Staining Techniques Used in Microbiology

By-Dr. Ekta Khare

- Structural details of organisms cannot be seen under a light microscope due to a lack of contrast.
- Hence, we use dyes to stain cells.
- Dyes bind with cellular constituents producing color contrast and increasing their visibility.

TYPES OF STAINS

- ACIDIC

Negatively charged acid radicals imparts color in eosin, acid fuchsine, malachite green, Indian ink.

- BASIC

Positively charged basic radicals combines with negatively charged particles in cytoplasm and gives color.

e.g., Haematoxillin, Methylene blue, Crystal violet.

- NEURTAL

Both positively and negatively charged imparts different colors to different components.

e.g., Geimsa's stain and Leishman's stain.

STAINING TECHNIQUES

POSITIVE STAINING

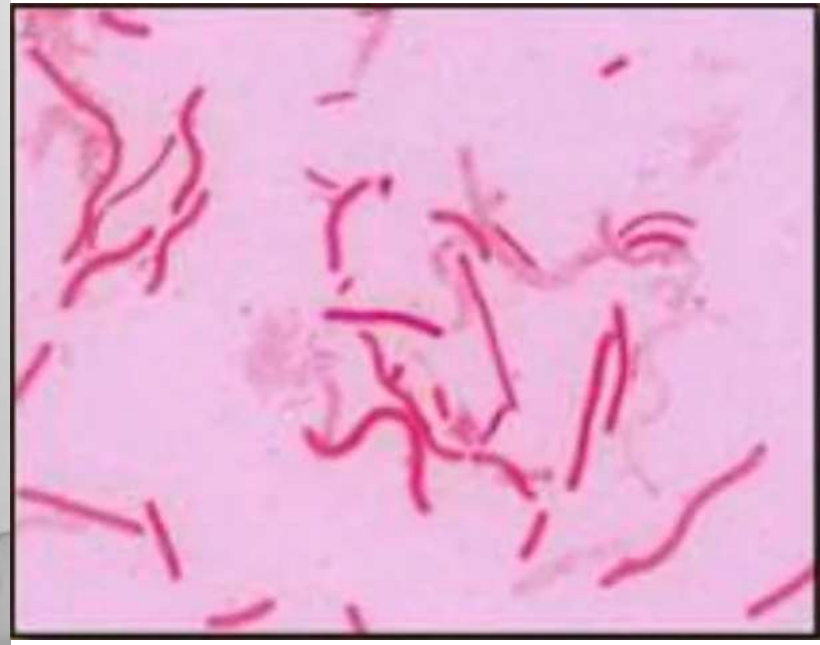
Where the actual cells are themselves colored and appear in a clear background.

- Simple staining: a stain which provides color contrast but gives same color to all bacteria an cells.

  e.g., **Methylene blue** and Crystal violet.

- Differential staining: a stain which imparts different colors to different bacteria is called differential stain (which contains more than one stain).

  e.g., Gram's stain, Acid fast staining and Endospore staining.

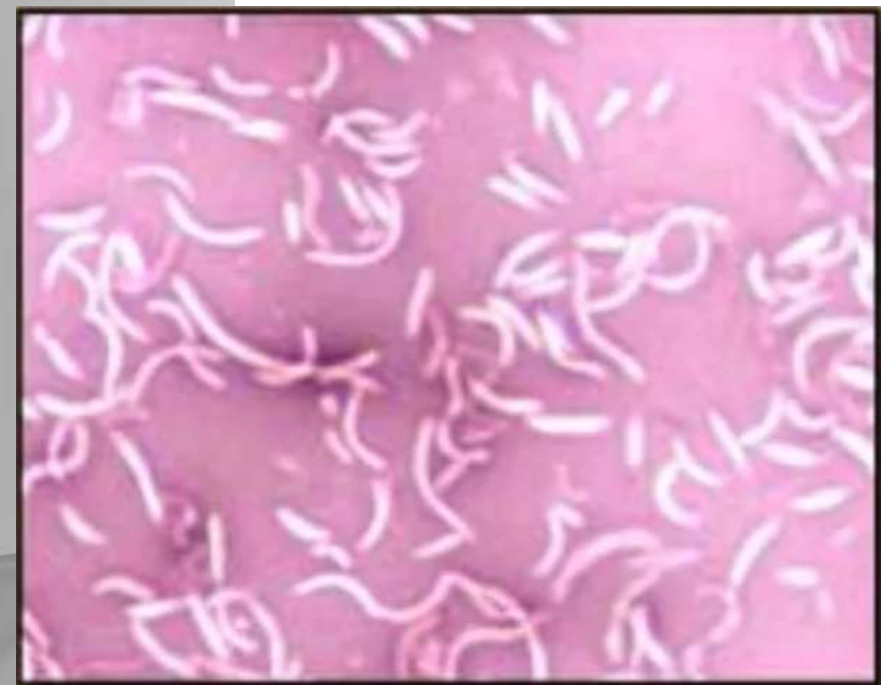


- NEGATIVE STAINING :

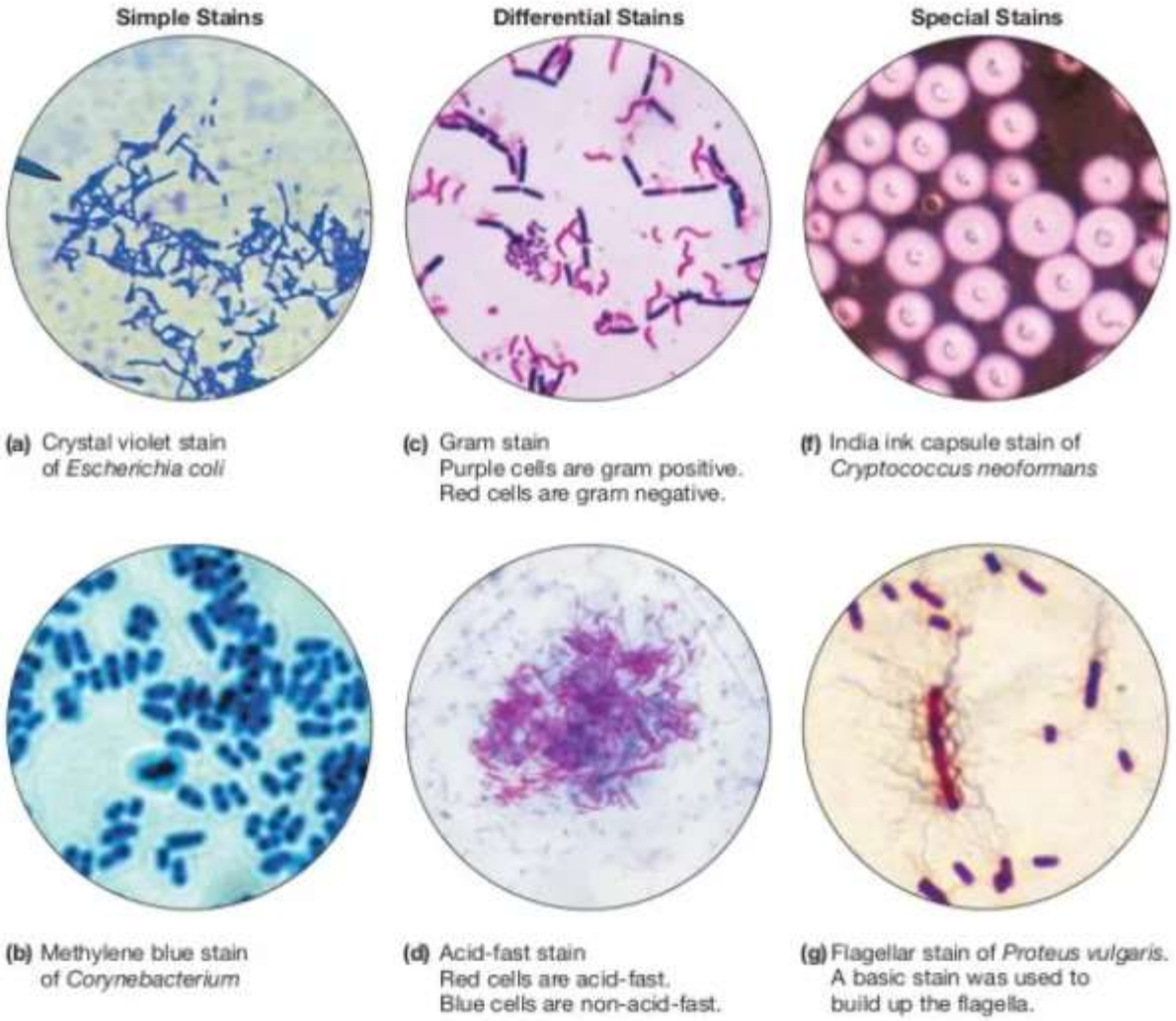
Where the cells remain clear (uncolored) & the background is colored to create a contrast to aid in the better visualization of the image.

