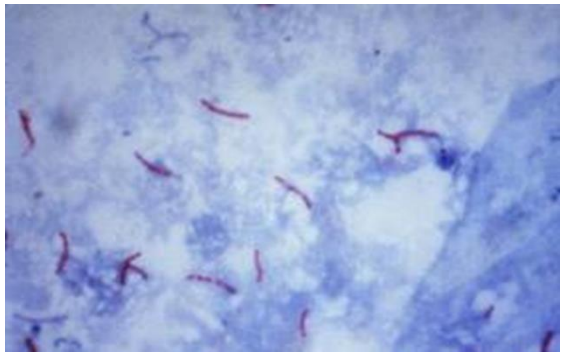
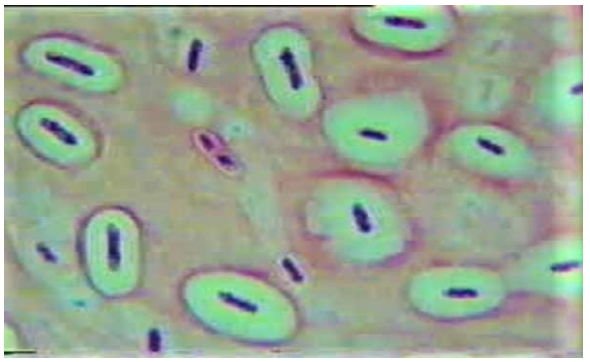
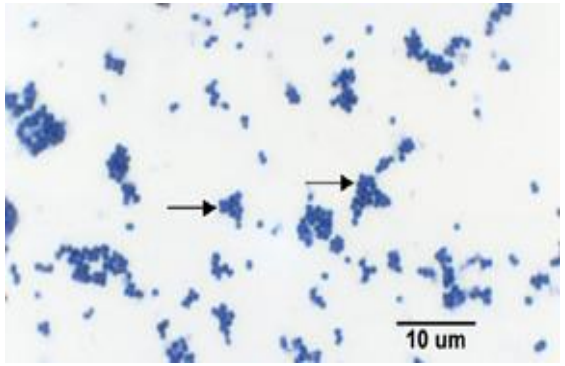
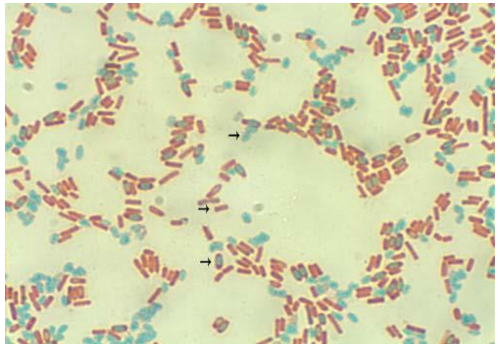
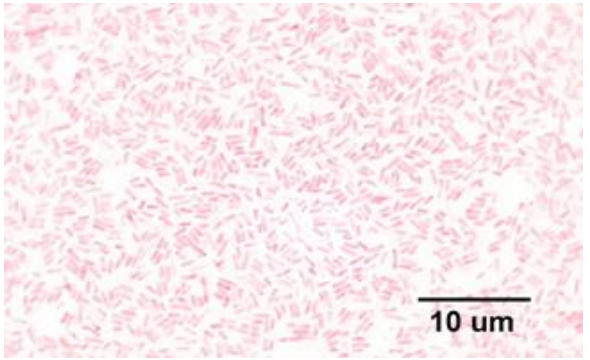




Staining of bacteria
(Gram stain & Acid fast stain)







OBJECTIVES

- Describe reagents used in Gram stain & purpose of these reagents
- Color expected of Gram Positive & Gram Negative after performing the procedure
- Explain reason of differential stain by Gram Positive & Gram Negative
- Describe cell wall structure of Gram Positive & Negative bacteria

Types of staining techniques:

Simple stains: is used only one dye

Differential stains: This staining method divided bacteria into two groups

Special stains: These are specialized staining methods to demonstrate certain bacterial components, e.g. spore, capsule, flagella stain.

Simple staining methods

Simple staining methods are used to demonstrate the morphology of bacteria like cocci, bacilli and arrangements of bacterial cells like chains, clusters, pairs.

They used a single basic stains e.g. crystal violet .

