National University-Sudan

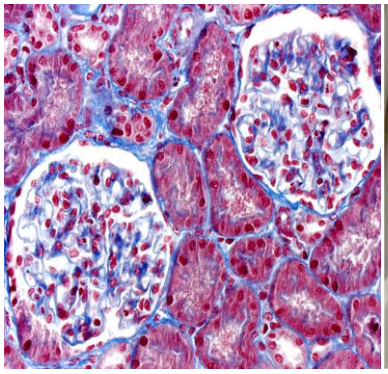
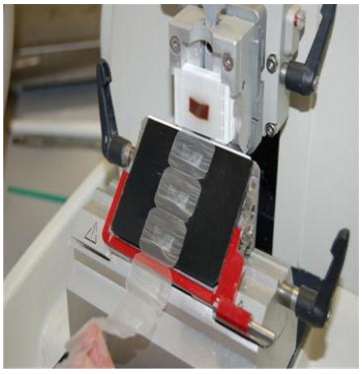
Faculty of Medical Laboratory Sciences

Student Practical Manual
Histopathology and Cytology Department

Forth Year, Semester (7)
Histopathological Techniques (MLS-HIST-412)

Student Name: ……...

ID: ……**Batch**…….....…...


PPE must be
worn beyond
this point

Practical N-1

preparation of routine fixative & special fixative

10% N.B Formalin

Principles:

It seems that fixatives have the property of forming cross- links in tissue proteins by forming a gel, ideally keeping everything in their invivo relations to each other(preserve the tissue as life as possible). Soluble proteins are fixed to structural protein rendered them insoluble.

Aim:

Used for fixation of routine work in histopathology lab

Advantage &property:-

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Requerment :-

Constituent &preparation:-

1- 10% buffered formalin:

- 40% formalin…….…… 100ml
- Tap water……..……. 900ml
- Acid sodium phosphate monohydrate….. 4g
- Anhydrous disodium phosphate……... 6.5g

Compound fixative

Special fixatives

Picric acid containing fixative:-

Bouin's fixative

Advantage &property:-

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Preparation of Bouin's fixatives:

- Saturated aqueous picric acid……. 75ml
- 40% Formalin…… 25ml
- Acetic acid……..…… 5ml.

Method of Bouin's fixation:

1. Place block of tissue in Bouin's, 6-24 hrs.
2. Transfer to 70% alcohol.
3. Proceed .

Practical NO.2

Alcoholic containing fixative

Carnoys Fixative

Advantage &property:-

1-Good preservative for nucleic acid DNA,RNA

2-

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Preparation of Bouin's fixatives:

- Ethanol ……. ??

- chloroform…… ??

- Glacial acetic acid…….…… ??

Method of carnoys fixation:

1. Place block of tissue in carnoys from 4-?? hrs.
2. Transfer to clearing reagent immidiatly eg xyelen .
3. Proceed .

Practical NO.3

Decalcification

Chemical test

Acid decalcification

Aim:

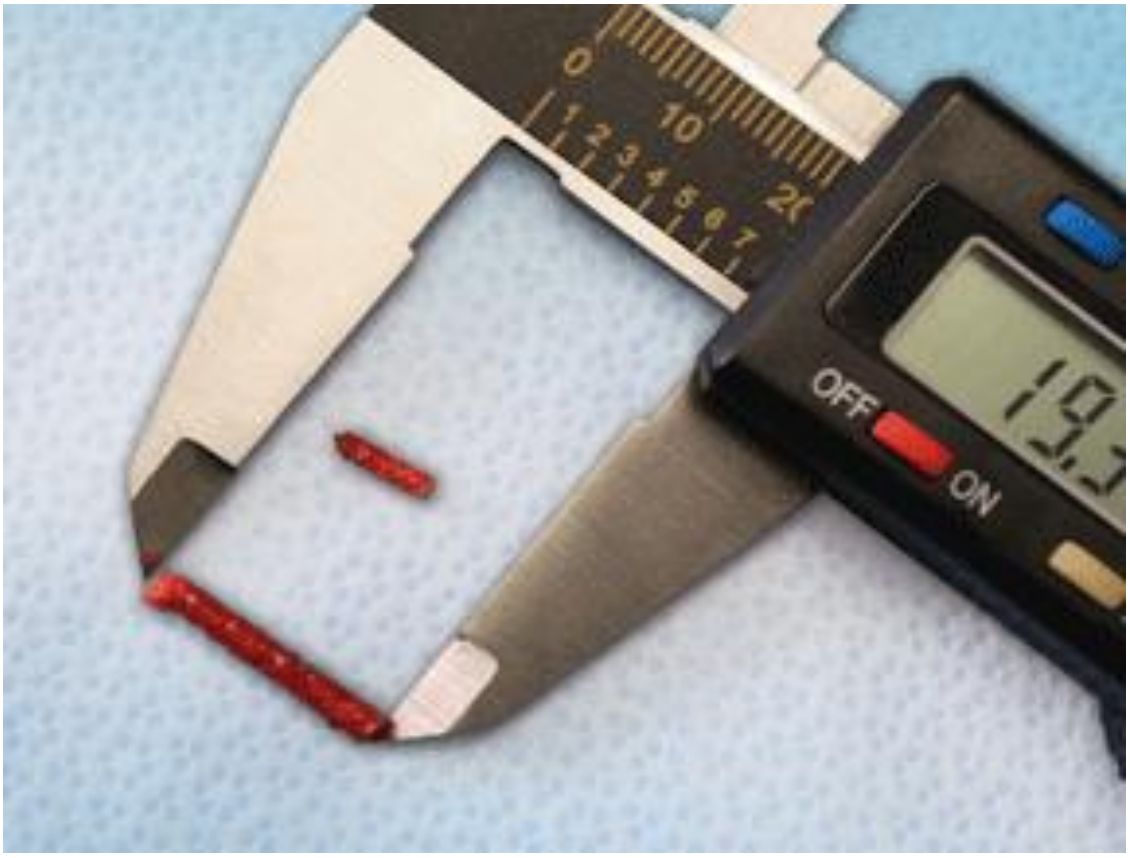
To remove inorganic calcium from calcified tissue.

Principle:

Acid decalcifier reacts with the calcium salts in the bone resulting in the formation of soluble calcium in an acid solution.

Example for different biopsy need decalcification:-

Bone marrow biopsy (trephine biopsy), lymph node biopsy(TB infection),any calcified tissue.



Core needle biopsy for bone marrow (trephine biopsy).

Requerment:-

Solution:

1- 10 % Hcl. Or **5% nitric acid (Decalcifier).**

Method:

1. Decalcified the tissue in 10 % Hcl for 4- 48 hrs. or in %5nitric acid for 1-2hrs.
2. The specimen is then placed in a fresh calcium free decalcifying fluid.
3. Check completion of decalcification. Chemical test (end point of decalcification)
4. Neutralization by alkaline tap water (running tab water for 10-15 min)
5. Then the specimen was ready to be process in processing machine.

Result:

Decalcified tissue ready for tissue processing

Notes:

1. Tissue should be fixed completely.
2. Large size specimen should be sawed to 3-5 mm.
3. Suitable container should be used (metal containers should be avoided).
4. Insufficient solutions should be avoided.
5. Avoid high temperature or low temperature.
6. Incomplete decalcification not recommended.

Result of decalcified bone marrow by H&E :-

The color is pink due to acidity of section.

H&E stain after the decalcification by acid solution :-

Method:-

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Practical NO.4

Detection of complete decalcification by Chemical test

End point of Decalcification

(Calcium oxalate test)

Aim:

To insure complete decalcification before tissue processing by

Detect the presence of calcium salt in the already used decalcifying fluid.

Principle:

Ammonium oxalate solution will react with the decalcifying fluid, if the result is clear solution that means complete decalcification, if the result is white precipitate or turbid solution (calcium oxalate formation) that means incomplete decalcification.

Solutions:

1. The decalcifying fluid
2. Concentrated ammonia
3. Saturated ammonium oxalate

Method:

1. Take 5 ml of decalcifying fluid.
2. Add litmus paper.
3. Add concentrated ammonia drop by drop until alkaline to litmus.
4. Add 0.5 ml of saturated ammonium oxalate
5. Incubate for 30 minutes.

Results:

- Clear solution indicates complete decalcification.

-if there is a present of turbidity after ammonium oxalate that mean we most renewing the decalcification fluid.

-White precipitate or turbid solution indicates incomplete decalcification.

Notes:

1. Calcium oxalate test done every 15 minutes until completion decalcification.
2. The decalcified tissue (soft tissue) must be transfer directly to 70 % alcohol after completion decalcification.
3. Proceed to tissue processing without neutralization not recommended.

Practical NO.5

Selection

Grossing (cut up)

Aim

To select a representative sample from the whole tissue specimen (3-5 mm) to facilitate tissue processing

Requirements:

most were :-biological hazard (contamination area)

PPE:personal protective equipment shoud be wered.

1. Already fixed tissue sample,or freah tissue .
2. **Request form** :-
   - ❖ important to demonstrate type of biopsy and the data of patient and history,
   - ❖ diagram is important for any remark eg to indicate right and left for eg in histroctomy for fallopian tube.
   - ❖ Date of collection,Name of patient, Type of specimen
   - ❖ Type of procedure,Physition,Sergune
   - ❖ Clinical history ,previous surgery,treatment
   - ❖ **Requirement :-**
3. **dissection set.**
4. **gloves**
5. **stickers**
6. **filter paper**:-used for preservation of tiny &fragment biopsy
7. **Indian ink** :-for labeling margin
8. **Eosin :-**for labeling tiny biopsy
9. Cassettes: used to hold the selected biopsy with labeling and sub labeling Exg : 556/ 15 / A1.
10. Pencil.
11. Gauze.
12. Scalpels and their holders.

13.Piece of wood.

14.10% N.B Formalin for routine biopsy

15. Bouin's fixatives: for fatty tissue.eg breast,testis.

*cut up

- Longitudinal
- Cross sectional all of biopsy ex kidney ,liver

Type of routine general biopsy received in histopathology lab:

1/ core needle biopsy (CT or UT guided) .

2/ true cut biopsy.

3/histroctomy biopsy(uterus , fallopian tube ,services) .

4/ mastectomy biopsy (breast).

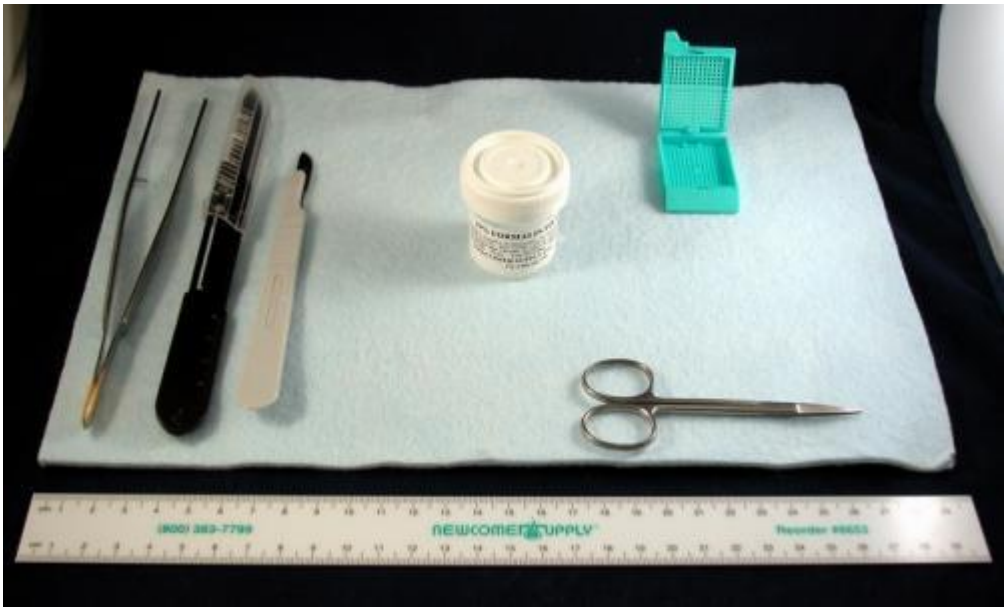
5/ spleenomectomy biopsy (spleen).

6/gall bladder

7/Appendix

Grossing area:-

Example for grossing small biopsy:-



Practical NO.5

GROSSING PROCEDURE

Give an example of grossing in a large biopsy with labeling in block

Specimen:-

MACRO:- morphology

1-SIZE & LENGTH :-

……

2-Weithg if it largespecimen

……

3-Number of fragment& size

4-Number of core needle& size: -

5-Color of specimen and other comment :

……..

6--Number of block taken from the large specimen Total:-

……

7-Number of block taken from the normal tissue:-

…….

9-Number of tissue taken from the tumor :-

…….

10-Number of the block for lymph node :- to detect invasion metastasis

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Method:

1. Select 3-5 mm tissue specimen.
2. Post fixation in 10% N.B Formalin and Bouin's fixatives.
3. Proceed in tissue processing.
4.

Notes:

1. Tissue size should not be more than 3mm or less than 1mm

Ather comment :-

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practical NO.6

Routine tissue processing

Through paraffin method

Automated tissue processing

Aim of tissue processing:

- To infiltrate the tissue in a solid medium firm enough to support the tissue, give it sufficient rigidity and suitable consistency to enable thin sections to be cut without damaging knife or tissue.
- To replace tissue water and fixative by wax to enhance & facilitate the cutting of tissue.

Principles of tissue processing:

The rate of solution diffusion through tissues is proportional to the difference between the concentrations of the fluids inside and outside the tissue.

Reagent needed:-

1. 70% alcohol (dehydrant)
2. 90% alcohol (dehydrant)
3. Absolute ethyl alcohol (dehydrant)
4. Chloroform or Xylene (clearing agent)
5. Paraffin wax (impregnation &embedding media)

METHOD:-a routine schedule for processing machine :-

***Steps:**

beaker :-1-Fixation: to complete action of fixation → 1 HOUR

***Grades of alcohol to remove water**

- **6 beaker** for Dehydration

2. 70% alcohol → 1hour
3. 90% alcohol → 1hour
4. 90% alcohol → 1hour
5. 100% alcohol → 1 hour
6. 100% alcohol → 2 hours
7. 100% alcohol → 2 hours:

3 beaker for Clearing

8. Xylene → 1hour
9. Xylene → 1hour
10. Xylene → 1hour

- **2beaker** :-Impregnation by paraffin wax : melting point (65)

11. Wax → 2hours
12. Wax → 2hours

TOTAL OF HOURS :-……

Results:

Solid impregnated tissue ready for embedding

Notes:

1. Tissue size not more than 3mm or less than 1mm
2. In sufficient solution should be avoided
3. Processing time should be within the ideal time
4. Proper agitation recommended
5. Proper labeling is important
6. Cassette should be closed well

1-GIVE AN EXAMPLE FOR A ROUTING, CHUDLE OF AUTOMATEDCPROCESSING MACHINE

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give an example for Urgent tissue processing schedule:-

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Practical NO.7

Embedding unit

Aim:

To obtain a solid block containing the tissue

To provide support and suitable consistency for sectioning

Principle:

Each biopcy were Oriented according to the side of which wonted to examieneded under microscope :-

1. (skin = at the right plane,
2. tubular organs = cross section,
3. muscles = longitudinal or transverse).
4. ……..
5. ……..
6. ……
7. ……..

By surround the tissue with suitable embedding media.

Method& equipment ;-

Metal mold smeared with glycerin to prevent sticking.

8. Fill the mold with fresh molten filtered wax.
9. With warm (not hot) smooth tipped forceps gently without squeezing orientate the specimen ,Cover the mold with the cassette containing label.
10. Cool at room temperature then in freezer or melting ice for at least 15 min.
11. Separate your block.

