

MODULE

Microbiology



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COMMON STAINING TECHNIQUE

2.1 INTRODUCTION

Staining is a technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells.

Bacteria have nearly the same refractive index as water, therefore, when they are observed under a microscope they are opaque or nearly invisible to the naked eye. Different types of staining methods are used to make the cells and their internal structures more visible under the light microscope.

Microscopes are of little use unless the specimens for viewing are prepared properly. Microorganisms must be fixed & stained to increase visibility, accentuate specific morphological features, and preserve them for future use.



OBJECTIVES

After reading this lesson, you will be able to:

- describe the need for staining techniques
- explain terms related to staining techniques
- discuss the substances used as stain
- enlist various staining techniques
- classify & explain various stains

2.2 TERMS RELATED TO STAINING

Stain

A stain is a substance that adheres to a cell, giving the cell color. The presence of color gives the cells significant contrast so they are much more visible. Different stains have different affinities for different organisms, or different parts of organisms. They are used to differentiate different types of organisms or to view specific parts of organisms

Staining

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes.

Fixation

Fixation by itself consists of several steps—aims to preserve the shape of the cells or tissue involved as much as possible. Sometimes heat fixation is used to kill, adhere, and makes them permeable so it will accept stains

What can be used as stain

The substance be used as a stain must be colored or it should react in the system to give a colored product, because of which some portion of the system becomes colored and the rest remains colorless. Staining renders the organism more visible, it displays the structure and finer details of bacteria and it helps to differentiate between organisms

Staining techniques

Direct staining - The organism is stained and background is left unstained

Negative staining - The background is stained and the organism is left unaltered



INTEXT QUESTIONS 2.1

Fill in the blanks:

1. Staining is primarily used to enhance in the image
2. Substances that adhere to cell giving colour to cell are called as
3. aims to preserve the shape of the cells
4. The organism is stained and background is left unstained in staining technique
5. Background is stained and the organism is left unchanged in staining technique



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2.3 KINDS OF STAINS

Stains are classified as

- Simple stain
- Differential stain
- Structural or special stains



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Simple Staining

The staining process involves immersing the sample (before or after fixation and mounting) in dye solution, followed by rinsing and observation. Many dyes, however, require the use of a mordant, a chemical compound that reacts with the stain to form an insoluble, coloured precipitate. When excess dye solution is washed away, the mordanted stain remains. Simple staining is one step method using only one dye. Basic dyes are used in direct stain and acidic dye is used in negative stain. Simple staining techniques is used to study the morphology better, to show the nature of the cellular contents of the exudates and also to study the intracellular location of the bacteria

Commonly used simple stains are

- Methylene blue
- Dilute carbol fuchsin
- Polychrome methylene blue

Loeffler's Methylene Blue

Method of Staining

Flood the smear with methylene blue, allow for 2 minutes, pour off the stain and allow the air to dry by keeping in a slanting position and by this the organism will retain the methylene blue stain

Use

Methylene blue staining is used to make out clearly the morphology of the organisms eg. *H.influenzae* in CSF, Gonococci in urethral pus

Polychrome Methylene Blue

Preparation

Allow Loeffler's Methylene blue to 'ripen' slowly. Methylene blue stain is kept in half filled bottles, aerate the content by shaking at intervals, Slow oxidation of methylene blue forms a violet compound and Stain gets polychrome property. The ripening nearly takes 12 months and this is hastened by addition of 1% potassium carbonate

Common Staining Technique

Use

Polychrome Methylene Blue is used to demonstrate Mc Fadyean reaction of *B.anthraxis* and in this the blue bacilli is surrounded by purple capsular material

Dilute Carbol Fuchsin

Preparation

Prepare carbol fuchsin and dilute it to 1/15 using distilled water

Method of staining

Flood the smear and let stand for 30 seconds, wash with tap water and blot gently to dry

Use

To stain throat swab from patients of suspected Vincent's angina, (*Borrelia* are better stained), it is used as a counter stain in Gram stain and to demonstrate the morphology of *Vibrio cholerae* (comma shaped)



INTEXT QUESTIONS 2.2

Fill in the blanks:

1. Chemicals that reacts with stain to form precipitates are called as
2. dyes is used in direct stain
3. dyes is used in negative stain
4. Commonly used simple stains are, &

2.4 DIFFERENTIAL STAINS

Gram Staining

Differential Stains use two or more stains and allow the cells to be categorized into various groups or types. Both the techniques allow the observation of cell morphology, or shape, but differential staining usually provides more information about the characteristics of the cell wall (Thickness). Gram staining (or Gram's method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell wall. The Gram stain is almost always the first step in the identification of a bacterial organism, While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming Gram variable and Gram

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Common Staining Technique



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indeterminate groups as well. The word Gram is always spelled with a capital, referring to Hans Christian Gram, the inventor of Gram staining

Gram staining Principles

Gram staining is used to determine gram status to classify bacteria broadly. It is based on the composition of their cell wall. Gram staining uses crystal violet to stain cell walls, iodine as a mordant, and a fuchsin or safranin counterstain to mark all bacteria. Gram status is important in medicine; the presence or absence of a cell wall will change the bacterium's susceptibility to some antibiotics. Gram-positive bacteria stain dark blue or violet. Their cell wall is typically rich with peptidoglycan and lacks the secondary membrane and lipopolysaccharide layer found in Gram-negative bacteria

Gram Staining Technique

1. Crystal violet acts as the primary stain. Crystal violet may also be used as a simple stain because it dyes the cell wall of any bacteria.
2. Gram's iodine acts as a mordant (Helps to fix the primary dye to the cell wall).
3. Decolorizer is used next to remove the primary stain (crystal violet) from Gram Negative bacteria (those with LPS imbedded in their cell walls). Decolorizer is composed of an organic solvent, such as, acetone or ethanol or a combination of both.)
4. Finally, a counter stain (Safranin), is applied to stain those cells (Gram Negative) that have lost the primary stain as a result of decolorization

Gram Reaction

Gram-positive bacteria are those that are stained dark blue or violet by Gram staining. This is in contrast to Gram-negative bacteria, which cannot retain the crystal violet stain, instead taking up the counter stain (safranin or fuchsin) and appearing red or pink. Gram-positive organisms are able to retain the crystal violet stain because of the high amount of peptidoglycan in the cell wall. Gram-positive cell walls typically lack the outer membrane found in Gram-negative bacteria.

Gram-negative bacteria are those bacteria that do not retain crystal violet dye in the Gram staining protocol. In a Gram stain test, a counter stain (commonly safranin) is added after the crystal violet, coloring all Gram-negative bacteria with a red or pink color. The test itself is useful in classifying two distinct types of bacteria based on the structural differences of their cell walls. On the other hand, Gram-positive bacteria will retain the crystal violet dye when washed in a decolorizing solution.



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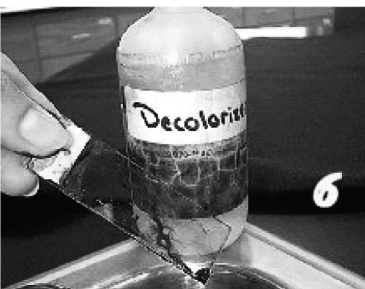


Fig. 2.1: Gram Reaction.



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**INTEXT QUESTIONS 2.3**

Fill in the blanks:

1. Gram staining uses to stain cell walls, as mordant & as counter stain
2. Gram positive bacteria stains colour
3. Gram negative bacteria stains colour
4. Gram positive organism are able to retain crystal violet stain because of high amount of in the cell wall

2.5 ACID-FAST STAINING

The Ziehl–Neelsen stain, also known as the acid-fast stain, widely used differential staining procedure. The Ziehl – Neelsen stain was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894) a pathologist. In this type some bacteria resist decolourization by both acid and alcohol and hence they are referred as acid-fast organisms. This staining technique divides bacteria into two groups namely acid-fast and non acid-fast. This procedure is extensively used in the diagnosis of tuberculosis and leprosy. Mycobacterium tuberculosis is the most important of this group, as it is responsible for the disease called tuberculosis (TB) along with some others of this genus

Principle

Mycobacterial cell walls contain a waxy substance composed of mycolic acids. These are β -hydroxy carboxylic acids with chain lengths of up to 90 carbon atoms. The property of acid fastness is related to the carbon chain length of the mycolic acid found in any particular species

