

# Picrosirius Red Staining: Revisiting Its Application to the Qualitative and Quantitative Assessment of Collagen Type I and Type III in Tendon

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## Summary

Collagen has a major role in the structural organization of tendons. Picrosirius red (PSR) staining viewed under polarized light microscopy is the standard method to evaluate the organization of collagen fibers in tissues. It is also used to distinguish between type I and type III collagen in tissue sections. However, accurate analysis and interpretation of PSR images are challenging because of technical factors and historical misconceptions. The aim of this study was to clarify whether collagen types I and III can be distinguished by PSR staining in rat Achilles tendons, using double immunohistochemistry as the positive control. Our findings showed that PSR staining viewed with polarized light microscopy was suitable for qualitative and quantitative assessment of total collagen but was not able to distinguish collagen types. We found it critical to use a polarizing microscope equipped with a rotating stage; tendon section orientation at 45° with respect to crossed polarizers was optimal for the qualitative and quantitative assessment of collagen organization. Immunohistochemistry was superior to PSR staining for detection of collagen type III. We also compared formalin and Bouin solution as fixatives. Both produced similar birefringence, but formalin-fixed tendons provided higher quality histological detail with both hematoxylin–eosin and immunostaining. (J Histochem Cytochem 69:633–643, 2021)

## Keywords

Achilles tendon, collagen type, immunostaining, microscopy, polarization, Sirius red

## Introduction

Collagen fibers are important for the composition and normal architecture of extracellular matrices of connective tissues such as tendons and ligaments. Tendons are largely composed of different types of collagen, which help determine the biomechanical properties of these tissues.<sup>1</sup> Collagen makes up 60–80% of the total extracellular matrix protein of normal tendons.

Collagen type I is the major fibrillar collagen in tendon and normally forms parallel fiber bundles to

provide the major tensile resistance to mechanical forces.<sup>2</sup> It may also be involved in regulating cell proliferation, migration, and differentiation of mesenchymal stem cells.<sup>3</sup> Tendons also contain collagen type III, which is organized as a meshwork of smaller collagen

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bundles. Collagen type III is also found in early-stage granulation tissue formation in wound healing, and it is believed to have a prominent role in regulating tissue regeneration.<sup>4,5</sup> It normally accounts for less than 10% of the total weight of healthy tendons<sup>6,7</sup> and is restricted to tendon sheaths.<sup>8</sup> The tendon repair tissue that forms after tendon injury, however, frequently shows increased collagen type III content, although the specific role of collagen type III in repair has yet to be defined. For instance, collagen type III content is markedly increased in patients with complete Achilles tendon rupture.<sup>9,10</sup> Importantly, the repaired tendon rarely achieves functionality equal to that of the preinjured state, with up to a 30% decrease in final tensile strength.<sup>11</sup> Similarly, the collagen type III:type I ratio has been reported to be increased in shoulder tendinopathies,<sup>12,13</sup> which suggests that a high collagen type III:type I ratio might account for poor matrix organization. This may not only affect tendon structure but also lead to inferior tendon repair.

As research on tendons increases, so does the need for standardized histochemical methods to identify changes in the composition and organization of collagens within tendons. This is particularly important when assessing the outcomes of tendon regeneration, tendon repair strategies, tissue engineering approaches, and the altered mechanical properties created by tendinopathies.

The van Gieson and Masson trichrome histochemical stains have been used to visualize collagen in paraffin-embedded tissue sections. However, collagen type I and type III cannot be distinguished by these stains.<sup>14</sup> Moreover, they may fail to stain thin fibers such as collagen type III,<sup>15</sup> leading to underestimation of the actual collagen content of the tissue.

In contrast to trichrome staining, picosirius red (PSR, also called Sirius red) staining has been considered the standard for collagen detection and quantitative estimation in histological sections of normal and abnormal tissues.<sup>14,16–20</sup> PSR F3BA is an anionic dye whose sulfonic acid groups react with high specificity with basic amino acids in collagen molecules. Because PSR molecules align parallel to the long axis of each collagen fiber, PSR enhances the birefringence of collagen under polarized light microscopy.<sup>17,21,22</sup> In addition to detecting bulk collagen, PSR staining has been used to distinguish collagen type I from collagen type III in several tissues, such as those of the musculoskeletal system,<sup>23–25</sup> liver,<sup>26</sup> gastrointestinal tract,<sup>27</sup> skin,<sup>28,29</sup> and myocardium.<sup>30</sup> With visualization of PSR-stained tissues under polarized light, termed *PSR-polarization*, thick collagen type I fibers appear yellowish-orange, orange, to red, whereas thin collagen type III fibers are supposed to appear green to yellowish-green against a black background.<sup>23,31</sup>

Although many studies have confirmed that PSR staining is useful for studying the collagen network under normal conditions and in pathological processes in different tissues,<sup>23–28,32,33</sup> other studies have questioned whether this staining can identify collagen types by their colors under polarized light.<sup>29,34–36</sup> For instance, one study using PSR-stained skin sections from patients with Ehlers–Danlos syndrome type IV showed fibers displaying the birefringence of collagen type III (greenish), although this condition is characterized by collagen type III deficiency.<sup>29</sup> This questions the ability of the technique to accurately distinguish between collagen types.

With this as background, while preparing for a large-scale rat tendon study, we evaluated our own tendon collagen data using the PSR-polarization method. We aimed to investigate the ability of this method to distinguish collagen type I from collagen type III in rat tendons.

