Modified Periodic Acid-Schiff (PAS) Staining of Bone-Cartilage Interfaces

(Yi Zhu and Yonghui Wang @ 02/14/2024; commented by TCH)

REAGENTS:

1) Periodic Acid (1g/dL) (***Sigma-Aldrich, cat. no. 3951-100 ML***)
2) Schiff's Reagent (Pararosaniline HCl, 1%, and sodium metabisulfite, 4%, in hydrochloric acid, 0.25 mol/L) (***Sigma-Aldrich, cat. no. 3952-50 ML***)
3) Weigerts' Iron Hematoxylin, including Solutions A and B. (***Sigma-Aldrich, cat no. 1159730002***)
4) Light green solution (1%) (***Sigma-Aldrich, cat. no. L1886***)

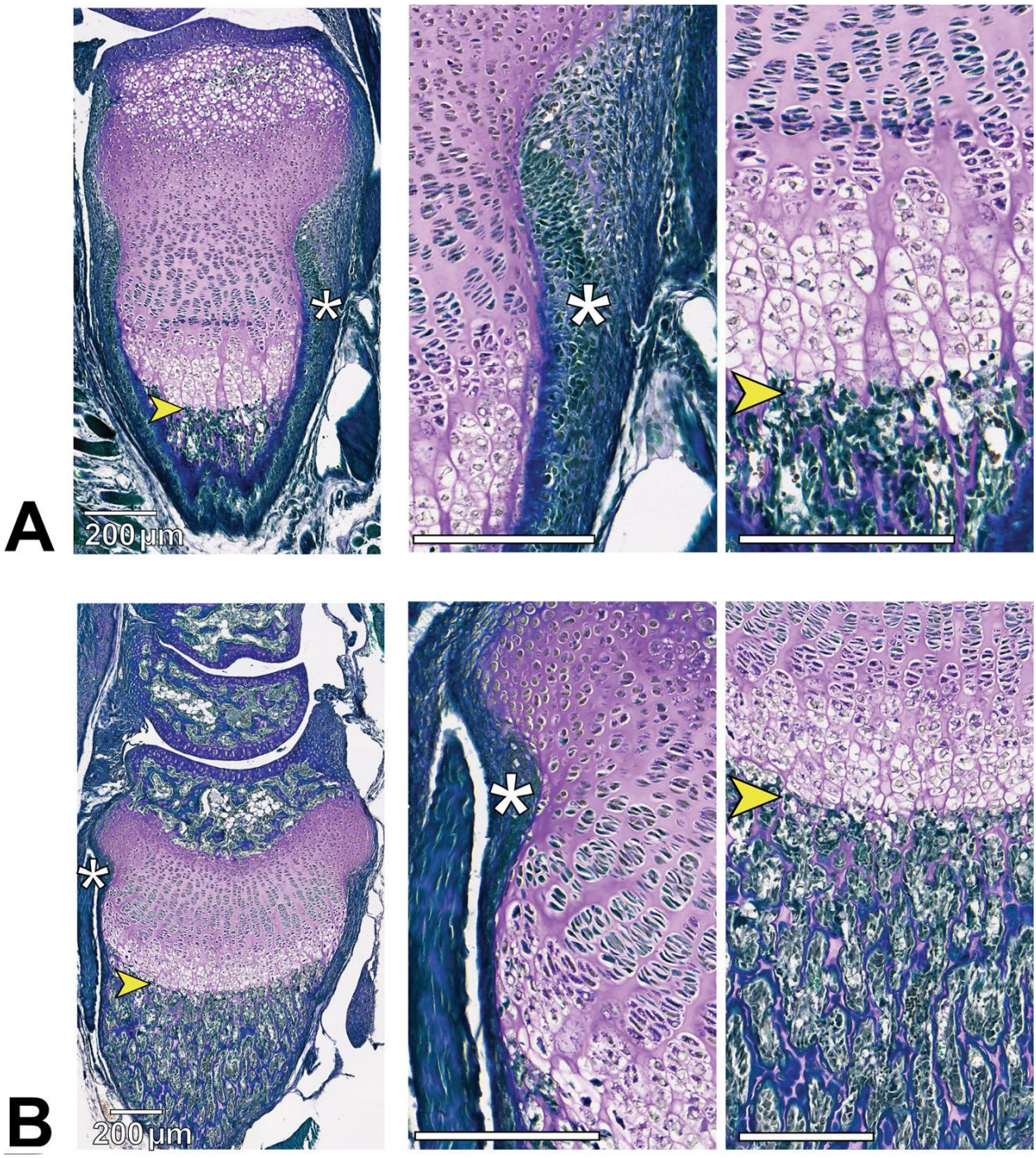
STAINING PROCEDURE:

1) Deparaffinize and hydrate sections. (Refer to **H & E Protocol**)
2) Stain with Periodic Acid for 5 mins.
3) Rinse in distilled water for several times.
4) Stain with Schiff's Reagent for 10 mins.
5) Wash with running tap water for 5 mins, and rinse in distilled water.
6) Mix Weigerts' Iron Hematoxylin Solution A and B (equal parts) when washing with tap water. Normally, 4ml (2ml A+ 2ml B) is sufficient for 8 slides.
7) Stain with mixed Weigerts' Iron Hematoxylin for 5 mins.
8) Wash with distilled water for 2 mins * 2.
9) "Blued" in 1X PBS for 20s, and briefly rinsed in distilled water.
10) Stain with 1% light green solution for 30s-2mins. Normally 30s is enough. Don't leave too long, otherwise you will get a total green section.
11) Rinse in distilled water for 2 mins *2.
12) Dehydrate, clear in xylene, add Permount, and cover slips. (Refer to **H & E Protocol**)

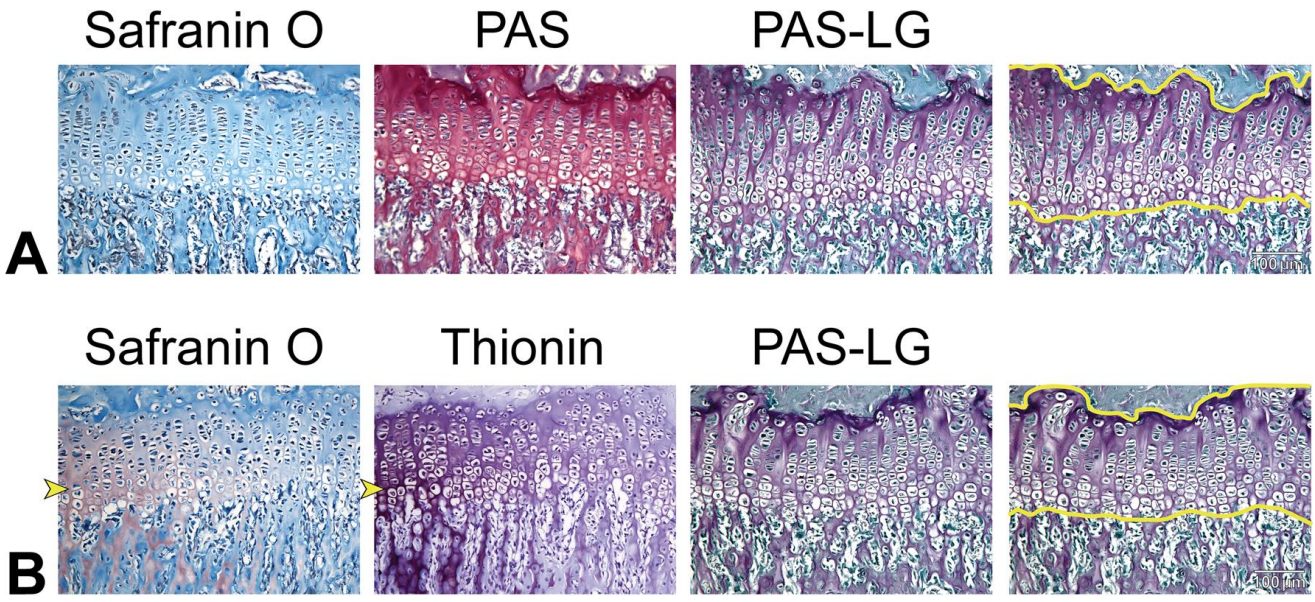
EXPECTED RESULT: **Cartilage** = Purple; **Surrounding bone and soft tissue** = blue-green

