Procedures - Histology

Amyloid

For the microscopic demonstration of amyloid, the Congo Red stain has stood the test of time It can be mounted from Xylol and is permanent, whereas other methods using Methyl or Crystal Violet require mounting in water soluble media and may fade.

Congo Red Method: (Highman's variant)

Use: For the demonstration of amyloid degeneration.
Fixation: The best results are obtained after using a fixative containing mercury.

1. Stages 1-5 of the preliminary steps.
2. Stain in alum hematoxylin, differentiate and blue as usual.
3. Place in Congo Red solution for 1-5 minutes.
4. Without washing differentiate in the above solution 1-3 minutes. Control under the L.P of microscope until amyloid is bright pink and the background is only slightly pink.
5. Dehydrate quickly through 95% and absolute alcohol.
6. Clear in Xylol and mount in Canada Balsam or Xam.

Results:
Amyloid - red
Nuclei - blue

Crystal Violet Method

Aim: For the demonstration of Amyloid degeneration.
Fixation: Mercury containing fixatives are preferred.

1. Stages 1-5 of the preliminary steps.
2. Stain in Crystal Violet solution 1-5 minutes.
3. Wash in water.
4. Mount in modified Apathy's gum syrup.

Results:
Amyloid - red
Other tissues - various shades of blue.

Bacteria

Gram's stain

Use: For the demonstration of bacteria
Fixation: The best results are obtained after any fixative containing corrosive sublimate, but good results may be obtained after any fixative.

1. Stages 1-5 of the preparatory steps.
2. Stain in Crystal Violet solution - 30 seconds.
3. Wash off stain with Gram's Iodine
4. Pour on fresh Gram's iodine and leave for 30 seconds.
5. Differentiate with equal parts of Acetone and absolute alcohol until no more stain leaves the slide.
   (This is a bit of an art).
6. Wash in tap water - 15-30 seconds.
7. Counterstain in Neutral Red or 0.5% Safranin - 3 minutes.
8. Dehydrate quickly through 95% and absolute alcohol.
9. Clear in xylol and mount in C.B or Xam.

Results Gram positive bacteria - blue-black Nuclei - red Other tissues - pink

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Acid Fast Bacilli

Ziehl-Neilson Method

Use:For the demonstration of Mycobacterium tuberculi and Mycobacterium lepri.
Fixation: Formalin is directly inhibitory to the staining of acid fast bacteria by Carbol-Fuchsin. Results in formalin fixed tissue are quite untrustworthy. Fixatives containing mercuric chloride are recommended.

1. Stages 1-5 of the preparatory steps.
2. Transfer to Ziehl-Neilson's carbol-fuchsin solution.
   Place the slide on staining rods, pour on the carbol fuchsin covering the whole slide. Heat the slide from below until steam starts to rise. Sections may be placed in the staining solution and left in an oven at 56°C for 15-30 minutes.
3. Wash briefly in water.
4. Differentiate in 20% aqueous Sulfuric acid. The section is differentiated when the microscope background is colourless, the naked eye appearance of the section being a faint pink. 5-10 minutes.
5. Transfer to Scott's blueing solution - 1 minute. Then wash with water for 2-5 minutes.
6. Stain lightly (0.5 minute) in a 0.1% aqueous solution of Methylene blue. Alternatively the section may be stained in alum hematoxylin for 1-2 minutes, blued in Scott's solution, without any application of acid alcohol then stained in a saturated solution of Tratrazine in ethylene glycol monoethyl ether (Cellosolve), 15-30 seconds.
7. Dehydrate with 2 changes of absolute alcohol.
8. Clear in xylol and mount in Canada Balsam or Xam.

Results

Organisms - bright red
Nuclei - light blue
Background when stained with Tratrazine - clear yellow

Staining of Bone Marrow Sections

The study of the bone marrow by using long and complicated stains does not seem to be justified. The detailed structure of blood cells has to be studied immediately after death, as autolysis rapidly obscures fine detail. Even if this is achieved, paraffin processing shrinks and distorts the cytoplasm of such cells. Also to be taken into consideration is the effect, when necessary, of decalcification which does not treat marrow cells kindly enough for detailed study.

Bone marrow sections are therefore unsuitable for the study of fine cytology, but observations of general architecture, fat absorption and fibrous replacement can give useful information.

For this purpose a well differentiated hemalum and eosin or a Giemsa stain is adequate.

Giemsa Stain

Use: For the demonstration of bone marrow structures.
Fixation: Zenker Fixative or any mercury containing fixative is essential.

1. Stages 1-5 of the preliminary steps.
2. Transfer the sections to the Giemsa staining solution until overstained, 5-10 minutes.
3. Examine under low power of microscope. Sections should be purple with the Nissl granules stained heavily.
4. Rinse in water.
5. Dehydrate and leave in absolute alcohol until Nissl granules show up plainly.
6. One quick change in absolute alcohol.
7. Clear in xylol and mount on Canada Balsam or Xam.

