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Three Dimensional Mesoscale Analysis of Translamellar Cross-Bridge Morphologies in the Annulus Fibrosus using Optical Coherence Tomography

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Abstract

The defining characteristic of the annulus fibrosus (AF) of the intervertebral disc (IVD) has long been the lamellar structures that consist of highly ordered collagen fibers arranged in alternating oblique angles from one layer to the next. However, a series of recent histologic studies have demonstrated that AF lamellae contain elastin- and type VI collagen-rich secondary “cross-bridge” structures across lamellae. In this study, we use optical coherence tomography (OCT) to elucidate the three-dimensional (3D) morphologies of these translamellar cross-bridge in AF tissues. Mesoscale volumetric images by OCT reveal a highly heterogeneous spatial network and distribution of 3-D translamellar cross-bridges. The results of this study confirm the translamellar cross-bridge is identified as a distinguishable structure, which is laid in the interbundle space of adjacent lamellae and crisscrosses multiple lamellae in the radial direction. In contrast to previously proposed models extrapolated from 2-D sections, results from this current study show that translamellar cross-bridges exist as a complex, interconnected network. We also found much greater variation in lengths of cross-bridges within the interbundle space of lamellae (0.8-1.4 mm from the current study versus 0.3-0.6 mm from 2-D sections). OCT-based 3-D morphology of translamellar cross-bridge provides novel insight into the AF structure.

Keywords

translamellar cross-bridge; annulus fibrosus; 3-D morphology; optical coherence tomography

1. Introduction

The intervertebral disc (IVD) is a highly heterogeneous organ, and serves to transmit, cushion, and distribute forces between adjacent vertebrae in the spine.¹ Complex interplay

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among the cartilaginous endplates, annulus fibrosus (AF), and nucleus pulposus determines the mechanical function of the IVD. The AF consists of lamellar structures that possess arrays of highly ordered collagen fibers. In the healthy IVD, the AF has a complex functional role of resisting tension, compression and shear.¹ The mechanical function of the AF largely depends on the structural integrity of the tissue, and its failure is associated with the onset of IVD degeneration.²

The primary structure of the AF, lamellae containing collagen arranged in alternating oblique angles from one layer to the next, has long been its defining characteristic (Figure 1). Structural proteins of the lamellae are mainly type I and type II collagen in the outer and inner AF, respectively.³ However, several recent histological studies have demonstrated that interdigitated within the annular lamellae are distinct secondary structures rich in elastin, proteoglycans and type VI collagen.⁴⁻⁶ In contrast to the lamellar matrix, fibers in the so-called translamellar “cross-bridge” structures are oriented in the radial direction of the AF.

These secondary structures in the AF have been reported in several species including human,⁷ ovine,^{4; 5; 8} and bovine caudal discs.⁹⁻¹¹ The origin and formation of translamellar cross-bridges in the AF have been attributed to vascular regression during growth and maturation.^{4; 7} It was proposed that penetrating blood vessels, which regress during the early postnatal years,^{12; 13} do not become incorporated into the lamellar matrix. Rather, the translamellar space becomes occupied with structural components similar to that of the interlamellar matrix.^{4; 7; 10; 11; 14} Schollum et al. (2008, 2009) schematically illustrated a three-dimensional (3-D) microstructure of translamellar cross-bridge deduced from serial planar histology images taken at 30 micron intervals and visualized using differential interference contrast (DIC) microscopy. They proposed a microstructural architecture in which radial translamellar fibrils tunnel between collagen fibers in the AF, and cross multiple lamellae.

