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Novel image analysis methods for quantification of in situ 3-D tendon cell and matrix strain

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Abstract

Macroscopic tendon loads modulate the cellular microenvironment leading to biological outcomes such as degeneration or repair. Previous studies have shown that damage accumulation and the phases of tendon healing are marked by significant changes in the extracellular matrix, but it remains unknown how mechanical forces of the extracellular matrix are translated to mechanotransduction pathways that ultimately drive the biological response. Our overarching hypothesis is that the unique relationship between extracellular matrix strain and cell deformation will dictate biological outcomes, prompting the need for quantitative methods to characterize the local strain environment. While 2-D methods have successfully calculated matrix strain and cell deformation, 3-D methods are necessary to capture the increased complexity that can arise due to high levels of anisotropy and out-of-plane motion, particularly in the disorganized, highly cellular, injured state. In this study, we validated the use of digital volume correlation methods to quantify 3-D matrix strain using images of naïve tendon cells, the collagen fiber matrix, and injured tendon cells. Additionally, naïve tendon cell images were used to develop novel methods for 3-D cell deformation and 3-D cell-matrix strain, which is defined as a quantitative measure of the relationship between matrix strain and cell deformation. The results support that these methods can be used to detect strains with high accuracy and can be further extended to an in vivo setting for observing temporal changes in cell and matrix mechanics during degeneration and healing.

Keywords

Tendon; 3-D strain; Cell deformation; Extracellular matrix strain; Digital volume correlation

1. Introduction

Tendons are load-bearing tissues that are essential for joint motion and stability. Macroscopic loads modulate cellular responses prompting an array of biological outcomes

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Conflict of interest

The authors have no conflict of interest.

such as maintained homeostasis, degeneration, or repair. However, a major gap in our understanding of mechanotransduction pathways stems from the fact that the influence of macroscopic deformations on the microstructural response remains unknown. Our overarching hypothesis is that the unique relationship between extracellular matrix strain and cell deformation dictates biological outcomes. A first step towards addressing this hypothesis is the ability to characterize the local strain environment. Two-dimensional methods have been used to determine matrix strain by applying speckle patterns and photobleached lines to the surface (Andarawis-Puri et al., 2009; Cheng and Screen, 2007; Luyckx et al., 2014), or by tracking the planar movement of nuclei centroids (Arnoczky et al., 2002; Screen et al., 2004). Additionally, nuclear deformation methods continually identify the 2-D focal plane of maximum cross-sectional area to observe changes in aspect ratio (Arnoczky et al., 2002). Despite the value gained from established 2-D methods, 3-D methods are integral for capturing the complexity of the local strain environment that can arise from out-of-plane motion, particularly in the highly cellular, injured state. Therefore, the objectives of this study were (1) to assess the accuracy of digital volume correlation (DVC) to quantify 3-D matrix strain using images of the innate tendon microstructure, (2) to develop and validate a method for 3-D cell deformation and (3) to develop and validate a method for cell-matrix strain, a measure of the relationship between matrix strain and cell deformation.

2. Methods

2.1. Image acquisition and processing

Patellar tendons from C57BL/6 mice ($n = 3$) were stained with Hoechst 33258 (Thermo-Fisher) nucleic acid stain and nominally loaded to 100 g. Z-stack images ($20\times$) through a depth of 50 μm were acquired using multiphoton microscopy for stained nuclei imaging (Fig. 1A) and second harmonic generation (SHG) imaging of the collagen fiber matrix (Fig. 1B). For the purpose of cell deformation and cell-matrix strain method development, cell nuclei images were assumed to represent those of cell membranes. Image resolution was 2048×2048 pixels, spanning an area of $425.1 \mu\text{m} \times 425.1 \mu\text{m}$ (Fig. 2A).

2.2. Surgical procedure

To test the extensibility of the matrix strain method to the highly cellular, disorganized injured state, images of the healed midsubstance of surgically punched tendons (Lin et al., 2006) were also evaluated. Briefly, under IACUC approval, left patellar tendons were surgically exposed, and a 0.75 mm biopsy punch was used to create a full thickness injury. Mice resumed cage activity until sacrifice two weeks post-surgery. Image acquisition (Fig. 1C) was conducted as previously described at four locations within the injury site.