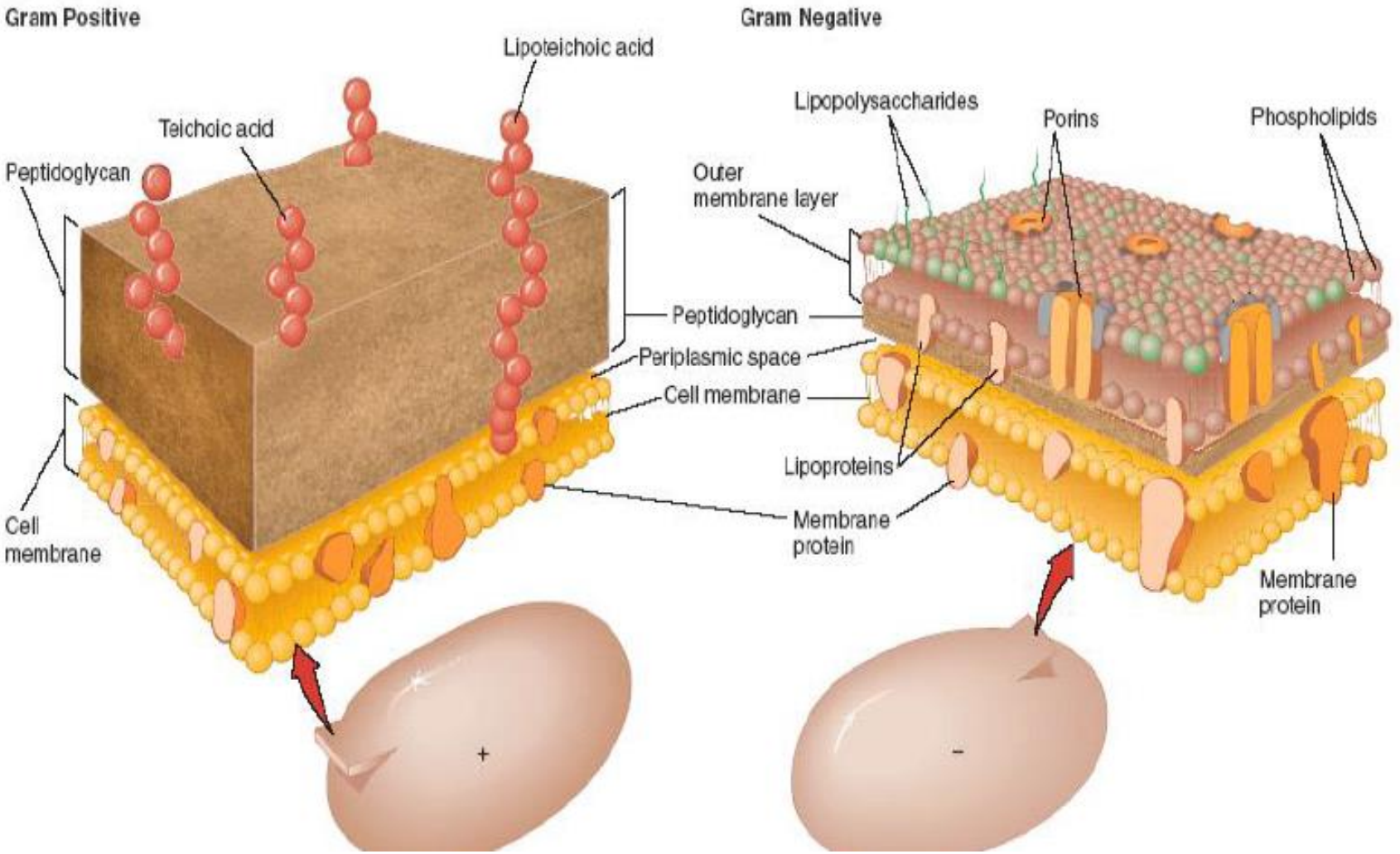
Differential staining methods:

1. Gram's stain

In 1884, Christian Gram, a Danish bacteriologist, described this staining method which is the most important stain in routine bacteriology.

It divided bacteria into two large groups the Gram positive and Gram negative bacteria.

CELL WALL OF GRAM POS & NEG



The Gram's stain reaction is based on the difference in the chemical composition of bacterial cell wall, and ability of the organism to resist decolourization with acetone, alcohol after the initial staining with one of the basic dyes and then treating with a mordant.

This shows the importance of cell wall in Gram stain reaction.

The primary basic dyes: commonly used are crystal violet.

The second dye: Iodine is the mordant used.

Third dye: A counterstain different in colour with the primary stain is used to stain Gram negative bacteria which are decolorized.

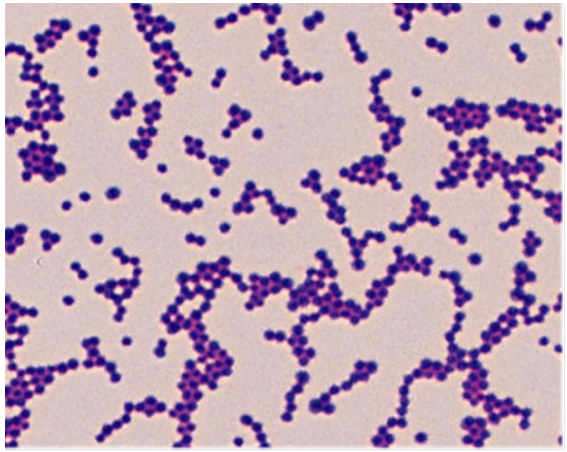
In a Gram stained smear Gram positive bacteria which retain the primary stain appear violet or blue-black or deep purple in colour.

The Gram positive reaction is due to the presence of thicker peptidoglycan layers in the cell wall.
thicker peptidoglycan layers which prevents the primary stain-mordant complex from being washed away by the decolorizing agent.

- **Gram negative bacteria**: These appear red. That have a high lipid content in cell wall which dissolves in the decolorizing agent.

- This allows the primary stain-mordant complex to be washed off.
- The red counter stain makes the decolorized Gram negative bacteria visible in a contrast colour.

