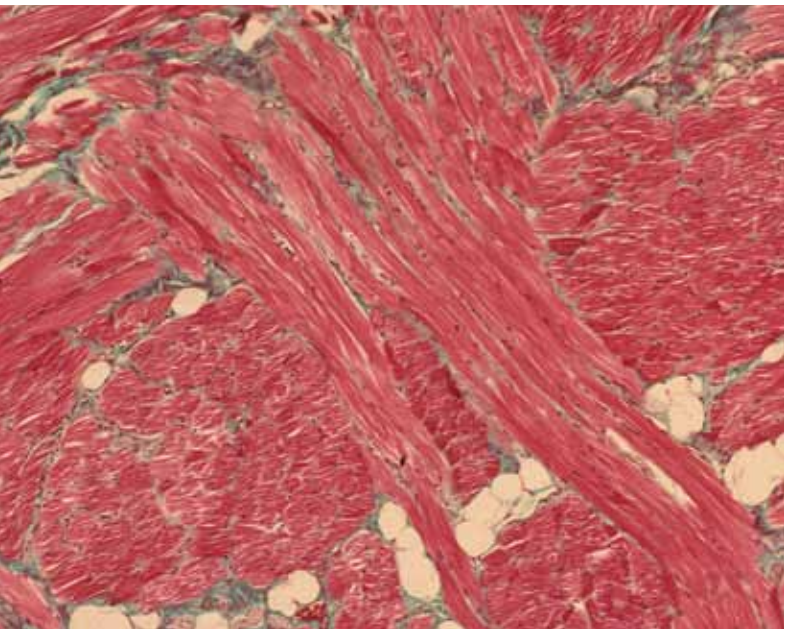
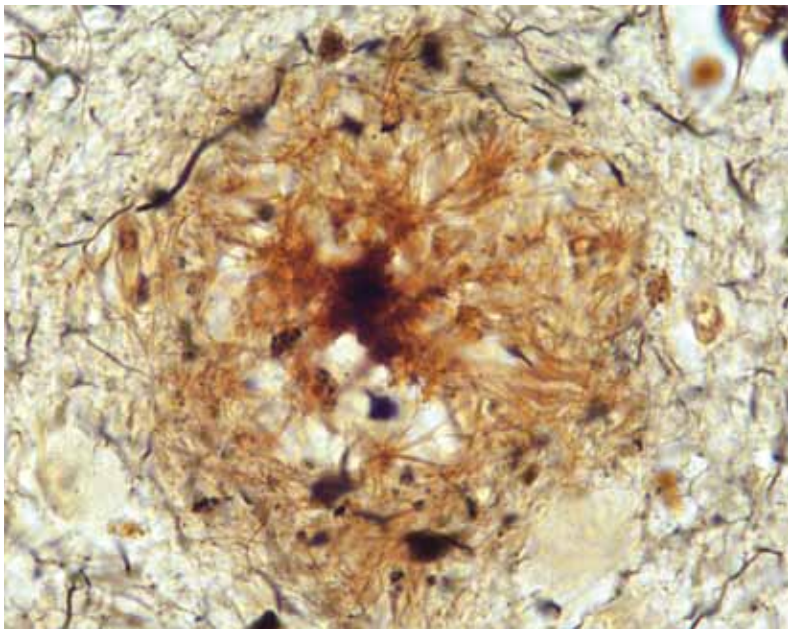
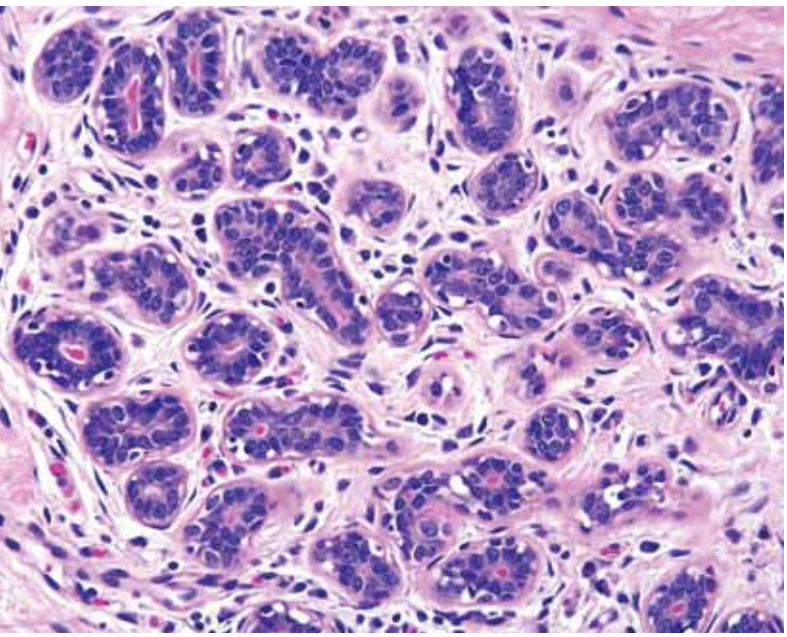
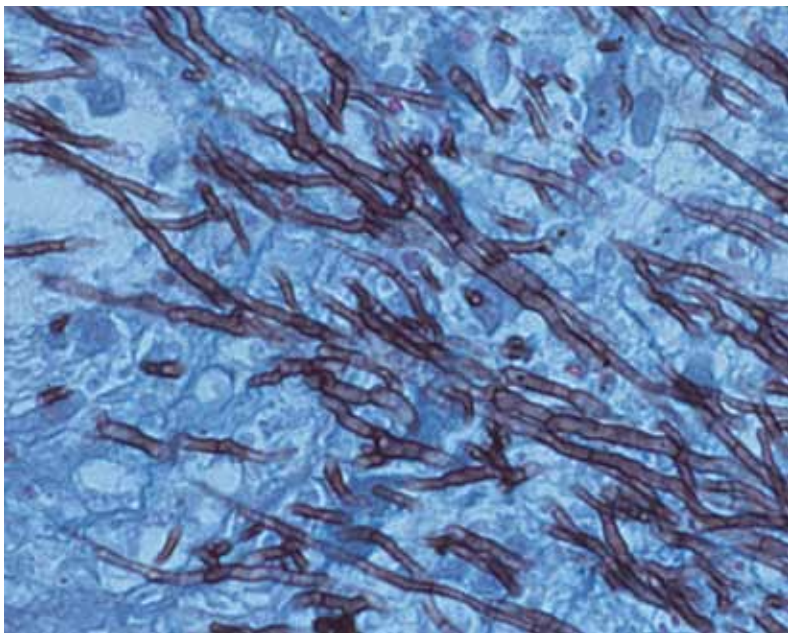
PATHOLOGY

# Education Guide | Special Stains and H & E Second Edition









## Cover Credits

*Top left:*
Muscle patterns demonstrated with trichrome stain. 20X.

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*Top right:*
Modified Bielschowsky silver stain. Alzheimer disease. High magnification image showing the actual plaque structure.

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*Bottom left:*
H&E staining. Histological changes in breast tissue with menstrual cycle.

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*Bottom right:*
Grocott's Methenamine Silver (GMS) staining of *Aspergillus*.

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# Education Guide | Special Stains and H & E

**Updated and Expanded Second Edition**


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# Preface

Dako introduces this second edition of “Education Guide - Special Stains and H & E”.  The second edition is a thorough update of the previous edition and gives readers a conceptual framework of Special Stains and H & E.

The origin of the term “special stains” is unknown, but one can be certain that it began to be used after 1876 when the combination of hematoxylin and eosin (H & E) was introduced and soon became the oversight method of first choice for most practitioners of normal histology and histopathology. The “special stain” terminology is most commonly used in the clinical environment, and simply means any technique other than the H & E method that is used to impart colors to a specimen. This also includes immunohistochemical and in situ hybridization stains. On the other hand, the H & E stain is the most popular staining method in histology and medical diagnosis laboratories.

Although H & E is very popular among histotechnologists and pathologists for looking at biopsies and surgical and post mortem specimens, the method has some limitations. For example, H & E does not identify microorganisms (e.g., *H. pylori*), or for that matter, specific tissue molecules in cancers (e.g., glycoproteins or glycosaminoglycans) or abnormal increase of interstitial fibers in some neoplastic clonal proliferations of the haematopoietic stem cell diseases (e.g., myelodysplastic syndromes). This is where special stains come in handy: for identifying microorganisms or specific structures or molecules in cells or in tissue sections. Immunohistochemistry provides supplementary information by identifying molecules on the basis of the specificity of antigen-antibody binding. In the case of human tumors, a diagnosis can usually be made with a combination of H & E and special stains, whereas immunohistochemical tests can provide valuable indications of prognosis and guidance for treatment.

Both H & E and special stains may be applied either manually or through automated methods using specialized instruments. Manual procedures are used when staining only a few slides per day by any particular method. Mechanized staining is valuable for processing much larger numbers of slides. Although these cost- and time-saving results have been documented, to a certain extent, it is still not clear as to how special stains are used in different parts of the world, or for that matter, in what modifications they are used. There is also a shortage of easily available information regarding the evolution of special stains, standardization and quality control.

In order to address some of these issues, we have dedicated this Guide to include topics that relate to history, chemistry, microscopy, quality control, technical details of staining mechanisms, applications of special stains and H & E, technology overview, users’ perspectives, troubleshooting and technical tips and glossary. This edition also includes the thoughts of some experts in the field that address some common questions with respect to the chemistry, troubleshooting and intelligent use of instruments and stains used in pathology. This is not a manual with instructions for carrying out staining methods; references to appropriate technical books and articles are given at the ends of the chapters.

We hope this edition will be as useful as the previous edition, and we ask that you share your experiences with us and with your colleagues.

The task of bringing a new edition requires the talents and efforts of many dedicated people. On behalf of Dako, we would like to thank all the participating authors and supporting staff for making this new edition possible.

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# Chapter 1 | Introduction to Special Stains

**George L. Kumar, PhD, MBA and Gary W. Gill, CT(ASCP)**

Special stains are “special” because they are not routine. They are applied to tissue sections in addition to hematoxylin and eosin (H&E)-stained sections to answer questions that arise above and beyond those that can be answered by interpreting H&E-stained tissue morphology (Fig. 1). The term “special stains” is of uncertain provenance, but one can be certain that it began to be used after 1876 when H&E was introduced.

Special stains (Fig. 2) can answer these questions:

- Is a certain class of molecules present or absent?
- Where are the molecules located in the preparation?
- How many of the molecules are present?

Answering the last question requires sophisticated instrumentation and computation methods and, to our knowledge, this aspect of special stains is neither well-documented nor understood.

In this article, we will describe some commonly used non-immunohistochemical stains. In the first part of the article, we will compare some key aspects between H&E and special stains, and certification of special stains by the “Biological Stain Commission”. In the second part of the article, we will delve into the technical details of special stains.

## H&E and Special Stains

Comparing key aspects of H&E and special stains is instructive.

## Classification of Special Stains by the Biological Stain Commission

The Biological Stain Commission (see Appendix) certifies biological stains. Among its objectives, the Biological Stain Commission strives to ensure the quality of dyes through independent testing according to appropriate rigorous chemical and performance criteria. The authors are unaware of comparable efforts elsewhere in the world to ensure the quality of dyes used as special stains and other applications. Sixty-four stains are on a certification basis with the Biological Stain Commission (Table 1). All but two, hematoxylin and orcein, are synthetic dyes. Twenty-nine of the 62 synthetic dyes were first used before 1909, and are highlighted in bold letters.

Fifty-two dyes have Colour Index (C.I.) numbers; 12 do not. C.I. numbers are 5-digit numbers assigned by The Society of Dyers and Colourists in England to uniquely identify stains that are the same chemically, but have different names. These 5-digit numbers must be specified when purchasing dyes or publishing articles in which the dyes are cited to ensure using the same dye, even if identified by different names.

| Aspect | H&E | Special Stains |
|---|---|---|
| Questions that can be answered | Many | One too many |
| Primary interest | Nucleus and cytoplasm<br><br>Medical diagnosis (e.g., growth activity) | Nucleus or cytoplasm<br><br>Mostly in the diagnosis of infectious diseases and cancer based on chemical composition |
| Basis of interpretation | Morphology | Morphology/Color |
| Frequency of use | Routine | As needed |
| Quantitative | No | No |
| Controls needed | No | Yes |
| Substrate specific | No | Yes |

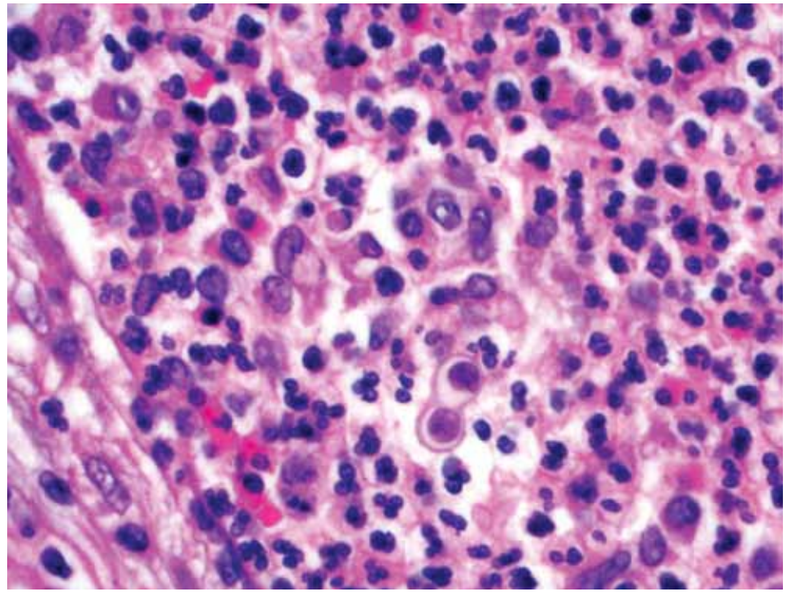

**Figure 1.** H&E-stained section of skin with cutaneous blastomycosis. High magnification (40x) view showing budding yeast with the inflammatory infiltrate. Hematoxylin stains the nuclei of cells blue to bluish-purple, and eosin stains other cellular elements in the tissues from pink to red (Figure courtesy: Sunil Badve, MD, FRCPath. Indiana University School of Medicine, Indianapolis, IN, USA).

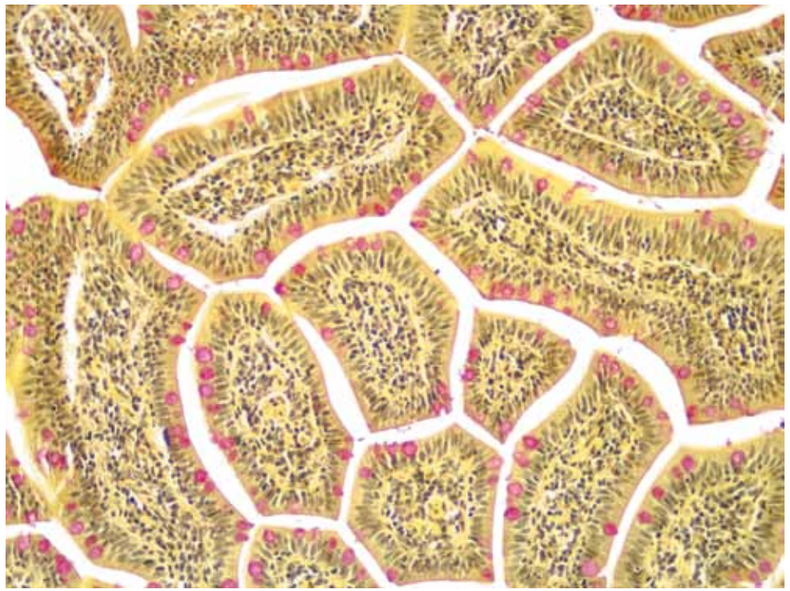

**Figure 2.** Special stained section of small intestine. The special stain mucicarmine is used for visualization of neutral epithelial mucins in small intestine. The mucins are stained rose to red, nuclei are blue/black (Weigert's iron hematoxylin), and other tissue elements are yellow (metanil yellow or tartrazine).





**Table 1.** 2009 biological stains certified by the Biological Stain Commission.

| | | | |
|---|---|---|---|
| **Acid fuchsin, C.I. 42685** | **Cresyl violet** | **Malachite green, C.I. 42000** | Phloxin B, C.I. 45410 |
| Alcian blue 8 GX, C.I. 74240 | **Crystal violet, C.I. 42555** | Martius yellow, C.I. 10315 | Protargol S |
| **Alizarin red S, C.I. 58005** | Darrow red | Methyl orange, C.I. 13025 | Pyronine B, C.I. 45010 |
| **Aniline blue WS, C.I. 42755** | Eosin B, C.I. 45400 | **Methyl violet 2B, C.I. 42535** | Pyronine Y, C.I. 45005 |
| Auramine O, C.I. 41000 | **Eosin Y, C.I. 45380** | **Methylene blue** | Resazurin |
| **Azocarmine B** | **Erythrosin, C.I. 45430** | Methylene blue, C.I. 52015 | Rose Bengal, C.I. 45435 |
| **Azocarmine G, C.I. 50085** | Ethyl eosin, C.I. 45386 | Methylene violet (Bernthsen), C.I. 52041 | **Safranine O, C.I. 50240** |
| Azure A, C.I. 52005 | Ethyl green, C.I. 42590 | **Neutral red, C.I. 50040** | Sudan black B, C.I. 26150 |
| Azure B, C.I. 52010 | Fast green F C F, C.I. 42053 | **Nigrosin, C.I. 50420** | **Sudan III, C.I. 26100** |
| Azure C, C.I. 52002 | Fluorescein Isothiocyanate | **Nile blue A, C.I. 51180** | **Sudan IV, C.I. 26105** |
| **Basic fuchsine, C.I. 42510** | **Giemsa Stain 1902, modified in 1904.** | Nuclear fast red, C.I. 60760 | Tetrachrome stain (MacNeal) |
| Bismarck brown Y, C.I. 21000 | **Hematoxylin, C.I. 75290 (Bohmer 1865)** | Oil Red O, C.I. 26125 | **Thionine, C.I. 52000** |
| Brilliant cresyl blue, C.I. 51010 | **Indigo carmine, C.I. 73015** | **Orange G, C.I. 16230** | **Toluidine blue, C.I. 52040** |
| Brilliant green, C.I. 42040 | **Janus green B, C.I. 11050** | Orange II, C.I. 15510 | Weigert 1878 |
| Carmine, C.I. 75470 | Jenner stain 1899 | Orcein | **Wright stain (1908)** |
| Chlorazol black E, C.I. 30235 | **Light green SF, C.I. 42095** | Pararosaniline, C.I. 42500 | |
| **Congo red, C.I. 22120** | | | |

## Special Stains

Special stain is a term that is mostly used in a laboratory setting. Special stains have two broad areas of application: research and diagnostic. In research, special stains are used as probes to identify certain chemical constituents in normal and abnormal cells. The information so obtained is used as a basis for further study and also as a baseline against which the results of special staining can be compared in diagnostic applications. On the basis of such a comparison, the significance of the findings can be interpreted.

Special stains can be applied to cell biology and histology. Some useful applications are: (1) the determination of DNA and RNA content, (2) the mode of action of drugs, hormones or of potentially toxic food additives, (3) metabolic biochemistry, (4) biochemistry of disease processes, (5) primary sites of many metastatic tumors, (6) identification of non-pigmented metastatic melanomas, (7) detection of early invading tumors, (8) definition of the margins of surgically resected tumors, (9) identification of Barr bodies, (10) staining cells in ways that can be used as a basis for cell separation by appropriate instrumentation (e.g., fluorescence), and (11) identification of micro-organisms (e.g., *Cryptococcus neoformans, Helicobacter pylori*). See Table 2.

The material, methods and interpretation of these special stains can be found in references 5-7. When working with special stains, keep in mind the following considerations:

- Special staining often requires the use of unusual stains and reagents that are available from only a few sources. Knowledge of such sources is essential to overcome technical bottlenecks.
- Be aware of special stains that contain colored and colorless impurities (e.g., salts) as these substances may interfere with the staining.
- Special staining requires broad knowledge of the tissue or cells targeted.
- When working with special stains, care should be taken to collect, fix and prepare the specimen in a manner that will maintain the molecule of interest within cells or tissues. For example, it is important to work with frozen sections when attempting to identify enzymes and to avoid fat solvents such as alcohol and xylene when attempting to identify lipids.
- With cell suspensions, it is essential to determine by microscopy whether cells are present, and how many cells are to be used when making the slides. Using this quality control step will improve the cellular preparations.
- Control preparations must be run in parallel with experimental preparations for one or more of the following reasons: (1) to determine if the special stain is working, (2) to assess the degree of non-specific staining, (3) to determine whether a reagent is still active, and (4) to serve as a standard in fractional reduction of staining procedures. If a positive reaction is noted when a control is not used, it can still be determined that the reaction is at least working (how well or how specifically is open to speculation). However, a negative reaction in the absence of a control may indicate either that the sought constituent is not present or the reaction is not working.
- Control slides should be: (1) sections of tissue/cell high in a particular molecule/constituent, (2) purified samples of a particular molecule in smears, (3) samples of the same specimen pretreated with solvents or enzymes to remove the sought constituent, (4) samples of the same specimen with the omittance of essential reagents or steps in the staining procedure, or (5) run as a duplicate cell spread in the same manner as the experiment minus one essential step.
- The amount of special stains within a cell or tissue represents the difference between the amount taken up during staining and the amount removed by the rinses following staining. To ensure the optimal amount, the user must employ those materials and methods that promote stain uptake during and following staining (e.g., dye concentration, suitable solvent, control of favorable pH, addition of salts, if necessary, control of ionic concentration, if necessary, time and temperature).
- To maintain the right amount and hue of the special stain, mount the stained specimen in a medium that does not promote bleaching or leaching.
- To ensure optimal image quality of the stained specimen, use the right amount of mounting medium, cover with a No. 1 cover glass, and use a clean microscope with the illumination adjusted according to the method of Köhler (see Appendix, page 283).





## Manual vs. Automated Special Staining Protocols

Depending on the financial situation of the laboratory, specimen sample size, and the number of personnel available, special stain protocols are performed either manually or by using automated systems. Manual staining of slides work well in a research setting, especially, when the number of processed slides are few per day. However, with increasing numbers of slides to be stained, the manual method becomes prone to error resulting in decreased flexibility and productivity. With the medical community demanding faster turnaround times, increased flexibility and productivity as well as greater standardization, automated instruments have replaced some manual methods of staining thus becoming an integral part of the laboratory. Automation combined with specialized software applications and connectivity have made many instruments capable of multiprogramming runs resulting in standardized protocols, manageable work schedules, enhanced workflow, cost-effectiveness and the ability to adapt to regulatory requirements.

**Table 2.** Commonly used special stains.

| Special Stain | Clinical Application | Staining Specificity |
|---|---|---|
| **For detecting micro-organisms and *Helicobacter pylori*** | | |
| Acid-Fast Bacteria (AFB) (Ziehl-Neelsen Stain) | Detects nocardioform-actinomycete groups of bacteria, including *Mycobacterium* Spp (acid fast), *Rhodococcus equi* and *Nocardia* Spp (weakly acid fast) Fig. 3 | Acid-fast bacilli retain a cationic dye that is extracted from all other types of bacteria and animal cells by acidified alcohol. The waxy wall (with mycolic acid) of mycobacteria retains the dye |
| Alcian Yellow / Toluidine Blue (Leung) Stain | Used for the detection of *H. pylori* See Fig. 4 for an electron micrograph and illustration of *H. pylori* | The yellow dye stains oxidized and sulphonated gastric mucus providing contrast for suspended Helicobacter organisms that are stained with toluidine blue |
| Dieterle's Stain | Identifies *Borrelia burgdorferi, Legionella pneumophila, Treponema pallidum* | Stains whole organisms |
| Diff-Quik Stain (Diff-Quik is the formerly trademarked name for a proprietary rapid Giemsa-like stain) | Detects *H. pylori* and some fungi (e.g., *Pneumocystis jiroveci)* | *H. pylori* and *Pneumocystis jiroveci* |
| Giemsa Stain | Used for staining *H. pylori, Plasmodium vivax, Rickettsia prowazekii, Rickettsia rickettsii, Rickettsia tsutsugamushi, Trypanosoma cruzi, Giardia lamblia;* Fig. 5a, b and c | Stains polyanions blue and polycations pink<br><br>Bacteria show up blue on account of their nucleic acids. Acidic capsules (e.g., Anthrax Bacilli, *Cryptococcus*) would be expected to be blue or purple |
| Gram Stain (Named after its inventor, the Danish scientist Hans Christian Gram, who developed the technique in 1884 to discriminate between two types of bacteria with similar clinical symptoms) | Used for the detection of Gram-positive *(Clostridium botulinum, Clostridium tetani, Staphylococcus aureus and Corynebacterium diphtheriae)* or Gram-negative bacteria (Salmonella, *Shigella dysenteriae, Escherichia coli* and *Pseudomonas aeruginosa).* Also used for the detection of *Actinomyces Israeli, Legionella pneumophila, Neisseria gonorrhea, Neisseria meningitidis, Nocardia asteroides* | Stains whole organisms |

**Table 2.** Commonly used special stains.

| Special Stain | Clinical Application | Staining Specificity |
|---|---|---|
| Grocott's Methenamine Silver (GMS) Stain | Useful in identifying a variety of pathogenic fungi, including *Aspergillus fumigatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Nocardia asteroids, Pneumocystis carinii, Pneumocystis Jiroveci* (human) and *Sporothrix schenckii;* Fig. 6-8 | Polysaccharide components of the fungal cell wall |
| Mayer's Mucicarmine Stain | Detects encapsulated yeast-like fungus *Cryptococcus neofarmans* | Polysaccharides on the capsule |
| Periodic Acid-Schiff (PAS) Stain | Used for the identification of *Aspergillus fumigatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Cryptococcus neofarmans, Sporothrix schenckii* | Polysaccharide components of the fungal cell wall |
| Sayeed's Stain (Schiff's reagent, 0.5% periodic acid, Mayer's hemalum) | Detects *H. pylori* | *H. pylori* |
| Steiner & Steiner Staining Method | Detects spirochetes and legionella, and pneumophila bacteria, e.g., *Borrelia burgdorferi, H. pylori, Legionella pneumophila, Treponema pallidum;* Fig. 9 | Stains whole organisms |
| Warthin-Starry Stain (these are reduced silver methods) | Identifies *Alipia feles, Bartonella henselae, Borrelia burgdorferi, H. pylori, Legionella pneumophila, Treponema pallidum;* Fig. 10 | Stains whole organisms |
| **For demonstrating connective tissue, muscle, collagen, lipid and fibrin** | | |
| Gomori's One-Step Trichrome Stain | Used for distinguishing collagen and smooth muscle fibers; Fig.11 | Collagen and smooth muscle fibers |
| Jones' Basement Membrane Periodic Schiff-Methenamine Silver (PASM) Stain | Used for the identification of basement membranes (of the glomerulus in the kidney or in tissue samples); Fig. 12 | Basement membranes |
| Masson's Trichrome Stain (TRI) | Used for distinguishing cells from surrounding connective tissue which has several variants and is probably the trichrome most used in histopathology. Black nuclei, red cytoplasm (including muscle), blue or green collagen (including fine fibers), cartilage and mucus; Fig. 13 | Muscle, collagen fibers, fibrin and erythrocytes |
| Russel-Movat Pentachrome Stain | Used for simultaneous demonstration of muscle, elastic fibers, collagen/reticular fibers, ground substance and fibrinoid in tissues | Muscle, elastic fibers, collagen/reticular fibers |
| Oil Red O and Sudan Black B Stains | Used for staining lipids in frozen sections and some lipoproteins on paraffin sections | Lipids, including triglycerides (which necessarily are neutral). Oil Red O stains only the most hydrophobic lipids (triglycerides and cholesterol esters). Sudan Black B stains these and also phospholipids and sphingomyelins, which are less hydrophobic |
| Orcein Stain | Used for staining elastic fibers | Elastic fibers |
| Lendrum's Method (Picro-Mallory Stain) | Fibrin | Fibrin |
| Phosphotungstic Acid-Hematoxylin (PTAH) Stain | Used for demonstrating striated muscle fibers<br>Also used to stain abnormal neuroglia (reactive astrocytosis) | Muscle fibers, collagen |
| Silver methods for reticulum and basement membranes (e.g., Reticulin/ Nuclear Fast Red Stain) | Used for the identification of reticulin fibers in tissue samples; Fig.14 | Reticulin (collagen with high level of hexosylation, including Type IV) |
| Verhoeff Stain<br>Van Gieson Stain | Used for the identification of elastic laminae and fibers in tissues; Fig.15 | The Verhoeven Stain is specific for elastic fibers. The Van Gieson Stain is specific for collagen. Verhoeff's iron-hematoxylin stains elastin and nuclei black. Van Gieson's picro-fuchsine gives yellow cytoplasm and red collagen fibers |
| **For detecting nucleic acids** | | |
| Ethyl Green-Pyronine Stain | Used for differential demonstration of DNA and RNA | A buffered mixture of the two dyes gives blue-green DNA and red RNA (rRNA in cytoplasm, nucleoli) |
| Feulgen Stain | Used for the identification of chromosomal material or deoxyribonucleic acid (DNA in paraffin-embedded tissue or cell specimens); Fig.16 | Deoxyribonucleic acid (DNA) |

**Table 2.** Commonly used special stains.

| Special Stain | Clinical Application | Staining Specificity |
|---|---|---|
| **Neuropathology** | | |
| Bielschowsky Silver Stain | Used for diagnosing Alzheimer's Disease to show neuritic components of plaques and tangles | Neurofilament protein. Normal axons are also stained |
| Congo Red | Used for the detection of amyloidal plaques in brain; Fig. 17 | Extracellular amyloidal deposits |
| Cresyl Violet Stain | Useful in identifying cell bodies of neurons in tissue sections; Fig. 18 | Nissl substance in neurons. The Cresyl Violet Stain shows cell bodies of neurons by virtue of their abundant rough ER and ribosomes (rRNA) |
| Phosphotungstic Acid-Hematoxylin (PTAH) Stain | Used to stain abnormal neuroglia (reactive astrocytosis) | Abnormal neuroglia (reactive astrocytosis) |
| **For demonstrating myelin** | | |
| Luxol Fast Blue (MBS) Stain | Used for demonstrating myelin; Fig. 18 and 19 | Myelin |
| Page's Eriochrome Cyanine R | Used for demonstrating myelin | Myelin |
| **Dermatopathology, hematology, pigment detection, minerals and bone** | | |
| Alizarin Red S Stain | Calcium detection in tissues | Complexes with calcium |
| Chloroacetate Esterase (Leder) Stain | Useful as a marker of neutrophils | Histochemical detection of an enzyme of neutrophil leukocytes |
| Hall's Stain | Used for the detection of bile pigment | Bilirubin |
| Masson-Fontana Stain | Used for the detection of melanin and some neuroendocrine cells | Serotonin, melanin and other silver-reducing (argentaffin) substances |
| Perls' Prussian Blue Stain | Demonstrates hemosiderin in bone marrow macrophages and within erythroblasts | Hemosiderin (iron storage complex) |
| p-dimethylaminobenzylidenerhodanine Stain | Used for the detection of copper in tissues | Copper or copper-associated protein |
| Villanueva Osteochrome Bone Stain | Gives uniform and reproducible results for mineralized or undecalcified bone | Mineralized or undecalcified bone |





| Special Stain | Clinical Application | Staining Specificity |
|---|---|---|
| **Miscellaneous and multipurpose stains** | | |
| Alcian Blue | Used in identifying mucins and glycosaminoglycans. At pH 2.5, Alcian Blue stains sulphated and non-sulphated acidic carbohydrates. At pH 1.0, only sulphated carbohydrates are stained; Fig. 20 and 21 | Mucins |
| Giemsa Stain | Used in hematology, e.g., for the detection of erythroidcolonies, binucleate normoblast, megaloblasts, mast cells, etc. Giemsa is also used for chromosome staining; Fig. 22a, 22b and 23 | Specific for phosphate groups of DNA |
| Gomori's Silver Stain | Used for the detection of reticulin in bone marrow | Reticulin |
| Mucicarmine Stain | Detects mucins; Fig.1 | Mucins |
| Periodic Acid-Schiff (PAS) Stain | Used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen and glycoprotein), basement membranes, collagen and primary cell types; Fig. 24 and 25 | Carbohydrate macromolecules by virtue of their content of galactose, glucose, fucose and mannose |
| Periodic Acid-Silver Methenamine (PEM) Stain | Used for the delineation of basement membranes | Carbohydrate macromolecules by virtue of their content of galactose, glucose, fucose and mannose |

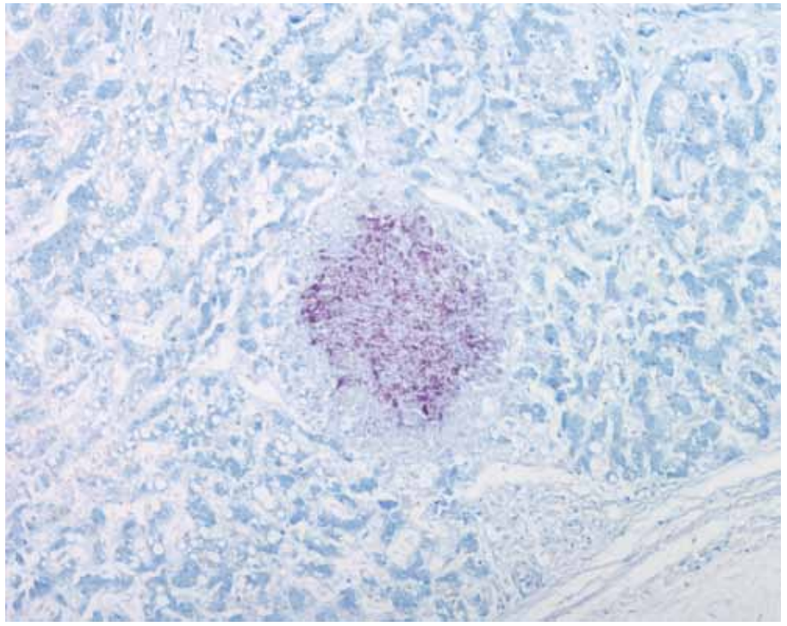

**Figure 3.** Lung stained with Acid-Fast Bacteria (AFB) Stain, Dako Code AR162. This AFB stain is suitable for the visualization of acid-fast bacteria belonging to the *Mycobacterium* genus on the Artisan™ Staining System. Application of carbol-fuchsin stains acid-fast bacteria fuchsia, followed by decolorization of all tissue elements except the acid-fast bacteria. A methylene blue counterstain is then applied to impart a blue color to all background tissue elements.

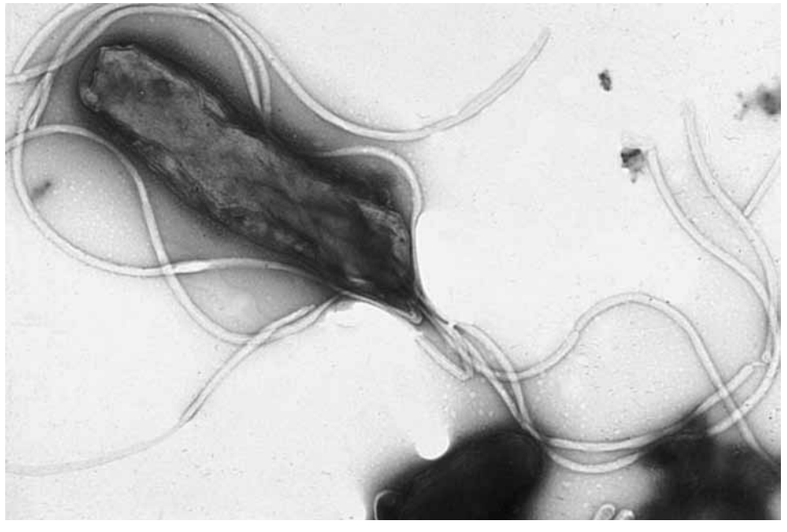

**Figure 4a.** Electron micrograph (EM) (negative staining) of *H. pylori* possessing multiple flagella. Courtesy of Wikimedia. Prof. Yutaka Tsutsumi, MD, Department of Pathology, Fujita Health University School of Medicine, Japan.





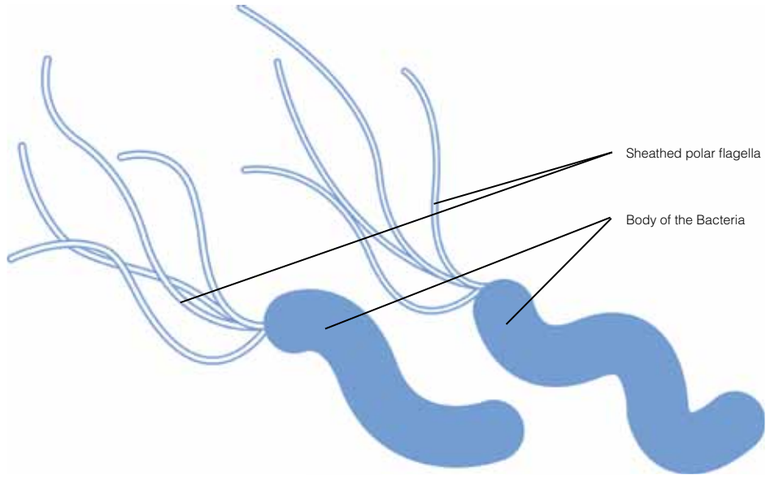


**Figure 4b.** Illustration of S-shaped *H. pylori* with four sheathed polar flagella. The majority of helicobacters possess this basic morphology of an S-shape with polar, sheathed flagella, though variations in size and the number of spirals are seen in a number of other species. These bacteria are usually around 0.5 × 5 µm, and the S-shaped morphology has been correlated with maximum in vitro motility. Thin sections of *H. pylori* revealed through an electron microscope show an outer and inner membrane separated by the periplasm of approximately 30 nm thickness (see EM picture above). The dense cytoplasm contains nucleoid material and ribosomes (Source: Jani O'Rourke and Günter Bode. Morphology and Ultrastructure of *Helicobacter pylori*. Physiology and Genetics. Eds. Harry L. T. Mobley, George L. Mendz, and Stuart L. Hazell. ASM Press. 2001). Illustration by Rashmil Saxena, BFA, HT(ASCP)$^{CM}$.

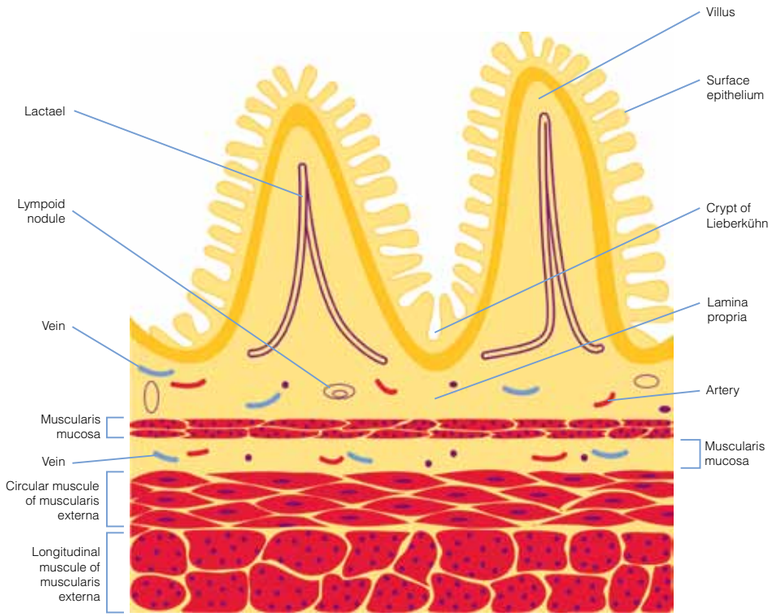


**Figure 5a.** Schematic diagram of intestinal wall. Illustration by Rashmil Saxena, BFA, HT(ASCP)$^{CM}$.

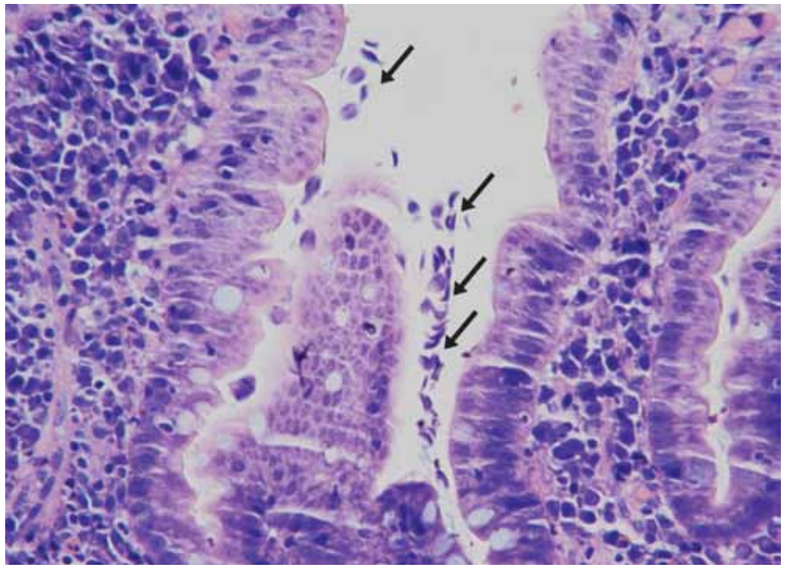

**Figure 5b.** Giemsa-stained section of small intestinal mucosa showing clusters of Giardia that stain purple (arrows) in the crypts. The background is stained faint pink by eosin. (Courtesy of Rashmil Saxena, BFA, HT(ASCP)$^{CM}$, FRCPath, Indiana University School of Medicine, Indianapolis, IN, USA).





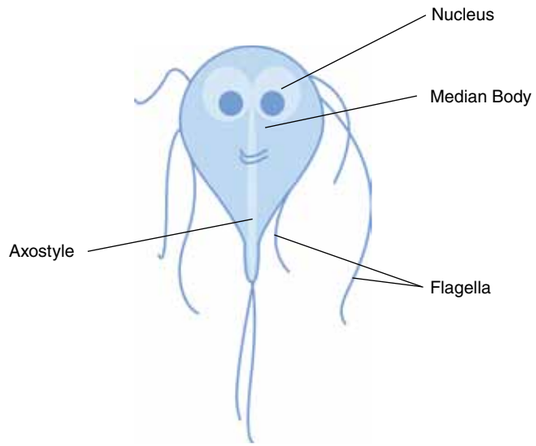


**Figure 5c.** Giardia intestinalis trophozoite: After ingestion of contaminated food or water within the small intestine, the trophozoites reproduce asexually and either float free or are attached to the mucosa of the lumen. Some trophozoites then encyst in the small intestine. Encystation occurs most likely as a result of exposure to bile salts and fatty acids, and a more alkaline environment. Both cysts and trophozoites are then passed in the feces, and are infectious immediately or shortly afterward (Legend courtesy: Centers for Disease Control (CDC, Atlanta, GA, USA). Illustration by Rashmil Saxena, BFA, HT(ASCP)$^{CM}$.

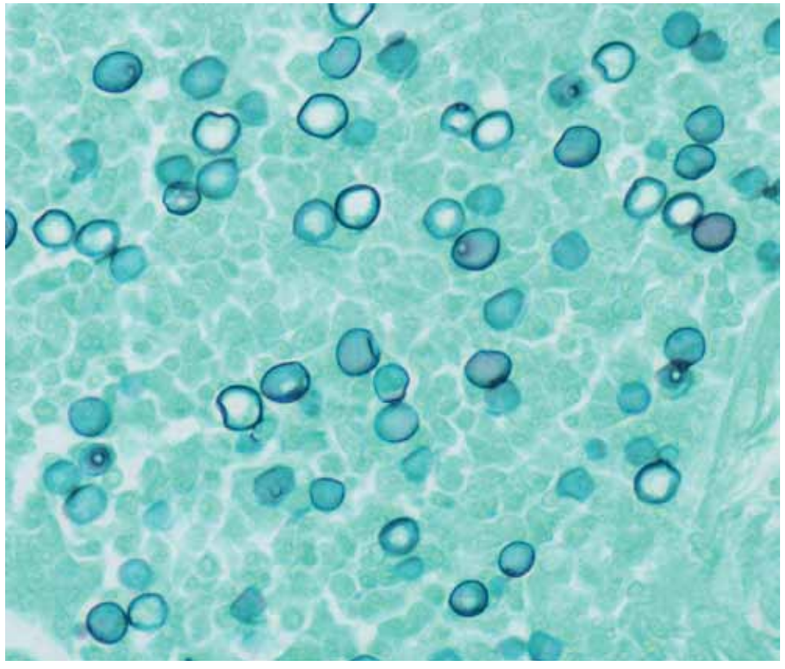

**Figure 6.** Biopsy stained with GMS, Dako Code AR176. The Grocott's Methenamine Silver method is utilized for the visualization of fungi and *Pneumocystis jiroveci* in tissue sections using the Artisan™ Staining System. Fungi and *P. jiroveci* are stained black while other tissue elements are stained green. This stain can be used on both tissue and aspirates or smears.

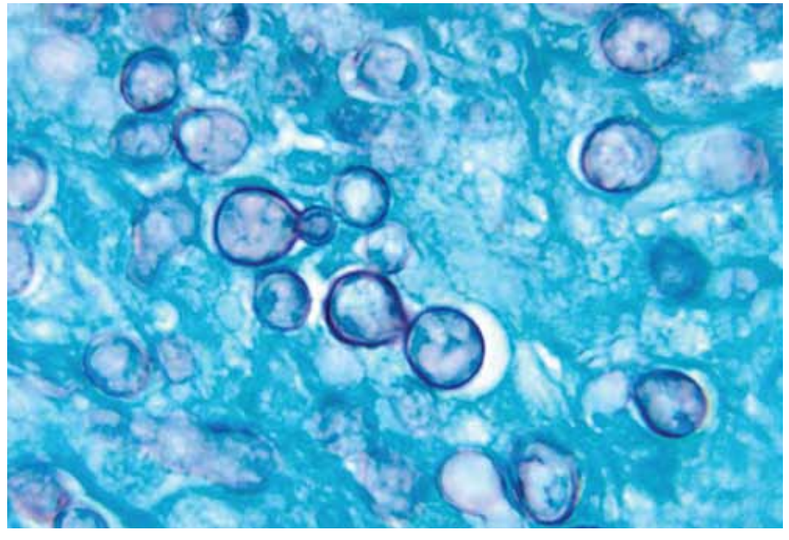

**Figure 7.** Methenamine Silver Stain. Histopathologic changes seen in histoplasmosis due to *Histoplasma capsulatum* var. *duboisii.* Note the presence of typical yeast cells, some of which are undergoing replication by "budding". Courtesy of Libero Ajello, PhD, The Centers for Disease Control and Prevention, Atlanta, GA, USA/Wikimedia.

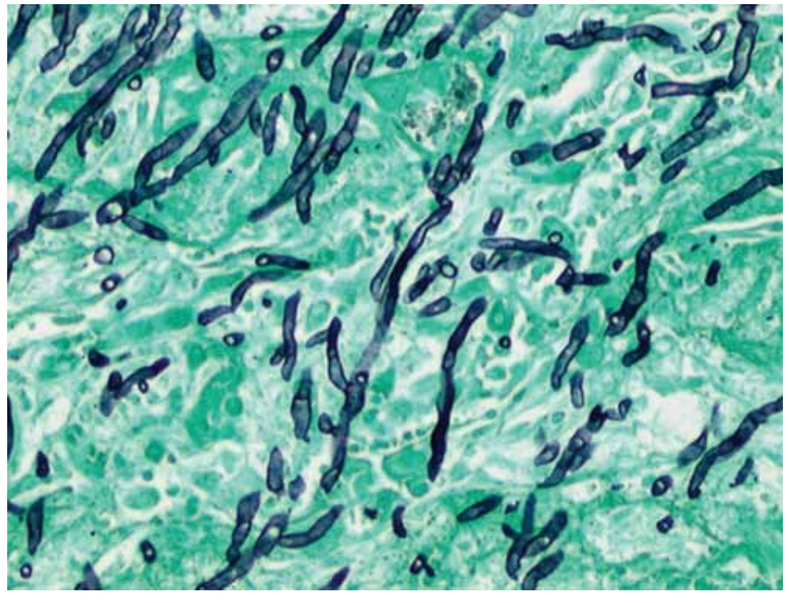

**Figure 8.** Grocott's Methenamine Silver (GMS) staining of fungi.





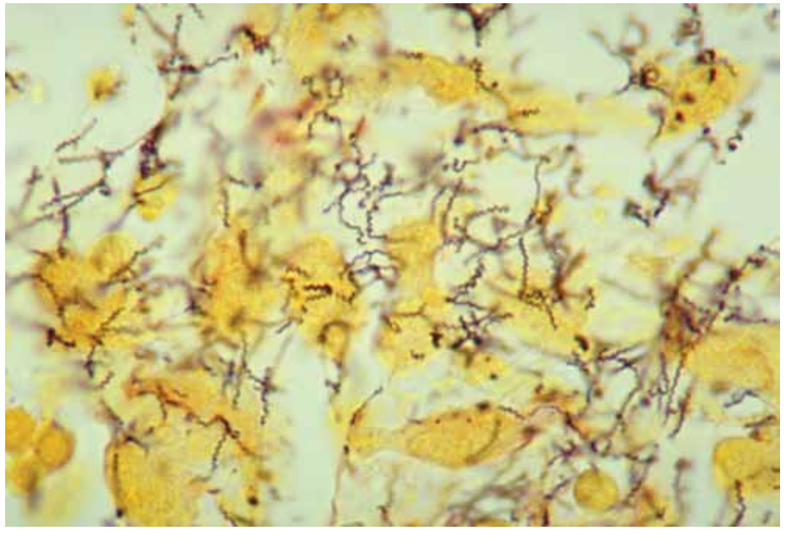

**Figure 9.** Histopathology of *Treponema pallidum* spirochetes using a modified Steiner Silver Stain. Image credit: Dr. Edwin P. Ewing, Jr., The Centers for Disease Control and Prevention, Atlanta, GA, USA/Wikimedia.

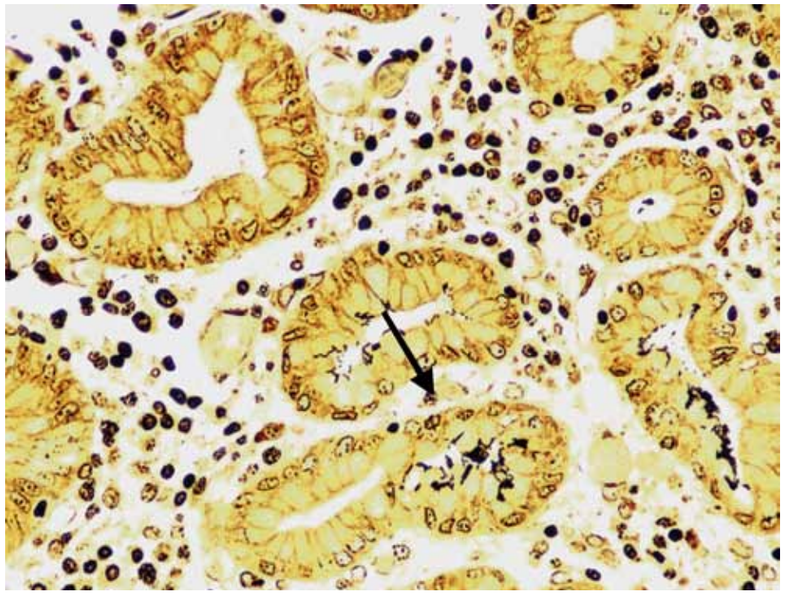

**Figure 10.** Helicobacter stained with Warthin-Starry, Dako Code AR181. The arrow points to some black *H. pylori* organisms in yellow mucus.

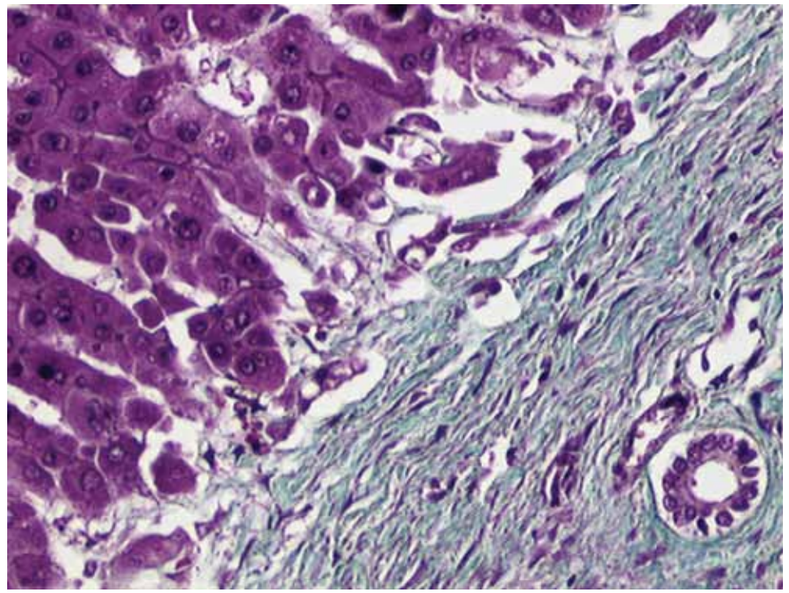

**Figure 11.** Liver section stained with a modification of Gomori's One-Step Trichrome method that colors collagen green rather than blue, Dako Code AR166.

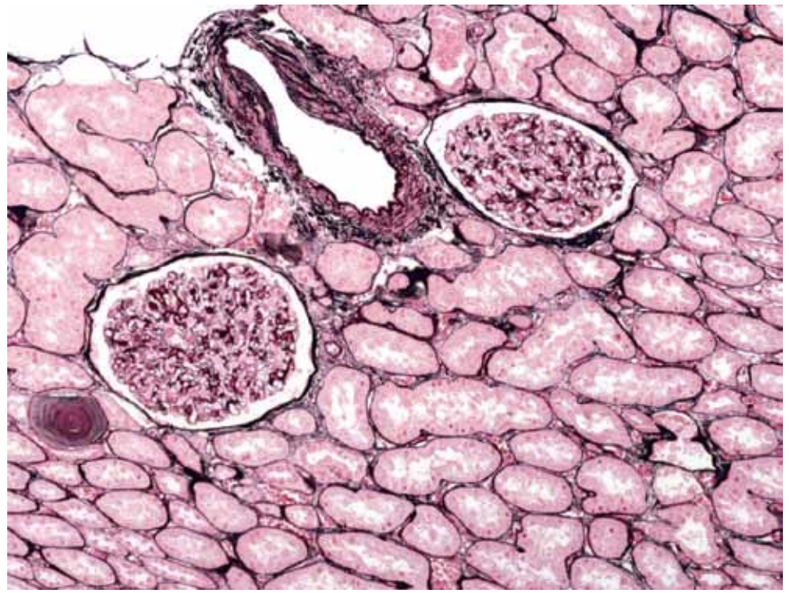

**Figure 12.** Kidney stained with Jones' Basement Membrane, Dako Code AR180. The Jones' Basement Membrane stain is used for visualization of basement membranes, specifically glomerular and tubular basement membranes in renal tissue. The Bowman's capsule is stained black, inner basement membrane - black to gray, nuclei - red, collagen - rose, and cytoplasm and other tissue are stained pink. This stain has been optimized for use on 2 µm thick tissue sections.





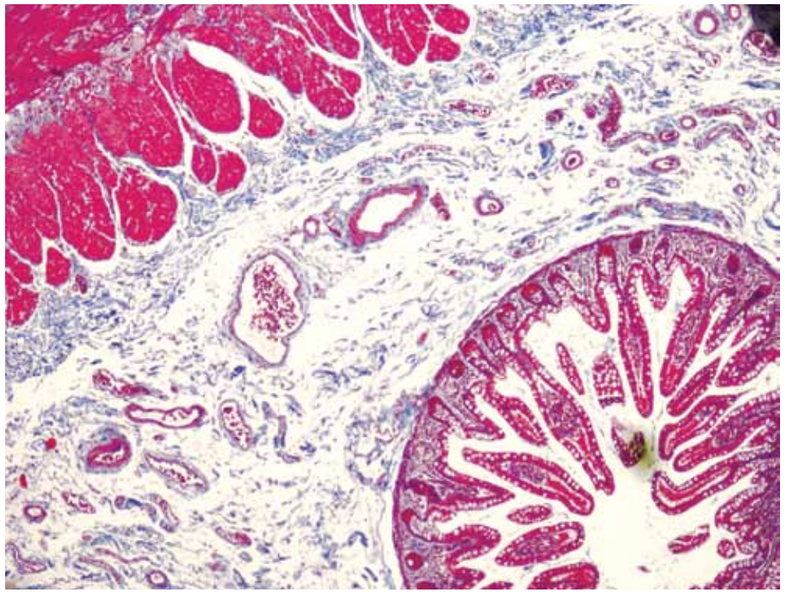

**Figure 13.** Biopsy stained with Masson's Trichrome, Dako Code AR173. This stain is used to distinguish collagen from muscle in tissue specimens using the Artisan™ Staining System. The Trichrome stain is often used to differentiate between collagen and smooth muscle and to identify an increase in collagenous tissue. With the Masson's Trichrome stain, muscle is stained red, collagen - blue, fibrin - pink, erythrocyte - red and nuclei - blue/black.

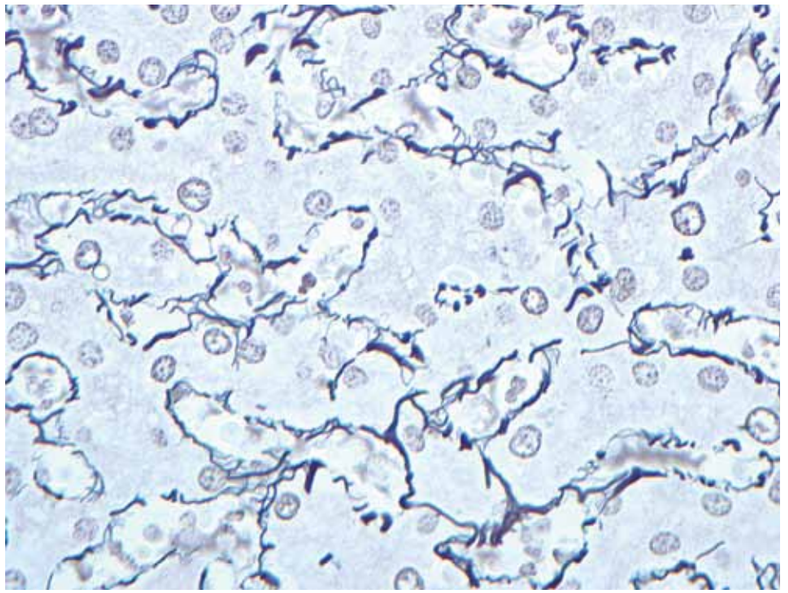

**Figure 14.** Liver stained with Reticulin/No Counterstain, Dako Code AR182. The Reticulin/No Counterstain stain is used for the visualization of reticulin fibers in tissue sections using the Artisan™ Staining System.

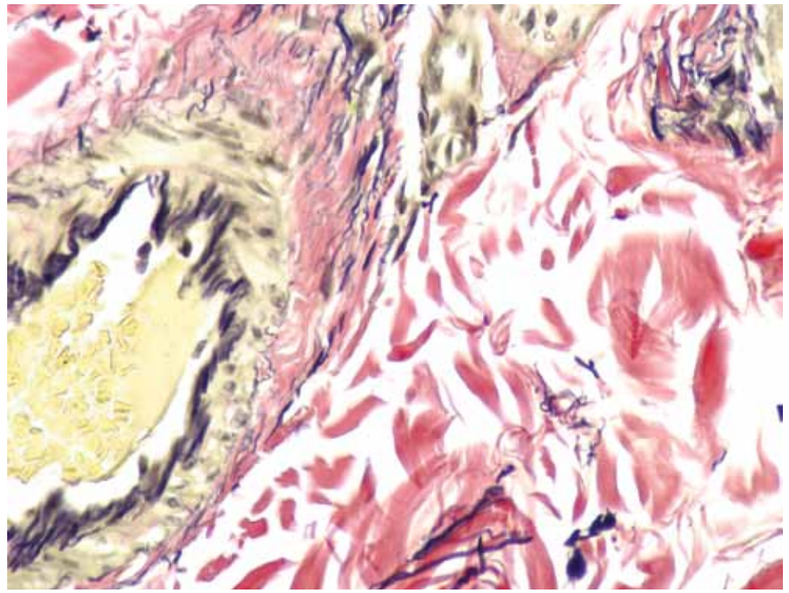

**Figure 15a.** Skin stained with Elastic stain, Dako Code AR163. In this section Verhoeff's hematoxylin method has been counterstained with Van Gieson's picro-fuchsine. The Elastic stain is based on Verhoeff's technique optimized for the Artisan™ Staining System. Elastin fibers and elastic lamina in histological specimens are stained black, while remaining tissue elements are stained as follows: nuclei - blue/black, collagen - red, other tissue elements - yellow.

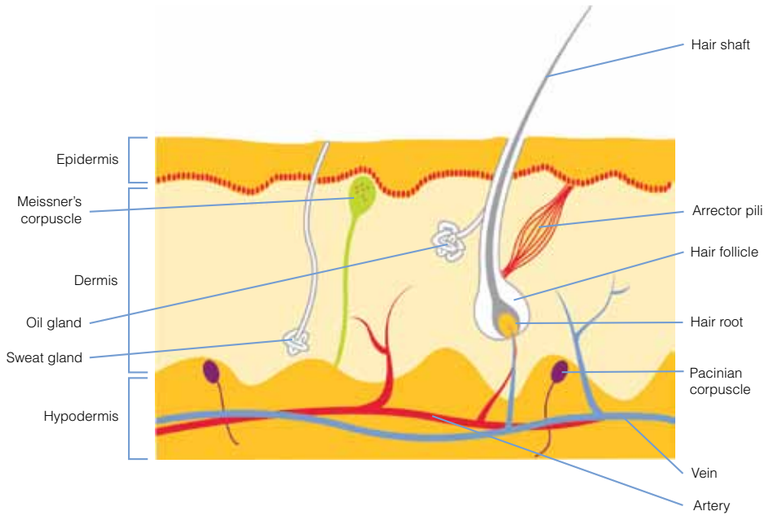


**Figure 15b.** Schematic diagram of skin: Cross-section. Dermis contains collagen and elastin which give the skin its form, shape and elasticity. Illustration by Rashmil Saxena, BFA, HT(ASCP)$^{CM}$.





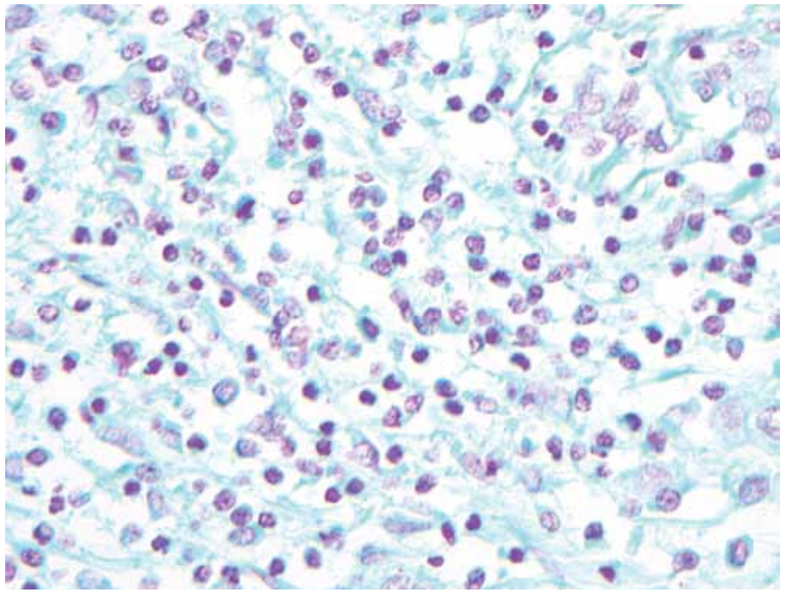

**Figure 16.** Breast tissue stained with Feulgen, Dako Code AR174. The Feulgen stain is used to demonstrate DNA in tissue sections. RNA is not stained by this procedure. The DNA is stained magenta with Schiff's reagent. The stained DNA is contrasted against a light green counterstain to allow better visualization by light microscopy or image analysis.

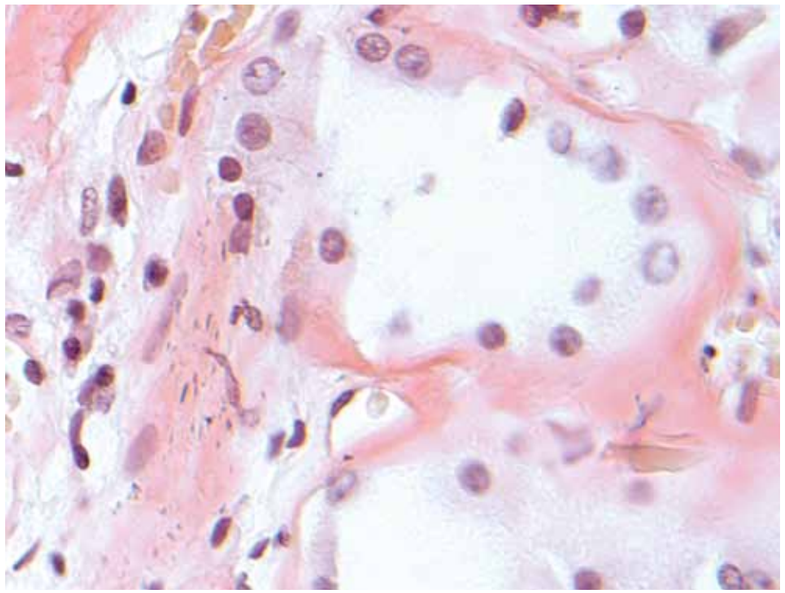

**Figure 17.** Amyloid stained with Congo Red, Dako Code AR161. The Congo Red stain is used to detect amyloid, an abnormal protein product that can be found in various pathologic conditions. This stain is based on Benhold's and demonstrates amyloid in pink to dark salmon with light microscopy or the characteristic "apple-green birefringence'' with polarized light. Mayer's hematoxylin is used as a counterstain. The preferred method for visualization of amyloid is under polarized light.

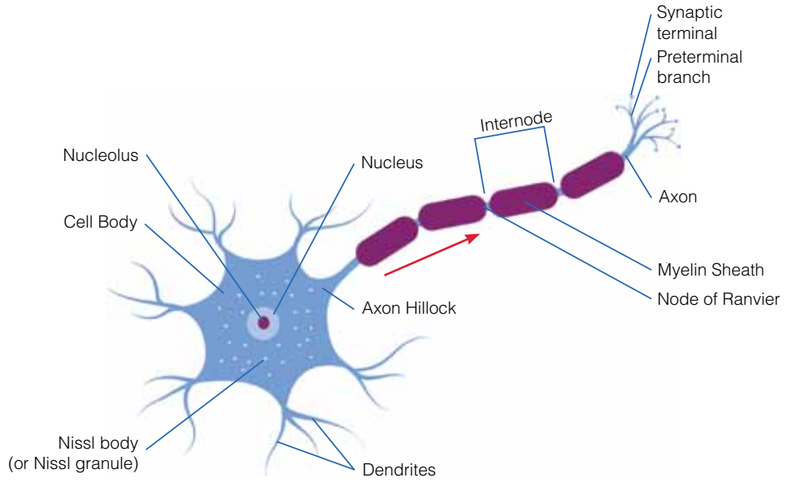


**Figure 18.** Schematic diagram of a generalized neuron with a myelinated axon. The arrow indicates the direction in which signals are conveyed. Axons conduct signals away from the cell body, while dendrites receive signals from the axons of other neurons. Around the cell body are dendrites that receive signals from other neurons. The end of the axon has branching synaptic terminals that release neurotransmitters into a gap called the synaptic cleft (not shown) between the terminals and the dendrites of the next neuron.

The axons of vertebrate neurons are insulated by a myelin sheath which greatly increases the rate at which axons can conduct a nerve impulse. The myelin sheath is interrupted at regularly spaced "Nodes of Ranvier" where Na + channels in an axon are concentrated. A myelin sheath is a many-layered coating, largely composed of a fatty substance called myelin that wraps around the axon and very efficiently insulates it. Nissl bodies or granules are clumps of free ribosomes attached to portions of rough endoplasmic reticulum. These are sites for protein synthesis. Illustration by Rashmil Saxena, BFA, HT(ASCP)$^{CM}$.





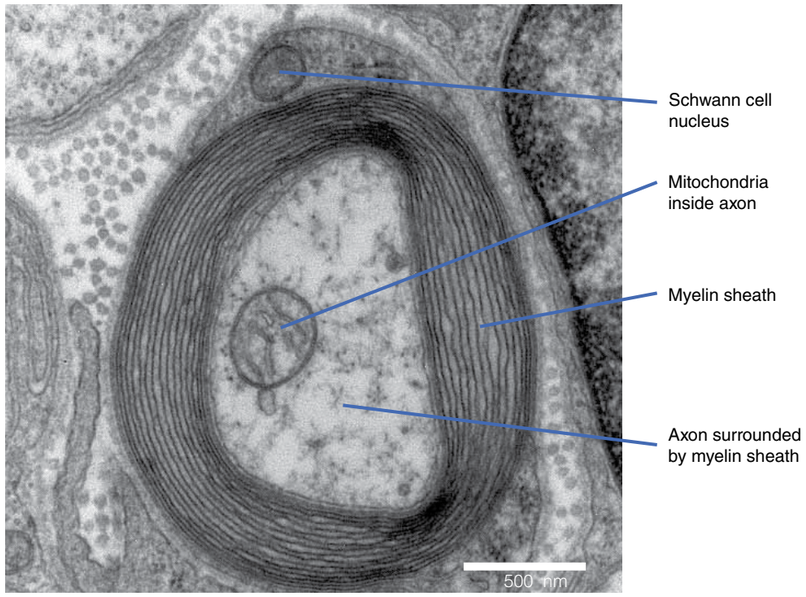


**Figure 19.** Transmission electron micrograph of a myelinated axon. Each Schwann cell wraps its plasma membrane concentrically around the axon to form a segment of myelin sheath. Generated at the Electron Microscopy Facility at Trinity College, Hartford, CT. (Courtesy of Wikipedia).

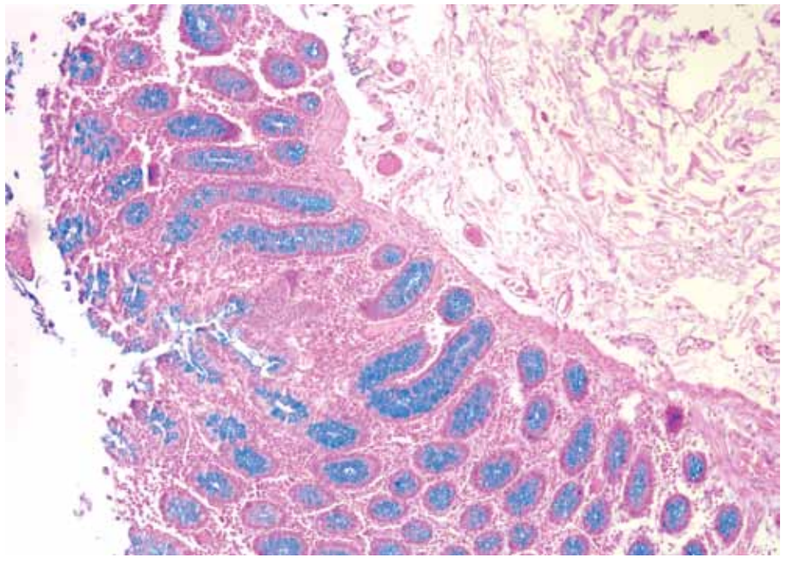

**Figure 20.** Small intestine stained with Alcian Blue pH 2.5, Dako Code AR160. Alcian Blue pH 2.5 stains weakly sulphated mucins, acidic mucopolysaccharides, sulphomucins, hyaluronic acid and sialomucins at pH 2.5, blue in color. All nuclei are stained red, and all other tissue elements are stained pink to red.

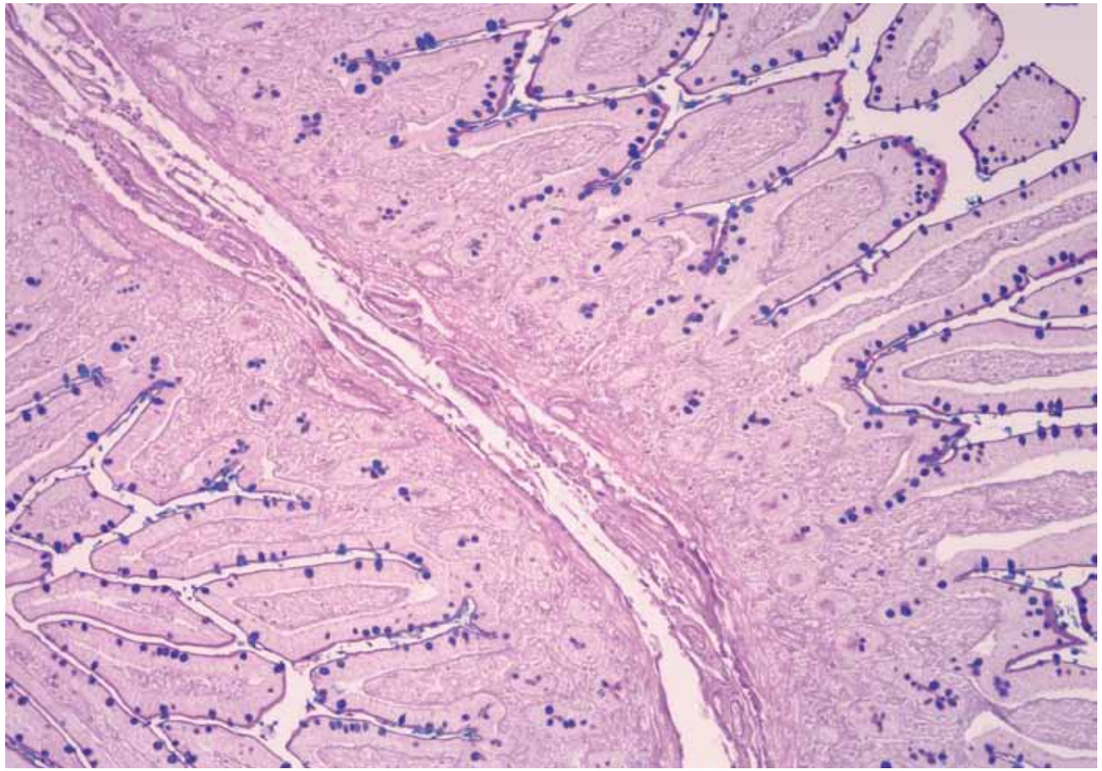

**Figure 21.** Small intestine stained with Alcian Blue/PAS, Dako Code AR169. This stain is used for the demonstration of neutral and acidic mucosubstances on the Artisan™ Staining System. Alcian Blue pH 2.5 imparts a blue color to the acidic mucins and other carboxylated or weakly sulphated acid mucosubstances. The periodic acid-Schiff (PAS) reaction is then used to stain basement membranes, glycogen and neutral mucosubstances pink to red. Mixtures of neutral and acidic mucosubstances will appear purple due to positive reactions with both Alcian Blue and PAS.





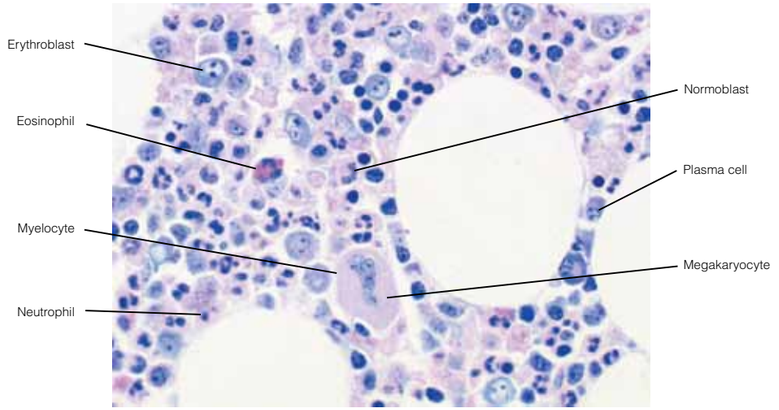


**Figure 22a.** Cell types seen in normal bone marrow. Giemsa staining. (Figure from Dako Education Guide, "The Illustrated Guide to Bone Marrow Diagnosis," 2nd Edition (2009). Editors: Carlos Martin, MD, and George L. Kumar, PhD).

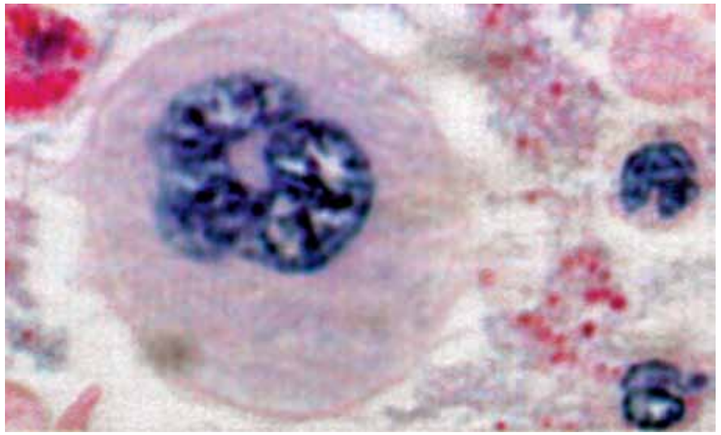

**Figure 22b.** Giemsa staining. Atypical mononuclear megakaryocyte in chronic myeloid leukemia. (Figure from Dako Education Guide, "The Illustrated Guide to Bone Marrow Diagnosis," 2nd Edition (2009). Editors: Carlos Martin, MD, and George L. Kumar, PhD).

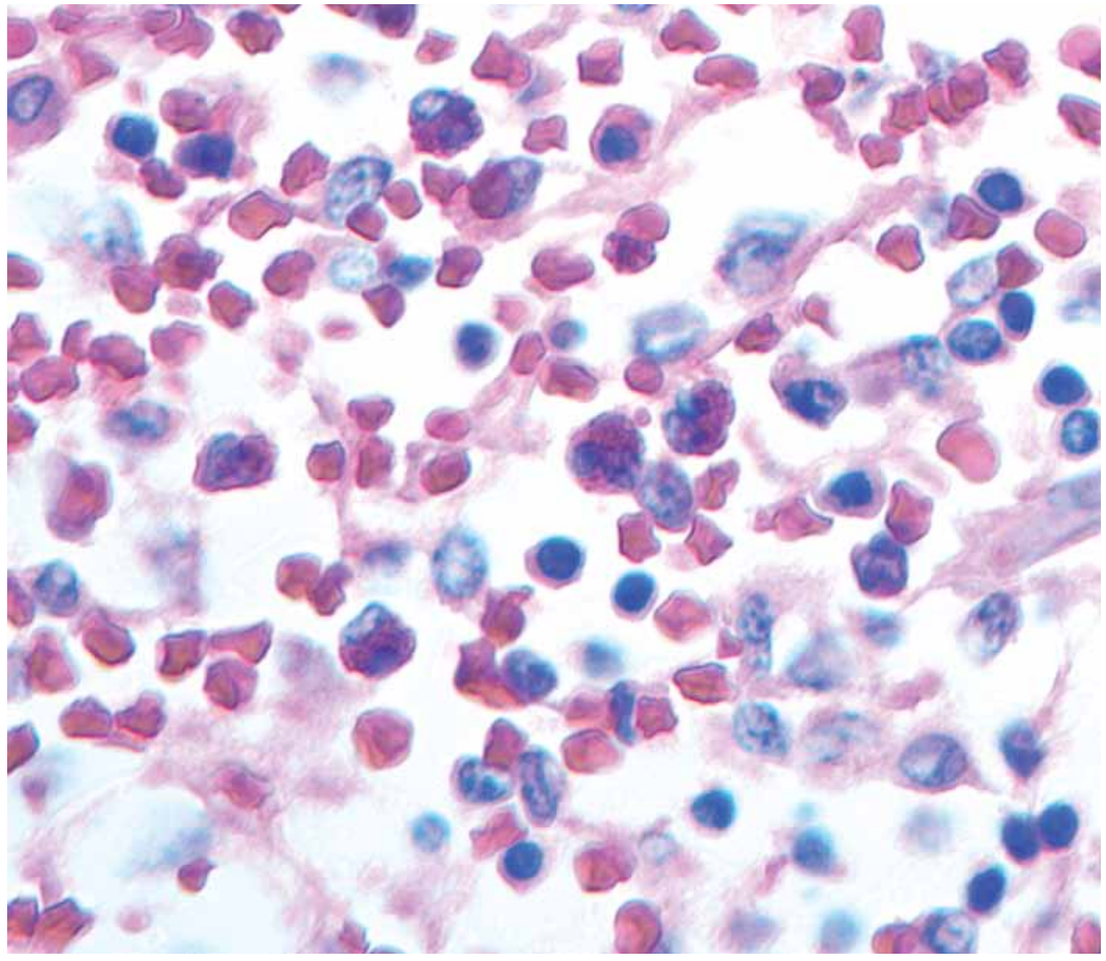

**Figure 23.** Spleen stained with Giemsa, Dako Code AR164. Cell types are stained as follows: mast-cell granules and basophils - purple, eosinophils - bright pink, lymphocytes - blue.





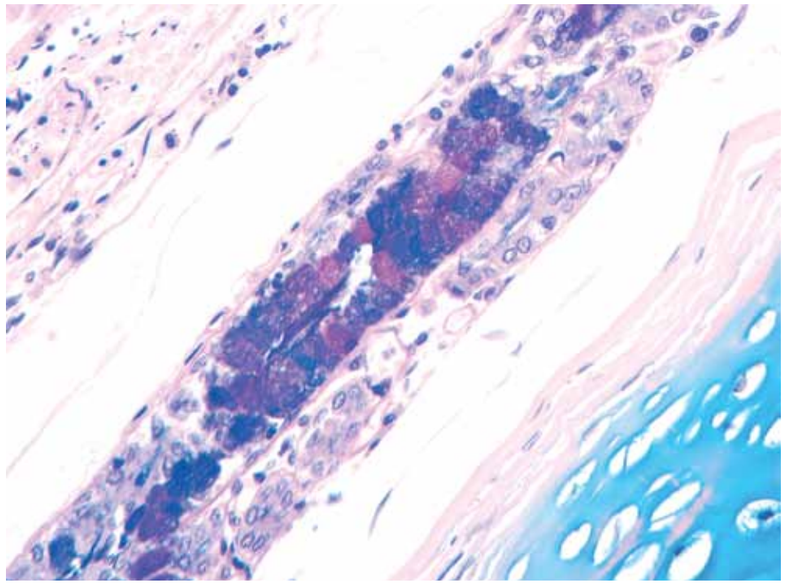

**Figure 24.** Trachea stained with Alcian Blue/PAS/Hematoxylin, Dako Code AR178. This stain is used for the demonstration of neutral and acidic mucosubstances on the Artisan™ Staining System. Alcian Blue pH 2.5 imparts a blue color to the acidic mucins and other carboxylated or weakly sulfated acid mucosubstances. The periodic acid-Schiff (PAS) reaction is then used to stain basement membranes, glycogen and neutral mucosubstances pink to red. Mixtures of neutral and acidic mucosubstances will appear purple due to positive reactions with both Alcian Blue and PAS. A hematoxylin counterstain is then applied to impart a blue/black color to the nuclei.

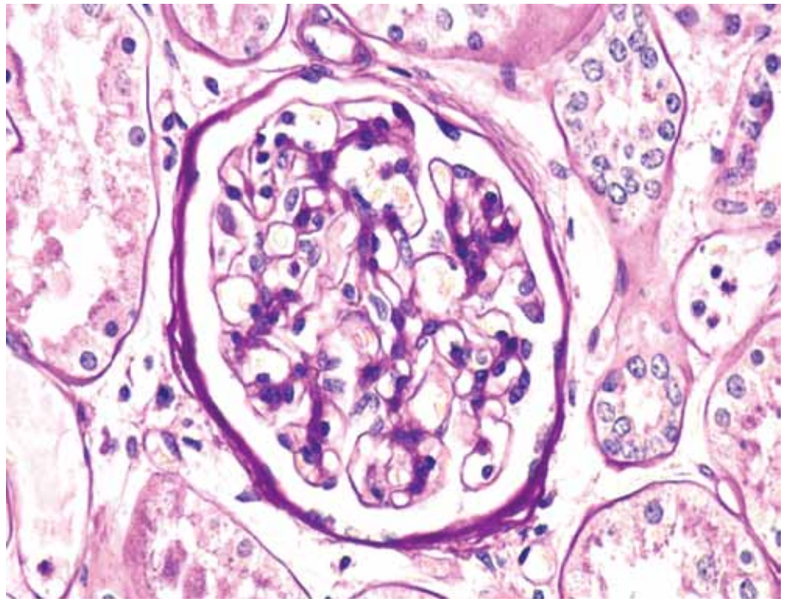

**Figure 25.** Kidney stained with PAS, Dako Code AR165.

## Conclusion

Special stains belong to an assorted family of stains for microscopic visualization and general identification of cells, tissues and micro-organisms. Special stains remain an important tool for many pathologists and technologists providing a powerful complement to immunohistochemistry, flow cytometry, in situ hybridization and other diagnostic technologies that define a patient's medical profile. With the medical community demanding greater standardization and quality control, special stain protocols have become increasingly automated resulting in higher levels of productivity and flexibility. Automation is no substitute for a solid understanding of the principles and practices of a quality staining. We anticipate that this technology will continue to evolve in the foreseeable future and expect it to form an integral part of pathologic diagnosis. In a nutshell, this introduction was intended to provide guidance to help interested readers acquire proficiency in selecting and performing special stains faster than they might have otherwise done.

## Appendix
## Biological Stain Commission

The US-based Biological Stain Commission was an indirect consequence of World War I. During the Great War there was a blockade of German products, including dyes. By 1920, the supply of pre-war dyes was almost exhausted, foreign supplies were erratic, and the domestic dyes were still often unsatisfactory. As a consequence, several concerned groups and individuals came together, which resulted in two key conferences in 1921 on the standardization of stains. From this activity, the Commission on the Standardization of Biological Stains originated. By 1923, the Commission already had a constitution that is recognizably the forerunner of the aims of the present Commission. In parallel with this, co-founder Dr. Harold J. Conn, while Chairman of the Commission, published the first edition of Biological Stains in 1925. This book has become a standard source of reference in technical and research histopathological and biological laboratories using dyes. The book has been revised regularly with a 10th edition (2002) as the most recent version. In 1944, the Commission on the Standardization of Biological Stains became the Biological Stain Commission.

The objectives of the Biological Stain Commission are: 1) to ensure an uninterrupted supply of dyes used in biological and medical applications, 2) to promote cooperation and dialogue among manufacturers, vendors and users of dyes for histochemical applications, 3) to ensure the quality of dyes through independent testing according to appropriately rigorous chemical and performance criteria, 4) to educate users of biological stains about sources of reliable dyes and how they might best be used, and 5) to publish information concerning new or improved uses for biological dyes and related histochemical techniques.

These objectives are met by way of: 1) analyzing dye content and composition of samples supplied voluntarily by dye manufacturers or vendors, 2) testing the performance of dye samples in rigorous, standardized procedures known to be discerning tests of the staining quality of the dye, 3) issuing certification labels to be attached to the containers used by companies marketing accepted dyes to assure consumers that these dyes have met the performance criteria of the Biological Stain Commission, 4) conducting and supporting research on biological dyes and histochemical techniques dependent on dyes, 5) Publishing books concerning biological dyes and histochemical techniques, and publishing Biotechnic & Histochemistry, a bimonthly journal of microtechnique and histochemistry, and 6) maintaining an active dialogue among scientists, manufacturers and vendors concerned with biological stains.

Interested readers can learn much more about the Biological Stain Commission at its Web site: http://www.biologicalstaincommission.org/.

## Anatomic Pathology Checklist by College of American Pathologists

Several thousand US anatomic pathology laboratories are inspected by the College of American Pathologists for accreditation purposes required by the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88). They can expect to be asked these two questions about special stains (see text in bold, page 27):





### SPECIAL STAINS (HISTOCHEMISTRY)

*The inspector must examine and evaluate sample case(s) of all special stains (with controls) routinely prepared by the histology laboratory. The histochemical stains listed below are neither compulsory for every laboratory nor all inclusive, but simply intended to represent some common stains.*

**ANP.21400** **Phase II** **N/A YES NO**

**Are positive controls run routinely on all special stains, with reactivity results documented, and are they verified for acceptability before reporting results?**

*NOTE: A positive control slide must be run at the same time as any single or group of slides stained with the same special stain. The tissue chosen for the special stain control slide must be appropriate in type and amount. Both the control slide and the test tissue slide must be judged technically acceptable before the results of the special stains are reported.*

REFERENCES
1) Sundblad AS. A simplified multitissue block. *Am J Clin Pathol.* 1994;102:192-193

**ANP.21450** **Phase II** **N/A YES NO**

**Are the following special stains of high quality, and do they satisfactorily demonstrate (on each day of use), the tissue characteristics for which they were designed?**

1. **Acid fast organisms**
2. **Iron**
3. **Bacteria**
4. **Elastic tissues**
5. **Fungi or pneumocystis**
6. **Mucin**
7. **Connective tissue**
8. **Myelin**
9. **Nerve fibers**
10. **Periodic acid Schiff (PAS)**
11. **Glycogen**
12. **Reticulin fibers**
13. **Amyloid**
14. **Methyl green-pyronine (MGP)**

*NOTE: This list is neither all-inclusive nor exclusive of other "special stains" used in a given histopathology laboratory. For Gram stains, control slides must demonstrate both Gram-positive and Gram-negative organisms. With the mucicarmine method, the stain must be sufficiently carminophilic. If the myelin stain is luxol fast blue, the tissue must be properly differentiated, i.e., myelin must be turquoise and clearly distinguishable from unmyelinated structures. For nerve fiber stains, axons must be distinct and black with a Bodian or related stain. With MGP, the control slide must demonstrate sufficient pyroninophilia.*

*The Inspector must provide specific details of which stains (this list or others) are deficient in Part B (Deficiency Summary) of the Inspector's Summation Report.*

REFERENCES
1) Department of Health and Human Services, Centers for Medicare and Medicaid Services. Clinical laboratory improvement amendments of 1988; final rule. *Fed Register.* 2003(Jan 24):7166 [42CFR493.1256(e)(2)]
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# Chapter 2 | General Oversight Stains for Histology and Histopathology

**John A. Kiernan, MB, ChB, PhD, DSc**

For normal or diseased tissues of humans and other vertebrate animals, a routinely used staining method for use on paraffin or frozen (cryostat) sections 4-7 µm thick is expected to provide intense blue, purple or black coloration that is largely confined to chromatin in the nuclei of cells, together with a contrasting and paler color such as pink or yellow in the surrounding cytoplasm and in extracellular structures (notably collagen fibers).

## Haemalum and Eosin

For about 130 years the oversight nuclear stain of first choice has been **haemalum**, which may be one of many solutions containing hematein, aluminum ions and usually other ingredients. Haemalum stains nuclear chromatin blue. For the second color, called the **counterstain**, red dyes are generally preferred, especially **eosin**, which can provide a range of orange hues. Eosin also changes the color of haemalum-stained nuclear chromatin from blue towards purple. Sections stained with haemalum and eosin are the first ones to be examined by human and veterinary pathologists examining surgical or post mortem specimens, and often are sufficient for diagnosis. Other staining methods are used as required, especially to show cytoplasmic and extracellular components that do not stain distinctly with either haemalum or eosin.

### Haemalum

The combination of haemalum and eosin is called **H & E**. This method is also called **hematoxylin and eosin** because haemalum solutions are made by dissolving hematoxylin in water or alcohol, oxidizing some of it to hematein, and adding an aluminum salt. Hematoxylin (colorless when pure) and hematein (yellow in acid, red-violet in neutral and alkaline solutions, changing at pH 5– 6) probably are not directly involved in the staining of nuclei by haemalum.

There are many formulations of haemalum. Almost all contain an excess of aluminum ions over haematein molecules and also contain an organic acid such as acetic or citric; the pH is usually in the range 2.0–3.5. Other substances in haemalum solutions may include an oxidizing agent, often sodium iodate, to accelerate hematein generation, and an organic liquid such as glycerol or ethylene glycol, which does not affect staining properties but may extend the shelf-life by retarding evaporation and precipitation of insoluble materials.

Mixtures with a high aluminum:hematein ratio stain sections slowly, with selective coloration of nuclear chromatin typically being achieved in 5 to 15 minutes. This is called **progressive staining**. Solutions with lower aluminum:hematein ratios rapidly color all components of the tissue. Selective coloration of nuclei is then achieved by **differentiation** (also called **destaining**) in a dilute mineral acid such as 0.1 M HCl (pH 1.0). This removes dye-metal complexes from the tissue, decomposes the complexes into hematein and aluminum ions, and accelerates the further oxidation of hematein to other compounds. Aluminum-hematein complexes attached to nuclear chromatin are more resistant to differentiation than those attached to other substances. In practice, differentiation is usually done with **acid-alcohol**, which is 70% or 95% ethanol with 1% v/v concentrated HCl. Selective nuclear staining achieved by differentiation is called **regressive staining**.

The chemical compositions of solutions containing haematein and aluminum ions have been investigated by electrophoresis and by spectrophotometry. These studies show the existence of cationic hematein-aluminum (HmAl) complexes, principally the red $[HmAl]^{2+}$ at pH 2.6, which is the acidity of practical staining solutions. A soluble blue complex, $[HmAl_2]^{3+}$, exists at pH 4.7 and is changed at higher pH to insoluble blue, presumably polymeric, material that can be re-dissolved by acidification. The equilibria are summarized in Figure 1. Anionic complexes, including $[Hm_2Al]^-$, have been shown to be involved in textile dyeing by complexes of hematein with various metal ions, and their involvement in nuclear staining by haemalum has been postulated.

In tissues stained by haemalum, progressively or regressively, cell nuclei are dull brownish-red. The color is changed to blue (Figure 2) by rinsing in tap water (if its pH is above 5) or distilled water that has been made slightly alkaline, as with a few drops of ammonium hydroxide. This "blueing" converts the red Hm-Al complex ions to blue polymers that are insoluble in water and organic solvents and also are remarkably resistant to fading. The blue product resists extraction by weakly acidic counterstains, such as eosin in 0.5% acetic acid, but it is removed by solutions of stronger acids such as

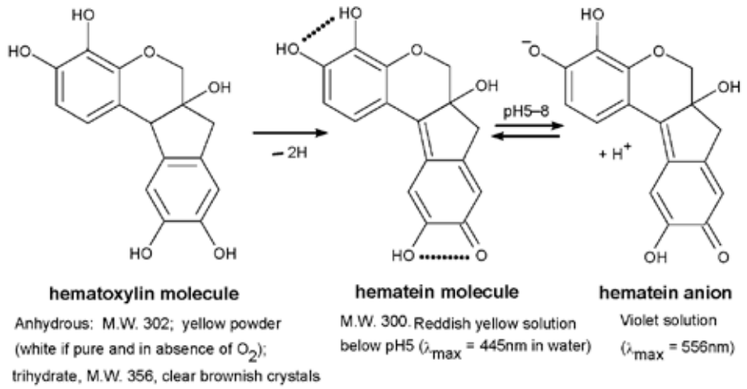
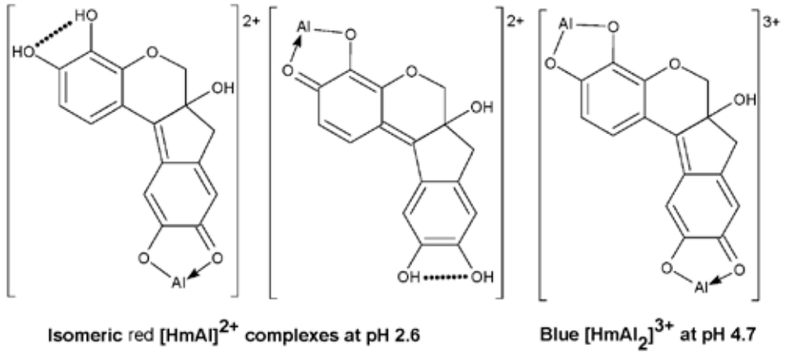



**Figure 1.** Chemical structures of hematoxylin, hematein and two hematein-aluminum (HmAl) complexes. (Bettinger and Zimmerman 1991a,b).



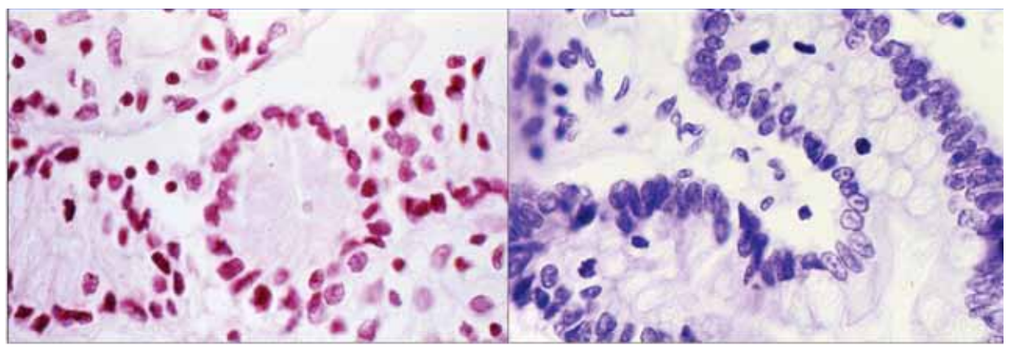



**Figure 2.** Nuclei stained with haemalum before (left) and after blueing. Reproduced with permission from Gill (2010b).





picric or phosphotungstic, used in mixtures for staining cytoplasm and collagen in different colors.

## Mechanism of Nuclear Staining by Haemalum

The mechanism traditionally put forward for attaching haemalum to cell nuclei is one involving coordinate (covalent) bonds between phosphate oxygens of DNA and aluminum atoms, and between aluminum atoms and haemalum molecules. This mechanism, often called **mordant dyeing**, is illustrated in Figure 3. In textile dyeing, the word mordant (present participle of the French *mordre*, to bite) was introduced in the late 18th Century to denote substances used to fix colorants to fabrics. Chromium is the metal most often used in industrial dyeing, because it can form stable cyclic complexes (chelates) with dyes having suitably placed coordinating groups. Tannic acid, a polyphenolic compound, has been used as a mordant for cationic dyes, with which it form insoluble salts of the form $(Dye^{+})_{n}(Tan^{n-})$.

These mechanisms are supported by experiments in which, for example, sections are treated first with a solution of an aluminum salt and then with a solution of hematein. Everything is colored. If the sections are rinsed in a dilute mineral acid after exposure to $Al^{3+}$, subsequent staining by hematein is confined to nuclei. These observations suggest that acids break the bonds between tissue and metal, and that tissue-metal bonds resist acids more strongly in nuclei than elsewhere. The phosphate groups of DNA are more strongly acidic (existing as anions at relatively lower pH) than the carboxyl groups of proteins in cytoplasm and connective tissue. The implication is $Al^{3+}$ ions from the solution are attracted to phosphate anions of DNA, with which they form coordination complexes. Each bound aluminum atom also forms a complex with two adjacent oxygens of haematein, so that the metal atom comes to be interposed between the DNA and the dye. More recent interpretations have the dye-metal complex cation $[HmAl]^{2+}$ binding directly to DNA phosphate, giving the same product (Figure 3). The mechanisms may not be mutually exclusive. DNA is known from chemical investigations to bind $Al^{3+}$ with high affinity and free hematein is present in haemalum staining solutions, albeit at very low concentration.

A greatly diluted haemalum at pH 3.2 (made with $2\times10^{-4}$M hematein and $2\times10^{-3}$M $Al^{3+}$) is known from spectrophotometric studies to contain the complexes $[HmAl]^{+}$, $[HmAl_2]^{3+}$ and $[HmAl]^{2+}$. Such a solution progressively stains nuclear chromatin and cytoplasmic RNA of methanol-fixed monolayer cultures of HeLa cells in about two hours. The nuclear staining is prevented by prior treatment of preparations with DNase. RNase prevents the cytoplasmic staining, and a combination of the two nucleases prevents all staining of the cells. The same solution stains nuclei of cells in paraffin sections of formaldehyde-fixed animal tissue in about 30 minutes, and this staining is also prevented by prior enzymatic or acid extraction of nucleic acids. These observations support the notion (Figure 3) of ionic attraction of dye-metal complex cations by DNA anions, followed by formation of strong metal-to-phosphate covalent bonds. Ionic attractions probably account for the staining of mucus (anionic glycoproteins) by haemalum solutions that are less acidic than formulations giving selective nuclear staining.

