MICROBIOLOGICAL STAINS & STAINING TECHNIQUES

Microbiology I

27.1.21

Types of stains & principles of staining

- Microorganisms are colorless as their cytoplasm is transparent.
- Although living microorganisms can be directly examined by wet-mount and hanging-drop techniques
- They are commonly stained to **increase visibility** through bright-field microscope.
- **Staining:** coloring the microorganisms with a dye.
- Used to study:
  - **Shapes**
  - **Cellular arrangements**
  - **Internal cellular components**
  - **Differentiating microbial species**

STAINS OR DYES

- ***STAINS or DYES are organic compounds which carries either positive charges or negative charges or both.***
- ***They adheres to a cell, giving the cell color as different stains have different affinities for different organisms, or different parts of organisms so they are used to differentiate different types of organisms.***

- ***Based on the charges:*** *the commonly used stains are salts.*
  - ***Basic stain/dyes:*** *stain with +ve charge, i.e., colored cation + colorless anion. E.g., methylene blue chloride*
  - ***Acidic stain/dyes:*** *stain with –ve charge, i.e., colored anion + colorless cation. E.g., Eosin- + Na+*
  - ***Neutral stain/dyes:*** *stain with both charges. E.g., Eosinate of Methylene blue*

- ***Bacterial cells are slightly negatively charged at pH 7.0***
  - *So +ve charged Basic dye stains bacteria*
  - *And Acidic dye stains background*

Nature of stains:

- Stains have complex molecular structure & are benzene derivatives.
- Stains has 3 constituents:
- **Benzene**: organic colorless solvent
- **Chromophore:** chemical group that imparts color to benzene
- **Auxochrome:** chemical group that intensifies the color of the chromogen.

Classification of stains:

- Based on origin:
  - natural stain
  - synthetic stain
- Based on purpose of use:
  - Direct or general
  - Indirect stains
  - Selective stains
  - Differential stains
- Based on staining activity:
  - Nuclear stains
  - Cytoplasmic stains
  - Histological stains
- Based on charges:
  - Acidic stains
  - Basic stains
  - Neutral stains

Types of stains / dye

- Basic dye:
- Methylene blue
- Crystal violet
- Safranin
- Basic fuchsin
- Malachite green.

- Acidic dye:
- Eosin
- Nigrosin
- Rose Bengal
- Congo red
- Acid fuchsin

Neutral dye:

- Eosinate of methylene blue

Basic dyes

Methylene Blue

Crystal violet

Safranin

Malachite green dye

Basic Fuchsin

Acidic dyes

Eosin

Nigrosin

Acid Fuchsin

Rose Bengal

Congo Red

PRINCIPLE OF STAINING:

- *Each staining methods have own principles but the following steps may be common:*

- *Stains → combine chemically with the bacterial protoplasm.*

- *Basic stain(+ve charge) –*

  To stain -ve charged molecules of bacteria

  Mostly used because cell surface is –ve charge.

- *Acidic Stain(-ve charge)*

  To stain +ve charged molecules of bacteria.

  Used to stain the bacterial capsules.

- *As cell surface is –ve charged: Basic dyes are mostly used.*

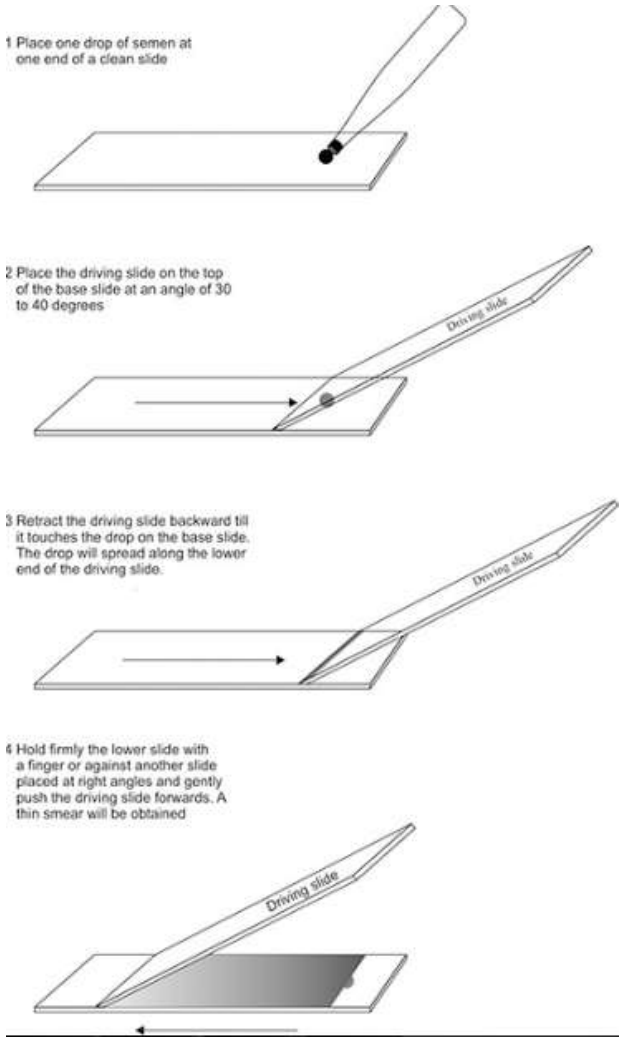
Preparation of Materials for Staining

- The essential steps in the preparation of materials to be observed are

1. Preparation of smear
2. Fixation
3. Application of one or more staining solutions

Preparation of smear

- Place a droplet of bacterial suspension on a glass slide and spread it to form a thin smear.
- The smear is air dried to attach or fix the microorganisms to the glass slide.



Fixation

- Fixation kills the microorganisms and attaches them to the slide.
- This prevents washing away of microorganism in further steps of staining procedure.
- It also preserves various parts of microorganisms in their natural state with only minimal distortion.

