Fluorescent Labeling with 5-DTAF Reduces Collagen Fiber Uncrimping in Loaded Tendons

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Abstract

A fluorescent dye commonly used to image tissues under load (5-DTAF) has previously been shown to stiffen tendons. This study hypothesized that 5-DTAF staining stiffens tendons through reduced fiber sliding, altering the rate at which crimped collagen fibers straighten under load. This was tested by using reflected cross-polarized light microscopy to measure fiber crimp period of cervine extensor digitorum longus tendon specimens under axial load. Specimens were treated with either phosphate buffered saline (negative control), genipin (positive control), or 5-DTAF. In saline treated specimens, crimp period (relative to unstretched) increased at approximately 2.5 times the applied axial strain, indicating substantial fiber sliding. In both 5-DTAF and genipin treated specimens, this ratio was reduced to 1:1, indicating no fiber sliding. These results add further evidence that care should be taken when using 5-DTAF to stain tissue for studying microscale deformations in tissues.

Introduction

5-(4,6-Dichlorotriazinyl) Aminofluorescein (5-DTAF) is a fluorescent dye frequently used for imaging microscale deformations in musculoskeletal tissues including tendon[1], cartilage[2], meniscus[3], and intervertebral disc[4–6]. However, treatment with 5-DTAF has been shown to increase stiffness of both tendons[7] and hydrogels[8, 9]. While the physical mechanism by which this occurs is unknown, it is believed to be the result of reduced sliding between adjacent collagen fibers due to either aggregation of dye molecules or nonspecific binding of charged fluorescein [10] to adjacent fibers.

Extensibility of tendons and ligaments is accomplished through a combination of the straightening of crimped fibers and sliding between fibers which do not run the full length of the tissue. The relative contributions of these two mechanisms contribute to observable changes in fiber crimp period with length change, as schematically described in Figure 1. Given two parallel fibers with initial length, L_0, and initial crimp period, P_0, application of axial stretch, ΔL, will result in increased crimp

period, ΔP. If interfibrilar sliding is restricted, the relative change in crimp period should equal the relative change in length. However, if sliding between discontinuous fibers occurs[11], the relative change in crimp period will be greater than the relative change in length. This phenomenon has been observed in a number of different tissues[12–14] with ratios of crimp strain to grip strain ranging from 2.7 to 10. While it is presumed that treatment with 5-DTAF will reduce this effect, it has yet to be tested directly. Furthermore, comparison of uncrimping behavior following 5-DTAF staining to that following treatment with genipin (which introduces crosslinks between adjacent fibrils[15]) may provide additional confirmation of this mechanism.

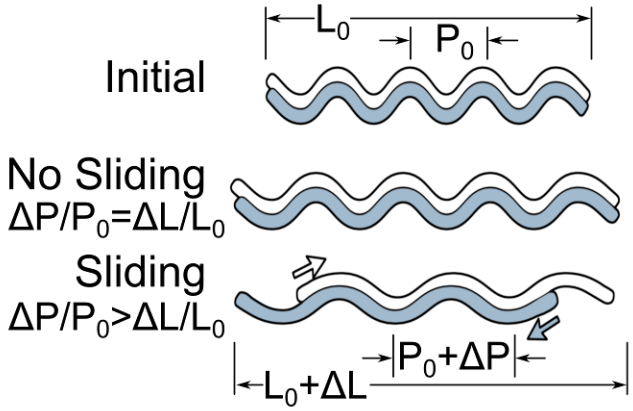

Figure 1: Given parallel fibers with initial length, L_0, and crimp period, P_0, sliding will dictate the increase in crimp, ΔP, relative to an applied increase in ΔL.

The primary difficulty in directly assessing the impact of 5-DTAF on microscale deformations is the fact that deformations are typically measured using optical techniques which either require, or may be affected by, 5-DTAF labeling. Confocal methods for measuring microscale deformations require fluorescence and necessarily preclude comparison to an unlabeled control. Second harmonic generation does not require a fluorescent label, but typically emits between 400 and 500nm[16], which may interfere with the peak absorption of 498nm and peak emission of 517nm of 5-DTAF. A recently proposed reflected cross polarized light technique[14] has demonstrated the ability to measure crimp strain on the surface of thick specimens of various tissues under tensile stretch. This technique uses light with a peak intensity of 410nm, which, while intended to minimize sub-surface scattering in the specimen, is expected to also limit excitation of 5-DTAF labeling.

