



Detection of rupture-prone atherosclerotic plaques by time-resolved laser-induced fluorescence spectroscopy

Laura Marcu^{a,*}, Javier A. Jo^{d,1}, Qiyin Fang^{e,1}, Thanassis Papaioannou^{f,1}, Todd Reil^c, Jian-Hua Qiao^b, J. Dennis Baker^c, Julie A. Freischlag^g, Michael C. Fishbein^b

^a University of California Davis, Department of Biomedical Engineering, One Shields Avenue, Davis, CA 95616, United States

^b University of California Los Angeles, David Geffen School of Medicine, Department of Pathology and Laboratory Medicine, United States

^c University of California Los Angeles, David Geffen School of Medicine, Department of Vascular Surgery, United States

^d Texas A&M University, Department of Biomedical Engineering, United States

^e McMaster University, Department of Engineering Physics, United States

^f Cedars-Sinai Medical Center, Department of Surgery, United States

^g Johns Hopkins University School of Medicine, Department of Surgery, United States

ARTICLE INFO

Article history:

Received 17 June 2008

Received in revised form 6 August 2008

Accepted 11 August 2008

Available online 6 September 2008

Keywords:

Atherosclerosis

Diagnosis

Fluorescence spectroscopy

ABSTRACT

Objective: Plaque with dense inflammatory cells, including macrophages, thin fibrous cap and superficial necrotic/lipid core is thought to be prone-to-rupture. We report a time-resolved laser-induced fluorescence spectroscopy (TR-LIFS) technique for detection of such markers of plaque vulnerability in human plaques.

Methods: The autofluorescence of carotid plaques (65 endarterectomy patients) induced by a pulsed laser (337 nm, 0.7 ns) was measured from 831 distinct areas. The emission was resolved spectrally (360–550 nm range) and temporally (0.3 ns resolution) using a prototype fiber-optic TR-LIFS apparatus. Lesions were evaluated microscopically and quantified as to the % of different components (fibrous cap, necrotic core, inflammatory cells, foam cells, mature and degraded collagen, elastic fibers, calcification, and smooth muscle cell of the vessel wall).

Results: We determined that the spectral intensities and time-dependent parameters at discrete emission wavelengths (1) allow for discrimination (sensitivity >81%, specificity >94%) of various compositional and pathological features associated with plaque vulnerability including infiltration of macrophages into intima and necrotic/lipid core under a thin fibrous cap, and (2) show a linear correlation with plaque biochemical content: elastin ($P < 0.008$), collagen ($P < 0.02$), inflammatory cells ($P < 0.003$), necrosis ($P < 0.004$).

Conclusion: Our results demonstrate the feasibility of TR-LIFS as a method for the identification of markers of plaque vulnerability. Current findings enable future development of TR-LIFS-based clinical devices for rapid investigation of atherosclerotic plaques and detection of those at high-risk.

© 2008 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Numerous studies [1] have suggested that acute cardiovascular events are most commonly triggered by the structural weakening and disruption of the thin fibrotic cap of fibroatheroma followed by thrombus formation. Generally, this process occurs in atherosclerotic lesions known as rupture-prone or vulnerable plaques. Plaque “vulnerability” has been associated with several pathologic features including a thin fibrous cap, a large lipid pool, and increased

macrophage and other inflammatory cell infiltration within the cap. Early detection and treatment of such plaques before rupture depend upon the availability of tools able to characterize in vivo plaque composition through minimally invasive and nondestructive ways. Most clinical techniques identify luminal diameter (stenosis), wall thickness, and plaque volume, but are inefficient in identifying the rupture-prone plaque [1–6]. New diagnostic techniques (including catheter-based) to localize and characterize vulnerable plaques are needed. Potential intravascular diagnostic techniques for assessment of plaque vulnerability include: magnetic resonance (MR) spectroscopy [6–8], intravascular ultrasound (IVUS—including high-frequency, elastography) [6,9], optical coherence tomography (OCT) [10–13], thermography [14,15], and optical spectroscopy methods (Raman, near-infrared (NIR), diffuse reflectance, and steady-state fluorescence) [5,16,17]. We report here

* Corresponding author. Tel.: +1 530 752 0288; fax: +1 530 754 5739.

E-mail address: lmarcu@ucdavis.edu (L. Marcu).

¹ These authors were with L. Marcu's research group at Cedars-Sinai Medical Center when this study was conducted.

an innovative time-resolved fluorescence spectroscopy approach that has potential to detect distinct markers of plaque vulnerability as a stand-alone technique or to complement the other imaging or spectroscopy modalities in detection of vulnerable plaques.

This study describes a fiber-optic-based time-resolved laser-induced fluorescence spectroscopy (TR-LIFS) technique that can be used *in vivo* to recognize the biochemical makeup of tissues including structural proteins, enzyme cofactors and lipid components. In previous studies, we validated this technique on fluorescent biomolecules constituent of normal and diseased arteries (elastin, collagens, free cholesterol, cholesteryl oleate and cholesteryl linoleate, LDL) [18–21], in human coronary and aortic postmortem specimens [21,22], and *in vivo* in an atherosclerotic rabbit model [23]. Depending upon the light excitation wavelength used and fiber-optic excitation-collection geometry, TR-LIFS facilitates evaluation of tissue composition within small tissues volume (~0.6–1.5 mm diameter \times 150–250 μ m penetration depth), thus feasible for assessing the intimal composition of atherosclerotic plaques. Overall, TR-LIFS has potential for direct evaluation of relative changes of the elastin/collagen and collagen/lipid contents; and indirect assessment of cap thickness and infiltration of macrophage and other inflammatory cells affecting the collagen/lipid content within the excitation-collection tissue volume.

Carotid plaque represents an optimal study model for the validation of new optical devices for detection of plaque composition patients. This allows for direct access of plaque *in vivo* during carotid endarterectomy (CEA) using fiber-optic probes (with or without distal rigidity) and without the need of intravascular procedures. The routine removal of the plaque during CEAs permits extensive TR-LIFS studies *ex vivo* in fresh plaque specimens that account for the plaque composition heterogeneity. Furthermore, it facilitates a direct validation of optical results against histopathological analysis. Consequently, we conducted this study in human carotid atherosclerotic plaque both *in vivo* in patients undergoing endarterectomy and *ex vivo* in the explanted plaques.

The goals of this study were (1) to use TR-LIFS to evaluate the composition of human carotid atherosclerotic plaques and to determine whether a TR-LIFS technique can distinguish fibrotic caps rich in macrophages and inflammatory cells and plaques with a necrotic/lipid core under a thin cap (rupture-prone) from plaques with caps rich in collagen (stable) and (2) to determine the TR-LIFS derived spectroscopic parameters that can be correlated to features of plaque vulnerability.

