

## Measuring Collagen Concentration in Histology Slides of Mice Skin Using the Adobe® Photoshop® Software

Osama S. Abbadi

*Department of Biochemistry, Faculty of Medicine, Omdurman Islamic University, Sudan*

**\*Correspondence to:** Dr. Osama S. Abbadi, Department of Biochemistry, Faculty of Medicine, Omdurman Islamic University, Sudan. Current address: Riyadh city, Saudi Arabia. P.O.Box: 22490, Riyadh 11426.

### Copyright

© 2019 Dr. Osama S. Abbadi. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received: 13 April 2019

Published: 17 May 2019

**Keywords:** *Collagen; Software; Histology; Slide Imaging*

### Abstract

#### Background

Collagen is the most abundant protein. It is distributed in almost all body tissues and serves structural and functional roles. The measuring techniques of collagen do not display a percentage of the protein unless it is in a soluble form. It would be useful to obtain a percent measurement of collagen level within the tissue of interest.

#### Study Objective

To estimate collagen concentration in histology slides by the use of Adobe® Photoshop® software, and comparing the results between three different histological stains.

#### Materials and Methods

Skin samples were taken from twelve albino mice and stained with masson's trichrome, van Gieson, and Verhoeff van Gieson's stains to visualize collagen fibers. Slides were photographed with high

resolution digital cameras at (x40) magnification. The software program enabled us to isolate the part of the photos with collagen and to calculate the percent of that part from the total photo.

### Results

The mean percent of collagen were for the Masson's trichrome 39.98%, for the van Gieson 41.3%, and 35.97% for the Verhoeff van Gieson stain. Statistically the differences between mean percents were not significant.

### Conclusion

Adobe® Photoshop® software can present a simple method to measure collagen concentration in digitally photographed histology slides.

## Introduction

Collagen is the most common protein to be found in human body; it represents almost one-third of the protein bulk [1,2]. Collagen could be found as fibers, filamentous mesh, or in spherical shapes. Under electron microscope, collagen appear as straight fibrils of different lengths and diameters [1]. Collagen serves as basement membrane element, giving strength to tissues such as tendons, skin, and bones. It also gives shape to tissues, and acts as cell signaling molecules [3]. The biochemical structure of collagen is comprised of the amino acid glycine alternating with a proline or hydroxyproline, and any amino acid, in the main fiber; this structure was designated as the Gly-X-Y structure of Collagen [3,4]. Nowadays, there are 28 different proteins designated as collagens [2,3,5].

Under light microscopy, collagen appears as thin fibers of red or blue color, according to the used staining method. The tri-chrome stain of Masson gives collagen a blue color [6], whereas it appears bright red with van Gieson stain [7]. While originally designed to visualize elastic fibrils, Verhoeff van Gieson stain will also demonstrate collagen in red [8]. Because van Gieson's stain is simpler, it is more preferable than the Masson's trichrome [9]. Picrosirius red stain is still used in collagen stains, and proved to be more effective in visualizing the thin fibers [10,11]. The concentration of a soluble form of collagen could be measured by the Lowry's method [12-14], or modified Lowry's method [15]. Lowry's method involves precipitation of proteins from a solution, re-hydrolyzing the protein using alkaline solution, colorimetric analysis, and blotting the wavelength of the test result in a standard curve comparing protein color development against time [12]. Lowry's modified technique adds initial heating of protein [15].

Histological stains give visual data about collagen pattern and content, but they don't give numerical information. Despite that Lowry and modified Lowry's method gives a percent of collagen; they require that it is presented in a tube, also the precision of pH and reagent concentration is important to achieve an accurate measurement.

Few computer soft-wares have been used recently to quantify and isolate molecules in the pathological slides. Boone *et al.* (2000) used a computer image program to measure the neoplastic levels in rats; they

