Rationale: Lipids are a key component of atherosclerosis. However, their physiological role on the progression of atherosclerosis including plaque vulnerability has not been clearly understood, because of the lack of appropriate tools for chemical assessment.

Objective: We aimed to develop a label-free chemical imaging platform based on multiplex coherent anti-Stokes Raman scattering (CARS) for the correlative study of the morphology and chemical profile of atherosclerotic lipids.

Methods and Results: Whole aortas from atherosclerotic apolipoprotein E knock-out mice were en face examined by multiplex CARS imaging and 4 distinctive morphologies of the lipids (intra/extracellular lipid droplets and needle-/plate-shaped lipid crystals) were classified. The chemical profiles of atherosclerotic lipids depending on morphologies were firstly identified from intact atheromatous tissue by multiplex CARS. We demonstrated that needle-/plate-shaped lipid crystals in advanced plaques had undergone a phase shift to the solid state with increased protein contents, implying that lipid modification had occurred beforehand. The validity of lipid-selective multiplex CARS imaging was supported by comparative results from oil red O staining and whole-mount immunohistochemistry. By spatial CARS analysis of atherosclerosis progression, we found greater accumulation of lipid crystals in both the lesser curvature of the aortic arch and the innominate artery. Furthermore, multiplex CARS measurement successfully demonstrated the effect of a drug, statin, on atherosclerotic lipids by showing the change of their chemical profiles.

Conclusions: Multiplex CARS imaging directly provides intact morphologies of atherosclerotic lipids with correlative chemical information, thereby suggesting its potential applications in the investigation of lipid-associated disorders and the preclinical drug screening. (Circ Res. 2010;106:00-00.)

Key Words: atherosclerosis • imaging • lipids • statins • multiplex coherent anti-Stokes Raman scattering

Lipids are an essential component in the pathogenesis of atherosclerosis. As atherosclerosis advances, lipids play an important role in determining plaque vulnerability.1–3 Vulnerable plaques are characterized by distinct morphological features: thin fibrous caps and enlarged lipid cores.3–6 The latter is composed of free and modified cholesterols, phospholipids, triacylglycerol, and fatty acids.7 Cholesterols, which comprise the main component of these lipid cores, can exist in a soft gruel-like phase and in various crystallized forms such as plates, needles, and sometimes helices.8,9 Recently, Virmani et al10 reported that ruptured plaques contain greater amounts of cholesterol clefts or crystals in their necrotic cores than stable plaques, potentially indicating that these cholesterol clefts and crystals may be partly responsible for plaque vulnerability. However, little is known about the role of lipid crystals in the pathogenesis of atherosclerosis and plaque vulnerability, most likely because of the lack of currently available non–tissue-destructive imaging techniques and ex vivo chemical profiling tools.

Nonlinear spectroscopic imaging modalities have the potential to visualize cellular organelles and tissue architecture with molecular specificity. Coherent anti-Stokes Raman scattering (CARS) microscopy has recently emerged as the most viable means for 3D chemical imaging of tissues.10–12 It works by probing intrinsic molecular vibrations, which obviates the need to label target molecules and fix specimens.13
CARS microscopy has been used in a full-scale biological study of lipid metabolism in a living organism after direct evidence of the undesirable bias associated with fluorescence labeling techniques was demonstrated. Recently, a video-rate CARS microscopy system has been developed for imaging skin tissue in vivo. Because of the nonlinear nature of the CARS process, rapid scanning of the tight focal spot over the specimen permitted real-time acquisition of vibrational contrast images with 3D submicron resolution, which is not possible with conventional Raman microscopes.

CARS microscopy is particularly ideal for selective imaging of lipids because of the abundance of carbon–hydrogen (CH) bonds that exist in lipids as compared to the surrounding tissues. Lipids exhibit strong and distinct vibrational signatures in CARS spectra from 2700 to 3100 cm⁻¹. Because of these unique characteristics, CARS microscopy is a suitable tool for atherosclerosis which is one of lipid-related diseases. Le et al applied CARS to image lipid-laden cells in atheroma, and the morphological components of arterial walls were imaged as well by integrating other nonlinear optics such as second harmonic generation and 2-photon auto-fluorescence (TPAF) into CARS. However, detailed chemical analysis of lipid composition beyond mere vibrational contrast images with 3D submicron resolution is still limited in the currently available CARS imaging modalities.

In the present study, we developed a multiplex CARS imaging system to image atherosclerotic lipids and to concomitantly analyze their chemical profiles. This system was used to characterize 4 types of atherosclerotic lipids using en face microscopic imaging and to identify their distinct chemical compositions. To determine the applicability as a promising method of studying atherosclerotic lipids, the results obtained by CARS were compared to results obtained with oil red O staining, which is currently the most conventional histological method used to study these lipids. The structural cellular components of the plaques were validated by comparative whole-mount immunohistochemistry. We further investigated the effect of statins on increasing plaque stability by analyzing changes in the chemical compositions of lipid crystals that occurred after statin treatment using multiplex CARS, which could be used in the future as a novel preclinical drug screening methodology.

Methods
The expanded Methods section in the Online Data Supplement (available at http://circres.ahajournals.org) includes information about the following: animal procedures, the principles and experimental setup of multiplex CARS, morphometric validation (whole-mount immunohistochemistry, cross-sectional and en face oil red O staining, and 3D image rendering), and statistical analysis.

Results

Four Distinct Morphologies of Atherosclerotic Lipids Imaged by CARS
We developed a multiplex CARS spectroscopic imaging system that could be readily switched between a lipid-selective imaging mode for fast tissue examination and a wideband multiplex spectroscopic analyzing mode for detailed chemical profiling of points of interest. Previous approaches used Raman shift fixed around 2845 cm⁻¹ to image only CH₂ symmetrical vibrational mode. However, in our study, the probing bandwidth was expanded to the range of 2650 to 3050 cm⁻¹ to allow imaging of the entire CH vibrational region, because atherosclerotic lipids undergo biochemical modifications during the progression of atherosclerosis.

