouraging results for the detection of early stage bladder tumours [103]. Finally, an integrated detection instrument combining fluorescence bronchoscopy and a spectrally-resolved fluorescence spectrometer has been reported in patients with malignancies in the respiratory tract with high sensitivity for early stage cancer, including CIS [104].

The most extensive study of tissue autofluorescence in clinical diagnosis was presented in 1987 [17], using a Xe-ion laser (365 nm). Autofluorescence emission spectra in 50 tissue specimens (stomach, oesophagus, tongue, mandible and bladder) showed characteristic peaks near 630 and 690 nm in malignant lesions, especially around the margins. The central necrotic regions did not yield any characteristic fluorescence and the peaks were also absent in spectra from normal tissues. Further fluorescence measurements were performed in more than 100 patients with oral malignancies : 80/90 measurements were judged as true positive or negative, while 10/90 were not in accordance with histopathology. However, it has been shown [105] that red fluorescence results from microbial synthesis of porphyrins in necrotic areas of tumours, so that the spectra changes may not reliably identify malignant transformation. In vitro fluorescence emission spectra from normal and malignant breast and lung tissue with Argon-ion excitation (457, 488 and 514 nm) have been presented [106], showing differences between tumour and normal tissue. However, the differences were mainly due to haemoglobin absorption rather than to the fluorescence emission per se.

A study of in vivo tissue autofluorescence spectra (337 and 405 nm excitation) from normal and tumour tissue of the bronchial tree, brain and mouth [107] showed no systematic differences in the spectral shape between tumours and normal tissues. By contrast, autofluorescence spectra from normal, pre malignant and malignant bronchial tissue following Kr-ion (405 nm), HeCd (442 nm) or Argon-ion (488 nm) laser excitation showed significant reduction in the fluorescence intensity in the green region in all malignant tissues, possibly due to a change in the balance between oxidized and free reduced forms of flavins [80].

Several reports of autofluorescence in colonic mucosa have recently been presented. All studies found an overall weaker fluorescence from adenomatous and, to some extent, hyperplastic polyps as compared with normal tissue. One such investigation used HeCd laser excitation (325 nm) and multichannel detection to record the in vitro fluorescence emission spectra from normal colonic specimens and adenomatous polyps [108]. The spectra were used as teaching data for a discrimination algorithm and, subsequently, new samples were classified with accuracies of 100 %, 100 % and 94 % for normal mucosa, adenomatous and hyperplastic polyps, respectively. Similar results have been reported in a second group of patients [109], where adenomas could be diagnosed with an accuracy of 97 %.

Two studies have used an excitation-emission matrix (EEM) technique (λex = 250 – 500 nm, λem = 260 – 700 nm) as in Fig. 1a to identify the fluorophores responsible for autofluorescence of normal colonic mucosa and adenomatous tissue as tryptophan, elastin, collagen, NADH, fluorophores related to vitamin B_6

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and endogenous porphyrins [9, 18]. Fluorescence EEMs from normal bladder, CIS and invasive transitional cell carcinoma in the bladder showed similarities between fluorophores from colonic and bladder tissue.

Finally, in a study using low dose HpD and 337 or 405 nm excitation, significant differences in tissue autofluorescence between normal bladder, mild dysplasia, severe dysplasia and tumour tissue werre found, possibly due to reduced elastin and a NADH content of dysplastic and malignant tissues [10].

Outstanding issues and future directions

There are a number of generic and application-specific issues in considering the limitations and possible impact of fluorescence techniques in clinical oncology. Perhaps the most difficult overall aspect in assessing the future of in vivo techniques is that of identifying the potential clinical applications and the possible impact on cancer control. In practice this will be strongly dependent on the technical capabilities of fluorescence instruments.

