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Picrosirius Red Staining: A Useful Tool to Appraise Collagen Networks in Normal and Pathological Tissues

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Summary

Specific staining of the extracellular matrix components is especially helpful in studying tissue remodeling, particularly in the case of connective tissue pathologies. As developed by Junqueira and colleagues in 1979, specific staining by Picrosirius red is one of the most important stains to study collagen networks in different tissues. Under polarized light, collagen bundles appear green, red or yellow, and are easily differentiated from the black background, thus allowing for quantitative morphometric analysis. As Junqueira and colleagues point out, many studies use color staining to differentiate collagen bundles and to specify collagen types, yet other studies report that polarized colors only reflect fiber thickness and packing. Using a simple histological example, our study illustrates the inability of Picrosirius red staining to differentiate collagen types, since the absorbed amount of polarized light by this dye strictly depends on the orientation of the collagen bundles. (J Histochem Cytochem 62:751–758, 2014)

Keywords

picrosirius red staining polarized light, birefringence, collagen fibers, Ehlers-Danlos syndrome.

Introduction

Extracellular matrices are complex networks composed of four intimately associated families of proteins—collagens, elastin, adhesive glycoproteins and proteoglycans (Royce and Steinman 2003)—which are present in one form or another in all connective tissues and basement membranes. Therefore, inherited or acquired pathologies in which at least one of these components is impaired usually affect several connective tissues, and are often regarded as multisystemic diseases. Examination of the skin is the most beneficial means to identify connective tissue disorders because it is easily biopsied and encompasses at least one member of each of the extracellular matrix protein families (Holbrook and Byers 1989).

Collagens are the most abundant macromolecules in extracellular matrices and naturally occur in the cutaneous extracellular matrix. Thus, mutations in human collagen genes give rise to numerous connective tissue diseases and disturb macromolecular skin architecture (Holbrook et al. 1982). For example, type IV Ehlers-Danlos syndrome (EDS type IV) is a collagenopathy characterized by vascular dissection or rupture, with patients exhibiting thin translucent skin and easy bruising (Pepin and Byers 1993). Diagnosis is generally based on clinical findings and confirmed by the identification of a causative mutation in COL3A1, the only gene in which mutations are known to cause EDS type IV (Hamel et al. 1998). Furthermore, biochemical studies from cultured fibroblasts of EDS type IV-affected individuals show that structural abnormalities disturb pro-collagen III synthesis and cause intracellular retention and reduced secretion (Chiodo et al. 1995). Approximately 90% of patients with classic Ehlers-Danlos (type I and II) develop

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COL5A1/COL5A2 mutations (Symoens et al. 2012; Ritelli et al. 2013). Thus, in this case, specific collagen network staining of skin sections could be a rapid procedure for a clinical diagnosis.

Using Picrosirius red (F3BA) staining, Junqueira et al. (1979) developed a simple and sensitive method that identifies fibrillar collagen networks in tissue sections (Junqueira et al. 1979). Picrosirius red (F3BA) is a strong, linear anionic dye comprising six sulfonate groups that can associate along cationic collagen fibers, and enhance their natural birefringence under cross-polarized light (Junqueira et al. 1979; Montes and Junqueira 1991).

We have previously shown that the use of Picrosirius red staining associated with morphometric image analysis allows for qualitative and quantitative characterization of collagen network alterations in degenerative pathologies (Eijel et al. 2003; Sansivelstri Morel et al. 2007) and inherited or acquired diseases (Ghomrasseni et al. 2001; Ghomrasseni et al. 2002). Thereafter, several studies demonstrated that this specific staining is particularly useful to observe collagen network abnormalities occurring in connective tissues, specifically in the dermis of patients suffering from EDS (Junqueira and Roscoe 1985). Until recently, many studies relied on Picrosirius red staining to identify collagen types according to their colors under polarized light (Binnebösel et al. 2010; Bayounis et al. 2011; Peeters et al. 2013; Coen et al. 2013; Cavallo et al. 2014). For example, yellow-red strong birefringence would be assigned to collagen type I, whereas collagen type III would display a weak birefringence associated with a greenish color (Junqueira et al. 1979; Montes and Junqueira 1991).

