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# Quantification of collagen organization using fractal dimensions and Fourier transforms

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ABSTRACT

Collagen fibers and fibrils that comprise tendons and ligaments are disrupted or damaged during injury. Fibrillogenesis during healing produces a matrix that is initially quite disorganized, but remodels over time to resemble, but not replicate, the original roughly parallel microstructure. Quantification of these changes is traditionally a laborious and subjective task. In this work we applied two automated techniques, fast Fourier transformation (FFT) and fractal dimension analysis (FA) to quantify the organization of collagen fibers or fibrils. Using multi-photon images of collagen fibers obtained from rat ligament we showed that for healing ligaments, FA differentiates more clearly between the different time-points during healing. Using scanning electron microscopy images of overstretched porcine flexor tendon, we showed that combining FFT and FA measures distinguishes the damaged and undamaged groups more clearly than either method separately.



## Introduction

Tendons and ligaments are composed of a hierarchical system of collagen fibrils, fibers, and bundles of fibers, which are approximately parallel in their organization. Injured tendons and ligaments or those subjected to sub-failure damage by overstretching exhibit a loss of organization in the ruptured fibers and fibrils. During healing, fibrillogenesis produces a matrix that is quite disorganized in the early granulation tissue, but remodels over time to resemble (but not replicate) the original microstructural organization. The collagen in excised specimens can be visually examined using variety of imaging techniques including scanning electron microscopy (SEM) and multi-photon imaging to identify these signs of damage. With the aid of a microscope, an observer can easily recognize these signs of damage, but it is difficult to quantify results for scientific inquiry. Ranking systems are subjective and often not repeatable between researchers. Therefore, it would be advantageous to have an objective and repeatable method to quantify disruption and damage in fibers and fibrils. Subjectivity can be removed from image characterization by removing the element of human judgment. This requires the implementation of an image analysis program capable of recognizing and quantifying patterns. One method often employed to remove subjectivity when analyzing sample organization is the two-dimensional (2D) fast Fourier transform algorithm (FFT). A single dimension FFT algorithm breaks an electronic signal into its component frequencies and then counts how often those frequencies occur. A 2D FFT performs a similar operation on a two-dimensional signal, or image computing spatial frequencies within the image in radial directions. Using this technique, the 2D FFT can quantify the distribution of fiber orientation in an image of collagen fibers. The shape of the spatial distribution of the data, i.e. the plot of spatial frequencies in orthogonal, planar

*Abbreviations:* 2D, two-dimensional; AR, aspect ratio (of 2D FFT); FA, fractal dimension analysis; FFT, fast Fourier transform; MCL, medial collateral ligament; SEM, scanning electron microscopy.

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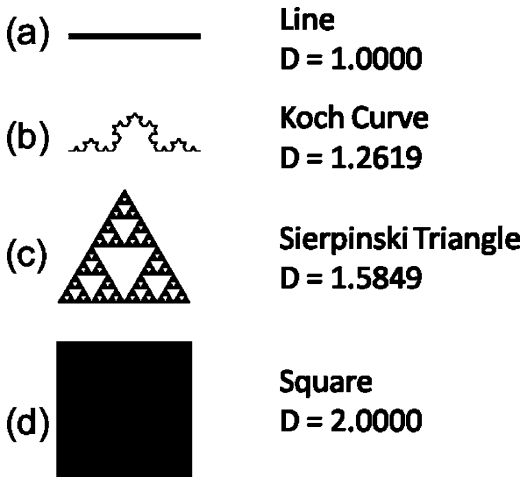


**Fig. 1.** Examples of fractals with dimensions. (a) A line is one-dimensional [Fractal Dimension = 1.0000]. (b) The Koch Curve is somewhere between one and two dimensions [Fractal Dimension = 1.2619]. (c) The Sierpinski Triangle is closer to two dimensions that the Koch Curve [Fractal Dimension = 1.5849]. (d) A solid rectangle is two-dimensional [Fractal Dimension = 2.0000].

