Department of Mechanical Engineering

A Three-Dimensional Study on the Microstructure of Achilles Tendons

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

Date: 04/09/2017

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Abstract

A human Achilles tendon connects and transfers the contractive force of the gastrocnemius and soleus muscles to the calcaneus bone, which enables normal activities of a foot. The Achilles tendon is the largest and strongest tendon in human body. Despite this, it is one of the tendons prone to injury, tendinopathy and tears, causing chronic pain and sufferings. The mechanical function of an Achilles tendon is derived from its microstructure and composition. Therefore, there have been intensive studies of the microstructure and composition of an Achilles tendon in the past decades. However, there are still contradictory views about the microstructure and composition of Achilles tendons. Using a rabbit model, this PhD project aims to develop a 3D imaging technique and conduct a systematic study of the spatial microstructure of healthy Achilles tendons. This study also seeks to develop computer imaging analysis techniques to quantitatively describe the morphological characteristics of the collagen, elastin and cells in Achilles tendon.

Confocal and second harmonic generation (SHG) microscopy have higher imaging resolution than conventional optical and polarised microscopy. These methods offer a 3D imaging capability for studying the internal microstructure of bulk biological tissues without tissues dehydration. The SHG is a specialised microscopy that allows imaging in 3D collagen structures of biological tissues without tissue staining and dehydrating. Using a multiphoton microscope integrated with confocal and SHG microscopy, in this study, we have developed a 3D imaging technique and examined systematically the 3D microstructure of collagen, elastin and cells of rabbit Achilles tendons from the mid-portion to enthesis as well as from the tendon proper to the paratendineous tissues. Quantitative imaging analysis methods have also been developed and applied to provide a numerical description of the microstructural characteristics of the collagen fibrils, elastic fibres and cells. Fast Fourier Transform (FFT) alignment analysis and coherency analysis have been found to be useful methods for numerically identifying the orientation features of the collagen fibrils and elastic fibres of tendons.

The morphology of cells and the microstructure of the collagen fibrils and elastic fibres have been found to change significantly from the mid-portion to enthesis. The collagen fibrils, elastic fibres and elongated tenocytes show a great concordance in the mid-portion of Achilles tendons (Chapter 3). The most strike findings are the discovery of the series connection between the elastic fibres and tenocytes, the prominent crimp of the collagen fibrils and elastic fibres, and the spirals within the fibril bundles in the mid-portion of Achilles tendons. From the mid-portion to the enthesis (Chapter 4), the 3D microstructure of collagen, elastin and cells shows a gradual transition to adapt to the functional requirements of the Achilles tendons. The layered structure of the enthesis fibrocartilage found in this study demonstrates how the mid-portion anchor to the bone and how the stress accumulated at the interface of the soft tendon and hard bone is dissipated. Particularly, two types of fibrocartilage cells, either containing a thin elastin membrane or thick elastin cloud, were found in the uncalcified fibrocartilage.

The paratendineous tissues in the mid-portion and region near enthesis of Achilles tendons also have a distinctive microstructure that reflects the requirements of the mechanical function (Chapter 5). In the mid-portion, the endotenon partitions the longitudinal fibril bundles into different levels of fascicles to facilitate interfascicular sliding and prevent the tendon from being a rigid tissue. The epitenon firmly wraps the fascicles together to provide the tendon with the structural and functional integrity. In comparison, the paratendineous tissues in the region near the enthesis show great microstructural alterations. The deep surface of the Achilles tendons near the enthesis is covered by sesamoid fibrocartilage with an undulating surface visible under a microscope. More specifically, the concave regions of the undulating surface are featured with a thin sesamoid fibrocartilage superficial layer and a thick transitional zone. In contrast, the convex regions are characterised by an elastin superficial surface covering on a thick sesamoid fibrocartilage. Underneath the sesamoid fibrocartilage, there is a dense endotenon meshwork partitioning the longitudinal fibrils into small fascicles.

In conclusion, the PhD project has developed a 3D imaging technique and studied the microstructure of the collagen fibrils, elastic fibres and cells in the mid-portion and enthesis of Achilles tendons. The study provides new information about the

microstructure of Achilles tendons for understanding the physiology and mechanical function of the tissue. The computer imaging analysis techniques have been proven to be effective tools for objectively and quantitatively identifying the microstructural characteristics of Achilles tendons and the future assessment and diagnosis of the physiological changes of tendon tissues.

Publications

Published Paper

1. **Pang, X.**, Wu, J.P., Allison, G.T., Xu, J., Rubenson, J., Zheng, M.H., Lloyd, D.G., Gardiner, B., Wang, A. and Kirk, T.B., 2017. Three dimensional microstructural network of elastin, collagen, and cells in Achilles tendons. Journal of Orthopaedic Research.

Manuscripts in Preparation

2. **Xin Pang**, Jian-ping Wu, Brett Kirk, Garry Allison, Jiake Xu, The 3D Microstructure of Achilles Tendons and the Enthesis.
3. **Xin Pang**, Jian-ping Wu, Brett Kirk, Garry Allison, Jiake Xu, The Study on the Microstructure of the Paratendineous Tissues of the Mid-Portion and Region near the Enthesis of Achilles Tendons.

Conference

4. **Pang, X.**, Wu, J.P., Kirk, B., Xu, J. and Allison, G., 2017. Application of Confocal and Second Harmonic Generation Microscopy to Study the 3D Network of Elastic Fibres, Collagen Fibrils and Cells in Achilles Tendons. *Bone Joint J*, *99*(SUPP 1), pp.43-43.

Abbreviations

3D	Three-dimensional
SHG	Second harmonic generation
GAG	Glycosaminoglycan
AFM	Atomic force microscopy
PG	Proteoglycan
MRI	Magnetic resonance imaging
US	Ultrasound
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
FFT	Fast Fourier transform
2D	Two-dimensional
SRB	Sulforhodamine B
AO	Acridine orange
PBS	Phosphate-buffered saline
NA	Numerical aperture
UCF	Uncalcified fibrocartilage
CF	Calcified fibrocartilage
H&E	Hematoxylin and eosin

AR	Aspect ratio
TM	Tidemark
CL	Cement line
MPM	Multiphoton microscope
ECM	Extracellular matrix

Contents

CHAPTER 1 Introduction

1.1 Background and motivation

As the largest and strongest tendon in human body, the Achilles tendon makes a significant contribution to body movements. The delicate microstructure and composition of Achilles tendons are responsible for their biological and mechanical functioning in the human body. Degeneration and dysfunction of Achilles tendons severely impact the quality of life among athletes as well as the general population (Schepsis et al., 2002). Research focusing on the microstructure and the mechanical characteristics of healthy and pathological Achilles tendons has progressed for decades. However, the actual microstructure of Achilles tendons remains unclear, and the pathology of Achilles tendinopathy is still not fully understood.

Improved knowledge of the human body largely relies on the development of technologies that examine the body from macro to micro scales. The rapid development of imaging techniques promises to acquire high resolution 3D images of biological tissues at the micron scale for a better representation of the true structure of tissues in the body. Three dimensional imaging techniques contribute greatly to the integration of the structural segments shown by 2-dimensional techniques into a spatial network. Biological and medical research benefits from these techniques. These techniques have facilitated the development of a 3D imaging technique to study the microstructure of Achilles tendons within the current study.

Tendons consist of fibrous tissue and is predominated by collagen (OBrien, 1997). The orientation of the fibrillar components largely reflects the functional properties and state of health of a tendon. Based on the observed characteristics of tendon tissues, it has been found in this study that confocal microscopy and Second harmonic generation (SHG) microscopy are effective imaging techniques for studying the spatial microstructure of Achilles tendons. Confocal microscopy and SHG microscopy are normally integrated within a multiphoton microscopic system. Both techniques can acquire Z-series optical section images, which can be reconstructed into 3D images. With the utilisation of fluorescent staining techniques, confocal microscopy is able to

acquire high resolution 3D images of the microstructure of many tissue components, such as cell nuclei, bone matrix and plasma membrane (Takaku et al, 2010; Zipfel et al, 2003). SHG microscopy is a unique tool for imaging in 3D the collagen structure of biological tissues without tissue staining and dehydrating as collagen can generate SHG signals inherently (Theodossiou et al, 2006). These techniques have been used separately in many microstructural studies on different tissues and led to the increase of our knowledge about the biological tissues at a micro level (Williams et al, 2001). These applications have stimulated the development of a new technique combining both confocal and SHG microscopic techniques to examine the microstructure of Achilles tendons in different regions subjected to different mechanical regimes. It will help to provide a more comprehensive understanding of the microstructure of Achilles tendons.

Elastin has been found to be widely distributed in the extracellular matrix of many tissues and it is of significant importance for the biological and mechanical function and structural integrity (Kielty et al, 2002). It also exists in tendon tissues although it accounts for only 1-2% of the dry weight when compared to collagen (Kannus, 2000). Subsequently, less attention has been paid to study the elastin in tendons. The structure of elastin in tendons still remains unclear. In order to build a comprehensive view of the spatial microstructure of Achilles tendons, this PhD project has paid particular attention to the examination of the 3D architecture of elastin, and the microstructural relationship of the elastin with the cells and collagen in Achilles tendons.

The high resolution images acquired by confocal and SHG microscopy contain more than only morphological information. The orientation of the fibrillar components in Achilles tendons can be analysed to provide quantitative information using image analysis methods for systematically and consistently measure the health statue of the tendons. Image analysis not only contributes to the digital characterisation of the microstructure of normal Achilles tendons, but also provides a potential objective method to assess the pathology of tendons. The current research has developed image analysis methods to quantitatively study the structural characteristics of Achilles tendons.

In summary, the current research has provided an in-depth 3D microstructural study using confocal and SHG microscopy. New techniques developed during this research are capable of visualizing the 3D microstructure in rabbit Achilles tendons and quantitatively assessing the relationships between its components. The serial studies in the thesis have provided a first step to introduce advanced imaging techniques and demonstrate quantitative research findings in healthy Achilles tendons. Information gathered in the current study has established a robust foundation for potential application in pathological Achilles tendon research and eventually for clinical examination.

1.2 Objectives and signification of research

The current research aims to study the 3D microstructure of healthy rabbit Achilles tendons using the confocal and SHG microscopic techniques. In addition, multiple regions (mid-portion and enthesis, tendon proper and the paratendinous tissues) in Achilles tendons were chosen to demonstrate the interactions between tissues and their mechanical environment. The specific objectives of this research are outlined below:

1. To study the 3D microstructure of the tendon proper and the paratendinous tissues in the mid-portion and the enthesis of healthy rabbit Achilles tendons.
2. To identify the forms of elastin present in Achilles tendons and image the 3D microstructure.
3. To study the relationship between the fibrillar components (collagen fibrils, elastic fibres) and the cells (tenocytes, fibrocartilage cells) within Achilles tendons.
4. To compare microstructural changes in regions with different mechanical environments, and analyse the possible mechanism of how the tissues in Achilles tendons adapt to their mechanical environment.

This research is significant in several areas. The results of the serial studies add to knowledge of the 3D microstructural network in different regions in Achilles tendons. The orientation of collagen, elastin and tenocytes in the mid-portion of Achilles tendons shows how the general tendon tissues bear tensile forces (chapter 3). The 3D microstructural study of Achilles tendon enthesis reveals how load-bearing tendons

anchor to bones and dissipate the stress accumulated at the interface of soft and hard tissues (chapter 4). The paratendinous tissues in the mid-portion of Achilles tendons add to knowledge of the hierarchical structure of tendon tissues, and the characteristics of the paratendinous tissues near the enthesis reveals how the tissues adapt to compression and shear forces (chapter 5). The structural connection between the cells and the extracellular matrix provides useful evidence to facilitate understanding of the bio-mechano-transduction mechanism within tendon tissues. The imaging techniques and the image analysis methods used in this research provide a promising strategy that could very possibly become the next generation methods for imaging and assessing tendon pathology in clinical practice.

1.3 Structure of thesis

The thesis consists of six chapters. One chapter reviewed the literature and three articles demonstrated the methods and results of the serial studies comprising the research which forms the basis of the thesis. The thesis structure is outlined below:

1. Chapter 1 introduces the background and motivation, objectives and significance, and structure of this research.
2. Chapter 2 provides an up-to-date literature review on the basic science of tendons, the anatomy of human Achilles tendons, the pathology of Achilles tendons, imaging techniques for tendon tissues and image analysis techniques.
3. Chapter 3 demonstrates the techniques of confocal and SHG microscopy to study the 3D orientation of the fibrillar components and the morphology of tenocytes in the mid-portion of healthy rabbit Achilles tendons.
4. Chapter 4 provides an in-depth study on the spatial microstructure of the enthesis in healthy rabbit Achilles tendons, and how the structures smoothly change from tendon tissue to fibrocartilaginous tissue and finally anchor to the bony attachment.
5. Chapter 5 investigates the 3D microstructure of paratendinous tissues in the mid-portion and near the enthesis which contains sesamoid fibrocartilage in healthy rabbit Achilles tendons. A discussion is also included regarding how the mechanical environment affects the structure of paratendinous tissues and how paratendinous tissues adapt to different mechanical environments.

6. Chapter 6 presents a general conclusion relating to the serial studies and discusses both the findings and limitations of this research, and recommendations for future work.

1.4 References

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CHAPTER 2 Literature review

2.1 The basic science of tendons

Tendons are dense connective tissues linking muscles and bones. They function to transmit force generated by muscles to bones in the musculoskeletal system. The dense fibrillar structure, which is dominated by collagen, endows tendons with the ability to sustain tremendous tensile forces. The tendons connect to muscles and bones through the myotendinous junction and the osteotendinous junction. The osteotendinous junction is also called the "insertional site" or "enthesis". Besides the tensile forces, tendons must withstand compressive and shear forces when they insert into bony attachments or wrap around some bony anatomical structures. The delicate hierarchical microstructure of tendons and their paratendinous tissues co-ordinate to provide tendons with unique mechanical properties to fulfil their functional roles.

2.1.1 The hierarchical structure of tendons

Tendons are constituted predominately of water and collagen. Collagen has been reported to account for 65-80% of the dry mass of the tendons (Kannus, 2000). It is the most abundant protein in tendons. Besides collagen, there is also a small proportion of elastin (approximately 2% of the dry mass) and a variety of ground substances such as proteoglycans and glycosaminoglycans (GAGs). The extracellular matrix is produced and maintained by the tenocytes and tenoblasts (Thorpe & Screen, 2016). Type I collagen is the most abundant collagen type in normal tendons (Kannus, 2000). The hierarchical microstructural organisation of tendons is based on the structure of the principal protein in tendon tissues, type I collagen. The collagen fibrils, the smallest functional unit in tendons, have a diameter from 20 nm to 500 nm (Christiansen et al, 2000; Dyer & Enna, 1976; Jozsa et al, 1984; Moeller et al, 1995). A collagen fibril also has its own hierarchical organisation (Provenzano & Vanderby, 2006), as schematically shown in **Fig. 2.1**.

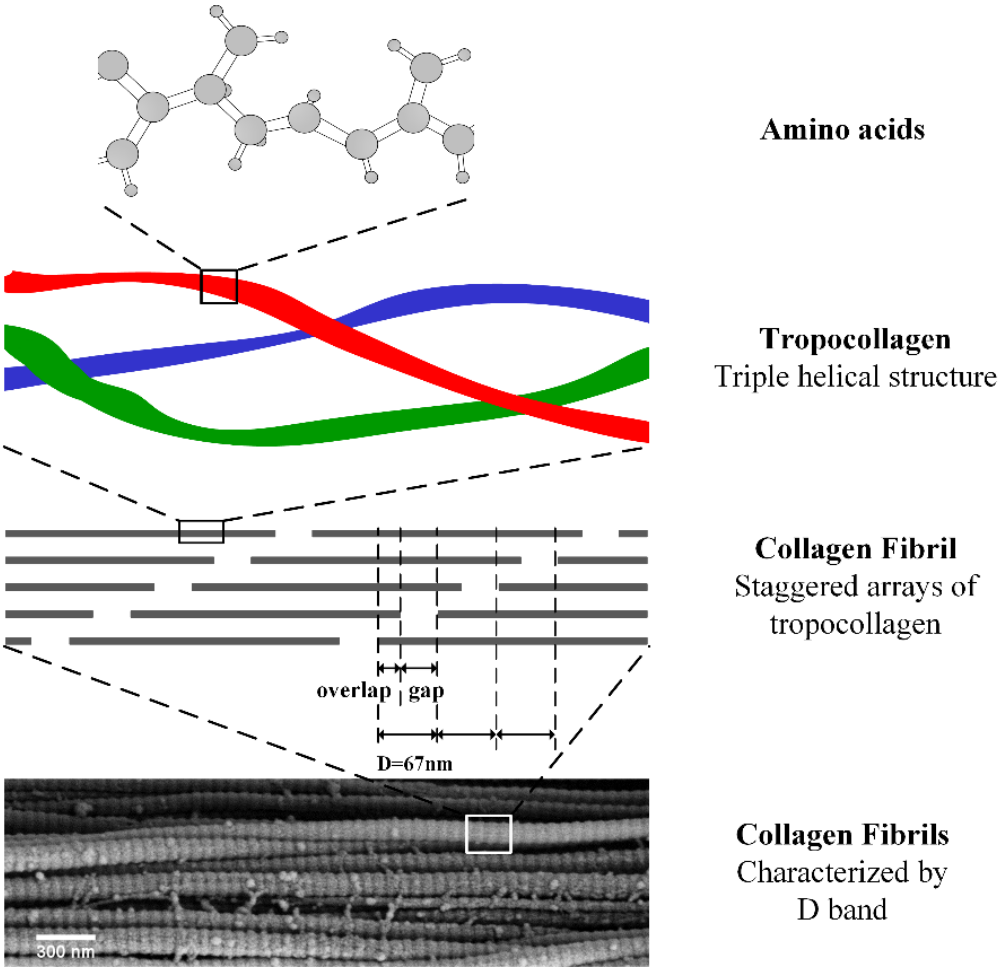

Fig. 2.1 A schematic illustration of the hierarchy of the structure of a collagen fibril. Collagen fibrils are formed by large numbers of tropocollagen molecules. The tropocollagen molecule is formed by three polypeptide chains, which are comprised of an amino acid sequence (Fratzl, 2008).

Collagen fibrils can be characterised by D-period bands of approximately 67 nm spacing under electron microscopes (Kadler et al, 1996). A fibril is formed by staggered arrays of tropocollagen molecules. A tropocollagen molecule has a length of approximately 300 nm and a diameter of 1.5 nm (Kadler et al, 1996). The axial periodic array of the tropocollagen molecules explains the formation of the D-period banding. A tropocollagen molecule is formed by triple helical polypeptide chains. A polypeptide chain is comprised of amino acid sequences (Kadler et al, 1996). It has been suggested that tendon extension involves the molecular stretching and slippage (Pins et al, 1997), while molecular slippage may result in an increase in D-period beyond 2% (Folkhard et al, 1987; Sasaki & Odajima, 1996).

The nomenclature used to describe the hierarchy of a tendon differs amongst scholars. This may result from the complicated structure of tendons and variations of the

hierarchy within different tendons to some extent. The widely accepted hierarchical structure of a tendon tissue was proposed by Kannus (Kannus, 2000), as shown in **Fig. 2.2**. A bunch of collagen fibrils form a collagen fibre. A bunch of collagen fibres are enveloped by a fine sheath of connective tissue, which is called the endotenon, to form a primary fibre bundle named a sub-fascicle. A group of sub-fascicles form as secondary fibre bundles named fascicles. A group of fascicles forms a tertiary bundle. A tendon is composed of several tertiary bundles. A tendon is normally wrapped by the epitenon as a functional unit. Outside the epitenon is the paratenon, which is the sleeve of a tendon and has been reported to connect with the epitenon via fibre bundles (Nisbet, 1960). Some large tendons in the human body, such as Achilles tendons, are formed by several tendons, and the hierarchical structure could display local variations (Handsfield et al, 2017; Pekala et al, 2017).

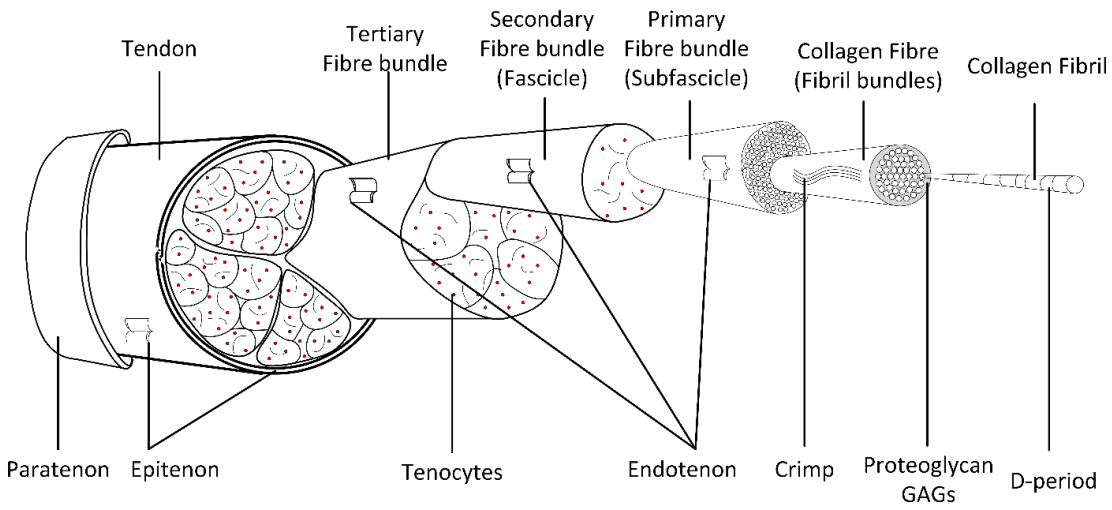

Fig. 2.2 A Schematic view of the hierarchical structure of a tendon, which is adapted from Jozsa (Józsa & Kannus, 1997).

Many contradictory findings regarding the existence of collagen fibres in tendons have been published. When discussing the hierarchical structure of tendons, any ambiguity must be avoided by using clear terminology to clearly distinguish fibres from other features such as fascicles. Some authors have suggested that a collagen fibre is a bunch of closely packed collagen fibrils (Ushiki, 2002). Jozsa and Kunnus (Józsa & Kannus, 1997) have defined the collagen fibres as a bunch of collagen fibrils, which are surrounded by proteoglycans and glycosaminoglycans (GAGs). In comparison, a fascicle is a group of collagen fibrils surrounded by the endotenon. The definition of

fascicles is consistent and well accepted in the literature. Due to the advancements in microscopic technology, collagen fibrils can be clearly shown in microstructural observations (Pang et al, 2016), and their mechanical properties can be tested individually (Miyazaki & Hayashi, 1999). Researchers have subsequently started focussing more attention to the smallest microscopic visible unit, the fibrils. Using high resolution second harmonic generation microscopy (SHG) and atomic force microscopy (AFM), a group of scholars have paid particular attention to the distinction of the collagen fibrils and fibres in bulk Achilles tendons without tissue dehydration and labelling (WU et al, 2017). This study suggested that a fascicle is composed of a group of collagen fibrils with a diameter of about 0.5μm. Collagen fibres were not reported in tendon tissues but they are made of collagen fibrils. Indeed, so far, no studies within the literature have directly shown images of a collagen fibre comprising a group of collagen fibrils. It appears that the suggestion of the existence of collagen fibres within tendons was mainly based on the use of optical microscopy for the observation of tendon microstructure in early studies. Optical microscopy does not have sufficient image resolution to distinguish collagen fibrils. Some features of tendon tissues are also embodied in the aggregation of collagen fibrils, for example, the crimps of the fibril bundles, and the spatial spiral of collagen fibril bundles (Kannus, 2000; Smith et al, 2013).

The size of tendons varies greatly, as does the size of fascicles. Different studies on the size of the fascicles within different tendons have been conducted (Józsa & Kannus, 1997). The data demonstrates that the size of fascicles is highly concordant with the size of the tendon, which means a large tendon is normally composed of larger diameter fascicles. Also, the hierarchical fibrillar system comprising sub-fascicles, fascicles and tertiary fibre bundles varies from tendon to tendon (Józsa & Kannus, 1997; Smith et al, 2013). Different levels of fascicles in a tendon are surrounded by the endotenon, which largely prevents the tendon from being a rigid tissue (Blasi et al, 2014; Franchi et al, 2007b). The epitenon of a tendon is a dense fibrous tissue that serves to improve the integrity of the tendon (Jozsa et al, 1991). However, some big tendons (like the Achilles tendon) are formed by several sub-tendons that are wrapped by loose connective tissues and can be easily separated from each other (Ballal et al, 2014; Szaro et al, 2009). These sub-tendons have their own names and muscle origins, and are much bigger than some small tendons.

2.1.2 The paratendinous tissues of tendons

Paratenon, epitenon and endotenon in a tendon are called the paratendinous tissues, which are the fundamental structures other than the longitudinal fascicles of most tendons. These tissues contribute to the hierarchical structure of tendons. Endotenon inserts into the longitudinal fibre bundles and groups the fibre bundles into fascicles. Interfascicular sliding is very important for force transmission and deformation of tendons (Fallon et al, 2002). Endotenon is a thin layer of a loose connective tissue between fascicles to facilitate interfascicular sliding. Endotenon is derived from the epitenon, which lies on the surface of the tendon proper. Epitenon of a tendon is formed by dense fibrillar components dominated by collagen fibrils, and it firmly adheres to the tendon proper to promote the integrity of the tendons (Stecco et al, 2014). Paratenon is a loose woven tissue that surrounds the whole tendon outside of the epitenon (Stecco et al, 2014; Strocchi et al, 1985). Some scholars exclude the paratenon from the structure of the tendon proper because of the loose connection between the paratenon and the tendon proper (Nisbet, 1960). Indeed, the main functions of paratenon are to facilitate sliding of the whole tendon and reduce friction between the tendon and its surrounding structures (Stecco et al, 2014). The paratenon of a tendon is well vascularized and innervated, and is the main source of nutrition for a tendon (Jozsa et al, 1991; Józsa & Kannus, 1997).

The five structure categories described by Ippolito and Postacchini do not appear in every tendon (Perugia et al, 1986). (1). Fibrous sheaths, which surround tendons to facilitate their gliding; (2) Reflection pulleys, which help to keep tendons to stay inside their sliding bed; (3) Synovial sheaths, which normally form a closed duct to facilitate tendons to slide at the bone surfaces; (4) Peritendinous sheet or paratenon, which functions as an elastic sleeve to enable tendon gliding and reduce the friction between the tendon and surrounding structures; (5) Tendon bursae, which functions to reduce friction at the sites where there are bony prominence that might compress or wear the tendons.