Result:

Solid block containing the tissue

Notes:

1. Wax should be completely molten
2. Wax should be filling the mold
3. Correct orientation is recommended
4. Formation of two layers of waxes should be avoided
5. Ice cracking should be avoided

Practical NO.8

Mayer's Hematoxylin

Aim:

- **Microanatomical stain**
- To demonstrate nuclei

Principle of hematoxylin:

Hematoxylin is a cationic dye positive charge react with tissue negative charge (nucleus) tissue dye mordant complex.

Hematoxylin its self is not a dye, haematein is the major oxidation product, haematein is an acid dye have a poor affinity for tissue, so mordant substance is added to form mordanted dye, mordanted dye react with the tissue to form tissue mordant dye complex(acid base reaction)

Solutions:

1- Mayer's Hematoxylin

-1 g hematoxylin……..stain

-1000 ml D.W……solvent

-0.2 g sodium iodate……..oxidizing agent

-50 g potassium alum……..mordant

-1 g citric acid……..sharpen nuclear stain

-50 g chloral hydrate……preservative

2- Eosin Y:

- 1 g eosin Y…….Counter stain

- 100 ml D.W……solvent

-0.05 ml GAA……...sharpen cytoplasm stains

-Small amount of crystal thymol or few drops of formalin …preservative

Method:

1. Take section to water
2. Stain with Mayer's hematoxylin for 5-7min.progressive
3. Rinse in water
4. Blue in running tap water for 10 min.
5. Counter stain in eosin Y for 1-3 min.
6. Dehydrate, clear and mount.

Note:-

Bluing is the process of converting the initially red soluble hemalum to a final blue insoluble form.
Tap water alone can blue cells satisfactorily; chemically defined bluing agents are unnecessary

Results:

Nuclei: Blue

Cytoplasm: Pink

Others: Pink-Red,

Notes:

1- Structures stained:

Many structures are not clearly demonstrated or are not stained by hematoxylin and eosin method. Thus neuroglia fibers, axons, nerve endings, much reticulin, cell constituents such as Golgi bodies and mitochondria and mucopolysaccharides and glycogen.

2- Duration of staining with hematoxylin:

Staining time have to be prolonged with tissues given long treatment with chrome salts and following chrome- osmium fixatives, tissues subjected to long acid decalcification, prolonged storage of tissue in acid formalin or in 70%

alcohol, storage of the stain. Warming accelerates while low temperature retards staining with hematoxylin.

3- According to the staining time Mayer's hematoxylin can be used either progressive (5-10min) or regressively (10-20min).

4- Mayer's hematoxylin is alum hematoxylin affected by subsequent treatment in acid stains.

5- Bleuing of Hematoxylin:

In some places, the tap water is not sufficiently alkaline, or is even acid, and unsatisfactory for bleuing hematoxylin, so Scott tap water or alkaline solution can be used.

6- Eosin y used commonly as counter stain because it colors back ground by colors vary from pinkish to red

Practical NO.9

Harris's hematoxylin and Eosin

Aim:

- Cytological stain and microanatomicla stain
- To demonstrate nuclei and cytoplasm

Principle of hematoxylin:

Hematoxylin its self is not a stain, haematein is the major oxidation product, haematein is an acid dye have a poor affinity for tissue, so mordant substance is added to form mordanted dye, mordanted dye react with the tissue to form tissue mordant dye complex(acid base reaction)

Solutions:

1- Harris's Hematoxylin

2.5 g hematoxylin……..stain

25 ml absolute ethanol……solvent

500 ml D.W……solvent

0.2 g sodium iodate……..oxidizing agent

50 g potassium alum……..mordant

25 glacial acetic acid……..sharpen cytoplasmic stain

2- Eosin Y:

1 g eosin Y……Counter stain

100 ml D.W……solvent

0.05 ml GAA……...sharpen cytoplasm stain.

Small amount of crystal thymol or few drops of formalin …preservative

Method: for cytological smear

1. Wash mouth by water
2. Scrape cells from buccal mucosa by wooden spatula
3. Smear directly on a microscopic glass slide
4. Fix the smear immediately while it's wet in 95% ethanol
5. Hydrate in 90% alcohol for 2 min
6. Hydrate in 80% alcohol for 2 min
7. Hydrate in 70% alcohol for 2 min
8. Rinse in water for 2 min
9. Stain in Harris Hematoxylin for 5 min
10. Differentiate in 0.5 % aqueous hydrochloric acid for 10 second or in 1% acid alcohol for 5-10 second
11. Rinse in water
12. Blue in running tap water for 10 min.
13. Counter stain in eosin Y for 1-3 min.
14. Dehydrate, clear and mount.

Results:

Nuclei: Blue

Cytoplasm: Pink

Others: Pink-Red

Notes:

1. Duration of staining with hematoxylin:
2. Bluing of Hematoxylin:
   In some places, the tap water is not sufficiently alkaline, or is even acid, and unsatisfactory for bluing hematoxylin, so Scott tap water or alkaline solution can be used.
3. Eosin y used commonly as counter stain because it colors back ground by colors vary from pinkish to red

Practical NO.10

Weigert's Hematoxylin and Eosin

Aim:

To demonstrate nuclei and cytoplasm & CNS tissue .

Principle of hematoxylin:

Hematoxylin is self is not a stain, haematein is the major oxidation product, haematein is an acid dye have a poor affinity for tissue, so mordant substance is added to form mordanted dye, mordanted dye react with the tissue to form tissue mordant dye complex(acid base reaction)

Solutions:

1- Weigert's Hematoxylin (nuclear stain)

A) 1% alcoholic hematoxylin:

1 g hematoxylin……..stain

100 ml alcohol……solvent

B) Iron solution:

4ml 30% ferric chloride

1ml conc Hcl

95ml D.W

Working solution: Take equal volume from A & B.

2- 1% acid alcohol(differentiation fluid):

99 ml of 70% alcohol

1ml of conc Hcl

3- Eosin Y(counter stain):

- 1 g eosin Y…….Counter stain

- 100 ml D.W……solvent

-0.05 ml GAA……...sharpen cytoplasm stain.

-Small amount of crystal thymol or few drops of formalin …preservative

Method:

1- Take section to water.

2- Stain with Weigert's hematoxylin for 10-15min.regressive stain .

3- Rinse in water.

4- Differentiate in 1% acid alcohol.

5- Wash in water 5 mins.

6- Counter stain in eosin Y for 1-3 min.

7- Dehydrate, clear and mount.

Result:

Nuclei: Blue black.

Cytoplasm: Pink

Others: Pink-Red

Notes:

1. The working solution must be violet black if its brown discards it and prepare new one.

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Practical NO.11

Spesial staining techniques

Special stains remain an important tool for many pathologists and technologists providing a powerful complement to immunohistochemistry,flow cytometry, in situ hybridization and other diagnostic technologies that define a patient'smedical profile.

Special Stains

Special stain is a term that is mostly used in a laboratory setting.
Special stains have two broad areas of application: research and diagnostic.
In research, special stains are used as probes to identify certain chemical constituents in normal and abnormal cells.

The information so obtained

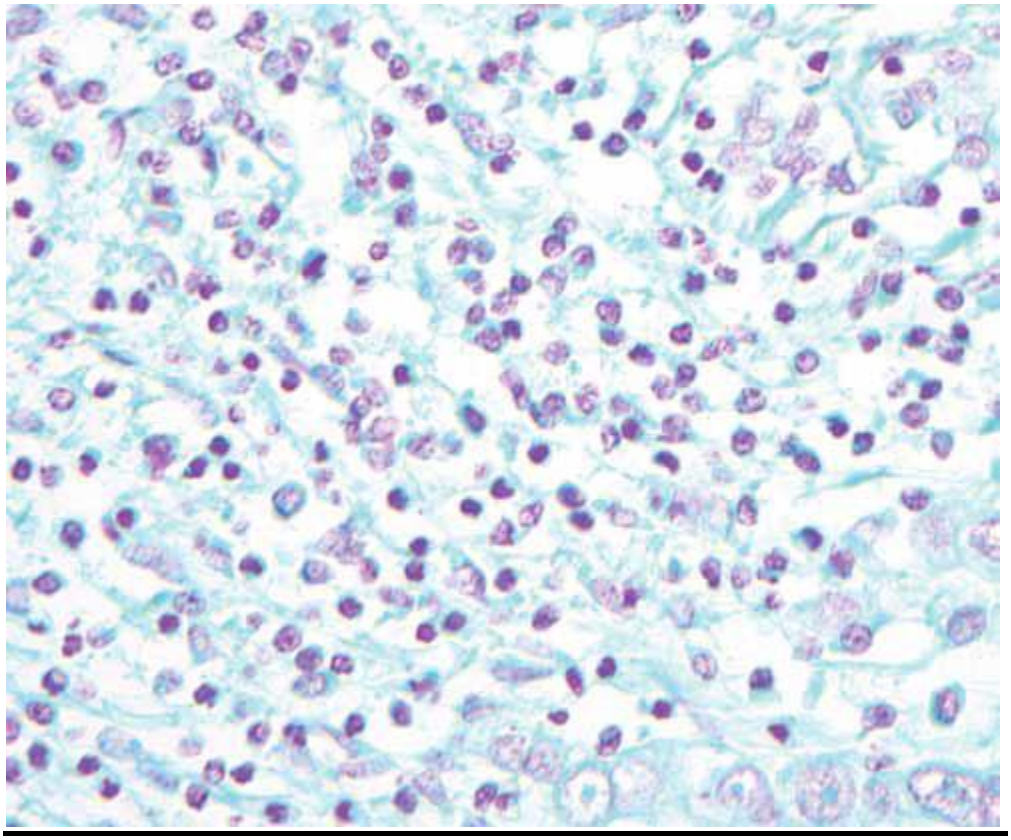
is used as a basis for further study and also as a baseline againstwhich the results of special staining can be compared in diagnostic applications. On the basis of such a comparison, the significance of the findings can be interpreted. Special stains can be applied to cell biology and histology.

Someuseful applications are:

(1) the determination of DNA and RNA content,(2) the mode of action of drugs, hormones or of potentially toxic foodadditives, (3) metabolic biochemistry, (4) biochemistry of diseaseprocesses, (5) primary sites of many metastatic tumors, (6) identificationof non-pigmented metastatic melanomas, (7) detection of early invadingtumors, (8) definition of the margins of surgically resected tumors, (9) identification of Barr bodies, (10) staining cells in ways that can be used as a basis for cell separation by appropriate instrumentation(e.g., fluorescence), and (11) identification of micro-organisms (e.gCryptococcus neoformans, Helicobacter pylori).

Pactical NO.12

Feulgen Reaction for DNA



- **Breast tissue stained with Feulgen, The Feulgen stain is used to demonstrate DNA in tissue sections.**
- **RNAis not stained by this procedure.**
- **The DNA is stained magenta with Schiff's reagent.**
- **The stained DNA is contrasted against a lightgreen counterstain to allow better visualization by light microscopy or image analysis.**

Aim:

For demonstration of Deoxyribonucleic acid (DNA)

Fixation:

Beast fixative carnoys , alcoholic fixative ,avoid fixation by picric acid containing fixative ,mercuric fixative which resulting to denaturation of DNA.

Preparation &function of reagent :

1-1M HCL (Hydrolysis):

Concentrated HCL…….. 8.5 ml

D.W……...……. 91.5 ml

2-Schiffs reagent: constituent and function :

Basic Fuchsin…...…….…… Stain

D.W ……..…… Solvent

Potassium metabisulphite…….. Reducing agent

Conc HCL…….…….…… Maintain PH

Activated Charcoal……..….precipitate & Remove any excess Sulpher dye.

3-1% Light green (counter stain)

Principle:

Mild acid hydrolysis employing 1M HCL at 60^0c, is used to break down the purine deoxyribose bond, the resulting exposed Aldehydes are then demonstrated visualized by the reaction with Schiff's reagent resulting to magenta color.

Controle :-

3hydroxynaphathoic acid

Method:

1. Bring section to water.
2. Rinse sections in 1M HCL at room temperature for 1 min.
3. Place sections in 1M HCL at 60^0c for 8 min (according to fixative used).

4. Rinse in 1M HCL at room temperature for 1 min.
5. Transfer sections to Schiff's reagent for 45 min.
6. Rinse sections in R.T W for (5-10) min.
7. Counter stain in 1% Light green for 1-2 min.
8. Wash in D.W.
9. Dehydrate, clear and mount.

Results:

DNA: red- purple.

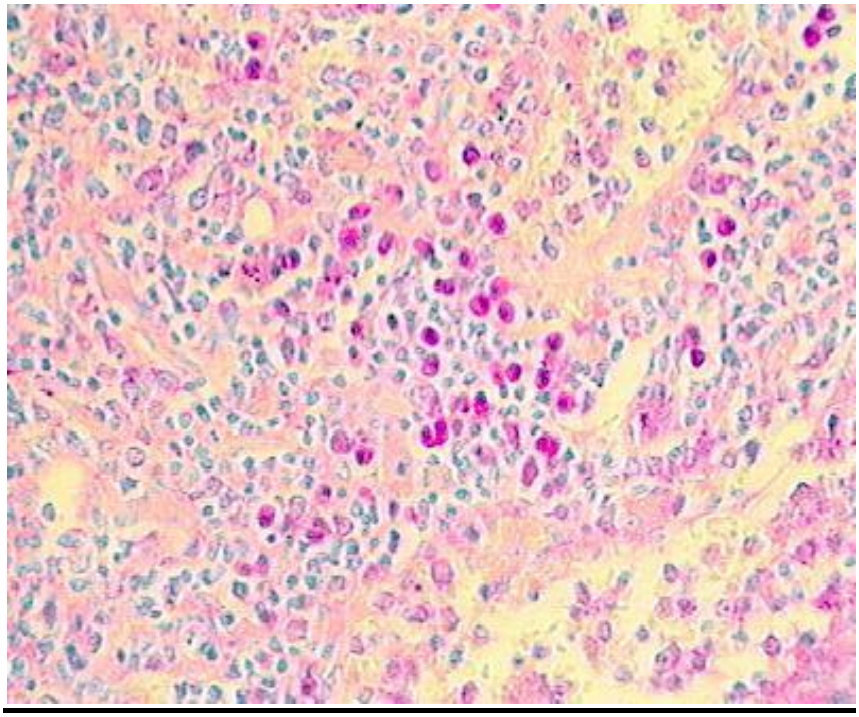
Cytoplasm: green.

Notes:

1. Bouin's fixative is not suitable as it causes over hydrolysis.
2. Decalcification, strong inorganic acid should be avoided, and organic acid for short time can be used.
3. Optimum hydrolysis time should be recommended.
4. The hydrolysis time is important, and the correct time for the fixative must be used.
5. The 1M HCL should be preheated to 60˚C.
6. 5M HCL at room temperature may be used but it need longer time .

Practical NO.13

Methyl Green – Pyronin



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Aim:

For the demonstration of DNA and RNA

Fixation:

Carnoy preferred but formalin acceptable

Solutions:

Methyl green pyronin Y

- 2% aqueous methyl Green (chloroform extracted)……..... 9 ml
- 2% aqueous pyronin y…….……..…….. 4 ml
- Acetate buffer pH 4.8……...……...… 23 ml
- Glycerol…..……..… 14 ml

Mix well before use.

Preparation of acetate buffer pH 4.8:

Stock A: 0.2 M acetic acid:

1.2 ml glacial acetic acid in 100 ml of D.W

Stock B: 0.2 M sodium acetate:

1.64 g of sodium acetate in 100 ml of D.W

Composition of buffer:

8 ml of A + 12 ml of B

Principle:

Methyl green specificity is attributed to the spatial alignment of the NH_2 groups of the dye to the phosphate radicals on the DNA, pyronin lack this spatial affinity and any negatively charged tissue will stain red.

