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## RESEARCH ARTICLE

# HISTOPATHOLOGICAL STAINS - A REVIEW

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**ABSTRACT**

Various ways of histopathological staining has been carried out in today's laboratories through advancements by way of chemical, molecular biology assays and immunological techniques. At early years, only the readily commercially available chemicals available for staining purpose were Potassium dichromate, alcohol and the mercuric chloride to harden the cellular tissue varieties. Staining techniques available during those times were Carmine, Silver nitrate, Gram's, Giemsa, Trichrome and Hematoxylin. In modern histopathological procedures, it has been proved that various combination of the different staining procedures would enhance the staining process efficiency and results. In this article, we have analyzed the different varieties of stains and its efficiency.



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## INTRODUCTION

In the biopsy/tissue-based diagnostics research study at present times the routine H & E (Hematoxylin & Eosin) staining plays a vital role. These stains allow a highly experienced pathologists and researchers to view under microscope; a tissue morphology for the presence of particular architecture/ phenomenon of importance which lead to a proving diagnosis. In routine laboratory, the term 'routine staining' refers to the H & E stain which is used to observe all tissue specimens to reveal the underlying structures and their variations from abnormality. The term 'Special Stains' has long been used to refer a large number of alternative staining techniques to be used when H & E doesn't create the information to the pathologist for confirmative diagnosis (Anderson, 2011).

**Different varieties of stains**

- Stains for Carbohydrates.
- Stains for Amyloid
- Stains for Nucleic acids.
- Stains for Lipids.
- Stains for Micro-organisms.
- Stains for Connective Tissues.
- Stains for Pigments and Minerals.

**STAINS FOR CARBOHYDRATES:** Simple Carbohydrates are the molecules composed purely of carbohydrates. They are three types:

a) Monosaccharides. (Glucose, Mannose, Galactose).
b) Oligosaccharides. (Sucrose, Maltose).
c). Polysaccharides. (Glycogen, Starch).

Glycoconjugates are molecules composed of carbohydrates and other molecules such as protein and lipid. They are three types:

a) Proteoglycans compose 90-95% of the molecular weight due to carbohydrate component. The carbohydrate component is known as glycosaminoglycans(GAG) which is repeating disaccharide units, made of 2 different monosaccharides. Each disaccharide is composed of a carboxylated uronic acid (glucuronic or iduronic acid) and a hexosamine such as N-acetylglucosamine or N-acetylgalactosamine. Types of

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glycosaminoglycans are chondroitin sulfate, Dermatan sulfate, Keratan sulfate, Heparin sulfate, Heparin, Hyaluronic acid.

Mucins are composed of Neutral Mucins, Sialomucins, Sulfomucins. Neutral mucins cover surface of epithelia of gastric mucosa, brunner's glands, prostatic epithelia. Sialomucins are bronchial submucous glands, goblet cells, salivary glands. Sulfomucins are composed of bronchial mucous glands. Sialomucins and sulfomucins are acidic mucins (Bancroft, 2013).

**Other glycoproteins:** The fixation of carbohydrate tissue is promptly after removal since it is capable of autolytic reaction. In case if immediate fixation is not possible, tissue is refrigerated until adequate fixation possible. The recommended fixatives are Rossman's fluid, Alcoholic formalin with picric acid. Glutaraldehyde fixatives are avoided since they contain free aldehyde groups which are capable of undergoing Schiff reaction, so there are chances of increased background staining. The fixation must be carried out at 4 degree centigrade to minimize streaming artifact (Black, 2012). Various carbohydrate staining techniques are Periodic acid schiff (PAS), Alcian blue method, Combined technique(Alcian blue & PAS), Mucicarmine technique, Colloidal iron technique, Metachromatic staining, Iodine staining for glycogen, Enzymatic digestion technique - Diastase digestion, Sialidase digestion, Hyaluronidase digestion.