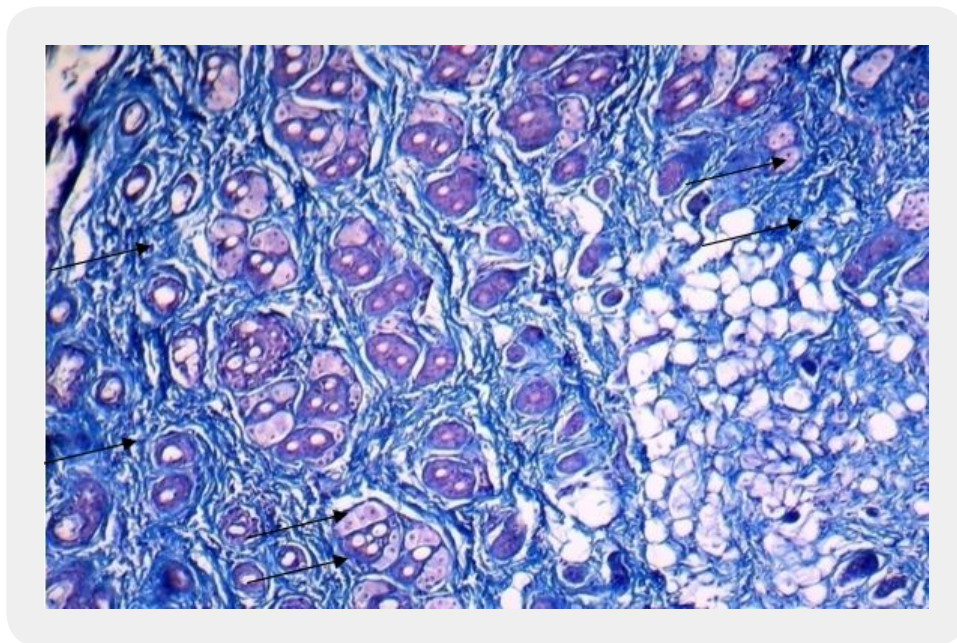
agreed to that tile grading of histological slides through software is superior to the pathologist's experienced eyes in grading rats oesophageal neoplasias [16]. The 2006 work of Xu and Pitot used software programs to study hepatocytes, and further improvement in the picture quality was achieved by background manipulation and pixel refinement [17]. Calvi *et al.* studied collagen in muscle for morphometric analysis using the UTHSCSA Image Tool 3.0 software. The software enabled the researchers to quantify muscle collagen from slides stained with Immunohistochemical stains and routine collagen stains [18].

## Study Objective

In this study we tried to test Adobe® Photoshop® ability, as a software program, to provide both figurative distribution and concentration of the protein collagen in histology slides.

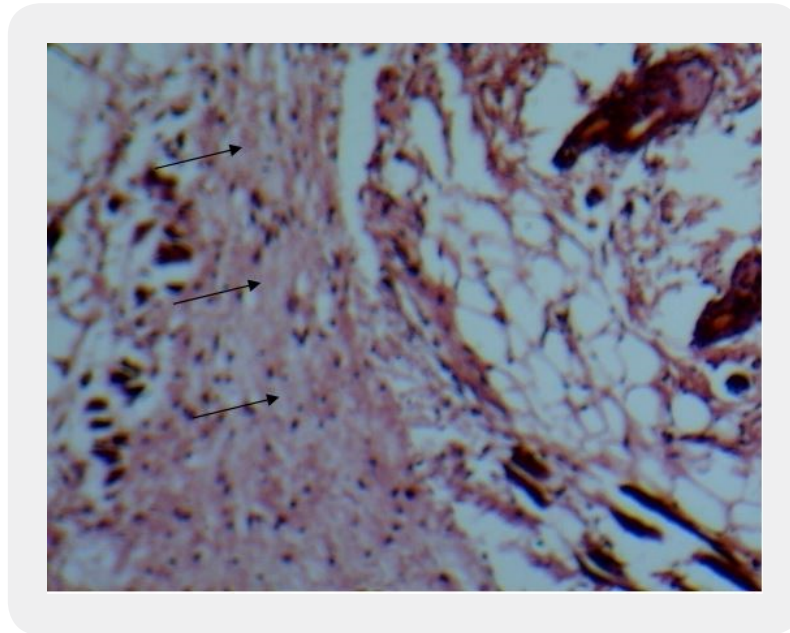
## Materials and Method

Twelve albino mice were used to obtain skin specimens. The mice were euthanized after tissue extraction. Initially the mice were anaesthetized by inhalation of Diethyl ether solution in an isolated chamber. A skin sample, with an area of 1 cm<sup>2</sup> each, was taken from each mouse. Samples were preserved in 10% formaldehyde, and two days later they were immersed in Paraffin blocks. Tissues were cut using Leica® microtome. Histological staining was performed to the samples: Five Masson's trichrome stain slides. This method is used for the detection of collagen fibers in tissues. The collagen fibers will be stained blue and the nuclei will be stained black and the background is stained red [6]. See figure (1)



