

QUANTITATIVE CHARACTERIZATION OF COLLAGEN ORIENTATION IN THE SUPERFICIAL ZONE FOR STUDYING EARLY DEGENERATIVE CHANGES IN ARTICULAR CARTILAGE

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ABSTRACT

Collagen fibres in the superficial zone are critical to the durability and stability of articular cartilage. Early osteoarthritis is often characterized by lesions on the surface of articular cartilage as a result of deterioration or degeneration of the collagen meshwork in the superficial zone. Therefore, traditional histology employing visual inspection of the microstructure of collagen fibres is one of the methods frequently used to evaluate the physiological state of articular cartilage in the medical field. However, traditional histology is often limited to 2D observations. It requires physical sectioning and dehydration of articular cartilage for stereological study. In addition, visual inspection is subjective and time consuming. Confocal microscopy provides a way to study the three-dimensional collagen structure in bulk hydrated articular cartilage. Utilizing fibre optical laser scanning confocal microscopy, the aim of the present study was to apply the spectral moment as a method for quantitatively describing the orientation of the collagen fibres in the superficial zone in relation to the physiological status of the cartilage, such as distinguishing normal cartilage from the early osteoarthritic cartilage.

Keywords: Spectral moment; Confocal microscopy; Collagen orientation; Early osteoarthritis.

INTRODUCTION

During normal activities, the superficial zone of articular cartilage is subjected to various compressions, wear and shear from unpredictable directions. The orientation of the collagen fibres in the superficial zone is critical to the tensile strength, wear resistance and other mechanical properties of the articular cartilage.^{3,5,17,22} Disruption of the articular surface is a typical feature of early osteoarthritis,¹⁶ and it closely related to interruption of the collagen structure and deterioration of type II collagen fibres in the superficial zone.^{4,7,11,12,20} Therefore, studying the collagen structure offers understanding of the pathological changes in articular cartilage, and can be used to determine the physiological and biomechanical properties of articular cartilage in clinical fields.

However, traditional histology often involves using 2D microscopy to observe the 3D collagen structure. Important information about the collagen matrix could be lost and misinterpreted in the 2D observations.²⁹ For stereological study, two-dimensional microscopy requires cutting the cartilage into hundreds of micro-sections following the dehydrating and embedding of the cartilage specimens. These processes cause artefacts in the tissue, which will bring errors to the following analysis.

Confocal microscopy permits studying the internal structure of bulk articular cartilage when the tissue is in a hydrated state. Using fibre optic laser scanning confocal microscopy (FOCM, Optiscan Pty Ltd, Australia), we previously developed a 3D imaging technique to visualize the 3D collagen orientation in the superficial zone.²⁹ However, the method requires visual inspection, which is subjective and time consuming. In order to overcome this limitation, 2D Fast Fourier Transform (FFT) and Power Spectral Analysis (PSA) have been used to characterize the collagen orientation in the superficial zone of normal cartilage, aged cartilage, cartilage with surface disruption and fibrillated cartilage.²⁸ It has been

found that FFT and PSA are effective tools for identifying the collagen orientation in the superficial zone of the cartilage with typical physiological status (for example, normal and early degenerated cartilage). However, FFT and PSA require visual inspection of the spectral distributions of the collagen fibres in their frequency domain. Therefore, they are not genuine quantitative techniques.

The spectral moment γ^2 is a numerical descriptor which has been used for distinguishing texture patterns of a surface, such as isotropy or anisotropy.^{8,18,23} The present study uses the spectral moment γ^2 to quantitatively characterize the collagen orientation in the superficial zone of normal cartilage, aged cartilage, cartilage with surface disruption and fibrillated cartilage, and to explore a quantitative methodology for measuring the physiological state of articular cartilage (AC).

METHODS

Sampling and Staining of Cartilage

- Forty-three normal cartilage specimens (N) were obtained from ten femoral condyles and five femoral heads of approximately two-year-old cows within 24 hours of slaughter.
- Eleven cartilage specimens (PM) were obtained by physically peeling off the semitransparent membrane covering the surface of AC of normal bovine cartilage using the method described in a previous study.²⁹
- Twenty-two human cartilage specimens (aged cartilage) (AG), which showed a generally glossy surface and few macroscopic arthritic signs, were obtained from five femoral heads of cadavers aged from 40 to 60 years old.
- Twenty-eight human cartilage specimens (SM) were obtained from regions of fifteen human arthritic femoral heads with a slightly matte surface.

- Six fibrillated human cartilage specimens (F) were harvested from the arthritic femoral heads removed during joint replacement surgery.