To determine whether CARS could be applied to the study of atherosclerotic lipids, we performed ex vivo CARS imaging of atherosclerotic aortas from ApoE−/− mice fed a high-fat diet for 16 weeks (n=9). Figure 1A shows the en face CARS images, which represent the typical features of atherosclerotic lipids. The bright yellow color identifies CH-rich regions of lipids. The typical microanatomical components of the lesions were visualized and subsequently categorized into 4 appearances: lipid-laden foam cells, extracellular lipid deposits, needle-shaped lipid crystals, and plate-shaped lipid crystals.

Intact Volumetric Visualization of Atherosclerotic Plaques by En Face 3D CARS
To characterize individual atherosclerotic plaques, we performed en face 3D chemical imaging of mouse atherosclerotic ApoE−/− mice (n=9), the lumen side of the lesser curvature of the aorta was serially imaged by CARS toward the deep intima. Figure 1B (Online Video 1) shows the 3D reconstructed z-stack CARS images, representing the typical features of atherosclerotic plaques by lesion depth. In the superficial layers (5 to 10 μm in depth from the lumen), foam cells containing intracellular lipid droplets were clearly imaged, whereas needle-plate-shaped lipid crystals were observed usually in the deep intima region (≥20 μm in depth). These crystallized lipids had a structure-conserved appearance.

Morphometric Validation by Whole-Mount Immunohistochemistry
To validate the lipid-selective CARS images, particularly for lipid-laden foam cells, we integrated a confocal microscopic set-up into the developed multiplex CARS imaging system, and this newly developed system was used to compare the images obtained by fluorescence and CARS signals from identical sites. We performed whole-mount immunohistochemistry to examine major structural cell markers for endothelial cells (CD31), smooth muscle cells (SMCs)
After confocal imaging of cell markers, the identical site was imaged by CARS. Then, the level of colocalization between fluorescence and CARS signals was statistically analyzed (Figure 2). At the resonance of the symmetrical CH2 for lipids (2850 cm\(^{-1}\)), macrophages (identified by CD68 staining) were found to be the major contributor to the CARS signal, whereas endothelial cells and smooth muscle cells only slightly contributed to the signal (Pearson’s correlation coefficients for endothelial cells: \(-0.048\); SMCs: \(-0.01\); macrophages: 0.33). This result indicates that the objects in CARS images containing intracellular lipid droplets with a dark nucleus-like void express CD68, the cell marker of macrophages, demonstrating that these objects are lipid-laden foam cells.

**Spatiotemporal Assessment of Atherosclerosis Progression by CARS: The Development of Lipid Crystals**

**Atherosclerotic Lipid Features Assessed by CARS in Relation to Time**

To assess the progression of atherosclerosis using CARS, various levels of atherosclerotic plaques were obtained from ApoE\(^{-/-}\) mice (n=28) fed a high-fat diet for 2 to 20 weeks. As controls, ApoE\(^{-/-}\) mice fed a normal chow diet were assessed at the same time points. Every other week, we performed en face CARS imaging from the lumen to a depth of 50 to 80 \(\mu\)m in mouse aortas (Figure 3A). In the 2- and 4-week-old ApoE\(^{-/-}\) mice, few of the imaged lipid droplets were bound to the extracellular matrix (ECM) in the very superficial layer (<10 \(\mu\)m in the penetration depth). At 6 weeks, the number of lipid droplets was significantly increased as compared to the number present at 2 weeks (the number of lipid droplets at 2 weeks=420±255, and at 6 weeks=1620±110 in the total analyzed volume, 250×250×60 \(\mu\)m\(^3\); \(P<0.05\)). Notably, extracellular lipid droplets were retained in the ECM up to 30 \(\mu\)m in the penetration depth at this time. At 8 weeks, the atherosclerotic lesions exhibited advanced pathological features, such as crystallized lipid structures. Foam cells were imaged only in the superficial intima with the clear cell shape containing intracellular lipids (white arrows). At 10 weeks, the geography of atherosclerotic lipids in a single atherosclerotic plaque showed depth-dependent features: foam cells were distributed in the luminal surface, and extracellular lipid deposits, including crystal structures, were embedded in the deep intima. Additionally, the size of the necrotic core was measured to be 100 to 120 \(\mu\)m in lateral diameter in 3D CARS imaging down to \(\approx\)50 \(\mu\)m in depth. At 12 weeks, some lipid droplets were found to be deposited on the well-defined multiple layers of plate-shaped crystallized lipids in the deep intima (blue arrows). However, foam cells were still imaged at the same z-depth (white arrows). At 16 weeks, the necrotic core had enlarged and projected toward the lumen. Its size had increased to \(\approx\)250 \(\mu\)m in diameter. Interestingly, crystallized lipid layers were predominantly imaged. At 20 weeks, the complicated lipid crystals, the complex of multiple layers of plate-shaped lipid crystals, extracellular lipid droplets and some cells entangled with ECM were observed (white dotted line). The progression of atherosclerosis was analyzed by quantifying accumulated lipids at 3 stages depending on the period of high-fat diet consumption: initial (2 to 6 weeks),...
intermediate (8 to 12 weeks), and advanced (16 to 20 weeks) (Figure 3B).

Atherosclerotic Lipid Features Assessed by CARS in Relation to Vessel Geometry

The progression of lipid accumulation was spatially analyzed by 3D CARS imaging. The spatial analysis of lipid accumulation was designed based on the following hypothesis: lesional characteristics might differ across sites in the murine aorta. Disturbed and low laminar flows, both of which are proatherosclerotic, could induce different features of atherosclerotic lipids.19,20 Five main sites were examined: the aortic sinus, the lesser curvature of the aortic arch influenced by low laminar shear flow (segment 1), the innominate artery influenced by disturbed flow (segment 2), the left common carotid and left subclavian arteries (segment 3), and the thoracic descending aorta, which is relatively less proatherosclerotic (segment 4). All of these segments were used for spatial analysis (a schematic diagram of the segmentation is shown in the Online Data Supplement). Therefore, the different lipid subfractions for each of the 3 stages were quantified. The results of spatial evaluation are shown in Figure 3B (CARS images for the aortic sinus are shown in Online Figure III).

We observed a significantly increased amount of total lipids in segments 1 and 2 ($P<0.001$ and $P=0.003$, respectively). Interestingly, the crystallized lipids were observed in segments 1 and 2, but few crystal lipids were observed in segment 4. The subfraction of needle-shaped lipid crystals was <1% in all segments (detailed information for lipid subfractions is shown in Online Tables I and II).

