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**In vivo** fluorescence imaging for tissue diagnostics

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Abstract. Non-invasive fluorescence imaging has the potential to provide *in vivo* diagnostic information for many clinical specialities. Techniques have been developed over the years for simple ocular observations following UV excitation to sophisticated spectroscopic imaging using advanced equipment. Much of the impetus for research on fluorescence imaging for tissue diagnostics has come from parallel developments in photodynamic therapy of malignant lesions with fluorescent photosensitizers. However, the fluorescence of endogenous molecules (tissue autofluorescence) also plays an important role in most applications. In this paper, the possibilities of imaging tissues using fluorescence spectroscopy as a mean of tissue characterization are discussed. The various imaging techniques for extracting diagnostic information suggested in the literature are reviewed. The development of exogenous fluorophores for this purpose is also presented. Finally, the present status of clinical evaluation and future directions are discussed.

1. Introduction and principles

The biomedical use of fluorescence-based techniques is increasing. Frequently used techniques are fluorescence microscopy, flow cytometry and cell sorting (Dressler and Bartow 1989; Herman and Lemasters 1993). These techniques are frequently based on fluorescence marking utilising externally added fluorophores which selectively bind to specific targets in tissues.

The purpose of this paper is to review the work performed for *in vivo* fluorescence imaging for bulk tissue diagnostics, a much less developed area, and to point to possible future developments in this field. We limit the scope to imaging techniques, as the field of point spectroscopy is covered by Bigio and Mourant (1997, p 803 this issue). The various imaging techniques for extracting diagnostic information suggested in the literature, from visual examination following UV excitation to advanced multispectral imaging, are included. Primary fields of application of *in vivo* fluorometry include tissue metabolic studies (Tamura *et al* 1989, Horvath *et al* 1994, Cordeiro *et al* 1995), cardiovascular diagnosis (Perk *et al* 1991, 1993, Deckelbaum 1994, Deckelbaum *et al* 1995, Papazoglou 1995, Warren *et al* 1995), ophthalmology (Docchio 1989) and oncology. Most of the published *in vivo* fluorescence imaging studies deal with oncological applications; to identify early malignant lesions, for defining tumour extent and spread to adjacent tissues and as a guide for optimizing localized treatments of solid tumours.

It is important to recognize that there are many alternative fluorescence imaging techniques available to extract the relevant diagnostic information, and, in many of the potential applications, it is not yet clear which will be optimal. In analysing this we would like to address some parameters that differ between the approaches:
(i) Firstly, the fluorescent molecule, the fluorophore, marking lesions to be visualized, should be considered. Monitoring could be based on tissue autofluorescence, that means fluorescence resulting from endogenous fluorophores, or on an externally administered marker selectively accumulated in lesions of diagnostic interest. Many drugs investigated for their photodynamic activity in the field of photodynamic therapy (PDT) have properties of interest for fluorescence tissue diagnostics. Obviously, a technique offering sufficient diagnostic information based on pure tissue autofluorescence would be preferable, as it does not require the use of any exogenous fluorophore potentially associated with risks of unwanted side-effects.

(ii) Secondly, this fluorophore must somehow alter the fluorescence characteristics of the bulk tissue examined. This difference might be found in the fluorescence excitation or emission spectrum, or in the fluorescence lifetime. The difference may be due to a change in concentration of the fluorophore between the lesion and surrounding normal tissue, but it might also be due to an alteration in the fluorescence properties of that fluorophore due to variations in the microenvironment (Gudgin et al. 1981, Cubeddu et al. 1989). Other parameters in fluorescence recordings, such as polarization, could possibly also be utilized (Rigler et al. 1992).

(iii) Another issue to discuss is that the optical properties of the lesion might differ from those of the surrounding tissue. As the fluorescent light generated within the tissue is filtered by the tissue on its way to the detector, the tissue absorption and scattering properties and the detection geometry become important in addition to the primarily emitted fluorescence (Wu et al. 1993, Ahmed et al. 1994, Durkin et al. 1994). This makes it difficult to compare results obtained using different illumination and detection geometries.

In order to extract diagnostic information using fluorescence, several approaches have been suggested and examined. In the simplest case, fluorescence imaging can be performed at a single excitation wavelength, \( \lambda_{\text{ex}} \), and a single emission wavelength, \( \lambda_{\text{em}} \). However, extracting the maximum information may require spectroscopic imaging fully utilizing the differences in the fluorescence properties. Clinical evaluations are required to find out the best compromises between information content and technical complexity for different applications. The principle of several of the suggested methods and the results generated with the techniques will be briefly described below. The fluorophores used for this purpose are also discussed.

