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Identification of cartilage injury using quantitative multiphoton microscopy

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SUMMARY

Objective: Cartilage injury can lead to post-traumatic osteoarthritis (PTOA). Immediate post-trauma cellular and structural changes are not widely understood. Furthermore, current cellular-resolution cartilage imaging techniques require sectioning of cartilage and/or use of dyes not suitable for patient imaging. In this study, we used multiphoton microscopy (MPM) data with FDA-approved sodium fluorescein to identify and evaluate the pattern of chondrocyte death after traumatic injury.
Method: Mature equine distal metacarpal or metatarsal osteochondral blocks (OCBs) were injured by 30 MPa compressive loading delivered over 1 s. Injured and control sites were imaged unfixed and *in situ* 1 h post-injury with sodium fluorescein using rasterized z-scanning. MPM data was quantified in MATLAB, reconstructed in 3-D, and projected in 2-D to determine the damage pattern.
Results: MPM images (600 per sample) were reconstructed and analyzed for cell death. The overall distribution of cell death appeared to cluster into circular ($n = 7$) or elliptical ($n = 4$) patterns ($p = 0.006$). Dead cells were prevalent near cracks in the matrix, with only 26.3% (SE = 5.0%, $p < 0.0001$) of chondrocytes near cracks being viable.
Conclusion: This study demonstrates the first application of MPM for evaluating cellular-scale cartilage injury *in situ* in live tissue, with clinical potential for detecting early cartilage damage. With this technique, we were able to uniquely observe two death patterns resulting from the same compressive loading, which may be related to local variability in matrix structure. These results also demonstrate proof-of-concept MPM diagnostic use in detecting subtle and early cartilage damage not detectable in any other way.

Introduction

Osteoarthritis (OA) is a degenerative, multifactorial disease affecting the world population[1–3]. Knowledge of severe cartilage damage has led to therapies targeting symptom management, but understanding the early events in OA can facilitate the development of effective modalities in disease intervention. However, limited high resolution imaging of cartilage damage in the clinical setting has hindered early disease identification. Current medical imaging modalities including magnetic resonance imaging (MRI) and computed tomography (CT) do not have the resolution necessary to detect small cartilage injuries that can occur after joint trauma which lead to post-traumatic osteoarthritis (PTOA). Without the ability to detect these micron-sized structural or cellular changes, it is difficult to understand the progression of PTOA and facilitate its prevention. Developing methods for detecting early cartilage damage *in vivo* can expand the understanding of cartilage degeneration immediately after tissue damage.

Multiphoton microscopy (MPM) is capable of imaging live biological samples, including cartilage at submicron resolution, yielding structural details at depths greater than possible with confocal microscopy[4]. Importantly, MPM can be adapted for *in vivo* imaging with diagnostic application, including distinguishing normal vs abnormal or cancerous tissue[5–8], and it is being developed into a fast-scanning endoscope[9]. Diagnostic *in vivo* MPM can be adapted to cartilage because of the high collagen content of the cartilage extracellular matrix (ECM). Collagen emits second

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harmonic generation (SHG), making it readily detectable without staining[10]. Collecting SHG signal allows the general collagen matrix of the cartilage to be easily observed. Additionally, many molecules autofluoresce with MPM[4], and additional dyes may be used to further characterize tissue. Although fluorescent techniques have greatly expanded our understanding of cell biology, fluorescent labeling typically uses dyes that are unsuitable for clinical use. Sodium fluorescein is FDA-approved and is used ophthalmologically or intravenously to diagnose blood vessel disorders and corneal abrasions. Sodium fluorescein has been shown to stain cells[11] by binding non-specifically to proteins[12], making it an attractive method to label cells that have compromised membranes and may be dead. Imaging in a highly fluorescent solution like fluorescein can be difficult, but the optical capabilities of MPM make it possible. SHG and fluorescein can be used to collect high resolution information from live tissue to study the mechanisms involved in the progression of cartilage damage.

The importance of understanding the early sequence of events between cartilage trauma and OA is at the forefront of arthritis research[2,13–15]. Cell death has been shown to occur after traumatic injury[2,15], but the immediate effect of compressive trauma on chondrocyte viability[15] and the resulting dispersion of cell death and matrix damage around the injury site have not been studied in detail. The spatial distribution of chondrocyte death may be influenced by many factors. The superficial zone has been shown to be more susceptible than deeper cartilage layers to compressive injury[16]. This phenomenon may result from zonal ECM composition or collagen orientation, which distribute load when force is applied[17,18]. Evaluating chondrocyte death distribution in the superficial zone with MPM can further elucidate these underlying structural factors.

The goal of the current study was to use MPM to evaluate cellular damage after cartilage injury *ex vivo* in live tissue using an FDA-approved dye to detect chondrocyte death. Specifically, by using MPM with sodium fluorescein, we evaluated the pattern of chondrocyte death in the superficial zone immediately following traumatic injury like that which could contribute to the development of early PTOA.

