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Quantitative assessment of myocardial collagen with picrosirius red staining and circularly polarized light*

P. Whittaker[1], R. A. Kloner[1], D. R. Boughner[2], and J. G. Pickering[2]

[1] The Heart Institute, Hospital of the Good Samaritan and Department of Medicine, Section of Cardiology, University of Southern California, Los Angeles, USA. [2] Robarts Research Institute and the Departments of Medical Biophysics and Medicine, University of Western Ontario, London, Ontario, Canada.

Summary: Collagen plays a major role in the structural organization of the heart and therefore direct visualization of collagen fibers is a crucial component of cardiac analysis. Although linearly polarized light has proven an effective tool for the examination of myocardial collagen in histologic sections, the use of circularly polarized light may offer advantages and additional possibilities. We examined the potential enhancement of collagen analysis using circularly polarized light in two ways. We first measured the brightness, and hence indirectly assessed the birefringence, of collagen fibers in scars examined at different times after myocardial infarction. Secondly, we measured collagen content in myocardial tissue and compared results obtained from brightfield analysis of trichrome stained sections with those obtained from circularly polarized light analysis of picrosirius red stained sections. We observed a progressive increase in the maximum brightness of collagen fibers in the scar with time, and a time-dependent shift in the relative distribution of collagen fiber brightness from lower to higher levels. We found consistently lower values of collagen content in trichrome stained versus picrosirius red stained tissue, and concluded that trichrome staining underestimated collagen content. The information provided by these studies could not be obtained by brightfield analysis and could be only partially obtained from linearly polarized light analysis. Thus, analysis using circularly polarized light has the ability to enhance histologic assessment of tissue and can provide additional insights into the composition and structure of myocardial collagen.

Key words: Collagen, heart, histology, polarized light microscopy, stains and staining

Introduction

There has been increased interest in the role played by collagen in normal, injured, and diseased hearts (3, 24, 26). Direct visualization of collagen fibers in histologic sections is an important method of evaluation and provides information difficult to obtain by other means. Polarized light microscopy has proven effective in qualitative and quantitative histologic examination of collagen in the heart (17, 25, 28, 29) and other tissues (30, 31, 32). This effectiveness derives from the ability to assess collagen organization at both fiber and molecular levels by examination of polarized light's interaction with collagen. Such studies have used linearly polarized light. Circularly polarized light, offers advantages and additional possibilities by eliminating some of the orientation-related effects associated with linearly polarized light. Although circularly polarized light

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has been used for qualitative examination (1, 6, 21), its quantitative use has been limited to orthopedics (2). Our aim was to assess the potential of this optical technique in cardiovascular tissue by considering two specific problems; 1) measurement of changes in the optical properties of collagen fibers in scar tissue during wound healing, and 2) measurement of collagen content in histologic sections.

The optical properties of collagen change with fiber age. Specifically, as scar collagen matures there is an increase in its birefringence and hence an increase in the retardation of polarized light passing through it (12, 13, 27). However, the methods used to measure these changes are time consuming and each measurement is made on a single fiber. We therefore sought to demonstrate how circularly polarized light, in conjunction with digital image analysis, could be used to enable rapid analysis of relatively large areas. In addition, because the optical changes in scar collagen are well documented, we used this part of the study to validate the circularly polarized light technique.

We then addressed the problem of collagen content measurement in histologic sections. Such measurements are usually done in conjunction with trichrome staining (8, 9, 16); however, trichrome can yield variable staining results and may not be specific for collagen. We therefore sought to compare collagen content assessed from brightfield analysis of trichrome stained sections with that obtained from circularly polarized light examination of sections stained with picrosirius red, a stain thought to be specific for collagen when used with polarized light (10).

Methods

All of the tissue used in this study came from animals that were part of control groups in other ongoing or completed experiments in this laboratory. The myocardial tissue was fixed in a 10 % solution of formalin, embedded in paraffin, sectioned at a thickness of either 5 or 7 μm, and stained with either picrosirius red (20) or Masson's trichrome.