✓Nigrosin

✓Indian ink



Based on the types and number of dyes used, staining can be categorized into different types:



BACTERIAL SMEAR PREPARATION

SMEAR : is a distribution of bacterial cells on a slide for the purpose of viewing them under the microscope.

METHOD :

- Aseptically a small sample of the culture is spread over a slide surface.
- This is then allowed to air dry.
- The next step is heat fixation to help the cells adhere to the surface.
- The smear is now ready for staining.

SMEARING TECHNIQUES

- HEAT FIXATION

a) Pass air dried smears through a flame two or three times. Do not over heat.

b) Allow slide to cool before staining.

- METHANOL FIXATION

a) Place air dried smears in a coplin jar with methanol for one minute. Alternatively, flood smear with methanol for one minute.

b) Drain slides and allow to dry before staining.

Types of Microbiological Stains

- Simple stain
- Negative staining
    - India ink preparation
- Impregnation methods
    - Flagella stain
- Differential staining
    - Gram staining
    - Acid-fast stain (Ziehl-Neelsen technique)
    - Endospore stain
    - Capsule stain
    - Giemsa stain: Giemsa stain was a name adopted from a Germany Chemist scientist, for his application of a combination of reagents in demonstrating the presence of parasites in malaria. (It is a neutral stain made up of a mixture of oxidized methylene blue, azure, and Eosin Y)
    - Acridine orange Stain
    - Cytoplasmic inclusion stains
- Other staining methods
    - Auramine-Rhodamine technique
    - Calcofluor White Staining
    - Lactophenol cotton blue (LPCB) wet mount

IMPREGNATION METHODS

- Bacterial cells and structures that are too thin to be seen under the light microscope are thickened by impregnation of silver or other salts on their surface to make them visible, e.g., for demonstration of bacterial flagella and spirochetes.

Flagellar Stains

- To visualize the presence and arrangement of flagella for the presumptive identification of motile bacterial species.
- Most motile bacteria possess flagella, the shape, number, and position of which are important in the identification, particularly when biochemical reactions are weak or equivocal.

METHOD

1. LEIFSON METHOD

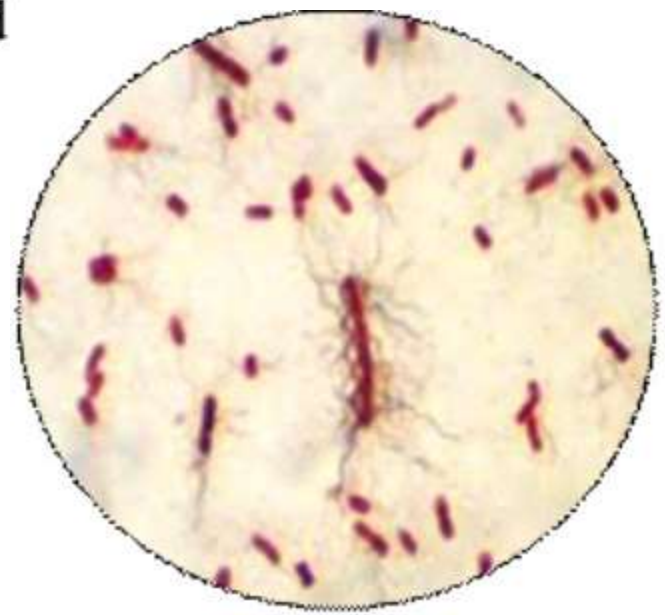
Most commonly used Method

- **Reagents**

1. Primary Stain- Basic fuchsin (para rosaniline acetate) 1.2% in 95% alcohol
2. Mordant-tannic acid 3% in water
3. Sodium chloride, 1.5% in water
4. Final Stain-1:1:1

Counterstain-methylene blue for better visualization

- Stain for 5–15 minutes by alcoholic solutions of rosaniline dyes , allowing a precipitate to form as the alcohol evaporates
- Observe the stained slide under the oil-immersion (100×) objective of the microscope -staining red to blue-black flagella should be observed



2. RYU METHOD

Easy to perform and gives good results

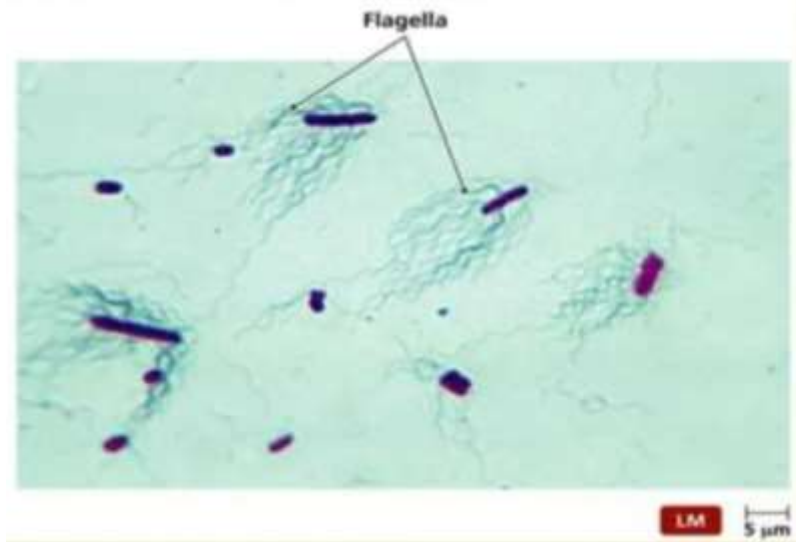
- **Solution I-**

a. 5% phenol, 10.0 ml
b. Powdered tannic acid, 2.0 g
c. Saturated aluminum potassium sulfate 12-hydrate (crystals)

- **Solution II-** Saturated solution of crystal violet in alcohol
- **Final Stain**: Solution I: II in 10:1 ratio

Method

- Flood the air-dried smears with the staining solution for 1–5 minutes.
- Wash the staining solution off in tap water. After the smears have dried, examine them under the oil-immersion objective of the microscope. Cell bodies & flagella stain violet.



Differential staining

- Here, two stains are used which impart different colors to different bacteria or bacterial structures, which help in differentiating bacteria. The most commonly used differential stains are:
  - Gram’s Staining
  - Acid-fast staining
  - Endospore staining
  - Capsule staining

Gram Staining

- 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.

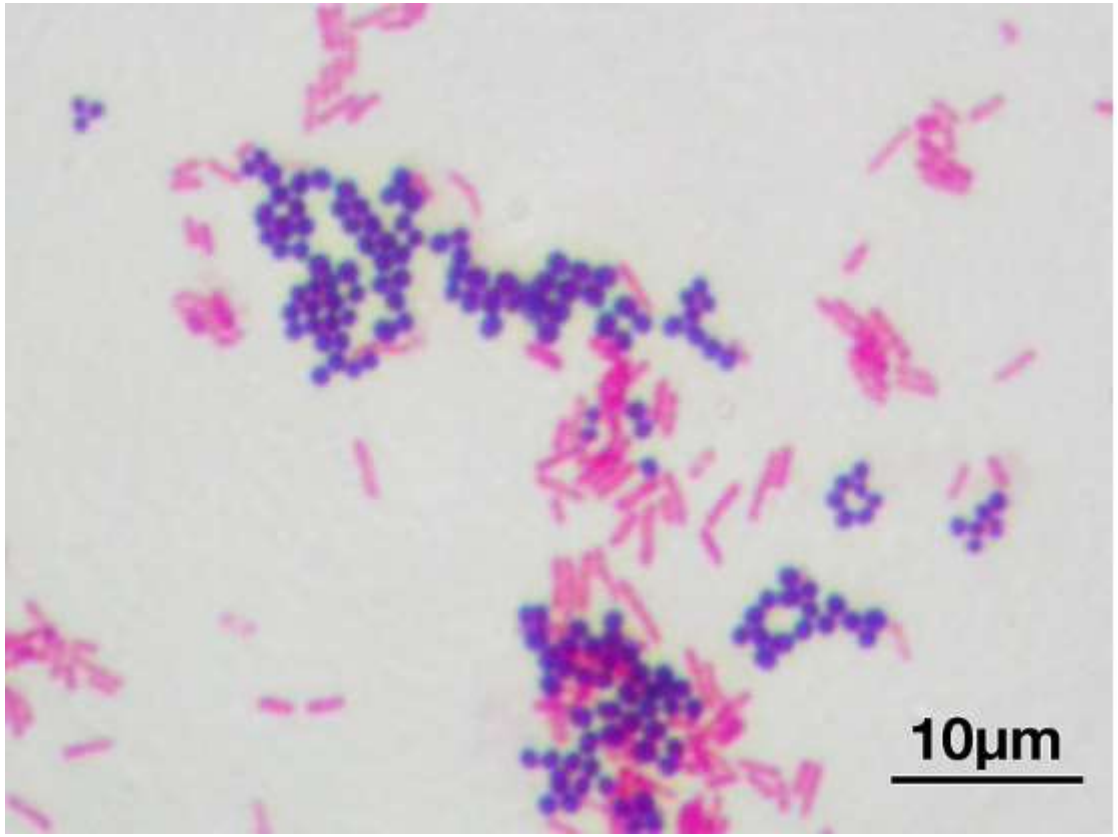
Reagents used:

- Crystal Violet, the primary stain
- Iodine, the mordant
- A decolorizer made of acetone and alcohol (95%)
- Safranin, the counterstain

Principle of Gram staining

- 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.
- 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.

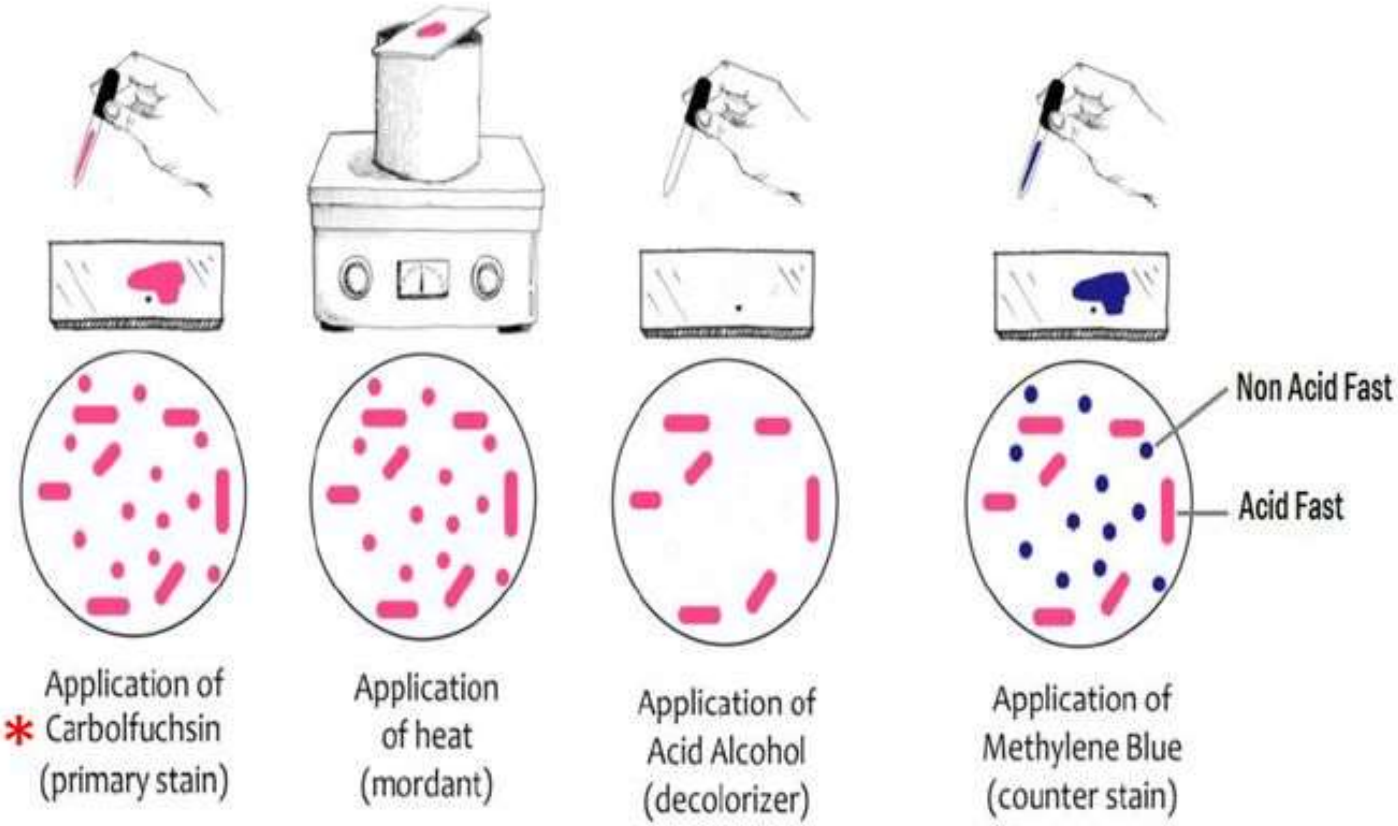
Gram stain showing Gram negative rods (pink) and Gram positive cocci (purple)



Acid-fast staining (Ziehl–Neelsen Staining)

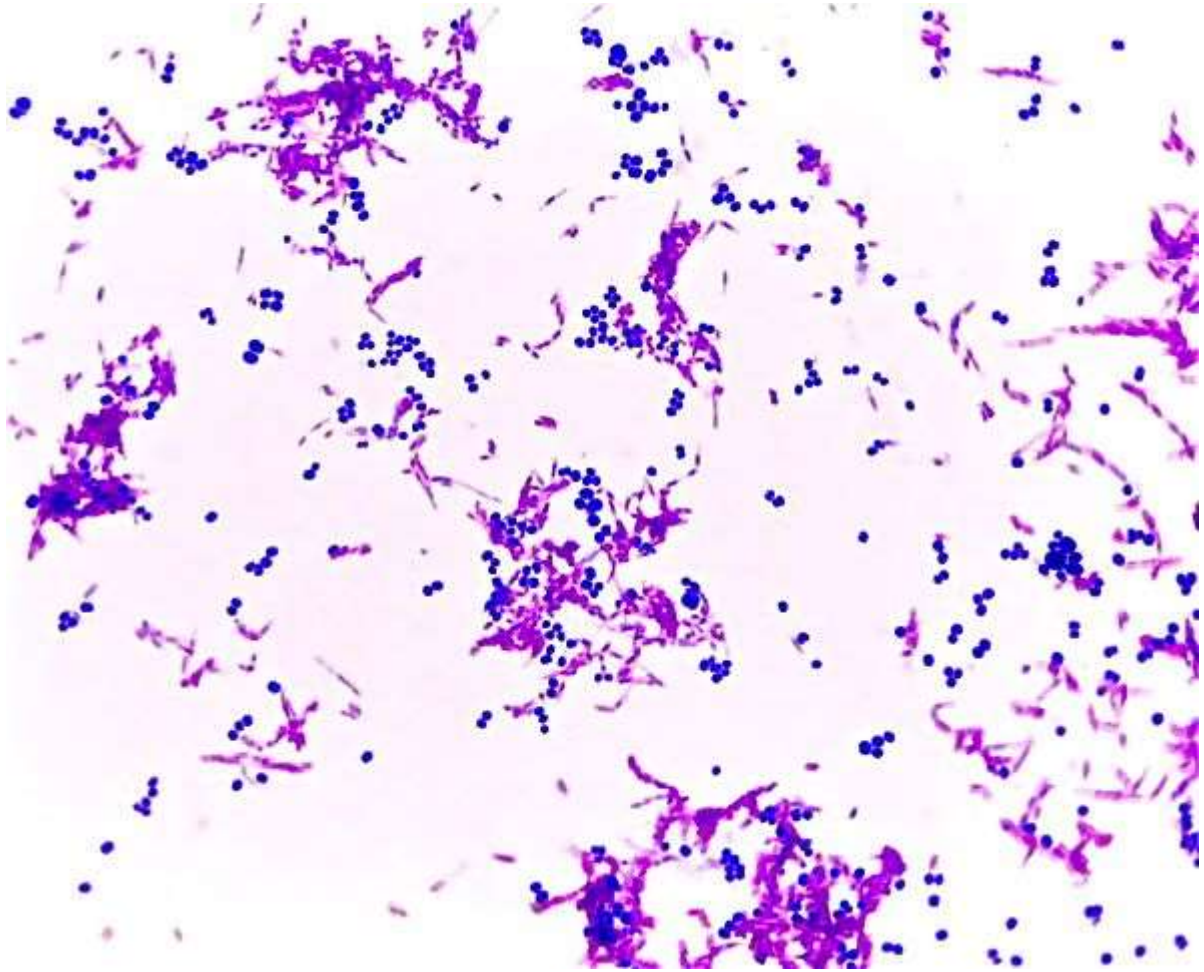
- The acid-fast stain is a differential stain used to identify acid-fast organisms such as members of the genus *Mycobacterium* .
- Acid-fast organisms are characterized by wax-like, nearly impermeable cell walls; they contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids. Acid-fast organisms are highly resistant to disinfectants and dry conditions.
- Because the cell wall is so resistant to most compounds, acid-fast organisms require a special staining technique.
- The primary stain used in acid-fast staining, carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall.
- This is further assisted by the addition of heat.
- The smear is then rinsed with a very strong decolorizer, which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms.
- The decolorized non-acid-fast cells then take up the counterstain.
- Primary stain – Carbol fuchsin
- Decolorizer – acid alcohol
- Counterstain – Methylene blue
- A differential stain used to detect bacteria with mycolic acid cell walls (genera *Mycobacterium* and *Nocardia*)
- Developed to detect the bacterial species that causes tuberculosis
- Acid-fast organisms resist decolorization with acid-alcohol

