View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Osteoarthritis and Cartilage 18 (2010) S113–S116

Osteoarthritis and Cartilage

Basic methods in histopathology of joint tissues

N. Schmitz †, S. Laverty ‡, V.B. Kraus §, T. Aigner ‖ *

† *Institute of Pathology, University of Leipzig, Liebigstrasse 26, D-04103 Leipzig, Germany*
‡ *Box 3416, Duke University Medical Center, Durham, NC 27710, United States*
§ *Département de Sciences Cliniques, Faculté de médecine vétérinair, Université de Montréal, CP 5000, St Hyacinthe, Quebec J2S 7C6, Canada*
‖ *Institute of Pathology, Medical Center Coburg, Ketschendorferstr. 33, D-96450 Coburg, Germany*

ARTICLE INFO

Article history:
Received 14 May 2010
Accepted 17 May 2010

Keywords:
Cartilage
Osteoarthritis histochemistry
Toluidine blue
Safranin O
Picrosirius red Goldner's trichrome stain
hematoxylin-eosine

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).

© 2010 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

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

* Address correspondence and reprint requests to: T. Aigner, Medical Center Coburg, Institute of Pathology, Ketschendorferstr. 33, 96540 Coburg, Germany. Tel: 49-9561-226213; Fax: 49-9561-226211.
E-mail address: thomas.aigner@klinikum-coburg.de (T. Aigner).

1063-4584/$ – see front matter © 2010 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.joca.2010.05.026

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.

Acknowledgements

We wish to thank Dr Christiane Girard DVM, Dipl ACVP, Faculté de médecine vétérinaire, Université de Montréal for constructive criticism of manuscript. Printing costs were supported by an unrestricted educational grant to OARSI by Bayer, Expanscience, Genzyme, Lilly, MerckSerono, Novartis, Pfizer, SanofiAventis, Servier, and Wyeth. The work performed was not influenced at any stage by the support provided.

References

1. Richardson CD, Bae WC, Fazeli B, Filvaroff EH, Sah RL. Quantitative characterization of osteoarthritis in the guinea pig. Trans Orthop Res Soc 2001;26.
2. Rosen A. End-point determination in EDTA decalcification using ammonium oxalate. Stain Technol 1981;56:48–9.
3. Camplejohn KL, Allard SA. Limitations of safranin 'O' staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies. Histochemistry 1988;89:185–8.
4. Alers JC, Krijtenburg PJ, Vissers KJ, van Dekken H. Effect of bone decalcification procedures on DNA in situ hybridization and comparative genomic hybridization. EDTA is highly preferable to a routinely used acid decalcifier. J Histochem Cytochem 1999;47:703–10.
5. Shepard N, Mitchell NS. Simultaneous localization of proteoglycan by light and electron microscopy using toluidine blue O – A study of epiphyseal cartilage. J Histochem Cytochem 1976; 24-5:621–9.
6. Fini M, Giavaresi G, Torricelli P, Cavani F, Setti S, Cane V, *et al*. Pulsed electromagnetic fields reduce knee osteoarthritic lesion progression in the aged Dunkin Hartley guinea pig. J Orthop Res 2005;23:899–908.
7. Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979;11:447–55.