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COLLAGEN AND PICROSIRIUS RED STAINING: A POLARIZED LIGHT ASSESSMENT OF FIBRILLAR HUE AND SPATIAL DISTRIBUTION

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ABSTRACT

Collagen plays a vital role in maintaining structural integrity and in determining tissue function. Therefore, methods to detect, quantify, and analyze collagen are valuable. Nevertheless, stains historically employed to detect collagen have disadvantages, principally a poor specificity for thin fibers. Conversely, picrosirius red, which has the capability to detect thin fibers, although frequently used, is seldom exploited to the fullest extent. Our goal was, using picrosirius red staining, circularly polarized light, and image-analysis software, not only to identify fibers and quantify collagen content, but also to assess fiber hue and the spatial distribution of the different colors. To assess collagen content, we used a subtraction technique to remove interstitial space and non-collagen elements from images of skin wounds, myocardial scars, and arterial tissue. The hue component of the resulting image was obtained, and the number of red, orange, yellow, and green (the colors of collagen fibers in order of decreasing thickness) pixels calculated. Finally, we assessed the spatial distribution of individual colors by the application of color threshold filters. Skin wound analysis demonstrated good inter-observer agreement for collagen content and fiber color. In myocardial scars, collagen content increased from 1 (61%) to 5 weeks (95%) after injury. The proportion of green (thin) fibers decreased (43 to 4%), while the proportion of orange (thick) fibers increased (13 to 65%). Color threshold application revealed regional variation in fiber color within subintimal arterial lesions. These methods increase the amount of structural information obtained from picrosirius red-stained sections.

Key words: Birefringence, collagen, histology, picrosirius red, polarized light

INTRODUCTION

Collagen fibers play a vital role not only in maintaining structural integrity, but also in determining tissue function. For example, collagen degradation and loss after myocardial infarction is associated with infarct expansion and subsequent functional decline [33]. On the other hand, although collagen confers tensile strength, excess accumulation is often detrimental. For instance, increased fibrosis after kidney transplant leads to a decrease in renal function and eventual graft failure [6,9], and hence quantification of fibrosis has been suggested as a means to predict graft survival. In such examples, insight into pathological structure-function relationships depends upon accurate identification of collagen fibers.

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Traditionally, stains such as van Gieson and the various forms of trichrome have been used to detect collagen fibers in tissue sections. Although the mechanisms of these methods, which combine two or more anionic dyes, are not completely understood, they rely on differential binding by tissue components. Such differentiation is determined by a combination of differences in the relative size of the dye molecules, differences in the physical structure of the tissue (for example, tightly versus loosely packed), and differences in the amino acid composition of tissue elements [15]. The lack of precise selectivity of these factors suggests that van Gieson and trichrome stains may not be ideal for collagen detection. Moreover, both of these methods fail to reveal very thin collagen fibers, a disadvantage which can, under certain circumstances, lead to a substantial underestimation of collagen content [15,35].

This confounding issue (poor and variable staining of thin collagen fibers by van Gieson's stain) combined with the stain's tendency to fade,

prompted Puchtler and colleagues [29] to seek a better method. They found that Sirius red F3BA (Colour Index 35780) dissolved in a saturated picric acid solution consistently stained thin collagen fibers, did not fade, and was suitable for use with polarized light microscopy. The potential structural insight that could be revealed using the latter technique was emphasized in a subsequent publication from the same group almost 10 years later in 1973 [25]. Nevertheless, these two groundbreaking studies were, for many years, only sporadically cited. Although the combination of picrosirius red (PSR) and polarized light microscopy for the detection and analysis of collagen was also subsequently described and investigated by the Brazilian groups of Vidal [30] and Junqueira [12], it was not until the work of Pick and Weber in the 1980s that the method became widespread [11,31].

Despite the specificity conferred by the combination of PSR and polarized light, the method is not as widely exploited as it might be. Moreover, even when the combination is used, it is often only to derive qualitative [e.g., 8] or limited quantitative information. One explanation for this under-utilization is that it is impossible to visualize all of the collagen when linearly polarized light is used; because those fibers, or parts of fibers, aligned parallel to the transmission axis of either polarizing filter appear dark [34]. In contrast, the use of circularly polarized light removes this problem and allows visualization of every portion of every fiber [35]. In the current study, we capitalized on this advantage to derive additional information from PSR staining and assessed three structural parameters; collagen fiber content, hue, and spatial distribution.