Controversially, other authors reported that the polarized colors of Picrosirius red staining depend only on fiber thickness and packing, not on the composition of the specific collagen type within collagen bundles (Pierard 1989; Dayan et al. 1989; Rich and Whittaker 2005; Coleman 2011). But, associated with morphometric image analysis, this dyeing procedure remains the most powerful method to study and quantify collagen network remodeling (Malkusch et al. 1995; Berton et al. 2000; Séguier et al. 2000)

As establishing collagen type by picrosirius red staining is still largely reported, we wanted to illustrate through simple and clear examples that phenotypic collagen determination by this method is unsuitable. To this end, indirect immunodetection of collagen type III and picrosirius red staining were performed on skin sections from clinically ascertained patients with Ehlers-Danlos type IV. Furthermore, picrosirius red-stained reconstructed connective tissue sections (collagen lattice) were observed under polarized light before and after rotation of the microscope stage.

Materials & Methods

Patients

Three test patients (2 males and 1 female; aged 21, 26 and 22 years) with clinically proven Ehlers Danlos type IV, and three control patients (2 males and 1 female; aged 25, 27 and 18 years) were included in the study. Skin biopsies were retrieved from non-sun-exposed areas after the patients gave their informed consent. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki.

Histological Studies

All specimens were fixed in Bouin's solution for 48 hr and embedded in paraffin. Serial tissue sections, 8-µm thick, were prepared with a manual microtome, and then stained for 30 min with Sirius red (0.1% of Sirius red in saturated aqueous picric acid), as described by Junqueira et al. (1979) for collagen bundle staining. Sections were then mounted for observation under polarized light microscopy (Zeiss AXIOPLAN; Oberkochen, Germany): Pictures of the same areas were taken under the same conditions (exposure time) before and after 90° stage rotation and compared. Other skin tissue sections (6-µm thick) were prepared for indirect immunodetection of collagen type III, with the cell nuclei stained with hemalun.

Immunodetection of Collagen Type III

Paraffin-embedded sections (6-µm thick) were deparaffinized through xylene and a graded alcohol series. Sections were rinsed for 5 min in distilled water. Endogenous peroxidase activity was blocked by incubation with 3% (v/v) H_2O_2 for 5 min. Tissue sections were washed in PBS for 5 min, incubated for 10 min with non-fat dried milk and washed twice in PBS for another 5 min. Tissue sections were further incubated for 1 hr with a primary monoclonal antibody against collagen type III (1:30 dilution; tebu-bio; le-Perray-en-Yvelines, France) in PBS then washed three times for 10 min in PBS. Tissue sections were then incubated with anti-mouse IgG conjugated with peroxidase (Amersham Biosciences; Freiburg, Germany), for 30 min and were the washed three times for 5 min in PBS. Finally, the sections were incubated in peroxidase substrate solution (Diaminobenzedine, Sigma-Aldrich, Saint-Quentin Fallavier, France) in a dark chamber for 20 min, rinsed in distilled water, counterstained with hematoxylin for 1 min, cleared, and then mounted for observation under a light microscope. The absence of primary antibody was used as a blank and controls were performed using non-immune mouse serum.

