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Original Article

A convenient method for quantifying collagen fibers in atherosclerotic lesions by ImageJ software

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Abstract: *Background and aim:* Collagen fibers are the main component of atherosclerotic lesions and should therefore be used as an index to evaluate plaque stability. Although Masson's trichrome stainis usually employed to identify the extracellular matrix, collagen fibers identified using this staining method are difficult to analyze because the three main colors often co-localize in same area within the atherosclerotic lesion. To solve this problem, we presented a convenient method for quantification of the collagen fibers in atherosclerotic lesions with Masson's trichrome stain. *Methods:* This method is based on the open-resource image software ImageJ and an associated color deconvolution plugin. Original images of atherosclerotic lesions were converted into RGB images, and these images were deconvolved by ImageJ using the color deconvolution plugin. *Results:* The resulting monochromatic image obtained showed collagen fibers in maximal separation from background tissues. To calculate the area of the green component, collagen fibers were quantified in an accurate and efficient manner. *Conclusions:* Because of the simplicity and accuracy of this method, it may be widely applied for studying atherosclerosis in humans or various animal models.

Keywords: Collagen fiber, atherosclerotic lesion, smooth muscle cells, animal models

Introduction

The primary components of an atherosclerotic lesion are extracellular matrix proteins, including collagen and elastin, which account for more than 60% of the lesion [1]. Collagen degeneration results in atherosclerotic plaque vulnerability; thus, the collagen content is considered an indicator of plaque stability [2]. Notably, Shiomi *et al*. introduced the morphometric 'vulnerability index', which is the ratio of macrophages and extracellular lipid deposits to smooth muscle cells (SMCs) and collagen fibers in the atherosclerotic plaque [3]. This index is usually employed to evaluate the degree of plaque destabilization in animal models. Considering that collagen fibers are responsible for the mechanical strength of the plaque, the number of collagen fibers determines the mechanical properties of the plaque [4]. Unlike the identification of macrophages and SMCs in an atherosclerotic lesion, immunohistochemical quantification of collagen fibers in an atherosclerotic lesion is not practical because at least four distinct types of collagen have been found. For this reason, numerous studies have used Masson's trichrome stain to identify the collagen fibers regardless of collagen type [5, 6]. However, with this three-color staining, colors usually co-localize on the surface of tissue and strongly interfere with the identification of a specific component within the tissue [7]. If the quantification of the collagen fibers relies on the calculation of specific staining based on visual inspection, this procedure could cause a considerable number of errors. Therefore, to overcome this obstacle, we developed a convenient method to quantify collagen fibers using Masson's trichrome stain and the open-resource image software ImageJ with its color deconvolution plugin.

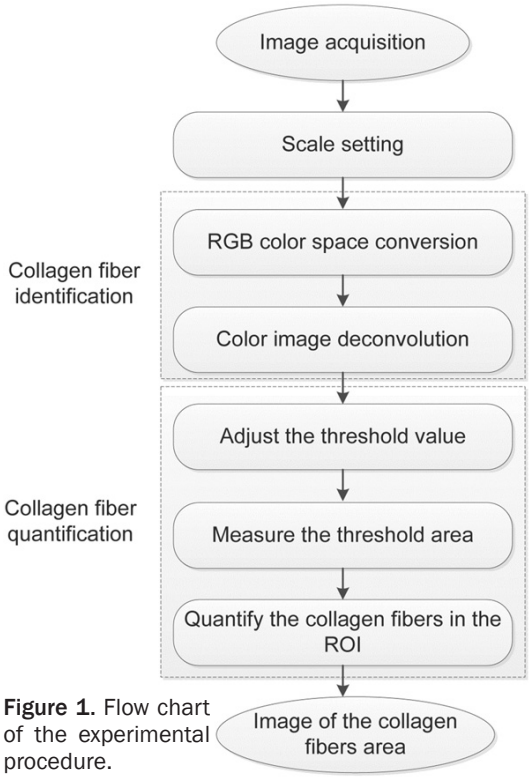

Figure 1. Flow chart of the experimental procedure.

Materials and methods

Sample preparation

Japanese white rabbits were fed with a high-cholesterol diet (HCD; containing 0.3% cholesterol and 3% corn oil) for 16 weeks to develop atherosclerosis as previously described [8]. After the rabbits were euthanized, the aortas were isolated and then fixed with 4% formaldehyde solution. For histological analysis, the aortic arch of each rabbit was cut into 10 cross sections (3 μm) as previously described [9]. Frozen cross sections of the aortic root in apoE deficient mice were kindly gifted by Dr. Hua Guan (Research Institute of Atherosclerotic Disease, Xi'an Jiaotong University Cardiovascular Research Center). The sections were stained with Masson's trichrome stain.

