

# Collagen fiber arrangement in normal and diseased cartilage studied by polarization sensitive nonlinear microscopy

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**Abstract.** Second harmonic generation (SHG) and two-photon fluorescence (TPF) microscopy is used to image the intercellular and pericellular matrix in normal and degenerate equine articular cartilage. The polarization sensitivity of SHG can be used directly to determine fiber orientation in the superficial 10 to 20  $\mu\text{m}$  of tissue, and images of the ratio of intensities taken with two orthogonal polarization states reveal small scale variations in the collagen fiber organization that have not previously been reported. The signal from greater depths is influenced by the birefringence and biattenuance of the overlying tissue. An assessment of these effects is developed, based on the analysis of changes in TPF polarization with depth, and the approach is validated in tendon where composition is independent of depth. The analysis places an upper bound on the biattenuance of tendon of  $2.65 \times 10^{-4}$ . Normal cartilage reveals a consistent pattern of variation in fibril orientation with depth. In lesions, the pattern is severely disrupted and there are changes in the pericellular matrix, even at the periphery where the tissue appears microscopically normal. Quantification of polarization sensitivity changes with depth in cartilage will require detailed numerical models, but in the meantime, multiphoton microscopy provides sensitive indications of matrix changes in cartilage degeneration. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2950318]

Keywords: cartilage; two-photon fluorescence microscopy; second-harmonic microscopy; intercellular; multiphoton microscopy.

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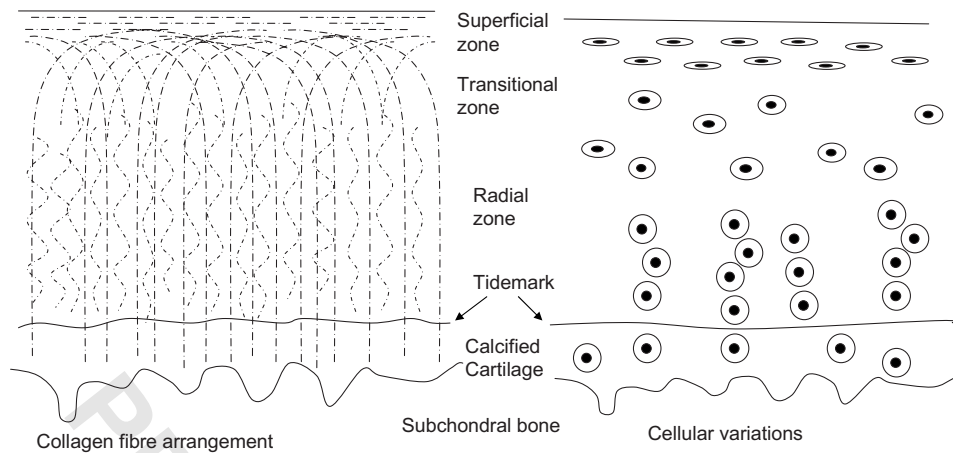
## 1 Introduction

Osteoarthritis is a widespread, painful, and debilitating joint disease characterized by the degeneration and eventual loss of the cartilage from the joint surface. Healthy cartilage has a well-ordered structure, containing few cells, but an extensive extracellular matrix comprising a network of collagen fibers immersed in an amorphous, highly hydrated gel rich in proteoglycans (see Fig. 1). This structure is lost as the disease progresses, and many lines of research into its pathophysiology and treatment are critically dependent on the development of techniques for quantifying these changes.<sup>1,2</sup> Particular interest attaches to the organization of the network of collagen fibers that are the principal determinant of the structure, and associated mechanical properties that are central to the biomechanical function of the tissue and a number of physical methods have been applied to this problem. Polarized light microscopy, small and wide-angle x-ray scattering, and polarization sensitive optical coherence tomography (PSOCT) have been employed in a number of important and revealing studies.<sup>3-5</sup> The latter has the great attraction, because it can provide

depth imaging on tissue attached to subchondral bone, thus opening the prospect of imaging in the intact joint. However, the depth and lateral resolution of PSOCT are too low to address many structural issues pertinent to cartilage pathophysiology, and in the present work, we investigate the ability of multiphoton microscopy to fulfill this need.

Multiphoton microscopy has been applied to biological systems ranging from living cells<sup>6,7</sup> to whole tissues,<sup>8,9</sup> and even living organisms,<sup>10</sup> and it has a number of inherent advantages. Its nonlinearity allows the use of infrared excitation wavelengths that maximize tissue penetration while maintaining intrinsically high spatial resolution.<sup>11</sup> It relies on two nonlinear processes that do not require the introduction of exogenous probe molecules or fixation and staining of the tissue. The first is two-photon fluorescence (TPF). A number of endogenous fluorophores have been identified in cells<sup>12</sup> and, particularly relevant to the present study, the extracellular matrix,<sup>13</sup> although their characterization is still incomplete. The second mechanism, second harmonic generation (SHG), occurs in molecules that do not possess inversion symmetry. It is again fortunate for the study of the extracellular matrix that collagen fibers are a strong source of SHG, although the

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**Fig. 1** A schematic diagram showing the different zones found in transverse sections of articular cartilage. The left-hand image represents the collagen fiber arrangements within the tissue, and the right-hand side shows changes in cell shape and arrangement that have been observed to occur with depth in the cartilage. Cartilage thickness is typically 1 to 4 mm, depending on the species and joint examined.

mechanisms of its generation are still under debate.<sup>14,15</sup> The SHG signal is polarization sensitive and its intensity depends on the angle between the fibers and the polarization of the exciting radiation. This relationship has been explored in a number of studies on rat tail tendon,<sup>15-19</sup> a tissue that is composed primarily of highly ordered fibrils of type-1 collagen. To a first approximation, the maximum signal intensity is generated when the polarization is parallel with the fiber axis, and the minimum intensity signal is generated when the polarization is perpendicular to the fiber axis. Therefore, polarization sensitivity studies of SHG from tissues can provide information on the fiber organization.<sup>20</sup> Cartilage contains type-2 collagen, which has important but small biochemical differences from type 1 and forms morphologically similar, but rather finer, fibers with a complex organization that varies through the depth of the cartilage, as shown in Fig. 1. This level of organization would lead to the expectation that the SHG from cartilage would be polarization sensitive, although a previous study only detected in diseased tissue where the collagen fiber organization had been disrupted.<sup>21</sup>

Further to explore this unexpected observation was the starting point of the present investigation. Variations in SHG polarization sensitivity with depth have previously been measured in tendons<sup>18,22</sup> and other tissues such as dentine, dermis and bone.<sup>22</sup> The study investigates the polarization sensitivity with depth in tendon and cartilage, and also considers the effects of the biattenuance and birefringence of the overlying tissue on the measure polarization sensitivity curve.

Our approach is based on comparisons of the variations in the polarization sensitivity of TPF and SHG with depth. We first study tendon as an example of a highly ordered collagenous structure whose birefringence and biattenuance have been characterized in some detail.<sup>3,23</sup> We then analyze normal cartilage and lesions in the equine metacarpophalangeal joint. This tissue was selected because its structure has been extensively characterized by other methods,<sup>3,4</sup> and the lesions that develop spontaneously share many of the characteristics of human disease.<sup>24</sup>

## 2 Materials and Methods

### 2.1 Methods

The experiments were carried using a home-built microscope, whose essential features are illustrated in Fig. 2. A reflection mode configuration was chosen, as this enabled the en-face examination of cartilage samples still attached to the subchondral bone, minimizing the disruption of the collagen architecture.

