Microstructural analysis of collagen and elastin fibres in the kangaroo articular cartilage reveals a structural divergence depending on its local mechanical environment

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SUMMARY

Objective: To assess the microstructure of the collagen and elastin fibres in articular cartilage under different natural mechanical loading conditions and determine the relationship between the microstructure of collagen and its mechanical environment.

Method: Articular cartilage specimens were collected from the load bearing regions of the medial femoral condyle and the medial distal humerus of adult kangaroos. The microstructure of collagen and elastin fibres of these specimens was studied using laser scanning confocal microscopy (LSCM) and the orientation and texture features of the collagen were analysed using ImageJ.

Results: A zonal arrangement of collagen was found in kangaroo articular cartilage: the collagen fibres aligned parallel to the surface in the superficial zone and ran perpendicular in the deep zone. Compared with the distal humerus, the collagen in the femoral condyle was less isotropic and more clearly oriented, especially in the superficial and deep zones. The collagen in the femoral condyle was highly heterogeneous, less linear and more complex. Elastin fibres were found mainly in the superficial zone of the articular cartilage of both femoral condyle and distal humerus.

Conclusions: The present study demonstrates that the collagen structure and texture of kangaroo articular cartilage is joint-dependent. This finding emphasizes the effects of loading on collagen development and suggests that articular cartilage with high biochemical and biomechanical qualities could be achieved by optimizing joint loading, which may benefit cartilage tissue engineering and prevention of joint injury.

The existence of elastin fibres in articular cartilage could have important functional implications.

Key words: Collagen, Articular cartilage, Elastin fibre, Mechanical environment, Orientation, Texture.

Introduction

Collagen is an important component of the matrix of articular cartilage and plays an important role in the function of articular cartilage in diarthrodial joints. It accounts for about two-thirds of the dry weight of articular cartilage and is distributed in a zonal pattern with tissue depth[1]. Generally, the collagen is parallel to the articular surface in the superficial zone, more random in the middle zone and perpendicular to the articular surface in the deep zone[1]. The collagen fibres in the superficial zone contribute to the tensile and shearing strength of the articular cartilage. Furthermore, they integrate with the collagen fibres in the middle and deep zones to form a three-dimensional collagenous framework, which entraps the hydrated proteoglycans (PGs) and constrains the expansion of the PGs so that the articular cartilage is afforded loading capacity[1, 2]. Disruption of the collagenous framework results in unconfined expansion of PGs, increased water concentration, softening of the articular cartilage, and hence mechanical failure of the matrix with less capacity to support load[3]. The structure and integrity of the collagen network are believed to be one of the key factors in maintaining the normal functions of articular cartilage[1, 3].

Despite the highly organized network of collagen in adult cartilage, articular cartilage at birth is biochemically and biomechanically homogenous[4-8]. The mechanical environment to which the articular cartilage is subsequently exposed is thought to play a crucial role in the development of the biochemical and biomechanical constitution of articular cartilage[5-7]. During growth and development, the articular cartilage in different joints is subjected to different mechanical forces. By regulating biosynthetic activities, these mechanical forces shape the heterogeneous composition and microstructure of articular cartilage, which is crucial to the mechanical function of articular cartilage[9, 10].

Although many previous studies have tested the effects of mechanical forces on the structure and composition of articular cartilage, the effects of mechanical stimuli are not fully understood, particularly in the early stages of articular cartilage development. In addition, the complex mechanical environment of articular cartilage cannot be simulated in experiments. In this study, we used kangaroos as an animal model in which the elbow and knee were exposed to significantly different mechanical regimes during their activities. By studying the collagen structure in kangaroo articular cartilage, we assessed the role of mechanical forces in the development of the collagen structure, and the relationship between the collagen structure and mechanical function of articular cartilage. As recent studies have revealed the presence of elastin fibres in the superficial zone of articular cartilage of bovine[11, 12] and equine[13], the current study aimed to verify the presence of elastin fibres in the extracellular matrix (ECM) of kangaroo articular cartilage. The present

study could also provide valuable information regarding the level and influence of mechanical forces on the growth and structure of engineered articular cartilage, and also with regard to prevention of cartilage injury.

Materials and Methods

Specimen preparation

Three elbow and three knee joints from three male kangaroos aged approximately 5 years were collected from a local butcher (King River International Company, Perth, Australia). The cartilage surfaces were checked for the absence of osteoarthritis (OA) (Fig. 1). Cylindrical articular cartilage samples connected to the subchondral bone were then harvested using 5 mm diameter punches from the weight-bearing areas of the medial femoral condyle and the medial distal humerus (dash square in Fig. 1). After removal, each cylindrical sample was cut in half from the surface of the cartilage to the subchondral bone. One semi-cylindrical cartilage sample was used to assess elastin fibres near the cartilage surface. The other half was used to assess the zonal arrangement of collagen and was chemically fixed in 10% buffered formalin solution (BFS) for 24h. After being processed and embedded, it was longitudinally sliced into 5-μm-thick sections for full-thickness analysis.

Picrosirius red staining

A method described by Miller[14] was used to stain the collagen. Following de-waxing, rehydration and a 2-min incubation in 0.2% phosphomolybdic acid (PMA), the 5-μm-thick sections were stained with a solution of 0.1% picrosirius red (PSR; Sirius Red F3B and saturated picric acid) for 90min. The samples were rinsed for 2 min in 0.01 N HCL (hydrogen chloride), followed by 1-min rinses in 70% ethanol, 100% ethanol (3 times), and toluene (3 times). Samples were then mounted for LSCM imaging.

Sulforhodamine B staining

Sulforhodamine B (SRB) is a low-molecular-weight polar fluorescent molecule, belonging to the xanthenes fluorescent dye family. The maximum absorption and maximum emission wavelengths are 565 nm and 590 nm, respectively[15]. The specificity of SRB staining to elastin was testified by the strong colocalization observed between the SRB staining and immunostaining of elastin[15]. In the present study, SRB powder (Sigma-Aldrich) was dissolved in 0.9% saline water and 1mg/ml SRB solution was used for staining of kangaroo articular cartilage for 1min. After thorough washing in phosphate buffed saline (PBS, pH 7.2), articular cartilage samples were immersed in PBS to maintain tissue hydration and mounted between a coverslip and a glass slide.

Imaging

All images of collagen and elastin were collected using LSCM (inverted TCS SP2, Leica) with a plan apochromat × 63 / 1.4 oil-immersion objective lens. For PSR stained slides, a 514 nm argon ion laser was used and the emission was recorded through a 570-700 nm bandpass filter; collagen images were taken in a longitudinal view of femoral condyle and distal humerus articular cartilage. For the SRB stained articular cartilage sample, a 561 nm DPSS laser was used and the emission signal was recorded at 565-590 wavelengths; images of elastin fibres were taken in a transverse view of articular cartilage plugs. Laser power and detector sensitivity were adjusted to provide optimum image quality without excessive dye bleaching or pixel saturation. For noise reduction, images with 1024×1024 pixel were obtained using a 1-μm step and frame averaging 4 scans per image.

