Topic Introduction

Nuclear Stains

John Alan Kiernan

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INTRODUCTION

For the study of microscopic anatomy and of pathological material, it is usual to stain sections of tissue in such a way as to impart a dark color to the nuclei of cells and a lighter, contrasting color to the cytoplasm and extracellular structures. Nuclear stains, including cationic, anionic, and metal complexing dyes, are considered in this article. The rationales of the techniques are discussed, but methods of higher chemical specificity (e.g., for nucleic acids, carbohydrates, and functional groups of proteins) are not covered.

RELATED INFORMATION

Articles describing **Anionic Counterstains** (Kiernan 2008) and **Hematoxylin and Eosin Staining of Tissue and Cell Sections** (Fischer et al. 2008) are also available.

NUCLEAR STAINS

The nucleus of a eukaryotic cell contains the two nucleic acids: DNA in the chromosomes, and RNA in the nucleolus. Both are associated with strongly basic nucleoproteins: These are histones in diploid cells or protamines in haploid cells such as spermatozoa. Nucleoproteins are rich in the amino acids arginine and lysine. The cations of these amino acids are neutralized by phosphoric acid residues of the nucleic acids. The DNA and nucleoprotein of the chromosomes together constitute the material known as chromatin, named for its prominence in most stained preparations. In interphase cells, the chromosomes are extended and cannot be seen individually. The chromatin seen in stained preparations of interphase nuclei may be evenly distributed through the nucleoplasm or aggregated in a pattern characteristic of the cell type. The dyes used as nuclear stains impart color to the chromatin by binding to the nucleic acids, the nucleoprotein, or both these substances.

Cationic Dyes

Cationic dyes are applied from acidified solutions in order to ensure the ionization of their primary, secondary, or tertiary amine groups. Three types of anions capable of binding cationic dyes occur in tissues. These are the phosphates of nucleic acids, sulfate esters of certain macromolecular carbohydrates, and carboxylate ions of carbohydrates and proteins. The ionization of carboxyl groups is suppressed if the concentration of hydrogen ions in the staining solution is too high (in practice, pH <3-4), while sulfate groups remain ionized even in strongly acidic media. Phosphoric acid is of intermediate strength. A basic protein, such as hemoglobin, contains an excess of free amino over carboxyl groups, so that the latter can be made available for binding cationic dyes only from alkaline solutions (e.g., at pH 8). The staining properties of cationic dyes are profoundly influenced by the pH of their

solutions. If they are too acid, only the structures rich in sulfated carbohydrates will be colored; if they are neutral or alkaline, everything will be stained.

The interpretation of the results obtained with cationic dyes at various pH levels has been studied in some depth (see Gabe 1976; Lillie and Fullmer 1976; Prento 2001 for reviews and discussion). It is convenient to think of staining by cationic dyes as a process of ion exchange, in which small cations such as H^+ are competitively displaced from the tissue by the larger cations of the dye (see Horobin 1982). When the tissue and the dye solution are together for a long enough time, there is an equilibrium:

$$\text{Tissue}^- \cdots H^+ + \text{Dye}^+ \leftrightarrow \text{Tissue}^- \cdots \text{Dye}^+ + H^+$$

Binding of dye by nonionic forces favors staining (pulling the above reaction from left to right), whereas a high concentration of H^+ or Na^+ in the solution opposes staining (pushing the reaction from right to left). This simple explanation applies only to dilute solutions of dyes with small molecules. In most real staining techniques, non ionic forces cause aggregation of dye, so that more than one colored molecule is bound at each anionic site in the tissue.

The following list will serve as a rough guide to the use of dilute (e.g., 0.01%-0.2%) solutions of cationic dyes. It applies especially to those of the thiazine series.

pH 1.0 Only sulfated carbohydrate components are stained. Sulfonic acid groups or sulfuric acid esters can be artificially produced in the tissue and these, too, will bind cationic dyes from strongly acid solutions.

pH 2.5-3.0 Staining of the above, and also phosphate of nucleic acids (DNA and RNA), and of phospholipids (in frozen sections).

pH 4.0-5.0 Staining of the above, and also carboxyl groups of carbohydrates and of the more acid proteins. Carboxyl groups of free fatty acids may be stained in frozen sections.

pH >5.0 Staining of the above, with increasing staining of neutral and basic proteins as pH increases.

With higher concentrations of dye, the rules set out above are not followed, and staining is generally less specific. The duration of exposure to the dye has only minor effects on the results, though longer times are needed to achieve the greatest possible depth of staining (equilibrium) if very dilute solutions are used. The end result is also affected by treatments after staining.

