In vivo imaging of atherosclerotic plaques in apolipoprotein E deficient mice using nonlinear microscopy

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Abstract. Structural proteins such as elastin and collagen can be readily imaged by using two-photon excitation and second-harmonic generation microscopic techniques, respectively, without physical or biochemical processing of the tissues. This time- and effort-saving advantage makes these imaging techniques convenient for determining the structural characteristics of blood vessels in vivo. Fibrillar collagen is a well-known element involved in the formation of atherosclerotic lesions. It is also an important component of the fibrous cap responsible for structural stability of atherosclerotic plaques. High resolution in vivo microscopic imaging and characterization of atherosclerotic lesions in animal models can be particularly useful for drug discovery. However, it is hindered by the limitations of regular microscope objectives to gain access of the tissues of interest and motional artifacts. We report a technique that facilitates in vivo microscopic imaging of carotid arteries of rodents using conventional microscope objectives, and at the same time avoids motional artifacts. As a result, collagen, elastin, leukocytes, cell nuclei, and neutral lipids can be visualized in three dimensions in live animals. We present and discuss in vivo imaging results using a flow cessation mouse model of accelerated atherosclerosis. © 2007 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2800337]

Keywords: Second-harmonic generation; intravital microscopy; atherosclerosis; collagen; two-photon excitation; elastin; lipids.

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1 Introduction

Atherosclerosis is the underlying cause of myocardial infarct, the main cause of death in Western society,¹ and hence it is the primary subject of many ongoing drug discovery efforts.² Atherosclerotic lesions consist primarily of macrophages, T cells, and smooth muscle cells, as well as structural components of extracellular matrix including collagen and elastin. Accumulation of neutral lipids (cholesterol esters) in macrophages and extracellular space is a salient feature of atherosclerosis that leads to the formation of the necrotic core retained by a fibrous cap when the lesion becomes mature.³⁴

Conventionally, drug efficacy is evaluated by postmortem pathology of the arteries in experimental animals, which can be time consuming. Developing in vivo imaging modalities would dramatically enhance drug discovery by accelerating analysis, making the evaluation process more objective as well as reducing the number of animals used. Significant efforts and progress have been made in the past few years in applying macroscopic imaging techniques, e.g. magnetic resonance imaging (MRI) and SPECT, to visualize mouse arteries in vivo⁵⁶ as well as microscopic imaging techniques for characterizing arterial walls ex vivo.⁹¹⁰ However, to better understand the molecular and cellular events associated with the formation of atherosclerotic lesions, microscopic images with submicron and subcellular resolutions in vivo are highly desirable.

Therefore, intravital microscopy represents a complementary approach that potentially offers both three-dimensional and high-resolution visualization of a lesion useful for investigations of lesion formation, progress, and understanding the associated molecular and cellular events. However, imaging of atherosclerotic plaques within a major vessel in vivo is extremely challenging. The major challenges include the physical restrictions of the microscope objectives, the accessibility of the major blood vessels, and movements associated with the heart beat, breathing, and other animal motions.

To mitigate the problems of sample accessibility, one can use an imaging system with objectives having a stick-like tip and additional degrees of motional freedom (such as the IV100 laser scanning microscope recently developed by Olympus) specifically designed for in vivo imaging.¹¹¹² One practical problem of using such an objective lens is the contact between the optics of the lens with the tissue or blood, which could complicate the measurement processes achieving...
uncompromised performance of the objective lens. Also, it
does not eliminate potential artifacts from animal motions,
especially imaging at locations near the heart.

On the other hand, in vivo imaging using two-photon ex-
citation laser scanning fluorescence microscopes in organ sys-
tems such the brain,\textsuperscript{13,15} kidneys,\textsuperscript{16,18} and small blood vessels
of superficial tissue, e.g., the skin,\textsuperscript{19} can be achieved without
the need of a special objective lens. In previous work, we
demonstrated that images of internal organs such as the kid-
ney can be obtained without motional artifacts by stabilizing
the tissue, and functional characteristics of the organs and the
kinetics of cellular processes can be studied and quantitatively
evaluated from these in vivo images.\textsuperscript{18,20} Typically with sub-
micron spatial resolutions. Based on the same idea, in this
work we have developed a technique to support and stabilize
the artery for obtaining fluorescence and nonlinear micro-
scopic images of atherosclerotic lesions in vivo, free of mo-
tional artifacts using a two-photon excitation laser scanning
microscope.

