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High-resolution study of the 3D collagen fibrillary matrix of Achilles tendons without tissue labelling and dehydrating

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Summary

Knowledge of the collagen structure of an Achilles tendon is critical to comprehend the physiology, biomechanics, homeostasis and remodelling of the tissue. Despite intensive studies, there are still uncertainties regarding the microstructure. The majority of studies have examined the longitudinally arranged collagen fibrils as they are primarily attributed to the principal tensile strength of the tendon. Few studies have considered the structural integrity of the entire three-dimensional (3D) collagen meshwork, and how the longitudinal collagen fibrils are integrated as a strong unit in a 3D domain to provide the tendons with the essential tensile properties. Using second harmonic generation imaging, a 3D imaging technique was developed and used to study the 3D collagen matrix in the midportion of Achilles tendons without tissue labelling and dehydration. Therefore, the 3D collagen structure is presented in a condition closely representative of the *in vivo* status. Atomic force microscopy studies have confirmed that second harmonic generation reveals the internal collagen matrix of tendons in 3D at a fibril level. Achilles tendons primarily contain longitudinal collagen fibrils that braid spatially into a dense rope-like collagen meshwork and are encapsulated or wound tightly by the oblique collagen fibrils emanating from the epitenon region. The arrangement of the collagen fibrils provides the longitudinal fibrils with essential structural integrity and endows the tendon with the unique mechanical function for withstanding tensile stresses. A novel 3D microscopic method has been developed to examine the 3D collagen microstructure of tendons without tissue dehydrating and labelling. The study also provides new knowledge about the collagen microstructure in an Achilles tendon, which enables understanding of the function of the tissue. The knowledge may be important for applying surgical and tissue engineering techniques to tendon reconstruction.

Background

The Achilles tendon is the largest tendon in the human body that is subjected to repetitive use and substantial mechanical forces, potentially leading to injury, degeneration and tendinopathy (Schepsis *et al.*, 2002; Wren *et al.*, 2003; Huang *et al.*, 2004; Kujala *et al.*, 2005; Hess, 2009; Aparecida de Aro *et al.*, 2012). Collagen (mainly type I collagen) is the most abundant constituent of the extracellular matrix (ECM) of tendons and plays a central role in the tissue's mechanical function (Fratzl *et al.*, 1998; Gelse *et al.*, 2003; Kuijpers *et al.*, 2004; Provenzano & Vanderby, 2006). Thus, the collagen structure has been extensively studied to comprehend the physiology and function of tendons (Harvey *et al.*, 2009;

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Liu *et al.*, 2011), resulting in establishing nomenclature systems to describe the hierarchical characteristics of the collagen structure in the tendon (Kastelic *et al.*, 1978; Kannus, 2000; Screen *et al.*, 2005; Doroski *et al.*, 2007; Harvey *et al.*, 2009; Smith *et al.*, 2013). However, the nomenclature systems existing in the literature mainly depict the collagen orienting longitudinally in the inner region. They are inferred by excluding the collagen comprising the epitenon and paratenon. The epitenon and paratenon also have an important role in the physiology and function of tendons (Stecco *et al.*, 2014).

Also, the hierarchical collagen structure has been described differently in various studies. There are still some unanswered questions such as whether tendons are made up of continuous collagen fibrils and whether there are collagen fibres of 10–50 μm thick in tendons which are made up of collagen fibrils. So far, no study has used an image to demonstrate that there are collagen fibres in a tendon that consist of collagen fibrils. The hypothesis that tendons contain collagen fibres which are made up of collagen fibrils was mainly derived from linking different studies using microscopy with different imaging resolution.

A tendon acts to transfer tensile stresses, which are largely supported by the collagen in the inner region aligning longitudinally to the direction of the principal tensile stresses in the tissue (Benjamin *et al.*, 2008). Like many other tissues constituting the musculoskeletal system, the collagen in a tendon forms into a 3D network which must possess essential structural integrity to allow the tissue to perform its function. Despite this, there have been relatively few studies investigating how the longitudinal collagen fibrils are connected or bonded strongly into a single unit that enables the tendon to sustain the tensile loads and possible transverse and torsional stresses (Provenzano & Vanderby, 2006). Therefore, the collagen structure in the Achilles tendon requires more comprehensive investigation.

There are many microscopic techniques available for studying the microstructure of a tendon. Optical and polarized microscopies do not possess sufficient imaging resolution to distinguish the collagen fibrils in a tendon. Electron microscopy (EM) has ultrahigh imaging resolution for studying the characteristics of collagen fibrils but requires an extreme imaging environment including excessive tissue dehydration in scanning EM (SEM) or ultrathin tissue sectioning in transmission EM (TEM), potentially causing artefacts. Also, EM does not possess three-dimensional (3D) imaging capabilities for direct studies of the internal collagen microstructure of a tendon. Confocal microscopy offers a method for studying the collagen structure of a tendon in 3D but requires specific fluorescent labelling of collagen. The degradation of fluorescent dyes can cause inaccuracy and inconsistency (Pawley, 1995).

Collagen fibrils are polymerized tropocollagen molecules of about 300 nm long and 1.5 nm diameter (Fratzl *et al.*, 1998; Gelse *et al.*, 2003; Cisneros *et al.*, 2006; Provenzano & Vanderby, 2006; Liu *et al.*, 2011). A tropocollagen consists of right-handed triple helices of coiled procollagen polypeptide α chains that are composed of left-handed triple helices of amino acid sequence chains. There are staggers of approximately 67 nm long between the tropocollagen molecules, which are believed to enhance the tensile strength of the collagen fibrils (Torchia *et al.*, 1982). Thus, collagen fibrils present distinctively periodic regions called 'D bands' or 'periodic bands' under EM and AFM.

Due to the noncentrosymmetrical molecular structure and crystalline assembly (Prockop & Fertala, 1998), collagen possesses birefringent or polarizing properties that disperse the passing through light in the long wavelength spectrum into short wavelength light (Campagnola & Loew, 2003; Cox *et al.*, 2003; Pena *et al.*, 2005; Chen *et al.*, 2012). Under a high-intensity laser in a near-infrared spectrum, collagen generates very strong second harmonic generation (SHG) resultant light with a wavelength in the visible spectrum for SHG imaging (Pena *et al.*, 2005; Chen *et al.*, 2012; He *et al.*, 2014). Fibrillar form collagen such as types I and II collagen have a much more crystalline structure; hence, they produce strong SHG signals, which enables SHG imaging (Cox *et al.*, 2003; Chen *et al.*, 2012). As SHG is an intrinsic optical property of noncentrosymmetrical and crystalline substances, SHG imaging does not cause photobleaching nor does it require tissue labelling and dehydration. Moreover, SHG imaging requires multiphoton laser excitation. Therefore, it naturally inherits 3D imaging capabilities to acquire high-resolution images of the internal collagen microstructure of biological tissues without need of a pin hole (Chen *et al.*, 2012).

It is hypothesized that the midportion of Achilles tendons contains an intrinsically complex 3D collagen matrix that can be studied comprehensively using SHG imaging, and the collagen fibrils in the epitenon uniquely orient to enhance the microstructural integrity of the inner collagen matrix, endowing the tendon with the essential microstructural integrity and tensile strength. SHG microscopy would provide high-resolution images of the tendon collagen fibrils for quantitative 3D characterization of the orientation of the collagen fibrils using computer imaging analysis techniques. This study would provide new knowledge regarding the collagen meshwork in Achilles tendons for comprehending the function of the tissue and improvement of surgical or tissue engineering strategies for tendon repair. Potentially, the study could lead to development of a novel imaging technique for assessing the physiology of Achilles tendons without tissue dehydration and labelling.

Methods

Specimens

Five Achilles tendons were harvested on a daily basis from five white New Zealand rabbits (three are male) of about 20 weeks old immediately after the animals' euthanasia. The specimens

had a normal appearance and were from both left ($n = 2$) and right ($n = 3$) hind limbs. The use of the animals was approved by the animal ethic committee of the University of Western Australia (RA/3/100/1049).

After carefully cleaning the sheath materials surrounding a tendon, a glistening white tendon specimen of about 5 mm long was dissected from the midportion of the tendon. The specimen was embedded in Tissue-Tek optimum cutting temperature compound (OCT compound, Sahura Finetek USA. Inc., Torrance, CA, USA) for cryo-microtoming. Seven longitudinal cryosections were microtomed consecutively from the specimen using a Leica cryo-microtome (Leica Cryosta CM3050S, Leica Microsystems, Wetzlar, Germany) at −20°C. The cryosections were placed gently on normal nonadhesive glass slides (ensuring a zero external stress state) and covered with glass coverslips before they were sealed using sticky tape and numbered consecutively. A cryosection was 50 μm thick.

The first five cryosections from each specimen were used for studying the 3D collagenous structure in the Achilles tendon using SHG imaging. The remaining cryosections (total $n = 2 \times 5 = 10$) were divided into two groups. The first group was used to validate whether SHG reveals the 3D collagen microstructure at a fibril level using AFM. The second group was used to assess whether the collagen fibrils in Achilles tendons are continuous filaments. Sections that could not be imaged immediately were sealed in a container and stored at −20°C for no more than 2 days. Five additional midportion specimens (each of about 5 mm long) were also cut from the five Achilles tendons for parallel traditional H&E histology. Figure 1 summarizes the experimental method used in this study.

SHG imaging

A Leica multiphoton confocal microscope (Leica TCS SPII AOBS, Leica Microsystems) equipped with a 63 X/NA1.40 oil immersion objective lens (HCX PL APO CS) was used in this study. The microscope offers lasers of different wavelengths including a near-infrared multiphoton (NIMP) laser tuneable from 710 to 990 nm (Spectra Physics Main Tai Sapphire).

