Raman Arthroscopy for *In vivo* Quantitative Monitoring of Cartilage Composition in Equine Stifle Joint

Erik E Ersland[1], Madeline Boyes[2], Keming Yan[1], Hsin Han[3], J. Todd Lawrence[4], Thomas P Schaer[2],
Mark W Grinstaff[1], Brian D Snyder[5], Mads S Bergholt[6], Michael B Albro[1]

[1]Boston University, MA; [2]University of Pennsylvania Department of Clinical Studies New Bolton Center, PA; [3]Drexel University, PA; [4]The Children's Hospital of Philadelphia, PA; [5]Beth Israel Deaconess Medical Center, MA; [6]King's College London, UK

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Introduction: Hyaline cartilage is a viscoelastic, biphasic, composite material optimized for its mechanical performance, comprised of a type-II collagen (COL) fibril network (5-20% wet weight) that affords structure and tensile strength to constrain a negatively charged sulfated glycosaminoglycan (GAG) hydrogel matrix (5-15% wet weight) that retains interstitial water (interstitial fluid load support, *IFLS*). These components act synergistically, bestowing the compressive, rheological and tribological material properties essential to cartilage function. Osteoarthritis (OA) is an incapacitating condition in which hyaline cartilage progressively breaks down as a consequence of mechanical overloading that promulgates structural failure (fissuring/fibrillation/superficial zone delamination), tissue swelling, GAG depletion and COL network derangement. GAG depletion reduces *IFLS*, transferring load to the COLII fibril network, which subsequently breaks down, culminating in increased hydraulic permeability, decreased cartilage stiffness and lubricity. Development of disease-modifying OA therapies is impeded by a lack of non-destructive clinical assessments of cartilage composition and mechanical properties germane to tissue function. Quantitative MRI (T1r, T2*) and arthroscopic-based cartilage grading (Outerbridge) are only moderately correlated with metrics characterizing cartilage composition and material properties[1]. Raman spectroscopy is an inelastic optical light scattering technique that provides an optical fingerprint of a tissue's molecular building blocks, allowing quantification of the predominant molecular constituents of cartilage (GAG, COL, H_2O) that account for the material properties intrinsic to its function. We developed a novel Raman spectroscopy needle probe and real-time spectral analysis platform capable of performing both benchtop *ex vivo* and arthroscopic *in vivo* measurement of ECM-specific compositional biomarkers for cartilage with a high degree of accuracy[2] (R^2=0.8-0.94). Using phantoms comprised of prescribed proportions of GAG, COL and H2O that serve as biochemical standards, absolute measures of cartilage content can be derived from the Raman spectra[3]. Here we demonstrate that *in-vivo*, real-time Raman spectroscopy performed in the operating room using a needle probe to measure cartilage composition at discrete anatomical sites along the equine trochlear groove provided compositional data equivalent to *ex vivo* analysis performed on an excised equine trochlear groove and validated by biochemical assay.

Methods: A custom <u>Raman probe</u> (In Photonics) coupled to a threaded needle tip (2.75mm) with a distal 2mm sapphire ball lens (AWI), fiber-coupled to a 785nm laser (100mW output; IPS), and high-performance spectrometer (Eagle; Ibsen). The spectrometer, laser, and a computer were battery-powered allowing for portability. High SNR <u>Raman spectra</u> in the fingerprint (800-1800 cm-1) and high wavenumber (2700-3800 cm-1) ranges were acquired. Spectra were preprocessed via background subtraction and area-under-curve normalization. The cartilage spectra (800-1800cm-1) was fit to a multivariate linear regression model: $Cart_{Spectra}=GAG_{Score}*(GAG_{REF})+COL_{Score}*(COL_{REF})+H2O_{Score}*(H_2O_{REF})$, where GAG_{REF}, COL_{REF}, and H_2O_{REF} are the reference spectra of purified chemicals for each ECM constituent; the "scores" are the regression coefficients reflecting the relative contribution of each constituent (Fig.2A,B). The high-wavenumber range spectra (2700-3800cm-1) was used to compute the area under the OH peak, reflecting tissue hydration[4]. The Raman spectral analysis was performed in real time, with ECM biomarker scores and quality metrics displayed to the operator via a custom GUI. Tissue phantoms comprised of prescribed ratios of GAG (chondroitin sulfate [0-10%w/v]), COL (gelatin [0-20%w/v]), and H2O (PBS [70-100%w/v]) were used to convert Raman scores to equivalent %w/v by applying a 2nd order polynomial function of Raman scores fit to the known %w/v of the tissue phantoms to map "scores" and derive %w/v estimates of ECM composition[3]. <u>In Vivo Spectra Analysis</u>: After IACUC approval Raman needle probe spectral analysis was performed on the vertical wall of the lateral trochlear groove of two 5-year-old TB horses through a 5–6 cm mini-arthrotomy between and parallel to the middle and lateral patellar ligaments before and after creating two 15mm focal defects removing uncalcified and calcified cartilage down to the subchondral bone (Fig 1). The needle tip was autoclaved and the optics draped in sterile plastic film. Measures were performed at defined anatomical sites using a PDMS template with 4mm diam. holes at sites along a defined polar coordinate system overlaying the trochlear facet. At each site, the probe lens gently contacted the cartilage surface, with spectra acquired over a 5 sec integration time. <u>Ex-vivo validation</u>: 3mm full thickness cartilage plugs were harvested at 169 discrete anatomical sites over the surface of an excised 5-y/o equine femoral trochlear groove. The composition of each plug was determined by Raman spectral analysis and DMMB GAG assay.