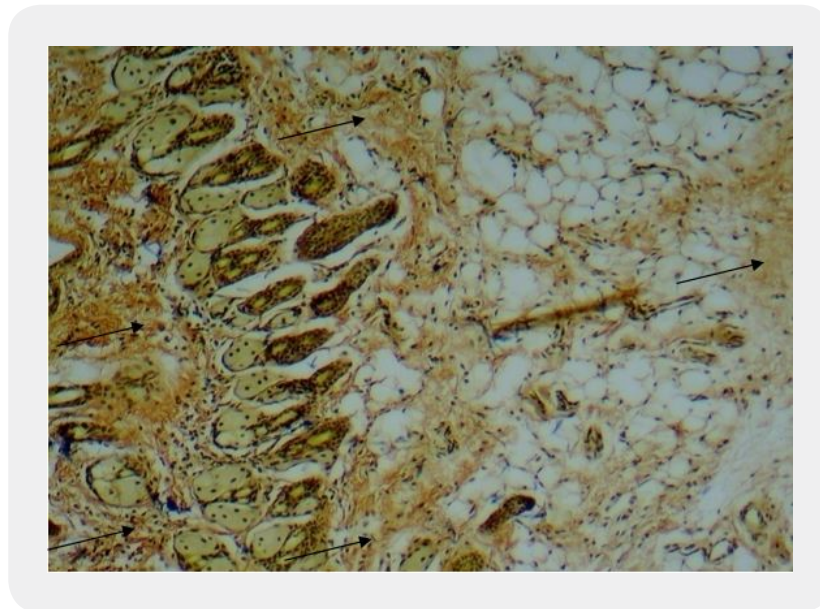
**Figure 1:** *The characteristic appearance of the blue collagen (arrowed) in Masson's trichrome stain. Other skin components such as the hair follicles, fat cells, and sebaceous glands, are also apparent.*

Three Van Gieson's stain slides. This stain is used to visualize collagen that appears as a bright red color, while nuclei will appear blue [7]. See figure (2)



**Figure 2:** Appearance of collagen (arrowed) in van Gieson's stain

Four Verhoeff van Gieson's stain slides. Usually used for demonstration of elastic fibers that appears blue-black to black, in distinction from collagen which appears in red [8]. See figure (3)



**Figure 3:** Appearance of collagen as bright red lines (arrowed) in Verhoeff van Gieson's stain.

The slides were visualized using light microscope and photographed, in four random sites, through an attached digital camera. Imaging sites were chosen randomly, provided that it will contain all the skin layers. The Lens magnification was x40.

The collagen that appeared in the images was measured as follows: Using Adobe® Photoshop® CS. version 8.0, the target field for measurement was selected, then through the (magic wand) tool on the tool bar, the area to be measured is right-clicked by the mouse. If the area was extensively irregular, the (magnetic lasso) better to be used instead because it allows the operator to manually select his target. The area will be shaded by intermittent dots, and the (Info) window on the right side will show how much-in percent- that area covers of a space in the W (Width) and H (Height) axis as part of the entire slide. The areas of interest are selected repeatedly and subtracted from the total slide area, and the number obtained will represent the concentration of the collagen in that slide, in percent.

## Results

Using Masson's trichrome stain, the collagen concentration scored a mean of 39.9% of the total slide size, while van Gieson's stained slides gave a mean Collagen concentration of 41.32%, and Verhoeff van Gieson's stain was 35.97%; statistically, the differences between these means were insignificant, and the critical F value was remarkably higher than the calculated F value ; see table (1).

**Table 1:** Comparing results of the Collagen concentration within slides of different stains (One-Way ANOVA test was performed to compare the three means ( $F = MMSB \div MSSW = 113.52/447.08 = 0.2539$ )).

