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Review Article

Connective Tissue Stains - A Review

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Abstract

Over the years, a wide range of dyes has been used for histological staining methods. Most of these have been adapted from those used in the textile dyeing industry. The common histological stains appeared during the second half of the 19th century. Routinely, hematoxylin and eosin stain is widely used for histopathological examination of tissue sections. Although they have plethora of advantages, they lack to distinguish the various connective tissue components. This review aims to highlight the special stains used to describe the connective tissue elements which contribute in diagnosing the tumors of connective tissue origin.

Keywords: *Connective tissue, Masson trichrome, phosphotungstic acid hematoxylin, special stain, Verhoeff–van Gieson*

Introduction

Over the years, a wide range of dyes has been used for histological staining methods. Most of these have been adapted from those used in the textile dyeing industry. The dye with probably the greatest claim to antiquity is indigo. It is extracted from the *Indigofera* plant, and there is evidence of its use to dye cloth by the ancient Egyptians some 3000 years ago. Coincidentally, the Arabic term for indigo is Annil, from which the word *Aniline* derived. It is a substance extracted from indigo in the early 19th century. The aniline group of dyes had become the dominant force in the growth of the textile dyeing industry and histological stain technology. In the early 1860s, one-step staining had been used in which the excess stain was washed off in alcohol or water before the slide was mounted. In 1867, *Schwartz* introduced two-dye sequential staining interspersed with a simple washing stage. The technique was further refined when in 1869, *Bottcher* incorporated an alcohol differentiation step.[1]

The common histological stains appeared during the second half of the 19th century. These included *fuchsin* and *carmine*; *methyl violet*, *alizarin* still used for histochemical localization of calcium; *methyl green*; *malachite green* was until recently widely used to treat pond fish with parasitic diseases; *indigo* used as the basis for indigo techniques in the 1950s to localize histochemical activities of enzymes; and *congo red* still used to localize amyloid. Several of these dyes, such as *rhodamine* and *malachite green*, have been "rediscovered" and found new value in fluorescence microscopy. *Orcein* is still widely used for the localization of elastin. *Weigert's elastin stain* was introduced in 1898. *Osmium tetroxide*, which stains myelin, found a new lease of life as a contrast stain for transmission electron microscopy.[2] This review aims to highlight the different special stains used for the detection of various connective tissue components which are important as accurate diagnosis is the need of this era.

Connective Tissue

Connective tissue is the most abundant tissue and can be found all over the body. It offers organs and other body structures a protective and supportive framework. Some cells make up connective tissue, which has a higher proportion of the extracellular matrix than other tissues.[3]

The three types of fibers present in the connective tissue matrix are reticular, collagenous, and elastic fibers.

- Collagen protein is used to make reticular fibers, which are coated with glycoprotein. They form a delicate framework around nerve fibers, fat cells,

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lymph nodes, and smooth and skeletal muscle fibers, as well as a network around nerve fibers, fat cells, lymph nodes, and smooth and skeletal muscle fibers
- Collagenous fibers are made up of the protein collagen and they are the strongest of the three fiber types. Ligaments, tendons, cartilage, and bone all contain collagenous fibers
- Elastic fibers which are made up of the protein elastin have the most flexibility of all the fiber types. Elastic fibers are found in the skin and the walls of blood vessels, and they allow the tissue to stretch.

Reticular Fibers

The stains containing silver solutions are commonly used to demonstrate reticular fibers. These stains work by impregnating fibers with silver ions and then reducing those silver ions to their visible metallic form. Reticular fibers are argyrophilic in the manner that they can adsorb silver from solution but are unable to reduce it to visible metallic form without the aid of a reducing solution. There are many different silver stains that can be used to show reticular fibers such as Gomori, Snook, Gordon, and Sweets [Figure 1].

The mechanism of action involves that a strong base (ammonium hydroxide) is added to an aqueous silver nitrate solution to form a silver diamine complex in an ammoniacal silver solution. Before the application of this complex, it is common practice to oxidize and sensitize the tissue. While the sensitizing agent (uranyl nitrate/dilute silver nitrate) initially binds to the tissue component of interest, oxidation (potassium permanganate/periodic acid) improves subsequent staining. The fibers are impregnated with silver ions from the ammoniacal silver solution, which replace the sensitizer in the metal–organic compound.[4] Further, the silver diamine complex is then reduced to a visible metallic form by the action of a reducing agent (formalin). Metallic silver is converted to metallic gold with the use of a toning reagent (gold chloride), which is more stable and provides better contrast and clarity. Excess gold chloride and unreduced silver are removed (sodium thiosulfate), and the tissue section is counterstained if desired. Counterstains such as nuclear fast red or light green are frequently used. The fibers are black and appear in a fine linear pattern.

