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Biochemical characterization of atherosclerotic plaques by endogenous multispectral fluorescence lifetime imaging microscopy

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Abstract

OBJECTIVE—To investigate the potential of endogenous multispectral fluorescence lifetime imaging microscopy (FLIM) for biochemical characterization of human coronary atherosclerotic plaques.

METHODS—Endogenous multispectral FLIM imaging was performed on the lumen of 58 segments of postmortem human coronary artery. The fluorescence was separated into three emission bands targeting the three main arterial endogenous fluorophores (390±20 nm for collagen, 452±22.5 nm for elastin, and 550±20 for lipids). The fluorescence normalized intensity and average lifetime from each emission band was used to classify each pixel of an image as either “High-Collagen”, “High-Lipids” or “Low-Collagen/Lipids” via multiclass Fisher’s linear discriminant analysis.

RESULTS—Classification of plaques as either “High-Collagen”, “High-Lipids” or “Low-Collagen/Lipids” based on the endogenous multispectral FLIM was achieved with a sensitivity/specificity of 96/98%, 89/99%, and 99/99%, respectively, where histopathology served as the gold standard.

CONCLUSION—The endogenous multispectral FLIM approach we have taken, which can readily be adapted for *in vivo* intravascular catheter based imaging, is capable of reliably identifying plaques with high content of either collagen or lipids.

Keywords

atherosclerosis; intravascular imaging; optical imaging; biochemical imaging; fluorescence imaging; fluorescence lifetime

1. INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in the United States (1). It is characterized as a systemic, progressive disease in which the arterial wall thickens through a

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process of inflammation (2), oxidative stress, and dyslipidemia (3). This process leads to plaque formation and flow restriction in the vessel lumen. These arterial plaques may also rupture leading to sudden thrombosis and occlusion of the vessel, and ultimately to myocardial infarction, stroke, or limb injury (4).

The development of systemic or localized therapies for atherosclerosis must rely upon a detailed understanding of plaque biochemical and morphological development. In vivo imaging of morphological, biochemical and functional/molecular changes accompanying plaque formation and response to treatment (5, 6) is important for building out understanding of plaque formation and will likely play a significant role in clinical diagnosis, monitoring and treatment of the disease. Important morphological biomarkers for plaque discrimination include plaque area/thickness, fibrous cap thickness, and percentage area of necrotic core and calcification (4). Important biochemical markers include the relative content of collagen and lipids within plaques (4). In vivo monitoring of molecular biomarkers related to endothelial activation, macrophage recruitment and activation, apoptosis among other mechanisms, is also crucial for improving our understanding of plaque development (7).

Noninvasive cardiovascular imaging modalities enable quantification of morphological biomarkers but provide little or no information about the biochemical composition of plaques. Recent advances in exogenous molecular probe development are facilitating noninvasive molecular and functional imaging of atherosclerotic plaques (8); however, the limited spatial resolution of noninvasive imaging modalities (e.g. MRI, CT, PET) and the intrinsic complications inherent to exogenous contrast agents dampens their utility (6, 8).

Intravascular imaging modalities, in particular intravascular ultrasound (IVUS) and optical coherence tomography (OCT), provide superior spatial resolution and improved quantification of coronary morphological biomarkers relative to noninvasive modalities (9). Other intravascular imaging technologies have also been proposed for functional and biochemical imaging. Spectroscopic IVUS can distinguish fibrotic from fibro-lipid plaques, but its sensitivity and specificity has recently been questioned (10). Statistical analysis of OCT images has the potential to detect superficial foam cell infiltration, but it has not been fully validated (11). Thermography has the potential to detect inflammation, but its specificity and spatial resolution is limited (12). Magnetic resonance spectroscopy has the potential to detect lipids, but it has limited spatial resolution and has not been fully validated (13). Near-infrared spectroscopy has the potential to detect lipids, but its ability to detect other biochemical markers has not yet been demonstrated (14). Raman spectroscopy has the potential for detailed biochemical characterization, but its intrinsic low signal has limited its intravascular use (15).