2.1.3 The osteotendinous junction of tendons

The osteotendinous junction of tendons is also known as “enthesis” or “insertional site”. It is the interface between soft tissues (the tendons) and hard tissues (the attached bones). This region sustains local peaks in tension because stresses concentrate at the interface between two materials with different mechanical properties (Benjamin et al, 2002). Tendons connect with their bony attachments through two kinds of entheses: the fibrous enthesis and the fibrocartilaginous enthesis (Benjamin et al, 2006; Doschak & Zernicke, 2005; Shaw & Benjamin, 2007). The fibrous entheses normally exist at the attachments of tendons to the diaphysis or metaphysis of the long bone. The fibrocartilaginous entheses are more commonly seen at the attachments of tendons to the epiphyses and apophyses of long bones. The fibrous entheses are subdivided into two types: the periosteal fibrous enthesis and the bony fibrous enthesis (Shaw & Benjamin, 2007). In the periosteal fibrous enthesis, the tendon connects to the periosteum and then indirectly connects to the bone (Benjamin et al, 2002). Whilst in the bony fibrous enthesis, the tendon directly connects to the bone.

Fibrocartilaginous entheses of a tendon are characterized by the formation of fibrocartilage tissue that connects the tendon proper to the bone. As shown in **Fig. 2.3**, a fibrocartilaginous enthesis of a tendon is often constituted of the tendon proper, the uncalcified fibrocartilage, the calcified fibrocartilage and the bone (Benjamin & Ralphs, 2001; Cooper & Misol, 1970). The layered structure helps to dissipate the stress accumulated at the interface of soft and hard tissue (Thomopoulos et al, 2003). There is a legible basophilic line observed in histological observations that separates the calcified and uncalcified fibrocartilage, which is termed the “tidemark”. It is a relatively straight line when compared with the “cement line” lying between the calcified fibrocartilage and the bone. The interdigital cement line is a convoluted interface between the calcified fibrocartilage and the bone (Clark & Stechschulte, 1998). The tendon proper and the uncalcified fibrocartilage show a gradual transition of the structure without an obvious boundary. The crimps in the tendon proper gradually disappear as the tissue gradually transforms into uncalcified fibrocartilage. The fibre bundles in the uncalcified fibrocartilage are parallel to each other and aligned in a direction perpendicular to the tidemark. There are rows of round fibrocartilage cells lying in the lacunae between the fibre bundles. The fibrocartilage cell rows are

parallel to the fibre bundles. The fibrocartilage cells are sparsely distributed in the calcified fibrocartilage. Near the tidemark, some of them align in a row that is continuous with the fibrocartilage cell row in the uncalcified fibrocartilage. The bone tissue distinctively consists of marrow cavities. The osteocytes are stellate that lie in the lacunae.

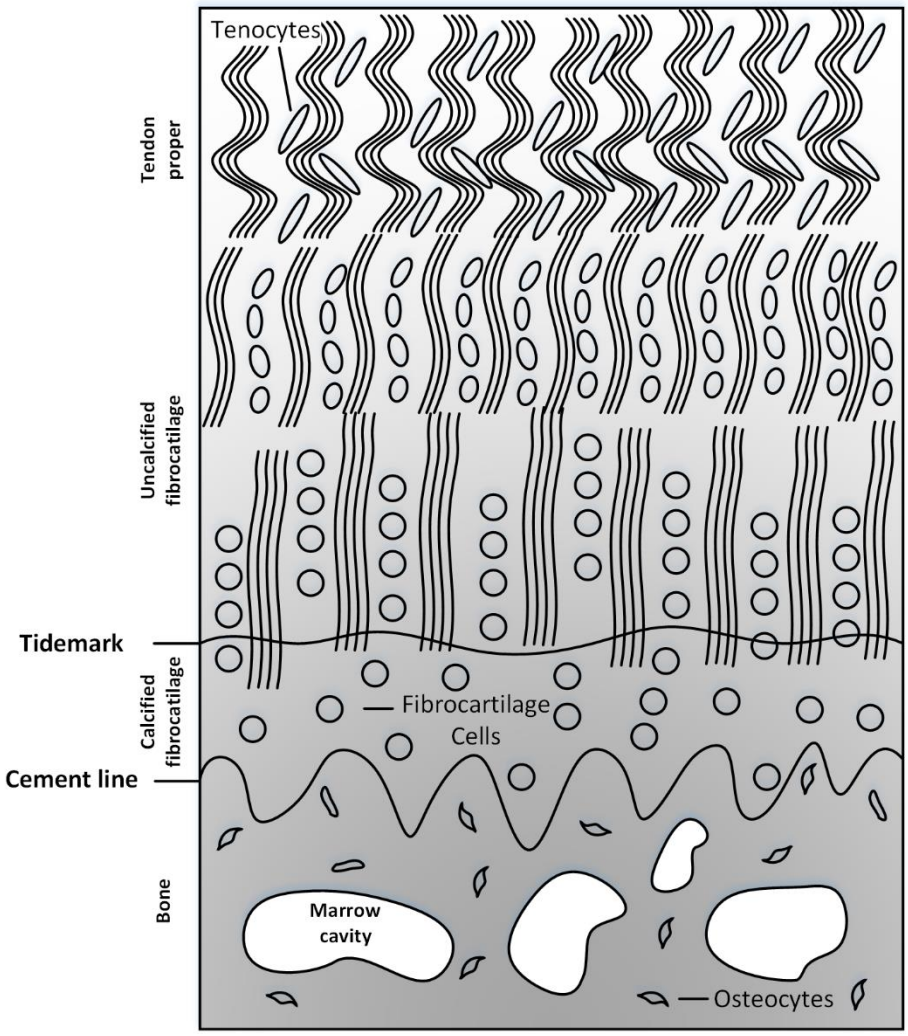

Fig. 2.3 The layered structure of the tendon fibrocartilaginous enthesis comprises the tendon proper, the uncalcified fibrocartilage, the calcified fibrocartilage and the bone (Deymier-Black et al, 2015).

2.1.4 The myotendinous junction of tendons

The myotendinous junction of a tendon is a region where tendons attach to the muscles. Muscles generate contractive forces, which are transferred to the bone through the tendon to enable movements. A robust attachment between muscles and tendons

ensures an efficient force transmission. The muscle-tendon attachment is achieved by connection of tendon fibrils to finger-like processes of muscle cells (**Fig. 2.4**). The finger-like processes of muscle cells significantly increase the contact surface between the muscle cells and the tendon fibrils. There are two types of muscle fibres. Type I fibres are responsible for fast voluntary movements while type II fibres are responsible for the slower movements and supporting body posture. A previous study (Kannus et al, 1992) has reported that the contact surface of type II muscle fibres with the tendon fibrils is 30% to 40% larger than that of type I muscle fibres. Type II fibres increase the contact surface by forming smaller subunits on the muscle cell processes. The muscle cell processes create tunnel-like perforations that permit the tendon collagen fibrils to penetrate within them and insert into the basement membrane of the muscle cells. The collagen at this region are primarily thin fibrils with diameters of 10 to 20 nm (Kannus et al, 1992). It has been reported that not all of the collagen fibrils in the region align parallel to the long axis of the tendons (Kannus et al, 1992). The basal lamina at the myotendinous region is approximately 3 times thicker than that in other regions. In addition, the basal lamina in type I muscle fibres is also thicker than in the type II muscle fibres (Kvist et al, 1991).

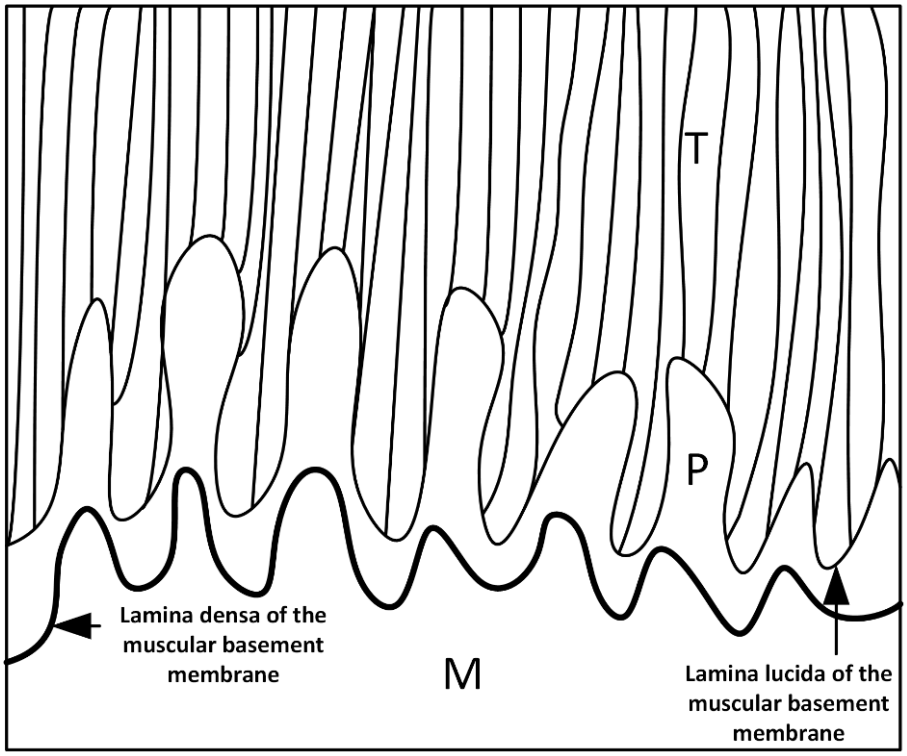

Fig. 2.4 The structure of the myotendinous junction of tendons. T: tendon. P: Muscle cell precesses. M: Muscle cell (Józsa & Kannus, 1997).

Compared to the osteotendinous junction, the interface of the myotendinous junction involves two soft tissues. It does not sustain a similar stress concentration to that of the osteotendinous junction where two materials with different mechanical properties meet. However, the myotendinous junction is still a fragile region in the muscle-tendon system. The contact surface of the myotendinous junction changes with the condition of the muscles (Curzi et al, 2016). The contact surface decreases when the muscle atrophies and it increases with exercise and training (Curzi et al, 2013). It has been shown that the health of the myotendinous junction is highly associated with the state of health of the muscles.

2.1.5 The components of tendons

Tendons consist of a dense fibrillar extracellular matrix and ground substance, which are produced and maintained by cells such as tenocytes, tenoblasts, fibrocartilage cells and the cells in the paratendinous tissues. The dense fibrillar extracellular matrix is constituted predominately by collagen, a small amount of elastin, ground substance

such as proteoglycans, glycan, and decorin. The composition and concentration of the proteins may also vary at different regions in a tendon (Thorpe & Screen, 2016).

Cells in tendons

Tenocytes and tenoblasts are the predominant cells within tendons. They account for 90%-95% of all cell types found in tendons (Józsa & Kannus, 1997). Aside from this, there are fibrocartilage cells at insertional sites, and some other cells, such as the synovial cells in the tendon sheaths, the vascular endothelial cells in the paratendinous tissues.

Tenocytes and tenoblasts are characteristic cells that are widely observed in the dense fibrillar extracellular matrix of tendons. The tenoblasts have diverse shapes and sizes in newborns. Tenocytes are transformed from the tenoblasts to be the resident cells in the mature tendons (Ippolito et al, 1980). Tenocytes are elongated cells and have a high nucleus-to-cytoplasm ratio. They have long and thin cellular processes extending into the fibrillar bundles to facilitate the communication with the fibrillar bundles (Ralphs et al, 1998). It is the tenocytes that mainly manage the secretion of the extracellular matrix as they can perceive the change of the mechanical environment and response to different mechanical loads, which allows the adaption of tendon tissues to different mechanical environments (Benjamin et al, 2008). Studies (Grant et al, 2013; Pang et al, 2016) have shown that the elastin has close connection with the tenocytes. This structural relationship between the tenocytes and elastin may imply that the elastin fibres participate in mechanotransduction within tendon tissues.

Fibrocartilage cells are found in the fibrocartilage that mediates tendons to adapt to compressive loads, for example the entheses. They are very important in maintaining the homeostasis of fibrocartilage tissues. The fibrocartilage cells are round or oval. They are often organised in rows. Each fibrocartilage cell lies in a lacunae and is isolated from the surrounding matrix (Benjamin & Ralphs, 2004). The lacunae of a fibrocartilage cell is formed by a thick pericellular matrix, helping to minimize cell deformation when the fibrocartilage is under compression (Martin et al, 2003).

It is worthy of mention that tendon progenitor cells demonstrate characteristics and capabilities of stem cells for reproducing tendon like, cartilage like and bone like tissues (Bi et al, 2007). They are cells that could be potentially investigated and used to generate tendon-like tissues for repairing deteriorated tendon, cartilage and bone (Zhang & Wang, 2010).

Collagen in tendons

Collagen accounts for 60%-85% of the dry weight of tendons (Kastelic et al, 1978). Type I collagen is the most abundant collagen type in normal tendons, comprising up to 95% of all collagen in the tissue. Tendons also contain a small amount of types III, V, XII and XIV collagen (Thorpe et al, 2013). These different types of collagen participate in forming the fibrillar structure and bearing tensile forces within tendons. Type III collagen is closely associated with the tendon rupture and healing process (Eriksen et al, 2002). Since type III collagen is weaker than the type I collagen, accumulation of type III collagen at the rupture site of a tendon is believed to weaken the mechanical properties of tendons. In comparison, the fibrocartilage found at the entheses or wrap around regions of a tendon contain mainly type II (Benjamin & Evans, 1990; Benjamin & Ralphs, 1998). The type II collagen are the predominant collagen type in cartilage that provide the cartilage with its tensile and compressive properties by forming a three dimensional collagen network that constrains the swelling of proteoglycans (Gelse et al, 2003). Thus, type II collagen presented in fibrocartilage tissues is crucial for resisting compression. Besides type II collagen, fibrocartilage also contains type IX, type X and type XII collagen (Thomopoulos et al, 2003).

Elastin in tendons

Elastic fibres have an elastin core with surrounding fibrillin-rich microfibrils as observed by transmission electron microscopy (Kielty et al, 2002). Even though elastic fibres widely distribute in the extracellular matrix of many tissues, tendons only consist of a small proportion of elastic fibres at approximately 1-2% of the dry weight of tendons (Kannus, 2000). There have been a very few early studies (Cooper & Misol, 1970) that have reported the existence of elastic fibres in the tendon-bone insertion site. Due to its low volume in tendons, the architecture of elastic fibres in tendon tissues has not been well studied. The elastic fibres functionally endow tissues with

elasticity and resilience. However, the functions of elastic fibres in tendon tissue are not fully understood. Recent studies have demonstrated that elastic fibres are broadly distributed in tendons and they have an intimate connection with tenocytes (Grant et al, 2013; Pang et al, 2016). The close relationship between tenocytes and elastic fibres has been suggested to be a possible way that the tenocytes communicate with the extracellular matrix (Grant et al, 2013; Pang et al, 2016). Studies on the functions of elastic fibres using elastase treatment on tendons and ligaments have shown that elastic fibres are associated with stabilizing collagen crimps and maintaining the integrity of tendon tissues (Fang & Lake, 2016; Grant et al, 2015; Henninger et al, 2013). Elastic fibres have also been observed in the interfascicular space and the sheath system of tendons and ligaments (Caldini et al, 1990; Smith et al, 2011). The functions of the elastic fibres in these regions have been suggested to facilitate the recoil of interfascicular sliding and protect blood vessels and nerves from extreme stretching and compression. Although many theories have been developed to explain the possible functions of elastic fibres, the function of elastic fibres is still not yet fully understood.

The ground substance in tendons

Besides the cells, collagen and elastin, a tendon tissue also consists of essential ground substances, such as proteoglycans (PGs), glycosaminoglycans (GAGs) and decorin that are vital for the mechanical and biological functioning of the tendon (Screen et al, 2015). PGs and GAGs have been found widely distributed within and between fibrous components although the distribution of them varies in regions. The PGs have a bottle brush-like structure containing a protein core that has one or more GAG chains attached covalently. Under electron microscopy, PGs appear as ‘bridges’ to bind the collagen fibrils in a tendon (Cribb & Scott, 1995; Scott, 1980; Scott & Thomlinson, 1998). PGs have active interactions with collagen fibrils and participate in transmitting forces from one collagen fibril to another (Cribb & Scott, 1995). Both the PGs and GAGs in a tendon have a considerable water-binding capacity, which can attract and retain water up to 50 times their own weight (Karpakka et al, 1991). In addition to the ability to bind water molecules, they are highly negative charged macro molecules that generate repulsion forces, which may aid a tendon to resist compression and tension (Kannus, 2000). PGs are also play an important role in spacing and lubricating tendon tissues (Kjaer, 2004).

2.2 The anatomy of human Achilles tendons

The Achilles tendon is the largest and strongest tendon in human body. It locates superficially and can be easily palpated along almost its whole length. The anatomical structure of Achilles tendons in the human body varies. The main body of Achilles tendon is composed of the tendons originating from the medial and lateral heads of the gastrocnemius and soleus muscles. Approximately 65% of the population has been found to possess the tendon of the plantaris muscle in the Achilles tendon (Ballal et al, 2014). The fascicles run parallel with each other at the beginning of the myotendinoust junction and rotate distally to form a macroscopic spiral before they finally insert into the tuber of the calcaneus (Ballal et al, 2014; Doral et al, 2010; Szaro et al, 2009). The cross-section of an Achilles tendon is flat at its origin, and it gradually becomes more rounded in the mid-portion (Apaydin et al, 2009). As the tendon tissue approaches the calcaneus, the rounded cross-section flares out to create a larger area for attachment to the calcaneus (Shaw & Benjamin, 2007). A study has shown that the Achilles tendon inserts into the calcaneus in a crescent shape (Lohrer et al, 2008). This crescent shape is believed to be important in controlling the movement of inversion and eversion of the heels.

The Achilles tendon inserts into the tuber of the calcaneus, where forms the enthesis. The stress accumulates at the interface of Achilles tendon and the calcaneus due to the different mechanical properties of soft tissue and hard tissue. The Achilles tendon enthesis functions as a typical fibrocartilaginous enthesis to anchor the soft fibrous tissue to the bone and to dissipate the stress (Thomopoulos et al, 2003). There are several surrounding structures that work together to reduce wear and tear at the insertional site and the adjacent regions of an Achilles tendon, which are termed as the ‘enthesis organ’ (Benjamin & McGonagle, 2009; Benjamin et al, 2004; Benjamin et al, 2005). These structures include enthesis fibrocartilage, sesamoid fibrocartilage on the deep surface of Achilles tendon, periosteal fibrocartilage on the posterior surface of the tuber of calcaneus opposite to the sesamoid fibrocartilage, the retrocalcaneal bursa and the tip of Kager’s fat pad. This concept of the enthesis organ helps to explain why some patients have symptoms within regions adjacent to the Achilles tendon enthesis.

The Achilles tendon proper is avascular tissue but the paratendinous tissues convey capillaries that penetrate into the tendon proper within the endotenon. The blood supply in an Achilles tendon comes from the myotendinous junction, the osteotendinous junction and the surrounding paratendinous tissues (Ahmed et al, 1998; Carr & Norris, 1989; Schmidtrohlfing et al, 1992). The blood vessel branching into the paratendinous tissues are primarily from the posterior tibial artery. The nerve systems of an Achilles tendon are mainly from the sural nerve but the tibial nerve has also been suggested to contribute a small fraction of the nerves in an Achilles tendon (Obrien, 1992; Stilwell, 1957).

2.3 The pathology of Achilles tendons

2.3.1 The terminology of Achilles tendon disorder

The Achilles tendon is one of the most easily injured tendons in human body. The causes of Achilles tendon pathology can be injuries, diseases and some other forms of disorder (Józsa & Kannus, 1997). Achilles tendon problems are commonly seen in both athletes and the general population. The terminologies used to describe Achilles tendon conditions are inconsistent and confusing (van Dijk et al, 2011). Achilles tendinopathy is proposed by Maffulli et al (Maffulli et al, 1998) to describe the pathological condition of Achilles tendon that has clinical symptoms including pain, swelling and impaired movement (van Dijk et al, 2011). Achilles tendinopathy can be classified as insertional and non-insertional tendinopathy based on the anatomical location. Achilles tendon injuries include partial or complete rupture.

2.3.2 Insertional Achilles tendinopathy

Insertional Achilles tendinopathy accounts for 20%-25% of all Achilles tendon disorders (Kvist, 1991). It normally occurs in overused Achilles tendons subjected to repetitive loads that cause microtears accumulated over time. Insertional Achilles tendinopathy is commonly found in runners and ballet dancers (Nunley, 2008). The pathological characteristics of insertional Achilles tendinopathy are often shown as ossification of the fibrocartilage tissue, formation of a bone spur at the enthesis and tearing of the tendon proper at the enthesis (Irwin, 2010). Posterior heel pain is the

main complaint resulting from insertional Achilles tendinopathy, and patients normally have a palpable bone spur at the tendon insertional area (van Dijk et al, 2011).

2.3.3 Non-insertional Achilles tendinopathy

Non-insertional Achilles tendinopathy accounts for more than half of Achilles tendon problems (Paavola et al, 2002). The cause is multifactorial but it is similar to insertional Achilles tendinopathy in that excessive loads over the physical limit and repetitive usage are the main stimulus for non-insertional Achilles tendinopathy (Kvist, 1991; Pearce & Tan, 2016). However, there are also some tendinopathy patients who are not involved in excessive physical activities (Rolf & Movin, 1997). Non-insertional Achilles tendinopathy often involves lesions in different regions, which include the mid-portion, the paratendinous tissues and the retrocalcaneal bursa. The tendon proper in the mid-portion is the main region to suffer pathological tendon degeneration (van Dijk et al, 2011). The main pathological structural alterations affect the collagen, tenocytes and the neovascularization (Astrom et al, 1996; Khan et al, 1999; Śmigielski & Zdanowicz, 2016). The degeneration of collagen can be seen as collagen bundles experience disorganization, microtears and collagen bundle separation. Birefringence is an important optical property of collagen. The birefringence of the collagen in pathological tendons becomes weaker under polarised light microscopy. Tenocytes with round nuclei increase in number, and round tenocytes can form clusters within some regions. Neovascularization is mainly in the paratenon, and this can be viewed as a body response to microtrauma within the avascular tendon proper (Alfredson et al, 2003). The neovessels are normally accompanied by nerves, which possibly directly cause pain to affect daily activities (Śmigielski & Zdanowicz, 2016). Furthermore, inflammation of the paratendinous tissues and the retrocalcaneal bursa are also often found in pathological tendons. The swelling of the tendon tissue is normally observed 2-7 cm from the insertional site (Nunley, 2008).

2.3.4 Achilles tendon rupture

The Achilles tendon is one of the most frequently ruptured tendons (Jozsa et al, 1989; Lipscomb & Weiner, 1956). The rupture is highly associated with sport activities such

as ball games that require repetitive abrupt jumping and sprinting (Jozsa et al, 1989; Leppilahti et al, 1996). People aged from 30-45 years who are leisure athletes or have sedentary occupations are prone to have acute Achilles tendon ruptures (Jozsa et al, 1989). The rupture normally happens within the region 2 to 6 cm proximal to the Achilles tendon enthesis as this region has the smallest cross-sectional area (Hess, 2010). Since the Achilles tendon has multiple muscle origins and the structure is not uniform, when an asymmetrical load is applied to the Achilles tendon, a partial rupture may happen to the most loaded fascicles (Śmigielski & Zdanowicz, 2016). Due to the poor self-repair capability of tendons, a rupture often leads to on site hypoxic degeneration, mucoid degeneration, tendolipomatosis and calcification in the Achilles tendon. These degenerative changes to Achilles tendons indicate that the rupture of an Achilles tendon can lead to tendon degeneration (Fox et al, 1975; Jozsa et al, 1989).

2.4 Imaging techniques for tendon tissue

2.4.1 Clinical imaging techniques

Imaging techniques play an important role in clinical diagnosis and monitoring the health of a tendon. Magnetic resonance imaging (MRI) and ultrasound (US) are non-invasive imaging techniques which are widely used in current clinical practice for pathological assessment of a tendon (Del Buono et al, 2013). Based on the MRI and US techniques, novel clinically available imaging modalities have emerged to provide more efficient and sensitive structural information, such as power Doppler, sonoelastography, ultrashort echo time MRI, dynamic contrast-enhanced MRI and T2 mapping (Hodgson et al, 2012; Weinreb et al, 2014). Lateral radiographs and multidetector computed tomography (CT) are also available for imaging the structure of tendons and their surrounding tissues (Harris & Peduto, 2006). Video arthroscopy, as a minimally invasive imaging technique which can provide real-time images of a tissue, has been regarded as the ‘gold’ standard clinical imaging technique to locate and measure the size of an actual lesion in a tendon, and guide tendon surgery (Teefey et al, 2004).

MRI shows normal Achilles tendon to have homogeneous low signal intensity on all imaging sequences, whereas the surrounding structures are shown as a distinct layer

with different intensities. When the Achilles tendon undergoes inflammation, degeneration and tears, these pathological alterations will appear as thickening, foci of mildly increased intensity and the discontinuity in tendon tissue (Haims et al, 2000; Movin et al, 1998; Reinig et al, 1985; Schweitzer & Karasick, 2000).

A normal Achilles tendon is shown in US as tightly packed thin echogenic bright lines, and the paratenon is shown as a slightly more echogenic border. Pathology of a tendon such as thickening of a tendon or the paratenon can be clearly shown in US images. A degenerated tendon is shown as hyperechoic signals (Nadeau et al, 2016). A power Doppler is able to show the vascularity of tissues. Therefore, it is helpful to identify the neovascularization in pathological Achilles tendons (Richards et al, 2001; Zanetti et al, 2003). CT imaging and radiographs do not offer as high quality contrast resolution for soft tissues as the MRI and US do, but the shape of a tendon can be bordered by the adjacent structures. CT and radiographs are useful imaging modalities for accurately detecting abnormities of the bony attachments of tendons and the subtle calcification in tendon tissues (Harris & Peduto, 2006).

