ANATECH LTD

H&E Staining

INNOVATOR

The Hematoxylin & Eosin stain remains the cornerstone of tissue diagnostics despite advances in tissue staining, i.e., IHC, ISH, etc. So, the importance of good H&E staining cannot be overlooked. Figure 1 shows a properly prepared H&E. If your staining is less than optimal, this *Innovator* may hold the solution to your staining dilemma.

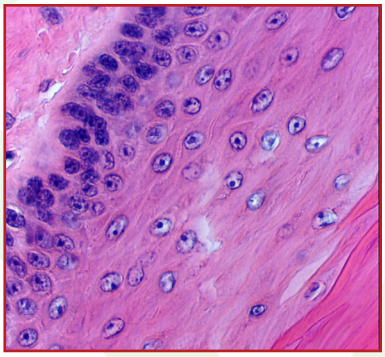

Figure1. Good nuclear staining exhibits stained nuclear membranes, well defined chromatin and unstained karyoplasm. Skin, Anatech Harris Hematoxylin and Eosin Y.

DEPARAFFINIZATION

In routine practice, the histology lab produces tissues embedded in paraffin wax. But, the wax needs to be removed in order for Hematoxylin & Eosin solutions to stain the tissue. Sounds like a simple chore, right? It should be. However, time limitations and space restrictions on automatic stainers have many labs taking shortcuts that do not allow sufficient time to completely remove the wax from the specimen. One of the most neglected cardinal rules of deparaffinization is to use three clearant changes for three minutes each. Consider that the first station of clearant will always be contaminated with wax. How can it not be when each slide has a 3-4 micron layer of wax on it? The second clearant station will contain wax carryover leaving the third station to assure complete deparaffinization. Many labs believe that time is the important factor and will only use two stations, for five minutes each, to deparaffinize the slides. Following the three stations-three minutes each rule is less time overall and will provide better deparaffinization.

HYDRATION

After removal of the wax it is necessary to hydrate the tissue to enable staining in the aqueous hematoxylin solution. But, due to insolubility, it is impossible to go directly from clearant to water. Clearant steps are followed by anhydrous alcohol (100%) and then diluted alcohols (95%, 70%) gradually take the tissue section to water.

Again, this sounds easy but urgency in the lab has introduced an artifact from improper hydration. Quickly passing through the anhydrous alcohol does not completely remove the clearant from the tissue section. The subsequent diluted alcohols cannot displace clearant because of the water incompatibility. The clearant left on the section forms a barrier, which prevents aqueous hematoxylin from attaching to the tissue. Figure 2 , page 2 shows the result of an oily clearant (limonene), which was not sufficiently removed.

The cardinal rule to assure adequate removal of clearants is to have two stations of anhydrous alcohol followed by a 95% alcohol, for one minute each. The number and dilution of additional alcohol rinses varies between laboratories. Personal experience has confirmed equal success at the extremes of using dilutions down to 50% alcohol before water as well as going directly from 95% alcohol to water.

For over 25 years I've given workshops at NSH and state seminars as a way of teaching and sharing my technical knowledge. My presentation of "Troubleshooting Hematoxylin and Eosin" has evolved over the years. I began with use of an overhead projector, then upgraded to Kodachromes and now use an animated PowerPoint. Just as the presentation mode has kept pace with the times, so has the workshop content. Although the chemistry of the hematoxylin and eosin stains remains constant, continual administrative and technical changes are the new challenge we face. We must learn how to adapt to the variables created by time constraints, automation and alternative processing and staining reagents. Although the written word may seem archaic in this age of electronic communications, it seemed appropriate for sharing the workshop information with a larger audience while providing a reference source. Of course, it will also be available for download from Anatech's website. I hope you find it instructive. Ada Feldman, CEO

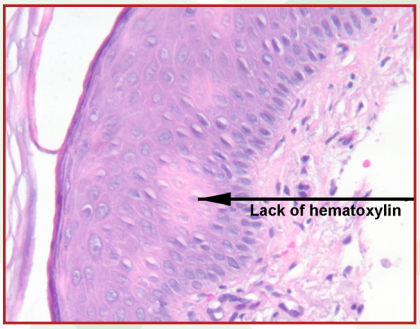

Figure 2. A single station of anhydrous alcohol for one minute was insufficient to displace the oily limonene clearant. Oil droplets remaining on the tissue section blocked hematoxylin staining (light spots in the epithelium).