Method:

1. Bring section to water.
2. Rinse in acetate buffer solution pH 4.8.
3. Place in staining solution for 25 min.
4. Rinse in acetate buffer solution pH 4.8.
5. Blot, dry.
6. Rinse in 93% Alcohol, then in Absolute Alcohol.
7. Clear and mount.

Results:

DNA: green-blue.

RNA: red.

Notes:

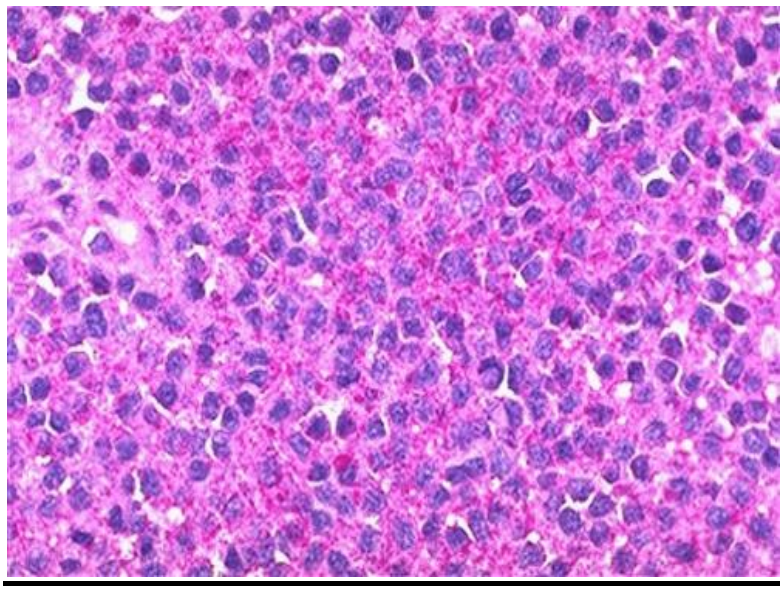
1. PH and staining solution concentration, final dehydration optimizes.

2. Methyl green is an impure dye containing Methyl violet removed by washing in chloroform.
3. Some mucous cells may be stained by pyronin.
4. Acid decalcification is said to interfere with the technique (to solve, by adjusting the two dyes the methyl green increased and the pyronin y decreased).
5. The final dehydration is also important, and washing in water should be avoided.

Practical NO.14

Periodic Acid Schiff's Reaction (PAS)

PAS :-for carbohydrate (glycogen)



Aim:

For demonstration of Glycogen

Fixation:

Bouins &alcoholic fixative is the best.

Controle:

Diastase enzyme digestion before treated with shiffs reagent.

Solutions:

1. **1%periodic acid for oxidation**
2. **Schiff's reagent.**
    - Basic fuchsin……..……..stain.
    - D.W……..……..solvent.
    - Potassium metabisulphite……..…….Reducing agent.
    - Conc HCL.…… PH.

- Activated Charcoal……....Remove any excess Sulpher.

3. **Mayer's Hematoxylin (nuclear counter stain).progressive**
    - 1 g hematoxylin……..stain
    - 1000 ml D.W……solvent
    - 0.2 g sodium iodate……..oxidizing agent
    - 50 g potassium alum……..mordant-
    - 1 g citric acid……..sharpen nuclear stain
    - 50 g chloral hydrate……preservative

Principle:

1%Periodic acid will bring about oxidative cleavage of the Carbon to Carbon bond in 1-2 glycol or their amino or alkyl-amino derivatives, to form dialdehydes. Then these aldehydes will react with Schiff's reagent to form magenta color compound.

Method:

1. Bring section to water.
2. Oxidize in 1% P.A for 5 min.
3. Wash well in different changes of D.W
4. Place in Schiff's reagent for 15 min.
5. Wash in R.T W for (5-10) min.
6. Stain nuclei with M.H and bluing.
7. Wash in water, rinse in absolute alcohol.
8. Clear and Mount.

Results:

Glycogen: / Magenta.

Nucleus: Blue.

Notes:

1. Periodic acid is the oxidant of choice.

2. Other oxidants like potassium permanganate and chromic acid substitute periodic acid
3. They tend to over oxidize the formaldehyde to carboxylic acid which result in a weak Schiff's reaction.
4. Tap water after Schiff's solution to intensify the color of reaction.
7. 60 ˚C.

Practical NO.15

PAS with Diastase

Aim:

To specify the demonstration of Glycogen by PAS

Solutions:

1. **1%diastase:**
    - Diastase: 1g
    - D.W: 100 ml
2. **1% periodic acid.**
3. **Schiff's reagent.**
4. **Mayer's hematoxylin.**

Principle:

Diastase enzyme break down the glycosidic bonds (converting the glycogen into glucose units), the resulting glucose units dissolve in water so that the Schiff's reaction appear negative after employing of PAS staining procedure.

Method:

1. Dewax two positive control and two test sections and bring to distilled water.
2. Treat one test section and one positive control section, for 1 hour at 37^0c in diastase solution.
3. Wash in R.T.W for (5-10) min.
4. Stain all sections with the PAS technique for glycogen.
5. Dehydrate, clear and mount.

Results:

Presence of glycogen will be evidenced by loss of staining after enzyme treatment when compared to the untreated sections.

Treated section: colorless.

Untreated section: Red.

Glycogen present in sections.

Notes:

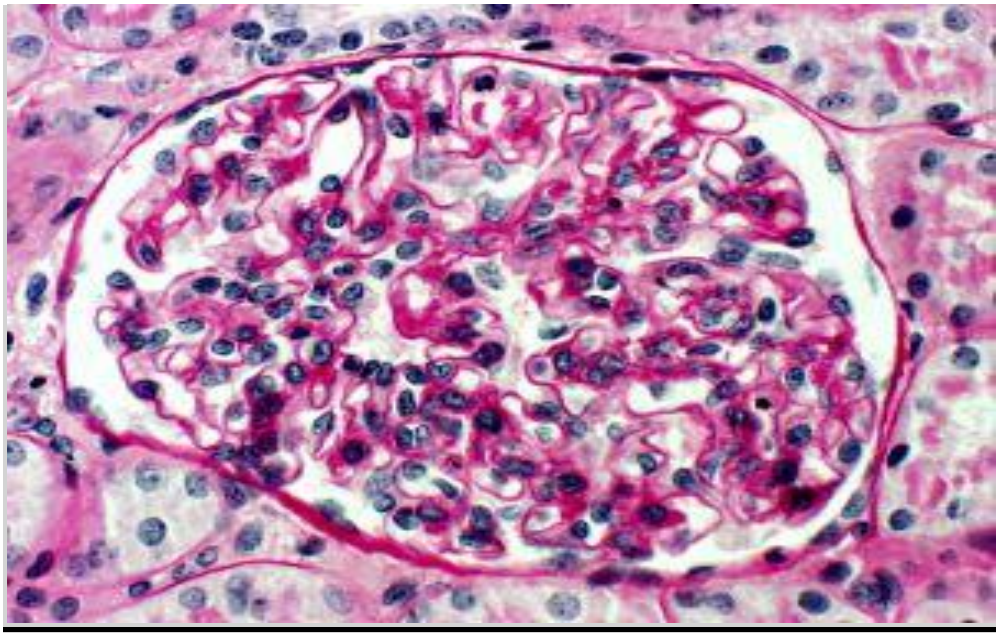
1. In addition to glycogen, starch is also digested but at a lower rate.
2. The type of fixation will affect digestion, as Gender or glutaraldehyde resist diastase digestion and longer times will be needed.
3. Celloidinization of sections prior to digestion may slow down or inhibit the enzyme action.

practical NO.16

Periodic Acid Schiff's Reaction (PAS)

PAS :-for carbohydrate (mucoprotein)

basement membrane



Aim:

Demonstration of basement membrane mucoprotein carbohydtrate

Fixation:

10%NBS,10% foemalin

Solutions:

4. **1%periodic acid.**
5. **Schiff's reagent.**
    - Basic fuchsin……..……..stain.
    - D.W…….……..solvent.
    - Potassium metabisulphite……..…….….Reducing agent.
    - Conc HCL.…… PH.
    - Activated Charcoal……....Remove any excess Sulpher.

6. Mayer's Hematoxylin (nuclear counter stain).

- 1 g hematoxylin……..stain
- 1000 ml D.W……solvent
- 0.2 g sodium iodate……..oxidizing agent
- 50 g potassium alum……..mordant-
- 1 g citric acid……..sharpen nuclear stain
- 50 g chloral hydrate……preservative

Principle:

1%Periodic acid will bring about oxidative cleavage of the Carbon to Carbon bond in 1-2 glycol or their amino or alkyl-amino derivatives, to form dialdehydes. Then these aldehydes will react with Schiff's reagent to form magenta color compound.

Method:

9. Bring section to water.
10. Oxidize in 1% P.A for 5 min.
11. Wash well in different changes of D.W
12. Place in Schiff's reagent for 15 min.
13. Wash in R.T W for (5-10) min.
14. Stain nuclei with M.H and bluing.
15. Wash in water, rinse in absolute alcohol.
16. Clear and Mount.

Results:

Basement membrane mucoprotein :magenta color, Nucleus: Blue.

Notes:

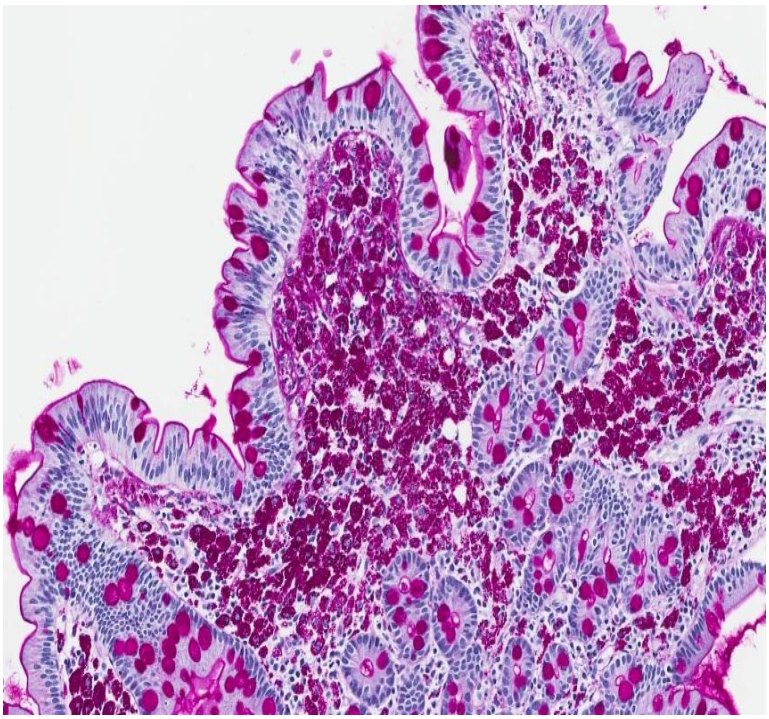
5. Periodic acid is the oxidant of choice.
6. Other oxidants like potassium permanganate and chromic acid substitute periodic acid
7. They tend to over oxidize the formaldehyde to carboxylic acid which result in a weak Schiff's reaction.
8. Tap water after Schiff's solution to intensify the color of reaction.
9.

Practical N17

PAS techniques for

carbohydrate material

Neutral mucin



Aim:

For demonstration of neutral mucin

Fixation:

Not critical ,alcoholic fixative ,avoid mercuric fixative

Controle:-

Phenyl hydrazine techniques

Solutions:

7. **1%periodic acid.**
8. **Schiff's reagent.**
    - Basic fuchsin……..……..stain.

- D.W……..……..solvent.
- Potassium metabisulphite……..…….…Reducing agent.
- Conc HCL.…… PH.
- Activated Charcoal……....Remove any excess Sulpher.

9. Mayer's Hematoxylin (nuclear counter stain).

- 1 g hematoxylin……..stain
- 1000 ml D.W……solvent
- 0.2 g sodium iodate……..oxidizing agent
- 50 g potassium alum……..mordant-
- 1 g citric acid……..sharpen nuclear stain
- 50 g chloral hydrate……preservative

Principle:

1%Periodic acid will bring about oxidative cleavage of the Carbon to Carbon bond in 1-2 glycol or their amino or alkyl-amino derivatives, to form dialdehydes. Then these aldehydes will react with Schiff's reagent to form magenta color compound.

Controle :-

Phynile hydrazine technique;-

……

……

Method:

17. Bring section to water.
18. Oxidize in 1% P.A for 5 min.
19. Wash well in different changes of D.W
20. Place in Schiff's reagent for 15 min.
21. Wash in R.T W for (5-10) min.
22. Stain nuclei with M.H and bluing.
23. Wash in water, rinse in absolute alcohol.
24. Clear and Mount.

Results:

Neutral mucin :magenta color

Nucleus: Blue.

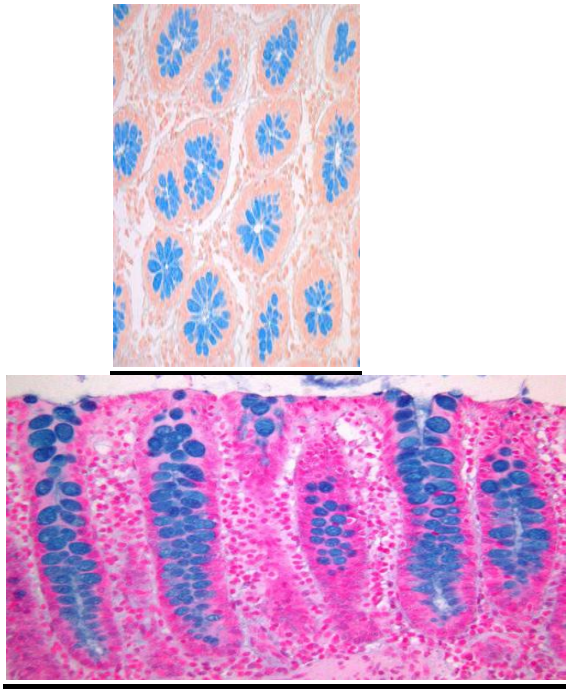
Notes:

10. Periodic acid is the oxidant of choice.
11. Other oxidants like potassium permanganate and chromic acid substitute periodic acid
12. They tend to over oxidize the formaldehyde to carboxylic acid which result in a weak Schiff's reaction.
13. Tap water after Schiff's solution to intensify the color of reaction.

Practical NO.18

Carbohydtrate Acid mucin mucopolysacharide

Alcian Blue dye.



Aim:

For demonstration of Acid mucins and connective tissue mucins

Preparation:

1. **1% Alcian blue.**
    - Alcian blue……. 1g
    - 3% acetic acid…….…….…… 100ml
2. **1% Eosin or neutral red (counter stain).**

Principle:

The Alcian blue is cationic dye, and at pH 2.5 bond via electrostatic linkages with polyanionic molecules within tissues (acid mucins and connective tissue mucins).

Method:

1. Bring section to water.
2. Treat with working solution for 30 min.
3. Wash in running tap water for 5 min.
4. Counter stain with 0.5% Neutral Red or Safranine or Eosin for 1-2 min.
5. Wash in D.w.
6. Dehydrate, clear and mount.

