The role of laser-induced fluorescence spectroscopy in the detection of human atherosclerosis

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Identification and characterization of atherosclerotic plaques is a major topic of interest in cardiovascular medicine. At present there are no reliable diagnostic tools available to characterize the chemical composition of atherosclerotic plaques *in vivo*. Laser-induced fluorescence spectroscopy (LIF) has shown to provide accurate diagnostic information on plaque development *in vitro*. This article reviews the current status of LIF research on atherosclerosis. In addition, preliminary new results are presented and future directions and possible cardiovascular applications are discussed.

Background and significance

Atherosclerosis, the major etiology of obstructive coronary artery disease, has become the leading cause of mortality in the western economically developed countries[1,2]. It accounts for the majority of the acute ischemic syndromes as sudden cardiac death, acute myocardial infarction and unstable angina. As such, it is responsible for at least 500,000 deaths per year and approximately $ 120 billion in direct medical costs in the United States alone[3]. Although the mortality from these diseases is decreasing in the United States[4,5], in many parts of the world it is rapidly increasing. Atherosclerosis, therefore, represents one of the major public health hazards of the new millennium.

Several therapies for treating atherosclerosis are available but a technique to image lesion composition or reliably predict or prevent lesion progression does not exist[6,7]. Clinical techniques capable of evaluating atherosclerosis in a safe, valid, and reproducible manner are of critical importance for diagnosis, for the selection and evaluation of the effects of various interventional or pharmaceutical therapies, and for use in epidemiological and clinical research relating to the pathophysiology of atherosclerosis[8]. In principle, early recognition of any abnormality or disease allows the physician an opportunity to predict the course of the patient's disease, re-examine the patient more frequently, or initiate an appropriate preventive therapy at the time of diagnosis. In cardiovascular medicine, there is a pressing need for instruments to detect and study the composition of early atherosclerotic lesions[6–9].

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Rapid advances in laser spectroscopy are leading to new methods for the diagnostic imaging of human artery tissue[10–12]. Optical spectroscopy provides detailed information about arterial disease. The incident light, interacting with tissue constituents in a variety of ways provides information about the chemical and structural composition of a lesion encoded in the spectrum of the re-emitted light. These features can be measured accurately with sensitive equipment that collects quality spectra in less than a second. Within the variety of techniques available in the field of optical spectroscopy, laser-induced fluorescence in particular may prove useful in the early detection of disease[10,13,14]. This relies on the fact that the chromophore content of the tissue varies depending on the state of the disease.

The possibility of exploiting laser-induced fluorescence spectroscopy (LIF) for tissue diagnostics has been increasingly studied by many authors in the past few years in a variety of fields, ranging from urology[15,16], gastroenterology and oncology[17–20] to dentistry[21]. One of the most promising applications of LIF in medicine, however, is the detection of atherosclerosis. In the present paper, a brief review of the detection of atherosclerosis by using fluorescence spectroscopy is given. In addition, new results are presented and future directions and possible cardiovascular applications are discussed.

Single wavelength excitation of arterial tissue

Kittrel *et al.*[22] used excitation at 480 nm wavelength to excite whole human cadaver carotid artery samples and showed a difference in fluorescence lineshape and intensity between normal and atherosclerotic artery (Figure 1). This work provided an empirical means of determining whether a given arterial wall specimen was normal or contained a fibrous atherosclerotic plaque. On the basis of this finding, the direct, *in vivo*, real time diagnosis of atherosclerosis via catheterization seemed to be feasible.

After the initial observation by Kittrel *et al.*[22], several groups went on to develop *in vitro* algorithms for the detection of atherosclerosis based on lineshape and/or intensity differences of the fluorescence spectrum. Sartori *et al.*[23] showed that fluorescence at visible wavelengths could be used to differentiate normal aorta from calcified plaque. Several groups[24–28] showed that using ultraviolet

excitation wavelengths (308 nm, 325 nm, 337 nm), fluorescence spectroscopy could be used to differentiate normal aorta from white fibrous plaques and/or yellow fatty plaques. With 325 nm laser excitation, using the total fluorescence intensity (defined as the sum of arterial fluorescence emission at 380 nm and 440 nm) as a parameter, Deckelbaum *et al.*[29] demonstrated 97% correct classification of non-atherosclerotic tissue *in vitro*. Andersson-Engels *et al.*[30] found that, at 337 nm excitation, very prominent differences between atherosclerotic lesions and normal arterial wall could be found in the fluorescence spectra of both the large elastic arteries, such as the aorta and femoral artery, as well as in small muscular arteries, such as the coronary arteries. Deckelbaum has suggested that the differentiation of normal and atherosclerotic coronary artery is not as successful with 325 nm excitation as it is with of normal and atherosclerotic aorta[29]. Leon *et al.*[28] noted that with ultraviolet excitation, calcified plaques were more similar to normal tissues in their fluorescence properties[28].