directions, often projects into an elliptical shape. This allows for quantification by an aspect ratio (AR) of the major and minor axes of the ellipse. An image with a preferred direction of fiber orientation has a higher AR. Two-dimensional Fourier analysis has been used to quantify organization of collagen fibrils in the dermis (de Vries et al., 2000), the cochlea (Tsuprun and Santi, 1999), and ligament (Bashford et al., 2008; Cicchi et al., 2010; Vidal Bde and Mello, 2010). The technique has been extensively validated in ligament, with the fiber directionality corresponding directly with regions of damage (Sereysky et al., 2010).

A second technique for objectively quantifying organization in an image is fractal analysis (FA). First used by Benoit Mandelbrot in 1975, the word "fractal" refers to a figure with a fractional dimension. It is necessary to point out that a true fractal has smaller structures that ramify endlessly. Physical objects can only be approximated by fractals because the atomic size limits the size of the smallest structure. Images are limited by their resolution. A simple example of this repeating self-similarity is the Koch Curve (also known as the Koch Snowflake), in which four lines of equal length are arranged so that they are the length of only three segments and then four of the line groups are treated the same way (Fig. 1b). There are many well-known fractals occurring in multiple dimensions, including the Sierpinski Triangle (Fig. 1c) and the more complex Julia and Mandelbrot Sets. Fractals are also commonly seen in nature: fjords, cloud shapes, trees (Mandelbrot, 1982), and artery branching (Gazit et al., 1997). Each of these fractal patterns has a degree of self-similarity that can be measured using a fractal number. It is necessary to point out that a true fractal has smaller structures that ramify endlessly. Physical objects can only be approximated by fractals because the atomic size limits the size of the smallest structure. A fractal dimension describes the amount of space and self-similarity of the structure. For example, a line exists in a single dimension, therefore it has a fractal dimension of 1 (Fig. 1a), while a square exists in two dimensions and has a fractal dimension of 2 (Fig. 1b). A simple fractal like the Koch Curve has a larger "dimension" than a line but not as much as a square; its fractal dimension is 1.26, between 1 and 2 and the Sierpinski Triangle has a greater fractal dimension of 1.58. Fractal analysis has been applied in several different biological and medical applications (Lopes and Betrouni, 2009). For collagen and related structures it has been used to quantify the periodontal bone-ligament interface (Wagle et al., 2005; Madan et al., 2007; Bosshardt et al., 2008), muscle attachment sites (Zumwalt, 2005), and ACL bone-ligament interface (Buckland-Wright et al., 2000).

In this study we perform both Fourier (FFT) and fractal (FA) analysis on images of tendon and ligament collagen at various levels of organization. One group of specimens was evaluated by multiphoton images of rat medial collateral ligament (MCL) healing. The other group of specimens is comprised of SEM images of porcine digital flexor tendon following overstretch injury. We hypothesize that both the aspect ratio and the fractal number will be altered as a result of injury and these differences can be used to differentiate between normal and damaged or healing tissues.

## Materials and methods

### Fourier analysis

The grayscale images were subjected to 2D fast Fourier transformation analysis using Matlab (Mathworks, Natick, MA, USA). Each line of the image was broken down into its spatial frequency components. Then histograms of both the horizontally occurring frequencies and the vertically occurring frequencies were examined. For each direction, the spatial frequencies occurring between the 9th and 10th percentiles were selected and plotted in 2D frequency space (horizontal frequency values vs. vertical frequency values). An ellipse was fit to the resulting data distribution. The ratio of the long axis to the short axis was calculated for each image and compared between groups.