Results

Nissl granules - dark blue
Other tissues - various shades of light blue.

Connective Tissue Fibres

Collagen may be stained selectively by two methods:

1. The first depends on the mixture of Picric Acid with Acid Fuchsin in such proportions that the collagen only is stained by the fuchsin. The Van Gieson stain is based on this principle.
2. The second depends on the special action of phosphotungstic or phosphomolybdic acid when combined with a red aniline dye. These acids lift the colour first from the collagen and only later from the cytoplasm. The differentiation is stopped at the point where the collagen only is colourless, then another aniline dye is added to stain the collagen. The Masson stain given here is based on this principle.

Masson's Stain

Use: For the differentiation of collagen and muscle fibres.
Fixative: The most brilliant results are obtained after using Zenker's fixative. Fair results may be obtained after 10% formal-saline or formalin material which has been post-mordanted.

1. Stages 1-5 of the preliminary steps.
2. Stain nuclei in Harris hematoxylin and Celestin blue for 5 minutes. Differentiate, wash etc. (see Nuclei)
3. Stain in Ponceau - acid fuchsin for 1-5 minutes.
4. Rinse in water. Do not leave for any length of time.
5. Mordant and differentiate in 5% aqueous phosphotungstic acid. Control differentiation under the low power of a microscope until the muscle is a bright red and the collagen fibres are pink. If possible select a large blood vessel and use this to gauge the differentiation.
6. Rinse in water.
7. Stain in Light Green solution for 0.5 - 2 minute. Do not overstain.
8. Rinse in water, blot dry.
9. Dehydrate quickly with 2 changes of absolute alcohol.
10. Clear in 2 changes of xylol and mount with Canada Balsam or Xam.

Results:

Collagen - green
Muscle - red
Nuclei - purple
Mucin - green
Fibrin - red

Van Gieson's Stain

Use: For the differentiation of collagen and muscle fibres.
Fixative: The most brilliant results are obtained after using Zenker's fixative. Fair results may be obtained with the material fixed in 10% formal-saline or formalin material which has been post-mordanted.

1. Stages 1-5 of the preliminary steps.
2. Stain nuclei heavily with Weigert's iron hematoxylin or the Celestin Blue-hemalum combination (see Nuclei).
   DO NOT DIFFERENTIATE
3. Rinse briefly in water.
4. Stain, and differentiate the nuclear stain, in Van Gieson's solution for 1-5 minutes. Control the length of staining time by observation under the low power objective of a microscope.
5. **DO NOT WASH**. Dehydrate rapidly in absolute alcohol with 2 changes.
6. Clear in xylol and mount in Canada Balsam or Xam.

Results:

Collagen - red
Muscle - deep yellow
Nuclei - brown

Elastic Tissue

Various methods have been produced to demonstrate elastic fibres in tissue. of these, Verhoff's iodine-ferric chloride-hemotoxylin mixture followed by a Ferric Chloride differentiation, seems to give the most consistent results.

Verhoff's iodine-ferric chloride-hemotoxylin

Use: For the demonstration of Elastic fibres.
Fixative: No specific fixatives preferred.

1. Stages 1-5 of the preliminary steps.
2. Stain in hematoxylin solution for 15-30 minutes or until sections are perfectly black.
3. Differentiate in 2% aqueous Ferric Chloride until the Elastic fibres show up black against a colourless background.
   Rinse off ferric chloride solution with tap water and examine under the low power objective of a microscope. If differentiation has been carried too far the section may be restained, providing it has not been washed in alcohol.
4. Wash in tap water for 5-10 minutes.
   NOTE: After the slide has been washed the section should be re-examined under the microscope. The alkalinity of the tap water tends to develop the colour of the deposited dye-lake and more differentiation may be needed.
5. Counterstain lightly in Eosin or in Van Gieson stain as required.
   NOTE: If Van Gieson stain is required after Verhoeff's elastic stain then the differentiation with 2% Ferric Chloride should not be carried as far as in step 3. Some background deposit of dye should be left and the final differentiation carried out in the Van Gieson solution then the section taken directly to absolute alcohol.
6. Do not wash in water.
7. Dehydrate, clear in xylol and mount in Canada Balsam or Xam.

Results:
Elastic fibres - jet black
NOTE: If Mercuric Chloride fixative has been used prior to this stain, it is not necessary to remove the mercury deposit before staining as it is removed by the iodine in the staining solution.

Fat

Fats may be demonstrated in frozen sections by oil-soluble dyes such as Sudan IV (Sharlach, R.), Oil Red O or Sudan Black B.

Use: For the demonstration of fats in frozen sections.
Fixation: Tissues should be fixed in 10% formal-saline.

Solutions:
Saturated solution of Sudan IV, Oil Red O or Sudan Black in equal parts of acetone and 50% alcohol.

