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Automatic segmentation of fibrosis in histological images of *Picrosirius red* stained tissues using supervised and unsupervised machine learning algorithms

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ABSTRACT

Fibrosis results from the excessive deposition of collagen in organs after chronic inflammation, impairing their function. The detection and quantification of collagen are essential for diagnosis and treatment, and Picrosirius Red (PSR) staining is a gold standard technique due to its high efficacy. This study aims to develop machine learning methods to target collagen in histological images obtained with simple microscopy, comparing supervised and unsupervised techniques.

Keywords: Fibrosis, Collagen, Machine learning.

INTRODUCTION

Fibrosis is characterized by the deposition of collagen or connective tissue in an organ after a process of chronic inflammation. This phenomenon results in an excessive and abnormal increase in the production of the extracellular matrix (ECM), resulting from scarring or reactional processes, causing undesirable effects to the body and compromising the function of the affected organs [1,2]. Thus, the detection of collagen in histological samples is fundamental in the clinical diagnosis of fibrosis, as in cases of hepatic [3,4], pulmonary [5], and renal [6,7] fibrosis. In addition, the quantification of collagen in healthy and pathological conditions can help in understanding the mechanisms of some diseases, prognosis, and treatment [8].

Several staining techniques have been developed to detect and quantify collagen deposition in histological sections, with varying degrees of efficacy. Among histochemical methods, traditional trichrome stains, such as the methods of Mallory, Masson, and van Gieson, have been shown to underestimate collagen content [9, 10]. Alternatively, PicroSirius-Red (PSR) staining was developed by introducing a more selective method for detecting collagen fibers. This method exhibits less fading over

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time compared to van Gieson staining and allows for better visualization through polarized light microscopy [11], as collagen fibers become birefringent. In addition, it allows a qualitative analysis of the element by differentiating thicker collagen fibers from thinner ones through the different birefringence colors ranging from red (thicker fibers) to green (finer fibers). In fact, this method is currently considered the gold standard for collagen quantification [12].

Obtaining histological images under polarized light (PL) allows segmenting and demarcating collagen exclusively using specific software, enabling the quantification of fibrosis [6,9]. However, acquiring these images requires more time and more expensive equipment. Therefore, analyzing images without polarized light is a faster and more affordable alternative. However, differentiating collagen fibers from the rest of the image is a major challenge, as the pixel intensities are very close together and do not differ as much as they are under polarized light. In order to mitigate this challenge, in recent decades, different computer vision techniques and methodologies have been employed to solve problems of classification and segmentation of objects such as this one.

In this context, several techniques have been employed, including machine learning algorithms, neural networks, and deep learning (*DL).* Among these, DL stands out as one of the most modern, using multiple layers of processing to identify patterns and structures in large data sets. This method does not require prior processing of the data, as it automatically extracts the attributes or characteristics from the raw image. However, studies involving LD require a large volume of training data, which may not be feasible for some analyses [13, 14, 15]. Other algorithms, such as the Multilayer Perceptron (MLP), follow similar principles to DL, but the attributes are not automatically extracted. However, they do not require as high an amount of training data as deep learning [16, 17].

Another alternative is unsupervised machine learning algorithms, which do not require prior classification of data. This approach is primarily feasible for small, unclassified datasets. Among the unsupervised algorithms widely used in the field of classification is K-Means. It is an unsupervised clustering algorithm that classifies input data by its characteristics or *features.* Therefore, it is necessary to provide as input of the algorithm some characteristics that characterize the object of interest and the method groups the patterns according to their similarity [18,19].

Thus, the objective of the present project is the development and standardization of a robust, cost-effective and easy-to-apply method for quantitative analysis of collagen in histological images stained with Picrosirius Red, and obtained under simple light microscopy (brightfield). To this end, two methods will be tested, one of supervised learning and the other of unsupervised learning, seeking to obtain reliable and reproducible results, optimizing and reducing the analysis time.

OBJECTIVE

Development and validation of a method for collagen segmentation in histological images stained with Picrosirius Red, using supervised (Multilayer Perceptron) and unsupervised (K-Means) machine learning algorithms, from photomicrographs obtained by simple microscopy, without the use of polarized light, for fibrosis detection.