2. Methods and materials

2.1. Patients and plaque samples

A total of 65 patients were included in this study. The patients were scheduled for CEA and underwent the planned operation. When clinical conditions of the patient allowed (14 patients), during the operation the TR-LIFS fiber optic probe was introduced through the common carotid arterotomy and the plaque was spectroscopically investigated (total: 28 locations, one to three measurements per plaque). During *in vivo* investigations the blood was evacuated from a cross-clamped artery. The plaque was then removed surgically and immediately spectroscopically re-evaluated in detail *ex vivo*. When investigations *in vivo* were not possible, the TR-LIFS studies were conducted only in *ex vivo* specimens. To account for the heterogeneity within plaque composition and structure, TR-LIFS data were acquired from several areas of the plaque (total: 813 locations, approximately 10 measurements per plaque). The time between plaque resection and TR-LIFS examination was less than 2 h. The specimens were kept moist

with saline (intermittently delivered drops) prior to and during TR-LIFS investigation. The location of each spectroscopic measurement was marked using India ink and the plaque specimen sent for histopathologic analysis. This study was approved by the institutional review board and all patients provided informed consent.

2.2. TR-LIFS instrumentation, measurements and data analysis (Fig. 1)

The detailed description of this section (TR-LIFS apparatus, experimental data acquisition protocols and analysis of the TR-LIFS data) is provided in [Appendix of this paper and in our earlier publications \[18,23,24,26,27\]](#). To characterize the fluorescence emission a set of parameters were used. This includes discrete spectral intensity values (I_λ) and two sets of parameters time-resolved parameters: the average lifetime (τ_f) values and the normalized value of the corresponding Laguerre expansion coefficients (LEC) c_j . Thus, a complete description of each sample fluorescence as a function of emission wavelength, λ_E , was given by the variation of a set of spectroscopic parameters (I_λ , τ_f , and LECs).

2.3. Histopathological analysis

The processing and analysis of the histopathological samples including hematoxylin and eosin (H&E), trichrome/elastin, CD68 (for macrophages) and CD45 (for leukocytes) are detailed in [Appendix of this paper](#). The histopathologic sections were categorized based on intima thickness (TH), cap thickness (CTH), the presence of calcification, necrosis, and inflammation (lymphocytes and/or macrophage infiltration). The following six groups were defined following the nomenclature similar with that reported by Virmani et al. [25]: (a) intimal thickening (IT) with TH < 200 μ m and no necrosis, inflammation, or calcification; (b) fibrous plaque/thick-cap (FP) with TH/CTH > 200 μ m and no necrosis, inflammation or calcification; (c) fibrocalcific plaque/thick-cap (FC) with TH/CTH > 200 μ m and significant calcification ($\geq 20\%$), but no necrosis or inflammation; (d) low-inflamed plaque/thick-cap (low-INF) with TH/CTH > 200 μ m and some necrosis/inflammation (<20%); (e) inflamed plaque/thick-cap (INF) with TH/CTH > 200 μ m, some necrosis (<30%), and significant inflammation ($\geq 20\%$); and (f) superficial necrotic core covered by a thin fibrotic cap (NEC) with TH > 200, CTH < 200 μ m and significant necrosis ($\geq 30\%$).

2.4. Statistical analysis and classification

A set of spectroscopic parameters (I_λ , τ_f and LECs at specific λ_{ES}) likely to provide means of discrimination among different compositional features of the carotid plaque were identified by following methods we previously reported [23,27,28] and reviewed in [Appendix](#). In addition, in order to investigate if these spectroscopic parameters reflect plaque composition, a correlation analysis was performed between the selected parameters and the relative content of the main plaque components (elastin, collagen/SMC, inflammatory cells and necrotic areas). For this correlation analysis, the mean value of the spectroscopic parameter was computed for each concentration value from 0 to 100%, in steps of 10% (Fig. 4). A linear correlation analysis was then applied between the spectroscopic parameter mean values and the plaque component concentrations. Correlation coefficients r and P -values were reported for each parameter-concentration correlation analysis. A P -value of <0.05 was assumed to indicate a statistically significant correlation. All statistical analysis was conducted using SPSS Version 12.0. A stepwise linear discriminant analysis (SLDA) approach was adopted to generate a classification model (discriminant functions) and for sample classification. This approach (also described

