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Indian Journal of Pathology and Oncology

Journal homepage: www.innovativepublication.com

Review Article

Reexploring picrosirius red: A review

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ARTICLE INFO

Article history:
Received 21-09-2019
Accepted 15-11-2019
Available online 25-05-2020

Keywords:
Birefringence
Collagen fibres
Picrosirius red stain
Polarized microscopy
Picric acid

ABSTRACT

Picrosirius red stain has been increasingly used for collagen studies in dental and medical research. Sirius red is an acidic dye which binds specifically to collagen and distinguishes type I and type III collagen fibres. This review depicts earlier uses of picric acid, preparation of the picrosirus red stain, mechanism of action, hazards and safety protocols of handling picric acid in laboratory.

1. Introduction

Picrosirius red (PSR) has proved to be a progressively more useful and accepted histological stain owing to the ease of staining procedure. The stain is everlasting and almost perfect. Since staining procedure is carried out at room temperature, results are relatively spectacular and provide more information than routine H & E staining and it can be on par with the complex trichrome staining methods.[1]

Picric acid was introduced as a first synthetic dye for silk in the year 1771. Picric acid name is derived from Greek word “pikros” which means “bitter”. During world war I, workers who used to prepare picric acid were called as canaries mainly because their skin stained yellow in colour. Picric Acid is a trinitro-aromatic compound commonly used in forensic and histology laboratories as a staining and fixative agent.[2] In the field of histology it is used as connective tissue stain (Jullien’s picroindogocarmine and Van Gieson’s picro-acid fuchsin), cytoplasmic stain (Van Gieson’s with iron hematoxylin) and woody sections (picro aniline blue). It is also used in the treatment of malaria, trichinosis, herpes, smallpox and burns.[3]

In 1889, Ira Van Gieson introduced a staining technique that combined picric acid with acid fuchsin, which was the most successful single histological technique ever devised. The main drawback was, the red color of the stained connective tissue faded quite rapidly after mounting and after a few months the red color totally vanished.[1]

Sweat et al in 1964, combined Sirius red F3BA (also known as F3B or Direct Red 80) with picric acid.[1,4] The staining technique was introduced to verify sites of amyloid, but it was soon obvious that it also stained collagen fibers.[1]

PSR staining in conjunction with polarizing microscopy is of value in histo pathological studies, as PSR greatly enhances birefringence of birefringent structures. The polarizing microscopy facilitates visualizing anisotropic

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https://doi.org/10.18231/j.ijpo.2020.038

structures which appear bright or shiny on a dark background.[5] Polarized microscopy is useful tool for studying the tissue components (Table 1).

Collagen is birefringent and this property is mainly due to ground substance acid mucopolysaccharides which are also anisotropic. In routinely stained or unstained sections, collagen can be distinguished from striated muscle which shows birefringent striations, smooth muscle which are weakly birefringent and elastic fibres which are weak or not birefringent. The birefringence could be due to positive intrinsic and form birefringence of the fibres. The intensity of birefringence of collagen depends on number of factors which are considered important in diagnostic pathology. Usually young collagen fibrils are more hydrated and less perfectly aligned than those of mature collagen. Puett et al states that cross links between the fibrils determine the intensity of birefringence.[5]

Collagen being rich in basic amino groups reacts with acidic dyes exclusively. It is seen that Sirius red dye has anisotropic molecular organization and when bound to collagen in an ordered method, enhances collagen birefringence. Thus it is this optical property of collagen that distinguishes PSR from trichrome staining.[6] The stained fibers then shows a spectrum of colours when viewed under polarized light depending on the fibre size, packing density and thus shows clear orientation of collagen fibres.[7] PSR stains collagen type I, II, III and complement component C1q (protein analogous to collagen) and increases their normal birefringency which is specific for collagen.[8–10]

