Imaging and Quantitative Analysis of Atherosclerotic Lesions by CARS-Based Multimodal Nonlinear Optical Microscopy

Han-Wei Wang, Ingeborg M. Langohr, Michael Sturek, Ji-Xin Cheng

**Objective**—The purpose of this study was to assess the ability of label-free multimodal nonlinear optical (NLO) microscopy to characterize, and thus enable quantitative in situ analyses of, different atherosclerotic lesion types, according to the original scheme suggested by the AHA Committee.

**Methods and Results**—Iliac arteries were taken from 24 male Ossabaw pigs divided into lean control and metabolic syndrome groups and were imaged by multimodal NLO microscopy where sum-frequency generation (SFG) and 2-photon excitation fluorescence (TPEF) were integrated on a coherent anti-Stokes Raman scattering (CARS) microscope platform. Foam cells, lipid deposits, matrices, and fibrous caps were visualized with submicron 3D resolution. Starting from the adaptive intimal thickening in the initial stage to the fibrous atheroma or mineralization in the advanced stages, lesions were visualized without labels. Histological staining of each lesion confirmed the lesion stages. Lipid and collagen contents were quantitatively analyzed based on the CARS and SFG signals. Lipid accumulation in thickened intima culminated in type IV whereas the highest collagen deposition was found in Type V lesions. Luminal CARS imaging showed the capability of viewing the location of superficial foam cells that indicate relatively active locus in a lesion artery.

**Conclusions**—We have demonstrated the capability of CARS-based multimodal NLO microscopy to interrogate different stages of lesion development with subcellular detail to permit quantitative analysis of lipid and collagen contents.

**Key Words:** coherent antistokes Raman scattering ■ multimodal nonlinear optical microscopy ■ Ossabaw miniature swine ■ atherosclerosis ■ histopathology

Atherosclerosis, the major cause of cardiovascular diseases, has been a leading contributor to morbidity and mortality in the United States, and it has been on the rise globally. The statistics that account for the rise in incidence consequently call for new imaging techniques to advance the research and diagnosis of atherosclerosis. Current imaging methods, such as x-ray angiography, MRI, intravascular ultrasound (IVUS), computed tomography, and optical coherence tomography (OCT) allow exquisite delineation of advanced lesions. Notably, MRI can achieve molecular imaging using contrast agents. Also, by using spectral analysis to identify lesion components, IVUS can perform virtual histology. Nonetheless, these techniques have not yet reached submicron resolution. As the gold standard, histology provides high performance in biopsy studies, but it is not feasible for live tissue imaging. In addition, with technical advances, fluorescence microscopy with 1- and 2-photon excitation has been applied to vascular and atherosclerosis studies with the capability of identifying cellular and molecular compositions in vivo with labeling. However, these techniques are subject to possible compromises to labeling, such as photobleaching, the requirement for extra incubation, or limited circulation lifetime, all of which could be less optimal for arterial studies. Thus, it is intriguing to explore label-free imaging methods which can also provide chemical selectivity and submicron resolution.

Nonlinear optical (NLO) microscopy has become a powerful tool for imaging biological samples by its unique advantages of inherent 3D resolution, near-IR excitation for superior optical penetration, and lower photodamage. By using endogenous sources of NLO signals, label-free NLO imaging of unstained tissues has been intensively studied, and several examples of label-free imaging methods have been reported. Specifically, 2-photon excited fluorescence (TPEF) microscopy has been applied to tissue imaging with intrinsic fluorescence. Being sensitive to noncentrosymmetric structures, second harmonic generation (SHG), also called frequency doubling, has been used for imaging membranes.
and protein fibrils. Sum frequency generation (SFG) derives its signal from noncentrosymmetric molecules at the sum frequency of 2 excitation sources with imaging capability similar to that of SHG for biological tissues. Third harmonic generation (THG) has been demonstrated for imaging of interface heterogeneities and lipid bodies.

