Which Histochemical Staining Technique Should I Choose for Biological Specimens

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This question has been frequently asked due to the great increase and renovation in histochemical methods. The answer of this question depends on what you need to emphasis. The structure you would like to demonstrate may sometimes have various histochemical methods and related modifications. At this point, methods may get complicated. To illustrate, if you need to display axon or neuronal extensions in a neural tissue, you should apply Bielschowsky's silver stain method. If you want to show axons in the peripheric nervous system, then you should apply Palmgren's method and to indicate the degenerative axons you should apply Eager's method [1, 2]. Additionally, there are several methods for neuroglia's. As seen above, it is possible to find various methods peculiar to a tissue.

Recently, choosing the effective tissue stain has been an important issue in our studies due to the fact that unless you choose the suitable and effective staining method, you may not be able to reach the possible findings in your study. As a result, choosing the most appropriate staining method to show your studies at microscopic level may be crucial for your studies. The chapter has been designed as a practical guide for those preparing for examinations in histopathology. It aims to help laboratory technicians, researchers, students, histologist, pathologist with their choice of the accurate histochemical method in their studies.

Keywords: histochemistry; staining techniques; biological specimen

1. Introduction

Since most cell structures are transparent, very little detail of the structure can be seen, unless the cells are stained. The great majority of routine histology is done with hematoxylin and eosin (H&E) staining, because it is quick, cheap and informative. Staining with H&E is very reliable although it does show some variation depending on the exact formulation of the stain, and the stain density is considerably affected by the thickness of the sections – thicker sections take up more stain. It is also generally done before any additional staining techniques. A wide variety of other histochemical stains are also available, each of which can help identify particular structures. Some are relatively simple to perform, merely requiring that the section is dipped in the stain for a set time. Others require a number of sequential steps, and in some cases the results can be surprisingly variable or unpredictable.

2. Effects of fixation on staining

Fixation is the very important part of staining tissues because it allows the protection tissue which closely represents living original tissue. The effects of fixation depend on some factors such as pH, temperature, penetration, osmolarity, concentration and duration. The pH values vary according to fixatives and usually buffers adjust the hydrogen ion concentration in fixative solution. Appropriate fixation occurs at pH 6-8. For metachromatic dyes, the pH of fixation is important especially in methylene blue and toluidine blue. They work well in acidic pH levels for examination of mast cell in urinary bladder [3].

The most commonly used buffers are; phosphate, bicarbonate, citrate, tris and cocodylate. Additionally, the most dangerous one is cocodylate buffer due to its toxicity. Moreover, you should take into consideration that when you choose the wrong buffer that react with the fixative, it can reduce buffering power and fixation ability. For example phosphate inhibits glucose-6-phosphate dehydrogenase so you should not choose phosphate buffer when you make glucose-6-phophate dehydrogenase histochemistry [1].

The most effective temperature for mostly used fixative formaldehyde is at +4^{o}C. The suitable infusibility and penetration of formaldehyde occurs 2-6 hours in +4^{o}C. Fixation with glutaraldehyde for electron microscopy, is longer than the one night, it may be disadvantageous. Long fixation in these aldehydes is known to severely inhibit enzyme activity and immunological reactions. Prolonged fixation with oxidizing fixatives is likely to degrade the tissues by oxidative cleavage of the proteins and the loss of peptides. For this reason, glutaraldehyde duration is short and osmium tetroxide is immediately used as immersion fixation to take maximum result for ultrastructural evaluation by electron microscopy [1].

