Supplementary Information for

Three-dimensional Structural Interrelations between Cells, Extracellular Matrix and Mineral in Normally Mineralizing Avian Leg Tendon

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Figures S1 to S5 and legends

Movies S1 to S7 legends

Other supplementary materials for this manuscript include the following:

Movies S1 to S7

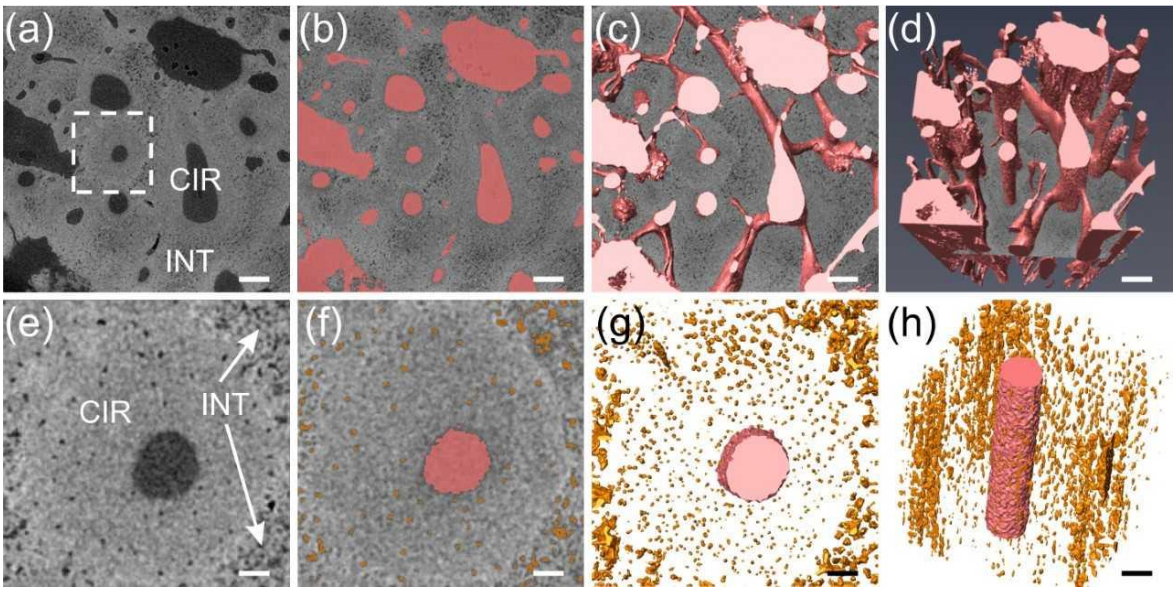

Fig. S1: Micro-CT images of a distal tendon specimen prepared by chemical methods and stained with osmium tetroxide. a, A cross-sectional slice from 3D micro-CT data of the tendon with a voxel size of 1.2 μm^3 shows a series of dark canals of various diameters traversing the tissue. The canals are surrounded by mineralized circumferential (CIR) tissue and such CIR tissue is itself separated by mineralized interstitial (INT) tissue. **b,** Unmineralized canals in **a** are highlighted in red. **c, d,** 3D surface rendering of unmineralized canals, presented at slightly different enlargements and angular aspects to demonstrate extensive interconnections or channels between canals. **e,** Enlarged image of the area marked in **a**, showing a single canal and numerous tenocyte lacunae. **f,** Unmineralized canal and lacunae in **e** highlighted in red and tangerine, respectively. **g, h,** 3D surface rendering of the canal (red) and lacunae (tangerine) in **f** viewed in transverse and approximately longitudinal profiles, respectively. Lacunae are elongated in longitudinal view and the lacunae long axes lie principally parallel to the canal. Both transverse and longitudinal views of the volume indicate lower density of lacunae within the circumferential zone compared to that within the interstitial region. Scale bars in panels **a**-**d**: 100 µm; in panels **e**-**h**: 25 µm.

