



FACULTY OF
HEALTH SCIENCE

School of
Human Life Sciences

CXA222

HISTOPATHOLOGY

STAINING TECHNIQUES

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TAKING PARAFFIN SECTIONS TO WATER

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

xylene	2 minutes
xylene	2 minutes
absolute alcohol	1 minute
absolute alcohol	2 minutes
70% alcohol	30 seconds
water	

Take the slides through the various solutions as follows:

1. Place slides in slide holder.
2. Lift lid off staining dish, and immerse the slides in the first solution with agitation.
3. Replace lid and allow slides to remain in solution for the specified time with periodic agitation.
4. Remove slides from the solution (with agitation) and tilt slide holder to allow excess solution to drain before transferring it to the next solution.
5. Continue in this manner through the remaining solutions for the specified times

Times can be reduced if slides are agitated constantly.

DEHYDRATION AND CLEARING OF SECTIONS IN XYLENE BEFORE MOUNTING

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Dehydration	70% alcohol	15 seconds
	absolute alcohol	1 minute
	absolute alcohol	2 minutes

Clearing	xylene	15 seconds
	xylene	15 seconds

Take the slides through the various solutions as follows:

1. Place slides in slide holder.
2. Lift lid off staining dish, and immerse the slides in the first solution with agitation.
3. Replace lid and allow slides to remain in solution for the specified time with periodic agitation.
4. Remove slides from the solution (with agitation) and tilt slide holder to allow excess solution to drain before transferring it to the next solution.
5. Continue in this manner through the remaining solutions for the specified times.

Times can be reduced if slides are agitated constantly.

BLOTTING DRY AND CLEARING OF SECTIONS

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Blotting Dry

1. Place slide face down carefully on blotting paper.
2. Fold blotting paper over slide over and apply gentle pressure to dry slide.
3. Lift slide and move to dry section of blotting paper and repeat until section is completely dry.
4. Allow section to air dry if necessary before clearing.

Clearing xylene 15 seconds
 xylene 15 seconds

Clear dried section by dipping the slide in xylene for specified times and mount in DPX.

MOUNTING OF SECTIONS

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

NB If section is dry, dip in xylene before mounting the coverslip

1. Remove slide from slide holder.
2. Carefully dry the BACK of the slide with a tissue.
3. Lay the slide on blotting paper or bench-coat on the fume bench.
4. Place a drop of DPX mounting medium on the section.
5. Carefully place a cover-slip over the DPX ensuring that it covers the section.
6. Remove any bubbles by jiggling the coverslip gently.
7. Place mounted slide on small cardboard slide holder and place in 50°C oven for a few hours.
8. Allow mounted slide to dry lying flat for at least 1 week.

ZIEHL-NEELSEN TECHNIQUE FOR ACID-FAST BACILLI (ZN) (Ziehl, 1882; Neelsen, 1883)
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Safety Note: *Turn on the exhaust system before staining.*
Wear protective clothing, gloves and safety glasses during the staining procedure.

Principle

The histological method is similar to the classical bacteriological technique that depends upon the resistance of certain bacilli to decolorization by acid alcohol after being stained with hot carbol-fuchsin.

Fixation

Most fixatives can be used. Avoid Carnoy which removes lipid from the bacilli which makes them less acid-fast. Formalin, especially when prolonged, is said to reduce acid-fastness, but specimens usually stain perfectly satisfactorily. The treatment of formalin fixed sections with 0.5% ammonium hydroxide before staining may improve the brightness of colour of acid-fast bacilli, but this is seldom necessary and may detach the sections from the slide.

Sections

Thin (3-5 µm) sections.

Staining Technique

1. Take sections to water.
2. Either

Slide Method

Place a rectangle of filter paper over the section (to prevent precipitation of the stain) and flood the slide with carbol-fuchsin.

Warm the slide until the stain begins to steam; this can be conveniently done by the flame from a throat swab soaked in alcohol.

Leave for 5 minutes.

Or

Coplin Jar Method

Place the slide in a warmed vessel of carbol-fuchsin for *at least* 1 hour in a 37°C or *at least* 30 minutes at 60°C.

3. Rinse in tap water.
4. Decolourise with acid alcohol until the section is pale pink and no more colour comes away (about 1-3 minutes). A further decolourisation is recommended in neutral 70% alcohol for the exclusion of non-pathogenic acid-fast bacilli (see note 2).
5. Rinse in water.
6. Counterstain in acidified methylene blue for 1 minute.
7. Rinse in water.
8. Blot dry.
9. Clear in xylene and mount in synthetic resin eg DPX.

Results

Acid-fast bacilli: red

Other bacteria: blue

Cells and their nuclei: blue

Red blood cells should retain a slight red colour.

Notes

- 1 Since this method involves the use of both acid and alcohol decolourisation, the risk of mistaking the non-pathogenic acid-fast bacilli for the tubercle bacillus is decreased. In sections the tubercle bacilli will be found in the abnormal areas (tubercles) and this risk is slight. The non-pathogenic acid-fast

bacilli that are found in butter, milk, or cerumen are seldom acid-fast, but the smegma bacillus may require prolonged alcohol decolourisation.