MATERIAL AND METHODS

The use of animals in this study was in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals and all of the experiments were approved by the Institutional Animal Care and Use Committee. The sections used in this study came from ongoing or completed experiments in this laboratory, and were selected from a variety of tissues (rat skin and myocardium and rabbit iliac arteries) to illustrate differences in collagen content, fiber color, and fiber distribution. Skin was obtained from 5 female Sprague-Dawley rats 1 week after closure (with an interrupted suture line of 2-0 silk) of a left lateral thoracotomy. The myocardial tissue, also from female Sprague-Dawley rats (n=3), was obtained at 1, 3, and 5 weeks after a narrow band of transmural necrosis had been created using a frequency-tripled neodymium:yttrium-aluminum-garnet laser. The iliac artery tissue was examined 4 weeks after balloon angioplasty in New Zealand White rabbits (n=2) that had been subjected to 6 weeks of an atherogenic diet and de-endothelialization, a combination that produces variable amounts of arterial stenosis containing variable amounts of collagen [3].

The tissues were fixed in a solution of 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with PSR [25]. The sections were analyzed using an Olympus BX51 microscope (Melville, NY, USA) equipped with filters to provide circularly polarized illumination [7,26,35]. The lower filter (3M circular polarizer; Edmund Industrial Optics, Barrington, NJ, USA) was placed above the microscope's field iris diaphragm ring, while the upper filter was constructed from a combination of a quarter-wave plate (U-TP137, Olympus) placed below a linear polarizer aligned such that its transmission axis was at 45˚ to the fast axis of the wave plate [28,35,36]. These two filters were aligned so that the background in the field of view was as dark as possible (that is, the filters were "crossed"). Tissue images were obtained with a 40X objective lens, recorded on a digital camera (DP11, Olympus), displayed on a high-resolution monitor (Trinitron, Sony Corp., Park Ridge, NJ, USA) and analyzed using SigmaScan Pro image analysis software (SPSS Inc., Chicago, IL, USA).

Collagen content and fiber color

Collagen content was calculated as a percentage of the area of each image (expressed in pixels). A complication can arise because collagen is not the only birefringent material found in the tissues; for example, fibrin, which is weakly birefringent and appears green when stained with PSR, was present in both the skin and arterial tissue samples. So, the next step was to remove as much of the birefringent non-collagen tissue as possible, which was achieved using an image subtraction method. We first performed a color separation on the original (circularly polarized) images, resolving each into its cyan, yellow, magenta and black components (using an automated function provided by the image-analysis software). Of these, only the black component was required and so the other 3 components were discarded. In the black-component image, collagen fibers appeared dark gray/black, while the interstitial space and most non-collagen elements appeared light gray/white. In contrast, in the circularly polarized image, the collagen fibers appear bright while the interstitial space and non-collagen elements appear darker. Therefore, subtracting the black-component image from the polarized image will eliminate interstitial space and non-collagen elements, but not the collagen. The brightness level of the black-component image was adjusted prior to subtraction to ensure that the thinnest collagen fibers were not also eliminated (confirmed by inspection). Examples of original, black component, and final subtracted images,

obtained from arterial tissue, are shown in Figure 1. In practice, a subtraction that retained the thinnest green collagen fibers also often retained a small amount of interstitial space and fibrin.

The final step was to determine the hue (color) of each pixel within the subtracted image. It is known that the color of collagen fibers stained with picrosirius red and viewed with polarized light depends upon fiber thickness; as fiber thickness increases, the color changes from green to yellow to orange to red [10,14]. To determine the proportion of different colored collagen fibers, we resolved the subtracted image into its hue, saturation and value components (also an automated function provided by the image-analysis software). Only the hue component was retained and a histogram of hue frequency was obtained from the resolved 8-bit hue images, which contain 256 colors. We used the following hue definitions; red 2-9 and 230-256, orange 10-38, yellow 39-51, green 52-128 (a modification of the definitions used by MacKenna *et al.* [16]. The hue range 129-229 consisted of interstitial space and non-birefringent tissue elements such as red blood cells (confirmed by inspection). The number of pixels within each hue range was determined and expressed as a percentage of the total number of collagen pixels, which in turn was expressed as a percentage of the total number of pixels in the image. The analysis of fiber content and hue was applied to sections from the skin, myocardial, and arterial samples.

