LABORATORY STUDY

A Semi-automated Method for Measuring Collagen Area Fraction and Size Distribution using Picrosirius Red

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Abstract: A method to quantify collagen area fraction, fibril size distribution and local orientation was developed using the collagen specific stain, picrosirius red. Collagen area fraction was determined from the brightness channel of a linear cross-polarized image and the saturation channel of the brightfield image, while corresponding colors (and hence sizes) of birefringent collagen were obtained from the hue channel of the polarized image. In unloaded rat left ventricular myocardium, collagen fibers were wavy with 54 ± 9% visible when muscle was extinguished, and 70 ± 7% visible when it was illuminated. The proportion of collagen measured in either view was repeatable, but the former view was more consistent to define and easier to analyze since the muscle was dark. The uncorrected collagen area fraction for 5 hearts averaged 3.0 ± 0.6% (5.6% when corrected for extinct collagen) primarily localized in large red (0.8 ± 0.5%) and orange (1.7 ± 0.6%) fibers. A model analysis revealed that at any particular orientation, the collagen fibers were illuminated along their lengths and extinct at positions where the local orientation was < ± 17° from the polarizing axes.

INTRODUCTION

The cardiac collagen extracellular matrix can undergo considerable remodeling in various disease states. For example, late stages of pressure overload hypertrophy and treatment with isoproterenol have been shown to lead to increases in matrix proliferation and individual collagen fibril diameter [1,2]. In contrast, ischemia increases collagenase activity which leads to a disruption of the collagen primarily localized in the smaller fibers [3,4]. Further, in myocardial infarction, the myocardium remodels dramatically and myocytes are replaced by large collagen fibers (scar). The degree of organization of the scar may vary depending on the experimental model, but the collagen fibers tend to be aligned with the previous muscle fiber architecture [5,6]. Consequently, it is clear that a quantitative method to distinguish differences in collagen fiber size and local orientation would be useful in many areas of cardiovascular research.

Quantifying the complex microstructural organization of the cardiac collagen extracellular matrix is not an easy task. Collagen content in tissues can be quantified biochemically using a colorimetric assay for the amino acid hydroxyproline [7] which comprises 13-14% of the collagen molecules. In the ventricular myocardium, this assay has yielded varying results for collagen concentration from 0.02-0.15 mg/mg protein [8,9]. However, this technique does not provide information about the structural organization of the extracellular matrix. Scanning electron microscopy (SEM) allows visualization of the 3-dimensional structure but is difficult to use for quantitative analysis [10]. Transmission electron microscopy (TEM) allows examination of detailed cellular structures in ultra-thin sections [11]. However, the images represent such small portions of the tissue that comprehensive measurements are exceedingly time consuming.

The collagen extracellular matrix in heart tissue has been examined by light microscopy using trichrome
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[12], silver impregnation [11], immunohistochemistry (for collagens type I and III) [13] and picrosirius red [14]. In the present study, we exploit the special features of picrosirius red, which is specific for collagen [15]. When viewed with polarized light, picrosirius red enhances the birefringence of collagen molecules and the different birefringence colors are associated with different fibril sizes (i.e., larger fibrils = more dye molecules). In tissue samples, large fibrils appear orange-red while small fibrils appear green. Many investigators have used picrosirius red to examine cardiac tissue [16,17], but few have quantitatively assessed the microstructure [6,18] or taken advantage of the different colors to quantitatively examine the distribution of fibril sizes.

In this study, a new semi-automated method was developed and tested to quantify the collagen area fraction and fibril size distribution in cardiac tissue. Color images were separated into hue, saturation and brightness components, and distributions of area fraction corresponding to fibril size extracted from the images. A statistical analysis showed that at 400× magnification, 25 views yield a good estimate of the overall collagen area fraction for a normal sample of heart tissue. However, more views are needed to have a similar confidence in the individual color distributions. When using linear cross-polarized light, the collagen fibers are extinguished when aligned with the polarizing filters. Consequently, previous analyses using picrosirius red and polarized light have underestimated collagen area fraction. We have estimated correction factors for this extinction and also predicted precisely which orientations of collagen are being illuminated at a given polarization angle. Hence, this new approach to quantify collagen is valuable in the normal heart, and can be used for determining the effect of various interventions on the organization of the fibrillar collagen and the distribution of different fiber sizes.