Results:

Acid Mucins, proteoglycans and hyaluronic acid: Blue

Nucleus or other structures: according to counter stain in use

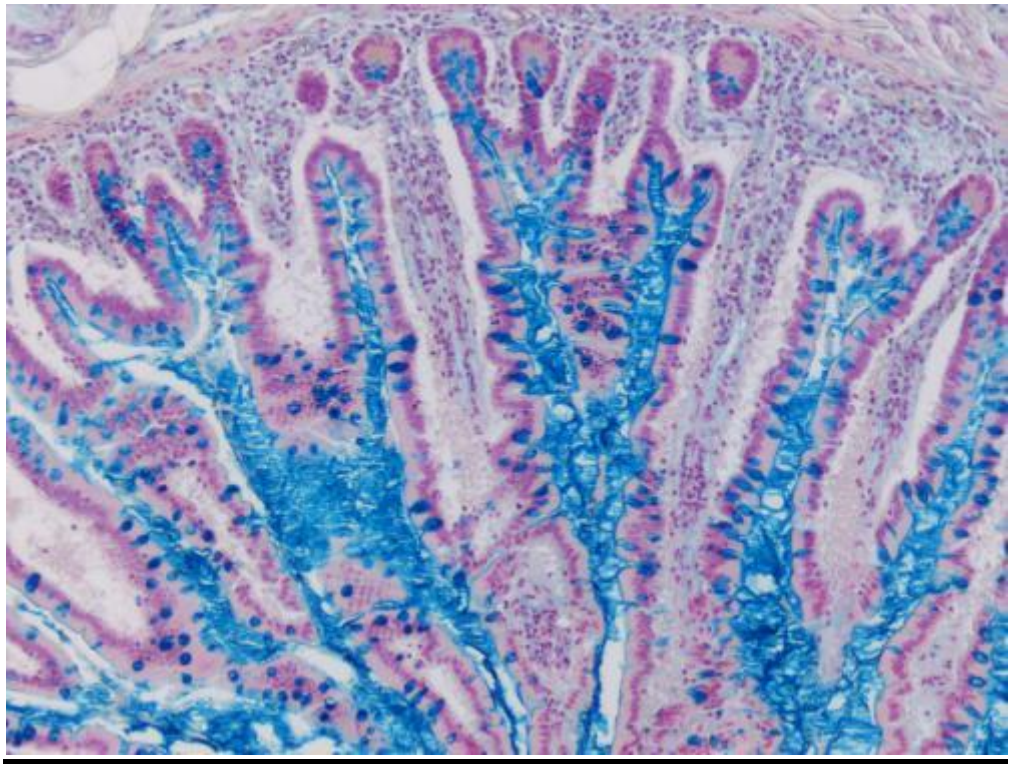
Notes:

1. It is important to counter stain with a weak solution of Neutral Red to avoid masking the Alcian blue staining.
2. It is best to filter the staining solution before uses it, particularly if the age of solution is more than month or so old.
3. Avoid celloidinization of slides as the Alcian dyes are strongly retained by the celloidin.
4. 3% of glacial acetic acid uses to adjust the ph of staining solution at 2.5.
5. Van Gieson solution may be used as a counter stain.

6. Alcian blue at different Ph level may differentiate the sub types of acid mucins.

Practical NO.19

Carbohydtrate Colloidal iron technique for acid mucin



Aim:

For demonstration of acid mucopolysaccharides

Principle:

The ferric cations in a colloidal ferric oxide solution are attracted and bound to acidic mucins; the ferric ions bound to the mucins are then treated with potassium ferrocyanide to form bright blue deposits of ferric ferrocyanide or Prussian blue.

Solutions:

Stock colloidal iron solution:

29% ferric chloride…… 4.4 ml

D.W…… 250 ml

Working solution:

Stock colloidal iron solution……..……. 20 ml

Deionized water……...… 15 ml

Glacial acetic acid……...… 5 ml

Prepare just prior to use.

12% Acetic acid solution

Potassium ferrocyanide hydrochloric acid solution:

A. 5% potassium ferrocyanide solution.
B. 5% Hydrochloric acid solution.

Take equal volume from A and B mix just prior to use.

Van Gieson solution:

1% acid fuchsin…….. 5 ml

Saturated picric acid……... 95 ml

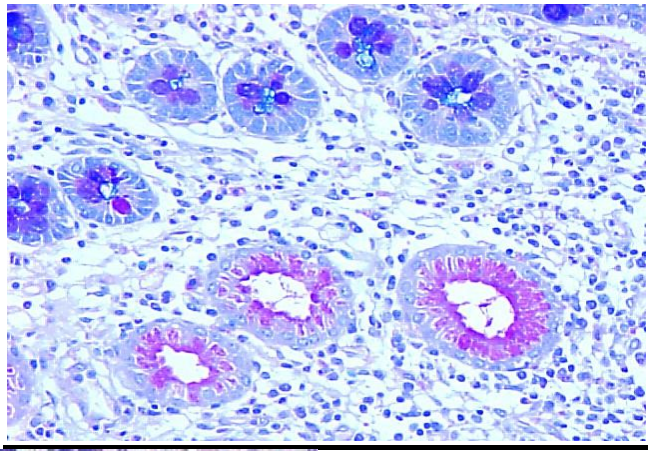
Method:

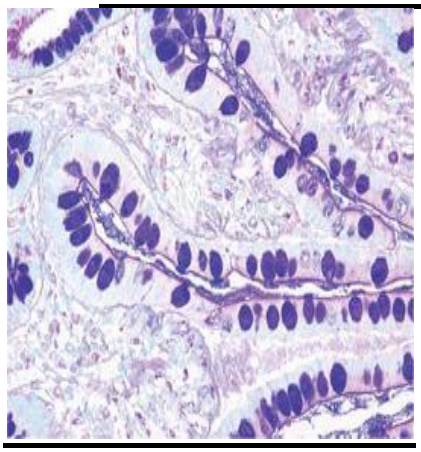
1. Take section to D.W
2. Rinse in 12% acetic acid solution for 1 min.
3. Cover the section with the working colloidal iron solution for 30 min.
4. Rinse in 12% acetic acid solution 2 or 3 changes each 3 min.
5. Place in the potassium ferrocyanide hydrochloric acid solution for 20 min.
6. Rinse in R.T.W for 5 min.
7. Counter stain with van Gieson solution for 5 min.
8. Dehydrate in absolute alcohol, clear, and mount.

Practical NO.20

Carboyhydtrate acid mucin neutral mucin

Combined alcian blue PAS





Aim:

To differentiate neutral mucins from acidic mucins within a tissue section

Also it may be used as a broad means of detecting mucins

Preparation:

1%Alcian blue.

Stain the acid mucopoly saccharides

1% periodic acid

Oxidizing agent

Schiff's reagent Main stain

Mayer's hematoxylin Counter stain nucleus stain.

Principle:

The section is first stained with Alcian blue (stains all acid mucins even those are PAS positive), then the subsequent treatment with PAS staining procedure demonstrate only the neutral mucins.

Method:

1. Bring section to water.
2. Stain with Alcian blue at pH 2.5 for 30 min.
3. Wash in water and then wash again in D.W
4. Oxidize in 1% P.A for 5 min.
5. Wash well in different changes of D.W
6. Place in Schiff's reagent for 20 min.
7. Wash in R.T W for (5-10) min.
8. Stain nuclei with M.H and bluing.
9. Wash in water, rinse in absolute alcohol.
10. Clear and Mount.

Results:

Acid Mucins: Blue

Neutral Mucin: magenta

Mixed type ;purple color.

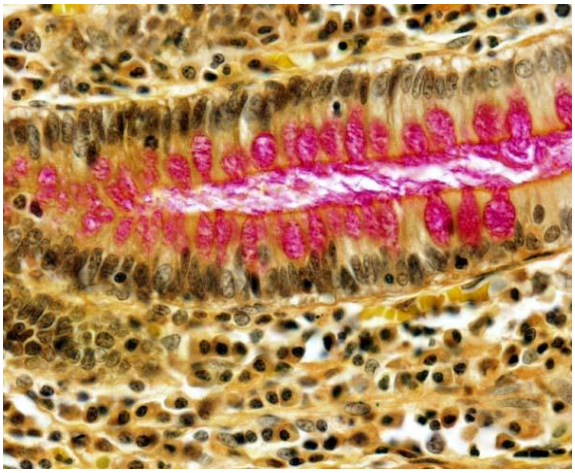
Notes:

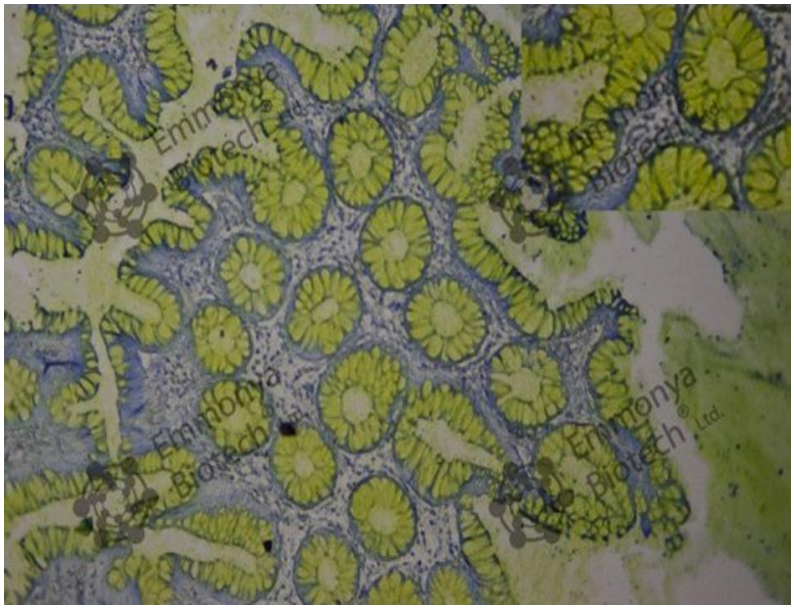
1. It is important to stain only lightly with Hematoxylin, as otherwise it may be difficult to distinguish B/w this and the Alcian blue staining.
2. Ehrlich's Hematoxylin should be avoided as a counter stain, as it well also stains some mucins.
3. It's very important to apply alcian blue first then followed by PAS.
4. Glands that contain acid and neutral mucins stain purple.

Muscle and cytoplasm: yellow

Notes:

1. The PH of the staining solution is critical.
2. Nuclear fast red or eosin may
3. be used as a counter stains.





Alcian yellow for mucopolysaccharide.

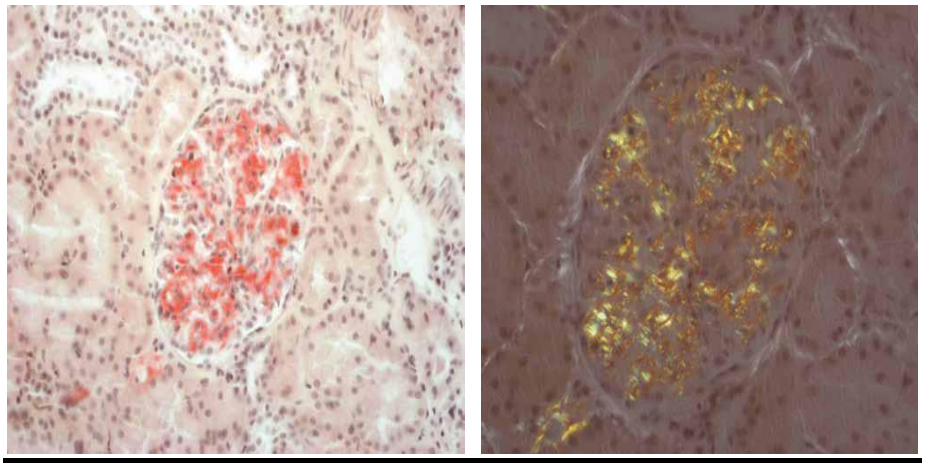
practical NO.21

Highman's Congo red

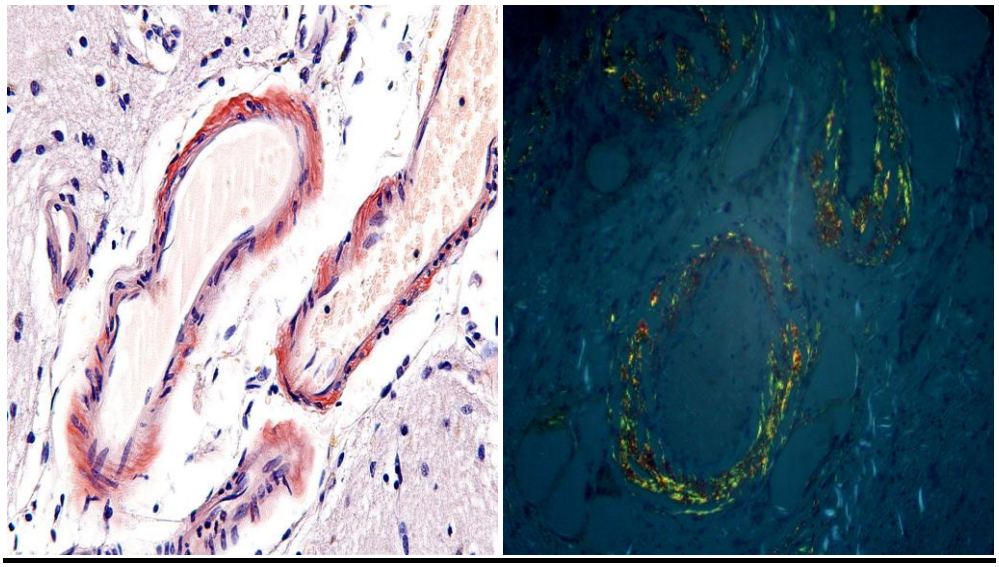
Amyloid deposition

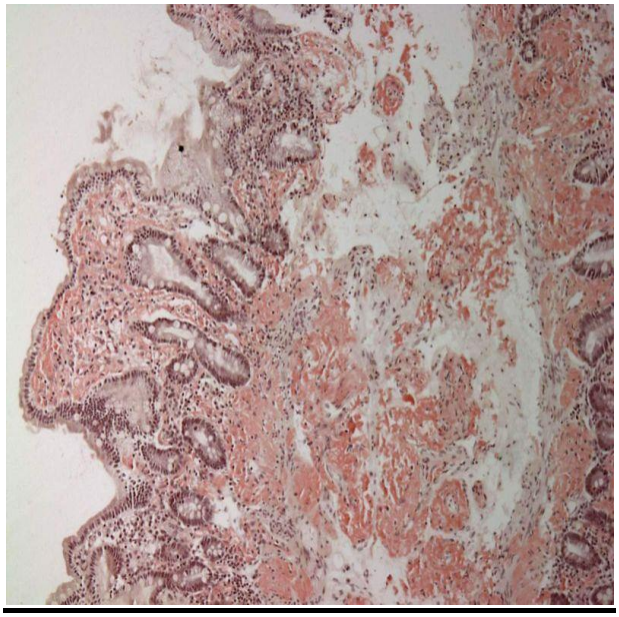
Congo red stain. Amyloidosis:

Amyloid deposits stain red with Congo red, demonstrate the characteristic apple-green birefringence under polarized light in the same section of kidney.



Cerebral amyloid angipathy by congo red





Small bowel Amyloid

Aim:

For the demonstration of Amyloid

Solutions:

Solution (a): Congo red working solution.

o.5% Congo red in 50% alcohol

Solution (b): Differentiator.

o.2%KOH in 80% Alcohol

Principle:

Congo red is an acidic diazo dye; the staining of Amyloid by Congo red is through hydrogen bonding formation between the amyloid and Congo red as opposed to the electrochemical bonds formed between the dye and most tissue components.

Method:

1. Bring section to water.
2. Stain with working solution for 5 min.
3. Differentiate in 0.2% KOH for (3-10) seconds.
4. Wash in water.

5. Stain nucleus with Alum hematoxylin.
6. Bluing in R.T.W.
7. Dehydrate, Clear and Mount.

Results:

Amyloid, elastic fibers, eosinophilic granules: orange Red

Nucleus: Blue

Notes:

1-Differentiation in step ‘’3’’ can be arrested in water and resumed if necessary-over differentiation can occur.