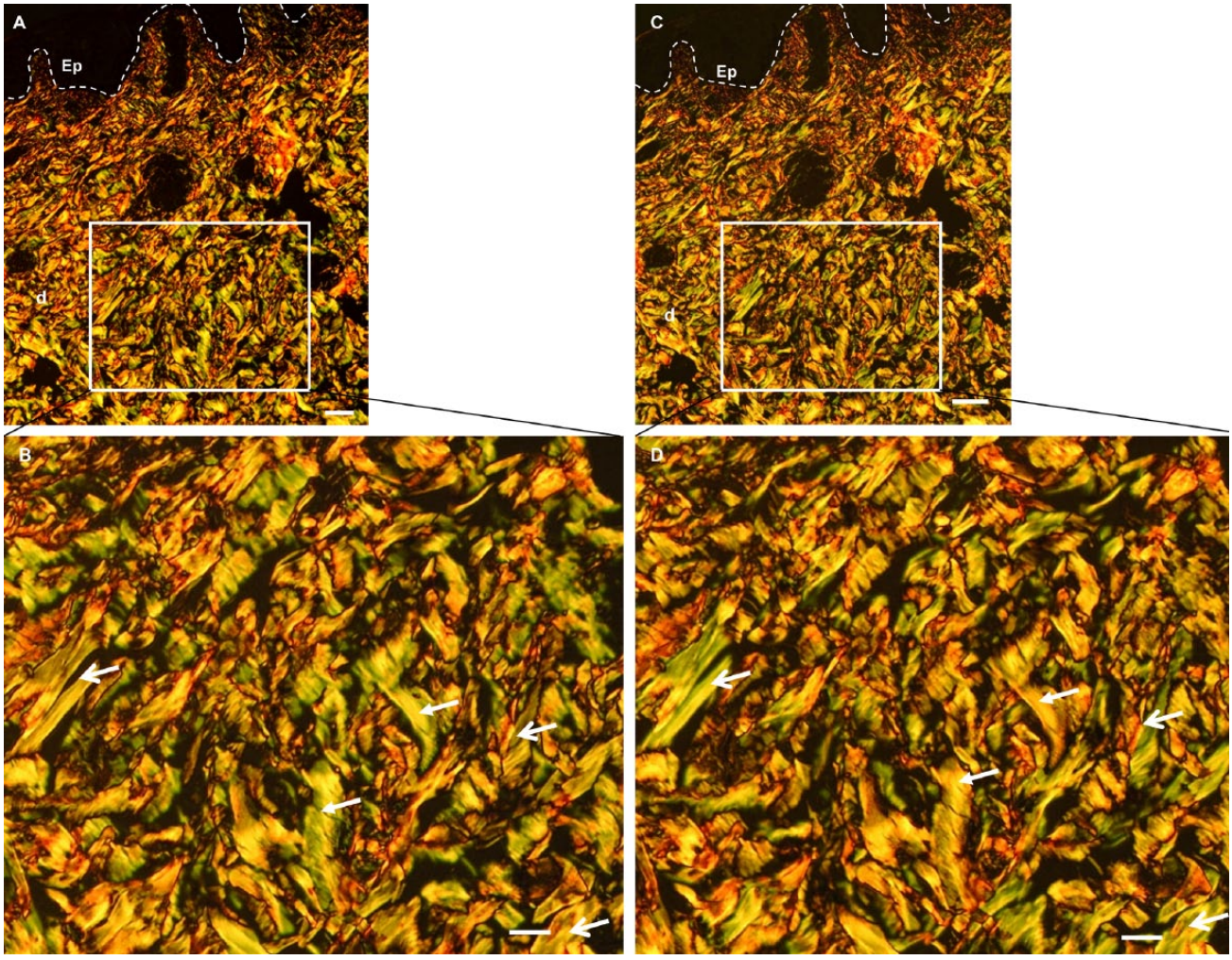

Figure 1. Normal human skin stained with Picrosirius red. (A) Normal human skin is observed under polarized light. (C) The same section observed after microscope stage rotation of 90°. (B) and (D) are enlargements of the boxed regions in (A) and (C), respectively. White arrows point to collagen bundles. Ep: epidermis; d: dermis. Bar=1 µm (A, C); Bar = 0.5 µm (B, D).

Reconstructed Connective Tissues (RCT)

RCT were performed in 60-mm diameter bacteriologic Petri dishes, as previously described (Chaussain-Miller et al. 2002) by mixing 2.75 ml of concentrated Dulbecco's modified essential media with FCS, 1.5 ml of type I collagen solution (3 mg/mL in 0.1% acetic acid; from rat tail tendon) (Institut Jacques Boy; Reims, France), 0.25 ml of 0.1 M NaOH and 0.5 ml of fibroblast suspension (3×10^5 cell/ml). The collagen gel forms rapidly when the dishes are placed at 37°C in a CO_2 incubator. After 1 hr, the dishes were slightly shaken in order to detach the RCT from the edges of the dish. Because of the active organization of collagen fibrils by fibroblasts, the collagen matrix gradually contracts over a few days. After 14 days, when the contraction phase is finished, the RCT is considered stable.