METHODS

Slide preparation

Five normal isolated, arrested rat hearts were perfusion-fixed with glutaraldehyde (1.5% in phosphate buffer) at zero left ventricular (LV) pressure. From each, an equatorial ring was dehydrated in a series of graded ethanol (70%, 90%, 95%, and 100%) and embedded in paraffin. Ten 10-μm sections were cut from the LV free-wall parallel to the epicardium at 200-μm spacing through the wall. The paraffin was cleared from the sections with three 3-min washes of histoclear (National Diagnostics). The sections were rehydrated in 3-min washes of 100%, 90%, and 70% ethanol followed by a 10-min wash in water. The tissue was stained with picrosirius red (0.1% w/v sirius red F3BA (Roboz Surgical Co.) in saturated picric acid) for 90 minutes, which is enough time to saturate the staining sites [15]. The sections were dehydrated and coverslips mounted with Permount adhesive (Fisher Chemical Co.).

Image analysis

The area fraction and color distribution of collagen in tissue sections was measured with a semi-automated image analysis method. The sample was transilluminated with polarized light, and the transmitted light viewed through an analyzer polarized at 90° to the incident plane (cross-polarization microscopy) with a 40× objective. The sample was rotated on a circular stage until the autobirefringence of the muscle was extinct (see Orientation Testing section). A 24-bit 640×480 pixel color image was acquired on a computer (Data Translation DT-2255 on Macintosh host) with an RGB camera (Sony CMA-D1) both for polarized (Figure 1a) and brightfield (Figure 1b) views. The RGB images were transformed into three 8-bit grayscale images corresponding to hue (dominant frequency), saturation (color purity), and brightness (HSB) of the image using a standard HSB separation algorithm (Adobe Photoshop) [19]. The area of birefringent collagen was obtained by thresholding the 8-bit grayscale brightness channel into a binary black-and-white image. The threshold level was chosen and recorded such that the illuminated collagen was separated from the tissue with a small but consistent amount of background noise present (0.03-0.25% of image area). The corresponding color of each birefringent collagen pixel was obtained using the thresholded brightness image as a mask overlaying the hue image of the polarized view. The 8-bit hue image defines a spectrum of 256 different colors with red at both 0 and 255 and the wavelength of the color varying inversely with the hue value.

Table 1 gives the approximate location of standard colors in this hue spectrum. The pixel area of tissue

Figure 1. Picrosirius red stained rat myocardium (~400×). (A) In the polarized view, collagen is birefringent with different colors representing different fibers sizes (red = largest; green-teal = smallest). (B) In the brightfield view, muscle is yellow and collagen is red.
was obtained by thresholding either the saturation channel from the HSB segmentation or the blue channel from the RGB images. These views were chosen because they provided the highest contrast between the tissue and collagen (yellow and red) and the extracellular space (gray and white). Again, this threshold level was chosen by the user such that the tissue was separated from the extracellular space. The area fraction of collagen was then determined as pixel area in the thresholded polarized view/pixel area of thresholded tissue. This semi-automatic process could be performed at 10-15 views/hour.

Systematic testing

Several tests were performed to assess the reliability of the image analysis system. The area fractions of 8 views measured with point counting were compared with the computer image analysis. Point counting was performed with a 20×20 etched grid reticle. The area of tissue and birefringent collagen was measured with both techniques.

The color separation accuracy of the image-analysis system was tested with several computer-generated test patterns including: (I) a grid of 25 distinct colors (holding saturation and brightness constant), (II) a continuous shading of hue (again holding brightness and saturation constant) and (III) a graded brightness for a single color (holding hue and saturation constant). The patterns were created with a drawing software package (Canvas 3.03) and imaged as 35 mm slides (Microsoft Powerpoint and Lasergraphics LFR). The film recorder has a resolution of 3000 lines/slide and the resulting image patterns were 3×3 mm. The slides were viewed with the microscope under low-power (2× objective) and brightfield images acquired and analyzed for the hue content of the image.