While this basically empirical approach often leads to a reasonably accurate diagnosis *in vitro*[27–29,31], it produces little biophysical information regarding tissue composition. To develop a meaningful diagnostic algorithm, it is important to understand the physical and biochemical basis for the observed emission lineshapes and to use this knowledge to extract specific, quantitative biochemical and morphological tissue information from the fluorescence spectra. Research, therefore, went on to investigate the morphological basis for discrimination of normal artery and atherosclerotic plaques, based on their fluorescence emission spectra. The autofluorescence of arterial wall appeared to be largely based on contributions of the proteins collagen, elastin and tryptophan[27,32,33]. NAD(P)H makes also minor contributions[34]. Atheromatous plaques showed intensely fluorescent, ring-shaped or discrete solid deposits in its necrotic core. The color of this fluorescence was yellow, when excited by both ultraviolet and visible wavelengths[33,35]. These deposits were attributed to ceroid, since they stained positively with lipid stains, but could not be extracted with alcohol. The excitation, emission maxima of the arterial fluorophores are listed in Table 1. Laifer *et al.*[27] noted that carotenoids could also be partially responsible for the fluorescence of yellow plaque.

Since absorption is frequency-dependent, the fluorescence of fluorophores depends strongly on the wavelength of the exciting light. The choice of excitation wavelength determines which fluorophores can contribute to the tissue emission spectrum in two ways[10]: only those chromophores that absorb at the excitation wavelength can be excited; and only those chromophores contained in tissue layers to which excitation light penetrates and from which emission light can escape will produce fluorescence that can be measured from the surface.

For the UV wavelength excitations, Deckelbaum *et al.*[36] and Andersson-Engels *et al.*[34] showed that at 325 nm and 337 nm the fluorescence signal of normal and atherosclerotic aorta could be attributed to the structural proteins collagen and elastin. However, at these excitation wavelengths calcified plaques could not be detected since *in situ* calcium phosphate and calcium hydroxyapatite deposits did not exhibit fluorescence. Clarke *et al.*[24] empirically correlated the fluorescence signal at 325 nm excitation to the intimal thickness and the relative proportions of fibrous, fatty and calcified tissue within coronary artery atherosclerosis. Laifer *et al.*[27] developed a 90% positive predictive classification algorithm based on

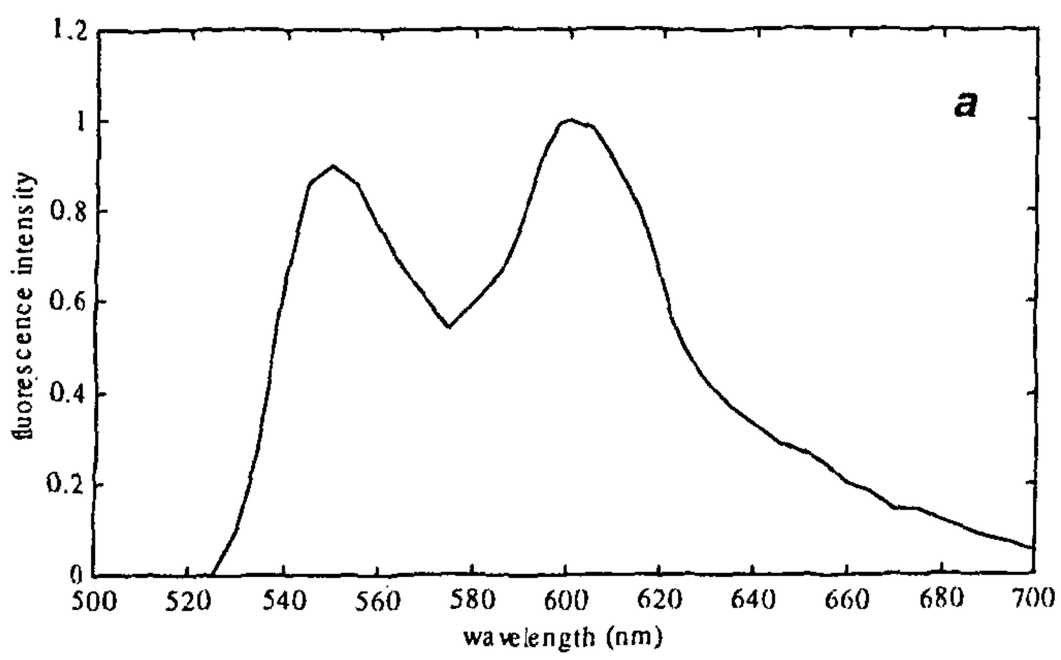

Figure 1. Fluorescence spectra of normal (*a*) and atherosclerotic (*b*) aorta at 480 nm, as observed by Kitrell *et al.* (adapted from ref. 22).