REFERENCE

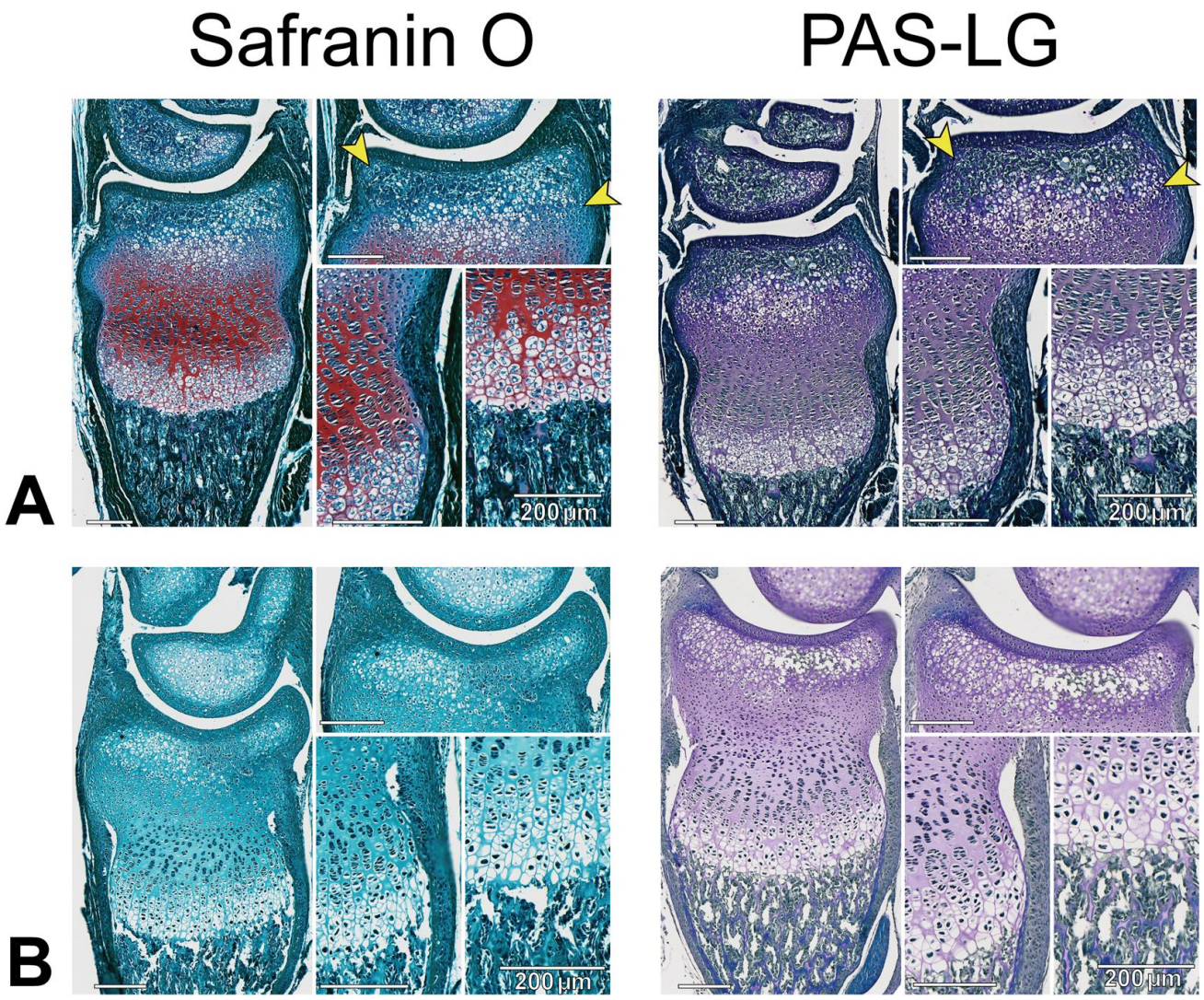
Kelsey M Kjosness, Philip L Reno, Maria A Serrat: Modified Periodic Acid-Schiff (PAS) Is an Alternative to Safranin O for Discriminating Bone-Cartilage Interfaces. ***JBMR Plus*** *. 2023 Apr 21;7(6):e10742. doi: 10.1002/jbm4.10742. eCollection 2023 Jun PMID:* ***37283654*** *PMCID: PMC10241084 DOI: 10.1002/jbm4.10742*



Modified periodic acid-Schiff (PAS) staining of distal radial growth plates from (A) 9-day-old and (B) 15-day-old mice stained as part of our protocol validation. Articular cartilage of the radiocarpal joint (distal) is at the top in all images. Magnified panels show demarcation of cartilage at the perichondrium (asterisks) and metaphyseal chondro-osseous junction (yellow arrowheads). Calcified cartilage at the metaphyseal chondro-osseous junction is also stained purple. The secondary center of ossification is present in the 15-day-old mouse in (B), further delineating the growth plate between epiphyseal (top) and metaphyseal (bottom) bone. Scale bar = 200 µm for all images.



Comparison of the modified periodic acid-Schiff (PAS) method with other cartilage stains on serial sections of proximal tibial growth plates from two different 6-week-old mice. (A) Safranin O (left) did not stain the growth plate, making the bone cartilage interface indistinct. Standard PAS staining (middle) did stain cartilage, but the color was difficult to discriminate from the surrounding bone because all tissues stained reddish-purple. The modified PAS protocol using Weigert's iron hematoxylin and light green (PAS-LG, right) stained cartilage purple and surrounding bone and soft tissue a blue-green color. This enabled multiple users to consistently trace the boundaries of the cartilage (far right) in a way that was not possible in the failed safranin O image. (B) In a different mouse, the tibial section was weakly stained with safranin O toward the lateral edge of the growth plate as indicated by the yellow arrowhead (left), whereas safranin O was absent toward the inner edge of the growth plate. Serial sections stained with thionin (middle) match this pattern of metachromasia, suggesting an uneven loss of proteoglycans across the tissue (more proteoglycans retained toward the lateral edge marked by the arrowheads). The PAS-LG section shows a clear bone-cartilage distinction across the entire image (right) and reliable demarcation of the growth plate boundary for data collection (far right). Scale bar = 100 µm for all images.



Comparison of safranin O and periodic acid-Schiff protocol using Weigert's iron hematoxylin and light green (PAS-LG) staining in distal radial growth plates of two different 9-day-old mice. Orientation and morphology match that described in Fig. 1. (A) Although safranin O did render the expected red staining of cartilage, the stain did not extend to boundaries of the perichondrium or epiphyseal cartilage nearest the articular surface (yellow arrowheads). The same cartilaginous areas did stain purple using the modified PAS protocol as shown by the yellow arrowheads on the right. A different mouse (B) did not stain red with safranin O, but all cartilage did stain purple with a blue-green bone interface using the modified PAS protocol. Magnified panels demonstrate articular cartilage, growth plate, perichondrium, and developing secondary center of ossification as in Fig. 1. Scale bar = 200 µm in all images.

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Modified Periodic Acid-Schiff (PAS) Is an Alternative to Safranin O for Discriminating Bone–Cartilage Interfaces

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ABSTRACT

Cartilage histomorphometry is often performed on decalcified, paraffin-embedded bone sections, which provide versatility in staining applications from basic morphology to immunohistochemistry. Safranin O is a cationic dye that binds to proteoglycans in cartilage and is routinely used to assess growth plate dynamics and/or fracture repair at bone–cartilage interfaces. When used with a counterstain such as fast green, safranin O can offer exquisite differentiation of cartilage from surrounding bone. However, various decalcification and processing methods can deplete proteoglycans, rendering inconsistent, weak, or absent safranin O staining with indiscriminate bone–cartilage boundaries. We sought to develop an alternative staining methodology that preserves the contrast of bone and cartilage in cases of proteoglycan depletion that can be applied when other cartilage stains are unsuccessful. Here, we describe and validate a modified periodic acid-Schiff (PAS) protocol that we developed using Weigert's iron hematoxylin and light green stains as an alternative to safranin O for discriminating bone–cartilage interfaces of skeletal tissues. This method provides a practical solution for differentiating bone and cartilage when safranin O staining is not detected after decalcification and paraffin processing. The modified PAS protocol can be useful for studies in which identification of the bone–cartilage interface is essential but may not be preserved with standard staining approaches. © 2023 The Authors. *JBMR Plus* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research.

KEY WORDS: BONE AND CARTILAGE; GROWTH PLATE; HISTOLOGY; HISTOMORPHOMETRY; STAINING METHODS

Introduction

Safranin O is frequently used with fast green counterstain to visualize cartilage proteoglycans[1,2] because it can produce clearly demarcated bone–cartilage interfaces (Fig. 1).[3] Safranin O is a cationic dye that binds stoichiometrically to the anionic glycosaminoglycan component of proteoglycans in solution.[4] However, this stoichiometric relationship (color intensity of stain proportional to proteoglycan concentration) is not always maintained in histological sections because of glycosaminoglycan loss.[5,6] Weak or absent safranin O staining can result from glycosaminoglycan depletion, even when proteoglycans are still detectable in the cartilage matrix with antibodies, suggesting that safranin O may not be a sensitive indicator of proteoglycan content at low levels.[6,7]

Proteoglycans and/or their glycosaminoglycan component can be lost from cartilaginous tissues in multiple ways. Proteoglycans are soluble and prone to leaching during submersion in fixatives, buffers, and decalcifying solutions. Many studies have reported strong safranin O staining regardless of fixation, decalcification, or sectioning procedures.[8,9] However, others have experienced weak or absent safranin O staining, presumably attributed to depletion of proteoglycans and/or their glycosaminoglycan component during tissue processing.[10–13] For example, glycosaminoglycan loss was evident after fixation in 4% formaldehyde and decalcification with 10% EDTA, with most of the loss having occurred during the decalcification step.[12] Other studies have shown that safranin O staining intensity varies with decalcification solution and/or temperature,[14,15] as well as fixation time,[13] suggesting that glycosaminoglycans can progressively leach from samples. Even short exposure to isotonic buffers can extract proteoglycans from tissues. In fact, most of this leaching, ranging from 10% to 30% of total proteoglycan content, can occur within the first 30 minutes of rinsing time.[16,17]

While performing routine processing and staining of juvenile mouse bones, we detected inconsistencies in safranin O intensity in some of our specimens. Although safranin O staining typically renders a clear demarcation of the cartilage–bone interface