Effect of thresholding

To evaluate the errors in the overall area fraction and the individual colors, 6 images were analyzed by varying the threshold over a wide range. Two different operators processed three images each as they would normally, and these threshold levels were defined as the a priori values. The same images were then reanalyzed over a large range of threshold levels. The “errors” for each color were then calculated by normalizing the pixels at each color level by the a priori value.

Sample orientation

The picric acid in picrosirius red not only enhances the birefringence of the collagen fibrils, but also that of the A-bands of the sarcomeres in cardiac muscle (this was verified by staining tissue with picric acid in the absence of the sirius red which yielded similar myocyte birefringence patterns to the complete stain). As the sample is rotated through various angles, the degree of this muscle illumination changes. Since, in the unloaded configuration, the collagen fibrils exhibit crimp along the cell axis, changing orientation of the sample may affect measured collagen area fraction. This was examined in 12 images by acquiring and analyzing data at different orientations. To assure superposition of the two images, the polarizing filters were rotated rather than moving the tissue section or rotating the microscope stage.

Variation analysis

To estimate the number of fields required in a single sample to have reasonable statistical power, the normal variation of collagen area fraction between fields was systematically evaluated in 52 views in a single section. A low-power (~15×) brightfield micrograph of an entire section was obtained and printed as a half-tone image. The positions of the high-power (400×) views were recorded on the print based on location of regional anatomic markers. The area fraction and relative standard error (RSE) were examined for various subsets of these 52 views, where RSE for the ratio x/y is:

$$RSE = \left( \frac{1}{n-1} \left( \frac{\sum x^2}{\sum x} \right) + \frac{\sum y^2}{\sum y^2} \right)^{1/2} \left( \frac{\sum xy}{\sum x \sum y} \right)$$

where x is the area of illuminated pixels in the polarized view, and y is the area of tissue. In biological samples, it is generally accepted that an RSE of <10% is desirable which results in a statistical power of 0.5 for changes in the mean of <25% [20]. To examine the...
transmural distribution of collagen, ten views were evaluated in each of 9 additional sections from the same heart. For this purpose, low-power micrographs (≈15×) were again obtained and printed. In these sections, a standard 10 point grid was drawn on the central portion of the section (where the long axis of the cells are in the plane of the section), and the views acquired after positioning the stage based on the regional markers.

Additionally, to estimate the variation between animals, the area fraction and size distribution from five images from each of five sections through the thickness of the left ventricular wall were measured in the remaining four hearts.

RESULTS

The hue-area fraction distribution for a typical image is shown in Figure 2. To get a better approximation of the area fraction for each size of fiber, the continuous distribution was grouped into 32-hue segments shown with dashed lines with the median colors characterized as red, orange, yellow, green and teal. The peaks and valleys observed in the distribution were common to all images examined and consistently occurred at the same hue levels.

Verification

Area fraction data from the computer image analysis on the Macintosh showed excellent correlation with the area fraction data from point counting methods (Mac(%) = 0.915*Pcount(%) + 0.17; r² = 0.910). The brightfield sections were more variable than the polarized views (Mac(%) = 0.936*Pcount(%) + 0.50; r² = 0.839 vs. Mac(%) = 0.948*Pcount(%) + 0.014; r² = 0.896, respectively). The range of area fraction analyzed was small with collagen comprising <7% in all views. To assure that the same region of the view was measured with both techniques, only the 361 grid points that were within the boundary of the camera view were used for point counting, resulting in an optimal resolution of 0.27% of the image area. The average absolute difference between the two techniques of measurement was 0.37% ± 0.39%.

The image-analysis system had a fairly accurate response for the computer-generated 35mm test patterns. Figure 3 shows the resulting signals acquired for the 3 test patterns with the input signal shown above each graph. Figure 3a shows the resulting peaks in the hue content and how they corresponded to the input peaks for the 25 color pattern (Pattern I) which were equally spaced over the hue range.