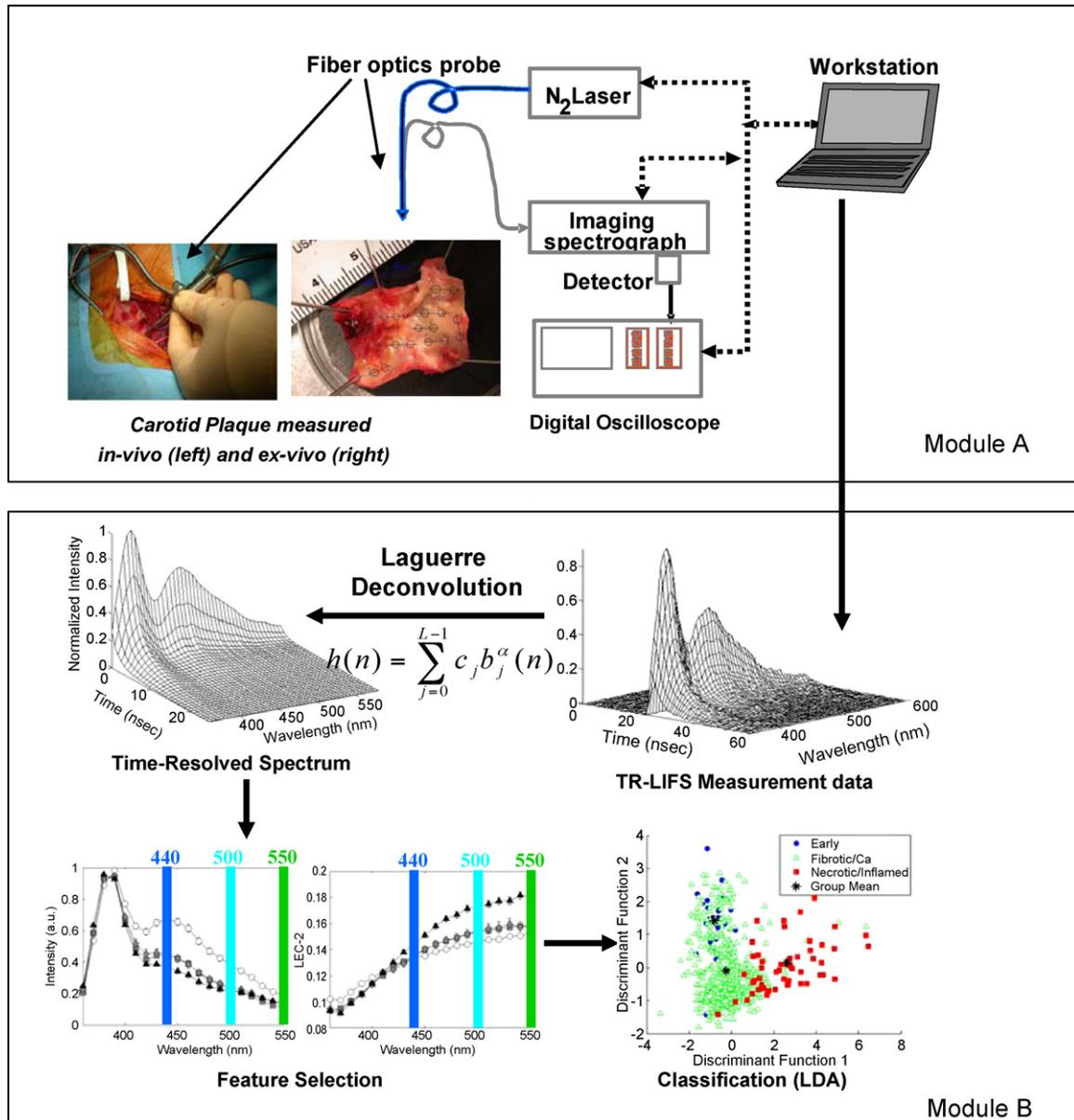


Fig. 1. Schematic representation of the TR-LIFS system used to induce, collect, record and analyze the autofluorescence emission of the carotid plaques.

in Appendix) was applied previously by our group to the classification of arterial plaque composition in a rabbit atherosclerotic model [23,28,29].

3. Results

3.1. Histopathology

The plaques TR-LIFS investigated ex vivo were histopathologically classified as follows: IT ($n=28$), FP ($n=420$), FC ($n=38$), low-INF ($n=273$), INF ($n=34$), and NEC ($n=20$). Representative features/sections for each histopathological group are shown in Fig. 2. The IT samples were characterized by a thin intima ($88 \pm 39 \mu\text{m}$) overlaying a normal media rich in elastin fibers and SMC (Fig. 2a). The composition of the FP samples was dominated by collagen fibers with various degrees of SMC accumulation (Fig. 2b). The FC samples presented various degrees of calcification (Fig. 2c). The low-INF samples were characterized by collagen fibers with scat-

tered infiltration of lymphocytes and/or macrophages (Fig. 2d). The INF samples presented a significant amount of inflammatory cells, mainly macrophages (Fig. 2e). The NEC samples were characterized by a necrotic core with a thin fibrous cap ($70 \pm 37 \mu\text{m}$) with or without inflammatory cell infiltration (Fig. 2f). Adjacent CD45 and CD68 stained sections (not shown) confirmed the presence of lymphocytes and macrophages, respectively. Out of the 28 TR-LIFS measurement conducted in vivo only 10 of them could be correlated with the histopathological analysis of plaques and classified as follows: IT ($n=3$) and FP ($n=7$). This was due to difficulties on accurately marking in vivo the location from where the TR-LIFS measurement was taken.

3.2. Time-resolved fluorescence plaque characterization

The time-resolved fluorescence spectrum of the atherosclerotic plaques can be fully described by the variation of the spectroscopic parameters I_λ , τ_f , and LECs as a function of

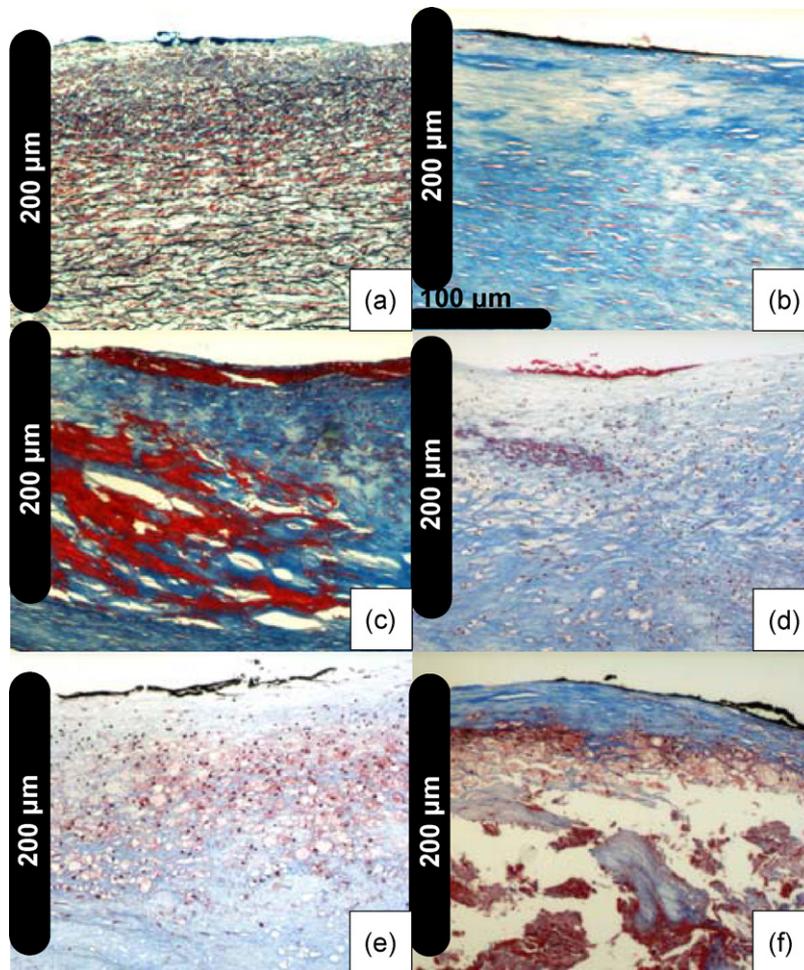


Fig. 2. Representative carotid plaque histological sections (elastic trichrome stain, 20× magnification). (a) Intima thickening (IT); (b) fibrous plaque (FP); (c) fibro-calcified (FC); (d) low-inflammation (low-INF); (e) inflammation (INF); (f) necrotic (NEC).

λ_E . Fig. 3 depicts these parameters for each lesion type/group investigated.

