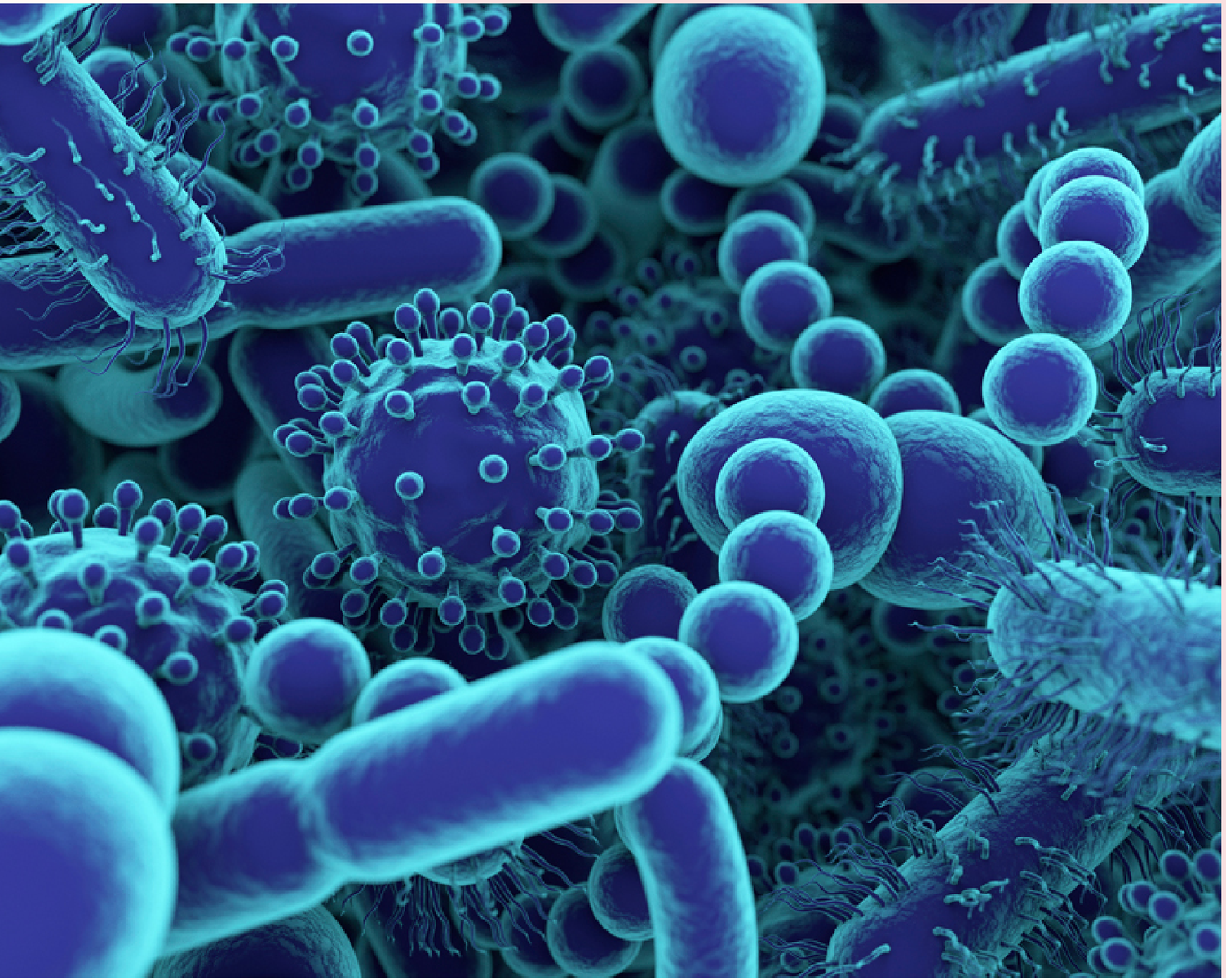


AFB & GRAM STAINING

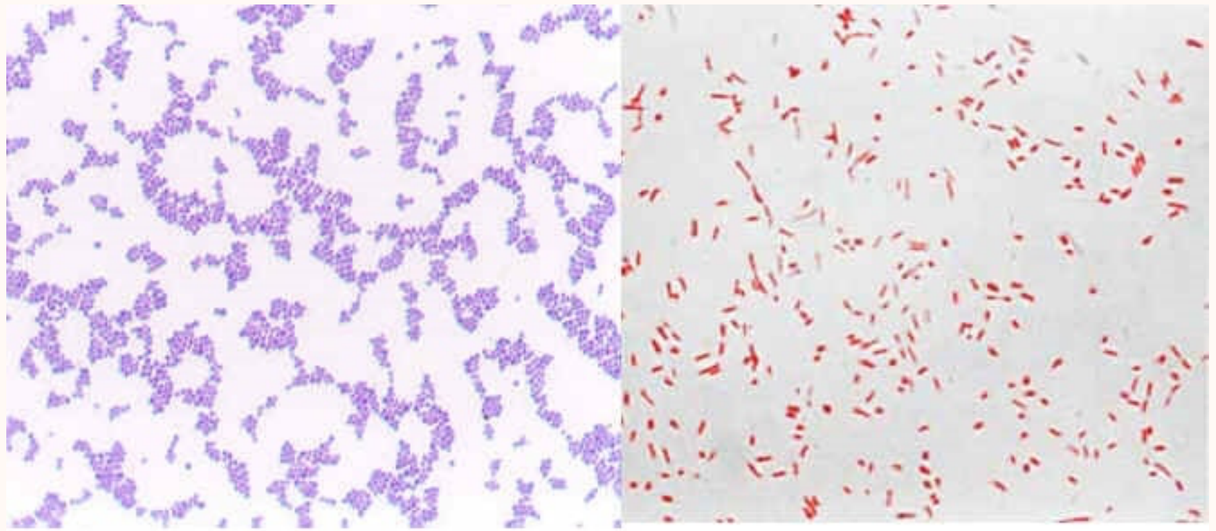
Prepared by Lai Xiang Yin

GRAM STAIN

OBJECTIVES

1. TO DIFFERENTIATE BETWEEN TWO MAJOR CATEGORIES OF BACTERIA: GRAM POSITIVE AND GRAM NEGATIVE

2. TO UNDERSTAND HOW GRAM STAIN REACTION AFFECTS GRAM POSITIVE AND GRAM NEGATIVE BASED ON THE BIOCHEMICAL AND STRUCTURAL DIFFERENCES OF THEIR CELL WALLS



Gram +ve Bacteria

Gram -ve Bacteria

PRINCIPLE

- Staining is an auxiliary technique used in microscopic techniques to enhance the clarifty of microscopic image

- Stains and dyes are widely used in the scientific field to highlight the structure of the biological specimens, cells, tissues etc

- The most widely used staining procedure in microbiology is the Gram stain, discovered by the Danish Scientist and physician Hans Christian Joachim Gram in 1884

PRINCIPLE

- Gram staining is a differential staining technique that differentiates bacteria into two groups: gram positive and gram negative