Unfortunately, the preceding fairly simple account of haemalum nuclear staining is incorrect because it applies only to dilute haemalum solutions that work too slowly for practical use and are not acidic enough to be selective stains for nuclear chromatin. The routinely used haemalum solutions, with high concentrations of all ingredients, require only a few minutes to stain nuclei. They are too darkly colored for spectrophotometry, and may therefore differ in composition from dilute solutions. In practe, haemalums pH is in the range 2.0 to 2.8. Low pH favors selective nuclear staining. At pH 2.5-2.8 there is also light coloration of mucus. The molar ratio of aluminum ions to the total hematein content ([Al]:[Hm]) also affects staining. For example, solutions with [Al]:[Hm]=16 are progressive nuclear stains, whereas solutions with the same concentration of hematein but only half the concentration of aluminum, [Al]:[Hm]=8, rapidly color most parts of a tissue and must be differentiated in acid-alcohol to obtain selective nuclear staining. Microspectrophotometry shows no correlation between the quantities of blued haemalum in stained nuclei and their DNA content determined with the Feulgen reaction, which is specific for DNA. Nucleic acids can be removed from sections of tissue, either by acid hydrolysis or by the action of the enzymes RNase and DNase. Extraction of nucleic acids prevents the staining of nuclei by the Feulgen reaction and by cationic dyes such as toluidine blue. Extraction of nucleic acids causes only slight weakening of nuclear staining by haemalum solutions of the routinely used type. Similar observations have been made with other pre-formed cationic dye-metal complexes that are used as progressive and regressive nuclear stains. It is evident that the attachment of haemalum and

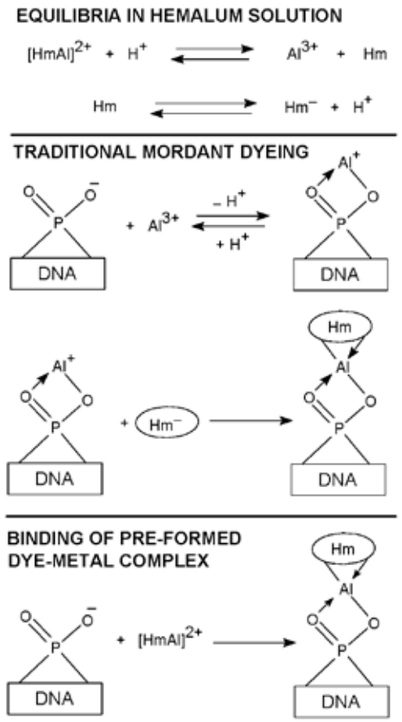



**Figure 3.** Postulated Equilibria in the mordant dyeing mechanism of progressive nuclear staining by haemalum. The staining solution is assumed to contain haematein (Hm), $[HmAl]^{2+}$ complexes and an excess of free $Al^{3+}$ ions. Based on the work of Baker (1962) and Bettinger and Zimmermann (1991b).

some other dye-metal complexes to chromatin does not require the presence of DNA. Among the few non-nuclear structures stainable with haemalum, the keratohyalin granules of the epidermis do not contain DNA; they are, however, stained by histochemical methods for arginine-rich proteins.

In the absence of DNA the most abundant materials in nuclear chromatin are histones and some other strongly basic proteins that contain much lysine and arginine. The guanidino side-chain of arginine, which accounts for some 15% of the amino acids in histones, has been proposed as a substrate for haemalum staining. Chemical modification (cyclization) of guanidino groups, by reaction with benzil or diacetyl, prevents nuclear staining by some dye-metal complexes, but not by those of hematein with aluminum or iron. Nuclear staining by haemalum is not prevented by chemical removal of amino groups (using nitrous acid) or by esterification of carboxy groups with methanol-HCl or methyl iodide. In the absence of any identifiable chemical substrate for haemalum, it must be assumed that ions or molecules of a dye-metal complex fit closely to the surfaces of histones and similar nucleoprotein molecules, and that they are held in place by short-range attractions such as van der Waals forces and possibly also by hydrogen bonds involving the phenolic hydroxy groups of hematein.

### Eosin Counterstaining

The dye most often used as a counterstain to haemalum is eosin Y, which is a tetrabromofluorescein (Figure 4). In eosin B, used in some blood stains, two of the bromines are replaced by nitro groups. The Y and B are for the yellowish and bluish shades of these red anionic dyes. Erythrosins and phloxins are related xanthene dyes with iodine and chlorine substituents; phloxin B is sometimes added





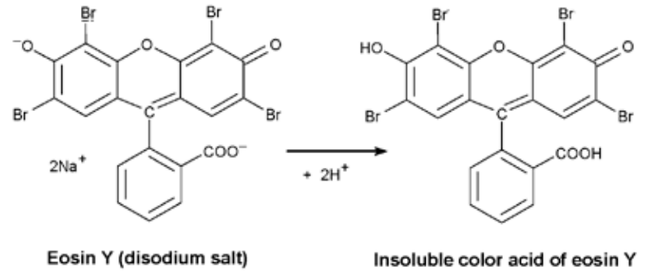



**Figure 4.** Structure of eosin Y and the effect of acidification.

to eosin Y solutions to increase the range of colours introduced by counterstaining. Eosin is usually applied from a slightly acidified solution to ensure protonation of amino groups of proteins, which then attract the negatively charged dye ions. Excessive acidification, however, causes precipitation of the color acid (Figure 4). Some workers, especially in the USA, prefer to counterstain with a solution of eosin in 70% alcohol.

Eosin is attracted to tissue proteins by ionic forces, and then held in place by van der Waals forces. In sites where the bound eosin molecules are close together, notably red blood cells, and the cytoplasmic granules of eosinophil leukocytes and Paneth cells, the color is shifted from red towards orange. Cytoplasm is red or dark pink. Collagen fibers, which contain relatively less protein and more water than cytoplasm, are lighter pink. Eosin should impart at least three colors to a correctly stained section.

Counterstaining with eosin changes the color of haemalum-stained nuclei from blue to purplish. This additive color change may be due to attraction of eosin anions to positively charged amino acid side-chains of basic nucleoproteins. If the polymeric blue Hm-Al complex bound to chromatin is cationic, this too can be expected to attract and bind eosin.

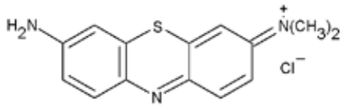
## Other Blue and Red Oversight Stains

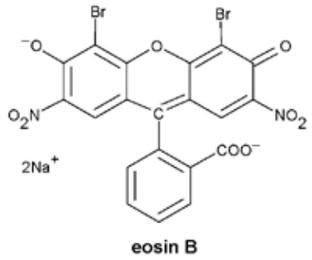
The advantages of H & E staining are the permanence of the preparations and the familiarity of the color scheme, especially among pathologists. Shortcomings of H & E include the uncertainty concerning the mechanism of the nuclear stain and the fact that many cytoplasmic structures cannot be discerned. Objects not seen with H & E include thin collagen (reticulin) fibers, mitochondria, the Golgi apparatus (even when preserved by the fixative), mast cell granules, and most of the glial and neuronal cytoplasmic processes of nervous tissue. Cytoplasm rich in rRNA (as in plasma cells and neurons) is stained blue if the pH of a haemalum approaches 3, but the organization of material within the cytoplasm cannot be clearly seen. Combinations of a blue cationic dye with an eosin form the basis of the azure-eosin techniques, which clearly show DNA and rRNA in blue, acidic glycoconjugates in purple, and proteins in red.

### Azure-eosin methods

Techniques of this type are derived from methods originally introduced for staining smears of blood or bone marrow. They are often named after Albrecht Eduard Bernhard Nocht (1857-1945) who published a method of this kind for staining malaria parasites in 1898, and after Ralph Dougall Lillie (1896-1979), who refined the composition of the staining solution to obtain, in 1944, a reliable method for routine application to paraffin sections of formaldehyde-fixed human tissues. Ordinarily, the two oppositely charged dyes in the staining mixture (Figure 5) would combine to form a water-insoluble “azure eosinate.” Precipitation is prevented by the inclusion of acetone. The color balance is greatly influenced by the fixative and is determined the pH of the staining solution. After formaldehyde fixation the optimal pH is 4.0.

The azure-eosin staining solution contains blue cations (attracted to nucleic acids and acid glycoconjugates, including cartilage matrix, mast cell granules and most types of mucus), and red anions (attracted to proteins). DNA and rRNA are stained blue, but structures




**LILLIE'S AZURE-EOSIN STAIN FOR PARAFFIN SECTIONS OF FORMALDEHYDE-FIXED TISSUE**

| | |
|---|---|
| Azure A (C.I. 52005), 0.1% in water: | 16 ml |
| Eosin B (C.I. 45400), 0.1% in water: | 16 ml |
| 0.2 M acetic acid: | 6.8 ml |
| 0.2 M sodium acetate: | 1.2 ml |
| Acetone: | 20 ml |
| Distilled water: | 100 ml |

Stain for one hour in freshly made mixture.
Replace staining solution with acetone, two changes, each 45-60 seconds with agitation.
Clear in xylene, 2 changes, and coverslip.

**Figure 5.** Structures of azure A and eosin B and the composition of an azure-eosin stain suitable for routine use in histopathology.

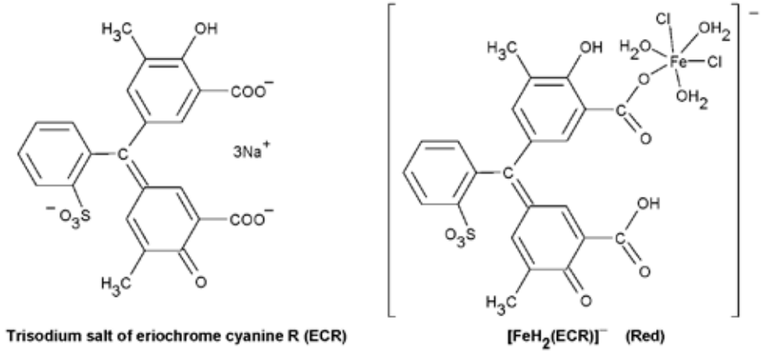



**Figure 6.** Structure of eriochrome cyanine R (ECR) and its iron (III) in aqueous solutions containing ferric chloride. ECR is also known as chromoxane cyanine R, solochrome cyanine R and C.I. 43820, Mordant blue 3. It also has several trade names.





with a high density of anionic sites, such as mast cell granules and cartilage matrix, are coloured reddish-purple, an effect known as **metachromasia**, attributed to stacking of the planar dye cations. Nocht-type stains can be made by combining any blue cationic thiazine with a red anionic xanthene dye, and they are used by the Biological Stain Commission in tests for the certification of azures A, B and C, toluidine blue, and eosins B and Y.

### Mann's Eosin-methyl Blue

In this method, sections are stained with a mixture of two anionic dyes, one with smaller ions (eosin Y, anionic weight 646) than the other (methyl blue, anionic weight 754). Methyl blue (not to be confused with methylene blue) is the principal component of dyes sold with the name aniline blue, which may also be used in this technique. Gustav Mann, in 1894, described two variants of this method, short and long. The short method is an oversight stain; the long one is used for showing intracytoplasmic objects such as secretory granules and viral inclusion bodies. With most methods using combinations of anionic dyes, the results are more colorful if the tissue has been fixed in a mixture containing picric acid or mercuric chloride than after fixation in neutral formaldehyde, but Mann's methods work well after almost any fixation.

In the short method, which is a progressive technique, hydrated sections are stained for 10 minutes in a solution containing 0.25% eosin Y and 0.2% aniline blue in water, rinsed in water to remove excess dyes, dehydrated in three changes of 100% alcohol, cleared in xylene and mounted. Nuclei, collagen fibers and mucus are stained blue; cytoplasm (including erythrocytes) and nucleoli are red. The differential staining is usually attributed to the larger blue dye ions penetrating collagen and nuclei more rapidly than the smaller red ions, which enter the supposedly more densely textured cytoplasm and nucleoli. This mechanism is supported by experiments involving staining of films made from concentrated and dilute gelatin solutions. The concentrated gelatin is colored red and the dilute gelatin blue.

Mann's long method is regressive. The sections are stained for 12 to 24 hours in the same solution used in the short method, rinsed in water and dehydrated in 100% ethanol. The slides are then placed in a dilute (0.0025%) solution of potassium hydroxide in 100% ethanol until the sections change color from dark purple to red. This color change is due to conversion of the aniline blue anion to a red color base, which is more soluble in alcohol than the blue form of the dye. Washing in 100% alcohol to remove alkali is followed by immersion in water, which removes most of the eosin from components of the tissue other than nucleoli, erythrocytes and some cytoplasmic granules and inclusions. Nuclear chromatin, collagen and some secretory products become blue again with removal of the alkali. Finally, the sections are rinsed in 0.1% aqueous acetic acid, which stops the differentiation of both dyes, dehydrated in three changes of 100% alcohol, cleared and coverslipped.

**Pituitary cytology.** The long version of Mann's technique was popular until the mid-1950s for showing cell-types in sections of the anterior lobe of the pituitary gland, which may contain red- or blue-staining granules. The red cells were usually called acidophils and for more than half a century the blue cells were very wrongly called basophils. In fact, both cell types are acidophil because both eosin and aniline blue are acid (anionic) dyes. Correct nomenclature had the blue cells **cyanophil** and the red ones **erythrophil**. These names respectively indicate staining by blue and red dyes. The "rhodocyan" method of Glenner and Lillie is a variant of Mann's stain for showing endocrine cell-types in the pituitary gland and pancreatic islets. The naming of anterior pituitary cell-types became more complicated in the late 1950s with the advent of rational but complex techniques that combined simple carbohydrate and protein histochemistry with the use of anionic dyes. The anterior lobe of the pituitary gland secretes at least six well understood protein or polypeptide hormones, and there are benign tumors that secrete only one hormone. In the 1970s and 1980s immunohistochemistry brought an element of certainty to the localization of pituitary hormones in normal glands and adenomas.

## Oversight Staining with One Dye

It is possible to see structural details by staining with a single dye if the sections are sufficiently thin. **Heidenhain's iron-hematoxylin** is the classical method of this type. Sections are immersed for several hours in an approximately 0.05M aqueous solution of a ferric salt, rinsed in water and then immersed for several hours in a 0.5% hematoxylin solution. The latter contains some hematein, formed by atmospheric oxidation, which forms a black complex with the ferric ions bound from the first solution, in all parts of the tissue. Careful differentiation in the 0.05M iron solution reveals nuclei and, in suitably fixed material,

cytoplasmic structures such as mitochondria, muscle striations and characteristic organelles of protozoan parasites. The method is too time consuming for routine use.

For some objects, such as renal and nerve biopsies, the routine requirement is for semi-thin sections (0.5-1.5 µm), which can be cut only from fixed specimens embedded in a hard plastic such as poly(methylmethacrylate), poly(glycolmethacrylate) or an epoxy or polyester resin. The 0.5-1.5 µm sections are called “semi-thin” because they can be used to locate structures seen in adjacent really thin sections (around 0.05 µm) that are examined by transmission electron microscopy. For semi-thin sections, one color usually suffices for an oversight stain. **Alkaline toluidine blue** has served well for more than 50 years. It colors everything, but with variations in intensity. 0.05% toluidine blue in 1% borax (0.05M $Na_2B_4O_7$) is commonly used. Penetration of the dye is enhanced by prior etching of the resin with sodium ethoxide (made by dissolving NaOH in ethanol) and by staining at 60°C.

**Eriochrome cyanine R** is an anionic hydroxytriarylmethane dye that has various uses as an industrial colorant, a reagent in analytical chemistry and a biological stain. Used alone, this dye stains everything red, in much the same way as eosin. Solutions containing ferric salts can be used as substitutes for haemalum, and in a staining method for myelin sheaths of axons in nervous tissue. In **Hyman and Poulding’s method** advantage is taken of the existence of red and blue dye-iron(III) complexes (see Figure 6) with affinities for different components of animal tissues.

The staining solution 0.2% of the dye, in a 0.21M solution of ferric chloride in 0.09M sulfuric acid (0.5 ml of 96% $H_2SO_4$ in 100 ml of water). The pH must be 1.5 in order to give the correct colors; it needs adjustment from time to time. Otherwise, the solution can be kept and used repeatedly for several years. Hydrated sections are stained for three minutes, washed in three changes of distilled water, each 20 seconds with agitation, dehydrated in 95% and two changes of 100% ethanol, cleared in xylene and coverslipped. Nuclei are blue-purple, erythrocytes orange-red, and cytoplasm pink to red. Collagen fibers are mostly pink but thinner ones are purple. Myelin sheaths are blue-purple. This color scheme is similar to that of H & E, but not identical.

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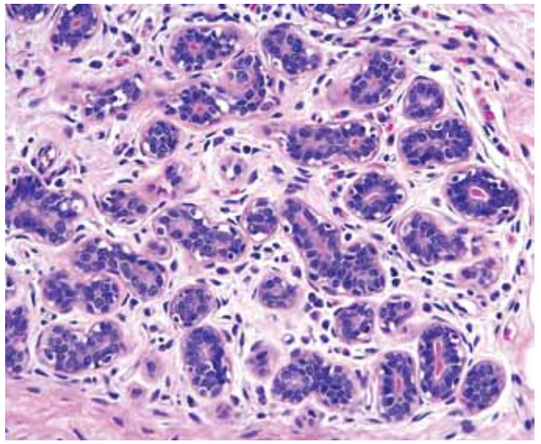
# Chapter 3 | Studying Histological Changes in Breast Tissue with Menstrual Cycle using H&E Staining

**Sunil Badve, MBBS, MD (Path), FRCPath**

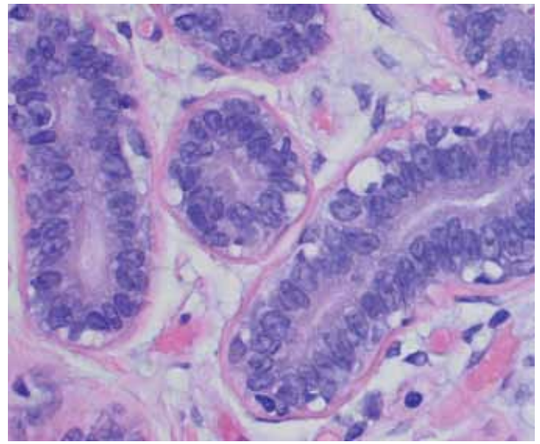
Breast cancer is a disease of both premenopausal and post-menopausal women. In premenopausal women the disease is often associated with poor histologic grade, high stage, presence of metastases at diagnosis and exhibits an aggressive course leading to significant morbidity and mortality. The biologic basis underlying the aggressive course is not well understood and attributed to the hormonal milieu in premenopausal women. In order to obtain clues about the nature of these effects, several studies have attempted to analyze the clinical course of breast cancer in relation to the phase of the menstrual cycle during which surgery was performed.

Timing of treatment seems to play a role in modulating the outcome of breast cancer. A prognostic benefit of timing of surgery during luteal phase of menstrual phase has been seen in some but not all studies. The reasons for the different outcome could be cyclic variations in cellular enzymes with menstrual cycle and/or chemotherapy induced amenorrhea.

The assessment of the impact of menstrual cycle on the biology of breast cancer in population-based or randomized studies is difficult. They are dependent on several factors including obtaining accurate menstrual history, a task often made difficult by irregular cycles in older perimenopausal women. Alternative strategies include assessment of hormone levels in serum or even in saliva; however, it must be noted that there is a marked variation in hormone levels in premenopausal women. A significant drawback of these strategies is that they require prospective design and collection of materials leading to often long expensive trials. Additionally, circulating hormone levels may not reflect tissue hormone status either due to physiological lag or due to local hormone resistance. Our group has



**Stage 1: (Menstrual days: 0-5)**
Distinction between the epithelial and the myoepithelial layers was not conspicuous. The cells had round nuclei with minimal and lightly stained cytoplasm. Minimal edema and infiltrate in the intralobular stroma were noted representing “left-over” changes from the previous cycle. Although rare cells can show vacuolation, it is not a feature. Sharp luminal borders with eosinophilic intraluminal secretions were common. Apoptosis and mitosis were, by and large, absent in this phase.



**Stage 2: (Menstrual days: 6-15)**
This phase was characterized by an increase in the distinction between the epithelial and the myoepithelial layers of the acini. Well-formed double-layered acini were appreciated within lobules. Similarly, there was an increasing tendency for the acini to show basal layer vacuolation; however, fewer than 30% of the lobules showed this feature. Stromal edema and infiltrate were absent and mitoses or apoptotic bodies were not seen.

been exploring the possibility of using histological changes in breast tissue as a means of assessing the phase of menstrual cycle at the time of surgery; the salient features of which are described here and is based on the work of several authors including our own.

## Morphological Changes

Distinct alterations in the morphology of the lobule and the intralobular stroma of the breast with some degree of overlap were identified in accordance to the phase of the cycle. Interlobular stroma was relatively unaltered in all these phases. Summarized here are the correlative hormonal response in each of the stages.

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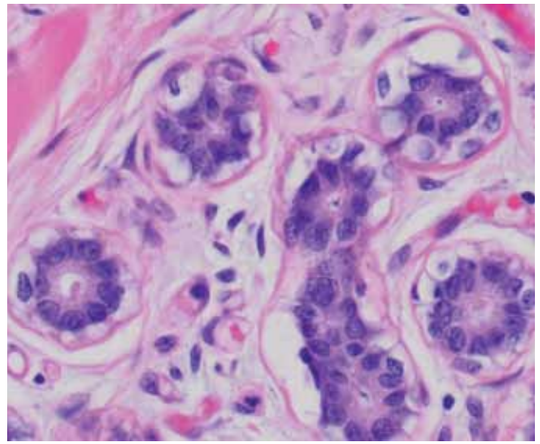
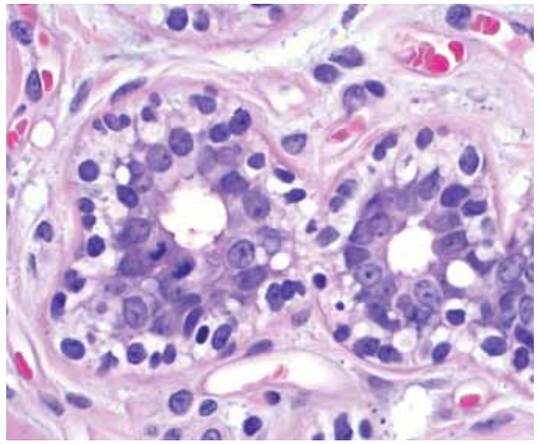


**Stage 3: (Menstrual days: 16-24)**

This phase was characterized by larger lobules with increased numbers of terminal duct units, which were lined by two distinct layers of cells. The basal layer showed marked vacuolations with a centrally or apically placed nucleus with clear and at times vesicular cytoplasm. The epithelial cells were more oval in nature with basophilic cytoplasm. The interlobular stroma showed increased edema and a mixed inflammatory infiltrate. Mitotic figures were rare and apoptotic cells infrequent.



**Stage 4: (Menstrual days: 25-28)**

This phase is morphologically characterized by the presence of extensive vacuolation within lobules. The epithelial cells have prominent nuclei with large nucleoli and a distinctive basophilic hue to the cytoplasm. The most characteristic feature is the presence of frequent mitotic figures as well as an increase in apoptosis. The latter are located towards the base of the lobule. There is extensive stromal edema with an increase in the inflammatory cell population.



# Chapter 4 | Evolution of Use of Special Stains

**Alton D. Floyd, PhD**

What is special about a stain, and why do we call some stains “special”? We all think we know what is meant when the term “special stain” is used, yet we all have different views of precisely what a special stain is. In the hospital based histology laboratory, special stains are generally those that are part of the laboratory certification checklists, as documented in the Introduction to Special Stains by Gill and Kumar in this publication.

It is unclear when the term special stains first entered the histology/pathology literature. An early documented use of the term is in the publication by Gomori in 1941 (1). In this paper, the term is used to describe a stain specifically created to differentially color the insulin containing cells of the pancreas (beta cells). Gomori considered this stain to be “special” since it was created specifically for a single purpose, and was different from the routine stains in use at the time. In this case, “special” could be considered to be a “targeted” stain that is designed to identify a single cell or tissue constituent. It is important to remember that histological staining at this time was based almost entirely on empirically developed protocols, and the guiding principle was the differential coloration of cell and tissue constituents. Only one histochemical procedure was in common use at this time, that being the Feulgen procedure (2) for deoxyribonucleic acid (DNA).

One of the first texts written for histologic technicians was by Ann Preece (3). This text, which first appeared in 1959, divided stains into three categories: Vital stains, Routine stains, and Special stains (Fig. 1-3). Using the definitions given in that text, vital stains are those that are applied to living cells or tissues. Routine stains are those used to differentiate cellular constituents, particularly cytoplasm versus nuclei of cells. Special stains are those that have a “more limited range” and that demonstrate special features. Cited examples include bacteria, fungi, particular cell products and microscopic intracellular and intercellular products.

The definitions provided by Preece are in some ways carried over to current usage, but are not totally accurate, even at the time they were written. Stain protocols were developed to provide differential contrast to cell and tissue constituents, with the goal of understanding cell structure and function. Stain procedures were necessary simply because cell and tissue specimens, when prepared for microscopic examination are essentially transparent, particularly after dehydration and mounting with media whose refractive index closely matches glass. Without staining, microscopic examination is not productive. As stain protocols evolved, more and more cell and tissue elements were identified. Many protocols were developed with four, five or more different colors, each targeting some particular element within the specimen. The rational for this multiple color staining was simply to gain a better understanding of the complex interrelationships between elements within specimens, and thereby gain a better understanding of function.

The definition given by Preece for a “routine stain” is much broader than current usage. In the Preece definition, all of the connective tissue stains, such as reticulum and trichromes, would be considered to be “routine”. In the modern histopathology laboratory, it is the hematoxylin and eosin (H&E) stain that is considered to be “routine”. In fact, the H&E stain is often referred to as the “Gold Standard”, and is the first stain performed on almost all specimens. All subsequent stains fall under the definition of “special stains”. There is one exception to this, and that is the immunohistochemical stains (IHC). Although IHC stains meet every criteria of the definition of “special stains” the FDA specifically excluded them from this category when they first regulated IHC stains (4), even though strong arguments were made for placing them in the category of special stains during the public hearings on these new regulations.

It is important to remember the purpose of stains. They are a way to define morphology of cells and tissues, by providing different colors to specific elements of the specimen. As an example, it is often difficult to differentiate smooth muscle from connective tissue in some specimens, based on microscopic structure alone, if both elements are stained the same general color. The trichrome connective tissue stains were developed to assist with this morphological differentiation. It should also be remembered that in the time period when many stain protocols were developed, it was common to utilize experimental animals that demonstrated some exaggerated morphology, as it was easier to determine the effectiveness of the stain protocol in obtaining the specific differentiation desired. A second result of this use of a

variety of experimental subjects was that many stain protocols were developed that provided confirmation of morphology that had subtle differences from one species of animal to another.

It should also be remembered that this “golden age” of complex stain protocols occurred prior to the advent of both electron microscopy and histochemical staining. Electron microscopy provided detailed confirmation of intracellular structure, much of which had been documented, although disputed, by conventional stain protocols. Histochemistry provided direct chemical and functional confirmation of intracellular and extracellular events that correlated directly to biochemical investigations and led to better understanding of cellular function (cell biology). As understanding has continued to develop, cell biology has expanded into the realm of molecular biology. The foundations of cell and molecular biology were generated by stain protocols, many of which were the “special stains” still in use today.

It should be noted that morphology and stain protocols are closely linked in this discussion. During the period when many morphological elements of both cells and tissues were still being discovered and their functions ascertained, stain protocols were a key tool providing confirmation of the existence of “something” within examined specimens that needed explanation, whether this was in normal specimens or those examined for diagnostic purposes. The introduction of the microscope to pathology was based on the observation that many disease processes result in morphological alterations in cells and tissues. Therefore the development of stain protocols that could clearly demonstrate morphology was a critical event in the advancement of understanding of disease processes.

Variants of the H&E stains have been utilized almost since the beginning of microscopic pathology. As a consequence, the development of microscopic pathology, and the terminology utilized to describe disease processes is based on descriptions of H&E stained preparations. There have been obvious changes in the appearance of H&E stains over the years, partly due to changes in stain formulations, and partly due to variations in fixation and processing of tissues. Regardless, the H&E stain is the primary or initial stain used on sectioned tissue specimens. It is for this reason the H&E stain is regarded as the “gold standard” for diagnostic purposes, even though any suggestion that the H&E stain is “standardized” is far from true. Clear evidence of this is seen both in publications and in presentations, where the color balance between the red and blue components of the stain is highly variable. As an empirical stain, the final stain result is “tuned” to the preferences of the observer. The high variability of H&E stained specimens complicates referral of specimens from institution to institution.

Another factor which has contributed to the use of H&E as the primary, or routine stain, and relegated other stains to the category of “special” was the adoption of Diagnostic Related Groups (DRG’s) which were introduced to the diagnostic laboratory several decades ago. This is an excellent illustration of how regulatory bodies can significantly influence laboratory practice. Over time, DRG’s have influenced the way specimens are handled, with an emphasis on rapid diagnosis and turnaround times. While in many cases, the initial clinical history provides guidance for the histological staining necessary for a diagnosis, in some specimens, it is the morphology of the H&E section that guides additional staining tests. Another consequence of increased regulatory oversight and attention to total costs of diagnosis are restrictions on reimbursement. In other words, once a diagnosis is indicated, there are strict limits on the stain tests which can be done, if the laboratory wishes to be reimbursed. Effectively the laboratory no longer has the luxury of investigating cases that show deviations from “normal” for a particular diagnosis.

Special stains, as currently defined in diagnostic pathology, consist of several types of stains. Many of these were developed strictly as morphological stains, although a few are derived from histochemical investigations. Morphological stains are those that demonstrate some particular morphology. Among special stains based on morphology are stains for bacteria and fungi, for myelin and nerve fibers, and for connective tissues including reticular fibers. Special stains for specific tissue components (mainly histochemical) are stains for iron, mucins and glycogen, amyloid, and nucleic acids. IHC stains, which are also a “secondary” stain, just like all other special stains, are not considered “special stains” for reimbursement purposes, and are a separate category of diagnostic tests (their own unique reimbursement codes).

IHC stains have replaced many traditional “special stains” simply because they have great specificity. Specificity means that the stain is exceptionally precise in the recognition of a specific target or epitope.





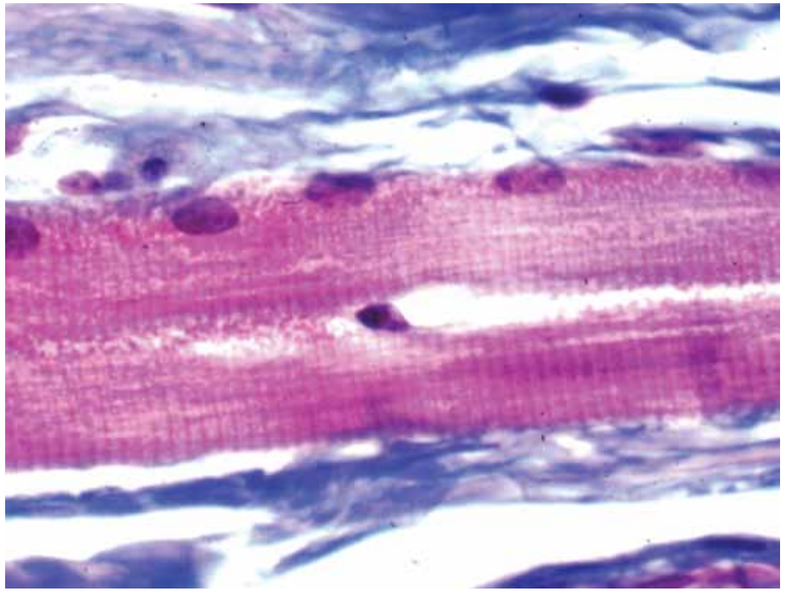


**Figure 1.** Masson Stained Skeletal Muscle and example of a “general oversight” stain. This stain clearly demonstrates muscle, connective tissue and nerve in contrasting colors.

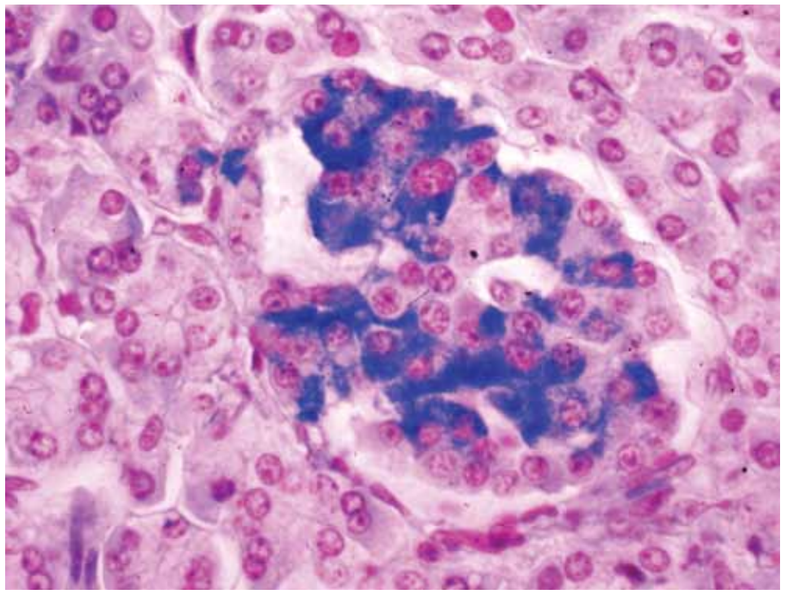


**Figure 2.** An example of a “special stain”, using Victoria blue in a protocol specific for pancreatic islet Beta cells.

A primary antibody, which provides the specificity for an IHC stain, can detect an epitope of three amino acids. In fact, a series of primary antibodies can be manufactured which can detect the sequential arrangement of three amino acids. While this degree of specificity is astonishing, it should be remembered that other special stains may also be exquisitely sensitive, for example, the iron stain actually detects ions of a single element. Not to be forgotten, the Periodic Acid-Schiff stain detects exceptionally small amounts of glycogen and mucopolysaccharides. The Feulgen reaction can detect DNA accurately enough to detect the gain or loss of a single one of the larger chromosomes (the basis of Ploidy measurements).

With continued advances in IHC staining (availability of new primary antibodies), it might be expected that IHC staining could eventually replace all traditional special stains. Since IHC staining is based on biological molecular recognition, it is ill suited for identification of elemental inclusions, such as iron. Also, the various special stains utilized for bacteria and fungi, when combined with clinical laboratory data, provide sufficient information for diagnosis. While IHC tests can be used for certain infectious agents, these tests are expensive, and are vulnerable to specimen processing as to reliability. Due to cost factors alone, IHC staining will most likely not replace all special stains.

Microscopic morphology of specimens was the driving force behind the development of special stains. For a number of reasons, morphology is of less concern in the diagnostic histopathology laboratory. There are many factors which have brought this about, including the explosion of knowledge in diagnostic pathology, the increased number of specimens submitted, and changing emphasis in education of pathologists. With severe time constraints in medical curricula, microscopic morphology does not receive the same emphasis it once did. Pathology residence programs still occupy essentially the same amount of time as in years past, yet the total amount of diagnostic information has increased by several orders of magnitude. The result of these time pressures is that classical microscopic morphology skills may not be as strong as in years past. Thus the modern practice of pathology has come to depend on certain of the special stains, and on IHC stains. Specimen submissions continue to increase, as would be expected in an aging population which tends to live longer. It is quite clear the use of special stains and IHC stains will continue to increase, and remain a fundamental element of diagnostic pathology.

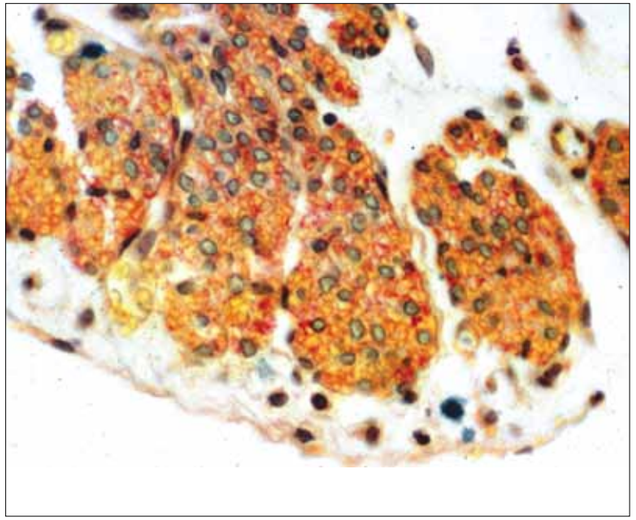
A clear case can be made for increased use of “traditional” multicolor stain protocols in research. First, traditional stains provide excellent overviews of specimens, and can easily define tissue organization. In many cases, histochemical stains can be combined with specimen survey stains. As an example, a classic trichrome stain can be replaced with a combination of histochemical stains which provides detailed information about the distribution of specific tissue constituents, as well as an overview of tissue organization. One such stain combination is the Blue Feulgen, PAS, Alcian Blue and Naphthol Yellow stain, which demonstrates DNA, Glycogen, Acidic mucopolysaccharides and general protein distributions. The individual colors are easily seen, and the stain is simple to perform.

## Continuation of Change – Where Special Stains May be Headed

It is clear that special stains including IHC stains will continue to be a key in accurate diagnosis of microscopic specimens. It should also be expected that changing usage patterns will continue. As an example, consider the case of cell proliferation. It is quite simple to identify mitotic figures in any stain, including H&E. However, in most normal tissues and early stage tumors, cell division is tied to a circadian rhythm and occurs in the time period between 1:00 AM to 6:00 AM. This is not the time when most specimens are collected. The introduction of a proliferation marker, KI-67 (IHC) made it possible to identify cells preparing for division, even when mitotic figures were not seen. This IHC test is both more convenient and gives a better indication of proliferation in specimens, and has essentially replaced purely morphological assessments. Another example is seen in breast carcinoma. In this case, it is easy to tell that a malignant process is occurring, using simple morphology. However, with the development of targeted drug therapy, it has become clear that there are different types of malignancies, even though they may resemble each other based on morphology alone. Thus the use of prognostic markers, which are cellular characteristics identified by specific IHC tests. In specimens positive for (expressing) estrogen receptor, the patient becomes a candidate for Tamoxifin (AstraZeneca) therapy. In specimens positive for Her-2-neu, the patient may respond







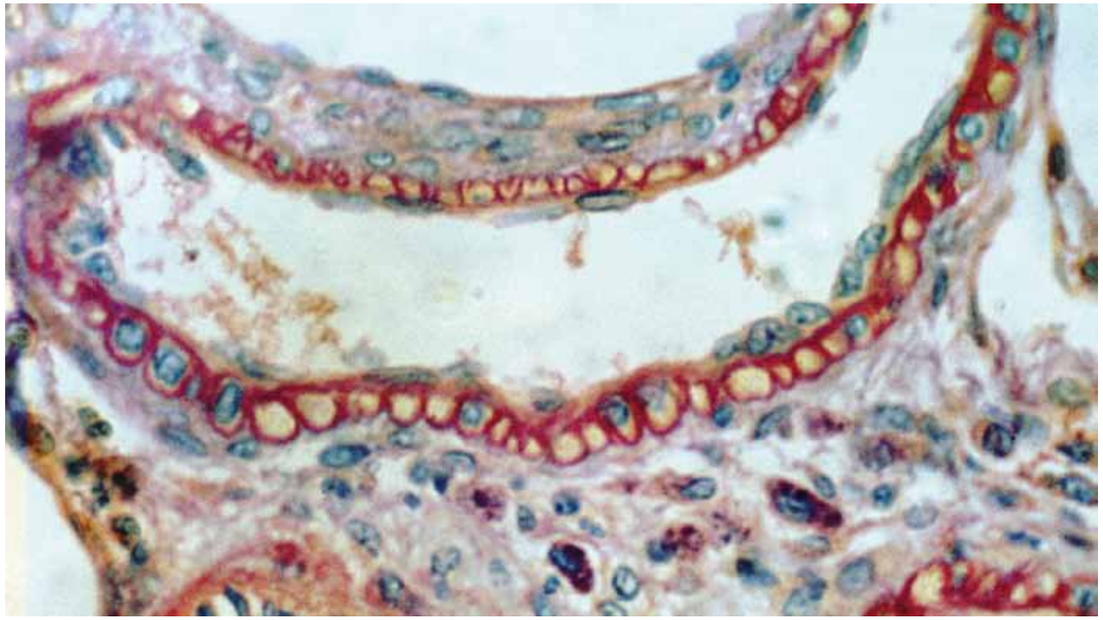


**Figure 3.** Blue Feulgen, Periodic Acid-Schiff, Alcian Blue and Naphthol Yellow S, a combined histochemical stain. Note the nuclei in blue, the cell coat stained with PAS, and the distinct granules of Mast cells. The photomicrograph on the left shows cross-sectioned smooth muscle cells in the intestinal wall. The section below is a higher magnification view of cross-sectioned blood vessel.

**Procedure: Blue Feulgen, PAS, Naphthol Yellow**

Demonstrates DNA, acid mucopolysaccharides, carbohydrates and proteins.

1. Remove paraffin and hydrate slides to water
2. Hydrolyze 1 hour in 5 N HCl at room temperature
3. Rinse slides well in running water
4. Stain 20 minutes in Cresyl violet Schiff

**Cresyl Violet Schiff Solution**

Dissolve 0.02 grams Cresyl Violet Acetate in 1 Liter distilled water as stock solution. Store in refrigerator.

For use, add 3 grams sodium dithionite to 150 ml of stock solution.
Use in covered staining dish.

5. Rinse well in running water
6. Stain in Alcian Blue (0.1% in 0.01 N HCl, pH 2.0) for 10 minutes
7. Differentiate in 0.01 N HCl for 2 - 3 minutes
8. Wash in running water 1 - 2 minutes
9. Hydrolyze 10 minutes in 0.5% periodic acid
10. Wash 5 minutes in running water
11. Stain 10 minutes in Standard Schiff (Basic Fuchsin Schiff)
12. Wash 5 minutes in running water
13. Stain 1 minute in Naphthol Yellow S (0.01% ion 1% acetic acid)
14. Differentiate in two changes 1% acetic acid, two minutes each
15. Dehydrate in tertiary butanol, 2 changes, 3 minutes each
16. Clear in xylene and coverslip

Steps 6, 7 and 8 may be omitted, if acidic mucopolysaccharides are not of interest. If these steps are omitted, then final dehydration can be carried out in a standard ethanol series.

to treatment with Herceptin (Genentech). Clearly, these are the beginning of significant change in microscopic diagnosis, and will certainly generate changes in the use of special stains. As IHC stains can be much more specific than the empirical multi-color stains of the past, it would be expected that over time, many of the general oversight stains, such as the trichrome stains, will be of less use in routine diagnosis, although this may not be so in research settings. As the examples given demonstrate, when a targeted therapy becomes available, based on a unique expression of the diseased cells or tissues, then targeted visualization tests will also be required. However, there will always be a need for special stains for bacteria, fungi, iron, and general tissue architecture.

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# Chapter 5 | Dye Quality and Implications for Biomedical Staining

**Richard W. Dapson, PhD**

## Introduction

Most of the dyes used in histology and cytology (H&C) are manufactured for use in textiles, printing, food, cosmetics, drugs and other colorant industries. The market for biomedical dyes is small, so there is little leverage when it comes to specifying quality. We get what we can get and the result is not always satisfactory. The issue of quality for us has always been problematic and will continue to be so into the foreseeable future. In part, this is due to differing needs of the various major consumers, which in turn determines standards of quality. There are other factors as well, however, and it is instructive to study trends in textile dyeing and the textile market (one of the major user of dyes) to appreciate why quality and availability of H&C dyes is always precarious.

## The Needs of Most Colorant Markets

Colorists in nearly all fields have a narrow goal: to produce, and perhaps then reproduce multiple times in the future, a very particular hue. A certain drug must be color coded in a precise way. Fashion designers specify an exact shade of color for garments, home furnishings and wallpaper. The list goes on, but the need is the same: an exact, reproducible color. Dye manufacturers have never been able to furnish pure dyes of exact hue (the color itself) and shade (how light or dark that color is). The cost would be prohibitive and the problem is more readily solved in other ways. The balance of color attributes may be manipulated in manufacturing by altering the manufacturing process (raw materials, reaction conditions) or by blending in one or more different dyes of comparable functionality but with slightly different optical characteristics. Other ingredients may be added for increased stability for particular dyeing processes, or as fillers to decrease intensity. Once in the hands of the dyers, further alterations can be made to get just that right shade on Ralph Lauren's latest seasonal fashion item.

In these fields, dyes must have appropriate solubility. They must attach firmly to the intended substrate, but that rarely means much more than having an affinity for one of the major classes of substrates (proteins like wool and silk, carbohydrates like cotton and linen, nonpolar and weakly polar substances like polyesters, acrylics, acetate and Nylon). No one is trying to discriminate between various types of protein, for instance.

## Defining Quality for Textile Dyes

Modern industrial dyeing operations generally have strict standards for incoming dye shipments (Park and Shore, 2007): homogeneity of characteristics, stability in storage, commercial form (powder, paste or liquid), solubility (if not liquid), and a variety of health and safety attributes: dustiness, trace metals, toxicological profile, biodegradation and ecotoxicity. These dyes are evaluated for moisture content, strength of reflectance of dyeings (comparable to running a control slide), strength of transmission of dye solutions, chromatography, sensitivity to pH, solution stability and many other tests not germane to our topic (Park and Shore 2007).

## The Needs of the H&C Market

By contrast, laboratory applications generally demand far greater specificity. In a finished slide, a good nuclear stain like alum hematoxylin will only color DNA and RNA. We may even want to distinguish between DNA and RNA, as when we use methyl green and pyronin in sequence. Schiff's reagent (in the PAS test) reacts with most carbohydrates bearing vic-glycols while Best's carmine stains glycogen specifically. Pap staining incorporates 3 acid dyes, light or fast green, eosin and orange G, to create a broad palette of colors on various cellular proteins. In short, we depend upon a rather precise and reproducible chemical composition for our dyes. The exact hue is really of secondary importance.

## Defining Quality for H&C Dyes

Dyes must be what we think they are, in the right strength, with no interfering contaminants. To verify this, end users rarely do more than stain a control slide, merely confirming that it works in that procedure, not that it is useful in other intended applications. Stain manufacturers ideally would perform chemical and physical tests to determine if incoming dyes are of the proper color, intensity and purity, but cost constraints mitigate against such exhaustive analyses. The problem is one of scale. For a commercial textile dyer, testing a sample from thousands of pounds of a textile dye is cost effective and realistic. A small stain manufacturer may buy only a few pounds (perhaps 100 pounds) at a time. For the relatively small number of bottles of stain sold from that batch and the high analytical costs, exhaustive testing is not feasible and may be beyond the expertise of the company.

## Why Dyes are so Variable

Dyes are highly variable for many reasons. They are not pure substances. Slight variations in manufacturing will have a profound effect on the final product. Each manufacturer has its unique way to make a particular dye. Constantly evolving health and safety regulations cause alterations in manufacturing protocols. Recent geographic shifts in where dyes are made has had a major impact on the nature of dyes.

## How Dyes are Made

Dyes are complex molecules that are synthesized in stepwise fashion. Starting with impure raw materials, a core segment is created. The reaction produces a major component but also leaves unreacted and partially reacted entities. Another reaction is begun with similarly impure reagents to add one or more new segments onto the core. Isomers may now form in addition to the other products. Clearly, each time an addition reaction occurs, the complexity of the mixture increases.

Further complicating this picture is the variety of ways some dyes can be made. Alizarin Red S (CI 58005) is a good example. The Colour Index (SDC/AATCC 1971 lists 8 different starting chemicals from which alizarin (CI 58000) can be synthesized, none of which is pure. While only one is used in a given process, different manufacturers may create very different mixtures, all of which are sold as alizarin. Sulfonation of any of them produces the water soluble dye alizarin red S. Obviously, purchasing the dye from different sources may yield quite a variety of mixtures, all of which are labeled alizarin red S and may actually contain a preponderance of that chemical, although the other components will be different.

Some reactions are prone to be incomplete, as in phloxine B (CI 45410). Theoretically it should contain 4 bromine atoms and 4 chlorine atoms. Samples submitted to the Biological Stain Commission (BSC) for certification vary in the degree of bromination and chlorination (Lillie 1977).

As if that weren't enough confusion, names from different manufacturers may be completely dissimilar: thus, eriochrome cyanine R (CI 43820), used as a hematoxylin substitute (among other things), is also known as chromoxane cyanine R, cyanine R, chrome cyanine R and solochrome cyanine R. It is easy to see how the wrong dye might enter our supply chain simply because of confusion over names. The use of Colour Index (CI) numbers reduces chances for this type of error.

## Historical Trends in Textile Dyes

Since the advent of synthetic dyes in 1856, quality has been a persistent problem and concern. Prospects for the future show no sign of abatement as major shifts in dye manufacturing occur. A brief look at that history offers clues to the supply side of our problem with quality.

Synthetic dyes invented in England in 1856 spawned a thriving industry there for a few years. Germany and Switzerland soon gained ascendancy in the market, however, by obtaining hundreds of patents and by acquiring expertise. Many British firms were driven out of business by the turn of the century. World War I caused a serious disruption in global trade, particularly from Europe. The United States encouraged local businesses to enter the dye business, but that effort essentially failed because quality was so poor. After the war, little was learned from the lessons of history: most dye manufacturing shifted back to Germany and Switzerland, thus setting the stage for a repeat of the inevitable when WW II began. At least by then the Biological Stain Commission (then called the Committee on the Standardization of Biological Stains) was certifying dyes for biomedical use (Penney 2002).





Environmental and safety issues became the driving force for change in the 1960's, beginning in Europe and eventually sweeping North America and Japan (Dapson 2009). Heavy metal catalysts were replaced. Production of carcinogenic dyes was voluntarily halted. Imports of goods colored with carcinogenic dyes were banned in some countries. Costs rose to accommodate stringent workplace and wastewater regulations.

European, Japanese and American dye makers quickly invented hundreds of new, less hazardous dyes to replace those that had to be abandoned, but their efforts came to naught. Textile dyers did not want to change and kept demand high for older dyes. They also resisted cost increases. From the 1970's to the 1990's, nearly all dye manufacturing by the standard companies was lost to less developed nations in Asia. With that came an entirely new supply chain paradigm (Park and Shore 2007). Instead of well known giants of the field with long-established reputations and respected R&D facilities, dyes are now being made by smaller operatives whose products are collectively marketed to developed countries via trading companies. Tracking a specific dye back to its ultimate source is now practically impossible. The track record of this type of supply chain is not good, as anyone knows who follows the news about adulterated, misbranded toothpaste, toxic infant formula, and melamine-laced pet food. There is great cause for concern now about dye quality. Is the dye purchased really the one wanted? Is it good enough to work properly? Does it contain toxic ingredients?

## Why Availability is so Often an Issue

Every histologist and cytologist is aware of at least one example where a critical dye has been in short supply or not available. Companies stop production for health, safety or environmental reasons (light green SF, CI 42095; alcian blue, CI 74240). In the case of hematoxylin, the latest shortage came about because the sole extractor of logwood simply dropped it off its production schedule to concentrate on more lucrative botanical extracts for the skin care industry (Dapson et al 2009). Fashion also plays a significant role. The principle use of nuclear fast red (CI 60760) is to color wall paper. Once that shade of pink is out of fashion, production ceases until the pendulum swings back. In other words, availability will always be a companion issue to quality: they often go hand in hand.

## Assuring Quality: How the BSC Operates

Participating vendors of dyes and stains submit samples of a given batch of powder to the Commission Laboratory. These are put through a variety of tests that might include (depending upon the dye) absorption characteristics with a spectrophotometer to verify color, amount of colored material in the powder (percent dye content) by physical or chemical methods to assess purity, and biological tests on appropriate control specimens in a variety of plant, animal and/or microbiological procedures (Penney et al 2002).

## Pure is Not Always Best

One would think that most of the problems discussed would simply go away if only we could get pure dyes. Many of us in the dye field have had similar ideas, and have even tried synthesizing or purifying dyes to achieve that goal. In all of the cases with which I am personally familiar, the dye did not work. Some were stubbornly insoluble while others simply failed to stain anything or, conversely, stained everything indiscriminately. Ingredients other than the dye itself may aid solubility, condition the tissue to be more receptive, or serve in the complex process known as ion exchange staining (Prentø 2009).

## New Dyes May Not be the Same as the Old Ones

Most of our staining protocols are decades old. Even recently modified versions usually assume that the dye is the same old material everyone has always used. Don't make that assumption. Dye composition changes as manufacturing processes supersede the originals. For example, thionine (CI 52000) originally was supplied as a chloride salt, but is now the acetate salt. This changes the molecular weight and hence any calculations based upon that (Lyon and Kiernan 2008).

## Final Advice

For laboratory professions: buy dyes and stains only from reputable suppliers. If the powders are on the list of Commission-certified dyes, insist that material supplied is certified and is accompanied by an authentic certification label. With stains, insist that they be made with certified dyes.

For stain manufacturers, use only certified dyes or perform extensive tests to verify identity, level of purity and effectiveness in common staining procedures. It would not hurt to include such information on labels or in package inserts. Since a vendor cannot anticipate how a customer will use the product, a listing of verified procedures would be helpful.

For everyone: when shortages occur, or when there is a sudden change in supplier, be especially cautious. When demand rises, there is always someone willing to make the sale with any material available. Irresponsible parties will slide merchandise down the supply chain hoping it is the right stuff. Disreputable suppliers will substitute a look-alike intentionally. Both types have operated in the past and will certainly do so again.

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# Chapter 6 | Standardization and Standards for Dyes and Stains Used in Biology and Medicine

**Hans O. Lyon, MD and Richard W. Horobin, PhD**

You don't think dyes and stains used in biomedicine need standardizing? And yet the most widely used stain for histological tissue sections, hematoxylin and eosin, can give remarkably varied staining. Even "identical" procedures can yield bluish or pinkish overall coloration. Why? Did the pHs of aluminium-haematein, blueing agents, or eosin solutions vary? Or were new dye lots used, with different concentrations of coloured materials? What can we do about this? Perhaps more rigorous standardization can help.

## What is Standardization?

"Standardization" requires agreement on a set of rules providing appropriate reproducibility of quality and/or quantity. Experts representing manufacturers, users and regulatory bodies reach consensus concerning the standard(s). A standard is thus a document establishing agreed uniform technical specifications, criteria, methods, processes, or principles for procedure. Formal standards organizations – e.g., the International Organization for Standardization (ISO), the European Committee for Standardization (CEN), or the American National Standards Institute (ANSI) – are independent of the manufacturers of products for which they publish standards.

## Who Makes the Standards?

Most countries have organizations for national standards, and such organizations cooperate regionally and/or internationally. Groups directly involved in biological staining standardization include ANSI, CEN, the USA Clinical Laboratory Standards Institute (CLSI), and ISO. Certain other organizations – e.g., the World Health Organization (WHO) and the European Diagnostic Manufacturers Association (EDMA) – are affiliated to Technical Committee(s) of CEN and ISO, and send representatives to meetings of these bodies and their Working Groups (see Box 1).

**Box 1. Organizations Concerned with Standardization**

*American National Standards Institute* – ANSI is a non-governmental, non-profit organization administering and coordinating USA voluntary standardization and conformity assessment system.

*Clinical Laboratory Standards Institute (CLSI)* – develops globally applicable, voluntary consensus documents for healthcare examination. Uses its own or ISO consensus processes, as appropriate.

*European Committee for Standardization* – CEN involves standardization bodies from European Union (EU) member countries plus EFTA (CEN 2009). Technical Committees (TC) include TC 140: In vitro diagnostic medical devices. CEN/TC 140 has produced harmonized standards concerning in vitro diagnostic devices (European Parliament 1998). "Harmonized" implies EU countries adopt these standards, replacing national standards on the subject, to provide products lawfully produced and marketed in one Member State, which are admitted to the market of all other Member States, eliminating trade barriers.

*International Organization for Standardization* – ISO, with a membership of 162 national standardization bodies (ISO 2009), facilitates coordination and unification of industrial standards...This is achieved through consensus between national delegations representing all the economic stakeholders concerned. Consensus includes consistent specifications and criteria for classifying materials, manufacture/supply of products, testing/analysis, terminology, and provision of services. International Standards provide a framework linking suppliers and customers, facilitating trade and technology transfer.

## Product Certification or Product Qualification

This entails certifying products that have passed performance and quality assurance tests or qualification requirements stipulated in regulations and nationally accredited test standards; or that they comply with regulations governing quality and minimum performance requirements. Examples of certification organizations concerned with biological stains and dyes are given in Box 2.

**Box 2. Organizations Concerned with Certification of Biological Stains and Dyes**

*American National Standards Institute* – ANSI facilitates development of American National Standards by accrediting procedures of other national standards-developing organizations, involved in developing voluntary national consensus standards in the USA. Accreditation signifies that procedures used meet the Institute's essential requirements for openness, balance, consensus and due process.

*Biological Stain Commission* – A Commission objective is standardizing and certifying selected biological stains and dyes i.e., dyes or products and preparations of dyes used to enhance visual contrast in biological specimens – for any and all laboratory purposes (Penney 2005). This objective is achieved by analysis of dye content and composition, plus testing performance of dye lots in standardized procedures, dyes being submitted voluntarily to the Commission's laboratory by manufacturers or vendors.

## Accreditation

Accrediting a laboratory requires independent evaluation of its competence and ability to perform certain tasks. Requirements are specified in international standards, e.g., ISO 15189:2007 Medical laboratories – Particular requirements for quality and competence. Although accreditation is usually by national organizations, a coordinating group exists, the International Laboratory Accreditation Cooperation. ILAC aims to increase use and acceptance of results from accredited laboratories and inspection bodies, nationally and transnationally, furthering the free-trade goal of "product tested once and accepted everywhere".

## Standards for Dyes and Stains used in Biology and Medicine

A primary objective in bacteriology, clinical cytology, haematology and histopathology is the development of procedures providing stained material whose microscopic appearance is reproducible from sample to sample, and indeed country to country. This goal applies when staining with dyes, fluorochromes, antibodies or nucleic acid probes. To achieve this, biological specimens must be obtained in a controlled manner, with samples resulting also treated in a controlled manner. For example, with paraffin sections sample processing involves fixation, dehydration, clearing, embedding and section cutting. All steps must be standardized, including pre-analytical, analytical and post-analytical procedures. Tables 1–3 show steps that already have associated standards and those which require standards.

**Table 1.** Pre-analytic procedures for biological samples, with available relevant standards.

| Pre-Analytic Procedures | Currently Available Standards |
|---|---|
| Conditions for taking biological samples | CLSI: AUTO 0-A[1]; CLSI: GP 23-A[2]; CLSI: GP 33-P[3] |
| Time delay from obtaining sample to first processing step | Standard not available |
| Fixation | CLSI: GP 28-A[4] |
| Dehydration | Standard not available |
| Clearing | Standard not available |
| Embedding in paraffin | Standard not available |
| Section cutting and handling of sections prior to the analytic procedure | CLSI: I 02-A2[5] |

[1]CLSI: AUTO 0-A: Laboratory automation: Bar codes for specimen container identification; [2]GP23-A: Nongynecologic cytologic specimens: Collection and cytopreparatory techniques; [3]GP33-P: Accuracy in patient and sample identification; [4]GP28-A: Microwave device use in the histology laboratory; [5]I 02-A2 Temperature calibration of water baths, instruments, and temperature sensors.





**Table 2.** Analytic procedures for biological samples, with available relevant standards.

| Analytic Procedures | Currently Available Standards |
|---|---|
| Dewaxing of paraffin sections | Standard not available |
| List of reagents used in selected procedure with information on necessary purity and use of in vitro diagnostic reagents | EN 12376:1999[1]; ISO 19001:2002[2]; EN 13640:2002[3]; CLSI: C 03-A4[4]; CLSI: GP 06-A[5] |
| Selected procedure listing sequential steps with information on temperature and duration | CLSI: EP 25-P[6]; CLSI: GP 05-A2[7]; CLSI: GP 20-A2[8]; CLSI: H 22-P[9]; CLSI: I 03-A[10]; CLSI: I 08-P[11]; CLSI: MM 04-A[12]; CLSI: MM 05-A[13]; CLSI: ILA 28-A2[14] |

**Table 3.** Post-analytic procedures for biological samples, with available relevant standards.

| Post-Analytic Procedures | Currently Available Standards |
|---|---|
| Microscopic evaluation | Standard not available |
| Report with differential diagnostic considerations | Standard not available |

Are dyes and stains in vitro diagnostic reagents? – Most dyes in biomedical laboratories were originally textile dyes. Nevertheless biomedical users should understand that when they perform diagnostic work on material from humans, this work is in vitro diagnostic. Consequently, reagents used should be classified as in vitro diagnostic, and if marked "for in vitro diagnostic use" should conform to quality standards, including ISO 19001:1999 and EN 12376:1999.

Standards concerning dyes and stains – These include the practically identical EN 12376 and ISO 19001 both named In vitro diagnostic medical devices – Information supplied by the manufacturer with in vitro diagnostic reagents for staining in biology. Their aim is to ensure that dyes, stains, chromogenic reagents, antibodies, nucleic acid probes and other reagents actually contain what is on their labels and inserts; and are present in appropriate states, amounts and concentrations to give acceptable and reproducible results when used in valid procedures. When applied to human material for diagnostic purposes, reagents listed are in vitro diagnostic reagents.

## What Demands do these Standards Place on the Manufacturer?

EN 12376/ISO 19001 specify requirements for reagents used in staining in biomedicine, and apply to manufacturers, suppliers and vendors. Information supplied by manufacturers, as specified in the standards, is a prerequisite for achieving comparable and reproducible results. Informative annexes accompany standards, demonstrating how supplier's information may be presented for several in vitro diagnostic reagents.

[1]EN 12376:1999 In vitro diagnostic medical devices - Information supplied by the manufacturer with in vitro diagnostic reagents for staining in biology; [2]ISO 19001:2002 In vitro diagnostic medical devices - Information supplied by the manufacturer with in vitro diagnostic reagents for staining in biology; [3]EN 13640:2002 Stability testing of in vitro diagnostic reagents; [4]Preparation and testing of reagent water in the clinical laboratory; [5]Inventory control systems for laboratory supplies; [6]Evaluation of stability of in vitro diagnostic method products; [7]Clinical laboratory waste management; [8]Fine-needle aspiration biopsy (FNAB) techniques; [9]Histochemical method for leukocyte alkaline phosphatase; [10]Standards for relating spectrophotometer performance characteristics to analytical goals; [11]Determining performance of volumetric equipment; [12]Quality assurance for immunocytochemistry; [13]Nucleic acid amplification assays for hematopathology; [14]Quality assurance for design control and implementation of immunohistochemistry assays; Approved guideline.

## The Standards Require Manufacturers to Provide

- Product name and other identifiers
- For each batch
  - > Molecular formula and molar mass, including counter-ion
  - > If coloured:
    - Molar absorbance, or content of pure dye
    - Wavelength/wave number of maximum absorbance
    - TLC, HPLC or HPTLC data
- Intended use
  - > Types of biological material, and pre-staining handling:
    - Whether cell or tissue samples can be used
    - Whether frozen or chemically fixed material can be used
    - Tissue processing protocol
    - Which embedding media can be used
  - > Manufacturers procedure for testing reactivity of reagent
  - > Expected results using reaction procedure on suggested type of material, as outlined by the manufacturer
  - > Notes: positive and negative control tissues, and interpretation of results
  - > References to published results
- Additional requirements for specific reagent types
  - > Fluorochromes:
    - Selectivity
    - Excitation/emission wavelengths
    - Fluorochrome/protein ratio for fluorochrome-conjugated antibodies
  - > Metal salts:
    - Systematic name
    - Purity
  - > Antibodies:
    - Antigen for raising antibody, if appropriate antibody CD number
    - Type and part of (macro)molecule detected
    - Intracellular, cellular and tissue localizations
    - Cross reactivity with other epitopes
    - Monoclonal antibody: clone, production method, immunoglobulin subclass and light chain identity
    - Polyclonal antibody: animal host, whole serum or gammaglobulin fraction used
    - Physical state, total protein, specific antibody; if in solution, nature/concentration of diluent/medium
    - If applicable, molecular linkers or extenders present
    - Declaration of purity, purification techniques and impurity detection methods
    - References to published results
  - > Nucleic acid probes:
    - Base sequence, strandedness
    - Molar mass or number of bases; if applicable, number fraction of GC base pairs
    - Marker; if non-radioactive, point(s) of attachment to the probe, substance fraction of probe marked
    - Target gene (DNA or RNA sequence) detected
    - Physical state (lyophilized powder or solution) and amount (pg/ml or pmol/ml): if in solution, nature/concentration of diluent/medium
    - Declaration of purity, detection and purification methods, e.g., HPLC
    - Publications concerning source description of DNA sequence, existence of known patents, information on applications





## Future Expectations

Standards ISO 19001:2002 and EN 12376:1999 are being revised, and will become ISO 19001:20xx, where 20xx represents the year for final approval. Informative Annex A, with examples of information expected from manufacturers for different procedures, is being reworked into product insert format.

As its name implies, this standard concerns information supplied by the manufacturers deemed necessary when the reagents in question are to be used as in vitro diagnostic reagents in medical laboratories. Discussions between ISO/TC 212 Clinical laboratory testing and in vitro diagnostic test systems experts indicated that routine users will profit from being informed how to select correct reagents for performing in vitro diagnostic work in the fields of microscopic bacteriology, cytology, haematology and histology. Consequently a group will operate under ISO/TC 212/WG 3 In vitro diagnostic products with the assistance of ISO/TC 212/WG 1 Quality and competence in the medical laboratory to prepare a new standard.

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# Chapter 7 | Pigments and Minerals

**Susie Smith, BS, HTL (ASCP) and Laurie J. Hafer, PhD**

**Revised and updated by John Kiernan MB, ChB, PhD, DSc**

This chapter is concerned with some non-living intracellular inclusions and extracellular deposits, especially those that are commonly encountered in human histopathology.

## Pigments

In the science of colorants, a pigment is an insoluble white or colored substance that can be suspended in a liquid for application to a surface (Latin *pigmentum*, paint) or incorporated into a solid material such as plastic, rubber or wax. Examples are carbon in Indian and printer's inks and in rubber, and titanium dioxide in white paint. In biology the word pigment is much more loosely used; it includes insoluble materials that may be colored or visible by virtue of being refractile or birefringent. Pigments in the biological sense include chlorophyll and the anthocyanins and carotenoids responsible for colors other than green in plants. These plant "pigments" dissolve in alcohol and water, and are not seen in paraffin sections. Hemoglobin, the colored oxygen-carrying metalloprotein of blood, is insolubilized by fixation. As a basic protein, hemoglobin stains strongly with anionic dyes such as eosin.

In pathology, abnormal insoluble deposits, yellow, brown or black without staining, and not distinctively stained with H&E, are frequently encountered. Pigments play an important part in the diagnosis of diseases and conditions such as gout, kidney and gallstones, jaundice, melanomas, albinism, hemorrhage and tuberculosis. In a section of tissue, the term pigment refers to a material that has color and can be seen without staining. It can be either normal or pathological. Pigments are identified either by their color, size and shape or by chemical testing. For example, if a chemical test that gives a blue product is applied to a yellow pigment, the result may be a green color. Pigments can be placed in three categories: artifacts of fixation, exogenous and endogenous (Table 1). Some fixative-induced and endogenous pigments are described in this chapter.

**Table 1.** Common Pigments.

| Fixation Artifacts | Exogenous Pigments | Endogenous Pigments |
|---|---|---|
| **Formalin pigment** *(in and near blood)* Black<br><br>**Mercury pigment** *(everywhere in the tissue)* Black<br><br>**Picric acid** *(everywhere in the tissue)* Yellow<br><br>**Osmium dioxide** *(in most parts of tissues; darkest in fat cells, lipid droplets)* Gray to black | **Carbon** *(in lungs and associated lymph nodes, especially of city dwellers, coal miners)* Black<br><br>**Inks used for tattoos** *(skin)* Various colors<br><br>**Melanosis coli** *(lipofuscin-like deposits in colonic mucosa of habitual users of anthraquinone purgatives)* Brown | **Melanins** *(in normal skin, eye, some neurons; melanomas)* Brown to black<br><br>**Hemosiderin** *(in cells that have phagocytosed blood; liver in diseases of iron metabolism)* Dark yellow to brown<br><br>**Lipofuscin** *(in older people, in cardiac muscle cells, neurons etc.)* Yellow to light brown<br><br>**Sodium urate** *(in lesions of gout)* Not a pigment but included here for convenience |

## Fixation Artifacts

**Formalin pigment,** also called acid hematin, is formed in specimens fixed in formaldehyde solutions at pH below 5.5, especially after several weeks in such a solution. Alkaline formaldehyde (pH above 8) may cause the same problem. This “pigment”, a product of degradation of hemoglobin, diffuses for a short distance from its site of formation and settles out as an insoluble product: abundant small black dots wherever blood is present in a section. This artifact does not occur when the fixative is a neutral, buffered formaldehyde solution. Formalin pigment can be removed by treating the sections with a saturated solution of picric acid in ethanol for two hours.

**Mercury pigment** consists of abundant uniformly distributed tiny dark crystals, probably containing mercurous chloride. These deposits occur in all tissues fixed in liquids containing mercuric chloride, including “B5", Heidenhain’s SUSA and Zenker’s fluid. The material is removed by brief treatment of sections with an iodine solution, followed by sodium thiosulfate to remove the brown iodine stain.

## Endogenous Pigments

### Melanins

Melanins are brown to black (eumelanin) or yellowish (pheomelanin) polymeric pigments formed from the amino acid tyrosine by a series of oxidations and other reactions (Figure 1) in skin cells, hair, eyes (retina, iris and choroid) and in the cell bodies of some neurons, notably in the substantia nigra and locus coeruleus of the brain stem. In skin, **melanocytes**, branched cells at the junction of the epidermis with the dermis, synthesize the pigment and package it into protein-containing granules called melanosomes. The melanosomes are then extruded and taken up by epithelial cells in the deepest layer of the epidermis, an event known as pigment donation. Pathologically, melanin is found in the cells of malignant melanomas and various benign nevus tumors derived from melanocytes.

Melanins are not extracted by acid treatments that remove formalin pigment. Melanins are, however, bleached by oxidation with an aqueous solution of either hydrogen peroxide or potassium permanganate, allowed to act for 12 to 24 hours. The latter reagent must be followed by oxalic acid, to remove the brown manganese dioxide that deposits on the sections. Histochemical staining of melanin is accomplished by exploiting the chemical reducing properties of this pigment. Two methods are commonly used: The Masson-Fontana silver method and Schmorl’s ferric ferricyanide reaction.

In the Masson-Fontana technique, the slides are immersed in a solution containing silver diammine, $Ag(NH_3)_2^+$, ions. This is made by adding ammonium hydroxide (strong ammonia solution) dropwise to 5% aqueous silver nitrate. A brown precipitate of silver oxide is first formed, but dissolves as the diammine is formed by addition of more ammonia. Finally, a drop of silver nitrate is added, making the solution opalescent (from AgO) and ensuring that there is no excess of $NH_3$. Reducing groups in tissues convert $Ag(NH_3)_2^+$ to colloidal metallic silver, which is brown or black. Optionally the color can be intensified by immersion in “gold chloride”, replacing the silver with gold. The “gold chloride” of histology is either tetrachloroauric acid , $HAuCl_4$ or its sodium salt. A contrasting counterstain such as neutral red or the aluminum complex of nuclear fast red, is applied to show the tissue architecture. The reactions of formation and reduction of silver diammine and gold toning are summarized in Figure 2.

Substances in tissues that reduce silver diammine are said to be **argentaffin**, they include melanin and phenolic compounds, notably 5-hydroxytryptamine in certain endocrine cells of the epithelium of the stomach and small intestine, known as enterochromaffin cells.

In Schmorl’s reaction, sections are immersed in a solution containing ferric chloride and potassium ferricyanide. Melanin reduces ferric ions ($Fe^{3+}$) to ferrous ions ($Fe^{2+}$), and the ferrous iron then combines with ferricyanide. The expected product is ferrous ferricyanide, a blue pigment known as Turnbull’s blue. In fact, the product is the same as Prussian blue, which is formed when ferric ions combine with ferrocyanide ions. Prussian blue is ferric ferrocyanide, $Fe_4[Fe(CN)_6]_3$, occurring in crystals that also contain water molecules and sodium or potassium ions. The color is associated with the occurrence of iron in both oxidation states (+2 and +3) in the same molecule. The final step is application of aluminum-nuclear fast red or a similar counterstain. As with the Masson-Fontana method, this reaction is not melanin-specific and may stain other elements, such as argentaffin and chromaffin cells and some types of lipofuscin.





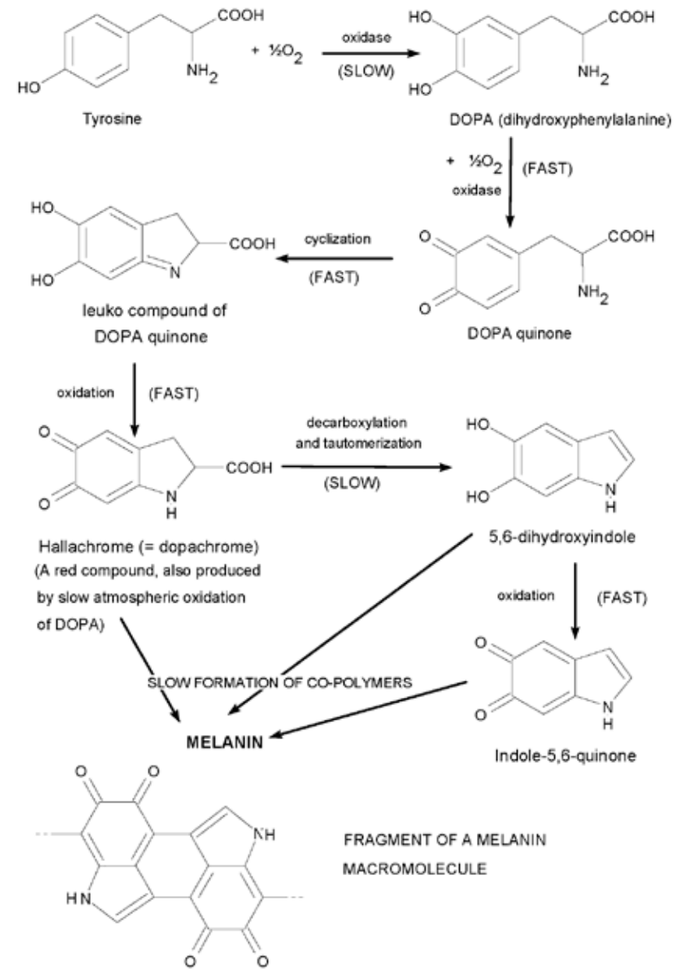


**Figure 1.** Biosynthesis of melanin, starting with the amino acid tyrosine. This simplified scheme omits several intermediates and alternative metabolic pathways. Several of the reactions occur without the need for catalysis by enzymes. This scheme applies only to eumelanin, the pigment in fair, brown and black skin and hair. Related pigments such as neuromelanin (in aminergic neurons) and pheomelanin (in “red” hair) are formed by other metabolic transformations of dopa and dopachrome.

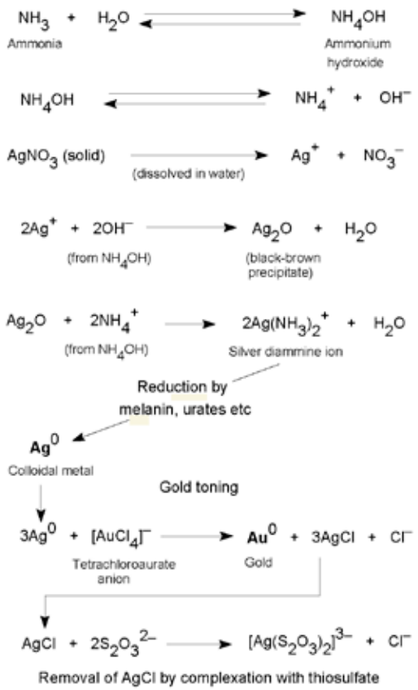


**Figure 2.** Chemical reactions in the formation of silver diammine ("ammoniacal silver nitrate"), its reduction in sections of tissue, and gold toning.

## Hemosiderin

Hemosiderin, an aggregate of proteins containing iron ions, is a yellow to brown pigment seen in cells that have phagocytosed and degraded hemoglobin. A major component is ferritin, a protein that forms hollow particles about 10 nm in diameter, each containing 2000-4500 $Fe3^{+}$ ions. Ferritin, which is not colored, is not the only component of hemosiderin. Glycoproteins are also present, and the deposits give a positive periodic acid-Schiff reaction. Some of the pathological conditions with hemosiderin deposits are hemorrhages of any kind, hemolytic anemia, some liver diseases, the lungs in congestive heart failure, and in the liver, pancreas and skin in hemochromatosis. This last condition is a group of metabolic diseases in which iron absorption from the small intestine, normally regulated by demand, is uncontrolled. Iron cannot be excreted, and the excess accumulates as hemosiderin in macrophages.

Hemosiderin is insoluble in alkalis and soluble in either 0.4 M aqueous oxalic acid (six hours) or 0.06M sodium dithionite in acetate buffer, pH 4.5 (five minutes). These treatments remove the visible unstained pigment and prevent histochemical staining, which is based on detection of iron by formation of Prussian blue, using the method of Perls.

The histochemical reaction for protein-bound iron traditionally had two stages: release of ferric ions by denaturing the binding proteins with hydrochloric acid, followed by treatment with potassium ferricyanide solution to produce Prussian blue. Diffusion of the released $Fe^{3+}$ resulted in blue deposits around the pigment-containing cells. In 1867, Max Perls devised a stable reagent containing concentrations of acid and ferrocyanide that optimally precipitated the $Fe^{3+}$ within the cells. Prussian blue is insoluble in acids but soluble in alkalis. The commonly used red counterstains are applied from acid solutions; neutral red and the aluminum complex of nuclear fast red both contrast well with Prussian blue. Eosin can be used if contrast between nuclei, cytoplasm and collagen is not needed. Figure 3 shows iron-containing cells in a section of bone marrow.





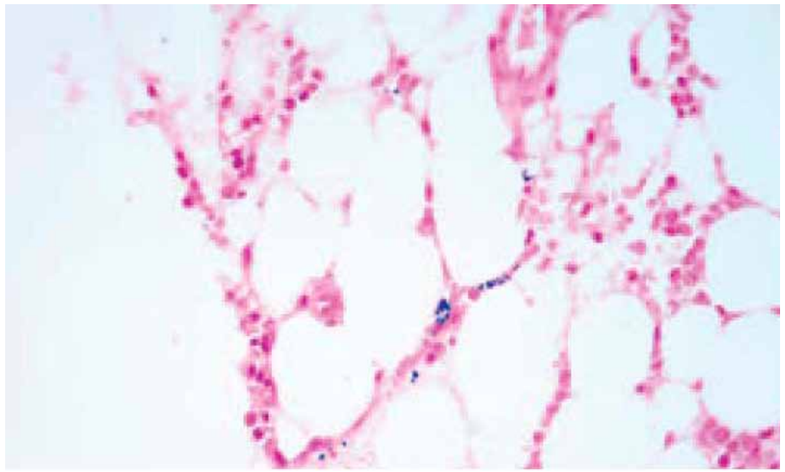

**Figure 3.** Perls' method demonstrates iron (blue) in this bone marrow specimen. The counterstain is aluminum-nuclear fast red.

Most of the iron in any vertebrate animal is in the heme of hemoglobin, so tightly bound that it cannot be released for staining by any method that would not destroy a section on a slide. Histochemical staining for iron with Perls' method is therefore tantamount to staining hemosiderin. There are chemical tricks to enhance the sensitivity, allowing staining of normal cells containing iron-binding and iron-transporting proteins, such as the duodenal epithelial cells through which iron is absorbed. The simplest of these is Quincke's method, which dates from 1887. Slides are first immersed in an ammonium sulfide solution (malodorous, alkaline, removes sections from slides), which attacks the metal-binding proteins, reduces $Fe3^{+}$ to $Fe^{2+}$ and immediately precipitates ferrous sulfide, FeS, at the sites of reduction. FeS is an almost black compound, but it shows as gray in sections 5-10 µm thick. Subsequent immersion in a potassium ferricyanide solution generates a blue pigment, simplistically Turnbull's blue but actually the chemically identical Prussian blue. It is also possible to amplify the blue deposits, taking advantage of the fact that they catalyze the oxidation of 3,3'-diaminobenzidine (DAB) by hydrogen peroxide. The brown polymer formed by oxidation of DAB is permanent, whereas Prussian blue fades after several months.

## Lipofuscin

Lipofuscin is a yellow-brown to reddish-brown pigment, found within cells in many parts of the body. The nickname "wear-and-tear" pigment (*Abnutzungspigment*) reflects the accumulation of lipofuscin with advancing age in cells that are either terminally differentiated (e.g. cardiac muscle fibers, neurons) or are infrequently replaced (e.g. adrenal cortex, liver). The pigment is formed from fragments of membranous organelles, which become permanently sequestered in lysosomes. This accumulation of lipofuscin does not appear to interfere with cellular function. Pathologically, lipofuscin is present in the neuronal ceroid lipofuscinoses, a group of several rare inherited disorders within the large category of lysosomal storage diseases.

Normal lipofuscin contains fatty acids that are closely associated with protein. This association allows most of the pigment to remain in place during passage through the solvents used in paraffin embedding. The staining properties of lipofuscin are attributable to hydrophobic character, the presence of unsaturated (—CH=CH—) linkages and of glycols and aldehydes produced by their oxidation, and the presence of weak acid (carboxy, phosphate) groups. Different types of lipofuscin, including adrenal lipofuscin, cardiac lipofuscin, hemofuscin and ceroid, have been described on the basis of applying a panel of staining methods that detect these physical and chemical properties.

It is recommended that more than one special staining technique be performed to confirm the presence of lipofuscin and to distinguish it from hemosiderin or other pigments that might also be present in the tissue.

Some staining techniques applicable to paraffin sections are listed in Table 2.

**Table 2.** Some properties of lipofuscin.

| Staining Method | Appearance of Lipofuscin | Explanation |
|---|---|---|
| None | Brown, autofluorescent; not bleached by $H_2O_2$ | Possibly from compounds formed by reaction of aldehydes with amino groups, and from flavoproteins |
| Oil red O | Red | Affinity of hydrophobic dye for lipid |
| Sudan black B | Black | Affinity of hydrophobic dye for lipid |
| Cationic dyes (azure A, ethyl green etc, pH 4. | Positive | Carboxy groups of fatty acids |
| PAS | Pink-purple | Aldehydes from oxidized sites of unsaturation |
| Ziehl-Nielsen stain (basic fuchsine with phenol, followed by acid-alcohol) | Ordinary lipofuscin negative. Red acid-fast staining of ceroid[1] | Lipoprotein retards extraction of cationic dye bound to fatty acids |
| Masson-Fontana | Brown; variable | Aldehydes from oxidized sites of unsaturation |
| Schmorl's reaction | Blue-green | Uncertain identity of reducing groups |
| Oxidation followed by aldehyde-fuchsine | Purple (Figure 4) | Probably carboxy groups generated from oxidized unsaturated sites |

[1]Ceroid is a type of lipofuscin with the additional property of acid-fast staining.

## Urates

Two purines, adenine and guanine, constitute half of the nucleotide bases of DNA and RNA. The purines released from dead cells are largely salvaged and incorporated into new nucleotides, but small quantities are transformed to xanthine, which is oxidized to uric acid. These enzyme-catalyzed reactions occur principally in the liver; the uric acid is released into the blood and excreted in the urine. An increased circulating level of uric acid (hyperuricemia) can result from metabolic errors in the recycling of purines or from insufficient excretion by the kidneys. In saline solutions at physiological pH, 99% of uric acid is present as the biurate ion. Sodium biurate (monosodium urate) is, however, sparingly soluble (5 to 10 mg/100 ml, depending on the method of measurement). This is not much higher than the normal concentration in blood and extracellular fluids (3 to 7 mg/100 ml), and hyperuricemia is associated with the precipitation of crystals of sodium biurate, which form principally in joint and in the kidneys, a condition known as gout. Larger accumulations of the crystals in soft tissues, especially near joints, are known as tophi.

Despite their low solubility, sodium biurate crystals can be dissolved by aqueous fixatives, so specimens are fixed in 95% ethanol. Examination with polarizing optics shows needle-like birefringent crystals.

Histochemical identification is possible because uric acid is a strong reducing agent (Figure 5). It can therefore be demonstrated using the two methods discussed for melanin; urate crystals reduce silver diammine or ferric ferricyanide more rapidly than other tissue components. Gomori's methenamine-silver method, can also be used to identify urate crystals in tissue sections. Its action is slower.





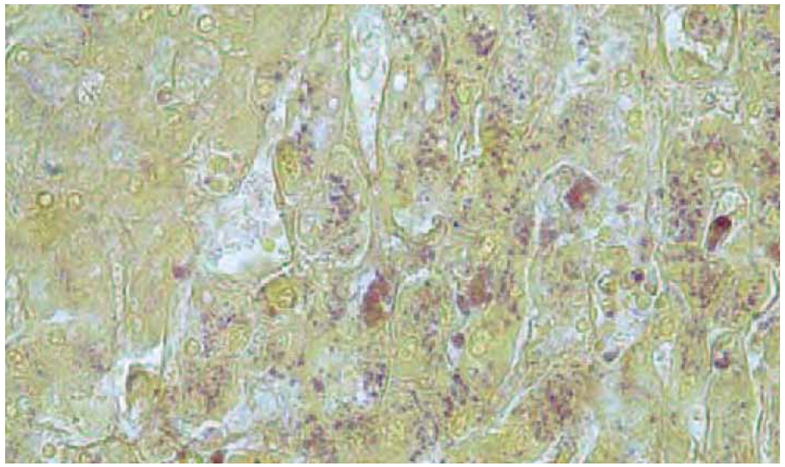

**Figure 4.** Lipofuscin in cells of the liver. Gomori's aldehyde-fuschsine, applied after oxidation with potassium permanganate and treatment with oxalic acid to remove deposited $MnO_2$. The counterstain is tartrazine, a yellow anionic dye.

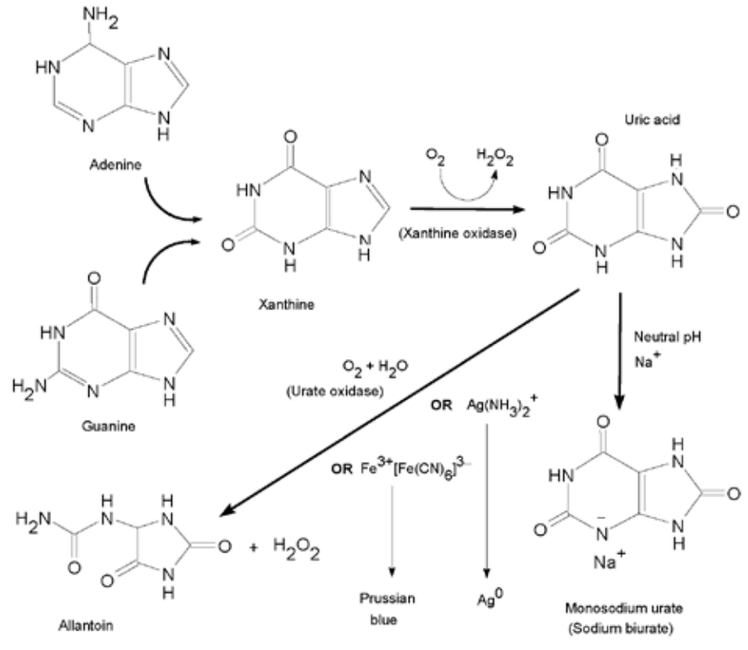


**Figure 5.** Formation of uric acid and sodium biurate from purines, and the chemical oxidation of uric acid. The enzyme urate oxidase occurs in all mammals other than monkeys, apes and man. Sodium urate crystals in tissues are easily oxidized by silver diammine or ferric ferricyanide, with concomitant deposition of silver (black) or Prussian blue.

### Bile Pigments

Human red blood cells have an average lifespan of 120 days, after which they are degraded in the spleen and bone marrow, and most of their components are recycled and used in the production of new red blood cells. Removal of iron from the heme of hemoglobin results in the formation of biliverdin, a green compound. Biliverdin is transported to the liver, where is it further reduced to bilirubin, which has an orange color. Bilirubin is then removed from circulation in the blood and secreted into the duodenum as a component of bile.

Biliverdin and bilirubin are considered bile pigments. Bile pigments can vary in color from yellowish-brown to green. Pathologically, excess bile pigment is seen in patients with liver failure, hemolytic anemia, or when there is an obstruction in the flow of bile from the liver. All these conditions are associated with jaundice, a yellow coloration of the skin due to bilirubin. In the liver, bile pigments appear in hepatocytes as yellow-brown globules. For the pathologist, it is sometimes necessary to distinguish bile pigments from lipofuscin, particularly in cases of possible sepsis in patients with liver transplants. Hematoidin is a brown pigment similar but not identical to bilirubin, found in sites of hemorrhage or infarction.

Traditional tests for bile pigments in tissue were based on the test developed by Leopold Gmelin (1788-1853) for detecting bilirubin in urine: transient colors (green, blue, violet, red, yellow) form during oxidation by concentrated nitric acid. Another traditional method is André Fouchet's reaction, first introduced in 1917, also for urine. Adapted as a histochemical method by M.J.Hall in 1960, this provides the most reliable and reproducible special staining technique for demonstrating bile pigments. It is applied to paraffin sections of formaldehyde-fixed tissue. Bilirubin is oxidized to biliverdin by treating the sections with Fouchet's reagent, a solution containing ferric chloride and trichloracetic acid. The sections are then counterstained with Van Gieson's solution. Only bile and bile pigments in the liver are detected, when stained green with this method. Hematoidin in other locations does not give the reaction. (Van Gieson's solution is 0.1% acid fuchsine in saturated aqueous picric acid; it colors collagen fibers red and everything else in the section yellow.)

Small amounts of bile pigments are lost during routine tissue processing and staining because of their slight solubility in organic solvents. Large deposits of bile pigments, however, can resist these processing procedures. It is recommended that two known positive control sections be processed with the test section. Both sections should be oxidized with Fouchet's reagent, but only one should be counterstained with Van Gieson's mixture. Fouchet's reagent should be prepared on the day it is to be used.





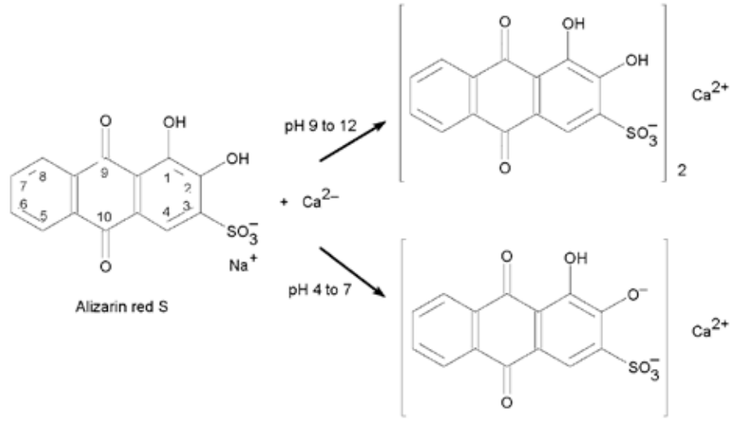


**Figure 6.** Combinations of calcium ions with alizarin red S. An earlier notion of chelation of $Ca^{2+}$ by oxygen atoms on carbons 1 and 9 is no longer accepted.

## Minerals

In histology and histopathology the word minerals is applied to substances detected by forming colored reaction products specific to metal ions or inorganic anions. The most common minerals that can be demonstrated by special staining techniques are calcium, iron and copper. The previous section detailing identification of hemosiderin encompasses the detection of iron. Special staining techniques for calcium and copper are now discussed.

### Calcium

Calcium is present in hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, the insoluble mineral of bones and teeth. Abnormal deposits of calcium phosphate or carbonate can be associated with necrotic tissue in lesions of atherosclerosis, hyperparathyroidism, nephrocalcinosis, sarcoidosis, tuberculosis, and in some tumors. Calcium phosphate crystals can form in the cartilage of joints in a condition known as chondrocalcinosis or pseudogout. Acidic fixatives such as Bouin's fluid have the potential to dissolve calcified deposits and must therefore be avoided. Neutral buffered formaldehyde is suitable. There are many ways to stain calcium, but only two methods are routinely used in histopathology. These are alizarin red S and the von Kossa technique.

Alizarin red S is an anionic anthraquinone dye that forms sparingly soluble salts with calcium ions (Figure 6). In the original techniques published in the 1950s (methods of Dahl and of McGee-Russell) alizarin red S is used at pH 4.8 or 6.1 and gives an orange color with calcified deposits. The yellow component is attributed to impurities in the dye. Moreover, diffusion of the colored product is usually evident, indicating partial dissolving of calcium phosphate or carbonate before precipitation of $Ca^{2+}$ by the dye. Amino groups of proteins in the tissue also bind the dye and must be removed by differentiation, leaving a pink background stain.

Application of alizarin red S at pH 9 (method of Puchtler) allows both the sulphonate group on carbon 3 and the ionized hydroxy group on carbon 2 to participate in salt formation with calcium (Figure 6). The impurities do not react at this higher pH, and the resulting calcium salt has a deep red color. An alkaline pH also suppresses protonation of amino groups, preventing most of the background staining and obviating the need for differentiation. A disadvantage of staining with any alkaline solution is the risk of sections detaching from the slides.

If staining is carried out at pH 4.8 or 6.1, the reaction must be monitored microscopically and stopped, usually after one or two minutes, before diffusion artifacts appear. With the stain at pH 9 diffusion does not occur and the slides may, with advantage, be left in the solution for an hour.

The von Kossa method indirectly localizes calcium in tissue by detecting phosphate or carbonate ions. The sections are placed in 1% aqueous silver nitrate. Calcium cations are replaced by silver, with transformation of $Ca_3(PO_4)_2$ to $Ag_3PO_4$ and of $CaCO_3$ to $Ag_2CO_3$. Both silver salts are easily reduced to the metal, a reaction most easily accomplished by placing the staining dish under a 100W light bulb or on a window sill. Calcified material is blackened in about 15 minutes and is sharply delineated. The sections are next immersed for a few minutes in a sodium thiosulfate solution, which removes silver that has complexed with protein and would eventually darken with storage of the slides. A light red counterstain such as neutral red or aluminum-nuclear fast red contrasts well with the silver deposits.

The only objects likely to be confused with sites of calcification in a von Kossa preparation are sodium biurate crystals, which reduce silver nitrate even in the absence of light. Urate crystals can be extracted with alkali before staining; a saturated aqueous solution of lithium carbonate (30 minutes) is used for this purpose because lithium biurate is more soluble in water than the corresponding sodium or potassium salts.

Silver nitrate is an expensive compound, but the solution may be reused and kept for several years in a brown glass bottle, provided that it is not contaminated with organic matter (such as bits of tissue sections) or with chloride, bicarbonate, carbonate, hydroxide, phosphate or sulfide ions (from inadequately cleaned glassware). Tap water kills silver nitrate solutions.

Another calcium compound that can form crystals in tissues is calcium oxalate. This condition, oxalosis, may be an inherited metabolic disease (rare), a consequence of renal failure or of poisoning by oxalate or ethylene glycol. Calcium oxalate crystals also occur in association with *Aspergillus* fungal infections in people with impaired immune function. The crystals are birefringent and not dissolved by acetic acid. The calcium is so tightly bound that it does not stain with alizarin red S. The von Kossa reaction is positive, but the best staining method is Yasue's technique. Sections are treated with 5% acetic acid to remove calcium carbonate and phosphate, then transferred to 5% aqueous silver nitrate. Silver displaces calcium ions and the resulting silver oxalate is stained with dithiooxamide, a reagent that combines with silver ions to form a dark brown polymeric chelate. Dithiooxamide will be discussed later as a histochemical reagent for copper.

## Copper

Copper is an essential nutrient, being a component of cytochromes and many oxidoreductase enzymes. Pathologically, the accumulation of copper is associated with Wilson's disease. This is a recessively inherited metabolic disorder in which a transporter protein in liver cells fails to move copper into the bile and fails to combine copper with ceruloplasmin, the copper-binding protein of blood plasma. Copper, associated with albumin and other proteins, accumulates in cells of the liver, cornea and corpus striatum of the brain in patients with Wilson's disease. Copper accumulations are seen also in primary biliary cirrhosis and some other liver disorders.

Two reagents are suitable for histochemical demonstration of copper by virtue of formation of colored complexes. Dithiooxamide (also known as rubeanic acid) gives a stable dark green polymeric product that can be mounted in a resinous medium. *p*-dimethylaminobenzylidene rhodanine (DMABR) gives a red product that dissolves in organic solvents and therefore requires an aqueous mounting medium. The structures of the compounds are shown in Figure 7. Both require long (overnight) incubation to develop the colors. DMABR is a more sensitive reagent than dithiooxamide and is generally preferred (Figure 8). The sensitivity of the dithiooxamide method can be increased by prolonging the incubation to 72 hours.





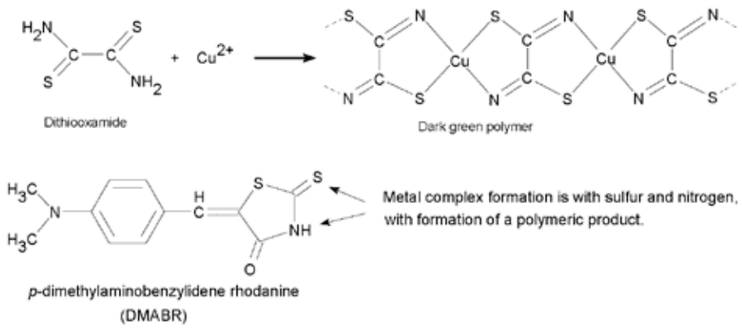


**Figure 7.** Dithiooxamide (rubeanic acid) and DMABR. The latter reagent is frequently and wrongly called "rhodanine", but rhodanine is a different compound that cannot be used for histochemical staining of copper. Several xanthene dyes with names that include "rhodamine" are likewise unrelated.

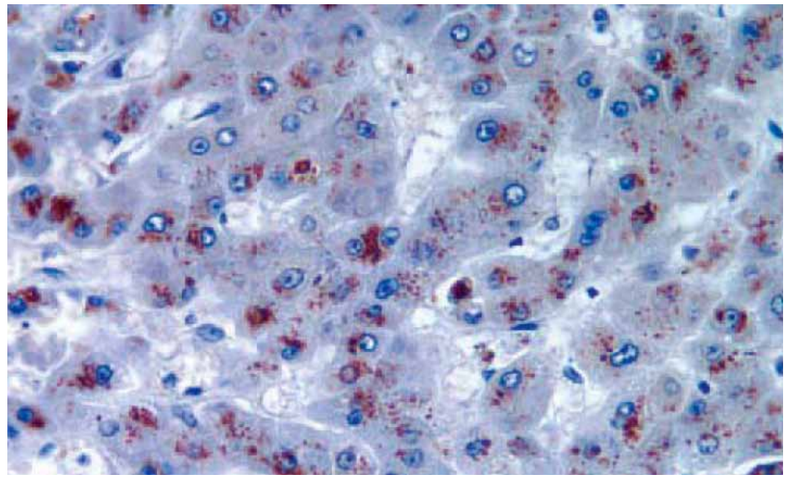

**Figure 8.** A section of liver stained by the DMABR method for copper (red) and counterstained with hemalum (blue).

**Further Reading**

This list includes books, review articles and a small selection of original papers.

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# Chapter 8 | Staining Sections of the Central Nervous System

**John A. Kiernan, MB, ChB, PhD, DSc**

Sections well stained with hemalum and eosin (H&E) display the cellular and microanatomical features of most normal and diseased organs. The method is valued especially by pathologists (1), who examine great numbers of slides and appreciate the simple rendition in two colors: a bold blue largely confined to nuclear chromatin, but insipid shades of pink for nearly everything else (2). H&E-stained sections of pieces of normal central nervous system (CNS) provide hardly any information about the cytoplasm of neurons and glial cells, though it is possible to recognize several cell-types on the basis of their stained nuclei. Special staining methods are needed to characterize different types of neurons and to reveal such elements as dendrites, axons, myelin sheaths and the cytoplasmic processes of glial cells (3, 4) (Fig. 1). Some of the traditional techniques, especially for glia, have largely been replaced by immunohistochemistry, but there is still a need for dye-based staining of neuronal cell bodies and myelin sheaths in neuropathology, research in neuroscience and the preparation of teaching materials for human and animal neuroanatomy.

