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New Trends in Photobiology (Invited Review)

Malignancies and atherosclerotic plaque diagnosis—is laser induced fluorescence spectroscopy the ultimate solution?

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Abstract

A non-invasive diagnostic tool that can identify diseased tissue sites in situ and in real time could have a major impact on the detection and treatment of cancer and atherosclerosis. A review of the research performed on the utilization of laser induced fluorescence spectroscopy (LIFS) as a means of diseased tissue diagnosis is presented. Special emphasis is given to problems which were raised during clinical trials and recent experimental studies. The common origin and possible solution of these problems are shown to be related to, firstly, the identification of the fluorescent chemical species, secondly, the determination of the excitation/collection geometry and its effect to the method and, finally, the further elaboration on the laser–tissue interaction.

Keywords: fluorescence; Atherosclerosis; Cancer; Detection

1. Introduction

Optical spectroscopy has the potential of being selective as well as sensitive with respect to the detection of various molecular species and it can be used for semi- or non-invasive medical diagnostics. Laser induced fluorescence spectroscopy (LIFS) in particular may prove useful in the early detection of diseased tissue. This relies on the fact that the tissue's chromophore content varies depending on the state of the disease.

Nowadays, various studies on both neoplastic and atherosclerotic tissue are performed. In these studies, LIFS is applied to both the naturally occurring and the so-called fluorochromization-induced fluorescence. In the first case, the detection–discrimination algorithms are based on the varying concentration of biological chromophores whereas in the second case the detection of the diseased tissue relies on the fact that the exogenous chemical probe has the ability to accumulate selectively in the diseased areas.

Numerous models and experimental techniques have been suggested regarding the most effective acquisition and processing of LIF signals. The main disadvantages of these techniques arise from the fact that both natural and exogenous chromophores possess broad and partly overlapping absorption and emission spectra, making quantitative measurements an almost impossible task. Furthermore, scattering and absorption complicate the acquired signal in the detection of solely superficial tissue layers. In this case the goal of the LIFS technique application is the correct interpretation of spectral variations which are linked to the change of the relative concentrations of chromophores between normal and diseased tissue.

2. Fluorescence agents: sources and mechanisms

2.1. Malignancies—naturally occurring fluorescence (autofluorescence)

Initial studies by Ghadially et al. [1] suggested that ulcerated squamous carcinoma exhibited a red fluorescence when exposed to UV light. They also concluded that this red fluorescence may be due in part to the action of bacteria on a protoporphyrin precursor such as δ-aminolaevulinic acid (δ-ALA).

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Table 1
Optical properties of common fluorescent drugs during photodynamic detection (PDD)

Drug	λ_{abs} (nm)	λ_{fluor} (nm)	Absorbance (cm^{-1} M^{-1})	Fluorescence quantum yield
Photofrin (DHE)	420, 530	630, 690	3×10^3	<0.1
Benzoporphyrin	413	690	5×10^3	0.1–0.2
Pyropheophobide	660–670	>670	–	<0.2
Phtalocyanine	670	740	10^5	0.3~0.5
Naphthalocyanine	740–780	>800	3×10^5	0.2

The most extensive studies using excised tumor tissue have been those of Alfano et al. [2–4], who reported results obtained by using three different excitation wavelengths (457.9 nm, 488 nm, and 514.5 nm). In all cases they reported discriminatory spectral features between normal lung tissue and lung tumors. In normal tissue there was a minimum at 580 nm and a maximum at about 600 nm (two additional maxima were excitation wavelength dependent). The malignant tissue, however, showed a single peak, broad band spectrum (peak at 512 nm for 457.9 nm and 488 nm excitation and at 530 nm for 514.5 nm excitation).

A systematic study on the naturally occurring fluorescence of malignancies in vivo was performed by various groups such as Lam et al. [5–7] and Cothren et al. [8]. The common result of these studies is the marked decrease of the overall fluorescence intensity in the case of pre-cancerous and cancerous tissue. They also observed spectral differences in the case of 488 nm excitation.

The chemical basis of these changes is not known. Differences in the relative concentrations of oxidized and reduced flavins in the malignant and normal tissue may account for the variations of the autofluorescence intensity [9].