Image analysis

Orientation analysis

Digital image analysis software ImageJ (NIH, Maryland, USA) was used to conduct image analysis. The orientation of the collagen fibres was analysed using OrientationJ (an ImageJ-plug-in) which was validated for study of collagen orientation in a previous study[16]. The angles of the oriented structures could be

characterized by hue-saturation-brightness (HSB) colour coded image outputs in which the colours indicated the orientation of the collagen. To quantitatively evaluate the local organization and isotropic properties of the collagen fibres, orientation and energy were selected as output parameters. Five regions of interest (ROIs) for every zone of each of three kangaroos were investigated. By quantitatively evaluating every pixel of the image, the degree of collagen fibre orientation could be determined from − 90° to 90°. Pixels with higher energy values correspond to less isotropic and more clearly oriented structures.

Texture Analysis

Texture in an image refers to the distribution of brightness and darkness within the image and describes a group of image properties related to intuitive notions of coarseness, smoothness, and similar properties[17, 18]. It contains important information about the structural arrangement of surfaces and their relationship to the surrounding environment[17]. Texture analysis methods evaluate the spatial location and signal intensity characteristics of the fundamental structural elements (pixels) of digital images[19]. In order to quantify the contrast and spatial distribution of selected ROIs, images were firstly converted to gray-scale, and the gray-level co-occurrence matrix (GLCM), the texture analyzer ImageJ plug-in was then used to calculate texture features in the X, Z plane. The GLCM is a statistical approach of texture analysis and has been widely used to analyse medical images[20]. In the present study, five regions of

interest (ROIs) for every zone of each of three kangaroos were investigated. Three most important parameters were selected for characterizing collagen structure: angular second moment (ASM), correlation, and entropy. ASM is a measure of homogeneity of an image and its value increases with texture homogeneity[17, 19, 21]. Correlation is a measure of gray tone linear dependencies in the image region, where high values (i.e., close to unity) imply a linear relationship between the gray levels of pixel pairs[17, 19]. The correlation value is 1 or -1 for a perfectly positively or negatively correlated[17, 19]. Entropy is a measure of disorder or complexity of intensity distribution and its value is large when the image is not texturally uniform[17, 19].

Statistical Analysis

Differences in orientation parameters (orientation and energy) and texture features (ASM, correlation and entropy) between the zones of articular cartilage from the femoral condyle and distal humerus were statistically examined. The linear mixed effects model was chosen for statistical comparison. The benefit of this model is that samples with potential interrelations can be reliably compared[22]. In the model, joints and zones of articular cartilage were set as fixed variables, and the animal was coded as the random variable. Multiple comparisons were achieved by Least Significant Difference (LSD) post-hoc analysis. Dot plots were used to compare the differences of texture features between the femoral condyle and distal humerus. Estimated means of orientation and energy values for the different zones were obtained and the main

effects between the zones were compared. 95% confidence intervals with LSD adjustment were finally presented. Differences were considered significant at *P* values less than or equal to 0.05. All statistical analyses and graphs were performed using statistical package for the social sciences (SPSS), version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Zonal arrangement of collagen

In order to reveal the zonal arrangement of collagen in kangaroo articular cartilage, LSCM and HSB colour coded images were first employed to compare the collagen structures of femoral condyle and distal humerus. Collagen fibres in the articular cartilage of the femoral condyle showed a clear zonal organization [Fig. 2(A)−(C)]. In the superficial zone, symbolized by the discoid chondrocytes within the top 40-50 μm depth, the collagen fibres aligned predominantly parallel to the cartilage surface [Fig. 2(A)]. In the middle zone, symbolized by the round chondrocytes, no apparent orientation of collagen was observed [Fig. 2(B)]. In the deep zone, symbolized by the vertical chondrocyte columns, the collagen fibres were oriented predominantly perpendicular to the cartilage surface [Fig. 2(C)]. In contrast, the collagen in the articular cartilage of distal humerus was relatively fine and did not show an apparent zonal organization from visual assessment [Fig. 2(D)−(F)].

To quantitatively evaluate the orientation of collagen, OrientationJ was used to generate HSB colour coded images based on the LSCM images and the orientation and energy values were calculated. In the superficial zone, collagen in the femoral condyles [Fig. 3(A)] was more clearly parallel to the articular surface than in the distal humerus [Fig. 3(D)], as indicated by the predominant green colour displayed in the images of the femoral condyle. In the middle zone, both femoral condyle [Fig. 3(B)] and distal humerus samples [Fig. 3(E)] displayed a randomly organized collagen network, indicated by a mix of colours. In the deep zone, the predominant red colour in both femoral condyle [Fig. 3(C)] and distal humerus samples [Fig. 3(F)] indicated that collagen fibres were perpendicular to the articular surface. Quantitative orientation values further confirmed the results from HSB colour coded images (Table Ⅰ). Both femoral condyle and distal humerus samples showed a sharp increase of collagen orientation from 0 to 15 degree in the superficial zone to more than 80 degree in the deep zone. Comparison of energy values revealed less isotropic and more clearly oriented collagen fibres in the superficial zone ($P < 0.0001$), middle zone ($P < 0.0001$) and deep zone ($P < 0.0001$) of femoral condyle articular cartilage than in the respective zones of distal humerus articular cartilage.

Texture features of collagen in different zones

To further characterize the collagenous differences, texture analysis was applied to reveal the textural features of the collagen fibres. Collagen in the superficial, middle

and deep zones of cartilage was more homogenous in the distal humerus than in femoral condyle, as indicated by higher ASM values in distal humerus cartilage [Fig. 4(A)]. No zonal variation of homogeneity was found between zones of the femoral condyle cartilage [Fig. 4(B)]. However, distal humerus articular cartilage showed a more homogenous collagen structure in the deep zone than in the superficial and middle zones [Fig. 4(B)]. Higher correlation values were found in the superficial, middle and deep zones of distal humerus articular cartilage [Fig. 5(A)], which implied a higher correlation of collagen fibres in the distal humerus than in the femoral condyle. Within the femoral condyle cartilage, the superficial zone differed with the middle and deep zones, but no statistically difference was found between the middle and the deep zones [Fig. 5(B)]. Within the distal humerus, gray-tone linearity of collagen structure differed between zones [Fig. 5(B)] and the deep zone had the highest correlation [Fig. 5(A)]. Higher entropy values were present in all three zones of femoral condyle cartilage [Fig. 6(A)], indicating that the collagen structure was more complex in the femoral condyle than in the distal humerus cartilage. No zonal variation of entropy values was found in the femoral condyle, but differences were found between zones of distal humerus articular cartilage [Fig. 6(B)]. These texture parameters demonstrated that the collagen was more homogeneous and linear in the distal humerus cartilage than in the femoral condyle cartilage, and that distal humerus cartilage had a zonal variation with respect to texture parameters while femoral condyle was more consistent with respect to texture characteristics.

