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Detection of morphological markers of vulnerable atherosclerotic plaque using multimodal spectroscopy

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Abstract. Vulnerable plaques, which are responsible for most acute ischemic events, are presently invisible to x-ray angiography. Their primary morphological features include a thin or ulcerated fibrous cap, a large necrotic core, superficial foam cells, and intraplaque hemorrhage. We present evidence that multimodal spectroscopy (MMS), a novel method that combines diffuse reflectance spectroscopy (DRS), intrinsic fluorescence spectroscopy (IFS), and Raman spectroscopy (RS), can detect these markers of plaque vulnerability. To test this concept, we perform an MMS feasibility study on 17 human carotid artery specimens. Following the acquisition of spectra, each specimen is histologically evaluated. Two parameters from DRS, hemoglobin concentration and a scattering parameter, are used to detect intraplaque hemorrhage and foam cells; an IFS parameter that relates to the amount of collagen in the topmost layers of the tissue is used to detect the presence of a thin fibrous cap; and an RS parameter related to the amount of cholesterol and necrotic material is used to detect necrotic core. Taken together, these spectral parameters can generally identify the vulnerable plaques. The results indicate that MMS provides depth-sensitive and complementary morphological information about plaque composition. A prospective in vivo study will be conducted to validate these findings. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2187943]

Keywords: atherosclerosis; vulnerable plaque; morphology; spectroscopy; multimodal.

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1 Introduction

The severity of atherosclerotic lesions has traditionally been assessed by imaging their location and percentage of intraluminal stenosis. However, landmark studies over the past ten years have shown that up to 70% of acute cardiac ischemic events result from the rupture of previously subclinical lesions, ending in thrombotic occlusion and, often, myocardial infarction.1,2 The vast majority of these thrombosed coronary artery plaques exhibit less than 75% stenosis,3 the figure often used to define a clinically significant lesion. Thus, plaques that are not critically stenotic may still cause acute ischemic events.4,5

These vulnerable plaques often exhibit rupture of a so-called thin cap fibrous atheroma, i.e., a plaque with a thin (<65 μm) fibrous cap overlying a large (>2 mm) necrotic core.6 A recent consensus paper by cardiovascular pathologists6 has reported that additional morphological features may be associated with thrombosis, such as erosion or denudation of the intimal endothelial layer. In both ruptured and eroded plaques, the fibrous cap3,7 or superficial intima3 is frequently infiltrated by inflammatory cells, most often macrophages and foam cells. Exposed calcifications3 and acute intraplaque hemorrhage or dissection2 are other common features of thrombosed plaques.

There is currently great interest in developing new techniques for clinical imaging of vulnerable plaque, including advances in intravascular ultrasound, optical coherence tomography, and thermography.7 Optical spectroscopy is also being studied for evaluation of these critical lesions. Several groups have explored the use of near-infrared spectroscopy to characterize atherosclerotic plaque,8,9 with a recent paper reporting promising results in detecting markers of plaque vulnerability.10 Fluorescence spectroscopy has also shown potential to detect features of vulnerable plaque, including a thin fibrous cap.11

The present work explores the potential of combining complementary spectroscopic methods, reflectance, fluorescence, and Raman spectroscopy to evaluate the morphological features of vulnerable plaques in vivo.
features of atherosclerotic plaque and assess plaque vulnerability, particularly by the detection of intraplaque hemorrhage, density and depth of superficial foam cells, fibrous cap thickness, and necrotic core size. This combination provides both depth sensitivity, important for identifying thin fibrous caps, and complementary morphological information. We term this method multimodal spectroscopy (MMS).

Using diffuse reflectance spectroscopy (DRS), we study the spectrum of near-UV-visible light (300 to 700 nm) traversing turbid biological tissue. The resulting spectrum exhibits features due to scattering and absorption of the incident light by the tissue. We have developed a model to analyze DRS spectra based on an analytical expression for diffusion of multiply scattered light to extract the wavelength-dependent coefficients of scattering and absorption. In atherosclerotic plaque, the main absorbers are hemoglobin, associated with thrombus or acute intraplaque hemorrhage, and beta-carotene, whose absorption has been used previously for spectroscopic detection of atherosclerosis in an animal model.

