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The Basis of Dye Chemistry and Techniques Available For Identification of Adulterated or Mislabeled Dyes PART I

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The material contained herein was submitted as an essay for histotechnology, New South Wales Institute of Technology. Ms. Barisic is now working on a Biomedical Degree.

Introduction

This essay deals briefly with the basic ideas behind "dye chemistry" and the methods available for the identification of mislabeled or adulterated dyes. Dye chemistry is based on the structure of the dye molecule, the coloration properties of the dye and how the dye imparts its color to certain materials to produce staining. The biological staining of tissues will be considered and therefore it is necessary to include the ways in which dyes and tissues interact in dye chemistry.

Methods used in the identification of dyes such as chromatography, chemical methods and spectrophotometry to mention but a few, have enabled the identification of mislabeled and adulterated dyes. Normally these adulterated or impure dyes would produce inconsistent and abnormal staining, but by methods of analysis, dyes can be tested for impurities or mislabeling and hence would be classified as either fit or unfit for biological staining.

Inside This Issue

The basis of dye chemistry lies behind the structure of the dye molecule and how it interacts with a particular material to produce staining. The structure of the dye will enable us to understand why the dye is colored and hence how it imparts its color to the material it is staining.

Before discussing the nature of a dye, let us first distinguish between a dye and a stain and the process of staining.

Dye: A dye is "a colored substance, generally aromatic in nature, used for imparting color to various materials and producing color in plant and animal tissues to elucidate their gross microscopic structure and nature".[1]

Staining: Coloration of substances by organic dye stuffs. A means of conferring a color reaction on tissue elements and their stainable components; metabolic, functional or pathological.[1]

To understand the basis of dye chemistry, we will concentrate on such topics as why dyes are colored, the interactions involved in the binding of dyes to specific sites and factors affecting staining.

Why Are Dyes Colored?

According to Bancroft, *et al.*[2] dyes appear colored because they absorb radiation in the visible region of the electromagnetic spectrum, between 400-750 mμ. Therefore, if a dye absorbs a specific wavelength of light from white light, the resultant light will then be colored, the color depending on the particular wavelength absorbed, see Table I.

Culling[3] has suggested that dyes act by virtue of their resonance systems and selectively absorb components of white light. Therefore the light that is transmitted or reflected from a given substance will have a color that is complimentary to that which is absorbed. The chromophoric groups of the dye molecule, discussed later, give the dye these transmitting properties, hence imparting color to the dye and the tissue to which it has bound. For example, consider picric

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acid, it absorbs predominantly blue-violet light and transmits yellow light. Table I shows the various colors that can be absorbed and the resulting colors that are transmitted, or the complimentary colors.

TABLE I [1]

SPECTRUM-COMPLEMENTARY COLOURS

SPECTRUM	WAVELENGTH	COMPLEMENTARY COLOURS
Violet	400–430	Yellow
Blue	430–490	
Blue-green	490–510	Orange
		Red
Green	510–570	Purple
Yellow	570–600	Violet
		Blue
Orange	600–620	Blue-Green
Red	620–750	

Let us now consider the basic structure of a dye and its relation to color.

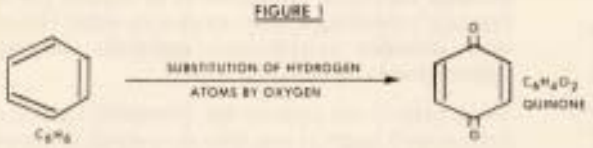
Structure of a Dye

A typical dye contains chromophoric and auxochromic groups attached to aromatic compounds, or benzene derivatives.

A. Benzene Derivatives

The benzene derivatives are an important part of the dye because if substituted with certain groups, they can produce colored substances. One type of substitution is important in dye chemistry, that is the formation of the quinoid compound.

FIGURE 1



This change from benzene to quinone is of significance in the production of color, since benzene derivatives absorb light only in the ultra-violet region and are colorless, but quinoid compounds absorb light in the visible spectrum and are therefore colored.[1]

B. Chromophores

Chromophores are the chemical structures that absorb certain wavelengths of light and therefore confer the color on a dye. The structure containing chromophores is known as the chromagen, and represents the colored component of the dye.

