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A comparative analysis of the collagen architecture in the carotid artery: Second harmonic generation versus diffusion tensor imaging

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ABSTRACT

Collagen is the main load-bearing component of the artery. The 3D arrangement of the collagen fibers is crucial to understand the mechanical behavior of such tissues. We compared collagen fiber alignment obtained by second harmonic generation (SHG) microscopy with the alignment obtained by diffusion tensor imaging (DTI) throughout the wall of a porcine carotid artery to check the feasibility of using DTI as a fast and non-destructive method instead of SHG. The middle part of the artery was cut into two segments: one for DTI and one for the SHG measurements. The tissue for SHG measurements was cut into 30 μm tangential sections. After scanning all sections, they were registered together and the fiber orientation was quantified by an in-house algorithm. The tissue for DTI measurement was embedded in type VII agarose and scanned with an MRI-scanner. Fiber tractography was performed on the DTI images. Both methods showed a layered structure of the wall. The fibers were mainly oriented circumferentially in the outer adventitia and media. DTI revealed the predominant layers of the arterial wall. This study showed the feasibility of using DTI for evaluating the collagen orientation in native artery as a fast and non-destructive method.

1. Introduction

The arterial wall consists of extracellular matrix (ECM) and several types of cells organized into different layers. Collagen, a major structural compound of the ECM, is largely responsible for the mechanical behavior of the artery [1]. Collagen orientation in native artery has been expressed as circumferential [2–5], helical [2,6], or axial [2,7]. Although there is variation between findings and it might be because different arteries were analyzed, most studies proved that the fiber orientation differs between media and adventitia [7,8]. Further studies indicated a variation in fiber orientation in the inner media region, which is assumed to be due to the transmission of shear stress from the flowing blood into the inner layers of the media [9,10]. These architectures were observed using different methods, such as polarized light microscopy [11], X-ray diffraction [12] and nonlinear optical microscopy (NLOM) [13,14]. These methods either have a limited imaging depth into the arterial wall like polarized light microscopy or have too elaborate set up like NLOM. The search for a non-invasive and fast imaging methodology to assess the three-dimensional (3D) fiber architecture in vascular tissue is still ongoing.

SHG is a high resolution nonlinear optical microscopy technique, which has been used to visualize non-centrosymmetric biological molecule structures [15–18]. Collagen has a non-centrosymmetric crystalline triple helix structure, which means that it is capable of emitting SHG light [19]. This technique enables volume reconstruction from serial sections through thick specimens. Moreover, it provides the information regarding 3D fiber structure in biological tissues with a high spatial resolution.

DTI is a non-invasive magnetic resonance imaging (MRI) technique, which measures water diffusion and gives important information about the tissue's microstructural organization [20]. The water molecules motion is normally random and isotropic, but anisotropic in tissues with fibrous architecture as the water molecules diffuse more easily along fibers than across them. The diffusion direction of water molecules is expressed by a 3D diffusion tensor in each voxel. The principal direction of water is represented by the first eigenvector of the diffusion tensor. This process of determining fiber structure from diffusion tensor is named tractography [21]. This method has been used for cartilage [22,23], bone [24], heart [25,26], muscle [27], and also for cartilage under compression to evaluate the fiber architecture changes under loading [28].

Second harmonic generation microscopy (SHGM) and DTI are both suitable for evaluating collagen architecture, but in different ways. The resolution of SHGM images is higher than DTI and it is widely accepted to be used as a method to measure collagen fiber

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alignment [19,29–31]. On the other hand, DTI is a non-invasive and non-destructive technique to observe the microstructural organization of the tissue [22,25,32]. Moreover, a DTI scan is much less time-consuming than SHGM when scanning the whole tissue.

The aim of this work is to assess the feasibility of visualizing the collagen structure of native arteries using DTI. To validate the DTI results, SHGM is performed, which measures comprehensively the 3D collagen structure within the tissue across the whole thickness of the artery wall.