Histological staining

Masson's trichrome stain was performed according to a standard protocol [10]. Briefly, sections were stained with Hansen's iron hematoxylin for 5 min. After the sections were washed under running tap water for 5 min, they were stained with Biebrich scarlet-acid fuchsin for 10 min. After the sections were further rinsed in distilled water, they were treated with phosphomolybdic acid for 10 min. Then, sections were stained with light green instead of Methyl Blue for 10 min to avoid any possible confusion between blue nuclear staining and collagen staining. In Masson's trichrome stain, stained components were identified as the following: nuclei were stained black or blue; cytoplasm, muscle, and erythrocytes were stained red; and collagen fibers were stained green.

Quantification of collagen fibers by ImageJ

As shown in **Figure 1**, four steps are considered necessary for quantifying collagen fibers: installation of the software and plugin, setting the scale, deconvolution of the color images and quantification of the collagen fibers. The algorithm described below was used in conjunction with version 1.8 of ImageJ.

Image processing

Image acquisition is a prerequisite for further analysis, and four steps are required as previously reported [11]. First, all images should be acquired under identical conditions. To avoid a short range on the grayscale (0-255), the automatic exposure and white balance should be turned off. Then, the blank part of images should be adjusted to white, and the false-positive staining should be excluded from images. Last, all images should be digitized and stored in an uncompressed tagged image file format (tiff) with 24-bit RGB and 640 × 480 pixel resolution.

Scale setting

As shown in **Figure 2A**, the scale bar was measured by the "Straight" line tool in the "Analyze" menu, which allowed us to set the length of scale bar in the "Known Distance" box and set the unit (μm) in the "Unit of Length" box under the "Set scale" dialogue box.

Deconvolution of color images and identification of collagen fibers

Separation via color deconvolution provides a means of separating collagen fibers from the overlapping regions. The basis of this method is to separate the component stains by performing an orthonormal transformation of the image's RGB information [12]. During color image deconvolution, the images were opened,

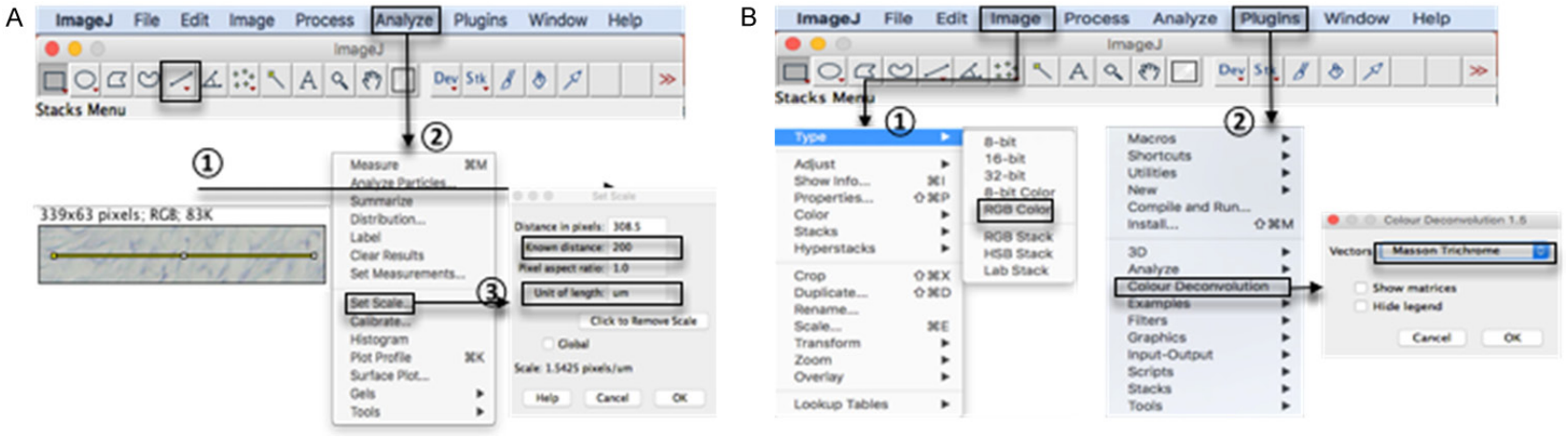

Figure 2. Procedure for processing images with ImageJ. Scale setting (A): 1) The scale bar was measured using the "Straight" line tool and 2) entered into the "Analysis" menu. 3) The length of scale bar was entered into the "Known Distance" box, and the unit (μm) was set in the "Unit of Length" box. (B) Color deconvolution: 1) The image was converted to an RGB color space and 2) processed using the "Color Deconvolution" plugin.