2. Fluorophores

The phenomenon of fluorescence was first observed by Stokes (1852). That tissue fluorescence potentially could be used for diagnostic purposes was recognised much later (Stüb 1911). Much effort has been spent on investigating the origin of this tissue autofluorescence. Many fluorophores have been identified using various spectroscopic techniques, such as excitation–emission matrices, time-resolved fluorescence spectroscopy, and comparing the results obtained from fluorophores in solution and in tissues. Some of the identified fluorescent substances in various tissues are tryptophan, collagen, elastin, nicotinamide adenine dinucleotide, reduced form (NADH), flavin mononucleotide (FMN) and porphyrins (Blankenhorn and Braunstein 1958, Chance et al. 1962, Aubin 1979, Benson et al. 1979, Visser et al. 1984, Laifer et al. 1988, Lohmann et al. 1989, Baraga et al. 1990, Hubmann et al. 1990, Rava et al. 1991). For a more detailed discussion and presentation of published research regarding the origin of tissue autofluorescence, we refer to the review by Andersson-Engels and Wilson (1992).
The development of exogenous fluorophores as tumour markers for fluorescence diagnostics is closely associated with that of photodynamic therapy (PDT), a tumour treatment modality utilizing production of cytotoxic radicals in photoinitiated chemical reactions. Experiments with fluorescent substances to photosensitize tissue were first performed at the very end of the nineteenth century (Raab 1899, 1900). Porphyrins were among the first naturally occurring compounds used to sensitize living organisms to visible light (Hausmann 1908, Meyer-Betz 1913).

The first quantitative study of fluorescence in vivo with exogenous fluorophores was performed by Winkelman and Rasmussen-Taxdal (1960) using fluorometry and spectrophotometry of porphyrins chemically extracted from tissue. In the same year, Lipson and Baldes (1960) reported on a derivative of haematoporphyrin (HpD) as a fluorescent tumour marker and photosensitizer of malignant tumours. HpD was first tested clinically in 15 patients with bronchial or oesophageal tumours and in a further 51 patients, 31 of whom had malignant lesions of cervix or vagina. All bronchial and oesophageal tumours and 29 of the cervical or vaginal lesions were examined with the unaided eye and observed to exhibit positive reddish fluorescence from the HpD following violet light excitation (Lipson et al 1961 1964b). In one of the two cervical or vaginal lesions in which no red fluorescence was seen the malignant lesion was found histopathologically to be covered with normal tissue.

Several other clinical fluorescence visualization studies with HpD during the period 1964–76 also showed encouraging results:

(i) Fluorescence was observed in 80% of 35 patients with bronchial or oesophageal carcinomas (Lipson et al 1964a).

(ii) In a study involving 226 patients, of whom 173 had malignant lesions of various types, positive HpD fluorescence was obtained in 132 (77%) (Gregorie and Green 1965, Gregorie et al 1968) and 22% of 53 patients with benign lesions also showed positive fluorescence.

(iii) Epithelial carcinomas of the mouth, hypopharynx, larynx or trachea all showed HpD fluorescence in a study involving 40 patients (Leonard and Beck 1971).

(iv) Eighteen of 23 patients with invasive or in situ cervical cancer showed positive HpD fluorescence (Gray et al 1967).

(v) All 12 patients with carcinoma in situ or dysplasia of the cervical uterus and three of four patients with squamous metaplasia showed reddish fluorescence in the lesions (Kyriazis et al 1973).

(vi) In a study of bladder carcinoma, lesions of 11 of 11 patients showed HpD fluorescence, and no normal tissue showed any HpD fluorescence, although a slight fluorescence was observed in the oedematous submucosa around the tumours in three patients (Kelly and Snell 1976).

It was also shown that intraocular tumours in animal models could be detected using HpD fluorescence (Cunningham and Henderson 1966, Krohn et al 1974). In this case interfering lens fluorescence had to be rejected to allow fluorescence to be seen in the positerior parts of the eye (Krohn et al 1974).

By the end of the 1970s 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 (Dougherty et al 1972, 1975). For this latter application many questions arose regarding the composition of HpD, which is a complex mixture of various porphyrins in both monomeric and oligomeric form.