### Fractal analysis

Prior to fractal analysis, the images were converted from grayscale to binary images using a threshold value that was automatically determined using Matlab's graythresh command (Mathworks, Natick, MA, USA). To improve contrast and avoid scaling issues, the full-size image was divided into 32 equally sized regions and the automatic threshold value for each region was determined independently. The image sections were then reaggregated to form a binary representation of the original grayscale image. Fractal analysis of each binary image was performed using a Matlab routine that calculated the fractal dimension using the Minkowski–Bouligand dimension, also known as the "box counting" dimension (Moisy, 2006). The box counting dimension is determined by covering the image in successively smaller grids of boxes and at each level counting how many boxes are required to cover the image. The number of boxes is then plotted as a function of the box size and the slope of the line is computed. If the slope of the line remains constant as the boxes get smaller that slope value is the fractal dimension. We confirmed that the slope of the line was approximately constant for each image and then averaged the slope value for the three smallest box sizes to calculate the fractal dimension for each image.

### Image preparation

#### Multiphoton

Four groups of three skeletally mature male Wistar rats (purchase weight 275–300 g, age two months) underwent surgical transection of the MCL according to a procedure approved by the University of Wisconsin-Madison Animal Care and Use Committee. Animals were then allowed to heal for 7, 14, 21, or 28 days, according to group. A fifth group was used as an intact control. After collection, the ligament was longitudinally cryosectioned into 5 μm thick sections, mounted on microscopy slides and stained with Hematoxylin and Eosin (H&E). The healing region of the tendon was then imaged using multiphoton microscopy to record the organization of collagen fibers. Multiphoton microscopy is a non-linear

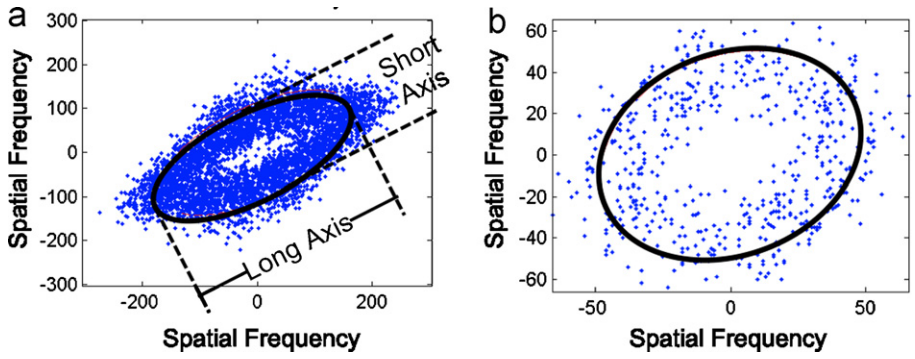


**Fig. 2.** Spatial frequency (arbitrary units of inverse length) distribution for intact vs. damaged tendon SEM images. Note the long and short axes of the ellipse used for the quantification ratio.

imaging technique that excites endogenous fluorescent proteins such as collagen. In this application, multiphoton images demonstrate collagen organization more clearly than light microscopy. The multiphoton system was designed around a Nikon Eclipse TE300 microscope and used a 5 W mode-locked Ti:sapphire laser (Spectra-Physics-Millennium/Tsunami, Mountain View, CA, USA) at a wavelength of 890 nm to generate multi-photon excitation (Wokosin et al., 2003) with a 445 nm band pass filter to allow only second harmonic generation (SHG) through. Images were collected using a Nikon 40× SuperFluor lens (numerical aperture = 1.3). Three images from the transected region were used for analysis.

### Scanning electron microscopy

Five porcine digital flexor tendons obtained from an abattoir were excised and preconditioned (to 2% strain) using a sinusoidal strain wave in a mechanical testing system (MTS Bionix, Minneapolis, MN, USA). Control specimens ($n = 3$) were removed following preconditioning, whereas damaged specimens ($n = 2$) were stretched to 13% strain at 10 mm/s prior to removal.

