The structural organization of the human CEP matrix across multiple length scales

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INTRODUCTION: Nutrients and metabolites entering and exiting the nucleus pulposus must pass through the cartilage endplate (CEP) [1], and hence, CEP permeability is strongly related to disc nutrition. For example, CEPs with low permeability impair nutrient transport to the disc cells *in vitro* [2] and associate with severer disc degeneration *in vivo* [3]. While much is known about the relative amounts of various CEP matrix constituents (e.g., collagen, glycosaminoglycans, and water) in relation to permeability [4, 5] , much less is known about the multiscale structural organization of CEP matrix constituents, such as the nanoscale porosity, matrix anisotropy, and cellularity. Measuring these characteristics is important for understanding the mechanisms of nutrient transport and for revealing changes in matrix turnover with aging and disease may impact nutrient transport. **The goal of this study was to discover the multiscale structural organization of the human CEP using multimodal imaging spanning the micron-to-nanoscales.**

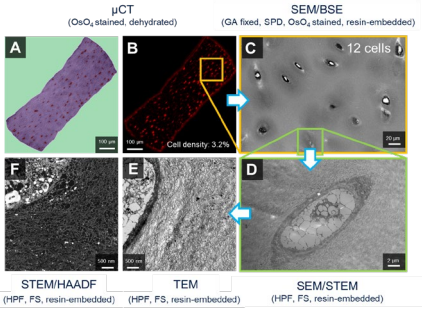

Figure 1. Multiscale structure of the CEP. (A-B) Micro-CT micrographs, (C) an SEM/BSE micrograph, (D) an SEM/STEM imaging, and a TEM (E) and STEM/HAADF (F) micrographs, respectively.

METHODS: The acquisition of human cadaver spines and surgical waste tissues used in this study was approved by our IRB. *Tissues.* Two fresh human lumbar spines (53 and 55 year-old, 1 male and 1 female) were obtained <72 hr post-mortem from UCSF's Willed Body Program. Intact cadaveric CEP samples (2-mm diameter) from the central region of the L3-L4 discs were removed from the subchondral bone and trimmed of NP tissue with a razor blade, resulting in a total of 6 full-thickness CEP samples (3 per donor). A third CEP sample was obtained from surgical waste tissues removed during spinal fusion (60 year-old female). *Micro-computed tomography (micro-CT).* CEP samples were scanned with micro-CT (Scanco μCT50): tube voltage 50 kVp, current 114 mA, integration time 250 ms, and isotropic voxel size of 12 μm. *High-pressure freezing*. Samples were frozen in a high-pressure freezer (Bal-Tec HPM 010) to prevent ice crystal damage on the chondrocytes and matrix. To achieve higher contrast, we used a freeze substitution protocol that involved 4% osmium tetroxide with 0.1% uranyl acetate and 5% deionized distilled. Finally, each sample was embedded in EPON resin in a BEEM capsule. *Electron microscopy imaging*. Ultramicrotome-prepared sections (up to 90 nm thickness) were imaged with a dual-beam system that combines scanning electron microscope with a focused ion beam (FIB; FEI Helios G4) and with a transmission electron microscope (TEM, FEI Tecnai F20). To help identify the interface between mineralized and non-mineralized regions of the CEP tissues, we employed electron dispersion X-ray spectroscopy (EDS) in TEM. A scanning and transmission electron microscope (STEM) with a high angle annular dark field (HAADF) image was also obtained to measure matrix porosity due to its sensitivity to chemical composition. *Image processing.* Nanoscale porosity, matrix anisotropy, and overall cellularity were measured using ImageJ. These characteristics were compared between regions of interest (ROIs) placed in territorial vs. inter-territorial matrices using paired t-tests.