Most cationic dyes are rather readily extracted from sections by 70% alcohol. Absolute ethanol, *n*-butanol, and acetone are usually less active in this respect. In any critical work with cationic dyes, the conditions of rinsing and dehydration must be carefully standardized. The H_2O used for rinsing the sections after staining should be buffered to the same pH as the dye solution. Fixatives generally do not directly affect sulfate, phosphate, and carboxylate groups, but combination with or removal of amine groups has the effect of lowering the pH at which proteins are stained. Thus, fixation by osmium tetroxide, which causes oxidative deamination, renders almost all components of tissues stainable by cationic dyes at pH 4 or higher.

For general purpose staining of nuclei, cationic dyes are used in dilute solution (0.1%-0.5%) at pH 3-4.5. The aqueous solution of the dye is usually acidified by addition of acetic acid or a suitable buffer. Objects that are colored by cationic (basic) dyes are said to be basophilic (Baker [1958] argued in favor of "basiphil"). When nuclei are stained by cationic dyes, basophilia will also be evident at sites of accumulation of cytoplasmic RNA (such as the Nissl substance of neurons) and in structures containing sulfated carbohydrates (such as mast cell granules, cartilage matrix, and many secretory products). In this propensity, cationic dyes differ importantly from some of the metal complexing (mordant) dyes used as nuclear stains. As explained above, cationic dyes will not stain nuclei specifically. The following solutions are suitable for use either alone or as counterstains to other methods.

Toluidine Blue (C.I. 52040)

Prepare a 0.5% aqueous solution, acidified by adding ~1.0 mL of glacial acetic acid to each 100 mL of dye solution. Alternatively, make up in 0.1 M acetate buffer, pH 4.0. The optimum pH may vary between 3.0 and 5.5 according to the fixative and the tissue to be stained. Stain in this solution for 3 min, rinse in H_2O, dehydrate in 95% ethanol followed by two changes of 100% ethanol, clear in xylene, and mount in a resinous medium.

If too much color is lost during washing or alcoholic dehydration, move the slides directly onto filter paper and blot firmly with two or three layers of filter paper. Transfer to a clean, dry staining rack

or coplin jar and dehydrate in two changes (each 4-5 min) of *n*-butanol. Clear and mount as above. If detachment of the sections seems imminent, the clearing in xylene may be omitted, but the resinous medium will then take longer to become fully transparent.

The dye may also be immobilized in stained structures by converting it to an insoluble molybdate. After staining, wash in H_2O and immerse for 5 min in 5% aqueous ammonium molybdate (ammonium paramolybdate, $[NH_4]_6Mo_7O_{24}\bullet 4H_2O$) solution (which may be used repeatedly until it becomes cloudy; usually stable for 1 or 2 wk). Wash in running tap H_2O for 2-3 min. The molybdate of toluidine blue is not extracted by H_2O or alcohols and even resists counterstaining by acidic mixtures of dyes, such as that of van Gieson.

Nuclei and acid carbohydrate components are stained blue. Some carbohydrate-containing structures are metachromatically stained (red). Cytoplasmic RNA (e.g., Nissl bodies of neurons) is also blue. Other thiazine dyes (e.g., Borrel's methylene blue, azure A) may be substituted for toluidine blue.

Neutral Red (C.I. 50040)

Use a 0.5% aqueous solution for 1-5 min. Acidification with acetic acid (as for toluidine blue) is usually desirable. Wash, dehydrate, and clear as described for toluidine blue.

Nuclei and other basophilic structures are stained red. Safranine O (C.I. 50240) gives a similar result, but longer staining times (often 30 min) are usually required.

Neutral red can also be used as a fluorochrome. Stain hydrated paraffin sections for 5 min in 0.002% aqueous neutral red, then dehydrate, clear, and cover in the usual way. With excitation by near UV or blue light (325-500 nm), nuclei and cytoplasmic RNA emit yellow-orange fluorescence (Allen and Kiernan 1994).

Ethyl Green (C.I. 42590) and Methylene Green (C.I. 52020)

These unrelated dyes are useful counterstains when objects of greater interest than nuclei have been colored red, purple, or brown. Ethyl green is still sold as "methyl green," a dye that has been obsolete for more than 30 yr.

For staining with ethyl green, immerse slides for 15 min in a 0.2% solution of the dye at pH 4.0-4.5. An acetate buffer is a suitable solvent. Rinse quickly in H_2O, blot the slides dry, and dehydrate in two changes of acetone or of *n*-butanol (as described above for toluidine blue). Clear in xylene and cover, using a resinous mounting medium. Nuclei and other basophilic structures are stained bluish green.

A methylene green counterstain for nuclei (McNulty et al. 2004) is a 0.5% (w/v) solution of the dye in 2.4% aqueous boric acid. After 5 min in this solution, the slides are washed in two 2-min changes of H_2O followed by three 3-min changes of 100% ethanol, clearing in xylene, and application of a resinous mounting medium.

