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Comparative Evaluation of Masson's Trichrome and Picrosirius Red Staining for Digital Collagen Quantification Using ImageJ in Rabbit Wound Healing Research

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ABSTRACT

The therapeutic potential of Pluronic F127 (PF127) hydrogel loaded with adipose-derived stromal vascular fraction (AdSVF), mesenchymal stem cells (AdMSC), and conditioned media (AdMSC-CM) for repairing full-thickness skin wounds was evaluated using a rabbit model. The rabbits were randomly divided into eight groups with six animals each and treatment was given as per the predetermined protocol (3 doses at one-week interval): Group A (Control), Group B (AdSVF), Group C (AdMSC), Group D (AdMSC-CM), Group E (PF127), Group F (AdSVF + PF127), Group G (AdMSC + PF127), and Group H (AdMSC-CM + PF127). Skin tissue samples were collected from the healing wounds on day 28 for staining and collagen quantification. Collagen density (Area %) was quantified using tissue sections stained with Masson's Trichrome (MT) and Picrosirius Red (PSR) stain using the Colour Deconvolution plugin of ImageJ and RGB stack method, respectively. These techniques function based on

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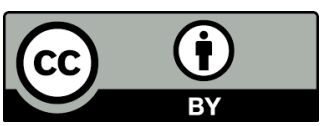

Wound healing

separating different colour channels in the stained tissue sections to isolate the collagen fibers and then quantifying them through thresholding and image analysis. Across the treatment groups, both staining methods generally showed a trend of increased collagen density compared to the control group. For most groups, PSR staining consistently indicated slightly lower collagen densities than MT staining. However, the overall trends were similar in both staining. The comparison between PSR and MT staining methods revealed that both techniques effectively assess collagen density in healing wounds. However, there were subtle differences in the absolute values obtained, with PSR staining tending to yield slightly lower collagen density measurements than MT. These differences can be attributed to the distinct mechanisms of these staining methods. Therefore, both staining methods can digitally quantify collagen density in wound healing research.

1 Introduction

Wound healing is a complex and dynamic process involving various cellular and molecular events to restore tissue integrity and functionality (Bist et al. 2021; Banu et al. 2023). Collagen, a major extracellular matrix (ECM) component, is pivotal in tissue repair, providing structural support and guiding cellular activities (Kular et al. 2014). In this study, PF127 hydrogel is loaded with adipose-derived stromal vascular fraction (AdSVF), mesenchymal stem cells (AdMSC), and conditioned media (AdMSC-CM). These components are chosen for their regenerative potential and ability to influence the wound-healing microenvironment. Accurate and reproducible quantification of collagen density is imperative for evaluating the success of wound healing interventions. Masson's Trichrome (MT) and Picrosirius Red (PSR) staining methods are selected for their well-established roles in visualizing and quantifying collagen fibers in tissue sections (Marcos-Garcés et al. 2017; Costa et al. 2019).

MT staining is a widely used histological technique to visualize collagen fibers in tissue sections (Van De Vlekkert et al. 2020). This staining method is precious because it can differentiate collagen from other tissue components, providing enhanced contrast and detailed information about collagen morphology and distribution. The principle behind MT staining lies in the differential affinity of tissue components for specific dyes (Chang and Kessler 2008). MT staining finds its applications in various realms of research and diagnostics. Researchers often rely on its ability to assess collagen density, with the intensity of the blue or green colouration acting as a quantitative indicator. Beyond quantification, the method detects fibrotic changes, making it a go-to tool for identifying pathological conditions like cardiac fibrosis, liver cirrhosis, and pulmonary fibrosis (Chang and Kessler 2008; Van De Vlekkert et al. 2020).

PSR staining is a widely used histological technique that provides excellent visualization of collagen fibers within tissue sections (Lattouf et al. 2014; López De Padilla et al. 2021). While its true power is often showcased under polarized light, PSR staining is also valuable when observed under regular brightfield microscopy (López De Padilla et al. 2021). This technique enhances the contrast between collagen and surrounding tissues, offering detailed insights into collagen distribution, density, and morphology (Lattouf et al. 2014). The principle of PSR staining involves the interaction between the dye and collagen fibers. Picrosirius Red, derived from the synthetic dye Sirius Red F3BA, has a specific affinity for collagen (Sharma et al. 2015). When applied to tissue sections, PSR binds to collagen molecules, resulting in distinct colouration under brightfield microscopy (Lattouf et al. 2014; López De Padilla et al. 2021). Both methods offer distinct advantages, and their comparative evaluation in this study aims to elucidate their efficacy in the specific context of digital collagen quantification.

In wound healing research, accurately quantifying collagen density is essential for assessing the efficacy of therapeutic interventions. This study evaluates two widely used staining methods, MT and Picrosirius Red, for digitally quantifying collagen density in a rabbit model of full-thickness skin wounds. The rationale behind this investigation lies in the need for robust and reliable techniques to measure collagen levels precisely, aiding in the comprehensive understanding of wound healing responses to various treatments.

Understanding the comparative performance of these staining methods is critical for researchers and clinicians involved in wound healing studies. Reliable collagen quantification contributes to optimizing therapeutic strategies and enhances the translatability of preclinical findings to clinical applications (Marcos-Garcés et al. 2017). We hypothesize that both MT and PSR staining methods will effectively quantify collagen density in healing wounds but may yield subtle differences in absolute values due to their distinct mechanisms of action. The results of this comparative evaluation will guide researchers in selecting the most suitable staining method for their specific wound-healing research contexts.