Organization of elastin fibres

Elastin fibres indicated by SRB florescence were revealed in kangaroo articular cartilage. From a femoral condyle articular cartilage sample, large elastin fibres bundles were found in the ECM [Fig. 7(A)–(C)]. These bundles were large and mainly linear [arrow head in Fig. 7(A)–(C)] with minor waviness in the form of the elastin fibres [arrow in Fig. 7(A)]. However, in the chondrocyte surface or the pericellular matrix, only fine elastin but no resolvable bundle was found [Fig. 7(D)–(F)]. This fine elastin surrounded the chondrocyte and provided a microenvironment for the entrapped cells.

To further characterise the elastin fibre and the fine elastin, articular cartilage were assessed in terms of different zones and a comparison was made between femoral condyle and distal humerus. In the most superficial layer of femoral condyle articular cartilage, which was acellular layer of about 10 μm in thickness, dense elastin fibres were found [Fig. 8(A)]. These fibres were lightly corss-linked with general direction. In the superficial zone of femoral condyle articular cartilage, elastin fibres were highly oriented to the longitudinal direction of chondrocytes [Fig. 8(B)]. However, it could not be determined whether chondrocytes determined the orientation of elastin fibres. In the deep zone of femoral condyle articular cartilage, elastin fibres were not observed and only fine elastin was found around the chondrocytes [Fig. 8(C)]. In the distal humerus, dense and relatively short elastin fibres were found within the most superficial zone [Fig. 8(D)]; highly oriented elastin fibres were found in the

superficial zone [Fig. 8(E)]; and only fine elastin was found around chondrocytes in the deep zone [Fig. 8(F)]. It appeared that the most obvious difference between femoral condyle and distal humerus was in the most superficial zone.

Discussion

This current study is the first to analyse kangaroo articular cartilage in the femoral condyle and the distal humerus to determine the collagen and elastin structure in the superficial, middle and deep zones. It was found that the structure, orientation and texture features of the collagen varied significantly between zones and joint types. Collagen in the femoral condyle was more clearly oriented, and more heterogeneous and irregular in texture when compared to collagen in the cartilage of the distal humerus. Collectively, these results suggest that the pattern and magnitude of mechanical forces play a crucial role in the development of collagen fibres and overall articular cartilage architecture.

This work used kangaroos as an animal model as it has several fundamental characteristics that are advantageous compared to other animal models[6, 8, 23]. Generally, conventional studies involve variations in individual animals with different genetic and environmental background. In contrast, the kangaroo can provide two significantly different types of loading in one individual. The knee joints of kangaroos are subjected to both dynamic and static loading during movement, while the elbow joints are rarely used for jumping and are mainly subjected to static loading[24]. By

comparing these two types of joints from the same individual kangaroo, intrinsic experimental errors could be avoided and experiments were expected to be more accurate and consistent than other animal models.

The present study revealed zonal variation and joint-dependent variation in the collagen network of kangaroo cartilage. Zonal organization of collagen was observed both in the femoral condyle and in the distal humerus, although zonal arrangement of collagen in the distal humerus was slight. Compared with the distal humerus, the collagen fibres in the femoral condyle were more clearly oriented and less homogenous. Previous studies have shown that articular cartilage responds to varied mechanical stimuli by functional adaption during cartilage development[5-7, 25]. In this process, the heterogeneity of chondrocytes and PGs in the articular cartilage of mature individuals was developed from a homogeneous composition of a less mature status[6, 26]. The present study shows that, compared with elbow joint, dynamic loading in the knee joint assists in establishing the heterogeneous organization of collagen, with more clearly oriented fibres in the superficial and deep zones. The collagenous difference due to mechanical stimuli demonstrated by this study provides an implication of how to optimize the loading in articular cartilage engineering, which is a promising strategy for the treatment of cartilage diseases such as OA. This work also emphasises loading to be a crucial variable in collagen development, and necessitates the optimization of joint loading during early life to create optimal biomechanical characteristics of articular cartilage, which may contribute to prevention of OA later in life.

Texture analysis has traditionally been applied in fields such as material science, geography and satellite image analysis. As the mathematically quantified "texture features" are very sensitive, texture analysis is increasingly being used in the medical area ranging from diagnostic[27-29] to prognostic applications[30]. A recent study examined the influence of mechanical loading on the organization of collagen in articular cartilage and found that the structural modification of collagen in the ECM of articular cartilage could be distinguished by texture analysis[31]. The present study confirms that the texture analysis is a valuable and quantitative tool for the study of collagen in articular cartilage. Further application of texture analysis could promote more future work to compare textural differences in the ECM of diseased and health cartilage.

Collagen structural alterations have been observed in OA by laboratory methods, but cannot be clinically diagnosed. Generally, OA can be diagnosed by X-rays, MRI (magnetic resonance imaging) or arthroscopy at the more advanced stages, at which time the options for therapeutic intervention without surgery are limited. It is therefore crucial to develop new diagnostic strategies targeting the early stages of OA. As damage of collagen fibrils or collagen network disorganization is one of the early signs of cartilage injury and OA[32, 33], testing of alteration in collagen structure could be a valuable tool for early OA diagnosis. Texture analysis of the collagen could be used to discover the textural differences which may not be detected by conventional methods. The changes in textural features of collagen during early degenerative changes are a subject for future investigations. Combining texture analysis with

non-invasive imaging techniques (such as laser scanning confocal arthroscopy)[34] could be promising to diagnose cartilage diseases such as OA at early stage.

Early histological results indicated that little elastin fibres or elastin existed in articular cartilage[35-37]. However, recent studies carried out with more sensitive methods have observed the existence of elastin fibres in the superficial zone of articular cartilage[11-13]. The present study revealed that an extensive elastin fibre network was present within the kangaroo cartilage surface, including most superficial layer and superficial zone, and that fine elastin surrounded chondrocytes throughout the whole cartilage depth. Due to its significant volume, elastin fibres and fine elastin should no longer be ignored in the future studies regarding articular cartilage. The mechanical function of elastin fibres is believed to endow critical properties of elasticity and resilience to tissues, such as skin, lung, blood vessels and elastic cartilage[35]. Investigation of the mechanical functions of elastin fibres in articular cartilage and their relationship with other components of the ECM would be important, but is beyond the scope of the current study.