Lillie[1] states that the functional atomic groupings involved in chromagens are, C=C, C=O, C=S, C=N, N=N, N=O, NO_2 and the more that are present, the more defined the color. The quinoid ring contains C=C and C=O groupings in duplicate and is hence a very important chromagen.

Dyes are divided into two main groups according to the type of chromophores present.

Group 1 - Quinonoid Group: Color variation is due to other groups attached to the the quinone rings.

1. Triarylmethane - Cationic (e.g. methyl violet)
   - Anionic (e.g. methyl blue)
2. Anthraquinonoid - Anionic (e.g. alizarin)
3. Xanthene - Cationic (e.g. rhodamine 6G)
   - Anionic (e.g. eosin)

Group 2 - Azo Group: Contains one or more azo groups (N=N).

1. Monoazo - Anionic (e.g. orange G)
2. Diazo - Anionic (e.g. biebrich scarlet)

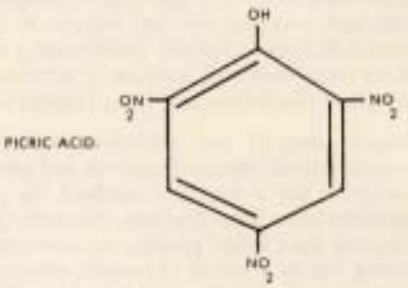
C. Auxochromes

Auxochromes are the ionizable portion of dyes which enables the dye to bind to any ionizable tissue structure by combining electrostatically or by covalent bonding to tissue end groups.[3] It gives the dye the salt forming property of electrolytic dissociation.

Auxochromic groups can either be acidic, e.g. COOH, or basic, e.g. NH_2, and hence dyes are classified as either acidic or basic depending on the auxochromic group. (See Acid Dyes and Basic Dyes below.)

An example of how an auxochrome group combined with a chromagen can produce a colored substance, is picric acid. The OH group is an auxochrome and when combined with the chromagen Trinitrobenzene (NO_2 group is the chromophore), a dye is produced.

FIGURE 2

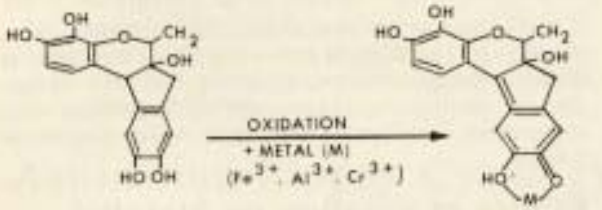


The auxochrome groups of a dye can combine directly to a tissue or by combination with a di or trivalent metal ion known as Mordants.

Mordants: Mordants chelate or combine with both dye radical and tissue end groups, therefore forming a link between tissues with dye. The dye and mordant chelate to form a "dye lake" which is then able to attach itself firmly to the tissue. Mordants are salts of metals usually sulfates of Cr, Al and Fe. To better explain the function of a mordant, let us consider a mordanted dye as an example — Hematoxylin.

Hematoxylin is a natural dye, but it has no staining properties until it is ripened by oxidation into Hematein, which has the quinonoid arrangement in one of the rings. However, Hematein alone has no affinity for tissue structures, therefore a mordant is combined to it prior to staining or incorporated into the staining solution to enable the dye to bind to the tissue. As a result, the dye is strongly bound to the tissue site due to dye-mordant combination and is resistant to washing in water.

FIGURE 3



HAEMATOXYLIN MORDANT - (HAEMATEIN + METAL)

Hematein plus mordant, has a strong affinity for phosphate groups of D.N.A., but by the use of different metal mordants, many tissue components can be stained.

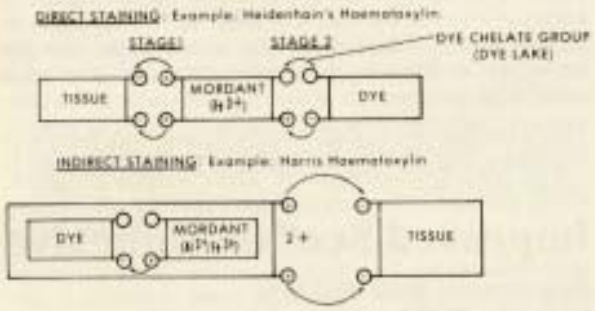
Table II gives a few examples of tissue components that can be stained by different metal mordants.