Oil Red O Staining Versus CARS Imaging for the Demonstration of Atheromatous Lipid Crystals

To determine the applicability of CARS to investigate the lipids, we compared images obtained by CARS imaging and oil red O staining, which is the technique commonly used for quantification of lipids in both cross-sectional and en face tissues. The same sections of tissue were sequentially submitted to label-free multiplex CARS examinations and then oil red O staining. When we compared cross-sectional results, the lipid distributions in the CARS and oil red O staining images were similar from a macroscopic viewpoint (Figure 4). However, the lipid crystals in the CARS images did not completely match the cholesterol clefts observed in the oil red O staining images. The 3 inserted boxes in the oil red O staining image (Figure 4A) correspond to CARS images (Figure 4B through 4D) as indicated. The lipid crystals in Figure 4C and 4D were clearly imaged by CARS, whereas they were displayed as empty imprints in oil red O staining. We also compared the results from en face CARS imaging to conventional oil red O staining. En face oil red O staining provided quantitative information regarding total accumulated lipids (Online Figure IV), whereas CARS imaging was complementarily used to provide microscale z-stack images of lipid distribution. Figure 4E shows 3D reconstructed CARS images with 4 representative serial z-stack images from the superficial (foam cell layer located at a depth of 3 $\mu$m) to the deep intima (plate-shaped lipid crystals located at a depth of 29 $\mu$m; Online Video II, corresponding to Figure 4E).
Chemical Profiling Analysis of Imaged Atherosclerotic Lipids Using Multiplex CARS

Chemical differences between the 4 categories of atherosclerotic lipids were further investigated by wideband multiplex CARS covering the entire CH vibrational region. After completing fast imaging of the 3D morphologies of lipids, the spectra of the imaged atherosclerotic lipids were simultaneously acquired from the point of interest (analyzing volume: \(0.4 \times 0.4 \times 1.5 \ \mu m^3\)) on the atherosclerotic lesions. Figure 5 shows the normalized CARS spectra from the 4 types of lipids as well as the surrounding ECM, which was included as a nonlipid control. The spectra of both extracellular lipid droplets in the ECM and intracellular lipid droplets from lipid-laden foam cells exhibited one main peak resonating at the symmetrical CH2 vibration, 2850 cm\(^{-1}\). The chemical profile of the plate-shaped lipid crystal, however, was significantly different from that of lipid droplets, because it exhibited 3 extra peaks at 2870, 2910, and 2950 cm\(^{-1}\) on the CARS spectrum. The extra peaks were assigned as CH2 asymmetrical, CH3 symmetrical, and CH3 asymmetrical vibrations, respectively. Conversely, the needle-shaped crystallized lipids showed weaker peaks at 2910 and 2950 cm\(^{-1}\) as compared to the spectra of plate-shaped lipid crystals. The resulting spectra were highly reproducible based on the appearance of the lipid, irrespective of their depth (total number of sites analyzed, \(n=187\) and total mice, \(n=9\)).

The Effects of Statins on the Phasic Changes of Atherosclerotic Lipids Demonstrated by Multiplex CARS

To address the potential applications of multiplex CARS spectra analysis, we attempted to examine the changes in atherosclerotic lipids that occurred after statin therapy. The accumulated lipids in both statin-treated (40 mg/kg of simvastatin every other day for 8 weeks) and nontreated high-fat, high-cholesterol (HFHC)-fed ApoE\(^{-/-}\) mice were assessed. The accumulated lipids in the aortic sinus of statin-treated mice were decreased compared to the nontreated group, confirmed by cross-sectional oil red O staining (\(P<0.05\), Online Figure V). The amount of lipids in the aorta, however, at 8 weeks (white arrows), and inert crystallized lipid structures (blue arrows) after 12 and 16 weeks. At 20 weeks, a fibrous matrix was imaged along with lipid crystals (dotted line). We presented the z-depth at each figure. Some partial z-stacking information combining several slices is expressed to clearly show the features. The CARS images of aortic sinus are in Online Figure III. B, Spatial and temporal assessments of the progression of atherosclerosis by CARS. The volume of accumulated lipids is quantified by the volume measurement via CARS in 250×250×60 \(\mu m^3\) (=3.75×10\(^6\) \(\mu m^3\)) of tissue. Three main stages, initial (stage 1), intermediate (stage 2), and advanced (stage 3), of atherosclerosis were demonstrated. The spatial analysis was performed by segmentation; the aorta segment containing the lesser curvature of the aortic arch (segment 1), the aorta segment containing innominate artery (segment 2), the aorta segment containing left common carotid and left subclavian arteries (segment 3), and the segment of the thoracic descending aorta (segment 4). The subfraction of plate-shaped lipid crystals are shown in blue. The subfraction of needle-shaped lipid crystals, generally <1%, are in the Online Data Supplement. *\(P<0.01\).

Figure 3. Spatiotemporal assessment of atherosclerosis progression by CARS: the development of lipid crystals. A, Atherosclerotic lipid features assessed by CARS in relation to time. En face CARS images show atherosclerotic lipids as the brighter spots. Note the increase in the number of lipid droplets at
was not significantly decreased when examined by en face multiplex CARS (P=0.403; HFHC: 126±71×10^3 μm^3; HFHC plus simvastatin: 120±101×10^3 μm^3). The distribution of 4 types of lipids was not changed by simvastatin treatment. In contrast to the unaltered morphologies, the chemical profiles of lipids were changed. For a qualitative assessment, the chemical profiles of 4 subtypes of aortic lipids were analyzed by multiplex CARS. The increased ratio between the asymmetrical (2870 and 2950 cm\(^{-1}\)) and symmetrical peaks (2850 and 2910 cm\(^{-1}\)) shows the more solidified phases of lipid. Interestingly, the spectra of needle-shaped and plate-shaped lipid crystals exhibited attenuated peaks at 2870, 2910, and 2950 cm\(^{-1}\) in statin-treated group compared to nontreated group (Figure 6). This result implies that the crystallized lipids are less solidified than in the nontreated group. These spectral differences were confirmed by analysis of multiple independent points (n=105 for 5 mice in nonmedicated group and n=70 for 4 mice in simvastatin-treated group).