3.2.1. Emission spectra and intensity values

The emission spectra of all histopathological groups measured ex vivo (Fig. 3a) were largely overlapped, except for the IT group which presented a higher intensity in the 400–500 nm spectral range ($I_\lambda = 0.69 \pm 0.03$ at 440 nm) when compared with all other groups (e.g. I_λ smaller than 0.5). The I_λ values at 440 nm (Fig. 3b) also showed slightly higher values for the FP (0.46 ± 0.01) and FC (0.47 ± 0.03) groups when compared to the INF (0.38 ± 0.03) and NEC (0.37 ± 0.03) groups. Characteristics indicating that IT could be distinguished using intensities values in red-shifted wavelength range (Fig. 3a and b). The characteristics of the emission spectrum of the in vivo measurements for the IT and FP samples were similar to those measured ex vivo (Fig. 3c). Nevertheless, the valley at ~ 415 nm, well recognized to correspond to the Soret band of hemoglobin absorption [30], was accentuated in the in vivo measurements.

3.2.2. Fluorescence average lifetimes

For ex vivo measurements, the analysis of average lifetime values along the emission wavelength (Fig. 3d) showed that for the blue-shifted (below 420 nm) emission wavelengths IT samples were characterized by short lifetime values ($\tau_f = 1.87 \pm 0.09$ ns at 380 nm) when compared with those of all other groups (τ_f longer

than 2.4 ns at 380 nm). The ITs lifetime values remained nearly constant along the emission spectrum while a decrease of lifetime with emission wavelength was observed for all other groups. The largest variation of the average lifetime with wavelength was observed for NEC samples. For example, the emission of NEC in the red-shifted (above 440 nm) wavelength range was characterized by the shortest lifetime values ($\tau_f = 1.38 \pm 0.04$ ns at 550 nm) when compared with those of all other groups (τ_f longer than 1.55 ns at 550 nm). Characteristics indicating that NEC plaques could be distinguished using lifetime values in 500–550 nm range (Fig. 3d and e). The average lifetimes of IT and FP measured in vivo (Fig. 3f) followed similar trends to those measured ex vivo. However, the averages lifetime values of IT were shorter above 400 nm ($\tau_f = 1.3$ –1.5 ns) in vivo relative to ex vivo measurements.

3.2.3. Laguerre expansion coefficients

The variation of the first four LECs (zero-order: LEC-0 through the third-order: LEC-3) as a function of emission wavelength provided an additional means of characterizing and comparing each tissue group. The LEC-0 changes with emission wavelength followed similar trends as those described above for the average lifetimes (Fig. 3g and h). For the blue-shifted (below 420 nm) wavelengths IT samples were characterized by smaller LEC-0 values (e.g. 0.77 ± 0.01 at 380 nm) when compared with those of all other groups (above 0.83 at 380 nm). For the red-shifted (above 440 nm) wavelengths NEC samples were characterized by small

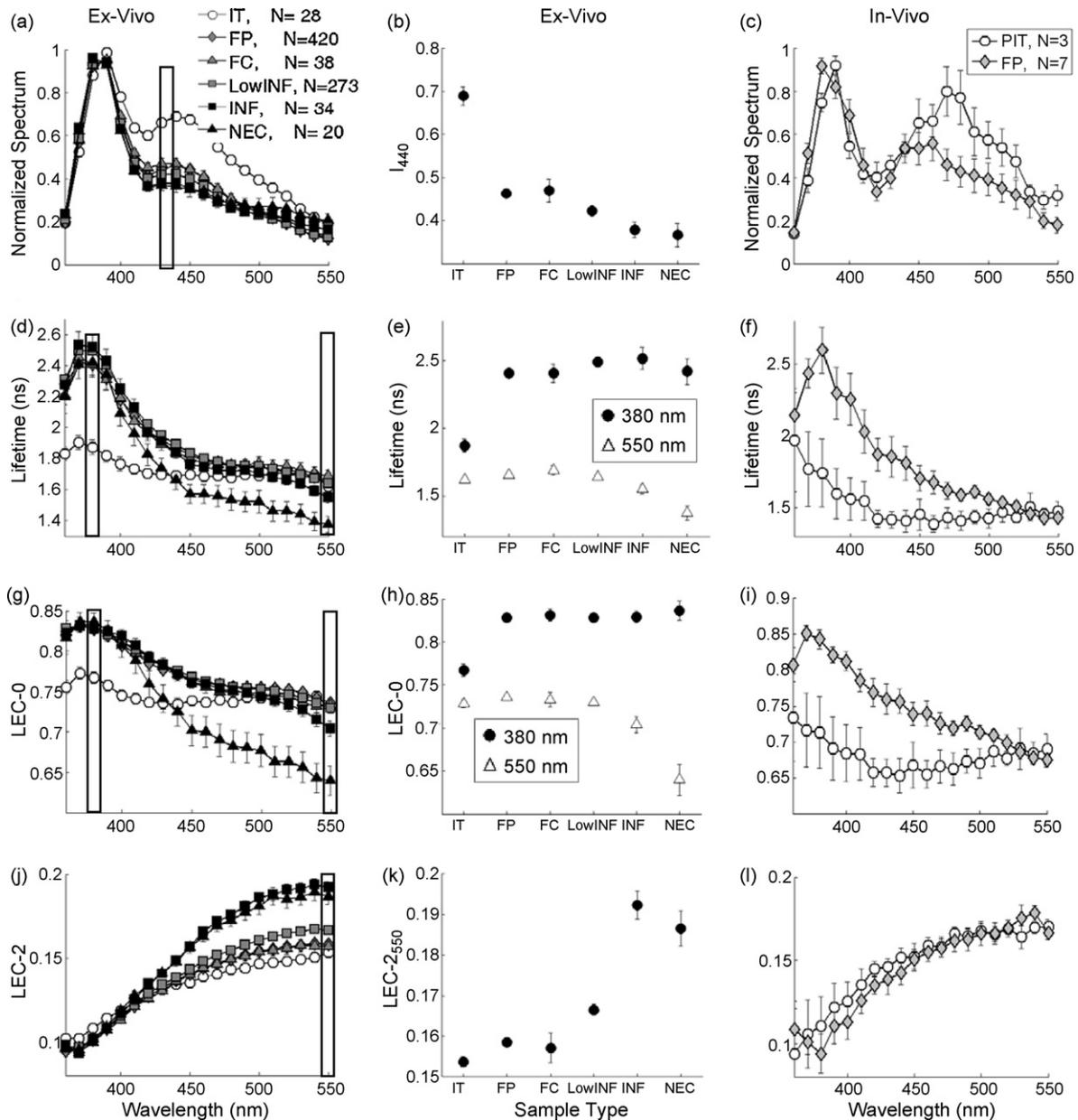


Fig. 3. Left panels: spectroscopic parameters as a function of emission wavelength derived from the time-resolved spectra measured in ex vivo samples. Middle panel: statistical group comparisons (mean \pm S.E.) for specific wavelengths—data corresponding to measurements ex vivo. Right panels: spectroscopic parameters as a function of emission wavelength derived from the time-resolved spectra measured in vivo. Spectroscopic parameters: normalized intensity (a)–(c), average lifetime (d)–(f), LEC-0 (g)–(i), and LEC-2 (j)–(l).