HEMATOXYLIN

A discussion of the transformation of hematoxylin dye powder into the hematoxylin staining solution could be a thesis of its own. For our purpose here, suffice to say that hematoxylin dye powder, alone in a solution, has little attraction to nuclei. So, certain steps must be taken to produce a solution that will actually stain the nuclei. Those who have ever made hematoxylin from scratch know the complexity of the steps needed to complete the process. The first step is to oxidize the hematoxylin dye powder to hematein. In the 'old' days this was called 'ripening' because the solution was slowly oxidized by exposure to oxygen in the air. It would take weeks to months before the hematoxylin was ready to use (or 'ripe') when oxidized in this manner. Today hematoxylin solutions are chemically oxidized in minutes. Mercuric oxide had been the oxidizer of choice but, because of the negative environmental impact, it has been replaced with the safer sodium iodate. While hematein has the ability to stain the tissue a red-yellow color it is not the desired result. So, the next step is complexing hematein with a mordant. A mordant is a substance that is capable of binding the dye to the tissue. In hematoxylin formulations for anatomical pathology applications, aluminum and iron salts are the chemicals most commonly used as the mordant. The dye (hematein) and mordant complex is called a dye lake, and it carries a positive charge due to the positively charged alum or iron mordant (Figure 3). While it appears to be just two simple steps of oxidation and the addition of a mordant that result in the working hematoxylin stain, chemists know it is actually the precise ratios, proper sequence and amount of mixing of each ingredient (hematoxylin powder, oxidizer, mordant and additives) that all play a role. Chemical manufacturers, like Anatech Ltd., have learned the chemistry of blending to provide the perfect end product. This is the reason why the majority of laboratories purchase ready to use hematoxylins rather than make their own.

Hematoxylin staining is mainly an ionic reaction between the dye and tissue components. The positively charged hematoxylin complex attaches to phosphoric acids of DNA and RNA and other negatively charged tissue elements. To assure attraction to the phosphoric acid groups of the nucleic acids, the pH of the hematoxylin solution should be between pH 2.5-2.9. The acetic acid ingredient of the hematoxylin solution is responsible for acquiring this correct pH. A pH above 3.0 will result in non-specific or muddy staining. Changing the pH of a perfect hematoxylin solution to above 3.0 can happen easily because of contamination from the rinse water preceding the hematoxylin stain. Eventually the carryover of water, which has a pH near 7.0, can increase the pH of the hematoxylin to above 3.0. When this happens, tissue carboxylic acid groups become ionized and will bind with the hematoxylin stain. So, elements that normally would not stain with hematoxylin will, making the tissue appear muddy. If reoccurring muddiness appears in your H&E staining and checking the pH reveals a solution with a pH above 3.0, you may have found a possible source of the problem. Check the (M)SDS that came with the product to know the recommended pH. Lowering the pH to below pH 3.0 with a small addition of acetic acid can eliminate the problem.

Another source of muddy slides is inadequate removal of weakly bound hematoxylin. The differentiation step of acid alcohol removes weakly bound hematoxylin from the tissue and glass slide. Formulations of acid alcohol vary from acid concentrations, type of acid (acetic acid, hydrochloric acid) and dilution of alcohol solvent. Generally a 0.1% hydrochloric acid in 95% alcohol solution is used with quick dips (lasting 2–3 seconds) while 10% acetic acid in 95% alcohol is used for one minute. The latter is popular with automatic stainers since hydrochloric acid is too strong for even the minimum programmable time.

