

Research Article

The Three Dimensional Microstructural Network of Elastin, Collagen and Cells in Achilles Tendons[†]

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Ms Xin Pang: conducted the experiment, collected and analysed the experimental data and drafted the manuscript. Jian-Ping Wu: study conception, designed and supervised the study, critical data integration and revisions of the manuscript. Garry T Allison, Jiake Xu, Jonas Rubenson, Ming H Zheng, Bruce Gardener, David G Lloyd, Allan Wang contributed to critical discussions and revisions of the manuscript. Thomas Brett Kirk: supervised the study, critical discussions and revisions of the manuscript. All authors have read and approved the final submitted manuscript.

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Abstract

Similar to most biological tissues, the biomechanical and functional characteristics of the Achilles tendon are closely related to its composition and microstructure. It is commonly reported that type I collagen is the predominant component of tendons and is mainly responsible for the tissue's function. Although elastin has been found in varying proportions in other connective tissues, previous studies report that tendons contain very small quantities of elastin. However, the morphology of and the microstructural relationship among the elastic fibres, collagen and cells in tendon tissue have not been well examined. We hypothesize the elastic fibres, as another fibrillar component in the extracellular matrix, have a unique role in mechanical functions and microstructural arrangement in Achilles tendons. Using confocal and Second Harmonic Generation (SHG) imaging techniques, this study examined the 3-dimensional microstructure of the collagen, elastin and cells in the mid-portion of hydrated rabbit Achilles tendons. It has been shown that elastic fibres present a close connection with the tenocytes. The close relationship of the three components has been revealed as a distinct, integrated and complex microstructural network. Notably, a "spiral" structure within fibril bundles in Achilles tendons was observed in some samples in specialized regions. This study substantiates the hierarchical system of the spatial microstructure of tendon, including the mapping of collagen, elastin and tenocytes, with 3-dimensional confocal images. This article is protected by copyright. All rights reserved

Keywords

Achilles Tendon; Confocal and SHG microscopy; Elastin; Collagen; 3-dimensional

Introduction

In humans, the Achilles tendon is the thickest and strongest tendon that sustains some of the largest tensile loads in the body ¹. Dysfunction and injuries are commonly seen in the Achilles tendon.

Various studies ¹⁻⁶ have used a range of imaging techniques to reveal the tendon's architecture, and elucidate biomechanical and functional characteristics in healthy and pathological states. It is generally believed that the fibrous matrix of tendons mainly consists of collagen and a small amount of elastin, which are produced and maintained by tenoblasts and tenocytes ⁷. Tendon consists primarily of collagen (70-80% of the tissue's dry weight) and less than 5% tenocytes and tenoblasts ⁸. These insoluble elements are embedded within a hydrated environment containing ground substance of proteoglycans, glycosaminoglycan (GAG) and some other small molecules ⁹.

The detailed hierarchical structural organization of the tendon has been well defined by Kannus ⁷, which includes fascicles (15-3000 μm in diameter) that are composed of bundles of collagen fibres (5-300 μm in diameter) surrounded by the endotenon. The collagen fibres are constituted of collagen fibrils with diameters ranging between 20 and 150 nm. At rest, the tendon fascicles, collagen fibres and fibrils are characterised with crimping as observed under microscopy. This unique composition and structure of tendon enables it to transmit the force between muscle fibres and their bony attachment, modulate different joint movements, and buffer forces of various directions to prevent injury.

Many microscopic techniques have been used to study the microstructure of tendons. Traditional optical microscopy, commonly used in histology, does not have sufficient imaging resolution to distinguish the detailed fibril structure of tendons but reveals general fibre texture and the morphology of tenocytes. Electron microscopy possesses superior imaging resolution and has been used intensively to study the ultra-structure of tendon ². While stereoscopic techniques can reveal some 3-dimensional features using electron microscopy, the depth of field is often limited.

Meanwhile, Scanning Electron Microscopy imaging techniques require excessive tissue dehydration and are limited to surface imaging, while Transmission Electron Microscopy (TEM) imaging

techniques require ultra-sectioning of tendon tissue and also have limitations for imaging the 3D microstructure of bulk tendon tissue ¹⁰.

Collagen is the predominant component in tendon and is therefore the most commonly investigated passive structural sub-element considered responsible for tensile resistance and stiffness ¹¹.

Collagen type I is the main type existing in tendons, and small amounts of collagen types II, III, IV, V and VI are also present ¹². Collagen is a triple-helical structure ¹³ and when observed using optical methods, it displays a birefringent characteristic ¹⁴, which means that the resultant refractive index and image are dependent on the polarization and the direction of propagation of light.

Therefore, alternative optical techniques are needed to observe collagen, or multiple staining techniques are required. Second harmonic generation (SHG) microscopy has emerged as a powerful platform offering high resolution for visualizing birefringent materials without staining. Hence, SHG techniques are well suited for the investigation of tendons that contain abundant collagen ¹⁴, and the use of these techniques opens up opportunities for inspection of other additional components such as elastin and tenocytes.

Elastin plays an important role in tissues and organs like large arteries, skin, lung and cartilage ¹⁵.

As an essential component in extracellular matrix, elastin ensures tissues with elastic stretch and recoil, cooperates with collagen for tensile resistance ¹⁶, and regulates the interactions between cells and extracellular matrix ¹⁵. However, elastin is sparsely distributed in tendons, accounting for approximately 1-2% of the dry mass of the tendon ⁷. Given its functional importance, the investigations of morphology of elastin can increase the knowledge of mechanism of its functional contribution to tissues. The substructures of elastin can be well displayed by TEM images ¹⁷, but it is difficult to image the elastic fibres, which work as the functional unit of elastin structure, by traditional optical microscopy and electron microscopy ¹⁶. As a consequence, the distribution of elastin, and more importantly the concurrent location of elastin in relation to the collagen and tenocytes in tendons, is yet to be studied.

The confocal laser scanning microscope combined with fluorescent techniques is well suited for 3D biomedical imaging with an appropriate resolution for the examination of cells and fibres¹⁸, and this system has been utilised by numerous studies^{14; 18-20} to reveal 3D microstructural detail.

Modern confocal microscopy is normally equipped with lasers of different wavelengths and can be integrated with SHG microscopy. Such systems provide an excellent platform for examining tendon structure. Clément Ricard et al.²¹ discovered that Sulforhodamine B (SRB) specifically stains elastic fibres. Combined with confocal microscopy, this convenient and inexpensive fluorophore has been demonstrated to stain elastic fibres in lung and articular cartilage by some researchers²²⁻²⁴.