**Discussion**

Here we report a multiplex CARS spectroscopic imaging that allows the simultaneous use of label-free lipid-selective imaging and chemical profiling. Using the system we developed, we identified the distinct chemical profiles of 4 categories of atherosclerotic lipids (intracellular/extracellular lipid deposits and needle-/plate-shaped lipid crystals). This is the first report in which ex vivo lipid crystals have been imaged by CARS with simultaneous chemical profiling analysis, rather than by in vitro or dissection imaging using polarized light microscopy. The representative CARS images of atherosclerotic lipids were validated by whole-mount immunohistochemistry, showing the potential of CARS to image lipid-laden macrophages in a label-free manner. Additionally, we demonstrated the superiority of this methodology in comparison to oil red O staining. The CARS technology was applied to assess the spatial and temporal progression of atherosclerosis. We also demonstrated the statin effect on plaque stability by analyzing the changes in lipid phase (from solid crystal to liquid phase). Of note, multiplex CARS is able to early monitor chemical profile changes induced by medication before quantitative decreases in atherosclerotic lipids can be observed.

The imaging of atherosclerotic lesions based on multimodal nonlinear microscopy has been previously attempted. Label-free lipid histological analysis was performed on cross-sectional tissue specimens using single Raman-shift CARS imaging, where TPAF was additionally necessary. Wang et al recently reported the quantitative analysis of different types of atherosclerotic lesions by CARS-based multimodal nonlinear optical microscopy. To assess atherosclerotic lesions, collagen and elastin components were visualized through sum-frequency gen-
eration and TPAF, respectively, and lipids were observed by CARS at a single Raman shift. Atherosclerotic lipids present in various types, however, should be characterized in detail for better understanding of atherogenesis. Particularly lipid crystals, as a key marker of advanced lesions, need to be assessed with the knowledge how they are chemically different with other lipids. This can be accomplished by analyzing the chemical profiles using multiplex CARS. In previous studies, spectral CARS measurements have been carried out by stepwise tuning of the Raman shift one by one. However, many biomedical applications requiring prompt tissue examination would significantly benefit from the multiplex CARS technique, which allows single-shot acquisition of vibrational spectra. To date, it would be best to combine the point-wise multiplex technique with fast full-field CARS imaging to provide a wealth of chemical information correlated with pathophysiological anatomy.

The microanatomical components imaged by CARS were corroborated by whole mount immunohistochemical analysis. The cellular shape of intracellular lipid droplets with a dark void in the center, presumably a nucleus, obtained from CARS imaging positively overlapped with CD68, the cellular marker of macrophages, thereby suggesting that these structures were lipid-laden foam cells. However, we encountered an unexpected complication, in that the CARS images of lipid-laden foam cells were not identically generated after tissue fixation of the same tissues for whole mount immunohistochemical analysis. It is possible that the process of tissue fixation during whole mount immunohistochemical analysis uses molecular crosslinking, which disturbs the original molecular structures contained in that tissue specimen.

The multiplex CARS lipid analysis was used to assess the progression of atherosclerosis from initial to advanced stages (Figure 3). In the initial stage, subendothelial lipid retention (<10 μm in depth) was observed with significantly increased number of lipid droplets in ECM at 6 weeks, suggesting that this might be a critical time point in the progression of atherosclerosis. In the advanced stages of atherosclerosis, however, the plate-shaped lipid crystals were dominantly imaged in the shape of multiple vertical stacks. Interestingly, several foam cells exhibiting morphological characteristics of SMCs were observed in advanced plaques, which could be explained by transdifferentiation via lipid uptake by SMCs (Online Figure VI). To understand the correlation between extracellular lipid deposition and cell death, we performed an apoptosis assay and observed that the apoptotic cells were distributed around the liquid phase of extracellular lipids, rather than around the crystallized lipids (data not shown).

Additionally, we found that the type of accumulated lipids were dependent on the vessel geometry by spatial and temporal analysis. As the progression continued, the total accumulated lipids were significantly increased in both the lesser curvature of the aortic arch (segment 1) and the innominate artery (segment 2). Interestingly, the increased fraction of plate-shaped lipid crystals in segment 2 may reveal the site-specificity of atherosclerosis. However, this study may have some limitations to reflect the progression of atherosclerosis as a chronic disease, because the high fat diet feeding has started from young ages (8 weeks).

The role of lipid crystals as significant markers of advanced atherosclerotic lesions has been underestimated, possibly because of limitations in the methods available for investigation atherosclerotic lipids. Even though oil red O staining is the most popular method used to study histological lipids, some limitations remain: 1) a lack of chemical selectivity and 2) the inability to image locations in which cholesterol crystals used to be, which are displayed by empty imprints called cholesterol clefts. To test the applicability of a multiplex CARS imaging system for overcoming these limitations, we compared the CARS...
imaging results with those of oil red O staining from both cross-sectional and en face tissue specimen analysis (Figure 4). For the cross-sectional imaging, the distribution of lipids macroscopically overlapped between CARS and oil red O staining. In the microscopic images, however, the lipid crystals were differentially imaged: both needle-shaped and plate-shaped lipid crystals were clearly imaged by CARS, whereas on oil red O staining, it was not possible to morphologically discriminate between these 2 types of lipid crystals. This can be explained by the procedure of oil red O staining, which includes the use of strong solvents to dissolve the lipid crystals, leading to underestimation of the lipid content. Because the quantification of lipid content produced by en face oil red O staining is based on the area of colorimetric signal obtained from 2D images, the multiple layers of accumulated lipids in the vessel can be counted as a single area, possibly leading misevaluation of the lipid content on microscopic analysis. However, oil red O staining is still considered as a practical method to quantify total lipids in the macroscopic scale, whereas CARS can provide the microscopic information of intact samples. Therefore, both imaging methodologies can complement each other for accurate evaluation.

