Crimp characterization of porcine posterior cruciate ligament by second-harmonic generation imaging

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ABSTRACT INTRODUCTION: Ligament, which connects bone to bone and transmits the load between them, is mostly composed of collagen fiber bundles similar to tendon. These collagen fibers have a complex three-dimensional crimping pattern [1] that is related to the mechanical strength of the ligament [2]. Typically, polarized light microscopy has been used to image and analyze ligament crimping, but it is often not applied to thick samples and does not provide 3D structural information. In this work, we evaluated the capacity for second-harmonic generation (SHG) imaging [3], a novel nonlinear microscopy technique, to quantitatively characterize the ligament regions based on the direction of the crimping. SHG is a process that is specific to collagen type I, and as an imaging modality, is capable of producing 3D images of collagenous tissues with sub-micron resolution. Therefore, we show that SHG imaging provides label-free images of collagen crimp organization in 3D that otherwise cannot be observed using either standard bright field or polarized light microscopy. Moreover, using this technique we observe 3 distinct types of crimp organization in ligament that may add insight to the mechanical properties.

METHODS: Five porcine knee specimens (age=six months) were collected from the Meat Science Laboratory of the University of Illinois at Urbana-Champaign. All soft tissue except the posterior cruciate ligament (PCL) were dissected leaving a bone (femur)–ligament (PCL)–bone (tibia) complex. Each specimen was secured and then subjected to 5 cycles of loading-unloading before being elastically loaded to 100N within a materials test machine. After testing, the PCL from all samples were harvested. The ligaments were then cut into thirds along the main ligament direction and the middle region was sectioned at 100-µm thickness by a cryostat. After mounting the sliced tissue on microscope slides, the samples were imaged by SHG microscopy and compared with traditional bright field and polarized light microscopy. The size of the obtained SHG 3D image stacks were 200x200x30-µm in the x-y-z-dimensions with a step size of 500 nm along the z-axis. Spatial Fourier-transform (FT) analysis, which decomposes an image into its sinusoidal-wave building blocks of various spatial frequencies, was utilized to determine the preferred orientation of collagen fibers and crimps. This process was carried out for each image in a 3D stack and was subsequently converted to a normalized histogram.

RESULTS SECTION: From our SHG images we were able to define three distinct types of ligament regions, referred to as crimp-associated tag (CAT) A, B, or C, which could be distinguished by simple observation and confirmed using FT analysis. CAT A was defined to have little or no observed crimps in the fibers. Crimps that were confined in-plane were defined as CAT B. Crimps that were out-of-plane, along the third spatial dimension, were defined as CAT C. For further analysis, CAT C was divided into C-1 and C-2, which exhibited irregular and regular crimp patterns, respectively. On the FT histograms (Figure 1) CAT A had a single, narrow peak, while CAT B exhibited a single peak with a broader distribution, corresponding to more varied orientations of fibers in the associated tissue volume. On the other hand, CAT C-2 had two peaks. The identification of the type depended on the location in the ligament and also along the depth (z-plane) within a fixed region.

DISCUSSION: Straight fibers along a certain direction can be interpreted as a sinusoidal wave by plotting the intensity profile perpendicular to the fibers. The x-axis of the FT histogram represents the direction of the sinusoidal waves which also means the orientation of the fibers. Therefore, the narrow single peak on the FT histogram for CAT A indicates the general orientation of the fiber bundles in a volume. This also applies to CAT B, however the peak has a broader distribution due to the fact that the in-plane crimping permits neighboring angles to be added to the primary fiber orientation. For CAT C, one peak indicates the orientation while the other shows the crimp direction. This is because the out-of-plane crimps form a repeating line pattern that adds another propagating sinusoidal wave in a different direction with the fiber orientation. It is worth noting that there was a variation of the type along the z-axis from type A to C or B to C. According to previous studies, it has been discovered that fibers in ligament not only have planar crimps but also have features such as three dimensional helical crimps and knots [4], shown by scanning electron micrographs, which are not identified in this research. However, our novel approach using SHG imaging with FT analysis allows us to define crimp types, and this modality can potentially be related to the strength and toughness of the ligament.

SIGNIFICANCE: The capacity to quantitatively analyze the complexity of the fibrous structures in ligament has been an impediment to investigations of their contribution to the mechanical strength of ligaments. Utilizing SHG imaging and simple FT analysis, we believe that the defined crimp types can be used as a parameter connecting the structure and mechanical properties and can be used to design and develop improved ligament allographs.

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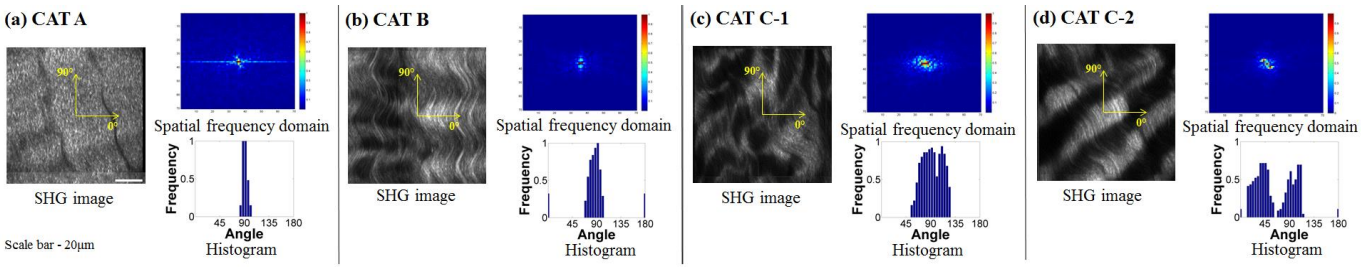

Figure 1. A representative SHG image and the corresponding FT converted image for each crimp type (CAT A, B, C-1, C-2). The FT image is integrated radially across different angles and plotted as the orientation angle verses radial amplitude strength. The FT histogram is the normalized sum of the plot from the entire stack.