LEC-0 values (0.70 ± 0.01 at 550 nm) when compared with those of all other groups (larger than 0.73 at 550 nm). The changes of the second-order LEC (LEC-2) with the wavelength of emission (Fig. 3j and k) showed that for red-shifted wavelengths (above 450 nm) the INF and NEC plaque areas are characterized by higher LEC-2 values (0.192 ± 0.003 and 0.187 ± 0.004 , respectively, at 550 nm) with respect to all other groups (smaller than 0.17 at 550 nm). In addition, higher LEC-2 values were observed for Low-INF (0.167 ± 0.001) when compared to the fibrotic groups FC (0.157 ± 0.003) and FP (0.159 ± 0.001); and the IT (0.154 ± 0.004) group. The variation of LECs of IT and FP measured in vivo (Fig. 3i and l) followed similar trends to those measured ex vivo. Nevertheless, in vivo relative to ex vivo investigations show that the LEC-0 values of IT samples were smaller in vivo (0.65–0.7).

3.3. Correlation between plaque composition and spectroscopic parameters

Fig. 4 depicts the linear correlation between plaque biochemical content and spectroscopic parameters within the ROI measured ex vivo. Plaque elastin content, ranging from 0% (in the advanced FP, FC, INF and NEC) to 50% (in IT) showed a positive correlation with the I_{440} (Fig. 4a). Necrotic area, ranging from 0% (in stable plaques) to 70% (in NEC), presented a negative correlation with the LEC-0_{550nm} (Fig. 4b). A similar correlation was determined for the average lifetime values (data not shown). The inflammatory cell (lymphocyte/macrophage) content, ranging from 0% (in stable plaques) to 60% (in INF and NEC), showed a positive correlation with the LEC-2₅₅₀ (Fig. 4c). In contrast, LEC-2₅₅₀ (Fig. 4d) was negatively correlated to the collagen/SMC content.

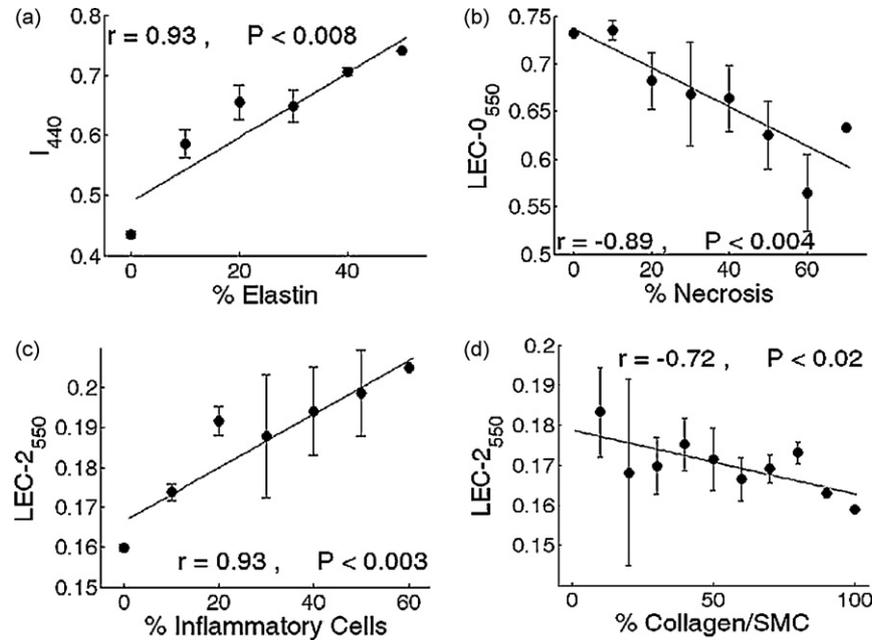


Fig. 4. Correlation analysis between spectroscopic parameters and plaque biochemical constituents: (a) I_{440} positively correlated with elastin content; (b) $LEC-0_{550}$ negative correlated with necrosis content; (c) $LEC-2_{550}$ positively correlated with inflammation; (d) negatively correlated with collagen/SMC content.

3.4. Feature selection

The application of one-way ANOVA to multidimensional TR-LIFS data sets (I_{λ} , τ_f , and LECs values at each emission wavelength) to ex vivo data sets have resulted in a reduction of this set to 10 statistically significant features: I_{440} , τ_{380} , τ_{550} , $LEC-0_{380}$, $LEC-0_{550}$, $LEC-1_{380}$, $LEC-1_{550}$, $LEC-2_{500}$, $LEC-2_{550}$, and $LEC-3_{380}$. The wavelengths with the lowest P -value were chosen for each parameter to form the new feature space.

3.5. Classification

Two classification goals were defined aiming to discriminate: (a) IT, FP/FC (as one group) and INF/NEC (as one group) lesions; and (b) IT, FP/FC/low-INF (as one group) and INF/NEC (as one group) lesions. The FP and FC lesions showed significant overlap in all the spectroscopic parameters. This indicated that classification between these two groups was not feasible based on TR-LIFS alone. Therefore, for classification purpose, FP and FC lesions were grouped together. Also, inflammation and necrosis represent two key markers of vulnerability often present simultaneously in plaques, thus for classification purpose, INF and NEC were grouped together.

Results of the classification aiming to discriminate IT, FP/FC and INF/NEC lesions are shown in Table 1. The overall cross-validation classification performance was 74.3%. IT was discriminated with sensitivity (SE) and specificity (SP) larger than 80%. However, there was an appreciable overlap between IT and FP/FC. Also, the INF and NEC lesions were detected with high SE (larger than 80%) and high

Table 1
Classification accuracy (sensitivity and specificity): discrimination of IT, FP/FC and INF/NEC.

	Fluorescence spectroscopy			SE (%)	SP (%)
	IT	FP/FC	INF/NEC		
IT ($n = 28$)	25	3	0	89.3	80.7
FP/FC ($n = 458$)	99	332	27	72.5	84.2
INF/NEC ($n = 54$)	0	10	44	81.5	94.4

SP (larger than 90%). Results of the classification aiming to discriminate IT, FP/FC/low-INF and INF/NEC lesions are given in Appendix (Table 2 in supplementary material).

4. Discussion

This is the first study to demonstrate that a TR-LIFS technique allows for detection of markers of vulnerability in human atherosclerotic plaques, including infiltration of macrophage and other inflammatory cells into the intima and lipid/necrotic core under a thin fibrous cap. Also, we report here a rather comprehensive analysis of time-resolved fluorescence emission features that allows for discrimination of various compositional and pathological features of human atherosclerotic plaques. Current findings represent the ground base for further development of intravascular TR-LIFS diagnostic devices.