Following removal from the testing system, all specimens were stretched flat under mild tension and fixed in a 2% glutaraldehyde solution. Specimens were then dehydrated in ethanol, freeze-fractured in liquid nitrogen, dried in a critical point drying system, and sputter-coated with gold prior to SEM imaging. Images were taken at 2000× magnification using a Hitachi S570 LaB6 scanning electron microscope with 10 keV excitation (Hitachi High Technologies, Schaumburg, IL, USA). Fifteen representative images from each group (damaged and normal) were chosen.

### Statistics

All groups were compared using unpaired, two tailed Student's *T*-tests. Significance was set at $p = 0.05$.

## Results

### Multiphoton microscopy

Multiphoton images of healthy ligaments (the control group of rats) showed well-organized collagen with fibers running in the same axial direction (Fig. 3c). After ligaments had been transected and allowed to heal for one week, images of the healing region of the ligament showed disorganized collagen fibrils, with no preferred direction of alignment (Fig. 3d). As healing progressed the collagen structure began to preferentially align along the direction of loading, until at four weeks post injury it looked similar to the tissue before injury (not pictured). *p*-Values are shown in Table 1.

The change in fractal number mimicked the differences described both qualitatively and quantitatively using FFT analysis for the multiphoton images. For all intermediate healing time points the FFT aspect ratio was significantly lower than the intact ligament and at day 28 the ligament had recovered to levels similar to the intact ligament (Fig. 4a and Table 1). However, the intermediate healing time points did not show significant differences relative to one another (Table 1).

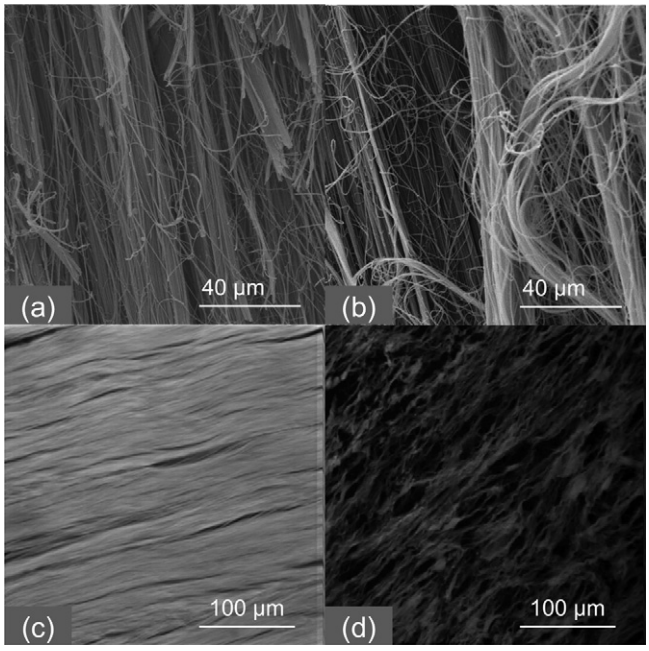


**Fig. 3.** SEM images of (a) undamaged and (b) damaged tendon at 2000× magnification and multi-photon images of (c) healthy and (d) 7 days post injury tendon at 40× magnification.

The fractal analysis technique showed significant differences between time points for the healing multiphoton images (Fig. 4b). The fractal number for intact ligament was 1.807 (standard deviation ±0.011). At 7 days post-injury the fractal number dropped significantly to 1.728 ($p < 0.001$). As the healing progressed the fractal number increased significantly relative to each of the proceeding

**Table 1**
Statistical significance for FFT aspect ratio analysis of multiphoton images (*$p < 0.001$).

| Day | Intact | 7 | 14 | 21 | 28 |
|---|---|---|---|---|---|
| Intact | × | 0.000* | 0.000* | 0.000* | 0.057 |
| 7 | | × | 0.584 | 0.273 | 0.000* |
| 14 | | | × | 0.574 | 0.000* |
| 21 | | | | × | 0.000* |
| 28 | | | | | × |