Polychrome Methylene Blue

The staining solution is a mixture of thiazine dyes produced by oxidation and demethylation (polychroming) of methylene blue. The polychroming may be accomplished in various ways. The following method, in which silver oxide is used as an oxidizing agent, is recommended for its simplicity and speed.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**R**>Borrel's methylene blue
Ethanol
Mounting medium (resinous)
<**!**>Xylene

Method

1. Stain frozen sections or hydrated paraffin sections for 2 min in the diluted solution of Borrel's methylene blue.
2. Rinse rapidly in running tap H_2O, blot slides dry, and dehydrate in two changes (each 30 sec, with agitation) of 100% ethanol.
3. Clear in xylene and cover, using a resinous mounting medium.

Results

All components of the tissue are stained in various shades of blue. Strongly acid carbohydrates stain metachromatically (red), especially if some differentiation is allowed to occur in the H_2O wash. With deliberate overstaining, as described, the objects that are normally metachromatic will be dark blue or purple.

Anionic Dyes

Anionic dyes are applied from acidic solutions. Used alone, an anionic dye will color almost all the components of a tissue, but differential staining effects can sometimes be obtained when two or more dyes are applied simultaneously or in sequence. A method of this type is Mann's eosin-methyl blue. Nuclei, cartilage, and collagen acquire the blue color, and cytoplasms are stained pink or red by eosin. The reasons for these results are not fully known, but it is widely held that the large molecules of methyl blue are excluded by the smaller ones of eosin from the supposedly "dense" network of cytoplasmic protein molecules. Collagen and chromatin, with their presumed "looser" textures, would admit molecules of both dyes, but the eosin would diffuse out more rapidly than the methyl blue when the sections are washed following immersion in the mixture. Nuclear staining by methyl blue was studied by McKay (1962), who found that the presence of nucleic acids was necessary. He was unable to obtain evidence for binding of the dye by ionic attraction and suggested that the staining of nuclei was analogous to the direct dyeing of cotton. McKay compared methyl blue with chlorazol black E, a direct azo dye. Chlorazol black E was found to behave like most other anionic dyes, including eosin, and was, therefore, thought to be bound to tissue principally by electrovalent forces.

Mann's Eosin-Methyl Blue

The procedure given below is the "short method" of Mann (1902). The "long method" (Mann 1902; Cook 1974; also discussed at length by Gabe 1976) is more controllable and is used for demonstrating intracellular objects such as secretory granules and viral inclusion bodies. Fixation is not critical, but superior contrast is obtained if mixtures containing mercuric chloride, potassium dichromate, or picric acid have been used.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Ethanol (95%, 100%)
<**R**>Mann's eosin-methyl blue
Mounting medium (resinous)
<**!**>Xylene

Method

1. De-wax and hydrate paraffin sections.
2. Stain in Mann's eosin-methyl blue for 10 min.

3. Wash in running tap H_2O for a few seconds to remove excess dyes from slides.
4. Dehydrate in 95% ethanol and two changes of 100% ethanol.
5. Clear in xylene and cover, using a resinous mounting medium.

Results

Nuclei and collagen are stained blue; erythrocytes, cytoplasm, and nucleoli are stained red. With this "short" method, the colors obtained are rather variable. Like Borrel's methylene blue, this is a "quick look" stain for determining which of a series of sections or specimens are worthy of more critical examination.

Chlorazol Black E

Useful staining of nuclei and cytoplasm can be obtained with chlorazol black E. This dye also stains elastin, but probably by a nonionic mechanism (Goldstein 1962).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**R**>Chlorazol black E
Ethanol (70%, 95%, 100%)
Mounting medium (resinous)
<**!**>Xylene

Method

1. De-wax paraffin sections and take to 70% ethanol.
2. Stain in the chlorazol black E solution for ~10 min.
   The time is not critical.
3. Rinse in 95% ethanol, two changes, 1 min in each.
   Overstaining seldom occurs if the time at Step 2 is <20 min. Differentiation occurs slowly and controllably in 95% ethanol.
4. Complete the dehydration in two changes of 100% ethanol.
5. Clear in xylene and cover, using a resinous mounting medium.

Results

Nuclei and elastin are stained black. Other components of tissue are visualized in various shades of gray. Cytoplasm often has a greenish tinge and cartilage matrix is usually pinkish gray. Collagen is rather lightly stained. Cytoplasmic organelles such as mitochondria and secretory granules are well displayed in suitably fixed material. The color does not fade. The most informative preparations are thin sections (5 µm or less) examined at high magnification.

