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A COMPARISON OF FOUR PREPARATION METHODS FOR THE MORPHOLOGICAL STUDY OF ARTICULAR CARTILAGE FOR SCANNING ELECTRON MICROSCOPY

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Abstract

This study compared 4 fixation methods of rabbit articular cartilage. Tibial plateau samples were prepared either by chemical fixation, with or without microwave enhancement, freezing at ambient pressure followed by freeze-substitution or high-pressure freezing methods. Following the initial preservation, samples were freeze-fractured to expose their internal structure. Microwave enhanced chemical fixation provided preservation of the morphological structure within seconds. Conventional immersion fixation required hours to give comparable preservation. Ambient pressure cryofixation preserved samples in their hydrated stage within seconds and the subsequent freeze-substitution required 10 days. The collagen structure was qualitatively as well preserved as by the other methods, but the chondrocytes showed severe ice crystal damage. The shrinkage of the samples cryofixed at ambient pressure was significantly less than conventional or microwave methods. Cryopreservation at high pressure resulted in matrix preservation that was presumably closest to the *in vivo* state. Collagen fiber structures were difficult to differentiate, which may be due to the presence of frozen hydrated proteoglycans throughout the tissue. Each technique available to preserve cartilage has it's advantages and disadvantages and the methods best suited for the specific research goal must be chosen.

Key Words: Articular cartilage, collagen, scanning electron microscope, fixation.

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Introduction

Scanning electron microscopy (SEM) is an appropriate method for imaging the three-dimensional arrangement of collagen fibers and chondrocytes in intact and pathological articular cartilage. The appearance of cartilaginous tissue, being highly aqueous, is highly influenced by the fixation technique. For example, Cameron *et al*. [7] compared different solutions for dehydration and drying including air-drying, vacuum drying or freeze-drying which showed differences in the quality of fixation. Conventional chemical fixation, with glutaraldehyde and formaldehyde mixtures followed by dehydration and critical-point drying (CPD) with processing times from several hours to weeks is the most widely used method [8, 9, 13]. However, there are also other methods available. Microwave assisted chemical fixation has recently been applied to articular cartilage [37], with the benefits of reduced fixation time and reproducibility in the preservation quality of fine structures.

Cryopreservation at ambient pressure [1, 14, 35] can cause crystallization artifacts in the chondrocytes of the highly hydrated tissue. The technically more demanding cryopreservation at high pressure (HP) [17, 24, 25] can result in vitrification (ice crystal free fixation). These cryomethods are used in an attempt to preserve articular cartilage in a condition as close to its natural state as possible. HP freezing is a powerful method for preservation of the ultrastructure. However, a limiting factor is the sample size where ice crystal formation can still destroy structural relationships in larger samples [15]. Unfortunately, these different fixation and preparation methods involve various degrees of artifact formation. For instance, cartilage surface phenomena such as undulations, pits and humps may be shrinkage artifacts due to dehydration during cartilage processing for SEM [12] but also may be structural characteristics [33].

Currently, SEM is the method of choice to show the three-dimensional structure of articular cartilage. With respect to numerous demanding tasks of cartilage research, such as osteoarthosis research or evaluation of cartilage defects replacement, SEM is rarely used. One reason for this may be the demanding techniques for cartilage fixation

Table 1. Characteristics of the various fixation methods.

Method	*Max. sample size used*	*Duration of fixation until dehydration*	*Collagen structure*	*Ultrastructure chondrocytes*	*Characteristic advantages (A) & disadvantages (D)*
Conventional	12x7x3 mm^3	4 hr (9 hr)	well preserved, Fig. 2a,b	slight shrinkage Fig. 2b	A: standard method, sample size relatively large D: duration, shrinkage, artifacts from chemicals
Microwave enhanced to 40°	12x7x3 mm^3	1 min (1 hr) Fig. 3a	well preserved Fig. 3b	slight shrinkage	A: rapid fixation D: sample size limited, shrinkage,artifacts from chemicals
Plunge freezing at ambient pressure / freeze-substitution	12x7x3 mm^3 and whole rabbit knee joints	30 sec (8 days)	well preserved Fig. 4a,b	ice crystal damage Fig. 4b	A: relatively large sample size possible, immediate fixation (e.g., under loading conditions), low shrinkage D: severe ice crystal artifacts in cells, time consuming
High pressure freezing / Cryo-SEM	< 1 mm^3	5 sec	well preserved Fig. 5a	well preserved Fig. 5a,b	A: fixation closest to in vivo state D: sample size very small, technically demanding, expensive, masking effect of proteoglycans

