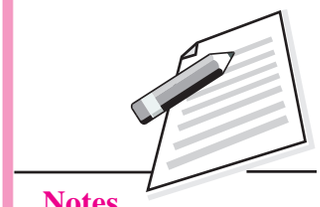


11

STAINING METHODS TO DEMONSTRATE SPECIAL/ SPECIFIC TISSUES

11.1 INTRODUCTION

Biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used.

OBJECTIVES

After reading this lesson, you will be able to:

- describe various staining methods for demonstrating special tissues.
- demonstrate various staining methods.

11.2 TRICHROME STAIN

A combination of three different dyes is used to identify different cells and tissue elements.

Aim: To identify the collagen and muscle fibers in a histological section.

Reagents

1. Bouin's solution
   - Saturated picric acid 75ml
   - Formaldehyde (37-40%) 25ml
   - Glacial acetic acid 5ml

Notes

- Mix all the reagents well. This solution improves the trichrome stain quality.

2. Weigert's iron hematoxylin stock solution

Stock solution A

- Hematoxylin 1gm
- 95% alcohol 100ml

Stock solution B

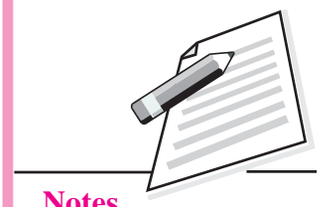
- 29% Ferric chloride in water 4ml
- Distilled water 100ml
- Hydrochloric acid, concentrated 1.0ml

3. Weigert's iron hematoxylin working solution - Mix equal parts of solution A and B (This solution works for three months.)
4. Biebrich scarlet acid fuchsin solution
   - 1% Biebric Scarlet-Acid Fuchsin solution (aqueous solution) 90ml
   - 1% Acid Fuchsin (Aqueous) 10ml
   - 1% Glacial acitic acid 1ml
5. Phosphomolybdic acid-Phosphotungstic Acid Solution
   - 5% Phosphomolybdic Acid 25ml
   - 5%phosphotungstic Acid 25ml
6. Aniline blue solution
   - Aniline blue solution 2.5gm
   - Glacial acitic acid 2ml
   - Distilled water 100ml

Control: skin

Procedure

1. De-paraffinize and rehydrate through graded alcohol.
2. Wash in distilled water.
3. Fix the slides in Bouin's solution for one hour at 56^0C.
4. Rinse in running tap water for 5 to 10 minutes to remove yellow color.
5. Stain in Weigert's Iron Hematoxylin solution for 10 minutes.
6. Rinse in warm tap water for 10 minutes.
7. Wash in distilled water.



8. Put Biebric Scarlet Acid Fuchsin solution for 10 to 15 minutes.
9. Wash in distilled water.
10. Differentiate in Phosphomolybdic-Phosphotungstic Acid solution for 10 to 15 minutes.
11. Put the sections in Aniline blue solution for 5-10 minutes.
12. Rinse in distilled water briefly.
13. Differentiate in acetic acid solution for 2-5 minutes.
14. Wash in distilled water.
15. Dehydrate quickly through 95% alcohol and absolute alcohol. (These steps will wipe off Biebric Scarlet acid Fuchsin staining)
16. Clear in xyline and mount in DPX.

Result

- Glycogen, muscle fibre and keratin — red
- Collagen and bone — blue/green
- Nuclei — brown/black

Note: This stain can be used on frozen sections also.

11.3 VERHEOFF STAIN FOR COLLAGEN

Aim: To identify collagen and elastic tissue in the same section.

Principle: In the presence of ferric salts (oxidizers) elastic fibers stain with hematoxylin, along with the nuclei.

Control: skin

Reagents

1. Verhoeff's solution: Freshly prepared solution gives best result.

   Solution A

   - Hematoxylin — 5gm
   - Absolute alcohol — 100ml
   - Dissolve hematoxylin with the aid of heat, cool and filter.

   Solution B

   - Ferric chloride — 10gm
   - Distilled water — 100ml

Notes

Solution C

- Iodine 2gm
- Potassium iodide 4gm
- Distilled water 100ml
- Add 8ml of solution B into 20ml of solution A and then add 8ml of solution C.