## Materials and Methods

### *Animals and Collection of Achilles Tendon Specimens*

Achilles tendons were obtained from Sprague Dawley rats aged 3–6 months (Charles River Laboratories; Raleigh, NC) and fixed immediately after the animals were euthanized. To determine the effect of histological fixative type, tendon specimens were fixed in either Bouin fixative (Polysciences, Inc; Warrington, PA), which has traditionally been used for PSR staining, or 10% neutral-buffered formalin solution (Thermo Scientific Richard-Allan Scientific; Waltham, MA) for 24 hr. Bouin-fixed tissues were washed thoroughly in tap water until the water ran colorless. Fixed specimens were dehydrated in a series of graded ethanols and embedded in paraffin. Specimens were sectioned longitudinally at a thickness of 5  $\mu\text{m}$  using an automatic microtome (HM 355S; Thermo Scientific, Kalamazoo, MI), mounted onto positively charged slides (Fisherbrand Superfrost Plus Microscope Slides; Fisher Scientific, Pittsburgh, PA), and used for PSR, hematoxylin–eosin (H&E), and immunohistochemical staining.

### *PSR Staining*

Serial paraffin-embedded sections of tendon tissue were stained with PSR using a commercial kit (Polysciences, Inc), according to the manufacturer's protocol. Briefly, paraffin-embedded sections were deparaffinized and rehydrated through a series of xylene and graded alcohol washes, followed by staining with Weigert hematoxylin for 7 min. Slides were washed for 10 min in running tap water, treated with a solution of

phosphomolybdic acid for 2 min (solution A), and then rinsed in distilled water. Next, the sections were stained in PSR F3BA (pH 2) (solution B) for 1 hr and then washed in 0.1 N hydrochloric acid (solution C) for 2 min. Slides were submerged in 70% ethanol for 45 sec, quickly dehydrated in graded ethyl alcohols, cleared with xylene, and mounted with xylene-based mounting medium (Thermo Scientific Richard-Allan Scientific).

H&E staining of Achilles tendon was performed using standard protocols for the histological assessment of tendon structure and collagen fiber organization.

### *Polarized Light Microscopy and Imaging*

The tissue sections were analyzed under linear polarized light or brightfield microscopy using a microscope (IX83; Olympus, Waltham, MA) equipped with a digital camera (DP80; Olympus) with its dual charge-coupled device sensors, a rotating polarizer (IX-LWPO) built into the condenser with a fixed analyzer (U-ANT), and a high-luminosity and high-color-rendering LED. The IX83 also has a fully motorized stage with a circular rotating slide holder (H224ROTS; Prior Scientific, Cambridge, UK) capable of 360° rotation. The sub-stage condenser height and the field iris diaphragm were aligned for proper Köhler illumination to ensure even illumination of the specimen. The optimal exposure time setting was determined by acquiring a series of images at varied lengths of time with constant illumination, choosing the exposure time that guaranteed capture of the brightest yellow/red and green birefringence possible without pixel saturation against a dark background. The exposure time was readjusted every time the objective and the slide changed.

To evaluate how the tendon orientation on the slide may influence the polarizing color of PSR-stained collagen fibers, the slide holder was rotated in the axial plane in increments of approximately 22.5° counterclockwise, with the tendon long axis oriented at 22.5°, 45°, 67.5°, and 90° and then back to the original 0° position. Images were stitched at 4× magnification using the cellSens imaging software (Olympus, version 3.2) to capture the entire cross-sectional area of the tendon. Anatomic landmarks (e.g., paratenon) on the tendon sections were used as points of reference to acquire the same region of interest in each tendon orientation, which represented the area of best tendon morphology. Two consecutive slides of each specimen were examined independently by three investigators (C.M.L.D.P., S.A.M., M.J.C.).

### *Immunohistochemistry*

As a positive control, collagen type I and collagen type III were identified on Achilles tendon sections by double