MRI and US have similar accuracy in the detection of Achilles tendon disorders (Khan et al, 2003) but they also have their own advantages and disadvantages. US can provide clear images of the internal structure of a tendon and it is also easy to get images from different angles or under stress (Hodgson et al, 2012). Since a power Doppler is able to detect the neovascularization, it is very useful in the diagnosis of tendinopathy (Richards et al, 2001; Zanetti et al, 2003). However, US is unable to assess deep-lying tendons and image the adjacent bony structures. MRI possesses a high spatial resolution and is able to image tendons at almost any location. MRI also permits visualizing the tendon tissues as well as the soft and hard tissues surrounding a tendon. However, MRI has contraindication for patients who have metal implant devices in the body (Weinreb et al, 2014).

2.4.2 Laboratory imaging techniques

Light microscopy is the most frequently used imaging modality for studying the histology of a tendon (de Almeida et al, 2010; Glazebrook et al, 2008; Harvey et al, 2009; Oryan & Shoushtari, 2008; Smith et al, 2014; Stecco et al, 2014; Stecco et al,

2013). Using histological staining, light microscopy offers the capability to study the 2-dimensional morphology of the cells and the extracellular matrix in tendon tissues. As the dominant component, collagen has a birefringent characteristic. The configuration and crimping of collagen fibril bundles can be visualised using polarized light microscopy (Franchi et al, 2007a; Grant et al, 2013; Raspanti et al, 2015; Thorpe et al, 2013). To specifically study some components in tendon tissue, an immunohistological technique can be used for optical light microscopic or fluorescent microscopic observations of tendon microstructure (Ippolito et al, 1980; Kvist et al, 1992; Russo et al, 2015; Sodersten et al, 2013; Stecco et al, 2014; Stecco et al, 2013).

Confocal laser scanning microscopy has recently emerged as a powerful microscopic imaging technique possessing 3-dimensional (3D) imaging capability for biological studies. The 3D imaging capability of confocal microscopy enables observation of the spatial microstructure of biological tissues. It has greatly improved the understanding of the spatial network of many micro components of tendon tissues (Grant et al, 2013; Harvey et al, 2009; Pang et al, 2016; WU et al, 2017). Fibre optic imaging technologies have enabled the development of confocal arthroscopy. Confocal arthroscopy uses a probe to collect spatial microstructural information from tissues. It has emerged as a minimally invasive imaging modality for real-time visualization of the microstructure of a tendon tissue (Wu et al, 2015). Moreover, SHG microscopy enables imaging the collagen of biological tissue without tissue dehydration and staining. The integration of confocal and SHG imaging technologies has enabled the development of a powerful imaging technique to study the microstructural relationship of the micro components and collagen network of tendons in 3D (Fung et al, 2010; Gerson et al, 2009; Gusachenko et al, 2013; Hase et al, 2016; Theodossiou et al, 2006).

Electron microscopic imaging techniques also play an important role in studying the ultrastructure of tendons. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have ultrahigh image resolution for studying the size of the collagen fibrils, subtle twists of collagen fibrils, detailed organelles of tenocytes and the substructure of the collagen and elastic fibres in a tendon (Abrahamsson et al, 1992; Cetta et al, 1982; Ippolito et al, 1980; Oryan & Shoushtari, 2008; Pingel et al, 2014; Raspanti et al, 2015; Scott & Thomlinson, 1998; Strocchi et al, 1985). AFM is a novel imaging technique that enables studying the ultra-structure of a tendon at a

nanoscale without tissue staining and dehydration (Kahn et al, 2013; Rigozzi et al, 2013; WU et al, 2017). Using a tapping mode, AFM is able to test the mechanical properties of individual collagen fibrils (Graham et al, 2004; van der Rijt et al, 2006).

2.4.3 Confocal and second harmonic generation imaging techniques

Confocal microscopy and SHG microscopy are based on different imaging principles for acquiring the internal microstructure of tissues but they can be integrated in one multiphoton microscopic system. A multiphoton microscopic system normally has multiple laser sources to meet the requirements of a wide range of excitation wavelengths. Both confocal microscopy and SHG microscopy have higher imaging resolution than normal optical microscopy and a 3D imaging capability by collecting Z-series optical section images. 3D high-resolution images are achieved by the point-to-point scanning modality of confocal microscopy and SHG microscopy (Williams et al, 2001). Confocal microscopy uses a pinhole to block the fluorescence emissions from out-of-focus planes (**Fig. 2.5**). In this way, only the in-focus imaging information is collected by the photodetector (Paddock, 1999). The imaging mechanism of SHG microscopy is based on the theory of the nonlinear optical effect, the second harmonic generation optical property. It requires an intense SHG laser source and a material with noncentrosymmetric molecular structure. When a highly intensive SHG laser passes through a material that has an asymmetrical molecular structure, the material acts as an optical media such that two near-infrared incident photons are changed into one photon with precisely half of the wavelength of incident laser source (Campagnola & Loew, 2003). This phenomenon happens only at intense SHG laser points, which enables point-to-point scanning in SHG microscopy.

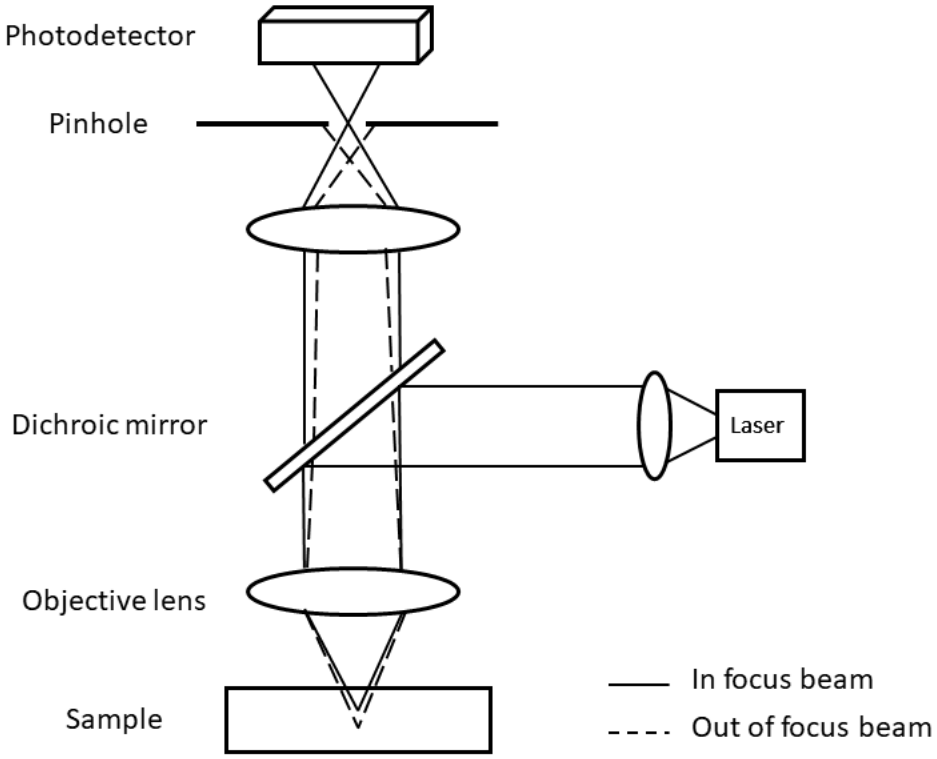

Fig. 2.5 The simplified schematic of the optical setup of confocal microscopy.

As the predominant component of tendons, collagen has a triple-helix molecular structure, which has long been known for generating efficient SHG signals (Freund et al, 1986). This intrinsic SHG characteristic of collagen makes SHG microscopy to be an optimal candidate for studying the collagen structure in a tendon without tissue staining and dehydration. In addition, both confocal and SHG imaging techniques are capable of imaging the internal microstructure of bulk tissues that are thick enough to retain the original microstructure of the tissues without dehydration. By using a specific staining solution targeting the micro components in a tendon, confocal and SHG microscopy enables acquiring of image stacks of the micro components in the tendon simultaneously through independent imaging channels. These image stacks are ready to be constructed into an integrated 3D image to show the spatial relationship of the micro components with the collagen, and analysed quantitatively using computer imaging analysis software.

2.5 Imaging analysis techniques

With the development of high resolution 3D microscopy techniques, the internal microstructure of biological tissues can be quantitatively and systematically analysed using computer imaging analysis software. There are numerous imaging analysis software packages and algorithms for biological imaging processing and analysis. Each of the software packages has their own specialized capabilities (Eliceiri et al, 2012). ImageJ (NIH, Maryland, USA) stands out as an open source imaging analysis system maintained and evaluated for its accuracy by researchers and software developers worldwide. With its diverse plugins, ImageJ can cover the functions of imaging visualization, processing, segmentation, registration and tracking (Schindelin et al, 2015). In addition, there are also other outstanding free or commercial programs with their own specialties that can be used in biological imaging processing and analysis, such as Voxx, Imaris, and matlab image processing toolbox (Clendenon et al, 2006).

Tendons are a fibrous tissue composed of cells and an abundant fibrillar extracellular matrix. The orientation of the fibrillar components is closely related to the state of health of tendon tissue. Fast Fourier transform (FFT) alignment analysis and fibril coherence analysis are useful imaging analysis algorism traditionally used for measuring the isotropic or anisotropic characteristics of an image (Ayres et al, 2008; Frisch et al, 2012). They can be used to quantify or semi-quantify the anisotropy and concordance of the fibrillar structure in a tendon. These imaging analyses can be performed using OrientationJ, FibrilTool and Oval profile in ImageJ (Ayres et al, 2008; Boudaoud et al, 2014; Fonck et al, 2009). The data extracted from analysis of the orientation of the fibril components can be used to build an intelligent system for a systematic study of the pathology of tendons.

2.5.1 2D fast Fourier transform alignment analysis

2D FFT is a spectral analysis technique which can objectively characterize anisotropic or isotropic features in an image (Peng & Kirk, 1997). It transfers spatial structural information in an image into a spectral frequency domain. The FFT frequency plot is a greyscale image that displays the original image information as pixels with different

intensities. The low frequency signals at the centre of the plot reflect the background and the general shape in the origin image, whilst the high frequency signals at the image periphery reflect the edge and structure details utilising ImageJ software (Ayres et al, 2008). The structure of the high frequency signals contains much information regarding the orientation of the original images. The FFT frequency plot can be analysed quantitatively using a number of methods. A previous study (Frisch et al, 2012) measured and compared the structure of the high frequency signals in the FFT frequency plot to quantitatively analyse the collagen orientation in the rat ligaments. Ayres et al (Ayres et al, 2008) introduced the oval profile ImageJ plugin to extract the information in FFT frequency plot to show the principle angle of the fibre alignment in the electrospun scaffolds.

2.5.2 Coherence analysis

The fibrillar components in healthy tendon tissues have a highly ordered architecture. The orientation of the fibrillar components in pathological tendons is normally interrupted. The coherence of the fibrillar components is an important index to quantify the state of health of tendons. OrientationJ in ImageJ has been developed to objectively and quantitatively identify the fibres coherence in biological tissues and some engineering scaffolds (Boudaoud et al, 2014; Fonck et al, 2009). The plugin OrientationJ is developed to calculate the directional coherency coefficient of the fibres in biological tissues (Fonck et al, 2009). Another ImageJ-based plugin named FibrilTool is able to measure the anisotropy or isotropy of biological tissue structures presenting in an image (Boudaoud et al, 2014). Both OrientationJ and FibrilTool in ImageJ are based on the measurement of image features in a region of interest and provide a value between 0 and 1 to quantitatively indicate the orientation of fibrillar components. A value of 0 indicates no predominant direction. A value of 1 indicated a strongly coherent orientation of fibrillar components.

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CHAPTER 3 The three dimensional microstructural network of elastin, collagen and cells in Achilles tendons

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3.1 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 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 (3D) 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 3D confocal images.

Keywords: Achilles Tendon; Confocal and SHG microscopy; Elastin; Collagen; 3D

3.2 Introduction

In humans, the Achilles tendon is the thickest and strongest tendon that sustains some of the largest tensile loads in the body (Yang et al, 2010). Dysfunction and injuries are commonly seen in the Achilles tendon. Various studies (Glazebrook et al, 2008; Kahn et al, 2013; Obst et al, 2014; Pingel et al, 2014; Raspanti et al, 2005; Yang et al, 2010) 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 (Kannus, 2000). Tendon consists primarily of collagen (70-80% of the tissue's dry weight) and less than 5% tenocytes and tenoblasts (Wang et al, 2013). These insoluble elements are embedded within a hydrated environment containing ground substance of proteoglycans, glycosaminoglycan (GAG) and some other small molecules (Hess et al, 1989). The detailed hierarchical structural organization of the tendon has been well defined by Kannus (Kannus, 2000), 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 (Pingel et al, 2014). While stereoscopic techniques can reveal some 3D 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 (He et al, 2014).

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 (Benjamin et al, 2008). 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 (de Aro et al, 2012). Collagen is a triple-helical structure (Kadler et al, 1996) and when observed using optical methods, it displays a birefringent characteristic (Cox et al, 2003), 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 (Cox et al, 2003), 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 (Baldwin et al, 2013). As an essential component in extracellular matrix, elastin ensures tissues with elastic stretch and recoil, cooperates with collagen for tensile resistance (Green et al, 2014), and regulates the interactions between cells and extracellular matrix (Baldwin et al, 2013). However, elastin is sparsely distributed in tendons, accounting for approximately 1-2% of the dry mass of the tendon (Kannus, 2000). 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 (Kielty et al, 2002), 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 (Green et al, 2014). 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 fibres (Zipfel et al, 2003), and this system has been utilised by numerous studies (Cox et al, 2003; Sands et al, 2006; Takaku et al, 2010; Zipfel et al, 2003) 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. (Ricard et al, 2007) 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 researchers (Gaertner et al, 2011; He et al, 2013a; He et al, 2013b).

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 tissues (He et al, 2013b), it is suggested that elastic fibres may have a crucial role in the mechanical function of Achilles tendons.

3.3 Methods

3.3.1 Sample preparation

Ten Achilles tendons from left hind limbs were freshly harvested from ten New Zealand white rabbits. 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 optimum cutting temperature (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. Both the longitudinal and transverse

slices had approximately 50 sections to be examined. The sections were adhered to slides, labelled, wrapped in cling film, and stored at -80℃ 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.

3.3.2 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.

3.3.3 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 beam was used for acquiring the images of cells stained by AO, while a 561 nm diode-pumped solid-state laser beam 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×, numerical aperture (NA) 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.

3.3.4 Image processing and 3D image reconstruction

The image processing is summarized in **Fig. 3.1**. 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).

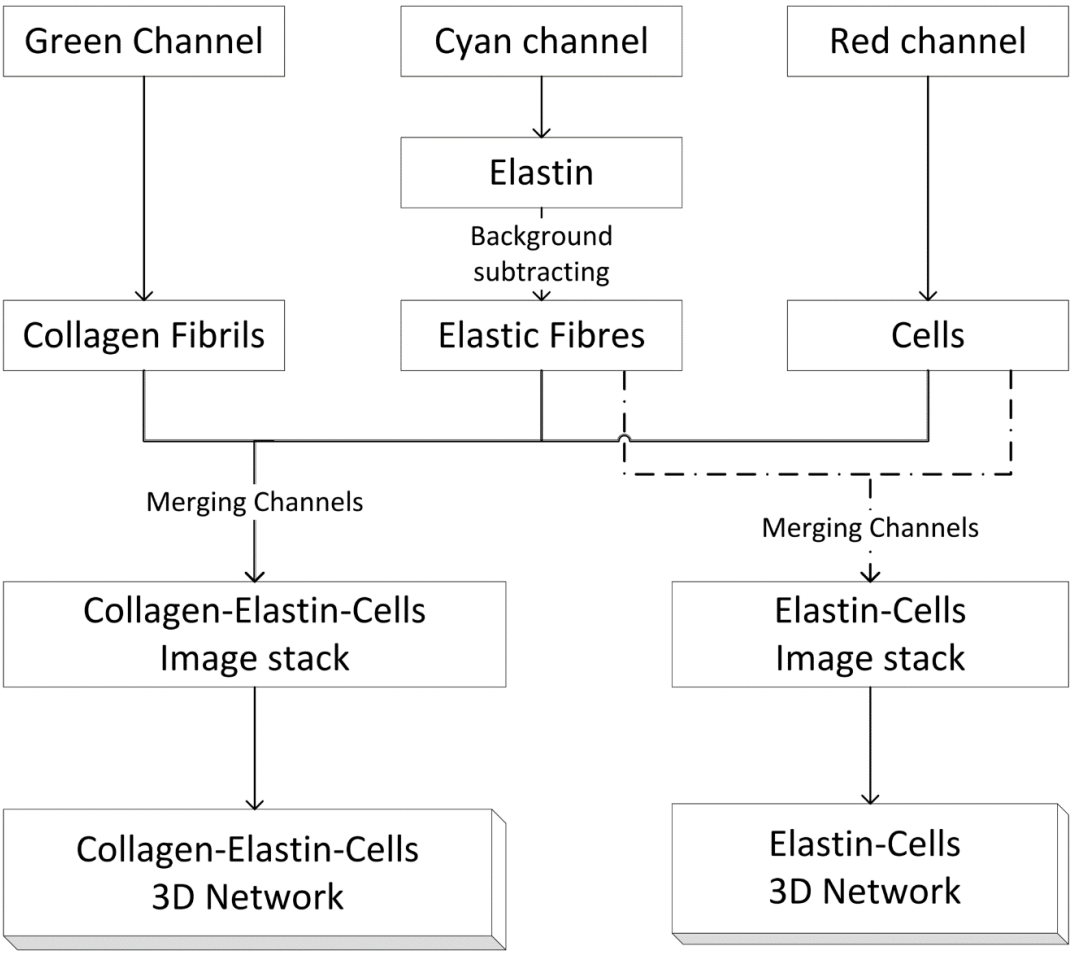


Fig. 3.1 The procedures of image processing and 3D image construction.

In order to get clear images of elastic fibres, the background was subtracted using ImageJ (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.

3.3.5 2D fast Fourier transform and alignment analysis

A 2-dimensional 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 image (Wu et al, 2005). 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 image (Ayres et al, 2008). 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.

3.4 Results

3.4.1 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. 3.2**e) 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. 3.2**a, e), while the elastin and cell images (**Fig. 3.2**b, 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. 3.2**b, f).

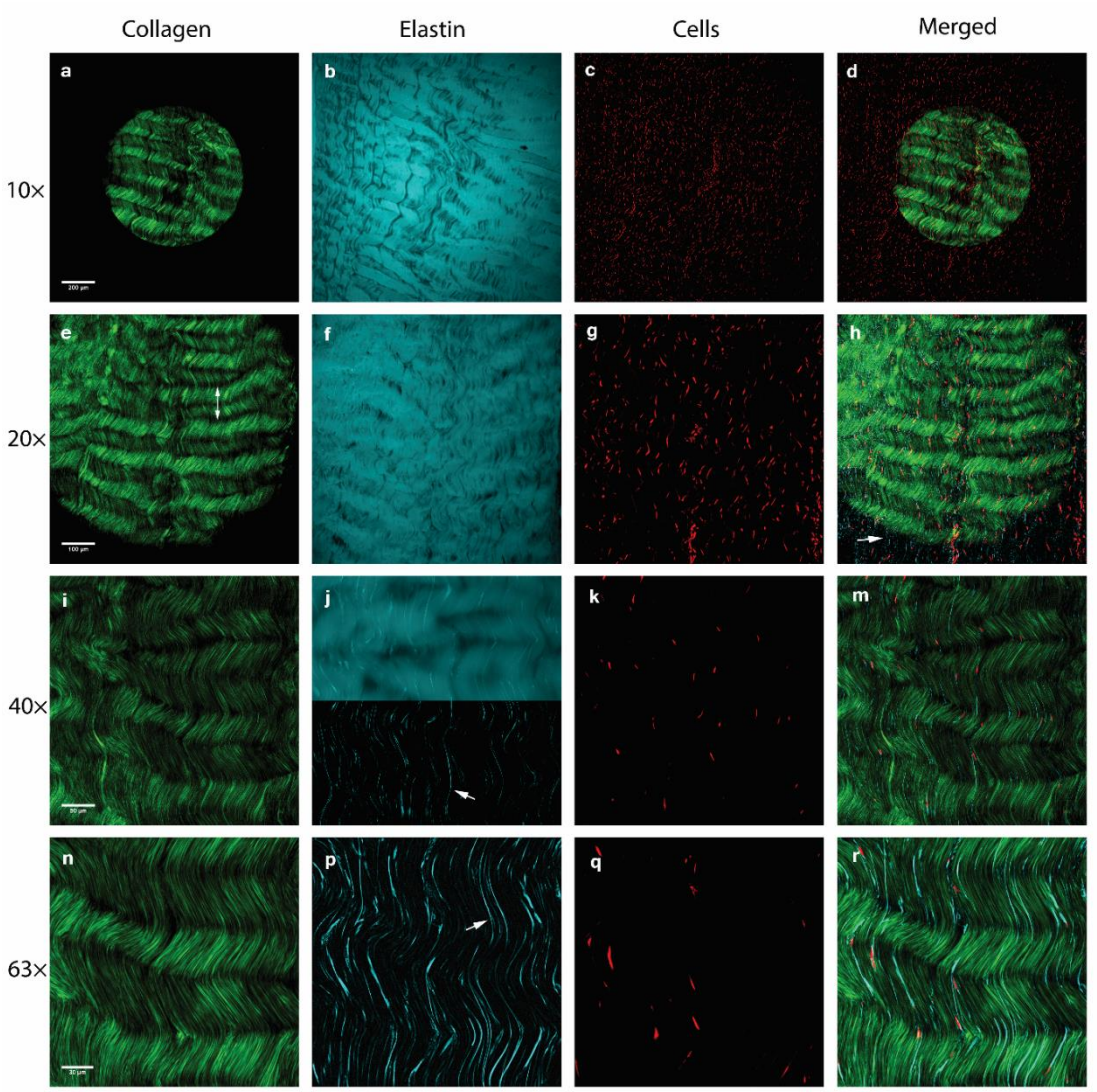


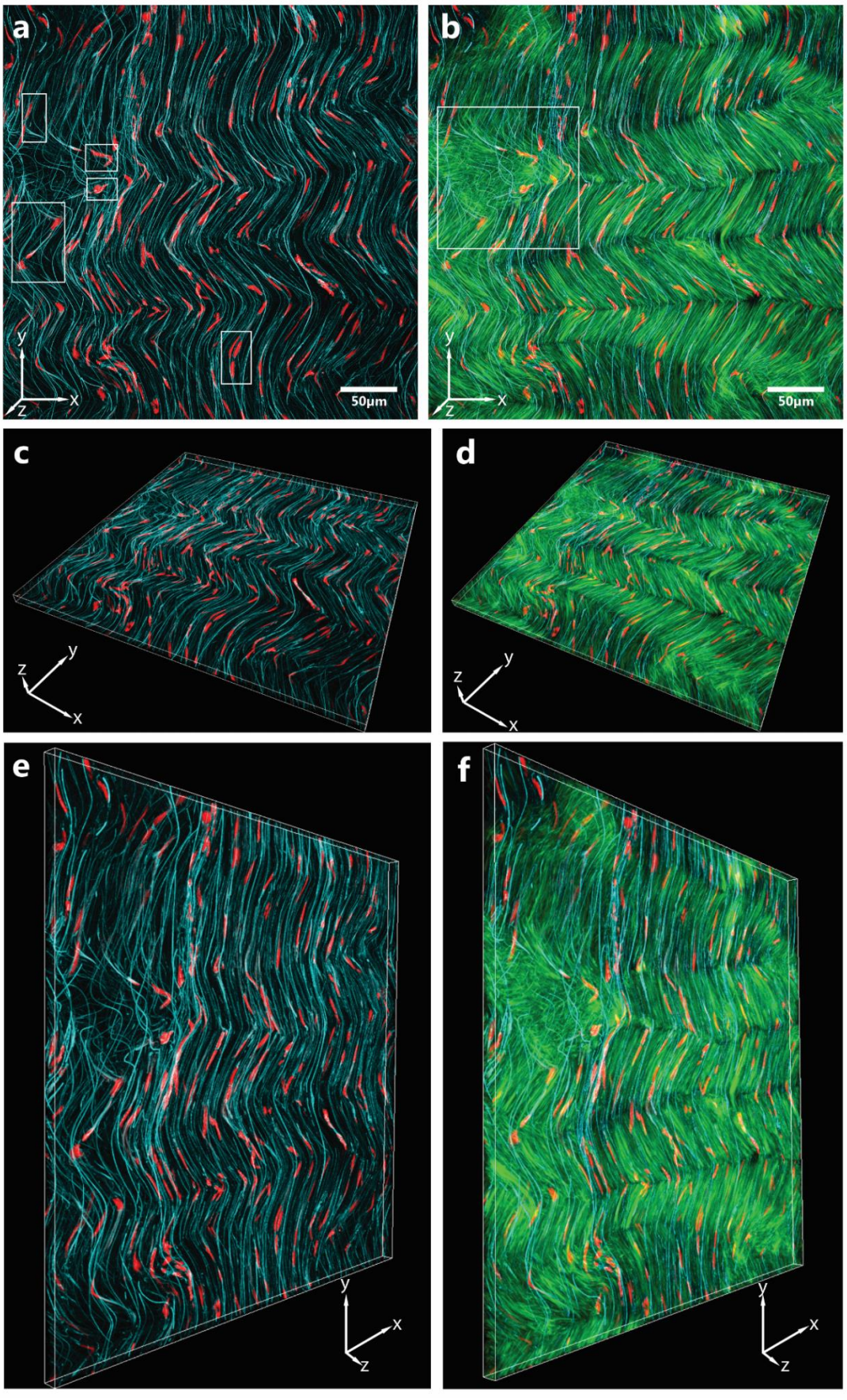
Fig. 3.2 Representative images of longitudinal 2D images of collagen (green), elastin (cyan) and cells (red) with different magnifications using confocal and SHG microscopy. Arrows indicate the elastic fibres. "j" shows the comparison of elastic fibres signal before and after background subtracting. The double headed arrow in "e" shows a crimp.

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. 3.2**i, n), which can be verified by the higher magnification observations in the transverse images (**Fig. 3.7**). The elastic fibres signals in higher magnifications are strong enough to be distinguished from the background noise (**Fig. 3.2**j, p), and **Fig. 3.2**j 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. 3.2**j, p) are discontinuous in the 2D images. In the merged images (**Fig. 3.2**m, r), elastic fibres and tenocytes within a layer align with the collagen fibril bundles and conforms to the collagen orientation.