2 Materials and Methods

2.1 Masson's Trichrome staining

The MT staining procedure was performed on paraffin-embedded tissue sections. These sections were initially dewaxed by

immersion in xylene until the paraffin was removed entirely. Subsequently, a series of descending alcohol concentrations (100%, 90%, 70% and 50% alcohol) were used for rehydration, followed by a rinse in distilled water. The nuclei of the tissue sections were stained with Weigert's iron haematoxylin for approximately 5-10 minutes, and the excess stain was removed by rinsing with tap water. Cytoplasm and muscle fibers were stained using Biebrich scarlet-acid fuchsin solution for 15-30 minutes, followed by another rinse in distilled water. The sections were treated with a phosphomolybdic-phosphotungstic acid solution to differentiate collagen and muscle fibres for 15 minutes. Collagen fibers were stained blue by immersing the sections in an aniline blue solution for 5-10 minutes, followed by a brief rinse in distilled water. After acid differentiation with 1% acetic acid solution, sections underwent gradual dehydration through ascending alcohol concentrations (70%, 80%, 90%, and 100% alcohol) and were cleared in xylene. Finally, a mounting medium (DPX) was applied to each section, covered with a glass coverslip, and allowed to dry. The stained tissue sections were then examined under a light microscope with appropriate filters for MT staining, facilitating the distinction of collagen fibers (blue) in histological analysis.

2.2 Collagen quantification using the Colour Deconvolution plugin in ImageJ software

Collagen density (Area %) was quantified using tissue sections stained using MT stain based on the protocol described by Chen et al. (2017) with modifications. The principle behind quantifying collagen in skin stained with MT stain using the Colour Deconvolution plugin in ImageJ is based on the separation of different colour channels in the stained tissue sections to isolate the collagen fibers and then quantifying them through thresholding and image analysis (Ruifrok and Johnston 2001; Landini et al. 2021).

The first step involves installing the ImageJ software on a computer. To achieve this, the user downloads and installs the software opens it, and loads the digital image of the MT-stained skin section by navigating through "File" > "Open" and selecting the image file. Moving on to the second step, the user is provided with two methods for installing and setting up the colour deconvolution plugin. In Method 1, the Color Deconvolution plugin is downloaded from the ImageJ website, saved in the "plugins" folder within the ImageJ directory, and ImageJ is restarted to ensure plugin recognition. In Method 2, the user is guided to download the JAR file from a specified link, and in ImageJ, the plugin is installed by going to the "Plugins" menu, selecting "Install...," and following the prompts. The user may need to restart ImageJ to complete the installation.

Select the "Straight Line" tool from the ImageJ toolbar. This tool allows us to measure the length of the scale bar (Figure 1). By clicking at one end of the scale bar in the image and without releasing the mouse button, drag the line to the other end of the scale bar. Release the mouse button to draw a straight line along

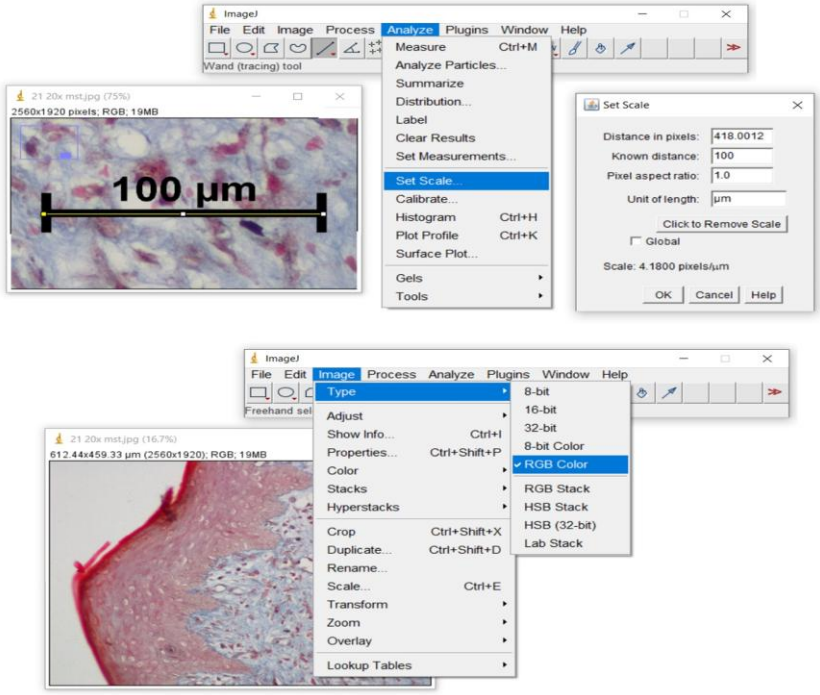

Figure 1 Steps for performing collagen quantification in ImageJ. The scale has to be calibrated to ensure accurate measurements. Use the straight-line selection tool to draw a line along the scale bar and set the known measurement. The input images should be converted to RGB images before performing the analysis