The main inaccuracies in color separation occurred in the blue-magenta range where three peaks (#16-18) grouped together. Acquisition and analysis of the rainbow spectrum of graded hues (Pattern II) yielded a fairly uniform distribution of hues, again with large peaks in the blue range (Figure 3b). The most accurate regeneration of the input image occurred for the single hue image of varying brightness (Pattern III), which yielded a dominant single hue (Figure 3c) with relatively uniform distribution of brightness (Figure 3d). While any of these variations may have been introduced by the test film recorder, it appears that this camera and image acquisition system can accurately distinguish discrete colors in the range of hues for the picrosiris red stain (from red to green) over a range of brightness values.

![Figure 2. Example area fraction-color distribution for a single image. The range of hues defining the “standard” collagen birefringence colors are shown with the dashed lines. The majority of the collagen was observed in the larger red and orange fibers. The remaining signal (hues: 135-231) was extracellular background noise which revealed a large peak in the blue range. This background signal can be used to assure that thresholding is consistent for different preparations.](image-url)
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Figure 3. Pixel distributions for 3 color test patterns generated by a 35 mm film recorder. In each panel, the inset above each graph shows a schematic of the input signal. Pattern I (A) was a 5×5 grid of different hues equally spaced over the 256 hues. Pattern II (B) was a linear gradation of hue over the field. Both I and II were created at a single saturation and brightness value. Pattern III represents a (C) single hue value and (D) a continuous shading of brightness.

Thresholding

Adjusting the threshold in the brightness channel had a significant, but highly repeatable effect on the area fraction of colors measured as shown in Figure 4. The background pixels changed the most rapidly from <50% to >300% of the final value over only 6 intensity values. This non-linearity continued over the entire range evaluated (25 intensity values). We utilized this feature to provide an independent criterion for thresholding. Over the small range of ±3 intensity values, the area fraction of collagen did not change much, varying < ± 15% for the large (red) or medium (orange and yellow) fibers. The small (green and teal) fibers were more sensitive to thresholding ~ ± 75%.

Sample Orientation

Figure 5 demonstrates the effect of rotating the sample relative to the planes of polarization. When the sample is oriented such that the axis of optical anisotropy of the muscle corresponds to one of the polarization axes, the muscle is extinguished (Dark-muscle; Figure 5a). Alternatively, if the sample is oriented 45° between the polarization axes, the A-bands of the muscle become maximally birefringent (Bright-muscle; Figure 5b). Changing the orientation also affects the birefringence of the collagen which is crimped and oriented in varying directions. In Figure 5a, the portions of the collagen fibers oriented parallel to the muscle fibers are extinct, but become illuminated when the muscle is birefringent (Figure 5b). Similarly, obliquely oriented regions of the collagen fibers that are illuminated in Figure 5a, become extinct when the muscle is birefringent in Figure 5b. Finally, there are some regions that do not coincide with either direction and are illuminated at both orientations.

To measure the collagen area fraction in the bright-muscle views, the images were processed using the same method as the dark-muscle views, however the background criterion for thresholding was loosened and birefringent pixels of the muscle included. The collagen fibers were then visually identified and extracted from the final images by manually deleting the background pixels leaving only the illuminated collagen in the bright-muscle image. The area fraction (±SD) in 12 random dark-muscle and corresponding bright-muscle images was 36.9 ± 1.85% and 4.03 ±
1.35%, respectively. Since the microscope stage was not rotated between views, it was possible to superimpose the two images and obtain a composite image. The total area fraction of collagen referred to the tissue area for the combined images was $5.84 \pm 2.21\%$. The fraction of the total collagen measured in the bright-muscle and dark-muscle images averaged $53.8 \pm 8.7\%$ and $70.1 \pm 6.9\%$, respectively. This corresponded to an overlap of $19.0 \pm 4.5\%$ of all the collagen between the two images. These results were very consistent and independent of the area fraction.

Figure 6 shows an example of grayscale hue image for a collagen fiber in (a) dark-muscle and (b) bright-muscle image, while Figure 6c shows the resulting combined image. Note that the collagen fiber is more continuous in the composite image while blank regions exist in the dark- and bright-muscle images corresponding to their points of extinction. The dark-muscle image was chosen for overall analysis because it was easier to define consistently and can be used in the semi-automatic mode. This method also provides a correction for the degree of underestimation in these unloaded samples.