Reproducibility: To establish the method's reproducibility, both co-authors independently analyzed 5 randomly selected dermal wound images. We determined the total percent collagen and the relative content of each hue in these images and calculated the gradient and regression coefficient of the resulting correlation plot.

Spatial distribution of fibers using Color Threshold analysis

We applied the software's "color threshold" function to the iliac artery sections to isolate fibers of different hue. Such sequential isolation facilitated assessment of the spatial distribution of various fiber populations. The color threshold for each range described above was applied (all other fibers and interstitial space were removed) and the resulting images examined.

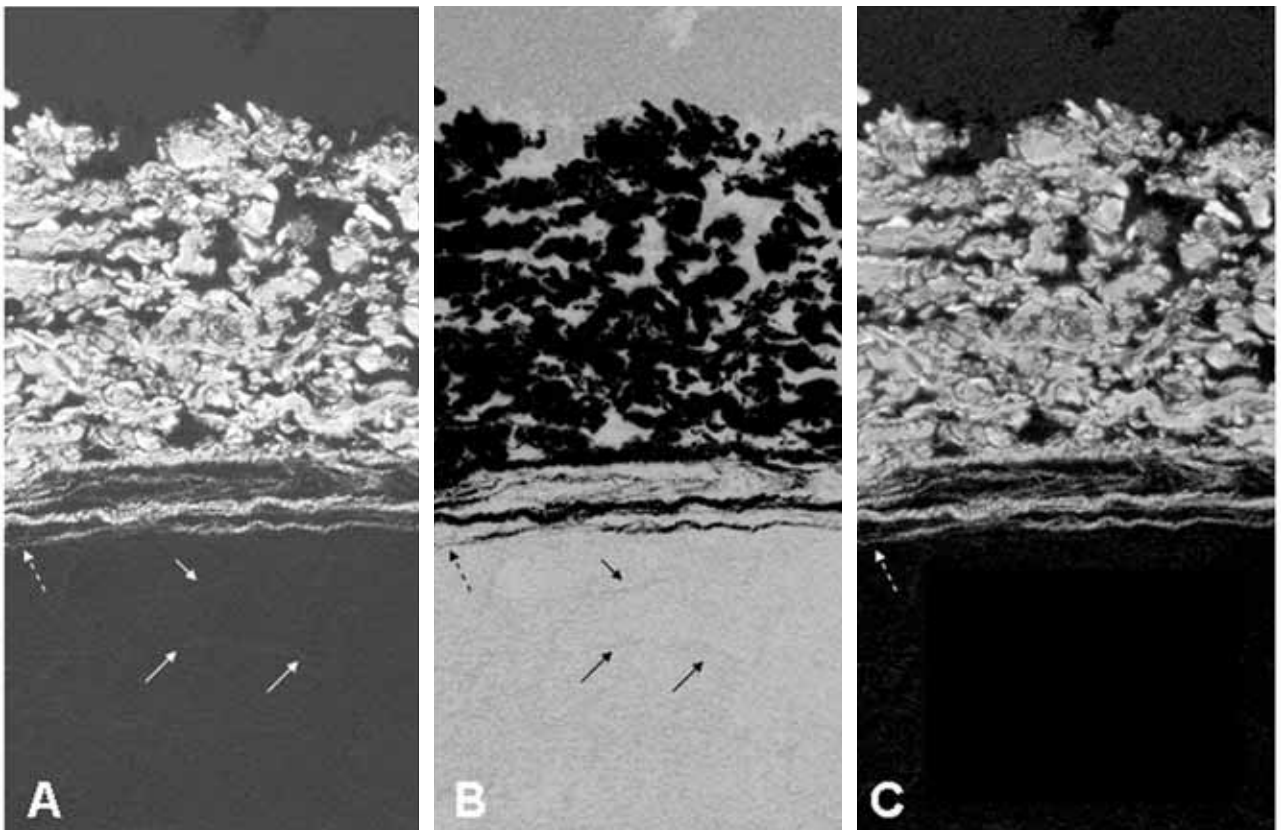

Figure 1. Series of images illustrating the image subtraction method in an arterial sample. (**A**) Original circularly polarized image. The solid arrows point to faint strands of weakly birefringent fibrin, while the dashed arrow points to thin green collagen fibers. (**B**) The black component image obtained after color separation. The fibrin appears a light grey (brighter than they were in panel A), while collagen appears either black or dark grey (darker than in panel A). (**C**) The final subtracted image. Although the fibrin has been removed, the thin green collagen fibers remain.

RESULTS

Collagen content and fiber color reproducibility

There was good correlation between the values obtained by both investigators for total collagen content (r = 0.993; P = 0.0003) and also fiber color (r = 0.999; P < 0.0001) in the skin samples. The gradient of both lines was very close to 1 (1.090 and 0.996, respectively) indicating good inter-observer agreement (Fig. 2). In addition, both regression lines passed close to the origin and thus were equivalent to the line of identity (not plotted for clarity).