The variation of the results of the previously mentioned studies could be based on their different experimental conditions (in vitro vs. in vivo) as well as the different types of tissue (Lung parenchyma vs. bronchial tissue). If the difference in the natural fluorescence intensity between normal and malignant tissue was indeed due to the lower concentration of oxidized riboflavin in tumor tissue, this might not be observable under in vitro conditions, where re-oxidation of reduced riboflavin, in normal tissue, occurs.

2.2. *Malignancies – exogenous chemical agent induced fluorescence*

The utilization of exogenous tumor binding fluorescing agents for tumor treatment (photodynamic therapy) preceded their utilization as a means of demarcation. Lipson et al. [10], at the Mayo Clinic in the 50s, noted that hematoporphyrin derivative (HpD) localizes in or on malignant cells, depending on their viability. Living tumor cells showed a characteristic pink fluorescence confined to the cytoplasm, whereas non-viable cells exhibited diffuse fluorescence from the cell membrane. Various groups then indicated the possibility of the usage of porphyrins as a diagnostic means while cautioning, at the same time, on their limitation. Figge et al. [11–14] revealed the porphyrin accumulation in neoplastic (induced and transplanted sarcomas, mammary carcinomas), embryonic and regenerating tissues via their fluorescence. The same group observed a tendency of porphyrins to accumulate in lymph nodes, a fact that might complicate their therapeutic and diagnostic use. These observations led to a series of clinical studies during which the initial optimism was reduced due to the lack of technological means which would facilitate the clinical application of the technique [15–19]. With the introduction of lasers in the clinical field, similar studies were performed in the early 80s [20,21]. The use of HpD and a purified version Photofrin II (containing mainly dihematoporphyrin ether or ester, DHE, with a large amount of aggregated compounds) gradually increased during the 80s. Fig. 1 depicts the fluorescence spectrum from a metastatic lymph node after intravenous administration of Photofrin II (0.75 mg per kg of body weight, 48 h), and during its exposure to the He–Cd laser (442 nm). The spectrum is characterized by a broad band section related to natural tissue fluorescence and the typical dual peak emission spectrum of Photofrin II.

The tumor localizing components of HpD or Photofrin II, however, were proven to differ from the components showing maximum photosensitizing efficiencies (useful in PDT). Dimer and aggregated species were reported to be the main tumor-localizers, whereas dimer and monomer species had the best photosensitizing properties [22–24]. It was also noted that the monomer substances hematoporphyrin, hydroxyethyl–vinyl-deutero–porphyrin and protoporphyrin had high fluorescence yields [24]. Andreoni [25], Cubeddu et al. [26–28], Svanberg et al. [29], and other groups [30,31] used time resolved techniques to confirm these discrepancies. The goal of these studies was to clarify whether certain components accumulate selectively in the tumor tissue, or if a tumor-specific conversion (monomerization and dimerization) might take place.

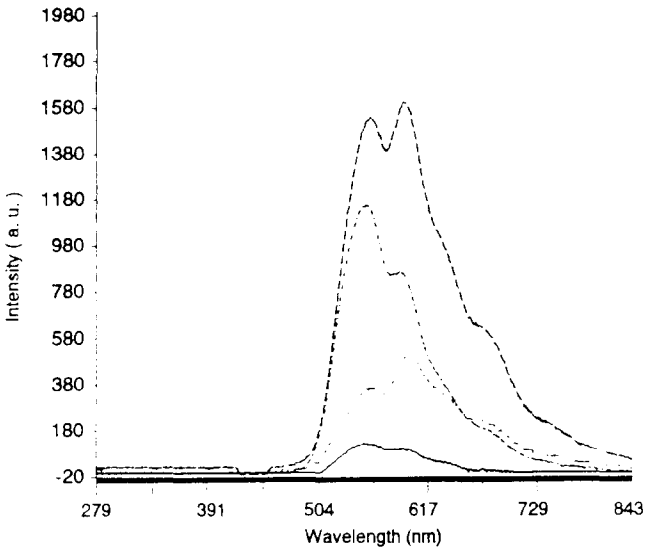

Fig. 1. Fluorescence spectrum from a metastatic lymph node after intravenous administration of Photofrin II (0.75 mg per kg of body weight, 48 h), and during its exposure to a He–Cd laser (442 nm). The spectrum is characterized by a broad band section related to natural tissue fluorescence and the typical dual peak emission spectrum of Photofrin II. —, Normal; – – –, fibrous; · · ·, aneurism; – · –, calcified.