The bovine cartilage specimens were fixed with 10% buffered formalin solution (BFS) for 24 hours. The human cartilage specimens had been prefixed before being obtained. All cartilage specimens were immersed into 0.2% phosphomolybdic acid for 24 hours and stained with 1g/L picrosirius red for approximately 72 hours.

3D Imaging Technique

The cartilage specimens were washed with 9 g/L physiological saline and imaged by FOCM. An Olympus PlanApo 60×/1.4 oil immersion lens, and a reflectance channel with a combined laser wavelength of 488 nm and 514 nm, were utilized to acquire an image stack of collagen fibres in the superficial zone of AC. The optical sectioning-depth was set between 0.541 and 0.689 μm .

Using computer software (VoxBlast, VayTek Inc, Iowa, USA), a 3D image of the collagen network was reconstructed from the image stack acquired from FOCM and used for 3D visual inspection of the collagen network. Utilizing a computer program (F900e, Optiscan Pty Ltd, Melbourne, Australia), the image stack was reconstructed into a maximum brightness image (MBI) and a height encoded image (HEI). The former is an image projection, which possesses the maximum pixel value for each xy location from all the 2D image slices and represents a view of all of the data in the image stack. The latter is a topographical image map in which the intensity of the each pixel in the image stack acquired from reflectance channel represents the height.

Spectral Moment and Statistic Analysis

The spectral moment analysis is based on the concept that all the relevant characteristics of a

random Gaussian surface can be obtained from the spectral moment. The spectral moment γ^2 is derived from the power spectrum of a surface and it measures mean square slope, the average length of a contour, and the average density of maximum and minimum per unit area to numerically indicate the dominant characteristics of the surface.^{18,23} A quantity of the spectral moment γ^2 is defined with the second order the spectral moment:

$$\gamma^2 = \frac{(m_{20} + m_{02}) - \sqrt{(m_{20} - m_{02})^2 + 4m_{11}^2}}{(m_{20} + m_{02}) + \sqrt{(m_{20} - m_{02})^2 + 4m_{11}^2}}.$$

Mean squares slope in x -direction can be expressed as $m_{20} = E\left[\frac{\partial h(x,y)}{\partial x} \frac{\partial h(x,y)}{\partial x}\right]$, mean squares slope in y -direction can be expressed as $m_{02} = E\left[\frac{\partial h(x,y)}{\partial y} \frac{\partial h(x,y)}{\partial y}\right]$ and mean squares slope in x - and y -direction can be expressed as $m_{11} = E\left[\frac{\partial h(x,y)}{\partial x} \frac{\partial h(x,y)}{\partial y}\right]$, where $E[h(x,y)h(x + \tau_x, y + \tau_y)] = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \exp(j\tau_x f_x + j\tau_y f_y) G(f_x + f_y) df_x df_y$, and $h(x,y)$ is a residual surface at location (x, y) . The spectral moment γ^2 is valued between 0 and 1. A value approaching 0 indicates that the surface texture is strongly anisotropic while a value approaching 1 indicates the surface texture is strongly isotropic.

Using a previously developed computer program¹⁸ based on Optimas 6.5 (Media Cybernetics, MD, USA), the MBI was used for performing the spectral moment analysis of the orientation of the collagen fibres in the superficial zone. The HEI was used to plot the 3D topography of the surface of the collagen matrix for visual inspection of the surface contour of the collagen matrix.

Student's t -test for assuming the mean of two populations with equal variances within Excel was used to test the difference in the means of the spectral moment between the groups of specimens.

RESULTS

The collagen fibres in the superficial zone demonstrated distinctive morphological characteristics in normal cartilage (N), aged cartilage (AG), cartilage with surface disruption (PM, SM) and fibrillated cartilage (F), as shown in Figs. 1 to 5. These morphological characteristics of the collagen fibres in the cartilage types have been summarized in Table 1. The spectral moment of the collagen fibres in the superficial zone of the normal cartilage is significantly different from that of the cartilage with surface disruption/early arthritic cartilage (at a 99% confidence level).

As shown in Fig. 1(a), normal cartilage contained a collagen network with little disruption. As a result of this, the 3D surface contour of the collagen network plotted from the topography

(HEI) of the collagen network presented even amplitudes, as shown in Fig. 1(b). Elsewhere, interwoven collagen bundles frequently appeared in the region near the articular surface, as the arrows show in Figs. 1(a) and 1(d). The collagen fibres in the superficial zone were oriented predominantly in a spatially oblique direction to the AC surface, as shown in Fig. 1(a). Thus, in the MBI [Fig. 1(c)] which is the 2D projection of Fig. 1(a), these collagen fibres appeared to be aligned predominantly in a direction parallel to the cartilage surface. The 3D plot of FFT of the collagen fibres, therefore, demonstrates a directional distribution of the spectrum, as shown in Fig. 1(d). The spectral moment γ^2 of the collagen fibres of the cartilage is mainly distributed in the lower regions, as shown in Fig. 6. The average the spectral moment γ^2 of

Table 1 Morphological Characteristics of Collagen Fibres in the Superficial Zone of AC with Typical Physiological Status.

