Matrix Biology 39 (2014) 33–43

Contents lists available at ScienceDirect

Matrix Biology

journal homepage: www.elsevier.com/locate/matbio

Mini review

How best to preserve and reveal the structural intricacies of cartilaginous tissue

Ernst B. Hunziker *, Kurt Lippuner, Nahoko Shintani

Departments of Osteoporosis, Orthopaedic Surgery and Clinical Research, Inselspital, University of Bern, Murtenstrasse 35, P.O. Box 54, 3010 Bern, Switzerland

ARTICLE INFO

Available online 27 August 2014

Keywords:
Articular cartilage
Matrix
Fine structure
Preservation

ABSTRACT

No single processing technique is capable of optimally preserving each and all of the structural entities of cartilaginous tissue. Hence, the choice of methodology must necessarily be governed by the nature of the component that is targeted for analysis, for example, fibrillar collagens or proteoglycans within the extracellular matrix, or the chondrocytes themselves.

This article affords an insight into the pitfalls that are to be encountered when implementing the available techniques and how best to circumvent them.

Adult articular cartilage is taken as a representative *pars pro toto* of the different bodily types. In mammals, this layer of tissue is a component of the synovial joints, wherein it fulfills crucial and diverse biomechanical functions. The biomechanical functions of articular cartilage have their structural and molecular correlates. During the natural course of postnatal development and after the onset of pathological disease processes, such as osteoarthritis, the tissue undergoes structural changes which are intimately reflected in biomechanical modulations. The fine structural intricacies that subserve the changes in tissue function can be accurately assessed only if they are faithfully preserved at the molecular level. For this reason, a careful consideration of the tissue-processing technique is indispensable. Since, as aforementioned, no single methodological tool is capable of optimally preserving all constituents, the approach must be pre-selected with a targeted structure in view. Guidance in this choice is offered.

Contents

1. Introduction

"Believe only half of what you see": The morphological appearance of cartilage is a *fata morgana*. The structural organization of the tissue as we perceive it in the microscope depends greatly upon the nature of the technique that is implemented to preserve it for observation. No single processing technique is capable of optimally preserving each and all of

* Corresponding author at: Center of Regenerative Medicine for Skeletal Tissues, Departments of Osteoporosis,Orthopaedic Surgery and Clinical Research, Inselspital, University of Bern, Murtenstrasse 35, P.O. Box 54, 3010 Bern, Switzerland. Tel.: +41 31 632 86 85; fax: +41 31 632 49 55.
E-mail address: ernst.hunziker@dkf.unibe.ch (E.B. Hunziker).

http://dx.doi.org/10.1016/j.matbio.2014.08.010

the structural entities of cartilaginous tissue. Hence, the choice of methodology must necessarily be governed by the nature of the component that is targeted for analysis, for example, fibrillar collagens or proteoglycans within the extracellular matrix, or the chondrocytes themselves.

This article will begin with a general overview of the available preservation techniques (1. preservation techniques for cartilage: specific chemical and physical properties of the tissue are a source of multiple artifacts). The techniques that are best suited to preserve and reveal each of the structural components of cartilaginous tissue will then be delineated and briefly discussed in separate subsections: 2. chondrocytes and their membranous systems; 3. the network of fibrillar collagens; 4. the proteoglycans; 5. the tissue-water pool; and 6. the "best of the rest". Special techniques will be summarily dealt with at the end of the article in a separate subsection (7. special procedures), which will be followed by a few concluding remarks (8.conclusions).

In this review, adult hyaline articular cartilage will be taken as a representative *pars pro toto* of the different bodily types. In mammals, this layer of tissue is a component of the synovial joints, wherein it fulfills crucial and diverse biomechanical functions: it absorbs and distributes loads, transfers these to the opposing bony shafts, and ensures that the movements of the latter are executed in a practically frictionless manner (Hasler et al., 1999; Buckwalter et al., 2005). Since cartilaginous tissue owes 70% of its wet weight to its aqueous component, human beings may be envisaged as "walking on water" (Padalkar et al., 2013). Naturally, the water does not flow out of the tissue when we are walking. But this seemingly banal observation should by no means be taken for granted. On the contrary, it is a small wonder, the secret of which lies in the strong water-binding capacity of the highly-soluble proteoglycans, and in the fact that these macromolecules are trapped in an underhydrated state within an insoluble network of anisotropically-organized collagenous fibrils of high tensile strength (Heinegård et al., 1982; Tobias et al., 1992; McLeod et al., 2012; Padalkar et al., 2013). The fibrillar collagens and the proteoglycans, together with minor components, such as non-fibrillar collagens, glycoproteins and signaling peptides, constitute the remaining 30% of the wet weight of cartilaginous tissue (Anderson et al., 1964).

It is in the well-defined anisotropic organization of both the chondrocytes and the extracellular matrix of adult joint cartilage that the key to its capacity to counteract the deleterious effects of externally-applied mechanical forces lies. An externally-applied mechanical force induces a flow of water within and through the extracellular matrix. The densely-packed glycosaminoglycan-chains of proteoglycanous aggrecans resist this fluid flow in a manner that is proportional to the rate of loading. In practical terms, this means that water cannot move through the matrix with sufficient rapidity to relieve the hydrostatic pressure that builds up during the compression of the tissue. The consequence is, that the compressive stiffness of the cartilaginous tissue increases. It has recently been shown that the loss of glycosaminoglycans—which is a hallmark of the early stages of osteoarthritis—causes a dramatic increase in the hydraulic permeability of the tissue. This finding suggests that early osteoarthritic cartilage may be more vulnerable to loading rate than to loading magnitude, which is the conventionally-studied parameter (Nia et al., 2013). Hence, over the wide frequency-range of joint motion that is the norm during daily activities, hydraulic permeability would appear to be the most sensitive marker of early tissue degradation.

Compression-propagated fluid flow also induces the movement of mobile positive counterions relative to the positions of the fixed, negatively-charged groups on the immobilized glycosaminoglycan-chains of the aggrecan molecules. This separation of the mobile from the fixed charges generates localized electrical streaming-potential fields, which slow down the movement of the counterions and thus also the flow of water (Frank and Grodzinsky, 1987). The generation of the localized electrical streaming potentials is an energy-absorbing process. This absorption of energy further limits the flow of fluid through the tissue during its compression—in such a manner that its dynamic stiffness is maintained and its effective hydraulic permeability reduced by 10 to 40% (Frank et al., 1990). Although the dominant protective effect of the proteoglycans is attributable to the impeding influence of the closely-packed glycosaminoglycan-chains on fluid flow (Nia et al., 2013), the generation of electrical streaming-potential fields is also a contributing factor (Frank and Grodzinsky, 1987).

By virtue of the unique sequestered microenvironment that is generated by the entrapment of underhydrated proteoglycans within a network of collagenous fibrils of high tensile strength, the internal osmotic pressure of adult articular cartilage is permanently maintained at a level of about two atmospheres (Maroudas, 1976; Urban et al., 1979; Maroudas and Bannon, 1981; Horkay, 2013). Other features that are characteristic of the tissue include the absence of a blood-vascular supply, of lymphatic vessels and of nerve endings (Eggli et al., 1988; Hunziker et al., 2007).

2. Preservation techniques for cartilage: specific chemical and physical properties of the tissue are a source of multiple artifacts

The preparation of blocks of cartilaginous tissue for preservation necessitates a violation by cutting of the integrity of the sequestered internal microcompartments. By this mechanical act of excision, the high internal pressure of the tissue drops explosively to the atmospheric level. Water gushes into the cartilage in a tsunami-like fashion, drawn there by the hydrophilicity of the proteoglycans, whose state now changes from an underhydrated to a hydrated one (Hunziker and Graber, 1986). The tissue swells (Hunziker and Schenk, 1989). Even maintenance in a humid aeric atmosphere suffices to induce this phenomenon, which is accelerated in an aqueous environment (Thyberg et al., 1973). Molecules of the extracellular matrix undergo dislocation and disruption, thereby leading to a destruction of the native structural intricacies of the tissue (Hunziker et al., 1983; Hunziker and Schenk, 1987).

Upon immersion in an aqueous solution, irrespective of whether this is a cell-culture medium or a chemical fixative, such as formaldehyde or glutaraldehyde, or whether it is buffered or not, low-molecular-weight proteoglycans within the extracellular matrix of cartilaginous tissue are immediately extracted; the higher-molecular-weight ones first undergo a process of shifting, which has a knock-on effect, leading to the secondary dislocation of other macromolecules that lie in their wake and of those with which they interact chemically (Hunziker et al., 1983; Hunziker and Schenk, 1987). 10 to 30% of the proteoglycan-population is extracted from the tissue during the first 10 to 15 minutes of its immersion in an aldehyde-based medium (Hunziker and Graber, 1986). The processes of molecular dislocation and extraction lead to changes in the structural organization of the tissue of such a high order of magnitude as to yield a morphological image that bears little resemblance to that of native cartilage (Hunziker et al., 1982, 1983). This "image" of articular cartilage is the one that has been impressed on our mind's eye since the advent of the classical textbook reproductions in the 19th century (Davies et al., 1962).

