Raman Microscopy with Phantom Calibration for Quantitative Imaging of Cartilage Biochemical Composition

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INTRODUCTION: Hyaline cartilage is a specialized connective tissue responsible for low-friction load transmission in synovial joints. Extracellular matrix (ECM) components impart the mechanical characteristics essential for cartilage function; the type-II collagen (COL) fibril network constrains negatively charged sulfated glycosaminoglycans (GAGs) that retain interstitial fluid, enabling compressive load support. Osteoarthritis (OA) is a chronic, debilitating synovial joint arthrosis characterized by the progressive degradation of hyaline cartilage, initiating with GAG depletion and breakdown of the COL network. The advancement of OA therapies through preclinical testing is burdened by a lack of standardized, quantitative cartilage assessment platforms that reflect the composition and mechanical function of cartilage in response to chondroprotective and chondrogenerative therapies. Histological scoring (e.g., OARSI, Mankin) remains the 'gold standard' *ex vivo* characterizations of cartilage pathology, but require laborious sample processing, are subject to staining artifacts, and are unable to provide quantitative measures of the distribution of cartilage ECM constituents [1]. Raman spectroscopy is an inelastic light scattering technique that shows the vibrational modes of the biochemical bonds in key cartilage ECM constituents [2]. Raman microscopy imaging offers a non-destructive, water-compatible, and label-free method for high spatial resolution evaluation of tissue biochemistry with high molecular specificity. Prior cartilage Raman imaging studies utilize semi-quantitative Raman peak analysis for classifying cartilage degenerative states, but do not offer quantitative evaluations of the concentration of ECM constituents in the tissue. Here, we present a novel quantitative Raman microscopy platform, combining multivariate spectral analysis coupled with biochemical phantom-based calibrations, to enable high-resolution fully quantitative mapping in absolute units (% per wet weight) of the distribution of cartilage ECM constituents.

METHODS: ***Tissue Preparation:*** Immature bovine stifle joint cartilage explants (∅3×2mm) were sterilely harvested with the trochlear articular surface intact and either frozen immediately (day 0; n=2) or subjected to a 7-day treatment with 50ng/mL IL-1α (n=2). Full-thickness human tibial plateau chondral explants (~∅6×3mm) were harvested from 2 human cadaver knees (NDRI, age/sex: 13M, 27F). The 13M knee appeared healthy, while the 27F knee had sustained prior meniscal trauma, repaired surgically. Samples were fixed overnight in formalin, bisected, embedded in OCT, cryosectioned to 100μm slices, mounted on steel plates, and submerged in PBS for Raman imaging (Fig.1). ***Raman Imaging***: The confocal Raman microscopy system consists of a 785nm laser (120mW; Toptica IBeam), 60x water immersion objective (Nikcon), motorized stage (Zaber), and fiber-coupled spectrometer (Eagle; Ibsen). Spectra maps were collected through the depth of bovine (650×1500μm^2) and human sections (650×3000μm^2) at 10×10 μm^2 resolution with a 3sec integration. Higher resolution maps were collected on two regions on the human cartilage (50×50 μm^2) at 1×1μm^2 resolution. ***Spectra Analysis***: Spectra were processed by background subtraction and area-under-curve normalization for fingerprint (800-1800cm^{-1}) and high wavenumber (2250-3600cm^{-1}) ranges. The cartilage fingerprint spectra were fit to a multivariate linear regression model: $Cartilage_{spectra} = GAG_{score}*(\mathbf{GAG_{REF}}) + COL_{score}*(\mathbf{COL_{REF}}) + H_2O_{score}*(\mathbf{H_2O_{REF}})$, where: $\mathbf{GAG_{REF}}$, $\mathbf{COL_{REF}}$, and $\mathbf{H_2O_{REF}}$ are spectra of purified reference chemicals of GAG, COL, and water, and 'scores' represent regression coefficient biomarkers that reflect the weighted contribution of each constituent to the measured tissue specra [3]. The high-wavenumber spectra were analyzed by measuring the areas under the organic-content-associated carbon-hydrogen ($\mathbf{CH_{area}}$) peak, and water-associated oxygen-hydrogen ($\mathbf{OH_{area}}$) peaks [4]. GAG, COL and H2O % per wet weight at each imaged pixel were calculated from the Raman scores using a data set of ECM phantoms of known chemical composition. Raman spectroscopy was performed on a set of phantoms emulating cartilage ECM, prepared by mixing known concentrations of GAG (bovine trachea chondroitin sulfate; 0-10%ww), COL (bovine skin gelatin; 0-20%ww), and PBS (70-100%ww). A multidimensional polynomial relationship was derived relating the biomarker scores to the known constituent concentrations in the phantom data set [5].