Types	Matrix	Collagen Orientation	FFT	γ^2 (mean \pm STD)
N	Integrity, WCB	//	Directional	0.4353 \pm 0.0423
AG	Integrity 50% Disrupted 50%	// (50%); \perp (50%) -	Directional (50%)	0.4893 \pm 0.0619
PM	Disrupted	\perp	Even	0.6116 \pm 0.1315
SM	Disrupted, rough	\perp	Even	0.6123 \pm 0.0796
F	Abnormal	Abnormal	Even	0.6723 \pm 0.0948

Col: Collagen.

N: normal cartilage.

AG: aged cartilage.

PM: cartilage physically peeled off the membrane.

SM: cartilage obtained from regions of arthritic cartilage which showed slight matte.

F: fibrillated cartilage.

WCB: interwoven collagen bundles.

//: collagen fibres were predominantly oriented in a spatially oblique direction to the AC surface.

\perp : collagen fibres were predominantly oriented in a direction perpendicular to the AC surface.

Col-orientation: the orientation of the collagen fibres in the superficial zone.

Col- matrix: the collagen matrix in the superficial zone.

γ^2 : The spectral moment of the collagen fibres in the MBIs.

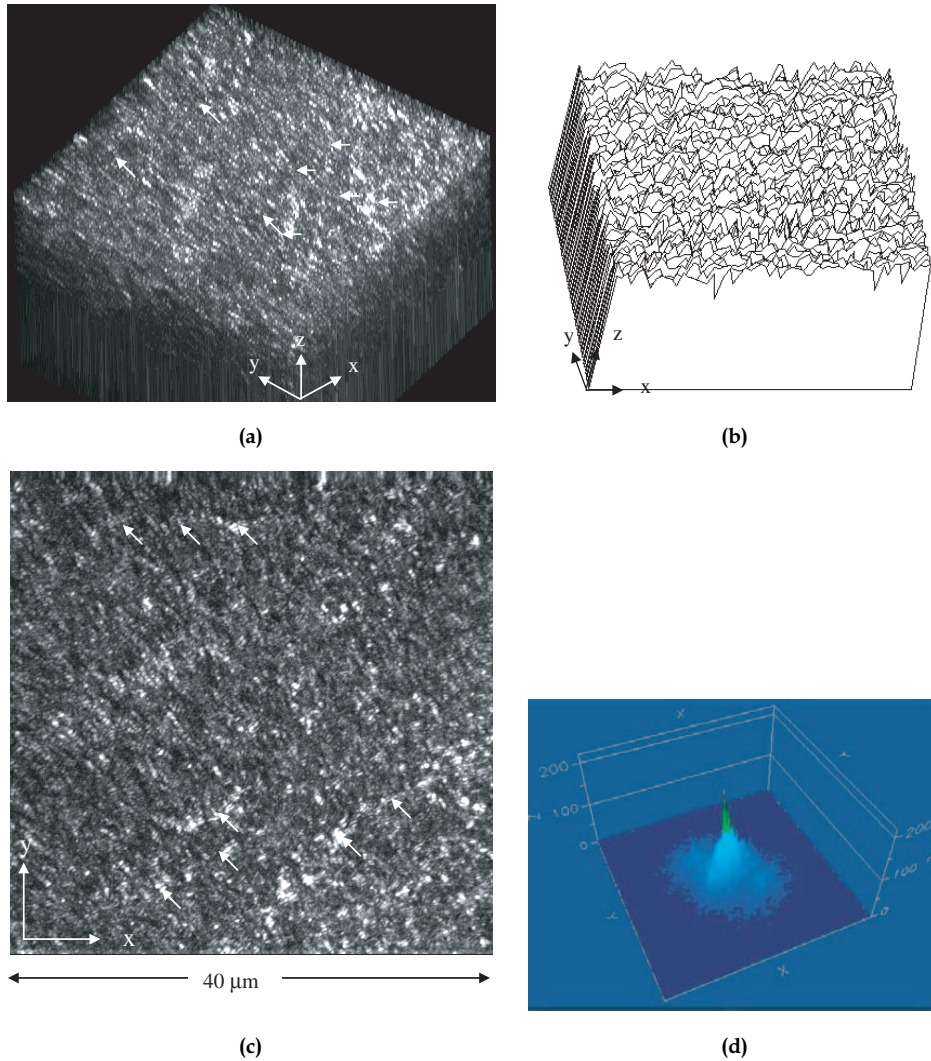


Fig. 1 The collagen matrix in the superficial zone of normal cartilage (N) contained interwoven collagen bundles (arrows in Fig. 1a) while the collagen fibres were oriented predominantly in a direction oblique to the AC surface. **(a)** 3D image of the collagen fibres. **(b)** 3D plot of the surface topography (HEI) of the collagen network in the superficial zone. **(c)** MBI of the collagen fibres. **(d)** 3D plot of the FFT of the collagen fibres in the MBI.