1. Prepare frozen sections by the method given in the notes.
2. Rinse sections in water.
3. Rinse in 50% alcohol.
4. Transfer to staining solution for 5-15 minutes. Care should be taken to see that the staining solution is kept covered to prevent evaporation. It should be filtered before use.
5. Differentiate in 50% alcohol until only the fat is stained.
6. Rinse in water.
7. Stain in alum hematoxylin for 3 minutes (Sudan IV or Oil Red O).

8. Differentiate, blue and rinse in water.
9. Mount in Apathy's water soluble medium or Kaiser Jelly.

Results:
Fat - red in Sudan IV or Oil Red O, black in Sudan Black

Fungi and Yeasts

These may be shown to advantage in most instances by the use of Periodic Acid - Schiff technique. Other methods used to demonstrate fungi and yeasts are the Gram-Weigert method and Azure-eosinate techniques.

Gomori's Methenamine Silver Nitrate Technique

1. Stages 1-5 of the preliminary steps.
2. Wash in water - 1 min.
3. 5% Chromic acid - 60 min
4. Wash under running tap water - 10 min
5. Sodium bisulphite - 1 min
6. Water - 5 min
7. Three washes in distilled water
8. Gomori's Methenamine Silver Nitrate - 60 min @ 45-50°C
9. Three washes in distilled water
10. Gold Chloride 0.1% - 5 min
11. Distilled water - 1 min
12. 2% Sodium Thiosulphate - 2 min
13. ater - 1 min
14. Counter stain with Light Green
15. Wash clear and mount

Result:
Fungi - black
Mucin - rose red
Other - green

Periodic Acid - Schiff technique for the demonstration of Fungi and Yeasts.

Use: For the demonstration of tissue fungi and yeasts in paraffin sections.
Fixation: Any fixative gives good results

The solution and method of use is the same as for Mucin and Glycogen, except that on some occasions the time in the periodic acid solution can be extended to 5-20 minutes and the strength of the solution increased to 1%.

Glycogen

Glycogen is a poly saccharide derived from, and decomposing into, glucose. It is stored in the liver cells, certain cells of the parathyroid, muscle, cartilage and other locations. It is prone to hydrolysis, so prompt fixation is necessary.

Periodic Acid - Schiff Technique for Glycogen

Use: For the demonstration of glycogen.
Fixation: Ordinarily strong alcoholic fixatives are recommended as glycogen is said to be readily soluble in water. For good preservation, Carnoy's, Bouin's and Absolute alcohol, although the latter fixes well only in the surface layers. Appreciable amounts of glycogen can usually be shown after normal fixation. Float paraffin sections out on 70% alcohol.

Staining Procedures: The method is that for Periodic Acid - Schiff Technique. When glycogen is to be stained, it pays to dilute the Schiff reagent with an equal amount of distilled water, to use the minimum time even in the dilute reagent, and to rinse in running tap water as little as possible afterward. Sections should be covered with 0.5% celloidin, after removal of the paraffin, drained and hardened in 70% alcohol. This helps prevent the diffusion of glycogen out of the section when it is rinsed in water.

Results:
Glycogen - red.

Best's Carmine Stain

Use: For the demonstration of Glycogen
Fixation: The same as given for P.A.S. staining. It is recommended, when cutting paraffin sections to be stained for glycogen, to float out on warn 70% alcohol as glycogen is soluble in water.

1. Remove paraffin with xylol in the usual manner.
2. Transfer through 2 changes of absolute alcohol.
3. Transfer to 0.5% celloidin in equal parts of absolute alcohol and ether for 5 minutes.
4. Drain and wipe the back of the slide, then harden celloidin in 70% alcohol - 5 minutes.
5. Rinse in water.
6. Stain in alum hematoxylin - 2 minutes.
7. Wash briefly in tap water.
8. Without blueing stain for 10-20 minutes in freshly diluted staining solution.
9. Differentiate if necessary in the above differentiating solution or quickly with 3 changes of fresh methyl alcohol, preferably using a dropper bottle.
10. Dehydrate and remove celloidin with 2 or 3 changes of acetone.
11. Clear in xylol and mount in Canada Balsam or Xam.

Results:
Glycogen - red
Nuclei - blue

NOTE: The section is covered with celloidin to prevent the glycogen from diffusing out. Should the glycogen stain poorly it is advisable to use undiluted stock solution. The stock solution should be kept in a refrigerator, it usually keeps for only a few weeks.

It is always advisable to control the stain with a section known to contain glycogen.

Saliva Digestion
Usually for positive identification of glycogen, saliva digestion is carried out before staining on one section, while another is taken through the staining procedure untreated.

Cover the section with saliva for 15 minutes. This is usually done before the celloidin is applied as the celloidin film hinders the penetration of the enzyme. It is often more convenient to use a 1% solution of ptyalin or malt diastase in a buffered neutral saline solution.