There was no initial, at least, experimental evidence of the latter scenario [29]. It was suggested though that if the photosensitizing efficiency in vivo was mainly attributed to dimer compounds, the similar time dependence of fluorescence for dimer and monomer species could lead to a correlation between the fluorescence emitting sites and the ones of optimum photosensitization [30].

During the past few years an alternative concept has been introduced. It is based on the initial observations of Ghadially et al. [1], suggesting the involvement of δ-aminolaevulinic acid, as precursor of protoporphyrin, in the creation of the tumor related fluorescence emission. δ-ALA itself is not fluorescing but it is converted to heme via protoporphyrin, a well-known effective photosensitizer which also emits strongly in the red [31]. Table 1 lists the optical properties of some common exogenous fluorescent agents.

2.3. *Atherosclerotic lesions—naturally occurring fluorescence (autofluorescence)*

Early chromatographic experiments reported on the pale green fluorescence in atheromas [32]. This was related to the presence of various carotenoids and/or vitamin A in these sections. Both carotenoids (B-carotene, lutein) and vitamin A are large lipid molecules which are not synthesized by human tissue and their distribution may serve towards the localization of dietary lipids in atherosclerotic lesions. The concentration of these chromophores seems to be related to the age of the lesion. Banga et al. [33] at a later stage supported the hypothesis that the fluorescent substances which accumulate in the human atherosclerotic aorta are tightly bound in the intimal plaque and take the form of "atherofluorescent" component (AFC) pigments which are directly related to the degeneration of elastin. The maximum activation/emission peaks were determined to be 350/405 nm and 380/450 nm respectively.

Leon et al. [34] introduced the concept of the filtering of the emitting species from the superimposed atheroma which acts as an inner filter by both attenuating the excitation of media residing fluorophores and the resulting fluorescence. In the previously mentioned studies the measurements were performed on extraction products. Laifer et al. [35] suggested that elastin is the major fluorophore in atherosclerotic aorta. He also suggested that the key element is its relative concentration in the tissue and not its presence per se. Feld's group has suggested [36,37] that there are also variations of the autofluorescence between coronary arteries and aortas. The media of coronary arteries exhibited less intense fluorescence than that of aortas. This was due to the scarcity of elastic fibers in these media. Another reason is that the internal and external elastic laminae of the coronary arteries were the sites of the fluorescing fibers. In Table 2 a summary of excitation–emission maxima of selected biologically important molecules is shown. However, the most distinctive autofluorescence of atherosclerotic arteries occurs from the lipid and calcified deposits on the atheroma core. In Fig. 2, fluorescence spectra from various types of atherosclerotic tissue are shown.

Table 2
Summary of excitation–emission maxima in selected biologically important molecules

Chromophore		(λ_{exc}, λ_{em}) maximum
Tryptophan	Solution	(275, 350 nm)
NADH	Solution	(350, 460 nm)
NADPH	Solution	(350, 460 nm)
4-Pyridoxic acid	Solution	(300, 435 nm)
Pyridoxal 5′-phosphate	Solution	(305, 375 nm)
		(410, 520 nm)
Collagen I	Powder	(340, 395 nm)
		(270, 395 nm)
		(285, 310 nm)
Collagen III	Powder	(275, 310 nm)
		(330, 390 nm)
		(370, 450 nm)
Elastin	Powder	(460, 520 nm)
		(360, 410 nm)
		(425, 490 nm)
		(260, 410 nm)

