Novel techniques in determining macroscopic 2D and microscopic 3D collagen orientation in articular cartilage of the tibia plateau
An experimental study based on the split-line technique and 2-photon confocal laser scanning microscopy

Jasper Foolen
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Supervisor:
C.C. v. Donkelaar

Special thanks:
M.A.M.J. v. Zandvoort
M.P.F.H.L. v. Maris
W. Wilson

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Summary
Data concerning the collagen orientation in articular cartilage is needed in establishing numerical models that assist in understanding cartilage pathology. Over the years, several models of collagen orientation have been proposed, however it is unknown if the applicability of the models is site dependent. Besides, techniques that have been used are based on visualisation in a two-dimensional manner where it is believed that collagen orientation is essentially three-dimensional. The well-known split-line technique has been applied to cross-sectional porcine samples to establish the macroscopic collagen orientation throughout the articular cartilage. Furthermore, 2-photon confocal laser scanning microscopy (2-photon CLSM), the first technique able to create three-dimensional reconstructions of collagen orientation in articular cartilage samples, has been used to visualise the orientation at a microscopic level. From the split-line technique, it appears that three distinct collagen orientation patterns can be indicated. Collagen arises from lateral end of the cartilage and curves towards the subchondral bone in an oblique manner, a model that is formerly proposed by MacConaill (MACCONAILL, 1953). In the centre, Benninghoff’s model (Benninghoff A., 1925) is applicable, where collagen arises from the subchondral bone running perpendicular to the cartilage surface, where collagen arches in the transitional layer and runs parallel to the cartilage surface in the superficial layer. At the medial side, collagen orientation is random, a model proposed by for instance Reinert et al (Reinert et al., 2001). 2-Photon CLSM has revealed new insights in collagen architecture. Furthermore, collagen is constructed of a very complex network of splitting and congregating fibres and bundles that is essentially three-dimensional.
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1 Introduction

Articular cartilage is composed of a relatively low density of chondrocytes, within a matrix of proteoglycans and collagen fibres (Muehleman et al., 2004). The primary collagen components are collagen type II, IX and XI, where the latter two are proteins that cross-link the collagen type II (Eyre, 2002). Collagen fibrils have a very high tensile strength, with little ability to withstand compression. The hydrated proteoglycan has this capability, provided it is immobilised and contained in the collagen matrix (Muehleman et al., 2004). Therefore, the collagen network resists strains, tensile and shear stresses while the fluid component resists the high hydrostatic compressive stresses that are generated with external loading (Wong and Carter, 2003).

The hyaline cartilage consists of four zones: superficial, transitional, deep and calcified (Muehleman et al., 2004). In the superficial zone, cells are flattened and subjected to fluid flow and matrix consolidation in addition to hydrostatic pressure. They synthesise and maintain higher amounts of collagen relative to proteoglycans (Wong and Carter, 2003) (Fragonas et al., 1998). In contrast, chondrocytes of the middle and deep zones, which are loaded primarily under hydrostatic pressure and experience little strain or fluid flow, synthesise and maintain high amounts of glycosaminoglycans and collagen (Wong and Carter, 2003) (Clark, 1991). This general zonal organisation changes with location in joints, for instance the centre of the tibia plateau lacks a thick collagen-rich surface. The superficial zone is relatively thin (10 – 50 µm) and constitutes less than 10% of the total cartilage thickness. The periphery, however, contains less water and the fibre density is lower (Clark, 1991). The tangential zone is thicker (100-400 µm) and contributes up to 50% of the cartilage thickness (Clark, 1991). Clark observed that at the periphery, fibres that rise radial from the deep and middle layer either turned away from or towards the tibial edge (figure 1). In the latter case, the radial fibres clearly interdigitated with the group arising from the periosteum (Clark, 1990).

The structure of the different layers was further investigated by Benninghoff using polarised light microscopy (PLM) (Benninghoff A., 1925).

