Orientations of collagen fibers in aortic histological section

L. Horny, J. Kronek, H. Chlup, R. Zitny, J. Vesely, M. Hulan

Abstract— **Histological sections of male abdominal aorta were analyzed with the aim to obtain collagen fibrils and smooth muscle cells nuclei (SMCN) orientations. The sections were stained with van Gieson and digitalized. Digital images of the sections were reduced to binary pixel maps with target component represented in white (collagen or SMCN). One image was used to assess the sensitivity to threshold in binary conversion. Consecutively images were processed by in house developed software BinaryDirections, which works with the algorithm of the rotating line segment to determine significant directions in digital images. The algorithm operates in the way that in each target pixel a line segment is rotated step by step to explore neighborhood of the pixel. Exploring the neighborhood, the number of unity pixels coincident with the line segment is determined in each rotating step. This operation is conducted based on matrix formulation of the neighborhood and the line segment. The distribution of orientations in the entire image is obtained either as averaged density distribution from all pixels or as histogram of the most frequent directions. It was found that both collagen fibrils and SMC nuclei analyses give unimodal distributions. The peak ranges from -50° to -20° (defined as declination from circumferential direction of an artery) depending on the method. It implies that preferred direction in aortic medial layer was oriented circumferentially rather than longitudinally. However, aligning with circumferential direction is not as close as commonly referred in the literature. This conclusion was independent of the threshold setup. Results suggest that the orientations of SMC nuclei and collagen fibrils are mutually correlated and determination of collagen fibril orientations, which may be stained in insignificant manner, could be supported with SMC nuclei orientations to obtain more realistic models.**

Index Terms— **aorta, anisotropy, collagen, fiber distribution, histology, probability density**

I. Introduction

Constitutive models of biological tissues based on microstructure considerations are in extensive development because mechano-biological phenomenon and processes, such as for instance remodeling and adaptation, may not be satisfactorily described neglecting them. However, biological tissues comprise large number of different cells, matrix proteins and bonding elements. Moreover, there are complicated interrelations between these constituents.

When arterial wall is considered, there are three main layers usually distinguished, Holzapfel et al. [1]. Each layer has different function and composition. Briefly, innermost layer called *tunica intima* consists of endothelial cells which rest on basal lamina. There is also subendothelial layer dominated with collagen and smooth muscle cells (SMC). Tunica intima and subendothelial layer are very thin and compliant in physiological state; however they may become mechanically considerable in pathological situation, see Holzapfel et al. [3]. *Tunica media*, middle layer of an artery, has distinctive three-dimensional network structure. It is arranged into so-called musculoelastic fascicles which consist of SMC, bundles of collagen fibrils and elastin. They are separated with fenestrated elastin laminae and repeat through medial layer [2]. Directional arrangement of fibrillar components in media is helical with small pitch (almost circumferential orientation) [1,2]. Tunica adventitia is the outermost layer where especially fibroblasts, fibrocytes and collagen fibers are presented. Collagens form two helically arranged families of fibers with significantly dispersed individual fibers. Above mentioned layers are separated by elastic laminae. However, described structure is valid only in the case of elastic artery. Histological structure of muscular arteries may differ significantly. For more details see [1] and [2] and references therein.

Models of internal structure are being implemented in constitutive theories. Nowadays constitutive theories based on continuum mechanics can incorporate information about fibrillar structure to predict appropriate anisotropic behavior of a material. The anisotropy may rise from either finite number of preferred directions or their continuous distribution. For example of finite number of orientations see Holzapfel et al. [1,6] or Lanir [4]. Examples of continuous distribution of orientations can be found in Gasser et al. [2], Lanir [4] or Driessen et al. [5]. Aforementioned approaches homogenize arterial wall to anisotropic continuum. However, it is also possible to build up a model with composite structure

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incorporating mixture theory. Such models are intrinsically multiphase and may concern interrelations between particular wall constituents. Well known is the fact that fibrillar components of biological tissues are usually crimped and their load-carrying capacity is employed not before straightening. Models accounting for an unfolding and successive straining within a loading process have also been proposed; see Lanir [4], Freed and Doehring [7] or Cacho et al. [8].