Fluorescence spectroscopy relies on the excitation of molecular electronic energy levels, giving rise to re-emission at wavelengths longer than the exciting light. The emission spectrum provides information about the fluorophores excited. The primary fluorophores in arterial tissue are elastin, collagen, tryptophan, ceroid, and oxidized low-density lipoprotein (LDL). A number of research groups, including our own, have employed continuous wave and time-resolved fluorescence spectroscopy to diagnose atherosclerosis. However, the broad overlapping spectral features of tissue fluorophores, further confounded by absorption and scattering, are a barrier to extracting spectral information with sufficient accuracy for quantitative analysis of vulnerable plaque. One recent study using fluorescence showed promising results in identifying thin fibrous cap atheromas, but a number of samples had to be eliminated from the analysis due to the inability to compensate for the spectral distortions caused by scattering and absorption. We have developed a method to remove these distortions using the information from the DRS spectrum, and thus extract the intrinsic fluorescence, which can be decomposed into a linear combination of the spectra from fluorophores associated with morphological structures in the tissue. Intrinsic fluorescence spectroscopy (IFS) has been successfully employed in our research for cancer diagnosis.

Raman spectroscopy (RS) detects molecules by exciting vibrations among bonds that are unique to each molecule, and has been used extensively in biomedicine. Previous work by our group on RS of atherosclerosis identified eight key morphological components in arterial pathogenesis that could be identified by their Raman spectral signatures: collagen fibers, cholesterol crystals (CC), calcium mineralization (CM), elastic lamina (EL), adventitial adipocytes (AA), foam cells/necrotic core (FC/NC), beta-carotene crystals (β-CC), and smooth muscle cells (SMC). A diagnostic algorithm, using morphological information extracted with a linear combination model similar to that used for IFS, is able to classify in vitro coronary artery specimens as nonatherosclerotic, non-calcified plaque and calcified plaque with 94% accuracy. The development of a small-diameter, high-throughput, filtered Raman probe has resulted in the first in vivo clinical application of the RS diagnostic algorithm in real time during carotid endarterectomy and femoral bypass surgery.

MMS, the combined use of DRS, IFS, and RS, yields complementary biochemical and morphological information about arterial tissue that no individual modality can provide by itself. Furthermore, the information obtained by these modalities is depth-sensitive due to the inherently different tissue penetration by the various incident wavelengths employed. The results of the following feasibility study indicate that MMS has the potential to accurately assess plaque vulnerability in vivo, in real time, and as a guide to future treatment.

2 Methods

2.1 Depth Sensing

Light of different wavelengths penetrates tissue to different depths. The effective penetration depth is defined as the depth at which the power of light incident on a tissue sample falls to 1/e of its incident value, and can be calculated from diffusion theory:

\[ \delta = \frac{1}{\mu_{\text{eff}}} = \left( \frac{1}{3\mu_a(\mu_a + \mu_s'')} + 1 \right)^{1/2}, \]

using the values for the reduced scattering coefficient \( \mu_s' \) and absorption coefficient \( \mu_a \) of tissue. Based on this equation, the literature on the optical properties of aorta indicates effective penetration depths of about 90, 140, and 1200 \( \mu \)m for light of wavelengths 308, 340, and 830 nm, respectively. Another study indicates values 70, 90, and 800 \( \mu \)m for the effective penetration depths of 308, 340, and 830 nm in aortic tissue.

We performed experiments to verify these values using our instruments and probes. The penetration depths at different excitation wavelengths were measured by incrementally stacking 20-\( \mu \)m-thick sections of aortic media. An optical fiber probe, placed in contact with the tissue, delivered the light, and a power meter measured the transmitted power as a function of tissue thickness. Note that in the single-ended geometry of our artery studies (i.e., the probe both delivers and collects light from the same side of the tissue), we need to account for the propagation of both the excitation and emission light. Thus the sampling depth \( \delta_s \) can be related by

\[ \frac{1}{\delta_s} = \frac{1}{\delta_{\text{ex}}} + \frac{1}{\delta_{\text{em}}}, \]

where \( \delta_{\text{ex}} \) and \( \delta_{\text{em}} \) are the penetration depths of the excitation and emission light, respectively. Other groups have defined the sampling depth, or probing depth, as the depth above which 90% of the remitted fluorescence originates.

