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Multi-modal characterization of collagen fibril orientation in human cortical bone by a combination of quantitative polarized Raman spectroscopy, nanoscale X-ray computed tomography and 360° electron tomography

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Bone is one of the most exquisite materials of natural origin and astounds researchers with its morphological complexity. At the micro-scale, human cortical bone structural components are represented by arrays of parallel mineralized collagen fibrils (MCF) with a diameter of 10-300 nm, respectively. Variations of MCF orientation can be connected to degenerative metabolic changes in the bone structure [1] and are known to affect the mechanical properties of the bulk material [2]. Developing reliable and representative methods for quantitative assessment of MCF orientation poses considerable interest for medical diagnostics and therapy as well as for material development.

In previous studies, quantitative polarized Raman spectroscopy (qPRS) revealed quantitative information concerning the MCF orientation in bovine bone [3]. Following these promising results, the present study seeks to obtain correlative information in human cortical bone from the aforementioned pioneering technique and advanced microscopy techniques such as high-resolution X-ray tomography (Nano-CT) and 360° electron tomography (ET) by employing a correlative 3D workflow over several length-scales.

Combining these techniques appears promising to verify their accuracy and accordance as well as to overcome the limitations and expand the abilities of each of the characterization methods. On the one hand, qPRS provides information on the local MCF orientation and chemical composition of larger areas with a spatial resolution of ~$(0.3\ \mu m)^3$. On the other hand, Nano-CT allows obtaining statistically relevant data of individually segmented MCFs in a large sample volume of $(16\ \mu m)^3$ featuring a spatial resolution of $(50\ nm)^3$, hence enabling to directly resolve important features like fibrils and canaliculi channels (Figure 1e and 1f). Besides, it can serve as a navigation tool for ET. Transmission electron microscopy (TEM), in general, provides access to particularly interesting regions down to the atomic scale and also offers a flexible toolset concerning chemical composition using energy-dispersive X-ray spectroscopy (EDXS) and structural analysis by electron diffraction.

In this study, four freestanding bone micropillars (about 25 µm in diameter and 55 µm in height) were fabricated using femtosecond laser ablation and investigated with qPRS. The results are plotted as area maps with in-plane and out-of-plane MCF orientations (see Figure 1a,b and Figure 2c). Following up, pillars are investigated with non-destructive Nano-CT utilizing a ZEISS Xradia 810 Ultra with a quasi-

monochromatic 5.4 keV X-ray beam. For organic materials with a low variety of constituent elements, the image quality is typically not limited by the attenuation length but by the contrast difference of relatively small variations in the material. Consequently, we utilize Zernike phase contrast imaging to enhance edge contrast. The acquisition of tilt series and the subsequent reconstruction provides a 3D volume with structural information down to a resolution of $(50\ nm)^3$; these steps are depicted in Figure 1c-f. From the 3D reconstruction the local orientation of MCFs can be evaluated using machine-learning segmentation in Arivis Vision4D [4] (see Figure 2a and 2b). Further, regions of interest are selected to prepare pillars with a size suitable for ET with analogous reconstruction and segmentation routines for higher resolution data.

The multi-modal correlative characterization approach applied in this study makes it possible to analyze the structural organization of bone from the level of single MCFs with high spatial resolution to statistically relevant volumes with tunable size. The acquired complementary information can be used to identify new biomarkers of bone quality and, in the future, to improve the accuracy of fracture risk assessment in patients. [5]