4.1. TR-LIFS features in plaque characterization and qualitative discrimination of rupture-prone plaques

Intrinsically, TR-LIFS measurements are rich in information content. The measurement allows recording of complementary features of the fluorescent emission from tissue that encompasses a detailed description of fluorescence intensity decay along the emission spectrum of each sample. Moreover, the application of the Laguerre deconvolution approach to TR-LIFS data, developed by our group and used in this study, generated not only conventional fluorescence lifetime value(s) but also a new set of spectroscopic-related parameters (set of Laguerre coefficients). Consequently, numerous fluorescence parameters are available for characterization of the fluorescence emission from atherosclerotic plaques and for identification of plaque type. As exemplified in Fig. 3, upon 337 nm excitation the emission spectrum and the discrete intensity values within 420–500 nm wavelengths range can be used to delineate the early plaques. The average lifetime and $LEC-0$ values in two emission wavelength ranges, 360–400 nm and 450–550 nm, permit separation of early plaques and necrotic plaques, respectively. In addition, the $LEC-2$ coefficients in 450–550 nm range facilitate

discrimination of both inflamed and necrotic plaques. Important to note is the use of LECs to describe the fluorescence intensity decay of the atherosclerotic plaques. These coefficients are key parameters in the discrimination of inflamed and necrotic plaques, the two most common forms of vulnerable plaque. Also, we identified that the trends of certain LECs values (e.g. LEC-0 and LEC-2 at 550 nm emission, Fig. 3h) follow the plaque transition from stable characteristics (FP, FC) to features indicating weakening of the fibrotic cap (low-INF) and then to features associated with prone to rupture plaques (INF/NEC).

The large number of spectroscopic parameters generated by a TR-LIFS measurement provides a means for discrimination of distinct compositional features of atherosclerotic plaques. However, acquisition and analysis of a large database is time expensive and thus may preclude translation of TR-LIFS systems to practical applications. This study has identified pathways that can overcome such drawbacks and enable development of TR-LIFS systems with the near real time display of diagnostic information, thus leading to solutions that are clinically relevant. For example, we determined that a small subset of spectral and time-resolved parameters (10 parameters) can provide a means for delineating early from advanced lesions, and more important, the presence of necrosis and inflammatory cells (foam cells and lymphocytes) in the plaques. Moreover, our results indicate that these spectroscopic parameters are retrieved from four discrete emission wavelengths (380 nm, 440 nm, 500 nm and 550 nm). This finding is particularly important as it allows for the design of TR-LIFS systems that collect fluorescence decay at a limited number of wavelength bandwidth as recently reported [30]. Consequently, it will allow for construction of TR-LIFS devices with even shorter data acquisition time (less than 1 s for each point measurement). Also, relevant to practical applications are the analytical methods employed in this study (Laguerre expansion of kernels combined with linear discriminant analysis) that can facilitate near real-time TR-LIFS data analysis. In a previous study, we demonstrated the ability of the Laguerre expansion technique for fast (less than 35 ms per wavelength) deconvolution of the fluorescence impulse function and analysis of biological systems [27,28]. Thus, a fast real-time classification algorithm can be developed by assigning a discriminant score for different types of tissue or plaques. The discriminant score can then be compared with the acquired fluorescence from the unknown tissue so as to classify the tissue and display the diagnostic information in near real-time.

4.2. TR-LIFS features in the characterization of arterial wall composition and pathway for quantitative discrimination of rupture-prone plaques

The current TR-LIFS study of carotid plaques complements and is in agreement with previous work conducted in human aorta and coronary arteries that identified relationships between the fluorescence emission of normal and diseased arterial wall and the emission of the intrinsic fluorescent constituents in arterial tissue [18–21,31,32]. Upon UV irradiation, the fluorescence emission of arteries originates from several intrinsic fluorescent constituents which we summarized previously [20,21]. These include structural proteins (elastin and collagens) and lipid components (free cholesterol, cholesterol esters, and lipoproteins). Nevertheless, structural proteins elastin and collagen types I and III are the primary fluorophores in normal and diseased arteries. Elastin (broad band: 70 nm full width at half maximum (FWHM) with peak at ~400 nm; average lifetime: ~2 ns), largely found in the media and internal elastic lamina, dominates the fluorescence emission of normal arterial wall and early plaques; while collagen type I (narrow band: ~40 nm FWHM with peak ~385 nm; average lifetime: 3–4 ns)

is the main contributor to the fluorescence of fibrotic plaques [20,21]. In current the study (Fig. 3) the spectral signature (broad band: 80 nm FWHM) and the fluorescence lifetime (~1.9 ns) values obtained for carotid IT samples demonstrate the significant contribution of elastin fluorescence to their overall fluorescence emission; whereas, the fluorescence emission signature (narrow band: ~40 nm, lifetime ~2.5 ns) of all other types of arterial plaques was dominated by collagen fluorescence.

The atherosclerotic stages between normal and advanced fibrotic are characterized by accumulation within the intima of various types and amounts of lipids localized either in macrophage foam cells or in the extracellular space. These lipids alter the intimal composition and consequently modulate the elastin–collagen fluorescence signature, in particular, the time-domain features. Lipids, typically, are characterized by a shorter lived fluorescence when compared with structural proteins fluorescence [19–21]. Accumulation of macrophage, smooth muscle cells, cellular debris and other plaque constituents can alter the intimal morphology, dislocate the structural proteins, and change the constituent relative content within a particular ROI. Thus, in turn these conditions can also alter the elastin–collagen fluorescence signature. It was shown that human coronary and aortic plaques with large lipid pools and thin fibrotic caps have a shorter fluorescence lifetime (~2.7 ns) than that of fibrotic plaques (~3.2 ns) [20,21]. Also, a shorter lived fluorescence was reported for fatty streaks in human arteries (~1 ns) when compared to that of normal arterial wall (~2.2 ns) [21]. Similar trends were observed in a rabbit atherosclerotic model, where macrophage-rich lesions presented a faster decay dynamic than the collagen-rich lesions or normal artery [23]. In this study, the fluorescence lifetime of INF and NEC samples with relatively high lipid and macrophage vs. collagen content showed a faster fluorescence intensity decay dynamic (at 550 nm emission ~1.5 ns and ~1.3 ns, respectively) than that of FP and FC with a relatively low lipid vs. collagen content (~1.7 ns). Albeit no direct comparison between current fluorescence lifetime values and the values previously reported can be made due to differences of experimental conditions and methods of data analysis, current results confirm that the presence of lipid components in plaque generally decreases the overall fluorescence lifetime of the atherosclerotic tissue.