Microscope setup

We examined the sections on a Nikon Optiphot-pol microscope (Nikon Inc., Garden City, NY), using either brightfield or circularly polarized light. Circularly polarized light was obtained as described by Frohlich (6) and Schnabel (21). The analyzer (upper filter) was rotated so that its transmission axis was aligned at 45° to the fast axis of a quarter-wave plate placed above it. The polarizer (lower filter) was replaced by a circular polarizer (Meadowlark Optics, Longmont, CO) aligned so that the field of view was dark. Images were observed on a monochrome, solid-state charge-coupled device video camera (4800 series, Cohu Inc., San Diego, CA), mounted on the vertical tube of the microscope along with a X1 relay lens. The images were digitized by a videoframe-grabber (Truevision Inc., Indianapolis, IN), displayed on a high resolution monitor (PVM 1342Q trinitron, Sony Corp., Japan) and analyzed using the Java video analysis software package (Jandel Scientific, Corte Madera, CA).

Scar maturation

We examined tissue from 15 dogs that had been part of a previous study (27). We examined collagen in myocardial scar tissue at 1 (n=4), 3 (n=4), and 6 (n=7) weeks

after permanent coronary artery occlusion in sections (7 μm thick, one section per heart) stained with picrosirius red.

Measurement of collagen birefringence correlates with collagen brightness when viewed with polarized light (19). We therefore measured collagen brightness in two regions of longitudinally sectioned fibers in the midmyocardium of each section viewed with circularly polarized light using a X20 objective lens. In addition , we measured the brightness of longitudinally sectioned collagen in two regions of visceral pericardium distant from the scar. This latter tissue was assumed to represent normal, mature collagen. To eliminate effects from the interstitial space, an image of the background (a field of view with no tissue present) was subtracted from each image of scar collagen examined. A histogram of pixel brightness was plotted (brightness was expressed on a 0-255 gray scale, 0=black and 255=white). All of the interstitial space had a gray level of zero after subtraction. We calculated the mean gray level of the collagen for each field examined and expressed the average gray level of collagen fibers in the scar as a percent of the average gray level of collagen fibers in the pericardium.

Collagen content

We analyzed serial sections (5 μm thick) from 7 rat hearts, selected to provide a range of collagen contents. Specifically, we examined tissue from the free wall of the left ventricle, right ventricle, and intraventricular septum from mature (9 months old, n=2) and aged (24 months old, n=2) female, Fisher 344 rats. We also analyzed tissue from scars one week after left coronary artery occlusion in female Sprague-Dawley rats (n=3). Collagen content was assessed by two methods; point counting and digital image analysis. In total, we analyzed 18 fields of view; one each in the left ventricle, right ventricle, and interventricular septum of each Fisher 344 rat and 2 fields in the scar of each rat examined one week after coronary artery occlusion.

Point counting

We used an eyepiece reticle containing a 100-point rectangular grid. The number of intersections of the grid with collagen fibers was recorded when the tissue was examined using a X20 objective lens. The percent volume fraction of collagen (C_{vf}) was calculated according to the equation $C_{vf}=(P_c/P_r)$ X 100 % (where P_c equals the number of intersections with collagen and P_r the number of intersections within the reference area). Two consecutive sections from each sample were stained with trichrome and picrosirius red. First a region of tissue in the trichrome stained section was examined in brightfield and the number of grid intersections with collagen noted. The same region was located on the picrosirius red stained section viewed with circularly polarized light, and the measuring process repeated.

Digital image analysis

After point counting, we examined the same areas using digital image analysis. For trichrome stained sections, we illuminated the field of view via a red glass filter (Melles Griot, Irvine, CA). In trichrome stained sections, muscle appeared red and collagen fibers blue/green. The resulting red-filtered monochrome image consisted of dark collagen

fibers and bright muscle. The tissue was then illuminated via a red absorbing, blue glass filter (Melles Griot, Irvine, CA) to produce a monochrome image that contained bright collagen fibers and dark muscle. The red-filtered image was digitally subtracted from the blue-filtered image to produce an image composed of gray collagen fibers on a black background. A histogram of the brightness of each pixel in the image was plotted. All of the muscle and interstitial space was removed by the subtraction and had a gray scale level of zero. So any pixel with a gray scale level greater than zero represented collagen. Thus the collagen content could be expressed as the area fraction of pixels with a gray scale level greater than zero.