Ziehl- Neelsen Procedure

1. Make a smear. Air Dry. Heat Fix.
2. Flood smear with Carbol Fuchsin stain
 - Carbol Fuchsin is a lipid soluble, phenolic compound, which is able to penetrate the cell wall
3. Cover flooded smear with filter paper
4. Steam for 10 minutes. Add more Carbol Fuchsin stain as needed
5. Cool slide
6. Rinse with DI water

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7. Flood slide with acid alcohol (leave 15 seconds). The acid alcohol contains 3% HCl and 95% ethanol, or you can decolorize with 20% H₂SO₄
8. Tilt slide 45 degrees over the sink and add acid alcohol drop wise (drop by drop) until the red color stops streaming from the smear
9. Rinse with DI water
10. Add Loeffler's Methylene Blue stain (counter stain). This stain adds blue color to non-acid fast cells. Leave Loeffler's Blue stain on smear for 1 minute
11. Rinse slide. Blot dry.
12. Use oil immersion objective to view.

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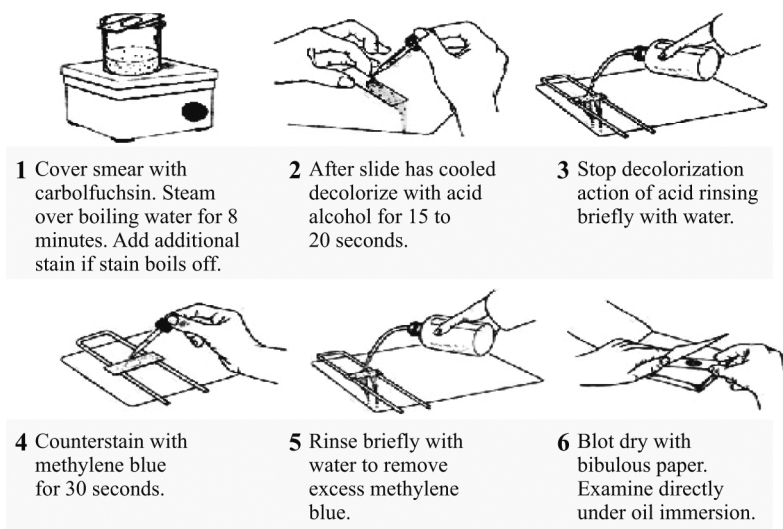


Fig. 2.2: Ziehi-Neelsen acid fast staining procedure

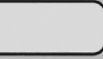
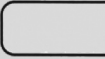
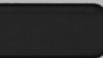

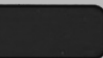
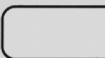
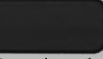
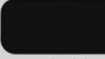
Acid-fast (+) bacilli	Steps for staining	Acid-fast (-) bacilli
1 	Cells on slide	
2  Stain red	Primary stain (carbolfuchsin red)	 Stain red
3  Remain red	Decolorizer, (HCl, alcohol)	 Become colorless
4  Remain red	Counterstain, (methylene blue)	 Stain blue

Fig. 2.3



Notes



INTEXT QUESTIONS 2.4

Fill in the blanks:

1. Organisms that resist decolourisation by acid and alcohol are called as
2. staining is used in the diagnosis of Tuberculosis and Leprosy
3. Acid fastness is related to length of mycolic acid
4. Acid fast staining groups bacteria into two groups namely &

2.6 SPECIAL STAINS

- Stains for Metachromatic granules
- Stain for spores
- Stain for capsules
- Stain for spirochetes
- Stain for flagella

Albert's Staining for *C. diphtheriae*

In all cases of suspected Diphtheria, stain one of the smears with Gram stain. If Gram stained smear shows morphology suggestive of *C.diphtheriae*, proceed to do Albert staining which demonstrates the presence or absence of metachromatic granules.

C.diphtheriae are thin Gram positive bacilli, straight or slightly curved and often enlarged (clubbing) at one or both ends and are arranged at acute angles giving shapes of Chinese letters or V shape which is characteristic of these organisms. Present in the body of the bacillus are numerous metachromatic granules which give the bacillus beaded or barred appearance. These granules are best demonstrated by Albert's stain.