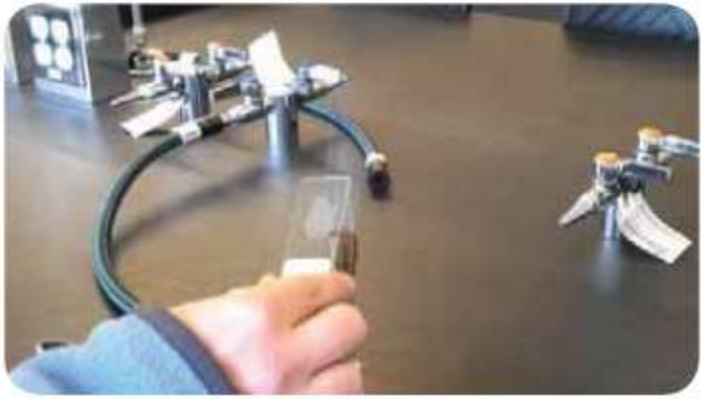
- Fixation methods:

➢ Heat fixation

➢ Chemical fixation

Heat fixation

- In this method the slide is gently heated by passed through a flame.
- Heat fixation will preserve the overall morphology of the cell without destroying the internal structures.



Chemical fixation

- It involves the use of chemical fixative to protect the fine cellular structures of delicate microorganisms.

- Chemical fixatives:

➢Ethanol

➢Acetic acid

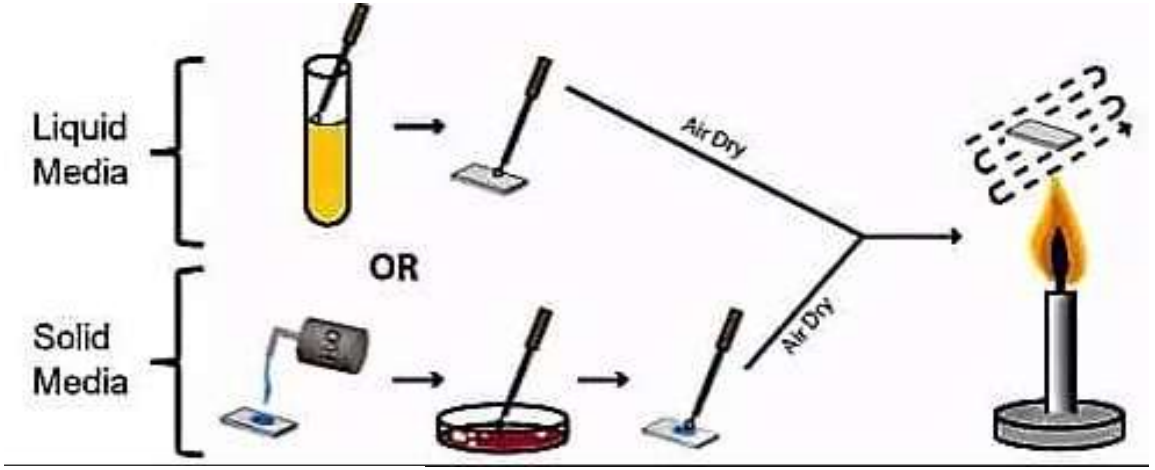
➢Formaldehyde

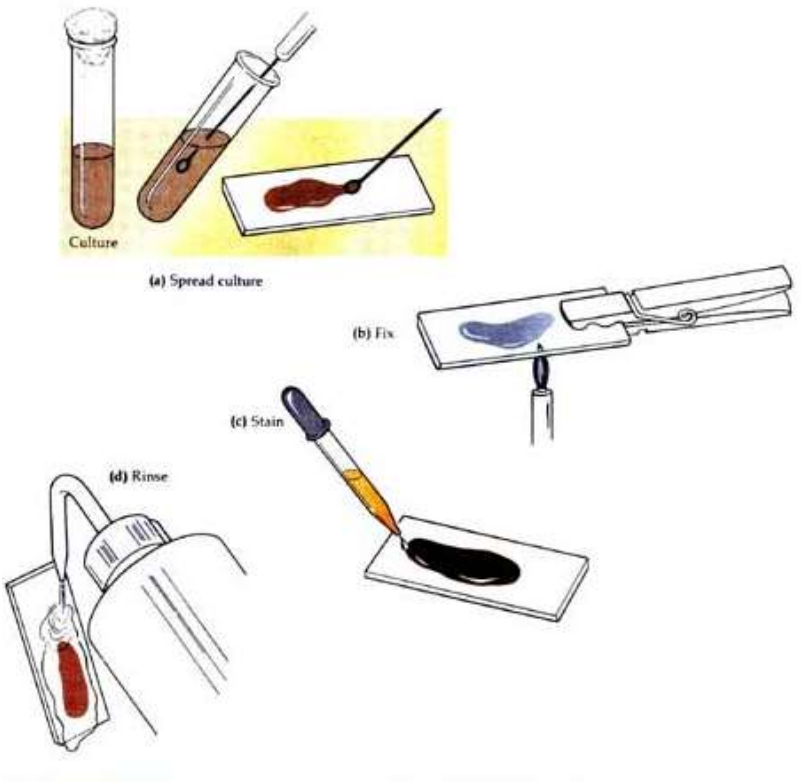
➢Glutaraldehyde

➢Mercuric chloride

- After fixing the smear stain is applied for a specific time period rinsed-off with water and then blotted dry with absorbant paper for microscopic examination
- Staining increases the contrast between the specimen and the background.

