Discrimination of Human Coronary Artery Atherosclerotic Lipid-Rich Lesions by Time-Resolved Laser-Induced Fluorescence Spectroscopy

Laura Marcu, Michael C. Fishbein, Jean-Michel I. Maarek, Warren S. Grundfest

Abstract—Lesion composition plays a significant role in atherosclerotic lesion instability and rupture. Current clinical techniques cannot fully characterize lesion composition or accurately identify unstable lesions. This study investigates the use of time-resolved fluorescence spectroscopy for unstable atherosclerotic lesion diagnosis. The fluorescence of human coronary artery samples was induced with nitrogen laser and detected in the 360- to 510-nm wavelength range. The samples were sorted into 7 groups according to the AHA classification: normal wall and types I, II, (fatty streaks), III (preatheroma), IV (atheroma), V (fibrous), and VI (calcified) lesions. Spectral intensities and time-dependent parameters [average lifetime, decay constants: \( \tau_1 \) (fast-term), \( \tau_2 \) (slow-term), \( A_1 \) (fast-term amplitude contribution)] derived from the time-resolved spectra of coronary samples were used for tissue characterization. We determined that a few intensity values at longer wavelengths (>430 nm) and time-dependent parameters at peak emission region (390 nm) discriminate between all types of arterial samples except between normal wall and type I lesions. The lipid-rich lesions (more unstable) can be discriminated from fibrous lesions (more stable) on the basis of time-dependent parameters (lifetime and fast-term decay). We inferred that features of lipid fluorescence are reflected on lipid-rich lesion emission. Our results demonstrate that analysis of the time-resolved spectra may be used to enhance the discrimination between different grades of atherosclerotic lesions and provide a means of discrimination between lipid-rich and fibrous lesions. (Arterioscler Thromb Vasc Biol. 2001;21:1244-1250.)

Key Words: atherosclerosis ■ lesion instability ■ time-resolved laser-induced fluorescence ■ spectroscopy

Rupture of coronary atherosclerotic lesions leads to the acute coronary syndromes of unstable angina, acute myocardial infarction, and ischemic sudden death. Evidence suggests that lesion composition plays a crucial role in lesion instability and that lipid-rich lesions are more prone to rupture than fibrous lesions. Current clinical techniques (angiography, angioscopy, ultrasound) are limited in their ability to characterize lesion composition and identify lipid-rich lesions. Various techniques are currently under study as potential new tools for identification of lipid-rich lesions (MRI, near-infrared spectroscopy, Raman spectroscopy) or markers of instability, such as macrophage activation in fibrous cap (local thermography). Several groups have investigated laser-induced fluorescence spectroscopy (LIFS) as a tool for analyzing plaque composition in the attempt to guide laser angioplasty and to evaluate the likelihood of restenosis. The research was carried out for both ex vivo and in vivo conditions. These early studies have demonstrated the potential of LIFS to characterize a few types of atherosclerotic lesions (fibrous, atheromatous, calcified) and to discriminate those from the normal arterial wall. These studies, however, have neither fully explored nor demonstrated the potential use of LIFS as a clinical tool for identification and characterization of unstable or lipid-rich atherosclerotic lesions. Previous work, including research from our group, suggested that time-resolved LIFS (TR-LIFS) improves the specificity of fluorescence measurements in tissue and enhances the ability of LIFS to characterize atherosclerotic lesions and to evaluate lesion composition. The use of TR-LIFS for arterial tissue characterization is suitable for several reasons. Time-resolved fluorescence measurement (1) can resolve the spectral overlap of endogenous fluorophores in tissue; (2) is independent of fluorescence emission intensity as long as the signal-to-noise ratio is commensurable, and thus independent of the presence of the endogenous chromophores in tissue (hemoglobin) or of excitation-collection geometry (optical assembly); and (3) is sensitive to microenvironmental parameters in tissue (pH, enzymatic activity, temperature), and thus may reflect inflammatory activity at the arterial wall level. These are important features for in vivo diagnostics.
The purpose of this research was to investigate the use of TR-LIFS as a diagnostic tool for assessment of unstable atherosclerotic lesions. Using a time-domain time-resolved technique, this work attempts to (1) determine the spectrotemporal fluorescence emission characteristics of excised human coronary tissue (normal and atherosclerotic vascular wall); (2) identify the main spectrotemporal fluorescence features that provide a means of discrimination between arterial tissue types, in particular the fluorescence parameters able to distinguish the lesions with lipid-rich core and thin collagenous cap (more unstable) from those with thick collagenous cap (more stable); and (3) infer correlations between fluorescence features and morphological and compositional changes that occur during the atherosclerotic process.

Methods

Instrumentation
The experimental setup has been described by our group. For details, please see Figure I and the instrumentation description at http://atvb.ahajournals.org.