Table 1. Excitation emission maxima of the major fluorophores in normal and atherosclerotic arteries (adapted from ref. 38).

Arterial component	Excitation emission maxima (nm)
Tryptophan	(290, 330)
Collagen type I	(350, 420)
II	(340, 390)
III	(450, 510)
Elastin	(460, 520)
	(360, 410)
	(425, 490)
Ceroid	(340, 440)
	(460, 520)
NAD(P)H	(350, 465)

elastin and collagen spectral decomposition. Each aortic spectrum was normalized to unit area and then mathematically resolved into a linear combination of pure elastin and collagen spectra, thereby assuming that elastin and collagen are the only fluorescent properties at this excitation wavelength. Baraga *et al.*[37,38], using 308 nm excitation, has established diagnostic algorithms for the presence of non-calcified and calcified atherosclerosis in human aorta based on differences in the contributions of the fluorophores tryptophan, collagen and elastin to the spectra of these tissues. He noted that other studies at 325 nm or 337 nm excitation, where tryptophan is not excited, were unable to differentiate normal aorta and ulcerated calcified plaques[28] and suggested that excitation at 308 nm avoids this problem. *In vivo* application of fluorescence spectroscopy using 308 nm may not be safe, however, due to the mutagenic character of such short ultraviolet excitation wavelengths[39].

Multiple excitation spectra are required in order to fully evaluate the fluorescence diagnostic potential of a given type of tissue. An excitation-emission matrix (EEM), a three-dimensional plot displaying the fluorescence intensity (vertical axis) as a function of the excitation and emission wavelengths (horizontal axis), is a convenient way of exhibiting such information (Figure 2). Such EEMs provide a full characterization of the fluorescent properties of a tissue sample. To define the optimal excitation wavelength, Richards-Kortum *et al.*[13] collected *in vitro* EEMs of normal and diseased aorta and coronary arteries. They concluded that normal, non-calcified atherosclerotic and calcified atherosclerotic coronary artery lesions could best be differentiated using excitation at 476 nm (ref. 13). By varying the excitation, Oraevsky *et al.*[40] observed that the maximum qualitative differences in emission spectra could be found within excitation range of 450–490 nm, which is in correspondence with the observation by Richard-Kortum.

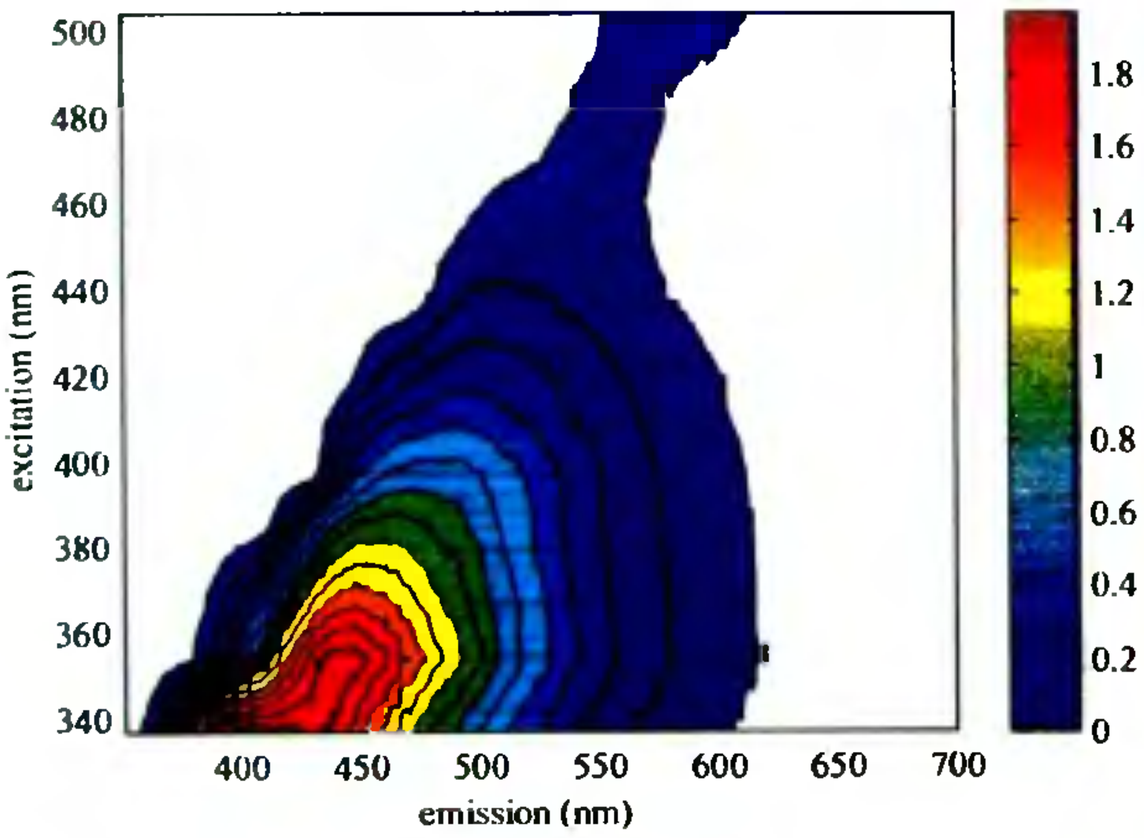

Figure 2. Excitation emission matrix (EEM) of a sample of normal aorta. The color bar represents the intensity of the fluorescence in arbitrary units. This EEM was collected within half a second, using the clinical fast-EEM system, as described in the text.