- The procedure is based on the ability of microorganisms to retain colour of the stains used during the gram stain reaction

- Gram negative bacteria are decolourized by the alcohol, losing the colour of the primary stain, purple. Gram positive bacteria are not decolourized by the alcohol and will remain as purple

- After decolorisation step, a counterstain is used to impart a pink colour tothe decolourized gram negative organism

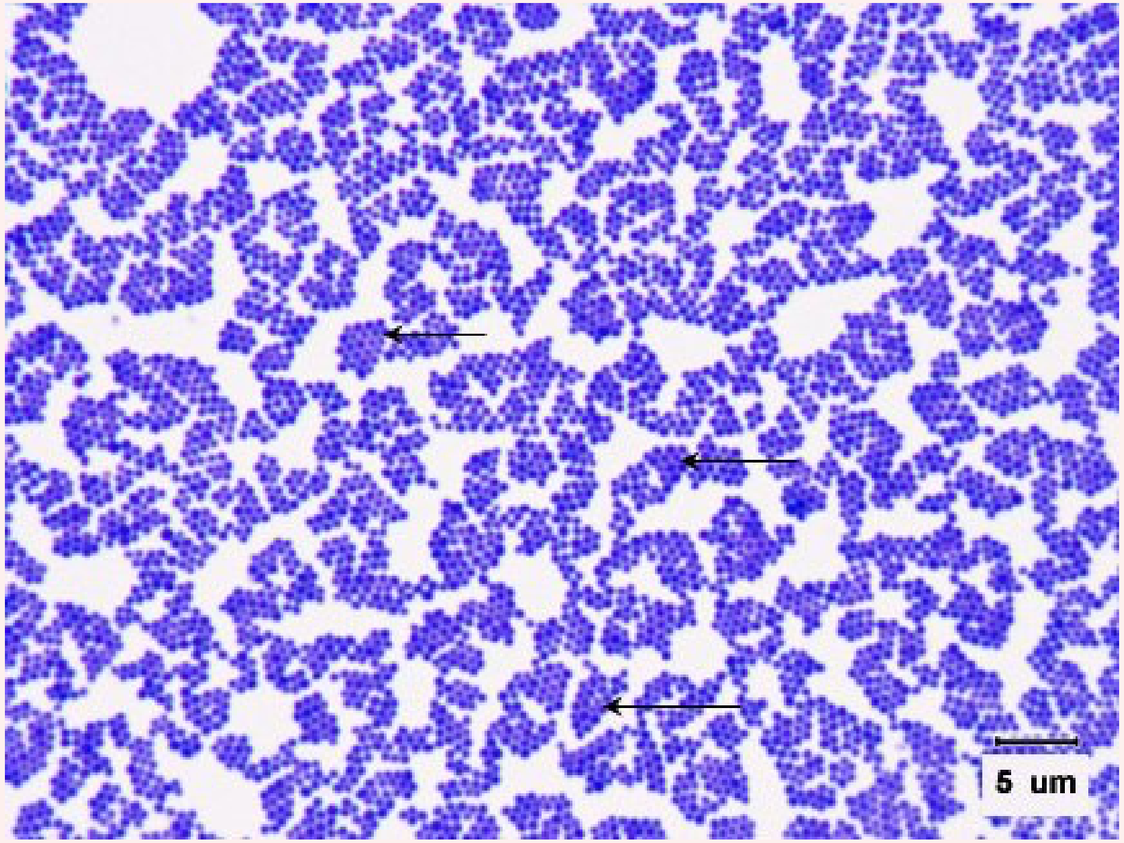
IMPORTANCE

- Gram stain is a very important preliminary step in the initial characterization and classification of bacteria
- Once stained, the morphology and arrangement of the bacteria may be observed as well
- Furthermore, it is also an important step in the screening of the infectious agents in clinical specimens such as direct smears from a patient
- The Gram stain procedure enables bacteria to retain colour of the stains , based on the differences in the chemical and physical properties of the cell wall