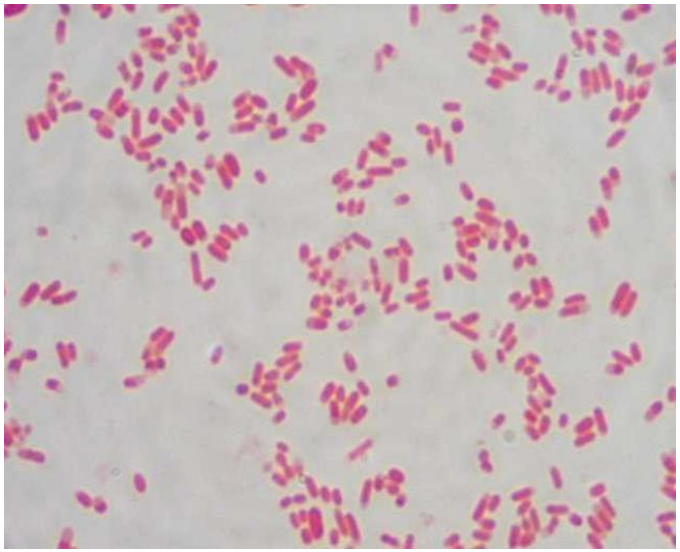
REAGENTS USED IN GRAM STAIN



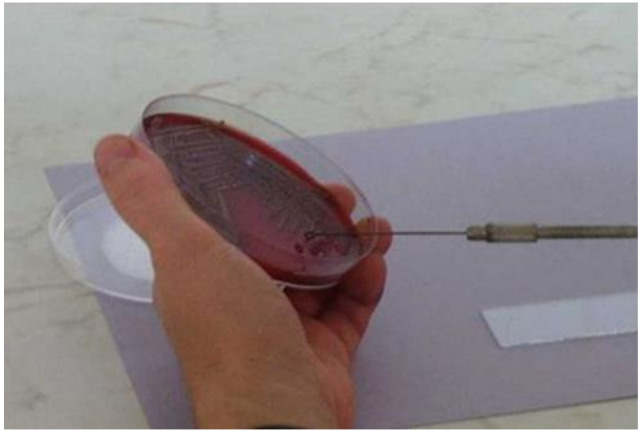
1. CRYSTAL VIOLET
   - Primary stain
   - Violet colored, stains all bacteria
2. Logus IODINE
   - Mordant
   - Forms Crystal violet iodine complexes
3. DECOLORIZER
   - Acetone + Methanol
   - Removes Crystal violet iodine complex from thin peptidoglycan layers
   - Dissolves outer layer of Gram negative bacteria

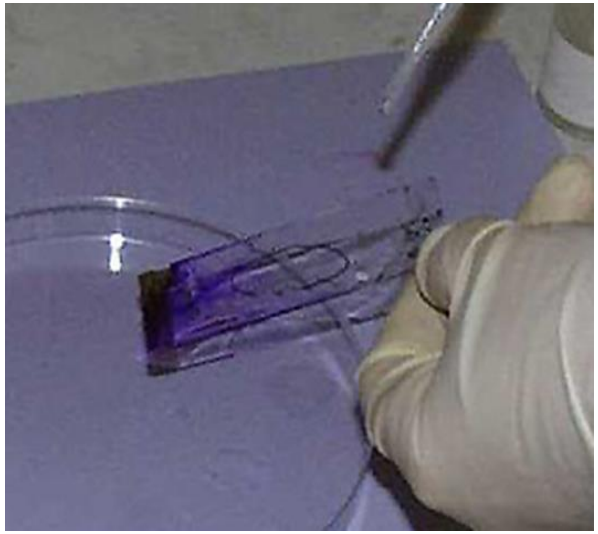
REAGENTS USED IN GRAM STAIN

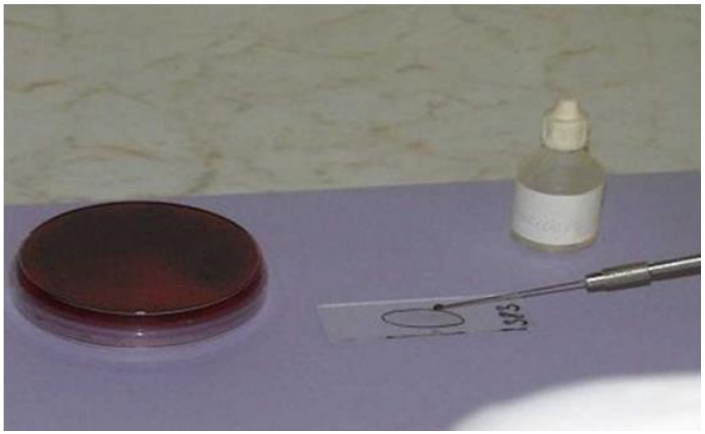
4. Carbol fuchsin or SAFRANINE
   - Counter stain
   - Red colored
   - Stains thin walled Gram negative bacteria red color