Albert staining

Albert stain I

- Toluidine blue 0.15 gm
- Malachite green 0.20 gm
- Glacial acetic acid 1.0 ml
- Alcohol(95%) 2.0 ml
- Distilled water 100 ml

Albert stain II

- Iodine 2.0 gm
- Potassium iodide 3.0 gm
- Distilled water 300 ml

Albert staining Procedure

- Cover the heat-fixed smear with Albert stain I. Let it stand for two minutes.
- Wash with water.
- Cover the smear with Albert stain II. Let it stand for two minutes.
- Wash with water, blot dry and examine.
- To demonstrate metachromatic granules in *C.diphtheriae*. These granules appear bluish black whereas the body of bacilli appear green or bluish green.

Capsule staining

The purpose of the capsule stain is to reveal the presence of the bacterial capsule, the water-soluble capsule of some bacterial cells is often difficult to see by standard simple staining procedures or after the Gram stain. The capsule staining methods were developed to visualize capsules and yield consistent and reliable results

Capsule may appear as clear halo when a fresh sample is stained by Grams or Leishman stain, Negative staining- using - India ink, Nigrosin

India ink

Commercially available India ink is used undiluted

Procedure

- Place a loop full of India ink on the slide
- A small portion of the culture is emulsified in the drop of ink
- Place a clean cover slip over the preparation without bubbles. Press down gently
- Examine under dry objective

Uses

India ink is used to demonstrate capsule which is seen as unstained halo around the organisms distributed in a black background eg. *Cryptococcus*

Endospore Staining

Bacterial **endospores** are metabolically inactive, highly resistant structures produced by some bacteria as a defensive strategy against unfavorable



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environmental conditions. **Primary stain** - is **malachite green**, which stains both vegetative cells and endospores and **heat** is applied to help the primary stain penetrate the endospore. **Decolorized** with **water**, which removes the malachite green from the vegetative cell but not the endospore, **Safranin - 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

Flagella stain

- Flagella are fragile appendages
- Cannot be seen under ordinary microscope
- Hence the surface is coated with a precipitate to form a colloidal substance
- This precipitate serves as a layer of stainable material

Components

1. 1% Osmic acid
2. Mordant
 - 10% Tannic acid
 - Sat.potassium alum
 - 10% Ferric chloride
3. Fontana's silver solution

Use

This is used to demonstrate the flagella and the organisms stain black and flagella appear light brown



INTEXT QUESTIONS 2.5

Fill in the blanks:

1. Presence or absence of metachromatic granules is demonstrated by staining
2. chemical is used in capsule staining
3. is used as primary stain in Endospore staining
4. is the counter stain in endospore staining



WHAT YOU HAVE LEARNT

- Staining is technique used in microscopy to enhance contrast in the microscopic image.
- Stain is a substance that adheres to a cell, giving the cell colour.
- Stains are classified as Simple stain, Differential stain and Special stains.
- Gram staining is used to differentiate bacterial species as Gram-positive and Gram-negative based on the chemical and physical properties of cell wall.
- Acid Fast staining technique or Ziehl Neelsen stain divides bacteria into acid fast and non-acid-fast and this is used in diagnosis of tuberculosis and Leprosy.
- Albert staining technique demonstrates the presence or absence of metachromatic grannules which is used in identification of *C. diphtheria* bacilli.



Notes



TERMINAL QUESTIONS

1. List staining techniques
2. Describe different kinds of stains
3. Explain gram staining
4. Explain Acid fast staining



ANSWERS TO INTEXT QUESTIONS

2.1

1. Contrast
2. Stain
3. Fixation
4. Direct
5. Negative

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2.2

1. Mordant
2. Basic
3. Acidic
4. Methylene blue, Polychrome methylene blue & Dilute carbol fuchsin

2.3

1. Crystal violet, Iodine & Fuchsin
2. Dark blue or violet
3. Red or pink
4. Peptidoglycan

2.4

1. Acid fast organism
2. Acid fast staining
3. Carbon chain
4. Acid fast and Non acid fast

2.5

1. Albert's
2. Indian ink
3. Malachite green
4. Safranin