2.2 Tissue Study

We have performed a preliminary in vitro study to establish the effectiveness of DRS, IFS, and RS for providing information about plaque morphology and vulnerability. Spectra were collected from excised human carotid endarterectomy specimens (n=17) from 12 patients, obtained from the Cooperative Human Tissue Network. The snap-frozen specimens were thawed and rinsed in phosphate-buffered saline. The DRS and IFS spectra were obtained using a fast excitation-emission matrix (FastEEM) instrument and probe, described in detail elsewhere. The FastEEM probe was placed in gentle con-
dependent absorption coefficient which Raman system and probe, described elsewhere (33–36) signal was then acquired from the same location using a clinical/H20849 found and a density grade beneath the fibrous cap, if present. The foam cells were evaluate- size was recorded as the maximum dimension of the core/thickness found underneath the ink dot. The necrotic core The intimal or fibrous cap thickness was recorded as the range perficial foam cells, intraplaque hemorrhage, and ulceration. morphological features associated with plaque vulnerability was assessed: fibrous cap thickness, necrotic core size, super- ficial foam cells, intraplaque hemorrhage, and ulceration. The intimai or fibrous cap thickness was recorded as the range of thicknesses found underneath the ink dot. The necrotic core size was recorded as the maximum dimension of the core beneath the fibrous cap, if present. The foam cells were evalu- ated based on the most superficial depth at which they were found and a density grade (0=none; 1+=isolated single foam cells; 2+=small clusters of foam cells; 3+=confluent sheets of foam cells). Intraplaque hemorrhage was identified as an accumulation of blood within the lesion, usually associated with the core. Plaque ulceration was graded based on whether the defect in the fibrous cap did (rupture) or did not extend (fissure) into the underlying atheroma core.

DRS spectra were used to extract the wavelength-dependent absorption coefficient $\mu'_a(\lambda)$ and reduced scattering coefficient $\mu'_s(\lambda)$ based on a diffusion theory model. We modeled $\mu'_a(\lambda)$ as a linear combination of two absorbers, oxy-hemoglobin and $\beta$-carotene:

$$\mu'_a(\lambda) = [Hb]^* \epsilon_{Hb}(\lambda) + [\beta c]^* \epsilon_{\beta c}(\lambda),$$

with $[Hb]$ and $[\beta c]$ the concentrations and $\epsilon_{Hb}(\lambda)$ and $\epsilon_{\beta c}(\lambda)$ the extinction coefficients of oxy-hemoglobin and $\beta$-carotene, respectively [Fig. 1(a)]. Deoxy-hemoglobin was not included in the model because this is an in vitro study of frozen-thawed tissues in which the hemoglobin was oxygenated by exposure to room air. We modeled the $\mu'_s(\lambda)$ spectrum as an inverse power law, $\mu'_s(\lambda) = A \lambda^{-\beta}$, as reported elsewhere.

IFS spectra were obtained by correcting the raw fluorescence for the effects of scattering and absorption. Based on literature values, IFS spectra excited at 308 and 340 nm have the shallowest sampling depths and were used to assess the composition of the fibrous cap. Multivariate curve resolution (MCR), a chemometric technique used to extract the individual spectra of a known number of components from a mixture spectrum, was performed on the IFS spectra at each of the two excitation wavelengths. For IFS at 308 and 340 nm, a linear combination of two MCR components [MCR1 and MCR2, Figs. 1(b) and 1(c)] resulted in good fits to all 17 spectra. The fit coefficient of the blue-shifted and narrower MCR component (MCR1) is reported as $C_{308}$ and $C_{340}$ for IFS at 308 and 340 nm, respectively.

Raman spectra were fit using a linear combination of basis spectra components of the eight morphological structures (CF, and reduced scattering $\mu'_s(\lambda)$ based on a diffusion theory model. We modeled $\mu'_a(\lambda)$ as a linear combination of two absorbers, oxy-hemoglobin and $\beta$-carotene:

$$\mu'_a(\lambda) = [Hb]^* \epsilon_{Hb}(\lambda) + [\beta c]^* \epsilon_{\beta c}(\lambda),$$

with $[Hb]$ and $[\beta c]$ the concentrations and $\epsilon_{Hb}(\lambda)$ and $\epsilon_{\beta c}(\lambda)$ the extinction coefficients of oxy-hemoglobin and $\beta$-carotene, respectively [Fig. 1(a)]. Deoxy-hemoglobin was not included in the model because this is an in vitro study of frozen-thawed tissues in which the hemoglobin was oxygenated by exposure to room air. We modeled the $\mu'_s(\lambda)$ spectrum as an inverse power law, $\mu'_s(\lambda) = A \lambda^{-\beta}$, as reported elsewhere.

