

ORIGINAL CONTRIBUTION

A New Pentachrome Method for the Simultaneous Staining of Collagen and Sulfated Mucopolysaccharides

Kevin Doello

School of Medicine, University of Granada

Collagen is one of the most common fibers in the extracellular matrix, where sulfated mucopolysaccharides are also located. In addition, sulfated mucopolysaccharides are present in some goblet cells and secretory glands. The objective of this article is to develop a new staining method that detects these two macromolecules simultaneously in the same sample. The method described stains tissues in five fundamental colors: collagen in red; sulfated mucopolysaccharides in violet; red blood cells in yellow; muscle in orange; and nuclei in green.

As a conclusion, it will be interesting in the future to evaluate whether this method could be used as a basic histological method, as a histology teaching tool, or even in histopathological and cytopathological studies.

INTRODUCTION

Collagen and sulfated mucopolysaccharides are two tissue elements that form the extracellular matrix, even in cartilage. Additionally, sulfated mucopolysaccharides are present in some goblet cells and secretory glands [1,2,3]. In basic histology, there are many methods to stain them. Traditionally, collagen is stained with different colorants like light green (component of the Masson Trichrome staining) or Sirius red. On the other hand, sulfated mucopolysaccharides are colored with dyes like toluidine blue or alcian blue [4,5,6].

However, a staining method that selectively and simultaneously stains collagen and sulfated mucopolysaccharides does not exist. Therefore, the objective of this article is to describe a new staining method that detects these two elements in the same sample.

METHOD

In this section, the histological processing of the samples and the staining protocol are presented.

Each one of the solutions or reagents is placed into independent containers of 50

To whom all correspondence should be addressed: Kevin Doello, School of Medicine, University of Granada; Email: kevindoello@gmail.com.

Keywords: collagen, sulfated mucopolysaccharides, toluidine blue, picrosirius, pentachrome

Table 1. Solutions and reagents, with their amounts and concentrations, needed in this histological technique.

Reactive or solution	Composition and concentration	Amount
Formaldehyde 10%	10 mL of formaldehyde + 90 mL of distilled water	Depends on the capacity of the container. Normally, 50 to 100 mL
Xylene	Pure xylene	“
70% Ethanol	70 mL of absolute ethanol + 30 mL of distilled water	“
95% Ethanol	95 mL of absolute ethanol + 5 mL of distilled water	“
Absolute ethanol	Pure ethanol	“
Distilled water	Pure distilled water	“
Nitric acid at 6%	6 mL of nitric acid + 94 mL of distilled water	“
Toluidine blue staining solution	0.5 g of toluidine blue dissolved in 100 mL of distilled water at 1 – 1.5 pH	“
Picrosirius staining solution	0.5 g of sirius red dissolved in 500 mL of distilled water saturated of picric acid	“

Note: Commercial solutions (for example, formaldehyde or nitric acid) are not often pure, so in those cases calculations will be needed in order to maintain the shown proportions.

to 100 mL and samples are introduced, depending on the step of the processing.

All the necessary solutions and reagents with their amounts and concentrations are described in Table 1.

Histological Processing of the Samples

1. Fix in 10% formaldehyde for 48 hours using a volume 10 times that of the sample.

2. Hydrate in alcohols of crescent concentrations (50%, 70%, and 96%, 50 to 100 mL of solution) for 1 hour at each concentration. Next, treat with absolute ethanol for 2 hours.

3. Process in xylene (50 to 100 mL) for 30 minutes.

4. Treat with liquid paraffin (50 to 100 mL) for 2 hours (repeat three times), in order to eliminate the residual xylene.

5. Immobilize in paraffin blocks. In this process, the samples are immersed in molds

with liquid paraffin that completely solidifies after 8 hours in ice.

6. Slice the blocks in sections with a thickness of 5 μ m with a rotary microtome, Microm HM325 (Microm, Walldorf, Germany).

7. Place the sheets over microscopy slides and dry at 36°C for 24 hours.

Staining Protocol

1. Deparaffinize in xylene (50 to 100 mL) for 10 minutes (repeat twice).

2. Hydrate with three solutions: absolute ethanol for 5 minutes, 95% ethanol for 5 minutes, and distilled water for 5 minutes (50 to 100 mL).