Autofluorescence spectroscopy has the potential to quantify the elastin, collagen and lipid contents in plaques (16). Although steady-state fluorescence spectroscopy is more simple to implement, it is less robust and less sensitive than time-resolved spectroscopy (17). Time-resolved autofluorescence spectroscopy has the potential to distinguish between intimal-thickening, fibrotic and fibro-lipid plaques (18); however, point-spectroscopy has limited spatial resolution and slow acquisition speed. Both spatial resolution and acquisition speed can be significantly improved by implementing time-resolved fluorescence spectroscopy in imaging-mode, most commonly known as fluorescence lifetime imaging (FLIM) (19). Two recent studies have shown some of the potential of FLIM for biochemical imaging of atherosclerotic plaques; however, its ability to distinguish intimal-thickening, fibrotic and fibro-lipid plaques (as in point-spectroscopy) has not yet been established (20, 21).

Here, we report the first demonstration of 1) luminal multispectral FLIM imaging of coronary arteries at practical spatial resolution and acquisition speed, and 2) FLIM based biochemical imaging of coronary arteries with sufficient sensitivity and specificity to distinguish plaques with high content of either collagen or lipids.

2. MATERIALS AND METHODS

2.1 Tissue preparation and imaging

Human coronary artery segments were obtained from 8 autopsy cases within 48 hours of the time of death, according to a protocol approved by the Texas A&M University Institutional Review Board. A total of 58 arterial segments were longitudinally opened and imaged from the lumen side. The imaged field of view (FOV) had an area of $2 \times 2 \text{ mm}^2$. Immediately after imaging, each segment was ink marked (for correlation with histopathology), fixed in 10% formalin, and sent for histopathology analysis.

2.2 Histopathology analysis

Each imaged plaque segment was consecutively sectioned every 500 μm . The sections were stained with Movat pentachrome and evaluated by a cardiovascular pathologist (F.C.). Based on the histopathological evaluation, each section was classified as: intimal thickening (IT), pathological intimal thickening (PIT), fibroatheroma (FA), thin-cap fibroatheroma (TCFA), calcified plaque (CA), and/or plaque with significant foam-cell infiltration. A total of 23 segments showing similar and uniform histopathological characteristics in all consecutive sections were selected and grouped as “High-Collagen” (including fibrotic FA and PIT), “High-Lipids” (including TCFA and PIT with significant foam-cell infiltration or FC-PIT), and “Low-Collagen/Lipids” (including IT and CA).

2.3 FLIM instrumentation

The coronary segments were imaged using a recently developed high-speed multispectral FLIM imaging system shown schematically in Fig. 1A (22). Briefly, the scanning FLIM system was implemented following a direct pulse-recording scheme, in which the pixel rate could be equal to the laser repetition rate. A frequency tripled Q-switched Nd:YAG laser was used as the excitation source (355 nm, 30 kHz max. repetition rate, 1 ns pulse FWHM). The fluorescence emission was separated into three bands using a set of dichroic mirrors and filters (390 $\pm$ 20 nm for collagen, 452 $\pm$ 22.5 nm for elastin, and 550 $\pm$ 20 nm for lipids). Each band was launched into a fiber with different lengths (1 m, 10 m and 19 m, respectively) chosen to provide $\sim$ 45 ns intervals between each emission band decay. The three consecutive decays were detected with a MCP-PMT (rise time: 150 ps) and sampled with a high bandwidth digitizer (1.5 GHz, 4 GS/s). The system lateral resolution was measured to be 100 μm . Each multispectral FLIM image (FOV: $2 \times 2 \text{ mm}^2$ at 60×60 pixels) was acquired in $\sim$ 7 s.

2.4 FLIM data processing

The tissue autofluorescence emission has been quantified in terms of the relative fluorescence intensity and the average lifetime at each pixel of the image. Two-dimensional maps for each emission band were thus estimated from the multispectral FLIM data as follows. First, the intrinsic fluorescence decays at the three emission bands for each pixel of the image were estimated using a computationally efficient time-deconvolution algorithm (23) (Fig. 1B). The fluorescence intensity maps for each spectral channel (I_1 , I_2 and I_3) were then calculated by time integration of the intrinsic fluorescence decays, and normalized (pixel-by-pixel) by dividing the intensity of each channel by the sum of intensities of the three channels ($I_{n1} = I_1/(I_1 + I_2 + I_3)$, $I_{n2} = I_2/(I_1 + I_2 + I_3)$ and $I_{n3} = I_3/(I_1 + I_2 + I_3)$). The

average lifetime maps (τ_1 , τ_2 and τ_3) for each channel were calculated from the intrinsic fluorescence decay using its general mathematical definition (19). A total of 6 fluorescence feature maps (I_{n1} , I_{n2} , I_{n3} , τ_1 , τ_2 and τ_3) were thus computed for each imaged arterial segment (Fig 1C).