In conclusion, the present work assessed microstructural and textural characteristics of collagen and revealed the presence of elastin fibres in articular cartilage under different natural mechanical environments. Significant differences observed between the knee and elbow with respect to the structural, orientation and textural features of collagen suggest that the type and magnitude of mechanical forces play a crucial role in collagen structure development. The existence of elastin fibres and fine elastin around chondrocytes suggests it as another important component for

articular cartilage besides collagen and PGs. This work suggests that articular cartilage with optimal biochemical and biomechanical qualities could be achieved by optimizing mechanical forces, which may benefit cartilage tissue engineering and prevention of joint injuries. Our findings also promote the application of texture analysis as a promising method for future collagen structural studies and early diagnosis of OA.

Author contributions

All authors significantly participated and were involved in the (1) conception and design (2) drafting the manuscript or revising it critically for intellectual content, and (3) all authors approved the final version of the paper before submission.

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The sponsor had no role in the study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Conflict of interest

The authors declare that they have no competing interests.

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References

1. Eyre D. Articular cartilage and changes in Arthritis: Collagen of articular cartilage. Arthritis Res 2002;4:30–5.
2. Kuhn K. Structural and functional domains of collagen: a comparison of the protein with its gene. Coll Relat Res 1984;4:309–22.
3. Mow VC, Holmes MH, Lai WM. Fluid transport and mechanical properties of articular cartilage: a review. J Biomech 1984;17:377–94.
4. Brama PA, Karssenberg D, Barneveld A, van Weeren PR. Contact areas and pressure distribution on the proximal articular surface of the proximal phalanx under sagittal plane loading. Equine Vet J 2001;33:26–32.
5. Brama PAJ, TeKoppele JM, Bank RA, Barneveld A, van Weeren PR. Development of biochemical heterogeneity of articular cartilage: influences of age and exercise. Equine Vet J 2002;34:265–9.

6. Brommer H, Brama PA, Laasanen MS, Helminen HJ, van Weeren PR, Jurvelin JS. Functional adaptation of articular cartilage from birth to maturity under the influence of loading: a biomechanical analysis. Equine Vet J 2005;37:148–54.
7. Brama PAJ, Tekoppele JM, Bank RA, Barneveld A, Van Weeren PR. Functional adaptation of equine articular cartilage: the formation of regional biochemical characteristics up to age one year. Equine Vet J 2000;32:217–21.
8. Brama PA, Tekoppele JM, Bank RA, van Weeren PR, Barneveld A. Influence of different exercise levels and age on the biochemical characteristics of immature equine articular cartilage. Equine Vet J Suppl 1999:55–61.
9. Palmoski MJ, Brandt KD. Effects of static and cyclic compressive loading on articular cartilage plugs in vitro. Arthritis Rheum 1984;27:675–81.
10. Klein-Nulend J, Veldhuijzen JP, van de Stadt RJ, van Kampen GP, Kuijer R, Burger EH. Influence of intermittent compressive force on proteoglycan content in calcifying growth plate cartilage in vitro. J Biol Chem 1987;262:15490–5.
11. Yeh AT, Hammer-Wilson MJ, Van Sickle DC, Benton HP, Zoumi A, Tromberg BJ, *et al.* Nonlinear optical microscopy of articular cartilage. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 2005;13:345–52.
12. Yu J, Urban JP. The elastic network of articular cartilage: an immunohistochemical study of elastin fibres and microfibrils. Journal of anatomy 2010;216:533–41.
13. Mansfield J, Yu J, Attenburrow D, Moger J, Tirlapur U, Urban J, *et al.* The elastin network: its relationship with collagen and cells in articular cartilage as visualized by multiphoton microscopy. Journal of anatomy 2009;215:682–91.

14. Miller TA, Lesniewski LA, Muller-Delp JM, Majors AK, Scalise D, Delp MD. Hindlimb unloading induces a collagen isoform shift in the soleus muscle of the rat. Am J Physiol Regul Integr Comp Physiol 2001;281:R1710–7.

15. Ricard C, Vial J-C, Douady J, Sanden Bvd. In vivo imaging of elastic fibers using sulforhodamine B. Journal of Biomedical Optics 2007;12:064017.

16. Rezakhaniha R, Agianniotis A, Schrauwen J, Griffa A, Sage D, Bouten C, *et al*. Experimental investigation of collagen waviness and orientation in the arterial adventitia using confocal laser scanning microscopy. Biomechanics and Modeling in Mechanobiology 2011:1–13.

17. Robert M. Haralick KS, and Its'Hak Dinstein. Textural Features for Image Classification. IEE Transactions on System, Man, and Cybernetics 1973;3:610–21.

18. Haralick RM. Statistical and structural approaches to texture. Proceedings of the IEEE 1979;67:786–804.

19. Mathias JM, Tofts PS, Losseff NA. Texture analysis of spinal cord pathology in multiple sclerosis. Magn Reson Med 1999;42:929–35.

20. Castellano G, Bonilha L, Li LM, Cendes F. Texture analysis of medical images. Clin Radiol 2004;59:1061–9.

21. Rolauffs B, Muehleman C, Li J, Kurz B, Kuettner KE, Frank E, *et al.* Vulnerability of the superficial zone of immature articular cartilage to compressive injury. Arthritis Rheum 2010;62:3016–27.

22. Ranstam J. Repeated measurements, bilateral observations and pseudoreplicates, why does it matter? Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 2012;20:473–5.

23. Murray RC, Birch HL, Lakhani K, Goodship AE. Biochemical composition of equine carpal

articular cartilage is influenced by short-term exercise in a site-specific manner. Osteoarthritis and Cartilage 2001;9:625–32.

24. Dawson TJ, Taylor CR. Energetic Cost of Locomotion in Kangaroos. Nature 1973;246:313–4.

25. Helminen HJ, Hyttinen MM, Lammi MJ, Arokoski JP, Lapvetelainen T, Jurvelin J, *et al.* Regular joint loading in youth assists in the establishment and strengthening of the collagen network of articular cartilage and contributes to the prevention of osteoarthrosis later in life: a hypothesis. J Bone Miner Metab 2000;18:245–57.

26. Jadin KD, Bae WC, Schumacher BL, Sah RL. Three-dimensional (3-D) imaging of chondrocytes in articular cartilage: Growth-associated changes in cell organization. Biomaterials 2007;28:230–9.

27. Mommers EC, Poulin N, Meijer CJ, Baak JP, van Diest PJ. Malignancy-associated changes in breast tissue detected by image cytometry. Anal Cell Pathol 2000;20:187–95.

28. Sutton RN, Hall EL. Texture Measures for Automatic Classification of Pulmonary Disease. Computers, IEEE Transactions on 1972;C-21:667–76.

29. Harms H, Gunzer U, Aus HM. Combined local color and texture analysis of stained cells. Comput. Vision Graph. Image Process. 1986;33:364–76.

30. Sudbo J, Lippman SM, Lee JJ, Mao L, Kildal W, Sudbo A, *et al.* The influence of resection and aneuploidy on mortality in oral leukoplakia. N Engl J Med 2004;350:1405–13.

