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THE USE OF THE PICROSIRIUS-POLARIZATION METHOD FOR THE STUDY OF THE BIOPATHOLOGY OF COLLAGEN

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The histochemical study of collagen has permitted a better understanding of the biology of this family of macromolecules in the last decade. Two methods, namely: the immunohistochemical and the Picrosirius-polarization methods, contributed significantly to our better knowledge of collagen function and pathology. In this paper we outline the theoretical basis and applications of the Picrosirius-polarization method as developed and applied in this laboratory. This short article has not been designed as a comprehensive reference work: thus, the reader is encouraged to consult a recent review on the subject for additional information (Montes & Junqueira, 1988).

The term collagen relates to a family of glycoproteins which are contained in different histological entities such as *collagen fibers, reticulin fibers*, basement membranes, etc. A frequent fault among pathologists is to speak of collagen when in reality they mean one of the above-mentioned tissue components itself.

Several staining techniques have been devised to differentiate between collagen and muscle fibers, and some are satisfactory for this application. The majority fall into the category of *trichrome stains*.

Still in constant use are Mallory, Masson, and van Gieson methods (Luna, 1968; Bradbury & Gordon, 1977). Although *collagen fibers* are usually stained intensely by these methods, other collagen-containing structures such as *reticulin fibers* and basement membranes are not selectively stained by the trichrome methods, the density of color produced being insufficient to resolve these fine structures. Thus, the various genetically distinct collagen types cannot be distinguished in tissue sections by collagen stains such as van Gieson (Horton, 1984). Though it was designed for demonstrating collagen, Mallory's method is not especially useful for this purpose either (Luna, 1968). In addition, as it has been reported previously (Junqueira & Montes, 1983; Junqueira et al., 1986a), and is common knowledge, the trichrome methods do not show consistent results even with *collagen fibers* themselves which may take on different colors in a same tissue section.

The above-mentioned staining techniques therefore do not readily differentiate between *collagen fibers* and *reticulin fibers*. Metal impregnation techniques, whilst capricious, do provide contrast although they do not distinguish *collagen fibers* from other tissue components (Montes et al., 1980).

THE PICROSIRIUS-POLARIZATION METHOD

Specificity — Because the bulk of the collagen molecules in mammalian tissues are orderly disposed in a parallel orientation, a normal birefringence is one of the classic characteristics of those collagenous entities whose molecules aggregate forming structures visible in the optical and electron microscopes.

Collagen molecules, being rich in basic aminoacids, strongly react with acidic dyes. Sirius Red is an elongated dye molecule which reacts with collagen and promotes an enhancement of its normal birefringence due to the fact that many dye molecules are aligned parallel with the long axis of each collagen molecule (Junqueira et al., 1979a). ***The enhancement of birefringence promoted by the Picrosirius-polarization method is therefore specific for collagenous structures composed of aggregates of orientated molecules.***

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Sensitivity, stoichiometry, and collagen quantification — Sirius Red is a strongly acidic azo dye which contains six sulfonic groups (Fig. 1), with a molecular weight of 1372, that has been much used to stain collagen in tissue sections (Montes et al., 1984a). It has four azo chromophoric groups and has consequently a high extinction coefficient that permits the detection of small amounts of collagen in tissue sections and in acrylamide gel electrophoresis to the extent that it can detect 5.76 μg of collagen/mg of protein (López-de León & Rojkind, 1985).

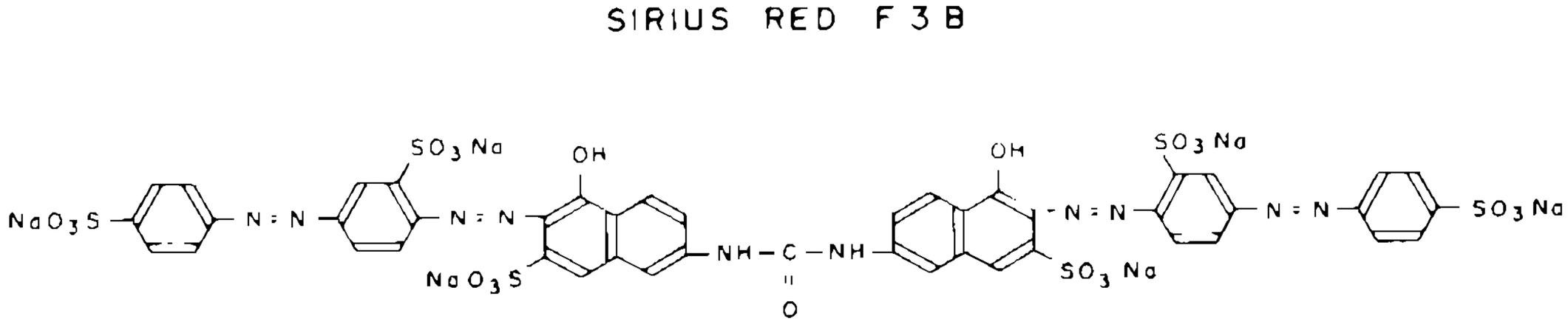

Fig. 1: diagram depicting the molecular formula of the dye Sirius Red (C.I. 35780) which has been extensively used in the commercial dyeing of cellulosic fibers (hydrogen atoms on aromatic rings have been omitted). This is an elongated sulfonated azo dye molecule which contains six auxochromic (sulfonic) radicals, by which the dye molecule interacts with the basic groups of collagen, and four azo (-N=N-) chromophores.