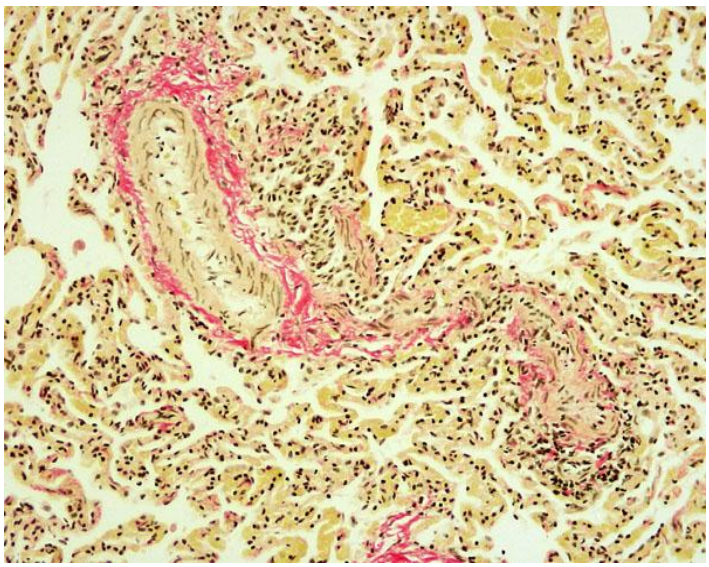
2-The solutions are relatively stable and the method affords a high degree of selectivity in practical hands.

3-This is simple method, has found wide application.

Practical No.22

Demonstration of connective tissue

Weigert's Van Gieson



Aim:

For selective demonstration of muscles, collagen fibers, fibrin, and erythrocytes

Solutions:

Van Gieson solution:

1. Saturated aqueous picric Acid.
   (Small dye molecule)
2. 1% acid Fuchsin. 1 gm in 100 ml D.W
   (Large dye molecule)

Take 10 ml of 1% acid fuchsin and add to 90 ml Picric acid.

Weigert's hematoxylin (Nuclear stain)

A. 1% alcoholic hematoxylin
B. Iron solution:

30% aqueous ferric chloride…….....4 ml

HCL (Conc)……..……1 ml

D.W…….95 ml

Take equal parts of solution A and B and mix them immediately before use.

Principle:

Collagen fibers have strong affinity for high molecular weight of dyes, while muscle, RBCs, and fibrin have affinity for low molecular weight of dyes (according to the tissue permeability and dye size molecule).

Method:

1-Bring section to water.

2-Stain nucleus with Weigert's solution for (10-15) min

3-Wash in water for (7-10) min

4-Stain with V.G working solution for (3-5) min

5-Blot with Filter Paper

6-Dehy, Clear and Mount.

Results:

Nucleus: Black color.

Collagen fiber: Red color.

R.B.Cs, Muscles: Yellow color.

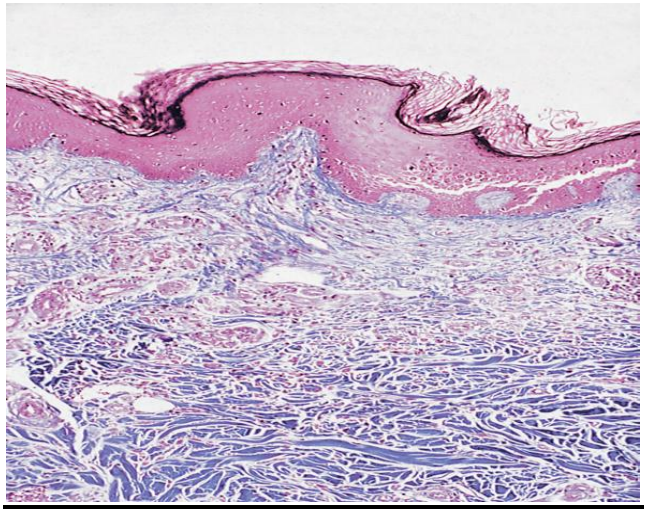
Notes:

1. Washing in water after V.G should be avoided, the color balance being impaired by this.
2. Nuclear staining should be before application of V.G.

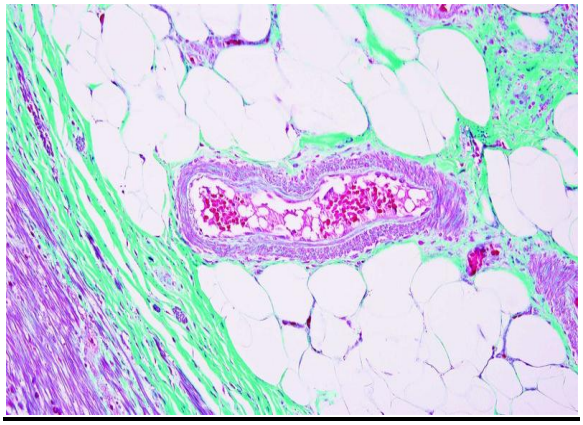
3. Celloidin sections washed in D.W. after V.G solution.
4. Differentiation step after Weigert's hematoxylin is not needed because picric acid in vangieson solution do this.

practical NO.23

Masson Trichrome Technique



Masson trichrome on a section of skin demonstrating, collagen in the dermis.



Masson trichrome: collagen fibers green, muscles and RBCs red, nucleus black

Aim:

For selective demonstration of muscles, collagen fibers, fibrin, and erythrocytes

Solutions:

Nuclear stain:

Weigert's Hematoxylin

Cytoplasmic stain:

1% Acid Fuchsin in 1% Acetic Acid

Fiber stain:

2% Light Green in 1% Acetic Acid or

2% Fast Green in 1% Acetic Acid or

2% Methylene Blue in 1% Acetic Acid

Differentiation:

1% Aqueous PMA or PTA

Principle:

Collagen fibers have strong affinity for high molecular weight of dyes, while muscle, RBCs, and fibrin have affinity for low molecular weight of dyes.

Method:

1. Bring section to water.
2. Stain Nucleus with Weigert's .H for (10-15) min
3. Wash in water.
4. Differentiate with 1% Acid Alcohol.
5. (Rinse) Keep in R.T.W for 5 min.
6. Stain with Cytoplasmic stain for (3-5) min.
7. Wash.
8. Differentiate with 1% aqueous PMA or PTA until the collagen decolorize while the RBCs and Muscle still stain or differentiate for 5 minutes.
9. Drain.
10. Stain with Fiber stain for (3-5) min.
11. Treat with 1% Acetic Acid for (1-2) min, to remove any excess of light green.

12.Dehydrate, Clear and Mount.

Results:

Collagen Fiber: Green.

RBCs, Muscle: Red.

Nucleus: Black.

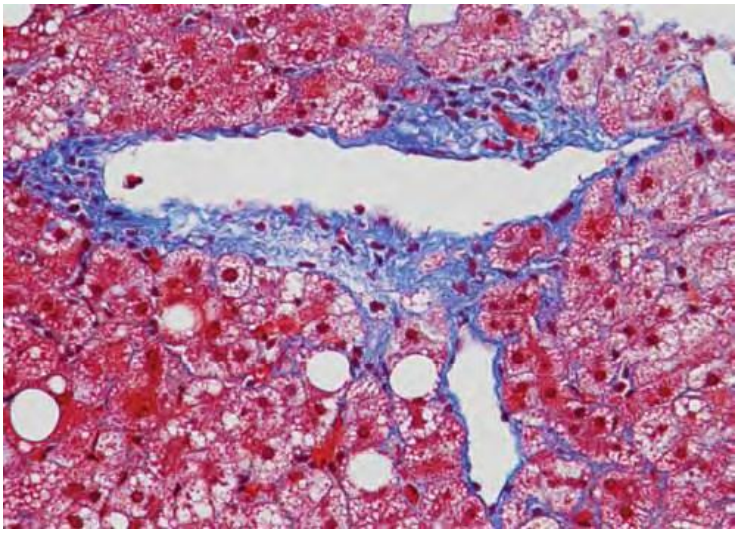
Notes:

1-If a section is first treated with PMA or PTA solution and then with a low concentration of a dye in the same solution, the dye will color nothing but the erythrocytes.

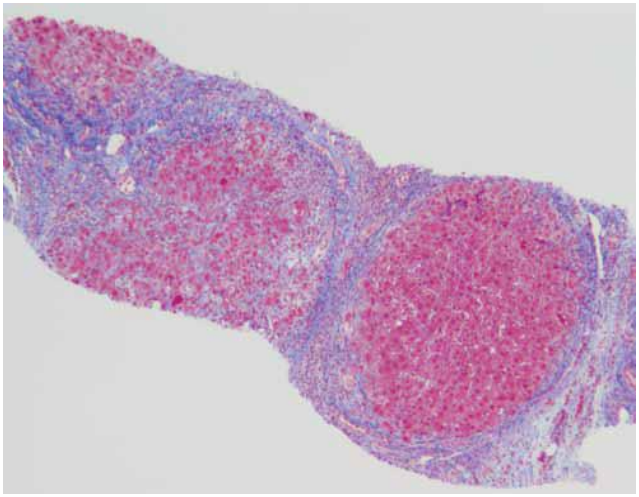
2-PTA is unstable at a pH greater than about 2.

Note:-

- Masson trichrome is Special histochemical stains are routinely used for the interpretation of liver biopsies
  - The panel of special stains varies from laboratoryto laboratory, depending on tumor versusnon-tumor cases, or transplant versusnon-transplant liver biopsy specimens.
- trichrome stain is used to assess fibrosis, which gives important information about stage and progression of disease.
- The stain is used to make treatment decisions; utilized to assess the effect of therapy, medications in clinical trials and is needed for all liver biopsy specimens.



A-Trichrome stained liver showing fibrous tissue. The fibrous tissue is stained blue while the cytoplasm of hepatocytes arestained red.
The nuclei can be seen as dark red to black structures within cells; Collagen is the fibrous tissue are stained Blue (withaniline blue) or very light green (by anilinelight green).

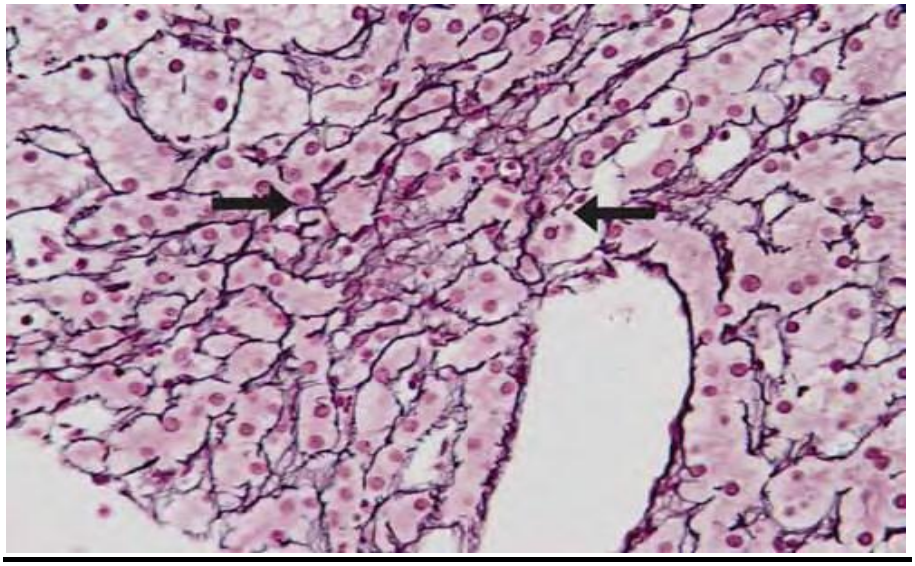


B-Trichrome stain showing cirrhotic liver.
As it is evident, the normal architecture of the liver is destroyed in this disease and the liver shows nodules surrounded by fibrous bands.

Practical NO.24

Silver impregnation method

Gordon an d sweet technique



Reticulin stain shows an area of collapse of reticulin fibers (between arrows)

Aim:

For demonstration of reticular fibers

Solutions:

Silver solution

- 10% $AgNo_3$
- 3% NaOH
- Concentrated Ammonia

Working solution:

- To 5ml of 10% Ag No_3, add ammonia drop by drop until the precipitate first formed is dissolve, then add 5 ml of 3% Na OH, and redissolves by using ammonia drop by drop till the solution retain fine opalescence, if not, you can add a drop of 10% Ag No_3.

- Make up the volume 50 ml with D.W; means complete the volume to 50 ml by adding distilled water.

1% potassium permanganate (oxidizing agent)

1% oxalic acid (bleaching agent)

2.5% iron alum (sensitized agent)

10% formalin (reducing agent)

5% sodium thiosulphate (removes the unreduced silver)

Principle:

Reticular fiber reduces ammonical silver solution to metallic silver with help of reducing agent (argyrophilic).

Method:

1. Bring section to water.
2. Oxidize with 1% potassium permanganate for 5 min.
3. Wash well in D.W.
4. Bleach with 1% oxalic acid until the brown color become colorless.
5. Wash well in D.W.
6. Treat with 2.5% iron alum for 15 min.
7. Wash well in D.W.
8. Treat with silver solution for 30 seconds to 1 min.
9. Was well with D.W.
10. Reduce with 10% formalin for 2 min.
11. Wash well.
12. Treat with 5% sodium thiosulphate for 5 min.
13. Wash well.
14. Counter stain as desire, either 1% neutral red, Safranin or Eosin.

15.Dehydrate, Clear and Mount.

Results:

Reticular fiber: Black.

Other: Red.

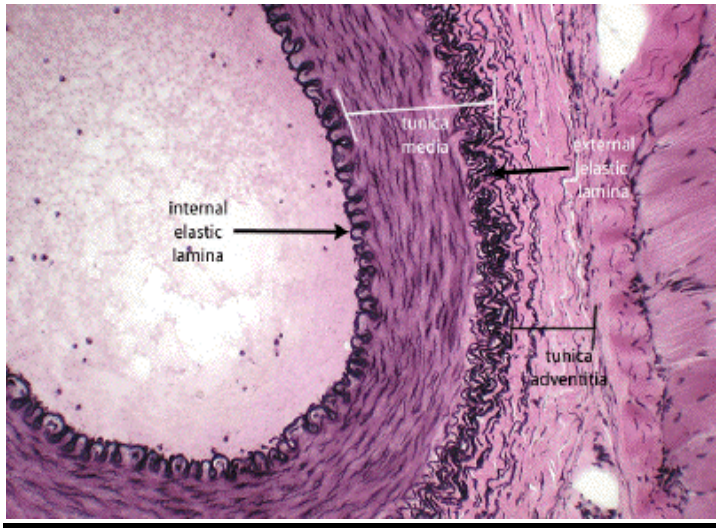
Notes:

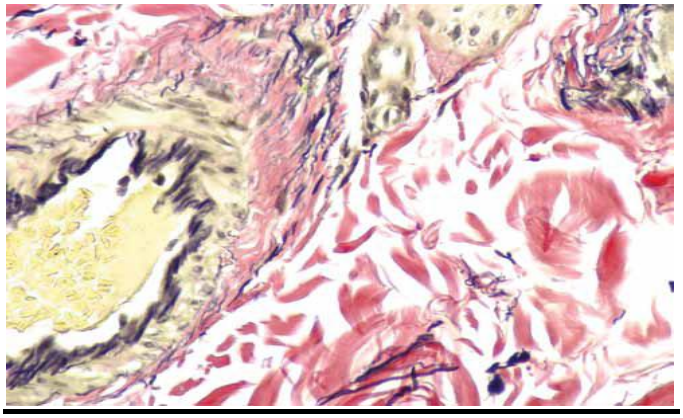
1. A short treatment with iron alum of less than 5 min. gives less staining of nuclei.
2. The use of Coplinjars for the silver solution greatly reduces the possibility of precipitation on the slide.
3. Silver solution, mainly Alkaline frequently causing sections to become detach from the slide, adhesive media must be used.