## Nissl Staining

H&E shows the shapes and sizes of the cell bodies of neurons but little detail is visible in the perikaryon – the cytoplasm surrounding the nucleus. (Most of the cytoplasm of a neuron is in the dendrites and axon.) In 1884 Franz Nissl (1860-1919), then a medical student in Munich, discovered darkly colored granules in neuronal perikarya in sections of brain stained with methylene blue, a cationic (basic) dye. The abundance, size and distribution of the granules varied among different cells, providing data for the description of different types of neurons. Nissl published his technique and results in a series of papers in the mid-1890s (5,6). Within less than 10 years Nissl's name became an eponym for the cytoplasmic granules in neuronal perikarya and for most of the methods used for staining them with cationic dyes. The terms Nissl body and Nissl substance also evolved. In the early decades of the 20th Century some authors, perhaps disliking eponyms, used the word tigroid, from the striped appearance often seen in the perikarya of large neurons. The science of cytoarchitectonics – parcelation of the gray matter of the CNS into cell groups (nuclei) and layers (laminae) containing distinctive types of neuronal somata – is based on the study of Nissl-stained sections. Advances in electron microscopy and biochemistry in the 1950s showed that the Nissl bodies represented aggregations of rough endoplasmic reticulum, containing numerous ribosomes. Here, mRNA is translated and proteins are synthesized (7). The staining of these structures is due to high concentrations of rRNA.

Cationic dyes currently in favor for Nissl staining are cresyl violet, neutral red, thionine and toluidine blue, typically applied as 0.1% to 0.5% aqueous solutions adjusted to about pH 3. Some differentiation occurs during dehydration, which needs to be carefully carried out. A pale contrasting anionic counterstain is optional and preferably applied before the cationic dye. Blood stains such as Giemsa (8) can also be used to demonstrate Nissl substance.

Figures 2 and 3 show paraffin sections of human brain stem, 10 µm thick, stained with toluidine blue.

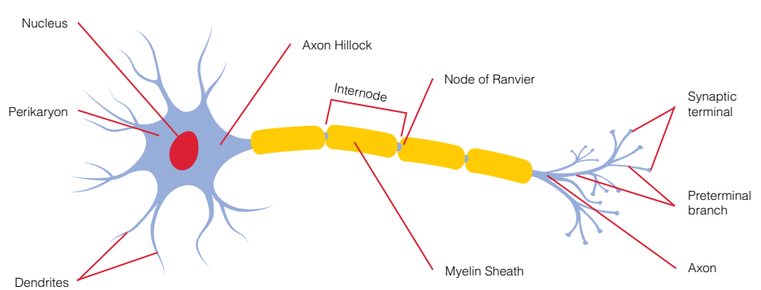


**Figure 1.** Diagram of a generalized neuron with a myelinated axon. The term "nerve fiber" means an axon together with its myelin sheath. Synaptic terminals may contact the dendrites, penkarya or preterminal axonal branches of other neurons.

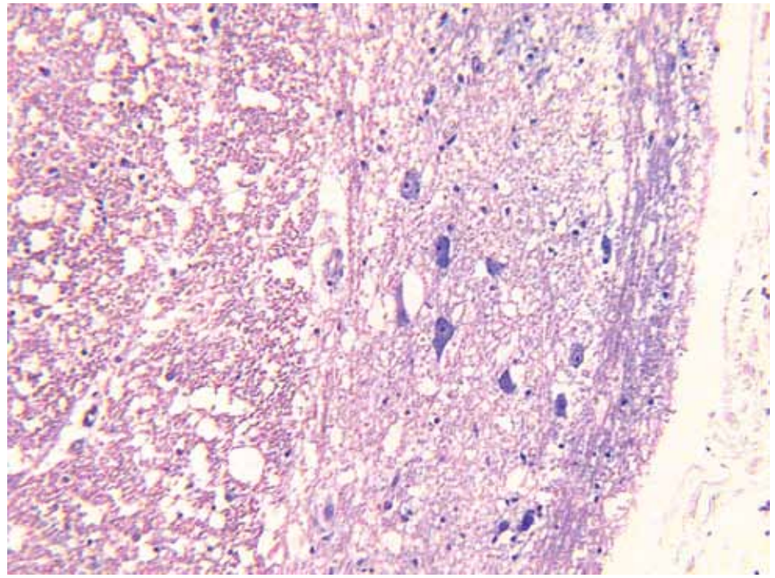

**Figure 2.** Near the ventral surface of the medulla, neurons of the arcuate nucleus are sandwiched between transversely sectioned descending fibers from the cerebral cortex (above) and transversely oriented arcuato-cerebellar fibers (below). Stained with toluidine blue to show nucleic acids and lightly counterstained with eosin Y.

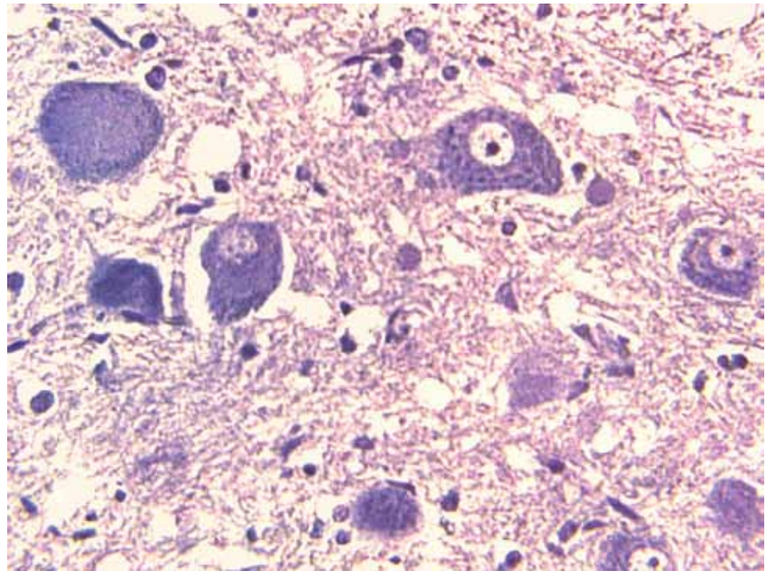

**Figure 3.** Neurons of the external cuneate nucleus in the medulla of the human brain, characterized by rounded cell bodies, often with eccentric nuclei. The axons of these neurons carry proprioceptive signals, derived from the upper limb, to the cerebellum. Stained with toluidine blue to show nucleic acids and lightly counterstained with eosin Y (pink background).





## Myelin Stains

The myelin sheath consists of compacted spirally wrapped layers of neuroglial surface membranes surrounding the axon of a neuron (Fig. 4). Myelin is rich in phospholipids and basic proteins. Each glial cell (a Schwann cell in the peripheral nervous system, or an oligodendrocyte in the CNS) provides myelin for a length of axon known as an internode. The intervening nodes are the only points at which the axonal surface membrane is in contact with extracellular fluid. Action potentials therefore "jump" from node to node, providing much more rapid conduction than is possible in an unmyelinated axon (7, 9). Although it is not part of the neuron, the myelin sheath degenerates if the cell body of its neuron dies, or if the axon is severed.

Sections stained for myelin are valuable aids in teaching the normal anatomy of the CNS. With no or low magnification the tracts of white matter show as dark areas whereas nuclei and other bodies of gray matter are colorless or palely stained. Myelinated fibers are present in gray matter, but at a lower density than in white matter because dendrites and the terminal branches of axons lack myelin sheaths. The principal pathological changes seen in stained white matter are regions of pallor, which may indicate bundles of degenerated axons (as, for example, following destruction of their cell bodies by a stroke) or foci of demyelination containing mostly intact but non-functional axons (as in lesions of multiple sclerosis).

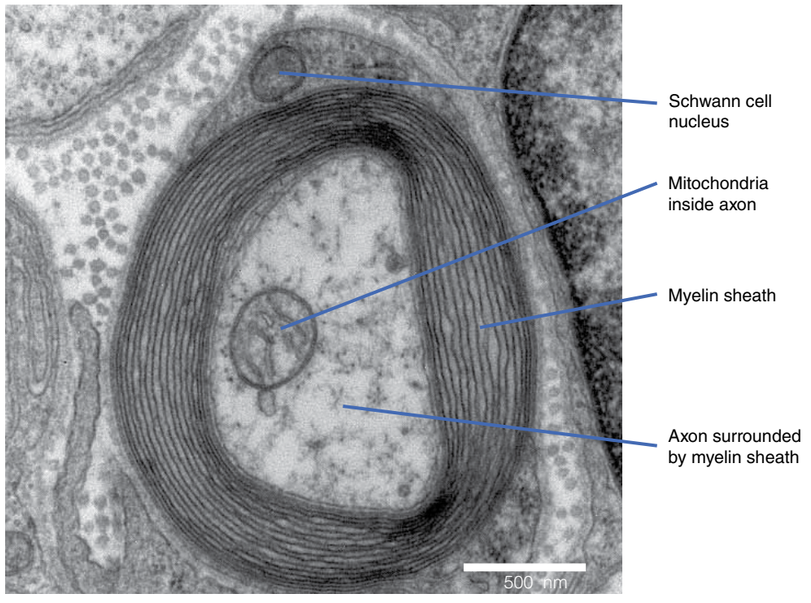


**Figure 4.** Transmission electron micrograph of a myelinated axon. Generated at the Electron Microscopy Facility at Trinity College, Hartford, CT. (Courtesy of Wikipedia).

**Figure 5.** Bundles of myelinated axons leaving the optic tract (lower left) and entering the lateral geniculate body of the thalamus. Stained by Weigert's chromium-hematoxylin method.

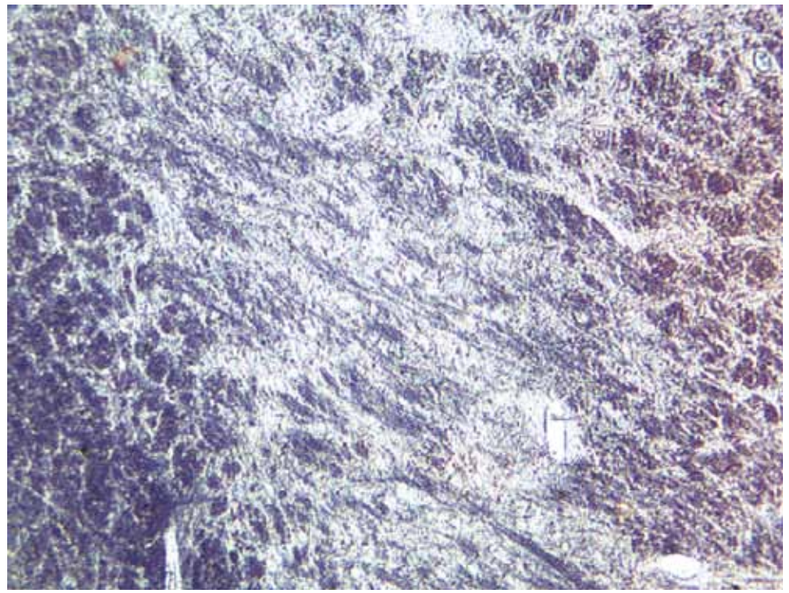

**Figure 6.** Cerebellothalamic fibers passing around and through the red nucleus of the human midbrain. Stained by Weigert's chromium-hematoxylin method.





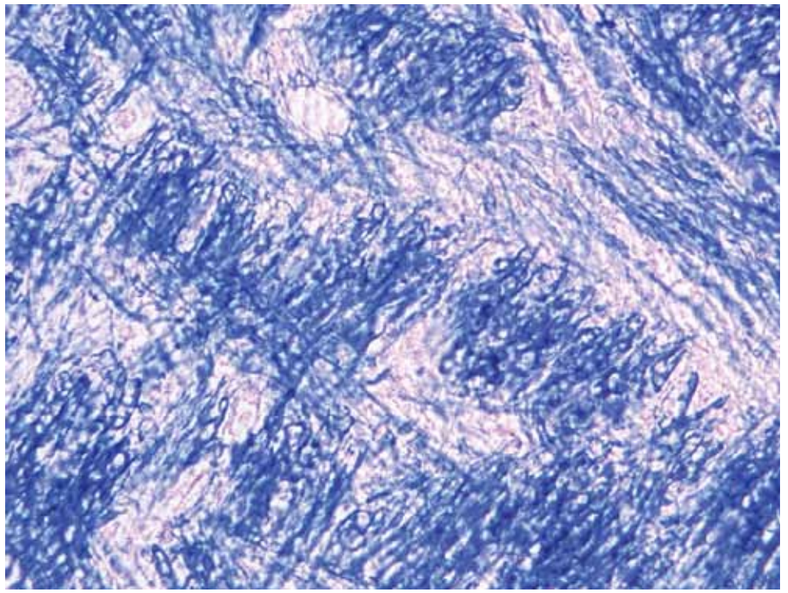

**Figure 7.** Myelinated axons in the decussation of the medial lemnisci in the midline of the human medulla. Stained with luxol fast blue MBS.

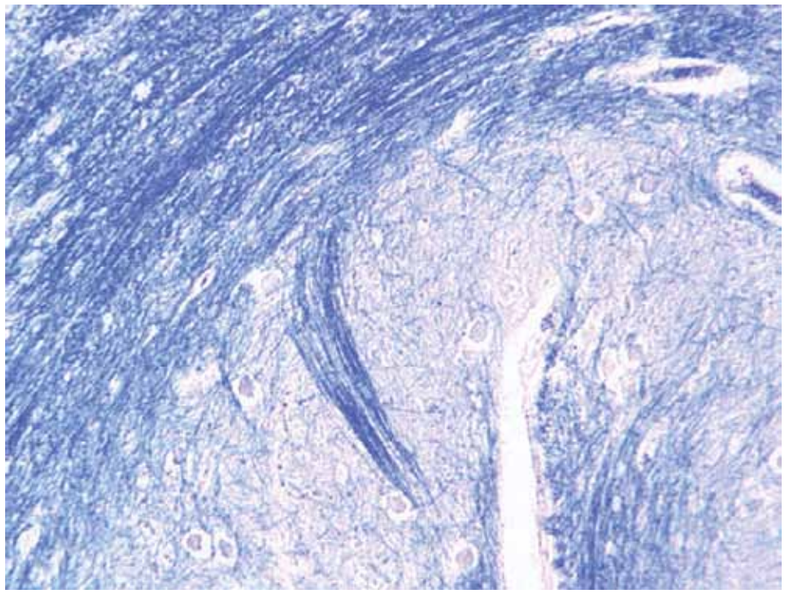

**Figure 8.** Myelinated axons in the human inferior olivary nucleus. At the top of the picture is the hilum of the nucleus, composed of fibres that will end in the contralateral cerebellar hemisphere. Stained with luxol fast blue MBS.

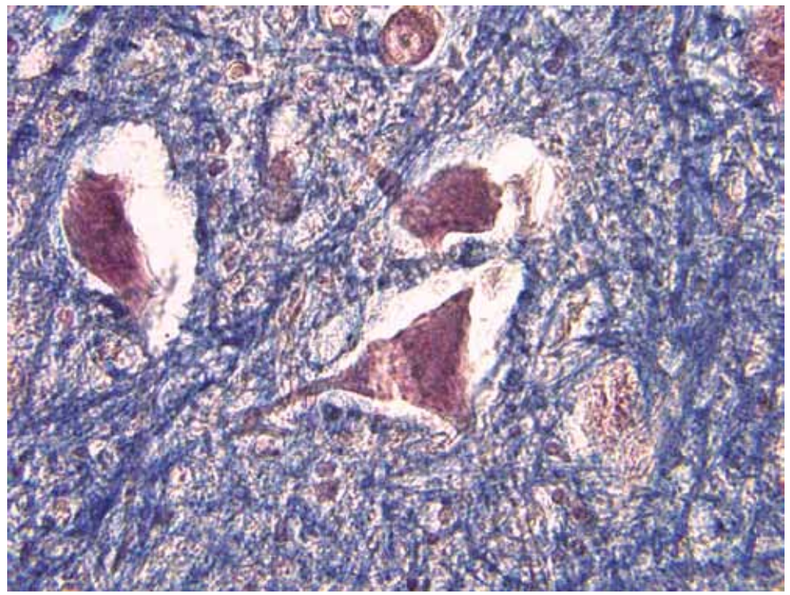

**Figure 9.** Motor neurons in the hypoglossal nucleus of the human brain stem. Stained with Page's iron-eriochrome cyanine R to show myelinated axons and neutral red to show DNA and cytoplasmic rRNA (Nissl substance). The spaces around the neuronal cell bodies are artifacts due to differential shrinkage that commonly occurs when formaldehyde-fixed central nervous tissue is dehydrated and embedded in paraffin wax.

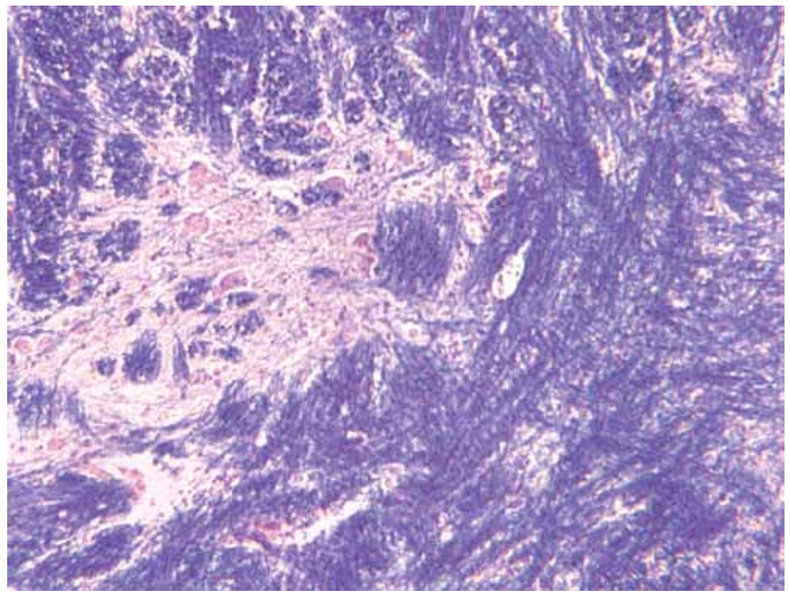

**Figure 10.** In the midline of the medulla of the human brain, the nucleus raphes pallidus contains neuronal cell bodies with scanty, peripherally located Nissl substance. The nucleus is named from its appearance as a pale area in a region otherwise occupied by tracts of myelinated axons. Stained with Page's iron-eriochrome cyanine R to show myelinated axons and neutral red for Nissl substance and nuclei.





There are many ways to stain myelin (9); three are illustrated here. In the original (1885) method of Carl Weigert (1845-1904), pieces of nervous tissue were fixed by immersion for several weeks in a potassium dichromate solution, which acts by rendering proteins and many lipids insoluble in water and organic solvents. (Formaldehyde, first used for fixation in 1891, does not have this action on lipids.) The Cr(VI) in dichromate ions is reduced to Cr(III), which is deposited at sites of oxidation of the choline-containing phospholipids of myelin (10). The chromated specimens were dehydrated, embedded in nitrocellulose, sectioned and stained with a solution of hematein ("ripened" hematoxylin), which formed a dark blue complex with the bound Cr(III). Simpler techniques have been used in more recent years, using Fe(III)-haematein ("iron hematoxylin") formulations (11, 12). These later methods, which are applicable to paraffin sections of formaldehyde-fixed tissue, probably demonstrate the basic proteins of myelin rather than residual phospholipids (9), but they are frequently called Weigert stains. Figures 5 and 6 show anatomical details of two areas in the human brain.

The myelin stain most frequently used in American laboratories is probably luxol fast blue MBS (13). This compound is the salt formed by an anionic dye (disulfonated copper phthalocyanine) and a ditolylguanidinium cation. This hydrophobic ion pair is insoluble in water but soluble in ethanol. Paraffin sections are stained overnight at 55-60°C. The dye anions are attracted to cationic sites in the tissue, coloring all components. The ditolylguanidinium cations remain in solution. Differentiation in aqueous alkali, which extracts loosely bound dye anions, is continued for 15 to 30 seconds, until gray matter is almost unstained. Basic proteins probably account for retention of the dye in myelin (9). There is a one-hour variant of this technique, in which the dye is dissolved in acidified methanol (12). In pathology laboratories staining with luxol fast blue is usually followed by counterstaining with cresyl violet or neutral red to show Nissl substance. Figures 7 and 8 show staining of the brain stem with luxol fast blue alone.

Myelin sheaths can be stained in 30 minutes with the iron-eriochrome cyanine R method, introduced in Britain by Kathleen Page in 1965 (14). The staining mechanism is probably similar to that postulated for luxol fast blue MBS (9). Unlike many of the dyes used in microtechnique, this one, a hydroxytriarylmethane with metal-binding properties, has many commercial uses, so it is inexpensive and likely to be encountered with any of a wide range of other names, including chromoxane cyanine R, solochrome cyanine R and CI Mordant blue 3. The last name identifies the dye unequivocally, as does its Colour Index number: 43820. Hydrated sections are immersed for 30 minutes at room temperature in a solution containing the dye and a ferric salt. All components of the tissue become strongly colored. Differentiation may be in either alkali, which acts in seconds, or a solution of a ferric salt, which takes 5-10 minutes to remove color from material other than myelin. Figures 9 and 10 show 10 µm sections of brain stem stained by Page's method for myelin, with neutral red as a Nissl counterstain.

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# Chapter 9 | Carbohydrate Histochemistry

**John A. Kiernan, MB, ChB, PhD, DSc**

Histochemically detectable carbohydrates occur as large molecules (constituting the **glycocalyx**) on the outside surfaces of all types of cell, as stored or secreted substances within some cells, and in the extracellular matrix. They are all macromolecules in which sugar units (monosaccharides) are joined in chains. Some of the structural features of sugars are summarized in Figure 1, which also shows an abbreviated nomenclature that is commonly used to show how the units are connected by glycoside linkages. The classification of macromolecular carbohydrates can be quite complicated because it has to take into account materials as diverse as cellulose, gastric mucus and cartilage matrix. Some human pathogens, notably the yeast *Cryptococcus*, are detected in tissues by histochemical methods for carbohydrates. This chapter is adapted from an article in the Dako *Connection* scientific magazine (Kiernan 2010), which may be consulted for references to review articles and original papers that I respect and trust. Some practical histopathological applications of traditional carbohydrate histochemistry, not mentioned in the *Connection* article, are included in this chapter. The Further Reading list at the end contains some relevant books and review articles.

## Mammalian Macromolecular Carbohydrates: Classification and Some Names

Three main groups are recognized. **Glycans** (polysaccharides) consist of straight or branched chains of the same monosaccharide. The only mammalian example is glycogen, which consists of D-glucose molecules joined by α-1→ 4 linkages. **Glycosaminoglycans (GAGs)** have long chains of repeating units of two or more different sugars, with each unit including a nitrogen-containing and an acid monosaccharide. An example is hyaluronan, also called hyaluronic acid, with the repeating unit -glcUAβ-3glcNAc β-4- . It occurs in extracellular matrices. The compositions of some other GAGs are shown in Figure 2. These substances were formerly known as mucopolysaccharides. They are connected with matrix proteins to form **proteoglycans**, which, together with associated collagen fibrils, determine the physical properties of connective tissues. With the exception of hyaluronan, the GAGs all include sugars with half-sulfate ester groups. **Glycoproteins** have multiple short chains (oligosaccharides), each composed of a wide variety of sugars, attached to a protein molecule. They occur on the outside surfaces of all cells and in secreted products such as **mucus** and some hormones. The glycosidic linkage to protein in proteoglycans and glycoproteins may be with the side-chain nitrogen of asparagine or the side-chain oxygen of serine or threonine. In a **glycolipid**, a chain of sugar molecules is joined to the terminal oxygen atom of sphingosine, a long-chain amino alcohol of cell membranes. Large molecules with carbohydrate and protein or lipid moieties are collectively called **glycoconjugates** by biochemists. The term **mucosubstance** is controversial but it conveniently embraces all glycans and glycoconjugates that can be localized in sections of tissues. The word **mucin** is used very widely, but inconsistently, and for this reason has been deprecated and is not used again in this article. Some terminology is reviewed in the **Glossary**.

## Immobilizing Macromolecular Carbohydrates in Tissues

All carbohydrates have abundant hydroxy and other polar groups (Figure 1) and consequently are hydrophilic. Glycogen is soluble in water but insoluble in alcohols. The hydroxy groups and the anionic, ester and amide side chains of carbohydrates do not react with formaldehyde, ethanol or other compounds used for fixation and tissue processing. Retention of mucosubstances in fixed animal tissues is due largely to insolubilization of associated proteins by coagulation or covalent cross-linking of nearby protein molecules. The histochemically interesting parts of sugars are the anionic groups and diols (Figure 1), which are unchanged in paraffin sections. Glycogen frequently diffuses within cells of the liver before being immobilized during fixation by an aqueous formaldehyde solution, giving rise to an artifact known as polarization, illustrated on page 144 (Chapter 16: Fixation and Tissue Processing, Figure 2). This artifact can sometimes, but not always, be avoided by using a non-aqueous fixative.

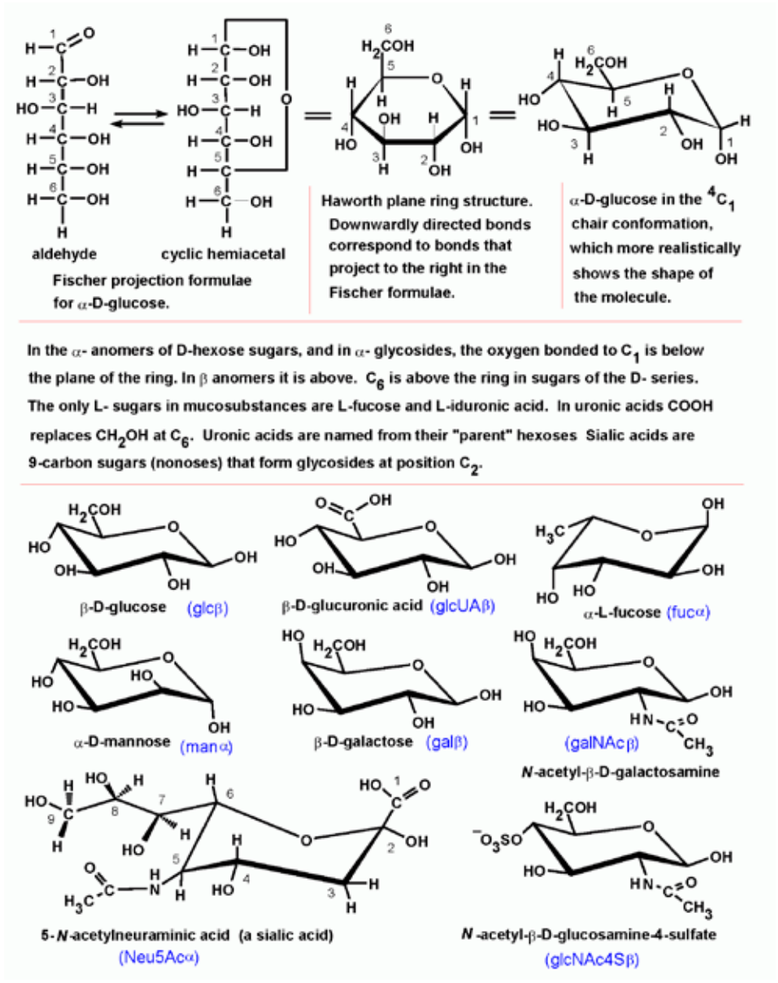


**Figure 1.** Some monosaccharides that occur commonly in mucosubstances. The upper part of the figure summarizes depictions of structural formulae. In the examples in the lower half of the figure, ring hydrogens are omitted from the hexose structures. Glycoside linkages are from C1 (C2 in sialic acids) usually to C3, C4 or C6 of the next sugar in the chain. The shorthand notation for glycoside chains takes forms such as L-fuc(α-1→4)D-man(α-1→6)D-galNAc(β-1→)thr, which describes a trisaccharide attached to a threonine side-chain of a protein. A simpler notation (4) shown in blue omits the D or L, the unnecessary parentheses and 1→, so the preceding example becomes fucα-4manα-6galNAcβ-(thr).

The shape of an oligo- or polysaccharide is markedly different with α or β glycosides, even though the chemically reactive groups (hydroxy, carboxy, sulfate) are the same.





**GLYCANS (Polysaccharides)**

Glycogen -4glcα-4glcα-4glcα-4glcα- with occasional -4glcα-6glcα- giving branched chains.

**GLYCOSAMINOGLYCANS (GAGs) (Repeating units shown)**

Hyaluronic acid -4glcUAβ-3glcNAcβ-

Chondroitin-4-sulfate -4glcUAβ-3galNAc4Sβ-

Chondroitin-6-sulfate -4glcβ-3galNAc6Sβ-

Dermatan sulfate -4idoUAα-3galNAc4Sβ-

Keratan sulfate -3galβ-4glcNAc6Sβ-

Heparan sulfate -4glcNAc6Sα-4idoUA2Sα-4glcNAc6Sα-4glcUAβ-4-glcNAcNSα-

Variable. Heparin (in mast cells) has more N-sulfates and more iduronic acid

PROTEOGLYCANS are like bottle brushes, with many GAGs joined to a core protein.

Link proteins attach proteoglycans to hyaluronan.

**GLYCOPROTEINS (A few examples of oligosaccharides attached to proteins)**

Blood group A antigens galNAcα-3 / fucα-2 gal-

Blood group B antigens galα-3 / fucα-2 gal-

Blood group O(H) antigens fucα-2gal-

There are three or more antigens for each ABO blood type, but their oligosaccharides have these terminal sequences in common.

Structures in glycoproteins of mucus

galNAcα-(ser/thr)

neu5Acα-6galNAcα-(ser/thr)

galβ-3galNAcα-(ser/thr)

glcNAcβ-2manα-3 / glcNAcβ-2manα-6 manβ-4glcNAcβ-4glcNAcβ-(asn)

In collagen: glcα-2galβ-(hyl/hyp)

**Figure 2.** Sugar sequences of some macromolecular carbohydrates.

## Histochemical Methods for Carbohydrates

There are three approaches to the histochemical study of mucosubstances (Figure 3).

- The ionized acidic groups (anions of sialic acids, uronic acids and half-sulfate esters) can be detected with **cationic dyes**.
- Adjacent hydroxy groups (diols) can be selectively oxidized to aldehydes, which are then localized by a chromogenic chemical reaction. The **periodic acid-Schiff (PAS) reaction** is the principal method of this type.
- Terminal monosaccharide or oligosaccharide saccharide units may be labeled by virtue of their specific affinity for carbohydrate-binding proteins known as **lectins**, which are used in much the same way that labeled antibodies are used in immunohistochemistry.

These three groups of methods will now be reviewed.

### Cationic Dyes

In another chapter of this Guide, Richard Horobin explains the mechanisms whereby dye molecules or ions are attracted towards particular substrates and then held in place there. The subject has recently been reviewed in greater detail elsewhere. A dye composed of colored organic cations (balanced by small inorganic ions such as chloride) diffuses into a section of tissue and is attracted towards negatively charged groups of macromolecules. In an animal tissue these are most notably phosphates of nucleic acids, half-sulfate ester groups of glycoconjugates and carboxylate groups of glycoconjugates and proteins. The pH of the staining solution determines which of these acidic groups is ionized. Sulfuric acid is a strong acid, so the half-sulfate esters exist as anions even in a highly acidic medium (pH 1 or less). Phosphoric acid is weak ($pK_1$ = 2.1); the single negative charges of the phosphate groups interposed between the ribose units of RNA or the deoxyribose units of DNA are mostly protonated at pH < 2 and therefore do not attract cations. Carboxy groups in tissues are even weaker acids, though varying greatly in strength. Sialic acids (pK 2.0-2.8) are stronger than glucuronic and galacturonic acids (pK 3.0-3.5), which in turn are stronger acids than the carboxy groups of proteins (pK 3.9-4.3).

A cationic dye with small planar molecules stains the nuclei of cells (DNA) and RNA-rich cytoplasm in addition to acidic mucosubstances. The latter are often colored differently (red with such blue dyes as thionine and toluidine blue, or orange with neutral red, for example). This effect, known as **metachromasia**, is attributed to stacking of dye cations at sites of high density of anionic groups in the tissue. Stacking shortens the wavelength of maximum absorption – a hypsochromic shift – so that the maximum wavelength in the spectrum of the transmitted light is longer, making the observed color red instead of blue (Figure 4). Mast cells were first recognized by virtue of their metachromasia in 1877 by Paul Ehrlich (1854-1915, Nobel laureate 1908), who was then a medical student in Leipzig. The compound responsible for the metachromasia was later identified as heparin, a heteroglycan rich in half-sulfate esters.





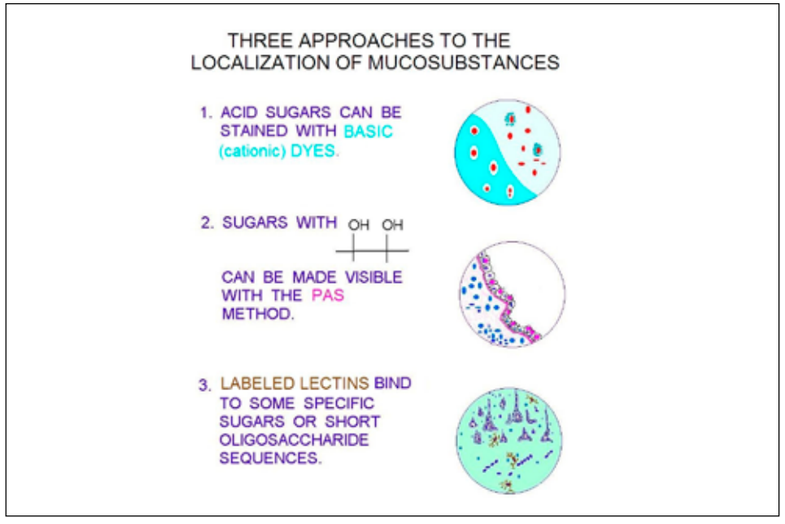


**Figure 3.** Approaches to mucosubstance histochemistry.

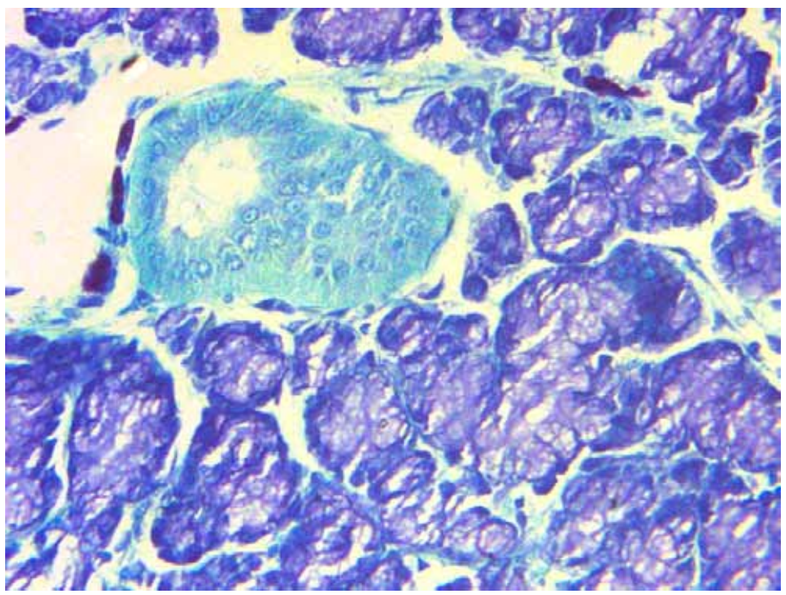

**Figure 4.** Section of salivary gland stained with thionine at pH 4.0, showing metachromatic (reddish purple) staining of mucous acini and of mast cells in connective tissue around a duct. Nuclei have the orthochromatic (blue) color of the dye.

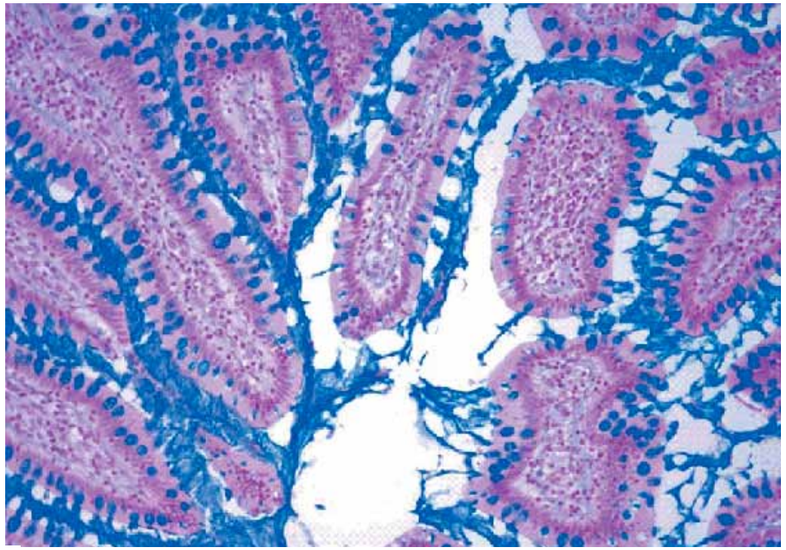

**Figure 5.** Section of small intestine stained with alcian blue at pH 2.5 to show mucus in goblet cells and in the spaces between the villi. Counterstaining with the aluminium complex of nuclear fast red provides red nuclei and pink cytoplasm.

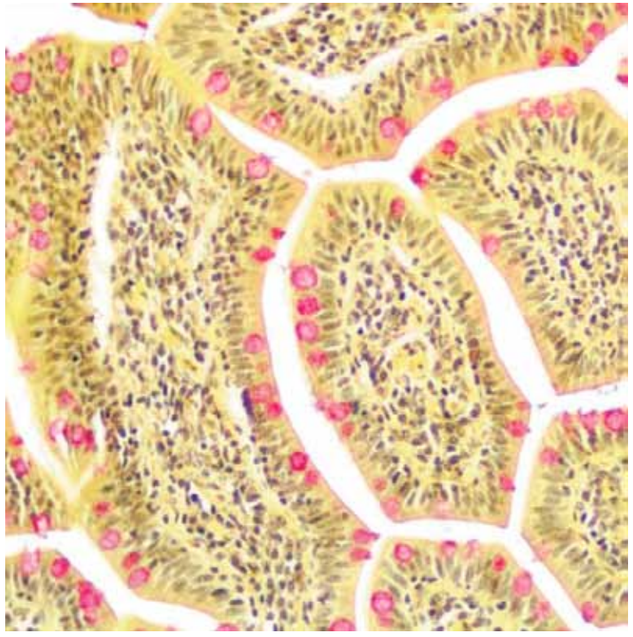

**Figure 6.** Section of small intestine stained with hemalum (blue nuclei), Mayer's mucicarmine (red goblet cells) and metanil yellow (yellow cytoplasm and collagen).





**Alcian blue** is the cationic dye most often used to stain acidic mucosubstances. This dye is not metachromatic but it stains acid mucosubstances selectively, because its cations are too large to associate closely with nucleic acid molecules. **At pH 1.0**, alcian blue stains only sulphated GAGs and glycoproteins. **At pH 2.5** this dye stains, in addition, hyaluronic acid and glycoproteins that owe their acidity to sialic acids (Figure 5). GAGs are colored more intensely at PH 2.5 than at pH 1.0 because their ionized uronic acid carboxy groups add to the negative charges of the half-sulfate esters. In another group of methods, alcian blue is used at pH 5.5 and the staining is restrained by adding different concentrations of an inorganic salt, magnesium chloride. The $Mg^{2+}$ ions may compete with the dye for anionic binding sites in the tissue and each acidic mucosubstance can be assigned a **"critical electrolyte concentration" (CEC)** above which it fails to stain. CECs are highest for the most highly sulfated mucosubstances. The reason may be higher porosity rather than greater strong acid concentration in the materials being stained, though this explanation is controversial.

If one wants to stain only the carboxy groups of GAGs and glycoproteins, the following chemical trick may be used. The sections are treated for several hours with methanol, acidified with HCl. This converts carboxy groups to methyl esters and removes sulfate-ester groups. Staining with alcian blue at any pH is prevented. The sections are next treated with a dilute solution of KOH in ethanol. This procedure, commonly but inaccurately called "saponification" splits the methyl esters, regenerating the tissue carboxy groups, which can then be stained by alcian blue at pH 2.5.

Anionic mucosubstances are also stained by cationic colloids containing iron(III), which do not also bind to the nuclei of cells. The bound particles containing ferric ions are detected by treatment with aqueous potassium ferrocyanide, which reacts to form Prussian blue (ferric ferrocyanide), an insoluble inorganic pigment. This **colloidal iron stain** is often named for C.W. Hale but a simplified version of the method by R.W. Mowry is usually used.

A traditional stain for mucus is **mucicarmine**. This contains a large 2:1 dye-aluminum cationic complex, which is red. The mucicarmine solution is applied to sections after staining nuclei blue with a hemalum. The red dye-metal complex is attracted to anionic sites in the tissue (mucus, cartilage matrix, etc.) but it does not displace the nuclear stain (Figure 6). A yellow anionic dye such as picric acid or metanil yellow may optionally follow mucicarmine, as a cytoplasmic counterstain.

## Glycols, Periodic Acid and the PAS Method

Most sugars include at least one glycol formation: a pair of adjacent carbons with hydroxy groups, usually at positions 2 and 3. The periodate ion (in periodic acid or sodium metaperiodate) selectively oxidizes glycols, yielding two aldehyde groups. In a macromolecular carbohydrate in a section, the ring structures of the monosaccharide units are broken, but the chain is still intact (Figure 7), so the aldehydes are firmly anchored to the tissue, where they can be detected with one of several chromogenic reagents. The one most frequently used for the purpose is Schiff's reagent, which combines covalently with aldehyde groups to form a red-purple compound. The **periodic acid-Schiff (PAS)** method is one of the most frequently applied special stains in histopathology.

It might be expected that all mucosubstances would stain strongly with the PAS method, but this is not the case. Glycogen (Figure 8) and some types of mucus stain strongly. Basement membranes are clearly shown; other collagen fibers stain less strongly. Sites rich in GAGs, such as cartilage and mast cells, stain weakly or not at all. The sugar units from which aldehydes can be formed by reaction with periodic acid for 10-60 minutes at room temperature have been shown to be glucose, galactose, mannose, fucose and some sialic acids. Even though they contain glycols the uronic acids of hyaluronan and GAGs are not oxidized to aldehydes unless periodate is used at a raised temperature and for a much longer time. The low reactivity of uronic acid glycols is attributed to electrical repulsion of periodate ions by nearby carboxylate and sulfate of the GAG. It is possible to stain GAGs with PAS by applying the following steps:

1. Periodate oxidation sufficient to oxidize the glycols of all neutral sugars and sialic acids to aldehydes
2. Reduction of the aldehydes to primary alcohols by sodium borohydride
3. A second periodate oxidation, warmer and for longer, to oxidize the glycols of uronic acids
4. Schiff's reagent to reveal the aldehyde groups derived from GAGs

Uses of the PAS method in histopathology include detection of **glycogen**, which is stored in increased amounts in some inherited diseases, staining abnormally thick, glucose-rich renal glomerular basement membranes in diabetes mellitus and some other diseases, and characterizing mucus in cells of carcinomas originating in different parts of the digestive tract. Pre-treatment of sections with amylase (diastase, an $\alpha$-glucosidase) removes glycogen, confirming that PAS staining is due to this polysaccharide and also revealing other neutral mucosubstances such as glycoproteins in basement membranes and reticular fibers.

Oxidants other than periodic acid and aldehyde-reactive reagents other than Schiff's are used for some applications. Examples are traditional **stains for reticulin** and for pathogenic fungi. Reticulin comprises fine fibers and thin basement membranes with collagen Type III fibrils embedded in a carbohydrate-rich matrix. Although it is PAS-positive, reticulin can be shown with greater contrast by oxidizing with periodate (or, in the commonly used traditional methods, permanganate) and detecting the resulting aldehydes with an ammoniacal silver nitrate solution, which deposits black colloidal silver. The method is not selective but it gives high contrast and the blackening of reticular fibers and basement membranes is prevented by either acetylation of hydroxy groups before oxidation or blocking aldehydes with phenylhydrazine after oxidation. In **Grocott's method for staining fungi** in animal tissues the oxidant is chromium trioxide, which over-oxidizes much of the carbohydrate content of connective tissue, glycogen and mucus (yielding carboxy groups) but generates abundant aldehyde in the cell walls of fungal hyphae. These are then stained with methenamine-silver nitrate, which is similar to ammoniacal silver nitrate but can be kept for longer for reactions that occur more slowly.

An old staining method for showing glycogen is **Best's carmine**, which is an alkaline solution containing an aluminum complex of carminic acid (a red anthraquinone glycoside) in 20% methanol. In this technique, which is used only to detect glycogen, the carmine ions are not attracted by polyanions of the tissue. Instead, the dye is probably held in contact with glycogen by hydrogen bonding to the numerous hydroxy groups of the polysaccharide.





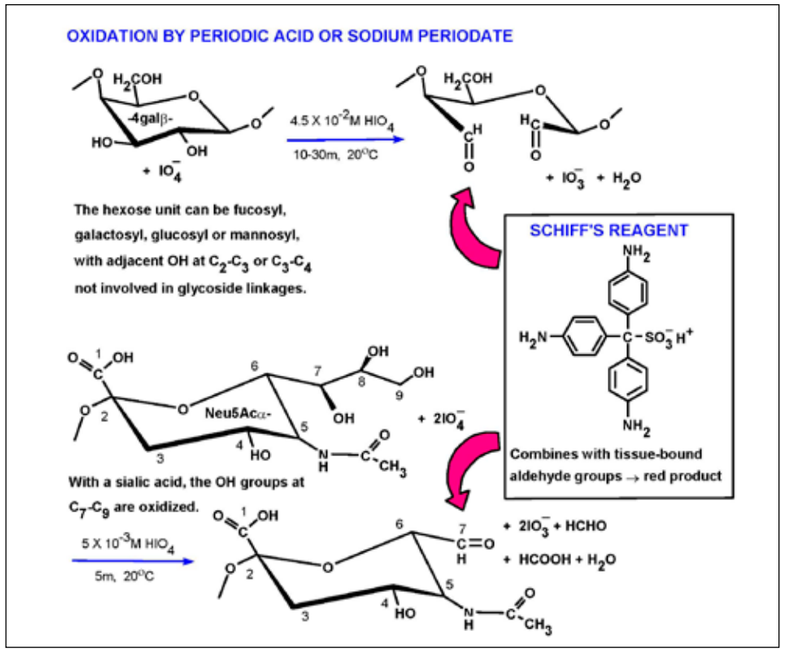


**Figure 7.** Periodate oxidation of glycols in hexosyl units (above) and sialyl groups (below) of mucosubstances. Colorless Schiff's reagent forms a covalently bound red-purple compound with the resulting tissue-bound aldehydes. A sialic acid with O-acetylation at $C_9$ cannot be oxidized by periodate unless the acetyl group is first removed by alkaline hydrolysis.

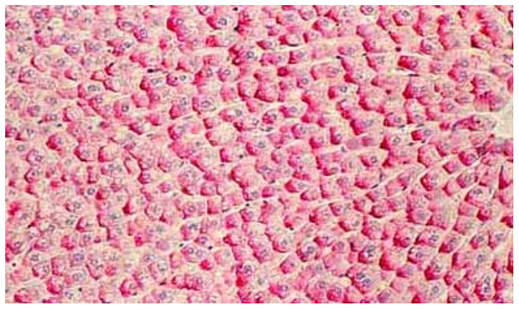

**Figure 8.** Section of liver stained by the periodic acid-Schiff (PAS) method to show glycogen (red) in hepatocytes. Within each cell the glycogen has displaced to one side of the cytoplasm. This common artifact, known as glycogen polarization, may be due to movement or partial dissolution of glycogen particles during penetration of the tissue by the fixative. Nuclei have been lightly counterstained (blue) with hemalum.

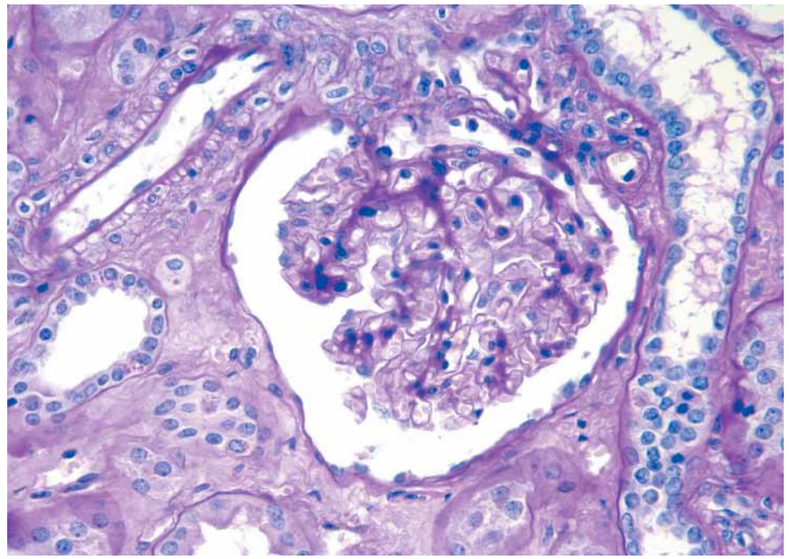

**Figure 9.** Section of kidney stained with PAS and hemalum.

## Alcian Blue and PAS in Histology and Histopathology

Because their colors contrast and add in understandable ways, alcian blue and the PAS reaction (red) are frequently applied to the same section. Usually alcian blue staining is done first, either at pH 2.5 (for all acidic mucosubstances) or at pH 1.0 (selective for sulfated GAGs). The combined stain shows neutral mucus, notably that of the esophagus and stomach, in red. Occasional mucous neck cells of gastric glands have sialic acid-containing mucus and therefore stain purple with alcian blue pH 2.5 and PAS. The secretory cells of the duodenum, jejunum and upper (proximal) ileum produce both neutral and sialic acid-containing mucus, and also are purple after staining with alcian blue pH 2.5 and PAS. This staining combination is useful for recognizing **Barrett's esophagus**. This is a possibly pre-malignant metaplasia in which reflux of acid from the stomach causes the normally multi-layered squamous epithelium to change to a columnar intestinal form, with goblet cells containing stainable sialic acids.

**Sulfated mucus** accompanies sialylated mucus in goblet cells of the lower (distal) ileum and in the crypts of the colon and rectum. In the ascending and transverse colon, sialylated mucous cells predominate in the lower one-third of the crypts, and sulphated mucous cells in the upper two-thirds. This distribution is reversed in the descending and sigmoid colon and rectum. The PAS reaction in the epithelium of the lower alimentary canal is due entirely to the presence of sialic acids in the mucus. Alcian blue is therefore more informatively used at pH 1.0 to stain only the sulfated glycoproteins. Variants of the PAS method, developed by Culling and Reid in the 1970s and 1980s, provide ways to recognize acylation sites (at combinations of C7, C8 and C9 of sialic acids, see Figure 1). These methods can often identify the colonic epithelial cell-types from which primary tumors and metastases were probably derived.





The kidney is another organ in which structural details are clearly revealed by carbohydrate histochemistry. The **glomerular basement membrane** is clearly seen with either PAS or the related periodic acid-methenamine silver technique (Jones' method for basement membranes), as also are the basement membranes of renal tubules. These staining properties are attributable to relatively high concentrations of hexose sugars, including glucose, in the collagen of basement membranes. The collagen fibers of ordinary connective tissue stain pink with PAS. Glomerular basement membranes become thickened and irregular in the various types of glomerulonephritis, and in diabetic nephropathy much of the volume of the glomerulus is taken up by extracellular PAS-positive material between dilated capillaries.

**Fungi** have cell walls containing the polysaccharides chitin (-glcNAcβ-4-glcNAcβ-) and zymosan (-glcβ-3-glcβ-), which do not have glycol groups or acidic substituents, and mannose-rich glycoproteins, which do. Thus, pathogenic fungal hyphae (such as *Aspergillus* or *Mucor*) or yeast cells (such as *Coccidioides* or *Histoplasma*) in tissue can be stained with the PAS method or, for greater contrast, with methenamine-silver following oxidation with chromic acid (**Grocott's method**). The yeast *Cryptococcus* also has an external capsule rich in glucuronic acid, which is stained by alcian blue at pH 2.5, or alternatively by the colloidal iron method or with mucicarmine.

## Carbohydrate-binding Proteins

In 1888 Peter Hermann Stillmark described a toxic protein extracted from castor beans, which he named ricin. One property of ricin was its ability to agglutinate mammalian red blood cells. In the ensuing decades many other plant proteins with this property were discovered, and in the 1940s some were found to be selective for particular human blood groups. They were called phytohemagglutinins until, in 1954, William Boyd adapted the supine form of the Latin verb *legere*, to choose or pick out, and coined the term **lectin**, a word that came into widespread use in the 1970s. A lectin molecule resembles an antibody in having at least two sites that can bind to specific receptors. In the case of a lectin the receptor is a specific glycan configuration; when this is on the surfaces of blood cells, agglutination is brought about by cross-linking. Although the first lectins to be studied were from seeds, these proteins are now known from organisms of all kinds, including vertebrate and invertebrate animals, protozoa, bacteria and viruses. The largest quantities of lectins are found in the seeds of certain plants, where they may serve as storage proteins. The best understood functions of lectins, however, are defensive. They constitute parts of the innate immune systems of all organisms. In contrast to conventional antibodies, which are produced in response to antigens, lectins are there all the time, waiting to bind to the surfaces of invading cells. Most of the lectins used in carbohydrate histochemistry are derived from plants. They can be labeled by covalent conjugation to fluorochromes, biotin or histochemical enzymes such as horseradish peroxidase (HRP), in the same ways that labeled antibodies for immunohistochemistry are prepared. Large numbers of labeled lectins have been commercially available for many years.

The modern classification of lectins is based on homologies in their amino acid sequences. For histochemical purposes, however, it is more convenient to follow the older system in which lectins are recognized by the sugar units to which they principally bind. These specificities (Table 1) were determined principally by determining which monosaccharides (or oligosaccharides or synthetic glycosides) competitively inhibited the agglutination of human red blood cells. Thus, lectins that agglutinate group A cells are inhibited by *N*-acetylgalactosamine. For groups B and O(H) the inhibitory sugars are galactose and fucose respectively. These are terminal

**Table 1.** Some lectins used as histochemical reagents.

| Source of Lectin (Name, if Applicable) | Abbreviation | Specific Affinity[a] |
|---|---|---|
| **Group 1 - Affinity for glucose and mannose** | | |
| *Canavalia ensiformis* (concanavalin A) | Con A | manα- > glcα- > glcNAcα- |
| *Galanthus nivalis* (snowdrop lectin) | GNL | Terminal manα-3manα- |
| *Lens culinaris* (lentil lectin) | LCA | manα- > glcα- > glcNAcα- |
| *Narcissus pseudonarcissus* (daffodil agglutinin) | NPA | -6manα-6manα-6-man |
| *Pisum sativum* (pea lectin) | PSA | manα- > glcα- > glcNAcα- |
| **Group 2 - Affinity for *N*-acetylglucosamine** | | |
| *Griffonia simplicifolia* (= *Bandeiraea simplicifolia*; Griffonia lectin II) | GSL-II or BSL-II | glcNAcα- and glcNAcβ- |
| *Lycopersicon esculentum* (tomato lectin) | LEL or TL | glcNAc oligomers |
| *Phytolacca americana* (pokeweed mitogen) | PAA or PWM | glcNAcβ-4glcNAc = galβ-4glcNAc |
| *Solanum tuberosum* (potato lectin) | STA | glcNAcβ-4glcNAc |
| *Triticum vulgare* (wheat germ agglutinin) | WGA | glcNAcβ-4glcNAc > glcNAcβ- > sialic acids |
| **Group 3 - Affinity for galactose and *N*-acetylgalactosamine** | | |
| *Arachis hypogaea* (peanut agglutinin) | PNA | galβ-3galNAc- > galβ- and galα- |
| *Artocarpus integrifolia* (jacalin, jackfruit lectin) | Jac | galβ-3galNAcα- |
| *Bauhinia purpurea* (Bauhinia lectin) | BPL | galβ-3galNAcα- > galNAcα- |
| *Dolichos biflorus* (horse gram lectin) | DBA | galNAcα-3galNAc >> GalNAcα- |
| *Glycine max* (soybean agglutinin) | SBA | galNAcα- and galNacβ- > galα- and gal-β |
| *Griffonia simplicifolia* (= *Bandeiraea simplicifolia*; Griffonia lectin I) | GSL-I, BS-1 | galNAcα- (isolectin A) and galα- (isolectin B) |
| *Helix pomatia* (Edible snail agglutinin) | HPA | galNAc > glcNAc >> gal |
| *Maclura pomifera* (osage orange lectin) | MPA | galNAcα- > galα- |
| *Phaseolus vulgaris* (kidney bean lectins) | PHA-E & PHA-L | Complex sequences, including galβ-4glcNAcβ-2manα- (isolectins E and L agglutinate erythrocytes and leukocytes respectively) |
| *Ricinus communis* (castor bean agglutinin I) | RCA-I or RCA120 | galβ- > galα- >> galNAc |
| *Vicia villosa* (hairy vetch lectin) | VVA | galNAcα-(ser/thr) > galα-3galNAcα- > galNAcβ- |
| **Group 4 - Affinity for fucose** | | |
| *Anguilla anguilla* (eel lectin) | AAA | fucα- |
| *Lotus tetragonobolus* (=*Tetragonobolus purpureus*; asparagus pea lectin) | LTA | fucα- |
| *Ulex europaeus* (gorse lectin I) | UEA-I | fucα- |



Carbohydrate Histochemistry

**Table 1.** Some lectins used as histochemical reagents.

| Source of Lectin (Name, if Applicable) | Abbreviation | Specific Affinity[a] |
|---|---|---|
| **Group 5 - Affinity for sialic and/or uronic acids** | | |
| *Aplysia depilans* (sea-hare gonad lectin) | AGL, SHL | Galacturonic acid >> D-Gal |
| Bovine or porcine lung, pancreas, salivary glands (aprotinin; bovine trypsin inhibitor) | | Uronic and sialic acids[b] |
| *Limax flavus* (slug lectin) | LFA | Sialic acids: *N*-acetylneuraminic acid > *N*-glycolylneuraminic acid |
| *Limulus polyphemus* (limulin *or* horseshoe crab lectin) | LPA | Sialic acids: Neu5Ac(*or* Neu5Gc)α-6galNAc > Neu5Ac |
| *Maackia amurensis* (Amur maackia lectin) | MAL | Sialylα-3gal |
| *Sambucus nigra* (elder bark lectin) | SNA | Neu5Acα-6(gal *or* galNAc) |

**Footnotes to Table 1.**

[a] The information on specificity is from various sources. For lectins that bind to more than one sugar, ligands are listed in order of decreasing affinity (>> indicates much greater affinity; > greater, and = equal affinity for the lectin).

[b] Aprotinin is not a lectin, but this basic polypeptide, fluorescently labelled, can be used to stain glycoproteins and gycosaminoglycans that owe their acidity to carboxyl rather than to sulphate groups.





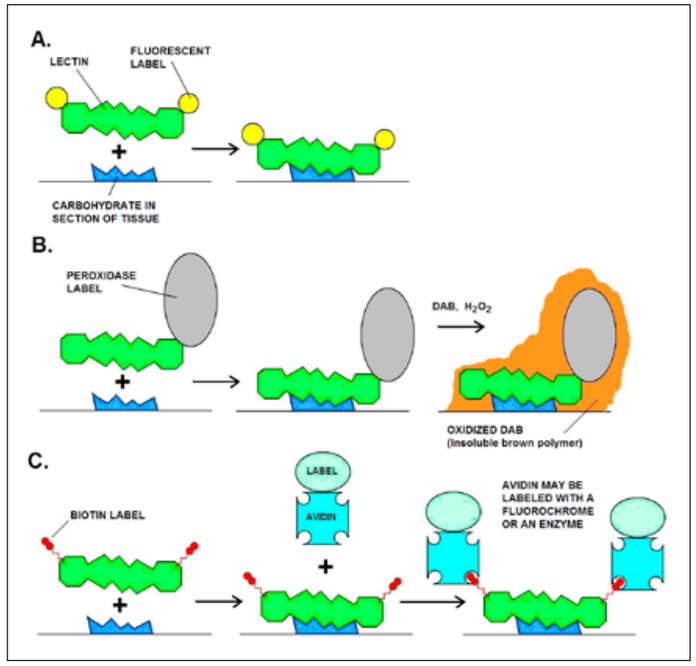


**Figure 10.** Three ways to visualize a lectin bound to its carbohydrate ligand in a section of a tissue. **A.** Direct method, using lectin that has been conjugated with a fluorochrome such as a reactive fluorescein or rhodamine derivative. There is no amplification, but the fluorescent sites are conspicuous against a dark background. **B.** Using a peroxidase-conjugated lectin. The enzyme catalyzes the oxidation of 3,3'-diaminobenzidine (DAB; soluble, colorless) by hydrogen peroxide. The visible reaction product accumulates (amplification). **C.** Even after conjugation to a lectin, biotin (vitamin $B_7$) retains its specific affinity and strong avidity for avidin, a protein of egg-white that has four binding sites. This diagram shows some amplification. Much greater amplification can be achieved with subsequent application of a biotinylated enzyme.

monosaccharides of the oligosaccharide chains of the corresponding glycoproteins that comprise the blood group antigens (see Figure 2). There are three other groups of lectins, which can agglutinate all types of red blood cell and are inhibited by *N*-acetylhexosamines, sialic acids, or either α-D-glucose or α-D-mannose. It can be seen in Table 1 that many lectins have affinity for more than one monosaccharide, and often the highest affinity is for a sequence of two or three sugar units.

In general, lectins bind most readily to the terminal few sugars of a chain because, being large molecules, their deeper penetration is sterically blocked. The affinity of a lectin for a sugar is similar to that of an antibody for its specific antigen, involving a combination of electrostatic and van der Waals forces. The manner of application of a labeled lectin is closely similar to the use of an antibody in immunohistochemistry (Figure 10). The main difference is that many lectins require metal ion ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ or more than one of these). These metals have insoluble phosphates, so a TRIS buffer is usually used, at pH 7.2-7.6, containing chlorides of all three metals, at $10^{-4}$M. Lectin affinity is, however, robust over a wide pH range. The concentration of the reagent is seldom critical, with 10 µg/ml usually being satisfactory for most lectins, whether they be unlabelled, fluorescently labelled or conjugated with biotin or peroxidase.

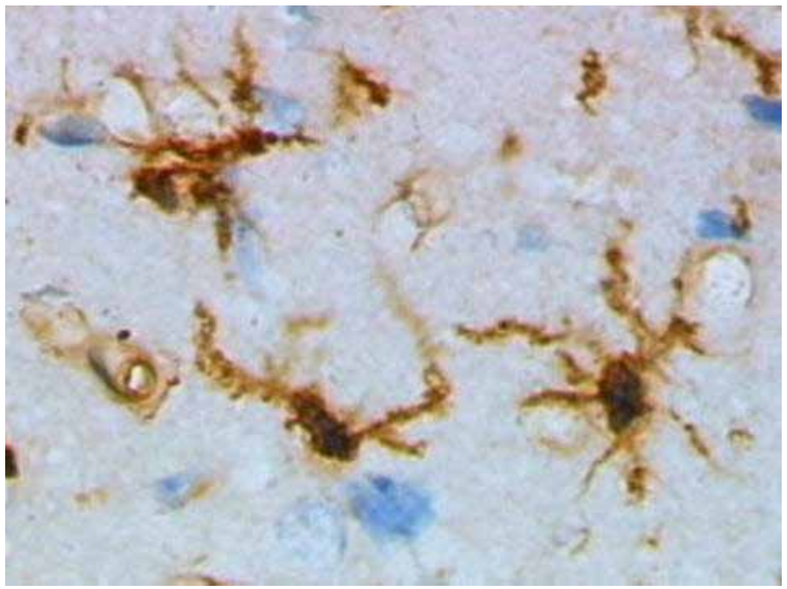

**Figure 11.** Microglial cells in the brain, stained by virtue of their affinity for the lectin RCA-1 from *Ricinus communis*. (Micrograph by Grzegorz Wicher)





## Applications of Lectin Histochemistry

For nearly 20 years histochemistry with labeled lectins was a technique with few applications other than descriptive and comparative studies of the staining properties of various tissues. Certain cell-types can be fairly selectively stained with lectins, and in some cases this provides an alternative to traditional staining methods that are difficult or capricious for the non-expert. Examples are the use of labeled RCA-I to stain microglial cells (Figure 11), labeled GSA-I, PNA or UEA-I for small neurons of sensory ganglia and their axons in the central nervous system and peripheral nerves, and LEL, SBA and VVA to distinguish between the axons of olfactory and vomeronasal neurosensory cells. Lectins have also been used to show specific cell-types in the stomach, kidney and eye and to recognize different populations of connective tissue mast cells. Immunohistochemistry can, of course, be used for these and similar purposes. Lectins are less specific than antibodies but have the advantages of being cheaper and effective across wider ranges of species.

More recent applications of lectin histochemistry have been in the fields of tumor biology and pathology. Malignant transformation has long been known to be associated with increased carbohydrate content of the glycocalyx or cell-coat, associated with increased binding of lectins to the cell surface. Notable changes include the appearance of repeating lactosamines (-3galβ-4glcNAcβ-3-), detectable with LEL or PNA, -1β-6-glcNAc branching, detectable with PHA-L, and the expression of numerous neu5Acα-6galNAcα- and galNAcα-, *O*-linked to serine or threonine. Staining with labeled HPA identifies tumors that metastasize, and LPA, MAL and SNA have been used to mark 2-6- and 2-3-linked sialic acids in developing, adult and neoplastic epithelia. Mammalian lectins have also been used in these studies.

Finally, if frozen sections of unfixed tissue are used, it is possible to localize lectins in the cells of plants and animals by means of synthetic glycoproteins, known as neoglycoproteins, which are biotinylated to allow detection with labeled avidin. Such studies assist in the investigation of the roles of lectins in development, intercellular communication and neoplasia.

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# Chapter 10 | Mastering the Trichrome Stain

**M. Lamar Jones, BS, HT(ASCP)**

## Introduction

The trichrome stain is one of the most utilized special stains in the Histopathology Laboratory. Most of the common uses for requesting a trichrome stain are liver biopsies, renal biopsies, dermatopathology, cardiac biopsies and muscle and nerve biopsies. Widely utilized techniques are the Masson, Gomori One Step, Martius Scarlet Blue and Mallory. One of the accepted basis for these methods is an application of mass action coupled with observations on the physical properties of various tissue components and their impact on staining. At the onset it must be made clear that the methods control how ionized acid dyes react with the ionized basic tissues. This is the fundamental principle on which they depend and the explanation is only about how that fundamental reaction can be manipulated.

## History

The first account of a triple stain was by H. Gibbs in 1880 followed by B. W. Richardson in 1881. Animal tissues were stained with picro-carmine, iodine, malachite green, atlas scarlet and soluble blue. The triple stain became known as the trichrome stain: Tri - 3; Chrome - Color. Frank Burr Mallory developed a method especially suitable for studying connective tissue. Tissue sections were stained in acid fuchsine, aniline blue-orange G solution and phosphotungstic acid; fibrils of collagen stained blue, fibroglia, neuroglia and muscle fibers stained red and fibrils of elastin stained pink or yellow.

## Purpose

The purpose of the trichrome stain is primarily to demonstrate collagen and muscle in normal tissue or to differentiate collagen and muscle in tumors. It is also used to identify an increase in collagenous tissue or indicate fibrotic change in cirrhosis of the liver or in a renal disease such as pyelonephritis. The trichrome stain is also used to distinguish tumors that have arisen from muscle cells and fibroblasts. Gomori's trichrome is the trichrome stain of choice for distinguishing histological changes that occur in neuromuscular diseases.

## Entities

**Nuclei:** Usually the nuclear stain in the trichrome stain is an iron hematoxylin. The classic nuclear stain is either Weigert's or Heidenhain's hematoxylin. Due to the acidity of the staining solutions following the staining of the nuclei the standard aluminum-based hematoxylins are decolorized. Iron hematoxylins are more resistant to these acid solutions. An additional acid resistant nuclear stain is Celestine blue or solochrome prune in iron alum followed by a conventional alum hematoxylin (Fig. 1).

**Collagen:** Collagen is a common protein found in the body, has slender thread-like structures intertwined in varying degrees of density referred to as "collagen fibers". These fibers are composed of even finer thread-like structures observed at the EM level called "microfibrils". Collagen appears white in the fresh state, is birefringent when polarized with light microscopy and is eosinophilic (Fig. 2). The more the collagen in the extracellular matrix, the stronger the tissue. There are about 7 types of collagen used in the clinical setting out of the 29 identified – with Type I being the most common.

**Muscle:** Muscle is one of the four primary types of tissue. It is composed of muscle cells called muscle fibers due to their fiber-like appearance. The three types of muscle are: smooth, skeletal and cardiac. Smooth muscle is also known as non-striated muscle that is involuntary, and usually associated with viscera (Fig. 3). These muscles lack striations, are elongated, have tapered ends with a centrally located nucleus. Skeletal or striated muscle is voluntary and has distinctive striations. This type of muscle is composed of long cylindrical fibers surrounded by a membranous sheath, the sarcolemma. The fibers are elongated and multinucleated with the nucleus located at the periphery of the cell. Cardiac muscle is involuntary and striated with a centrally located nucleus composed of branching or anastomosing fibers connected by junctions known as "intercalated discs".

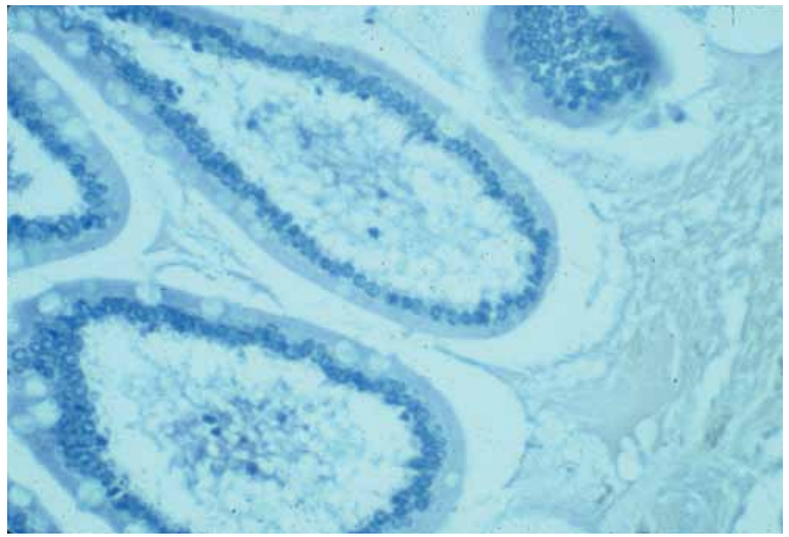

**Figure 1.** Weigert's Iron Hematoxylin demonstrating nuclear detail prior to muscle and collagen staining. 20X

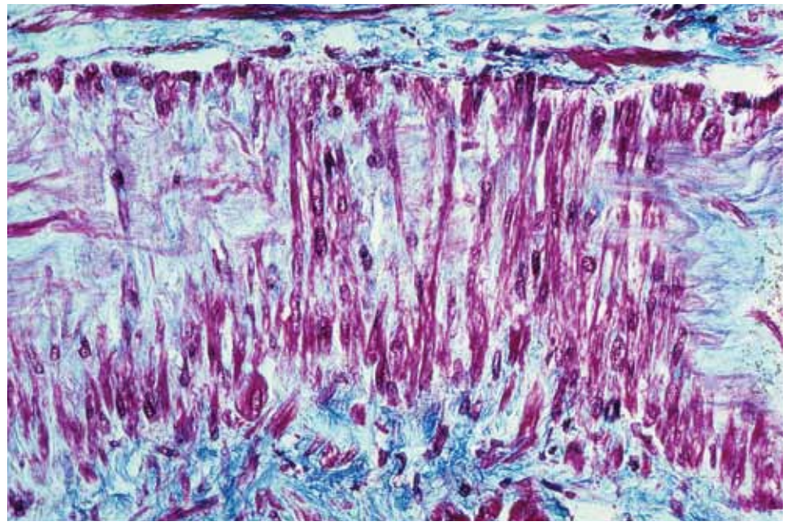

**Figure 2.** Muscle and collagen demonstrated by Masson Trichrome in gastrointestinal tract. 20X





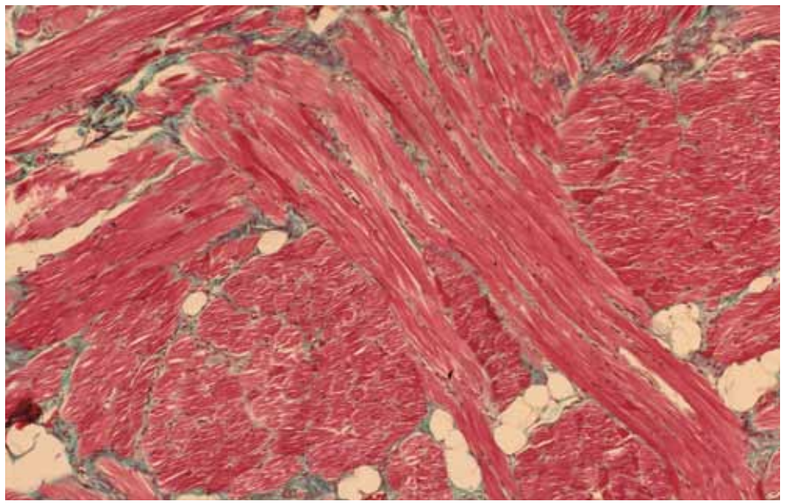

**Figure 3.** Various muscle patterns being demonstrated with trichrome stain. 20X

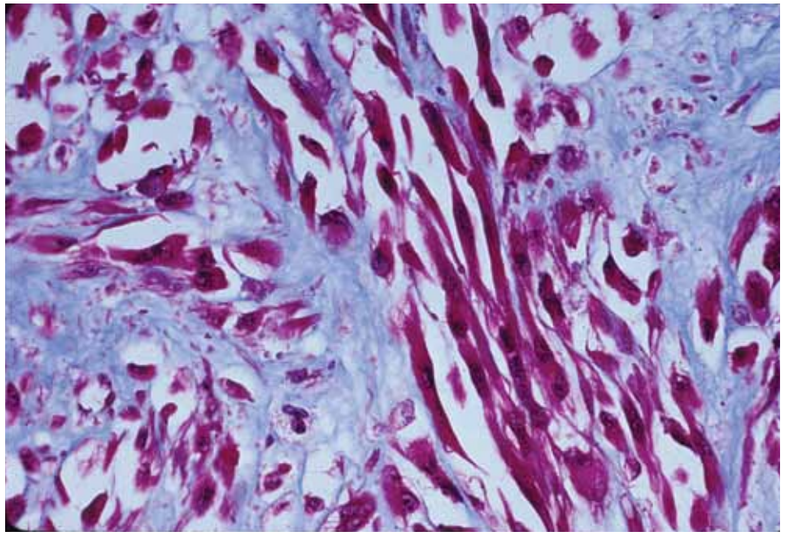

**Figure 4.** Embryonal Rhabdomyosarcoma. 40X

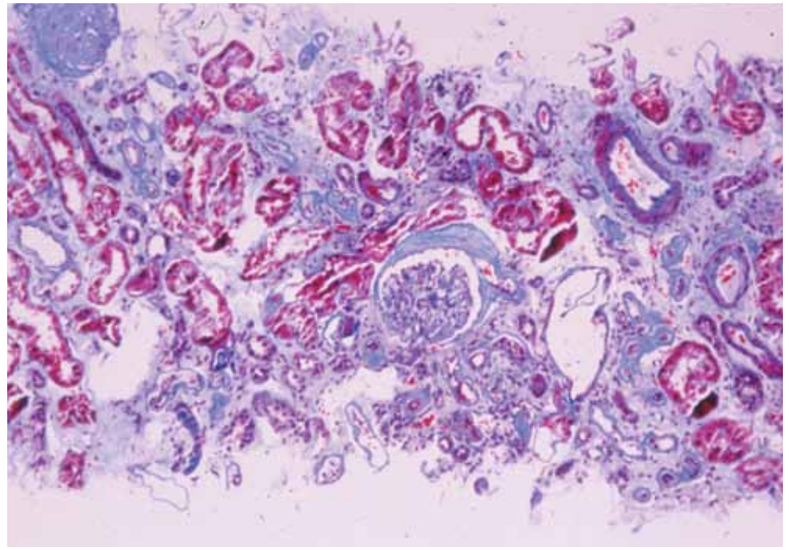

**Figure 5.** Interstitial fibrosis in kidney. 10X

## Factors That Effect Trichrome Staining

**Fixation:** 10% neutral buffered formalin will not yield optimal trichrome staining results. The longer those tissues remain in formalin fixatives, the less optimal the staining results. After formalin fixation mordanting the tissue sections with a picric acid solution such as Bouin's will enhance the trichrome staining intensity and radiance. The recommended fixatives for trichrome staining are: Bouin's, Zenker's, Formal-mercury, Zinc formalin and Picro-mercuric alcohol.

**pH:** In order to achieve both adequate and even staining of connective tissue fibers the dyes utilized in the trichrome techniques are prepared as low pH solutions, usually in the range of 1.5 - 3.0.

**Tissue Permeability and Dye Molecule Size:** When the protein component of a tissue is exposed to a fixative agent an interaction between the protein chains and the fixative occurs. Usually a 3 dimensional, insoluble protein "network" is formed. Different proteins will form networks with different physical features. Red blood cell protein will produce a dense network with small pores between protein elements. Muscle cells will form a more open structure with larger pores. Collagen will demonstrate the least dense network with more pores. This structure and the density of the protein network may relate to the staining reactions of the tissue components. The smaller molecule dyes will penetrate muscle and collagen but will not react with the red blood cells. With trichrome staining usually the smaller dye molecule will penetrate and stain a tissue element. When a larger dye molecule can penetrate the same element then the smaller molecule will be replaced by the larger molecule.

## Utilization of Phosphotungstic (PTA) and Phosmolybdic Acid (PMA)

PTA and PMA do not produce the same reactions in trichrome staining. Research demonstrates that the principles utilized are the same but the substances are not interchangeable. In trichrome staining there are three stages at which PTA or PMA can be utilized:

1. Before treatment with the small molecule dye
2. Combined in solution with the small molecule dye
3. Before treatment with the large molecule dye





Any of these combinations are acceptable. In the first method when a tissue section is treated with PTA or PMA and then with a low concentration of a "leveling dye" in the same solution, the leveling dye will stain only the red blood cells. Leveling dyes are relatively small molecules which form a salt like bond with the protein fiber. When a tissue section is treated with the smaller molecule dye followed by the PTA or PMA then the PTA or PMA competes with the dye in the process and gains access to the collagen. If this treatment is stopped at the proper time only the collagen will be free to stain when treated with a large molecule dye.

Then if the treatment with the large molecule dye is prolonged, some staining of the muscle and cytoplasm may occur. Both PTA and PMA act as conventional acidifying agents. A 10% solution should have the pH of less than 1. PTA is unstable at a pH of greater than 2.

## Generally Speaking

The most common techniques for trichrome staining are the Masson (multi-step) and Gomori One Step. The more traditional Masson technique incorporates what often is referred to as polyacids, (PTA or PMA). This is the primary reason the multi-step and one-step differ.

The Masson utilizes all of the mordanting and staining steps individually whereas the one-step incorporates all of the staining steps in one staining solution except the mordant (Bouin's) and the nuclear stain.

## Multi-Step Technique

The Masson is utilized to differentiate between collagen and muscle or to demonstrate a change in the amount of collagen present. Lendrum's Picro-Mallory technique will demonstrate fibrin in sharp contrast to the red blood cells and other tissue entities. Either of the stains for collagen can be utilized. For aniline blue staining the polyacid mixture of PTA/PMA must be utilized which will demonstrate small amounts of collagen. For light green the polyacid PTA must be utilized. The final staining results are:

1. Erthrocytes – yellow or red
2. Cytoplasm, fibrin and muscle – red
3. Collagen, bone – blue or green, depending upon the collagen stain utilized

## One-Step Technique

The one-step techniques such as the Gomori's One-Step and Van Gieson's method combine all of the dyes and reagents into one single solution that is applied for a specific amount of time. The various tissue entities are stained differentially. The one-step techniques are protocol dependant and will perform well if everything is standardized. If changes or alterations need to be made to the one-step method, it is suggested that one changes only one factor at a time and record. Since all of the dyes are in one staining solution it can be difficult to adjust the plasma stain over the fiber stain or vice versa. The basis is the trend toward reaching equilibrium created by the reaction products participating in a mass action type reaction. The one-step techniques must be standardized since changing any of the parameters may result in a different equilibrium. Proper staining is reached by interrupting the progress toward equilibrium at a specified, repeatable point and therefore is stopped by removing the solution when the desired results are achieved. The final staining results are:

1. Erythrocytes – yellow or red
2. Cytoplasm, fibrin, muscle – red
3. Collagen – light green

## Conclusion

The trichrome stain is utilized as the stain of choice of distinguishing histologic changes in tumors, connective tissue diseases, muscle and fibroblast tumors, renal diseases and dermatology cases. Even the disciplines of forensics, archaeology and hematopathology incorporate the trichrome stain for specific tissue entities and structures. With the utilization of immunohistochemistry expressions, the trichrome techniques still offer a great deal of diagnostic results (Fig. 4 and Fig. 5).

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# Chapter 11 | Role of Special Histochemical Stains in Staining Microorganisms

**Rashmil Saxena, BFA, HT(ASCP)CM**

Microorganisms encountered in routine pathology specimens include bacteria, fungi, protozoa and viruses[1]. Several histochemical stains help to visualize the first three groups of organisms; however, histochemical stains do not offer an advantage over H&E in the visualization of viruses and immunohistochemistry is the preferred method for this purpose. Histochemical stains also help to identify and classify bacteria, fungi and protozoa.

The Giemsa and Gram's stains help to visualize bacteria as well as classify them on their morphological characteristics. Thus bacteria can be classified into cocci or bacilli and cocci can be further classified into diplococci, staphylococci and streptococci based on their appearances on the Gram and Giemsa stains. The Gram stain also classifies bacteria into Gram-positive and Gram-negative organisms depending upon whether they take up the Gram stain or not; this classification is clinically useful and helps in therapeutic decisions. Some bacteria may not be adequately visualized with the Gram's and Giemsa stains. Of these, the clinically most significant ones are mycobacteria and spirochetes. Mycobacteria stain with carbol fuschin and resist decolorization with acid-alcohol, leading to their designation as "acid-fast bacilli". Spirochetes can be stained with a variety of silver stains such as the Warthin-Starry, Dieterle and Steiner stains. Finally, due to the large number of gastrointestinal biopsies in routine practice, a large number of stains are available for visualization of the Gram-negative bacillus, *Helicobacter pylori*. These include Giemsa, Alcian yellow - toludine blue, Diff-Quik, Genta, and Sayeed stains. A large number of laboratories prefer immunohistochemistry for identification of *Helicobacter pylori*.

The Giemsa stain highlights several protozoa such as toxoplasma, leishmania, plasmodium, trichomonas, cryptosporidia and giardia. Ameba can be highlighted by the PAS stain due to their large glycogen content. Histochemical stains for fungi are discussed separately in this publication.

## Special Stains for Detection of Bacteria

### Gram Stain

**Utility of the Stain:** The Gram stain is used to stain both bacillary and coccal forms of bacteria (Fig. 1). The most basic classification of bacteria consists of dividing them into Gram-positive and Gram-negative bacteria based on whether they take up the Gram's stain or not. Although the exact mechanism of staining is not known, bacteria that have large amounts of peptidoglycan in their walls retain the methyl violet stain, i.e., they are Gram-positive, whereas those that have more lipids and lipopolysaccharides in their cell walls are Gram-negative. The definite diagnosis of a bacterial species requires culture but the Gram stain provides a good initial indication of the nature of infection.

**Principles of Staining:** The method consists of initial staining of the bacterial slide with crystal violet or methyl violet which stain everything blue. This is followed by Gram's or Lugol's iodine made up of iodine and potassium iodide, which act by allowing the crystal violet to adhere to the walls of Gram-positive bacteria. Decolorization with an acetone-alcohol mixture washes away the methyl violet which is not adherent to bacterial cell walls. At this stage, Gram-positive bacteria stain blue while the Gram-negative bacteria are colorless. A carbol fuschin counter-stain is then applied which stains the Gram-negative bacteria pink.

**Modifications:** The Brown-Hopps and Brown-Brenn stains are modifications of the Gram stain and are used for demonstration of Gram-negative bacteria and *rickettsia*.

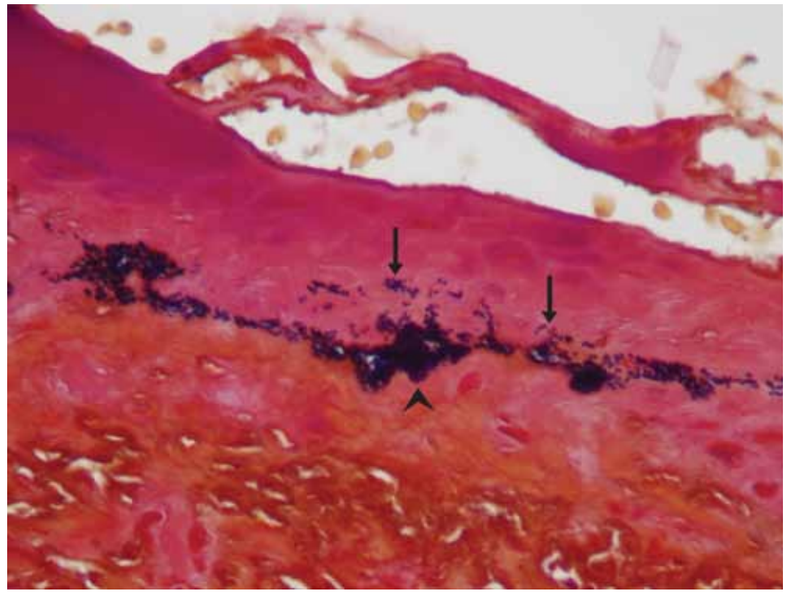

**Figure 1.** Photomicrograph of ulcerated skin stained with Gram's stain. The purple stain represents Gram-positive bacteria which are seen as clumps (arrowhead) or as separate clusters of cocci (arrows). Everything other than Gram-positive bacteria is stained pink by the carbol fuschin counterstain. The underlying structure of the skin cannot be seen.

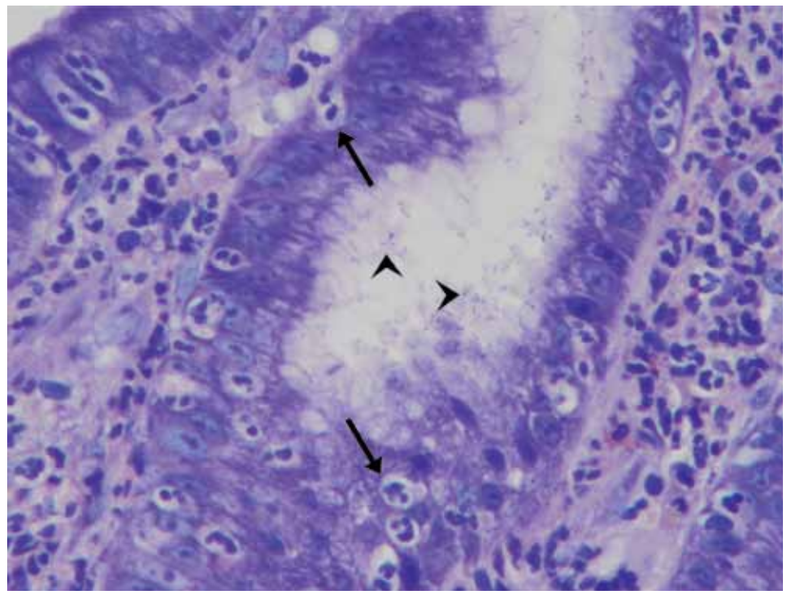

**Figure 2.** Giemsa stained section showing a gastric pit containing *Helicobacter pylori* which appear as delicate, slightly curved rod-shaped purple organisms (arrowheads). The stomach is inflamed and shows many neutrophils (arrows). The background is stained light pink by the eosin counterstain.





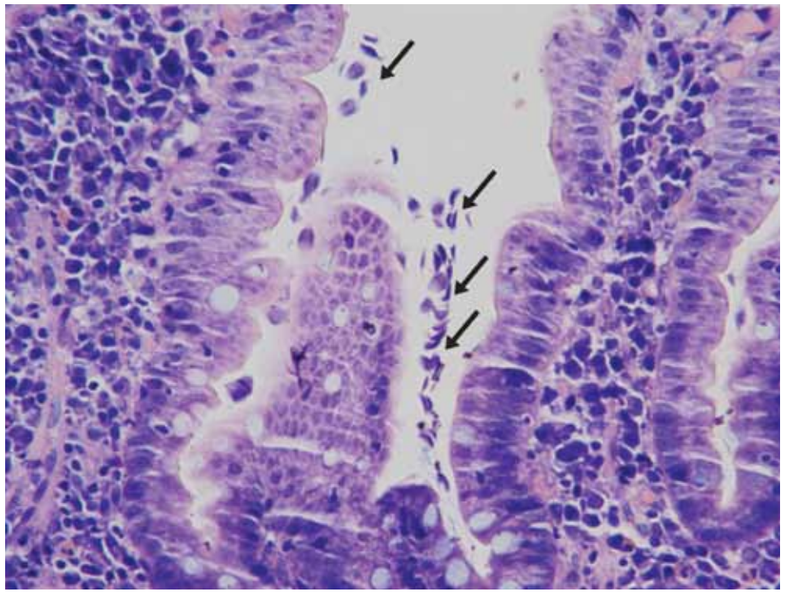

**Figure 3.** Giemsa stained section of small intestinal mucosa showing clusters of Giardia which stain purple (arrows) in the crypts. The background is stained faint pink by the eosin counterstain.

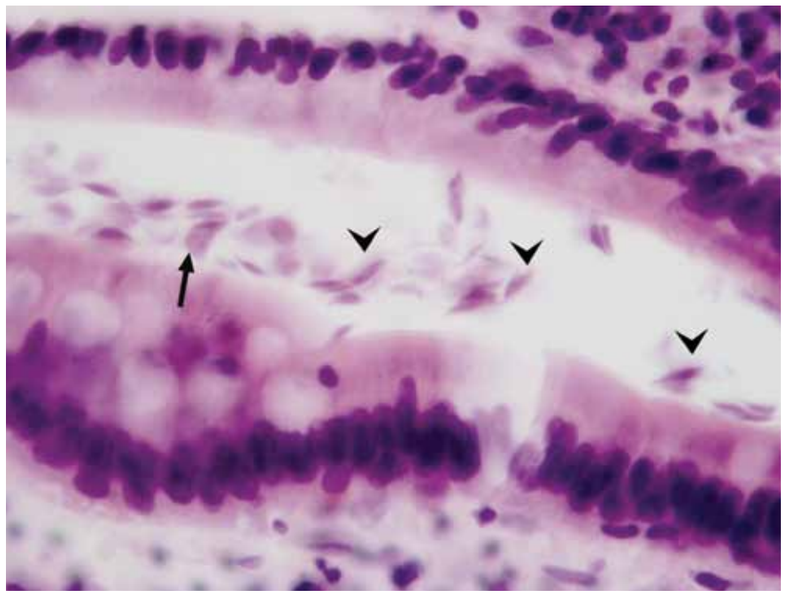

**Figure 4.** An H&E section of an intestinal crypt showing clusters of Giardia (arrowheads). The oval shape and clustering gives them a "tumbling leaves" appearance. Faint nuclei can be seen in some organisms (arrow).

## Giemsa Stain

**Utility of the Stain:** The Giemsa is used to stain a variety of microorganisms including bacteria and several protozoans. Like the Gram stain, the Giemsa stain allows identification of the morphological characteristics of bacteria. However, it does not help further classification into Gram-negative or Gram-positive bacteria. The Giemsa stain is also useful to visualize *H. pylori* (Fig. 2, 3; See also Fig. 4 for a high resolution H&E stain showing Giardia). The Giemsa also stains atypical bacteria like *rickettsia* and chlamydiae which do not have the peptidoglycan walls typical of other bacteria and which therefore do not take up the Gram stain. The Giemsa stain is used to visualize several protozonas such as toxoplasma, leishmania, plasmodium, trichomonas, cryptosporidia and giardia.

**Principles of Staining:** The Giemsa stain belongs to the class of polychromatic stains which consist of a mixture of dyes of different hues which provide subtle differences in staining. When methylene blue is prepared at an alkaline pH, it spontaneously forms other dyes, the major components being azure A and B. Although the polychromatic stain was first used by Romanowsky to stain malarial parasites, the property of polychromasia is most useful in staining blood smears and bone marrow specimens to differentiate between the various hemopoeitic elements. Nowadays, the Giemsa stain is made up of weighted amounts of the azures to maintain consistency of staining which cannot be attained if methylene blue is allowed to "mature" naturally.

**Modifications:** The Diff-Quick and Wright's stains are modifications of the Giemsa stain.

## Carbol Fuschin Acid-Alcohol Stain

**Utility of the Stain:** The carbol fuschin stain helps to identify mycobacteria which are bacilli containing thick waxy cell walls (Latin, *myco*=wax). Several mycobacteria can cause human disease; the two most significant ones are *M. tuberculosis* and *M.leprae* causing tuberculosis and leprosy respectively. Mycobacteria have large amounts of a lipid called mycolic acid in their cell walls which resists both staining as well as decolorization by acid-alcohol once staining has been achieved. The latter property is responsible for the commonly used term "acid-fast bacilli". Mycobacteria cannot be stained by the Gram stain because it is an aqueous stain that cannot penetrate the lipid-rich mycobacterial cell walls.

**Principles of Staining:** The mycobacteral cell walls are stained by carbol fuschin which is made up of basic fuschin dissolved in alcohol and phenol. Staining is aided by the application of heat. The organisms stain pink with the basic fuschin. Staining is followed by decolorisation in acid-alcohol; mycobacteria retain the carbol fuschin in their cell wall whereas other bacteria do not retain carbol fuschin, which is extracted into the acid-alcohol. Counterstaining is carried out by methylene blue. Mycobacteria stain bright pink with basic fuschin and the background stains a faint blue. Care has to be taken to not over-counter stain as this may mask the acid-fast bacilli.

**Modifications:** The 2 commonly used methods for staining of *M.tuberculosis* are the Ziehl-Neelsen and Kinyoun's acid-fast stains. The Fite stain is used for staining of *M.leprae* which has cell walls that are more susceptible to damage in the deparaffinization process. The Fite procedure thus includes peanut oil in the deparaffinization solvent to protect the bacterial cell wall. The acid used for decolorization in the Fite procedure is also weaker (Fig. 5).

## Silver Stains (Warthin Starry Stain, Dieterle, Steiner Stains)

**Utility of the Stains:** Silver stains are very sensitive for the staining of bacteria and therefore most useful for bacteria which do not stain or stain weakly with the Grams and Giemsa stains. Although they can be used to stain almost any bacteria, they are tricky to perform and are therefore reserved for visualizing spirochetes, legionella, bartonella and *H. pylori.*

**Principles of Staining:** Spirochetes and other bacteria can bind silver ions from solution but cannot reduce the bound silver. The slide is first incubated in a silver nitrate solution for half an hour and then "developed" with hydroquinone which reduces the bound silver to a visible metallic form. The bacteria stain dark-brown to black while the background is yellow (Fig. 6).





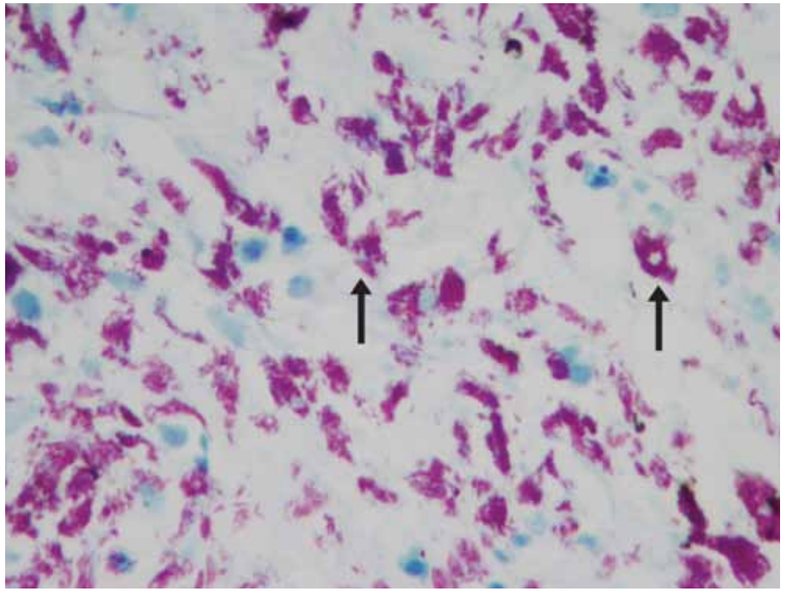

**Figure 5.** Ziehl-Neelsen stained section of lymph node. The pink color demonstrates clusters of mycobacteria stained with carbol-fuschin (arrows). The stain has resisted decolorisation by acid-alcohol. Other cells in the background are stained light blue by the methylene blue counterstain.

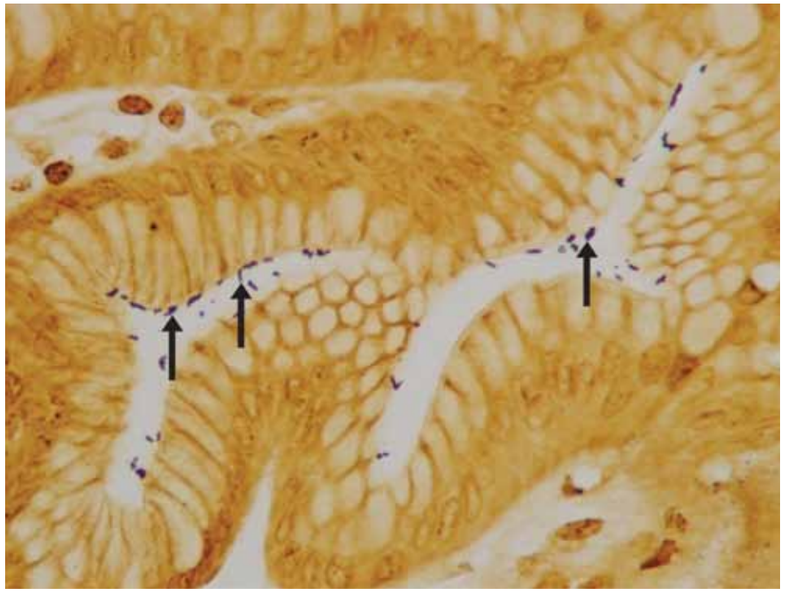

**Figure 6.** Warthin-Starry stain of stomach containing *Helicobacter pylori* which appear as black and slightly curved, rod-like bacteria (arrows). The background is stained light yellow.

**Table 1.** Provides a summary of some special stains used in detecting microorganisms.

| Microorganism | Preferred Stains | Disease |
|---|---|---|
| **Bacteria** | | |
| Bacteria, usual | Gram, Giemsa | Wide variety of infections |
| *Mycobacteria tuberculosis* | Ziehl-Neelsen, Kinyoun's | Tuberculosis |
| *Mycobacteria lepra* | Fite | Leprosy |
| *Rickettsia* | Giemsa | Rocky Mountain Spotted fever, typhus fever |
| Chlamydia | Giemsa | Sexually transmitted disease, pneumonia |
| Legionella | Silver stains | Pneumonia |
| Spirochetes | Silver stains | Syphilis, leptospirosis, Lyme's disease |
| Bartonella | Warthin-Starry | Cat-scratch disease |
| *Helicobacter pylori* | Giemsa, Diff-Quik, Alcian-yellow Toludine blue, Silver stains | Inflammation of stomach, stomach ulcers |
| **Protozoa** | | |
| Giardia | Giemsa | "traveller's diarrhea" |
| Toxoplasma | Giemsa | Toxoplasmosis in immunocomprised hosts |
| Cryptosporidium | Giemsa | Diarrhea in AIDS patients |
| Leishmania | Giemsa | Skin infections, severe generalized infection with anemia and wasting |
| Plasmodium | Giemsa | Malaria |
| Trichomonas | Trichomonas | Vaginal infection |
| Ameba | PAS stain | Diarrhea, liver abscess |





### Auramine O-Rhodamine B Stain

The auramine O-rhodamine B stain is highly specific and sensitive for mycobacteria. It also stains dead and dying bacteria not stained by the acid-fast stains. The mycobacteria take up the dye and show a reddish-yellow fluorescence when examined under a fluorescence microscope.