Per-iodic Acid Schiff method is the first histochemical stain use by McManus for demonstration of mucin. The reagents used are Periodic acid and Schiff reagent. Here 0.5-1% solution of periodic acid(oxidant) used for 5-10 minutes which causes oxidation of hydroxyl group within the carbohydrate by forming two free aldehyde groups and thses free aldehyde react with the schiff reagent to form bright red magenta end product where glycogen and mucins represent magenta color and nuclei blue color (Cai, 2014). In mild PAS technique 0-0.01% periodic acid used for shorter period for N-acetyl sialic acid containing mucins as the hydroxyl groups are highly susceptible to periodic acid oxidation. To note further PAS reactive cells and tissue components are glycogen, starch, mucin, basement membrane, alpha anti-trypsin, reticulin, fungi(capsules), pancreatic zymogen granules, thyroid colloid, corpora amylacea, russell bodies. PAS technique is used for diagnosis of several medical conditions: glycogen storage disorder, staining macrophages in whipple's disease, mucins in adenocarcinoma of large intestine, demonstration of fungi, Seminoma, Rhabdomyosarcoma, Ewing's sarcoma containing glycogen.

Alcian blue 8GX is another stain used in carbohydrate detection comprising of copper containing pthalocyanine ring linked to 4 isothiouronium groups containing strong bases on account for cationic nature of the dye. Sulfate and carboxylate groups of proteoglycans ionised at pH 2.5 and carry a negative charge. Sialo- and sulfo mucins also reactive at pH 2.5 and they stain with alcian blue. Neutral mucins are not reactive with alcian blue. The reagents used are alcian blue, aluminium sulfate, nuclear fast red. The results obtained are sulfomucin, sialomucin, proteoglycans, hyaluronic acid give blue color and nucleus stained red color (Costa, 2010). In combined alcian blue and PAS technique demonstrate presence of mucin. Here differentiation between acid and neutral mucin takes place. They first stain all acid mucin with alcian blue i.e. blue color. Those acid mucin which are PAS positive will not be stained on PAS reaction. Only neutral mucin will be stained magenta color. This technique is based on phenomenon known as Critical Electrolyte Concentration (CEC), which is defined as point at which amont of electrolyte such as $MgCl_2$ is sufficient to prevent staining from alcian blue. There is competition between cations of salt and dye occuring for polyanionic sites within tissue. Different acidic carbohydrates have different CEC value and can differentiate acidic mucins and proteoglycans. Mucicarmine stain for carbohydrates: Here active dye molecule is aluminium-carminic acid complex known as Carmine. Carminic acid produced from dried bodies of female coccus Cacti insects. Carmine complex has a positive charge and so attracts polyanions such as sialomucins and sulfomucins. Useful for identification of adenocarcinoma(escpecially of GIT). Capsule of fungus Cryptococcus neoformans can also be detected. The reagents used are southgate's mucicarmine solution, alcoholic hematoxylin, acidified ferric chloride solution, weigert's iron hematoxylin solution, metanil yellow solution. The results obtained are acidic mucins stained deep rose to red color, Nuclei are stained black color, other tissue elements are stained light yellow color (Godwin, 2014; Harris, 2010; Iyiola, 2011; Jackson, 2013; Loreto, 2013)

Demonstration of nucleic acid depends upon either the reaction of the dyes with the phosphate groups or production of aldehydes from the sugar. There is no histochemical methods available to demonstrate the nitrogenous base. DNA is demonstrated by Feulgen techniqe(demonstrate sugar), Methyl green pyronin technique(demonstrate phosphate), acridine orange( by fluorescent method), Gallocyanin-chrome alum method which don't separate the two nucleic acids staining both DNA and RNA blue and suitable extraction technique must be utilised. The extraction techniques include Digestion methods(pure deoxyribonuclease will digest DNA and pure ribonuclease will digest RNA); Chemical methods(Perchloric acid, Trichloroacetic acid, Hydrochloric acid) (Lyon, 2014; Morelli, 2013).