31. Werkmeister E, de Isla N, Marchal L, Stoltz JF, Dumas D. Interest of second harmonic generation imaging for diagnosis in thick and opaque tissue. Biorheology 2008;45:375–83.

32. Kempson G. Relationship between the tensile properties of articular cartilage from the human knee and age. Ann Rheum Dis 1982;41:508–11.

33. Hollander A, Heathfield T, Webber C, Iwata Y, Bourne R, Rorabeck C. Increased damage to type II

collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 1994;93:1722–32.

34. Jones C, Keogh A, Smolinski D, Wu J, Kirk T, Zheng M. Histological assessment of the chondral and connective tissues of the knee by a confocal arthroscope. J Musculoskeletal Res 2004;8:75–86.

35. Kielty CM, Sherratt MJ, Shuttleworth CA. Elastic fibres. J Cell Sci 2002;115:2817–28.

36. Ushiki T. Collagen fibers, reticular fibers and elastic fibers. A comprehensive understanding from a morphological viewpoint. Arch Histol Cytol 2002;65:109–26.

37. Cotta-Pereira G, Del-Caro LM, Montes GS. Distribution of elastic system fibers in hyaline and fibrous cartilages of the rat. Acta Anat (Basel) 1984;119:80–5.

Table Ⅰ. Mean (95% confidence interval, CI) orientation and energy values of collagen in articular cartilage. A linear mixed effects model was used to perform statistical comparison.

Zones	Cartilage	Orientation (95% CI)		Energy	
Superficial	Femoral condyle	-2 (-6 - 2)	$P < 0.0001$	134 (125 - 142)	$P < 0.0001$
	Distal humerus	11 (7 - 15)		44 (35 - 52)	
Middle	Femoral condyle	67 (63 - 71)	$P < 0.0001$	86 (77 - 94)	$P < 0.0001$
	Distal humerus	80 (76 - 84)		63 (54 - 71)	
Deep	Femoral condyle	86 (82 - 90)	$P = 0.433$	126 (117 - 134)	$P < 0.0001$
	Distal humerus	84 (80 - 88)		21 (12 - 29)	

Fig. 1. A photograph of a kangaroo femoral condyle and distal humerus showing sampling of articular cartilage. The femoral condyle was connected by the anterior cruciate ligament (ACL) and the posterior cruciate ligament (PCL) to the femoral-tibial joint (A). Articular cartilage samples were collected from the central load bearing area of the medial femoral condyle (dashed square in B) and distal humerus (dashed square in C).

Fig. 2. LSCM images of picrosirius red stained collagen in the articular cartilage of the femoral condyle and the distal humerus (longitudinal view). In the femoral condyle, collagen was mainly horizontally oriented in the superficial zone (A), randomly oriented in the middle zone (B) and vertically oriented in the deep zone (C). In the distal humerus, no apparent organization of collagen was found from visual assessment in the superficial zone (D), middle zone (E) and deep zone (F). Scale bar=10 μm.

Fig. 3. HSB colour coded images of collagen in the articular cartilage of the femoral condyle and the distal humerus. In the femoral condyle, the predominant green colour indicated horizontally oriented collagen in the superficial zone (A); a mix of colour indicated randomly oriented collagen in the middle zone (B); and the predominant red colour indicated vertically oriented collagen in the deep zone (C). In the distal humerus, the horizontally oriented collagen in the superficial zone (D) and randomly organized collagen in the middle zone (E) and perpendicular collagen in the deep zone (F) were also found but not as obvious as in the femoral condyle. Scale bar=10 μm.

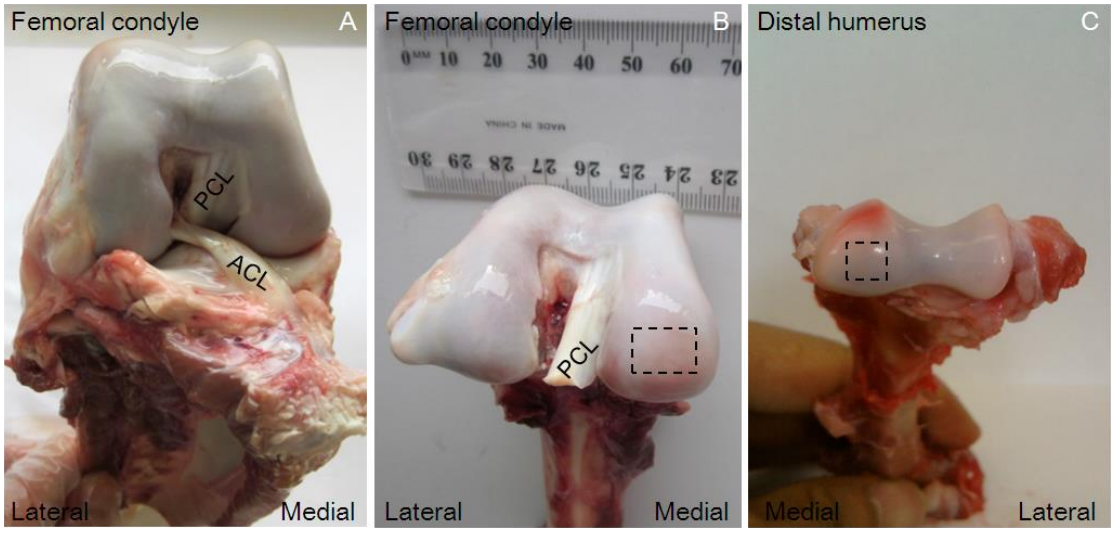
Fig. 4. A comparison of homogeneity of the collagen structure indicated by ASM values. Compared with the femoral condyle articular cartilage, the distal humerus articular cartilage showed more

525 homogenous structure of collagen in the superficial, middle and deep zones, as indicated by higher
526 ASM values in respective zones (A). Within the femoral condyle, no zonal variation was observed in
527 terms of homogeneity of collagen (B). Within the distal humerus, the homogeneity of collagen differs
528 between zones, and the collagen structure is significantly more homogenous in the deep zone than in
529 superficial and middle zones (B).
530
531 **Fig. 5.** A comparison of correlation of collagen in the femoral condyle articular cartilage and the distal
532 humerus articular cartilage. Higher correlation of collagen in cartilage were found in the distal humerus
533 than in the femoral condyle (A). Within the femoral condyle, the superficial zone differed with the
534 middle and deep zones in terms of correlation, but there was no significant difference between the
535 middle and deep zones. (B). Within the distal humerus, correlation of collagen differed between zones
536 (B) and the collagen in the deep zone was most highly correlated (A).
537
538 **Fig. 6.** A comparison of complexity of collagen structure indicated by entropy values. Collagens of the
539 superficial, middle and deep zones were more complex in femoral condyle than in distal humerus, as
540 indicated by higher entropy values in femoral condyles (A). Within the femoral condyle, no zonal
541 variation was observed in terms of complexity of collagen (B). Within the distal humerus, collagen
542 differed between zones (B), and the middle zone was the most complex layer as indicated by higher
543 entropy value (A).
544
545 **Fig. 7.** Sulforhodamine B staining revealed the existence of elastin fibres in kangaroo femoral condyle
546 articular cartilage (transverse view). Both straight elastin fibres (arrow head in A, B and C) and wave
547 elastin fibres (arrow in A) were observed in the extracellular matrix. Fine elastin was observed in

pericellular matrix (D, E and F). Scale bar = 10 μm.

