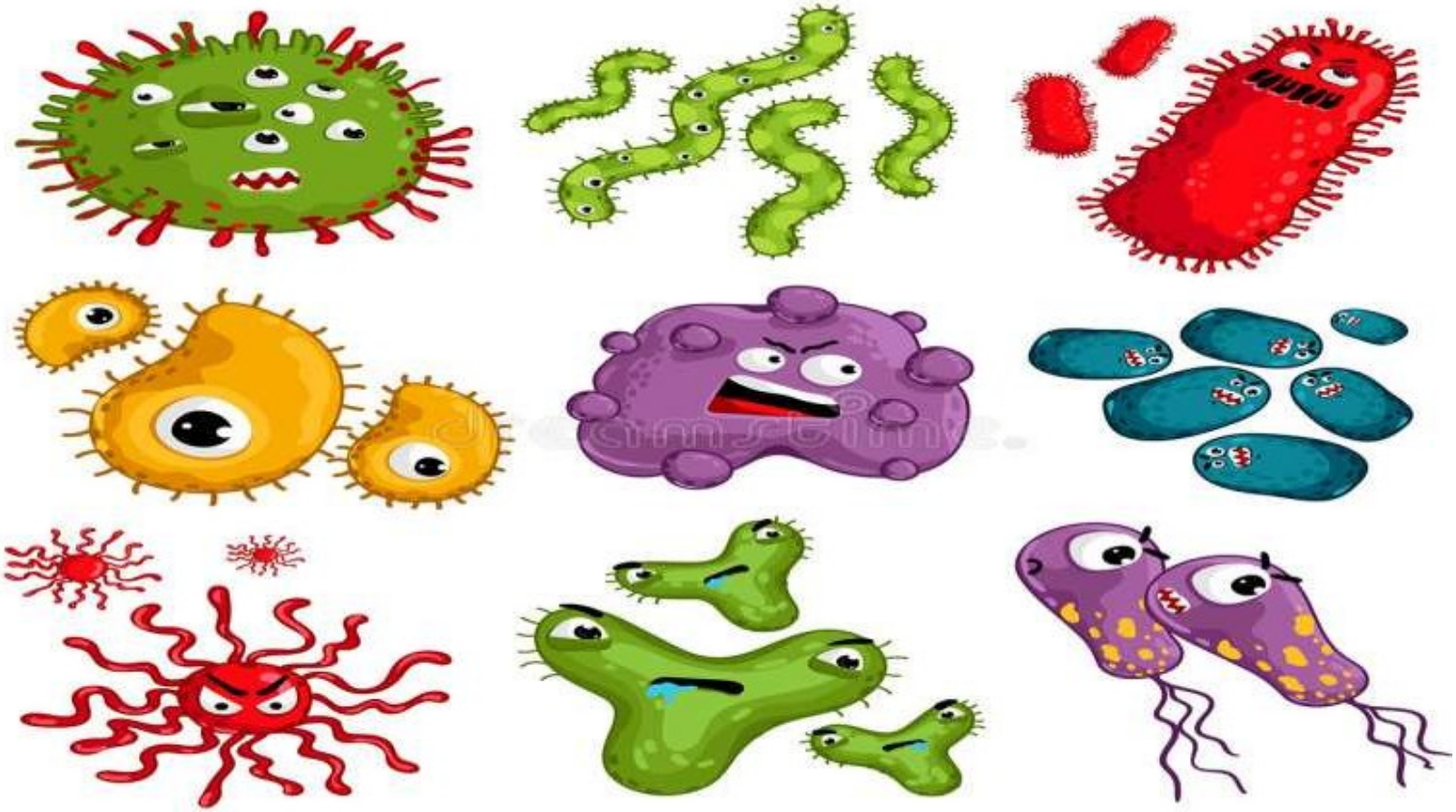
IDENTIFICATIO N OF BACTERIA



INTRODUCTION

- Identification of unknown bacterial culture is one of the major responsibilities of a microbiologist
- Samples of blood, water, food and cosmetics are examined daily in laboratories throughout the world for the presence of contaminants and pathogenic microorganisms.
- Pharma industries and research institutes are constantly screening soil, water, marine samples to isolate new antibiotics, enzymes and vitamins producing microorganisms.