The use of two-photon excitation microscope systems is
beneficial for imaging blood vessels in vivo because: 1. elas-
tin, a major structural protein of the blood vessel, produces
bright autofluorescent signals under two-photon excitation,
and elastin fibers within the vessel wall can be readily
imaged\textsuperscript{21,22}; 2. collagen fibers produce strong second-order
nonlinear effects (second-harmonic generation)\textsuperscript{23-27} under
high photon flux illumination, a condition similar to that of
generating two-photon excited fluorescence, and can be visu-
alized in the microscope with proper barrier filters; 3. the
increased penetration depth by the near-infrared excitation
light is particularly beneficial for imaging through the vessel
wall from outside to reveal the structural features of the ath-
erosclerotic lesions at the luminal side of a vessel, a necessary
process for visualizing plaques in vivo.

To facilitate in vivo imaging of atherosclerotic plaques, we
used a carotid ligation model of accelerated atherosclerosis in
apolipoprotein E (ApoE)-deficient mice.\textsuperscript{28} This model offers
fast lesion development in the common carotid artery, a pe-
ripheraly located vessel with thin walls and simple geometry,
which is convenient for in situ microscopic imaging. In this
work, we explored and demonstrated the feasibility of in vivo
two-photon excitation fluorescence and second-harmonic im-
aging of mouse carotid arteries using a conventional micro-
scope objective.

2 Materials and Methods

2.1 Animal Preparation

Experimental procedures using animals were approved by
the Institutional Animal Care and Use Committees and performed
in accordance with the Guide for the Care and Use of Lab-
oratory Animals (Washington, D.C., National Academy Press,
1996). 8-week-old ApoE-KO mice and C57Bl/6J mice were
obtained from Taconic (Hudson, New York). The animals (n
=6 per group) were prefed with a Western diet containing
0.21% cholesterol and 21% fat for 14 days prior to any sur-
gical interventions. To accelerate lesion formation, the left
carotid artery was ligated under isofluorane anesthesia,
and this configuration was used for both the two-photon
excitation fluorescence and second-harmonics imaging. A
custom-made biochamber was used to cover the whole imag-
ing space around the microscope sample stage, so that the
temperature inside the chamber was kept at 37°C. All intra-
vital images were acquired using a 40×/1.2 NA water emul-

The fluorescent dye we used for intravenous injection in-
cluded Nile Red for staining the lipids, and nuclear probe
Hoechst 33342, was purchased from Invitrogen (Eugene,
Oregon) and used directly by dissolving the dyes in 0.9% saline.

2.2 Fluorescent Probes

The exposed carotid artery of the animal being imaged was
supported by a custom-made stainless steel shovel-like vessel
holder, as shown in Fig. 1. On the shovel head there are mul-
tiple 500-μm-wide groves about 200 μm in depth for posi-
tioning the artery so that a regular 22×50 mm number 1.5
cover glass can be laid flat against the surface of the shovel
body. A custom-made stage with a vertical rail having a
mechanism for holding the vessel holder horizontally via its
handle was placed on top of the sample stage of the Axioplan
2 (upright) microscope base, which is part of the LSM 510
Meta NLO microscope system (Carl Zeiss, Inc., Thornwood,
New York) we used. The vertical and horizontal positions of
the vessel holder are made adjustable to facilitate positioning
of the artery to be imaged. The animal was laid back on a
piece of poly styrene foam with a flat surface for comfort.

A 0.5-ml saline solution containing 250 μg of Nile Red
with or without 50 μg of Hoechst 33342 was infused through
either the tail vein or the jugular vein catheter approximately
10 min before microscopic imaging when imaging the lipids
together with or without cellular nuclei, respectively.