GRAM POSITIVE BACTERIA

- Stain dark purple due to retaining the primary dye called crystal violet in the cell wall

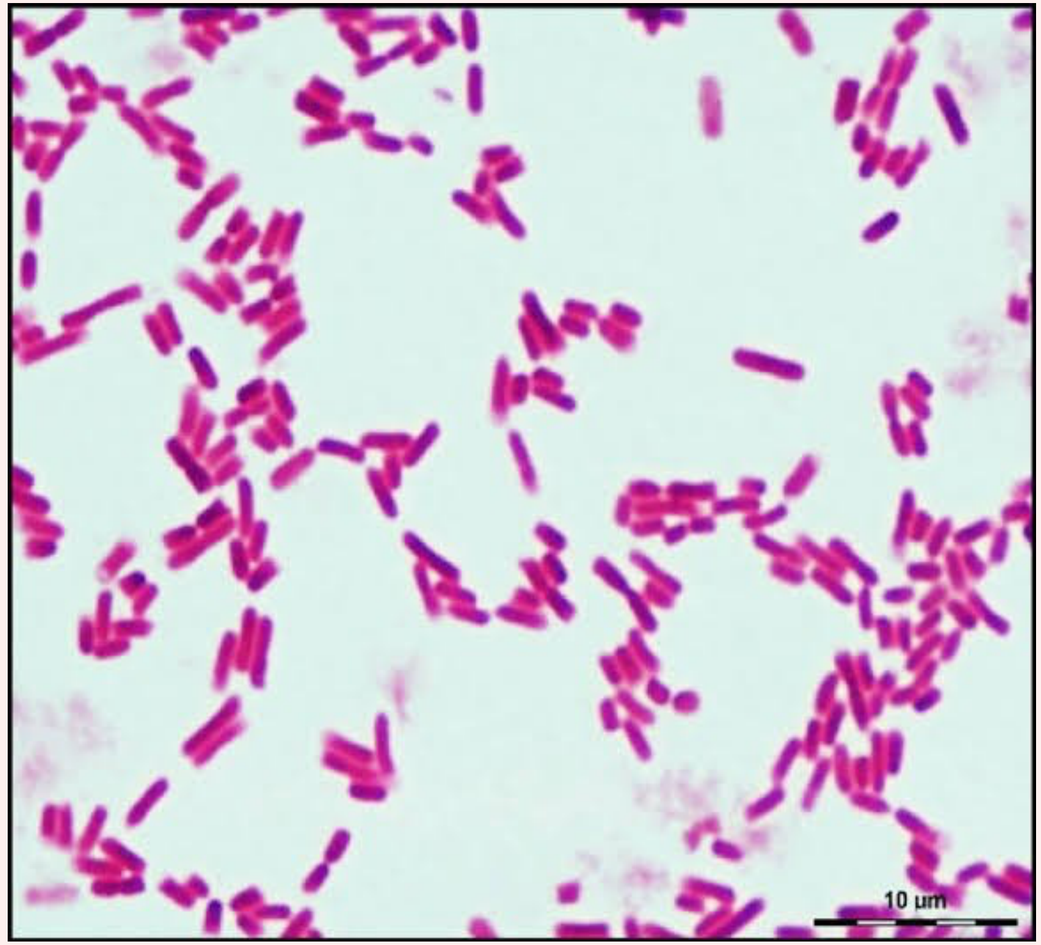
- Example: *Staphylococcus aureus*



GRAM NEGATIVE BACTERIA

- Stain red or pink due to retaining the counter staining dye called safranin

- Example: *Escherichia coli*



BACTERIAL MORPHOLOGY

The most common morphologies are:

- Coccus
  Spherical bacteria, may occur in pairs (diplococci), in group of four (tetracocci), in grape shape-like clusters (staphylococci), in chain (streptococci) or in cubical arrangement of eight or more (sarcinae)

- Bacillus
  Rod shape bacteria, generally occur singly but occasionalbe found in pairs or chains

- Spirilium
  Spiral shaped bacteria

BACTERIAL MORPHOLOGY

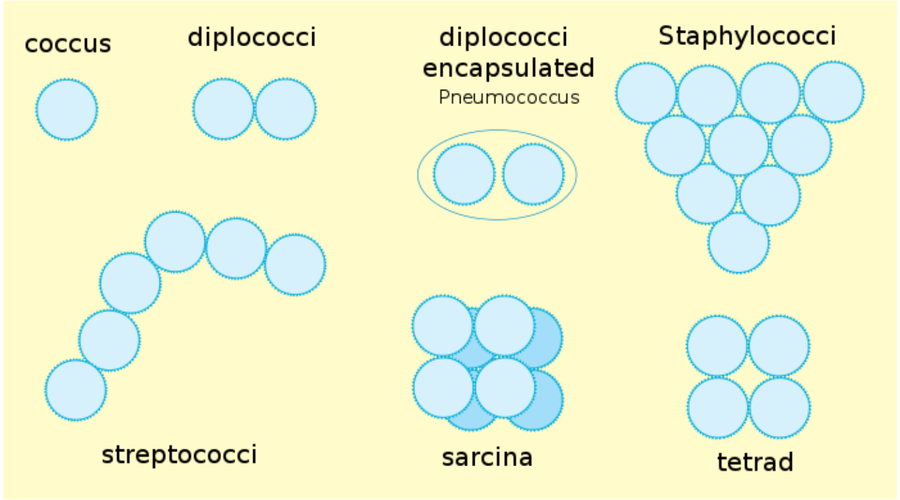
Some bacteria have other shapes such as:

- Coccobacilli
  Elongated spherical or ovoid form

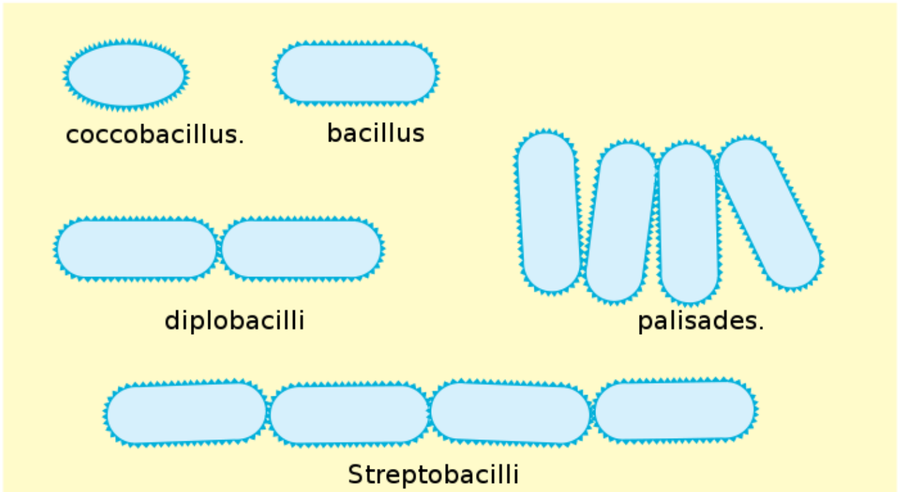
- Filamentous
  Bacilli that occur in long chains or threads