3. Pre-treat with 6% nitric acid (50 to 100 mL) for 5 minutes.

4. Stain with toluidine blue solution (50 to 100 mL) for 5 minutes. The toluidine blue solution is prepared by dissolv-

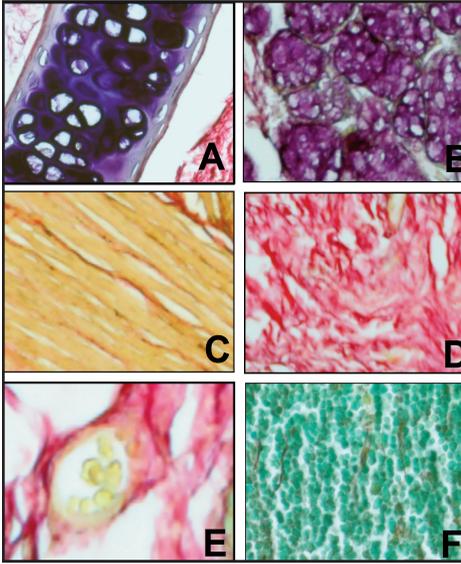


Figure 1. Each one of the main structures stained with the new pentachrome method. A: cartilage in violet; B: sulfated mucopolysaccharides of secretory glands in violet; C: muscle in orange; D: collagen in red; E: red blood cells in yellow; F: lymphocytes in green.

ing 0.5 g toluidine blue in 100 mL distilled water at pH 1 to 1.5 (adjusted with 0.5 % HCl) [7].

5. Wash in distilled water.

6. Stain with picosirius solution (50 to 100 mL) for 5 minutes. Picosirius solution is composed of 0.5 g Sirius red dissolved in 500 mL distilled water saturated with picric acid [8].

7. Wash in distilled water.

8. Dry the samples at room temperature.

9. Wash the samples three times in xylene.

10. Cover with resin and cover slip.

RESULTS

All the samples showed five fundamental colors, which were attributed to different structures. Collagen fibers were stained in red; sulfated mucopolysaccharides were stained in violet; red blood cells were stained in yellow; and muscles were stained in orange. Due to their prominent nuclei, leukocytes, like lymphocytes and plasma cells, were stained in green (Figures 1, 2, 3 and 4).

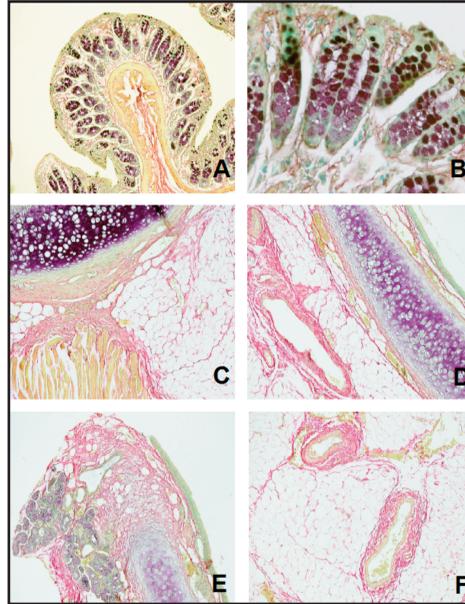


Figure 2. This image shows examples of tissues stained with the new pentachrome method. A and B are histological samples of gut stained with this method. Globet cells are colored in violet; collagen fibers, of corium and submucosa, in red; smooth muscle in light orange; nuclei of enterocytes and lymphocyte in dark green. C, D, and E are tissue samples of epiglottis. Cartilage and secretory glands are stained in violet; skeletal muscle in orange; collagen fibers in red; red blood cells in yellow. F is a microphotograph of connective tissue with many adipose cells and blood vessels. In the adipose cells, collagen fibers are stained in red. In the blood vessels, smooth muscles are stained in light orange, collagen fibers are in red, and inner red blood cells are in yellow.