Since the simple routine chemical fixation of cartilaginous tissue in an aldehyde-based medium leads to such a severe distortion of its macromolecular organization, this mode of proceeding is totally inadequate for a fine structural analysis in the transmission electron microscope. The lateral resolution is so greatly reduced—being at a par with that achieved in the light microscope (micrometer-range), even on thin (30- to 40-nm-thick) sections—as to render any measurements other than coarse, elementary ones completely spurious (Poole et al., 1982). Tissue that has been preserved in this manner could be used to estimate the numerical density of chondrocytes, or to ascertain whether a network of collagenous fibrils is present or not, but information of a more sophisticated nature would not be forthcoming (Aszodi et al., 1998, 2001; Gustafsson et al., 2003). An example will illustrate the dangers

of attempting any such analysis. Fibrillar collagens are not preserved in their entirety: those with a comparatively low tensile strength and with a diameter below 20 nm are simply destroyed or lost to the aqueous medium (Studer et al., 1995; Hunziker et al., 1997). Consequently, we are presented with a biased optical image, which carries the danger of mis- or over-interpretation. And these dangers are heightened if the tissue is subjected to selective enzymatic digestion with a view to facilitating the diffusion of antibodies in the context of an immunohistochemical analysis (Poole et al., 1982; Poole and Pidoux, 1989).

The classical histological image of cartilaginous tissue as seen after preservation in aldehyde-based media is personified in the appearance of the chondrocytes, which, owing to the disruption of the interactions that their surfaces establish with proteoglycans in the pericellular matrix, undergo retraction and shrinkage (Figs. 1A and 3A, Table 1). The space that they occupied in a native, expanded state remains "empty". This halo was long ago designated as the chondrocytic lacuna. Owing to their collapsed state, the chondrocytes become intensely stained after exposure to a standard cationic dye, such as Alcian Blue. However, in light micrographs, the positions of the chondrocytes are most readily identified by their large, stain-free lacunae. This classical image of chondrocytes (Fig. 3A) has given rise to a number of misinterpretations. For example, in immature articular cartilage and in cartilage of the epiphyseal plate, the shrinkage of the hypertrophic chondrocytes is more "impressive" than that of the proliferative ones (Fig. 1A), since their 10-fold larger volume (Hunziker and Schenk, 1987; Hunziker and Schenk, 1989) leads to the generation of 10-fold larger lacunae, thereby giving rise to the belief that they were degenerative (Hunziker et al., 1984). In the due course of time, this belief led to the postulate that the hypertrophic chondrocytes underwent programmed death (apoptosis) during the process of matrix mineralization (Gibson et al., 1995; Roach et al., 1995). In the 1980s, appropriate methodologies were implemented to preserve growth-plate cartilage in a near-native state. The hypertrophic chondrocytes were thereby revealed to be structurally intact and functionally active right up to the point of contact with the vascular-invasion front (Hunziker et al., 1982, 1984).

During the preservation of cartilaginous tissue in aldehyde-based media, only the protein core of the macromolecules undergoes chemical fixation, viz., crosslinkage; the carbohydrate chains, which constitute 80% of the bulk of the molecules, are not fixed. Consequently, the degree to which proteoglycans are extracted into the aqueous medium depends upon their specific make-up, and will thus vary unpredictably. This variability has been observed (Engfeldt and Hjertquist, 1968; Hunziker and Graber, 1986) and is often falsely interpreted as a tissue-specific effect, whereas in reality it is no more than a fixation-associated artifact (Hunziker and Graber, 1986; Handley et al., 2002).

In the 1960s and 1970s, the addition of a cationic dye, such as Toluidine Blue (Shepard and Mitchell, 1976a), Alcian Blue (Sames, 1974),

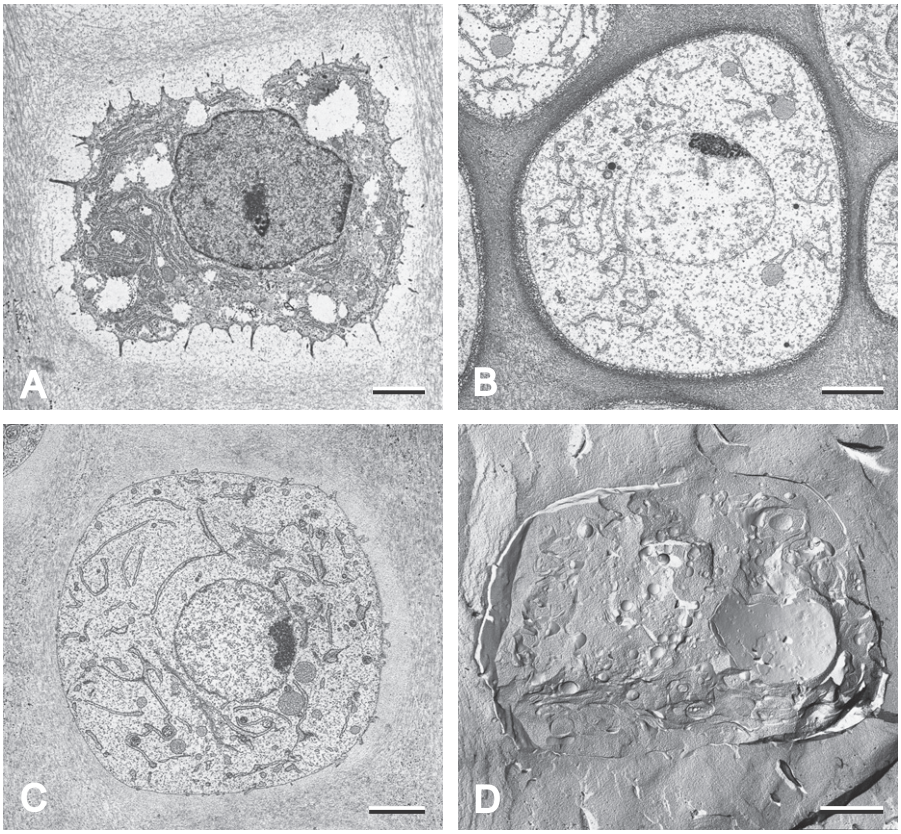
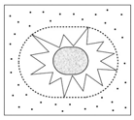
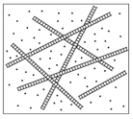
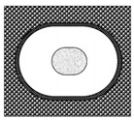
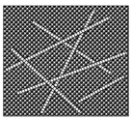
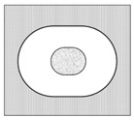
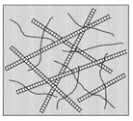
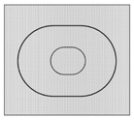
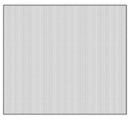


Fig. 1. Transmission electron micrographs of thin sections through the growth-plate cartilage of rats, depicting chondrocytes of the hypertrophic zone after (A) conventional, primary chemical fixation in 2.5% glutaraldehyde and post-fixation in 1% osmium tetroxide, (B) glutaraldehyde-based fixation in the presence of 0.7% RHT and post-fixation in 1% osmium tetroxide, likewise in the presence of 0.7% RHT, (C) high-pressure freezing, freeze-substitution and low-temperature embedding, and (D) high-pressure freezing, freeze-etching and shadowing. In (A), the depicted chondrocyte has a shrunken appearance and is surrounded by an "empty" (unstained) lacuna. The mechanical stability that is afforded to the plasmalemma by its interactions with proteoglycans in the pericellular matrix is lost owing to the extraction of these macromolecules. In consequence, the cell retracts and maintains only focal contacts with the surrounding matrix. In (B), the presence of RHT prevents the extraction of proteoglycans from the extracellular matrix. Consequently, the chondrocytic plasmalemma maintains its hold on the pericellular compartment and is preserved in an expanded state without a mantling lacuna. Owing to the precipitation and consequent condensation of the proteoglycans that interact with the cell surface, the plasmalemma is drawn towards the matrix, which leads to a compensatory expansion (swelling) of the chondrocytes. Likewise as a consequence of the precipitation and condensation of the proteoglycans, the matrix assumes a coarse, granular appearance. In (C), the depicted chondrocyte is similarly preserved in an expanded state, but has undergone no swelling. Consequently, the organelles undergo no distortive movements and are preserved *in situ*. In (D), the native dimensions of the depicted chondrocyte are preserved, but no ultrastructural details relating to either the cell or the surrounding matrix are revealed in such a fractured relief. Bars = 2.5 μm (A), 5 μm (B), 6 μm (C) and 2 μm (D). Reproduced with the publishers' permission from Hunziker, E.B. Microsc Res Tech 24, 457-464, 1993 (A-C) and Hunziker, E.B. et al., J Cell Biol 98, 267-276, 1984 (D).

Table 1
Preservation quality of cartilaginous tissue after the instigation of different fixation protocols.

General features	Chondrocytes (schematic views)	ECM	ECM (schematic views)	Preservation technique	Observation technique
• Chondrocytic lacunae • Significant loss of PGs • Only large-caliber collagenous fibrils are preserved intact		PM • ≈ 100% of the PGs are extracted TM/ITM • ≈ 60% of the PGs are extracted • Network of collagenous fibrils is dislocated and disrupted		Chemical • Formaldehyde • Glutaraldehyde Chemical • Glutaraldehyde (PF) →osmium tetroxide (SF)	LM TEM
• No chondrocytic lacunae • Precipitation and condensation of PGs throughout the entire ECM • Only large-caliber collagenous fibrils are visible; the finer ones are partially masked by PGs		PM • 100% of the PGs are retained • Collagenous network is preserved		Chemical • Formaldehyde + CD • Glutaraldehyde + CD Chemical • Glutaraldehyde + CD (PF) →osmium tetroxide + RHT (SF)	LM TEM
• No chondrocytic lacunae • Homogeneous distribution of PGs • Collagenous fibrils of all dimensions are preserved and visible		• PGs are retained and homogeneously distributed • Collagenous network is preserved in its entirety		Physical/Chemical • HPF/ FS/ LTE	TEM
• No chondrocytic lacunae • PGs are not visible (too fine) • Collagenous fibrils are generally not visible				Purely Physical • HPF/FE/Shadowing	TEM

Key to abbreviations: PGs: proteoglycans; ECM: extracellular matrix; PM: pericellular matrix; TM: territorial matrix; ITM: interterritorial matrix; PF: primary chemical fixation; SF: secondary chemical fixation; CD: cationic dye; RHT: ruthenium hexaammine trichloride; HPF: high-pressure freezing; FS: freeze-substitution; LTE: low-temperature embedding; FE: freeze-etching; LM: light microscopy; TEM: transmission electron microscopy.