The excitation laser was tuned to 890 nm to generate the SHG signals of the collagen in an unstained tendon section. The selection of the laser was determined from previous studies (He *et al.*, 2013b; He *et al.*, 2014). The resultant SHG signals were collected by a photomultiplier tube (PMT) detector at 445 nm (exactly half the wavelength of the excitation laser) via a transmission channel. An SHG image stack was

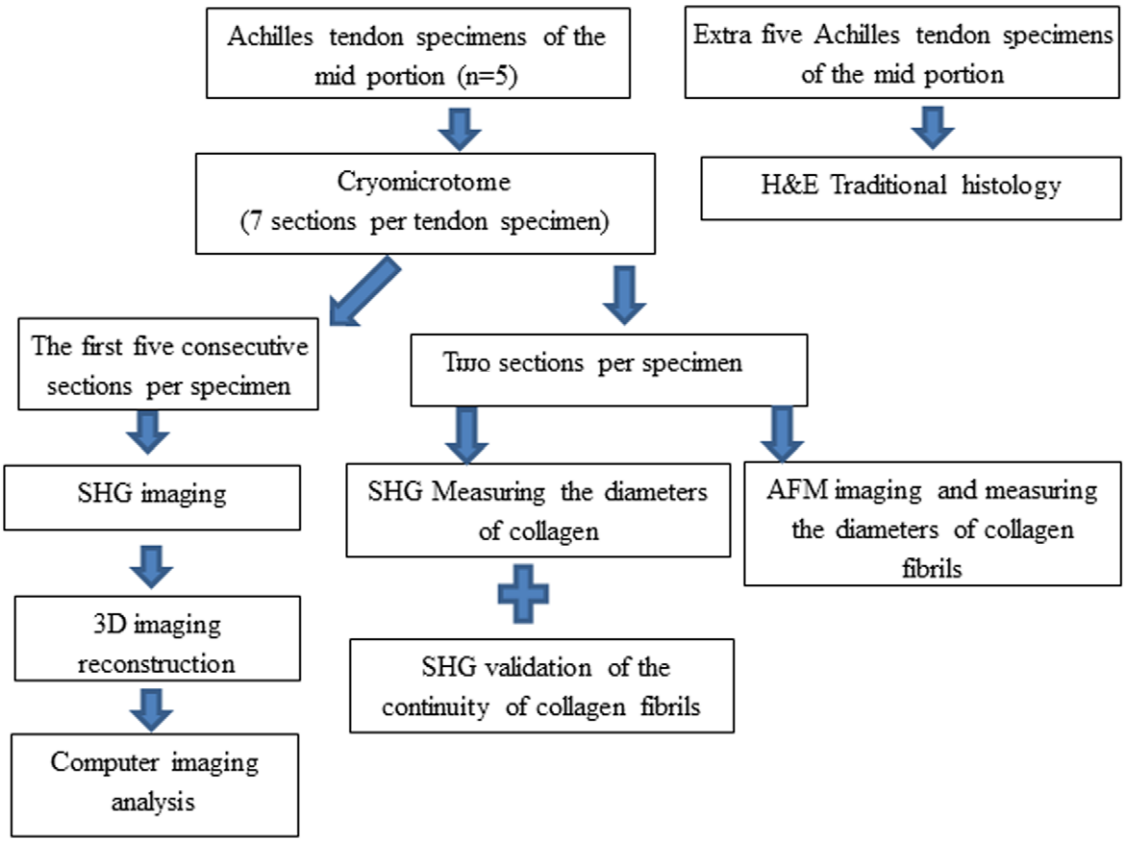

Fig. 1. Schematic diagram of the experimental procedure.

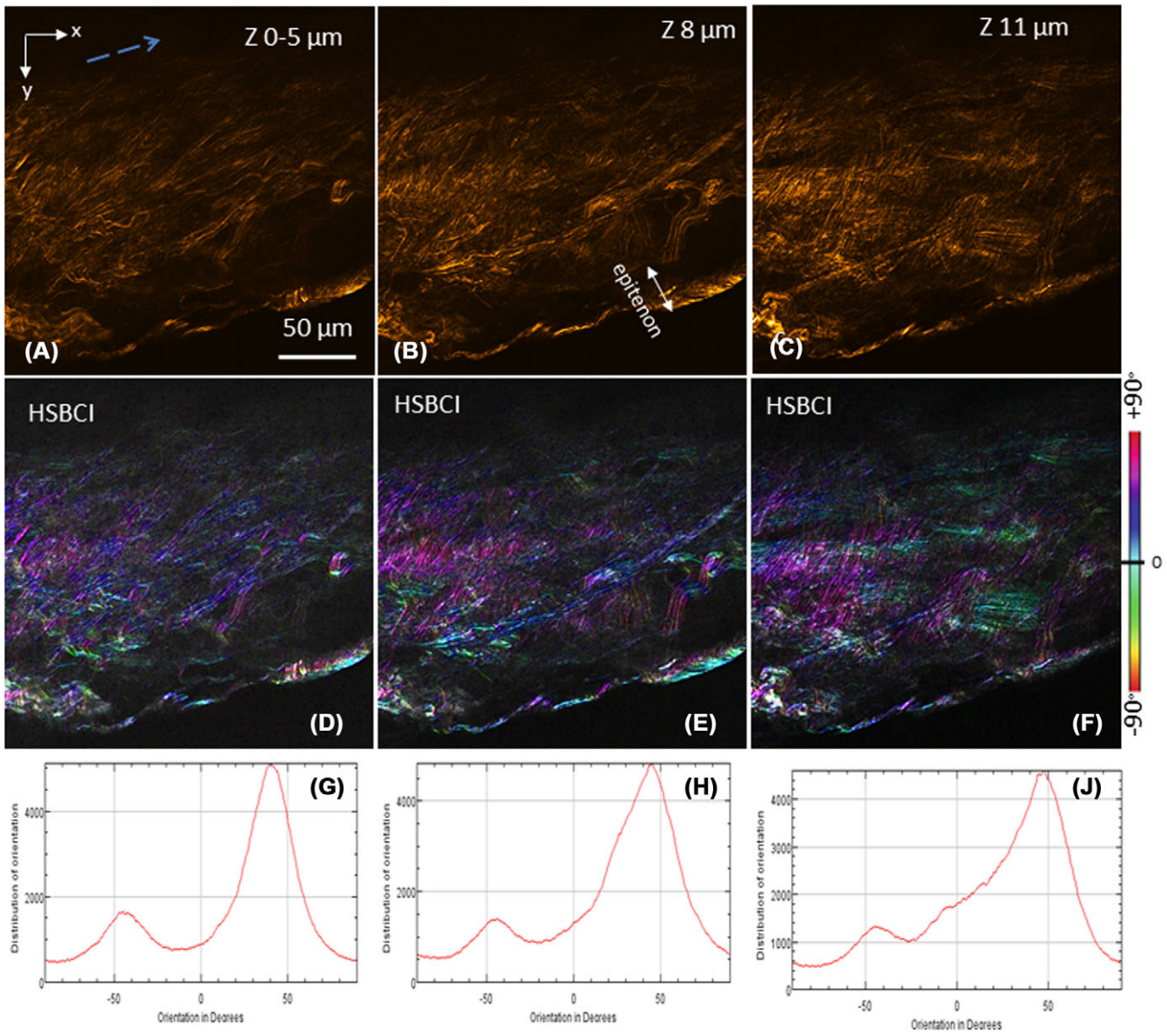

Fig. 2. (A) and (B) Representative images of the collagen fibrils at the edge of the epitenon of an Achilles tendon. (C) A representative image of the collagen fibrils adjacent to the longitudinal collagen fibrils. (D)–(F) Corresponding HSBCIs in which colours are used to highlight the orientation of the collagen fibrils. (G)–(J) Corresponding fibril directional analysis shows that the orientation of the collagen fibril in the region is spread from −90° to 90° with respect to the *x*-axis.

acquired using the Leica Confocal Software supplied within the microscope at an image step size of 1 µm. An image stack composed of a 2D image series was acquired at 1024 × 1024 pixels over an imaging field of the view (FOV) of a 240 µm × 240 µm. Four image scans were averaged to produce a 2D image to eliminate noise, which was determined to be appropriate from preliminary tests. To achieve a comprehensive understanding of the collagen microstructure in a tendon, SHG image stacks were acquired at five different locations in a cryosection.

3D image reconstruction and quantitative imaging analysis

ImageJ (Rasband, 1997–2014) was used to reconstruct the SHG image stacks into 3D images for visually studying the 3D arrangement of the collagen fibrils in the midportion of Achilles tendons. In cases where an image stack contained collagen fibrils from the epitenon to inner region of a tendon (e.g. Figs. 2 and 3), the image stack was optically divided into several smaller imaging stacks for 3D reconstructing and visual examining so that the critical orientation characteristics