2. Materials and methods

2.1. Sample preparation

A carotid artery was harvested as slaughterhouse material of a one-year old pig. The artery was excised from the surrounding tissue and carefully cleaned. The middle part of the artery was cut into two segments: the proximal portion was used for SHGM and the distal portion was used for DTI. The proximal part was placed in a cryomold (Sakura, USA), with the longitudinal side of the artery parallel to the face of mold, and embedded in tissue-Tek O.C.T compound (Sakura, USA). Three 0.3-mm diameter graphite leads were embedded next to the artery as a spatial reference system for later 3D reconstruction of the artery from individual cryosections (Fig. 1A). The block was mounted on cryotome chucks and was cut in 30 μm tangential sections from the outer edge to the lumen since the fibers are expected to run in the x-y plane [33]. At the end, there were 40 sections visualized by SHGM. The sections were thaw-mounted onto polylysine slides (Thermo Scientific). The distal part was placed in a cryovial (Sigma, USA) and embedded in 4% type VII agarose (Sigma, USA) for DTI measurements.

2.2. SHGM

Collagen visualization was conducted on a Zeiss LSM 510 Meta laser scanning microscope (Carl Zeiss, Germany) attached to an inverted Axiovert 200 motorized microscope (Carl Zeiss, Germany). SHG imaging was performed using a mode-locked chameleon ultra 140 fs pulsed Ti-Sapphire laser (Coherent, USA). Collagen SHG signal was collected from the sample excited at 800 nm.

Tile scan images were taken to cover the whole area of each section with the graphite leads and the artery. Each tile was also scanned along the z-direction separately to create a z-stack (Fig. 1B). All tiles have the same size of 450 × 450 μm. On average, 18 × 13 tiles were needed to cover the whole area containing sample and graphite leads. The distance between subsequent images in the z direction was 2 μm. The scanning time for one section was about 7 h and total scanning time for the vessel was about 280 h. Positioning of the microscope stage was done automatically by programming the computerized x–y stage (Zeiss Multi Time Series software).

2.3. DTI

DTI scanning was performed with a 6.3 T horizontal-bore MRI scanner (Bruker, Ettlingen, Germany) using a home-built send-and-receive solenoid radiofrequency coil with a diameter of 10 mm and a length of 15 mm. Diffusion imaging was done with a three-dimensional spin-echo sequence with unipolar diffusion sensitizing pulsed field gradients placed symmetrically around the 180° radiofrequency pulse. Sequence parameters were: TE = 20 ms, TR = 1000 ms, NA = 1, field-of-view = 25.6 × 25.6 × 25.6 mm, acquisition matrix = 128 × 64 × 64, reconstruction matrix = 128 × 128 × 128, 10 diffusion directions with b-value = 900 mm^2/s, 1 image without diffusion weighting, total acquisition time = 12 h 30 min.

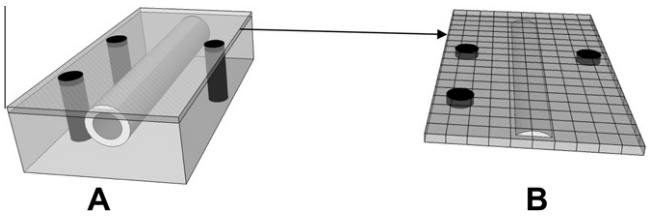

Fig. 1. (A) A schematic of the artery and graphite leads embedded in Tissue-Tek block with one slice on top as an example, (B) Representative stack mosaic of one entire slice.

2.4. Data and image analysis

The image stacks obtained from SHGM were stitched to make a stack mosaic of the entire slice. Next, the markers were registered together to align the stacks. Subsequently, the stack mosaics containing all images were rotated and aligned horizontally to calculate the orientation of fibers (θ) with the vertical axis ($\theta = 0$ indicates that the fiber is aligned in the circumferential direction and $\theta = 90$ indicates that the fiber is aligned axially). All the above steps were performed in Matlab (Mathwork, USA). A sub-image of 715 × 715 pixels was cropped through the whole volume from the total image volume for further analyses. An algorithm was developed in Mathematica (Wolfram, USA) as fully described by Daniels et al. (2006), in order to quantify the fiber orientations in the microscopy images with the cropped images as inputs [34]. The principal curvature directions, determined from the Hessian's matrix, were calculated per pixel. Furthermore, a histogram showing fiber angle percentage is exported from the program.