To estimate which orientations of collagen fibers were being illuminated in the dark- and bright-muscle views, the angle of extinction was predicted based on the physics of the polarized light. Briefly, we used the results from the proportions of collagen observed in the bright- and dark-muscle views for fibers in various configurations to predict what regions of a circular piece of collagen would be illuminated (Figure 7). The properties of the illumination can easily be described analytically, and the data were obtained from a separate study where hearts were fixed at different ventricular pressures and the collagen fibers were in various states of coil configuration (from wavy to virtually straight) [21]. Using a regression of the proportion of collagen in the dark-muscle vs. either proportion in the bright-muscle or overlap, ($%\text{Brightness} = -0.565\%\text{Dark} + 101.36\%\text{Dark} + 0.25\%$; $r^2 = 0.65$) it is found that a circle would be illuminated 62.5% in each view. Furthermore, extinction angle is found to be $\sim 28^\circ$ from the polarizing axis. Consequently, the semi-automated image analysis technique, which uses the dark-muscle view, measures collagen fibers oriented between $(\pm)$ 17$^\circ$ and 73$^\circ$ from the muscle fiber axis. Similarly, the collagen fibers illuminated in the bright-muscle configuration are oriented between $-28^\circ$ and $28^\circ$ or between $62^\circ$ and $118^\circ$ from the muscle fiber axis. Finally, the overlap is a measure of the collagen fibers oriented from $(\pm)$ 17$^\circ$ to 28$^\circ$ and from $(\pm)$ 62$^\circ$ to 73$^\circ$ from the muscle fiber axis.

**Variation analysis**

*Single section:* The mean collagen area fraction for the 52 views examined in one section was 3.04% with a relative standard error of 9.9%. To estimate the population variation and determine the number of views necessary for each sample, views were systematically eliminated and mean and RSE calculated (Table 2). As the number of views decreased, the RSE of the measurement increased with a large RSE (18.8%) for 10 views.

*Single heart-multiple sections:* The area fraction measured in ten slides from epicardium to endocardium did not vary with depth ($p > 0.4$ using linear regression). The mean values ranged from 1.4 to 4.0% with the overall mean of 2.99% and an RSE of 7%. Again,
Figure 6. Grayscale image of the digitally acquired data demonstrating the effect of rotating the axes of polarization. Panels A and B demonstrate the portions of the fiber that are illuminated in the Dark and Bright muscle orientations, respectively. Panel C demonstrates the composite fiber when panels A and B are superimposed on one another producing a continuous collagen fiber.

Figure 7. Results for the orientation of illumination for bright- (dashed lines) and dark- (solid lines) muscle views. In any configuration collagen is extinct <17° from the polarizing axes. The collagen illuminated in the bright-muscle view is oriented from −28° to 28° and from 62 to 118° from the muscle axis. Since the bright- and dark-muscle axes are oriented 45° apart, the illuminated collagen in the dark-muscle view is oriented from (±) 17° to 73°. This leads to an overlap of 11° at the end of each range or from (±) 17° to 28° and from (±) 62° to 73°.

the number of views was systematically reduced to determine the minimum number of views necessary for the desired accuracy (Table 3). Since there was not a significant transmural variation, 25 views—5 from each of 5 sections—was selected as a reasonable sampling criterion for future studies.

Normal population: The overall area fraction for the group of 5 normal rat hearts was 3.02 ± 0.57%. The collagen area fraction-size distribution was similar to that found in the single heart with the mean area fraction (±SD) of the individual colors presented in Figure 8. For these hearts, the majority of collagen observed was comprised of red and orange fibers.

DISCUSSION

Picrosirius red used with polarization microscopy has been employed by many investigators to examine collagen structure and organization [15,22,23]. However, few have developed quantitative measures of area fraction using the stain or utilized the unique feature of birefringence colors to quantitatively describe fibril size distribution. The present study describes a new method to semi-automatically quantify collagen fi-

ber sizes in normal cardiac tissue that could readily be applied to various cardiac pathologies known to alter collagen structure.