At 476 nm excitation wavelength, normal arteries fluoresced in the green, and this was attributed to the structural proteins. In non-calcified plaques, an increase in intensity of the collagen band was observed, which was attributed to its denser packing in the intima. When an atheromatous core was present, intense yellow fluorescence was observed, and this was attributed to ceroid deposits. In calcified plaques, the intense yellow and green fluorescence is mainly due to ceroid and an increased amount of collagenous fibrous tissue[41,42].

The penetration depth of this wavelength in normal human aorta is approximately 300 μm (ref. 42). This is greater than the intimal thickness of normal muscular and elastic arteries and is also greater than the intimal thickness in intimal fibroplasia and in many early atherosclerotic plaques, implying that with this excitation wavelength both structural and histochemical information can be extracted from the resulting emission spectra. This is better than at 325 nm excitation, where Gmitro *et al.*[43] showed that 95% of the arterial fluorescence was produced by the top 150 μm (ref. 10).

A fluorescence model at the 476 nm excitation was described by Richards-Kortum *et al.* They described the fluorescence spectrum of arterial tissue (see equation) as a composite of autofluorescence (F) from structural protein (β_{sp}, F_{sp}) and ceroid (β_{Ce}, F_{ce}) as altered by the attenuation effects (A) of structural proteins (x_{sp}, A_{sp}) and hemoglobin (x_{Hb}, A_{Hb}). In this equation, F_{sp} and F_{ce} are fluorescence lineshapes and A_{sp} and A_{Hb} are absorption lineshapes. P is the power of the excitation light and k is a constant.

$$S(476, \lambda_m) = kP(476) * \frac{\beta_{sp}(476) F_{sp}(476, \lambda_m) + \beta_{Ce}(476) F_{Ce}(476, \lambda_m)}{x_{sp}(476) A_{sp}(476, \lambda_m) + x_{Hb}(476) A_{Hb}(476, \lambda_m)}$$

In analysing the spectrum, the variables (β_{sp}, β_{Ce} and X_{Hb}) are varied to optimize a fit, while X_{sp} is maintained constant, assuming that the structural protein attenuation is approximately constant in different tissues. An algorithm, based on the parameters β_{sp}, β_{Ce} was then developed[42].

Richards-Kortum *et al.*[44] showed that even by using optical fiber probes with lengths of 80 to 120 cm instead of conventional lenses to couple the light in the spectrometer, the *in vitro* diagnostic algorithm had an overall accuracy of 92% in classifying coronary artery as normal, atherosclerotic, or calcified plaque[13].

Most of the research described above has been performed *in vitro*, in a controlled laboratory environment. Space and time limitations are generally not overly restricted for *in vitro* studies. However, there is a high premium for space in operating rooms, catheterization

labs and other clinical settings. In general, the systems must be compact and fast. The MIT Laser Biomedical Research Center and other institutes are developing instruments to conduct such studies in a clinical setting. However, the goal of all this research is to perform laser spectroscopy *in vivo*, and then further develop spectroscopy into a powerful tool that can be used in the detection of disease in human beings.

The present clinical systems contain four main elements; (1) a light source, (2) an optical fiber spectral probe or catheter, (3) a spectrometer and (4) a detector. The light source can be either a laser or narrow-band conventional source for fluorescence, and a white light lamp for reflectance. The detector is typically an intensified photodiode array with 1024 elements. The catheter contains long, flexible, optical fibers that deliver the excitation light to the tissue and return the re-emitted light (either fluorescence or reflectance) to the spectrometer. In our catheters the distal tip contains several collection fibers, circularly arranged around a single delivery fiber, and terminated by a short transparent 'optical shield' which expands and overlaps excitation and collection spots and has certain other advantages[45,46]. The outside diameter of these catheters is typically about 1 mm (3–5 F). Light from the excitation source is focused into the proximal end of the catheter, the distal tip of which is brought into contact with the tissue site to be sampled. The re-emitted light from the tissue is collected and returned to the spectrometer where it is spectrally dispersed and then detected. The resulting spectrum is usually then transmitted to a computer, where it can be stored and analysed. For fluorescence detection, a long wavelength-pass filter with a sharp cut-off is used to prevent the excitation light from flooding the detector. Because of differences in light–tissue interaction due to differences in excitation and collection optics, it is however believed that algorithms designed for a particular collection system may be valid only for that particular system[10,44]. For a detailed description of present single wavelength fluorescence systems for *in vivo* research, one is referred to the work by Richard-Kortum *et al.*[44] and Brennan *et al.*[46].