Fourteen- and 21-day reconstructed connective tissues (RCTs) were fixed for 24 hr with a paraformaldehyde solution (4%), then dehydrated and embedded in paraffin. Tissue sections (8 µm) were prepared for histochemistry with picrosirius red F3BA.

Results

Normal Human Skin Sections Stained by Picrosirius Red Dye

Skin sections from control subjects were stained by picrosirius red staining and observed under polarized light microscopy (Fig. 1). As shown in Fig. 1A and 1B, the section background appears black, and the collagen bundles are red, yellow, and green. The epidermis overlying the continuous and undulated basement membrane can be observed, and collagen bundles appear regularly ordered with multiple orientations within the dermis. The same skin section was then observed after microscope stage rotation of 90°

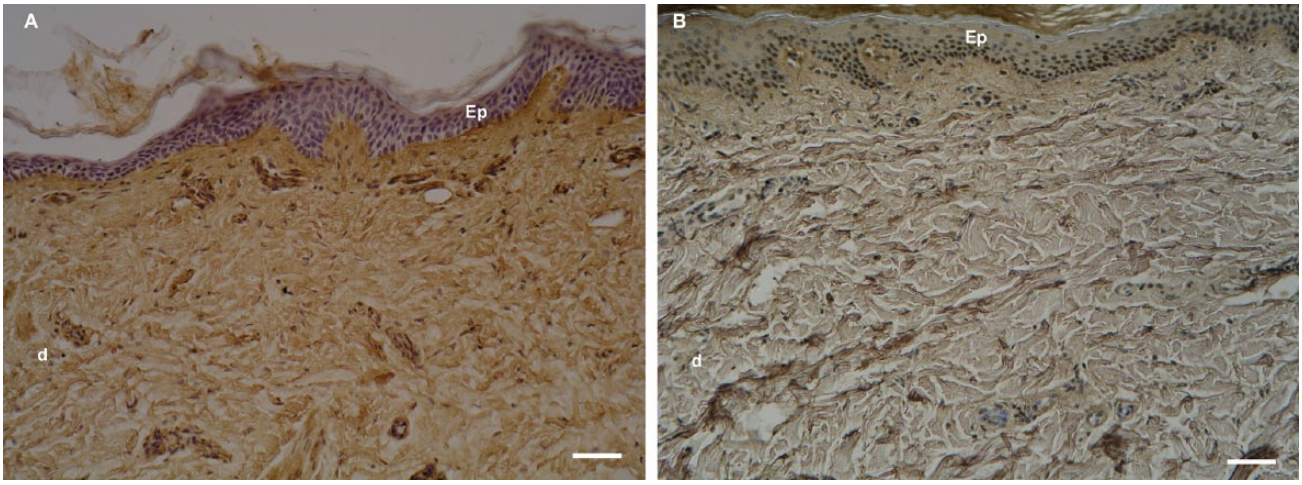

Figure 2. Immuno-detection of collagen type III. (A) Normal human skin and (B) Human skin from a patient with EDS type IV human skin. Ep: epidermis; d: dermis. Bar=1 µm.

(Fig. 1C and 1D, respectively). Thus, after microscope stage rotation, many of the collagen bundles displaying primarily red-yellow color became greenish, and in reverse, the green bundles became red-yellow (white arrows, Fig. 1C and 1D).

Immuno-detection of Collagen Type III on Normal and EDS Type IV Human Skin Sections

Immunodetection of collagen type III was performed on normal skin sections from healthy subjects and on skin sections from patients with Ehlers Danlos type IV, as illustrated in Figure 2. Normal skin sections displayed a specific labeling of the whole extracellular matrix, which is particularly stronger in the upper region of the dermis underlying the dermo-epidermal junction. The cytoplasmic compartment of the cutaneous fibroblasts and the lining of the blood vessels are also labeled (Fig. 2A). No or very faint collagen III-specific labeling was observed in the extracellular matrix of the EDS IV skin section. However, positive labeling was observed in skin fibroblasts of the upper and middermis regions, which may indicate intracellular retention of this collagen (Fig. 2B). On the negative control sections, no labeling was observed.