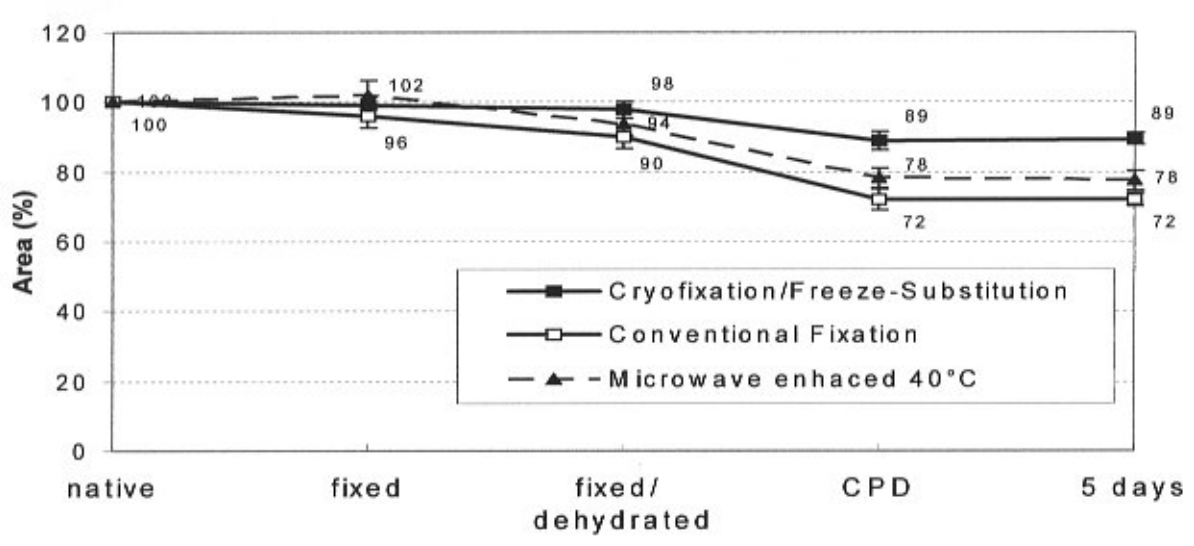

Figure 1. Shrinkage of the samples fixed with three different methods. Relative dimension changes are shown in area %. Repeated measurements were made, of two dimensional changes in size which occurred during the different stages of sample preparation, using a stereomicroscope and digitizing tablet. CPD: critical point drying.

and preparation for SEM. The intention of this paper is to review our experience with different fixation techniques and to describe and discuss the advantages and disadvantages of four different fixation methods [27, 35, 37].

Material and Methods

Sample preparation

Full thickness samples of articular cartilage were obtained from tibial plateaux of adult rabbits immediately after sacrifice. Vertical knife cuts were used to remove squares of the cartilage (up to 4x4x3 mm^3, small samples), which included the overlying articular cartilage and a layer of underlying bone. During preparation the cartilage was carefully prevented from drying by the use of pH 7.2 buffered Ringer's solution [6]. To assess the fixation quality in larger samples, rabbit tibial plateau halves (12x7x3 mm^3, except for freezing at high pressure) were also used. For each method analyzed a minimum of 10 samples were used. Shrinkage of samples was measured during fixation [27] (except for freezing at high pressure). Briefly, repeated measurements were made of two dimensional changes in size which occurred during the different stages of sample preservation and were compared to the initial measurement of the native, unfixed sample. Measurements were performed using a stereomicroscope (Wild, Heerbrugg, Switzerland) and a digitising tablet (Kontron Electronics, Munich, Germany).