Most used fixative as Formaldehyde:

10% Formalin
Formaldehyde100 ml
Tap water 900 ml

10% Formalin-Saline
Formaldehyde100 ml
NaCl 8.5 g
Tap water 900 ml

10% Buffered Neutral Formalin (pH=7.0)
Formaldehyde100 ml
Tap water 900 ml
NaH_2P0_4. H_20 4 g
Na_2HP0_4 6.5 g

2.1 Some important tips for tissue fixation [5]

- Put your specimens into fixatives immediately after death or operation. Specimens should be placed in fixative for maximum 30 min for light microscopy, 4 min for electron microscopy after cardiac arrest.
- The chemicals used in fixatives should be fresh, measured and mixed meticulously.
- Tissue pieces should be as small as possible.
- Fixatives volume should be minimum 10 times more, for formalin 20 times more than tissue volume.
- Fixation is offered at +4 ^{0}C. Lower temperature is decreased the infusion of fixative to the tissue but it leads good fixation in order to prevent autolysis of tissue.
- Luminal organs are filled with fixative to preserve tissue from both sides.
- Tissues within possibility for corrugation and folding should be stretched on cork plates.
- First the fixatives then tissues should be put in container. Otherwise, tissues stick on container and fixative cannot infuse into the tissue from stick part.
- Tissues which are rich in lipid and air do not sink. So, this type of tissues should be first placed into cassette then put into fixative to sink.
- Avoid from speeding procedures to shorten the fixation time. Warming increase the infusion of fixative as well as it increases autolysis.
- The infusion time of fixatives into tissues should be known. For instance; alcohol fix the 5mm tissue for 5 hours, 4% formalin fix the 4 mm tissue in +4 ^{0}C for 5 hours.
- Avoid from fixing tissue more than 24 hours except for some special methods.
- Fixative solution should not be hypotonic or hypertonic. And adjust the osmolality of fixative. Do not wash the tissue with water before fixation.
- pH of fixative solution should be close to tissue pH (pH:6-8). For this reason buffered formalin is more suitable.
- The fixative pots should not be narrow.
- Tissues becoming hard due to staying in the fixative is not preferred. If tissues become hard, sectioning will be difficult.

3. Interactins of Dye and Cells

Proteins have an important role in staining of tissues. Proteins have both acidic and basic groups. At the isoelectric point, the dye is forming a salt linkage with the residue of an acidic group, it is termed a basic dye and the substance stained is termed basophilic. Conversely, basic groups (primarily amino) are positively charged so that at a pH below their isoelectric point they will bind anionic dyes such as eosin. Such dyes are therefore called acid dyes and the substance stained is termed acidophilic, or eosinophilic in the case of cell components that stain with eosin. Basophilic substances have biological importance. The phosphate groups place in nucleic acids abundantly (in both ribose and deoxyribose forms). Thus, nuclei, nucleoli, and ribosomes (free and ER-bound) are basophilic. Negatively charged carboxyl and sulfate groups are found abundantly in glycosaminoglycans, which are linear sugar polymers that are associated with core and linkage proteins to form complex branched proteoglycans. The weakest binding (weakest basophilia) is by phosphates; the strongest is by sulfates. Likewise, a number of acidophilic substances (positively charged substances) are also important: examples include collagen, keratin, hemoglobin, and muscle proteins. Some tissues are stained cationic below the certain pH and anionic above the certain pH. Typical basic dye is positively

charged; acidic dye is negatively charged in histologically used pH ratio; amphoteric dye has neutral charged in certain pH (isoelectric point) and it is acidic above or basic below that pH [1]. There are some most used acidic and basic dyes in Table 1.

Table 1 Some acidic and basic dyes with representative colors.

Basic Dyes	Color	Acidic Dyes	Color
Methyl Green	Green	Acid fuschin	Red
Methylene Blue (Metachromatic dye)	Blue (Purple)	Anilin blue	Blue
Pyronin G	Red	Eosin	Red
Toluidine blue (Metachromatic dye)	Blue (Purple)	Orange G	Orange
Hematoxylen	Dark blue	Congo Red	Pink-red

3.1 Types of Hematoxylin

The reason of dark blue staining of nucleus is the polyanionic DNA and basic hematein-mordant strongly interact. Also the reason of dark blue staining of cartilage with hematoxylin is polyanionic glicosaminoglycans. Although cytoplasm is expressed as pink, it is seen as blue-pink in microscope because of the hematein merging with mordant in free RNA [6]. You can find the list of most used hematoxylin as below:

3.1.1 Alum Hematoxylin

The types of alum hematoxylins contain potassium alum or ammonium alum as mordant. Alum hematoxylins are not used with counter dyes which include acid. For example; van Gieson which includes acidic counter stain replaces alum hematoxylin from tissue. For this reason, alum hematoxylins are not used in van Gieson dye.