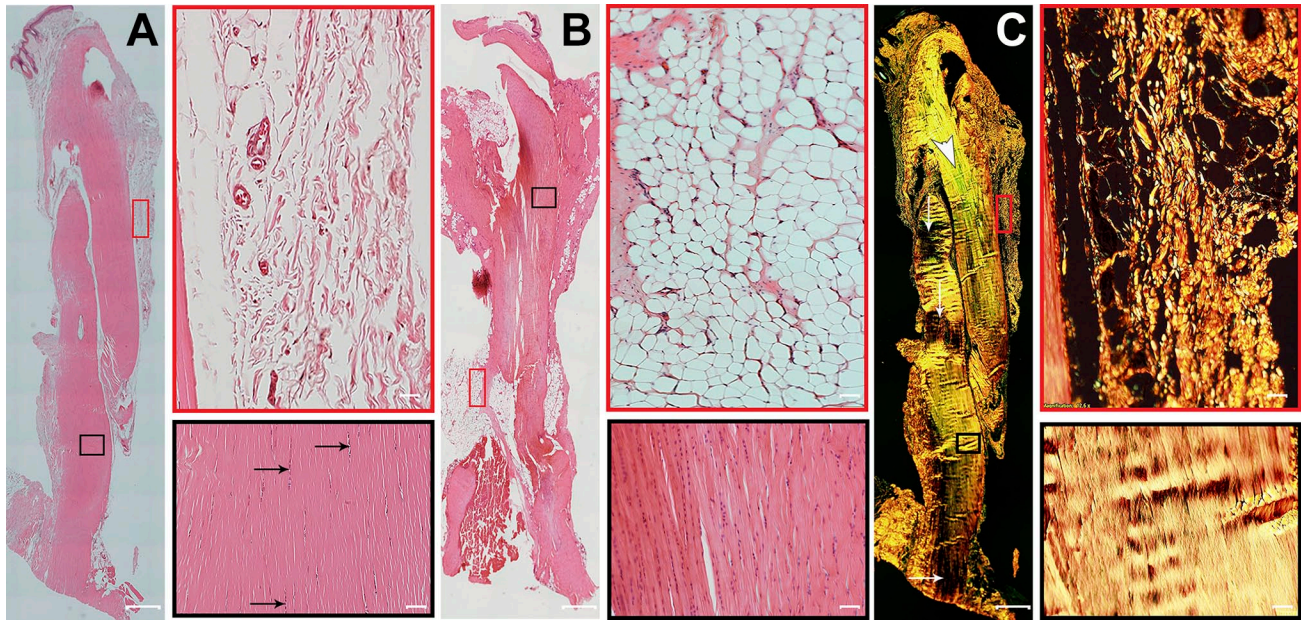
immunohistochemistry. Briefly, formalin-fixed tissue sections (5 μm) were deparaffinized and rehydrated as described above, and then washed twice for 5 min in phosphate-buffered saline (Sigma; St. Louis, MO). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, followed by treatment with 0.1% hyaluronidase with 0.1% pronase in tris-buffered saline (all from Sigma) in a water bath for 30 min at 37C to unmask the collagen antigens. Slide sections were washed 3 times in tris-buffered saline/0.1% Tween-20 (TBS-T) for 5 min each. Nonspecific binding was blocked with 5% normal horse serum (Abcam; Cambridge, MA) and 1% bovine serum albumin (Sigma) in TBS-T for 30 min. Slides were incubated with a specific mouse monoclonal anti-collagen type I antibody [COL1 (ABIN957814), 1:50; Antibodies-Online, Limerick, PA] in a humidified chamber at room temperature for 1 hr, washed 3 times in TBS-T for 10 min, and incubated for 20 min with the 2-step HiDef Detection HRP Polymer System (954D-10; Cell Marque Millipore Sigma, Rocklin, CA). This was followed by incubation with horseradish peroxidase detector reagent for 10 min.

The antigen-antibody complex was detected by reaction with 3,3'-diaminobenzidine peroxidase substrate (ImmPACT DAB; Vector Laboratories, Burlingame, CA), which yields a brown product. Diaminobenzidine-stained slides were washed and subsequently incubated with the second primary antibody, a rabbit polyclonal specific to collagen III (ab7778, 1:300; Abcam) in a humidified chamber at 4C overnight. The next day, slides were washed 3 times in TBS-T for 10 min and treated with the 2-step HiDef Detection alkaline phosphatase polymer system (962D-10; Cell Marque Millipore Sigma), incubated with the amplifier solution for 10 min at room temperature, and then washed 3 times in TBS-T for 10 min, followed by incubation with alkaline phosphatase polymer detector solution for 10 min at room temperature. Collagen type III staining was developed with permanent red chromogen (960D-10; Millipore Sigma) that gives a red precipitate. Nuclei were counterstained with hematoxylin (Vector Laboratories), then quickly dehydrated in a graded ethyl alcohol series, cleared with xylene, and mounted with xylene-based mounting medium.

To verify the specificity of collagen immunolabeling, isotype controls consisting of mouse IgG1 kappa (1:50) and rabbit IgG polyclonal isotype (1:300) were included in a set of sections. Positive controls consisted of formalin-fixed, paraffin-embedded rat skin (collagen type I) and umbilical cord (collagen type III).

### *Quantitative Imaging*

Images were quantified using the Count & Measure module of cellSens Dimension software (Olympus).



**Figure 1.** Histological structure of healthy Achilles tendon. Representative serial longitudinal sections of Achilles tendons, with two fields of view of paratenon (red boxes) and a region of the mid tendon section (black boxes). (A) Formalin-fixed, hematoxylin–eosin (H&E)-stained section shows uniformly organized parallel collagen bundles and elongated tenocyte cell nuclei (black arrows). The paratenon is seen as loose connective tissue surrounding the tendon. (B) Bouin-fixed, H&E-stained tendon shows collagen fibers appearing intensely eosinophilic with nuclei stained darkly. In addition, the shrinkage effect of picric acid in Bouin solution causes artifactual changes in the normal appearance of tendon. (C) Formalin-fixed, picrosirius red staining on a sequential tissue section from the same tendon as panel A shows thick, bright yellowish-orange fibers. Another tendon area with greenish collagen fibers (white arrowhead) also contains some fibers that are at extinction (white arrows). The paratenon is seen with both thin fibers and thick fibers of yellowish-orange birefringence by polarizing microscopy. Tendon overview images were obtained with a 4× magnification lens (scale bar: 500 μm). Higher magnification images within the boxes were obtained with a 20× magnification lens (scale bar: 50 μm).

Total collagen was quantified using the minimum/maximum threshold settings of 47/256 for the red channel, 15/256 for green, and 0/128 for blue. The background was quantified using minimum/maximum thresholds of 0/47, 0/29, and 0/5 for the red, green, and blue channels, respectively. Sum areas of total pixels were automatically generated via the Count & Measure module for the above settings and used to calculate the percentage of total collagen and background for each tissue.