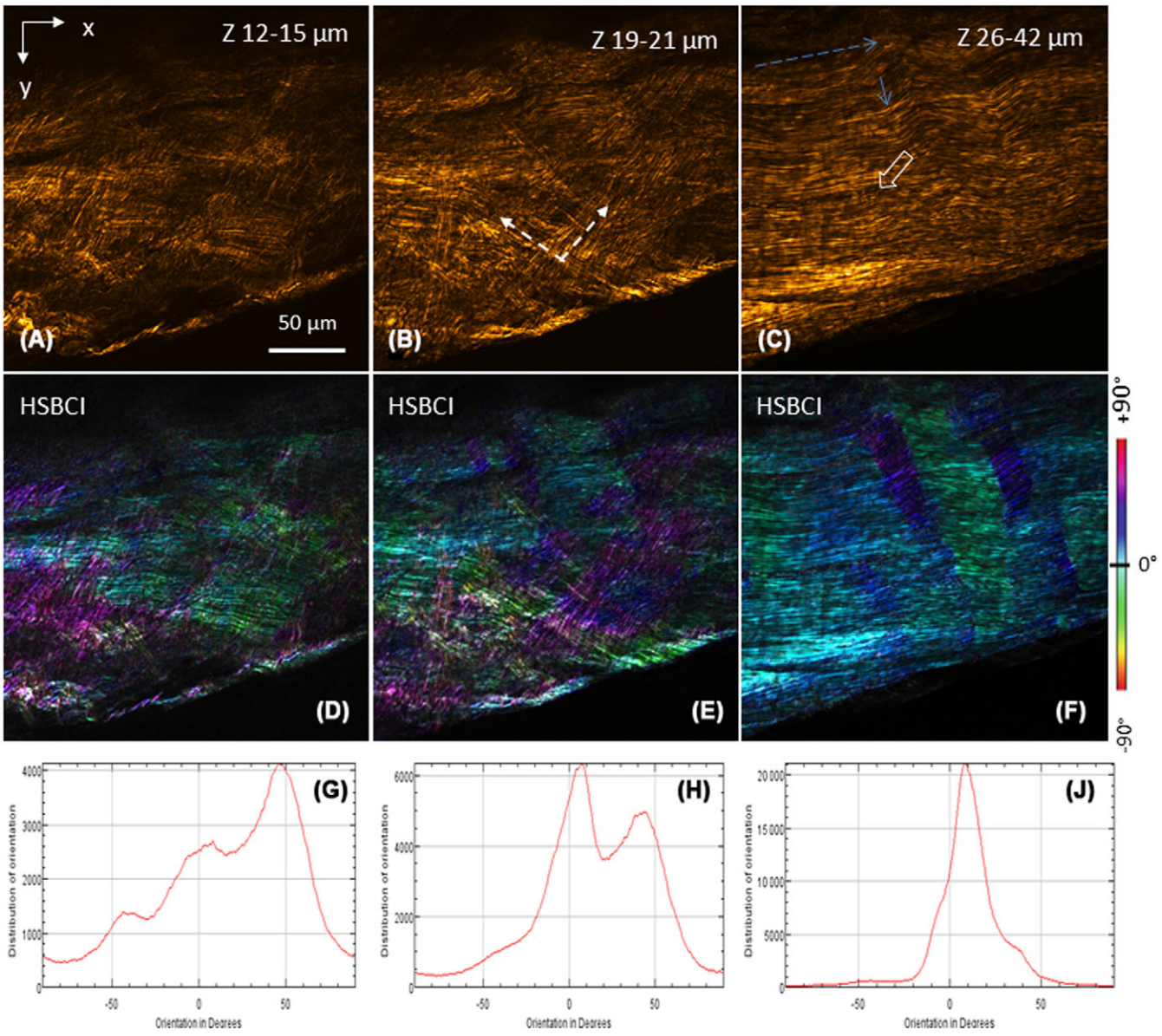

Fig. 3. (Continuing from Fig. 2). (A) and (B) The collagen fibrils immediately adjacent to the longitudinal collagen fibrils align in distinctive patterns to wrap around the longitudinal collagen fibrils. (C) The longitudinal collagen fibrils in the inner region of an Achilles tendon responsible for the principal tensile strength of the tendon. (D)–(F) Corresponding HSBCIs highlight the orientation of the collagen fibrils in the region. (G)–(J) Corresponding fibril directional analysis.

of the collagen fibrils in the relevant region could be clearly revealed (e.g. Figs. 4A–D).

OrientationJ plug-in (Rezakhaniha *et al.*, 2012) in ImageJ was used to quantitatively analyse the orientation characteristics of the collagen fibrils from the epitenon and inner regions. OrientationJ applies a structure tensor to examine the grey-level value of pixels in the local neighbourhood of a digital image to identify if the pixels have isotropic or anisotropic features (Jähne, 1993; Jahne, 1997; Rezakhaniha *et al.*, 2012). Pixels with a high-energy value denote anisotropic and directional features. Conversely, pixels with lower energy levels represent isotropic and less directional characteristics. Thus, OrientationJ measures the orientation features of the objects in the digital image (Jähne, 1993; Bigun *et al.*, 2004). OrientationJ has been proven to be a very useful tool for quantifying in 3D the orientation characteristics of the collagen fibrils in arterial walls and articular cartilage (Rezakhaniha *et al.*, 2012; He *et al.*, 2013a).

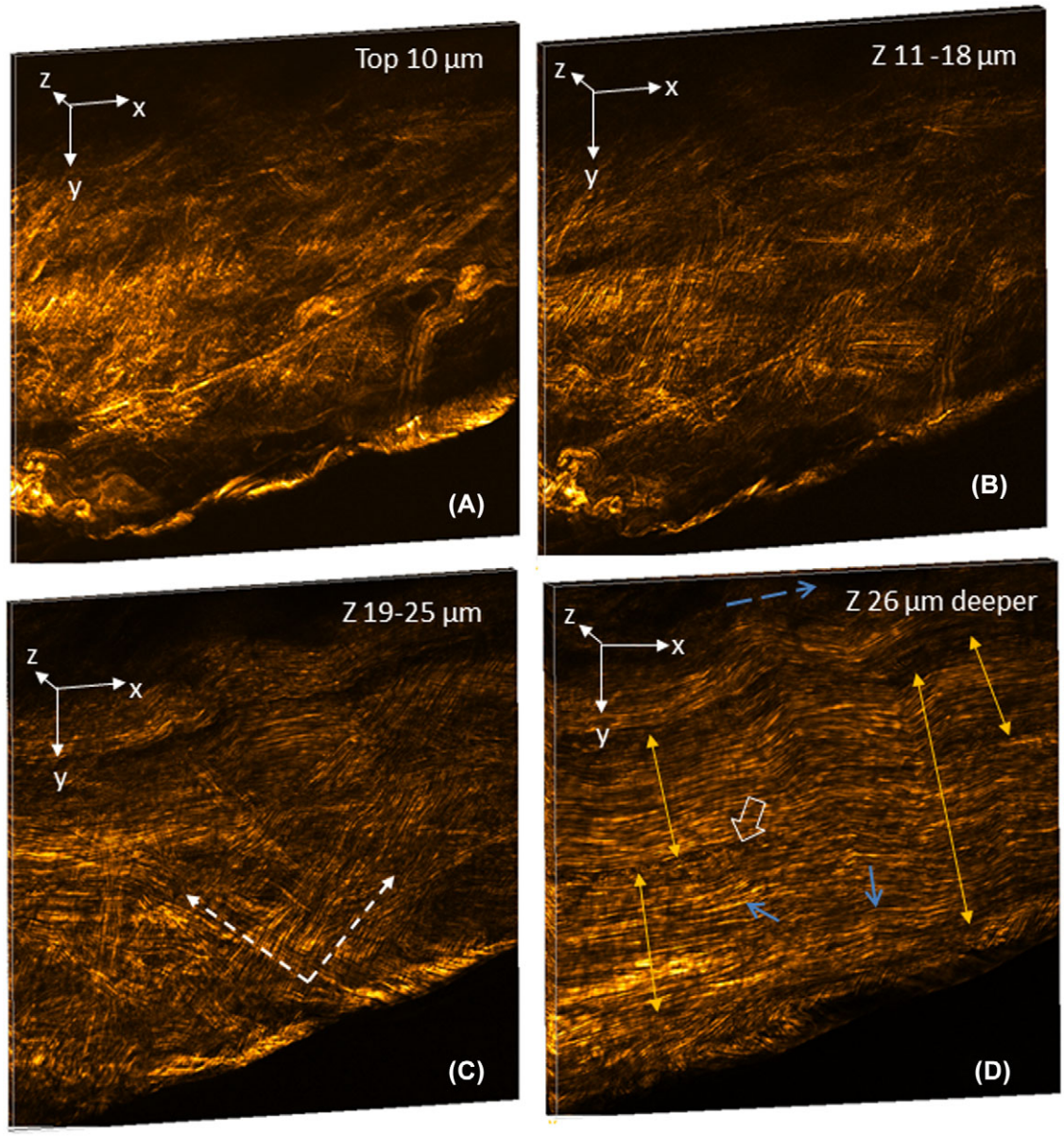

Fig. 4. Corresponding 3D images reconstructed show the characteristics of the collagen fibrillar matrix in the epitenon (A–C) and inner region (C and D) of an Achilles tendon. (The images are reconstructed from an SHG image stack shown in Figures 2(A)–(C) and Figures 3(A)–(C).

The Distribution function within OrientationJ was used to map the orientation of the collagen fibrils of Achilles tendons in the epitenon and inner region. A hue-saturation-brightness colour-coded image (HSBCI) in which colours were used to highlight the orientation of collagen fibrils from −90° to + 90° with respect to the *x*-axis (e.g. Figs. 2D–F and 3D–F). The predominant orientation of the collagen fibrils was also shown using an orientation graph (e.g. Figs. 2G–J and 3G–J).

The dominant-direction algorithm within OrientationJ (Rezakhaniha *et al.*, 2012; He *et al.*, 2013a) was used to quantify the depth-dependent orientation and coherency of the collagen fibrils from the epitenon to inner region (e.g. Figs. 6 and 7C and D). The fibril orientation was expressed in degrees from 0° to 180° with respect to the *x*-axis in the 2D image series of an image stack. The coherence parameter has a value between 0 and 1. A value of 1 indicates that the collagen fibrils exhibit strongly anisotropic and coherent characteristics. Conversely, a value of 0 indicates that the collagen fibrils display isotropic and less coherent features.

Validating SHG reveals the collagen fibrils in tendons

Although SHG microscopy is a unique imaging technique for visualizing collagen without tissue dehydrating and labelling,

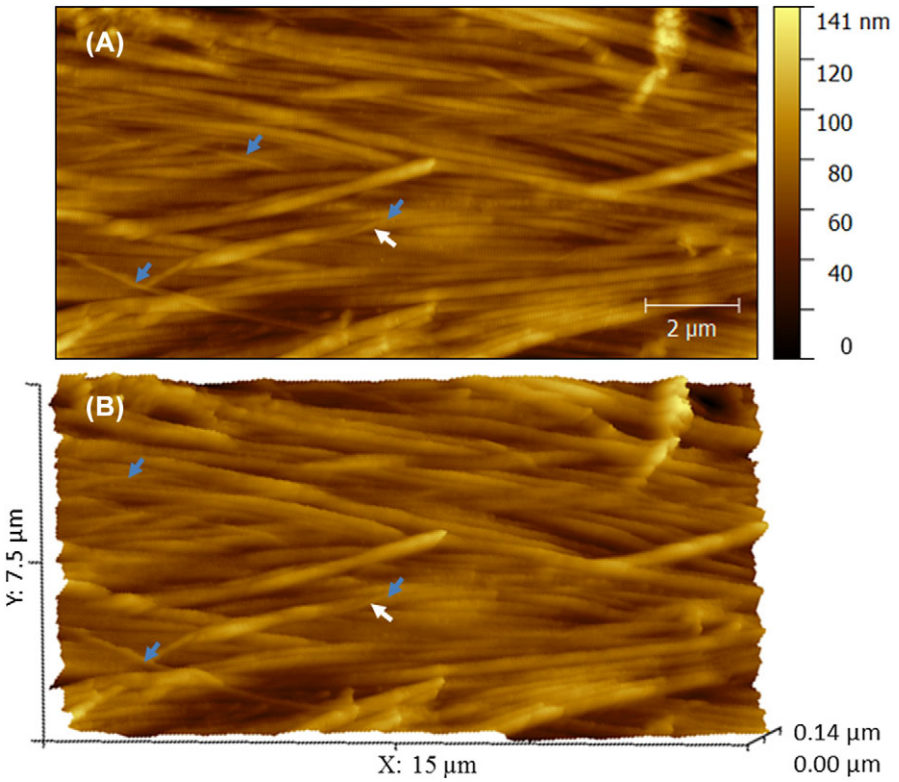

Fig. 5. (A) AFM shows the dense basket woven longitudinal collagen fibrils in the inner region of an Achilles tendon. (B) A 3D topography of the AFM image.

it was unknown whether it reveals the collagen fibres or fibrils of a tendon as the collagen is displayed as ordinary (or featureless) wires or threads in SHG images. Since AFM has a superior imaging resolution for distinguishing collagen fibrils without tissue dehydration and labelling, it was used to measure the diameter of the collagen fibrils of the tendon. After the diameter of the collagen displayed by SHG was measured, it was compared to the diameter of collagen fibrils measured from AFM. Thus, we know if SHG reveals the internal collagen structure of Achilles tendons at a fibril level or not. The method is detailed below.

