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## Multiphoton Microscopy for 3-Dimensional Imaging of Lymphocyte Recruitment Into Apolipoprotein-E–Deficient Mouse Carotid Artery

Pasquale Maffia, PhD; Bernd H. Zinselmeyer, PhD; Armando Ialenti, PhD; Simon Kennedy, PhD; Andrew H. Baker, PhD; Iain B. McInnes, MRCP, PhD; James M. Brewer, PhD; Paul Garside, PhD

Two recent elegant studies have shown that in apolipoprotein-E–deficient mice, the lamina adventitia is a major site of arterial wall inflammation associated with lymphocyte infiltration into atherosclerotic arteries and with formation of adventitial lymphoid-like tissues.<sup>1,2</sup> These results suggest that lymphocyte responses in the lamina adventitia may play a crucial role in atherosclerosis development.<sup>1,2</sup>

However, in both studies, the detection of immune cells within the artery was limited to conventional analysis systems (flow cytometry, immunohistochemistry), which require the removal, disruption, and processing of tissue at defined time points to give a “snapshot” of the immune response to vascular damage *in vivo*. For evaluation of atherosclerotic processes and their relationship to immune activation, imaging of 3-dimensional structures in intact vascular tissues and functional aspects of the diseased artery (eg, details of the interactions of immune cell with vessel wall structures) are required. A potential solution to this problem is offered by the development of multiphoton laser-scanning microscopy,<sup>3</sup> with the ability to penetrate directly into tissues at sufficient depths to image tissues *in situ* in the absence of significant phototoxicity. By moving the focal point axially, a stack of optical sections at various depths can subsequently be viewed as a movie. Recently, multiphoton imaging has been applied in atherosclerosis to image murine vascular structures.<sup>4</sup> However, homing of cells to the vessel wall was not addressed. In our laboratory, we have established multiphoton scanning microscopy to allow imaging of lymphocytes in real time, *in situ*, *in vivo*.<sup>3</sup> Here, we report the 3-dimensional imaging of the entire structure of an isolated intact apolipoprotein-E–deficient mouse carotid artery, identifying homed, fluorescently tagged, adoptively transferred lymphocytes.

Cell suspensions were prepared from peripheral and mesenteric lymph nodes of female C57BL/6 mice and were labeled with Cell Tracker Red CMTPX (Molecular Probes Inc.) at  $10^7$  cells/mL of CO<sub>2</sub>-independent medium (GIBCO BRL) plus 0.5  $\mu$ L of 20 mmol/L CMTPX stock solution in DMSO for 45 minutes at 37°C. Cells were washed with complete CO<sub>2</sub>-independent medium, and 2 to  $5 \times 10^6$  lymphocytes were injected intravenously into 8-week-old female apolipoprotein-E–deficient mice or C57BL/6 recipients. Twenty-four hours after transfer, excised intact mouse carotid arteries were imaged by 2-photon microscopy, as described previously.<sup>3</sup> The excised vessel was transferred into CO<sub>2</sub>-independent medium at room temperature. The vessel was bound (Vetbond, 3M) on a coverslip. The coverslip was adhered with grease to the bottom of the imaging chamber, which was continuously supplied with warmed (37°C) and gassed (95% O<sub>2</sub> and 5% CO<sub>2</sub>) medium (RPMI 1640 with 25 mmol/L HEPES) before and throughout the period of microscopy. The 2-photon excitation source was an all-solid-state, tuneable, titanium sapphire laser system (Chameleon, Coherent Laser Group). The laser beam was routed into a multiphoton excitation laser scanning system (Radiance 2000MP, Bio-Rad Laboratories). The output laser beam from the scan head was aligned through an upright microscope (E600-FN, Nikon). The objective lens used for all imaging investigations was a CFI-60 Nikon Fluor 40 $\times$ /0.8 NA water-dipping objective lens. The sample was illuminated with 830 nm, approximately 210 fs in pulse duration and 76 MHz in repetition frequency. The excited fluorescence passed through a laser blocking filter (E625SP, Chroma Technologies) and was detected with a multialkali cathode photomultiplier tube (S20 PMT) in an external or nondescanned configuration as part of the Radiance 2000MP system. A custom-built filter block was used to separate fluorescence at 500 to 550

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**The online-only Data Supplement, consisting of movies I and II, is available with this article at <http://circ.ahajournals.org/cgi/content/full/115/11/e326/DC1>.**