EDS Type IV Skin Sections Stained by Picrosirius Red Dye

Skin sections from clinically and histologically confirmed EDS type IV patients were stained by picrosirius red dye and observed under polarized light (Fig. 3). Collagen networks appeared to be clearly impaired in the EDS type IV patient samples, displaying thinner and shorter fibers than in the control skin sections. Although the red-yellow collagen bundles typically seen in the control sections were observed in EDS type IV skin sections, green collagen bundles were also present, despite the established lack of collagen III. Furthermore, as previously demonstrated, Figure 3A and 3B show the same skin EDS type IV sections observed before and after 90° rotation of the microscope stage. As observed in normal skin sections, most of the red-yellow-stained collagen bundles became green, and, in reverse, the green bundles became red-yellow (Fig. 3A and Fig. 3B).

Reconstructed Connective Tissues

Reconstructed connective tissues containing normal human fibroblasts were created using acido-soluble collagen type I, and were cultured for 21 days before histology. Tissue sections, 8-µm thick, were stained with picrosirius red and then observed under polarized light. As shown in Figure 4, the section background appears black, whereas the RCT appears as a well-structured extracellular matrix containing thin collagen bundles which appear as red, yellow or green according to their orientation. The same area of the RCT section has been compared before and after 90° rotation of microscope stage. As previously observed in the skin sections, red or yellow fibrils turned in green in RCT after rotation, and conversely green fibrils become red or yellow, as highlighted by the white star in Figures 4A and 4B.

Discussion

Combining the technique by Junqueira and colleagues with automated image analyses, we have previously shown that