- Measuring the diameter of collagen in SHG

The dense microstructural arrangement of the collagen in tendons (Figs. 4 and 7) prevents accurately measuring the diameter of individual collagen filaments directly from the SHG images. Therefore, the individual collagen filaments composing a tendon cryosection ($n = 5$) were separated (isolated) from the tendon to obtain SHG images of the collagen (Fig. 8A). Specifically, after a cryosection was thawed completely on a glass slide at room temperature, a volume of 1000 μL Phosphate buffered saline (PBS) was dropped on the sample to wash and separate the collagen construction from the cryosection for SHG imaging, as shown in Figures 8(A)–(C). The plot profile function in ImageJ (Rasband, 1997–2014) was then used to acquire intensity profiles containing the width information of collagen filaments (Fig. 8D) from a series of 2D SHG images (Fig. 8A). Since the intensity profiles displayed a typical Gaussian normal distribution (Fig. 8D), the diameter or thickness of the collagen filaments was determined at the full width at the half maximum intensity (FWHM), as shown in Table 1.

Table 1. Diameter of collagen and collagen fibrils in Achilles tendons measured using SHG and AFM.

Diameter of collagen in SHG	0.41–0.50 µm
Diameter of collagen fibrils in AFM	0.37–0.45 µm

- Measuring the diameters of collagen fibrils using AFM

Five cryosections were fully thawed at room temperature, respectively, for acquiring AFM images of the collagen fibrils *in-situ* in physiological saline solution using a Bruker FastScan AFM (Dimension FastScan, Bruker, CA, USA). AFM imaging was performed in Tapping Mode with FastScan-B probes (spring constant: 1 N/m, resonant frequency: 300 kHz).

The AFM images were rendered and analysed using Gwyddion software (Nečas & Klapetek, 2012), as shown in Figure 8(E) where the colour-encoded scale bar (Fig. 8E) shows the vertical profile of the collagen fibrils. After levelling the

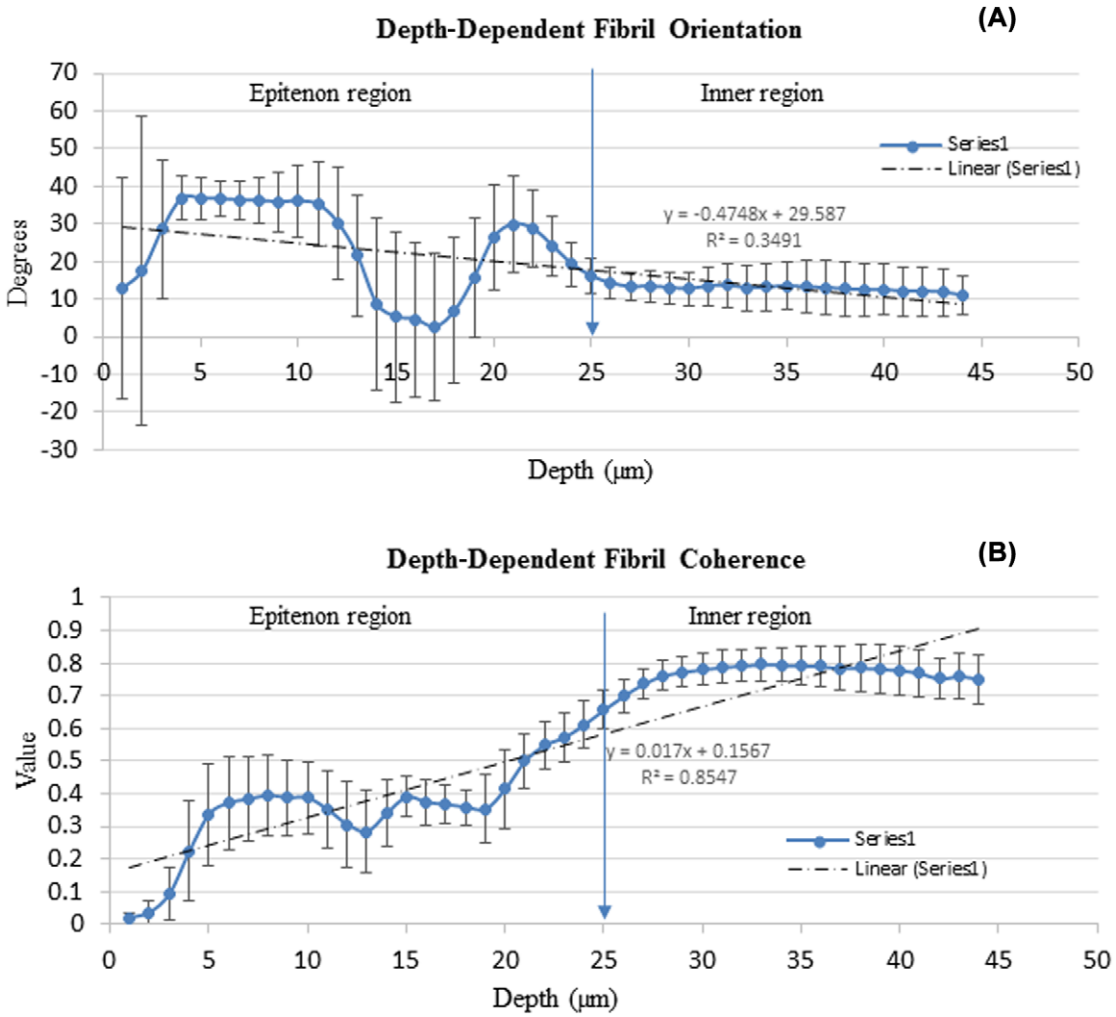

Fig. 6. (A) Quantitative analysis of the orientation of the collagen fibrils in the midportion of an Achilles tendon (from the epitenon to inner region). (B) Quantitative analysis of the fibril coherence of the collagen fibrils from the epitenon to inner region.

data, the diameters of the collagen fibrils were measured using the distance measurement tool within the Gwyddion software. To study the spatial morphology and arrangement of the collagen fibrils, 3D topographies were also reconstructed from the AFM images using the 3D display and visualization tool within the GWYddion software, as shown in Figure 8(F).

Comparing the collagen diameters measured from both SHG and AFM images, as shown in Table 1, it can be determined whether SHG permits observing the 3D collagen structure at a fibril level or not. AFM images at a low magnification are also acquired to show the basket weave characteristics of the longitudinal collagen fibrils in tendons (Fig. 5).

- Validation of the continuity of collagen fibrils

To further determine if the collagen fibrils are long continuous filaments in tendons, a small portion of 2D image sequences in an SHG imaging stack containing the morphology or dimensions of collagen fibrils were optically extracted to reconstruct the maximum intensity projection image (MII) (e.g. Fig. 8B) and 3D image (e.g. Fig. 8C) using ImageJ (Rasband, 1997–2014). An MII is made by combining pixels with the maximum intensity values of the 2D image sequences along the *z* direction. It represents a view of all the data in the 2D image sequences as if all of the 2D images were combined into a single image showing only in focus information. Therefore, from the MII and 3D image, we can determine that the collagen fibrils in the Achilles tendons are long continuous fibrils.

Results

Imaging techniques and collagen substance in SHG

SHG and AFM permit studying the collagen fibrils in tendons at different microscopic scales. SHG does not have imaging resolution to resolve the symbolic periodic bands of collagen

