Published in final edited form as:

J Biomech. 2014 March 21; 47(5): 935–943. doi:10.1016/j.jbiomech.2014.01.027.

A Custom Image-Based Analysis Tool for Quantifying Elastin and Collagen Micro-Architecture in the Wall of the Human Aorta from Multi-Photon Microscopy

Ryan G. Koch, BSc^{#1}, Alkiviadis Tsamis, PhD^{#1,4,6}, Antonio D'Amore, PhD^{6,7,8}, William R. Wagner, PhD^{1,3,4,6}, Simon C. Watkins, PhD⁵, Thomas G. Gleason, MD^{1,2,4,6}, and David A. Vorp, PhD^{1,2,3,4,6}

¹Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, United States

²Department of Cardiothoracic Surgery, University of Pittsburgh, Pittsburgh, PA, United States

³Department of Surgery, University of Pittsburgh, Pittsburgh, PA, United States

⁴Center for Vascular Remodeling and Regeneration, University of Pittsburgh, Pittsburgh, PA, United States

⁵Center for Biologic Imaging, University of Pittsburgh, Pittsburgh, PA, United States

⁶McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, United States

⁷Fondazione Ri.MED, Palermo, Italy

⁸DICGM University of Palermo, Palermo, Italy

[#] These authors contributed equally to this work.

Abstract

The aorta possesses a micro-architecture that imparts and supports a high degree of compliance and mechanical strength. Alteration of the quantity and/or arrangement of the main load-bearing components of this micro-architecture the elastin and collagen fibers leads to mechanical, and hence functional, changes associated with aortic disease and aging. Therefore, in the future, the ability to rigorously characterize the wall fiber micro-architecture could provide insight into the complicated mechanisms of aortic wall remodeling in aging and disease. Elastin and collagen fibers can be observed using state-of-the-art multi-photon microscopy. Image-analysis algorithms have been effective at characterizing fibrous constructs using various microscopy modalities. The

© 2014 Elsevier Ltd. All rights reserved

Corresponding author: David A. Vorp, PhD Associate Dean for Research, Swanson School of Engineering William Kepler Whiteford Professor of Bioengineering Professor of Cardiothoracic Surgery, and Surgery University of Pittsburgh 123 Benedum Hall 3700 O'Hara Street Pittsburgh, PA 15261, United States Business Telephone: 412-624-8503 Fax: 412-624-0412, vorp@pitt.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST STATEMENT The authors do not have any financial interest or other relationship (grant, research support, consultant, etc.) with any manufacturer(s) of any commercial product(s) to disclose.

objective of this study was to develop a custom MATLAB-language automated image-based analysis tool to describe multiple parameters of elastin and collagen micro-architecture in human soft fibrous tissue samples using multi-photon microscopy images. Human aortic tissue samples were used to develop the code. The tool smooths, cleans and equalizes fiber intensities in the image before segmenting the fibers into a binary image. The binary image is cleaned and thinned to a fiber skeleton representation of the image. The developed software analyzes the fiber skeleton to obtain intersections, fiber orientation, concentration, porosity, diameter distribution, segment length and tortuosity. In the future, the developed custom image-based analysis tool can be used to describe the micro-architecture of aortic wall samples in a variety of conditions. While this work targeted the aorta, the software has the potential to describe the architecture of other fibrous materials, tube-like networks and connective tissues.

Keywords

Multi-photon Microscopy; Collagen; Elastin; Micro-architecture; Quantification; Fiber Orientation

INTRODUCTION

Soft fibrous tissues – e.g., ligaments, the heart, blood vessels, gastrointestinal tract and the urethra – perform functions which rely on a high degree of elasticity or compliance (Lentle *et al.*, 2013; Mijailovich *et al.*, 2007; Westerhof *et al.*, 2005). The elastic properties of these tissues are supported by a complex underlying fiber micro-architecture (Halloran *et al.*, 1995; Sokolis and Sassani, 2013; Wu *et al.*, 2011). The two main load-bearing constituents, elastin and collagen, impart the elasticity and strength of soft fibrous tissues. Alteration of the content and/or arrangement of these fibers leads to the mechanical, and hence functional, changes associated with diseases or disorders of these tissues. Therefore it is important to extract detailed microstructural information from soft fibrous tissue to reveal possible mechanisms of diseases or disorders; i.e., relating changes in micro-architecture to tissue function and dysfunction.