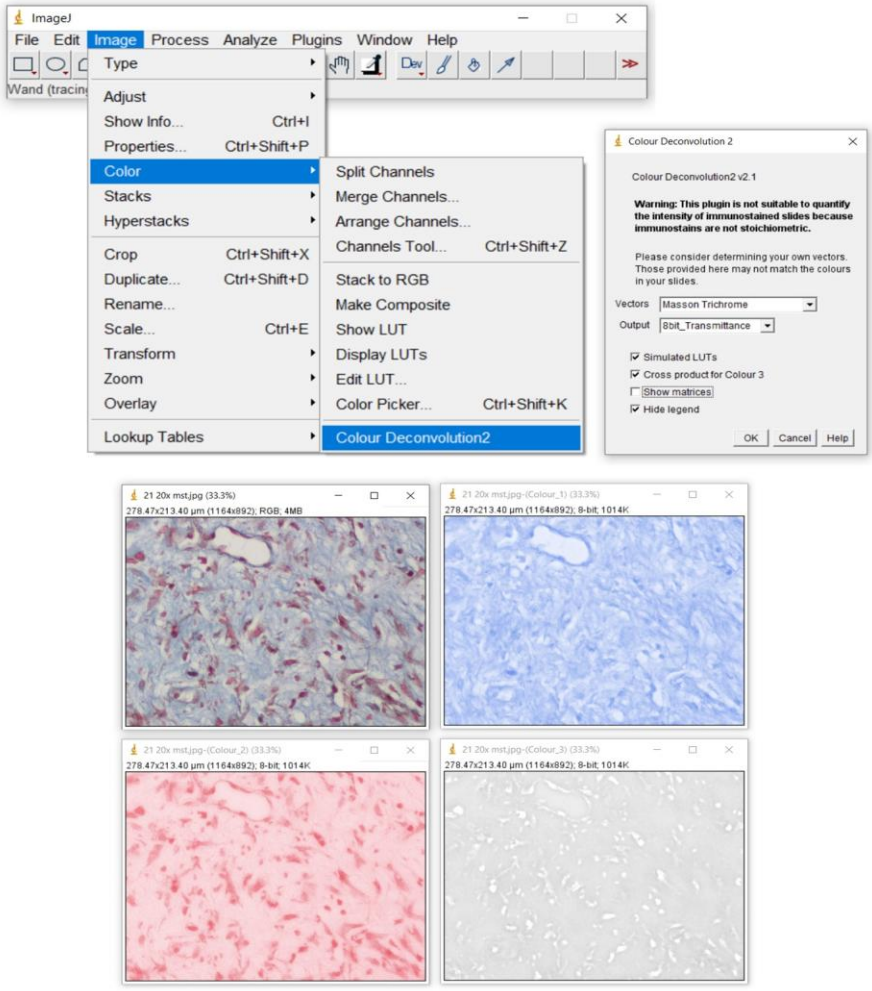

Figure 2 Steps for performing collagen quantification using image deconvolution technique in ImageJ. Choose the Masson's Trichrome stain vector in the Colour Deconvolution dialogue box. This will create separate grayscale images corresponding to each stain (red, blue and green components)

the scale bar. In the ImageJ main menu, go to "Analyse." Select "Set Scale." This will open the "Set Scale" dialog box. In the "Set Scale" dialog box, Enter the known distance of the scale bar (in micrometres) in the "Known Distance" box. Set the unit of length (e.g., µm) in the "Unit of Length" box. Click "OK" to set the scale. Open the image to analyze. The input images were converted to RGB images by clicking on the "Image" menu (Figure 1), clicking on the "Type" box, and then "RGB Color." Click on "Image" > "Color" in the menu bar and select "Colour Deconvolution2." In the Colour Deconvolution dialog box, the Masson's Trichrome stain vector should be chosen (Figure 2) (Ruifrok and Johnston 2001; Landini et al. 2021).

Parameters were set as indicated in Figure 3. This was followed by clicking "OK" to perform the colour deconvolution. This will create separate grayscale images corresponding to each stain (red, blue and green components) and select the grayscale image corresponding to the collagen stain (blue). Click on "Image" > "Adjust" > "Threshold" to open the Threshold dialog box (Figure 3). Adjust the threshold settings to segment the collagen fibers accurately. The settings mentioned above are appropriate to achieve the best results. Click "Apply" to apply the threshold to the image, making the collagen fibers appear as a black and white binary image. In the ImageJ main menu, click on "Analyze." From the dropdown menu, select "Set Measurements." This will open the "Set Measurements" dialog box. In the dialog box, make sure the following options are checked: "Area" to measure the area of selected regions, "Standard Deviation" to calculate the standard deviation of pixel values within the selected regions (Ruifrok and Johnston 2001; Landini et al. 2021), "Area Fraction" to determine the fraction of the total area covered by the selected regions, "Display Label": to display labels or names for the measured regions, and "Limit to Threshold" to limit measurements to areas within the threshold (Figure 4).

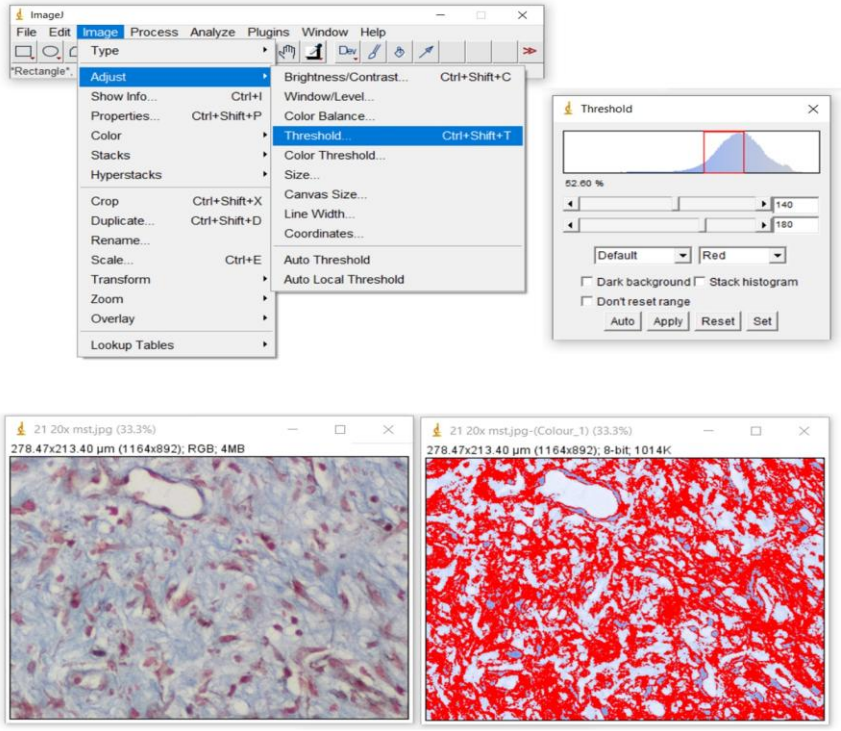

Figure 3 Steps for performing image thresholding in ImageJ. The grayscale image corresponding to the collagen stain (blue) will be selected. The threshold settings should be adjusted to segment the collagen fibers accurately