Figures 1-4 show different tissues, including intestine, epiglottis, ear, salivary gland, tongue, or thymus with some of their basic structures (collagen, sulfated mucopolysaccharides, red blood cells, muscles, and leukocytes) stained in five fundamental colors: red, violet, yellow, orange and green, respectively. The principles underlying this novel pentachrome staining technique are outlined below.

First, it is important to mention that this new pentachrome method permits the simultaneous staining of collagen and sulfated mucopolysaccharides in the same sample

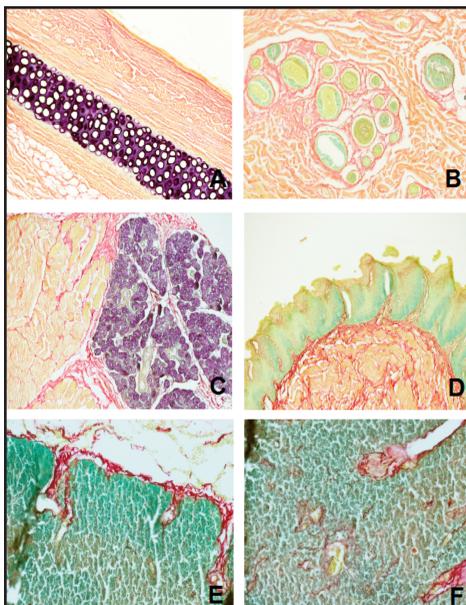


Figure 3. A and B are two images of histological samples of the ear. A shows the elastic cartilage in violet and B shows hair follicles in green and yellow. C is an oral mucosa sample. Secretory glands are stained in violet; skeletal muscle in orange; and collagen fibers in red. D is a sample of tongue in which collagen fibers are stained in red, muscle in orange, and the basal stratum of the epithelium in light green and the rest of the epithelial layers in light brown. E and F are two images of thymus. Thymocytes (immature T lymphocytes) are stained in dark green, and collagen fibers at the capsule and trabeculae are stained in red.

(the intestine, epiglottis, and salivary gland samples in Figures 2 and 3).

Moreover, this method not only stains collagen and sulfated mucopolysaccharides, but also red blood cells, muscle, and leukocytes in the same sample.

Finally, this method provides a clear image of multi-layered tissues like intestine because it can distinguish among epithelium, corium, muscularis mucosae, submucosa, muscularis propria, and adventitia/serosa by staining them in different colors.

DISCUSSION

The five fundamental colors of collagen, sulfated mucopolysaccharides, red

blood cells, muscles, and leukocytes using the new pentachrome method arise from the use of three reagents: 6 percent nitric acid, toluidine blue solution, and picrosirus solution. Toluidine blue solution normally stains cells and tissues in blue; however, sulfated mucopolysaccharides are not stained in blue using this new pentachrome method because pre-treatment with 6 percent nitric acid converts the majority of anionic groups to their protonated form. The protonation of carboxylic groups, which are present in the majority of cell and tissue structures, impedes the binding of toluidine, the cation in toluidine blue solution [4,5,6]. Therefore, sulfated mucopolysaccharides do not stain with the original blue color. However, treatment of nitric acid does not protonate sulfate ions. As a result, sulfated mucopolysaccharides are stained in violet because of an interesting characteristic of toluidine called metachromasia, where its color changes from blue to violet when it binds to substances with concentrated negative charges, like glycosaminoglycans. It is due to toluidine polymerization when it binds to compounds/structures with many anions. The new “polymer” of toluidine has a violet color [4,5,6,7,9]. Since the 6 percent nitric acid treatment does not protonate sulfate ions in the sample, sulfated mucopolysaccharides tend to concentrate many of the toluidine polymers. For this reason, toluidine blue stains only structures that contain sulfated mucopolysaccharides, such as cartilage, mastocytes, and some secretory glands, in violet. Therefore, cartilage, goblet cells and secretory glands, which contain sulfated mucopolysaccharides, are stained violet [4,5,6,7,9].