2.5 Statistical analysis and classification

One goal of this study was to classify each image pixel using the multispectral FLIM features into one of the three histopathological groups defined above. To design a proper statistical classifier following a supervised approach, a training set is required, in which each pixel is labeled to one of the groups. This requirement is extremely hard to meet, since perfect co-registration between histopathology sections and FLIM maps is virtually impossible. To mitigate this problem, we restricted the training set to the pixels of 23 plaque segments showing uniform histopathological characteristics as described above.

The capacity of the six FLIM features (I_{n1} , I_{n2} , I_{n3} , τ_1 , τ_2 and τ_3) to distinguish between the three histopathological classes was assessed via the following statistical analysis. A 15×15 pixel block ($500 \times 500 \mu\text{m}^2$) in each FLIM image was counted as a single data point in order to reduce the effect of non-normality of our data. The nonparametric Mann-Whitney U-test was performed between every pair of classes, wherein $P < 0.01$ was considered significant. For statistical classification, multiclass Fisher's linear discriminant analysis (M-FLDA) was applied with the six multispectral FLIM parameters as the feature vector for each pixel (24). A 10-fold cross-validation approach was adopted (Fig. 1D) in order to assess the classification performance. Finally, a biochemical map was generated after classifying each pixel of an image to one of the three histopathological groups (Fig. 1E).

3. RESULTS

3.1 Histopathology Analysis

Each of the 23 imaged arterial segments showing approximately uniform histopathological characteristics were grouped as either "High-Collagen" (HC, $n = 4$, $4 \times 60 \times 60 = 14400$ pixels), "High-Lipids" (HL, $n = 4$, $4 \times 60 \times 60 = 14400$ pixels) or "Low-Collagen/Lipids" (LCL, $n = 15$, $15 \times 60 \times 60 = 54000$ pixels). This subgroup served as the training set for the statistical classification design and cross-validation. The remaining 35 imaged arterial segments showed a combination of high collagen, high-lipid and/or low collagen/lipid content and were used to further validate the classifier (Fig. 5).

3.2 Spectral Characteristics of Training Set

Three sets of multispectral FLIM maps corresponding to HC, HL and LCL plaques from the training set are shown in Fig. 2. Each set consists of the normalized fluorescence intensities and the average lifetime maps for each of the three spectral bands or channels. Representative histopathological sections are also shown. For the HC plaque (Fig 2A), the fluorescence intensity was stronger in channel 1 (0.57 ± 0.02) than in channels 2 (0.28 ± 0.01) and 3 (0.15 ± 0.02), and the average lifetime varied between ~ 5 -6 ns. In the histopathological sections (PIT and FA) light blue indicates collagen. For the HL plaque (Fig 2B), the fluorescence intensity, as compared to the HC plaque, was lower in channel 1 (0.42 ± 0.05), similar in channel 2 (0.30 ± 0.02) and higher in channel 3 (0.28 ± 0.04). The averaged lifetime gradually increased from 5.3 ± 0.2 ns in channel 1 to 8.3 ± 1.1 ns in channel 3. In the histopathological sections (TCFA and PIT with significant foam-cell infiltration) empty areas indicate lipids. For the LCL plaque (Fig 2C), the fluorescence intensity was similar in channels 1 and 2 (0.48 ± 0.02 and 0.38 ± 0.01), while the averaged lifetimes were similar in all channels (~ 5 ns). This fluorescence pattern was characteristic of both IT and calcified plaques.