This paper deals with internal structure of arterial wall based on observations made within an inspection of histological sections. Several approaches may be used to obtain such data. Analysis of histological sections employing transmitted light microscopy (TLM) is probably the oldest and simplest one. Nevertheless certain drawbacks exist. First of all a section is mechanically loaded during slicing. Chemical fixation (in formalin) may also change internal structure. Moreover this method does not seem to be suitable for an investigation of rearrangements in internal structure under a loading, what is of cardinal importance, because of complicated fixation of deformed geometry. Although papers reported successful strain-fixation in histological sections exist. See Sokolis et al. [9], they reported straightening of elastic lamellae under uniaxial tension. The polarized light microscopy (PLM) may be used in the same way because of the collagen and SMC birefringence, Canham et al. [10], [11]. Both studies revealed approximately circumferential orientations of collagen fibrils and SMC in coronary arteries and internal mammary artery.

Small-angle scattering methods may be used as an alternative to the light microscopy. Small-angle X-ray scattering (SAXS) seems to be capable to reveal a relation between fibers orientation and external load in a nano-scale, Schmid et al. [12]. Small-angle light scattering (SALS) method may also be employed rather than for nano-scale in micro-scale investigations of collagen organization in a tissue, Sacks et al. [13], Billiar and Sacks [15]. It should be noted that techniques of nonlinear optics such as multi-photon excited fluorescence (MFM) may also be engaged in histo-morphometrical analysis of arterial wall. See Wang et al. [16] who combined MFM with coherent anti-Stokes Raman scattering, which allows chemically selective imaging, in carotid artery extracellular matrix inspection. Also Zoumi et al. [17] reported MFM to be suitable within histological analysis of coronary arteries.

Present study reports results gained within the investigation of histological sections of human abdominal aorta based on computer-aided analysis of digital images. In house developed software BinaryDirections with implemented algorithm of the *rotating line segment* (RoLS), see Horny et al. [14], was used to quantify the organization of collagen fibrils and nuclei of SMC. As first RoLS algorithm is described and obtained results follow.

II. HISTOLOGY

Histological sections were obtained from abdominal aorta of 36-years-old male donor. They were stained with van Gieson and digitalized under 10 x magnifications. Four images were the same as in [14] and were used to confirm previous results. It was due to the fact that the algorithm in BinaryDirections (RoLS) has been improved in 2009. Additional image from medial layer was indicated to examine if the correlation between collagen fibers and SMC nuclei orientations exists. This image was also used in the analysis of threshold sensitivity to elucidate an influence of a binary conversion on results; threshold of RGB filter which transforms stained collagen to white (logical unity) pixels and non-collagen components to black (logical zero) pixels.

III. ROLS ALGORITHM

Exact mathematical formulation of the Rotating Line Segment (RoLS) was described in details in [14]. We have to mention that the algorithm was originally [14] abbreviated as RLS. However, in order to avoid confusing with Recursive Least Squares filter, which is common to abbreviate as RLS, the acronym was changed.

Histological section converted to binary pixel map may be viewed as a matrix with elements uniquely corresponding to pixels in binary image. Elements are equal to either zero or unity depending on the pixel color. The algorithm explores neighborhood of each non-zero pixel in the image using the rotating line segment. In details, consider any non-zero pixel in the image; the pixel is further referred as the target pixel. In the next step the neighborhood of the target pixel must be chosen. To achieve simple matrix representation, this neighborhood is chosen to be square. It means that neighborhood of the target pixel is represented via sub-matrix (NxN) in full-image matrix. Here N must be odd prime number greater than one. It will ensure the existence of midpoint in the neighborhood occupied with target pixel. Let denote the neighborhood matrix $\mathbf{M}$. Now, imaginary line segment is rotated step by step around the midpoint of neighborhood. Each rotating step, α, of the line segment is represented via additional matrix, say $\mathbf{L}^{\alpha}$. $\mathbf{L}^{\alpha}$ has only non-zero elements in positions corresponding to rotated imaginary line segment. This matrix is sparse and rank N.