Melanin

Found normally in various sites through the body as yellow-brown to black granules. The demonstration of melanin is of pathologically importance when there is an increase of this substance due to disease and in tumors containing melanin producing cells.

The cells responsible for the production of melanin are melanocytes. Melanin is formed when the oxidation of the amino acid, tyrosine (substrate) in the presence of the enzyme tyrosinase and molecular oxygen. Absence of any one of these results in decrease of melanin formation and excess of all three substances results in melanin production. The enzyme produced by the melanocytes can be demonstrated by the DOPA (dehydroxyphenylaline) reaction which converts the precursor of melanin in the cells to a black melanin (dope melanin).

Melanin granules have the property of being capable of reducing silver nitrate solution to metallic silver, without the aid of an external reducer. This Argentaffin reaction can be demonstrated by other cells in the body, such as the enterochromaffin of Kultschitsky cells due to their reaction with silver and the tumors which are said to derive from enterochromaffin cells are called Argentaffinoma or carcinoid tumors.

Argyrophilia is the reaction between tissue and silver solution which requires some form of external reducing agent to produce metallic silver. Armoniacal silver is the familiar complex and is reduced by formalin to a colloidal metallic silver solution containing negatively charged particles. Tissue structures can be charged either to repel or absorb the silver and this can be affected by fixation and pit which is a strong factor in determining charges.

It is worth noting that while all substances showing the argentaffin reaction are argyrophilic, not all argyrophilic reactions are argentaffin.

Examples

- Melanin or enterochromaffin will reduce silver nitrate solutions to metallic silver.
- Reticulin or nerve fibres need some form of sensitising (potassium permanganate) to initiate silver deposition and a reducing agent such as formalin to obtain metallic silver.

Fontana Silver Reduction Technique

1. Bring the sections to water and wash in distilled water.
2. Place in the ammoniacal silver solution for 3 to 12 hours in the dark. This step may be shortened by the application of heat eg., 45°C for 1 hour.
3. Wash the slides in distilled water.
4. Tone in 0.2% gold chloride, 3-5 minutes.
5. Rinse in distilled water.
6. Counterstain in 0.1% neutral red made up in 0.1% acetic acid, 1-2 minutes. Then wash in alcohol.
7. Dehydrate rapidly and mount.

Results

Melanin - black
Nuclei - red

Schmorl's reaction

Melanin has the property of reducing ferricyanide to ferrocyanide and in the presence of ferric salts produces a Prussian blue reaction.

This is the Schmorl's reaction shared by lipofuscin, enterochromaffin, chromaffin and thyroid colloid.

Solution:
Mix 3 parts of 1% ferric chloride with one part of freshly prepared 1% ferricyanide. This solution should be used within 30 minutes.

1. Bring the sections to water.
2. Immerse for 1 hour in 2.5% ferrous sulphate.
3. Wash for 20 minutes in distilled water with 4 changes.
4. Transfer to 1% potassium ferricyanide in 1% acetic acid for 30 minutes.
5. Rinse in 1% acetic acid.
6. Counterstain in Van Gieson's picric acid, fuchsin stain.
7. Dehydrate, clear and mount.

Result

Melanin - dark green
Lipofuscin - no reaction.

Mucin

Southgate's Mucicarmine Stain

Use: For the demonstration of mucin
Fixation: Alcoholic and acid fixatives are preferred, formal saline may be used with much less brilliant staining.

1. Stages 1-5 of the preliminary steps.
2. Stain in alum hematoxylin, differentiate and blue in the usual manner.
3. Stain in Southgate's mucicarmine diluted stock solution for 20-30 minutes.

4. Rinse briefly in distilled water.
5. Dehydrate directly to absolute alcohol with 2 changes.
6. Clear in xylol and mount in Canada Balsam or Xam.

Results:
Mucin - red
Nuclei - blue

Periodic Acid - Schiff Technique for the demonstration of Mucin.

Fixation: Alcoholic and acid fixatives are preferred, formal saline may be used with much less brilliance of staining.

The staining technique is that for the Periodic Acid - Schiff Technique The P.A.S. technique sometimes and unavoidably produces a diffusely pink background which may be a disadvantage in precise localisation of mucin. In this case, it is recommended that the section be placed in chromic instead of periodic acid.

The sections are placed in 54% chromic acid for 1 hour and washed in running water for at least 5 minutes. Thereafter the procedure is the same as when Periodic Acid is used.

Muscle

The best general stain for detecting smooth muscle fibres is the Masson's stain method. However, for the detection of striations in primitive muscle cells, such as occur in rhabdomyosarcoma, special methods are needed. The best is Heidenhain's Iron-Hematoxylin . The sections should be stained for a prolonged time and the final iron alum differentiation should be carried out to the point where nuclei are beginning to appear and the cytoplasm in the wet section still looks greyish-black. If the sections are passed to xylol at this stage, the striations should become evident. Phosphotungstic acid hematoxylin will also stain striations and has the additional advantage of not requiring differentiation.