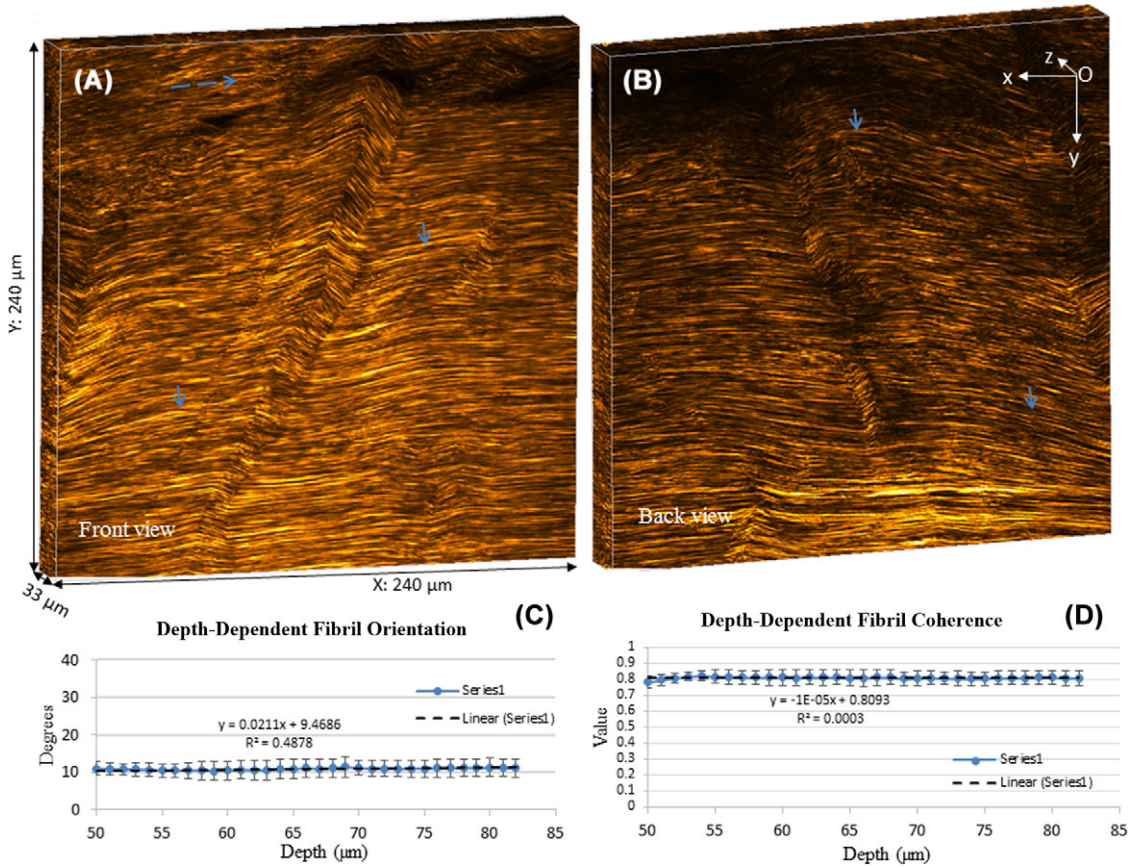

Fig. 7. The front (A) and back view (B) of a typical 3D image of the longitudinal collagen fibrils in the inner region of Achilles tendons. As shown, the collagen fibrils braid spatially (small blue arrows) to form a rope like network, which increases the stability of the collagen fibrils and endows the tendon with the tensile strength. (C) Orientation analysis shows the collagen fibrils in the inner region orient in one direction at approximately 11° (with respect to the *x*-axis). (D) Coherence analysis shows that the collagen fibrils in the inner region of an Achilles tendon have a high coherence value of about 0.8.

fibrils (collagen fibrils are shown as strings in SHG images) but it offers visualizing the collagen fibrillar arrangement of the collagen network in tendons without a need of tissue dehydration and labelling (Figs. 2–4).

Although AFM does not have a vertical imaging range comparable to that of SHG for studying the internal microstructure of the collagen fibrils, it has imaging resolution for distinguishing the periodical bands and measuring the diameter of collagen fibrils without dehydrating and labelling the tissues (Figs. 8E and F). The measurement data from AFM imaging indicate that the collagen fibrils primarily composing an Achilles tendon have a diameter range from 0.37 to 0.45 µm (Table 1). In comparison, the collagens revealed in SHG images have a diameter range of 0.41–0.50 µm (Table. 1). The comparable measurement of the collagen in SHG with that of collagen fibrils in AFM indicates that SHG reveals the 3D collagen microstructure of an Achilles tendon at a fibril level. In addition, the 3D AFM topography (Fig. 8F) shows that as the primary constituent of the ECM of an Achilles tendon, the collagen fibrils have an approximately cylindrical shape.

The 3D collagen meshwork of an Achilles tendon

SHG images show that the midportion of an Achilles tendon contains an intrinsically complex collagen meshwork, as shown in Figures 2(A)–(C), 3(A) and (C), 4 and 9(A) and (B). The collagen fibrils, which orient longitudinally in a direction compliant to the principal tensile stresses in the tendon (long blue dash arrows in Figures 2A, 3C, 4D, 6A and 9A), occupy the core and majority volume of the 3D tendon collagen matrix (Figs. 4C and D, 7A and B and 9A and B).

The longitudinal collagen fibrils spatially braid or basket weave (small blue arrows in Figs. 3C, 4D, 7A and B and 9A) into a dense collagen matrix (Figs. 3C, 4D and 7A and B), which is entrapped or wound by the collagen fibrils emanating from the epitenon (Figs. 2A–C, 3A and B, 4A–C and 9A). Hereby, a robust 3D collagen meshwork forms in the tendon. With the microstructural arrangement of collagen, the longitudinal collagen fibrils are endued with the essential structural integrity to function as a unit responsible for the tensile

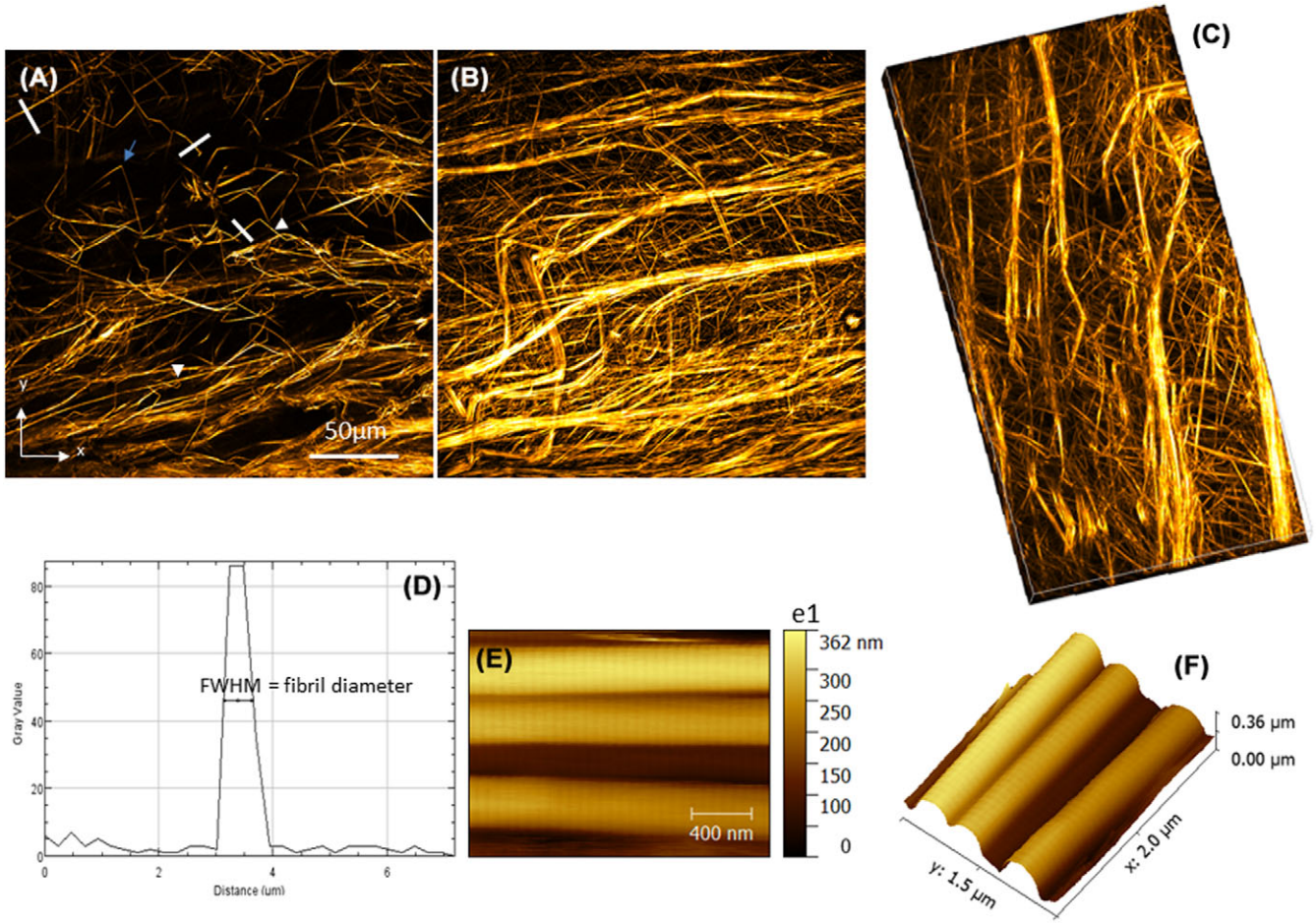

Fig. 8. (A) A typical 2D image in an SHG image stack used for measuring the diameters of the collagen filaments. The image also shows that the collagen fibrils are continuous filaments in Achilles tendon (white arrow heads). (B) A maximum intensity image reconstructed from a small portion of 2D image sequences in the SHG image stack further confirms that the longitudinal collagen fibrils are long continuous filaments. (C) A typical 3D image shows the continuity of the longitudinal collagen fibrils in the inner region of an Achilles tendon (the fibrils were purposely separated using PBS for the SHG imaging). (D) The intensity output of the diameter of collagen fibrils derived from Figure 8(A) was used to measure the collagen diameter using FWHM. (E) An AFM image shows the longitudinal collagen fibrils in an Achilles tendon. (F) A 3D AFM topography shows the 3D profile of the longitudinal collagen fibrils.

strength of the tendon. Basket weaving of the collagen fibrils aligning longitudinally was also confirmed by AFM (Fig. 5).

It is worthy of a note that AFM also shows that the tendon collagen matrix is also blended with a very small number of collagen fibrils (presenting D-band features) whose diameter is smaller than the primary collagen fibrils (blue arrows in Fig. 5). Occasionally, the thinner collagen fibrils physically entwine with the primary collagen fibrils (white arrow in Fig. 5).

Due to the unloaded state, the longitudinal collagen fibrils exhibit typical crimps in various sizes (Figs. 3C, 7A and B and 9A), which are highlighted in dark blue in the corresponding HSBCIs (Figs. 3F and 9B). The narrow peak in the analytic graph of fibril orientation (Fig. 3J) indicates that the orientation of the longitudinal collagen fibrils is strongly directional and anisotropic.