By means of the PLM technique, Benninghoff (Benninghoff A., 1925) examined the orientation of collagen fibres lying within the cartilage matrix. He proposed the well-known arcade model in which collagen fibres were described as arising in the subchondral bone then passing towards the surface in a radial manner before arching over to run tangential to the surface and finally returning to the subchondral bone (Jeffery, 1994). Benninghoff’s findings have generally been confirmed using scanning electron microscopy (SEM) in various studies, with slight variations on the arcade concept (Clark, 1990) (Clark, 1991) (Kaab et al., 1998a) (Hunziker et al., 1997) (Clarke, 1971) (Muehleman et al., 2004). Recently it was shown by high intensity X-ray imaging that several striations that extend from the bottom of the deep zone to the superficial zone, begin to arc a bit deeper in the cartilage than others, but all arc within the superficial 25% (Muehleman et al., 2004). Clarke observed that at lower magnification range, SEM revealed the arcade pattern, while at higher magnifications the fibrillar organisation correlated well with a random network of fibrils overlaid at the articular surface by a membrane-like system of bundled fibrils (Clarke, 1971). Hunziker et al. used electron microscopy and observed an orientation similar to Clarke, namely that the collagenous component appears to be organised into a dual system. One system is composed of the classical arcade-like architectural arrangement, whereas the other is organised isotropically (Hunziker et al., 1997).

Figure 1: Fibre orientation at the periphery (Clark, 1990)
This random orientation has been confirmed by researchers that propose a much more disorganised, almost isotropic, arrangement overlaid by a distinct superficial zone of orientated fibrils or bundles using SEM (Hunziker et al., 1997) (Clarke, 1971) (DAVIES et al., 1962) (Ruttner and Spycher, 1968) (Silverberg et al., 1964) (Silvergeberg et al., 1966) (Silverberg, 1968) (Weiss et al., 1968). Arsenaull et al. proposed that the specific matrix components appear intermixed beyond the capacity to appreciate a fundamental pattern of distribution in whichever layer (Arsenault and Kohler, 1994). Reinert and Reibetanz observed that the collagen fibrils in the tangential layer lose their orientation with increasing distance from the articular surface using magnetic resonance imaging (MRI) and PLM (Reinert et al., 2001).

A model proposed by Clarke (Clarke, 1971), based on observations using SEM, describes thin fibrils in the superficial zone that run primarily parallel to the plane of the articular surface, a random organisation in the deeper zones and a preferred orientation of fibril bundles orthogonal to the surface in the radial zone. This model has been confirmed by Eyre using transmission electron microscopy TEM (Eyre, 2002), by Muehleman using Diffraction Enhanced X-ray Imaging (DEI) (Muehleman et al., 2004) and by Reinert using scanning transmission ion microscopy (STIM) (Reinert et al., 2001).

A study by MacConaill (MACCONAILL, 1953) did not support Benninghoff’s arcade model. Using PLM and phase contrast microscopy, collagen fibres were described as forming a dense network with fibres running obliquely between the articular surface and the subchondral bone. This pattern has also been encountered by Ohnsorge (Ohnsorge et al., 1970), and more recently using in vitro MR imaging by Goodwin et al. (Goodwin et al., 2000).

Obviously, the organisation of the collagen structure varies between point and with location in a joint. In addition it may well be that observations with SEM or TEM depend on the orientation of the slice through the cartilage. Such differences may explain the differences between the various proposed models. Insights in the three-dimensional collagen organisation could also assist in the understanding and prediction of articular cartilage pathology and repair (Clark, 1990). For instance, it is assumed that integrity loss of the network could be an initial pathological process for cartilage degradation (Reinert et al., 2001). Numerical analysis of the effect of external mechanical loading on the loading of the collagen network may assist in the understanding of such cartilage degradation. Knowledge of the collagen orientation throughout the cartilage for the use of predictive numerical models is essential.

The aim of the present study is to assess the three-dimensional collagen fibre organisation in porcine articular cartilage from the medial side of the tibia plateau. The medial tibia plateau is of particular interest because it is known to be a common site of cartilage degradation in humans (Kobayashi et al., 1995) (Aston and Bentley, 1986). In order to obtain this three-dimensional collagen network information, two different methods are used. First, the well-known split-line technique was used to establish the collagen orientation at the surface. From this surface orientation framework, different cross-sections are selected and split-lines have determined the collagen orientation throughout different planes in articular cartilage of the tibia plateau. Specifications of the locations from cross-sectional harvest site are included. SEM has been used to justify whether or not the split-line technique is a valid one to determine collagen orientation in cross-sections of articular cartilage. This is done mainly because split-line research is outdated as a result of which the images that form the justification (Jeffery et al., 1991) (Clarke, 1971) are of poor quality. Nevertheless, Clarke (Clarke, 1971) and Jeffery (Jeffery et al., 1991) concluded that split-lines made on articular surfaces are in fact surrounded by parallel aligned collagen fibres. So far, studies on collagen orientation research are based on two-dimensional visualisation techniques, like SEM and the split-line technique. Recently, a new technique called 2-photon confocal laser scanning microscopy (2-photon CLSM) is developed that is ideal to study the three-dimensional orientation of collagen in fresh tissue. To the author’s knowledge, this has not been done yet.
2 Methods

