⟨1285.1⟩ HEMATOXYLIN AND EOSIN STAINING OF SECTIONED TISSUE FOR MICROSCOPIC EXAMINATION

INTRODUCTION

Histologic methods involve the preparation of organs, tissues, or cells for microscopic analysis of constituent elements that have been exposed to particular chemistries or immunochemical staining procedures. Histologic methods have many goals, and are often used to evaluate native and processed therapeutic tissue-based products. For example, these methods can be used to establish the integrity of the tissue with regard to cellular content and extracellular constituents. Nuclear staining intensity and nuclear shape may be altered if tissue recovery or handling is compromised. Furthermore, extracellular structural and accessory components such as collagen, elastin, and glycosaminoglycans can be specifically stained so their presence and location can be determined.

Histologic methods are particularly useful for detecting trends in content (e.g., cell numbers and amounts of tissue constituents). Therefore, they have been valuable in the development and verification of tissue-processing methods intended to reduce or eliminate cellular content (decellularization detected by reduced nuclear staining, for example with hematoxylin) while maintaining extracellular matrix structure and components (e.g., retention of glycosaminoglycans or other carbohydrates assessed by persistence in Alcian blue staining).

Tissue histologic analysis typically begins with a routine staining procedure using hematoxylin and eosin (H&E) to detect cellular chromatin and cytoplasm and extracellular structures (e.g., collagen and muscle). Hematoxylin acts as a basic dye. In a metal complex it binds to acidic structures such as cellular nucleic acids (DNA or RNA), producing the blue–purple stain typically noted as nuclear staining. Additionally, in calcified tissues it forms a blue or purple precipitate. Eosin acts as an acid dye and stains basic materials red (muscle) to pink (collagen). Eosin staining is responsible for the detection of the cytoplasm and intracellular and extracellular proteins, but it does not allow identification of specific intracellular structures or proteins. H&E does not stain fat itself, but fatty areas can be identified by the staining of the stroma, yielding an outline of adipocytes.

H&E staining is often used to demonstrate the reduction of nuclei following tissue decellularization processes, but H&E staining also provides structural information. H&E staining reveals that Type I collagen fibrils have a particular arrangement and wave pattern depending on the tissue observed and the orientation of the specimen. Altered collagen fibril patterns, e.g., smearing and loss of fibrils, may suggest disruption of the native structure of the tissue. Decellularization methods, which can alter the structural integrity of tissue, may reduce the staining intensity of collagen. Alternatively, the staining intensity can be artificially increased if residual charged processing chemicals remain in the tissues. H&E staining can help identify noncollagen protein structures. For example, elastin fibers are not specifically stained by this method, but thick elastin fibers such as the internal elastic lamina of arteries are easily detected by their profound waviness and subintimal location shown by staining. A mineralized matrix (e.g., in bone) can be seen as dark red to purple (depending on the hematoxylin choice) granular staining within fields of structural protein fibers.

H&E staining is a starting point for more detailed histologic analysis. Differential staining of collagen (typically colored blue) is achieved with Masson Trichrome stain, and Verhoeff's Van Gieson and Movat Pentachrome stains highlight elastin (black). The other major components of connective tissues are the glycosaminoglycans, and these can be detected and to some extent differentiated with various cationic dyes such as Alcian blue in Movat's Pentachrome stain, for example.

More detailed compositional analysis of tissues and observations of the effects of decellularization methods require structure-specific probes. At the level of light microscopy, this specificity is provided by immunohistochemical staining using antibodies to targeted structural proteins and extracellular matrix constituents or by using labeled lectin probes to detect certain sugar structures. The latter are of particular utility for evaluating the removal of important xeno-antigenic epitopes.

The purpose of this chapter is to identify the factors that should be controlled to optimize the consistency of the results of tissue staining with H&E. In addition, the chapter provides a common H&E staining procedure.

BASIC PRINCIPLES

Hematoxylin is oxidized to hematein, which complexes with a metal cation, binds to negatively charged groups, and stains basophilic substances such as nucleic acids. The most common cation for this application is aluminum (III), but iron (III) and tungsten also are important. Hematoxylin also stains calcium deposits. The most common metal used in hematoxylin staining is aluminum, and the resulting stain produces a blue color. This aluminum salt form of hematoxylin requires a pH >5 to form an insoluble blue aluminum hematein complex. The high pH is obtained in a bluing step using a weak alkali solution such as ammonium water. Because anionic materials often are used to process tissue (e.g., anionic detergents), these materials should be removed from the tissue first, or nonspecific hematoxylin staining may be seen.

Eosin is an acidic analine dye with affinity for cationic amino acids such as arginine and lysine in proteins. Cytoplasm, muscle, connective tissue, colloid, red blood cells, and decalcified bone matrix all stain pink to pink/orange/red with eosin. Eosin methods use solutions containing various proportions of water, ethanol, and acetic acid. The uptake of eosin in cells is promoted by higher eosin concentration, a higher proportion of water than ethanol, and the inclusion of acetic acid.