A similar subtraction method was used for the picrosirius red stained sections. Initially, a blue-filtered brightfield image was obtained. When picrosirius red stained sections are viewed in brightfield, muscle appears yellow and collagen red. Thus the blue-filtered, monochrome, brightfield image consisted of dark collagen fibers and bright muscle. This image was subtracted from a circularly polarized image, in which both muscle and collagen appeared bright. The result of the subtraction was an image consisting of bright collagen fibers on a black background. Collagen content was calculated as above. To eliminate muscle from the subtracted image, the brightness of muscle in the brightfield image was adjusted (by manipulation of the microscope lamp voltage) so that it exceeded the brightness of muscle in the circularly polarized image.

Statistics

For analysis of collagen maturation, comparisons between groups were done using one-way analysis of variance followed by simultaneous multiple comparisons using the Bonferroni method (23). Data were expressed as mean $\pm$ standard error of the mean. We used the Wilcoxon matched paired t-test and regression analysis for comparison of collagen content. Means were considered to differ significantly if $p<0.05$.

Results

Circularly polarized light

Figure 1 shows the same region of myocardium observed with linearly polarized (Fig. 1A) and circularly polarized light (Fig. 1B). Portions of collagen fibers viewed with linearly polarized light appear dark because they are aligned parallel to the transmission axis of one of the polarizing filters. In contrast, all birefringent tissue elements appear bright when viewed with circularly polarized light.

Scar maturation

We observed an increase in the maximum gray scale level with time, and a time-dependent shift in the relative distribution of collagen fiber brightness from lower to higher gray scale levels. Representative examples of individual brightness histograms from each time period are shown in Fig. 2. We found no changes in the frequency distributions obtained from pericardial collagen at 1, 3, and 6 weeks after infarction. Thus, in contrast to scar collagen, the optical properties of the pericardial collagen were constant. We found a progressive increase in the mean gray level of picrosirius red stained collagen

