Nonlinear Optical 3-Dimensional Method for Quantifying Atherosclerosis Burden

Susana A. Sánchez, PhD; Nerea Méndez-Barbero, BSc; Antonio M. Santos-Beneit, PhD; Vanesa Esteban, PhD; Luis J. Jiménez-Borreguero, MD; Miguel R. Campanero, PhD; Juan Miguel Redondo, PhD

Changes in atherosclerosis burden provide an experimental measure of the effectiveness of therapeutic strategies. Unfortunately, none of the methods used in mice to assess atherosclerosis burden "ex vivo" is sufficiently accurate and fast enough to process the number of samples required in preclinical studies. We aimed to design an easy-to-implement and relatively fast method for accurate volumetric quantification of atheroma plaque burden in mice.

The apolipoprotein E-deficient mouse (Apoe\(^{-/-}\)) is a widely used preclinical model that reproducibly develops hypercholesterolemia and atherosclerosis.\(^1\) Two widely used methods for "ex vivo" assessment of atherosclerosis burden in mice are histopathologic analysis\(^2\) and the en face method.\(^3\) The histological method requires serial axial sectioning of arteries and subsequent histopathologic staining. This approach is labor intensive, and its inherent destructiveness undermines the accuracy of lesion-volume determinations. In en face analysis, the aorta is opened longitudinally and stained to reveal lipid-laden plaques; the stained area is quantified from photographs. The speed and ease of en face analysis have made it the most widely used method for measuring atherosclerotic plaques "ex vivo," but this method gives only a 2-dimensional (2D) measure of plaque burden.

Here, we describe the nonlinear optical 3D (NLO-3D) method. Aortas from euthanized Apoe\(^{-/-}\) mice fed a high-fat diet were dissected, stained with oil red O as described,\(^4\) and placed in a holder to permit measurement by either NLO-3D imaging or the en face method (Figure 1A in the Data Supplement). We used a single excitation wavelength (800 nm) of a commercially available 2-photon instrument to simultaneously generate 3 confocal signals from these aortas (Figure 1B in the Data Supplement): second harmonic generation from collagen and 2-photon excitation fluorescence from elastin and from oil red. A Zeiss 780 upright microscope equipped with a \(\times10\) air objective (Zeiss Plan Apochromat/0.45 NA) and coupled to a Spectra-Physics Mai Tai Deep Sea Ti: Sapphire laser system with an 80-MHz repetition rate and a 70-fs pulse width was used for these studies. Optical sections were taken every 3 \(\mu\)m, and Imaris was used for 3D image reconstruction and volume determination from the voxel information provided.

A 3D/3-color image reconstruction of a single atheroma plaque is shown in Figure 1A and Movie I in the Data Supplement. The shape of each atheroma was defined by collagen (red) and elastin (green) signals, the Oil Red signal (pink) identified the damaged area, and the combination of these signals (merge) allowed full 3D/3-color reconstruction (Figure 1A). Plaque cross-sections reveal a well-defined structure with a black core (Figure 1B; Movie I in the Data Supplement) because of the penetration limit of infrared light in this tissue (50–70 \(\mu\)m). This issue, far from being problematic, provides an additional reference parameter for localizing the structure and determining its size. The top of each plaque was defined by the 3 signals (Figure 1B, z1), middle sections were defined by the 3 signals surrounding the black core (Figure 1B, z2 and z3), and the base of the structure by the elastin and collagen signals from the vessel, the presence of the black core, and the absence of oil red labeling (Figure 1B, z4). To obtain 3D/3-color images of a full aortic arch, a 4×4 mosaic imaging protocol was used: each image in the mosaic corresponded to 512×512 pixels of an area of 1212.2 \(\mu\)m\(^2\) (Movie II in the Data Supplement).

The manual option on the Imaris program was used to draw the atheroma perimeter of each z-section, starting from the top and continuing through all sections (z spacing=3 \(\mu\)m) to the base of the atheroma, defined by the vessel wall (Figure 1C, z1–z3). Data processing generates a 3D structure (Figure 1C, volume [z planes]). Imaris determines the height and calculates total volume (159 \(\mu\)m and 6.15×10\(^5\) \(\mu\)m\(^3\), respectively, in this example) from the area occupied by the atheroma in each z plane and the z spacing between the images (Figure 1D). For atheromas located in vessel folds, we confirmed the volume obtained with z sections by generating y sections (Figure 1E).
After NLO-3D imaging (Figure 1F), the same plaques were sectioned and histologically stained, and plaque volume was determined (Figure 1G). The image of a 3D reconstructed aortic arch obtained by the NLO-3D method shows, in yellow, the reconstructions generated by the software for the 2 plaques present (Pa and Pb) (Figure 1F). The dotted line indicates the approximate plane of sectioning of the paraffin block used for histological analysis. We obtained 96 (Pa) and 84 (Pb) 5-μm sections from these atheromas and stained them with Masson trichrome. The lesion area was easily distinguished for quantification (Figure 1G, left). Images of these sections were realigned to adjust for shape distortion inherent to sample processing (fixing and cutting), and total atheroma volume was calculated (Imaris) from the 3D reconstructed image generated from the stack of photographs (Figure 1G, right). Plaque volumes estimated by NLO-3D and from cross-sectional histology images broadly agreed (Figure 1H).