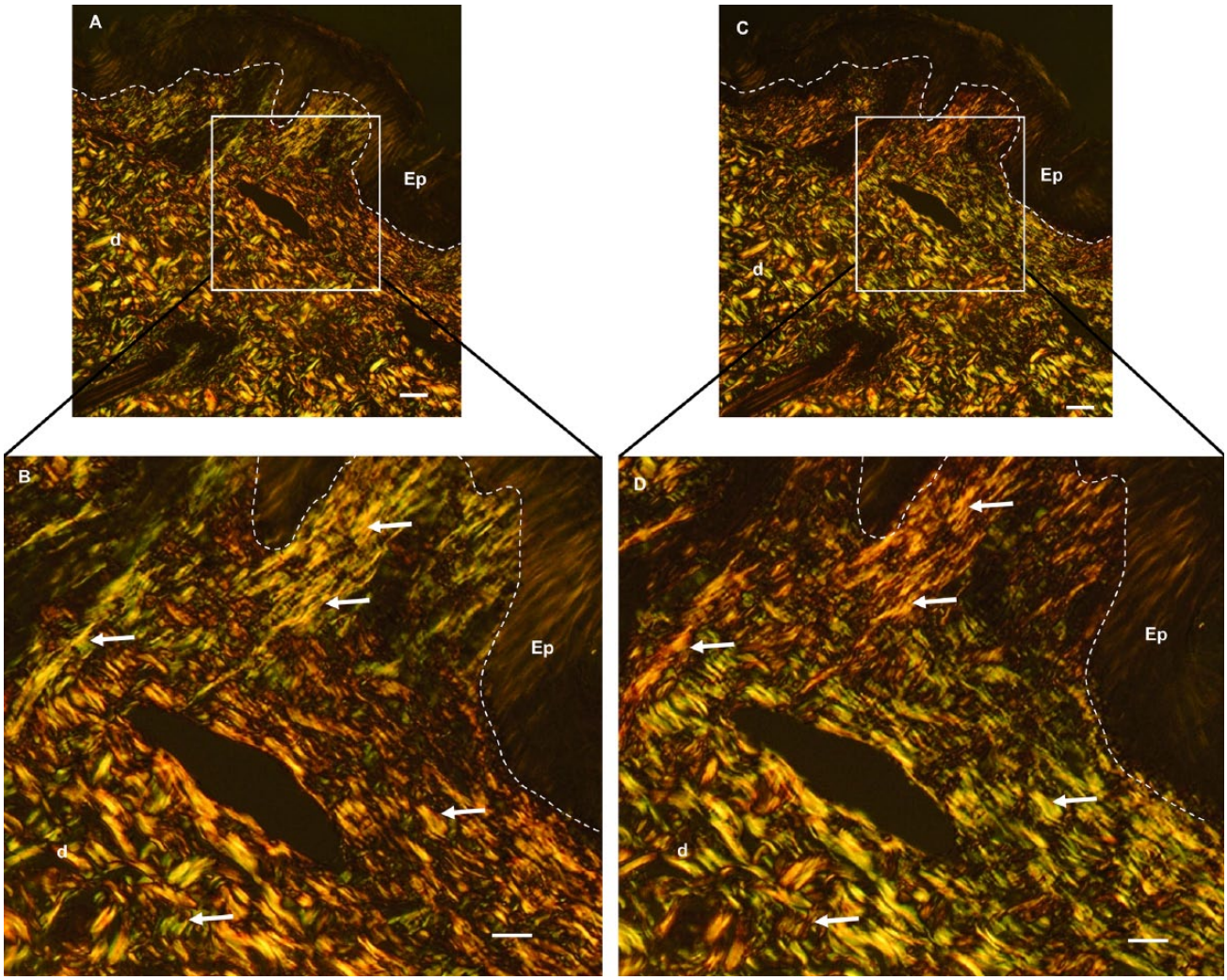

Figure 3. Patient EDS type IV skin section stained by Picrosirius red. (A) Patient EDS type IV skin observed under polarized light. (C) The same section after microscope stage rotation of 90°. (C) and (D) are enlargements of the boxed areas on (A) and (C), respectively. White arrows point to collagen bundles. Ep: epidermis; d: dermis. Bar=1 µm (A,C); Bar= 0.5 µm (B, D).

collagens are the major components of the human skin (Gogly et al. 1997). Confirming clinical evaluation and, as reported previously (Holbrook and Byers 1982; Pepin and Byers 1999), the indirect immunodetection performed in the present study showed little or no expression of collagen type III in the dermis of patients suffering from EDS type IV, although it is well observed in control human skin sections. Under polarized light, the skin sections of patients with EDS type IV stained by Picrosirius red displayed similar collagen network alterations as that previously reported (Junqueira and Roscoe 1985). In these conditions, with the exception of structural alterations, EDS type IV as well as normal skin sections displayed different collagen bundles with multiple orientations and colors.

Astonishingly, 90° rotation of the microscope stage changed the color of the collagen fibers, appearing initially as red or yellow then transforming into green; and in reverse, green fibers changed to yellow. Consequently, it is apparent that under these conditions, and despite what Junqueira et al. (1979) hypothesized, color changes do not reflect any changes in the composition of collagen fibrils.

Moreover, the presence of green fibers under polarized light in skin sections of patients with EDS type IV should imply, according to Junqueira et al. (1979), that collagen type III is present as an extracellular component, which is certainly inconsistent with the disease characteristics of this syndrome (Steinmann et al. 2002). It could also be hypothesized that these color changes were due to particular spatial organization of the collagen type III fibrils in the skin. However, this argument does not hold true, as the same color changes to the collagen bundles were observed with