Cartilage samples of the lateral tibia plateau of porcine and calf knees, obtained from the slaughterhouse, were produced within 4 days after slaughter. Porcine cartilage samples were obtained by opening the joint without damaging the cartilage of the tibia plateau. Porcine cartilage is most similar to human and contains a more leaf-like arrangement (Kaab et al., 1998a). Ligaments, menisci, fat and muscle were removed, after which the lateral side was separated from the medial side and excessive subchondral bone removed using a jigsaw, leaving approximately 15 mm to support the cartilage. From the remaining manageable tissue, samples of desired geometry were produced using an accutom (Struers Accutom-5, Rødovre Denmark) with PBS as refrigerant to prevent excessive swelling of the cartilage. Visualisation of collagen around split-lines is established using calf samples because their collagen is more fibrous and therefore provides more distinct images on SEM (Kaab et al., 1998a).

2.1 SEM

SEM has been used frequently to visualise the architecture of collagen (Clark, 1990) (Clark, 1991) (Jeffery, 1994) (Clarke, 1971) (ap Gwynn et al., 2000) (ap Gwynn et al., 2002) (Kaab et al., 1998a) (Riesle et al., 1998) (Teshima et al., 1995). In spite of the conclusion drawn by Jefferey et al. (Jeffery et al., 1991) and Clarke (Clarke, 1971) that split-line pattern in articular cartilage is determined by the three-dimensional structure of collagen, confirmation of this proposal is desired due to poor quality of the presented results. Therefore, in order to further justify the split-line technique, SEM images were made to visualise the collagen alignment around split-lines. Samples from the lateral side of a calf tibia plateau were sawed from medial to lateral with a thickness of 2 mm to prevent artefacts that occur during rapid freezing. Before samples were frozen, split-lines were applied by inserting a dissecting needle at a 90° angle (perpendicular to the cross-sectional surface), approximately 5 mm from each other over the complete length of the sample. Subsequently, the freezing protocol (ap Gwynn et al., 2000) (Kaab et al., 1998b) (Kaab et al., 2000) is followed. Images are produced using a Philips XL30 ESEM FEG.

2.1.1 Freezing protocol:

- A container filled with isopentane was cooled in liquid nitrogen. The iso-pentane was left to cool for approximately 10 minutes before proceeding.
- Prepared and sectioned samples were placed in the cooled iso-pentane and left for at least 2 minutes.
- Samples were removed from the iso-pentane and placed directly in liquid nitrogen, where they were left for 4-5 minutes.
- After freezing, the samples were removed from the nitrogen, and placed in containers containing anhydrous, or ‘dry’, acetone that had been pre-cooled in the deep freezer to 193K.
- The containers with acetone and bone/cartilage fragments were sealed in sample bags to prevent any leakage, and placed in the freezer at 193K.
- After 6 days, the samples were transferred into fresh, pre-cooled, acetone and left for a total of at least 8 days at 193K.
- After substitution, the sample containers were removed from the freezer, and placed in a freezer at 248K for approximately 24 hrs.
- After this, containers were transferred to a conventional refrigerator at 277K, and left for another 24h.
- In the final step, containers were removed from the cooling-unit and were allowed to come up to 293K.
- Once the samples had reached room temperature, they were removed from the acetone and placed in petri dishes for air-drying. The samples were left for at least 48 hrs to ensure they had completely dried.
2.2 Split-lines