To compare the NLO-3D and en face methods, we evaluated atherosclerosis burden in the aortic arch of Apoe−/− mice. Samples were held in the same sample holder for both experiments to maintain their position. Assessment of area and volume of each atheroma from a single aortic arch suggested that these parameters might not necessarily correlate (Figure 2). Discrepancies could be explained by height differences in some cases (plaques P1, P2, and P3) but not others: P5 is 40% taller than P4 but has a smaller volume, whereas P7 is similar in area to P6 but is 51% smaller. Differences could also be explained by the complex 3D structures revealed by NLO-3D. For example, atheromas P1 to P3 are near cylindrical, with height correlating directly with volume, whereas P5 has a pronounced restriction that might explain its lower volume compared with P4, despite its greater height (Figure 2). P6 and P7 present a more extreme case: P6 resembles a flattened cone, whereas P7 emerges from the vessel wall as a single structure before splitting into 2 inverted cone-shaped structures (Figure 2).

We exploited the accuracy of plaque volume determination and shape definition by NLO-3D to create a computer-generated reconstruction and visualize the degree of stenosis. The image obtained by NLO-3D was bent until the vessel closed, and stenosis was assessed in transverse slices of the sample (Figure 3) or in a virtual trip along the vessel (Movie III in the Data Supplement).

NLO-3D has high spatial resolution and reproducibility, is non-destructive, accurately determines atherosclerosis burden, is fast enough to process numerous samples, and allows visualization of stenosis. NLO-3D is likely to become the method of choice for quantifying atherosclerosis burden in preclinical studies.

Acknowledgments
We thank Dr Bartlett for English editorial work, R. Alberca for technical support, and Dr Pompa and A.L. Armesilla for critically reading the article.

Sources of Funding
Dr Redondo is supported by the Spanish Ministry of Economy and Competitiveness (MINECO; SAF2009-10708 and SAF2012-34296) and Fundación La Marató TV3 (264/C/2012). The Red de Investigación Cardiovascular of the Spanish Ministry of Health (Ministerio de Sanidad) supports Dr Redondo (RD12/0042/0022) and Dr Jiménez-Borreguero (RD12/0042/0056). Dr Campanero was supported by MINECO (SAF2010-15126). The Centro Nacional de Investigaciones Cardiovasculares (CNIC) is supported by the Spanish Ministry of Science and Innovation and the Pro-CNIC Foundation. N. Méndez-Barbero holds a Formacion del Profesorado Universitario fellowship (FPU2008-1500).

Disclosures
None.

References

Keywords: 2-photon excitation ▪ atherosclerosis burden ▪ infrared pulsed laser ▪ plaque volume ▪ second harmonic generation
Figure 1. Volume determination by the nonlinear optical 3-dimensional (NLO-3D) method. A, NLO-3D reconstruction (83 optical sections at 3-μm intervals) of a single atheroma plaque using separate signals for collagen (red), elastin (green), oil red (pink), and mixed signals. Bar, 100 μm. B, Optical z sections taken at the top of a plaque (z1), through the center (z2 and z3), and at the base (z4). Collagen in red, elastin in green, and oil red in pink. The merging of collagen and elastin signals produces a yellowish color over most of the vessel wall. Bar, 500 μm. C, z plane volume determination: starting from the top of the atheroma (z1), the contour of the structure was hand drawn at each z plane (z2 and z3). The plaque is reconstructed with Imaris software (volume [z planes]), and its volume is calculated from the contour (area) and the slice spacing (3 μm). D, Representation of atheroma volume as the summed volumes of slices with area A and thickness Δz. E, y sections (y1–y3) were created from the same data set used in C. The base of the atheroma was interpolated to determine the surface of each section. The plaque was reconstructed with Imaris (volume [y planes]), and values of volume determination of the same atheroma using z and y planes are shown. F, NLO-3D image showing 2 reconstructed plaques (Pa and Pb) in yellow. G, Left, Representative histological sections of atheromas Pa and Pb stained with Masson trichrome, in which the lesion (dotted outlines) can be distinguished from the media layer. Images were acquired with a Leica DM2500 microscope fitted with a ×5 HCX PL Fluotar objective. Right, Three-dimensional reconstruction performed using Imaris software (shown in yellow) of images from the histological analysis of Pa (96 sections) and Pb (84 sections). H, Volume of plaques Pa and Pb determined by NLO-3D and histological analysis (Paigen). AF indicates autofluorescence; and SGH, second harmonic generation.