In addition, this study shows that specific spectroscopic parameters are linearly correlated to the biochemical content of plaques; thus it opens a new pathway for discrimination of plaques based on their biochemical composition (quantitative classification). We determined that parameters derived from the red-shifted wavelength range such as I_{440} (Fig. 3b) are linearly correlated (positive) to that of elastin content within the ROI. Thus, the analysis of intensity values has potential to identify normal arterial wall. LEC-2₅₅₀ (Fig. 4) was found positively correlated with the macrophage and inflammatory cells content (markers of plaque instability) and negatively correlated with collagen and smooth muscle cell content (markers of plaque stability). This is an important finding suggesting that LEC-2 values represent key parameters in the discrimination of rupture-prone plaques. Yet another Laguerre coefficient LEC-0₅₅₀ was found linearly correlated (negative) with the necrotic area within the ROI, suggesting that analysis of LEC-0 values may allow for identification of necrotic areas under the fibrotic cap. As the penetration depth of irradiation at about 337 nm in the artery ranges between 150 and 250 μm [21], detection of plaques with thin fibrotic caps and large lipid or necrotic core is possible.

Calcium deposits were histopathologically identified in numerous plaques investigated in this study. Calcium is known to exhibit sharp fluorescence emission peaks in the 350–660 nm range upon 308 nm excitation [31], but calcium does not fluoresce upon 335 nm. The latter condition was also observed in the current study

(excitation 337 nm). The fluorescence emission of plaques with calcification was dominated by the collagen fluorescence typically present in calcified plaques in the samples investigated here. Thus, calcified plaques cannot be distinguished from fibrotic plaques using 337 nm. As a result, in the current study we were not able to distinguish fibrotic from fibrocalcified lesions. The use of excitation wavelengths within the calcium absorption bands, however, can overcome this limitation.

4.3. *In vivo* vs. *ex vivo* TR-LIFS measurements

The TR-LIFS study of surgical removed plaques permitted acquisition of a large number of measurements that accounted for the heterogeneity of composition, capturing the most clinically relevant biochemical features (including inflammation), and enabling accurate correlation with the histopathological features at each measurement. While the main chemical components and morphological structures of atherosclerotic plaques are expected to undergo minimal changes after surgical removal of the plaque, morphometric effects such as shrinkage may affect the relative content of fluorescent constituents within a particular ROI. Current results showed that TR-LIFS features (spectral feature, average lifetimes, Laguerre coefficients) retrieved from *in vivo* measurements during endarterectomy resemble those obtained *ex vivo*. Nevertheless, some effects were identified, primary on the spectral profile. As expected, much stronger hemoglobin absorption was observed *in vivo* when compared to *ex vivo* measurements, demonstrated as an enhanced spectral valley at ~415 nm. This effect was more enhanced on near normal areas (IT) rather than fibrotic/fibrous cap areas. However, the trends of the time decay parameters along emission wavelengths remained virtually unchanged. This demonstrates, as expected, that time-resolved measurements are minimally influenced by fluctuation in fluorescence intensity due to the presence of the endogenous absorbers such as blood; and thus a TR-LIFS approach is more robust for investigations in clinical environment. We also determined that the actual values of time decay parameters of IT appear to change more after plaque explantation than those of the fibrotic plaques. We plan to study this aspect further and to identify if such differences (*in vivo* vs. *ex vivo*) reflect changes in the tissue thickness between *ex vivo* and *in vivo* samples due to lack of longitudinal stress in the *ex vivo* case. Challenges with accurate identification of the location of the spectroscopic measurement after plaque explantation and limited time to collect data during surgery have resulted in only a few point measurements per plaque.

4.4. TR-LIFS in atherosclerotic plaque diagnostics: clinical translation perspective and current study limitations

As a first step in the development of intravascular catheters to be used in characterization and diagnosis of atherosclerotic plaques, the present study demonstrated that TR-LIFS measurements can be easily conducted via a fiber-optic interface with biological tissue. As noted above, modalities for fast data acquisition/analysis were also identified which demonstrate the feasibility of TR-LIFS technique for near-real time analysis and display of diagnostic data. However, engineering of flexible catheters and methodologies for TR-LIFS intravascular application has to be further addressed. The relatively shallow UV light penetration of excitation light makes TR-LIFS a suitable method for detection of markers of plaque vulnerability in thin fibrotic caps, but also limits the ability of this technique (1) to assess plaque morphology associated with other markers of plaque vulnerability such as the size of the lipid pool or expansive (positive) remodeling or (2) to distinguish other important features involved in plaque pathology including pathologic intimal thickening [25]

vs. fibrous thick-cap atheroma. Such limitations, however, can be further addressed through complementary imaging techniques. As earlier suggested earlier for other spectroscopic methods currently evaluated for clinical diagnostic of vulnerable plaques (e.g. NIR and Raman spectroscopy) an optimum approach to vulnerable plaque detection would need to incorporate structural definition of a high-resolution modality (e.g. OCT, intravascular MRI or high-frequency IVUS) with biochemical processes detected by TR-LIFS. Concurrent studies in our lab are investigating the potential of combining TR-LIFS and IVUS modalities for simultaneous discrimination of the morphological and compositional features of atherosclerotic plaques.

5. Conclusion

The results of this study have demonstrated the ability of TR-LIFS to detect biochemical features in atherosclerotic plaques which are clinically relevant. Due to TR-LIFS high specificity in the detection of inflammation in human atherosclerotic plaques, a potential application of this technique is clinical diagnosis of high-risk plaques through intravascular fiber-optic catheters. Moreover, future optimization of TR-LIFS data acquisition/analysis approaches, as identified here, will enable near-real time (less than 1 s) tissue diagnosis. Such catheters could work in conjunction with either conventional catheterization procedures (angiography and IVUS) or newly developed optical techniques currently evaluated in clinical settings such as OCT, NIR, and Raman [5,11,16]. Due to its ability to detect the compositional makeup of the arterial wall, another potential application of TR-LIFS is the clinical management of stenting procedures. Knowledge of arterial wall composition at the time of percutaneous coronary interventions may allow the physician to “personalize” the selection of the type of stent (e.g. uncoated or drug-eluting), thus more effectively reducing the risk of restenosis and target-vessel revascularization. Yet, the sensitivity of TR-LIFS measurement to the arterial wall content in structural proteins suggests the potential application of this technique in assessing the effect of experimental drugs in plaque regression and stabilization. Thus, TR-LIFS may also play an important role in drug-discovery studies *in vivo* in animal models.

Acknowledgements

This study was supported by the National Institute of Health Grant R01 HL 67377. We thank the vascular surgeons and nurses at the UCLA Vascular Center for their support with data collection from patients; and Dr. Pramod Butte for his contribution to data acquisition.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.08.035.