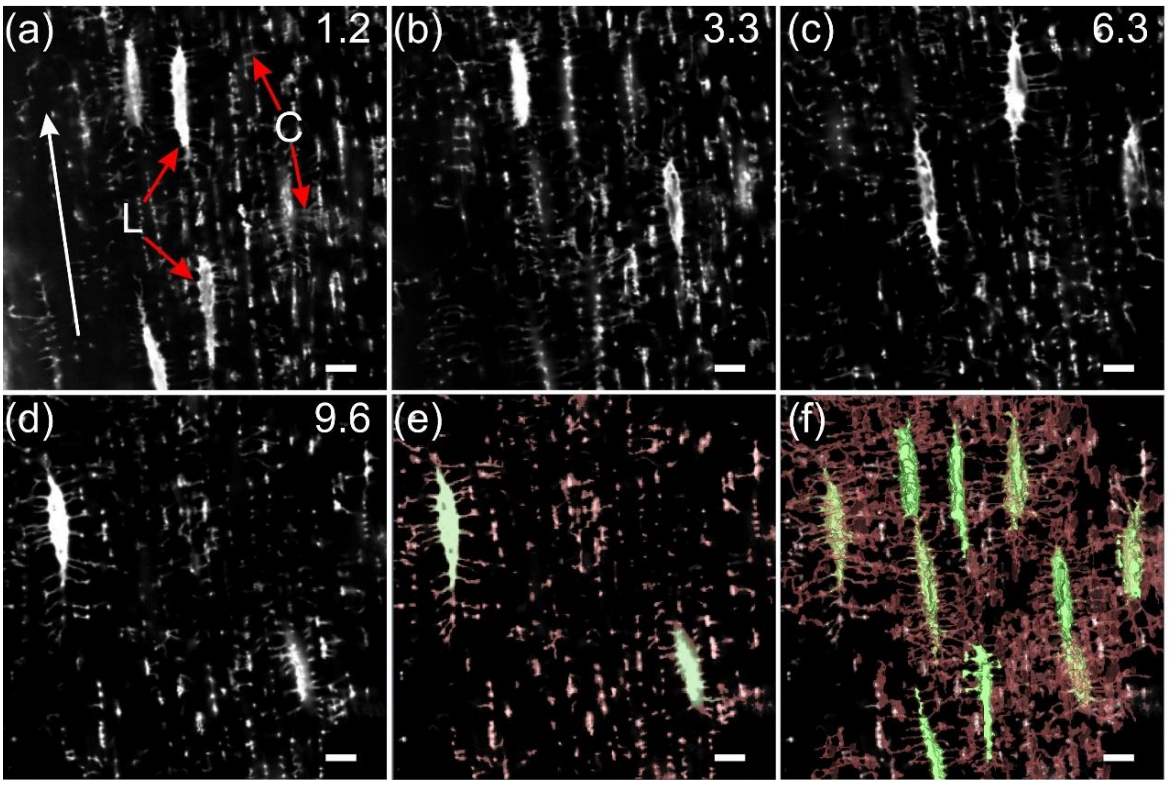

Fig. S2: 3D confocal images of a turkey distal tendon prepared by chemical fixation and osmium tetroxide staining. a-d, Representative slices of images from the 3D confocal volume show long, narrow tenocyte lacunae (L) and smaller canaliculi (C). The longitudinal direction of the tendon is marked by a white arrow. Slices are marked (upper right corner of an image) to designate the depth in microns from the tendon surface being viewed. **e,** Lacunae and canaliculi in **d** are highlighted in green and red, respectively. **f,** 3D surface rendering of the full volume of slices including **a-d** of lacunae and canaliculi demonstrates an extensive network resembling the lacuno-canalicular system in bone. Scale bar = 10 µm for all panels.