Continuity of collagen fibrils

Due to the basket weave status of the longitudinal collagen fibrils in the Achilles tendons, the collagen fibrils were shown as short fibrils in SHG (only the in focus portion of the fibril can be seen in each SHG image. The part under another collagen fibril cannot be seen) (Figs. 4D and 7A and B). However, the SHG images of the collagen fibrils isolated from the extra cellular matrix (ECM) of Achilles tendons (using PBS) show that the longitudinal collagen fibrils are long continuous filaments (Figs. 8A–C). The fibrils appear to be the smallest independent units directly responsible for the tensile property of the tendons. SHG images also show that the collagen fibrils assemble directly into the tendon fascicles with a diameter from 44 μm to more than 300 μm (yellow long arrows in Fig. 4D). There are not collagen fibre suggested to be 10–50 μm

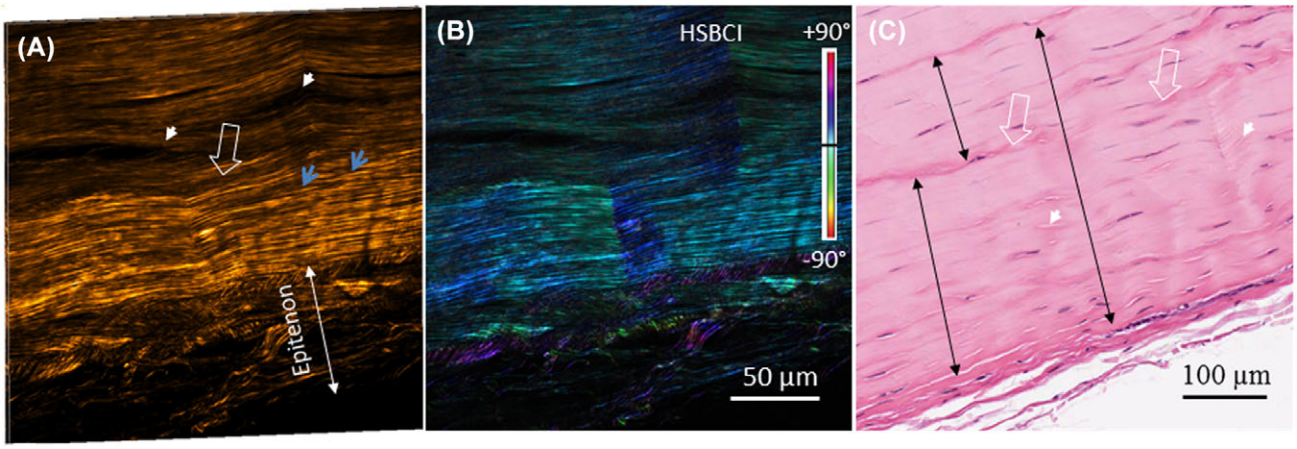

Fig. 9. (A) A 3D image shows the microstructural characteristics between the collagen fibrils in the inner and epitenon region (white double head arrow). (B) Corresponding HSBCI highlights the distinctive arrangement of the collagen fibrils in the inner and epitenon region. (C) A histological image. Both SHG and histology show splitting of the tendon fibrous matrix (white arrows in Figs. 9A and C) after loss of the reinforcement of the collagen fibrils in epitenon. The splitting of the collagen matrix shown in Figure 9(A) appears to be resulted from the crimping of the collagen fibrils (highlighted as dark blue in Fig. 9B). The hollow white arrows indicate the end of fascicles.

thick and composed by collagen fibrils with a diameter of 0.37–0.50 µm in the tendons. In other words, the collagen fibrils do not assessable into collagen fibres but directly into the fascicles. However, the fascicles do not necessarily extend the full length of Achilles tendons (white hollow arrows in Figs. 3C, 4D and 9A), which is consistent with the traditional histology (white hollow arrows in Fig. 9C).

The collagen fibrils in epitenon

The collagen fibrils in the epitenon constitute a smaller fraction of the tendon collagen matrix (Figs. 2A–C, 3A and B, 4A and D and 9A and B). The diameter of these fibrils is in the same range of the longitudinal collagen fibrils. However, the orientation of the collagen fibrils in the epitenon is clearly not compliant to that of the longitudinal collagen fibrils (Figs. 3B and C, 4C and D and 7A and B) and the principal tensile stresses in a tendon. Near the edge of the tendon collagen matrix, the orientation of the collagen fibrils is much less directional (Figs. 2A–C), which is demonstrated by the colours of the HSBCIs (Figs. 2D and E). The analytic graphs of the fibril orientation distribution (Figs. 2G and H) also confirm that the orientation of the collagen fibrils varies from −90° to 90°. Although there is a peak at about 40° to 50° in the graphs (Figs. 2G and H), it does not correspond to the orientation of the longitudinal collagen fibrils in the inner region (Figs. 3J). Also, the intensity of the peak (which is at about 4500) is almost five times lower than that of the longitudinal fibrils (Fig. 3J), further indicating that the orientation of the collagen fibrils near the edge of the tendon collagen matrix is strongly isotropic.

It is worth noting that in the region immediately adjacent to the longitudinal collagen fibrils, the collagen fibrils are interwoven into distinctive patterns perpendicular to each other (the white dash arrows in Figs. 3B and 4C) to firmly entrap the longitudinal collagen fibrils. The structural characteristics of the collagen fibrils in this region are highlighted by the corresponding HSBCI (Fig. 3D and E). The analytic graph of the fibril orientation distribution (Figs. 3G and H) in this region also indicates that the collagen fibrils are oriented from −90° to 90°. There are two intensity peaks shown in the graph (Fig. 3H). The first one at about 8° corresponds to the orientation of the longitudinal collagen fibrils (coloured as cyan in Fig. 3E). The second one at about 45° is related to the collagen fibrils aligning at about 45° (coloured as purple in Fig. 3E). The intensity of the peaks is much weaker than that of the longitudinal fibrils (Fig. 3F).

The collagen fibrils in the epitenon region appear to act to strengthen the microstructural stability of the longitudinal collagen fibrils. A loss of the reinforcement derived from the collagen fibrils in the epitenon, such as occurs due to physical tissue sectioning, leads to disruption or 'splitting' of the collagen meshwork composed of longitudinal fibrils (white arrows in Fig. 9A). Splits in the collagen meshwork are also commonly seen in traditional histology (white arrow in Fig. 9C) due to the requirement of physically sectioning tendon tissues.

Quantitative analysis of the orientation of collagen fibrils

SHG microscopy provides sufficiently high imaging resolution for quantitative analysis of the orientation and coherency of the tendon collagen fibrils in 3D. Numerical analysis of the fibril orientation characteristics along the *z* direction indicates that the collagen fibrils in the epitenon and inner region have distinctive orientation (e.g. Fig. 6A). In the epitenon region (e.g. depth < approximately 25 µm in Fig. 6A), the collagen fibrils orient randomly in a range from 3° to near 40°,

corresponding with the visual observations (Figs. 4A–C). In the region immediately adjacent to the longitudinal collagen fibrils (a depth range of 10–25 μm in Fig. 6A), the orientation value of the fibrils fluctuates markedly as the collagen fibrils align into two distinctive directions perpendicular to each other (Figs. 4B and C). Approaching the inner region (depth > 25 μm), the orientation value of the collagen fibrils becomes almost a constant (at about 10°).

Analysis of the coherency value of the collagen fibrils along the *z* depth indicates that the coherency value of the collagen fibrils displays a clearly increasing trend from the epitenon to inner region (Fig. 5B). Near the edge of the tendons, the collagen fibrils have the lowest coherency value. Towards the inner regions, the fibril coherency value consistently increases, and becomes almost a constant at a maximum value of about 0.8 at the inner region (Figs. 6B and 7D). In particular, there is a linear increase of the fibril coherency value (from about 0.35 to about 0.8) in the depth range of 20–30 μm (Fig. 6B). The coherency value reaches a maximum plateau at about 0.8 in the inner tendon region (Figs. 6B and 7D) as the collagen fibrils in the inner region of tendons align predominantly in one direction (Figs. 4D and 7A and B).

Discussion

A tendon is naturally designed to primarily transfer tensile stresses. However, in sports, exercise and accidents, the stress exerted on a tendon may vary enormously including transverse shearing and torsional stresses in addition to the tensile stresses. The stress magnitude may exceed the physical tolerance of the tissue and cause a tendon rupture. Collagen, the main constituent responsible for the mechanical properties of tendons, possesses a tensile strength equivalent to a steel rope when compared weight for weight (Ghadially, 1983). Despite this, the tensile strength derived from an individual collagen fibril is very limited. The formation of these fibrils as a collagen network provides the collagen with the structural stability to function as a unit. Therefore, both the physical properties and structural arrangement of collagen influence the mechanical performance of tendons. Despite intensive studies on the collagen structure of tendons, knowledge about the collagen fibrillary arrangement remained uncertain.

SHG and AFM are advanced microscopic techniques permitting studies of the collagen structure in tendons without tissue dehydration and labelling, which is desirable in research and clinical applications. By using SHG and AFM, we have investigated the 3D microstructural characteristics of collagen fibrils in the midportion of Achilles tendons. The study has provided new knowledge regarding how the longitudinal collagen fibrils are structurally integrated as a strong unit to bestow the tendons with the essential mechanical properties.

Collagen is capable of self-assembly (Cisneros *et al.*, 2006; Doroski *et al.*, 2007). Hydroxylation condensation in the lysine residue and disulphide bonds at the cysteine have been suggested to crosslink the tendon collagen fibrils (Doroski *et al.*, 2007), which bonds the collagen fibrils together. Many scholars disagree that proteoglycans play a role in cross-linking collagen fibrils in tendons but suggest the macroproteins only act as a lubricant for fibril sliding and hydrating the tissue (Provenzano & Vanderby, 2006). In this study, we have not examined the covalent or molecular-level crosslinks or hydroxylation condensation adhesions between the collagen fibrils. We have studied and demonstrated how the collagen network in a tendon establishes the microstructural integrity to provide the tissue with the mechanical function.

In a 3D domain, the longitudinal collagen fibrils interweave into a dense rope-like collagen framework (Figs. 4G and 7), which instils the structural stability of the collagen fibrils and endows tendons with the tensile properties. The structural integrity of the longitudinal collagen fibrils appears to be further enhanced by the entrapment of the oblique collagen fibrils from the epitenon. Clearly, the formatting characteristics of collagen fibrils discovered in this study permit the longitudinal collagen fibrils to act as a strong unit to withstand the tensile loads, and potential transverse and torsional stresses. As shown in Figures 8(A)–(C) and 9(A) and (C), with the loss of the reinforcement from the collagen fibrils in the epitenon, the inner rope-like collagen framework made up of longitudinal collagen fibrils responsible for carrying the tensile stresses of a tendon can be easily disrupted. The cracks or splits within the collagen matrix shown in Figure 9(A) (white arrows) appear to be caused by crimping of the longitudinal collagen fibrils after the absence of the entrapment of the collagen fibrils in the epitenon. In fact, cracks or splits of the ECM of tendons are also commonly shown in traditional histology because the physical sectioning requirement causes the loss of reinforcement from the collagen fibrils in the epitenon, as shown in Figure 9(C) (white arrow).