The method will work after any fixation but, as with most other dye staining methods, greater contrast is obtained after fixation in a mixture containing picric acid, mercuric chloride, or potassium dichromate than after fixation in formaldehyde alone. Cannon (1937), the originator of the method, recommended terpineol as a differentiator. He also commented on the similarity of the end result to that obtained with Heidenhain's iron-hematoxylin, a traditional but time-consuming technique involving a critical differentiation. For other methods of staining with chlorazol black E, see Clark (1981).

For nuclear staining, mixtures of mordant dyes with appropriate metal salts are applied to sections of tissue. Some dye-metal complexes, such as that of chromium with gallocyanine, behave as if they were simple cationic dyes. For others, including hematein, brazilin, and eriochrome cyanine R, the interactions of dye, metal, and substrate are more complicated than the attraction of oppositely charged ions. These latter dyes are used to obtain selective staining of chromatin.

Hematein (the principal product of oxidation of hematoxylin) is used in solutions containing ferric, aluminum, or more rarely, chromium ions. In other mixtures, used only for specialized purposes, the metal ion may be lead, copper or zirconyl, or phosphotungstic or phosphomolybdic acid. For nuclear staining, the solution must contain a considerably greater proportion of ferric or aluminum ions than of hematein and it must be acidified. The word "hematoxylin" is commonly used in the names of staining methods and mixtures, even though "hematein" would be more correct.

Solutions containing oxidized hematoxylin (hematein) and Al^{3+} are the "H" (hemalum) of H and E, and are used everywhere to stain the nuclei of cells. The reactions of aluminum ions with hematein have been studied by Bettinger and Zimmermann (1991a,b), who found that a cationic dye-metal complex was present in acid solutions. The complex was bound by DNA in sections of tissue, even though the pH was lower than that at which nucleic acids can be stained by ordinary cationic dyes. The hemalum mixtures in common use contain a large excess of Al^{3+} ions over hematein molecules. Aluminum ions have considerable affinity for DNA, and can prevent its subsequent staining by cationic dyes. Acids used to increase the selectivity of nuclear staining probably disrupt the bonding between Al^{3+} and parts of the tissue other than chromatin, rather than between Al^{3+} and the dye (Baker 1960; 1962).

Unfortunately, affinity for Al^{3+} or the Al-hematein complex for DNA does not account for the selectivity of nuclear staining by the commonly used hemalum solutions. This is inhibited only slightly by prior extraction of DNA from the tissue, but it is considerably reduced after chemical blocking of lysine and arginine residues (Lillie et al. 1976). Furthermore, measurements of the color intensity in hemalum-stained nuclei do not vary in proportion to the content of DNA (Schulte and Fink 1995). These observations suggest that the dye-metal complex attaches mainly to a component of the nucleus other than DNA. The non-DNA component of chromatin is the basic nucleoprotein, which would be expected to bind an anionic rather than a cationic dye-metal complex. However, alum-hematein does not stain other tissue components that have affinity for anionic dyes, so there must be a high affinity for chromatin that is unrelated to DNA or the basic amino acids of its associated nucleoprotein.

Horobin (1988) suggests that dye-metal complexes are bound to chromatin by both ionic and nonionic forces. The latter are likely to be enhanced by the other substances present in alum-hematein staining solution. Most formulations contain a highly polar substance such as glycerol, ethylene glycol, or chloral hydrate, which would be expected to associate by hydrogen bonding with hydrophilic components of the tissue and to interfere with short-range forces (van der Waals, hydrophobic, etc.) that would hold the dye-metal complex to some potential substrates. A shape of the dye-metal complex ion that favorably conforms with the nucleoprotein and nucleic acid molecules would be bound closely enough to resist disruption by hydrogen bonding substances. Any colorant intimately adherent to DNA or the histone molecules of chromatin would not easily be removed by aqueous or other hydrogen-bonding rinses.

Alum-hematein staining may be progressive or regressive. The former is usually preferred, but if overstaining must be corrected, the sections are differentiated in 70% or 95% ethanol containing a little hydrochloric acid. Aqueous acid may also be used, but the differentiating action is then slower. Addition to the staining bath of more acid or more of the aluminum salt also suppresses the tendency to overstain, thereby making the mixture more selective as a progressive nuclear stain. The acid-lability of nuclei stained by alum-hematoxylin precludes counterstaining with solutions that are more than slightly acidic. Eosin is suitable but the van Gieson mixture is not. The aluminum-hematein complex changes color from reddish brown to blue at ~pH 6. The latter color is the one desired, so stained sections are washed in tap H_2O, to which a trace of alkali may have to be added. This process is called blueing. An eosin counterstain imparts pink and red colors to all components of the tissue, including nuclei previously made blue by alum-hematein. Consequently, the nuclei in a typical H and E preparation are purple, from mixing of the colors.