It has been shown that this dye binds to collagen through a strong interaction of its acid sulfonic groups with the basic groups of collagen molecules. Soluble purified collagen types I, II and III bind 126 dye molecules per collagen molecule (Junqueira et al., 1979a).

Evidence obtained comparing differences in color equivalence found with native or denatured collagen with native fibrous collagen indicate that the degree of polymerization and three-dimensional organization of the collagen fibers can also be of importance regarding the amount of dye uptake in the collagenous components of tissue sections (López de León & Rojkind, 1985).

Staining procedure — Due to its high molecular weight, Sirius Red does not stain well sections covered with celoidin nor plastic-embedded tissues.

Five μm sections of isotonic 10% formalin- or Bouin-fixed material are deparaffinized, hydrated to water, and stained during one hour in a 0.1% solution of Sirius Red (Sirius Red F 3 B 200, Mobay Chemical Co., Union, New Jersey, U.S.A.) dissolved in aqueous saturated picric acid. The sections are then rapidly washed in running tap water and counterstained with fresh (less than two months' old) Harris hematoxylin for 6 min. Thin (1-2 μm) sections are not suitable for studying collagen distribution in tissues with the aid of this method. A conventional optical microscope with a strong light source (preferably with a halogen lamp), and two polaroid filters, should be used to study the birefringence of the stained collagen.

Sirius Red solutions are very stable and can be used during several months. Samples of the stain, however, have deteriorated in our laboratory when kept for more than four years on the shelf. Under these conditions the solution loses its specificity and, besides staining collagen, it also stains muscle and epithelia.

Collagenase digestion — Collagen is extremely stable and resists to the action of nonspecific proteases. Collagen turnover, which is necessary for its growth and remodelling, requires specific enzymes to initiate degradation. These enzymes, known as animal collagenases, nick the alpha chain at a very specific and limited site promoting denaturation of collagen. Once denatured, nonspecific proteolytic enzymes take over and readily digest the

collagen. Collagenases of bacterial origin cut collagen polypeptide alpha chains at several sites forming small segments and can, thus, hydrolyze collagen easily. Collagen degradation has been comprehensively reviewed by Pérez-Tamayo (1978).

Combined with the Picrosirius-polarization method, collagenase digestion has been applied to further confirm the specificity of the method. In order to submit histologic material to collagenase digestion, tissue sections are deparaffinized, hydrated to water, and incubated for 24 to 48 hrs at 37°C in a 0.1% solution of collagenase (Collagenase Type V, Sigma Chemical Co., St. Louis, Mo., U.S.A.), dissolved in a 0.05*M* Tris buffer solution containing 1m*M* $CaCl_2$ and 1m*M* sodium azide. Control sections should be incubated under the same conditions without the enzyme. Under these conditions collagenase degrades collagen specifically.

Characterization of the different collagen types — Interstitial collagens display different interference colors, and intensities, of birefringence in tissue sections studied by the Picrosirius-polarization method. The comparative study of vertebrate organs by this method disclosed a striking correlation between the localization of the different colors and intensities of birefringence, and the thus far described biochemical distribution of collagen types I, II, and III, which led Junqueira et al. (1978) to postulate that the above-mentioned different collagen types could be distinguished in tissue sections by the Picrosirius-polarization method. Their results demonstrated that collagen type I (which corresponds to the bulk of the collagenous component of what has been classically called *collagen fibers* by histologists) shows up as thick, strongly birefringent, yellow or red fibers; while collagen type III appears under the form of thin, weakly birefringent, greenish fibers that could be identified as *reticulin fibers* (Montes et al., 1980). The chemical nature of *reticulin fibers* is still a subject of controversy and it has been suggested that they may also contain other types of collagen besides type III (Konomi et al., 1981). Collagen type II, which is present in hyaline and elastic cartilages, does not form fibers and displays a weak birefringence of a varying color (Zambrano et al., 1982).

Although it is generally accepted that basement membrane collagens do not form visible fibrils or fibers, very thin microfibrils can be seen under the electron microscope. Several authors were of the opinion that collagen molecules were arranged randomly in basement membranes forming a feltwork-like structure (Kefalides et al., 1979). However, a clear birefringence has been detected in all basement membranes studied so far by means of the Picrosirius-polarization method (Fig. 2), showing that collagen molecules are orderly disposed in basal laminae (Junqueira et al., 1983b).