In the present study, en face 3D CARS images of individual atherosclerotic plaques showed microanatomic features with depth-dependent distribution: foam cells in superficial layer and lipid crystals in the deep intima, which is consistent with previous reports. In addition, we verified distinct chemical profiles associated with the 4 types of atherosclerotic lipids via multiplex CARS, implying that lipid crystals underwent various types of biochemical modifications during the progression of atherosclerosis. Among 4 types, lipid droplets exhibited a single dominant resonance at the symmetrical CH2 vibration point (2850 cm\(^{-1}\)), whereas needle- and plate-shaped lipid crystals showed additional peaks at 2870 and 2910 cm\(^{-1}\), respectively. These spectral differences reflect different states of lipid including liquid, liquid crystal, and solid crystal. This is because, in Raman spectroscopy, the ratio of asymmetrical to symmetrical CH2 vibrations (2870 and 2850 cm\(^{-1}\), respectively) indicates the ordering of polyethylene chains, with a higher ratio corresponding to highly ordered methyl chains. In Figure 5, the ratio of asymmetrical/symmetrical CH2 of lipid droplets was the lowest, meaning that lipids were in the liquid phase, whereas needle-shaped and plate-shaped lipid crystals exhibited higher ratios than droplets (plate-shaped<needle-shaped), corresponding to an ordered phase such as a solid or gel. In addition, the increased ratio of symmetrical CH2/CH3 (corresponding to bands 2910/2850 cm\(^{-1}\)) in plate-shaped and needle-shaped lipid crystals (plate-shaped<needle-shaped) indicated that these regions had higher protein contents, because CH2 groups are less frequent in amino acids side chains than in lipids. Therefore, these spectral results suggest that atherosclerotic lipids undergo physiochemical transformation from liquid to solid and from pure lipid to various posttranslationally modified lipids during the progression of atherosclerosis. It is also noteworthy that the presence of lipid crystals in the solid state may enhance plaque vulnerability by inducing a nonuniform mechanical stress distortion in an atherosclerotic plaque, leading to ECM fracture.

We extensively applied a distinguishing feature of CARS to monitor chemical profile changes of atherosclerotic plaques by testing the effects of statin therapy on atherosclerotic lipids (Figure 6). The pleiotropic role of statins on plaque stabilization has been reported by various approaches based on antiinflammatory function, an overall reduction of lipids, or decrease of matrix metalloproteinases. The plaque composition changes seen following statin therapy have been studied by Raman microscopy, showing a decrease in atherosclerotic plaque size. These studies, however, have not demonstrated how statins directly alter lipid crystals, thereby stabilizing adverse components of stable plaques. We demonstrated that the chemical profiles of lipid crystals were changed by statin therapy using multiplex CARS. After 8 weeks of
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Multiplex CARS Imaging of Atherosclerotic Lipids

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simvastatin therapy, the chemical profiles of both types of lipid crystals had changed, with attenuated peaks observed at 2870, 2910, and 2950 cm⁻¹, which could be interpreted as lipid phase changes and changes in protein content. This means that statin-treated lipid crystals were less solidified and had lower protein contents than nontreated atherosclerotic ones. These results can be supported by the previous report that the inhibition of posttranslational lipid modification is one of the pleiotropic effects of statins.28,34 It is noteworthy that the effect of statins was screened at the early stage qualitatively. In terms of future clinical applications, CARS may be a promising clinical diagnostic tool that could be used in combination with catheter-based endoscopy,35,36 to improve the current limited penetration depth and allow in vivo chemical assessment.

In this report, we clearly demonstrate that a multiplex CARS spectroscopic imaging system can be effectively used as a novel label-free biochemical imaging methodology for investigation of ex vivo atherosclerotic lesions to allow more chemical details on important lipids. It can also potentially be used for preclinical drug screening.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

● Acute coronary syndromes are often triggered by rupture of atherosclerotic plaques.

● A large lipid core is a characteristic of vulnerable plaques.

● Oil red O staining is the most popular methodology used to study atherosclerotic lipids ex vivo, but its ability to provide microscopic and depth-dependent information of intact lesions is limited.

● Statin therapy reduces acute cardiovascular events, but there are no reliable nondestructive clinical modalities to assess its effects on the composition of the lipid core.

What New Information Does This Article Contribute?

● Microspectral CARS imaging is a nondestructive, label-free modality that allows morphological and chemical characterization of plaque lipid core ex vivo.

● Four distinct types of lipid compartments are identified in intact atherosclerotic lesions.

● Multiplex CARS microspectroscopy demonstrated a change of lipid crystals from solid to liquid phase in atherosclerotic plaques of mice treated with statins.

Although various imaging modalities have been introduced to assess the presence and size of the atherosclerotic lesion lipid core, these do not provide sufficient morphological and chemical details. Recently, coherent anti-Stokes Raman scattering (CARS) microscopy has received considerable attention as a viable means for label-free chemical imaging of biological samples. We have developed a state-of-art CARS imaging platform that allows the visualization and nondestructive characterization of the lipid content of unstained ex vivo atherosclerotic lesions. The multiplex CARS microspectral imaging enabled us to classify atherosclerotic lipids into 4 representative categories according to their morphology (intracellular droplets in foam cells, extracellular deposits, and needle- and plate-shaped lipid crystals) and to assess changes attributable to statin therapy. We also found that statin treatment induced softening of lipid crystals. Our study is the first to simultaneously provide lipid chemical composition and structural information in atherosclerotic lesions, thereby furnishing new insights into the pathological role of atherosclerotic lipids. Our observations support the utility of further developing label-free imaging for clinical diagnosis and evaluation of atherosclerosis.
Multiplex Coherent Anti-Stokes Raman Spectroscopy Images Intact Atheromatous Lesions and Concomitantly Identifies Distinct Chemical Profiles of Atherosclerotic Lipids

Se-Hwa Kim, Eun-Soo Lee, Jae Yong Lee, Eun Seong Lee, Bok-Soo Lee, Jeong Euy Park and Dae Won Moon

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Multiplex coherent anti-Stokes Raman spectroscopy images intact atheromatous lesions and concomitantly identifies distinct chemical profiles of atherosclerotic lipids

In this SUPPLEMENT MATERIAL:
1. Extended Materials and Methods
2. Online Figures I through VI
3. Online Tables I and II
4. Legends for Online Video Files I and II

1. Extended Materials and Methods

Animal procedures

Apolipoprotein E knock-out (ApoE\(^{-/-}\)) mice (n=53) were purchased from the Jackson Laboratory (Bar Harbor, ME) and adapted for one week at the Samsung Biomedical Research Institute under specific pathogen-free conditions. Eight-week-old male ApoE\(^{-/-}\) mice were fed a 0.15% high-fat high-cholesterol (HFHC) diet for 2 – 20 weeks (CRF-1, Research Diets, Inc., New Brunswick, NJ). Mice fed normal chow were used as a control group. Every other week after the first two weeks of the study, 4 – 6 mice were sacrificed via CO\(_2\) inhalation. The heart and aorta were perfused with PBS for 10 min and then promptly removed for CARS imaging. To study the effect of statins (Simvastatin, Zocor, MSD), 8-week-old mice were orally administrated (40 mg/kg, every other day) simvastatin for another 8 weeks and fed the same high fat diet used following the previous protocol (n=6). All animal studies conformed to the tenets of the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Institute.

