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 over-stain 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 hematoxins 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 over-staining or under-staining. 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 (bluing, if you prefer the English spelling) are essential to satisfactory staining by hematoxylin. Differentiation is used only with regressive hematoxylin formulations, while bluing is used with both progressive and regressive hematoxylin formulations. Differentiation effects quantitative changes; bluing, qualitative.

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.

Formatin-fixed tissues or solutions 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 initiating 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.

On Chemical Reactions and Staining Mechanisms

Chromosome Banding

Giemsa (G-banding) is the stain most commonly used to display chromosomes (telomeres). A variant known as T-banding emphasizes identifying chromosomes. R-banding is useful for showing the ends of chromosomes (telomeres). In the Giemsa mixture (azure B and methylene blue) are attracted by phosphate anions of DNA and are then held more closely by van der Waals forces, which are regions rich in adenine and thymine. Regions of a chromosome rich in guanine-cytosine pairs do not bind the dye. 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 with 1.0 M NaH2PO4 (pH about 4) at 88°C for 20 minutes. The histochemical rationales of the various pretreatments have not been systematically investigated (26).

1. Woronzoff- dashkoff KpK. The ehrlich- chenzinzky- plehn-Malachowski-pretreatments have not been systematically investigated (26).

REFERENCES

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H&E Staining

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 (i.e., hydration and dehydration)
- stop action of previous solution (post-hematoxylin rinses)
- 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)

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

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Progressive</th>
<th>Regressive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemalum concentration</td>
<td>Less (i.e., 1 to 4 gm/L)</td>
<td>More (i.e., 5 gm/L or more)</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Rate of uptake</td>
<td>Slow</td>
<td>Rapid</td>
</tr>
<tr>
<td>Easily controlled?</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Overstaining?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Differentiation required?</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2. Differentiation and bluing: comparisons and contrasts.

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

Figure 1. Hypothetical uptake of aluminum-hematein in cells: progressive vs. regressive staining.
Table 3. Troubleshooting hematoxylin staining problems.

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

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 rinses. 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. QA 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.
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 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 nucleic acids and some carbohydrates; the lipids for the most part have been dissolved out. The protein is naturally transparent, but if the 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.

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 may prefer to use prepared commercial MST that have been dissolved out. The protein is naturally transparent, but if

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.

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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).

What factors influence the effectiveness of H&E stains and their stains?

- Stain-laden rinses
- Use clean alcohol rinses
- Insufficient subsequent alcohol rinses
- Increase rinse time, dip more

What is “clearing” and what does it accomplish?

“Clearing” is the term applied to immersing fixed tissue sections in a solution of nearly matching refractive index and the transparency it enables. Clearing is used to remove water, oils, and other materials that may interfere with the staining process or affect the appearance of the final product. Clearing agents, such as xylene, are used to increase the transparency of tissues, making them easier to see under the microscope. Xylene is a clearing agent that is commonly used in histology and cytology to prepare tissue sections for staining.

How does “clearing” work?

“Clearing” involves the use of organic solvents to remove water from tissue sections. Organic solvents have a lower refractive index than water, making them ideal for use in clearing. As the tissue sections are exposed to the clearing agent, the water is replaced by the organic solvent, resulting in a more transparent appearance.

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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.

Suggested Reading