Elastin and collagen are easily visualized based on their intrinsic fluorescence and second-harmonic generation, respectively (Cahalan *et al.*, 2002; Jiang *et al.*, 2011; Konig *et al.*, 2005; Strupler *et al.*, 2007). Multi-photon micrographs of these extracellular matrix components could be processed using an appropriate image-based analysis tool to obtain micro-architectural characteristics. Prior image-analysis paradigms such as Hough transforms (Chaudhuri *et al.*, 1993), intensity gradient-based texture analysis algorithms (Chaudhuri *et al.*, 1993; Courtney *et al.*, 2006; Karlon *et al.*, 1998), direct-tracking methods (Pourdeyhimi *et al.*, 1999; Pourdeyhimi *et al.*, 1996a; Pourdeyhimi *et al.*, 1996b), and fast Fourier transform (FFT)-based image-analysis (Ayres *et al.*, 2006; Ayres *et al.*, 2007; Ayres *et al.*, 2008) have been successfully implemented to quantify fiber architecture of native and engineered tissues. However, these image-analysis methodologies do not offer a complete description of the fiber-network topology as they focused primarily on orientation-based information. D'Amore *et al.* (2010) successfully developed an image-based analysis tool to effectively characterize the architecture of engineered-tissue fiber-networks viewed with standard scanning-electron microscopy (SEM) based on a Delaunay-network skeleton and

image-gradient information. The detected and quantified micro-architectural features included the fiber angle distribution, node connectivity, spatial-intersection density, and fiber diameter.

Multi-photon images of elastin and collagen, containing different levels of noise and fluorescence when compared with images from different imaging modalities such as SEM, require an optimized image-analysis tool to provide a rigorous description of micro-architectural characteristics. Characterization of fiber orientation in soft fibrous tissues would be possible using FFT, direct-tracking or texture-based methods (D'Amore *et al.*, 2010). However, these methods would need to be modified to be compatible with the image quality from multi-photon microscopy to sufficiently describe the complicated micro-architecture of soft fibrous tissues.

The goal of this study was to develop a custom, image-based analysis tool to describe multiple parameters of elastin and collagen micro-architecture in human soft fibrous tissue samples using multi-photon images. This goal was achieved using a MATLAB language-based code that utilizes a novel fiber skeletonization approach optimized for multi-photon images of collagen and elastin. Human aorta samples were used to develop the code.

METHODS

Human Aorta Specimens

To illustrate the utility of our tool on soft fibrous tissues, we used human ascending thoracic aorta (ATA) tissue specimens as a test bed. ATA specimens were obtained after informed consent according to the guidelines of our Institutional Review Board and the Center for Organ Recovery and Education (Pittsburgh, Pennsylvania). In brief, segments of ATA were either collected from organ donor/recipient subjects or obtained from patients undergoing elective ATA repair at the University of Pittsburgh Medical Center. Tissue specimens from patients aged 39–81 and aortic diameter 46–68 mm (8 males/10 females) were first perfusion-fixed in 4% paraformaldehyde. After 1.5hr, the solution was replaced with PBS and stored at 4°C.

Multi-Photon Microscopy

An Olympus multi-photon microscope (Model FV10, ASW software) was used to observe elastin and collagen fibers in the ATA (Cahalan *et al.*, 2002; Jiang *et al.*, 2011; Konig *et al.*, 2005). Elastin (green) and collagen (red) were automatically detected and visualized based on intrinsic fluorescence (channel RXD1, wavelength $525\pm 25\text{nm}$) and second-harmonic generation (channel RXD2, wavelength $400\pm 50\text{nm}$), respectively. Figure 1 shows a representative multi-photon image ($500\times 500\mu\text{m}^2$) from ATA.

Image-Processing

To analyze the multi-photon images of ATA, a custom MATLAB image-analysis tool was developed utilizing the Image-Processing Toolbox. The input image (Fig. 2-A) was selected through a user-interface dialog box. Information for a given fiber type was saved in one

channel (red, blue or green). Accessing that channel provided the information to start processing (Fig. 2-B).

Intensity-equalization and fiber-extraction—The image was smoothed and fiber edges preserved using the MATLAB function (MF) for median filtering (3×3 window, MF *medfilt2*), then contrast-enhanced using contrast-limited adaptive histogram equalization (3×3 window, MF *adapthisteq*) and a histogram adjustment (MF *imadjust*), where 1% of the image was saturated at the extremes (Fig. 2-C). The image was adjusted to equalize the noise and intensity levels between inputs and to ensure the maximum available dynamic range was utilized. Noise and background information was suppressed and fibrous information enhanced using multi-scale vessel-enhancement filtering (Frangi *et al.*, 1998; Kroon, 2010). The Frangi algorithm analyzed intensity gradients, looking for tube-like vessels. The filter emphasized these components while suppressing non-tube-like information. Because of the similarity of fibers to vessels, the technique isolated the fiber content from the background material. Threshold-filtering was then performed on the image using a value of 0.4 as the cutoff-intensity by creating a binary, black and white image, and segmenting the fiber information from the background (Fig. 2-D). The value 0.4, fixed for all images, was qualitatively determined to minimize fiber diameter deviations from the grayscale to binary image. A static value was able to be used because of the intensity equalization steps preceding this threshold. A Euclidean-distance transform (MF *bwdist*) was performed on the complement of the binary image to find the distance from every pixel within the fibers to the nearest edge of the fiber. A watershed-transform (MF *watershed*) was then performed to identify ridges in the distance-transformed image marking an approximation of the center of the most well-defined fibers. This image was then used as a mask on the distance-transformed image to aggregate initial fiber diameter values. The median of the fiber diameter values was chosen as the representative fiber diameter (FD). Due to artifacts at the edges of the image from the filtering techniques, FD-pixels were cropped from the four image edges. The result was a binary, black and white (BW) image.