TABLE II [3]

ELEMENT	MORDANT (METAL)
Nuclei	Aluminum, Iron, Tungsten
Myelin Sheaths	Chromium, Copper, Iron
Elastic Fibres	Iron and Iodine
Fibroglia, Myoglia, Neuroglia and Epithelial Fibrils	Tungsten
Axis Cylinders	Lead
Mucin	Aluminum
Fibrin	Tungsten
Mitochondria	Iron

Mordants bind to tissues by two methods, either directly or indirectly, therefore giving Direct and Indirect staining.[4]

FIGURE 4



All ordinary dyes are salts composed of an acid and a base. This salt forming property of dyes enables them to bind to specific tissue sites depending on the nature of their auxochromic groups; that is, whether they are acidic or basic. Dyes are classified as either acid or basic, neutral and amphoteric.

Acid Dyes: Salts of color bearing acids with colorless basic radicals. The colored ion in acid dyes is the anion, which is negatively charged through normal pH 1-9, and is attracted to the positive charged or acidophilic tissue components, e.g., structures rich in carboxylated or sulfated mucosubstances, e.g., proteins.

Basic Dyes: Salts or color bearing bases with colorless acid radicals. The colored ion in basic dyes is the cation, which is positively charged through normal pH range and will have a high affinity for anionic or basophilic tissue structures, e.g., nucleic acids.

Neutral Dyes: Composed of an acid and basic dye in which both the anion and cation contain chromophoric groups and are colored. Neutral dyes stain different portions of the tissue according to its ionization at a particular pH; e.g., fat and lipid stains such as Oil Red O and Sudan dyes.

Amphoteric Dyes: Possess both positive and negative charged groups, hence dye can function as either an acid or basic dye depending on the pH of the staining solution; e.g., hematein. Table III provides a summary of the ionization of acid, basic and amphoteric dyes at a particular pH.

TABLE III [4]
EFFECTS OF pH ON DYE CHARGE

pH	3 4 5 6 7 8 9 10	
BASIC DYE	+ + + + + +	e.g. Methylene Blue
ACID DYE	− − − − − − − −	e.g. Eosin
AMPHOTERIC	+ + + + + − −	e.g. Haematein

Now that the basic structure of a dye is known and a few different types of dyes described, the next step in considering the basis of dye chemistry is the nature of the forces that enable dyes to bind specifically to tissues and therefore to confer a color reaction on the tissue components. Such a concept can be called Dye-Tissue-Affinity.

Dye-Tissue-Affinity

Affinity is the measure of the tendency of a dye to transfer from a dye-bath onto a section.[2] Affinity is influenced by factors that aid and hinder the process, such as solvent-solvent, dye-solvent, dye-dye, and dye-tissue interactions. Only a few factors aiding the affinity of a dye for a tissue will be considered.

A. Electrostatic or Ionic Bonding

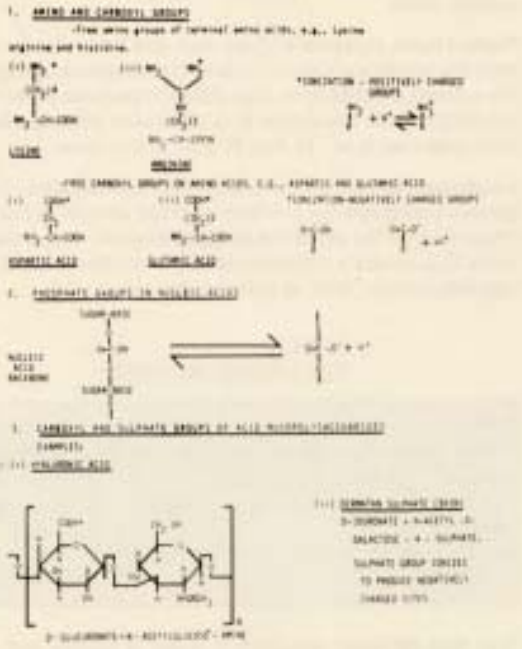
This is the most common linkage, where dyes form salt linkages to tissue components having the opposite charge (due to electrostatic attraction between oppositely charged ions).

The pH of the staining solution has a marked affect on the types of tissue components that can be stained at the same pH. Since dyes must ionize before they can produce colored cations and anions in solution, any material in a tissue that ionizes at that same pH will take up the oppositely charged dye molecule due to electrostatic attraction. Table IV gives a few examples of common ionizable tissue groups.