Important

- **Medical diagnostics & clinical significance — identifying a pathogen isolated from a patient.**
- **Food & brewage industries — identifying a microbial contaminant responsible for food spoilage and fermentation.**

IDENTIFICATION METHODS

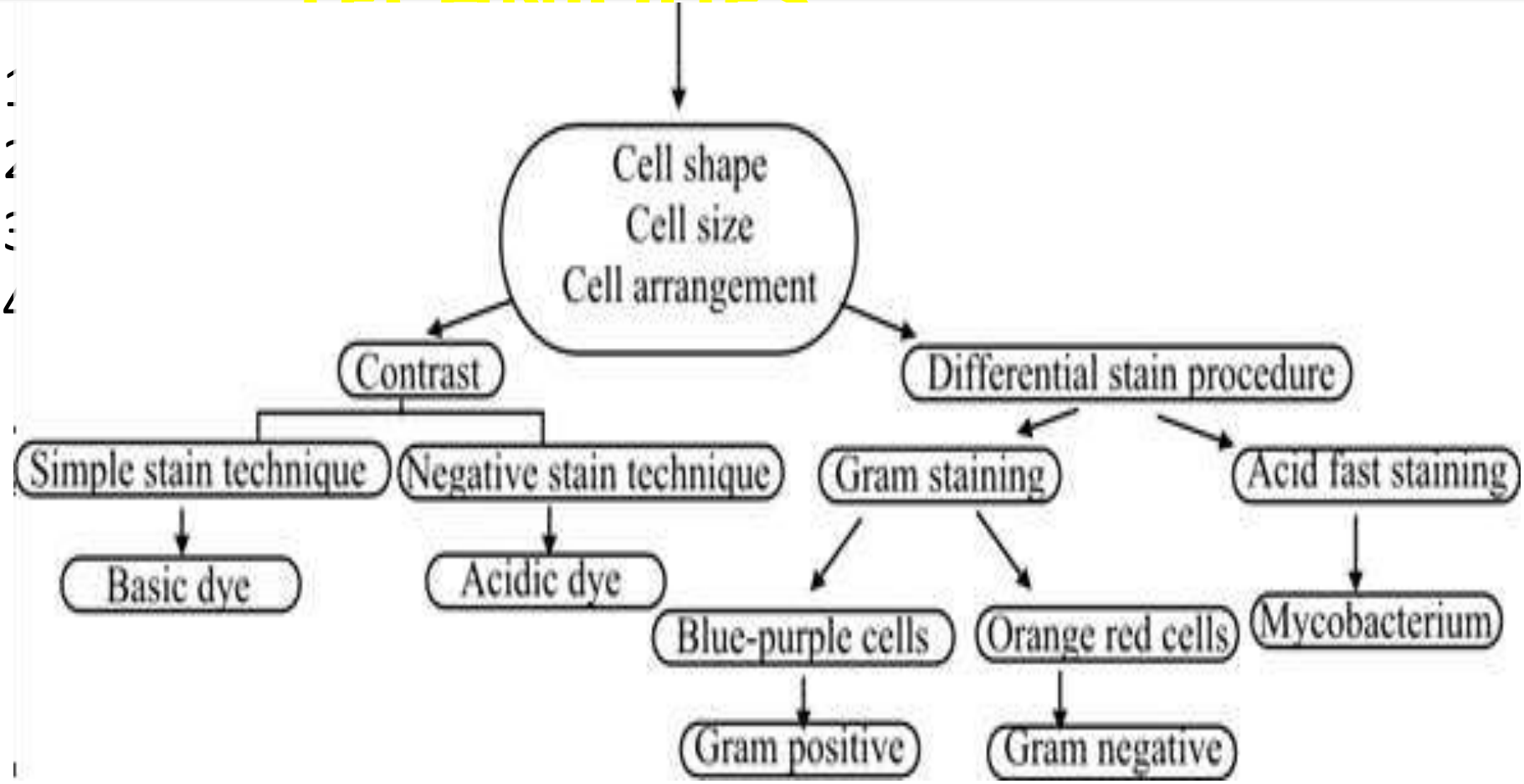
- The methods fall intotwo categories:

- **Staining Techniques**
- **Biochemical reactions**

STAINING REACTIONS

- To study **size, shape, arrangement of microorganisms.**
- **Stains is an organic compounds containing a benzene rings with chromophore.**
- **Different types of staining techniques are used to study the morphological and structural properties of microorganisms.**

STAINING TECHNIQUES



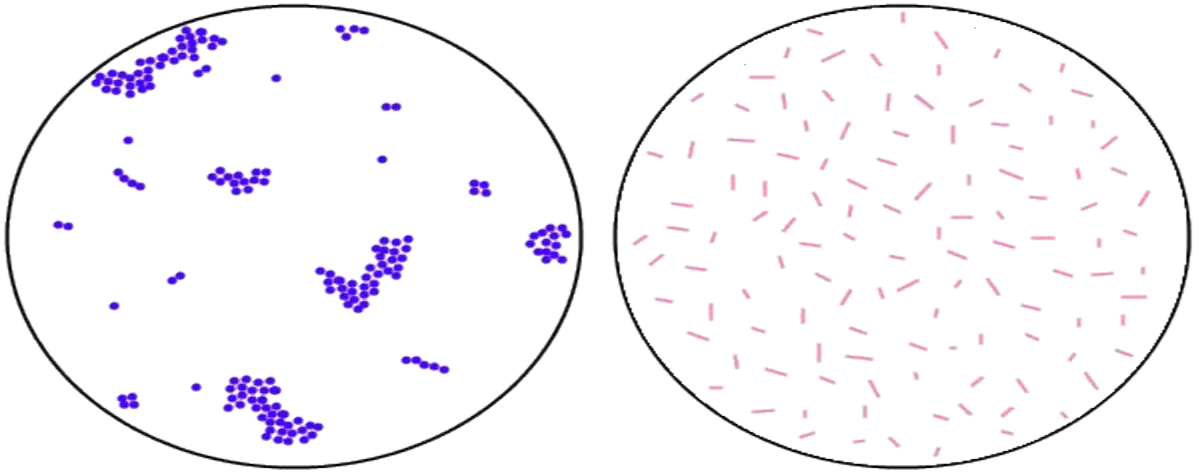
Dyes

- **Basic dyes:** Methylene blue, Basic fuchsin, Crystal violet, Safranin, Malachite green.
  - Have **positively charged groups**.
  - Basic dyes bind to negatively charged molecules such as nucleic acids, many proteins and surfaces of bacteria.
  - Used for Positive staining

- **Acidic dyes:** Eosin, Nigrosin (Indian ink), Congo red and Acid fuchsin possess groups such as carboxy (-COOH) and phenolic hydroxy (-OH).
  - Acidic dyes, in their ionized form, have a negative charge and bind to positively charged cell structures.
  - Used for negative staining