Samples
The coronary artery samples (total of 58 coronary segments from 11 subjects; subject age 11 to 85 years; median age 48 years) were obtained at autopsy (24 to 48 hours postmortem). The histological sections were examined by a pathologist and classified according to the American Heart Association (AHA) classification as follows: normal, type I (early lesion), type II (progression-prone type II corresponding to type II, in Stary’s classification), type III (preatheroma), type IV (atheroma), type V (fibroatheroma), and type V (complicated lesions with advanced calcification close to the intimal surface but not exposed calcification). For tissue and histological section processing and assessment, please see http://atvb.ahajournals.org.

Experimental Procedures
The fluorescence response pulses derived from each excitation laser pulse were measured at 5-nm intervals for the 360- to 510-nm spectral range. For details, please see http://atvb.ahajournals.org.

Data Analysis
The time-integrated fluorescence spectrum (conventional spectral emission) was computed from the measured fluorescence response pulses. The time-resolved fluorescence spectrum [fluorescence impulse response function If (t) (FIRF)] was constructed by deconvolving the measured laser pulse from the measured fluorescence pulse at each wavelength across the spectrum. Spectral intensities and time-dependent parameters [average lifetime \( t_f \); decay constants: \( t_1 \) (fast-term), \( t_2 \) (slow-term), \( A_1 \) (fast-term amplitude contribution)] derived from the time-resolved spectra of coronary samples were used for tissue characterization. For details, please see http://atvb.ahajournals.org.

Results

Histology
Of 58 histological samples that underwent spectroscopic examination, 46 sections were histopathologically classified as normal, \( n = 13 \); type I, \( n = 4 \); type II, \( n = 5 \); type III, \( n = 4 \); type IV, \( n = 9 \); type V, \( n = 7 \); and type V, \( n = 4 \). Representative histological sections are depicted in Figure 1. For descriptions of sample composition and morphology, please see http://atvb.ahajournals.org. The median thickness of intima for each tissue type is shown in Figure II (http://atvb.ahajournals.org). Although the median thickness gradually increased as a function of lesion type, we recorded similar ranges of intimal thicknesses for normal and type I samples and for types IV, V, and V samples. The thickness of the intimal cap overlying the lipid pool ranged between 150 and 350 \( \mu \text{m} \) for type IV and 450 and 600 \( \mu \text{m} \) for type V samples. For type V, the cap above the calcium deposit varied in the 50- to 150-\( \mu \text{m} \) range.
**Time-Integrated Fluorescence Spectra**

The fluorescence spectra of coronary specimens are shown in Figure 2a. For a detailed description, please see http://atvb.ahajournals.org.22 The variation of emission intensity with increased atherosclerotic level was observed primarily at the longer wavelengths. Figure 2b depicts the changes of emission intensity for 430 and 470 nm ($I_{430}, I_{470}$). The intensity decreased considerably from normal coronary wall ($I_{470}$ ~60%) to type V lesions (~23%), except for type II lesions (~70%).

**Time-Resolved Fluorescence Spectra**

Figure 3 shows typical time-resolved spectra (FIRF) for (a) normal arterial wall, (b) type II lesion, (c) type IV lesion, and (d) type V lesion.

Representative FIRFs of coronary samples are shown in Figure 3. The emission of normal coronary wall (Figure 3a) lasted for ~14 ns (5% of initial intensity) at the wavelength region of the main peak emission (390 nm). Similar values were observed across the spectrum. The emission of type I lesions (not shown) presented characteristics similar to those of normal wall. The emission of type II lesions (Figure 3b) lasted for a shorter time (~10 ns at 390 nm, ~8 ns at 470 nm) than that of normal wall. For type III (not shown), the emission at the main peak region was comparable to that of normal wall, but it diminished as a function of wavelength (~12 ns at 470 nm). The emission of type IV (Figure 3c) lasted ~13 ns at 390 nm and decreased as a function of wavelength (~10 ns at 470 nm). The emission of type V (Figure 3d) lasted ~20 ns at the region of the main peak and for ~13 ns at longer wavelengths. For type V lesions, the fluorescence decayed faster than that of type V. Their emission (not shown) lasted ~15 ns at peak range and ~12 ns at a longer spectral range. Note that for deconvolution, we used a weighting factor proportional to the inverse of experimental variance. This factor is probably not optimal for some experimental data, thus resulting in artifacts (oscillations) in time in their FIRFs.