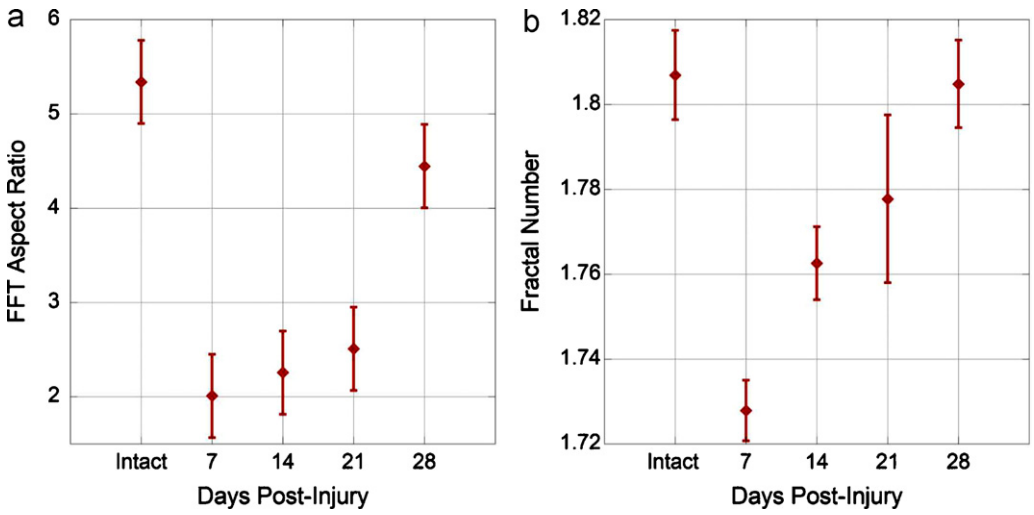


**Fig. 4.** (a) The average FFT aspect ratio decreases after injury, then recovers four weeks after injury. Statistical significance is shown in Table 1. (b) The average fractal number for multi-photon images decreases after injury, and then increases over healing time. Error bars in both figures represent standard deviation. Statistical significance is shown in Table 2.

**Table 2**
Statistical significance for fractal analysis of multiphoton images ($^{*}p < 0.001$, $\hat{}\ p < 0.01$).

| Day | Intact | 7 | 14 | 21 | 28 |
|---|---|---|---|---|---|
| Intact | × | 0.000* | 0.000* | 0.002^ | 0.698 |
| 7 | | × | 0.000* | 0.000* | 0.000* |
| 14 | | | × | 0.066 | 0.000* |
| 21 | | | | × | 0.004^ |
| 28 | | | | | × |

time points with the exception of 21 days relative to 14 days. The fractal number over healing time also remained significantly lower than that of the intact tendon until four weeks post injury (day 28) when it had returned to 1.805. *p*-Values are shown in Table 2.

Fractal analysis differentiated more clearly among the different time points, showing significant differences relative to preceding time points (Table 2) while the FFT aspect ratio method showed a trend (Fig. 4b) but no significance for the days relative to one another (Table 1).

## Scanning electron microscopy

SEM images of control specimens showed organized, intact fibrils without breaks (Fig. 3a). The fibrils were mainly oriented along the long axis of the tendon. Images of overstretched specimens showed broken fibrils, disrupted groups of fibers, and general disorganization (Fig. 3b). While many fibrils were still oriented along the long axis of the tendon, the ends of broken fibrils followed no pattern, giving the damaged tendon an overall disorganized appearance. For normal tendon tissue the ellipse generated by the two-dimensional FFT analysis was elongated (Fig. 2a), giving an aspect ratio greater than one (average 2.36, standard error 0.21). The ellipse generated by the analysis for the damaged tendon tended to be more circular (Fig. 2b), leading to an aspect ratio closer to one (average 1.57, standard error 0.06). The aspect ratio was significantly higher in images from normal specimens than in comparable images from damaged specimens ($p < 0.001$).

The fractal dimension algorithm returned significantly higher ($p < 0.001$) values for images of the damaged specimens, with an average value of 1.76 (standard error 0.012), than for the images of normal specimens, with an average value of 1.71 (standard error 0.007).

