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Using multimodal femtosecond CARS imaging to determine plaque burden in luminal atherosclerosis

Alex C-T. Ko¹, Leila B. Mostaço-Guidolin¹, Andrew Ridsdale², Adrian F. Pegoraro², Michael S.D. Smith¹, Aaron Slepko², Mark D. Hewko¹, Elicia K. Kohlenberg¹, Bernie Schattka¹, Albert Stolow², Michael G. Sowa¹

¹ Institute for Biodiagnostics, National Research Council Canada, Winnipeg, Canada

² Steacie Institute for Molecular Sciences, National Research Council Canada, Ottawa, Canada

Abstract

Luminal atherosclerosis imaging was demonstrated by multimodal femtosecond CARS microscopy (MM-CARS). Using a myocardial infarction-prone rabbit model of atherosclerosis, this study demonstrated the utility of multimodal CARS imaging in determining atherosclerotic plaque burden through two types of image analysis procedures. Firstly, multimodal CARS images were evaluated using a signal-intensity parameter based on intensity changes derived from the multi-channel data (e.g. TPEF, SHG and CARS) to classify plaque burden within the vessel. Secondly, the SHG images that mainly correspond to collagen fibrils were evaluated using a texture analysis model based on the first-order statistical (FOS) parameters of the image histogram. Correlation between FOS parameters of collagen images with atherosclerosis plaque burden was established. A preliminary study of using spectroscopic CARS in identifying the different lipid components within the plaque was also discussed.

Keywords: multimodal, femtosecond CARS, atherosclerosis, luminal imaging, plaque burden, texture analysis, first-order statistics, myocardial infarction-prone rabbit

1. Introduction

In North America, atherosclerotic coronary artery disease is the leading cause of death in both men and women. It is estimated that more than 7 million North Americans will experience atherosclerosis-related symptoms during their lifetime. Current clinical imaging modalities to visualize vulnerability within the atherosclerotic plaque include angiography, intravascular ultrasound (IVUS), multi-detector CT (MDCT) and more recently cardiac MRI, intravascular OCT and PET. Each of these techniques may provide some unique insight in determining plaque burden and associated vulnerability however none can provide high resolution compositional information within lesions and thereby help in understanding plaque development and predicting the risk of plaque rupture.

Recently arterial/atherosclerosis imaging was demonstrated by multimodal CARS-based microscopy. [1-4] These studies showed the power of label-free CARS imaging in differentiating plaques from healthy arterial surfaces as well as the potential of multimodal CARS to interrogate atherosclerotic plaque burden. In this study, multimodal femtosecond CARS was used for ex vivo epi-luminal imaging of extra-cellular proteins (elastic lamina

and collagen fibrils) and lipid-rich structures (foam cells, extracellular lipid-deposit) within intact aortic tissue obtained from myocardial infarction-prone rabbits. Based on the extracellular changes observed in the multimodal CARS images of arterial tissue harvested from rabbits at different ages, we developed a signal intensity-based numerical parameter for differentiating atherosclerotic plaque burden. This parameter consists of contributions from the individual CARS, SHG and TPEF signal intensities and intensity differences between these imaging channels. An age-dependent correlation of the proposed parameter in quantifying atherosclerotic plaque burden was observed. Histology of the arterial sections were compared with label-free multimodal CARS imaging. Texture analysis of the SHG images from the luminal surface of the artery was used to examine the structural organization of collagen fibrils in relation to plaque burden.

2. Materials and Imaging Methods

2.1 Arterial tissue samples and sample preparation

All animal experiments conformed to the guidelines set out by the Canadian Council on Animal Care were approved by the local Animal Care Committee of the National Research Council of Canada. This study uses the myocardial infarction prone Watanabe heritable hyperlipidemic (WHHL-MI) rabbit model [5] which develops atherosclerotic plaques without the requirement of a diet modification due to a hereditary defect in LDL processing. A total of eight rabbits between 4 and 24 months-old were sacrificed.

The rabbit aorta was dissected from the ascending aorta to the external iliac artery and then rinsed in heparinized saline. The exterior of the aorta was delicately cleaned of connective tissue prior to being subdivided into ~20-30 mm sections resulting in 5-7 pieces. Additionally, some short segments were set aside for histology at this point. Each section was cut open longitudinally exposing the luminal surface. The samples were placed in petri dishes with the luminal surface facing up on a moist surface and hydration was maintained throughout the measurements by applying phosphate buffered saline (PBS) solution periodically. Digital photos of the luminal surface were acquired and regions of interest were identified prior to measurements.