Most notably, a third-order NLO imaging technique known as coherent antistokes Raman scattering (CARS) microscopy has been successfully applied to live tissue imaging with vibrational selectivity. To achieve such selectivity, the CARS process involves a pump laser beam at frequency $\omega_p$ and a Stokes laser beam at frequency $\omega_s$. By tuning the beating frequency ($\omega_p - \omega_s$) to be resonant with the symmetrical CH$_2$ vibration, CARS shows high sensitivity and selectivity to lipid droplets and lipid membranes. This unique characteristic makes CARS microscopy an attractive tool for atherosclerosis studies. Before the use of CARS for vascular studies, other NLO methods have been used to visualize the arterial wall without labeling, but these were limited to viewing collagen and elastic fibril structures. Therefore, beyond the fibrils, cells, such as endothelial cells, smooth muscle cells, and foam cells, must be detected by TPEF with vibrational selectivity. To achieve such selectivity, a third-order NLO imaging technique known as coherent antistokes Raman scattering (CARS) microscopy has been successfully applied to live tissue imaging with vibrational selectivity. Bandpass filters (600/65 nm, Ealing Catalog) were used to transmit the CARS signal of $\approx 588$ nm. The average powers of the master and slave laser beams at the sample were 40 mW and 20 mW. The same lasers were used to produce the SFG signal of $\approx 393$ nm. Two bandpass filters (HP375/50m-2p, Chroma) were used to transmit the backward SFG signal. For TPEF imaging of nonlabeled samples, 2 bandpass filters (hp520/40m-2p, Chroma) were used to transmit autofluorescence. For TPEF imaging of Doxorubicin-labeled samples, 2 600/65-nm filters were used. The fluorescence was generated by only using the master laser ($\approx 707$ nm) to avoid the CARS signal.

**Histology Analysis**

Artery sections adjacent to those imaged by NLO imaging were immersion-fixed in formalin and routinely processed with paraffin embedding for histological staining. A detailed description of staining can be found in the supplemental materials. Histology samples were blindly examined by pathologist and coauthor, Dr. Ingeborg Langohr, using a Nikon Eclipse E400 microscope (Nikon Corp) equipped with air objectives and a Spot Insight Camera (Diagnostic Instrument).

**Analysis of Area Percentage of Lipid and Collagen**

Collagen content in the thickened intima of each lesion was calculated according to the SFG signal. By setting a signal threshold of 30 in the 8-bit (255) range, area percentage of collagen in intima was measured. Area percentage of lipid droplet content in the thickened intima was calculated according to the CARS signal. A threshold was used so that the CARS signals from cell membrane and other structures, such as elastin, were not counted (for details see the supplemental materials). Images for quantitative analysis were derived with the use of the 20x air objective. The measurement was conducted at the most-affected sectors within the plaque shoulder of lesions. The ImageJ software was used for the percentage measurement. Data are presented as mean±SD ($P<0.01$). One-way ANOVA was used to identify the significant difference among lesion types. $P<0.05$ was considered significant.

**Results**

NLO images of the cross-sectional view of iliac arteries were analyzed and classified into different atherosclerotic lesion types according to the scheme suggested by the AHA Committee and modified by other authorities (supplemental Table I). The NLO images were compared with histological evaluations. Figures 1 to 5 illustrate multimodal NLO images of atherosclerosis lesions. Images of normal arteries (data not shown) and the early lesions, such as type I and II, were obtained from the 11 lean control pigs. Intermediate to advanced lesions, including types III, IV, V, and VII, were derived from the pigs of the metabolic syndrome group. Types VI and VIII were not found in the samples collected for...
this study. NLO images were representatively demonstrated around the most severe portion of the eccentric plaques or partial media-intima thickening lesions.