3.4.2 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. 3.3**. 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. 3.3**a) with normal and twisted shapes were examined at higher magnifications to show the fine structure of the elastin-tenocyte network (as shown in **Fig. 3.4**). 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. 3.3**a, c, e). Elastic fibres and tenocytes are embedded in the collagen matrix and mostly conform to the collagen fibril orientation (**Fig. 3.3**b, 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. 3.5**.


a
b
c
d
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f
50μm
50μm
y
x
z

Fig. 3.3 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. 3.4** and **Fig. 3.5**. The volume size is 375 µm × 375 µm × 28 µm.

Connections between elastin and the tenocytes are shown in **Fig. 3.4**. The elastic fibres and tenocytes are connected in series. In **Fig. 3.4**a and b, the elastin forms a sparse peri-cellular meshwork around tenocytes. The same structure can be observed in **Fig. 3.4**c and d. A tenocyte in a twisted shape (**Fig. 3.4**d) appears to be dragged by the elastic fibres (arrow indicated in **Fig. 3.4**d). 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. 3.4**e, f).

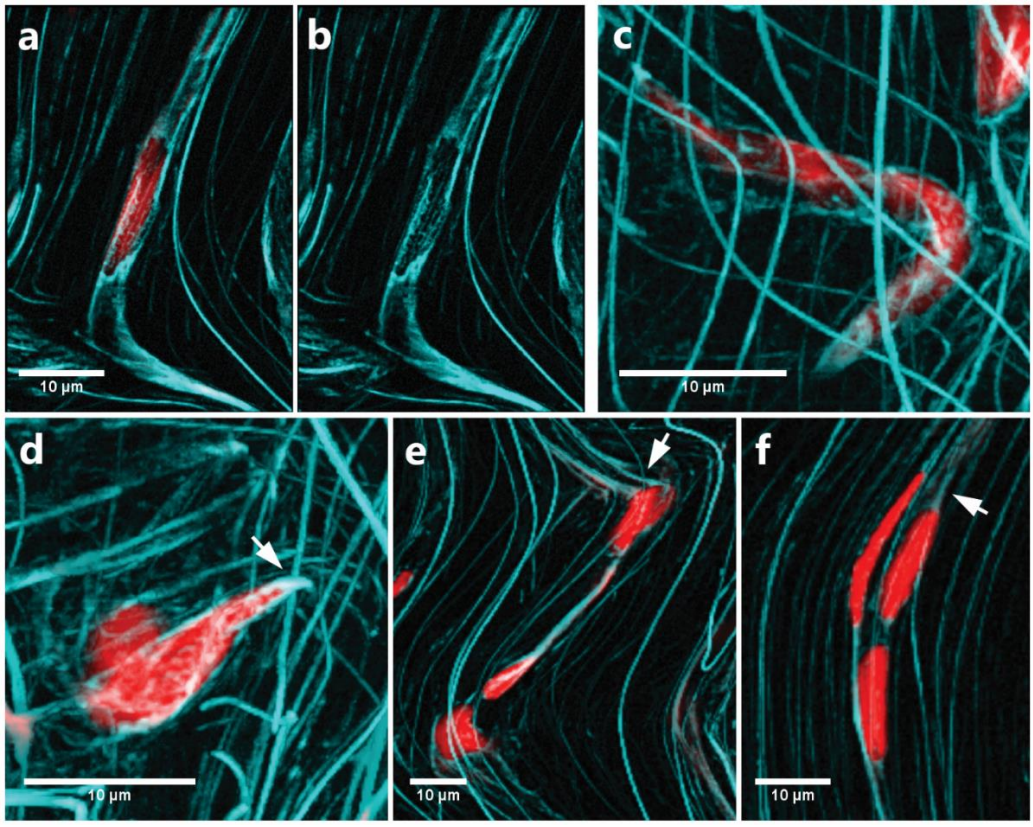

Fig. 3.4 The localisation of the elastin-tenocytes network shows the close relationship between tenocytes (red) and the elastin (cyan). The images correspond to the regions highlighted in **Fig. 3.3**a. "a" and "b" are the same location, while "b" shows an elastin pericellular meshwork which can also 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.

The extraordinary structure of the complex area in **Fig. 3.3**b is presented in detail in **Fig. 3.5**. The elastic fibres in this area (**Fig.3.5**a) 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. 3.5**b). The 2D merged images (**Fig. 3.5**c-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. 3.5**f).

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. 3.7**. The prevalence of these oblique nodes in the whole Achilles tendon warrants further investigation.

3.4.3 2D fast Fourier transform and alignment analysis

The intensity distribution of the 2D FFT of the collagen fibrils (**Fig. 3.6**b), elastic fibres (**Fig. 3.6**e) and tenocytes (**Fig. 3.6**h) 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. 3.6**c, 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. 3.6**d, g, j), the FFT alignment graphs of the tenocytes and elastic fibres exhibit a great similarity (**Fig. 3.6**f, i), which also can be confirmed from the normalised FFT alignment graph (**Fig. 3.6**k).

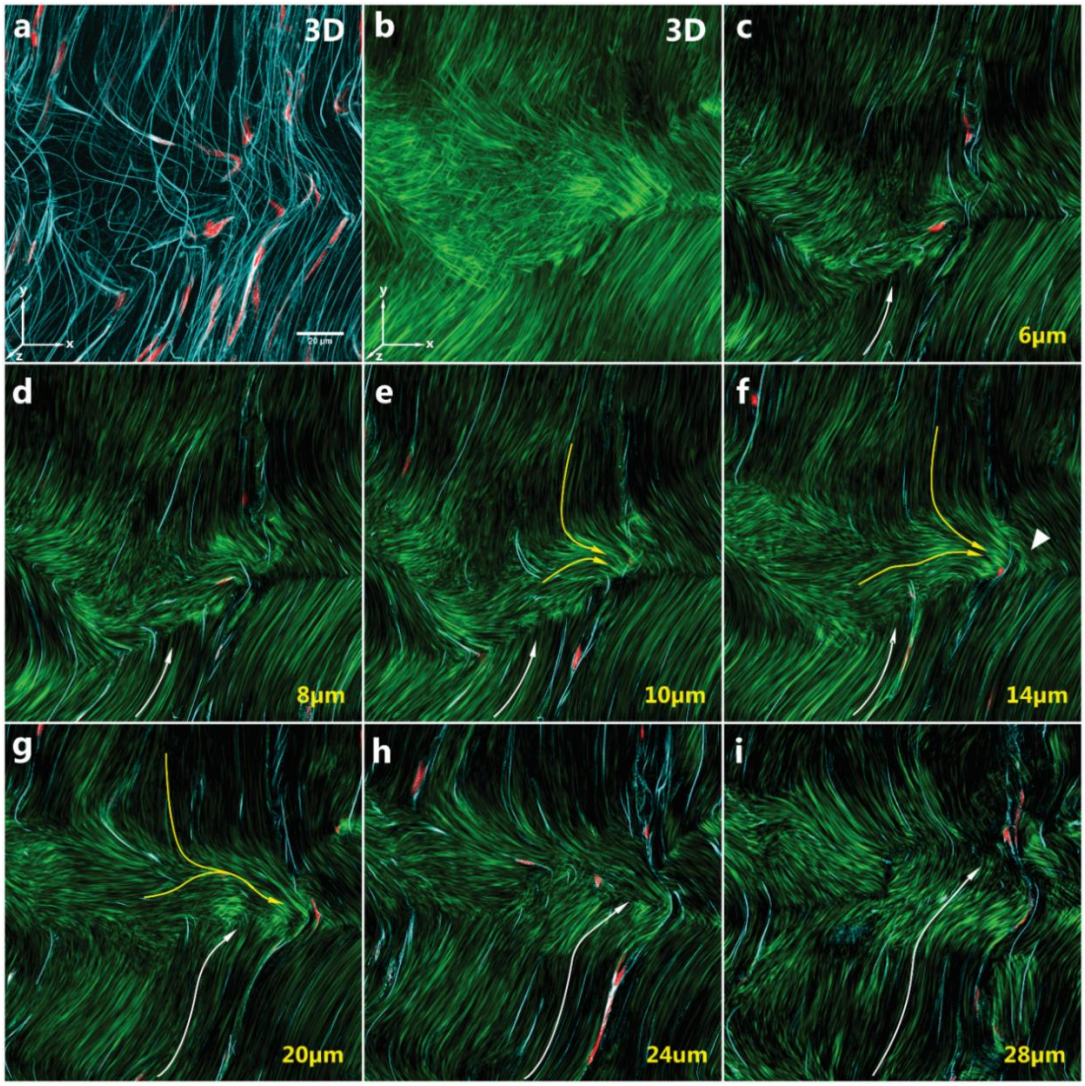

Fig. 3.5 The longitudinal observation of a spiral corresponds to the region highlighted in **Fig.3.3**b. Collagen fibrils are green, tenocytes are red and elastic fibres are cyan. All the images are in the same scale. “a” and “b” are 3D images. “c”-“i” are 2D images and the numbers at the lower right 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.

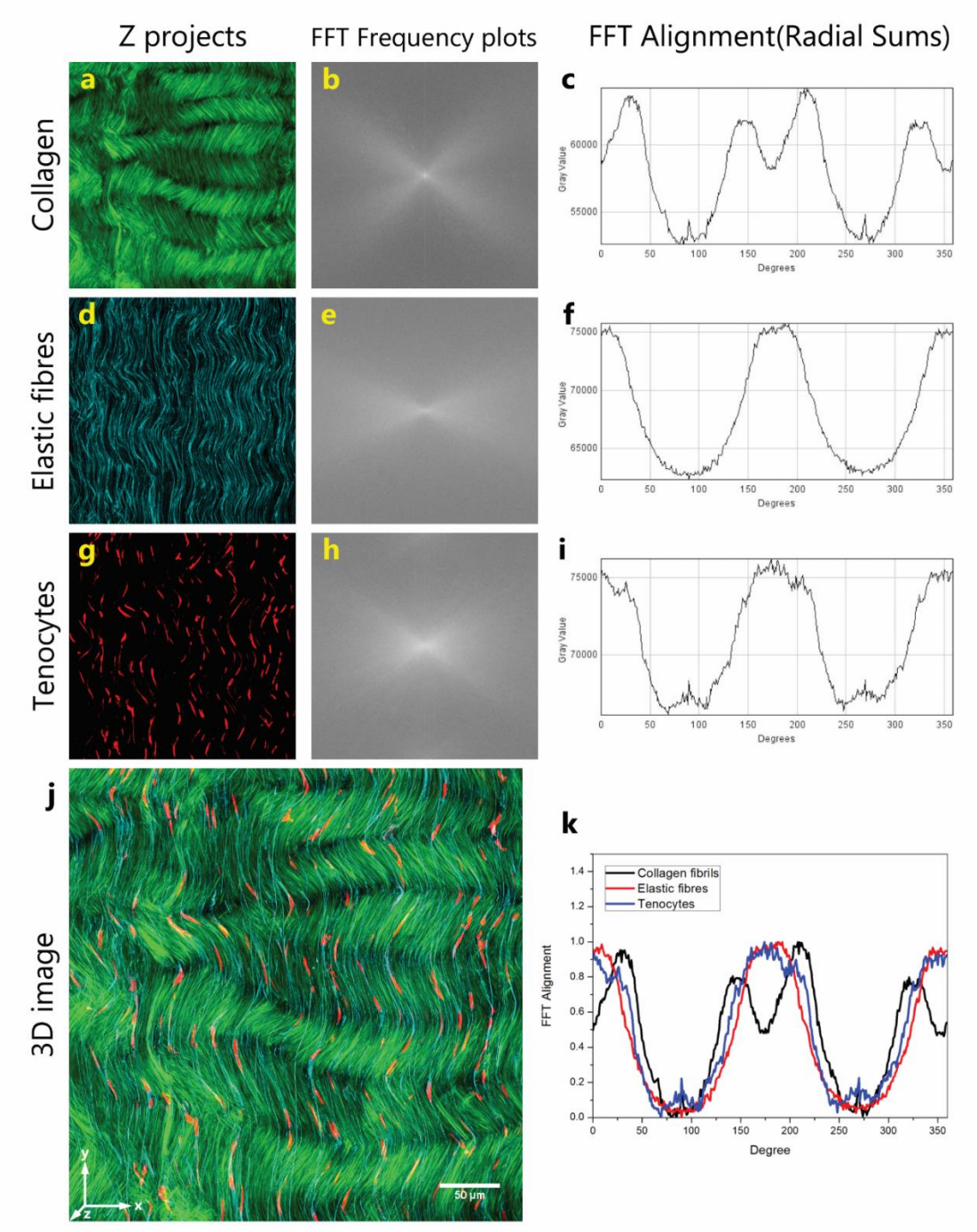

Fig. 3.6 2D z-project of collagen fibrils (a), elastic fibres (d) and tenocytes (g) was reconstructed from the corresponding image stack, respectively. The corresponding 2D FFTs of the collagen (b), elastic fibres (e) and tenocytes (h) were used to objectively indicate the predominant alignment of the 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.

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

The 3D transverse images (**Fig. 3.7**) 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. 3.7**), the diameter of collagen fibrils is at sub-micron level, which corresponds to the size of tendon collagen fibrils (Kannus, 2000). 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. 3.7**a, e) and elastin (yellow arrowhead in **Fig. 3.7**b, 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. 3.7**b) that conform to the collagen orientation in endotenon, and elastin that forms a thin membrane (yellow arrowhead in **Fig. 3.7**f) at the interface between the tendon matrix and the endotenon. Skinny tenocytes (white arrowhead in **Fig. 3.7**b) 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. 3.7**b) have a larger diameter range than the tenocytes.

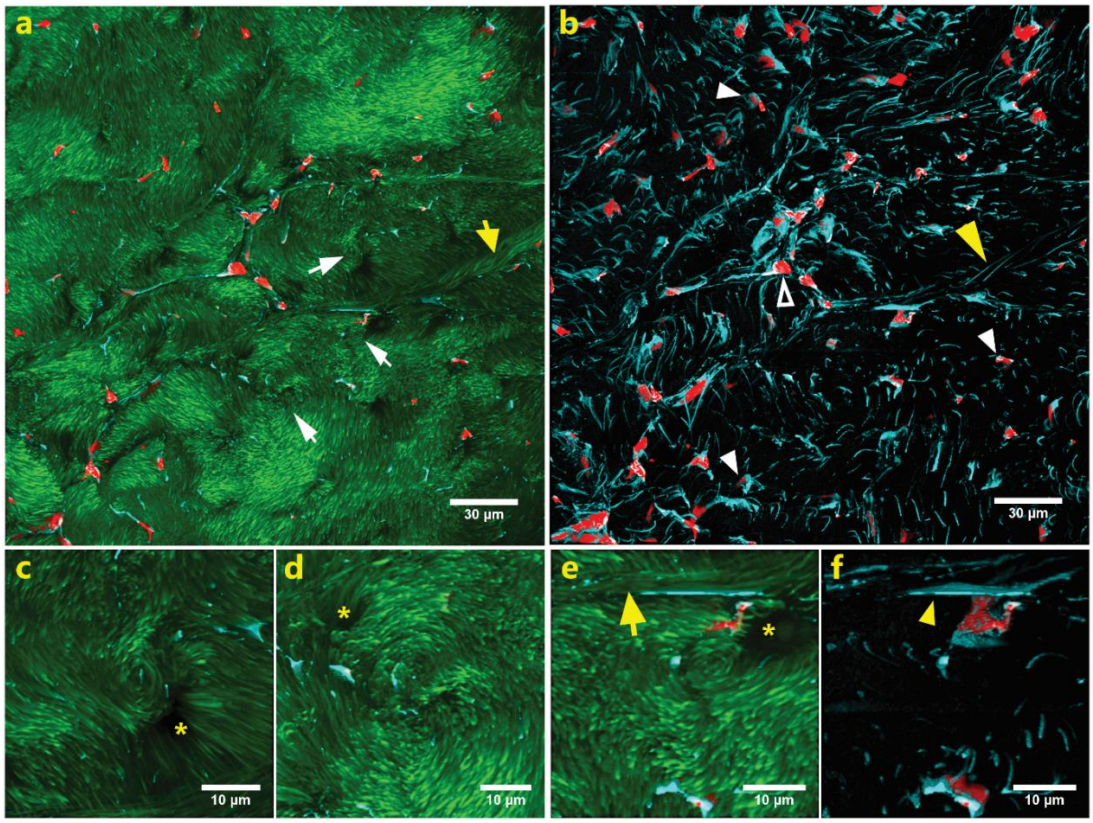

Fig. 3.7 Representative 3D transverse observation of the microstructure of Achilles tendons. Collagen fibrils are green, tenocytes are red and elastic fibres are cyan. "a" and "b" are from the same location. "c"-"e" are magnified spiral structures from white arrows indicated areas in "a". "f" is the 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 μm × 238 μm × 20 μm.

From the transverse view in **Fig. 3.7**a, 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 (Kannus, 2000; Smith et al, 2013). 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. 3.5**i 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 (Ayres et al, 2008; Grant et al, 2015; Kannus, 2000; Smith et al, 2013), 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. 3.7**a as indicated by white arrows, and further magnified images are provided (as shown in **Fig. 3.7**c-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. 3.5**. Meanwhile, some spaces in the matrix ("*" indicated in **Fig. 3.7**c-e) appear to accompany the spirals, which warrants further study.

3.5 Discussions

As important components in the extracellular matrix, elastin and collagen usually have been studied conjointly (Grant et al, 2015; Henninger et al, 2013; Koch et al, 2014; Thakkar et al, 2014). 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 (Green et al, 2014; Konig et al, 2005). 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 (Henninger et al, 2015; Konig et al, 2005). By utilizing these techniques, observations of elastin fibre networks within different tissues are increasing (Dunkman et al, 2013; Grant et al, 2013; He et al, 2013a; He et al, 2013b; Mansfield et al, 2009; Yu et al, 2007).

3.5.1 Elastin

In this study, three forms of elastin were observed in the mid-portion of Achilles tendon: (1) peri-cellular elastin meshwork that enveloped the tenocytes, which is consistent with the studies of bovine deep digital flexor tendons (Grant et al, 2013) and human rotator cuff (Thakkar et al, 2014); (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 cells (Kannus, 2000). 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 ligaments (Thorpe et al, 2013). However, future studies are required to investigate the mechanotransduction process between tenocytes and the extracellular matrix.

Some most recent studies (Grant et al, 2015; Henninger et al, 2013; Henninger et al, 2015; Smith et al, 2014) 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 (Grant et al, 2015; Henninger et al, 2013; Henninger et al, 2015). Using immunofluorescent methods, Ritty (Ritty et al, 2002) 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.

3.5.2 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 (Franchi et al, 2007; Magnusson et al, 2002; Raspanti et al, 2005). 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 reported (Jozsa et al, 1991) that collagen fibres which are oriented longitudinally and transversely run cross each other forming spirals. From the 2D longitudinal image series in **Fig. 3.5** and the 3D transverse images in **Fig. 3.7**, 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. Study (Ippolito et al, 1980) 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 (Grant et al, 2015; Henninger et al, 2013) 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 (He et al, 2014; Wu et al, 2015). 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 (Duan et al, 2013; Duan et al, 2015), 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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CHAPTER 4 The 3D microstructure of Achilles tendons and the enthesis

(Manuscript in preparation)

4.1 Abstract

An Achilles tendon inserts into the calcaneus through a fibrocartilaginous enthesis. A secure connection between the Achilles and the calcaneus is essential for the Achilles tendons to transfer the muscle contractive forces to the calcaneus bone and movement of the foot. The enthesis of the Achilles tendon is the weakest point and can be subject to tearing and breakage. Degeneration of the Achilles tendon enthesis leads to insertional Achilles tendinopathy, which is a common disorder that causes pain and mobility difficulties for patients. From fibrous soft tissue to stiff hard tissue, the Achilles tendons and the enthesis experience dramatic alterations in their microstructures and mechanical properties. However, it is still not fully understood how the Achilles tendons and calcaneus are microstructurally connected through the enthesis. Using confocal and second harmonic generation (SHG) microscopy, this study has examined the 3-dimensional (3D) microstructure of collagen fibrils, elastic fibres and cells in Achilles tendons and the enthesis, particularly the microstructural arrangement of collagen and elastin near the tidemark. The results demonstrate a layered microstructure of the enthesis in 3D, and a gradual transition of the collagen fibrils, elastic fibres and cell shapes from the mid-portion to the enthesis of Achilles tendon. This study provides new information about the adaption of the microstructure and mechanical properties of an Achilles to the enthesis. This knowledge will increase understanding of the function of the muscle-tendon-bone system and the mechanism of anchorage and stress dissipation of Achilles tendons to the calcaneus.

Keywords: Achilles tendons; Enthesis; Microstructure; Fibrocartilage; 3D

4.2 Introduction

Tendons function as a force transmission tissue in a muscle-tendon-bone system. With the involvement of the tendons, the forces generated by muscle are transmitted efficiently to the bone to enable body movements (Kannus, 2000; Lavagnino et al, 2015; Smith et al, 2013). In the chain of force transmission, the interface between the soft and hard tissue in tendons, which is often called the tendon-bone insertion or osteotendinous junction or 'enthesis', possesses a unique composition and a microstructure that permits the tendon to securely connect to the bone and perform its force transmission role. It is one of the weakest points, subjected to huge stress concentration and prone to tearing (Shaw & Benjamin, 2007). Therefore, studying the microstructure of the region is important to understand the function of the muscle-tendon-bone system and aetiology of enthesitis.

Tendons insert into bones through two kinds of entheses: a. the fibrous enthesis, which normally presents at the attachment site of tendons to the diaphysis; b. the fibrocartilaginous enthesis, which normally presents at the attachment site of tendons to the epiphysis or the apophysis (Benjamin et al, 2006). The enthesis of a human Achilles tendon is a typical fibrocartilaginous enthesis. The Achilles tendon enthesis transfers contractive forces generated by the gastrocnemius and soleus muscles to the calcaneus bone to enable normal use of the foot.

In comparison with the mid-portion, the enthesis of Achilles tendon experiences very complex mechanical loads including normal tensile forces, torsional shearing stresses and compression from the calcaneus (Rufai et al, 1995). The microstructure is correspondingly organised to allow the tissue to adapt to its mechanical environment. The enthesis can be classified into four distinct microstructural regions: the tendon proper, the uncalcified fibrocartilage (UCF), the calcified fibrocartilage (CF) and the bone. Histologically, there is a visible boundary called the 'tidemark' that clearly separates the UCF from the CF (Benjamin et al, 2002; Shaw & Benjamin, 2007). An interdigitating line called the "cement line" distinguishes the CF from the lamellar bone. The fibrocartilage is characterized by rows of round fibrocartilage cells lying in the lacunae between collagen bundles. Both the cell rows and collagen bundles are

oriented in a direction perpendicular to the tidemark (Doschak & Zernicke, 2005; Shaw & Benjamin, 2007).

Gradation of the microstructure and composition of the tendon enthesis endows the tissue with graduated mechanical properties to dissipate the stresses accumulated at the interface of the fibrous tendon and rigid bone (Thomopoulos, 2011). A tendon contains primarily type I collagen that aligns longitudinally to allow the tendon to withstand tensile stresses (Screen et al, 2015). A bone contains highly mineralised type I collagen (Thomopoulos et al, 2006). In contrast, the UCF and the CF that mediate the tendon to the bone contain predominantly types II, III and X collagen (Waggett et al, 1998). Indeed, there have been intensive studies of the microstructure of Achilles tendons and the enthesis for comprehending the tissues' function and pathogenesis (Benjamin & Ralphs, 2001; Clark & Stechschulte, 1998; Cooper & Misol, 1970; Cury et al, 2016; Thomopoulos et al, 2003). However, the microstructure and composition of the tendon and enthesis are not yet fully elucidated. There are still questions regarding how the tendon and bone are integrated as a functional unit.

SHG microscopy has been recognised as a powerful tool for imaging the collagen fibrils of Achilles tendons and articular cartilage without tissue dehydration and labelling (He et al, 2013b; Pang et al, 2016; WU et al, 2017). Previous studies of tendons and articular cartilage using confocal and SHG microscopy have led to discovery of a unique microstructural network of collagen fibrils, elastic fibres and cells in the tendons and articular cartilage to facilitate understanding of the functions of the tissues (He et al, 2013a; He et al, 2013b; He et al, 2014; He et al, 2013c; Pang et al, 2016). In a spatial domain, the longitudinal collagen fibrils and elastic fibres form a fibrillary matrix in tendons, which is critical for the homeostasis of the elongated tenocytes and the tensile and elastic properties of the tendon (Pang et al, 2016). It is hypothesized in this study that there is a gradual microstructural transition of the collagen, elastin and cell characteristics from the mid-portion to the fibrocartilaginous enthesis of Achilles tendons. The application of confocal and SHG microscopic techniques (He et al, 2013a; Pang et al, 2016; WU et al, 2017) allowed visualisation of the 3D microstructure of the collagen, elastin and cells within four distinct regions of the tendon proper, the UCF, the CF and the bone. This study will lead to systematic understanding of the function of Achilles tendons and the muscle-

Achilles tendon-bone chain. Thus, the purpose of this study is to observe and understand the microstructural characteristics of Achilles tendons and the enthesis.

4.3 Methods

4.3.1 Samples

A total of ten Achilles tendon complexes with the attachment of the mid-portion and calcaneus were dissected from 5 healthy New Zealand white rabbits aged 18 to 19 weeks (3 males and 2 females). The first group of six Achilles tendon-calcaneus complexes were used for confocal and SHG observation. The specimens were immediately embedded in optimal cutting temperature (O.C.T) compound (VWR International Ltd., Leuven, Belgium) and stored in -80 °C freezer until cryo-sectioning for confocal and SHG imaging. The second group of two Achilles tendon-calcaneus complexes used for histological study were immediately fixed in 4% formaldehyde PBS (phosphate buffer solution) solution for 24 hours and proceeded with normal standard hematoxylin and eosin (H&E) histology. The third group of two Achilles tendon-calcaneus complexes used for scanning electron microscopy (SEM) imaging were snap frozen and embedded in O.C.T compound for cryo-sectioning and fixation in 2.5% glutaraldehyde for imaging.