Ruthenium Red (Luft, 1971; Shepard and Mitchell, 1977a,1977b), Cupromeronic Blue (Scott, 1980; Scott and Orford, 1981) or Safranin O (Shepard and Mitchell, 1976b), to chemical-fixation media led to a significant improvement in the preservation quality of cartilaginous tissue, as seen in the light- and transmission-electron microscopes. These agents precipitate the proteoglycans and the glycoproteins, as well as any other negatively-charged molecules, more or less *in situ* (Lammi and Tammi, 1988; Jortikka et al., 1993), thereby preventing their aqueous extraction. However, tissue that had been further processed for inspection in the transmission electron microscope by post-fixation in an aqueous solution of osmium tetroxide—to improve the contrast of the membranous systems (Palade, 1952)—was not improved in appearance. When cartilaginous tissue is exposed to an aqueous solution of osmium tetroxide, 70% of the precipitated (immobilized) proteoglycans are secondarily extracted (Engfeldt and Hjertquist, 1968). So far, only one compound—ruthenium hexaammine trichloride (RHT)—is known to have sufficient proteoglycan-precipitation power to overcome this problem (Hunziker et al., 1982). In cartilaginous tissue that has been pre- and post-fixed in the presence of RHT, the chondrocytes are uniform in size and shape (Figs. 1B and 3B). The so-called "dark" cells (Shepard and Mitchell, 1976b; Nissila et al., 1977; Carlson et al., 1985) that have been observed after the instigation of routine chemical-fixation protocols (Fig. 3A) are never encountered, thereby intimating that they are fixation-associated artifacts (Hunziker et al., 1982, 1984). Albeit so, some authors are still convinced of their reality, and speculate on their plausible functions (Ahmed et al., 2007; Chen et al., 2010a).

Using these RHT-based chemical-fixation protocols, it became possible for the first time to map the distribution and density profiles of the proteoglycans within cartilaginous tissue—the latter parameter at a subcellular level of resolution (Hunziker and Herrmann, 1987; Buschmann et al., 2000). However, owing to the RHT-induced precipitation and collapse of these macromolecules, the fine intermolecular relationships are destroyed (Fig. 2B) (Hunziker and Schenk, 1987). Omission of the post-fixation step in osmium tetroxide also yields reproducible preservation results in terms of cell size and shape. Moreover, in the absence of osmium tetroxide, cationic dyes other than RHT can be used, for example, Ruthenium Red or one of its derivatives (Hunziker et al., 1992). However, the contrast of the membranous systems does not suffice for a detailed ultrastructural analysis.

As far as the geometrical configuration of the chondrocytes and the immobilization of the proteoglycans are concerned, the introduction of a suitable cationic dye into aldehyde-based chemical-fixation media effects a substantial qualitative improvement in the preservation quality of cartilaginous tissue. However, the proteoglycan-precipitation process *per se* generates another set of problems and is an additional source of artifacts. For example, the cationic-dye-induced precipitation of proteoglycans induces their collapse. During this process, those molecules that have established interactive relationships with the surfaces of the chondrocytes will draw the plasmalemmae towards the matrix, thereby leading to a compensatory expansion of the cells and to the concomitant formation of intracellular vacuoles (Hunziker et al., 1982, 1983). In consequence, the chondrocytes will occupy larger volumes and their pericellular-matrix compartments smaller ones than they do in reality (Hunziker et al., 1983). Hence, quantitatively, the dimensions of the former will be over and those of the latter underestimated.

The dehydration and embedment of cartilaginous tissue can also generate artifacts. For example, if the process of dehydration is commenced in 40% ethanol, the cartilaginous tissue will undergo swelling. This problem can be circumvented if the initial concentration of the ethanol is raised to 70% (Hunziker and Schenk, 1989). However, if the native tissue is in a mechanically-compressed state at the time of its excision, the re-swelling pressure after chemical fixation in the presence of a cationic dye will be so high that, even after dehydration in 100% ethanol, its expansion during the process of embedment cannot be avoided (Patwari et al., 2004).

By now, it must be clear to the reader that the preservation of cartilaginous tissue in a near-native state at the molecular level cannot be achieved using chemical-fixation protocols. This ideal can be attained only if physical, cryoprocessing techniques are implemented, and even then, only if the process is effected with great rapidity (at a cooling rate of 10,000 °C per second or higher) (Studer et al., 1995; Keene and Tufa, 2010), whereby an instantaneous inactivation of the molecules will be procured. Such conditions can be realized; but, in practice, only down to a tissue depth of a few micrometers. If extremely high pressures in the order of 2,000 atmospheres can be generated for a few milliseconds, then the formation of destructive ice crystals and their subsequent growth can be avoided or at least greatly impeded down to a deeper tissue level, viz., approximately 200 to 300 μm (Studer

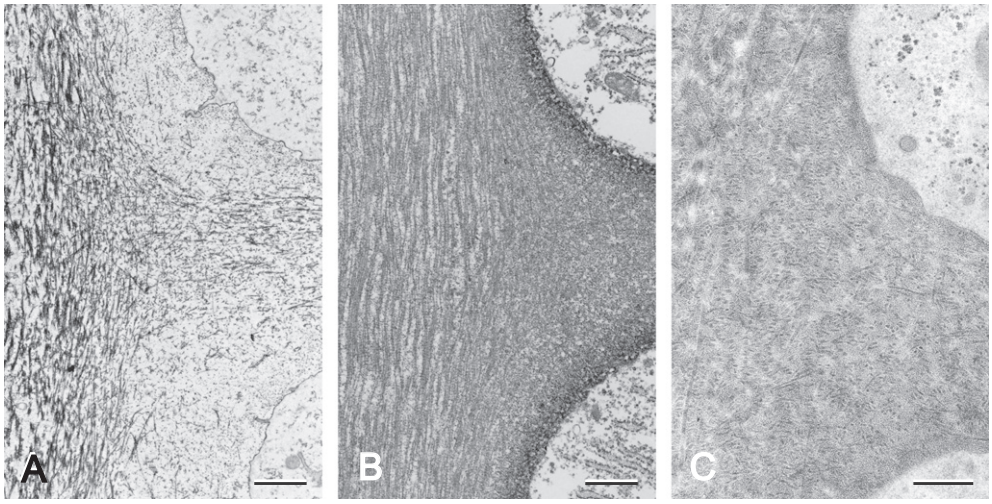


Fig. 2. Transmission electron micrographs of thin sections through the growth-plate cartilage of rats, depicting the extracellular-matrix compartments in the zone of hypertrophic chondrocytes after (A) conventional, primary chemical fixation in 2.5% glutaraldehyde and post-fixation in 1% osmium tetroxide, (B) glutaraldehyde-based fixation in the presence of 0.7% RHT and post-fixation in 1% osmium tetroxide, likewise in the presence of 0.7% RHT, and (C) high-pressure freezing, freeze-substitution and low-temperature embedding. In (A), most of the proteoglycans have been extracted. In consequence, the skeleton of collagenous fibrils is revealed with great clarity. In (B), the presence of RHT has prevented the loss of proteoglycans. Owing to their precipitation and consequent condensation, the matrix has a coarse, granular appearance, which reduces the lateral resolution of the imaging process: the fine ultrastrucutural features of the collagenous fibrils are but poorly revealed. In (C), the proteoglycans are preserved *in situ* and in their native, expanded state. The lateral resolution of the imaging processes is thus in no way compromised: the fine ultrastructural details of the collagenous fibrils are clearly visible. Bars = 1.5 μm (A, B) and 420 nm (C). Reproduced with the publisher's permission from Hunziker, E.B. Microsc Res Tech 28, 505-519, 1994.

et al., 1995). Even the molecules of the tissue's water pool (constituting 70% of its volume) will be preserved in their physiological, randomly-orientated state, rather than in an artificially crystalline one. The process whereby water molecules are preserved in such an amorphous (non-crystalline) state is referred to as vitrification. The vitrification of water molecules can be achieved only at very high pressures (and very low temperatures) (Studer et al., 1995; Hunziker et al., 1996; Keene and Tufa, 2010). At atmospheric pressure, ice crystals will be formed even in the presence of an antifreeze agent, albeit on a finer scale. Moreover, these agents themselves exert a destructive influence not only on the membranous systems of the chondrocytes but also on the fine molecular structure of the cartilaginous matrix, the smaller soluble compounds (such as peptides and glycoproteins) being particularly vulnerable.

Using a high-pressure-freezing technique (Moor et al., 1980; Hunziker et al., 1984; Studer et al., 1995), the chondrocytes and their membranous systems, as well as the macromolecular organization of the extracellular matrix, can be preserved in a near-native state by the vitrification of the water pool (Figs. 1C and 2C). However, visualization of the vitrified tissue at the ultrastructural level in the transmission electron microscope necessitates further processing, which, by its very nature, will give rise to artifactual changes. These changes can be completely circumvented (or at least minimized) if the tissue is further processed by purely physical means, viz., by freeze-etching, followed by shadowing with platinum (Hunziker et al., 1984), which yields a replica of the fractured surface (Fig. 1D). However, the information that can be gleaned from such replicas is confined to parameters such as the size and the shape of the chondrocytes and to the organization of their membranous systems (Hunziker et al., 1997). Using this technique, chondrocytes of the hypertrophic zone in growth-plate cartilage have been confirmed to be structurally intact and in a functionally-active state down to their point of contact with the vascular-invasion front. And when high-pressure freezing is followed by freeze-substitution and low-temperature embedding (see below), instead of by freeze-fracturing, etching and shadowing, hypertrophic chondrocytes within growing (immature) articular cartilage have likewise been demonstrated to be structurally and functionally integral throughout the entire zone of mineralization (Hunziker et al., 1984, 1996, 1997; Keene and McDonald, 1993).

