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Three-dimensional micro-scale strain mapping in living biological soft tissues

Eng Kuan Moo [a,1], Scott C. Sibole [a,b,1], Sang Kuy Han [c], Walter Herzog [a,b,*]

[a] Human Performance Laboratory, Faculty of Kinesiology, University of Calgary, Calgary, Alberta, Canada
[b] Schulich School of Engineering, Biomedical Engineering Graduate Program, University of Calgary, Calgary, Alberta, Canada
[c] Korea Institute of Industrial Technology, Cheonan-si, South Korea

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A B S T R A C T

Non-invasive characterization of the mechanical micro-environment surrounding cells in biological tissues at multiple length scales is important for the understanding of the role of mechanics in regulating the biosynthesis and phenotype of cells. However, there is a lack of imaging methods that allow for characterization of the cell micro-environment in three-dimensional (3D) space. The aims of this study were (i) to develop a multi-photon laser microscopy protocol capable of imprinting 3D grid lines onto living tissue at a high spatial resolution, and (ii) to develop image processing software capable of analyzing the resulting microscopic images and performing high resolution 3D strain analyses. Using articular cartilage as the biological tissue of interest, we present a novel two-photon excitation imaging technique for measuring the internal 3D kinematics in intact cartilage at sub-micrometer resolution, spanning length scales from the tissue to the cell level. Using custom image processing software, we provide accurate and robust 3D micro-strain analysis that allows for detailed qualitative and quantitative assessment of the 3D tissue kinematics. This novel technique preserves tissue structural integrity post-scanning, therefore allowing for multiple strain measurements at different time points in the same specimen. The proposed technique is versatile and opens doors for experimental and theoretical investigations on the relationship between tissue deformation and cell biosynthesis. Studies of this nature may enhance our understanding of the mechanisms underlying cell mechano-transduction, and thus, adaptation and degeneration of soft connective tissues.

Statement of Significance

We presented a novel two-photon excitation imaging technique for measuring the internal 3D kinematics in intact cartilage at sub-micrometer resolution, spanning from tissue length scale to cellular length scale. Using a custom image processing software (*lsmgridtrack*), we provide accurate and robust micro-strain analysis that allowed for detailed qualitative and quantitative assessment of the 3D tissue kinematics. The approach presented here can also be applied to other biological tissues such as meniscus and annulus fibrosus, as well as tissue-engineered tissues for the characterization of their mechanical properties. This imaging technique opens doors for experimental and theoretical investigation on the relationship between tissue deformation and cell biosynthesis. Studies of this nature may enhance our understanding of the mechanisms underlying cell mechano-transduction, and thus, adaptation and degeneration of soft connective tissues.

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1. Introduction

Most cells in the human body, such as those in the heart, blood vessels, lungs, and connective tissues, regulate proliferation, differentiation, and matrix production in response to the mechanical micro-environment [1]. Understanding how mechanical signals control *in situ* cellular biosynthesis is particularly important for

* Corresponding author at: Human Performance Laboratory, University of Calgary, 2500 University Drive N.W., Calgary, Alberta T2N 1N4, Canada.

E-mail addresses: ekmoo@ucalgary.ca (E.K. Moo), ssibole@ucalgary.ca (S.C. Sibole), shan@kitech.re.kr (S.K. Han), wherzog@ucalgary.ca (W. Herzog).

[1] These authors contributed equally to this work.

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avascular connective tissues, such as articular cartilage and annulus fibrosus, because these tissues are maintained exclusively by the resident cells. Within connective tissue, cells are typically surrounded by matrix proteins, such as proteoglycans (PGs) and collagen fibres. Since the spatial distribution of PGs and collagen fibres often is not uniform throughout connective tissues, strain fields can be highly heterogeneous [2–6]. Furthermore, strains applied externally to connective tissues often are amplified or attenuated at the cellular level in a location-dependent manner [6–10]. Thus, quantification of strains from the tissue to the cellular level becomes important for an understanding of the molecular events associated with meccano-transduction in cells [2,3,6].

Non-invasive imaging techniques, such as confocal laser scanning microscopy (CLM) or magnetic resonance imaging (MRI) have been developed for analysis of *in situ* tissue strains. Using CLM, fluorescently-labelled cells or cell nuclei have been used as fiducial markers for tracking tissue deformations during loading [2,8,11,12]. However, the distribution of cells in tissues is unpredictable, thus, the region of interest may not contain the required cell triads or quadrangles for optimal local tissue strain measurements. Also, the resolution of tissue determinations is limited by the cell density, which for some articular cartilages can be as low as 2–5% [13,14]. The dependence on resident cells for tissue strain measurements can be eliminated by using a localized photo-bleaching approach to spatially define two-dimensional (2D) grid lines in fluorescently-labelled tissue [5,15,16]. Photo-bleached grid lines can then be tracked prior to and during tissue loading for instantaneous strain analyses. Although this technique improves the spatial resolution from a few hundred micrometers to 50 μm [16], conventional CLM excites all fluorescent agents along the light path [17], thus making it impossible to define grid lines in three-dimensional (3D) space. MRI can be used for 3D strain analysis, but its spatial resolution is poor (400 μm) and it requires long imaging time, as the grid lines have to be repeatedly imprinted because they only persist for less than a second [4].