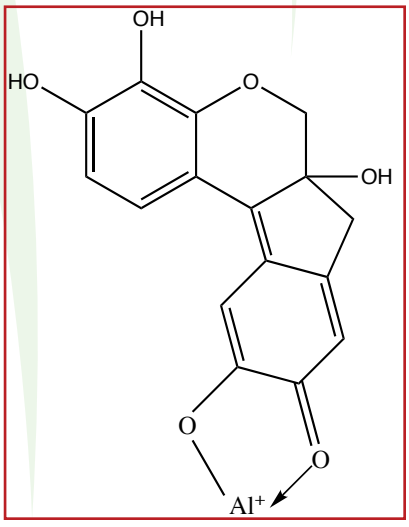

Figure 3. Bettinger (1991) suggested this molecular structure for hematoxylin solution (hematein complexed with aluminum).

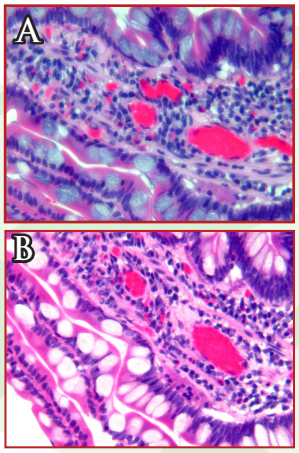

Figure 4: Sections of the same small intestine. (A.) Anatech Hematoxylin Normal – Gill II equivalent, showing stained goblet cells and (B.) Anatech Harris Hematoxylin with unstained goblet cells.

Sometimes what is perceived as non-specific staining is actually specific staining! Each formulation of hematoxylin (named for the originator, e.g. Harris, Mayer, Delafield, Weigert, Gill) vary in composition and concentrations and therefore produce different staining results. A notable difference in staining is often observed between Harris and Gill hematoxylins. Both solutions have a positive charge due to the aluminum mordant, allowing the hematoxylin to bind with the negatively charged nuclei. However, other negatively charged elements in the tissue are also capable of staining. For example, Gill hematoxylin is notorious for staining the acid mucopolysaccaharide component of intestinal goblet cells. While Harris hematoxylin, because of the formulation's excess mordant (aluminum ammonium sulfate), is less likely to stain goblet cells*. Excess positively charged aluminum, which is not attached to the hematoxylin complex, binds to the acid mucopolysaccharides. Since the mucus no longer has an available negative charge, hematoxylin bonding cannot occur. Figure 4 demonstrates the staining properties of Gill and Harris hematoxylin, respectively. So, it's easy to see the importance of understanding the characteristics of the hematoxylin formulation that you choose.

BLUING

Another important influence in the hematoxylin appearance is the bluing step. The aluminum-hematein complex behaves as a pH indicator. Below pH 3.0 alum hematoxylin turns red, and above pH 3.0 it turns blue/purple. Exposing the alum hematoxylin (pH 2.5-2.9) to bluing solutions, typically having an approximate pH of 8.0, raises the pH to yield the color change. Bluing solution formulations vary from tap water (see caution on using tap water in the next section), ammonia water, saturated lithium carbonate solutions and Scott's tap water. Highly alkaline aqueous solutions (e.g., ammonia water and saturated lithium carbonate) are capable of loosening tissue sections from glass slides and should be used with that in mind.

** Acidity of mucus, fixation, processing, staining protocols , etc can affect goblet cell staining regardless of Hematoxylin formulation.*

WATER RINSES

The importance of water rinses in the H&E procedure is often underestimated. As the name states these are water rinses; their role is to remove and stop the action of the previous solution the slide was exposed to: either the hematoxylin solution, acid alcohol or bluing reagent. Before using tap water for rinsing, be sure to know its quality. Tap water consists of inherent minerals and additives that make it drinkable. These ingredients may not be compatible with chemical staining. For example, using tap water containing iron as a post-hematoxylin rinse can decrease the stain's intensity, since using excess mordant to decolorize hematoxylin from the tissues is a known procedure. Also be sure, particularly with automatic stainers, that the flow rate and fluid level are adequate to rinse the slides. Inadequate removal of excess hematoxylin stain, acid alcohol and bluing reagent can alter the H&E stain appearance.