RESULTS: Multiscale/multimodal imaging of the CEP (Fig. 1A and 1B) revealed high chondrocyte density (lacunar volume/total volume = 3.2%). From SEM imaging (Fig. 1C), the territorial and inter-territorial matrices were delineated using the density- and matrix-sensitive capabilities of the BSE detector. From the SEM/STEM imaging (Fig. 1D), CEP chondrocytes and their pericellular (territorial) matrices were visualized without the loss of sGAG that would have accompanied standard chemical fixation protocols. High-magnification TEM (Fig. 1E) and STEM/HAADF images (Fig. 1F) confirmed the bright-field and dark-field contrast needed for measuring matrix porosity and anisotropy. Montage images (Fig. 2A, a total of 256 stitched images, each at 9,300X magnification) were used to visualize neighboring chondrocytes and the territorial (Fig. 2B) and the inter-territorial matrix (Fig. 2C). Dispersion of the inter-territorial matrix was significantly higher than the territorial matrix ($p < 0.0001$), while the porosity was similar (Fig. 2D). Diffraction patterns from STEM/HAADF confirmed minimal electron beam damage on the sample (Fig. 2E and its inset), and EDS maps of showing the spatial distribution of various biological elements (Fig. 2F – C, O, N, Ca, P, and S) revealed sGAG-rich (high S) territorial matrices without mineralization (low Ca & P).

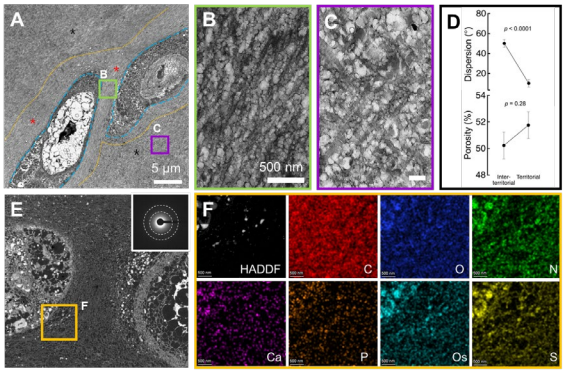

Figure 2. (A) TEM montage image of an ultramicrotome sectioned sample. Note that distinct characteristics of the territorial matrix and the inter-territorial matrix. (D) Porosity and dispersion between matrix types. (E) A STEM/HAADF image with its SAED pattern. (F) EDS maps of essential biological elements (C, O, N, Ca, P, and S) to confirm specific spatial elemental distribution from sGAG (S) and mineralization (Ca & P).

DISCUSSION: We found that the territorial matrix of the CEP is more highly aligned (lower dispersion) than the inter-territorial matrix. This difference suggests that inter-territorial and territorial matrix organization have distinct functional roles. Specifically, the more isotropic (higher dispersion) inter-territorial matrix would be expected to better facilitate nutrient transport through the CEP than the territorial matrix since the more anisotropic organization of the territorial matrix may confer greater *resistance* to solute transport perpendicular to the fiber direction (fibers are primarily organized transversely in the CEP [6]). Conversely, the more anisotropic organization of the territorial matrix is expected to better transmit mechanical signals to the embedded chrondrocytes than a more isotropic organization. We also observed compositional differences between the territorial and inter-territorial matrices: EDS mapping revealed more collagen-rich inter-territorial matrices and sGAG-rich territorial matrices. Interestingly, despite differences in organization and composition, inter-territorial and territorial matrices had similar overall porosity (50-52%), which suggests that measures of porosity are insensitive to important organizational and compositional characteristics of the CEP, and that age and degeneration-related changes to the composition and organization of the inter-territorial and territorial matrices could impact nutrient transport and mechanical behavior independently of any changes in matrix porosity.

SIGNIFICANCE: Structural and compositional motifs of the human CEPs may be altered by changes in matrix turnover that occur with aging and disease. This new multiscale and multimodal imaging approach enables mechanistic insights into these important characteristics impacting disc nutrition.

REFERENCES: [1] Maroudas A+, J Anat 1975;120. [2] Wong J+, Osteoarthr. Cartil. 2019;27. [3] Bonnheim NB+, Eur. Spine J 2022;31. [4] Wu Y+, J Biomech 2016;49. [5] Sampson SL+, J Biomech 2019;83. [6] Paietta RC+, J. Struct. Biol. 2013;184.

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