Mallory's Phosphotungstic Acid - Hematoxylin Stain (modified)

Use: This stain can be used to demonstrate nuclei, mitochondria, fibrin, fibrils of neuroglia (astrocytes and glis fibrils are well shown) and the myofibrils and striations of muscle. These all stain blue.
It stains collagen, reticulum, elastin, cartilage and bone matrix a yellowish to brown red, depending on the age of the staining solution.
Fixation: Zenker's fixation was recommended by Mallory, but if material has been fixed in formalin the sections should be bought down to water and mordanted in saturated solution of mercuric chloride for 3 hours at 56°C. Sections so mordanted are then treated as though Zenker's fixation has been employed.

1. Stages 1-5 of the preliminary steps.
2. Place section in Mallory's bleach.
3. Rinse in tap water
4. Mordant for 1 hour in 4% Ferric Alum
5. Rinse in tap water.

6. Place section in Mallory's staining solution for 2-24 hours. The staining times can be shortened by placing in a 56°C oven for 45-60 minutes. Examine under low power microscope. **DO NOT WASH**
7. Dehydrate rapidly in absolute alcohol, 2 changes.
8. Go to Xylol and mount in Canada Balsam or Xam.

Results:

This stain can be used to demonstrate nuclei, mitochondria, fibrin, fibrils of neuroglia (astrocytes and glis fibrils are well shown) and the myofibrils and striations of muscle. These all stain blue.
It stains collagen, reticulum, elastin, cartilage and bone matrix a yellowish to brown red, depending on the age of the staining solution.

Notes:

- Mordanting in Ferric Alum intensifies the colour of the blue staining elements.
- The use of the bleach is to give the necessary sharp contrast between the various stained elements and reduces the background staining.
- The time in the staining solution should be watched as the solution acts quickly (as compared with the original method of Mallory) usually taking from 2-3 hours to stain at room temperature.

Myelin

The staining of myelin sheaths is a useful procedure to show certain degenerative and inflammatory diseases of the Central Nervous System.

The classical technique is the Weigert-Pal, however a quicker and less troublesome method is the Weil's Rapid Method for Myelin Sheaths.

Luxol fast Blue Myelin Stain

This method is based on the use of an alcohol-soluble amino salt of sulphonated copper phthalocyanine as Luxol Fast Blue MBS. This dye is characterised by the great beauty and intensity of the blue or bluish-green staining, it's specificity for myelin, and it's remarkable fastness to light, heat acids and alkalies. It has the main advantage of being applicable to formalin fixed tissues without the usual mordants required for the traditional myelin methods.

1. Take sections to 95% alcohol.
2. Place in Luxol fast blue for 1 hour, this may be extended to overnight, in an oven at 56°C.
3. Rinse off stain in 70% alcohol and bring to water.
4. Rinse in 0.05% Lithium carbonate.
5. Differentiate in 70% alcohol - 20-30 seconds.
6. Rinse in water.
7. Differentiate in 70% alcohol.
8. Wash in water and counterstain as desired, eg. Cresyl violet, P.A.S.,

   Possible Counterstains:
   Van Gieson, Neutral red, safranine.

Celestin Blue - 5 minutes
Wash in water
Harris's Hematoxylin - 5 minutes
Wash with water
Differentiate
Blue (Scotts)
Wash with water
Counterstain with Van Gieson - 2-3 minutes

9. Dehydrate, clear and mount.

Results

Phospholipids, eg. myelin sheaths, red cells - dark blue
Other tissues - as per counterstain.

Weil's Rapid Method for Myelin Sheaths.

Use: To stain myelin sheaths of nerve fibres in the brain, spinal chord, peripheral nerves and ganglia.
Fixation: 10% formal-saline. Cut sections at 15-25 microns and attach to slide using the gelatin method.

1. Stages 1-5 of the preliminary steps.
2. Stain sections in solution A of Weil's Rapid Method for 30 minutes at 56°C. If the solution commences to turn brown remove the sections immediately.
3. Wash in water and place in solution B of Weil's Rapid Method, to partly decolourise (about 20-30 seconds). This is usually done without the aid of a microscope. The section is held over a white background to view and when the grey and white matter can be recognised the section is placed in water for a few minutes.
4. Differentiate in solution C of Weil's Rapid Method. The time for this step is variable and is controlled by microscopic observation. Differentiation is complete when the grey matter appears as a yellow grey colour and the myelinated fibres stand out as blueish-black bands.
5. When the differentiation is complete wash off the ferri-cyanide-borax solution and place the section in water containing a few drops of ammonia.
6. Wash in water for a few minutes then dehydrate, clear and mount in Canada Balsam of Xam.

Results

Myelin sheaths - blue black
Other structures - unstained.
NOTE: The staining solution should be filtered before use. It may also deteriorate and should be discarded when a white precipitate forms.