1. SIMPLE STAINING

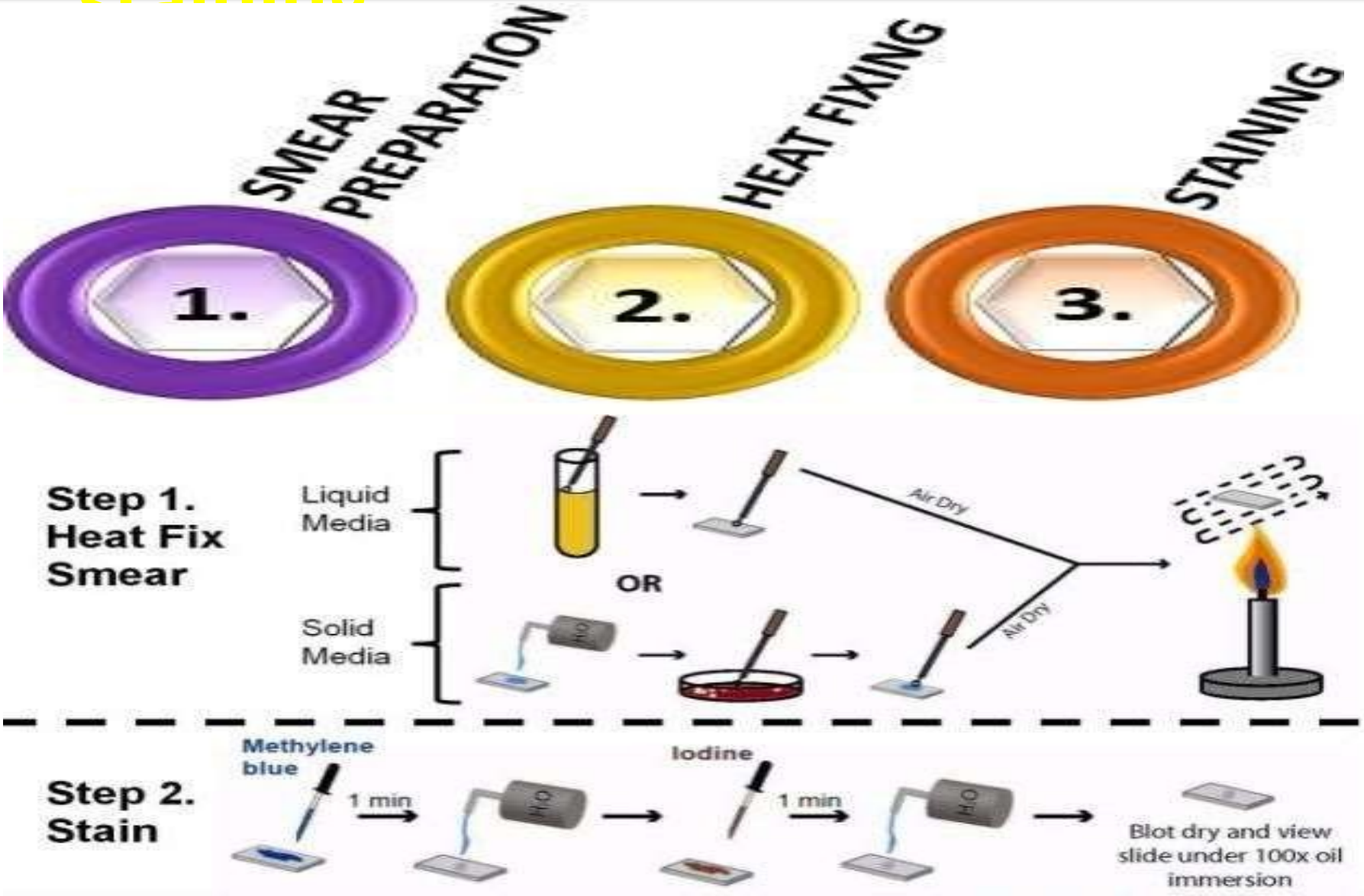
- Used only **single stain**
- Ex: Any one at a time- Methylene blue (2-3mins), Crystal violet(1-2mins), Carbol fuchsin(15-30secs), Safranin (1mins), etc.
- **Used to study Size, shape and bacterial cell arrangements.**
- **Basic stains** with a **positively charged** chromogens are used.



PROCEDURE

- Select oil/grease free slide. Do it by washing with detergent and wiping the excess water and then dry the slide by passed through flame.
- These slide is allowed to air dry and smear of sample is applied.
- After air drying these slide is rapidly passed through a flame for three to four times for heat fixation.
- After heat fixation the slide is flooded with a particular stain and these stain is allowed to react for two-three minutes.
- Further the slide is washed under running distilled water.
- The slide is air dried and observed under oil immersion lens in microscope.