The aim of RoLS is to find dominant directions in an image. This procedure is based on so-called matching coefficient, $C(\alpha)$. The matching coefficient is normalized number of non-zero pixels shared with the line segment and neighborhood of target pixel at given rotating step α. This is obtained via (1) and (2).

$$C(\alpha) = \frac{\sum_{i=1}^{N}\sum_{j=1}^{N} M_{ij} \cdot L_{ij}^{\alpha}}{Nl} \tag{1}$$

Normalization procedure is related to length of square neighborhood N and number of pixels creating the line segment, l.

$$l = \sum_{i=1}^{N}\sum_{j=1}^{N} L_{ij}^{\alpha} \tag{2}$$

We point out that geometrically it is a sector of pixels under $[\alpha,\ \alpha+\Delta\alpha)$, which is for simplicity called *line segment*. $\mathbf{M}$

and $\mathbf{L}^{\alpha}$ are never disjoint due to the midpoint, thus $C(\alpha) \in (0;1]$.

There are two ways to reveal relevant information about directional frequency of non-zero (collagen) pixels in the neighborhood of target pixel. First, one can reduce information from pixel's neighborhood to the most abundant angle (rotation step with greatest $C(\alpha)$) and create histogram over entire image (all non-zero pixels). Dominant directions distribution (distribution of orientations with highest content of collagen) is obtained. Second way is to consider $C(\alpha)$ as a function of α in each pixel and averaged this function through all non-zero pixels. Thus average density distribution of collagen pixels frequency over entire image is obtained. In order to obtain reliable results, analyses should be repeated with different values of N. It is obvious that results may depend on N. Thus, it should be chosen with respect to characteristic dimension of structures observed at images.

The algorithm is implemented in BinaryDirections in the way that multiple maxima in the matching coefficient, if happened, are added to their corresponding orientations weighted with multiplicative inverse of their multiplicity. This was newly added in 2009. However, it was not clear how frequently it happens. Hence previous analyses, reported in [14], were performed in new version of BinaryDirections again. Averaging procedure in the new BinaryDirections was also featured with a penalty function which suppresses contribution from pixels with almost uniform distributions.

Image-based determination of tissue architecture may employ many kinds of algorithms and mathematical methods. Presented algorithm, RoLS, is similar to so-called volume orientation (VO) method which operates with point grid and seeks for longest intercept in target volume. VO was first described in Odgaard et al. [18]. It was found to be suitable within an analysis of bone architecture. Interested reader can track details in [19] or recent review [20].

IV. Results

Selected results for processed images are shown in Fig. 4–6. Fig. 1 shows original digital image of used histological section (abdominal aortic media). Filtering this image to collagen bundles is in Fig. 2. Smooth muscle cell nuclei, easy distinguishable in Fig. 1, are filtered in Fig. 3. Fig. 2 and Fig. 3 are overlaid with orange grid spaced every 100 pixels to highlight length scales. A distribution of orientations is build up using information from a pixel neighborhood, thus its dimension (N) should be chosen with respect to dimensions of image texture. Fig. 4 shows small sensitivity of the texture on N. Density peak is located approximately at -30°.

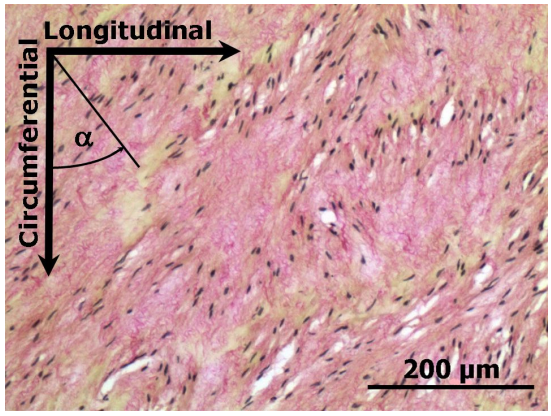

Fig 1 Additional histological section obtained from abdominal aortic media; stained with van Gieson. Bundles of collagen fibrils are stained in red. Black nuclei of smooth muscle cells are also apparent. White field are cracks probably caused by a manipulation.