Collagen when stained with PSR and when viewed under polarized light microscopy normally shows thin collagen fibres (type III) which are green to greenish yellow, while thick collagen fibres (Type I) range from yellowish orange (YO) through orange red polarization colours.[11–13]The green to greenish yellow (GY) colour of both thin and thick fibres suggest that the collagen is loosely packed and orange red colour originates from tightly packed fibres.[11,12] The particular colours produced by polarization microscopy of PSR stained section could be due to fibre size, alignment and packing, cross linking of fibres, interstitial ground substance and water content. It is also seen that in tightly packed and better aligned collagen molecules, a shift to the longer wavelength of polarization colours were seen.[7,10] Predominance of green to greenish -yellow of thin and thick fibres indicates that the collagen molecules are loosely packed and could be composed of procollagens, intermediates, or pathological collagen rather than tightly packed normal fibres.[7,11,12,14–18] Type II Collagen is usually present in hyaline and elastic cartilages and it exhibits a weak birefringence and type IV fibres are thin, amorphous and weakly birefringent.[19]

Studies on different species have also shown that all structures that stained red and their birefringence enhanced by Sirius red correspond to areas that contain collagen. However the staining with Sirius red alone is not specific for collagen and three exceptions were observed. First was keratohyaline granules of cornified epithelia, second were mucous glands and the third exception being the heart of fishes. Though these tissue stained well with PSR, bifringency was observed with polarizing microscope as these proteins do not have molecular structure as oriented as collagen. It was also observed that PSR stains amyloid lightly and promotes a faint birefringency and thus can be easily distinguished from collagen because amyloid does not have fibrous arrangement characteristics of collagen.[10]

Polarization colours of collagen fibres in the fibrotic process have shown that during maturation of fibres, the proteoglycan content changes, dehydration occurs which increases the number of cross links and stainable side groups thus the diameter of collagen fibres grow markedly. All these factors enhances the intensity of birefringence and at the same time change their polarization colours. Thus a young, very fine type I collagen fibres with weak birefringence appears green in colour similar to mature type III collagen fibres whereas they show orange or red birefringence in the further maturative stage.[20]

Further ^{2}H Double Quantum Filtered (DQF) Nuclear Motional Resonance (NMR) spectrum studies by Sharf et al have shown that green to greenish yellow colour of thin and poorly packed collagen fibres correlates the narrow component of ^{2}H DQF NMR spectrum. While yellow orange red colour pattern of thick well packed collagen fibres co-relates the broad component of spectrum.[21]

The uses of PSR staining are:

1. To differentiate differing forms of collagen fibers.[1]
2. It works well on decalcified tissues and also helps to demonstrate bone canaliculi.[1]
3. It also helps in staining of amyloid, keratohyaline granules of cornified epithelia and mucous glands.[1,10]
4. Stains dental tissues like dentin (Figure 1), Cementum (Figure 2), periodontal ligament (Figure 3) and bone (Figure 4) red in colour.[8]

The Picrosirius polarization method has been extensively used in dental and medical research studies to express pathological changes in collagens (Tables 1 and 3).

2. Methods of Preparation of Picrosirius Red Stain

Picrosirius red stain for paraffin sections are prepared by dissolving 0.1 gm of Sirius red F3BA in 100 ml of saturated picric acid. Leave to stand overnight and filter.

The iron and haematoxylin solutions are prepared separately and mixed immediately before use eg: Weigert's iron haematoxylin solution. The Weigert's iron haematoxylin solution consist of Weigert's iron haematoxylin A (haematoxylin solution) i.e. haematoxylin 1g dissolved in 100 ml of absolute alcohol and Weigert's iron haematoxylin B (iron solution) i.e. 30% aqueous ferric

Table 1: Polarized microscopic observation of tissue components

Enamel	Enamel is intensely birefringent than dentin & cementum, Dental fluorosis & caries are associated with variations in the birefringence of enamel.[5]
Dentine	Similar to bone ie both organic & hydroxyapatite crystals are birefringent.[5]
Cementum	More intense birefringence than dentin[8]
Periodontal ligament	More intense than cementum[8]
Bone	The organic matrix & hydroxyl apatite crystals are anisotropic the collagen is more intensely birefringent in old animals than young animals.[5]
Osteoid in	
a. Primary bone	Weakly birefringent[5]
b. Secondary bone	Strongly birefringent
Striated muscle	Mainly show birefringent transverse striation[5]
Smooth muscle	Weakly birefringent[5]
Elastic fibres	Weakly birefringent or absence of birefringence[5]
Amyloid	Faintly birefringent[5]
Fibrin	Weakly birefringent compared to collagen[5]
Formalin pigment	Appears as birefringent needle[5]