Conventional fixation

Conventional aqueous fixation was carried out at 20°C. Samples were rinsed for 10 minutes in 0.1 mol l^{-1} piperazine-NN´-bis-2-ethane sulphonic acid (PIPES) buffer pH 7.4. Primary fixation was in 2.5% glutaraldehyde with 4% paraformaldehyde in PIPES pH 7.4 for 4 hours. Samples were rinsed two times for 10 minutes each in PIPES pH 7.4 before postfixation in 0.2% osmium tetroxide in PIPES pH 6.8 for 60 minutes. They were then rinsed twice for 10 minutes each in 0.1 mol l^{-1} PIPES pH 6.8 and stained with 2% aqueous uranyl acetate for 60 minutes. Each fixed sample was dehydrated using graded ethanol solutions of 50%, 60%, 70%, 80%, 90%, 100%, 100% for 15 minutes each.

Microwave enhanced fixation

Samples were rinsed as described above and then fixed with an optimized method in the stated fixing solutions in a conventional microwave oven (Miele M696, Gütersloh, Germany) [37]. Microwave irradiation was cyclic (450W). An automatic temperature probe was placed in a fixation vessel which had 5 ml of solution in it. When the desired temperature of 40°C was attained, microwave irradiation was stopped. The sample was immediately placed in buffer at 4°C with one change for a total time of 20 minutes. Primary fixation was in 2.5% glutaraldehyde with 4% paraformaldehyde in 0.1 mol l^{-1} PIPES pH 7.4. Postfixation was in 0.2% osmium tetroxide in 0.1 mol l^{-1} PIPES pH 6.8 and staining in 2% uranyl acetate. Dehydration was performed as described with the conventional technique.

Cryofixation at ambient pressure

Samples were frozen by immersion, at ambient pressure for 60 seconds, in isopentane slush pre-cooled with liquid nitrogen (LN_2) to below -160°C [35]. Samples were then removed and fixed by freeze-substitution. The initial fixative was a solution of 10% acrolein and 0.2% tannic acid in a mixture of 30% methanol and 59% acetone maintained at -80°C for 7 days. Specimens were rinsed in acetone twice at -80°C for 20 minutes each and then transferred into a second fixation solution of 5% glutaraldehyde (from a 50% aqueous solution) in a mixture of 10% methanol and 85% acetone at -23°C for four days. The temperature was then slowly increased to 4°C and samples remained at that temperature for 24 hours. The samples were then washed with 100% ethanol and slowly brought to room temperature. The samples were rinsed again twice each time for 30 minutes in 100% ethanol prior to secondary fixation in 1% osmium tetroxide for 120 minutes at 4°C.

High-pressure freezing

Tibial plateau articular cartilage (15 samples) were prepared into approximately 500 mm^3 sized pieces. Samples were then placed in the cavity (diameter 2 mm, depth 0.2 mm) of a cylindrical aluminum plate and sandwiched with a second platelet with no cavity, that matched the specimen thickness as close as possible. The space between the sample and the plates was filled with liquid 1-hexadecane. This method was performed according to Walther *et al.* [46, 47]. A commercial high pressure freezer HPM 010 (Balzers Union, Balzers, Liechtenstein) was used. The pressure of 2500 bar was reached within 15 msec. The samples were transferred into LN_2 immediately after freezing. The aluminum sandwiches were opened under LN_2. The samples were then fractured in a freeze etching device (BAF 300, Balzers at a vacuum of 1 x 10^{-7} mbar and a temperature of -150°C for 2 minutes. Immediately after fracturing, the samples were coated by electron beam evaporation with 1.5-3 nm of platinum-carbon (unidirectional at an angle of 45°) and with 5-7 nm of carbon at an angle of 80°. During carbon evaporation, the samples were rotated in order to obtain a more uniform coat. During cryo-transfer to the SEM, the specimen was shielded by the shutter of the Gatan cryo-holder (Munich, Germany). A Hitachi S-900 in-lens field-emission SEM (Hitachi Ltd., Tokyo, Japan) was used. The cryo-holder was inserted into the microscope and the specimen chamber was pumped for about 10 minutes to remove water vapor. Specimens were investigated at -160°C. The primary accelerating voltage was 30 kV for high magnification and 5-16 kV for intermediate magnifications.