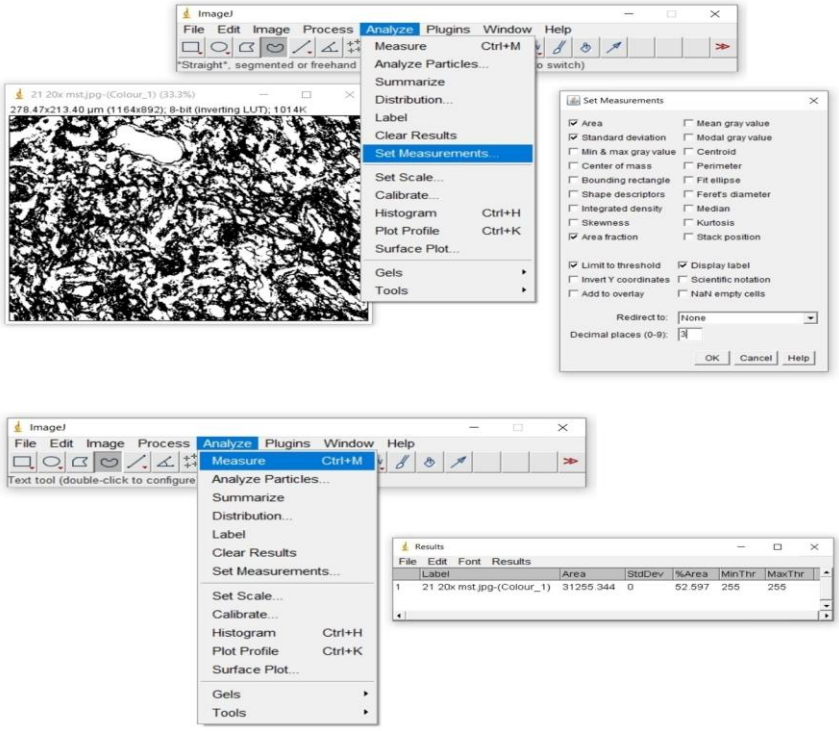

Figure 4 Steps for performing collagen quantification in ImageJ. Once the threshold is applied, the collagen fibers appear as a black and white binary image. Once the measurement parameters are set, ImageJ will calculate and measure the area, standard deviation, and area fraction of the regions of interest (ROIs) within the threshold

Go back to the ImageJ main menu and select "Analyze." From the dropdown menu, choose "Measure" (or "Ctrl + M" can be pressed on the keyboard). ImageJ will calculate and measure the area, standard deviation, and area fraction of the regions of interest (ROIs) within the threshold. The measurement results will be displayed in the "Results" window. To further analyze or quantify the ROIs, they can be manually selected and extracted by using tools such as the "ROI Manager" in ImageJ ("Analyze" > "Tools" > "ROI Manager"). Once the ROIs are selected, area-based analysis can be performed. The results table will show measurements of the collagen area in the selected image. To quantify collagen in multiple images, the steps for each image can be repeated (Ruifrok and Johnston, 2001; Landini et al. 2021). Export or save the measurement results for further analysis if needed. Import the measurement results into a spreadsheet program for data analysis and visualization.

2.3 Picrosirius red staining for collagen estimation

PSR stain (Solution A) was prepared by mixing 0.5 g of Direct Red 80/Sirius red F3B (Sigma-Aldrich, Catalog # 365548) in 500 ml of saturated aqueous solution of picric acid. This solution was considered stable and can be stored for at least 3 years and used multiple times. Solution B is acidified water made by mixing 5 ml of glacial acetic acid in 1 litre of distilled water. The tissue sections were deparaffinized by incubating them in xylene (two changes) and then rehydrated by passing them through a graded ethanol series (100%, 90%, 70%, 50% and distilled water). The nuclei were stained using Weigert's iron haematoxylin (8 min) and the slides for 10 minutes in running tap water. The sections were stained in PSR (Solution A) for one hour. It has to be noted that longer staining time with PSR did not improve the staining quality, but shorter staining times were discouraged, even if the colours appeared satisfactory.

After staining, the sections were washed in two changes of acidified water (Solution B). Most of the water was removed from the slides either by vigorous shaking or, in the case of a few slides, by blotting with damp filter paper. The sections were then dehydrated in three changes of 100% ethanol. Finally, the sections were cleared in xylene and mounted using a mounting medium and coverslip. Once the slides were prepared, the collagen fibers were visualized using a light microscope.

2.4 Collagen quantification in picrosirius stained sections

Open the image of the picrosirius stained tissue section in ImageJ. Measure the scale bar in the image using the straight-line selection tool. Create a line selection along the length of the scale bar. Go to "Analyze" in the menu bar and select "Set Scale." In the "Set Scale" dialog box, Ensure that the "Distance in Pixels" field is automatically populated with the length of the line selection. Enter the known distance (in µm) in the "Known Distance" field. Set the "Unit of Length" to "µm" (micrometres). Click "OK" to apply the scale to the image. In the menu bar, go to "Image" and select "Type," then choose "RGB Stack" to split the image into its red, green, and blue channels (Figure 5). After splitting, go to "Image" and select "Stacks," then choose "Make Montage" to view all three