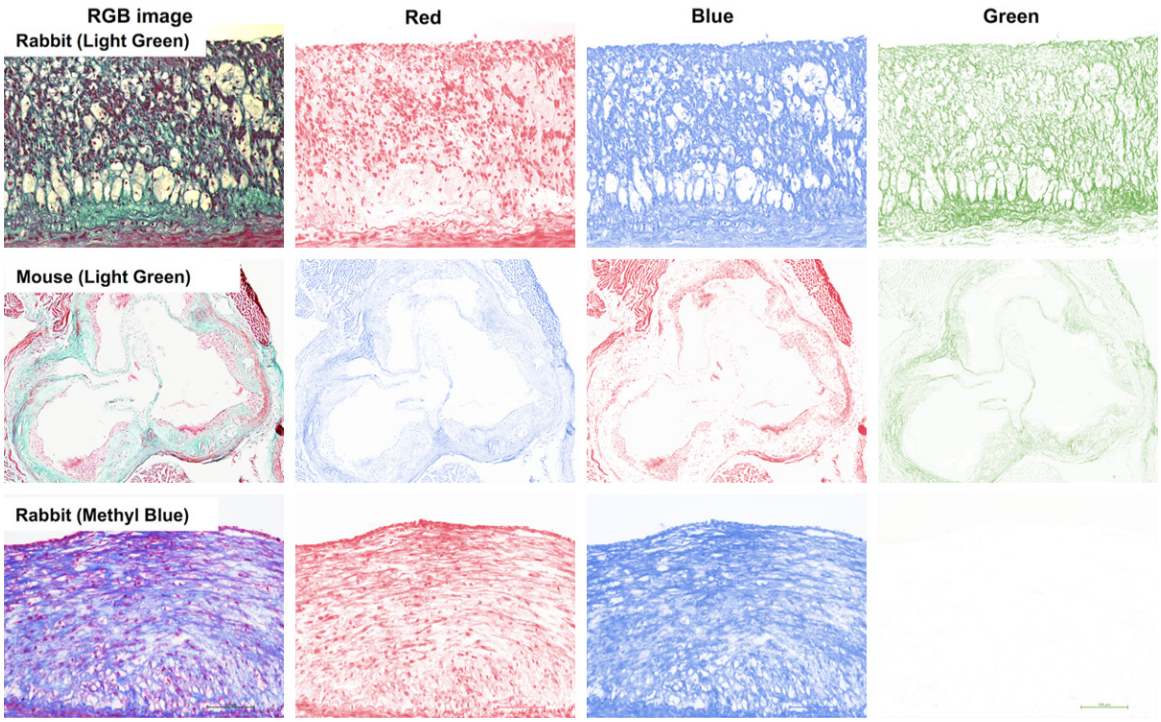

Figure 3. Images of atherosclerotic lesions processed by color deconvolution. The original RGB imagesof atherosclerotic lesions from rabbit (Light Green, upper), mouse (Light Green, middle) and rabbit (Methyl Blue, lower) were split into their red, blue and green components.