3.3 Statistical analysis and classification

Results of the statistical analysis are summarized in Figs. 3A and 3C (the bar plots denote the median value and the error bars denote the first and second quartile). For comparison, the normalized emission spectrum and average lifetime (as a function of emission wavelengths) from (purified) collagen, elastin and LDL (Sigma Aldrich) measured with a calibrated time-resolved spectrometer are also shown in Figs. 3B and 3D. Overall, the fluorescence normalized intensity and average lifetime median values of the HC and HL plaques reflected the fluorescence characteristics of collagen and LDL, respectively. In particular, HC plaques showed the characteristic strong emission at 390 nm (channel 1) of collagen, while HL plaques showed the characteristic long lifetime at 550 nm (channel 3) of LDL. The horizontal brackets above the bars indicate a significant difference among pairs ($P < 0.01$).

The six FLIM features were also used to design M-FLDA classifier. The scatter plot in Fig. 4A shows the projection of the data points onto the 2-D linear discriminant score space. Data belonging to different classes are denoted by color. Good performance of M-FLDA for class separation is indicated by the weak overlap of the classes in the discriminant score space in Fig. 4A. The pixel classification performance assessed by 10-fold cross-validation is summarized in the confusion matrix shown in Fig. 4B. Sensitivity (96% (HC), 89% (HL) and 99% (LCL)) and specificity (98% (HC), 99% (HL) and 99% (LCL)) were measured, and the overall classification was estimated to be 96.79 %.

3.4 Validation in plaques with heterogeneous histopathology

The M-FLDA classifier developed above was also used to classify pixels of plaques showing heterogeneous histopathology. Four examples of such validation are shown in Fig. 5. A plaque showing regions of LCL (top) and HC (bottom) is shown in Fig. 5-A; a histology section corresponding to the middle of the plaque confirmed that the LCL region was IT and the HC region was fibrotic PIT. A plaque showing regions of HL (left) and HC (right) is shown in Fig. 5-B; a histology section corresponding to the left half confirmed that the HL region was PIT highly infiltrated by foam cells, and a histology section corresponding to the right half confirmed that the HC region was fibrotic PIT. A plaque showing regions of HL (top-left) and LCL (bottom-right) is shown in Fig. 5-C; a histology section corresponding to the middle of the plaque confirmed that the HL region was PIT highly infiltrated by foam cells, and the LCL region was IT. Finally, a plaque showing regions of HC, HL and LCL is shown in Fig. 5-d; a histology section corresponding to the middle of the plaque confirmed that the HC region was fibrotic PIT, the HL region showed foam cell infiltration, and the LCL region was IT.

4. DISCUSSION

The primary endogenous fluorophores in normal and atherosclerotic arteries are elastin, collagen and lipids (16). Thus, it is expected that the artery autofluorescence will provide means for identifying the plaque content of these molecules. This has already been demonstrated for time-resolved fluorescence spectroscopy (18); however, due to its intrinsic limited spatial resolution and acquisition speed, the translation of point-spectroscopy to catheter based intravascular *in vivo* applications will be difficult. FLIM can overcome these two limitations, but its potential for biochemical imaging of atherosclerotic plaques has not yet been fully demonstrated, in particular for detecting lipid content (20, 21). The results reported here, on the other hand, indicate that the multispectral time-resolved autofluorescence signal of plaques (at three emission bands specifically targeting elastin, collagen and lipids) enables the discrimination of high and low collagen and lipid content. Furthermore the classification using the derived M-FLDA classifier based on these signals

has shown for the first time that multispectral FLIM can provide accurate biochemical imaging of the lumen of coronary arteries.

The estimation of both lipid and collagen content is important for characterizing plaques as well as estimating the propensity for developing into a high-risk “vulnerable plaque” (4). Early stage inflammation is characterized by the accumulation of foam cells. Intermediate stages are characterized by both foam cell accumulation as well as an increase in collagen content. Late stage vulnerable plaques are characterized by a lipid filled lesion with a necrotic core. In all three stages noted the relative content of collagen and lipids could help to stage the plaque development. A number of different imaging modalities have thus been proposed for the identification of lipids and/or collagen in plaques (25). Spectroscopic IVUS (in particular, the “Virtual Histology” technology developed by Volcano Corp.) has shown some potential for detecting fibrotic and fibro-lipid plaques, but the sensitivity and specificity of this technology has recently been questioned in several studies (10, 26, 27). NIR spectroscopy (in particular, the intravascular platform developed by InfraRex Inc.) provides accurate identification of lipid-rich plaques, but its capability for assessing the collagen content has not been fully demonstrated (28). Our results, on the other hand, demonstrate that multispectral FLIM can assess both the collagen and lipid content of coronary atherosclerotic plaques. We are currently exploring novel chemometric methods for multispectral FLIM image processing that will potentially provide direct quantification of the relative content of collagen and lipids within plaques.