Gram Stain







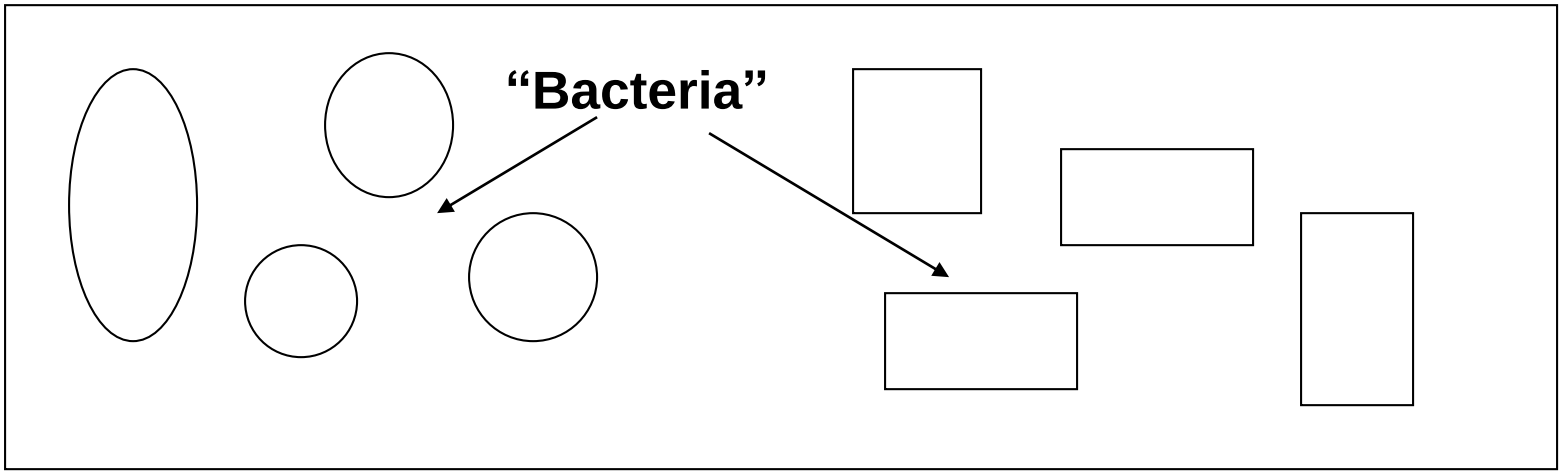
The Gram Stain Procedure

Step 1 - **Prepare a Smear**

Suspend some of the material to be stained in a drop of water on a microscope slide, spread the drop to about the size of a nickel.