PRINCIPLE OF ZIEHL-NEELSEN STAIN



* Carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall

Acid-fast staining

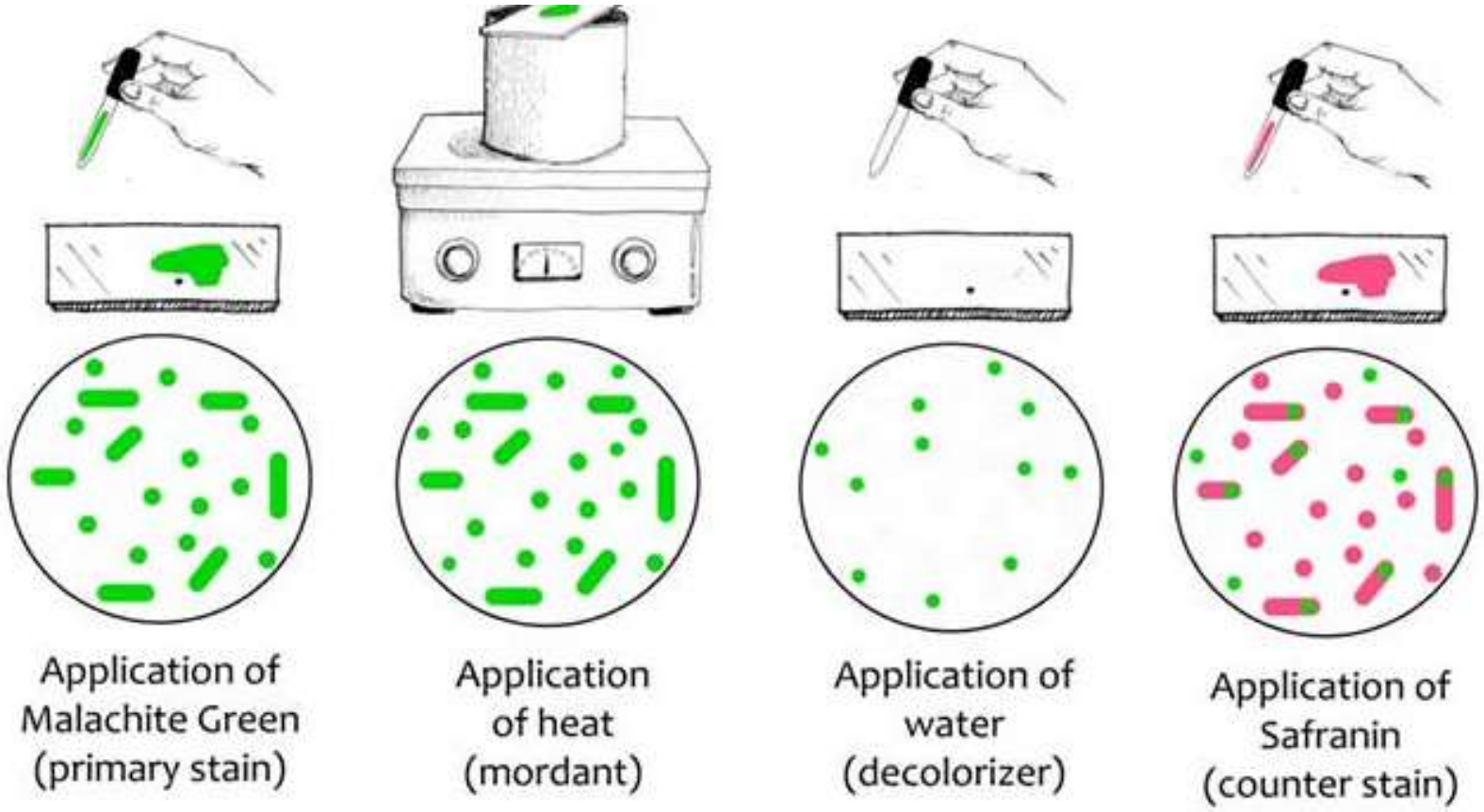


Endospore Staining

- 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.

Shaeffer and Fulton Method of endospore staining

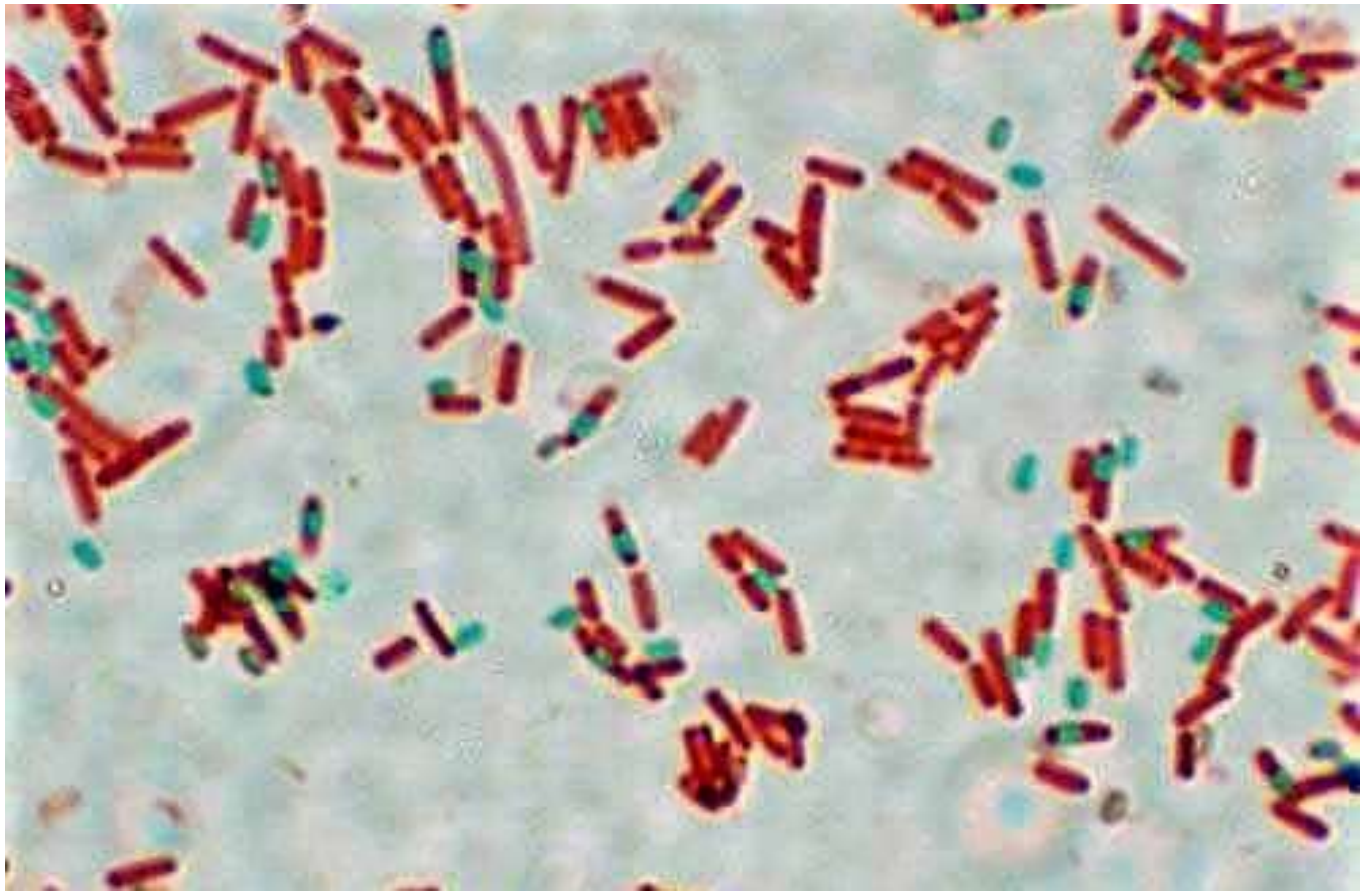
- Reagents used for Endospore Staining
- **Primary Stain: Malachite green (0.5% (wt/vol) aqueous solution)**
  0.5 gm of malachite green
  100 ml of distilled water
- **Decolorizing agent**
  Tap water or Distilled Water
- **Counter Stain: Safranin**
  Stock solution (2.5% (wt/vol) alcoholic solution)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