Practical NO.25

Verhoeff's technique

For Elastic fiber





Aim:

For demonstration of Elastic Fibers

Preparation:

Solution "A": 5% Alcoholic Hematoxylin.

Solution "B": 10% Ferric chloride.

Solution "C": Lugol's Iodine.

Working solution

Solution "A": 20 ml

Solution “B”: 8ml

Solution “C”: 8ml

Add in the above order, mixing between additions.

Counter stain:

Van Gieson solution:

- Saturated aqueous picric Acid.
- 1% acid Fuchsin. 1 gm in 100 ml D.W

Take 10 ml of 1% acid fuchsin and add to 90 ml Picric acid.

Or: 1% Eosin.

Principle:

Elastic Fibers are highly linked with disulphide Bridges, iodine oxidizes disulphide bonds to anionic sulphonic acid derivatives, and these derivatives react with mordanted hematoxylin to form black color.

Method:

1. Bring section to water.
2. Stain section with Verhoeff’s solution for (15-20) min.
3. Wash with water.
4. Differentiate with 2% $Fecl_3$, until Elastic fiber appears black color.
5. Wash in D.W.
6. Rinse in 95% Alcohol to remove iodine deposit from back ground.
7. Counter stain with eosin for (2-3) min.
8. Dehydrate, Clear and Mount.

Result:

Elastic Fiber: black.

Collagen fibers: Red.

Muscles, cytoplasm, fibrin, and RBCs: yellow

Notes:

1. Prepared working solution has a usable life of only (2-3) hours, satisfactory results have been obtained using solution up to 48 hours old.
2. The differentiation step is critical to the success of this method, so control this step by microscope and washing to stop the reaction of differentiation solution, so will give good results and to prevent loss of fiber by over differentiation.
3. Removal of mercury pigment is unnecessary, this being carried out by the iodine in the staining solution.

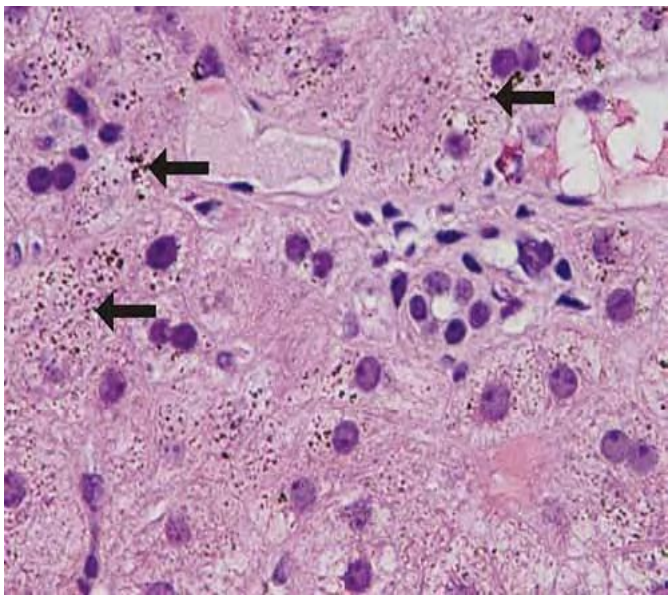
Practical NO.26

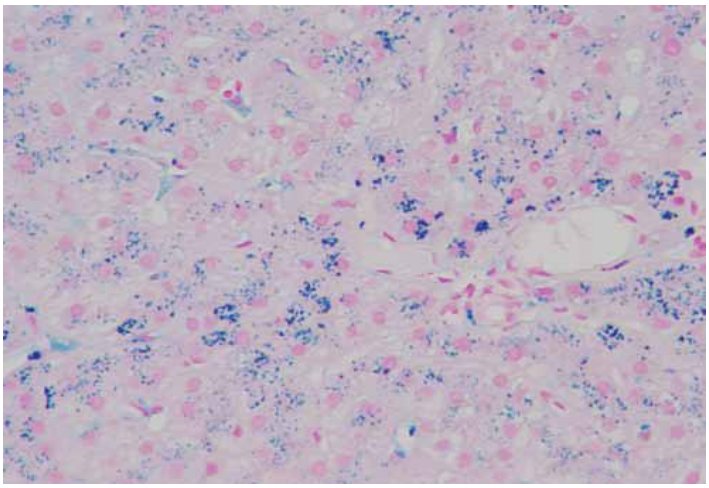
Demonstration of pigment

Heamatogenous endogenous pigment

Perl's Prussian blue Method

Hemosiderin appears on an H&E stain as coarse, dark-brown, refractile granules.





Perl's iron stain shows accumulation of dark blue granules of hemosiderin withinhepatocytes. This pattern of iron deposition occurs in genetic

hemochromatosis. The coarse blue granules are hemosidern, and the bluish blush is Ferritin.

Aim:

For the demonstration of haemosiderin and Inorganic Iron

Fixation
Avoid the use of acid fixatives. Chromates will also interfere with the preservation of iron.
Sections
Works well on all types of section, including resin.
Solutions:

Perls solution

Ferrocyanide solution
1% aqueous potassium ferrocyanide 20 ml
2% aqueous hydrochloric acid 20 ml
Preferably freshly prepared just before use.
Stock solution:

A: 2% K. Ferrocyanide.

B: 2% Hcl.

Working solution:

Mix equal parts of solution A and B just immediately before use.

Eosin: as a counter stain.

Principle:

- **Perls' Prussian blue reaction for ferric iron *(Perls 1867)*This method is considered to be the first classical histochemical reaction.**

Treatment with an acid ferrocyanide solution will result in the unmasking of ferric iron in the form of the hydroxide, Fe(OH)3, bydilute hydrochloric acid. The ferric iron then reacts with a dilute potassium ferrocyanide solution to produce an insoluble blue compound, ferric ferrocyanide (Prussian blue).

2% Hcl removes the masking of ferric iron to form ferric hydroxide, and then ferric hydroxide will react with K. Ferrocyanide to form ferric-ferrocyanide (Prussian blue).

Method

1. Take a test and control section to water.
2. Treat sections with the freshly prepared acid ferrocyanide solution for 10–30 minutes (see Note a below).
3. Wash well in distilled water.
4. Lightly stain the nuclei with 0.5% aqueous neutral red or 0.1% nuclear fast red.
5. Wash rapidly in distilled water.
6. Dehydrate, clear, and mount in synthetic resin.

Results:

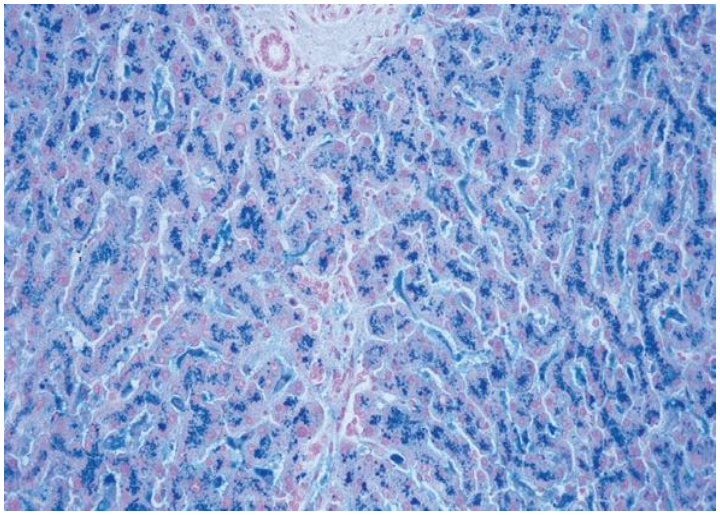
Haemosiderin and Ferric salts: Blue color.

Other pigment: Their natural color.

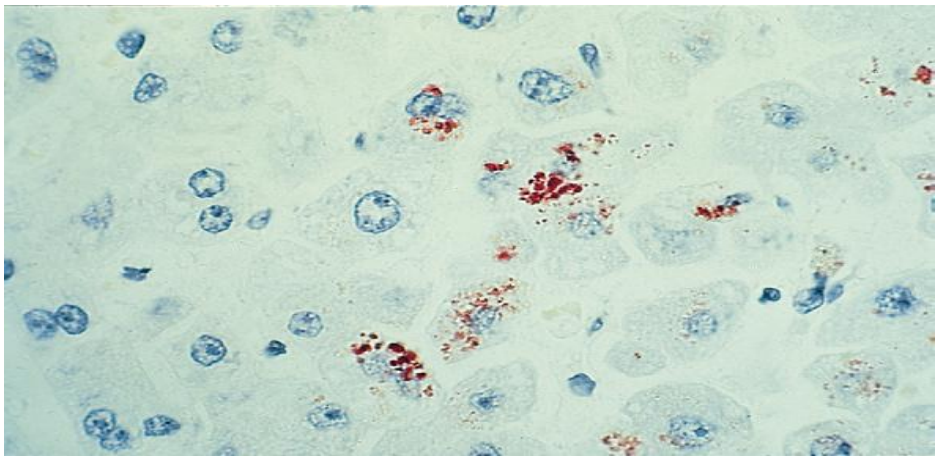
Other tissue structures: pink to Red color.

Notes:

1. It is better to keep the two stock solutions made up separately and stored in the refrigerator, but must be stored for prolonged periods, this will ensure that the solutions retain their viability.
2. A positive control is used with all test sections.
3. Avoid the use of acid fixatives; chromates will also interfere with the presentation of Iron.
4. Necessary to vary the staining time



A section of liver from a patient with hemochromatosis stained for ferric iron with Perls' method. Ferric iron is stained blue.

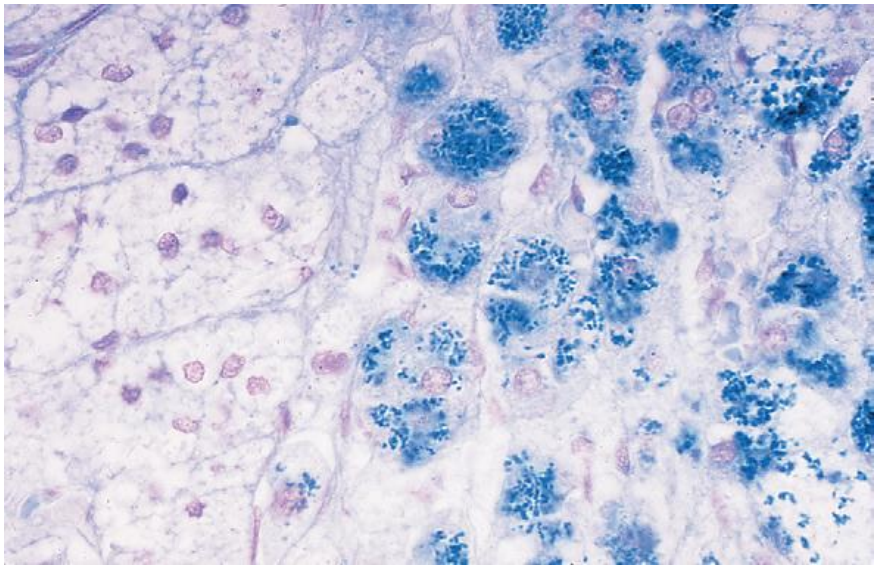


A section of fetal liver of the third trimester stained with Lindquist's method for copper. Copper is stained red to orange-red.

Practical NO.26

Schmorl's Reaction

For melanin endogenous non hematogenous pigment



A section of adrenal stained with Schmorl's method for reducing substances. Chromaffin is stained blue.

Aim:

For demonstration of Melanin pigment

Solutions:

Stock solution:

A: 0.4% aqueous K.Ferricyanide

B: 1% aqueous Ferric Chloride or Ferric sulphate

Working solution:

Add 4ml of K.Ferricyanide to 30ml of Fe Cl_3, and mix gently.

Principle:

Melanin reduces potassium ferricyanide to ferrocyanide, and then ferrocyanide will react with ferric chloride to form ferric ferrocyanide dark/ blue in color (Prussian blue).

Method:

1. Bring section to D.W
2. Treat with working solution from 5-10min.
3. Wash in R.T.W for 5 min, so as to remove any ferricyanide remains.
4. Lightly counter stain with 1% eosin (or o.5 % neutral red or Safranine) for 1-2 min.
5. Dehydrate, Clear and Mount.

Results:

Melanin: Dark/Blue color.

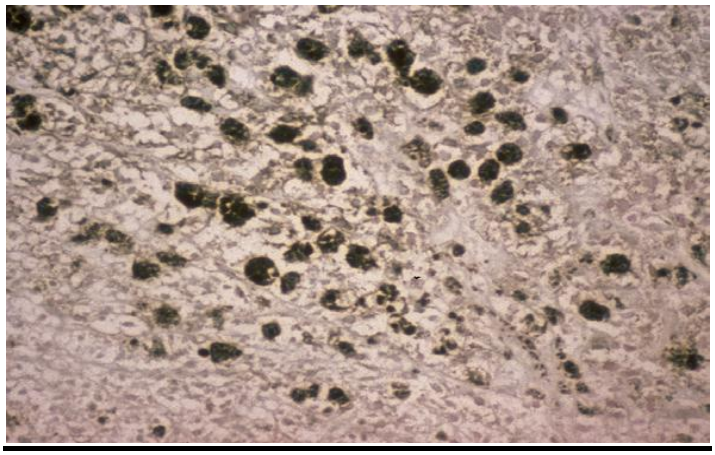
Nuclei: Red color.

Notes:

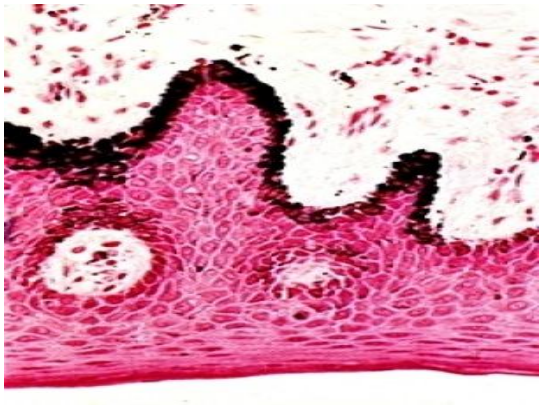
1. The time for the reaction to take place depends on the substance to be demonstrated, with melanin generally reacting more quickly than lipofuscins.
2. When choosing a control section, remember that melanin reduces the ferric-ferricyanide more quickly than other reducing substances, so control which could match the test should be used.
3. This modification is preferred to the more traditional method because it is easier to control and gives less background staining.

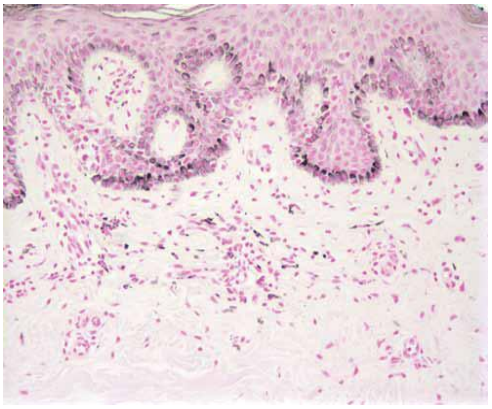
Practical NO.27

Masson Fontana for melanin endogenous non hematogenous pigment



A section of liver from a patient with malignant melanoma stained with Lillie's ferrous ion uptake method for melanin. Melanin is stained black.