**Fluorescence Decay Dynamics Characteristics**

The average lifetime varied as a function of both wavelength and lesion type. The lifetime of normal wall and all lesion types except type II was increased at the peak emission region (390 nm: 1.9- to 3.25-ns range) compared with longer wavelengths (470 nm: ~1.4 ns). For type II, the lifetime averaged 0.9 ns and appeared to be constant along the emission spectrum. At the peak region, the lifetime varied significantly with lesion progression. Figure 4 describes the variation of lifetime for 390-nm emission. Except for type II lesions, the lifetime values measured at 430 and 470 nm did not change with lesion type. The average lifetimes along the emission spectrum for each lesion type are depicted by Figure III (please see http://atvb.ahajournals.org).
The decay constant (fast- and slow-term time constants; fast-term amplitude constant) variation as a function of lesion type for 390-nm emission is depicted in Figure 5. Both $\tau_1$ and $\tau_2$ values increased significantly from normal ($\tau_1=1.05$ ns, $\tau_2=5.75$ ns) to type $V_a$ lesions ($\tau_1=1.95$ ns, $\tau_2=7.70$ ns), whereas $A_i$ did not vary significantly with lesion progression. Type II lesions were different from these trends. They were associated with a reduced $\tau_1$ and increased $\tau_2$ and $A_i$ values compared with normal and type I. Also, type $V_b$ lesions were characterized by lower time-decay constant values than those observed for type $V_a$, suggesting a faster emission decay for calcified lesions than that of collagenous lesions. The time constants at 430 and 470 nm (not shown) did not vary significantly with lesion progression. Table I (http://atvb.ahajournals.org) summarizes the decay characteristics ($\tau_1$, $\tau_2$, and $A_i$) for 390-nm emission and fluorescence intensity at 430 nm ($I_{430}$) and 470 nm ($I_{470}$) values able to discriminate (post hoc test) between various types of arterial tissue. The variation of decay constants as a function of wavelength is represented in Figure IV and described at http://atvb.ahajournals.org.

**Discussion**

This study examined the time-resolved spectra of ex vivo human coronary artery wall (normal and graded levels of atherosclerotic lesions types I, II, III, IV, $V_a$, and $V_b$) to characterize them and to determine whether features of their fluorescence provide a means for lesion classification. We determined that 6 parameters derived from spectral emission at longer wavelengths (>430 nm) and time-resolved emission at the peak emission region (390 nm) discriminate between all types of arterial samples except between normal wall and type I lesions. Furthermore, the lipid-rich lesions can be discriminated from fibrous lesions primarily on the basis of time-dependent parameters, the lifetime and the fast-term decay component. The lipid-rich lesions exhibit faster emission decay than fibrous lesions.

**Spectral and Temporal Fluorescence Characteristics**

The spectral emission of coronary artery has been reported for both ex vivo and in vivo investigation and for various excitation wavelengths: 308 nm, 325 nm, 337 nm, and 476 nm. The time-integrated spectra of normal coronary artery described by our study are in agreement with the emission spectra reported by the research groups that used 337 nm and 325 nm for excitation. Their emission was characterized by a broad spectrum (360- to 550-nm range; peak 380 to 390 nm), modulated by a valley at ~415 nm. Furthermore, Andersson-Engels et al. showed that the emission of atherosclerotic lesions was reduced at longer wavelengths compared with the emission of normal coronary arterial wall. Our results confirm these early findings by displaying a gradual decrease of the intensity at wavelengths >420 nm from normal tissue to type V lesions. In the early studies, the atherosclerotic specimens were differentiated from the normal samples on the basis of their intimal thickness (<200 $\mu$m normal, >200 $\mu$m atherosclerotic). In our study, the lesions were classified into 6 groups on the basis of their composition and morphology (AHA classification). The discrimination between various lesion types provides a means for interpretation of spectral data in correlation with lesions in diverse clinical situations. For instance, the spectral emission data from the red range allow discrimination (Figure 2, Table I) between early lesions from progression-prone areas (type $I_a$) and normal coronary artery wall, collagenous (type $V_a$), and calcified (type $V_b$) lesions but not between lipid-rich (type IV) and collagenous (type $V_a$) or calcified (type $V_b$) lesions.

The time-resolved fluorescence emission of coronary artery has been described by only a few studies, in particular that by Andersson-Engels et al. This research group investigated the fluorescence of human aorta and coronary artery samples generated by 337-nm picosecond laser pulses and recorded with a photon counting detection technique. Three exponentials were used for characterization of fluorescence decay at 3 wavelengths: 380, 437, and 480 nm. The samples were grouped on the basis of their intimal thickness: normal, thin plaque (<500 $\mu$m), thick plaque (>500 $\mu$m), and calcified. Our results agree with the previous findings, although no extensive comparison can be made between the present study and the early observations, because different experimental methods, data analysis techniques, and criteria for sample classification were used. For instance, we observed an increase of fast- and slow-term time constant values (390 nm) from normal tissue to type $V_a$ lesions (Figure 5) that resemble the trends reported earlier, such as the increase of time decay constants from normal (380 nm: $\tau_1=6.0$ ns, $\tau_2=1.7$ ns, $\tau_3=0.2$ ns) to thick plaque ($\tau_1=7.3$ ns, $\tau_2=2.4$ ns, $\tau_3=0.3$ ns). The present study complements the previous work by reporting the characteristics of fluorescence decay for graded levels of atherosclerotic lesions, thus providing information regarding changes of time-resolved fluorescence in relationship to the morphogenetic sequence of lesion progression. We determined that progression-prone lesions (type $I_a$) can be distinguished from normal wall and advanced lesions on the basis of their fast decay, reflected by short lifetime (<0.9 ns), short fast-term time constants ($\tau_1<0.7$ ns), and large values of $\tau_1$ contribution to the overall decay ($A_1>0.8$). Furthermore, the lifetime and the fast-term time constants derived from the peak spectral range can provide discrimination between lipid-rich (type IV) and collagenous (type $V_a$) lesions. Because the intimal thicknesses of type IV and $V_a$ lesions were not resolved (Figure II), our results suggest that compositional differences between the 2 types are reflected in their time-dependent parameters. Overall, the time-dependent parameters provide means of discrimination between the majority of tissue groups investigated in this study (Table I) and complement the parameters.