## Results

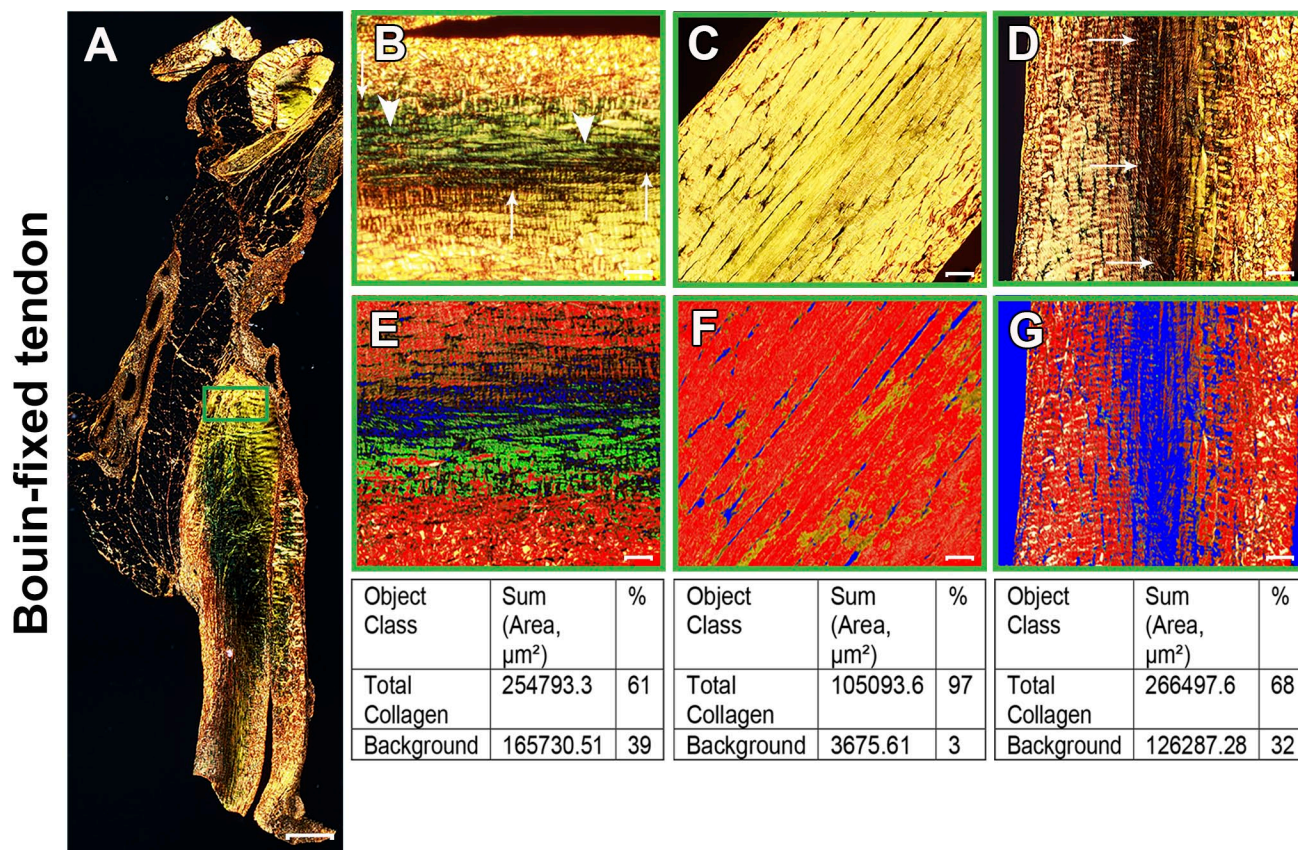
### *Histological Assessment of Tendon Structure*

The Achilles tendon structure was evaluated by H&E staining. Overall, light microscopic examination showed that the normal tendon structure was maintained in both formalin- and Bouin-fixed tendon tissues. However, the formalin-fixed tendons showed higher histological quality, characterized by uniformly bright eosinophilic collagen fibers with crisper and better nuclear staining (Fig. 1A). In Bouin-fixed tendons, in contrast, the eosinophilic and basophilic cell structures were stained with

a different intensity. For instance, the collagen fibers appeared intensely eosinophilic and nuclei stained darkly. In addition, shrinkage of the collagen fibers in Bouin solution caused artifactual changes in the normal appearance of tendon; the collagen fibers were more separated and assumed a wavy appearance (Fig. 1B). All slides, regardless of the fixative, were of appropriate quality for the PSR evaluation.

### *Evaluation of Collagen Fibers in PSR-stained Rat Achilles Tendon*

We configured the image capture program to crossed polarized light and Köhler illumination to achieve proper light extinction and the brightest yellow/red and green birefringence without pixel saturation, as shown in Fig. 1C. In the evaluation of polarizing colors and birefringence intensities of the collagen fibers, the whole-tendon sections showed that the interference color of the collagen was not distributed homogeneously throughout the entire section, giving different patterns of color and intensity of birefringence in both formalin- and Bouin-fixed tendons, which is due to the orientation of the collagen fibers. For optical detection,