PROCEDURES—POINTS TO CONSIDER

It is important to control the consistency and quality of the stain reagents and process. Each new lot of stains should be qualified with known control specimens to ensure consistent staining processes. Solution color should be monitored, and if colorless solutions become colored they should be discarded (especially the alcohols and xylene). Staining solutions can age,

and their staining effectiveness changes over time. Thus analysts should establish or assign a shelf life to critical staining reagents. Some reagents form precipitates or undergo overt color changes over time. Best practices indicate that such solutions should be replaced. Labeled expiration dates of commercial stains should be followed closely. If reagents are mixed in the laboratory, analysts should monitor their performance over time using control specimens to assess staining intensity and differentiation. Expiration dates should be added for each laboratory reagent. Best practices require that to the extent possible samples to be compared should be stained at the same time. To facilitate comparison, staining should be monitored and corrected if necessary. Regressive staining can be performed if overstaining with hematoxylin occurs. In this case, excess stain is removed with acid (e.g., 0.5% HCl in 70% ethanol) in a step called differentiation. Excessive hematoxylin staining can block eosin staining. Ideally, progressive staining, or real-time monitoring of the staining process to the desired intensity, should occur.

As with any tissue-staining method, H&E staining intensity may be affected by steps taken before the staining procedure, the chemicals used, and the conduct of the staining itself. Section thickness can affect the overall specimen depth and can influence the apparent stain. Inadequate removal of paraffin wax can prevent interaction of the aqueous solutions of the staining steps with the tissue. The color can be too strong if certain hematoxylin reagents are not diluted before use, if the stain is applied for too long, if the differentiation step is too short, or if the acid is too dilute. Weak staining can be the result of exhausted hematoxylin, short staining time, overdifferentiation, or low pH during the bluing step.

SYSTEM SUITABILITY

Control tissue should be used during each run to ensure that all the reagents are working appropriately and the processing regime does not adversely affect staining. Untreated fresh tissue makes a good positive control, because there are no processing reagents that could affect the staining process. An appropriate negative control is an unstained sample from a previous lot that met all the product quality criteria of the H&E analysis. Controls are important because processing methodologies such as decellularization or cross-linking may positively or negatively affect the ability of the reagents to stain the tissue as expected and can contribute to lot-to-lot variability in staining intensity.

QUANTITATION

A physical count of positively stained cells in a given area (cells/mm^2) is preferable to simply comparing a sample to a previously submitted visual reference, particularly for products that undergo a decellularization process that yields a greatly reduced (yet nonzero) number of positively stained cells. Many, though not all, samples of tissues that have undergone a process of cellular removal still have identifiable cellular debris after processing. It is difficult to compare these nonzero samples to a visual reference, so a standardized count per area is a better comparison of process effectiveness.

Software-based automated cell-counting systems that are available on many microscopy systems must be validated to confirm that the software appropriately counts cells and sample area. When the tissue has not been decellularized, there should be a high value of positive signals, and the system should be able to accurately differentiate that tissue from other samples with low cell counts. Validation is important when analysts attempt to quantify the number of cells in a given area on irregularly shaped samples because the algorithm used to estimate areas may have limitations when applied to these types of samples. Sample area should be measured accurately when there is a low cell count, because a large error in the denominator can cause a significant error in the reported cell density.

EXAMPLE PROTOCOL

A common H&E method is described below. Analysts can follow this example after preparing and deparaffinizing tissue sample slides as described in *Preparation of Biological Specimens for Histologic and Immunohistochemical Analysis* ⟨1285⟩. This protocol is commonly used to study the effectiveness of tissue decellularization.

Bluing reagent: 3 mL of 28% ammonium hydroxide in 1 L of *Purified Water*

Staining method: After deparaffinizing tissue-containing slides, place the slides in a hematoxylin solution[1] for 1.5–3 min. [NOTE—Staining times can vary depending on hematoxylin brands (and type) and the tissues being stained, so optimization of staining time is required in this step. For this reason, laboratories should track the reagent sources to ensure consistency.] Rinse slides under running tap water for 0.5–3 min. If necessary, perform a differentiation step to remove excess stain so the desired element or structure is left stained by placing the slide in a clarifying solution[2] for 30–60 s followed by rinsing in tap water for 30–60 s. Next, place slides in *Bluing reagent* for 1–2 min. Dip slides 12 times in *Purified Water*. Rinse thoroughly in tap water for 0.5–2 min to remove the *Bluing reagent*. [NOTE—Insufficient rinsing affects subsequent staining because of a change in pH.] Next, place the slides in the following solutions for the specified times: 95% ethanol for 30 s to 1 min [NOTE—The time will vary depending on eosin type and components.] then place the slides in eosin solution[3] for 45 s to 3 min, 70% ethanol for 3 min, 95% ethanol for 3 min, and finally 100% ethanol for 3 min. [NOTE—Alcohol washing must be closely monitored to avoid excessive removal of eosin stain. Excessive removal of eosin from the extracellular matrix can be seen as pale pink staining.] Place slides in three changes of fresh xylene or xylene substitute for 1–3 min each. Place a couple drops of mounting medium on the slide, and apply the cover slip.

Expected results: Nuclei should be stained blue. Cartilage and calcium deposits should stain various shades of blue. Cytoplasm and other tissue constituents stain various shades of pink. Erythrocytes and eosinophilic granules stain bright pink to red.

[1] Richard Allan Scientific #7231 or suitable equivalent.
[2] Clarifier 2 from Richard Allan Scientific or suitable equivalent.
[3] Richard Allan Scientific #7111 or suitable equivalent.

CONCLUSIONS

It is critically important to test and verify staining conditions for each new tissue being studied. Staining intensity may be altered by tissue treatment before processing. A notable example is the treatment of tissues to decellularize or antigenically modify them, because these procedures may affect tissue components and may alter the staining of the remaining constituents.