Although our results clearly demonstrate the potential of FLIM for biochemical characterization of coronary atherosclerotic plaques, this study was performed *ex vivo* and it will be imperative to validate these results in intravascular *in vivo* conditions. Before that, however, an intravascular FLIM platform has to be first developed. The current paradigm for catheter based *in vivo* intravascular optical imaging is a rotating endoscope that is pulled along the artery lumen while a saline flush is administered to clear the blood, creating a spiral image. Motion artifact and the risk of ischemia from the flush place a practical lower limit on pullback velocity of several mm/s. FLIM imaging of atherosclerotic arteries at a sufficient pixel rate to achieve reasonable sampling along the lumen under these conditions has not yet been demonstrated. Phipps et al. reported acquisition times of 150 s per FLIM image and per emission band (circular FOV of ~4 mm in diameters) on postmortem human aortic arteries (21), and Thomas et al. reported acquisition times of 60-120 s per FLIM image and per emission band (circular FOV of ~2 mm in diameters) on postmortem human coronary arteries (20). Our reported imaging speed of ~7 s per multispectral FLIM image (514 pixels/s) is significantly faster than those reported, but still ~100 times (assuming 3 mm diameter lumen) slower than needed for intravascular imaging. However, the approach we have taken should allow pixel rates equal to the laser repetition rate as long as the pulse energy is sufficient for obtaining adequate levels of fluorescence signal (22). In our current FLIM instrumentation, the maximum available pulse energy was 240 nJ at 10 kHz, and a total of 20 averages were needed to generate sufficient signal to noise. If we make the reasonable assumption that the noise is uncorrelated between temporal traces and the improvement in the signal to noise ratio due to averaging is $n^{1/2}$ (n, number of samples averaged) then a single pulse of ~1.1 μ J would be sufficient for single pulse acquisition. If we assume uniform 50 μ m sampling of the artery lumen then a laser repetition rate of 38 kHz would be necessary to image the artery with a pullback velocity of 10 mm/s. There are already a number of laser systems that are capable of delivering >10 μ J per pulse at repetition rates exceeding 100 kHz. Hence a fairly straightforward upgrade of the current system should provide the performance necessary for *in vivo* intravascular FLIM. We are currently evaluating this catheter design in combination with our developed high-speed multispectral FLIM implementation, and believe that our proposed design will allow intravascular FLIM with adequate imaging speed for practical in-vivo applications.

In addition to fast acquisition, practical intravascular imaging also requires fast image processing for immediate feedback to the clinician/researcher during the procedure. As noted above, accurate estimation of the fluorescence lifetime values from the FLIM data requires performing time-deconvolution of the instrument response from the pixel fluorescence decays. Time-deconvolution methods are in general computationally expensive, since they involved applying non-linear least-square optimization. We have proposed a novel time-deconvolution method in which linear least-square estimation can be used instead of non-linear least-square optimization, resulting on at least two orders of magnitude increase in computation speed compared to conventional methods (23). Fisher's linear discriminant analysis is one of the simplest statistical classification methods, and our classification results indicate that LDA provides sufficient accuracy for identifying collagen-rich and lipid-rich plaques. Once the LDA classifier is trained, classification of new pixels involves only simple linear functions evaluations. Thus, we expect that the adoption of both our new time-deconvolution method for lifetime estimation and LDA for statistical classification will facilitate real-time and accurate intravascular biochemical imaging based on multispectral FLIM.

5. CONCLUSION

In conclusion, we have demonstrated the feasibility of 1) luminal multispectral FLIM imaging of coronary arteries at practical spatial resolution and acquisition speed, and 2) FLIM based biochemical imaging of atherosclerotic coronary plaques, in particular, the identification of collagen-rich and lipid-rich plaques. When coupled to another imaging modality capable of high-resolution plaque morphological characterization, this technology will facilitate in-vivo intravascular identification of most histopathological types of atherosclerotic plaques.