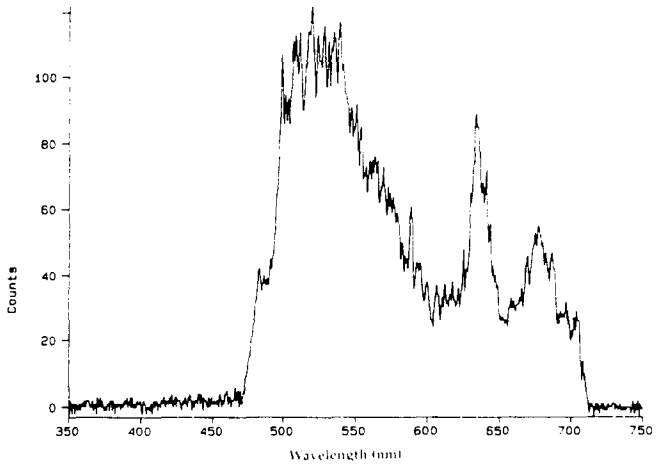

Fig. 2. Fluorescence spectra of arterial samples with various degrees of atherosclerosis (fibrous plaque, calcified plaque, aneurism) compared with normal arterial tissue. Curves are not plotted on the same scale.

2.4. Atherosclerotic lesions—exogenous chemical agent induced fluorescence

In addition to the autofluorescence-based diagnostic technique, it has been shown that certain exogenous drugs bind preferentially to atherosclerotic lesions in vitro. Extended work has been performed with chlortetracycline (CTC-HCl) [38,39], HpD [40] and other substances [41]. Different modes of porphyrin accumulation to plaques have been suggested. The smooth muscle cell (SMC) is often proposed as the site of porphyrin uptake by the lesions. However, several other mechanisms have also been postulated. These include the increased endothelial permeability at the site of atherosclerotic lesions, the binding to collagen and elastin fibers and the interaction with other cells such as macrophages.

3. Diagnostic accuracy of fluorescence spectroscopy

The lack of structure, except the few broad-band peaks, in the fluorescence spectrum permits low-resolution spectroscopy to be an adequate method of diagnosis. The major diagnostic component is the enhancement of contrast between the diseased and the healthy tissue. This may be strongly affected by technical parameters such as the uniformity of illumination of the target area (especially in the case of imaging), the concentration of the exogenous fluorescing agent (if it is the case), the light transmission and detection optics as well as the detector's response to the in vivo environment. The "signal" from the target must be comprehended and integrated by the physician into diagnostic information, i.e. "true fluorescence or not?" Perception is increased by continuous practice, following a certain learning curve. Accuracy of perception may increase by comparing fluorescence based information with other conventional diagnostic modalities. Ultimately, the histologic evaluation of the irradiated sites will determine the characteristics of the diseased site and its relevance to the "signal".

The final determination of the diagnostic-localizing ability of LIFS will depend on the values of the following indices:

(i) true positive: number of diseased lesions with positive fluorescence (TP);

(ii) false positive: number of non-diseased lesions with positive fluorescence (FP);

(iii) false negative: number of diseased lesions with no fluorescence (FN);

(iv) true negative: number of non-diseased lesions with no fluorescence (TN).

The accuracy for positive prediction is determined as TP/(TP + FP), while the accuracy for negative prediction is determined as TN/(TN + FN).

The physician's goal is to diagnose positively or rule out the presence of the disease. In order to diagnose the disease requires high specificity, whereas to determine absence of the disease requires high sensitivity. This rigorous approach was introduced in the early 80s by Balchum et al. [42] and was used in all recent preclinical and clinical trials.

4. Tumor detection and localization

4.1. Clinical studies—present trends

Technological improvements in the clinical instrumentation and the simultaneous introduction of lasers in clinical practice gave a new boost to LIFS-based diagnostic work. The result of the research effort of the Mayo Clinic in the late 50s and early 60s was reproduced and improved by various groups. Profio and coworkers played an important role during this period [43–49], focusing on lung carcinoma in situ (CIS) detection. This particular type of malignancy is hardly detectable by X-ray or conventional white light bronchoscopy. The introduced methodology included the use of Kr^+ laser for excitation, the administration of HpD and the use of intensified video camera detection. The target in these studies was the detection–monitoring of the characteristic dual peak fluorescence intensity of HpD at 630 nm and 690 nm. There were other groups, mainly in Europe, which targeted on other types of tumor, such as Spinelli et al. [50], Andersson-Engles et al. [51], and clinical groups shifting towards the use of δ-ALA [52] and other chromophores [53]. In Baumgartner's study [52], δ-ALA has been successfully used in the detection of CIS and pre-cancerous types of hyperplasia or dysplasia in the bladder (mucosal lesions).