2.3. Method development and validation

A custom MATLAB (Mathworks, Natick, MA) program was developed to calculate 3-D matrix strain, 3-D cell deformation, and 3-D cell-matrix strain between unloaded and loaded configurations, simulated through digital stretching. Using bicubic interpolation in ImageJ (Schneider et al., 2012), images were scaled to local tissue strains ranging from 0.5 to +10% and -10 to -0.5% in the X- and Z-directions, respectively. The direction parallel to fiber

orientation was denoted as the X-direction. Error, the difference between the expected and average normal strain, was calculated for each level of applied strain. Because image resolution in the X- and Y-directions is equivalent, errors were expected to be the same. Accuracy of the matrix strain method was assessed for all three image types in the X- and Z-directions, while cell deformation and cell-matrix strain methods were evaluated using naïve tendon cell images in the X-direction to simulate strains along the collagen fiber direction. Spearman rank correlation tests were conducted between error and level of applied strain. Mann-Whitney *t*-tests were used to test for differences in error between the X- and Z-directions at low (0.5%), moderate (5%) and high (10%) levels of applied strain. Significance was set at $p < .05$.

2.4. 3-D matrix strain

Z-stacks were constructed into 3-D volumes (Fig. 2B), and DVC was implemented to calculate matrix strain (Bay, 2008). Briefly, a digital grid of nodes was placed within a region of interest on unloaded and loaded image pairs (Fig. 2C). Each node on the unloaded image served as the center of a sub-volume template, and at the matched node on the loaded image, a search volume was established (Fig. 2D). At each node, variance was calculated for the sub-volume template to indicate the extent of texture. If variance was low (threshold determined via visual inspection), the sub-volume template increased by 20 voxels in the X- and Y-directions. If insufficient variance persisted following two increases, the node was marked as poor. For valid nodes, the cross-correlation algorithm was executed between template and search volumes. The cross-correlation algorithm uses the convolution theorem and fast Fourier transforms between template and search volumes to calculate a normalized correlation coefficient matrix at each node, and the location of maximum correlation was noted as the initial displacement.

In a second iteration, volumes were interpolated to identify sub-voxel displacements. Final displacements in the X, Y, and Z-directions, and the maximum correlation coefficient were recorded. Poor nodes were removed, and a tetrahedral mesh was imposed on the 3-D volume using remaining nodes. Tetrahedra were treated as constant strain elements (Screen & Evans, 2009), and the Lagrangian strain tensor was calculated, resulting in a final strain map (Fig. 3). Average and standard deviations of ϵ_{xx} , ϵ_{yy} , and ϵ_{zz} were recorded.

2.5. 3-D cell deformation

3-D volumes were converted to binary through region-specific thresholding. A low-threshold mask was created to distinguish between voxels definitively part of a cell and background. A boundary threshold was then identified for each mask region. Briefly, a region intensity histogram was generated, and the transition zone between the low and high intensity peaks formed an exponential curve. The threshold was identified as the intensity where the derivative of the least-squares curve fit equals the slope of the line through the extrema of the transition zone (Fig. 4A). For each cell, principal axes were calculated from eigenvalues and eigenvectors of a 3-D covariance matrix. Cell deformation was quantified as the change in aspect ratio between matched cells in unloaded and loaded configurations (Fig. 4B).

2.6. Cell-matrix strain

Only cells fully contained within the strain map were included (Fig. 5A). A cell and its surrounding matrix strain elements were isolated (Fig. 5B). Each voxel corresponds to an element and matrix strain value, dividing the cell into regions (Fig. 5C). Cell-matrix strain is calculated using a weighted average of the cell volume within each matrix strain element.

