Dermal type I collagen assessment by digital image analysis[*]

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Abstract: Type I collagen is the main dermal component, and its evaluation is relevant to quantitative studies in dermatopathology. However, visual gradation (0 to 4+) has low precision and high subjectivity levels. This study aimed to develop and validate a digital morphometric analysis technique to estimate type I collagen levels in the papillary dermis. Four evaluators visually quantified (0 to 4+) the density of type I collagen in 63 images of forearm skin biopsies marked by immunohistochemistry and two evaluators analyzed the same images using digital morphometric techniques (RGB split colors (I) and color deconvolution (II)). Automated type I collagen density estimation in the papillary dermis (two techniques) were correlated with visual evaluations (Spearman´s rho coefficients of 0.48 and 0.62 (p<0.01)). With regard to the inter-observer repeatability, the four evaluators who used visual classification had an intraclass correlation coefficient (for absolute agreement) of 0.53, while the other two evaluators who used digital analysis (algorithm II) had an intraclass correlation coefficient of 0.97.
Keywords: Collagen; Collagen Type I; Image Processing, Computer-Assisted; Immunohistochemistry

Type I collagen, the most common type in the post-fetal human body, is the main component of the dermis and it is also found in tendons, bones and cartilage. The aggregation of its molecules makes up fibrils, named collagen fibers.[1]

Many diseases are characterized by direct injury to or insufficient or abnormal formation of collagen fibers. Therefore the study of the density of type I collagen in the papillary dermis is important for better understanding photoaging, rheumatic diseases, skin repair and healing.

In research, collagen fiber density calculations are usually performed visually by trained evaluators (ranking from 0 to 4+). However, visual gradations suffer systematic interference of subjectivity and show low precision, which encourages the use of automated techniques of image analysis to estimate these results.[2-5]

Digital morphometric techniques are widely employed in the objective measurement of microscopic structures. Nevertheless, they must be properly validated before use in quantitative research. Moreover, the availability of freeware for image analysis supports wider use of this technology for quantifying these structures.[6-9]

This study intended to validate a technique of digital morphometric analysis for type I collagen estimation in the papillary dermis.

Sixty-three pictures from adult (50 to 75 years-old) skin biopsies (healthy ventral forearm skin) were marked by immunohistochemistry with anticollagen I antibody (purified rabbit anti-human type I collagen, Dako, d-13, 974), incubated with diaminobenzidine (DAB) and counterstained with Hematoxylin. The study was approved by the institutional review board.

Images of interfollicular spaces were acquired in a standardized way, with a light microscope (Olympus BX 40) under 400x magnification, with 1280x960 pixels, 24-bit color pixels, ISO 80, speed 1/2000, and stored as JPG files (high quality).

Qualitative assessments (ranking from zero to 4+) of collagen type I density in the papillary dermis seen in the images were performed by two senior dermatopathologists (Ev3 and Ev4) and two boarded dermatologists (Ev1 and Ev2) (Figure 1).

ImageJ 1.46 software was used for morphometric analysis. Labeled areas associated with the papillary dermis were evaluated following automatic segmentation (ISODATA) after using two algorithms.[6,10] The first algorithm uses the RGB color decomposition tool for channel "blue", and the second algorithm uses the color deconvolution technique (H&E-DAB) (Figure 2).[11]

The statistical analysis was carried out using the SPSS 20 software.[12] Agreement between measurements was assessed by the intraclass coefficient of correlation (ICC - for complete agreement). The correlation between the morphometric estimate and the median of all four estimates calculated by the evaluators was determined by Spearman's rank correlation coefficient. All tests considered a two tailed p value of 0.05 as significant.[13,14]

The gradations of all four evaluators are shown in table 1. The intraclass coefficient of correlation (ICC) between the visual scores of the four evaluators was 0.52, (p <0.01). The 2x2 concordance between evaluators is shown in table 2.
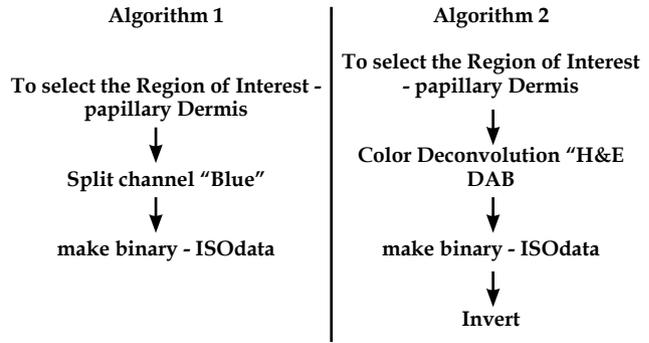
Algorithm 1

To select the Region of Interest - papillary Dermis

↓

Split channel "Blue"

↓

make binary - ISOdata

Algorithm 2

To select the Region of Interest - papillary Dermis

↓

Color Deconvolution "H&E DAB

↓

make binary - ISOdata

↓

Invert

FIGURE 2: Algorithms used in the image processing. Algorithm 1 – split channels "blue", algorithm 2 – colors deconvolution "H&E DAB"

