Deep imaging of traditional histological staining in cartilage tissue using optical clearing and confocal microscopy

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INTRODUCTION: The understanding of native 3D tissue structure is limited by the penetration depth of light in confocal microscopy, due to absorption and scattering of photons during imaging. Fructose-based optical clearing replaces the water within tissues with a solution that matches the refractive index of the tissue permitting deep tissue imaging [1]. We recently demonstrated that this technique is possible with fluorescently-tagged antibodies and cell stains in multiple types of dense connective tissues including cartilage [2, 3], but the use of traditional histological stains has been largely unexplored. Combining the optical clearing with traditional histological stains to visualize deep tissue in confocal fluorescence microscopy can be very useful in the assessment of osteoarthritis (OA) severity [4] *in situ*, without compromising tissue integrity. The objectives of this study were to (1) assess the feasibility of fluorescence imaging with traditional histological stains, (2) demonstrate the ability to fluorescently visualize structural changes with increased osteoarthritis severity, as a parallel to light microscopy, and (3) image deep tissue by exploiting the fluorescence of those stains in tandem with fructose-based optical clearing.

METHODS: ***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. Samples were excised from medial femoral condyle regions and placed in 10% (v/v) buffered formaldehyde (Ricca, Arlington, TX). Additional human tissue sections from total knee replacement subjects were also obtained for study [5]. ***Staining:*** Fixed tissues were treated with ethanol and subsequently stained with either Fast Green FCF (Sigma) or Aqueous Eosin Y solution (with glacial acetic acid) (Sigma) solution overnight with gentle rocking at room temperature. Additional common stains (*e.g.* hematoxylin, Safranin-O) were also utilized, with similar results, but not presented here due to brevity. The tissue sections were thoroughly washed in tap water equivalent (TWE). ***Optical Clearing:*** Osteochondral tissues were cleared using a fructose-based optical clearing agent. 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 [1]. Tissues were placed in ~2 mL of each concentrated fructose solution overnight at room temperature, under a gentle rocking motion. As a control, phosphate buffer saline (PBS) was used.
Microscopy of Cartilage Tissue Explants: Light transmission through cartilage was assessed through the bulk (control/cleared, unstained/stained) using a stereo dissecting microscope (Leica M80). Tissues were imaged on a printed grid pattern. To image deep inside the tissue, confocal microscopy was used (Nikon Eclipse Ti). Imaging parameters were: field of view= 316×316 μm^2; matrix = 1024×1024 $pixels^2$; number of slices=1001; interslice spacing=0.5 µm. The same imaging and postprocessing settings were used for uncleared and cleared samples. One sample was intentionally damaged prior to Eosin staining by inducing an artificial fissure to test the feasibility of visualizing internal features. ***Microscopy of cartilage histological slides:*** To assess the capability of fluorescently visualizing standard histological stains, paraffin slides of human cartilage sections (from 2 different osteoarthritis grades, [5]), stained with Safranin O, counterstained with Fast Green, were imaged using light microscopy and fluorescence confocal microscopy.
RESULTS: Optical clearing enhanced light transmission through cartilage, even with the addition of histological stains (Figure 1A). Fluorescent labeling was preserved through sample preparations, enabling standard confocal imaging to depths approaching 400 µm, only limited by the working distance of the microscope objective. Detailed features inside tissue at the cell level were visible deep within cleared tissue. Fluorescence microscopy of histological slides showed difference in fluorescence intensity between samples with different OA severity (Figure 1B). In higher OA grade tissue, characterized by the lack of proteoglycan [4], the intensity was low at the same imaging settings, and surface abnormalities were visualized. Cracks in deep tissue were visible in Eosin stained cleared cartilage (Figure 1C).
DISCUSSION: *In situ* characterization of OA severity is possible utilizing optical clearing and fluorescence microscopy, without the need for traditional histological sectioning and preparations. Recent developments in optical clearing and microscopy have enabled the high-resolution imaging of large volumes of intact tissues to characterize cells and ECM microstructures. 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, especially in OA. Fluorescent imaging of traditional histological stains in intact 3D tissue can open a direction to histologists to assess tissue pathophysiology without compromising tissue integrity and over short periods of time. Further studies will include the combined applications of stains to visualize multiple structural features in deep tissue.
SIGNIFICANCE: We anticipate that 3D visualization of complex osteochondral tissues enhanced by optical clearing will better enable the diagnosis of pathologies such as OA. The capability of doing so with traditional histological stains can be used in research/laboratory setting to minimize the time consuming and elaborate histology processes without compromising with tissue integrity.
REFERENCES: [1] Ke et al., *Nat. Neuro*, 2013; [2] Neu et al. *Osteo Cart*, 2015; [3] Calve et al., *PLoS ONE*, 2015; [4] Pritzker et al., *Osteo Cart*, 2006. [5] Neu et al., *Arth Rheum*, 2010.
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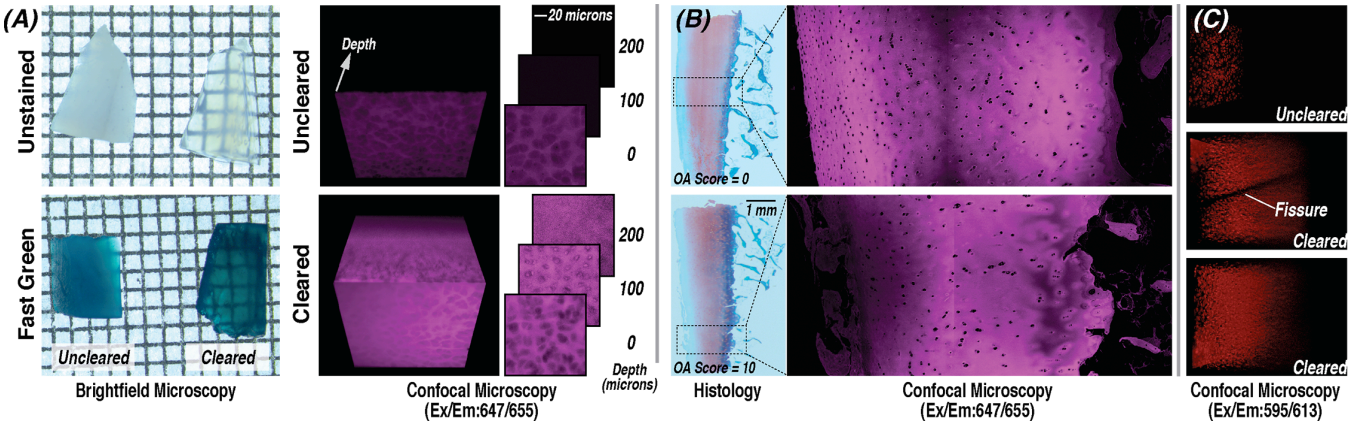

Figure 1: (A) Optical clearing achieved in bovine articular cartilage tissue sections visualized in unstained and Fast Green stained tissue. Detailed features are visible in cleared deep tissue using confocal microscopy. (B) Histological slides from cartilage stained with Safranin O / Fast Green visualized using confocal microscopy for different OA severity [4]. Fluorescence reveals structural features at the articular surface that are also visualized by standard histology, suggesting OA severity grading of intact biopsies is possible. (C) An internal fissure deep within the tissue could be resolved with when stained with Eosin and optically cleared.