Initial clinical studies have shown that atherosclerotic tissue can be differentiated from normal tissue *in vivo* using 325 nm excitation light[27,28]. Successful percutaneous detection of atherosclerotic plaque has also been reported[47]. Leon *et al.* showed that using 325 nm light for fluorescence excitation and 480 nm light for tissue ablation, occluded superficial femoral artery could be recanalized and subsequently reopened by balloon angioplasty. Another *in vivo* fluorescence spectroscopy study[47] demonstrated the ability of *in vivo* plaque recognition in coronary artery sites, in a blood-filled environment. Despite this initial success, calcified tissue remains difficult to detect at 325 nm excitation and, therefore its clinical use is limited.

Clinical studies using 476 nm excitation light, based on the algorithm successfully developed *in vitro*[13], have unfortunately not been successful in differentiating[48]. It is believed that this is due to issues related to catheter-probe geometry and tissue contact[10,48]. Successful application of fluorescence spectroscopy requires that the spectral catheter be in good contact with the arterial wall, without the presence of intervening blood. Hemoglobin does not fluoresce, but its frequency-dependent reabsorption can severely distort the observed fluorescence spectrum. This suggests developing clinical catheters with improved contact, and/or the use of an approach which combines information obtained from diffuse reflectance (which monitors absorption) and fluorescence. Interestingly, little work has been reported to date on the use of diffuse reflectance as a spectroscopic tool for diagnosing atherosclerosis[10,40], although its use to identify cancer and dysplasia in various organs has been studied extensively[12,15].

Fluorescence is a potentially powerful tool for detecting atherosclerosis. However, its *in vivo* capability depends strongly on developing improved instruments, catheters and more powerful diagnostic algorithms.

Multiple wavelength excitation of arterial tissue

By using multiple wavelength excitation, a broader diagnostic algorithm may be developed, combining the information from both the ultraviolet and visible fluorescence spectra. In the past, collecting tissue fluorescence at multiple excitation wavelengths was a tedious process, requiring collection times of the order of an hour. Collection times of this duration precluded clinical applications.

Recently, Zangaro *et al.*[49] have developed a compact, portable clinical instrument called the Fast-EEM. This system can generate *in vivo* EEMs and reflectance spectra in less then one second. Though this system is successfully being used in several hospitals for *in vivo* studies involving esophagus, colon, bladder and the oral cavity, its potential in arteries is yet to be tested. At present this system is being tested on arteries *in vitro* at the Cleveland Clinic Foundation. Soon *in vivo* studies will commence. A brief description of our *in vitro* work is presented here.

The portable Fast-EEM used in these experiments and designed for *in vivo* human tissue fluorescence studies has been previously described in detail[49]. Briefly, the system (Figure 3) uses a sequence of dyes (Table 2) placed in cuvettes on a rotating wheel. As the wheel rotates, the dyes are successively pumped by a pulsed nitrogen laser (Laser Science Inc.) at 337 nm, producing 9 different excitation wavelengths in less than one second. The pumping laser light (at 337 nm) is also used, and during each rotation of the wheel, a flash of white xenon light is produced by means of a xenon flash lamp (EG&G Park)