Smear preparation & fixation

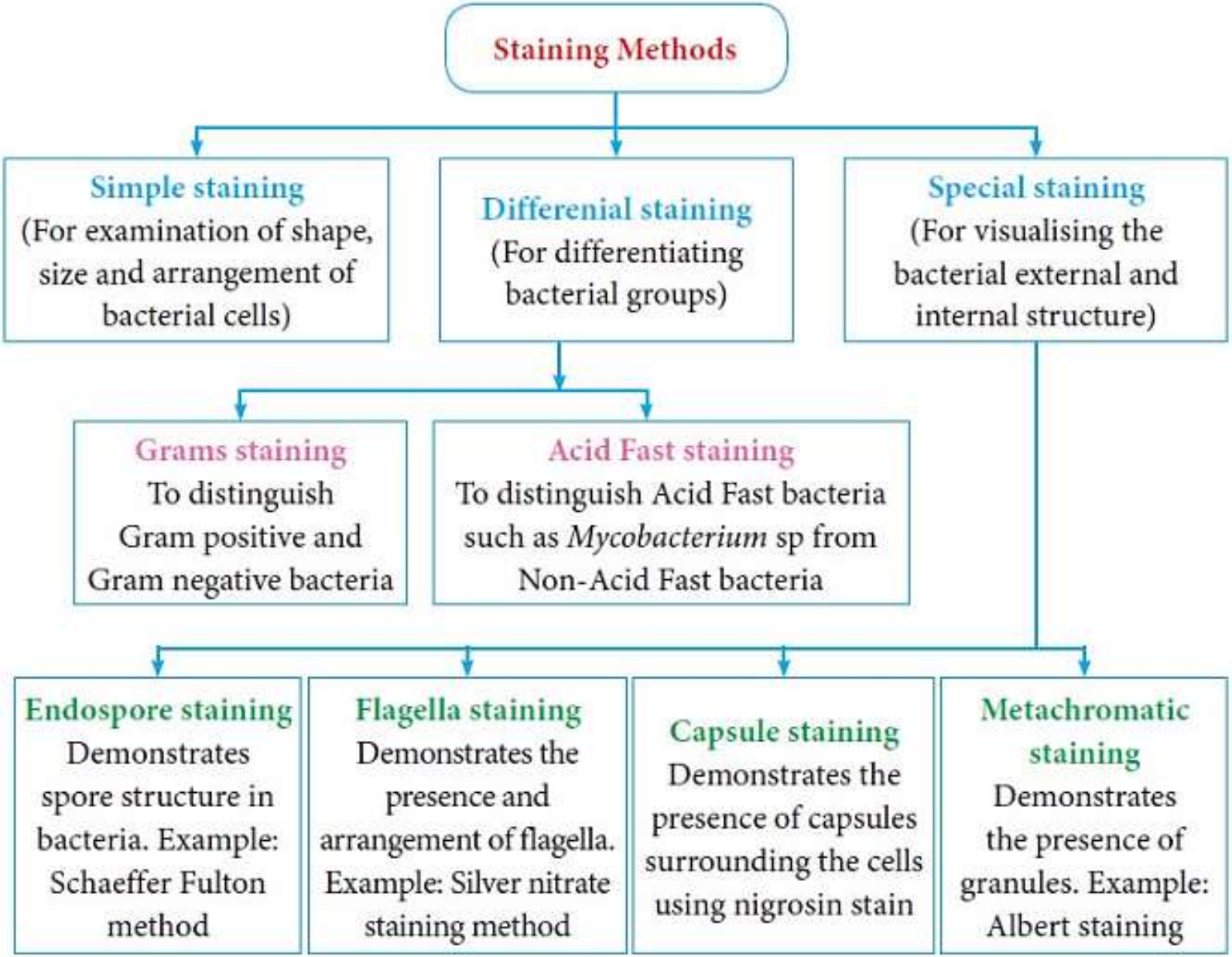




Staining

Bacterial Staining Methods

- Different staining methods are employed to study the bacterial morphology and to identify bacteria.
- Some methods are used for general purposes and others are used for special purposes.
- Staining methods:
  - Simple staining
  - Differential staining
  - Special staining


Staining Methods
Simple staining
(For examination of shape, size and arrangement of bacterial cells)
Differenial staining
(For differentiating bacterial groups)
Special staining
(For visualising the bacterial external and internal structure)
Grams staining
To distinguish Gram positive and Gram negative bacteria
Acid Fast staining
To distinguish Acid Fast bacteria such as *Mycobacterium* sp from Non-Acid Fast bacteria
Endospore staining
Demonstrates spore structure in bacteria. Example: Schaeffer Fulton method
Flagella staining
Demonstrates the presence and arrangement of flagella. Example: Silver nitrate staining method
Capsule staining
Demonstrates the presence of capsules surrounding the cells using nigrosin stain
Metachromatic staining
Demonstrates the presence of granules. Example: Albert staining

Simple staining

1. Direct staining
2. Indirect staining (Negative staining)

SIMPLE STAINING:

- Simple to perform- only one basic stain used.

E.g. Crystal violet, Methylene blue, Basic fuschin etc.,

- **Principle:**

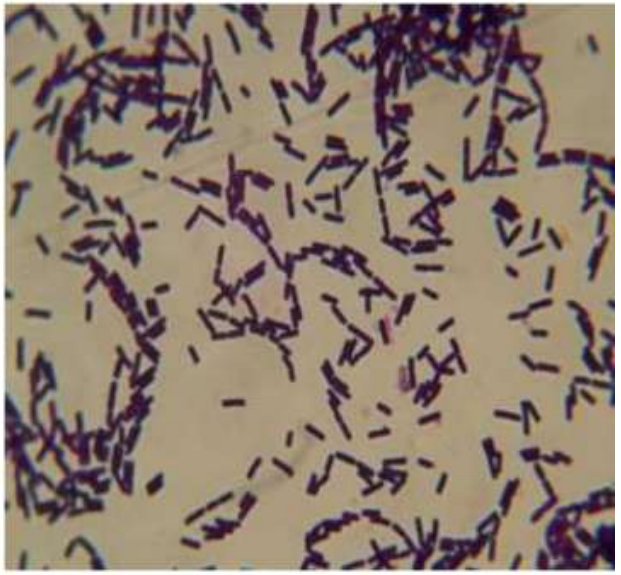
  - All bacteria in smear takes stain and appears in colour of stain.
  - Basic stain more affinity towards bacterial surface & stains the bacteria.

- **Uses:**

  To study morphology and arrangement of bacteria.

PROCEDURE:

- A bacterial smear is prepared, air-dried and heat-fixed.
- A Heat-fixed smear is flooded with either one of the basic stain and allowed to react for 1-2 minutes and then washed under running tap water.
- Air dried and focused with 10x,45x & 100x.