**Figure 2.** Bouin-fixed normal Achilles tendon stained with picrosirius red (PSR). (A) Polarized view of representative entire longitudinal Achilles tendon section. (B–D) Magnifications of approximately the same region as the green box in panel A at three different orientations: 0° (B), 45° (C), and 90° (D). At 0° (B), collagen fibers showed a mixture of birefringence with yellowish-orange and greenish-yellow (arrowheads). As the microscope stage was rotated, the birefringence of collagen changed gradually. At 45° (C), the tendon appeared uniformly bright yellow. At 90° (D), birefringence changed to yellowish-orange with more collagen fibers at extinction (arrows in B and D). (E–G) PSR images acquired in the same regions of interest as panels B–D were quantitatively compared for the percentage of total collagen using the Count & Measure module of the cellSens software. Blue indicates background; the sum of red and yellow is total collagen, represented as red in the quantification panel. Tendon overview image (A) was obtained with a 4× magnification lens (scale bar: 500  $\mu\text{m}$ ). Higher magnification images within the boxes (B–F) were obtained with a 20× magnification lens (scale bar: 50  $\mu\text{m}$ ).

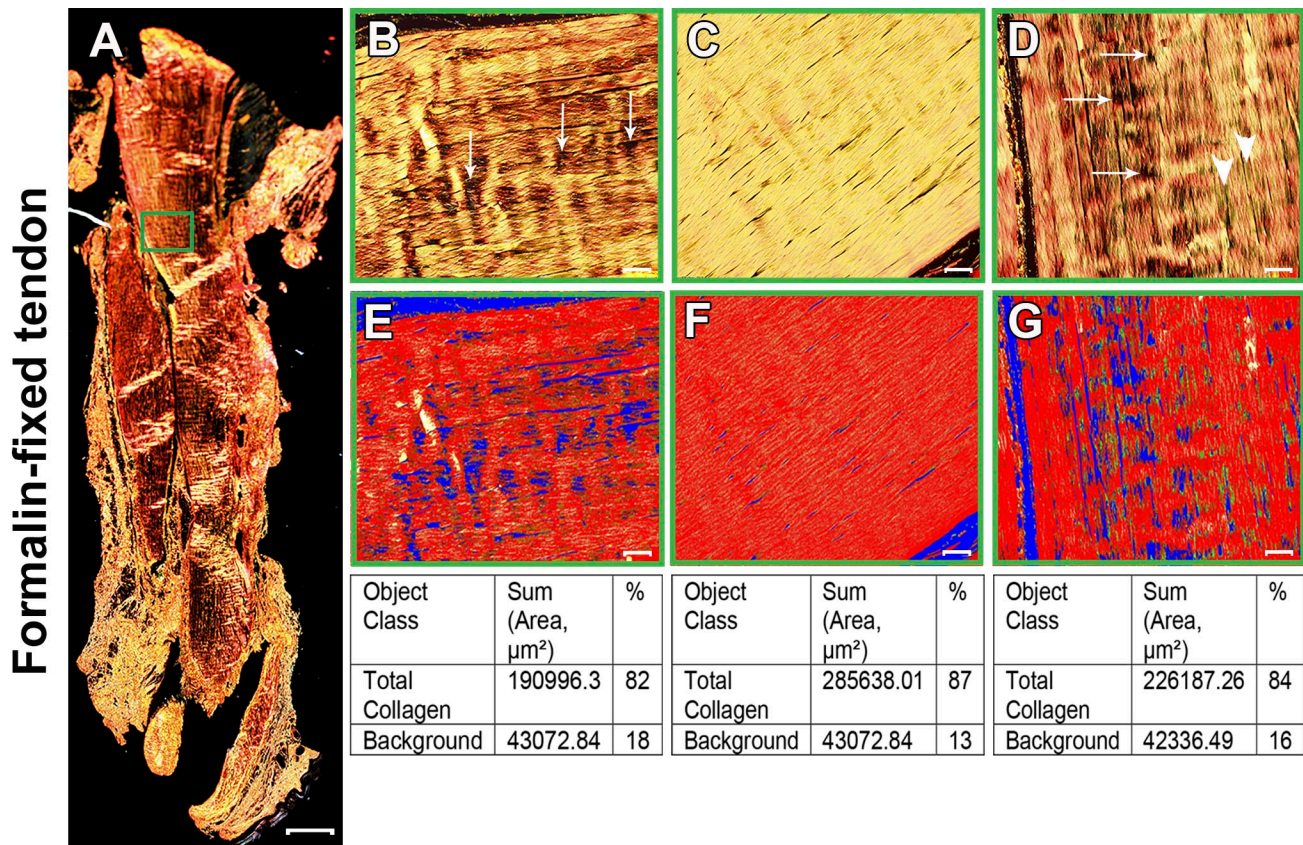
collagen fibers must be aligned in line with the plane of the polarized light, which is why a rotating stage is essential for imaging.

In Bouin-fixed tendons, collagen fibers showed predominantly greenish-yellow birefringence interspersed with some amount of yellowish-orange birefringence (Fig. 2A), and few sections displayed only yellowish-orange birefringence. In formalin-fixed tendons, collagen fibers showed predominantly yellow or yellowish-orange birefringence (Fig. 3A), with some areas of greenish collagen fibers colocalized with yellowish-orange fibers in a few tendons (Fig. 1C). Overall, Bouin-fixed tendons tended to present a darker hue that was more green, whereas formalin-fixed tendons tended toward a lighter hue that was more yellow. The paratenon showed both thin and thick collagen fibers with a brilliant yellowish-orange birefringence in both

Bouin- and formalin-fixed tendons (Fig. 1C); no greenish fibers were observed under polarized light.