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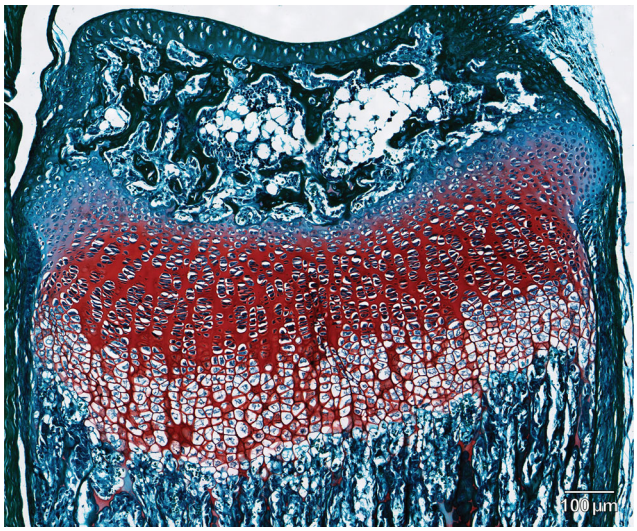

Fig. 1. Successful safranin O staining in a 17-day-old mouse distal radial growth plate illustrating distinction of cartilage (red) from surrounding bone and soft tissue (green). Scale bar = 100 µm.

when counterstained with fast green (Fig. 1), we found that some specimens exhibited unexpectedly weak or absent safranin O stain and it was not always possible to predict when this failure of safranin O stain would occur. These findings were problematic for morphometric analyses of the growth plate because we relied on the metachromatic color of safranin O to consistently discriminate the cartilage boundary, and many of our samples were irreplaceable. Although we followed the same protocols, our literature review revealed that minor differences in pH between batches of reagents for fixation, decalcification, and washes, as well as differences in submersion time to accommodate different-sized samples and/or time between fixation and processing, could underlie the weak or absent safranin O staining that we observed. For example, there was a delay in processing some of our samples because of equipment failure and several batches of 4- to 6-week-old decalcified mouse knees were kept in 70% ethanol for approximately 6 weeks. When the samples were processed, most exhibited weak to absent safranin O staining despite otherwise following all the same protocols.

Metachromatic staining methods, such as safranin O, create a color change in certain tissues because the dye forms high-affinity salt linkages with polyanions, such as the chondroitin sulfate groups in cartilage.[6] For example, toluidine blue also has a high affinity for sulfate groups in proteoglycans and turns cartilage a deep purplish-blue color in contrast to the light blue of surrounding noncartilaginous tissues.[18] Such metachromatic approaches may therefore be unreliable when polyanions leach out during fixation and processing protocols.[18] Results of diminished safranin O staining are seldom published when not a direct result of decreased proteoglycan content due to genetic mutation or disease state. We are aware, however, of other colleagues who have experienced this problem through conversation and requests for help troubleshooting when standard safranin O methods failed to stain their tissues as expected. To address this problem, we sought to develop an alternative staining methodology that preserves the metachromatic contrast of bone and cartilage even in cases of proteoglycan depletion.

Periodic acid-Schiff (PAS) is a carbohydrate-specific staining reaction used to demonstrate glycogen and other polysaccharides,[6,19] such as the mucopolysaccharides in cartilage.[2] This two-step protocol involves exposure to periodic acid, which oxidizes glycol groups to aldehydes, followed by Schiff's reagent, which turns the aldehydes a rose-violet color.[2,6,19] Although the glycosaminoglycan component of proteoglycans does react with PAS after prolonged incubation, PAS preferentially targets glycol groups with a neutral charge, making this reaction more sensitive than safranin O for staining cartilage matrix.[8,20] Here we describe a simple and novel modification of a standard PAS protocol using Weigert's iron hematoxylin and light green stains that produces a contrast between bone (blue-green) and cartilage (purple). This technique provides a clear distinction of the bone–cartilage interface even when safranin O staining does not.

Materials and Methods

All animal procedures were approved by and followed all ethical guidelines of the Institutional Animal Care and Use Committees at Northeast Ohio Medical University (formerly Northeastern Ohio Universities College of Medicine, protocol 05-008) and Pennsylvania State University (protocol 44735-1). A total of 87 juvenile mice were used in this study. Knees were harvested from 37 male CF-1 outbred mice (Charles River Laboratories, Wilmington, MA, USA) between 4 and 6 weeks old as part of another investigation,[21] and their tibial growth plate sections were used to develop the PAS protocol described here. In a separate laboratory, forelimbs were harvested from 50 C57BL/6 and FVB/NJ juvenile mice (The Jackson Laboratory, Bar Harbor, ME, USA) between 9 and 40 days old as part of other investigations. Their distal radial growth plate sections were used for subsequent protocol validation.

Bones for protocol development were fixed in 10% neutral buffered formalin for 24 hours and decalcified in 10% EDTA for 4 weeks, rinsed, and processed following our published methods.[22] Because of unexpected equipment failure, decalcified and rinsed samples were placed in 70% ethanol for approximately 6 weeks before processing. Bones used in protocol validation were fixed in 4% paraformaldehyde in 1× phosphate buffered saline (PBS) for 24 to 48 hours and decalcified in 10% EDTA for 1 to 4 weeks (based on tissue size and specimen age).[23] Samples for both protocol development and validation were then rinsed in PBS, dehydrated in a graded series of ethanol, cleared in Histoclear II (National Diagnostics, Atlanta, GA, USA), CitriSolv (Thermo Fisher Scientific, Waltham, MA, USA), or xylene, embedded in Paraplast Plus paraffin, and cut into 6 to 8 µm sections using a rotary microtome. Because of weak or absent safranin O staining in growth plate cartilage, sections were stained using a modified PAS protocol that we developed to provide sufficient contrast for visualizing the cartilage–bone interface as detailed below. Additional sections were stained using a standard PAS protocol following manufacturer instructions for a commercially available kit (Sigma-Aldrich, St. Louis, MO, USA; Periodic Acid-Schiff Kit, cat. no. 395B) and with 0.1% thionin to visualize cartilage histology.[19] Safranin O staining followed previously published methods.[23]

Modified PAS staining was performed at room temperature using a commercially available kit (Sigma-Aldrich, Periodic Acid-Schiff Kit, cat. no. 395B) with several modifications to the manufacturer's protocol. For this protocol, the key reagents in

the staining kit are periodic acid (1 g/dL; Sigma-Aldrich, cat. no. 3951-100 ML) and Schiff's reagent (pararosaniline HCl 1% and sodium metabisulfite 4% in hydrochloric acid 0.25 mol/L; Sigma-Aldrich, cat. no. 3952-50 ML). Care must be exercised when applying this method as periodic acid is corrosive, Schiff's reagent is toxic, and hematoxylin is harmful (flammable liquid and skin irritant), according to the manufacturer SDS information. Solutions should also be prepared fresh and/or used within manufacturer expiration dates for optimal results.

The first part of the procedure closely followed the manufacturer's instructions: Deparaffinized and hydrated sections were exposed to periodic acid for 5 minutes, rinsed in three changes of distilled water, immersed in Schiff's reagent for 10 minutes (time was reduced from the manufacturer protocol of 15 minutes to enable a better counterstain), and then rinsed under running distilled water for 5 minutes. The remaining steps were novel to this study: Sections were then counterstained with Weigert's iron hematoxylin (detailed in Presnell and Schreibman[19]) for 5 minutes, rinsed in two changes of distilled water (2 minutes each), "blued" in 1× PBS for 20 seconds, briefly rinsed in distilled water, and stained with a 1% light green solution (Sigma-Aldrich, cat. no. L1886) for 30 seconds to 2 minutes, depending on the preferred intensity of the stain. Slides were then rinsed in distilled water for 2 minutes, allowed to briefly air dry, then finally dehydrated, cleared, and cover-slipped.

Slides for protocol development were imaged using a Leica (Buffalo Grove, IL, USA) DM2500 microscope coupled with a QImaging Retiga R6 6.0 megapixel color camera (Surrey, BC, Canada) interfaced to a PC running Ocular Software (version 2.0). Sections used for protocol validation were imaged with a Motic (San Francisco, CA, USA) Easy Scan digital slide scanner and Motic VM 3.0 Motic Digital Slide Assistant for image capture.