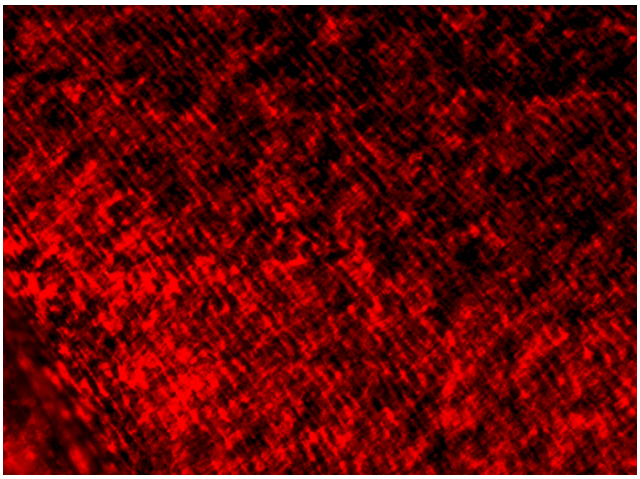

Fig. 1: Photomicrograph of PSR stained sections under polarized light microscopy (20x) showing dentin

chloride, Concentrated HCL and distilled water. The iron and hematoxylin solutions are prepared separately and are mixed immediately before use. The mixture should be a violet-black colour.

3. Method of Staining

1. Deparaffinize and hydrate paraffin sections to distilled water.
2. Stain in Weigert's Hematoxylin for 8 minutes
3. Rinse the slides well in running water for 10 minutes
4. Stain in 0.1% PSR for one hour
5. Wash in two changes of acidified water quickly
6. Physically remove most of the water from the slides by shaking and blotting sections.
7. Dehydrate in three changes of 100% isopropyl alcohol for 1 minute each.
8. Clear in xylene and mount in DPX (Di butyl phthalate polystyrene xylene)[11,12,22]

4. Results (Table 2)

4.1. Mechanism of staining

The basic mechanism of action of PSR is that Sirius red being an acidic dye reacts with basic amino acids present in the collagen molecule. Junqueria et al had studied the mechanism of staining and quantification of increase in birefringence as well as conditions for optimal staining which includes influence of fixation, dye concentration, picric acid concentration, time of staining, pH and washing.[23,24]

Sirius red F3BA (C.I 35780) is a elongated sulfonated azo dye which contains four chromophoric azo groups and six auxochromic sulfonic radicals which interact with the basic groups of collagen.[8,20,25] The molecular weight is approximately 1372 and length is 46 Å.[10,25] At low pH the sulphonic group of the dye interacts with the basic amino groups of lysine, hydroxylysine and guanidine groups of arginine. Picric acid prevents the haphazard staining of non-collagenous structure by Sirius red.[10]

It was observed that about 126 molecules of Sirius red binds to each molecule of collagen type I, II and type III.[9] The enhancement of birefringence is due to the parallel arrangement of axis of collagen and Sirius red molecules.[8,10] Increased light intensity up to 700% was seen due to birefringency of collagen stained by PSR.[10]

The picric acid concentration is not vital for Sirius red collagen interaction (Constantine and Mowry), mostly a saturated solution is used to prevent staining of other tissue components.[26] But the concentration of Sirius red is important and should be 0.1% as recommended Sweat et al. The staining of the sections with Sirius red was maximum in 1 hr and the highest staining was recorded at an acidic

Table 2: Results of staining

Light Microscopy[22]		**Polarization Microscopy**[9–11]	
Nuclei	Black	Type I (Thick collagen fibres) 1.6-2.4μm	Tightly packed - Yellow or Orange birefringence (normal) Loosely packed - Greenish yellow birefringence (pathological)
Collagen	Red	Type III (Thin collagen fibres) 0.8 μm or less	Greenish yellow birefringence (normal)
Cytoplasm	Yellow		

pH of 2.[8,10] In terms of acid fastness (fading of dye), it is rated between 4 to 5 in a scale of 5 i.e. prolonged fading compared to acid Fushin which has a rapid fading in scale range of 1-2.[4] The tissues stained by PSR were stable for at least 15 months. Prolonged fixation in 10% acid formalin diminishes PSR staining by blocking hydrogen bonding or dipole attractions between the sulphonic groups of Sirius red and amine groups of lysine or the guanidine group of arginine.[4,8]