- Fusiform
  Bacilli with tapered ends

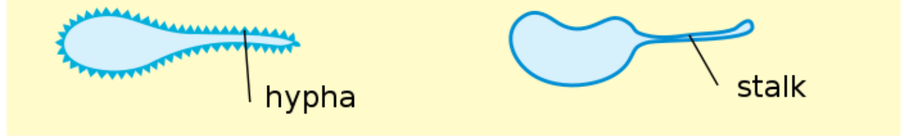
Cocci



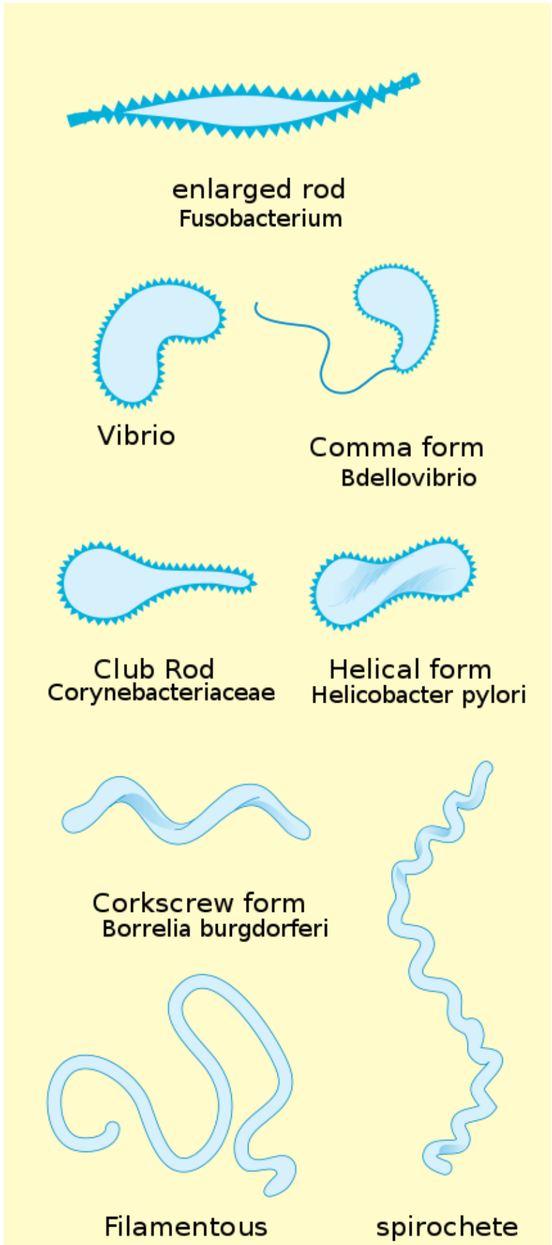
Bacilli



Budding and appendaged bacteria



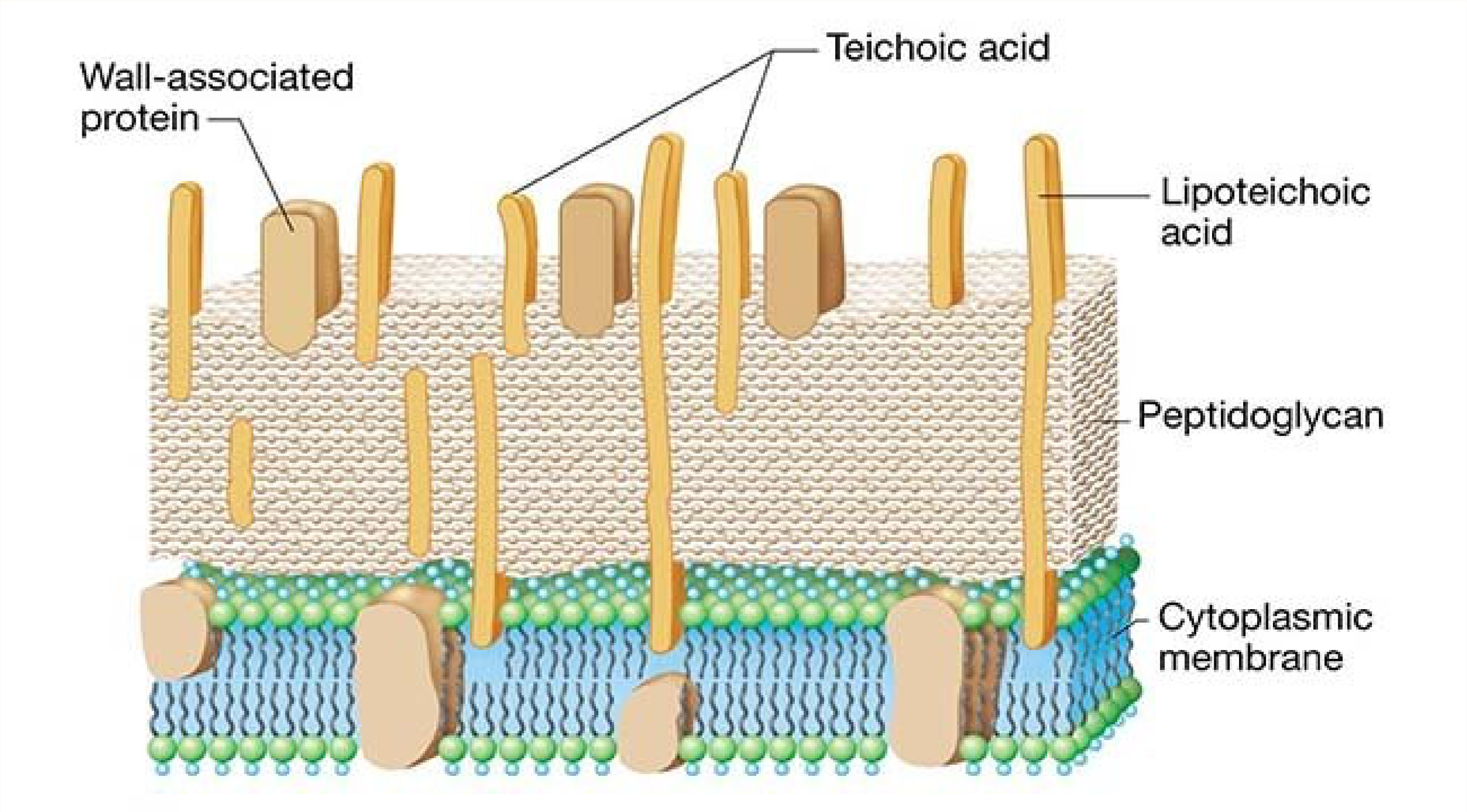
Others



GRAM STAIN MECHANISM

Gram positive cell wall:

- Gram positive bacteria have a thick mesh-like cell wall which is made up of peptidoglycan (50-90% of cell wall), which stains purple
- Peptidoglycan is mainly a polysaccharide composed of two subunits called N-acetyl glucosamine and N-acetyl muramic aicd
- As adjacent layers of peptidoglycan are formed, they are cross linkedby short chains of peptides by means of a transpeptidase enzyme, resulting in the shape and rigidity of the cell wall
- The thick peptidoglycan layer of gram positive organisms allows these organisms to retain the crystal violet-iodine complex and stains the cells as purple