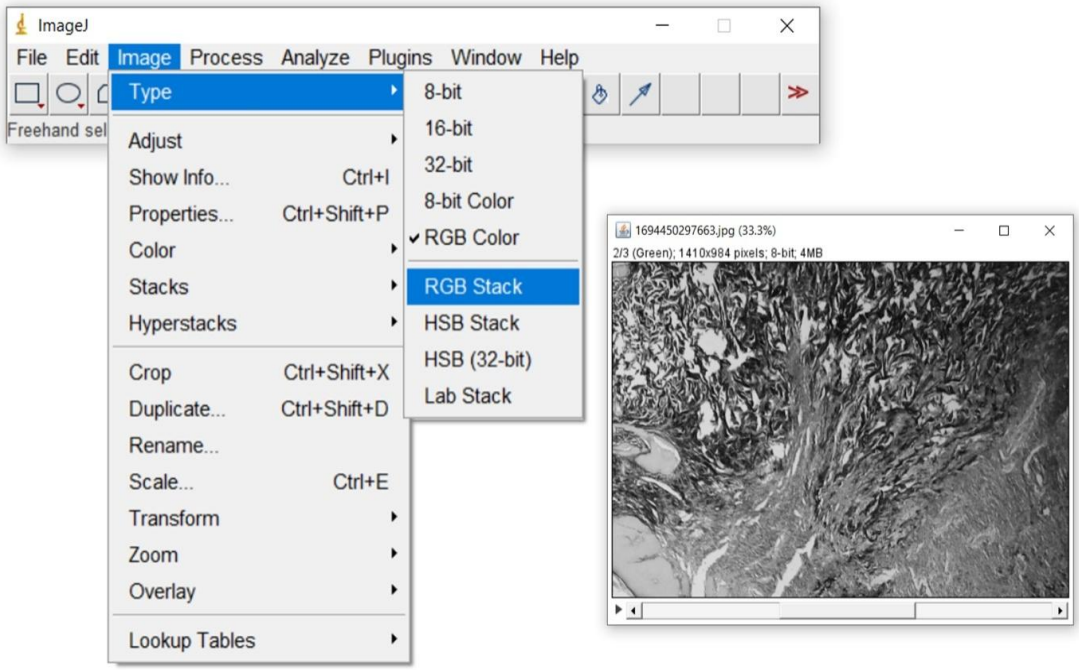

Figure 5 Steps for performing collagen quantification using RGB stacking technique in ImageJ

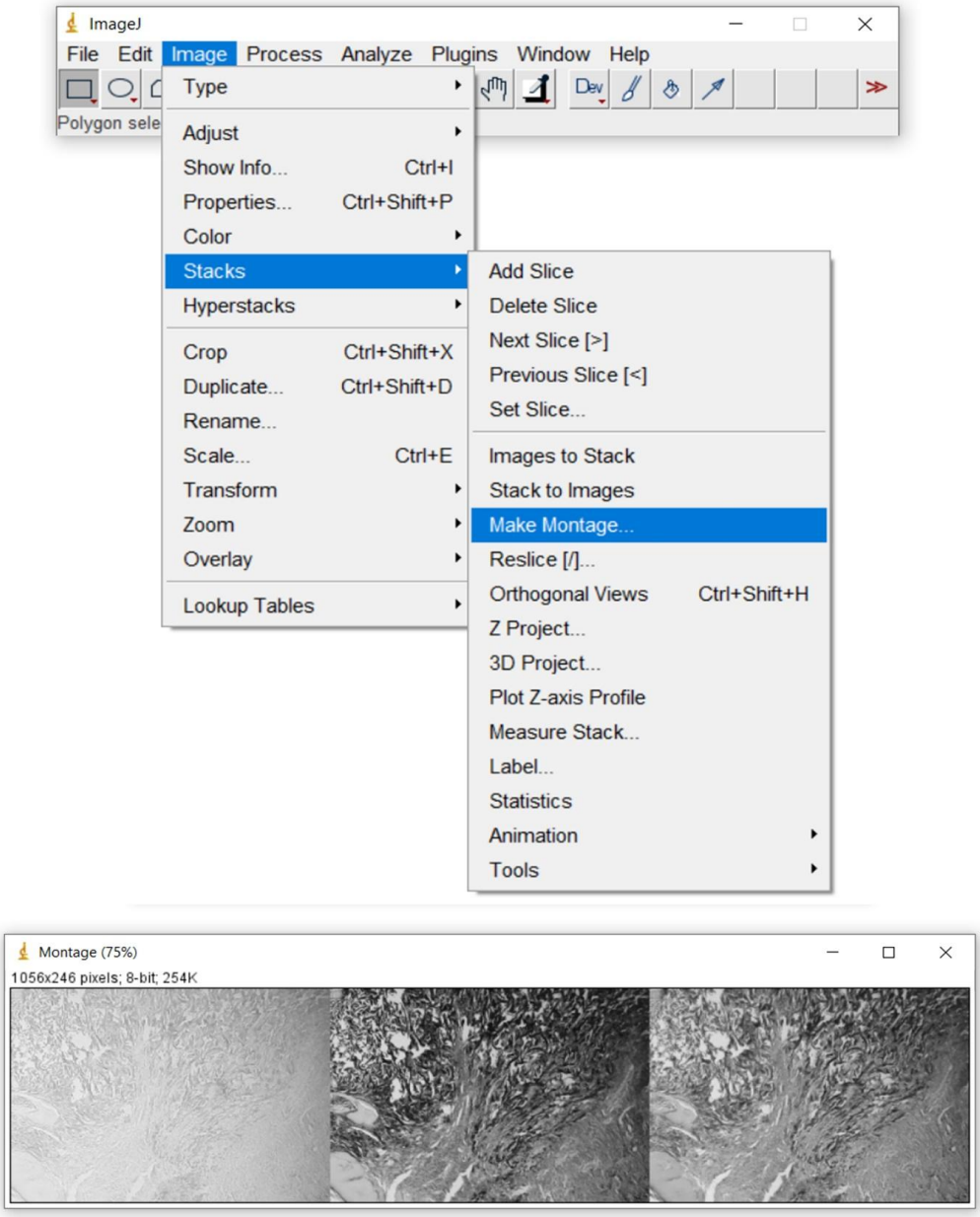

Figure 6 Steps for performing collagen quantification using RGB stacking technique in ImageJ. This will split the image into its red, green, and blue channels. A montage was done to view all three channels simultaneously

channels simultaneously (Figure 6) (Ruifrok and Johnston 2001; Landini et al. 2021). Select the RGB stack, making sure the green channel is chosen. While the Green channel is selected, go to "Image," then select "Adjust" and choose "Threshold." The "Threshold" tool will open, and the green channel will be thresholded automatically.