NLO Identification of Early Atherosclerotic Lesions

Figures 1 and 2 show the representative results of early lesions. In Figure 1A through 1C, the stratification of arterial composition is similar to a normal artery except the observation of the adaptive intima thickening with scattered lipid-laden cells viewed by CARS. The adaptive thickening, ordered collagen fibrous tissues, and elastic lamella, which were imaged by CARS, SFG, and TPEF, respectively, elucidated the status of the artery as a Type I lesion. In comparison, the histology observations also showed the intimal thickening (Figure 1D through 1F) referring to the location of the internal elastic lamina (Figure 1F), which is also in keeping with the observation in Figure 1C and 1G. The scattered collagen staining in Figure 1D and 1E highly support the SFG signal shown in Figure 1B and 1H. The adaptive thickening milieu.35,36

Figure 2A through 2C shows the NLO images of the cross-section of a typical Type II lesion. The CARS image (Figure 2A) shows a fatty streak lesion characterized by the accumulation of closely spaced foam cells around the luminal area. This observation compares favorably to the histology images in Figure 2D through 2F, which also show the thickening milieu. The bubbly and granular scenario in the thickened part, which implies lipid accumulation, agrees with the same lesion viewed in NLO imaging. Moreover, the Masson trichrome staining in Figure 2F agrees with the collagen distribution in Figure 2B and 2G. Figure 2G shows a smaller collagen deposit in the intima in the proximity of lumen. These conditions match the observation stipulating that collagen secretion is mainly attributed to modified smooth muscle cells.39 Along with the foam cell accumulation (Figure 2G through 2I), the adaptive thickening is suggested to associate with pathological thickening and future lesions.40 When CARS imaging of lipids is combined with TPEF imaging of internal elastic lamina, the results show that the foam cells resided inside the intima layer (Figure 2H). The TPEF signal around foam cells and arterial cells is likely attributed to the auto-fluorescence of oxidized-LDL41 and NAD(P)H species.42 Because the TPEF signal from...
foam cells only minimally contributes to the CARS channel, the strong CARS signal from the foam cells specifically shows the distribution of granulated lipid (Figure 2I).

**NLO Identification of Intermediate to Advanced Atherosclerotic Lesions**

The intermediate lesion of atherosclerosis, which is defined as Type III, contains scattered lipid pools (Figure 3A) in a relatively thicker intima (Figure 3A through 3C). Images of histological morphology (Figure 3D through 3F) are in agreement with this scenario. The accumulation of interstitial lipid droplets, which can be viewed in CARS imaging (Figure 3A, 3H, and 3I), has not yet formed a confluent and well-defined lipid core within the thickened intima (as compared to Type IV and V). The disordered collagen fibrils around the lipid pools were detected (Figure 3B and 3H). The randomly distributed lipid droplets underscored the observation of a Type III lesion.

Compared with a Type III lesion, a Type IV lesion, as shown in Figure 4, consists of a well-defined lipid core which renders a strong CARS signal (intense, bright) starting from the shoulder region of the plaque (Figure 4A). Intimal disorganization is obvious in the images (Figure 4A through 4C). The lesion episodes and fibril distributions viewed in the NLO images are confirmed by Masson trichrome (Figure 4D and 4E) and H&E staining (Figure 4F). Furthermore, the predominant cellular milieu with abundant foam cells can be viewed at the shoulder (Figure 4A and 4G) and around the luminal regions of the atherosclerotic plaque (Figure 4A and 4H). The foam cells appeared much more circular and densely packed with lipid at this stage (arrows). Doxorubicin labeling (Figure 4I), which is specific to nuclei, highlights the extracellular lipid accumulation within the lipid core viewed by CARS.

Figure 5 shows a typical type V (or Va) lesion. The dense core of lipid and necrotic debris accumulation viewed by CARS (Figure 5A) and the surrounding collagen fibrous cap imaged by SFG (Figure 5B), together with stronger auto-fluorescence viewed by TPEF in the core area (Figure 5C), illustrate a Type V lesion. The NLO imaging result is consistent with histology analyses of serial sections shown in Figure 5D through 5F. The surrounded collagen cap identified by the Masson trichrome staining (Figure 5D and 5E) and the light-microscope features of lipid gruel, which give
the bubbly, granular, and anucleate necrotic debris (Figure 5E and 5F), highly support the NLO imaging results. The location of internal elastic lamina (Figure 5A and 5C) and cell organizations (Figure 5G and 5H) viewed in NLO images are in keeping with the histology observation. The lipid gruel inside the lipid core viewed by CARS (Figure 5I) suggests that the relatively higher TPEF signal in the core area (Figure 5C) probably arose from oxidized-LDL.

