A NEW TECHNIQUE TO INVESTIGATE *IN SITU* COLLAGEN MICROMECHANICS IN THE ANNULUS FIBROSUS UNDER TENSION

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INTRODUCTION

Similar to other tensile bearing connective tissues, the nonlinear mechanical properties of the annulus fibrosus can be attributed to complexities in the collagen matrix: uncrimping, sequential recruitment and the relative sliding of collagen fibers. However, direct evidence of these mechanisms has been limited to date. Although collagen sliding has been demonstrated during ligament wound healing (1), it has never been directly observed during normal tissue loading in any connective tissue. Collagen sliding is expected to play an integral role in the local mechanical environment of the annulus cells (2). The objective of this study was to develop a non-destructive method to investigate *in situ* collagen fiber sliding within the annulus fibrosus under physiologic levels of tensile strain.

MATERIALS AND METHODS

Specimen Preparation

A thin (<1 mm) strip of outer annulus fibrosus, approximately 5 mm in width, was dissected from three bovine upper caudal discs along the collagen fibre direction (Figure 1a). The physiologic attachment of the collagen network to the vertebral bodies was preserved by including small sections of bone on either side of the specimen. The specimens were then incubated in dicholorotriazynl fluorescein (5-DTAF, Molecular Probes) for 15 min at room temperature. The DTAF was prepared using 0.2 M sodium bicarbonate buffer (pH 9.0) at a concentration of 2mg/ml, and has been used previously to fluorescently label collagen fibres *in vivo* (1). A 2-mm^2 grid of black markers (rubber shavings approximately 0.1mm in diameter) was then fixed to the central portion of the interior surface of the annulus with cyanoacrylate (Figure 1b).

Load Apparatus

The specimen was placed in a custom-built load apparatus (3) that was mounted on the stage of an inverted laser scanning confocal microscope (LSM 510, Carl Zeiss Germany). The exterior surface of the specimen lay against a coverslip positioned over the microscope objective (Figure 2a). Uniaxial strain in the direction of the collagen fibers was applied to the specimen through wire staples threaded through each of the bone ends (Figure 1b). The grips were displaced by precision stepping motors (TMG, Clovis, CA) along linear guides (Thompson, Port Washington, NY). Forces were recorded by a load cell (Transducer Techniques Inc., Temecula, CA) mounted to one of the grips. A dissection microscope (Zeiss Stemi 2000-C, Germany) and CCD camera (Coho 4910, San Diego, CA) mounted above the specimen, in combination with custom written particle tracking software (LabVIEW & IMAQ Vision, National Instruments, Austin, TX) captured displacements of the markers on the interior surface of the specimen (Figure 2b). Using a Lagrangian strain formulation, the marker displacements were translated into real-time, localised measurements of surface strain (accuracy ± 0.11%), which were then used to drive the motors in a positive feedback loop to generate controlled strains in the central portion of the soft tissue. During the entire test the specimen was kept in a bath of 0.2M sodium bicarbonate.

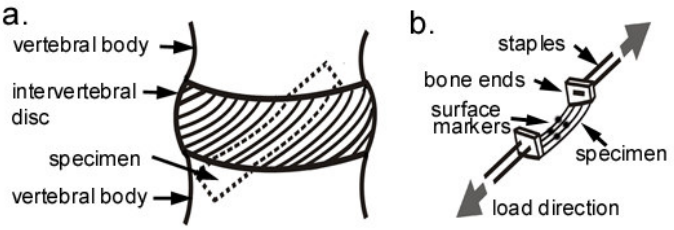

Figure 1. a. Orientation of the specimen preparation b. Prepared specimen and direction of applied load

Strain Protocol

A preload of 0.1 N was placed on the specimen. Using the 25x objective, the specimen was scanned within the region defined by the surface marker grid for a section where the oriented collagen fibers appeared straight and continuous. Using the 488 line of the Kr/Ar laser at full power (100%) a single line scan was performed continuously for 1 min in a direction perpendicular to the collagen fibers. The resulting photobleached line was approximately 1 μm in

width (length: 300 μm). Three lines, 60 μm apart, were bleached in each specimen (Figure 2c). Preliminary studies demonstrated that after 1 hr in the equilibrium position the line remained stable.

An image of the three lines was captured at the zero strain position with the x25 lens at a laser intensity of 10% (1024 x 1024; LP 505 filter with the pinhole set at 1.0 airy unit). Each specimen was then strained successively to 2% and 4% (0.150±0.003%/s) and similar images were captured at each level. The specimen was then returned to 0%, allowed to equilibrate for 30 min, and a final image was captured. During loading, the lines were tracked with the laser intensity set at 1% and the pinhole fully opened to reduce any further photobleaching.