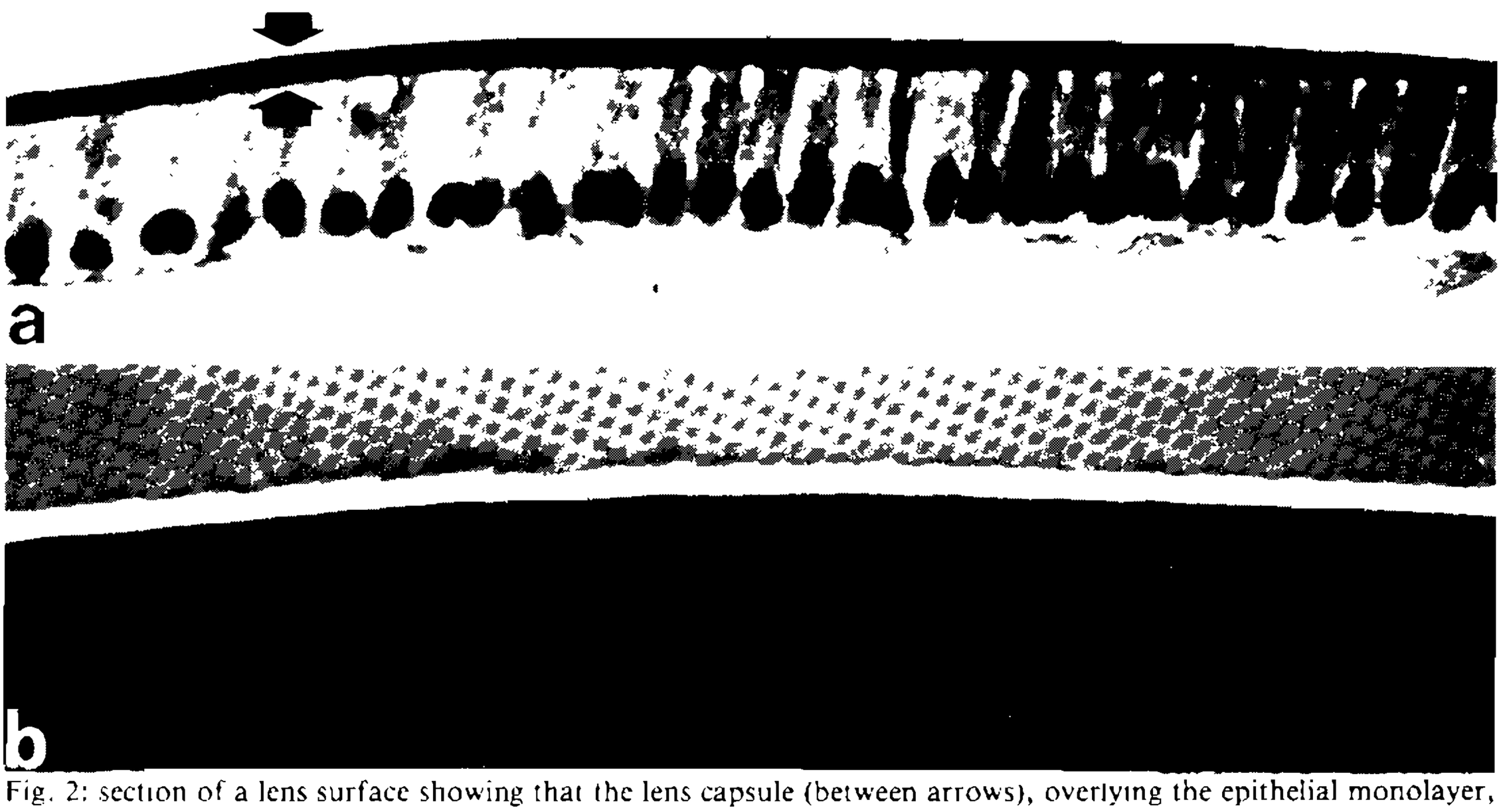

Fig. 2: section of a lens surface showing that the lens capsule (between arrows), overlying the epithelial monolayer, is darkly stained by Sirius Red (a) and displays a distinct birefringence when viewed by polarized light (b). The intense birefringence of the lens capsule (horizontal white band) denotes that the collagen molecules are orientated in this thick basal lamina. Picrosirius-hematoxylin stain: x 1,000.

Fibrillogenesis — It is very well known that, during fibrillogenesis, collagen molecules aggregate forming a collagen ***fibril*** which can be resolved by the electron microscope. Bunches of these fibrils associate to form the ***fibers*** which are visible by optical microscopy. Fibers clumped together in a parallel array result in bundles of fibers (Montes & Junqueira, 1982). This complete sequence of hierarchical levels of supramolecular organization is only attained in the case of collagen type I (Table).

TABLE

Histochemical and ultrastructural characteristics of the most common collagen types

Collagen Type	I	III	II	IV
Maximum level of physical organization attained	Bundles of thick (2-10 μm) fibers	Individual fibers (0.5-1.5 μm in diameter)	Fibrils (20-30 nm in diameter)	Microfibrils containing ordered molecules (1.5nm wide)
Histologic features when studied by the Picrosirius--polarization method	closely packed, thick, non-argyrophilic, strongly birefringent, yellow or red fibers. ***Collagen fibers***	loose network of thin, argyrophilic, weakly birefringent, greenish fibers. ***Reticulin fibers***	loose collagenous network displaying a weak birefringence of a varying color	thin, amorphous, weakly birefringent basal laminae
Ultrastructure	densely packed, thick (75 nm) fibrils with marked variation in diameter	loosely packed thin (45 nm) fibrils with more uniform diameters	no fibers, very thin fibrils embedded in abundant amorphous ground substance	neither fibers nor fibrils; only microfibrils are detected

Theoretical Basis for the Characterization of the Different Collagen Types with the Aid of the Picrosirius-Polarization Method — The observation that collagen types I, II, and III show different interference colors and intensities of birefringence in a same tissue section (Junqueira et al., 1978), can be explained by the widely known fact that these different interstitial collagens display distinct patterns of physical aggregation (Lapiere et al., 1977; Bornstein & Sage, 1980; Eyre, 1980; Minor, 1980; Pérez-Tamayo & Montfort, 1980).