Results

Our modified PAS protocol using Weigert's iron hematoxylin and light green stains (PAS-LG) produced a distinctive purple staining

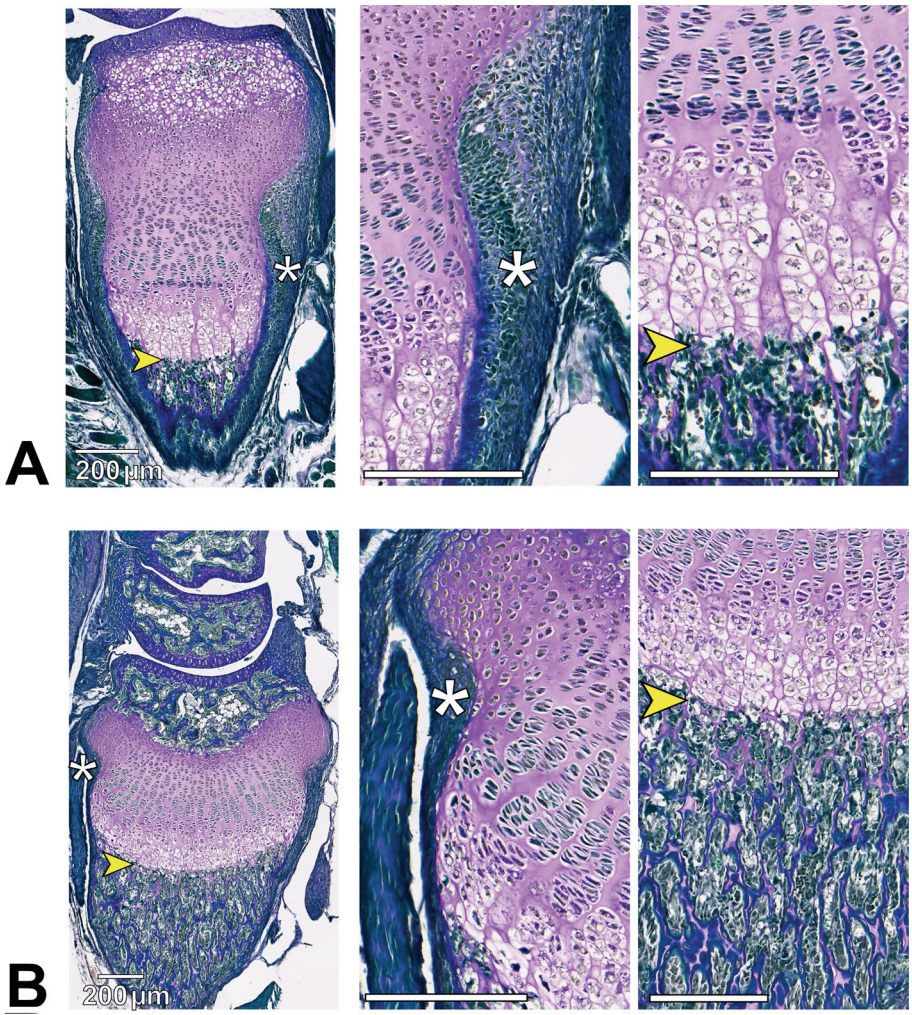

Fig. 2. Modified periodic acid-Schiff (PAS) staining of distal radial growth plates from (*A*) 9-day-old and (*B*) 15-day-old mice stained as part of our protocol validation. Articular cartilage of the radiocarpal joint (distal) is at the top in all images. Magnified panels show demarcation of cartilage at the perichondrium (asterisks) and metaphyseal chondro-osseous junction (yellow arrowheads). Calcified cartilage at the metaphyseal chondro-osseous junction is also stained purple. The secondary center of ossification is present in the 15-day-old mouse in (*B*), further delineating the growth plate between epiphyseal (top) and metaphyseal (bottom) bone. Scale bar = 200 µm for all images.

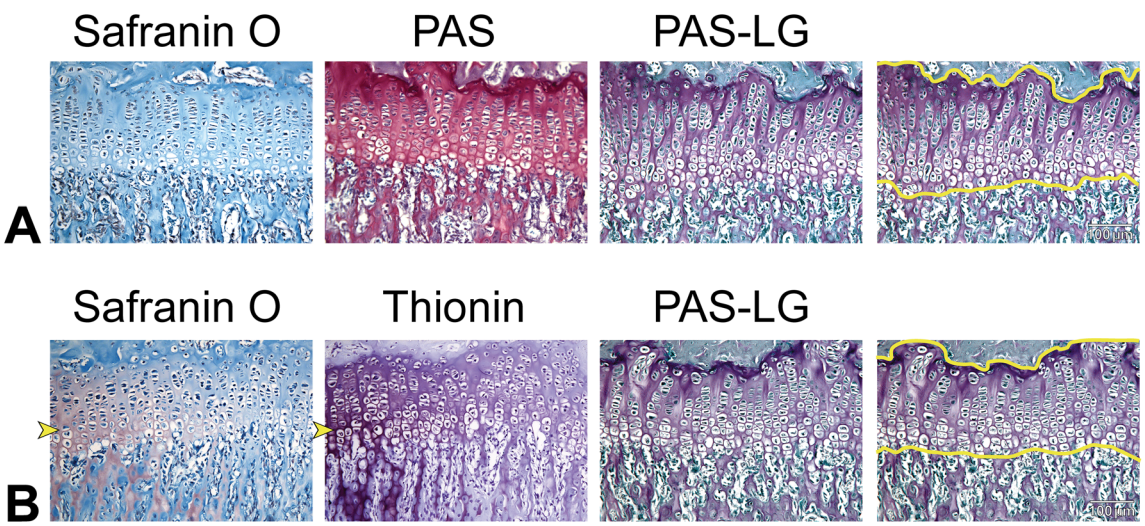

Fig. 3. Comparison of the modified periodic acid-Schiff (PAS) method with other cartilage stains on serial sections of proximal tibial growth plates from two different 6-week-old mice. (*A*) Safranin O (left) did not stain the growth plate, making the bone cartilage interface indistinct. Standard PAS staining (middle) did stain cartilage, but the color was difficult to discriminate from the surrounding bone because all tissues stained reddish-purple. The modified PAS protocol using Weigert's iron hematoxylin and light green (PAS-LG, right) stained cartilage purple and surrounding bone and soft tissue a blue-green color. This enabled multiple users to consistently trace the boundaries of the cartilage (far right) in a way that was not possible in the failed safranin O image. (*B*) In a different mouse, the tibial section was weakly stained with safranin O toward the lateral edge of the growth plate as indicated by the yellow arrowhead (left), whereas safranin O was absent toward the inner edge of the growth plate. Serial sections stained with thionin (middle) match this pattern of metachromasia, suggesting an uneven loss of proteoglycans across the tissue (more proteoglycans retained toward the lateral edge marked by the arrowheads). The PAS-LG section shows a clear bone-cartilage distinction across the entire image (right) and reliable demarcation of the growth plate boundary for data collection (far right). Scale bar = 100 μm for all images.

in cartilage, which was clearly demarcated from surrounding bone and soft tissue that appeared a blue-green color (Fig. 2). Images shown in Fig. 2 are distal radial growth plates from 9-day-old (Fig. 2*A*) and 15-day-old (Fig. 2*B*) mice stained with PAS-LG as part of our protocol validation. Magnified panels in Fig. 2 show that cartilage is clearly demarcated from perichondrium (asterisks in Fig. 2) as well as the metaphyseal chondro-osseous junction (yellow arrowheads in Fig. 2), validating the utility of this method for discriminating cartilage from bone and other surrounding tissue. Calcified cartilage at the metaphyseal chondro-osseous junction is also stained purple (Fig. 2).

Most importantly, we were able to discriminate bone–cartilage interfaces using modified PAS staining on sections that were not successful with other cartilage stains. We found during protocol development that our modified PAS-LG protocol renders a clear bone–cartilage distinction and robust staining in specimens for which safranin O failed and standard PAS staining produced limited distinguishing features between cartilage and bone (Fig. 3). Under standard protocols, a positive PAS reaction produces a rose to purplish-red color.[19] When applied to our bone sections, we found that growth plate cartilage appeared a deep red-purple color and surrounding bone was pale red-purple with a somewhat indiscriminate boundary (Fig. 3*A*, middle).

Figure 3 shows the application of our PAS-LG method on serial sections of proximal tibial growth plates from two different 6-week-old mice with weak and/or absent safranin O staining. In Fig. 3*A*, safranin O (left) did not stain the growth plate at all, making it nearly impossible to differentiate cartilage from bone. However, standard PAS staining (middle) showed a clear staining pattern in cartilage, but the color distinction from bone was vague because all parts of the tissue had a purple-red hue. When we applied our modified PAS protocol using Weigert's iron hematoxylin and light green, cartilage in the PAS-LG image (right) appeared purple, whereas the surrounding bone and soft tissue was blue-green. This allowed independent investigators to outline the same boundaries of the growth plate (Fig. 3*A*, far right) for consistent data collection, which we were unable to do reliably in the failed safranin O sections because of the vague cartilage–bone interface. In Fig. 3*B*, the tibial section was weakly stained with safranin O toward the lateral edge of the growth plate (shown by the yellow arrowhead on the left), while safranin O was absent toward the inner edge of the growth plate. Serial sections stained with thionin (middle) match this pattern of metachromasia at the same lateral edge (shown by yellow arrowhead) with no color distinction in cartilage toward the inner edge of the growth plate, suggesting an uneven loss of proteoglycans across the tissue (more proteoglycans retained toward the lateral edge marked by the arrowheads). However, the serial-stained PAS-LG section in Fig. 3*B* shows a clear bone–cartilage distinction across the entire image, enabling consistent tracing of the growth plate boundary for data collection (far right). Proximal tibial growth plates of all 37 mice used in our protocol development successfully stained with PAS-LG in a similar pattern, despite weak or absent safranin O staining in all individuals. Furthermore, not only did all 50 sections used in protocol validation stain with PAS-LG regardless of safranin O success, we found that our modified PAS-LG protocol actually provided a better distinction of the bone–cartilage interface in some cases (Fig. 4).

