New Methodology to Quantify Collagen Orientation and Organization in Bone

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Introduction: Bone is a biological composite that acquires distinctive mechanical properties of strength and toughness due to its highly organized hierarchical structure. While the mineral reinforces the collagen framework by conferring stiffness, the collagen organization at the fibers and fibrils level provides elasticity and toughness to bone. In diseases, such as osteogenesis imperfecta (OI), collagen type I moleculae are altered and that ramifies at the tissue level in a disorganized collagen fiber microarchitecture [1], which may contribute to the low fracture toughness of OI bone [2]. Second Harmonic Generation (SHG) imaging is a powerful technique that captures collagen-specific images without the need of sample staining. Quantitative SHG has been mainly based on the search for the preferred orientation of the fibers with respect to the horizontal axis [3]. Distribution of orientation has been suggested to describe well-organization of a bone surface. However, these methods miss to detect the continuous organization of collagen in bone, particularly in the areas around the pores, where the fiber arrangement follows the cavities organization. Therefore, in this study, we developed a new methodology for the quantification of collagen orientation and organization in bones based on SHG polarimetry.

Methods: SHG images of mouse bone cross-sections were collected at a excitation wavelength of 920nm using a 40X 0.8 N.A. water immersion objective (Prairie Tech. Ultima IV Multiphoton Microscope, Bruker). The images were processed, and the data were extracted using a custom-made MATLAB code. Specifically, in our approach, we divided the images into small facets of 32.83μm² size. We selected and removed the noise facets first to avoid their effect in the subsequent analysis (Fig. 1A). We then rotated a line fragment and performed morphological image apertures to calculate the regional orientation map per pixel (Fig. 1B). The principal orientation of the tissue within each pixel was calculated as the mode of the rotation values that gave the maximum intensity for aperture operations at each pixel (Fig. 1C). Based on this information, a vector field was displayed over the image of the bone section to show the preferred orientation of the collagen fibers within each facet (Fig. 1D). Subsequently, the orientation connectivity with the 8-neighbors facets was estimated for every facet. Two facets were considered connected if the orientation of their facets differ between +/- 20 deg. This allows to maintain the connection of symmetrical or U-shaped collagen regions. Connected regions were labeled, and small areas with less than 50 facets were discarded. The different surfaces were displayed with different colors on top of the original image (Fig. 1E). Only surfaces greater than 1/6 of the total bone area were considered to contribute to the well-organized bone surface (Fig. 1F), which was estimated as a percentage of the total area of bone in the image.

Results: Working with denoised images boosted the performance of the custom code, reducing computational costs and removing imperfections in the results. By rotating a line segment and performing an image aperture and subsequently saving the angle at which the signal was maximal, the preferred orientation of the collagen fibers was correctly identified in all the analyzed images (Fig. 1B-D). Our approach correctly detects aligned neighbors, identifying bone regions with continuous patterns of collagen fibers (Fig. 1E). After eliminating small patches that followed a particular orientation, bone areas with a continuous arrangement of collagen were correctly identified as well-aligned zones for different bone images (Fig. 1F).

Conclusions: This new methodology of quantification of bone collagen orientation reveals differences in preferential collagen organization in WT bone compared to *oim/oim*. Our results indicate that this code can be used to correctly identify continuous collagen fiber patterns in cortical bone, even in the presence of vascular canals, pores, and in darker regions characterized by lower signal intensity. The reported orientation maps were able to locally track the preferred orientation of collagen in a more quantifiable and consistent manner than the ones generated by OrientationJ (ImageJ (NIH)), a software package that has been commonly used for analyzing SHG images of different connective tissues [3]. This quantitative assessment provides a way of consistently characterizing regions in bone matrix where collagen fibers are continuously aligned contributing to a better bone mechanistic behavior [4].

References:

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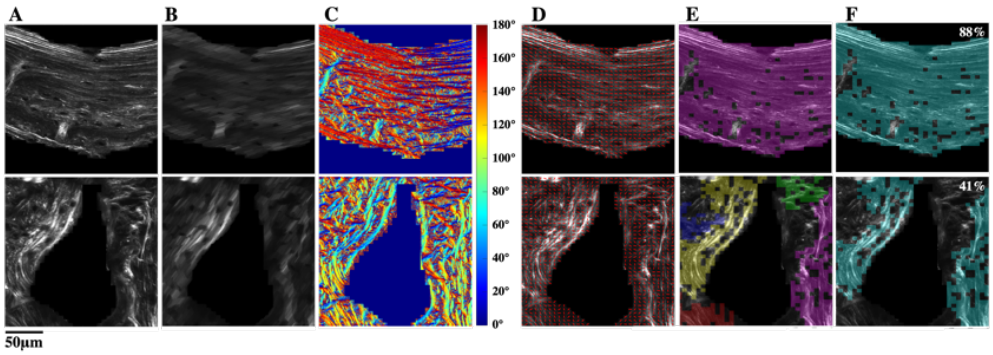

Figure 1. SHG image processing pipeline for two representative bone images. **A** Original images without noise. **B** Morphological opening of the grayscale images. **C** Local orientation maps. **D** Vector fields showing the preferred orientation of the collagen fibers in each facet. **E** Regions in bone with different principal orientations. **F** Area of bone with well-organized collagen orientation