METHODOLOGY

OBTAINING HISTOLOGICAL IMAGES

The histological slides used in this study were obtained from different research centers and produced in previous experimental protocols. In total, 120 photomicrographs of kidney, heart and tendon tissues were captured, from both mice and rats. The entire protocol for the use of histological slides was approved by the Ethics Committee on the Use of Animals (CEUA 6210010316; CEUA 056/2010). The tissues were processed following a standard protocol: fixation in 10% buffered formalin; cross-sections of approximately 4 μm of the paraffin tissues; and coloring with *Picrosirius red.* The images were obtained at the Physiology Laboratory of the Institute of Science and Technology of UNIFESP, using the ZEN 3.7 software, an AxioLab 5 microscope and an Axiocam 208 color camera attached (Carl Zeiss Microscopy, GmBH). The photos were captured with a 20x lens, without the use of polarized light and also with the use of it, in order to obtain gold-standard images.

The image bank consisted of 60 histological images of mouse kidneys. Of these, 40 were intended exclusively for training the neural network used, while 20 were reserved for testing, both the neural network and the K-Means algorithm. Additionally, for the test group, 20 histological images of kidney, 20 heart images and 20 tendon images of rats were captured. Each test group included 10 high-quality images and 10 images containing some type of artifact, either from the preparation of the slide (such as bubbles or dirt) or from the image capture itself (such as lack of focus). The objective was to evaluate the versatility of algorithms in the segmentation of images under different conditions.

IMAGE PROCESSING

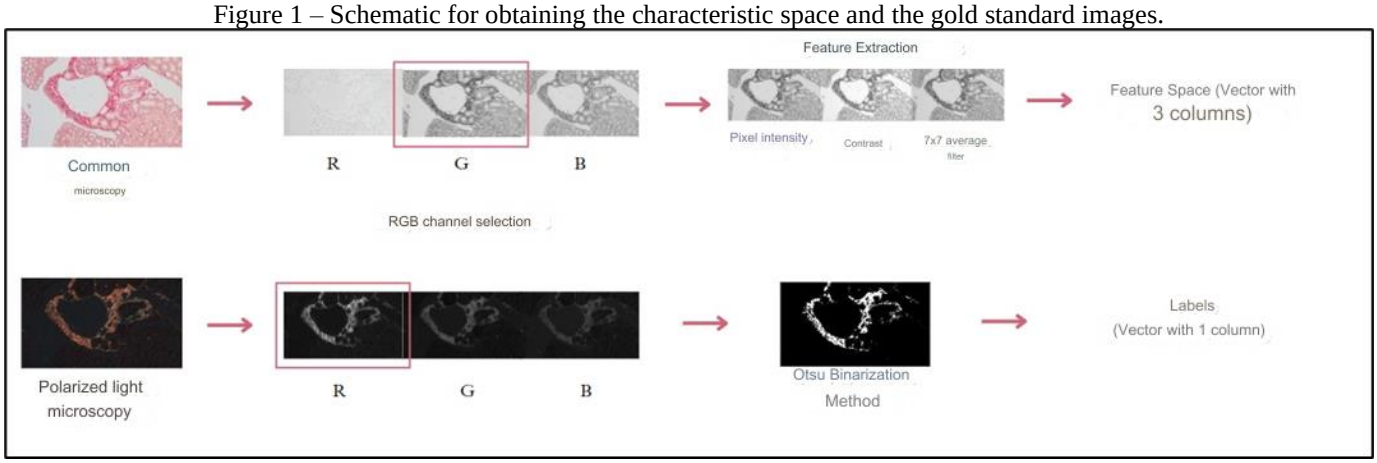
The processing and analysis of the images were carried out in Python language using the Spyder software version 4.1.5, free of charge, on a computer with an Intel Core i7 processor and 16GB of RAM. Thus, initially, a separate analysis of the RGB channels of each image was performed in order to verify which one favored the demarcation ofcollagen. For the images obtained in the absence of polarized light, the G channel presented the best contrast. In the images obtained using polarized light, the R channel better demarcated the collagen. Thus, the pixel intensities were normalized between 0 and 1 to standardize

the subsequent calculations and these channels (G - brightfield and R - darkfield) were used in the subsequent processing steps.

For both the supervised and unsupervised algorithms, it was necessary to provide some characteristics to distinguish the objects of interest from the other regions of the image. These *features* were found empirically and based on performance tests of the algorithms used. Thus, the *features* extracted or used were: original intensity of each pixel in the G channel; intensity of each pixel in the G channel after contrast elongation; image resulting from the correlation between the G channel and a 7x7 average filter. Each of these attributes was converted into a vector and placed in a column forming the input set of the algorithms.