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The Raman fit coefficients were normalized to sum to unity so that each fit coefficient specifies a percentage contribution to the fit by that respective basis spectrum, as previously described. The hemoglobin contribution to the Raman spectra was zero in all of our samples, as hemoglobin is a relatively weak Raman scatterer at 830-nm excitation. The spectral parameters obtained were then correlated with the presence or absence of the morphologic features of vulnerable plaque.

### 3 Results

#### 3.1 Depth Sensing

In our experiments, the effective penetration depths at 308 and 340 nm were measured as 85 and 105 μm, respectively. The corresponding fluorescence emission peaks were 400 and 410 nm, respectively, with effective penetration depths of 128 and 147 μm. Therefore, the sampling depths for the IFS at 308 and 340 nm are 51 and 62 μm, respectively, accounting for the longer emission wavelength of the fluorescent light. Similarly, there were different wavelength regions of the DRS spectra sample tissue at different depths (50 to 500 μm). A previous experiment estimated the sampling depth of 470 μm for 830-nm Raman excitation. The prior penetration depths, measured on aortic media, may vary with different types of lesions. Nevertheless, the trend of deeper sampling depth for longer wavelength excitation light still holds for each sample or atherosclerotic lesion.

#### 3.2 Tissue Study

The histopathologic parameters relating to plaque vulnerability are summarized in Table 1 for each of the 17 specimens. The last four specimens exhibit the hallmarks of vulnerable plaque and are so classified: specimen 14 contains an intraplaque hemorrhage, specimens 15, 16, and 17 all have thin fibrous caps, some with presence of ulceration, superficial foam cells, and necrotic core. The remaining specimens (1 through 13) do not have the necessary combination of features to be deemed vulnerable.

### Table 1: Morphological features of the 17 specimens. IF= intimal fibroplasia, ATS= atherosclerotic plaque, ATM= atheromatous plaque, FS= fibrotic-sclerotic plaque, C= calcified.

<table>
<thead>
<tr>
<th>Specimen number</th>
<th>SNOMed classification</th>
<th>Intimal or fibrous cap thickness (microns)</th>
<th>Necrotic core thickness (microns)</th>
<th>Foam cell depth (microns)</th>
<th>Foam cell grade (0–3+)</th>
<th>Intraplaque hemorrhage</th>
<th>Ulceration</th>
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<tbody>
<tr>
<td>1</td>
<td>IF</td>
<td>24 to 64</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>IF</td>
<td>40 to 80</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>3</td>
<td>ATS</td>
<td>480 to 500</td>
<td>—</td>
<td>480</td>
<td>3+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>ATS</td>
<td>240 to 440</td>
<td>—</td>
<td>40</td>
<td>1+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>ATS</td>
<td>456 to 536</td>
<td>—</td>
<td>456</td>
<td>2+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>ATM</td>
<td>200 to 320</td>
<td>400</td>
<td>280</td>
<td>2+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
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<td>560</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>ATM</td>
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<td>4800</td>
<td>440</td>
<td>2+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>ATM</td>
<td>1000 to 1500</td>
<td>6400</td>
<td>1800</td>
<td>1+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>ATM</td>
<td>520 to 640</td>
<td>1340</td>
<td>640</td>
<td>2+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>CATM</td>
<td>140 to 160</td>
<td>1840</td>
<td>68</td>
<td>1+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>CATM</td>
<td>120 to 480</td>
<td>4000</td>
<td>120</td>
<td>1+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13</td>
<td>CATM</td>
<td>1440 to 1600</td>
<td>240</td>
<td>256</td>
<td>1+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>CFS</td>
<td>0 to 400</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Acute Fissure</td>
<td>—</td>
</tr>
<tr>
<td>15</td>
<td>FS</td>
<td>40 to 80</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Rupture</td>
</tr>
<tr>
<td>16</td>
<td>ATS</td>
<td>27 to 52</td>
<td>—</td>
<td>0</td>
<td>1+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17</td>
<td>ATM</td>
<td>0 to 280</td>
<td>1600</td>
<td>28</td>
<td>2+</td>
<td>—</td>
<td>Rupture</td>
</tr>
</tbody>
</table>