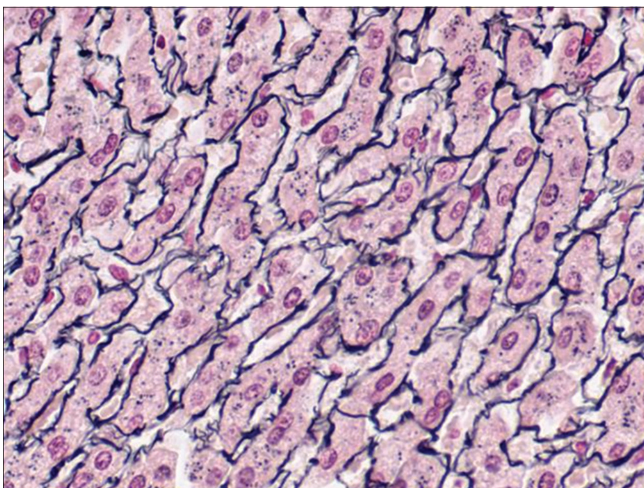

Figure 1: Gordon and Sweet stain demonstrating reticulin fibers

Gordon and Sweet's Staining Procedure

1. Deparaffinize sections with xylene then take through alcohols to water
2. Oxidize in acidified potassium permanganate for 3 min
3. Rinse in distilled water
4. Decolorize with 2% oxalic acid for 1 min
5. Rinse in distilled water
6. Mordant in 4% iron alum for 10 min
7. Rinse in distilled water
8. Impregnate in ammoniacal silver solution for 11 s
9. Rinse quickly in distilled water
10. Immediately reduce with 10% aqueous formalin for 2 min
11. Wash in running tap water for 2 min
12. Tone in 0.2% gold chloride for 2 min
13. Rinse in distilled water
14. Fix with 2% aqueous sodium thiosulfate (hypo) for 2 min
15. Wash in water for 2 min
16. Counterstain with neutral red for 2 min
17. Dehydrate, clear, and mount with DPX.

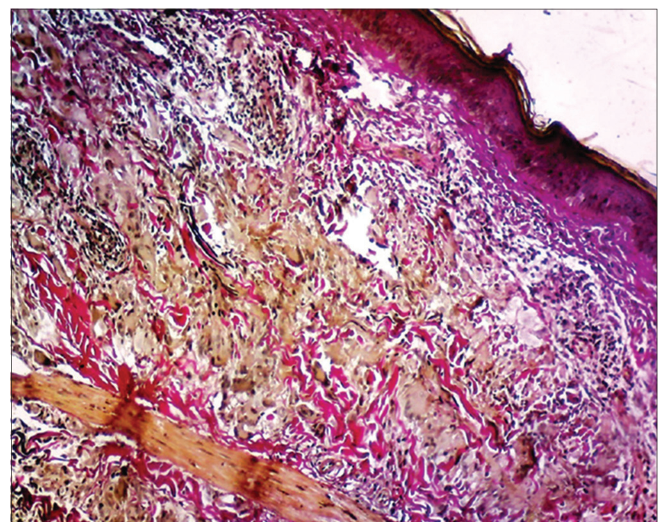

Figure 2: Verhoeff–van Gieson stain showing loss of elastic fibers in the upper and mid-dermis

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Elastic Fibers

The Verhoeff-van Gieson stain, commonly used to demonstrate elastic fibers [Figure 2], is an example of a regressive staining method. The tissue section is initially overstained with a solution of hematoxylin-ferric chloride-iodine and then differentiated for optimal demonstration of elastic fibers.[5]

Hematoxylin dye molecules are linked to the tissue components using ferric chloride and iodine as a mordant. They then convert hematoxylin to hematein by acting as oxidizers. The tissue-mordant-dye complex is then broken with a dilute solution of ferric chloride, allowing structures within the tissue to be distinguished. Elastic fibers retain dye molecules because they have a strong affinity for the iron-hematoxylin complex, whereas other structures dissociate dye molecules. The result is a stain reaction that is positive. Excess iodine and ferric chloride are removed from tissue sections using distilled water washes followed by sodium thiosulfate. As a counterstain, the van Gieson solution is used.[6]

The elastic fibers and nucleus appear as blue to black. Sometimes, the collagen fibers are also appreciated in red.