The acquisition of the produced protoporphyrin IX laser induced fluorescence (Kr^+ laser excitation) revealed the power of this technique by detecting precancerous or malignant lesions which were not recognized during routine cystoscopy. Keeping the rate of false positive diagnosis to low levels (16%) and not exhibiting any false negative results these studies open new perspectives in bladder cancer diagnosis and treatment. Recently, Lam's group [54] has also presented impressive results (94% specificity, 72.5% sensitivity) in the application of LIF imaging in bronchoscopy indicating that, at least in the case of lung cancer, this method is slowly becoming a candidate for routine clinical use.

4.2. Laboratory work—future trends

It is evident that the future of LIF based detection techniques will depend firstly on the improvement of the instrumentation and signal analysis algorithms and secondly, on the development of new photosensitizing agents with higher selectivity/emission bands and lack of side effects, such as skin photosensitization.

The improvement of endoscopic instrumentation has created a tendency in research groups to try to combine LIF and 2-D imaging [55–61]. Some of the groups use dual wavelength excitation for enhancing the contrast between the photosensitizer's emission and the autofluorescence [54]. The search for chromophores other than HpD and Photofrin II is continuing, and other porphyrins (Uroporphyrin I) or aluminium phthalocyanine TS and acridine red have been extensively tested [56]. Finally, time gating of the CCD camera's intensifier could also improve the signal-to-noise ratio in fluorescence images of tumors. By delayed observation of LIF it is possible to identify the contribution of the photosensitizer fluorescence to the overall emission signal [59–61]. A prerequisite of this technique is the use of fast pulsed lasers and a relatively expensive detection system.

Numerous groups continue the research on single point measurements of LIF, using simpler instrumentation and looking at the LIF distribution as a function of wavelength [62–66]. Their prediction rates reached impressive levels in some cases (94%, [64]) but the same groups cautioned on the measurement of a relatively high number of false positives [62].

5. Atherosclerotic plaque identification

5.1. Clinical studies—present trends

The success of laser angioplasty in patients with peripheral vascular disease has been limited by a high frequency of vascular wall perforation. Leon et al. [67,68] have used a LIF-guided angioplasty system in clinical trials and using an atheroma detection algorithm, analysis of aorta and coronary spectra showed a specificity of 100% for recognizing normal vessel wall and a sensitivity of 73% for recognizing atherosclerotic sites. The latter was related to the heterogeneous composition of atheromatous tissue compared with normal vascular tissue. These studies, along with similar studies in Europe [69], introduced the possibility of incorporating spectroscopic guidance into clinical multi-fiber systems but also raised new issues which are directly related to the clinical practice. These issues concern the assurance of uniform and adequate physical contact between a larger catheter tip and the plaque surface in a blood environment and the interpretation of LIF spectra obtained from a large area when the fiber is not in contact.

5.2. Laboratory work—future trends

As a result of the extensive laboratory development on new detection methodology which was performed during the late 80s by various groups [70–81] progress was rapid during the 90s with the improvement of fiber optic delivery of laser light for laser angioplasty and the resulting Phase II and III clinical trials in the USA. From the initial studies, it was evident that a "smart" laser angioplasty system might incorporate low-power laser radiation for arterial LIFS to guide the delivery of the high-power laser needed for plaque ablation. The latter was suggested by Sartori et al. [71,73,75], Deckelbaum et al. [72,77,80], and was experimentally tested by Laufer et al. [76,81]. In Laufer's studies the ablation was performed by a KrF Excimer laser (248 nm) and later by a XeCl excimer laser which was also used in clinical trials.

In the 90s, research has progressed and the use of time resolved techniques was introduced by Andersson-Engles et al. [82,83]. Their data revealed a fluorescence band peaking at 380 nm with a lifetime of $\simeq 7$ ns to be specific for atherosclerotic plaque. They also suggested that time resolved techniques are relatively insensitive to blood interference.

Fluorescence intensity rationing has been extensively used by Feld et al. [37], postulating that the two peaks at 340 nm and 380 nm were attributed to tryptophan and collagen/elastin respectively. By combining these parameters in a binary classification scheme, normal and atherosclerotic aorta samples were distinguished in 93% of the cases and various types of plaques could be successfully classified.