Principle of Endospore Staining

- 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 decolourized 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.

Endospore Staining



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

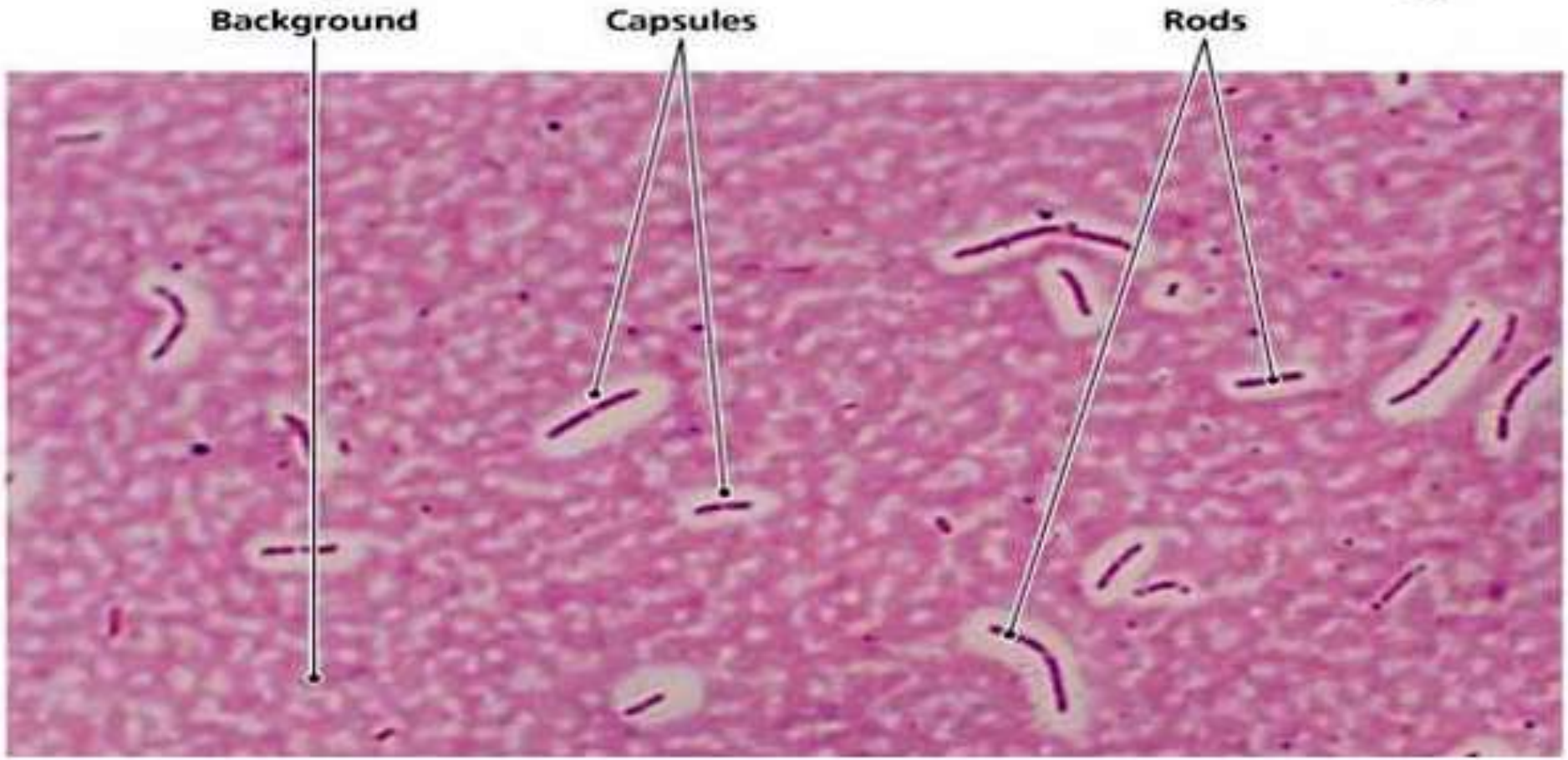
- Capsules stain very poorly with reagents used in simple staining and a capsule stain can be, depending on the method, a misnomer because the capsule may or may not be stained.
- Negative staining methods contrast a translucent, darker colored, background with stained cells but an unstained capsule.
- The background is formed with **india ink or nigrosin or congo red**.
- 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.

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.

Capsule Staining



Acridine Orange Staining

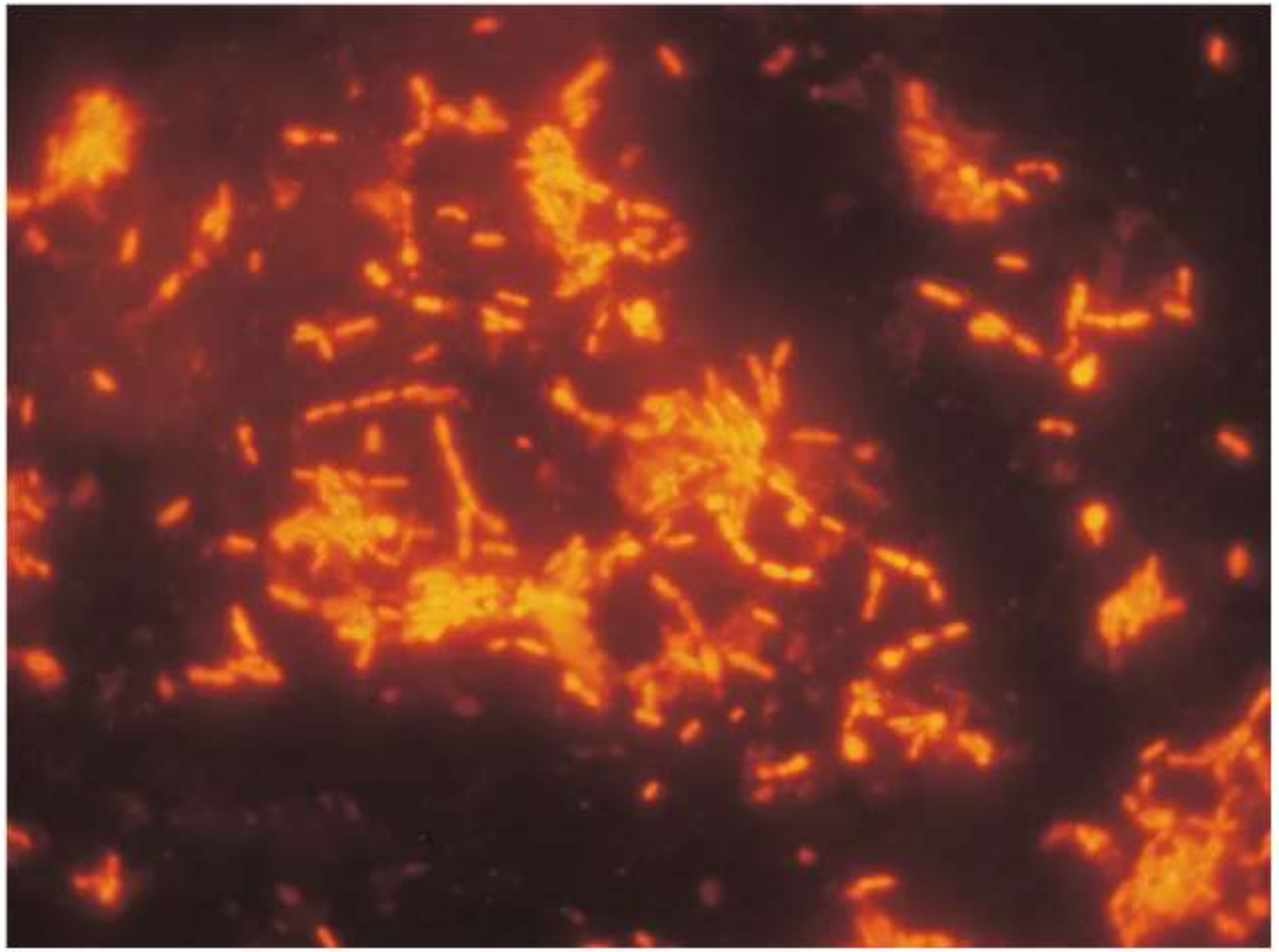
- Acridine orange is a dye that intercalates or binds with the nucleic acid (either DNA or RNA) present in organisms and fluoresce to emit various colors that help in the differentiation of cellular organelles.
- This binding is the result of the electrostatic interactions of acridine molecules between the nucleic acid-base pairs.
- Acridine orange (AO), due to its metachromatic properties, is commonly used in fluorescence microscopy and flow cytometry analysis of cellular physiology and cell cycle status, including the fluorescent microscopic examination of microorganisms.