In a calcific atheroma classified as Type VII (or Vb),35,36 the intima-media interface is dominated by mineralization, where no significant NLO signals were obtained (supplemental Figure II). A complex lesion milieu can be observed. Staining by the von Kossa method confirmed the calcification lesion.

Quantitative Analysis of Lipid and Collagen Contents in Different Lesion Types

It is established that CARS microscopy permits label-free quantitation of lipid droplets in cells and tissues.26,44 To validate the SFG signal, we performed a correlation study of collagen content measured by SFG and that by Masson trichrome staining and obtained a correlation coefficient of 0.75 (supplemental Figure III). Based on the CARS and SFG intensities, we have calculated the lipid and collagen contents in thickened intima of different lesions (see Materials and Methods). The quantitative results are summarized in the Table. As expected,45 the area percentage of lipid deposition was lower in early-stage lesions than in advanced lesions. The Type IV lesion demonstrated the highest percentage of lipid accumulation (>45% of intima). The lower lipid deposition found in Types V and VII implies the likelihood of retrogression of lipid accumulation and the development of fibrous deposition and mineralization. With respect to collagen content, the type V lesion showed a higher area percentage of collagen (>40% of intima) than earlier stages. Notably, the type IV lesion with a necrotic core and a low degree of fibrosis formation33,35 showed a lower collagen-to-lipid ratio than early lesions.

Discussion

We have shown that CARS-based multimodal NLO microscopy is capable of viewing the various pathological components of atherosclerotic lesions and, as such, can act as an in situ histological tool that is free from the labeling requirement of conventional methods. Moreover, based on the CARS and SFG signal intensities, we were able to carry out a quantitative analysis of collagen and lipid deposition in intima of different lesions. Thus, NLO microscopy gives us both a morphological, as well as quantitative, tool by which to characterize lesions and the percentage contents of lipid and collagen, respectively. The importance of this capability arises from the fact that lipid and collagen contents dominate the formation of soft and hard tissues that affect the mechanical properties of atherosclerotic arteries.46,47 In this context, our results show the potential of NLO microscopy in provid-
longer excitation wavelength, the imaging depth could be
rupture, however, depend not only on the fibrous cap thick-
Imaging the whole cross-section will be very helpful to identify lesions and for quantitative studies. This technical challenge can be overcome by combining laser-scanning with sample scanning on a stepping motor stage.\textsuperscript{46–50} Photodamage effects in a femtosecond NLO system\textsuperscript{51} were not observed in our CARS-based NLO system. Instead of femtosecond pulse excitation, we used 2 5-ps lasers at 707 nm and 884 nm with 40 mW and 20 mW at sample, respectively. At constant damaging potential, it was demonstrated that signals may even be larger with ps pulses because the exponent of the power law of damage is higher than that of the signal.\textsuperscript{52}

Lilledahl and colleagues recently demonstrated that SHG and TPEF imaging of fibrous caps can be used for identifying vulnerable plaques.\textsuperscript{53} The vulnerability and tendency to rupture, however, depend not only on the fibrous cap thickness, but also on its superimposition with other complex factors, such as accumulation of lipid and macrophage foam cells, infiltration of inflammatory cells, and secretion of collagen degradation factors.\textsuperscript{53,54} By adding the capability of viewing foam cells and lipid deposition, CARS-based multimodal NLO imaging provides a significant improvement for mapping the superficial area of a plaque and assessing its vulnerability to rupture and exposure of the lipid core. Luminal scanning of subendothelial scenario (supplemental Figure IV) could potentially be used for assessing instability or vulnerability. In this work, however, such imaging was restricted by the penetration depth of around 60 μm. Nonetheless, with adaptive optics,\textsuperscript{55} microprobe objective,\textsuperscript{56} or longer excitation wavelength,\textsuperscript{25} the imaging depth could be remarkably increased to hundreds of microns. Because the empirical definition of plaque cap was 60 to 65 μm in unstable lesion\textsuperscript{43,45,57} and about 25 to 35 μm in disrupted coronary arteries,\textsuperscript{43,45,58} the improved penetration depth is expected to meet the need for vulnerability studies.