CC, CM, EL, AA, FC/NC, β-CC, and SMC) and hemoglobin [Fig. 1(d)]. The Raman fit coefficients were normalized to sum to unity so that each fit coefficient specifies a percentage contribution to the fit by that respective basis spectrum, as previously described. The hemoglobin contribution to the Raman spectra was zero in all of our samples, as hemoglobin is a relatively weak Raman scatterer at 830-nm excitation. The spectral parameters obtained were then correlated with the presence (or absence) of the morphologic features of vulnerable plaque.
atheromatous plaque. The difference between the measured spectrum and the model fit, the residual, is shown below each spectrum. Lack of significant structure in the residuals demonstrates that the model accounts for the majority of spectroscopic features observed and robustly describes the data.

The following spectroscopic parameters showed the best correlation with morphologic features of vulnerable plaque:

- **DRS hemoglobin concentration** [Hb]/H2O
- **DRS scattering parameter** A
- **IFS parameter** C308/C340, and **Raman parameter** CC+FC/NC.

The DRS spectra are composed of contributions from absorption and scattering. The absorption is primarily due to oxy-hemoglobin, with a small contribution due to β-carotene. The scattering parameter A is related to the total amount of scattering produced by the tissue. The parameters [Hb] and A are given in Figs. 3(a) and 3(b), respectively, for the 17 specimens.

Components MCR1 and MCR2 of IFS at both 308 and 340 nm (Fig. 1) exhibit features similar to the IFS spectra of collagen and elastin, respectively, as reported in previous studies at similar excitation wavelengths.11,17,20 Both IFS MCR1 and the fluorescence spectrum of collagen are blue shifted and more narrow when compared to IFS MCR2 and the fluorescence spectrum of elastin, which has a longer tail. Thus, we conclude that the corresponding fit coefficients C308 and C340 of MCR1 are related to the amount of collagen present within the tissue volume sampled. We define an IFS parameter ρ as the ratio of the fit coefficients of MCR1 at 308- and 340-nm excitation (ρ=C308/C340), which is related to the amount of collagen present and the depth at which it is found. Since the sampling depth at 340-nm excitation is greater than that at 308 nm (see Depth Sensing in Sec. 3.1), C340 provides information about collagen distributed over a greater depth compared to that provided by C308. Values of ρ are shown in Fig. 3(c) for each of the specimens. A value of ρ was undefined for specimen 1, as C340 was smaller than the error in this case (see Error Analysis in Sec. 3.3), implying a potential division by zero.

Previous diagnostic algorithms32 were able to discriminate plaque content using the sum of the Raman fit coefficients for CC and FC/NC. Similarly, we defined a parameter Σ=CC+FC/NC, which is given for each specimen in Fig. 3(d). We use Σ to measure the presence of necrotic core.

### 3.3 Error Analysis

To determine the error in our spectral parameters, we performed a chi-square analysis and applied the error propagation formula.46 Chi-square-analysis is a standard method for calculating the goodness of a fit and the error associated with fitting parameters. Errors reported for ρ and Σ are based on

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**Fig. 2** Representative spectra (dotted black line), fits (solid red line) (color online only), and residuals between the data and fit (lowermost gray line) of the MMS modalities for three specimens with different pathologies: (a) intimal fibroplasia; (b) atherosclerotic plaque (not vulnerable); and (c) vulnerable atheromatous plaque. Note the progression of an increased presence of hemoglobin (420-nm absorption dip in DRS) for the three specimens. The IFS spectra for intimal fibroplasia are broader (characteristic of elastin) when compared to the more narrow spectra for the plaques (characteristic of collagen in the fibrous cap). The Raman spectrum of (c) is noisy due to the decreased signal intensity associated with hemoglobin absorption.
propagating error from the individual components. The error bars in Fig. 3 are generated from this analysis.

4 Discussion

We compare the extracted spectroscopic parameters with the measured morphologic features, and demonstrate how the former can be used to make inferences about the latter, and thus serve to characterize plaque vulnerability. The small size of this sample set is somewhat offset by our ability to directly compare spectroscopic features with those of pathology.

