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Label-free imaging of arterial cells and extracellular matrix using a multimodal CARS microscope

Han-Wei Wang [a], Thuc T. Le [a], Ji-Xin Cheng [a,b,*]

[a] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, United States
[b] Department of Chemistry, Purdue University, West Lafayette, IN 47907, United States

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Abstract

A multimodal nonlinear optical imaging system that integrates coherent anti-Stokes Raman scattering (CARS), sum-frequency generation (SFG), and two-photon excitation fluorescence (TPEF) on the same platform was developed and applied to visualize single cells and extracellular matrix in fresh carotid arteries. CARS signals arising from CH_2-rich membranes allowed visualization of endothelial cells and smooth muscle cells of the arterial wall. Additionally, CARS microscopy allowed vibrational imaging of elastin and collagen fibrils which are also rich in CH_2 bonds. The extracellular matrix organization was further confirmed by TPEF signals arising from elastin's autofluorescence and SFG signals arising from collagen fibrils' non-centrosymmetric structure. Label-free imaging of significant components of arterial tissues suggests the potential application of multimodal nonlinear optical microscopy to monitor onset and progression of arterial diseases.
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1. Introduction

With intrinsic 3D resolution and relatively large penetration depth, nonlinear optical (NLO) microscopy has opened up a new avenue to tissues imaging [1]. First demonstrated in 1990 [2], two-photon excited fluorescence (TPEF) microscopy has been extensively applied to biological imaging by utilizing intrinsic fluorescence or extrinsic labeling of bio-molecular structures [3]. Being sensitive to non-centrosymmetric structures [4], second harmonic generation (SHG) imaging was first demonstrated in 1970s [5]. Both SHG and electronic sum-frequency generation (SFG) have been utilized for imaging biological samples such as membranes [6] and protein fibrils [7–9]. TPEF and SHG were simultaneously generated by a single femtosecond laser in many tissue imaging studies [10–12]. In addition to these methods, a third-order NLO microscopy based on coherent anti-Stokes Raman scattering (CARS) allows chemically selective imaging [13]. In CARS microscopy, a pump beam at ω_p and a Stokes beam at ω_s are collinearly combined and tightly focused into a sample. CARS signal can be significantly enhanced by tuning ω_p–ω_s to a Raman-active vibration band, thus providing chemical selectivity. Although CARS microscopy was first experimentally reported in 1982 [14], its robust potential for biological research was not realized until its revival in 1999 [15]. Significant technical advances including integration of near-IR picosecond pulse excitation [16], collinear beam geometry [15], epi-detection [16,17], and laser scanning [18] have produced a new generation of CARS microscope with high sensitivity, sub-micron 3D spatial resolution, and high acquisition speed [13]. CARS microscopy has demonstrated the capability for label-free imaging of a wide range of bio-molecular structures including single lipid bilayers

* Corresponding author. Address: Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, United States.
E-mail address: jcheng@purdue.edu (J.-X. Cheng).

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[19–21], myelin sheath in nervous systems [22,23], native drug molecules [24], tumor cells and adipose tissues [25], and skin [26].

With cardiovascular diseases becoming a prevalent malady in both developed and developing countries [27,28], there is a strong demand for technologies that allow high-resolution imaging of arterial components in the *ex vivo* and *in vivo* conditions. In general, an elastic artery, such as carotid artery or iliac artery, comprises three layers (Fig. 1). The innermost layer is tunica intima which consists of endothelial cells and an internal elastic lamina. The intermediate layer is tunica media which consists of smooth muscle cells, collagen fibrils, and elastin lamellas. The outermost layer is adventitia which consists of collagen and elastin fibrils. In recent years, TPEF and SHG microscopy has been applied to vascular imaging. Being sensitive to non-centrosymmetric structures, SHG microscopy has been applied to visualize collagen fibrils of the arterial wall [11,29]. Relying on intrinsic autofluorescence, TPEF microscopy has been applied to visualize elastin fibers [11,30,31]. Moreover, TPEF microscopy has been used to image fluorescently labeled endothelial cells, smooth muscle cells, and macrophages [32]. However, imaging arterial components with fluorescent labels faces tremendous technical challenges including non-specific binding and inefficient diffusion into arterial wall. It is known that endothelial cells and smooth muscle cells play leading roles in normal arterial function [33–35] as well as in arterial disease onset [36–38]. Therefore, label-free visualization of endothelial cells and smooth muscle cells would be crucial for investigating the early stages of cardiovascular diseases.