B. Polar Attractions of Van Der Waals Attractions

These occur between dyes and tissues in close proximity, since attractions are only weak polar forces which become stronger as polarizability of molecules increases. They are important with large dye molecules such as those used in many elastic fiber stains.

TABLE IV
COMMON IONIZABLE GROUPS (4)



C. Hydrogen Bonding

Important in non-aqueous staining solutions, since it involves bonding between two electronegative molecules. An example is the interaction between Carminic acid and polysaccharides such as glycogen.

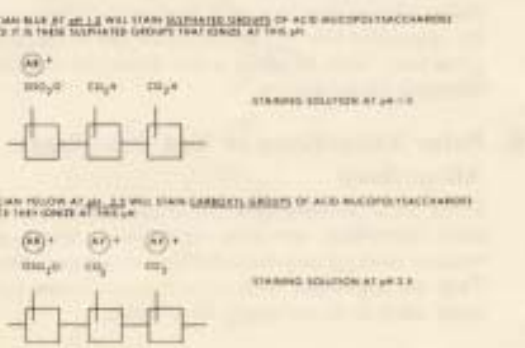
D. Hydrophobic Bonding

Hydrophobic groupings of tissues and dyes have a tendency to unite in an aqueous environment, due to hydrophobic attractions. Example, the staining of nonpolar elastin fibers by dyes containing hydrophobic groups such as biphenol and naphthyl. (Also lipid staining by neutral dyes.)

E. Covalent Bonding

Mordant dyes act by covalent bonding since they attach to tissues by metal-tissue covalencies. Also consider the staining of tissues by the Schiff Reagent in PAS. The Schiff Reagent forms covalent bonds with aldehyde groups on tissues that have been previously oxidized.

There are many factors that affect the uptake of a dye by a tissue. Such factors as pH of the staining solution, previously discussed, effects of fixatives prior to staining, and the metachromatic nature of some tissue components, as well as the presence of impurities, will influence the uptake of dyes.



A further note on the *effect of pH*, is that small changes in pH of the staining solution causes amphoteric substances in tissues to change to basic or acidic, whereas large changes alter the electrical charge balance of acidic and basic tissue components. Consider the staining by Alcian Blue/Alcian Yellow:

Effects of Fixation on Staining

Different tissue components are retained in tissues to varying degrees after different fixatives. Therefore staining will be different for different components and different fixatives. Fixation can have many effects on dye-tissue interaction, such as: a) changing the acidophilic-basophilic balance of tissues, e.g. formalin fixation reduces the number of primary amino groups therefore inducing acidophilia[2]; b) fixation times, different fixatives and fixation rates result in different staining rates, e.g. after Carnoy fixation, overstaining tends to occur by the large dye in Masson's trichrome staining, where the collagen fiber stain, light green, tends to give overstaining.

Metachromasia affects the color produced by certain dyes. Varying colors are produced from a single dye due to certain tissue components which exhibit metachromasia, such as mucin, cartilage, amyloid and mast cell granules. These are called Chromotropes or "color turners". Culling[3] has shown that the presence of high molecular weight substances in tissues, with free anionic groups, is essential for the production of metachromasia. Such groups as SO_3H-sulfonate, COOH-carboxyl and PO_4-phosphate are chromotropes. Dyes exhibiting metachromasia are toluidine blue, azure A, methylene blue and methyl violet.

Finally, the presence of *impurities* in a dye or solvent may affect the solubility of the dye as well as influencing the intensity of staining. The effects of impurities will be better explained in the following section which deals with the techniques available for the identification of adulterated or mislabeled dyes.

This is the first of a two-part article dealing with Basic Dye Chemistry and Techniques Available for Identification of Adulterated Dyes.

Improved Section Adhesive

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Gelatin, egg albumin and various other substances are used for adhering tissue sections to microscopic slides. When performing special staining procedures on gelatinized slides it was noticed that excess gelatin on the slides picks up certain dyes. This has often become a troublesome problem and therefore its prevention becomes important. The following solution has proven helpful for elimination of this problem.

Gelatin Adhesive Solution

Gelatin	0.5 g
5% Potassium dichromate	1 ml
Distilled water	200 ml

(Add a few crystals of thymol as a preservative.)

