# Sirius Red for Collagen Staining Protocol

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**Description:** It's one of the best understood techniques of collagen histochemistry. Technical details follow, and are followed by some comments and a few references. You should come to grips with the theory, advantages and limitations of this method before using it on a large scale. Picro-sirius red method (after Puchtler et al., 1973; Junqueira et al., 1979). Step 4 is an addition that prevents the loss of dye that happens if the stained sections are washed in water.

**Fixation:** Fixation is not critical, The method is most frequently used on paraffin sections of objects fixed adequately (at least 24 hours but ideally 1 or 2 weeks) in a neutral buffered formaldehyde solution.

**Solutions and Reagents:**

Solution A. Picro-sirius red

Sirius red F3B (C.I. 35782) ---------- 0.5 g
Saturated aqueous solution of picric acid ---------500 ml
Add a little solid picric acid to ensure saturation (This is important). ***NOTE: This is a dangerous suggestion. Just add only the liquid solution. – Deb Martinson***
(Keeps for at least 3 years and can be used many times)
(Sirius Red is available from Sigma-Aldrich under the name of "Direct Red 80", Catalog # 36-554-8)

Solution B. Acidified water

Add 5 ml acetic acid (glacial) to 1 litre of
water (tap or distilled).

**Procedure:**

1. De-wax and hydrate paraffin sections.
2. (Optional, and not usually done) Stain nuclei with Weigert's haematoxylin (as for the van Gieson method, but more strongly, then wash the slides for 10 minutes in running tap water).
3. Stain in picro-sirius red (Solution A) for one hour (This gives near-equilibrium staining, which does not increase with longer times. Shorter times should not be used, even if the colours look OK.)
4. Wash in two changes of acidified water (Solution B).
5. Physically remove most of the water from the slides by vigorous shaking or (for a few slides only) blotting with damp filter paper.
5. Dehydrate in three changes of 100% ethanol.
6. Clear in xylene and mount in a resinous medium.

**Results:**

In bright-field microscopy collagen is red on a pale yellow background. (Nuclei, if stained, are ideally black but may often be grey or brown. The long time in picro-sirius red causes appreciable de-staining of the nuclei. This is not a problem with traditional van Gieson or with picro-aniline blue, with their 1-minute staining times.)

When examined through crossed polars the larger collagen fibers are bright yellow or orange, and the thinner ones, including reticular fibers, are green. According to Junqueira et al. (1979) the birefringence is highly specific for collagen. A few materials, including Type 4 collagen in basement membranes, keratohyaline granules and some types of mucus, are stained red but are not birefringent. It is necessary to rotate the slide in order to see all the fibres, because in any single orientation the birefringence of some fibres will be extinguished. This minor inconvenience can be circumvented by equipping the microscope for use with circularly rather than plane polarized light (Whittaker et al., 1994; Whittaker, 1995), but then you don't get a completely black background. (Search Images)

**Comments and References:**

Although this method is technically very easy, it is important for the person doing it and (if it's someone else) the person using the stained slides, to know what it does and how it works. Even without a polarizing microscope, picro-sirius red shows things like reticular fibres and the basal laminae of cerebral capillaries, which are missed by van Gieson and may be obscured by masses of other stained details in trichrome methods (Mallory, Masson, Heidenhain etc).

To the best of my knowledge, most users of picro-sirius red are doing research that exploits the enhancement by sirius red of the birefringence of collagen fibres, which is largely due to co-aligned molecules of Type I collagen. It is also used to stain amyloid.

If you are using only polarized light it does not matter if you lose the "yellow background" of picric acid staining. If you use picro-sirius red as a "better" van Gieson and want to keep the yellow cytoplasm, be hasty with the dehydrating - even more so than with the original van Gieson method.

About 4 years ago, someone (sorry, I've forgotten who, so I can't shout your name) posted to HistoNet an excellent bibliography of staining methods using sirius red F3B. This should be findable in the Archives (www.histosearch.com)

Nobody should do (or order to be done) a picro-sirius red stain without reading at least one of the first two items listed below.

1. Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979; 11, 447-455
2. Puchtler H, Waldrop FS, Valentine LS. Polarization microscopic studies of connective tissue stained with picro-sirius red FBA. Beitr Path 1973; 150, 174-187
3. Whittaker P. Polarized light microscopy in biomedical research. Microscopy and Analysis 1995; 44, 15-17
4. Whittaker P, Kloner RA, Boughner DR, Pickering JG. Quantitative assessment of myocardial collagen with picrosirius red staining and circularly polarized light. Basic Research in Cardiology 1994; 89, 397-410
5. Kiernan. J.A., (1999) Histological and Histochemical Methods: Theory and Practice, Ed. 3 Butterworth Heinemann, Oxford, UK.

Finally, it's important to get the right dye. Sirius red F3B is C.I. 35782 (Direct red 80). There are other "sirius red"s that are quite different. At least one that I've used a lot is OK but does not carry any C.I. designation on the label. With this kind of dye (a tetra-azo direct cotton dye) the manufacturing process necessarily generates more than one coloured product, and other compounds are added to precipitate the dye and adjust its colour intensity. Test your sirius red on sections of muscle, brain and kidney before using it for research or diagnosis. In normal kidney the glomerular basement membranes should be red but not birefringent. Every muscle fibre should be surrounded by red and birefringent collagen. I could continue, but this is already too long.