**Stains For Lipids:** Lipids with melting point below staining temperature can be stained with fat stains. So only lipids which are liquid at staining temperature are stained. Those in solid or crystalline state remains unaffected. Melting point of a lipid is inversely related to its fatty acid chain length. Simple lipid is best demonstrated with fresh frozen sections and best fixative is Formal calcium (2% calcium acetate + 10% formalin). Sudan black B is most common special stain for lipids. First sudan dye was Sudan 3. Most sensitive of all fat dyes is Sudan black B. Sudans must be dissolved in organic solvents to penetrate fats. Some organic solvents used are 70% ethanol, isopropanol, propylene glycol, triethyl phosphate. Sudan black B has two fractions - First stains neutral fats blue-black color, Second stains the phospholipids gray color. This gray reaction can be enhanced as a bronze dichroism if section is viewed in polarised light. It fails to stain crystalline cholesterol, lecithin and free fatty acids. Bromine pre-treatment converts crystalline cholesterol to oily derivatives and hence permeable to sudan dye (MacKenzie, 2010; Nadworny, 2010). Congo-red stain is an acidic dye and composed of two identical halves. Each half has a phenyl ring bound to a naphthalene moiety by a diazo group. Two phenyl groups bound by a diphenyl bond gives a linear dye molecule. It stains amyloid by hydrogen bonding and other tissue components by electrochemical bonds.

Electrochemical staining of other tissues can be suppressed by using alkaline-alcoholic solvents; using competitive inhibition by salt solution. The factors which are important to the congo-red-amyloid reaction are the linearity of the dye molecule and beta-pleated sheet configuration of the amyloid. If the spatial configuration of either is altered, the reaction fails. The solutions used are 0.5% Congo-red in 50% alcohol, 0.2% Potassium hydroxide in 80% alcohol. The results are amyloid stains red color, nuclei stains blue color. Under polarized light, congo-red stained amyloid exhibits apple-green birefringence. It is most reliable diagnostic test for amyloid currently (Ntziachristos, 2010).

**Stains for Micro-Organisms:**Gram staining of bacteria is most common procedure followed with reagents crystal violet stain, gram's iodine solution, ethyl alcohol-acetone solution(decolorizer), acetone-xylene solution, basic fuchsin, picric acid, 0.1% in acetone. Results obtained are Gram positive bacteria stain blue color, Gram negative bacteria stain red color, Nuclei stain red color, other tissue elements stain yellow color (Rotimi, 2014; Rodrigues, 2009; Russell, 2009).
In acid fast staining/Ziehl Neelson staining for bacteria the mycobacteria cannot be demonstrated by gram's stain since they possess a capsule containing long chain fatty acid(mycolic acid) that makes them hydrophobic. Can be stained by a strong stain like carbol fuchsin. Fatty capsule resist the removal of stain by acid-alcohol solution. Mycobacteria are PAS positive due to carbohydrate content of their cell wall. Reagents used are Carbol fuchsin solution, 1% acid alcohol, 0.1% methylene blue solution. The results obtained are acid fast bacilli/mycobacterium tuberculosis stain bright red color, other tissue stain pale blue color, caseous material very pale grayish blue color. Blue counterstain may be patchy if extensive caseation is present. Avoid over counterstaining since scant organism can easily be obscured. There are chances of decalcification using strong acids which may destroy acid-fastness, so formic acid is recommended.

For identification of mycobacterium leprae, modified fite technique reagents used are carbol fuchsin solution, 5% sulphuric acid in 25% alcohol, methylene blue solution. The results obtained are mycobacterium leprae stain bright red color, nuclei and other tissue elements stain pale blue color. For identification of spirochetes, Warthin-starry method is used. Reagents used are acetate buffer with pH 3.6 and 1% silver nitrate. The results obtained are spirochetes stain black color, background stain golden-yellow color (Safeena et al., 2010; Shostak, 2013; Serrano et al., 2010; Sine, 2014).

