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Optical clearing in collagen- and proteoglycan-rich osteochondral tissues

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SUMMARY

Objective: 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 three-dimensional (3D) cellular characterization within brain and heart, study of dense connective tissues of the musculoskeletal system have been largely unexplored. 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 osteochondral tissues, followed by imaging with transmission and confocal microscopy. To confirm preservation of tissue structure during the clearing process, samples were mechanically tested in unconfined compression and visualized by cryo-SEM.

Results: Optical clearing enhanced light transmission through cartilage, but not subchondral bone regions. Fluorescent staining and immunolabeling was preserved through sample preparations, enabling imaging to cartilage depths five times deeper than previously reported, limited only by the working distance of the microscope objective. Chondrocyte volume remained unchanged in response to, and upon the reversal, of clearing. 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. In addition, cryo-SEM-based analysis of cartilage showed no ultrastructural changes.

Conclusion: 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.

Introduction

Articular cartilage and bone exhibit a gradient in structural heterogeneity through the depth of the layered osteochondral tissue that gives rise to unique functional and biological properties[1]. At the articular surface, where cartilage counterfaces contact in the joint to permit normal daily activities like walking and running, type II collagen fibrils run parallel to the surface, and are covered by a molecularly (i.e., nanometer) thin boundary lubricating material, including superficial zone protein (aka SZP/lubricin/PRG4), that contributes to low friction and wear[2]. Beneath this superficial zone, the extracellular matrix (ECM) of the middle zone (MZ) is characterized by randomly oriented type II collagen fibrils and localization of cartilage oligomeric matrix protein (COMP)[3]. Type X collagen is a characteristic marker of hypertrophic chondrocytes at or near the deep zone[4,5], while type II collagen fibrils orient perpendicular (and anchor) to the calcified cartilage and subchondral bone. Cell and pericellular matrix morphology, proteoglycan (e.g., aggrecan) expression, and water content all vary by tissue depth from the surface, giving rise to zonal cellular subpopulations within a load-bearing (LB) viscoelastic and anisotropic tissue. Importantly, cartilage tissue structure is altered – degraded, worn, and softened – during aging and through the progression of osteoarthritis (OA), a debilitating disease affecting tens of millions of people in the United States alone[6]. Moreover, recapitulation of the normal osteochondral tissue structure remains an elusive target for

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scientists designing regenerative medicine and tissue engineering strategies[7].

Cartilage and bone microstructure has historically been visualized *in situ* by optical microscopy. Unfortunately, the penetration depth of light in confocal microscopy is influenced by the tissue structure of the collagen-, proteoglycan-, and mineral-rich tissue, which limits the absorption of excitation energy and increases scattering of excitation and emission fluorescent photons, and therefore also limits the understanding of native osteochondral tissue structure. In cartilage, chondrocytes have been visualized in three dimensions by confocal microscopy, although only to depths of less than ~100 μm[8,9]. Two-photon microscopy is capable of penetrating deeper in tissue owning in part to the use of longer wavelength excitation light[10,11]. In a direct comparison of optical imaging of cartilage, two-photon microscopy provided visualization of calcein-loaded chondrocytes to a depth of ~300 μm (or approximately a two-fold increase over one-photon excitation), depending on the wavelength and setup of the optical system[12]. Nonlinear optical microscopy, including second harmonic generation, provides increased depth of imaging in thick tissues, to ~400 μm, though collagen and ECM are typically visualized without revealing detail of embedded cells or cellular substructures[13,14]. Moreover, histomorphometry techniques are widely used to study osteochondral tissues, but they require invasive (~5 μm thick) sectioning that limit a full three-dimensional (3D) characterization of cells throughout the complex osteochondral tissue, and in the context of disease and regeneration.