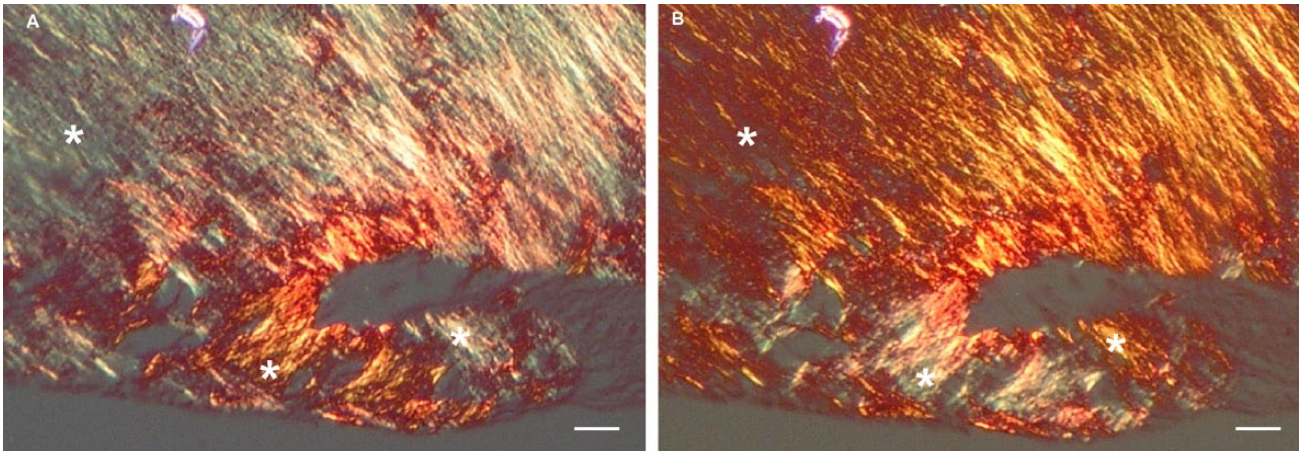

Figure 4. Reconstructed Connective Tissue (RCT) stained by Picrosirius red. (A) RCT observed under polarized light. (B) The same RCT area after microscope stage rotation of 90°. White stars point out areas with color modification. Bar=250 nm.

reconstructed tissues when the microscope stage was rotated.

Finally, as in skin and other numerous connective tissues, collagens bundles are heterotypic fibrils composed of type I, III, and V fibrillar collagens intimately associated (Ricard-Blum 2011). Thus, it seems difficult to differentiate between hypothetic bundles composed only of type III or type I collagens. Furthermore, the mechanisms of the molecular interactions between picrosirius red dye and collagens bundles cannot explain any specific interaction with one or another collagen type. Collagen molecules are known to be rich in basic amino acids, which strongly interact with acidic dyes like sirius red, which contains six sulfonate groups (Montes et al. 1984; Montes and Junqueira 1991). Thus, sirius red is an elongated molecule binding alongside the great collagen fiber axis via a strong interaction of its acidic sulfonic groups with the basic groups of each collagen molecule. Subsequently, binding of the sirius red dye promotes an enhancement of the natural collagen bundles birefringence under light waves vibrating in only one plane: polarized light (Junqueira et al. 1979). Polarizing microscopy involves the use of a polarizer in the optical path between the light source and the specimen on stage, and an analyzer is inserted between the specimen stage and the eyepieces. When just a polarizer or just an analyzer is inserted without a specimen, the field appears bright even if the polarizing filter is rotated. When the planes of polarization of the polarizer and analyzer are at right angles, the filter being crossed the field appears dark. If an oriented, colored material is on the stage of a microscope filter with a polarizer or an analyzer, and either the stage or the polarizing filter is rotated, the appearance of the material should change from its initial color to colorless. This means that a material has different amounts of absorption of polarized light in different planes, or the material varies from colored to colorless depending on the plane of polarization (Born and Wolf 1999). The data presented in this work concerning human skin tissue sections stained with picrosirius red and observed under polarized light with or without rotation of microscope stage resulting in different colors of cutaneous collagen bundles, are a good illustration of this physical phenomenon.

So, How Should We Use the Picrosirius Polarization Method?

It is difficult to claim that a specific chemical interaction occurs between picrosirius red stain and the different collagen types; this was clearly mentioned by Montes and Junqueira (1991). Nevertheless, the Picrosirius polarization method is simple, sensitive and specific for collagen staining. It is particularly useful to reveal the molecular order, organization and/or heterogeneity of collagen fiber orientation in different connective tissues. Furthermore, it supplies accurate information to study pathological tissues. Last, but not least, picrosirius red staining can be a precious tool in measuring the amount of collagen content in normal or pathological tissues (Gogly et al. 1997; Séguier et al. 2000; Séguier et al. 2001; Ejeil et al. 2003; Sansilvestri-Morel et al. 2007).

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Declaration of Conflicting Interests

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