2. 2% Ferric chloride solution
3. 1% aqueous solution of acid fuchsin
4. Saturated aqueous solution of picric acid
5. Van Gieson's stain
   - Acid Fuchsin 1% (aqueous) 5ml
   - Saturated aqueous solution of picric acid 100ml
6. Sodium thiosulphate, 5% (aqueous solution)

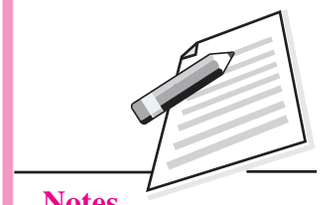
Procedure

1. Deparaffinize and take the section to water.
2. Stain in Verhoeff solution until the section is black.
3. Wash in distilled water.
4. Differentiate in 2% Ferric chloride with agitation for few minutes. Check differentiation by rinsing in distilled water. Under the microscope the elastic fibers and nuclei should stain black and rest of the tissue should be light grey.
5. Put in 5% sodium thiosulphate for 1 minute.
6. Wash in tap water for 5minutes.
7. Counter-stain with Van Gieson's stain for 1-2 minutes.
8. Differentiate in 95% alcohol.
9. Dehydrate in absolute alcohol two times.
10. Clear in xylene and mount in DPX.

Result

- Elastic fibres black
- Nuclei black
- Collagen red
- Other tissues yellow

Note: It is a rapid method but fails to demonstrate fine fibers.



11.4 PRUSSIAN BLUE STAIN

Aim: To demonstrate the presence of iron in tissues.

Principle: The ferric iron in tissue combines with potassium ferro cyanide to form ferric-ferro cyanide. This compound has bright blue color (prussian blue). Prussian blue precipitate is insoluble, hence it can be combined with other staining methods.

Control: Hemosiderin positive tissue

Reagents

1. 2%Hydrochloric acid
   - concentrated hydrochloric acid 2ml
   - Distilled water 98ml
2. 2% potassium ferrocyanide
   - Potassium ferrocyanide 2mg
   - Distilled water 100ml
3. 0.15% Basic fuchsin
   - Basic fuchsin 0.15gm
   - 50% ethyl alcohol 100ml

Procedure

1. Bring section to water.
2. Mix equal volume of 2% potassium ferrocyanide and 2% hydrochloric acid. Pour the solution on the slide and keep it for 20 minutes.
3. Wash thoroughly with water.
4. Counter-stain with basic fuchsin or eosin for 30 seconds.
5. Wash with water, dehydrate, clear in xylene and mount in DPX.

Result

- Ferric iron blue
- Nuclei red
- Other tissues shades of pink

Note: All traces of ferrocyanide should be removed before it is counter-stained, otherwise a dark red fine precipitate will form.

Notes

11.5 MASSON FONTANA SILVER STAINING

Aim: To demonstrate the presence of argentaffin granules.

Principle: Granules in argentaffin cells reduces ammoniacal silver solution to metallic silver. This histo-chemical reaction is due the presence of 5 hydroxy tryptamine(5HT).The 5HT must be converted to tetrahydri-carbolin derivative by formalin fixation before reactions can be demonstrated.

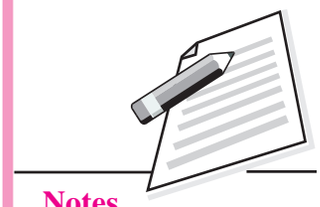
Control: skin or any positive tissue

Reagents

1. Stock 10% silver nitrate solution
   - Silver nitrate A R grade 10gm
   - Distilled water 100ml
2. Fontana masson silver nitrate solution
   - To 50 ml of 10% silver nitrate solution, add one or two drops of ammonium hydroxide. The first drop itself will cause a brown precipitate. Continue to add ammonia solution drop by drop just until the solution is clear. From stock 10% silver nitrate solution, add a little more solution drop by drop dissolving the initial precipitate and stop when a permanent faint turbidity is attained. Let it stand overnight to settle. Before use, decant silver solution, filter and dilute with an equal amount of distilled water. Prepare the fresh solution each time.
3. Gold chloride solution
   - Gold chloride 1gm
   - Distilled water 500ml
   - Keep the solution in refrigerator.
4. Sodiumthiosulfate 5gm
   - Distilled water 100ml

Procedure

1. Bring sections to distilled water
2. Treat with Fontana silver nitrate solution for 1 hour at 56-58IN0C ammoniacal silver solution in a closed jar 15 mins
3. Check microscopically and repeat step 2 if necessary