4.3.2 Cryo-sectioning and fluorescent staining

The samples were cryo-sectioned sagittally into 50 μm thick sections and placed on adhesive glass slides. 50 sections were prepared for confocal and SHG microscopic examination. The cryo-sections were gently washed with PBS to remove the O.C.T compound completely and were stained with 0.03 g/L acridine orange (AO) for 3 min. After washing with PBS, the cryo-sections were stained with 1 mg/mL sulforhodamine B (SRB) for 1 min. After washing thoroughly with PBS and covered by coverslips, the sections were used for confocal and SHG imaging. AO is a specific fluorescent dye that stains cell nucleus, and SRB has been proved to fluorescently label elastin (He et al, 2013a; Ricard et al, 2007).

4.3.3 Confocal and SHG imaging

A Leica TCS SP2 multiphoton microscope was used to acquire confocal and SHG image stacks showing elastin, cells and collagen (He et al, 2013a; Pang et al, 2016; WU et al, 2017). Image stacks displaying elastin, collagen and cell nuclei were collected separately in three independent imaging channels. The elastin stained by SRB solution was excited by a 561 nm laser beam to collect signals from elastin at 590-680 nm. The cell nuclei stained by AO were excited by a 514 nm laser beam to collect signals from cells at 565-600 nm. Collagen generates a large quantity of SHG signals under intensive pulse lasers for SHG imaging. The signals of the collagen were collected by a secondary non-descanned detector at 445 nm using an 890 nm laser excitation (Spectra Physics Mai Tai Titanium Sapphire). The image stacks were acquired using a step size of 0.5 μm.

Since the confocal images of elastin images contained homogeneous background noise, the function of 'subtract background' within ImageJ (NIH, Bethesda, MD) was used to remove homogeneous background noise. After the background noise subtraction, the image stacks acquired from the three independent imaging channels were reconstructed into 3D images containing collagen, elastin and cells using Imaris 7.4.2 (Bitaplane, Concord, MA) (Pang et al, 2016). In this study, the cell nucleus, elastin and collagen were assigned as red, cyan and green respectively in the 3D confocal and SHG images.

4.3.4 Hematoxylin & Eosin histology

After thoroughly washing with PBS to remove the formaldehyde solution, the two Achilles tendon-calcaneus complexes scheduled for traditional histology were decalcified by the RDO rapid decalcifier (Apex Engineering Products, Plainfield, IL) and embedded in paraffin for sagittally microtoming into 5 μm thick sections. The sections were stained with hematoxylin and eosin for histological imaging using Scanscope (Leica Aperio).

4.3.5 Scanning electron microscopy

The two Achilles tendon-calcaneus complexes used for SEM studies were cryo-sectioned into 100 µm thick sections. The sections were washed with PBS to remove the O.C.T compound. The sections were fixed in 2.5% glutaraldehyde for 24 hours and dehydrated in 50%, 70%, 95% and 100% ethanol. The sections were immersed in 100% ethanol and transferred to a critical point dryer (KE3100) to be dried at a critical drying point, as required for SEM. The critical point dried sections were mounted on stubs and coated with gold for SEM imaging using a Zeiss 1555 VP-FESEM.

4.3.6 Image analysis

The analyze particles function within ImageJ (NIH, Maryland, USA) was used to quantify cell morphology. The 2-dimensional fast Fourier transform (2D FFT) function, the ImageJ plugins oval profile and orientationJ were used to quantify the orientation characteristics of the collagen, elastin and cell distribution in four distinct regions of Achilles tendons prone to a change in microstructure: the mid-portion, the transitional region near the mid-portion, the transitional region near UCF, and the UCF region.

2D fast Fourier transform alignment analysis.

The 2D fast Fourier transform (2D FFT) transfers spatial information of a digital image into a mathematically defined frequency domain to objectively identify the anisotropic or isotropic features of an image (Ayres et al, 2008). In this study, image stacks displaying cells, collagen and elastin were processed into z-projections using ImageJ for 2D FFT alignment analysis. The oval profile plugin within ImageJ was used to measure grey scale intensities of the pixels of the frequency spectrums of the 2D FFT, which quantified the orientation characteristics of the collagen, elastin and cell arrangement in the four distinct regions of Achilles tendons prone to a change of microstructure.

Coherency analysis.

The OrientationJ plugin in ImageJ was also used to measure the orientation of collagen fibrils, elastic fibres and cell arrangement in the four regions of Achilles tendons. Coherency analysis was performed on the image stacks showing collagen, elastin and cells to quantify the orientation characteristics of the micro components. Coherency analysis evaluates anisotropic or isotropic features in an image and assesses them using a coherency coefficient ranging from 0 to 1 (Fonck et al, 2009). A coherency coefficient close to 1 indicates a strongly coherent orientation of fibrillar components in an image whilst a coherency coefficient close to 0 indicates there is no dominant direction in the image.

Cell morphology analysis.

The 'analyze particles' function in ImageJ was used to measure the morphology of the nuclei. The morphology parameters, the circularity and aspect ratio (AR), were used to carry out the measurement. The circularity was calculated by equation:

$$Circularity = \frac{4\pi \times \text{area}}{perimeter^2} \quad . \qquad 4.1$$

The circularity is a value ranging from 0 to 1. A value of 1 indicates a perfect circle while a value close to 0 indicates an extremely elongated shape. The AR of a nucleus/cell is the ratio of the major axis divided by the minor axis. These morphological analyses were conducted on the Z-projections of the nuclei derived from the image stacks.

4.4 Results

4.4.1 The layered structure of Achilles tendon enthesis

Confocal and SHG microscopic observations (**Fig. 4.1**a, b) show broad views of the layered microstructural arrangement of the collagen bundles (green), elastin (cyan) and cells of bone, CF, UCF and tendon proper in the Achilles tendon enthesis. There was no obvious microstructural boundary of the collagen, elastin (**Fig. 4.1**b) and cells

between the UCF and tendon proper but the collagen became less crimped from the tendon proper to UCF. However, the collagen, elastin and cells are clearly connected between the UCF and CF (oval shaped dash circle in **Fig. 4.1**a). The layered structure observed by confocal and SHG (**Fig. 4.1**a, b) is comparable with H&E histology (**Fig. 4.1**c). Bone is identified by the bone marrow (arrow heads in **Fig. 4.1**a, b).

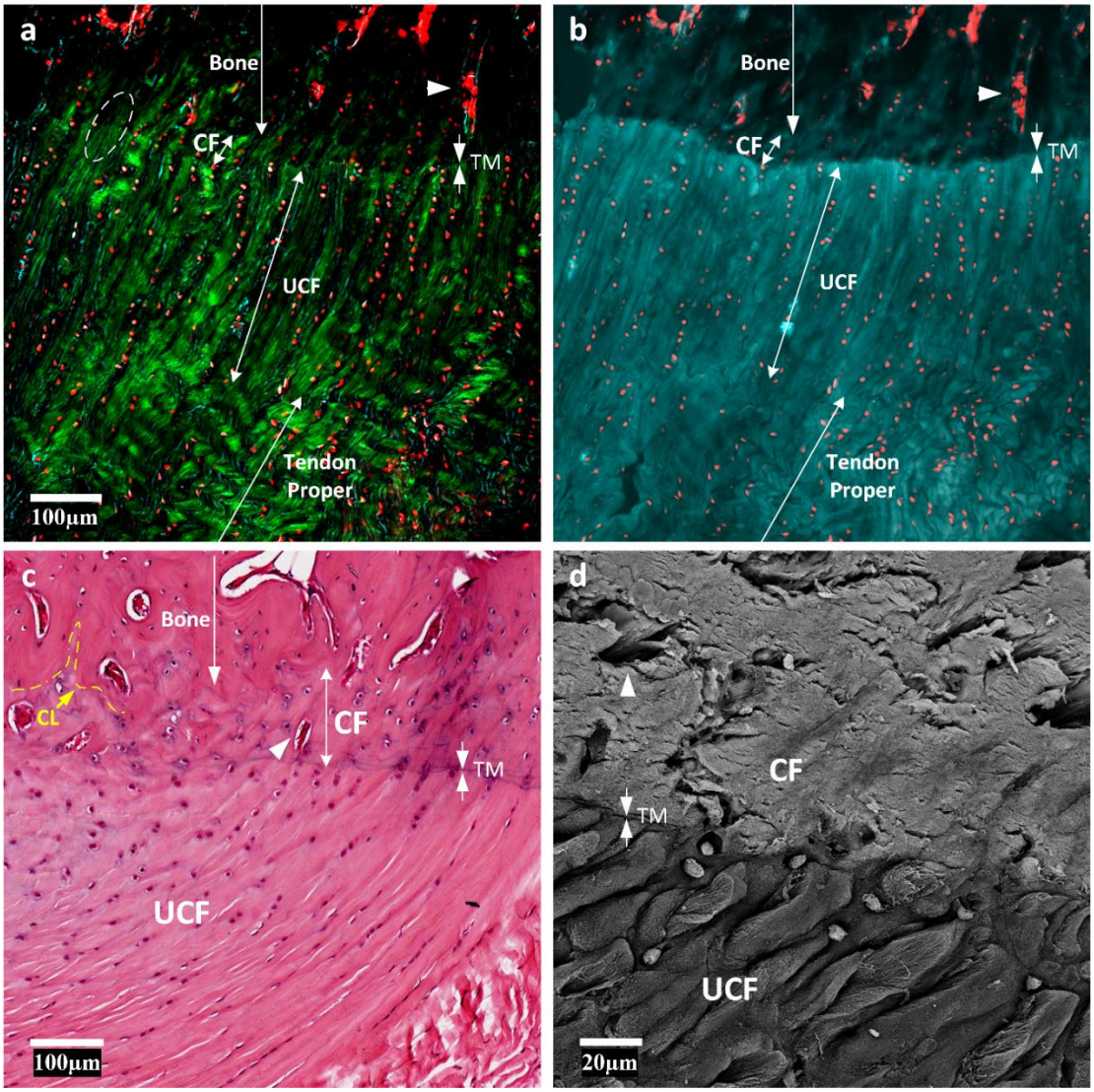

Fig. 4.1 Confocal and SHG images (a, b) show the layered-microstructure of Achilles tendon enthesis: cells (red), elastin (cyan) and collagen (green). The corresponding H&E histological image (c) and the SEM image (d) show the enthesis of Achilles tendons. The arrowheads in a-d show bone marrow cavities. CF: calcified fibrocartilage. UCF: uncalcified fibrocartilage. TM: tidemark. CL: cement line.

SEM images (**Fig. 4.1**d) in the backscattered mode show in more detail the transition between CF and UCF. The calcified tissue is rich in calcium. The heavier atomic weight of calcium makes the CF light grey in contrast to the dark grey UCF.

4.4.2 Structural alterations of collagen, elastin and cells from the mid-portion to the uncalcified fibrocartilage

3D images (**Fig. 4.2-4.3**) show the gradual microstructural alterations of cells, elastic fibres and collagen fibrils from the mid-portion to the UCF. Cells and elastic fibres form as longitudinal series which conform to the longitudinal collagen fibrils but the elastic fibres and collagen fibrils become straight and have less crimps from the mid-portion to UCF of Achilles tendons. Although there is no noticeable change in the content of the collagen from the mid-portion to the UCF, the content of elastic fibres decreases from the mid-portion to the UCF. The cell morphology also alters from an elongated shape in the mid-portion to be a round shape in the UCF.

The fibre structure in the transitional region (**Fig. 4.2**c, **4.3**a-b) is particularly interesting. Some of the longitudinal elastic fibres and collagen fibrils (arrows indicated in **Fig. 4.2**c, **4.3**a-b) are wound into bunches by transverse fibrils. This phenomena is more prominent in the transitional region near the UCF (**Fig. 4.3**a, b). The elastin in fibrocartilage (**Fig. 4.3**c) presents as amorphous (arrow in **Fig. 4.3**c) and fibrillar forms. The amorphous elastin mainly appears around the peri-cellular matrix of the round fibrocartilage cells (**Fig. 4.3**c).

4.4.3 Quantitative analysis

FFT alignment analysis (**Fig. 4.4**a-d) shows the orientation characteristics of the elastic fibres, cells and collagen fibrils in the four regions from the mid-portion to UCF of Achilles tendons. It confirms that the collagen fibrils, elastic fibres and cells orient longitudinally (at about 90° or 270°). The orientation curves indicate a great concordance of cell arrangement with the orientation of the elastic fibres and collagen fibrils in the four regions. Coherency analysis (**Fig. 4.4**e) shows that the collagen fibrils and cell arrangement have a high coherency value of about 0.8 in the transitional regions. The elastic fibres have a similarly high coherency value of about 0.6 in the

mid-portion and transitional regions. There is an increase of the coherency value of the collagen from the mid-portion to the UCF. The cell circularity and AR analysis (**Fig. 4.4**f) show that, in general, the cells in the transitional region near mid-portion (tenocytes) had the lowest circularity of about 0.1 and highest AR of about 11. The cells in the UCF had the highest circularity of about 0.7 and lowest AR of about 2.5.

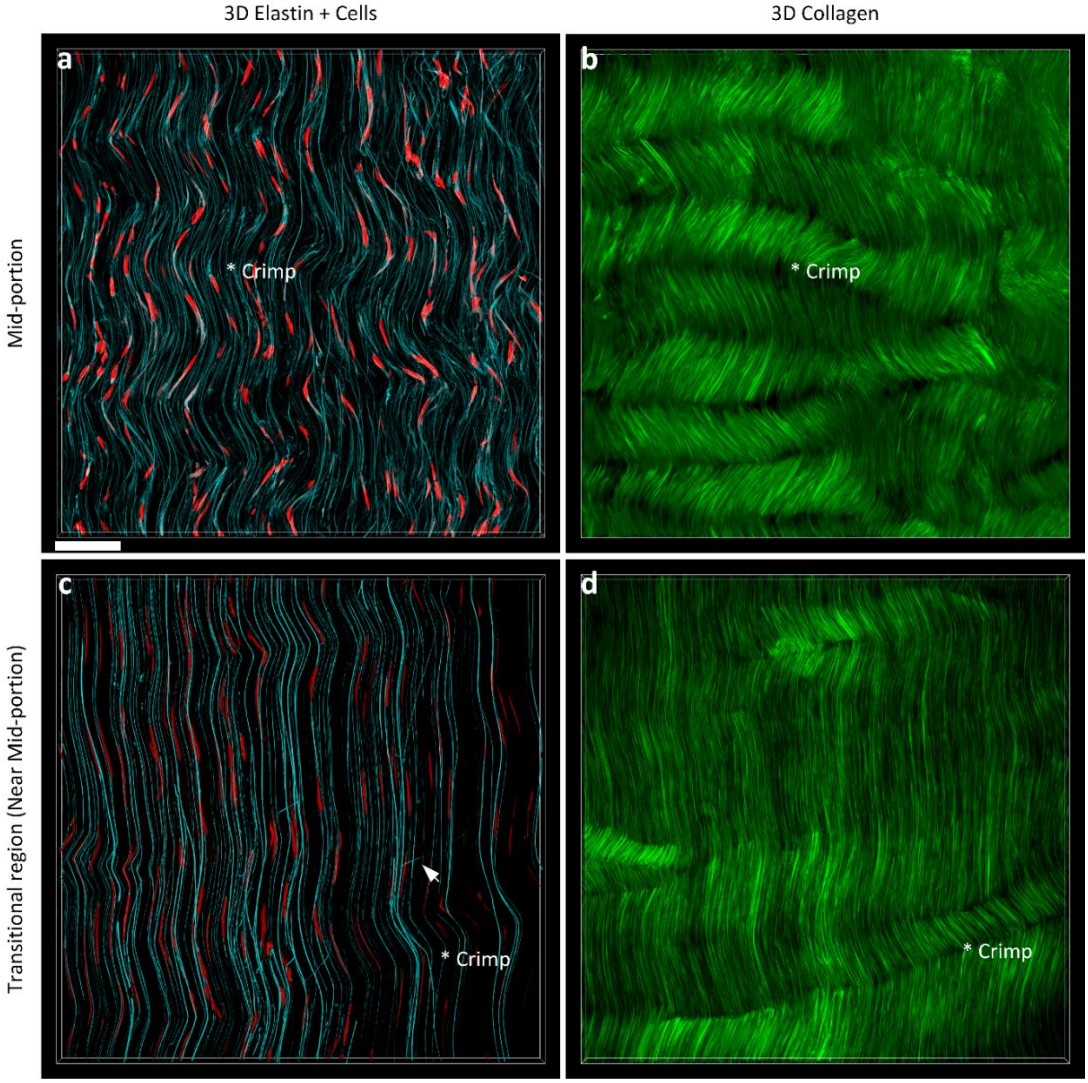

Fig. 4.2 The tenocytes (red), the elastic fibres (cyan) and corresponding collagen (green) in the mid-portion (a, b) and the transitional region near mid-portion of Achilles tendons (c, d). All images are of the same scale. Scale bar: 50 µm. The white arrow in c shows transverse elastic fibres.

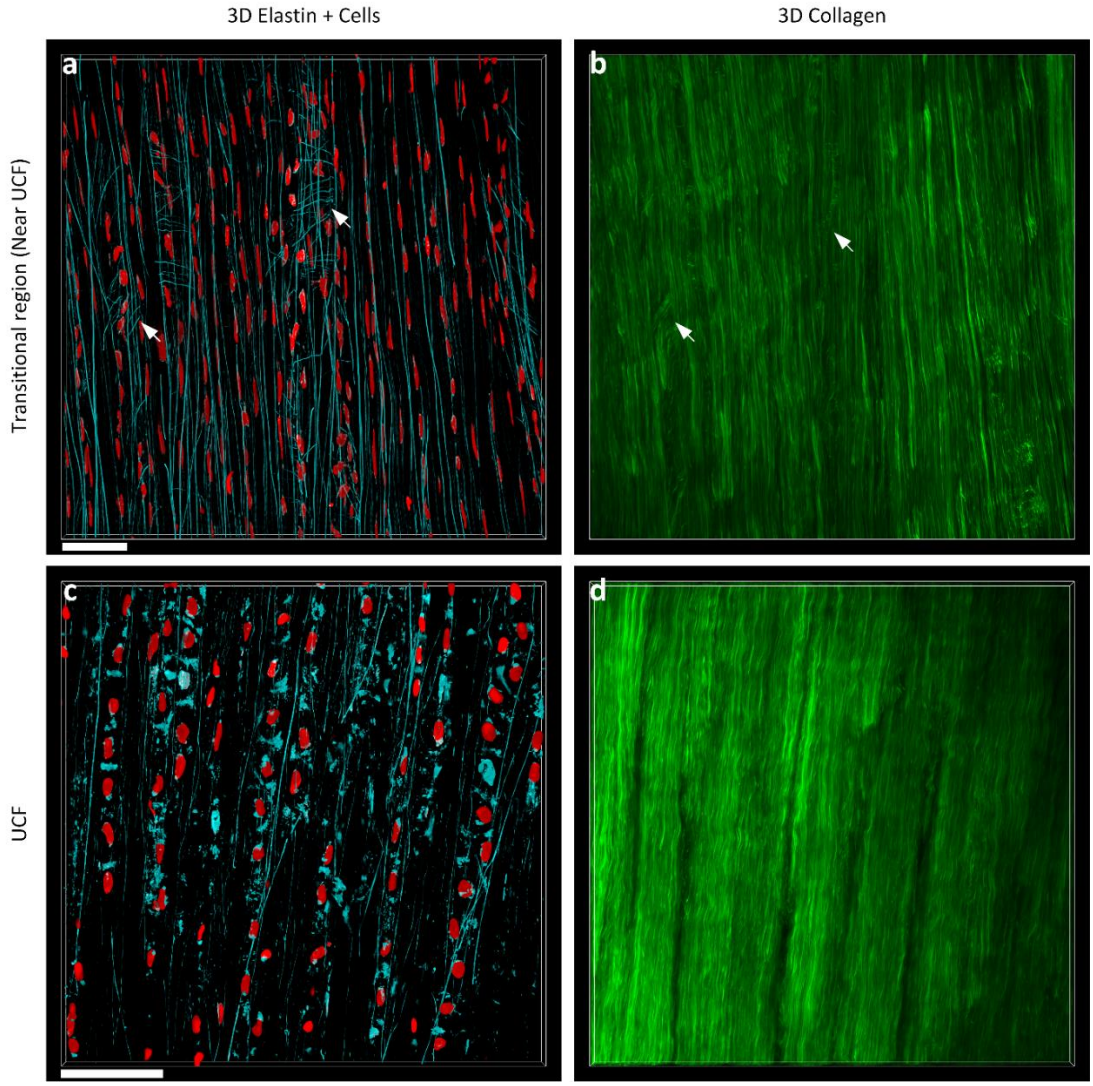

Fig. 4.3 The cells (red), the elastic fibres (cyan) and corresponding collagen (green) in the transitional region near the UCF (a, b) and the UCF (c, d) of Achilles tendons. Scale bar: 50 µm. The white arrows in a-b show transverse elastic fibres and the corresponding transverse collagen fibrils.

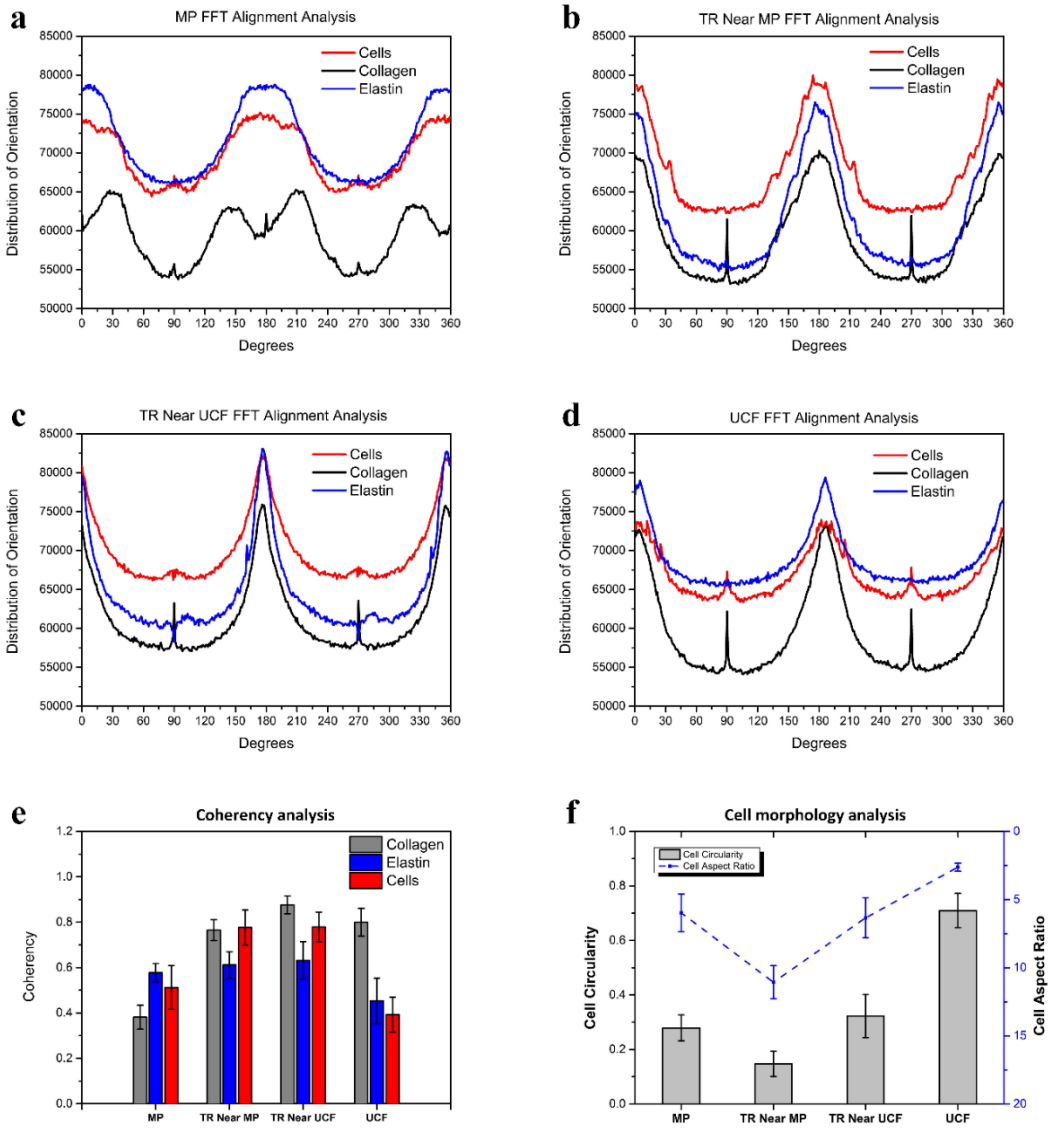

Fig. 4.4 FFT alignment analysis (a-d) and coherency analysis (e) of the collagen, elastin and cell organisation in the mid-portion, transitional region near the mid-portion, transitional region near the UCF region and UCF region of Achilles tendons. The morphology analysis of the cells in the corresponding regions (f).

4.4.4 Collagen, elastin and cells from the uncalcified fibrocartilage to bone

Fig. 4.5 shows in detail the microstructural arrangement of the collagen, elastin and cells from the UCF to CF. The collagen forms into bundles in the UCF region (shown in **Fig. 4.5**b, d) that pass the tidemark to continue in the CF (white arrow in **Fig. 4.5**d). As shown in **Fig. 4.5**a, elastic fibres and cell columns are sparsely distributed within the abundant collagen bundles in the UCF to pass through the tidemark to continue in

the CF region (white arrows in **Fig. 4.5**a, c). The fibrocartilage cells align in the UCF tissue and become sparse in the CF. The continuity of the fibrillar matrix in the UCF and CF has been confirmed by SEM (**Fig. 4.5**e). This demonstrates that the anchorage at the tidemark of Achilles tendons is mainly achieved by the continuity of the collagen fibrils and elastic fibres between the two regions.

On the other hand, the interface of the CF tissue and the bone is shown in **Fig. 4.5**f. It is clear that the collagen fibrils in the CF are not connected to the bone. This supports the view that irregular interdigitating of the cement line (CL in **Fig. 4.1**c) serves a role in the anchorage between the CF tissue and the bone.

4.4.5 Fibrocartilage cells in the uncalcified fibrocartilage tissue near the tidemark

Figs. 4.6 a-c show the detailed microstructure of the collagen, elastin and cells near the tidemark. Both the elastic fibres and collagen in the UCF orient approximately perpendicularly to the tidemark (**Fig. 4.6**a). It is noticeable that there are two forms of fibrocartilage cells. A small volume of fibrocartilage cells are prominently caged by thick elastin 'clouds' (arrows in **Fig. 4.6**a-c). The remaining majority of cells are enclosed by a thin pericellular elastin membrane (white arrowhead in **Fig. 4.6**b, c). The existence of these two types of cells in the UCF near tidemark are confirmed by SEM observations (white arrow and arrow head in **Fig. 4.6**d).