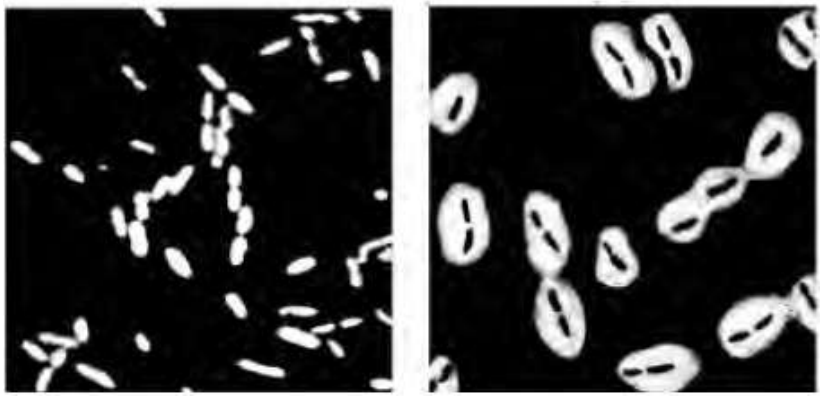


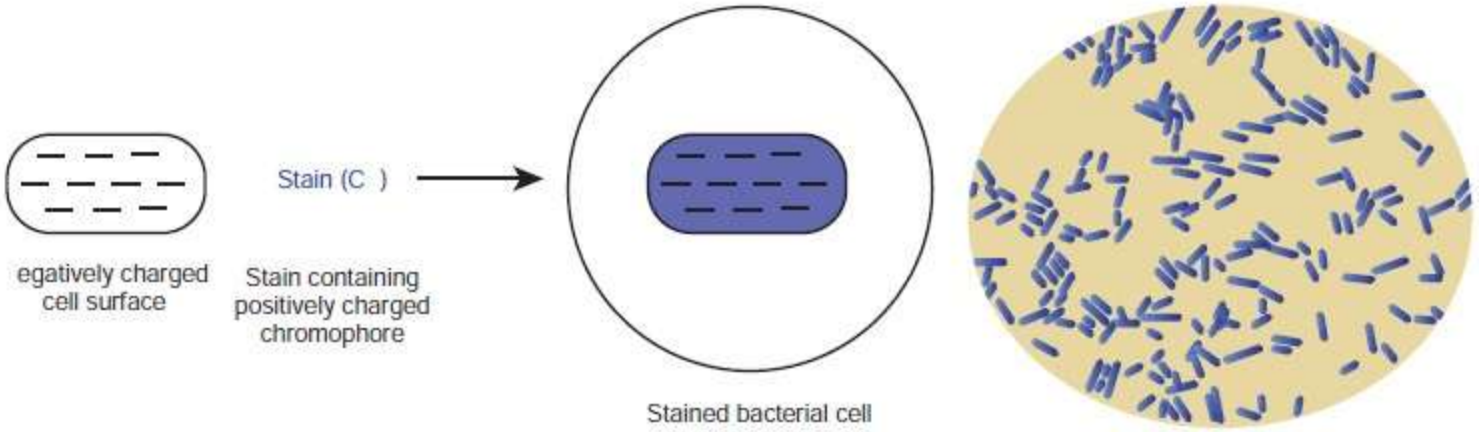
Negative or indirect staining technique

- This technique is also used for light microscopy
- Bacteria are mixed with an acidic dye such as nigrosin (a black stain) or Congo red (a red dye).
- The mixture is then spread out in a thin film on a slide and allowed to air-dry.
- Acidic dye has a negatively charged chromophore that is repelled by

the negatively charged microorganisms so that it gathers in the background.

- This results in the negative or indirect staining of the microbial cell observed as clear or white cells against a contrasting dark background.
- Since this technique avoids heat fixing and chemical reactions, the cell size and shape appear less shriveled and less distorted.





Direct Staining

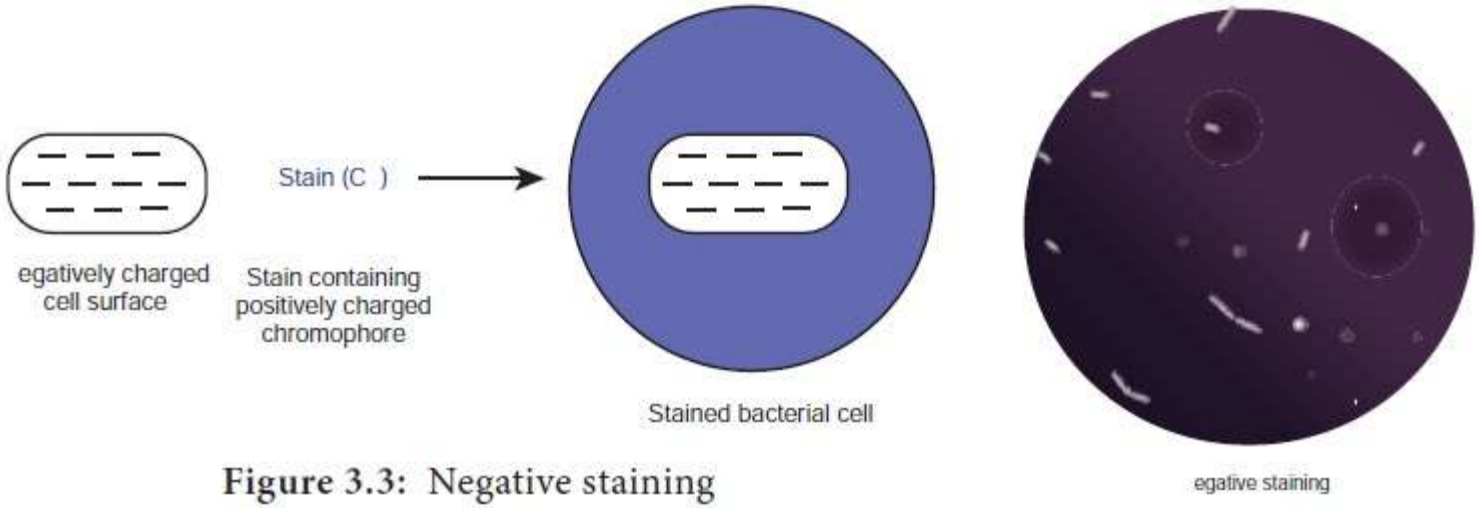


Figure 3.3: Negative staining

28.1.21

Differential staining

Differential staining

- In this method more than one stain is employed.
- Differences b/w the cells or parts of a cell
- Used for identification

Two techniques:

- **Gram staining**
- **Acid Fast staining**

Table 3.3: Differences between Simple and Differential Staining

Simple staining	Differential staining
1. This method uses only one stain.	This method uses more than one stain.
2. It imparts only one colour to all bacterial cells.	It imparts two or more different colours to bacterial cells.
3. It reveals the size, shape and arrangement of bacterial cells.	It reveals the size, shape and arrangement. In addition, it differentiates two groups of bacteria.
Example: Methylene blue staining method.	Example: 1. Gram's staining method 2. Acid Fast staining method