Thus, collagen type I forms thick fibers (***collagen fibers***), composed of closely packed thick fibrils (Junqueira et al., 1979b; Montes et al., 1980; Carrasco et al., 1981; Montes et al., 1984a, b) and consequently presents an intense birefringence of yellow to red color. Collagen type III forms thin fibers (***reticulin fibers***) composed of loosely disposed thin fibrils (Junqueira et al., 1979b; Montes et al., 1980; Carrasco et al., 1981; Montes et al., 1984a, b) and thus displays a weak birefringence of a greenish color. Collagen type II (which is present in hyaline and elastic cartilages) does not form fibers, and its very thin fibrils are disposed as a loose mesh that strongly interacts with the ground substance. This type of physical aggregation results in a weak birefringence, and a varying color (Figs 3 and 4), both of which depend on the interaction of collagen molecules with the ground substance (Zambrano et al., 1982).

It is thus evident that, in the 5 to 7 μm sections routinely used in histology, the different colors and intensities of birefringence displayed by collagen types I, II or III, are due to differences in their patterns of physical aggregation. *This is the reason that it has never been claimed that a specific chemical interaction occurs between Picrosirius and the different collagen types* (Junqueira et al., 1978; Junqueira et al., 1979a; Junqueira et al., 1982; Montes & Junqueira, 1982; Montes et al., 1984a, b).

This method has proved to be useful for the study of collagen distribution in tissue sections obtained from normal specimens (for a review, see Montes et al., 1984a) and in various pathological models (Junqueira et al., 1980b; Junqueira et al., 1981; Junqueira et al., 1986a, b). However, in the early stages of the development of this method, it became evident that tissue section thickness is of importance on the color and intensity of the birefringence displayed by collagen, for folds in tissue sections are more strongly birefringent and present a different color when compared to the surrounding tissue. The influence of tissue section thickness on the color and intensity of birefringence displayed by collagen when observed by the Picrosirius-polarization method, has been reported (Junqueira et al., 1982). These observations have clearly demonstrated that color and intensity of birefringence are a function of collagen thickness.

The fact that color and intensity of birefringence depend on the thickness of each collagenous entity has also been reported by Perez-Tamayo and Montfort (1980), and later on confirmed by Vidal et al. (1982) and Szendroi et al. (1984), when using the Picrosirius-polarization method on different experimental models.

This discussion shows the usefulness of this method for studying the distribution of the different interstitial collagen types in tissue sections from normal adult organs. *It also explains why, as has been stated previously (Pérez-Tamayo & Montfort, 1980), care should be taken when interpreting pictures obtained in situations of rapid turnover of collagen, as in a healing wound, and embryonic or pathologic material in which processes involving intense collagen production or degradation are frequent.*