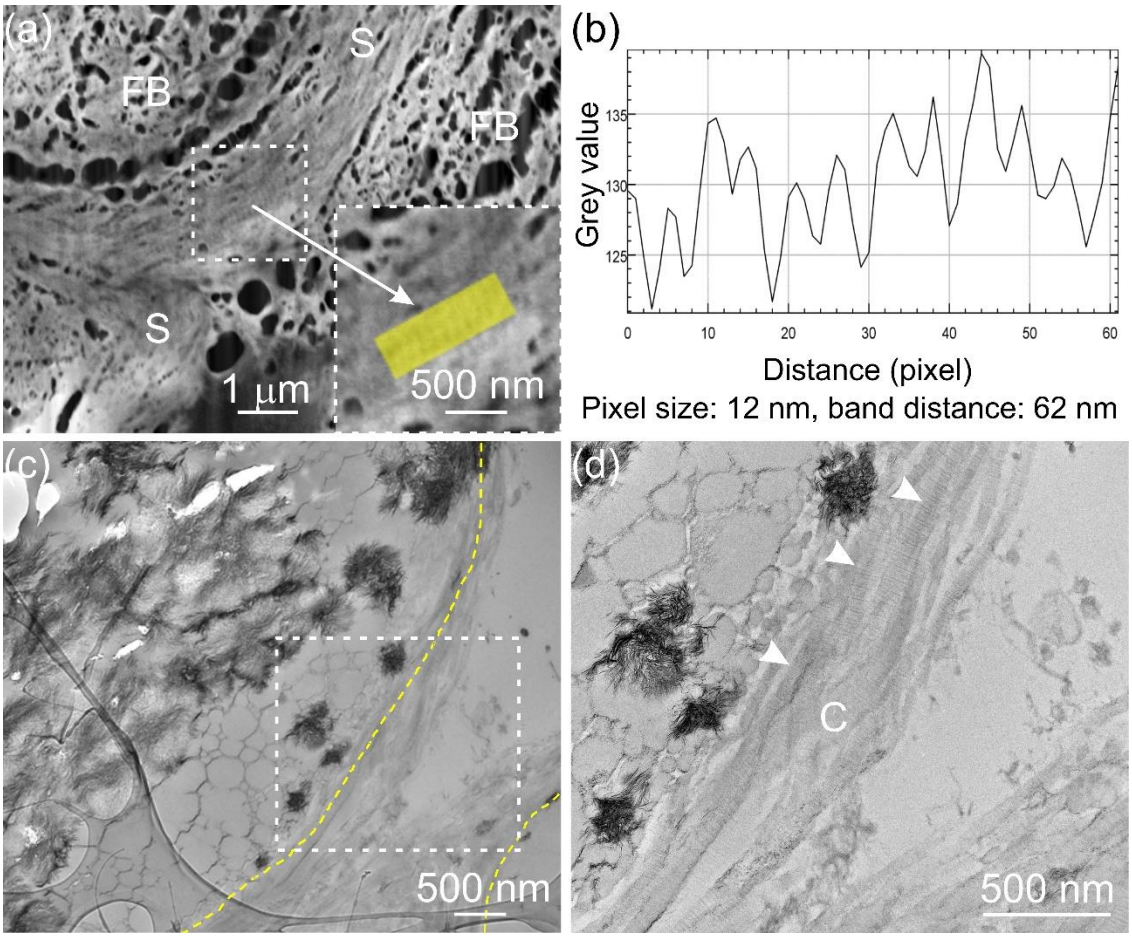

Fig. S3: FIB-SEM and TEM images of the sheath region of a distal tendon specimen prepared by HPF and stained with osmium tetroxide-uranyl acetate. a, A transverse SEM image showing the sheath region (S) containing collagen fibrils oriented in the circumferential direction with respect to the long axes of several fibril bundles (FB). An area of the sheath (outlined by white dashes) was enlarged (arrow, lower right aspect of **a**) and a portion was examined (following a longitudinal midline through the yellow rectangular region) by Fiji to determine changes in image grey values. **b,** The characteristic periodic banding pattern of collagen fibrils (~62 nm) was clearly observed on grey value analysis of this region. **c, d,** TEM images of a different region of the same tendon specimen shown in **a** illustrates the sheath region between two collagen fibril bundles. Yellow dashed lines denote the bundle boundaries. **d,** The enlarged TEM image of the region in **c** (white dashes) shows multiple collagen fibrils (C) with clear banding patterns (arrowheads) in the sheath region.