Previously, SEM and TEM studies on tendons have also reported interwoven collagen fibrils aligning longitudinally (Provenzano & Vanderby, 2006) but the observations were much more localized and in 2D. Since SEM and TEM imaging involves physically sectioning tissues into ultrathin sections, the process could potentially reorganise the ultrastructure of the collagen fibrils, leading to a debate regarding whether the interweaving of the collagen fibrils observed was genuine or artefacts related to tissue preparation.

SHG imaging possesses an intrinsic 3D imaging capability for studying the internal microstructure of a tendon, eliminating artefacts possibly caused by ultrasectioning of a tissue. The imaging results from the reaction of photons with anisotropic crystalline structures (Cox *et al.*, 2003). It is a unique imaging technique for studying collagen, microtubules and myosin, especially fibrillar forms of collagen such as types I and II (Cox *et al.*, 2003; Pena *et al.*, 2005; Chen *et al.*, 2012). There is no chemical reaction such as in immunohistology nor fluorescent process such as in confocal and multiphoton fluorescent imaging (He *et al., 2014*). Although the tissues in this study were

cryosectioned into 50-μm-thick sections for SHG imaging via a transmission channel, the sections are hydrated and thick enough to allow observation of the internal microstructure of the collagen network close to the natural status in a tendon. Therefore, this study has provided new evidence to support the findings using TEM and SEM that the longitudinal collagen fibrils interweave in tendons (Provenzano & Vanderby, 2006), which increases the structural stability of the longitudinal fibrils.

The experiments carried out in this study to isolate the collagen fibrils from the tendon ECM for SHG imaging of individual collagen fibrils (Fig. 8A) further indicate that the crosslinks via hydroxylation condensation and disulphide bonds between the longitudinal collagen fibrils are very weak. This finding is reasonable because very strong cross-linkages between the longitudinal collagen fibrils would build a strong resistance to sliding between the collagen fibrils during loading. However, interfibril sliding has been proven to exist when tendons are under loads (Screen *et al.*, 2005; Provenzano & Vanderby, 2006). Therefore, the structural integrity of the collagen meshwork of tendons derived from braiding of individual collagen fibrils orienting longitudinally and reinforcement of the collagen fibrils in the epitenon on the longitudinal fibrillar structure is crucial for the function of tendons. The microstructural arrangement discovered in this study ensures the longitudinal collagen fibrils to possess the essential structural integrity to function as a unit to support tensile loads, whereas the intersliding of the collagen fibrils during loading is litter interfered.

It is well accepted that collagen forms a hierarchical structure in tendons but the hierarchical structural order has been reported differently by different authors (Benjamin *et al.*, 2008; Liu *et al.*, 2011; Smith *et al.*, 2013). Some scholars claimed that there are collagen fibres with a diameter from 10 to 50 μm in tendons that compose tendon fascicles. The collagen fibres are the smallest unit that can be visualized under light microscopy and are made up of collagen fibrils of 0.41–0.50 μm thick. However, the observations made using SHG and AFM in this study demonstrate that there are not collagen fibres made up of collagen fibrils in Achilles tendons. This is consistent with the report that tendon fascicles are made directly of collagen fibrils (Liu *et al.*, 2011), and longitudinal collagen fibrils of about 0.50 μm thick are the primary building blocks of the hierarchical collagen microstructure and the tensile property of an Achilles tendon (Aparecida de Aro *et al.*, 2012). If there were collagen fibres in an Achilles tendon, they would have been shown to be made up of collagen fibrils in SHG images with an FOV of 240 μm × 240 μm in this study as SHG has imaging resolution to reveal collagen fibrils (about 5–24 collagen fibres would have been displayed in an SHG image).

The reason that tendon fascicles were suggested to be made up of collagen fibres comprising collagen fibrils by some scholars (Kastelic *et al.*, 1978; Smith *et al.*, 2013) could be due to the use of microscopic techniques (e.g. light microscopy and confocal microscopy) which do not possess sufficient imaging resolution for resolving individual collagen fibrils in a tendon. In fact, to our best knowledge, there is not a study that directly uses an image or a range of images to show that tendon fascicles are made up of collagen fibres, which are subcomposed of collagen fibrils. So far, most reports about the hierarchical collagen structure are based on linking information from different studies using different microscopy. Nevertheless, future studies will further examine whether all tendons contain collagen fibres or not.

Furthermore, the diameters of collagen fibrils measured in this study (Table 1) agree with the data reported by other studies (Kastelic *et al.*, 1978; Cox *et al.*, 2003, Williams *et al.*, 2005; Cisneros *et al.*, 2006). The comparable diameter range of the collagen fibrils with the wavelength of visible light (445 nm) used in this study played a key role in acquiring high-resolution SHG images of the collagen fibrils (Williams *et al.*, 2005; Chen *et al.*, 2012).

It has been confirmed in this study that the collagen fibrils composing the Achilles tendon fascicles are long filaments (Craig *et al.*, 1989; Birk *et al.*, 1997; Graham *et al.*, 2000; Provenzano & Vanderby, 2006). However, the fascicles composing Achilles tendons do not necessarily extend over the entire length of the tissue (hollow arrow in Figs. 3C, 4D and 9A), which is supported by the traditional histology shown in this study (Fig. 9C) and the literature (Benjamin *et al.*, 2008). Since the bone–cartilage surface to which a tendon connects is irregular, the forces applied to individual collagen fibrils or a group of collagen fibrils may be heterogeneous during physical activities (Arnoczky *et al.*, 2002; Screen *et al.*, 2005). The formation of the fascicles in tendons (particularly a thickest tendon) may serve as a buffer that allows individual collagen fibrils or groups of collagen fibrils to slide and adjust position easily to take or share the forces applied, which would reduce stress concentration in certain groups of collagen fibrils.

It is understood that a normal tendon also contains a small volume of types III, IV and VI collagen (Aparecida de Aro *et al.*, 2012). The thin collagen fibrils shown using the AFM (Fig. 5) may be minor collagen in the Achilles tendons as they also showed periodic bands. However, the SHG images show mainly the structure of type I collagen fibrils in tendons as type I collagen produces a large quantity of SHG signals sufficient for imaging, whilst types III and IV collagen generate little SHG signal for imaging (Cox *et al.*, 2003; Pena *et al.*, 2005; Chen *et al.*, 2012). Since the replacement processes of type I collagen by type III collagen represent changes of age and pathology of a tendon (Wu *et al.*, 2011), the imaging technique developed in this study may potentially be used as a technique for assessing early tendinopathy or evaluating tissue engineering techniques for restoring the collagen structure of a tendon without tissue staining.

Traditional histology is a well-accepted clinical and pathological assessment method for tendons. Meanwhile, mechani-

cal tests are also used for numerically evaluating the functional characteristics of tendons. Future studies using SHG imaging, AFM, traditional histology and mechanical testing of normal and pathological tendons may lead to better understanding of the structure and functional relationship of tendons.

Conclusions

This study developed a 3D imaging technique for examining the collagen matrix at a fibril level without tissue labelling and dehydrating. The study has also provided new knowledge about the 3D collagen structure of an Achilles tendon.

Computer imaging analysis techniques have been well acknowledged for their consistency and efficiency for measuring feature sizes and quantitatively identifying abnormal tissues (Chen *et al.*, 1989; Wu *et al.*, 2005; Duan *et al.*, 2009; Duan *et al.*, 2015, Duan *et al.*, 2013). Although studying the orientation and structure of the collagen fibrils provides a way for evaluating the physiology of a tendon, currently, this is mainly carried out by visual inspection and in 2D (Chen *et al.*, 2007; Wu *et al.*, 2011; Wu *et al.*, 2015). SHG microscopy offers high-resolution imaging of collagen fibrils in tendons for quantitatively assessing their orientation characteristics in 3D using computer imaging analysis methods. This study could potentially lead to development of an artificial-intelligence-based imaging analysis system for consistently quantifying the collagen structure and physiology of tendons.

SHG emissions contain components in the transmission and backscattered direction (Cox *et al.*, 2003). In this study, we only explored SHG using a transmission channel for the examination of the collagen structure in Achilles tendons. Since physiological changes of biological tissues such as tissue ageing and healing can alter SHG signals in the transmission and backscattering direction (Williams *et al.*, 2005; Abraham *et al.*, 2012), future studies using SHG imaging in the transmission and reflectance channel may lead to the development of a more advanced imaging technique for evaluating the physiology of tendons. Also, since it is feasible to integrate confocal and SHG imaging into one microscope (Cox *et al.*, 2003; Williams *et al.*, 2005; de Dood, 2006; Chen *et al.*, 2012), with the successful development of confocal arthroscopy for detecting early tendinopathy without biopsy (Wu *et al.*, 2015), future studies in this area could lead to development of an integrated confocal and SHG endoscope with an artificial-intelligence-based imaging analysis system for consistently assessing the collagen structure and subtle physiological changes of a tendon without tissue labelling and biopsy.

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References

Abraham, T., Kayra, D., McManus, B. & Scott, A. (2012) Quantitative assessment of forward and backward second harmonic three dimensional images of collagen type I matrix remodeling in a stimulated cellular environment. *J. Struct. Biol.* **180**, 17–25.

Aparecida de Aro, A., de Campos Vidal, B. & Pimentel, E.R. (2012) Biochemical and anisotropical properties of tendons. *Micron* **43**, 205–214.

Arnoczky, S.P., Lavagnino, M., Whallon, J.H. & Hoonjan, A. (2002) In situ cell nucleus deformation in tendons under tensile load; a morphological analysis using confocal laser microscopy. *J. Orthop. Res.* **20**, 29–35.

Benjamin, M., Kaiser, E. & Milz, S. (2008) Structure-function relationships in tendons: a review. *J. Anat.* **212**, 211–228.