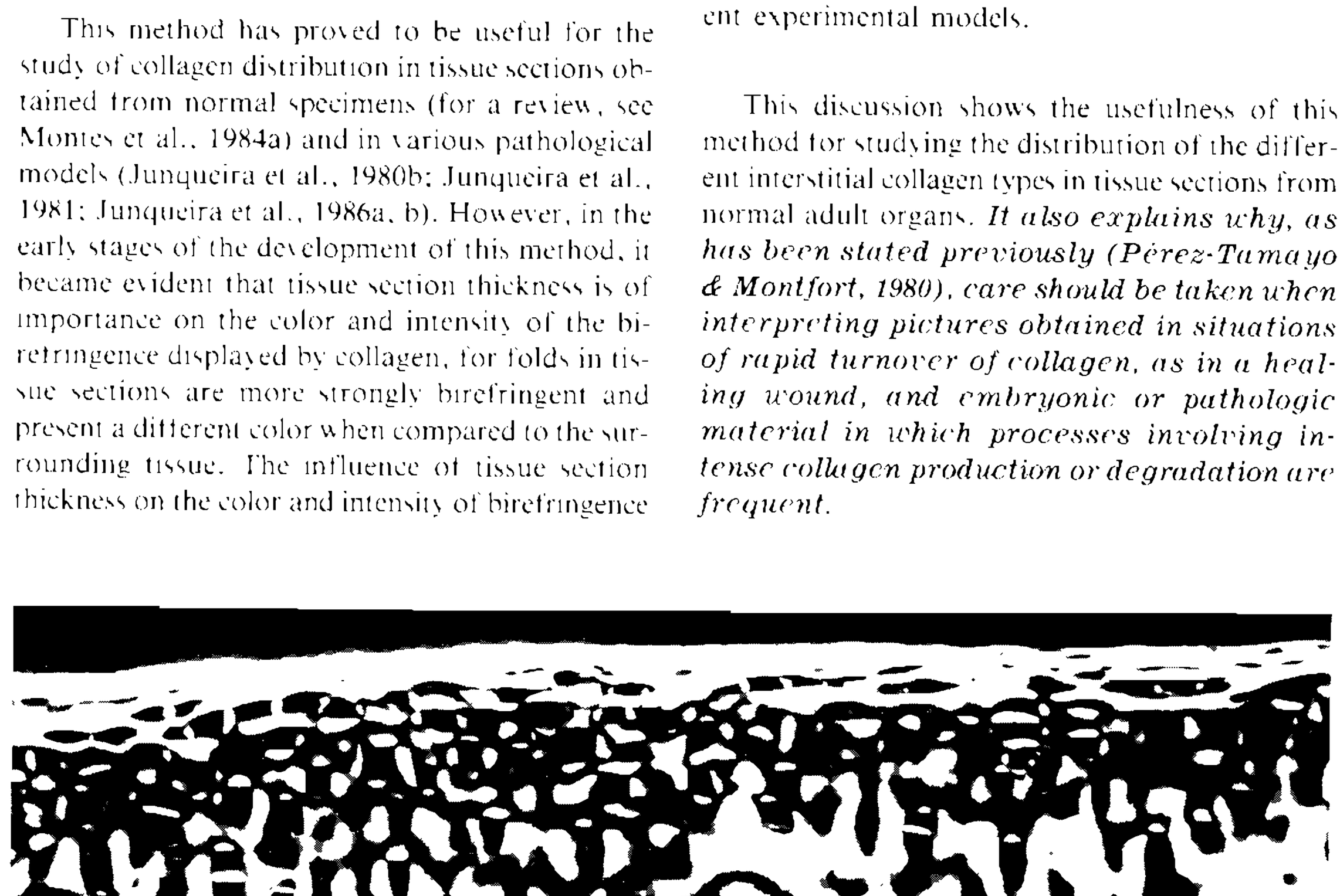

Fig. 3: photomicrograph of an articular cartilage stained with Picrosirius and observed under the polarizing microscope. All brightly birefringent structures, which shine against a dark background, contain orientated collagen molecules. Observe that collagen is aligned parallel with the articular surface (top), whereas the collagen in the deeper layers (bottom) displays a perpendicular orientation. No "fibers" can be distinguished as such; however, the preferential orientation of collagen fibrils promotes a distinct birefringence that is characteristic of structures containing collagen type II. × 500.