Intraplaque hemorrhage. Intraplaque hemorrhage is an unambiguous marker of plaque vulnerability. Histopathology indicates that specimen 14 is a site of acute intraplaque hemorrhage [Table 1 and Fig. 4(a)], the other specimens are not hemorrhagic. MMS indicates that specimen 14 also exhibits a high value of \[Hb\] (9.5 mg/mL) as assessed by DRS [Fig. 3(a)], whereas all other nonhemorrhagic specimens have relatively low \[Hb\] values (\(\leq3\) mg/mL). This indicates that a high concentration of hemoglobin inside the plaque, as measured by DRS, is consistent with intraplaque hemorrhage. The intraplaque hemorrhage in sample 14 was not detected by Raman spectroscopy (through the hemoglobin basis spectrum) because of the presence of calcification in the tissue. The contribution of CM dominates the Raman spectrum of specimen 14 and marginalizes the contribution from all other components.

Foam cells. The presence of superficial foam cells is associated with plaque ulceration/erosion, and their detection is important in assessing plaque vulnerability.\(^{27}\) We compared the magnitude of the scattering parameter \(A\) for specimens rich in foam cells and those without foam cells. It appeared

![Fig. 3](Fig. 3 Spectral parameters for each of the 17 specimens: (a) hemoglobin concentration (mg/mL) obtained from DRS, used to detect intraplaque hemorrhage; (b) scattering parameter \(A\) (relative units) obtained from DRS, used to detect the presence of foam cells; (c) \(p\) parameter (relative units) extracted from IFS\(_{340}\) and IFS\(_{308}\), used to detect a thin fibrous cap [the value of \(p\) for specimen 1 (\(\ast\)) is undefined as C\(_{140}\) is within the error for this sample]; and (d) the \(\Sigma\) parameter (relative units) extracted from the Raman fit coefficients, used to obtain information about the presence of necrotic core. The error bars indicate one standard deviation.)

![Fig. 4](Fig. 4 Photomicrographs of representative tissue sites: (a) specimen 14, an ulcerated vulnerable plaque with acute intraplaque hemorrhage (inset; arrows indicate red blood cells) and hemoglobin fit contribution of 9.5 (mg/mL) (H and E; 4\(\times\)); (b) specimen 17, a vulnerable plaque with superficial foam cells (arrows) and scattering parameter \(A>2\) (H and E; 20\(\times\)); and (c) specimen 9, a nonvulnerable plaque with deep foam cells (inset) and scattering parameter \(A<2\) (H and E; 4\(\times\)).)
that a threshold value of $A$ could serve as a parameter for assessing the presence of foam cells. To enable an accurate comparison in establishing this threshold, we only considered specimens with a relatively thick fibrous cap. Two representative specimens with superficial foam cells (11 and 12) had a mean value of $A$ of 2.3, whereas two specimens without superficial foam cells (7 and 9) had 1.7 as the mean value of $A$. Based on this, a threshold value of $A=2$ was selected.

Foam cells are present in all ten specimens with $A>2$ [Fig. 3(b)], and they occur at an average depth of 250 $\mu m$ below the surface of these plaques [Table 1 and Fig. 4(b)]. Foam cells are observed in only two of the seven specimens with $A<2$, and these foam cells tend to reside deeper in the plaque at an average depth of 1100 $\mu m$ [Table 1 and Fig. 4(c)].

Given the range of several hundred micron sampling depth for DRS excitation wavelengths, we do not expect to sense such deep-lying foam cells; moreover, these deep-lying foam cells are not clinically relevant to plaque vulnerability. The inverse relationship of $A$ with foam cell depth suggests that foam cells, which contain a high concentration of lipid vacuoles, are strong light scatterers and that their presence near the surface should markedly enhance tissue scattering. Thus, the intensity of scattering, as measured by DRS, can be used to indicate the presence of superficial foam cells.

**Thin fibrous cap.** A thin fibrous cap is the hallmark of a vulnerable plaque. As discussed earlier, the parameter $\rho = C_{308}/C_{340}$ assesses the amount of collagen present in the top layer of tissue. A large value of $\rho$ indicates a thinner layer. Hence, $\rho$ can provide information about the thickness of the fibrous cap. To study this, we performed a two-layer Monte Carlo simulation in which we varied the thickness of a top collagen-rich layer, modeling the collagen that comprises the fibrous cap. We assumed collimated delivery of excitation light in a uniform beam of 200 $\mu m$ diam; fluorescence was generated in the tissue proportional to the fluence of the excitation light within the varied top layer. The fluorescence collected at the surface was compared for 308- and 340-nm excitation wavelengths. As expected, the ratio of the collected fluorescence excited by 308- versus 340-nm light is a monotonically decreasing function of the thickness of the fluorescing layer, asymptotically leveling off. For a 65-$\mu m$-thick layer, the value of the ratio is approximately in the middle of the range. This suggests that the value of $\rho$ in the middle of our experimental range of $\rho$’s can separate specimens with thin fibrous caps from those with thicker caps. We thus chose $\rho=1.8$ as the threshold value.