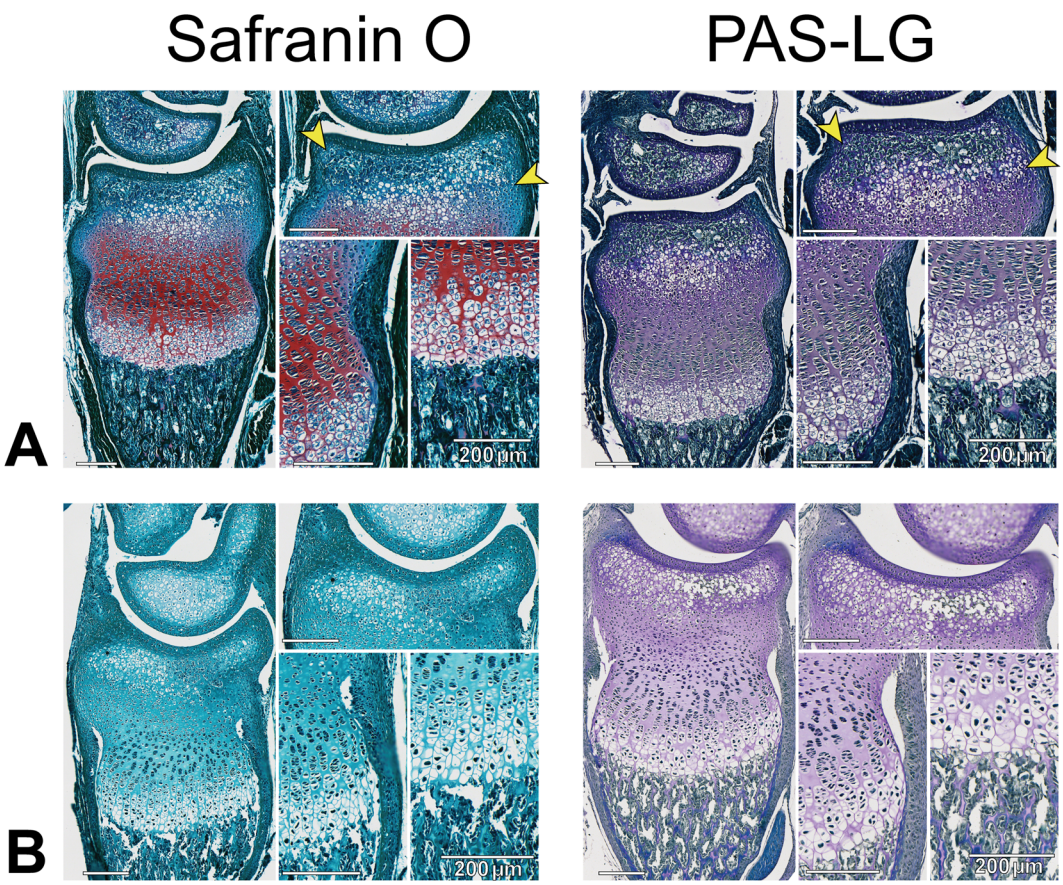

Fig. 4. Comparison of safranin O and periodic acid-Schiff protocol using Weigert's iron hematoxylin and light green (PAS-LG) staining in distal radial growth plates of two different 9-day-old mice. Orientation and morphology match that described in Fig. 1. (*A*) Although safranin O did render the expected red staining of cartilage, the stain did not extend to boundaries of the perichondrium or epiphyseal cartilage nearest the articular surface (yellow arrowheads). The same cartilaginous areas did stain purple using the modified PAS protocol as shown by the yellow arrowheads on the right. A different mouse (*B*) did not stain red with safranin O, but all cartilage did stain purple with a blue-green bone interface using the modified PAS protocol. Magnified panels demonstrate articular cartilage, growth plate, perichondrium, and developing secondary center of ossification as in Fig. 1. Scale bar = 200 µm in all images.

In specimens where safranin O staining was successful and produced the expected red staining of cartilage (Fig. 4*A*), this staining did not always extend to boundaries of the perichondrium or epiphyseal cartilage nearest the articular surface (yellow arrowheads in Fig. 4*A*). However, these cartilaginous areas did stain purple using the modified PAS protocol (yellow arrowheads in Fig. 4*A*). In specimens for which no red staining occurred with safranin O (Fig. 4*B*), all cartilage did stain purple using the modified PAS protocol (Figs. 3 and 4*B*). We could not always predict when safranin O would fail in our samples, yet we had a 100% success rate using the PAS-LG protocol.

Discussion

This protocol was established as an operable alternative to the safranin O method for differentiating bone and cartilage, wherein cartilage appears purple and the surrounding bone and soft tissue stains blue-green. We were able to capitalize on the sensitivity of the PAS reaction for staining cartilage with low proteoglycan content, while incorporating a novel combination of Weigert's iron hematoxylin and light green counterstains to provide a distinctive bone contrast.

This modified PAS protocol provides a suitable and consistent method for assessing cartilage morphology independent of proteoglycan content, and importantly, under different fixation (neutral buffered formalin and paraformaldehyde) and processing/clearing (CitriSolv, Histoclear II, xylene) conditions that we tested. It produces reliable visualization of the bone–cartilage interfaces at the metaphyseal chondro-osseous junction, subchondral bone interface, and perichondrial bone collar. We believe this method is successful because PAS targets glycol groups that are not impacted by tissue fixation and processing protocols.

Other studies have reported novel combinations of staining methods to distinguish bone from cartilage, including the bone-inflammation-cartilage (BIC) stain, which is intended to distinguish cartilage degradation and inflammation from bone erosion[24]; however, the BIC protocol utilizes safranin O to stain cartilage and is therefore sensitive to proteoglycan depletion. We specifically designed the modified PAS protocol here to stain cartilage for morphological studies, regardless of proteoglycan content.

While the absence of safranin O staining is typically only reported when it is an indicator of proteoglycan loss due to disease or genetic mutation, it is a surprisingly common and underrecognized artifact of many tissue processing protocols. This can be particularly problematic when samples span a range of ages and sizes in developmental studies that would otherwise require time-consuming optimization to avoid potential proteoglycan leaching between ages during decalcification. In addition to the data presented here for development and validation of this modified PAS protocol, we have shared our methods with several other colleagues who have successfully stained their specimens using the modified PAS technique when safranin O did not stain cartilage. Although there are commercially available kits using light green with PAS, such as Abcam (Cambridge, MA, USA) Periodic Acid-Schiff (PAS) Stain Kit (Mucin Stain) (ab150680), our protocol is distinct in the application of Weigert's iron hematoxylin and light green counterstains to specifically highlight the bone–cartilage interface.

In summary, this modified PAS protocol provides a novel method for staining cartilage when other cartilage-specific stains are unsuccessful. Although not intended to replace classic methods such as safranin O, our protocol is an alternative for discriminating bone–cartilage interfaces when other approaches fail. The PAS method consistently renders purple staining in cartilage and blue-green staining in bone, independent of proteoglycans, which are the target of most other cartilage stains. The protocol could have important uses for studies in which identification of the bone–cartilage interface is essential, but may not be preserved because of proteoglycan depletion, whether from tissue processing or genetic disease. This approach offers an especially useful alternative for staining cartilage when standard methods fail in irreplaceable samples.

Author Contributions

Kelsey M Kjosness: Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing - review and editing. **Philip L Reno:** Data curation; formal analysis; funding acquisition; methodology; supervision; validation; visualization; writing – review and editing. **Maria A Serrat:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing – original draft; writing – review and editing.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Peer Review

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Histochemistry

Limitations of safranin 'O' staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies

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Summary. The intensity of safranin 'O' staining is directly proportional to the proteoglycan content in normal cartilage. Safranin 'O' has thus been used to demonstrate any changes that occur in articular disease. In this study, staining patterns obtained using monoclonal antibodies against the major components of cartilage proteoglycan chondroitin sulphate (anti CS) and keratan sulphate (anti KS), have been compared with those obtained with safranin 'O' staining, in both normal and arthritic tissues. In cartilage where safranin 'O' staining was not detectable, the monoclonal antibodies revealed the presence of both keratan and chondroitin sulphate. Thus, safranin 'O' is not a sensitive indicator of proteoglycan content in diseases where glycosaminoglaycan loss from cartilage has been severe.

Introduction

Chondroitin sulphate and keratan sulphate are large polyanionic extracellular matrix molecules present in abundance in cartilage (Muir 1980). The glycosaminoglycans are found in vivo linked covalently by acylosyl-O-serine to a core protein. Cartilage proteoglycans are capable of binding in a specific fashion to hyaluronic acid at a globular domain of the core protein known as the hyaluronic acid binding region. Such associations produce large multimolecular aggregates which are maintained within the extracellular matrix by a network of type II collagen.

The concentration of the glycosaminoglycans chondroitin sulphate and keratan sulphate in normal human articular cartilage has been shown to increase from surface to deeper zones (Bayliss et al. 1983). The glycosaminoglycans play an essential role in the cartilage affording it the necessary elasticity and resilience (Kempson et al. 1971; Scott 1975) as a result of the Donnan osmotic pressure which they generate.

Safranin 'O' is a cationic dye which under certain circumstances exhibits metachromasia (Lillie and Fullmer 1976). In dehydrated cartilage sections stained with safranin 'O' it is in the orthochromatic form. In such a form it is bound to the tissue glycosaminoglycan stoichometrically (Rosenberg 1971). This stoichometric binding infers that safranin 'O' may be used to quantitate the amount of proteoglycan present in cartilage (Rosenberg 1971; Mitchell and Shepard 1978; Kiviranta et al. 1985). In a number of disease states, cartilage degradation is accompanied by changes in distribution or amount of glycosaminoglycan in the tissue. Under such circumstances, cationic dyes such as safranin 'O' and toluidine blue 'O' have been used to assess the amount of proteoglycan present in diseased tissue (Mankin 1971; Mitchell and Shepard 1978; Getzy et al. 1982).