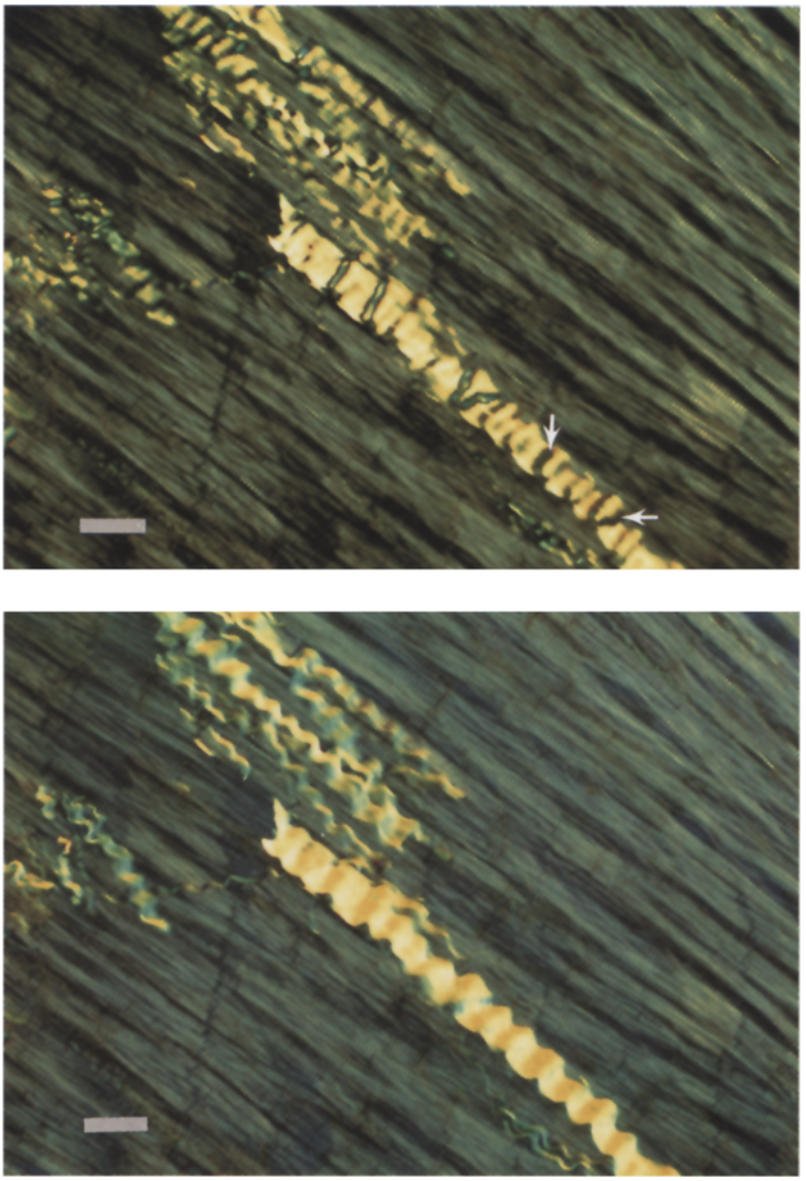

Fig. 1. Section (5μm thick) of rat myocardium stained with picrosirius red. Cardiac muscle appears green and the cross-striations can be seen. Collagen fibers are highly birefringent with thin fibers appearing green, and thicker fibers appearing yellow, or orange. A) Tissue viewed with linearly polarized light. Arrows indicate portions of collagen fibers that appear dark because they are aligned parallel to the transmission axis of one of the polarizing filters (the transmission axes are parallel to the edges of the micrograph). B) Same field viewed with circularly polarized light. All of the collagen fibers appear bright. [bar=25μm]

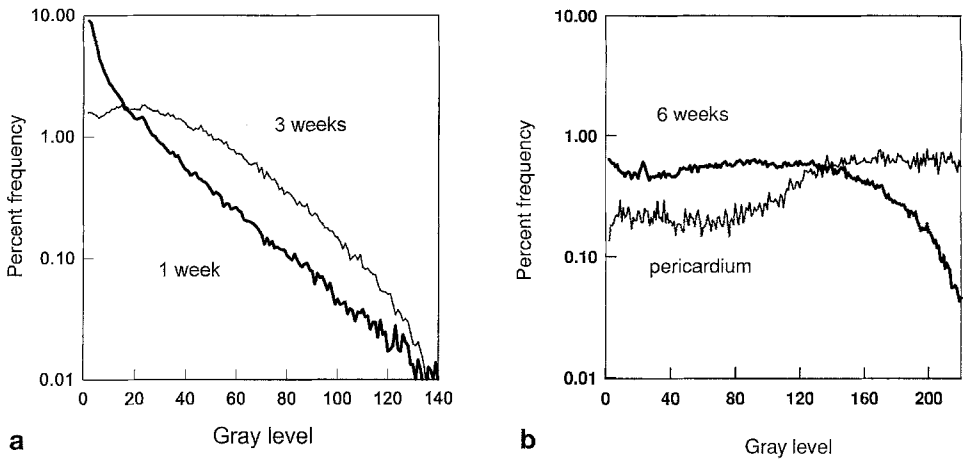

Fig. 2. Representative collagen fiber brightness histograms obtained from A) scar tissue at 1 week and 3 weeks after occlusion, and B) from visceral pericardium and scar tissue 6 weeks after myocardial infarction. The y-axis denotes the relative percent frequency of pixels with a particular gray level, while the x-axis denotes the gray scale level.

fibers in the scar, expressed as a percent of the mean gray level of collagen in the visceral pericardium, from 1 (21±7%) to 3 (45±2 %, $p<0.05$ versus 1 week), to 6 weeks (71±3 %, $p<0.01$ versus 3 weeks) after infarction (Fig. 3). In our study, trichrome stained collagen fibers were only weakly birefringent and changes in birefringence could not be followed.

Collagen content

The tissue selected provided a wide range of collagen content (an approximate 10-fold difference between minimum and maximum values). There was no significant difference between collagen content assessed by point-counting and that obtained by digital image analysis for either trichrome (5.3±1.2 % point-counting, 4.3±1.2 % video analysis) or picrosirius red (19.6±3.0 % point-counting, 17.9±3.3 % video analysis). In addition, there was good correlation (Fig. 4) between the values obtained by both methods for both stains (picrosirius red $r=0.94$, $p<0.0001$; trichrome $r=0.81$, $p<0.0001$). However, when we plotted collagen content obtained from trichrome stained sections against that obtained from picrosirius red stained sections for both methods (Fig. 5), we found consistently higher values of collagen content in picrosirius red stained sections ($p<0.0001$ for both point-counting and video analysis). We observed that thick collagen fibers could be easily identified with trichrome staining, but thin fibers were harder to identify. In contrast, thin collagen fibers were easily seen in picrosirius red sections viewed with polarized light (Fig. 6).