Specimens for CARS study

After harvesting the heart and aortas, the samples were prepared for CARS imaging according to following procedures: the connective tissue of the aorta was carefully removed and the aorta was stored in cold PBS to allow analysis of its lipid chemical profile by CARS. The aortas were incised longitudinally from the ascending aorta to the thoracic descending aorta and dissected into four segments for further
assessment as follows: 1) the aorta segment containing the lesser curvature of the aortic arch, 2) the aorta segment containing innominate artery, 3) the aorta segment containing left common carotid and left subclavian arteries, and 4) the segment of the thoracic descending aorta.

Schematic diagram of the segmentation of the aorta

Prepared segments were mounted lumen side down on a cover-slip using PBS with no chemical mounting solution or fixatives for subsequent CARS study.

The aortic root for CARS was prepared by cryo-sectioning. The top half of the heart containing the aortic root was frozen in OCT (Optimal Cutting Temperature) embedding medium in liquid nitrogen. The frozen block of the heart was cut until the aortic valve leaflets appeared. The serial frozen sections (7 μm of thickness) were applied for CARS imaging.

The principles of CARS

Nonlinear spectroscopic imaging modalities have the potential to visualize cellular organelles and tissue architecture with molecular specificity and to allow chemical analysis in detail. Among them, coherent anti-Stokes Raman scattering (CARS) microscopy has recently emerged as the most viable means for 3-dimensional (3D) chemical imaging of tissues in vivo by probing intrinsic molecular vibrations, obviating the need for labeling of the target molecules as well as for fixation of the specimen. The CARS process is induced by three laser beams at the pump (ω_p), Stokes (ω_s), and probe (ω_p') frequencies, which simultaneously interact at a tight focus within the sample. When the beating frequency ω_p − ω_s of the pump and the Stokes beams is tuned to a molecular vibrational frequency Ω, the resonantly driven
molecular oscillations scatter off the probe beam (with frequency $\omega'_p$) to produce a strong CARS signal at the anti-Stokes frequency of $\omega_m = \Omega + \omega'_p$. Due to such nonlinear nature of CARS process, rapid scanning of the tight focal spot over the specimen could permit real-time acquisition of vibrational contrast images with sub-micron resolution in 3D.\textsuperscript{1,2} CARS microscopy has been proven ideal particularly for selective imaging of lipids because of its abundance in carbon-hydrogen (CH) bonds compared to the surroundings, exhibiting strong and distinct vibrational signatures in the CARS spectra from 2700 cm$^{-1}$ to 3100 cm$^{-1}$. Typical lipid concentration mapping has been successfully demonstrated by probing the symmetric CH$_2$ vibration with Raman shift at 2845 cm$^{-1}$ for optimal contrast.\textsuperscript{3,4} Beyond the single-frequency vibrational histology, detailed chemical profiling of lipid composition necessitates CARS spectral measurements with a full coverage of CH-related molecular vibrations. For this purpose, we developed a CARS imaging setup to implement wideband multiplex CARS microspectroscopy covering the Raman shift from 2650 cm$^{-1}$ to 3050 cm$^{-1}$. On this CARS measurement platform, a fast lipid-sensitive imaging based on integrated CH stretching vibrational signals is readily switchable with a multiplex CARS spectral analysis at points of interest in the sample.

**Experimental setup for a CARS-based spectroscopic imaging system**

Lipid-selective 3-D microscopic imaging and point-wise spectral analysis of cardiovascular tissue with atherosclerotic lesions were carried out concurrently on a same wideband multiplex CARS microspectroscopy platform (Schematic diagram in Online Figure I). The bimodal CARS measurement setup consists of a modified commercial laser-scanning microscope (IX81/FV300; Olympus, Japan) equipped with a bandpass CARS detector and a grating spectrometer (Triax320; Horiba Jobin Yvon) using CARS excitation beams from a three-color near-infrared (near-IR) pulsed laser system. Experimental details of our three-color multiplex CARS setup can be found in the literature published recently\textsuperscript{5}.

We used a 1064-nm mode-locked Nd:vanadate (Nd:YVO$_4$) laser (picoTrain; High Q Laser Production GmbH, Hohenems, Austria) with 7-ps pulse duration and 76-MHz repetition rate, to generate a Stokes beam by splitting off 10% of its total output of 10-W average power and guiding it into the microscope through a pulse delay line. The remaining portion (9 W) of the laser output synchronously pumped an intracavity-doubled optical parametric oscillator (Levante; APE GmbH, Berlin, Germany) to generate a 1.3-W probe beam in a 6-ps, 76-MHz pulse train at 776-nm wavelength. A multiplexed pump beam of 30-nm bandwidth centered at 817 nm was produced from a
femtosecond Ti:sapphire laser (Micra-10; Coherent, Inc., Santa Clara, CA) with 
average power of 900 mW, whose output pulse train was synchronized with that of the 
1064-nm picosecond laser by using an electronic cavity feedback module 
(SynchroLock-AP; Coherent, Inc.).

The synchronized three-color laser beams were collinearly combined in series 
and sent to an inverted optical microscope (IX81; Olympus, Japan) through a two-axis 
beam scanning unit (FV300; Olympus) with a pair of gold mirrors of ~95% reflectivity 
for near-IR wavelengths. The CARS excitation beams were then focused into a 
sample by a 1.2NA, 60 water-immersion microscope objective (UPlanSApo UIS2; 
Olympus). The total power of the laser beams illuminating the sample was attenuated 
to less than 40 mW to avoid the laser-induced damage in the tissue sample.