This study hypothesized that 5-DTAF labeling results in tendon stiffening by reducing fiber uncrimping. This hypothesis was tested by imaging fiber uncrimping under strain in 5-DTAF labeled tendon specimens along with negative and positive (genipin) controls.

Methods

Four extensor digatorum longus tendons were taken from white tail deer legs (three female, one male), with an average age of approximately three years. The knees were obtained from a local processer and frozen at -20°C until testing. Upon the knees being thawed to room temperature, explants were cut from each tendon in an area between five and fifteen millimeters from the femoral enthesis. The explants were then trimmed transversely to an average length of 8.7±1.9mm, with care taken to cut the faces at right angles to the tendon axis. The samples were then cut longitudinally into three pieces of approximately equal size. Samples were placed in 1.5mL vials containing one of the following: 1.54M phosphate-buffered saline (PBS), PBS with 10mg/mL genipin (Wako, Richmond, VA), and PBS with 0.2mg/mL 5-DTAF (Sigma-Aldrich, St. Louis, MO). The samples were left to soak for a minimum of

seventy-two hours at 4°C. Before observation, excess 5-DTAF and genipin was removed by rinsing with PBS for at least two hours.

Immediately after being rinsed, specimens were mounted, using cyanoacrylate glue, to a loading device as previously described. The device was then placed on a glass slide wetted with PBS on a microscope (IMT2, Olympus, Waltham, MA) equipped with reflected cross-polarized lighting at a peak wavelength of 410nm and a digital camera (E-M5 III, OM Digital Solutions, Tokyo). In order to avoid interference from the grips of the device, images were taken as close to the center of the sample as possible. Grip strain was applied manually at ten intervals from 0 to 3% of the sample length using a micrometer to adjust grip separation and the panel meter attached to a potentiometer to record it. At each interval, an image was acquired. After ensuring that the adhesive was still intact, the specimen was returned to its original length, and this procedure was repeated a second time with images acquired at a different location on the specimen.

Each image was analyzed using a custom MATLAB script as previously described [14] to measure crimp period. The code ran by taking a user-specified line that followed the orientation of the fibers and applying a Fast Fourier Transform in order to determine crimp frequency, the reciprocal of which yielded crimp period. This was repeated at approximately the same location in each frame of the test, and all crimp periods were normalized by the first frame to yield crimp strain. This was repeated for one to three lines of interest in each test. Additionally, the signal to noise ratio was calculated for two user-selected lines of interest on images of each specimen with 0% applied strain.

For each test, a linear regression was used to calculate the average ratio of crimp strain to grip strain. Strain ratios were then compared between treatment groups and between each treatment group and 1:1 using a Wilcoxon rank sum test. The Wilcoxon rank sum test was also used to compare signal to noise ratios of different treatment groups.

Results

The reflected light crossed-polarization technique yielded measurable crimp banding in all specimens, with a non-significant reduction in mean signal to noise ratio for both 5-DTAF (2.8±3.3dB) and genipin (2.6±3.3) treated specimens relative to PBS (6.5±4.4dB). This confirms minimal interference from either 5-DTAF labeling (with peak absorption of 500nm) or genipin crosslinking (with peak absorption of 600nm) when imaging with polarized light at a peak of 410nm. Crimp period increased linearly with applied grip strain in all tests (Figure 2).

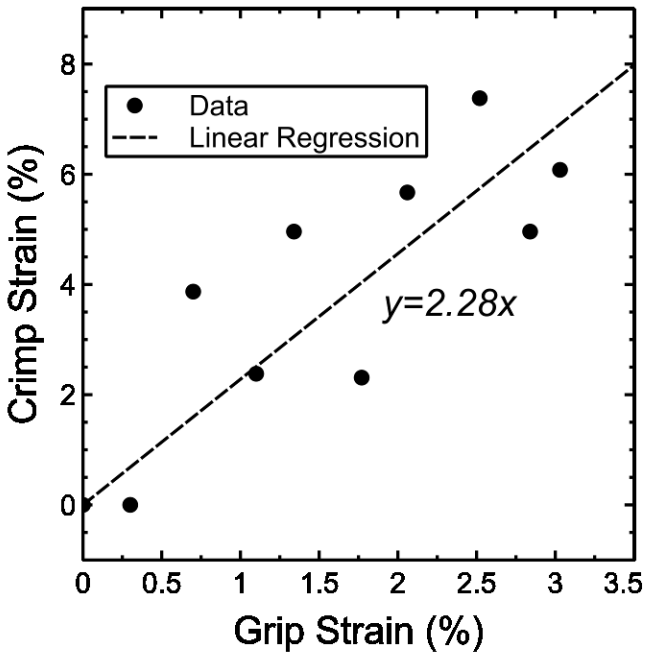

Figure 2: Representative observed crimp strain relative to applied grip strain with linear regression.