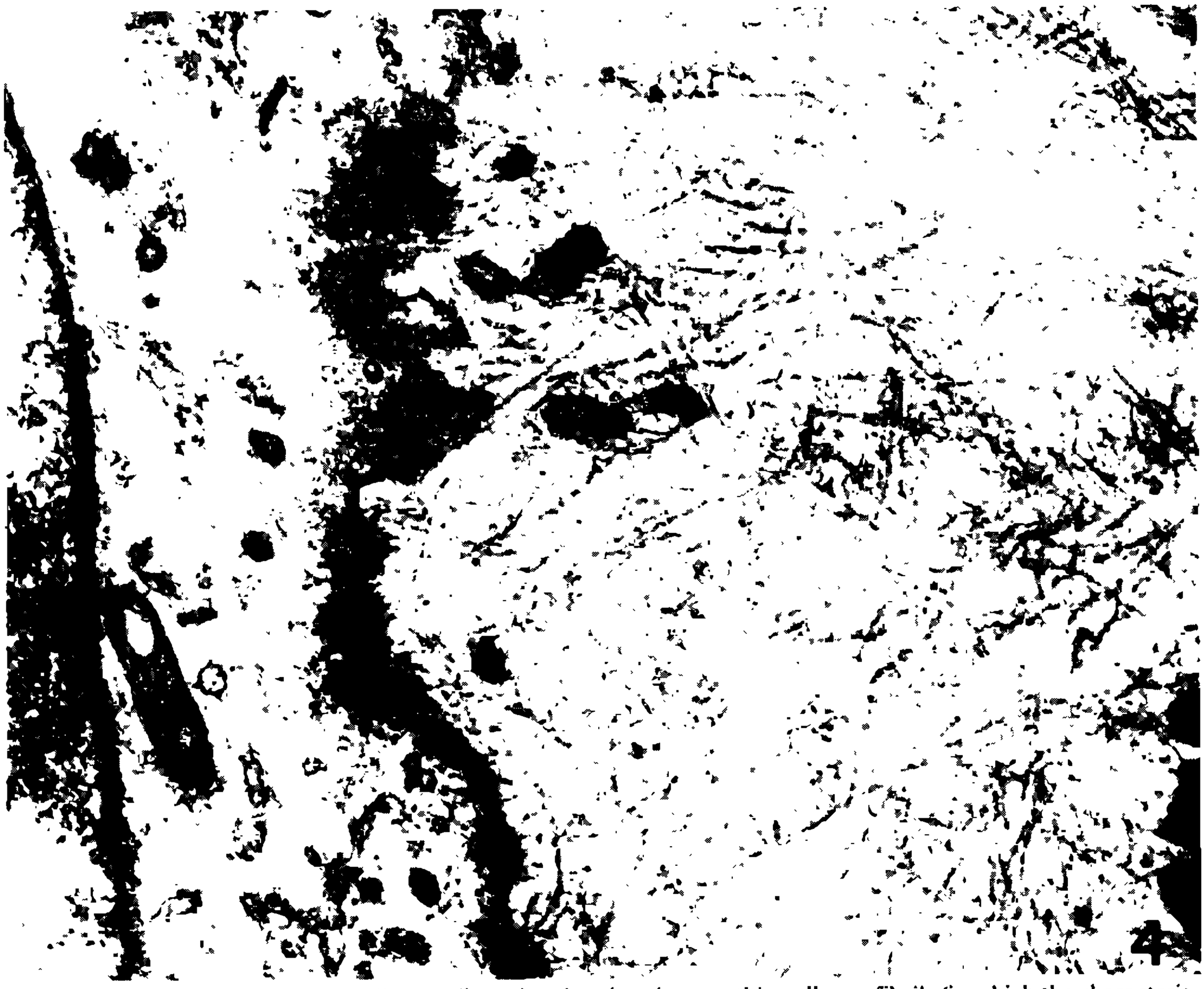

Fig. 4: electron micrograph of a hyaline cartilage showing that the very thin collagen fibrils (in which the characteristic banding pattern is not clearly evident) are orientated in several directions forming a three-dimensional network. Part of a chondrocyte is seen at left. x 35,000.

However this method can also be of value for the study of collagen degradation (Junqueira et al., 1980d; Pérez-Tamayo & Montfort, 1980; Luque & Montes, 1989) provided that, under these conditions, one should be cautious regarding the characterization of collagen types.

ULTRASTRUCTURAL CORRELATIONS

Electron microscopic studies have shown that collagen type I forms coarse fibers (which correspond to the structures that have been classically called *collagen fibers* by histologists) composed of closely packed thick fibrils, whereas collagen type III forms thin fibers (that have been characterized as *reticulin fibers*) composed of loosely disposed thin fibrils (Montes et al., 1980). However, collagen typing by means of fibril diameter morphometry is likely to remain a controversial issue since a recent report claims that type III collagen can be present on banded collagen fibrils regardless of fibril diameter (Keene et al., 1987).

In certain anatomical locations, for example in nerves (Junqueira et al., 1979b; Luque et al., 1983; Montes et al., 1984b), in arteries (Carrasco et al., 1981; Montes et al., 1989), in smooth muscle (Montes et al., 1980), and in skin (Junqueira et al., 1983a), the above-mentioned distinct fibril populations were found to be segregated into different compartments. Thus collagen type I was localized to the epineurium, the adventitial layer of blood vessels, and the deeper layers of the dermis; whereas collagen type III was found in the endoneurium, in the tunica media of arteries, in the inner muscular layer of the small intestine, and in the adventitial dermis.

The histochemical results obtained coincide with the electron microscopic observations in showing the presence of two distinct collagen populations segregated into different compartments in nerves (Junqueira et al., 1979b; Luque et al 1983; Montes et al., 1984b), in arteries (Carrasco et al., 1981; Montes et al., 1989), and in skin (Junqueira et al., 1983a); and these findings are in agreement with immunohistologic (Gay et al., 1975; Meigel et al., 1977; Shellswell et al., 1979) and biochemical (Trelstad, 1974; Seyer et al., 1977; Tajima & Nagai, 1980) findings on the distribution of collagen types I and III in the same organs.

REVISITING THE CLASSICAL HISTOLOGIC STRUCTURES IN THE LIGHT OF THE CURRENT HISTOCHEMICAL CONCEPTS

The results discussed so far demonstrate that collagen type I forms the *collagen fibers*, and collagen type III-rich structures the *reticulin fibers*, of the light microscopists; while collagen type II is identified with hyaline and elastic cartilages and type IV is localized to basal laminae.

Therefore, in all sites where *collagen fibers* have been described (such as tendons, fasciae, organ capsules, adventitial layer of blood vessels, dermis, and submucous layer of the digestive tube), closely packed, thick collagen fibrils of varying diameters with a clearly evident banded staining pattern, have been observed under the electron microscope. On the other hand, in all structures to which *reticulin fibers* have been localized (such as endoneurium, arterial tunica media, mucosal lamina propria, smooth muscle layers of the gastrointestinal tract, and the reticular network of parenchymatous organs such as the liver and kidney), loosely disposed, thin collagen fibrils of more uniform diameters have been detected by means of electron microscopy (Figs 5, 6 and 7).

Collagen fibers show up in the form of thick, brilliant (strongly birefringent), yellow or red fibers against a dark background when studied by the Picrosirius-polarization method; whereas *reticulin fibers* display a weak birefringence and are characterized by their thinness and greenish colour.