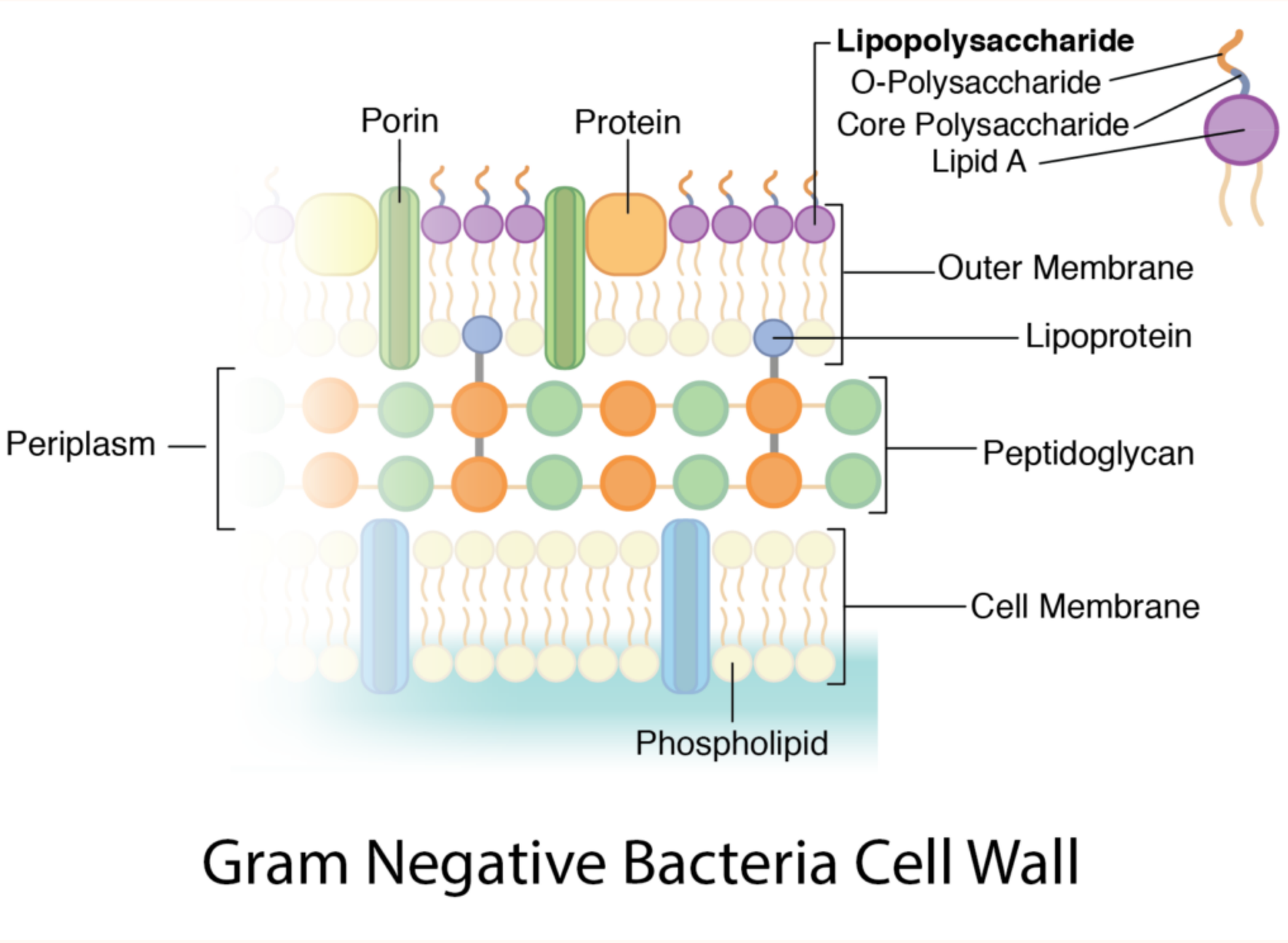
Gram positive bacteria cell wall

GRAM STAIN MECHANISM

Gram negative cell wall:

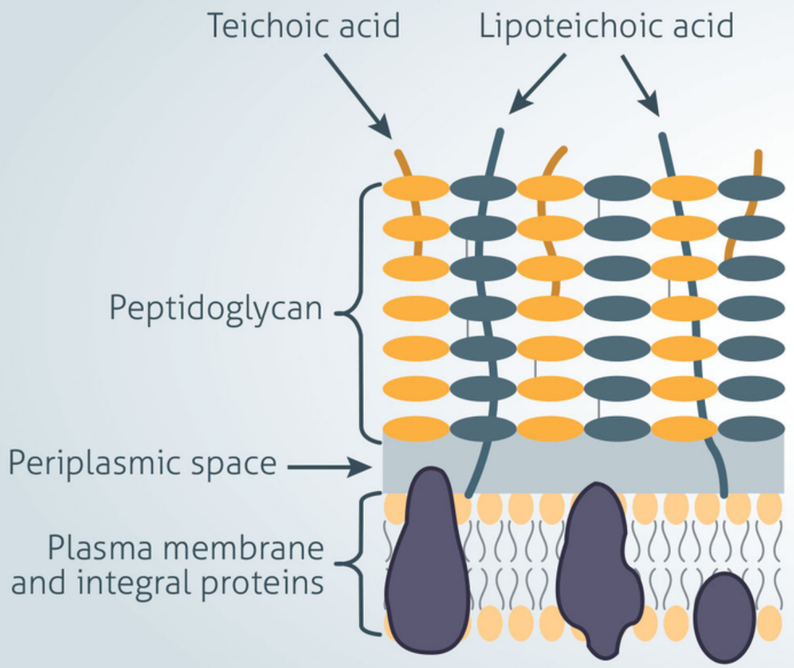
- Gram positive bacteria have a thinner layer of peptidoglycan (10% of the cell wall) and lose the crystal violet-iodine compels during decolourization with the alcohol rinse, but retain the counter stain Safranin, thus appearing reddish or pink

- They also have an additional outer membrane which contains lipids, which is separated from the cell wall by means of periplasmic space