Collagen has been well acknowledged to play an important role in the physiological and mechanical functions in Achilles tendons. Although elastin accounts for a very small fraction of the extracellular matrix of Achilles tendons, its existence and the orientation is potentially extremely valuable. We hypothesize that there is a close connection between tenocytes and the fibrillary extracellular matrix in the mid-portion of Achilles tendons, and that elastic fibres and collagen fibrils play important roles in the function of Achilles tendons. Given the unique mechanical properties of elastic fibres in chondral and connective tissues²³, it is suggested that elastic fibres may have a crucial role in the mechanical function of Achilles tendons.

Methods

Sample Preparation

Ten Achilles tendons from left hind limbs were freshly harvested from ten New Zealand white rabbits. Animal ethics approval was granted by the University of Western Australia. The rabbits were 18 to 20 weeks old, and weighed from 2.4 to 2.8 kg. The tendons were visually glistening and normal in appearance.

The specimens cut from the mid-portion of the Achilles tendons were embedded in O.C.T compound (VWR International Ltd, UK) for cryostat section. Longitudinal slices and transverse slices were sectioned with thicknesses of 50 μm and 20 μm respectively. The sections were adhered to slides, labelled, wrapped in cling film, and stored at -80°C until fluorescent staining was conducted. Cryo-sections were carefully prepared to avoid any possible tissue tearing and compression during and prior to imaging. Torn sections were excluded from imaging. Regions of interest were randomly selected within the tissue section for imaging.

Fluorescent staining

The sections were stained with the nucleic acid-selective fluorescent dye Acridine Orange (AO) for imaging the nucleus of tenocytes, and the fluorescent dye SRB was used to label the elastin fibres. Prior to staining, sections were thawed at room temperature and washed gently in phosphate-buffered saline (PBS, Ph 7.2) to remove the O.C.T thoroughly. The slides were stained in 0.03 g/L AO solution for 3 min, and then washed thoroughly in PBS before it was stained in 1 mg/ml SRB solution for 1 min. After thoroughly washing with PBS, the slides were covered by coverslips and sealed by clear nail polish, and imaged immediately.

Confocal laser scanning microscopy and SHG imaging

A Leica TCS SP2 multiphoton microscope was used to acquire the images of collagen, elastin and tenocytes in Achilles tendons through three independent channels. The system is integrated confocal microscopy with SHG microscopy, which possesses an Acousto-Optical Beam Splitter and multiple laser excitation sources. A 514 nm Krypton-Argon ion laser was used for acquiring the images of cells stained by Acridine Orange, while a 561 nm diode-pumped solid-state laser was used to acquire the images of elastin stained by SRB. A Spectra Physics Mai Tai Titanium Sapphire laser, which is tuneable from 710 to 990 nm, was set at 890 nm and used to acquire the SHG signals from the collagen. The elastin and cells fluorescent signals were collected by photomultiplier tubes at 565-600 nm and 590-680 nm, respectively. The SHG signals were directly collected by a

secondary non-descanned detector at 445 nm for transmitted lights. Oil-immersion objectives used in this study were: (a) 10×, NA (Numerical Aperture) 0.40; (b) 20×, NA 0.70; (c) 40×, NA 1.25; (d) 63×, NA 1.40. Image acquisition was conducted by Leica Confocal Software with 1024×1024 pixels in each image. Z-stacks were obtained with a step of 0.5 μm between each field of view.

Image processing and 3D image reconstruction

The image processing is summarized in Fig. 1. Collagen, elastin and cells were assigned green, cyan and red, respectively. The only channel that displayed significant homogeneous background noise was that of the elastin (cyan channel). In order to get clear images of elastic fibres, the background was subtracted using Image J (NIH, Maryland, USA) within the elastin images. After background subtraction, the stacks from the three channels were merged. In this study, elastin-cell image stacks were primarily formed along with collagen-elastin-cell image stacks. A voxel-based 3D rendering function in computer software Voxx (Indiana University, USA) and Imaris 7.4.2 (Bitplane, USA) were used to render the merged image stacks into 3D images for three-dimensionally studying the microstructural relationship of tenocytes, elastic fibres and collagen.

2D Fast Fourier Transform and Alignment Analysis

A 2D fast Fourier transform (2D FFT) transfers the spatial information contained in a digital image into a mathematically defined frequency domain for objectively studying the anisotropic or isotropic features in the image²⁵. By measuring the grey value of the pixels in a 2D FFT image derived from the original digital image of fibrillary objects, Oval profile plug-in was used to numerically evaluate the alignment characteristics of the objects in the image²⁶. 2D FFT and Oval profile plug-in within Image J were employed to analyse the alignment characteristics of elastic fibres, collagen fibrils and tenocytes in Achilles tendons. Image stacks of collagen fibrils, elastic fibres and tenocytes were used to reconstruct the corresponding 2D z - projects for conducting 2D FFT. For easily assessing and comparing the orientation characteristics, the grey values in the FFT alignment graphs were normalized into digits ranging from 0 to 1 using feature scaling method.

Results

Collagen, Elastin and Cells

Low magnification observations using 10× and 20× objective lenses show the general structure of Achilles tendon. The collagen bundles exhibit the typical crimped pattern (double headed arrow indicated in Fig. 2e) along the tendon's long axis. An inherent technological limitation in the system for SHG imaging prevents observing the collagen in a full field of view at low magnifications (Fig. 2a, e), while the elastin and cell images (Fig. 2b, c, f, g) display in full. The sparse elastin signals are submerged in the background noise and the elastic fibres cannot be easily distinguished (Fig. 2b, f).

Images with higher magnifications, using 40× and 63× objective lenses, show more detailed microstructure of the three components. The size of the collagen observed by SHG microscopy are at fibril level (Fig. 2i, n), which can be verified by the higher magnification observations in the transverse images (Fig. 7). The elastic fibres signals in higher magnifications are strong enough to be distinguished from the background noise (Fig. 2j, p), and Fig. 2j shows the comparison of image quality before and after background subtraction, which successfully enhanced the visibility of elastic fibres. Elastic fibres (white arrow indicated in Fig. 2j, p) are discontinuous in the 2D images. In the merged images (Fig. 2m, r), elastic fibres and tenocytes within a layer align with the collagen fibril bundles and conforms to the collagen orientation.

The longitudinal spatial relationship of collagen fibrils, elastic fibres and tenocytes

Collagen fibrils, elastic fibres and tenocytes have a distinctive spatial relationship in Achilles tendons, as shown in Fig. 3. The 3D networks of elastin-tenocytes and collagen-elastin-tenocytes are observed from different angles of view. The alignment of the three components shows high concordance and is consistent with the long axis of the tendon.