Although the 3-D morphology and mechanical role of translamellar cross-bridges structures in AF have not yet been fully characterized, it has been proposed that the distinctly different fibrillar structure and orientation of a cross-bridge relative to its surrounding lamellar matrix could affect local tissue deformations of AF.^{9; 15} A more comprehensive understanding of the translamellar cross-bridge network could provide important insight into the physiology, pathology, and mechanics of the AF. In this study, we investigate the use of optical coherence tomography (OCT) for IVD imaging in mesoscale tissue level. In comparison with microscale level imaging techniques, such as confocal microscopy, OCT provides larger image field (2-6 mm × 2-6 mm) and deeper image depth (~ 1 mm), allowing for the visualization of 3-D lamellar connectivity and the cross-bridge network across multiple lamellar layers. We found that OCT was able to capture detailed 3-D morphologies of cross-bridges and, therefore, is particularly suited for non-destructive visualization of the AF mesostructure. Using this approach, we were able to demonstrate marked heterogeneity and interconnectedness among cross-bridges within the AF.

2. Methods

2.1. OCT imaging

OCT is an emerging medical imaging technology which enables micron scale, cross-sectional and three-dimensional imaging of microstructure of biological tissues in situ, non-invasively, and in real time. Such performance approaches those of standard excisional biopsy and histopathology¹⁶⁻¹⁸. OCT is analogous to ultrasound B mode imaging, except that imaging is performed by measuring the echo time delay of back-reflected light rather than sound. An optical beam is delivered on the tissue and echoes of backscattered light are measured. The measurements, through transformations, renders an axial profile of the sample. 2D cross-sectional and 3D volumetric OCT images of tissue are constructed by juxtaposing a series of such axial measurements over transverse directions.

OCT takes two advantages of light. One, light penetrates into and reflects back from material. For biologically relevant material, such penetration is about 1-2mm. Second, OCT uses the interference property of light to establish micron level resolution. One key is the ability to control where to form constructive interference and everywhere else is destructive interference. In other words, it is the ability to see the region of interest and only the region of interest. This is what has been called the coherence gating¹⁹⁻²¹, and such ability can be realized in either time-domain or frequency-domain. To date, frequency-domain OCT has been proven outperforming 100 times in sensitivity and speed than its time-domain counterpart²²⁻²⁴.

A swept-source OCT system (Thorlabs, Newton, NJ) was used in this study. Its center wavelength was 1.31 μm , and the full-width-half maximum (FWHM) bandwidth was 0.1

μm , yielding a theoretical dispersion-free axial resolution of $7.6 \mu\text{m} (= \frac{2\ln 2}{\pi} * \frac{1.31^2}{0.1})$ in air, or 5.6 μm in the IVD where an index of refraction $n=1.35$ was assumed. The objective in the OCT sample arm was an achromatic doublet (AC254-030-C, Thorlabs; NA=0.04), achieving

a theoretical lateral resolution of $\sim 12.12 (= 0.37 * \frac{1.31}{0.04}) \mu\text{m}$. Independently, a phantom-based measurement²⁵ was conducted to experimentally determine the achieved resolution (FWHMs of the PSF) being $14.1 \times 14.1 \times 11.2 \mu\text{m}$ in x, y, z directions. Imperfect dispersion compensation could explain why the actual axial resolution was slightly higher than the theoretical.

Each A-scan of the OCT mapped 2.55 mm penetration depth in air (or 1.89mm in tissue) in 512 pixels, and the OCT operated at a sweep rate of 16 kHz (16k A-scans per second), scanning $6 \times 6 \text{ mm}^2$ wide. Therefore, a data cube of $512 \times 512 \times 512$ pixels captured a $6 \times 6 \times 1.89 \text{ mm}^3$ field of view (FOV) in about 16.3 seconds. The effective penetration depth was however about 1 mm (compared to 1.89 mm), which could result from at least the scattering nature of the AF and the OCT sensitivity. The OCT delivered 6.3 mW (averaged over the swept spectra) to the sample. By using the adjustable neutral densities as the sample and collecting the reflected light, the sensitivity (signal-to-noise ratio, SNR) was measured being 95 dB.