In vivo images of the artery from the animals were cap-
tured with a two-photon laser scanning fluorescence micro-
scope system (LSM 510 Meta NLO, Carl Zeiss, Inc., Thorn-
wood, New York). The internal and the Meta detectors were
configured and used for acquiring fluorescence and second-
harmonic signals from 450 to 500 nm (for Hoechst 33342),
500 to 550 nm (for elastin autofluorescence), 560 to 650 nm
(for Nile Red), and 388 to 409 nm (for second harmonics)
simultaneously, as well as fluorescence emission spectra of
the tissues. The polarization of the excitation light is approxi-
mately parallel with the orientation of the artery being im-
aged, and this configuration was used for both the two-photon
excitation fluorescence and second-harmonics imaging. A
custom-made biochamber was used to cover the whole imag-
ing space around the microscope sample stage, so that the
temperature inside the chamber was kept at 37°C. All intra-
vital images were acquired using a 40×/1.2 NA water emul-
sions objective. The Ti-sapphire laser (Spectra-Physics, Mountain View, California) was tuned to 800 nm for excitation. The power of the laser was attenuated to between 3 to 30 mW on the sample using an acoustic optical tunable filter (AOTF) of the LSM 510 system. During all imaging procedures, the body temperature of the animal was maintained at 37°C. After imaging, animals were sacrificed by lethal injection of pentobarbital followed by a bilateral pneumothorax.

2.4 Histology

For conventional histology, the mice were perfused and fixed in Zinc-Tris fixative. Paraffin-embedded sections were stained with Masson’s Trichrome. Macrophages were immunohistochemically stained using MAC-2 antibody (clone M3/38 from Cedarlane Laboratories, Burlington, North Carolina). Smooth muscle cells were immunostained with anti-α-smooth muscle actin antibody obtained from DAKO (Glostrup, Denmark).

2.5 Image Data Analysis

Intravital images were analyzed by using the Meta Imaging Series (version 6, Universal Imaging Corporation, West Chester, Pennsylvania), Zeiss LSM 510 imaging software, and Voox developed at the Indiana Center for Biological Microscopy.

3 Results and Discussion

Based on conventional histology, no spontaneous lesions occurred in the carotid arteries of the control age-matched mice. In comparison, 14 days after ligation of the left common carotid artery near its bifurcation, atherosclerotic plaque-like lesions were developed along the length of the artery and observed with a cross sectional view under a regular light microscope (Fig. 2). Trichrome staining [Fig. 2(b)] indicates that an extracellular matrix (shown in dark blue) was accumulated predominantly in the peripheral part of the lesion along the lumen side, forming a fibrous cap-like structure. Matrix deposition colocalized with smooth muscle cells [Fig. 2(d)]. The central part of the lesion was occupied by lipid-laden macrophages [Figs. 2(b) and 2(c)]. No lesions were developed in the contralateral common carotid artery Fig. [2(a)], which did not undergo a ligation procedure.

By using the setup described earlier (Fig. 1), we were able to image the carotid artery in vivo and effectively eliminate the problem of animal motions. This is demonstrated in various 3-D in vivo images of the arteries without signs of motional artifacts. Figure 3 is a 3-D image stack of a normal artery in an orthogonal view showing elastin autofluorescence (in green) and the second-harmonic generation of collagen fibers (in red). No fluorescent dyes were used in the experiment. It can be seen that the elastin fibers are well organized, relatively straight, and aligned approximately parallel with the direction of blood flow. This is consistent with the fact that in major arteries elastin fibers are predominantly longitudinally orientated. The fenestrations of the internal elastic lamina are visible as black holes in the xy, xz, and yz planes, and are most clear in the xy plane. The distance between the vessel intima to the outside surface of the vessel in this case is about 60 μm, very close to what is reported in the literature using ex vivo imaging. The distribution of the fenestrations appears to extend from the tunica intima through tunica media to the adventitia of the vessel wall. The second-harmonic signals of collagen fibers appear to be primarily located within the adventitia.

To visualize cell nuclei and neutral lipid accumulation in vivo, we acquired image stacks of the artery after intravenous infusion of Hoechst dye (nuclear staining) and Nile Red (lipid staining). Elongated smooth muscle cell nuclei within the media as well as the endothelial cells can be clearly observed [Fig. 4(a)]. The smooth muscle cells have an orientation that is perpendicular to the vessel axis, while the endothelial cells are aligned parallel with the longitudinal axis of the carotid artery [Fig. 4(a)], e.g., two cells going through by the blue line in the xz plane and three cells slightly below the green line in the xy plane are likely to be endothelial cells aligned horizontally (parallel to the vessel). This cellular orientation is as expected for an artery with normal blood flow, since endothelial cells are typically aligned along the direction of the blood flow. On the contrary, in ligated arteries, intimal cells are randomly orientated [Figs. 4(b) and 4(c)], which is consistent with having an altered pattern of blood flow in the vessel. These randomly orientated intimal cells can be the endothelial cells as well as other cell types, including macrophages recruited to the lesion site.