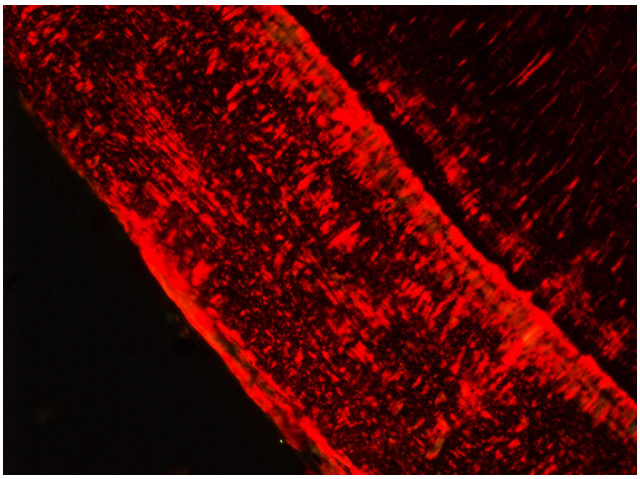

Fig. 2: Photomicrograph of PSR stained sections under polarized light microscopy (20x) showing cementum

4.2. Potential hazards of picric acid

Picric acid is a yellow, odourless crystal which is slightly soluble in water. On hydration, it is harmless, but once dry it is a powerful explosive. The hazards include:

1. Dry picric acid is extremely unstable & highly sensitive to shock, friction or heat. On contact with concrete, amines, bases and metals they can also form explosive picrate salts. The salts are even more reactive and shock sensitive than the acid.[3,27,28]

2. Picric acid causes permanent damage to the eyes and skin due to its corrosive effect. Yellow tainting of vision and yellowing of skin is also sometimes seen. On contact with skin can cause allergic reaction like redness, itching, swelling and is harmful if swallowed. Chronic exposure could cause renal and liver damage.[3,27,28]

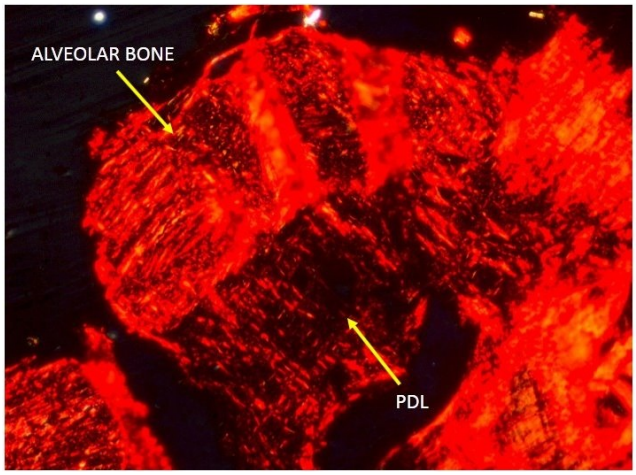

Fig. 3: Photomicrograph of PSR stained sections under polarized light microscopy (4X) showing both PDL & alveolar bone

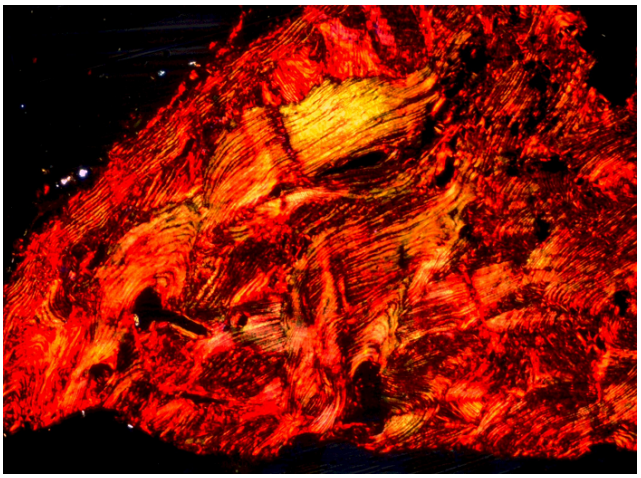

Fig. 4: Photomicrograph of PSR stained sections under polarized light microscopy (4x) showing cortical bone