Slide no.	a (Masson's trichrome) %	b ( van Gieson's) %	c ( Verhoeff van Gieson's) %
1	47.91	28.72	74.64
2	52.23	25.77	42.75
3	21.96	26.31	56.25
4	47.69	23.67	74.89
5	44.33	57.32	28.09
6	50.27	52.34	20.54
7	38.48	33.35	36.03
8	26.62	58.57	24.95
9	85.65	60.47	11.44
10	45.65	25.38	56.05
11	20.92	15.07	43.47
12	56.3	88.82	9.94
13	29.5	*	39.53
14	86.1	*	4.47
15	24.97	*	27.54

<b>16</b>	57.68	*	24.98
<b>17</b>	17.08	*	*
<b>18</b>	16.72	*	*
<b>19</b>	15.24	*	*
<b>20</b>	14.37	*	*
<b>Sum</b>	799.67	495.79	575.56
<b>Mean (%)</b>	39.98	41.32	35.97
<b>Sample size</b>	20	12	16
<b>Total size</b>	48		
<b>Overall mean</b>	38.97		
<b>Groups number</b>	3		
$\Sigma(X-X)^2$	8759.01	5186.46	6172.97
<b>Critical F v.</b>	3.20430		
<b>Calculated F v.</b>	0.2539		

## Discussion

The study aimed to measure collagen concentration in histological slides of albino mice skin by Adobe® Photoshop software. Thirty eight slides with different histological stains were used in the experiment. The staining procedures were performed by expert personnel, and the collagen concentration difference measured through the software was statistically insignificant, which suggest that the software properly isolated collagen in the slides. Sensitivity of this test depends mostly on the staining procedure, while the specificity is dependent on the software and the person operating it. The measured concentrations of collagen slides recorded a total mean of (39.1%), but since there were no previous studies concerning collagen concentrations in albino mice skin, this result couldn't be compared and certified. The above method can be useful in measurement of collagen, or any other molecule, in a particularly targeted site within a slide or a tissue sample. It also can provide a method of follow up in treatment progress, as in scar healing trials, or in disease prognosis. It also offers a mean for concentration measurements of molecules in skin and other tissues.

The software used in Boone *et al.*, in 2000, was able to calibrate different image of both normal and tumor bearing histological tiles, using CAS 200 system and a multi-scan image recorder bound to the microscope [16]. The study adopted four tumor characters to be detected through the program: piling of nuclei, presence or absence of what is known as (absorbance holes), increased DNA density, and chromatin dysmorphism16. The end result was obtained by calculating each feature occurrence in comparison with normal oesophageal epithelium. In the Adobe® Photoshop® study, the measurement of collagen was direct and manual, there was no control comparison, single molecule was targeted, and the result was displayed in percent. The Calvi *et al.*, study, held in 2012, was targeting both quantitative and morphometric analysis of muscle collagen in human cadavers [18]. They used the UTHSCSA Image Tool 3.0 that has the ability to calculate collagen concentration in histology slide, in addition to performing morphometric analysis. The similarity between Image tool 3.0 and Adobe® Photoshop® is that both programs can isolate certain sections of the slide image

and represent that area as a percent of the total image [18]. Steps of our study in order to reach a mean percent of collagen concentration could be summarized in three main steps: digital imaging of the slides, manual selection of collagen patches within the slides and sequentially record the area of the selected patch, and thirdly, the summation of the total area of collagen, which will be divided by 100. Unlike the 2006 Xu and Pitot study, there were no image enhancement, no background rectification, object confinement, or pixel splitting. In fact, Adobe® Photoshop® was also used by Xu and Pitot as a reserve for the manipulated photos [17]. The purifying steps would offer more accurate results, and pixel separation would provide optimum outlining for collagen particles. However, background correction is of limited value in collagen molecules measurement, since the Adobe® Photoshop® (magic wand) and (magnetic lasso) tools distinguish the background perfectly. The step by step summation of areas of collagen from the slides in Adobe® Photoshop® software could be a time consuming process that masks out the simplicity of the procedure, but time could be markedly lessened by the use of two calculators simultaneously, and a help from a colleague.

Drawbacks of this method include that its invasiveness for human subjects, since an area of skin should be extracted. The sensitivity and specificity of the software should be re-evaluated. The accuracy of the result depends solely on the experience of the histologist and the slides photographer. Moreover, there was no distinction between different subtypes of the protein.