the collagen fibres in the superficial zone of the cartilage is 0.4353 ± 0.0423 , as shown in Table 1. As the data in Fig. 6 shows that about 80% of the normal cartilage specimens had the spectral moment less than 0.47.

With increasing age, the most noticeable change in the collagen matrix in the superficial zone of the cartilage is the gradual disappearance of the interwoven collagen bundles near the cartilage

surface (the aged cartilage specimens rarely contained interwoven collagen fibres), as shown in Fig. 2(a). About 50% of the aged cartilage specimens demonstrated little or slight disruptions of the collagen matrix in the superficial zone, as shown in Fig. 2(a). Therefore, the surface contour of the collagen network plotted from the HEI showed slightly irregular amplitudes, as shown in Fig. 2(b). The collagen fibres in the superficial

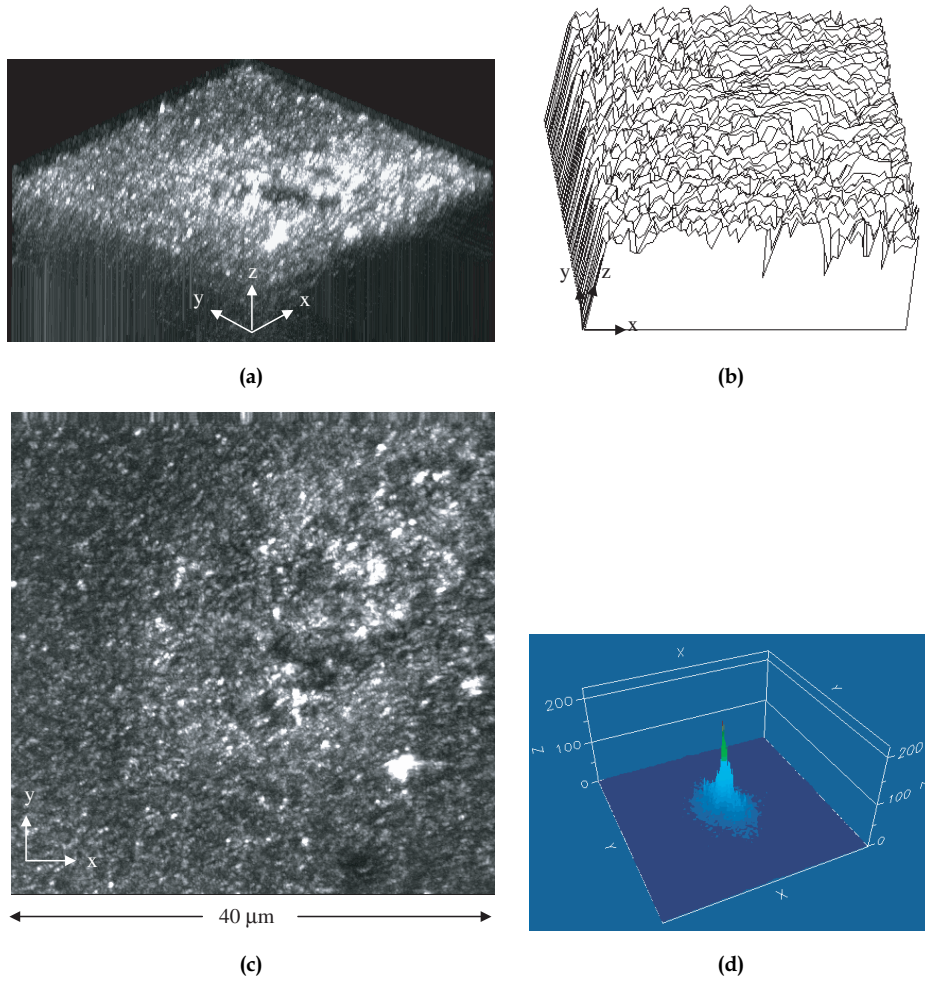


Fig. 2 Approximately 50% of the aged cartilage specimens (AG) still had the collagen fibres in the superficial zone were aligned predominantly in a direction oblique to the AC surface. **(a)** A 3D image of the collagen fibres. **(b)** 3D plot of the surface topography of the collagen network. **(c)** MBI of the collagen fibres. **(d)** 3D plot of FFT of the collagen fibres in the MBI.