Optical clearing by sugar-based and hydrogel replacement techniques has emerged as a powerful tool to characterize cellular volume, morphology, and connectivity in a range of complex tissues, including brain and heart[15–18]. Moreover, optical clearing by refractive index matching using BABB or glycerol-based solutions in conjunction with second harmonic generation has imaged tendon[19], skin[20], muscle[21], cartilage and bone[22] at the tissue scale. However, the previous studies utilized reagents that precluded the ability to identify and resolve individual cells within the musculoskeletal system due to significant specimen shrinkage (BABB) or disruption of ECM morphology (glycerol). In this study, we explore the possibility that optical clearing of dense connective tissues will reveal unique microstructures and cell characteristics that are not easily observed or quantified using standard microscopy or histomorphometry techniques. Our primary objective is to determine the effect of morphology-preserving and fructose-based optical clearing agents[15] *in situ* on collagen-, proteoglycan-, and mineral-rich osteochondral tissues. Secondarily, we characterize the connectivity of chondrocytes throughout cartilage and near the complex osteochondral interface within tissues *in situ*. Multiple assays, including mechanical testing and scanning electron microscopy, are used to verify the reversibility and morphology-preserving characteristics of optical clearing for osteochondral tissues.

Materials and methods

Tissue acquisition

Osteochondral tissues (5–7 month-old) were harvested from bovine knee (stifle) joints obtained from a local abattoir (Dutch Valley Foods, Holland, IL) within 48 h of slaughter. Cylindrical samples (ø = 5.0 mm, 5.0 mm height) were excised from medial femoral condyle regions and trochlear groove that naturally experience relatively high and low *in vivo* contact pressures (i.e., LB and non-load-bearing (NLB) regions, respectively; Fig. 1(A))[23]. Samples were immediately placed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) or PBS alone for subsequent comparative analyses. Samples in PFA or PBS were placed on a rocker for 1–2 days at 4°C, then rinsed 2 × 1 h with PBS at room temperature with gentle rocking.

Optical clearing

Osteochondral tissues were cleared using a fructose-based optical clearing agent[15] [Fig. 1(D); sample sizes for each assay defined subsequently]. 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 and under a gentle rocking motion. Unclearing of osteochondral tissues was achieved by reversing the clearing process, through daily equilibration in tissues in fructose solutions of decreasing concentrations, to PBS.

Transmission and confocal microscopy

Cartilage and chondrocyte morphology were imaged by light microscopy. Light transmission was assessed through the bulk

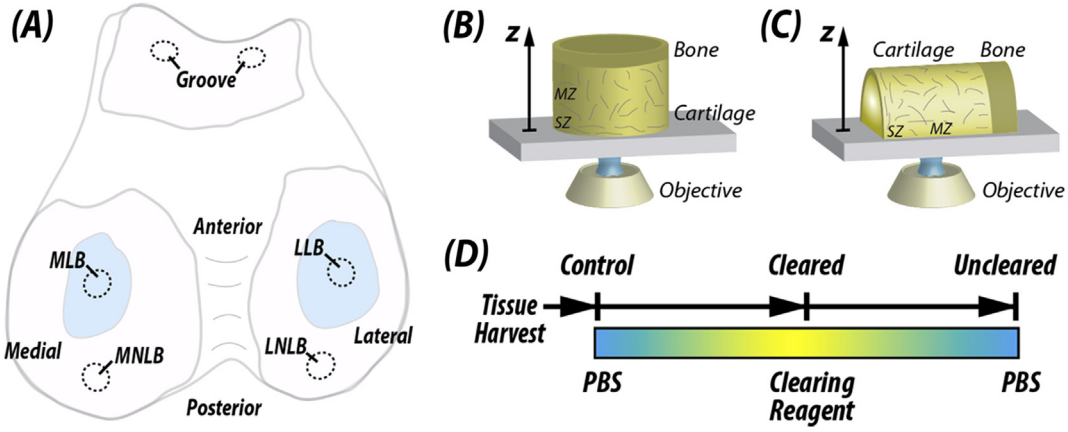

Fig. 1. Tissue harvest locations and experimental overview. Osteochondral samples were harvested from the femoral condyles of bovine knee (stifle) joints. (A) Samples were selected from the LB and NLB regions of the medial (MLB and MNLB, respectively) and lateral (LLB and LNLB, respectively) condyles. For mechanical testing, samples were selected from the trochlear groove. Imaging of samples was performed from either the articular surface through the depth of the samples (B; Fig. 3 and 4) or transverse to the depth direction (C; Figs. 2, 5 and 6) using inverted confocal microscopy (drawing not to scale). (D) Samples were subjected to equilibration in concentrated fructose-based solutions for optical clearing[15] or PBS in a three-stage reversible process.