Skin: Although these samples were suitable for the reproducibility test, they were from immature wounds that provided limited color differences; the fibers were mainly green. To illustrate the method's capability to discern color changes, we required samples with greater range. This range was provided by the heart and arterial samples.

Myocardium: There was, as expected, a temporal increase in scar collagen content; from 61% to 83% to 95% at 1, 3, and 5 weeks after injury. Furthermore, we found that the percentage of green (thin) fibers decreased at 5 weeks versus 1 week (from 43% to 4%), while the percentage of orange (thick) fibers increased (from 13% to 65%); consistent with scar collagen maturation (Fig. 3).

Arterial tissue: Evaluation of the iliac arteries revealed that the tunica adventitia contained the most collagen (89%) - primarily yellow fibers (55% versus 25% orange and 17% green), while the tunica media contained the least collagen (54%), but a higher proportion of green fibers (50% versus 8% orange and 41% yellow). In contrast, the majority of fibers in the subintimal lesion (68% collagen) were orange (52% versus 36% yellow and 10% green) (Fig. 4).

Color threshold analysis

The arterial lesion image (Fig. 5A) indicated a complex relationship between fiber color, alignment, and content. However, color threshold analysis revealed structural information. Specifically, fibers directly beneath the elastic lamina were mainly green and circumferentially aligned (Fig. 5B), while deeper regions contained radially aligned orange fibers (Fig. 5C).

DISCUSSION

We describe several techniques that augment the structural information obtained from analysis of collagen fibers in picrosirius red-stained sections.

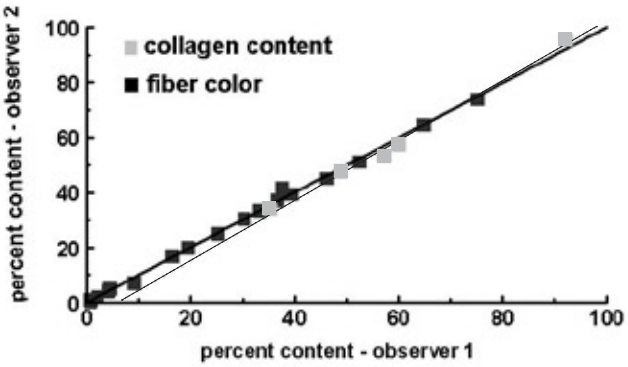

Figure 2. Reproducibility of collagen content and fiber hue analysis. There was a correlation between the values obtained by each co-author for total percent collagen content (dashed line; r = 0.9963; P = 0.0003) and percent fiber color (solid line; r = 0.9987; P < 0.0001). The gradient of both regression lines was close to 1.