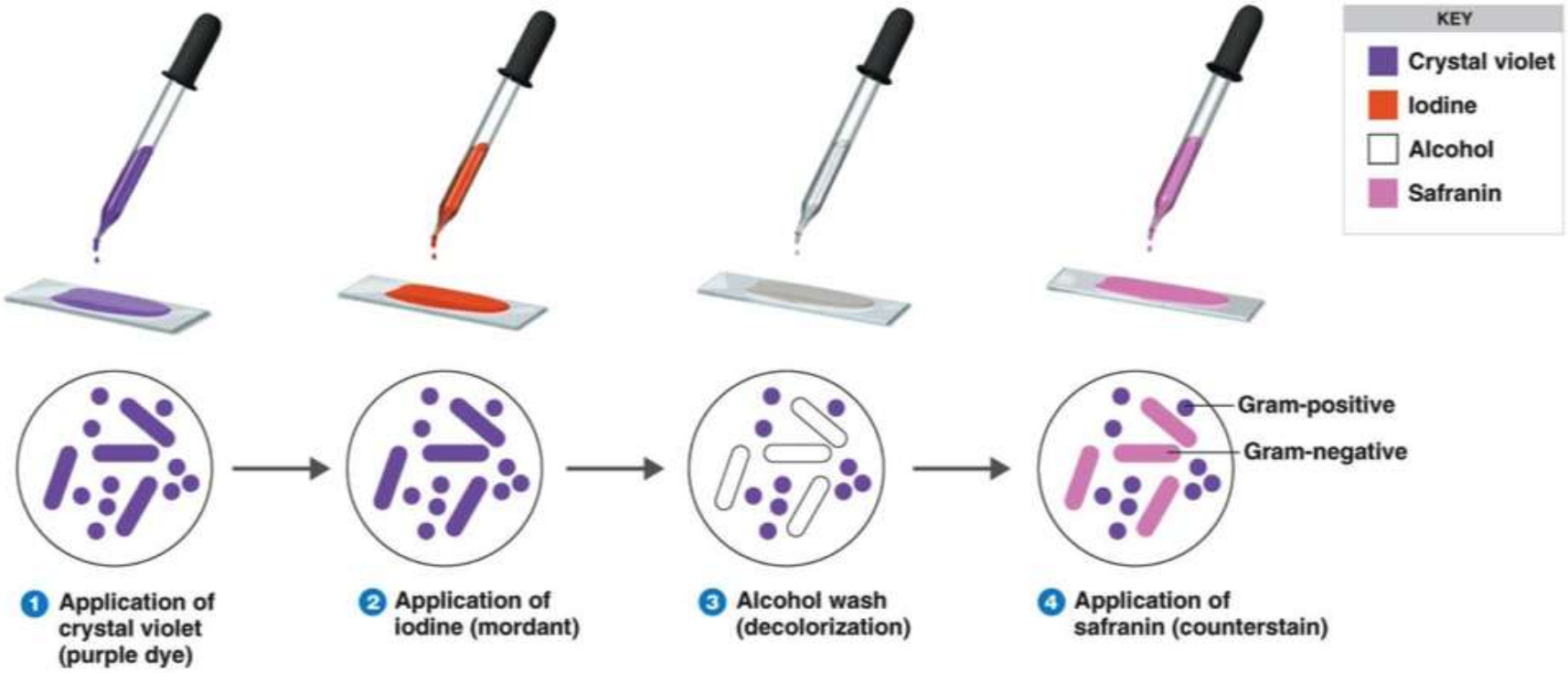
Grams staining Technique

- Developed by Danish Bacteriologist Hans Christian Gram in 1884.
- Most widely employed staining method in bacteriology.

- It classifies bacteria into two large groups:
  - **Gram positive** and
  - **Gram negative**.

Gram staining method:

- Fixed bacterial smear is subjected to staining reagents in the order of sequence:

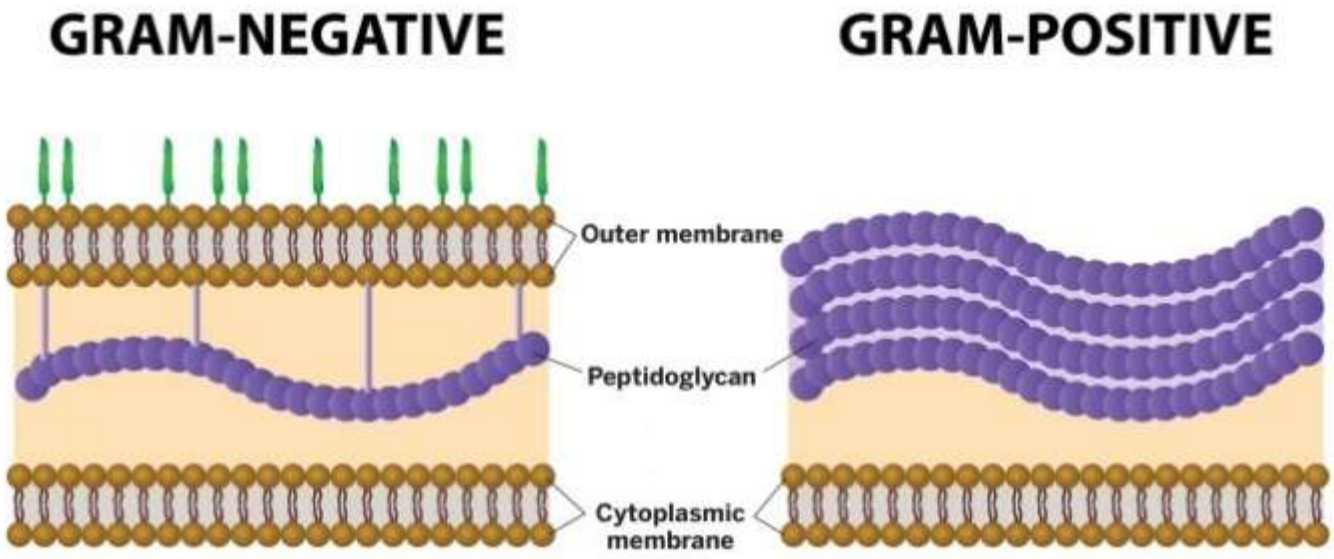


- **Gram positive:**
- The organisms that retain the colour of the **primary stain** are called Gram positive
- **Gram negative:**
- Organisms that do not retain the primary stain when decolorized and take on the colour of the **counter stain** are called Gram negative.