Methods

Tissue collection and injury model

The distal third metacarpus ($n = 3$) or metatarsus ($n = 8$) was harvested from the left ($n = 5$) or right ($n = 6$) limb of young adult horses ($n = 10$, ages 4–6 years) immediately after being euthanized for reasons unrelated to this study under the guidelines and approval of the Institutional Animal Care and Use Committee. The limb was chosen at random. Cartilage was grossly evaluated and scored using the International Cartilage Repair Society (ICRS)

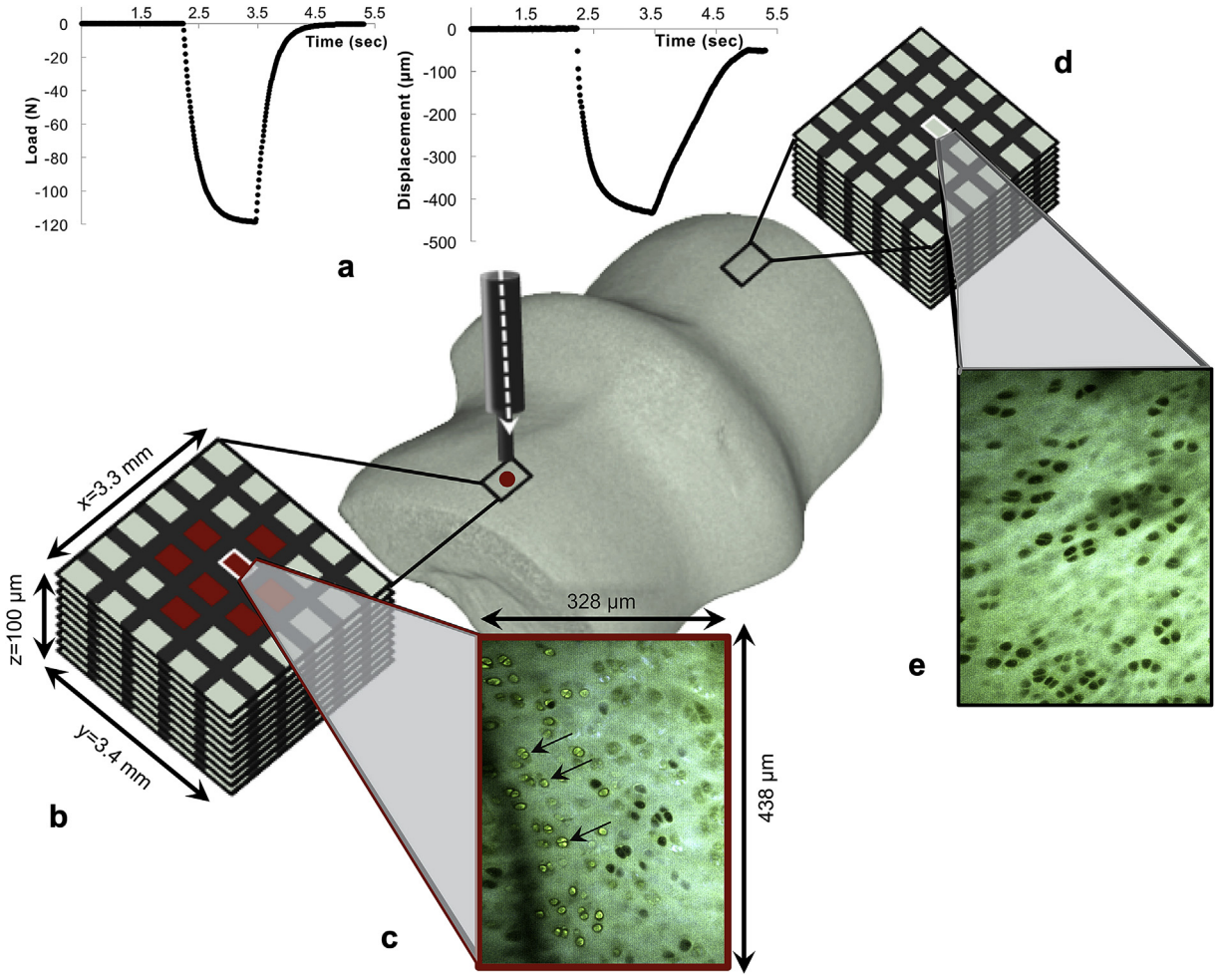

Fig. 1. Schematic of methods. (**a**) An OCB is injured with compressive loading. The injured site (**b**) and corresponding control site (**c**) are each scanned with MPM using a rasterized pattern, alternating scanned and non-scanned regions to encompass a 3.3 mm × 3.4 mm area. Individual 328 µm × 438 µm images that comprise the larger rasterized scanned area are shown in (**d**) and (**e**), with arrows denoting fluorescein-labeled dead cells.