Steps involved in simple staining

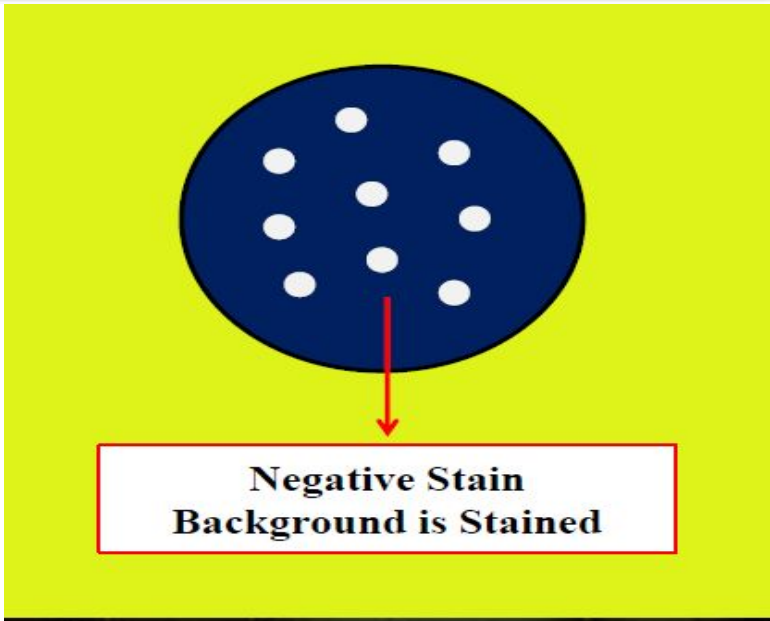


NEGATIVE STAINING

- Colouring the Background of Object
- Not stain but made visible against dark background
- Bacteria are mixed with **acidic stains** such as Eosin or Nigrosin that provide a uniformly coloured background against which the unstained bacteria stand out in contrast.
- Useful to observed bacteria that are difficult to stain **(Spirili & spirochetes– *Trepanoma palladium*)** and in demonstration of bacterial capsule.
- **Acidic stains** has negative charge; therefore, it doesn't combine with negatively charged of bacteria cell surface.

Advantages over Simple:

1. Natural size and shape of microorganism can be seen
2. Heat fixation is not required
3. Doesn't need physical and chemical treatment.
4. It is possible to observe bacteria that are difficult to stain.



HERO OF GRAM STAINING

Dr. HANS CHRISTIAN GRAM:

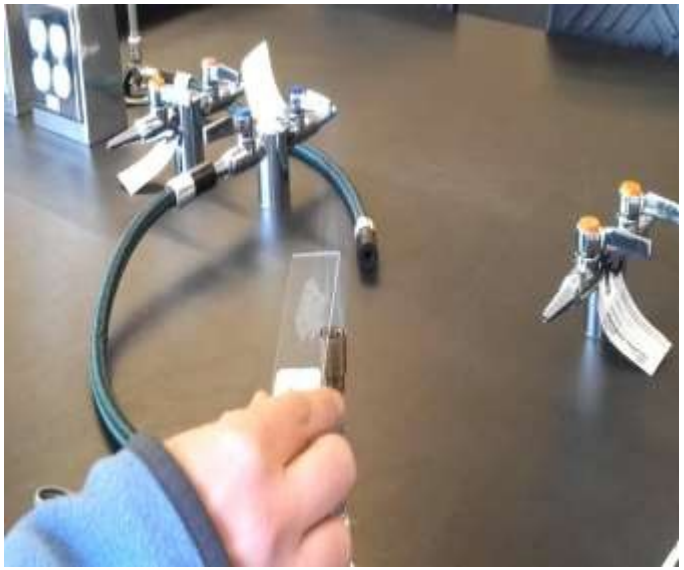
- Danish bacteriologist noted for his development of the Gram stain in 1884.
- It is used to differentiate bacterial species into two broad groupes , Gram positive & Gram negative based on the physical property of their cell wall.

GRAM STANING

- It is not only reveals the size and shape of bacteria but also used to differentiate bacteria into Gram positive and Gram negative cells. Hence, called **differential staining.**
- It is **first** and usually the only method employed for the diagnostic identification of bacteria in clinical specimens.
- Provides more information about the characteristics of the cell wall (Thickness).
- A stain is a chemical substance that adheres to a cell, giving the cell colour.

Why heat fixation?

- To preserve the shape of the cells or tissue
- To prevent autolysis of the cell
- To adhere the bacterial cell on the slide properly
- To kill unwanted microbe attached to the edge of the slide.