The great benefits of tissue vitrification are jeopardized if due thought is not given to the subsequent processing steps. For example, if the high-pressure-frozen tissue is dehydrated at ambient temperature, it will thaw, and the vitrified water will crystallize during the thawing process before the liquid state is attained, thereby leading to the disruption and dislocation of the fine molecular intricacies of the extracellular matrix. And if the dehydration process is then initiated in 70% ethanol, the vitrified water will be very rapidly substituted with the alcoholic solution; proteoglycans within the extracellular matrix will be lost to the medium, and the tissue blocks will undergo considerable dimensional changes. Only when the vitrified water is substituted very slowly with a purely organic solvent, such as acetone, at very low temperatures (e.g., at −90 °C) can the process of molecular shifting be minimized (Studer et al., 1995; Hunziker et al., 1997). The embedment step should likewise be conducted very slowly at sub-zero temperatures (Hunziker et al., 1984; Keene and McDonald, 1993).

If cartilaginous tissue is preserved by high-pressure freezing, it is expedient to confirm that the water pool has indeed been vitrified. This information can be obtained by subjecting cryosections through the tissue to an electron-diffraction analysis in the transmission electron microscope (Studer et al., 1995; Hunziker et al., 1996). The technical demands of preparing cryosections from the high-pressure-frozen tissue and of manufacturing a cryostage for their electron-diffraction analysis in the transmission electron microscope were not overcome until the 1980s. Before this time, the vitrification-check by electron diffractionometry was conducted on freeze-fractured reliefs. However, the level of resolution did not suffice to yield the necessary information (Hunziker et al., 1984; Keene and McDonald, 1993; Studer et al., 1995; Keene and Tufa, 2010).

3. Chondrocytes and their membranous systems

In routinely-prepared histological sections through cartilaginous tissue (chemical fixation in formaldehyde and embedment in paraffin: see Table 1), the chondrocytes have a typically shrunken appearance, are intensely stained, and are characteristically mantled by an “empty” (unstained) pericellular lacuna. Although the lacuna is a processing artifact, it is such a patent structure and so characteristic a feature of chondrocytes in routinely-prepared specimens as to be a

useful landmark in distinguishing cartilage from connective and other tissue types in engineered constructs (Chen et al., 2010b; Liu et al., 2012). The chondrocytic plasmalemma and the organellar membranes are usually disrupted, giving the cells an apoptopic appearance (Hunziker et al., 1983). In extreme cases, the chondrocytes and their membranous systems may undergo complete collapse, imparting to the cells a necrotic appearance, which may be misinterpreted as such (Fig. 3A, Table 1) (Nissila et al., 1977; Carlson et al., 1985; Chen et al., 2010a). This phenomenon can occur even when chemical fixation is conducted in the presence of a cationic dye, if the choice of agent is inappropriate, and also during post-fixation in osmium tetroxide (when the tissue is destined for an ultrastructural analysis in the transmission electron microscope). It can be likewise encountered if, during the act of tissue excision, the tools implemented for this purpose exert high local mechanical forces along the cutting edge. Owing to the collapsed and thereby condensed state of their membranous systems, these grossly shrunken cells are rendered remarkable by the manifestation of a strong staining signal. This feature, coupled with the circumstance that irregular scatterings of the cells are consistently observed, has led to their being specially designated as "dark" chondrocytes (Nissila et al., 1977; Hunziker et al., 1983; Carlson et al., 1985; Chen et al., 2010b). Not all authors concede to the opinion that these so-called "dark" cells are an artifact; indeed, several groups of investigators have attributed specific functions to them (Shepard and Mitchell, 1976b; Gibson et al., 1995; Ahmed et al., 2007; Chen et al., 2010b). If chemical fixation is conducted in the presence of a cationic dye, such as RHT, with a high binding affinity for negatively-charged molecules and thus with a strong proteoglycan-precipitation power, then these collapsed cells are not observed unless other conditions at an earlier or a later stage of the tissue-processing sequence are suboptimal. Such will be the case if an unwontedly high mechanical force is applied along the cutting edge of the tissue specimens during their excision, which will occur if a blunt razor blade rather than a gentle sawing device is used for the purpose; and likewise if the cartilage is excised together with a portion of the adjacent bone, the cutting of which will generate a momentarily very high compressive force (Loqman et al., 2010). That suitable cutting tools must be employed should, one would have thought, be a matter of course. But a surprisingly large number of authors are negligent in this matter. False economy! : "Truly [*not*] a cutting of blocks with fine razors while we scrape our chins so uncomfortably with rusty knives!" (Anthony Trollope: *The Warden*). Apoptosis can be readily induced up to a distance of about 200 μm from a mechanically-traumatized cutting edge, and can be misinterpreted as an observation of functional significance (Walker et al., 2000; Hunziker and Stahli, 2008).

The native shape of chondrocytes can be consistently and reproducibly preserved only if the cationic dye is present throughout the entire course of chemical fixation. If the tissue is subjected only to primary fixation in an aldehyde-based medium, as will be the case if it is destined solely for a histological analysis, then a cationic dye other than RHT could be utilized (Hunziker et al., 1983). An RHT-analogue (Hunziker et al., 1992), Ruthenium Red or Toluidine Blue would be suitable alternatives. But using several others, such as Alcian Blue (Ruggeri et al., 1975; Schofield et al., 1975), either the tissue-penetration properties of the dye are too poor, or its binding affinity for negatively-charged molecules is too weak, to yield a homogeneous proteoglycan-precipitation result. An appropriate positive control should be included to validate the preservation results that are achieved using the cationic dye of choice.

A suitable cationic dye will be one with a low molecular weight, the capacity to diffuse rapidly through the tissue, and a high electrical-charge density. If the choice is appropriate, then its inclusion in the chemical-fixation medium will prevent the extraction of proteoglycans and of other negatively-charged extracellular compounds. In consequence, the interface between the extracellular matrix and the chondrocytes will be conserved: the native shape of the cells will be maintained; so, too, will the plasmalemma and the intracellular membranous systems.

Tissue that is destined for an ultrastructural analysis in the transmission electron microscope is usually subjected to post-fixation in osmium tetroxide to enhance the contrast of the membranous systems. A cationic dye that has been demonstrated to be effective in preventing the extraction of proteoglycans into a primary-fixation (aldehyde-based) medium may not be likewise effective in the presence of osmium

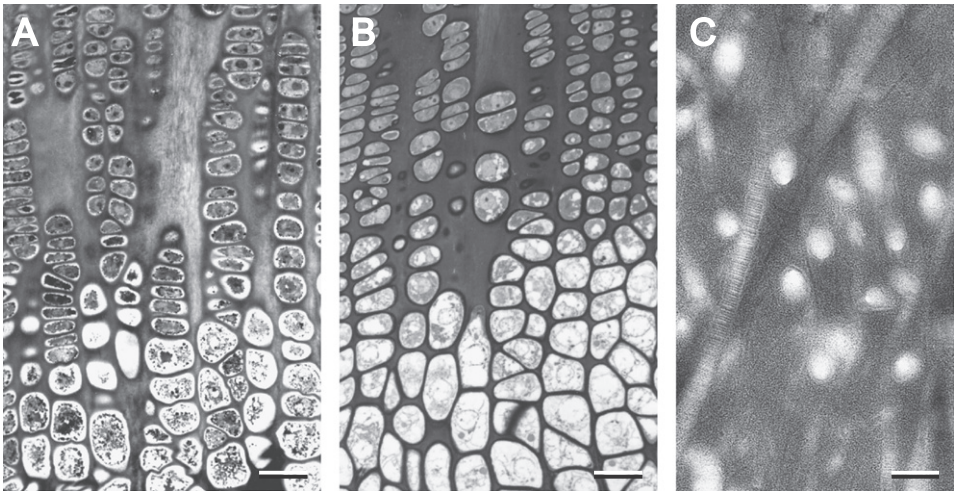


Fig. 3. (A, B): Light micrographs of semi-thin sections through the proximal tibial growth-plate cartilage of rats after conventional gultaraldehyde-based fixation in the absence (A) or presence (B) of 0.7% RHT. (C): High-resolution transmission electron micrograph of a thin section through bovine articular cartilage, depicting the interterritorial matrix of the radial zone. In (A), the cells have retracted from the surrounding pericellular matrix, thereby giving rise to the formation of "empty" (unstained) lacunae. The cells have undergone shrinkage and condensation to variable degrees, thereby enhancing their affinity for the cationic stain Toluidine Blue. In extreme cases, so-called "dark" cells arise. In (B), the chondrocytes maintain their intimate contact with the surrounding pericellular matrix. Owing to the precipitation and consequent condensation of the proteoglycans that interact with the cell surfaces, the plasmalemmae are drawn towards the matrix, which leads to a compensatory expansion (swelling) of the chondrocytes and to their vacuolization. In (C), an expansive network of collagenous fibrils in the nanometer-diameter range is revealed with great clarity between the fibrils of larger caliber (50 nm to 2 μm). Under conventional chemical-fixation conditions, the nanofibrillar network is destroyed and never seen. In the longitudinally-orientated fibrils of both dimension-classes, a distinct cross-banding is apparent. Bars = 30 μm (A, B) and 200 nm (C). Reproduced with the publishers' permission from Hunziker, E.B. et al., J Histochem Cytochem 31, 717-727, 1983 (A, B) and Hunziker, E.B. et al., Histochem Cell Biol 106, 375-382, 1996 (C).

tetroxide (Hunziker et al., 1983). Even if the binding affinity of the cationic dye is sufficiently high to immobilize (precipitate) the proteoglycans in an aldehyde-based medium, the interaction may be too weak to resist the harsh extraction forces of an osmium-tetroxide-containing one (Hunziker and Graber, 1986). RHT and several of its derivatives are the sole exceptions (Hunziker et al., 1982, 1992). Moreover, during their cationic-dye-induced precipitation, the proteoglycans undergo collapse (Hunziker and Schenk, 1987). During this process, those molecules that have established interactions with the surfaces of the chondrocytes will draw the plasmalemmae towards the matrix, thereby leading to a compensatory expansion (swelling) of the cells, the concomitant formation of intracellular vacuoles, and a consequent narrowing of the pericellular-matrix compartment. The resulting increase in cell volume has been estimated to lie in the order of 17%, as measured at the end of the processing sequence, even in the face of the tissue shrinkage that is associated with the process of dehydration in ethanol at concentrations exceeding 70% (Hunziker et al., 1987; Hunziker and Schenk, 1989).

