Differences in 3D Collagen Fibril Structure with Tendon Type and Health are Revealed Using SBF-SEM Combined with Machine Learning Automated Segmentation and Reconstruction

Kelsey Y. Gsell[1], Lily M. Lin[1], Jamie M. Benson[1], Chandran Sabanayagam[2], Jeffrey Caplan[2,3], Dawn M. Elliott[1]
[1]Department of Biomedical Engineering, [2]Delaware Biotechnology Institute, [3]Department of Plant and Soil Sciences, University of Delaware, Newark, DE
kgsell@udel.edu

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INTRODUCTION: Collagen fibrils are the primary structural unit of connective tissues like tendon. At the nanoscale, the ability of collagen to bear load depends directly on fibril structure [1]. Accordingly, there are structural differences between tendons with distinct functions [2], and structural changes can occur with damage and injury that alter mechanical function, leading to further injury risk [3,4]. Assessing fibril structure within a tissue remains challenging. Electron microscopy provides sufficient resolution but is traditionally limited to a single plane, and fibril segmentation remains a time-consuming and difficult step. While 2D imaging allows for fibril diameter, shape, and density to be measured [5], 3D fibril geometry and organization are required to fully characterize tendon structure and infer its contribution to mechanics. Using serial block face scanning electron microscopy (SBF-SEM), sequential cross-sections can be imaged through a sample volume. We previously demonstrated that tendon fibrils throughout a 3D image stack can be automatically segmented using a U-Net machine learning algorithm [5]. The aim of the current study was to reconstruct the fibrils, creating volumetric renderings to quantify 3D fibril structure, and to assess potential differences between tendon type and tendon health.

METHODS: Three rat tendons were utilized: tail, plantaris, and a plantaris following 8 weeks of chronic mechanical overload in a synergist ablation model. Animal use was approved by IACUC and animal sex (M,F,F) and sample size are justified for method development. SBF-SEM images were taken from our previous study [5], with an example volume shown in Fig 1A. Fibril segmentation and 3D reconstruction were optimized and validated against manual methods. The highest performing method can be seen in Fig 1B: U-Net segmentation with cylinder tracing using Amira (ThermoFisher). While Amira provides useful visualizations, such as for tortuosity and orientation (seen in Fig C&D respectively), calculations are not always relevant or customizable. Therefore, centroid coordinates (x, y, z) of each fibril were extracted and analyzed using a custom MATLAB pipeline to calculate and compare fibril length, tortuosity, and alignment across the 3 tendon samples. Length was quantified by summing the Euclidean distances between consecutive centroids in each z-plane. Individual fibril lengths were normalized to a bulk fibril average (calculated with average centroids at each plane). Similarly, fibril tortuosity was calculated by normalizing individual fibril lengths to the straight-line distance between the first and last centroid, termed the "chord", where a value of 1 is a perfectly straight fibril and larger values indicate greater waviness/twist. Fibril alignment was calculated first as the chord length angle created between the straight-line chord of the individual fibril and that of the bulk average, representing the general relative orientation a fibril. Finally, the average fibril angle quantifies the degree of off-axis twist, i.e. the local angular deviation created between every pair of consecutive centroids relative to the bulk fibril.

RESULTS: Three-dimensional fibril structure varied by tendon type and health (Fig 2). While the peak frequency of all 4 measures was the same between tendons (with one exception), the magnitude of the peak frequency and spread varied between tendons. Rat tail fibrils had the highest peak magnitude of all 4 measures: 52% of fibrils had a relative length of 1 (Fig 2A), 66% had a tortuosity of 1.05 (Fig 2B), 80% had a chord length angle of 5° (Fig 2C), and 77% had an average fibril angle of 24° (Fig 2D). This indicates that rat tail fibrils were relatively straight and aligned. Compared to the rat tail tendon, the healthy plantaris tendon had more fibrils with shorter relative lengths but had a similar tortuosity distribution. Healthy plantaris fibrils were also less aligned, with peak magnitudes almost half those in the rat tail: 44% and 46% for chord length angle and relative angle, respectively, indicative of greater deviation in global and local fibril alignment. Overloaded plantaris fibrils were less organized than their healthy counterpart. More fibrils had longer relative lengths, had higher tortuosity (peak magnitude of only 43%), and less alignment by way of larger chord length angles and average angles (lower peak magnitude of 29%). Chord length angle of overloaded plantaris fibrils differed from both healthy tendons as there was no distinct peak. Most fibrils fell between 5° and 30° of deviation from the bulk chord axis.