Collagen and red blood cells are stained in red and yellow, respectively. These colors are the result of the picrosirus solution, which contains two colorants: Sirius red and picric acid. Sirius red stains collagen, while picric acid stains cytoplasm. So collagen is stained in red, and red blood cells are stained in yellow [4,5,6,8].

The green color of lymphocyte and plasma cells are due to the mixing of toluidine cation and picric acid. Toluidine cation

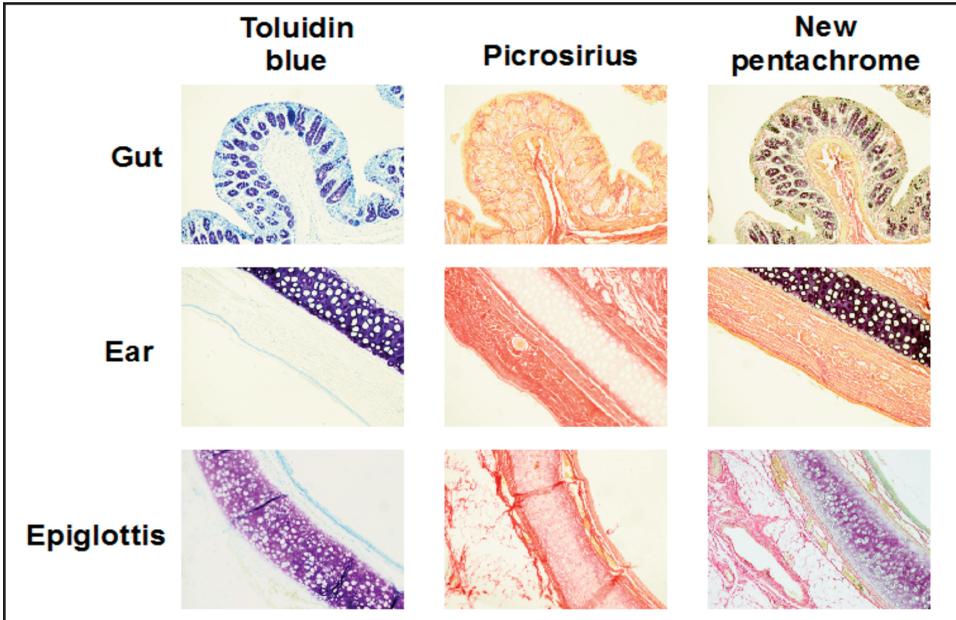


Figure 4. Comparative images between the new pentachrome method and the toluidine blue and picrosirius methods.

binds to phosphate groups in the nucleus, providing some weak orthochromatic blue staining in the nucleus [4,5,6]. Afterward, picric acid, which has a negative charge, binds to tolonium cation. Since tolonium has a blue color and picric acid has a yellow color, they mix together to create a green color.

Muscles are stained in orange as a result of the mixing of the red color of Sirius red and yellow color of picric acid. Cytoplasm is stained in light brown, except in the basal stratum of epithelia in which they are stained in light green [4,5,6]. Further histochemical studies are needed to understand the basis for this result (Figure 5).

The pentachrome method described employs a pre-treatment with nitric acid followed by toluidine blue as the first staining step and picrosirius as the second staining step. However, there is another pentachrome method, the Movat's pentachrome method, that employs different dyes. The Movat's pentachrome method employs alcian blue, alkaline alcohol, resorcin — fuschin, Weigert's hematoxylin, and Woodstain Scarlet — acid fuchsin, acetic acid, phosphotungstic acid, and alcoholic safran solution. There are also other variants of Movat's pen-

tachrome method but they lie beyond the scope of this paper. Other than the use of different dyes and treatment, staining times are also different between these two pentachrome methods [10,11].

The new pentachrome method and Movat's pentachrome method stain different elements in the sample. The new pentachrome method stains collagen in red, sulfated mucopolysaccharides in violet, red blood cells in yellow, muscle in orange, and nuclei in green. Movat's pentachrome stains collagen and reticulin in yellow, glycosaminoglycans in light blue, muscle in red, elastic fibers in dark purple, and nuclei in black [10,11] (Figure 6 and Table 2).