Fig. 8. Comparison between femoral condyle and distal humerus with respect to sulforhodamine B stained elastin fibres in different zones (transverse view). Dense elastin fibres were found in the most superficial zone of articular cartilage from femoral condyle (A) and distal humerus (D). Less dense elastin fibres were observed to parallel to the adjacent chondrocytes in the superficial zone of articular cartilage from femoral condyle (B) and distal humerus (E). Only fine elastin was found in the deep zone of articular cartilage from femoral condyle (C) and distal humerus (F). Scale bar = 30 μm.

557 Figure. 1



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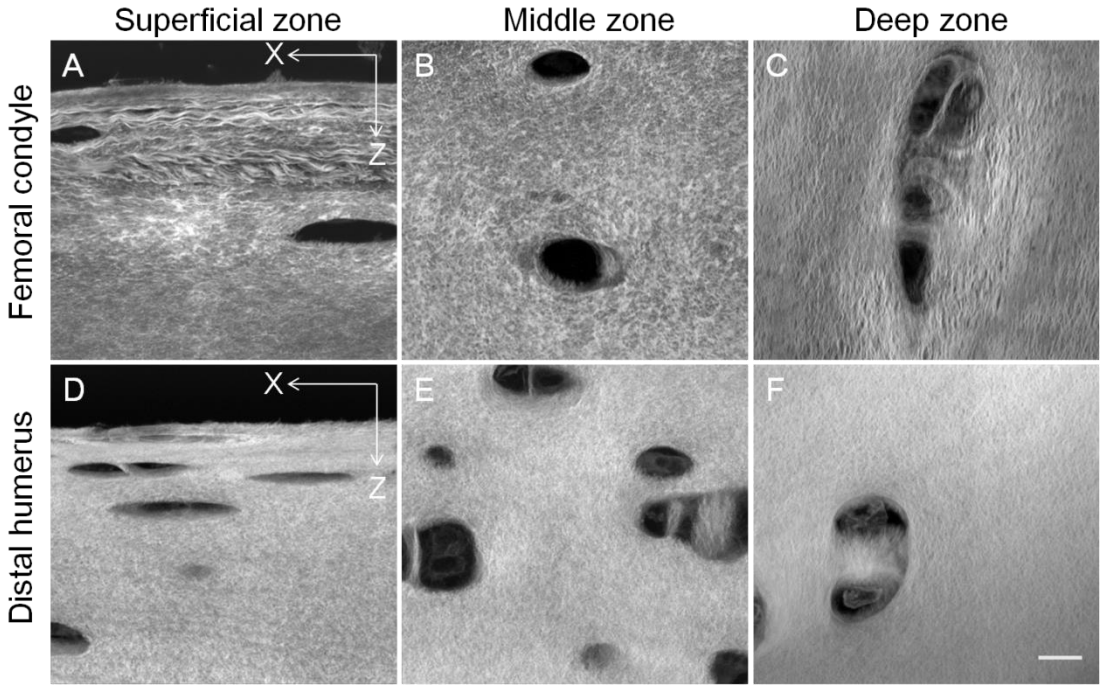
561 Figure. 2



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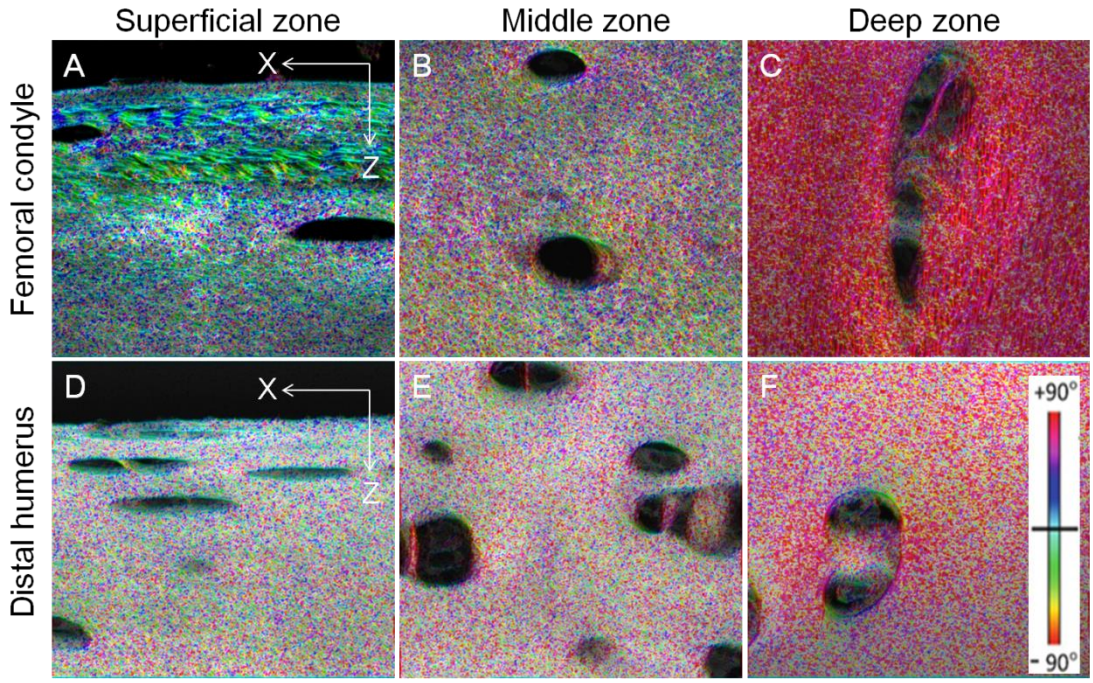
564 Figure. 3