Picrosirius red as a collagen specific stain

Picrosirius red was first identified as a collagen-specific stain by Sweat in 1964 [24] with the use of polarization microscopy quick to follow in 1968 [25]. Since then, picrosirius-polarization microscopy has been used extensively for examination of collagen in a wide variety of tissues from blood vessels [26], skin [27], cartilage [28,29], nerve tissue [30,31], myenteric plexus [32], dental structures [33] to myocardium [6,16,17,18,34,35]. Sirius red F3BA enhances the birefringence of collagen because its six sulphonic acid groups interact with the lysine and hydroxylysine residues of the collagen molecules at low pH, and the long axis of the stain molecule aligns with the axis of the collagen [15]. Junqueira and colleagues [15,30] stained many tissues to examine the specificity of picrosirius red to collagen.
Table 2. Relative standard error and area fraction for different combinations of the 52 views measured in a single section

<table>
<thead>
<tr>
<th>Iteration</th>
<th>Numbers of Views</th>
<th>Area Fraction (%)</th>
<th>RSE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Views</td>
<td>52</td>
<td>3.04</td>
<td>9.9</td>
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<tr>
<td>Inner Views</td>
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<td>13.1</td>
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<tr>
<td>Even # Views</td>
<td>26</td>
<td>2.81</td>
<td>13.5</td>
</tr>
<tr>
<td>Center Views</td>
<td>10</td>
<td>1.56</td>
<td>18.8</td>
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They found enhanced birefringence of virtually all structures that stained red and were known to contain fibrillar collagen types I and III. Line et al. [28] have stained asbestoid cartilage which contains mostly collagen type II and Dayan et al. [22] have stained in-vitro samples of collagen types I, II, III, and V which all show positive staining patterns and enhanced birefringence. Junqueira et al. [15] showed that the current staining protocol saturates dye binding sites; hence it can be used to quantitate collagen density.

Birefringence colors

Initially, the birefringence colors were considered to represent different collagen types (i.e., red-orange was type I while green was type III), but have since been shown to be more likely associated with size [22]. As the fiber diameter increases, more dye molecules can bind to the collagen yielding different birefringence colors. The discrepancy between fibril size and collagen type appears to have arisen from the fact that, in general, collagen type III is localized in smaller fibers (i.e., reticulin fibers) and collagen type I is localized in larger fibers (such as tendon) [36]. However, in the cardiac muscle, both collagen types I and III can be found in the same fibers such as the small struts which would appear green or teal with this technique [37].

Unfortunately, the quantitative relationship between color and size is difficult to determine because the birefringence colors may be affected by different factors. Dayan et al. [22] attempted to measure this relationship by staining various types and sizes of collagen extracted from tissues. Using a qualitative scheme where the color distribution was assigned by the user based on 20% intervals, they found that the small fibers (<0.8 μm) appeared green to green-yellow and the larger fibers (1.6-2.4 μm) had a shift to a longer wavelength appearing yellow to red. These larger fibers had a much wider range of colors depending the embedding substrate. In tissue samples, section thickness also affects birefringence colors with a gradual increase in wavelength as section thickness increases [38]. Hence, if the current approach is to be utilized, samples should be of uniform thickness. Also, comparisons between different tissue types should be examined carefully. Most importantly, it is possible to determine relative changes in small and large fibers with different experimental interventions.

The 24-bit image acquisition allows definition of 256 distinct hues or colors each with 256 possible saturation and brightness values yielding 16.6 million different combinations. Analysis of both tissue sections and 35mm test patterns revealed that these 256 hues were more than sufficient for the current application because there was potential noise introduced by the system as reflected by the consistent location of the peaks and valleys in the acquired signal. Hence, it was

Table 3. Relative standard error and area fraction for different combinations of the 100 views measured in single heart. When the number of views was between 25 and 50, the RSE was approximately 10%. Hence a value of 25 views was chosen as a reasonable estimate of the number of views required for a single sample