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Figure 5. Decay constants $\tau_1$, $\tau_2$, and $A_i$ (mean±SEM) variation for 390-nm as a function of lesion type.
inferred from spectral information for lesion evaluation. In addition, this study describes the variation of time-dependent parameters as a function of wavelength.

**Fluorescence of Coronary Artery: Interpretation in Terms of Intrinsic Fluorescent Components**

Fluorescence of human arteries on UV irradiation has been attributed to several intrinsic constituents. The main fluorescent components of normal and diseased arterial wall, together with their emission characteristics, are depicted in Table II (http://atvb.ahajournals.org). The fluorescence of the structural proteins elastin and collagen was related primarily to artery fluorescence. Previous research25 demonstrated that the fluorescence of elastic fibers within the internal lamina or media predominates over the emission of normal coronary artery or aorta, respectively, whereas the fluorescence of collagen (30% to 60% dry weight) prevails over the emission of advanced collagenous lesions (type V; or fibrous plaques). The spectrototemporal fluorescence of both proteins is well documented for 337-nm excitation (Table II).

The atherosclerotic stages between normal and advanced collagenous lesions are characterized by accumulation within arterial intima of various types and amounts of lipids (20% to 25% in type II, 35% to 30% in type III, ∼60% in type IV) that alter the intimal morphology.33 Although reported by several studies, the contribution of lipids to arterial wall fluorescence is still not well understood. Blood-derived particles that undergo transendothelial diffusion, such as LDL or VLDL and their oxidative products, fluoresce, and are thus likely to contribute to the fluorescence emission of matrix structural proteins in arterial tissue.28,30 Cholesteryl esters (cholesteryl oleate and linoleate) account for >60% of the lipid composition of types II and III lesions.20 Cholesteryl oleate is the major lipid component of type II lesions, and the oleate/total ester (oleate and linoleate) ratio decreases with lesion progression,20,34,35 whereas free cholesterol accumulates largely within the necrotic core of type IV lesions, and the free/total cholesterol ratio increases with lesion progression. Previous LIFS studies reported characteristics of cholesterol fluorescence emission (Table II). Both free and esterified cholesterol exhibits emission trends that were distinct from those of elastin and collagen. Thus, the cholesterol emission features are likely to affect the fluorescence dynamics of lesions with high cholesterol content. Also, 2 lipopigments, ceroid and carotenoids, were related to atherosclerotic lesion fluorescence emission.15,31,32 Ceroid, an end product of lipoprotein oxidation, is found in macrophages and in lesions with lipid-rich necrotic cores. Carotenoids are present in lipid-rich lesion cores. Their peak emission, however, is >510 nm, a spectral range not explored by this study.

Other components, such as glycosaminoglycans, tryptophan, and calcium, are also reported to fluoresce. Glycosaminoglycans represent <2% of the organic matrix within normal arterial wall and ∼0.4% of the organic matrix within atherosclerotic wall; thus, it has been suggested20 that these components may not contribute significantly to the fluorescence of arterial wall. Tryptophan fluorescence induced by excitation wavelengths <310 nm was shown to strongly influence the emission of arterial wall at wavelengths <360 nm,8 but it had minimal influence on the emission at >360 nm.8,15 Calcium exhibits sharp fluorescence peaks in the 350- to 650-nm range on 308-nm excitation,28 but its emission on longer excitation wavelengths (325 nm) was not reliably detected.25 Consequently, these components are less likely to significantly influence the fluorescence characteristics reported in our study (excitation 337 nm).

Early work reported a penetration depth of irradiation at ∼337 nm of 150 to 200 μm.8 Therefore, the fluorescence of normal-wall and type I specimens in our study is likely to originate not only from intima but also from media (Figure I), whereas the fluorescence of advanced lesions originates entirely from diseased intima. Furthermore, the fluorescence of type IV lesions is likely to be generated by both the thin cap and the top layers of the lipid-rich core, in contrast to type V lesions, in which the emission may be yielded only by the thick collagenous cap.

