H&E Staining

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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 2 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

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

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

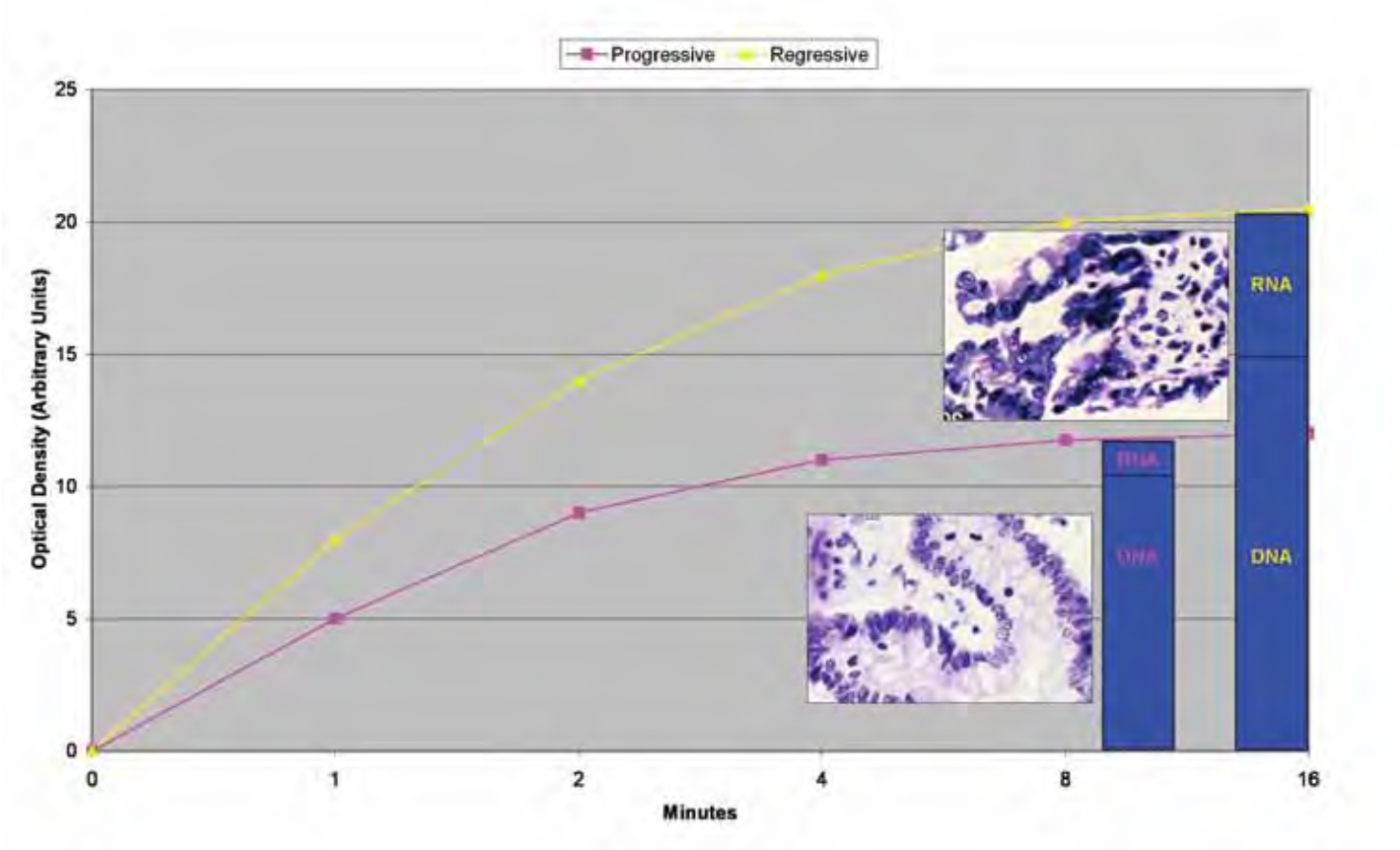

Figure 1. Hypothetical uptake of aluminum-hematein in cells: progressive vs. regressive staining.

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

Table 2. Differentiation and bluing: comparisons and contrasts.

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.

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.

Complaint	Cause	Correction
Hyperchromatic	Strong hematoxylin (e.g., Harris full-strength without acetic acid)	Use lesser strength hematoxylin; Dilute 3:1 with ethylene glycol; Stain for less time; Differentiate in 0.25% HCl
	Staining time too long	Stain for less time
	Inadequate differentiation in HCl	Differentiate more; 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; Underdifferentiation in HCl	Differentiate more; 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 3. Troubleshooting hematoxylin 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; Eosin nearly exhausted	See Table 1 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

Table 4. Troubleshooting eosin staining problems.

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.

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 ($MgSO4 \cdot 7H2O$ [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 3, 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

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.