A. Harris Hematoxylin [7]:

Harris hematoxylin provides strong nuclear stain. It can be most useful in your routine laboratory specimens for H&E stainning.

Table 2 Ingredients and amounts of materials in Harris Hematoxylin Staining.

Ingredient	Amount
Hematoxylin	2.5 g
Absolute alcohol	25 ml
Potassium Alum	50 g
Distilled water	500 ml
Mercuric oxide or sodium iodate	1.25 g or 0.5 g
Glacial acetic acid	20 ml

Protocol: Hematoxylin is dissolved in alcohol on heater with lower temperature. And add alum and mix. The mixed solution is rapidly brought to the boil and mercuric acid or sodium iodate is slowly added. Then solution is plunged into cold water. When the solution is cold, acetic acid is added. Solution is ready for immediate use for staining.

B. Mayer's Hematoxylin [8]:

It is mostly preferred as a nuclear counterstain in the demonstration of various enzyme histochemistry and immunohistochemistry techniques.

Table 3 Ingredients and amounts of materials in Mayers Hematoxylin Staining.

Ingredient	Amount
Hematoxylin	1g
Distilled water	1000 ml
Potasium or amonium alum	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate SLR or Chloral hydrate AR	50 g or 30 g

Protocol: Dissolve hematoxylin, potassium alum and sodium iodate in distilled water with stirring and warming. Add chloral hydrate and citric acid, mix and boil for 5 min. Cool and filter before use. The dye is ready for immediate use.

C. Ehrlich's Hematoxylin [9]:

Ehrlich's hematoxylin is used for the structures of general tissue with eosin. It stains mucopolisaccaharides and cements lines of bone and tooth as well. The glycerin content helps to stabilize the stain and prevent over oxidation. The glacial acetic acid increases pH and sharpens nuclear staining. It protects its staining ability for several years.

Table 4 Ingredients and amounts of materials in Ehrlich's Hematoxylin Staining.

Ingredient	**Amount**
Hematoxylin	4 g
95% ethyl alcohol	200 ml
Distilled water	200 ml
Glycerin	200 ml
Amonium or 4unsen4um alum sulfate	6 g
Glacial acetic acid	20 ml

Protocol: Maturation can occur in four weeks or more with exposing sun light and air. Immediate maturation can make by adding 0.6 g sodium iodate.

Dissolve hematoxylin in alcohol. Dissolve alum in hot water and add glycerol and cool. Add gradually hematoxylin to alum solution. Move solution to maturation in clean pot. Maturation takes 6-8 week. Staining occurs 4-5 min.

3.1.2 Iron hematoxylins [1]:

You can find brief explanations of iron hematoxylins as below in Table 2.

Table 5 Table of Iron hematoxylins giving results and preferability of stainings.

Iron Hematoxylins	**Prefer for**	**Result**	
Weigert's Hematoxylin	Useful stain with eosin for CNS; with Van Gieson which shows collagen fibers	Nuclei	Brown to black
Heindenhain's Hematoxylin	Cross striations of muscle fiber, mitochondria, chromosomes, chromatin, centrioles, nuclear membrane	Color of grey to black depends on differentiation and fixation.	
Loyez Hematoxylin	Myelin (paraffin, frozen and nitrocellulose sections can be used)	Brown to black	
Verhöeff's Hematoxylin	Coarse elastic fibers	black	

3.2 Special Stains

The most commonly used staining combination is H&E but sometimes there is need for many other staining combination to show specific tissue structures. We can use more specific dye combination or solutions for getting the best and most identifiable results such as PAS (Periodic acid-Schiff) reaction for carbohydrates staining, Feulgen reaction for deoxyribonucleic acid (DNA), Masson trichrome for connective tissue, silver impegration technique for neurofibrils or reticular fibers located in lymphoid organs, liver, lung etc. You can find friendly protocols of special stains as below.