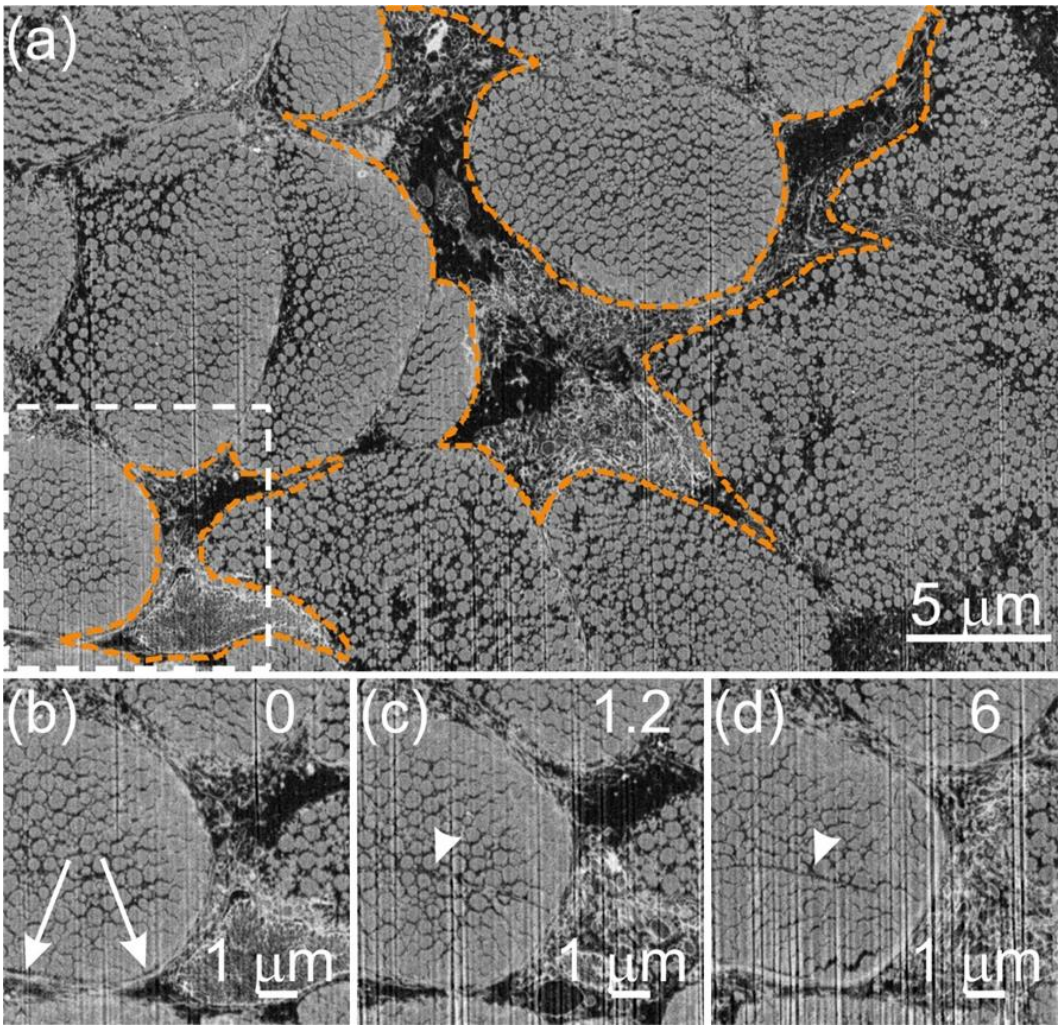

Fig. S4: Cross-sectional FIB-SEM images of an unmineralized distal region of a tendon specimen prepared by HPF and stained with osmium tetroxide-uranyl acetate. a, An SE image showing ultrastructural aspects of two tenocytes (tangerine dashed lines) situated between multiple collagen fibril bundles. **b,** Enlarged SE image in **a** (white dashed frame) reveals possible cell processes (arrows) surrounding collagen fibril bundles. **c**,**d,** Representive SE images obtained from the same volume of tissue as **b** but at different distances along the longitudinal direction of collagen fibril bundles. Numbers (top right corner of **c, d**) designate distance in µm from **b**. Putative canaliculi (arrowheads) were found occasionally traversing the collagen fibril bundles.