## Summary

Histochemical stains available for demonstrating microorganisms include Giemsa stain, Grams stain, carbol fuschin acid-alcohol stain and a variety of silver stains such as Warthin-Starry, Dieterle and Steiner stains. The Gram stain allows classification of bacteria into Gram-positive and Gram-negative bacteria. The acid-alcohol stain allows classification of bacilli into acid-fast and non-acid-fast bacilli. These are both clinically useful classifications. The silver stains are very sensitive and help to visualize difficult-to-stain bacteria. Most protozoans are stained by the Giemsa stain.

# Chapter 12 | Special Stains in Interpretation of Liver Biopsies

**Rashmil Saxena, BFA, HT(ASCP)CM**

Special histochemical stains are routinely used for the interpretation of liver biopsies. Most laboratories have standard protocols for liver biopsies so that special stains are automatically performed for these specimens without having to be specially ordered by the pathologist. The panel of special stains varies from laboratory to laboratory, depending on tumor versus non-tumor cases, or transplant versus non-transplant liver biopsy specimens.

## Commonly Used Special Stains

Most anatomic pathology laboratories perform a trichrome stain along with two or three H&E stained sections. The remaining panel of special stains varies depending on the laboratory and consists of a variable combination of reticulin silver stain, Perl's iron stain and Periodic Acid-Schiff (PAS) stain with and without enzyme digestion.

## Special Stains for Special Circumstances

Some stains are ordered for special purposes when indicated by the clinical situation. For instance, the Ziehl-Neelsen stain is ordered for mycobacteria, and Grocott's silver methanamine stain is used when granulomas are seen or infection is suspected. The Congo Red stain is requested when amyloid is suspected to be present. Rhodanine stain, Victoria blue or orcein stain is ordered to detect copper deposition when there is clinical suspicion of Wilson's disease (see Glossary). The latter two may also be used for the detection of hepatitis B surface antigen; but most pathologists prefer to use immunohistochemistry to detect hepatitis B surface antigen. The Oil Red O can only be performed on frozen sections and therefore is not widely applied in routine practice. In clinical practice, the Oil Red O stain is mainly ordered on frozen sections of liver biopsy specimens to assess the amount of fat in donors for liver transplantation. This stain is also popular with researchers when they are looking for fat in the liver. The Sirius red and aniline blue stains are used for selective staining of collagen when quantitative measurement of fibrosis by morphometry is required. The Hall's stain for bile pigment seems to be obsolete today and is hardly used in clinical practice or research applications.

## Trichrome Stain

**Principles of Staining:** As the name implies, the trichrome stain uses three dyes to impart three different colors, on collagen, cytoplasm and nuclei respectively. The three most common trichrome stains are Masson's trichrome, Gomori's one-step trichrome and the Van Gieson stain. Of these, the first two are used in the majority of laboratories. The Masson's trichrome stain consists of sequential staining with iron hematoxylin which stains nuclei black; Biebrich scarlet which stain cytoplasm red and aniline blue or aniline light green which stain collagen blue or green respectively.

The Gomori's One-Step method employs the same principles except that all dyes are present in a single solution together with phosphotungstic acid and glacial acetic acid. The red color in this Gomori's one-step method is imparted by chromotope 2R.

**Utility of Trichrome Stain:** The trichrome stain is performed on medical liver biopsies to assess the degree of fibrosis in the liver. A large number of liver diseases such as hepatitis B and C viral infections, fatty liver disease, alcoholic liver disease and chronic biliary diseases show the formation of fibrous tissue including bridging fibrous septa leading to the end-stage process called cirrhosis (Fig. 2). The aim of the treatment in these disease processes is to halt the progression of fibrosis. Therefore, every liver biopsy report that comes from a pathologists office contains a statement about the degree of liver fibrosis, also known as the stage of disease. The degree of liver fibrosis provides hepatologists the necessary information regarding the advancement of the disease thereby helping them to make the required therapeutic decisions. Comparison of the degree of fibrosis in pre- and post treatment biopsies indicate if the treatment has been effective or to what degree has it been effective. Comparison of fibrosis is important in clinical trials to assess the success of different medications. The assessment of fibrosis is mostly carried out with a trichrome stain making this staining procedure a preferred method on medical liver biopsies.

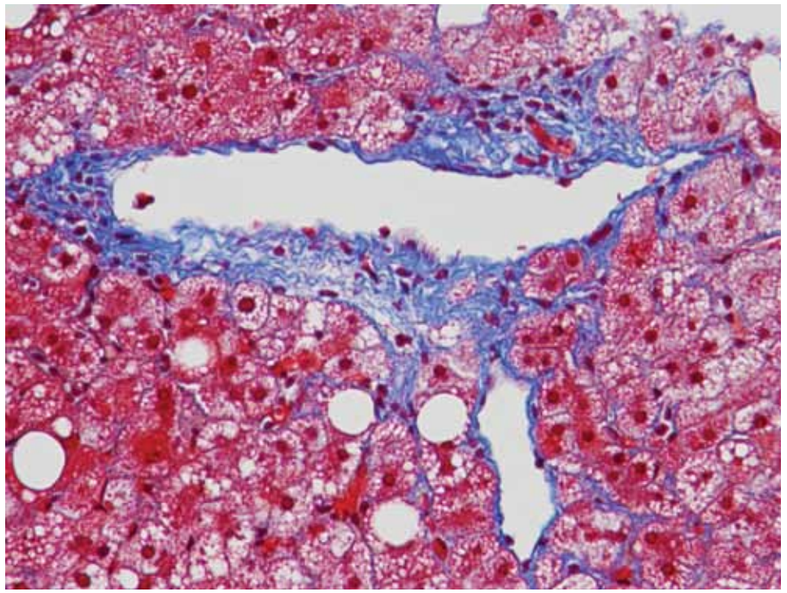

**Figure 1.** Trichrome stained liver showing fibrous tissue. The fibrous tissue is stained blue while the cytoplasm of hepatocytes are stained red. The nuclei can be seen as dark red to black structures within cells. Collagen in the fibrous tissue are stained blue (with aniline blue) or very light green (by aniline light green).

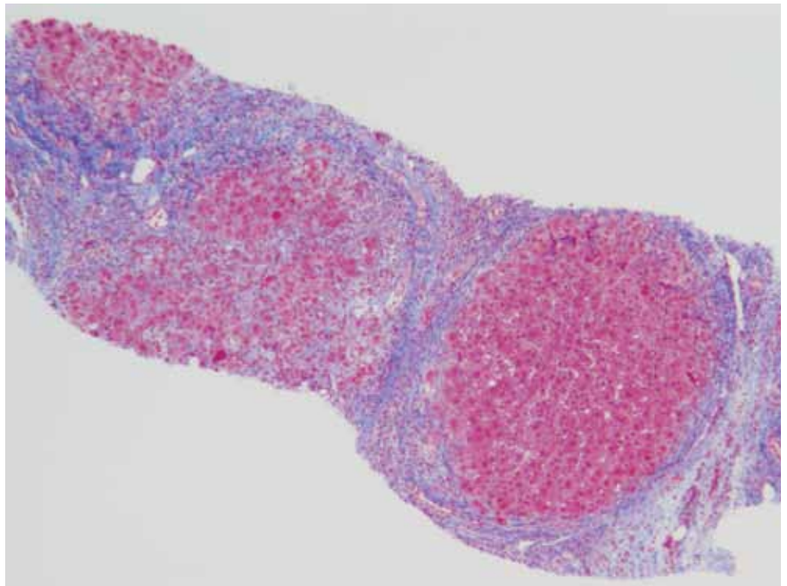

**Figure 2.** Trichrome stain showing cirrhotic liver. As it is evident, the normal architecture of the liver is destroyed in this disease and the liver shows nodules surrounded by fibrous bands.





In summary, a trichrome stain is used to assess fibrosis, which gives important information about stage and progression of disease. The stain is used to make treatment decisions; utilized to assess the effect of therapy, medications in clinical trials and is needed for all liver biopsy specimens.

## Reticulin Stain

**Principles of Staining:** Reticulin stains are silver stains based on the argyrophilic properties of reticulin fibers. The two most common reticulin stains are the Gomori's stain and Gordon & Sweet's stain. The first step in the staining procedure consists of oxidation of the hexose sugars in reticulin fibers to yield aldehydes. The second step is called "sensitization" in which a metallic compound such as ammonium sulfate is deposited around the reticulin fibers, followed by silver impregnation in which an ammonical or diamine silver solution is reduced by the exposed aldehyde groups to metallic silver. Further reduction of the diamine silver is achieved by transferring the sections to formaldehyde; this step is called "developing". The last step consists of "toning" by gold chloride in which the silver is replaced by metallic gold and the color of the reticulin fibers changes from brown to black.

**Utility of Reticulin Stain:** Reticulin fibers are thin fibers composed of collagen III which form a delicate stromal network in many organs. The reticulin network is particularly rich in the liver and can be seen along hepatic trabecula. Since reticulin provides the stromal support for the parenchyma, the reticulin stain provides important information about the architecture of the liver. When hepatocytes are damaged and undergo necrosis, the reticulin fibers surrounding them collapse in the empty space left behind. Areas of reticulin crowding thus indicate focal hepatocyte loss (Fig. 4). Large areas of cell necrosis appear as reticulin collapse. On the other hand, when hepatocytes regenerate, the reticulin fibers show thickening of the hepatic cell plates which appear as two or three- cell thick plates instead of the usual 1-cell thick plates (Fig. 5). Fibrous tissue composed of collagen type I appear brown on a reticulin stain and thus can be distinguished from reticulin fibers (Fig. 3).

In summary, a reticulin stain is useful for demonstrating liver architecture; hepatocyte necrosis and hepatocyte regeneration.

## Iron Stain

**Principles of Staining:** The Perl's stain is the most commonly used method for staining of iron. It depends on the Prussian blue reaction. The tissue is first treated with dilute hydrochloric acid to release ferric ions from binding proteins. The freed ions then react with potassium ferrocyanide to produce ferric ferrocyanide which is an insoluble bright blue compound called Prussian blue.

**Utility of Iron Stain:** The liver is one of the main organs for storage of excess iron. Iron may be stored in cells as a soluble compound called ferritin or an insoluble form called hemosiderin. Ferritin cannot be seen on an H&E stain while hemosiderin appears as coarse golden-brown refractile granules (Fig. 7). On the Perl's stain, ferritin appears as a faint bluish blush while hemosiderin appears as coarse blue granules. Hemosiderin may accumulate in hepatocytes or Kupffer cells. Accumulation in Kupffer cells usually occurs in secondary hemosiderosis in which there is an underlying cause for iron accumulation such as hemolysis and transfusions (Fig. 8). Deposition in hepatocytes occur in primary hemochromatosis which is due to a genetic mutation in a key gene involved in iron metabolism (Fig. 6). The iron stain helps to define the pattern of iron deposition and provides a clue to the possible underlying causes of excess iron. Iron deposition also occurs in a wide variety of other diseases such as hepatitis C and fatty liver disease. In the former, iron deposition has been thought to affect response to therapy. The iron stain also gives an estimate of the degree of the iron deposition and various grading methods exist to grade the extent of deposition in the liver.

In summary, the iron stain is useful for demonstrating excess iron deposition in the liver providing information about the degree of iron deposition and clue to the underlying causes leading to the iron deposition.

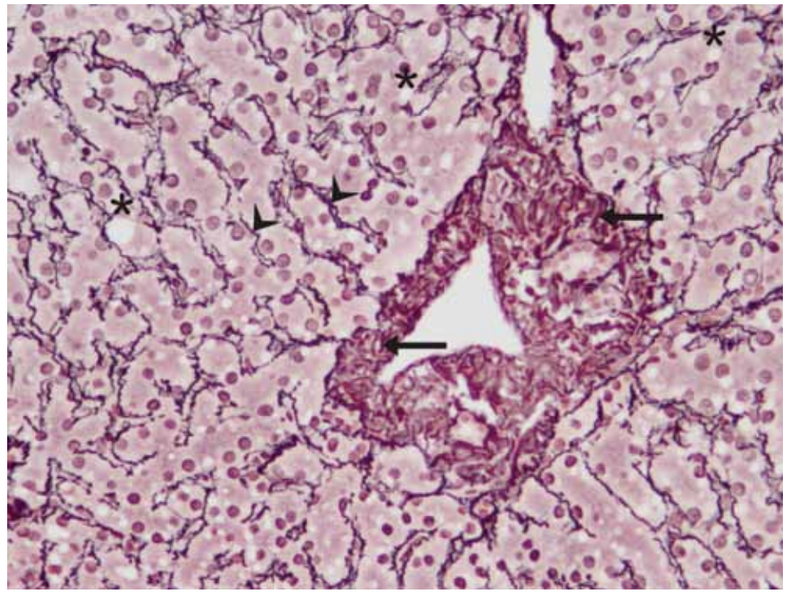

**Figure 3.** Dark black staining of the reticulin fibers (arrowheads) with reticulin stain. Collagen fibers are stained brown (arrows), nuclei red and the background in grey, or light pink if overtoned. The other white circular structures near the reticulin fibers are fat globules (asterix*).

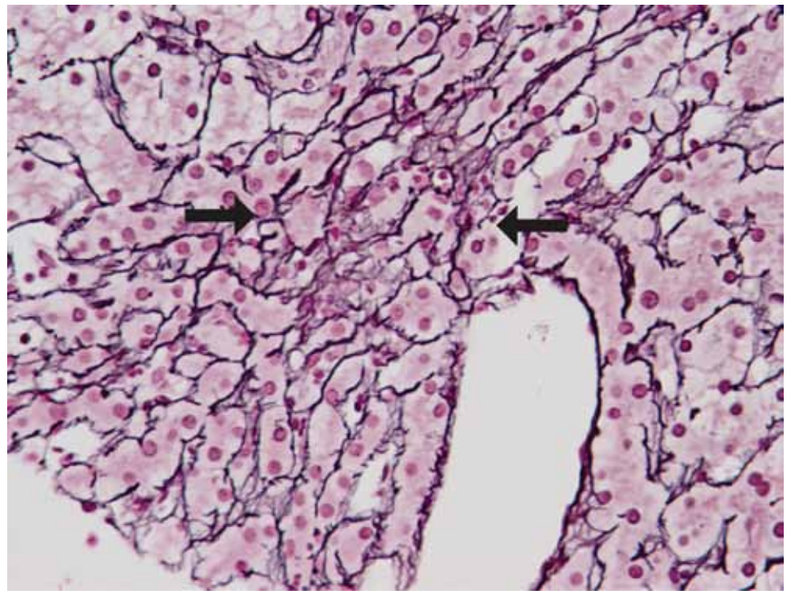

**Figure 4.** Reticulin stain shows an area of collapse of reticulin fibers (between arrows) corresponding to an area of cell loss.





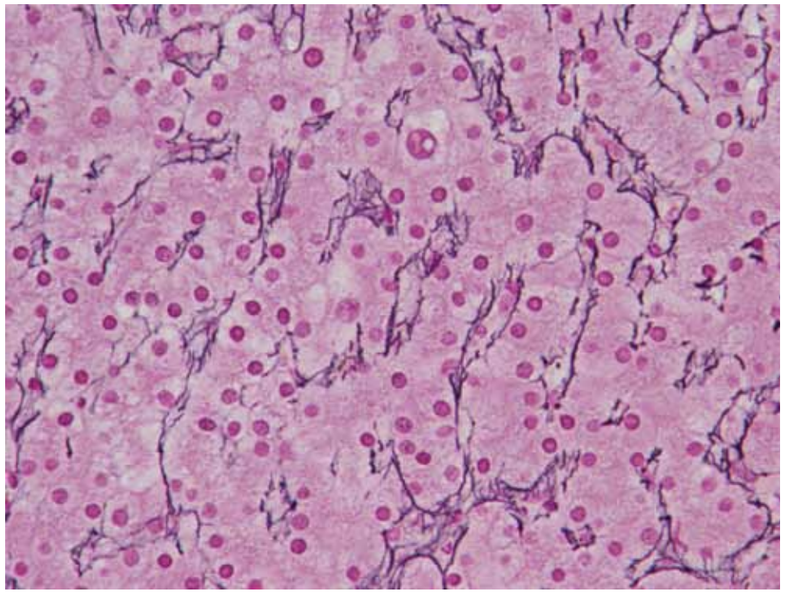

**Figure 5.** Reticulin stain shows hepatic plates which are more than 1-cell thick indicating regeneration of hepatocytes.

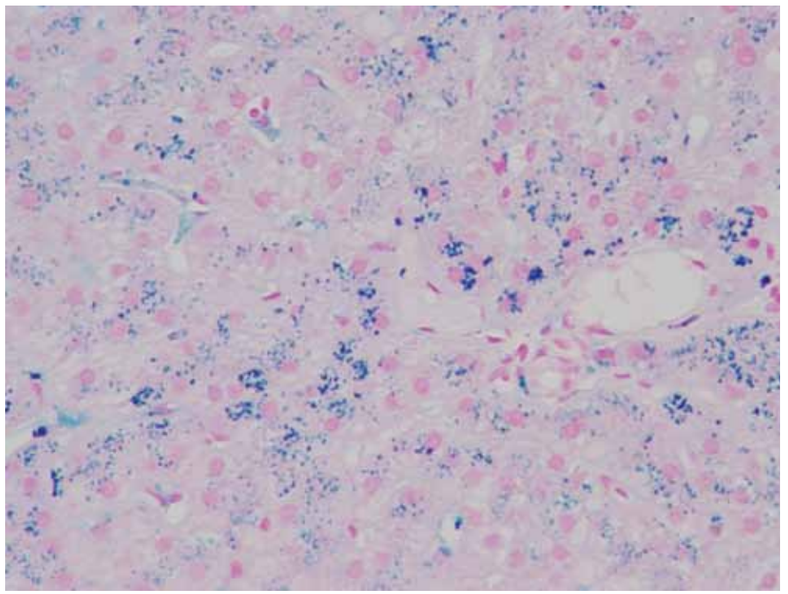

**Figure 6.** Perl's iron stain shows accumulation of dark blue granules of hemosiderin within hepatocytes. This pattern of iron deposition occurs in genetic hemochromatosis. The coarse blue granules are hemosidern, and the bluish blush is ferritin.

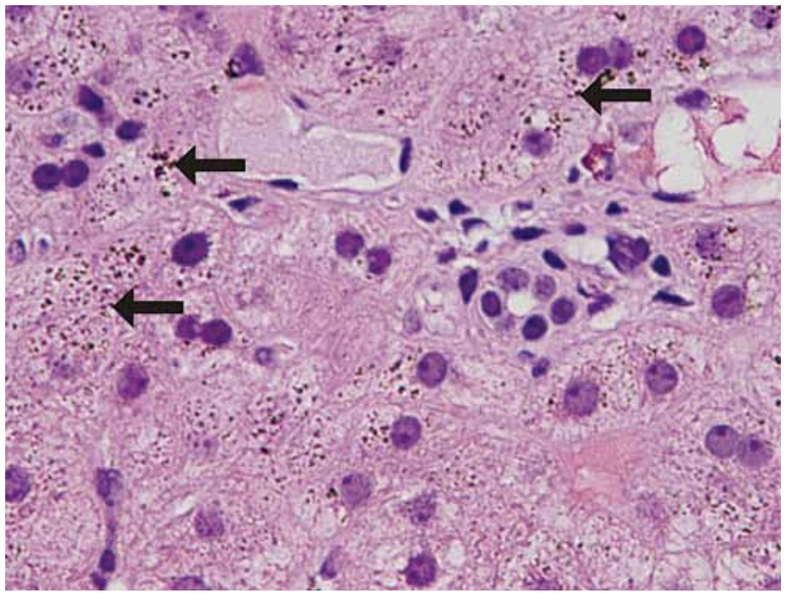

**Figure 7.** Hemosiderin appears on an H&E stain as coarse, dark-brown, refractile granules.

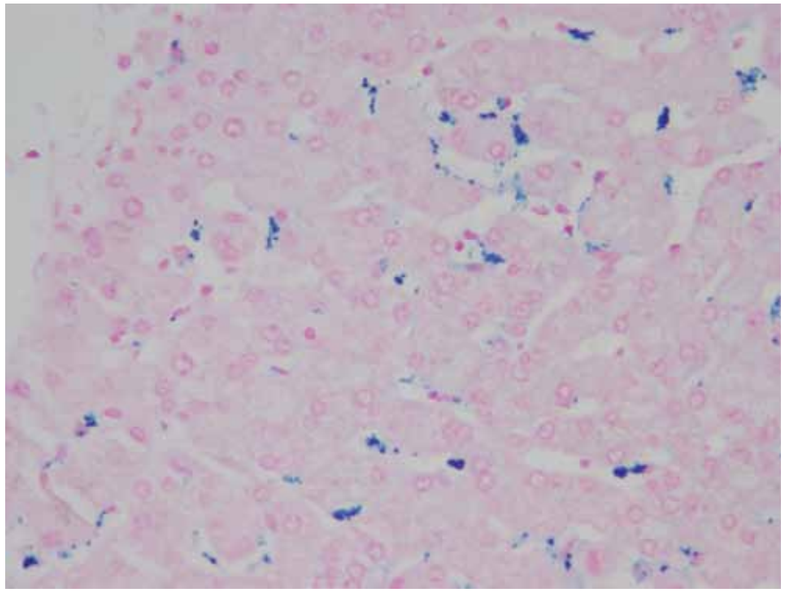

**Figure 8.** Perl's iron stain showing hemosiderin accumulation in Kupffer cells. This pattern is seen in secondary hemosiderosis.





## PAS Stain With and Without Enzyme Digestion

**Principles of Staining:** The PAS stain is based on exposing aldehyde groups in sugars by oxidation with periodic acid. The exposed aldehyde groups react with the chromophores in Schiff's reagent (produced by treating basic fuschin with sulfurous acid) to produce a bright pink color. The PAS stain demonstrates glycogen, neutral mucosubstances and basement membranes. Treatment with alpha-amylase digests glycogen and the PAS staining performed following enzyme digestion shows no staining. Enzyme digestion is also called diastase digestion. The word diastase comes from malt diastase which contains both alpha and beta amylase. However, alpha-amylase derived from various sources is preferred to malt diastase.

**Utility of PAS Stain:** The normal liver contains a large amount of glycogen. This, therefore leads to the staining of hepatocytes intensely pink with a PAS stain (Fig 9). This staining is abolished following treatment with alpha-amylase as this enzyme selectively digests glycogen. The major utility of the PAS stain with enzyme digestion is for demonstration of alpha-1 antitrypsin globules within hepatocytes (Fig. 10). Alpha-1 antitrypsin is a major serum anti-protease which protects the body from the destructive actions of endogenous enzymes. Some individuals have genetic mutations that lead to an abnormal alpha-1 antitrypsin which accumulates in hepatocytes and can be demonstrated by the PAS stain. PAS stain with enzyme digestion also highlights storage cells in Gaucher's disease and Niemann-Pick disease. Finally, the stain is useful for the demonstration of basement membranes around bile ducts which may be destroyed or thickened in biliary diseases.

In summary, the PAS stain is useful for demonstrating alpha-1 antitrypsin globules in hepatocytes; storage cells in Gaucher's and Niemann-Pick disease, and abnormalities of the bile duct basement membrane in biliary diseases.

## Oil Red O Stain

**Principles of Staining:** The Oil Red O stain is based on the greater solubility of the dye in neutral fats than in the solvent in which it is dissolved. Oil Red O dissolved in an alcohol is used for staining. In tissues containing fat, the Oil Red O moves from the staining solution to the tissue fat because of its greater solubility in the latter than in alcohol. Oil Red O staining can only be performed on frozen sections since tissue fat is removed by the alcohols and clearing agents used for paraffin processing. The sections are counterstained with hematoxylin and mounted in an aqueous medium or a synthetic medium that will not dissolve the tissue fat.

**Utility of Oil Red O Stain:** Oil Red O is used to demonstrate neutral fats in liver tissue. It is used to assess the presence and extent of neutral fat in two main situations. First, it is used on frozen sections of liver donors for transplantation and second in experimental studies which require the evaluation of the presence and extent of fat in liver tissue.

## Rhodanine Stain

The rhodanine stain is used to demonstrate excess copper in hepatocytes in Wilson's disease, a disease in which a genetic mutation leads to an excessive storage of copper within the body. The stain is also used to demonstrate copper deposition in chronic biliary diseases. The stain is based on the reaction of 5 p-dimethylaminobenzylidine rhodanine with copper-binding protein associated with deposited copper.

## Orcein Stain

Orcein is a natural dye obtained from lichens which are found to stain copper-associated protein, elastic fibers and hepatitis B surface antigen, all of which stain dark brown.

The orcein stain is used for diagnosis of hepatitis B infection as well as copper accumulation in Wilson's disease or in chronic biliary diseases.

## Victoria Blue

The Victoria Blue stain demonstrates hepatitis B surface antigen, copper-binding protein and elastic fibers, all of which stain blue.

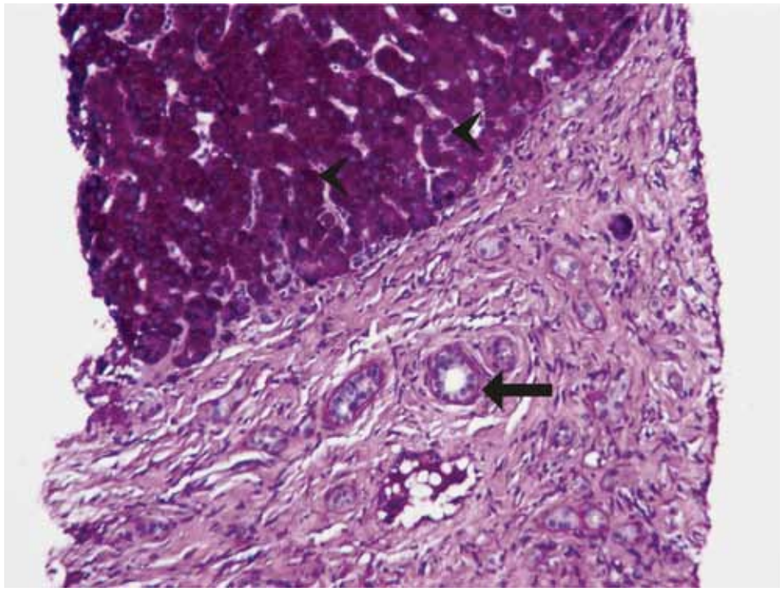

**Figure 9.** PAS stain shows intense staining of hepatocytes (arrowheads) and basement membranes of bile ducts (arrow). Glycogen, neutral polysaccharides and basement membranes are stained bright pink.

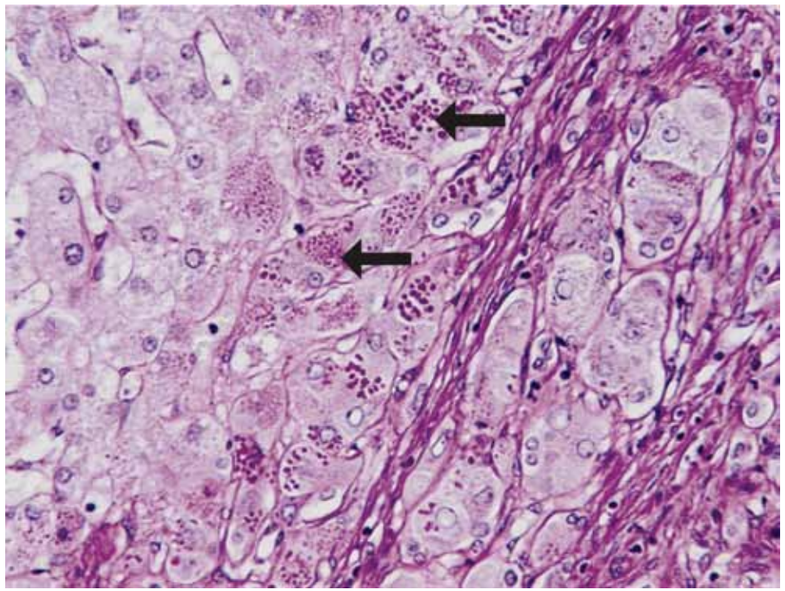

**Figure 10.** A PAS stain with enzyme digestion shows globules of alpha-1 antitrypsin (arrows). Neutral polysaccharides and basement membranes are stained bright pink and glycogen is seen as colorless areas.





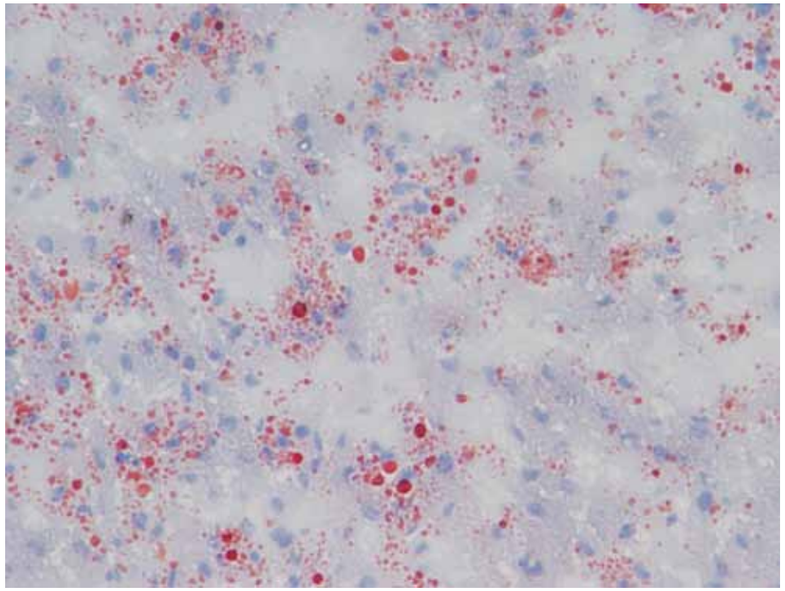

**Figure 11.** Oil Red O stain shows orange-red droplets of fat of varying sizes. Neutral fats are stained orange-red and the nuclei are seen in blue.

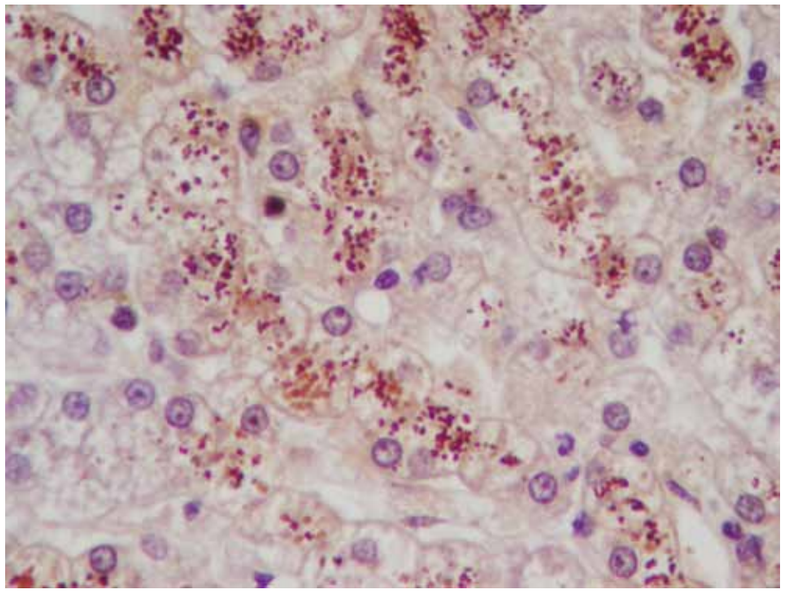

**Figure 12.** Rhodanine stain shows bright orange to brown granules within hepatocytes. Copper is stained bright orange to brown and nuclei in blue.

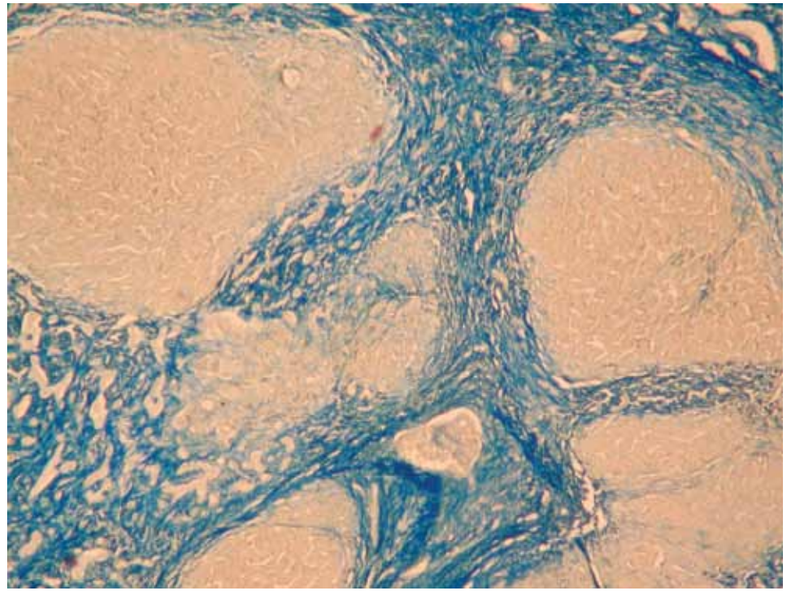

**Figure 13.** Methylene blue staining of a cirrhotic liver. Collagen appears dark blue with methylene blue.

## Sirius Red Stain

Sirius Red F3B (Direct Red 80) is an extremely hydrophilic dye which stains collagen I fibers, reticulin fibers, basement membranes and some mucins. The stain is most often used in research applications to enhance the natural birefringence of collagen. When observed by brightfield microscopy, collagen, reticulin fibers, basement membranes and some mucins appear red, while the counterstained nuclei appear grey-brown to black. However, under polarized light, collagen is selectively visualized as bright yellow to orange fibers.

## Methylene Blue Stain

The methylene blue stain is used to selectively stain collagen in cirrhotic liver for digitized morphometry applications (Fig. 13).

## Summary

In summary, special stains are very important in the interpretation of liver biopsies. The trichrome stain imparts valuable information about the stage of chronic liver diseases and effect of treatment. Reticulin stain is used for assessment of hepatic microarchitecture. The PAS stain with diastase highlights storage of abnormal metabolites and alpha-1 antitrypsin. The Perl's and rhodanine stains allow detection of intracellular deposits of iron and copper respectively.





Summary of Special Stains in Diagnostic Liver Pathology.

| Stain | Staining Characteristics | Utility |
|---|---|---|
| Masson's Trichrome | Collagen: blue | To assess the degree of fibrosis |
| | Hepatocytes: red | To make treatment decisions |
| | Nuclei: dark red to black | To assess the effect of therapy |
| | | To assess medications in clinical trials |
| Gordon and Sweet's silver | Reticulin fibers: black | Demonstrates the liver architecture |
| | Collagen fibers: brown | Demonstrates hepatocyte loss or necrosis |
| | Background: grey to light pink (depending on toning) | Demonstrates proliferation of hepatocytes |
| Perl's iron stain | Hemosiderin: coarse dark blue granules | Demonstrates presence of hemosiderin deposition |
| | | Demonstrates extent of hemosiderin deposition |
| | | Demonstrates the site of hemosiderin deposition (primary vs secondary) |
| PAS stain | Glycogen, neutral polysaccharides, basement membranes: bright pink | Demonstrates the presence of storage cells in Gaucher's disease and Niemann-Pick disease |
| | | Demonstrates bile duct basement membrane damage in biliary diseases |
| | | Demonstrates the presence of alpha-1 antitrypsin globules |
| Rhodanine | Copper-associated protein: bright orange to red | Demonstrates copper deposition in Wilson's disease and chronic biliary diseases |
| Orcein | Copper associated protein, elastic fibers, hepatitis B surface antigen: dark brown | Demonstrates the presence of hepatitis B infection |
| | | Demonstrates copper deposition in Wilson's disease and chronic biliary diseases |

Summary of Special Stains in Diagnostic Liver Pathology.

| Stain | Staining Characteristics | Utility |
|---|---|---|
| Victoria Blue | Copper associated protein, elastic fibers, hepatitis B surface antigen: blue | Demonstrates the presence of hepatitis B infection |
| | | Demonstrates copper deposition in Wilson's disease and chronic biliary diseases |
| Sirius Red | Collagen, reticulin fibers, basement membranes, some mucins: red | Enhances the natural birefringence of collagen under polarized light |
| Aniline Blue | Collagen: blue | Exclusively stains collagen for digitized morphmetric analysis |
| Oil Red O | Neutral fats: orange-red | Demonstrates the presence of fat in frozen sections of liver biopsies from donor livers for transplantation |
| | | Demonstrates the presence of fat in liver in experimental studies |

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# Chapter 13 | H&E Staining: Oversight and Insights

**Gary W. Gill, CT(ASCP)**

Böhmer and Fischer independently introduced the stains hematoxylin and eosin in 1865 and 1875 respectively (1, 2). In 1867, Schwarz introduced the first double staining technique using successive solutions of picric acid and carmine (1867) (3). With the idea of a double staining technique already published, it wasn't difficult for Wissowzky to describe the combination of the hematoxylin and eosin (H&E) dyes in 1876 (4). All four authors published their articles in German journals; two in the same journal, which may account for the relative rapidity of communication and development in those pre-Internet times more than a century ago.

As the common saying goes, "simple is best", and so it is that H&E has stood the test of time. Even today, 134 years after its introduction, it is still the most frequently used staining method in anatomical pathology worldwide (5). Simple though it may be, however, H&E staining doesn't always produce satisfactory outcomes. In this article, therefore, I describe what it takes to stain tissue sections well.

## Hematoxylin

Hematoxylin is extracted from the heartwood of the Central American logwood *Haematoxylon campechianum* Linnaeus (Fig. 1 and 2). *Haematoxylon* is derived from Greek, *haimatodec* (blood-like) and *xylon* (wood). Hematoxylin by itself cannot stain. It must first be oxidized to hematein, which process is referred to as ripening. Ripening can proceed spontaneously and slowly by exposure to atmospheric oxygen, or rapidly by added chemical oxidants such as mercuric oxide (Harris) or sodium iodate (Gill).

Since virtually everyone buys hematoxylin and eosin stains ready-made, I am not providing instructions how to prepare these solutions. Suffice it to say, hematoxylin formulations are more alike than different. All contain hematoxylin, oxidizing agent, mordant, often glacial acetic acid, and sometimes citric acid instead; all dissolved in water or 25% ethylene glycol.

Hematoxylin powder or crystals in a screw-cap jar already contain hematein. Numerous oxidants have been used historically, but sodium iodate is preferred because it oxidizes at room temperature and is environmentally friendly (and does not contain mercury).

Using half the sodium oxide required to oxidize the entire amount of hematoxylin is intended to ensure that it is not overoxidized initially, which would produce a product with a short useful shelf-life. Figure 3 shows the stoichiometric oxidation of a gram of anhydrous hematoxylin

**Figure 1.** *Haematoxylon campechianum*, which can grow up to 50 feet tall. Inset: leaves on branch.

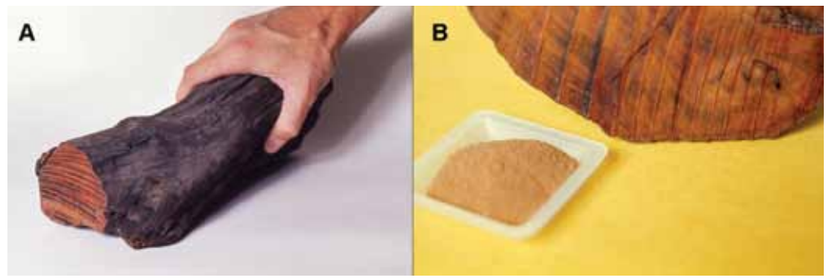


**Figure 2. A.** Logwood. **B.** Cut end of same logwood and hematoxylin powder.

by 217.7 mg sodium iodate. Nominally half that amount, 100 mg, is used to “half-oxidize” the hematoxylin. Furthermore, the remaining hematoxylin can continue to be spontaneously oxidized slowly by atmospheric oxygen to maintain the strength of the solution. Users should expect, therefore, possible variation in the strength of each new lot of hematoxylin.

Not only is hematoxylin useless as a dye, but its oxidation product hematein also cannot stain. Negatively-charged, hematein cannot become attached to the negatively charged phosphoric acid groups along DNA and nucleoproteins that are collectively known as chromatin. To stain and to introduce color, positively charged metallic ions are added as a mordant (French–*mordre*, to bite). In fact, it is the mordant plus the hematein that does the staining. In the case of Gill and Harris hematoxylins and many other formulations, aluminum ions are used. The combination is known as hemalum, which is the chemically correct nomenclature, and is specifically responsible for the blue color. Other mordants produce other colors. For example, potassium produces a purple color; iron, a black color; and copper, a green color. As a matter of conventional practice and simplicity, we talk about hematoxylin staining rather than hemalum staining even though the latter term is chemically correct.

Gill hematoxylin formulations use as a mordant the simple salt aluminum sulfate as recommended by Baker (14). The simple salt is responsible for Gill hematoxylin’s staining mucin. Harris hematoxylin uses as a mordant the complex salt aluminum ammonium sulfate, which results in its not staining mucin. Depending on the amount of hematein present – which always varies – the ratio of mordant ions to hematein molecules ranges from 8 to 16. Baker identified this range as the critical mordant quotient, which demonstrably promotes progressive staining.

Glacial acetic acid is added routinely to Gill hematoxylin and to some Harris formulations. It should be added until the deep purple color of the hematoxylin solution (i.e., hematein + mordant) changes to a deep cherry red. Acetic acid separates aluminum ions from hematein molecules. A cherry red color serves as a subjective visual endpoint that assures, albeit qualitatively, the same starting concentration of aluminum-hematein from batch to batch. Stated another way, hematoxylin formulations with acetic acid will stain less intensely than an identical formulation without acetic acid when applied for the same length of time.

Progressive hematoxylin stains color primarily chromatin and to a much less extent cytoplasm to the desired optical density, regardless

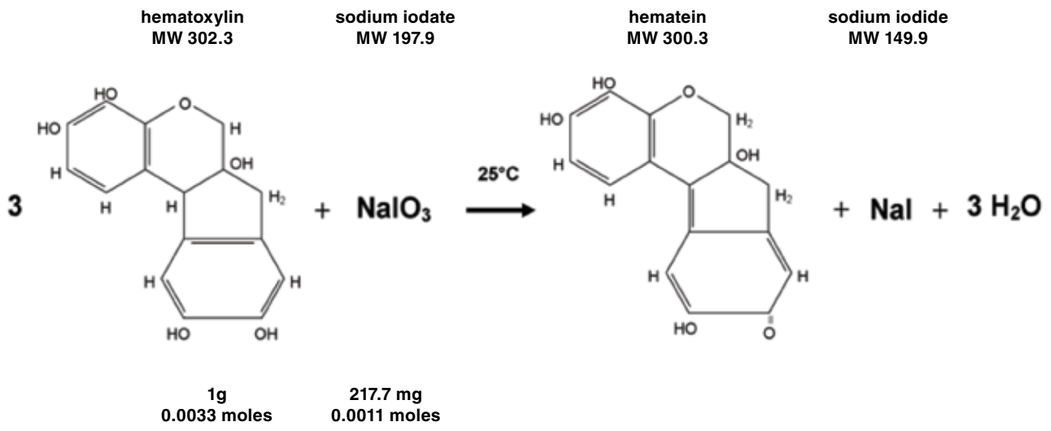


**Figure 3.** Stoichiometric oxidation of hematoxylin.





**Table 1.** Progressive and regressive hematoxylin formulations: similarities and differences.

| Aspect | Hematoxylin | |
|---|---|---|
| | Progressive | Regressive |
| Hemalum concentration | Less (ie, 1 to 4 gm/L) | More (ie, 5 gm/L or more) |
| Acetic acid | Present | Absent |
| Rate of uptake | Slow | Rapid |
| Easily controlled? | Yes | No |
| Overstaining? | No | Yes |
| Differentiation required? | No | Yes |

of the length of staining time. Regressive hematoxylin stains overstain chromatin and cytoplasm and require subsequent immersion in dilute acid to pull out the excess color from the chromatin and cytoplasm (Table 1). If differentiation is omitted or incomplete, residual hematoxylin visually obscures fine chromatin detail and can prevent the uptake of eosin entirely.

Gill hematoxylins No. 1 and 2 contain 2 and 4 gm hematoxylin per liter respectively and 25% ethylene glycol. They are progressive stains that can be applied for many minutes without overstaining and without differentiation in a dilute acid bath. Harris hematoxylin contains 5 gm hematoxylin per liter of water. It overstains within minutes and requires differential extraction in dilute HCl to decolorize the cytoplasm (differentiation) and to remove excess hematoxylin from chromatin. Figure 4 illustrates the difference between the two approaches.

Hematoxylin formulations initially color cells and tissues red, which users do not see in the normal course of events. To see the red color of hematoxylin, it is suggested that one immerse hematoxylin-stained slides immediately in 95% ethanol, which is pH neutral; followed by the immediate removal of the slide to examine it microscopically. This red color represents the starting color of the bluing process (Fig. 5).

Why blue the red dye? According to Baker: “It colors the same objects as the blue, and is equally insoluble in ethanol. The color itself is probably the reason. The one is a dull looking red, the other bright blue. It is easy to choose an anionic dye that will provide a striking contrast with the blue, but this is difficult with the red (14). Anionic dyes are also known as acid dyes. The latter term does not relate to pH, but it is a fact that an acid pH promotes the uptake of acid dyes such as eosin[1].

The hemalum is converted to an insoluble blue color by immersing the hematoxylin-stained sections in a bluing solution. Bluing can occur over a wide range of pH, beginning at about pH 5 and up. The lower the pH of a bluing solution, the slower the rate of bluing, and vice versa (Fig. 5).

With the possible exception of some acidic tap waters, most public tap waters are sufficiently alkaline (pH 5.4 to 9.8) relative to Al-hematein to convert the color from red to blue (ie, bluing). For bluing, two minutes in tap water is satisfactory. However, to convince oneself, staining two tissue sections routinely in hematoxylin; rinsing one section in tap water without bluing *per se*, and the other with bluing (for example by using Scott’s tap water substitute) will do the job[2].

[1]Baker’s explanation may not be compelling, but it is the only one I have ever seen published. For readers not familiar with Baker, he is considered by many to be a giant in the field of biological microtechnique. His many publications are timeless and scholarly expositions. [2]At this point, you could skip the counterstains, dehydrate, clear, mount, and compare.

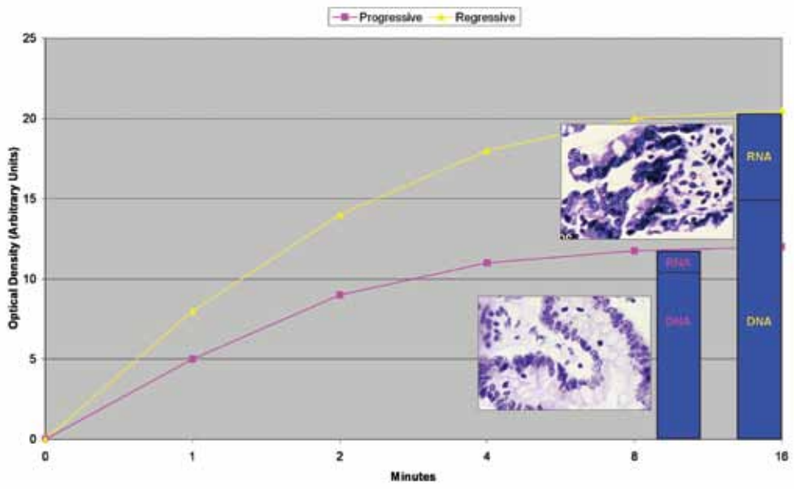


**Figure 4.** Hypothetical uptake of aluminum-hematein in cells: progressive vs. regressive staining.

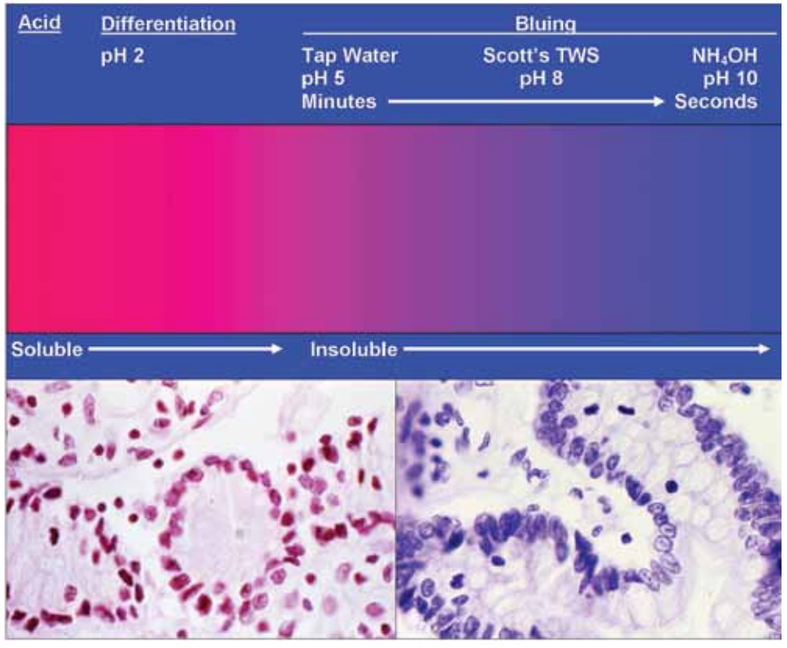


**Figure 5.** Bluing is the process of converting the initially red soluble hemalum to a final blue insoluble form. Tap water alone can blue cells satisfactorily; chemically defined bluing agents are unnecessary.





The higher the pH of a bluing solution, the faster the rate of bluing. Ammonium hydroxide in alcohol, for example, blues hemalum within seconds. Longer immersion can loosen cell adhesion to glass and result in cell loss.

## Eosin

Eosin Y is the basis for eosin stains (Fig. 6). Although its classical name is eosin (*eos*, meaning dawn; Y, yellowish), it is also known as eosin G, Bromo acid, and tetrabromofluorescein. Its solubility in water at room temperature far exceeds the amount used in any eosin stain solution. Similarly, its solubility in alcohol also exceeds the amount ever used in alcohol, but is a fraction of its solubility in water (i.e., 2.18% vs. 44.2%). This difference can be used to advantage when preparing stain solutions by making concentrated aqueous stock solutions of eosin. American companies have access to eosin dyes that have been certified by the Biological Stain Commission, which requires – among other things for eosin Y—minimum dye content of 90%.

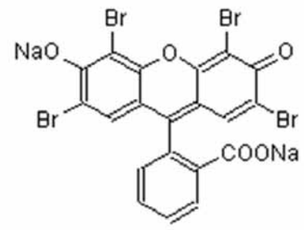


**Figure 6.** Eosin Y (tetrabromofluorescein).

To ensure quantitative consistency, the amount of dye used for any particular eosin formulation must be based on total dye content (TDC) and be adjusted as needed. For example, if 100 liters of 0.5% TDC eosin Y is being prepared commercially using 90% dye content eosin, 555 g—not 500 g—must be added The extra 55 g is an unknown mix of salts and impurities that are found in most, if not all, biological stains and dyes. Otherwise, using 500 g of 90% dye content will result in 450 g eosin Y being used, which equals a 0.45% (w/v) eosin Y solution.

To those rare individuals who prepare their eosin solutions from scratch, it is recommended making no less than 500 mL of an aqueous 20% (w/v) TDC eosin Y stock solution. Using aqueous stock solutions saves time and facilitates dissolving the dye in alcohol. Table 2 shows a variety of eosin formulations.

Composition differences that promote eosin uptake in cells:

- Increasing eosin concentration
  (from 0.5 to 2.5 gm [note a 5-fold range])
- Water rather than alcohol,
- Acetic acid, and
- Staining times (1 dip to 3 minutes). Acetic acid acts as an accentuator that dramatically shortens staining times. If eosin overstains, it can be removed by differentiation in alcohol until the desired color density is reached.

Note: Glacial acetic acid is included in three of the six eosin stain formulations in Table 2. The impact of acetic acid on the uptake of acid (negatively charged) dyes such as eosin is immense. Figure 7 illustrates the mechanism.

Phloxin B (CI No. 45410)[3] is sometimes added to eosin formulations (i.e., 0.5 gm/L eosin stain) to increase the range of red colors. However, phloxin B is exceedingly “bright” and can be visually overpowering if too much is used. Therefore, one needs to be cautious when using phloxin B. One successful formula for preparing eosin is as follows (20):

| | | |
|---|---|---|
| ▪ Biebrich scarlet (ws [water soluble]) | CI No. 26905 | 0.4 gm |
| ▪ Eosin Y | CI No. 45380 | 5.0 gm |
| ▪ Phloxin B | CI No. 45410 | 2.1 gm |
| ▪ 95% ethanol | | 200 mL |
| ▪ Distilled water | | 800 mL |

[3]C.I. numbers are 5-digit numbers assigned by The Society of Dyers and Colourists (http://www.sdc.org.uk/) to uniquely identify stains with the same chemical composition but different names. These 5-digit numbers must be specified when publishing or purchasing dyes to ensure using the same dye, even if identified by different names.

**Table 2.** Eosin Y stain solution variants in order of increasing staining strength.

| Reference | Eosin Y (gm) | Water (mL) | 95% Ethanol (mL) | Acetic Acid (mL) | Staining Time |
|---|---|---|---|---|---|
| McManus (15) | 0.5 | 30 | 70 | 0 | 2-3 min |
| Lillie (16) | 0.5 | 100 | 0 | 0 | 1 min |
| Disbrey/Rack (17) | Slow | Rapid | 0 | 0 | 5-10 dips |
| AFIP (18) | Yes | No | 80 | 0.5 | 15 sec |
| JHMI* | No | Yes | 70 | 0.5 | 1 dip |
| Carson/Hladik (19) | No | Yes | 67 | 0.5 | 10-20 dips |

*JHMI = The Johns Hopkins Medical Institutions, Baltimore, Maryland. References are in parenthesis.

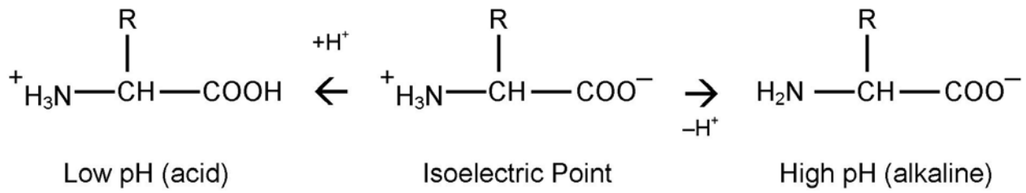


**Figure 7.** Shows a generalized amino acid. In a protein most of the amino and carboxy groups are on side chains (R in the structures shown) – which outnumber the alpha-NH2 (only at the N-terminal) and the C-terminal -COOH. Amino acids united by peptide linkages make up proteins, which are all that remain in cytoplasm following fixation in formalin. If an amino acid in solution is placed in an electric field, as in electrophoresis, the molecules will migrate to one pole or the other in accordance with the pH of the solution. At a certain pH, which is unique to the particular protein, the amino acid does not migrate to anode or cathode. This pH is the isoelectric point. Adding glacial acetic acid (ie, low pH) neutralizes the COO– groups and leaves relatively more positively charged $H_3N$ groups. As a result, eosin Y molecules, which are negatively charged, are attracted to the positively charged groups, and thus are taken up faster and in greater total amounts per given amount of time. Ref: Singer M. Factors which control the staining of tissue sections with acid and basic dyes. *Intern Rev Cytol.* 1952; 1:211-56.





To see what H&E stains should look like when applied together to the same tissue section at the same time, it is useful to stain a section in hematoxylin only and another in eosin only using the same solutions and times as in the routine method. This approach allows one to see what each stain looks like without any interference from the other. Sections stained in H&E that don't display the pure colors seen in singly-stained sections should trigger troubleshooting of the method. When H&E outcomes go awry, it is usually because too much of one stain or the other has been taken up, or removed, or a combination of the two.

Hematoxylin may be applied progressively or regressively, depending on the concentration of the hematoxylin formulation. Apart from the particular hematoxylin formulation and associated differences in staining times, as well as the addition of an acid bath and related rinses, H&E staining methods are almost identical. Table 3 begins with the staining procedure starting with paraffin-embedded fixed tissue sections that have been deparaffinized (also referred to as dewaxed or decerated), and are ready to be stained (Table 3).

## Notes

- Rinses may also be referred to as baths or washes.
- Rinses remove traces of previous solutions; they prepare the sections for the next solutions that are different.
- Dipping sections in each rinse promotes the exchange of solutions. Standing rinses are discouraged. A dip is fully submersing sections in, and removing them from, each rinse.
- For maximum effectiveness, rinses should be in sets of three, kept deep, and clean.
- If used repeatedly without being changed, rinses become less effective. For example, rinses unchanged following eosin become dye solutions themselves. When the concentration of dye in the rinse equals that in the tissue, the eosin cannot escape the tissue, which results in "muddy" staining results.
- The amount of stain that remains in a tissue represents the difference between the amount deposited by the stain solution and the amount removed by the rinse.
- One-step hydration and dehydration work satisfactorily. Graded alcohols are unnecessary.
- Gill hematoxylin – No. 2 is recommended. It is a progressive stain with high slide throughput.
- Harris hematoxylin[4] is available in 4 different formulations of decreasing strength in the following order: 1) full-strength without acetic acid, 2) full strength with acetic acid, 3) half-strength without acetic acid, and 4) half-strength with acetic acid. The stronger formulations (1 and 2) stain regressively; the weaker formulations (3 and 4), progressively.
- Regardless of the hematoxylin, whether it is Gill, Harris, Mayer, Ehrlich, Delafield, etc., the finished results should be virtually identical in terms of color, optical density (i.e., light, dark), and distribution (i.e., nucleus vs. cytoplasm).
- Gill and Harris hematoxylins are used as examples because the author is familiar with them.
- Thin sections will stain less optically dense than thick sections when both are stained for the same length of time.
- Differentiation is a portmanteau for differential extraction.
- 0.5% HCL in 70% ethanol is prepared by adding 5 mL concentrated HCl to 995 mL 70% ethanol. Using a higher concentration of HCL (e.g., 1%) can extract excess hematoxylin rapidly and result in understaining, especially if the acid is mixed with water only. Seventy percent ethanol slows the rate of decoloration.
- Overdifferentiation in HCL is a potential limitation of using regressive hematoxylin formulations.
- Most tap water sources will "blue" hematoxylin. A chemically defined bluing agent (e.g., Scott's tap water substitute) isn't necessary.
- There are many eosin stain formulations (Table 2). The one described here is comprised of 5 gm (total dye content) eosin Y (CI No. 45380), 5 mL glacial acetic acid, and 995 mL 70% ethanol.
- No appreciable fading occurs in preparations stained and rinsed well. Well-kept slides do not fade even after more than 35 years. Fading is defined as any change in color, not merely a weakening of the shade.

[4]Harris and Gill are the only currently marketed hematoxylin formulations – in America. Further, their names are the only ones cited in the Clinical Laboratory Improvement Amendments (CLIA '88) interpretive guidelines: "Stains used (ie, Harris, Gill or other type of hematoxylin, OG-6, modified OG-6, EA36, EA50, EA65, modified EA) or the identity of a combination counterstain." (6)

**Table 3.** Progressive and regressive H&E staining methods. * See Notes for details.

| Step | Progressive Times | Solution | Regressive Times | Purpose |
|---|---|---|---|---|
| 1 | 10 dips | Tap water | 10 dips | Hydrate |
| 2 | 10 dips | Tap water | 10 dips | |
| 3 | 10 dips | Tap water | 10 dips | |
| 4 | Gill-2 × 2 min | Hematoxylin | Harris × 6 min* | Color nuclei |
| 5 | NA | Tap water | 10 dips | Rinse |
| 6 | NA | Tap water | 10 dips | |
| 7 | NA | 0.5% HCl in 70% EtOH* | 10 dips | Differentiate |
| 8 | 10 dips | Tap water | 10 dips | Rinse/blue/rinse |
| 9 | 10 dips | Tap water | 10 dips | |
| 10 | 10 dips | Tap water | 10 dips | |
| 11 | 10 dips | Tap water | 10 dips | |
| 12 | 1-2 dips | 0.5% (w/v) eosin Y* | 1-2 dips | Color tissue and nucleoli |
| 13 | 10 dips | Tap water | 10 dips | Rinse |
| 14 | 10 dips | Tap water | 10 dips | |
| 15 | 10 dips | Tap water | 10 dips | |
| 16 | 10 dips | Absolute ethanol | 10 dips | Dehydrate |
| 17 | 10 dips | Absolute ethanol | 10 dips | |
| 18 | 10 dips | Absolute ethanol | 10 dips | |
| 19 | 10 dips | Xylene | 10 dips | Clear |
| 20 | 10 dips | Xylene | 10 dips | |
| 21 | 10 dips | Xylene | 10 dips | |





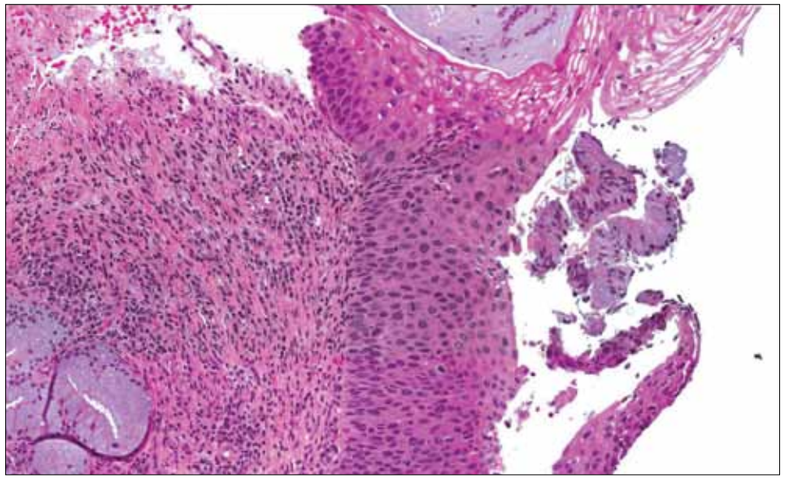

**Figure 8.** H&E stained sectioned biopsy of uterine cervix with marked dysplasia (precancerous changes) ×100 (original magnification).

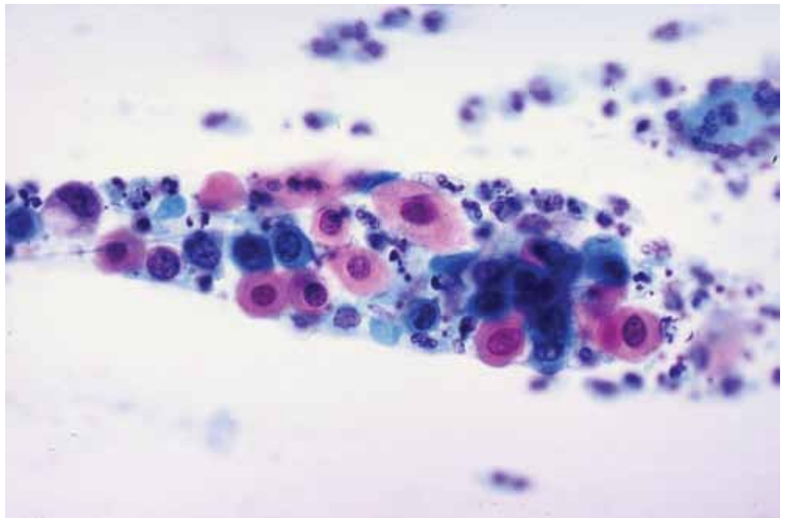

**Figure 9.** Carcinoma *in situ* in conventional Pap smear, modified Papanicolaou stain ×400 (original magnification).

## Results

Ideally, hematoxylin should color chromatin blue. Depending on the mordant, mucin may also be colored blue. The depth of color (i.e., optical density) should be deep enough to make small particles visible and shallow enough to not obscure fine details. Cytoplasm should be colored scarcely at all.

Eosin should color nucleoli red, and stain cytoplasmic structures varying shades of red to pink. When present, erythrocytes and cilia should also be colored varying shades of red to pink (Fig. 8).

It is interesting that few laboratories, if any nowadays, prepare hematoxylin and eosin staining solutions from “scratch.” Stains are bought readymade. They are prepared by vendors with varying degrees of staining knowledge and quality assurance programs. Not all stain solutions with the same name prepared by different vendors perform the same. It is also to be noted that those who perform H&E staining are not the same individuals who interpret the microscopic morphology. Given these two practical realities, the opportunities for things going wrong are plentiful.

While the exact appearance of an H&E-stained section will vary from lab to lab, results that meet the individual user’s expectations are considered satisfactory. This means, of course, that others with different expectations may conclude otherwise. Quality has many definitions and is context-dependent, but a practical working definition is “the result useful for its intended purpose.” If the user can see what s/he needs to see to interpret the tissue, the H&E results are functionally satisfactory. This is not necessarily the same as technically satisfactory, in which an experienced observer can see no technical deficiencies in the results.

## Conclusion

The widespread use of commercially-prepared stain solutions such as hematoxylin and eosin has increased user reliance on the manufacturers and decreased user reliance on basic knowledge. An unintended consequence has been a reduced recognition of satisfactory results, an increased tolerance for marginal satisfactory or unsatisfactory results, and an inability to troubleshoot problems. Hence, it is essential that users immerse themselves in basic knowledge about staining materials and methods so they can control the quality of results.





**Table 4.** Composition of Gill Hematoxylin.

| No. | Component | Gill Hematoxylin | | |
|---|---|---|---|---|
| | Mix in order at room temperature | No. 1 | No. 2 | No. 3 |
| 1 | Distilled water | 730 mL | 710 mL | 690 mL |
| 2 | Ethylene glycol | 250 mL | 250 mL | 250 mL |
| 3 | Hematoxylin, anhydrous | 2.0 gm | 4.0 gm | 6.0 gm |
| 4 | Sodium iodate | 0.2 gm | 0.4 gm | 0.6 gm |
| 5 | Aluminum sulfate | 17.6 gm | 35 gm | 54 gm |
| 6 | Glacial acetic acid | 20 mL | 40 mL | 60 mL |

## Appendix

I introduced Gill hematoxylin at the 20th annual scientific meeting of the American Society of Cytopathology in New Orleans in 1972. That introduction was followed by a 1974 paper that also described Gill hematoxylin No. 2 (7). Gill hematoxylin No. 3 is a tripling of components 3-6 in Table 4. It was introduced by Lerner Laboratories, the first company to make Gill hematoxylins available commercially in 1973 (13).

Since 1865, when Böhmer introduced the first successful use of hematoxylin to stain cells, more than 60 formulations have been introduced. (Suggested Reading 7) All the formulations include a solvent (usually water, sometimes with ethanol, glycerol, or ethylene glycol), hematoxylin, oxidizing agent, mordant (usually ammonium alum), and sometimes acid. Amounts of solutes per liter range from 0.4 to 20 gm hematoxylin, 3 to 140 gm mordant, and 50 to 500 mg oxidant. Not having read the original publications, I cannot comment on the thinking behind these various formulations. Few, however, have stood the test of time. Relatively few are available commercially today.

H&E is used universally on sectioned materials, most often in histopathology but also in cytopathology. After cell spreads have been prepared from non-gynecological specimens for Papanicolaou staining, residual cell suspensions of body cavity fluids, for example, can be centrifuged. The pellet can be processed as a cell block that is fixed, embedded in paraffin, sectioned, and stained in H&E. Such preparations sometimes contain abnormal cells that were not in the cell spreads.

The Papanicolaou stain is basically an H&E stain with three additional dyes: orange G as the first counterstain (OG-6) dye solution, and in a second dye solution known as EA, light green SF yellowish, eosin Y, and Bismarck brown Y. Because of chemical incompatibility with phosphotungstic acid (PTA), Bismarck brown is usually omitted from EA formulations today. PTA is essential for the differential staining by eosin and light green, but it precipitates Bismarck brown and renders it useless. The additional colors differentiate certain cell types from one another and facilitate the detection of abnormal cells during microscopic screening by cytotechnologists (Fig. 9).

## Acknowledgment

*We thank Michael D. Glant, MD for his excellent digital photo-micrograph (Fig. 8).*

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# Chapter 14 | Microscopy and Special Stains

**Alton D. Floyd, PhD**

Cell and tissue specimens placed on microscope slides are stained to provide contrast so that specimen details can be seen with the microscope. Staining is necessary simply because cells and tissues are essentially transparent (excluding melanin containing specimens) when viewed with transmitted light as is commonly employed in routine microscopy. "Special" stains are simply stain mixtures or combinations that provide selective or enhanced contrast for specific structures within the cell or tissue. This is desirable to either confirm the presence of specific structures or substances, or to permit detailed morphological study of these materials.

The microscope itself is a highly developed instrument, and is still under active development. While the theoretical resolution for transmitted light as predicted by Abbe has been achieved for over 100 years, recent developments have permitted resolution of structures well below these transmitted light resolutions. While these developments promise to provide much new information in the future, they rely on methods other than traditional staining of the specimen being examined.

For optimum results when examining stained specimens, the microscope must be used in a proper manner. All optical surfaces must be clean and debris-free. The microscope must also be in optical alignment and have a light source that can effectively illuminate the field of view for all objectives on the microscope. For purposes of this discussion, we will consider the traditional, upright transmitted light microscope. Be aware that many research laboratories, particularly those involved in fluorescence studies, use *inverted* microscopes, as these provide more flexibility in types of objects that can be placed on the specimen stage.

The laboratory microscope consists of a stand to which other parts are attached (see Figure 1 and Appendix on page 279). The stand gives the unit stability, and also carries the various controls necessary for use of the instrument. Attached to the stand is the stage, used to hold the specimen which is most commonly mounted on a glass or plastic microscope slide. The stage generally has some type of holding device to ensure the slide is held firmly down to the stage surface. In addition, most stages also have some type of movement device that permits the specimen slide to be moved in both side to side and front to back movements. These movement devices are designed to move the specimen smoothly, even at high magnifications, while keeping the specimen slide firmly against the stage surface (important to ensure the specimen remains in focus as it is moved).

The base of the microscope stand also carries the illuminator, which consists of some type of housing with a light source. The light source may be an incandescent light bulb, a tungsten halogen bulb, or an LED light source. For specialized examinations, i.e. fluorescence, arc lamps may be used. In all cases, the light source will have some type of lens arrangement to provide a beam of collimated light to the microscope. For some types of light sources, the illuminator may have concentrating mirrors as well as lenses to further enhance light efficiency. Most illuminators attach to the rear of the microscope stand, and the collimated light is projected through the base of the microscope to a mirror mounted in a line below the stage which turns the light 45 degrees and directs it upwards through the stage and specimen.

Just below the stage is a condenser. This is an integrated series of lenses which serve to concentrate the collimated light from the illuminator into a cone of light that just fills the capture cone of the objective lens, which is above the specimen. The condenser typically contains a diaphragm, which can be opened and closed to provide a larger or smaller cone of light. Often, a mechanical support is included on the bottom of the condenser to support a removable filter. This filter can be colored glass, plastic, or constructed of thin films (dichroic filters). In addition, the filter support may be used for a dark field stop or Rheinberg differential color filters.

Modern upright microscopes are *compound* lenses systems, which means there are at least two sets of lenses in the magnification system. The primary lens is the objective lens. There is generally more than one objective lens on a microscope, and these objectives are mounted on a rotating nosepiece. A common set of objectives would include a low power, 4 to 5 x lens, a 10 x, a 40 x and an oil immersion lens of approximately 100 x. If the microscope has five objective lenses positions, a 20 x lens is generally inserted between the 10 and the 40 x objective. Above the objective is a tube through which the image of the specimen is transmitted. At the top of the tube

is the ocular. The ocular is a compound lens and may be of several specific magnifications. Most common are 10 x oculars, but 5 x, 15 x and 20 x are also available, as well as some other magnifications. Total magnification of a compound microscope is the product of the objective and the ocular, i.e., a 20 x objective and a 10 x ocular gives a magnification of 200 times. Be aware that many microscopes include a tube lens between the objective and the ocular. If this is present, it is generally so marked on the tube, as well as the magnification factor. These tube lenses typically have magnification of 1.5 to 2, and this magnification factor would be included in the total magnification. For example, a 20 x objective and a 10 x ocular with a 1.5 x tube lens would give a total magnification of 300.

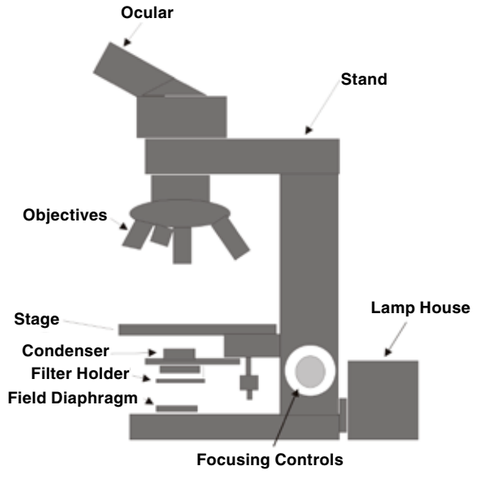


**Figure 1.** Generalized microscope.

Lenses are not perfect, and introduce certain distortions to the image. Manufacturers build corrections into the lens system to correct many of these distortions. However, each manufacturer may make these corrections in different ways. For this reason, it is imperative that a user never mix objective and ocular lenses from different manufacturers. As lenses are made of glass, they bend light of different wavelengths at different angles. This results in chromatic (color) distortion. Lenses can be corrected for this distortion to some degree, and the amount of correction applied has become standardized into three categories. The least corrected system is the common achromat lens system. Achromat lenses are the cheapest lenses, but do give enough color distortion that color fringes can be seen around the edges of objects. These types of lenses are not suitable for photography or image analysis. The second grade of lenses are fluorite lenses, which are made from a different type of glass. These lenses significantly reduce color fringing, but do not eliminate it entirely. Fluorite lenses are more expensive than achromat lenses. The most highly correct lenses are apochromat lenses. These lenses have negligible color fringing, and can be used for high quality image collection and image analysis. Because of the complexity of construction of these lenses, they are quite expensive. It should be noted that color correction is not an issue if the specimen is examined with light of a single color, such as that produced by a filter in the light path.

Resolution of a microscope is governed by the quality of the lenses used, the wavelength of light, and the alignment of the microscope. Since ultimate resolution occurs at shorter wavelengths of light, it should be understood that theoretical resolution is never achieved with white light, which is a mixture of all colors of light. Regardless, many microscopists never achieve the resolution of which their microscope is capable simply because they do not align the microscope. Alignment consists of centering the light source, and adjusting the condenser to fully fill the capture cone of the objective. The method of alignment with modern light sources is called Köehler illumination, and is quite simple to achieve (also refer to pages 279-283 in the Appendix). First, the illumination system should contain a field diaphragm. This is an adjustable diaphragm, generally located in the base of the microscope. It serves to reduce the diameter of the collimated beam of light coming from the lamp house.

To align a microscope, first place a specimen on the stage and focus the microscope until the specimen is in focus. While looking though the oculars, reduce the size (close down) of the field diaphragm until only a small illuminated spot is seen. Focus the condenser by moving the condenser up or down. The goal is to have the edge of the bright spot as sharp as possible. Using the centering screws of the condenser, move the condenser until this spot is in the center of the field of view. Slowly open the field diaphragm, and note that if the condenser is centered accurately, the edge of the field diaphragm disappears evenly all the way around the field of view. At this point, do not touch the condenser height adjustment as long as you





continue to use the same objective. Open the field diaphragm only until it just disappears from view. Opening further simply adds to stray, scattered light in the microscope, and reduces contrast. The only further adjustment is for the condenser diaphragm. To adjust this, pull the ocular out of the microscope and look straight down the tube. Close the condenser diaphragm until it covers the edge of the field of view down the tube. Now replace the ocular, and the microscope is fully aligned ***for the objective used during alignment***. As one goes to objectives of higher magnification, the condenser diaphragm will have to be opened further. Also, it will be found that most rotating nosepieces are not all that accurate in centration, so when one changes objectives, the condenser may need readjustment. As a practical matter, one generally aligns the microscope for the highest power objective being used, and simply ignores the decreased resolution at lower magnifications, unless one is collecting images, either photographically or digitally. Under no circumstance should the condenser height or condenser diaphragm be used to create contrast in the image. This practice invariably introduces distortions in the image and degrades resolution. If the illumination is too bright, then the illumination source should be turned down, or the intensity reduced by placing neutral density filters in the light path.

The filter holder, or a flat surface on the top of the field diaphragm, is an important item for a microscope used to evaluate special stains. Since many special stains are composed of complex mixtures of dyes of distinctly different colors, or in some cases subtle differences in colors, use of filters to change the color of light used for evaluation is quite useful. By selecting a color filter on the opposite side of the "color wheel" from the dye color of interest, the contrast of that dye is dramatically enhanced. This is quite useful when there is a question as to whether a particular structure has stained with a particular component of a special stain mixture. For those who engage in a considerable amount of special stain evaluation, it is advantageous to construct a filter holder permitting use of a circular continuous color wheel. Such a device can be rotated through the entire range of visible colors, all the while observing the effects on the microscopic image.

A filter holder is also quite useful to support a dark field stop. Dark field is a technique where the direct light that passes through the specimen is blocked by a solid disk, but the disk is surrounded by clear areas to permit light to pass around the central dark stop. The result is a cone of light illumination, none of which is captured directly by the objective. What is seen is *scattered* light. Small particles light up like stars in a dark sky, as do linear arrays of objects, assuming they have some degree of refractility. In fact, dark field images can be exceptionally dramatic when the specimen is mounted with an aqueous mountant that does not closely match the refractive index of glass (hardening mounting media are designed to closely match the refractive index of glass).

A unique refinement of dark field microscopy is Rheinberg differential color contrast. In this technique, the dark field stop is constructed of colored filters. If the central stop area is colored a deep blue, and the surrounding ring is yellow, then refractile objects will show up as bright yellow on a deep blue background. Rheinberg filters are easily constructed these days using a color printer and transparency film. Interesting results can be obtained by using various colors in the outer ring, and in segmenting the ring into two or more colors. As an example, if the outer ring is segmented into fourths, with two opposite sides in one color, and the other two sides in a contrasting color, the result in the image will be that refractile objects aligned vertically in the field will have one color, while those oriented horizontally will have the other color.


**Figure 2.** Examples of dark field and Rheinberg color stop.

When constructing dark field stops of Rheinberg color stops, a different sized central ring will be required for each objective used, as the capture cone for each objective is different. As one goes to objectives of higher magnification, the objective front lens has to be placed closer to the specimen, and this means that a larger (in diameter) cone of light is captured.

Microscope lenses are quite delicate, and the actual lens is quite small. A tiny bit of debris can cause serious problems in use of the microscope. Most lenses are coated with antireflection coatings, and

these are susceptible to scratching. Gentle air is useful to move lose materials from these surfaces. If a wipe must be used, it should be of lens quality fiber, and gently moistened with alcohol. If slides are examined that have been freshly prepared, and the coverslip medium is not yet dry, extra care must be taken to avoid getting mounting medium on the objective lens. This is a particular problem with the 40 x objective, as it must be very close to the slide surface to focus the specimen (this is why a 40 x objective cannot focus on the specimen if the slide is turned upside down - the slide glass is simply too thick to permit the objective to get close enough to the specimen).

The Picrosirius Red special stain is unique in enhancing birefringence of collagen. Birefringence is the property of an object to selectively interact with polarized light. While it is possible to obtain a microscope equipped for polarization, this is an expensive and highly specialized instrument. For examination of the Picrosirius stain, it is possible to simply utilize a simple polarizing filter in the light path, much as was done with the dark field stop or the Rheinberg filter. Polarizing filters for this use can be easily obtained from a camera shop, and these generally are mounted in a frame which makes handling easy. In use, the filter is slowly rotated through 360 degrees while observing the specimen. Areas containing collagen are easily seen with this procedure. The stain is so sensitive that one can detect basement membrane collagen, as well as reticular fibers.

The evaluation of special stains includes many different types of stains. Some of these are strictly empirical stains, developed for the sole purpose of imparting differential coloration to various cell and tissue elements. Other special stains are quite specific for a single molecular species, and are therefore histochemical in nature. An example of a strictly empirical stain is any of the trichrome procedures for connective tissue. Because of the various mordants and differentiations used in these stains, they are highly variable in terms of reproducibility of a specific color density. A histochemical stain, since it is actually a chemical reaction to a specific compound, can be standardized and repeated with sufficient accuracy to enable quantitative spectrophotometry through the microscope. Examples of such histochemical stains are the Periodic Acid-Schiff (PAS) stain for mucopolysaccharides and the Feulgen procedure for Deoxyribose Nucleic Acid (DNA). Quantitative microscope spectrophotometry has contributed greatly to our understanding of biological systems, and was used to demonstrate the DNA constancy hypothesis which simply stated says that all cell nuclei (other than mature germ cells) in an individual organism contain the same amount of DNA.

Image collection with the microscope is made possible by an addition of a third port to the head of the microscope (a trinocular microscope). This third port permits the use of a camera system. If the camera is closely matched to the microscope, one can obtain a parfocal system, that is, a system where the image seen by the camera is in focus when the image seen in the oculars is in focus. The camera attached to the microscope may be either a film camera or a digital camera. It is now rare to find film cameras, as digital cameras offer the advantage of instant image availability, and eliminate the cost of film processing. Digital images are also easily distributed, stored, and retrieved, and can be added to publications and reports much more readily than can film images.

Collecting images through a microscope requires careful alignment of the microscope. While the eye is quite tolerant of changing light intensity at the edges of the field of view, the camera is not. It is quite common to see “brown corners’ in images, whether digital or film. This is because the camera captures as much of the field of view as possible, and as the field of view approaches the edge of the lenses, there is a decrease in light intensity. For digital cameras, this is easily corrected by collecting “blank” images (nothing in the field of view) and then combining this blank image with the actual specimen image to correct the uneven illumination. While many camera systems have some type of correction built into their “capture” program to accomplish this, a very simple procedure is to collect the blank image, invert it (make it a “negative”), and then add it back to the actual specimen image. The result is totally even illumination in the resulting image.

For additional details on microscopy, a variety of texts can be consulted. Excellent chapters are available in all editions of Bancroft and Gamble “Theory and Practice of Histological Techniques”. An excellent internet resource is the Optical Microscopy Primer found at http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html.

An interactive Java tutorial of Rheinberg illumination can be found at http://www.microscopy.fsu.edu/primer/java/rheinberg/index.html



# Chapter 15 | Microscopic Quality Control of Hematoxylin and Eosin – Know your Histology

**Anthony Henwood, MSc, CT(ASC)**

Producing quality microscopic sections goes a long way to achieving a confident, accurate diagnosis. Unfortunately tissues can be distorted post-operatively, through fixation, during processing, microtomy and staining. A competent histological scientist needs to be able to minimise the distortion and correct it when the quality process goes out of control. Quality staining is highly dependent on fixation and processing and it is difficult to appreciate histochemical quality control (QC) in isolation (1). Recognising microscopic quality is directly connected with knowledge of basic histology and recognition of the common microscopic disease entities. This review analyzes the quality requirements for Hematoxylin and Eosin (H&E) staining.

Hematoxylin and eosin (H&E) is the “bread and butter” stain in most histopathology laboratories. It is appreciated that the H&E stain varies from laboratory to laboratory and that personal preference plays a large part in the final results. Some general guidelines in assessing a good H&E are:

1. Nuclear detail – how effective is the stain in demonstrating nuclear membranes, nucleoli, chromatin and nuclei of both vesicular (open) and hyperchromatic (small, dense) nuclei?
2. Cytoplasm and background substance – can cell cytoplasm, collagen, muscle, red blood cells and mucin be easily discerned?
3. In sections of appendix, lung or gut is the mucin of epithelial cells blue or clear? The mucin staining is a function of the Hematoxylin pH. Lowering the pH (usually by adding acetic acid) can significantly reduce mucin staining.
4. Do the hematoxylin-stained elements of the section appear brown (indicating an over-oxidised solution)?

Luna (1991) (2) believes that an H&E-stained slide should contain the following tinctorial qualities:

1. The nuclear chromatin should stain blue to bluish purple and be very distinct.
2. Nucleoli should appear reddish purple if the hematoxylin is well decolourised. If too much hematoxylin remains in the nucleoli, there is no doubt, that there is excess hematoxylin in the section. Excess eosin will produce a purple monochromatic nuclear chromatin and ill-defined nucleoli.
3. Collagen and muscle should stain well but in a delicate fashion. One should be able to see some form of fibrillar pattern by moving the microscopic focal plain up and down. The same pattern is observed by reviewing muscle except that muscle should have a slightly redder colour than collagen.
4. Eosinophilic granules should be well-defined and appear orange-red. Lack of well-defined eosinophilic granules suggests over-staining by either hematoxylin or eosin, or both.

These are general guidelines but are there guidelines for individual tissues?

When assessing H&E-stained sections from differing organs, features that are usually present are arteries. One should be able to differentiate muscle and collagen with muscle appearing as dark red. Red blood cells should be bright red. Assessment of nuclear staining will depend on the cell-types in the tissue being stained and the following is an attempt to set some standards according to the tissue being assessed.

## Nervous Tissue

The Nissl substance of neurones and Purkinje cells should be well demarcated and stain dark blue. The large nuclei should be open with a visible nucleolus. The fibres should be delicately eosinophilic which will allow easier demonstration of senile plaques if present. The pale pink staining of neurones will also allow easier detection of “red” neurones, an important indicator of antemortem injury (3).

## Pituitary

The pituitary contains three principle cell types that should be apparent with a good H&E: acidophils (40%) with pink cytoplasm, basophils (10%) with a pale blue cytoplasm and chromophobes (50%) with clear cytoplasm. The nuclei of acidophils should have relatively prominent nucleoli (3).

## Thyroid

Colloid should be pale pink. The cytoplasm of follicular cells should be a slightly darker pink. The nuclei should be delicately stained with discernible small darker chromatin. The basement membrane binding the follicular cells should be visible and appear as deep red. In well-fixed and processed tissue, Clear cells may be seen admixed with the follicular cells (4).

## Parathyroid

The two cell types should be recognisable. Chief cells should have clear to faint pink cytoplasm with even blue chromatin, and rare small nucleoli. The cell membrane should be poorly defined. Oxyphil cells should show an eosinophilic granular cytoplasm, pyknotic nuclei and should have a prominent cell membrane (3).

## Respiratory

The bronchioles contain smooth muscle and collagen in their walls. The lung parenchyma is rich in small blood vessels and thus eosin balance between muscle and collagen can be assessed. In the alveoli, type II pneumocytes should have pale nuclei with prominent nucleoli. The nuclei of type I pneumocytes are small and dense (4).

## Heart

The heart is rich in blood vessels as well as cardiac muscle and collagen enabling good eosin differentiation. Purkinje fibres, if present, should have pale cytoplasm with open vesicular nuclei. Cardiac nuclei should have evenly distributed dust-like chromatin with a clear (non-staining) perinuclear sarcoplasm. The eosin should demonstrate the cross-striations adequately (5).

## Lymph Node

In the germinal centres, centroblasts have large, round, vesicular nuclei with 1-3 small but conspicuous nucleoli. Small lymphocytes have dark staining, irregular nuclei. Plasma cells with their “spoke-wheel” chromatin pattern caused by small clumps of chromatin on the nuclear membrane in a clear nucleus may be present (Fig 1). A clear “hof” should be seen (3).

## Gastrointestinal Tract

The gastrointestinal (GI) tract contains epithelial, connective, neural and lymphoid tissues that need to be easily recognisable using a routine H&E. Lymphoid nuclei, especially those of plasma cells, should be assessed. The usual colour differentiation should exist between muscle and collagen. Ganglion cells should be apparent in between the muscle layers. The nuclei should contain a single, prominent, eosinophilic nucleolus and the cytoplasm should be pink (a lighter hue than that of the surrounding muscle). It is expected that the epithelial mucin should be clear. Blue staining of the mucin usually indicates that the pH of the hematoxylin is too high (Fig. 2). It has often been found that in these cases, the hematoxylin often appears over-stained resulting in difficulty in assessing the nuclear component. Throughout the GI tract there are unique cells that exist and it is important that they be recognised in a good H&E:

- In the stomach, the parietal cell cytoplasm should be light pink, where as that of the chief cells should appear purplish. Small nucleoli should also be apparent in the chief cells (3).
- Paneth cells, showing intensely eosinophilic cytoplasmic granules, should be present in the small intestine.
- In the large bowel, the nuclei of the goblet cells should appear darker than those of the absorptive cells. Endocrine and Paneth cells should be discernible by the eosinophilic granules present in the cytoplasm.

## Liver

The predominant cell is the hepatocyte. The nuclei should have small nucleoli visible. The pale red-pink cytoplasm of the hepatocytes should contrast well with the pink of collagen around blood vessels. There should also be small blue granules (RER) in the cytoplasm. Cuboidal bile duct cells have open nuclei and dark pink cytoplasm (3).

## Spleen

The cell population in the spleen is similar to the lymph node so you can recognise similar microscopic quality control features. Both medium-sized and small lymphocytes are present. Plasma cells are often found near the arteries. The endothelial cells lining the sinus should have bean-shaped nuclei containing a longitudinal cleft (3).



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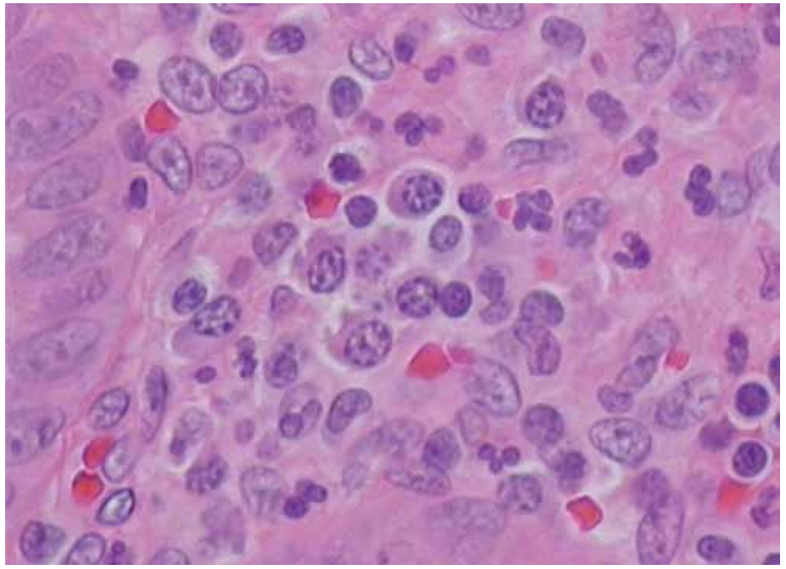

**Figure 1.** High power H&E showing plasma cells with characteristic cart-wheel nuclei.

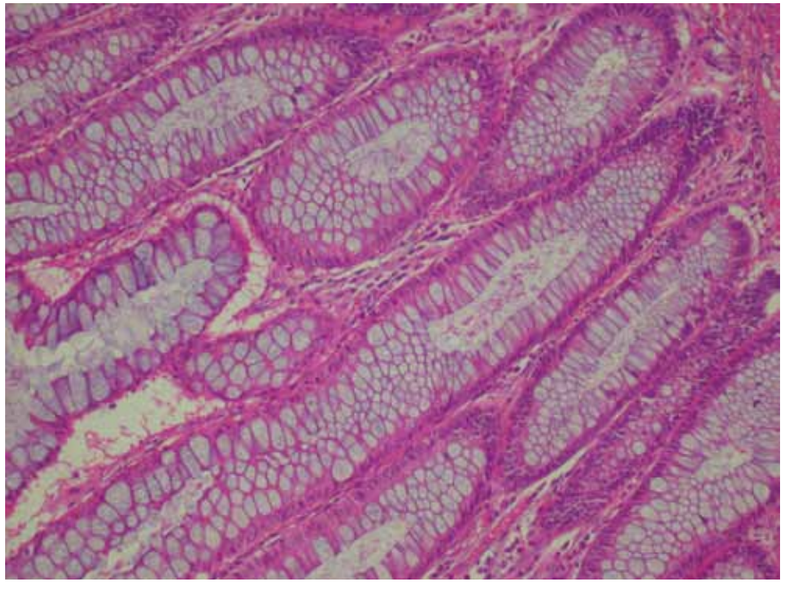

**Figure 2.** Colon section stained with H&E showing blue mucin staining. Decreasing the hematoxylin pH should remove this staining.

## Pancreas

The pancreas has a wide variety of cells to meet the exocrine and endocrine functions of the organ:

- Acinar cells have dark nuclei with a few tiny clumps and strands of chromatin. Nucleoli should be evident. The cytoplasm should have intense eosinophilia at the apical with strong hematoxylinophilic staining at the basal end.
- Duct cells have clear to pale eosinophilic cytoplasm and lightly stained nuclei (less dense than those of acinar cells).

Islet cell nuclei should have evenly stained nuclei and cytoplasm a paler pink than acinar cells. Within the islet, differing cells should be discerned with darker cytoplasm cells probably being glucagon containing cells (3).

## Kidney

The kidney contains a diverse population of cells from those exhibiting dense chromatin (in the glomerular tufts) to those exhibiting a dusting of chromatin material (cuboidal cells of the collecting tubules) (2).

The cytoplasm of the cells of the Proximal Convoluted Tubules have eosinophilic cytoplasm where as those of the Distal Convoluted Tubules have paler cytoplasm (fewer granules). The cells of the Proximal Convoluted Tubules should have a discernible brush border. The basement membranes of both proximal and efferent tubules should be visible (5).

## Adrenal Gland

Under low magnification, the cortex and medulla of the adrenal gland should be apparent with acidophilic (pink) cortex and basophilic (blue) medulla.

In the medulla, the chromaffin cells should have an overall blue cytoplasm. The nuclei should have coarsely clumped chromatin with visible clear areas. The nuclear material of the ganglion cells should be speckled and have prominent nucleoli.

Three zones should be visible in the cortex. In the zona glomerulosa (closest to the capsule), the cells should have clear cytoplasm containing a scattering of pink granules. The nuclei should have a longitudinal groove present in many of the cells. In the middle zone (zona fasciculata) the nuclei should be more vesicular and less chromatic than in the previous zone. A single small nucleolus should be apparent and the cytoplasm should be clear. In the zona reticularis, the cells have cytoplasm that is solid, granular and eosinophilic. Lipofuscin should be discernible especially in cells adjacent to the medulla (3).

## Prostate

The stroma of the prostate contains both collagen and smooth muscle and these should be easily differentiated on an H&E. The secretary epithelial cells of the central zone of the prostate should have darker cytoplasm and slightly darker nuclei compared to those in the peripheral zone (3).

## Testis

The main features of the testis that need to be recognised include (3):

- Sertoli cells – nuclei should have a slightly wrinkled nuclear membrane and have prominent nucleoli.
- Germ cells – You should be able to easily distinguish each of the differentiating elements based on the presence or absence of nucleoli and chromatin pattern. A good H&E is imperative.
- Leydig cells – have prominent nucleoli, the cytoplasm is intensely eosinophilic and the eosinophilic crystalloid of Renke should be visible.

## Endometrium

The endometrium contains epithelial and mesenchymal elements and these need to be differentiated with a good H&E. Prominent nucleoli should be apparent in the proliferating glandular epithelium. The smooth muscle of the myometrium should contrast well with the supporting collagen (4).



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## Bone

Bone, apart from the rigors of fixation and processing, is also subjected to acidic decalcifying solutions. This will adversely affect H&E quality. As a guide, osteoblasts have a clear to blue cytoplasm often containing a perinuclear halo. The nuclei often have a prominent nucleolus (3).

## Skin

Skin, apart from a squamous cell covering, contains hair, sebaceous and sweat glands, melanocytes, nerves, collagen and other cells that should be seen in a good H&E (Fig. 3a and 3b) (3):

- Keratohyalin granules in the cytoplasm of keratinocytes in the Stratum Granulosum should appear densely basophilic.
- Basal cells should show a coarse chromatin.
- Collagen, nerves and muscle should show differing shades of red-pink.
- The basement membrane should be seen.
- Within the Eccrine glands, three cell types should be present: Clear cells, Dark cells bordering the lumen of the glands and Myoepithelial cells.

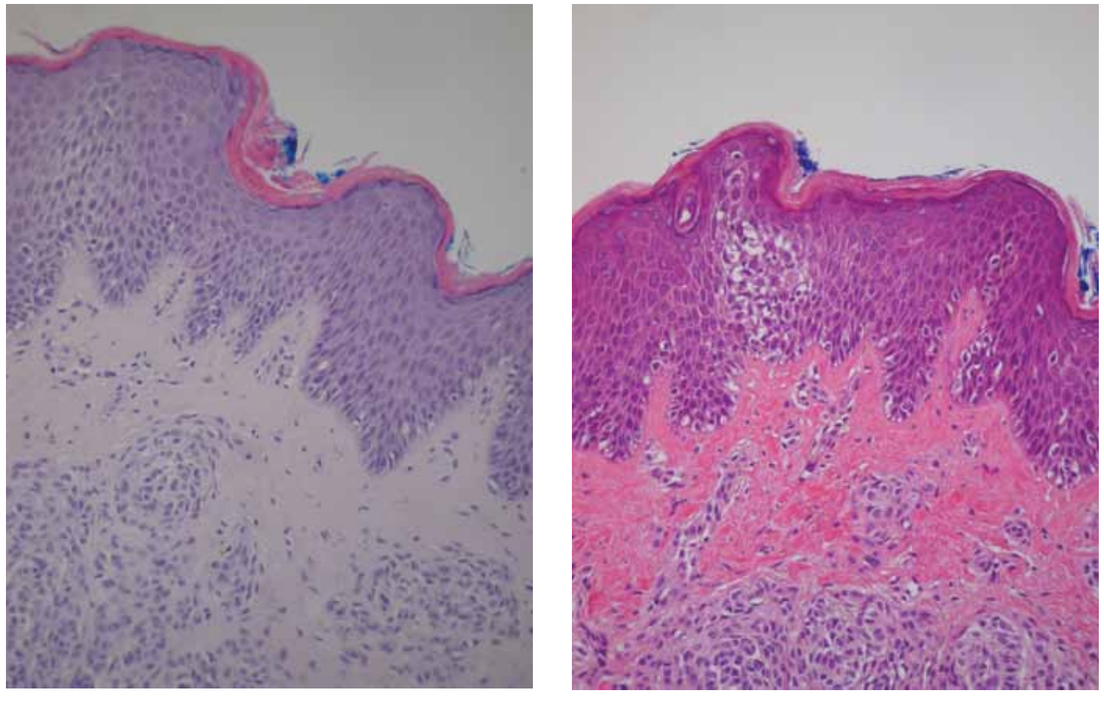

**Figure 3.** Skin section stained H&E. **A.** (Left) pH of eosin too high. **B.** (Right) Acidic eosin showing differentiation of collagen and neural tissue.

## Breast

Breast tissue contains blood vessels, collagenous stroma, myoepithelial cells and acinar epithelial cells. The latter cells are affected by the menstrual cycle, for example, myoepithelial cells have clear cytoplasm and small dense nuclei during the follicular phase and such changes need to be considered when assessing H&E quality control (3).

## H&E Quality Control Material

For the daily QC of the H&E stain I would suggest the following:

- Colon – differentiation of muscle and collagen, inappropriate staining of mucin, clear staining of epithelial vesicular nuclei. Assessing plasma cell staining is quite helpful in that a clear clock face arrangement of nuclear chromatin will indicate an adequate differentiation of the hematoxylin.
- Skin – demonstration of blue keratohyaline granules, differentiation of keratin from collagen and nerves, definition of the reticular/papillary border of the dermis.
- Kidney – identification of basement membranes and both proximal and efferent tubules. The kidney contains a wide diversity of cells from those exhibiting dense chromatin (glomerular tufts) to those having a dusting of chromatin material (cuboidal cells of collecting tubules) (Luna 1988).

## Conclusion

It is apparent that quality H&E sections are important in obtaining a confident, accurate diagnosis. The above review is an attempt to list those microscopic features that histotechnologists should assess in order to obtain a quality stain. Unfortunately, fixation and processing play a huge role in section quality and it is difficult to assess staining quality in isolation.

Many of the above recommendations are based on personal experience and as such should be open for debate. Critical discussion is appreciated and encouraged. It is also apparent that knowledge of routine histology and reference to classic histology texts (such as those listed in the References) is important.