Recently developed immunohistochemical techniques using monoclonal antibodies (Pearse 1980) has made possible more accurate estimations of the distribution of a great number of tissue components within histological specimens.

A number of monoclonal antibodies to specific epitopes of cartilage protoglycan have been raised and characterised (Caterson et al. 1982; Caterson et al. 1983; Zanetti et al. 1985). We have used these monoclonal antibodies to investigate the distribution of chondroitin sulphate and keratan sulphate in diseased cartilage. Staining patterns obtained using the monoclonal antibodies were compared with those obtained using safranin 'O'.

Materials and methods

Operative specimens of articular cartilage from a total of 24 patients with arthritic disease were obtained. Of these, 13 showed classical or definite rheumatoid arthritis (RA) as defined by the ARA criteria (Ropes et al. 1958). The ages of patients ranged from 23 to 82 years, with a mean age of 52.0. Nine joints were also obtained from patients with generalised osteoarthritis (GOA) with an age range of 58 to 80 years, the mean being 68.3. These GOA patients all had unilateral hip/knee disease and Heberden's nodes. The remaining two cases were normal cartilage obtained at autopsy.

The tissue was fixed in alcohol and processed to paraffin wax. Serial sections were cut at 5 μm. Sections were stained in one of three ways. Safranin 'O' staining was carried out using a 0.1% aqueous solution at pH 5.3 for 5 min and counter-stained with light green and haematoxylin (Lillie 1965). In this study four monoclonal antibodies were used. MZ15, which recognises keratan sulphate in articular cartilage and cornea only (Zanetti et al. 1985), a gift from Dr. F.M. Watt. This antibody has been shown to recognise a heptasaccharide subunit of keratan sulphate (Mehmet et al. 1986), and was used without prior digestion by chondroitinase ABC. Three monoclonal antibodies, 1B5, 9A2 and 3B3, were donated by Prof. B. Caterson. These three antibodies recognise the disaccahride residues of chondroitin sulphate (Couchman et al. 1984; Vertel and Barkman 1984) after digestion with chondroitinase ABC (Sigma Chemical Co.) (Derby and Binter 1978). These antibodies were used together in a cocktail. An indirect immunoperoxidase method (Van Noorden 1986) was used employing a rabbit anti-mouse IgG horse raddish peroxidase conjugate second-

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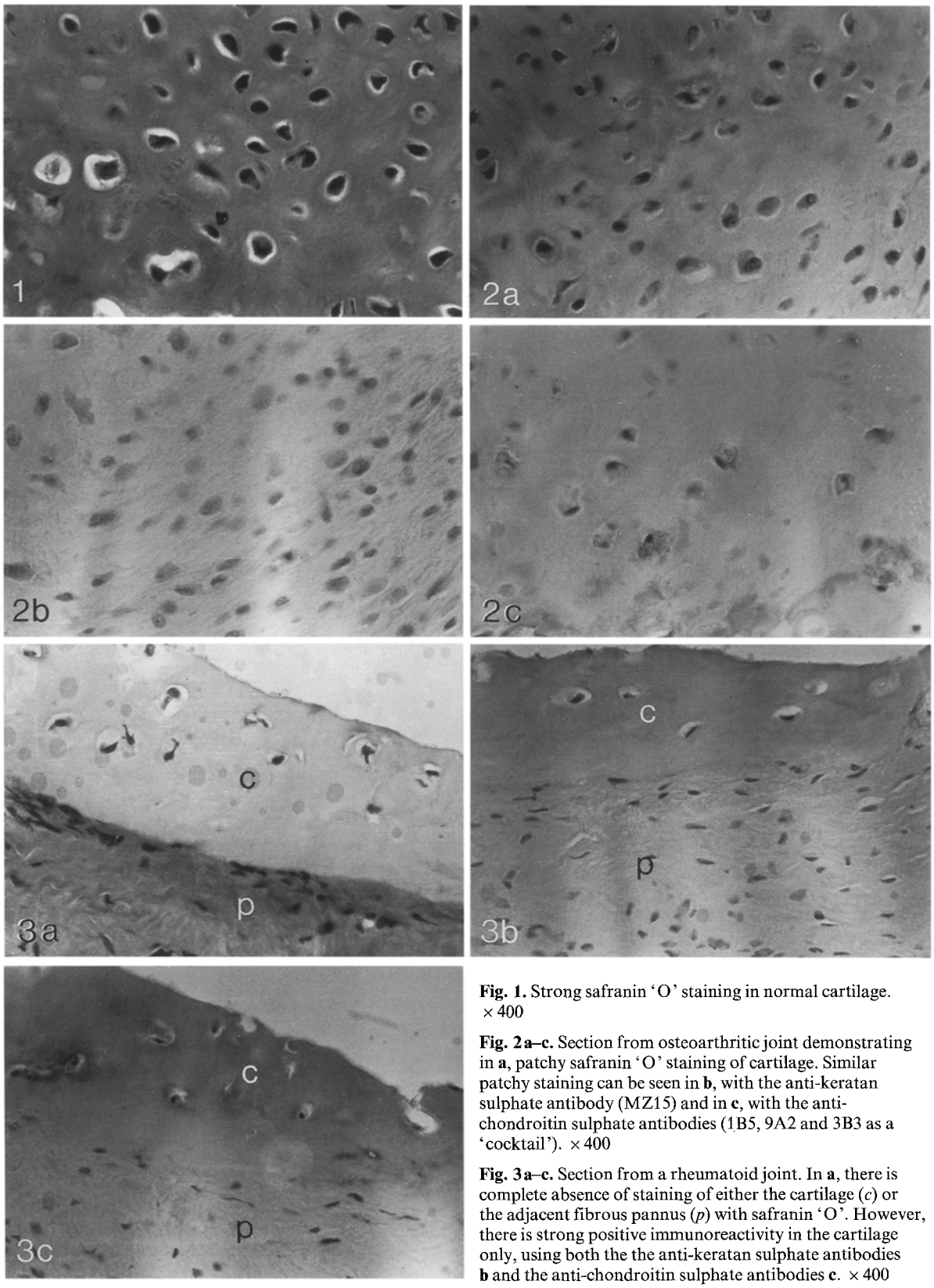

Fig. 1. Strong safranin 'O' staining in normal cartilage. × 400

Fig. 2a–c. Section from osteoarthritic joint demonstrating in **a**, patchy safranin 'O' staining of cartilage. Similar patchy staining can be seen in **b**, with the anti-keratan sulphate antibody (MZ15) and in **c**, with the anti-chondroitin sulphate antibodies (1B5, 9A2 and 3B3 as a 'cocktail'). × 400

Fig. 3a–c. Section from a rheumatoid joint. In **a**, there is complete absence of staining of either the cartilage (*c*) or the adjacent fibrous pannus (*p*) with safranin 'O'. However, there is strong positive immunoreactivity in the cartilage only, using both the the anti-keratan sulphate antibodies **b** and the anti-chondroitin sulphate antibodies **c**. × 400

ary antibody (Sigma Chemical Co.). Negative controls using non-immune serum in place of the primary antibody were carried out for all tissues.

Specimens were assessed at 400 times magnification without reference to clinical details.

Results

From the 24 cases a total of 34 blocks were assessed. These consisted of 19 rheumatoid arthritic, 13 osteoarthritic and 2 normal blocks. Tissues were assessed for the amount of

Table 1. Samples from rheumatoid joints

Score	Number of blocks staining with		
	Safrarin 'O'	anti CS	anti KS
+	4	15	18
±	7	1	1
−	8	3	0

Table 2. Samples from osteoarthric joints

Score	Number of blocks staining with		
	Safranin 'O'	anti CS	anti KS
+	8	12	9
±	4	0	3
−	1	1	1

safranin 'O' stain present in the section. Scoring of the staining was as follows:−
+ strong staining throughout matrix (Fig. 1)
± slight or patchy matrix staining (Fig. 2a)
− no discernible staining of cartilage (Fig. 3a)
Scoring of the monoclonal antibody staining was carried out using the above criteria (Figs. 2b, c and 3b, c). A summary of the results is shown in Tables 1 and 2. From this data it is apparent that many of the blocks that did not stain with Safranin 'O', or had only patchy matrix staining, stained strongly with the monoclonal antibodies.

Of the 34 blocks assessed 9 were completely devoid of safranin 'O' stain in the cartilage matrix. Of these, 8 were from rheumatoid and only 1 from a patient with osteoarthritis. All 9 blocks were positive for either chondroitin sulphate or keratan sulphate, and 6 showed positive staining with both antibodies. Slight or patchy safranin 'O' staining of the matrix was observed in 11 blocks, 9 of which had strong staining for both chondroitin sulphate and keratan sulphate antibodies, the other 2 showing positive staining with one of the antibodies (either anti CS or anti KS). Strong pericellular staining in addition to matrix staining with antibodies was present in 2 of 7 blocks of rheumatoid tissue and in 1 of 4 osteoarthritic blocks. In 1 block of osteoarthritic cartilage, safranin 'O' and antichondroitin sulphate staining was observed but anti-keratan sulphate staining was negative. The remaining 13 blocks, 7 osteoarthritic, 4 rheumatoid and 2 normals showed strong staining with all the methods used. The negative controls which were carried out in parallel to the antibody staining showed no staining of the cartilage matrix.

In tissue where safranin 'O' staining was patchy with both strongly staining and negative areas (Fig. 2a), the corresponding antibody staining was positive throughout the matrix, however areas showing strong safranin 'O' staining were also strongly immuno-positive (Fig. 2b and c).