Toward the goal of intravital NLO biopsy, multiphoton fluorescence endoscopy has been reported.\textsuperscript{59,60} It is also notable that SHG contrast-enhanced OCT is emerging for in situ cross-sectional imaging.\textsuperscript{61–63} More recently, the preliminary concept of CARS endoscopy has been proposed.\textsuperscript{64} An Advantage of CARS and SHG imaging is that tissues are not required to undergo slicing, paraffin embedding, or freeze-thaw processes, thus maintaining the potential of intravital imaging. With the capability of quantifying lipid body and collagen fibril contents as shown in the present study, it is foreseeable that a multimodal NLO intravascular catheter with miniaturized probing devices could facilitate and benefit in vivo studies and diagnosis in the years to come.

Acknowledgments
The authors cordially thank Thuc Le for the insightful discussion.

Sources of Funding
This work was supported by a NIH R01 grant EB007243 to Cheng, NIH grants RR013223 and HL062552 to Sturek, and the Comparative Medicine Program.

Disclosures
None.

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Arterioscler Thromb Vasc Biol. 2009;29:1342-1348; originally published online June 11, 2009;
doi: 10.1161/ATVBAHA.109.189316

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material

Online Methods

Tissue Specimen

Male Ossabaw pigs\textsuperscript{1} were divided into two diet groups. The first group was healthy lean control pigs (n=11) fed with a normal maintenance diet (8% kcal from fat). The second group was obese pigs (n=13) fed excess calorie atherogenic diet (45% kcal from fat and 2% cholesterol) for 57 weeks. Pigs were sacrificed in age order from January 2006 till August 2007. The genetic predisposition of Ossabaw pigs to obesity and metabolic syndrome renders the development of cardiovascular disease.\textsuperscript{1} Due to the physiological variation, atherosclerotic lesions with different severity can be obtained in the metabolic syndrome group.

Iliac arteries in the vicinity of the bifurcation of the caudal aorta were collected from the pigs. Presence of plaques in iliac arteries from metabolic syndrome pigs was verified by intravascular ultrasound. Arteries were rinsed with saline and then preserved by zinc formalin fixation immediately after harvested. Phosphate buffered saline (PBS) was used to wash the samples before imaging to avoid the background signal from fixation solution in CARS imaging. The arteries were sliced horizontally (~1mm in thickness) for cross-sectional imaging (Figures IA and IB), and were incised longitudinally for luminal imaging. The plaque section of each lesion artery was collected and sliced serially for imaging. Slices and whole arterial rings were checked with CARS and SFG. Representative NLO and histology images were shown around the most-affected sector according to the thickening severity, plaque and core size, and/or stenosis condition. Images of different lesions were from different animals, but not derived from serial sections of a single artery.

Multimodal NLO Imaging

CARS, TPEF, and SFG imaging modalities (Figure IC) were integrated into a laser-scanning microscope system (Figure ID). The laser source comprised of two mode-locked 5-ps Ti:sapphire lasers (Tsunami, Spectra-Physics) synchronized to each other through an electronic module controller (Lok-to-Clock, Spectra-Physics). Wavelengths of the master and slave lasers, measured by a wavemeter (WA-
1100, Burleigh), were tunable from 690 to 810 nm and from 690 to 1025 nm, respectively. The two parallel-polarized laser beams were collinearly combined and sent into a laser scanning confocal microscope (FV300/IX71, Olympus America). A 20x air objective with a 0.75 numerical aperture (NA) or a 60x water-immersion objective with a 1.2 NA was used to focus the laser beams onto the artery sample. The backward CARS signal was detected by an external photomultiplier tube (PMT, H7422-40, Hamamatsu) at the back port of the microscope (Figure ID). The forward CARS signal was collected by using a 0.55 NA condenser and detected by a second PMT (R3896, Hamamatsu). The CARS signal in forward direction was used for previewing sample slices. Images were taken according to the backward CARS signal that would not be blocked in turbid arterial samples and would represent lipid content under the control of sample thickness. The same imaging routines were used for each sample.