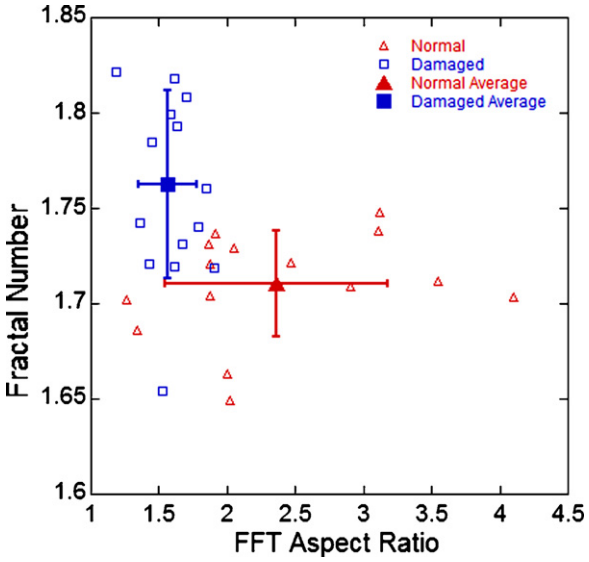


**Fig. 5.** Fractal number as a function of FFT aspect ratio for intact vs. damaged SEM images of pig flexor tendon. Note the distinction between groups that appears when the two are plotted together.

When the fractal number is plotted as a function of the aspect ratio for each image (Fig. 5), the mean values for normal vs. damaged tendons are very different.

## Discussion

We hypothesized that both the aspect ratio and the fractal number would be altered after injury and indeed, our results show that both Fourier analysis and fractal analysis are capable of differentiating between healthy and healing or damaged collagenous tissue.

With the multiphoton images we found that during healing both the FFT aspect ratio and the fractal number initially decreased, then recovered over the healing time course. The reorganization of collagen fibers over the time course of healing is well established, however, it is usually characterized using a tedious and subjective visual ranking process (Chamberlain et al., 2009). Recently it has been shown that quantification of collagen organization can be

performed using Fourier analysis (Sereysky et al., 2010). Our results show that the fractal analysis technique has a greater sensitivity to the progression of healing using the same set of healing tendon images. This suggests that fractal analysis individually or in combination may be a stronger tool for analyzing the organization of collagen fibers.

SEM images of normal and damaged tendon show an increase in fractal number and a decrease in aspect ratio after damage. Using FFT analysis and fractal analysis in combination works well for quantifying overstretch damage in porcine flexor tendon. Our results suggest that FFT analysis by itself would be problematic to distinguish between the normal and damaged tissue, however, when the two techniques are combined they clearly distinguish the two groups.

We found that SEM images of normal tendon have a lower fractal number than damaged tendon, since damaged tendon is less regular with bent and broken fibrils. This is the opposite of the effect observed in the multi-photon images, where the fractal number decreases with fiber disorganization. This discrepancy occurs because of differences in what is being imaged. The SEM images of the overstretched tendon are looking at broken fiber ends and irregularities in a field of tendon fibrils. Multiphoton images are examining only collagen fiber organization, thus early on, while the collagen is developing (days 7–21), there is more space between the newly forming collagen fibrils (Fig. 3c and d), leading to a lower fractal number in the healing images than in healthy images, where the collagen fills the image in a regular pattern. If fractal analysis were to be applied to the H&E images of the healing ligament the fractal number would increase because it would measure general disorganization, instead of collagen only.

In conclusion, we found that both Fourier analysis and fractal analysis have potential to significantly decrease the burden of manually and subjectively quantifying collagen organization in damaged, diseased and healing tissues. These techniques also have potential value for other imaging modalities, including medical imaging with ultrasound or MRI. As such, these methods may be useful objective tools for tissue characterization and diagnostics, but efficacy must be interpreted individually by modality and application.

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