Verhoeff-van Gieson Staining Procedure

1. Deparaffinize and hydrate slides to distilled water
2. Stain in Verhoeff's solution for 1 h. Tissue should be completely black
3. Rinse in tap water with 2–3 changes
4. Differentiate in 2% ferric chloride for 1–2 min
5. Stop differentiation with several changes of tap water and check microscopically for black elastic fiber staining and gray background. It is better to slightly under differentiate the tissue since the subsequent van Gieson's counterstain can extract the elastic stain somewhat
6. Wash slides in tap water
7. Treat with 5% sodium thiosulfate for 1 min. Discard solution
8. Wash in running tap water for 5 min
9. Counterstain in van Gieson's solution for 3–5 min
10. Dehydrate quickly through 95% alcohol and 2 changes of 100% alcohol
11. Clear in 2 changes of xylene for 3 min each
12. Coverslip with resinous mounting medium.

Muscle

Muscle cells are characterized by the ability to contract, providing the movement necessary for locomotion of an organism, and movement of materials within an organism.

Three types of muscle tissue are identified based on their arrangement of actin and myosin fibers.[7]

- Skeletal muscle cells are multinucleated and have striations, which are bands. Conventionally, these cells are arranged in bundles and attached to skeletal elements. They are responsible for locomotion and are controlled by motor neurons
- Within the interconnecting fibers of cardiac muscle cells, there are centrally located nuclei (intercalated discs). Only heart tissue contains cardiac muscle, which provides constant rhythmic contraction without the need for external stimulation
- Smooth muscle cells have a central nucleus and are spindle-shaped. Smooth muscle can be found in the skin as well as a variety of internal organs, such as the digestive and reproductive systems. They are in charge of involuntary movements such as peristalsis.

Trichrome Stains

Masson's Trichrome Stain

With Masson's trichrome stain [Figure 3], Bouin's solution is used initially as a mordant to link the dye molecules to the tissue components of interest. Nuclei are stained with Weigert's hematoxylin, an iron hematoxylin, which is resistant to decolorization by subsequent acidic staining solutions. Application of Biebrich scarlet-acid-fuchsin stains all acidophilic tissue elements such as cytoplasm, muscle, and collagen. Subsequent treatment by phosphomolybdic/ phosphotungstic acid serves as a decolorizer causing the Biebrich scarlet-acid-fuchsin to diffuse out of the collagen fibers while leaving the muscle cells red. Subsequent application of aniline blue will stain the

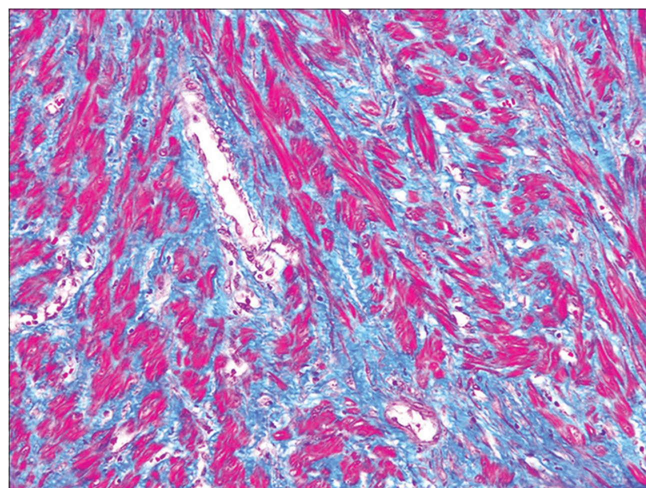

Figure 3: Masson trichrome stain (high power field). The marginal area shows increased collagen fibers with hypertrophic smooth muscle cells

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collagen after which, 1% acetic acid is employed to properly differentiate the tissue section. Most tissue will contain an internal quality control, but the appendix, Fallopian tube, uterus, or small intestine may be used. By this staining, muscle appear as red, nucleus as black, and the surrounding collagen and other materials such as mucin appear as blue.[8]