To obtain the gold standard images, the images obtained using polarized light in the R channel were processed using the Otsu Method [20], which performs the binarization automatically by analyzing the image histogram. Thestep-by-step process for obtaining the feature space and the formation of the gold standard images can be seen below in Figure 1.

Figure 1 – Schematic for obtaining the characteristic space and the gold standard images.



IMPLEMENTATION OF THE K-MEANS METHOD

Before the application of the K-Means algorithm, tests were carried out to determine the ideal number of classes for fiber segmentation, obtaining better results with a number of classes equal to five. The KMeans function of the sklearn.cluster library was used with the standard parameters to implement this step. After classifying each pixel by separating it into classes, the object of interest was segmented.

NEURAL NETWORK IMPLEMENTATION

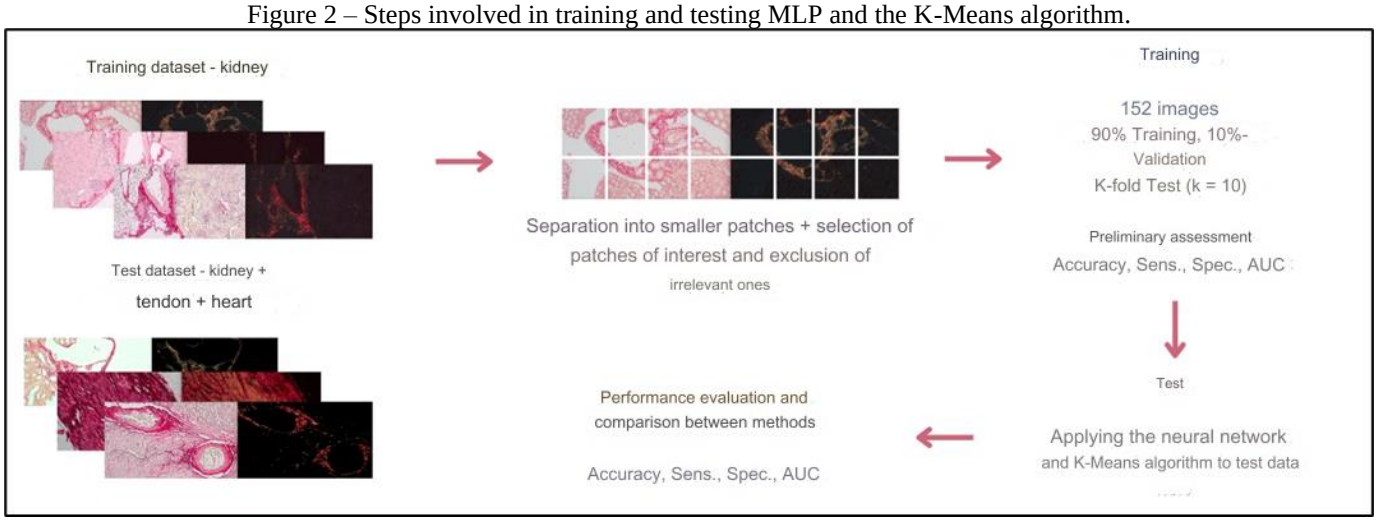
The neural network implemented is of the MLP type, built empirically after performance tests. It has three connected layers: the first is the input layer with 3 neurons and a 'linear' activation function; the second layer has 20 neurons with a 'relu' activation function; and the third layer has 1 neuron with

'*sigmoid*' activation. The model was compiled using the Adam optimizer, with a learning rate of 0.001, and the loss function chosen was '*binary_crossentropy*'.

To compose the training data, we used 40 mouse kidney images, all with their respective gold-standard (GS) images and dimensions of 1920x1080 pixels. These images were divided into *smaller patches, resulting in 320 images (480x540 pixels),* as can be seen in Figure 2. After visual analysis, we selected 152 images with a minimally satisfactory collagen ratio to compose the final training database, discarding those without marking. The previously mentioned features of interest were then calculated for each of these images, and the results were vectorized and allocated in a *Data Frame* to compose the neural network input data.

For the training and validation of the neural network, we used k-fold cross-validation with k=10, since the dataset was not extremely large. In each training iteration, 10% of the data was reserved for algorithm testing, while the remaining 90% was used for training. This process ensured that all data was used for both training and testing at least once. To evaluate the performance of the network, we calculated parameters such as accuracy, sensitivity, specificity, and the AUC of the ROC curve. The average of these values was calculated after the 10 iterations, and then the data and network weights were saved to be used in the classification of each of the test sets.