However, the threshold for accurate collagen detection was manually adjusted. Move the lower slider to the right until the red-stained collagen is highlighted in red while the background is mostly black. Once the threshold is set correctly, click "Apply" in the "Threshold" tool. Open the "Analyze" menu and select "Set Measurements." In the "Set Measurements" dialog box, check the following options: "Area," "Area Fraction," "Limit to Threshold," and "Display Label." Close the "Set Measurements" dialog. To measure the thresholded collagen area, go to "Analyze" and choose "Measure." The area and percent area of the thresholded collagen will be displayed in the "Results" window (Figure 7) (Ruifrok and Johnston 2001; Landini et al. 2021).

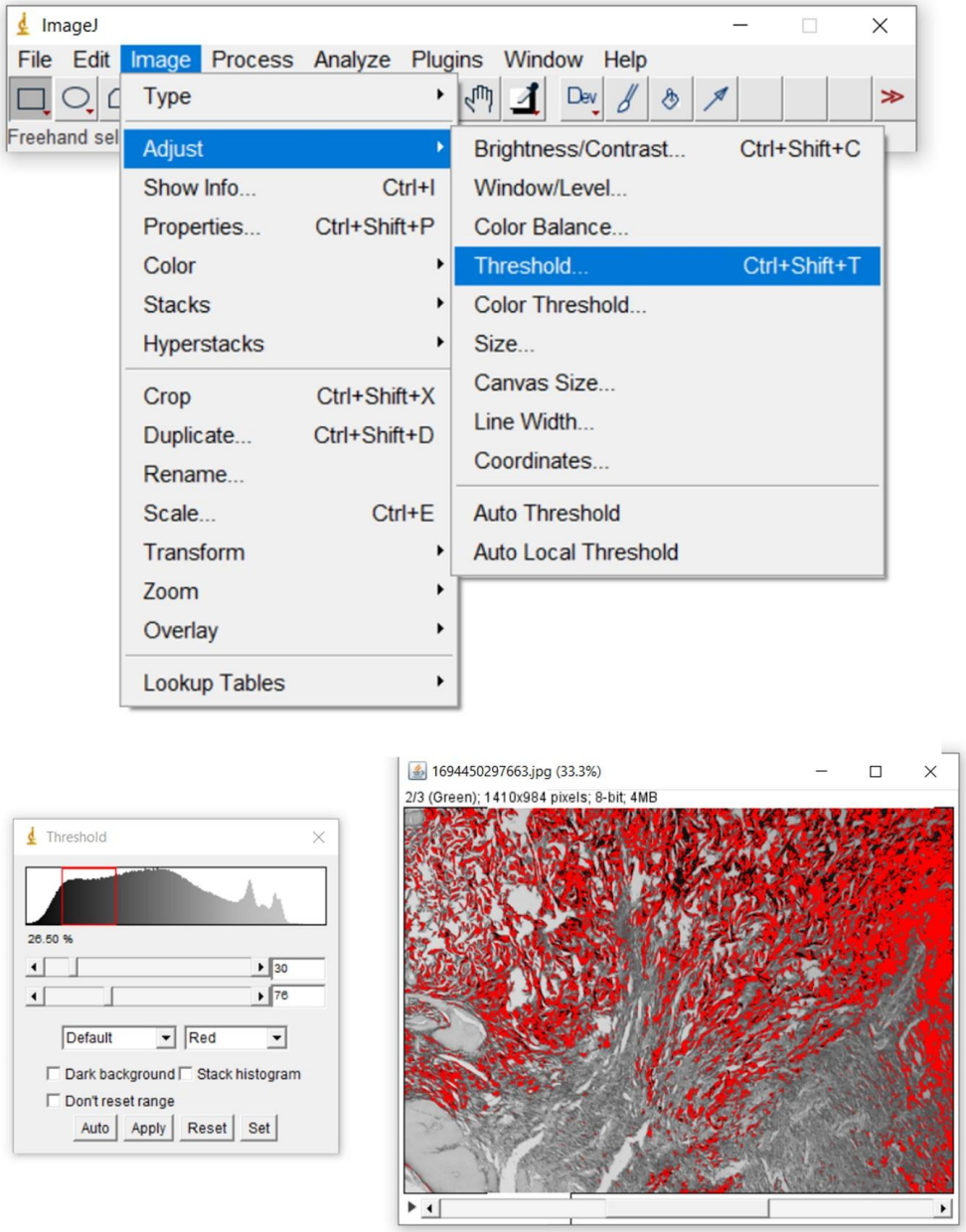

Figure 7 Steps for performing collagen quantification using RGB stacking technique in ImageJ. The threshold settings should be adjusted to segment the collagen fibers accurately

3 Results

3.1 Masson's Trichrome versus Picrosirius Red Staining

In MT staining, collagen fibers are stained blue. By staining collagen blue, this technique allows for the visualization and assessment of collagen distribution and content in tissue samples (Figure 8). Blue-stained collagen fibers are often seen as tissues' structural framework or scaffold. Muscle fibers, cytoplasm, and keratinized structures (such as keratinized skin cells) are stained red or pink in MT staining. This staining is achieved using Biebrich scarlet-acid fuchsin solution. The dark black colouration of the nuclei results from the staining with Weigert's iron haematoxylin.