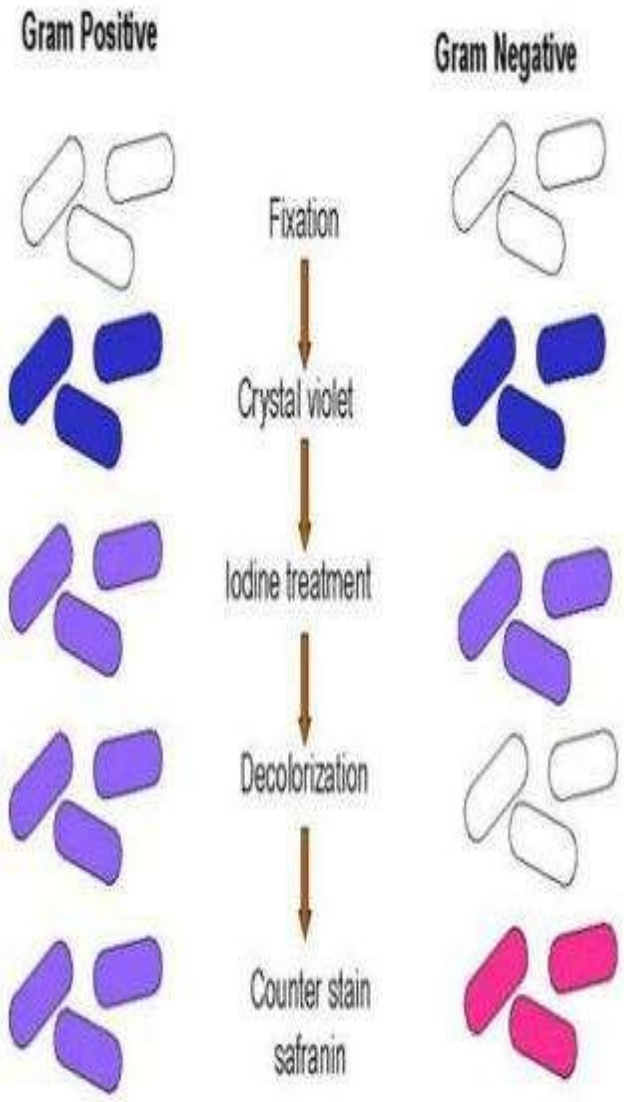


Procedure

- Preparation of smear
- Air dry and Heat fixation

- Step 1- Crystal violet (primary stain) for 1 minute. Water rinse.
- Step 2- Iodine (mordant) for 1 minute. Water rinse.
- Step 3 – Alcohol (decolorizer) for 10-30 seconds. Water rinse.
- Step 4 - Safranin (counterstain) for 30-60 seconds. Water rinse. Blot dry.

- Cells stain purple.
- Cells remain purple.
- Gram-positive cells remain purple. Gram negative cells become colorless.
- Gram positive cells remain purple. Gram-negative cells appear red.



Mechanism

- This peculiar response is related to physical and chemical difference in the cell walls of the two groups of bacteria.
- In gram negative bacteria, the cell wall is thin, multilayered containing high lipid contents
- which are readily dissolved by alcohol, resulting in pore formation in the cell wall leads to leakage of the crystal violet-iodine complex
- and resulting in discoloration of gram negative bacteria which takes safranin and appears red.

- On the other hand, cell wall of gram positive bacteria are thick, composed mainly proteins and cross linked mucopeptides.
- On the application of decolorizing agent, dehydration is caused resulting in closure of pores of cell wall thereby retaining the crystal violet-iodine complex and do appear blue or purple color.

Result

Gram positive organism produce violet or blue color
Gram negative organism produce red color

Ex of gram positive bacteria –Staphylococcus, streptococcus, Corynebacterium, nocardia sp.

HERO OF ACID FAST STAINING

- **Dr. PAUL ERHLICH,** was a German physician
- He is credited with finding a cure for syphilis
- In 1908, he received the Nobel Prize for his contributions to immunology.

ACID FAST STAINING

- Another widely used differential staining procedure in bacteriology developed by **Ehrlich in 1882.**
- Also known as **Ziehl–Neelsen staining.**
- Some bacteria(specially ***M. Tuberculosis, Actinomycetes, and M. leprae***) resist toward the gram staining or other staining due to high lipid content in the cell wall, hence they are called **ACID FAST BACTERIA.**
- **Because of high lipid content, acid fast cell have low permeability to dyes and hence difficult to stain.**
- **Heat(physical intensifier) are used to enhance the penetration of primary stain(chemical intensifier)**
- Classified into two group-
  - **A) ACID FAST BACTERIA**
  - **B) NON ACID FAST BACTERIA**
- Acid-fast cells contain a large amount of lipids and waxes in their cell walls
- **Primarily mycolic acid**

PROCEDURE

- A clean sterile glass slide was taken.
- A thin uniform smear was prepared in the slide using a inoculation loop.
- The smear was allowed to air dry and then heat fixed.
- The smear was flooded with Ziehl-Neelsen Carbol Fuchsin (ZNCF) and heated until steam arises.
- Preparation was allowed to stay for 5-7 min. The stain must not be allowed to evaporate or dry in the slide. Pour more carbol fuchsin on slide is necessary.
- It is then washed under a low steam of running water.
- The smear was decolorized with 3% HCl + 95% ethanol.
- The slide is then washed again under running distilled water.
- Counter stain the smear with methylene blue for the 2 min.
- Washed under running tap water.
- Slide was blot dried and examined under microscope.