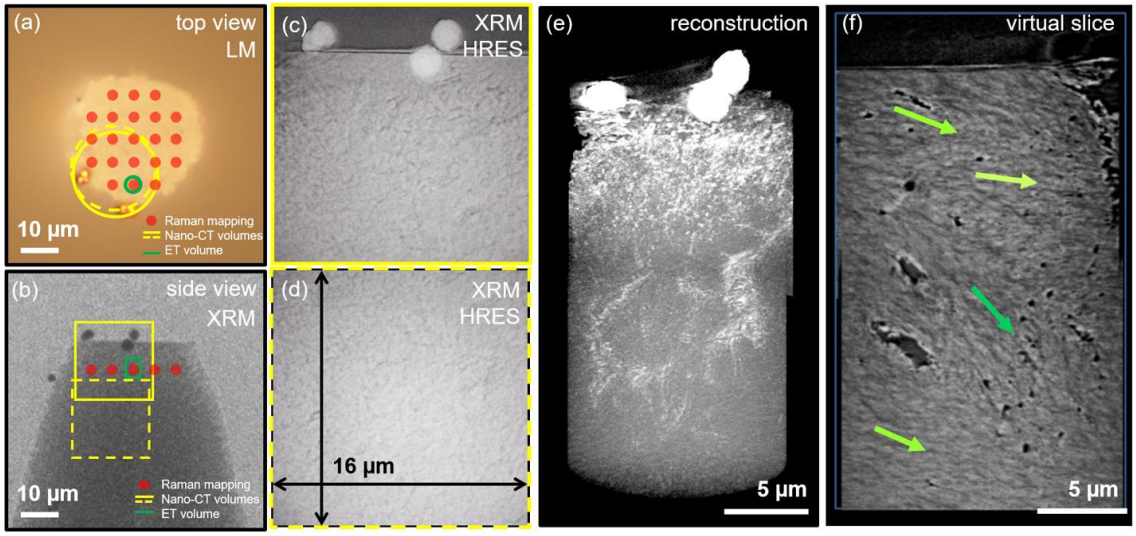

Figure 1. Multi-modal microscopy and spectroscopy. (a),(b) Light microscopy image (reflection mode) of one of four investigated bone pillars in top view and in side view (transmission mode) in the X-ray microscope (phase contrast). The specific regions of interest for qPRS, Nano-CT and 360°-ET are partialy overlapping. (c),(d) Upper (solid line) and lower (dashed line) Zernike phase contrast tilt series for correlation and in-depth tracing. Bright dots are artificial gold particles introduced to improve tilt series alignment and reconstruction. (e) The reconstructed 3D volumes of the upper and lower region are stitched together in the overlapping part (yellow circles in (a) and squares in (b)). Canaliculi channels and inner cavities are clearly visible (bright regions). (f) In the virtual vertical slice through the reconstructed volume, the local fibril orientation is visible as density variations of the fibrils. Exemplary local orientations are pointed out by arrows. Furthermore, regions of darker contrast can be indentified as cuts through Canaliculi channels.

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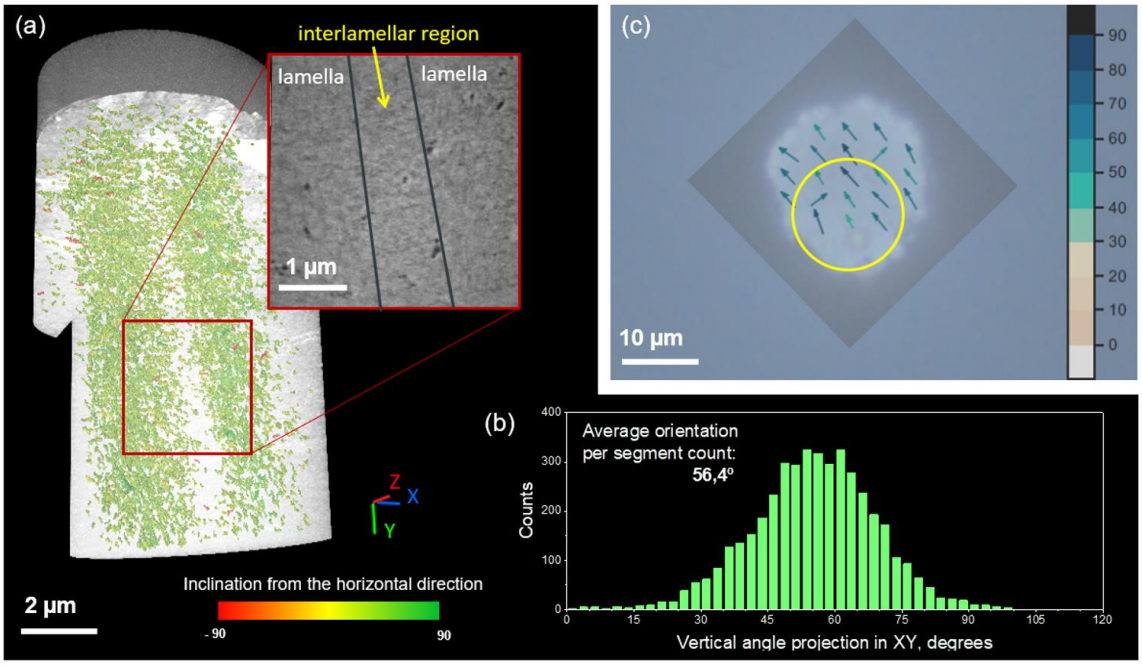

Figure 2. Preliminary results of fibril orientation analysis. (a) Machine-learning segmentation in Arivis Vision4D of fibrils in another bone pillar sample (color code represents the inclination from the horizontal plane). The estimated parallel lamella structure of the fibrils is clearly visible by the intersecting region (virtual slice magnified as an inset); (b) Histogram of fibril orientation angle distribution in the volume. The displayed angle represents a projection of the inclination from the vertical axis (pitch angle in Tait-Bryan notation) onto the common segmentation plane XY. The graph demonstrates a preferred orientation of 56.4° throughout the segmented volume. (c) Raman map obtained for a third pillar sample that features the in-plane (XZ) fibril orientation (arrow direction) and inclination from the vertical Y-axis also known as the pitch angle (color-coded on the right). Data is measured in elliptical points ~1 µm at a depth of 5 µm below the surface. The yellow circle indicates the position of the reconstructed Nano-CT volume.

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