Fontana-Masson stain highlighting melanin in epidermal keratinocytes,melanocytes, and dermal melanophages

Aim:

- for demonstration of melanin pigment
- Occasionally, Fontana-Masson is used in the evaluation ofvitiligo and post-inflammatory hyperpigmentation.
- Some observers report that the Fontana-Masson stain is difficult to interpret when only rare granular staining is present

Solutions:

Silver solution

Stock solution:

10% Ag No_3

Ammonia

5% Na. thiosulphate

Working solution:

1. To 20 ml of 10% silver nitrate, add ammonia drop by drop until a fine precipitate remains.
2. Add 20 ml of D.W to the above solution, and keep in a dark bottle in cold place.

Principle:

Melanin directly reduces silver to metallic black silver without help of reducing agent (Argentaffin).

Method:

1. Bring section to water.
2. Treat section with working solution for (3-4 min) at 56^0 c in water bath or oven.
3. Wash well in several changes of D.W.
4. Treat with fix with 5% sodium thiosulphate for 1min.
5. Wash in R.T.W for 3 min.
6. Lightly counter stain with neutral red, Safranin, or eosin for 1min.
7. Wash, Dehydrate, Clear and Mount.

Result:

Melanin: Black color.

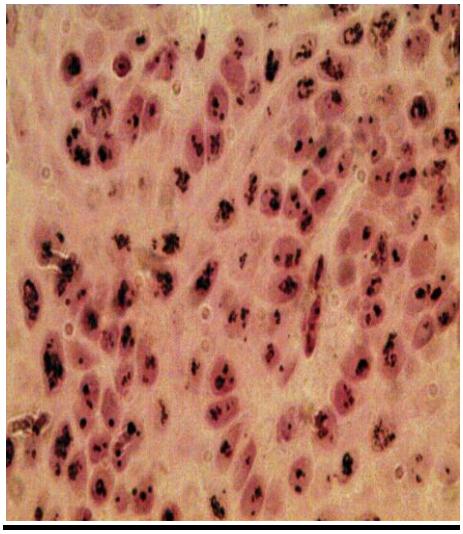
Nucleus: Red color.

Notes:

1. Prolonged exposure to 56^0c, may give rise to a fine deposit over the section.
2. Friable material may need to be coated with celloidin as the ammonia in the silver solution could lead to sections lifting off the slide.
3. Adhesive media uses in preparation of section to avoid detach of tissue from slide.
4. Only use clean glassware, as the silver solution will react with any residual contaminant left on glassware.

Practical NO .28

Silver nitrate method NOR technique



Aim:

For AgNOR protein sites

Solutions:

50% silver nitrate solution:

Silver nitrate……..….. 50 g

Distilled water……..100 ml

Gelatin solution:

Gelatin……2 g

Formic acid……1 ml

Distilled water……100 ml

Working solution:

Silver nitrate solution…….2 parts by volume

Gelatin solution……...1 part by volume

Mix immediately before use.

Principle:

AgNOR protein sites directly reduce silver to metallic silver without help of reducing agent (Argentaffin).

Method:

1. Take section to water.
2. Rinse in D.W.
3. Incubate in freshly prepared working solution for 45 min at room temperature.
4. Wash in D.W for 1 min.
5. Dehydrate, clear, and mount.

Results:

AgNOR sites: Intranuclear black dots

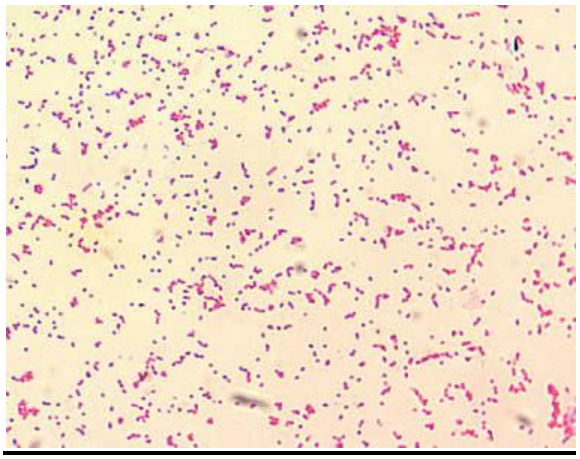
Background: pale yellow

Notes:

1. Section may be lightly counter stain with neutral red or car malum.
2. Section may be toned using 1% gold chloride.
3. The working solution must be used immediately.

Practical NO.29

Gram- Twort Stain



Aim:

To demonstrate bacteria

Solution:

1- Crystal violet solution:

- 0.5% crystal violet in 25% ethanol

2- Gram's iodine:

- Iodine: 1 g

- Potassium iodide: 2 g

- D.W: 10 ml

(Shake together D.W and potassium iodide until dissolved, add iodine. Make up to 300 ml with D.W)

3- Twort stain:

- 1% neutral red in ethanol: 9 ml.

- 0.2% fast green in ethanol: 1 ml

- D.W: 30 ml.

(Mix immediately before use)

4- 2% acetic alcohol:

(Preheated to 56c)

Method:

1- Take section to water.

2- Stain in crystal violet solution, 3 min.

3- Rinse in gently running tap water.

4- Treat with gram iodine, 3 min.

5- Rinse in tap water, blot dry, and complete drying in a warm place.

6- Differentiate in preheated 2% acetic alcohol until no more color washes out.

7- Rinse briefly in D.W.

8- Stain in Twort, 5 min.

9- Wash in D.W.

10- Rinse in 2% acetic alcohol until no more red color runs out of the section.

11- Dehydrate in absolute alcohol, clear and mount.

Result:

- Gram positive organisms: blue-black.

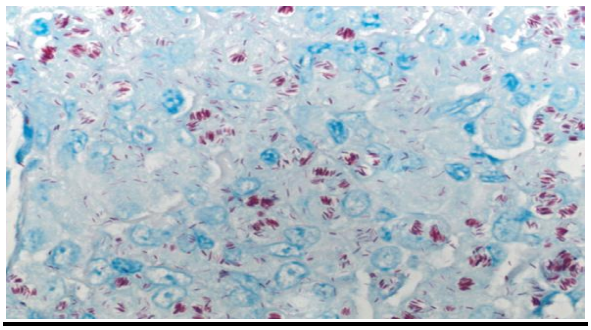
-Gram negative organisms: pink-red.

- RBCs, most cytoplasmic structures and elastic fibers: black.

The modified Fite's procedure is necessary to demonstrate *Mycobacterium leprae* due to the organism's fragile,fatty capsule.

Practical NO.29

Cold Ziehl-Neelsen (ZN) stains for leprosy



Aim:

To demonstrate mycobacterium bacillus (Leprosy)

Principle:

Phenolic acid and frequently heat, are used to reduce surface tension and increase porosity, thus forcing dyes (basic fuchsin) to penetrate the mycobacterium capsule (mycolic acid= long chain fatty acid= acid fast).

Solutions:

1- Carbol-fuchsin:

Basic fuchsin…… 4g

95% alcohol…….. 20ml

Phenol crystal…… 5g

D.W…… 100ml

2- 0.2% methylene blue:

Methylene blue…… 0.2 g

D.W……..100ml

3- 1% acid alcohol:

70% alcohol…….…… 99 ml

Conc Hcl…….. 1 ml

Method:

1. Take section to water.
2. Flood section with freshly filtered Carbol-fuchsin, 20 min at R.T
3. Wash well in tap water.
4. Differentiate in 1% acid alcohol, control microscopically
5. Wash well in tap water, 5-10 min.
6. Counter stain in methylene blue solution, 30 seconds
7. Blot, dehydrate, clear and mount

Result:

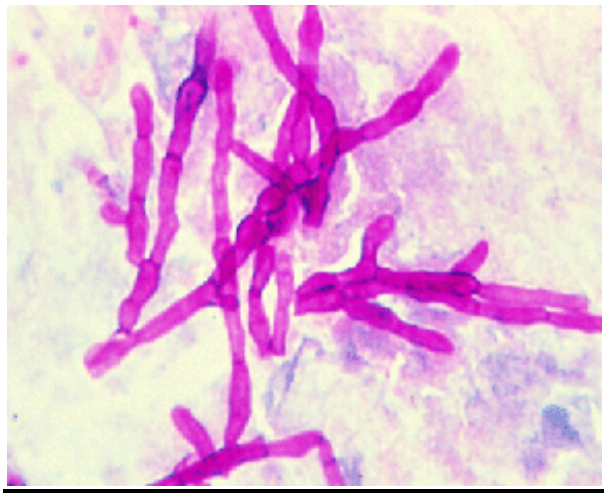
Mycobacterium: Red.

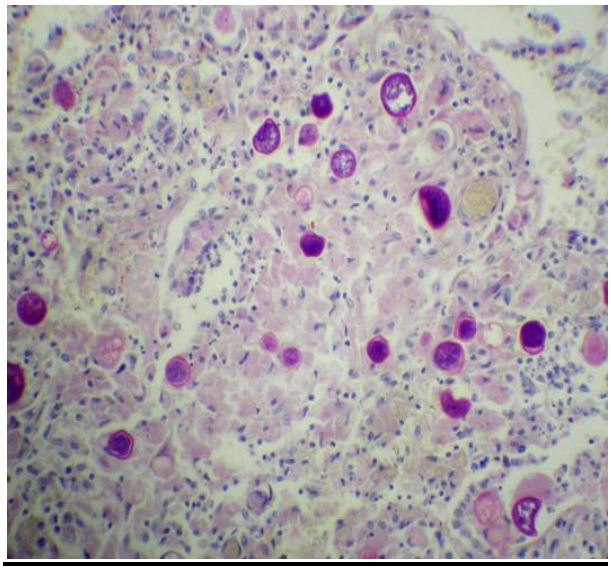
Back ground: Pale blue.

Some fungi: Red.

Practical NO.30

Periodic acid Schiff's for fungi





Aim:

To demonstrate fungi

Principle:

The mucopolysaccharide components of the fungal cell wall are oxidized

To release dialdehydes then these aldehydes is recolor the Schiff's reagent purple or magenta.

Solution:

1-1% periodic acid

2-Schiff's reagent.

3-light green.

Method:

1- Bring section to water.
2- Oxidize in 1% P.A for 5 min.
3- Wash well in different changes of D.W.
4- Place in Schiff's reagent for 15 min.
5- Wash in R.T W for (5-10) min.
6- Counter stain in light green 1 min.
7- Wash in water, rinse in absolute alcohol.
8- Clear and Mount.

Results:

Fungi: Red/ Magenta

Background: Green

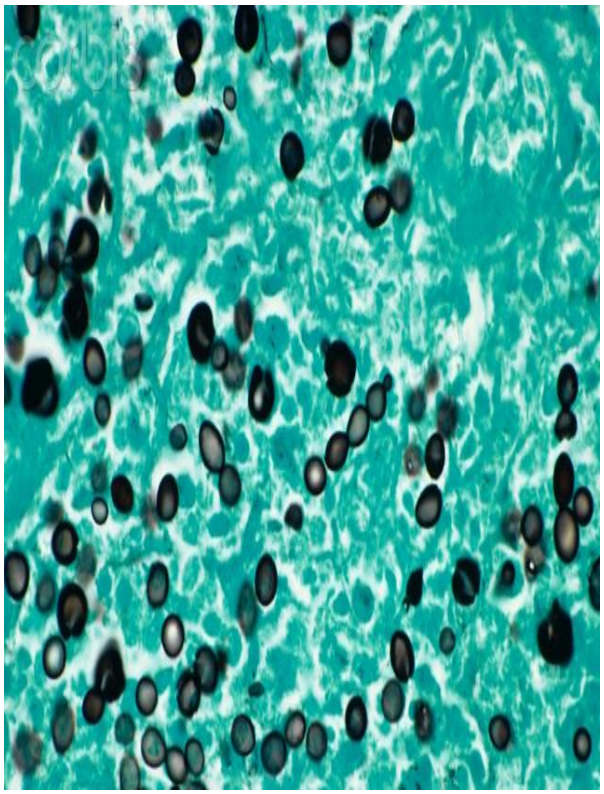
Mucins and glycogen: Magenta.must use control.

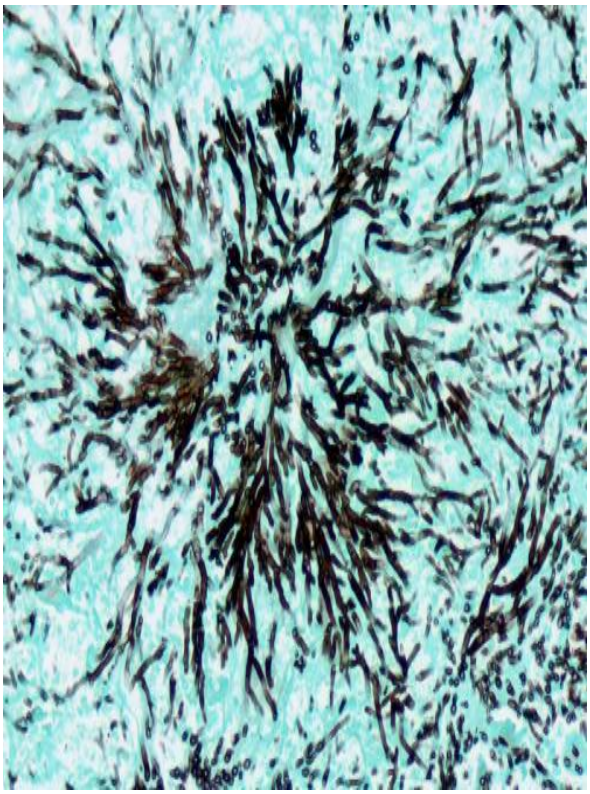
Note:

1. Periodic acid is the oxidant of choice, so there is other oxidants they over oxidizes the form Aldehyde to carboxylic acid which result in a weak or negative Schiff's reaction.

Practical NO.31

Grocott's methenamine (hexamine) silver





Grocott's silver technique: spores and hyphi of fungi black, background green

Aim:

To demonstrate fungi

Solution:

1- Silver incubating solution:

a- 5% sodium tetra borate in D.W.(Insure alkaline pH).

b- Methenamine silver:

- 5% silver nitrate in D.W……5 ml.

- 3% methenamine in D.W……..100 ml.

(Add silver nitrate to methenamine silver, gently shaking until formed precipitate dissolves. Mixture will keep for 1-2 months at 4c.

**Incubating solution:

- Solution a (borax)……5 ml.

- D.W……..25 ml.

- Solution b (methenamine silver)…….25 ml.

(The methenamine silver / water solution and the borax should be preheated to 56c and mixed prior to use, as the silver solution starts to degenerate once borax is added).

2- Arzac's counterstain:

Orange G…….0.25 g

Light green……..1 g

Phosphotungstic acid……...0.1 g

50% alcohol…….100 ml

Glacial acetic acid……....1 ml

(Keep solution well).

3- 5% aqueous chromic acid:(oxidizing agent).

4- 1% sodium metabisulfate:(removes excess chromic acid).

5- 3% sodium thiosulfate :(removes any un reactive silver).

Principle:

Chromic acid oxidizes the carbohydrate contents of the fungal cell wall resulting in the formation of aldehydes then these aldehydes reduce the silver solution into black metallic silver.

Method:

1- Take section to D.W.

2- Oxidize in 5% chromic acid, 1 hr.

3- Wash in tap water.

4- Rinse in 1% sodium metabisulfate.

5- Wash in tap water, 5 min.

6- Rinse in D.W.

7- Place in preheated to 56c silver incubating solution in a dark place, up to 1 hr

8- Rinse well in D.W.

9- Place in 3% sodium thiosulfate, 5 min.

10- Counter stain in Arzac's or 1% light green in 0.1% acetic acid,15-30 sec.

11- Blot, dehydrate, clear and mount.

Results:

Fungi, pneumocystis, melanin: Black.

Mucins and glycogen: Gray/Black.

RBCs: Yellow.

Back ground: Pale green

Notes:

1- Incubation time is variable and depends on the type and duration of fixation and the organism being demonstrated.