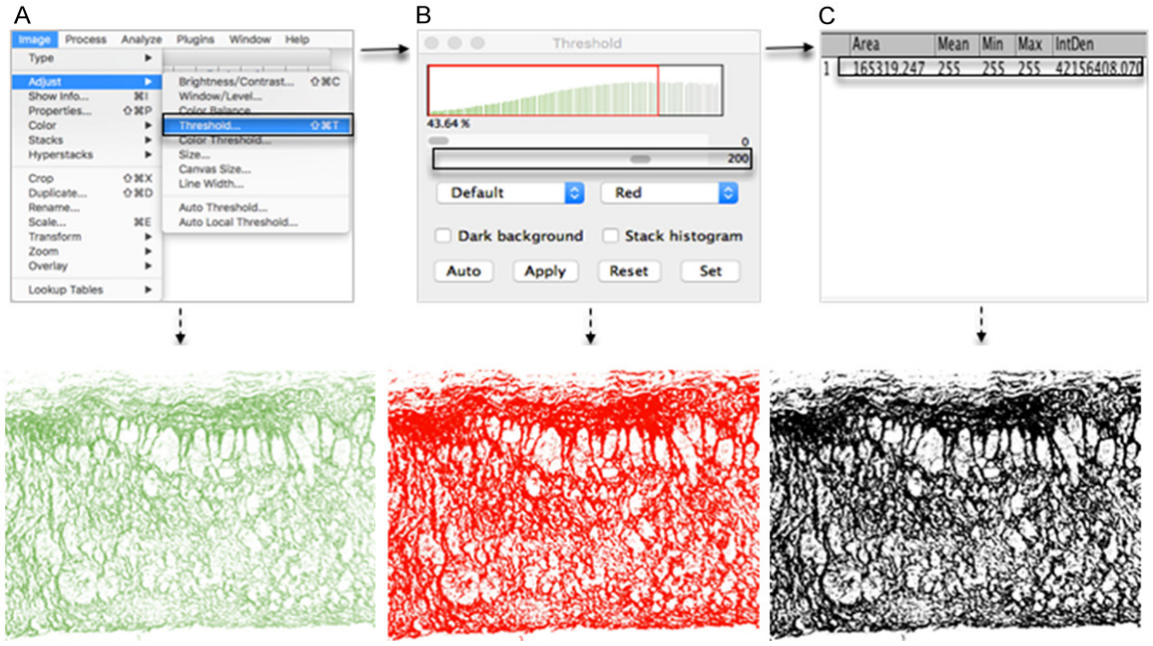

Figure 4. Quantification of the green component using ImageJ software. The threshold (A) was set to a constant value (B) for the green component. The results were shown as the area and integrated intensity (C).

and the following steps were applied. First, the input images were converted to RGB images by entering the "Image" menu, clicking on the "Type" box, and choosing the "RGB Color" com-

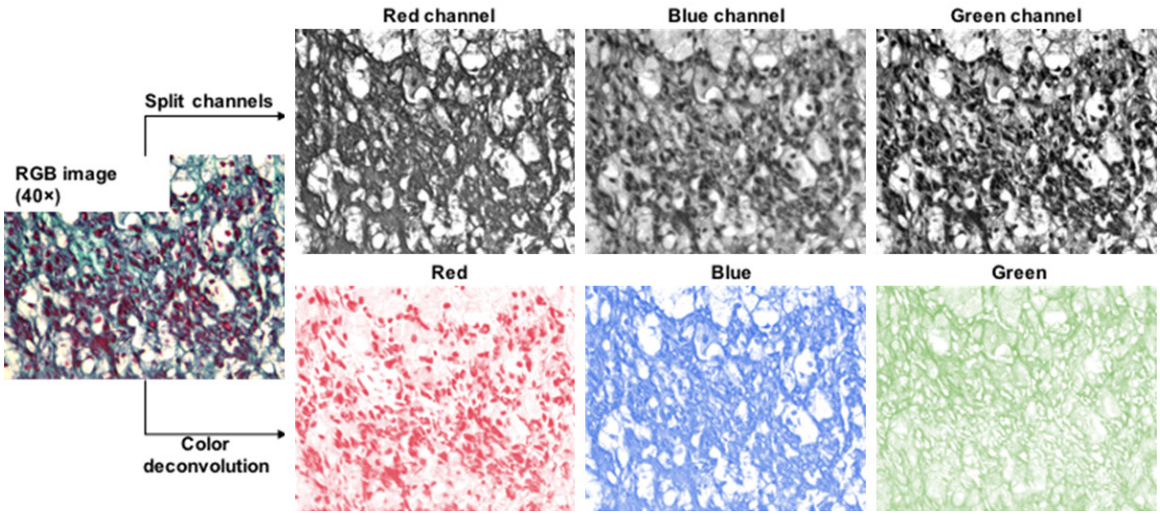

Figure 5. Comparison of the image processing by RGB image splitting and color deconvolution. The split channels function separated the RGB image into red, blue and green channels (upper). The deconvolution plugin separated the RGB image into a red component, blue component and green component (lower).

mand to convert the images (**Figure 2B**). Second, images with Masson's trichrome stain were processed using the "Color Deconvolution" plugin by clicking "Color Deconvolution" in the "Plugins" menu to deconvolve the images into their red, blue and green components; the green component was identified as the collagen fibers (**Figures 2B**, **3**).