In PSR staining, collagen appeared red against a pale-yellow background under brightfield microscopy (Figure 8). The nuclei ideally appeared black, although they might often appear grey or brown due to the extended exposure to picrosirius red, which caused some degree of de-staining of the nuclei.

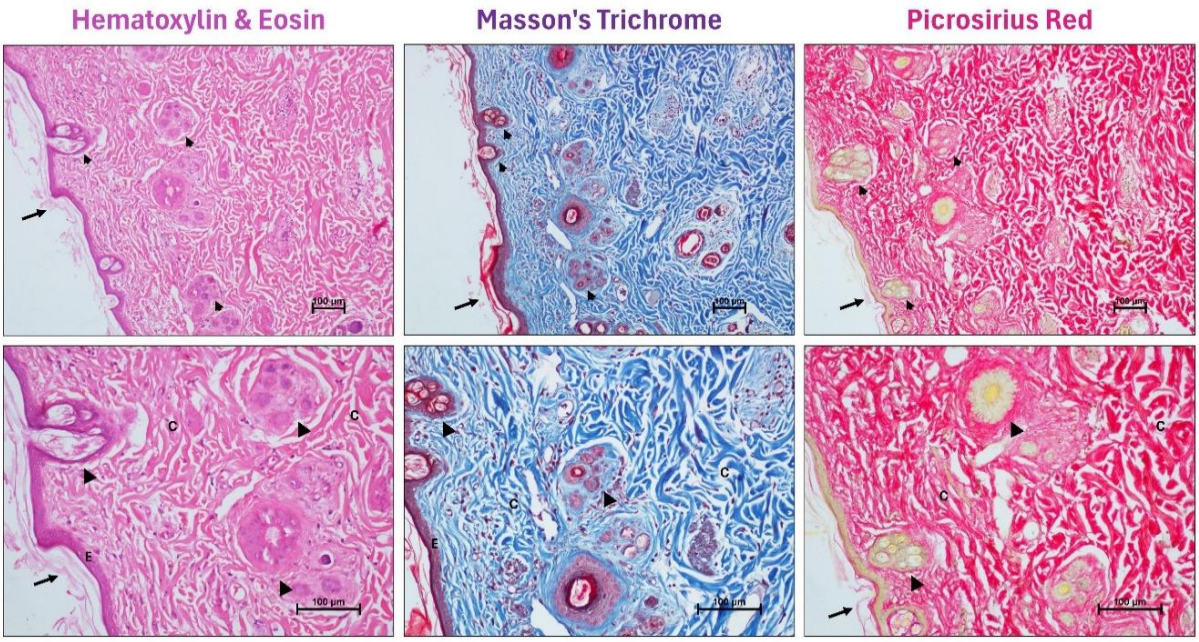

Figure 8 Photomicrographs of healthy rabbit skin from dorsum showing normal histological structures (Top panels - x100 and Lower panels - x200). The stained section of normal rabbit skin shows a well-organized structure with intact epithelium, hair follicles, and a loose connective tissue dermis. Note: keratin layer (black arrow), epidermis (E), collagen fibers (C), hair follicles (black arrowhead)

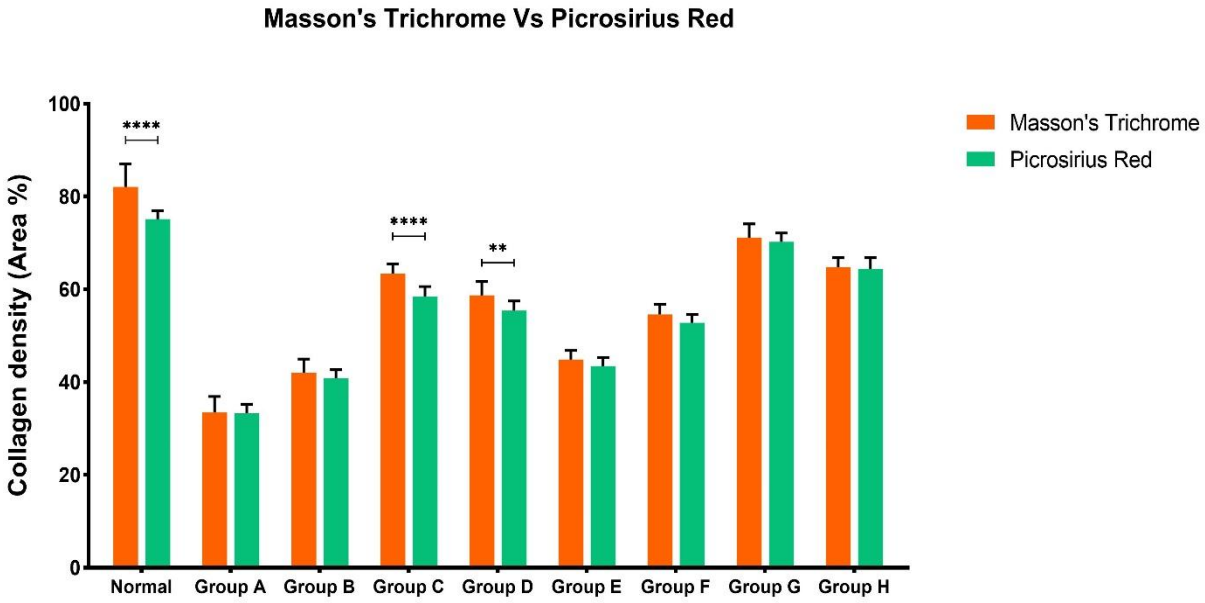

Figure 9 Comparative analysis of collagen density (Area %) values generated from MT and PSR stained sections using colour deconvolution and RGB stack methods. Note: ** - $p < 0.01$and **** - $p < 0.0001$