Split-lines are applied to the articular surface in order to visualise collagen orientation in the superficial layer and on cross-sections of tibia plateau samples to visualise the orientation in the transitional and deep layer. The first method is applied to obtain surface collagen orientation and to get a reference framework for the cross-sections. This framework will also determine the cross-section that is examined with 2-photon CLSM. The cross-sectional samples will now contain a superficial collagen orientation parallel to the surface as well as to the cross-sectional surface. The method is based on the fact that after disruption of the collagen network a split will occur in the direction where the resistance is lowest. From this, it is postulated that the majority of the fibres are aligned in the direction of the split-line. It is not said that in the direction perpendicular to the split-line no collagen fibres can be found. This proposal is supported by the findings of Kempson et al., who examined that the tensile properties of articular cartilage parallel to the split-line is two or three times larger than that of a specimen loaded at right angles to the split line. In addition, stress at fracture was larger (Jeffery et al., 1991). Below et al. verified that a “preferential” orientation of the collagen fibres exists in the superficial layer of articular cartilage (Below et al., 2002).

2.2.1 Orientation in the superficial layer

On the lateral side of pig tibia plateaus, split-lines are made within 1 hour after the joint is exposed. Split-lines are applied a few millimetres from each other in order to indicate specific orientation patterns. Afterwards the surface is cleaned from excessive ink with a tissue and images are made using a digital camera. In total, four different knees are supplied with split-lines to test reproducibility.

2.2.2 Orientation in the transitional and deep layer

Cross-sectional samples from the tibia plateau were produced with a thickness of approximately 10 mm. Split-lines were made and cover the complete cross-section. Excessive ink is removed after which images are taken using a light microscope (Zeiss Axioplan 2) connected to a digital camera (Zeiss Axiocam). A total of 5 cross-sections are examined, where the cross-sections are sawed in the direction parallel to the fibre orientation in a radial manner towards the centre of the plateau (figure 9). It is not established whether or not there exists a collagen orientation perpendicular to the examined cross-sections, which will be determined with 2-photon CLSM.

2.3 2-Photon CLSM

2-Photon CLSM is a technique that allows making three-dimensional reconstructions up to a depth of approximately 62 µm. A single tissue component or multiple components, dependent on their specific binding properties to a dye, can be visualised three-dimensionally. Hereby, fresh tissue in combination with eosin, specific for both collagen and cells in 2-photon CLSM, is used. The use of 2-photon CLSM has advantages compared to ordinary CLSM:

− The tissue is examined at a laser wavelength of 800 nm.
− A high penetration depth and therefore ideal to make 3-D reconstruction movies that allow to follow collagen fibres through the tissue.
− Photo bleaching and damage effects are limited to the focal plane because 2-photon excitation only occurs at focal plane. Therefore, the tissue can be examined for a prolonged time.
− No diaphragms are needed.
− To the author’s knowledge, no collagen type II staining method for ordinary CLSM has been found up to now.

Pig samples from the lateral side of the tibia plateau are produced with a thickness of approximately 10 mm. Samples were made from lateral to medial and placed in PBS before and during examination. Eosin is used to serve as a fluorescent dye at a concentration of 2 µM, where samples were exposed to the dye for at least 30 minutes. Longer exposure-time
did not provide better results. A standard BioRad 2100MP was used in TPLSM (two photon laser scanning microscope) mode. The excitation source was a Spectra Physics Tsunami Ti:Sapphire laser, tuned and mode-locked at 800 nm. The microscope objective consists of a water-dipping lens with a 60× magnification and a 1.2 numerical aperture, connected to an upright Nikon E600FN microscope. Sequences of images have been taken with slice spacing of 0.45 µm, and a z-resolution of approximately 1.25 µm. Three photomultipliers detected the fluorescence. For TPLSM, pinholes were fully opened. Each photomultiplier accepted a different wavelength region: (1) 420-490 nm; (2) 500-540 nm and (3) 560-610 nm. The third bandwidth is based on the autofluorescence properties of collagen (Hanley *et al.*, 1999). The hereby-obtained three separate images (coded red, green and blue, respectively), were combined into a 3D reconstruction or movie consisting of 80 up to 138 single images (slices). Generally, generating movies or 3D reconstructions is essential to visualise collagen because the cross-sections sporadically run perfectly parallel to the collagen arrangement.