Quantification of collagen fibers

The area of the green collagen fibers was measured after we entered the "Image" menu, clicked on the "Adjust" box, and isolated the green area of collagen fibers using the "Threshold" tool. The threshold was manually adjusted until the entire green area was highlighted in red (**Figure 4**). Then, measurement of the threshold area was performed as follows: we entered the set measurement dialog under the "Analyze" menu, and after we checked the "Area", "Integrated Intensity" and "Limit to Threshold", when we clicked the "Measurement" button under the "Analyze" menu, the results were presented in the "Results" window (**Figure 4**). Finally, area-based analysis was used to extract and quantify the regions of interest (ROIs) from the image. This analysis allowed the performance of the algorithm to be evaluated. To quantify the collagen fibers in the ROI (i.e., the atherosclerotic plaque), the "ROI manager" was chosen in the "Tools" box under the "Analyze" menu, and the region containing the atherosclerotic plaque was identified along the internal elastic lamina and the surface of the plaque.

Results

Collagen fibers were maximally separated from the background tissue

As shown in **Figure 3**, the images of atherosclerotic lesions from both rabbits and mice were separated into their red, blue and green components. However, if Methyl Blue was employed to stain collagen, the image was split into only its red and blue components (**Figure 3**). In the blue component, blue staining of the nucleus made it difficult to quantify the collagen fibers. However, when light green was used to identify collagen fibers, a green image was obtained, and the collagen fibers were shown with maximal separation from the background tissue in the green component (**Figure 3**).

Collagen fibers were conveniently quantified by ImageJ

In the green image, the collagen fibers could be quantified from the interested area (**Figure 4**). When a constant threshold was set, the collagen fibers in different sections could be accurately and consistently quantified by ImageJ (**Figure 4**). To compare with staining separation into three channels, image with deconvolution-indeed made the collagen fibers to obtain maxi-

mal separation from the background tissue (**Figure 5**).

Discussion

In this study, we present a method to estimate the area and integrated density of collagen fibers in an atherosclerotic lesion; this method is based on image analysis of a sample stained with Masson's trichrome. As previously reported, a wide range of collagen types are present in an atherosclerotic lesion, which increases the difficulty in quantifying collagens via immunohistochemical staining [12, 13]. Thus, staining against various collagens within a lesion may help researchers to better quantify the collagen density, and Masson's trichrome stain is a potential option.

For more than eighty years, Masson's trichrome stain has been frequently used to stain collagens in various tissues with either blue or green, depending on the dyes available (aniline blue or light green) [14]. However, because the three main colors often co-localize to the same area, it is difficult to analyze or quantify tissues stained with Masson's trichrome stain [15]. In conventional digital imaging, bright field images are a composite of three 8-bit monochromatic channels (red, green, and blue) that form a 24-bit color image [16]. Based on this convention, the RGB images can be split into three channels (red, green, and blue), and using one of these three channels can result in selective contrast between common stains. Using this separation technique, on a monochromatic image allows maximal separation between positive-stained pixels and background tissue, which facilitates accurate calculations of both the area and intensity of immunohistochemical staining [17]. However, staining separation is not suitable for analysis of Masson's trichrome stain because this technique has inefficient separation of the collagen fibers from the blue or green staining of other tissues. As previously reported, this incomplete separation was ascribed to the color spectrum of the available stains, with the common colors partially spread over all three channels [7]. To overcome this problem, Ruifrok et al. developed a plugin named "color deconvolution", which separates a three-channel image into three colors [18]. Although some limitations should be considered in further applications, this technique can effectively separate the contributions of two co-localized stains [19].

As above description, a green image was obtained after image deconvolution, showing that collagen fibers were shown with maximal separation from the background tissue.

Moreover, monochrome imaging also helps researchers use this image for calculating the area and integrated intensity. Therefore, these techniques offer the feasibility of automatically extracting collagen fiber data from atherosclerotic lesion images, which radically improve the methodology of collagen analysis based on Masson's trichrome stain. This method is more objective, sensitive and accurate compared to the traditional Masson's trichrome staining method.

In conclusion, we presented a convenient method to assess collagen fibers in atherosclerotic lesions using Masson's trichrome stain. This method was based on the cross-platform software ImageJ and its associated color deconvolution plugin. Because of the simplicity and accuracy of this method, it may be widely applied for studying atherosclerosis in human or various animal models.

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Disclosure of conflict of interest

None.

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