Procedure:

1. Heat the gelatin solution on low heat until it steams, but not boiling.
2. Allow it to cool for 10 minutes.
3. Dip slides quickly in gelatin solution.
4. Drain on cloth towel and allow to air dry. This step eliminates the excess accumulation of gelatin on slides.
5. Perform desired stain.

Aspiration Biopsy Cytology

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Aspiration biopsy cytology (ABC) is the study of cells obtained by a fine needle under vacuum. The specimen consists of a minute quantity of tissue or fluid.[2] ABC is a branch of diagnostic cytology that interprets changes in cells extracted from within organs, tumors, and non-neoplastic abnormal tissues.[1] All tissues, as well as cystic fluids, can be aspirated. The lack of adhesion of tumor plugs to the enveloping lymphatic walls and to the stroma of the tissue spaces invaded by the tumor allows one to withdraw large numbers of cells.[1] Aspiration biopsy is especially valued because:[2]

1. it can be performed in an office, done without advance preparation or anesthesia;
2. it is safe and virtually painless;
3. both biopsy and interpretation are rapid;
4. it's accuracy is high and preps give a distinct cellular and nuclear detail;
5. preps are suitable for decolorization and special stains;
6. it is a cost-contained procedure.

Aspiration of Tumor Cells Using Cytocentrifugation

Cytocentrifugation forcefully sediments cells from suspension onto a vertical microslide as the specimen's suspension medium is absorbed by a blotter. Cytocentrifugation is performed in a bench top centrifuge with a specially designed rotor and sample chambers that together constitute a cytocentrifuge. In simplest terms, cytocentrifugation is but another way to separate cells from their suspension medium and recover them on a microslide.

In our laboratory, we have incorporated our cytocentrifugation technique to tumor aspirations. When our hospital first started performing aspirations for rapid diagnosis of tumor masses, we employed the Direct (conventional) Method of preparing smears from the aspirations performed in radiology. This method was adequate but had many pitfalls. Many cells were lost during fixation and staining and the whole slide had to be screened. Many times the aspiration was a clear fluid which revealed very few cells.

The cytocentrifuge is used mainly for our cytology preparations. The main function of the cytocentrifuge is to forcefully sediment cells onto a vertical microslide. It has definitely enhanced our cytology preparations and increased the quality of our aspirations. Some advantages are:

1. It takes little time for the operator to prepare fluids for cytocentrifugation.
2. It is particularly useful when the aspirate has too few cells to form a cell button.
3. Cells are sedimented within a 32 mil area that is *38 times smaller* than the collections-display area of a conventional cell spread, thus markedly reducing screening time.
4. Centrifugal force constructively flattens individual cells to enhance the display of their chromatin distribution patterns.
5. A large number of cells are recovered.
6. It reproducibly yields high quality results.
7. Preparations require no special handling after cytocentrifugation.

We have compared slides prepared using the Direct Method to those prepared using the cytocentrifuge preparations and have concluded that the quality, number of cells present, and area to screen *greatly* surpasses the conventional method of preparing aspirate smears.

Method of Preparing Slides for Use with the Cytocentrifuge

The morning of a scheduled aspiration, radiology notifies histology personnel of the time the aspirate is planned. It has been found advantageous to use *small* glass disposable culture tubes to rinse the syringe and, if needed, to dilute our specimen. A small amount of Balanced Electrolyte Solution (BES-10 drops) is placed in the tube and taken to radiology at the time the aspirate is performed. After the radiologist has completed the aspiration, he will draw up the BES from the culture tube, rinse the syringe and deposit the aspirate back into the tube. The tube is then returned to the histology laboratory where the preps are made. Upon arrival into the lab:

1. Place the culture tube in a holding rack.
2. Take 4 clear, sterile tubes and place them in the holding rack. Our laboratory routinely prepares at least four preps on each aspirate.
3. Place 2 drops of albumin (adhesive) in each of the four tubes.
4. If needed, depending upon the consistency of your aspirate, dilute the specimen with BES. If your aspirate is clear, do not dilute.
5. Add 4 to 8 drops of aspirate to each tube.
6. If necessary add more BES to each tube.
   Note: There should always be a total of 10 drops of solution to each tube—whether it be 8 drops of aspirate with 2 drops of albumin or 4 drops of aspirate, 4 drops of BES and 2 drops of albumin. The amount of aspirate added to each tube depends upon the consistency of the specimen. We have always added 2 drops of albumin to each tube.
7. Label the 4 slides with patient's name or log number.
8. With 4 sample chambers, 4 spring-loaded assemblers and 4 blotters assemble the sample chambers to go into the cytocentrifuge following the instructions in the operation manual.
   Note: Disposable cytofunnels with attached filter paper are commercially available.
9. Remove the chamber lid.
10. Take one sample chamber and pour one aspirate sample into the vial.
11. Snap on the plastic lid and place into the chamber. Do this with each of the remaining culture tubes and place them in the centrifuge chamber in a balanced position.
12. Spin specimens. We spin ours for 10 minutes at 120 rpm.
13. After spinning, unload the slides carefully from the