Nonligated arteries, as expected, did not accumulate any neutral lipids [Fig. 4(a)]. However, one week after ligation, we observed small lipid droplets and aggregated clumps of elastin [Figs. 4(b) and 4(c)]. After two weeks of ligation, as the atherosclerotic lesions grew in size [Figs. 5(a) and 5(b)], both the size and number of lipid droplets were increased. Often one can observe lipid cores sandwiched between the two collagen layers (Fig. 5). The atherosclerotic lesion seen in Fig. 5 was developed quite extensively along the vessel as judged by the continuous extensions of the core in the x direction [xz planes of Figs. 5(a) and 5(b)]. In the yz plane, one can see a classical cross section view of the artery containing a plaque (Fig. 5, indicated with white arrows). In comparison, there was more collagen in the vessel ligated for two weeks than that of the controls (Fig. 4).

Remarkably, morphological features of collagen are very different in the fibrous cap as compared to that within the adventitia [Figs. 5(a) and 5(b)]. In the adventitia, collagen can be seen as individual fibers organized in an overall mesh-like structure, as generally observed in the adventitia of arteries. Collagen of the fibrous cap, on the other hand, does not show a clear fiber structure and does not resemble what is observed from that of the adventitia. Since we use second-harmonic generation for collagen visualization, it is reasonable to suggest that the morphological differences we observed here reflect differences in structural organization of the collagen molecules due to the fact that visualization of second harmonics is highly dependent on the structural characteristics of collagen. To the best of our knowledge, this is the first time morphological differences between collagen in the plaque and adventitial collagen are demonstrated in vivo. It is still unclear whether the observed structural differences of the plaque collagen reflect the lack of proper fiber maturation or partial degradation of the collagen. Spectroscopic and quantitative analysis are underway to further evaluate the observed morphological or structural differences of collagens.
Fig. 1 Prototype of an artery holder and a setup for in vivo imaging of carotid artery.

Fig. 2 Lesion development in carotid arteries of ApoE−/− mice. (a) Nonligated artery, Masson trichrome staining; (b) artery 14 days after ligation, Masson trichrome staining showing atherosclerotic lesion with a fibrous cap and lipid core; (c) Mac-2 immunostaining showing macrophages enriched with lipids; and (d) α-actin immunostaining showing actin filaments of smooth muscle cells. Images were acquired with a 20× objective.
We have also detected atherosclerotic lesions with large lipid droplets and clumps of elastin, but without the collagen fibrous cap in vessels ligated for two weeks [Fig. 6(a)]. The observations of aggregated elastin are consistent with vessel wall remodeling processes related to lesion formation.\textsuperscript{33,34}

There are several reasons for the lack of second-harmonic signals in these samples. First, lesion development and maturation could vary in different animals as well as along the length of the same carotid artery.\textsuperscript{35} Second, to detect second harmonics, one needs to have a molecule with noncentrosym-
metry that satisfies the phase matching conditions with regard to the molecule interacting with the laser light.\textsuperscript{26,36} It is generally agreed that fibrillar (predominantly type 1) collagen is the main source for generating second harmonics and mouse atherosclerotic plaques do contain fibrillar collagen.\textsuperscript{40} However, newly synthesized collagen may not possess structural features required for generating the second-harmonic signal. Alternatively, the amount of collagen may not be high enough for generating sufficient second-harmonic signals in the backward direction, the detection scheme we can use for in vivo applications at the present time.

We have verified spectroscopically that the fluorescence emission spectrum from the lipid droplets (labeled with Nile Red) within the atherosclerotic lesion [Fig. 6(c)] is similar to that from the foam cells found at the lesion site [Fig. 6(b)], and both resemble the spectrum from adipose tissues stained with the Nile Red molecules [Fig. 6(a)].

In a pilot manner, we have also explored if in vivo two-photon microscopy is capable of detecting smaller spontaneous lesions in carotid arteries of ApoE-deficient mice. After 12 weeks on the high fat diet (without ligation), we found lipid droplets in both the intima and media [Fig. 7(b) $xz$ and $yz$ planes]. Other than the smaller size, the images of the spontaneous atherosclerotic lesion resembled those of ligated arteries [Fig. 7(a)]. Interestingly, the adventitial collagen layer [Fig. 7(b) in red] was significantly thicker than that of a normal artery [Figs. 3 and 4(a)]. In fact, the thickening of the collagen layer was observed in both the two-week ligated [Figs. 5 and 7(a)] and nonligated vessels that spontaneously developed atherosclerotic lesions [Fig. 7(b)]. This could be a result of fibrosis known to occur in mice lacking apoE.\textsuperscript{41,42} The average thickness of the collagen layer in the arteries that developed atherosclerotic lesions was $14\pm2\mu m$, measured under our experimental conditions. In comparison, the collagen layer thickness of the normal artery has an average thickness of $8\pm2\mu m$. We should point out that these measurements are likely to be different from the “native” thickness, since the arteries may have been stretched slightly due to the insertion of the vessel holder.