4. Wash well in distilled water
5. Tone with gold chloride 2 minutes
6. Wash well with distilled water
7. Fix in 2% aq sodium thiosulphate 2 minutes
8. Wash well with distilled water
9. Counter-stain with neutral red stain 1 minutes
10. Rinse in distilled water
11. Rapidly dehydrate well in absolute alcohol, clear and mount

Results

- Melanin — black
- Argentaffin cell granules — black
- Some lipofuscins — black
- Chromaffin — black
- Nuclei — red

Note

- Formalin fixation is essential for argentaffin substances, but not critical for melanin.
- A known positive control section must be used to ensure correct demonstration has been achieved.
- Time of the ammoniacal silver impregnation depends upon the tissue component to be demonstrated. At room temperature, melanin will require 12 hrs, argentaffin 24 hrs. At 60°C melanin blackens within 20 minutes, argentaffin requires approximately 40 minutes. Excessive heat over long periods may cause the silver solution to precipitate, give non-specific background deposits, and cause precipitation of silver on connective tissue fibres.
- Ammoniacal silver solutions can be explosive when allowed to dry. Immediately after use neutralise the silver solution with saturated sodium chloride and discard.

11.6 VON-KOSSA STAIN

Aim: To demonstrate calcium in paraffin sections.

Principle: Tissue sections are treated with silver nitrate solution, the calcium is reduced by the strong light and replaced with silver deposits, visualized as metallic silver.

Notes

Reagents

5% silver nitrate solution

- Silver nitrate 25gm
- Distilled water 500ml

Mix well, pour into acid bottle. Store in refrigerator (stable for one year).

Control: Positive control

Procedure

- Bring sections to water. One section works as negative control.
- Immerse one section in citrate buffer (pH4.5) for 20 minutes to remove calcium if it is present.
- Wash both the slides in distilled water.
- Put 5% silver nitrate solution.
- Expose the slides to bright sun light for 10 to 20 minutes.
- Wash in distilled water several times.
- Treat with 5% sodium thiosulfate for 2 to 3 minutes.
- Wash well in water.
- Counter-stain with neutral red or Van Gieson stain.
- Dehydrate, clear in xylene and mount in DPX.

Result

- Calcium Dark green or black
- Back ground Depends on counter stain.

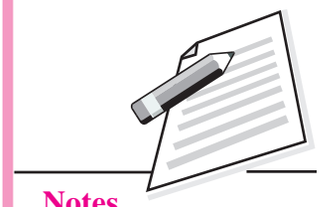
Note: Silver nitrate is tumorogenic and oxidizer. It is strong skin and eye irritant.

11.7 PHOSPHO-TUNGSTIC-ACID-HEMATOXYLIN STAIN

Aim: It is a mix of hematoxylin and phosphotungstic acid. Muscle cross striations, fibrin and glial fibers can be demonstrated by this stain.

Principle: The phosphotungstic acid all of the available hematin to form a blue lake pigment. This lake stains the muscle cross striations, fibrin and nuclei. The rest of the phosphotungstic acid stains red brown, components like collagen.

Control: Skeletal muscle, cardiac muscle, fibrin, cerebral cortex for glial fibers.



Reagents

- Hematoxylin 1gm
- Phosphotungstic acid 20gm
- Distilled water 1000ml

Dissolve the solid ingredients in separate portions of the water, the hematoxylin with aid of gentle heat. When cool mix it. Allow the mixture to ripen or add 177gm of potassium permagnate for immediate use. Properly ripened stain is rich purple in color and opaque.

0.25% potassium permagnate

- Potassium permagnate 0.25gm
- Distilled water 100ml

5% oxalic acid

- Oxalic acid 5gm
- Distilled water 100ml

Procedure

1. Dehydrate and bring section to water.
2. Oxidize in potassium permagnate for 5 to 10 minutes. Discard the solution.
3. Wash in water.
4. Bleach in oxalic acid for 5 minutes or until the sections are colorless.
5. Wash thoroughly. Rinse in distilled water.
6. Put PTAH stain for 12 to 24 hour.
7. Transfer the section into 95% alcohol, followed by absolute alcohol.
8. Dehydrate, clear in xyline and mount in DPX.

Result

- Striated muscle fibers blue
- Astrocytes blue
- Fibrin blue
- Nuclei blue
- Cytoplasm brown red
- Collagen brown pink

Notes

11.8 STAIN FOR RETICULIN FIBRES

Aim: To identify reticulin fibers in sections.