Excitation was at 800 nm using a tuneable 100-fs pulsed Ti:sapphire laser with a repetition rate of 82 MHz (Tsunami, Spectra-Physics) The selection of the excitation wavelength was based on a number of considerations. Previous studies have produced conflicting results as to which wavelength most efficiently excites SHG in collagen,<sup>7,25</sup> but we found that in our system, the signal of both SHG and TPF peaked between 780 and 820 nm, and the use of longer wavelengths increases tissue penetration and minimizes cellular damage.<sup>6</sup> A 1.0-NA water dipping objective with a 2-mm working distance was employed (Fluor 60 $\times$ ) and data acquisition was via a photon-counting photomultiplier tube and a PC-based photon counting card (Hamamatsu H7360-02 and M8784). The laser fundamental was removed by a short pass dichroic beamsplitter (CVI Laser) and a bandpass filter (CVI Laser). Blocking of the fundamental was greater than 9 OD and the transmission of the TPF and SHG light about 30%. To change between SHG and TPF imaging, the filter directly below the PMT was changed, with a 450 to 520-nm bandpass filter being used for TPF detection and a 410 to 390-nm filter for SHG detection (CVI Laser). For polarization measurements, two 800-nm half wave plates were added to the system. The first wave plate (CVI Laser) was placed before the dichroic beamsplitter, orientated to ensure that completely p-polarized entered the beamsplitter. The second (Meadowlark, Frederick, Colorado) was placed directly behind the back aperture of the microscope objective to allow the polarization of the light incident on the sample to be rotated. This arrangement was necessary because our dichroic beamsplitter introduced differ-

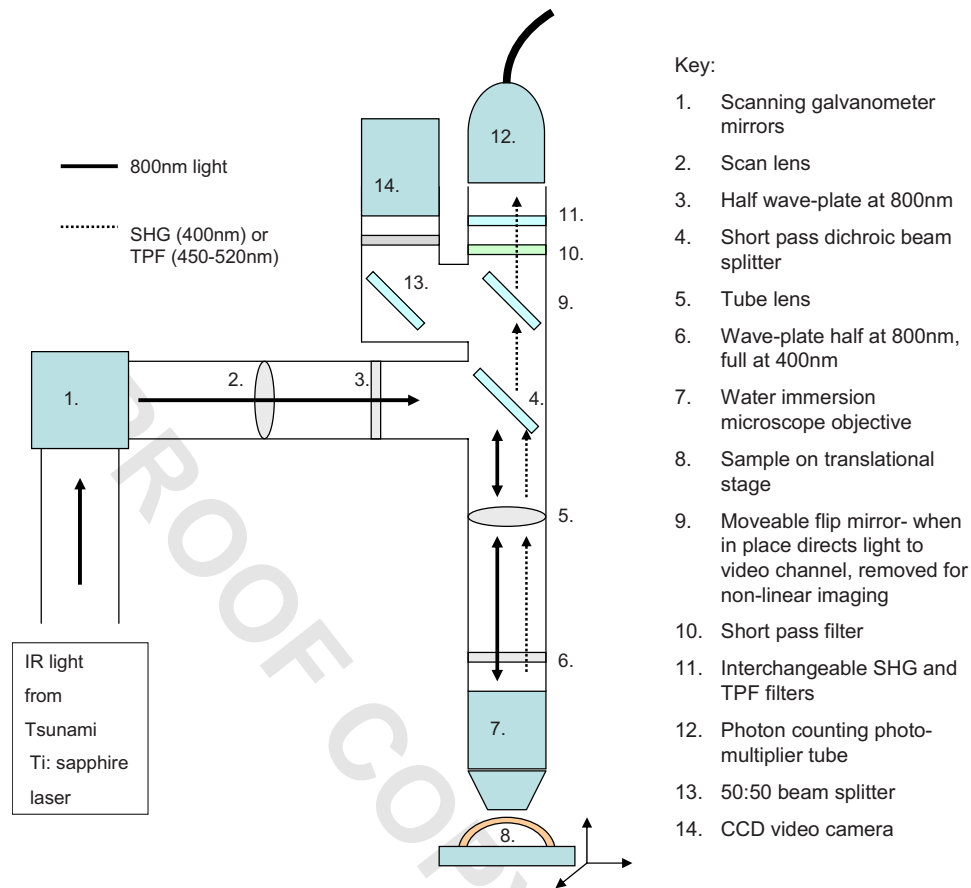


Fig. 2 The optical elements of the nonlinear microscope (not to scale).

120 ential phase-shifts between s and p-polarized states when used  
 121 in a reflection mode, which would convert the incident linear  
 122 polarization state to an elliptical state if the polarization was  
 123 rotated before the dichroic. The photon-counting PMT used  
 124 for the detection of the TPF and SHG signal was polarization  
 125 insensitive, and therefore the effect of the wave-plate had on the  
 126 TPF light passing back through the objective would not affect  
 127 the experimental results.

128 Axial imaging was performed by raster scanning the beam  
 129 using two scanning galvanometer mirrors (Cambridge Tech-  
 130 nology Incorporated). An axial scan was generated by moving  
 131 the sample upward on a motor stage with an accuracy of  
 132 about  $1\ \mu\text{m}$  (Physik Instrumente). The field of view of the  
 133 microscope objective allowed images up to  $125 \times 125\ \mu\text{m}$  to  
 134 be acquired, the size being controlled by the voltage applied  
 135 to the galvanometer mirrors. The images reported in this study  
 136 were  $100 \times 100\ \mu\text{m}$  and composed of  $500 \times 500$  pixels. The  
 137 pixel dwell time was  $10\ \mu\text{s}$ .

138 The laser power used for imaging was approximately  
 139  $40\ \text{mW}$  exiting the microscope objective. For the polarization  
 140 sensitivity measurements, a power of  $30\ \text{mW}$  was used for  
 141 acquisition of surface images, but power was increased with  
 142 scanning depth so that at  $200\text{-}\mu\text{m}$  depth, the power exiting  
 143 the objective was  $140\ \text{mW}$ .

144 In polarization sensitivity experiments, the polarization of  
 145 the light incident on the sample was rotated in  $10\text{-deg}$  steps  
 146 using the half wave plate behind the microscope objective.

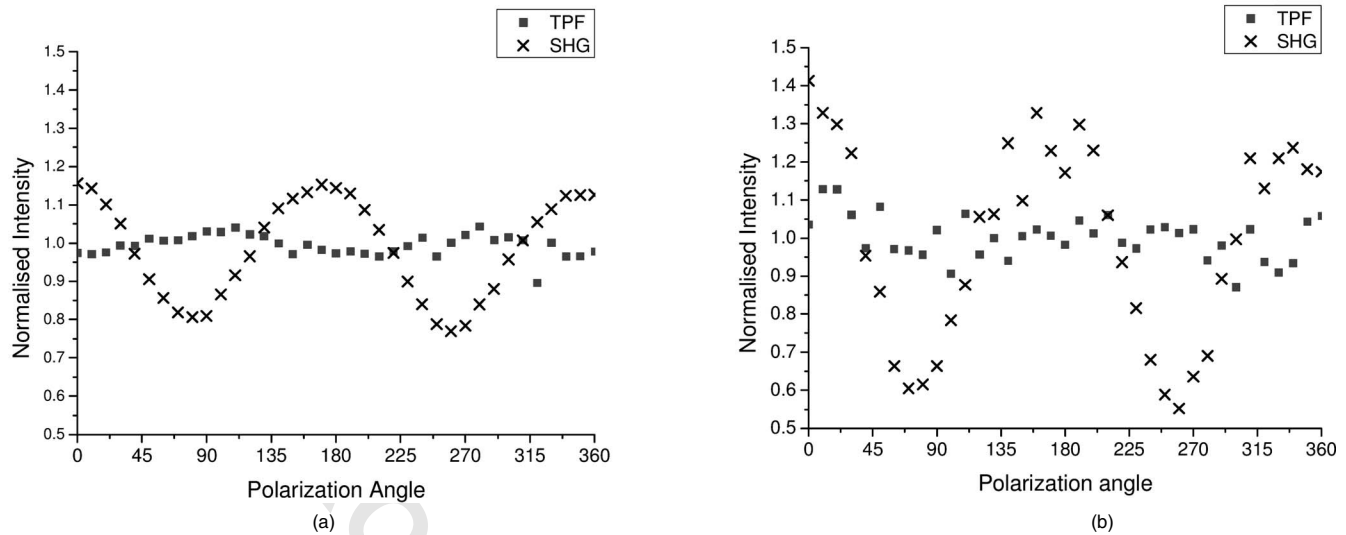
The total intensity from the area scan was measured, and the  
 intensities for each angle were combined to create the polariza-  
 tion sensitivity curve. This process was repeated at depth  
 steps into the tissue of  $10\ \mu\text{m}$  in tendon and healthy cartilage  
 and  $15\ \mu\text{m}$  in cartilage lesions. The depth steps were chosen  
 as a compromise between collecting a very detailed dataset  
 and minimizing the total scan time.