4.4.6 Cells morphological alterations from the mid-portion to the bone

The SEM images in **Fig. 4.7** show in detail significant morphological alteration of the cells in Achilles tendons from the mid-portion to the bone. The tenocytes in the tendon proper are elongated and lie directly inside the fibrillar matrix (**Fig. 4.7**a). Towards the UCF, the cells become round and are enclosed in lacunae and rest in the fibrillar matrix (**Fig. 4.7**b, c). The round fibrocartilage cells in the UCF and CF region generally align in rows but the osteocytes in the bone matrix are evenly distributed (arrow in **Fig. 4.7**e, f). The osteocytes are stellate or oval (white arrow in **Fig. 4.7**f) lying in the lacunae.

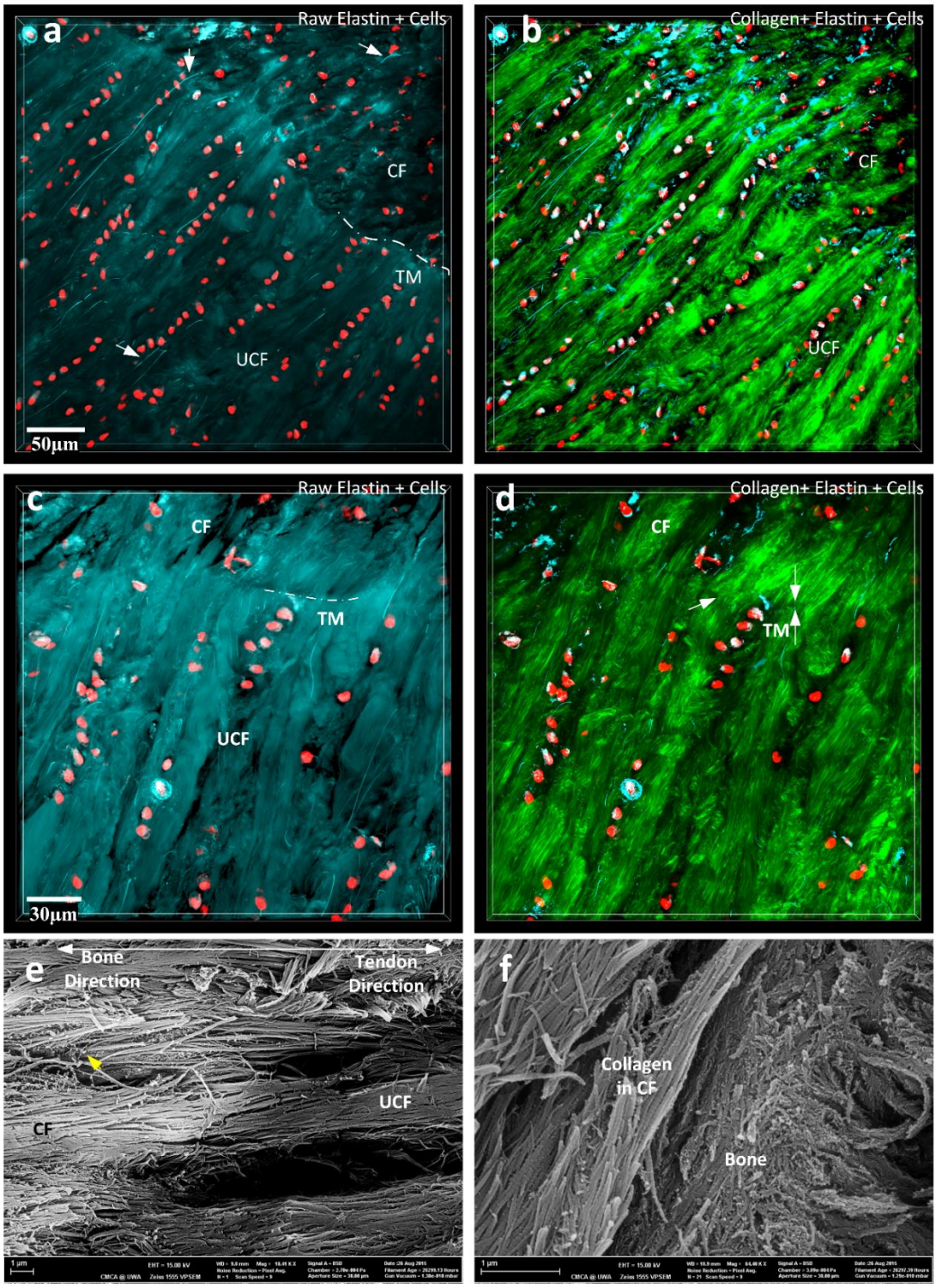

Fig. 4.5 3D images of the fibrocartilage cells (red), elastic fibres (cyan) and collagen (green) in the tidemark region at the enthesis of Achilles tendons. The SEM image (e) shows the fibres in the UCF and CF to be continuous within the tidemark region. The SEM image (f) at the cement line showed the collagen fibrils in the CF region is disconnected from the bone. (a) and (b), (c) and (d) are of the same scale.

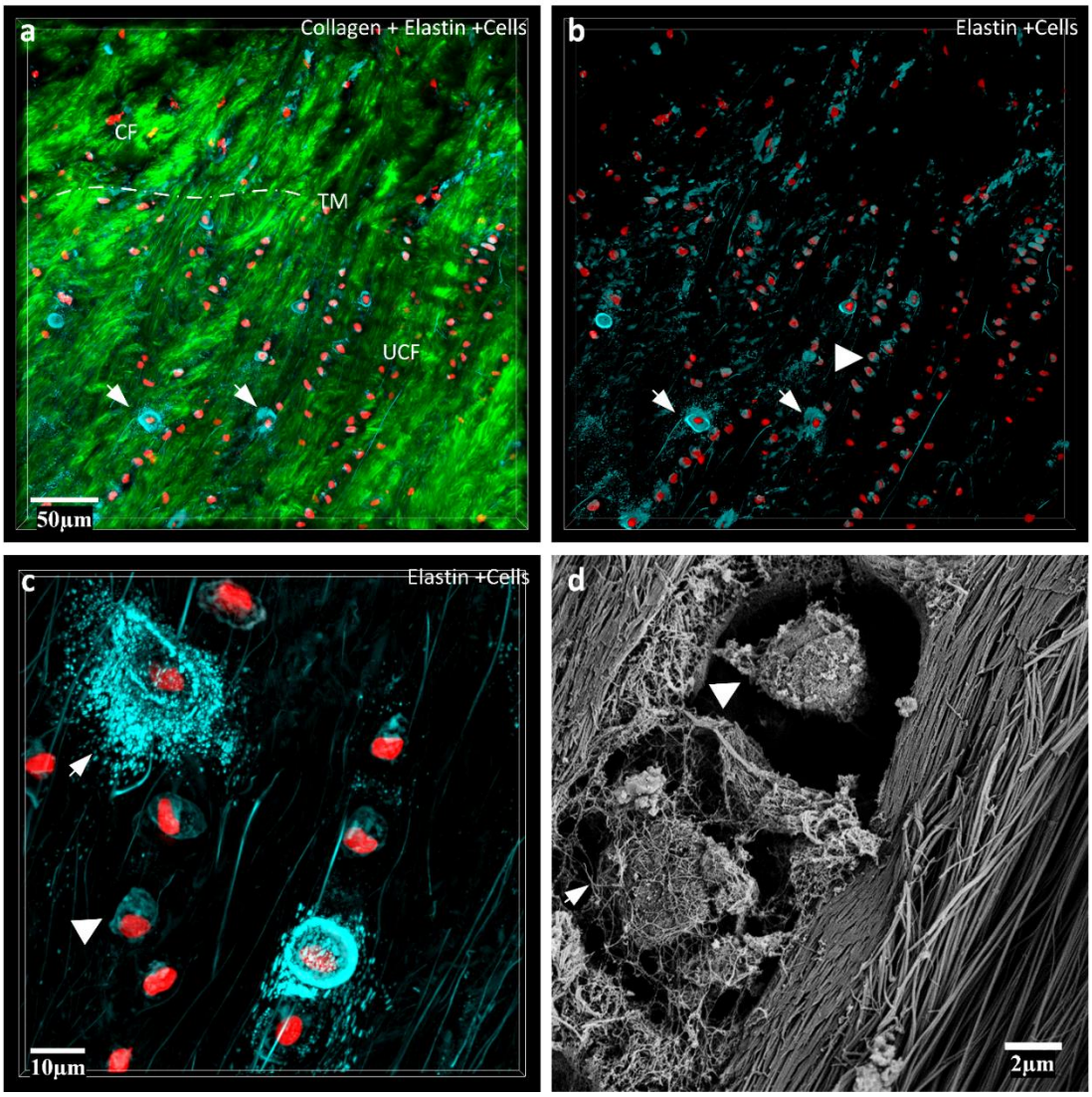

Fig. 4.6 At the region of UCF near the tidemark, fine elastin formed a pericellular matrix (arrow head) around most of the fibrocartilage cells. It is worthy of note that some of the fibrocartilage cells were surrounded by a thick elastin 'cloud' (arrows), while some were surrounded by a thin elastin membrane (arrowheads). (a) and (b) are of the same scale.

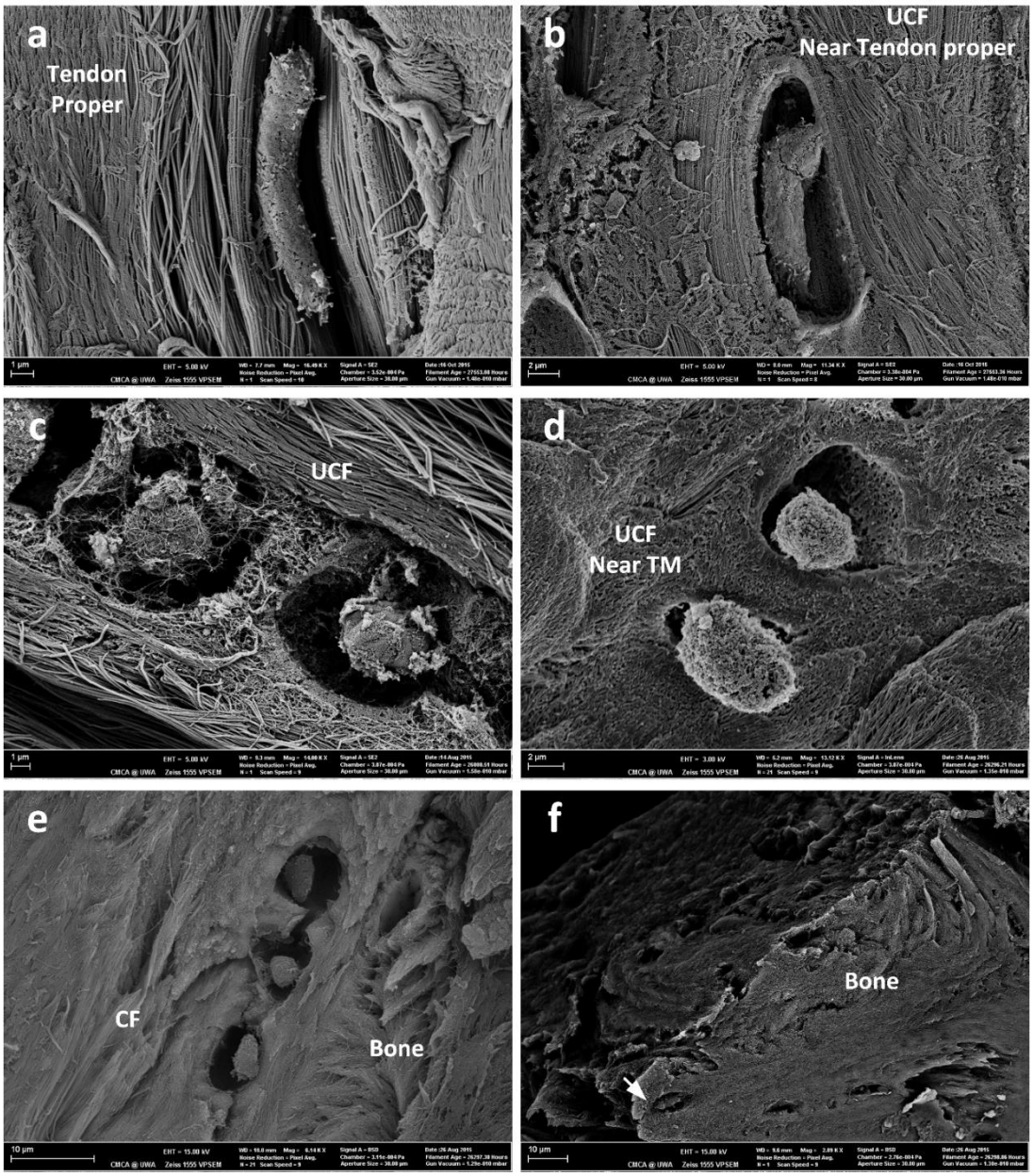

Fig. 4.7 SEM images confirm that the morphology of cells changes from the tendon proper towards the bone: a. cells in tendon proper; b. cells in the UCF region near tendon proper; c. cells in the majority of the UCF region; d. cells in the UCF region near the tidemark; e. cells in the CF region; f. cells in the bone (arrow).

4.5 Discussions

Securely anchoring on the calcaneus bone is a prerequisite for Achilles tendons to transfer the muscular contractive forces and movement to a foot. This study has systematically examined the 3D microstructure of collagen fibrils, elastic fibres and nuclei/cells of Achilles tendons from the mid-portion to the enthesis using confocal and SHG microscopic techniques without tissue dehydration. Therefore, the findings closely represent the native state of the tissue. It has been clearly demonstrated in this study that there is a gradual microstructural transition of the collagen, elastin and cells from the mid-portion to the bone of Achilles tendons (Figs. 1-3). As shown in **Fig. 4.2** and **Fig. 4.3**, the collagen fibrils and elastic fibres become less crimped towards the fibrocartilage enthesis. Since the crimp waveforms of collagen fibrils and elastic fibres are crucial to the flexibility and initial deformation of a tendon (Franchi et al, 2007; Miller et al, 2012), the gradual decrease of the crimp waveforms of the collagen and elastin observed in this study may imply the tissue becomes more rigid as moves close to the enthesis. The change in microstructure and composition would ensure a smooth transition of mechanical properties from the mid-portion to the enthesis, and an adaption of the tissue from being primarily responsible for resisting tensile forces to withstanding compressive and shear forces.

The main function of elastin is to endow a tissue with its elastic properties for a rapid recovery from deformation (Ritty et al, 2002). Thus, the gradual decline of elastin concentration from the mid-portion to the UCF (**Fig. 4.2**a, c, **4.3**a, c) observed in this study also indicates that the tissue become more and more rigid when close to the enthesis of a Achilles tendon. The extremely low concentration of elastic fibres in comparison with the predominant collagen in an Achilles tendon limits the observation of elastic fibres at low magnification (**Fig. 4.1**a, b) by confocal microscopy but they are clearly seen in high magnification observations (**Fig. 4.5**a-d, **Fig. 4.6**a-c).

It has been observed in this study that a close microstructural relationship between elastin and tenocytes (Pang et al, 2016) extends from the mid-portion to the fibrocartilage enthesis (**Fig. 4.2**a, c, **Fig. 4.3**a, c). Approaching the enthesis, the content of fibrocartilaginous tissue showed an obvious rise (**Fig. 4.2-4.3**). Studies (Thomopoulos et al, 2003; Waggett et al, 1998) have demonstrated that the cartilage-

like molecules in the fibrocartilaginous tissue around the fibrocartilage cells plays an important role in retaining water and withstanding shear force.

The main functions of the enthesis are anchorage and stress dissipation (Benjamin et al, 2006). Anchorage, as the basic function of enthesis, is achieved by the architecture of two important interfaces, the cement line and the tidemark. The CF has been viewed as 'metaplastic bone' by some scholars (Haines & Mohuiddin, 1968). The cement line can be viewed as an interface between two hard tissues, the CF and the subchondral bone. The observations in this study indicate that the extracellular matrix in CF were not continuous with the bone (**Fig. 4.5**f). This supports the view that anchorage at the cement line is achieved by the interlocking of the bone matrix into the CF tissue. These observations were in accord with many studies (Benjamin et al, 2000; Clark & Stechschulte, 1998; Cury et al, 2016; Zhao et al, 2014), especially the study by Milz et al. (Milz et al, 2002) that demonstrated the interlocking interface of bone and CF to be important in promoting attachment. On the other hand, the collagen and elastin in the mid-portion of an Achilles tendon extend along the length of the tendon and pass through the tidemark and UCF to anchor to the bone (arrows in **Fig. 4.5**. a, b). This indicates that collagen fibrils and elastic fibres play an important role in the anchorage of Achilles tendons from the UCF and CF (**Fig. 4.5**). Furthermore, the significant decrease of the content (**Fig. 4.1**a, b) and mineralisation of the collagen and elastin (**Fig. 4.1**d, **Fig. 4.5**e) from the CF to UCF of the tendon-bone chain may indicate that the enthesis is vulnerable to breakage.

Two forms of elastin have been observed within the Achilles tendon enthesis: amorphous (arrow in **Fig. 4.3**c) elastin and elastic fibres (**Fig. 4.2**-**4.3**, **4.5**-**4.6**). The amorphous elastin 'cloud' observed in this study is similar to the intermediate filaments attached to the surface of chondrocytes in articular cartilage that provide a microenvironment for chondrocytes to sense mechanical changes while protecting the chondrocytes from harsh shearing and compressive forces (Benjamin et al, 1994; He et al, 2013a). The function of the elastin 'cloud' around fibrocartilage cells in the Achilles tendon enthesis could play the similar function to that of the chondrocytes.

There were two types of fibrocartilage cells observed in the UCF (**Fig. 4.7**), the fibrocartilage cells with a thin elastin membrane and the fibrocartilage cells with a

thick elastin ‘cloud’ around. The biological function of the two types of fibrocartilage cells have not been examined in this study, and remains a potential focus for future studies.

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CHAPTER 5 The microstructure of the paratendineous tissues in the mid-portion and region near the enthesis of Achilles tendons

(Manuscript in preparation)

5.1 Abstract

A human Achilles tendon connects the gastrocnemius and soleus muscles to the calcaneus bone. It also transfers the muscle contractive stresses to the bone, enabling the normal use of a foot. The Achilles tendon is the largest tendon in humans and is subjected to frequent uses and a wide range of dynamic stresses leading to injuries, tears and pathological degeneration. The main function of an Achilles tendon is to transmit muscle contractive forces to calcaneus. However, due to the unique anatomy, different regions of an Achilles tendon can be exposed to different mechanical environments. The mid-portion of an Achilles tendon mainly withstands for tensile forces, whilst the region near the enthesis of an Achilles tendon experiences more complex mechanical forces including the tensile, shearing and compressive stresses when articulating with the calcaneus bone. This leads to development of the different forms of microstructural degeneration. Despite the paratendineous tissues are essential to the function of an Achilles tendon, their microstructure has not received much of attention in previous studies. Utilising a confocal and second harmonic generation (SHG) microscopic technique, this study systematically examined the 3-dimensional (3D) microstructure of the tendon proper and paratendineous tissues in the mid-portion and region near the enthesis. The results show that the 3D microstructure of the paratendineous tissues is distinctive in the two regions. The information provided in this study allows understanding of the influence of the mechanical environment on the microstructure, function and pathology of an Achilles tendon.

5.2 Introduction

A human Achilles tendon connects the gastrocnemius and soleus muscles to the calcaneus bone and transfers the muscle contractive forces to the bone to facilitate normal use of a foot. The main function of the tendon is to withstand for tensile stresses. Despite this, tissues in different regions/portions of an Achilles tendon bear different mechanical stresses. The microstructure of the tissues is naturally designed to allow them to fulfil the functional requirements. The mid-portion of an Achilles tendon is mainly exposed to tensile forces. The collagen fibrils align longitudinally to maximize the tensile strength of the tissue (Kannus, 2000; Smith et al, 2013). The cells, whose function is to synthesize and regulate the extracellular matrix to meet the physiological requirements, have an elongated shape and are arranged in rows parallel to the collagen fibrils. Recent studies (Gosline et al, 2002; Grant et al, 2013; Pang et al, 2016; Smith et al, 2014) have reported that the mid-portion of a tendon is composed of a small volume of elastic fibres to endow the tissue with the elastic properties, which increases the storage of elastic energy and permits the tissue to recover rapidly from deformation. The elastic fibres, orienting to conform to the orientation of longitudinal collagen fibrils, have been found to have a close microstructural relationship with the tenocytes (Pang et al, 2016). The series connection between the elastic fibres and tenocytes has been suggest to be a possible way that the tenocytes perceive changes of the mechanical environment (Pang et al, 2016).

In comparison with the mid-portion, regions where a tendon wraps around the pulley or attaches to a bone, for example, the regions near the enthesis and at the enthesis of an Achilles tendon, experience more complex mechanical forces than just the simple one directional tensile loads (Benjamin et al, 2004; Benjamin & Ralphs, 1998). The tissues in the regions possess a unique microstructure to allow them to adapt to the mechanical environment. Near the enthesis of an Achilles tendon, the tendon tissue is covered by a sesamoid fibrocartilage surface in contact with the periosteal fibrocartilage covering on the calcaneus bone. The tendon tissue is under both tensile and compressive stresses during normal activities.

Paratendineous tissues are often referred to as the endotenon, epitenon and paratenon of a tendon (Stecco et al, 2014). They are not responsible directly for the primary

mechanical properties of a tendon but they are essential to the function of a tendon. Endotenon primarily contributes to construct the hierarchical structure of tendons (Franchi et al, 2007), which provides the tendons with the flexibility to extend under tensile stresses (Thorpe & Screen, 2016). The hierarchical fibrillary arrangement fundamentally prevents a tendon from being a rigid tissue. The endotenon is essential to the interfascicular sliding within a tendon (Blasi et al, 2014; Franchi et al, 2007). Therefore, the endotenon, which is also referred as the interfascicular matrix/tissues in some studies, has been suggested to provide a tendon with the elastic recoil property for interfascicular sliding (Screen et al, 2004; Thorpe et al, 2015; Thorpe et al, 2016; Thorpe et al, 2012). The interfascicular sliding within a tendon is crucial to the extension of a tendon and storage of elastic energy within the tissue (Screen et al, 2004; Thorpe et al, 2015; Thorpe et al, 2016; Thorpe et al, 2012).

Although there is a divergence in the terminology describing the hierarchical structure of a tendon (Harvey et al, 2009; Kannus, 2000; Pang et al, 2016), it has been generally reached a consensus that a fascicle is a bunch of longitudinal collagen fibrils which are enveloped by endotenon (Kannus, 2000; Smith et al, 2013). The endotenon of a tendon can further penetrate into a fascicle and divide the longitudinal fibrillar matrix into different subunits (Blasi et al, 2014; Stecco et al, 2014; Thorpe et al, 2015; Thorpe et al, 2016). Furthermore, a group of fascicles in different sizes gathers together to make up a tendon, which is wrapped tightly by the epitenon (WU et al, 2017). The epitenon of a tendon physically adheres to the surface of the tendon proper (Nisbet, 1960). It moves together with the tendon proper (Nisbet, 1960) and has a critical role in keeping the microstructural integrity of the longitudinal collagen fibrils (WU et al, 2017). It has been suggested that the fibrillar matrix of the endotenon is derived from the epitenon (Blasi et al, 2014; Stecco et al, 2014; Thorpe et al, 2015; Thorpe et al, 2016).

The paratenon of a tendon refers to the loose areolar connective tissue or sleeve outside the epitenon (Kannus, 2000). The paratenon is connected to the epitenon via loosely coiled fibres (Jozsa et al, 1991; Nisbet, 1960; Stecco et al, 2014; Strocchi et al, 1985). The paratenon permits near friction free movement of a tendon amongst the surrounding tissues (Jozsa et al, 1991; Kannus, 2000). In comparison with the avascular, alymphatic and aneural tendon proper, the paratenon conveys blood vessels,

nerves and lymphatics, which deliver nutrients into and discharges the waste outside the tendon proper (Blasi et al, 2014; Franchi et al, 2007; Stecco et al, 2014).

Despite the paratendineous tissues have an important role in the structure, biology and function of a tendon, most of the studies have only concentrated on examining the microstructure and degeneration of the tendon proper that are responsible for the principal tensile property of a tendon. The paratendineous tissues, especially those in regions exposed to different environments, have received little attention in previous studies.

Multiphoton microscopy possesses a 3D imaging capability for studying the 3D microstructure of collagen, elastin and cells in chondral and connective tissues without tissue dehydration (He et al, 2013a; He et al, 2013b; Pang et al, 2016; WU et al, 2017). Collagen generates SHG signals under an intensive laser pulse. SHG microscopy allows study of the collagen microstructure of biological tissues without tissue staining and dehydration (Campagnola & Loew, 2003; Theodossiou et al, 2006). Using confocal and SHG microscopy within a multiphoton microscopic system, a previous study has shown that the mid-portion of an Achilles tendon possesses a unique 3D structure of collagen and elastin matrix that is critical to the tensile properties and homeostasis of tenocytes of the tissue (Pang et al, 2016).

It is hypothesized in this study that the tendon proper and paratendineous tissues in the mid-portion and region near the enthesis of an Achilles tendons possess a unique collagen and elastic fibrillar microstructure. The application of the confocal and SHG microscopic technique (Pang et al, 2016) to systematically study the 3D microstructure of collagen, elastin and cells of the paratendineous tissues will facilitate better understanding of the function and pathogenesis of Achilles tendons. Using a rabbit model, the aim of this study was to utilise the confocal and SHG microscopy and scanning electron microscopy (SEM) to systematically investigate the microstructure of the paratendinous tissues in the mid-portion and region near the enthesis of Achilles tendons. The study will lead to a better understanding of the 3D microstructure and function of Achilles tendons.

5.3 Methods

5.3.1 Sampling

Six Achilles tendons including the tendon enthesis and entire sheath were dissected from 3 healthy New Zealand white rabbits of 18 to 19 weeks old. The tissues were washed with phosphate buffered saline (PBS) to thoroughly clean blood from the surface. Six tendon blocks of about 5 mm thick were cut transversely from the mid-portions of the Achilles tendons (the large dash box in **Fig. 5.1**a). Another six blocks of about 3 mm thick were cut transversely from the region near the enthesis (the small dash box in Fig. 1a). Four blocks were randomly chosen from each of the sample groups and used for confocal and SHG study. The remaining samples were used for SEM examinations.