Tenocytes generally display as spindle or elongated shape and deform into various shapes as the bundles crimp. Some tenocytes (rectangle indicated in Fig. 3a) with normal and twisted shapes were examined at higher magnifications to show the fine structure of the elastin-tenocyte network (as shown in Fig. 4). The elastic fibres are continuous in the 3D network and appear to have a very close relationship with the tenocytes. They are attached to the two ends of the elongated tenocytes, and appear to connect the tenocytes into wavy lines along the tendon's long axis (Fig. 3a, c, e). Elastic fibres and tenocytes are embedded in the collagen matrix and mostly conform to the collagen fibril orientation (Fig. 3b, d, f). However, the collagen fibrils are crimped more sharply than the elastic fibres. An area with complex structure which is indicated by a rectangle in Fig. 3b shows a twisted geometry of collagen fibril bundles, where a similar twisted geometry can be observed within the elastic fibres and tenocytes. This area is shown in further detail using 2D images from a stack at different depths in Fig. 5.

Connections between elastin and the tenocytes are shown in Fig. 4. The elastic fibres and tenocytes are connected in series. In Fig. 4a and b, the elastin forms a sparse peri-cellular meshwork around tenocytes. The same structure can be observed in Fig. 4c and d. A tenocyte in a twisted shape (Fig. 4d) appears to be dragged by the elastic fibres (arrow indicated in Fig. 4d). Sharp and gentle crimps within the collagen fibril bundles can alter the shape of tenocytes and the directions of the elastic fibres (arrow indicated in Fig. 4e, f).

The extraordinary structure of the complex area in Fig. 3b is presented in detail in Fig. 5. The elastic fibres in this area (Fig.5a) show a circuitous trail, which could indicate a complex architecture of the entire matrix. Due to the different orientations, collagen fibrils in different layers appeared as a braided fabric in the 3D collagen network (Fig. 5b). The 2D merged images (Fig. 5c-i) from different depths show: a) a group of longitudinal collagen fibrils (white lines with arrow head marked fibril bundle) running deeper toward an upper right direction; b) a group of longitudinal collagen fibrils and a group of transverse collagen fibrils form into one group (yellow lines with arrow head marking fibril bundles), then inserts into a deeper layer through a gap (white arrowhead

in Fig. 5f). These images display the spatial traversing trail of fibril bundles. The images also show connections between the fibrils at different depth and between fibrils oriented in different directions. It could also be interpreted that several collagen fibril groups with different directions spatially run across each other at a certain point and form spirals and plaits, which can be verified in transverse images in Fig. 7. The prevalence of these oblique nodes in the whole Achilles tendon warrants further investigation.

2D FFT and alignment analysis

The intensity distribution of the 2D FFT of the collagen fibrils (Fig. 6b), elastic fibres (Fig. 6e) and tenocytes (Fig. 6h) displays a very similar butterfly pattern which aligns approximately with the horizontal axis. The corresponding FFT alignment analysis using Oval profile plug-in shows very similar graphs with distinctive and harmonic peaks and troughs (Fig. 6c, f, i). These indicate that the collagen fibrils, elastic fibres and tenocytes align similarly and approximately to the vertical axis direction. As the alignment of tenocytes is much more compliant to that of elastic fibres (Fig. 6d, g, j), the FFT alignment graphs of the tenocytes and elastic fibres exhibit a great similarity (Fig. 6f, i), which also can be confirmed from the normalised FFT Alignment graph (Fig. 6k).

The transverse spatial structure of collagen fibrils, elastic fibres and tenocytes

The 3D transverse images (Fig. 7) show a very clear structure of the tendon's hierarchical system as well as the spiral structure of the fibril bundles that supports the longitudinal observations.

As shown in the transverse view (Fig. 7), the diameter of collagen fibrils is at sub-micron level, which corresponds to the size of tendon collagen fibrils⁷. An Achilles tendon is made by a massive number of collagen fibrils and a small quantity of elastic fibres which are evenly distributed within the collagen fibril framework. Endotenon is made of collagen fibrils (yellow arrow in Fig. 7a, e) and elastin (yellow arrowhead in Fig. 7b, f) in a direction perpendicular to the tendon's long axis. It binds groups of collagen fibrils and elastin together. Two forms of elastin are observed in the endotenon: elastic fibres (yellow arrowhead in Fig. 7b) that conform to the collagen orientation in

endotenon, and elastin that forms a thin membrane (yellow arrowhead in Fig. 7f) at the interface between the tendon matrix and the endotenon. Skinny tenocytes (white arrowhead in Fig. 7b) sparsely distribute in the fibril matrix, and it can still be observed that elastin exists around the tenocytes. Clearly, the cells in the endotenon (white hollow arrowhead in Fig. 7b) have a larger diameter range than the tenocytes.

From the transverse view in Fig. 7a, collagen fibrils are the basic unit in a fascicle that can be observed by SHG microscopy. The fibrils show different tendency of running directions, and fibrils with a same tendency form a secondary unit — fibril bundles. It is widely accepted that collagen fibres are constituted of a group of collagen fibrils in the tendon's hierarchical system, and no endotenon exists between collagen fibres^{7; 27}. Therefore, it can be assumed that the definition of collagen fibres in tendon's hierarchical system actually refers to a group of collagen fibril bundles which have similar orientation. This structural feature can be related to the longitudinal observations in Fig. 5i that collagen fibrils run in groups with different orientations. The orientation differences between collagen fibril bundles, or “collagen fibres”, are not obviously distinguishable most of the time, because the general orientations are the same. However, when the sectioning occurred at the spiralled or plaited areas, the orientation differences can be observed clearly in transverse sections.

The spatial spirals between collagen fibril bundles have been reported^{7; 26-28}, but to date there are few studies that have provided clear images of this proposed model. Three very obvious spirals have been observed in Fig. 7a as indicated by white arrows, and further magnified images are provided (as shown in Fig. 7c-e). There is a centre in each spiral, and the orientation of collagen fibril bundles around the centre forms a typical “twirl” structure. This transverse appearance can correspond to the longitudinal spiral or plait structure shown in Fig. 5. Meanwhile, some spaces in the matrix (“*” indicated in Fig. 7c-e) appear to accompany the spirals, which warrants further study.

Discussion

As important components in the extracellular matrix, elastin and collagen usually have been studied conjointly²⁸⁻³¹. Compared with collagen, the elastic fibres in connective tissues have received less attention. This is likely to be a methodological issue related to the difficulty of elastic fibre detection using traditional optical microscopy and electron microscopy^{16; 32}. Due to the development of microscopy and staining techniques, the fine structure of elastic fibres, together with the collagen fibres and cells can now be well presented in a 3-dimensional network^{32; 33}. By utilizing these techniques, observations of elastin fibre networks within different tissues are increasing^{23; 24; 34-37}.