Research groups in collaboration with medical excimer laser producing companies attempted to incorporate LIF based diagnosis on the angioplasty systems. They also presented real-time feedback systems that could control the ablation process [84,85].

With respect to highly calcified areas, Klug et al. [86] have used Raman spectroscopy analysis, irradiating the suspected tissue with a long wavelength laser (710 nm). This method is still hindered by problems though, such as the weakness of the signal, leading to long integration times, and the attenuation of the scattered Raman signal by the smooth layer of tissue which covers the atheroma.

Finally, various groups studied the ablation process via LIFS. The analysis of the temporal behavior of the resulting plume luminescence could become a useful technique for the destructive characterization of plaque [87,88]. Similar studies on the ablation produced plasma emission revealed high sensitivity (91%) and specificity (100%) for calcified atherosclerotic plaque [89].

6. Deliver/collection and tissue optics interference

An important parameter of the design and development of LIFS based diagnostics instrumentation is the effect of the excitation/collection geometry. From the experimental work and simulations of Keijzer et al. [90,91], Chaundhry et al. [92] and Richards-Kortrum et al. [93], it was shown that, for example, laser delivery/emission collection through the same fiber, in contact with the tissue, produces fluorescence spectra different from those obtained when the excitation and collection fibers differ. Another consideration in a single fiber system is whether the excitation area is smaller than the collection area, leading again to different spectra. Monte Carlo simulations also demonstrated that the excitation light dose strongly depends on the diameter and the profile of the incident beam, indirectly affecting the actual collection of the emitted fluorescence. Recently, a new semi-analytical Monte Carlo simulation technique (SAMC) was presented by Avrillier et al. [94] where the computational time is decreased dramatically by the a posteriori evaluation of the result of every simulation step on the physical quantity of interest.

It was also shown that LIF spectra in the case of arterial tissue can be altered by excessive exposure to laser irradiation. Reversible changes in the spectral line-shape were detected, postulated to be related to the dissociation of oxyhemaglobin, whereas irreversible decreases in the fluorescence intensity were linked to photochemical interaction with the tissue fluorophores. Similar time dependent alterations were observed in the fluorescence spectrum of Hp in a semi-solid environment [95], related to the combined thermal and photodynamic effect and the resulting denaturation of the environment. More importantly, variations in HpDs decay properties have been reported during its conversion to photoproducts [96], and photobleaching [97].

Finally, in a series of studies on the pharmacokinetics of Photofrin II and m-THPC by van den Bergh et al. [98] LIFS ability to monitor the photosensitizer's distribution in the body is demonstrated. These results agree in the case of HpD with similar studies that were presented, simultaneously, on an animal model by Vari et al. [99]. In den Bergh's work it is also pointed out that the spectral signature of the photosensitizer changes dramatically by the collection geometry (tissue–fiber distance) due to both variations of mean free path of the photons in the red with respect to the blue and green but also to the absorption of HbO_2 which interferes with the fluorescence spectrum of the photosensitizer (HpD). This observation leads to a simple algorithm of data correction from the tissue's emission background.

7. Epilogue

The advance of optical clinical instrumentation during the second half of the 80s has boosted LIF related research. Continuing experimental studies have demonstrated that laser induced fluorescence spectroscopy (LIFS), especially when it is an adjunct to standard endoscopic techniques (laparoscopy, angioscopy), could plausibly operate in a synergetic manner with conventional techniques (biopsy, arteriography). Long term and tedious clinical studies should further establish the sensitivity and specificity of LIFS, particularly when it is combined with imaging. Both time-resolved and continuous LIFS exhibit promising results, with the former requiring more expensive instrumentation.

The responsibility for the fate of this novel technique is slowly shifting to the clinicians. Their experience during the clinical evaluation will be the determining factor of the survival of this potential diagnostic tool. The numerous parameters that may affect the diagnosis are still under investigation and it is rather surprising that 30 years after the initial clinical trials of Lipson et al. [16] the skepticism of clinicians can be expressed by his comments then: "Despite the simplicity of the technique and the relative safety, we do not think its routine use in the study of patients.....would offer much advantage over presently employed procedures."

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