

Structural Analysis of the Rat Intervertebral Disc Interface Regions Utilizing Fourier Transform Infrared Spectroscopy Imaging and Atomic Force Microscopy

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INTRODUCTION: Recent work in the field suggests that the intervertebral disc (IVD) and surrounding tissue plays a large role in the onset of disc degeneration (DD) and related lower back pain [1]. The structure and function of the annulus fibrosus (AF) and nucleus pulposus (NP) regions of the IVD have been extensively studied. However, the cartilage endplate (CEP), bony endplate (BEP), and the transitional region from soft to hard tissue in the IVD has been comparatively understudied [2-4], despite evidence demonstrating their crucial role in the stability and function of the disc [5]. Furthermore, in considering a tissue-engineered (TE) replacement of the disc, the addition of an endplate (EP, referring to the CEP and BEP together) region has demonstrated better integration with native tissue [5]. This work aims to better understand the structure of this transitional region to highlight intrinsic properties of the CEP and BEP that are critical to the long-term stability and efficacy of a TE total disc replacement. To do this, we utilized Fourier Transform Infrared Spectroscopy Imaging (FTIR-I), which allows for high-resolution quantitative mapping of matrix compositional differences [6], as well as Atomic Force Microscopy (AFM), which provides resolution of the mechanical properties of biological tissue at the microscale [7].

METHODS: Lumbar spine of skeletally mature Sprague-Dawley (SD) rats (n=4, 4 months – 12 months old) were dissected to retrieve motion segments and then embedded along the coronal plane. Only male rats that have undergone secondary ossification in BEP were utilized. Females are not yet included due to a lack of an established relationship for vertebral bone skeletal maturity with age and rat weight. L2-L3 motion segments were fixed, decalcified and embedded for Safranin-O/Fast green and Picosirius red staining. L4-L5 motion segments for FTIR-I analysis were fixed as previously described [6], decalcified, embedded, sectioned and placed on barium fluoride optical windows (Spectral Systems). L4-L5 motion segments for AFM analysis were placed in 30% sucrose solution before cryo-embedding. Samples were sectioned at 20 μm using cryofilm adhesive (Section-Lab). **FTIR-I:** Spectra were obtained using a FTIR spectrometer (Spectrum 100, Perkin Elmer) and microscope imaging system (Spotlight 400, Perkin Elmer). 4 AF regions were analyzed using a 6.25 μm pixel size for a 60 x 60 pixel size region of interest (ROI), with the interface between EP and IVD aligned in the center of the image. Collagen distribution was mapped using the Amide I peak (1720 – 1590 cm^{-1}), and proteoglycan distribution using the carbohydrate peak (1140 to 985 cm^{-1}) [5]. Spectroscopic composition maps were generated using ISys 3.1.1 chemical imaging software (Spectral Dimensions Inc.) and MATLAB code. Spectra were baseline corrected. Line graphs show mean and SD. **AFM:** 6.1 μm diameter colloidal silicon AFM probes (NanoandMore) were used to indent on ROIs with trigger force of 300 nN. 2 ROIs of AF interface (AF-EP), NP interface (NP-EP), and BEP were selected, with 5 separate regions analyzed per ROI. Force indentation curves were fit using the Hertz model. Data were log-transformed [8] and analyzed with Kruskal-Wallis test followed by Dunn's multiple comparison test.

RESULTS: Polarized light images revealed that the AF lamellae structure is continuous through the EP and inserts into the mineralized BEP region. Safranin-O staining showed that the BEP has undergone secondary ossification and has no positive staining, whereas there is a spatial gradient in the AF, with more concentrated staining towards the inner AF region. FTIR-I analysis of the AF-EP interface showed that proteoglycan distribution does not significantly differ across the interface, although an increase in collagen can be seen when transitioning from the BEP to the AF (Figure 1). AFM brightfield images demonstrated that there is a distinct morphological difference in the transition region of the AF-EP and NP-EP region. Although NP data was not collected for these samples, comparison between the NP-EP region (avg. 129 KPa) and the AF-EP region (avg. 86.2 KPa) shows that the NP-EP region and the AF-EP region did not have significant differences in stiffness. The BEP (avg. 760.7 KPa) had a significantly higher modulus than the soft tissue regions. AF-EP modulus values were significantly higher than that of the AF region (avg. 30.2 KPa) (Figure 2).

DISCUSSION: Overall, FTIR-I results matched histological results, but provided more insight on gradients and quantified changes in matrix composition, particularly the collagen content. Preliminary results demonstrated that morphologically and structurally, the AF-EP interface is more continuous with the AF region compared to that of the NP-EP interface. This was further highlighted in histology and brightfield images which showed a distinct layer between the NP and BEP adjacent to the NP, but not adjacent to the AF. Notably, the indentation modulus of the AF-EP interface was higher than that of the AF alone, despite no visible difference in morphology. However, as this was not reflected in either the FTIR-I or the histology analysis for collagen or proteoglycans, this suggests that other matrix compositional differences or matrix organization may be contributing to these findings. Future work analyzing this interface region for mineral content and matrix organization, such as collagen alignment, may provide further insights. Focusing on the NP-EP region for FTIR-I as well as obtaining mechanical properties of the NP region will define differences in the soft tissue regions of the disc. In addition, further subdividing by age may elucidate any changes in matrix properties.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding the composition and mechanical properties of the interface regions will be critical in determining the structure and related function of the endplate and will guide TE design of a total disc replacement.

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FIGURES:

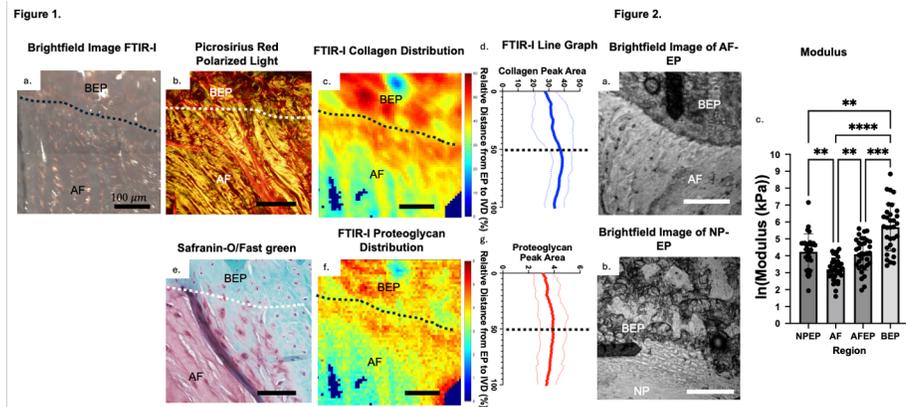


Figure 1. FTIR-I analysis on AF-EP interface region. A) Brightfield image of ROI B) Picosirius red stain of ROI C) FTIR-I Image of collagen content D) Line graph of collagen peak area across ROI, starting with the BEP to the IVD. (CI) E) Safranin-O/Fast green stain of ROI F) FTIR-I Image of proteoglycan content G) Line graph of proteoglycan peak area across ROI. **Figure 2.** AFM analysis. Brightfield image of ROI under AFM microscope, with representative images of ROI for A) AF-EP and B) NP-EP region. C) Bar graph of indentation modulus values for each ROI plotted on natural log axis. Technical replicates plotted. Kruskal-Wallis test followed by Dunn's multiple comparison test performed; ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. All scale bars are 100 μm .