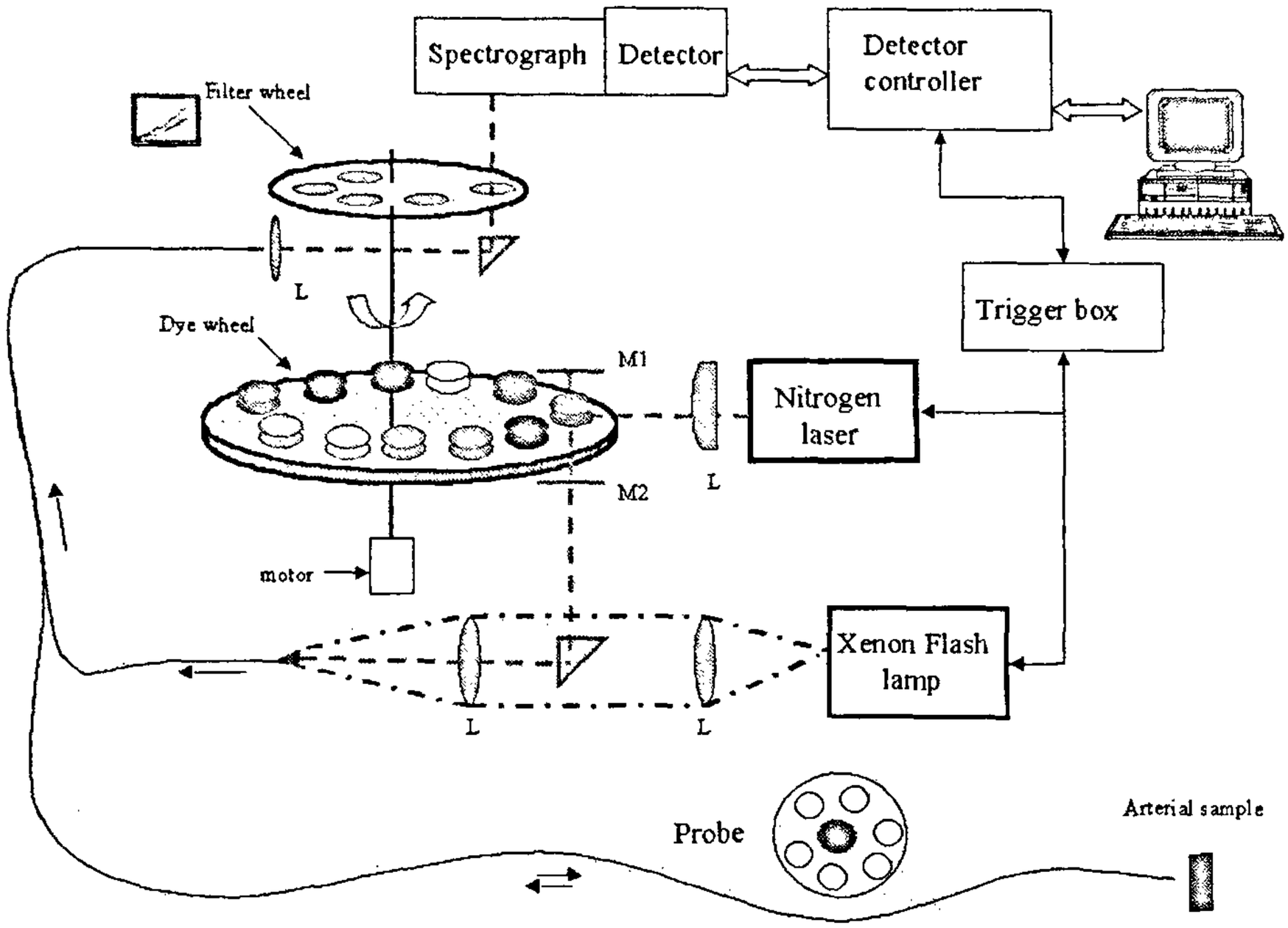

Figure 3. Schematic diagram of the clinical fast-EEM system. (L, lenses; M, mirrors). The distal tip of the optical-fiber spectral probe is illustrated in the inset.

Table 2. Characteristics of the dyes and filters used in the Fast-EEM system

Dye	Emission peak (nm)	Bandwidth (nm)	Solvent	Long-pass filters
PBD	359	5	*p*-dioxane	L 40*
Exalite 384	382	6	*p*-dioxane	L 42*
Exalite 398	397	6	*p*-dioxane	L 42*
LD 425	413	7	*p*-dioxane	GG 435
Stilbene 420	429	9	methanol	GG 555
Coumarin 440	440	10	methanol	Y 46*
Coumarin 460	463	12	methanol	Y 48*
LD 490	485	12	methanol	OG 515
Coumarin 500	505	15	ethanol	OG 530

*These filters were procured from HOYA, and the rest from CVI Optics (adapted from ref. 48).

to generate a reflectance spectrum. The laser light and white light is then focused into an optical fiber. The optical fiber probe (diameter ± 1.1 mm) consists of a central excitation fiber surrounded by 6 collection fibers, contained in a catheter terminating in a transparent 'optical shield'. The distal end of the probe is brought into contact with the coronary artery wall where excitation light is delivered and tissue fluorescence and diffuse reflectance is collected. A wheel of long pass filters is used to prevent the detector from flooding due to the excessive, reflected excitation light present in the collection fibers. After collection of the spectrum by a spectrograph (CP 200, Jobin Yvon) and detector (1420/1024 U, EG&G Park), the resulting fluorescence and reflectance spectra can then be displayed and stored on a personal computer. This computer also controls the excitation-collection process, using specially developed software (OMAVIS, Princeton Instruments, 1990) and a Detector Interface (1461 E with 1303 gate pulse interface EG&G Park).