Nerve Fibres

It should be made clear that the staining of nerve fibres, or any structure of the Central Nervous System, with silver nitrate solutions is an art that can only be perfected with experience.

As with any metallic stain care should be taken to ensure that all glassware is chemically clean and that the staining solutions should be protected from light to prevent reduction.

Rogers Silver Nitrate Impregnation Technique Use: For the demonstration of nerve fibres in the central nervous system.
Fixation: 10% formal-saline for fine non-myelinated fibres. Carnoy's fluid for the coarser myelinated fibres.

Pre-treatment:
After deparaffinising, place sections in a 2% solution of strong ammonia made up in 95% alcohol, for 12 hours.

1. Rinse in 80% alcohol.
2. Impregnate in 40% silver nitrate solution in an incubator at 56°C for 30 minutes. This solution can be used over long periods if covered to prevent evaporation and contamination.
3. Rinse in distilled water.
4. Treat with 20% neutral formalin for 5 minutes, then transfer to 5% neutral formalin for 5 minutes or longer. Then wash in distilled water.
5. Blot section and flood with a few drops of diammoniacal silver solution for about 1 minute. Wash in distilled water.
6. Blot section and place in 20% neutral formalin for 5 minutes or until the section is a rusty orange. If impregnation is insufficient, step 5 may be repeated.
7. Rinse in distilled water.
8. Tone in 1:300 gold chloride bath acidified with 2% glacial acetic acid, for 10 minutes.
9. Rinse in distilled water.
10. Intensify with 1% neutral formalin which contains 2% oxalic acid and wash well with distilled water.
11. Fix in 5% sodium thiosulfate for 5 minutes, wash, dehydrate, clear and mount.

Results

Myelinated and amyelinated fibres - black
Neurofibrils show well.

Nuclei

Alum Hematoxylin

Use: To stain nuclei prior to methods such as Van Gieson's or Masson's technique. This stain is far more resistant to the effects of acids in subsequent solutions than Weigert's iron hematoxylin.
Fixative: Good results after any fixative.

1. Steps 1-5 of preliminary steps.
2. Stain in Celestin Blue staining solution for 10 minutes.
3. Rinse in water.
4. Stain in Alum Hematoxylin (Ehrlich's, Mayer, Harris), for 10 minutes.
5. Rinse in water.
6. Differentiate in 0.5% Acid Alcohol as required.

7. Wash in water - 5 minutes.
8. Counter stain as desired.
9. Dehydrate, clear and mount in Canada Balsam or Xam.

Results:
Nulcei - brown to blue black.

Heidenhain's Iron Hematoxylin

Use: For staining nuclear and cytoplasmic elements, striated muscle, protozoa, etc.

Fixative: Gives good results after all fixatives. For the demonstration of mitotic figures, mitochondria etc. material must be placed in fixing solution as soon as possible.

1. Stages 1-5 of the preliminary steps.
2. Heidenhain's Iron Hematoxylin - solutions.
3. Mordant in 5% iron alum;
    1. nuclear staining 10-20 minutes.
    2. protozoa 3-12 hours.
4. Rinse rapidly in distilled water.
5. Stain in Solution B;
    1. nuclear staining 10-20 minutes.
    2. protozoa 3-12 hours.
6. Differentiate in 2.5% iron alum, controlling under microscope, rinse in tap water to stop decolorization.
7. Wash in running tap water 5-10 minutes to remove all traces of the iron alum.
8. Counterstain as desired.
9. Dehydrate, clear and mount in Canada Balsam or Xam

Results:
Nuclei - blue to black,
Red Blood Cells - blue
Tissue - tone of blue and grey according to differentiation.

Haemulum and Eosin

Use: This is the commonest histopathological stain used for general microanatomical features of tissues. As this is a routine staining method care should be taken to master the procedure as batches of Hematoxylin and Eosin vary widely, and it requires constant care to secure uniform results.
Fixative: Good results may be obtained from any fixative.
May use:

- Ehrlich's Acid Alum Hematoxylin
- Harris's Alum Hematoxylin
- Mayer's Acid Alum Hematoxylin

1. Stages 1-5 of the preliminary steps.

2. Stain in alum hematoxylin for 5 to 15 minutes according to the strength of the hematoxylin solution..
3. Wash in water, blue in Scott's Blueing solution for 1-2 minutes, rinse in water and examine under low power of microscope. Section should be overstained.
4. Differentiate in 0.5% Acid Alcohol to remove excess stain from background leaving only nuclei stained.
5. Rinse in water and transfer to Scott's Blueing solution for 1-2 minutes.
6. Examine under the microscope and if sufficiently decolourised proceed to the next step. If the section is not sufficiently differentiated then repeat steps 4 and 5.
7. Wash well in running tap water 5-10 minutes.
8. Counterstain in alcoholic eosin, leaving in the staining solution until the section is slightly overstained.
9. Rinse in 2 changes of 95% alcohol.
10. Differentiate in absolute alcohol until optimum staining has been obtained.
11. Dehydrate in a further change of absolute alcohol.
12. Clear in several changes of xylol.
13. Mount in Canada Balsam, Xam or any other neutral mounting medium which is miscible with xylol.