While all tests of 5-DTAF and Genipin treated specimens were successful, a total of three tests in the PBS group were omitted from analysis due to evidence of glue breakage. PBS treated specimens had a median crimp strain to grip strain ratio of 2.48, which is comparable to previous measurements of medial collateral ligaments[14], and indicative of substantial fiber sliding. Conversely, genipin treated specimens had a median ratio of 1.23, which was not significantly greater than 1:1, and indicates that crosslinking nearly eliminated fiber sliding. 5-DTAF treated specimens had a median crimp strain to grip strain ratio of 1.11, which was significantly lower than PBS and higher than 1:1, but not significantly higher than genipin (Figure 3).

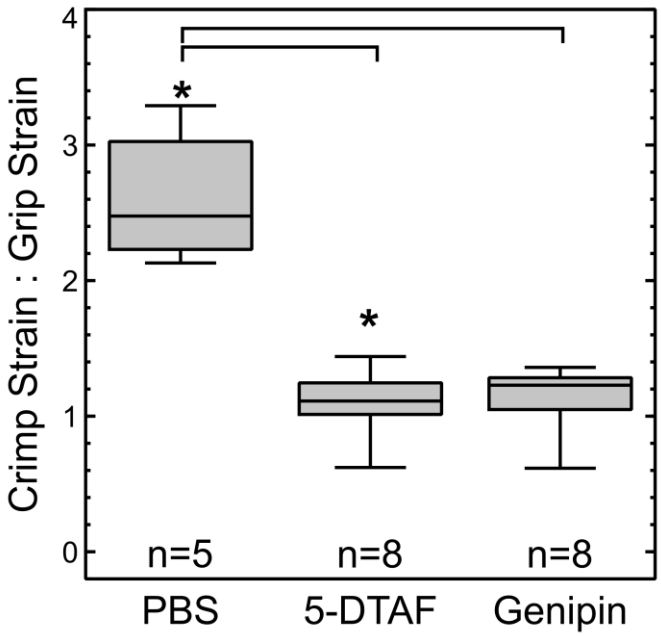

*Figure 3: Ratios of observed crimp strain to applied grip strain. Bars indicate significant (p<0.05) difference between groups. * indicates significant (p<0.05) difference from 1.*

Discussion

The presently reported ratio of crimp strain to grip strain for negative control (saline treated) cervine extensor digtorum longus tendons (median of 2.47) is comparable to that of previous measurements of cervine medial collateral ligament specimens of similar aspect ratio (median of 3.58)[14]. As hypothesized, treatment with a known collagen crosslinker (as a positive control) reduced this ratio nearly to one, confirming that crosslinking restricts interfibrilar sliding. As further hypothesized, labeling with 5-DTAF had a similar effect. This direct observation supports previously reported modeling work suggesting a decrease in fiber sliding following 5-DTAF labeling.

It should be noted that the present results contradict the findings of prior experiments, in which interfibrillar sliding was directly observed in 5-DTAF labeled intervertebral disc tissue [6]. Internal swelling of the disc in this previous study resulted in fibrils that were nearly fully straightened even in the unloaded state. The present study, however, was limited in its ability to test high strain behavior by both the strength of the cyanoacrylate attachment technique and by the extinction of collagen crimps at higher strain. This discrepancy suggests that 5-DTAF forms weak bonds between collagen fibrils that may be broken by interfibrillar shear stresses. This may be the result of either aggregation of 5-DTAF molecules[7] or an electrostatic interaction between negatively charged fluorescein [10] and positively charged argenine residues on adjacent collagen molecules. Further study into the mechanism underlying this phenomenon, is warranted.

The present study was performed using a saturating 5-DTAF staining protocol, with a moderately high dye concentration and a much longer incubation time than would typically be used to stain specimens for imaging. This approach was aimed at providing homogenous binding throughout the thickness of the tissue of both 5-DTAF and genipin. Surface staining protocols used in most imaging studies will result in a gradient with depth, and effect of that gradient on surface observations, along with the effect of sub-saturating staining, remain important areas of further study. The findings of this

work, however, add further to the growing body of evidence that care should be taken when using fluorescent dyes (particularly 5-DTAF) to study microscale deformations of musculoskeletal tissues.

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Author Contributions

Gianna T Voce: Investigation, Writing **Arthur J Michalek:** Conceptualization, Supervision, Funding Acquisition, Writing

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