3.2.1 Schiff's Reaction

The more useful dye is basic fuchsin, a red dye in solution that becomes colorless when reduced by acid. Hugo Schiff (1834-1915) was the first to discover that colorless solution named as basic fuchsin could be decolorized to bright magenta by the addition of aldehydes [10]. This reaction called as Schiff's reagent, is widely used in histochemistry to show aldehydes by forming the stable red-magenta color product. Moreover, it is significant to demonstrate DNA and complex carbohydrates.

A. PAS (Periodic acid-Schiff) Reaction [11]:

PAS reaction is useful indicator for the demonstration of tissue carbohydrates and glycogen. The principal of the reaction is that periodic acid react with 1-2 glycol and convert it into aldehydes and these aldehydes combine with Schiff reagent and form magenta color in the tissue. Thus this reaction has 2 step procedures as below.

Table 6: Ingredients and amounts of materials in PAS Reaction.

Ingredient	Amount
Periodic acid	1 g
Basic fuchsin	1 g
Distilled water	200 ml
Potassium metabisulfite	2 g
Hydrochloric acid	2 ml
Activated charcoal	2 g

Schiff's Reagent: Dissolve 1 g basic fuchsin in 200 ml of boiling distilled water, removing the flask of water from the 5unsen just before adding the basic fuchsin; this will avoid premature renovation of the laboratory in a deep magenta color. Allow the solution to cool 50^{o}C, and add 2 g potassium metabisulfite by mixing. Allow cooling to room temperature then adding 2 ml concentrated hydrochloric acid, mix, add 2 g activated charcoal and leave overnight in the dark at room temperature. Filter the solution and store in dark at 4^{o}C.

Protocol: Dewax section and bring to distilled water. Treat with periodic acid for 5 min. Wash well with distilled water. Treat with Schiff's reagent for 15 min. Wash with running tap water for 5-10 min. Stain nuclei with Harris's hematoxylin and blueing as usual. Wash in water. Rinse in absolute alcohol and clear in xylene and mount [1].

B. Feulgen Reaction [12]:

This reaction was developed by Robert Feulgen. This two-step staining technique specifically demonstrates DNA, not for RNA. Firstly weak HCl break the purin-deoxyribose bond and yield aldehyde groups, and then aldehydes are demonstrated by using Schiff's reagent. Bouin's fixative is not appropriate for Feulgen reaction because it leads over hydrolysis of the nucleic acid during fixation.

Table 7 Ingredients and amounts of materials in Feulgen Reaction.

Ingredient	Amount
Schiff's reagent	See above
10% potassium metabisulfite	5 ml
Distilled water	91,5 ml
Potassium metabisulfite	2 g
Hydrochloric acid	13,5 ml
1% Light green	

Bisulfide Solution: 10% potassium metabisulfite 5ml, 1M hydrochloric acid 5ml, distilled water 90 ml.

Protocol: Bring all section to water, Rinse sections in 1M HCl at room temperature, place sections in 1M HCl at 60 ^{o}C. Rinse in 1M HCl at room temperature for 1 min. Transfer sections to Schiff's reagent for 45 min. Rinse sections in bisulfite solution for 2 min. Wash two times in bisulfite solution for 2 min. Rinse well in distilled water. Counterstain if required in 1% light green for 2 min. Wash in water and dehydrate through alcohol to xylene and mount [1].

3.2.2 Osmic acid method for lipids

Osmium demonstrates unsaturated lipids because it has an ability to reduce its black lower oxide by fatty acid ethylene bonds. You should prefer unfixed cryostat sections; otherwise if you choose usual way, lipids dissolve in the alcohol series. Osmium is usually used for the demonstration of Golgi apparatus [1]. The reaction depends on a lengthy impregnation procedure. Also osmium method demonstrates the normal myelin [13]. Osmium is used as a secondary fixative for electron microscopy as well, because it preserves membrane lipids and gives contrast to them. Please pay attention to handle osmium solution inside a fume hood to avoid the effect of its toxicity to cornea and mucous membranes.