Further processing (not for high pressure frozen samples)

After dehydration, freeze-fracturing was performed on all specimens containing 100% ethanol, after cooling in liquid nitrogen for a few seconds. The cartilage was fractured with a broad sharp chisel from the subchondral bone perpendicular to the surface [22]. The samples from each fixation group were dried with a Polaron E3000 critical point drier (Agar Scientific Ltd., Stansted, UK), using CO_2 as a transitional fluid. The specimens were mounted onto stubs and coated with 8 nm of gold in a Baltec MED 020 unit (Baltec AG, Balzers, Liechtenstein) and examined with a Hitachi S-4100 field emission SEM (Hitachi Ltd., Tokyo, Japan). The images were collected in their original digital form with Quartz PCI image management system (Quartz imaging, corporation, Vancouver, Canada). The SEM was operated in secondary electron detection mode at an accelerating voltage of 1-2 kV and an emission current of 10 mA.

Results

The characteristics of the different fixation techniques are summarized in Table 1. There were significant differences in time required to fix the samples. Conventional aldehyde immersion fixation required a few hours to provide good fixation results.

Cryofixation at ambient pressure, followed by freeze-substitution, preserved cartilage samples in their hydrated stage within a few seconds. The subsequent freeze-substitution required a total of 11 days. There was a significant difference in the shrinkage behavior of articular cartilage of samples prepared by cryofixation followed by freeze-substitution and that of samples fixed by conventional or microwave techniques (Fig. 1). The average conventional fixed material, after all the steps of preservation, retained 72% of its initial size, whereas microwave enhanced fixation samples retained 78% and the cryopreserved sample 89%.

The cryofractures of all specimens that had been fixed either conventionally, with microwave-enhancement or cryopreservation, followed the natural cleavage patterns of the tissue and demonstrated relationships between the structures. In all cryofractures, there was a mixture of fractured and unfractured chondrocytes and also lacunae when the chondrocytes remained in the opposite face of the fracture. Formation of clefts was visible in all fixation methods except high pressure freezing, presumably due to shrinkage.

The collagen fiber structure of conventionally fixed samples (Figs. 2a,b) showed no difference in comparison to cryopreserved (Figs. 4a,b) or microwave fixed (Figs. 3a,b) samples. All three methods showed parallel collagen fibers, running perpendicular from the calcified cartilage through the radial zone towards the surface. In the intermediate (transitional) zone they became bent over in order to build the tangential zone at the surface (Fig. 2a).

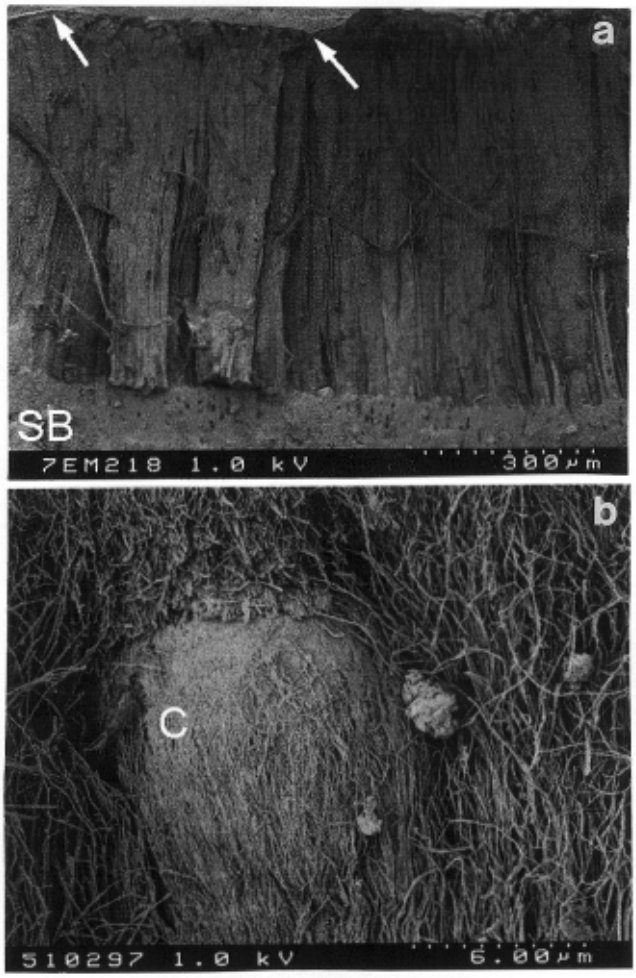

Figure 2. Conventional fixation (small sample). (**a**) Fracture through rabbit tibial plateau articular cartilage. The collagen fiber bundles extend vertically from the subchondral bone (SB) to the surface (arrows). (**b**) Fracture beside a chondrocyte (C). Intact chondrocyte extracellular matrix and more parallel collagen organization.