Fast 3D lipid-selective CARS imaging

Lipid-selective CARS images were obtained by fast raster scanning the focus 
of the collinear pump and Stokes laser beams over the specimen and collecting the 
anti-Stokes signal. For high throughput, we spectrally integrated the broadband CARS 
signals resulting from the lipid-associated Raman vibrations, including aliphatic CH₂, 
CH₃, vinyl CH stretches, etc.⁶ The wideband nature (30-nm bandwidth) of the 817-nm 
centered pump beam, incorporated with the 1064-nm Stokes beam, allowed multiplex 
access to the entire CH stretching vibrations in the range of 2650 cm⁻¹ – 3050 cm⁻¹, 
providing a high image contrast for lipid-rich structures.⁵ A similar approach has been 
attempted to demonstrate a signal-to-background ratio of more than 20:1 in lipid-
selective CARS imaging⁷, while many previous studies have exploited a single 
vibrational band of the symmetric CH₂ stretch³ ⁴.

The broadband anti-Stokes signal in the lipid window (644 nm – 683 nm) was 
collected in the forward direction through a 0.55NA condenser and detected by a 
photomultiplier tube (R3896, Hamamatsu), after reflecting off a long-pass dichroic 
mirror (750dcxr, Chroma Technology) and further isolation through a 660-nm 
bandpass filter (42-7120, Ealing). The CARS microscopy setup could acquire two-
dimensional (2D) en-face images having a maximum field of view of 250 × 250 µm² 
with spatial resolution of 0.4 µm in the lateral (x-y) plane and 1.3 µm along the axial 
(z) direction. Each depth-sectioned image slice consisting of 512 × 512 pixels could be 
obtained at a frame rate of 2.5 s/frame. For 3D imaging, a motorized focusing stage 
was actuated in micrometer steps along the axial direction to collect a z-stack of 2D 
slice images covering the sample thickness up to ~80 µm. Microscopic inspection and 
CARS image acquisition of the atherosclerotic tissue was facilitated using a two-axis
motorized translation stage (H117, Prior Scientific, UK). In the fast lipid-sensitive imaging mode, the CARS probe beam was excluded by blocking the 776-nm OPO output using a mechanical shutter since the pump beam can effectively serve as a probe beam as well for the broadband integral CARS detection.

The temperature and humidity were kept constant during the imaging and spectral analysis, since the formation of cholesterol crystals could be influenced by the temperature. Furthermore, local heating effects by the laser exposure were tested by focusing on a single intracellular lipid droplet in cultured foam cells in the medium. The irradiated intracellular lipid droplets did not show any deformation with the laser power used in this study.

Wideband multiplex CARS spectroscopy in the high-wavenumber Raman region

Once 3D lipid-selective imaging was carried out, the CARS microscope could be quickly switched into a wideband multiplex CARS setup for microspectral analysis of the sites selected in the atherosclerotic lesion. For high-resolution CARS measurements, the narrowband (∼5 cm⁻¹) probe beam prepared from the 776-nm picosecond OPO source was added in order to produce spectrally resolvable multiplex anti-Stokes signals residing in the wavelength range of 623 nm – 641 nm, which were separated away from the 2-color CARS contribution in 644 nm – 683 nm.

The focus of the 3-color laser beams was made to target the point of interest by operating the laser-scanning microscope in the point-scan mode and adjusting the sample position using the precision motorized translation stage. The forward-collected 3-color multiplex anti-Stokes signal from the sample was redirected to a spectrometer (Triax320, HORIBA Jobin-Yvon) equipped with a 600-groove/mm diffraction grating and a cooled EMCCD camera (DU970N-BV, Andor, UK) with a 1600×200 pixel image sensor. The spectrometer could record multiplex CARS spectra spanning the Raman shift from 2650 cm⁻¹ to 3050 cm⁻¹ with frequency resolution of ∼6 cm⁻¹. (Energy diagram of 3-color multiplex CARS in Online Figure II) The spectral recording required an exposure time of 20 ms – 150 ms, depending on the tissue sample thickness and lipid types under investigation. Measured spectral data were processed by a MATLAB (MathWorks, Natick, MA) routine, yielding normalized multiplex CARS spectra that were corrected for the nonuniform spectral nature of the wideband pump excitation as well as the overall transmission and detection characteristics of the anti-Stokes signals.

Without any technical difficulty involved (in the laser wavelength tuning, focal volume overlap adjustments, and sample re-mounting issues), the 3-color broadband CARS measurement platform used in this study allows a convenient bimodal
switching between the lipid-selective imaging mode for fast tissue examination and the wideband multiplex spectral analysis mode for detailed chemical profiling of points of interest.

**Morphometric validation: Whole-mount immunohistochemistry**

The specimens were also examined by whole-mount immunohistochemistry for validation of our findings obtained by CARS imaging. Four sections were fixed with 4% paraformaldehyde for 10 min and washed with PBS containing 0.1% bovine serum albumin. The whole tissues were permeabilized with PBS containing 0.1% BSA, 0.5% Triton X-100, and 10% goat serum for 10 min. Then, tissues were incubated with anti-CD68 (1:100, Abcam; UK), anti-CD31 (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-smooth muscle α-actin (1:200, Sigma, St. Louis, MO) antibodies at 4°C overnight. After washing with PBS, tissues were incubated with secondary antibody-conjugated Alexa488 or Alexa594 (Molecular Probe, Eugene, OR) for 1 h at room temperature and then loaded into Lab-Tek II two-well chambered coverglass (Nalge Nunc International, Rochester NY) for CARS and confocal co-measurements.

**Cross-sectional and en face oil red O staining**

Tissues were fixed with 4% paraformaldehyde for 10 min and washed twice with PBS. The tissues were pretreated with 60% isopropyl alcohol for 5 min and then incubated with 0.6% (w/v) oil red O (Sigma) solution in 60% isopropyl alcohol for 30 min. Before use, the oil red O solution was warmed to 55°C and passed through a 0.22 μm filter. Tissues were then washed in running tap water for 2 min. For analysis of cross-sectional tissue samples, tissues were treated with hematoxylin (Sigma) for 1 min and excess stain was removed with running tap water. Images were obtained using a stereoscope (SZX7, Olympus).