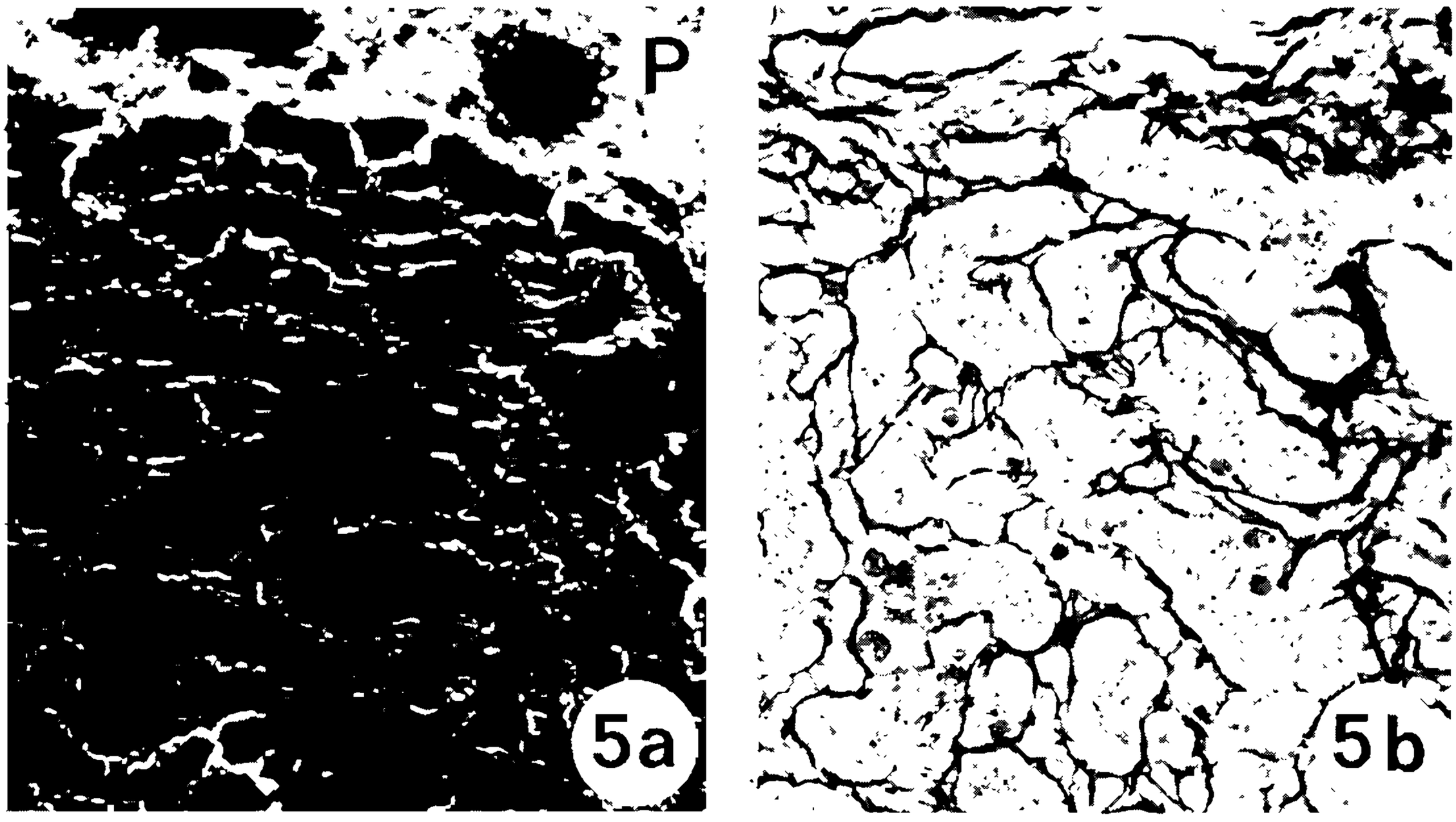

Fig. 5: adjacent sections from the liver of a mouse, studied by the Picrosirius-polarization method (a) and a silver impregnation technique (b). a: a portal space (P) contains thick, strongly birefringent fibers, contrasting with the weakly birefringent, thinner fibers that form a network surrounding the hepatic cell plates within the lobule. b: meshwork of black *reticulin fibers* (surrounding the hepatocytes) which contrast with the *collagen fibers*, in the portal space, that do not blacken with silver (asterisk). There is a striking correspondence between the *reticulin fibers* in *b* and the thin, weakly birefringent fibers in *a*. x 400.