Like previous studies,10,24 ours found spectral trends for normal coronary wall and type I lesions that reflect the emission of elastin (broad spectrum: ∼70 nm full width at half maximum, peak ∼410 nm) modulated by that of collagen (blue-shifted peak: ∼385 nm). In addition, we determined that time-resolved data complement the spectral information by displaying a slightly slower emission decay for arterial specimens (∼14 ns) along the wavelengths than for elastin (∼11 ns), but faster than for type I collagen (∼20 ns), thus suggesting the contribution of both elastin (more) and collagen (less) to the overall fluorescence of the samples. These results are in agreement with the histopathological analysis of arterial samples as well as with the early interpretation of the origin of arterial wall fluorescence.24 Also, it is known that both types I and III collagen are the major collagen types in the arterial wall. Immunological studies36,37 have shown that type III collagen is localized in the subendothelial space of the normal intima, but no type I collagen was found at that location. Consequently, for our study, another possible fluorophore is type III collagen. Previously,27 we found an emission decay of ∼15 ns for type III collagen. These early results also support our findings for normal and near-normal coronary wall.

The time-resolved spectra of type V lesions, characterized by a narrow-band emission focused in the blue spectral range, closely resembled the emission of type I collagen (Table II). These results are in agreement with our histopathological analysis, which identified thick layers of collagen fibers as a dominant feature of the type V lesion caps. Also, they agree with the chromatographic analysis of the composition of arterial wall collagen types, which identified type I collagen as prevalent (70%) in the fibrous cap of advanced lesions.3

To the best of our knowledge, this is the first study that describes the time-resolved spectra of early lipid-laden lesions (fatty streaks or type II lesions), early atheroma (type III), and atheroma (type IV) in coronary artery. The emission features of type II lesions, characterized by a faster fluorescence dynamics and red shift of secondary peak emission compared with that of normal specimens, suggest the contribution of a component with fast and enhanced fluorescence emission at longer wavelengths. These characteristics correlate well with the emission of free and esterified cholesterol that exhibited a fast dynamics and a secondary peak in the 470- to 490-nm range.29 Consequently, the emission of type II samples could be the result of the lipid emissions superimposed on the emission of elastin and collagen from the
underlying structures. This hypothesis correlates with the histopathological analysis of our samples. The analysis reveals accumulation in the intima of a large number of macrophages known to contain considerable amounts of cholesteryl oleate. The discrimination of macrophage deposits is particularly important, because macrophages (foam cells) are markers of plaque instability. The emission of type III and IV lesions probably originates from the competing fluorescence emission of the large amount of lipid components (small $\tau_L$ and $\tau_1$ values) located deeper in the intima and the collagen fibers (large $\tau_2$ values) observed in the proximity of the endothelial surface by histopathological analysis. The larger $\lambda_1$ values and decreased emission intensity at longer wavelengths for type III than type IV lesions correlates with the increased amount of collagen in type IV compared with type III lesions. Although very heterogeneous intimal structure characterizes the transition from normal to advanced atherosclerotic wall, our results demonstrate that TR-LIFS data reflect the compositional changes induced by lipid accumulation and offer the possibility of interpretation of coronary artery spectra as a function of lipid composition.

The emission of type $V_b$ lesions (calcified but not exposed cells) reflects the fluorescence of the thin intimal layers that covered the calcium deposits. Consequently, collagen, elastin, and lipids are likely to contribute to their fluorescence.

**Comparison Between Aortic and Coronary Artery Wall Fluorescence**

The morphology and composition of the arterial wall varies with the type of blood vessel and arterial wall layers. For instance, collagen-elastin ratio and distribution in normal coronary wall (more collagen and elastin fibers localized mainly in the internal elastic lamina) are different from those of normal aortic wall (less collagen and elastin distributed in the media). These morphocompositional differences have been reported to be reflected on the spectral emission trends of the 2 types of arterial tissue. Higher values of the emission intensity were reported for normal aortic wall (intensity in the red range: $\approx 80\%$ of main peak value) relative to those found for normal coronary artery ($\approx 65\%$) in this study. Also, the time-dependent emission of normal aorta was shorter ($\approx 11$ ns at the main peak region) than that of normal coronary artery ($\approx 14$ ns). These results suggest that the greater collagen/elastin content in coronary wall relative to that in aortic wall is reflected not only by the decreased fluorescence emission intensity at longer wavelengths but also by the long-lasting fluorescence at the region of main peak emission.

Unlike normal arterial wall, the histopathological analysis of the collagenous cap of advanced lesions reveals similar morphology and composition for both arterial beds, although the cap is much thinner in coronary artery than aortic wall. The spectroscopic results of this study show that the emission characteristics for type $V_b$ lesions in coronary artery are similar to those previously described for aorta (intensity in the red-range spectrum $\approx 20\%$, $\tau_L=2.1\pm0.2$, $\tau_1=7.45\pm0.4$). These findings suggest that the collagenous matrix of type $V_b$ lesions is characterized by unique spectroscopic features that resemble the emission of type I collagen. The fluorescence emission characteristics of the lipid-rich lesions in coronary artery show trends comparable to those determined for aorta in our early studies, albeit different thicknesses and heterogeneous composition and morphology of the 2 different types of arterial walls. For both tissue types, the emission was characterized by a faster fast-time decay component than the emission of type $V_a$ lesions. These observations suggest that lipid accumulation in the intima of distinct arterial beds generates particular fluorescence emission characteristics that provide a means of discrimination between lipid-rich (unstable) and collagenous (stable) lesions. It remains to be determined, however, how in vivo environmental conditions and tissue structure and geometry influence the time-resolved spectra measurements of arterial tissue.