The iron-hematein complex has a deep blue-black color and can be removed from stained sections only by strongly acid differentiating solutions. If the iron salt and the hematein are applied sequentially to the section, as in Heidenhain's method, a great variety of structural details can be revealed by careful differentiation. The progressive mode is the method of choice for pure nuclear

staining, so the dye solutions are then made with large excesses of ferric salt and acid (usually hydrochloric). Weigert's hematoxylin is a typical mixture of this type. The ferric ions eventually overoxidize the hematein, so the stain deteriorates gradually (over several days), until it is no longer usable. Some iron-hematoxylins have better keeping properties than Weigert's. The more stable iron-hematoxylin mixtures contain both ferric and ferrous ions. The reducing action of the latter is assumed to restrain the oxidation of the dye (see Lillie and Fullmer 1976). Little is known of the mechanisms of nuclear staining by iron-hematein. Extraction of DNA has no effect, and chemical blocking of lysine and arginine residues causes only partial inhibition of staining (Lillie et al. 1976).

Sections containing nuclei stained by iron-hematoxylin do not lose their color when treated with strongly acidic reagents, including the mixtures of anionic dyes used for differential coloration of cytoplasm and collagen.

It is also possible to stain nuclei with synthetic metal complexing dyes that are cheaper than hematoxylin. A suitable substitute is eriochrome cyanine R, used with a ferric salt. This behaves like alum-hematein when used as a nuclear stain. Several other alternatives to hematoxylin have been described by Lillie et al. (1976).

Often red is preferred to the blue and black imparted to nuclei by the metal-hematein stains. This may be achieved by using an aluminum salt with carminic acid (carmalum), brazilein (brazalum), or nuclear fast red. Red nuclear stains are most often employed as counterstains to histochemical or other specialized methods that impart other colors to the objects in which the microscopist is primarily interested.

Heidenhain's Iron-Hematoxylin

This simple classical technique is used principally to resolve structural details within cells, so the tissue should be fixed in a mixture that preserves cytoplasmic organelles. Helly's or Altmann's fixative meet this requirement. Paraffin sections should be no more than 5 µm thick. The sections are soaked in a solution containing ferric ions and then immersed in a hematein solution, which forms a black complex with the bound iron, everywhere in the tissue. The hematein serves only to demonstrate iron (Wigglesworth 1952), which is bound principally to carboxyl groups of proteins. The iron alum solution is then used again, to differentiate the preparation until the desired staining is obtained.

The differentiating solution acts partly because it is acidic and partly because its ferric ions form soluble complexes with hematein, thereby extracting the dye from the stained section. Differentiation by acid alone leaves the nuclear chromatin as the last material to be destained. A neutral or alkaline solution of a compound containing iron(III), such as potassium ferricyanide, extracts color first from the nuclei and last from membranous structures such as myelin and mitochondria. Iron alum is between these extremes, and allows the staining of nuclei, secretory granules, cilia, brush borders, centrosomes, mitochondria, the various transverse bands of striated muscle fibers, and bundles of cytoskeletal filaments. Even structures that can be properly resolved only by electron microscopy, such as the endoplasmic reticulum (ergastoplasm) and some of the organelles of protozoa, were described originally in preparations stained by Heidenhain's hematoxylin technique. Complete extraction of nucleic acids from sections has no effect on nuclear or cytoplasmic staining by this method; the binding of iron is principally to carboxyl groups of proteins (Puchtler 1958).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Ethanol series (graded)

<**R**>Iron alum (5%)

Iron alum is also called ammonium ferric sulfate.

<**R**>Matured 10% alcoholic hematoxylin

Prepare a working solution of matured hematoxylin by diluting 5 mL of matured 10% alcoholic hematoxylin in 95 mL of H_2O. This solution can be kept (in a dark place) and used repeatedly for ~1 yr.

Mounting medium (resinous)
<!>Xylene

Method

1. De-wax and hydrate paraffin sections. Remove mercury deposits if necessary.
   If the sections are stained with eosin or a similar red anionic dye before Step 2, there will be less staining of collagen by the iron-hematoxylin (Gabe 1976).
2. Put the slides in 5% iron alum solution overnight or for 24 h.
   It is a common practice to preheat the iron alum and the working solution of matured hematoxylin to ~55°C. Steps 2 and 4 can then be shortened to 1 h, but the black color will not be as strong as with the traditional long method.
3. Rinse in three changes of H_2O, each for 1 min.
4. Put the slides in the working solution of matured hematoxylin overnight or for 24 h.
5. Wash in running tap H_2O for 5 min.
6. Shake and wipe off excess H_2O from one of the slides. Put a few drops of the 5% iron alum solution on the sections. Put the slide on the stage of a microscope, and watch the destaining process through a 10X objective.
7. When the desired appearance has been achieved, put the slide into running tap H_2O, and leave it there for at least 30 min.
   A counterstain is not usually necessary or desirable, but may be applied after Step 7. Cook (1974) recommends eosin or light green.
8. Dehydrate through graded ethanols.
9. Clear in xylene and cover, using a resinous mounting medium.