While FLIM enables biochemical characterization of coronary plaques, little information about the plaque morphology can be inferred from the autofluorescence signal. For instance, our results demonstrated that FLIM can identify TCFA and plaques with significant foam cell infiltration as lipid-rich, but it cannot distinguish between them. Similarly, FLIM can identify pathological intimal thickening and fibroatheromas as collagen-rich, but it cannot distinguish among them. The integration of FLIM with an imaging modality capable of characterizing plaque morphology, however, should result in a powerful tool for in vivo intravascular identification of most histopathological types of atherosclerotic plaques. Recently, Sun et al. performed coregistered FLIM, ultrasonic backscattering microscopy (UBM) and photoacoustic (PA) imaging of two fresh postmortem human carotid atherosclerotic plaques (29). Their preliminary results suggested that FLIM and PA could provide biochemical characterization of the surface and inner volume of the plaque, respectively, while UBM can provide plaque morphological characterization. However, it was not clearly demonstrated how the individual imaging modalities would complement each other to provide a more comprehensive plaque characterization. Moreover, the complexity associated with integrating ultrasonic and optical imaging into a single catheter might limit the feasibility of this multimodality approach. Other multimodal approaches have been proposed based on the integration of IVUS with either OCT, NIR spectroscopy or fluorescence spectroscopy (8). Nevertheless, we believe that the integration of two optical modalities, such as OCT and FLIM, is more practical, since it will result in intrinsic registration of the different image modalities and more simple catheter and imaging platform designs. We have thus recently developed a multimodal optical imaging system that integrates multispectral FLIM and OCT and are currently evaluating its potential for simultaneous high-resolution biochemical and morphological imaging of atherosclerosis (30).

An intravascular imaging modality capable of in-vivo biochemical, functional and morphological plaque characterization, as the one we are currently developing, could initially be used as a research tool for studying the development of atherosclerotic plaques in animal models. As new treatments move to preclinical trials the technology would be used to evaluate the efficacy of the treatments. The technology could ultimately be used to image in humans for clinical trials or as a clinical tool to evaluate the progress and/or remission of the disease. Human in vivo imaging may be particularly appropriate for monitoring and deploying localized therapies. Moreover, such a technology could also be used to screen for particular plaque types during diagnostic catheterization procedures, and those plaques deemed “vulnerable” could then be treated. Similarly, a screening protocol could be established for at-risk patients that combined a lower resolution noninvasive imaging technology like CT or MRI with follow-up on suspicious lesions with intravascular imaging.

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Figure 1. Summary of the experimental methods. A) Schematic diagram of FLIM instrumentation, B) Multispectral FLIM data acquisition, C) Multispectral FLIM data processing, D) Statistical analysis and classification, and E) Generation of biochemical map.

Figure 2.

Sample multispectral FLIM maps (left panels) and representative histopathology (right panels) corresponding to A) High-Collagen (HC), B) High-Lipids (HL), and C) Low-Collagen/Lipids (LCL) plaques selected from the training set. (IT: intimal thickening, PIT: pathological IT, FA: fibroatheroma, FC-FA: FA with foam-cell infiltration, TCFA: thin-cap FA, CA: calcification).

Figure 3.

Results of the statistical analysis on the multispectral FLIM features. The values of the normalized fluorescence intensity at the three spectral channels from the HC, HL and LCL groups (A) resemble the fluorescence emission spectra from collagen, LDL and elastin, respectively (B). Similarly, the values of the fluorescence lifetime from the HC, HL and LCL groups (C) resemble the fluorescence lifetime from collagen, LDL and elastin, respectively (D).

Figure 4. Results of the statistical M-FLDA classification based on the six multispectral FLIM features. (A) 2-D Linear discriminant score space, and (B) Classification performance table assessed by 10-fold cross-validation.

Figure 5. Sample biochemical maps of plaques showing heterogeneous histopathology. A) Plaque showing regions of LCL and HL. B) Plaque showing regions of HL and HC. C) Plaque showing regions of HL and LCL. D) Plaque showing regions of LCL, HC, and HL.