**Conclusions**

Our results demonstrate that analysis of the time-resolved fluorescence spectra can be used to enhance the discrimination between different grades of atherosclerotic lesions. The lipid-rich lesions can be differentiated from the other lesion types (in particular, fibrous lesions) and normal arterial wall. On the basis of indirect evidence, an increased lipid/collagen content ratio has been related to decreased mechanical strength of the atherosclerotic lesions and thus to increased risk of lesion rupture. This research shows that spectroscopic features derived for lipid components are reflected in the emission of lipid-rich lesions, whereas characteristics of type I collagen are identified in the emission of fibrous lesions. By inferring such a relation, this study promotes the development of clinical instrumentation based on spectroscopic characterization of lipid/collagen content to predict and monitor the clinical evolution of individual lesions in vivo. Furthermore, the analysis of the time-resolved spectra provides a wealth of spectroscopic parameters. By use of suitable algorithms, the optimal parameters that provide a means of discrimination between lesion types could be identified. Our results suggest that a few parameters that combine spectral features at longer wavelengths and time-resolved characteristics from the peak emission region are the best selections for coronary artery lesion discrimination. Parameters derived from time-resolved spectra, such as the lifetime and the fast-time decay constants, are most likely to differentiate between lipid-rich and fibrous lesions and be used for diagnosis. In addition, by showing that parameters derived from time-resolved spectra can enhance the discrimination of atherosclerotic lesions, this study demonstrates that the TR-LIFS technique can be used for characterization of arterial tissue and advances a paradigm that may be applicable for other disease states, such as neoplasms.

**Acknowledgments**

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**References**

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Figure I. TR-LIFS experimental setup used to measure the fluorescence emission of coronary artery samples. PD 1 and PD 2, silicon photodetectors. PMT, multichannel plate photomultiplier tube. BS 1 and BS 2, beam splitters.
**Figure II.** Thickness of intima as a function of lesion type. Line in the middle of the box = intima thickness median (if the median is not centered in the box – indication of skewness). Lower and upper lines of the box = 25th and 75th percentiles of the thickness. Lines extending above and below the box = extend of thickness.
Figure III. Lifetime, $\tau_f$: average for each type of coronary samples.
Figure IV. Decay constants: average for each type of coronary samples. a) Fast-term, $\tau_1$ (clear symbols), and slow-term, $\tau_2$ (fill symbols) decay constants. b) Fractional contribution of the fast-term decay component ($A_1$). Note that, the decay constants below 1ns were probably overestimated.
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<td>Baraga et al.8</td>
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<td>360 - 500 / 390 / 40</td>
<td>380: 0.78 (24.8) / 4.99 (45.5) / 9.94 (29.7)</td>
<td>337</td>
<td>Andersson-Engels et al.15</td>
<td></td>
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<td>360 - 500 / 385 / 40</td>
<td>390: 3.9 (65.0)/ 9.9</td>
<td>337</td>
<td>Maarek et al.17</td>
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<td>Yan et al.26</td>
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<td></td>
<td>350 - 500 / 390 / 30</td>
<td>-</td>
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<td>Laifer et al.25</td>
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<td>360 - 500 / 385 / 60</td>
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<td>337</td>
<td>Marcu et al.27</td>
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<td>Free cholesterol</td>
<td>350 - 650 / 430 / 75</td>
<td>308</td>
<td>Morguet et al.28</td>
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<td>-</td>
<td>325</td>
<td>Laifer et al.25</td>
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<td>390: 0.53 (40) / 3.0</td>
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<td>Marcu et al.29</td>
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<td></td>
<td>360 - 500 / 375 / 40</td>
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<td>Verbunt et al.31</td>
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<td>Hunt et al.32</td>
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<td>377</td>
<td>Andersson-Engels et al.15</td>
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<td>350 - 600 / 405 / 55</td>
<td>325</td>
<td>Laifer et al.25</td>
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<td>340 - 600 / 405 / 55</td>
<td>325</td>
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<td>Heparan sulfate</td>
<td>340 - 600 / 400 / 55</td>
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<td>Laifer et al.25</td>
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* Full-width at half maximum measured on the long wavelength side of the peak emission.
Methods

Instrumentation. The pulses of a nitrogen laser (337 nm) were focused into a fiber optic probe and directed to the coronary artery sample from above. The resulting fluorescence emission was collected by a fiber optic bundle, directed into a scanning monochromator (bandwidth: 5 nm), and detected by a gated multichannel plate photomultiplier tube (rise time: 0.3 ns) placed at the monochromator exit slit. The photomultiplier output was amplified (rise time: 0.35 ns; bandwidth: 1 GHz) and the entire fluorescent pulse from a single excitation laser pulse was recorded with a digital oscilloscope (bandwidth: 500 MHz, sampling frequency: 2 Gsamples/s). A fraction of the excitation source output beam was directed toward two fast silicon detectors. One detector triggered the gate and delay generator, which in turn gated the photomultiplier. The second detector triggered the oscilloscope to begin sweeping the photomultiplier output and to monitor laser pulse-to-pulse shape and energy variation. A personal computer was used to control data acquisition, data transfer from the oscilloscope, and monochromator wavelength scanning.