Discussion

In this study we have shown that the absence of safranin 'O' staining in diseased cartilage is not an indication of complete proteoglycan loss from the cartilage matrix.

This difference in the staining patterns obtained with the two techniques can be explained by their varying modes of action within tissues. Safranin 'O' is a cationic (positively charged) dye and interacts with the negative charges of the tissue proteoglycans as reviewed by Scott (1985). This review gives an in depth account of the staining mechanisms occurring within tissues containing anions and cationic dyes and outlines reasons why cationic dyes such as safranin 'O', if not used in a carefully controlled way may produce misleading results. Where there is a normal amount of glycosaminoglycan present in the cartilage matrix, as in non-diseased tissue, the binding of safranin 'O' is stoichometric as indicated by Rosenberg (1971).

Our study indicates that where very low levels of glycosaminoglycan occur in the tissue, safranin 'O' staining may not be detectable. This may be due to reduced levels of chondroitin sulphate and keratan sulphate in the matrix. This loss results in a reduction of tissue anions, producing an apparent negativity in the staining of cartilage proteoglycan with safranin 'O', due to an inability to visualise the tissue/dye complex.

Monoclonal antibodies are considerably more sensitive than dyes and show high affinity for specific epitopes (Pearse 1980) enabling the detection of very low levels of the epitope, in this case, specific dissacharide subunits of chondroitin sulphate (Couchman et al. 1984) and heptasaccharide subunits of keratan sulphate (Mehmet et al. 1986) within the diseased tissue. It also has the ability to amplify the staining reaction (Van Noorden 1986). Such a method has enabled us to show the presence of proteoglycans in the matrix of diseased cartilage where a large amount of glycosaminoglycan loss has occurred, and the safranin 'O' reaction appears negative. This has practical applications, in that we have recently been able to show that a safranin 'O' negative fibrobastic zone of rheumatoid pannus contains cells and matrix with substantial quantities of proteoglycan, as demonstrated with these monoclonal antibodies (Allard et al. 1987). This zone therefore appears to originate from the underlying cartilage rather than the adjuvant synovial membrane as had been previously suggested.

Indirect immunoperoxidase staining using monoclonal antibodies has shown that none of the tissues investigated were completely proteoglycan deficient despite being negative for safranin 'O'. Safranin 'O' does not therefore always reflect the proteoglycan content of the cartilage matrix, especially in disease states in which proteoglycan loss is high.

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Osteoarthritis and Cartilage

Basic methods in histopathology of joint tissues

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SUMMARY

Histological and histochemical methods are important tools in the evaluation of joint tissue samples for degenerative joint diseases, both in humans and in animal models. In this respect, standardized, simple, and reliable techniques are mandatory. This chapter describes five basic staining procedures appropriate for macroscopic (Indian ink) and histologic (HE/hematoxylin - eosin) visualization and scoring of cartilage proteoglycan and collagen content (toluidine blue/safranin O and picrosirius red/Goldner's trichrome).

Macroscopic overview

The ease of the macroscopic evaluation depends of the species investigated and most importantly on the size of the joints to be scored. The joints of the smaller animals (mice, rats, guinea pigs?) are not usually assessed macroscopically whereas in the larger species (rabbit, dog, sheep & horse), macroscopic grading contributes to the overall staging of the OA disease process. Furthermore the macroscopic assessment in these larger species ensures that sections are made through the sites where the lesions are most severe. Digital photographs and schemas of lesions are recommended and can serve as valuable records.

Macroscopic evaluation – staining with Indian ink

The use of Indian ink on the cartilage surface can facilitate quantifying the depth of cartilage loss in the macroscopic scoring process. Indian ink adheres to fissured cartilage (black patches) and consequently enhances visualization of lesions by providing contrast with surrounding normal cartilage. It does not adhere to the subchondral bone and the latter appears white to yellow surrounded by blacker fissured cartilage when a deep lesion is present. Indian Ink can also be invaluable for orienting frozen sections of cartilage that are devoid of underlying bone (ink-stained surface). Although Indian ink itself is inert, its solvent could potentially interfere with certain analyses (e.g., immunohistochemistry, molecular biology) it would therefore be prudent to avoid its use when additional analyses are required or tested to insure compatibility with the particular analytic process.

Indian ink staining method: As published by Richardson *et al.*[1], the cartilage surface is painted, for 15 s, with a 20% (v/v) dilution of blue Indian ink (Parker, Quink) in phosphate buffered saline containing protease inhibitors. Any excess is blotted off with a moist cotton swab. For frozen joints, the specimens are thawed by immersion in phosphate buffered saline, pH 7.4 for 20 min prior to the application of Indian ink.

Methods of fixation, embedding, and decalcification

The most frequently used method for histological evaluation is fixation in 10% buffered formalin, decalcification in either a solution of 10% ethylenediaminetetraacetic acid (EDTA) (in 0.1 M phosphate buffer, pH 7–8 for approximately 8–10 weeks, solution changed once a week) or 5% formic acid (diluted in water, for approximately 1 week), and embedding in paraffin. In order to speed up the decalcification process in larger species sequential sections, 5 mm apart, may be cut (dog, sheep horse).

In small species, approximately halfway through decalcification, the process can be hastened by transecting the joints in half coronally with a sharp razor blade (using the tibial collateral ligament as a guide), then returning the joint halves to the decalcification solution. While EDTA decalcification is optimal for proteoglycan

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preservation and highly recommended for sections destined for *in situ* hybridization, formic acid decalcification has the advantage of being very rapid. Decalcification is adequate when an aliquot of the decalcification solution no longer produces a calcium precipitate upon addition of ammonium oxalate using the method of Rosen[2]. Briefly, remove a 0.5 ml aliquot of the EDTA decalcification solution and add 10 ml of a citrate–phosphate buffer (0.2 M citric acid and 0.16 M dibasic potassium phosphate, pH 3.2–3.6 achieved with NaOH), and 2.5 ml of saturated ammonium oxalate (5% solution) and vortex. The failure to form a cloudy white precipitate over the course of 20 min on two successive days of testing indicates that decalcification is complete. When decalcification is complete, the tissue is well rinsed in buffer (with several changes of buffer solution), and the whole joint is embedded in paraffin and sectioned (either coronal or sagittal); in the case of larger joints, the two halves of the joint are embedded (in paraffin) to facilitate production of central sections.

Methods for sectioning

Sectioning and sectioning levels

The orientation of sectioning (i.e., the plane of sectioning) is species-dependant and will also depend on the objectives of the study, i.e., which compartment of the joint is the focus of evaluation. For small animals (mice, rat, guinea pig) frontal (coronal) sections are feasible whereas for larger animals (rabbit, dog, sheep, horse) and humans, invariably sagittal sections are performed due to joint size.

Table I
HE (hematoxylin–eosin) staining protocol

Sections	Paraffin section	
Solutions	Eosin Y solution (1%) store at room temperature	Eosin: 10 g Distilled water: 200 ml 95% Ethanol: 800 ml
	Hematoxylin solution (Mayer):	Potassium or ammonium (alum): 50 g Hematoxylin: 1 g Sodium iodate: 0.2 g Citric acid: 1 g Distilled water: 1000 ml
	Eosin Y working solution (0.25%): store at room temperature	Eosin Y stock solution: 250 ml 80% Ethanol: 750 ml Glacial acetic acid (concentrated): 5 ml
Staining procedure	Solution	Time (min)
	1. Xylene	4
	2. Xylene	4
	3. Xylene	4
	4. 95% Alcohol	1
	5. 95% Alcohol	1
	6. 70% Alcohol	1
	7. Tap water rinse	1
	8. Mayer hematoxylin solution	8
	9. Running tap water	10
	10. 95% Alcohol	10 dips
	11. Eosin Y solution	1
	12. 100% Alcohol	5
	13. 100% Alcohol	5
	14. 100% Alcohol	5
	15. Xylene	5
	16. Mount with xylene based media	
Results	Cartilage matrix	Pink (bluish)
	Bone, fibrotic tissue	Pink to red
	Nuclei	Blue
	Cytoplasm	Pink to red
Reference	Mayer P. Mitt zool Stn Neapel 1896;**12**:303	

Similar orientation of all sections is desirable in order to allow comparability of the specimens. In particular, equal thickness of the sections is important for quantitative analysis e.g., of the proteoglycan content. It is also crucial to acquire enough sections to insure inspection of all potentially diseased areas. For the spontaneous model, such as the guinea pig, the central medial tibial plateau is the region of interest for sectioning due to the reliable localization of disease in this area; for other animal model systems, particularly surgically induced models, sectioning of the whole joint may be necessary to insure capture of information from the pertinent disease regions. In this case, the macroscopic inspection and localization of lesions can guide appropriate sectioning for the microscopic analyses. When performing investigations of potential disease modifying drugs it is very important to section through the most severe lesions of the joint.

Histological staining – overview with hematoxylin–eosin

The basic histological staining for the general assessment of cell and tissue morphology and distribution is the hematoxylin–eosin stain (Table I): hematoxylin–eosin stains cell nuclei blue–purple and the cartilage matrix pinkish with a bluish aspect in the areas of a high proteoglycan content.

Histochemical staining of proteoglycan content: Safranin O and Toluidine blue

Both Safranin O and Toluidine blue are cationic dyes that stain proteoglycans as well as glycosaminoglycans. Toluidine blue (Table II) has been reported to provide more intense staining, due to the fact that it has a higher affinity for the sulfur in cartilage compared to Safranin O[3] (Table III). Although Safranin O staining is proportional to proteoglycan content in normal cartilage, it has been reported that it is not a sensitive indicator of proteoglycan content in cartilage in which glycosaminoglycans have been depleted[3].