## 2.2 Materials

Tissue samples were obtained fresh from the abattoir, carti-  
 lage from the metacarpophalangeal joints and tendon from the  
 deep flexor tendon of horses. The age of the samples was  
 estimated by the abattoir staff and ranged from 5 to 20  
 + years. Some samples were frozen prior to imaging, but this  
 had no discernable effect on the images. At the laboratory, the  
 joint was opened and a full depth plug of cartilage and several  
 millimeters of subchondral bone was excised from the apex of  
 the joint using a jeweller's saw. During measurement, samples  
 were maintained at room temperature and kept moist by immer-  
 sion in  $0.15\text{-M}$  saline.

## 3 Results and Discussion

The first stage of the investigation was to examine the TPF  
 and SHG signals generated from the surface layer of both  
 tendon and cartilage. In the case of cartilage, we explored the  
 variations in collagen fiber orientations within the imaging



**Fig. 3** Polarization sensitivity curves for both TPF and SHG taken at the surface in (a) cartilage and (b) tendon, with the x axis showing the angle between the collagen fiber axis and the polarization of the laser fundamental. The intensities are normalized with respect to the mean intensity for each curve. Data were acquired at a depth of 10  $\mu\text{m}$  beneath the articular surface.

171 area by producing ratio images from the polarization sensitiv-  
 172 ity data. We then investigated signals emanating from deeper  
 173 in the tissue, first in tendon, which constitutes a simple model  
 174 system because its structure is uniform through the depth of  
 175 the tissue, and then in cartilage. The final section reports data  
 176 obtained from cartilage lesions.

177 **3.1 Surface Measurements on Cartilage and Tendon**

178 Figure 3 shows the angular variations in intensity of SHG and  
 179 TPF at the surface of cartilage and tendon. The images were  
 180 acquired at a depth of 10  $\mu\text{m}$  from the surface zone of fibers  
 181 aligned parallel to the surface (Fig. 1). The polarization angle  
 182 is defined as the angle between the collagen fiber and the  
 183 polarization of the incident radiation, and in both tissues TPF  
 184 varied only weakly with angle. The visibilities  $[(I_{\text{peak}}$   
 185  $- I_{\text{trough}})/(I_{\text{peak}} + I_{\text{trough}})]$  for tendon and cartilage were  
 186  $(0.045 \pm 0.005)$  and  $(0.04 \pm 0.02)$ , respectively, (mean  $\pm$  SD,  
 187 measurements from five samples). Variations in the intensity  
 188 with polarization angle were comparable in magnitude with  
 189 the measurement noise. It therefore appears that TPF genera-  
 190 tion in both tissues does not have any significant intrinsic  
 191 polarization sensitivity, or the generation is polarization sen-  
 192 sitive but the sources are randomly orientated. As found by  
 193 previous researchers,<sup>15-19</sup> the SHG signal from tendon showed  
 194 a strong polarization dependence, with the greatest intensity  
 195 when the laser excitation was parallel with the collagen fibrils.  
 196 The peak in the SHG signal corresponded with the long axis  
 197 of the collagen fibers visible in the SHG images to within  
 198  $\pm 7$  deg. The SHG signal from the cartilage surface also de-  
 199 pended on polarization and had a period of 180 deg, suggest-  
 200 ing a preferred orientation of the collagen fibers in the super-  
 201 ficial zone of the cartilage. Though this was not observed in  
 202 previous studies on SHG from cartilage,<sup>21</sup> it is consistent with  
 203 well-established histological evidence.<sup>1,26</sup>

204 The visibility of the intensity-polarization angle plots was  
 205 calculated as a measure of the degree of collagen fiber orga-  
 206 nization in the tissue. For tendon, the visibility was

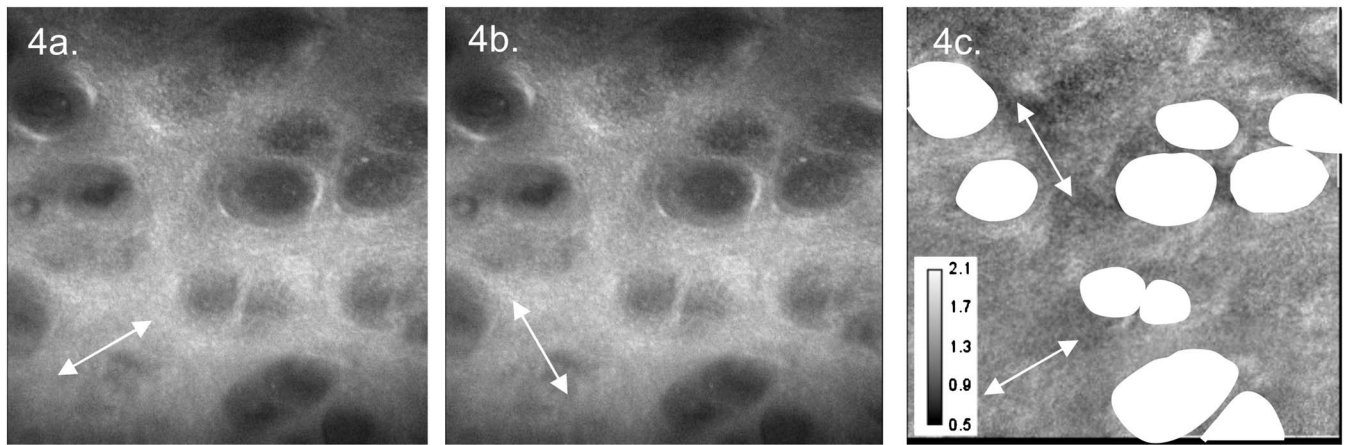
0.29  $\pm$  0.04, and for cartilage the visibility was 0.11  $\pm$  0.05 207  
 (mean  $\pm$  SD, measurements from six samples). These differ- 208  
 ences are consistent with the perceived differences in the level 209  
 of collagen fiber organization in the two tissues, but different 210  
 levels of intrafibrillar organization or optical properties bet- 211  
 tween type-1 and -2 collagen may also contribute. 212

To investigate whether there were differences in collagen 213  
 fiber organization within the 100- $\mu\text{m}^2$  scanning area, we 214  
 compared SHG images with the polarizer set to provide maxi- 215  
 mum signal with those with the polarizer rotated through 216  
 90 deg. Representative images and the resulting ratio image 217  
 are shown in Fig. 4. Histological studies have described the 218  
 pericellular matrix as basket-like and containing finer colla- 219  
 gen fibrils.<sup>1,26,27</sup> Therefore, we were expecting less polariza- 220  
 tion sensitivity and consequently a ratio close to 1 in these 221  
 regions. However, these differences were not apparent in the 222  
 ratio image, and although the ratio varied between 0.5 and 2 223  
 on a length scale of a few microns, the variations showed no 224  
 obvious correlation with position. On the basis of these obser- 225  
 vations, it was felt that averaging over a 100  $\times$  100- $\mu\text{m}$  226  
 image area was suitable for subsequent polarization sensitivity 227  
 investigations. A smaller area will provide information on the 228  
 collagen fiber arrangement on lower hierarchical levels inste- 229  
 ad of the zonal architecture described in Fig 1. Averaging 230  
 over longer length scales is not required, as the polarization 231  
 sensitivity results averaged over the 100  $\times$  100- $\mu\text{m}$  image 232  
 area are repeatable, and therefore a larger imaging area will 233  
 produce the same results. 234