For identification of fungal stains Gomori methanamine silver nitrate(GMS) technique is used. Reagents used are 4% chromic acid, 1% sodium bisulfite, 5% sodium thiosulfate, 0.21% silver nitrate, gold chloride 0.1% aqueous solution, light green solution. The results obtained are fungi, pneumocystis, melanin stain black color, mucin and glycogen stain dark grey color, background stain pale green color, hyphae and yeast form are sharply delineated in black color against green background. Other miscellaneous stains are Cresyl violet acetate method for helicobacter pylori, Macchiavello's stain for rickettsia and viral inlusions, Lendrum's phloxine-tartrazine stain for viral inclusions, Giemsa stain for parasites.

**Connective tissue stains:** The stains used for collagen fibres are masson's trichrome stain, van-gieson's stain, mallory's phosphotungstic acid hematoxylin, MSB stain, PAS stain, Heidenhain's azan stain, Lillie's allochrome stain, Luxol fast blue G stain. Masson's Trichrome technique demonstrates collagen and muscle in normal tissue. They differentiate collagen and muscle in tumors. They identify an increase in collagenous tissue. Useful in indicating fibrotic changes in cirrhosis of liver, pyelonephritis. Helpful in distinguishing tumors that have arisen from muscle cells and fibroblasts. The reagents used are weigert's iron hematoxylin, acid fuchsin, glacial acetic acid, phosphomolybdic acid, methyl blue. The results show nuclei being blue/black in color, cytoplasm along with muscle and RBC (red blood cells) representing red color, collagen blue color. Van-Gieson technique represents demonstration of muscle fibres. The reagent used are weigert's iron hematoxylin, saturated picric acid solution, acid fuchsin. The results show collagen bright red color, nuclei blue/black in color, cytoplasm, muscle, RBC, elastin and reticulin show yellow color (Titford, 2009; Titford, 2012).

**Stains For Pigment And Minerals:** Hemosiderin is the breakdown product of hemoglobin composed of ferric iron and protein. They are seen as yellow-brown granules. There are three methods of demonstration of hemosiderin pigment. First is Perl's prussian blue reaction used for ferric iron, Lille's method used for ferrous iron, Hukill and Putt's method used for both ferric and ferrous iron. Perl's stain is used for unmasking of ferric iron in hydroxide form by dilute hydrochloric acid. The reagents used are 2% aqueous potassium ferrocyanide, 2% Hydrochloric acid, Counterstaining with 1% neutral red or saffranin. The results obtained are ferric iron show blue color, Nuclei stain red color. The best positive control is seen in post-mortem lung tissue that contains a reasonable amount of iron positive macrophages(heart failure cells). In hemoglobin and myoglobin, iron bound tightly within protein complex cannot be demonstrated by traditional technique. Treatment with hydrogen peroxide releases iron stained with perl's stain.

Melanin normally occur as light brown to black granules in substantia nigra, hair, skin and eye. Found pathologically throughout the body in benign nevus and malignant melanoma. Melanin is demonstrated by Masson Fontana silver reduction technique, Schmorl's ferric-ferricyanide reduction test, Enzyme -DOPA method, Solubility and bleaching characteristics, Fluorescent method, Immunohistochemistry. In Masson Fontana stain there is reduction of ammoniacal silver solution to form metallic silver without the use of extraneous reducer. Masson's method rely on melanin's argentaffin property. Melanins are blackened by acid silver nitrate solution. The results display melanin to be black in color, nuclei red in color. Schmorl's ferric-ferricyanide reduction test produce melanin with production of prussian blue in the presence of ferric salts. The results obtained are melanin stain dark blue color, nuclei red in color (Victor, 2013; Weiss, 2010; Young, 2010).

## Conclusion

Special stains enhance detection and localization of individual tissue components but should not be substituted for routine H & E stain methods.

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