The problems that are associated with the chemical fixation of cartilage in aldehyde-based media, namely, cell collapse and the formation of a pericellular lacuna, cannot be overcome by cryofixation according to conventional methodologies, for example, using carbon dioxide or liquid nitrogen. When these techniques are implemented, the formation and growth of ice crystals leads to cell collapse and to a destruction of the fine structural intricacies of the extracellular matrix. Such material is suitable only for a coarse histological evaluation in the light microscope; an ultrastructural analysis in the transmission electron microscope is out of the question.

4. The network of fibrillar collagens

In adult mammalian articular cartilage, the matrix is characterized by an arcade of fibrillar collagens. This architectonic feature was first described by Benninghoff in 1922 (Benninghoff, 1922), and its existence has since been repeatedly confirmed (Hunziker et al., 1997; van Turnhout et al., 2010b). The arcade of fibrillar collagens is not to be seen unless the articular layer is sectioned in a precisely vertical direction relative to the plane of the underlying bone. In obliquely-sectioned specimens, its anisotropic organization will not be clearly reflected, and such images are open to misinterpretation. This danger will also arise if the images hit artifactual, processing-induced undulations in the surface contour of the bony plate. These circumstances serve to illustrate the importance to image interpretation of instigating not only a suitable technical-processing methodology but also an appropriate and well-defined sectioning protocol.

A close scrutiny of the arcade of fibrillar collagens reveals a gradient of increasing diameter from the superficial zone down to the lower radial one, which is of biomechanical import (Jurvelin et al., 2003; van Turnhout et al., 2010a). The collagenous network can be most readily identified in low-power transmission electron micrographs after chemical fixation in the absence of a cationic dye (Table 1). In this respect, we can take advantage of the biased structural appearance of cartilaginous tissue from which the proteoglycans have been extracted, in so far as the collagenous fibrils are thereby unmasked and rendered more visible (Fig. 2A). This methodological approach has been applied in a number of studies with knock-out mice whose aim has been to assess the impact of selective gene-deletion on the structure of the collagenous network (Aszodi et al., 1998, 2001). However, only the coarser fibrils are revealed using this tactic. The population of nanofibrils does not survive chemical fixation in aldehyde-based media. These entities can be preserved only if sophisticated cryotechnical approaches—involving high-pressure freezing, freeze-substitution and low-temperature embedding (Hunziker et al., 1984; Li et al., 2005)—are implemented (Fig. 3C). Moreover, the native tissue must be processed immediately upon its excision, without even transient immersion even in a buffered solution, which would lead to the extraction of proteoglycans and a consequent disruption of the macromolecular organization of the nanofibrils (Hunziker and Graber, 1986; Hunziker et al., 1996; Keene and Tufa, 2010). This phenomenon can occur even during freeze-substitution if the organic solvent is ill-chosen. Under these suboptimal conditions, an artifactual optical impression is gained, and the nanofibrils appear as hollow cylinders (ap Gwynn et al., 2000). When freeze-substitution is executed under optimal conditions, the nanofibrils are revealed to be compact structures, which are more electron dense centrally than peripherally, and are surrounded at a distance by a water-rich compartment (Studer et al., 1996).

The anisotropic organization of the coarser collagenous fibrils can be revealed also by implementing specific stains, such as Sirius Red (Junqueira et al., 1980), as well as by polarized light microscopy (Mittelstaedt et al., 2011) and by magnetic resonance imaging (Momot et al., 2010; Koller et al., 2014), or by an analysis of birefringence (Panula et al., 1998). Scanning electron microscopy is the least useful methodology. The critical-point-drying step generates artifacts of such a crude and severe nature as to greatly compromise an analysis of the fibrillar architecture. Furthermore, after the tissue has been bombarded with particles of gold in a vacuum to enhance the electron-conductivity of its surface, the characteristic cross-banding of all but the coarsest collagenous fibrils can no longer be distinguished (Changoor et al., 2011; Fujioka et al., 2013); nor can the different types of fibril. Moreover, as may be the case when high-pressure-frozen tissue undergoes freeze-substitution in an Ill-chosen organic medium, the collagenous fibrils may artifactually appear as hollow tubes (ap Gwynn et al., 2000).

Hence, using conventional methodological approaches, little useful information can be gleaned respecting either the “backbone” of coarser fibrils or the intricacies of the intervening network of finer ones. At best, the data thereby accumulated can play a supportive role in helping to explain the mechanical properties of cartilaginous tissue (Studer et al., 1995; Hunziker et al., 1997).

5. The proteoglycans

The matricular population of proteoglycans within articular cartilage is unique in its organization, which is a key to the biomechanical attributes and functions of the tissue (Heinegård, 2009). Its specific structural, electrical and osmotic properties, as well as its density and state of underhydration, change during the course of development, and vary as a function of age and disease (Heinegård, 2009).

The routine, aldehyde-based chemical fixation of cartilaginous tissue in the absence of a cationic dye is to be regarded as a proteoglycan-extraction rather than a preservation step, and the situation is exacerbated if the tissue is subjected to post-fixation in osmium tetroxide for an ultrastructural analysis (see Subsection 2). Notwithstanding, such methodologies are still widely implemented in research and pathology laboratories. The stained tissue-sections thereby produced for routine inspection can reveal only the extraction-and the redistribution profiles of the proteoglycans, and, as such, yield biased information of very limited use. Moreover, it makes no sense to subject such specimens to a semi-quantitative histological analysis using scoring systems (Glasson et al., 2010).

The introduction of a given cationic dye into an aldehyde-based fixation medium was shown in the pioneering histochemical studies of Szirmai (Szirmai and Doyle, 1961; Szirmai, 1963) to induce the selective precipitation of specific proteoglycan- (hitherto known as mucopolysaccharide) classes [for review, see (Hyllested et al., 2002)]. Although the inclusion of an appropriate and powerful cationic dye during the course of primary and post-fixation (the latter for transmission electron microscopy) can prevent the extraction of matricular proteoglycans, the immobilization of these macromolecules by precipitation induces their collapse and condensation (Hunziker and Schenk, 1987)—which reduces the power of lateral

resolution (Hunziker and Schenk, 1987)—thereby leading to their uncontrolled, random displacement and a consequent destruction of their native distribution pattern and structural organization. Moreover, since the dimensions of the condensed proteoglycans lie within the range of the tissue-section thickness (approximately 40 nm), imaging in the transmission electron microscope is hampered by truncation and over-projection artifacts. At best, the distribution profiles of the proteoglycans in the different matricular compartments and tissue-zones can be compared and quantified. Such information can be helpful in an analysis of the intra-tissue strain forces that are generated under different mechanical-loading conditions (Buschmann et al., 2000).

The matricular proteoglycans can be preserved in their native, expanded state only if the tissue-water pool is vitrified, which can be achieved by implementing highly-sophisticated cryopreservation techniques (see Subsection 2). Under these conditions, the proteoglycans are revealed as a homogeneously-distributed, densely-packed mass of electron-dense entities between the collagenous fibrils (Hunziker et al., 1997; Keene and Tufa, 2010) (Fig. 2C). The individual molecular components of the mass of proteoglycans cannot be revealed without recourse to specific immunological tools. After post-embedment immunolabeling with a tagged antibody (or its Fab-fragment) against the carbohydrate moities of the proteoglycans, the individual molecular components can be visualized with a relatively high power of lateral resolution (Hunziker and Herrmann, 1987). As yet, a successful methodology for the protein epitopes has not been elaborated. Moreover, although the lateral resolution is reasonably high, it is reduced by the presence of the electron-dense tag and the antibody (or its Fab-fragment), by localized scattering around the epitope, and by image-analysis interferences (Hunziker and Schenk, 1987). Information respecting the structures and the sizes of the individual molecular components can be garnered only after these have been isolated and spread on a two-dimensional surface. Such analyses can be performed using the Kleinschmitt technique (Buckwalter et al., 1994), by rotatory shadowing (Morgelin et al., 1995), or—with the highest resolution—by atomic-force microscopy (Lee et al., 2012). The latter methodology is the only one that permits a visualization of the proteoglycans in a near-physiological state (Han et al., 2007).

6. The tissue-water pool

Given the avascular nature of articular cartilage, the tissue-water compartment is obviously an important medium for the transport of ions, nutrients and soluble substances of all kinds. But, in addition, it plays a crucial role in the biomechanical activities of the tissue (Frank and Grodzinsky, 1987; Frank et al., 1987). Although many of the metabolic activities of the extracellular matrix, such as its remodeling, its mineralization and the formation of its highly-organized, anisotropic network of collagenous fibrils, occur within this compartment, and often at some distance from the chondrocytes, they are nevertheless under cellular control (Okazaki and Sandell, 2004). The extracellular water is maintained in a bound or trapped state owing to the interactive activities of the chondrocytes, the collagenous meshwork and the proteoglycans (see Subsection 1).