235 **3.2 Depth Variations in Tendon**

To determine how the effects of biattenuance and birefrin- 236  
 gence in the overlying tissues affected measurements of 237  
 changes in polarization sensitivity with depth, we undertook 238  
 an initial study on tendon, whose structure and fiber orienta- 239  
 tions are constant with depth and can be observed directly in 240  
 the multiphoton images. The angular dependence of SHG and 241  
 TPF was measured at depths up to 200  $\mu\text{m}$  and values aver- 242





**Fig. 4** SHG images acquired with the polarizer at the position of (a) maximum intensity and (b) rotated through 90 deg, with the arrows indicating the polarization angle. (c) shows the ratio of the two images. The dark regions in (a) and (b) represent the locations of the chondrocytes. In the ratio image, these areas are circled. (Field  $100 \times 100 \mu\text{m}$ , arrows indicate the polarization of the laser light.) The dark region in the top right quarter of the image represents an area where the polarization sensitivity varies from that found in the rest of the scan area.

aged over a  $100\text{-}\mu\text{m}^2$  field in three samples are summarized in Fig. 5. Although the orientation of the collagen fibers remained constant with depth, the SHG polarization sensitivity pattern changed dramatically with depth, as is shown in Fig. 5(a). The TPF polarization sensitivity in Fig. 5(b) shows an increase with depth, with strong peaks in intensity appearing perpendicular to the collagen fiber orientations. This indicates that the optical properties of the overlying tissue are contributing to the measured patterns. Figures 5(c) and 5(d) show the natural log of the unnormalized data for the SHG and TPF polarization, respectively. These plots have been included because they show the attenuation of the light through the tissue. From the TPF data, it was possible to estimate an upper bound for the biattenuance of the tissue at the excitation wavelength. Surface imaging demonstrated that the TPF excitation efficiency has no intrinsic polarization sensitivity, which in turn suggests an isotropic orientation of fluorescence excitation dipoles within the focal volume. The variation in TPF emission at depth can thus be presumed to arise from “biattenuance” (a term introduced by Kemp et al.<sup>23</sup> to describe differential absorption or scattering of the excitation and/or emitted light for different incident polarization states). Let us assume that the superficial layer contains a well-defined transmission axis such that field strengths of light linearly polarized parallel and perpendicular to this axis experience a differential attenuation coefficient given by  $2\pi\Delta\chi/\lambda_0$ , where  $\Delta\chi$  is the biattenuance of the medium. Further, let us assume that these axes are coincident with the birefringence fast and slow axes of the superficial layer, i.e., we effectively assume that the superficial tissue can be described by a complex birefringence  $\Delta n + i\Delta\chi$ . Incident linearly polarized light aligned with either of these directions will thus remain polarized along these directions with increasing depth, and the differential attenuation of the excitation light between these directions will attain the maximum value. Linearly polarized light incident at other angles will undergo conversion to elliptical polarization states with increasing depth, and will thus experience an intermediate degree of attenuation. We thus identify the angles at which maximum and minimum TPF intensities

occur as defining the directions of the fast and slow axes. Recalling that our detection system is polarization insensitive, if the emitted TPF were itself unpolarized, then the emitted light would propagate to the detector with equal efficiency, regardless of the polarization direction of the excitation light. We could then use the difference in the maximum and minimum TPF intensities to infer  $\Delta\chi$  at the excitation wavelength a follows.

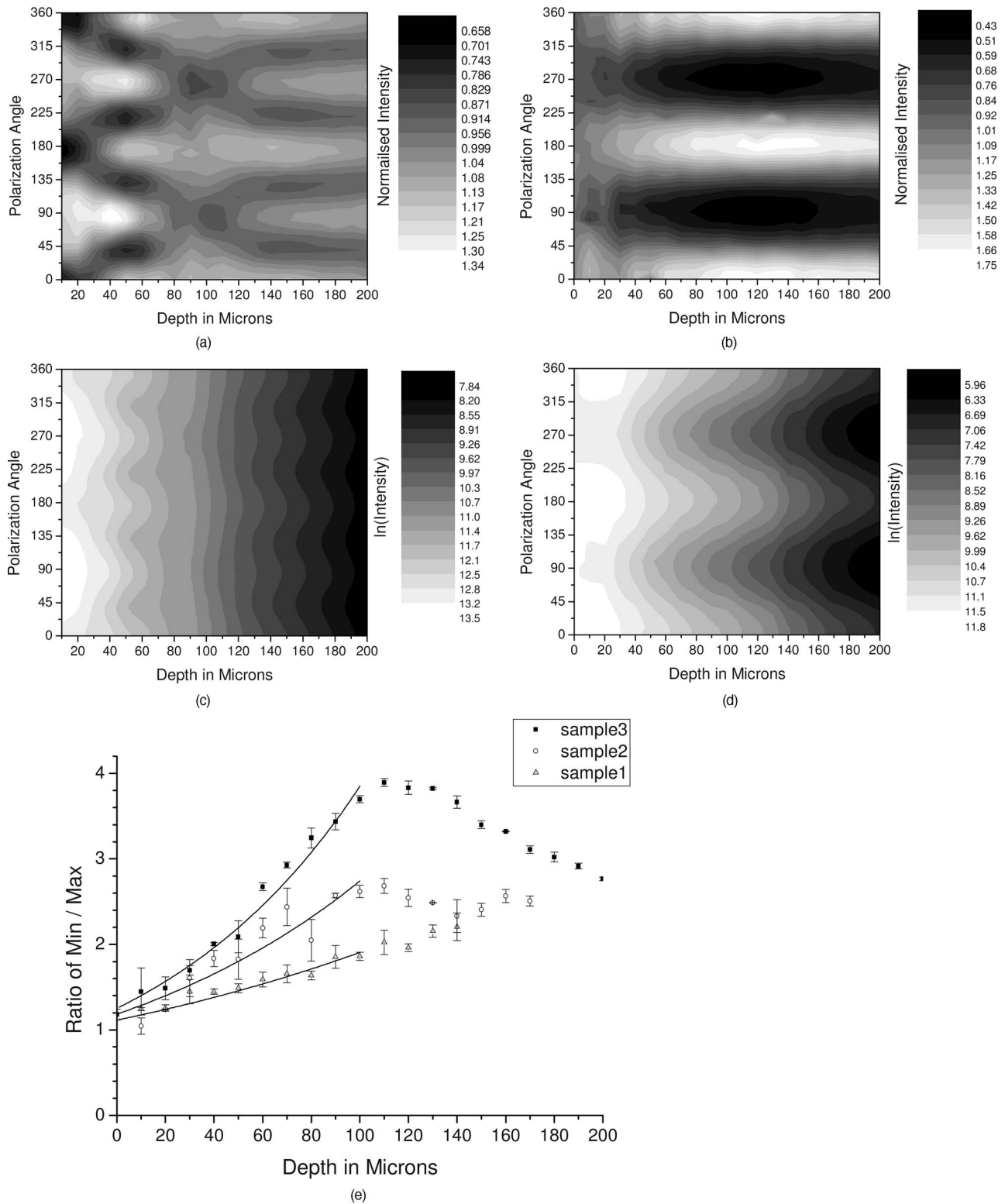
Following Kemp et al.,<sup>23</sup> we note that  $\Delta\chi$  defines the ratio of the field strengths polarized along the two axes at a depth  $\Delta z$ , such that

$$\frac{E_{\max}}{E_{\min}} = \exp\left(\frac{2\pi\Delta\chi}{\lambda_0}\Delta z\right). \quad 293$$