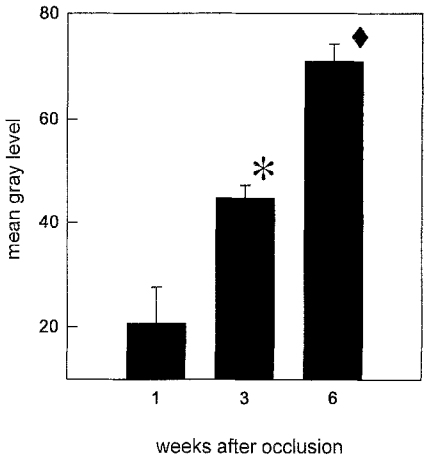

Fig. 3. Mean gray scale level of picrosirius red stained collagen fibers in the scar expressed as a percent of the mean gray scale level of collagen in the visceral pericardium and plotted as a function of time after myocardial infarction. *$p<0.05$ versus 1 week, ♦ $p<0.01$ versus 3 weeks.

Discussion

We found that circularly polarized light, in combination with digital image analysis, enhances quantitative light microscopic assessment of histologic tissue. We have also shown that 1) trichrome, a traditional stain used to identify collagen in histologic sections, may underestimate collagen content; and 2) confirmed using circularly polarized light that the optical properties of scar collagen change with time.

Circularly polarized light: benefits and limitations

Circularly polarized light has rarely been used to examine biologic tissue. Its limited application has been mainly as an aid to visualization (1, 6, 21), but it can also be used for quantitative analysis (2). In our study, we demonstrated that circularly polarized light facilitates quantitative image analysis of collagen. Circularly polarized light provides advantages over linearly polarized light. For example, birefringent materials viewed with linearly polarized light appear bright except when they are aligned with their optic axis parallel to the transmission axis of either polarizing filter. This property could result in underestimation of collagen content especially in tissues containing wavy fibers, where some portion of each fiber is likely to be aligned parallel to the filters' transmission axis and so appear dark. However, when viewed with circularly polarized light, every fiber will appear bright (Fig. 1).

Although birefringent materials appear bright viewed with linearly polarized light (except as discussed above), brightness intensity varies with orientation (4). Maximum brightness occurs when a fiber is aligned at 45° to the transmission axis of the polarizing filters. However, when the tissue is viewed with circularly polarized light, the dependence of brightness on orientation within the section plane is removed, and fibers will be equally bright regardless of orientation.

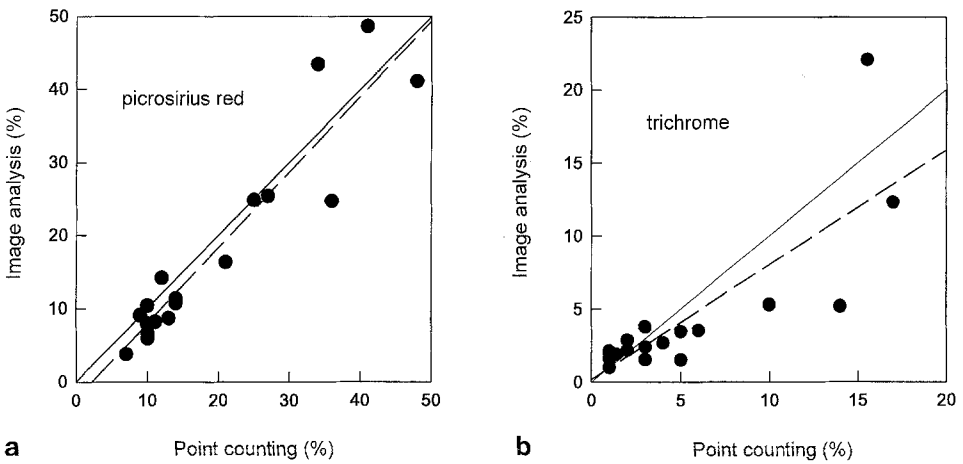

Fig. 4. Comparison of collagen content obtained from point-counting and image analysis. A) Data obtained after picrosirius red staining (r=0.94, gradient of regression line=1.03). B) Data obtained after trichrome staining (r=0.81, gradient of regression line =0.79). The dashed line represents the regression and the solid line the line of identity. Each point represents data from a single field of view.