Allow to air dry. Heat fix by gently warming

Watch what happens to the “Bacteria” at each step



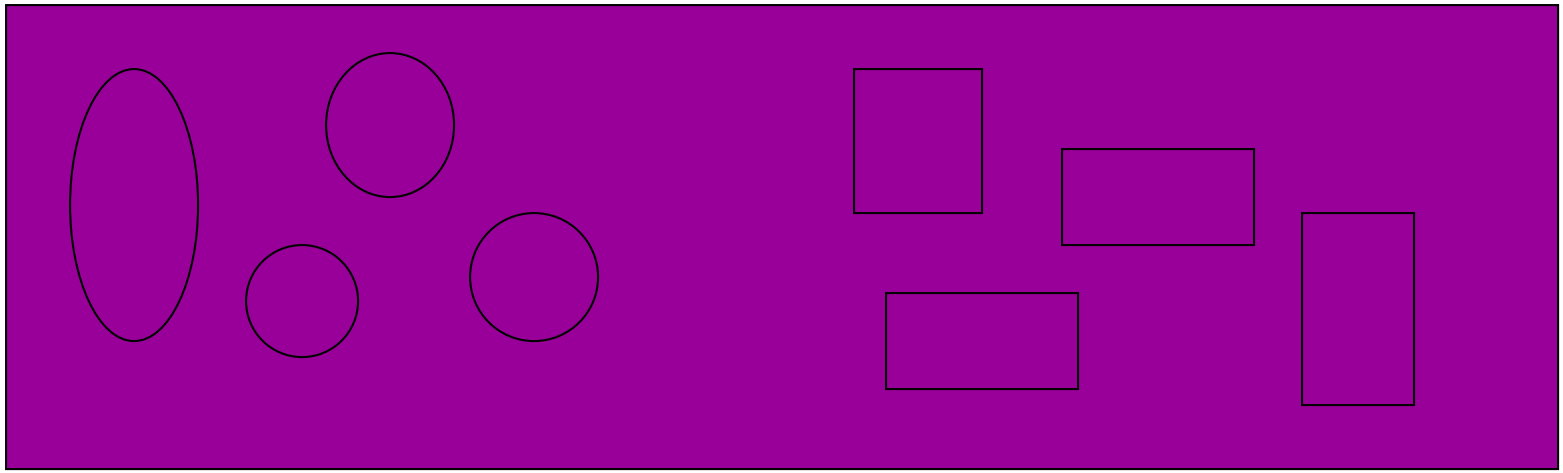
The Gram Stain Procedure

Step 2 - **Apply the Primary Stain**

Flood the Smear with **Crystal Violet**

Allow to stand for 1 min

Rinse with water to remove excess stain

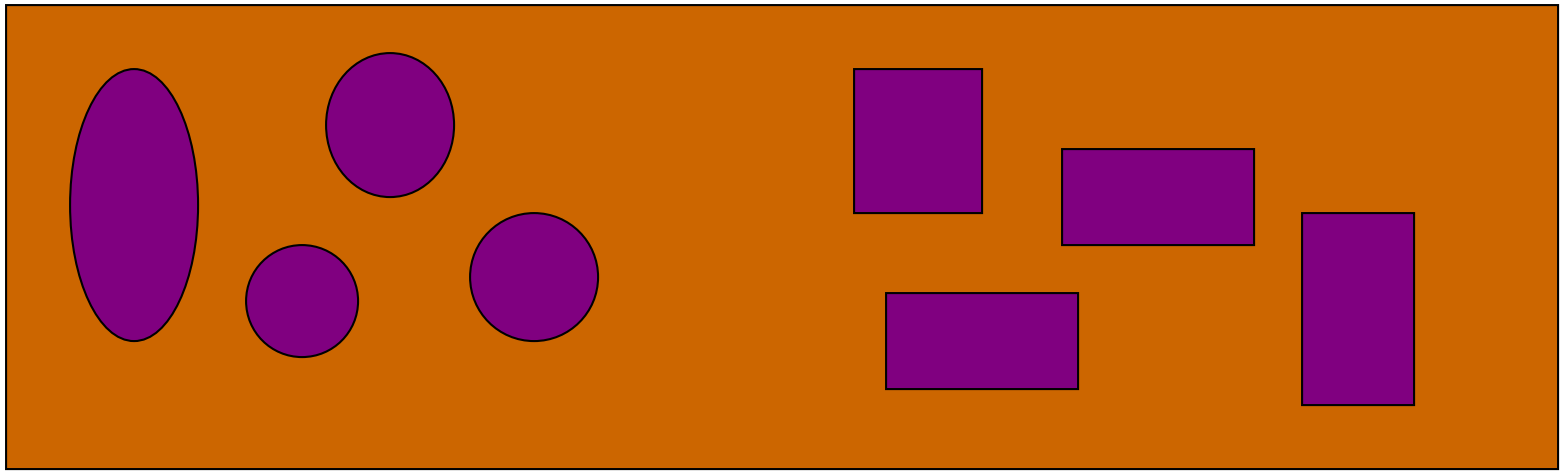


The Gram Stain Procedure

Step 3 - **Apply the Mordant**

Flood the Smear with **Iodine** solution

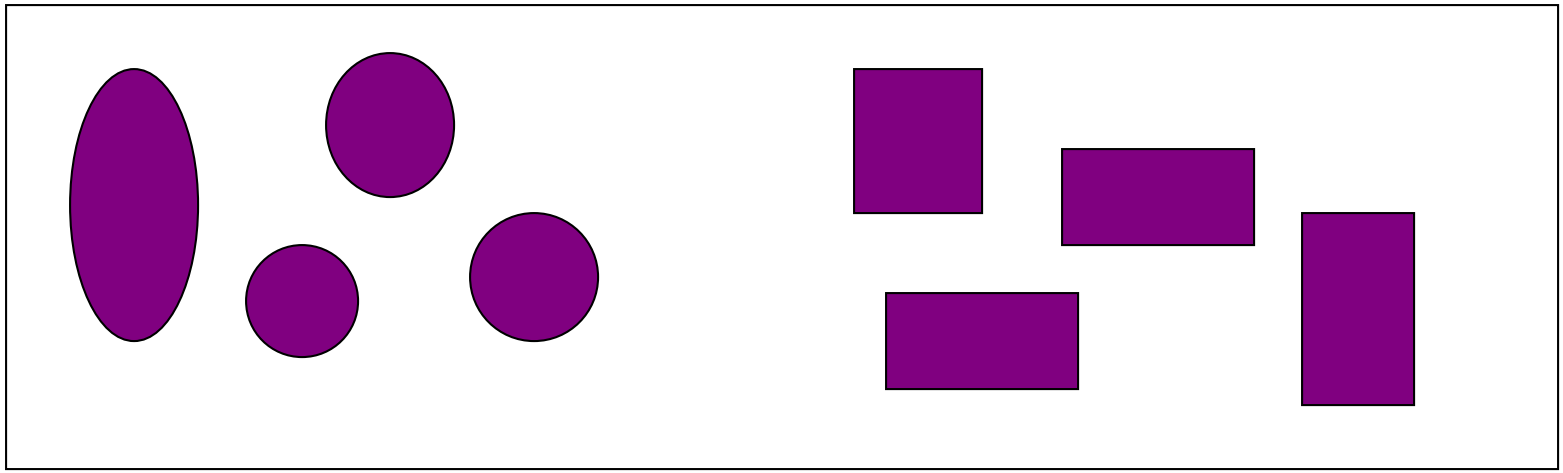
Allow to stand 2 min



The Gram Stain Procedure

Step 4 - **Rinse**

Rinse with water to remove excess Iodine

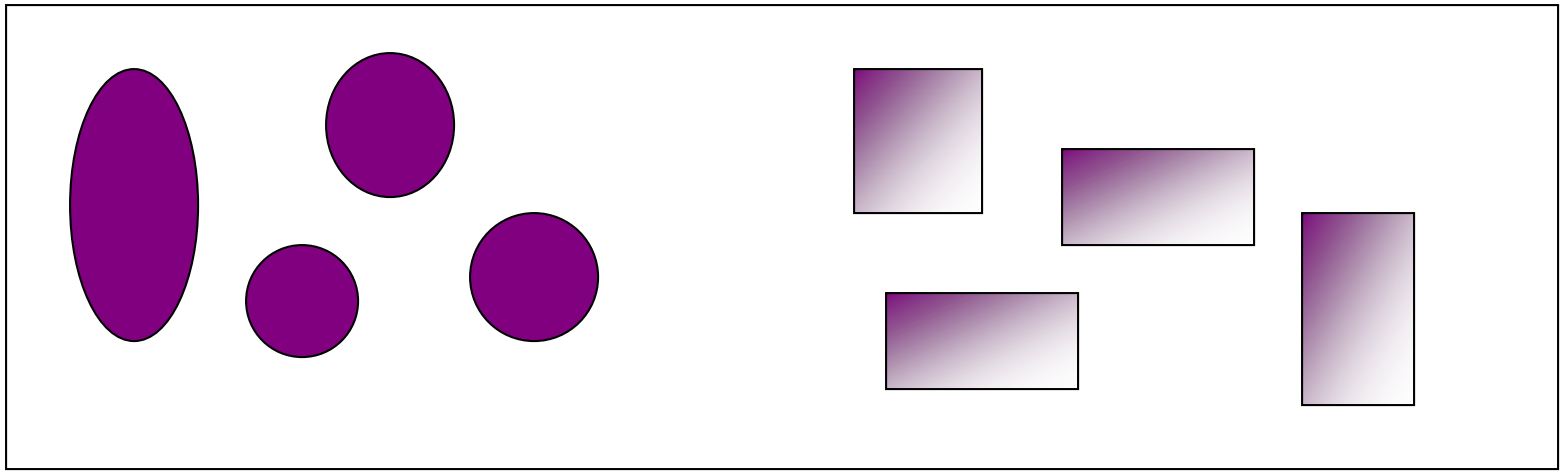


The Gram Stain Procedure

Step 5 - **Decolorize**