2.2. 3-D translamellar cross-bridge network visualization

Skeletally mature female ovine lumbar motion segments (two adjacent vertebrae with IVD, $N = 4$ animals, $n = 2$ motion segments per animal, L4-5, L-5-6) were obtained after euthanasia for an unrelated procedure, as approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park. Samples were isolated from the animals and fixed in 10% formalin solution for ~ 20 days before imaging. For OCT image acquisition, IVDs were cut in the transverse plane, and the anterior region of the AF was scanned by OCT (Figure 2A). AF samples were placed on a goniometer to ensure that the laser path from the OCT was perpendicular to the cut surface of the AF. All tissues were hydrated with physiologic saline during OCT imaging (Figure 2B).

2.3. Histological validation of translamellar cross-bridge imaging by OCT

AF samples from two of ovine lumbar IVDs used for OCT image acquisition were excised from the anterior region. The same samples were then processed in graded ethanol and xylene baths before paraffin embedding (TP1020/EG1160; Leica Microsystems, Buffalo Grove, IL, USA). Paraffin blocks were cut with a microtome (HM355; Microm/Thermo Fisher Scientific, Waltham, MA, USA) to obtain $10\text{ }\mu\text{m}$ thick transverse sections of the AF. Sections were stained with Safranin-O/Fast green and examined under brightfield. At precise locations of tissue samples, histology images were compared with OCT images to identify corresponding features between imaging modalities. For additional verification that formalin had little effect on cross-bridge structure during imaging, a freshly cut porcine AF sample was imaged using both a regular dissection microscope and OCT. After 10% formalin fixation and histological staining with Safranin-O/Fast green described above, the same AF sample was examined under brightfield microscopy.

2.4. Image data analysis

Even though the OCT A-scan contains phase information, only the intensity information was used in this study. A 3-D reconstruction of the AF from OCT was performed using ImageJ (National Institute of Health, Bethesda, MD, USA). Image segmentation of translamellar cross-bridges was conducted using a ITK-SNAP (Insight toolkit; <http://www.itksnap.org>). A 3-D rendering of segmented images was performed using a custom-written code (VTK, The Visualization Toolkit; Kitware Inc., Clifton Park, NY, USA). The average number of cross bridges per lamellae in the anterior region was calculated by counting the total number of cross bridges and dividing by total lamellae in the scanning volume of the AF region. A translamellar cross-bridge was defined as any radially oriented structure existing in at least two lamellae.

3. Results

3.1. 3-D characterization of the translamellar cross-bridge network

Heterogeneity in the morphologies, distribution, and interconnectedness of cross-bridges within any given 3-D volume of the AF was apparent from OCT scans. Optical sectioning along the radial direction of the AF produces axial-circumferential plane images showing the interbundle localization of translamellar cross-bridges, by virtue of the contrast between lamellar and translamellar cross-bridge matrices (Figure 3A). Within each lamella, cross-

bridges assumed the orientation of oblique collagen fiber angles that alternate from one layer to the next (Figure 3B).

Optical sections of the volume in the radial direction show that translamellar cross-bridges are connected across multiple lamellar layers (Figure 4), as typically observed in 2-D histology studies. Multi-planar views can be obtained at any point within the volumetric image to enable tracking of the intricate pathways of cross-bridges through the AF. These views highlight the heterogeneity and complexity of the interconnected translamellar cross-bridge network and their relationship with the inter-lamellar matrix (Figure 4).

The span of the translamellar bridging matrix within the interbundle space of collagen is highly variable. Within any given lamella, we measured interbundle spans from approximately 758 to 1420 μm in length (Figure 5). Based on our scans, the average number of cross-bridges per lamella was calculated to be 3.5 ± 1.1 in the anterior region of ovine lumbar discs (average volume of 11 mm^3 , $n=6$ lumbar discs).