When cartilage is immersed in an aldehyde-based fixation medium, the tissue undergoes considerable changes in mass and volume, which are largely a consequence of the aqueous extraction of the proteoglycans. These changes are lessened, although not altogether wanting, after chemical fixation in the presence of a cationic dye. Further changes in mass and volume, albeit to a lesser degree, occur also during the processes of dehydration and embedment.

The water pool can be preserved and visualized in a near-physiological state only by implementing a thoroughly physical approach, namely, by subjecting the tissue to high-pressure freezing, freezing-etching and rotatory shadowing (Hunziker et al., 1984). After high-pressure freezing under optimal conditions, the tissue water is preserved in a vitrified (amorphous) state, quite in the absence of ice crystals, the formation and growth of which have a distortive, disruptive and destructive influence on all cellular and matricular structures—without exception. After freeze-etching and rotatory shadowing, visualization of the tissue-reliefs in the transmission electron microscope yields useful information respecting the size and the structural integrity of the chondrocytes, but otherwise, such images are of limited value, representing as they do no more than shadowed reliefs of the tissue. Transmission electron microscopy of tissue sections is a much more powerful methodology, which can furnish information respecting the structural intricacies of the extracellular matrix. However, for this purpose, the high-pressure-frozen tissue must undergo freeze-substitution and low-temperature embedding. During these processes, the vitrified tissue-water is first substituted with a chemically-inert organic solvent at very low temperatures (−90 °C). The tissue is then infiltrated at sub-zero temperatures with a plastic resin, whose polymerization is likewise effected at sub-zero temperatures (Roth et al., 1981; Hunziker et al., 1984). Under optimal conditions, these processes are accompanied by only minor changes in the dimensions and the volume of the tissue. Transmission electron microscopy of cartilaginous tissue that has undergone high-pressure freezing, freeze-substitution and low-temperature embedding has revealed a phenomenon whose existence had hitherto not even been suspected, let alone observed, namely, the presence of a water-rich halo around the collagenous fibrils (Studer et al., 1996), the biological significance of which awaits clarification.

7. The “best of the rest”

The cartilaginous matrix contains diverse molecules and small macromolecules, which are quantitatively minor components. Their concentrations change during the course of development and vary with the level of tissue reactivity to trauma or disease (Heinegård, 2009). These components include small proteoglycans, glycoproteins, non-fibrillar collagens and signaling substances. Despite their low levels, many of these compounds play pivotal roles in the formation, the remodeling, the repair and the biomechanical activities of articular cartilage. During the course of development, the levels of some of these substances change predictably in a stage-specific manner (Wilson et al., 2011). And in cartilaginous tissue that has attained maturity, the levels of specific molecules can be indicative of ongoing remodeling or repair activities.

The precise localization of these quantitatively minor matricular components is a troublesome and often a frustrating undertaking. Data pertaining thereto are often open to mis-and over-interpretation owing to the instigation of inappropriate tissue-preservation techniques. The methodological approach that is most frequently implemented to localize these components and to map their distribution profiles is immunohistochemistry, which is commonly performed either on paraffin-embedded sections though tissue that has been conventionally fixed in aldehyde-based media, or on cryosections through conventionally-crofixed specimens. As with the quantitatively major matricular components, the quantitatively minor ones are likewise prone to dislocation and aqueous extraction during chemical fixation in aldehyde-based media and dehydration in ethanolic ones, and, owing to their generally smaller size, to a greater degree. Hence, the immunohistochemical images should be interpreted with great reservations. They can reveal no more than a very crude notion of the patterns of distribution of the targeted molecules, unless these happen to form stable interactions with water-insoluble structures, such as the chondrocytic plasmalemma or fibrillar collagen. The belief that the problems can be overcome by performing the immunohistochemical analysis on cryosections instead of on paraffin-embedded ones is ill-founded. Firstly, such cryosections are usually air-dried, which induces molecular dislocations. Secondly, the labeled antibodies are applied to the cryosections in aqueous media, which will lead not only to the

extraction of both the targeted and the untargeted molecules, but also to the dislocation of those that are spared. Hence, also using this approach, the images are open to mis- and over-interpretation, and the danger of deception is perhaps the greater in so far as many investigators suffer under the delusion that the aqueous-extraction problem is circumvented by conventional cryopreservation.

No attempts have yet been made to investigate these quantitatively minor matricular components in the transmission electron microscope after the immunostaining of sections through tissue-specimens that have been processed by high-pressure freezing, freeze-substitution and low-temperature embedding. This methodological approach would be the one best suited to yield meaningful ultrastructural information.

8. Special procedures

A number of special techniques can be implemented to yield complementary information relating to specific questions, which usually have a functional rather than a structural bearing. Examples of such methodological tools include nuclear magnetic resonance imaging (Koller et al., 2014), polarization light microscopy (Panula et al., 1998), laser-scanning microscopy and autoradiography (Buschmann et al., 1996; Kawaguchi et al., 2012). The value of the data gleaned using these approaches is compromised by the limited resolution of the images, which lies within the light-microscopic range.

Atomic force microscopy has been implemented to yield both structural and functional information appertaining to the surface and subsurface features of fresh cartilaginous tissue, which can be maintained under physiologically-relevant conditions during imaging in the subnanometer-resolution range. In one such study (Jurvelin et al., 1996), the articular surface of bovine cartilage was revealed to be characteristically smooth and amorphous, thereby confirming the long-held suspicion that the undulations disclosed by scanning electron microscopy were processing (dehydration)-induced shrinkage artifacts. Furthermore, simultaneous quantification of the degree of cantilever-tip indentation and contact force revealed local variations in surface stiffness which could reflect corresponding differences in mechanical behavior [see also (Allen and Mao, 2004)].

9. Conclusions

Adult human articular cartilage has a unique structure, the anisotropy of which is manifested at all levels—from the macroscopic down to the molecular. The structural features of the tissue layer faithfully reflect, and afford clues to, its biomechanical properties. The truth of this assertion is borne out by the changes in structural organization that occur during the course of the tissue's development from immaturity (fetal through postnatal stages) to maturity (adulthood). During the different phases of postnatal development, the tissue subserves a dual function, acting not only as an articular layer but also as a superficial growth plate (Hunziker et al., 2007). This double role of the tissue layer is minutely reflected in its structure, which changes in temporal synchrony with the changes in its functional needs.

As the human individual grows visibly, the elongation of the long bones proceeds at a rapid pace, peaking at the end of the prepubertal spurt, and then at a declining rate until the time of its cessation, which marks the attainment of skeletal maturity. These changes in growth rate are temporally reflected in the growth-plate-like structural features of the tissue (Hunziker, 1994; Hunziker et al., 2007). On the other hand, as the biomechanical loading of the long bones, particularly those of the lower limbs, increases—the most dramatic changes occurring during the transitions from non-crawling babyhood, through the crawling phase of infancy, to the verticality of walking childhood—the articular-cartilage-like structural features of the tissue are superimposed and become more prominent.

What has taken many words to explain but poorly is in nature effected smoothly and “effortlessly” in a beautifully-coordinated process (Hunziker et al., 2007). An insight into the structural intricacies that subserve the changes in function can be gained only if they are faithfully preserved at the molecular level. Unfortunately, no single methodological tool is capable of surmounting the Herculean difficulties that pave the way to this end. Only by instigating several different methodologies—each with its advantages and disadvantages—can we assemble, in a jigsaw-puzzle-like fashion, a comprehensive overview of the tissue's structure as a mosaic of the whole.

Acknowledgement

We would like to thank Barbara Krieger (Institute of Anatomy, University of Bern) for her assistance in digitizing the light and transmission electron micrographs.