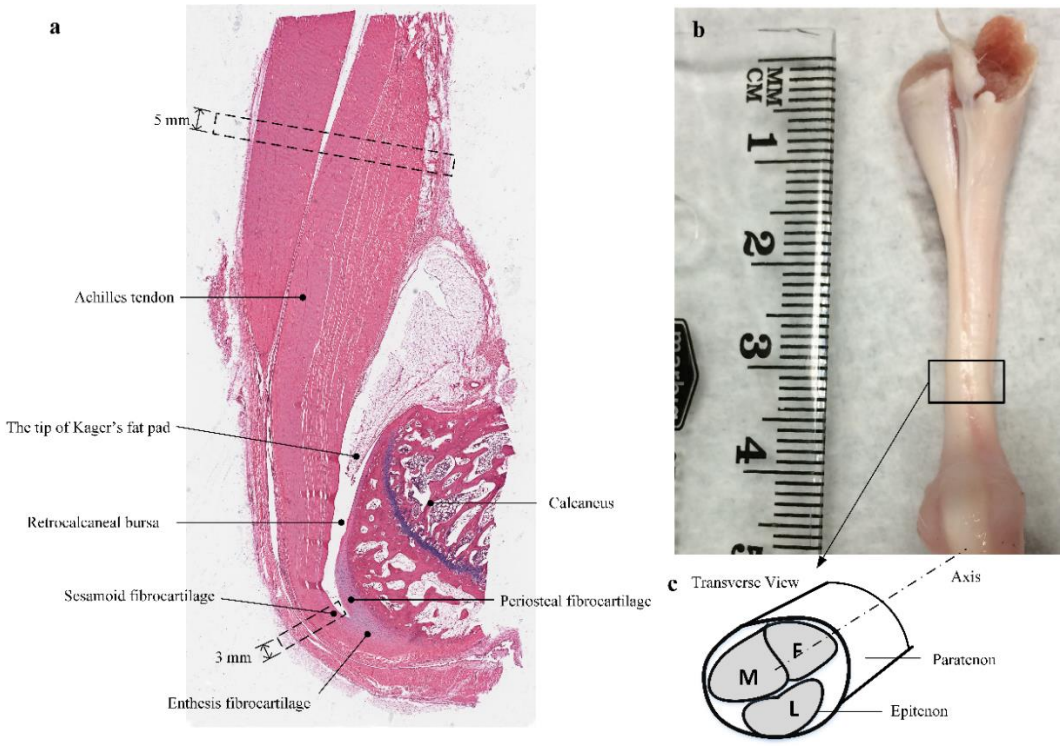

Fig. 5.1 a. A hematoxylin & eosin (H&E) histological image in the sagittal plane shows the regions (dash boxes) where the samples were harvested for this study. **b**. The mid-portion of a rabbit Achilles tendon comprises three independent sub-tendons: medial gastrocnemius tendon (M), flexor figitorum superficialis tendon (F) and lateral gastrocnemius tendon (L). **c**. A schematic diagram of the mid-portion of Achilles tendon in transverse view.

5.3.2 Cryo-sectioning

The samples used for confocal and SHG studies were embedded in optimal cutting temperature (O.C.T.) compound (VWR International Ltd., Leuven, Belgium) for cryo-sectioning transversely into 20 μm thick sections. There were 50 sections for each portion were prepared for confocal and SHG microscopic examination. The samples used for SEM imaging were cryo-sectioned transversely into 100 μm thick sections. All of the cryo-sections were placed on glass slides.

5.3.3 Confocal and SHG imaging and image processing

The sections used for confocal and SHG imaging were washed with PBS to remove the O.C.T compound (VWR International Ltd., Leuven, Belgium), and stained with 0.03 g/L Acridine Orange (AO) solution for 3 minutes to stain the cell nuclei. After washing with PBS thoroughly, the sections were stained with 1 mg/ml Sulforhodamine B (SRB) solution for 1 minute to stain the elastin. After washing with PBS thoroughly, the samples were covered with coverslips and sealed for imaging.

A Leica TCS SP2 multiphoton microscope (MPM) integrated with multiple laser sources, including a Spectra Physics Mai Tai Titanium Sapphire laser for SHG imaging, was used to acquire image stacks of the collagen, elastin and cells in Achilles tendons through three independent imaging channels simultaneously (He et al, 2013a; He et al, 2013b; Pang et al, 2016; WU et al, 2017). In this study, the wavelength of excitation laser used for SHG imaging was set at 890 nm. The SHG signals from the collagen were collected at 445 nm wavelength by the secondary non-descanned detector. The elastin stained by SRB was excited at a wavelength of 561 nm wavelength by a diode-pumped solid-state laser. The emission signals were collected at wavelengths of 565-600 nm via a photomultiplier to convert into digital images. The cell nuclei stained by AO were excited at a wavelength of 514 nm by the Krypton-Argon ion laser. The emission signals were collected at 590-680 nm. The image stacks were acquired using an image step size of 0.5 μm.

Since the confocal images of elastin contained homogeneous background noise, the subtract background function in ImageJ (NIH, Bethesda, MD) was used to subtract the background noise in the image stacks. After this, the image stacks, which were acquired from the three independent imaging channels for collagen, elastin and cell nuclei, were merged and reconstructed into 3D images using Imaris 7.4.2 (Bitaplane, Concord, MA) to study the 3D microstructure of the paratendineous tissues in Achilles tendons. In this study, the collagen, elastin and cell nucleus were assigned as green, cyan and red colours respectively in a merged image.

5.3.4 SEM sample processing and imaging

After cryo-sectioning, the sections used for SEM imaging were washed with PBS to thoroughly remove the O.C.T compound before being fixed in 2.5% glutaraldehyde for 24 hours. After fixation, the sections were washed with PBS again to remove the 2.5% glutaraldehyde, and dehydrated in ethanol solutions in a series of concentrations of 50%, 70%, 95% and 100%. The sections were then immersed in 100% ethanol and transferred into a critical point dryer (KE 3100) for critical point drying of the specimens. The dried sections were mounted on stubs and coated with gold for SEM imaging using a Zeiss SEM (Zeiss 1555 VP-FESEM).

5.4 Results

5.4.1 The paratendineous tissues in the mid-portion

Collagen, elastin and cells of the epitenon

As an important paratendineous tissue of a tendon, the epitenon in the mid-portion of Achilles tendon wraps tendon fascicles into an integrated unit that provides the tendon with the tensile strength. Anatomically, a rabbit Achilles tendon in the mid-portion contains three sub-tendons (**Fig. 5.1**b), which are separated by the epitenon (**Fig. 5.2**a). **Fig. 5.2**a shows the microstructural arrangement of the collagen fibrils (green), elastic fibres (cyan) and cells (red) of the epitenon (white double-headed arrow indicated in **Fig. 5.2**a) in the mid-portion of rabbit Achilles tendons. The fascicles (highlighted by

the dash circles) of the tendons are wrapped by the epitenon that is made of layers of collagen fibrils (white hollow arrows indicated in **Fig. 5.2**a), a small volume of elastic fibres (**Fig. 5.2**a-c) and cells (**Fig. 5.2**a, b, d). Clearly, the epitenon has an outer layer rich of elastin (white arrow in **Fig. 5.2**c) and cell (**Fig. 5.2**d). Both the 3D images (**Fig. 5.2**a) and SEM images (**Fig. 5.2**e, f) show that the endotenon matrix that divides the longitudinal collagen and elastic fibrillar matrix into fascicles (dash circles in **Fig. 5.2**a) is derived from the epitenon (**Fig. 5.2**a, e, f).

Endotenon fibrillary matrix

A large branch of endotenon matrix derived from the epitenon enters into the longitudinal fibrillar matrix (white arrows in **Fig. 5.3**a, c) and divides the longitudinal fibrils into a hierarchical structure (**Fig. 5.3**c, e, f).

As shown in **Fig. 5.3**a-d, the endotenon matrix that divides the longitudinal fibrillar matrix into various sized units contains collagen fibrils (green, **Fig. 5.3**a, e), elastic fibres (cyan, **Fig. 5.3**b, d) and cells (red, **Fig. 5.3**a, b, d). Some cells cluster at the collagen and elastic fibrillar matrix of endotenon (yellow arrow in **Fig. 5.3**b). It is interesting to see that some elastic fibres bend at their heads and hook onto the long continuous elastic fibres of the endotenon matrix (white arrowheads indicated in **Fig. 5.3**b, d). SEM images (**Fig. 5.3**e, f) confirm the endotenon collagen fibrils (characterised by the collagen D-bands, **Fig. 5.3**f) penetrate into the longitudinal collagen fibrillary matrix to divide it into a hierarchical structure. However, SEM images cannot distinguish the elastic fibres existing in the endotenon matrix.

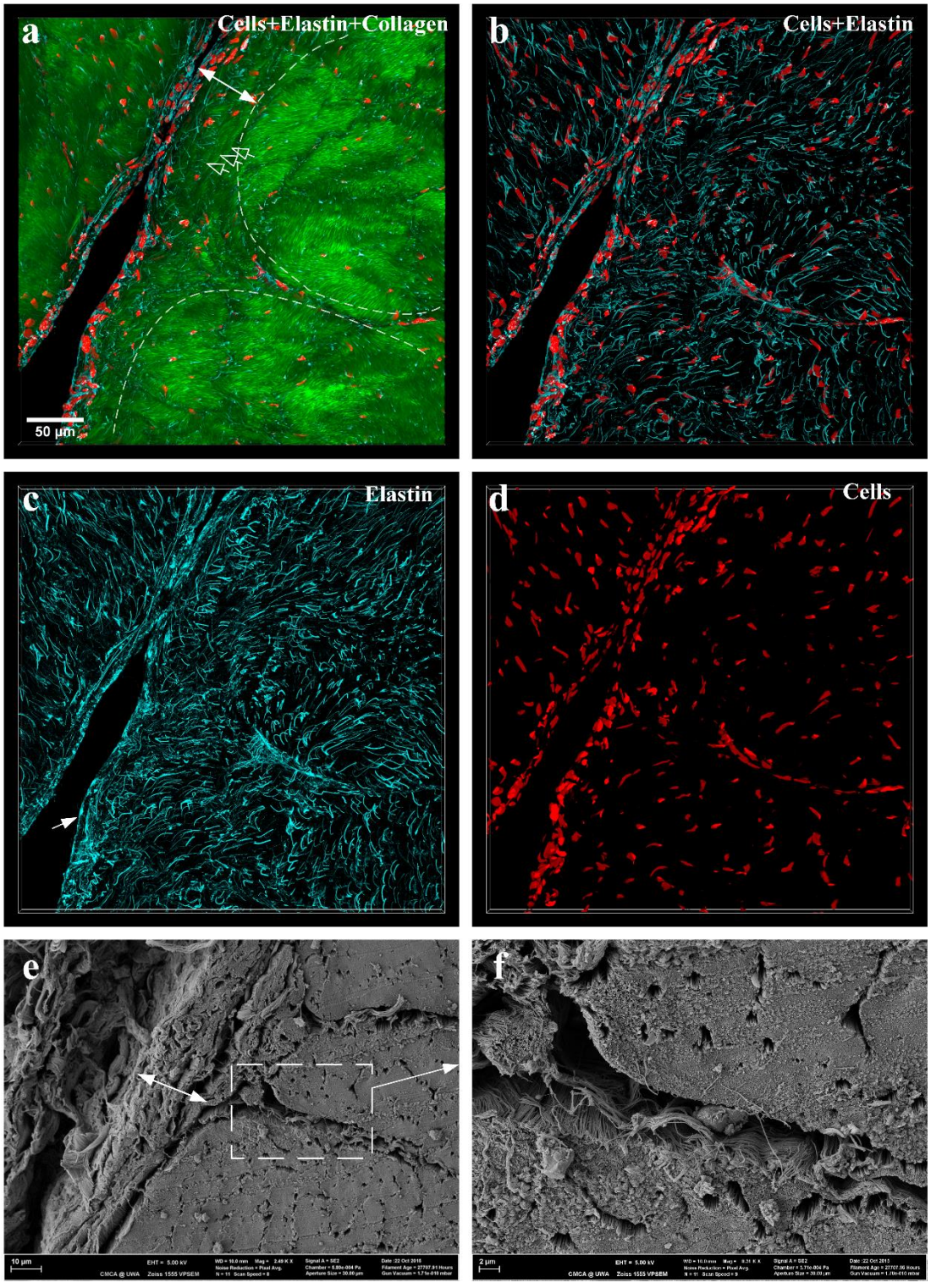

Fig. 5.2 a. A 3D confocal and SHG image in the transverse view shows the microstructure of the epitenon (white double-headed arrow) of the mid-portion of Achilles tendons that wraps the fascicles (white dash curve) in a place. Collagen (green), Elastin (cyan) and cells (red). **b**. The co-localisation of the cells and elastic network in image (**a**). **c**. The elastic network in image (**a**). **d**. The distribution of the cells in image (**a**). SEM images **e** and **f** confirm the fibrils in the endotenon is derived from the epitenon.

Hollow arrows in image (**a**) indicate the layers of the epitenon which are comparable to those shown in SEM (double headed arrow in image **e**).

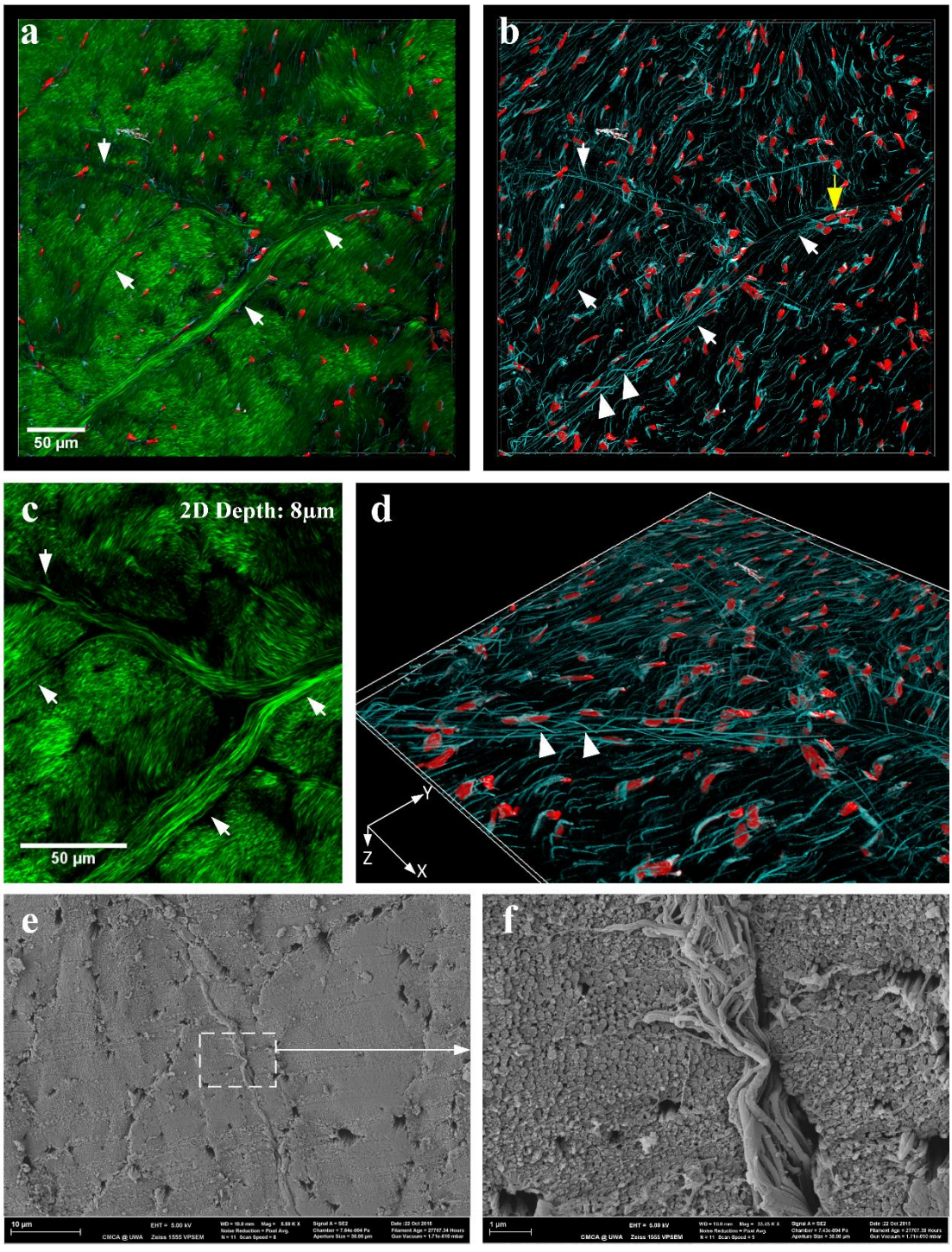

Fig. 5.3 a. A 3D image shows the microstructure of the endotenon (indicated by the white arrows) in the mid-portion of Achilles tendons that divides the longitudinal fibrils in a hierarchical order. Collagen (green), elastin (cyan) and cells (red). **b**. The corresponding elastic network and cells of image (**a**). **c**. A SHG image shows the continuity of the endotenon collagen fibril. Some bent elastic fibres (white

arrowheads indicated in **b** and **d**) are in the endotenon. SEM images **e** and **f** confirms the fibrils of endotenon divide the longitudinal fibrils in a hierarchical structure.

5.4.2 The paratendineous tissues in the region near the enthesis

Upon approaching to the enthesis of Achilles tendon, the tissue is gradually exposed to dynamic tensile, shearing and compressive stresses during normal activities. The microstructure of the paratendineous tissues is correspondingly constructed to allow the tissues to adapt to their sophisticated mechanical environment. As shown in **Fig. 5.4**a, **5.5**a, the tendon tissue near the enthesis of an Achilles tendon is covered by a sesamoid fibrocartilage surface (white double-headed arrows in **Fig. 5.4**a-b, **Fig. 5.5**a-b) to contact the periosteal fibrocartilage (**Fig. 5.1**) covering the calcaneus bone during normal use of a foot. Under a microscope, as shown in **Fig. 5.4**a and **5.5**a, the sesamoid fibrocartilage has an undulating surface. The microstructure of the concave (**Fig. 5.4**a) and convex (**Fig. 5.5**a) regions in the undulating surface is significantly different.

The concave regions

As shown in **Fig. 5.4**a, the paratendineous tissue in the concave region is composed of two contrastive zones: a sesamoid fibrocartilage surface of approximately 60 - 70 μm thick (white double-headed arrow indicated in **Fig. 5.4**a-b) and a transitional zone of about 300 μm (yellow double-headed arrows in **Fig. 5.4**a-c). The sesamoid fibrocartilage is characterized by the round fibrocartilage cells (**Fig. 5.4**a, b, e) and relatively solid matrix (white arrows in **Fig. 5.4**e). The collagen of the sesamoid fibrocartilage produces very weak SHG signals (**Fig. 5.4**b). The transitional zone underlying the sesamoid fibrocartilage surface, as shown in **Fig. 5.4**b-d, is a loose fibrillar matrix (yellow double-headed arrows in **Fig. 5.4**a-c) comprising collagen fibrils , a small volume of elastic fibres (yellow arrows in **Fig. 5.4**b, d) and rows of cells (red) align approximately parallel to the surface of the sesamoid fibrocartilage. Due to the small quantity, elastic fibres can only be observed clearly at a larger magnification (**Fig. 5.4**c-d). Although the cells in the transitional zone vary in shape, most of them are oval in shape. SEM images also confirm the concave regions contain a solid fibrocartilage surface (white double-headed arrow in **Fig. 5.4**e) and loose

matrix (yellow double-headed arrow in **Fig. 5.4**e). **Fig. 5.4**f shows the loose matrix in more detail.

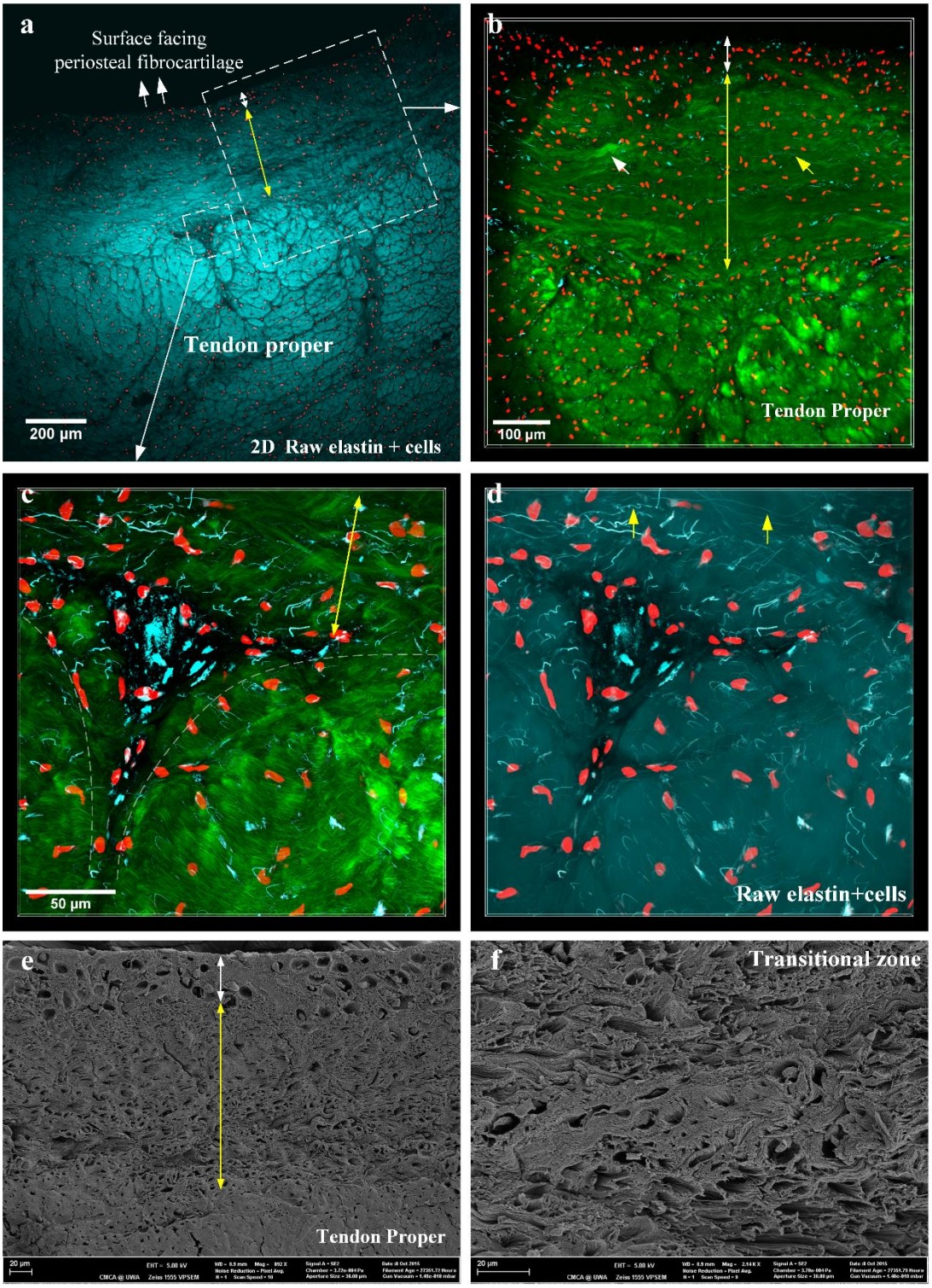

Fig. 5.4 The paratendinous tissues near the enthesis contain a sesamoid fibrocartilage layer (white double-headed arrow in **Fig. 5.4a**) to contact the periosteal fibrocartilage covering the calcaneus bone. The sesamoid fibrocartilage layer has an undulating surface containing concave regions (**Fig. 5.4**) and

convex regions (**Fig. 5.5a**). **a**. A confocal image shows that the microstructure of the concave regions is characterised by a sesamoid fibrocartilage layer (white double-headed arrow) and a thicker fibrillar transitional zone (yellow double-headed arrow) in which the fibrils orient perpendicular to the longitudinal fibrils of the tendon proper. **b**. A 3D confocal and SHG image shows in more detail the microstructural arrangement of the collagen (green), elastin (cyan) and cells (red) of the paratendinous tissue (double-headed arrows) and tendon proper in the concave regions. SEM images **e** and **f** confirm the paratendinous tissue of the concave regions to be composed a solid fibrocartilage layer (white double arrow) and loose fibrillar transitional zone (yellow double-headed arrow).

The convex regions

In contrast, the convex regions have a thick sesamoid fibrocartilage surface (**Fig. 5.5**a) which is approximately of 300 μm deep from the surface (white double-headed arrows indicated in **Fig. 5.5**). It is noticeable that there is an elastin superficial layer lying on the surface of the sesamoid fibrocartilage (cyan, white arrows in **Fig. 5.5**b). Under this elastin superficial layer, as shown in **Fig. 5.6**a-b, the fibrocartilage matrix (white double-headed arrows in **Fig. 5.5**) is composed of fine collagen fibrils and sparsely distributed elastic fibres and cells (**Fig. 5.6**a, b). Immediately underneath the thick sesamoid fibrocartilage is a dense endotenon matrix (yellow arrows in **Fig. 5.5**b; white arrows in **Fig. 5.7**a) that partitions the longitudinal fibrils into small dense fascicles (white hollow arrows in **Fig. 5.5**b; **Fig. 5.7**). The elastic fibres are sparsely distributed within the fascicles and around the tenocytes (white hollow arrow in **Fig. 5.5**b; yellow arrowheads in **Fig. 5.7**b). The endotenon matrix orients approximately parallel to the articular surface. Underneath the dense endotenon matrix is the tendon proper (**Fig. 5.5**a) containing larger fascicles.

SEM studies also confirm that a dense superficial matrix (yellow double-headed arrows in **Fig. 5.6**c-d) comparable to the elastin layer shown by confocal and SHG microscopy (cyan surface in **Fig. 5.5**a, b and **Fig. 5.6**a, b) lies on the most superficial surface of the sesamoid fibrocartilage.