- 2 Counterstaining should be light, especially in sections containing nuclear material, such as lymphoid tissue. Heavy counterstaining makes the identification of acid-fast bacilli difficult and may colour them purple.
- 3 Basic fuchsin specified for Schiff's reagent may not give satisfactory results.
- 4 Control sections of known positive tuberculous material with abundant acid-fast bacilli are valuable in that they indicate that the staining technique is satisfactory when several negative sections are being examined. They also give an idea of the colour of the acid-fast bacilli; this may vary from a light to a dark red.

The leprosy bacillus is more easily decolourised than the tubercle bacillus, and differentiation must be carefully controlled. A faint residual red colour in the tissues is especially important, and sections that do not show this may be unreliable for the exclusion of leprosy. The Fite-Faraco modification (Faraco, 1938; Fite, Cambre and Turner, 1947) for leprosy bacilli is similar to the standard Ziehl-Neelsen technique, but the paraffin wax is removed from the sections with two changes of one part of groundnut oil, cottonseed oil or olive oil and two parts xylene for 10 minutes each. Wade (1952) used two parts of rectified turpentine and one part of liquid petrolatum for the same purpose. After either of these variants the sections are drained, blotted until opaque and placed directly in water; the residual oil in the sections helps prevent shrinkage.

Reagents

- *Carbol Fuchsin*

Basic Fuchsin	1 g
Absolute Ethanol	10 mL
5% phenol in distilled water	100 mL

Dissolve the basic fuchsin in the alcohol, then mix with the phenol solution. Filter.

- *Acid Alcohol*

1% hydrochloric acid in 70% alcohol.

- *Acidified Methylene Blue Counterstain*

Methylene Blue	1 g
Glacial Acetic Acid	1 mL
Absolute Ethanol	20 mL
Distilled Water	80 mL

THE GRAM-TWORT MODIFICATION FOR BACTERIA IN PARAFFIN SECTIONS

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Principle

The following modification of the Twort (1924) method for bacteria has the advantages of easier differentiation and a better colour contrast compared with the other Gram techniques (Ollett, 1947, 1951). The sections are easy to examine for long periods without eye strain.

Fixation

Formalin; other fixative can be used.

Sections

Thin (3-5 μm) paraffin sections.

Reagents

- *Modified Twort's Stain - Stock Solution* (prepare fresh)
 - 0.2% absolute alcoholic Neutral Red 90 mL
 - 0.2% absolute alcoholic Fast Green FCF 10 mL
- *Modified Twort's Stain - Working Solution* (prepare fresh)
 - Dilute 1 volume of the stock solution with 3 volumes of distilled water.

Staining Technique

- 1 Stain in 1% methyl violet or aniline crystal violet for 3-4 minutes.
- 2 Wash quickly in distilled water.
- 3 Treat with Gram's iodine for 3 minutes.
- 4 Wash quickly in distilled water and blot dry.
- 5 Decolourise with 2% acetic acid in absolute alcohol until no more colour comes away (check after a couple of minutes). The section should be a dirty straw colour at this stage.
- 6 Wash quickly in distilled water.
- 7 Counterstain in modified Twort's stain for 5 minutes.
- 8 Wash quickly in distilled water.
- 9 Decolourise quickly and carefully in 2% acetic acid in absolute alcohol until no more red colour comes away (a few seconds).
- 10 Clear in xylene and mount in a synthetic resin medium eg DPX.

Results	Gram negative bacteria:	pink
	Nuclei:	red
	Cytoplasm:	light green
	Red blood cells:	green

If sections are exposed too long to alcohol, the Gram-positive bacteria will also decolourise. Most bacteria, especially in large numbers stain a pale grey colour with haematoxylin. With all stains for microbes, it is essential that known positive control slides are stained along with the section.

PERIODIC ACID SCHIFF TECHNIQUE (PAS)

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Principle

Sections are oxidised by the periodic acid resulting in the formation of aldehyde groups. These then react with Schiff's reagent (a leucofuchsin) to restore the quinoid chromophoric grouping, giving a magenta coloured final product to the PAS positive substances.

Reagents

- *0.5% Aqueous Periodic Acid*
- *Schiff's Reagent*

Staining Technique

- 1 Take sections to water.
- 2 Remove mercuric deposit (if present) with iodine thiosulphate.
- 3 Oxidize with Periodic Acid for 5 mins
- 4 Wash well in distilled water
- 5 Stain with Schiff's Reagent for 10-15 mins
- 6 Wash well in running tap water for 5 mins
- 7 Stain nuclei with Haematoxylin for 1 min
- 8 Wash in running tap water for 2 mins
- 9 Differentiate briefly with acid alcohol
- 10 Wash briefly in running tap water.
- 11 Wash and blue nuclei in ammonia water
- 12 Wash briefly in running tap water.
- 13 Dehydrate in alcohol, clear in xylene and mount in DPX.