Drip Decolorizer (80% Methanol +20% Acetone) across the slide about 5 sec

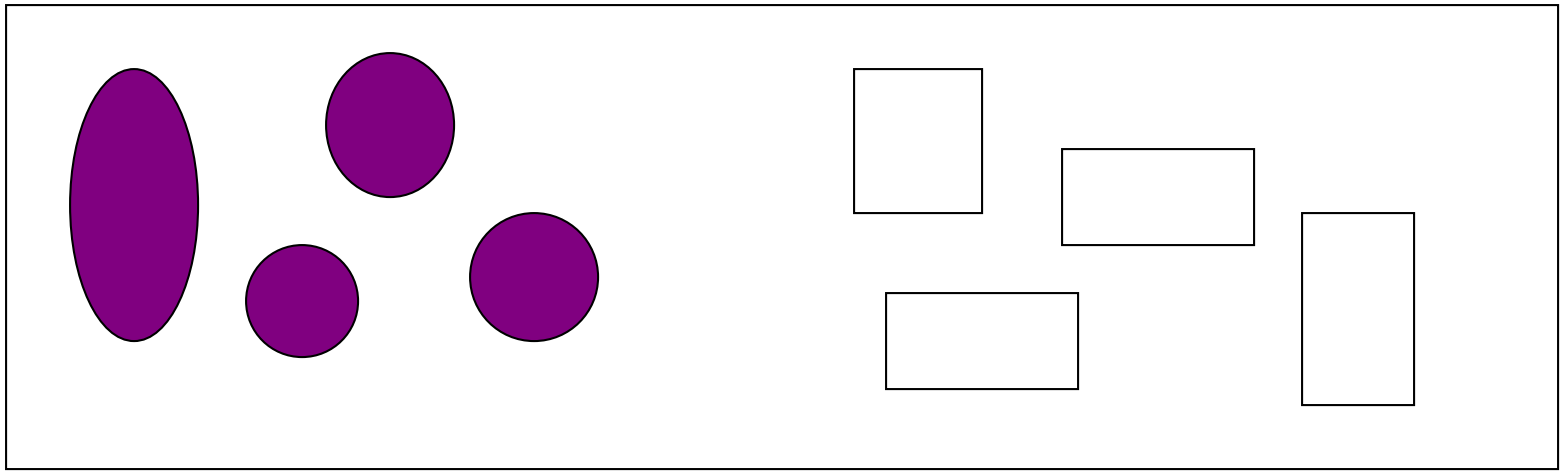
The smear should appear pale or clear



The Gram Stain Procedure

Step 6 - **Rinse**

Rinse with water to remove excess alcohol

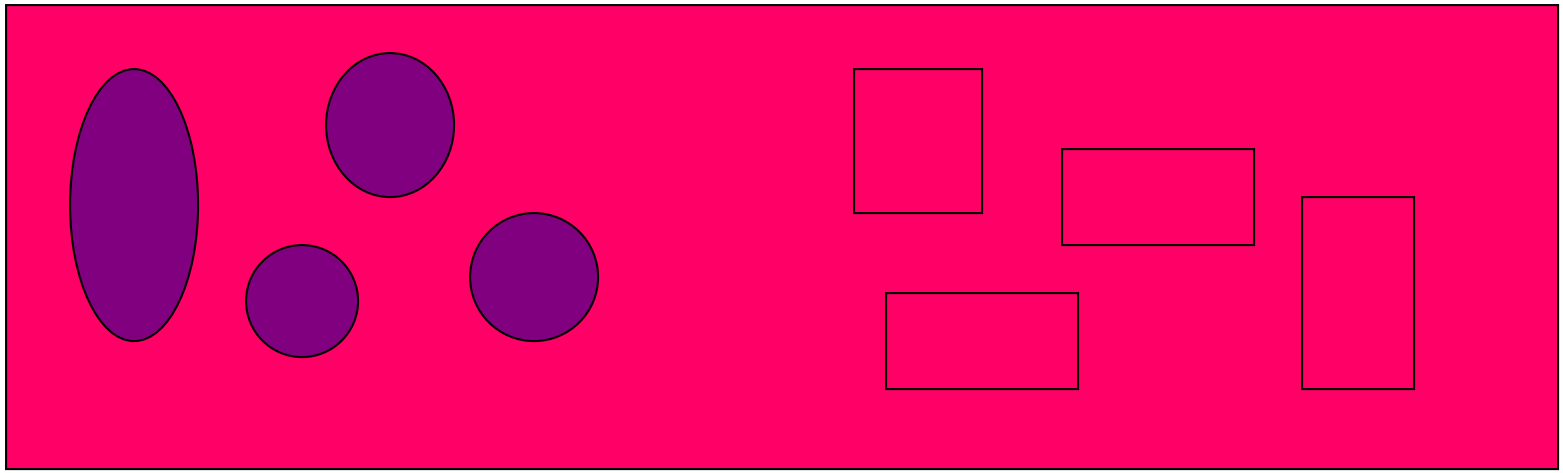


The Gram Stain Procedure

Step 7 - **Counterstain**

Flood the slide with **Safranin** solution

Let stand for 2 minutes



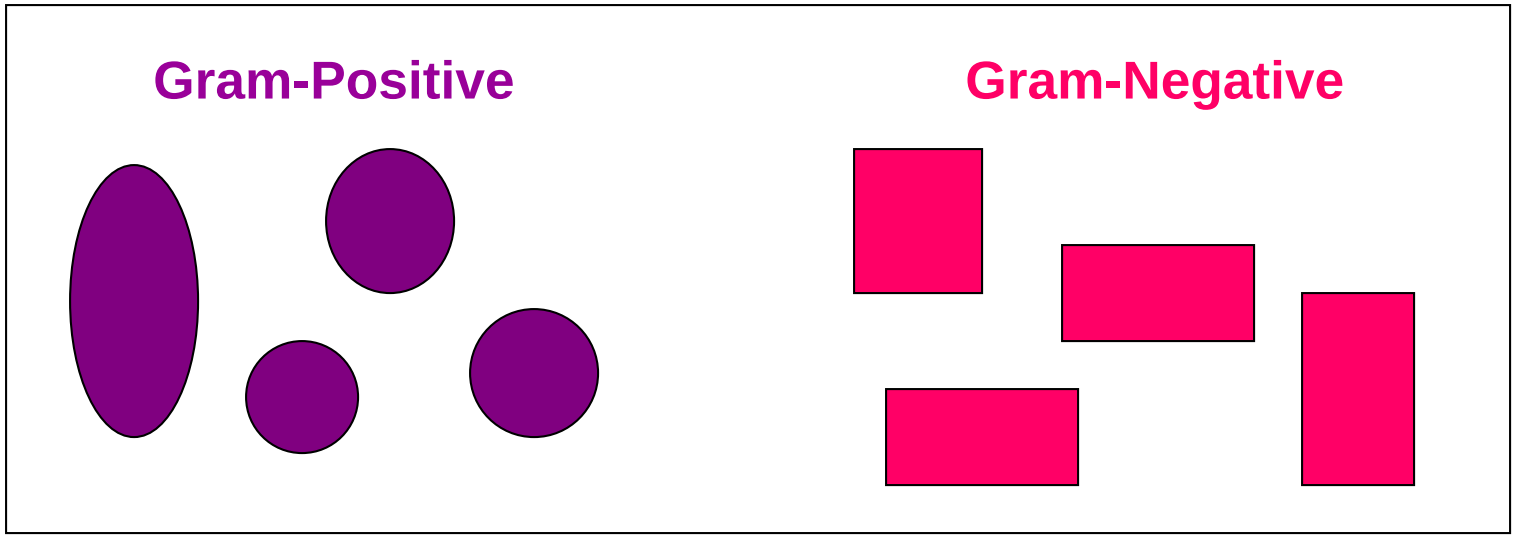
The Gram Stain

Step 8 - **Rinse, Dry and Observe**

Rinse with water to remove excess stain

dry the smear in air

Observe under Oil Immersion



2. (Zehiel-Nelseen method) **Acid-fast stain**

Acid-fast staining is another example of a differential stain used in Bacteriology.