Table 3: Picrosirius red polarization studies on collagen

No	Authors & year	Field of research	Findings
1	Soma Susan Varghese et al; 2015 [19]	Oral Epithelial dysplasias & inflammatory fibrous dysplasia	Epithelial dysplasias showed GY birefringence while inflammatory fibrous hyperplasia showed red polarisation hue.
2	Pillai Arun gopinathan et al; 2015 [14]	Oral squamous cell carcinoma	Change of birefringence of collagen from yellowish orange to greenish yellow from well to poorly differentiated squamous cell carcinoma
3	Benjamin Vogel et al ;2015 [29]	Determination of collagen with PSR using fluorescent microscropy	PSR stained collagen showed red fluorescence & live cells showed green autofluorescence which is mainly used to quantify collagen in healthy and fibrotic tissues like aorta, lung kidney.
4	Nazac A et al; 2015 [26]	Collagen fiber orientations in vaginal and uterine cervical tissues	PSR stained uterine & vaginal cervical tissues are visualized not only for collagen but also for fibrillar protein orientation by using second harmonic generation microscope.
5	Cristina Segnani et al; 2015 [30]	Normal & inflammed rat colon	Collagen was stained with 3 different stains ie. Vangieson, PSR, PSR/fast green. The Sirius red/fast green showed the best well defined red stained collagen by morphometric analysis in both normal & inflamed colon.
6	Gaganna et al; 2012 [15]	Oral submucous fibrosis	Significant change in birefringence of collagen between connective tissue stages and between mild, moderate to severe degree of epithelial dysplasia
7	Aggarwal P et al; 2011 [16]	Odontogenic cyst & tumours	In dentigerous cysts, odontogenic keratocysts and odontogenic tumors, the predominant birefringence was found to be orangish red, whereas in radicular cysts it was green colour.
8	Irrit Alon et al; 2006 [11]	Salivary gland tumours	The polarization colours of the collagen fibres in the stroma of PA (Pleomorphic Adenoma) were in the range of yellowish orange where as collagen fibres in PLGA (Polymorphous Low Grade Adenocarcinoma) & ACC were mainly greenish yellow.
9	Rumelia Koren et al; 2001 [7]	Follicular thyroid neoplasms	Capsular collagen fibres at the site of invasion exhibited an intense yellow green birefringence whereas capsular sites without invasion stained intensely orange red.
10	Hirshberg et al; 1996 [17]	Central odontogenic fibroma & hyperplastic dental follicle	Thin fibres (0.8 μm or less) in both the lesions showed polarization colours of green to yellow. Whereas, polarization colours of thick collagen fibres (1.6 μm-2.4 μm) in COF (Central Odontogenic Fibroma) were GY and in HDF (Hyperplastic Dental Follicle) they were found to be YO.
11	Nyska A et al; 1995 [31]	Ameloblastic fibroma in maxilla of a young cat	Predominantly green to greenish yellow for both thin and thick fibres compared to the ectomesenchyme of human desmoplastic ameloblastoma which were yellowish orange to orange red
12	Malkusch et al;1995 [32]	Morphometric collagen measurement in lungs	Collagen along with quartz dust were stained with PSR and were measured with quantitative image anlaysis.