Noting the TPF intensity scales as the fourth power of the field, the ratio of the emitted TPF fluxes is then described by

$$\frac{I_{\max}}{I_{\min}} = \exp\left(\frac{8\pi\Delta\chi}{\lambda_0}\Delta z\right). \quad 296$$

Thus, if the tissue has biattenuance, the ratio of the maximum to minimum intensity should increase exponentially with depth and a plot of  $\ln(I_{\max}/I_{\min})$  versus  $\Delta z$  would have a slope of  $8\pi\Delta\chi/\lambda_0$ , where  $\Delta\chi$  unambiguously represents the biattenuance at the excitation wavelength, i.e., 800 nm. We see from the data [Fig. 5(e)] that an exponential increase can be fitted to the first  $100 \mu\text{m}$  of the curves, but beyond this, the ratio remains constant or falls. The exponential increase appears quasi linear over this range due to the low value for the biattenuance of collagen. This may be because at these depths, the excitation light becomes depolarized due to scattering in the tissue,<sup>28</sup> or the collagen fibers are no longer perfectly aligned. However, restricting the analysis to the most superficial  $100 \mu\text{m}$ , we fitted the data to the equation



**Fig. 5** Polarization dependence of (a) SHG and (b) TPF in tendon at different depths [collagen fibers orientated at 90 deg to the original incident beam polarization (0 deg), intensities normalized by the mean intensity of the image field at each depth]. (c) and (d) show the natural log of the unnormalized polarization sensitivity. (e) shows the ratio of maximum intensity of the TPF/ the minimum intensity of the TPF as a function of depth in the tissue.

$$311 \quad \frac{I_{\max}}{I_{\min}} = \exp \left[ \frac{8\pi\Delta\chi}{\lambda_0} (z - z_0) \right],$$

312 where  $z$  is the depth into the tissue,  $z_0$  is a factor to allow for  
 313 any uncertainty in identifying the surface of the tendon,  $\lambda_0$  is  
 314 the wavelength of the incident light, and  $\Delta\chi$  is the biattenu-  
 315 ance using Microcal Origin. This gave a biattenuance of  
 316  $2.65 \times 10^{-4}$  (range  $1.7 - 3.6 \times 10^{-4}$ ,  $R^2 > 0.85$  for all fits),  
 317 which is comparable with values measured for different types  
 318 of tendon using polarization sensitive OCT (rat tail tendon  
 319  $5.3 \times 10^{-4}$ , rat Achilles tendon  $1.3 \times 10^{-4}$ , chicken patel-  
 320 lofemoral tendon  $2.1 \times 10^{-4}$ ).<sup>23</sup>

321 These estimates represent only an upper bound for the bi-  
 322 attenuance, however, because even with an isotropic distribu-  
 323 tion of fluorophores, the TPF may still be partially polarized.  
 324 This is because the incident polarization vector will most ef-  
 325 ficiently excite parallel-aligned dipoles, resulting in the TPF  
 326 being partially polarized in the direction of the incident laser  
 327 polarization (unless the fluorophores rotate on a time scale  
 328 fast compared with the fluorescence lifetime, which is un-  
 329 likely in collagen).<sup>29</sup> This means that the emitted light will be  
 330 transmitted to the detector with different efficiencies for the  
 331 two orthogonal polarization states, so that the intensity ratio  
 332 will then depend on the value of  $\Delta\chi$  at both the excitation and  
 333 emission wavelengths. The precise relationship between our  
 334 value and the true values at 400 and 800 nm will depend on  
 335 the degree of fluorescence anisotropy. This highlights the need  
 336 for further research into not only identifying but also charac-  
 337 terizing the properties of endogenous two-photon fluoro-  
 338 phores.

339 For the SHG, the pattern of polarization with depth in ten-  
 340 don represents the combined effects of the biattenuance, bire-  
 341 fringence, and the intrinsic polarization sensitivity of the col-  
 342 lagen SHG. Tendon is birefringent, with a higher refractive  
 343 index for light polarized parallel to the collagen fibers.<sup>19</sup> The  
 344 birefringence ( $\Delta n$ ) is approximately 0.0045 for equine flexor  
 345 tendon,<sup>3</sup> indicating that a 180- $\mu\text{m}$  thickness of tendon would  
 346 act as a whole wave plate at 800 nm. It should be possible to  
 347 use this information to recreate our polarization sensitivity  
 348 patterns, using either a Jones or a Muller matrix (if scattering  
 349 is found to be a significant effect) to describe the effects of the  
 350 overlying tissue, and the Freund model<sup>16</sup> to describe the po-  
 351 larization sensitivity of collagen fibers. However, the effects  
 352 of beam geometry, such as varying interaction lengths and  
 353 ray-to-fiber inclination angles across the beam profile, are  
 354 likely to be significant for the large numerical aperture objec-  
 355 tive we employed, and it would be a nontrivial task to include  
 356 these in the calculations. Such studies will form the basis of  
 357 further work to be reported in the future.

### 358 3.3 Depth Variations in Cartilage

359 TPF and SHG polarization sensitivity data were taken on the  
 360 same field in samples of healthy cartilage, and representative  
 361 plots are shown in Fig. 6. It proved possible to acquire data at  
 362 depths up to 200  $\mu\text{m}$ . This region is marked on the histologi-  
 363 cal section taken of the sample after imaging [Fig. 6(e)]. From  
 364 this we can see it corresponds to the superficial and transi-  
 365 tional zones shown in Fig. 1. The TPF polarization measure-  
 366 ments were affected by photobleaching, and although expo-  
 367 sure time was kept to a practical minimum, it was impossible

entirely to eliminate the effect. The photobleaching in carti- 368  
 lage was a more significant effect than in tendon. This may 369  
 indicate that a different fluorophore is responsible for the fluo- 370  
 rescence in the two tissues, or that the differences in the ex- 371  
 tracellular matrix environment effect the photobleaching rates. 372  
 In contrast to tendon, the TPF images did not show strong 373  
 polarization sensitivity. At 150 to 200  $\mu\text{m}$ , four peaks were 374  
 evident but their visibility was very small ( $< 0.05$ ) compared 375  
 to that in tendon ( $\sim 0.6$ ) at a similar depth. It is therefore 376  
 apparent that biattenuance is not as significant in cartilage as 377  
 it is in tendon, and the structural bases of this difference re- 378  
 quire further investigation. If this is interpreted as a null result 379  
 for detecting biattenuance in cartilage, we can place an upper 380  
 bound for the possible biattenuance of  $6 \times 10^{-5}$  by assuming 381  
 that periodic variations should be at least two times greater 382  
 than the variations due to noise or photobleaching. 383

The variation in SHG polarization with depth showed a 384  
 clear repeatable pattern in all the normal samples of cartilage 385  
 investigated. At the surface of the tissue, there were two in- 386  
 tensity maxima, but at greater depths two additional peaks 387  
 occurred between the original peaks. There was also an in- 388  
 crease in visibility of the peaks with depth into the tissue. The 389  
 depth at which the additional peaks appeared varied between 390  
 60 and 90  $\mu\text{m}$  in different samples. 391