Staining procedure:

- Prepare a smear in a clean grease-free slide and allow it to air dry.
- The slide is then fixed with methanol and dried again.
- It is then put in a trough with an acridine orange staining working solution (i.e 0.01 percent).
- After 2 minutes of staining, the slides are washed gently with water and dried, and then examined in a fluorescent microscope.

Observance: Bacteria stain orange against a green to a yellow background of human cells and debris.

Fluorescent acridine orange stain coryneforms bacteria



Cytoplasmic inclusion stains

- **Metachromatic (Volutin) granules** which are intracellular inclusion bodies, found in the cytoplasmic membrane of some bacterial cells for storage of complexed inorganic polyphosphate (poly-P) and enzymes.
- Staining methods:

- Albert stain
- Neisser stain
- Ponder's stain
- Loeffler's methyline blue

- **ALBERT'S STAIN**

Albert's staining is specially demonstrates the presence or absence of the metachromatic granules, a characteristic feature of Corny bacterium diphtheria. During gram staining if a smear appears as purple rods with straight or slightly curved with clubs at the end, with a characteristic V shape then it is suspected as Corny bacterium diphtheria. The further confirmation can be done by Albert's staining technique

How are these solutions prepared?

Albert Stain 1: Preparation of 100ml Albert stain 1

1. Into 100ml of water, add 0.1ml of glacial acetic acid
2. Add 2ml of 95% ethanol into the solution
3. Then, dissolve 0.15g of toluidine blue into the solution
4. Lastly, dissolve 0.2g of malachite green into the solution

Albert Stain 2: Preparation of 300ml of Albert stain 2

1. Dissolve 2g of iodine in 50ml of distilled water
2. Add 250ml of water to the solution
3. Dissolve 3g of Potassium Iodide into the solution in the solution

Procedure

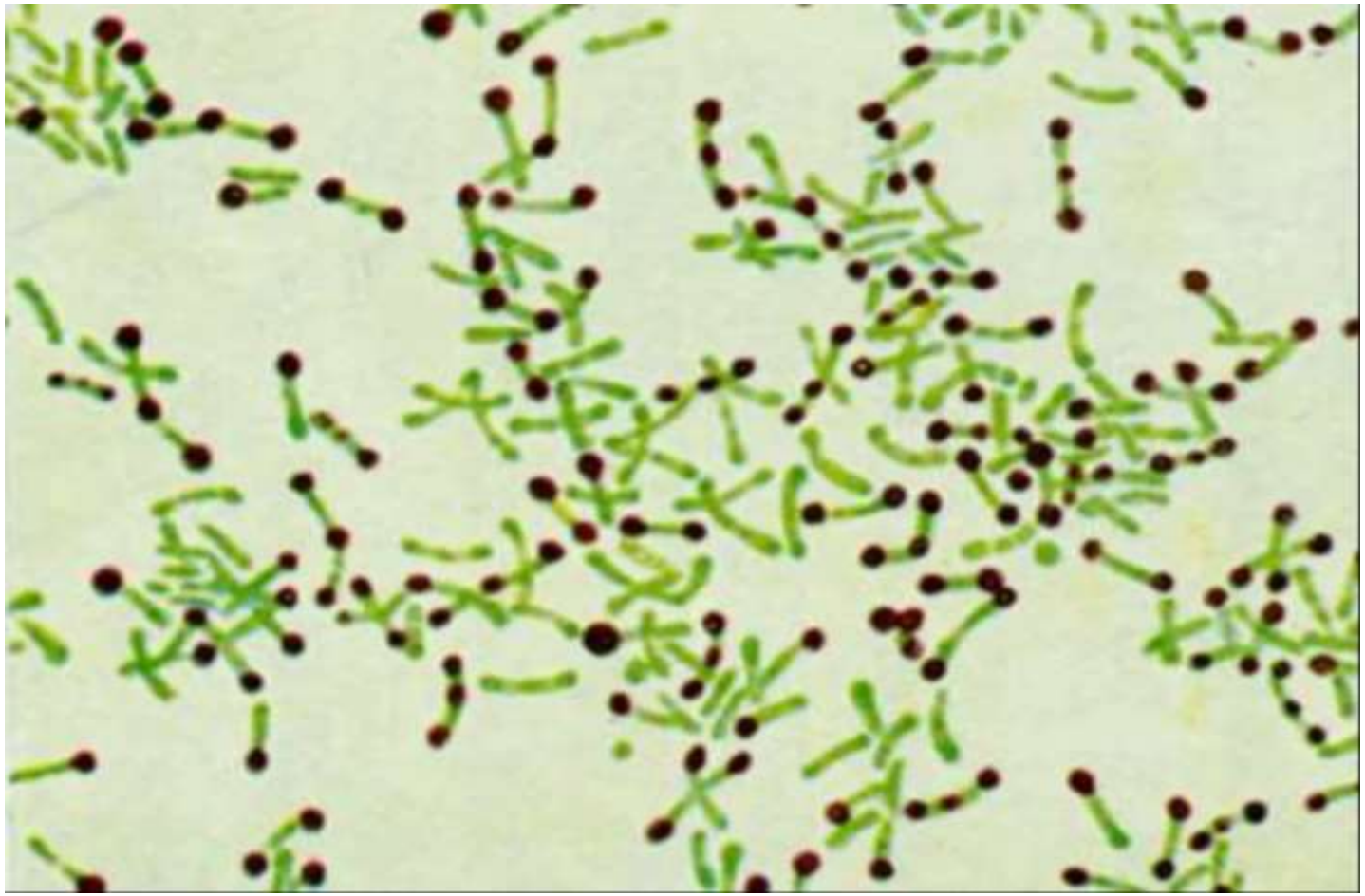
A. Staining:

1. Aseptically, take a loopful culture of *Corneybacterium diphtheriae*
2. Make a smear at the center of a clean sterile glass slide
3. Heat fix the smear, gently
4. On a staining rack, place the smeared glass slide.
5. Add Albert staining Solution 1 into the smear and leave it for 3-5 minutes
6. Wash the smeared slide with gently flowing tap water

B. Mordanting

1. Add Albert staining solution 2 and leave it for 1 minute
2. Wash the slide with gently flowing tap water.
3. Blot to dry the smeared glass slide
4. Add cedarwood oil on the smear
5. Then observe under a microscope by oil immersion at 1000x