3.2 Comparison of collagen density in Masson's Trichrome and Picrosirius Red stained sections

We compared the collagen density values obtained using two different staining methods, PSR and MT (Figure 9). This provides insights into how these techniques measure collagen content and organization in the healing wounds of the studied groups. The baseline collagen density in healthy skin, measured with PSR staining, was 75.15±1.78%. MT staining, on the other hand, indicated a slightly higher baseline collagen density of 82.08±4.95%. Across the treatment groups, both staining methods generally showed a trend of increased collagen density

compared to the control group. For most groups, PSR staining consistently indicated slightly lower collagen densities than MT staining. However, the overall trends were similar.

The comparison between PSR and MT staining methods revealed that both techniques are effective in assessing collagen density in healing wounds. However, there were subtle differences in the absolute values obtained, with PSR tending to yield slightly lower collagen density measurements than MT. These differences can be attributed to the distinct mechanisms of these staining methods. PSR specifically stains collagen fibers and provides an enhanced visualization of collagen organization, making it suitable for assessing collagen fiber quality and maturity.

4 Discussion

The histological assessment conducted by trained pathologists utilizing semi-quantitative scoring techniques has traditionally been employed for evaluating collagen fiber density. Nevertheless, the subjective nature of this histologic evaluation is susceptible to inter-observer bias, leading to diminished accuracy and reproducibility due to inherent methodological constraints (Lee et al. 2001). Previous studies have focused on assessing collagen fibers by categorizing them based on the different scoring systems. Regrettably, this system was limited to demonstrating qualitative improvements and couldn't provide quantitative data (Meyer et al. 2017). In contrast, the methodology used in this study facilitates the direct comparison of collagen fiber density using continuous values and rigorous statistical analysis, allowing for a quantitative assessment of the therapeutic effects. MT stain is a histological staining method that uses multiple dyes to distinguish various tissue components. The stain typically consists of Weigert's Haematoxylin (stains nuclei dark purple or black), Aniline blue (stains collagen fibers blue), and Biebrich scarlet-acid fuchsin (stains cytoplasm other cellular components red).

MT techniques effectively provide intense staining for collagen fibers in connective tissue. This technique is best for collagen fibres' quantitative visualization and analysis capabilities. However, they fall short in selectively staining specific structures, such as the basal membrane (Constantine and Mowry 1968). This lack of selectivity poses challenges in accurately differentiating structures, especially in the presence of thin collagen fibers that may exhibit different colours within the same tissue section. MT staining is hindered by a background stained red by the dye, complicating the distinction between true colours observed (Street et al. 2014).

PSR staining is a more straightforward and highly effective method for examining collagen arrangement. This technique offers results superior to routine staining techniques (Juengsomjit et al. 2022). This technique involves Sirius red reacting with basic groups in collagen molecules through its sulfonic acid groups, thereby effectively staining the collagens (Junqueira et al. 1979). Unlike trichrome staining, PSR staining has been widely regarded as the benchmark for detecting and quantitatively estimating collagen in histological sections of both normal and abnormal tissues (Segnani et al. 2015).

A study evaluated a porcine model for pathomorphological age assessment of surgically excised skin wounds. The results were assessed through special staining techniques involving MT and picrosirius red. MT and PSR stains confirmed newly formed collagen and effectively evaluated increased collagen deposition (Barington et al. 2018). Both these stainings have addable advantages in predicting the variation in levels of myofibroblast and collagen in wound healing (Owens et al. 2010).

The principle begins with colour deconvolution, which separates these individual stains into distinct grayscale channels. The Colour Deconvolution plugin in ImageJ takes advantage of the fact that each stain has a characteristic colour spectrum (Ruifrok and Johnston 2001; Landini et al. 2021). Using mathematical algorithms, the plugin separates the RGB (Red-Green-Blue) colour channels of the stained image into grayscale images corresponding to each stain. In this case, the blue collagen stain is isolated. After separating the collagen stain into a grayscale image, the next step involves setting a suitable threshold (Chen et al. 2017). Thresholding converts the grayscale image into a binary image, where the collagen fibers appear black against a white background. This is achieved by specifying intensity values above which pixels are considered part of the collagen (black) and below which they are not (white) (Chen et al. 2017). The choice of thresholding values depends on the image's staining intensity and background noise. After measuring the collagen area in one or more images, analysis was done to derive quantitative information (total collagen area) (Chen et al. 2017; Elshazly et al. 2023).

Conclusion

Picrosirius Red staining consistently indicated slightly lower collagen densities than Masson's Trichrome staining. However, the overall trends were similar in both staining. The comparison between Picrosirius Red and Masson's Trichrome staining methods revealed that both techniques effectively assess collagen density in healing wounds. However, there were subtle differences in the absolute values obtained, with Picrosirius Red staining tending to yield slightly lower collagen density measurements than Masson's Trichrome. These differences can be attributed to the distinct mechanisms of these staining methods. Therefore, based on our findings, both staining methods can digitally quantify collagen density in wound healing research.

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Data availability

Datasets supporting the conclusions of this article are included within the manuscript.

Declarations

Competing interests

All authors declare that no commercial or financial relationships exist that could, in any way, lead to a potential conflict of interest.

Ethics approval and consent to participate

All experimental protocols used in the study were approved by the Institutional Animal Ethics Committee (IAEC), ICAR-Indian Veterinary Research Institute, vide order No. 26-1/2022-23/JD(R) under protocol no. IAEC/07.07.2022/S31.

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