For CARS imaging, the master laser beam was tuned to \(~14140\) cm\(^{-1}\) (\(\omega_p\)) and the slave laser beam was tuned to \(~11300\) cm\(^{-1}\) (\(\omega_s\)), generating a frequency difference of \(~2840\) cm\(^{-1}\) that matches the symmetric CH\(_2\) stretch vibration.\(^2\)\(^,\)\(^3\) Bandpass filters (600/65 nm, Ealing Catalog, Rocklin, CA) were used to transmit the CARS signal around 588 nm. The average powers of the master and slave laser beams were attenuated by using neutral-density filter wheels to 40 mW and 20 mW at the sample. Zoomed-in images were taken with the use of the 60x objective, which provides better signal contrast than that of the 20x objective due to the tighter focal volume as a result of the larger NA. There was no noticeable photo-damage to the samples throughout the imaging processes. The PMT gain value and pixel dwell time were set identically for different samples. A lower gain value, comparing to that in SFG and TPEF imaging, was set for CARS imaging due to the stronger CARS signal from the tissues than the TPEF and SFG signals overall. The crosstalk from two photon autofluorescence was negligible at the CARS channel with the use of the specific filters as described above.

For SFG and TPEF imaging, the two laser beams at wavelengths identical to CARS imaging were utilized. The backward SFG signal around 393 nm was collected through the objective, filtered with two bandpass filters (HQ375/50m-2p, Chroma), and probed by the PMT at the back port of the microscope. For TPEF imaging of non-labeled samples, two bandpass filters (hp520/40m-2p, Chroma) were used to transmit the green fluorescence signal while blocking the CARS signal at 588 nm. The same PMT gain was used for TPEF and SFG. For TPEF imaging of doxorubicin labeled samples, fluorescence was generated by only using the master laser to avoid generating the CARS signal. Two 600/65 nm filters were used for a better coverage of its emission spectrum.
**Histology**

Iliac arteries adjacent to those imaged by NLO imaging were immersion-fixed in formalin and routinely processed with paraffin embedding for histological staining.\(^4,5\) Samples were sliced serially by microtome into \(5 \mu m\) in thickness. H&E (Hematoxylin and eosin) was used for identification of vasculature and nuclei. Masson’s trichrome was used to identify collagen (blue) and vascular structure (muscle cells are red, nuclei are brown to blue, erythrocytes are reddish-orange). Von Kossa’s method with 3% silver nitrate followed by a 0.1% nuclear fast red counterstaining was used to identify calcification (black deposition) in advanced lesions. After staining, 24-bit, full-color digital images were acquired using a Nikon Eclipse E400 microscope (Nikon Corp., Tokyo, Japan) equipped with air objectives and a Spot Insight Camera (Diagnostic Instrument, Inc., Sterling Heights, MI). Histology samples were blindly examined by a pathologist, Dr. Langohr.

**Analysis of Area Percentage of Lipid and Collagen**

Area percentage of lipid droplet content in the thickened intima of each lesion was calculated according to the CARS signal. Typically, lipid droplets give significantly higher contrast in CARS imaging due to its abundance of C-H bonds, in comparison with other structures in biological tissues. Thus, a germane selection of contrast level in CARS imaging exhibits specific visualization of lipid droplets. The specification has been verified by lipid stains, such as Oil Red O and Nile red.\(^6-8\) Other than lipid droplets, weaker CARS signals in arterial samples also come from cell membranes and fibrils, such as elastin and collagen,\(^9\) where CH2-rich amino acid side chains of lysines present predominantly at the cross-linking region.\(^10,11\) Meanwhile, the two-photon excited autofluorescence of elastin is negligible under the CARS bandpass filters.\(^9\) Therefore, for analysis of lipid droplet in lesion arteries, a threshold was set so that the CARS signals from cell membrane and other structures, such as elastin, were not counted.