RESULTS: Raman imaging with phantom-calibration yielded high-resolution, spatial distribution measures of GAG, COL, and H_2O consistent with prior bulk measures from biochemical assays [6]. Day 0 bovine cartilage sections exhibited a characteristic zonal biochemical distribution, ranging from 1-4%ww GAG in superficial zone (SZ), 4-6%ww in middle zone (MZ), and 6-10%ww in deep zone (DZ) (Fig.2c-d). IL-1α treatment induced significant GAG depletion through the cartilage depth (Fig.2c) but COL content remained relatively unchanged, 10%ww Day 0 and 11%ww Day 7 (not shown). Compositional maps of the human 13M specimen revealed characteristic depth-dependent concentrations of ECM constituents (Fig.3a), with COL increasing from 8%ww in SZ to 25%ww in DZ, GAG increasing from 3%ww in SZ to 5%ww in DZ, and H_2O decreasing from 80%ww in SZ to 70%ww in DZ. In contrast, the 27F specimen revealed significant ECM degeneration, marked by a loss of zonal biochemical delineation and depletion of COL (1-10%ww) and GAG (0-3%ww), and elevated H_2O (87-100%ww) on average through the entire tissue depth. Detailed scans of chondrocytes showed high intercellular water content and a GAG/COL-rich pericellular matrix (Fig 3a).

DISCUSSION: We demonstrate that the implementation of multivariate spectral analysis coupled with biochemical phantom-based calibrations enables the generation of high-resolution (1-10μm), fully quantitative Raman microscopy images of the distribution of the major ECM constituents (GAG, COL, H_2O) in cartilage tissue sections. The accuracy of quantitative Raman imaging is supported by measured biochemical distributions that are consistent with previously reported depth-dependent biochemical assay measures of ECM in cartilage zones. Untreated bovine explants exhibit a zonal distribution of GAG, COL, and H_2O, consistent with literature values [6]. In response to IL-1 treatment, explants exhibit a loss of GAG and increase in H_2O, while COL content remains nearly unchanged, consistent with the predominant effect of IL-1 in upregulating GAG degrading enzymes over the time course of culture [7]. The healthy 13M human cartilage specimen also exhibits a zonal distribution of GAG, COL, and H_2O, consistent with literature values of young, healthy human specimens [8]. In contrast, the injured 27F human specimen exhibited ECM degenerative changes, consistent with the OARSI grade 2 post-traumatic OA [8]. Quantitative Raman imaging provides significant benefits over conventional histological analysis, including offering label-free analysis of hydrated cartilage specimens and providing high-resolution, fully quantitative measures of the distribution of ECM constituents.

SIGNIFICANCE: Osteoarthritis is one of the leading causes of disability, yet methods for quantitative assessment of cartilage pathology are limited. Raman microscopy provides a standardized, fully quantitative platform to evaluate the distribution of key cartilage ECM constituents that determine tissue mechanical performance, addressing a critical need for quantitative assessment of matrix degradation.

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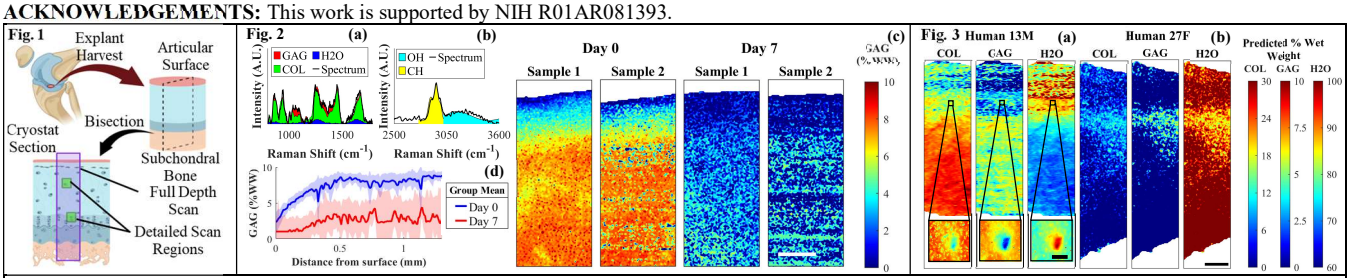

Fig. 1. Sample preparation diagram (BioRender, Brisebois, H., 2025, https://BioRender.com/9kmlooh). **Fig. 2.** Processed Raman fingerprint **(a)** and high-wavenumber **(b)** spectra with fitted references (GAG, COL, H_2O) and peak areas (OH, CH). **(c)** Predicted GAG maps of immature bovine cartilage: Day 0 controls (n=2) and IL-1α–treated Day 7 samples (n=2). Scale bar=300 μm. **(d)** Mean ± SD of GAG content across tissue depth. **Fig. 3.** COL, GAG, and H_2O maps of healthy (13M) **(a)** and arthritic (27F) **(b)** cartilage with a high-resolution chondrocyte scan. Scale bars=300 μm, 25 μm (inset).