zone of these cartilage specimens were predominantly oriented in a direction oblique to the AC surface, as shown in Fig. 2(a). Thus, their orientation in the MBI appeared directional, as shown in Fig. 2(c). The 3D plot of the FFT of the collagen fibres in the MBI exhibited a directional spectrum distribution, as shown in Fig. 2(d). In another 50% of the aged cartilage specimens, the collagen fibres in the superficial zone were oriented predominantly in a direction perpendicular to the AC surface, and the collagen network in the superficial zone is more seriously disrupted. The

collagen orientation in these cartilage specimens appeared similar to that of the cartilage with surface disruption shown in Fig. 3. Subsequently, the spectral moment γ^2 of these cartilage specimens fluctuated between that of normal cartilage and the cartilage with surface disruption (both PM and SM cartilage types), as shown in Fig. 6. The average spectral moment of aged cartilage increased approximately 12.4% in comparison to that of normal cartilage, as shown in Table 1. Approximately 50% of the aged cartilage specimens exhibited the spectral moments greater than 0.48.

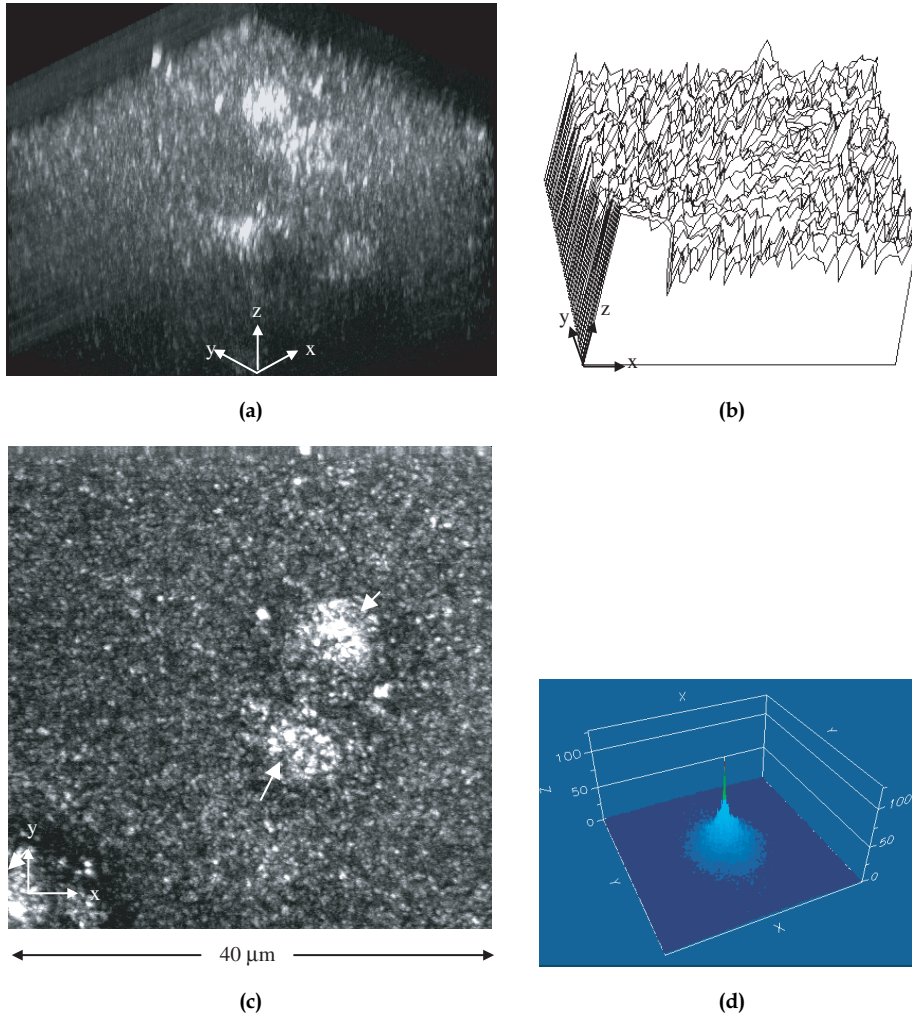


Fig. 3 Peeling off the most superficial layer altered the collagen orientation in the superficial zone (PM). **(a)** A 3D image of the collagen fibres in the superficial zone of the cartilage with the most superficial membrane peeled off. **(b)** 3D plot of the surface topography (HEI) of the collagen matrix in the superficial zone. **(c)** MBI of the collagen fibres (arrows indicate chondrocytes). **(d)** 3D plot of FFT performed in the MBI.