Current studies of in vivo fluorescence for screening or diagnosis of early cancer include detection of : bronchogenic lesions in sputum-positive, X-ray negative patients [95, 97-99] ; dysplastic or malignant transformation in patients with chronic ulcerative colitis [9, 18] ; vulvar or endometrial dysplasia [94] ; bladder cancer [10, 89, 96, 103] ; and (more speculatively) early breast cancer by fluorescence-enhanced transillumination spectroscopy or imaging [110]. A major unresolved question is whether such investigations can be done using tissue autofluorescence only, or if an exogenous fluorophore is required. This may be disease-site and stage dependent. For example, certain types of early bronchogenic tumours are detectable using autofluorescence alone, while for other tumour sites, although differences in autofluorescence spectra and/or lifetimes have been reported, it is not know if these alone will have sufficient sensitivity and specificity for reliable clinical diagnosis. On the other hand, development of applications based on exogenous fluorophores is complicated by selection of the appropriate dye and/or delivery vehicle to achieve adequate tumour selectivity, as well as by possible toxicities.

In vivo fluorescence techniques are also being strudied for in tumour localization, for defining the margins of tumours, for determining lymph node involvement and as a guide for surgical resection. In tumour localization, most work has been done for bronchogenic tumours using endoscopic imaging or point fluorometry, but other applications such as localization of bladder and colorectal tumours and defining tumour margins in head and neck tumours have also been proposed. Point interstitial fluorometry for determining lymph node spread of disease also shows promise, since it may be capable of detecting minimal involvement earlier than clinical signs or other diagnostic techniques [100], and encouraging results have recently been reported in animal models for fluorescence-guided brain tumour resection [111].

A major question with all these applications is how to obtain the maximum discrimination of the tumour fluorescence signal from the background fluorescence of the normal host tissue. In PDT patients, in which the objective is to define the required light treatment field, the obvious strategy is to use the photosensitizer fluorescence (if any). For non-PDT cases, with known tumour, the toxicity tolerance for using exogenous fluorophores is also much greater than in the case of tumour screening or diagnosis, allowing a higher fluorophore dose to increase the tumour-to-background and signal-to-noise ratios, and in-situ measurements may also be possible both before and after dye administration to improve autofluorescence subtraction.

In all cases it will be necessary to establish reliable limits for defining a positive fluorescence signal, by comparative studies against standard techniques such as histopathology. A universal algorithm for fluorescence detection of all tumour types seems unlikely. The main potential application of absolute fluorescence quantification is in PDT dosimetry, since there can be considerable subject-to-subject variation in the uptake of photosensitizers, affecting the treatment response. One possible limitation with the current clinical photosensititzer,

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HpD, is its complex porphyrin composition, which means that there is not, a-prior, a necessary correlation between fluorescence and photodynamic effectiveness in vivo. Even with pure, « second-generation » photosensitizers, however, the problem remains of accounting for the attenuation of the excitation and emission light by the tissue. It is not known if a fully rigorous solution to this problem is necessary, or if empirical light attenuation factors will suffice for clinical applications.

Since the fluorescence signal can be markedly influenced by the source-tissue-detector geometry and by the wavelength-dependent optical attenuation of the tissue, tumour detection based simply on the fluorescence intensity at a particular wavelenght can be subject to significant error, as illustrated in Fig. 6. Ratio-based techniques, using two or more excitation and/or emission wavelengths can reduce, but not eliminate, these errors. Use of fluorescence decay lifetimes is intrinsically more robust, and, if the technology for time-resolved fluorescence can be made feasible for routine clinical use, then combining spectral and lifetime information is likely to provide the most reliable fluorescence techniques.