Figure 2 – Steps involved in training and testing MLP and the K-Means algorithm.



EVALUATION OF THE METHOD

To evaluate the performance of the algorithms, they were applied to the test data and parameters such as accuracy, sensitivity, specificity, and the AUC of the ROC curve were calculated. In addition, before capturing all the test and training images from the study, we applied a questionnaire to four experts in the field of histology to evaluate and validate the training data. We presented 10 mouse kidney images to the specialists and they answered the standardized questionnaire on the quality of the images, both in

bright and dark fields. Through this instrument (questionnaire) data were collected on the correspondence between the gold standards produced and the collagen fibers observed in bright field, and on the evaluation of the result of the segmentation produced by a preliminary neural network. Based on the answers obtained, we proceeded with the collection of the other images, using the laboratory parameters indicated by the experts, making the necessary adjustments to meet them.

DEVELOPMENT

After separating the RGB channels in each of the images, both in brightfield and with polarized light, and creating the feature space and the gold-standard images, we used the training dataset composed of 152 mouse kidney images to train and validate the implemented MLP. For this process, we applied K-fold cross-validation with 10 iterations, where 10% of the data was used for validation and 90% for training in each iteration. After the 10 iterations, we calculated the averages of the parameters evaluated in each step, as shown in Table 1.

Table 1 - Results of the parameters evaluated with k-fold validation for MLP in the training data of mouse kidney images.

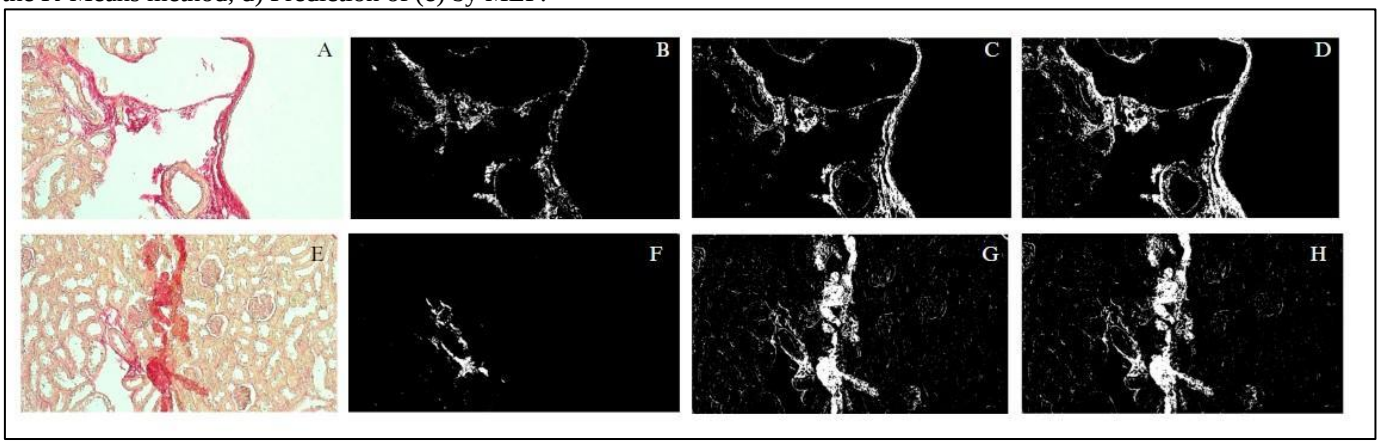
Acurácia (%)	Sensibilidade (%)	Especificidade (%)	AUC ROC
96,89	83,06	97,04	0,90

Analyzing the performance of the neural network, tested through cross-validation, we observed that it presented satisfactory results in the training images. The neural network demonstrated a high level of detection of collagen fibers and a good differentiation of non-collagen elements, evidenced by the high specificity of the method. Another positive point is the AUC (Area Under the Curve) of the ROC (Receiver Operating Characteristic) curve. The ROC curve is a graph that shows the relationship between the true positive rate (PV) and the false positive rate (FP) at different classification thresholds. The AUC is the total area under the ROC curve. It ranges from 0 to 1 and is an aggregate measure of the model's performance across all possible rating thresholds. The closer to 1, the better the classifier, that is, the better the correct classification of positive and negative examples.