Results

Simple polysaccharides, neutral mucosubstances, some macro mucosubstances and basement membranes are PAS positive (Magenta in colour)

PERIODIC ACID SCHIFF/ALCIAN BLUE TECHNIQUE (PAS/AB)

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Principle

Sections are oxidised by the periodic acid resulting in the formation of aldehyde groups. These then react with Schiff's reagent (a leucofuchsin) to restore the quinoid chromophoric grouping, giving a magenta coloured final product to the PAS positive substances.

By first treating the section with Alcian Blue the acid mucins will stain and therefore will not react when the section is subsequently stained with the PAS method. The PAS will stain neutral mucins and carbohydrates, red.

Reagents

- *1% Alcian Blue Solution (pH2.5)*

Alcian Blue	1 g
Water distilled	97 mL
Acetic acid (glacial)	3 mL

Check that the ph is 2.5
- *0.5% Aqueous Periodic Acid*
- *Schiff's Reagent*

Staining Technique

1. Take sections to water.
2. Stain in 1% Alcian Blue solution for 10-15 minutes.
3. Wash in running water for 2 minutes.
4. Rinse in distilled water.
5. Oxidize with Periodic Acid for 5 mins
6. Wash well in distilled water
7. Stain with Schiff's Reagent for 10-15 mins
8. Wash well in running tap water for 5 mins
9. Stain nuclei with Haematoxylin for 1 min
10. Wash in running tap water for 2 mins
11. Differentiate briefly with acid alcohol
12. Wash briefly in running tap water.
13. Wash and blue nuclei in ammonia water
14. Wash briefly in running tap water.
15. Dehydrate in alcohol, clear in xylene and mount in DPX.

Results

Neutral mucins	magenta
Acid mucins	blue
Mixtures of above	blue/purple
Nuclei	deep blue
Basement membranes	magenta
Background	yellow

BUFFERED CONGO RED METHOD FOR AMYLOID (EASTWOOD AND COLE 1971)

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Fixative

Buffered formal-saline

Reagents

- *pH 10.0 Sorensen-Walbum buffer*
0.1M Glycine (MW 72.07) 30 mL
0.1M Sodium Chloride (MW 58.5) 30 mL
0.1M Sodium Hydroxide (MW40.0) 40 mL
Mix together and check pH.
- *0.5% Congo Red Stain*
Congo Red 0.5g
Absolute alcohol 50ml
pH 10.0 Sorensen-Walbum Buffer 50ml
- *1% HCl Acid Alcohol*
- *70% Alcohol*
- *Haematoxylin*

Staining Technique

1. Take sections to water.
2. Stain in Harris's Haematoxylin for 30 seconds.
3. Rinse in tap water.
4. Differentiate in 1% HCl/alcohol for a few seconds (2 - 3dips)
5. Rinse in tap water.
6. Blue in ammonia water followed by running tap water.
7. Stain in 0.5% Congo Red for 10-20 minutes.
8. Differentiate in 70% alcohol for a few seconds (2 - 3 dips).
9. Blot dry.
10. Clear in xylene and mount in DPX.

Results

Amyloid: bright pink to red
Nuclei: blue

Notes

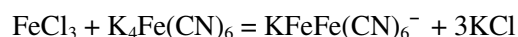
1. Dichroism is pronounced and can assist in distinguishing amyloid from other tissue components.
2. The stain has a permanent shelf life but may require occasional filtering.

PERLS' PRUSSIAN BLUE METHOD FOR HAEMOSIDERIN (Perls)

Safety Note: *Turn on the exhaust system before staining.
Wear protective clothing, gloves and safety glasses during the staining procedure.*

Principle

Ferric iron combines with potassium ferrocyanide to form the insoluble Prussian blue precipitate as follows:



Fixation

Neutral formalin (acid fixatives and potassium dichromate should be avoided).

Sections

Thin (3-5 μ) paraffin sections.

Reagents

- 2% Aqueous Solution of Hydrochloric Acid
- 2% Aqueous Solution of Potassium Ferrocyanide
- *Perls Working Solution:*
Mix equal parts of 2% hydrochloric acid and 2% potassium ferrocyanide solution JUST before use.
- 1% Neutral Red Stain
Neutral red (CI 50040) 1.0 g
Distilled water 99.0 mL
Glacial acetic acid 1.0 mL

Staining Technique

1. Take sections to distilled water
2. Transfer sections to a mixture of equal parts of 2% Potassium Ferrocyanide and 2% Hydrochloric Acid for 20-30 minutes.
3. Wash in distilled water.
4. Counterstain in filtered 1% neutral red for 1 minute.
5. Rinse in distilled water.
6. Rapidly dehydrate in absolute alcohol, clear and mount.

Results

Haemosiderin and ferric salts:	deep blue
Tissues and nuclei:	red
Cytoplasm	pink
Erythrocytes	yellow

Notes

- 1 More pronounced staining is obtained by heating the ferrocyanide to 37°C.
- 2 If the stain fades it may be revived by treating with 10 vol H₂O₂.
- 3 Tap water must be avoided at all times. The distilled water must be iron-free, and the hydrochloric acid must be of analytical grade, or it will contain iron.