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# Chapter 16 | Fixation and Tissue Processing

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**Revised and updated by John A. Kiernan, MB, ChB, PhD, DSc**

## Fixation

The structure of a tissue is determined by the shapes and sizes of macromolecules in and around cells. The principal macromolecules inside a cell are proteins and nucleic acids. In vertebrate animals, the macromolecules on the outside surfaces of cells and in the extracellular spaces are glycoproteins and proteoglycans, in which much carbohydrate material is covalently joined to protein molecules. Carbohydrates are hydrophilic; they hold much water in the extracellular space, by hydrogen bonding. There is also, of course, much water inside cells; water accounts for about 60% of the weight of the human body. (Guyton, Arthur C. (1976). *Textbook of Medical Physiology* (5th ed.). Philadelphia: W.B. Saunders. p. 424) In bones and teeth hydroxyapatite, a crystalline mineral containing calcium and phosphate ions, dominates the extracellular domain, together with collagen, a fibrous protein.

An essential part of all histological and cytological techniques is preservation of cells and tissues as they naturally occur. To accomplish this, tissue blocks, sections or smears are usually immersed in a fixative fluid, although in the case of smears, merely drying the preparation acts as a form of preservation. The fixatives employed prevent autolysis by inactivating lysosomal enzymes, and they stabilize the fine structure, both inside and between cells, by making macromolecules resistant to dissolution by water and other liquids. Fixatives also inhibit the growth of bacteria and molds that give rise to putrefactive changes.

In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds, or by a combination of coagulation and additive processes. A compound that adds chemically to macromolecules stabilizes structure most effectively if it is able to combine with parts of two different macromolecules, an effect known as cross-linking. The result is conformational changes in the structure of proteins and subsequent inactivation of most enzymes. Some lipids, proteins, carbohydrates and minerals are extracted by fixative liquids in which they are soluble. Fixation changes both chemical and antigenic profiles of proteins. The dilemma of fixation has always been that it introduces some artifact in order to have a protective effect. By definition, fixatives change the original chemical and physical compositions of tissues. In addition to altering the chemical nature of the cells and tissues to which they are applied, fixatives also cause physical changes to cellular and extracellular components.

Viable cells have surface membranes that are impermeable to large, hydrophilic molecules. Fixation, especially in organic liquids that dissolve or disrupt the lipids of the cell membrane, allows relatively large molecules to penetrate and escape. Furthermore, the cytoplasm becomes permeable to macromolecules, forming a proteinaceous network sufficiently porous to allow further penetration of large molecules. In this context, "large molecules" include those of paraffin wax, those of antibodies used for immunostaining, and larger dye molecules. Different fixatives result in different degrees of porosity. Coagulants such as mercuric chloride, picric acid, or zinc sulfate result in a larger pore size than do noncoagulant fixatives, such as formaldehyde, glyoxal or glutaraldehyde. Some fixatives are both coagulant and non-coagulant. For example, acetic acid coagulates nuclear chromatin but not proteins. Thus, coagulant fixatives are generally beneficial for subsequent paraffin sections. In addition to facilitating permeation of sections by antibodies, coagulant fixatives can also increase the exposure of antigenic sites, a consequence of the deformation of macromolecular shapes that constitutes coagulation. Cross-linking impedes penetration of paraffin but increases the physical strength of the tissue, especially if frozen sections are needed. Chemical addition of fixative molecules, with or without cross-linking, can modify antigenic sites and suppress immunostaining. This is a well known effect of formaldehyde, often reversible by one of many antigen retrieval procedures.

In attempts to combine the best properties of different compounds used for fixation, many mixtures have been developed. Most contain both coagulant and non-coagulant ingredients. Other substances in fixative solutions have actions such as controlling the osmotic pressure, adjusting the pH, and counteracting shrinkage or swelling caused by another ingredient. Shrinkage or swelling that occurs evenly through a specimen usually does not matter, but severe distortion results from changes in the sizes of some but not all parts of a tissue, as when tubular structures shrink within their surrounding connective tissue, or when artificial spaces are formed around cells.

## Fixation before Paraffin Embedding

Most samples used for staining normal and pathological tissues are embedded in paraffin, and a number of fixatives have been formulated with this in mind. The most commonly used fixatives are discussed here. There is an abundance of specialty fixatives that will not be covered here but may be found in the references given in the bibliography.

### Formaldehyde

Formalin is an article of commerce containing 40% w/v (= 40% w/w) formaldehyde (which is a gas) in water. Most of the formaldehyde is present as soluble polymers, which depolymerize on dilution. Formalin also contains about 10% methanol, which is added by the manufacturer to retard the formation of higher polymers, which eventually fall out of solution as paraformaldehyde. Old bottles of formalin, especially after storage in a cold place, often contain deposits of this white powder. Formaldehyde in solution also deteriorates by reacting with itself (Cannizzaro reaction) and changing into methanol and formic acid. Monomeric formaldehyde exists almost entirely as methylene hydrate, an addition compound formed by a reversible reaction with water. Formaldehyde itself is the compound that reacts with proteins; it is present at an extremely low concentration in a “4% formaldehyde” solution, but is produced instantly from methylene hydrate following removal from solution in the reactions of fixation. These chemical properties of formaldehyde are summarized in Figure 1.

The most commonly used fixative for histopathology is a 4% aqueous solution of formaldehyde, often called 10% formalin because it is made by tenfold dilution of formalin. For about 50 years this fixative has also included inorganic salts to maintain a near neutral pH and an osmotic pressure similar to that of mammalian extracellular fluid. The solution is called neutral buffered formalin, or NBF. It fixes not by coagulation, but by adding to the side-chains of basic amino acids, especially lysine, and to the amide nitrogen atoms of peptide linkages. Cross-linking methylene bridges are formed where two formaldehyde binding sites are close together (see Figure 1). This results in lowered permeability to macromolecules but the structures of protein molecules are not greatly altered. The small sizes of the methylene glycol and formaldehyde molecules allow rapid penetration. Consequently this fixative is suitable for large or small specimens.

Unfortunately, despite rapid penetration of tissues, the chemical reactions of formaldehyde with tissue proteins, especially the formation of methylene bridges, occur slowly. A commonly made mistake with formalin-containing fixatives is to under-fix the tissue. Small (10×10×3 mm) pieces fixed in NBF for 12-24 hours will generally show good cytoplasmic preservation and nuclear detail. Addition of formaldehyde is largely complete in 24 hours, but cross-linking reactions continue for at least two weeks. Large, soft specimens such as whole human brains require 2-6 weeks in NBF to become firm enough to cut into slices from which samples can be taken for histology. Variations in time and conditions of fixation cause the majority of problems in histochemistry.

Fixation profoundly affects histological and immunohistochemical staining, technicians, pathologists and research workers must therefore decide on the most appropriate method. Aspects to consider are temperature, size of the storage container, volume ratio, salt concentration, pH and incubation time. Formaldehyde fixation is typically performed at room temperature. Using a low and wide specimen container to allow for adequate penetration and ease of retrieval by a technician is the best choice for adequate volume ratio. In addition, 1:20 volume ratio of fluid to tissue and 3–4 mm specimen thickness are recommended for good penetration. To prevent swelling or shrinking of the cells, an isotonic solution buffered to pH 7.2–7.4 is recommended, to maintain ultrastructure and minimize cell distortion. The shorter the time elapsed between removing the sample from the body and immersing it in fixative, the better. The duration of exposure to the fixative must be optimized for each specimen type. For example, it has been noted in a CAP survey that glycogen preservation in liver can be subject to fixation artefacts. As NBF penetrates slowly through the hepatocyte membrane, the glycogen associated with the cytoplasmic protein matrix is displaced to one side of the cell. This observation is referred to as polarization and is considered part of the morphology when discerning the stain result. (Figure 2: glycogen polarization) This is a great example of how specimens that have prolonged exposure to fixatives can demonstrate extreme morphology changes. Glycogen polarization is less marked after fixation in a non-aqueous liquid, in which this polysaccharide is not soluble.





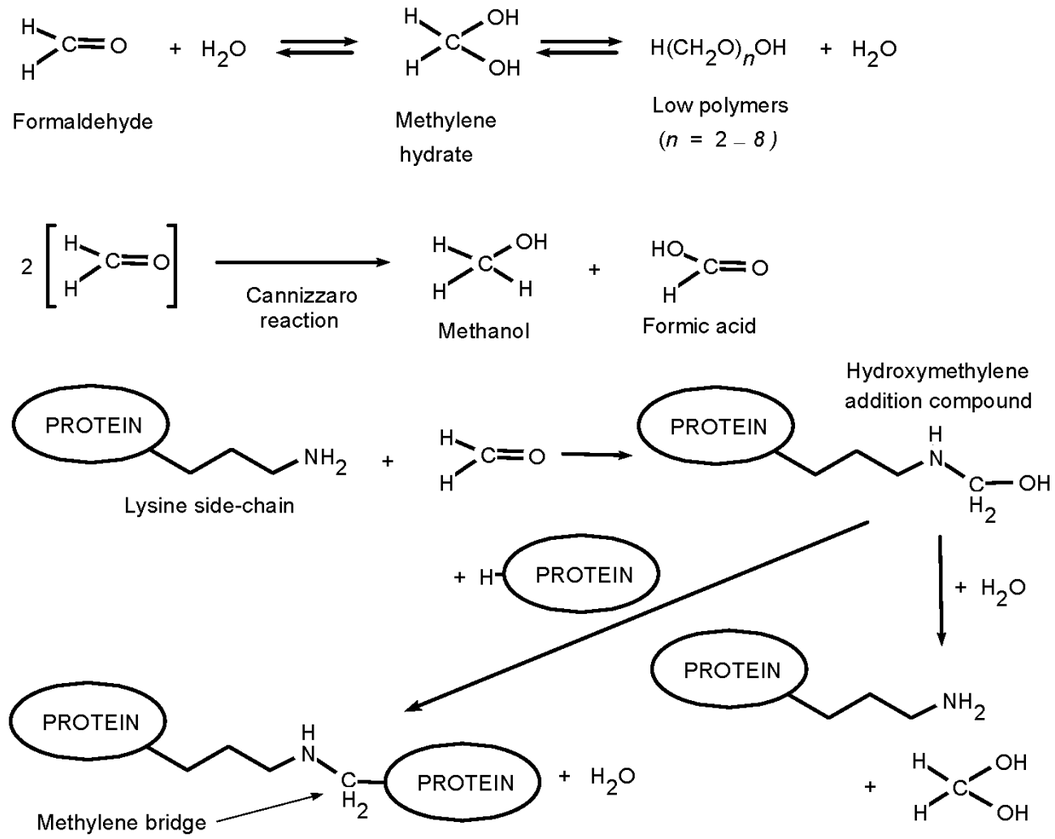


**Figure 1.** Some reactions of formaldehyde with water, with itself, and with proteins.

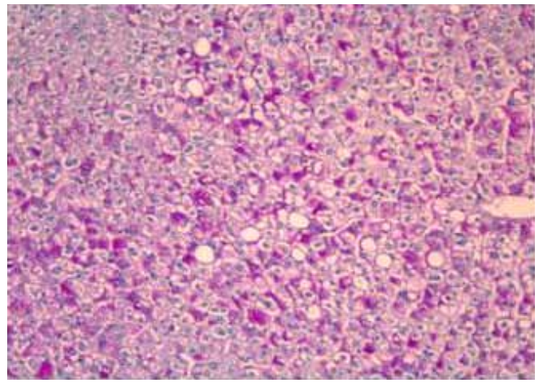

**Figure 2.** A paraffin section of formaldehyde-fixed liver stained by the periodic acid-Schiff method, illustrating polarization of glycogen (magenta) within the hepatocytes. Nuclei are counter-stained with hemalum (blue).

### Glyoxal

Glyoxal is the simplest dialdehyde, with the formula OHC—CHO. Like formaldehyde, it readily forms hydrates and polymers. Decomposition by way of the Cannizzaro reaction occurs rapidly in neutral or alkaline solutions. Aqueous solutions must be buffered to about pH 4 to be stable, and they must also contain a small proportion of ethanol, which catalyzes the reaction of glyoxal with proteins. Addition to and cross-linking of proteins is quicker than the equivalent reactions of formaldehyde. Adequate fixation of small specimens by glyoxal is achieved in one hour.

The glyoxal-based fixatives available to laboratories are sold as pre-made solutions with trade names and undisclosed compositions. Evidently there are no published recipes for fixatives of proven efficacy that contain glyoxal as the principal ingredient. Proprietary glyoxal fixatives are reported to be useful for general histology and immunohistochemistry. The differences from formaldehyde (NBF) fixation are:

(a) Suppression of staining of arginine-rich proteins by anionic dyes (this matters if you need to stain Paneth cells, eosinophil leukocytes or the tails of spermatozoa), and

(b) Antigen retrieval is less frequently required than after fixation in NBF.

The trade secrecy associated with glyoxal-containing fixatives may preclude their use in research and in diagnostic applications where the pathologist may be interrogated about his competence and knowledge of materials used in the laboratory.

### Bouin's Fluid

Pol André Bouin (1870-1962) invented several fixative mixtures in the years 1895-1900; the one most often associated with his name, first published in 1897, contains 10% formaldehyde (25% formalin), 0.9M acetic acid and 0.04M picric acid, in water. Picric acid penetrates tissues rather slowly, coagulating proteins and causing some shrinkage. It also dyes the tissue yellow. The acetic acid coagulates nuclear chromatin and opposes the shrinkage caused by picric acid. The reactions of formaldehyde have already been discussed; at the pH of Bouin's fluid (1.5-2.0) these occur more rapidly than in NBF. The complementary effects of the three ingredients of Bouin's solution work well together to maintain morphology. Specimens are usually fixed in Bouin's for 24 hours. Prolonged storage in this acidic mixture causes hydrolysis and loss of stainable DNA and RNA. Thorough washing after fixation is necessary.

Like mercuric chloride, picric acid enhances subsequent staining, especially with anionic ("acid") dyes. Paraffin sections of formaldehyde-fixed tissues are usually immersed for a few hours in a picric acid solution (Bouin's fluid is commonly used) before staining by the trichrome methods. Trichrome stains use combinations of anionic dyes with phosphotungstic or phosphomolybdic acid to impart contrasting colors to cytoplasm, collagen fibers and other components of tissues.





### Mercuric Chloride

Each fixative preserves morphology differently, thus there are multiple options. For some cells and tissues NBF does not provide adequate fixation. Examples are connective tissues, some structures containing mucins, and bone marrow. Mercuric chloride ($HgCl_2$) adds to proteins and brings about rapid coagulation with resulting granularity that is too fine to be visible with light microscopy. Lillie's B5 fixative is 4% aqueous formaldehyde with 0.22M $HgCl_2$ and 0.22M acetic acid. This mixture enhances nuclear detail, which is important for identifying normal and abnormal cell types in bone marrow (hematopoietic tissue) specimens. The coagulation of nuclear chromatin is an effect of the acetic acid. The mercuric chloride ensures rapid structural stabilization and also facilitates bright staining by many of the dyes used in microtechnique. The reasons for this effect on stainability are not understood.

A dirty looking brown crystalline precipitate, probably mercurous chloride ($Hg_2Cl_2$) forms in all parts of tissues fixed in mixtures containing $HgCl_2$; It is called mercury pigment and must be removed, by sequential treatments with iodine and sodium thiosulfate solutions, before staining. Because it contains mercury, B5 is subject to toxic waste disposal regulations, which apply to the fixative solution and every solution thereafter that has been contaminated with mercury. Records must be kept of all B5 volumes used in the laboratory.

### Zinc salts

The toxicity of mercury compounds has discouraged their use in laboratories. Of the other elements in Group 12 (IIb) of the periodic table (Zn, Cd, Hg), zinc is the least toxic. Zinc sulfate has been used for more than a century in astringent lotions and eye drops, which reduce the swelling of inflamed surfaces by coagulating extravasated plasma proteins. The first zinc-formalin fixative was probably the one published by P.A.Fish in 1895, only three years after the discovery that formaldehyde preserved tissue architecture very well. In recent decades several intelligently formulated fixatives have been developed. A few are entirely coagulant, with $Zn^{2+}$ as the only ingredient likely to immobilize proteins. Most zinc-containing fixatives also contain formaldehyde, making them comparable to B5 and other mercury-containing solutions.

## Alcoholic Fixatives

NBF is the most widely used fixative, despite not being the best one for every purpose. This is because the shortcomings of NBF are not very serious, provided that the specimens are fixed for sufficient time. Moreover, the appearances of formaldehyde-fixed tissues are familiar to pathologists, who have trained their visual systems to recognize normal and abnormal features in sections cut from routinely fixed materials.

Arguments have been made for changing the routine fixative to one of several non-aqueous mixtures, and strong cases can be made for Clarke's fluid (ethanol and acetic acid, 3:1 by volume), Carnoy's fluid (ethanol, chloroform and acetic acid, 60:30:10) and Puchtler's methacarn (Carnoy with methanol instead of ethanol). These liquids, which fix by coagulation of proteins and chromatin, contain no water. They almost completely dehydrate the tissue, so that the time for processing into paraffin wax is shorter than after NBF or other aqueous fixatives. There are also many mixtures containing an alcohol (usually ethanol), formalin, acetic acid and 10% to 70% water, often called AFA or FAA or named for their inventors, who include Tellyesniczky (around 1900), Bodian (1930s) and Davidson (1940s). In an AFA mixture the chemical reactions of formaldehyde with proteins are not retarded by buffering to a near-neutral pH. All these alcoholic fixatives contain acetic acid, which produces characteristic patterns of coagulated nuclear chromatin, facilitating the recognition of cell types. Nuclei that have been in NBF for a week or more exhibit less pronounced patterns of chromatin.

Alcohol (ethanol or methanol) alone instantly coagulates proteins but causes considerable distortion of the micro-anatomy in pieces of animal tissue. These unwanted changes are opposed by dilution of the alcohol with chloroform (immiscible with water), water, and/or acetic acid (which coagulates chromatin and opposes shrinkage, being miscible with water, alcohol and hydrocarbons. Alcohol alone (methanol is usually preferred) is suitable for fixing thin layer preparations such as blood films or cell cultures. Solid specimens taken from patients with gout are usually fixed in 95% ethanol for subsequent histochemical detection of sodium urate crystals, which can be dissolved out of the tissue by water.

## Decalcification

Following fixation, there are techniques available to the histotechnician to improve microtomy and staining quality. Each technique must be evaluated for each assay to preserve morphology and provide the best stain possible (Table 1). The first method, going in chronological order of histology, is decalcification of specimens that may be difficult to cut on a microtome because of calcium carbonate or phosphate deposits. Decalcification can be achieved either by acids or by chelating agents. First, make sure the tissue has been adequately fixed and rinsed well to prevent any undesired reaction with the decalcifying agent. Dilute mineral acids (hydrochloric or nitric) or formic acid can be used effectively if the end point of decalcification is monitored carefully. Nuclear and cytoplasmic detail are compromised if specimens are exposed for too long to acidic decalcifying agents, which can extract RNA and remove the purine and pyrimidine bases from DNA. It is also imperative to wash the acid out of the tissue. If preservation of nuclear DNA is important, or if histochemical methods for nucleic acids or enzyme activities are intended, a chelating agent is preferred to an acid. Usually the disodium salt of EDTA is used, with the pH adjusted to a level between 7 and 8. Decalcification by EDTA much longer than decalcification by acids – weeks rather than days.

**Table 1.** Stain optimization.

| Stain Technique | Thickness of Sections | Recommended Fixative | Fixatives to Avoid |
|---|---|---|---|
| AFB | 3–4 µm FFPE<br>smear | NBF<br>95% | |
| Bielschowsky | 3–4 µm FFPE<br>frozen | NBF | |
| Bodian | 3–4 µm | NBF | |
| Congo Red | 8–10 µm FFPE<br>frozen | NBF | |
| PAS | 3–4 µm FFPE<br>frozen 10 µm | NBF | |
| Jones | 2 µm FFPE | NBF | |
| GMS | 3–4 µm FFPE<br>smear | NBF<br>95% | |
| Urate Crystals | 3–4 µm | alcohol | aqueous, NBF |
| Iron | 3–4 µm<br>smear | NBF<br>air dry | |
| Reticulin | 3–4 µm | NBF | |
| Snook's | | | |
| Gram | 3–4 µm<br>smear | NBF<br>95% | |
| Warthin-Starry | 3–4 µm | NBF (critical) | |
| Helicobacter | 3–4 µm | NBF | |
| Masson's Trichrome | 3–4 µm | NBF, Bouin's | |
| Colloidal Iron | 3–4 µm | NBF | chromate |
| Copper | 3–4 µm | NBF, B5 okay | Bouin's, decal |
| Rhodanine | | | |
| TRAP | smear | citrate/acetone | |
| Sudan Black | 3–4 µm<br>frozen 10 µm | NBF | Bouin's |
| Leder | 3–4 µm | NBF, B5 | decal |
| Mast Cell | smear | CAF* | |
| MPO +/- Fluoride | smear | alcoholic formalin | |
| Alcian Blue | 3–4 µm | NBF | |
| Mucicarmine | 3–4 um | NBF | |
| Feulgen | 3–4 µm | NBF | Bouin's |
| Gomori's Trichrome | 3–4 µm | NBF | |
| Elastic Van Gieson | 3–4 µm | NBF | |
| Oil Red O | 3–4 µm | NBF | |
| Fontana Masson | 3–4 µm | NBF | |
| Calcium | 3–4 µm | NBF, alcohol | |
| Bile | 3–4 µm | NBF | |
| PTAH | 3–4 µm | B5 | |
| Mallory's | | | |
| Luxol Fast Blue | 3–4 µm | NBF | |

*CAF = citrate + acetone + formalin

## Frozen Sections

For histochemistry, cryostat sections give much faster results than paraffin sections. Additionally, fixative can be used with cryostat sections, allowing the histochemist to select a different and optimal fixative for each stain, all from the same sample. The morphological detail and resolution of frozen sections is usually considerably inferior to tissue that has been embedded in paraffin. In histopathology, frozen sections are commonly cut from muscle and nerve biopsies and from surgically removed tumors. Muscle and nerve biopsies are subdivided into specimens for formalin fixation and paraffin embedding, unfixed snap-frozen for cryostat sections, fixation and resin embedding for electron microscopy (EM) and, in some rare cases, biochemical immunoblotting studies. Multiple fixation processes are required because multiple techniques are to be used. The portion of a specimen portion intended for frozen sectioning should be transported on top of wet ice, on saline-dampened gauze, and rapidly frozen within two hours. Do not allow the tissue to freeze slowly or to soak up excess saline, as these will cause artifacts that can be seen microscopically and interfere with diagnostic interpretation.

It has been suggested that talc powder can alleviate the moisture absorbance of the muscle tissue. This procedure should be evaluated and quality tested before introducing it into a laboratory. Upon receipt in the histology lab, orient in OCT (optimal cutting temperature) compound and snap-freeze in liquid nitrogen/isopentane for optimal results. For complete instruction and illustration, refer to pg 312-314 in Carson & Hladik's textbook. Orientation, size, and expedient flash freezing are critical to obtaining undamaged sections of unfixed muscle fibers. The EM portion of a biopsy will be fixed in a buffered solution of glutaraldehyde and postfixed in osmium tetoxide, usually by a specialist in electron microscopy. In some muscular degenerative disorders, biochemical techniques may also be required.

## Smears

Histotechnicans sometimes perform special stains on cytology smears, blood films and cytopreps from other departments within the laboratory. Increasingly, the commonly received cytoprep is that of the "thin prep." These smears are wet-fixed in 95% ethanol immediately after preparation to preserve the fine structure of the chromatin and help in the evaluation of nuclear changes. The May-Grünwald or the Giemsa stain is routinely evaluated, and the more complicated Papanicolaou method is also widely used, especially on samples taken from the vagina and cervix. Air drying is avoided with smears for cytological detection of neoplasia because it changes the appearances of the cells. Slides bearing blood or bone marrow smears, on the other hand, are usually air-dried. Marrow smears are stained in parallel to sections of the bone marrow core biopsy.

## Specimen Processing

Staining quality can be depreciated by inadequate fixation and similarly by poor tissue processing. A good technician must evaluate and determine the processing of choice for each purpose, be it special stains on paraffin, frozen or cell smear preparations.

Paraffin processing has evolved and stabilized in the modern histology lab with the use of vacuum infiltration. It remains fundamentally important to remember the basics when processing and troubleshooting. The specimen processing methods presented in this chapter should be considered a brief introduction and do not include all the available procedures.

### Tissue Processing

In order to prepare a tissue for embedding, it must be infiltrated with paraffin. Because water and paraffin are not miscible, the specimens must be gradually dehydrated to achieve replacement of water with alcohol before the clearing agent is introduced. The size and penetrability of the tissue dictate how quickly this will occur. Once successfully dehydrated, a clearing agent that is miscible with alcohol and paraffin (i.e. xylene or substitute) is infiltrated through the tissue. Finally, the paraffin is introduced and completes the tissue for embedding. A vacuum automated system improves the efficiency of wax infiltration by speeding up the removal of the clearing agent.





Keep in mind that individual laboratories must optimize to their specimen types. In general, needle biopsies and bloody specimens should be incubated conservatively, whereas fatty specimens can be processed for longer than average.

In recent years efforts have been made to streamline processing time by using microwave heating techniques. The benefits and risks associated with the technique are being revealed as more laboratories employ the technology and publish their findings. For a list of easily available literature, review the bibliography section. At this time, the concept will be presented with supporting examples.

It has been reported that microwave processing can be achieved ~60% faster than conventional processing time. See Table 2 for a comparison of the two techniques, each using 1-3mm thick biopsy samples.

One critical aspect of using microwave techniques is ensuring the samples have been adequately fixed. Each laboratory should evaluate its fixation control methods to optimize the use of microwave processing. Although the decrease in processing time increases the specimen workflow, there has been some resistance to the implementation of microwave processing technology, partly for staffing reasons. Traditionally histopathology staffs are available in the morning hours when conventional overnight processing yields its highest volumes, whereas microwave processing may keep staff later in the day. Microwave-accelerated processing is as effective as slower traditional processing, and sections stain identically with several methods: Periodic Acid-Schiff's, Van Gieson, Congo red, Masson's trichrome, alcian blue, Mayer's mucicarmine, and silver methods for reticulum. Because staining methods can vary from lab to lab, it is recommended that individual labs validate microwave methods within their environments.

Whether the lab has time initially to validate the technique and train the staff accordingly, long term considerations are prevalent. Converting to the safer chemicals (ie. less fumes, non-regulated disposal) may include rotating smaller quantities more often, causing a net increase in chemical consumption. The safety benefits of removing undesired regulated waste in addition to calculating net volumes may offer immediate cost savings. Each lab must weigh its benefits to fit its needs.

Table 2.

| Process | Solution | Time | Retort |
|---|---|---|---|
| **a) Conventional, room temperature processing schedule, 3mm biopsy. 12 hrs.** | | | |
| Fixation | Formalin, 10% | 120 min | --- |
| Fixation | Alcoholic formalin | 60 min | --- |
| Fixation | Alcoholic formalin | 60 min | --- |
| Dehydrate | Alcohol, 95% | 60 min | vacuum |
| Dehydrate | Alcohol, 95% | 45 min | --- |
| Dehydrate | Alcohol, absolute | 45 min | vacuum |
| Dehydrate | Alcohol, absolute | 60 min | --- |
| Clearing agent | Xylene | 60 min | --- |
| Clearing agent | Xylene | 60 min | vacuum |
| Infiltrate | Paraffin | 30 min | --- |
| Infiltrate | Paraffin | 60 min | --- |
| Infiltrate | Paraffin | 90 min | vacuum |

Table 2.

| Process | Solution | Time | Retort |
|---|---|---|---|
| **b) Conventional processing schedule, 1mm biopsy. 2.5 hrs.** | | | |
| Dehydrate | Alcohol, 65% | 15 min | |
| Dehydrate | Alcohol, 95% | 15 min | |
| Dehydrate | Alcohol, 95% | 15 min | |
| Dehydrate | Alcohol, absolute | 15 min | |
| Dehydrate | Alcohol, absolute | 15 min | |
| Clearing agent | Xylene | 15 min | |
| Clearing agent | Xylene | 15 min | |
| Infiltrate | Paraffin | 15 min | |
| Infiltrate | Paraffin | 15 min | |
| Infiltrate | Paraffin | 15 min | |
| **c) Microwave processing schedule, 1mm biopsy. ~45 min.** | | | |
| Dehydrate | Ethyl alcohol, 100% | --- | fill |
| DRAIN | | | |
| Dehydrate | Ethyl alcohol, 100% | 5 min | 67 °C, microwave |
| DRAIN | | | |
| Dehydrate | Isopropyl alcohol, 100% | 3 min | 74 °C microwave |
| DRAIN | | | |
| Infiltrate | Liquid paraffin, 60 °C | | fill |
| DRAIN* | | | |
| Infiltrate | Liquid paraffin, 60 °C | 2 min | 65 °C microwave, agitate |
| | --- | 5 min | 85 °C microwave |
| DRAIN* | | | |

*Drain to 80 °C container





## Embedding and Microtomy

Once the tissue has been processed it is ready to be orientated into a paraffin block and subsequently sectioned. Orientation during embedding is crucial for the representation of proper morphology. Structures in skin, small gastrointestinal biopsies, and vas deferens are among those for which orientation is especially critical. Good microtomy techniques will minimize artifacts that lead to difficult diagnostic interpretation of special stains. One of the most directly correlated factors is the thickness in which a specimen is cut. Routine H&E stained specimens are cut 3–4 µm, but some morphology is best represented otherwise. For example, amyloid deposits are better represented at 8–12 µm, whereas kidney biopsies should be cut at 2 µm for optimal viewing of the structures of glomeruli. Techniques often used to aid in microtomy are water bath adhesive and positively charged slides. However, in some silver impregnation stains, the silver ions are attracted to the coating and produce an overall background to the slide. To avoid this artifact (Figure 3), use clean slides without a coating.

After sectioning, the tissue slide is drained and may be gently heated to evaporate the layer of water between the sections and the glass. When all the water is gone, it is permissible to heat the slide enough to melt the wax, a procedure that may improve adhesion. For optimal melting of paraffin consult the melting temperature and “plastic point” on the manufacturing product insert. The plastic point is usually a few degrees lower than the melting point and represents the lowest temperature at which permanent deformation can occur without fracture.

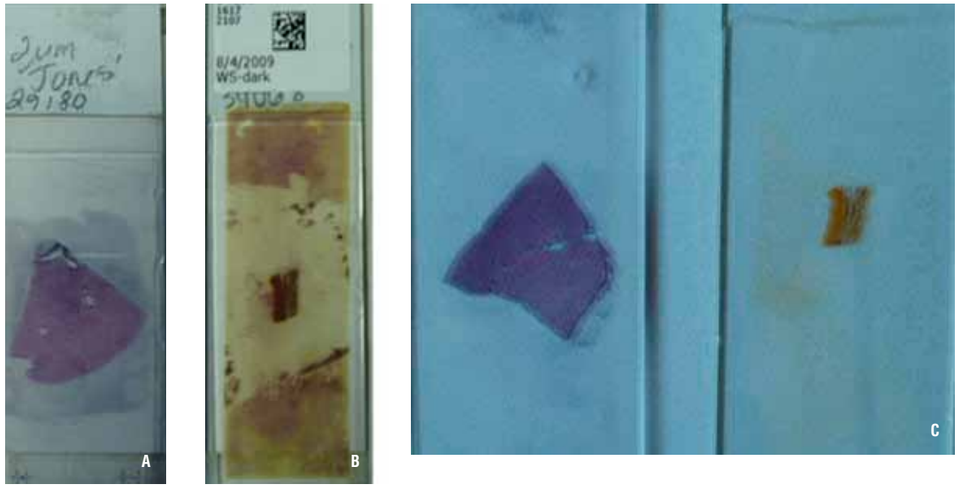


**Figure 3.** **A.** Jones’ silver method, kidney, 2µm adhesive residue stained by a silver method. **B.** Warthin Starry 4µm paraffin residue ghost (not stained by reduced silver). **C.** Jones’ and Warthin Starry slides demonstrating no mirroring artifact.

## Technical Considerations

There are some technical tips that can apply to all preparative procedures in microtechnique. Make sure glass slides are clean and free from debris. Gentle washing and minimal thickness of cell layers will prevent the cells from detaching during staining. Staining interpretation depends on adequate chemical spread and allocation. Make sure that there are enough sections to make a diagnosis, and that ensure that the reagents have been applied evenly to the slides. If counterstaining is required, be sure to not over-incubate.

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# Chapter 17 | “Special Stains” – Influence of Dye Chemistry on Staining

**Richard W. Horobin, PhD**

## What These Words Mean

Special stains are “non-routine” colorants of biological samples applied prior to microscopic examination (i.e., not hematoxylin & eosin staining). This usage of special versus routine works for histology, but is not so clear-cut when routine stains of other diagnostic specialties (e.g., the cytological Papanicolaou stain, or the hematological Wright-Giemsa stain, or the microbiological Gram stain) are used on histological preparations. Nevertheless, since stains such as Sudan black, the Periodic acid-Schiff procedure, or a Ziehl-Neelsen acid-fast stain are so widely termed “special stains” I retain this convention. Occasionally people refer to “usual special stains” to distinguish such methods from immunostaining or in situ hybridization. Here, the phrase “dye chemistry” has the restricted meaning of those physicochemical properties of dyes key to staining. Reactivity, of various types, is briefly considered in a later section on “complications”.

“Staining mechanisms” imply accounts of molecular processes involved in selective uptake of dyes into biological specimens during biological staining. Here we restrict discussion to explicating the role in some mechanisms of key physicochemical dye properties. Only simple mechanistic accounts are provided; and all examples illustrate more common special stains. For more substantive accounts of these, often complex, processes, see Dr. John A. Kiernan’s paper elsewhere in this Guide, which focuses on carbohydrate histochemistry; or recent reviews by Dapson (2005) and Prentø (2009); or, for thumbnail mechanistic sketches of most types of special stains, Horobin & Bancroft (1998).

## Key Dye Chemistry Factors – Definitions, Examples, Parameterization

Physicochemical factors influencing selective cell and tissue uptake of the special stains are electric charge; size, both overall and that of the conjugated/aromatic system; and the hydro- or lipophilicity. Traditionally some idea of these factors is gained from structural formulae. For instance Figures 1 and 2 provide formulae of dyes present in well known special stains. The colored species illustrated are, respectively, negatively charged (anionic or “acid” dyes) and positively charged (cationic or “basic” dyes). Figure 3 illustrates a non-ionic dye and a metal complex (“mordant”) dye.

A casual glance at such formulae does indicate dye size, and more careful inspection reveals their electric charge. However, assessment of the conjugated system size from an inspection of the structural diagrams is not obvious for non-chemists; and even if color-coding is used (see Fig. 1) “overall” hydrophilic or lipophilic character is hard to assess by anyone merely by eyeballing structures. Since structural formulae are limited in what they show us directly, how can we gain such information?

One approach is to use numerical structure parameters. Electric charge (abbreviated as Z) can be directly defined numerically. Other properties may be modeled – overall size by the relative molecular mass (or “molecular weight”, or MW), size of the aromatic/conjugated system by the conjugated bond number (CBN), and hydro- or lipo-philicity by using the logarithm of the octanol-water partition coefficient (log P). Table 1 gives structure parameters for the dyes shown in Figures 1-3. For more information on these parameters, and their derivation, see Horobin (2004); various alternative structure parameters are discussed by Dapson (2005).

How are these numbers useful to us? First, they let us readily compare dyes, in terms of size or lipophilicity and so on, and indeed compare dyes on the basis of multiple features. We see from Table 1 that azure B is little more than half the size of the orcein component, which in turn is less than half the size of alcian blue: significant differences. But we also see that whilst alcian blue is extremely hydrophilic, the orcein component is lipophilic. And these are not merely curiosities, they impact on mechanisms and practical usage, as seen in Table 1.

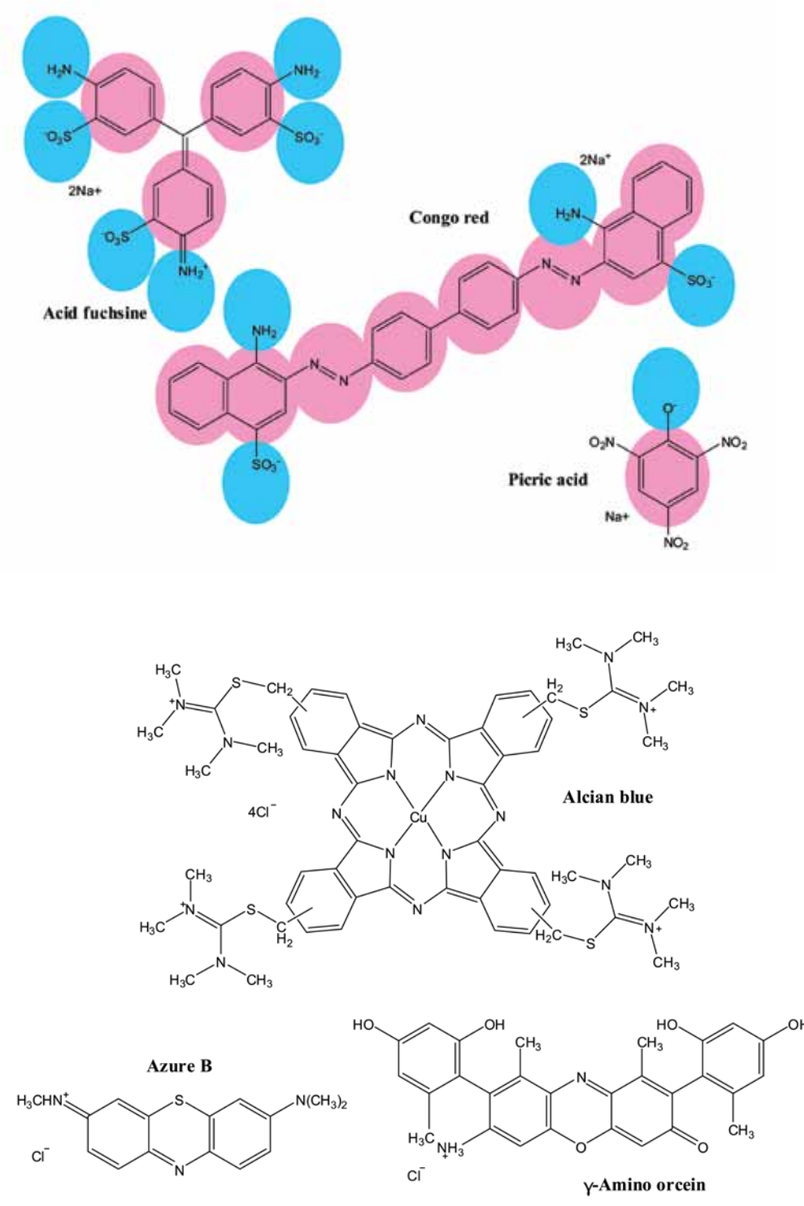


**Figure 1.** Structures of exemplar anionic (“acid”) dyes used in special stains. Blue blobs indicate the more hydrophilic structural fragments, pink blobs the more lipophilic. Counterions are shown as nominal sodium ions.

**Figure 2.** Structures of exemplar cationic (“basic”) dyes used in special stains. Counterions are shown as nominal chloride ions.





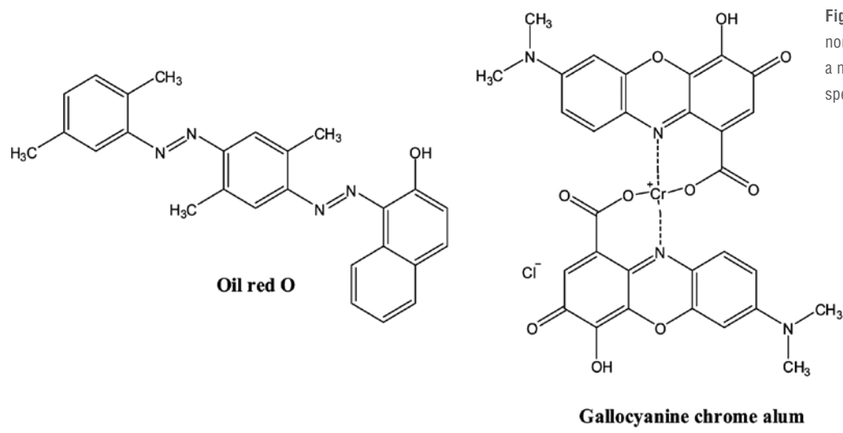


**Figure 3.** Structures of Oil Red O, a non-ionic, and gallocyanine chrome alum, a metal-complex (“mordant”) dye, used in special stains.

**Table 1.** Structure parameters specifying or modeling key dye chemistry properties. See text for abbreviations.

| Dye Name | Z | CBN | MW | log P |
|---|---|---|---|---|
| Acid fuchsine | 2- | 24 | 540 | -11.5 |
| Alcian blue 8G | 4+ | 48 | 1157 | -9.7 |
| Azure B | 1+ | 18 | 269 | -0.7 |
| Congo red | 2- | 43 | 651 | -1.5 |
| Gallocyanin chrome alum | 1+ | 44 | 618 | -3.9 |
| Oil Red O | 0 | 30 | 409 | 9.4 |
| γ-Amino orcein | 1+ | 36 | 485 | 1.8 |
| Picric acid | 1- | 16 | 229 | -1.9 |

## How Dye Chemistry Impacts on Staining Mechanism – Case Examples

**Example 1 – Acid and basic dyeing.** Most special stains use anionic ("acid") or cationic ("basic") dyes, one at a time or in multicolored combination. This enables selective staining of electrically charged cell and tissue components. Thus anionic biopolymers such as DNA, RNA and glycosaminoglycans are selectively stained by cationic dyes, such as azure B and alcian blue; and cationic polymers such as proteins in acidic dyebaths by anionic dyes, such as acid fuchsine and picric acid. Note that some metal complex ("mordant") dyes, such as gallocyanine chrome alum, are also cationic and also selectively stain anionic biopolymers. However their staining mechanisms are more complex and are not always dominated by electrical effects.

**Example 2 – Staining rates, dye size, and mucin staining.** Large dyes diffuse through the tissue sections or into cell smears much slower than small dyes. This influences selectivity of several special stains using staining protocols in which large dyes only have time to reach the faster staining tissue sites. An example, discussed further by Kiernan elsewhere in this Guide, is the selective staining of glycosaminoglycans by alcian blue. As indicated by Table 1 this dye is much larger than azure B; which latter also stains mucins but in addition stains polyanionic nucleic acids present in the less permeable nuclear chromatin and ribosomes. Other mucin stains, such as alcian yellow and mucicarmine, are also very large cationic species.

**Example 3 – Conjugated system size, the basis of amyloid and elastic stain selectivity.** Most acid (anionic) dyes used in special stains, including acid fuchsine and picric acid, stain proteins most strongly from acid dyebaths, when the targeted biopolymers are cationic. Analogously, basic (cationic) dyes stain proteins from alkaline (i.e. high pH) but not acid (low pH) dyebaths. However some dyes stain certain proteins strongly even from dyebaths of the "wrong" pH; indeed even when the solvent used is largely non-aqueous, which also usually inhibits acid and basic dyeing of proteins. Such unusual coloration patterns include the selective staining of amyloid by dyes such as Congo red, and the selective staining of elastin by dyes such as orcein. As seen in Table 1, these dyes have unusually large conjugated systems, and hence large conjugated bond numbers (CBNs). The atypical dye-protein binding is due to various non-polar attractive forces, which are stronger with the dyes possessing large aromatic (conjugated) systems.

This effect is illustrated in Figure 4, which compares "normal" acid dyes with acid dyes giving selective staining of amyloid and elastin. Analogous, unillustrated, effects arise with basic dyes. Of course amyloid and elastin are themselves unusual proteins. Amyloid forms β–pleated sheets, facilitating close approach by linear high CBN dyes, which give the best staining. Elastin is unusually hydrophobic, with numerous aromatic residues.

**Example 4 – Lipophilicity and staining of lipids.** Since staining of fat and lipid droplets by non-ionic dyes from aqueous-alcoholic solutions is mechanistically understood as partitioning of hydrophobic dyes between "wet" and "dry" environments, the log P parameter should predict which dyes are effective – and, from Figure 5, this is apparent. For currently recommended lipid stains of this type log P > 7; whilst quinoline blue, a dye so used in the nineteenth century, has a log P value of only 2.2; and note that for all fat stains log P > 0.





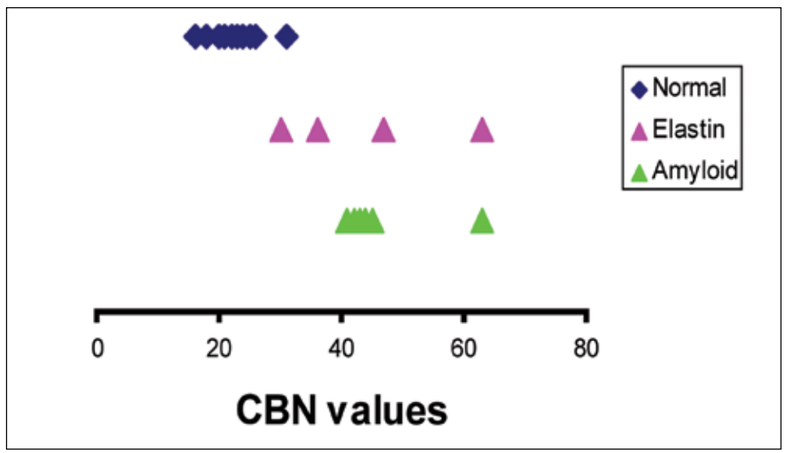


**Figure 4.** Influence of the size of the aromatic/conjugated system (modeled by CBN) of dyes on staining of proteins, in particular elastin and amyloid. Unbiased samples of "normal", elastin and amyloid staining "acid" (anionic) dyes were obtained from Lillie & Fullmer (1976: being the first 10 dyes listed on page 138, and relevant dyes from page 666 and page 707 respectively).

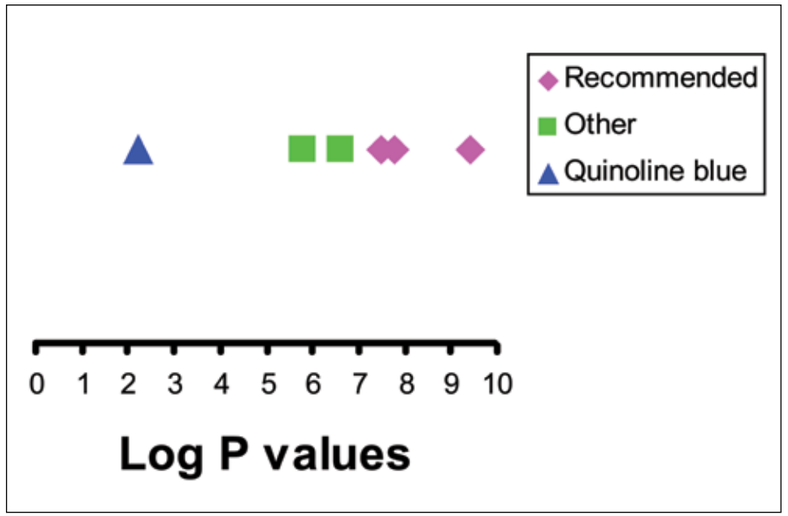


**Figure 5.** Influence of the lipophilicity (modeled by log P) of dyes on staining of fat and lipid droplets. Unbiased sample of fat staining dyes taken from Lillie & Fullmer (1976, p. 565).

## Is That It? Complications and Puzzles

Yes, it is more complicated! One complication is "reactive staining", involving making or breaking of covalent bonds, or significant oxidation-reduction changes, or formation of insoluble salts or complexes. This is not usual for the uptake of dyes – except for "metachromatic" dyeing, and the "Gram" and "Romanowsky" stains. However some special stains do involve reactivity, of various types. Procedures such as the "periodic acid-Schiff" or "Feulgen nucleal" stains are organic chemistry on a slide, with making and breaking of covalencies. Biochemical processes involved in "enzyme histochemistry" also involve changes in covalencies. Formation of Prussian blue in the "Perls'" stain for iron involves polar covalencies. "Metal impregnation" or "silver stains" involve substantial redox changes, and precipitation of metal microcrystals or insoluble metal sulfides. Mechanistic details of phenomena within quotes in this paragraph can be found via the indices of various monographs, e.g., Horobin & Bancroft (1998), Kiernan (2008) and Lyon (1991).

But don't let this appeal to documentation deceive you, because there are still puzzles concerning mechanisms of special stains. Consider the trichromes. In sequence stains such as Masson's, it is typically the larger acid dyes which stain collagen fibers. The experimentally-grounded interpretation, that this is because access of slow diffusing dyes is limited to the most readily penetrated tissue substrate, dates back to Mann (1902). Application of this mechanism to one-bath trichromes, such as that of Gomori, has been argued elegantly by Baker (1958) and, using the structure-parameter approach, by Horobin & Flemming (1988). Nevertheless this is not a universal mechanism, as clearly demonstrated by Prentø (1993) for the widely used picro-Sirius red variant of van Gieson's stain.

## Conclusion

The general principles of the staining mechanisms of most special stains are now understood. Staining selectivity is surprisingly often dominated by a limited number of dye chemical factors – such as electric charge (Z) for acid and basic dyeing; overall molecular size (MW) and charge for mucin stains; size of the aromatic/conjugated system (CBN) for amyloid and elastin stains; and lipophilicity (log P) for fat stains. Nevertheless some complications exist, and some puzzles remain, even for some widely used methods.

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# Chapter 18 | How Do Dyes Impart Color to Different Components of the Tissues?

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The purpose of imparting color (to "stain") certain components of the tissues is to assist making diagnostic judgements. The diversity of these components, the targets of staining, is indicated in Table 1.

You may be surprised that small-molecule reagents such as dyes can impart color differentially to such a variety of targets, so this chapter provides a broad-brush picture of the physical principles underlying this achievement.

**Table 1.** Some diverse targets of staining.

| How Targets are Defined by the Pathologist | Examples of Such Targets | Examples of Dyes Used to Stain Such Targets |
|---|---|---|
| **By their chemical character** | | |
| | Calcium | Alizarin red S |
| | Carbohydrates | Periodic acid-Schiff stain |
| | DNA and RNA | Feulgen nucleal stain, methyl green-pyronin |
| | Glycosaminoglycans (GAGs) | Alcian blue, colloidal iron |
| | Hemosiderin | Perls stain |
| | Lipids | Oil red O, Sudan black |
| **By their biological character** | | |
| Tissue components | Smooth muscle and collagenous or elastic fibers | Gomori's and Masson's trichromes, Verhoeff's stain |
| | Cartilage matrix | Alcian blue |
| Whole cells | Mast cells | Toluidine blue metachromasia |
| | Mucus goblet cells | Alcian blue |
| Intracellular entities | Cytoplasms | Eosin, light green |
| | Myelin sheaths | Luxol fast blue |
| | Nuclei | Al hematoxylin, nuclear fast red Al complex |
| | Nissl substance (RER) | Cresyl violet |
| **By their pathological character** | | |
| | Amyloid | Congo red |
| | Microorganisms | Carbol fuchsine, Giemsa and Gram stains |
| | Viral inclusion bodies | Feulgen nucleal stain |

## Background Concepts

To answer the question of the chapter title, two more specific questions must be addressed. Firstly, how do any components become colored? Secondly, how do certain components (targets) become colored whilst the background remains unstained? Physicochemically this is equivalent to the questions: what are the sources of affinity and selectivity? Given the variety of detectable targets, the variety of sources of affinity and selectivity needed to explain the actions of most special stains is surprisingly small. It is in fact the multiplicity of possible combinations of chemical and physical features of dyes, dye solutions and tissue components that provide the bases of selective staining. A third term which can be clarified here is sensitivity: a highly sensitive stain is one which demonstrates small amounts of a tissue component.

A few complications will be mentioned before proceeding. Firstly, a classical thermodynamic property such as entropy applies rigorously only to systems at equilibrium. Fortunately we are not using such properties quantitatively, so this is not a problem. Secondly, when considering intermolecular effects, dye uptake is often thought of as involving some type of “bonding”, of strong and directed attractions. This is sometimes so, as with covalent bonds. However other intermolecular attractions, such as van der Waals forces, are neither strong nor directed. Finally, note that case examples are presented in summary form. Readers seeking more complete accounts should look elsewhere, eg Horobin (1982), Lyon (1991), Horobin & Bancroft (1998) and Prentø (2009).

## How Do Any Components Become Colored?

In physicochemical terms, staining occurs because a dye has an affinity for the tissue components. Affinity is itself dependent both on increasing entropy, with the overall system becoming more disordered, and on decreasing enthalpy, involving such phenomena as dye-tissue attractive forces. Dyes move from their solutions (“dyebaths”) into the unstained tissues and cells because they are moving from regions of high dye concentration to regions containing less dye. Thermodynamically, entry of dye into tissue sections or cell smears is always driven in part by an increase in system entropy, disorder increasing as dye distributes between dyebath and tissue components.

This partial explanation does not explain why dyes can reach higher concentrations in the tissues than in the dyebaths. Such dye accumulation requires additional sources of affinity. Some of these factors are sketched below, starting with those which involve useful decreases in enthalpy.

The most general are the non-directed, short-range intermolecular attractions deriving from weak electronic coupling, e.g., dipole-dipole and dispersion forces. These weak forces are generically termed **van der Waals attractions**. Although occurring with all dyes, they are most significant with dyes having large conjugated systems, such as Congo red shown in Figure 1. Van der Waals attractions can contribute to affinity increases in an intuitively obvious way, by providing dye–tissue attractions. Less intuitively obvious are increases in affinity due to induction of dye–dye binding at certain sites, e.g., metachromatic staining of mast cell granules occurring with basic dyes such as toluidine blue, which dye is shown in Figure 2 .

Non-directed, longer-range attractions are the strong **electrical forces** arising between ions. These can contribute to affinity when dye and tissue component have charges of opposite sign. For instance tissue components containing glycosaminoglycans (GAGs) can stain metachromatically with basic (cationic) dyes such as toluidine blue. However affinity of routine acid and basic dyeing — involving ionic tissue components becoming preferentially stained by dyes with the opposite charges — is not primarily driven by the electrical effects. Rather, such processes are entropy driven (see above) with the electrical effects controlling selectivity (see below Figure 3). Electrical forces are procedurally adjusted by altering electrolyte content or pH of dyebaths.





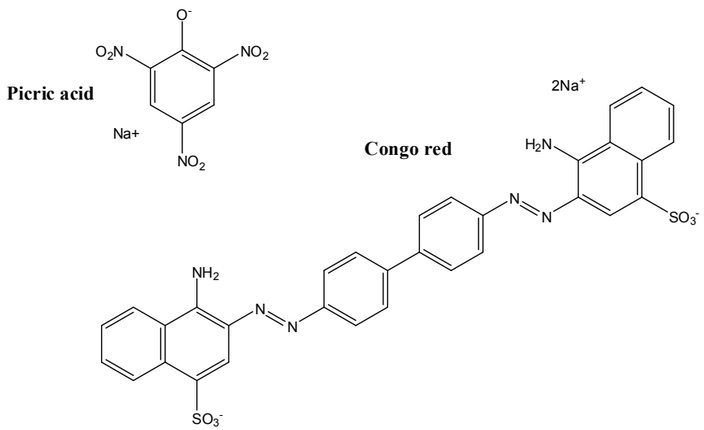


**Figure 1.** Acid dyes, small and large.

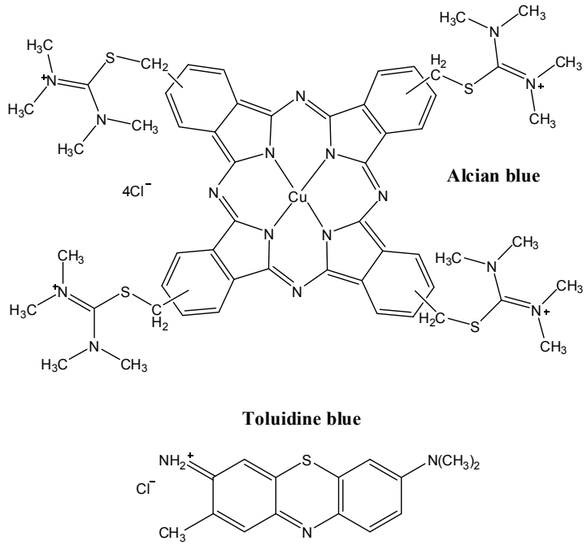


**Figure 2.** Basic dyes, large and small.

Strong directed attractions arise when dyes interact with tissue components to form **covalent bonds**, e.g., between Schiff reagent and tissue aldehydes; or **polar covalent** bonds, e.g., between alizarin red S and tissue calcium ions. **Hydrogen bonds** are weaker directed bonds, occurring when dye and tissue component carry suitably oriented hydrogen donors and acceptors, as in staining of glycogen with carminic acid.

An additional source of affinity, due to increase in entropy of the aqueous solvent, arises during **hydrophobic bonding**. This occurs when both dyes and tissue biopolymers have substantial hydrophobic domains, and when the solvent is wholly or largely aqueous (Horobin and Bennion 1973).

## How Are Targets Colored, Whilst Backgrounds Are Not?

If a tissue component has a high affinity for a dye whilst its surroundings do not, selective staining may occur. An example is staining of elastin — a protein carrying numerous unusual aromatic aminoacid residues — by dyes with large conjugated systems, such as Congo red, which bind by strong van der Waals attractions. Another example is the staining of metal ions such as calcium or copper by reagents which react to form polar covalent bonds with the metal ions, e.g., alizarin red S (discussed below) and p-dimethylaminobenzylinenerhodanine respectively.

However there are additional and very different sources of selectivity. Widely discussed are various **rate of staining effects**. For instance, even in the absence of affinity differences, if a tissue component takes up dye more rapidly than does its surroundings, and if staining is terminated before equilibrium is reached, then the component will become selectively colored. This is **progressive staining**, and an example is the selective coloration of goblet cell mucin by a large dye such as alcian blue. Conversely, both a tissue component target and its surroundings may be stained. Then exposure to a dye solvent (differentiator) results in selective staining if the target loses dye more slowly than its surroundings, and if destaining is terminated before too much dye has been lost. This is **regressive staining**, and an example is the staining of mycobacteria with carbol fuchsine.

Such effects depend on differing rates of dye diffusion through different tissue components. This is predictable, since tissue components can be ranked in terms of their permeabilities, see Table 2. Rates of staining may be accelerated by various procedural manoeuvres such as increasing dye concentration or temperature, adding swelling agents such as phenols, or using extremes of pH in staining solutions. For more detail see Horobin (1982, pages 49 ff and 97 ff).

**Table 2.** Permeabilities of some common tissue components.

| Type of Component | |
|---|---|
| **Acidophilic components** | |
| Collagen fibers | Most permeable |
| Smooth muscle and other cytoplasms | ↕ |
| Red blood cells | Least permeable |
| **Basophilic components** | |
| Goblet cell mucin | Most permeable |
| Chromatin | ↕ |
| Nissl substance | |
| Elastic fibres | Least permeable |





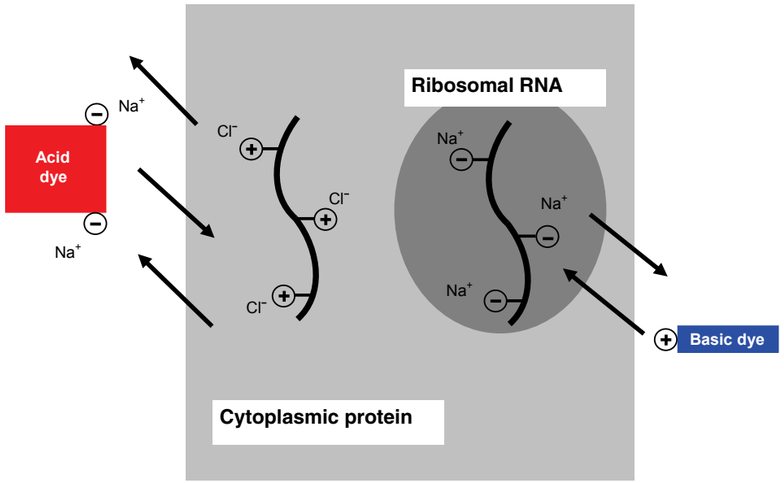


**Figure 3.** The ion-exchange selectivity mechanism of acid and basic dyeing. The phosphate groups of the RNA and the protonated amines of the protein are represented by their charge signs. The mobile ions are shown as nominal chloride and sodium ions, although the actual ionic species present will depend on the composition of the staining solution.

## Case Examples Illustrating the Bases of Affinity and Selectivity

As entropy effects contribute to affinity in all staining procedures, they are not always mentioned. Another common effect which does call for comment is that selective staining often depends both on factors favoring staining of the target **and** on factors favoring the non-staining of the background.

### Acid Dyes Giving General Oversight Staining of Cells and Tissues

Such dyes are anionic, vary from small (picric acid, see Figure 1 for structure) to moderate (e.g, light green) in size; and also vary from very hydrophilic (acid fuchsine) to weakly lipophilic (eosin Y). The generalised background staining of tissue resulting from binding of such dyes to proteins can be conceptualized as follows.

**Sources of affinity —** Dye–protein van der Waals attractions contribute significantly, and in aqueous solutions hydrophobic bonding also occurs.

**Sources of selectivity —** Such dyes bind to most proteins, and the near-universal distribution of these biopolymers results in non-selective background coloration. Staining is typically carried out from acidic dyebaths, in which proteins are protonated and carry overall positive charges. Acid dye anions exchange with mobile tissue anions associated with the protein polycations, a process termed “acid dyeing”. This ion-exchange is entropy driven, as it increases the randomness of the system, and is illustrated in Figure 3.

### A Small Basic Dye Giving Metachromatic Staining of Mast Cell Granules — Toluidine Blue (TB)

TB is a small, weakly hydrophilic cationic dye. Attached to DNA or RNA, in chromatin or Nissl substance, this dye has a blue color. Attached to GAGs, in mast cell granules or cartilage matrix, TB displays a purple "metachromatic" color. The dye's structure is shown in Figure 2.

**Sources of affinity —** Van der Waals attractions between TB and polyanions contribute to affinity when binding to DNA and RNA, as does hydrophobic bonding. However flexible, high charge density GAGs permit charge neutralization of dye aggregates or "stacks" formed due to dye–dye van der Waals attractions and hydrophobic bonding.

**Sources of selectivity for mast cell granules —**TB is typically applied from weakly acidic aqueous solutions. DNA, RNA and GAGs are then polyanionic, whilst most proteins are protonated and so polycationic. Thus basic dye cations exchange with mobile tissue cations associated with the various polyanions, a process termed "basic dyeing". This ion-exchange is entropy driven as it increases the randomness of the system. Since polycationic proteins are not associated with mobile cations, ion exchange cannot occur, minimising background staining. For a cartoon illustrating basic dyeing see Figure 3.

TB does however distinguish GAGs from other polyanions. TB stacks, only occurring with GAGs, are of a different "metachromatic" color to the monomeric dye present in nuclei and Nissl substance.

### A Large Acid Dye which Stains Amyloid Selectively — Congo Red (CR)

CR is a large, hydrophilic anionic dye with a linear molecule, see Figure 1a.

**Source of affinity —** The large conjugated system of CR results in substantial dye-tissue van der Waals attractions.

**Sources of selectivity for amyloid —**The staining solution (and/or pre-staining wash and post-staining differentiator, dependent on variant) is typically of high pH. Consequently most tissue proteins are anionic, and acid dyeing of background proteins does not occur since proteins contain no mobile exchangeable anions. In some variants, acid dyeing is also inhibited by the presence of large amounts of sodium chloride, and hydrophobic bonding inhibited by use of a non-aqueous solvent. Proteins stain under these conditions only if: they are cationic at high pH (the basic proteins of eosinophil granules); they contain unusual aromatic aminoacid residues (elastin); or have linear binding sites matching the linear structure of CR so facilitating van der Waals attractions, as do the β-pleated sheet proteins of amyloids.

### A Large Basic Dye Giving Selective Staining of GAGs — Alcian Blue (AB)

AB is a large, hydrophilic cationic dye; see Figure 2 for the structural formula.

**Source of affinity —** The large conjugated system of AB results in a significant contribution from dye–tissue van der Waals attractions.

**Source of selectivity for GAGs —** AB is applied from acidic aqueous solutions. Various tissue polyanions are present — DNA, RNA and GAGs — whose associated mobile cations could exchange with AB in a basic dyeing process. However AB stains GAGs very much faster than other polyanions, as tissue components containing GAGs are more permeable than chromatin or Nissl substance, see Table 2. Background staining of proteins is minimal as these biopolymers are protonated, and hence have no mobile exchangeable cations.





### A Reactive Dye Selective for Calcium Deposits — Alizarin Red S (ARS)

ARS is a small, hydrophilic anionic dye which can chelate a variety of metal ions; see Figure 4 for the structural formula.

**Source of affinity —** This is usually regarded as the formation of dye–metal ion polar covalent bonds.

**Sources of selectivity for calcium deposits —** Background acid dyeing of proteins is reduced by the small conjugated system size and hydrophilic character of ARS minimising van der Waals attractions and hydrophobic bonding. In the pH 6.5 variant, acid dyeing by anionic ARS is further reduced, since tissue proteins then have reduced cationic character and so contain few mobile exchangeable anions.

**Sensitivity for calcium deposits —** Increasing pHs and staining times increases staining intensity, since these maximise the number of dye phenolate groups and accommodate the slow staining reaction rate respectively.

### A Non-ionic Dye Selective for Lipids — Sudan Black B (SBB)

SBB is a non-ionic, hydrophobic dye of moderate size; see Figure 4 for the structural formula.

**Source of affinity —** Staining is primarily entropy driven. In some variants affinity is enhanced by manoeuvres such as increasing the proportion of water in the aqueous-alcoholic solvent, or using supersaturated SBB solutions.

**Source of selectivity for lipid —** The non-lipid components of tissues, being largely comprised of polysaccharides, proteins and nucleic acids, are significantly hydrated. Consequently entry of SBB would be entropically unfavorable — it would result in increased water structure — so accumulation does not occur. Unbound background is readily removed by post-staining solvent washes.

**Sensitivity for lipids —** Triglyceride deposits near their melting point are fluid and permeable, and stain readily; whereas crystalline deposits such as cholesterol remain unstained in routine procedures.

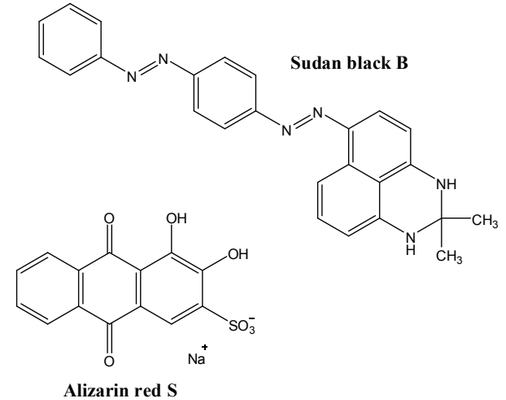


**Figure 4.** A non-ionic dye (Sudan black B) and a "reactive" dye (alizarin red S).

### A Tailpiece: Hematoxylin & Eosin (H & E)

A special case, though not a special stain. H & E is so widely used that no account of staining mechanisms can fail to mention it — the more so as H & E still puzzles us in various ways.

**What is H & E? —** The easy bit is to say what "eosin" is. Eosin Y is a normal acid (anionic) dye of moderate size and well studied chemistry. "Hematoxylin" however is a misnomer, hematoxylin being a colorless natural product whose oxidation can produce hematein. And "hematoxylin" in H & E is the product of the reaction of hematein with aluminium ions, so is better termed Al-hematein. But to ask "what is this?" is to encounter puzzle number one. The nature of the Al-hematein complex or complexes in staining solutions remains uncertain. Both cationic and anionic complexes could be present; for experimental data and summary of prior work see Bettinger & Zimmermann (1991) and Puchtler et al (1986). Moreover the Al-hematein complex present in tissue sections following "blueing" is probably different again, perhaps a poorly soluble olation polymer (Puchtler et al 1986).

**How does H & E work? —** Again starting with the easy bit: eosin stains sections by acid dyeing. The Al-hematein staining mechanism however is puzzle number two. Its staining behavior differs from nuclear and Nissl staining basic dyes such as cresyl violet or toluidine blue. Extraction of DNA from cell nuclei inhibits nuclear staining by such basic dyes, but does not eliminate Al-hematein staining. Routine basic dyes readily wash out of sections if over-enthusiastically dehydrated by alcohol, but "hematoxylin" does not. Nuclear staining by hemalum after extraction of DNA may be attributable to histones, the strongly basic proteins of eukaryotic nuclei. It has been argued (but not proved) that hemalum solutions contain anionic dye-metal complexes that would be attracted to the protonated lysine and arginine side-chains of histones. It has also been suggested that Al-hematein is a large hydrophilic basic dye, with resistance to alcohol extraction due to insolubility in that solvent; an alternative speculation notes that olation polymers are typically insoluble.

**Conclusions —** "H" remains a puzzle in some respects, although "E" is easy. Turning speculations into knowledge will require renewed chemical investigation of the actual Al-hematein complexes present in the dyebaths, and of the actual nature of "blued hematoxylin".

**Further Reading**


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**John A. Kiernan, MB, ChB, PhD, DSc**

## What is the difference between argentaffin and argyrophilic reactions?

Both terms relate to the formation of colloidal metallic silver (Latin argentum, Greek argyros) (1), which may range in color from yellow through brown to black depending on particle size and density, in specific structural components of tissues. The metal is formed at sites of reduction of silver ions derived from a staining solution. These ions may be simple $Ag^+$, as in a solution of silver nitrate, or they may be complex ions such as silver diammine, $[Ag(NH_3)_2]^+$ or silver methenamine, $[Ag_3(C_6H_{12}N_4)_2]^{3+}$, which are formed respectively by adding ammonium hydroxide or hexamethylenetetramine to solutions of silver nitrate. (Hexamethylenetetramine, the reaction product of formaldehyde and ammonia, is also used as a urinary antiseptic; its pharmaceutical name is methenamine in the USA and hexamine in the UK.) These complex silver ions are more easily reduced than $Ag^+$. By convention, argentaffin is used when the reducing agent is present in the tissue. Structures are said to be argyrophilic when bright light or a solution of a reducing agent is applied to the object after exposure to a silver solution.

An example of an argentaffin reaction with silver nitrate as the reagent is the histochemical demonstration of ascorbic acid in such sites as the adrenal cortex and the embryonic eye. Tiny fragments of fresh tissue are immersed in an acidified solution of silver nitrate and then in a solution of sodium thiosulfate (2, 3, 4). The latter reagent removes unreduced silver, which otherwise would be slowly reduced by the action of light, discoloring all parts of the tissue. This slower reduction, which forms the basis of several traditional methods for showing intercellular clefts and spaces (5), is an argyrophil, not an argentaffin reaction, because the light serves as an external reducer, acting probably on silver chloride precipitated in extracellular fluid. The same holds for von Kossa's stain for calcified material. In this, silver ions from aqueous $AgNO_3$ displace calcium from deposits of calcium carbonate or phosphate, forming insoluble silver carbonate or phosphate, which is quickly decomposed by the action of light to form black colloidal silver.

The only argentaffin reaction of importance in histopathology is the reduction of an alkaline solution containing silver diammine in the Masson-Fontana stain, which detects small quantities of melanin and, in suitably fixed tissues, the amines derived from tyrosine and tryptophan: dopamine, noradrenaline, adrenaline and serotonin (6). Most types of melanoma and some tumors of intestinal endocrine cells give positive Masson-Fontana argentaffin reactions (7, 8).

Nearly all the cell-types of the diffuse endocrine system (DES) are argyrophilic (9, 10), as are the axons of neurons and reticulin, a term that applies to thin collagen fibers and basement membranes. The technical details of argyrophil silver methods vary with the structures to be demonstrated, but in all cases there are at least two steps. In the first, which is often called impregnation, the tissue (whole pieces or sections) is exposed to a silver-containing solution. Silver ions bind non-specifically to the tissue and at certain significant sites some of the ions are reduced to silver "nuclei", each consisting of several atoms of the metal and far too small to be visible. In the second step, known as development, a reducing agent changes dissolved silver ions (which may or may not be derived from those non-specifically bound by the tissue) into deposits of colloidal metal. This development reaction is catalyzed by the silver nuclei, which are thereby enlarged enough to form visible deposits. Thus, the second (development) step of an argyrophil method is a chemical amplification of the invisible signal generated in the first (impregnation) step (11). Two examples of argyrophil methods will serve to illustrate different determinants of specificity and types of catalytic amplification.

In the Grimelius technique, for showing cells of the diffuse endocrine system (10), sections are impregnated with 0.004 M AgNO3 in a 0.02 M acetate buffer at pH 5.6 for three hours at 60 °C. This solution has been found empirically to provide for nucleation of silver in and around the secretory granules of cells of the DES. The slides are then transferred to a 0.09 M solution of hydroquinone in 0.4 M sodium sulfite, in which they remain for one minute. Sulfite ions react with silver ions (partly derived from residual impregnating solution and partly from silver non-specifically bound to the tissue) to form a complex anion, $[Ag(SO_3)_2]^{3-}$. This is reduced by hydroquinone and the resulting black colloidal silver accumulates around the initially formed nuclei in the cytoplasms of cells of the DES. A negative result with this simple, inexpensive technique may avoid multiple expensive immunohistochemical tests for the various peptides and proteins that characterize different DES cell-types and tumors derived from them (8).

There are several silver methods for reticulin that have in common three major steps (12). Gomori's technique (13) is typical. The sections are treated for one minute with 0.06 M aqueous potassium permanganate. This oxidizes the neutral sugar components of glycoproteins to aldehyde groups. (Reticular fibers and basement membranes contain relatively more carbohydrate than larger collagen fibers.) Brown manganese dioxide is deposited all over the sections and is removed by rinsing in 0.09 M potassium metabisulfite. After washing well with distilled water the sections are placed for two minutes in an alkaline silver diammine solution (approximately 0.4 M total Ag). This reagent is reduced to metal by aldehydes and some of the complex silver ions are nonspecifically bound to the tissue. After a brief rinse in water the slides are placed in 20% formalin (2.7 M formaldehyde) for 4 minutes. This reacts with residual and loosely bound silver diammine ions and the resulting silver is deposited preferentially upon the catalytic silver "nuclei" formed at the sites of aldehyde groups produced by the initial permanganate oxidation (14).

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## How does pH influence the staining mechanism of Schiff's reagent in biological tissue samples?

Schiff's reagent is a solution made by reaction of basic fuchsine, a red dye, with sulfurous acid. The reagent ideally is colorless, but it may be light yellow on account of impurities in the dye. It was introduced by Hugo Schiff in 1866 as a chromogenic test for aldehydes. Traditionally, basic fuchsine was a mixture of four cationic red triphenylmethane dyes (pararosaniline, rosaniline, magenta II and new fuchsine) made by oxidizing a crude mixture of aniline and mixed ortho- and para-isomers of toluidine (1). Pure aniline, p-toluidine and o-toluidine have been articles of commerce for many years. Consequently, pararosaniline and new fuchsine are available as individual dyes, whereas products sold as basic fuchsine typically are mixtures (2, 3). All four components of basic fuchsine are suitable for making Schiff's reagent, but pararosaniline has been the one used in chemical studies. Triphenylmethane dyes are commonly formulated as shown in Figure 1 (I - IV) with the positive charge on a nitrogen attached to a quinonoid ring, but other resonance forms are equally valid. The carbonium ion structure (V) allows for the simplest explanations of the formation and reactions of Schiff's reagent. The balancing anions, not shown in Figure 1, may be chloride or acetate.

The reaction with sulfurous acid (Fig. 1, V) decolorizes the dye. The structure of the colorless product was debated for many years (4), but it is now generally agreed to be an alkylsulfonic acid as shown (for Schiff's reagent made from pararosaniline) in Figure 1 (5, 6, 7).

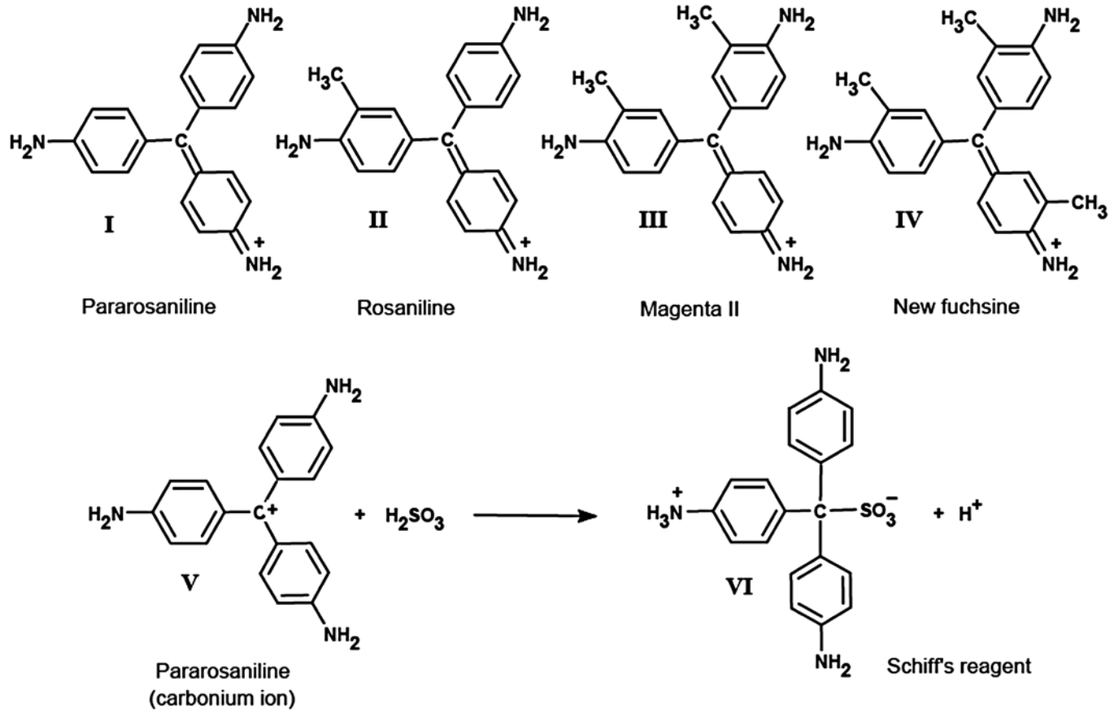


**Figure 1.** The components of basic fuchsine (I - IV) and the formation of Schiff's reagent by reaction of pararosaniline with sulfurous acid (V,VI).

Studies with NMR spectroscopy indicate that the major component is the zwitterionic form shown (VI) in which one of the amino groups is protonated (6).

The colorless solution is stable only at low pH and in the presence of an excess of sulfurous acid. Raising the pH or reducing the concentration of sulfurous acid increases the sensitivity of the reagent for detecting aldehydes but also leads to regeneration of the original dye. Some of the equilibria that account for this instability (6, 8) are summarized in Figure 2 (VII, V-VI). For histochemical purposes high sensitivity is not usually needed and the reagent must be stable while exposed to the air and losing sulfur dioxide for 15 to 20 minutes. Accordingly, Schiff's reagent for use as a biological stain must be acidic (pH about 2 is best for most applications) and it must contain more sulfurous acid than was needed to decolorize the basic fuchsine. If the pH approaches 3 a white precipitate may form after a few months of storage (4, 9). In equation VII it is seen that a solution at pH 2 contains predominantly bisulphite ions and sulfurous acid molecules. Reversal of the sulfonation, VI → V, occurs if there is loss of $SO_2$ into the atmosphere (see VII) or if the pH is raised towards neutrality. $SO_2$ can escape from a loosely capped bottle. The Schiff's reagent then becomes pink and is unfit for use because it

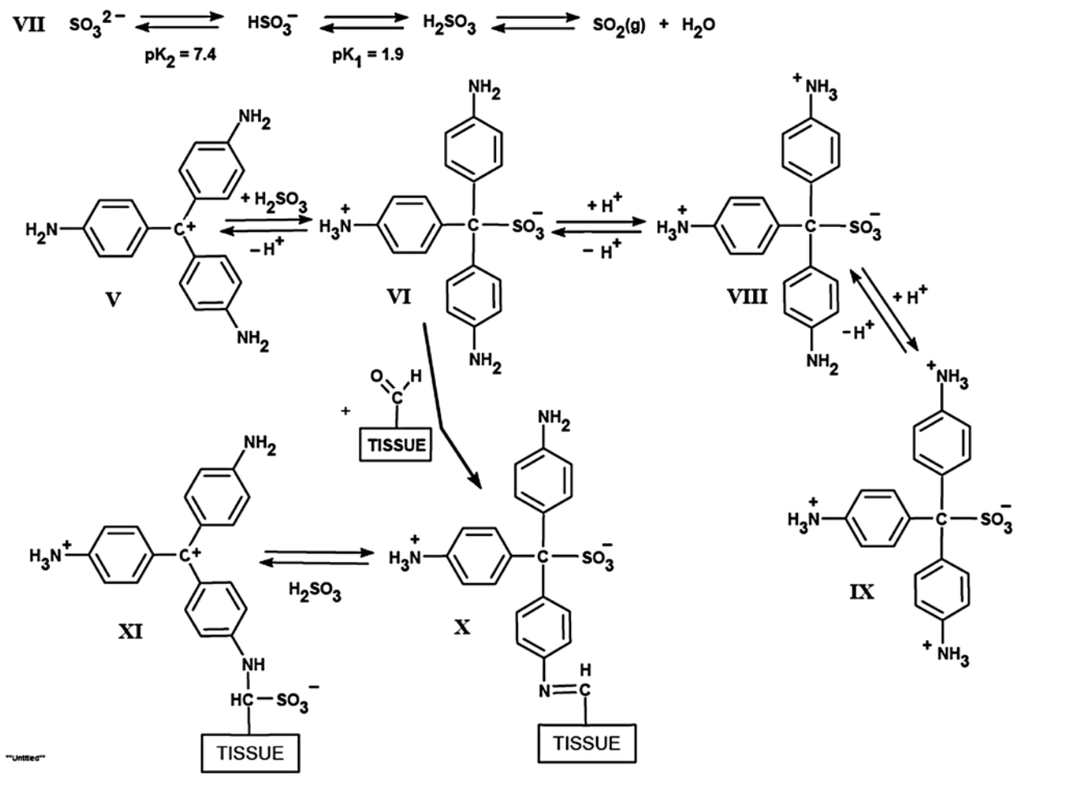


**Figure 2.** Effects of pH on equilibria in solutions containing sulfite, bisulfite, sulfurous acid and sulfur dioxide (VII). Effects of $H_2SO_3$ and H+ on the protonation of Schiff's reagent (VI, VIII, IX) and on the product of reaction with tissue-bound aldehyde groups (X, XI).





now contains some regenerated basic fuchsine, which could bind to polyanions in the tissue and give false-positive staining in cartilage matrix, mast cell granules and other materials that are stainable by cationic dyes at low pH. Reaction VI → V of Figure 2 is reversible, so Schiff's reagent that has become pink from losing $SO_2$ can be regenerated by adding a source of sulfurous acid, such as thionyl chloride or sodium metabisulphite. Excessive acidification causes additional protonation, leading to the formation of IX, which does not react with aldehydes (6, 8).

The simplest (theoretically) and oldest way to make Schiff's reagent is to bubble gaseous sulfur dioxide through a 0.3-1.0% (0.01-0.03M) aqueous solution of basic fuchsine. This is the method implied in Figure 2. The acidity of the solution is then due only to ionization of sulfurous acid (pK1 = 1.9) but it is almost impossible to adjust the amount of added $SOCl_2$ in the range between the minimum needed to decolorize the dye and the maximum dictated by the solubility of $SOCl_2$ gas in water.Another technique is to add some thionyl chloride, $SOCl_2$, to the dye solution. This liquid reacts instantly with water: $SOCl_2 + 2H_2O \rightarrow H_2SO_3 + 2H^+ + 2Cl^-$ providing both sulfurous and hydrochloric acids in a 1:2 molar ratio. Schiff reagents made with thionyl chloride can be stored for a few years in securely capped bottles (4, 9). Probably the most popular recipes for Schiff's reagent (4, 7, 10) are those in which the sulfurous acid is formed by dissolving sodium metabisulfite in the dye solution and then adding hydrochloric acid, allowing control of both the pH and sulfurous acid content as well as avoiding the use of more hazardous chemicals. Many vendors sell Schiff's reagent as a ready-made solution. A survey of 20 material safety data sheets (MSDS) shows that all were made with sodium metabisulfite, bisulfite or sulfite (0.1-2.0%), and hydrochloric acid, with concentrations of basic fuchsine ranging from 0.01 to 1.0%. The pH was reported in only seven of the MSDS, usually as a range such as 1.1-1.5 or 1.5-2.0.

The two most frequent histochemical applications of Schiff's reagent are the periodic acid-Schiff (PAS) method for neutral mucosubstances and the Feulgen reaction, a specific stain for DNA. Investigations in the 1950s indicated that maximum staining intensity was achieved at pH 2.4 for the PAS method, whereas pH optima ranging from 2.3 to 4.3 were reported for the Feulgen technique (4). Current practice favors more acidic and therefore more stable solutions. The ambient pH changes when slides are washed after immersion in Schiff's reagent. Rinses in 0.03 M sulphurous acid to remove unbound reagent were formerly commonplace (10, 11) but they weaken the final color. Rapid washing in water does not result in false-positive staining (12) and is currently recommended (9, 13). The rise in pH associated with washing in water for 10 minutes reduces the protonation of the stable dye-tissue complex (X in Figure 2), with resultant intensification of the color. The same effect can be achieved in 15 seconds with 0.3% sodium tetraborate, an alkaline solution (14).

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## What is Giemsa's stain and how does it color blood cells, bacteria and chromosomes?

### Stains for Blood

Probably the first use of a staining solution containing methylene blue (a cationic thiazine dye) and eosin (a red anionic xanthene dye) was in 1888 by C. Chenzinsky, to stain malaria parasites in blood films. F. Plehn in 1890 and E. Malachowski and D. L. Romanowsky in 1891 all independently described similar staining solutions that imparted a variety of colors to erythrocytes, leukocytes and malaria parasites (1). Notably, leukocyte nuclei were purple and the parasite nuclei were bright red; these colors could not be obtained with any technique in which the two dyes were used sequentially (2). Romanowsky's thesis (in Russian) and publication (in German) noted that the desired colors were obtained only when the methylene blue was derived from a stock solution old enough to have mold growing on its surface. The improved staining properties of "ripened" methylene blue solutions were studied by Unna (3), who also published in 1891 and found that the aging process could conveniently be accelerated by heat and alkalinity. Artificially ripened or polychrome methylene blue was used by most later inventors of blood stains, including L. Jenner (1899), B. Nocht (1899), W. Leishman (1901), R. May & L. Grunwald (1902), and J. Wright (1902) (1, 2). The products of polychroming, studied by Kehrmann (4) and later by Homes and French (5), result from demethylation of methylene blue and also from oxidation of the resulting dyes (Fig. 1).

Staining solutions made by mixing solutions of polychrome methylene blue and eosin are unstable because the oppositely charged dye ions combine to form salts, known as azure eosinates, that are insoluble in water. Azure eosinates are soluble in alcohols – a mixture of methanol and glycerol is commonly used – and they dissolve in water in the presence of an excess of either thiazine dye or eosin. Furthermore, continued oxidation results in the deposition of other water-insoluble products such as methylene violet (Bernthsen) (Fig. 1). For the production of commercial blood stains the precipitated eosinate is collected and dried (6, 7). Blood stains devised after 1902, such as Macneal's "tetrachrome" (1922) and the formulation of Roe, Lillie & Wilcox (1941) made use of eosinates of azures A and B and methylene blue. Later work showed that all commercially produced thiazine dyes were mixtures of the compounds shown in Figure 1, with the nominal dye sometimes not the most abundant component (6, 8).

In most blood stains the anionic dye is eosin Y (tetrabromofluorescein), which is easily purified. The stains of Leishman and of Macneal have eosin B, which is often a mixture of related dyes.

### Giemsa's Formulation

Gustav Giemsa (1867-1948) endeavored to produce mixtures of dyes that would reliably provide the Romanowsky color scheme (6). These formulations were published in 1902-1904, with the most advanced version being an aqueous solution containing a large excess of azure II over eosin Y. Azure II was a deliberate mixture of unoxidized methylene blue with azure I. Azure I was the name used at that time for the dye now called azure B. Modern formulations of Giemsa's stain are made by mixing azure B eosinate with methylene blue and are commercially available as Giemsa powder, which is dissolved in a 50/50 mixture of methanol and glycerol to provide a concentrated stock solution (9). For staining, the stock solution is diluted in water that is buffered to a pH appropriate to the intended application – pH 6.5-7.0 for alcohol-fixed blood films, lower for sections of formaldehyde-fixed tissues (2).

### Dyes Needed for the Romanowsky-Giemsa Effect

The compositions of blood stains were critically studied, using modern chromatographic and analytical methods, in the 1970s, notably by Paul Marshall in the UK (7, 8) and Dietrich Wittekind (2, 10) in Germany. These investigations revealed that the nuclear and all other colors constituting the "Romanowsky-Giemsa effect" could be obtained with solutions containing only azure B and eosin Y. Pure azure B, made by direct synthesis rather than from polychromed methylene blue, has been commercially available (as chloride, tetrafluoroborate or thiocyanate) since 1980 (11) and is used in a "Standardized Romanowsky-Giemsa method" (12) in which a stable methanolic solution of the eosinate is diluted with an aqueous buffer to make the working staining solution.

Pure azure B is more expensive than the crude variety, and there is still considerable demand for Giemsa's, Wright's, Macneal's and related blood stains made from polychrome methylene blue and from impure azures A and B. The chemical compositions and staining properties of these traditional mixtures have been shown to vary





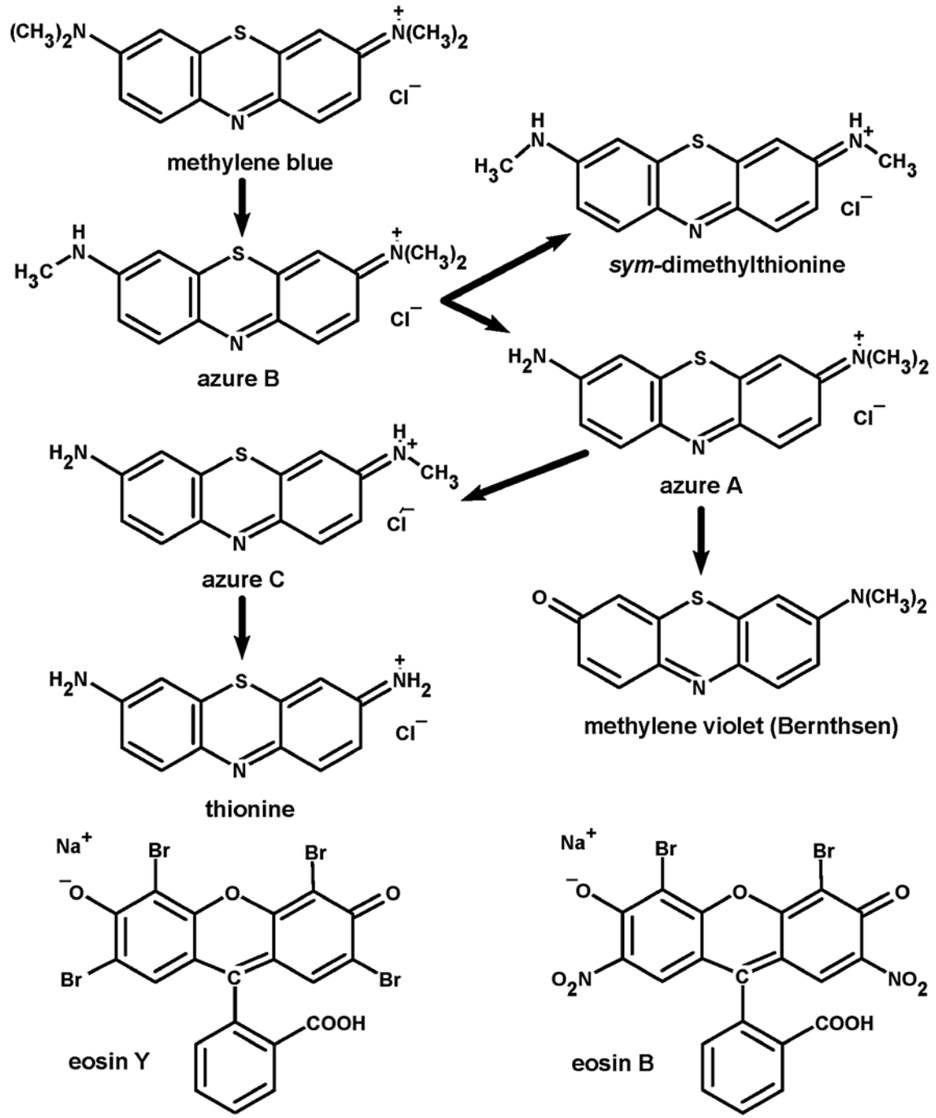


**Figure 1.** Dyes present in Giemsa's and other blood stains. The arrows indicate the progressive demethylation and oxidation that constitute polychroming of methylene blue.

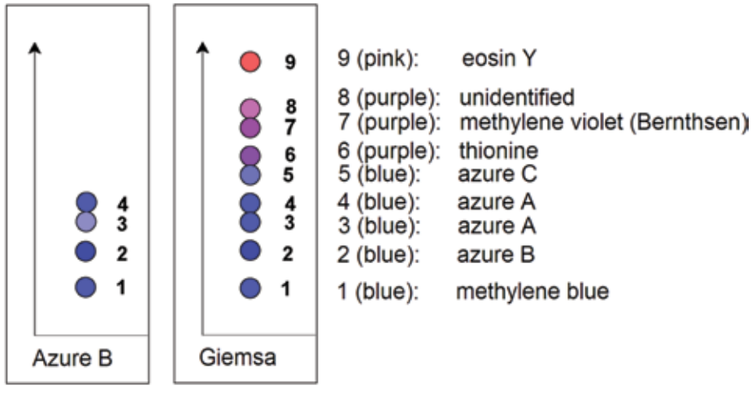


**Figure 2.** Thin layer chromatography of certified commercial samples of azure B and Giemsa's stain, on Unisil™ silica gel plates. Solutions in methanol were applied to the origin and development was in butanol-water-acetic acid for 120 minutes. Transcribed from cromatograms prepared by Mr Matthew Frank in the Biological Stain Commission's laboratory, Rochester NY. Spots 3 and 4, both labeled azure A, probably represent azure A and its isomer sym-dimethylthionine respectively (7, 8).

among different suppliers and even among different lots from the same supplier (7, 8). In the USA, samples of batches of methylene blue, azures A, B and C, methylene violet (Bernthsen) and powders for Giemsa's Jenner's and Wright's stains are tested by the Biological Stain Commission (BSC) by assaying dye content, spectrophotometry, thin-layer and high performance liquid chromatography and use in standardized staining procedures (13). Satisfactory batches are certified, and each bottle of dye powder carries a small additional label, provided by the BSC, attesting to this fact. Certified dyes should always be used for preparing solutions of Romanowsky-Giemsa stains. Azure B, the single most important ingredient of these stains, does not have to be the ultra-pure directly synthesized dye to meet the BSC's requirements for certification (Fig. 2).

## Staining Mechanisms

Studies of the interaction of DNA with azure B (14, 15) indicate that in solution the dye cations are present as dimers, each with the two planar ring systems held together by van der Waals forces. The dimers are attracted to the negatively charged phosphate groups of DNA and adhere to the macromolecule as a result of hydrophobic interactions with the purine and pyrimidine rings of the DNA bases. Attraction of eosin anions by un-neutralized positive charges of bound azure B dimers changes the color of the stained DNA from blue to purple (16). Eosin is also attracted to proteins with excess protonated amino and guanidino groups (mainly side chains of lysine and arginine) over ionized carboxy groups (mainly glutamic and aspartic acids); these proteins are hemoglobin in erythrocytes and the major basic protein of eosinophil granules (17). The red coloration of malaria parasite nuclei may be a due to a preponderance of basic proteins (histone) over DNA in the nuclei of protozoa (18, 19) and some invertebrates (20).

## Giemsa for Tissue Sections

The expected colors in cells of blood or bone marrow are seen only in alcohol-fixed films or smears and when the diluted staining mixture is close to neutrality - usually pH 6.8. Wright's or Leishman's stain is usually used, being allowed to act for about 3 minutes. Giemsa's stain, which is much more stable when diluted, can be allowed to act for 15-45 minutes. After fixation with formaldehyde, which reacts with amino and other basic groups, nearly everything stains blue at pH 6.8. Paraffin sections can be stained with Giemsa at pH 4 to 5. The sections show blue (not purple) nuclei, pink erythrocytes, cytoplasm and collagen, and metachromatic (red-purple) mast cells and cartilage. The colors are not the same as those seen in alcohol-fixed blood films (21-23). Giemsa is often used to stain bacteria in sections of formaldehyde-fixed gastric biopsies. The organisms are dark blue against an unstained or light pink background (23). The substrates of staining are presumably bacterial DNA and RNA, which differ from their animal counterparts in not being associated with basic proteins (24). The teichoic acids of the cell walls of Gram-positive bacteria probably also bind cationic dyes, and the phosphate groups of lipopolysaccharides can be expected to have a similar role in Gram-negative organisms.





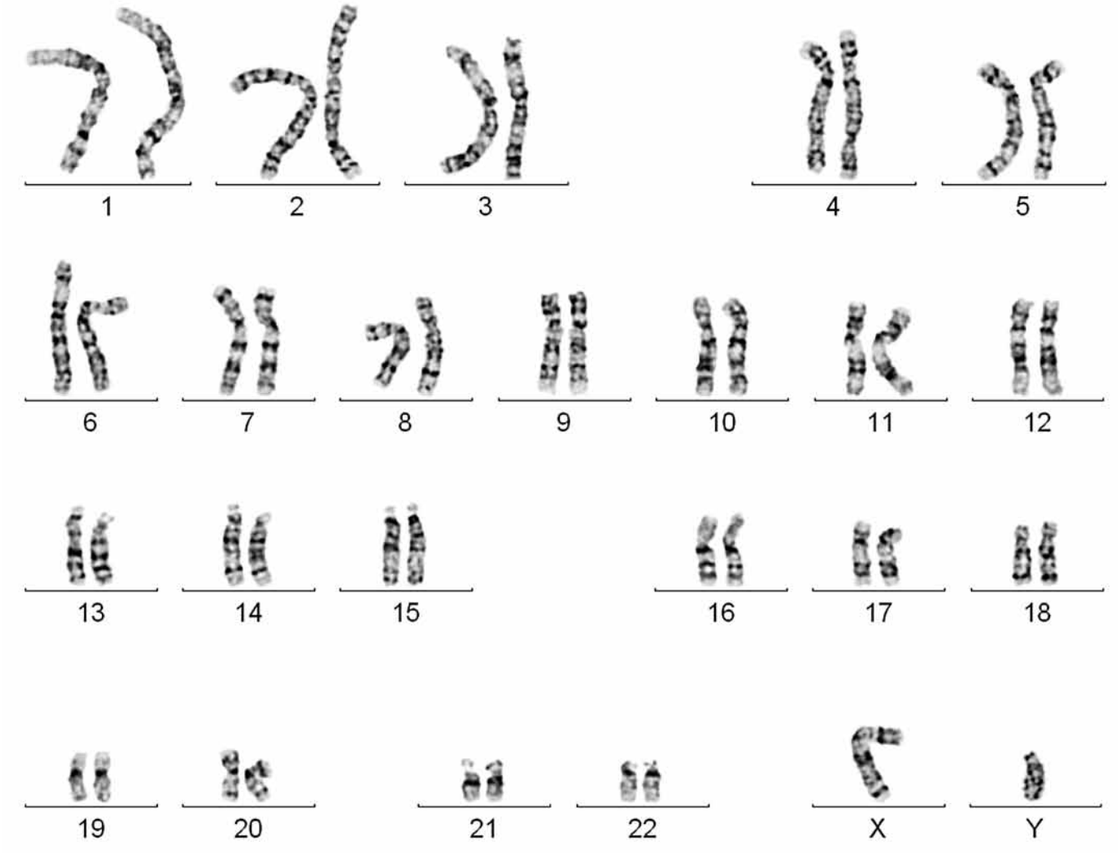


**Figure 3.** Karyogram of GTG-banded human chromosomes (♂) (46 chromosomes, including XY): GTG = G-banding with trypsin-Giemsa. Courtesy of Dr. Stefan Mueller, Ludwig-Maximilians-University, Munich, Germany.