<table>
<thead>
<tr>
<th>Iteration</th>
<th>Numbers of Views</th>
<th>Area Fraction (%)</th>
<th>RSE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Views</td>
<td>100</td>
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<td>7.0</td>
</tr>
<tr>
<td>All Views (each slide averaged)</td>
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<td>2.99</td>
<td>8.0</td>
</tr>
<tr>
<td>Even Views</td>
<td>50</td>
<td>3.23</td>
<td>10.7</td>
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<tr>
<td>5 Views per slide</td>
<td>25</td>
<td>3.39</td>
<td>12.3</td>
</tr>
<tr>
<td>for 5 slides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Every 4th view in 10 slides</td>
<td>25</td>
<td>2.68</td>
<td>13.0</td>
</tr>
<tr>
<td>(odd set of 25)</td>
<td>13</td>
<td>2.93</td>
<td>12.8</td>
</tr>
<tr>
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necessary to group the distribution into smaller segments which we defined arbitrarily to include 32 hue levels. The computer-generated patterns tested the response of the camera over a wide range of colors and intensities. Our camera appeared to introduce a large gain in the blue range that was also observed as a spike in the distribution from the tissue sections and was identified as extracellular background. Fortunately, that range of color is not included in the range of birefringence colors for picrosirius red. In fact we were able to utilize this range of colors to assure repeatability between different samples and operators. In the range of colors observed with picrosirius red (red to teal), the system yielded a good differentiation of color even for a large range of brightness values.

Agreement with other methods and effect of orientation

Pickering and Boughner [18,35] have utilized computer-based image analysis methods for quantifying image intensity to find area fraction in cardiac tissue. This is similar to thresholding the brightness channel as described in the current method. The area fraction measurements they reported spanned a larger range due to infarct-induced fibrosis. There was excellent agreement of the area fraction measurements with biochemical hydroxyproline analysis (r = 0.98). The area fraction in normal heart tissue was 2.9 ± 0.6% which was very similar to the results from the dark-muscle views in the current study. Whittaker et al. [6,39] have quantified area fraction with picrosirius red by masking a monochrome brightness image.

As shown with the present method, a simple application of a standard color video camera and acquisition board would allow extraction of color and size information from these images. The techniques of Jalil et al. [1] involve manually identifying the yellow and red birefringent collagen fibers and tracing them on a digitizing pad yielding a normal collagen area fraction of 2.7 ± 0.2%. The current method again yields similar values while introducing a more objective selection criterion and semi-automated method. Furthermore, the color data allow examination of the full spectrum of sizes. While small fibers may not contribute significantly to the collagen area fraction measurements at this magnification, certain interventions may differentially affect a specific range of fiber sizes. Pickering and Boughner [18] discussed the use of color image analysis and assert that it suffers from imperfect segmentation of features. This could have been a problem in the current study, but its effects were reduced because the threshold brightness signal was utilized as a mask for the hue distribution.

Figures 5 and 6 demonstrate the importance of proper orientation of the cardiac muscle sample with respect to the polarization axes in this method. By choosing to orient the tissue such that the autobirefringence of the muscle is extinct, one consistently underestimates collagen area fraction by 46%. Similarly, orienting such that the muscle is bright results in an underestimation of 30%. It is the nature of cross-polarized light to result in such a behavior, but these results indicate that the orientation of collagen is relatively uniform with a relatively small difference between the two orientations. By examining the distribution of the proportion of collagen illuminated at each orientation over a large range of collagen fiber configurations, an extinction angle was calculated and it was shown that the cross-polarization illuminates 62% of the possible orientation angles in each view, or collagen oriented more than 17° from the polarizing axis.

While the measurements of other investigators using linearly polarized light have all agreed well with the automated measurements of the present study [1,18,35,40], it appears that they have all underestimated the collagen area fraction by as much as 46% because they used linearly polarized light and did not account for the extinguished collagen fibers. For any intervention, the degree of underestimation with the dark-muscle approach should be examined to assure that the difference measured with the dark-muscle scheme represents changes in overall collagen area fraction. Further, this ability to objectively assess collagen-muscle alignment may provide useful information.