3. Results

3.1. 3-D matrix strain

For naïve tendon cell images (Fig. 1A), level of applied strain positively correlated with error in the X-direction ($r = 1$, $p = .0004$) and standard deviation within each sample ($r = 1$, $p = .0004$) (Fig. 6). In the Z-direction ($r = 0.4286$, $p = .3536$), however, there was no correlation between level of applied strain and error (Fig. 7A). Results for injured tendon cell images (Fig. 1C) in the X- ($r = 0.9286$, $p = .0067$) and Z-directions ($r = 0$, $p > .999$) were similar (Fig. 7C). In contrast, for SHG matrix images (Fig. 1B), there was no correlation in the X-direction ($r = 0.3929$, $p = .3956$), but there was a positive correlation in the Z-direction ($r = 0.7857$, $p = .048$) (Fig. 7B).

At low levels of applied strain, error in the Z-direction was greater than the X-direction for naïve tendon cell images ($p = .100$) and the SHG matrix ($p = .100$), and significantly greater for injured tendon cell images ($p = .0286$) (Fig. 8A). At moderate levels, error in the Z-direction was greater than the X-direction for naïve tendon cell images ($p = .100$) and the SHG matrix ($p = .100$), while no difference was found for injured tendon cell images ($p = .200$) (Fig. 8B). And at high levels, there were no significant differences between error in the X- and Z-directions for naïve tendon cell images ($p = .200$) or the SHG matrix ($p = .700$), while error was significantly greater in the Z-direction for injured tendon cell images ($p = .0286$) (Fig. 8C).

Normalized correlation coefficients negatively correlated with level of applied strain in the X- (Fig. 9A) and Z-directions (Fig. 9B) for images of naïve tendon cells ($r = 1$, $p = .0004$; $r = -0.9643$, $p = .0028$), SHG matrix ($r = 0.8571$, $p = .0238$; $r = -0.9643$, $p = .0028$) and injured tendon cells ($r = 1$, $p = .0004$; $r = -0.9643$, $p = .0028$).

3.2. Cell deformation and cell-matrix strain

There was a negative, moderate correlation between level of applied strain and error for cell deformation ($r = -0.75$, $p = .0663$) (Fig. 10A). Conversely, there was a positive, moderate correlation between level of applied strain and error for cell-matrix strain ($r = 0.7143$, $p = .0881$) (Fig. 10B).

4. Discussion

We developed image analysis methods for calculating 3-D matrix strain, 3-D cell deformation, and 3-D cell-matrix strain in tendon. This 3-D approach is unique because it considers out-of-plane motion, which can occur from misalignment with the uniaxial loading direction or anisotropy. 2-D methods have been used extensively to quantify strain transfer from the macro to microscales (Han et al., 2013; Upton et al., 2008), but many studies

acknowledge that results are confounded by artifacts from excluding the effect of strain in the third dimension. Our matrix strain and cell deformation methods capture the 3-D microstructural response and how cells reorient or deform multi-dimensionally, providing a comprehensive picture of the strain environment. Cell-matrix strain connects these methods to quantify a cell's response to its surrounding matrix strain, and this relationship is a vital step towards unraveling the underlying mechanotransduction pathways. With errors across all three methods no more than 0.25%, results support that these analyses can detect a large range of strains with high accuracy. However, more consistent results at lower levels of applied strain, shown by increased correlation coefficients and reduced sample standard deviations, suggest that if large strains are expected, consecutive analyses at smaller increments should be conducted. Additionally, larger errors in the Z-direction are likely caused by lower resolution and greater use of interpolation. Results can be improved by acquiring Z-stacks at smaller intervals.

DVC is a promising non-contact method for calculating strain but requires a high-contrast, random pattern. Because the observed cell pattern is characteristic of an individual tendon and cannot be controlled as with an applied speckle, the use of varying template sizes and normalized cross-correlation are necessary for establishing confidence in calculated displacements. The unique use of a variance-dependent template ensures that cells are included in analyzed regions and excludes those lacking a sufficient pattern. Additionally, the normalized cross-correlation algorithm was chosen because it is optimal for intensity-independent template matching, and the maximum coefficient represents how closely templates match (Bay, 2008).