Fiber-skeletonization and locations of intersections—The BW image was subjected to a series of morphological operations. These operations further smoothed the edges of the fibers and removed small holes and fiber fragments that might have been background or incomplete information. First the BW image was eroded (structuring element (SE) disk radius = FD/8, MF *imerode*), then dilated (SE disk radius = FD/4, MF *imdilate*), then eroded (SE disk radius = FD/8). The image was eroded before dilation to allow for smoothing without closing small gaps between fibers. The re-erosion returned the fibers to their original size, but with smoother edges. The resulting image was morphologically thinned (MF *bwmorph*('thin')) to form a 1-pixel thick representation of the fibers. Segments with areas smaller than $2 \times \text{FD}$ were considered negligible fragments and removed (MF *bwareaopen*). The skeleton was dilated (SE disk radius = FD/2) and holes smaller than $\pi \times (\text{FD}/2)^2$ were removed. Small branches were pruned from the skeleton and neighboring intersections were merged by dilating the intersection points (SE disk radius = FD+FD/2) and re-thinning the image. The resulting image was a 1-pixel thick fiber skeleton (FS) (Fig. 2-E). To calculate a fiber diameter distribution in the image, a Euclidian-distance transform was performed on the final BW image. The resulting image was then masked using the FS to

collect fiber radii values that corresponded to the FS. The FS was overlaid on the original image (Fig. 2-F) to qualitatively assess the accuracy.

Segmented skeleton, tortuosity and orientation—The intersections were removed from the FS by dilating intersection points (SE square length = 3), to create a segmented skeleton (SS) consisting of individual fiber segments. The SS provided clear, definable fiber segment endpoints for calculations. The SS was used to calculate fiber tortuosity (Medyukhina *et al.*, 2011), defined as the length of a segment divided by the distance between the segment's endpoints. Each segment of the SS was sampled every FD-pixels along its length. The sub-segments orientation was determined by the angle of the tangent line between the first and last points of the subsegment. The resulting histogram's main bin count can be anticipated as shown in Appendix I. The FD was chosen as the sampling distance, because it is large enough to provide angular degrees of freedom and small enough to capture fiber tortuosity.

Concentration, porosity and density of intersections—A region of interest was selected by the program for area-based calculations to avoid human bias when selecting concentrated areas. First, each row and column in the BW image was summed. The image was cropped on all sides at the four corners where a summed value first surpassed a threshold value. The cropped image was broken into 100×100-pixel frames and the concentration (ratio) of white to the total pixels was determined for each frame. Frames with a concentration within ± 1 standard deviation from the average for the image were selected as concentrated areas. The 'concentrated' areas were the portions of the final BW image used to calculate concentration and porosity (ratio of black to the total pixels) of fibers in addition to fiber intersection densities (ratio of intersection points to area of the region of interest).

RESULTS

Validation Using Phantom Images

To confirm outputs from the skeleton-finding tool, phantom images were used. Phantoms ranged in complexity from simple lines to representative fiber images. Figures 3-A,C,E,G show the results of four simple phantoms used to validate orientation measurements (Fig. 3-B), concentration/porosity, tortuosity (Fig. 3-D) and fiber diameter estimations (Fig. 3-F,H). The phantom images in Fig. 3-A,C,E,G went through the same process as Fig. 2, but because they lack the noise of real multi-photon images, the first few steps did little to the image. Figure 3-A shows a simple orientation phantom. The orientation histogram (Fig. 3-B) has expected peaks at 45°, 90°, 135° and 180°. Counts for 135° (306) were almost double the counts for 45° (169), which was expected, because the length of the 135° line was twice that of the 45° line. The concentration determined for this image was 7.1% (porosity 92.9%), while the true concentration was 8.4% (porosity 91.6%). Variation was likely due to the code's expectation of noise. Darker edges of the phantom were treated as background, effectively lowering the concentration. Tortuosity was tested using a segmented quarter circle (Fig. 3-C). Breaks were inserted to provide endpoints for tortuosity calculations. Actual tortuosity for the two straight lines was 1, and for the curved line 1.11 ($(0.5 \times \pi \times \text{radius}) / (\text{radius} \times 2) = 1.11$). The estimate found by the script for straight lines was 1

and for the curved line was 1.17 (Fig. 3-D). The slight overestimation was due to the SS formation, which, when subtracting the endpoints, also subtracted a few pixels of the line segments in order to assure full segmentation of the FS.