Continued on next page

Table 3 continued

13	M.Y Rabau et al; 1994[18]	Collagen pattern in normal & healing sutured intestinal anastomosis	The collagen in the intestinal anastomotic area showed predominantly greenish yellow birefringence i.e. both the thick fibres and thin fibres were greenish yellow. Thus these finding suggest vulnerability of anastomotic site and also can be helpful to assess the quality of collagen in different pathological conditions in the intestine
14	P Whittaker et al; 1994[6]	Myocardial collagen	Maximum brightness was seen in the scar collagen during infarct healing with time when PSR was used and this technique is superior to trichrome staining for collagen dectection.
15	Trau H et al ;1991[33]	Connective tissue nevi collagens	Both thick & thin fibres showed GY birefringence compared to normal human debris which showed YO birefringence.
16	James K et al; 1988[8]	Decalcified dog mandible	It was observed that cementum, periodontal ligament, dentin, dentinal tubules, sharpey fibres, korff's fibres and small reticular fibres stained red. While cells of pulp like odontoblast, fibroblast and mesenchymal cells stained blue. Cementum staining had the same intensity as that of the cortical bone
17	Luiz C.U Junqueira et al ;1986[34]	Differential diagnosis of osteoid in osteosarcomas	The normal osteoid in immature (primary) bone showed up as a network of randomly oriented, thin, short, weakly birefringent collagen fibres & osteosarcoma osteoid showed collagen fibres appeared as long, thick, strongly birefringent fibres of uniform thickness
18	G.S Montes et al ;1983[35]	Dermal collagen distribution	Weakly birefringent greenish fibres (collagen type III) in the advential dermal region, and coarse collagen type I fibres in deeper layers
19	M Szendroi et al; 1984[20]	Maturation process of collagen fibres	During maturation the proteoglycan content changes, dehydration occurs and number of cross links increases and all these factors increases the diameter of collagen thus increasing the intensity of birefringency. Thus the young, fine type I collagen with weak birefringence appears green like the mature type III fibres of reticulin and the same type I fibres become orange/red when matured.
20	G.S Montes et al; 1980[36]	Histochemical & morphological characterization of reticular fibres	In Light microscopy with PSR collagen fibres appeared thick, deep and red while reticular fibres showed up as a thin reddish network. Polarized microscopy, collagen fibres revealed thick, strongly birefringent yellow or red structures whereas reticular fibres appeared thin, pale (weakly birefringent) greenish fibres
21	Junqueira LCU et al; 1979[10]	PSR & Collagen	Staining with PSR for collagen is specific & it also enhances birefringency

5. Protocols for handling picric acid

1. Minimum quantities of picric acid have to be used in the laboratory. Try to eliminate solid form of picric acid and instead prefer premixed stain or 1% solution which can be used in stain preparation.[27]
2. Solid picric acid should always be stored in 10% moisture content and should be regularly inspected. If at all moisture content is below 10% the bottle shouldn't be moved or opened because of the risk of explosion exist. Thus the picric acid must be hydrated always and must not dry out.[3]
3. Metal spatulas should not be used to remove the material from the bottle container. Always remember to clean the bottle neck, cap and threads with a wet cloth before closing the lid of the cap.
4. Personal protection equipment's.
   a) Students and employees who deals with picric acid must thoroughly read the material safety data sheet.
   b) Proper eye protection gears like safety glasses or splash goggles must be worn.
   c) Gloves like nitrile, neoprene, butyl and vilton along with proper lab coat with long sleeves, pants and shoes are mandatory while working with picric acid.
   d) One should be prepared for accidents i.e location of eye wash, first aid kit, emergency contacts and coworkers must be informed about your activities with picric acid.
   e) The last but not the least never work alone in the laboratory.[27,28]

5.1. Safety storage & disposal

1. Picric acid should be stored in a cool, dry and well-ventilated area and should be out of the reach of direct sunlight and heat sources.
2. Storage area must be clearly identified and should be accessed by trained personnel only.
3. Picric acid should always be kept in clearly labelled compatible containers and must be always immersed in water. The container should be made of polyethylene, polpylene, Teflon or glass and should be closed and must not be stacked.
4. Periodic monitoring of the picric acid container must be maintained to check for the evidence of crystallization or leaks.
5. The disposal can be done by reducing the picric acid to non-explosive form of sodium hydroxide and sodium sulfide following disposal as hazardous chemical waste.
6. The most important point one must follow is the picric acid should not be poured down the drain as it could react with copper or iron piping's and could form explosive salts.[3,28]

6. Conclusion

The picrosirius red polarization method is a reliable, precise and an economic method for localization and characterization of collagen fibres. Due to its characteristics, picrosirius red and polarization technique will unquestionably carry on its legacy of understanding collagen in the study of connective tissue biology and pathology.

7. Source of Funding

None.

8. Conflict of Interest

None.

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Cite this article: Gopinathan PA, Kokila G, Siddeeqh S, Prakash R, Pradeep L . Reexploring picrosirius red: A review. *Indian J Pathol Oncol* 2020;7(2):196-203.