Fiber tractography was performed on the DTI data by an in-house-developed software program, referred to as the DTI tool [35]. In order to analyze the fiber orientation, the fiber coordinates as generated by the DTI tool were imported in Matlab for further analysis. First, the fiber coordinates were converted from the image reference system to a cylindrical coordinate system. Then, the orientation of fibers (θ) with the horizontal angle was obtained for a piece of the artery with the same dimensions as for the SHG method.

3. Results

Representative SHGM examples of collagen orientation histograms with their depicted locations within the arterial wall are shown in Fig. 2. Most fibers are aligned in circumferential direction in the outer adventitia region (Fig. 2A, E and I). The collagen orientation changed to almost axial direction from outer adventitia towards the lumen (Fig. 2B, F and J). Then, there were few images in which there are fibers in both axial and circumferential directions, most likely cause by the curvature of the artery. The fibers located in the next layer, the media, are mostly aligned in the circumferential direction (Fig. 2C, G and K). Lastly, there is a thin layer under the endothelial cells at the lumen where some fibers are oriented in the axial direction (Fig. 2D, H and l).

Fig. 3A shows fiber-tracking, representing the fibrous structure, generated by the DTI tool. Fibers are color-coded based on their directions: red (x direction), green (y direction) and blue (z direction). The fibers as imported in Matlab for further analyses are shown in Fig. 3B. After converting the coordinate system from the image reference system to a cylindrical coordinate system, a piece of artery, as depicted in Fig. 3C, having the same dimensions as the SHG sample, was chosen for the orientation analyses. As it is shown in the Fig. 3, the fibers are predominantly oriented in the circumferential direction.

The collagen orientation, as demonstrated by 3D histograms through the arterial wall, shows a transition layer between media and adventitia with both DTI and SHG (Fig. 4A and C). The fiber