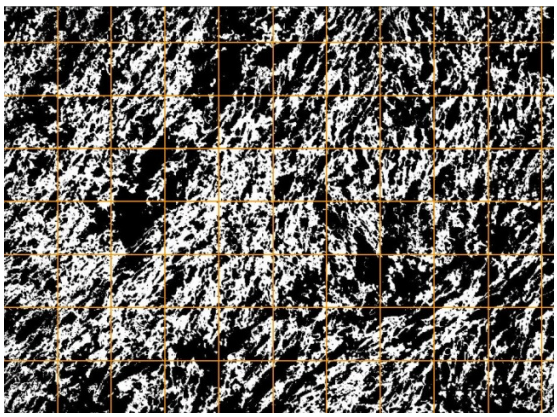

Fig. 2 Binary pixel map of the section from Fig. 1 filtered with respect to collagen (white). Orange grid spacing is 100 pixels; The scale for image is 0.64μm/pix.

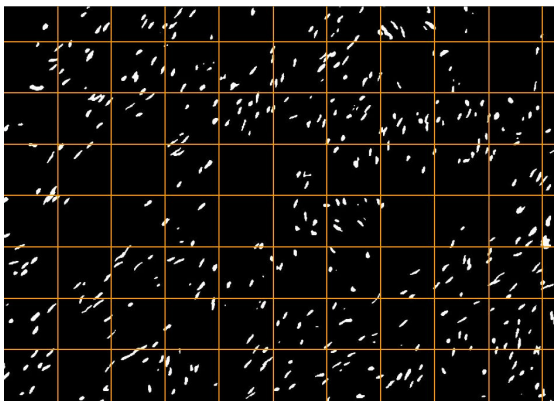

Fig. 3 Binary pixel map of the section from Fig. 1 filtered with respect to SMC nuclei (white). Orange grid spacing is 100 pixels; 0.64μm/pix.

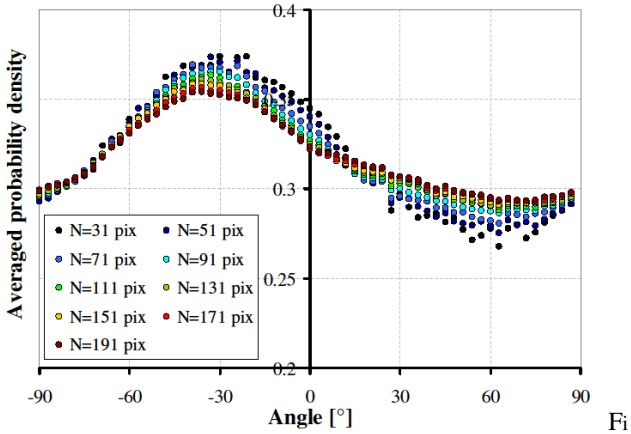

Fig. 4 Empirical probability density distribution of collagen fiber orientations obtained from the binary map in Fig. 2. Small sensitivity to neighborhood dimension *N* is shown. Data were obtained within averaging local distributions from all collagen pixels.

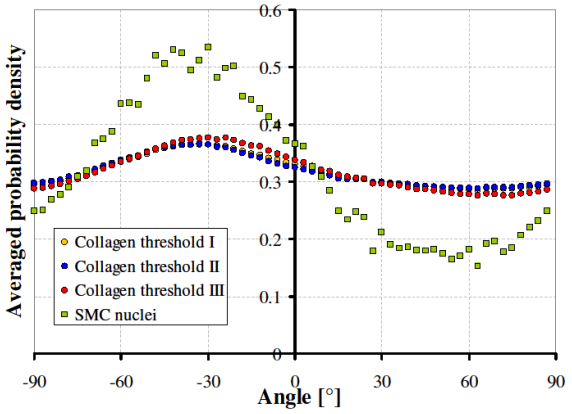

Fig. 5 Comparison between empirical probability density distributions of collagen fibers orientations obtained from Fig. 2 with different filter thresholds. The distribution of orientations for smooth muscle cell nuclei identified in Fig. 3 is also presented. Data were obtained within averaging local distributions from all pixels. Results support hypothesis of collagen-SMCn correlation. Small sensitivity on threshold perturbations is documented.