Collagen content in the thickened intima of each lesion was calculated according to the SFG signal. By setting a signal threshold of 30 in the 8-bit (255) range, area percentage of collagen in intima was measured.

Images for quantitative analysis were derived with the use of the 20x air objective. The measurement was conducted at the most-affected sectors within the plaque shoulder of lesions. The ImageJ software was used for the percentage measurement. Data are presented as mean ±SD (P<0.01).
One-way ANOVA was used to identify the significant difference among lesion types. A threshold value of $P<0.05$ was considered significant.

**Correlation Study of Collagen Content between SFG and Histological Evaluations**

Correlation of collagen content between SFG and histology images was performed to support the validation of the SFG signal. The study was carried out with 5 arterial samples, including 2 normal arteries and 3 lesion arteries. Each sample was sliced consecutively for NLO imaging and histological staining. Collagen content was then measured at corresponding areas in the tunica media, adventitia, and lesion locations. The matching of NLO to histology sectors was completed by landmark identification, such as the relative geometry, distance, and morphological features. Measurements were made according to the SFG signal in NLO imaging and the morphometric color in Masson’s trichrome staining. To determine the area percentage of collagen content in SFG images, the same routines as described above were used. For histological images, colorimetric analysis was performed with the adventitia as the reference color template. Pixels presented the color of collagen stain were retrieved from the 24-bit, full color images, and then transferred into gray scale. Representative area percentage of collagen content can be derived by using a routine threshold and settings. Background threshold, color code, and color scale were set identical for each measurement. Area percentage of collagen was derived according to the percentage of pixels that gave positive value over threshold. Each data point in the correlation graph is an average value of three rectangular, $>100 \times 100 \, \text{um}^2$, areas of an arterial layer or lesion site. The correlation result is shown in Figure III.
Online Figure Legends

Figure I. Imaging methods and system schematic diagram. A, Sections of iliac artery collected from the vicinity of the bifurcation of the caudal aorta were sliced before imaging. B, Cross-sections of the sliced artery samples were imaged with 20x and 60x objectives. C, Energy diagram of multiphoton processes including CARS, SFG, and TPEF. D, System setup of a multimodal NLO microscope. Inset window shows the simplified light path. For multimodal imaging, tightly synchronized picosecond (ps) laser beams were aligned into a laser scanning microscope for generating 2-D imaging area with the excitation beams focused by a selected objective. M: mirror; D.M: dichroic mirror.

Figure II. Representative images of a Type VII lesion inspected by CARS (A), SFG (B), and TPEF (C). The mineralized region, which gave no NLO signals at the inner part of the intima, evidenced that the lesion should be classified as a calcific atheroma. The co-existence of a fibrous cap can be viewed by SFG imaging in B. The close proximity to the luminal area showed strong TPEF signals corresponding to the accumulation of lipid-laden cells, which could be detected by CARS. Bar: 80 μm for A-C. D, H&E staining of a serial cross-section of the lesion. The black rectangular shows the relative location viewed in A-C. E and F, Von Kossa’s method confirmed the large area of calcification deposit (black) within the plaque. Bars: 300 μm in D-F. G, Colocalization of CARS and SFG image within the yellow rectangle in A shows that collagen fibrils circled around cells in the middle of the intimal layer. H, CARS and the colocalization of CARS and TPEF in I show the appearance of lipid-laden cells at the region close to the vascular lumen. L: lumen; CA: calcification; FC: fibrous cap; IE: internal elastic lamina; gray for CARS; blue for SFG; green for TPEF. Bars: 30 μm in G-I.