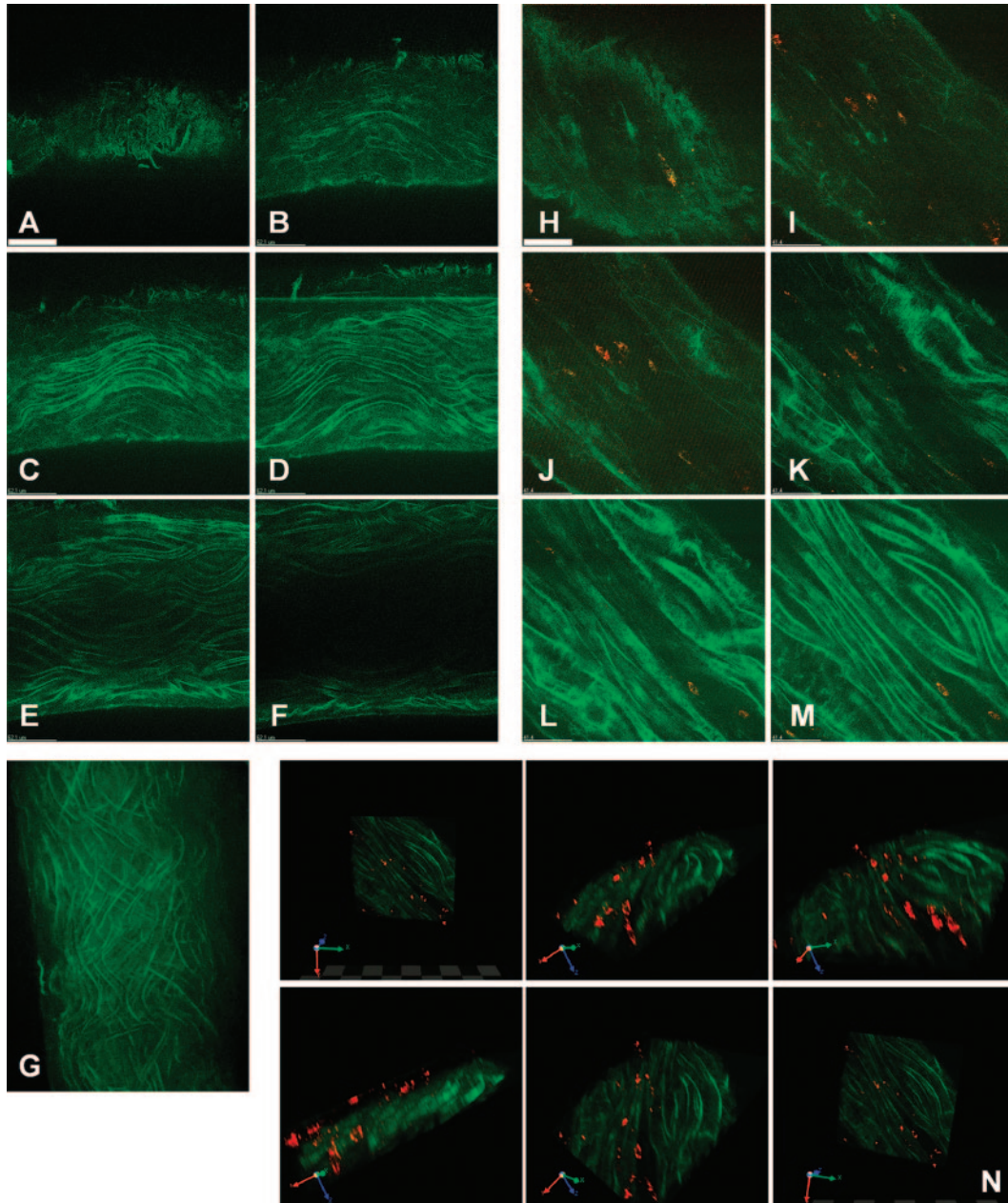
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In an unlabeled sample, the whole structure of the vessel, with adventitial connective tissue and elastin fibers in the media, was visible by autofluorescence.<sup>4</sup> A set of longitudinal sections of intact mouse carotid artery was imaged to demonstrate the large penetration depth and resulting potential of 2-photon imaging in visualization of 3-dimensional structural information in intact arteries. A, The first image ( $\sim 10 \mu\text{m}$  below the outer surface of the intact artery) is of the outer layer of the adventitia with autofluorescent connective tissue. B, The next image ( $\sim 30 \mu\text{m}$  below the outer surface) originates from somewhat deeper in the adventitia, revealing more fibers. C, D and E, Entering the media further, elastin bands became clearly discernible. At approximately  $80 \mu\text{m}$  below the surface (F), the empty lumen finally was reached. For panels A through F, bar= $62 \mu\text{m}$ . G, Three-dimensional reconstruction of an intact mouse carotid artery. Three-dimensional reconstruction of whole-vessel wall structure (Movie I) is available as online supplemental data. At 24 hours after transfer, no lymphocytes were detectable in C57BL/6 mice carotid arteries (A-F). H-J, Several red-labeled lymphocytes were detectable, mainly in adventitia ( $\sim 10$  to  $30 \mu\text{m}$  below the outer surface) and within elastin fibers in media (K-M,  $\sim 30$  to  $50 \mu\text{m}$  below the outer surface), as shown by the set of longitudinal sections of carotid arteries from apolipoprotein-E-deficient mice. For panels H through M, bar= $41 \mu\text{m}$ . N, Three-dimensional reconstruction of lymphocyte infiltration in the entire mouse carotid artery, also available as online supplemental data (Movie II). Each imaged volume consisted of 16 to 50 planes,  $2.5 \mu\text{m}$  apart.

nm (autofluorescence) from that above  $600 \text{ nm}$  (red label). The scans were acquired with 500 lines per second and between  $256 \times 256$  and  $1024 \times 1024$  pixel boxes, for a frame rate of 1.95 frames per second.

As shown in Figure 1 and in the accompanying online

3-dimensional movies, this system enabled us to clearly image lymphocyte infiltration mainly within the adventitia of an intact mouse carotid artery. Consequently, this system could be a powerful tool to study immune cell behavior in artery disease.

## Disclosures

None.

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