3 Results

3.1 Scanning Electron Microscopy

From figure 2, it appears that the split-line method causes the tissue to cleave in a certain direction. Unfortunately, collagen fibres at the cross-sectional area are not visible (figure 2 and 3), where it is believed that they are denaturised as a consequence of sawing. Samples were produced using an accutom where, in spite of cooling, the heat produced during the sawing process probably denaturises the protein based collagen. Therefore, no orientation was observed at the surroundings of the split-lines. Nevertheless, the collagen orientation at the side of a split-line slightly deeper into the tissue did become visible (figures 3 and 4), because this surface is not exposed to excessive heat or rotating blades. The odd geometry of the split-line in figure 3 is believed to arise from bifurcating collagen fibres or bundles. This phenomenon is observed frequently using the split-line technique. Bifurcation of collagen bundles is also observed with 2-photon CLSM, paragraph 3.3.1.

![Figure 2: Surface of cross-section with split-line.](image)

![Figure 3: Surface of cross-section with split-line; side is slightly overexposed.](image)
Figure 4: Side in a split-line from figure 3 at higher magnification.

From figure 4, it is clear that the collagen orientation is aligned with the direction of the split-line. The darker area at the right, with absence of a visible collagen orientation, shows the cross-sectional surface.

3.2 Split-lines

3.2.1 Orientation in superficial layer

Collagen orientation at the surface is visualised at four different lateral tibia plateaus, where a remarkable similarity is observed, with exception of the area indicated with a dashed circle in figure 6. The orientation appeared to be clear and fibre direction could be followed throughout the plateau. Three views are depicted; see figures 5 to 7, in order to reflect the overview.

In accordance with the pictures that are not depicted, a collagen orientation for the lateral tibia plateau is proposed (figure 8). The collagen orientation laterally was clearer and more obvious compared to medially, where split-lines are not successively aligned. Medially, split-lines more frequently have a crossed pattern. This implies that collagen arrangement in this specific area at the surface is oriented in two directions, where this area by approximation is restricted to the site where the cartilage is in direct contact to the femur articular surface and not segregated by the meniscus. This could be an explanation for this proposed dual orientation, because the cartilage is subjected to higher loads and this location of the joint may be more susceptible for forces that change position during joint movement.
3.2.2 Orientation in the transitional and deep layer

As mentioned before, no literature has been published concerning the application of the split-line technique to a cross-sectional sample. Therefore, a test sample was made in order to check whether it was possible to obtain split-line patterns on a cross-section. A sample was cut from medial to lateral (conform #3 in figure 9) and split-lines were applied. Results were obtained using a light microscope (Zeiss Axioplan 2) and showed that it is possible to obtain split-lines at cross-sectional samples (figure 10) with little damage by the needle and that collagen orientation can be followed throughout different layers (figure 11), including the superficial layer (figure 12).

Figure 9: Direction of the samples cut from the medial tibia plateau.
Subsequently, a choice has been made for the direction of the cross-sectional samples in which split-lines had to determine the transitional and deep orientation. By examining samples that are sawed parallel to the collagen fibre orientation from the superficial layer conform figure 8, conclusions can be drawn concerning the complete cross-section. As mentioned before 5 cross-sections were evaluated (figure 9). Due to the remarkable reproducibility of the split-line technique, three cross-sections are evaluated from the centre (indicated #3 in figure 9) and only one sample of each cross-section outside the centre is evaluated (indicated with #1, 2, 4 and 5). The results of the split-lines are depicted in figures 13 to 20 from lateral (left) to medial (right), where the topside represents the cartilage surface and the bottom side depicts bone.
In each cross-section (figure 13 to 17), three different areas with clear orientation can be pointed out: the periphery (at the lateral side), the centre and the medial side, where the similarity in collagen orientation at different cross-sections is remarkable. At the periphery (figure 18), fibres tend to originate perpendicular from the lateral end of the cartilage into the tissue ending obliquely orientated to the subchondral bone. This oblique pattern is consistent in one third of the total cartilage cross-section. In the centre (figure 19), fibres run perpendicular from the subchondral bone towards the cartilage surface where arching is expected but rarely observed, probably due to the relative small thickness of the layer in which arching occurs. At the inside (figure 20), a more random orientation is observed where no distinct pattern could be indicated. This area corresponds roughly to the area indicated in figure 8 where the meniscus does not segregate the articular surfaces and a dual collagen system in the surface layer seems to occur. Therefore, besides the surface also the deeper layers in this area have a more complex collagen orientation which is hard to determine and can be described best as being random. 
In accordance with the images obtained from the split-line, a cross-sectional fibre pattern is proposed. It is postulated that this pattern is applicable to each cross-section that is in alignment to the collagen orientation proposed in figure 8.