Elastin

In this study, three forms of elastin were observed in the mid-portion of Achilles tendon: (1) pericellular elastin meshwork that enveloped the tenocytes, which is consistent with the studies of bovine deep digital flexor tendons³⁶ and human rotator cuff³¹; (2) elastin fibres that were distributed along the fascicles and the endotenon; (3) elastin that formed a thin membrane in the endotenon. Even though the elastin content is much less than collagen, the longitudinal and transverse distributed elastin appears to play an important role in Achilles tendon.

Firstly, the attachment between elastic fibres and tenocytes appear to be in series. As is known, the extracellular matrix of tendon is synthesized and maintained by tenocytes and tenoblasts, and when load is applied on the tendon, the extracellular matrix transfers the load information to the cells⁷. Considering the anatomical relationship — the series connection between the elastin and tenocytes, the elastin could act as a medium to transfer mechanical information to cells and also act as a direction guide to the cells during movement. Secondly, the elastin meshwork around tenocytes could not only provide physical protections to tenocytes, and could also modulate the force transmitted to tenocytes to ensure the force can be transmitted more evenly. Moreover, the two forms of elastin in the endotenon enable sliding and recoil between adjacent bundles and fascicles,

and this feature of elastin fibres is similar to ligaments³⁸. However, future studies are required to investigate the mechanotransduction process between tenocytes and the extracellular matrix.

Some most recent studies^{28; 29; 33; 39} have also ascertained that elastin plays an important role in the microstructure and mechanical properties of tendons and ligaments. Elastin has also been suggested to be responsible for retaining the collagen crimp within tendons and ligaments, and has a crucial role in the resistance of the tensile and transverse shear forces within ligaments^{28; 29; 33}. Using immunofluorescent methods, Ritty⁴⁰ reported the distribution of elastin and elastic fibre-related proteins in flexor tendons. These findings and methods could be utilized in further studies to get a better understanding of elastin's role in Achilles tendons.

Collagen

Collagen fibres are the most abundant and important component in tendons. The representative phenomenon 'crimp' can be easily observed by optical microscopy and electron microscopy, and it has been studied in depth regarding the morphology together with other parameters^{4; 41; 42}. In this study, the high quality of collagen fibril images has confirmed the present understood hierarchical system of tendon tissue, and it is presumed that the collagen fibres are actually a group of collagen fibrils with the same orientation. The reason that the terminology of "collagen fibres" is widely used in many tendon studies could be due to the limited capabilities of optical microscopy and traditional staining techniques, which are widely used in tendon studies. Meanwhile, it has been reported⁴³ that collagen fibres which are oriented longitudinally and transversely run cross each other forming spirals. From the 2D longitudinal image series in Fig. 5 and the 3D transverse images in Fig. 7, the spatial spirals which are formed by collagen fibril bundles can be observed in the Achilles tendon. Apparently, compared to the organized crimp areas, such orientations which can be called "twists", "spirals", "plaits", or "twirls" in transverse sections, may improve the mechanical properties of the tendon. Functionally, they may increase the tensile strength of the tendon and optimise transmission of the tensile forces of muscle during activities. It may be the first time that this structure has been displayed in detailed 3D images containing both collagen and elastin in Achilles tendons. However,

future studies are required to determine the patterns of the occurrence of the twisted areas along the longitudinal and lateral directions of Achilles tendons. Simultaneously, the spirals appear to be normally accompanied by small spaces, which might be filled by ground substance rich in proteoglycans and other proteins that cannot be imaged by the SHG imaging technique. These spaces possibly work as buffer areas to absorb the tension created by twisting. Future studies could explore the distribution of the solid components within the ground substance in this buffer area.

Using SHG and confocal microscopy, a close spatial relationship between tenocytes and the elastic fibres has been detected in micron scale in this study. Study⁴⁴ that utilized electron microscopy to show the ultra-microstructure of rabbit Achilles tendons in nanometre scale matches our observation perfectly that elastin has a close relationship with tenocytes' plasma membrane. Studies in different scales and dimensions may build up a comprehensive view of the tendon's structure, which may contribute to the understanding of its functional mechanism. On the other hand, the elastic fibres localized along the tendon's axis are consistent with the collagen bundles, but show gentle curves rather than the collagen bundles' sharp kinks. This appearance can be related to the elastic fibres' mechanical function^{28; 29} of retaining the collagen crimp pattern. The gently curved elastic fibres appear to be able to hold the collagen bundles' crimp, but more evidence is still needed to fully assess and explain the mechanism.

In conclusion, a strong association of orientations between the elastin and tenocytes within the longitudinal collagen fibril framework has been demonstrated, and the techniques can show this association remains when the collagen fibrils become oblique and spiral. This anatomical structural knowledge may enrich the theory of mechanical and biological information transduction in tendon tissue, and may pave the way to develop novel imaging techniques for investigating tendon pathology^{10; 45}. This study has shown that the structure of the collagen fibrils and elastic fibres in the longitudinal and transverse sections of tendons are more complex than previously reported.

With these imaging techniques and suitable analysis methods^{46; 47}, further studies can focus on the quantitative evaluation of elastic fibre meshwork in relation to tenocytes and collagen matrix, like

the density analysis and their interconnectivity, and the structure and texture deviation in aged tendons or under different pathological conditions.

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Accepted Article

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Figure Legends

Figure 1

The procedures of image processing and 3D image construction.

Figure 2

Representative images of longitudinal 2D images of Collagen (green), elastin (cyan) and cells (red) with different magnifications using Confocal and SHG microscopy. Arrows indicate the elastic fibres. “j” shows the comparison of elastic fibres signal before and after background subtracting.

The double headed arrow in “e” shows a crimp.

Figure 3

Representative longitudinal observations of 3D network of elastin-tenocytes and collagen-elastin-tenocytes from different angles of views. At rest state, the collagen fibrils (green) and elastic fibres (cyan) are crimped. The tenocytes (red) and elastic fibres show series connections and are consistent with the long axis of the tendon. The area highlighted by rectangle in “b” shows the spatial spiral or plait of the tendon texture. Rectangles highlighted areas in “a” and “b” are shown in detail in Fig. 4 and Fig. 5. The volume size is $375\ \mu\text{m} \times 375\ \mu\text{m} \times 28\ \mu\text{m}$.

Figure 4

The localisation of the elastin-tenocytes network shows the close relationship between tenocytes (red) and the elastin (cyan). The images correspond to the regions highlighted in Figure 3a. “a” and “b” are the same location, while “b” shows an elastin pericellular meshwork which can also be seen in “c” and “d”. Arrows in “d”-“f” indicate the way that elastic fibres attach to the tenocytes in the twist area, crimp peaks and flat region.