DISCUSSION: Three-dimensional structural measures allowed us to characterize fibril organization within the tendon and identify distinctions between types of tendons. Plantaris fibrils were shorter, with similar twist, but less aligned than rat tail fibrils indicating a less uniform structure, potentially due to reported differences between the fiber structure of rat tail and plantaris tendons [6]. Two-dimensional U-Net analysis has also shown rat plantaris to have smaller diameter and denser fibrils than rat tail [5]. When the plantaris is subjected to overload, fibrils are longer, more twisted, and less aligned, indicative of disorganization that has been reported with similar models in animals [3] and human tendinopathy [4]. Again, this offers more information than the 2D analysis, where only a decrease in circularity for overloaded fibrils was found [5]. By utilizing machine learning to segment and reconstruct collagen fibrils from SBF-SEM images we were able to trace 90% of fibrils through the stack. This greatly improved throughput compared to manual methods: 42 rat tail fibrils compared to ~1,800 using our methods, with comparable tortuosity values [7]. Our methodology allows for the measurement of structural features that support and better characterize native fibril differences found between different tendons [2,6] and with disease/injury [3,4]. Further development of this tool could allow for investigation of different tendon regions, aging, other connective tissues, and the shape and influence of cells.

SIGNIFICANCE: SBF-SEM with fibril reconstruction allows for fast, robust assessment of 3D collagen fibril structure in tendon. Comprehensive characterization of fibril geometry and organization is necessary to infer mechanical function in healthy, adapted, or degenerated tissue.

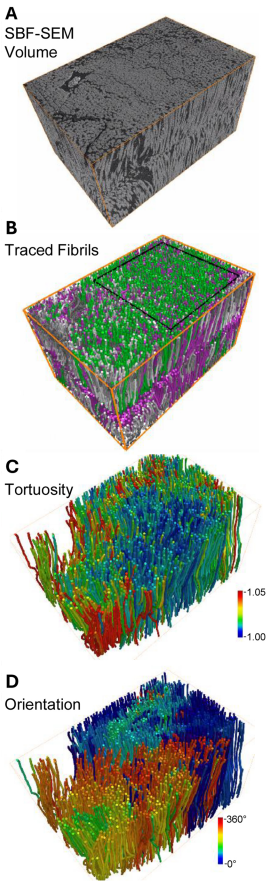

Fig 1: A) 3D SBF-SEM volume of plantaris tendon. **B)** Fibrils reconstructed in Amira with color representing % of sample spanned (gray<50%, purple>50% but less than full-length, green=full-length), **C)** tortuosity (red indicates higher tortuosity), and **D)** orientation (color shows the direction each fibril points in the imaging plane) for full-length fibrils.

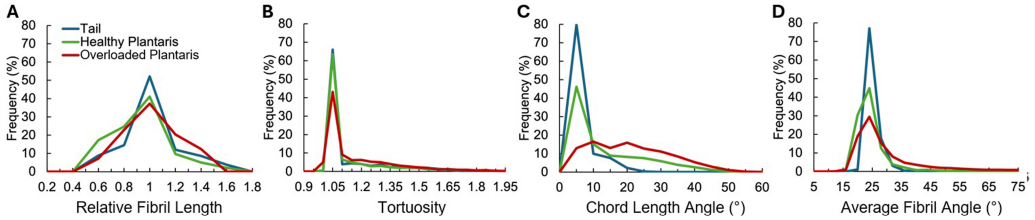

Fig 2: Frequency distributions for 3D structural fibril measures in 3 rat tendons: tail, healthy plantaris, and overloaded plantaris. **A)** Relative fibril length, **B)** tortuosity, **C)** chord length angle, and **D)** average angle.

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