### Chromosome Banding

Giemsa (G-banding) is the stain most commonly used to display transverse bands in mitotic (metaphase) chromosomes, having largely replaced quinacrine and other fluorochromes (Q-banding) that were formerly used for this purpose. There are various empirically developed G-banding techniques in which chromosome preparations – typically cell cultures that have been arrested in metaphase, spread onto slides, and fixed in 3:1 methanol-acetic acid – are subjected to pre-treatments before staining with Giemsa (25, 26). The dye cations in the Giemsa mixture (azure B and methylene blue) are attracted by the phosphate anions of DNA and are then held more closely by van der Waals forces to regions rich in adenine and thymine. Regions of a chromosome rich in guanine-cytosine pairs do not bind the blue dyes. The pretreatment determines the type of banding pattern. In typical G-banding the preparations are treated either with a sodium chloride and citrate solution at 60°C for one hour or with trypsin for 10 seconds at room temperature before staining in dilute Giemsa at pH 6.8 for 45 minutes (Fig. 3). R-banding is a method that shows bands that are complementary to the G-bands; that is, the dark and light regions of the G-banded chromosome are reversed. The pretreatment for R-banding is with 1.0 M NaH2PO4 (pH about 4) at 88°C for 20 minutes, and the staining is with a more concentrated Giemsa solution, for 10 minutes (27). G-banding is the method usually used for identifying chromosomes. R-banding is useful for showing the ends of chromosomes (telomeres). A variant known as T-banding emphasizes these terminal segments; after treatment with a phosphate buffer, pH 5.1 at 87°C for 20-60 minutes the preparations are stained in Giemsa, also at 87°C (25). The histochemical rationales of the various pretreatments have not been systematically investigated (26).

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# Chapter 20 | H&E Staining

**Gary W. Gill, CT(ASCP)**

## What is the difference between progressive vs. regressive hematoxylin staining?

Progressive hematoxylin stains color primarily chromatin and to a much less extent cytoplasm to the desired optical density, regardless of the length of staining time. Regressive hematoxylin stains overstain chromatin and cytoplasm and require subsequent immersion in dilute acid to pull out the excess color from the chromatin and cytoplasm (Table 1). If differentiation is omitted or incomplete, residual hematoxylin visually obscures fine chromatin detail and can prevent the uptake of eosin entirely.

Gill hematoxylins No. 1 and 2 contain 2 and 4 gm hematoxylin per liter, respectively, and 25% ethylene glycol. They are progressive stains that can be applied for many minutes without overstaining and without differentiation in a dilute acid bath. Harris hematoxylin contains 5 gm hematoxylin per liter of water. It overstains within minutes and requires differential extraction in dilute HCl to decolorize the cytoplasm (differentiation) and to remove excess hematoxylin from chromatin. Figure 1 illustrates the difference between the two approaches.

## Do you have a preference for progressive or regressive hematoxylin staining?

I prefer progressive hematoxylin staining because it does not require differentiation. Under- or over-differentiation can produce overstaining or understaining. Depending on the degree of timing control exercised in a given laboratory, the results may be satisfactory one day, hyperchromatic another day, and hypochromatic the next. Extreme hyperchromasia can block entirely the uptake of eosin so that H&E becomes simply H.

## What is the difference between differentiation and bluing?

Differentiation and bluing (blueing, if you prefer the English spelling) are essential to satisfactory staining by hematoxylins. Differentiation is used only with regressive hematoxylin formulations, while bluing is used with both regressive and progressive hematoxylin formulations. Differentiation effects quantitative changes; bluing, qualitative. See Table 2.

## Are there reasons to prefer water or alcohol as the solvent for eosin formulations?

I prefer alcohol-based eosin formulations: 1) they are chemically more stable 2) they minimize, if not eliminate entirely, the unpredictable effects of various impurities such as water-soluble salts that in water may interfere with dye uptake, and 3) they tend to stain more slowly than water-based formulations (promotes a wider range of shades of eosin colors).

## Is there a simple way to perform quality assurance (QA) on hematoxylin and eosin stains before using a batch for the first time?

Yes. Whether buying or making hematoxylin eosin solutions, one cannot be absolutely certain the product will perform. Apart from unsound methods, limitations in ingredients, incorrect formulations (e.g., precipitated mordant crystals in commercial Harris hematoxylin formulations), and errors in formulating (e.g., weighing out too much oxidizing agent) can contribute to unsatisfactory results. It doesn't happen often, but it does happen. Regulatory documentation does not guarantee efficacy.

Formalin-fixed tissue sections or alcohol-fixed buccal smears are invaluable probes to: 1) determine the performance of each new lot of stain, 2) select suitable staining times, 3) find out how many slides can be stained satisfactorily by a given volume of each stain, 4) learn when rinses should be changed, and 5) troubleshoot whether a given stain already in use is the cause of an observed staining problem. Once experience imparts confidence to selected staining times, stain and rinse change schedules, the use of control sections or smears is not necessary for the remainder of the life of the particular stain that has been validated. However, control preparations should be used when new containers of the same stain with different lot numbers are opened to confirm that the stain does indeed perform as expected. Manufacturers occasionally make bad batches of stain.

Simply looking at one of the first slides stained daily and initialing a stain quality log sheet is of no value if a laboratory has not defined its standards. It is not uncommon to see such sheets dutifully maintained and also to see unsatisfactory staining results.

**Table 1.** Progressive and regressive hematoxylin formulations: similarities and differences.

| Aspect | Hematoxylin | |
|---|---|---|
| | Progressive | Regressive |
| Hemalum concentration | Less (ie, 1 to 4 gm/L) | More (ie, 5 gm/L or more) |
| Acetic acid | Present | Absent |
| Rate of uptake | Slow | Rapid |
| Easily controlled? | Yes | No |
| Overstaining? | No | Yes |
| Differentiation required? | No | Yes |

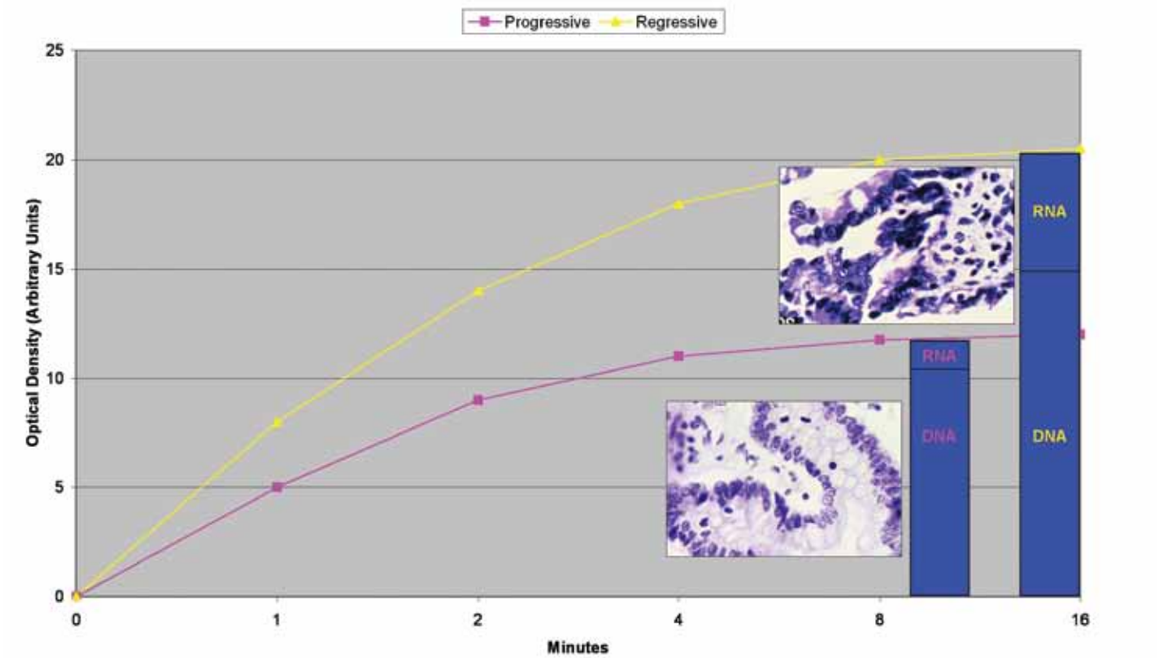


**Figure 1.** Hypothetical uptake of aluminum-hematein in cells: progressive vs. regressive staining.





**Table 2.** Differentiation and bluing: comparisons and contrasts.

| Property | Differentiation | Bluing |
|---|---|---|
| Purpose | Differentially extract excess hematoxylin from chromatin and cytoplasm; quantitative | Convert soluble red color to insoluble blue color; qualitative |
| Function | Attacks tissue/mordant bond | Oxidizes Al-hematein |
| Used with | Regressive hematoxylins | Progressive and regressive hematoxylins |
| Working pH | About 2.5 | 5-11 |
| Common example | 0.5% HCl in 70% ethanol | Scott's tap water substitute |
| Timing | Dips | Minutes |
| Timing accuracy | Critical | Forgiving |
| Risk if too brief | Hyperchromasia | Purple color |
| Risk if too long | Hypochromasia | Decolorization if pH ≥ 11 |
| Possible negative impact | Low contrast = less detail | Section loss if pH ≥ 11 |

## What role do rinses play in staining?

Rinses are the neglected step-child of staining. Defined here somewhat arbitrarily as all the non-coloring solutions, rinses constitute 90% of all the solutions in an H&E stain and outnumber the stains more than 10 to 1 if the initial xylene and alcohol rinses are included, yet their purposes vary and their contributions often go unrecognized. Consequently, rinses not uncommonly are overused to extremes – even becoming dilute staining solutions themselves, usually in an effort to save money. Not appreciated, unfortunately, is the hidden cost of extended, tedious microscopic examinations.

Among the many purposes of rinses are the following:

- remove paraffin
- effect transition from organic solvents to aqueous solutions and vice versa (i.e., hydration and dehydration)
- stop action of previous solution (post-hematoxylin waters)
- differentially extract excess hematoxylin (i.e., differentiation)
- convert hematoxylin from red to blue color (i.e., bluing)
- promote redistribution of dyes within tissue (i.e., uniformity)
- allow expression of differential staining
- dehydrate (with absolute alcohol)
- clear (with xylene)

Of the post-stain rinses, therefore, it may generally be said that the amount of stain that remains within cells represents the difference between what the staining solutions put in and the rinses take out. The post-eosin rinses perform most effectively when clean. Clean simply means that there is the maximum difference in concentration gradient between the dyes in the cells and the rinse. When stained tissue is immersed in clean alcohol, the dyes diffuse effectively into the surrounding rinse. As the rinses become dye-laden, the concentration gradient is reduced and diffusion slows. When the concentration of dye in the tissue equals that in the rinse, there is zero concentration gradient and the benefits of rinsing are lost.

To promote effective rinsing: (1) keep the rinses deep for maximum dilution (not just simply covering the tops of the slides when the slides are oriented horizontally, as opposed to vertically), (2) use in sets of three, (3) dip racks at least 10 times in each, and (4) change as needed. As needed means when the third rinse becomes colored with carryover dye, discard the contents of the first dish, move rinses 2 and 3 back one step to become rinses 1 and 2, and refill the third dish with fresh rinse. The third dish in each series of three post-eosin rinses should remain color-free. Maintaining this level of quality allows the absolute alcohols and xylene rinses to remain color-free.

## Is there a difference between quality control (QC) and quality assurance (QA) and measures for staining?

I've read many definitions of QA and QC. QC is any material or method that is introduced prospectively into every procedure to promote a desired outcome. If a QC material or method doesn't make a visible difference, it doesn't make a difference and shouldn't be used. QA, on the other hand, samples outcomes to assess whether the intended outcome was achieved. By its nature, QA is retrospective. In a word, QC imparts credibility to results; QA assesses impact.

## Can you describe a microscopical approach to evaluating stained sections?

When microscopically examining a preparation, one must remember that one is looking not at the object itself but an image of the object that is projected onto the retina. Therefore, one needs to separate the effects of the materials and methods that interact with the object itself (i.e., fixative, sectioning, possible artifacts, stain etc.) and those that influence the image of the object (i.e., mounting medium thickness, cover glass thickness, microscope cleanliness, and optical alignment (i.e., Köhler illumination).

A knowledgeable observer can assess whether the preparation is technically satisfactory and/or functionally satisfactory. If deficiencies are noted, one should be able to identify the likely cause and implement a solution. A technically satisfactory preparation exhibits no technical deficiencies. Such preparations are also likely functionally satisfactory, but not always. A functionally satisfactory preparation may exhibit technical deficiencies but still be useful for its intended purpose. This means the preparation does not have to be redone, but a solution should be implemented to ensure technically satisfactory preparations in the future. Examples of technical deficiencies include incomplete differentiation, no eosin, and excessively thick mounting medium and cover glass that cause image-degrading spherical aberration.

## Are there ways to systematically troubleshoot staining problems?

Yes. Whether the problems are seen in hematoxylin, eosin, or any stain, wayward results can be categorized as: 1) too much stain, 2) too little stain, 3) wrong color, or 4) wrong site. See Tables 3 and 4. If the problem is too much stain, put less in by using a less concentrated stain for the same staining time or staining for less time with the same concentration. It's vice versa when the problem is too little stain.





**Table 3.** Troubleshooting hematoxylin staining problems.

| Complaint | Cause | Correction |
|---|---|---|
| Hyperchromatic | Strong hematoxylin (e.g., Harris full-strength without acetic acid) | Use lesser strength hematoxylin;<br>Dilute 3:1 with ethylene glycol;<br>Stain for less time;<br>Differentiate in 0.25% HCl |
| | Staining time too long | Stain for less time |
| | Inadequate differentiation in HCl | Differentiate more;<br>Use more concentrated HCl |
| | Differentiator exhausted | Replace more frequently |
| Hypochromatic | Hematoxylin nearly exhausted | Replace hematoxylin |
| | Staining too briefly | Increase staining time |
| | Overdifferentiation in HCl | Differentiate less; Use weaker HCl |
| | Progressive stain differentiated | Do not differentiate |
| | Paraffin sections very thin | Cut thicker; stain longer |
| | Regressive stain overdifferentiated | Differentiate less |
| | Acid tap water, rare (e.g., West Virginia) | Use distilled water |
| | Chlorine in tap water (rare) | Use distilled water |
| | Bluing in acid tap water | Use Scott's tap water substitute (TWS) |
| Wrong color: purple | Bluing too briefly | Blue longer |
| | Bluing solution exhausted | Change bluing solution daily |
| | No blue filter in microscope | Use microscope's "daylight" blue filter |
| Wrong color: gray | Colored impurities | Use BSC-certified hematoxylin |
| Wrong color: brown | Too much oxidizing agent | Use less (e.g., 0.1 gm/gm hematoxylin) |
| | Overoxidized by long-term air exposure | Store with no air space and replace |
| Wrong site: cytoplasm | Hematoxylin too concentrated;<br>Underdifferentiation in HCl | Differentiate more;<br>Stain less time or dilute |
| | RNA-rich cytoplasm | |
| Wrong site: nucleoli | Staining time too long | Stain less time |
| | Ineffective eosin Y | Use effective eosin Y |

**Table 4.** Troubleshooting eosin staining problems.

| Complaint | Cause | Correction |
|---|---|---|
| Hyperchromatic | Exceeds user expectations | Adjust expectations |
| | Insufficient subsequent alcohol rinses | Increase rinse time, dip more |
| | Stain-laden rinses | Use clean alcohol rinses |
| Hypochromatic | Al-hematein in eosin bonding sites;<br>Eosin nearly exhausted | See Table 1<br>Replace eosin |
| | Eosin staining time brief | Double staining time to start |
| Wrong color: purple | Cytoplasm has retained hematoxylin applied regressively and partially differentiated | Use progressive hematoxylin or differentiate completely |
| | Insufficient subsequent alcohol rinses | Use three 95% EtOH baths, dip 10 times each |
| | Stain-laden rinses | Use clean alcohol rinses |

## What is Scott's tap water substitute?

Scott's tap water substitute (TWS) is an aqueous bluing solution with pH 8, which is an intermediate value along the range of pH within which bluing can occur (i.e., 5 to 11). Scott's TWS is prepared by dissolving in 1 liter of water 2 gm sodium bicarbonate and either 10 gm anhydrous magnesium sulfate (MgSO4) or 20 gm hydrated magnesium sulfate (MgSO4 · 7H2O [Epsom salts]). If you prepare this solution, be aware that dissolving magnesium sulfate is an exothermic reaction that can get unpleasantly warm. For safety, wear goggles and gloves. To minimize risks, add the magnesium sulfate slowly to the water so it dissolves rapidly and dissipates the heat produced. Alternatively, laboratorians who prefer to use Scott's TWS can purchase it readymade. Scott's TWS was described in an article published nearly a century ago (Scott SG. On successive double staining for histological purposes (preliminary note). *J Path Bact.* 1911-1912;16:390-398).

## How often should one replace in-use hematoxylin and eosin?

Predictably, the answer is "it depends." On the supply side, it depends on: 1) concentration of dye, 2) volume of staining bath, 3) number of dips, and 4) how well pre-stain rinses are maintained. On the demand side, it depends on the number of intracellular bonding sites for hematoxylin and eosin, which is a function of: 1) thickness and area of tissue sections, 2) relative proportion of chromatin and non-chromatin (e.g., malignant tissue has higher concentrations of nuclear chromatin than normal tissue), and 3) number of slides.

If your laboratory maintains a steady workload, you might want to replace hematoxylin and eosin at regular intervals or *ad hoc*. It is helpful to keep a well-stained section available as a visual reference against which stained sections can be compared.





## Can 1-step hydration and dehydration replace graded alcohols of hydration and dehydration?

Yes. Historically it has been customary to use series of graded percentage alcohols to hydrate or dehydrate specimens (e.g., 95%, 80%, 70%, and 50%, and vice versa). The rationale has been that shrinkage-distortion of cells and tissue sections is minimized by such stepwise displacement of alcohol or water. Another suggested reason is that gradual decreases or increases in alcohol concentration minimize the convection currents that otherwise occur, and are plainly visible, when specimens go directly from alcohol to water or vice versa.

In practice, neither event is observed. Regardless of the closeness of alcohols in percentage, shrinkage inevitably occurs. The final amount is neither greater nor lesser than that which results from 1-step hydration and 1-step dehydration procedures (i.e., 95% ethanol to water in one step and vice versa). Nor does increased cell loss occur. The currents seen moving around a slide that has gone from alcohol to water, or water to alcohol, are diffusion currents, not convection currents. Diffusion currents are concentration gradient-based; and convection currents are temperature gradient-based. Differences in the refractive index of water and alcohol exaggerate the visibility of the diffusion currents when dehydration or hydration takes place in a single step.

To blend the diffusion currents immediately and minimize any agitation effects they might produce, repeatedly dip the slides as soon as they are immersed. Dipping also ensures uniform rates of shrinkage or swelling in tissue, and thus minimizes the likelihood of poorly affixed sections becoming detached from the glass surface. The pH of the water has no effect on subsequent staining. The pH of the stain solutions influences the uptake of dyes.

To maintain the effectiveness of 1-step hydration and dehydration rinses: 1) keep the solutions deep for maximum dilution (not merely covering the tops of the slides when the slides are oriented horizontally, as opposed to vertically), 2) dip racks immediately at least 10 times in each, 3) use in sets of three, and 4) change as needed.

## What does "clearing" accomplish?

Suffusing fixed protein with a solution such as xylene that has a similar refractive index minimizes light diffraction and promotes nearly optically perfect images. Fixed protein has a refractive index of about 1.536; histological grade xylene, 1.5. (Baker JR. *Cytological Technique - The Principles Underlying Routine Methods.* Methuen, London, 1960 (4th edition); Crossmon GC. Mounting media for phase microscope specimens. *Stain Technol.* 1949;24(4):241-7). Clearing is the term applied to immersing fixed tissue sections in a solution of nearly matching refractive index and the transparency it enables. Formaldehyde-fixed tissue is comprised mostly of proteins and nucleic acids and some carbohydrates; the lipids for the most part have been dissolved out. The protein is naturally transparent, but if allowed to remain dry it will diffract light waves that pass through it.

## Can a lab "go green" with its H&E staining?

Yes. Relative to the 3 Rs of saving the environment: reduce, reuse, recycle:

- Reduce alcohol use: substitute 0.5% glacial acetic acid in water for 95% ethanol as a rinse wherever possible. This concentration of acetic acid is weaker than that of household vinegar. It is also less expensive than alcohol and not flammable.
- Reuse xylene forever: use water scavenging beads in xylene (see URL in next bullet).
- Recycle alcohol or xylene using Creative Waste Solutions and Suncycle Technologies products (http://www.cwsincorp.com/; http://www.suncycletech.com/).

**Suggested Reading**

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# Chapter 21 | Biological Microtechniques

**Gary W. Gill, CT(ASCP) and Debra M. Wood, MS, HT(ASCP)**

## What is the correct pH for the hematoxylin staining solution?

**GWG:** Other than the pH of a particular hematoxylin formulation, there is no universally correct pH to my knowledge. For example, Gill hematoxylin–No. 1, which I introduced in 1972 at the annual scientific meeting of American Society of Cytopathology in New Orleans, is pH 2.54. The pH of any hematoxylin formulation is correct when that hematoxylin performs as expected.

## What are the "rocks" at the bottom of some bottles of the Harris hematoxylin?

**GWG:** The "rocks" are aluminum sulfate crystals (Fig. 1). Ammonium aluminum sulfate (ammonium alum) is used as a mordant in Harris hematoxylin. Ammonium alum is slightly soluble in room temperature water. At 100 gm/L water, ammonium alum appears to be pushing its solubility limits. Containers of Harris hematoxylin that sit undisturbed for a long time often form these crystals. I recall encountering a gallon jug of commercially-prepared Harris hematoxylin with crystal formation so extreme that I could hear the crystals moving about when I shook the container. In the latter instance, I surmised the manufacturer had used more ammonium alum than called for. Diluting Harris hematoxylin with ethylene glycol 3 parts to 1 produces a stain that can hold more ammonium alum in solution, and crystal formation no longer occurs.

**Figure 1.** Crystal of aluminum sulfate that grew at the bottom of a flask of Harris hematoxylin with mordant that exceeded its solubility limit in water.

Some questions in this section are from personal communications from Anatech, Ltd, Battle Creek, MI. GWG =Gary W. Gill, DMW = Debra M. Wood

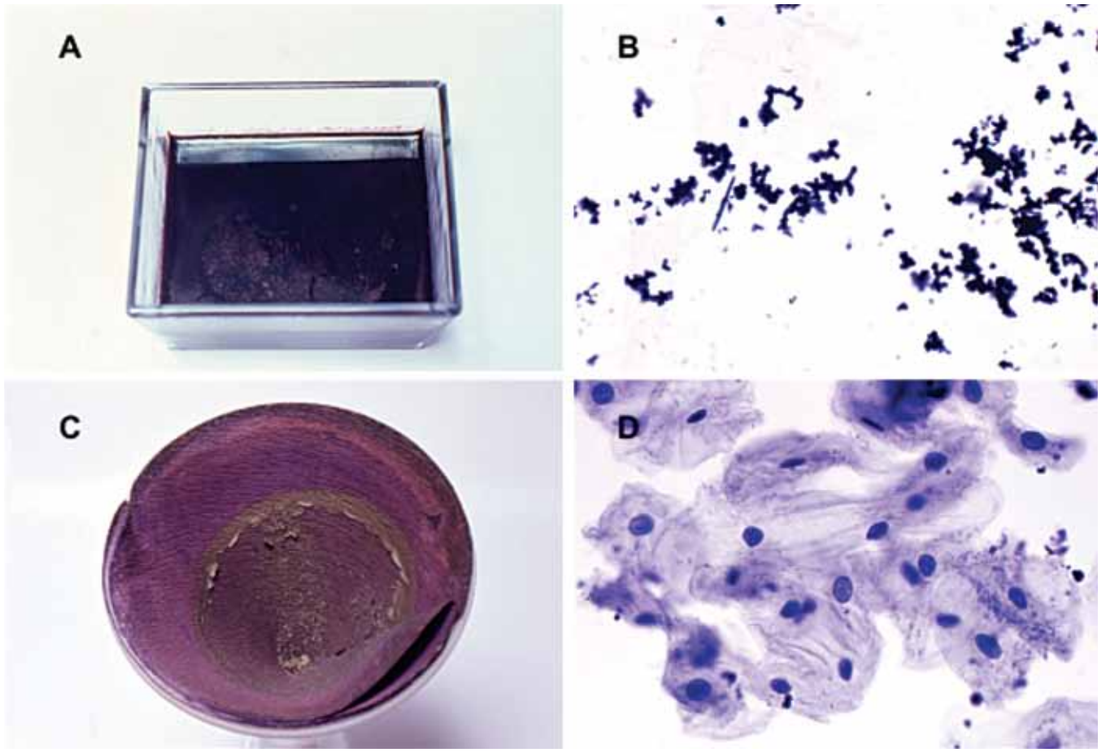



**Figure 2.** Genesis and identity of surface precipitate on Harris Hematoxylin.

**A.** Harris hematoxylin with greenish-golden surface precipitate.
**B.** If the greenish-golden surface precipitate is not filtered before staining, the precipitate is deposited on the microscope slide.
**C.** The precipitate can be captured by filtration.
**D.** When the precipitate is redissolved in 25% ethylene glycol, its identity as aluminium hematein is confirmed by its ability to stain cells.





## What Is the metallic-appearing material that precipitates on the surface of Harris hematoxylin?

**GWG:** Although often identified as an overoxidation product, it is not; the precipitate is aluminum-hematein. Figure 2 illustrates the explanation.

## How do bluing reagents work?

**GWG:** Hematein, the oxidation product of hematoxylin, exists in solution in three forms: 1) free yellow hematein, 2) partially-linked red hematein linked to 1 aluminum ion per molecule, and 3) fully-linked blue hematein with each molecule attached to 2 aluminum ions. Below pH 5, hydrogen ions compete with aluminum ions. As the pH rises, competition by hydrogen ions decreases, and the blue aluminum-hematein replaces the red. In my experience, tap water will blue hematoxylin in about two minutes.

## What is the correct pH for an eosin dye solution? What is the purpose of the alcohol rinse prior to the eosin stain?

**GWG.** I am unaware of any pH that is considered to be correct for eosin formulations. Many eosin formulations include glacial acetic acid, which increases eosin uptake dramatically by increasing the number of positive charges on protein molecules to which negatively-charged eosin molecules can bind. One could, I suppose, measure the pH of an eosin formulation in water, but pH is not so easily measured in non-aqueous formulations. I doubt that adjusting the pH to some predetermined value would make a real difference.

Historically, an alcohol rinse or rinses preceded alcohol-based eosin stains. Apparently, it was thought that the alcohol concentration of the eosin solution would be maintained, which would somehow translate into more reliable staining results. In practice, whether alcohol or water rinses precede an alcohol-based eosin makes no visible difference. Therefore, I always use water rinses before stains of any kind to save money.

## What causes poor nuclear detail in tissue sections?

**GWG:** Poor nuclear detail may originate in nuclei *per se* or in the image of the nuclei. In nuclei, the poor detail may be the result of tissue degeneration before fixation, inadequate fixation, unsatisfactory nuclear staining, or any combination of the three possibilities. In images of nuclei, poor detail may be due to excessively thick mounting medium + cover glass (i.e., spherical aberration), light scattering dirt on glass surfaces (i.e., glare), excessively wide substage condenser aperture diaphragm (i.e., flare), or any combination of three possibilities. Whether the limitation resides in the specimen can be ruled-in if the poor nuclear detail persists when the preparation has been covered with a very thin layer of mounting medium, covered with a No. 1 thickness cover glass, and viewed through a clean microscope adjusted by Köhler illumination.

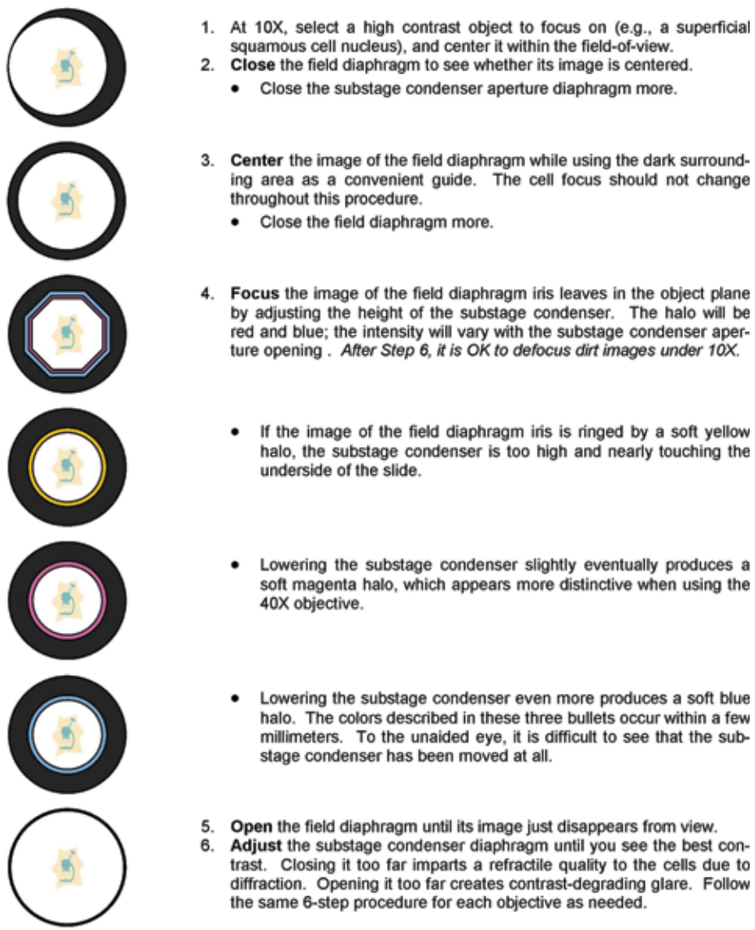
## What is Köhler illumination?

**GWG:** Köhler illumination is a simple, systematic method of aligning the lens elements along the microscope's optical axis to promote uniform illumination and optimal resolution. The method is named after Professor August Köhler who, as an employee of Carl Zeiss, published his description of the method in 1893. See Figure 3 and page 283 in the Appendix.

## Stained sections sometimes appear sharp microscopically when viewed using the 10× objective, but hazy when seen using the "high-dry" 40× objective. What is responsible for the difference?

**GWG:** Assuming the microscope is clean and adjusted for Köhler illumination, the combined thickness of mounting medium and cover glass exceeds the cover glass thickness tolerance limits of the 40X objective.

Cover glasses, and by extension mounting media, are the *de facto* front lens of every microscope objective. Those who coverslip are, in effect, completing the assembly of a microscope objective with every cover glass they apply. So while coverslipping is a tedious process, its visibly appreciable contribution should not be minimized.



**KÖHLER ILLUMINATION**

Köhler illumination[1] ensures uniform illumination and maximum microscopic resolution. Before beginning, interpupillary eyepiece distance must be adjusted and each eyepiece focused. The entire procedure is completed within seconds. The openings of the field diaphragm and substage condenser diaphragm are different for each magnification. For best results the microscope must be clean.

1. At 10X, select a high contrast object to focus on (e.g., a superficial squamous cell nucleus), and center it within the field-of-view.
2. **Close** the field diaphragm to see whether its image is centered.
   - Close the substage condenser aperture diaphragm more.
3. **Center** the image of the field diaphragm while using the dark surrounding area as a convenient guide. The cell focus should not change throughout this procedure.
   - Close the field diaphragm more.
4. **Focus** the image of the field diaphragm iris leaves in the object plane by adjusting the height of the substage condenser. The halo will be red and blue; the intensity will vary with the substage condenser aperture opening . *After Step 6, it is OK to defocus dirt images under 10X.*
   - If the image of the field diaphragm iris is ringed by a soft yellow halo, the substage condenser is too high and nearly touching the underside of the slide.
   - Lowering the substage condenser slightly eventually produces a soft magenta halo, which appears more distinctive when using the 40X objective.
   - Lowering the substage condenser even more produces a soft blue halo. The colors described in these three bullets occur within a few millimeters. To the unaided eye, it is difficult to see that the substage condenser has been moved at all.
5. **Open** the field diaphragm until its image just disappears from view.
6. **Adjust** the substage condenser diaphragm until you see the best contrast. Closing it too far imparts a refractile quality to the cells due to diffraction. Opening it too far creates contrast-degrading glare. Follow the same 6-step procedure for each objective as needed.

[1] Köhler A. A neues Beleuchtungsverfahren für microphotographische Zwecke. *Z wiss Mikr.* 1893;10:433-40.

**Figure 3**. Köhler Illumination. Reproduced with permission from: Gill, Gary W (2005). *Köhler Illumination.* Laboratory Medicine; 36(9):530. Copyright: 2005 American Society for Clinical Pathology and 2005 Laboratory Medicine.





Objective lens designers assume cover glasses will be used. According to ASTM (American Society for Testing and Materials) E211 - 82(2005) Standard Specification for Cover Glasses and Glass Slides for Use in Microscopy, cover glasses should have a refractive index of 1.523 ± 0.005, a dispersion value of 52.0, fall within precise thickness ranges, be planoparallel and free of optical pits and imperfections. Lens designers have long incorporated such data into their calculations, but it was not until 1953 that Setterington gathered the proprietary optical specifications used by various microscope manufacturers and proposed a uniform standard. Cover glasses were used first in 1789 and first made commercially in 1840 by Chance Brothers of Birmingham, England.

The tolerance of an objective to deviations from the recommended 0.180 mm thickness cover glass (ie, No. 1½ [0.17-0.19 mm]) is a function of its numerical aperture (NA). Low power objectives such as a 10X with an NA of 0.2 are highly tolerant of deviations from the recommended thickness. On the other hand, high-dry achromat objectives with NA 0.65 are much less forgiving. When the combined thickness of the cover glass and mounting medium exceeds ±15µm from the ideal thickness, spherical aberration makes object images look washed out, hazy, cloudy, milky, and low in contrast. Greater deviations degrade images greater.

The metal jacket of a microscope objective is known as the “boot.” On the boot of every professional quality microscope there are three numbers engraved: 1) magnification, 2) numerical aperture, and 3) cover glass thickness. For example, on my Olympus high-dry fluorite objective, the numbers are 40×, 0.75, and 0.17. The latter number equals a No. 1½ cover glass, which is what many people recommend.

Such recommendations are wrong when the specimen is mounted on the glass slide surface and is overlaid with mounting medium that distances the underside of the cover glass from the specimen. Light is an equal opportunity transparency employer; it cannot tell the difference between transparent mounting medium and glass. As far as light is concerned, mounting medium is an extension of the cover glass thickness. Therefore, in the rare circumstance where the specimen is in direct contact with the cover glass, No. 1½ cover glasses should be used. Otherwise, No. 1 (013-0.16-mm) cover glasses should be used with as little mounting medium as will remain intact after the solvent evaporates.

**Table 1.** Destaining H&E sections.

| Steps | Solution | Time | Note |
|---|---|---|---|
| 1-2 | Xylene | 1 min/each | Removes traces of mountant. Dip repeatedly; inspect surface. A wavy rather than a smoothly glistening surface denotes incomplete rinsing and indicates further dipping |
| 3-5 | Absolute alcohol | 1 min/each | Prepares slides for next step |
| 6 | 1.0% HCl* | min-1 hr | Removes hematoxylin. The exact time depends particularly on the type of tissue, its thickness, and how much hematoxylin is to be removed. Monitor the progress of decolorization by periodic microscopic inspection |
| 7 | 1.5% NH4OH in 70% ethanol** | 1 min | Time required may vary and should be adjusted as needed. Repeated dipping aids uniform decolorization |
| 8-9 | Tap water | 1 min/each | To intended stain |

*0.23 mL HCl (concentrated: 36.5-38% w/w, S.G. 1.1854-1.1923) or 5.5 mL N/2 HCl in water q.s. 100 mL

**5.7 mL NH4OH (concentrated: 29.2% w/w, S.G. 0.900) in 75 mL 95% ethanol, and water q.s. 100 mL

## Can you recommend an effective way to destain H&E sections?

**GWG:** Yes. After removing the cover glass, remove all traces of residual mounting medium by xylene-immersion twice for at least 1 minute each. The basic approach is immerse-and-reverse: immerse the sections in solutions with the opposite pH that promoted stain uptake in the first place (see Table 1).

## What is formalin pigment?

**GWG:** Formalin pigment, also known as acid formaldehyde hematin, is a dark-brown to black microcrystalline deposit found intracellularly and extracellularly in blood-rich tissue that occurs in simple formalin solutions. This hematin should not be confused with hematein, the oxidation product of hematoxylin. At acid pH, formaldehyde acts on hemoglobin to form the pigment. Formalin pigment can be prevented by using neutral buffered formalin. Once formed in tissue, it can be removed by immersing sections in alcoholic picric acid solution or alkaline alcohol. Since it is birefringent, formalin pigment can be identified microscopically using crossed polaroid filters. Additional information can be found at http://stainsfile.info/StainsFile/prepare/fix/agents/formalin-pigment.htm.

## How do fixatives that contain metal additives affect special stains, particularly silver stains?

**DMW:** Fixation plays an important role in tissue staining. In addition to the stabilization of proteins, a fixative also brings out differences in refractive indexes and maintains conditions that allow for good staining. Some fixatives actually enhance certain staining techniques. There are a number of fixatives used in histology that contain metal salts and other metallic compounds that are preferred specifically for the staining results. Heavy metal fixatives include chromium, mercury and osmium, all of which are cations that combine with anionic groups of proteins. The result is an excess charge of cationic charges. Some groups that combine with these excess positive charges are the sulfhydryl (-SH), carboxyl (-COOH), and phosphoric acid ($-PO_4$).

Chromium and osmium are not widely used in routine histology and will be mentioned briefly. The cations of chromium attach to some anion charged groups of lipids. Chromium increases the reactive basic groups within the tissue proteins allowing for an increased affinity for acidic dyes. Chromate solutions form an insoluble pigment in the tissue if the fixed specimen is taken directly to an alcoholic solution. This pigment can be prevented by washing the fixed specimen in water before placing it on the tissue processor. Chromium is toxic and its use should be closely tracked.

Osmium tetroxide is primarily used as a secondary fixative in electron microscopy studies. Osmium chemically reacts with the double bonds of lipids and makes them insoluble. The lipids in cell membranes then become electron dense and can be viewed with the electron microscope. Osmium also reacts with small amounts of fat rendering them insoluble. Therefore, they can withstand paraffin processing,

Mercuric chloride is only used in compound fixatives because it is such a powerful protein coagulant. It is an additive fixative because it chemically combines with the tissue acting as a permanent mordant and leaving tissue more receptive to dyes. Mercuric fixatives best application is for fixation of hematopoietic and reticuloendothelial tissues (liver, lung, spleen, lymph nodes, thymus gland, kidney). The three most common mercury fixatives are: Zenker's, Helly's and B-5.

Zenker's and Helly's fluid share the same stock formulation: mercuric chloride has a mordanting effect on tissue, potassium dichromate has a binding effect on tissue and fixes the cytoplasm well, sodium sulfate is optional and distilled water. The working formulation of Zenker's requires the addition of glacial acetic acid. It's recommended for PTAH stain and for Feulgen reaction for nucleic acids (DNA). Zenker's lyses RBCs, yields brilliant trichrome stains, and because of the acetic acid, also decals small pieces of bone so it can be use as decal and fixative for bone marrow biopsies. Note: Zenkers should be avoided if stains for hemosiderin are necessary as it dissolves the iron. Zenker's requires washing in water before processing to avoid residual chrome pigment from being formed (dichromate). The working formulation of Helly's requires the addition of formaldehyde. Helly's preserves RBCs and is recommended for bone marrow specimens when the demonstration of iron is required. Helly's has an increased probability of formalin pigment. Both Zenker's and Helly's decrease nuclear basophilia, therefore may need to increase staining time of hematoxylin.





B-5 fixative is a compound fixative that contains mercuric chloride. In addition to mercuric chloride, it contains anhydrous sodium acetate (↑ pH to 5.8-6.0) and distilled water in stock solution. The working solution will also contain 37-40% formaldehyde (1 part formaldehyde solution to 10 parts B-5) and must be prepared immediately before its use. Mercuric chloride and formaldehyde are the fixatives while sodium acetate acts as a buffer. Fixation in B-5 can be completed in 24 hours, however, specimens cut in size for processing (no more than 3mm thick) can fix in 4-8 hours. Tissues should not remain in the B-5 fixative indefinitely as the tissue becomes very brittle. It isn't necessary to wash tissues in water after fixation is complete however, any wet tissue must be held in 70% alcohol. Specimens fixed in B-5 will have remaining mercury pigment that needs to be removed; this is accomplished with a solution of iodine followed by sodium thiosulfate. B-5 gives excellent results with many special stains, demonstrates great nuclear detail, and so is preferred for use in hematopoietic and lymphoreticular tissue.

Since mercury is such a powerful protein coagulant and because it chemically combines with the tissue acting as a permanent mordant and leaving tissue more receptive to dyes, mercury fixatives are recommended for rhabdomyoscaromas, Negri bodies, Feulgen plasma reaction, trichromes, Giemsa, and Mallory's PTAH stains.

## What is B5 fixative and how does it impact special stain performance quality for bone marrows smears and tissue specimens (e.g., Giemsa, Iron staining). What are the regulatory/safety implications in the United States that may affect the handling of "B5" fixative?

**DMW:** B-5 fixative is a compound fixative that contains mercuric chloride. B-5 is used less often in laboratories today than in the past due the mercuric-chloride found within the solution. B-5 gives excellent results with many special stains, demonstrates great nuclear detail, and so is preferred for use in hematopoietic and lymphoreticular tissues. Because it contains mercuric chloride, iron stains should be avoided as the mercuric chloride dissolves hemosiderin.

Since B-5 is a mercury-based fixative it is regulated by the Environmental Protection Agency. Nationally the EPA has limited the disposal of all mercury containing waste to 1 part per billion. Laboratories using any mercury-based fixative, including B-5, should realize the disposal of B-5 is not limited to excess fixative discarded from the specimen, but includes the tissue itself and anything it has come in contact with. During fixation some mercuric salts in the B-5 solution attach to the tissue but others remained unattached. During processing and staining the "free" mercuric salts are washed out of the tissue and into the subsequent solutions. In many cases the waste solutions are discarded down the drain. Even if these solutions are distilled, the remains must be hauled away by a licensed company as mercuric waste.

# Chapter 22 | What are the Special Stains used in Neuropathology?

**Roscoe Atkinson, MD**

## What are the special stains used in neuropathology?

Special stains are a fundamental component of neuropathology. Non-tumor neuropathology probably utilizes special stains more than any other pathology subspecialty. Most descriptions of special stains focus on listing and describing the stains one by one with appropriate examples. In this case, it is more appropriate to list the scenarios and discuss the various stains that would be utilized. However, before proceeding further, it must be emphasized that the hematoxylin and eosin (H&E) stain is the fundamental stain performed on all samples in every neuropathology case. It is also the stain that is examined by the neuropathologist – and also the best stain to show basic morphology. With this stain, nuclear detail is highlighted by hematoxylin while the cytoplasmic and extracellular proteinacious components are stained by eosin. What happens next and how the special stains are utilized depends on the type of sample and the disease category being evaluated.

**Neurodegenerative disease:** The neurodegenerative diseases include Alzheimer disease and Pick disease. These are both dementing diseases associated with cerebral atrophy. The clinical diagnosis is made with around 90% accuracy; however, because these two diseases have substantial overlap of symptoms not only among themselves, but also with other diseases, a neuropathological confirmation of the diagnosis is often requested. This confirmatory diagnosis is completely dependent on special stains, in particular one of the silver stains.

There are a variety of silver stains available to histologists. One of this is the Bielschowsky silver stain, which highlights the tangles and plaques of Alzheimer disease. In this method, catalytic “nuclei” of silver metal are formed in neurons and their axons. The specificity is determined mainly by the composition of the silver-containing solution. Other silver atoms are coordinately bound to proteins in general. The developing agent removes the chemically bound silver ions and reduces them to metal at sites of catalytic nuclei. In Ferenc Gallyas’s method, all the chemically bound silver is removed from the sections (by complexation) before putting the slides in the developer, which contains a controlled concentration of silver ions along with a reducing agent and a stabilizer that retards reduction in the solution. Such a developer is called a “physical developer”, a curious term derived from the early days of photography.

The original use of Bielschowsky's (1904) and Bodian's (1936) methods was for staining normal axons, especially in the central nervous system (CNS). Bodian’s technique has since been shown to be fairly specific for one of the neurofilament proteins. Pathologists use modified versions to show Alzheimer's pathology. The Gallyas (1970s) method that pathologists use (not usually the author's first stain of choice) was developed specially for showing neuritic plaques and neurofibrillary tangles (Fig. 1a, b).

**Infectious disease:** Many of the organisms that cause CNS infections can be highlighted with special stains. A common example is the Zeihl-Nielson stain (also known as the acid-fast stain) that is used to turn the acid-fast organisms of tuberculosis a bright red color on a blue background (Fig. 2 and 3). Another example is the use of Mucicarmine stain that highlights the gelatinous capsule of the fungus *Cryptococcus*. Fungal organisms in general can also be stained with “silver” stains such as Gomori Methanamine Silver (Fig. 4 and 5).

**Nerve biopsy:** Special stains are a major component of the nerve biopsy. Reactive fibrosis (scarring) in between the small nerve processes and in between groups of them can be assessed with the Mallory trichrome stain that turns the accumulated collagen fibers bright blue on an orange background. The Congo red stain is usually included to rule out amyloidosis. The Congo red stain turns the semi-crystalized beta-pleaded sheet protein a bright salmon orange (Fig. 6) that turns a shiny apple green during polarization (see Appendix, page 284).

**Pituitary biopsy:** Although immunohistochemical stains are the fundamental stains (in addition to H&E) used in the workup of pituitary adenomas, the distinction between adenoma and anterior pituitary gland is often required and often challenging. The reticulin stain can be very successful in demarcating the nests of normal anterior pituitary. The delicate normal stromal type of demarcation is completely lost in adenomas.

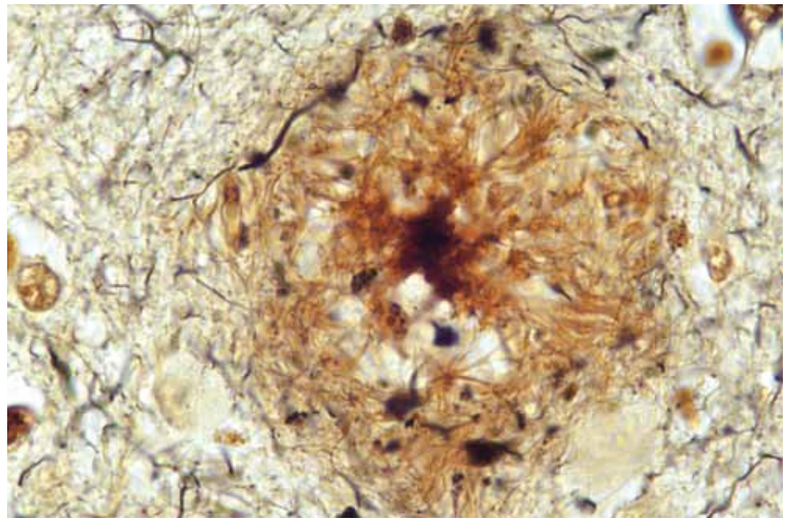


**Figure 1a.** At high magnification the modified Bielschowsky silver stain highlights the actual plaque structure.

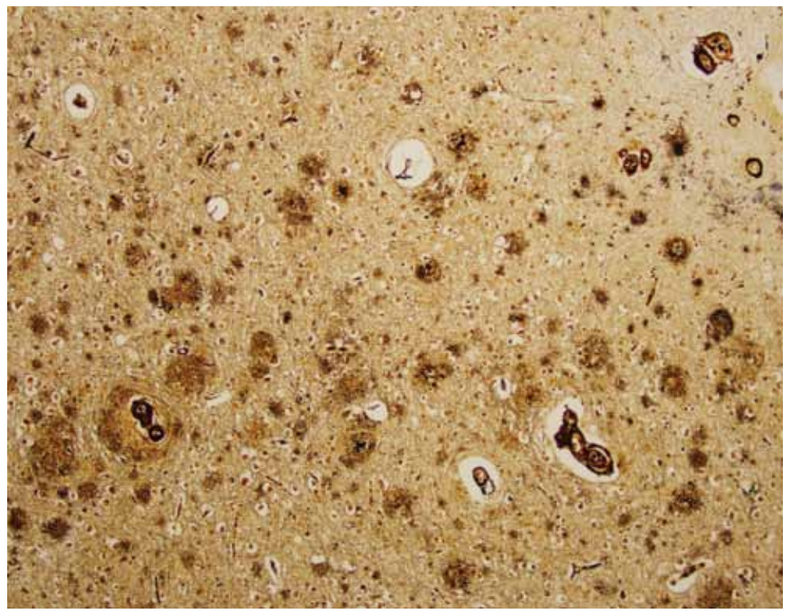


**Figure 1b.** Modified Bielschowsky silver stain showing scattered silver-positive neuritic plaques within a brown background. The density of these structures is used in making the diagnosis of Alzheimer disease (low magnification).





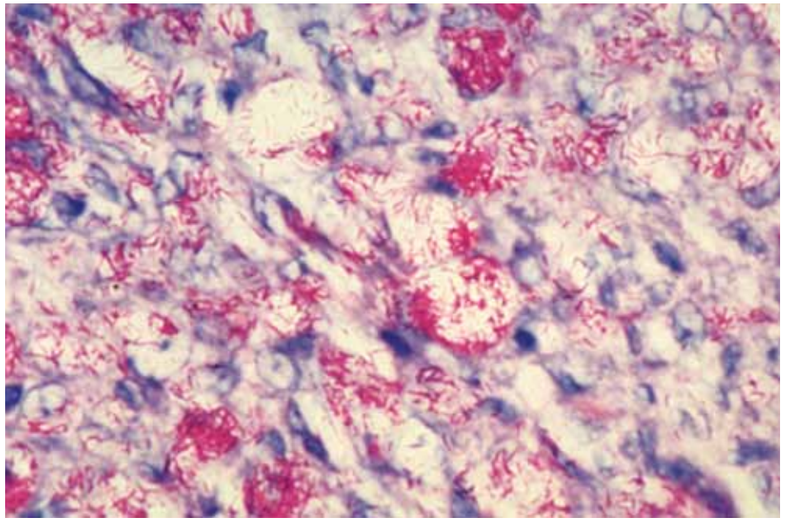


**Figure. 2**. Acid fast rods of *Mycobacterium tuberculosis* stained bright red using the Zeihl Neelsen stain. This density of microorganisms is more commonly encountered in stain-control tissue.

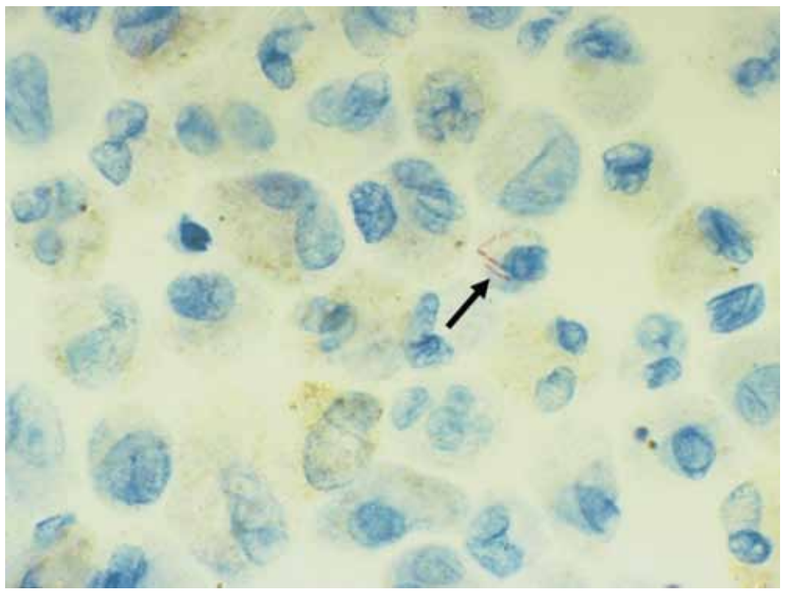


**Figure 3.** Two isolated acid fast rods of *Mycobacterium tuberculosis* stained red using the Zeihl Neelsen stain (arrow). This density of organisms is more commonly encountered in the tissue being tested. Only a single organism need be found to consider the tissue positive and make the diagnosis. Exhaustive searching using high magnification is required before considering a tissue section negative.

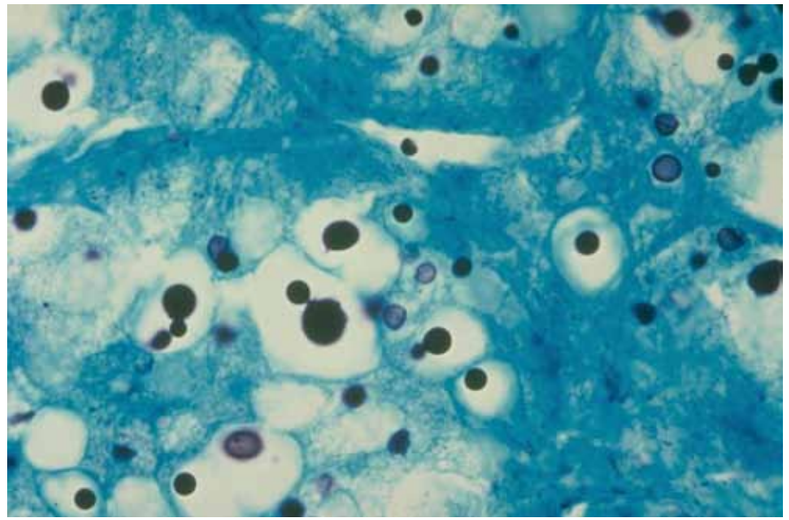


**Figure 4.** The Gomori Methanamine Silver stain at high magnification shows the yeast of *Cryptococcus neoformans* stained black by the silver component of the stain. The clear demarcation around the yeast is produced by the gelatinous cryptococcal capsule.

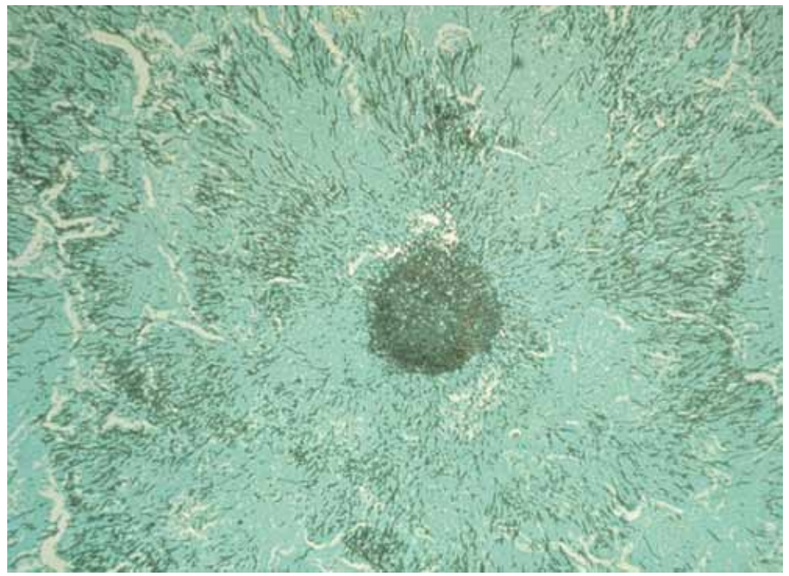


**Figure 5.** The Gomori Methanamine Silver stain at low magnification shows a characteristic starburst pattern of *Aspergillus fumigate*. The deposited silver produces a grey/black fungal coloration within a green counterstained background.





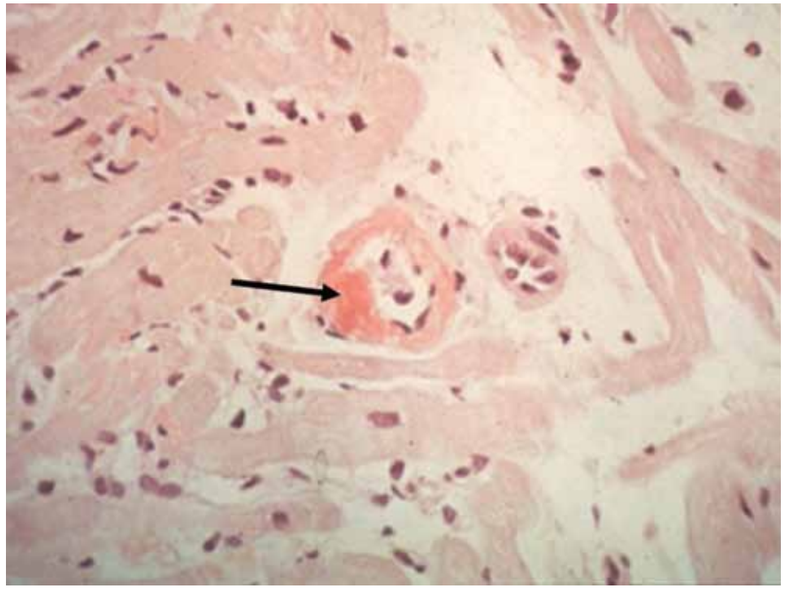


**Figure 6.** In this section the Congo Red stain highlights vascular amyloid deposition. Note the salmon orange coloration of the vessel wall (arrow).

**In summary,** special stains are very effective in the interpretation of neuropathology specimens. They are a fundamental component in neuropathology, and are usually used in adjunct to routine staining with hematoxylin and eosin. How the special stains are utilized depends on the type of sample and the disease category being evaluated. While some are easier to use and interpret than others, most find diagnostic utility in everyday practice.

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# Chapter 23 | Special Stains in Dermatopathology

**Jameel Ahmad Brown, MD and Bruce R. Smoller, MD**

## Histochemical Stains

Dual staining with hematoxylin, which stains nuclei blue, and eosin, a pink cytoplasmic stain, is the mainstay in routine examination of cutaneous tissue under light microscopy. The ability to delineate differences in tissue type and cellular components is greatly enhanced by exploiting the inherent uptake properties of various tissues with these stains. When particular characteristics are not easily identified with hematoxylin and eosin, histochemical staining becomes of great value. Its merit is recognized diagnostically and economically, as histochemical staining is much less expensive than immunohistochemical staining and is readily available in virtually all dermatopathology practice settings.

## Commonly Used Special Stains

### Periodic Acid-Schiff (PAS)

Periodic Acid-Schiff (PAS) is one of the most frequently employed special stains in the dermatopathology laboratory. A positive stain is perceived as purplish-red or magenta. PAS is used to demonstrate the presence of neutral mucopolysaccharides, especially glycogen. The process by which tissues are stained with PAS involves the oxidation of hydroxyl groups in 1,2 glycols to aldehyde and subsequent staining of the aldehydes with fuschin-sulfuric acid. PAS also has utility when predigestion with diastase (PASD) has been performed. The diastase removes glycogen from tissue sections but leaves other neutral mucopolysaccharides behind. PAS stain allows for the recognition and highlighting of basement membrane material and thickness, such as in lupus erythematosus. In addition, PASD accentuates fungal cell walls, helps identify the presence of glycogen in tumor cells versus lipid, and illuminates many PAS positive inclusion bodies, secretions, and granules. Non-cutaneous pathology finds PAS equally useful (Fig. 1).

### Alcian Blue

Alcian blue facilitates visualization of acid mucopolysaccharides or acidic mucins. The staining procedure is performed at pH of 2.5 and 0.5 respectively. Positive staining is perceived as blue. The mucopolysaccharide differential staining pattern is created by and based on the principle that sulfated mucopolysaccharides, like chondroitin sulfate and heparan sulfate, will stain at both pH values while non-sulfated ones stain at a pH of 2.5 only. The most common non-sulfated mucopolysaccharide of the skin is hyaluronic acid, found normally in scant amounts in the papillary dermis, surrounding cutaneous appendages, and encircling the vascular plexuses. Alcian blue is also an excellent special stain when attempting to detect acidic mucin in neoplasms, inflammatory conditions, and dermatoses including granuloma annulare, lupus erythematosus, dermatomyositis, scleromyxedema, pre-tibial myxedema, scleredema, and follicular mucinosis. Additionally, sialomucin produced in extramammary Paget's disease demonstrates positive alcian blue staining. Of note, colloidal iron demonstrates a staining profile which is essentially indistinguishable from alcian blue, and the use of one over the other will be laboratory-dependent; based on the familiarity of the operator with the nuances of each stain and the quality of results obtained.

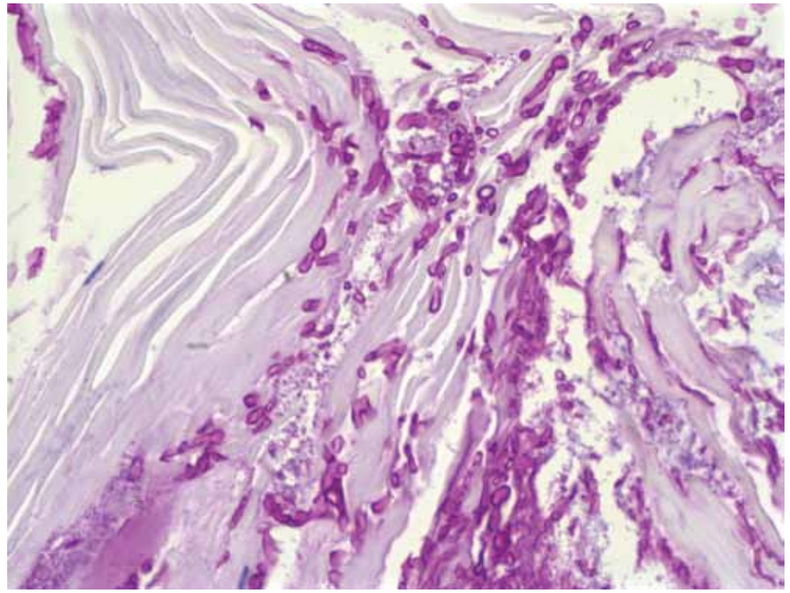

**Figure 1.** PAS-D showing fungal organisms in a case of onychomycosis.

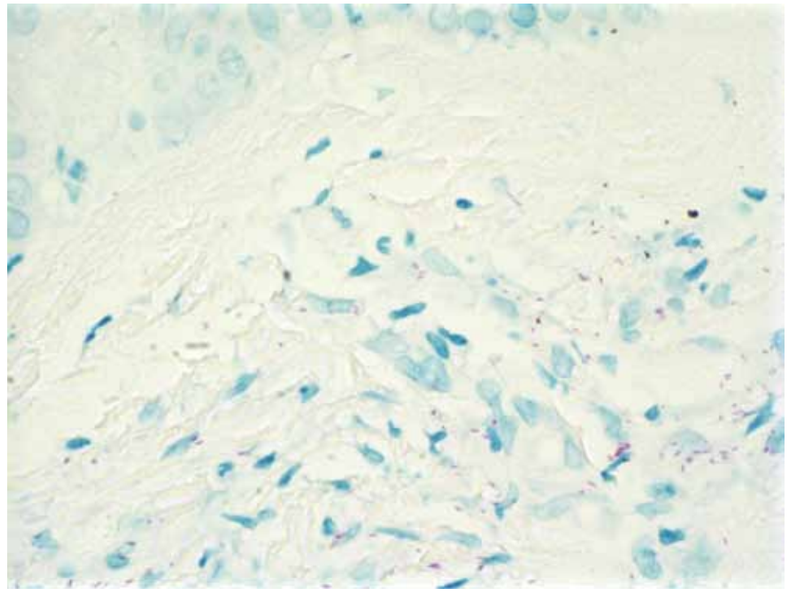

**Figure 2.** Fite stain demonstrating acid fast bacilli in a case of lepromatous leprosy.





## Elastic Tissue Stain (Verhoff von Gieson)

The elastic tissue stain (ETS), a silver stain, is used to demonstrate and evaluate the quantity and quality of tissue elastic fibers. Positive staining is perceived as black. ETS serves as a dependable adjunct when evaluating tissue sections for entities under the umbrella of elastolytic granulomata; these diseases include actinic granuloma, atypical facial necrobiosis lipoidica, and granuloma multiforme. Similarly, when assessing a patient for anetoderma (focal dermal elastolysis) and cutis laxa, the elastic tissue stain aids in identifying complete elastolysis, fragmented elastic fibers in the papillary and mid-reticular dermis, and the morphology of existing elastic fibers. Anomalous distribution and organization of elastic-rich tissue, as in collagenoma and other variants of connective tissue nevi, can also be detected. Most recently, the elastic tissue stain has received a new place in the spotlight of the dermatopathology literature, primarily because of its utility in differentiating the invasive component of melanoma from a benign underlying or adjacent melanocytic nevus when the two are associated.

## Brown and Brenn Stain

The Brown and Brenn stain is a tissue Gram stain that is a practical method of identifying and differentially staining bacteria and resolving them into two primary groups – Gram-negative and Gram-positive. The baceteria's staining characteristics are based on the physical properties of their cell walls. Gram-positive bacteria have thick cell walls composed of high proportions of peptidoglycan which retain the purple color during the staining process. Peptidoglycan comprises only a small fraction of the thin cell walls of Gram-negative bacteria which also have a lipid outer membrane; both characteristics contribute to their pink color as the purple dye (crystal violet) is washed away during the decolorization step. In addition to the color contrast created by the stain, morphology can also be elucidated and one can appreciate bacilli, cocci, or coccobacilli. The tissue Gram stain is not ideal for all bacterial species, as some have ambiguous staining patterns or are simply not highlighted. The Brown and Brenn stain is very difficult to interpret in skin sections, thus its diagnostic value is limited in dermatopathology practice. Additionally, cultures of suspected cutaneous infections are superior in sensitivity and reproducibility to the Brown and Brenn stain.

## Acid-Fast Stain (AFB)

The classic acid-fast bacilli stain used most commonly in dermatopathology settings is the Ziehl-Neelsen stain. Acid-fast organisms are those whose cell walls contain high lipid in the form of mycolic acids and long-chain fatty acids. These characteristics permit strong binding and retention of carbol fuchsin dye after decolorization with acid-alcohol. An AFB positive organism stains brightly red after completion of the procedure. Frequently, a counterstain of methylene blue is used to serve as a contrasting background. AFB stain is employed most often when there is a high clinical suspicion of a mycobacterial infection and when granulomatous inflammation is seen in the absence of foreign body material on histopathologic examination. The stain is an excellent adjunct to light microscopy when seeking the presence of *Mycobacterium tuberculosis*; however, not all mycobacterial species stain positively. Modified versions of the classic stain include modified bleach Ziehl-Neelsen, Kinyoun, Ellis and Zabrowarny, auramine-rhodamine (most sensitive of all), and Fite. The Fite stain highlights mycobacteria in general but is specifically used to identify *Mycobacterium leprosum*. The stronger acid used in the classic method is deemed too harsh for *M. leprae* and the lipid in the cell's membrane is washed away, making visualization of the organism difficult. Fite uses a weaker acid in the decolorization phase of the procedure, preserving the more delicate cell walls of the organism. The Fite and the Ziehl-Neelsen methods share their positive acid-fast profiles; red is read as a positive stain (Fig. 2).

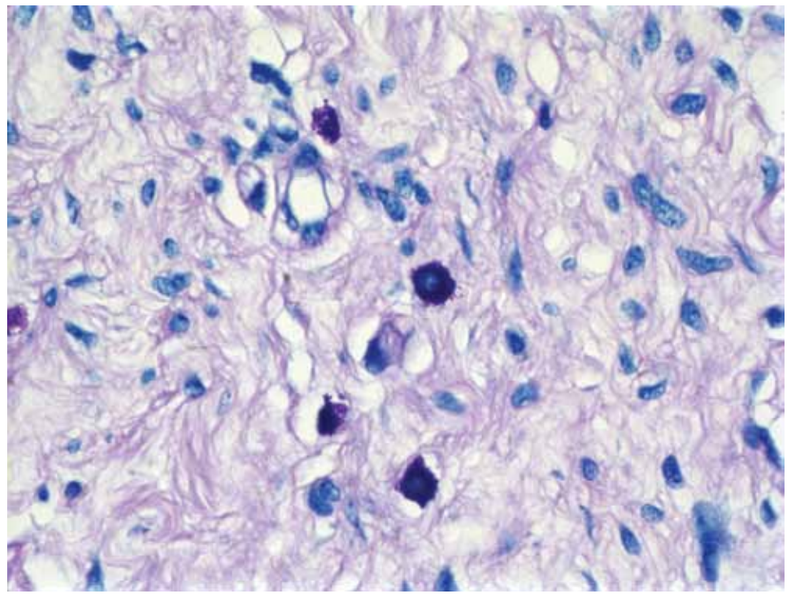

**Figure 3.** Mast cell tryptase highlighting secretory granules of mast cells.

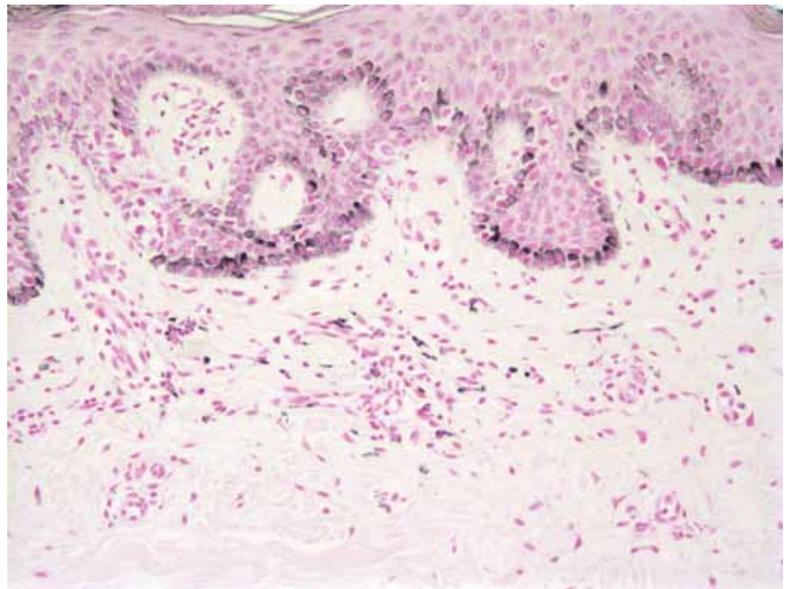

**Figure 4.** Fontana-Masson stain highlighting melanin in epidermal keratinocytes, melanocytes, and dermal melanophages.





## Giemsa

Giemsa is a metachromatic stain in that many tissues/organisms stain differently than the dye color itself. The dye mixture – methylene blue, azure, and eosin compounds – is blue, but positive staining is designated by a purplish hue. Giemsa is frequently used to identify mast cells; their granules stain positively. Of note, the immunohistochemical stains “mast cell tryptase” and “CD 117” are also used to detect mast cells. Urticaria and urticaria pigmentosa are among the disease entities marked by a significant increase in mast cell volume, thus Giemsa finds its utility here. Further contributing to the value of Giemsa is the positive staining of several infectious organisms; spirochetes, protozoans, and cutaneous Leishmania in particular.

## Warthin-Starry

Warthin-Starry stain is a silver nitrate stain. Tissue sections are incubated in a 1% silver nitrate solution followed by a developer such as hydroquinol. Warthin-Starry is used chiefly in the identification of spirochetes in diseases such as syphilis, Lyme disease, and acrodermatitis chronica atrophicans. A positive stain will reveal black spirochetes. Like the Verhoff von Gieson stain, the Warthin-Starry stain has the disadvantage of non-specific elastic tissue fiber staining, making it difficult to interpret in skin sections. Further complicating interpretation is positive staining of melanocytes. The use of this stain is often supplanted by antibody stains and/or serologies.

## Masson’s Trichrome

Masson’s trichrome is a special stain which is typically used to characterize and discriminate between various connective and soft tissue components. Smooth muscle and keratin stain pink-red, collagen stains blue-green, and elastic fibers appear black. Either phosphotungstic or phosphomolybdic acid is used along with anionic dyes to create a balanced staining solution. Masson’s trichrome stain is frequently employed when the histopathologic differential includes leiomyomatous and neural tumors. The finer characteristics of collagen in the dermis can be better appreciated, as evidenced by the highlighted and well-delineated pattern demonstrated in collagenomas. Perivascular fibrosis, scar formation, and sclerotic lesions are also better appreciated with the use of Masson’s trichrome.

## Congo Red

Detection of amyloid in tissue sections is greatly enhanced and confirmed by positive staining with Congo red. The stain itself is red-pink. Examination of tissue sections suspected of involvement by amyloidosis must be performed under both light microscopy and polarized light. When polarized, amyloid has a characteristic apple green birefringence. The color and tinctorial subtleties observed with Congo red staining are attributed to amyloid’s physical structural arrangement and antiparallel beta-pleated sheets. The elastolytic material in colloid milium and the degenerating keratinocytes in lichen amyloidosis also stain with Congo red. Other stains such as crystal violet, thioflavin T, and sirius red are also capable of staining amyloid but they are far less convenient and are less widely available.

## Fontana-Masson

Silver is often a component of special stains in dermatopathology. Not only is it used in the Verhoff von Gieson elastic tissue stain and the Warthin-Starry spirochete stain described above, silver is a constituent of the Fontana-Masson melanin stain as well. In the latter case, the observer relies on the reduction of silver to form a black precipitate. In the context of traumatized melanocytic lesions or melanocytic entities superimposed upon hemorrhagic processes, the Fontana-Masson can aid in the distinction between melanin and hemosiderin pigments. Similarly, secondary dermal pigment deposits produced by specific drug exposures can be distinguished from those with melanocytic origins. Occasionally, Fontana-Masson is used in the evaluation of vitiligo and post-inflammatory hyperpigmentation. Some observers report that the Fontana-Masson stain is difficult to interpret when only rare granular staining is present (Fig. 4).

### Chloroacetate Esterase

The lineage-specific cytoplasmic granules of myeloid cells are readily identified with the use of chloroacetate esterase. A positive stain is observed as an intense bright red hue. The stain finds its relevance in the investigation of malignant hematopoietic infiltrates in which the lineage of the suspicious cells is unclear. It can help distinguish acute lymphocytic leukemia from acute myeloid leukemia, although up to approximately one-quarter of myeloid cases stain negatively, with false negative findings attributed to excessive immaturity of the granulocytes and significant monocytic expression. Lastly, mast cells also stain positively with chloroacetate esterase.

In summary, special stains are an effective adjunct to routine staining with hematoxylin and eosin in the practice of dermatopathology. While some are easier to interpret than others, most find diagnostic utility in everyday practice. Special stains are economical tools and have rapid turn around times, giving them significant advantages to many more sophisticated ancillary diagnostic techniques.

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# Chapter 24 | Special Stains in Native and Transplant Kidney Biopsy Interpretation

**Licia Laurino, MD**

Renal percutaneous biopsy still represents a useful diagnostic tool. First, it can provide a precise diagnosis, therefore, providing the best rationale for a proper clinical decision making. Secondly, it represents a key indicator of the amount of chronic damage of the kidney allowing an estimation of the chances of survival of the organ. Furthermore, renal biopsy is the only way to report new entities, particularly the adverse effects of drugs, and therefore, is absolutely required in monitoring the acute or chronic impairment of graft excretory function. Finally, in clinical trials kidney biopsy serves as a baseline for evaluating therapeutic efficacy, as well as to compare disease severity among the study groups.

The traditional approach to renal biopsy is to identify the relevant pathologic features in the different compartments (i.e., glomeruli, tubules, interstitium and blood vessels) of the kidney, and to integrate them with those of fluorescence and electron microscopy studies, and most importantly, with clinical information. This approach seems to be very simple; however, there are a few limitations. First, the kidney tends to respond with limited as well as stereotyped morphologic patterns to different insults, and such responses are determined by the type of injury, host susceptibility, and therapeutic interventions. Second, deciding the primary site of injury is one of the major tasks in renal pathology since pathologic changes in one portion of the nephron may cause injury elsewhere. Adaptive changes may modify the normal anatomy leading to secondary injury and some diseases may affect more than one compartment. The third issue is related to the small size of the biopsy. Therefore, evaluation of renal biopsies of native and transplant kidney needs to maximize the efforts in order to provide as much information as possible from such a limited amount of tissue.

The histologic elements of the kidney (glomeruli, tubules, interstitium and blood vessels) are organized into a functional unit, the so called nephron (Fig. 1). A glomerulus consists of a tuft of capillaries with basement membranes lined by endothelial cells and covered by visceral epithelial cells. These capillaries are held together by specialized muscle cells called mesangial cell and are enclosed within a fibrous capsule called Bowman's capsule.

## Changes in glomeruli assessed by light microscopy are:

a. hypercellularity (predominantly or purely mesangial with four or more cells per mesangial area, mesangial and/or endocapillary with obliteration of capillary loops and extracapillary with crescents)

b. glomerular capillary abnormalities (capillary wall collapse, capillary wall thickening and capillary occlusion)

c. minimal light microscopic changes with no lesions and with limited differential diagnosis (such as minimal change, Alport's disease and some others)

## Changes in the tubulointerstitial compartment assessed by light microscopy are:

a. presence of significant interstitial infiltrate and/or presence of alteration of tubules due to degeneration/regeneration of tubular epithelium

b. presence of tubular casts

c. tubular atrophy and interstitial fibrosis

## Changes in the vascular compartment of the kidney can be divided in two categories:

a. those associated with necrosis of the vascular wall

b. those without necrosis; the latter, can be divided into occlusive lesions, with intraluminal thrombi or emboli, and those with wall remodelling due to intimal or medial changes.

Light microscopy remains the gold standard in evaluating kidney biopsies, and for a complete examination, hematoxylin-and-eosin (H&E) and special stains, i.e. Periodic-Acid Schiff stain (PAS), Jones' methenamine silver stain (JMS) and trichrome stain (TS) are routinely employed. H&E and special stains are performed on 2-3 µm thick sections of formalin-fixed paraffin-embedded tissue in order to exhaustively examine the different compartments of the kidney.

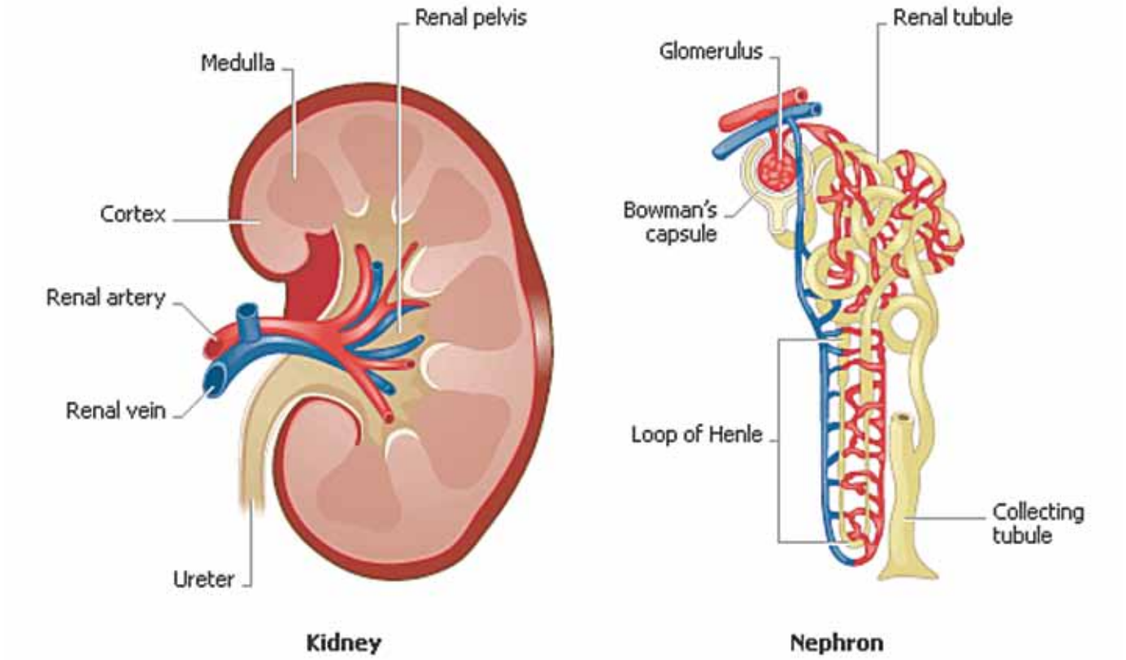



**Figure 1.** Bean-shaped kidney (left) and nephron (right). Approximately one million nephrons compose each bean-shaped kidney. The filtration unit of the nephron is called the glomerulus. Figure Courtesy: MSN (Microsoft) Encarta http://encarta.msn.com/media_461530114_761560819_-1_1/kidney.html.



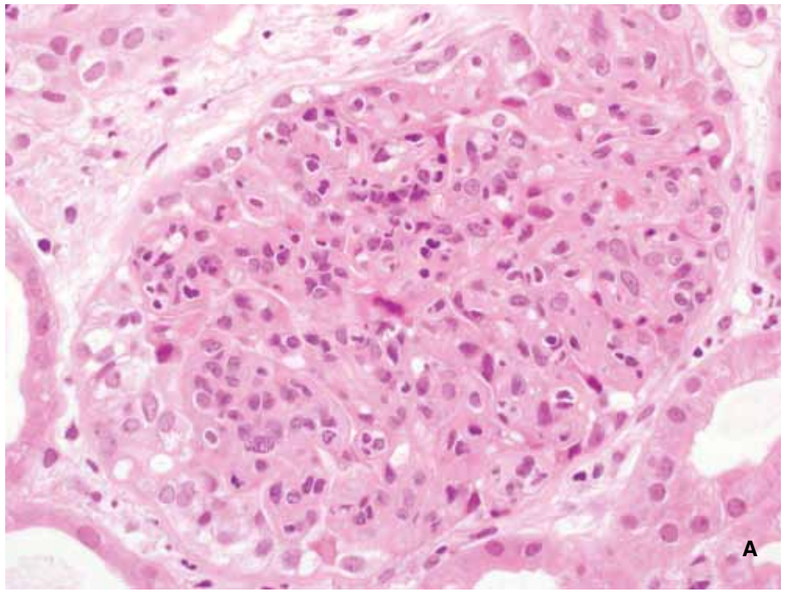


**Hematoxylin-and-Eosin (H&E).** Glomeruli appear falsely hypercellular on H&E since with this stain it is difficult to distinguish between endothelial and mesangial cells; glomerular capillary basement membranes (GBMs) appear slightly thickened too, due to the cytoplasm of glomerular visceral epithelial cells. The H&E is helpful in pointing out the wire loop deposits which appear hypereosinophilic, and the truly pathognomonic hematoxylin bodies which appear purple in lupus nephritis. Karyorrhexis in the form of blue nuclear fragments is also seen in necrotizing glomerulonephritis. Fibrin and platelets thrombi (shade of pink) and fibrinoid necrosis (deep pink) can be easily seen in glomeruli and vessels. Moreover, the H&E is the best stain in assessing interstitial oedema, inflammation, and tubular and epithelial damage (Fig. 2).

**Periodic-Acid Schiff Stain (PAS).** PAS defines very well GBMs and Bowman's capsule, and it is for sure the best stain to evaluate the glomerulus. Diffuse GBMs thickening is seen in membranous glomerulonephritis (due to immunocomplex deposition), in diabetic glomerulopathy and hypertension, and is very well depicted with PAS stain. Wrinkling and folding of GBMs with capillary wall collapse as seen in ischemia and hypertension are again easily seen with this staining. Hyaline deposits are typically PAS positive and JMS negative, whereas sclerosis is PAS and JMS positive. Mesangial matrix increase, and cellularity, endocapillary and extracapillary proliferation can be seen. Large wire loop subendothelial deposits in lupus glomerulonephritis and mesangiolysis are also detected. PAS positive droplets are often found in glomerular visceral epithelial cells and tubular epithelial cells in patients with proteinuria. Proximal tubules are readily identified by their PAS positive brush border. Tubular basement membrane thickening in diabetic nephropathy and tubular atrophy stand out clearly in PAS stained sections as well as Tamm-Horsfall casts in tubules; conversely, cast due to Bence-Jones nephropathy are virtually negative with PAS. The identification of tubulitis, especially in the evaluation of acute cellular rejection of renal allograft, is greatly helped by PAS stain. PAS stain also identifies glomerulitis. In addition, PAS stain is useful in the evaluation of blood vessels to detect diffuse hyaline subendothelial deposition in arteriolosclerosis and diabetes, and hyaline nodular deposition in transplant kidney due to calcineurin inhibitor toxicity (Fig. 3).

**Jones' Methenamine Silver Stain (JMS).** JMS is superior to PAS in the study of the fine structure of GBMs. With this stain it is very easy to demonstrate spikes of GBMs due to immune deposits in membranous glomerulonephritis; spicules in amyloidosis due to amyloid infiltration of peripheral capillary loops; and double contours (with tram-track appearance) due to mesangial interposition in membranoproliferative glomerulonephritis and to widening of subendothelial space and occasional mesangial interposition in chronic transplant glomerulopathy. Breaks in GBMs and Bowman's capsule in cases of crescentic glomerulonephritis are also well-demonstrated with this stain. Tubulitis and mitotic figures are easily seen because of the affinity of silver stain for chromatin. Tubular cast of Tamm-Horsfall protein appear gray to black. Since the internal elastic lamina of arteries is well-visualized with this stain, it is easy to identify vascular wall disruption as it occurs in vasculitic lesions (Fig. 4, 5).

**Trichrome Stain (TS).** The Trichrome stain is particularly helpful in evaluating the amount of fibrous connective tissue (such as deep green staining with Masson trichrome or dark blue staining with chromotrope-aniline blue) in a renal biopsy. These stains distinguish interstitial oedema (which shows a pale colour with reticular appearance) from interstitial fibrosis that stains green or blue. However, early or mild fibrosis may be impossible to detect when oedema is present. Double contours, Bowman's capsule and lobular accentuation of the glomerular tufts are easily evaluated with TS. Glomerular immune deposits may appear as small fuchsinophilic (red) dots on TS in slides examined at high magnification (Fig. 6). If located in the mesangium, these deposits must be distinguished from cytoplasm of mesangial cells. They should also not to be confused with protein droplets included in the cytoplasm of glomerular visceral epithelial cells which can be also fuchsinophilic. Hyaline thrombi in the glomerular capillary lumen are easily seen in cases of crioglobulinemia as well as fibrinoid necrosis which stains bright red orange. Tubular cast of Tamm-Horsefall protein stain pale blue. Amyloid stains light blue, whereas Bence-Jones casts stain fuchsinophilic. TS is also useful to demonstrate fuchsinophilic nodular deposits of hyaline material in the vessels. These deposits can be found either in the media of preglomerular arterioles and in most distal portions of the interlobular arteries in patients treated with calcineurin inhibitors (so-called cyclosporine arteriolopathy) or in



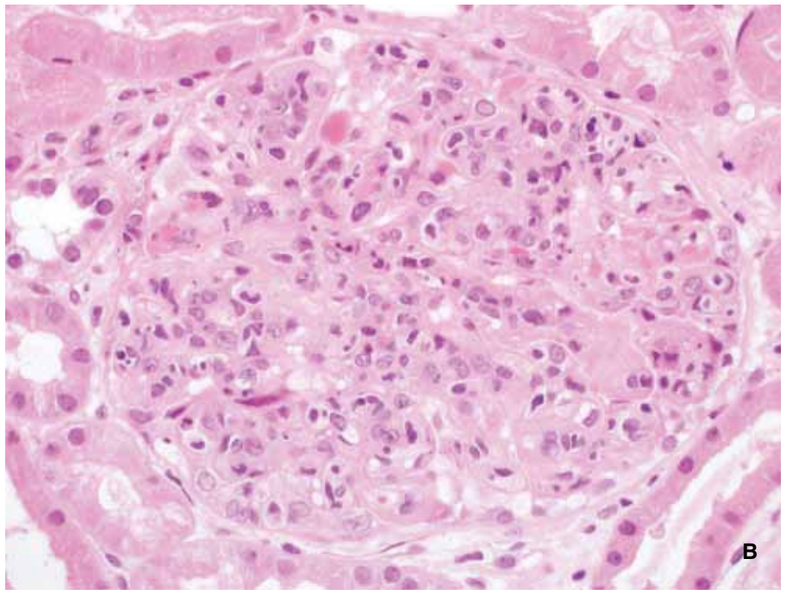




**Figure 2a and 2b.** Hematoxylin & eosin (H&E) stain. Lupus nephrithis class IV: endocapillary proliferation of mesangial and endothelial cells occludes capillary lumens. Infiltrating neutrophils and pyknotic karyorrhectic debris are also present. Few capillary walls have wire-loop thickening caused by suendothelial immune deposits.





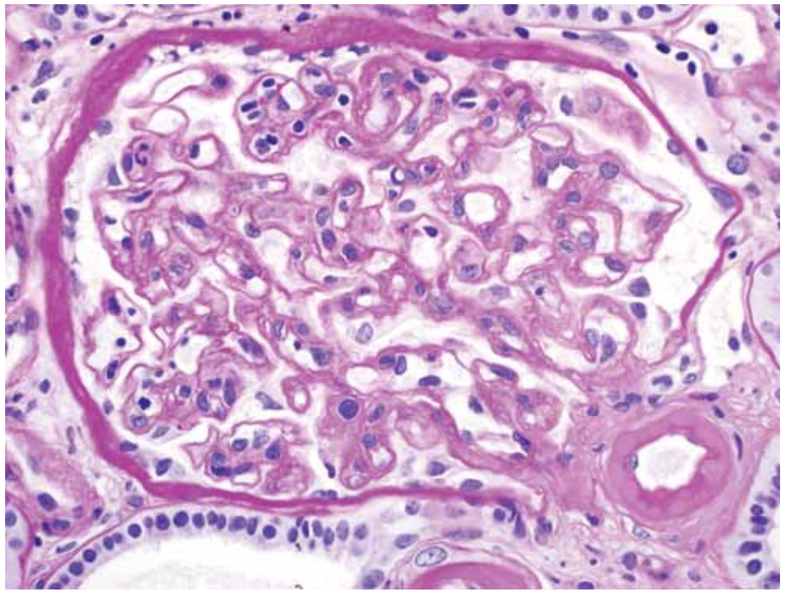


**Figure 3.** PAS stain. Transplant glomerulopathy: double contours, arteriolar hyalinosis, and intracapillary marginated leukocytes.

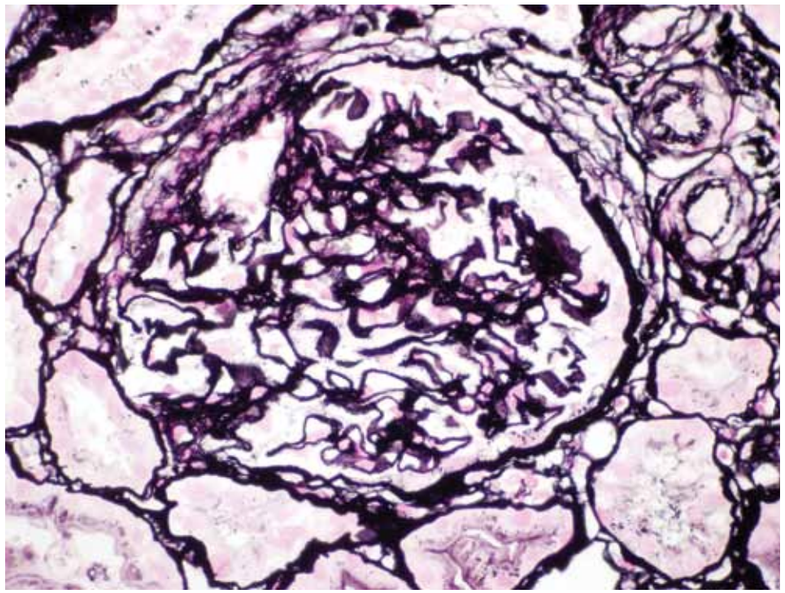


**Figure 4.** Jones' silver stain. Membranous glomerulonephritis: small spike-like projections representing the basement membrane reaction to the subepithelial deposits.

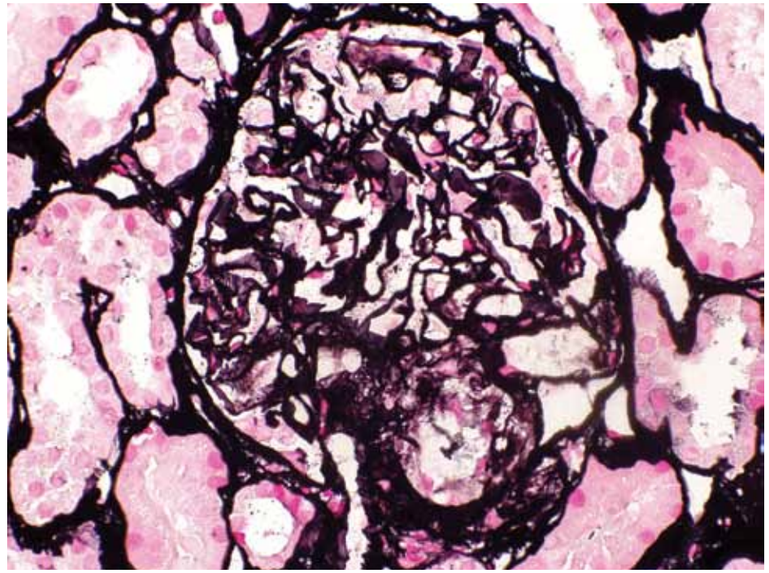


**Figure 5.** Jones' silver stain. Segmental spicules along the peripheral basement membrane.

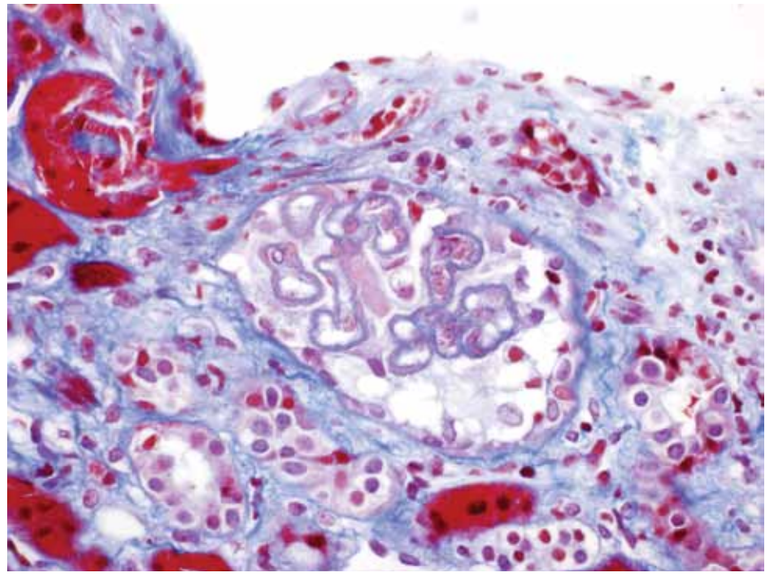


**Figure 6.** Trichrome stain. Membranous glomerulonephritis: subepithelial deposits stain pink-red.





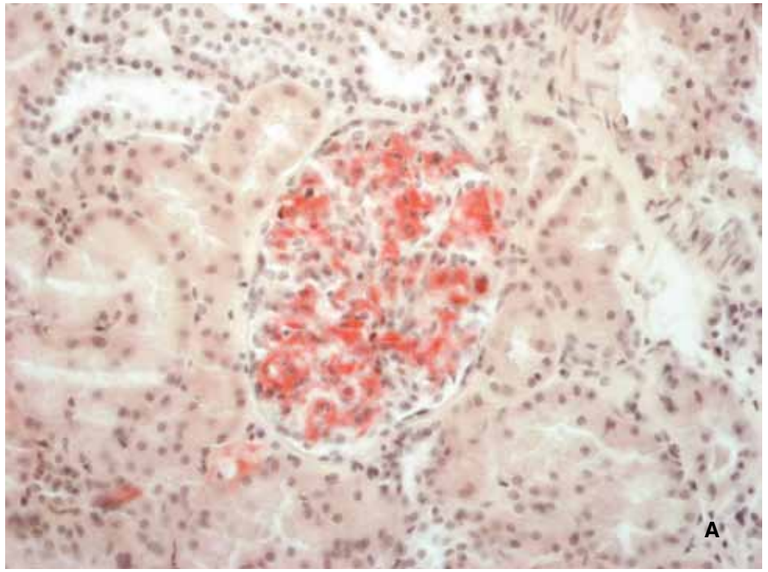



**Figure 7a.** Congo red stain. Amyloidosis: amyloid deposits stain red with Congo red.

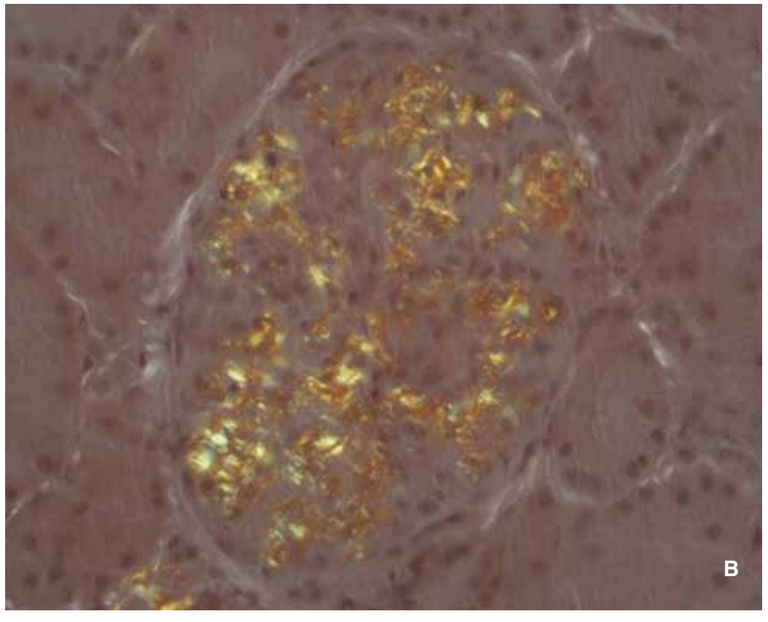



**Figure 7b.** Congo red stain. Amyloidosis: demonstrate the characteristic apple-green birefringence under polarized light (see Appendix, page 284).

the subendothelial location of small vessels, especially arterioles, in patients with hypertensive nephrosclerosis. A quite specific hyalinization of the glomerular efferent arterioles may also be found in diabetic patients.

Additional stains may be required for specific purposes and are employed whenever indicated. Deposits of amyloid can involve glomeruli (typically mesangial nodules), arteries and arterioles as well as tubules and interstitium. Congo red stain, performed on 8 µm thick sections under polarized light shows the peculiar apple-green birefringence diagnostic of amyloid deposition. This stain can also be helpful in the differential diagnosis of mesangial nodules. Mesangial nodules in nodular diabetic glomerulosclerosis (Kimmelstiel-Wilson nodules) or mesangial nodules in light chain deposition disease are not birefringent with Congo red (Fig. 7).

Finally, Lendrum stain differentiates fibrin thrombi from hyaline thrombi, Prussian blue demonstrates iron deposits, usually in tubular epithelial cells, and elastic stains highlight blood vessels. Stains to detect microorganisms when infection is suspected are: Acid-Fast stain for mycobacteria, and Grocott's methenamine stain (GMS) for detecting fungi.

To summarise, medical kidney is traditionally known to be one of the major fields of special stains application. In order to take best advantage, all the above mentioned stains should be applied on renal biopsy since each of them gives complementary information. In limited cases, special stains can substitute the information given by IF and EM when these two techniques are not available (for example, in the documentation of the characteristic spikes in membranous glomerulonephritis and the spicules in amyloid deposition). Automation contributes to an increase in the reproducibility of the staining.



# Chapter 25 | Urine Cytologic Analysis: Special Techniques for Bladder Cancer Detection

**Anirban P. Mitra, MD, PhD**

## Introduction

Carcinoma of the urinary bladder is a common cancer with high incidence and prevalence. With over 70,000 new cases estimated in 2009, bladder cancer has the fifth highest incidence of all cancer types in USA (1). According to the Surveillance, Epidemiology and End Results database, bladder cancer is the third most prevalent malignancy in men (2).

Based on clinical behavior, two types of diseases can be differentiated: a more aggressive and a less aggressive type. The depth of tumor infiltration and tumor grade reflect this biological behavior and recent molecular findings support divergent underlying molecular alterations (3). At initial presentation, about 75% cases are superficial (Ta and T1) while 25% cases invade the detrusor muscle (4) (Fig. 1). Superficial tumors are characterized by a high recurrence rate (50%-70%) within five years and a relatively low progression rate (approximately 10%), which results in relatively long survival (5-7). Tumors that do invade the muscularis propria have a far worse prognosis. A recent series reported a five-year recurrence-free survival rate of only 69% in all patients, and 39% in patients with regional lymph node metastasis (8).