Figures 3-E,G demonstrated the effect of different number of iterations (4 vs 2) of the primary segmentation step, the Frangi filtering, on the script diameter measurements. The resulting diameter distributions (Fig. 3-F,H) showed that increasing iterations increased the minimum fiber diameter found (artificial dilation) when there were not strong intensity gradients on the fibers. This was because more iterations of the Frangi filter resulted in more defined thick fibers, but also slightly increased the diameter of the smallest found fibers. These effects were more pronounced when the fibers did not have a strong, tube-like gradient. The benefit of gradients is seen in Fig. 6-C,F where diameters well below the average FD (8-pixels) were identified while using 4 iterations of the Frangi filter, when compared to the virtually gradient-free phantom in Fig. 3-E,G. While these settings generally did not affect the accuracy of the FS due to the robustness of the morphological image-processing steps, they might influence the found fiber diameters. This suggested that *a priori* knowledge of expected fiber diameters was needed to optimize the segmentation step.

More complex phantom images were used for qualitative evaluation and development of the algorithm in a less noisy environment than the real images. They provided simpler images to help ensure that image-processing steps had minimal negative impact on the accuracy of the resulting FS. In addition to real images, the complex phantoms were used to observe the locations of intersections and the area over which they reside for a semi-quantitative validation of intersection density. Results of an example phantom image of elastin can be seen in Fig. 4. Due to the skeleton creation, some intersection points might be broken into two neighboring intersection points. The code merges these points if they are within a FD's width of each other. Small areas were merged or removed as part of the skeletonization process, but these negative artifacts were qualitatively minimized. Noticeable in the phantom results was the removal of objects significantly smaller than the selected FD (FD=5, Fig. 4).

Implementation Using Multi-Photon Microscopy Image

Figure 5-A shows an example aortic wall multi-photon image ($500 \times 500 \text{ m}^2$) of collagen (red, first column) and elastin (green, second column). The image intensity, influenced by the microscopy technician's chosen parameters such as excitation power, detector gain, etc., can directly affect the image. However, the intensity equalization steps performed minimize these effects by making sure each input image utilizes a full 0–255 dynamic range. Figure 5-B shows the BW image obtained for collagen and elastin images with an overlay of the FS used for calculation of the diameter distribution. Figure 5-C shows the FS and intersection points generated by the program overlaid onto the collagen and elastin images from Fig. 5-A, respectively. Figure 5-D demonstrates the code's ability to filter fiber segments of particular orientation for additional analysis; here, radially-oriented components are shown.

In total, the code returned the following results for the collagen sample of Fig. 5-A (**left**): FS (Fig. 5-B **left**), intersection locations (Fig. 5-C **left**, in X-Y space), spatial-intersection

density (0.0031), orientation histogram (Fig. 6-A), mean orientation (90.2°), vertically (radially) and horizontally-oriented fiber percentages (4.9%, 19.7%), fiber concentration (45.7%), porosity (54.3%), mean segment length (27.1-pixels), mean tortuosity (1.11), tortuosity distribution histogram (Fig. 6-B) and fiber diameter distribution (Fig. 6-C). The following results were returned for the elastin sample of Fig. 5-A (**right**): FS (Fig. 5-B **right**), intersection locations (Fig. 5-C **right**), spatial-intersection density (0.0028), orientation histogram (Fig. 6-D), mean orientation (92.1°), vertically and horizontally-oriented fiber percentages (4.7%, 19.9%), fiber concentration (40.7%), porosity (59.3%), mean segment length (26.5-pixels), mean tortuosity (1.11), tortuosity distribution histogram (Fig. 6-E) and fiber diameter distribution (Fig. 6-F). Due to the discretization of the image when generating the FS, the orientation histogram is discrete. Resolution of orientation information is lost when removing fiber “thickness” to generate the FS and also when converting the 0–255 value grayscale image into a binary image. The effects of discretization on the potential fiber orientations are summarized in Appendix I and can be visualized in Fig. 7. The figure shows a 22.5° line, when sampled every 3-pixels, is actually comprised of a few possible orientations of 3-pixel line segments.

The ability of our tool to be used in multi-photon microscopy images of fibers with the circumferential-longitudinal plane facing up is demonstrated in Appendix II. Such images of collagen and elastin fibers from the wall of the human ascending thoracic aorta, at five different depths from the outer surface of adventitia, are shown in Figure II.1. The results of orientation analysis depicted in Fig. II.2 show that, close to the outer surface of the adventitia (10 μm), both collagen and elastin fibers seem to have preferred orientations in the circumferential and longitudinal directions. In deeper layers of the aortic wall (50 μm), elastin fibers maintain an almost orthotropic arrangement, whereas collagen fibers become more longitudinally oriented. The latter is in agreement with the results by Wan *et al.* (2012) on collagen fiber organization in mouse carotid arteries. For completeness, the output results of the image analysis for the collagen and elastin displayed in Fig. II.1 are shown in Tables II.1 and II.2, respectively.