Table 8 Ingredients and amounts of materials in Osmic acid Method.

Ingredient	Amount
%1 aqueous OsO_4	
Distilled water	100 ml

Protocol: Immerse sections in %1 aqueous OsO_4 for 1 hour at room temperature. Then wash well in distilled water. Mount in glycerin jelly or water based mounting medium.

3.2.3 Connective Tissue Stains for Collagen

It is hard to distinguish embedded cytoplasm of cell in extracellular collagen, and additionally, collagen from cellular material such as in nerve and tendon tissues after H&E staining. The connective tissue staining distinguishes these differences clearly. Most used connective tissue staining methods are Masson trichrome [14], Mallory [15] and van Gieson [16] techniques in order to demonstrate collagen fibers.

A. Masson's Trichrome [14]:

Table 9 Ingredients and amounts of materials in Masson's Trichrome Method.

Ingredient	Amount
Glacial acetic acid	3.5 ml
Acid fuchsin	0.5 g
Phosphomolybdic acid	1 g
Methyl blue or light green	2 g
Distilled water	300 ml
1% Acetic acid	

a. Acid Fucshin Solution: add 0.5 g acid fucshin and 0.5 ml glacial acetic acid to 100 ml distilled water and mix.
b. Phoshomolybdic Acid Solution: Add 1 g phospomolybdic acid to 100 ml distilled water and mix.
c. Methyl blue or Light green Solution: Add 2 g methyl blue (or light green) and 2.5 ml glacial acetic acid to 100 ml distilled water and mix.

Protocol: Deparaffinize sections and bring to water. Stain nuclei with iron alum hematoxylin, differentiate with 1% acid alcohol. Wash in tap water. Stain with acid fucshin solution for 5 min. Rinse in distilled water. Treat with phospomolybdic acid solution for 5 min. Stain with methyl blue (or Light green) Solution for 3 min. Rinse in distilled water. Treat with acetic acid for 2 min. Dehydrate with xylene or toluene and mount with mounting medium or entellan. If you use mercuric based fixatives, you should remove mercuric based precipitation with using iodine, thiosulfate sequence first after deparafinization.

B. van Gieson Tecnique [16]:

Table 10 Ingredients and amounts of materials in van Gieson Tecnique.

Ingredient	Amount
Aqueous Picric acid	50 ml
1% aqueous acid fuchsin	9 ml
Hematoxylin	100 ml
Distilled water	50 ml

Van Gieson Solution: Add 50 ml aqueous picric acid and 9 ml 1% aqueous acid fuchsin to 50 ml distilled water.

Protocol: Deparaffinize sections and bring to water. Stain nuclei with hematoxylin. Wash in tap water. Differentiate in 1% acid alcohol. Wash in tap water. Stain van Gieson solution for 3 min. Dehydrates with alcohol series. Clear in xylene or toluene and mount in mounting medium or entellan.

4. Some troubleshooting about dyes

- If your sections are not stain, or stain irregularly; first control your fixatives and embedding media. Moreover, duration of the fixative effect the production of artifacts and staining problems. Second, paraffin has not completely been cleared from the sections, so repeat dewax process carefully.
- If your sections are not uniform in thickness, you can see more dark or light colors as you expected.
- If the tissues are inadequately fixed, dehydrated or infiltrated, the staining problems can occur.
- The most frequent problem in histochemistry labs is the loss of sections on the slides during staining procedure. It can be the solution to prepare strong adhesive agent or to use positively charged slides. Moreover, move your slides softly when changing your solutions.
- Keep your solutions in dark, room temperature or $+4^{o}C$ according to instructions. Keep away some solutions from sun light. Improper keeping conditions of dye solutions can cause unexpected staining problems.

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