Suggested Reading

1. Brown RW (Ed.). Histologic Preparations: Common Problems and Their Solutions. College of American Pathologists, Northfield IL, 2009.
2. Dumas T. 'Going green' in the lab. *MLO.* 2009;41(5):48-49. Accessed November 1, 2009 at: http://www.mlo-online.com/features/2009_may/0509_education.pdf.
3. Kiernan JA. Histology FAQ Staining, Histochemistry and Histotechnology. Accessed October 5, 2009 at: http://www.ihcworld.com/_faq/histology-faq/misc/m6.htm.
4. Luna LG. Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts, American Histolabs, Gaithersburg MD, 1992 (767 pages).
5. Thompson SW, Luna LG. An Atlas of Artifacts Encountered in the Preparation of Microscopic Tissue Sections, Thomas, Springfield IL, 1978 (190 pages).
6. Wallington EA. Artifacts in Tissue Sections. *Med Lab Sci.* 1979;36(1):3-61.

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

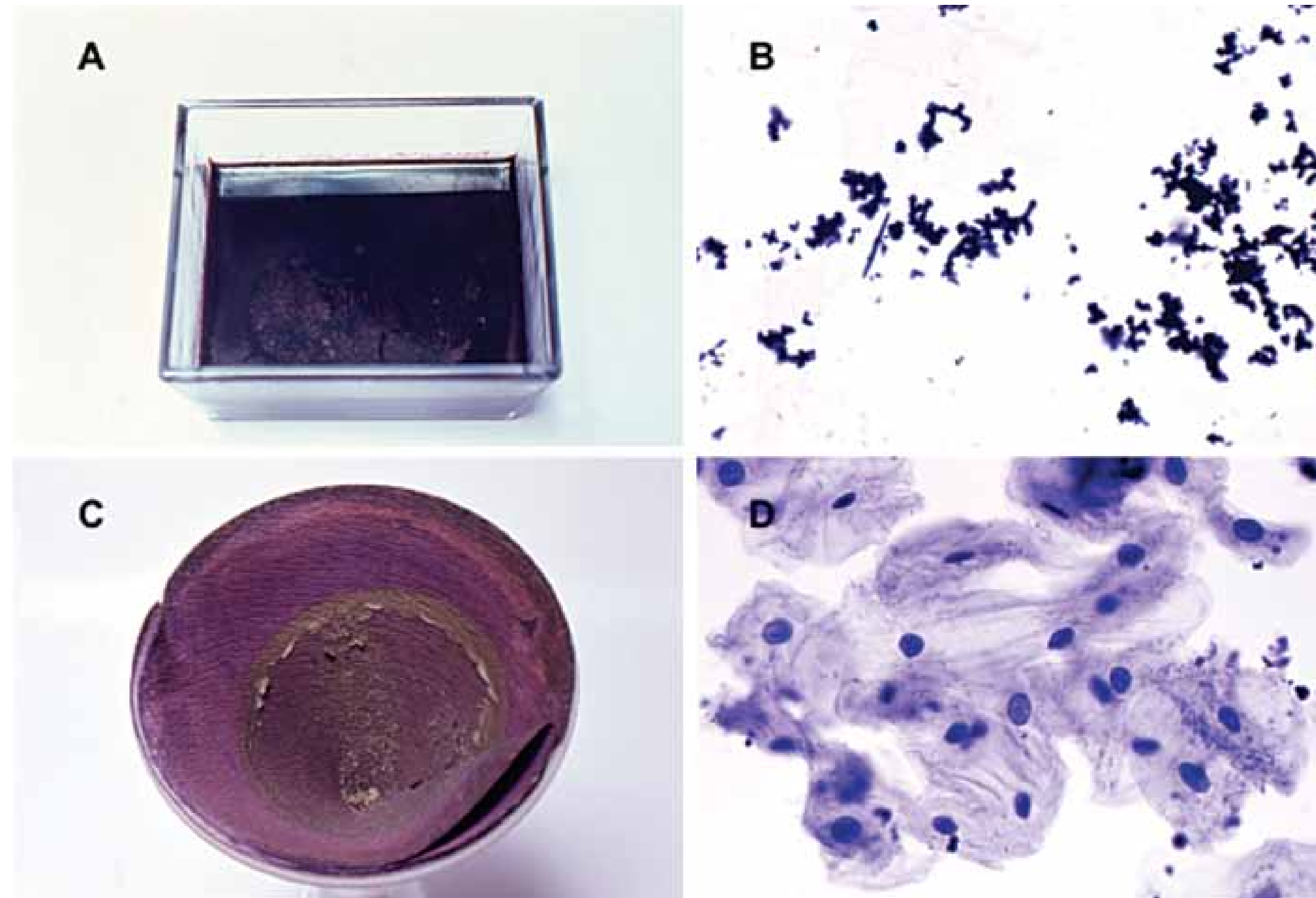

Figure 2. Genesis and identity of surface precipitate on Harris Hematoxylin.

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.

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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 sub-stage 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.

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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.