EOSIN

Eosin provides the cytoplasmic counterstain. As with hematoxylin, there are multiple formulations of eosin that differ in the dye (Eosin Y, Eosin B, addition of phloxine), dye concentration and solvent. Alcohol is the most popular solvent; both ethanol and methanol can be used with the latter providing more intense staining. Proper proportions of eosin dye concentration, solvent and pH will demonstrate a 'three-tone effect', demonstrating red blood cells, muscle and collagen with three distinct shades of eosin (Figure 5).

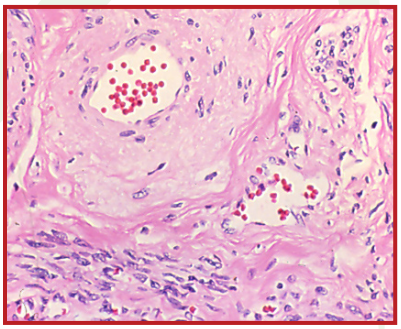

Figure 5. Eosin, three-tone effect. Red blood cells are intense pink, collagen fibers an intermediate shade and arterial wall smooth muscle is a lighter pink. Anatech Harris Hematoxylin and Eosin Y.

ALCOHOL RINSES

The alcohol rinses after eosin dehydrate and prepare the section for clearing and coverslipping. However, these alcohol rinses also play a critical role in obtaining the three-tone effect. Eosin is very water-soluble. Rinsing with a diluted alcohol following the eosin allows the water component to extract eosin faster than anhydrous alcohol and produces a lighter stain. Generally one change of 95% alcohol is used but the alcohol concentration, number of changes and amount of time all dictate the final color. Depending on the desired eosin intensity, H&E protocols will use 70%, 95% or even 100% after the eosin.

No matter what alcohol dilution is used after the eosin, every H&E staining procedure should have three changes of anhydrous alcohol, one minute each, to assure complete dehydration. As with deparaffinization, three changes have their merits. The first alcohol immediately following the eosin soon becomes very pink. The second alcohol (generally the first anhydrous) is also easily colored. Since eosin is typically formulated with diluted alcohol, not anhydrous, seeing pink color in the anhydrous alcohols means there is water contamination. Water that is not removed from the section results in a 'hazy' appearance microscopically; this is caused by the inability of water to mix with the clearant. While using three anhydrous alcohols assists in eliminating water carryover, high humidity is another potential source of moisture contamination. To remedy this problem, add a drying agent (such as Drierite™) to the final anhydrous alcohol and the first clearant at the end of the H&E procedure.

The hazy slide phenomenon is also commonly seen when xylene is replaced by a xylene substitute in the staining protocol. Once again, inadequate dehydration, and not the xylene substitute, is the culprit. Xylene can tolerate 0.5% moisture contamination while most xylene substitutes are unable to tolerate *any* moisture contamination. The drying agent remedy will solve this problem also. Another solution is to use a xylene substitute that has some water tolerance. Anatech's Pro-Par Clearant was formulated to withstand moisture contamination much like xylene.

CLEARING

The clearing step's purpose is to completely displace the alcohol and provide a compatible solvent for the mounting medium. Providing all the previous steps have been done correctly, this should occur without any difficulty. However, because xylene substitutes differ in composition, it's important to assure compatibility with the mounting medium before attempting to coverslip. Fortunately, there are multiple mounting media from which to choose! The xylene substitute's manufacturer should be able to provide compatibility information regarding mounting media.

The appearance of the stained H&E is the result of not only the staining procedure but also of how the tissue was fixed and processed. This article has dealt only with the staining procedure. Check out past issues of Anatech's *Innovator* to learn how fixation and processing can alter the appearance of the stained slide. ❖