## Conclusion

Collagen density could be measured in a histology slide using the software Adobe® Photoshop® CS version 8.0. It is convenient to say that similar programs are capable of detecting the collagen density in histology slides. Other visible biomolecules, such as Elastin and Fibrin, also could be measures in percents. The technique needs approval, and the software needs more development regarding color specificity and slide area definition.

## Bibliography

1. Richard-Blum, S. (2011). The collagen family. Cold Spring Harb. *Perspect Biol.*, 3(1), a004978.
2. Dutov, P., Antipova, O., Varma, S., Orgel, J. & Schieber, J. D. (2016). Measurement of Elastic Modulus of Collagen Type I Single Fiber. *PLoS One.*, 11(1), e0145711.
3. Kadler, K. E., Baldock, C., Bella, J. & Boot-Handford, R. P. (2007). Collagens at a glance. *J Cell Sci.*, 120(Pt 12), 1955-1962.
4. Murray, R. K., Granner, D. K., Mayes, P. A. & Rodwell, V. W. (2003). *Harper's Illustrated Biochemistry*. 26<sup>th</sup> ed. Toronto (Ontario): McGraw-Hill Inc, (p. 693).
5. Mienaltowski, M. J. & Birk, D. E. (2014). Structure, physiology, and biochemistry of collagens. *Adv Exp Med Biol.*, 802, 5-29.
6. IHC world website (2019). *Masson's Trichrome Staining Protocol for Collagen Fibers*.
7. IHC world website (2019). *Van Gieson Staining Protocol*.

---

Osama S. Abbadi (2019). Measuring Collagen Concentration in Histology Slides of Mice Skin Using the Adobe® Photoshop® Software. *CPQ Medicine*, 6(5), 01-08.

8. IHC world (2019). *Verhoeff Van Gieson (VVG) Staining Protocol for Elastic Fibers*.
9. Huang, D. K., Zhang, Y. X., Man, S. Q., Yu, F. Z. & Shen, J. J. (2012). Comparison of collagen fiber staining between Van-Gieson staining and Masson trichrome staining of hepatic specimens in mice with *Schistosoma japonicum* infection- article in Chinese [2012]. *ZhongguoXue Xi Chong Bing Fang ZhiZaZhi*, 24(4), 468-470.
10. Constantine, V. S. & Mowry, R. W. (1968). Selective Staining of Human Dermal Collagen: II. The Use of Picrosirius Red F3BA with Polarization Microscopy. *Journal of Investigative Dermatology*, 50(5), 419-423.
11. Rich, L. & Whittaker, P. (2005). Collagen and Picrosirius red staining: a polarized light assessment of fibrillar hue and spatial distribution. *Braz. J. Morphol. Sci.*, 22(2), 97-104.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with Folin Phenol reagent. *J. Biol. Chem.*, 193, 265-275.
13. Redmile-Gordon, M. A., Armenise, E., White, R. P., Hirsch, P. R. & Goulding, K. W. T. (2013). A comparison of two colorimetric assays, based upon Lowry and Bradford techniques, to estimate total protein in soil extracts. *Soil Biol Biochem.*, 67(100), 166-173.
14. Peterson, G.L. (1979). Review of the Folin Phenol Protein Quantitation Method of Lowry, Rosebrough, Farr and randall. *An. Biochem.*, 100(2), 201-220.
15. Komsa-Penkova, R., Spirova, R. & Bechev, B. (1966). Modification of Lowry's method for collagen concentration measurement. *J Biochem. Bioph M.*, 32(1), 33-43.
16. Boone, C. W., Stoner, G. D., Bucas, J. V., Kagan, V., Morse, M. A., Kelloff, G. J. & Bucas, J. W. (2000). Quantitative grading of rat esophagial carcinogenesis using computer-assisted image tile analysis. *Cancer Epidemiol Biomarkers & Prev.*, 9(5), 495-500.
17. Xu, Y. & Pitot, H. C. (2006). A software package to improve image quality and isolation of objects of interest for quantitative stereology studies of rat hepatocarcinogenesis. *Comp Method Prog Biom.*, 81(3), 236-245.
18. Calvi, E. N., Nahas, F. X., Barbosa, M. V., Calil, J. A., Ihara, S. S., Silva Mde, S., *et al.* (2012). An experimental model for the study of collagen fibers in skeletal muscle. *Acta Cir Bras.*, 27(10), 681-686.