We have studied human cadaver aorta, collected within 24 h of death and coronary arteries, obtained from recipient hearts within one hour of heart transplantation. Immediately after dissection of the specimens, the artery segments were rinsed in isotonic saline and snap frozen in liquid nitrogen. The samples were passively warmed to room temperature, cut open and positioned on a black, non-fluorescent surface. During examination, the arteries were kept moist with buffered saline. A perpendicular probe-tissue orientation was maintained and pressure was controlled at approximately 3 g of pressure, measured by a balance underneath the tissue. After spectral examination of both diseased and non-diseased spots of the tissue, the exact place of the optical probe on the tissue was marked with an ink dot and the specimens were sent for histopathological examination.

The resulting spectra were intensity calibrated by means of a standard rhodamine 610 solution, to correct for laser power fluctuations or changes in alignment of the system. For reflectance data, a barium sulfate spectrum was collected to correct for both intensity and spectral

features of the xenon-lamp. The final spectra are the first fast-EEM and reflectance data collected from arterial tissue in real time.

The preliminary results look promising (Figure 4). Lineshape and intensity differences between normal, atherosclerotic and calcified tissue can be easily detected. At 337 nm, a clear shift in fluorescence peak as well as a shift in peak intensity is observed, possibly due to an increase in the amount of collagen in the fibrous cap of atherosclerotic lesions. This is in agreement with the above described observations by Deckelbaum *et al.*[25], Andersson-Engels *et al.*[30] and Yova *et al.*[31]. As noted by Deckelbaum *et al.*, the differences are more clear in aortic tissue than in coronary artery, possibly due to a difference in contribution of elastin and collagen in elastic and muscular arteries. At visible excitation wavelengths, differences in fluorescence intensity can be observed in the yellow and red part of the emission spectrum, especially at the 460–500 nm region. This also agrees with previous work[13,40]. The reflectance spectra reveal important information about the absorption and attenuation of hemoglobin and other constituents of arterial wall, and the differences this imposes on the fluorescence spectra of the arterial wall. However, in order to fully understand the importance of reflectance in the detection of atherosclerosis, much remains to be investigated.

These observations indicate that an algorithm can be developed which combines the wealth of information in