The morphologic quality of the structure of the large samples was comparable to that of the small samples for all fixation methods. Higher magnification also revealed obliquely oriented fibers in addition to the parallel running collagen fibers, which might be displaced due to fracturing. The high pressure frozen samples revealed an organized matrix with chondrocytes embedded in a fibrous matrix. There were no clefts detectable in the cartilage. The matrix of the vitrified samples consisted of fibers, presumably collagen fibers, which were oriented in parallel (Fig. 5). The fibrous structure could be traced only over a relatively short distance. While the collagen structure was pre-

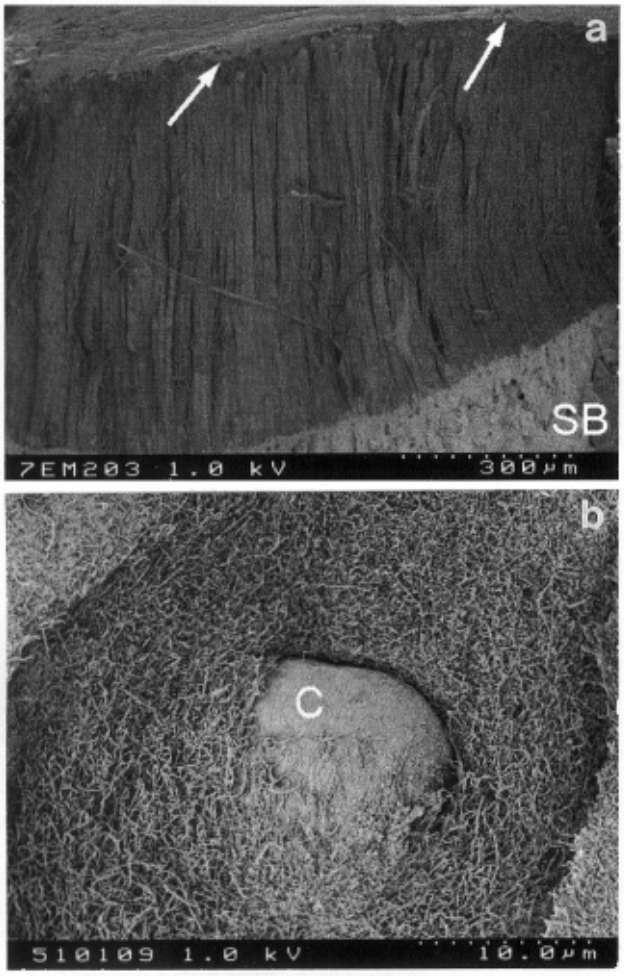

Figure 3. Microwave enhanced fixation (40°, small sample). (**a**) Parallel collagen fiber bundles arising from the subchondral bone (SB) to the cartilage surface (arrows). (**b**) Chondrocyte in the collagen matrix.