Figure 21: Proposed fibre orientation pattern

3.3 2-Photon CLSM

Because the split-line technique only indicates fibre orientation in two dimensions, while it is thought that this orientation is three-dimensional, cross-sectional samples from lateral to medial (indicated with #3 in figure 9) are produced for 2-photon CLSM to reveal the orientation in three dimensions. It appeared possible to visualise collagen fibres in the superficial and transitional layer, however fibres in the deep layer could rarely be observed. Images presented are either slices that are part of a large sequence of scanned images or z-projections that consist of all scanned images as a stack in a single image. The sequences of images have been reconstructed in a 3D projection or movie in order to follow collagen fibres through the tissue and determine collagen orientation. The focus using 2-photon CLSM has been the periphery. All images from paragraph 3.3 are depicted from lateral (left) to medial (right) and from cartilage surface (above) to bone (bottom). The location from where the images are taken is depicted in figure 22. From figure 23 and 24, it is clear that the majority of the fibres extend from the peripheral end of the cartilage and curve towards the subchondral bone in an oblique manner. Fibres that arch in the opposite direction are also observed. Arching of fibres is most frequently seen within the superficial 200 µm of the cartilage. The transitional layer at the periphery in which fibres have an orientation parallel to the surface is approximately 100 µm thick. Furthermore, images 25 to 31 are 206 µm x 206 µm in size.

Figure 22: Location of presented images; cross-sectional location conform section #3 in figure 9.
3.3.1 Splitting and congregation

From the majority of the images (for example figure 25) it appears that collagen fibres exist in bundles or packs where they are cross-linked to each other and where splitting of fibres appears a common event. Therefore, regions where fibres have a specific orientation can be identified.
Another example of the congregation and splitting of collagen fibres (figure 26 and 27) indicates the complexity of the collagen network where collagen cannot be characterised as single fibres but more like a strongly branched tissue component, where remarkable splitting and congregation are common. Figure 27 shows a triangular shaped collagen network where slice 15 together with slice 16 reveals that collagen either splits or congregates at three locations in the same plane.
3.3.3 The 3-D network

2-Photon CLSM images reveal the crosses obtained using the split-line technique as depicted in figure 28 from which it can be concluded that the main collagen orientation here is indeed two-ways. The second observation that can be made from figure 28 is that the collagen is constructed in a 3-dimensional network configuration where fibres from four corners originate (slice 40 and 44), congregate in the centre of the cross (slice 48 and 51) after which they extend towards 3 different directions again (slice 59, 62 and 66). The third observation is that the observed collagen fibres exist localised and not uniformly throughout the tissue.

![Z-projection](image)

(Image 1: Slices 40, 44, 48, 51, 54, 59, 62, 66 of 84)

Figure 28: Example of a three-dimensional collagen network.

A 3D reconstruction of the images is obtained by stacking the images using ImageJ. Views from different angles show that the collagen orientation is essentially three-dimensional and cannot be examined appropriately by only considering an orthogonal view as the z-projection (see figure 29). In the z-projection of figure 29, it seems that fibres run parallel to the cutting
plane. However, the $z$-projections that are rotated reveal that the fibre direction actually is almost perpendicular to the plane of the cross-section.

![Figure 29: Existence of a collagen orientation perpendicular to the cross-section.](image)

### 3.3.4 Long individual fibres

From figure 30 it appears that collagen in the superficial layer has an orientation parallel to the surface with long fibres that extend to a length over 200 $\mu$m. The superficial layer, when defined as fibres that run parallel to the articular surface, reaches up to 200 $\mu$m.