Results: Raman derived biomarkers accounted for 78% of the variance in GAG content (Fig 2E) and depicted a biochemical spatial gradient along the length of the trochlear groove, with GAG content increasing and H2O content decreasing from distal to proximal (Fig 2A-D). In the surgical setting, the needle probe achieved high quality spectral acquisition of cartilage, as evidenced by a high SNR and the strong fit of the multivariate regression model to the $Cart_{Spectra}$ (R^2=0.93+/-0.03). *In-vivo* Raman GAG measures exhibited a similar range and spatial distribution as the *ex vivo* analysis (Fig 3).

Discussion: This study establishes the capability of our Raman arthroscopic platform to achieve accurate quantitative *in vivo* measures of the biochemical content of cartilage in the surgical setting. The analysis is fully automated, which provides the clinician real time measurement of tissue composition, allowing diagnostic assessments at defined sites. The accuracy of ECM composition calculated from the *in-vivo* Raman spectral analysis is supported by *ex vivo* measures of tissue composition at discrete anatomical sites over the equine trochlea, where Raman derived GAG measures accounted for 78% of the variation in GAG content. While Raman derived GAG %w/v underestimated the GAG content by ~20% (Fig.2E), this is attributable to some disparately between the regions of Raman probe interrogation (~300µm depth of penetration) vs. the full thickness cartilage plug used for DMMB assay. The successful implementation of this platform *in-vivo* supports the use of Raman arthroscopy to objectively monitor standardized biomarkers that reflect cartilage function in health and disease and to (non-destructively) longitudinally monitor the efficacy of OA treatments that preserve or regenerate the cartilage ECM in pre-clinical animal models and human clinical trials. We will perform Raman arthroscopy assessments of the regenerate tissue generated by micro-fracture at the chondral defects at 3 & 6-month follow-up on these horses, providing the first-ever longitudinal *in vivo* Raman assessment of tissue response to a chondroregenerative therapy.

Significance: This work supports the use of Raman spectroscopy acquired through needle probes as a clinical tool to perform comprehensive diagnostics of the composition of articular cartilage in health and disease and as a non-destructive tool to monitor the tissue response to chondroprotective and chondroregenerative therapies *ex-vivo*, *in-vivo* and clinically using Raman arthroscopy.

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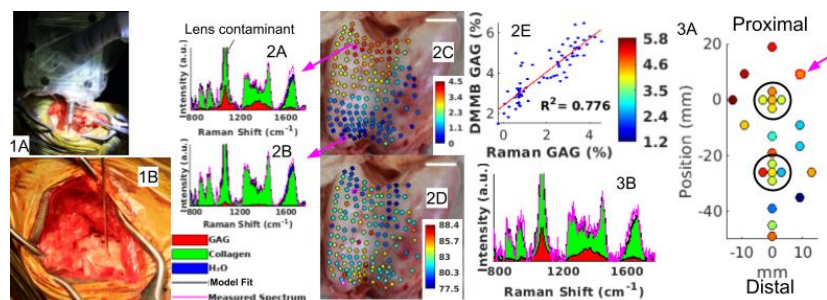
Fig 1: (**A**) Probe in contact with trochlea surface. (**C**) PDMS location guide aligned to tissue coordinate system.
Fig 2: (**A,B**) Representative 2D stacked area plot of cumulative contribution of ECM constituents to measured Raman spectra for GAG replete and depleted ex vivo tissue regions. *Ex vivo* Raman biochemical mapping of (**C**) GAG and (**D**) H_2O contents (% wet weight) over surface of equine trochlea. 20mm scale bar. (**E**) Regression Raman biochemical analysis vs. DMMB GAG content. **Fig 3**: (**A**) *In vivo* Raman biochemical mapping of GAG content over surface of equine trochlea. Chondral defects (made after measurement) marked. (**B**) 2D stacked area plot of cumulative contribution of ECM constituents to *in vivo* Raman spectra.