With the neural network parameters saved, we started experiments with the test dataset, both for the unsupervised algorithm and for the MLP. Initially, we conducted tests to determine the optimal number of grouping classes when using K-Means, obtaining the best results with five classes. With this parameter set, we applied both algorithms to the available kidney, heart, and tendon test images. As previously reported, the images were separated into two groups: images with good quality or without artifacts and images with some negative characteristic that could hinder the classification of the pixels. These characteristics consisted of dirt, bubbles, weak colorations, and even lack of focus. Thus, in Figure

3 it is possible to observe the result of collagen segmentation provided by the two machine learning methods in the classification of mouse kidney images and the performances related to each of the methods in Table 2.

Figure 3 – Results of collagen fiber segmentation by the K-Means method and MLP in mouse kidney slides: a) photomicrograph of a histological slide of a mouse kidney in good condition obtained in bright field; b) gold standard image referring to collagen segmentation; c) Prediction of (a) by the K-Means method; d) Prediction of (a) by MLP; e) photomicrograph of histological slide of mouse kidney with presence of artifact; f) gold standard image; g) Prediction of (e) by the K-Means method; d) Prediction of (e) by MLP.



It is observed that the Otsu Method segmented the collagen fibers in a satisfactory manner, without eliminating any information or adding undue data. Similarly, the results of the K-Means and MLP segmentation were largely successful, but some undue pixels were added to the segmentation. Another factor to highlight is the difficulty that the algorithms had in separating what was an artifact from what was really collagen (Figure 3 - Chart E).

Regarding the performance of the methods, a noticeable difference was noted between the results obtained with good quality images and those of inferior quality. Although the accuracy values were very close, the sensitivity parameters and AUC revealed a lower performance of both methods for the lower quality images. For good quality images, the MLP method stood out in relation to the K-Means algorithm, with a sensitivity of 78.81%.

Table 2 – Performance of MLP and K-Means in the segmentation of collagen fibers in histological images of mouse kidneys.

Mouse Kidney Test Group	Accuracy (%)		Sensitivity (%)		Specificity (%)		AUC	
	MLP	K-Means	MLP	K-Means	MLP	K-Means	MLP	K-Means
Good Images	95.92	95.01	78.81	68.47	96.21	95.46	0.88	0.82
Bad Images	95.77	94.70	56.53	58,58	96.63	95.49	0.77	0.77

For rat kidney imaging, MLP performed better on sensitivity and AUC metrics, while K-Means stood out on accuracy and specificity metrics. An important point to be highlighted was the better performance of the methods on lower quality images compared to higher quality ones. This may have been due to the weaker staining in some images, where the collagen does not become sufficiently birefringent under polarized light. As a result, the methods target more fibers than those present in the gold standard, achieving high levels of sensitivity (Figure 4, Table 3).

In the heart images of rats in good conditions, both the supervised and unsupervised learning methods showed similar performances. However, in the lower quality heart images, the MLP demonstrated better performance in the parameters of accuracy and specificity, while the K-Means stood out in the other parameters.

Similarly, there was no significant difference between the performance of the methods when comparing the two sets of test images. In fact, the heart images considered to be of lower quality were not so different from the good quality images and had fewer artifacts than the other images (kidney and tendon) tested in this study. This demonstrates that the methods are robust in situations where the image has some slight alteration, unlike images with extensive artifacts as shown in Figure 3 - Chart E.

Figure 4 – Results of collagen fiber segmentation by the K-Means method and MLP in rat kidney slides: a) photomicrograph of histological slide of rat kidney in good condition obtained in bright field; b) gold standard image referring to collagen segmentation; c) Prediction of (a) by the K-Means method; d) Prediction of (a) by MLP; e) photomicrograph of histological slide of rat kidney with weak stain and presence of artifact; f) gold standard image; g) Prediction of (e) by the K-Means method; d) Prediction of (e) by MLP.