Results:

Nuclei - blue
Red Blood Cells - red
Muscle - deep pink
Collagen fibres - pink

Basic analine dyes are employed in staining nuclei, mainly when a contrast is needed for the main tissue element stain. They are used to stain nuclei in such methods as Ziehl-Nealson stain for acid fast bacilli (methylene blue) and the Prussian Blue reaction for hemosiderin (safranine).

Weigert's Iron Hematoxylin

Use: To stain nuclei prior to methods such as Van Geison and Masson's technique.
Fixative: Good results from any fixative.

1. Stages 1 to 5 of the preliminary steps.
2. Stain for 10 minutes in Weigert's Iron Hematoxylin.
3. Rinse in water and examine under the microscope. Nuclei will show black and the background grey.
4. Differentiate with 0.5% Acid Alcohol to remove the grey differentiation background. **NOTE**: Differentiation is not always necessary as it can be carried out in subsequent staining, eg. Van Gieson's stain, in which picric acid will do the differentiation.
5. Wash in running tap water 5-10 minutes to remove all traces of acid.
6. Counterstain in eosin or other methods as required.
7. Dehydrate, clear and mount in Canada Balsam or Xam.

Results:

Nuclei - blue to black.

Pigments

Hemosiderin

Hemosiderin is an iron containing pigment which is produced by the degradation of haemoglobin. It appears brown and granular in unstained sections or those stained by ordinary methods.

Free ferric iron can be released from this pigment by the action of acid and this in turn combines with potassium ferrocyanide to form Prussian blue.

Gomori Technique

Use: For the demonstration of hemosiderin (free iron).
Fixation: Alcoholic fixatives give the best results. Any fixative containing acid will cause diffusion. Formalin may be used provided it has not become acidic.

Solutions:

- Hydrochloric acid - 20% in distilled water
- Potassium Ferrocyanide - 10% in distilled water

1. Stages 1-5 of the preparatory steps.
2. Wash in distilled water.
3. Transfer the sections to Gomori's solution for 20-30 minutes. This solution consists of equal parts of the HCl and KCn solutions. All these reagents must be fresh and filtered. It is recommended to renew the solutions at least once.
4. Rinse in distilled water and then wash in tap water - 5 minutes.
5. Counterstain lightly in
6. Eosin or in 0.5% Neutral Red.
7. Transfer to 95% then absolute alcohol.
8. Clear in Xylol and mount in Canada Balsam or Xam.

Results:
Hemosiderin - blue
Other tissues - red
Nuclei - red with Neutral Red/Safranin staining.

Melanin

Melanin is a granular dark-brown pigment occurring mainly in the basal layer of the epidermis, hairs, chorioid of the eyes and in certain tumors.

Melanin is usually demonstrated by a process of exclusion. It can be quite simply stained by applying a 2% silver nitrate solution to the section for 1-2 hours. Rinse in distilled water and counterstain lightly with methylene blue or thionin, then dehydrate, clear and mount.

Results:

Pigment - black
Cells - greenish blue

Formalin Pigment

These artefact pigment granules may be rapidly formed in relation to blood collections.

This formalin pigment is included among the staining procedures because it can be a source of error and so the method of removal is given below.

Method:

1. Bring sections to water in the usual manner and treat with a saturated alcoholic Picric Acid solution for 0.5 to 1 hour.
2. Place the sections in saturated aqueous lithium carbonate solution to rid sections of Picric acid.
3. Wash for 5-10 minutes and stain.

Reticulum

The silver methods are used to differentiate between reticulum and collagen fibres. The former are coloured black, the later brown, lavender and grey in varying shades and tones. These methods are often uncertain in their action and it needs experience to discern whether or not a preparation has given a reasonably complete demonstration.

Laidlaw's Silver Carbonate Method

Use: For differentiating between Collagen and Reticulum fibres.
Fixative: Bouin's or 10% formal-saline is recommended, sections should be attached to the slide by the gelatin method.

1. Stages 1-5 of preparatory steps.
2. Place sections in a 1% alcohol solution of iodine - 3 minutes.
3. Rinse in tap water.
4. Decolourise with 5% sodium thiosulfate - 3 minutes
5. Wash well in tap water.
6. Place in a 0.25% Potassium permanganate solution - 3 minutes
7. Rinse in tap water.
8. Place in 5% oxalic acid - 3 minutes
9. Wash in running tap water - 10 minutes
10. Wash in 3 changes of distilled water 5-10 minutes.
11. Stain in Silver Carbonate solution, pre-heated to 50°C and left for 5 minutes.
12. Rinse in distilled water.
13. Treat the section with 1% formalin - 3 minutes. Change the solution several times.
14. Rinse in distilled water.
15. Immerse the slide in a 0.2% gold chloride solution 1-5 minutes.
16. Rinse in distilled water.
17. Treat the section with 5% sodium thiosulfate - 10 minutes.