![Figure 30: Existence of long superficial collagen fibres.](image)

### 3.3.5 Localised presence of collagen

As mentioned before, collagen is not uniformly observed throughout the tissue, probably due to the limitations of the 2-photon CLSM technique. Fibres appear localised, where splitting of fibres occurs until they become too thin to visualise them. Figure 31 again illustrates that the collagen network, is sometimes detected very locally. Furthermore, the orientation in the superficial layer is not detected or not present.
Figure 31: Localised collagen fibres.
4 Discussion

Collagen orientation in articular cartilage has been studied intensively, however, authors constricted themselves frequently by reporting results without mentioning the area from which examined samples were taken, assuming that the collagen orientation is equal at all sites. Furthermore, techniques have been used that are based on visualisation in a two-dimensional manner, assuming that all collagen fibres and bundles observed in generated images, approximately run parallel to the cutting or fracture plane. Using different techniques, this study shows that both assumptions are wrong. The split-line technique shows that the harvest site is crucial. 2-Photon CLSM images obtained in this study show that collagen is essentially constructed of a three-dimensional network, which can barely be understood from two-dimensional techniques such as PLM and SEM.

The well-known split-line technique has proven to be a useful tool in determining collagen orientation at the articular surface and at different cross-sectional planes through the articular cartilage. Formerly, articular cartilage surfaces have been subjected to the split-line technique in several studies. However, results of cross-sectional samples have never been reported, to the knowledge of the author. This relatively simple and very fast tool enables to determine collagen orientation on fresh tissue without artefacts that frequently occur during sample preparation using various electron microscope techniques (Kaab et al., 1999) (Clarke, 1971) (Reinert et al., 2001) (Kaab et al., 1998a) (Arsenault and Kohler, 1994) (ap Gwynn et al., 2002) (Hunziker et al., 1997) (Muehleman et al., 2004). The split-line technique results in a remarkable similarity in the stress pattern of knee specimens examined, an observation that is consistent with conclusions drawn by Below et al. (Below et al., 2002), who applied split-lines to the articular surface of the distal femur. However, a site exists (figure 6) where collagen orientation contains a more random pattern, which is restricted to the site where no meniscus is found and femoral cartilage directly contacts the tibial cartilage. This is contradictory to the findings of Below et al., who observed that cartilage surface areas, exposed to significant contact forces, produce more consistent split-line orientation patterns (Below et al., 2002). Split-lines, applied to cross-sectional surfaces, reveal on a macroscopic level, three different collagen orientation patterns: the arcade model proposed by Benninghoff (Benninghoff A., 1925) at the central part of samples (figure 19), a more oblique pattern at the periphery (figure 18) proposed by MacConnaill (MACCONAILL, 1953) and a more random pattern at the lateral side (figure 20) formerly proposed by, among others, Arsenault et al. (Arsenault and Kohler, 1994). It appeared difficult, but possible, to visualise the arches from the transitional layer (figure 11 and 12) in cross-sectional samples. Along cross-section #3 in figure 9, which is a representative cross-section, the three-dimensional collagen architecture is assessed using 2-photon CLSM. The images obtained from 2-photon CLSM reveal new insights in the structure of the collagen network and several striking phenomenon appear. It is frequently encountered in 2-photon CLSM that collagen fibres do not perfectly run parallel to the cutting plane where the technique enables the 3D reconstruction to be rotated in order to visualise the actual collagen orientation, as shown in figure 29. This observation confirms that two-dimensional imaging techniques have their shortcomings in determining the collagen network organisation. From the numerical aperture of the objective (60 x magnitude; 1.2 numerical aperture) and used wavelength (800 nm) it can be calculated that fibres with a thickness of approximately 400 nm can be easily detected, where it is less plausible that smaller fibres are visualised, unless the surroundings are free from components sensitive to the fluorescent dye. Yet, it is believed that the visualised organisation of thicker fibres is representative for the organisation of smaller bundles of fibres. This thought is re-enforced by the similarity with respect to the split-line patterns in cross-sections. Several studies have indicated that collagen diameter does not exceed 150 nm (Riesle et al., 1998) (Clarke, 1971) (Hunziker et al., 1997) (Teshima et al., 1995), where others have reported collagen with a diameter up to 600 nm in the middle zone and even thicker in the deep zone, where a progressive increase in fibril diameter moving from the superficial and transitional zones towards deeper regions is reported (Hunziker et al., 1997).
(Clark, 1990). From the current study, it is concluded that articular cartilage contains a large amount of fibres with a diameter that range up to a few hundred nanometres. The increase in collagen diameter from the superficial to the deep zone has not been observed directly. However, from figure 23 and 24 it can be postulated that fibres congregate frequently as they move from the superficial to the transitional layer and therefore can be supposed to increase in thickness. Yet, collagen fibres in the deep zone are rarely detected with 2-photon CLSM. Even thick collagen fibres in the superficial layer are not always detected (figure 31). It is thought that the increase in proteoglycans and decrease in collagen moving towards deeper layers may contribute to the fact that the composition of the tissue, sensitive to the fluorescent dye, in deeper zones is too dense in order maintain resolving power. Therefore, emitted light from tissue components is scattered by surrounding components and no detail is observed. Clark’s findings concerning the existence of a thicker tangential zone, strictly defined as the layer in which fibres run parallel to the articular surface, at the periphery compared to the centre (Clark, 1991) cannot be confirmed. The opposite is found in this study where the tangential zone in the centre is thicker (200 µm) compared to the periphery (100 µm). The density of collagen fibres appeared to be greater at the superficial layer compared to the transitional layer, which is consistent with findings by Fragonas et al. (Fragonas et al., 1998).