Physically peeling off the semi-transparent membrane covering on the normal cartilage surface (PM) caused slightly structural changes to the collagen matrix, as shown in Fig. 3(a). Therefore, the surface contour plotted from the topography of the collagen matrix presented irregular amplitudes in some parts and appeared coarser than that of normal cartilage, as shown in Fig. 3(b). Also, the orientation of the collagen fibres in the superficial zone of the cartilage was altered to

align predominantly in a direction perpendicular to the AC surface, as shown in Fig. 3(a) (the arrows in Fig. 3(c) indicate the chondrocytes). This resembles that of the cartilage obtained from the regions of arthritic femoral heads which had a slightly matte surface (SM cartilage type), as shown in Fig. 4(a). It is worthy of notice that the collagen fibres in these two cartilage groups showed even distributions in the MBIs, as shown in Figs. 3(c) and 4(c) respectively. Consequently,

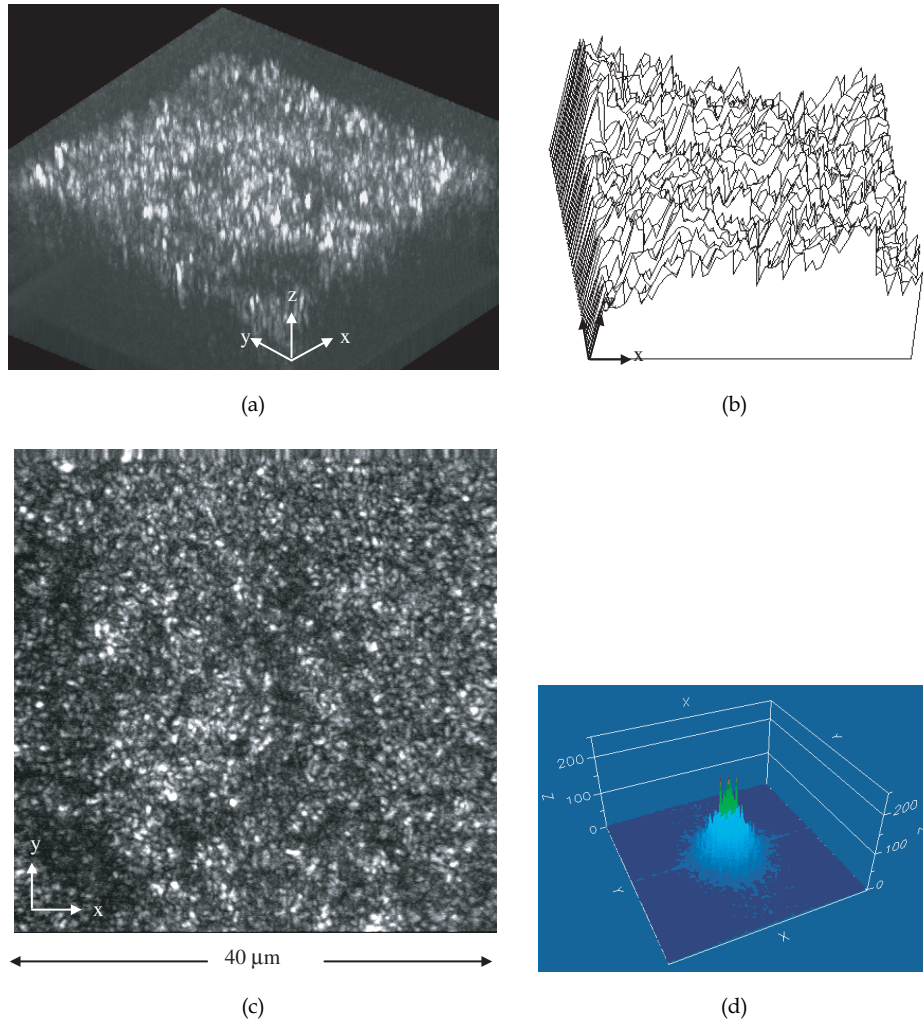


Fig. 4 The collagen orientation in the cartilage obtained from the regions of arthritic femoral heads with slightly matte appearance (SM) resembled that of cartilage with the most superficial layer peeled off. **(a)** A 3D image of the collagen fibres in the superficial zone of AC obtained from the regions of arthritic femoral heads with slightly matte surfaces. **(b)** 3D plot of the surface topography (HEI) of the collagen matrix in the superficial zone. **(c)** MBI of the collagen fibres. **(d)** 3D plot of FFT performed in the MBI.