Results

Nuclei, cytoplasmic structures, and erythrocytes are stained black, blue-black, or gray, depending on the degree of differentiation.

Alum Hematoxylin: Hemalum and Eosin

The method described here is typical of the many H and E procedures. The traditional recipe for Mayer's hemalum has been modified by reducing the amount of sodium iodate. Alternative alum-hemateins of equal value are also described. Do not expect a satisfactory result by following the numbered steps of this method uncritically: Careful attention to the included notes is essential.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**R**>Acid-alcohol (optional; see Step 2)
<**!**>Ammonium hydroxide (optional; see Step 3)

Alternatives include:

<**!**>*Calcium hydroxide*

<**!**>*Lithium carbonate (saturated aqueous)*

<R>Eosin solution
Ethanol (70%, 95%, 100%)
<!>Fast green FCF (0.5% aqueous) (optional; see Step 4)
<R>Mayer's hemalum

This is the 1901 variant of Langeron, which is ready to use immediately; Mayer's original mixture, published in 1891, did not include an oxidizing agent and it was necessary to wait several months for sufficient atmospheric oxidation of hematoxylin to hematein. (For more information, go to http://stainsfile.info/StainsFile/dyes/75290.htm.)

Mounting medium (resinous)
<!>*n*-Butanol (optional; see Step 6)
<!>Periodic acid (5% aqueous) (optional; see Step 3)
<!>Xylene

Method

1. De-wax and hydrate paraffin sections.

   Frozen sections should be dried onto slides.

2. Stain in Mayer's hemalum for 1-15 min (usually 2-5 min, but this should be tested before staining a large batch of slides).

   Overstained sections can easily be differentiated by agitating for a few seconds in acid-alcohol, then washing thoroughly in tap H_2O.

3. Wash in running tap H_2O for 2 or 3 min or until the sections turn blue. Examine the wet slide under a microscope to check that selective nuclear staining has been achieved.

   If the tap H_2O is not sufficiently alkaline to blue the sections, a few drops either of ammonium hydroxide (SG 0.9) or of saturated aqueous lithium carbonate, or a small pinch of calcium hydroxide may be added to ~500 mL of H_2O; the washed sections may be left in this for 30-60 sec, then rinsed in tap H_2O again.

   Any blue coloration of cytoplasm and connective tissue should be extremely faint.

   Poor nuclear staining may be due to prior excessive exposure of the tissue to acidic reagents (e.g., unneutralized formalin or decalcifying fluids). To restore the chromophilia, see the manual by Luna (1968), which recommends treatment of the hydrated sections with either 5% aqueous sodium bicarbonate ($NaHCO_3$) or 5% aqueous periodic acid, overnight, followed by a 5-min wash in H_2O before staining.

4. Immerse the slides in eosin for 30 sec with agitation.

   A satisfactory alternative to eosin is fast green FCF (0.5% aqueous), which is differentiated by H_2O more readily than by alcohol. This dye stains acidophilic elements a bluish-green color. It is valuable if some components of the section have already been stained pink or red with, for example, the periodic acid-Schiff method.

5. Wash (and differentiate) in running tap H_2O for ~30 sec.

6. Dehydrate in 70%, 95%, and two changes of 100% ethanol (with agitation, ~30 sec in each change; without agitation, 2-3 min in each change).

   For nitrocellulose sections, avoid 100% ethanol and complete the dehydration in two changes (each 10 min) of n-butanol. Frozen sections usually require shorter times in the dyes and longer times for differentiation, washing, dehydration, and clearing.

7. Clear in xylene and cover, using a resinous medium.

Results

Nuclear chromatin is stained blue to purple; cytoplasm, collagen, keratin, and erythrocytes are stained pink.

Discussion

The ideal balance between the two components of the H and E stain is a matter of personal taste and is determined by the intensity of coloration due to the eosin. For a weaker counterstain, use 0.2% eosin or prolong the differentiation (Step 5). Differentiation also occurs in the 70% ethanol used for

dehydration and, to a lesser extent, in the higher ethanol concentrations. A yellow to orange cast can be discerned in some objects stained by eosin Y. This is most easily seen in erythrocytes. Staining by eosin should never be so strong that the nuclei are obscured.