Whittaker et al. [6], on the other hand, used circularly polarized light with picrosirius red and found an area fraction in normal rat myocardium of 7.6 ± 0.7% which is substantially higher than the area fraction found with linearly polarized light, but comparable to that found by combining the dark- and bright-muscle images. The area fraction was probably larger overall because the rats were retired female breeders and collagen is known to increase with age [8]. In theory, circular polarized light can simultaneously illuminate the collagen oriented in all directions, but we encountered two major problems when testing it. First, circular polarizers are constructed by combining a standard linear polarizer with a quarter wave plate. However, these quarter wave plates only shift a specific frequency of light. Hence, white light is only circularly polarized at a specific frequency, and the rest of the spectrum is elliptically polarized. Therefore, while it did illuminate the collagen, it also resulted in unusual birefringence colors. Secondly, circular polarization not only illuminated collagen aligned at all orientations, but it also illuminated the muscle which created difficulties in the automated thresholding and col-
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Collagen extraction using the present method. Furthermore, the current approach provides additional information by quantifying local collagen fiber orientation.

Limitations

The autobreifringence of muscle in cardiac tissue may influence these measurements in other ways as well. Sectioning of muscle oblique or perpendicular to their axes also introduces difficulties in extinguishing the birefringence. The influence of this error on the measurements was observed in a single section because the outer regions of the section had larger area values. Due to the curvature of the heart wall and the muscle fibers, the edges were cut obliquely and hence were difficult to extinguish and may have artificially increased the area fraction. In all subsequent measurements, the fields were only chosen from the central region of the sections. This problem also precludes the use of this measurement technique for equatorially or longitudinally oriented sections unless the bright-muscle areas are excluded from the images or the muscle fibers are in the plane of the section.

To evaluate the potential error associated with the subjective nature of operator-defined threshold levels, the change in area fraction for a wide range of threshold values was evaluated. The number of background pixels changed most rapidly in the range of threshold levels examined. Hence we were able to use this as an independent criterion of the "best" threshold level. To determine what limits should be used to retain the greatest accuracy while maintaining a robust environment, the distribution of background pixels was obtained for a large data set (~800 views) from several studies in progress in our laboratory that included several operators—both experienced and inexperienced. The median background noise was 379 pixels with a mean (and SD) of 455 ± 333 pixels. Further, by examining the range of threshold values that any limits would include for the 6 test images (and hence the intrinsic accuracies), values of 100 and 800 pixels were chosen as the minimum and maximum allowable total pixels in the hue range from 135-231. Therefore, if at the end of an analysis sequence, the background pixels were not within those bounds, the operator would have to go back and adjust the threshold until the noise was within these ranges. At this level, the thresholding accuracy is about ±15-20% for the total area fraction with specific accuracies of about ±15% for the red, orange, and yellow fibers and about ±75-100% for the green and teal fibers. This may seem large until one realizes that the variation in these smaller fibers from view to view is also large (±90%) even when making 25 views per heart. Consequently, this method only has the statistical power to detect large changes in the green and teal area fraction (as much as ±100%). If a significant change is not observed, one must realize that there may be a possibility of making a statistical type II error (false negative). However, the technique is still very powerful for determining changes in the overall area fraction as well as the red, orange or yellow ranges.

CONCLUSION

A new semi-automated method to quantify collagen area fraction, fiber size distribution, and fiber orientation was developed using picrosirius red and polarization microscopy. The area fraction in the rat heart agreed well with previous methods using linear cross-polarized light. However, we found that in normal unloaded rat hearts the picrosirius-polarization method reproducibly underestimated the total collagen area fraction by approximately 45% due to extinguished collagen fibers aligned with the muscle. Any application of the quantitation of collagen area fraction with cross-polarized light should recognize this limitation. Additionally, this approach has the potential to quantitate changes in collagen fiber orientation with respect to the muscle fiber axis. The current method allows differentiation of area fraction by size based on color determined from a color scheme based on the hue (dominant wavelength), saturation (color purity) or brightness (image intensity). In normal rat hearts the majority of the fibers are red-orange which represent the large-sized perimysial fibers.

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