It divides bacteria into two groups, acid fast- bacteria and non acid fast bacteria.
Members of the genus Mycobacterium are acid-fast in nature. Mycobacteria have a high lipid content, especially mycolic acid, in their cell wall. The ordinary dye solutions cannot penetrate the mycobacterial cell wall.

Strong staining solutions with application of heat, are used for staining bacteria that resistant decolourization with acid alcohol. Therefore they are called acid-fast bacilli (AFB).

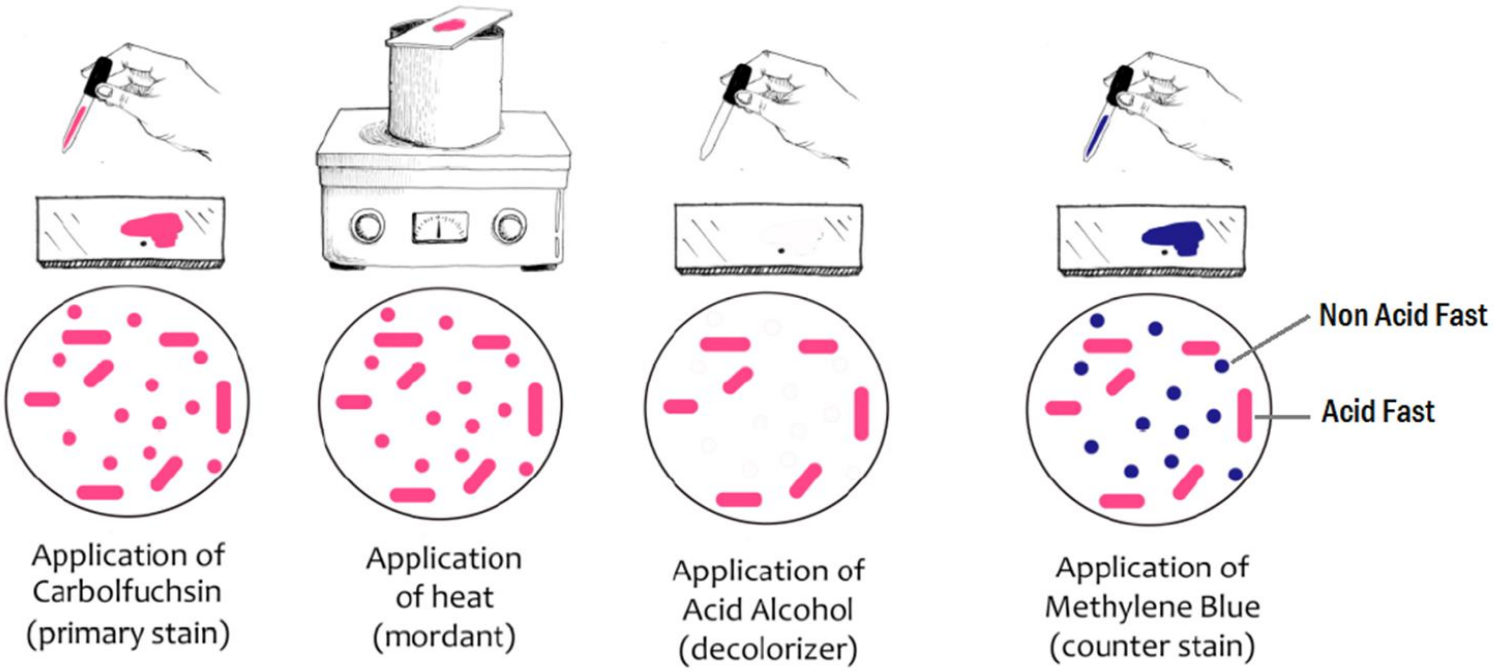
Mycobacterium tuberculosis can resist decolourization with 3% hydrochloric acid in 95% ethanol. Hence they are also called acid-alcohol-fast (AAFB).