The 3-D reconstruction of segmented OCT images around localized regions of translamellar cross-bridges accentuates how heterogeneously they are interconnected across lamellae and distributed within the AF (Figure 6). A volumetric image obtained from OCT (Figure 6A) that has been subjected to manual segmentation reveals a skeletal network of cross-bridge structures (Figure 6 B, C). From these 3-D reconstructions of segmented OCT images, it is apparent that the alignment and continuity of cross-bridges across lamellae varies with the lamellar layer. At times, a cross-bridge may bifurcate to form complex 3-D networks (Figure 6 C, D).

3.2. Histology validation of OCT-based translamellar cross-bridge imaging

Structural features of translamellar cross-bridges in the AF observed in planar optical slices from volumetric OCT images (Figure 7A) were consistent with those visualized by histology. Safranin-O/Fast green staining confirmed proteoglycan-rich interlamellar and translamellar cross-bridge matrix (Figure 7B). Comparison of dissection micrographs and OCT images obtained using freshly cut AF tissue samples with histological sections suggests that translamellar cross-bridge network structures are not appreciably altered by formalin fixation and paraffin embedding. Morphology appeared to be unchanged, and individual cross-bridge structures were readily identifiable across the range of imaging techniques (Figure 8).

4. Discussion

In this study, we elucidate the 3-D mesostructure of the translamellar cross-bridge network within an intact volume of anterior region of AF tissue, using a non-destructive imaging modality, OCT. This enabled us to capture the spatial heterogeneity and complexity of translamellar cross-bridge geometries and distributions through a relatively broad ($6\text{mm} \times 6\text{mm}$) region of tissue, a level of characterization that has not previously been reported. Additionally, the approach minimizes any adverse effects on cross-bridge morphology caused by tissue dehydration and processing steps, reconstruction artifacts, and potential sample loss inherent in traditional histological methods.

Our results confirm that the translamellar cross-bridge is a dominant secondary structure in the AF (Figure 9 A). Consistent with a previous study,⁸ the translamellar cross-bridge was identified as a distinguishable structure that lies between and parallel to bundles of collagen fibers within annular lamellae, and crosses radially through multiple lamellae. That same study proposed a conceptual 3-D model of cross-bridges based on micrographs of serial histologic sections within local regions of tissue. By examining larger tissue volumes using OCT, we were further able to discern extensive heterogeneity in both the distribution and span of the translamellar matrix within the collagen interbundle space. At times, cross-bridges bifurcated to form complex 3-D networks (Figure 9).

Evidence found in previous studies suggests that cross-bridge formation is due to vascular regression.^{4; 7} The thought is that blood vessels in the AF matrix start to disappear after birth because of reduced metabolic requirements and mechanical stress surrounding matrix, and those remnants of regressed blood vessel matrix develop into translamellar cross-bridges. Why these cross-bridge structures are not completely resorbed and remodeled into matrix similar to the surrounding lamellar AF after vascular regression remains unknown. It has been speculated that the slow metabolic activity of AF cells and mechanical pressure around the AF may inhibit the recapitulation of lamellar microstructure⁷ or that the biophysical stimuli in the prospective translamellar cross-bridge regions promote type VI collagen and proteoglycan accumulation.⁴

Differences in structural content and fiber angles support the biophysical mechanism, and suggest the translamellar cross-bridge matrix may experience a micromechanical environment that is distinct from the lamellar matrix. In fact, translamellar cross-bridges bear significant resemblance to the interlamellar matrix; they both possess higher content of type VI collagen, proteoglycans, and elastin compared to the lamellar AF matrix,⁴ which primarily consists of collagen types I and II. Furthermore, collagen fiber orientation deviates from the alternating oblique 24 degree angles that define the lamellar AF (Figure 9 B). Within the translamellar cross-bridge, collagen fibers tunnel through lamellae in the radial direction. Previous studies have shown that cells with distinctive morphologies reside within the interlamellar matrix.²⁶ These or similar cells may be responsible for translamellar cross-bridge matrix metabolism.