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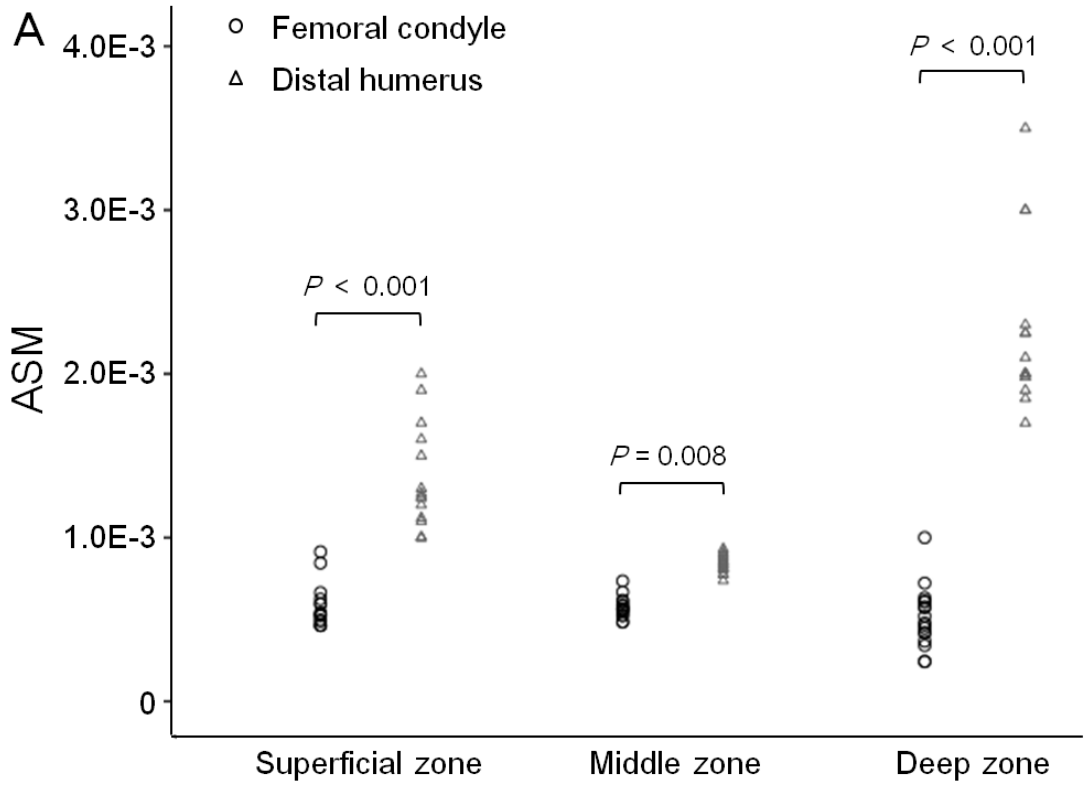
568 Figure. 4



B

Articular cartilage	zones	*P* values		
Femoral condyle	Superficial	0.934		
	Middle		0.490	0.439
	Deep			
Distal humerus	Superficial	<0.0001		
	Middle		<0.0001	<0.0001
	Deep			

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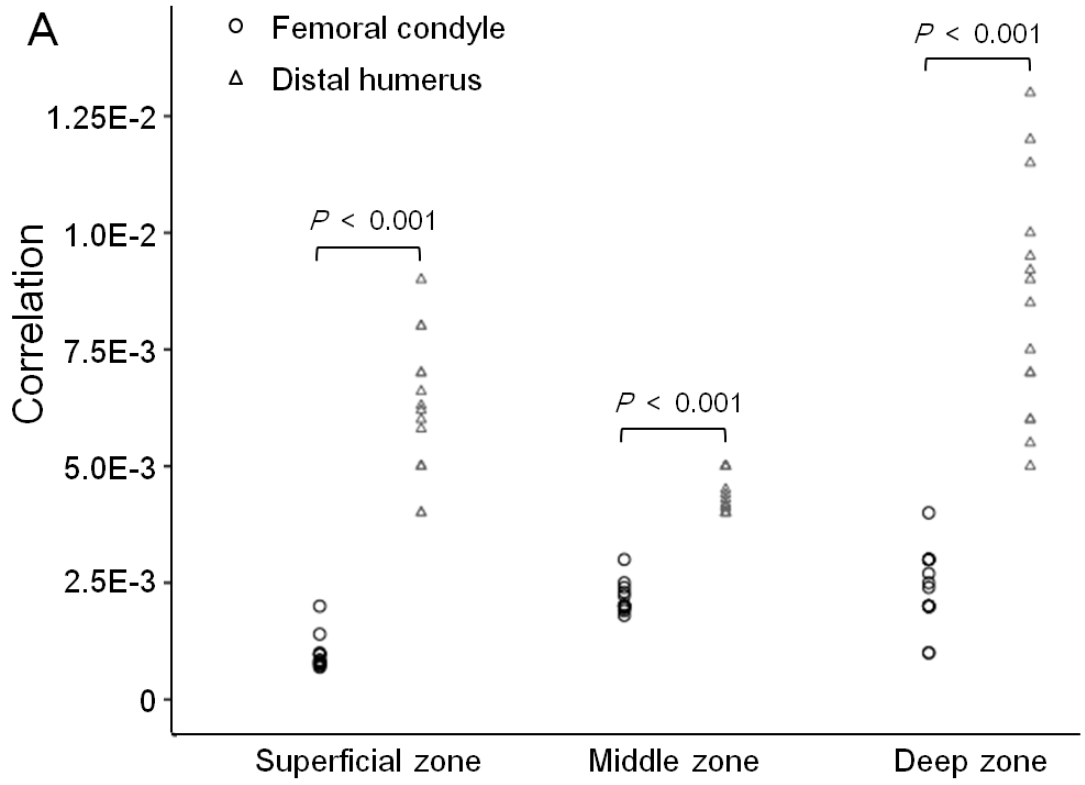
572 Figure. 5



B

Articular cartilage	zones	*P* values		
Femoral condyle	Superficial	0.010		
	Middle		0.569	0.001
	Deep			
Distal humerus	Superficial	<0.0001		
	Middle		<0.0001	<0.0001
	Deep			