### Tendon Orientation and Polarizing Colors of PSR-stained Collagen Fibers

We set up the microscope software user interface such that we could focus and acquire the same region on the same tendon section as the tendon slide was rotated on the microscope stage at different angles with respect to the transmission axis of either the polarizer or the analyzer. Representative examples of Bouin-fixed and formalin-fixed tendon tissues are shown in Figs. 2 and 3, respectively. The collagen fibers showed different birefringence colors according to the tendon orientation, particularly after slide rotation from 0° to 45° and 45° to 90°. In the formalin-fixed



**Figure 3.** Formalin-fixed normal Achilles tendon stained with picosirius red (PSR). (A) Polarized view of representative entire longitudinal Achilles tendon section. (B–D) Magnifications of approximately the same region as the green box in panel A at three different orientations: 0° (B), 45° (C), and 90° (D). Collagen fibers showed different birefringence colors depending on the tendon orientation. Overall, collagen was displayed predominantly as a parallel arrangement of thick, yellowish-orange birefringent fibers. At 45° (C), the collagen fibers appeared uniformly bright yellow. At 0° (B) and 90° (D), a striped pattern was seen, with some fibers at extinction (arrows). White arrowheads in D show some greenish collagen fibers colocalized with thick, yellowish-orange fibers. (E–G) PSR images acquired in the same regions of interest as panels B–D were quantitatively compared for the percentage of total collagen using the Count & Measure module of the cellSens software. Blue indicates background; the sum of red and yellow is total collagen, represented as red in the quantitation panel. Tendon overview image (A) was obtained with a 4× magnification lens (scale bar: 500  $\mu\text{m}$ ). Higher magnification images within the boxes (B–F) were obtained with a 20× magnification lens (scale bar: 50  $\mu\text{m}$ ).

tendons, at 0° and 90° orientations, collagen was displayed in a parallel arrangement of thick and wavy collagen fibers of predominantly yellowish-orange birefringence (Fig. 3B and D), with some amount of greenish-yellow thin fibers interspersed with yellowish-orange fibers showing at 90° (Fig. 3D). At 45° orientation, collagen fibers tended to present a uniform and lighter yellow color (Fig. 3C). In addition to the change of color as the slide was rotated, the tendon acquired a striped appearance, with some areas turning black along the long axis of the tendon section at 0° and 90° because in these orientations some collagen fibers were at their extinction angle (Figs. 2B and D, and 3B and D). At 45°, no fiber appeared to be in extinction.

In the Bouin-fixed tissue, two distinct colors could be seen frequently depending on tendon orientation. At 0°,

the collagen bundles showed predominantly greenish-yellow birefringence interspersed with some amount of yellowish-orange birefringence toward the edges of the tendon section (Fig. 2B). At 45°, the collagen fibers were straight, and all appeared bright yellow (Fig. 2C). At 90°, the color changed from the greenish-yellow birefringence seen at 0° to yellowish-orange (Fig. 2D), with more collagen fibers at extinction.

#### Quantitative Evaluation of Collagen Fibers

Because it has been claimed that PSR staining can distinguish between collagen type I (yellow-red) and collagen type III (green), areas exhibiting these colors were counted using the cellSens software. Depending on the tendon orientation, the ratio of yellow-red to

green differed. Therefore, both colors were counted together and compared with the background (no red, yellow, or green) (Figs 2E–G and 3E–G). This ratio was the highest at 45° and may serve to measure collagen density as the collagen to background ratio.

### *Immunolocalization of Collagen Type I and Type III in the Normal Achilles Tendon*

Immunostaining protocols were performed by double immunohistochemistry alone or combined with PSR. Combined immunolabeling followed by PSR staining was performed at the same time on the same section, as described by Gadd.<sup>37</sup> In our hands, however, PSR caused appreciable overstaining by the red chromogen, which made it difficult to assess the expression of type III collagen. Staining with PSR first, followed by immunolabelling, also did not work well because PSR faded from the slide during exposure to heat-induced antigen retrieval conditions. Therefore, the immunostaining and PSR staining were carried out separately on serial sections.

As shown in Fig. 4, the tendon core was strongly stained for collagen type I (thick brown collagen fibers) in formalin-fixed tendon, with low expression of collagen type III (thin reddish collagen fibers). Collagen type III was most highly expressed in the paratenon, endotenon, and blood vessels (Fig. 4). Bouin-fixed tendon sections lacked immunoreactivity for collagen type III in most tendons, which suggests that the antigen retrieval did not reverse the denaturing effect of Bouin fixation. None of the isotype controls showed any staining for either collagen type I or type III.