Although studies have investigated translamellar cross-bridge mechanics,^{9; 15} the functional role of translamellar cross-bridges during the biomechanical function of the IVD has not yet been fully elucidated. Preliminary studies that we have performed using OCT-based elastography indicate that, under an applied tissue deformation, the translamellar and interlamellar regions are subjected to significantly higher shear strain relative to the lamellar matrix.²⁷ These separate pieces of evidence support the conjecture that the inter-/translamellar AF may represent a distinct cellular and tissue compartment in the disc. Since translamellar cross-bridges comprise a relatively small volume in the AF, however, we hypothesize that they play a minor role in bulk AF mechanics but may have substantial influence in the micromechanical regulation of cell function. Further studies focusing on understanding the functional significance of translamellar cross-bridges in cellular mechanobiology are needed to substantiate this hypothesis.

Although the development of translamellar cross-bridges has been explored, it is interesting to speculate whether they might also contribute to disc aging and degeneration. In human discs, mucoid degeneration and clonal expansion of chondrocyte-like cells are hallmarks of age-related changes.²⁸ Similarly, various animal models have demonstrated increased proteoglycan content and shifts in cell shape toward more rounded chondrocyte-like morphologies within the interlamellar space.^{2; 29} It is possible that chronic changes mirroring those in the interlamellar matrix could be occurring in translamellar cross-bridges, though this has not yet been identified. Acute formation of annular clefts during disc pressurization can also occur in the interlamellar/translamellar matrix,³⁰ likely due to their putative role as stress risers.⁷ The susceptibility of these regions to shear strain and damage may underscore their potential significance in disc health and disease,¹⁴ and it has been postulated that clefts might facilitate vascular and nerve tissue ingrowth as a precursor to degeneration and pain.³¹

As other connective tissues, the structural integrity of AF matrix substantially determines health and functional properties of the IVD. With aging or injury related degeneration, AF matrix loses its structural integrity and functional properties.¹ Although the existence of translamellar cross-bridges in the degenerated human AF has been reported,⁶ there remains no detailed characterization as to how the translamellar cross-bridge network structure and function might be altered during the degenerative process. It can be speculated that the composition of translamellar cross-bridges may change accordingly relative to the surrounding annular lamellar matrix.

In this study, OCT was utilized to perform mesostructural imaging of anterior region of the AF and characterize the translamellar cross-bridge network. This imaging modality has been useful for studying various other tissues.^{16; 32-36} Although the mesoscale resolution of OCT (8 μm and 15 μm for axial and transverse directions, respectively) precludes analyzing microstructural features, OCT provides several advantages for examining AF tissue structures compared to conventional imaging techniques. In particular, the ability for OCT to generate a 3-D image volume and perform optical sectioning in three orthogonal planes on tissues enabled us to elucidate the anisotropy and inhomogeneity of the translamellar cross-bridges, without having to use multiple tissue samples or reconstruct images from different cutting angles.

Our primary goal was to establish the use of OCT for visualizing the morphology and interconnectedness of the cross-bridge network in the anterior AF. Previous studies indicate that the numbers of cross-bridges vary among different regions of the AF, and current knowledge of age- and degeneration-related changes in the AF suggest a strong likelihood that the cross-bridge network may be correspondingly disrupted. In order to make these comparisons, however, it would be necessary to develop and validate new OCT-based quantitative metrics to characterize cross-bridge network morphology, an area of current interest to us. Additionally, since the IVD was chemically fixed in this study and transversely cut without applying residual load, the structural characteristics of the imaged tissue may not be exactly as same *in situ*. To investigate the structure and function of translamellar cross-bridges in an intact disc, further development of OCT implementation techniques is needed.

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Figure 1.

Schematic drawing of the macrostructure of the IVD. The AF is characterized by lamellar layers of organized collagen oriented at oblique angles from one lamella to the next. The nucleus pulposus (NP) is radially contained within the confines of the AF.

Figure 2.