the 3D plots of the FFTs of these two group cartilage demonstrated even distributions of the spectrum, as shown in Figs. 3(d) and 4(d). The spectral moment γ^2 of the two cartilage types was distributed in higher ranges, as shown in Fig. 6. There was no significant difference in the mean of the spectral moment of these two cartilage categories (at a 99% confidence level). In comparison to that of the normal cartilage, the average spectral moment of the collagen fibres in the

two cartilage types increased about 40% (at a 99% statistical confidence level), as shown in Table 1. However, the cartilage with a naturally disrupted surface (SM) exhibited a rougher surface contour for its collagen matrix than that of the PM cartilage where the superficial membrane had been peeled off, as shown in Figs. 3(b) and 4(b), which are the 3D plots of the topographies of the collagen matrices. More than 84% of the cartilage specimens with

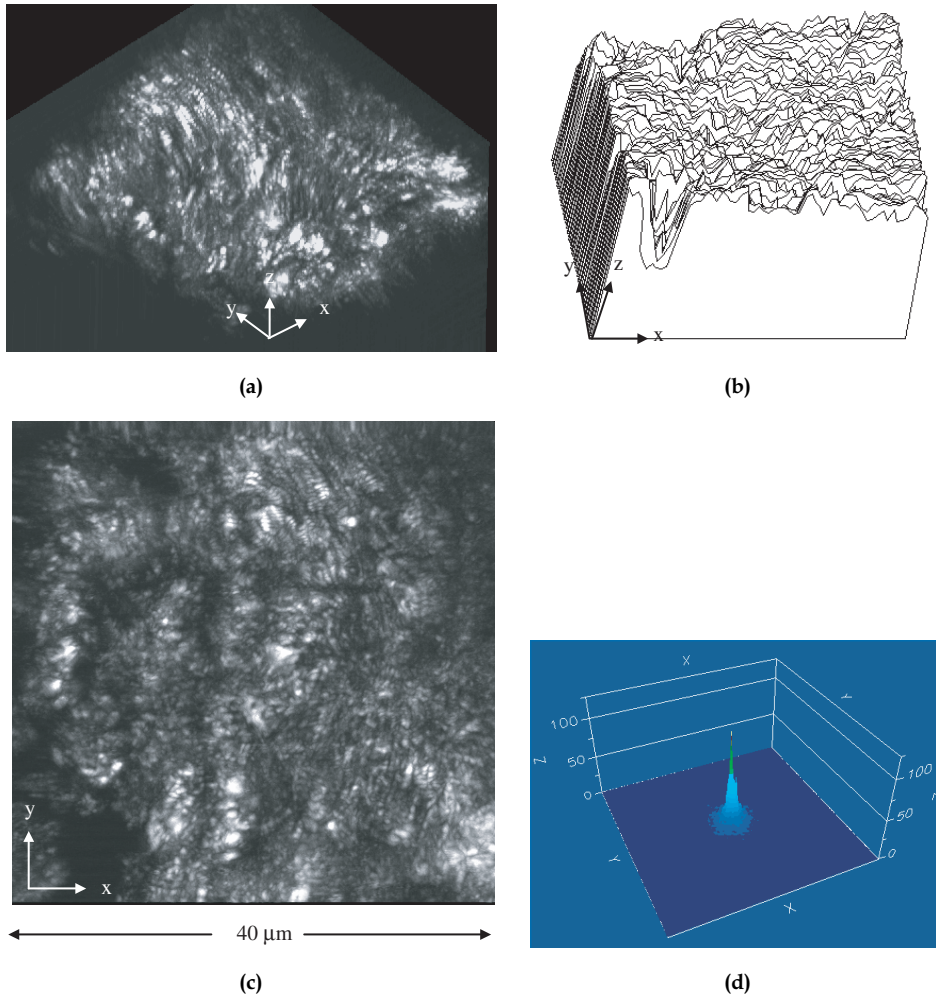


Fig. 5 Fibrillated cartilage (F) had a distinctive macro appearance and abnormal collagen orientation. **(a)** A 3D image of the collagen matrix in fibrillated cartilage. **(b)** 3D plot of surface topography of collagen matrix. **(c)** MBI of the collagen matrix. **(d)** 3D plot of FFT performed in the MBI.

surface disruption (including natural surface disruption and peeled superficial surface) had the spectral moments larger than 0.55.

Fibrillated cartilage (F) had a distinctive macroscopic appearance and abnormal collagen orientation, as shown in Fig. 5. The spectral moment γ^2 of the cartilage is scattered in the top region, as shown in Fig. 6. The average spectral moment γ^2 of the cartilage was about 54% greater than that of the normal cartilage (95% statistical confidence level). It was also slightly larger than that of PM and SM (95% statistical confidence level).

