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ijrset@gmail.com

www.ijrset.com

Microtomy, Sectioning and Different Staining Techniques Used in Laboratory Practicals

DR. RANA TAJ

Department of Zoology, Seth RL Sahariya Govt. PG College, Kaladera, Jaipur, India

ABSTRACT: The history of histology indicates that there have been significant changes in the techniques used for histological staining through chemical, molecular biology assays and immunological techniques, collectively referred to as histochemistry. Early histologists used the readily available chemicals to prepare tissues for microscopic studies; these laboratory chemicals were potassium dichromate, alcohol and the mercuric chloride to harden cellular tissues. Staining techniques used were carmine, silver nitrate, Giemsa, Trichrome Stains, Gram Stain and Hematoxylin among others. The purpose of this article was to assess past and current literature reviews, as well as case studies, with the aim of informing ways in which histological stains have been improved in the modern age. There has been a rising need for efficient, accurate and less complex staining procedures. Many stain procedures are still in use today, and many others have been replaced with new immunostaining, molecular, non-culture and other advanced staining techniques. Some staining methods have been abandoned because the chemicals required have been medically proven to be toxic. The case studies indicated that in modern histology a combination of different stain techniques are used to enhance the effectiveness of the staining process. Currently, improved histological stains, have been modified and combined with other stains to improve their effectiveness.

I. DETAILED REVIEW

Microtomy and Paraffin Section Preparation

Creating great paraffin sections using a rotary microtome takes a great deal of skill and experience. "Microtomy and Paraffin Section Preparation" is a great training aid for new microtomists and an excellent refresher for experienced operators. All of the essential aspects of cutting paraffin sections are covered, including:

Safety

Knife tilt angle is always optimized for each microtome and blade type.

Carefully Trim Blocks

Blocks are carefully trimmed to expose tissue. The last few sections are always cut at what will be the final thickness to polish the block face.

Avoid Freezing Damage

Blocks are chilled on a cold wet surface and are always cold when cut (the surface of melting ice is excellent).

Use Cold Blocks

Blocks are always cold when cut.

Cut Sections Slowly

The final sections from each block are cut gently with a uniform, slow rotation.

Use Clean Water

The water in the flotation bath is replaced regularly.[1]

Ensure Slides are Clean

The cleanliness of slides is always checked before they are used. Handling of slides is kept to a minimum to avoid contamination with squamous cells prior to flotation.

Avoid Cross-contamination

The water surface is always skimmed between specimens to avoid contamination of one section with cells from another.

Avoid Contamination with Squames

Care is taken not to brush hair or hands while floating-out sections (squames can contaminate sections).

Don't Float from Multiple Blocks

Sections from more than one block (case) are never simultaneously floated on the water bath.

Check Water Temperature

Flotation bath temperature is carefully checked. A temperature 4–5°C below the melting point of the wax is optimal.

Sections should readily flatten but the wax should not melt.

Avoid Wrinkles in Sections

Sections flatten readily on the flotation bath. [2]

Avoid Over-expanding Sections

Sections are left on the flotation bath for just long enough to flatten then promptly picked up on a slide.

Don't Damage Floating Sections

Extreme care is taken to avoid damaging floating sections when mechanically removing wrinkles with a brush or forceps

Carefully Choose Sections

The first one or two sections in a ribbon are never picked up on slides.

Prevent Bubbles Under Sections

Care is taken to avoid the formation of air bubbles in the flotation bath. Any visible bubbles are dislodged before the sections are laid on the water.

Prevent Section Lifting

The use of "sticky" (charged) slides or section adhesives such as AAS is considered and used appropriately.[3]

Drain Before Drying

Sections are drained briefly before being placed in the slide dryer or onto a hotplate.

Monitor Drying Temperature

The temperature of the slide dryer is carefully monitored.

Dry for Appropriate Time

The minimum and maximum slide drying time is monitored.



Semi Automatic Microtome

Gram Staining is the common, important, and most used differential staining techniques in microbiology, which was introduced by Danish Bacteriologist **Hans Christian Gram in 1884**. This test differentiate the bacteria into **Gram Positive and Gram Negative Bacteria**, which helps in the classification and differentiations of microorganisms.

When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to

dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears blue or purple in colour[4]

In case of gram negative bacteria, cell wall also takes up the **CV-Iodine complex** but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with safranin, they take the stain and appears red in color.