Many workers use a mixture of two xanthene dyes (such as eosin Y and phloxin) to increase the variety of shades of the background color. Garvey (1991) favors a combination of phloxin with saffron, a yellow dye that can be bought cheaply as a food coloring agent.

There are several alternatives to Mayer's hemalum. Baker's "hematal-16", which contains 16 ions of Al^{3+} for every one molecule of hematein, is a slow, progressive nuclear stain and should be applied to the sections for 10-30 min. Another popular solution is Gill's hematoxylin, which has higher concentrations of ingredients and a 1:8 dye:Al^{3+} ratio (approximately). It usually gives adequate staining in <10 min. Ehrlich's hematoxylin, one of the oldest of these mixtures, also has a low dye:Al^{3+} ratio.

Alternative alum-hemateins:
<**R**>Ehrlich's hematoxylin (Ehrlich 1886)
<**R**>Hematal-16 (Baker 1962)
<**R**>Gill's hematoxylin (Gill et al. 1974)

Weigert's Iron Hematoxylin

A 5-10 min stain in a working solution of Weigert's hematoxylin should give selective nuclear staining without the need for differentiating.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**R**>Acid-alcohol (optional; see Step 4)
Mounting medium (resinous)
<**R**>Solution A for Weigert's hematoxylin
<**R**>Solution B for Weigert's hematoxylin

Method

1. Prepare a working solution of Weigert's hematoxylin by mixing equal volumes of Solutions A and B. Put A in the staining jar first for more rapid mixing.
   The mixture should be made just before use, but can be kept for a few days on the bench or for ~2 wk at 4°C.
2. De-wax and hydrate paraffin sections.
3. Stain in the working solution of Weigert's hematoxylin for 5 min (10 min if the solution is more than a few days old).
4. Wash in running tap H_2O. Check with a microscope.
   If overstaining occurs, the sections can be destained by immersion in acid-alcohol, though the process is slow. Overstaining can be prevented on future occasions by using a higher proportion by volume of Solution B in the working mixture. Conversely, if staining is too slow or too weak, even with a freshly mixed working solution, the proportion of Solution A should be increased. Slight gray coloration of the cytoplasm does not usually matter; it disappears when a counterstain is applied.
5. (Optional) Apply other staining procedures, as desired.
6. Dehydrate, clear and cover, using a resinous mounting medium.

Results

Nuclei are stained black or blue-black (may be dark brown if the working solution has been stored for more than a few days).

Lillie's Modification of Weigert's Iron Hematoxylin

After preparation, this modified solution can be used immediately, in the same way as the original Weigert's iron-hematoxylin.

<R>Lillie's modification of Weigert's iron hematoxylin

Brazilin

This dye is closely related to hematoxylin. Its oxidation product (brazilein) forms with aluminum ions a complex that stains nuclei red. The color (like that imparted by alum-hematein) is extracted by acids, so alum-brazilein is best applied as a counterstain to follow a method that imparts green or blue colors to other components of the tissue.

A suitable mixture is Mayer's brazalum. This is made in exactly the same way as Mayer's hemalum, but substituting brazilin (C.I. 75280) for hematoxylin. It is more stable than Mayer's hemalum, perhaps because brazilin has only one pair of hydroxyl groups in the easily oxidized catechol configuration. The staining time is usually ~5 min, followed by differentiation, if necessary, in acid-alcohol. Nuclei are stained red. Brazalum is an excellent nuclear counterstain to follow alcian blue or Perls' Prussian blue method for iron deposits.

Mayer's Carmalum

Mayer's carmalum is often used for whole specimens such as embryos and small invertebrates. For these, dilute the staining mixture 20 times in 0.5% (v/v) acetic acid, and stain for 24-48 h.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**R**>Mayer's carmalum staining mixture (after the collection from Gatenby and Beams 1950)

Method

1. Stain hydrated sections for 10-30 min.
   Overstaining does not occur.
2. Wash in running tap H_2O for ~1 min.
3. Dehydrate, clear, and cover.

Results

Nuclei are stained crimson. Like alum-brazilein, carmalum is valuable as a counterstain. It gives pleasing appearances following alcian blue, indigogenic methods for esterases, or silver methods for nervous tissue.

Alum-Nuclear Fast Red

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<R>Nuclear fast red solution (after Luna 1968; Humason 1979; Presnell and Schreibman 1997)

Method

1. Stain hydrated sections for 5-10 min.
2. Wash in running tap H_2O, ~l min.
3. Dehydrate, clear and cover.

Results

Nuclei are stained crimson. Usually, there is also pale pink staining of cytoplasm and collagen. If staining is unsatisfactory, the cause may be a mislabeled or otherwise unsatisfactory batch of the dye (see Frank et al. 2007).