Macrophages play a critical role in plaque development and destabilization, while neutrophils may be involved in arterial response to injury,\textsuperscript{43} hence important in their in vivo visualization. Therefore, in separate experiments, we explored in vivo imaging of leukocyte accumulation in a mouse carotid artery by using transgenic MacGreen mice expressing enhanced green fluorescence protein (EGFP) in white blood cells.\textsuperscript{44} The injury of the vessel wall was induced by acutely overstretching the common carotid artery by gently pulling the artery with a stainless surgical tweezer. Within less than 30 min after stretching the vessel, attachment of green fluorescent leukocyte to the lumen of the vessel was visualized in vivo [Figs. 8(a) and 8(b)]. The relative intensity of the elastin fluorescence was considerably lower compared to that of the cells containing GFP [Fig. 8(a) $yz$ plane]. In the uninjured vessel, other than occasionally observing a GFP tagged cell flowing through the vessel, there were no cells attached to the vessel wall (data not shown). In these experiments, we were unable to identify white blood cell types, e.g., monocytes and neutrophils. However, this is an important initial
step for *in vivo* investigations of vessel wall responses to injury.

Finally, we want to emphasize that *in vivo* imaging facilitated by using a vessel holder provides a unique opportunity to visualize vascular structures in their native states, free from artifacts associated with sample preparation processes. Although one can obtain *ex vivo* images of the vessel wall by using excised arterial specimens [Figs. 9(a) and 9(b)], there are striking differences between the *ex vivo* and *in vivo* images. Specifically, adventitial collagen fibers of excised vessels lost their mesh-like network appearance from intact arteries [as in Fig. 5(a)] and became curled [Fig. 9(a)]. Elastin fibers appeared to be wrinkled and curled in the *ex vivo* images [Fig. 9(a) $xz, yz$ planes and Fig. 9(b) $xy, xz,$ and $yz$ planes] that were different from those observed *in vivo* (Fig. 3). These differences in the *ex vivo* images are likely to be the results of vessel contraction and elastic recoil associated with...
Fig. 7 Comparison between (a) ligated artery and (b) that which developed spontaneous atherosclerotic lesions without ligation. Elastin in green, lipid droplets in yellowish green, and collagen (SHG) in red. Scale bar: 40 μm.

Fig. 8 Luminal accumulations of leucocytes. Images obtained from MacGreen mouse with its leucocytes expressing enhanced green fluorescent protein (EGFP). Leucocytes accumulation was stimulated by acutely overstretching the artery. (a) Orthogonal view of a 3-D image stack. Image size: 230 × 230 × 64 μm³. Scale bar: 40 μm. (b) 3-D reconstruction of the same dataset showing EGFP-containing leucocytes (in green) and elastin (in white) of the vessel wall extracellular matrix.
excision of the artery. Ex vivo microscopy can be a useful alternative, though only when proper sample preparation techniques are used to preserve the native structural features of the arterial wall.

In summary, we have demonstrated that in vivo imaging of the carotid arterial wall and obtaining 3-D images without motion artifacts can be achieved using a conventional two-photon confocal microscope with the help of a custom-made vessel holder. We have visualized atherosclerotic lesions in mice with ApoE deficiency using both two-photon excited fluorescence and second-harmonic generation. Critical components of atherosclerotic plaques including collagen, elastin, and neutral lipids can be visualized in vivo. This opens up new possibilities for conducting an array of future studies that can answer important questions relevant to vascular diseases. In particular, it will facilitate translational research in drug discovery.

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References

Fig. 9 Orthogonal views of image stacks of ex vivo arterial sample of ApoE -/- mice. Elastin autofluorescence is shown in green and collagen SHG is shown in red. (a) xy plane closes to the adventitial. (b) xy plane closes to the lumen. Scale bar: 40 μm.
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