

MODULE

Histology and Cytology



Notes

25

CYTOLOGY : STAINING METHODS

25.1 INTRODUCTION

Consistency and reliability are most important in cytological interpretation. Cytologists rely heavily on the quality and appearance of the stain. The Papanicolaou stain is recommended for the staining of alcohol fixed cytology slides. Romanowsky stains may also be used for wet fixed slides, but are primarily applied to air-dried smears.

Special stains are used as per requirements: Modified Ziehl Neelson (for acid fast bacilli), Gram staining (Bacteria), Mucicarmine (mucins), PAS (for glycogen, fungal wall, lipofuscin, etc), Oil red O (lipids), Perl's Prussian blue (iron), modified Fouchet's test (bilirubin), etc. Recently, immunocytochemistry is also being increasingly used in cytology specimens. These special stains and immunocytochemistry will be discussed along with respective sections in histopathology as the principles and methods remain the same.



OBJECTIVES

After reading this lesson, you will be able to:

- describe the principle of cytology stains
- explain the methods of staining cytology specimens.

25.2 STAINING IN CYTOLOGY

The universal stain for cytological preparations is the Papanicolaou stain. Harris' hematoxylin is the optimum nuclear stain and the combination of OG6 and EA50 give the subtle range of green, blue and pink hues to the cell cytoplasm.

Papanicolaou stain

Papanicolaou formula

1. Harris' hematoxylin

Hematoxylin	5g
Ethanol	50ml
Potassium alum	100g
Distilled water (50°C)	1000ml
Mercuric oxide	2-5g
Glacial acetic acid	40ml
2. Orange G 6

Orange G (10% aqueous)	50ml
Alcohol	950ml
Phosphotungstic acid	0-15g
3. EA 50

0.04 M light green SF	10ml
0.3M eosin Y	20ml
Phosphotungstic acid	2g
Alcohol	750ml
Methanol	250ml
Glacial acetic acid	20ml

Filter all stains before use.

Original Papanicolaou staining method:

1. 96% ethyl alcohol 15 seconds
2. 70% ethyl alcohol 15 seconds
3. 50% ethyl alcohol 15 seconds
4. Distilled water 15 seconds
5. Harris hematoxylin 6 minutes
6. Distilled water 10 dips
7. Hydrochloric acid 0.5% solution, 1-2 quick dips
8. Distilled water 15 seconds
9. Few dips in 0.1% ammoniated water. The smear turns to blue.



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10. 50% ethyl alcohol 15 seconds
11. 70% ethyl alcohol 15 seconds
12. 96% ethyl alcohol 15 seconds
13. OG-6 (orange) 2 minutes
14. 96% ethyl alcohol 10 dips
15. 96% ethyl alcohol 10 dips
16. EA 50 eosin yellowish 3 minutes
17. 96% ethyl alcohol (10 dips)
18. 100% ethyl alcohol (10 dips)
19. Xylene (10 dips)
20. Mount: in DPX using coverslip

Results:

The nuclei should appear blue/black

The cytoplasm (non-keratinising squamous cells) – blue/green

Keratinising cells- pink/orange

Precautions:

1. Use stains only after filtering them
2. Change stains frequently
3. Check staining under microscope for good quality control

25.3 MAY-GRÜNWARD GIEMSA STAIN

This is one of the common Romanwsky stains used in cytology. It is useful for studying cell morphology in air-dried smears. It is superior to Papanicolaou to study the cytoplasm, granules, vacuoles, basement membrane material etc. For nuclear staining Papanicolaou is superior.

Contents of the staining reagents:

May-Grünwald solution	0.2%
Methanol	99 %
May-Grünwald's eosin-methylene blue	0.2 %

Contains: Eosin G, Methylene blue

Giemsa solution

Methanol	73 %
Glycerol	26 %
Giemsa's Azur-Eosin-Methylene blue	0.6 %

Contains: Azur I, Eosin G, Methylene blue

Phosphate buffer

Potassium dihydrogen phosphate/ disodium hydrogen phosphate x 2H₂O
67.0 mmol/l

Storage

Giemsa solution, May-Grünwald solution: protected from light at 2-25°C.

Unopened reagents may be used until the expiry date on the label.

Phosphate buffer: at 2-8°C. Unopened reagents may be used until the expiry date on the label.

Preparation of working solutions

1. Buffered water: Dilute phosphate buffer with deionised or distilled water 1:20, e.g. 30 ml phosphate buffer + 570 ml deionised or distilled water.
2. Giemsa working solution : Mix 84 ml of Giemsa solution into 516 ml of buffered water.
3. May-Grünwald working solution: Mix 360 ml of May-Grünwald solution into 240 ml of buffered water.