Figure 2. Comparison of atherosclerotic burden by en face and nonlinear optical 3-dimensional (NLO-3D). En face photographs and 3D reconstruction images of atheromas from a single aortic arch. Side views are shown for 3D images to better visualize height and shape of individual plaques. Height (h), area and volume determinations are shown.
Figure 3. Computer-generated image reconstruction of an apolipoprotein E–deficient mouse aorta presenting atheromic plaques. **A** to **E**, Sequential steps in the reconstruction with Maya software. **A**, The original 3-dimensional (3D)-3-color image obtained by nonlinear optical 3D (NLO-3D; gray) and the Imaris-generated volume structures (red) were used as the template. **B**, The template was overlaid with a net to set Z coordinates according to the volume determination values. **C** to **E**, The same net was used to pull the cut sides of the vessel together. **E**, The degree of stenosis can be appreciated by comparing transverse sections of the atherosclerotic aortic arch (1–4) and the non-atherosclerotic thoracic aorta (5–8). **F**, The trajectory shows the path followed by the virtual camera in Movie III in the Data Supplement.
Nonlinear Optical 3-Dimensional Method for Quantifying Atherosclerosis Burden
Susana A. Sánchez, Nerea Méndez-Barbero, Antonio M. Santos-Beneit, Vanesa Esteban, Luis J. Jiménez-Borreguero, Miguel R. Campanero and Juan Miguel Redondo

Circ Cardiovasc Imaging, 2014;7:566-569
doi: 10.1161/CIRCIMAGING.114.001753
Circulation: Cardiovascular Imaging is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-9651. Online ISSN: 1942-0080

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circimaging.ahajournals.org/content/7/3/566

Data Supplement (unedited) at:
http://circimaging.ahajournals.org/content/suppl/2014/05/15/CIRCIMAGING.114.001753.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Cardiovascular Imaging can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Cardiovascular Imaging is online at:
http://circimaging.ahajournals.org//subscriptions/
VIDEOS FILES

Movie S1. NLO-3D imaging of an Oil-Red stained atheroma from an ApoE<sup>−/−</sup> mouse. A 3D reconstructed zoomed image (scanned area, 600 µm<sup>2</sup>) of one of several atheromas detected in the aorta of an Apoe<sup>−/−</sup> mouse is shown as an example of the experimental approach. Several z-planes were imaged with a femtosecond (fs) pulsed IR laser at 800 nm and three signals were simultaneously obtained: SHG signal from collagen at 400 nm (red), elastin autofluorescence (AF) at 490-575 (green), and Oil Red fluorescence at 600-680 nm (pink). The scanned z-planes appear in the following order: collagen alone (red), collagen (red) + elastin (green), and collagen (red) + elastin (green) + Oil Red (pink). In the full reconstruction (three signals), different signal combinations are shown to highlight details: elastin alone (green), elastin (green) + Oil Red (pink), elastin (green) + Oil Red (pink) + collagen (red). The dark center observed in the core of the atheroma is the result of the ~100 µm penetration limit of IR light in this tissue. Images were acquired with a Zeiss 780 upright microscope (Zeiss, Germany) fitted with a 10X air objective (Zeiss Plan Apochromat/ 0.45 N.A,) and coupled to a Spectra-Physics Mai Tai Deep Sea Ti: Sapphire laser system (Irvine, CA. USA) with an 80 MHz repetition rate and a 70 fs pulse width. 3D images were reconstructed and movies assembled with Imaris software.

Movie S2. NLO-3D imaging of an Oil-Red stained aortic arch from an ApoE<sup>−/−</sup> mouse. 3D reconstructed image (scanned areas of 5 x 3.8 mm<sup>2</sup>) of a mouse aortic arch with extensive atherosclerosis. Several z-planes were imaged and three signals were simultaneously obtained: SHG signal from collagen at 400 nm (red), elastin autofluorescence (AF) at 490-575 (green) and Oil Red fluorescence at 600-680 nm (pink). The scanned z-planes appear in the following order: collagen alone (red), collagen (red) + elastin (green), and collagen (red) + elastin (green) + Oil Red (pink). In the full reconstruction (three signals), the presence of collagen (red) directly beneath the elastin can be readily observed on the several vessel folds and also on top of the large central atheroma. A zoomed view of an atheroma shows the z-sections (starting from the top) used for volume determination and the central black core due to the limit of laser penetration. Images were acquired with a Zeiss 780 upright microscope (Zeiss, Germany) fitted with a 10X air objective (Zeiss Plan Apochromat/ 0.45 N.A,) and coupled to a Spectra-Physics Mai Tai Deep Sea Ti: Sapphire laser system (Irvine, CA. USA) with an 80 MHz repetition rate.
rate and a 70 fs pulse width. 3D images were reconstructed and movies assembled with Imaris software.

**Movie S3. Virtual voyage through an atherosclerotic vessel.** The NLO-3D 3-color image was computer processed to generate an intact vessel to reveal the degree of stenosis. A virtual camera was passed through the vessel lumen, recording a video of the vessel interior along the trajectory indicated in Figure 3F. The atheroma surface is shown in red.