2.2 Multimodal femtosecond CARS imaging

Samples were investigated using an in-house built multimodal femtosecond CARS microscope which is capable of acquiring co-localized TPEF, SHG and CARS images of artery samples simultaneously, either in the forward- or in the epi-direction. A schematic diagram of this laser-scanning imaging microscope is illustrated in Figure 1. Technical details of this CARS imaging system can be found elsewhere [1,3]. In summary, the laser source is a Ti:Sapphire oscillator. The output fs pulses from the Ti:sapphire oscillator is split into the reflected pulses and the transmitted pulses using a beam splitter. The reflected pulse is transmitted through various optical components including a delay line stage and reaches the sample through an objective lens. The transmitted fs pulse is focused into a PCF (FemtoWhite CARS, NKT Photonics) to generate a broadband emission. The near-IR portion of the broadband emission is used as the Stokes pulses for generating the CARS

signal. The pump and Stokes pulses are combined and sent into the microscope co-linearly. Three non-descanned PMT detectors are mounted on the microscope for simultaneous detection of TPEF, SHG and CARS signals through arrays of dichroic and colour filters. Typically 25mW of pump and 8mW of Stokes (measured after the objective) were used for imaging. We used ScanImage (ver. 3.5) software for sample stage control and image acquisition. Data collection time per image was approximately 21 μ s per pixel for an average of 4 scans. Post image processing and image viewing were carried out in ImageJ ver 1.42b and Matlab.

For hyperspectral CARS measurements, data presented in this manuscript was obtained on another similar system which comprises of a modified Olympus Fluoview 300 (FV300) scanning microscope. In order to achieve desired spectral resolution of the CARS signal, both the pump and the Stokes pulses are optimally chirped using highly dispersive glass blocks based on a methodology presented previously. [6]

Figure 1. Schematic of the in-house built multimodal femtosecond CARS microscope. SF6 is the highly dispersive glass used to chirp the fs pulses for improving CARS spectral resolution. (see Ref.6 for detail description of methodology)

3. Results and Discussion

3.1 Differentiation between healthy and atherosclerotic lumen using MM-CARS imaging

Representative multimodal CARS images of healthy luminal surfaces and atherosclerotic lesions of aorta samples are shown in Fig. 2. On the healthy lumen, membrane-like structures giving rise to strong TPEF signal are evident without much SHG and CARS signal being observed. (2A and 2B) The structures observed in the images of healthy segments of

aorta correspond well to the thin internal elastic lamina (IEL) located within tunica intima. When imaging deeper into the tissue, a strong TPEF signal is observed from another fibre-structure (possibly the elastic fibers in the tunica media). This fibre-structure shows a very different morphology from those of IEL at a shallower layer of the aorta (compare Fig. 2E and 2F).

Representative multimodal CARS Images acquired from an early atherosclerotic lesion and an advanced lesion are illustrated in Fig. 2C and 2D, respectively. In these images, the IEL structure shown in the images of healthy lumen is no longer visible. Instead, the images show the presence of collagen fibrils, accumulated lipid-rich structures and strong fluorescent macromolecules. It is noted that the advanced lesion shows much denser network of collagen fibrils and higher concentration of fluorescent material than does the early lesion.

Figure 2. Multimodal epi-CARS images of (A) healthy arterial lumen of a 4 month-old rabbit (B) healthy arterial lumen of a 10 month-old rabbit (C) early atherosclerotic lesion (D) advanced atherosclerotic lesion collected using 20x air lens. (E) and (F) compare the images of healthy lumen acquired at shallower and deeper depth of the artery sample using a 40x WI. Green:TPEF, Blue:SHG, Red:CARS

3.2 Interrogating atherosclerotic plaque burden

In an effort to quantify the extracellular features shown in the MM-CARS images collected on the arterial lumen surfaces with plaque burden, two approaches were taken in analyzing the images-see Fig. 3. First we developed an intensity based summary parameter derived from multimodal CARS images to correlate with atherosclerotic plaque burden in the WHHLM rabbit model. This method used a phenomenological scoring method similar to that reported by Strupler et al [7]. While Strupler's model worked well for collagen fibril scoring in fibrotic kidney tissue sections, it appeared to be inadequate in accurately quantifying extracellular changes in complex system such as atherosclerosis lesions. A new

parameter, optical index for plaque burden (OIPB) was developed based on our prior observations reported earlier [1], and was defined by the equation,

$$OIPB = SS_{SHG} + SS_{CARS} + d(SS_{SHG}, SS_{TPEF}) + d(SS_{CARS}, SS_{TPEF}) + d(SS_{CARS}, SS_{SHG})$$

where SS_x is a signal significant score calculated for respective channel (TPEF, SHG or CARS) of the multimodal CARS image, while the terms $d(SS_x, SS_y)$ are the difference between two SS scores (SHG, CARS and TPEF), $d(SS_x, SS_y) = SS_x - SS_y$. Note that SS_{TPEF} score was not included in the calculation of OIPB indices because it provides no additional contrast in discriminating between atherosclerosis lesions from healthy vessel. The methodology used to calculate SS_x scores can be found elsewhere. [1]

Figure 3. Image analysis strategy for correlating extracellular features shown in the multimodal CARS images of arterial lumen surfaces with atherosclerosis plaque burden.