Albert’s Stain



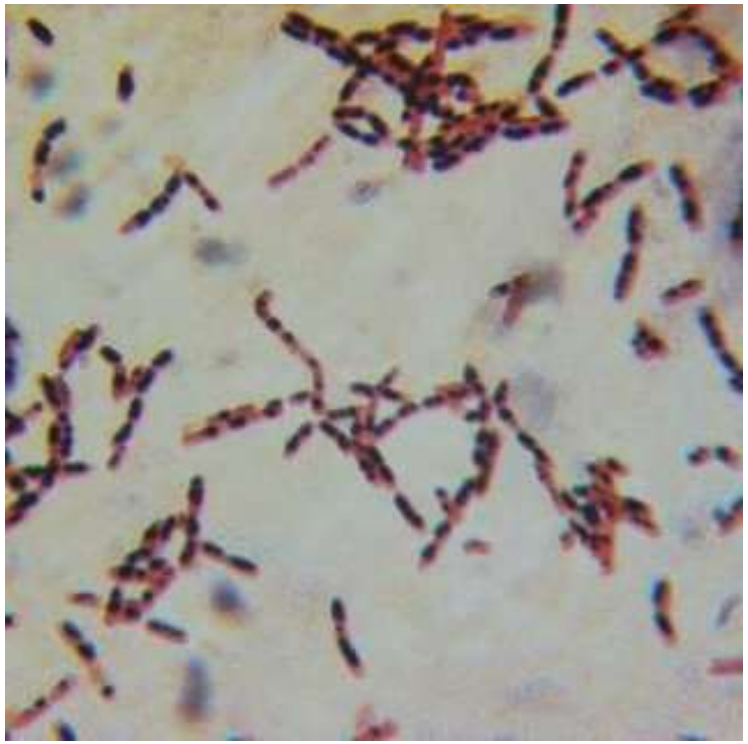
Polyhydroxybutyrate (PHB) staining

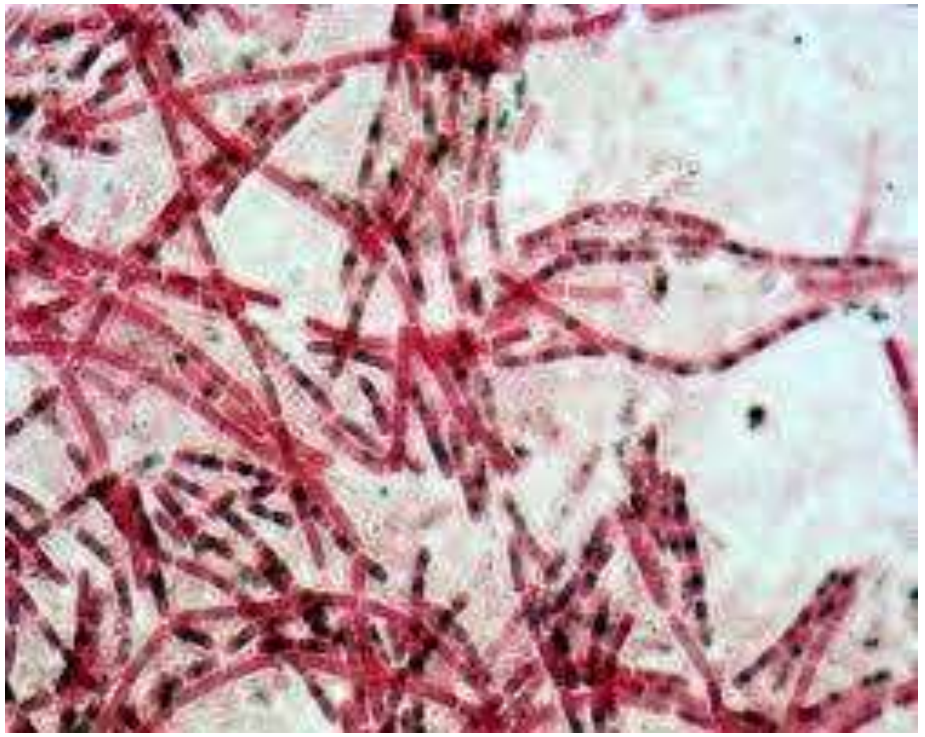
- Poly-3-hydroxybutyrate (PHB) is the most abundant naturally-occurring polyester produced by bacteria in response to carbon oversupply and other nutrient limitations, such as low nitrogen levels.
- PHB is stored as cytoplasmic granules with a diameter ranging from 0.2 to 0.5 µm, and can provide carbon and energy for the bacteria under certain conditions.

Staining method

- Bacteria was smeared onto clean slid, then stained with 0.3 g of Sudan black B in 70% (v/v) (ethanol) for 10 minutes.
- Subsequently, the smear was immersed with xylene to decolorize the cells, after that, 5 % (w/v) Safranin water solution was used for 10 seconds as counter stain.
- Finally, the slide was washed with distilled water and dried before observation under an optical microscope.

Polyhydroxybutyrate (PHB) staining





Auramine-Rhodamine technique

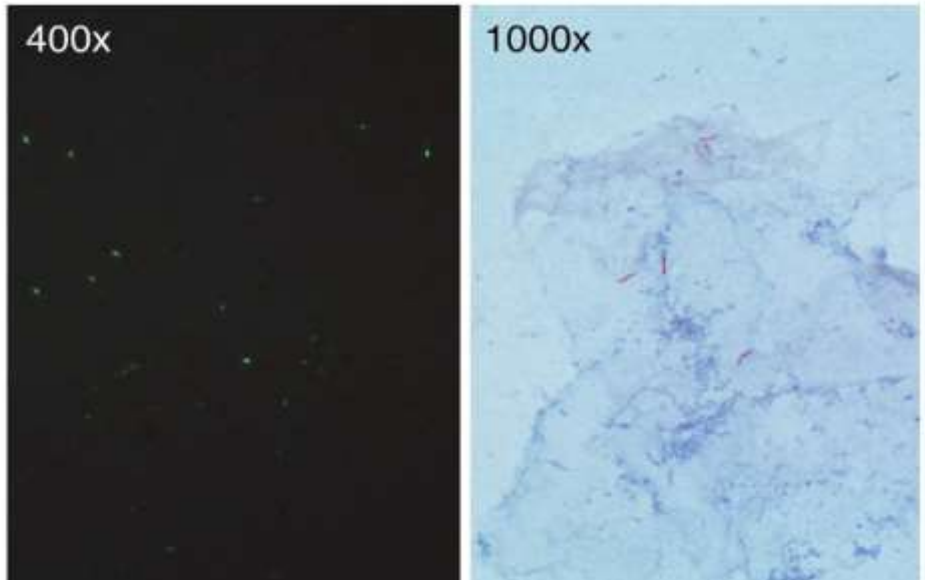
- Auramine-rhodamine fluorochrome staining also known as “Truant method of staining”, is used to visualize acid-fast bacilli (AFB).
- Fluorescent dyes like auramine-rhodamine bind to the mycolic acid present in them and impart bright **yellow or orange fluorescence against a greenish background** when viewed using a fluorescent microscope.
- It is also used to stain all acid-fast organisms including the sporozoan parasites.

Materials

Primary stain: auramine rhodamine solution (**caution**: possible carcinogen)

Decolorizer: 0.5% acid alcohol (5 ml HCl in 995 ml 70% alcohol). (**caution**: flammable, corrosive)

Counter stain: 0.5% pottassium permanganate (0.25 gm in 50 ml). (**caution**: corrosive)



Comparison of ZN Staining and Flurochrome staining

Calcofluor White Staining

- Calcofluor white staining technique was used as a comparative stain specifically for the identification of Onychomycosis fungal infection.
- The chitin containing structures that fluoresce bright white under ultraviolet light in a fluorescent microscope.
- Calcofluor White stain as a non-specific fluorochrome that binds to 1-3 beta and 1-4 beta polysaccharides on chitin and cellulose cell walls, has been described to be the most rapid technique for detection of pathogenic fungi and yeasts such as *Pneumocystis carinii, Microsporidium,* ***Acanthamoeba, Naegleria, and Balamuthia*** species.
- Calcofluor White Stain visualizes well when mixed with potassium hydroxide for fungal elements.
- The counterstain used is Evans Stain which forms a dark background that enables the tissues and cells to fluoresce using blue light excitation and not UV rays.
- Other elements fluoresce reddish-orange while fungal and parasitic elements fluoresce bright apple-green.