Nevertheless, one limitation of linearly polarized light microscopy is not mitigated by using circularly polarized light. The intensity of birefringence also depends on the orientation of the fiber with respect to the section plane. Maximum birefringence is obtained when the material is sectioned so that the fibers are aligned longitudinally within the section. As the angle between the fibers and section plane increases, the birefringence decreases until it is zero when the fibers are cut in cross-section. Therefore, comparison of fiber brightness by the methods described should be performed on tissue in which fibers have the same orientation relative to the section plane. In the current study, we always examined longitudinally sectioned collagen fibers. In contrast, such limitations will not affect assessment of collagen content. The only requirement for our measurement of collagen content was that the fibers appeared bright when viewed with circularly polarized light (the degree of brightness is unimportant). The only fibers excluded would be those cut in perfect cross-section. However, we were unable to locate any such fibers (fibers stained by picrosirius red but which appeared dark when viewed with circularly polarized light) in our sections.

Scar maturation

We found that scar collagen brightness increased with time after myocardial infarction in picrosirius red stained sections viewed with circularly polarized light. This finding is consistent with an increase in collagen birefringence that we had previously reported on eosin stained sections (27). The changes in optical properties of maturing scar derive from several factors; increase in molecular organization of collagen, increases in thickness and packing of collagen fibers, and changes in the amounts of type I and III collagen.

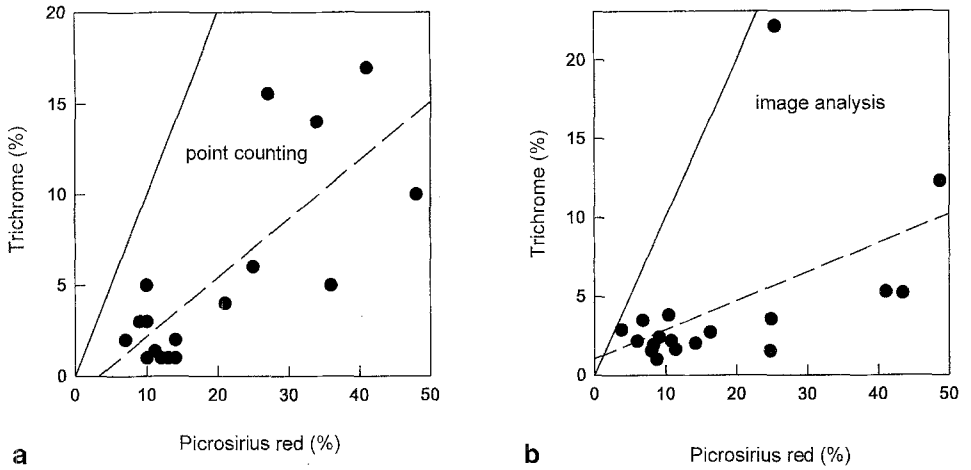

Fig. 5. Comparison of collagen content from trichrome and picrosirius red stained sections assessed by A) point counting (r=0.78, p=0.0002), and B) digital image analysis (r=0.50, p=0.04). The dashed line represents the regression and the solid line the line of identity. All of the data points were below the line of identity indicating higher collagen contents in the picrosirius red stained sections.

The latter change is particularly important because type I fibers are generally thicker than type III (15), and so would appear brighter when viewed with polarized light. The relative amount of type III collagen is known to be high in the early stages of wound healing and then decreases (7). Our results also confirm a similar analysis of healing in rat gracilis muscle (19), which did not use circularly polarized light but which minimized the orientation problems associated with the use of linearly polarized light because fiber orientation did not change with time in the regions analyzed. At 6 weeks after gracilis muscle injury, the median brightness of fibers in the scar was approximately 75 % of that of collagen fibers in normal, uninjured fascia. At 6 weeks after myocardial infarction, we found that the brightness of the scar was 71 % of that of normal pericardial collagen. We interpret these data to indicate that the healing process may not be complete even 6 weeks after injury. However, it is possible that there are differences in the type III:type I collagen ratio between scar and pericardial collagen, and so the brightness of scar collagen may never equal that of visceral pericardium.

In addition , our method allowed rapid analysis of relatively large areas of tissue. Previous measurement of retardation of linearly polarized light (27) was done on 10 individual fiber bundles each no larger than 50μm × 10μm, whereas each field analyzed in the video method was approximately 0.1 mm^2.