The new pentachrome method is simpler and faster than the Movat's pentachrome method, but the Movat's method is a much more "specific" method in terms of the chemical reactions involved. While the Movat's method stains the majority of the structures or compounds by chemical reactions that are specific for a concrete substance, the new pentachrome method stains some structures by reactions or physical phenomena that are not specific for a unique substance.

Movat's pentachrome method is used in the staining of the components of the extra-

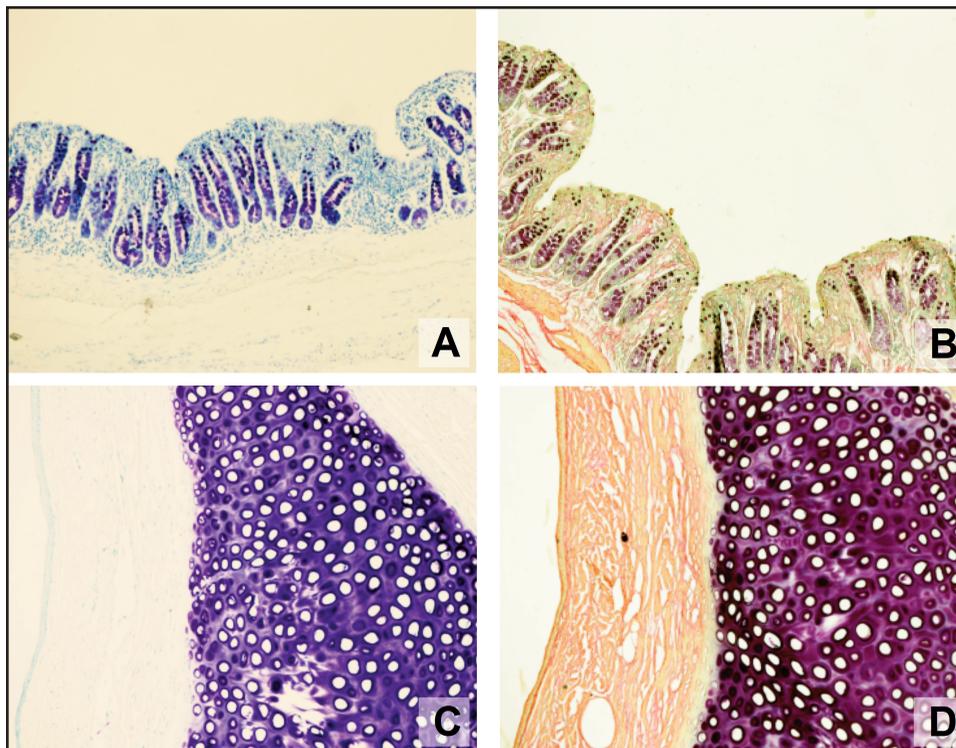


Figure 5. Two images of colon (A and B) and ear (C and D) samples stained with toluidine blue (A and C) and the new pentachrome method (B and D). A and C are samples of colon and ear stained with the traditional toluidine blue staining method which is used in histology laboratories for the demonstration of sulfated mucopolysaccharides. In A, goblet cells are stained in violet, and in C, cartilage is stained in violet as well, so it demonstrates the presence of sulfate mucopolysaccharides in these structures. B and C employ the new pentachrome method; in these images, goblet cells and cartilage are also stained in violet. So, it demonstrates that the new pentachrome method described specifically stains in violet sulfated mucopolysaccharides.

cellular matrix. It would be interesting to study the possibility of employing the new pentachrome method as a basic histology method, as a histology teaching tool, or in cytopathological and histopathological studies.

In conclusion, this new pentachrome method and the Movat's method are similar in that they stain some of the same structures (collagen and mucopolysaccharides). They also stain five fundamental structures (pentachrome). However, the protocols, reagents, histochemical basis, and the final colors are different. It is not a modified version of the Movat's method, but rather another method that coincides in some aspects.