Indeed, of the diseased specimens (3 through 17), all those with $\rho>1.8$ (14, 15, and 16) have a minimum fibrous cap thickness well below 65 $\mu m$ (Table 1), whereas the remaining diseased specimens have thicker fibrous caps. In specimen 17, the small value of $p$ is not consistent with the histologically observed thin cap and associated plaque rupture. However, pathology also indicates that some portions of the fibrous cap are thick (range 0 to 280 $\mu m$), which can explain the small value of $\rho$. With this caveat, the parameter $\rho$, an indicator of the amount of collagen present in the topmost tissue layer, can be used to estimate fibrous cap thickness.

**Necrotic core.** When a large necrotic core is exposed to luminal blood flow following rupture of the fibrous cap, the coagulation cascade is triggered, resulting in thrombosis. We used the diagnostic parameter $\Sigma$, defined as the sum of the Raman fit coefficients for cholesterol crystals (CC) and foam cells/necrotic core (FC/NC), to assess presence of a necrotic core [Fig. 3(d)]. 11 of 14 specimens with $\Sigma>0.4$ had either a necrotic core or were rich ($\geq 2$+) in foam cells; only 1 of 3 specimens with $\Sigma<0.4$ had a necrotic core. Specimen 8 has a large necrotic core but $\Sigma<0.4$; however, this plaque has a thick fibrous cap (>440 $\mu m$), so the penetration depth of 830-nm light may not sufficiently sample the necrotic core in this case, and such a deep necrotic core is not associated with plaque vulnerability. In contrast, specimens 14 and 15 have high values of $\Sigma$ but lack foam cells and necrotic core. It should be noted that these specimens are fibrotic-sclerotic plaques, demonstrating a well-developed fibrous cap but lacking a necrotic core and cholesterol crystals. Some investigators regard these as end stage plaques, in which the necrotic core may have been resorbed. Further studies will be needed to fully understand the significance of $\Sigma$, particularly in fibrotic-sclerotic plaques, but the results from this small sample set indicate the ability of $\Sigma$ to detect necrotic core.

**Identifying vulnerable plaques.** Fibrous cap thickness is a key parameter and, taken together with the presence of necrotic core or foam cells, can serve as an indicator of plaque vulnerability. In addition, the presence of intraplaque hemorrhage by itself indicates a vulnerable plaque. For our sample set, having $\rho>1.8$ (thin fibrous cap) taken together with $A>2$ (foam cells) and/or $\Sigma>0.4$ (necrotic core) correctly identifies three of the four vulnerable plaques (14, 15, and 16). A value of $[\text{Hb}]>5$ (intraplaque hemorrhage) by itself also correctly identifies sample 14 as vulnerable. As discussed earlier, sample 17, which exhibits rupture and is vulnerable, would be missed by this identification scheme. The ability to localize small regions of thinning of the fibrous cap is under investigation. All nonatherosclerotic samples (1 and 2) and nonvulnerable plaques (3 through 13) are correctly identified as such.

### 5 Conclusion

This pilot study demonstrates the feasibility of using a combination of diffuse reflectance, intrinsic fluorescence, and Raman spectroscopy to detect morphological markers of vulnerable plaque. The approach is based on the correlation of parameters obtained from different spectroscopic modalities with pathology features and follows from a simple physical picture of the way light probes biological tissue. These spectroscopic parameters allow depth sensing and provide information about intraplaque hemorrhage, superficial foam cells, a thin fibrous cap, and large necrotic core, morphologic features that are associated with plaque vulnerability. In view of the small size of the sample set, these results should be considered preliminary. However, given these promising results, a larger study is warranted to establish the full potential of MMS by assessing spectral variability across many different pathologies and patients. The larger study will permit regression and statistics to be employed to validate these initial conclusions. Advances in instrumentation and the development of small-diameter side-viewing probes will enable percutaneous access to vessels and potentially provide clinically relevant information about plaque morphology. Thus, this method has the potential to guide diagnosis and treatment of atherosclerotic cardiovascular disease.
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