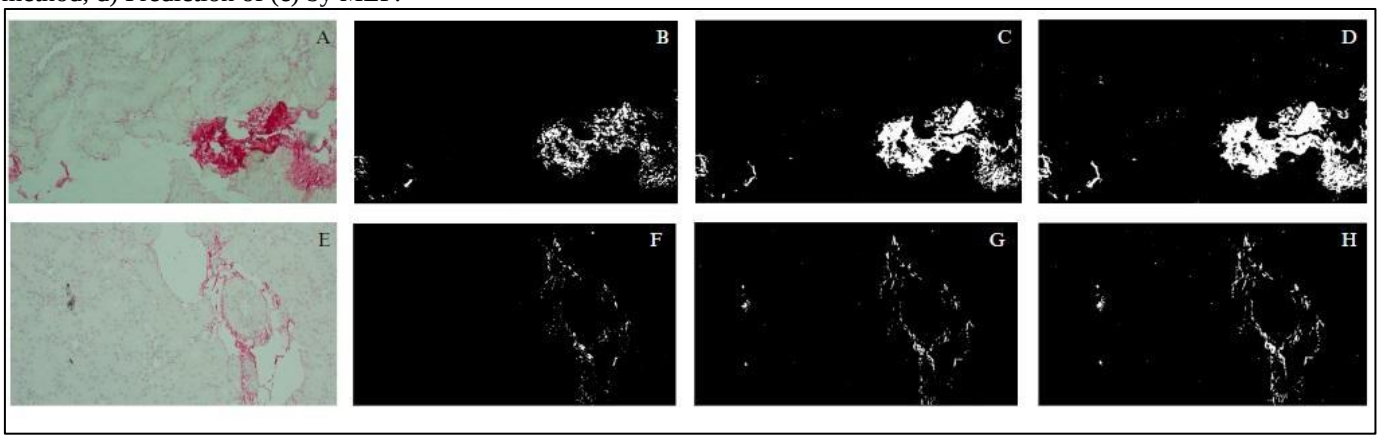


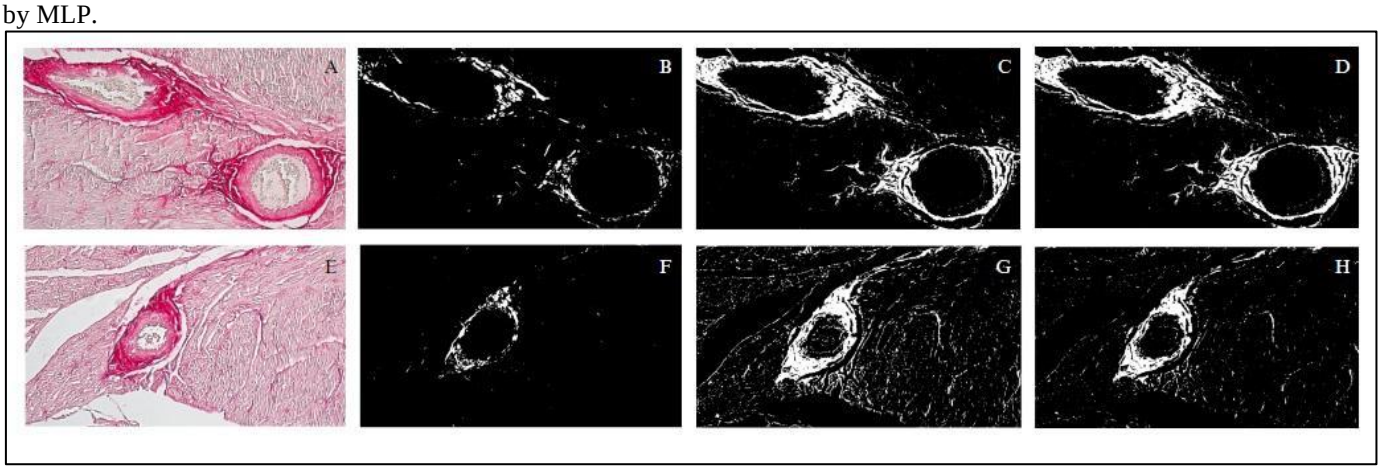
Table 3 – Performance of MLP and K-Means in the segmentation of collagen fibers in histological images of rat kidneys.

Rat Kidney Test Group	Accuracy (%)		Sensitivity (%)		Specificity (%)		AUC	
	MLP	K-Means	MLP	K-Means	MLP	K-Means	MLP	K-Means
Good Images	95.28	96.69	81.12	62.84	95.74	97.80	0.88	0.80
Bad Images	96.89	98.32	85.99	69.88	96.97	98.51	0.91	0.84

Table 4 – Performance of MLP and K-Means in the segmentation of collagen fibers in histological images of rat hearts.