## **Discussion**

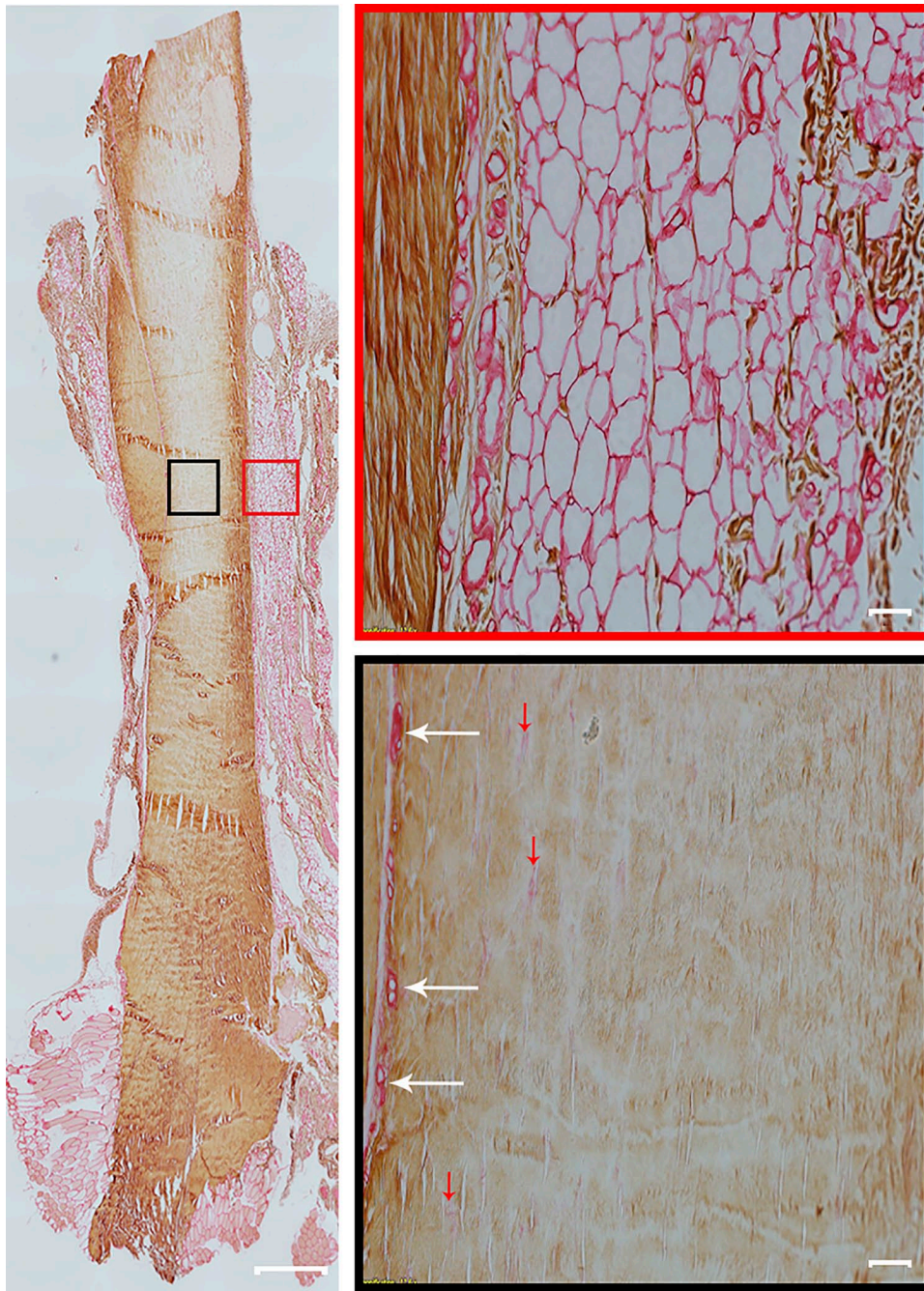
Collagen has an important role in maintaining the structural integrity of tendons and in determining their tensile strength.<sup>2</sup> Therefore, a better understanding of the relevant molecular and mechanical properties of collagen is an important objective in tendon research. For several decades, the group of Junqueira<sup>17,18,21–23</sup> promoted the use of PSR-polarization for the detection and analysis of collagen, and it has become widely used to distinguish between collagen type I and type III in different tissues. However, Pierard<sup>34</sup> questioned its utility for this purpose because PSR-stained collagen bundles in dermis sections changed from orange-red to green, and vice versa, in different slides originating from the same biopsy when the slides were rotated on the microscope stage. This observation was, for many years, cited only sporadically, and Piérard failed to report many aspects of his methods, such as the optical configuration and details about the

specimen orientation with respect to the polarizing filters, which are crucial for establishing the method's reproducibility. Lattouf et al.<sup>29</sup> also subsequently described how the polarization colors in skin strictly depended on the orientation of the collagen bundles and did not specifically reveal collagen types.

Nevertheless, the idea that a spectrum of yellow/orange/red birefringence accurately identifies collagen type I, and that green birefringence identifies collagen type III, has almost universally persisted since the early development of this method. At the time of this writing, querying PubMed for the phrase “picrorisius red staining AND collagen type I OR collagen type III” retrieved 4010 records, whereas searching for a conjunction of query terms (picrorisius red staining AND collagen type I AND collagen type III) retrieved 163 records. A cursory glance at these published studies shows that most identify a mixture of yellow, orange, and red for collagen type I and that some identify green fibers in tissue areas where collagen type III is not expected at all.

PSR-polarization can be considered the most direct method for collagen detection in tissue sections owing to the simplicity of the procedure. However, insufficient understanding of the polarization properties and polarization microscope configuration may introduce errors during image acquisition, which can lead to misinterpretation of the experimental data. In this study, we have made important observations that can be of practical value for the analysis of collagen fibers in PSR-stained tendons. We capitalized on acquiring stitched images of the entire tendon section, which allowed visualization of every portion of each collagen bundle.