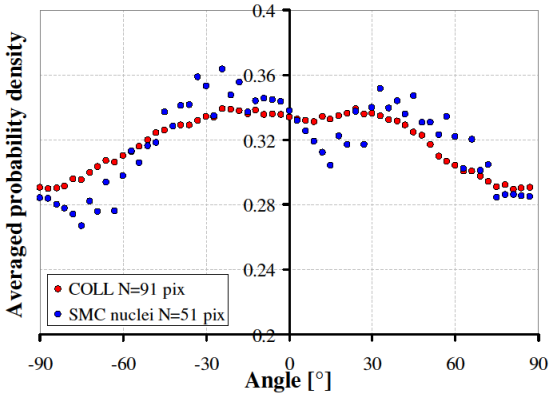

Fig. 6 Comparison between empirical probability density distribution for collagen orientations and smooth muscle nuclei orientations for adventitial section published in [14]. Data were obtained within averaging local distributions from all pixels. Results support hypothesis of collagen-SMCn correlation.

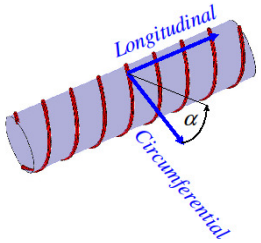

Fig. 7 The sketch illustrates the angle definition (from circumferential direction to longitudinal).

Filtering to target component is not unambiguous process and may depend on individual skills of an operator. To find out this influence, the section (Fig. 1) was binary converted three times with small perturbations in collagen threshold. Fig. 5 shows that all images gave similar results; the peak at ≈-30°.

Due to the existence of so-called musculoelastic fascicles in aortic media it was hypothesized that the correlation between collagen fibers and SMC orientations may exist. This hypothesis was confirmed; see results in Fig. 5. Both collagen and SMC nuclei analysis revealed density maximum at $\alpha \in$ [-50°;-20°]. In order to elucidate results, Fig. 7 depicts a blood vessel with one family of reinforcing fibers (collagen) aligned with mean value of determined dominant angle. Fig. 6 shows that the section previously analyzed in [14] also supports correlation between SMC and collagen orientations.

V. Conclusions

The results suggest that the rotation line segment may be powerful instrument in analyses of arterial architecture. It was shown that analyzed histological image from human aortic media (Fig. 1) gives continuous density distribution of orientations with maximum at approx. -30° (or +150°) for the collagen (the angle is defined as shown in Fig. 7). Similar result was obtained within the analysis of smooth muscle cell nuclei orientations. They gave continuous distribution with one peak located between [-50°;-20°]. This result is in agreement with collagen analysis and suggests mutual correlation in the arrangement. The correlation between SMC nuclei and collagen fibrils orientations may be expected especially within medial layer where so-called musculoelastic fascicles are presented. Fig. 6 presents result obtained in adventitial layer which also supports the hypothesis. However, additional analyses revealed that deviations exist. Prospective mismatches between SMC and collagen distributions may be attributed to finite thickness of histological section (5μm in this study) which may contain more than one fascicle.

Results show that preferred direction is oriented circumferentially rather than longitudinally, however the orientations density maximum does not meet exactly circumferential direction as in [6]. Unimodal distribution of collagen orientations indicates that continuum where anisotropy arises from presence of one preferred direction (so-called transversely isotropic material) may also be meaningful model for aortic wall. Within re-analysis of histological

sections processed in [14] the improved algorithm reported herein gave results similar in character with originally reported.

REFERENCES

[1] G. A. Holzapfel, T. C. Gasser, and R. W. Ogden, "A new constitutive framework for arterial wall mechanics and a comparative study of material models," *J. Elast.*, vol. 61, no. 1-3, pp. 1–48, July 2000.

[2] T. C. Gasser, R. W. Ogden, and G. A. Holzapfel, "Hyperelastic modelling of arterial layers with distributed collagen fiber orientations," *J. R. Soc. Interface*, vol. 3, pp. 15–35, Feb. 2006

[3] G. A. Holzapfel, G. Sommer, C.T. Gasser, and P. Regitnig,, "Determination of layer-specific mechanical properties of human coronary arteries with nonatherosclerotic intimal thickening and relative constitutive modeling," *Am. J. Physiol. Heart. Circ. Physiol.*, vol. 289, pp. 2048–2058, July 2005.

[4] Y. Lanir, "Constitutive equations for fibrous connective tissues," J. Biomech., vol. 16, no. 1, pp. 1–12, 1983.