Test group Rat heart	Accuracy (%)		Sensitivity (%)		Specificity (%)		AUC	
	MLP	**K-Means**	**MLP**	**K-Means**	**MLP**	**K-Means**	**MLP**	**K-Means**
Good Images	94.94	94.85	73.89	74.11	95.49	95.40	0.85	0.85
Bad Images	97.72	94.84	66.03	78.88	97.90	94.92	0.82	0.87

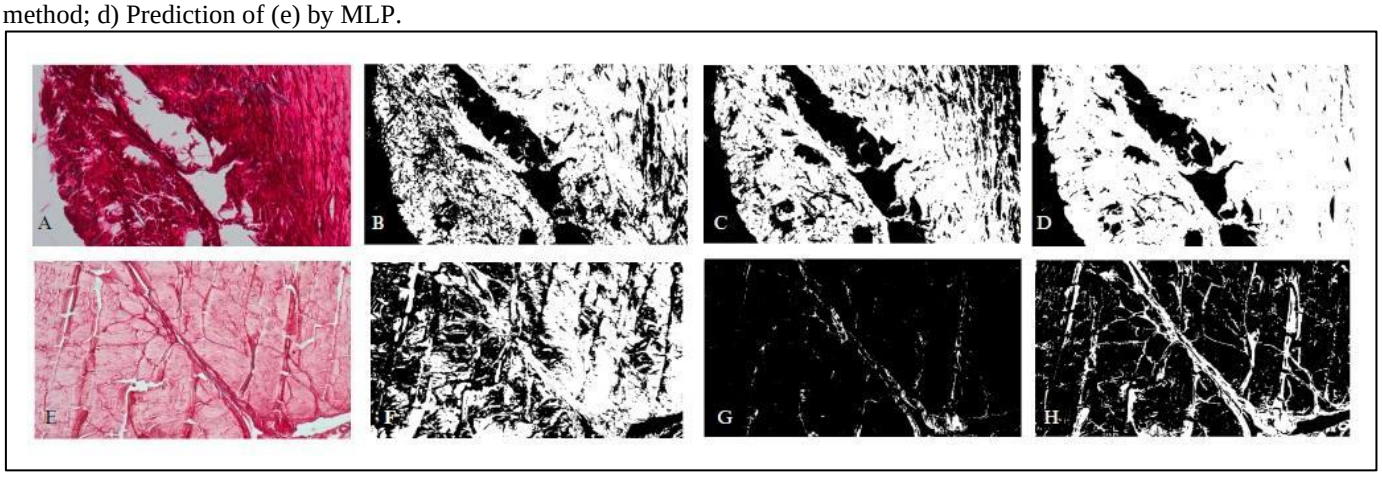
Figure 5 – Results of collagen fiber segmentation by the K-Means method and MLP in rat heart slides: a) photomicrograph of histological slide of rat heart in good condition obtained in bright field; b) gold standard image referring to collagen segmentation; c) Prediction of (a) by the K-Means method; d) Prediction of (a) by MLP; e) photomicrograph of a histological slide of a rat heart with lack of focus; f) gold standard image; g) Prediction of (e) by the K-Means method; d) Prediction of (e) by MLP.



The images of the tendon (Figure 6) differ from those shown above (Figures 3, 4 and 5), because almost its entire area is occupied by collagen fibers. Like the heart photos, the tendon photos did not have as many artifacts. Thus, to compose the group of 'bad' images, images were chosen not because they are of low quality, but because they present a greater challenge in their segmentation because they present thinner fibers that are stained with a less intense red than thicker fibers, as can be seen in the letter and figure below.

It is possible to observe in Figure 6, the difficulty of the algorithms in recognizing the collagen fibers that have a lighter color. In bright field, collagen can present darker shades, standing out from the other structures of the image, as well as it can present shades very similar to the other structures, making its differentiation and segmentation complex. In the same way, with gold-standard images, it is possible to analyze how collagen segmentation is not so trivial, since pixels of very close colors may or may not belong to the group of collagen fibers.

Figure 6 – Results of collagen fiber segmentation by the K-Means method and MLP in rat tendon slides: a) photomicrograph of the histological slide of a rat tendon in good condition obtained in a bright field; b) gold standard image referring to collagen segmentation; c) Prediction of (a) by the K-Means method; d) Prediction of (a) by MLP; e) photomicrograph of a histological slide of a rat tendon with collagen that is difficult to identify; f) gold standard image; g) Prediction of (e) by the K-Means method; d) Prediction of (e) by MLP.