In the calcific atheroma, where the intima-media interface is dominated by mineralization, the luminal fibrous region detected by SFG (Figure IIG) and the infiltrating foam cells detected by CARS (Figures II H-I) indicate the superimposition of features in this advanced lesion. An interesting feature is the fibrofatty mass under the calcification region (Figures IIA and IIB) that further implies the complexity in lesion progression. At the proximity of the vascular lumen, only CARS and TPEF signals can be detected (Figures II H-I). The lack of any SFG signal shows the absence of collagen in this region.

Figure III. Correlation analysis of collagen content between SFG and histological evaluations. Representative images of SFG (A) and Masson’s trichrome staining (B) exemplify the imaging results of two serial slices collected from an arterial sample. Arrows shows the adipocytes in the connective tissue out of the adventitia. C-D, Images of the analysis results of SFG and histology, respectively, after
performing the analysis routines (in the supplemental methods). Green and red boxes represent the areas selected for the calculation of average collagen percentage in adventitia and media, respectively. The average value accounted for a single data point in the correlation graph in E, in which the result of the correlation study is presented. The result shows a high correlation with a coefficient about 0.75.

**Figure IV. Superficial foam cells detected by multimodal NLO imaging.** CARS (A) and TPEF (B) images of an area full with many foam cells in the superficial aspect of the intima. C, CARS (red) and TPEF (green) overlaid images obtained at different depths with (right column) and without (left column) superficial lipid-laden cells. CARS and TPEF images taken at identical surface area of the endothelium show the locations of lipid droplets and autofluorescence, respectively. SFG signal (blue) inset at 20 μm layer shows the capability of collagen imaging for fibrous cap detection. Bar: 30 μm.

The result shows CARS imaging allows subendothelial inspection of the locations with or without lipid-laden cells. Meanwhile, the collagen network of locus in quo could be imaged by SFG. The TPEF signals could arise from the oxidized LDL and NAD(P)H species. The capability of imaging foam cells and collagen distribution in the pathological intima without any labeling can be important for the study of atherosclerosis, because the amounts of foam cell and collagen content in the subendothelial area could indicate active lesion locus and dictate the vulnerability of complicated lesions.12-14
### Online Table

Table I. Classification of atherosclerosis lesions according to the AHA’s Committee and authorities.15-17

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
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<tr>
<td>Type I</td>
<td>Initial lesion Isolated, scattered macrophage foam cells at the luminal proximity with adaptive intima thickening.</td>
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<tr>
<td>Type II</td>
<td>Fatty streak Layers of foam cells closely spaced in the thickened region of the intima. The term “intimal xanthoma” has been proposed for this alteration.</td>
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<tr>
<td>Type III</td>
<td>Intermediate lesion Pathological intimal thickening with aggregates of lipid-laden cells and scattered extracellular lipid droplets with no necrotic lipid core.</td>
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<tr>
<td>Type IV</td>
<td>Atheroma Confluent accumulation of extracellular lipid and debris forming a necrotic core.</td>
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<tr>
<td>Type V (Vₐ)</td>
<td>Fibrous atheroma Plaque with a well-formed necrotic core covered by a fibrous cap.</td>
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<tr>
<td>Type VI</td>
<td>Complex lesion Surface defect, hematoma, or thrombosis.</td>
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<tr>
<td>Type VII (V₇)</td>
<td>Calcific atheroma Collagen-rich atheroma with large mineralized area dominating the core region.</td>
</tr>
<tr>
<td>Type VIII (Vₐ)</td>
<td>Fibrotic lesion Fibrous tissue predominates.</td>
</tr>
</tbody>
</table>
Online Figures

Figure I
Figure II
Figure III

A

Adventitia Media

Lumen

B

Adventitia Media

Lumen

C

D

E

R² = 0.75

SFG (%) vs. Hist. (%)

0 10 20 30 40 50 60 70 80 90 100

0 10 20 30 40 50 60 70 80 90 100
Figure IV
Online References