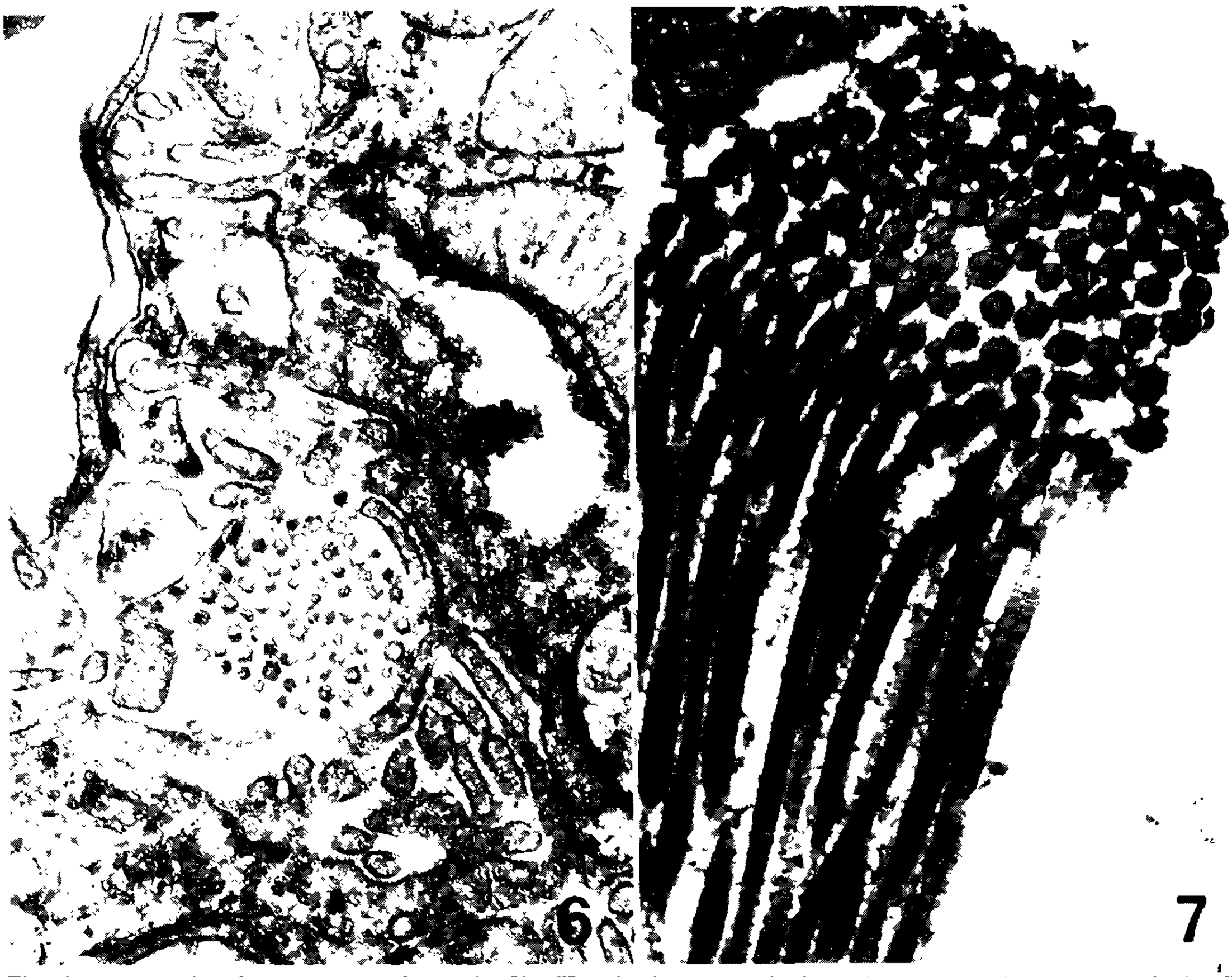

Fig. 6: cross-sectional appearance of a *reticulin fiber* in the space of Disse of the mouse liver. Each reticulin fiber is composed of a few fibrils; these thin fibrils are loosely disposed and reveal an abundant surface-associated granularity. Compare this picture with the electron micrograph in Fig. 7. x 42,000.

Fig. 7: electron micrograph of a *collagen fiber* of a mouse. Note that this fiber is composed of numerous fibrils. The constituent fibrils, of significantly larger diameter than those shown in Fig 6 at the same magnification, are closely packed and display the characteristic banded staining pattern (which is particularly evident in collagen type I fibrils). x 42,000.

The above-mentioned features provide a general background for understanding the distribution of the different collagen types in sections obtained from organs of adult vertebrates.

CONCLUSION

The Picrosirius-polarization method is an extremely simple, reliable, specific, sensitive, and cheap method. Besides these characteristics, it presents the advantage of permitting an easy and precise localization and characterization of the tissue components containing orientated collagen molecules, thus permitting a close correlation between morphological and histochemical observations. Furthermore, Picrosirius staining has also been used in the only method so far described to quantitate collagen-proteoglycan interaction in tissue sections (Junqueira et al., 1980a).

The use of this method in this laboratory permitted us to report significant contributions to the study of collagen biology: namely, the distribution and architecture of collagen was studied in cartilages (Zambrano et al., 1982), nerves (Junqueira et al., 1979b; Luque et al., 1983; Montes et al., 1984b), arteries (Carrasco et al., 1981; Montes et al., 1989) and skin (Junqueira et al., 1983a). This method was also used for studying collagen distribution in osteosarcomas (Junqueira et al., 1986a), in tumors of the peripheral nervous system (Junqueira et al., 1981), in mixed tumors of the parotid gland (Line et al., 1989), and in lesions of schistosomiasis (Junqueira et al., 1986b) and leprosy (Junqueira et al., 1980b).

This method was of the utmost importance for elucidating the process of uterine cervix softening at parturition (Junqueira et al., 1980d; Luque & Montes, 1989) and it was also employed for studying the rapid tissue degradation which occurs during autotomy in holothurians (Junqueira et al., 1980c).

Undoubtedly, due to its characteristics, the Picrosirius-polarization method will continue to contribute to a better understanding of the participation of collagen in tissue biology and pathology.

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