In considering exogenous dyes, besides low toxicity and high selectivity, the excitation and emission spectra are

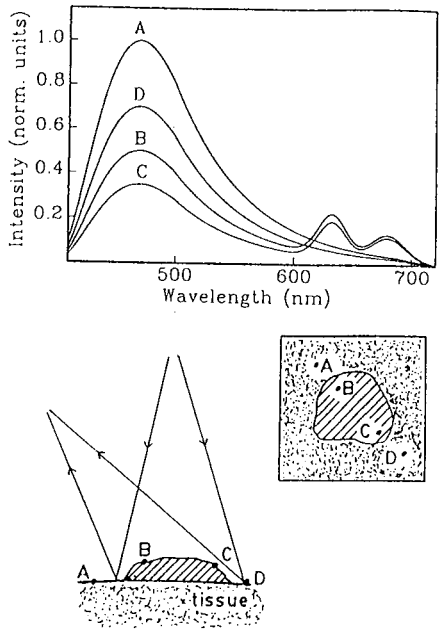

Fig. 6. Illustration of the effects of source — tissue — detector geometry and light attenuation in tissue on fluorescence measurements. $S_A < S_D$ due to the geometric factor. $S_B \neq S_C$ due to differential attenuation of the shorter wavelengths in transversing the intervening tissue

important to minimize the autofluorescence signal and the influence of tissue attenuation. Further, as illustrated in Fig. 7, the limited penetration of short wavelength light in tissues confines the measurement to the tissue surface of to the localized

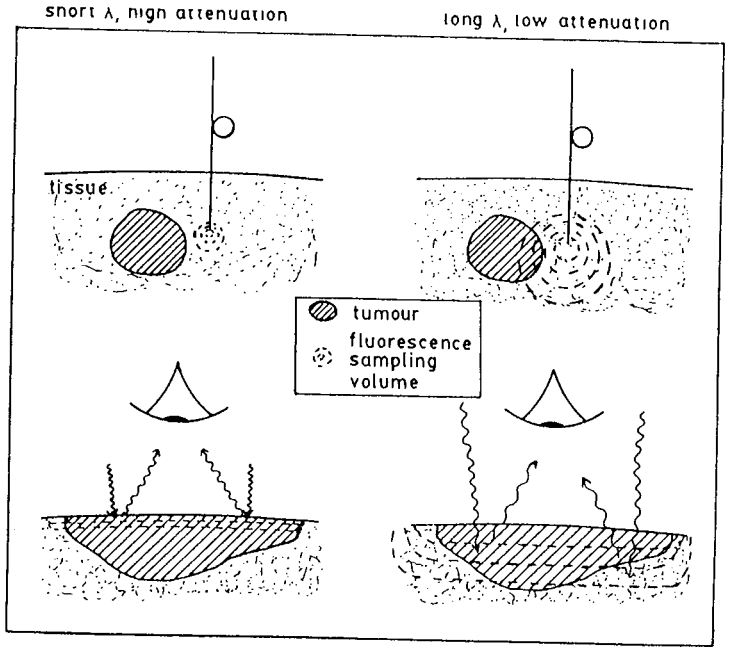

Fig. 7. Illustration of the influence of the light attenuation on the sampling volume in fluorometry and fluorescence imaging

volume at the fibre tip. The use of long wavelengths with exogenous fluorophores, allows subsurface depth and volume probing and so may increase the sensitivity, but a the cost of spatial resolution. Thus, a combination of short and long wavelength detection may be optimal.

Finally, current instruments for in vivo fluorescence range from simple and relatively inexpensive fibre-optic fluorometers using a filtered lamp or HeNe or diode laser sources and photodiode detectors, to highly complex and expensive multi-spectral imaging systems involving sophisticated laser sources, sensitive array detectors and computerized image processing. Equipment may become further complicated by the addition of fluorescence decay measurements. The components of such systems are, however, becoming cheaper, more reliable in clinical environment and simpler to use. At present the optimal configuration(s) for clinical use are undetermined, since there has been little systematic evaluation of the newer instruments.

In conclusion, it is clear that in vivo fluorescence as a clinical tool is embryonic, and caution is required to avoid the false assumption that, just because fluorescence *can* be measured in vivo, it should be measured. Innovative basic science, including the identification of tissue autofluorescence fluorophores and their relevance to fluorescence diagnostics, the search for new and better exogenous fluorophores as tumour markers, and the development of clinical fluorosensors, as well as rigorous clinical evaluation of techniques and instrumentations are essential if the technique is to play a significant future role in clinical oncology.

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