2- Impregnation controlled microscopically until fungi are dark brown. Background is colorless at this point.

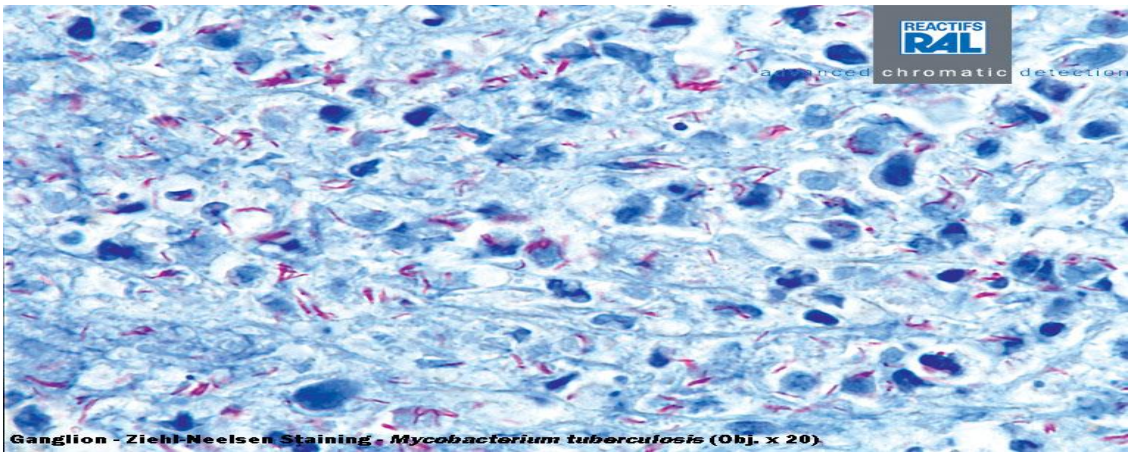
3- Over incubation produce intense staining of elastin, glycogen and fungi that may obscure final internal detail of the hyphal septa.

4- Light H&E counterstain recommended providing more details.

Practical N32

Ziehl-Neelsen (ZN) stains

for T.B



ZN stain: Tubercle bacilli: red, Cytoplasm: pale blue, Nuclei: blue, RBCs: pale pink.

Aim:

To demonstrate mycobacterium bacillus (TB)

Principle:

Phenolic acid and frequently heat, are used to reduce surface tension and increase porosity, thus forcing dyes (basic fuchsin) to penetrate the mycobacterium capsule (mycolic acid= long chain fatty acid= acid fast).

Solutions:

1- Carbol-fuchsin:

Basic fuchsin…….1g

Absolute alcohol…….. 10ml

5% aqueous phenol ……..… 100ml

(Mix well and filter before use)

2- Acidified methylene blue:

Methylene blue……. 0.25 g

1% acetic acid……..100ml

3- 1% acid alcohol:

70% alcohol…….. 99 ml

Conc Hcl…….. 1 ml

Method:

1- Take section to water.

2- Flood section with freshly filtered Carbol-fuchsin.

3- Heat to steaming with flaming for 15 minutes, or stain in Coplin jar at 60c, 30 minutes.

4- Wash well in tap water.

5- Differentiate in 1% acid alcohol, 3 minutes.

6- Wash well in tap water.

7- Counter stain in methylene blue solution, 10 seconds.

8- Blot and differentiate and rehydrate until the back ground is a pale blue.

9- Dehydrate, clear and mount.

Results:

Mycobacterium: Red.

Back ground: Pale blue.

Some fungi: Red.

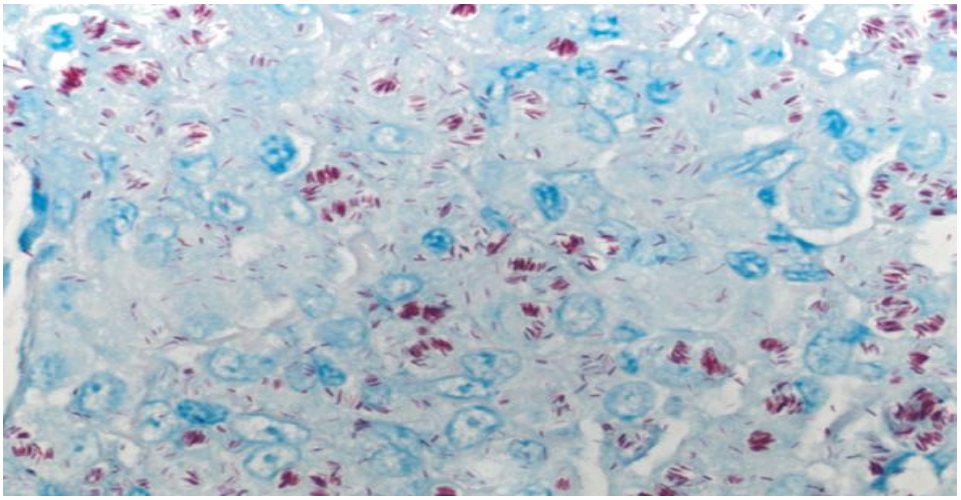
Notes:

- Avoid over- counter stain as scant organisms can easily be obscured.

2- Victoria blue can be substituted for Carbol fuchsin and picric acid for the counter stain.

1- Decalcification using strong acids destroys acid fastness; formic acid is recommended.

Practical N33
Modified Fite method
for *M. leprae* and *Nocardia*



The modified Fite's procedure is necessary to demonstrate *Mycobacterium leprae* due to the organism's fragile,fatty capsule.

Fixation
10% neutral buffered formalin (NBF).
Sections
Paraffin sections at 4–5 μm.
Solutions
Carbol fuchsin solution commercially available, or
0.5 g basic fuchsin dissolved in 5 ml of absolute alcohol; add 100 ml of 5% aqueous phenol. Mix well and filter before use. Filter before each use with #1 filter paper.
5% sulfuric acid in 25% alcohol
25% ethanol 95 ml
Sulfuric acid, concentrated 5 ml
Methylene blue (stock) commercially available, or
Methylene blue 1.4 g
95% alcohol 100 ml
Methylene blue, working
Stock methylene blue 5 ml
Tap water 45 ml
Xylene-peanut oil 1 part oil: 2 parts xylene

Method

1. Deparaffinize in two changes of xylene-peanut oil, 6 minutes each.

2. Drain slides vertically on paper towel and wash in warm, running tap water for 3 minutes. (The residual oil preserves the sections and helps accentuate the acid fastness of the bacilli.)

3. Stain in carbol fuchsin at room temperature for 25 minutes. (Solution may be poured back into bottle and reused).

4. Wash in warm, running tap water for 3 minutes.

5. Drain excess water from slides vertically on paper towel.

6. Decolorize with 5% sulfuric acid in 25% alcohol, two changes of 90 seconds each. (Sectionsshould be pale pink.)

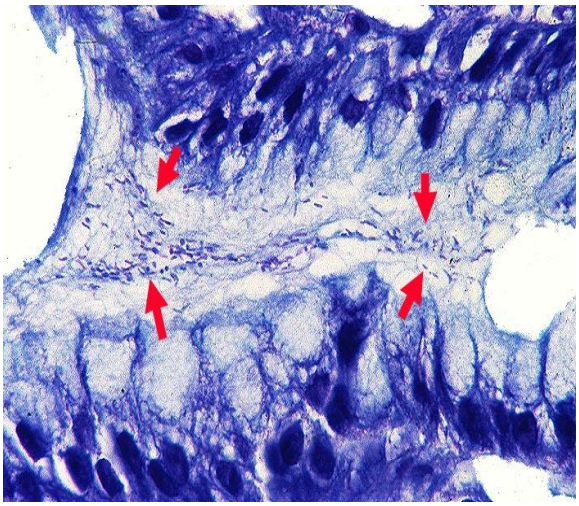
Result:-

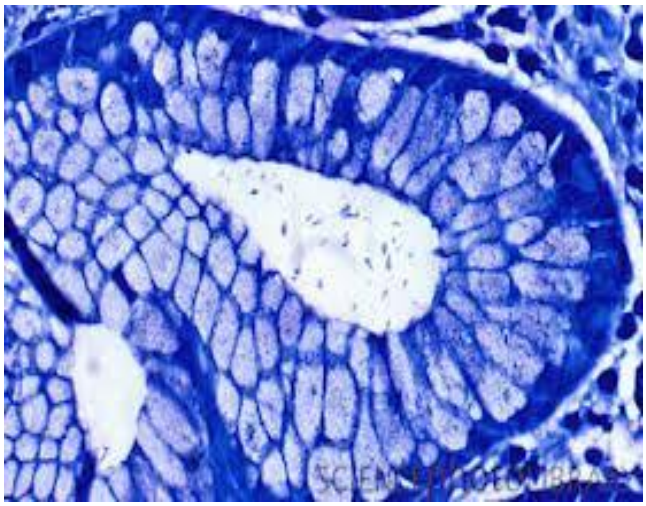
Bacilli red color ,Background blue.

practical N34

Demonstration of helicobacter Hpylori

Giemsa stain





AIM:

To demonstrate Hpylori stain

Principle:-

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Preparation and constituent:-

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Method:-

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Result:-

Microorganism:- blue Hpylori

NOTES:-

Silver Stains (Warthin Starry Stain, Dieterle, Steiner Stains)

Utility of the Stains:

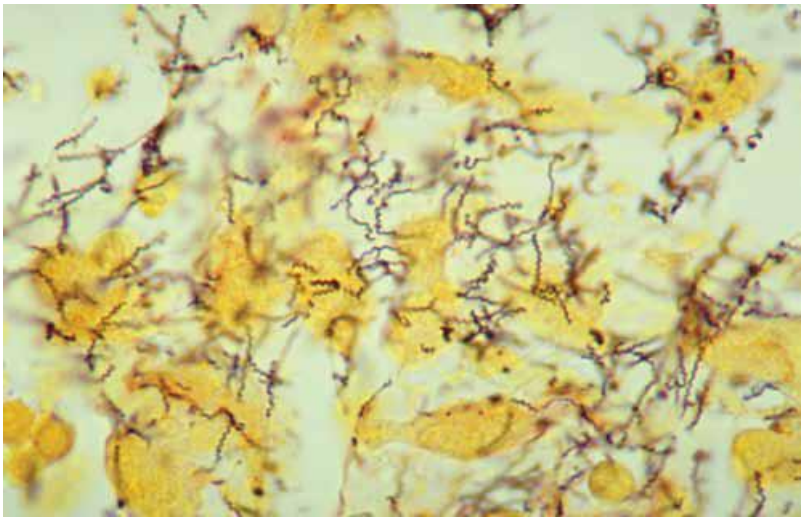
- ❖ Silver stains are very sensitive for the staining of bacteria and therefore most useful for bacteria which do not stain orstain weakly with the Grams and Giemsa stains.
- ❖ Although they can be used to stain almost any bacteria, they are tricky to perform and are therefore reserved for visualizing spirochetes, legionella, bartonella and *H. pylori*.

Principles of Staining: -

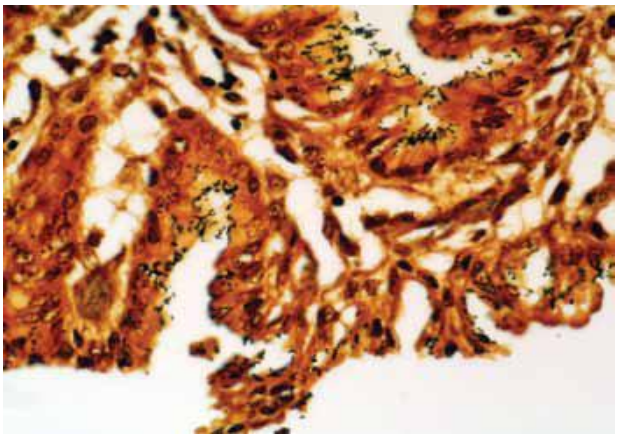
- ❖ Spirochetes and other bacteria can bind silver ions from solution but cannot reduce the bound silver.
- ❖ The slide is first incubated in a silver nitrate solution for half an hour and then "developed" with hydroquinone which reduces the bound silver to a
- ❖ visible metallic form.
- ❖ The bacteria stain dark-brown to black while the background is yellow

Auramine O- Rhodamine B Stain:-

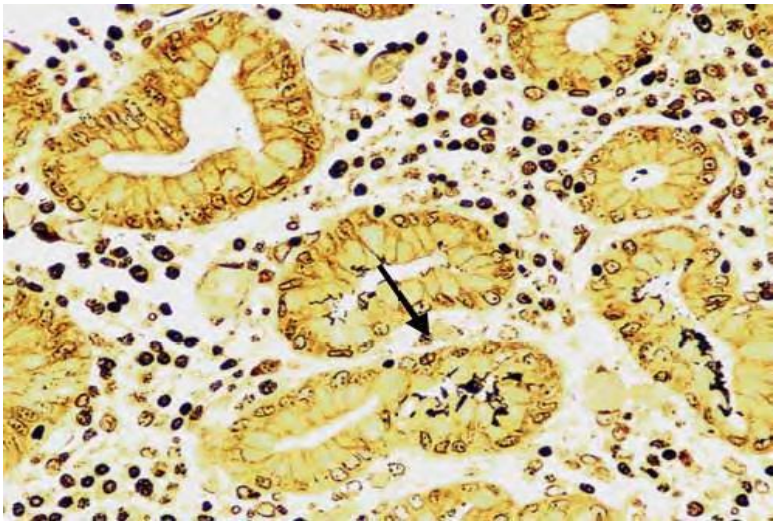
- ❖ The auramine O-rhodamine B stain is highly specific and sensitive for
- ❖ mycobateria. It also stains dead and dying bacteria not stained by the acid-fast stains.
- ❖ The mycobacteria take up the dye and show a reddishyellow fluorescence when examined under a fluorescence microscope.



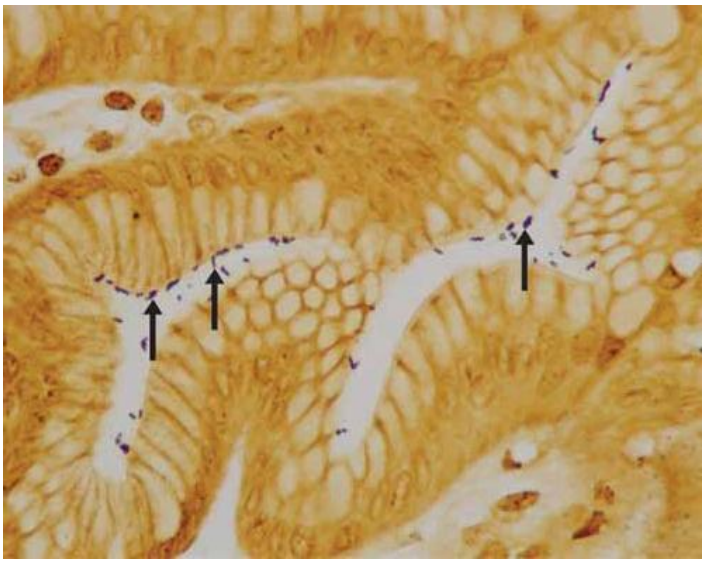
Histopathology of *Treponemapallidum* spirochetes using a modified Steiner Silver Stain. Image credit: Dr. Edwin P. Ewing,Jr., The Centers for Disease Control andPrevention, Atlanta, GA, USA/Wikimedia.



Mucous layer of gastric surface epithelium showing *H. pylori* (black-stained rods). Warthin-Starry silver stain (x40).



Helicobacter stained with Warthin-Starry, Dako Code AR181. The arrow points to some black *H. pylori* organisms in yellow mucus.



Warthin-Starry stain of stomach containing *Helicobacter pylori* which appearas black and slightly curved, rod-likebacteria (arrows). The background is stained