Arching of fibres is most frequently observed within the first 200 µm, which corresponds to the first 25% of the cartilage thickness, consistent with findings by Muehleman, et al. (Muehleman et al., 2004). The proposed tubular arrangement of collagen in articular cartilage proposed by various researchers (Reinert et al., 2001) (Malinin et al., 1998) (Kaab et al., 1998a) (ap Gwynn et al., 2002) (Reinert et al., 2002) (ap Gwynn et al., 2000) (Muehleman et al., 2004) has not been observed using 2-photon CSLM, even though this technique would seem the best option for observation of such three-dimensional structures. Images from superficial and transitional layers reveal that thick fibres are not located uniformly throughout the tissue but appear localised. Images produced with SEM by Clark (Clark, 1991) of human cartilage also reveal localised behaviour of collagen. Since it is reported that porcine cartilage is most similar to men (Kaab et al., 1998a), Clark’s findings (Clark, 1991) correspond to the results presented in this study. Images of calf, rabbit and dog reveal that the collagen is aligned in a columnar arrangement and distributed more homogeneous (Clark, 1991). By applying the 2-photon CLSM technique to samples taken from animals that contain this columnar arrangement, the similarity between SEM and 2-photon CSLM can further be proven.

It has been suggested that all cartilage surfaces have a unique orientation of stress lines, which are designed to optimally resist the tensile forces that are generated on the joint surface during motion and weight bearing (Below et al., 2002). This hypothesis ascribes to the assumption that split-lines are aligned in the direction where collagen alignment is highest. This has been confirmed by 2-photon CSLM where main directions of collagen can be indicated, however fibres in other directions were also found (figure 23 and 24). Nevertheless, they exist in a quantity that is too low in order to create a split-line. On the bases of results presented, no conclusions can be drawn concerning the quantity and direction of collagen in order to create a split-line. However, it does confirm the finding that split-lines appear in the direction where collagen alignment is highest, because fibre orientation is not uniform but anisotropic (figure 25). Arching of fibres is most frequently seen within the first 200 µm of the cartilage using 2-photon CLSM, which clarifies the difficulty of visualising arching fibres with the split-line technique. The split-line technique reveals the macroscopic collagen orientation in a single plane and is useful to determine an orientation framework, where 2-photon CLSM reveals a more complex three-dimensional network of collagen fibres. By using the combination of these two techniques, much is revealed concerning the three-dimensional collagen fibre network organisation in porcine tibia plateau cartilage. From the reconstruction of five z-projections using 2-photon CLSM (figure 23
and 24) a similar pattern compared to results using the split-line technique (figure 18) has been observed, where fibres arise from the lateral end of the cartilage and arch towards the subchondral bone in an oblique manner. This peripheral collagen orientation has similarities to the conclusions drawn by Clark (Clark, 1991), shown in figure 32. It is proposed that by this study the collagen orientation in alignment to the split-line pattern from the articular surface is elucidated, however the orientation perpendicular to this direction should in future work be established, for instance by assessing more split-line patterns in alternative cross-sections.

Figure 32: Proposed peripheral collagen orientation Clark (Clark, 1991). m=meniscus; b=bone; P=periphery; C=centre.
References