References

- [1] Waksman R, Serruys PW, Dunitz M. Handbook of the vulnerable plaque. London, UK: Taylor & Francis Group; 2004.
- [2] Libby P, Aikawa M. Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat Med* 2002;8:1257–62.
- [3] Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient—a call for new definitions and risk assessment strategies: Part I. *Circulation* 2003;108:1664–72.
- [4] MacNeill BD, Lowe HC, Takano M, Fuster V, Jang IK. Intravascular modalities for detection of vulnerable plaque: current status. *Arterioscler Thromb Vasc Biol* 2003;23:1333–42.
- [5] Moreno PR, Muller JE. Identification of high-risk atherosclerotic plaques: a survey of spectroscopic methods. *Curr Opin Cardiol* 2002;17:638–47.

- [6] Fayad ZA, Fuster V. Clinical imaging of the high-risk or vulnerable atherosclerotic plaque. *Circ Res* 2001;89:305–16.
- [7] Hatsukami TS, Ross R, Nayak L, Polissar, Yuan C. Visualization of fibrous cap thickness and rupture in human atherosclerotic carotid plaque in vivo with high-resolution magnetic resonance imaging. *Circulation* 2000;102:959–64.
- [8] Zimmermann GG, Erhart P, Schneider J, et al. Intravascular MR imaging of atherosclerotic plaque: ex vivo analysis of human femoral arteries with histologic correlation. *Radiology* 1997;204:769–74.
- [9] de Korte CL, Carlier SG, Mastik F, et al. Morphological and mechanical information of coronary arteries obtained with intravascular elastography—feasibility study in vivo. *Eur Heart J* 2002;23:405–13.
- [10] Pasterkamp G, Falk E, Woutman H, Borst C. Techniques characterizing the coronary atherosclerotic plaque: influence on clinical decision making. *J Am Coll Cardiol* 2000;36:13–21.
- [11] Tearney GJ, Yabushita H, Houser SL, et al. Quantification of macrophage content in atherosclerotic plaques by optical coherence tomography. *Circulation* 2003;107:113–9.
- [12] Jang IK, Tearney G, Bouma B. Visualization of tissue prolapse between coronary stent struts by optical coherence tomography—comparison with intravascular ultrasound. *Circulation* 2001;104:2754.
- [13] Bouma BE, Tearney GJ, Yabushita H, et al. Evaluation of intracoronary stenting by intravascular optical coherence tomography. *Heart* 2003;89:317–20.
- [14] Verheye S, De Meyer GR, Van Langenhove G, Knaepen MWM, Kockx MM. In vivo temperature heterogeneity of atherosclerotic plaques is determined by plaque composition. *Circulation* 2002;105:1596–601.
- [15] Stefanadis C, Toutouzas K, Vavuranakis M, et al. Statin treatment is associated with reduced thermal heterogeneity in human atherosclerotic plaques. *Eur Heart J* 2002;23:1664–9.
- [16] Motz JT, Fitzmaurice M, Miller A, et al. In vivo Raman spectral pathology of human atherosclerosis and vulnerable plaque. *J Biomed Opt* 2006;11:021003.
- [17] Angheloiu GO, Arendt JT, Muller MG, et al. Intrinsic fluorescence and diffuse reflectance spectroscopy identify superficial foam cells in coronary plaques prone to erosion. *Arterioscler Thromb Vasc Biol* 2006;26:1594–600.
- [18] Marcu L, Grundfest WS, Maarek JMI. Photobleaching of arterial fluorescent compounds: characterization of elastin, collagen and cholesterol time-resolved spectra during prolonged ultraviolet irradiation. *Photochem Photobiol* 1999;69:713–21.
- [19] Maarek JMI, Marcu L, Snyder WJ, Grundfest WS. Time-resolved fluorescence spectra of arterial fluorescent compounds: reconstruction with the Laguerre expansion technique. *Photochem Photobiol* 2000;71:178–87.
- [20] Marcu L, Grundfest WS, Fishbein MC. Time-resolved laser-induced fluorescence spectroscopy for staging atherosclerotic lesions. In: Mycek MA, Pogue B, editors. *Fluorescence in biomedicine*. New York: Marcel Dekker; 2003. p. 397–430.
- [21] Marcu L, Fishbein MC, Maarek JMI, Grundfest WS. Discrimination of human coronary artery atherosclerotic lipid-rich lesions by time-resolved laser-induced fluorescence spectroscopy. *Arterioscler Thromb Vasc Biol* 2001;21:1244–50.
- [22] Maarek JMI, Marcu L, Fishbein MC, Grundfest WS. Time-resolved fluorescence of aortic wall: use for improved identification of atherosclerotic lesions. *Lasers Surg Med* 2000;27:241–54.
- [23] Marcu L, Jo JA, Fang Q, et al. In-vivo detection of macrophages in a rabbit atherosclerotic model by time-resolved laser-induced fluorescence spectroscopy. *Atherosclerosis* 2005;181:295–303.
- [24] Fang Q, Papaioannou T, Jo JA, et al. Time-domain laser-induced fluorescence spectroscopy apparatus for clinical diagnostics. *Rev Sci Instrum* 2004;75:151–62.
- [25] Virmani R, Burke A, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. *J Am Coll Cardiol* 2006;47:13–8.
- [26] Marmarelis VZ. Identification of nonlinear biological systems using Laguerre expansions of kernels. *Ann Biomed Eng* 1993;21:573–89.
- [27] Jo JA, Fang Q, Papaioannou T, Marcu L. Fast model-free deconvolution of fluorescence decay for analysis of biological systems. *J Biomed Opt* 2004;9(4):743–52.
- [28] Jo JA, Fang Q, Papaioannou T, et al. Laguerre-based method for analysis of time-resolved fluorescence data: application to in-vivo characterization and diagnosis of atherosclerotic lesions. *J Biomed Opt* 2006;11:021004-1–13.
- [29] Duda RO, Hart PE, Stork DG. *Pattern classification*. New York: John Wiley and Sons; 2004.
- [30] Sun YH, Liu R, Elson DS, et al. Simultaneous time- and wavelength-resolved fluorescence spectroscopy for near real-time tissue diagnosis. *Opt Lett* 2008;33(6):630–2.
- [31] Richards-Kortum R, Sevick-Muraca E. Quantitative optical spectroscopy for tissue diagnosis. *Annu Rev Phys Chem* 1996;47:555–606.
- [32] Morguet AJ, Körber B, Abel B, et al. Autofluorescence spectroscopy using a simultaneous plaque ablation and fluorescence excitation. *Lasers Surg Med* 1994;14:238–48.