Results support that the matrix strain method can be used across all three types of microstructural images: naïve tendon cells, collagen fiber matrix, and injured tendon cells. Using naïve tendon cell images to calculate matrix strain is advantageous for compatibility with cell deformation and cell-matrix strain methods. Additionally, collagen matrix images are more uniformly patterned compared to stained cells, warranting further investigation to determine if these images are more susceptible to noise. In contrast, the disorganized cell pattern in the injured state is optimal for DVC methods. High levels of anisotropy and cellularity leading to unidentifiable cell boundaries also make cell deformation and 2-D methods difficult to use. Therefore, this matrix strain method would be aptly suited for the injured state.

Though nuclear staining was implemented, these methods can be extended to cell membrane staining applications. Specifically, the use of transgenic mice, in which cells are fluorescent, can provide in vivo temporal strain assessments during tendon healing. Additionally, previous studies have shown that sub-rupture fatigue damage is characterized by extracellular matrix changes (Andarawis-Puri et al., 2014; Bell et al., 2015), and these methods could be used to study resulting differences in cell and matrix mechanics.

While method validation results are promising, further investigation is necessary to determine how inherent noise during in vivo loading and use of interpolation affect the accuracy of matrix and cell strain measurements. Also, the matrix strain method considers translation in three directions, and inclusion of rotational degrees of freedom may be

required in a highly anisotropic state. Nevertheless, this analysis is the first to validate the use of DVC for tendon microstructural images and introduces novel methods for quantifying the relationship between three-dimensional cell and matrix strain.

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Fig. 1.
(A) Fluorescently stained naïve tendon cells acquired using multiphoton microscopy at 740 nm. (B) SHG image of naïve collagen fiber matrix acquired at 840 nm. (C) Fluorescently stained injured tendon cells acquired using multiphoton microscopy at 740 nm.

Fig. 2.

(A) Z-stack images acquired at 1 μm intervals through a depth of 50 μm . Morphological top-hat filtering was applied to reduce background noise and highlight stained nuclei. (B) Z-stack images constructed into a 3-D volume. (C) A digital grid of nodes within a user-specified region of interest. (D) An initial template and search region on the unloaded and loaded images, respectively.

Fig. 3.

Elements associated with poor nodes were removed from the final strain map. These regions were typically associated with those lacking cells in images of naïve and injured tendon cells.

Fig. 4. (A) Extraction of the transition zone from the intensity histogram (top right) and threshold (*) determination using the first derivative of an exponential curve-fit. (B) Threshold application to identify 3-D binary cell regions and calculation of change in aspect ratio between matched cells.

Fig. 5.
(A) Isolation of cells completely enclosed within the final strain map. (B) Extraction of a cell and its surrounding matrix strain elements. (C) Cell-matrix strain calculated as a weighted average of the encompassing volume regions.

Fig. 6.
Level of applied strain in the X-direction positively correlated with error ($r = 1$, $p = .0004$) and standard deviation within each sample ($r = 1$, $p = .0004$) for naïve tendon cell images.

Fig. 7.

Spearman rank correlation tests between error and level of applied strain in the X- and Z-directions for (A) naïve tendon cell images, (B) SHG images of the collagen fiber matrix, and (C) injured tendon cell images. Positive correlations were found for images of naïve tendon cells ($r = 1$, $p = .0004$) and injured tendon cells ($r = 0.9286$, $p = .0067$) in the X-direction and for SHG images of the collagen fiber matrix ($r = 0.7857$, $p = .048$) in the Z-direction.

Fig. 8.

Strain differences between the X-direction (left) and Z-direction (right) for each image type at (A) low, (B) moderate and (C) high levels of applied strain. (*: $p < .05$; #: $0.05 < p < .10$).

Fig. 9. Correlation coefficients negatively correlated with level of applied strain across all three image types in the (A) X-direction and (B) Z-direction.

Fig. 10. Moderate correlations between level of applied strain and error were observed for (A) cell deformation ($r = -0.75$, $p = .0663$) and (B) cell-matrix strain ($r = 0.7143$, $p = .0881$).