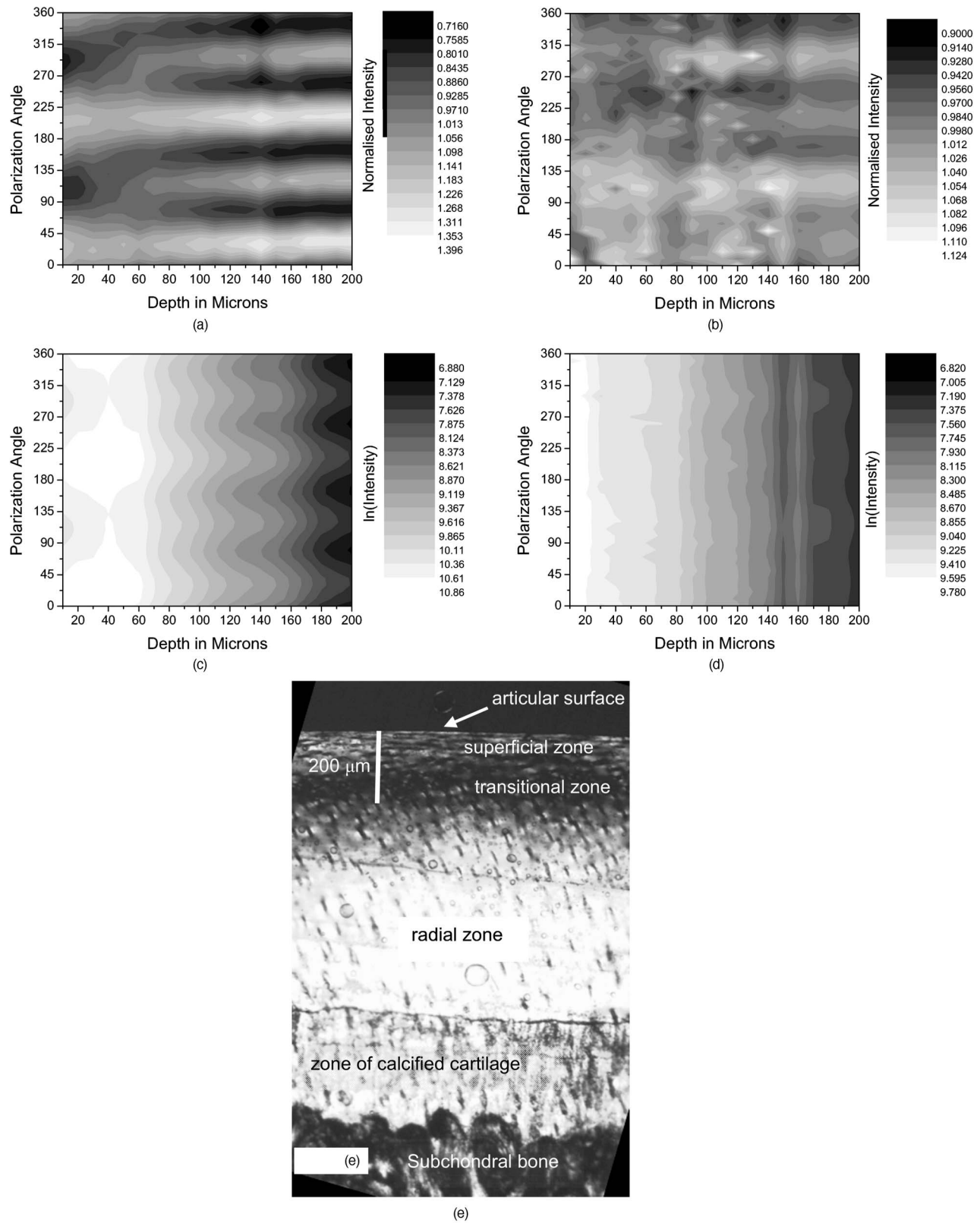
Relating the changes in polarization sensitivity with depth 392  
 to fiber orientation is a more complex problem in cartilage 393  
 than in tendon. In contrast to tendon, the birefringence and 394  
 biattenuance properties of the tissue are likely to change with 395  
 depth, and this level of complexity cannot simply be de- 396  
 scribed by a single Jones or Muller matrix. In addition, the 397  
 collagen fibers form a 3-D network, so the fibers in the deeper 398  
 zones no longer lie in the imaging plane, and the larger the 399  
 angle between the collagen fiber and the imaging plane, the 400  
 weaker the polarization dependence and the weaker the SHG 401  
 signal.<sup>19</sup> It seems therefore that it will be necessary to develop 402  
 a numerical model, perhaps based on measurements of biat- 403  
 tenuance and birefringence, in a series of thin slices of carti- 404  
 lage at increasing depth into the tissue. 405

### 406 3.4 Cartilage Lesions

Spontaneous lesions were found on the apex of a large num- 407  
 ber of the metacarpophalangeal joints examined. The histol- 408  
 ogy of these lesions has been discussed in detail elsewhere.<sup>30</sup> 409  
 We report here on the detailed examination of three such le- 410  
 sions. 411

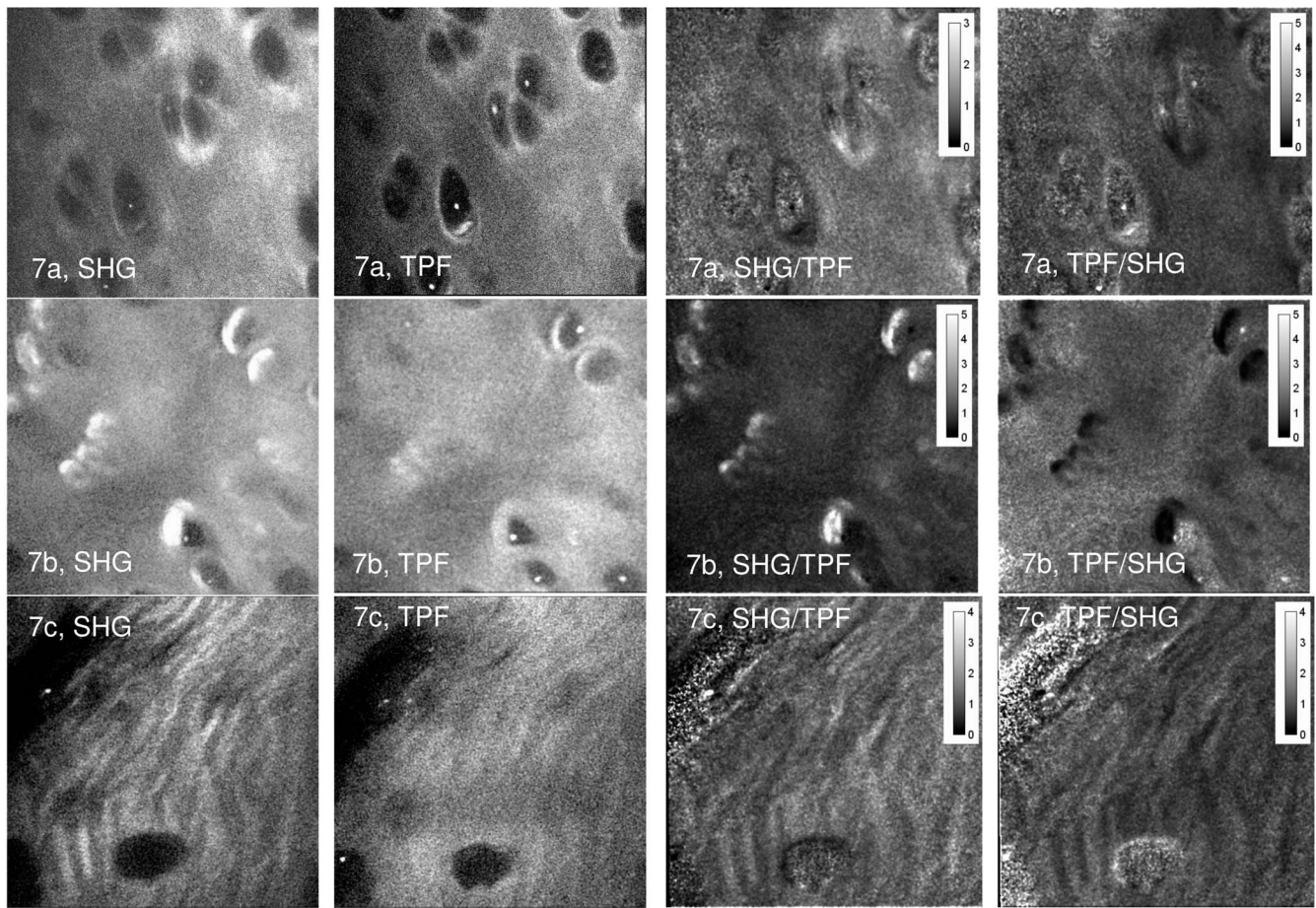
Figure 7 shows paired TPF and SHG images taken from 412  
 the surface of the cartilage in the histologically normal tissue 413  
 close to the lesion, at the periphery of the lesion, and in the 414  
 center of the lesion. The former images are very similar to 415  
 those described earlier and reported by previous authors.<sup>21,31</sup> 416  
 At the periphery of the lesion, where the histology showed an 417  
 intact surface but a loss of zonal structure, the TPF images 418  
 appeared normal, but the SHG images showed abnormally 419  
 high signal intensities from the pericellular matrix. In the core 420  
 of the lesion, there were far fewer cells and the matrix also 421  
 appeared abnormal with a rippling effect clearly visible. The 422  
 rippling appears more strongly in the SHG than the TPF and 423  
 so can be confidently ascribed to a collagen abnormality. Ra- 424  
 tio images emphasized the difference in the pericellular ma- 425  
 trix between the normal tissue and the tissue peripheral to the 426