Figure 5

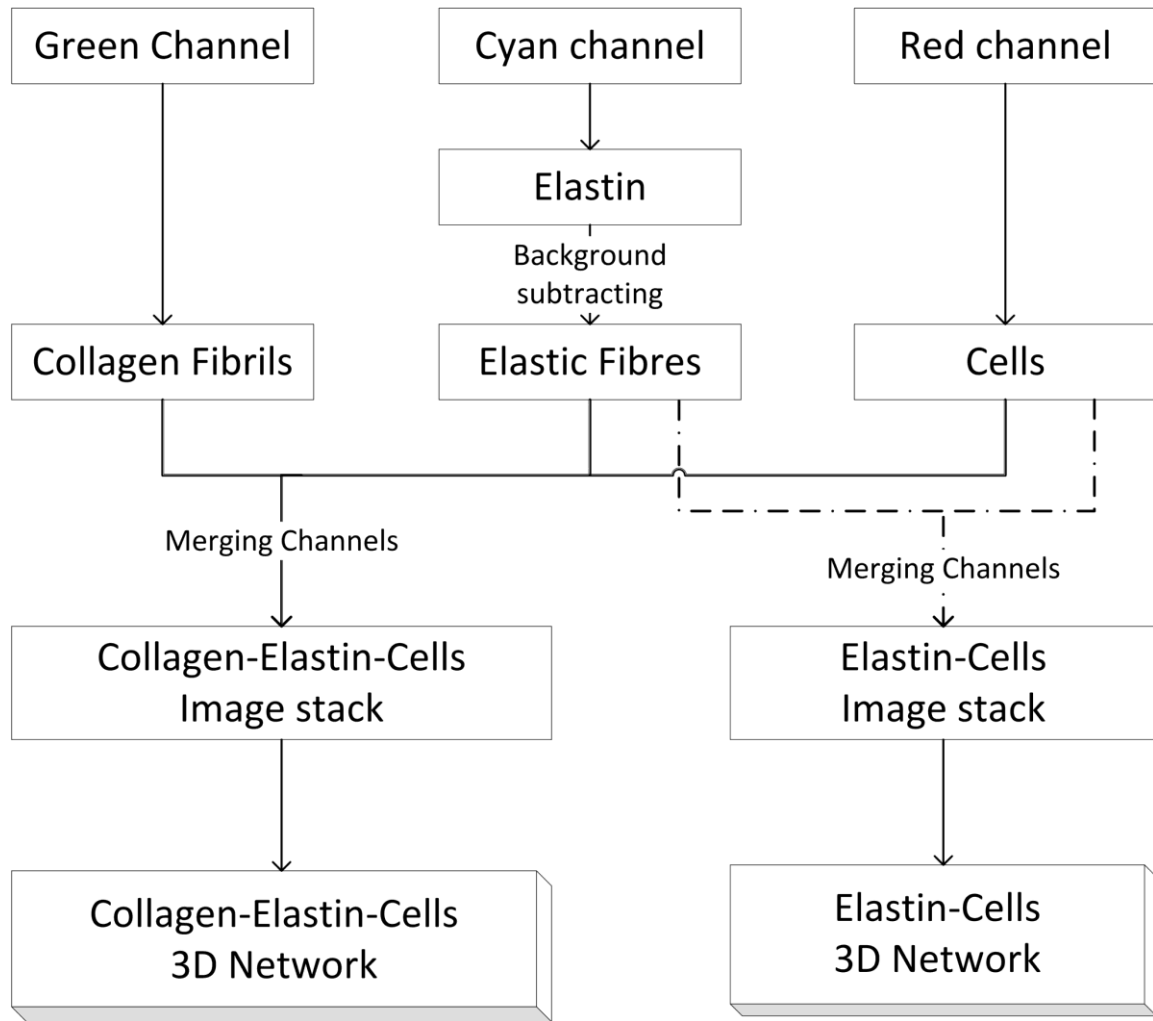
The longitudinal observation of a spiral corresponds to the region highlighted in Fig.3b. Collagen fibrils are green, tenocytes are red and elastic fibres are cyan. All the images are in the same scale. “a” and “b” are 3D images. “c”-“i” are 2D images and the numbers at the lower right corner indicate the depths of the images in the stack. White lines with arrowhead in “c”-“i” indicate a group of collagen fibrils going deeper toward an upper right direction. Yellow lines with arrowhead in “e”-“g” indicate two groups of collagen fibril bundles form into one group and go deeper through the gap. White arrowhead in “f” indicates the gap between bundles.

Figure 6

2D z - project of collagen fibrils (a), elastic fibres (d) and tenocytes (g) was reconstructed from the corresponding image stack, respectively. The corresponding 2D FFTs of the collagen (b), elastic fibres (e) and tenocytes (h) were used to objectively indicate the predominant alignment of the three components in Achilles tendons, which is along the vertical axis direction, but shown in the FFTs as along the horizontal axis. The two distinctive intensity distributions in the FFT of the collagen fibrils (b) at about 30° and 150° to the horizontal axis are related to the crimps of the collagen fibrils. The FFT alignment graphs of the collagen fibrils (c), elastic fibres (f) and tenocytes (i) were used to numerically study the alignment characteristics of the three components of Achilles tendons. “j” is a 3D image reconstructed from the merged image stacks of the collagen fibrils, elastic fibres and tenocytes. “k” is a FFT Alignment graph in which the grey value of the collagen fibrils, elastic fibre and tenocytes were normalised data for easily assessing their orientation characteristics. The volume size of the 3D image “j” is 375 μm × 375 μm × 28 μm.

Figure 7

Representative 3D transverse observation of the microstructure of Achilles tendons. Collagen fibrils are green, tenocytes are red and elastic fibres are cyan. “a” and “b” are from the same location. “c”-“e” are magnified spiral structures from white arrows indicated areas in “a”. “f” is the elastin-cell structure of “e”. Yellow arrow in “a” and “e” indicates the collagen fibrils in endotenon. Yellow arrowheads in “b” and “f” indicate the morphology of elastin in endotenon. White solid arrowheads in “b” indicate the elongated tenocytes. White hollow arrowhead in “b” indicates the cells in endotenon. The spiral structure appears to be accompanied by a small space that is indicated by “*” in “c”-“e”. The volume size of “a” and “b” is $238 \mu\text{m} \times 238 \mu\text{m} \times 20 \mu\text{m}$.