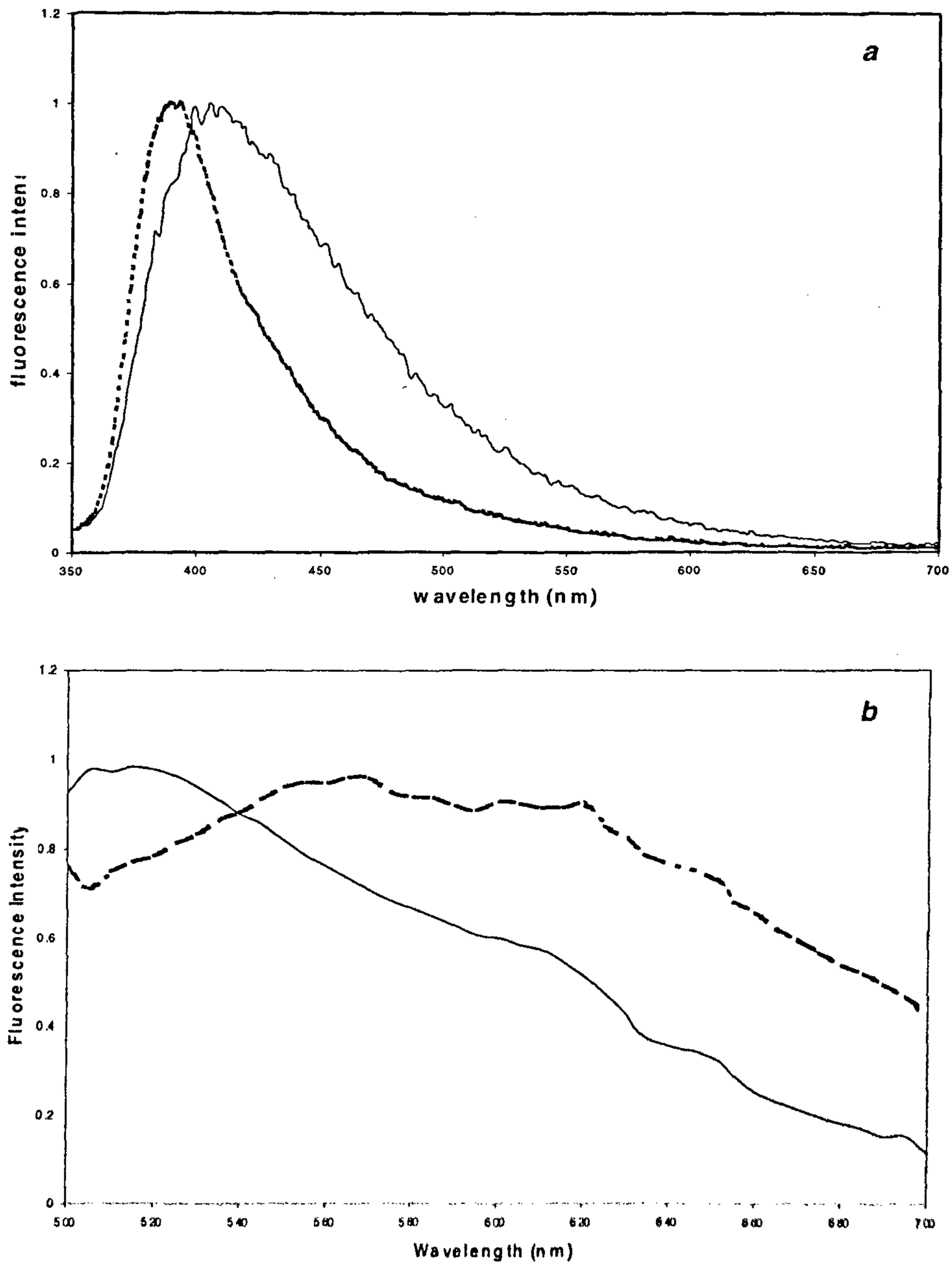

Figure 4. Differences between the fluorescence spectra of normal and atherosclerotic (- -) samples of aorta at 337 (*a*) and 460 nm (*b*) wavelength excitation. These spectra were collected with the clinical fast-EEM system.

the ultraviolet region with the information in the visible wavelength region. This may allow us to distinguish not only normal tissue from atherosclerotic and calcified tissue, but also to recognize different stages of atherosclerosis, such as intimal fibroplasia or atheromatous plaque.

Future directions

Much of the motivation to develop fluorescence-based methods for the detection of atherosclerotic plaque has been to develop a guidance system for laser angiosurgery catheters. Plaque recognition is important for the safe, precise intra-arterial atheroma ablation, to reduce the high incidence of vessel wall perforation. In contrast to other modalities that have been suggested (intravascular ultrasound, angioscopy), the advantage of spectroscopic guidance is that the lasers used for detection of atherosclerotic plaques and ablation may be incorporated in one system, without increasing catheter size or complexity. Fluorescence spectroscopy may also be important as a tool to predict lesion progression or plaque rupture, and it may help us in understanding the pathogenesis of atherosclerosis *in vivo*. Additionally, it may be useful in determining the optimum form of medical intervention for patients with atherosclerosis. However, fluorescence spectroscopy has its limitations. The spectral features are relatively broad and featureless, fluorophores are often difficult to distinguish, and fluorescence spectroscopy has difficulty in detecting calcium deposits.

Another spectroscopic technique, Raman scattering, has been proven to be successful in the *in vitro* diagnosis of atherosclerosis. It provides information about molecular and chemical composition, and structure of the arterial wall. A number of studies[50–52] have demonstrated the role of Raman spectroscopy as a means for obtaining quantitative chemical information from atherosclerotic plaques, which could be used for pathological staging of the disease. Moreover, Raman spectroscopy is able to detect calcium deposits, even small ones. Raman spectra, however, are not as easy to obtain as fluorescence spectra and it cannot detect ceroid, which is a good marker of necrotic core in atherosclerotic plaques.

Combining these techniques, perhaps in a single instrument, would combine the advantages of each spectroscopic technique and provide complementary information. A study will soon be started at the Cleveland Clinic Foundation and at MIT to evaluate the possibilities of combining the different spectroscopy techniques, in an *in vivo* clinical setting at the time of vascular and open heart surgery.

Combining spectroscopic techniques with contemporary medical diagnostics, such as intravascular ultrasound (IVUS), angioscopy or X-ray angiography, and MRI is also worthwhile to consider. This could lead to improved physico-chemical imaging techniques. Some research to evaluate these possibilities has already been performed. Warren *et al.*[53] used a combined ultrasonic and spectroscopic system to show that chemical cross-sections of aorta could be obtained that resembled the histologic cross-section. The chemical image successfully identified regions of ceroid, increased collagen deposition, and normal aorta tissue. In addition, Römer *et al.*[54] imaged the histochemistry of the arterial wall of mouse aorta *in vitro*, using both Raman spectroscopy and IVUS, thereby demonstrating the additional value of employing Raman histochemical information in combination with ultrasound for the purpose of diagnosing and studying atherosclerosis.

In summary, there is a pressing need for the development of reliable ways for the detection of atherosclerosis *in vivo*. Standard techniques can only be used after removal of tissue during autopsy or surgery. A safe technique of assessing the stage of atherosclerosis in a living patient, as in percutaneous *in vivo* fluorescence spectroscopy, can possibly provide this information. The development of a fast multi-excitation system will be a step forward in the real time detection of the fluorescent properties of the arterial wall. At present, *in vivo* experiments are being conducted to show that laser spectroscopic techniques can be a powerful tool for the nondestructive study of human atherosclerosis. Systems allowing the acquisition of a percutaneous optical biopsy should be a reality in the future.

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