[5] N. J. B. Driessen, C. V. C. Bouten, and F. P. T. Baaijens, "A structural constitutive model for collagenous cardiovascular tissue incorporating the angular fiber distribution," *J. Biomech. Eng. Trans. ASME*, vol. 127, no. 3, pp. 494–503, 2005.

[6] G. A. Holzapfel, T. C. Gasser, and M. Stadler, "A structural model for the viscoelastic behavior of arterial walls: Continuum formulation and finite element analysis," *Eur. J. Mech. A-Solids*, vol. 21, no. 3, pp. 441–463, 2002.

[7] A. D. Freed, and T. C. Doehring, "Elastic model for crimped collagen fibrils," *J. Biomech. Eng. Trans. ASME*, vol. 127, no. 4, pp. 587–593, Aug. 2005.

[8] F. Cacho, P. J. Elbischger, J. F. Rodríguez, M. Doblaré, and G. A. Holzapfel, "A constitutive model for fibrous tissues considering collagen fiber crimp," *Int. J. Non-Linear Mech.*, vol. 42, no. 2, pp. 391–402, March 2007.

[9] D. P. Sokolis, E. M. Kefaloyannis, M. Kouloukoussa, E. Marinos, H. Boudoulas, and P. E. Karayannacos, "A structural basis for the aortic stress-strain relation in uniaxial tension," *J. Biomech.*, vol. 39, no. 9, pp. 1651–1662, 2006.

[10] P. B. Canham, H. M. Finlay, J. G. Dixon, D. R. Boughner, and A. Chen, "Measurements from light and polarized light microscopy of human coronary arteries fixed at distending pressure," *Cardiovasc. Res.*, vol. 23, no. 11, pp. 973–982, 1989.

[11] P. B. Canham, H. M. Finlay, and D. R. Boughner, "Contrasting structure of the saphenous vein and internal mammary artery used as coronary bypass vessels," *Cardiovasc. Res.*, vol. 34, no. 3, pp. 557–567, 1997.

[12] F. Schmid, G. Sommer, M. Rappolt, C. A. J. Schulze-Bauer, P. Regitnig, G. A. Holzapfel, P. Laggner, and H. Amenitsch, "In situ tensile testing of human aortas by time-resolved small-angle X-ray scattering," *J. Synchrot. Radiat.*, vol. 12, no. 6, pp. 727–733, Nov. 2005.

[13] M. S. Sacks, D. B. Smith, and E. D. Hiester, "Small angle light scattering device for planar connective tissue microstructural analysis," *Ann. Biomed. Eng.*, vol. 25, no. 4, pp. 678–689, July 1997.

[14] L. Horny, M. Hulan, R. Zitny, H. Chlup, S. Konvickova, and T. Adamek, "Computer-Aided Analysis of Arterial Wall Architecture," in IFMBE Proceedings, vol. 25/4, O. Dossel, W. C. Schlegel, Eds. Berlin: Springer, 2009, pp. 1494–1497.

[15] K. L. Billiar, and M. S. Sacks, "A method to quantify the fiber kinematics of planar tissues under biaxial stretch," *J. Biomech.*, vol. 30, no. 7, pp. 753– 756, July 1997.

[16] H. W. Wang, T. T. Le, and J. X. Cheng, "Label-free imaging of arterial cells and extracellular matrix using multimodal CARS microscope," *Opt. Commun.*, vol. 281, no. 7, pp. 1813–1822, April 2008.

[17] A. Zoumi, X. Lu, G. S. Kassab, and B. J. Tromberg, "Imaging coronary artery microstructure using second-harmonic and two-photon fluorescence microscopy," *Biophys. J.*, vol. 87, no. 4, pp. 2778–2786, Oct. 2004.

[18] A. Odgaard, E. B. Jensen, and H. J. G. Gundersen, "Estimation of structural anisotropy based on volume orientation. A new concept," *J. Microsc.*, vol. 157, no. 2, pp. 149–162, Feb. 1990.

[19] A. Odgaard, "Three-dimensional methods for quantification of cancellous bone architecture," *Bone*, vol. 20, no. 4, pp. 315–328, April 1997.

[20] E. A. Sander, and V. H. Barocas, "Comparison of 2D fiber network orientation measurement methods," J. Biomed. Mater. Res. Part. A, vol. 88, no. 2, pp. 322–331, 2009.