Samples. Coronary segments were washed with buffered saline solution, snap frozen in isopentane and liquid nitrogen, and stored at -75°C until use. For spectroscopic examination, the frozen segments were passively warmed at room temperature, longitudinally opened, and kept moist with saline prior to study. Thereafter, the segments were flattened and pinned in place with intimal (endothelial) surface outward on a plastic board. Each segment was visually examined, and areas with normal and atherosclerotic characteristics were chosen for spectroscopic investigation. The samples were spectroscopically measured in air at room temperature. After spectroscopic investigation, areas from where fluorescence was recorded were marked with small incisions. Each sample was then fixed in 10% buffered formalin and two transversely oriented sections (4 µm thick) were cut from the marked areas. The sections were embedded in paraffin
and stained with hematoxylin-eosin (H&E) and Movat pentachrome. Movat stain was used to evaluate the
distribution and quantity of elastin, collagen, proteoglycans, and smooth muscle cells (SMC). Both H&E
and Movat stain were used to infer the presence of macrophages (foam cells), lipid components and cal-
cium within the specimens.

The histologic sections were evaluated by light microscopy. Following histological assessment, 12 samples
were excluded from spectroscopic interpretation (7 samples presented artifacts as a result of histological
processing, 5 samples showed morphological characteristics different from those of the lesion types inves-
tigated in this study).

*Experimental procedures.* To improve the signal-to-noise ratio, each recorded fluorescence pulse repre-
sented the average of 16 consecutive fluorescence pulses computed by the oscilloscope circuitry. Ten con-
secutive measurements of the fluorescence response were collected for three wavelengths (390, 430, 470
nm) to assess the variability of the fluorescence measurement across the emission spectrum. The energy
output of the laser at the tip of the excitation fiber was adjusted to 0.6 µJ/pulse. During a single measure-
ment sequence that lasted for 370 seconds, the energy total fluence delivered to the sample was <1.2 mJ/
mm². Previously we reported that this fluence minimizes the photobleaching of arterial components fluo-
rescence emission.¹⁶ After each measurement sequence, the monochromator was tuned to a wavelength
slightly below the laser line. The average of sixteen laser pulses reflected by the sample was used to repre-
sent the temporal profile of the laser pulse. The background noise was measured with the monochromator
tuned to 400 nm. During this measurement the sample was removed and the excitation-collection fiber
assembly was raise 30 cm above from optical bench.

*Data analysis.* The time-integrated fluorescence spectrum (conventional spectral emission) was computed
from the measured fluorescence response pulses by integrating each pulse as a function of time for each
investigated wavelength. The constructed fluorescence spectra was corrected for background noise, corrected for nonuniform instrumental system response, and normalized by dividing the florescence intensity at each emission wavelength by the peak fluorescence intensity. The reconstructed spectrum was characterized by discrete intensity values \( I_{n} \) that show the variation of fluorescence intensity as a function of wavelength. The time-resolved fluorescence spectrum (fluorescence impulse response function \( I_f(\tau) \) (FIRF)) was constructed by deconvolving the measured laser pulse from the measured fluorescence pulse at each wavelength across the spectrum. The deconvolution method was based upon Least–Square iterative reconvolution technique combined with Laguerre expansion of kernels technique. This method was developed by our group and previously described.\textsuperscript{17,21} Five Laguerre functions were used for expansion. The optimal values of expansion coefficients and order of expansion are determined by least-squares minimization of the weighted sum of residuals. For the present experimental technique, the weighting factor was determined experimentally and found proportional to the inverse of experimental variance at certain time for the majority of data. Deconvolution based on this technique separates the computation of the FIRF from the modeling of the fluorescent system, therefore it facilitates an unconstrained interpretation of time-resolved fluorescence data from complex biological media such as arterial tissue.

To characterize the dynamics of fluorescence decay, two sets of parameters were used 1) the average lifetime \( \tau_f \) estimated as the interpolated time at which the FIRF decays to \( 1/e \) of its maximum value, 2) the decay constants obtained by approximating the FIRF with a biexponential function.

\[
I_f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}
\]

In the biexponential model, parameters \( \tau_1 \) and \( \tau_2 \) represent the fast- and slow-term decay constants. Ratio \( A_1 = \frac{a_1}{a_1 + a_2} \) represents the fractional contribution of the fast-term component to the FIRF.