**Fig. 6** Polarization sensitivity with depth in cartilage, (a) SHG and (b) TPF (intensities normalized by the mean field intensity at each depth). (c) and (d) show the natural log of the unnormalized polarization sensitivity for SHG and TPF. (e) shows a histological section taken from the sample after the polarization sensitive measurements, viewed through crossed polarizers to reveal collagen organization.





Region with healthy looking matrix and cells in the TPF and SHG images similar to those shown in shown in 7a

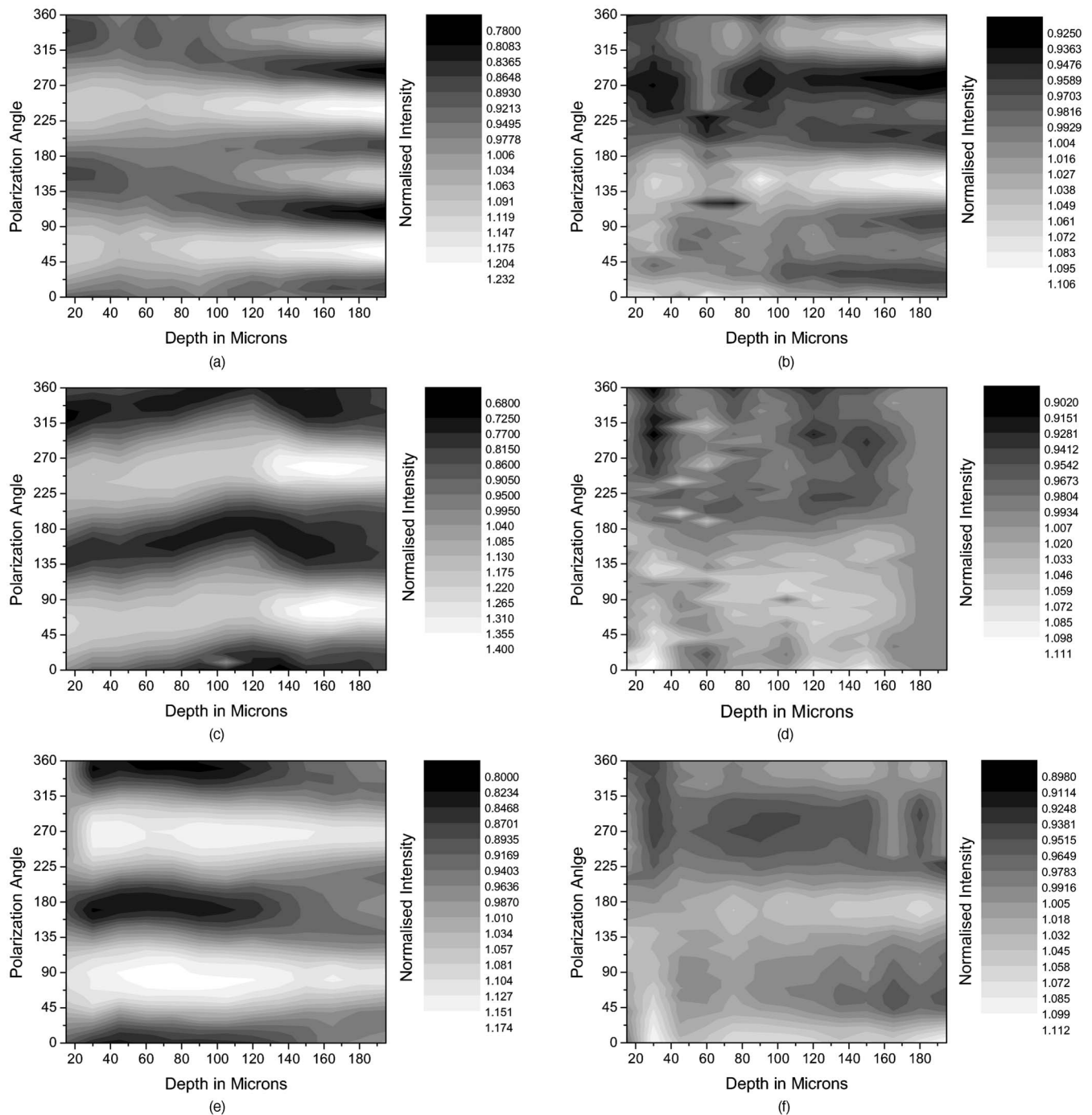
Lesion area: very strong birefringence due to high collagen content: images in the SHG and TPF show very few cells rippled looking matrix in the SHG as see 7c.

Region nearing the lesion histology shows normal arcade structure of matrix is being lost: SHG and TPF images show unusual looking cells see 7b.



7d. Histological slice of the lesion viewed between crossed polarizers

**Fig. 7** Cartilage lesion. (a) SHG and TPF images taken from a region 3 mm away from the center of the lesion, which appeared normal in histological sections. (b) Images from the periphery of the lesion, showing abnormalities in the SHG from the pericellular matrix, while the TPF image remains normal. (c) Images from the center of the lesion, showing loss of cells and rippling effect in the SHG image. (Field  $100 \times 100 \mu\text{m}$ ). The ratio images shown in the right-hand column highlight the changes in (b) the pericellular matrix and that (c) the rippling effect is stronger in the SHG image.