TABLE 1: Evaluators´ scores frequency

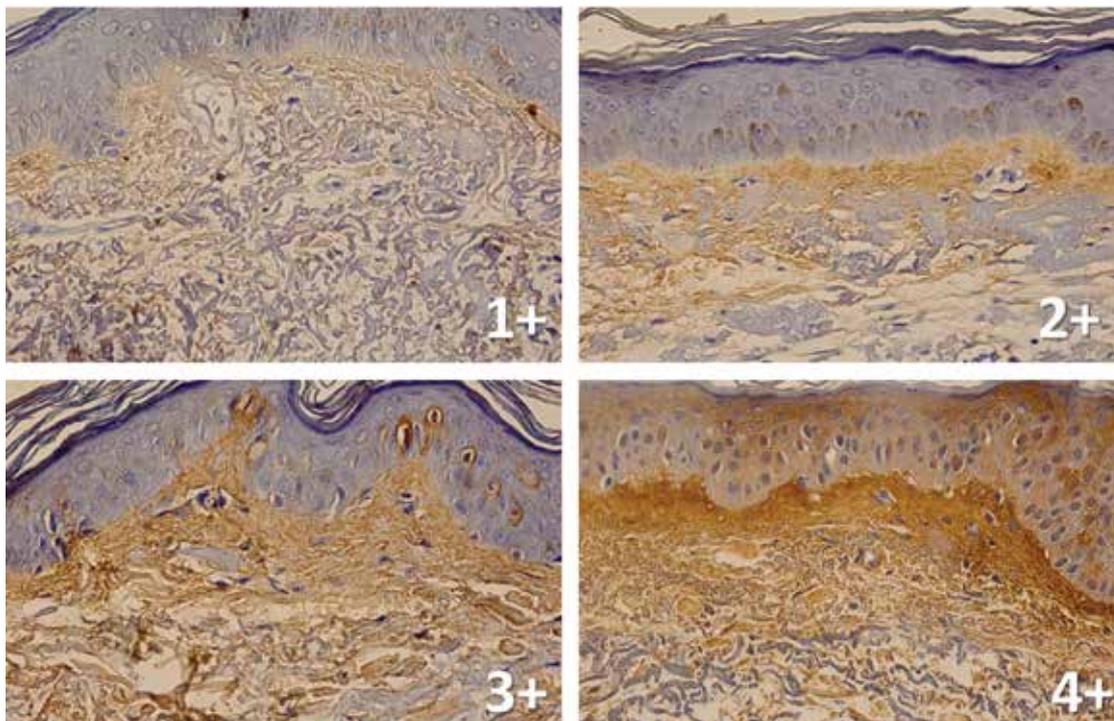| Scores	Ev1	Ev2	Ev3	Ev4
ZERO	- (-)	- (-)	- (-)	- (-)
1+	5 (8%)	20 (32%)	3 (5%)	7 (11%)
2+	22 (35%)	11 (18%)	21 (33%)	21 (33%)
3+	24 (38%)	18 (29%)	24 (38%)	23 (37%)
4+	12 (19%)	14 (22%)	15 (24%)	12 (19%)

Ev = Evaluator

FIGURE 1: Standards of visual gradation for papillary collagen type I (1+ to 4+)

The estimate densities of collagen type I by color decomposition technique (channel blue - algorithm I) and color deconvolution (algorithm II) are shown in figure 3. Correlation coefficients (Spearman's rho) between the image analysis techniques and the median of all four estimates calculated by the evaluators are shown in table 3.

The agreement (ICC) between the digital estimate of papillary dermal collagen calculated by two independent evaluators using the color deconvolution (algorithm II) method was 0.97 (p <0.01).

Usually, histological estimation of collagen is performed qualitatively by an experienced dermatopathologist using visual gradation for each microscopic field, stained by picrosirius "red" or labeled by immunohistochemistry. However, in scientific research, the low interobserver reproducibility and the low accuracy of visual grading encourage the use of computerized morphometric estimators for such evaluation, because they allow greater objectivity and agility of results, as well as greater reproducibility among evaluators.[5,15]

The reproducibility of the results provided by algorithm II showed greater concordance between estimates than the one found between different evaluators using visual classification, which suggests a superior reliability of the digital method. Moreover, the visual classification, even if performed by the same

TABLE 2: Intraclass coefficient of correlation (ICC) for complete agreement between evaluators

	Ev2	Ev3	Ev4
Ev1	0.39*	0.52*	0.83*
Ev2	-	0.65*	0.40*
EV3		-	0.46*

* p<0.01, Ev = Evaluator

TABLE 3: Correlation coefficients (Spearman's rho) between the image analysis techniques and the median of the evaluators´ scores