**Figure 1**

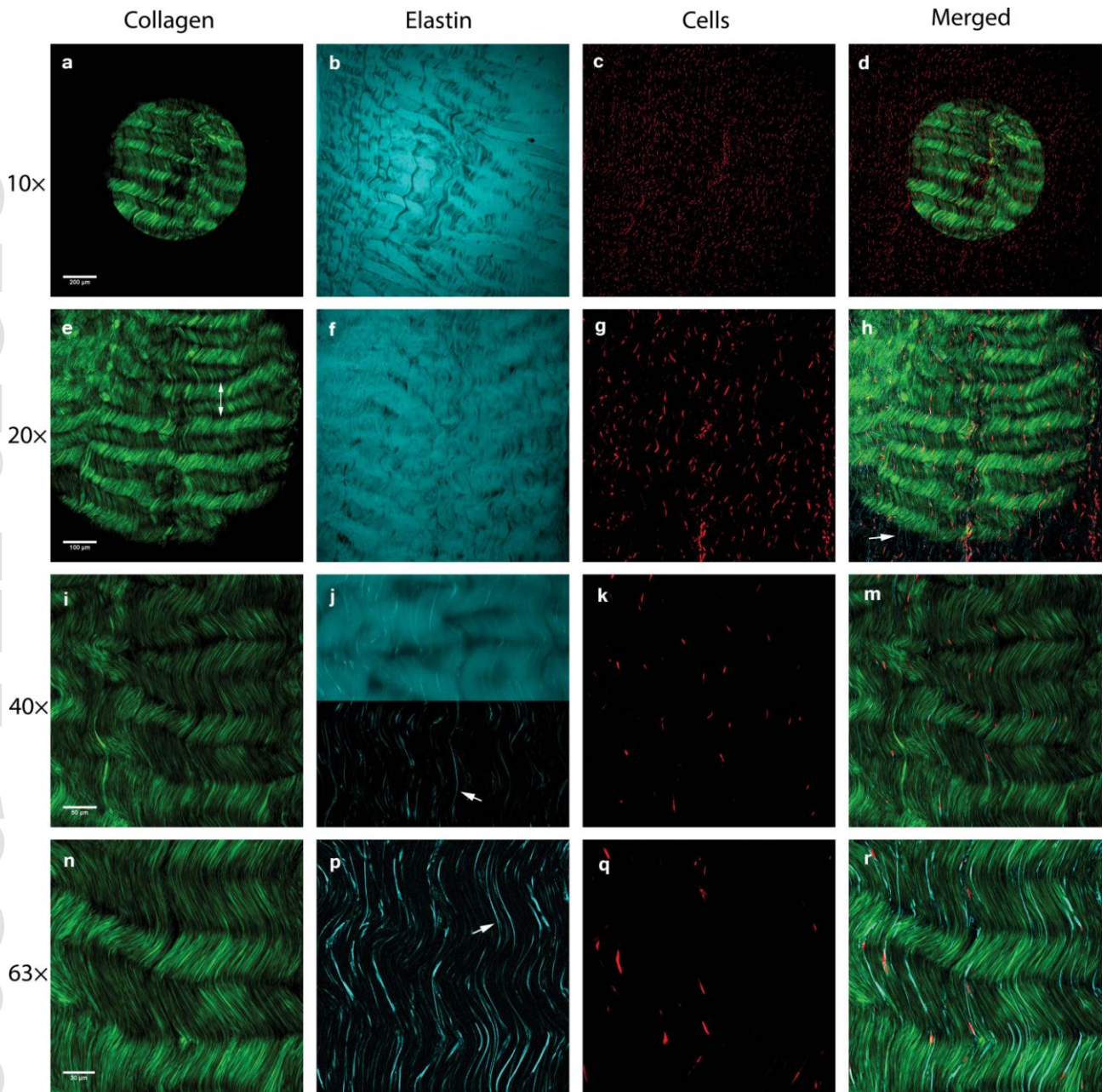


Figure 2

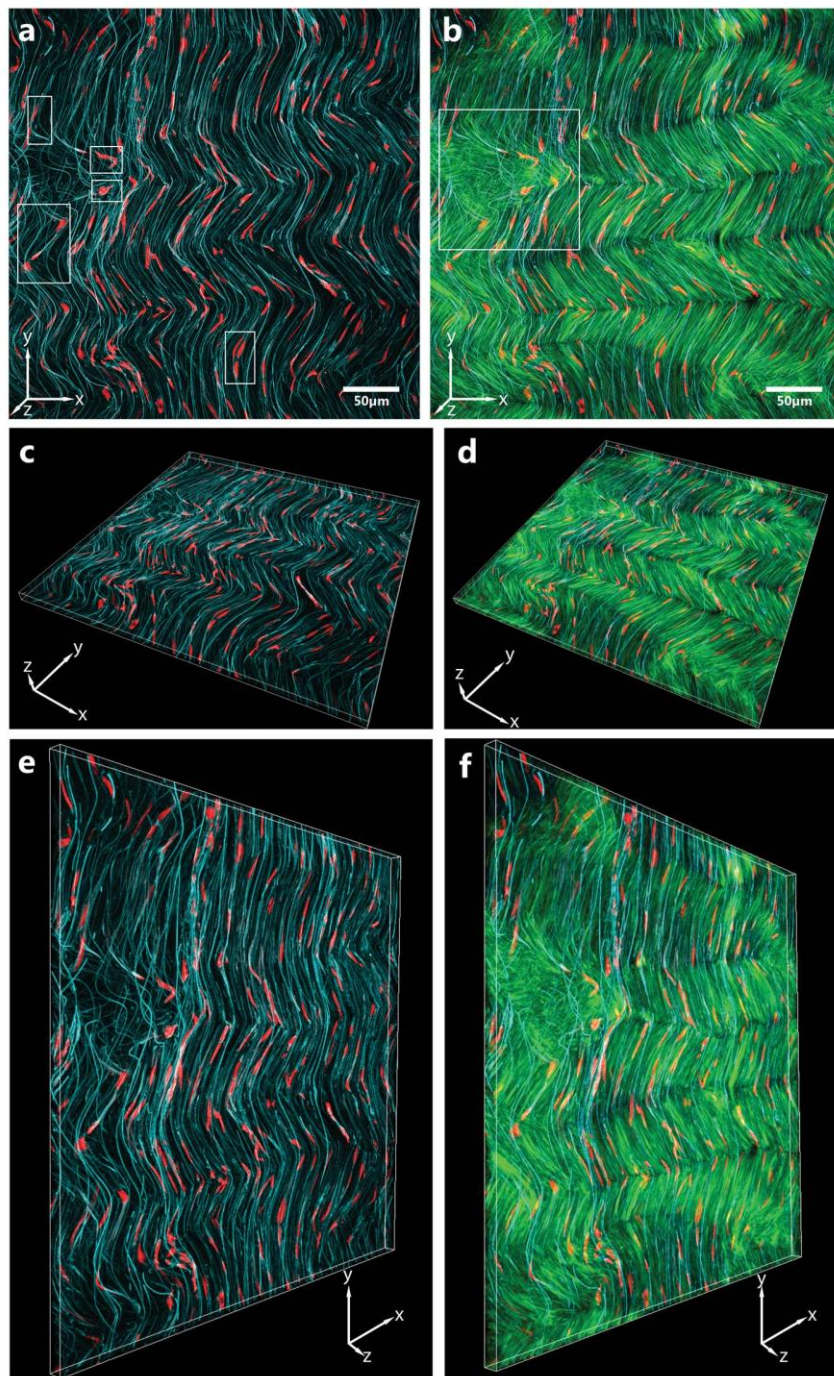