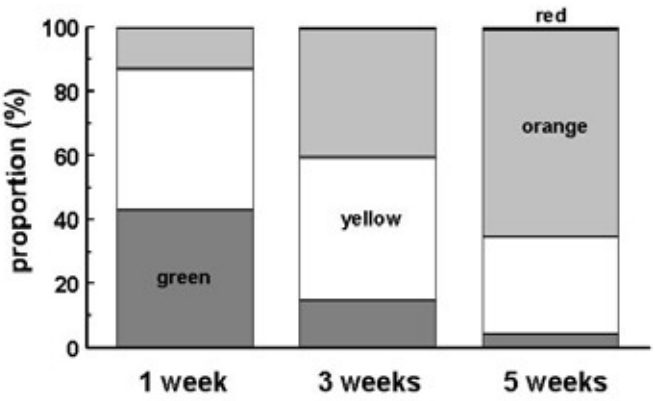

Figure 3. Collagen fiber hue changes in healing myocardial scars. The proportion of green (thin) fibers decreased with time, while the proportion of orange (thick) fibers increased.

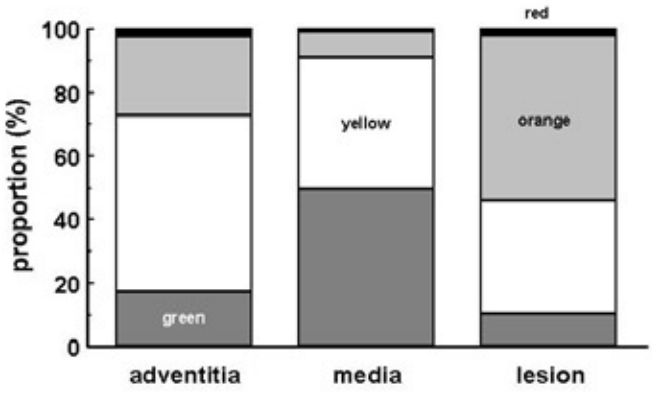

Figure 4. Regional variation in collagen fiber hue in an arterial section containing a subintimal restenosis lesion. The tunica adventitia contained mainly yellow fibers, while the predominant color was green in the tunica media and orange within the lesion.