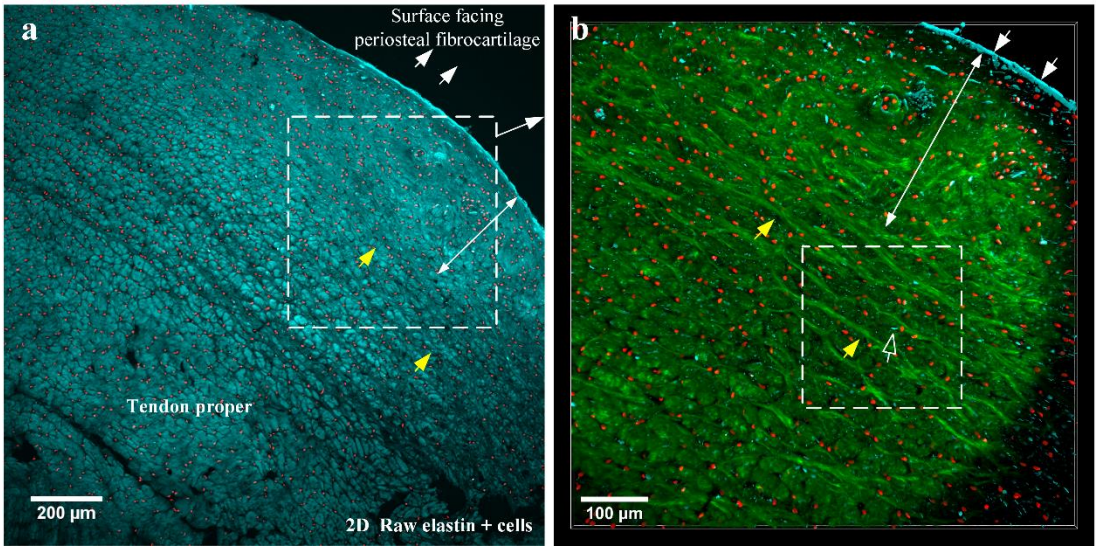

Fig. 5.5 a. A confocal image shows the convex regions are featured with a thicker sesamoid fibrocartilage (white double-headed arrow) covered by an elastin superficial surface (the cyan surface). **b**. A 3D confocal and SHG image shows the detailed microstructural arrangement of the collagen (green), elastin (cyan) and cells (red) of the paratendinous tissue in the convex regions (approximately the region indicated by the white box in **a**). A dense endotenon meshwork (yellow arrows in **a** and **b**) subjacent to the fibrocartilage (white double headed arrow) divides the longitudinal collagen fibrils into small fascicles. Elastic fibres (white hollow arrow in **b**) are sparsely distributed in the small fascicles.

5.4.3 The longitudinal fibrils

The longitudinal collagen fibrils in the mid-portion of an Achilles tendon are main tendon constitutents withstanding tensile stresses. Therefore, as shown in **Fig. 5.2**a and **Fig. 5.3**a, they appear to have a larger diameter than that of the longitudinal collagen fibrils of the tendon proper near the enthesis of Achilles tendons (**Fig. 5.4**c). Also, the tendon proper in the mid-portion has a larger volume of elastic fibres (**Fig. 5.2**c) than that near the enthesis (**Fig. 5.4**d). However, the cells of the tendon proper near the fibrocartilage enthesis of an Achilles tendon (**Fig. 5.4**c-d) are much larger and rounder than that in the mid-portion (**Fig. 5.2**a, b, d).

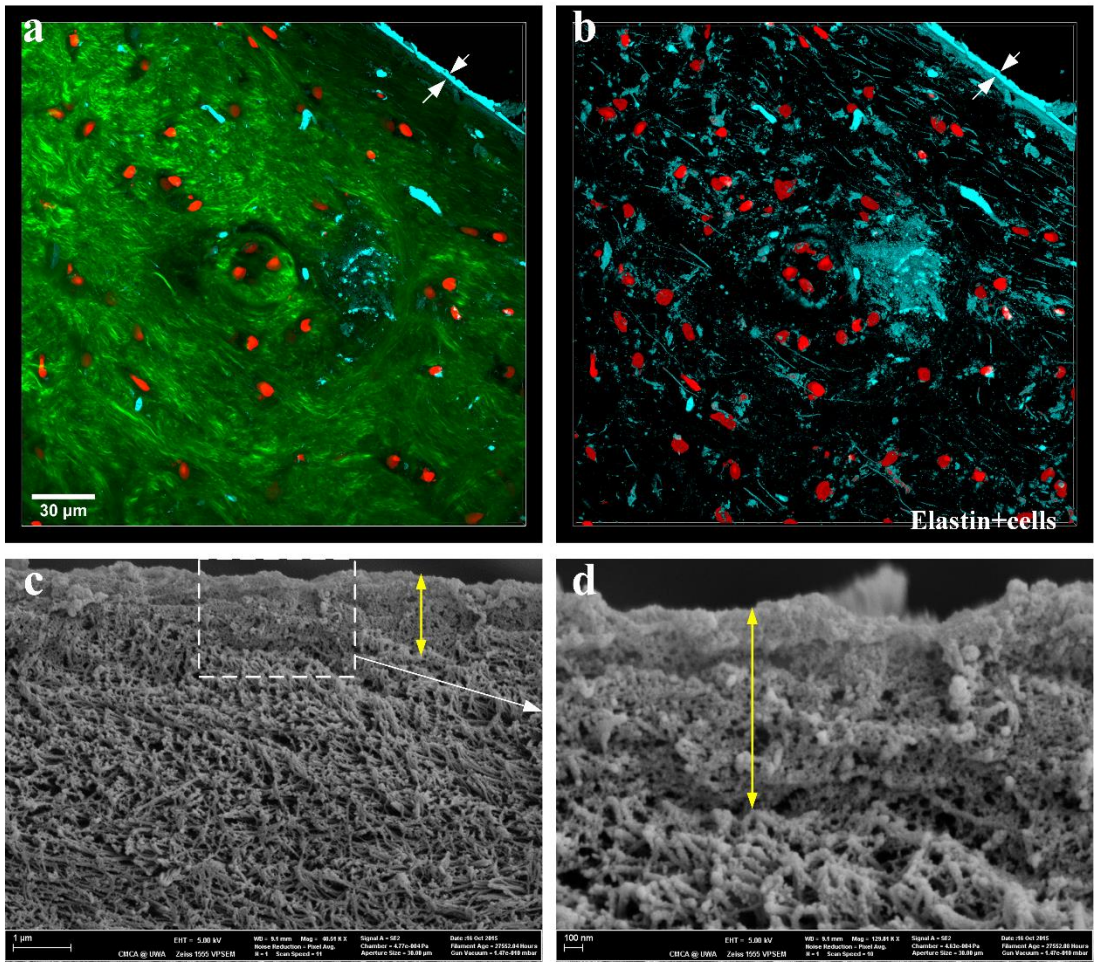

Fig. 5.6 High magnification observations of the microstructure of the sesamoid fibrocartilage in the convex regions (approximately region indicated by the double arrow in **Fig. 5.5**). **a**. A 3D confocal and SHG image shows that the sesamoid fibrocartilage is featured with an elastin superficial surface (the cyan surface) and contains fine collagen fibrils (green), a small volume of elastin (cyan) and round cell nuclei (red). **b**. A confocal image highlights the microstructural arrangement of the elastin and cell nuclei of the sesamoid fibrocartilage. SEM images **c** and **d** confirm that a dense superficial layer (indicated by the yellow double-headed arrows) is comparable to the elastic fibrillar matrix discovered by confocal and SHG microscopy (the cyan surface in **a** and **b**) which lies on the most superficial surface of the sesamoid fibrocartilage.

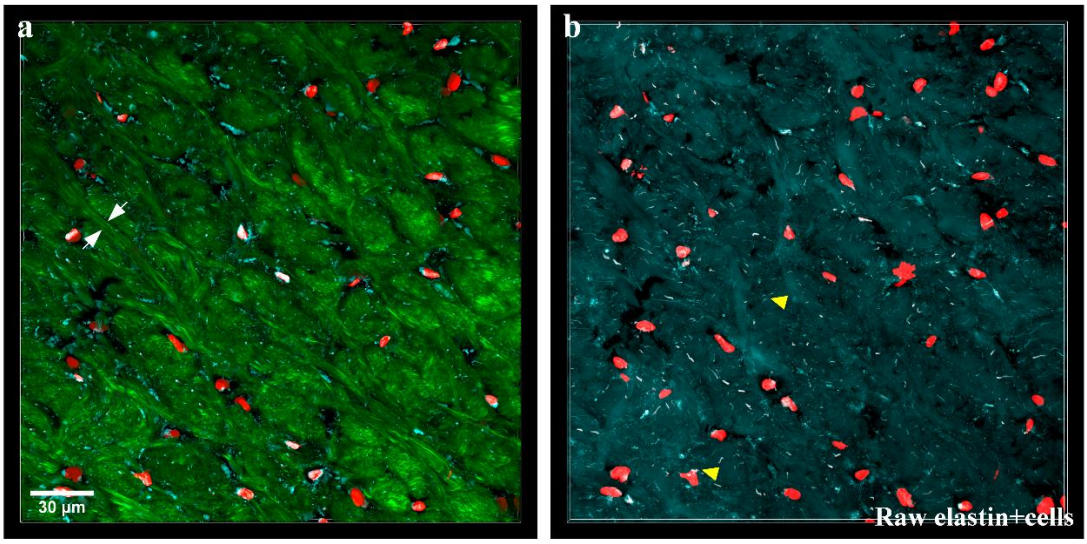

Fig. 5.7 High magnification observations of the microstructural arrangement of the collagen fibrils (green), elastin (cyan) and round cell nuclei (red) of the denser endotenon meshwork subjacent to the sesamoid fibrocartilage in the convex regions (approximately the region indicated by the white dash box in **Fig. 5.5**b). **a**. A 3D confocal and SHG image shows the dense endontenon meshwork (white arrows) partitions the longitudinal fibrillar matrix into many small fascicles. **b**. A confocal image highlights the elastin and cell arrangements of the dense endotenon meshwork. Clearly, the elastin (cyan) sparsely distributes amongst the densely packed fascicles and around the cells (yellow arrowheads in **b**).

5.5 Discussions

Unlike the mid-portion of Achilles tendons, which is designed for withstanding and transferring tensile forces, the region near the enthesis fibrocartilage adapts to endure the tensile force in the long axis direction, and compression and shear force from the tuberosity of the calcaneus (de Palma et al, 2004; Nisbet, 1960). Different mechanical environments lead to different types of injuries. Therefore, the degeneration mechanisms of the two regions are different. This study has investigated the 3D microstructure of collagen fibrils, elastic fibres and cells of the paratendineous tissues of Achilles tendons in the mid-portion and region near the enthesis. The two regions are subjected to different mechanical environments. Although the paratendineous tissues are not responsible directly for the principal tensile stress, they are the essential components of a tendon. Thus, by examining the 3D microstructure of the paratendineous tissues in regions subjected to different mechanical environments, this

study has offered a thorough understanding of the influences of the mechanical environment on the microstructure of Achilles tendons.

5.5.1 The paratendineous tissues in the mid-portion of Achilles tendons

With a relatively simple mechanical environment, the epitenon and the endotenon in the mid-portion of Achilles tendons have relatively simple microstructure organisation (**Fig. 5.2**-**5.3**). The 3D images (**Fig. 5.2**a-d) show that the epitenon of an Achilles tendon is composed mainly by collagen fibrils (green), a small amount of elastic fibres (cyan) and cells (red) that organise in a way to conform to the direction where the principal tensile stresses occur. The fibrillar matrix of the epitenon is found to be physically integrated with the longitudinal fibrils of the tendon proper (**Fig. 5.2**a), which is consistent with previous literature (Nisbet, 1960; Schlecht, 2012). The formation of an elastin outer layer (**Fig. 5.2**a) along the epitenon surface (**Fig. 5.2**c) would assist the tendon tissues to recover rapidly from deformation and reduce the sliding, shearing and wearing forces (Nisbet, 1960; Schlecht, 2012). The finding of a smooth elastin superficial layer and densely packed cell line along the surface of epitenon in this study confirms previous studies (Abrahamsson et al, 1992). The elastin outline together with the collagen fibrils will protect the densely packed fibroblast-like cells near the surface of the epitenon matrix (**Fig. 5.2**b, d) from excessive deformation. Although this current study has not examined the function of the densely packed fibroblast-like cells presenting along the surface of the epitenon, some previous studies have also reported that these densely packed fibroblast-like cells play an important role in tendon healing (Abrahamsson et al, 1992; Ackermann et al, 2016; Chang et al, 1998; Dellasanta, 1992; Kakar et al, 1998; Lyras et al, 2010).

Collagen fibrils possess great tensile properties. Their function in the epitenon is to increase the microstructural integrity of the longitudinal fibrils (WU et al, 2017) and the resistance of a tendon to the wearing and shearing force of a tendon. The endotenon of a tendon functions to enable smooth sliding and recoil between fascicles (Thorpe et al, 2015; Thorpe et al, 2016). Therefore, the combination of the layers of collagen fibrils and elastic fibres found in the ECM of epitenon and endotenon ensures a tendon with a unique tensile and elastic recoil properties during normal activities. The tensile properties of collagen fibrils allows a tendon to withstand tensile deformation while

the elastic properties of the elastic fibres allows the tendon to recover rapidly from the tensile deformation. Elastin is a highly extendible, fatigue resistant protein in tissues (Gosline et al, 2002; Lillie & Gosline, 2002; Thorpe et al, 2013; Thorpe et al, 2012). The fence-like elastin structure (**Fig. 5.3**b, d) in the endotenon may prevent the longitudinal fascicles from excessive sliding between each other. It may also enable sliding and recoil between fascicles (Thorpe et al, 2015; Thorpe et al, 2016).

The paratenon, epitenon and endotenon contain blood vessels, nerves and lymphatics (Jozsa et al, 1991; Kannus, 2000). The continuity of the fibrillar matrix between the epitenon and endotenon has been clearly demonstrated in this study (**Fig. 5.2**a, e, f). The continuity of the epitenon to the endotenon provides the anatomical basis that allows the communication of blood vessels, nerves and lymphatics in different areas.

5.5.2 The structure and function relationships of the paratendineous tissues near the enthesis fibrocartilage of Achilles tendons

As approaching to the enthesis of an Achilles tendon, the tissue gradually sustains both compressive and tensile stresses during normal activities. The sesamoid fibrocartilage surface found on the surface of the tendon prevents the longitudinal fibrillary matrix from directly taking compression and breaking into parts when articulating with the periosteal fibrocartilage capping the surface of calcaneus bone (Rufai et al, 1996). The formation of fibrocartilage on the surface of the tendon tissue indicates that the tissue endures compressive stresses (Benjamin & Ralphs, 1998).

The undulation surface of the sesamoid fibrocartilage may help storage of the lubricant for joint articulation. Since the convex regions directly contact their counterpart, they are covered by a thicker fibrocartilage surface (**Fig. 5.5**) in comparison with the concave regions. The denser endotenon collagen bundles underneath the thick fibrocartilage (**Fig. 5.5**b) orient approximately parallel to the cartilage surface while partitioning the longitudinal fibrils into small fascicles. This structural arrangement allows the fascicles to have the structural integrity to sustain a certain degree of compression without breaking into parts. The structure also endows the tissue with structural flexibility to endure deformation under the compression.

Since the concave regions are not subjected to large shearing and compressive stresses when compared to the convex regions during joint articulation, they are covered by a thin fibrocartilage surface (**Fig. 5.4**a, b). The loose parallel fibrils under the fibrocartilage surface may help the tissue to be flexible to extend during joint articulation.

While the convex regions would be under greater compression than the concave regions during joint articulation, an elastin superficial layer found on the surface will assist this convex region to recover from a deformation quickly (**Fig. 5.5**, **5.6**a, b). The dense fine fibrillar layer observed at the superficial surface of fibrocartilage in SEM images (**Fig. 5.6**c-d) is consistent with the findings of the existence of an elastin superficial surface by confocal and SHG microscopy. Other scholars have also reported the most superficial surface of the sesamoid fibrocartilage at the insertional angle region of rat Achilles tendons to be an amorphous electron dense layer (Rufai et al, 1996). However, this elastin superficial layer was not observed in the concave regions of the sesamoid cartilage in this study.

In conclusion, this study has examined the 3D microstructure of the collagen, elastin and cells in two regions of an Achilles tendon exposed to different mechanical forces. Due to the different mechanical environment, the paratendinous tissues in the mid-portion of an Achilles tendon have a different microstructure to the tissue near the enthesis. This has proven that the mechanical environment influences the formation of the paratendinous tissues of Achilles tendons. Thus, the information provided in this study will facilitate better understanding of the function and pathogenesis of an Achilles tendon.

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CHAPTER 6 Discussion and future directions

6.1 Discussions

The objective of this PhD project was to develop a confocal and second harmonic generation (SHG) microscopic method for studying the 3-dimensional (3D) microstructure and quantitatively analysing the morphological characteristics of the collagen, elastin and cells of healthy rabbit Achilles tendons.

- The 3D imaging technique developed in this project harnesses the advantages of confocal and SHG microscopy for studying the spatial microstructure of Achilles tendons simultaneously without tissue dehydration. Thus, the images closely show the microstructure of the tissues in a native state. SHG microscopy is a specialized imaging technique for studying the collagen fibrillar network in tendons without additional staining and tissue dehydration. With the aid of fluorescent staining techniques specific to a micro component of a tendon, confocal microscopy allows targeted visualisation of a micro component of interest for study. Acridine orange and Sulforhodamine B are specific fluorescent dyes for cell nuclei and elastin respectively (Ricard et al, 2007; Tatton & Kish, 1997) so that they have been used to study the cell nuclei and elastin of Achilles tendons in this study.

  This study has also developed a novel 3D imaging technique that led to the visualisation and study of the microstructural relationship of cells with collagen and elastic fibrillar matrix of Achilles tendons. Since this technique possesses higher imaging resolution than conventional optical microscopic techniques, SHG imaging has distinguished individual collagen fibrils within Achilles tendons. The most striking finding in this study was the close microstructural relationship of tenocytes with the elastic fibres, even though elastic fibres constitute only a very tiny proportion of tendons. In addition, the high resolution images have allowed successful exploration of the use of computer imaging technologies for quantitatively describing the morphology of tenocytes, and the orientation of collagen fibrils and elastic fibres.

- The mid-portion of Achilles tendons sustains mainly tensile forces. The 3D microstructure of the mid-portion, as shown in this study (Chapter 3), is correspondingly organised in a way to facilitate withstanding of the tensile stresses. The crimps of the fibrillar components in the mid-portion of Achilles tendons were visualised clearly using the 3D imaging technique developed in this study. Crimps of collagen fibrils have been well recognised to have an important role in the initial tensile deformation of tendons (Franchi et al, 2007). The measurement of the crimps of the fibrillary matrix in future studies may allow evaluating subtle functional and physiological changes in tissues.

  The collagen fibrils, elastic fibres and the elongated tenocytes show a great concordance in their orientation in the mid-portion of Achilles tendons. The series connection between the elastic fibres and the elongated tenocytes were also clearly shown in this study. The 3D imaging technique has also led to the discovery of an elastin peri-cellular meshwork around tenocytes. The close microstructural relationship between elastin and the tenocytes may suggest that elastic fibres may mediate the bio-mechano-transduction between tenocytes and the extracellular matrix (ECM). Moreover, collagen fibril spirals within the fibril bundles were visualized longitudinally and transversely in this study. The spirals create longitudinal, transverse and oblique fibril bundles which can resist the rotational forces possibly generated from different directions. The spatial arrangement of the tenocytes and the fibrillar components was well preserved and displayed by the confocal and SHG microscopic techniques, as shown in Chapter 3.
- An Achilles tendon connects to the calcaneus through a fibrocartilaginous enthesis. The Achilles tendon enthesis is a mechanical weak point but it has a very complex microstructure naturally arranged to adapt to mechanical changes. This study systematically examined 3D microstructural changes from the mid-portion to the enthesis of Achilles tendons, which has allowed a better understanding of how the micro components are arranged in a manner to support the requirement of a secure anchorage and a smooth transition from soft fibrous tissue to rigid hard tissue (Chapter 4). The findings that the crimps of the collagen and elastic fibrils/fibres gradually decrease in size and frequency, and they disappear towards the enthesis, may indicate a functional

change of an Achilles tendon from the mid-portion to the enthesis. This again implies an importance to the crimps of the collagen fibrils and elastic fibres in the function of a tendon. The content of elastic fibres also shows a decreased trend from the mid-portion to the enthesis of an Achilles tendon. This may suggest that the tissue gradually becomes more rigid and less elastic from the mid-portion to enthesis. Most importantly, these microstructural changes from the mid-portion to the enthesis of an Achilles tendon clearly demonstrate an adaption of the tissue from being responsible for tensile forces to withstanding compressive and shear forces.

Moreover, the layered microstructure of an Achilles tendon enthesis is crucial to the anchorage of an Achilles tendon to the calcaneus bone and the stress dissipation at the soft and hard tissue interface (Doschak & Zernicke, 2005). This study confirms that the anchorage of an Achilles tendon to the calcaneus bone is achieved by the continuity of collagen fibrils and elastic fibres at the tidemark and the interlocking of calcified fibrocartilage to the bone matrix at the cement line.

Cells function to regulate and maintain the extracellular matrix to meet the physiological requirements of a tissue. The morphology of cells plays a critical role in their bio-synthetic activities and gene expression (Watson, 1991; Zanetti & Solursh, 1989), for example, round chondrocytes actively synthesize type II collagen and proteoglycans but when chondrocytes are restricted from spreading, they no longer synthesize type II collagen and proteoglycans (Newman & Watt, 1988). The finding that the cell morphology changes from being elongated tenocytes in the mid-portion to the round fibrocartilage cells lying in the lacunae in the enthesis is consistent with the functional requirement of the tendon tissue. Furthermore, the fibrocartilage is a relatively rigid tissue compared to the mid-portion tendon so that the enclosure of the round fibrocartilage cells in the lacunae can protect the cells from an extreme deformation of the fibrocartilage during compression. Some fibrocartilage cells in the uncalcified fibrocartilage were observed to be surrounded by a thick elastin cloud. Their function is not clear and has not been examined in this study.

- This study has also paid particular attention to the 3D microstructure of paratendineous tissues in the mid-portion and the region near the enthesis of

Achilles tendons (Chapter 5). The mid-portion of Achilles tendons mainly sustains axial tensile forces, while the region near the enthesis sustains tensile, shearing and compressive forces in multiple directions. The paratendineous tissues in the mid-portion and region near the enthesis have contrasting microstructural arrangements that reflect the requirements of the mechanical environment. In the mid-portion, the endotenon is responsible for dividing the longitudinal fibre bundles into different levels of fascicles, which facilitates the interfascicular sliding and enables the tendon tissue being more flexible for a tensile deformation. The epitenon firmly wraps the fascicles of a tendon together to provide the tendon with its microstructural and functional integrity. By contrast, the deep surface of the Achilles tendons near the enthesis is covered by a sesamoid fibrocartilage to allow the tissue to contact with the periosteal fibrocartilage covering on the calcaneus.

Also, this study shows the sesamoid fibrocartilage has an undulating surface including concave and convex regions under a microscope. The paratendineous tissues at the concave regions have a thin sesamoid fibrocartilage superficial layer. Underneath this layer is a thick transitional zone formed by loosely oriented collagen fibrils, elastic fibres and oval shaped cells. The paratendineous tissues at the convex regions are covered by a thick sesamoid fibrocartilage layer included a thin elastin superficial surface. Underneath the thick sesamoid fibrocartilage are a dense endotenon meshwork that partitions the longitudinal fibrils into flat and small fascicles. Relatively, the convex regions sustain larger shear and compressive forces than the concave regions during joint articulation so that they have a thick sesamoid fibrocartilage layer. The different microstructural arrangement in the concave and convex regions, as found in this study, is consistent with the functional requirements of the two regions.

- This study has systematically examined the 3D microstructure of rabbit Achilles tendons from the mid-portion to the enthesis as well as from the tendon proper to the paratendineous tissues. It increases our understanding about the 3D microstructural system of healthy Achilles tendons. The 3D observations in this study have also enriched the knowledge about the spatial microstructure of Achilles tendons for understanding the mechanical and

biological roles of collagen fibrils, different forms of elastin components and the cells in tendons and the fibrocartilaginous enthesis.

- Although standard SEM and TEM have superior imaging resolution for studying the microstructure of tendons and provided important information about the collagen microstructure of tendons, they do not have a 3D imaging capability to reveal the complex spatial microstructure of the tendons in a hydrated status. SEM, TEM and traditional histology require tissue dehydration, which can cause unknown artefacts. The confocal and SHG microscopic technique possesses 3D capability and high resolution, and the hydrated samples can keep the native status of tissues. TEM is able to study the substructures of elastin in tendons, but it is difficult for traditional optical microscopy and electron microscopy to image the elastic fibres (Green et al, 2014), which work as the functional unit of elastin structure (Kielty et al, 2002). Consequently, 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. It is first time in this study, the spatial microstructural relationship of elastic fibres with the collagen fibrils and tenocytes have been revealed in high resolution 3D images. The finding of the close relationship of elastin with the tenocytes' plasma in this study could lead to develop new method for evaluating tendon pathology.
- The application of computer image analysis techniques has allowed quantitative description of the microstructural characteristics of Achilles tendons from the mid-portion to the enthesis. It promises the development of a computer imaging analysis system to quantitatively and consistently characterise the physiology of Achilles tendons in the future.

6.2 Future directions

The following directions are suggested for future studies of Achilles tendons using the developed imaging technique in this study.

- Great concordance of the collagen fibrils, elastic fibres and organisation of the elongated tenocytes has been observed in the mid-portion and enthesis of healthy rabbit Achilles tendons. Future studies could examine the possible

disruption of the concordance of the collagen fibrils, elastic fibres and the tenocytes in the mid-portion and enthesis in relation to the pathological alteration of Achilles tendons.

- Although elastin is a minor protein in Achilles tendons, it has been found in healthy rabbit Achilles tendons in different forms (elastic fibres, elastin pericellular meshwork, and elastin cloud around fibrocartilage cells). Importantly, elastin has been found to have a rather close microstructural relationship to the tenocytes than the collagen fibrils that play a crucial role in the mechanical function of Achilles tendons. Therefore, further studies could investigate if the degeneration or disappearance of the elastic fibre network in a tendon directly affect the physiological status of the tissue and disruption of the mechano-transduction process between the tenocytes and ECM.

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