The main challenge to formulating an optimal management strategy for newly diagnosed bladder cancer is to accurately and promptly identify patients whose disease is amenable to conservative management (i.e., low-risk) from those who require radical therapy (i.e., high-risk). Whereas intravesical therapy can reduce recurrence rates, it has limited benefit on stage progression or disease-specific survival. Unfortunately, bladder-sparing salvage therapy is often ineffective. Delaying cystectomy to preserve the bladder in patients with high-risk tumors can threaten survival.

The chronic nature of bladder cancer necessitates long-term surveillance in patients after initial diagnosis. Typically, most recurrences occur within the first five years, but late recurrences can occur at any time, making lifetime active surveillance standard practice. If the bladder is not removed, active surveillance, including sequential cystoscopies and urine cytologies must be performed every three-six months for three years and at least yearly thereafter. This makes a disproportionate impact on our healthcare system. The prevalence of bladder cancer in USA approximates 500,000 people and most still have intact bladders, but the estimated annual expenditures for this disease ($2.2 billion) notably exceed those for prostate cancer ($1.4 billion), a cancer that is nearly four times more common than bladder cancer (9). Moreover, repeated cystoscopies, which are currently considered the gold standard for bladder cancer detection, can have a significant impact on patient morbidity and quality of life.

Detection of bladder cancer using morphologic molecular tests could improve patient management in two ways. It would allow diagnosing tumors of the aggressive phenotype earlier and thus improve the prognosis of patients. In cases of less aggressive tumors, it could identify early disease onset and recurrences, thereby potentially reducing the need for expensive cystoscopic monitoring and surveillance procedures.

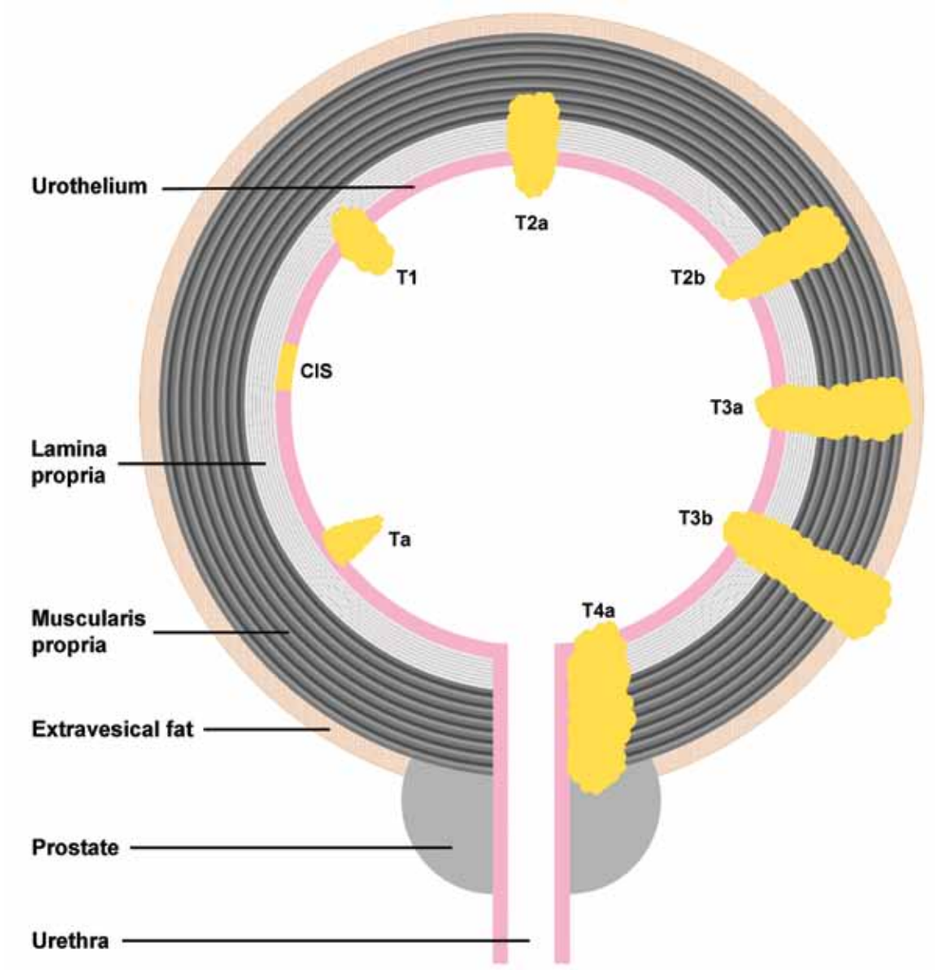



**Figure 1.** Tumor staging for bladder cancer. Ta and carcinoma in situ (CIS) represent forms of noninvasive bladder cancer. While Ta generally has a papillary appearance, CIS is a flat and aggressive lesion with a tendency to eventually invade the basement membrane. Invasive tumors (T1-4) have a poorer prognosis. T1 tumors are confined to the lamina propria, while T2 tumors invade to different depths of the muscularis propria. T3a and T3b tumors show microscopic and macroscopic invasion of the extravesical fat, respectively. T4a tumors invade adjacent organs (such as the prostate), while T4b tumors (not shown) invade the pelvic and abdominal walls.



Urine Cytologic Analysis: Special Techniques for Bladder Cancer Detection

## Urine Cytology

The urinary bladder serves as a reservoir for urine. Since few other organs are exposed to urine, this attribute makes it much more specific for identification of markers for urologic cancers than blood. The collection of voided urine to look for exfoliated tumor cells is the basis for cytologic examination and various molecular assays. This also represents a noninvasive detection method.

Cytology of voided urine or bladder washes is the most established noninvasive method in the work-up of hematuria (blood in the urine; the most common presentation of bladder cancer) and follow-up in patients with a history of bladder cancer and is used as an adjunct to cystoscopy. This involves microscopic identification of exfoliated tumor cells based on cytologic criteria (see Appendix). Briefly, exfoliated tumor cells obtained as a sediment after centrifugation of a midstream voided urine sample are fixed and stained using the Papanicolaou procedure (Fig. 2).

The method has a high specificity but relatively low sensitivity, particularly in well-differentiated bladder tumors (10). A meta-analysis that included data on 18 published series with 1,255 patients reported a sensitivity of 34% and specificity of 99% (95% confidence interval 20%-53% and 83%-99.7%, respectively) (11). Several factors contribute to this poor ability of urine cytology to detect cancer cells: only a small sample of urine can be processed and only a fraction of the sample can be used for final analysis which reduces the chance of capturing tumor cells. Background cells such as erythrocytes and leukocytes also confound the cytologic technique (12). Furthermore, cytologic criteria that differentiate between low grade tumors and reactive cells can be ambiguous. As the incidence of bladder cancer is even lower in asymptomatic patients, the low sensitivity of urine cytology limits its use as a detection tool.

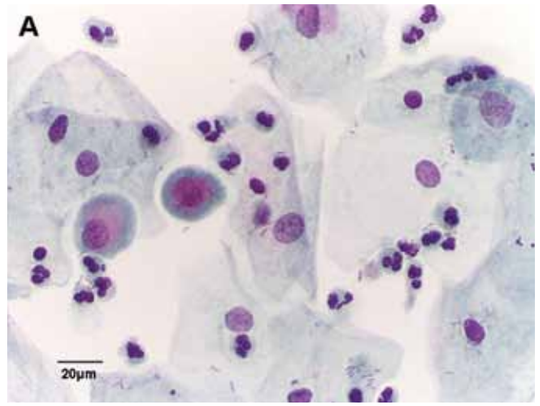
## Application of Molecular Tools in Urine Cytology

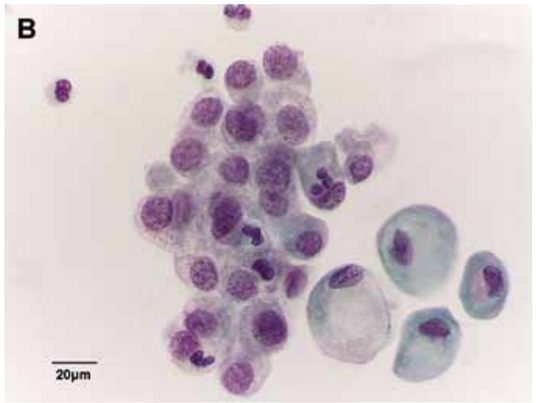
The advent of molecular technologies has enabled the detection of novel molecular markers on exfoliated cells in voided urine. These approaches harness the high specificity of urine cytology and its advantage as a noninvasive procedure. The markers are tumor-specific, which allows more focused detection of molecular alterations while avoiding the confounding effects of background erythrocytes and inflammatory cells.

## ImmunoCyt

ImmunoCyt (DiagnoCure, Inc., Quebec, Canada; marketed as uCyt+ in Europe) is based on immunocytofluorescence and uses a cocktail of three fluorescently labeled monoclonal antibodies to detect cellular markers of bladder cancer using exfoliated cells from voided urine. The manufacturer does not recommend the use of bladder washings for this test. Antibody 19A211 labeled with Texas Red detects a glycosylated form of carcinoembryonic antigen. Fluorescein-labeled antibodies M344 and LDQ10 detect mucin glycoproteins that are expressed on most bladder cancer cells but not on normal cells. The test requires urine fixation with ethanol or isopropyl alcohol before shipment to a reference cytopathology laboratory. A minimum evaluation of 500 epithelial cells is required, and presence of one fluorescent cell constitutes a positive test. ImmunoCyt is approved by the United States Food and Drug Administration (USFDA) as an aid in bladder cancer management in conjunction with urine cytology and cystoscopy.

The median test sensitivity and specificity are 81% and 75%, respectively (Fig. 3, Table 1). Interestingly, some studies have noted that including ImmunoCyt in the standard urine cytology protocol offers increased sensitivity (around 86%-90%) without significant loss of specificity (13). However, the presence of microhematuria, cystitis and benign prostatic hyperplasia can lead to false-positive results (14, 15). A steep learning curve, inter-observer variability, need for constant quality control, and relatively high test failure rate due to inadequate specimen cellularity are also compounding problems that need to be overcome to improve the general acceptance of this test.



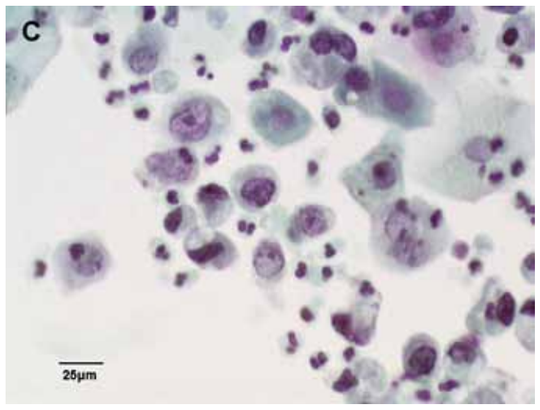




**Figure 2.** Urine cytology stained by the Papanicolaou procedure. (A) Normal cytology with no mitotic activity and normal nuclear-to-cytoplasmic ratio. (B) Urothelial cells showing slight atypia with increased nuclear-to-cytoplasmic ratio. (C) Severe urothelial atypia that is characteristic of bladder cancer, with varying cell size, increased nuclear-to-cytoplasmic ratio and an abnormal chromatin pattern. In all cases, note the presence of inflammatory cells in the field that can potentially interfere with the analysis. Slides at 400x magnification. Images courtesy of Alessandro Brollo (Hospital of Monfalcone, Italy).





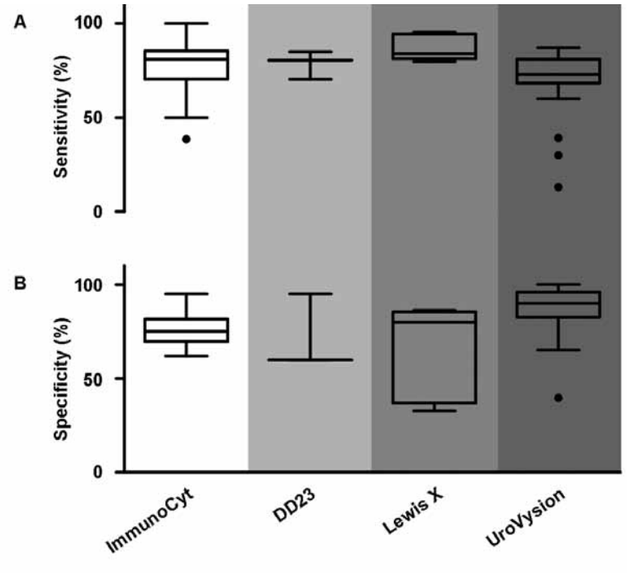



**Figure 3.** Performance metrics of major molecular cytologic tests for bladder cancer detection. Tukey boxplots show the median and range of (A) sensitivities and (B) specificities for ImmunoCyt, DD23, Lewis X antigen and UroVysion. The boxes represent median with interquartile range; whiskers go 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter. Dots represent outliers.

**Table 1.** Characteristics of several molecular cytologic tests for bladder cancer detection.

| Test | Principle | Detection | |
|---|---|---|---|
| | | Median sensitivity (range) | Median specificity (range) |
| ImmunoCyt | Immunocytofluorescence; detects a glycosylated form of carcinoembryonic antigen and mucin glycoproteins | 81% (39%-100%) | 75% (62%-95%) |
| DD23 | Immunocytochemistry; detects a tumor-associated protein dimer | 81% (70%-85%) | 60% (60%-95%) |
| Lewis X antigen | Immunocytochemistry; detects Lewis X antigen | 84% (80%-96%) | 80% (33%-86%) |
| UroVysion | Multitarget, multicolor fluorescent in situ hybridization assay | 73% (13%-87%) | 90% (40%-100%) |

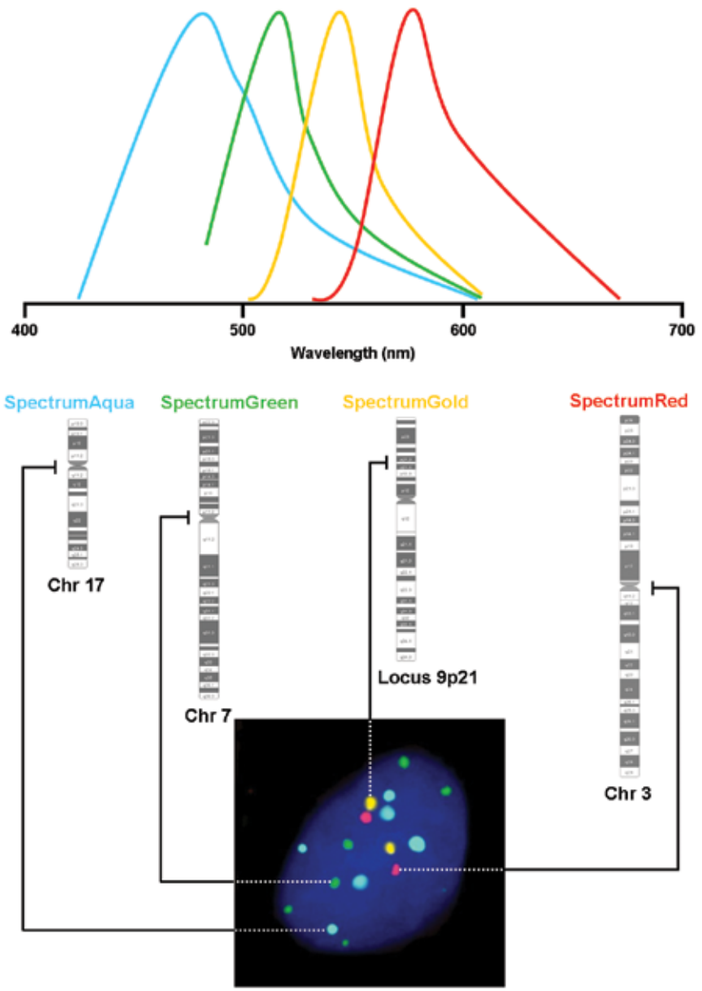



**Figure 4.** Principle of the UroVysion test. Against a DAPI nuclear counterstain, the test assays aneuploidy for chromosomes 3, 7, 17, and loss of 9p21 locus as shown. The visualized cell is abnormal as the nucleus shows a gain of two chromosomes (aneuploid for chromosomes 7 and 17). Note that wavelengths of the filter sets shown are approximate, and proprietary to Abbott Molecular, Inc.





## DD23

DD23 is a monoclonal antibody that detects a protein dimer expressed on bladder tumor cells. It has been integrated into an alkaline phosphatase-conjugated immunocytochemical assay for exfoliated tumor cells in the urine (UroCor Labs, Oklahoma City, OK). Few studies have evaluated this test, but initial reports suggest that the marker has high sensitivity (70%-85%) and modest specificity (around 60%) (Fig. 2, Table 1) (16, 17). The sensitivity also increases slightly when DD23 is used in combination with cytology (78%-85%). The marker is reasonably sensitive in detecting both low grade (55%-72%) and high grade (76%-87%) tumors.

## Lewis X Antigen

Glycoproteins are proteins that contain oligosaccharide chains (glycans) covalently attached to their polypeptide, and are important structural components of cells and also act as antigens. The most commonly studied glycoprotein antigen in the context of bladder cancer is the blood antigen, stage-specific embryonic antigen 1 or the sialyl Lewis X (commonly referred to as Lewis X). The Lewis X determinant is a tumor-associated antigen in the urothelium and is visualized in exfoliated tumor cells in urine by an immunocytochemical assay using anti-Lewis X monoclonal antibody (18). At least 100 cells are required for evaluation, and slides showing more than 5% positive cells for Lewis X are considered positive.

The median sensitivity and specificity of the test are 84% and 80%, respectively (Fig. 3, Table 1). It is reported that Lewis X immunocytology of two consecutive voided urine samples instead of one can increase the sensitivity of the test by almost 15% (19, 20). The sensitivity of the antibody also increases with increasing tumor stage and grade (21). More recent reports have indicated that the test has higher sensitivity (as high as 95%) than other molecular tests such as BTA stat, NMP22 and UroVysion, although the specificity was the lowest (range, 33%-37%) (22, 23). However, expression of Lewis X antigen by benign umbrella cells of the normal urothelium that may be found in the urine can interfere with the assay results (18, 24).

## UroVysion

Bladder cancers frequently exhibit well-defined chromosomal alterations. For example, loss of heterozygosity on chromosomes 9 and 17 are well documented in different stages of bladder tumor development, and deletion of the 9p21 locus that encodes for the p16 protein is a common early event in bladder tumorigenesis (25). Chromosomal abnormalities can be detected by fluorescent in situ hybridization (FISH) assays, wherein hybridization of fluorescently labeled DNA probes to chromosome centromeres or loci of exfoliated cells from urine sediments can be detected under a fluorescent microscope. The UroVysion test (Abbott Molecular, Inc., Des Plaines, IL) is a multitarget, multicolor FISH assay using pericentromeric fluorescent enumeration probes that are designed to detect aneuploidy for chromosomes 3 (SpectrumRed), 7 (SpectrumGreen), 17 (SpectrumAqua), and loss of the 9p21 locus using a locus specific identifier (SpectrumGold) (Fig. 4). The criteria for detecting bladder cancer by UroVysion are: >5 cells with a gain of >2 chromosomes, or >10 cells with a gain of 1 chromosome, or >20% cells with a loss of 9p21 locus. However, many studies have employed variations in these criteria to determine positivity. UroVysion is USFDA approved as an aid for initial diagnosis of bladder cancer in patients with hematuria and suspicion of disease, and in conjunction with cytoscopy to monitor bladder cancer recurrence.

The median sensitivity and specificity of UroVysion for bladder cancer detection are 73% and 90%, respectively (Fig. 3, Table 1). Better performance has been reported in identification of carcinoma in situ and high grade tumors (26). Another potential advantage is the apparent ability of the test in detecting occult tumors that are not initially visible on cystoscopy. Chromosomal abnormalities detected in exfoliated cells in urine of patients under surveillance have preceded cystoscopically identifiable bladder tumors by 0.25 to 1 year in 41%-89% patients (13).

## Automation: The Future of Cytologic Urinanalysis

### Improvements in Sample Preparation

Efforts are underway to design faster, inexpensive and more sensitive point-of-care sample collection and preparation techniques that will significantly improve platforms on which routine and specialized urine cytopathologic tests are performed. The recent fabrication of a parylene membrane microfilter device that can capture circulating tumor cells from the blood of cancer patients by employing the principle of inherent differences in tumor cell size compared to normal blood cells (27) is also being evaluated for capture and characterization of exfoliated urothelial tumor cells from patient urine. The unique and precise engineering of the microfilter device allows enrichment of exfoliated tumor cells on a small surface area while eliminating background erythrocytes and leukocytes that typically confound routine urine cytologic specimens. This platform has shown promise in bladder cancer surveillance, and it is conceivable that this can also be used for on-site bladder cancer detection protocols in the future.

### Automated Image Cytometry

Abnormalities in DNA ploidy and nuclear shape are characteristic of tumor cells. While cytologists rely on identification of these changes in urine specimens, automated image analysis now allows identification of these changes on a more objective scale. The Quanticyt system employs image analysis to identify DNA ploidy abnormalities and nuclear morphometry (28). The karyometric system is based on two nuclear features: the 2c-deviation index (2cDI) and the mean of a nuclear shape feature, MPASS. Samples are scored as low-, intermediate-, or high-risk that has been determined by correlation with histology data. A study has shown that prediction of a cystoscopic lesion by routine cytology and Quanticyt was comparable, and the latter was superior for predicting tumor recurrence after normal findings at cystoscopy (29). Another group has employed artificial intelligence to develop a neural network-based digitized cell image diagnosis system that identifies potentially abnormal cells in bladder washes by algorithmic analysis (30). The selected digitized cell images are reanalyzed by two independent neural networks; one is trained to select single cancer cells and the other one cancer cell clusters, regardless of cell shape, overlapping, or staining. The selected cells or cell clusters are displayed on a high-resolution computer screen for evaluation by a cytopathologist.

The Quanticyt system has shown a sensitivity of 59%-69%, and specificity of 72.5%-93% in detecting bladder cancer (31, 32). It has also been suggested that employment of Quanticyt to analyze consecutive bladder washes can increase the detection rate of invasive disease in samples that are labeled high-risk (33). In a pilot study, the neural network-based diagnosis system showed 92% sensitivity in diagnosing histologically confirmed tumors, as opposed to 69% for Quanticyt and 50% for routine cytology (30). While the major advantage of these cytometric analyses is label-free quantification that closely mimics the routine cytology procedure, a drawback is the need for a bladder wash sample instead of voided urine.



Urine Cytologic Analysis: Special Techniques for Bladder Cancer Detection

## Conclusion

Cystoscopy, the current gold standard for bladder cancer detection, exhibits high sensitivity and specificity but is not an ideal method for initial diagnosis due to its invasive nature. Urine cytology is an established method and there is sufficient evidence to show its high specificity. However the method suffers from low sensitivity. There are several noninvasive, urine-based molecular tests, some of which are USFDA approved, for the detection and follow-up of bladder cancer patients. These tests employ the high specificity of urine cytology, and use specific molecular determinants to identify cells with subcellular abnormalities that are characteristic for bladder cancer. Future detection and follow-up strategies will include the use of molecular diagnostic tests in combination with automated technologies for sample preparation and diagnosis for robust detection of bladder cancer presence or recurrence.

## Appendix

### Urine Cytology – Procedural Overview

Urine cytology is generally performed by the Papanicolaou procedure. The specimen utilized could be voided urine or a bladder wash. Catheterized specimens need to be labeled as such, as cellular findings may be altered by instrumentation. Voided urine is the specimen of choice in males, while catheterized specimens may be used in females. 24 hour collections are not recommended due to cytologic degradation.

For voided samples, a midstream collection into a clean container is best. No fixatives are required. However, an equal amount of 50% ethanol may be added as preservative and the specimen may be refrigerated if delay in delivery to the reference laboratory is anticipated. Laboratory specimen preparation methods vary and may include cytocentrifugation, membrane filter preparations and monolayer preparations. Cytocentrifugation is the most basic method where aliquots of urine are centrifuged to concentrate the cells, as urine normally contains few cells. In the ThinPrep technique (Hologic, Bedford, MA), cells are collected on a filter during processing, and then transferred to a glass slide before staining. The slide containing the cytospecimen is stained by the Papanicolaou method and examined under a light microscope. By this method, the cytoplasm of transitional cells stains greenish-blue and the nuclei are purple. The ThinPrep technique provides the added advantage of a clearer background, with the cells of interest concentrated and confined within a demarcated area of the slide.

The procedure is used for detection of inflammatory lesions including specific infections, urinary crystal and calculi disease, iatrogenic changes and neoplasms of the urinary tract. Accompanying clinical information is essential as instrumentation and presence of urinary calculi may result in cytologic changes that mimic malignancy. If malignancy is indeed present, the degree of cellular atypia and other cytologic features are noted. Interpretation of low grade urothelial neoplasms may mimic benign disease due to minimal nuclear abnormalities.

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# Chapter 26 | Renal Neoplasms: An Update on Immunohistochemical and Histochemical Features

**Stephen M. Bonsib, MD and Ami Bhalodia, MD**

## Renal Cell Carcinomas

The final two decades of the 20th century witnessed a major evolution in our concept of renal cell carcinomas. The extensive histological variability long noted in renal cell carcinoma (RCC) was shown to reflect the existence of a large number of distinct entities having differing prognoses and often harboring unique cytogenetic abnormalities (1-3). Advancement in understanding the molecular misadventures that result from these genetic abnormalities spurned development of promising targeted therapies for a disease in which surgical approaches were previously the sole option (4, 5).

The emerging therapeutic possibilities have raised the bar for pathologists mandating accurate classification in an increasingly complex arena. Although, histological assessment remains the mainstay of RCC classification, there are immunohistochemical (IH) and histochemical tools that serve as adjuncts to the histologic analysis. However, cautious interpretation of IH data is recommended since experience in the less common and most recently described tumors may be limited. In this brief review we discuss the use of ancillary immunohistochemistry in RCC classification but admit that space precludes fully addressing the myriad of diagnostic permutations that may arise.

## Clear Cell Renal Cell Carcinoma

Clear cell RCC is overwhelmingly the most common type of renal cancer accounting for 70% of cases (1, 6). The World Health Organization defines CC-RCC as “a malignant neoplasm composed of cells with clear or eosinophilic cytoplasm within a delicate vascular network” (6). As this definition implies, cytoplasmic clearing may not be present in every cell, or even in every neoplasm. Other types of RCC may have optically clear cytoplasm and appear indistinguishable. Granular cell, or eosinophilic variant of CC-RCC, a term historically applied to tumors with few or no clear cells, is terminology no longer acceptable since several RCC types are characterized by cells having eosinophilic cytoplasm. Since CC-RCC is so common and histologically diverse, most differential diagnoses are driven by comparative features relative to this tumor, a strategy employed below.

## Histochemistry of RCCs

The cytoplasmic clearing in CC-RCC results from extraction of abundant intracellular glycogen and lipid by organic solvents during tissue processing (Fig. 1). However, sufficient residual glycogen remains after processing that a PAS stain without diastase is usually positive. Demonstration of lipid by an Oil Red O stain is easily accomplished but requires frozen tissue. Although glycogen and lipid are characteristic findings in CC-RCC, these histochemical stains offer little differential diagnostic power in RCC classification since tumors other than CC-RCC may also harbor appreciable lipid and/or glycogen.

The single differentiating histochemical stain useful in RCC classification is the Hale’s colloidal iron (CI) stain (7, 8). The CI stain demonstrates the presence of abundant acid mucosubstances in chromophobe cell (Ch) RCC which are absent in other types of RCC (Fig. 2). The acid mucosubstances are located within complex cytoplasmic vesicles demonstrable by electron microscopy, a unique feature of this tumor. The CI is often essential to confirm the diagnosis in Ch-RCC because similar to CC-RCC, Ch-RCC shows histological extremes. Some tumors are composed of cells with somewhat clear, so-called transparent cytoplasm, eliciting the differential diagnosis of CC-RCC. Other Ch-RCCs are composed entirely of cells with eosinophilic cytoplasm, the so-called eosinophilic variant, which elicits the differential of a benign oncocytoma or a CC-RCC with eosinophilic cells.

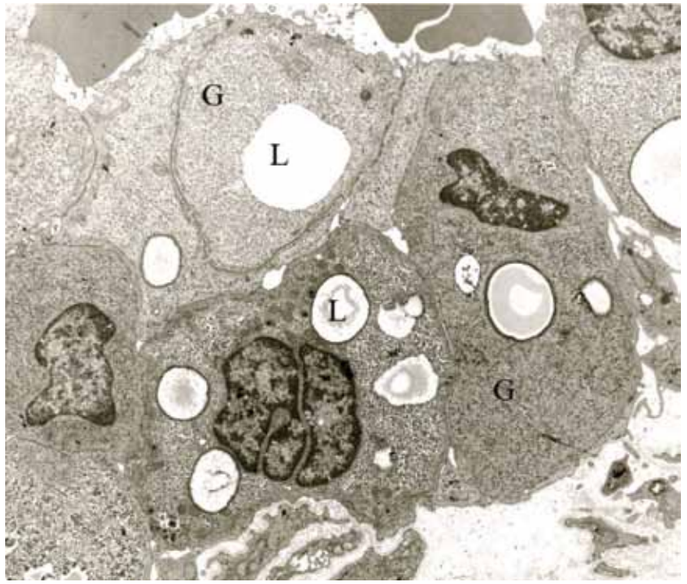


**Figure 1.** Electron microscopy of a clear cell renal cell carcinoma showing abundant lipid and glycogen. (L- lipid, G- glycogen).

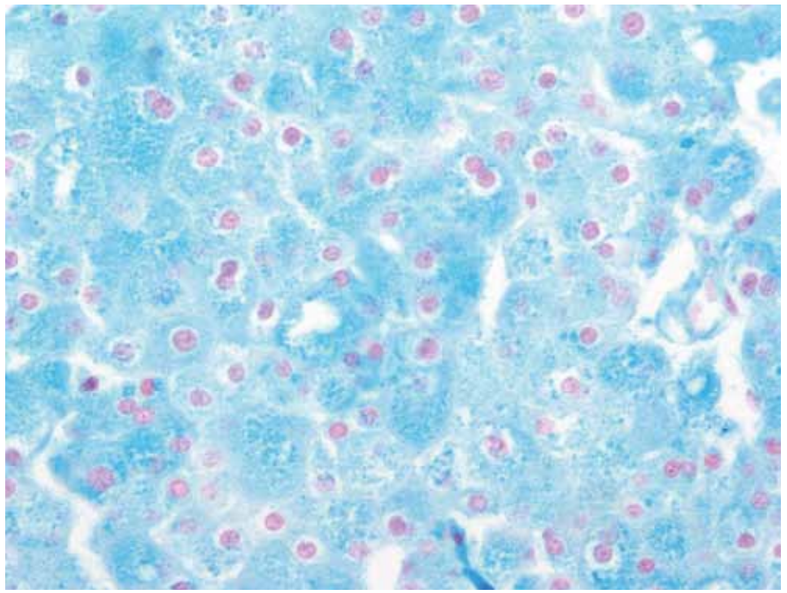

**Figure 2.** Colloidal iron stain of a chromophobe cell renal cell carcinoma demonstrating intense cytoplasmic staining.





**Table 1.**

| Clear Cell Carcinomas with Defining Gross, Architectural and Additional Features | | |
|---|---|---|
| Clear Cell Tumor | Gross or Architectural Features | Additional |
| von Hippel-Lindau disease | +/- Clear cell lined cysts | Extra-renal neoplastic disease |
| Tuberous sclerosis complex | +/- Polycystic kidney disease | Angiomyolipomas<br>Extrarenal disease |
| Constitutional chromosome 3 syndrome | Multiple and/or bilateral CC-RCC | Family history of CC-RCC |
| Multilocular cystic RCC | Consists entirely of cyst and septae | Renal limited<br>pT1 or pT2 |
| Clear cell papillary RCC | Papillae formation | Often ESKD |

## Immunohistochemical Differential Diagnosis of RCCs with Cytoplasmic Clearing

Although most cases of CC-RCC represent sporadic disease, identical neoplasms may arise in several hereditary and cystic diseases and two tumors are defined by their unique gross or architectural features (Table 1). Family history and presence of extra-renal findings are crucial to establishing the correct diagnosis in these various scenarios because the CC-RCCs that develop may show histological and immunohistochemical identity to sporadic cases.

The most common genetic cystic disease with CC-RCC is von Hippel-Lindau disease (9, 10). In this autosomal recessive syndrome CC-RCC is accompanied by clear cell-lined cysts and extrarenal neoplastic disease. CC-RCC also rarely arises in tuberous sclerosis complex (TSC) especially when associated with polycystic kidney disease, known as the contiguous gene syndrome (11, 12). Finally, there is a very rare non-cystic genetic disease known as constitutional chromosome 3 syndrome, an autosomal dominant syndrome defined by the presence of single or multiple, unilateral or bilateral CC-RCC in patients with a balanced chromosome 3 translocation (13, 14). Only seven families have been identified, each with a different translocation.

The multilocular cystic renal cell carcinoma, a relatively common (3-5% of RCCs) RCC and defined by its gross, is a tumor that consists entirely of clear cell lined cysts and cyst septae without solid nodules of tumor cells (15). Clear cell papillary RCC, a recently recognized type of RCC originally identified in patients with end stage kidney disease and acquired cystic kidney disease, is now recognized to also develop in native kidneys. This tumor contains clear cells arranged along papillary fronds with basally oriented nuclei (16-18). The papillary architecture is definitional of this entity; conversely true papillary architecture is an exclusionary criterion for CC-RCC.

Many entities with clear and/or eosinophilic cells enter into the differential of RCCs as noted in Tables 2 and 3. The laundry list of antigens provided includes both common and uncommonly employed immunohistochemical analytes. The scope of the differential is smaller with clear cell tumors compared to those with eosinophilic cytoplasm. It includes the two other most common RCCs, papillary RCC, especially when solid, Ch-RCC, translocation carcinomas and urothelial carcinoma which occasionally shows extensive cytoplasmic clearing.

**Table 2.**

| Immunohistochemical and Histochemical Evaluation of Renal Tumors with Eosinophilic Cytoplasm | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Antibody | Oncocytoma | CC-RCC, Eosin Cytoplasm | Chromophobe Carcinoma, Eosinophilic Variant | Epithelioid Angiomyolipoma | Papillary Renal Cell Carcinoma (Pap RCC), Type 2 | Translocation Carcinoma t(6:11) t(6;11) | Collecting Duct Carcinoma | Medullary Carcinoma | Tubulocystic Carcinoma | Tubular Mucinous Spindle Cell Carcinoma |
| CAM 5.2 | pos | pos | pos | neg | pos | neg | pos | pos | pos | pos |
| AE1/AE3 | pos | pos | pos | neg | pos | neg | pos | pos | pos | pos |
| Vimentin | neg | pos | neg | neg | pos | pos | pos | pos | - | pos |
| EMA | pos | pos | pos | neg | pos | neg | pos | pos | - | pos |
| CD10 | pos/neg | pos | pos/neg | neg | pos | pos | pos/neg | - | pos | pos/neg |
| RCC Ma | neg | pos | neg | neg | pos/neg | - | - | - | - | neg |
| CK7 | “patchy” | neg | pos | neg | pos | neg | pos | pos | pos | pos |
| CK20 | neg | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| CK19 | neg | neg | neg | neg | neg | pos/neg | pos | pos | pos/neg | pos |
| 34BE12 | neg | neg | neg | neg | pos/neg | neg | pos/neg | neg | pos/neg | pos/neg |
| AMACR | neg | neg | neg | - | pos | pos | neg | - | pos | pos |
| Parvalbumin | pos | neg | pos | - | neg | - | - | - | pos | - |
| S100A1 | pos | pos | neg | - | pos | - | - | - | - | - |
| C-kit | pos | neg | pos | neg | pos/neg | - | pos/neg | - | - | neg |
| E-cadherin | pos | neg | pos | - | pos/neg | pos | pos | - | - | - |
| Kid sp cad | pos | neg | pos | - | neg | neg | neg | neg | neg | neg |
| Caveolin-1 | neg | - | pos | - | - | - | - | - | - | - |
| Cathepsin K | neg | neg | neg | - | neg | pos | - | - | - | - |
| PAX-2 | pos | pos | neg | - | pos/neg | neg | neg | - | pos | pos/neg |
| TFE3 | neg | neg | pos | - | neg | pos | - | - | - | - |
| TFEB | neg | neg | - | - | neg | neg | - | - | - | - |
| UEA-1 | neg | neg | - | - | pos/neg | - | pos | - | pos | - |
| HMB 45 | neg | neg | neg | pos | neg | neg | neg | neg | neg | neg |
| Melan A | neg | neg | neg | pos | neg | neg | neg | neg | neg | neg |
| Coll iron | neg | neg | pos | - | neg | neg | neg | neg | neg | neg |



Renal Neoplasms: An Update on Immunohistochemical and Histochemical Features

**Table 3.**

| Immunohistochemical and Histochemical Evaluation of Renal Tumors with Clear Cytoplasm | | | | | |
|---|---|---|---|---|---|
| Antibody | Clear Cell RCC | Papillary RCC, Type 1 | Chromophobe RCC | Translocation Carcinoma | Urothelial Carcinoma |
| CAM 5.2 | pos | pos | pos | neg | pos |
| AE1/AE3 | pos | pos | pos | neg | pos |
| Vimentin | pos | pos | neg | pos | pos |
| EMA | pos | pos | pos | neg | pos |
| CD10 | pos | pos | pos/neg | pos | pos |
| RCC Ma | pos | pos | neg | pos | pos |
| CK7 | neg | pos | pos | neg | pos |
| CK20 | neg | neg | neg | neg | neg |
| CK19 | neg | neg | - | neg | neg |
| 34BE12 | neg | neg | neg | neg | pos |
| Parvalbumin | neg | neg | pos | - | - |
| S100A1 | pos | pos | neg | - | - |
| C-kit | neg | neg | pos | neg | neg |
| AMACR | neg | pos | neg | pos | neg |
| E-cadherin | neg | - | pos | pos/neg | neg |
| Kid sp cad | neg | neg | pos | neg | - |
| Cathepsin K | neg | neg | neg | pos | - |
| PAX-2 | pos | pos | neg | neg | neg |
| TFE3 | neg | neg | neg | pos | neg |
| TFEB | neg | neg | neg | neg | neg |
| HMB 45 | neg | neg | neg | neg | neg |
| Melan A | neg | neg | neg | neg | neg |
| Coll iron | neg | neg | pos | neg | neg |
| P63 | neg | neg | neg | - | pos |

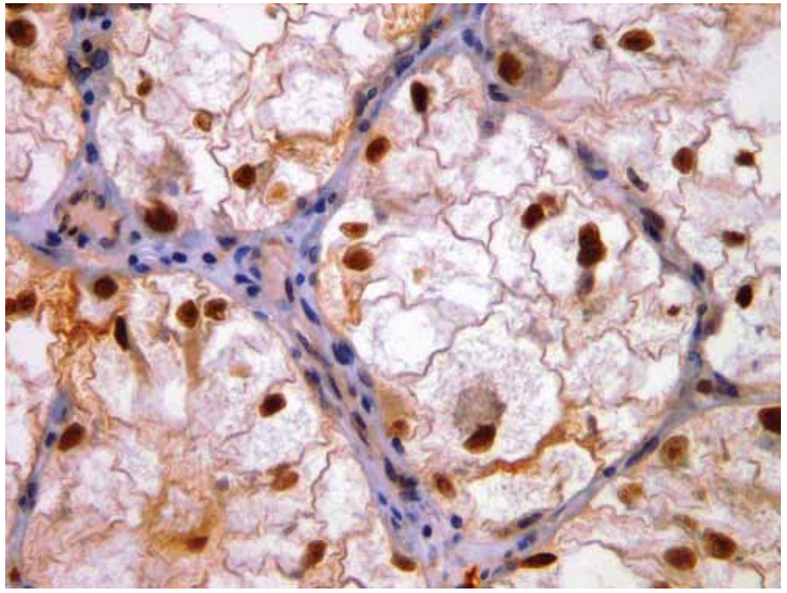

**Figure 3.** A Xp11.2 translocation carcinoma showing the characteristic TFE3 nuclear staining.

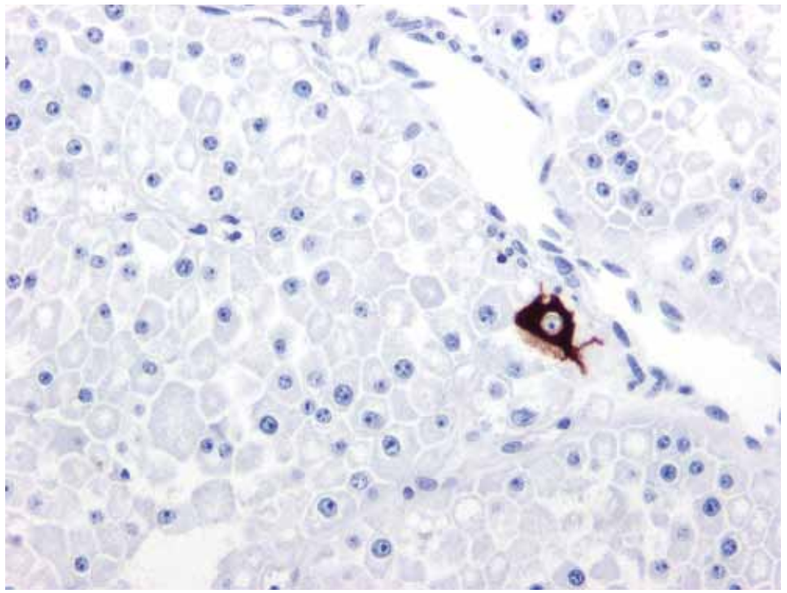

**Figure 4.** An isolated CK 7 positive cell in an oncocytoma.





Clear Cell RCC expresses vimentin, a variety of low molecular cytokeratins, EMA, CD10 and RCC MA (19, 20). The latter two have some diagnostic utility when a RCC is considered in a metastatic lesion (Table 2). Notable cytokeratin exceptions in CC-RCC are CK7 and high molecular weight cytokeratins. Cytokeratin 7 coupled with AMACR (a-methylacyl-CoA racemase), is useful when solid forms of Pap-RCCs are encountered (21, 22). Several antigens, some uncommonly stocked in immunopathology laboratories, such as parvalbumin, S100A1, caveolin-1, C-kit, PAX2 and E-cadherin have discriminatory efficacy in separating CC-RCC from Ch-RCC (23-30). However, the first line strategy for Ch-RCC should be to stain for CI. For urothelial carcinoma the presence of high molecular weight cytokeratin, CK 7 and p63 permit confirmation of the diagnosis (32-34).

Xp11.2 translocation carcinomas are a group of neoplasms characterized by a variety of break points at Xp11.2 with translocation to one of several other chromosomes forming fusion genes (35-37). They occur most frequently in children and young adults. Although histologically variable, the most common tumor cell phenotype are clear cells with voluminous cytoplasm and papillary architecture. Intracellular and stromal calcifications provide additional useful clues to the diagnosis. In general the absence of epithelial markers in translocation carcinomas are powerful clues to the diagnosis. However, a positive reaction for the nuclear transcription factor TFE3 is definitive in the absence of cytogenetic confirmation (Fig. 3). Some cases may also stain for melanocytic markers, but this is more common in TFEB positive t(6:11) translocation carcinomas.

## Immunohistochemistry in the Differential Diagnosis of RCC with Cytoplasmic Eosinophilia

The differential diagnosis for RCCs comprised entirely or predominately of cells with eosinophilic cytoplasm is broad, as reflected in Table 3. Although both the mucinous tubular and spindle cell RCC and tubulocystic carcinoma are listed, their distinctive histology drives the diagnosis rather than immunohistochemical phenotype (38-40). The differential diagnosis in tumors with eosinophilic cytoplasm extends beyond carcinomas to include a benign tumor, oncocytoma, and a RCC mimic, the epithelioid angiomyolipoma (41, 42). Epithelioid AML (EpAML), although not a carcinoma, demonstrates malignant behavior in 30% of cases and may lead assist in recognition of the hereditary disease, tuberous sclerosis complex.

Most cases of oncocytoma are recognized by their distinctive histology. The presence of mitoses or necrosis should prompt great hesitation in rendering an oncocytoma diagnosis regardless of an otherwise typical histology. Although there are several antigens that can be employed to separate oncocytoma from CC-RCC with eosinophilic cells and the eosinophilic variant of Ch-RCC, because of histogenetic similarity between oncocytoma and Ch-RCC, immunohistochemical stains in these two tumors show substantial overlap. Thus parvalbumin, S100A1, C-kit, caveolin-1, E-cadherin and kidney specific cadherin are usually positive in both (23-30). The CK 7, however, can be very helpful in oncocytoma where most cells are completely negative but a small population of strongly positive cells is invariably present imparting a distinctive staining pattern (Fig. 4). Recently PAX2 has been reported to discriminate between oncocytoma and Ch-RCC (25, 26). When combined with C-kit and CK this profile of three antigens is useful in the separation of the four most common RCCs with eosinophilic cytoplasm as noted in Table 4.

Table 4.

| Selected Immunohistochemical Profile for the Four Most Common Renal Neoplasms | | | |
|---|---|---|---|
| Antibody | CK 7 | C-Kit | PAX2 |
| Clear cell RCC | neg | neg | pos |
| Chromophobe cell RCC | pos | pos | neg |
| Papillary RCC | pos | neg | pos |
| Oncocytoma | neg | pos | pos |

Identification of a translocation carcinoma as previously described is facilitated by negative cytokeratin stains and EMA stain. The presence of a positive melanocytic marker is helpful, but a positive stain for the nuclear transcription factor TFEB or TFE3 is required for confirmation. Although, EpAML like translocation carcinomas is negative for cytokeratin and EMA stains and positive for melanocytic markers, its distinctive histology with large ganglion-like cells should permit recognition (40, 41). In uncertain case TFEB and TFE3 stains should be employed.

When collecting duct carcinoma (CDC) is considered the histologic features of a high grade tubulopapillary tumor with desmoplastic stroma and infiltrative growth among native nephron elements is characteristic and markedly distinct from all other renal cancers except for urothelial carcinoma (42, 43). The presence of high molecular weight cytokeratin and positive reaction for UEA-1 stain distinguish CDC from other RCCs. A related carcinoma, renal medullary carcinoma (RMC), is recognized by its mucinous or mucoid stroma and infiltrating inflammatory cells. Since development of RMC to date has been limited to patients with sickle cell trait or SC disease, this laboratory feature must be present to support the diagnosis. Immunohistochemistry rarely plays a role.

## Discussion

The classification of RCC has blossomed in recent years with the description of many new entities. The 20th century acceptance of an astounding degree of morphologic diversity within a single nosologic entity RCC has been replaced by a broad menu of diagnostic choices that the pathologist must consider. One member, CC-RCC, remains the most common of RCC. Although CC-RCC is usually sporadic, similar appearing tumors can arise in patients with several genetic cystic diseases. Furthermore, CC-RCC is histologically diverse and may resemble several other cytogenetic and prognostically different tumors. Therefore, the pathologist, must have pertinent history and be well versed in histological nuances of renal tumor classification.

Histology remains the cornerstone for diagnosis; immunohistochemistry serves as a useful adjunct to the diagnosis in selected circumstances. When considering immunohistochemistry in differential diagnosis, profiles of antigens are required since any type of RCC may not fully express the IH profiles listed in the tables provided where positive entries do not indicate 100% incidence, nor do negative entries indicate 0% incidence of reaction.

The IH profiles although useful are not a substitute for careful histologic assessment. In a practical sense, more blocks of tissue are often more cost effective than immediate initiation of IH. Lastly, do not feel obligated to place every tumor within a currently defined category. Renal cell carcinoma, unclassified is a valid designation and should account for several percent of RCC diagnoses rendered. It is from this category that new entities may emerge of academic interest that may demonstrate differing biologies with important prognostic or therapeutic implications.



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# Chapter 27 | Special Stains Use in Fungal Infections

**Abida Haque, MD**

Histologic evaluation of tissues is a quick and easy way to identify fungal organisms, and a strong adjunct to microbiologic culture for diagnosis of fungal infections. Histologic evaluation of granulomatous inflammation and granulomas must include special stains to exclude or include presence of fungi and acid-fast bacteria. Gomori Methenamine Silver (GMS) and Periodic acid-Schiff (PAS) are the two most common stains used to look for fungi in tissues and in cytology specimens in the daily practice of pathology. The presence of fungus in the tissue sections provides an indisputable evidence of invasive infection. Because of their size and morphologic diversity, many fungi can be seen in tissue sections by conventional light microscopic examination of Hematoxylin and Eosin (H&E) stained sections. In cytology specimens, fungi can be identified by their size and specific morphology.

In the tissues, fungi usually occur either as hyphae, budding yeast, endosporulating spherules, or a combination of these forms (1, 2). In some groups of fungi only one species of fungus is the cause of mycosis, and therefore when classic forms are observed, an etiologic diagnosis can be made. These groups of fungal diseases include adiaspiromycosis, blastomycosis, coccidioidomycosis, cryptococcosis, *Histoplasmosis capsulati, Histoplasmosis duboisii*, paracoccidioidomycosis, *Penicilliosis marneffei*, protothecosis, rhinosporidiosis, and sporotrichosis.

Other mycoses are caused by any of the several species of a genus, all of which are morphologically similar in tissue sections. Although these fungi cannot be identified as to the species by conventional histology, the disease that they cause can be diagnosed generically; for example, aspergillosis, candidiasis, and trichosporonosis. Still other mycoses are caused by any of a number of fungi belonging to different genera. These fungi appear similar, if not identical to one another in tissues. With these fungi, it is not possible to identify the etiologic agent, however, the mycosis can be named; for example, phaeohyphomycosis and zygomycosis.

Hematoxylin & Eosin is a versatile stain that enables the pathologist to evaluate the host response, including the Splendore-Hoeppli phenomenon, and to detect other micro-organisms (3). It is the stain of choice to confirm the presence of naturally pigmented fungi, and to demonstrate the nuclei of yeast-like cells. However, there are drawbacks to using just the H&E stain for fungal diagnosis. It is often difficult to distinguish poorly stained fungi from tissue components, even at higher magnifications. When sparse, fungi are easily overlooked in H&E stained sections. The morphologic features may not be evident and sometimes may be misleading. For example, *Histoplasma*, Blastomyces, and Paracoccidiodes may have cytoplasmic retraction artifact in the sections, making morphologic evaluation difficult. Some of the fungal variants may have different sizes, such as the large form variant (African) *histoplasma*, and microform blastomycosis. Some of the dimorphic fungi can form pseudohyphae in tissues. Sometimes the fungal morphology may be altered by therapy. Special stains for fungi are therefore essential for histopathologic evaluation of unexplained inflammatory processes (4, 5). Most fungi can be readily demonstrated with the common special stains, Gomori's methenamine silver (GMS), Gridley's fungus (GF), and periodic acid-Schiff (PAS), also referred to as "broad spectrum" fungal stains. GMS is preferred for screening, because it gives better contrast, and stains even degenerated and nonviable fungi that are sometimes refractory to the other two stains (Fig. 1, 2).

GMS also stains algae (Prototheca and Chlorella spp.), cyst walls of *Pneumocystis jiroveci* (Fig. 3, 4), pathogenic free living soil amebas, the spore coat of most microsporidian parasites, intracytoplasmic granular inclusions of Cytomyeolovirus, *Actinomyces Israeli* and related species, Nocardia spp., most *Mycobacterium* spp., and nonfilamentous bacteria with polysaccharide capsules such as *Klebsiella pneumoniae* and *Streptococcus pneumoniae*. Prolonged staining in the silver nitrate solution may be required to adequately demonstrate degenerated fungal elements such as the yeast-like cells of H*istoplasma capsulatum* var. *capsulatum* in granulomas.

The disadvantage of GMS and GF fungal stains is that they mask the natural color of pigmented fungi, making it impossible to determine whether a fungus is colorless hyaline or dematiaceous (pigmented). Such a determination is crucial in the histologic diagnosis of mycosis caused by dematiaceous fungi such as phaeohyphomycosis (6). Except for the PAS reaction, fungal stains GMS and GF do not

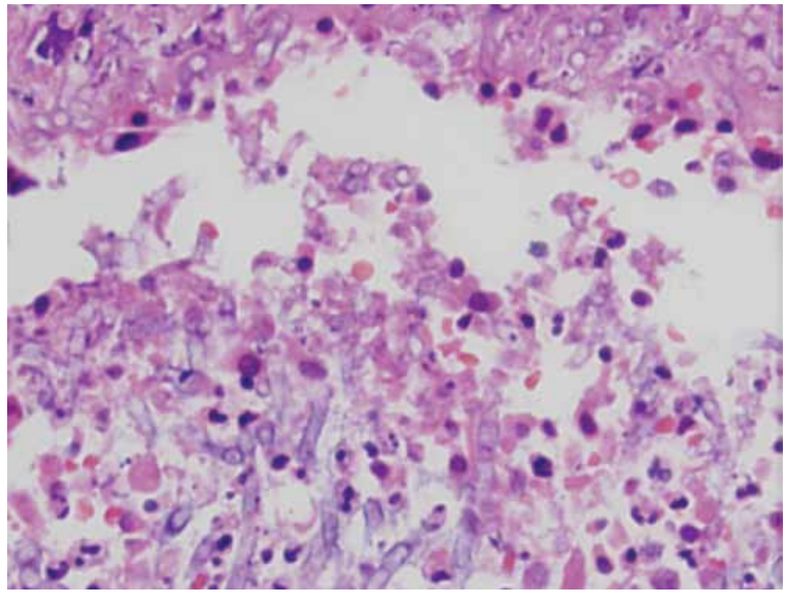

**Figure 1.** Hematoxylin and Eosin staining of *Aspergillus*.

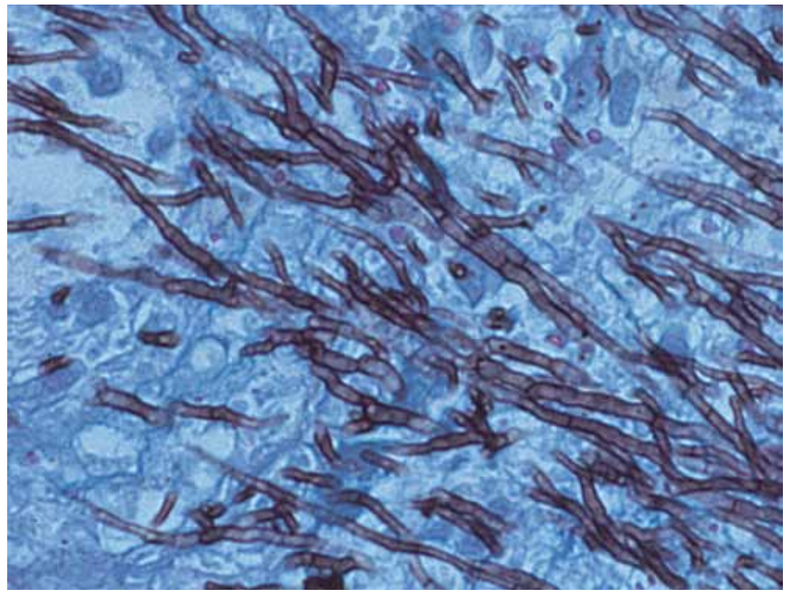

**Figure 2.** GMS staining of *Aspergillus*.





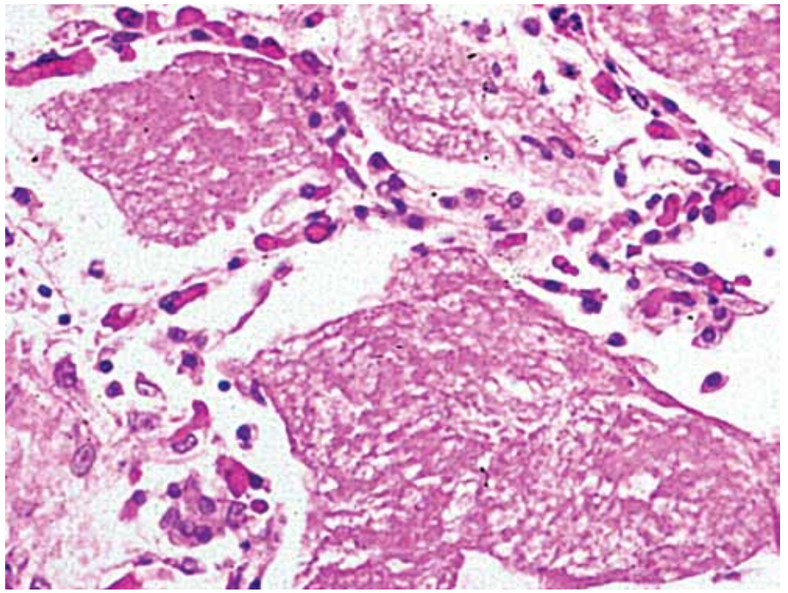

**Figure 3.** Hematoxylin and Eosin staining of *Pneumocystis jirovici*.

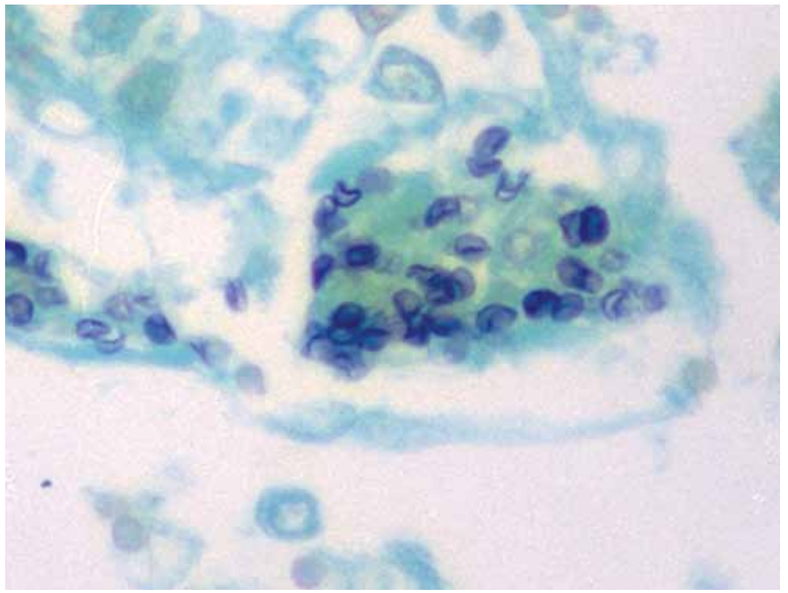

**Figure 4.** GMS staining of *Pneumocystis jirovici*.

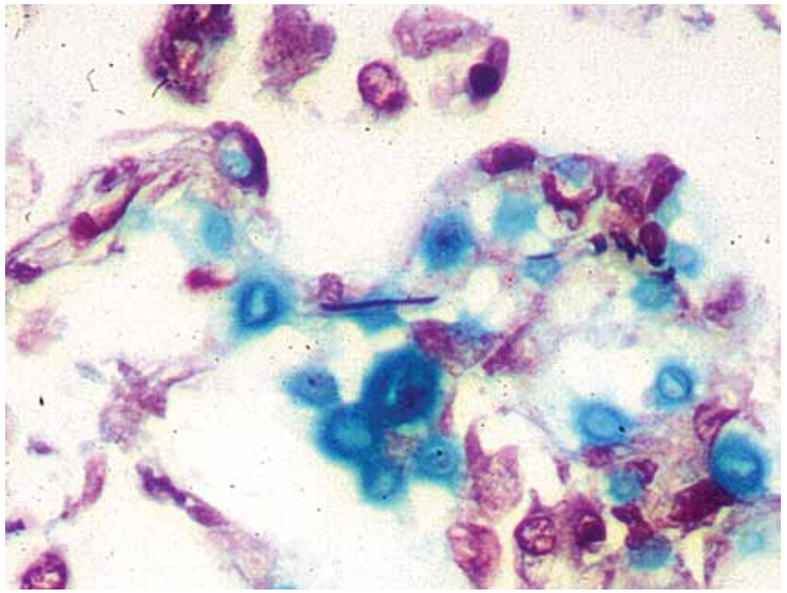

**Figure 5.** Alcian Blue staining of *Cryptococcus.*

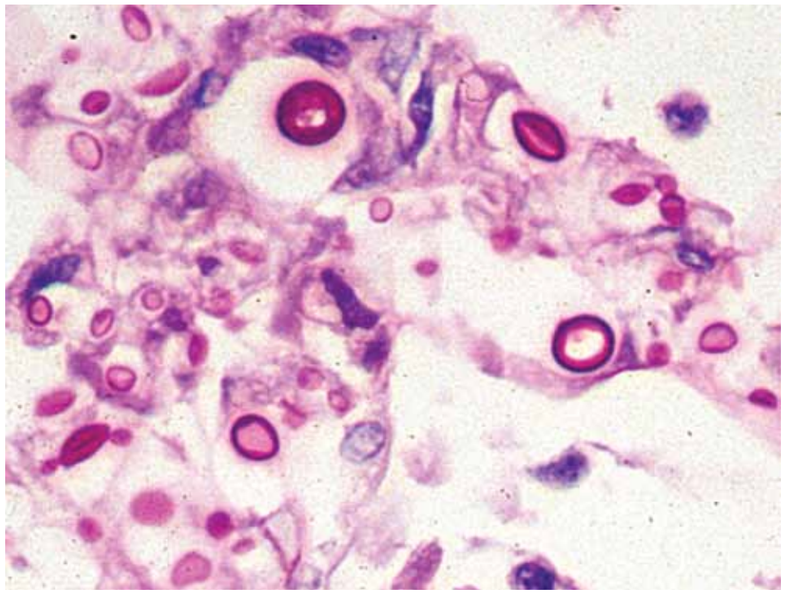

**Figure 6.** PAS staining of *Cryptococcus.*





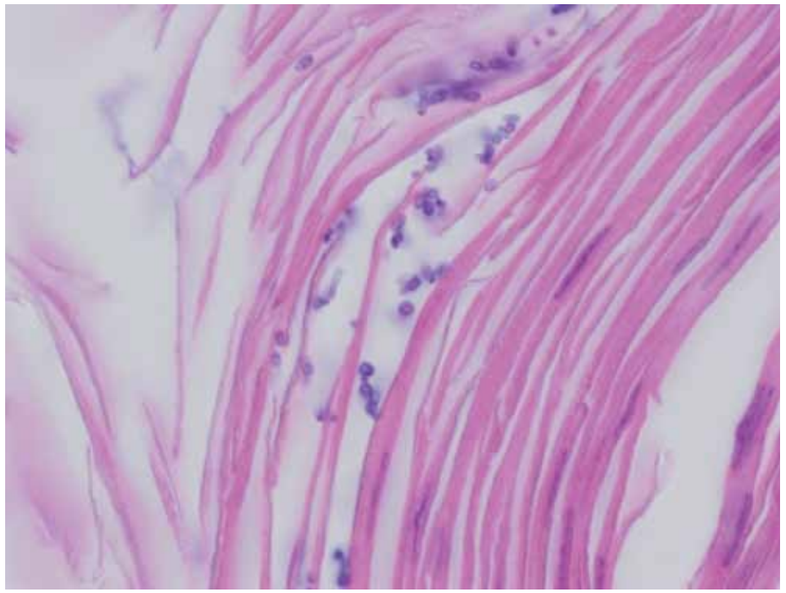

**Figure 7.** Hematoxylin and Eosin staining of *Malassezia.*

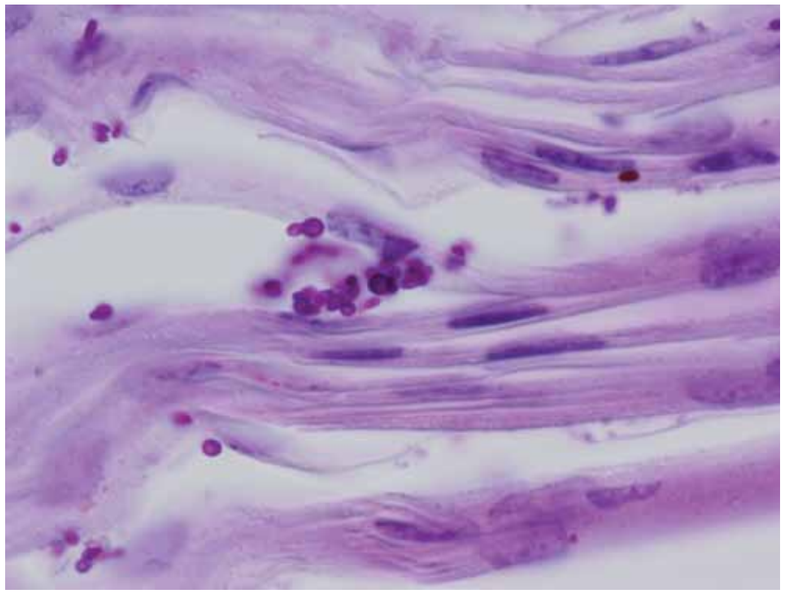

**Figure 8.** PAS staining of *Malassezia.*

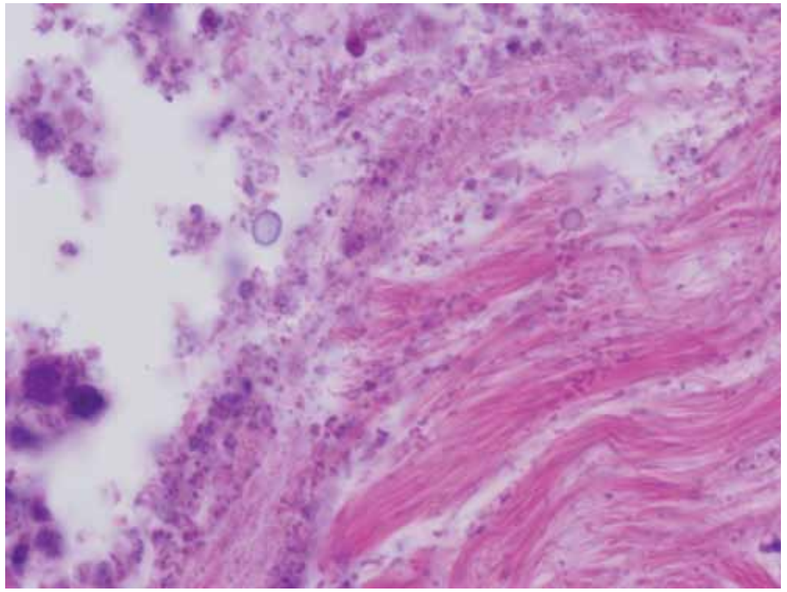

**Figure 9.** Hematoxylin and Eosin staining of *Histoplasma*.

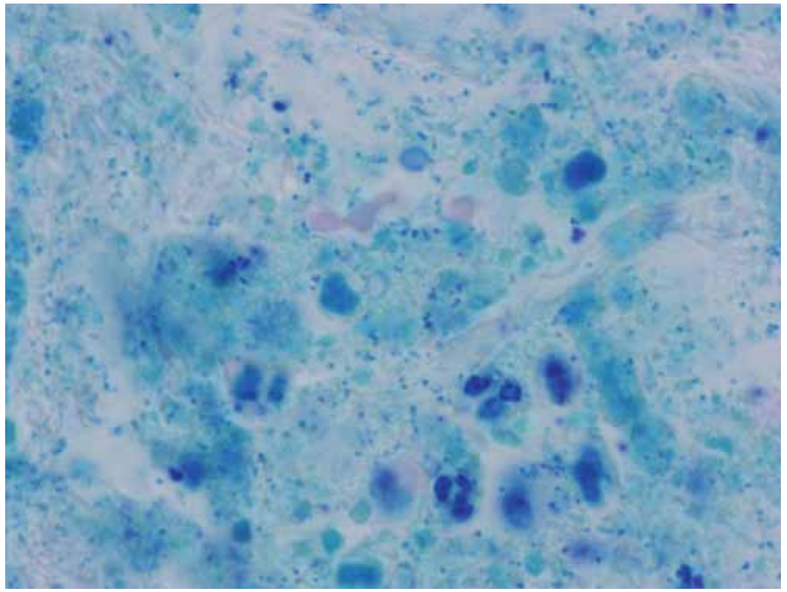

**Figure 10.** AFB staining of *Histoplasma*.





adequately demonstrate the inflammatory response to fungal invasion. To counteract this, a GMS-stained section can be counterstained with H&E for a simultaneous study of the fungus and the host response.

The PAS stain performs almost as well as GMS, in screening for fungi. It actually demonstrates fungal morphology better than the silver stains. PAS can stain degenerated fungi that may not be visible on H&E stain.

Calcific bodies that are sometimes found in caseating granulomas are also stained with PAS, and can be mistaken for yeast-like fungi. This is especially true when calcific bodies are apposed to give the false impression of budding yeasts, or when the bodies are laminated to give the appearance of a capsule or thick cell wall. Best stains to avoid this misinterpretation are GMS and GF stains, because the chromic acid used as an oxidizer in these stains dissolves the calcium, leaving the calcific bodies unstained. Conversely, there are artifacts that mimic fungi on GMS and GF stains that are not seen on PAS stain, therefore the use of both silver and PAS can reduce the incidence of false positive results.

## Narrow-Spectrum Fungal Stains

The differential diagnosis of fungi may require the use of additional special stains that stain some fungal organism and not others. These are sometimes referred to as “narrow-spectrum” fungal stains (7, 8). Some of the stains in this category are mucin stains such as alcian blue and Mayer’s, or Southgate’s mucicarmine, that readily demonstrate the mucoid capsule of *Cryptococcus neoformans* (Fig. 5, 6).

This staining reaction differentiates *Cryptococcus* from other fungi of similar morphology, such as *Coccidiodes*, *Candida*, and *Histoplasma*. These mucin stains are not specific for *C. neoformans*; the cell walls of *B. dermatitidis* and *Rhinosporidium seeberi* are often stained to varying degrees with mucin stains. However, these two fungi are nonencapsulated and morphologically distinct, and not ordinarily mistaken for *Cryptococcus*. In some cases, poorly encapsulated cryptococci in tissue sections may not stain positive with mucicarmine stain. In these cases, since the cell wall of *C. neoformans* contains silver reducing substances, possibly melanin precursors, it can be stained with Fontana-Masson’s silver procedure for melanin (9, 10). This stain is especially useful in those cases of Cryptococcosis with invasive yeast forms that do not have readily detectable capsules, the so-called dry variants. Such forms could possibly be confused with non-encapsulated yeasts of similar morphology. Fontana-Masson and Lillie’s ferrous iron stains for melanin can also be used to confirm and accentuate the presence of melanin or melanin-like pigments in the cell walls of poorly pigmented agents of phaeohyphomycosis in tissue sections (11). PAS may be used as a narrow-spectrum fungus stain. For example, in the differential diagnosis of small budding yeast forms, a weak PAS and a strong GMS staining favors a diagnosis of *Histoplasma*, since *Candida*, microforms of *Blastomyces*, and yeast forms of *Malassezia* show a strong cell wall staining with PAS (Fig. 7, 8).

Another narrow-spectrum fungus stain is Ziehl-Neelson (ZN). In one study, 60% of blastomyces and 47% of *histoplasma* organism showed positive cytoplasmic staining of the yeast-like cells with ZN stain (Fig. 9, 10).

No staining was seen in *Cryptococcus* or Candida, and very rare acid-fast staining was seen in coccidiodomyces endospores (12). However, these staining properties are inconsistent and should not be used for primary diagnosis. The cell walls of fungi are in general, not acid fast.

## Autoflourescent Fungi

Some fungi or fungal components in the H&E stained tissue sections are autofluorescent when examined under ultraviolet light source (13). *Candida* species, *Coccidioides immitis* and *Aspergillus* species can exhibit bright green to yellow-green autofluorescence (14). When sections of these fungi are stained with the PAS, bright yellow fungal autofluorescence against a deep red-orange background is seen (15). Autofluorescence may help delineate sparse or poorly stained fungi in H&E stained sections, however this property is inconsistent and should not be used for definitive diagnosis. Most fungi in frozen or paraffin embedded tissue sections also stain nonspecifically with Calcofluor white, a cotton whitener that fluoresces under ultraviolet light (16). This rapid and a simple fluorescence procedure can be routinely used in the intraoperative examination of fresh-frozen tissues for fungi.

Immunoperoxidase stains can be used to identify certain fungi in smears and in formalin fixed, paraffin embedded tissue section. This technique, however, has only limited diagnostic use (17).

## Direct Immunofluoresence (IF)

Direct immunofluoresence (IF) can improve the diagnostic capability of conventional histopathology in the diagnosis of fungal diseases (18). The IF procedure, which can be performed on smears and on formalin fixed paraffin embedded tissue sections is helpful in confirming a presumptive histologic diagnosis, especially when fresh tissues are not available for culture or when atypical fungus forms are seen. The Division of Mycotic Diseases, Center for Disease Control, Atlanta (United States) and others have a broad battery of sensitive and specific reagents available for identifying the more common pathogenic fungi.

The immunofluoresence procedure has several advantages. Final identification of an unknown fungus is possible within hours after H&E and GMS stained sections are initially examined. The need for time consuming and costly cultures is often obviated by IF, and the hazards of handling potentially infectious materials are reduced when microorganisms are inactivated by formalin prior to IF staining. Prolonged storage of formalin fixed tissues, either wet tissue or paraffin embedded, does not appear to affect the antigenecity of fungi. This antigenic stability makes possible retrospective studies of paraffin embedded tissue and the shipment of specimens to distant reference laboratories for confirmatory identification. Most service laboratories, however, do not routinely use IF, since the special stains have been very reliable for diagnosis in the day to day pathology practice.

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# Chapter 28 | Special Techniques Applied to Bone Marrow Biopsies in the Diagnosis of Myelodysplastic Syndromes

**Carlos Alberto Martín, MD, PhD and Marina Narbaitz, MD**

## Background

Myelodysplastic syndromes (MDS) are neoplastic clonal hematologic stem cell disorders which occur mainly in older adults (1). These are a heterogeneous group of diseases characterized hematologically by conspicuous cytopenias and possess a variable rate of progression to overt acute leukemia. (1, 2). Cytologic dysplasia, the morphologic hallmark of MDS, is defined in bone marrow aspirate samples with May-Grünwald Giemsa stain. The staining is so important in this field, that it formed the basis for the classification of MDS in 1982 by the French-American-British (FAB) group. Today, several additions to these cytologic findings – such as, refined morphology, risk evaluation, and cytogenetic data form the basis for the diagnosis of MDS. According to the current World Health Organization (WHO) classification, trephine biopsies are considered to be very important in the initial diagnosis of MDS (2, 3).

Histomorphological studies of core biopsies yield complementary data to those provided by bone marrow aspirates and blood smears. They also provide complementary information with respect to the amount of hematopoietic cellularity and topographic alterations of the progenies. The use of appropriate special techniques permits grading bone marrow fiber content and defines the presence of fibrosis. Both cellularity and fibrosis constitute parameters of significant prognostic importance (4-8).

## Histopathology Methods and Special Techniques

Hematoxylin and eosin stain (H&E), Giemsa, Gomori's silver impregnation technique and Perls stain in combination with immunostaining are some of the most common methods used to study MDS. Bone marrow cylinders that were optimally fixed, decalcified, embedded in paraffin and stained with H&E and other special stains were used for the quantitation of cellularity and the evaluation of histoarchitectural displacement of progenies (Fig. 1).

In general, hyperplastic bone marrows predominate in MDS (Fig. 2), although in some patients hypoplastic marrows were seen, (8, 9). Histotopography of different progenies with H&E were used to differentiate MDS from secondary dyshematopoietic changes produced by other neoplastic (lymphoma, metastasis) or reactive conditions (nutritional, toxic) (5). These changes consist of displacement of poorly formed erythroid groups to endosteal surface and, conversely, relocation of myeloid cells to centromedullary spaces (Fig. 2 and 3). In addition, dysmorphic megakaryocytes (hypolobated, microforms, atypical large cells) were frequently seen (Fig. 4) in MDS adopting a paratrabecular position.

Cytomorphological detail studied with Giemsa allowed us to discern fine nuclear details (such as the nuclear membrane, chromatin pattern, nucleoli) and also the presence of cytoplasmic granules, leading to a better definition of different cell populations (Fig. 5) (10,11). Giemsa stain was also helpful in the search for the presence of immature cell (blast) aggregates, formerly designed as ALIP (abnormal localization of immature precursors), which may represent an increase in blasts cells (Fig. 3, 6). Based on this finding, one can make a differential diagnosis with other immature cells that present themselves in the marrow, as groups of megaloblastic erythroblasts, promyelocytes, and monocytic accumulates. However, in this case, in order to confirm the blastic nature of the cells, there was a need for additional immunostains, which included not only appropriate lineage markers such as glycophorin A, myeloperoxidase and CD68, respectively, but also CD34 and CD117 (12). Cases with more than 5% of CD34+ blasts in the marrow were included in the Refractory Anemia with Excess Blasts (RAEB) category, namely RAEB 1 (5-9% blasts cells) and RAEB 2 (10 to 19% blast cells) respectively (1) (Fig. 7). Bone marrows with CD34+ cells forming aggregates or clusters (i.e., three or more CD34+ cells) are currently described as having "multifocal accumulations of CD34+ progenitor cells" (2) (Fig. 8). These patients were defined as having both overall lower survival rates and leukemia free survival in a multivariable analysis (4).

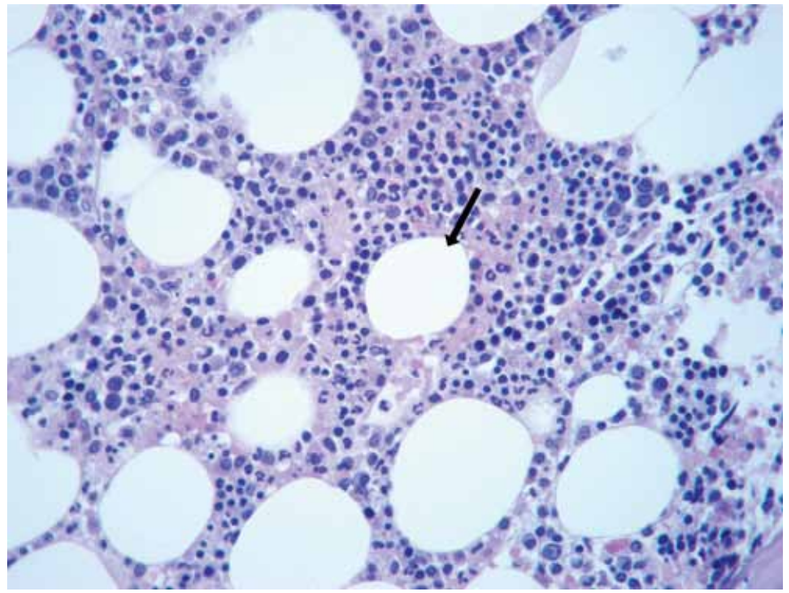

**Figure 1.** Normocellular MDS. Conspicuous dyserythropoiesis and increase in immature mononuclear cells (H&E, 250x). Arrow points to a fat cell.

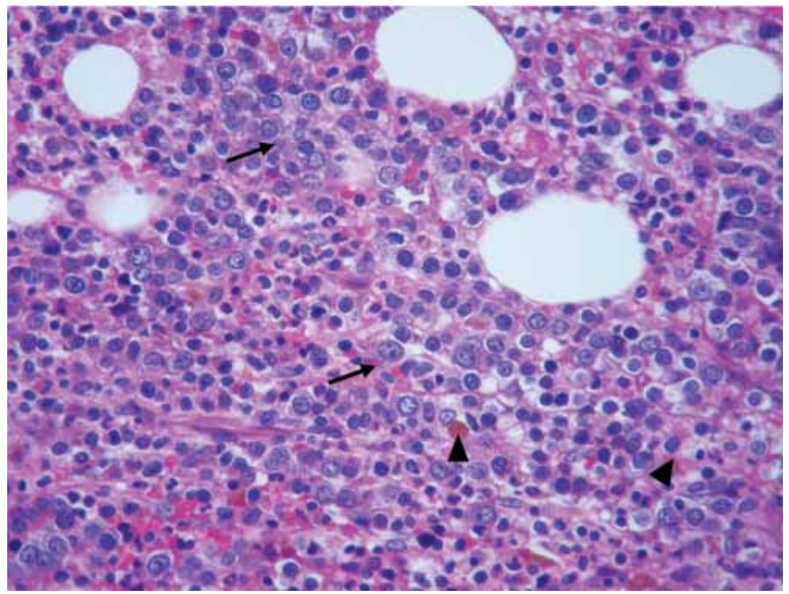

**Figure 2.** Hypercellular MDS. Dyshematopoietic features include dyserythropoiesis, increase in immature cells (presumably of myeloid lineage) (arrows) and granular deposits of hemosiderin (iron-storage complex) (arrowheads) (H&E, 400x).





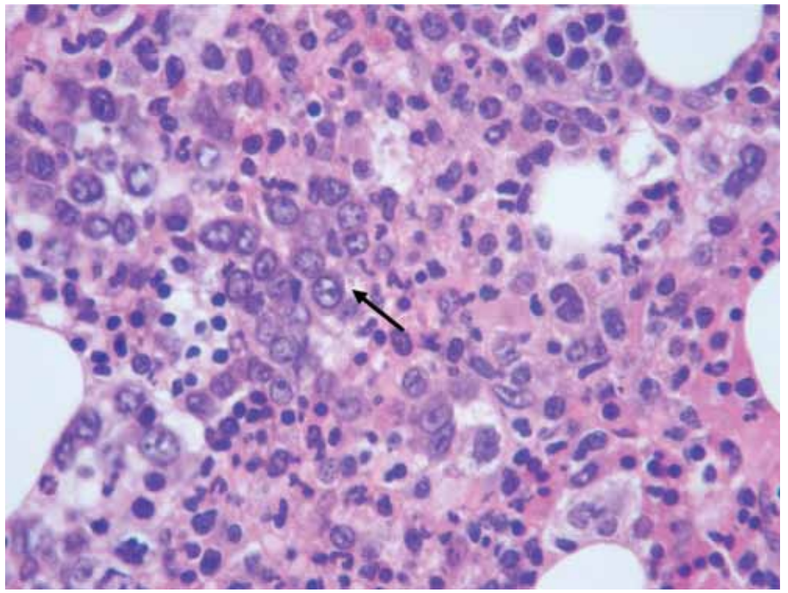

**Figure 3.** MDS. High power view showing aggregates of abnormally localized immature precursor (ALIP) cells (arrow). (H&E, 400x).

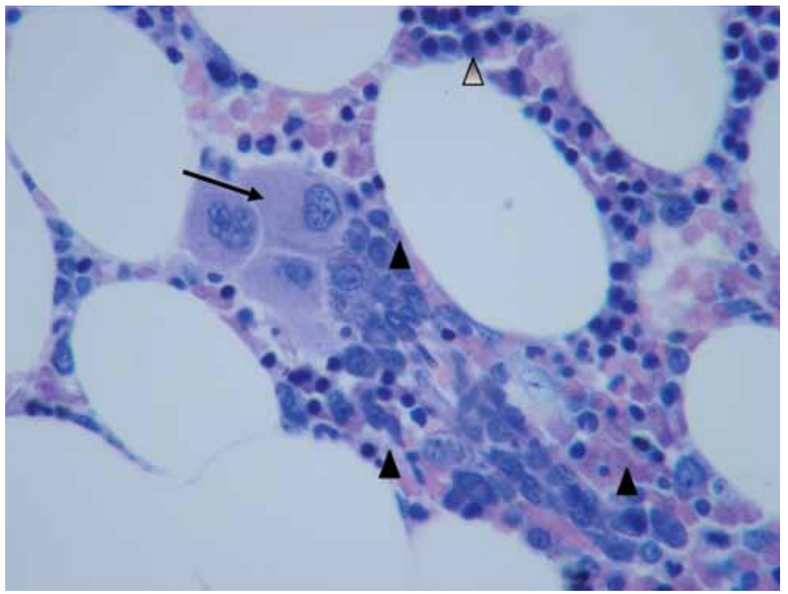

**Figure 4.** MDS. Grouped dysmorphic (hypolobated) megakaryocytes (arrow), immature cells with clear chromatin and finely granular cytoplasms (black arrowheads), and dark stained nuclei of erythroid cells (grey arrowhead) (Giemsa, 400x).

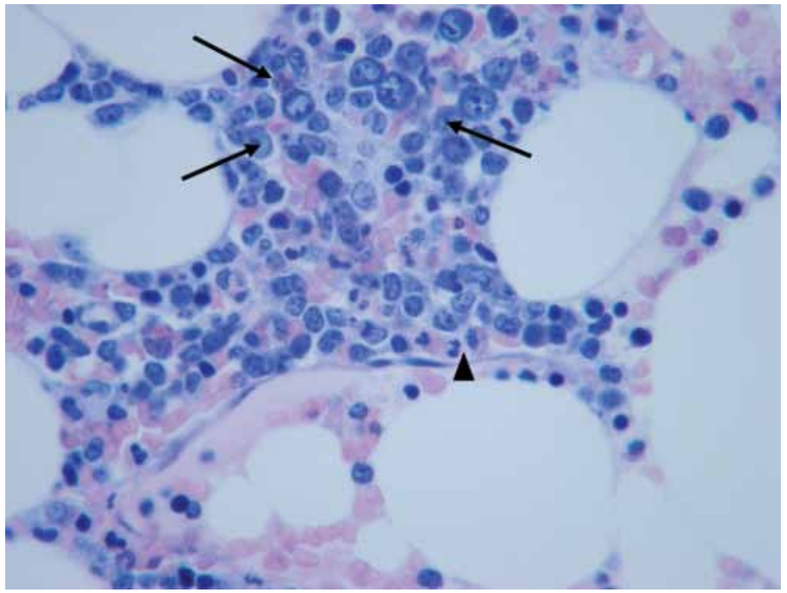

**Figure 5.** MDS Refractory anemia with excess blasts 2 (RAEB 2). There are numerous interstitial blasts cells (arrows) and isolated segmented granulocytes (arrowhead) (Giemsa, 400x).

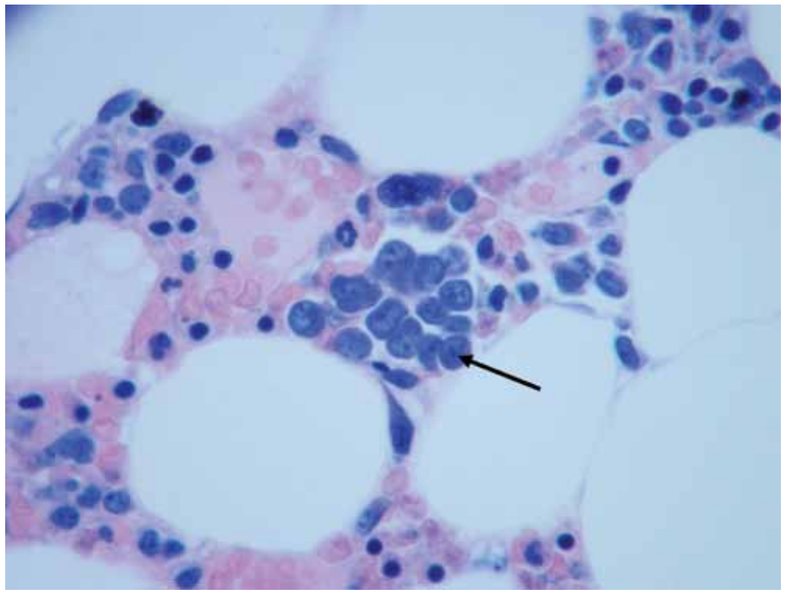

**Figure 6.** Hypocellular MDS (RAEB 2). A cluster of immature myeloid cells is depicted (arrow) (Giemsa, 400x).





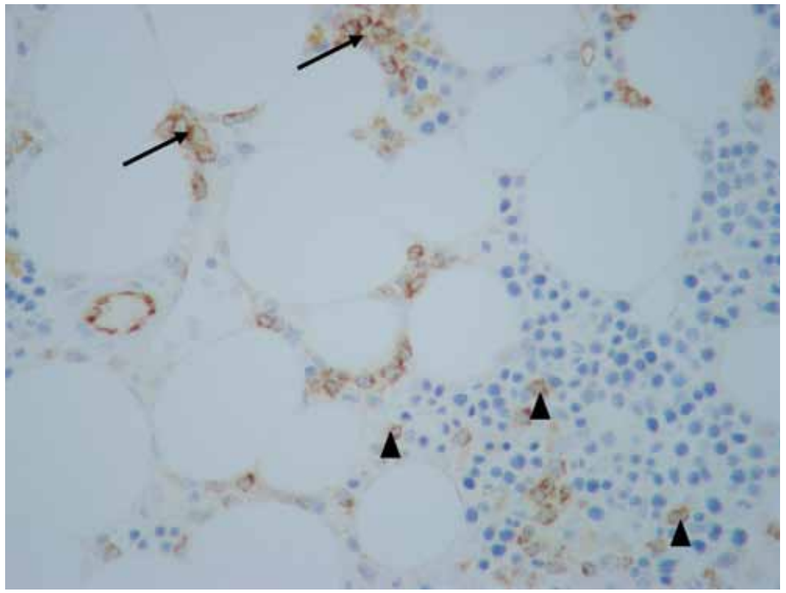

**Figure 7.** Hypocellular MDS (RAEB 2). Note the striking increase in CD34+ blast cells, which appear disperse (single cells) (arrowheads) and sometimes found as clusters (arrows). CD34+ is immunostained, 100x.

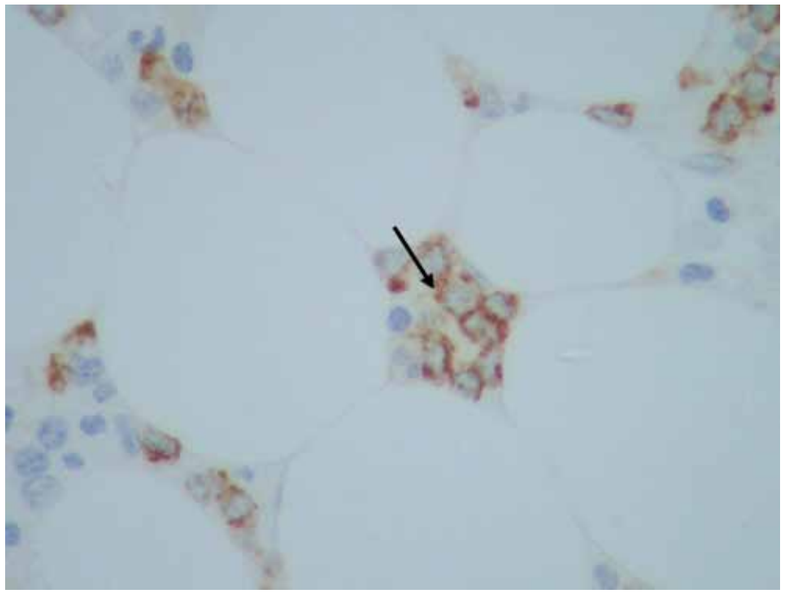

**Figure 8.** Hypocellular MDS (RAEB 2). High power view of an aggregate of CD34+ progenitor cells (arrow); Also refer to Figure 7 (CD34 immunostain, 400x).

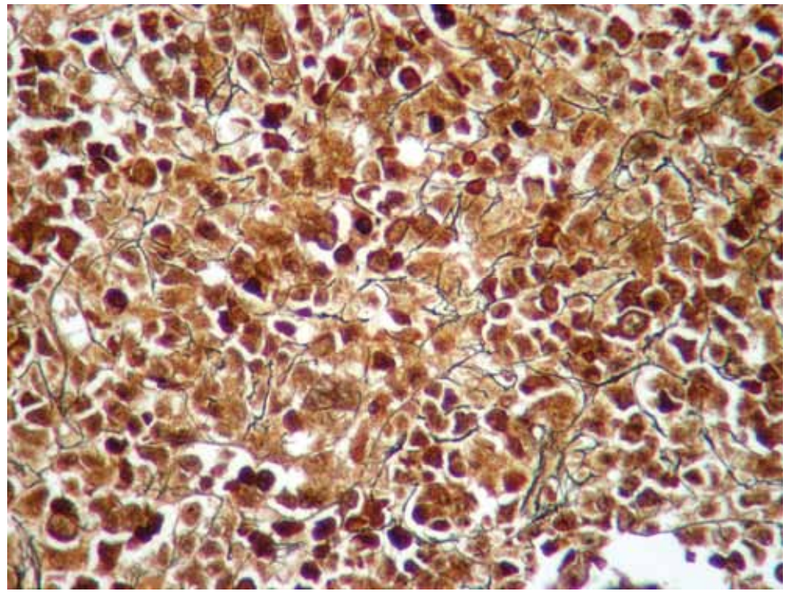

**Figure 9.** MDS. Grade 1 myelofibrosis in hypercellular marrow (Gomori, 400x).

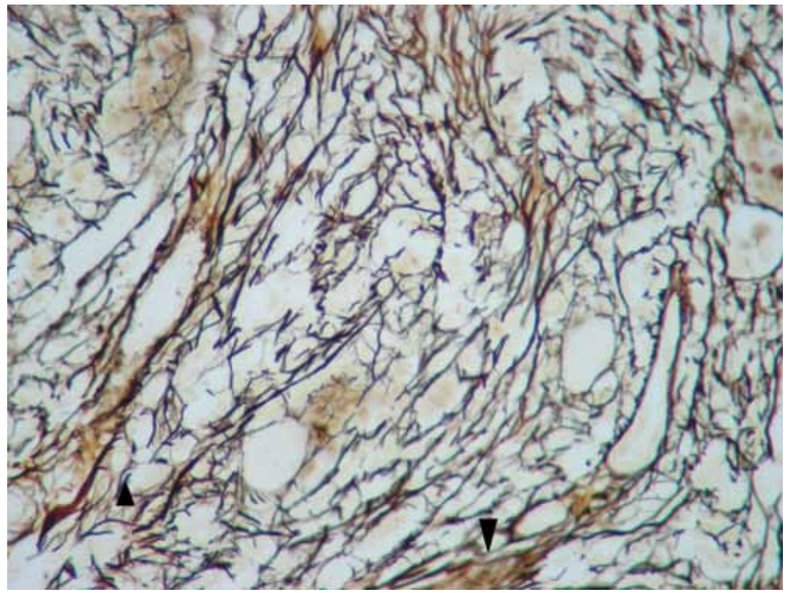

**Figure 10.** MDS. Grade 2 myelofibrosis with focal collagen deposition (arrowheads). (Gomori, 400x).





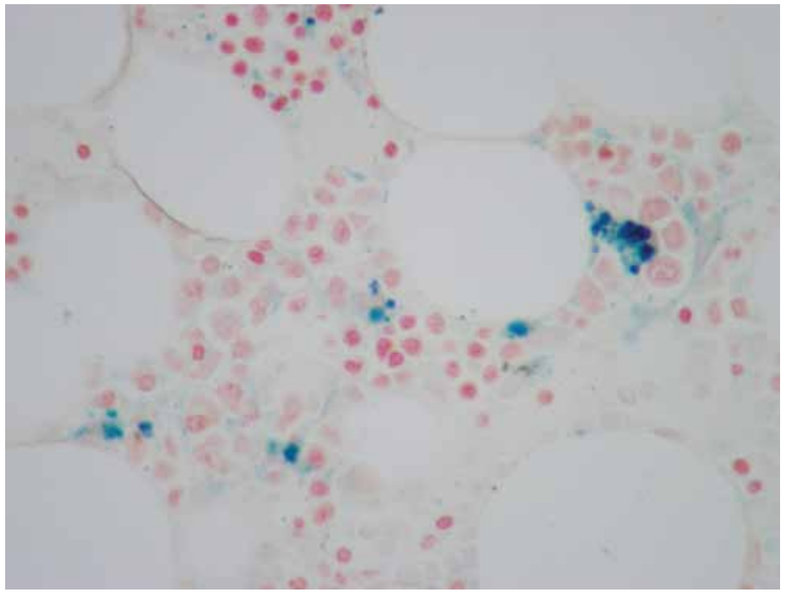

**Figure 11.** MDS. Marked increase in granular iron deposits (blue dots). (Perls, 400x).

In addition to Giemsa, the silver impregnation technique of Gomori (13) is also used in MDS in the detection of an abnormal increase of interstitial fibers or reticulin fibers . In fact, 60% of MDS marrows studied show some degree of an increase in reticulin fibers or myelofibrosis (MF) (4). According to the European Consensus guidelines, the silver impregnation technique allows one to semi-quantitatively grade the fiber content into four grades (13,14) (a) Grade 0 (or normal) defined as the presence of only scattered linear reticulin; (b) Grade 1 (mild fibrosis) as a loose network of reticulin with many intersections (Fig. 9); (c) Grade 2 (moderate fibrosis) with a diffuse and dense increase in reticulin with extensive intersections and sometimes focal collections of collagen (10); and (d) Grade 3 (severe fibrosis) as having a diffuse and dense increase in reticulin fibers with extensive intersections and coarse bundles of collagen.

MDS is also frequently associated with anemia caused by ineffective erythropoiesis, and increased hemosiderin (iron-storage complex) deposits that can be readily detected in H&E-stained sections. However, for a detailed semiquantitative analysis (1 to 4+), a special stain for iron such as Perls (Prussian blue) is needed. With Perls stain, blue hemosiderin granules can be easily seen in the bone marrow interstitium, or inside macrophages. Sometimes, ringed sideroblasts can also be observed in thin sections.

## Conclusion

Currently, the diagnosis of MDS is made with the integration of clinical data, cytomorphological data and genetics. The bone marrow biopsy is an essential tool in the diagnosis of MDS, for not only producing additional data on cellularity, but also in detecting the presence of topographical distortion and fibrosis, quantity of iron stores, an increase in blasts cells and / or aggregates, and other reactive or paraneoplastic conditions associated to cytopenias and dysplastic changes. To that end, the use of Giemsa, reticulin and Perl's stain is strongly recommended in order to better appreciate cell morphology and interstitial changes associated to this group of diseases.

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## Acknowledgment

*We would like to thank Maria Elena Gomez for invaluable technical assistance.*



# Chapter 29 | The Evolution of the Artisan™ Special Stains Instrument

**Ron Zeheb, PhD and Steven A. Bogen, MD, PhD**

"Special stains cannot be automated" was a common early objection to the Artisan™ project, when we started in the early 1990's. The highly diverse nature of the special stains protocols, the instability of freshly mixed working reagents, and the unpredictability of the color development time were all factors weighing against the feasibility of special stains automation. Since there was no previously established market for special stains automation, companies in the field were not interested in financing such an effort. At the time, immunohistochemistry automation was still in its infancy and struggling to find a market toehold. Against this backdrop, creating a special stains instrument was a daunting challenge. As the inventors of the Artisan™, we considered that the story of the instrument and how it was developed might be of general interest.

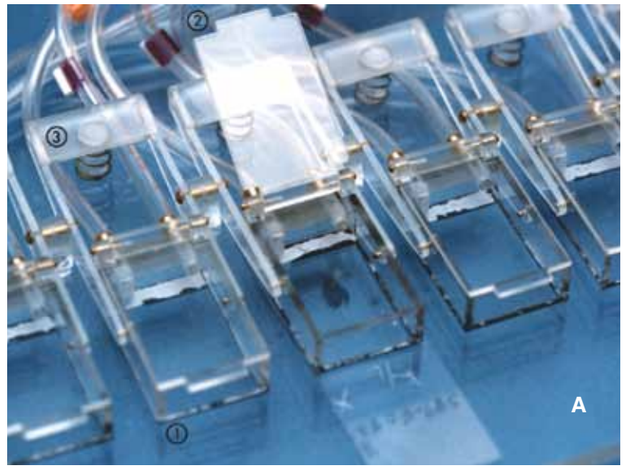
## Funding the Research and Development

The project was initiated by one of the authors (SAB) who, at the time, was in residency training at the Department of Pathology, Brigham & Women's Hospital, Boston, MA. Early simple breadboard prototypes were fabricated using out-of-pocket funds. However, it soon became clear that professional engineering expertise was required. In addition, the project needed funding. Both problems were solved by teaming up with Mr. Herb Loeffler who, at the time, was Principal of Loeffler-MacConkey Design Inc., Arlington, MA. Together, we submitted a grant application to the National Institutes of Health, under the Small Business Innovative Research (SBIR) program. In total, we received approximately $550,000 to fund the R&D for the first prototypes. Later, in 1996, Richard Foemmel, PhD, joined the team and we formed CytoLogix Corporation. Dr. Foemmel, as President & CEO, raised venture capital to fund the transition from R&D to manufacturing. The Artisan™ product line was transferred to Dako in 2002.