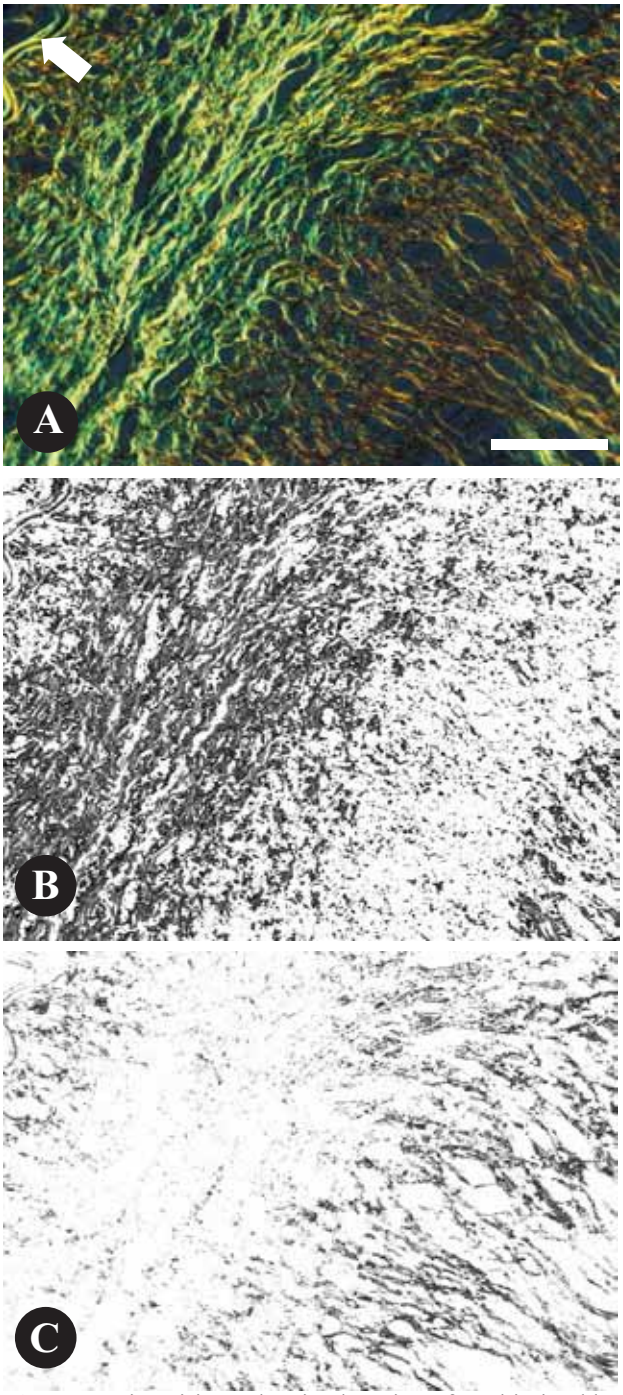

Figure 5. Picrosirius red-stained section of a subintimal lesion within a restenotic rabbit iliac artery. (**A**) Original image viewed with circularly polarized light. A short segment of the internal elastic lamina (IEL) can be seen in the upper left-hand corner (**arrow**) [Bar = 50 μm]. (**B**) Green threshold image. Only green fibers present in panel A are shown, everything else appears white. Most green fibers are located in the region immediately beneath the IEL and appear circumferentially aligned. (**C**) Orange threshold image. Only collagen fibers containing pixels with orange hues from panel A are shown. These fibers lie predominantly beneath the layer of green fibers and appear radially aligned.

Given the importance of collagen in multiple tissues and pathologies, these techniques may potentially be applied in many fields of medicine.

Confounding issues in collagen analysis

Staining methods such as van Gieson's and the various forms of trichrome have been regarded as specific for collagen and, despite their documented limitations, are still used. Perhaps more surprising is the continued use of hematoxylin and eosin to detect collagen [27], even though eosin has no specificity for collagen. Although most of the early published reports that used PSR staining for collagen did so in combination with polarized light, many recent studies employed brightfield illumination to detect collagen [18,19,22] (all 2004 publications). Nevertheless, with this approach, neither collagen fiber color nor brightness is uniform; thicker fibers appear deep red, while thin fibers appear bright pink and can be difficult to see. Some of these studies also attempted to quantify collagen content from brightfield images using methods ranging from time-consuming manual planimetry of red-stained areas [18] to a computer-generated conversion of the original color image to a black and white image based (presumably) on the assignment of positive (i.e., red) collagen staining to white and non-collagen and interstitial space assigned to black [19]. Both approaches have the potential to underestimate collagen content, especially in tissues containing large amounts of thin fibers. Moreover, these methods provide no structural information beyond assessment of collagen content. It is unclear why brightfield illumination rather than polarized light was used in these studies, and the authors provided no rationale.

Nevertheless, one disadvantage of polarized light may explain the persistence of brightfield illumination methods. If linearly polarized light is used, even birefringent PSR-stained fibers will appear dark if they are aligned parallel to the transmission axis of either of the two linearly polarizing filters. The use of a rotating microscope stage can minimize this problem by changing the orientation of the tissue section with respect to the transmission axes. Nevertheless, collagen fibers are frequently crimped or wavy and so, in most sections, some fibers will appear dark no matter how the microscope stage is rotated. Thus, total collagen content may be underestimated, especially in tissue containing large amounts of wavy fibers. Although

several groups have used linearly polarized light when measuring collagen content [9,16,20,21], this shortcoming is seldom acknowledged. The potential problem with this method is illustrated in a study that compared collagen content, assessed using three methods (Masson's trichrome and PSR with and without linearly polarized light), in biopsy samples obtained after kidney transplant [6]. The highest collagen content values were obtained in PSR-stained sections analyzed without polarized light. In contrast, the values obtained with PSR and polarized light and Masson's trichrome were 20-25% lower. Furthermore, the investigators found that collagen content measured without polarized light provided the best correlation with decreased graft function and concluded that the results validated the non-polarized method. Nonetheless, inspection of the figures in that paper reveals that the fibers, in addition to being wavy, encircled the renal tubules, which increases the likelihood of a relatively large proportion of the collagen being aligned parallel to the filters' transmission axis and hence the underestimation of collagen content. This potential orientation-mediated drawback is eliminated with circularly polarized light and even wavy collagen fibers will appear bright [35]. Although some have employed this approach [23,24], it is not, despite the advantages, widely used. Our current study emphasizes circularly polarized light's ability to reveal structural detail and extends the initial application (collagen content measurement) to assessment of the hue and spatial distribution of fibers.