However, a quick macroscopic inspection is sufficient to distinguish this type of cartilage from the other cartilage types mentioned above.

DISCUSSION

Quantitative measurements of the physiological status of articular cartilage are mainly dependent on macro testing the material properties of articular cartilage. This method, however, does not reveal information about the physiological change of the articular cartilage in relation to its

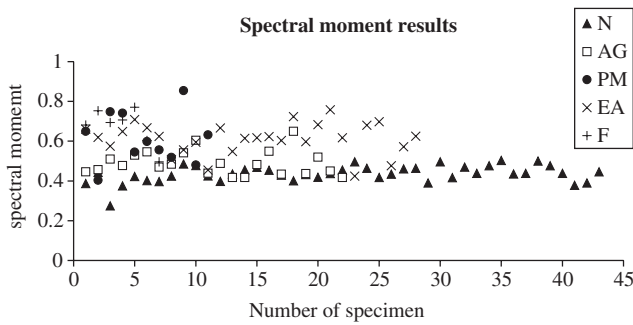


Fig. 6 The distribution of the spectral moment of the collagen fibres in the superficial zone of AC with different physiological states. N: normal cartilage. AG: aged cartilage. PM: cartilage from which the most superficial membrane was peeled off. SM: cartilage obtained from regions of arthritic femoral heads which showed slight matte. F: fibrillated cartilage.

microstructures. Therefore, it is not widely used in the medical field for pathologic study of cartilage.

Normal functions of articular cartilage are largely dependent on the integrity of the 3D collagen matrix.^{2,10} Therefore, standard histology using visual inspection of the collagen structure is often used to study the pathology of the cartilage in the medical field. However, the visual inspection is subjective so that it can open errors in assessments of the physiological states of articular cartilage.

Using Fourier transform techniques, a number of previous studies have developed techniques for objectively describing the orientation of collagen fibres to determine the physiological status of biological tissues.^{1,6,24–26,30} However, all these studies involved the use of electron microscopy (EM). EM offers superior image resolution but it requires dissecting the cartilage which could cause artefacts in the image analysis.^{13,27} Laser scanning confocal microscopy provides a minimally invasive way to image the collagen fibres in the superficial zone of bulk articular cartilage.²⁹ In particular, the characteristics of FOCM used in this study have allowed the development of confocal arthroscopy to study the microstructure

(mainly cellular morphology) of AC *in vivo*.^{14,15,21} Therefore, the methodology developed in this study has a potential to be used to quantitatively analyse the microstructure of articular cartilage for study of the early pathological changes in articular cartilage in the medical field.

In comparison to FFT and spectral analysis techniques developed in the previous study,²⁸ the spectral moment (γ^2) used in this study offers genuine quantitative information about the collagen orientation. Particularly, it has been found that there is a significant difference between the spectral moment (γ^2) of normal and that of early arthritic cartilage. Thus, the spectral moment analysis can be potentially used to numerically identify the cartilage with early arthritis. Since the FFT and power spectral analysis employ complex mathematical calculations/transformations, they are generally more time consuming when applied for image analysis.⁹ In contrast, the spectral moment analysis takes less time.^{18,19} Therefore, it can be a more efficient means of indicating the collagen orientation for measuring the physiological condition of the AC.

Furthermore, the surface topography of the collagen matrix in the superficial zone also showed a distinct difference between the surface contours of the collagen matrix in the normal cartilage and cartilage with surface disruption/arthritic cartilage. Therefore, future studies can further introduce numerical descriptors for more detailed enumeration of the surface contours of the collagen network in normal and early arthritic cartilage and for clearly identifying the subtle physiological differences in the cartilage. However, the measures for such surface characterizations have yet to be investigated in this study.

The abnormal orientation of the collagen fibres in fibrillated cartilage is difficult to be described using the spectral moment analysis. However, fibrillated cartilage demonstrated a clearly abnormal macroscopic appearance, which means this group

of specimens can be singled out from normal and early degenerated cartilage by a simple visual inspection.

CONCLUSIONS

Computer image analysis overcomes the subjective nature of human inspection. Confocal microscopy allows the visualization of the 3D collagen matrix in articular cartilage with minimal physical disruption of the tissue's natural state. Utilizing FOCM and the spectral moment analysis, this study focused on quantitatively measuring the collagen orientation in the superficial zone of articular cartilage with typical physiological status. The method developed in this study shows potential for use in routine histology for quantitatively identifying early arthritic changes in articular cartilage.

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