References

Ahmed, Y.A., Tatarczuch, L., Pagel, C.N., Davies, H.M., Mirams, M., Mackie, E.J., 2007. Physiological death of hypertrophic chondrocytes. Osteoarthr. Cartil. 15, 575–586.
Allen, D.M., Mao, J.J., 2004. Heterogeneous nanostructural and nanoelastic properties of pericellular and interterritorial matrices of chondrocytes by atomic force microscopy. J. Struct. Biol. 145, 196–204.
Anderson, C.E., Ludowieg, J., Harper, H.A., Engleman, E.P., 1964. The composition of the organic component of human articular cartilage. Relationship to age and degenerative joint disease. J. Bone Joint Surg. Am. 46, 1176–1183.
ap Gwynn, I., Wade, S., Kaab, M.J., Owen, G.R., Richards, R.G., 2000. Freeze-substitution of rabbit tibial articular cartilage reveals that radial zone collagen fibres are tubules. J. Microsc. 197, 159–172.
Aszodi, A., Chan, D., Hunziker, E., Bateman, J.F., Fassler, R., 1998. Collagen II is essential for the removal of the notochord and the formation of intervertebral discs. J. Cell Biol. 143, 1399–1412.
Aszodi, A., Hunziker, E.B., Olsen, B.R., Fassler, R., 2001. The role of collagen II and cartilage fibril-associated molecules in skeletal development. Osteoarthr. Cartil. 9 (Suppl. A), S150–S159.
Benninghoff, A., 1922. Ueber den funktionellen Bau des Knorpels. Anat. Anz. Erg. Heft 55, 250–267.
Buckwalter, J.A., Roughley, P.J., Rosenberg, L.C., 1994. Age-related changes in cartilage proteoglycans: quantitative electron microscopic studies. Microsc. Res. Tech. 28, 398–408.
Buckwalter, J.A., Mankin, H.J., Grodzinsky, A.J., 2005. Articular cartilage and osteoarthritis. Instr. Course Lect. 54, 465–480.
Buschmann, M.D., Maurer, A.M., Berger, E., Hunziker, E.B., 1996. A method of quantitative autoradiography for the spatial localization of proteoglycan synthesis rates in cartilage. J. Histochem. Cytochem. 44, 423–431.
Buschmann, M.D., Maurer, A.M., Berger, E., Perumbuli, P., Hunziker, E.B., 2000. Ruthenium hexaammine trichloride chemography for aggrecan mapping in cartilage is a sensitive indicator of matrix degradation. J. Histochem. Cytochem. 48, 81–88.
Carlson, C.S., Hilley, H.D., Henrikson, C.K., 1985. Ultrastructure of normal epiphyseal cartilage of the articular-epiphyseal cartilage complex in growing swine. Am. J. Vet. Res. 46, 306–313.
Changoor, A., Nelea, M., Methot, S., Tran-Khanh, N., Chevrier, A., Restrepo, A., Shive, M.S., Hoemann, C.D., Buschmann, M.D., 2011. Structural characteristics of the collagen network in human normal, degraded and repair articular cartilages observed in polarized light and scanning electron microscopies. Osteoarthr. Cartil. 19, 1458–1468.
Chen, K.S., Tatarczuch, L., Ahmed, Y., Huang, H.H., Mirams, M., Pagel, C.N., Mackie, E.J., 2010a. Identification of light and dark hypertrophic chondrocytes in mouse and rat chondrocyte pellet cultures. Tissue Cell 42, 121–128.
Chen, W.C., Yao, C.L., Wei, Y.H., Chu, I.M., 2010b. Evaluating osteochondral defect repair potential of autologous rabbit bone marrow cells on type II collagen scaffold. Cytotechnology 63, 13–23.
Davies, D.V., Barnett, C.H., Cochrane, W., Palfrey, A.J., 1962. Electron microscopy of articular cartilage in the young adult rabbit. Ann. Rheum. Dis. 21, 11–22.
Eggli, P.S., Hunziker, E.B., Schenk, R.K., 1988. Quantitation of structural features characterizing weight- and less-weight-bearing regions in articular cartilage: a stereological analysis of medial femoral condyles in young adult rabbits. Anat. Rec. 222, 217–227.
Engfeldt, B., Hjertquist, S.O., 1968. Studies on the epiphysial growth zone. I. The preservation of acid glycosaminoglycans in tissues in some histotechnical procedures for electron microscopy. Virchows Arch. B Cell Pathol. 1, 222–229.
Frank, E.H., Grodzinsky, A.J., 1987. Cartilage electromechanics–I. Electrokinetic transduction and the effects of electrolyte pH and ionic strength. J. Biomech. 20, 615–627.
Frank, E.H., Grodzinsky, A.J., Koob, T.J., Eyre, D.R., 1987. Streaming potentials: a sensitive index of enzymatic degradation in articular cartilage. J. Orthop. Res. 5, 497–508.
Frank, E.H., Grodzinsky, A., Phillips, S.L., Grimshaw, P.E., 1990. Physicochemical and bioelectrical determinants of cartilage material properties. In: Mow, V.C., Ratcliffe, A., Woo, S.L.-Y. (Eds.), Biomechanics of Diarthrodial Joints. Springer Verlag, New York, pp. 261–282.
Fujioka, R., Aoyama, T., Takakuwa, T., 2013. The layered structure of the articular surface. Osteoarthr. Cartil. 21, 1092–1098.
Gibson, G.J., Kohler, W.J., Schaffler, M.B., 1995. Chondrocyte apoptosis in endochondral ossification of chick sterna. Dev. Dyn. 203, 468–476.