holders and immediately place them in 95% ethyl alcohol.
14. The slides are fixed in the 95% ethanol for 5 minutes and are then stained using the Papanicolaou stain.
15. Slides are then coverslipped and taken to the Pathologist for review.

The only *disadvantage* that we have found in using this technique is that it takes a little longer (about 20 minutes from start to finish) for the finished product to be given to the Pathologist. The *advantages far outweigh* this one disadvantage. Your slides will be much more accurate, and if there are tumor cells present you can be assured they will be on your slide. The cells are much clearer and flatter. Screening takes place in a small circular area instead of having to search the entire slide and possibly miss tumor cells on the end or corner of your slide.

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Rapid Slide Drying With Microwave Heating

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The microwave oven has proven to be an extremely useful and time saving tool in histotechnology for the acceleration of metallic staining,[1,2] trichrome mordanting[3] and tissue fixation[4].

It has been found, in this laboratory, that microwave heating can be applied to dry (or attach) routine paraffin tissue sections on slides in glass trays in 2½ minutes. This can be used as an alternative to drying slides in a convection oven or forced air heater at 60 °C for 20-30 minutes. It is especially useful when preparing slides of tissue such as bone, which has a tendency to detach from the slide during staining. Consequently, it is usually dried on albumin or gelatin treated slides for several hours to overnight, prior to staining.

We have microwave (Samsumg-RE 515D) dried a wide variety of tissues and thus far not observed artifacts after hematoxylin and eosin or numerous special staining procedures. This has proven to be a fact when compared with conventionally dried slides from identical tissue blocks. Microwave bombardment is recommended as a rapid method for drying paraffin sections onto glass slides.

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Connective Tissue Staining in Water Soluble Methacrylate

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The following procedure for demonstrating connective tissue in water soluble plastic embedded specimens has proven useful and reproducible in our laboratory. It is being presented here, in the hope it will be helpful to other laboratories who have or are presently experiencing difficulties in staining connective tissue embedded in plastic. The procedure is a conglomeration of staining solutions from other staining procedures, which after much trial and error, has resulted in a very useful staining method. The water soluble methacrylate used, is from kits purchased from DuPont Company, Hitchin, Herts, United Kingdom and PolySciences, Moulton Park, Northampton, United Kingdom.

The extra strength Weigert's hematoxylin is similar to that reported by Johan Savesoderbergh, in Microtomy Report.[1] The Biebrich scarlet-acid fuchsin solution was obtained from a report in Stain Technology by R.D. Lillie.[2]

The light green solution being used is that reported by B.M. Jennings, *et al.*, in American Journal of Pathology.[3]

Fixation: 10% buffered neutral formalin

Microtomy: Cut sections at desired thickness (1-24).

Solutions

Picric Acid Solution (Saturated)

Picric acid	1.25 g
Distilled water	100 ml

Extra Strength Weigert's Hematoxylin Solution A (Stock)

Hematoxylin	10 g
Alcohol, 95% ethyl	100 ml

Solution B (Stock)

Ferric chloride	11.6 g
Distilled water	980 ml
Hydrochloric acid, 25% aqueous	10 ml

Extra Strength Weigert's Hematoxylin (Working)

Solution A	5 ml
Solution B	25 ml
Alcohol, absolute ethyl	20 ml

This working solution keeps for two weeks, but must be filtered prior to each use.

Aniline Blue Solution

Aniline blue	2.5 g
Distilled water	100 ml
Acetic acid	2 ml

Biebrich Scarlet-Acid Fuchsin Solution

Biebrich scarlet, 2% aqueous	45 ml
Acid fuchsin, 1% aqueous	5 ml
For use, add acetic acid	0.5 ml

This solution can be reused, but must be filtered prior to each use.