Collagen content

Trichrome staining: Whether we used point-counting or the video analysis method, we always obtained a higher value of collagen content in picrosirius red than in trichrome stained sections. Trichrome has been the traditional stain used to identify collagen in

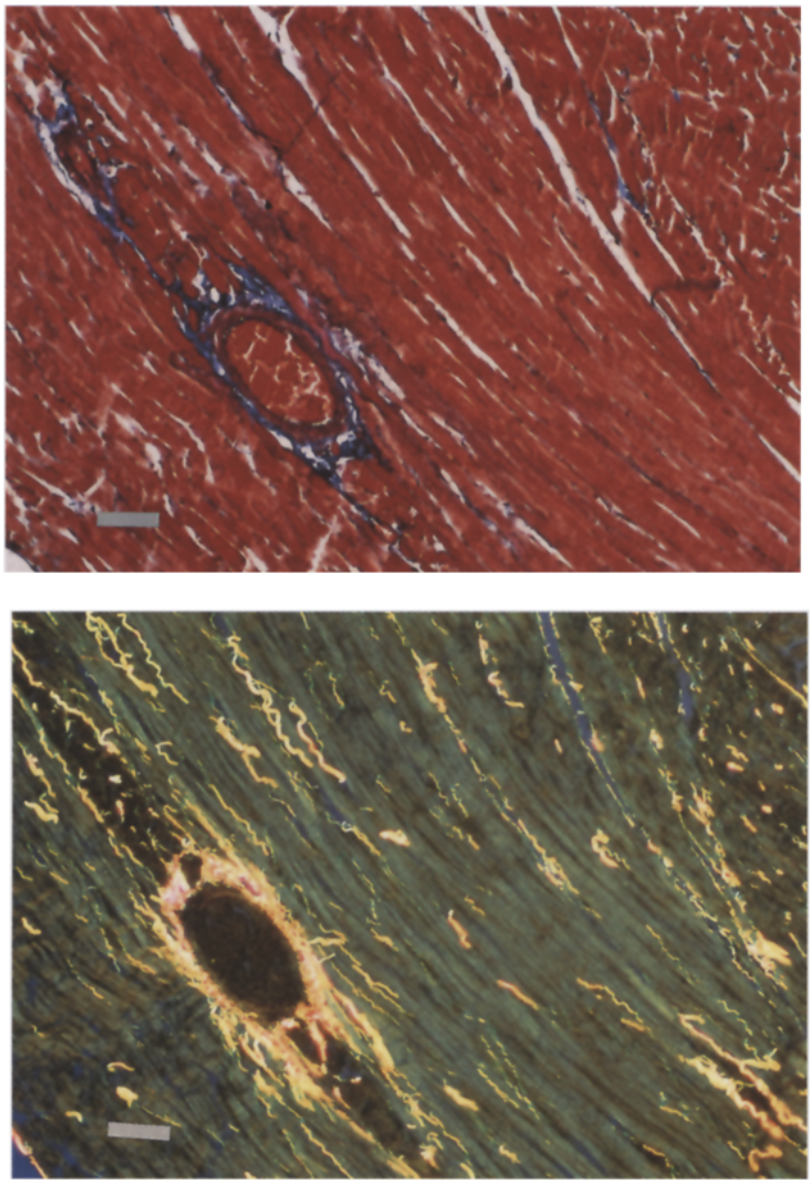

Fig. 6. Tissue from consecutive sections stained with A) Masson's trichrome viewed in brightfield, and B) picrosirius red viewed with circularly polarized light. The thick collagen fibers around the blood vessel are easily identified in both views; however, thin collagen fibers appear more frequently and more clearly in the picrosirius red stained tissue viewed with circularly polarized light. [bar=50μm]

histologic sections; fibers appear blue or green depending on the exact method used. To determine whether trichrome staining could give a false measure of collagen content, we must consider the mechanism of staining. Several studies have reported variability in collagen fiber color after trichrome staining. Flint et al. (5) found that collagen fibers fixed under tension stained red while non-strained fibers appeared blue/green, a difference they attributed to mechanically induced structural changes in the fibers. Lanir et al. (11) were unable to confirm this explanation and suggested that the density of collagen fiber packing may determine the color: densely packed fibers may not be easily accessible to the blue/green counterstain and so retain the red stain. Thus, the precise mechanisms of trichrome staining are unknown and variable results can be obtained.