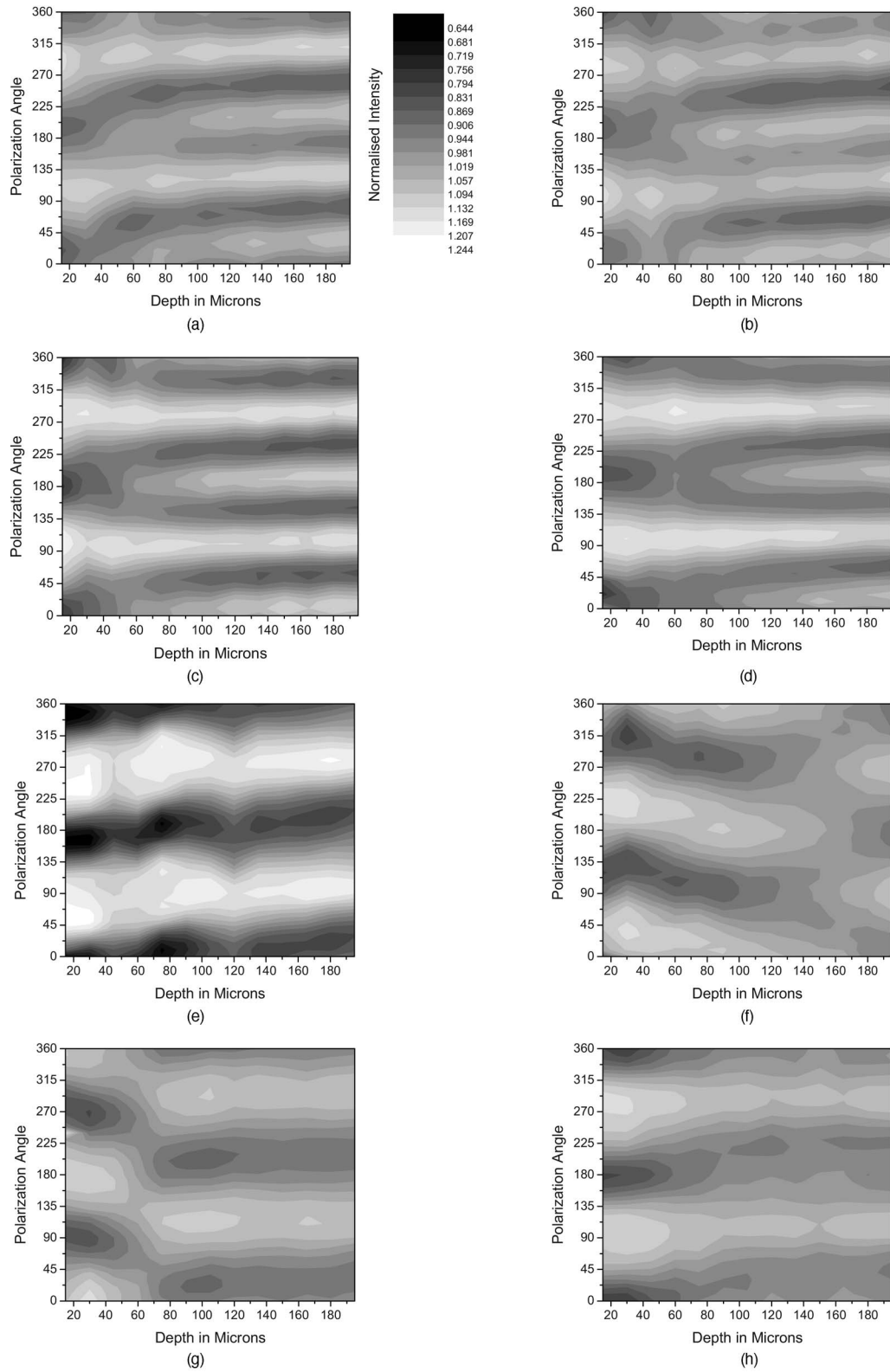
**Fig. 8** The polarization dependence of TPF and SHG in the vicinity of a lesion, with site 1 being macroscopically normal-looking tissue close to the lesion, site 2 being approximately central to the lesion and site 3 being tissue in the periphery of the lesion.

427 lesion, with the ratio of SHG/TPF much greater in the peri-  
428 cellular matrix at the periphery of the lesion.

429 Figure 8 shows the measurements of both SHG and TPF  
430 polarization as a function of depth, in the core of the lesion  
431 and at the periphery, in macroscopically normal tissue. At the  
432 latter sites, the tissue showed the weak biattenuance in the  
433 TPF signal reported earlier, but at the core of the lesion, no  
434 polarization sensitivity was observed. Again we interpret this  
435 as a null result and place an upper bound of  $6 \times 10^{-5}$  on the

biattenuance, as for the TPF from healthy cartilage. 436

The SHG depth profiles showed larger changes than the 437  
TPF in the vicinity of the lesion, and so Fig. 9 shows a set of 438  
nine depth scans taken at 1-mm intervals across the lesion. 439  
The first three scans covered regions that were histologically 440  
normal, and the scans were indistinguishable from those obtained 441  
in normal tissue at remote sites. However, at the site of the 442  
fourth scan, the histological structure was disrupted with 443  
the cartilage zones no longer clearly identifiable, and the SHG 444



**Fig. 9** Polarization dependence of SHG with depth for a series of ten sites at 1-mm lateral positions through the lesion. Position 1 is anterior to the lesion and position 9 is posterior. At position 6, at the center of the lesion, the structure was too broken to detect any pattern of polarization sensitivity with depth. [All scales as in (a)].



445 pattern in the four peak pattern beginning to be lost. The  
 446 remaining scans were grossly abnormal in showing only two  
 447 intensity maxima. In addition, the angle at which the peaks in  
 448 intensity occurred was not constant with depth. This indicates  
 449 a change in the angle of the fibers, as observed in x-ray dif-  
 450 fraction studies on similar lesions.<sup>4</sup> The intensity of the polar-  
 451 ization sensitivity was greatest in the center of the lesion. This  
 452 is consistent with a previous study that was able only to mea-  
 453 sure polarization sensitivity in diseased and not in healthy  
 454 cartilage.<sup>21</sup>

#### 455 4 Conclusions

456 We demonstrate that nonlinear microscopy is a useful tool  
 457 with which to study cartilage changes associated with disease,  
 458 with polarization sensitivity measurements in particular pro-  
 459 viding information on changes in collagen fiber organization.  
 460 The work is carried out in reflection mode, which enables  
 461 examination of the surface of cartilage still attached to sub-  
 462 chondral bone. Although the SHG signal from collagen is  
 463 propagated primarily in the forward direction, backscattering  
 464 was sufficiently strong to make this viable. The source of TPF  
 465 remains uncertain. The ratio of TPF to SHG in cartilage was  
 466 six times greater than that in tendon. TPF was not codistrib-  
 467 uted with the SHG, and so collagen cross links, at least some  
 468 of which are fluorescent, are unlikely to be the only source.  
 469 Elastin is a strong source of TPF, but it is present in articular  
 470 cartilage only in very small quantities, if at all, and certainly  
 471 not with the observed distribution. Proteoglycans are present  
 472 at much higher concentrations in cartilage than tendon, and so  
 473 are potential contributors to the tissue difference, but in our  
 474 hands, pure preparations of aggrecan monomers and aggre-  
 475 gates did not produce TPF at the wavelengths employed (data  
 476 not shown). Further work on this and other fundamental ques-  
 477 tions such as the mechanisms of generation of SHG in  
 478 collagen<sup>15</sup> is required if multiphoton methods are to provide  
 479 detailed information on tissue composition.

480 The SHG signal is polarization dependent, and surface  
 481 studies prove a sensitive means of detecting fiber orientation  
 482 in the superficial layers of cartilage. Since disruption of this  
 483 layer is believed to be one of the earliest indicators of disease,  
 484 the potential of these measurements for early *in-vivo* detection  
 485 is worth consideration. However, the interpretation of polar-  
 486 ization sensitivity data acquired from deeper regions of the  
 487 tissue requires care and further information. It is clear that  
 488 these signals are distorted by the birefringence and biattenu-  
 489 ance of the overlying tissue, and quantitative measurements of  
 490 these properties, together with detailed models of light propa-  
 491 gation in tissue, will be required to correct for these effects.  
 492 However, the detailed characterization of changes in the struc-  
 493 ture of living tissue on microscopic scales that could be ob-  
 494 tained would well reward such efforts.

495 In addition to detecting changes in the organization of the  
 496 intercellular matrix, multiphoton microscopy reveals changes  
 497 in the pericellular matrix that appear to precede changes in the  
 498 intercellular matrix. The ability to observe this structure in  
 499 living tissue should provide new avenues for studying the  
 500 underlying biological processes.

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