Reagents Used in Gram Staining

Crystal Violet, the primary stain

Iodine, the mordant

A decolorizer made of acetone and alcohol (95%)

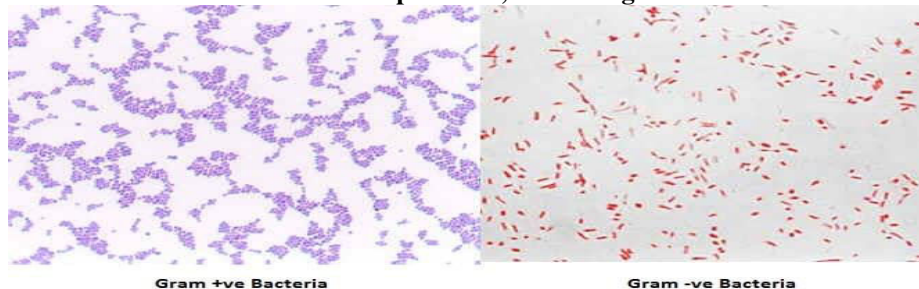
Safranin, the counterstain

Procedure of Gram Staining

- Take a clean, grease free slide.
- Prepare the smear of suspension on the clean slide with a loopful of sample.
- Air dry and heat fix
- Crystal Violet was poured and kept for about 30 seconds to 1 minutes and rinse with water.
- Flood the gram's iodine for 1 minute and wash with water.
- Then ,wash with 95% alcohol or acetone for about 10-20 seconds and rinse with water.
- Add safranin for about 1 minute and wash with water.
- Air dry, Blot dry and Observe under Microscope.[5]

Interpretation

Gram Positive: Blue/Purple Color, Gram Negative: Red Color



Examples

Gram Positive Bacteria: *Actinomyces, Bacillus, Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactobacillus, Listeria, Mycoplasma, Nocardia, Staphylococcus, Streptococcus, Streptomyces*, etc.

Gram Negative Bacteria: *Escherichia coli (E. coli), Salmonella, Shigella, and other Enterobacteriaceae, Pseudomonas, Moraxella, Helicobacter, Stenotrophomonas, Bdellovibrio, acetic acid bacteria, Legionella* etc

Endospore Staining

In 1922, **Dorner** published a method for staining endospores. **Shaeffer and Fulton** modified Dorner's method in 1933 to make the process faster. The endospore stain is a differential stain which selectively stains bacterial endospores. The main **purpose** of endospore staining is to differentiate bacterial spores from other vegetative cells and to differentiate spore formers from non-spore formers.

Principle of Endospore Staining

Bacterial endospores are metabolically inactive, highly resistant structures produced by some bacteria as a defensive strategy against unfavorable environmental conditions. The bacteria can remain in this suspended state until conditions become favorable and they can germinate and return to their vegetative state.

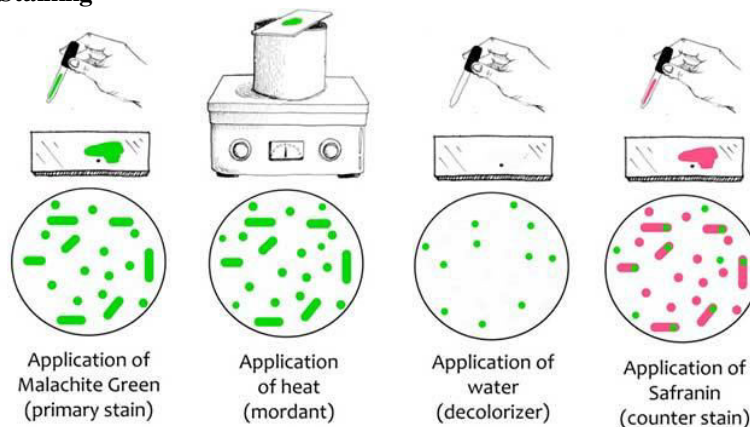
In the Schaeffer-Fulton's method, a primary stain-**malachite green** is forced into the spore by steaming the bacterial emulsion. **Malachite green** is water soluble and has a low affinity for cellular material, so **vegetative cells** may be **decolorized** with **water**. **Safranin** is then applied to counterstain any cells which have been decolorized. At the end of the staining process, **vegetative cells** will be **pink**, and **endospores** will be **dark green**.

Spores may be located in the **middle** of the cell, at the **end** of the cell, or **between the end and middle** of the cell. Spore shape may also be of diagnostic use. Spores may be **spherical** or **elliptical**.