**Three-dimensional image rendering and statistical analysis**

Three-dimensional images were reconstructed using FluoView software (FluoView 5.0 with O3D software, Olympus). Image analysis was performed using Image-Pro software (Media Cybernetics, Inc., Bethesda, MD). All imaging analyses of optical density measurements were conducted in duplicate by two different researchers. All probabilities were compared using ANOVA test. All p-values less than 0.01 were considered statistically significant.
Supplemental References


2. Online Figure I through VI

Legends for Online Figures

**Online Figure I.** Schematic diagram of the CARS microscopic measurement platform for fast 3D vibrational imaging and point-wise wideband multiplex CARS microspectroscopy.

**Online Figure II.** Energy diagram of 3-color multiplex CARS with a wideband pump laser excitation. (a) Without the probe laser beam, a wideband integrated detection of the 2-color-excited anti-Stokes signal generated by the multitude of lipid-related Raman resonances allows for a fast lipid-window imaging. (b) Addition of the separate probe laser produces the multiplex CARS spectra that can be spectrally resolvable.

**Online Figure III.** CARS imaging vs. oil red O (ORO) staining in relation to the assessment of atherosclerosis progression. Imaged area is the cross-sectioned aortic sinus of 2 – 13 weeks of high-fat-fed apoE⁻/⁻ mice. In CARS images on the left, the bright yellow color indicates carbonhydrogen (CH) bond-rich lipids. In ORO images on the right, the red color indicates neutral lipids. Scale bar in CARS and Oil-red O images indicates 20 μm and 200 μm, respectively. In the initial stage (2 – 4 weeks), the lipid crystals are not observed. At 6 weeks, the number of lipid droplets were increased, which is consistent with other areas of aorta. Using the capability of CARS to quantify lipid crystals, the sub-fractions of lipid crystals (lipid crystals/ total lipids) in 8 and 13 weeks are quantified, the results displaying in the picture as ‘Crystal in %’ out of total lipids.

**Online Figure IV.** *En face* total lipid quantification of four segments by Oil red O (ORO) staining. (a) Segmented aorta was applied for *en face* ORO staining for spatial analysis of. The red color indicates stained neutral lipids. Segment 1, the aorta segment containing the lesser curvature of the aortic arch, Segment 2, the aorta segment containing innominate artery, Segment 3, the aorta segment containing left common carotid and left subclavian arteries, Segment 4, the segment of the thoracic descending aorta. (b) The amount of accumulated lipids in each segment was quantified by the coverage of stained lipids out of each segmented area.

**Online Figure V.** The effect of simvastatin treatment on the lipid accumulation assessed by ORO staining. (a) Images of ORO staining. Scale bar indicates 200 μm.
HFHC indicates high-fat high-cholesterol fed ApoE\(^{-/-}\) mouse for 8 weeks. HFHC+SIM indicates simvastatin-treated and high-fat high-cholesterol fed ApoE\(^{-/-}\) mouse for 8 weeks. (b) The amount of accumulated lipids in the lesion was quantified by the coverage of stained lipids in red. Statistical significance was tested by t-test, resulting in \( p \) value = 0.025.

**Online Figure VI.** Various morphologies of lipid-laden cells containing intracellular lipid droplets in advanced stage of atherosclerosis, 16 weeks on high-fat high-cholesterol fed ApoE\(^{-/-}\) mice. The bright yellow color indicates carbonhydrogen (CH) bond-rich lipids. (a) Elongated morphology of lipid-laden cells, (b) Representative morphology of foam cells, (c) Rounded shape of lipid-laden cells. Scale bar indicates 20 \( \mu \)m.
Online Figure I.
Online Figure II.

(a) 3-Color Multiplex CARS Scheme
Incorporating a Wideband Pump Laser

(b) Narrow Band Stokes Wideband Pump Wideband Pump CARS

(c) Narrow Band Stokes Wideband Pump Narrow Band Probe 3-Color Multiplex CARS

\[ \epsilon_{\text{ST}} = (\epsilon_{\text{ST}} - \epsilon_{\text{PB}}) + \epsilon_{\text{PB}} \]
Online Figure III

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Online Figure IV

(a)

(b)

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Segment 1

Segment 2

Segment 3

Segment 4
Online Figure V

(a) HFHC  HFHC+SIM

(b) 

* (p value=0.025)
Online Figure VI.

(a) 

(b) 

(c)
### 3. Online Tables I and II

**Online Table I.** Quantification of the total accumulated lipid volume and sub-fraction of lipids

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<td>8.40 ± 11.18</td>
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*p < 0.01 comparing to stage 1

Note, a large standard deviation (SD) was drawn from the heterogeneity among individual lesions. The lesions where the crystals were not found were applied for this statistical test. The N number of each group was provided in Online Table II for better understanding the occurrence frequency.

Three main stages, initial (stage 1), intermediate (stage 2), and advanced (stage 3), of atherosclerosis were demonstrated.

The spatial analysis was performed by segmentation; the aorta segment containing the lesser curvature of the aortic arch (segment 1), the aorta segment containing innominate artery (segment 2), the aorta segment containing left common carotid and left subclavian arteries (segment 3), and the segment of the thoracic descending aorta (segment 4).
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4. Legends for online video files

Online Video I. Three-dimensional CARS imaging of a single atherosclerotic plaque showing micromorphological features. An entire atherosclerotic lesion imaged by CARS shows micro-anatomic features depending on z-depth; several superficial layers of foam cells and lipid crystals deeply embedded in the lesion (z-depth = 60 μm and scanning area = 250 x 250 μm²). These features were label-free imaged in original intact shapes. Intensity index indicates the abundance of CH bonding corresponding to its brightness. This video file corresponds to Figure 1B.

Online Video II. Label-free and en face CARS imaging of a atherosclerotic lesion showing depth-dependent morphological features. En face serial CARS images from lumen to deep intima (z-depth = 45 μm and scanning area = 250 x 250 μm²). The bright yellow color indicates CH bond-rich lipid. Notably, lipid-laden foam cells are imaged in superficial intima and plate-shaped lipid crystals are clearly visualized in deep intima. Z-depth indicates the distance from the surface of arterial wall. This video file corresponds to Figure 4E.