Regarding the evaluation of the methods, the MLP showed a better performance than the K-Means method in tendon images, especially in the sensitivity parameter, as shown in Table 5. Note that the accuracy parameters were lower in this experimental group, as well as the specificity of the method. Specificity is the ability of the method to classify negative pixels as non-collagen. Therefore, the methods showed a high false positive rate. Even with these complications, MLP proved to be a more robust method than K-Means in this test group.

Table 5 – Performance of MLP and K-Means in the segmentation of collagen fibers in histological images of rat tendons.

Test group Rat tendon	Accuracy (%)		Sensitivity (%)		Specificity (%)		AUC	
	MLP	**K-Means**	**MLP**	**K-Means**	**MLP**	**K-Means**	**MLP**	**K-Means**
Good Images	81.09	72.41	95.99	70,70	61.41	74.69	0.79	0.73
Bad Images	71.98	64.32	80.16	59.99	59.99	81.02	0.70	0.67

Thus, through the results obtained, it was noted that the methods presented have an easier time segmenting perivascular collagen than interstitial collagen or finer fibers. This is already a well-known topic and addressed in previous research. In [21], an automated method was proposed to quantify renal fibrosis using images obtained under polarized light. In this study, perivascular collagen was eliminated so that only interstitial collagen could be quantified. The authors state that most of the interstitial fibrotic pixels were far from the vessels and had intermediate intensity. For comparison purposes, the results were correlated with Masson's semiquantitative trichrome technique, presenting significant differences in fibrosis quantification between the methods, especially when the perivascular collagen content is added to the stones.

Another point to consider is the construction of gold-standard images for validation of the technique. PicroSirius Red dye binds to the tertiary grooves of collagen fibrils and enhances their natural birefringence. Under polarized light, it appears bright against a dark background, making it easy to see. However, there are studies that pay attention to the importance of standardization in capturing images under polarized light. In Street et al. [22] implemented a fluorescence-based method for collagen quantification. In the study, images are collected in a standard way, with polarized light, and in an alternative way, by fluorescence. The samples are rotated at different angles, but captures are taken from the same points in the image. Thus, the authors found differences in both the tonality and intensity of the collagen fibers when using linear polarized light, unlike fluorescent light that did not reflect changes in the samples even after rotation, which may be a possible alternative for creating gold standard images. In the study by Greiner et al. [23], images were collected with linear polarized light. However, the slides are rotated at 6 different angles and 6 images have been captured, which are then combined to form the final image.

In the present study, the gold-standard images were captured with a circular polarizer, which, according to the literature, is more suitable than the linear polarizer for the reason mentioned above. However, it was noticeable that in many images, the grading methods demarcated more pixels than those present in the gold-standard images. This indicates the need for further acquisition tests of the gold standard images to ensure that this validation method is adequate.

In addition to the points mentioned above, it is interesting to mention that the use of more modern techniques for segmentation and classification of images such as convolutional neural networks (CNN) has been growing. Fu et al. [24] propose a method for identifying fibrosis in images stained with Masson's trichcomic staining, where the performance of the proposed neural network was higher than the conventional U-Net network. In addition, Pham et al. [25] present the use of deep CNN for analysis of scar fibrosis in histopathological images of tissues stained with hematoxylin and eosin.

Thus, the methods proposed in this study showed satisfactory and promising performances, pointing to the need for improvements in the parameters evaluated and CNNs may be an alternative, despite requiring an extensive training database.

FINAL CONSIDERATIONS

It is concluded that the methodology proposed in this study presents satisfactory results for collagen segmentation, especially perivascular. New methods based on *machine learning algorithms* such as those presented can facilitate, cheapen, and improve the identification and quantification of collagen in healthy and pathological tissues, aiding in the diagnosis and prognosis of various diseases associated with

fibrosis. In this study, the MLP-type algorithm proved to be more robust than the K-Means algorithm, especially in the database of test images with lower quality.

Another point to highlight is the need for an adequate methodology for the construction of gold-standard images. This protocol involves several challenging steps , involving adjustments that must be standardized in the collection of images under polarized light to ensure a more robust method of validation, such as the acquisition of images from several different angles and subsequent overlapping of the captured images.

In addition, it was possible to perceive, especially in the tendon images, that there are some challenges regarding the segmentation of thinner or less compressed collagen fibers, because the pixels that compose them have characteristics similar to those of other elements of the image. Therefore, it would be necessary to explore other characteristics to better characterize collagen or apply more modern techniques that automatically recognize patterns, requiring a significant image database.

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