Deep Imaging of Collagen- and Proteoglycan-Rich Tissues using Optical Clearing

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Introduction: The understanding of native osteochondral tissue structure is limited by the penetration depth of light in confocal microscopy, which is influenced by the absorption of excitation energy and scattering of excitation and emission fluorescent photons in the collagen-, proteoglycan-, and mineral-rich tissue. In cartilage, chondrocytes have been visualized in 3D by confocal microscopy, although only to depths of less than ~100 μm [1]. Two-photon microscopy is capable of penetrating deeper in tissue owing to the use of longer wavelength excitation light [2]. Nonlinear optical microscopy, including second harmonic generation, provides increased depth of imaging in thick tissues, to ~400 μm, though collagen and extracellular matrix are typically visualized without revealing detail of embedded cells or cellular substructures [3]. Moreover, histomorphometry techniques are widely used to study osteochondral tissues, but they require invasive (~5 μm thick) sectioning that limit a full three-dimensional characterization of cells throughout the complex tissue, and in the context of disease and regeneration. Optical clearing by sugar-based techniques has emerged as a powerful tool to characterize cellular volume, morphology, and connectivity in a range of complex tissues, including brain and heart [4]; however, optical clearing has not been studied in the articular cartilage and bone. Here, we quantify how optical clearing impacted the cell and tissue morphology of collagen-, proteoglycan-, and mineral-rich cartilage and bone from the articulating knee joint.

Methods: Water-based fructose solutions were used for optical clearing of bovine tissues, followed by imaging with transmission and confocal microscopy. Tissue Acquisition: Osteochondral tissues (5-7 month-old) were harvested from bovine knee (stifle) joints obtained from a local abattoir within 48 hrs of slaughter. Cylindrical samples (ϕ = 5.0 mm, 5.0 mm height) were excised from medial femoral condyle regions and immediately placed in 4% paraformaldehyde in phosphate buffered saline (PBS) or PBS alone for subsequent comparative analyses. Optical Clearing: Osteochondral tissues were cleared using a fructose-based optical clearing agent [4]. Clearing solutions were prepared using D-(−)-fructose dissolved in ultrapure (milliQ) water of increasing concentrations (i.e., 20%, 40%, 60%, 80%, 100% and 115% wt/vol), with 0.5% α-thioglycerol added to prevent browning. Tissues were placed in ~1 mL of each concentrated fructose solution overnight at room temperature, under a gentle rocking motion. Unclearing of tissues was achieved by reversing the clearing process, through equilibration in fructose solutions of decreasing concentrations, to PBS. Microscopy: Light transmission through cartilage was assessed through the bulk (control, cleared, and uncleared) tissue (n=3) using a stereo dissecting microscope (Leica M80). Tissues were imaged on a printed grid pattern (spacing=2.1 mm). To image chondrocyte morphology deep within cleared cartilage, cell membranes (DiI) and nuclei (Hoechst) were labeled and visualized on a confocal microscope (Zeiss LSM 710) equipped with a 25× LD LCI Plan-Apochromat multi-immersion lens (NA=0.8, working distance=500μm, immersion oil n=1.518). Imaging parameters were: excitation wavelengths= 561 (DiI) and 405 (Hoechst 34580) nm; field of view= 340.1×340.1 μm²; matrix= 512 x 512 pixels²; number of slices=479; interslice spacing=1.0 μm. To determine the influence of fructose-based clearing on chondrocyte volume, cell membranes (DiI) were
labeled in separate samples and imaged following control, cleared, and uncleared treatments. Confocal imaging parameters were identical to those described previously. Cell volumes (n=10) were computed using image thresholding and region filling routines written in MATLAB (Mathworks) and compared by an analysis of variance. Mechanical Testing: To confirm preservation of tissue structure during the clearing process, samples were mechanically tested in unconfined compression.

Results: Optical clearing enhanced light transmission through cartilage, but not subchondral bone regions (Fig 1). In 2.5 mm thick samples from both load-bearing and non-load-bearing joint regions, cartilage became translucent following graded immersion in concentrated fructose solutions, revealing printed grid patterns on an underlying substrate. Translucent tissues regained normal (control) opacity following reversible graded immersion in PBS. Fluorescent labeling was preserved through sample preparations, enabling standard confocal imaging to depths approaching 500 μm, which was limited by the working distance of the microscope objective employed (Fig. 2). The outlines of individual chondrocytes (DiI, red) and their nuclei (Hoechst, blue) could be imaged through the superficial zone (z=1 μm) to deep within the cartilage middle zone (z=440 μm). Moreover, there was no significant change in average cell volume in control (399.6±69.2 μm³), cleared (390.6±69.1 μm³), and uncleared samples (402.4±68.6 μm³) (p>0.050). Normalized cell volume on average was slightly lower in cleared samples, though not significant, and qualitatively demonstrated improved definition of membrane morphology. Equilibrium moduli for unfixed/fixed samples were 0.4±0.1/0.5±0.1 MPa (control), 0.8±0.2/0.9±0.4 MPa (cleared), and 0.2±0.1/0.2±0.0 MPa (uncleared).

Discussion: Recent developments in optical clearing and microscopy technology have enabled the imaging of intact tissues at the millimeter scale to characterize cells via fluorescence labeling. While these techniques have facilitated the 3D cellular characterization within brain and heart, study of dense connective tissues of the musculoskeletal system have been largely unexplored. Fluorescent imaging was performed using standard (one-photon) confocal imaging with penetration depths limited only by the working distance of the microscope objective. Sample preparations did not alter fluorescent labeling, which included membrane and nucleic acid stains, in addition to cytoskeletal and immunolabeled extracellular matrix molecules (not shown). Equilibrium modulus increased in cleared samples, and was attributed to exchange of interstitial fluid with the more viscous fructose solution, but returned to control levels upon unclearing. Tissue morphology (assessed by light microscopy and scanning electron microscopy, not shown) remained unchanged and material properties returned to control levels in uncleared tissues, indicating that the structural integrity was conserved during the clearing process.

Significance: We anticipate large-scale microscopy of diverse connective tissues will enable the study of intact, 3D interfaces (e.g. osteochondral) and cellular connectivity as a function of development, disease, and regeneration, which have been previously hindered by specimen opacity.
Fig. 1. Optical clearing is achieved in collagen- and proteoglycan-rich osteochondral tissues.

Fig. 2. Visualization of fluorescent markers observed through the cartilage depth are limited by the working distance of the microscope objective.