Detailed research characterizing the properties of HpD were carried out during the 1980s. Some properties of interest are the dual-peaked fluorescence emission spectrum in
the 630–700 nm region and the relatively long fluorescence lifetime of about 16 ns, making its contribution to tissue fluorescence easily extractable. However, there also exist several drawbacks with this substance. As it is not a single stable chemical compound it is difficult to fully characterize. Furthermore, it has a low fluorescence yield, a poor selectivity in malignant tissue first 24 h after the drug administration, and the patient may suffer from a skin sensitivity up to several weeks afterwards.

For these reasons alternative drugs to be used as tumour markers for fluorescence diagnostics have been considered. Most of them, like HpD, are photodynamically active substances investigated primarily for their potential as photosensitizers for PDT. Phthalocyanines are interesting, as many of them have a much stronger fluorescence than HpD, and at longer wavelengths with less overlap with the tissue autofluorescence (Spikes 1986). A better selectivity for malignant tissues has also been found (van Leengoed et al 1990, Peng et al 1991). Other PDT drugs examined for fluorescence tumour marking capabilities include chlorins (mono-aspartyl chlorin e6 (MACE), di-aspartyl chlorin e6 (DACE), meso-tetra hydroxyphenyl chlorin (mTHPC) and benzoporphyin derivatives (Roberts et al 1988, Andersson-Engels et al 1993, Alian et al 1994). They all exhibit a strong fluorescence, but they have also a strong photosensitizing capability, which might sometimes be a drawback for pure diagnostic purposes. Also, they do not offer a much better selectivity to malignant tissue than HpD.

Several rhodamines have also been proposed (Haghighat et al 1992). The binding of these dyes to tissue is partly attributed to the positive charge of the rhodamines leading to attraction to transformed cells. Their drawback for this purpose is the emission wavelengths in the visible region, where the tissue autofluorescence is strong.

Recently, the introduction of δ-amino levulinic acid (ALA), a precursor to haem in the haem cycle, was a much needed boost (Malik and Lugaci 1987, Kennedy et al 1990, Kennedy and Pottier 1992, Svanberg et al 1994, Johansson et al 1997). Following administration of ALA, an excess amount of protoporphyrin IX (PpIX), the intermediate product just before haem in the intracellular chain reaction called the haem cycle, will build up in the tissue. Several advantages were found with this fluorescence tumour marking technique. Firstly, both ALA and PpIX are substances normally present in the body, making the toxicity issue less critical. Furthermore, the drug can conveniently be administered orally or applied topically (Kriegmair et al 1994, 1995, Svanberg et al 1994, Malik et al 1995, Rokahr et al 1995, af Klinteberg et al 1996). The ALA molecule is in itself a small non-photoactive substance, quickly metabolised to PpIX in tissue with a high selectivity to malignant tissue. The photophysical properties of PpIX are similar to those of HpD.

Some recent work has also been pursued to examine non-photosensitizing agents for fluorescence diagnostic of tissue. In this way one should totally eliminate the, for diagnostic purposes, drawback of light sensitization of tissues. Some compounds investigated with this idea in mind are carothenoporphyrins and chlorine derivatives (Nilsson et al 1994, Takemura et al 1994). Promising results have been obtained with these substances.

3. Fluorescence imaging techniques

At the end of the 1970s it was recognized that more sophisticated techniques than visual inspection and photometry were required (Sanderson et al 1972, Carpenter et al 1977, Profio et al 1977). Different types of fluorescence imaging instruments for diagnosis of malignancies are summarized below. 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. Earlier

Improving the detection techniques, it was recognized that electronic detection allows objective fluorescence measurement and time-sharing can also be used so that both visual inspection and fluorescence recordings can be performed in real time. Further, electronic detection makes it possible to record several fluorescence signals and form any function of those, such as to subtract tissue autofluorescence and/or to form a ratio of HpD to autofluorescence to reduce geometric effects (Ankerst et al. 1984, Profio and Balchum 1985). Alternatively, the ratio between the HpD fluorescence and the diffusely reflected excitation light (Profio et al. 1984, Lenz 1988), can be used, but care is needed to eliminate specularly reflected light (Lenz 1988). Pulsed light sources can be used to suppress ambient background light utilizing a time-gated detector (Ankerst et al. 1984).

The first clinical fluorescence bronchoscope using a mercury arc lamp (and later a CW krypton-ion laser) source and an image intensifier to amplify the faint fluorescence signal to a directly viewed image was presented in 1979 (Doiron et al. 1979, Profio and Doiron 1979). 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, and a video camera was also implemented to allow easy storage of the entire examination (Profio et al. 1983).