A robust and reliable imaging technique for multi-scale strain measurement in tissue should be non-invasive, require little imaging time, allow for strain analysis in three-dimensional space at high resolution, and allow for analysis of any tissue region of interest. Multi-photon laser microscopy, in theory, can fulfil the aforementioned requirements. Two-photon excitation (TPE) laser microscopy, in particular, excites fluorescein molecules simultaneously with two photons of higher wavelength and lower energy compared to the one-photon excitation used in conventional confocal microscopy, making its excitation volume highly localized to the focal plane [18–20].This property of TPE allows for the definition of grid lines in three-dimensional space by a novel localized photo-bleaching technique. However, the imaging protocol using TPE microscopy, and the subsequent image processing algorithm, has yet to be developed.

The objectives of this study were (i) to develop a multi-photon laser microscopy protocol capable of imprinting 3D grid lines onto living tissue at a high spatial resolution, and (ii) to develop image processing software capable of analyzing the resulting microscopic images and performing high resolution 3D strain analyses. Using articular cartilage as the biological tissue of interest, we present a novel two-photon excitation imaging technique in the Methods for measuring the internal 3D kinematics in intact cartilage at sub-micrometer resolution, spanning length scales from the tissue to the cell level (10–20 μm on average [10,21]). In the Results and Discussion sections, we showed that by using the custom image processing software, we can provide accurate and robust 3D micro-strain analysis that allows for detailed qualitative and quantitative assessment of the 3D tissue kinematics. This novel technique preserves tissue structural integrity post-scanning, therefore allowing for multiple strain measurements at different time points in the same specimen. This new imaging technique opens doors for experimental and theoretical investigation on the relationship between tissue deformation and cell biosynthesis.

2. Methods

2.1. Specimen preparation

Pig knee joints (n = 3, 10–12 month-old) were obtained from the local abattoir. 6 mm-diameter osteochondral blocks were harvested from the medial load bearing surface of femoral grooves and used within 24 h of harvest. Tissue thickness was determined using a dissection microscope at three locations around the cylindrical block. The extracellular matrix (ECM) of the cartilage was stained using 16 μM 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF, Thermo Fisher Scientific Inc, MA, USA) in a phosphate buffered saline solution for 45 min [22]. This concentration of 5-DTAF is low and induces minimal tissue stiffening effects [22]. Specimens were then washed three-times in a dye-free saline solution prior to attachment to a specimen holder using dental cement. Specimens were immersed in saline throughout the experiment to prevent dehydration.

2.2. Mechanical loading and multi-photon imaging protocols

Osteochondral specimens were compressed in a custom-designed indentation system [21], which consists of a light-transmissible indenter, piezo-actuator, load cell, and displacement transducer (see also Supplementary Material). This light-transmissible indentation system was mounted onto the stage of a laser scanning microscope (FVMPE-RS model, Olympus, Tokyo, Japan). A minimal tare load of 0.1 N was applied to the cartilage in order to establish initial contact between indenter and cartilage surface. The cartilage tissue was considered to be at its reference state for this initial contact condition.

At the reference state, three-dimensional (3D) evenly-spaced (10 μm inter-spacing in x-, y-, and z-directions) grid lines were photo-bleached onto a cartilage volume of $190 \times 190 \times 110\ \mu m^3$ using a laser intensity of ~35 mW (Fig. 2A, and C). Grid line photo-bleaching, and subsequent 3D imaging of cartilage tissue, was performed using upright TPE microscopy (FVMPE-RS model, Olympus, Tokyo, Japan) equipped with a wavelength-tunable (680–1300 nm), ultrashort-pulsed (pulse width: <120 fs; repetition rate: 80 MHz) laser (InSight DeepSee-OL, Spectra-Physics, CA, USA) and a 25×/1.05 NA glass-corrected water immersion objective (XLPLN25XWMP2 model, Olympus, Tokyo, Japan). The excitation laser wavelength was set at 800 nm. Since 5-DTAF labels the collagenous proteins [22], which are immobile in the extracellular matrix, the photobleached grid lines remained visible throughout the experiment (>3 h). It should be noted that dyes that are inert, uncharged and hydrophilic (e.g., fluorescein conjugated dextran) are not suitable for the current imaging protocol as the interstitial fluids are mobile in the cartilage and lead to the photo-bleached grid lines fading away quickly [23].

After the grid lines were imprinted onto the cartilage in its reference state, nominal compressive tissue strains of 10%, 20% and 30% were applied incrementally to the cartilage tissue using a low-friction, impermeable, 2 mm-diameter cylindrical indenter. At each tissue strain, the final displacement was held for 20 min prior to tissue imaging to achieve near steady-state conditions. Since the tissue underwent shear deformation during loading, the microscope stage was translated horizontally by approximately 20 μm to keep the photo-bleached grid at the centre of the imaging area. The resulting TPE signal emitted by the cartilage matrix was collected in the backward (epi-) direction using a band-pass filter

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