In order to illustrate the utility of the OIPB index Fig. 4 plots the mean OIPB index obtained from images of the

lumen surface from healthy (red circles) and atherosclerotic (black squares) regions for each rabbit presented in the order of the age of the rabbit. Two observations can be derived from this plot. First, $OIPB_{\text{plaque}}$ is significantly higher than $OIPB_{\text{healthy}}$ in all rabbits. Secondly, OIPB index derived from regions of plaque increases with an asymptotic fashion with the age of the WHHLM rabbit, representing the overall plaque burden in the aorta. The rate of increase in $OIPB_{\text{plaque}}$ seems to slow down beyond 16 months. This observation can be supported by the fact that most of the arterial lumen of rabbits older than 16 months show severe development of atherosclerosis and the aorta are almost entirely covered by thick lesions [8]. In this rabbit model, this age range is approaching the end of the animals' lifespan and they typically suffer from a severe burden of arterial plaque at this age. Further progression of the disease after 16 months does not change the fundamental structure of the lesions. The 24 month-old rabbit did in fact die of natural causes.

Figure. 4 Mean OIPB indexes of healthy and atherosclerotic lumen surfaces as a function of rabbits' age.

In addition to OIPB, we also tested the ability of texture analysis of each SHG image to track plaque burden. Five first-order parameters including mean, standard deviation, integrated density, skewness and kurtosis were calculated for each SHG image. Fig. 5A illustrates the relationship between these first-order parameters that capture various types of textures of collagen fibril network imaged by SHG with the age of the rabbit. It can be clearly seen that the collagen fibril changes both in density and shape with progression of atherosclerosis which is represented by rabbit's age. Such variations were captured by the statistics analysis presented in Fig. 5B and 5C, using the mean and kurtosis, extracted from the histogram of SHG images, respectively.

Figure 5 (A) Representative SHG images acquired on arterial lumen of rabbit at different ages, showing collagen fibrils (B) first-order mean (C) first-order kurtosis of image histogram as a function of rabbit's age. The yellow lines are highlighting the changes observed in the SHG channel as a function of rabbit's age.

The mean (Fig. 5B) provides measures of the average gray level within the defined window, showing the overall lightness/darkness of the image. While collagen density increases with plaque progression, the overall image shows more collagen thus becoming brighter, and

presenting a higher mean value in the image histogram. In the case of kurtosis, it measures the peakedness of the image histogram distribution relative to the length and size of its tails. In younger rabbits the SHG/collagen fibril image histograms are characterized by a narrow distribution with short tails around the mean value of gray levels. Images acquired from older rabbits (16, 18 and 24 month-old) tend to be represented by histograms with broader gray level distribution than images acquired from young rabbits. This difference in kurtosis is characterised by the sudden drop in kurtosis value starting from the 16 month-old rabbit (shown in Fig. 5C). Compared with actual images shown in Fig. 5A, we can conclude that this drop in kurtosis value is due to the presence of thicker collagen fibril bundles, a characteristic feature of late stage atherosclerosis plaque.

3.3 Spectroscopic study of lipids in excised artery sections using CARS

While imaging-CARS is useful in providing high resolution maps of lipid-rich structures within the sample, it cannot easily distinguish different forms of lipids due to limited spectral resolution. This challenge does exist for our PCF –based CARS system due to the broad bandwidth of PCF emission. Pegoraro *et al* [6] recently demonstrated a novel approach to acquire high resolution CARS spectra using chirped multimodal CARS microscopy based on a single femtosecond Ti:sapphire laser. We explored the potential of this new hyperspectral imaging technique in studying CARS spectra acquired on excised artery sections. Fig. 6 illustrates three CARS spectra collected at different locations on an unstained artery tissue section. These spectra represent the Raman transitions associated with C-H stretching vibrations (2700 to 3100 cm^{-1}) of the probed tissue matrix and are indicative of the underlining lipids/tissue structure. Such information will be very useful in determining the types of lipids and their correlation with other extracellular components within atherosclerotic plaques.

Figure 6. CARS spectra collected at 3 different locations on an excised artery section using chirped PCF-based CARS microscopy based on a single femtosecond laser.

4. Conclusion

In this paper we demonstrated label-free, *ex vivo* imaging of atherosclerotic tissue using multimodal femtosecond CARS. Not only can MM-CARS clearly distinguish differences between the extracellular composition of healthy and atherosclerotic arterial lumen, it also provides a means for interrogating atherosclerotic plaque burden using both a signal-intensity

based quantification parameter and collagen fibril texture discrimination. We also demonstrated that hyperspectral CARS imaging is a valuable tool in determining regional variation in the type of lipids within the vessel wall which in turn relate to local pathological features of the atherosclerotic plaques.

6. Acknowledgments

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