APPENDIX

Detection of Rupture-Prone Atherosclerotic Plaques by Time-Resolved Laser Induced Fluorescence Spectroscopy

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2. METHODS and MATERIALS

2.2. TR-LIFS Instrumentation, Measurements and Data Analysis

Instrumentation. The experiments were conducted with a prototype TR-LIFS apparatus, developed by our group and detailed elsewhere [24]. The apparatus allowed for both spectral- and time-resolved fluorescence measurements (Figure 1, module A). Autofluorescence of tissue was induced with a pulsed nitrogen laser (Lasertechnik, MNL200-ATM205, wavelength 337 nm, pulse width 700 ps, frequency 30 Hz) using a custom made sterilizable bifurcated fiber-optic probe (CeramOptec, East Longmeadow, MA). The probe consisted of a tapered fiber-optic central excitation channel (diameter 600 μm, numerical aperture 0.11) surrounded by a ring of 12 collection fibers (diameter 200 μm, numerical aperture 0.22). The collected fluorescence was dispersed by an imaging spectrograph/monochromator (Chromex Inc., model 250is/sm, F/4.4, 600 gr/mm grating, blazed at 450 nm) and detected with a gated microchannel plate photo-multiplier tube (MCP-PMT Hamamatsu, R5916-50; rise-time 180 ps). The fluorescence was temporally resolved using a digital oscilloscope (TDS5104, Tektronix; bandwidth 1 GHz, sampling rate 5 Gsamples/s) coupled to a preamplifier (Hamamatsu, C5594; bandwidth 1.5 GHz). A long-pass filter (360 nm) was used to remove the excitation light from the detection path at 337 nm. The laser triggering, wavelength scanning, and data acquisition, storage and processing were controlled using a computer and custom software written in LabVIEW (National Instruments) and MATLAB (Mathworks, Inc).

TR-LIFS measurements. The fiber-optic probe was placed perpendicularly to the intimal surface of the carotid plaque sample. TR-LIFS measurements were obtained with serial scanning of the monochromator across a spectral range from 360 to 550 nm in increments of 10 nm. The total acquisition time across the scanned emission spectrum was ~30 seconds. After each measurement sequence, the laser pulse temporal profile was measured at a wavelength slightly below the excitation laser line. This profile was later used as input to the deconvolution algorithm for the estimation of fluorescence lifetimes. Laser excitation output measured at the tip of the probe was set at 2 μJ/pulse (fluence 1.8 μJ/mm^2 per pulse, fluence rate

54 μW/mm^2 at tissue level). This output was found as being a reasonable compromise between an adequate signal-to-noise ratio and the photobleaching of the sample [18].

TR-LIFS Data Analysis. In the context of TR-LIFS, the intrinsic fluorescence impulse response function (FIRF), *h(n),* describes the real dynamics of the fluorescence decay. The FIRF were recovered by numerical deconvolution of the measured input laser pulse from the measured fluorescence response transients (Figure 1 – Module B). The Laguerre expansion technique [26] was used for deconvolution. This method allows a direct recovery of the intrinsic properties of a dynamic system from the experimental input-output data. The technique uses the orthonormal Laguerre functions $b_j^{\alpha}(n)$ to expand the FIRF and to estimate the Laguerre expansion coefficients (LEC) c_j. Once the FIRF's were estimated for each emission wavelength, the steady-state spectrum (I_λ), was computed by integrating each intensity decay curve as a function of time. Further, to characterize the temporal dynamics of the fluorescence decay, two sets of parameters were used: 1) the average lifetime (τ_f) computed as the interpolated time at which the FIRF decays to 1/e of its maximum value; and 2) the normalized value of the corresponding LECs. 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). This analytical approach for the characterization of fluorescence decay was recently developed by our research group and described in detail elsewhere [27].

2.3. Histopathological Analysis

Following TR-LIFS measurements, all spectroscopically investigated carotid areas were removed, fixed (10% buffered formalin), processed routinely, and embedded in paraffin. Four sequential 4 μm thick cross-sections were cut from each segment, and stained with hematoxylin and eosin (H&E), a trichrome/elastin method, CD68 (for macrophages) and CD45 (for leukocytes), respectively. Histopathological analysis was performed by two pathologists (J.H.Q., M.C.F.) specialized in

cardiovascular diseases and blinded to the TR-LIFS data. The composition of artery wall was assessed within a region of interest (ROI) under the ink mark, defined by the fiber-optic excitation-collection geometry (~1.1 mm illuminated diameter area at tissue surface) and the light penetration depth (~200 μm for 337 nm in arterial tissue) [21]. Each section was evaluated by light microscopy and digitized. The ROI was measured from the digitized pictures of the histological slides using the AxioVision image processing software (Carl Zeiss Inc, Germany). Digitized H&E and trichrome-stained sections were further analyzed to quantify the relative contents (%) of collagen, elastin, smooth muscle cells (SMC), calcification and necrosis within each ROI. Necrosis was defined as morphologically distinct extracellular tissue spaces composed of clear, needle-shaped cholesterol clefts (representing ghost outlines of dissolved crystals) and/or clear, bubbly, granular, mostly anucleate necrotic debris of foam cells. Macrophage and lymphocyte relative contents (%) within the ROI were assessed from the digitized CD68 and CD45 stained sections, respectively. Plaque thickness (and minimum thickness of a fibrous cap when a necrotic/lipid core was present) was measured from the digitized trichrome-stained sections.

Statistical Analysis. In order to identify a set of spectroscopic parameters that will best discriminate between different tissue types (feature selection – Figure 1 - Module B), a univariate statistical analysis (one-way ANOVA) was used to compare the spectroscopic parameters (I_λ , τ_f , and LECs) at every λ_E for each type of tissue as defined by histology. A P-value of <0.05 was assumed to indicate statistical significance. A systematic comparison of the P-values obtained for each parameter at every λ_E allowed the identification of a set of spectroscopic parameters (I_λ , τ_f and LECs at specific λ_E 's yielding the lowest P values) likely to provide means of discrimination among different compositional features of the carotid plaque.

Classification. Based on previous work [28], a stepwise linear discriminant analysis (SLDA) approach was adopted to generate a classification model (discriminant functions) and for sample classification. The

discriminant function analysis provides an effective means for classifying spectroscopic data of unknown origin [29].The set of features (spectroscopic parameters) selected from the statistical analysis were used for the optimization of discriminant functions. The classification accuracy was determined using a leave-one-out cross-validation approach [29], and then values of sensitivity (SE), specificity (SP) and overall classification performance (% of samples correctly classified) were computed.

3. RESULTS

Classification. Results of the classification aiming to discriminate IT, FP/FC/Low-INF and INF/NEC lesions are shown in Table 2. The overall cross-validation classification performance was 74.7%. For this case, IT lesions were also discriminated with high SE and SP (larger than 80%), although appreciable overlap between this group and FP/FC/Low-INF was also present. INF/NEC could still be detected with SE ~80% and high SP >90% from the rest of the more stable lesions (IT, FP, FC, Low-INF). However, the accuracy of detecting these vulnerable plaques slightly decreased when low-INF lesions were included in the analysis.

Table 2. Classification accuracy (sensitivity and specificity): discrimination of IT, FP/FC/Low-INF and INF/NEC

	Fluorescence Spectroscopy			SE	SP
	IT	FP/FC/Low-INF	INF/NEC	(%)	(%)
INF (N=28)	25	3	0	89.3	81.0
FP/FC/Low-INF (N=731)	149	519	63	71.0	81.7
INF/NEC (N=54)	0	12	42	**77.8**	**91.7**