- **Mordants:**
- Mordants are not dyes.
- It increases the interaction between the cell and the dye so that the cell is stained more strongly.
- Ex. **Iodine**.

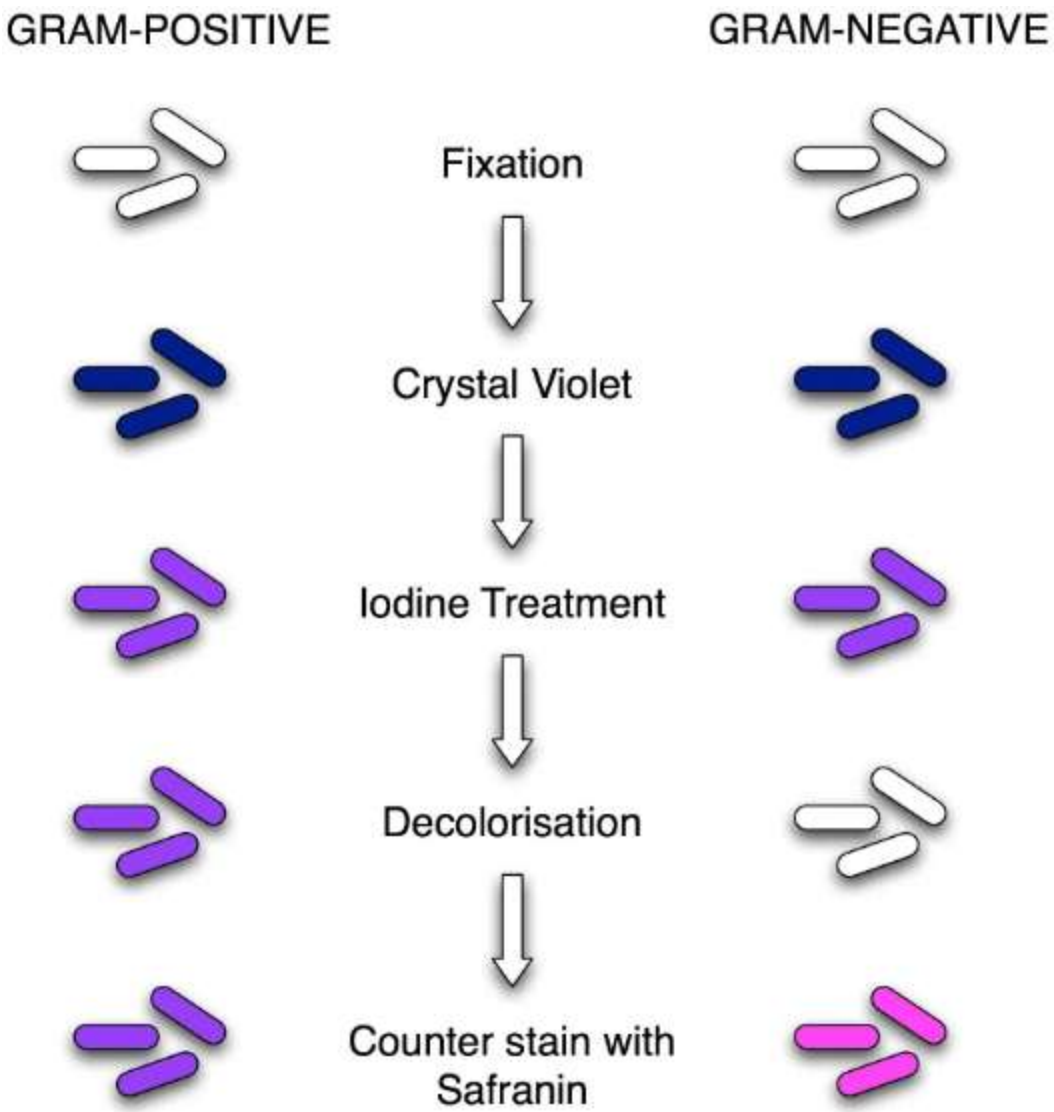
Principle of Gram's Staining

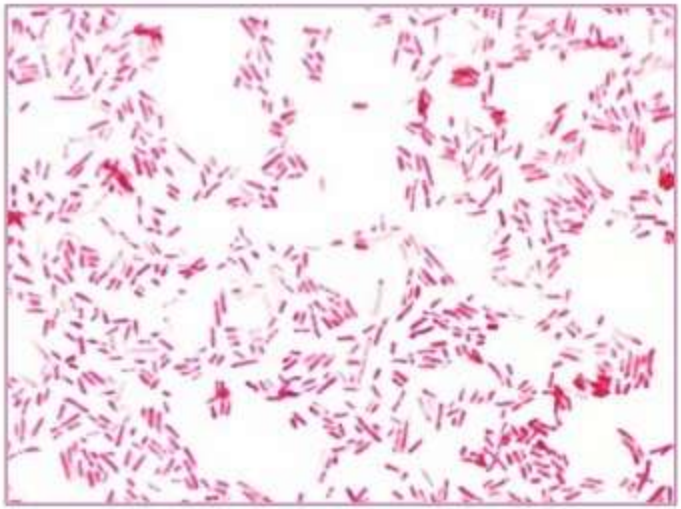
- Gram positive bacteria: have thick cell wall – **peptidoglycan**.
- Gram negative bacteria: contain **lipopolysaccahrides** layer and thinner cell wall.
- Crystal violet + iodine forms **CV-I complex**



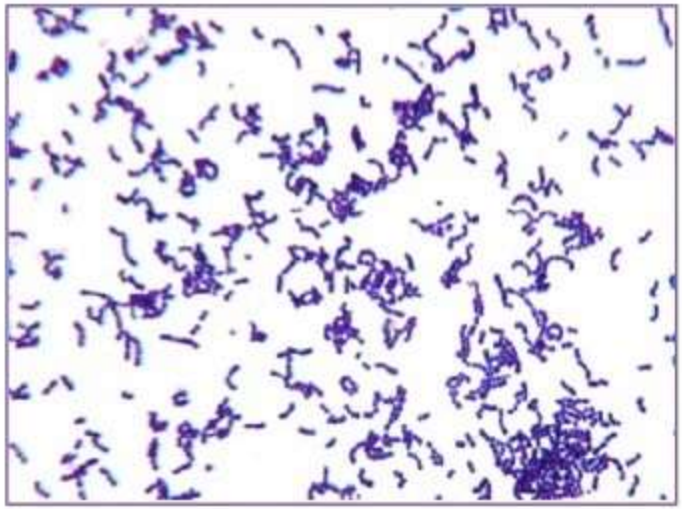
- **Gram positive bacteria**:
- Cell wall - lower lipid content, get dehydrated during alcohol treatment.
- pore size decreases, permeability is reduced.
- CV-I complex cannot be extracted and the cells remain violet.