It is expected that the fibrous structure will display differently when viewed with the circumferential-longitudinal plane facing up (Fig. II.1) as compared with the circumferential-radial plane (Fig. 5). The difference is due to the fact that the radial component of fiber undulation is not displayed in the circumferential-longitudinal plane. This is especially evident in the elastin fibers, which, when viewed with the circumferential-longitudinal plane facing up (Fig. II.1, green channel), they form more of a laminar-like structure especially in deeper locations with respect to the outer surface of the adventitia (Gasser *et al.*, 2006). When this laminar structure is projected onto the circumferential-radial plane, it shows as undulated fiber structure, for example see the green channel in Fig. 5-A.

DISCUSSION

The objective of this study was to develop a custom, image-based analysis tool to describe multiple parameters of elastin and collagen micro-architecture in human soft fibrous tissue samples using multi-photon images. Human aorta samples were used to develop the code. Multi-photon microscopy offers the advantage of viewing collagen and elastin individually

at a particular depth without the need for tissue-preprocessing. Our image-analysis tool used image-processing techniques to overcome the noise, saturation and complexity hurdles presented by these images to adequately describe important micro-architectural characteristics.

Prior image-processing methods primarily gather orientation information from microscopy images of soft fibrous tissues or manufactured biomaterial scaffolds. Intensity gradient-based texture analysis algorithms utilizing Hough transforms can obtain accurate orientation information (Chaudhuri *et al.*, 1993; Courtney *et al.*, 2006; Karlon *et al.*, 1998). However, the orientations are not connected in continuous segments presenting difficulties in obtaining other micro-architectural parameters. Direct-tracking methods are similar to the presented technique of finding fiber orientations (Pourdeyhimi *et al.*, 1999; Pourdeyhimi *et al.*, 1996a; Pourdeyhimi *et al.*, 1996b). This method presented problems in highly oriented and dense structures (Pourdeyhimi *et al.*, 1996b). Another common approach is FFT-based image-analysis which has accurately described overall orientation information about fiber-networks (Ayres *et al.*, 2006; Ayres *et al.*, 2007; Ayres *et al.*, 2008). However, the image as a whole was analyzed, not individual fibers.

Other limitations in the previously described methods were that they were typically applied to depth-limited imaging techniques such as light scattering (Sacks, 2003; Sacks, 2004) and SEM (Tan *et al.*, 2004). Of note, D'Amore *et al.* (2010) overcame most of these obstacles to develop an image-based analysis tool that effectively characterizes both orientation and non-orientation micro-architectural parameters of engineered-tissue fiber-networks viewed with SEM, using a Delaunay-network skeleton and image-gradient information. Multi-photon microscopy offers the inherent advantage of being able to visually penetrate into tissue depths of ~200 μm , allowing for optically sectioned 3-dimensional analysis. Cicchi *et al.* (2013) used multi-photon microscopy and Coherent anti-Stokes Raman spectroscopy to distinguish collagen from cholesterol in atherosclerotic arterial tissue. They further quantified fiber size, distribution and anisotropy of collagen in healthy arterial wall and in atherosclerotic plaque using image pattern evaluation algorithms. Here we utilized a unique skeleton-based approach to determine the orientation of collagen and elastin fibers and specific micro-architectural parameters including the location of fiber intersections, fiber concentration, porosity, diameter distribution, segment length and tortuosity.

The main limitation of our tool is the discretized orientation histogram. Figure 8 shows a comparison of orientation histogram found with our skeleton-based method and through an intensity-based edge detection method using D'Amore's modified (*not published*) Chaudhuri and Karlon technique (Karlon *et al.*, 1998). The discretization is noticeable in the skeleton-based method, because information is removed upon generation of the FS. As a result, the histogram has a more uni-modal trend as opposed to the distinct bi-modal trend seen in Fig. 8 (left). The simple phantom tests showed that the presented technique resulted in expected counts when certain orientations were present more than others, adding further validation to the orientation method. Another limitation of our tool is that it is currently optimized for fibers 5–15 pixels in diameter with low standard deviation from the average FD. Fibers larger than the FD might result in false FS, and fibers smaller than the FD might be treated as background. A third limitation is that the Frangi enhancement filter parameters should be

optimized according to the anticipated fiber diameter range to avoid diameter artifacts as seen in the simple phantoms.

The major advantage of our multi-photon-based image-analysis technique is the quantification of multiple micro-architectural properties. Orientation-based information alone is not sufficient for adequate modeling of the biomechanical response of soft fibrous tissues. In a structure-based approach, one needs additional parameters associated with the material properties of the individual wall constituents. Our skeleton finder tool can be very useful for this. The mass fractions of elastin and collagen can be approximated using concentrations and be used in a constrained mixture method to provide the effective wall stresses of the individual wall components (Gleason and Humphrey, 2004). Tortuosity can be used to fit the parameters of a probability density function that describes the gradual engagement of fibers as a function of strain (Zulliger and Stergiopoulos, 2007). Intersections density can impact the effective stiffness of the wall components and subsequently affect the mean and variance of the probability density function of fiber engagement. Attaching fibers to one another can stiffen the fiber ensemble by shifting the recruitment of the fibers to lower strains (decreased mean) and forcing the engagement to occur more abruptly (decreased variance) (Tsamis *et al.*, 2011). The end goal would be to model the fiber micro-architecture using finite-element methods. The previously-mentioned information can be combined with the fiber diameter distribution that will be applied to the section properties of individual finite elements to represent the network of the fiber skeleton.