Figure 3

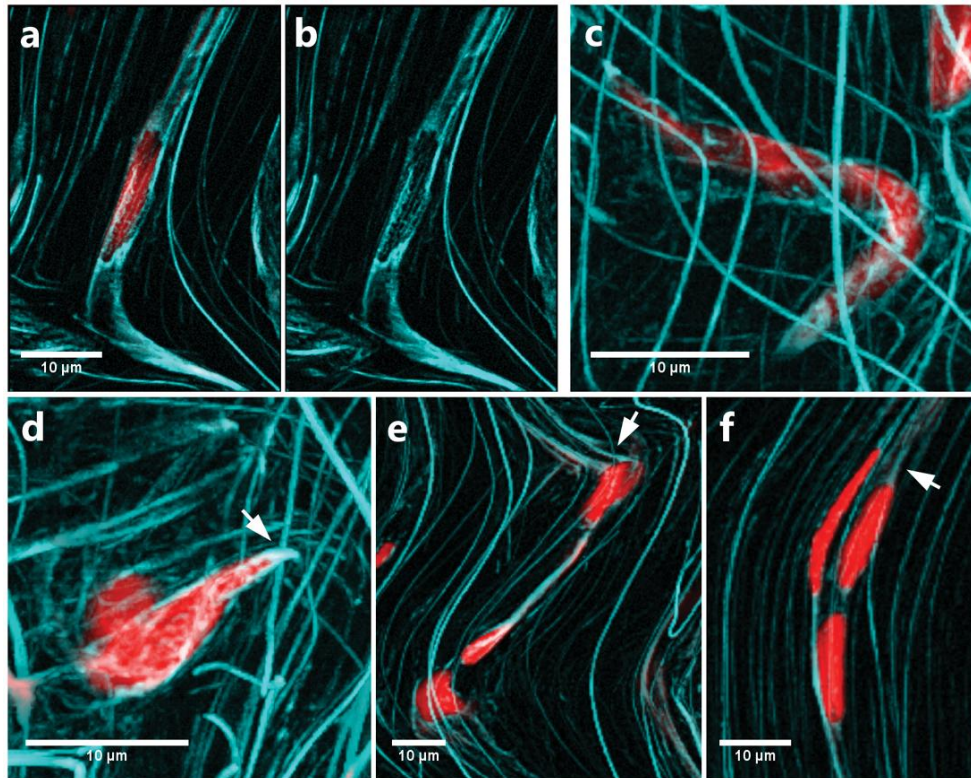


Figure 4

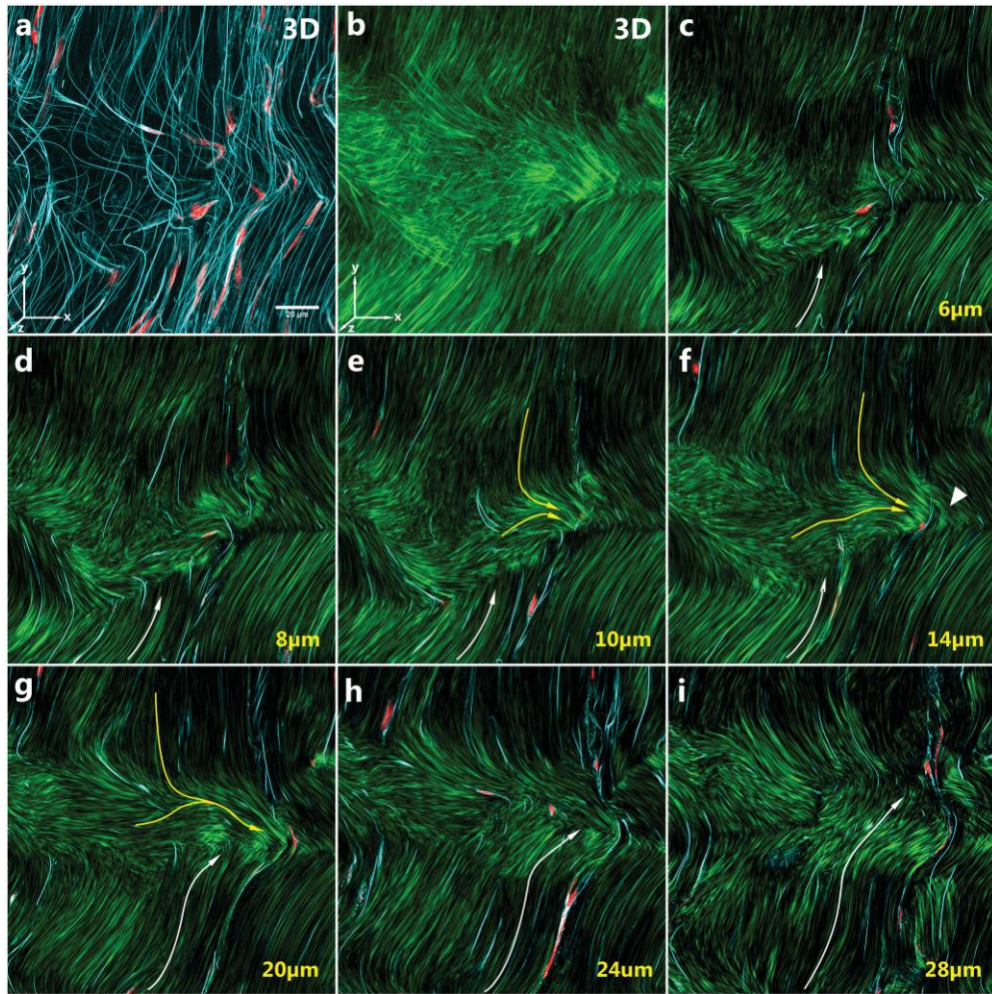