In this paper, we demonstrate label-free visualization of significant arterial components using a microscope that integrated CARS, SFG, and TPEF imaging modalities on the same platform. Spectrally resolved CARS, SFG, and TPEF signals were generated simultaneously from the same arterial sample by two synchronized picosecond lasers. We show that CARS imaging based on the CH_2 vibrations allowed visualization of endothelial cells, smooth muscle cells, elastin, and collagen fibrils of the arterial wall. CARS images were further confirmed by TPEF and SFG signals. The demonstrated label-free imaging capability of multimodal NLO microscopy suggests its potential application to the studies of arterial functions and cardiovascular diseases.

2. Experimental method and materials

2.1. Nonlinear optical imaging system

CARS, TPEF, and SFG imaging were integrated into the same microscope via the multimodal system shown in Fig. 2. Two mode-locked 5-ps Ti:sapphire lasers (Tsunami, Spectra-Physics, Mountain View, CA) were synchronized to each other (one as master and the other as slave) through an electronic module controller (Lok-to-Clock, Spectra-Physics). The two parallel-polarized laser beams were collinearly combined and sent into a laser scanning confocal microscope (FV300/IX71, Olympus America, Center Valley, PA). 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.

A 60× water immersion objective with a 1.2 NA (numerical aperture) and a 20× air objective with a 0.75 NA were used to focus the laser beams for imaging the sagittal-section and transection artery samples, respectively. Using neutral-density filter wheels, the average powers of the master and slave beams were attenuated to 40 mW and 20 mW at the sample. Two external photomultiplier tube (PMT, H7422-40, Hamamatsu, Japan) detectors were installed for receiving signals in both forward and backward (epi-) directions (PMT1 and PMT2 in Fig. 2). The epi-detected NLO signals were collected by the objective, while the forward CARS signal was collected by using a 0.55 NA condenser.

The mechanisms of forward and backward CARS have been thoroughly studied. For a thin sample, the forward-detected CARS (F-CARS) signal is mainly contributed by large objects and the bulk medium, while the

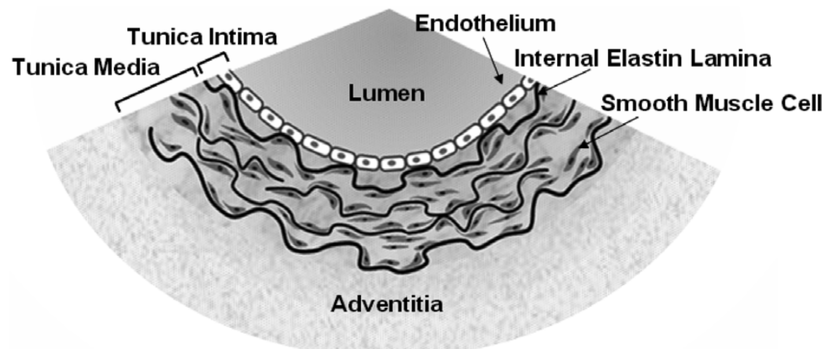

Fig. 1. Cross-sectional redition of an artery. An arterial wall consists of three layers, tunica intima, tunica media, and adventitia. Tunica intima consists of an endothelium and an internal elastic lamina. Tunica media consists of smooth muscle cells, elastin lamellas, and collagen fibrils (not shown). Adventitia consists mainly of collagen and elastin fibrils.

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