Table II
Toluidine blue staining protocol

Sections	Paraffin section	
Solutions	Acetate buffer: 0.2 M 0.04% Toluidine blue stain	Prepare in 0.2 M acetate buffer; mix on magnetic stirrer and filter before use; pH between 3.75 and 4.25
Staining procedure	Solution	Time (min)
	1. Xylene	4
	2. Xylene	4
	3. Xylene	4
	4. 96% Alcohol	1
	5. 96% Alcohol	1
	6. 70% Alcohol	1
	7. Tap water rinse	1
	8. 0.04% Toluidine blue	5–10
	9. Tap water rinse	1
	10. Warm air dry	9
	11. Xylene	5
	12. Mount with resinous mounting media	
Results	Cartilage matrix, nuclei	Deep violet
	Cytoplasm and other tissue elements	Various shades of light blue
Reference	Histotechnology: a Self-Instructional Text, 2nd edn., Freida L. Carson, 1996, pp. 154–156	

Table III
Safranin O staining protocol

Sections	Paraffin section	
Solutions	Weigert's hematoxylin working solution:	*Solution A*: 1% hematoxylin in 95% alcohol. *Solution B*: 30% ferric chloride (anhydrous) − 4 ml Concentrated HCl − 1 ml Distilled water − 95 ml Add equal parts of A + B mix and use immediately.
	0.001% Fast green (FCF) solution:	Fast green, FCF: 0.01 g Distilled water: 1000 ml
	1% Acetic acid solution:	Acetic acid, glacial: 1 ml Distilled water: 99 ml
	0.1% Safranin O solution:	Safranin O: 0.1 g Distilled water: 100 ml
Staining Procedure	Solution	Time (min)
	1. Xylene	4
	2. Xylene	4
	3. Xylene	4
	4. 96% Alcohol	1
	5. 96% Alcohol	1
	6. 70% Alcohol	1
	7. Tap water rinse	1
	8. Weigert's hematoxylin working solution	10
	9. Running tap water	10
	10. Fast green (FCF) solution	5
	11. 1% Acetic acid solution	10–15 s
	12. 0.1% Safranin O solution	5
	13. 100% Alcohol	5
	14. 100% Alcohol	5
	15. 100% Alcohol	5
	16. Xylene	5
	17. Mount with resinous mounting media	
Results	Cartilage matrix	Orange to red
	Underlying bone	Green
	Nuclei	Black
	Cytoplasm	Grey green
Reference	1. Kahveci Z, Minbay FZ, Cavusoglu L. Safranin O staining using a microwave oven. Biotech Histochem. 2000;**75**(6):264–8. 2. Camplejohn and Allard[3]	

Table IV
Picrosirius red staining protocol

Sections	Paraffin section	
Solutions	Sirius red F3B ("Direct Red 80")	Sigma–Aldrich Cat#365548 or Cat#43665
	Weigert's hematoxylin	*Solution A*: 1% hematoxylin in 95% alcohol. *Solution B*: 30% ferric chloride (anhydrous) − 4 ml Concentrated HCl − 1 ml Distilled water − 95 ml Add equal parts of A + B mix and use immediately.
	Acidified water	Add 5 ml acetic acid (glacial) to 1 litre of water (tap or distilled).
	Picrosirius red solution (keeps for at least 3 years and can be used many times)	Sirius red F3B: 0.5 g Saturated aqueous solution of picric acid (add some solid picric acid): 500 ml
Staining procedure	Solution	Time (min)
	1. Xylene	4
	2. Xylene	4
	3. Xylene	4
	4. 96% Alcohol	1
	5. 96% Alcohol	1
	6. 70% Alcohol	1
	7. Tap water rinse	1
	8. Weigert's hematoxylin	8
	9. Running tap water	10
	10. **Picrosirius** red solution	60
	11. Acidified water	5
	12. Acidified water	5
	13. 100% Alcohol	5
	14. 100% Alcohol	5
	15. 100% Alcohol	5
	16. Xylene	5
	17. Mount with resinous media	
Results	Bright-field microscopy	
	Collagen	Red on a pale yellow background
	Nuclei	Black, but may often be grey or brown
	Polarized light microscopy: larger collagen fibers	Bright yellow or orange
	Thinner ones, including reticular fibers	Green
Reference	1. Junqueira *et al.*[7] 2. Puchtler H, Waldrop FS, Valentine LS. Polarization microscopic studies of connective tissue stained with picro-sirius red FBA. Beitr Path 1973; **150**:174–87 3. Kiernan JA. Histological and Histochemical Methods: Theory and Practice, 3 edn., Butterworth Heinemann, Oxford, UK, 1999.	

The staining result is relative and ideally should be compared with a normal internal control as the staining intensity of articular cartilage tissue can vary between individuals of the same species.

With routine fixation techniques 15–20% of the proteoglycans may be washed out in the process[4]. An appropriate chemical fixation protocol when use of a cationic dye is necessary may be employed to preserve the proteoglycans[5]. Decalcification is also associated with further extraction of proteoglycans[6]. Use of chemical fixation in the presence of appropriate cationic dyes or plastic embedding employed without decalcification leads to the highest quality of proteoglycan preservation *in situ*, which is a precondition for quantitative assessments[3,4].

Histochemical staining of collagen fibers: picrosirius red and Goldner's trichrome

Two classical ways to visualize collagen fibers in histological sections are the staining with picrosirius red (Table IV) or the Goldner's trichrome staining method (Table V). In picrosirius red stained sections viewed under polarized light microscopy, the birefringence (i.e., light intensity) and the color observed are influenced by alignment of the collagen fibrils, their diameter and packing density[7].

Staining for specific molecular components: immunostaining using antibodies

Additional approaches for studying cartilage matrix composition and integrity, beyond histological and histochemical evaluations, are being developed and increasingly used, including immunolocalization of proteins, protein fragments, cleavage sites and other epitopes. Also, the quantification of mRNA expression levels within cells by *in situ* hybridization technologies will provide additional interesting insights into the behaviour and differentiation state of the cells. None of these methodologies were included in this overview of methods and scoring systems for evaluation of degenerative joint disease in animal models for various reasons: none of them are yet extensively validated, and results can

Table V
Goldner's trichrome staining

Sections	Paraffin section	
Solutions	Weigert's hematoxylin solution	*Solution A*: 1% hematoxylin in 95% alcohol. *Solution B*: 30% ferric chloride (anhydrous) − 4 ml Concentrated HCl − 1 ml Distilled water − 95 ml Add equal parts of A + B mix and use immediately.
	Fuchsin ponceau solution	Distilled Water: 1000 ml Acetic Acid: 2 ml Acid **Fuchsin**: 0.5 g Ponceau S: 2 g
	Molybdic orange G solution	Phospho-molybdic acid: 2 g Deionized water: 100 ml Add orange G: 2 g
	Light green solution	Light green: 0.1 g Deionized water: 100 ml Acetic acid (glacial): 2 ml
	Acidified water	Add 5 ml acetic acid (glacial) to 1 litre of water (tap or distilled).
Staining Procedure	Solution	Time (min)
	1. Xylene	4
	2. Xylene	4
	3. Xylene	4
	4. 96% Alcohol	1
	5. 96% Alcohol	1
	6. 70% Alcohol	1
	7. Tap water rinse	1
	8. Weigert's hematoxylin	10
	9. Running tap water	1
	10. Acetified water	1
	11. **Fuchsin** ponceau solution	20
	12. Running tap water	1
	13. Acidified water	1
	14. Molybdic orange G solution	6
	15. Running tap water	1
	16. Acidified water	1
	17. Light green solution	15
	18. Running tap water	1
	19. Acidified water	1
	20. 100% Alcohol	5
	21. 100% Alcohol	5
	22. 100% Alcohol	5
	23. Methylcyclohexan	5
	24. Mount between two microscope slides	
Results	Chondrocyte cytoplasm	Red
	Nucleus	Black
	Matrix collagen	Orange
	Calcified cartilage and bone	Green
Reference	Sabatini M, Pastoureau P, De Ceuninck F. Cartilage and Osteoarthritis: Cellular and Molecular Tools, Humana Press, 2004.	

potentially vary depending on many factors such as the use of a predigestion steps (i.e., antigen retrieval methodology), fixation methods, and decalcification procedures. Immuno-histochemical analyses should be considered to be informational, reflecting epitope distribution patterns in the tissue or semi-quantitative at best.

Overall, many more studies are needed in the future to identify reliable early molecular markers that could enhance grading and staging, particularly of early disease, when few if any macroscopic or histological alterations are present (i.e., the molecular phase of the disease). These early molecular processes are attractive OA therapeutic targets because they occur prior to the **physical alteration** of the articular cartilage matrix. These early phases of OA are of great interest for pharmacological studies and their evaluation in animal models would be greatly facilitated through the use of molecular markers in future that could overcome the relative insensitivity of classical histological scoring criteria.

Disclosures

Nicole Schmitz is employed by the University of Leipzig.
Sheila Laverty is employed by the Université de Montréal.
Virginia B Kraus is employed by Duke University Medical Center.
Thomas Aigner is employed by the Medical Center Coburg.

Conflict of interest

No author has any conflict of interest related to this work.

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