Acid fast staining procedure

Take a clean grease free slide and prepare a smear

Air dry and heat fix the smear

Flood the slide with ZNCF stain

Place the slide on boiling water bath for 3-5 minutes

Continuously add ZNCF stain to avoid drying of smear

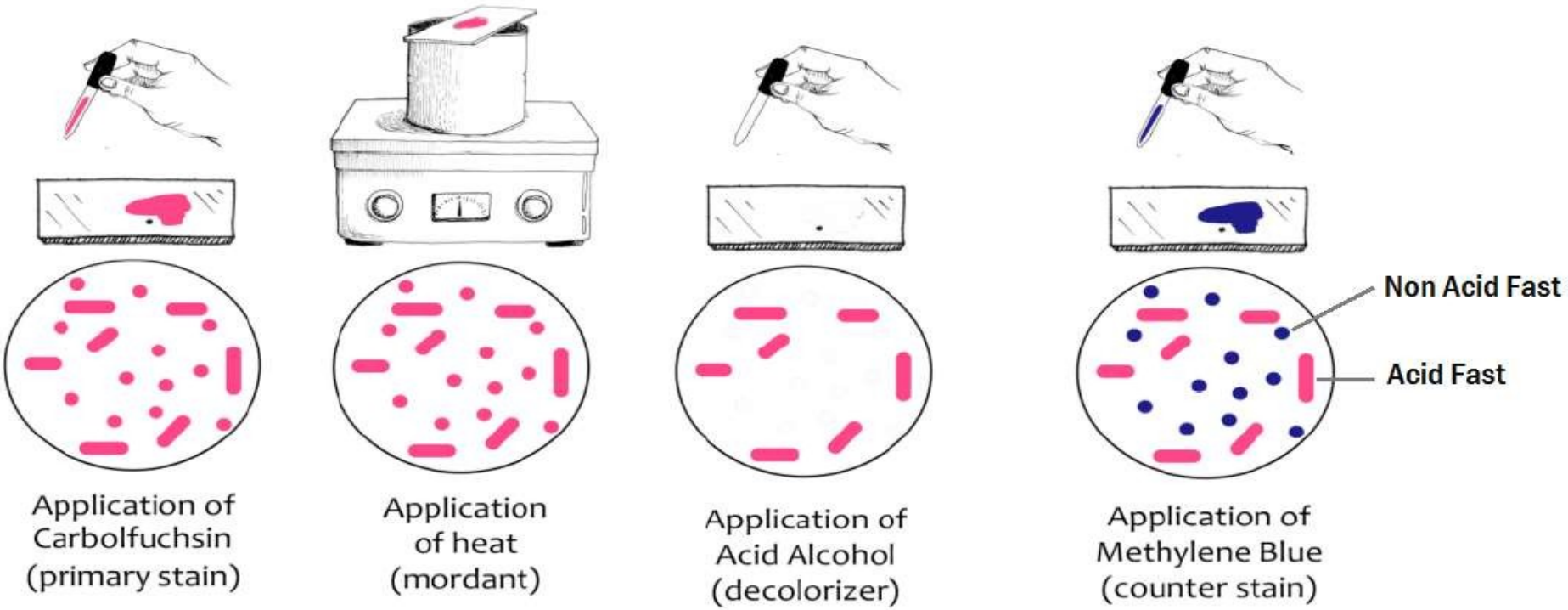
Treat the slide with decolourising agent

Give water wash treatment to the slide

Flood the slide with counter stain for 2-3 minutes

Give water wash treatment to the slide

Air dry and observe under oil immersion objective.



Application of Carbolfuchsin (primary stain)

Application of heat (mordant)

Application of Acid Alcohol (decolorizer)

Application of Methylene Blue (counter stain)