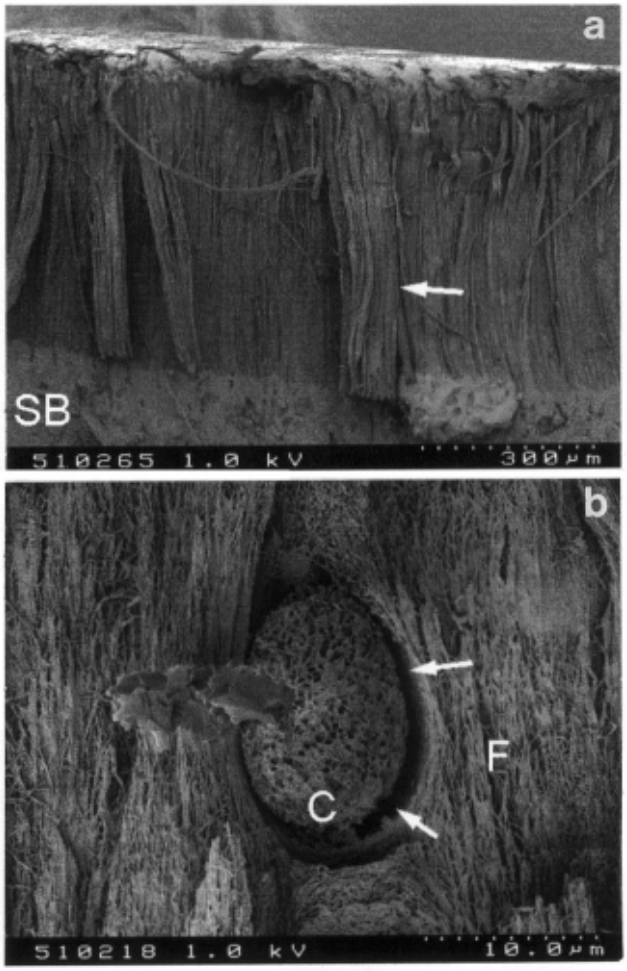

Figure 4. Plunge-freezing at ambient pressure, followed by freeze-substitution (large sample). (**a**) Fractured surface. Parallel running collagen fiber bundles (arrows) from the subchondral bone (SB). (**b**) The ultrastructure of the chondrocyte (C) is destroyed and shows a sponge pattern. The collagenous lacunae is maintained (arrows). The collagen fibers run parallel beside the chondrocyte.

served more or less identically by the first three fixation methods, different aspects of the chondrocyte structure were observed at higher magnification, depending on the fixation technique. In many conventionally fixed samples chondrocytes showed shrinkage from the walls of the lacunae. Those fixed by the cryotechnique (freezing at ambient pressure) showed a characteristic spongy pattern (segregation artifacts), presumably due to ice crystal damage in all sample sizes (Fig. 4b). Nonetheless, the contours of the cryofixed chondrocyte lacunae were maintained in the same condition as in the other fixation techniques; specifically the pattern of the surrounding collagen matrix fibers was intact and comparable to conventional and microwave fixed tissue (Fig. 4b). In the high pressure frozen samples, chondrocytes were identified without segregation artifacts, embedded in the collagen matrix (Fig. 5a). They were surrounded by the pericellular matrix and an interterritorial matrix, which appeared to have a less fibrous structure. There were no signs of shrinkage such as a gap between the chondrocytes and the pericellular matrix.

Discussion

The intention of this paper was to review our experience with different fixation techniques of articular cartilage and to present the strengths and weakness of four different fixation methods. The preparation of biological tissue for SEM imaging requires specific preparation and fixation techniques. Artifacts can be introduced into the cartilage tissue during different stages of preservation.