In general, the restriction to a single wavelength seriously limits the reliability of fluorescence diagnosis (Profio et al. 1983). Several approaches have been used to overcome this limitation. One is to incorporate a point-measuring device with spectral resolution into an imaging instrument, so that any suspicious region on the image can be investigated spectroscopically (Hirano et al. 1989). Another is to perform sequential or parallel imaging at several excitation or emission wavelengths to subtract autofluorescence or to form wavelength ratios (Profio and Balchum 1985, Profio et al. 1986, Baumgartner et al. 1987, Wagnières et al. 1990, Palcic et al. 1991, van den Bergh 1994). One of these systems, detecting two images in two emission bands, has been developed to a commercial product especially for endoscopic applications (Xillix Techn. Inc., BC, Canada, light-induced fluorescence endoscopy (LIFE)). This system utilizes a CW light source in the violet wavelength region, either a high-pressure mercury lamp at 405 and 436 nm or a HeCd laser at 442 nm. The diagnostic capability of this system, is based on the ratio between a red and a green autofluorescence emission band. The technique is reported to produce results of clinical interest in certain specialities (Lam et al. 1991, 1993a, b, Harris et al. 1995). The technique is therefore also approved by FDA for routine clinical use for endoscopic lung cancer examinations using the pure tissue autofluorescence. The reason for a change in this ratio for malignant tissues as compared with normal tissues is, however, not fully understood yet. Clinical evaluations for other specialities are also ongoing with such systems.

An alternative suggested technique combines multiple excitation and emission wavelengths. The rationale for this approach as compared with the previous ones is to be able to compensate for variations in tissue optical properties in the measurements, and thereby extract signals almost linearly dependent on an exogenous dye concentration (Sinaasappel and Sterenborg 1993, Sterenborg et al. 1994). This technique may allow quantitative measurements of fluorophores inside turbid media, as the idea is that the tissue optical properties should not affect the signals.

A slightly more advanced system using splitting optics to provide four images of an object, filtered at different emission bands has also been developed (Montán et al. 1985, Andersson-Engels et al. 1990, 1991, 1994, 1995, Svanberg et al. 1997). The four images can then be computer processed for viewing of the optimized contrast function image.
Another advantage with this system is the use of a pulsed light source and a gated detector. This concept allows the simultaneous use of fluorescence imaging and normal white light endoscopic examination. Many practitioners trained using normal white light examinations appreciate the possibility of adding the fluorescence information to the information they are used to working with rather than to replacing the white light with a fluorescence examination, or sequential examinations. As more detection wavelengths can be used, this system is more flexible in optimizing the diagnostic information extractable for various clinical applications. The system could work for tissue autofluorescence as well as for a combination for autofluorescence and fluorescence from an exogenous marker.

A further approach is to perform time-gated imaging to reduce the influence of autofluorescence. An amazingly good tumour demarcation capability has been demonstrated with very low concentrations of fluorescent tumour markers in animal studies (Cubeddu et al 1993, 1995). Time-gated imaging might also be interesting in the use of pure tissue autofluorescence as the diagnostic information.

Probably the most advanced and powerful system used for multispectral fluorescence imaging of tissue is presented by Malik et al (1996). This Fourier transform spectrometer allows recordings of the full fluorescence emission spectrum in each image pixel. With the spectral resolution provided with a system like this, it is possible to study very subtle changes in the fluorescence emission. The slight change in fluorescence emission of a fluorophore due to changes in the microenvironment can therefore be studied. Protoporphyrin bound to various subcellular compartments could thus be differentiated. This might be of specific interest for fluorescence tissue diagnostics.

Fluorescence imaging is a potential candidate for tissue diagnostics in a wide variety of clinical situations. Several techniques are suggested by which these examinations could be performed. Which one, if any, is best suited is probably going to vary with the application. All of them need to be examined separately in detail to be able to satisfactorily judge if a certain technique can provide valuable clinical information. We would therefore like to stress the need for well controlled clinical studies to evaluate the various techniques in detail. Also, since the methods vary significantly in what type of spectroscopic information they utilize, it might be difficult to judge how well the different methods would perform in a certain application, if only results from one of them have been studied. In particular, results from point-measuring systems are not directly transferable to the imaging geometry, as the tissue optical properties are much more important in the latter case due to self-filtration of the fluorescence from inside the tissue. It is thus important not to draw to general conclusions from results obtained with a certain technique.

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