Two studies reported a correlation between collagen content assessed by biochemical measurement of tissue hydroxyproline and morphologic assessment of trichrome stained sections (8, 9). However, even though these studies were performed by the same investigators, the slopes of the regression lines differed by a factor of two; in Hoyt et al. (8) the slope was 2.2; while in Hoyt et al. (9) the slope calculated from presented data was 1.0. A statistically significant correlation does not necessarily imply that the methods are accurate. For example, we found significant correlations between collagen content in trichrome and picrosirius red stained sections for both point-counting and image analysis, even though the actual numbers were quite different (Fig. 5).

We found that thin collagen fibers were difficult to see after trichrome staining. Hoyt et al. (9) attempted to correlate myocardial collagen content with integrated ultrasonic backscatter. Although they found a significant correlation between integrated backscatter and hydroxyproline content, there was a poor correspondence between local collagen content, assessed by trichrome staining, and integrated backscatter. Only in specimens with a discrete layer of subendocardial fibrosis (that is, a concentration of thicker collagen fibers) did they find good agreement between trichrome staining and ultrasonic backscatter. We suggest that underestimation of collagen content, specifically an inability to detect thin fibers, with trichrome staining was responsible for the observed discrepancy.

Thus, variability in fiber color, conflicting results from biochemical comparison, and possible inability to detect thin fibers suggest that trichrome is not an ideal stain for collagen detection.

Picrosirius red staining

Collagen content in picrosirius red stained sections has also been shown to correlate with tissue hydroxyproline content (18); however, this does not necessarily confirm the accuracy of the method. Why then do we think that picrosirius red provides a more accurate assessment of collagen content than trichrome staining? When examined in brightfield, collagen appears red and muscle yellow after picrosirius red staining. This brightfield contrast between collagen and muscle has been used, in conjunction with digital image analysis, to assess myocardial collagen content (14). However, it is the combination of picrosirius red and polarized light microscopy that provides a more powerful method of identifying collagen fibers (10). Collagen, because of its anisotropic molecular organization, is birefringent and appears bright when viewed with polarized light. Sirius red dye also has an anisotropic molecular organization, and when bound to collagen in an ordered manner, collagen birefringence is enhanced (20). Thus, even thin collagen fibers appear bright when viewed with polarized light and so are easy to identify. It is this optical

property of collagen that distinguishes picrosirius red from trichrome staining and probably accounts for the greater collagen content measured in picrosirius red stained sections.

Trichrome, versus picrosirius red staining

We were surprised at the magnitude of the difference between collagen contents; collagen content averaged almost six times greater in picrosirius red stained sections. Sweat et al. (22) stated that the color of thin collagen fibers is affected by the pH of the dye solution. It is possible that, in our trichrome stained sections, the pH was suboptimal for the staining of fine fibers, and so the underestimation of collagen content reported here may not be as large in every trichrome stained section. However, our results illustrate a key difference between the two methods; detection of collagen in trichrome stained sections depends on the observation of a specific color, while detection of collagen in picrosirius red stained sections depends on a fundamental physical property of collagen, its birefringence. Several factors such as; pH, duration of staining, fiber packing, and applied tension may affect collagen fiber color after trichrome staining (5, 11, 22), and thus confound collagen identification. Although some of these factors may affect picrosirius red staining, the collagen fibers will still appear birefringent. Thus, picrosirius red, used in conjunction with polarized light microscopy, is a more specific stain for collagen than trichrome and hence more likely to provide an accurate assessment of tissue collagen content.

Conclusion

Examination of picrosirius red-stained tissue sections with circularly polarized light provides a sensitive and specific means of evaluating myocardial collagen. This method eliminates disadvantages associated with the use of linearly polarized light, and is superior to trichrome staining for collagen detection. When this technique is combined with digital image analysis, it provides a convenient and quantitative histologic approach that can be used to quantify both collagen content and assess optical changes that occur in scar collagen during infarct healing. Changes in the content, organization, and nature of the myocardial collagen network play a significant role in many cardiac diseases and the methods of collagen analysis that we have described should provide a valuable means for studying these processes.

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Authors' address:

Peter Whittaker, Ph. D., Heart Institute-Research, Hospital of the Good Samaritan, 616 South Witmer Street, Los Angeles, California, 90017–2395, USA