Masson's Trichrome Staining Procedure

1. Deparaffinize and rehydrate through grades of alcohol
2. Wash in distilled water
3. For formalin-fixed tissue, refix in Bouin's solution for 1 h at 56°C to improve staining quality, although this step is not absolutely necessary
4. Rinse running tap water for 5–10 min to remove the yellow
5. 3. Stain in Weigert's iron hematoxylin working solution for 10 min
6. Rinse in running warm tap water for 10 min
7. Wash in distilled water
8. Stain in Biebrich scarlet-acid-fuchsin solution for 10–15 min. Solution can be saved for future use
9. Wash in distilled water
10. Differentiate in phosphomolybdic-phosphotungstic acid solution for 10–15 min or until the collagen is not red
11. Transfer sections directly (without rinse) to aniline blue solution and stain for 5–10 min. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2–5 min
12. Wash in distilled water
13. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these steps will wipe off Biebrich scarlet-acid-fuchsin staining) and clear in xylene
14. Mount with resinous mounting medium.

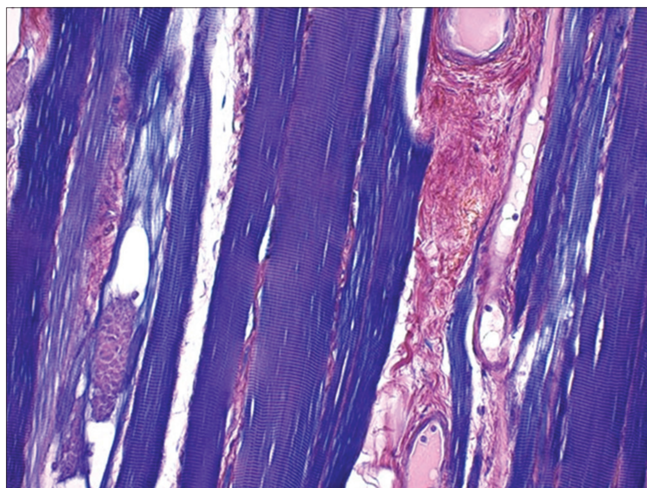

Figure 4: Phosphotungstic acid hematoxylin stain demonstrates collagen (red-brown) and muscle fibers (blue)

Phosphotungstic Acid Hematoxylin

Although trichrome techniques will demonstrate muscle fibers, phosphotungstic acid hematoxylin (PTAH), or phosphotungstic acid-hematoxylin, is preferred for demonstrating cross-striations of skeletal muscle, which may be lost in certain muscle diseases [Figure 4].[9]

Collagen and muscle fibers are demonstrated using a tungsten mordant provided by the phosphotungstic acid. This mordant binds hematein and stains selective tissue components blue, while the phosphotungstic acid is believed to stain other tissue components a red-brown. Tissue fixed in formalin is commonly postfixed in Zenker's solution, which is thought to intensify staining reactions. A section of skeletal muscle tissue can be used for quality control. Here, the muscle fibers appear in various shades of blue and collagen as red-brown.

Phosphotungstic Acid Hematoxylin Staining Procedure

1. Deparaffinize sections if necessary and hydrate to distilled water
2. Pour zinc chloride solution (10%) into plastic staining jar and set in 60°C. water bath for 10 min to equilibrate temperature
3. Place slide in warmed zinc chloride solution (10%) and incubate for 20 min at 60°C
4. During step 3, pour ferric ammonium sulfate aqueous solution into a second plastic staining jar and set in 60°C water bath for 10 min to equilibrate temperature
5. Rinse slide in running tap water for 1 min
6. Rinse in distilled water for 1 min
7. Place the slide in warmed ferric ammonium sulfate aqueous solution and incubate for 5 min at 60°C. 8. During step 7, pour PTAH solution into a third