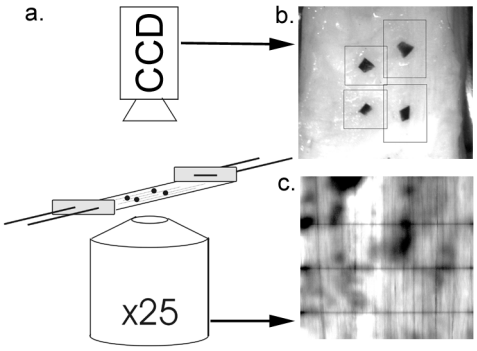

Figure 2. a. Test apparatus layout: confocal objective, specimen under uniaxial strain, and a CCD camera. b. Computer display of the surface marker image captured by the CCD camera. c. Confocal image of the labelled collagen with the photobleached lines.

RESULTS

The DTAF label, along with the 25x objective, provided a clear indication of fibrillar bundling and orientation. The laser was able to burn clean lines across the collagen with the typical hour glass bleach pattern through the depth (4). Focusing on the bleached line therefore allowed even small changes in depth (< 5 μm) of the marked collagen fibers to be detected. At the lower laser intensity scanning did not produce additional bleaching and the lines could be continuously tracked and high quality images repeatedly obtained at several intervals. The dark, blurry regions seen on the images are shadows produced by surface debris.

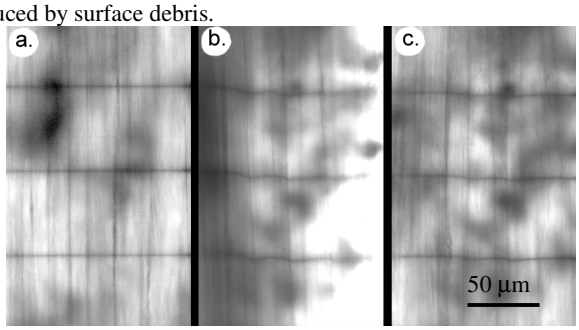

Figure 4. Confocal images of photobleached lines a. unstrained (0%) b. strained (4%) c. relaxed (0%)

The specimens were strained to 2.1 ± 0.3% and 4.2 ± 0.2% along the collagen fibre direction. At both strain states undulations appeared on the lines of all specimens (Figure 4b). Generally, the lines remained continuous with sharp dimples occurring at the edges of the fibrillar bundles. The line was then observed to slope gradually across the bundle (Figure 5a). Occasionally sharp dimples within a bundle (* in Figure 5b), or step discontinuities (Figure 5c) were observed. The pattern across all three lines was consistently matched. Relative differences in the depth of the strained collagen fibres were observed by changes in the focal position of the bleached line as well as by variations in the fluorescence intensity. After 30 min at the relaxed 0% strain position the line did not return to its original position, although the dimples were noticeably straightened (Figure 4c).

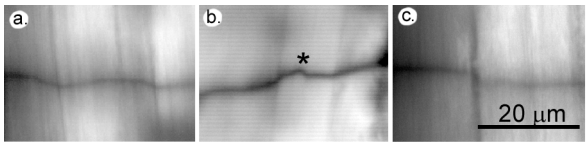

Figure 5. High magnification confocal images at 4% strain a. a sloping line b. a mid-fiber dimple c. a dislocated line

DISCUSSION

This technique enables direct in situ investigation of real-time collagen micromechanics within the annulus fibrosus. As well, it presents the first evidence of collagen sliding *in situ* at the microscopic level. If tissue deformation had been achieved by a uniform expansion of the matrix, the inscribed line would remain straight and simply increase in width with an elongation between the lines. The undulating line can only be created by the relative sliding of the collagen. This protocol can also be easily adapted to study collagen micromechanics within ligament and tendon.

While additional specimens will be required before an explicit understanding of the micromechanics of the annulus can be reported, these initial results confirm the behaviour suggested by a recent investigation of intercellular strain in our lab, namely: fibre sliding and relative changes in fibre depth (2). In addition, the continuity of the line suggests significant adhesion at the collagen bundle interface; while, the sloped line indicates shearing within the bundle.

The images obtained to date are all at the tissue surface due to the short working distance of the 25x lens. Although this objective provided a larger scan region for initial observations and testing of the technique, repeating the study with a 63x 1.2 NA lens (Carl Zeiss, Germany) will provide finer measurements of the collagen network mechanics at varying depths (0-80 μm) *in situ*. Future studies will also include an investigation of the micromechanics of the collagen matrix in relation to the cells, by use of a nuclear counterstain.

This technique will allow the investigation of collagen micromechanics at several hierarchal levels of the collagen structure, namely: bundle, fibre and fibril. It will also contribute to our understanding of the mechanical environment of the cells embedded within the collagen matrix.

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