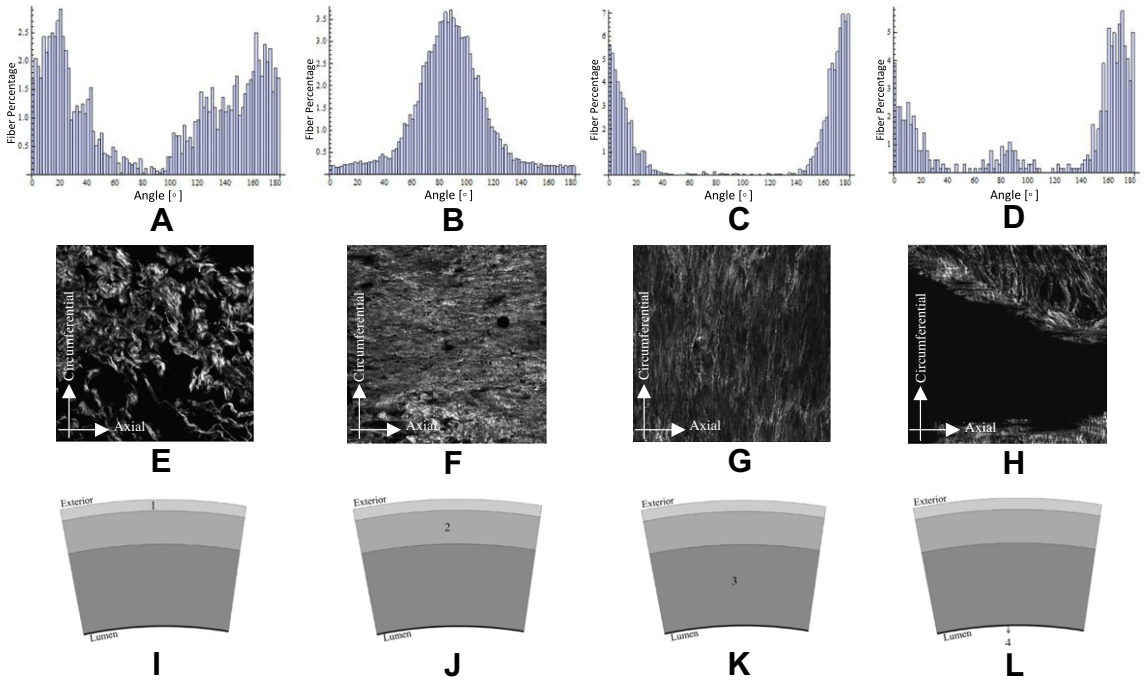

Fig. 2. Representative histograms (A,B,C,D) and corresponding images (628 × 628 μm, E,F,G,H) of the collagen orientation through the arterial wall from the adventitia (A,E,I) to the lumen (D,H,L). From outer adventitia towards the lumen, the fiber orientation changed from mainly circumferential direction (A,E,I) to the axial direction (B,F,J). The next layer, which is mainly media, contains fibers predominantly aligned in circumferential direction (C,G,K). Finally, there is a thin layer at the lumen side with fibers also in the axial direction (D,H,L).

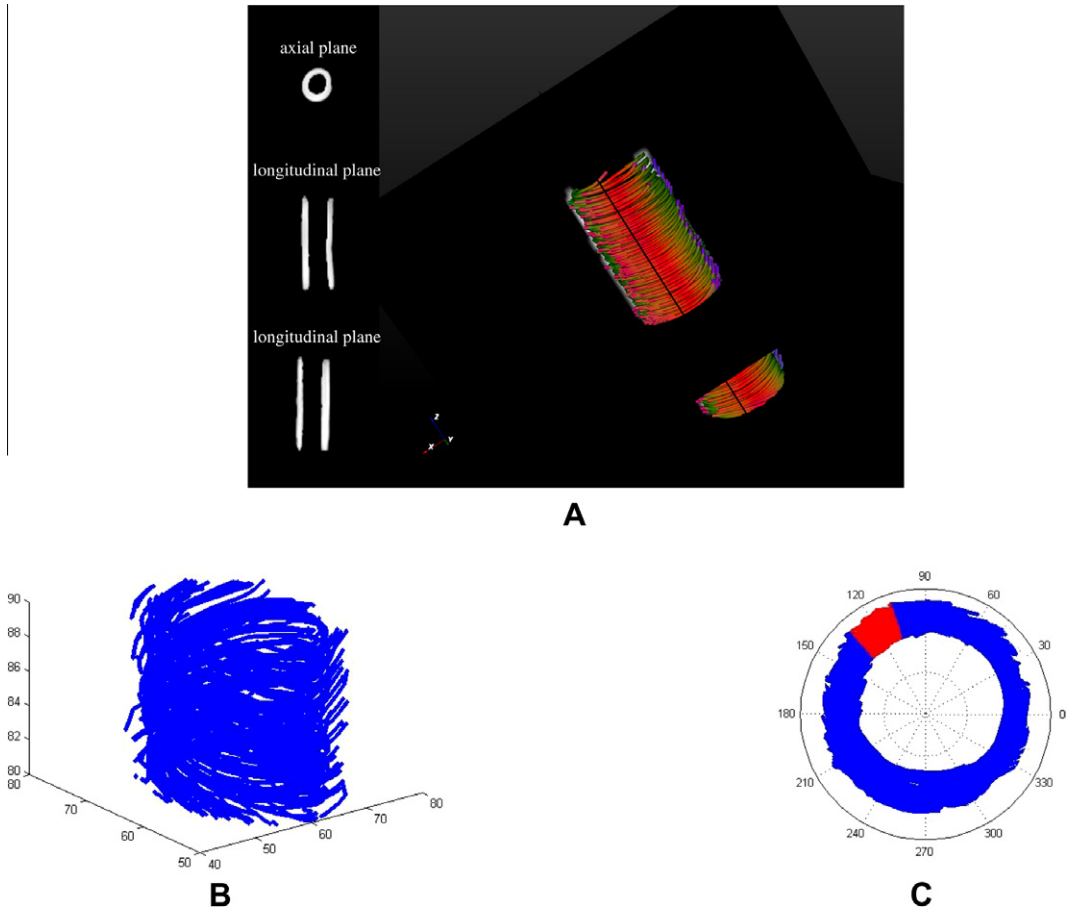

Fig. 3. (A) Fiber trajectories, as obtained by DTI, representing collagen fibers in the native artery: red (x direction), green (y direction) and blue (z direction), (B) imported fibers in Matlab before converting the coordinate system, (C) sub-volume of interest after converting the image reference system to a cylindrical coordinate system.