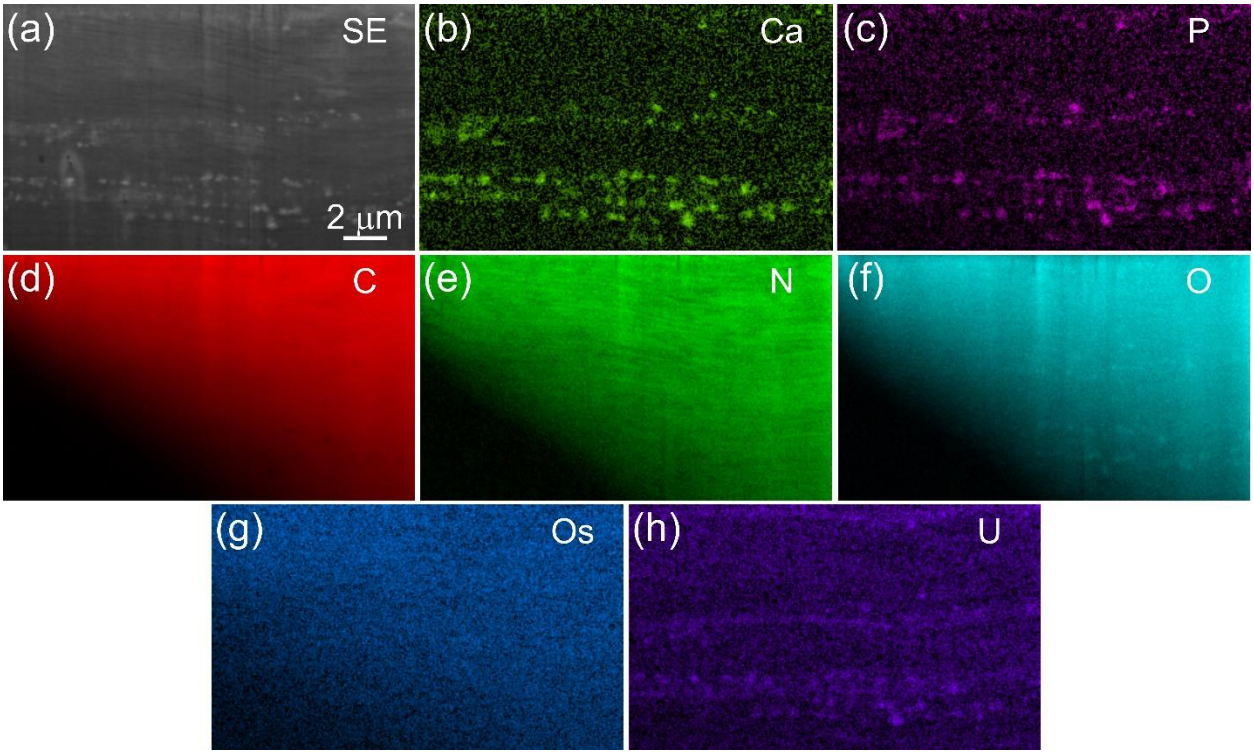

Fig. S5: FIB-SEM image and corresponding EDS mappings at the mineralization front of a proximal tendon specimen prepared by HPF and stained with osmium tetroxide-uranyl acetate. a, An SE image of a longitudinal section of a tendon tissue showing mineral deposits (white) and collagen fibrils (gray). **b-h,** Corresponding EDS mapping images of different elements in **a**. The mineral regions are rich in calcium **b** and phosphorous **c**. Nitrogen **e** shows slightly stronger signals in collagen while oxygen **f** has relatively higher intensity in mineral deposits. The signal intensities of carbon **d** and osmium **g** are homogeneously distributed across the tissue section. Uranyl **h** appears to have stronger association with mineral deposits.

Movie legends

Movie S1. 3D micro-CT imaging of a distal mineralized region of the tibialis cranialis tendon prepared by protocol 3. The movie starts with cross-sectional images advancing from mineralized to unmineralized portions of the tissue. At 1 min, the movie changes to longitudinal images. Numerous dark black (empty) canals are observed with white (mineralized) circumferential and interstitial regions surrounding them. Dark black and gray areas about black are thought to be regions of the tissue without Spurr embedding resin or only limited in resin infiltration, respectively. The region also shows extensive channels that interconnect canals. Voxel resolution is 4.5 μm^3.