Bigun, J., Bigun, T. & Nilsson, K. (2004) Recognition by symmetry derivatives and the generalized structure tensor. *IEEE Trans. Pattern Anal. Mach. Intell.* **26**, 1590–1605.

Birk, D.E., Zycband, E.I., Woodruff, S., Winkelmann, D.A. & Trelstad, R.L. (1997) Collagen fibrillogenesis in situ: fibril segments become long fibrils as the developing tendon matures. *Dev. Dyn.* **208**, 291–298.

Campagnola, P.J. & Loew, L.M. (2003) Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. *Nat. Biotechnol.* **21**, 1356–1360.

Chen, C.-C., Daponte, J.S. & Fox, M.D. (1989) Fractal feature analysis and classification in medical imaging. *IEEE Trans. Med. Imaging* **8**, 133–142.

Chen, J.M., Willers, C., Xu, J., Wang, A. & Zheng, M.H. (2007) Autologous tenocyte therapy using porcine-derived bioscaffolds for massive rotator cuff defect in rabbits. *Tissue Eng.* **13**(7), 1479–1491.

Chen, X., Nadiarynkh, O., Plotnikov, S. & Campagnola, P.J. (2012) Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. *Nat. Protoc.* **7**, 654–669.

Cisneros, D.A., Hung, C., Franz, C.M. & Muller, D.J. (2006) Observing growth steps of collagen self-assembly by time-lapse high-resolution atomic force microscopy. *J. Struct. Biol.* **154**, 232–245.

Cox, G., Kable, E., Jones, A., Fraser, I., Manconi, F. & Gorrell, M.D. (2003) 3-Dimensional imaging of collagen using second harmonic generation. *J. Struct. Biol.* **141**, 53–62.

Craig, A.S., Birtles, M.J., Conway, J.F. & Parry, D.A. (1989) An estimate of the mean length of collagen fibrils in rat tail-tendon as a function of age. *Connect. Tissue Res.* **19**, 51–62.

de Dood, M.J. (2006) Second-harmonic generation. *Huygens Laboratorium 909a*, http://users.uj.edu.pl/~ufdzierz/PracFot/SHG_Dood.pdf.

Doroski, D.M., Brink, K.S. & Temenoff, J.S. (2007) Techniques for biological characterization of tissue-engineered tendon and ligament. *Biomaterials* **28**, 187–202.

Duan, X., Wu, J. & Kirk, T. (2009) Identifying collagen bundles in the most superficial layer of normal articular cartilage. In *Proceedings of the IEEE International Conference on Computational Intelligence and Software Engineering, 2009 (CiSE 2009)*.

Duan, X., Wu, J., Swift, B. & Kirk, T.B. (2015) Texture analysis of the 3D collagen network and automatic classification of the physiology of

articular cartilage. *Comput. Methods Biomech. Biomed. Eng.* **18**, 931–943.

Duan, X., Wu, J.P. & Kirk, T.B. (2013) Rotated hough filtering for automatically distinguishing the collagen bundles in the most superficial layer of articular cartilage. *IEEE J. Biomed. Health Inf.* **17**, 922–927.

Fratzl, P., Misof, K., Zizak, I., Rapp, G., Amenitsch, H. & Bernstorff, S. (1998) Fibrillar structure and mechanical properties of collagen. *J. Struct. Biol.* **122**, 119–122.

Gelse, K., Von Der Mark, K. & Schneider, H. (2003) Cartilage regeneration by gene therapy. *Curr. Gene Ther.* **3**, 305–317.

Ghadially, F.N. (1983) *Fine Structure of Synovial Joints*. Butterworth & Co, London.

Graham, H.K., Holmes, D.F., Watson, R.B. & Kadler, K.E. (2000) Identification of collagen fibril fusion during vertebrate tendon morphogenesis. The process relies on unipolar fibrils and is regulated by collagen-proteoglycan interaction1. *J. Mol. Biol.* **295**, 891–902.

Harvey, A.K., Thompson, M.S., Cochlin, L.E., Raju, P.A., Cui, Z., Cornell, H.R., Hulley, P.A. & Brady, S.M. (2009) Functional imaging of tendon. *Ann. BMVA* **8**, 1–11.

He, B., Wu, J.P., Chen, H., Kirk, T. & Xu, J. (2013a) Elastin fibers display a versatile microfibril network in articular cartilage depending on the mechanical microenvironments. *J. Orthop. Res.* **31**, 1345–1453.

He, B., Wu, J.P., Chim, S.M., Xu, J. & Kirk, T.B. (2013b) Microstructural analysis of collagen and elastin fibres in the kangaroo articular cartilage reveals a structural divergence depending on its local mechanical environment. *Osteoarthritis Cartilage* **21**, 237–245.

He, B., Wu, J.P., Kirk, T.B., Carrino, J.A., Xiang, C. & Xu, J. (2014) High-resolution measurements of the multilayer ultra-structure of articular cartilage and their translational potential. *Arthritis Res. Ther.* **16**, 205.

Hess, G.W. (2009) Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot Ankle Spec.* **3**, 29–32.

Huang, T.-F., Perry, S. & Soslowsky, L. (2004) The effect of overuse activity on Achilles tendon in an animal model: a biomechanical study. *Ann. Biomed. Eng.* **32**, 336–341.

Jahne, B. (1997) *Practical Handbook on Image Processing for Scientific Applications*. CRC Press, New York.

Jähne, B. (1993) *Spatio-Temporal Image Processing: Theory and Scientific Applications*. Springer Science & Business Media, Berlin, Heidelberg, GmbH.

Kannus, P. (2000) Structure of the tendon connective tissue. *Scand. J. Med. Sci. Sports* **10**, 312–320.

Kastelic, J., Galeski, A. & Baer, E. (1978) The multicomposite structure of tendon. *Connect. Tissue Res.* **6**, 11–23.

Kuijpers, T., Van der Windt, D.A.W.M., Van der Heijde, G.J.M.G. & Bouter, L.M. (2004) Systematic review of prognostic cohort studies on shoulder disorders. *Pain* **109**, 420–431.

Kujala, U.M., Sarna, S. & Kaprio, J. (2005) Cumulative incidence of Achilles tendon rupture and tendinopathy in male former elite athletes. *Clin. J. Sport Med.* **15**, 133–135.

Liu, C.-F., Aschbacher-Smith, L., Barthelery, N.J., Dyment, N., Butler, D. & Wylie, C. (2011) What we should know before using tissue engineering techniques to repair injured tendons: a developmental biology perspective. *Tissue Eng. B Rev.* **17**, 165–176.

Nečas, D. & Klapetek, P. (2012) Gwyddion: an open-source software for SPM data analysis. *Open Phys.* **10**, 181–188.

Pawley, J. (1995) Fundamental limitation in confocal microscopy. *Handbook of Biological Confocal Microscopy* (ed. by J. Pawley), 2nd ed. Plenum Publishing Co, New York.

Pena, A.-M., Boulesteix, T., Dartigalongue, T. & Schanne-Klein, M.-C. (2005) Chiroptical effects in the second harmonic signal of collagens I and IV. *J. Am. Chem. Soc.* **127**, 10314–10322.

Prockop, D.J. & Fertala, A. (1998) The collagen fibril: the almost crystalline structure. *J. Struct. Biol.* **122**, 111–118.

Provenzano, P.P. & Vanderby, R. (2006) Collagen fibril morphology and organization: implications for force transmission in ligament and tendon. *Matrix Biol.* **25**, 71–84.

Rasband, W.S. (1997-2014) *ImageJ*. National Institutes of Healthy, Bethesda, MD, http://imagej.nih.gov/ij/.

Rezakhaniha, R., Agianniotis, A., Schrauwen, J.T.C., *et al.* (2012) Experimental investigation of collagen waviness and orientation in the arterial adventitia using confocal laser scanning microscopy. *Biomech. Model. Mechanobiol.* **11**, 461–473.

Schepsis, A.A., Jones, H. & Haas, A.L. (2002) Achilles tendon disorders in athletes. *Am. J. Sports Med.* **30**, 287–305.

Screen, H.R., Shelton, J.C., Chhaya, V.H., Kayser, M.V., Bader, D.L. & Lee, D.A. (2005) The influence of noncollagenous matrix components on the micromechanical environment of tendon fascicles. *Ann. Biomed. Eng.* **33**, 1090–1099.

Smith, D.W., Rubenson, J., Lloyd, D., Zheng, M., Fernandez, J., Besier, T., Xu, J. & Gardiner, B.S. (2013) A conceptual framework for computational models of Achilles tendon homeostasis. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **5**, 523–538.

Stecco, C., Cappellari, A., Macchi, V., Porzionato, A., Morra, A., Berizzi, A. & De Caro, R. (2014) The paratendineous tissues: an anatomical study of their role in the pathogenesis of tendinopathy. *Surg. Radiol. Anat.* **36**, 561–572.

Torchia, D., Batchelder, L., Fleming, W., Jelinskit, L., Sarkar, S. & Sullivan, C. (1982) Mobility and function in elastin and collagen. *Mobility Funct. Proteins Nucleic Acids, Cba Foundation Symposium* **93**, 98–115.

Williams, R.M., Zipfel, W.R. & Webb, W.W. (2005) Interpreting second-harmonic generation images of collagen I fibrils. *Biophys. J.* **88**, 1377–1386.

Wren, T.A., Lindsey, D.P., Beaupré, G.S. & Carter, D.R. (2003) Effects of creep and cyclic loading on the mechanical properties and failure of human Achilles tendons. *Ann. Biomed. Eng.* **31**, 710–717.

Wu, B., Chen, J., Rosa, T., Yu, Q., Wang, A., Xu, J. & Zheng, M. (2011) Cellular response and extracellular matrix breakdown in rotator cuff tendon rupture. *Arch. Orthop. Trauma Surg.* **131**, 405–411.

Wu, J.P., Kirk, T.B., Peng, Z., Miller, K. & Zheng, M.H. (2005) Utilization of two-dimensional fast Fourier transform and power spectral analysis for assessment of early degeneration of articular cartilage. *J. Musculoskeletal Res.* **9**, 1–13.

Wu, J.P., Walton, M., Wang, A., Anderson, P., Wang, T., Kirk, T. & Zheng, M. (2015) The development of confocal arthroscopy as optical histology for rotator cuff tendinopathy. *J. Microsc.* **259**, 269–275.