Figure 5

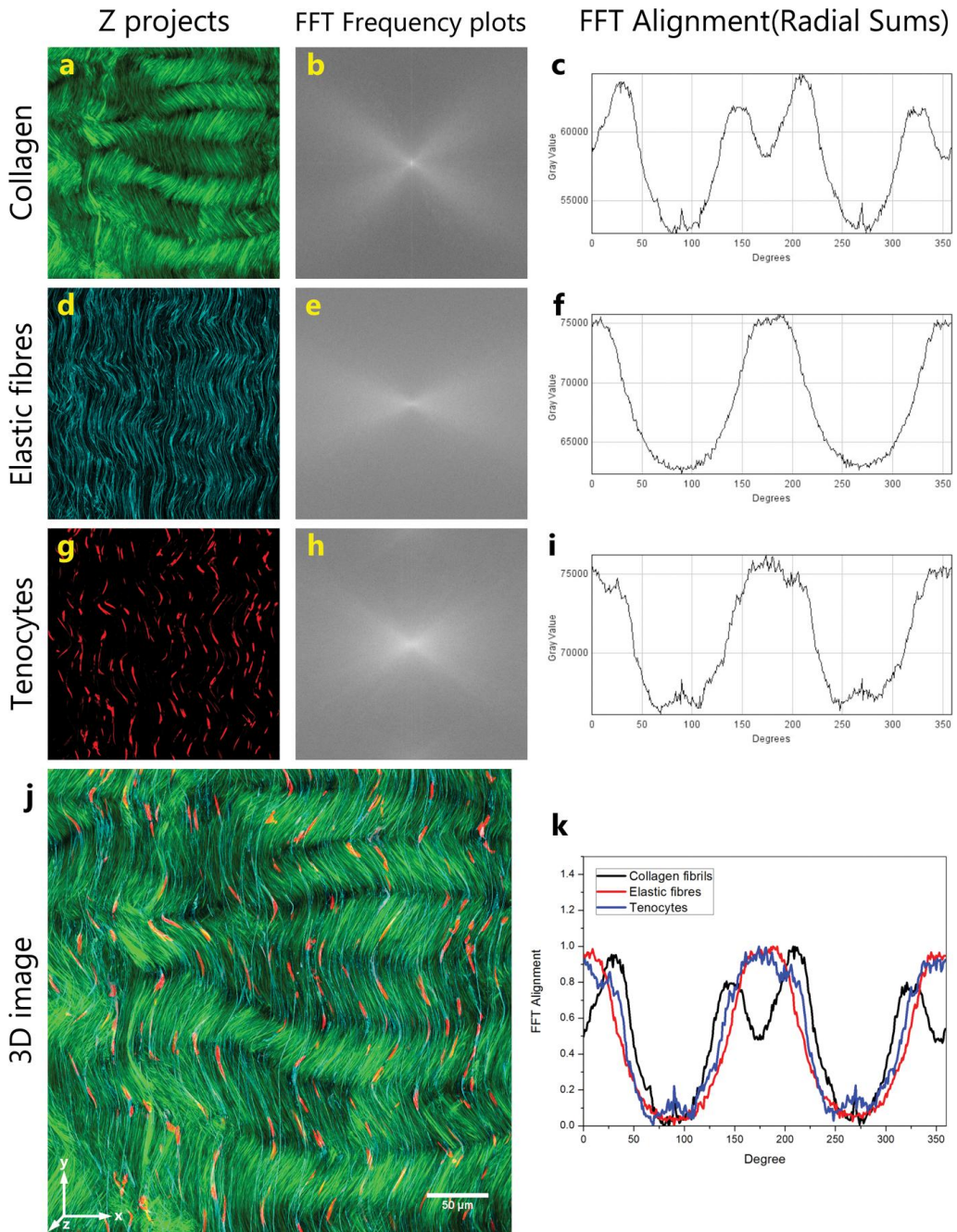


Figure 6

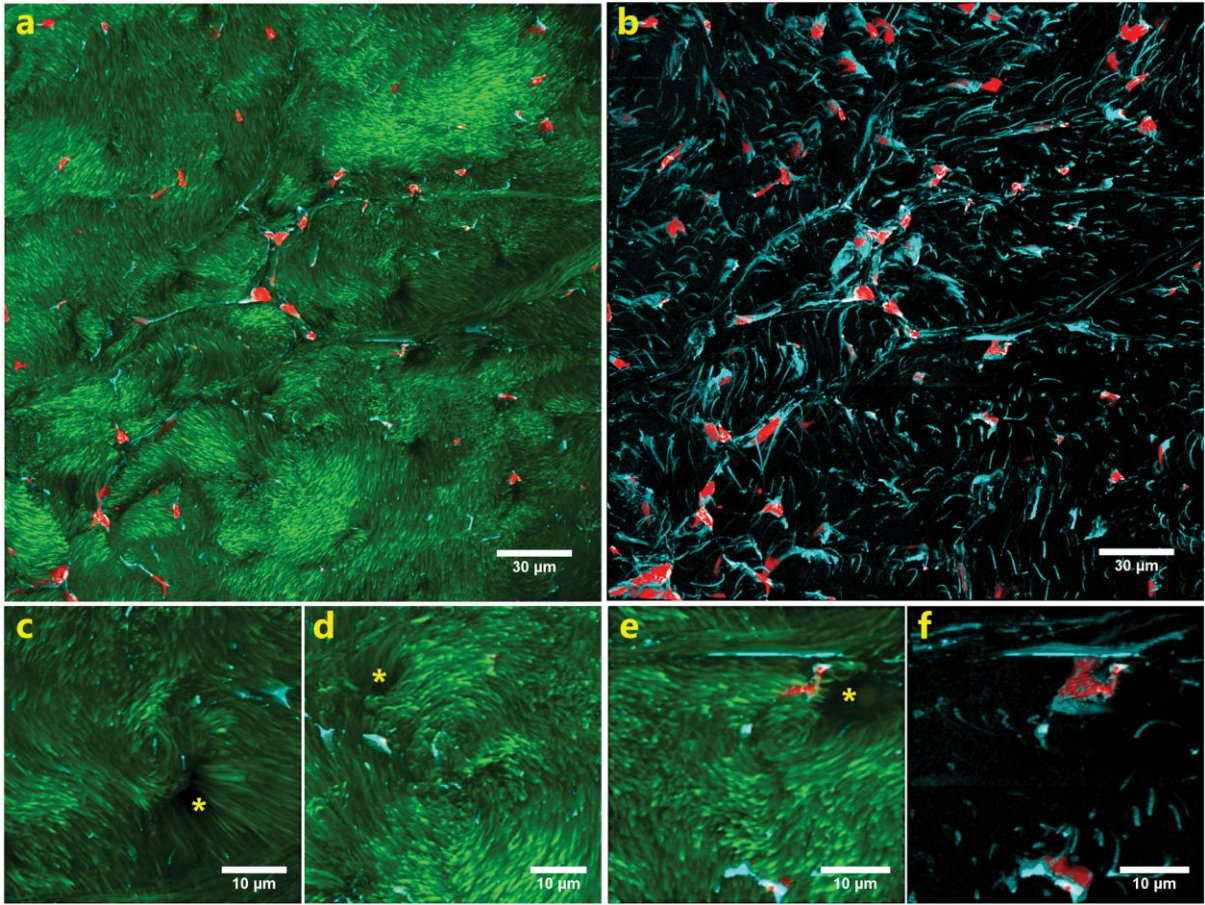


Figure 7