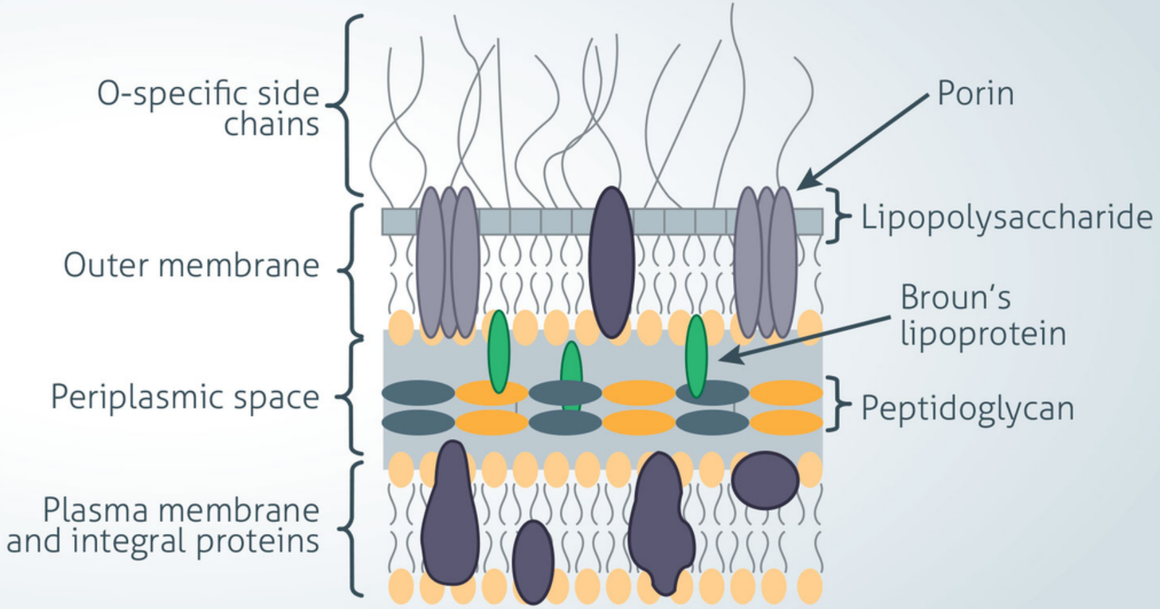


Gram Negative Bacteria Cell Wall

GRAM (+) CELL-WALL



GRAM (-) CELL-WALL



4 BASIC STEPS OF GRAM STAIN

1. Application of the primary stain crystal violet to a heat-fixed smear of bacterial culture for 1 minute

- Crystal violet dissociates in aqueous solutions into crystal violet+ and crystal violet- ions
- These two ions then penetrate through the cell wall and cell membrane of both gram positive and gram negative cells
- The crystal violet+ ions later interacts with negatively-charged bacterial components and
  stains the bacterial cells purple

4 BASIC STEPS OF GRAM STAIN

2. Addition of Gram's iodine for 1 minute

- Iodine (I-or I3-) acts as a mordant and as a trapping agent
- A mordant is a substance that increases the affinity of the cell wall for a stain by binding to the primary stains, thus forming an insoluble complex which gets trapped in the cell wall
- In gram stain, the crystal violet and iodine form an insoluble complex (CV-I) which serves to turn the smear a dark purple colour
- A this stage, all cells will turn purple

4 BASIC STEPS OF GRAM STAIN

3. Decolourization with 95% ethyl alcohol for 5-10 seconds

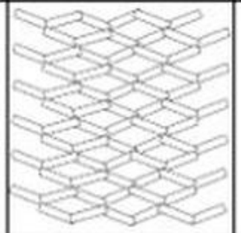
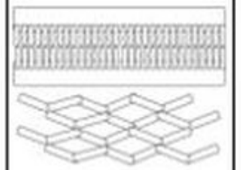
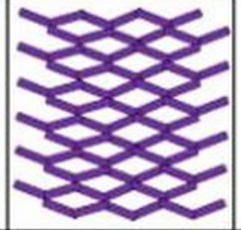
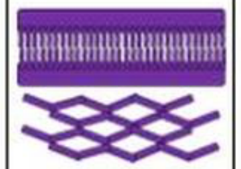
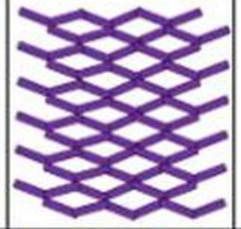
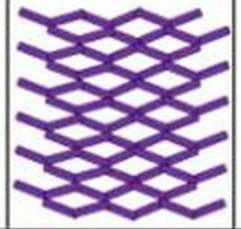
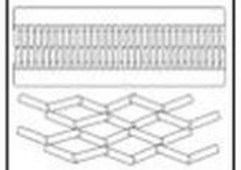
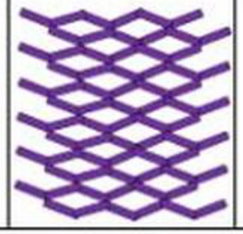
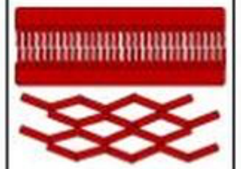
- Alcohol or acetone dissolves the lipid outer membrane of gram negative bacteria thus leaving the peptidoglycan layer exposed and increases the porosity of the cell wall
-The CV-I complex is then washed away from the thin peptidoglycan layer, leaving gram negative bacteria colourless

The decolourization step must be performed carefully, otherwise over-decolourization may occur. This step is critical and must be timed correctly otherwise the crystal violet stain will be removed from the gram positive cells. If the decolonizing agent is applied on the cell for too long time, the gram positive organisms to appear gram negative. Under-decolourization occurs when the alcohol is not left on long enough to wash out the CV-I complex from the gram negative cells, resulting in gram negative bacteria to appear gram positive.