Movie S2. LSCM images viewed following the longitudinal direction of a distal tendon prepared by protocol 3. Images progress from the tendon surface to deeper depths. In the movie with changing depth into the tissue, lacunae and canaliculi are visible and illustrate clearly the very extensive lacuno-canalicular network appearing similar to the osteocyte network in bone. Canaliculi are oriented principally perpendicular to the long axes of lacunae.

Movie S3. FIB-SEM images (EsB) and 3D reconstruction of a small, fully mineralized distal tendon region in a transverse profile to the longitudinal direction of the tissue prepared by protocol 3. Canaliculi originating from the lacunae of two representative tenocytes (tangerine) course through the ECM of the tendon composed here of mineralized (white) collagen fibril bundles. In 3D rendering, the canaliculi (gold) surround the bundles and clearly form circumferentially about them. Canaliculi from neighboring cells may interconnect.

Movie S4. FIB-SEM images (SE) and 3D reconstruction of a small, fully mineralized distal tendon region in transverse profile to the longitudinal direction of the tissue prepared by protocol 3. The movie shows numerous dark pores of various sizes and shapes throughout the ECM. These pores, reconstructed in 3D, comprise a vast system of secondary channels (turquoise) interconnected and oriented in a perpendicular direction to canaliculi (gold) and directed parallel to mineralized (white) collagen fibrils and fibril bundles.

Movie S5. FIB-SEM images (EsB) and 3D reconstruction of a small distal tendon region comprised of an interface between unmineralized and mineralized tissue zones. The specimen is viewed in transverse profile to the longitudinal direction of the tissue prepared by protocol 1. The region is comprised of mineralized (white) and unmineralized (grey) collagen bundles together with a few intervening cells. 3D rendering shows tenocytes (tangerine) in linear disposition closely associated with mineralized collagen (blue) as the protein is secreted from the cells to form fibril bundles. The movie also suggests that there are many individual mineralized collagen fibrils in apparent units separated within a fibril bundle by dark thin structures yet to be identified; they may originate from the canaliculi but further work is required to establish their source.

Movie S6. FIB-SEM images (SE) and 3D reconstruction of a distal tendon containing an interface between unmineralized and mineralized tissue zones. The specimen is viewed in transverse profile to the longitudinal direction of the tissue prepared by protocol 2. The region consists of collagen fibril bundles, one more heavily mineralized (right half of image and in white) than the others (left half of image). Bundles are separated by a continuous dark, narrow space representing the sheath about the mineralized bundle.

The more heavily mineralized bundle contains some fibrils yet to mineralize and which appear rather compact with indistinct borders. Fibrils comprising the less mineralized bundles are clearly separated from their neighbors by dark spaces which might be secondary channels. These same fibrils are not as compact as their counterpart unmineralized fibrils in the more heavily mineralized bundle. On 3D rendering of a portion of a less mineralized bundle, individual collagen fibrils (wine) of similar diameter, estimated to be ~250 nm, and unfused with each other are found with mineral deposits (blue) both within (intra-) and outside (interfibrillar) the fibrils.

Movie S7. FIB-SEM images (SE) and 3D reconstruction of a proximal tendon containing an interface between unmineralized and mineralized tissue zones. The specimen is viewed in longitudinal profile following the long axis of the tissue prepared by protocol 2. The region is characterized by numerous collagen fibrils of various diameters and separated from one another by dark spaces, some of which contain mineral deposits (white and pseudo-colored red) of different sizes and shapes. The dark spaces are thought to represent the secondary channels that pervade the tendon and provide the space for fluid transport of mineral ion and mineral precursors. The mineral deposits in the interfibrillar collagen spaces are presumed to originate in association with putative matrix vesicles residing in secondary channels. Sites of intrafibrillar mineral deposition are marked by blue. In this movie and several others reconstructed from stacks of FIB-SEM images, intrafibrillar mineral sites are found in very close proximity to interfibrillar mineral sites, which are arranged linearly and in abundance near each intrafibrillar mineral deposit.