orientation within the transition layer from adventitia to media are about 90° in SHG, while they are between 60 and 80° in DTI. The thickness was normalized for both methods to compare the orientation within the same regions. The means of the DTI and SHG histograms for each layer are shown schematically to summarize the identified collagen orientation through the wall using both SHG and DTI (Fig. 4B and D).

4. Discussion

To understand the mechanical behavior of the artery, it is crucial to get insight into the arterial structure and particularly collagen organization. In this study, we visualized the collagen structure of a pig carotid artery using two methods. DTI was used as a fast and non-destructive method and SHG was used as a validation method. As to our knowledge, comprehensive information showing collagen fiber orientation throughout the full thickness of a carotid artery has not yet been published. This study was aimed at the use of DTI for evaluating the fibrous architecture of a complete arterial wall in vitro and assessing the agreement of the DTI data with SHG measurements.

The SHG method has been used tremendously and is an accepted method for visualizing collagen fibers [18,36]. Due to the collagen non-centrosymmetric triple helix structure, it can be visualized without staining. In order to visualize collagen fibers through a thick tissue, such as an artery by SHG microscopy, the tissue has to be sectioned in slices to have a suitable thickness for the laser to penetrate. Moreover, for analyzing the SHG images, the images have to be stitched to make the 3D reconstruction by