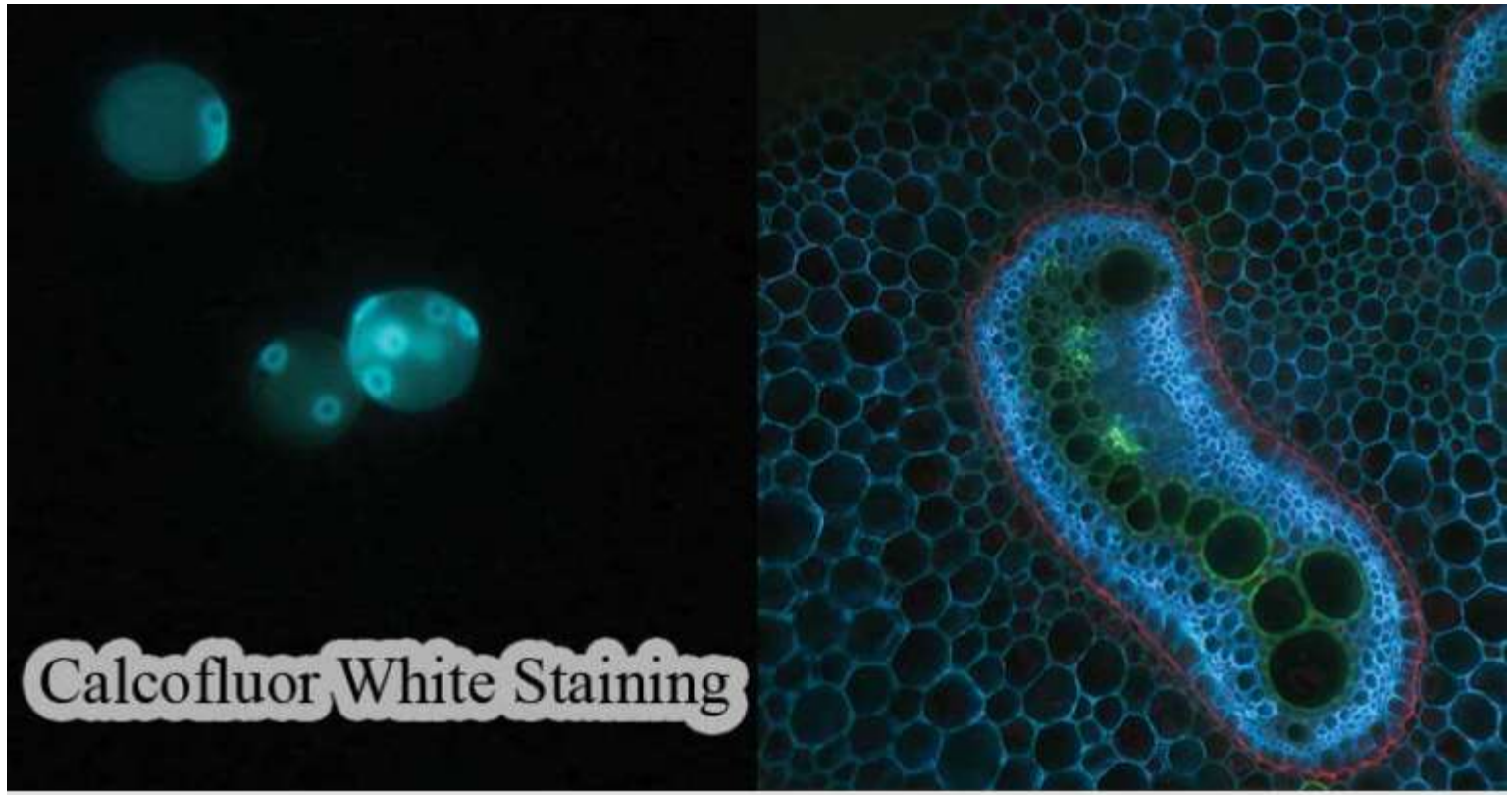


Figure: a: The fluorescent staining of yeast with calcofluor-white. The yeast shows a vivid blue color for the cell walls.

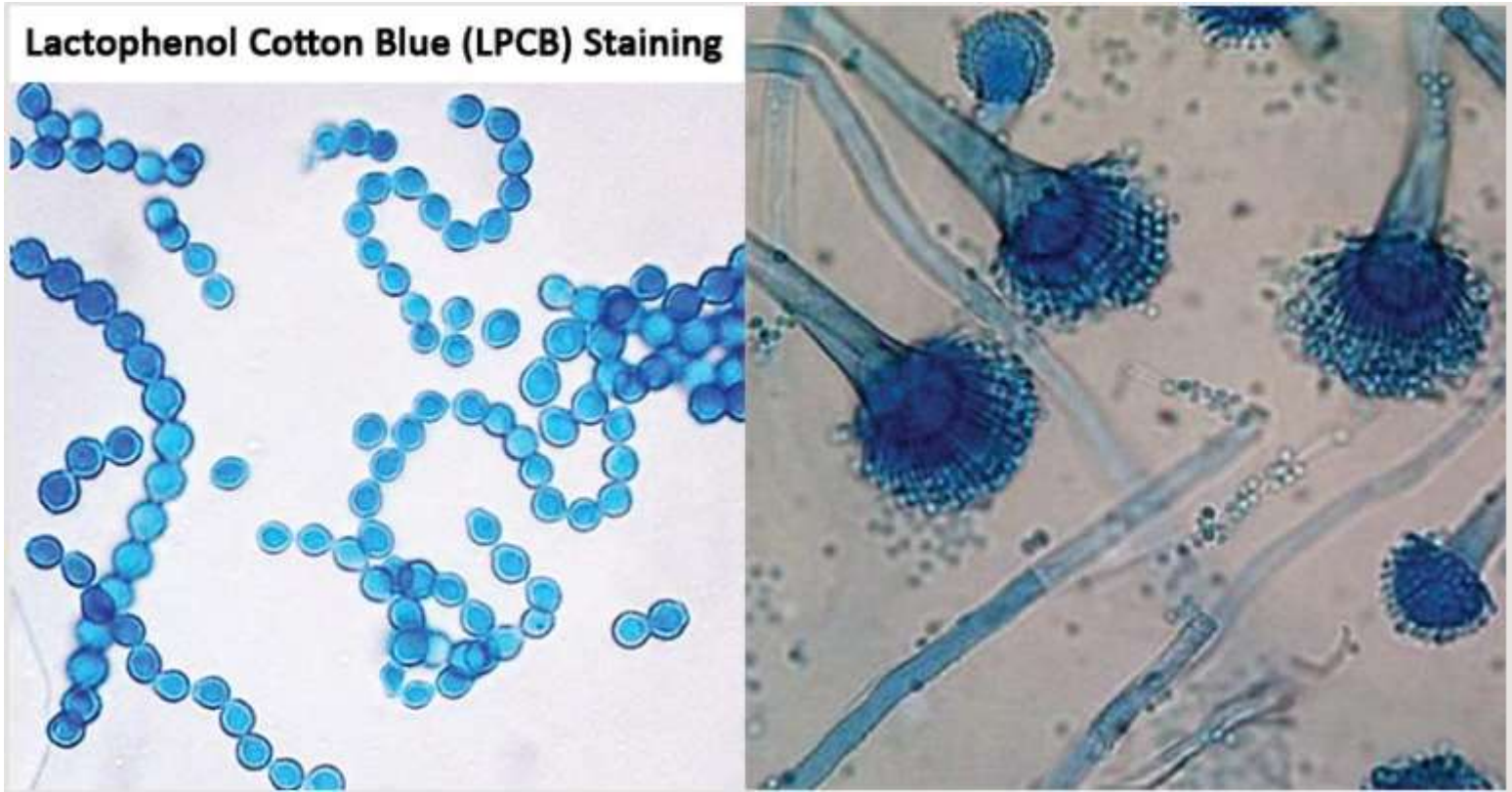
b: The cross-section of an Eagle Fern shows the bright green and bright blue fluorescence seen with CFW. Source: Calcofluor-white

Lactophenol cotton blue (LPCB) wet mount

- The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has the following constituents:
- **Phenol** kills fungus.
- **Lactic acid** acts as a clearing agent and helps preserve the fungal structures.
- **Cotton blue** is an aniline dye that **stains the chitin** in the fungal cell walls which adds colour to the fungal preparation thereby enhancing and contrasting the structures.
- **Glycerol** is a viscous substance that prevents drying of the prepared slide specimen.

Composition of LPCB mount

Ingredients	Amount
Lactic acid	20 mL
Glycerol (or glycerine)	40 mL
Phenol crystals or	20 g
phenol concentrate	20 mL
Distilled water	20 mL
Aniline blue or	0.05 g
1% aqueous solution (This is analogous to cotton blue.)	2 mL



THANKS