18. Wash in running tap water 2-3 minutes.
19. Dehydrate, clear and mount in
20. Canada Balsam or Xam. Counterstains may be used if desired before last step.

Results:

Reticulum fibres - Black to dark violet
Collagen fibres - red/purple
Nuclei - black (with formalin fixation).

The Periodic-Acid-Schiff Stain

1. Stages 1-5 of the preparatory steps.
2. Rinse in distilled water.
3. 0.5% periodic acid - 5 minutes (8 minutes max.)
4. Wash in running tap water - 5 minutes.
5. Rinse in distilled water.
6. Schiff's reagent - 15 minutes.
7. Wash in running tap water - 5 minutes.

   The colour reaction reaches it's maximum during this stage. Do not wash for longer than the recommended period. The colour may become more intense, but will also diffuse and become less specific.

8. Counterstain as desired, ie. Hemalum, Light Green or Tratrazine.. Stain with Hematoxylin and Scott's blue (as in H&E).
9. Dehydrate, clear and mount in C.B. or Xam.

Results:

It is by far the best method for glycogen and mucin. Among the other elements which take up this stain are:

1. Basophilia of the anterior pituitary
2. Thyroid colloid
3. Reticulum stands out in sharp contrast to the weakly reacting collagen.
4. Demonstration of fungi in tissues.

References:

Am.J. Clin. Path. Vol. **27**, No. 3, 1952
Stain Technology. No. **6**, Vol. 27, 1952.

Historical:

The Periodic-Acid-Schiff technique was developed by McManus (1946), Lille (1947), and Hotckiss (1948). It is generally believed to be two chemical reactions:

1. An oxidation to produce aldehyde groups.
2. A reaction of these aldehyde groups with the Schiff reagent to produce a violet colour.

Aldehydes can be demonstrated by a number of reagents of which the best known is the Shiff reagent (fuchsin-sulphurous acid). This reagent forms an intense red reaction with practically all known aldehydes.

The histologic oxidation by Periodic acid followed by Shiff's reagent was first used in 1946. In a few years it has become one of the most widely used staining methods. Its popularity is attributed to two main features:

1. It gives information of a chemical nature about the structures stained.
2. It discloses morphological details impossible to visualise with any other method.

What are the substances in the tissue that react? First it should be made clear that this stain is employed only on embedded tissue. In frozen sections of unfatted material, fatty aldehydes, which may be quite abundant, greatly interfere with the evaluation of the results.. Even in embedded tissues especially after fixation in dichromate containing mixtures, insoluble oxidation products of fats may give a fairly intense reaction. After most of the routine fixatives of moderate duration fats are not an important source of error.

As far as the technique is concerned, there are no special requirements of fixation. Any routine fixative which will preserve the structures to be demonstrated will do. The staining procedure proper is so insensitive to minor departures from any recommended routine that it is almost impossible to obtain failures. The strength of the periodic acid may vary between 0.2 an 1% and buffering is unnecessary. The time of exposure is from 3 minutes to 1/2 an hour or more. (Unlike chromic acid which on prolonged exposure destroys the aldehydes initially formed). Periodic acid does not oxidise beyond the aldehyde stage.

After oxidation the slides must be washed thoroughly to remove all trace of HIO_4. Neutralisation of the oxidiser by a short bath in bisulfite has been suggested (the so called reducing rinse). The effect of this treatment is a much more gradual development of colour in the next step. This may sometimes be desirable when the aim is the differentiation of the more rapidly reacting substances (glycogen and mucin) from carbohydrates of the connective tissue.

There are several formulas for the preparation of Schiff's reagent all of which give essentially the same results. The important point is to use a colourless solution or one that is slightly yellow. It should be kept in a refrigerator. The stability of the reagent is rather variable and unpredictable. For reasons completely unknown some batches will remain usable for months while others may deteriorate in a matter of weeks, even though they remain colourless. The more development of a pinkish shade does not indicate spoilage. On the addition of acid the reagent may decolourise once more and be as good as new. On the other hand it is best to discard solutions which develop a large amount of white precipitate.

In general it is a good practice to keep handy a few control slides known to contain glycogen, mucin or whatever substance you wish to stain.

Staining time will vary between 5-20 minutes, depending on the intensity of the colour desired.. The excess dye is removed by a rinse in bisulfite solution and the full intensity is developed by washing the slide in running tap water.

For the differentiation of glycogen from other polysaccharides the saliva test should be applied before oxidation. This involves covering the section with saliva for 15 to 30 minutes, with several changes. By this time all the glycogen will have been converted to glucose.

Back to the top