Our tool is currently shown to be effective at characterizing multi-photon images of elastin and collagen in the aorta. In the future, the tool could be used to quantify micro-architectural parameters of soft fibrous tissue samples in health and disease, such as different aortic phenotypes, with the potential to describe components of varying tortuosity. Architectural descriptors could provide insight into the micro-architecture's influence on biomechanical properties of the soft tissues. Our tool could be adapted to similar imaging modalities, such as confocal microscopy, characterized by different levels of noise. The tool could also be expanded to handle larger fiber diameter sizes and images with varying fiber diameter ranges. Allowing for characterization of 3-dimensional multi-photon datasets would further increase the script's usefulness in modeling. Future applications are diverse, due to the fact that collagen is the most abundant protein in the body and the proposed method could be used to quantify collagen as well as elastin information in other fibrous materials and connective tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the University of Pittsburgh's Swanson School of Engineering (Mr. Koch), the Swiss National Science Foundation Fellowships for Advanced Researcher Numbers PA00P2_139684 and PA00P2_145399 (Dr. Tsamis), the Ri.MED Foundation (Dr. D'Amore), the NIH R01 HL109132 (Drs. Gleason and Vorp), and the University of Pittsburgh Department of Cardiothoracic Surgery (Dr. Vorp). We would like to thank Dr. Julie Phillippi and Mr. Michael Eskay for assistance in collecting human ATA tissue for multi-photon imaging, and Mr. Kristofer Pomiecko for producing the complex image-phantom in Fig. 4.

REFERENCES

- Ayres C, Bowlin GL, Henderson SC, Taylor L, Shultz J, Alexander J, Telemeco TA, Simpson DG. Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform. *Biomaterials*. 2006; 27:5524–5534. [PubMed: 16859744]
- Ayres CE, Bowlin GL, Pizinger R, Taylor LT, Keen CA, Simpson DG. Incremental changes in anisotropy induce incremental changes in the material properties of electrospun scaffolds. *Acta Biomaterialia*. 2007; 3:651–661. [PubMed: 17513181]
- Ayres CE, Jha BS, Meredith H, Bowman JR, Bowlin GL, Henderson SC, Simpson DG. Measuring fiber alignment in electrospun scaffolds: a user's guide to the 2D fast Fourier transform approach. *Journal of Biomaterials Science-Polymer Edition*. 2008; 19:603–621. [PubMed: 18419940]
- Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nature Reviews Immunology*. 2002; 2:872–880.
- Chaudhuri BB, Pulak K, Nirupam S. Detection and gradation of oriented texture. *Pattern Recognition Letters*. 1993; 14:147–153.
- Cicchi, R.; Matthaus, C.; Meyer, T.; Lattermann, A.; Dietzek, B.; Brehm, BR.; Popp, J.; Pavone, FS. Characterization of collagen and cholesterol deposition in atherosclerotic arterial tissue using non-linear microscopy. *Journal of Biophotonics*. 2013. <http://dx.doi.org/10.1002/jbio.201300055>, in press
- Courtney T, Sacks MS, Stankus J, Guan J, Wagner WR. Design and analysis of tissue engineering scaffolds that mimic soft tissue mechanical anisotropy. *Biomaterials*. 2006; 27:3631–3638. [PubMed: 16545867]
- D'Amore A, Stella JA, Wagner WR, Sacks MS. Characterization of the complete fiber network topology of planar fibrous tissues and scaffolds. *Biomaterials*. 2010; 31:5345–5354. [PubMed: 20398930]
- Frangi AF, Niessen WJ, Vincken KL, Viergever MA. Multiscale vessel enhancement filtering. *Lecture Notes in Computer Science*. 1998; 1496:130–137.
- Gasser TC, Ogden RW, Holzapfel GA. Hyperelastic modelling of arterial layers with distributed collagen fibre orientations. *Journal of the Royal Society Interface*. 2006; 3:15–35.
- Gleason RL, Humphrey JD. A mixture model of arterial growth and remodeling in hypertension: altered muscle tone and tissue turnover. *Journal of Vascular Research*. 2004; 41:352–363. [PubMed: 15353893]
- Halloran BG, Davis VA, McManus BM, Lynch TG, Baxter BT. Localization of aortic disease is associated with intrinsic differences in aortic structure. *Journal of Surgical Research*. 1995; 59:17–22. [PubMed: 7630123]
- Jiang X, Zhong J, Liu Y, Yu H, Zhuo S, Chen J. Two-photon fluorescence and second-harmonic generation imaging of collagen in human tissue based on multiphoton microscopy. *Scanning*. 2011; 33:53–56. [PubMed: 21328394]
- Karlon WJ, Covell JW, McCulloch AD, Hunter JJ, Omens JH. Automated measurement of myofiber disarray in transgenic mice with ventricular expression of ras. *The Anatomical Record*. 1998; 252:612–625. [PubMed: 9845212]
- Konig K, Schenke-Layland K, Riemann I, Stock UA. Multiphoton autofluorescence imaging of intratissue elastic fibers. *Biomaterials*. 2005; 26:495–500. [PubMed: 15276357]
- Kroon, DJ. Hessian based Frangi Vesselness filter. Matlab Central File Exchange; 2010. <http://www.mathworks.com/matlabcentral/fileexchange/24409-hessian-based-frangi-vesselness-filter>
- Lentle RG, Reynolds GW, Janssen PW. Gastrointestinal tone; its genesis and contribution to the physical processes of digestion. *Neurogastroenterol Motil*. 2013; 25:931–942. [PubMed: 24028606]
- Medyukhina A, Vogler N, Latka I, Kemper S, Bohm M, Dietzek B, Popp J. Automated classification of healthy and keloidal collagen patterns based on processing of SHG images of human skin. *Journal of Biophotonics*. 2011; 4:627–636. [PubMed: 21595044]
- Mijailovich SM, Sullivan MP, Yalla SV, Venegas JG. Effect of urethral compliance on the steady state p-Q relationships assessed with a mechanical analog of the male lower urinary tract. *Neurourology and Urodynamics*. 2007; 26:234–246. [PubMed: 17039462]