- **Gram negative bacteria:**
- alcohol treatment – extracts lipid – increased porosity or permeability of cell wall.
- crystal violet iodine [CV-I] complex – extracted --- bacteria decolorized.
- Cells take up the counter stain – safranin – appear pink in color.


GRAM-POSITIVE
GRAM-NEGATIVE
Fixation
Crystal Violet
Iodine Treatment
Decolorisation
Counter stain with Safranin



Gram-Negative Bacteria



Gram-Positive Bacteria

- **Gram-variable reaction**:
- Old cultures (>24-48h) of Gram-positive bacteria lose the ability to retain the crystal violet
- hence will be stained by the counterstain (safranin).
- A similar effect may also sometimes be due to changes in the environment of the bacteria.

Uses:

- Gram's staining technique is very important in medical microbiology.
- It provides valuable information for the treatment of disease.
- Gram-positive bacteria are susceptible to penicillin and sulphonamide drugs.
- Gram-negative bacteria are resistant to these drugs
- but susceptible to streptomycin, chloramphenicol and tetracycline.
- Gram staining can also be useful in the diagnosis of many infectious diseases.

Acid fast staining

Acid fast staining

- Differential stain 1st developed – Paul Ehrlich in 1882
- Modified by **Ziehl – Neelsen**.

- Certain bacteria like *Mycobacterium & Nocardia* cannot be stained by simple stain or Gram stains.

- They have **waxy components (mycolic acid)** of cell wall, limits permeability.

- They are stained by acid-fast stain.

- Acid-fastness: due to high lipid contents, difficult to stain.
- Hence they require heating with strong dye.

- Once the acid-fast bacteria are stained – difficult to decolorize with acid or alcohol.

- The method uses carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue.

- It’s a good identification tool – no. of harmless saprophytic bacteria.

Principle

- When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the *Mycobacterial* cell wall

- Application of heat causes carbol fuchsin to further penetrate 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)

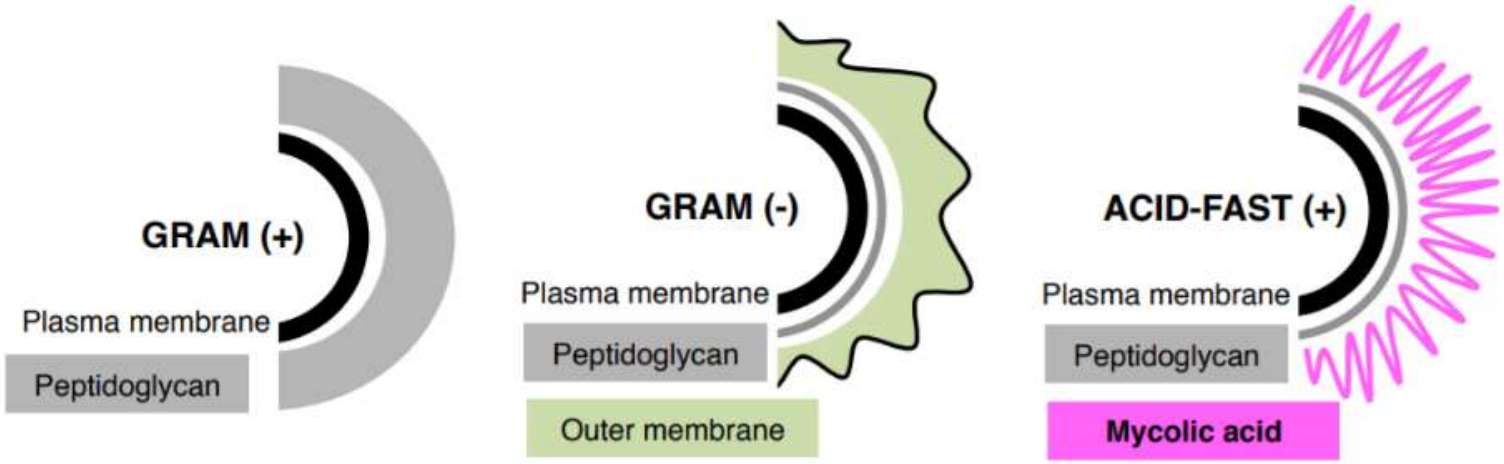
- The acid fast cells are resistant to decolorization as it contains lipidoidal material in large amount
- Thus prevents the entry of decolorizing solution.

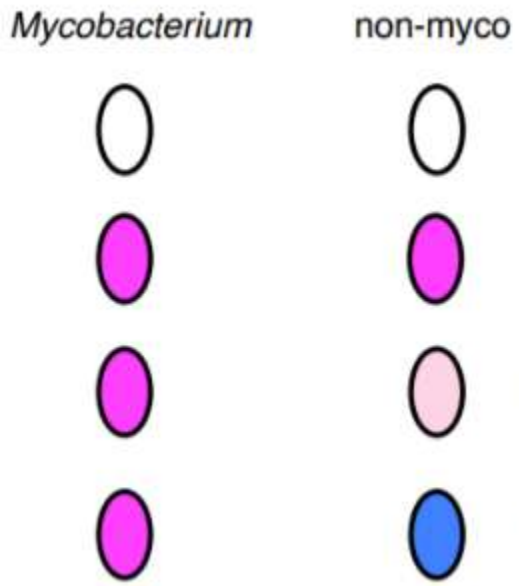
- The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless.