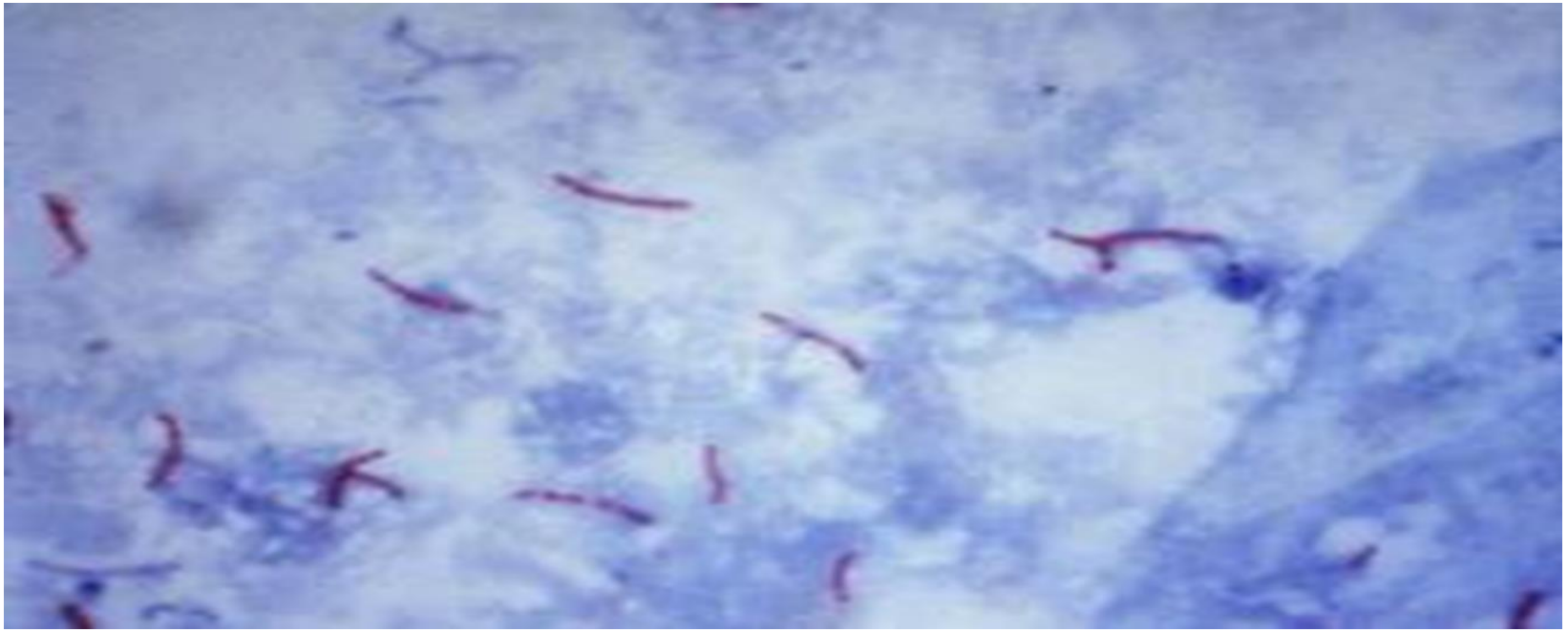
Procedure

- Make and fix smear by heat
- Flood the slide with primary stain: strong carbol fuchsin about 5- 10 minutes and heat gently until steam rises.
-Do not allow the stain to evaporate.
-Wash with tap water.
- decolorizing agent: acid alcohol (3% hydrochloric acid in 95% ethanol alcohol). For 5 minutes
- Wash well in water.
- Counter stain: methylene blue 2 minutes
- Wash well with tap water.
- dry and examine under oil immersion low.

Acid-fast Stain (Ziehl-Neelsen stain)

- It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria.





Results

red: Acid fast bacteria e.g. Tb

blue: non acid fast bacteria