- Pourdeyhimi B, Dent R, Jerbi A, Tanaka S, Deshpande A. Measuring fiber orientation in nonwovens part V: real webs. *Textile Research Journal*. 1999; 69:185–192.
- Pourdeyhimi B, Ramanathan R, Dent R. Measuring fiber orientation in nonwovens: part I: simulation. *Textile Research Journal*. 1996a; 66:713–722.
- Pourdeyhimi B, Ramanathan R, Dent R. Measuring fiber orientation in nonwovens: part II: direct tracking. *Textile Research Journal*. 1996b; 66:747–753.
- Sacks MS. Incorporation of experimentally-derived fiber orientation into a structural constitutive model for planar collagenous tissues. *Journal of Biomechanical Engineering*. 2003; 125:280–287. [PubMed: 12751291]
- Sacks, MS. Small-angle light scattering methods for soft connective tissue structural analysis. In: Bowlin, GL.; Wnek, G., editors. *Encyclopedia of biomaterials and biomedical engineering*. Informa Healthcare; 2004.
- Sokolis DP, Sassani SG. Microstructure-based constitutive modeling for the large intestine validated by histological observations. *Journal of the Mechanical Behavior of Biomedical Materials*. 2013; 21:149–166. [PubMed: 23545202]
- Strupler M, Pena AM, Hernest M, Tharaux PL, Martin JL, Beaurepaire E, Schanne-Klein MC. Second harmonic imaging and scoring of collagen in fibrotic tissues. *Optics Express*. 2007; 15:4054–4065. [PubMed: 19532649]
- Tan W, Sendemir-Urkmez A, Fahrner LJ, Jamison R, Leckband D, Boppart SA. Structural and functional optical imaging of three-dimensional engineered tissue development. *Tissue Engineering*. 2004; 10:1747–1756. [PubMed: 15684683]
- Tsamis A, Rachev A, Stergiopulos N. A constituent-based model of age-related changes in conduit arteries. *Am J Physiol - Heart C*. 2011; 301:H1286–H1301.
- Wan W, Dixon JB, Gleason RL Jr. Constitutive modeling of mouse carotid arteries using experimentally measured microstructural parameters. *Biophysical Journal*. 2012; 102:2916–2925. [PubMed: 22735542]
- Westerhof, N.; Stergiopulos, N.; Noble, MIM. *Snapshots of Hemodynamics: An Aid for Clinical Research and Graduate Education*. Springer Science + Business Media, Inc; New York: 2005.
- Wu K, Liu JJ, Adams W, Sonn GA, Mach KE, Pan Y, Beck AH, Jensen KC, Liao JC. Dynamic real-time microscopy of the urinary tract using confocal laser endomicroscopy. *Urology*. 2011; 78:225–231. [PubMed: 21601243]
- Zulliger MA, Stergiopulos N. Structural strain energy function applied to the ageing of the human aorta. *Journal of Biomechanics*. 2007; 40:3061–3069. [PubMed: 17822709]

Figure 1.