In this study only fresh samples were used, which were

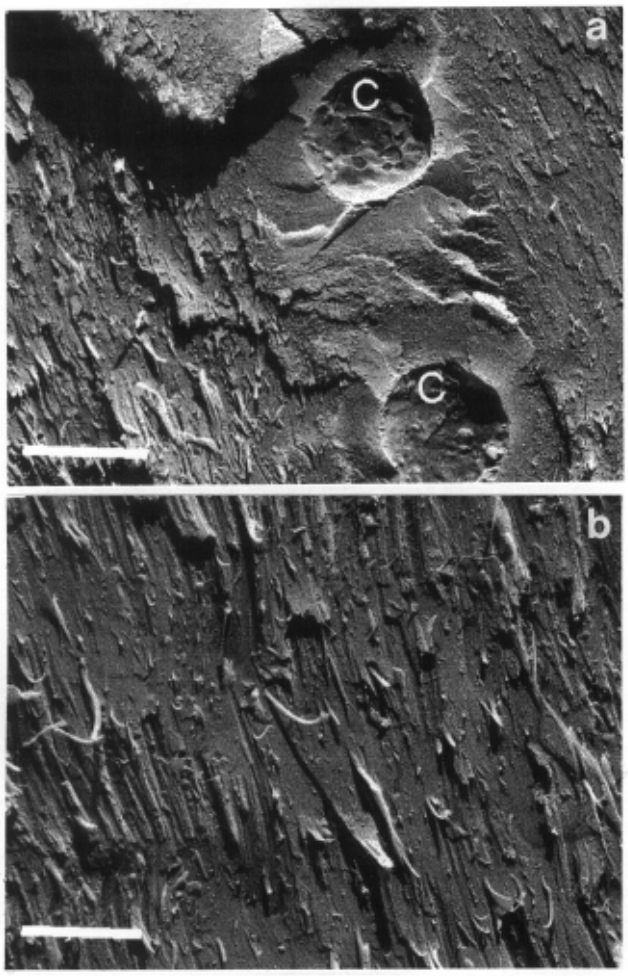

Figure 5. Cryo-SEM of a high-pressure frozen, then freeze etched sample. (**a**) Fibrous matrix structure with well preserved chondrocytes (C) are visible. Scale bar 10µm. (**b**) Fibrillar structure probably due to parallel running collagen fiber bundles in the radial zone. The collagen fibrils are probably masked by vitrified water and proteoglycans. Scale bar 1 µm.

taken from the animal within a few minutes after death. The use of frozen samples for structural studies is not recommended due to the possible damage to the ultrastructure due to freeze-thawing [42]. The mechanical properties of cartilage will be affected if the tissue has been frozen for storage and thawed for testing [3, 44]. The samples were always kept wet with buffered solution during preparation, since the drying of the surface might introduce drying artifacts such as specimen shrinkage [28, 32]. Ringer's solution can safely be used as an irrigation solution [6, 48]. The subchondral bone was left attached to the cartilage, because removing the subchondral bone leads to significant artifacts e.g. at the cartilage surface [10, 11, 18].

The production of surfaces by freeze-fracturing is an important step in the preparation of cartilage. Due to cooling down the sample to -196°C and fracturing in this condition, the internal structure of the collagen fibers can be exposed [8, 22]. Additionally, it was confirmed that the freeze-fracturing is an important step in exposing the internal arrangement of the collagen fibers and is best performed prior to drying, with the specimen immersed in 100% ethanol. The generation of ice crystals [41] during freezing prior to fracturing can be excluded since the water has already been extracted by the previous dehydration. However, additional to the parallel oriented collagen fibers, other fibers were also found running at oblique angles between the parallel fibers, and these might have been displaced during freeze-fracturing [8] or might represent fibril cross-links [36]. The general arrangement of the parallel running collagen fibers was not affected by these oblique fibers.

Microwave enhanced fixation showed similar good results when compared to conventional fixation. The results obtained with both microwave and conventional fixed samples were comparable to those published in other SEM studies [8, 9, 26, 37]. A rapid, reproducible good fixation for articular cartilage is enabled by the application of microwave irradiation to the solutions. Cryofixation also gave a rapid fixation of the samples. The time of fixation should be minimal to prevent extraction and shifting of diffusible ions and molecular components. The duration of the microwave fixation or plunge-freezing was less than 1 minute. This is much faster than any conventional immersion method used [8, 10, 13, 26].

Conventional and microwave enhanced fixation is based on the use of aldehydes. Glutaraldehyde fixes biological tissue by crosslinking [19, 20]. Since the effect of this aldehyde stabilization is almost exclusively limited to proteins, the postfixation by osmium tetroxide is useful [19]. This results in further staining as well.

Immersion-cryofixation at ambient pressure, followed by freeze-substitution, was used to preserve articular cartilage. It was shown to be a simple and effective method for immediate preservation also of large samples. The protocol tested was based on a previously used protocol [35], on recommendations for cryofixation and on numerous trials with fine tuning of the method. For cryofixation at ambient pressure isopentane precooled in LN_2 gives relatively rapid cooling rates [38]. Freeze-substitution for dissolving the ice from a frozen specimen is best performed between -80°C down to -90°C [2, 34, 40, 45], followed by slowly increasing the temperature. A mixture of organic liquids such as acetone and methanol seems to be best to substitute water at these temperatures [21, 40].