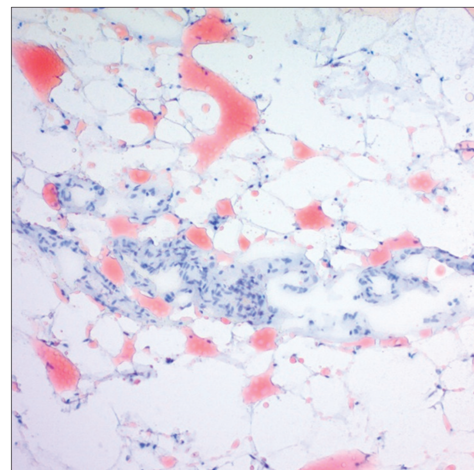

Figure 5: The Oil red O stain demonstrates fat (red) and nucleus (blue)

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plastic staining jar and set in 60°C water bath for 10 min to equilibrate temperature
8. Rinse slide in running tap water for 2 min
9. Rinse in distilled water for 1 min
11. Place the slide in warmed phosphotungstic acid hematoxylin solution and incubate for 60 min at 60°C. 12. Differentiate section in 95% reagent alcohol. Check section using microscope for proper differentiation
13. Note: graded alcohols will remove some stain
14. Dehydrate in 3 changes of absolute alcohol
15. Clear in 3 changes of fresh xylene or xylene substitute, and mount in DPX.

Lipids

Oil-Red-O and Sudan Black B stain[10]

Oil-Red-O and Sudan Black B stains are used to demonstrate simple lipids [Figure 5 and 6]. Most stains demonstrate chemical interactions, but these "staining" techniques demonstrate physical processes.

(A) lipid solvent, such as propylene glycol, is used to dissolve the dye (Oil-Red-O or Sudan Black (B). other fat solvents, such as isopropanol, can be used, but they cause a loss of lipids. Dye molecules are more soluble in cellular lipid than in dye solvent and will migrate from the solvent into the tissue lipid, giving the tissue lipid a positive stain color.

After that, tissue sections are stained with a counterstain (hematoxylin/nuclear fast red). Fat-containing tissue serves as a source of quality control material. Fat appears red and nucleus appears blue in Oil-Red-O stain, whereas fat appears blue-black and nucleus appears red in Sudan Black B stain.

Oil-Red-O staining procedure

1. Mount cryostat-cut sections of clean slides and fix in 10% buffered neutral formalin for 15 min. Formalin-fixed frozen sections may be floated in water and attached to albuminized slides. Allow to thoroughly dry (1to 2 h) to assure paper adherence. Fixation for touch preparations and material from cytology is not necessary
2. Rinse briefly with distilled water
3. A few dips in 60% isopropyl alcohol
4. Oil-Red-O working solution for 20 min
5. A few dips in 60% isopropyl alcohol
6. Rinse with four changes of distilled water
7. Counterstain with hematoxylin stain for 1 min
8. Rinse in three changes of distilled water
9. Blue hematoxylin in 0.3% sodium borate for 15 s
10. Rinse with four changes of distilled water
11. Mount with DPX.

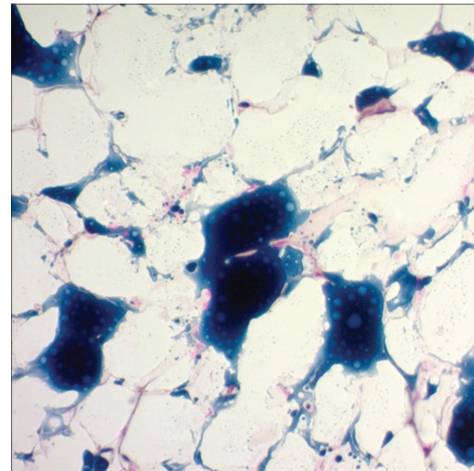

Figure 6: Fat appearing blue-black in Sudan Black B stain

Sudan Black B Staining Procedure

1. Deparaffinize and hydrate to 70% alcohol. When staining frozen sections, begin at step 3
2. Stand slides on end and allow to dry
3. Place in Sudan Black B solution for 1 to 3 h. Check after 1 h but first dip in 70% isopropyl alcohol. When staining frozen sections, stain for 20 min at room temperature
4. Rinse thoroughly in two changes of 70% isopropyl alcohol
5. Wash with six changes of distilled water
6. Nuclear fast red solution for 3 min
7. Rinse in two changes of distilled water
8. Mount with DPX.

Conclusion

Connective tissue stains have been used extensively for the diagnosis of tumors of varying origins. Understanding these staining techniques not only aids us in performing our staining procedures effectively but also can facilitate the innovation of new methods.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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