	Algorithm I	Algorithm II
MedEv	0.48*	0.62*
Algorithm I	-	0.80*

*p<0.01

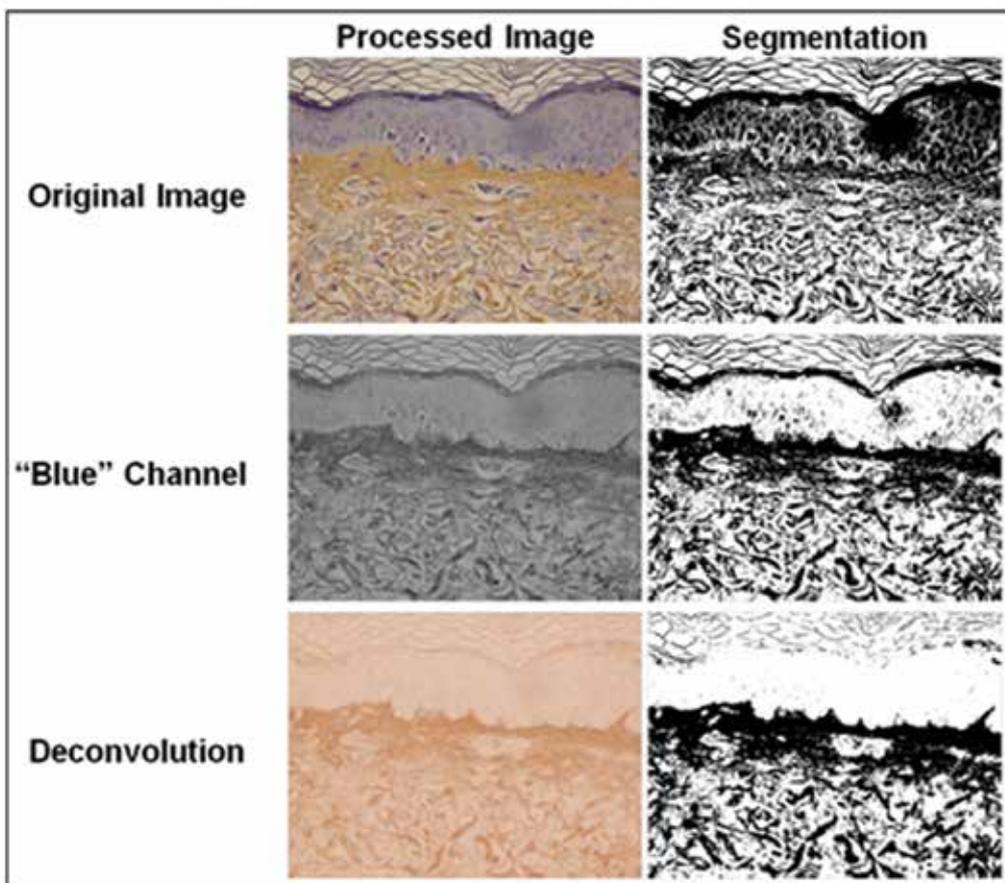MedEv = Median of visual scores among evaluators

FIGURE 3: Original Image, channel "blue" and color deconvolution products and the respective results of the automatic segmentation (ISOdata)

observer, can be influenced by psychological factors such as stress and fatigue. In addition, assessments performed on different days may present varying grading standards.

The automated morphometric analysis of the density of pixels representing type I collagen in digital images is more accurate and objective than visual qualitative gradation. It also allows the comparison of smaller samples, the statistical detection of less exuberant differences and earlier detection of phenomena; favoring blinding, reproducibility between laboratories and operationalization of the research process.[16]

The segmentation of the image directly from the pixel histogram has lower discriminating performance, underestimating the components of the study, which adds noise to the analysis. Therefore, it is important to use filters that extract the information and which are strictly enforced in a standardized way to ensure complete reliability of the results, besides the validation of the technique used, as presented in this study.

The first algorithm uses a RGB split color channel as a tool and the color channel "blue", which retains information of brown pigments. It is the most commonly used algorithm for the segmentation of immunohistochemical staining (DAB). However it has not shown the best correlation with visual classification. [16,17]

Algorithm II showed a better performance in the discrimination of pixels related to collagen type I, and also with regard to reproducibility and correlation to the scores given by the visual evaluators. Indeed, color deconvolution tools have been used in the analysis of DAB staining immunohistochemistry for research in other areas of knowledge.[18-20]

Color deconvolution algorithms use all pixel color information of the image and presuppose the effect of superimposed colors. They create a matricial operation that subtracts planes directed by vectors of predefined colors, providing an effective separation of stainings in histological specimens.[11]

This study proposes a digital technique for the analysis of type I collagen in the superficial dermis, based on color deconvolution. It correlates satisfactorily with the visual perception of the evaluators and can be used in this assessment, since adequate standardization of acquisition and processing of the specimens were warranted. This process can be extrapolated to other collagen markers labeled by Hematoxylin-DAB immunohistochemistry.

In conclusion, we developed and validated a technique for the assessment of type I collagen in the papillary dermis, using a color deconvolution algorithm. This technique provides adequate correlation with the evaluator's visual estimate and shows high reproducibility.❏

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