Staining Procedure

1. Mordant slides in saturated picric acid, 30 minutes in a 60°C oven.
2. Wash slides in running tap water for 10 minutes.
3. Rinse slides in distilled water.
4. Stain slides in extra strength Weigert's for 5 minutes.
5. Rinse slides in running tap water, 3-5 minutes.
6. Stain slides in Biebrich scarlet-acid fuchsin for 30 minutes.
7. Rinse slides quickly in 2-3 changes of distilled water.
8. Stain slides in aniline blue solution for 2-5 minutes. For bone stain in 2% light green in 2% acetic acid for 10-12 minutes.
9. Rinse slides in 2-3 changes of distilled water.
10. Rinse slides in 95% alcohol, 2 changes, until the stain is removed from the plastic media.
11. Rinse slides in 2 changes of acetone, xylene and coverslip.

Results:

Nuclei - black to dark blue
Mineralized Bone - green
Osteoid - red/orange
Collagen - green or blue (depending on counterstain)
Keratin, Muscle & Intercellular Fibers - red

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A Combination Myelin-Nissl Substance Stain

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Fixation: 10% neutral buffered formalin

Sections: 7 mu. or thicker, paraffin

Solutions

Solochrome Cyanine Solution

Solochrome cyanine RS	1.09 g
Sulfuric acid (conc.)	2.5 ml
10% Ferric ammonium sulfate	500 ml

Onto the dry solochrome cyanine powder, add drop-by-drop the sulfuric acid and mix into a slurry with a glass rod. Dissolve slurry in the 10% ferric ammonium sulfate solution. The resulting mixture is a very dark blue color.

10% Ferric Ammonium Sulfate Solution

Ferric ammonium sulfate	10 g
Distilled water	100 ml

2% Cresyl Fast Violet Solution (Stock)

Cresyl fast violet	2 g
Distilled water	100 ml

Buffer Solution

Sodium acetate	2 g
Distilled water	1000 g
Acetic acid	3 ml

Cresyl Fast Violet Solution (Working)

Cresyl fast violet (stock)	2 ml
Buffer solution	100 ml

Eosin Solution (Stock)

1% Aqueous eosin	68 ml
1% Aqueous phloxine	6.8 ml
95% Alcohol	500 ml
Glacial acetic acid	0.5 ml

Eosin Solution (Working)

Eosin (stock)	1 ml
Absolute alcohol	100 ml

Staining Procedure

1. Deparaffinize slides in usual manner and bring to water.
2. Stain slides in Solochrome cyanine solution for 1 hour.
3. Wash slides in tap water for 5 minutes.
4. Place slides in 10% ferric ammonium sulfate solution.
5. Wash slides in tap water at least 5 minutes.
6. Rinse slides in distilled water.
7. Stain slides in cresyl fast violet working solution for 1 hour.
8. Rinse slides in absolute alcohol.
9. Counterstain slides in eosin working solution until only the nuclei and nissl substance remain stained mauve/blue; myelin remains deep blue.
10. Dehydrate in 95% alcohol, absolute and clear in xylene 2 changes for 2 minutes each.
11. Mount coverslip with resinous media.

Comments

In the technique of Page,[1] 4 ml of 10% ferric ammonium sulfate per 100 ml of distilled water is used to make up a solochrome cyanine solution for staining of myelin. In my experience it is better to use a much stronger solution of ferric ammonium sulfate.

Washing in tap water for 5 minutes at Step 3 is mandatory as this seems to blue and 'fix' the stain.

Differentiation at Step 4 should be complete in 5 minutes. However, much longer times, e.g. overnight, will not remove the stain from the myelin.

Step 9 removes background mauve/blue staining and imparts a very pale pink background stain.

This combined procedure produces pleasing results and provides a method which is simple to perform.

References:

1. Bancroft, J.D. and Stevens, A. (1982) "The Solochrome Cyanine Technique for Myelin in Paraffin Sections", *Theory & Practice of Histological Techniques*, 2nd Edition, p.351.
2. Luna, L.G. (1968) "Vogt's Method for Nerve Cell Products", *Manual of Histologic Staining Methods of the AFIP*, 3rd Edition, p.212. McGraw-Hill:New York, N.Y.

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