## The First Breadboard Prototype

For a variety of special stains (when performed manually), it is common practice to monitor color contrast development under the microscope. When the tissue section achieves the appropriate level of contrast, the histotechnologist stops the reaction by immersing the slide in a liquid. Unfortunately, this protocol is incompatible with automation. To solve the problem, it seemed likely that if we tightly controlled all of the reaction variables, such as time, temperature, and reagent concentration, then the histochemical reaction should be reasonably predictable. If so, then visual inspection should not be necessary. As a first step, we needed to create a reaction chamber that could be applied to microscope slides, in order to contain the reagents that were added and removed. Without containment, the intensely colored stains and dyes might wreak havoc on the interior of the instrument.

Figure 1a and 1b show one of our first breadboards for testing this concept. Ten slide chambers were mounted on a clear plastic base. Each slide chamber was attached to a spring-loaded clamp, pressing the chamber down onto the glass microscope slide, thereby creating a seal. For early experiments, we added and removed reagents by pipette, simulating what an instrument would later do. The slide clip concept was kept throughout the Artisan™ design process and is incorporated into the present-day Artisan™ instrument (Fig. 1C).



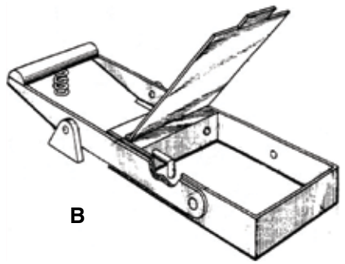




**Figure 1. A.** Early, breadboard-version slide chamber for containing staining reagents, with a slide inserted under one of the chambers. **B.** Line drawing of an individual slide chamber from the patent illustration, showing the spring-loaded mechanism for maintaining downward pressure. **C.** Picture of the current version of the slide clip, in the commercial version of the Artisan™.





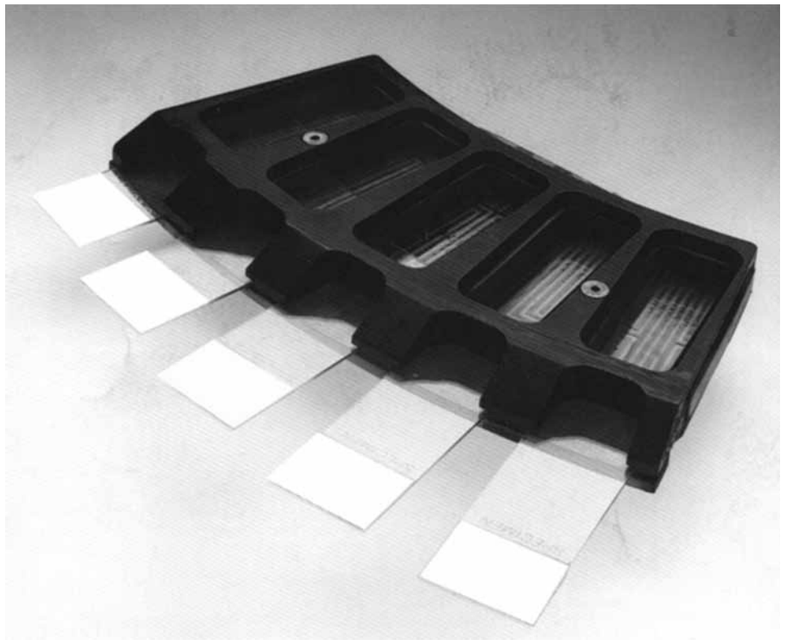


**Figure 2.** Photograph of a second-generation, development-version slide clip, capable of holding up to five slides at a time, with heating elements under each microscope slide. The heating elements can be seen in the photograph as the parallel lines etched on the base of the slide support.

## Development of Independent Slide Heating

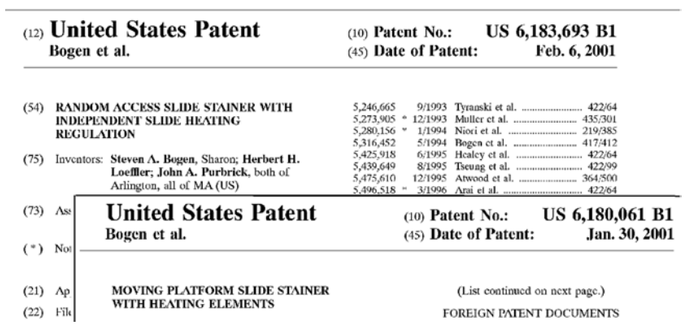
Many special stains require heat during one or more steps. A problem arose because the temperature needed for one stain will likely be different than the temperature needed for a second stain. Moreover, the times at which heat will be needed bore little relationship from one stain to the next. This created the need to independently control the temperatures of microscope slides. At the time, during the mid-1990's, immunohistochemistry stainers existed that used convective heating. Convective heaters, operating like a hair blower mounted inside the slide stainer, heat the air around the slides. All slides are heated to the same temperature. In addition, one of the authors (RZ) led project development of a slide stainer for in situ hybridization (gen II) that used radiant heating. A radiant heating element warmed the slides by radiating heat from above. Again, all the slides were heated to a single temperature.

Addressing special stains automation required the application of conductive heating to an automated slide stainer. A thin heating element was placed on the slide platform, underneath each slide. By applying an appropriate amount of electrical current to the heaters under selected slides, different slides could be warmed to different temperatures. An early prototype version of independent slide heating, underneath a slide clip for five slides, is shown in Figure 2. The heating elements can be seen through the opening in some of the slide chambers, as the thin parallel strips on the slide support surface.

Independent slide heating was more complex than it initially seemed. For example, controlling all of the heaters was a challenge. We initially intended to mount the heaters on a rotary carousel, with a wire bundle connecting each heater to a computer-driven temperature controller. The temperature controller was to have been positioned on the desktop, next to the computer. Since each heater required at least three wires (power, ground, and temperature sensor), the ultimate wire bundle for fifty slides (150 wires) would have been quite stiff, impeding the rotary motion of the carousel. A consulting electrical engineer, John Purbrick, helped solve this problem by placing some of the temperature controller circuitry on the rotary carousel itself. In this way, we only needed a few wires (power, ground, data, and a clock line) connecting the personal computer (PC) on the desktop and the temperature controller on the carousel. Through those wires, the PC instructed the controller circuitry on the carousel as to the desired temperature for each slide. The temperature controller, mounted on the moving rotary carousel, was then responsible for directly controlling each heater.

## Patents

For these discoveries, the inventors (and CytoLogix Corporation) were awarded patents. The patents proved to be important, were the subject of litigation, and the inventors and company were ultimately successful in enforcing them. Figure 3 shows an image of the front pages from the patent.



(12) **United States Patent**
**Bogen et al.**

(10) **Patent No.: US 6,183,693 B1**
(45) **Date of Patent: Feb. 6, 2001**

(54) **RANDOM ACCESS SLIDE STAINER WITH INDEPENDENT SLIDE HEATING REGULATION**

(75) Inventors: **Steven A. Bogen**, Sharon; **Herbert H. Loeffler**; **John A. Purbrick**, both of Arlington, all of MA (US)

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**United States Patent**
**Bogen et al.**

(10) **Patent No.: US 6,180,061 B1**
(45) **Date of Patent: Jan. 30, 2001**

**MOVING PLATFORM SLIDE STAINER WITH HEATING ELEMENTS**

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

**Figure 3.** The front pages of two slide heating patents developed for the Artisan™ staining instrument.





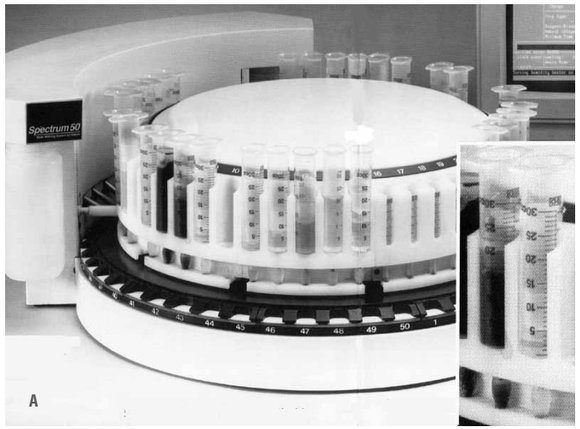




**Figure 4. A.** A second-generation prototype slide stainer. The inset (right) shows a higher magnification of the first proof-of-principle reagent cartridges. The reagent reservoirs were comprised of syringe barrels. **B.** The new **Artisan**Link Special Staining System. The unit consists of a slide processor, a computer system with workflow software and a printer (not shown).

## Uniformity of Temperature Across the Slide

Independent control of the temperature of each slide, while necessary, was not sufficient to achieve consistent results, particularly for some silver-based stains (e.g., Gomori methenamine silver stain and Jones methenamine silver basement membrane stain). To heat properly, the glass microscope slide had to maintain close contact with the underlying heater. This was achieved by making the slide platform out of high-quality stainless steel, manufactured to a strict flatness specification. Stainless steel also had the important attribute of being chemically resistant. The slide clip also helped, by pressing the slide firmly against the heater platform to ensure maximum heat transfer. Stainless steel is hard and chemically resistant but, unfortunately, it's a relatively poor conductor of heat. In prototype testing, it resulted in hot and cold spots on the slide. The engineering team solved this problem by laminating the stainless steel with an under-layer of copper, which is an excellent conductor of heat. This design change dramatically improved heat uniformity. However, even this modification was not enough.

Each slide platform is rectangular, like a glass microscope slide, and almost as thin. Heat radiates from the edges faster than the center. This resulted in a thermal gradient across the slide, with a hot center and cooler edges. We could see the gradient by using a thermal imaging camera. Our engineering group solved the problem by modifying the heating element design. Rather than heating evenly across the slide surface, we compensated for the heat loss at the edges by adding more heating capacity there. In the end, we achieved a chemically resistant slide platform that could be rapidly heated (and would also rapidly cool) to a uniform temperature across the working area of the slide.

## A Disposable Precision Reagent Dispenser

Special stains involve an extraordinary breadth of chemicals: acids, bases, oxidizers, reducing agents, alcohols, salts, dyes, etc. We were challenged to design reagent dispensers made of disposable materials that would safely hold each of these different reagents. The dispensers should not leak, even in the partial vacuum of the cargo hold of an airplane. The dispensers should also precisely dispense their liquids onto the slide. Figure 4 shows an early prototype of the instrument and latest model respectively.

The reagent reservoirs were comprised of a large syringe barrel. At the bottom, we attached a short length of flexible tubing with two one-way valves located at each end. When the tubing was squeezed, the contents already in the tubing were forced out onto the slide. The one-way valves ensured that the liquid only flowed downward, and not back into the reservoir. When the tubing was released, an equal volume of fresh reagent was drawn from the syringe barrel, refilling the tubing for the next dispense.

The reagent cartridge went through a series of changes, ultimately leading to the present-day dispenser. The flexible tubing that metered out reagents was eventually replaced with a diaphragm pump. Made of polypropylene and shaped like an oversized umbrella with a short handle, the diaphragm comprised one side of a chamber that held 0.25 mL of reagent. When depressed by the reagent dispense hammer, the diaphragm inverted, collapsing the space to near zero and dispensing reagent onto the slide with high accuracy and reliability. The syringe barrel was replaced with a sealed, collapsible flexible plastic bag held in a rigid plastic shell.





## Mixing it Up

Mixing stock solutions to form a working mixture is easy to do by hand. Robotics creates a new design problem. The Artisan™ design team faced the need to find a way for a robotic instrument to mix chemicals, with a minimum of parts. We felt that using air was the best bet, but the trick is to mix without drying the tissue section or splashing reagent. This problem was solved by directing a thin "curtain" of air downward through a narrow slit on a movable manifold. The manifold is positioned directly above the slide. When a slide is below, a small motor drives the manifold back and forth. The moving air curtain makes waves, mixing reagents together and distributing them uniformly over the slide.

## Scheduling the Artisan's Operation

Automated slide stainers like the Artisan™ generally perform a single operation at a time. Reagent can be added to a slide, removed from a slide, or mixed on a slide. To do this, the Artisan's slide carousel brings the slide in question either to the reagent addition station, the liquid aspiration station, or the mixing station. While one slide is being treated, other slides wait. This approach resulted in a flexible system capable of performing an incubation of any length. We were especially interested in being able to perform short incubations, such as less than one minute. This is particularly important for procedures such as AFB or Giemsa, which require brief incubations. For example, the manual protocol may state, "dip slides 5 times in acid alcohol". Being able to execute these short incubations, at any temperature, results in the best quality special stains. However, scheduling instrument operations to perform all these steps with maximum efficiency (shortest overall processing time) required the development of a highly sophisticated machine scheduling algorithm, probably the most advanced for a slide staining instrument.

To optimize throughput, the scheduling software algorithm sets out each slide protocol on a virtual timetable. Each slide demands the Artisan's attention at certain short periods of time for adding, removing, or mixing reagent. At other times, the Artisan™ can attend to other slides. The algorithm shuffles the many slides' schedules, examining thousands of combinations so as to find the best fit, executing all of the slide staining protocols in the shortest period of time.

## Chemistry Innovations for Special Stains

For a few stains, it was not possible to directly import the reagents as described for the manual procedure onto the Artisan™ platform. One of these related to the Warthin-Starry stain. The conventional protocol calls for preparing a gelatin solution in water at a temperature high enough to dissolve the solid gelatin. It is believed that gelatin serves a role in these stains that is somewhat similar to its role in black and white photography. Namely, it is believed that gelatin serves as a protective colloid. A common protocol in the conventional manual recipe is to dissolve the (solid) gelatin in water at 56°C for 15 minutes. The dissolved gelatin can then be mixed with a silver nitrate solution and hydroquinone, to form a developer solution for the Warthin-Starry stain. The problem we had to solve is that we could not provide a 5% gelatin solution in water as a liquid reagent. At room temperature, the gelatin "gels", making it impossible to dispense.

A staff colleague, Dr. Adrian Leek, solved the problem by replacing conventional gelatin with a solution of non-gelling gelatin, also sometimes referred to as "partially hydrolyzed gelatin". Non-gelling gelatin is hydrolyzed animal protein derived from collagen. It is produced from enzymatically modified gelatin. It has an approximate molecular weight of about 3,000 Daltons and an amino acid profile that is approximately the same as that of gelatin. Unlike gelatin, non-gelling gelatin is soluble at room temperature. After some experimentation, we found that non-gelling gelatin produces an excellent Warthin-Starry stain. A patent was awarded for this innovation (U.S. Patent 6,465,207).

## Conclusion

There were many more challenges and innovative solutions required to make the Artisan™ than could reasonably be discussed in this short article. The authors are gratified to see the result of their efforts, and the efforts of many talented individuals at CytoLogix and at Dako, in making the Artisan™ the premier special stains instrument. From its once humble beginnings, it now has a worldwide presence, helping clinical histopathology laboratories stain patient tissue samples all over the globe.

# Chapter 30 | Tissue Microarray Technology as a New Approach for Quality Control with **Artisan**Link

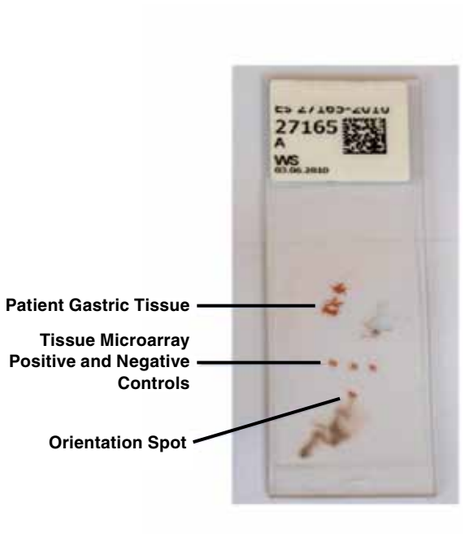
**Christiane Kahnt, Hanin El-Sayed and Herman Herbst, MD, FRCPath**

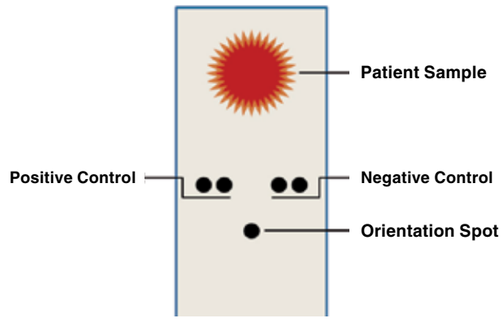
Special Stains is an established technique in many pathology laboratories and institutes. Quality control for special stains is one of the major problems in daily practice. Even when the protocols are standardized using automated instruments such as the **Artisan**Link™ , there is still a lot of variability left in inter-laboratory results. This is where tissue microarrays (TMA) become helpful when used as a method for quality control and in the facilitation of standardizing different staining procedures such as special stains (also see Appendix, page 285).

The detection of cellular and tissue structures or microorganisms by special stains is quite common and is often verified by qualitative or quantitative controls. The detection of bacteria, for instance by Ziehl-Neelsen stain or silver impregnation techniques such as Warthin-Starry or Grocott staining requires a control that definitely contains the characteristic microorganism(s) to be detected in the patient sample. In case of macromolecules, such as interstitial collagens or reticulin fibers, it is necessary to demonstrate characteristic staining patterns in a semi-quantitative approach to control a certain level of staining intensity. Usually such controls are performed on additional or separate slides. This implies that the result of the control stain documented on a separate slide requires extensive documentation and archiving in order to associate the special stain of a patient's tissue sample with the corresponding control slide. This issue can be avoided using TMA technology (For details about TMA technology, refer to the articles 1, 2 and 3; see references.)

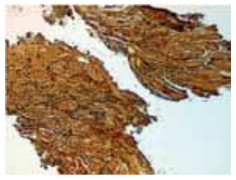
The TMA method not only enables a high throughput method of analysis of special stains sample, but also provides an economical alternative to conventional controls on separate slides in immuno-histochemistry (IHC) and in-situ hybridization techniques. The design of the TMA is so much different from the outlines of the patient tissue section, that an accidental mix up between TMA control and patient tissue is unlikely. In addition, no parallel slides are required for documentation as the TMA slide containing the patient sample has all the required material for staining and control. All of this makes the TMA technique a comfortable alternative in the daily routine work.

An example of “Warthin Starry” staining is shown below to demonstrate how TMA can be used as positive and negative controls in the same slide as a patient sample.



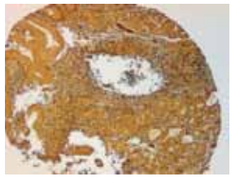




**Quality control for Warthin-Starry staining**

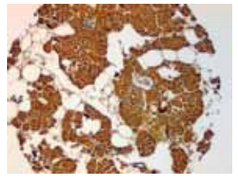


Patient sample (Gastric)

Patient sample (Gastric)



Positive control TMA



Negative control TMA





Other examples where TMAs can be used as controls are shown in the table below.

| Staining Kit | Dako Code No. | Clinical Application | Control Tissue | Typical Laboratory Performing the Stain |
|---|---|---|---|---|
| Artisan™ Acid-Fast Bacillius Stain Kit | AR162 | Most commonly used to demonstrate *Mycobacterium tuberculosis* in human tissue | Lung,<br>Mycobacteria (e.g., MAI)<br>Liver AFB+ | Microbiology |
| Artisan™ Gram Stain Kit | AR175 | Used in identifying Gram-negative bacteria in human tissue | Tissue containing Gram-positive or -negative bacteria | Microbiology or reference laboratory |
| Artisan™ Warthing-Starry Stain Kit | AR181 | Silver method used in the identification of *Helicobacter pylori* in human tissues, *H. pylori* has been associated with chronic gastritis and ulceration of the stomach | Spirochetes or *H. pylori*, Gastrointestinal tissue | Microbiology or reference laboratory |
| Artisan™ Alcian Blue/PAS Stain Kit | AR169 | Used as an aid in distinguishing adenocarcinoma from certain sarcomas | Liver, S.I., Colon, Kidney | Gastrointestinal laboratory |
| Artisan™ Reticulin/Nuclear Fast Red Stain Kit | AR179 | Used to outline the architecture in liver, spleen, bone marrow, and may be used to highlight the growth pattern of neoplasms | Liver | Renal pathology laboratory<br>Liver laboratory |
| Artisan™ Gomori's Trichrome Stain Kit | AR167 | May be used to differentiate normal structures and injured tissue as in chronic active hepatitis of liver cirrhosis | Normal or Zirrhotic Liver | Renal pathology laboratory<br>Liver laboratory |
| Artisan™ Elastic Stain Kit | AR163 | Use to demonstrate atrophy of elastic tissue in cases of emphysema, and the loss of elastin fibers in arteriosclerosis, differentiates collagen from elastic | Lung, Artery | Cardiovascular laboratory |

In summary, TMAs allow the performance of tissue-based assays on a large number of patient samples in an efficient and cost-effective manner. In addition, they provide an economical alternative to conventional controls on separate slides; thus circumventing extensive documentation and archiving in order to associate the special stain of a patient's tissue sample with a seperate corresponding control slide.

**References**


1. Battifora H (1986): The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. *Lab Invest*, 55:244-248.
2. Packeisen J, Korsching E, Herbst H, Boecker W, Buerger H (2003). Demystified tissue microarray technology. *Mol Pathol* 56(4):198-204.
3. Saxena R, Badve S (2009). Tissue microarray - Construction and quality assurance. IHC staining methods. Dako education guide. Pp 43-50.




# Chapter 31 | Automating Special Stains using Artisan™ and **Artisan**Link Special Staining System

**Christiane Kahnt and Herman Herbst, MD, FRCPath**

## Historical Perspective

The basis of histopathology is the application of natural and artificial stains to tissue sections. This results in visualization of cells, sub-cellular structures, extracellular matrix, minerals and microorganisms. The principal of staining is either to soak the tissue in the staining solution and interrupt the procedure when appropriate, or to saturate the section with a stain followed by a destaining procedure, or to apply metal salts which adhere to cellular and extracellular structures.

The staining procedure most widely applied in histopathology is the hematoxylin and eosin (H&E) technique which permits differentiation of staining between nucleus and cytoplasm. On the other hand, there are numerous other, less frequently applied stains, which are collectively called special stains.

Special stains reagents are diverse in their composition and chemistry. Acids, bases, solvents, metal salts and others, some of which are highly toxic, are commonly used in special staining techniques. Preparing these reagents is time-consuming and potentially hazardous, and the resulting reagents may not be of consistent quality. Many reagents must be mixed from various stock solutions seconds before use and may have a limited working stability. A laboratory that offers the full spectrum of special stains must procure and store a large stock of potentially hazardous chemicals in order to prepare the required staining reagents. Special stains protocols are as diverse as the reagents used to perform them. Some protocols have as few as two to three steps while others may have eight to ten. The incubation times are rather variable.

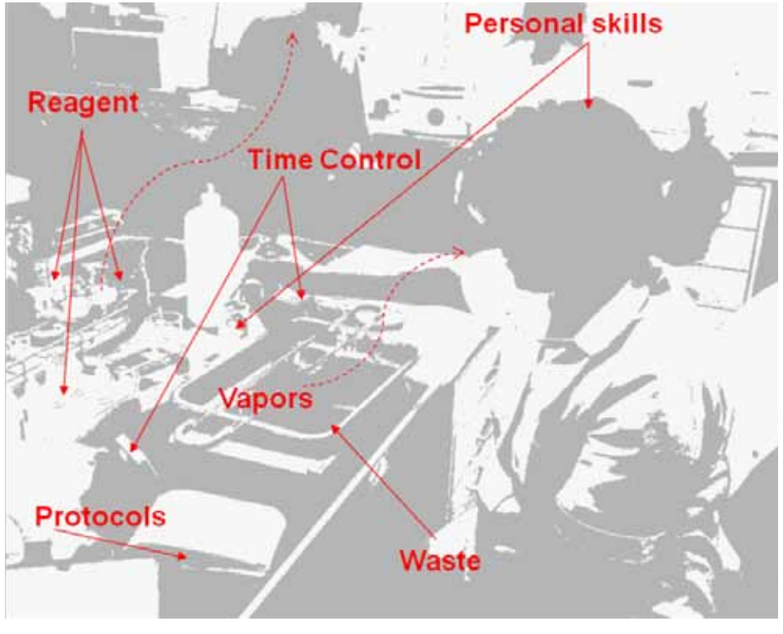
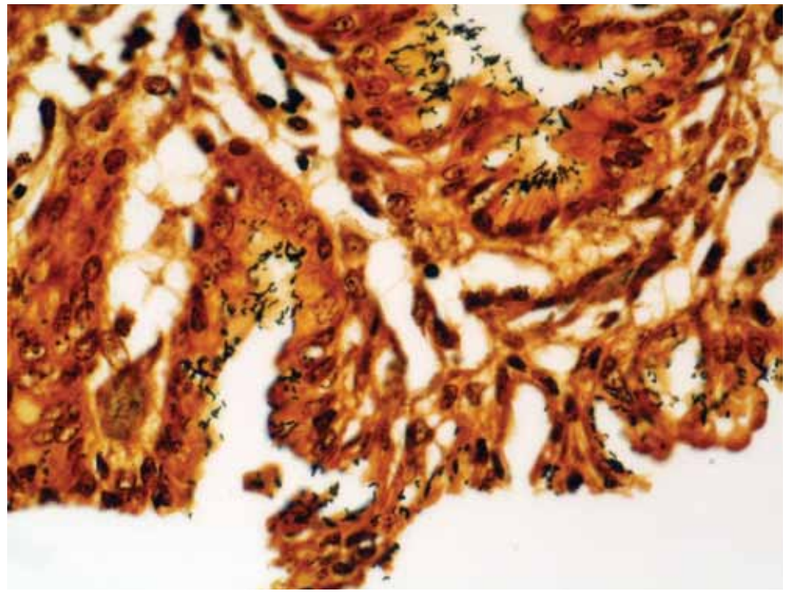
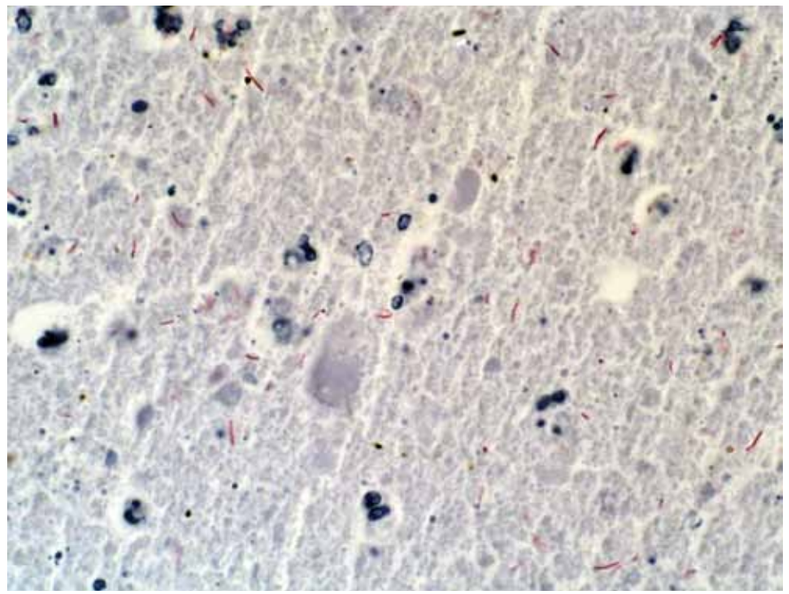



**Figure 1**. Manual staining of slides with special stains.



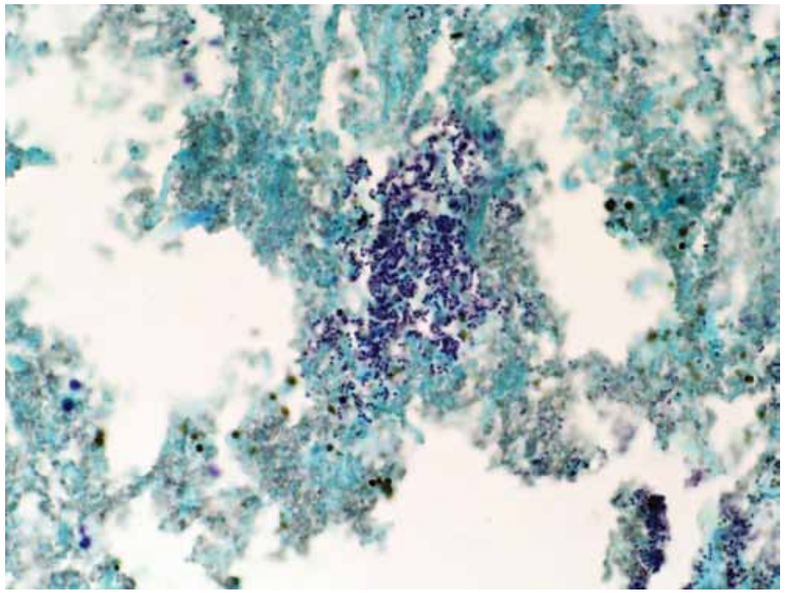
**Artisan™ Sample Staining.** Mucous layer of gastric surface epithelium showing *H. pylori* (black-stained rods). Warthin-Starry silver stain (x40).



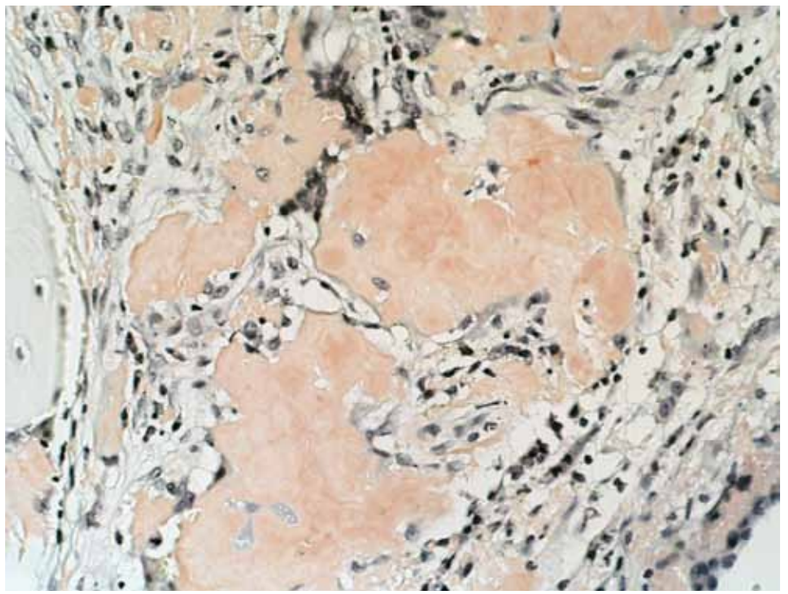
**Artisan™ Sample Staining.** Acid-fast bacteria demonstrated in a tuberculosis granuloma by a Ziehl-Neelsen Stain (x200).







**Artisan™ Sample Staining.** Bacteria visualized with the Gram Stain in a purulent infection (x60).



**Artisan™ Sample Staining.** Amyloid deposits in case of amyloidosis A visualized with the Congo Red Stain (x20).

The following table is an example of a Warthin-Starry silver staining for the detection of *Helicobacter pylori.*

| Manual Procedure | Artisan™ Procedure |
|---|---|
| Clean glass cuvette before using deionized water | |
| Deparaffinize in xylene x 2, 3 min each | Deparaffinize in xylene x 2, 3 min each |
| Absolute alcohol x 2, 3 min each | Absolute alcohol x 2, 3 min each |
| 95% alcohol x 1, 2 min | 95% alcohol x 1, 2 min |
| Submerge slides in deionized water | Pre-soak slides in wash solution <5 min |
| N/A | **Prepare Artisan™ Instrument** |
| | Bring reagents to room temperature for 45 min |
| | Prime and load reagents onto the instrument |
| | Select procedure to stain slides |
| | Apply printed labels to slides |
| | Load slides, apply wash solution, pre-soak |
| **Begin staining procedure** | **Select START to begin staining procedure** |
| Incubate in 1% AgNO3 30-60 min at 45-60°C | Instrument applies gelatin for 110 sec at 60°C |
| Prepare developer | Instrument applies AgNO3 for 200 sec at 60°C |
| Incubate in developer for 1 min at 56°C | Instrument applies hydroquinone 105 sec at 60°C |
| Microscopically select end development point | Wash solution x 6 changes at room temperature |
| Wash in warm tap water to stop reaction | Staining done ~7 minutes |
| Wash in distilled water at 56°C | N/A |
| Wash in cold distilled water | |
| Incubate in 2-5% sodium thiosulfate for 1 min | |
| Wash in distilled water | Remove slides from the instrument |
| Absolute alcohol x 2, 3 min each | Absolute alcohol x 2, 3 min each |
| Xylene x 2, 3 min each | Xylene x 2, 3 min each |
| Apply cover glass | Apply cover glass |



Automating Special Stains using Artisan™ and **Artisan**Link Special Staining System

Special staining procedures are more or less time-consuming and require manual labor for a considerable amount of the daily routine work. Even when reagents are supplied in kits, the manual application to tissue sections remains to be time-consuming and laborious. Therefore, automation of these procedures is most welcome to histopathology laboratories.

Following automation of special stains, the laboratory does not have to purchase and store raw materials, spend time on preparation, worry about the quality and stability or the potential toxicity of the reagents. On the other hand, it is nearly impossible to standardize these stains when performed manually on a day-to-day basis. Moreover, there is health risks associated with the manual staining method. The different dip tanks for the various stains must be cleaned every day. Most of them are not closed, which means that the toxic vapors leave the tanks without filtering. The incubation time is often overrun, temperatures must be kept stable and preparation and mix of the reagents must be done properly. Technicians can be much more efficient, if they do not have to do the special stains manually and can carry out other working procedures at the same time (Fig. 1).

## Automating Special Stains with Artisan™

Automation can significantly reduce many of the concerns associated with special stains reagents. Most of the platforms apply to ready-to-use reagents. This implies that the laboratory does not have to purchase and store raw materials, spend time on mixing and preparing reagents, and does not have to worry about the stability of the reagents.

The Artisan™ staining instrument is a new generation staining system that combines the flexibility of manual staining with the precision and consistency of automated staining. The reproducibility and quality of the staining system is very high, and the system also takes care of safety aspects. With this system, the toxic reagents are handled automatically. Thus the in-built waste management system separates the toxic from the non-toxic wastes, thereby reducing the volume of hazardous substances that may otherwise end up in the environment.

A big advantage of the new and improved **Artisan**Link Special Staining System is that the different staining reagents that come with the system are stable for a long period of time and do not require daily preparation of reagents. The cartridges are lightproof and equipped with precise reagent dispensing devices. There is no need for special slides, conventional glass slides may be used. The instrument also comes with software that has preprogrammed procedures, a reagent inventory management system and reporting templates.

## **Artisan**Link **Special Staining System**

Currently, histopathology laboratories are designed according to work-flow principles with an increasing degree of automation, integration of individual instruments, and tracking of all specimens. Unlike the classic Artisan™, which was a stand-alone instrument, the new **Artisan**Link Special Staining System can be utilized to operate either in a single or in a networked configuration mode using a Local Area Network (LAN)/Laboratory Information System (LIS) to connect up to three instruments via a specialized software. The unit provides 24/7 access to stainer processing status and delivers precision dispensing of reagents for optimal staining quality. In addition, the instrument can track specimens employing a two-dimensional (2D) barcoding system. Specimens, tissue blocks, and slides and tubes for nucleic acid extracts can be labeled with 2D barcodes which replace manual reading and transcription of numbers thus introducing new quality aspects of laboratory work with an increased degree of patient safety. The system can also read 2D barcodes printed on slides, including those subjected to special staining. Assignment errors such as allocating the wrong staining procedure to a slide are thus eliminated in this workflow procedure. In addition, the system locates case records using two-dimensional codes printed on the slide labels to assign the correct staining procedure to all slides.

The special stains that are available for the **Artisan**Link Special Staining System include:

| | |
|---|---|
| Acid-Fast Bacteria (AFB) Stain/ Ziehl-Neelsen Stain | Alcian Blue Stain pH 2.5 |
| Alcian Blue/PAS Stain | Gram Stain |
| Iron Stain | Alcian Blue/PAS Hematoxylin Stain |
| Jones' Basement Membrane (PAS-M) Stain/ Mucicarmine Stain | Congo Red Stain |
| Elastic Stain | PAS-Green Stain |
| Periodic Acid-Schiff (PAS) Stain | Giemsa Stain |
| Grocott's Methenamine Silver (GMS) Stain | Reticulin/No Counterstain |
| Gomori's Trichrome Stain | Masson's Trichrome (TRI) Stain |
| Gomori's Green Trichrome Stain | Warthin Starry Stain |
| Feulgen Stain | |

## Conclusion

In conclusion, the **Artisan**Link Special Staining System provides the flexibility of manual staining coupled with the precision and consistency of automation. Unlike the manual method, there is no mixing or monitoring of time dependent reaction steps. Furthermore, chemical hazards from mixing of reagents are eliminated and the variability of special stains results is greatly reduced. Last, but not least, the instrument allows the histotechnologist to perform other tasks while the slides are stained. The new **Artisan**Link Special Staining System also allows one instrument to communicate with two more instruments with specialized software.

**Suggested Reading**

White PI (2007). World Congress of Pathology Informatics. Brisbane, Australia.



# Chapter 32 | Troubleshooting Procedure for **Artisan**Link Special Staining System

**Debra Cobb, HT, MBA**

Automation in histology is here to stay. With the shortage of experienced histotechnologists, an increased workload, and demands for faster turnaround times, laboratories are looking for ways to produce consistent, quality-stained slides with less staffing in the most efficient time.

The arrival of automated instruments has forced histotechnologists to familiarize themselves with hardware, software and chemistry in order to adequately troubleshoot automated platforms. Understanding the differences and the similarities between manual and automated special staining is half the battle when it comes to troubleshooting.

## Back to Basics

Every histotechnologist understands that to achieve quality staining results performed manually the following basic factors should be in place in the laboratory:

1. Proper grossing, infiltration of tissue with fixative, and dehydration steps.
2. A certain tissue thickness recommended for a particular procedure. For instance, it is recommended to cut tissue for staining with Congo Red into a thickness of 8 µm and tissue for staining with Jones' Basement Membrane Stain is recommended to be cut into a thickness of 2 µm.
3. Drying slides at the right temperature because drying slides at temperatures above 62° C can cause damage to the tissue leading to a suboptimal staining.

The same basic factors are required for staining slides on an automated instrument such as **Artisan**Link. However, two more factors should be taken into consideration:

(a) The user must follow the procedures and instructions as provided by the instrument manufacturer.

(b) The user must follow a daily maintenance procedure in addition to the biannual or annual preventive maintenance procedure.

## Troubleshooting Issues

When troubleshooting **Artisan**Link Special Staining System, the histotechnologist will not only be required to rely on his/her knowledge of histology procedures and special stains chemistry, but also on his/her computer skills in order to optimize staining protocols using the **Artisan**Link software. Because the instrument applies reagents as well as heats and incubates according to the instructions given by the user, it is important that the user programs the instrument properly taking into consideration that all other processes such as grossing, sectioning, fixation, etc are optimized before the sample is placed on the instrument. In other words, how to optimize individual slides based on prestaining processes can only be defined by a user.

The most common staining issues on **Artisan**Link are:

- Staining artifacts
- Background staining
- Inconsistent staining

### Staining Artifacts

**Problem:**

(1) Tissue slides stored for long periods of time (months to years).

(2) Dust particles settling on slides, especially, those that are charged.

(3) Dispensing of reagents from dirty bulk liquid containers (Fig. 1).

**Solution:**

(1) For slides that may be dusty, it is recommended to try the following procedure after the deparaffinization and hydration steps:

- Place the slides in running tap water for 2-3 minutes.
- Rinse in deionized water.
- Place the slides in Artisan™ Wash Solution for 5 minutes before loading them on **Artisan**Link.

(2) For slides that have dispensing artifacts, it is recommended to use clean bulk liquid containers.

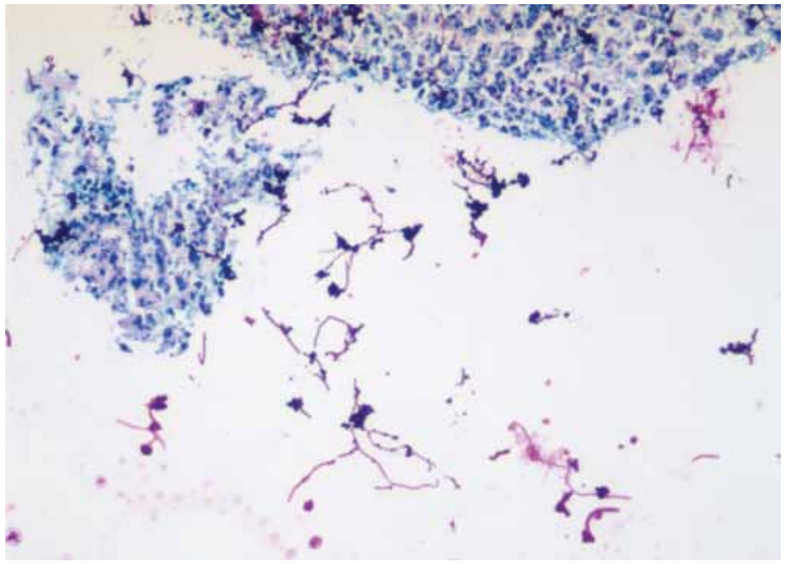


**Figure 1.** Artifacts on slides caused by dirty bulk liquid containers.

Reagent packs that are placed on the instrument need to be checked for any type of build-up of chemical deposits that can be dispensed on the slide during the staining run. It is recommended to check the dispenser tip and to mix and prime the packs before daily use (Fig. 2). It is also important to store the reagent packs tip down to prevent damage to the dispenser's internal parts.

Staining artifacts may also arise from reagent dyes. Dyes such as Nuclear Fast Red or Crystal Violet are known to cause artifacts. For instance, Nuclear Fast Red is sensitive to cold. If the reagent is stored in the refrigerator or left out on the loading dock in freezing weather, there is a risk that the reagent will start to precipitate. Once this reaction starts, it will continue regardless of correcting the storage temperature. This precipitate will then be dispensed onto the slide (Fig. 3a). Crystal Violet is another example of a dye that has a tendency to leave crystal-like shards on the tissue (Fig. 3b).

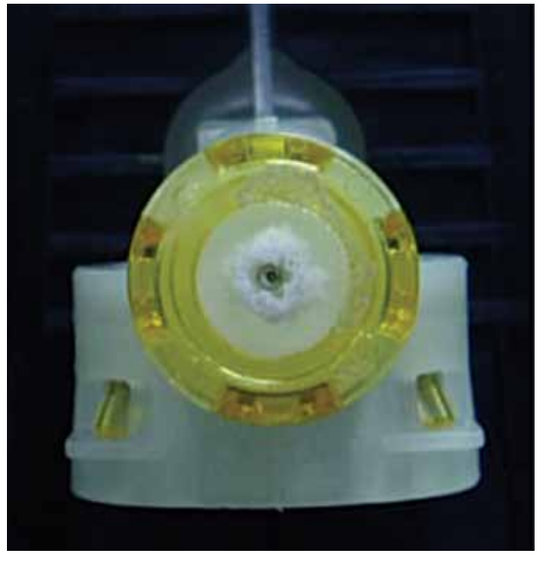


**Figure 2.** Dispenser showing build-up of chemical deposits.





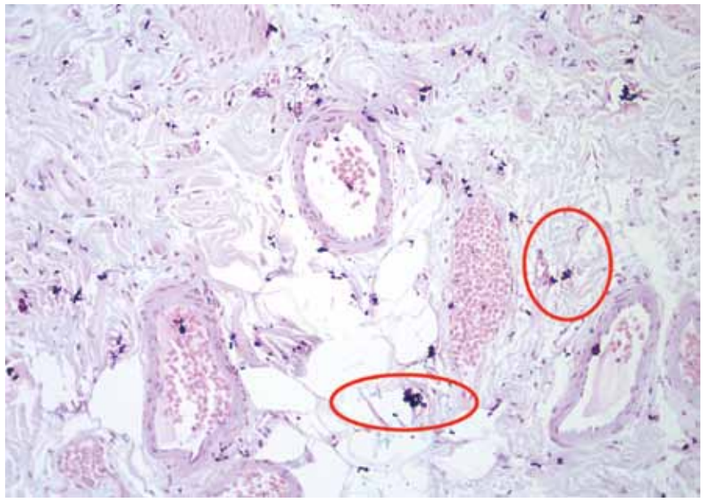


**Figure 3a**. Nuclear Fast Red precipitate.

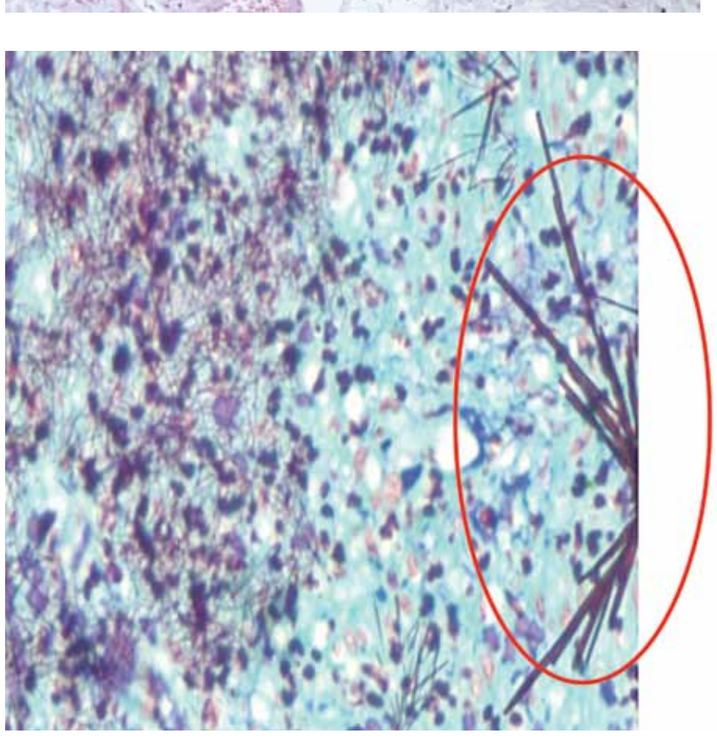


**Figure 3b.** Crystal Violet showing shards.

Reagents used on **Artisan**Link have been validated and optimized for staining quality as well as for correct storage and usage temperatures. It is important that the user follows the recommended temperature for storage and usage for each of the staining kits. All **Artisan**Link staining kits have been validated to be used at room temperature. Deviating from the recommendations may cause an uneven staining, shorten the shelf life, and possibly cause a false negative staining. Reagent storage and usage temperatures can be located on the reagent packs and in the package inserts.

## Background Staining

Background staining is a common problem when using charged slides or when there are additives in the water bath or by heat. When using a methenamine silver solution, a “mirroring” also know as grey haze can occur on the slide. “Mirroring” is defined as metallic silver deposited non-specifically over large areas of the front and back of the glass slide. The silver solution deposits a fine, brown-black, granular precipitate over the section and the glass portion of the slide (Fig. 4). Microscopically, this type of precipitate is usually not found on the same plane as the tissue (1).


**Figure 4.** Examples of “mirroring”/grey haze. The cause of this effect is over-heating of the methenamine solution.

To prevent “mirroring” or grey haze, it is important to check the temperature of the slide heater and/or the incubation time set in the software. Using non-charged slides (except for decalcified tissue) can also help lessen the amount of background staining when performing stains with silver nitrate solutions.





## Inconsistent Staining

This may be caused due to:

- Inconsistent tissue thicknesses from slide to slide. For instance, thicker sections will show a strong staining, whereas thinner sections will show a weak staining (Fig. 5). It is important to keep to the correct thickness throughout the microtomy sectioning to obtain a consistent staining.

**Figure 5.** Inconsistent tissue thicknesses from the same run. Warthin-Starry Stain.

- Cold reagent packs not brought to room temperature before use. All reagent packs used on **Artisan**Link Special Staining System must be brought to room temperature (22-25°C). Allow 45 minutes for the reagent packs to warm up before placing them on **Artisan**Link. Using a cold or cool reagent pack may prevent the chemical reaction from taking place, and will give the impression that the instrument is producing inconsistent staining results within the same run.
- Reagent packs not primed before use. This can cause either a non-dispense of reagent or an incomplete dispense of reagent. It is important to always prime the packs before daily use to ensure proper dispensing of reagents.
- Uneven staining. This can be caused by the reagent not spreading evenly across the tissue section or sections. This is particularly problematic with reagents containing acids such as acetic acid or periodic acid. The solution to this problem is to soak the slides in an Artisan™ Wash Solution for five minutes before loading the slides onto the slide carousel. This procedure conditions the slides for spreading the reagents (Fig. 6).

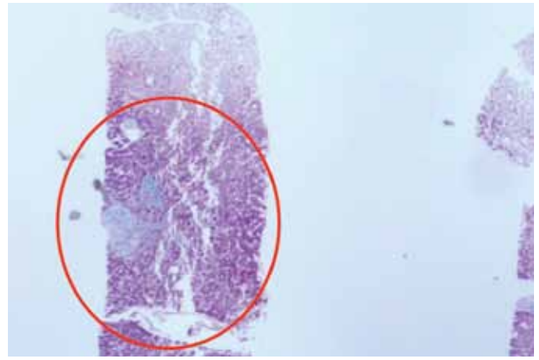


**Figure 6.** Periodic Acid-Schiff Diastase (m/D) staining of liver tissue. The uneven staining is due to amylase not spreading evenly- causing part of the liver section to stain undigested glycogen.

Another reason for an uneven staining may be the placement of the tissue sections on the slide close to the slide clip. This can be rectified by making sure that the tissue sections are centered on the slide. Placing the sections close to the slide clip causes a “wicking” effect which prevents complete aspiration of the reagents causing an uneven staining.

| Issue | Manual Procedure | **Artisan**Link Procedure |
|---|---|---|
| Staining Artifact | Store reagents at correct temperature; (See package insert for specification) | Store reagent packs at correct temperature; (See package insert for specification) |
| | Filter reagents before use | Mix and prime reagents before use |
| | Use clean slides | Use clean slides |
| | Clean glassware in soap and hot water | Clean bulk liquid containers |
| | Rinse in deionized water and air dry | Check dispenser tip for debri build-up |
| Background Staining | Use acid clean glassware | Use the default protocol for silver stains |
| | Do not use metal forceps | Do not add adhesives to the water bath |
| Inconsistent Staining | Employ proper fixation, dehydration and infiltration | Employ proper fixation, dehydration and infiltration |
| | Increase or decrease staining time by looking at individual slides under the microscope | Cut all sections into a thickness of 4 µm, except for Jones' Basement Membrane Stain for kidney which should be cut into a thickness of 2 µm and Congo Red Stain which should be cut into a thickness of 8 µm |
| | For an uneven staining, adjust the volume of reagent used to ensure proper spreading | Soak the slides in Artisan™ Wash Solution for five minutes before placing them on the instrument |

## Conclusion

The solution to staining artifacts, background staining and inconsistent staining for both the manual and the automated procedures is very similar. The table above shows the comparison.

**Artisan**Link Special Staining System provides the histotechnologist with a tool to produce the same diagnostic quality stains as found in a manual staining technique. However, unlike a manual staining, **Artisan**Link allows the histotechnologist to walk away from the special staining bench to pursue other tasks.

**Reference**

1. Brown RW (Ed.) (2009). Histologic Preparations: Common Problems and Their Solutions. College of American Pathologists, Northfield IL, 2009.



# Chapter 33 | Tips on using the **Artisan**Link Special Staining System

**Jamie M. Nowacek, BS, HT(ASCP)$^{CM}$, QIHC, PMP**

**Artisan**Link Staining System is an automated special stains slide processor that can be used as a stand-alone system or operated in a networked configuration using a Local Area Network (LAN) or a Laboratory Information System (LIS). The system uses special stains kits and accessory reagents packaged in patented cartridges that provide precision dispensing of reagents for optimal staining quality. **Artisan**Link utilizes a patented "reaction chamber" and can handle multiple tasks in a laboratory in a single run. With a broad menu of special stains, the system can optimize laboratory workflow through automation dramatically improving productivity. This article provides tips on hardware and software usage as well as some recommendations regarding the use of slides and reagents to get the most out of the **Artisan**Link Staining System[1].

## Hardware Tips

The following is a four-point inspection to keep **Artisan**Link running smoothly:

- **Waste management:** The **Artisan**Link Staining System utilizes a decrement system to track waste accumulation and capacity requirements. A special aspiration system allows the instrument to separate waste into four categories: metals, alcohol, combined dyes and water solubles. Depending on the laboratory workload volume, 2 L or 4 L bottles can be configured in the software. Upon completion of each session, a waste valve rinse should be performed to prevent contamination of the tubing and bottles. The user should inspect the inner orange waste gasket when replacing the cap to check that this has not been torn or ripped[2] (Fig.1).

  Keeping software and bottle inventory evenly matched at all times will prevent overflow and consequential aborted runs. Each time a bottle is removed, perform the Rinse valves (Fig. 2) from the **Artisan**Link bulk fluid screen to enable optimal aspiration.


**Figure 1.** Waste bottle showing the orange waste gasket.

[1]This article provides tips for experienced users. [2]A torn or ripped orange waste gasket may be the result of excessive torque applied when screwing/unscrewing the cap.

- **Bulk fluid maintenance:** Bulk liquids consist of liquids used in a staining run for rinsing or diluting. Dako Wash Solution, 100% ethanol and 95% ethanol, is required for use on the **Artisan**Link Staining System. Dako Wash Solution requires diluting and mixing prior to use. For bulk fluid maintenance, rotate the six bulk positions with adjacent bottles. When the alcohol bottle is rotated through each valve position routinely, it reduces the overall manual maintenance that will be needed outside slide sessions. Always unsnap the bottle tubing from the valve before unscrewing the cap to alleviate torque and pressure on the tubing connectors. When the caps are off, inspect the black o-ring inside the cap and return the cap with the notch to align and secure the cap tightly (Fig. 3). This will provide an even pressure and remove waste fluid during staining. Be sure to match software to bottle inventory. After reloading the bottle, prime three or more times to return the fluid into the tubes or lines.
- **Reagent handling:** At the end of each staining session, it is recommended to remove all reagent packs from the reagent carousel. Removing hazardous chemicals routinely will help maintain the life of the instrument.
- **Slide platform maintenance:** To ensure proper contact between the slides and the slide heater plates, the user should wipe the back of each slide with a dry gauze pad before placing it on the slide carousel. This will not only ensure a good slide clip seal, but will also optimize heating performance. Likewise, wiping residue from the slide platform upon removal of the slide and clip will prevent chemical erosion. For the same reasons, it is also recommended to regularly clean the spill tray and drip ring.

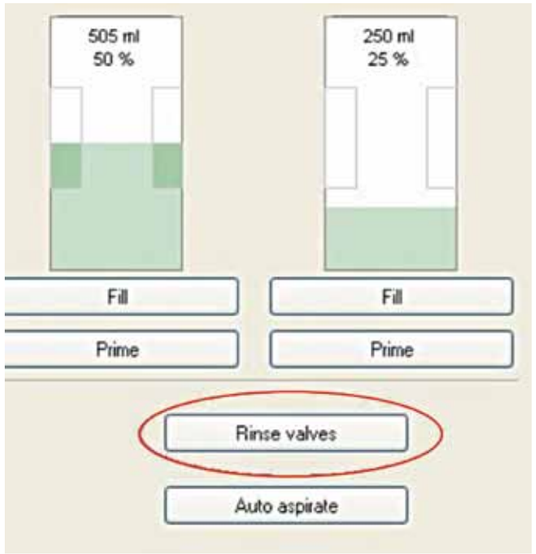



**Figure 2.** Fluid screen showing how to access the Rinse valves button referring specifically to waste valves.

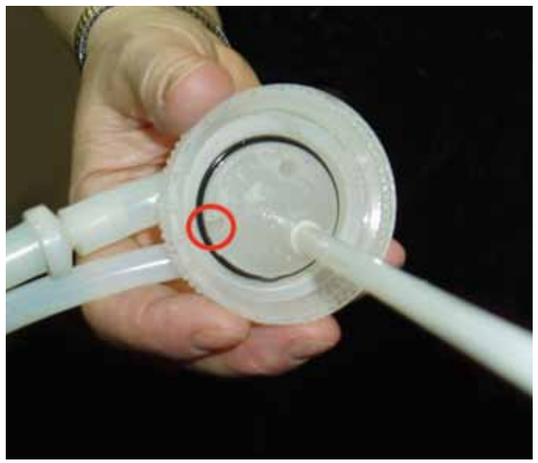


**Figure 3.** The inside of a bulk bottle cap showing the black o-ring. The red circle denotes the groove that should be aligned to the bottle neck notch.





## Software Tips

The **Dako**Link software allows review of workload information based on customized settings. Additionally, the software can track information previously tracked manually by the histotechnologist. It can measure and separate waste, track fluid inventory, maintain history of fluid lot numbers and expirations, provide customized audio alerts, perform automatic maintenance and, most importantly, identify slides. The handheld 2D barcode scanner can retrieve information about slides and reagents utilized during any given session. The reports generated can be exported while the stainer is still in use. Upon installation, the following suggestions may be helpful in maximizing the use of the **Dako**Link software:

- Customize user privileges allowing login access to inventory, report fields, system settings and procedure customizations. These are found under Administration/Configuration/USERS and GROUPS (Fig. 4).
- Set SYSTEM SETTINGS to “Automatically perform waste valve rinse after runs” and “Require confirmation before run begins”. This will automatically start the run and perform routine maintenance (Fig. 5).
- Customize the NEW SLIDES tab to fit the patient information that is pertinent to your Quality Control (QC) program. Slide accessioning will be easier, and a simple 2D scan of the slide label (see Fig. 6) will yield valuable information.

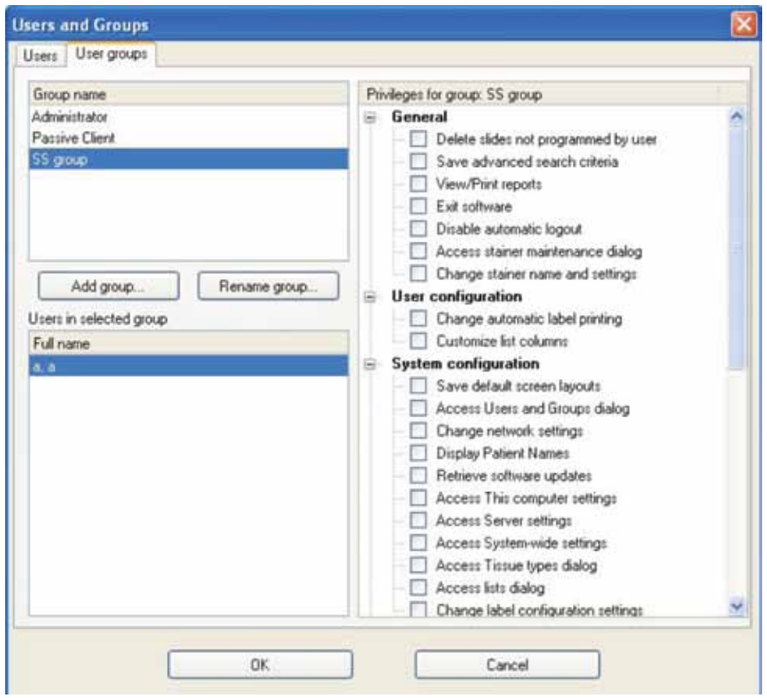



**Figure 4.** SYSTEM SETTINGS screen depicting possible privilege settings for User groups.

The **Artisan**Link software is part of the **Dako**Link software family.

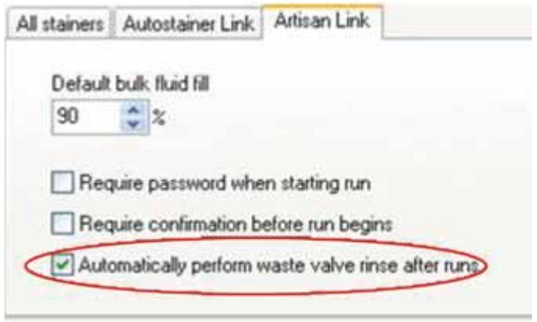



**Figure 5.** SYSTEM SETTINGS screen depicting automatic maintenance settings upon installation (default status).


**Figure 6.** A 2D slide label demonstrating customized information for quick identification.

- Set up slide labels to print customizable text. Maximize the text by utilizing "Slide notes" as a field option or placing Hospital institution as Case fields. Customizing slide labels using the Case fields option will ease slide accessioning and ultimately save time in the QC process that follows.
- Populate the bulk fluid lot numbers in the fluid inventory screen to ensure QC history reports are easily obtained upon demand. The assignment of bulk liquids automatically sets a 30-day reminder to rotate the fluids. The fluids are shown in red font when they have exceeded the 30-day reminder.
- While an instrument is staining, the user can set up the next session. Retrieve live updates on the reagent inventory utilizing the ADMINISTRATION report features. Multitasking by pulling out colder reagents needed for the next run can save valuable setup time.





## Tips on Slides and Reagents

### Slides

The type of glass slide used and how it is clipped into position can affect the staining procedure. Some tips to ensure a consistent staining result are:

- Avoid using slides with etched or painted surfaces. The uneven surface can prevent a consistent horizontal spreading of the fluid during sessions.
- Only use chemically resistant slide labels, e.g., Dako's S3393 (DL213), 0.75 x 0.75 mm labels. Larger labels will prevent the gasket from sealing tightly and as a consequence may leak fluid during the staining session.
- Slides, whether charged or uncharged, can be used for automation UNLESS "mirroring" occurs (see Reference). In this case, lowering the heat exposure to the slide or switching from ionically-coated slides to uncharged slides will reduce the phenomenon.
- Use clean slides: Wipe the back of the slide before placing it into position. This will not only ensure a good slide clip seal, but will also optimize heating performance.
- Make sure the slide is completely flat to seal the gasket firmly into place. This will ensure an even staining.
- Fill the slide clip with 2-3 mL of Dako Wash Solution (AR102) and presoak the tissue for a minimum of 5 minutes. This will condition the slide surface to aid in fluid spreading during the session.
- Store the slide clips in a clean and dry place to ensure a well-sealed gasket every time.

### Reagents

Every reagent pack specifies a storage temperature on the label. Each reagent kit comes with a package insert that includes the specifications for use. These include priming instructions, recommended control tissues, and intended use. Following the instructions given in the package insert is important for a good and consistent staining of the tissue. In other words, the staining quality of the last slide should be as good as the first one. Some tips for using reagents/reagent packs are:

- Reagent packs stored in a cold room should be brought to ambient room temperature before priming for staining use. This will usually take 30-45 minutes. If packs are cold at session startup, the chemicals continue to reach ambient temperature throughout the session. This can yield a varying temperature dispense from first to last slide.
- Reagent packs should not be shaken, but gently mixed prior to each session by inverting the pack several times. Some chemicals foam or release gases during motion which can damage plastic parts causing erroneous dispense behaviors.
- Each reagent pack should be primed until a steady stream is obtained. A good technique is to point the tip away from you into an approved waste container. This is done by holding the pack at a slight angle, tapping the side gently and priming with a plunge of the dispenser. Priming ensures that no air is left in the bottle neck during the dispense.

  NOTE: A steady prime will equal 250 µL. Dako provides extra reagent for each test pack to accommodate priming.
- Reagent packs should be placed on the carousel immediately after priming while maintaining them in a tip-down orientation. This will ensure a first full dispense and that no air is left.
- Reagent packs should be stored at their labeled temperature in a tip-down orientation, using Dako provided reagent storage trays, AR409. It is advised not to leave reagent packs on the carousel when the reagents are not in use. Proper storage will maintain manufacture label shelf life.
- Refrain from writing on the spine of the reagent packet labels as this may interfere with the barcode scanning. If the pack will not scan, the session will not begin.

  NOTE: The handheld 2D barcode scanner can be used at any time to verify inventory.

In summary, making the hardware and software work together along with other parameters such as using the right slides, reagents/ reagent packs and chemistry is the first step toward mastering the automated staining process. With the tips described in this article, you should be able to obtain consistent quality staining results without making extensive changes to the system or using additional time in the laboratory.

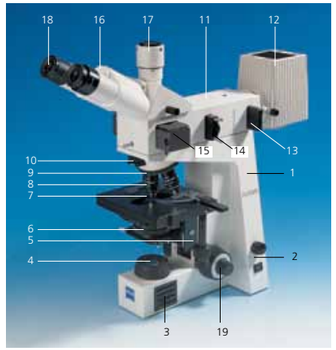
**Reference**

Brown RW (Ed.). Histologic Preparations: Common Problems and their Solutions. College of American Pathologists, Northfield IL, 2009, pp 86-91.





## Basic Components of a Light Microscope




1 Microscope stand
2 Power switch and light control
3 Built-in illuminator
4 Variable luminous-field diaphragm
5 Condenser mount with height adjustment
6 Transmitted-light condenser with variable aperture diaphragm
7 Specimen stage with object guide
8 Objective
9 Nosepiece
10 Filter mounts, in transmitted light on the observation side
11 Illumination equipment for epi-fluorescence
12 Lamp for epi-fluorescence
13 Filter slider for reflected-light fluorescence
14 Luminous-field diaphragm for epi-fluorescence
15 Reflector slider for epi-fluorescence
16 Binocular tube
17 Camera/TV port, switchable
18 Eyepieces
19 Focusing drive

**Color of the inscription:**
Contrasting method – see color chart above the objective. (A)

**Description of the objective:**
Objective category.
Explanation on page 31
plus special descriptions, such as "LD" for "Long (working) Distance", and others.

**Magnification / Numerical Aperture:**
plus: additional information on
– immersion medium ("W", "Oil", "Glyc")
– Adjustable cover slip correction ("Korr")

**Tube length / Cover slip thickness (mm):**
ICS-Optics: "∞"
Classical optics: "160"

Standard cover slip: "0.17"
Without cover slip: "0"

**Color coding for the magnification:**
See the color chart below the objective (B)

**Mechanical focusing ring: only for special objectives**
Allows the adaptation of the optical correction for different immersion media and / or the thickness of cover slip / chamber bottom ("Korr").
Also with objectives with variable aperture iris, e.g. for darkfield.

**Type of immersion liquid:**
See the bottom color chart (C)

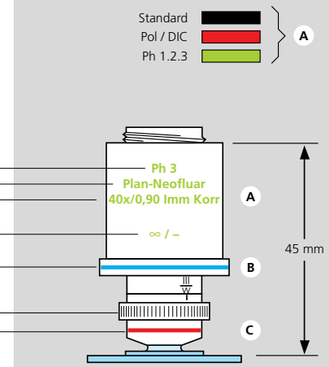
Other information on objectives *not* included in the inscription:

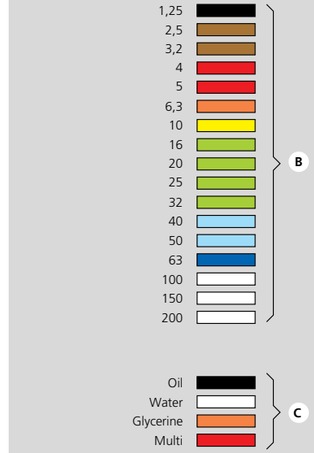
**Connector thread:**
W 0.8" or M 27

**Parfocal length:**
45 mm for all objectives
Covers the distance from the screw-on surface to the object plane including cover slips.

**Free working distances (AA):**
are indicated in mm and describe the distance from the tip of the objective to the specimen surface – or the cover slip surface, where applicable.

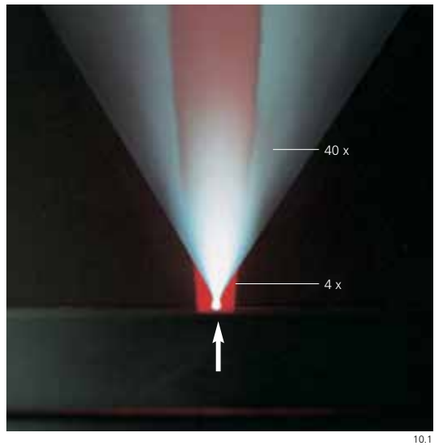
**Information by colors:**
**The labelling of the microscope objectives**



**Everything is under control: the path of the light rays – from the illuminator to the eye**

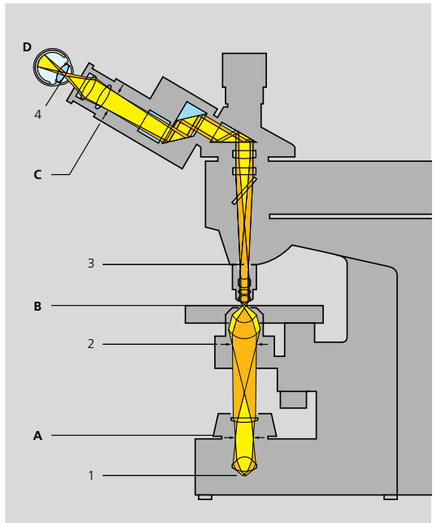
The design of a microscope must ensure that the light rays are precisely guided through the microscope. Only this will make it possible to obtain a bright image even with illuminators of a low wattage. A lack of brightness is no problem in simple brightfield microscopy, but if contrasting techniques such as phase contrast or polarization contrast are used, further optical elements which use up a great portion of the available light flow are inserted into the beam path. This leaves little light for observation, and, as a result, the images become dark.




10.1

A further important reason for the existence of diaphragms and filters in the microscope is that, strictly speaking, the illumination should be reset after every change of objectives for two reasons. Firstly, the size of the specimen section observed changes with every objective change. An objective with a low magnification, e.g. 4x, provides a large field of observation (with a diameter of as high as 5 mm in this case, provided that the eyepiece permits an intermediate image of dia. 20 mm). If a switch is made to the 40x objective, the diameter of the visible field of the specimen shrinks by the factor 10 (to only 0.5 mm). The viewed area becomes as much as 100x smaller. The second reason is that the numerical aperture increases from 0.12 to 0.65 or, expressed as aperture angles, from 15° to 80°. These two cases are illustrated in Fig. 10.1 above.

However, the Köhler guidelines require that nothing more than the viewed field in the specimen is illuminated, since the "excessive" light outside the field of view is disturbing, scattered light. At the same time, however, the light cone of the illumination should always be matched to the angular aperture of the objective to allow the numerical aperture of the optics to be utilized. This is the only way to achieve maximum resolving power.




10.2

The condenser containing the aperture diaphragm (2) and the luminous-field diaphragm (A) normally contained in the stand base are the aids which make all this possible. A closer look reveals that the luminous-field diaphragm is imaged on the specimen by means of the condenser. The luminous-field diaphragm determines which part of the specimen is illuminated. The aperture diaphragm, however, is imaged on the "pupil" of the objective (3) and regulates the illumination of this pupil. The entire optics are computed in such a way that aperture angles of the light cones are correctly set together with the aperture diaphragm.

Therefore, the microscope contains two different groups of optical planes which belong together.
The first group consists of:

1 = lamp filament
2 = aperture diaphragm
3 = objective pupil
4 = pupil of the observer's eye

This group defines the beam path of the pupils and determines the resolution of the microscope.

The other group contains:

A = luminous-field diaphragm
B = specimen plane
C = intermediate image in the eyepiece
D = retina of the observer's eye

A to D are the important optical planes in the image-forming beam path. The image becomes visible here and the image limits are set.
Within a group, the planes are always imaged one on the other. We speak of "conjugate" planes, which means that they are "connected with each other".





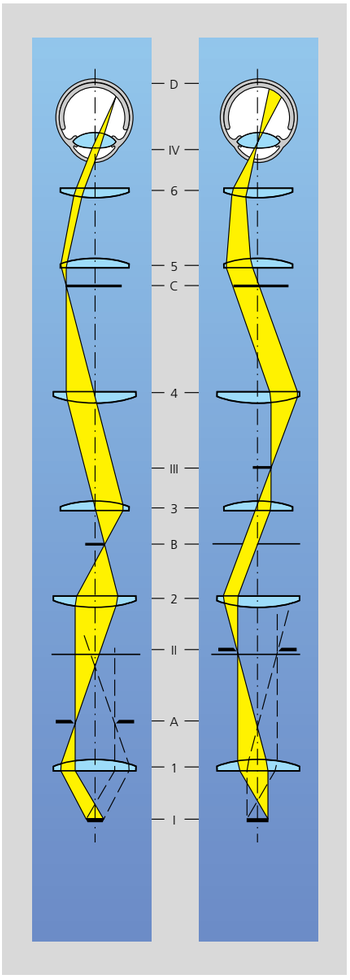
It is no exaggeration to say that almost the entire art of microscopy – if specimen preparation is not taken into account – consists in the correct use of the luminous field and aperture diaphragms. Thankfully, there are simple rules for this. The following pages describe in detail how to correctly set the microscope for Köhler illumination. It will be much easier for you to understand the Köhler principle if you made yourself familiar with the meaning of the special optical planes mentioned before. Their relationship with each other can be simplified as follows:

| | | |
|---|---|---|
| Planes "1" to "4" | **Pupils** | For resolving power, contrasting techniques, brightfield.<br><br>Components: light filter and contrasting.<br><br>Setting is made via the **aperture diaphragm**. |
| Planes "A" to "D" | **Fields** | For fields of view, illumination, intermediate images.<br><br>Components: reticles and scales.<br><br>Setting is made via the **luminous-field diaphragm**. |

The fact that the beams are superimposed may also have negative consequences. The best example of this are dust particles on a reticle in the eyepiece: these particles will be sharply imaged together with the microscope image and do not exactly embellish the microphoto.

The above makes it clear that the condenser – which concentrates the illuminating light ray on the specimen – plays a vital part in microscopy: it is as important as objectives and eyepieces. The condenser makes the specimen appear in the right light.

If you only want to get optimum intensity in the illumination, select the critical illumination system, where the light source is not imaged on the pupils, but on the object. The homogeneity of the illumination is lost, however. This type of illumination is obtained by the deliberate adjustment of the lamp collector (fluorescence).




**Schematic beam paths in a microscope with ICS optics (Infinity Color-corrected System)**

Left: **The image-forming beam path**

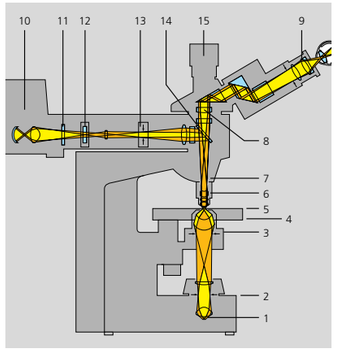
- A = Luminous-field diaphragm
- B = Specimen plane
- C = Intermediate image
- D = Retina of the observer's eye

Right: **The pupil beam path**

- I = Lamp filament
- II = Aperture diaphragm
- III = Objective pupil
- IV = Pupil of the observer's eye

The main imaging components are:

1 Collector
2 Condenser
3 Objective
4 Tube lens
5 Eyepiece

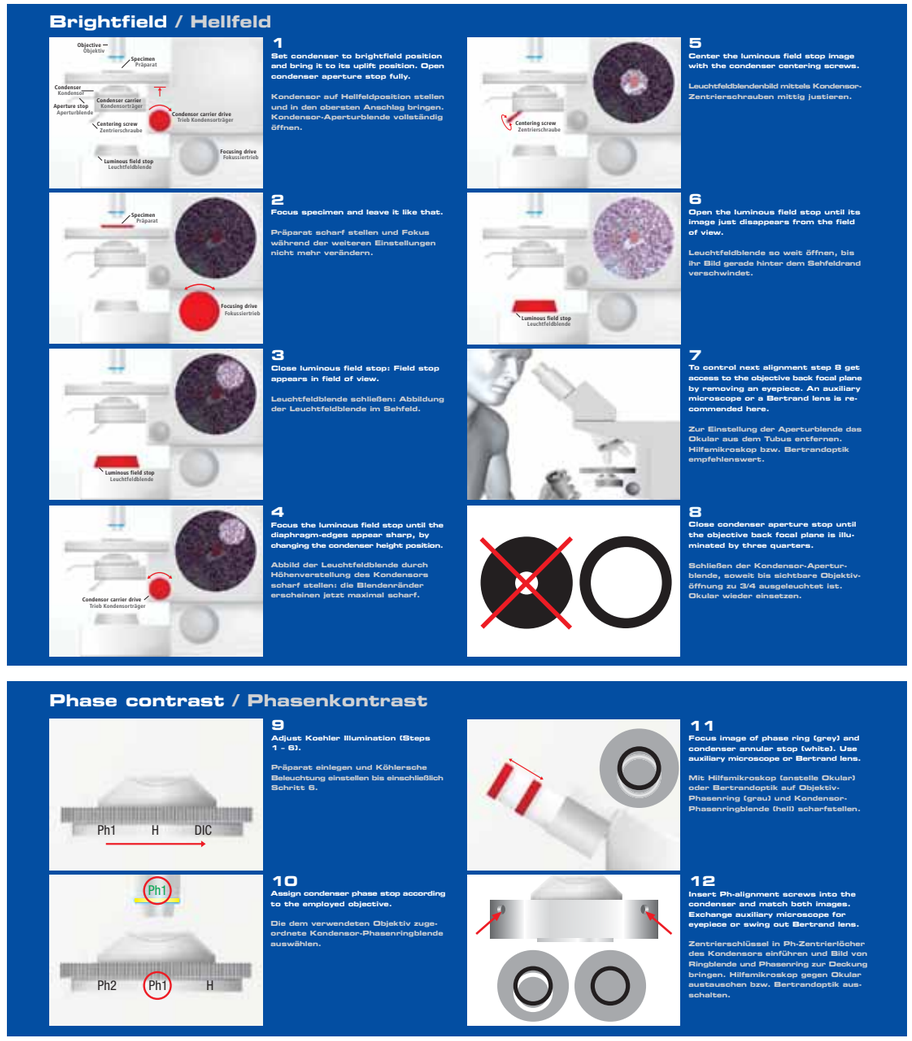



1 Built-in illuminator with collector
2 Luminous-field diaphragm for transmitted light
3 Aperture diaphragm in the transmitted-light condenser
4 Transmitted-light condenser (front optics)
5 Specimen plane (focal plane)
6 Objective
7 Pupil plane of the objective
8 Tube lens
9 Eyepiece
10 High-pressure lamp with collector
11 Heat-protection filter
12 Filter slider / Beam stop
13 Luminous-field diaphragm for epi-fluorescence
14 Filter combination with beam splitter for epi-fluorescence
15 Camera / TV adapter





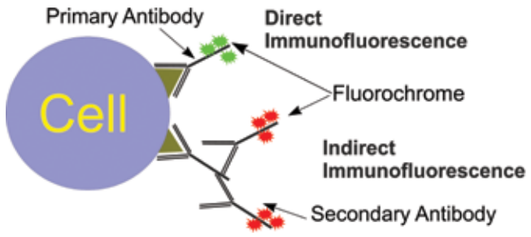
## Köhler Illumination




Dr. Jochen Thamn. Carl Zeiss MicroImaging, Inc. Thornwood, NY, USA.

Text Adapted from the book "Microscopy from the very beginning": Author Dr. H. G. Kapitz, Editor: Dipl.Bibl. Susanne Lichtenberg. Carl Zeiss Jena GmbH, 1997

## Direct Immunofluoresence

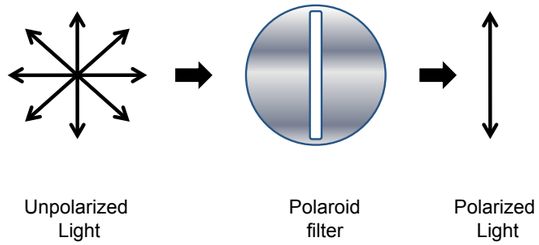



**Schematic of Direct and Indirect Immunofluorescence**

The two main methods of immunofluorescent labelling are direct and indirect. Less frequently used is direct immunofluorescence where the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect immunofluorescence, the antibody specific for the molecule of interest (called the primary antibody) is unlabeled and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye.

Figure: Courtesy of Prof. J.P. Robinson. Purdue University, West Lafayette, IN USA.

## Polarization




A light wave which is vibrating in more than one plane is referred to as unpolarized light (e.g., light emitted by the sun; see figure on the left). It is possible to transform unpolarized light into polarized light using a Polaroid filter (center). Polarized light waves are light waves in which the vibrations occur in a single plane (right). Microscopes that come equipped with accessories for observing polarized light are called polarized light microscopes.



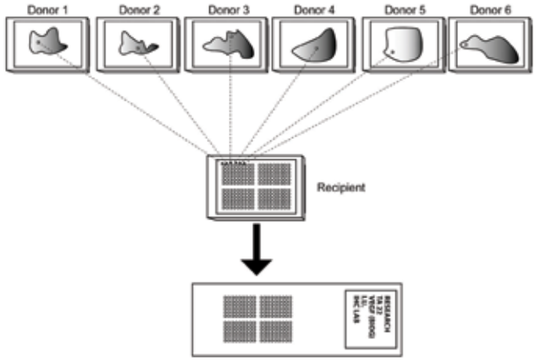


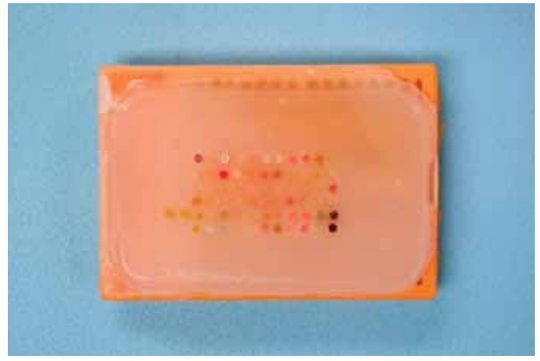
## Tissue Microarrays

Tissue Microarrays (also called TMAs) consist of paraffin blocks in which up to 1000 separate tissue cores are assembled in array fashion to allow multiplex histological analysis (Wikipedia).

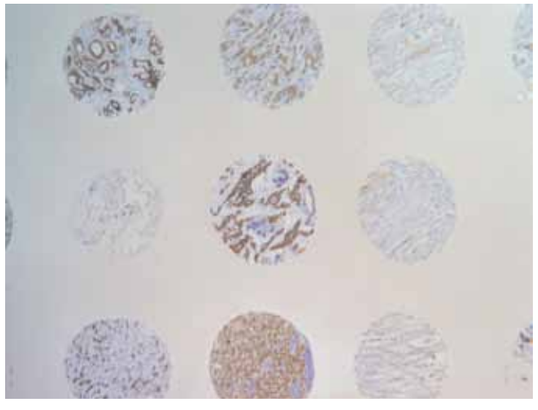
### Principle of Tissue Microarray (TMA) Analysis

Cylindrical cores are obtained from a number (up to a 1,000) of individual formalin-fixed paraffin embedded tissue blocks. These are transferred to TMA. Each TMA block can be sectioned up to 300 times. All resulting TMA slides have the same tissues in the same coordinate positions. The individual slides can be used for a variety of analyses saving labor and reagent costs while maintaining uniformity of assay.

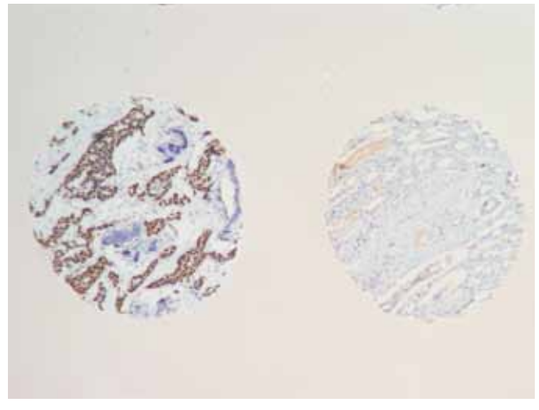


Photograph of a low density TMA block.



Low power photograph of a stained TMA.



High power photograph of a stained TMA.

Courtesy: Rashmil Saxena, BFA, HT(ASCP)CM ; Sunil Badve, MD, FRCPath. Indiana University, Indianapolis, IN, USA.

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# Glossary

This Glossary is not intended to be an all-encompassing list of terminology as used in special staining. Rather, it assumes a basic level of technical knowledge beyond which the included definitions were selected to help in clarifying the text of this Guide.

**Absorption** The penetration and coloring of a tissue element without chemical changes or reactions.

**Acetylation** of the carbohydrates in a section of tissue, done usually with acetic anhydride, blocks hydroxy groups, preventing the PAS reaction, lectin affinities etc.

**Acidic Dye** A negatively charged dye that easily stains acidophilic or eosinophilic elements, such as the cytoplasm, muscle and collagen.

**Acidophilic** A substance or tissue element, usually basic in nature, that is easily stained with acid dyes.

**Acylated** Having one or more hydroxy groups esterified by an organic acid. This is often acetic acid, $CH_3COOH$, in which the acyl group is acetyl, $CH_3C(O)$—. The acyl group replaces the H of —OH. Some sugars, notably sialic acids at C7, C8 or C9, are naturally acylated. Acetylation of the carbohydrates in a section of tissue, done usually with acetic anhydride, blocks hydroxy groups, preventing the PAS reaction, lectin affinities etc.

**Aldehyde** A reactive organic compound containing carbon, oxygen and hydrogen.

**Aldehyde Fuchsine** A mixture of rather hydrophobic purple cationic dyes produced by reaction of pararosaniline (a major component of basic fuchsine) with acetaldehyde. It binds with high affinity to elastin (a hydrophobic protein) and to locally high concentrations of carboxylic acids, sulfate esters, or sulfonic acids produced in tissues by oxidation. The shape of the aldehyde fuchsine cation is probably responsible for preventing staining of nucleic acids.

**Anion** A negatively charged ion (would be attracted to the anode in electrophoresis).

**Aminergic Neurons** Cells of the nervous system that secrete amines to communicate with other neurons or with smooth muscle or secretory cells. Metabolism of the catechol amines (notably dopamine and noradrenaline) results in accumulation of neuromelanin. This pigment accounts for the dark coloration of the substantia nigra in the midbrain and the locus coeruleus in the pons.

**Anionic** Having a negative electrical charge, which attracts cations to a positive electrode (anode) in an electrolytic cell. Anionic dyes have negatively charged colored ions that are attracted by positively charged materials (such as protein molecules with protonated amino groups) in objects being stained.

**Anthraquinone Dyes** Dyes that contain the anthraquinone structure, including alizarin red S and nuclear fast red.

**Anthraquinone Purgatives** A group of plant-derived drugs that are glycosides of anthraquinone compounds. Notable examples are extracted from *Cascara* and *Senna* species.

**Argentaffin** A reaction in which silver impregnation and subsequent reduction result in visualization of a tissue element.

**Argyrophilic** The ability to bind or be impregnated with silver ions.

**Artifact** An element that is not normally present but that is produced by an external action.

**Background** All nonspecific staining that results from procedural artifacts.

**Bacteria** are unicellular organisms that do not contain a nucleus or other membrane-bound organelles. Most bacteria have a rigid cell wall composed of peptidoglycan. Although there are exceptions, bacteria come in 3 basic shapes: round (cocci), rod-like (bacilli) and spiral (spirochetes). Bacteria cause a variety of infections in various organs.

***Bartonella*** is a Gram-negative bacillus which causes cat-scratch disease. The bacilli are transmitted to humans by cat-bite or cat-scratch.

**Basic Dye** A positively charged dye that easily stains basophilic elements.

**Basophilic** A substance or tissue element, usually acidic in nature, that is easily stained with basic dyes.

**Birefringence** The splitting of a light wave into two waves that are refracted in different directions.

**Birefringent** In microscopy, this means visible (bright) against the dark background that results from crossed plane polarizing filters.

**Carbohydrates** Compounds, including sugars, starches and cellulose, that contain only carbon, hydrogen and oxygen.

**Cation** A positively charged ion (would be attracted to the cathode in electrophoresis).

**Cationic** Having a positive electrical charge, attracted to the negative electrode (cathode) in electrolysis or electrophoresis. Cationic dyes have positively charged colored ions that are attracted towards negatively charged materials (including nucleic acids and the acidic carbohydrates of extracellular matrix and of many glycoproteins) in objects being stained.

**Chelate** A stable complex in which a metal atom forms part of a five- or six-membered ring of atoms, usually including carbon with oxygen or nitrogen.

***Chlamydia*** are Gram-negative bacteria which are unusual because they do not have typical bacterial cell walls. They are obligate intracellular parasites which means that they can only survive within cells. *Chlamydia* cause sexually transmitted diseases and pneumonia in humans.

**Corpus Striatum** The deep white matter of the center of a cerebral hemisphere, comprising, in the human brain, the caudate and lentiform nuclei. Functions include the encoding of movements and cognitive activities such as motivation and planning. Disorders typically include abnormal involuntary movements of the limbs or face.

**Chromophilic** Capable of being stained readily with dyes.

**Chromophore** The specific chemical grouping that bestows the property of a color on a compound.

**Chromotropic** Sometimes applied to materials that stain metachromatically.

**Clearing** The process of replacing alcohol with a reagent that can be mixed with paraffin.

**Connective Tissue Mucin** The carbohydrate-rich component of extracellular matrix, composed mainly of GAGs; stained by alcian blue and often weakly PAS-positive.

**Counterstain** A secondary stain that is applied to provide a visual contrast to the primary stain.

**Cytopenias** A deficiency or lack of cellular elements in the circulating blood.

**Decolorization** The removal of color from tissue.

**Dehydration** The removal of water from tissue.

**Deparaffinization** The removal of paraffin from a tissue section.

**Diammine** A cationic complex in which two ammonia molecules are bonded to a metal ion.

**Differentiation** The removal of excess stain from a tissue section so that only the desired element remains stained.

**Diol** A compound in which there are two —OH groups on the same or different carbon atoms.

**Dyserythropoiesis** Cytologic dysplastic features in erythroid cells.

**Dyshematopoietic** Dysplastic changes in hematopoietic cells.

**Eosinophilic** A substance or tissue element, usually basic in nature, that is easily stained with acid dyes.

**Epithelial Mucin** Mucus, either within secretory cells or already secreted and lying on the surface of an epithelium or in the cavity of a duct or hollow organ.

**Ester** The compound formed when an alcohol or a phenol combines with an oxygen-containing acid, with elimination of water. ROH + HO(O)X = R—(O)X + $H_2O$.

**Fixation** The stabilization of protein.





**Gaucher's Disease** Gaucher's disease is caused by an inherited deficiency of an enzyme that is involved in lipid metabolism. As a result, an abnormal biochemical metabolite accumulates in various tissue including the spleen, liver, bone marrow and kidneys.

**Genetic Hemochromatosis** Genetic hemochromatosis is a genetic disorder that causes the body to absorb excess iron from the diet. The excess iron accumulates in the liver, pancreas, heart and joints. Excess iron is toxic to these tissues.

**Glycol** An arrangement in which hydroxy groups are bonded to two adjacent carbon atoms. Glycols are sometimes called *vic*-diols (vicinal = neighboring).

**Goblet Cell** An epithelial cell containing a spherical or elliptical body of mucus awaiting extrusion from the cell's apical surface.

***Helicobacter pylori*** is a Gram-negative bacteria which causes inflammation (gastritis) and ulcers of the stomach. The name derives from the Greek "helix" for spiral and "pylorus" for the distal end of the stomach.

**Hematein** The oxidation product of hematoxylin; the active ingredient in hematoxylin solutions.

**Hexose** A monosaccharide with six carbon atoms.

**Impregnation** The deposition of metals on or around a tissue element of interest.

**Incubation** To maintain tissue sections at optimal environmental reactions for a desired chemical reaction to occur.

**Jaundice** Yellow discoloration of the skin and other tissues caused by an abnormally high concentration of bilirubin in the blood.

**Lake** The combination of a mordant with a dye.

***Legionella*** is a Gram-negative bacillus so named because it caused an outbreak of pneumonia in people attending a 1976 convention of the American Legion in Philadelphia. The organism was unknown till then and was subsequently named *Legionella*. It causes pneumonia.

**Lysosome** A cytoplasmic organelle with internal pH lower than that of the surrounding cytosol, containing enzymes that catalyze the breakdown of proteins, lipids and other materials.

**Macrophage** A cell with a round unsegmented nucleus that has emigrated from the blood (where it was a monocyte) into connective tissue where it serves as a phagocyte, ingesting foreign material and remnants of dead cells.

**May-Grünwald Giemsa Staining** Method commonly used in staining blood smears.

**Metachromasia, Metachromatic** Staining in a color different from the ordinary (orthochromatic) color of a dye. This is most frequently seen when substances rich in sulfate ester groups are red or purple after application of a blue cationic dye. Mast cell granules (heparin) and cartilage matrix (chondroitin sulfates) are conspicuously metachromatic with thiazine dyes.

**Methylation** In histochemistry, one of various ways to prevent staining by cationic dyes. Hot methanol containing HCl converts carboxy groups to their methyl esters and removes sulfate-ester groups. Methyl iodide esterifies carboxy groups but spares sulfate esters.

**Mordant** A reagent used to link a stain to a tissue.

**Mucin** A term sometimes applied to all carbohydrate-rich substances that can be shown by staining, with the exception of glycogen. Also used to mean mucus or epithelial mucin.

**Mucopolysaccharide** Older name for glycosaminoglycans (GAGs).

**Mucous** Secreting, containing, consisting of or resembling mucus.

**Mucus** A slimy secretion consisting of glycoproteins dissolved in water, and stainable with either PAS or alcian blue pH 2.5 or both.

**Mycobacteria** are bacilli that have a thick and waxy (Latin, *myco* = wax) cell wall composed of a lipid called mycolic acid. This cell wall is responsible for the hardiness of this organism as well as for its staining characteristics. The waxy cell wall is hydrophobic and resists staining with aqueous stains like the Gram and Giemsa stains. It also resists decolorisation once stained. Mycobacteria causes tuberculosis, leprosy and infections in patients with AIDS.

**Neutral Mucus (or Neutral Mucin)** Mucus stainable with PAS but not with cationic dyes such as alcian blue. Gastric mucus is an example.

**Neutral Red** A cationic dye of the azine series (CI 50040) with several uses as a biological stain. Applied from a solution at about pH 4 it colors nuclear DNA, cytoplasmic (ribosomal) RNA and acidic carbohydrates such as those of cartilage matrix, mast cell granules and glycoproteins of several types of mucus. This dye is also an indicator: red in acids, changing to yellow when the pH is above about 7.

**Niemann-Pick Disease** Niemann-Pick disease is caused by an inherited deficiency of an enzyme involved in lipid metabolism. As a result, an abnormal biochemical metabolite accumulates in various tissues such as the brain, liver and spleen.

**Normocellular MDS** Myelodysplasia with bone marrow cellularity according to patient's age.

**Nuclear Fast Red** An anionic dye of the anthraquinone series (CI 60760), also known by its German name *Kernechtröt*. Its aluminum complex is frequently used as a counterstain (red nuclei, pale pink background) following histochemical methods that yield blue products. The name nuclear fast red has been applied also to neutral red (as in the *Merck Index*) and this has caused confusion because the dyes are not interchangeable.

**Nucleotide** A compound formed by combination of a purine or pyrimidine base, a pentose sugar (ribose or deoxyribose) and one to three phosphate groups. Nucleotides with one phosphate group are the units from which DNA and RNA are built. Individual nucleotide molecules serve important functions in metabolism. A well known example is ATP (adenosine triphosphate).

**Oligosaccharide** A chain of 2 to about 20 monosaccharides, joined by glycosidic linkages.

**Osmium Dioxide** An insoluble black substance, $OsO_2$, formed when osmium tetroxide ($OsO_4$) reacts with lipids and other substances in tissues. $OsO_4$ was an ingredient of traditional fixatives for tiny intracellular structures such as mitochondria. It is still used for post-fixation of specimens to be examined by electron microscopy and in the study of teased preparations of peripheral nerve fibers.

**Oxidation** A chemical reaction involving the removal of electrons from a molecule.

**Periodic Acid** The compound $H_5IO_6$ ($HIO_4{\cdot}2H_2O$) or $HIO_4$ (metaperiodic acid). The metaperiodate ion, $IO_4^-$, oxidizes glycols to aldehydes.

**Polysaccharide** A glycan: a long chain of monosaccharide units not covalently linked to protein or lipid. Glycogen is an example.

**Progressive Staining** Staining to the desired intensity and stopping the stain. No differentiation is required.

**Protozoa** (Greek, *proton* = first; *zoa* = animal) are unicellular organisms that have a membrane-bound nucleus and other complex membrane-bound organelles.

**Regressive Staining** Overstaining followed by decolorizing or differentiation.

**Reduction** A chemical reaction involving the addition of electrons to a molecule.

***Rickettsia*** are Gram-negative bacteria that like *Chlamydia* lack typical cell walls and are obligate intracellular parasites. The rickettsial diseases are primary diseases of animals (zoonosis) such as the deer which are transmitted to humans by bites of insects like fleas and ticks. Rickettsial diseases include typhus fever and Rocky Mountain Spotty Fever.

**Ripening** Oxidation.

**Salt** A compound in which cations are electrically balanced by anions. A simple example is sodium chloride, NaCl. In the solid state, $Na^+$ and $Cl^-$ are held in place in the crystal structure. The cation and anio become widely separated when NaCl is dissolved in water. The ions of an insoluble salt such as calcium phosphate or sulfate stay together in the presence of water. In mineral histochemistry, insoluble colored pairs of ions include Prussian blue and the calcium-alizarin red S product.

**Saponification** Alkaline hydrolysis of esters. A reaction used in carbohydrate histochemistry to reverse methylation, restoring tissue carboxy (but not sulfate) groups, and to remove naturally occurring or artificially introduced acyl groups.





**Schiff's Reagent** A colorless solution made by reaction of the dye basic fuchsine with sulfurous acid. Chemical combination with aldehyde groups yields a red product.

**Sialomucin** Mucus stainable by virtue of its sialic acid content: with alcian blue at pH 2.5 but not at pH 1.0, and stainable with PAS unless acylated at C8.

**Sideroblasts** Erythroblast with five or more cytoplasmic iron granules.

**Spirochetes** are long Gram-negative bacilli with tightly-coiled helical shapes. Spirochetes cause syphilis, leptospirosis and Lyme's disease.

**Strong Acid** An acid that exists entirely as anions even at very low pH. An example is sulfuric acid: $H_2SO_4 \rightarrow H^+ + HSO_4^-$.

**Sulfated** Having one or more hydroxy groups esterified by sulfuric acid: $R{-}OH + H_2SO_4 = R{-}OS(O_2)O{-} + H^+ + H_2O$.

**Sulfate Ester** Strictly half-sulphate ester in carbohydrates: the group $-OS(O_2)O-$ is a strong acid and therefore anionic at low pH. Stained by alcian blue at pH 1.0 or 2.5. Methylation with hot methanol-HCl permanently removes sulphate ester groups, leaving —OH.

**Sulfomucin** Mucus containing glycoproteins rich in sugars with sulfate ester groups. Commonly such mucus also contains sialic acids. Conspicuous in glands of the descending colon and rectum.

**Uronic Acid** A hexose in which C6 is part of a carboxy group, —C(O)OH.

**Weak Acid** An acid present partly as molecules and partly as anions. The carboxy group of sialic and uronic acids ($-COOH = -COO^- + H^+$) is the only example in carbohydrates.

**Wilson's Disease** Wilson's disease is a genetic disorder that prevents the body from getting rid of extra copper. In Wilson's disease, copper builds up in the liver, parts of the brain and eyes. Excess copper is toxic to these tissues.

**Xanthenes** A large family of dyes that includes many important biological stains, including the eosins. Not to be confused with xanthine, a purine metabolite.

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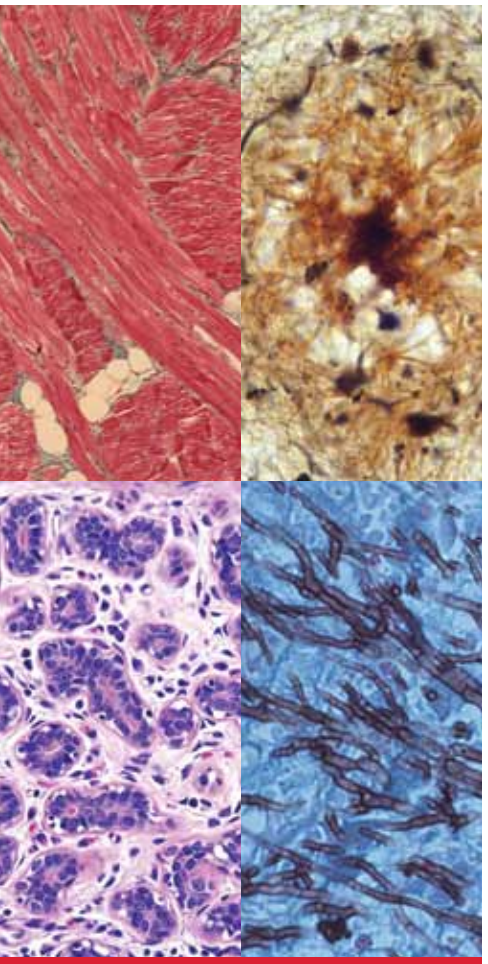

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