Example multi-photon microscopy image ($500 \times 500 \mu\text{m}^2$) of collagen (red) and elastin (green) fibers in the medial part of the wall of ATA. (A) Collagen and elastin fibers together. (B) Collagen fibers. (C) Elastin fibers. Pixel dwell time: $2 \mu\text{s}/\text{pixel}$; Number of pixels in the image: $1024 \times 1024 \text{ pixels}^2$; Laser wavelength: 830 nm ; Excitation power at the sample: 6%; Depth from surface: $20 \mu\text{m}$; PMT Voltage: 539V for elastin and 443V for collagen; Detector gain: 1; Offset (correction for background): 9%.

Figure 2.

A brief summary of the image-processing cascade is shown in **A–F**. A localized portion of a collagen image ($147 \times 147 \mu\text{m}^2$) is used to demonstrate the processing steps in more detail.

(**A**) A representative input collagen image from the medial part of the wall of ATA. Pixel dwell time: $2 \mu\text{s}/\text{pixel}$; Number of pixels in the image: $300 \times 300 \text{ pixels}^2$; Laser wavelength: 830 nm ; Excitation power at the sample: 6%; Depth from surface: $35 \mu\text{m}$; PMT Voltage: 451 V ; Detector gain: 1; Offset (correction for background): 9%. (**B**) The red channel information from the representative input image shown in grey scale. (**C**) The result of the intensity equalization steps. (**D**) The black and white image generated after the threshold of the Frangi filter result. (**E**) The final FS. (**F**) The final FS overlaid onto the red channel from Fig. 2-B.

Figure 3.

The FS (blue) and intersections (green) are overlaid on all the simple phantoms (A, C, E, G). The dimensions of each phantom are $122 \times 122 \mu\text{m}^2$ or $250 \times 250 \text{ pixels}^2$. (A) A simple orientation phantom. (B) The orientation histogram with the expected peaks and expected relative counts. (C) A simple tortuosity phantom. (D) The resulting tortuosity histogram showing the actual and expected values and counts. (E) A simple diameter phantom for Frangi settings of 4 iterations. (F) The resulting fiber diameter histogram showing the values aliased above 8 pixels due to multiple iterations of the Frangi filter. (G) The same diameter phantom for Frangi settings of 2 iterations. (H) The resulting fiber diameter distribution showing calculation of lower diameter values due to less interference from the Frangi filter.

Figure 4.

The FS (blue) and intersection points (red) of a phantom elastin image are shown ($245 \times 225 \mu\text{m}^2$, $500 \times 460 \text{ pixels}^2$). Small fibers (fiber diameter 1–2 pixels) that are much smaller than the automatically determined FD ($\text{FD} = 5$) are removed. Fibers running close together with small gaps (widths 1–2 pixels) are merged together and treated as a single fiber.

Figure 5.

(A) The multi-photon image ($245 \times 245 \mu\text{m}^2$) of collagen fibers from the medial part of the wall of ATA is shown in the first column. The second column contains the multi-photon image of elastin. Pixel dwell time: $2 \mu\text{s}/\text{pixel}$; Number of pixels in the image: 500×500 pixels²; Excitation power at the sample: 6%; Depth from surface: $35 \mu\text{m}$; PMT Voltage: 547V for elastin and 451V for collagen; Detector gain: 1; Offset (correction for background): 9%. (B) The BW images for collagen and elastin are shown respectively with the skeleton overlay to show the area sampled for diameter distribution. (C) The found FS (blue) with marked intersections (green for collagen, red for elastin) overlaid onto the respective images from Fig. 5-A. (D) The “radially-oriented” fiber components are shown in green for the collagen image and red for the elastin image.

Figure 6.

(A) Fiber orientation histogram for the representative collagen sample shown in Fig. 5-A **(left)**. (B) Fiber tortuosity histogram for the collagen sample shown in Fig. 5-A **(left)**. (C) Fiber diameter distribution for the collagen sample shown in Fig. 5-A **(left)**. (D) Fiber orientation histogram for the representative elastin sample shown in Fig. 5-A **(right)**. (E) Fiber tortuosity histogram for the elastin sample shown in Fig. 5-A **(right)**. (F) Fiber diameter distribution for the elastin sample shown in Fig. 5-A **(right)**.

Figure 7.

Sample discretized 22.5° line showing the digitization artifact angles of potential 3×3 line segments. Modified from Redon *et al.* (1998). This figure demonstrates that a long line, when sampled with 3 pixel segments, is represented by a few possible angle arrangements.

Figure 8.

(Left) The fiber orientation histogram using the D'Amore's modified Chaudhuri and Karlon method (Karlon *et al.*, 1998) (*not published*) to find the orientation of elastin fibers shown in Fig. 5-A. (Right) The fiber orientation histogram of elastin fibers shown in Fig. 5-A using the skeleton-based method presented here. The effects of the discretization of orientation data due to the skeletonization process are clearly visible. The number of main peaks in the right histogram were predicted in Appendix I, Table I.1 (29 peaks for fiber diameter equal to 8 pixels).