We found that it is critical to use a polarizing microscope equipped with a rotating stage. Our results showed that a tendon section oriented at 45° with respect to the crossed polarizers provided the best qualitative and quantitative assessment of collagen organization in both Bouin- and formalin-fixed tendon tissues. At 45°, each individual collagen bundle appears as uniformly bright yellow birefringence against the dark background, rather than collagen bundles with a mixture of yellowish and greenish birefringence observed on the same tendon area at different angles. Collagen bundles usually appear crimped or wavy with alternating transverse dark bands under polarized light microscopy; it is said that the dark collagen bands are at extinction.<sup>38</sup> This collagen crimp pattern was also seen in PSR-stained Achilles tendons, particularly in Bouin-fixed tendon sections, and could result in underestimation of total collagen content, especially in tendon sections containing a large amount of wavy fibers where portions of the fibers appear dark.



**Figure 4.** Collagen type I and type III expression in rat Achilles tendon. A representative entire longitudinal Achilles tendon section is double immunolabeled for collagen type I and collagen type III. Positive immunolabelling for collagen type I is brown (diaminobenzidine chromogen) and for collagen type III is red (permanent red chromogen). Two high-magnification fields of view are shown of paratenon (red box) and the mid tendon section (black box). Collagen type I is present in tendon. Collagen type III is found mostly in the paratenon (red box) and blood vessels (black box, white arrows). However, a small amount of collagen type III is also found in the tendon core and indicated with red arrows (black box). Tendon overview image was obtained with a 4 $\times$  magnification lens (scale bar: 500  $\mu$ m). Higher magnification images within boxes obtained with a 20 $\times$  magnification lens (scale bar: 50  $\mu$ m).

Figures 2 and 3 demonstrate that smaller areas of collagen bundles were at extinction when the long axis of the tendon sections was oriented at 45 $^{\circ}$ , compared

with 0 $^{\circ}$  and 90 $^{\circ}$ . In addition, higher total collagen content values were obtained in PSR-stained tendon sections analyzed at the 45 $^{\circ}$  orientation, which indicates



that a 45° orientation may be more favorable if total collagen quantification is intended. Because the birefringent colors of the collagen bundles varied in shades of yellow/orange and green when the Achilles tendon slide was rotated on the microscope stage with respect to crossed polarizers, we conclude that PSR staining cannot accurately identify collagen type I and collagen type III fibers in Achilles tendon. This conclusion was confirmed by immunohistochemical mapping of collagen type I and type III in the same tendons.

Bouin fixative has historically been recommended as a fixative for collagen on paraffin-embedded tissues.<sup>39,40</sup> Although both formalin and Bouin fixatives have occupational exposure risks, Bouin fixative consists of a mixture of saturated picric acid, acetic acid, and formaldehyde. Picric acid is related to trinitrotoluene, which has been shown to be a high-power explosive compound; thus, it poses a potential explosion hazard when it dries. Because of these safety concerns, our institution has encouraged its replacement with an alternative fixative whenever possible.

The quality of PSR staining was compared between tendon tissues prepared separately in both fixatives. On the basis of our findings, both Bouin- and formalin-fixed tendon sections produced similar birefringence. However, compared with Bouin fixative, formalin fixation of rat Achilles tendon showed superior quality for histological detail on H&E staining with regard to fiber structure, fiber arrangement, and morphology of the nuclei. In addition, Bouin- and formalin-fixed Achilles tendons were examined for immunohistochemical localization of collagen type I and collagen type III under similar conditions. The enzymatic antigen retrieval treatment of formalin-fixed Achilles tendon sections satisfactorily retrieved both collagen type I and type III antigens, but it was ineffective for unmasking collagen type III on Bouin-fixed tendon sections. Bedossa et al.<sup>41</sup> reported that enzymatic treatment with pepsin was also ineffective for retrieving collagen type I and III antigens but effective for visualizing the type IV collagen antigen on Bouin-fixed liver tissue. Gala et al.<sup>42</sup> reported a list of antibodies that work well in Bouin-fixed bone marrow biopsies. Therefore, a different antigen retrieval protocol may be needed to expose the collagen type III epitope in Bouin-fixed tissue. Our data showed that formalin fixation is a suitable alternative to Bouin fixation of tendon when various histochemical procedures are intended in the same specimen.

In summary, this study clarifies the distribution of type III collagen in tendon and demonstrates that PSR-polarization alone does not enable the distinction of collagen type I and collagen type III in tendon tissue. Even when the polarizer filters are accurately crossed, the core tendon, which mainly consists of

densely packed bundles of collagen type I, showed more than one color in the shades of yellow/orange and green. Only a small amount of type III collagen is located within the tendon core, whereas more type III collagen is found within the epitenon and paratenon, and blood vessels of uninjured tendons. PSR can be used along with double immunohistochemical labeling when precise distinction of collagen type I and type III is necessary. PSR staining is an effective and low-cost tool to investigate collagen fiber content, organization, and orientation and, possibly, total collagen density and quantification, depending on the orientation of the tendon section on the microscope stage. Correct and standardized use of PSR staining is important for reliable and reproducible results.

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### Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Author Contributions

CMLDP was involved in conceptualization, methodology, investigation, formal analysis, writing—original draft preparation, and visualization. MJC was involved in conceptualization, formal analysis, resources, and review and editing. AT was involved in methodology, and review and editing. REDV was involved in formal analysis. CHE was involved in conceptualization, formal analysis, writing—review and editing, visualization, supervision, and funding acquisition. SAM was involved in conceptualization, formal analysis, investigation, writing—review and editing, visualization, and supervision.

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### Data Availability Statement

More supplemental data that support the findings of this study are available at: <https://figshare.com/s/097a7ae29d5f75051608>.

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