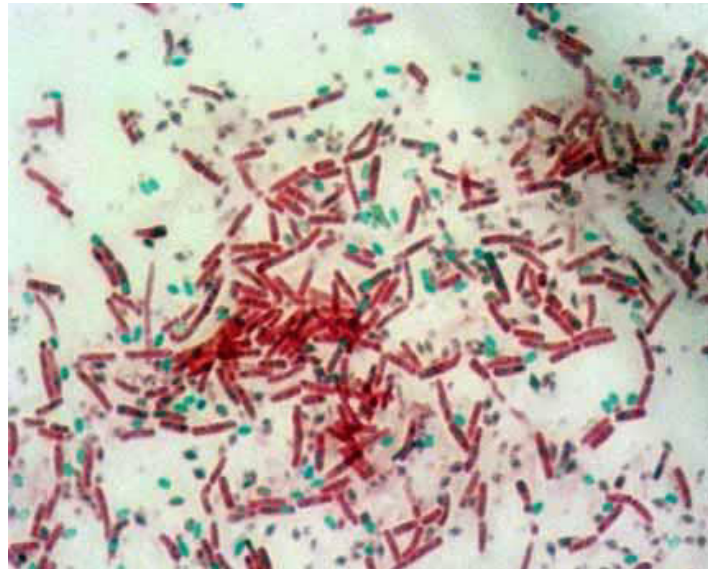
Reagents used for Endospore Staining

Primary	Stain:	Malachite	green	(0.5%	(wt/vol)	aqueous	solution)
0.5		gm	of		malachite		green
100 ml of distilled water							
Decolorizing							agent
Tap water or Distilled Water							
Counter							Stain: Safranin
Stock	solution		(2.5%	(wt/vol)	alcoholic		solution)
2.5		gm	of		safranin		O
100 ml of 95% ethanol							

Procedure of Endospore Staining



- Take a clean grease free slide and make smear using sterile technique.
- Air dry and heat fix the organism on a glass slide and cover with a square of blotting paper or toweling cut to fit the slide.
- Saturate the blotting paper with **malachite green** stain solution and steam for **5 minutes**, keeping the paper moist and adding more dye as required. Alternatively, the slide may be steamed over a container of boiling water.
- Wash the slide in **tap water**.
- Counterstain with **0.5% safranin** for **30 seconds**. Wash with tap water; blot dry.
- Examine the slide under microscope for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink.[6]



Endospores: Endospores are bright green.

Vegetative Cells: Vegetative cells are brownish red to pink.

Capsule Staining

The main purpose of capsule stain is to distinguish capsular material from the **bacterial cell**. A capsule is a gelatinous outer layer secreted by bacterial cell and that surrounds and adheres to the cell wall. Most capsules are composed of polysaccharides, but some are composed of polypeptides. The capsule differs from the slime layer that most bacterial cells produce in that it is a thick, detectable, discrete layer outside the cell wall. The capsule stain employs **an acidic stain** and a basic stain to detect capsule production.

Principle of Capsule Staining

Capsules stain very poorly with reagents used in simple staining and a capsule stain can be, depending on the method, a misnomer because the capsule may or may not be stained.

Negative staining methods contrast a translucent, darker colored, background with stained cells but an unstained capsule. The background is formed **with** india ink or nigrosin or congo red. India ink is difficult to obtain nowadays; however, nigrosin is easily acquired.

A positive capsule stain requires a mordant that precipitates the capsule. By counterstaining with dyes like crystal violet or methylene blue, bacterial cell wall takes up the dye. Capsules appear colourless with stained cells against dark background. Capsules are fragile and can be diminished, desiccated, distorted, or destroyed by heating. A drop of serum can be used during smearing to enhance the size of the capsule and make it more easily observed with a typical compound light microscope.[7]

Reagents used for Capsule Staining

Crystal Violet									
Crystal Violet		(85%	Violet dye	content)	=	1		(1%)	gm
Distilled Water = 100 ml									
Nigrosin									
Nigrosine,	water		soluble	=		10			gm
Distilled Water = 100 ml									

Procedure of Capsule Staining

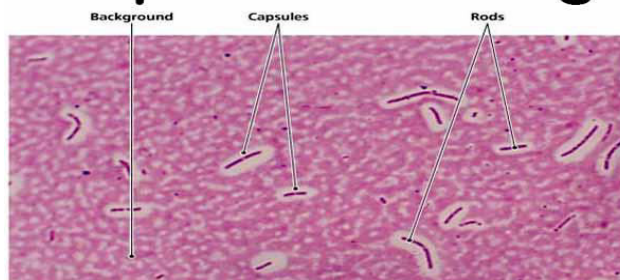
Place a small drop of a **negative stain (India Ink, Congo Red, Nigrosin, or Eosin)** on the slide. Congo Red is easier to see, but it does not work well with some strains. India Ink generally works, but it has tiny particles that display Brownian motion that must be differentiated from your bacteria. Nigrosin may need to be kept very thin or diluted. Using sterile technique, add a loopful of bacterial culture to slide, smearing it in the dye. Use the other slide to drag the ink-cell mixture into a thin film along the first slide and let stand for 5-7 minutes.