Glasson, S.S., Chambers, M.G., Van Den Berg, W.B., Little, C.B., 2010. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthr. Cartil. 18 (Suppl. 3), S17–S23.
Gustafsson, E., Aszodi, A., Ortega, N., Hunziker, E.B., Denker, H.W., Werb, Z., Fassler, R., 2003. Role of collagen type II and perlecan in skeletal development. Ann. N. Y. Acad. Sci. 995, 140–150.
Han, L., Dean, D., Mao, P., Ortiz, C., Grodzinsky, A.J., 2007. Nanoscale shear deformation mechanisms of opposing cartilage aggrecan macromolecules. Biophys. J. 93, L23–L25.
Handley, C.J., Winter, G.M., Ilic, M.Z., Ross, J.M., Anthony Poole, C., Clem Robinson, H., 2002. Distribution of newly synthesized aggrecan in explant cultures of bovine cartilage treated with retinoic acid. Matrix Biol. 21, 579–592.
Hasler, E.M., Herzog, W., Wu, J.Z., Muller, W., Wyss, U., 1999. Articular cartilage biomechanics: theoretical models, material properties, and biosynthetic response. Crit. Rev. Biomed. Eng. 27, 415–488.
Heinegård, D., 2009. Proteoglycans and more–from molecules to biology. Int. J. Exp. Pathol. 90, 575–586.
Heinegård, D., Paulsson, M., Sommarin, Y., 1982. Proteoglycans and matrix proteins in cartilage. Prog. Clin. Biol. Res. 110 (Pt B), 35–43.
Horkay, F., 2013. Interactions of cartilage extracellular matrix macromolecules. J. Polym. Sci. B Polym. Phys. 50, 1699–1705.
Hunziker, E.B., 1994. Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc. Res. Tech. 28, 505–519.
Hunziker, E.B., Graber, W., 1986. Differential extraction of proteoglycans from cartilage tissue matrix compartments in isotonic buffer salt solutions and commercial tissue-culture media. J. Histochem. Cytochem. 34, 1149–1153.
Hunziker, E.B., Herrmann, W., 1987. In situ localization of cartilage extracellular matrix components by immunoelectron microscopy after cryotechnical tissue processing. J. Histochem. Cytochem. 35, 647–655.
Hunziker, E.B., Schenk, R.K., 1987. Structural organization of proteoglycans in cartilage. In: Wight, T.N., Mecham, R.P. (Eds.), Biology of Proteoglycans. Academic Press, Inc., Orlando, FL, pp. 155–185.
Hunziker, E.B., Schenk, R.K., 1989. Physiological mechanisms adopted by chondrocytes in regulating longitudinal bone growth in rats. J. Physiol. 414, 55–71.
Hunziker, E.B., Stahli, A., 2008. Surgical suturing of articular cartilage induces osteoarthritis-like changes. Osteoarthr. Cartil. 16, 1067–1073.
Hunziker, E.B., Herrmann, W., Schenk, R.K., 1982. Improved cartilage fixation by ruthenium hexammine trichloride (RHT). A prerequisite for morphometry in growth cartilage. J. Ultrastruct. Res. 81, 1–12.
Hunziker, E.B., Herrmann, W., Schenk, R.K., 1983. Ruthenium hexammine trichloride (RHT)-mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chondrocytic plasma membranes in situ during cartilage fixation. J. Histochem. Cytochem. 31, 717–727.
Hunziker, E.B., Herrmann, W., Schenk, R.K., Mueller, M., Moor, H., 1984. Cartilage ultrastructure after high pressure freezing, freeze substitution, and low temperature embedding. I. Chondrocyte ultrastructure–implications for the theories of mineralization and vascular invasion. J. Cell Biol 98, 267–276.
Hunziker, E.B., Schenk, R.K., Cruz-Orive, L.M., 1987. Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. J. Bone Joint Surg. Am. 69, 162–173.
Hunziker, E.B., Ludi, A., Herrmann, W., 1992. Preservation of cartilage matrix proteoglycans using cationic dyes chemically related to ruthenium hexaammine trichloride. J. Histochem. Cytochem. 40, 909–917.
Hunziker, E.B., Wagner, J., Studer, D., 1996. Vitrified articular cartilage reveals novel ultrastructural features respecting extracellular matrix architecture. Histochem. Cell Biol. 106, 375–382.
Hunziker, E.B., Michel, M., Studer, D., 1997. Ultrastructure of adult human articular cartilage matrix after cryotechnical processing. Microsc. Res. Tech. 37, 271–284.
Hunziker, E.B., Kapfinger, E., Geiss, J., 2007. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. Osteoarthr. Cartil. 15, 403–413.
Hyllested, J.L., Veje, K., Ostergaard, K., 2002. Histochemical studies of the extracellular matrix of human articular cartilage–a review. Osteoarthr. Cartil. 10, 333–343.
Jortikka, M., Lammi, M.J., Parkkinen, J.J., Lahtinen, R., Tammi, M.I., 1993. A high sensitivity dot-blot assay for proteoglycans by cuprolinic blue precipitation. Connect. Tissue Res. 29, 263–272.
Junqueira, L.C., Bignolas, G., Mourao, P.A., Bonetti, S.S., 1980. Quantitation of collagen - proteoglycan interaction in tissue sections. Connect. Tissue Res. 7, 91–96.
Jurvelin, J.S., Muller, D.J., Wong, M., Studer, D., Engel, A., Hunziker, E.B., 1996. Surface and subsurface morphology of bovine humeral articular cartilage as assessed by atomic force and transmission electron microscopy. J. Struct. Biol. 117, 45–54.
Jurvelin, J.S., Buschmann, M.D., Hunziker, E.B., 2003. Mechanical anisotropy of the human knee articular cartilage in compression. Proc. Inst. Mech. Eng. H 217, 215–219.
Kawaguchi, T., Nanbu, P.N., Kurokawa, M., 2012. Distribution of prolylhydroxyproline and its metabolites after oral administration in rats. Biol. Pharm. Bull. 35, 422–427.
Keene, D.R., McDonald, K., 1993. The ultrastructure of the connective tissue matrix of skin and cartilage after high-pressure freezing and freeze-substitution. J. Histochem. Cytochem. 41, 1141–1153.
Keene, D.R., Tufa, S.F., 2010. Transmission electron microscopy of cartilage and bone. Methods Cell Biol. 96, 443–473.
Koller, U., Apprich, S., Domayer, S., Windhager, R., Trattnig, S., 2014. Magnetic resonance mapping of the rim of articular cartilage defects of the patella. Int. Orthop. 38, 67–72.
Lammi, M., Tammi, M., 1988. Densitometric assay of nanogram quantities of proteoglycans precipitated on nitrocellulose membrane with Safranin O. Anal. Biochem. 168, 352–357.
Lee, H.Y., Han, L., Roughley, P.J., Grodzinsky, A.J., Ortiz, C., 2012. Age-related nanostructural and nanomechanical changes of individual human cartilage aggrecan monomers and their glycosaminoglycan side chains. J. Struct. Biol. 181, 264–273.
Li, W.J., Tuli, R., Huang, X., Laquerriere, P., Tuan, R.S., 2005. Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold. Biomaterials 26, 5158–5166.
Liu, J., Liu, X., Zhou, G., Xiao, R., Cao, Y., 2012. Conditioned medium from chondrocyte/scaffold constructs induced chondrogenic differentiation of bone marrow stromal cells. Anat. Rec. (Hoboken) 295, 1109–1116.
Loqman, M.Y., Bush, P.G., Farquharson, C., Hall, A.C., 2010. A cell shrinkage artefact in growth plate chondrocytes with common fixative solutions: importance of fixative osmolarity for maintaining morphology. Eur. Cell Mater. 19, 214–227.
Luft, J.H., 1971. Ruthenium red and violet. II. Fine structural localization in animal tissues. Anat. Rec. 171, 369–415.
Maroudas, A.I., 1976. Balance between swelling pressure and collagen tension in normal and degenerate cartilage. Nature 260, 808–809.
Maroudas, A., Bannon, C., 1981. Measurement of swelling pressure in cartilage and comparison with the osmotic pressure of constituent proteoglycans. Biorheology 18, 619–632.
McLeod, M.A., Wilusz, R.E., Guilak, F., 2012. Depth-dependent anisotropy of the micromechanical properties of the extracellular and pericellular matrices of articular cartilage evaluated via atomic force microscopy. J. Biomech. 46, 586–592.
Mittelstaedt, D., Xia, Y., Shmelyov, A., Casciani, N., Bidthanapally, A., 2011. Quantitative determination of morphological and territorial structures of articular cartilage from both perpendicular and parallel sections by polarized light microscopy. Connect. Tissue Res. 52, 512–522.
Momot, K.I., Pope, J.M., Wellard, R.M., 2010. Anisotropy of spin relaxation of water protons in cartilage and tendon. NMR Biomed. 23, 313–324.
Moor, H., Bellin, G., Sandri, C., Akert, K., 1980. The influence of high pressure freezing on mammalian nerve tissue. Cell Tissue Res. 209, 201–216.
Morgelin, M., Paulsson, M., Heinegård, D., Aebi, U., Engel, J., 1995. Evidence of a defined spatial arrangement of hyaluronate in the central filament of cartilage proteoglycan aggregates. Biochem. J. 307 (Pt 2), 595–601.
Nia, H.T., Bozchalooi, I.S., Li, Y., Han, L., Hung, H.H., Frank, E., Youcef-Toumi, K., Ortiz, C., Grodzinsky, A., 2013. High-bandwidth AFM-based rheology reveals that cartilage is most sensitive to high loading rates at early stages of impairment. Biophys. J. 104, 1529–1537.
Nissila, M., Ahlqvist, J., Raunio, P., Isomaki, H., 1977. Morphological findings in joint cartilage after osmic acid treatment. Scand. J. Rheumatol. 6, 231–236.
Okazaki, K., Sandell, L.J., 2004. Extracellular matrix gene regulation. Clin. Orthop. Relat. Res. S123–S128.
Padalkar, M.V., Spencer, R.G., Pleshko, N., 2013. Near infrared spectroscopic evaluation of water in hyaline cartilage. Ann. Biomed. Eng. 41, 2426–2436.
Palade, G.E., 1952. A study of fixation for electron microscopy. J. Exp. Med. 95, 285–298.
Panula, H.E., Hyttinen, M.M., Arokoski, J.P., Langsjo, T.K., Pelttari, A., Kiviranta, I., Helminen, H.J., 1998. Articular cartilage superficial zone collagen birefringence reduced and cartilage thickness increased before surface fibrillation in experimental osteoarthritis. Ann. Rheum. Dis. 57, 237–245.
Patwari, P., Gaschen, V., James, I.E., Berger, E., Blake, S.M., Lark, M.W., Grodzinsky, A.J., Hunziker, E.B., 2004. Ultrastructural quantification of cell death after injurious compression of bovine calf articular cartilage. Osteoarthr. Cartil. 12, 245–252.
Poole, A.R., Pidoux, I., 1989. Immunoelectron microscopic studies of type X collagen in endochondral ossification. J. Cell Biol. 109, 2547–2554.
Poole, A.R., Pidoux, I., Reiner, A., Rosenberg, L., 1982. An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage. J. Cell Biol. 93, 921–937.
Roach, H.I., Erenpreisa, J., Aigner, T., 1995. Osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. J. Cell Biol. 131, 483–494.
Roth, J., Bendayan, M., Carlemalm, E., Villiger, W., Garavito, M., 1981. Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. J. Histochem. Cytochem. 29, 663–671.
Ruggeri, A., Dell'orbo, C., Quacci, D., 1975. Electron microscopic visualization of proteoglycans with Alcian Blue. Histochem. J. 7, 187–197.
Sames, K., 1974. Age related staining pattern of acid mucopolysaccharides in human rib cartilage by alcianblue-acridinorange following different fixation methods (author's transl). Histochemistry 39, 277–287.
Schofield, B.H., Williams, B.R., Doty, S.B., 1975. Alcian Blue staining of cartilage for electron microscopy. Application of the critical electrolyte concentration principle. Histochem. J. 7, 139–149.
Scott, J.E., 1980. Collagen–proteoglycan interactions. Localization of proteoglycans in tendon by electron microscopy. Biochem. J. 187, 887–891.
Scott, J.E., Orford, C.R., 1981. Dermatan sulphate-rich proteoglycan associates with rat tail-tendon collagen at the d band in the gap region. Biochem. J. 197, 213–216.
Shepard, N., Mitchell, N., 1976a. Simultaneous localization of proteoglycan by light and electron microscopy using toluidine blue O. A study of epiphyseal cartilage. J. Histochem. Cytochem. 24, 621–629.
Shepard, N., Mitchell, N., 1976b. The localization of proteoglycan by light and electron microscopy using safranin O. A study of epiphyseal cartilage. J. Ultrastruct. Res. 54, 451–460.
Shepard, N., Mitchell, N., 1977a. The localization of articular cartilage proteoglycan by electron microscopy. Anat. Rec. 187, 463–476.
Shepard, N., Mitchell, N., 1977b. The use of ruthenium and p-phenylenediamine to stain cartilage simultaneously for light and electron microscopy. J. Histochem. Cytochem. 25, 1163–1168.

Studer, D., Michel, M., Wohlwend, M., Hunziker, E.B., Buschmann, M.D., 1995. Vitrification of articular cartilage by high-pressure freezing. J. Microsc. 179, 321–332.
Studer, D., Chiquet, M., Hunziker, E.B., 1996. Evidence for a distinct water-rich layer surrounding collagen fibrils in articular cartilage extracellular matrix. J. Struct. Biol. 117, 81–85.
Szirmai, J.A., 1963. Quantitative approaches in the histochemistry of mucopolysaccharides. J. Histochem. Cytochem. 11, 24–34.
Szirmai, J.A., Doyle, J., 1961. Quantitative histochemical and chemical studies on the composition of cartilage. J. Histochem. Cytochem. 9, 611–615.
Thyberg, J., Lohmander, S., Friberg, U., 1973. Electron microscopic demonstration of proteoglycans in guinea pig epiphyseal cartilage. J. Ultrastruct. Res. 45, 407–427.
Tobias, D., Ziv, I., Maroudas, A., 1992. Human facet cartilage: swelling and some physicochemical characteristics as a function of age. Part 1: Swelling of human facet joint cartilage. Spine (Phila Pa 1976) 17, 694–700.
Urban, J.P., Maroudas, A., Bayliss, M.T., Dillon, J., 1979. Swelling pressures of proteoglycans at the concentrations found in cartilaginous tissues. Biorheology 16, 447–464.
van Turnhout, M.C., Kranenbarg, S., van Leeuwen, J.L., 2010a. Contribution of postnatal collagen reorientation to depth-dependent mechanical properties of articular cartilage. Biomech. Model. Mechanobiol. 10, 269–279.
van Turnhout, M.C., Schipper, H., Engel, B., Buist, W., Kranenbarg, S., van Leeuwen, J.L., 2010b. Postnatal development of collagen structure in ovine articular cartilage. BMC Dev. Biol. 10, 62.
Walker, E.A., Verner, A., Flannery, C.R., Archer, C.W., 2000. Cellular responses of embryonic hyaline cartilage to experimental wounding in vitro. J. Orthop. Res. 18, 25–34.
Wilson, R., Norris, E.L., Brachvogel, B., Angelucci, C., Zivkovic, S., Gordon, L., Bernardo, B.C., Stermann, J., Sekiguchi, K., Gorman, J.J., Bateman, J.F., 2011. Changes in the chondrocyte and extracellular matrix proteome during post-natal mouse cartilage development. Mol. Cell. Proteomics 11 (M111 014159).