- 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.

Acid-Fast bacteria:

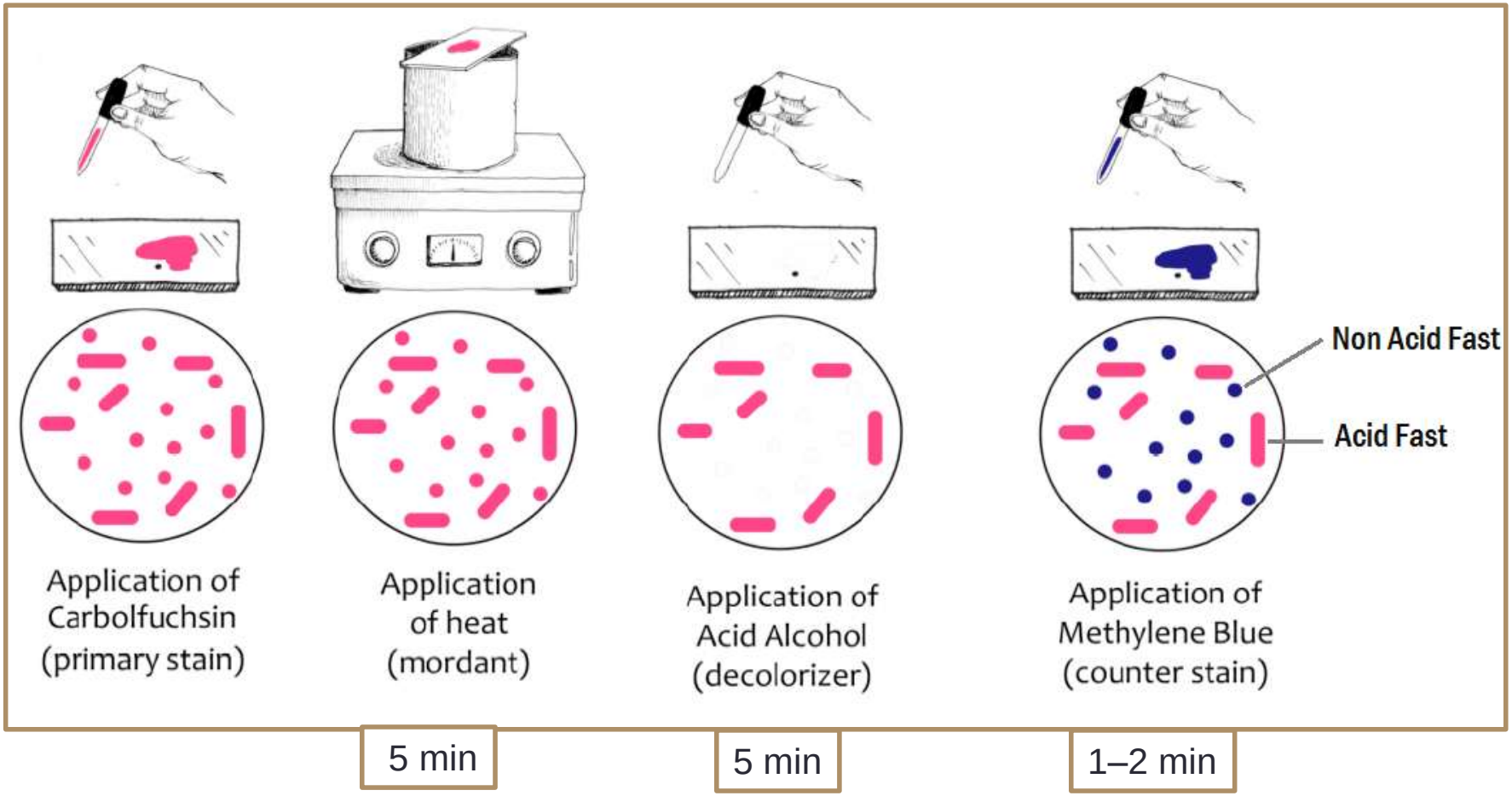




1. All bacteria are transparent prior to staining
2. Steam is used to force the CARBOL FUCHSIN into all the bacteria
3. Decolorization with acid removes the CARBOL FUCHSIN from the bacteria that do not have mycolic acid
4. Methylene blue is the counterstain

PROCEDURE (Ziehl – Neelsen method)

Prepare smear → Air dry → heat fix → staining procedure as follows:

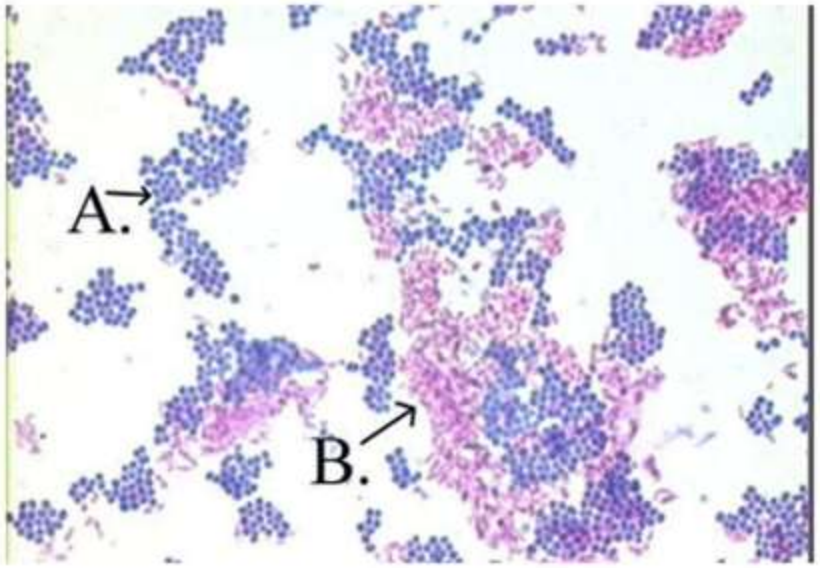


Examine the smear microscopically – 100 X oil immersion objective

Summary of Acid-Fast Stain

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

Microscopic observation of acid fast bacteria



A – non-acid-fast bacteria
(*Staphylococcus epidermidis*)

B – acid-fast bacteria
(*Mycobacterium gordonae*)

Uses

- 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.