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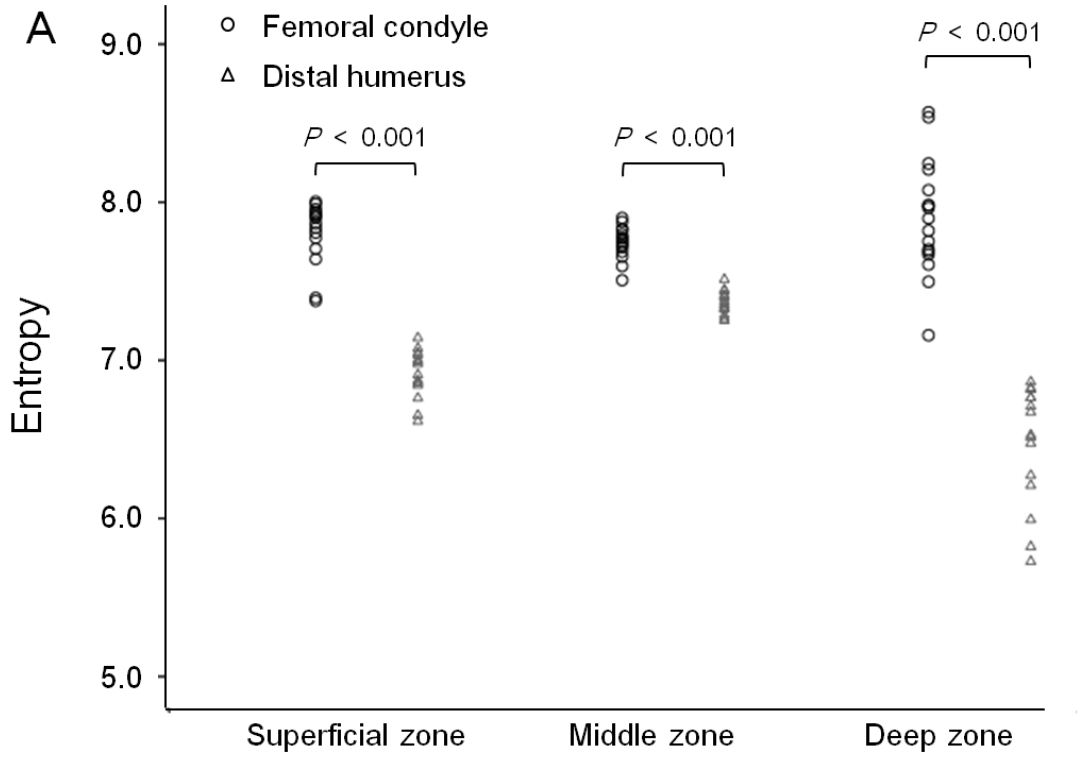
576 Figure. 6



B

Articular cartilage	zones	*P* values		
Femoral condyle	Superficial	0.520	0.070	0.246
	Middle			
	Deep			
Distal humerus	Superficial	<0.0001	<0.0001	<0.0001
	Middle			
	Deep			

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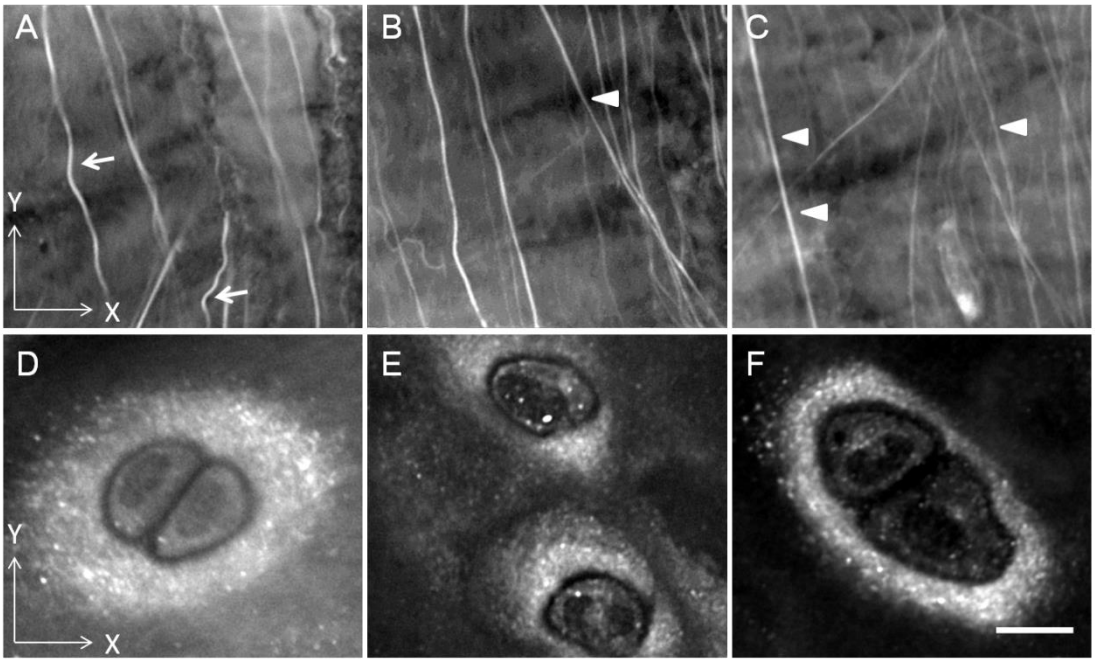
580 Figure. 7



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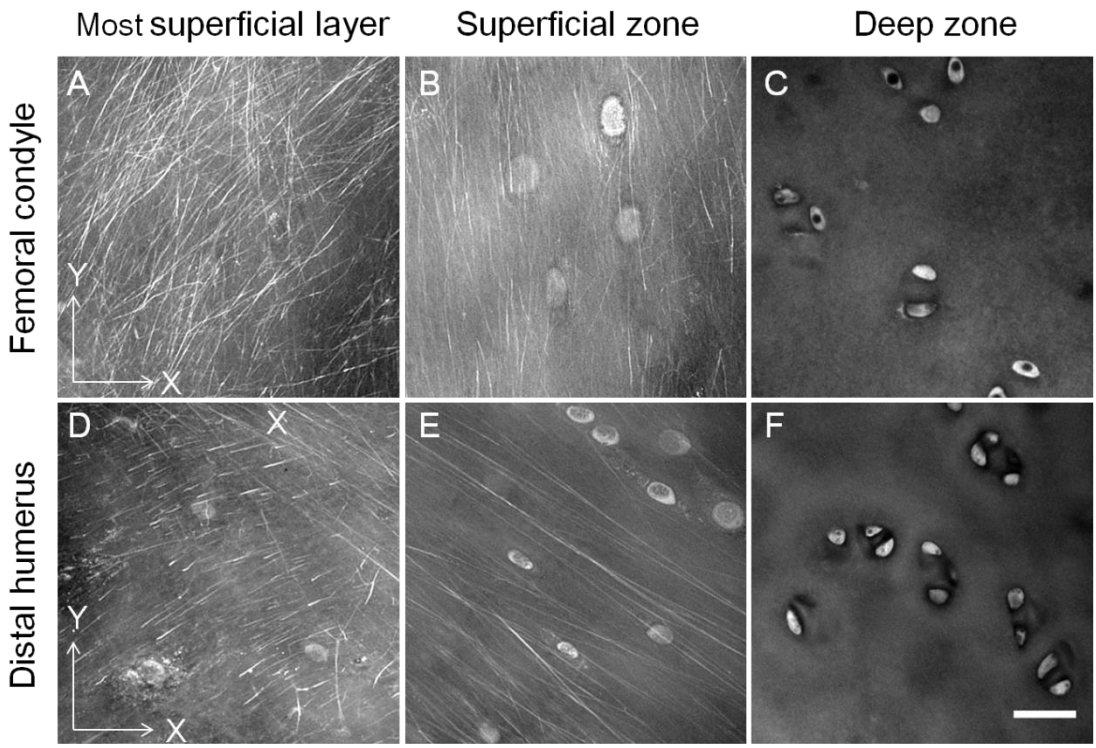
584 Figure. 8



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