Iron-Eriochrome Cyanine R for Nuclei or Myelin

This method (Kiernan 1984) is derived from the methods of Page (1965), Llewellyn (1974, 1978), Hogg and Simpson (1975) and Clark (1979a). It has two variants, and can be used either for selective nuclear staining (similar to that seen with aluminum-hematein) or for staining myelin sheaths of nerve fibers. The dye is also sold under the names solochrome cyanine R, chromoxane cyanine R, and mordant blue 3. The iron-dye complex stains everything. Differentiation in acid-alcohol removes color from everything except nuclear chromatin, and the blue color develops when the sections are washed in H_2O. Differentiation in alkali or a ferric salt removes color from everything except myelin and erythrocytes.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Counterstain

After a nuclear stain, use a red anionic dye such as eosin. Van Gieson's picro-fuchsine may also be used. After a myelin stain, use a red basic dye (0.5% aqueous neutral red or safranine is suitable) to stain nuclei and Nissl substance.

<R>Differentiating solution B(1) for nuclei

Differentiating solution B(2) for myelin

Any one of the following aqueous solutions may be used:

<**!**>*10% (w/v) ammonium ferric sulfate (iron alum, $NH_4Fe[SO_4]_2 \cdot 12H_2O$)*

<**!**>*5.6% (w/v) ferric chloride ($FeCl_3 \cdot 6H_2O$)*

<**!**>*7.3% (w/v) ferric nitrate ($Fe[NO_3]_3 \cdot 6H_2O$)*

In addition, Clark (1979b) recommended an alkaline differentiating solution for myelin staining:

<**!**>*ammonium hydroxide (a freshly prepared 1% [v/v] dilution)*

This acts in a few seconds, and it is easy to remove too much of the blue dye-metal complex.

The mechanisms of differentiation by Fe(III) or alkali have been discussed by Kiernan (1984, 2007).

Differentiating solutions keep for a few years, but may be used only once. Discard if they are cloudy or if there is a thick layer of pale insoluble material in the bottom of the bottle.

Mounting medium (resinous)

<R>Staining solution A

Method

1. Stain hydrated sections in Solution A.

   For nuclear staining, 5 min is sufficient. For myelin staining, 15-20 min are needed. (For either method, the slides may remain in the dye solution for 30 min without harm.)

2. Wash in running tap H_2O, 30 sec, or in three changes of H_2O.

   This is to remove unbound dye.

3. Perform differentiation:

   i. For nuclear staining, immerse in differentiating solution B(1) with continuous agitation for 10 sec, wash in running tap H_2O for ~30 sec and examine with a microscope to check that only the nuclei are stained. If other structures are also stained, put the slides back into differentiating solution B(1) for another 10 sec, and check again. (Differentiation usually takes 10-30 sec.) If too much color is extracted, go back to Step 1 and use a shorter differentiation next time.

   ii. For myelin staining, immerse in solution B(2) until only the myelin (white matter of central nervous system [CNS]) retains the stain.

   This usually takes 5-10 min. It is sometimes impossible to decolorize the nuclei completely without losing some intensity in myelin.

4. Wash in tap H_2O (running, or three or four changes) for ~5 min.

5. Apply a counterstain, as desired.

6. Dehydrate, clear, and cover using a resinous mounting medium.

Results

With the nuclear stain, nuclei are stained blue. The color is more resistant to aqueous acids than that of alum-hematein, allowing the use of a greater variety of counterstains.

With the myelin stain, myelin is stained blue. Erythrocytes are also blue.

Gallocyanine Chrome Alum

Gallocyanine chrome alum is a blue cationic complex of chromium(III) with an oxazine dye. It is a useful stain for nucleic acids that resist extraction by alcohols or acidic counterstains to a greater extent than simpler cationic dyes. Specimens may be fixed in any mixture that preserves DNA and RNA. The method is used mainly on paraffin sections, but it can also be used on frozen sections of formalin-fixed material.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Counterstain (optional; see Step3)
<**R**>Gallocine chrome alum solution
Mounting medium (resinous)

Method

1. De-wax and hydrate paraffin sections.

2. Stain either for 2 h at 50°C-60°C (coplin jar in oven), or for 24 h at room temperature.

3. Wash in tap H_2O, and counterstain (e.g., with eosin) if desired.
4. Dehydrate, clear, and mount in a resinous medium.

Results

Nissl substance of neurons and nuclear chromatin (including nucleoli) are stained blue. Deposits of ribosomal RNA in the cytoplasm of cells other than neurons are also blue. Proteoglycans (mast cells, cartilage matrix) and some types of mucus-containing acidic carbohydrates, such as intestinal goblet cells, are also stained.

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