Allow to air dry (do not heat fix).

Flood the smear with crystal violet stain (this will stain the cells but not the capsules) for about 1 minutes. Drain the crystal violet by tilting the slide at a 45 degree angle and let stain run off until it air dries . Examine the smear microscopically (100X) for the presence of encapsulated cells as indicated by clear zones surrounding the cells.

Result of Capsule Staining

Capsule Staining



Capsule: Clear halos zone against dark background

No Capsule: No Clear halos zone

Examples of Capsule Positive and Negative

Positive

Bacillus anthracis,	Klebsiella pneumoniae, Streptococcus pneumoniae
Neisseria meningitidis	Clostridium spp, Rhizaobium spp, etc.

Negative

Neisseria gonorrhoeae, etc.

Mneomonics to remember capsulated bacteria- Some Killers Have Pretty Nice Capsule

Streptococcus pneumoniae	Klebsiella pneumoniae
Haemophilus influenzae	Pseudomonas aeruginosa
Cryptococcus neoformans	Neisseria meningitidis

Acid Fast Staining

It is the differential staining technique which was first developed by Ziehl and later on modified by Neelsen. So this method is also called **Ziehl-Neelsen staining** techniques. Neelsen in 1883 used Ziehl's carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques was developed.

The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups.

This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus *Mycobacterium*, are resistant and can only be visualized by acid-fast staining.

Principle of Acid-Fast Stain

When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. Then after all cell appears red. Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the

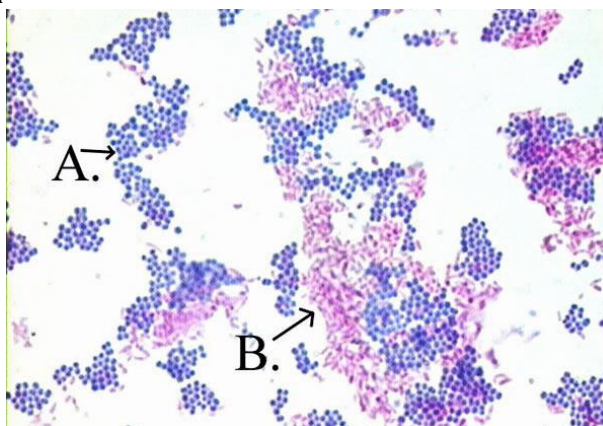
presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution. The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. Only decolorized cells absorb the counter stain and take its color and appears blue while acid-fast cells retain the red color.

Application of	Reagent	Cell colour	
		Acid fast	Non-acid fast
Primary dye	Carbol fuchsin	Red	Red
Decolorizer	Acid alcohol	Red	Colorless
Counter stain	Methylene blue	Red	Blue

Procedure of Acid-Fast Stain

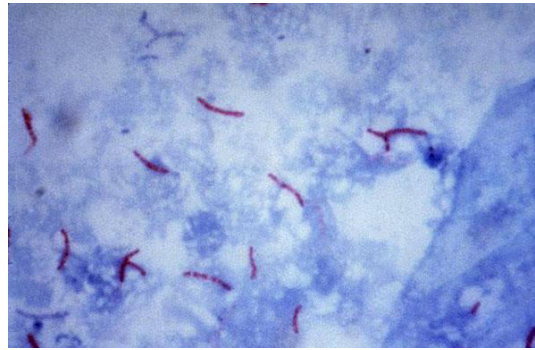
- Prepare bacterial smear on clean and grease free slide, using sterile technique.
- Allow smear to air dry and then heat fix..
- Cover the smear with carbol fuchsin stain.
- Heat the stain until vapour just begins to rise (i.e. about 60 C). Do not overheat. Allow the heated stain to remain on the slide for 5 minutes. Wash off the stain with clean water. Cover the smear with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolorized, i.e. pale pink.
- Wash well with clean water.
- Cover the smear with malachite green stain for 1–2 minutes, using the longer time when the smear is thin.
- Wash off the stain with clean water.
- Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry (do not blot dry).
- Examine the smear microscopically, using the 100 X oil immersion objective.[7]

Interpretation of Acid-Fast Stain



Acid fast: Bright red to intensive purple (B), Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded
 Non-acid fast: Blue color (A)

Examples of Acid-Fast Stain



Mycobacterium tuberculosis visualization using the Ziehl–Neelsen stain.

Acid-fast: *Mycobacterium*

tuberculosis,

Mycobacterium

smegmatis.

Non-Mycobacterial

bacteria: *Nocardia*

Coccidian Parasites: *Cryptosporidium*

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