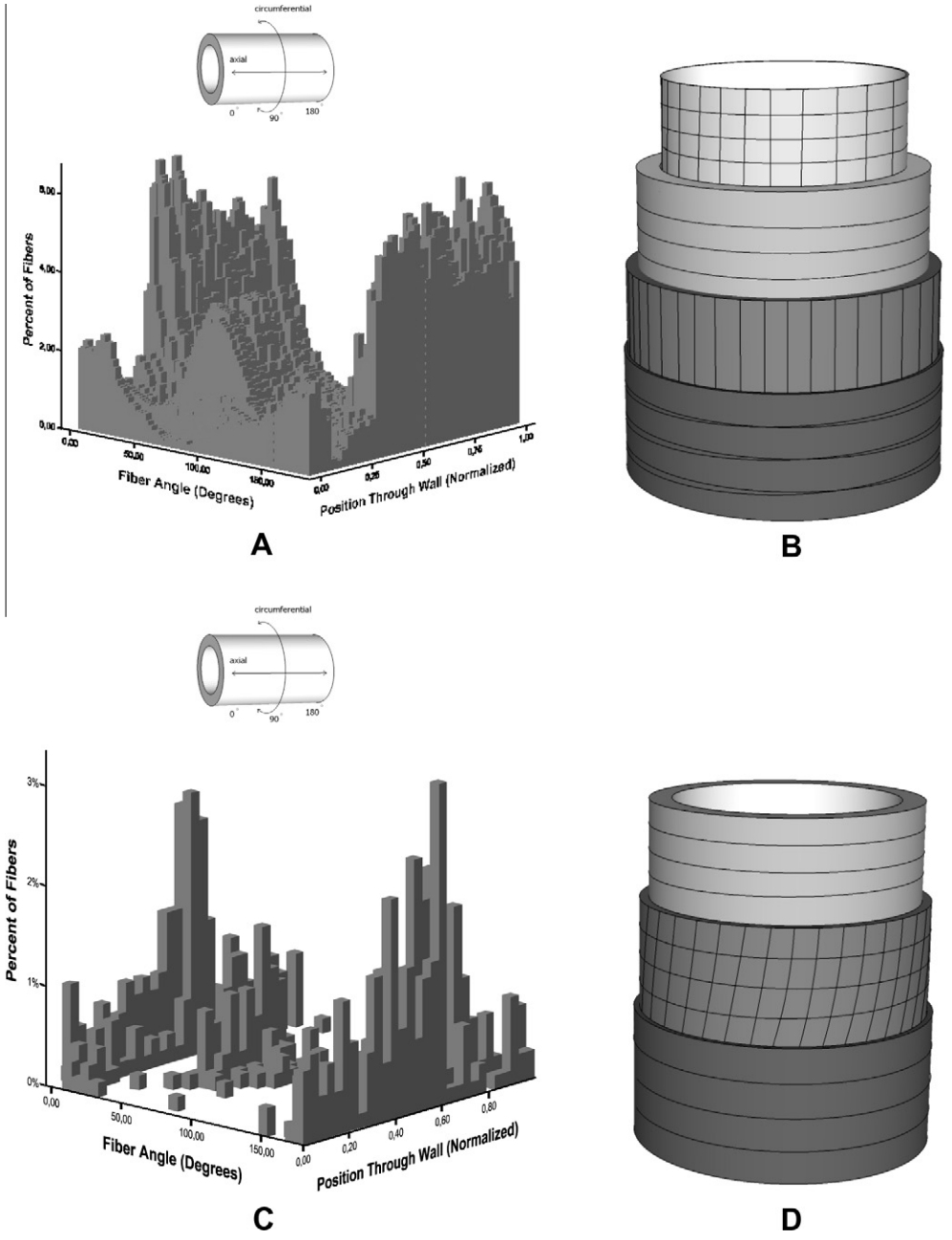

Fig. 4. (A and C) The collagen orientation shown by 3D histograms of SHG and DTI data through the arterial wall from the outer adventitia (0) to the lumen (1). As it is shown in the figure, 0 and 180° represent fibers aligned in circumferential direction and 90° represents axial direction. (B and D) Schematic drawing of the mean fiber orientation showing the different layers, next to their corresponding 3D bar charts.

the help of registration points. Then, all images need to be analyzed one by one. We assumed that the fibers are running in the cutting plane which might represent a limitation of this method as the out-of-plane fibers cannot be captured [33]. It has been shown recently that the main eigenvector in DTI images are representative of collagen orientation [22,23]. In this study, after performing fiber tractography on DTI data, the fibers eigenvalues were imported in MATLAB for further analyses.

Wicker et al. (2008) used NLOM to visualize the collagen structure in the basilar artery [13]. They observed that the orientation of fibers in the adventitia and media is mostly in circumferential direction. Furthermore, they showed a transition region between adventitia and media, similar to what we observed with both methods. Timmins et al. (2010) identified a very small region below the endothelial surface, in which there is a change in collagen fiber orientation, similar as observed in this study with SHGM [37]. O'Connell et al. (2008) demonstrated that the fibrous constituent of the artery are predominantly aligned in circumferential direction, similar to what we observed in the DTI results [38].

The results from both methods showed the architectural changes through the thickness of the arterial wall. Both methods show that there is a transition layer between the adventitia and the media of the artery. The orientation of collagen within this area is somewhat shifted in DTI from 90° compared to SHG to 60–80°. This difference might be due to the converting of the image reference system to a cylindrical coordinate system, which is inevitable. It is also important to consider how precise and for what aim one need to know the architecture. For instance, DTI shows the orientation in the predominant layers very well. In general, the results showed a good agreement for the collagen orientations for both methods.

The data as presented here shows the unloaded arterial wall architecture and is not representative for the in vivo loaded configuration. Now that the DTI is proved as a valid method to study collagen orientation in arteries, the method can be used as a fast method to visualize the in vivo state of the artery in future studies.

In conclusion, this study demonstrates the use of the DTI as a fast, non-destructive and reliable tool to study the arterial collagen structure. The validity of DTI method for quantifying the collagen architecture of more challenging aged and unhealthy artery should be studied. In future studies, DTI may be used to study changes in the arterial collagen structure by, for example, aging or disease condition.

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