Analysis of collagen fiber hue

The change in the color of PSR-stained collagen seen with polarized light as fiber thickness increases is well known and is often commented upon. Nevertheless, such descriptions tend to be qualitative rather than quantitative. A small number of studies have attempted to quantify fiber color; however, these attempts usually employ time-consuming evaluation of individual fibers using point-counting methods [1,10] – one computerized method [16] used linearly polarized light and hence underestimated collagen content. Our hue assessment not only allows rapid analysis of large areas, but also eliminates subjectivity associated with investigator descriptions of color. The hue ranges that we selected are empiric (our choice guided by the earlier work of MacKenna *et al.* [16]; however, they represent consistent objective criteria.

We used hue analysis to examine tissues in which we expected color changes to be present. Our assessment of arterial tissue was consistent with previous qualitative polarized light and electron microscopy reports that medial collagen fibers are thinner than those in the adventitia [2,17]. Similarly, prior qualitative studies have shown that collagen fiber thickness increases with time; either as a function of age [5] or as wound healing progresses [24,32]. Hence, as expected, we found a time-associated shift from green to orange in PSR-stained fibers within myocardial scars.

We propose that hue analysis could provide insight into wound healing therapies, efforts to limit fibrosis, and investigation of collagen degradation. However, we should emphasize that fiber hue does not permit identification of collagen fiber type as some have suggested [13]. Although type III fibers are usually thinner than type I fibers, it would not be valid to state that green fibers are type III and orange fibers type I. It is possible that a green fiber may be an immature, thin type I fiber or that a sectioning artifact "smeared" a thick type I fiber decreasing its thickness.

Color threshold as a tool for morphological analysis

The structure revealed by PSR staining and polarized light microscopy is often a complex mix of different collagen fiber densities and colors. In addition, both of these parameters frequently exhibit spatial variation. The use of color threshold analysis permits resolution of complex configurations into their constituent parts and thus facilitates visualization and assessment of structure. To our knowledge, such analysis has not been reported. In the specific example that we examined, color threshold analysis revealed spatial differences in arterial lesion structure. We anticipate that similar analysis could be used to study temporal changes in fiber organization; for example, in wound healing or in the development of fibrosis-related pathologies.

Limitations

Although materials such as keratin and fibrin are only weakly birefringent [4,36], their birefringence can equal that of the thinnest collagen fibers and hence their presence complicates analysis. In some cases, it is possible to crop such elements from the image; however, in cases where collagen comprises a relatively small proportion of the tissue (for

example, in non-infarcted myocardium), or when the other birefringent material is distributed throughout the image, cropping is not practical. In such cases, image subtraction techniques similar to the one that we employed may be effective. The requirements for success are that the material to be eliminated must appear brighter in the subtraction image than in the original circularly polarized image and, conversely, that collagen must appear darker in the subtraction image than in the polarized image. Hence, only the unwanted material is removed. Our subtraction method was specifically tailored to analyze the sections examined in this study. If collagen is to be assessed in another type of tissue, then a different color-separation component or the use of an appropriate filter might provide a better subtraction image. In some cases, it may not be necessary to perform a subtraction at all.

A key requirement is that the circular polarizers be aligned in the crossed position. Imprecise alignment can result in the appearance of orientation-dependent fiber colors; specifically a green-yellow change (that this does not occur is confirmed by rotating the microscope stage).

Conclusions

We have demonstrated that the combination of picrosirius red staining, circularly polarized light, and hue analysis provides a powerful tool for the structural analysis of collagen fibers. Many investigators have access to most of the components that we used and hence adoption of our methods would be straightforward. From published reports, it appears that the components most often missing are the circular polarizing filters; however, these are relatively inexpensive, readily available, and are not difficult to incorporate into an existing microscope system.

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