Apart from its ease and simplicity, this new pentachrome method distinguishes multi-layered tissues with different colors. On the contrary, the Movat's pentachrome method

permits a good study of the extracellular matrix because it distinguishes mucopolysaccharides, elastin, and collagen. In this last case, the advantage of the new pentachrome method is that it distinguishes sulfated mucopolysaccharides specifically, while the Movat's method stains acid and sulfated ones in the same color. They are two pentachrome methods that serve different purposes.

Finally, it would be interesting to study the combination of this method with others, like alcian blue (for acid and sulfated mucopolysaccharides), PAS (for neutral mucopolysaccharides), or orcein (for elastin) in order to identify more structures/compounds in the same sample. However, the combination of this new pentachrome method (a polichromic staining method) with immunoperoxidase would not yield good re-

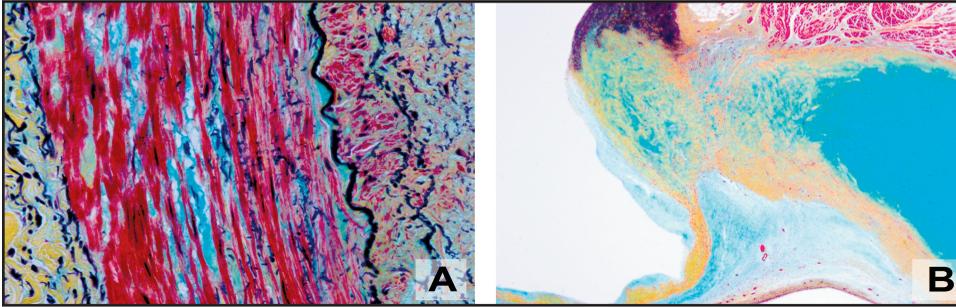


Figure 6. A and B are two Movat's pentachrome images. A is an artery wall sample and B a cardiac valve. In A, muscle is stained in red; collagen in yellow; glycosaminoglycans in light blue; and elastic fibers in black. In B, muscle in red; collagen in yellow; and glycosaminoglycans in light blue. Both images are reproduced with the permission of Histoserv, Inc.

Table 2. Comparative between the results of the pentachrome described and Movat's.

Structure or component	New pentachrome	Movat's pentachrome
Collagen	Red	Yellow
Mucopolysaccharides	Violet (sulfated)	Light blue (acidic and sulfated)
Nuclei	Green	Black
Muscle	Orange	Red
Other	Yellow (red blood cells)	Purple (elastin)

sults because the pentachrome could mask the staining signal of the immunoperoxidase.

CONCLUSIONS

The new pentachrome method described is a fast and simple method that stains five distinct tissue structures in different colors. It will be interesting in the future to evaluate whether this method could be used as a basic histological method, as a histology teaching tool, or even in histopathological and cytopathological studies.

REFERENCES

1. Leslie PG, Hiatt J. Color Textbook of Histology. 3rd edition. Philadelphia: WB Saunders Company; 2001.
2. Kierszenbaum AL, Tres LL. Histology and Cell Biology: An Introduction to Pathology. 3rd edition. Philadelphia: Elsevier Saunders; 2012.
3. Ross MH, Pawlina W. Histología. 5th edition. Buenos Aires: Panamericana; 2007.
4. Kiernan JA. Histological and histochemical methods. Oxford, UK: Butterworth Heinemann; 2008.
5. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 6th edition. Philadelphia: Churchill Livingstone; 2008.
6. Cook DJ. Cellular Pathology: An Introduction to Techniques and Applications. 2nd edition. Oxford, UK: Scion Publishing Limited; 2006.
7. Martoja M, Martoja-Pierson R. Técnicas de Histología Animal. 1st edition. Barcelona: Toray-Masson; 1970. p. 316.
8. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J. 1979;11(4):447-55.
9. Martoja M, Martoja-Pierson R. Técnicas de Histología Animal. 1st edition. Barcelona: Toray-Masson; 1970. p. 178.
10. Movat HZ. Demonstration of all connective tissue elements in a single section; pentachrome stains. AMA Arch Pathol. 1955;60(3):289-95.
11. Russell HK. A modification of Movat's pentachrome stain. Arch Pathol. 1972;94(2):187-91.