Additional crosslinking can be achieved by adding acrolein and glutaraldehyde to the solutions [4, 5, 40, 49]. Furthermore, the addition of tannic acid can improve the

preservation of surfaces by structural reinforcement of the collagen fibrils [30].

Morphological examination by SEM of cryopreserved samples revealed differences when compared to conventional or microwave fixed specimens. Tissue processing in a near-native condition using low temperatures can only be achieved if the water is frozen in its liquid state (true vitrification), as was done here with high pressure freezing. When ice crystals begin to form (as will occur when freezing at ambient pressure) tissue components become segregated into phases of pure water and of concentrated biological residues with consequent formation of segregation artifacts [25, 41]. However, the morphology of the fibrous lacunae of the chondrocytes was maintained. The morphological appearance of collagen structure obtained in cryopreseved cartilage corresponds well to the morphological findings of other authors [8, 9, 13, 26, 31, 39, 43]. This is probably due to the water binding capacity of the collagen fibers. The conventionally and microwave fixed samples did not show the characteristic segregation artifacts. The samples treated by high pressure freezing did not show any ice crystal damage when imaged with the SEM and were therefore superior for the ultrastructural preservation of chondrocytes when compared to those frozen at ambient pressure where ice crystal damage was evident. For studying the ultrastructure of chondrocytes and proteoglycans high-pressure freezing is a powerful method, as described previously [16, 23, 25]. However, here the sample size is a strictly limiting factor with these methods. In the present study the tissue matrix of the high pressure frozen and freeze-etched samples was similar to that seen with all other fixation methods. It consisted of a fibrous, parallel oriented structure. However it was not possible to trace single fibers over the same long distances as was done with the other fixed samples, which may be due to the retention of hydrated proteoglycans which mask the collagen fibers. This might also be due to the fact that the fracture does not follow the natural plane of the fibers. This can be probably explained by the presence of water in the vitrified tissue, which is not replaced.

There are various techniques available to preserve articular cartilage and each of the methods has advantages and disadvantages. The internal structure of articular cartilage was preserved in a condition close to its *in vivo* state, as judged by the high pressure freezing and Cryo-SEM results, by a number of the other fixation protocols. Conventional and microwave fixation allowed the study of cells and the collagen structure, though absolute measurements of structure size can not be made with these methods due to the amount of shrinkage that occurs. High pressure freezing also allowed the study of cells and, to a lesser degree, the collagen – which was limited probably due to the masking effect of hydrated proteoglycans. In a steady state situation, the low temperature technique with freezing at ambient pressure is suitable for the preservation of cartilage within seconds, with the possibility of having the cartilage under conditions of mechanical load. The freezing at ambient pressure approach allows for good collagen fiber preservation, but a very poor preservation of the chondrocyte ultrastructure.

The study of the three-dimensional structure of articular cartilage can provide useful information in various fields of articular cartilage research. For the preservation of the cartilage, a fixation method has to be chosen with respect to the specific research question being asked. For an understanding of the complete morphology of such tissues a compendium of results obtained by the application of a variety of different preparation techniques will be required.

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Discussion with Reviewers

C. Archer: To what extent does this study provide new information compared with that of Richards and Kääb [36]?
Authors: There was no high pressure freezing in this paper , the cryofixation at ambient pressure has been made more optimal for preparation ease, the best of the microwave enhanced fixation methods from the above paper was used and a longer conventional fixation.

J. Wroblewski: The paper would benefit from additional information about the different components of the extracellular matrix of cartilage and how these molecules behave during different steps of chemical or physical fixation.
Authors: We would like to refer to reference [27], as this area was discussed in depth there.

J. Wroblewski: What I miss in the present investigation, is a comparison of morphology after freezing at normal pressure and at high pressure and subsequent freeze substitution.
L. Edelmann: The high pressure frozen material should also be freeze substituted and freeze fractured afterwards in order to demonstrate more clearly the differences obtained with the different cryofixation techniques.
Authors: High pressure freezing followed by freeze substitution was not performed since we had access to using the high pressure method and viewing the samples frozen in Martin Müller's laboratory (probably the best for this technique). Why, possibly add more artefacts with the extra step of freeze substitution when the samples can be viewed using the original high pressure freezing method without this extra set of steps on a cooled stage.