Staining method

1. Fix the air-dried smear specimen in methanol for 10 -20 minutes
2. Stain with May-Grünwald working solution for 5 minutes
3. Stain with Giemsa working solution for 12 minutes
4. Wash with clean buffered water for 2, 5 and 2 minutes
5. Dry the slides in upright position at room temperature
6. Mount the slides with a coverslip using DPX

Any modifications to the staining procedure/working solutions may affect the staining result, and are subject to precise method validation



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**Notes****Sources of errors**

Irregular distribution of the blood smear on a glass slide may result in an erroneous cell counts. Alcohols used for wiping the skin may cause hemolysis and artifacts. Do not let the specimens dry at any stage of the staining procedure. Wash properly to avoid dye artifacts. Buffered water is strongly recommended for washing. Staining result is dependent on pH. Alkaline pH increases blue and acidic pH pink or reddish tinge in the stained specimen.

Ziehl-Neelsen stain**Reagents**

- (1) Carbol Soft Fuchsin

Basic Fuchsin 1 gm

Absolute alcohol 10 ml

Add the basic fuchsin to the alcohol in a 100 ml flask and mix, on a magnetic stirrer for 30 minutes. Add 100ml of 5% aqueous phenol. Mix well. Filter and store in a brown glass bottle.

- (2) Acidified Methylene Blue

0.25% methylene blue in 1% acetic alcohol

- (3) 0.5% Acid Alcohol

Distiller water 700 ml

Absolute alcohol 300 ml

Hydrochloric acid 5 ml

- (6) 5% Sulphuric Acid

Distilled water 475 ml

Sulphuric acid 25 ml

Staining Method

Place fixed slides on the staining rack in serial order, smeared side up. Slides should be separated by a 1 cm gap, and should never touch one another. Cover slides individually with filtered Ziehl's carbol fuchsin working solution. Heat slides from underneath with the flame of a Bunsen burner, an alcohol lamp or an alcohol soaked cotton swab until vapour starts to rise. Staining solution should never be allowed to boil. Do not allow the stain to dry. Keep slides covered with hot, steaming carbolfuchsin for 5 minutes by re-flaming as needed. Rinse slides gently with water to remove excess carbolfuchsin. Drain off excess rinsing water from slides. Sputum smears appear red in colour.

Decolourising: Cover slides with 25% sulfuric acid or acid-alcohol solution and allow to stand for 3 minutes, after which the red colour should have almost completely disappeared. If needed, repeat sequence until the red colour disappears, but do not overdecolourise. Gently wash away the sulfuric acid or acid alcohol and the excess stain with water. Drain off excess rinsing water from slides.

Counterstaining: Cover slides individually with 0.3% methylene blue counterstaining solution and allow to stand for 1 minute. Rinse slides individually with water. Drain water off the slides, which are then allowed to air dry.

A properly stained smear should show a light blue colour due to methylene blue.

Results: Tubercle bacilli, hair shafts, Actinomyces, some fungal elements- red.

Background: pale blue.



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INTEXT QUESTIONS 25.1

1. stain is recommended for stains of alcohol fixed cytology
2. stain is used for wet fixed slides
3. stain is used for identification of Glycogen, Fungal Wall
4. stain is used for identification of lipids
5. test is used for identification of Bilirubin
6. The universal stain for cytological preparations is the



WHAT HAVE YOU LEARNT

- Consistency and reliability are most important in cytological interpretation. Cytologists rely heavily on the quality and appearance of the stain.
- Special stains such as Modified Ziehl Neelson for acid fast bacilli, Gram staining for Bacteria, Mucicarmine for mucins, PAS for glycogen, fungal wall, lipofuscin, Oil red O for lipids, Perl's Prussian blue for iron, modified Fouchet's test for bilirubin
- The universal stain for cytological preparations is the Papanicolaou stain. Harris' hematoxylin is the optimum nuclear stain
- May-Grünwald Giemsa Stain is one of the common Romanowsky stains used in cytology. It is useful for studying cell morphology in air-dried smears.

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- Irregular distribution of the blood smear on a glass slide may result in an erroneous cell counts. Alcohols used for wiping the skin may cause hemolysis and artifacts



TERMINAL QUESTIONS

1. What are the various stains used commonly in cytology.
2. Write the basic principle of Papanicolaou staining.
3. What cell components are better seen in MGG staining?
4. Name the sources of error in Papanicolaou & MGG staining.
5. Enumerate the substances that get stained red with Ziehl Neelsen staining.



ANSWERS TO INTEXT QUESTIONS

25.1

1. Papanicolaou stain
2. Romanowsky
3. PAS
4. Oil red O
5. Modified Fouchet's
6. Papanicolaou stain