Principle: Reticulin fibers are treated with potassium permagnate to produce sensitized sites for silver deposition. Silver is in a form readily able to precipitate as metallic silver. Formalin, a reducing agent causes deposition of metallic silver at pH 9.0.Excess silver is removed by sodium thiosulphate solution .Treatment with gold chloride produces permanent precipitate.

Control: Normal liver.

Reagents

Acidified potassium permagnate

- 0.5% potassium permagnate 95ml
- 3% sulfuric acid 5ml
- Solution should be made fresh.

Silver nitrate solution

- To 5ml of 10% aqueous silver nitrate, add strong ammonia drop by drop until the precipitate which has formed initially is dissolved. Add 5ml of 3% sodium hydroxide. Again add strong ammonia drop by drop till the precipitate is completely dissolved. Add distilled water to make it 50ml and keep it in a jar.

2% Oxalic acid

- Oxalic acid 2gm
- Distilled water 100ml

4% aqueous iron alum

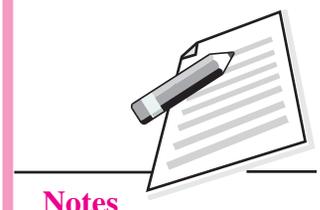
- Ferric ammonium sulphate
- Distilled water 100ml

10% Formalin

- Formaldehyde 10ml
- Distilled water 90ml

0.2% Gold chloride

- Gold chloride 0.2%
- Distilled water 100ml
- Store in refrigerator



2% Sodium thiosulphate

- Sodiun thiosulphate 2gm
- Distilled water 100ml

Neutral red (acidified)

- Neutral red 1gm
- Distilled water 100ml
- Glacial acitic acid 1ml
- Dissolve the dye in distilled water. Add the acid, mix, filter and store.

Procedure

1. Deparaffinize and bring the sections to water.
2. Oxidize in acidified potassium permagnate for 3 minutes.
3. Rinse in distilled water.
4. Decolorize with 2% oxalic acid for 1 minute.
5. Rinse in distilled water.
6. Put iron alum for 10 minutes.
7. Rinse in distilled water.
8. Put ammonical silver solution for 10 seconds.
9. Rinse in distilled water.
10. Immediately reduce with formalin for 2 minutes.
11. Wash in running tap water for 2 minutes.
12. Tone in 0.2% gold chloride for 2 minutes.
13. Rinse in distilled water.
14. Fix in 2% thiosulphate for 2 minutes.
15. Wash in water for 2 minutes.
16. Counter-stain with neutral red for 2 minutes.
17. Dehydrate, clear in xylene and mount in DPX.

Result

- Reticulin fibres black
- Nuclei red

Note

- All glassware should be cleaned thoroughly.

- Silver solution should be made fresh.
- Step 12 should be omitted in liver sections.
- Counter-stain may be omitted in trefine biopsies.

INTEXT QUESTION 11.1

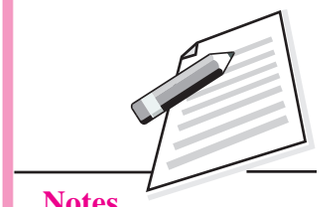
1. ..... stain is used for identifying collagen and muscle fibers in a histological section
2. ..... stain is used to demonstrate the presence of iron in tissues.
3. ..... is the control used in Prussian blue stain.
4. ..... staining is used to demonstrate the presence of argentaffin granules.
5. ..... is used to demonstrate calcium in paraffin sections.

WHAT HAVE YOU LEARNT

- Trichrome stain is used to identify the collagen and muscle fibres in a histological section.
- Trichrome stain uses a combination of three different dyes
- Stain is used as a control in trichrome stain.
- Verheoff stain is used for identifying collagen and elastic tissue.
- Prussian blue stain is used to demonstrate the presence of iron in tissues and hemosiderin positive is used as control.
- Argentaffin granules are demonstrating masson Fontana silver staining.
- Von-kossa stain is used to demonstrate calcium in paraffin sections.
- Muscle cross striations, fibrin and glial fibres can be demonstrated by phospho-tungstic acid hematoxylin stain.

TERMINAL QUESTIONS

1. Explain Prussian blue staining technique.
2. Explain briefly masson Fontana silver staining.



ANSWERS TO INTEXT QUESTIONS

11.1

1. Trichrome
2. Prussian blue
3. Hemosiderin positive tissue
4. Masson Fontana silver
5. Von – kossa stain