The temporal resolution of our system is determined by the resolution of experimental apparatus (0.5 ns) and deconvolution method described above. In a previous study,\textsuperscript{17} we reported that decay constants down
to 1 ns can be accurately retrieved employing our experimental apparatus and deconvolution method (using only the first five Laguerre function for expansion). We also reported that subnanosecond decays could be retrieved, however with overestimation of the decay constants.

Variance analysis (one-way ANOVA), applied to the time-dependent parameters ($\tau_f$, $\tau_1$, $\tau_2$, $A_1$), was employed to evaluate the effect of emission wavelength and lesion type on the dynamics of fluorescence decay. Differences among individual means were assessed with a post-hoc comparison test (Student-Newman-Keuls). Data are reported as mean±SE. The level of significance used was $p < 0.05$. 
Results

*Histology.* Normal coronary samples were characterized by sparse elastin and collagen fibers and SMC within the intima, well defined internal and external elastic lamina (10 out of 13 samples), and abundance of SMC within the media. Also, small amounts of proteoglycan matrix was observed within the intima. Type I lesions showed similar characteristics with those of normal artery. Additionally, these specimens presented evidence of isolated macrophages within the intima. Type II lesions differed from normal artery and Type I lesions by increased amount of macrophages, SMC, and proteoglycan and scarce presence of extracellular lipid inside the intima. The Type III lesions showed evidence of isolated small pools of intimal extracellular lipid located adjacent to layers of macrophages. The lipid pools were surrounded by proteoglycans matrix with scarce collagen fibers and variable number of SMC. Type IV lesions were characterized by a prominent extracellular lipid core. The lipid core was covered by a compact matrix of smooth muscle cells in 5 of 9 specimens. Layers of macrophages were more often adjacent to the lipid core rather than endothelial surface of the intima. Abundant collagen fibers were present in thin layers near the endothelium in 6 specimens and was more focal in the remaining specimens. Scattered elastic fibers were present within the intima. Four specimens contained fine calcium particles within the lipid core. Two types of morphology were observed for Type V<sub>a</sub> lesions. One type (4 specimens) was characterized by small lipid cores separated by closely packed layers of collagen. Thin layers of macrophages were observed within the structure. The second type (3 specimens) was characterized by one prominent lipid pool covered by thick layers of collagen fibers with aggregates of macrophages near the endothelium, and SMC within the intima. Type V<sub>b</sub> specimens had features similar to those of Type V<sub>a</sub> (first type), but in addition they contained sparse elastin fibers and large calcium deposits located below the endothelial surface.
Time-integrated fluorescence emission spectra. The main peak emission was at ~380 nm for normal wall, Type I, and V_b and slightly blue shifted (~375 nm) for Types II, III, IV, and V_a lesions. A valley modulates the spectra of all lesions at ~415 nm (more distinctive for normal wall and Types I to III lesions). This valley corresponds with the oxy-hemoglobin absorption spectra as previously reported. The normal and Types I to III samples showed a broader emission when compared to Types IV, V_a, and V_b. Also, the secondary peak emission for Type II and III lesions was red-shifted (~470 nm) when compared to that of the other lesions or normal wall (~450 nm). Type II lesions presented the most wide-ranging emission and enhanced intensity in 470-490 nm range. Overall, the emission at the longer wavelengths decreased with increased atherosclerotic level.

Fluorescence decay dynamics characteristics.

Decay constants (fast- and slow-term time constants; fast-term amplitude constant). The variation of decay constants as a function of wavelength (Figure IV) is summarized by the following. For normal coronary wall and Types I and III lesions, the fast-term time constants (\(\tau_1\)) had similar values (Figure IVa) over the investigated wavelength range (Normal: ~1.0 ns; Type I: ~1.0 ns; Type II: ~0.5 ns). In contrast for Types III, IV, V_a, and V_b, \(\tau_1\) values decreased at wavelengths above 400 nm. This feature was enhanced for Type V_a lesions (390 nm: 1.95±0.2 ns; 430 nm: 0.75±0.2; 470 nm: 0.8±0.3). The slow-term time constant (\(\tau_2\)) appeared to gradually increased as a function of wavelength for all tissue types except for Type V_a lesions. However, no statistical difference was observed between values of 390, 430, and 470 nm emission. The fast-term amplitude constant (\(A_1\)) increased significantly as a function of wavelength for all tissue types except Types I and II lesions (Figure IVb). The contribution of \(A_1\) to the overall decay was between 0.55-0.65 at 390 nm emission and reached 0.7-0.75 at 470 nm. For Type I lesions, \(A_1\) was ~0.66 along the entire emission spectrum. For Type II lesions, \(A_1\) was larger when compared to the other tissue lesion types and
varied around 0.85 value along the emission spectrum. For the emission at 360 and 365 nm we observed that both $\tau_1$ and $\tau_2$ were decreased whereas $A_1$ was increased when compared to the emission at the proximate wavelengths. These trends suggest that the recorded fluorescence pulses at 360 and 365 nm probably contained a fraction of the fast laser pulse. Furthermore, the time-decay constants significantly varied with increased atherosclerotic level, particularly in the main peak emission wavelengths band.