A: A representative sample of the transversely cut IVD sample used for OCT imaging. The black box indicates OCT scanning area in the anterior region of the AF. B: The testing AF samples were placed on the platform of a two rotational degree of freedom goniometer to ensure that the laser path from OCT was perpendicular to the cutting surface of the AF. C: The goniometer containing the tissue sample was then placed on the OCT system used for image acquisition.

Figure 3.

A. Representative 3-D volumetric OCT image of the AF ($4.1 \times 3.7 \times 1.3 \text{ mm}^3$ volume in circumferential, radial and axial directions, respectively). B. Optical sections (axial-circumferential plane) of the 3-D volume near the outer AF, showing the alternating directions of translamellar matrix localized in the space between collagen bundles of lamellae. Circled numbers in panel B indicate images obtained from the approximate position of corresponding numbers in panel A. Oblique lines were manually drawn to highlight the observed collagen orientations in each image. Scale bar = 500 μm .

Figure 4.

Multi-planar views of 3-D OCT images were useful for facilitating the identification of the complex paths of the translamellar matrix through the tissue volume. As evident from these images, cross-bridges do not exist as discrete channels through the AF, but as tortuous networks that permeate the entire tissue. Yellow arrows indicate cross-bridge networks. A volume of 3-D OCT in A: $2.0 \times 1.3 \times 1.1 \text{ mm}^3$ in circumferential, radial and axial directions, respectively. A volume of 3-D OCT in B: $2.5 \times 1.3 \times 0.3 \text{ mm}^3$ in circumferential, radial and axial directions, respectively.

Figure 5.

A representative image of a single lamellar layer that contains several localized regions of translamellar matrix that span different lengths. Scale bar = 500 μm .

Figure 6.

A: 3-D OCT volumetric image of the anterior AF ($5.4 \times 6.0 \times 1.15 \text{ mm}^3$ volume in circumferential, radial and axial directions, respectively). Red arrows indicate translamellar cross-bridges. B: Segmented image corresponding to the yellow dashed line region ($5.4 \times 4.0 \times 1.15 \text{ mm}^3$) of the same 3-D OCT volume shown in panel A. Green structures represent the cross-bridge network. Grey regions are manually segmented regions to illustrate the lamellar matrix. Red arrows point to the same translamellar cross-bridges labelled in panel A. C: More detailed view of the segmented translamellar cross-bridge network corresponding to the region encompassed by the blue dashed lines shown in panel B ($1.8 \times 4.0 \times 1.15 \text{ mm}^3$). The large white arrows shown in panels B and C are intended for orientation of viewing direction between the two panels. Black arrows indicate the bifurcation of a translamellar cross-bridge between two adjacent lamellae. D: Detailed image ($1.8 \times 2.2 \times 1.15 \text{ mm}^3$) around the translamellar cross-bridge bifurcation shown in panel C.

Figure 7.

A: A transverse optical section (radial-circumferential plane) from the 3-D OCT image volume showing a segment from the translamellar cross-bridge network in the anterior AF. B: The same tissue specimen was marked, processed, paraffin sectioned, and stained with Safranin-O/Fast green. The stained section from the same region of tissue was examined, and the corresponding translamellar cross-bridge indicated by red arrows shown in panel A was located. Scale bar = 1000 μm .

Figure 8.

Comparison of cross-bridge network structures from a single AF tissue sample using different imaging techniques. A: freshly cut tissue image taken under a regular dissection microscope. B: freshly cut tissue image acquired by OCT. C: Histological section stained with Safranin-O/Fast green and imaged under brightfield microscopy. Red arrows indicate identical cross-bridge network structures in the images. Scale bar = 1mm.

Figure 9.

Schematics of the translamellar cross-bridge network within the existing macrostructural framework of AF. A: The AF matrix containing cross-bridge networks. B: Fiber orientations of translamellar cross-bridges within the interbundle space of surrounding lamellar collagen; the connectivity to the interlamellar matrix is not shown in order to maintain clarity.