4 BASIC STEPS OF GRAM STAIN

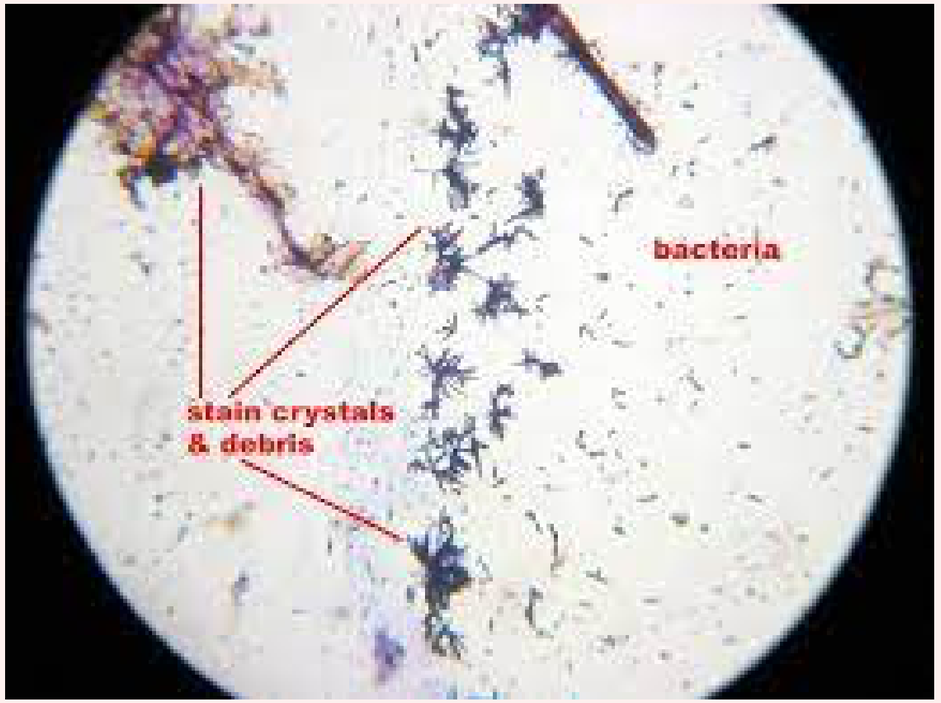
4. Counterstain with safranin for 1 minute

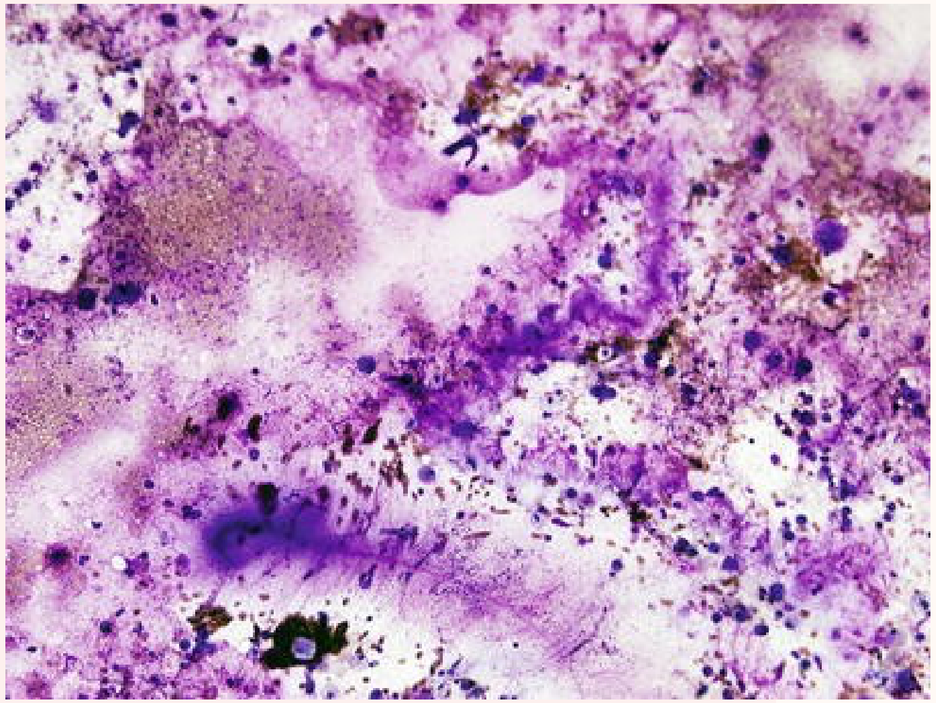
- The decolourized gram negative cells can be rendered visible with a suitable counterstain, which is usually positively charged safranin, which stains them pink
- Pink colour which adheres to the gram positive bacteria is masked by the purple of the crystal violet

Gram Staining Procedure		Gram Positive Cell Wall		Gram Negative Cell Wall	
Process of test	Appearance of Cells	Effect of Step	Effect on Cell Wall	Effect of Step	Effect on Cell Wall
Step 1: Begin with heat fixed cells		**Step 1:** Cell wall remains clear.		**Step 1:** Cell wall remains clear.	
Step 2: Flood slide with crystal violet dye for 1 min.		**Step 2:** Peptidoglycan cell wall is flooded with crystal violet and appears purple.		**Step 2:** Cell wall is stained purple from the crystal violet dye.	
Step 3: Add iodine solution for 1 min.		**Step 3:** A crystal violet – iodine complex is formed within the peptidoglycan cell wall trapping the purple stain.		**Step 3:** A crystal violet-iodine complex is formed but does not adhere to the cell wall due to the thin layer of peptidoglycan.	
Step 4: Wash slide with alcohol for 20sec.		**Step 4:** The crystal violet – iodine complex is trapped with the peptidoglycan cell wall and doesn't wash out.		**Step 4:** The crystal violet – iodine structure is washed out of the thin peptidoglycan layer.	
Step 5: Counter stain with safranin.		**Step 5:** As the peptidoglycan cell wall remains stained purple the red safranin has no effect.		**Step 5:** The red safranin stains the washed gram negative cells.	

ARTIFACTS IN GRAM STAINING

- Contaminated stain
- Dirty glass slide
- Contaminated water used during rinsing of the slide





TOURBLESHOOTING IN GRAM STAINING

There are several factors which could result gram positive organism staining negative:

- Method and technique used
  Over heating during heat fixation, over decolourization with alcohol, and even too much washing with water between steps may result gram positive bacteria losing the crystal violate-iodine complex

- The age of the culture
  Cultures more than 24 hours may loose their ability to retain crystal violate-iodine complex

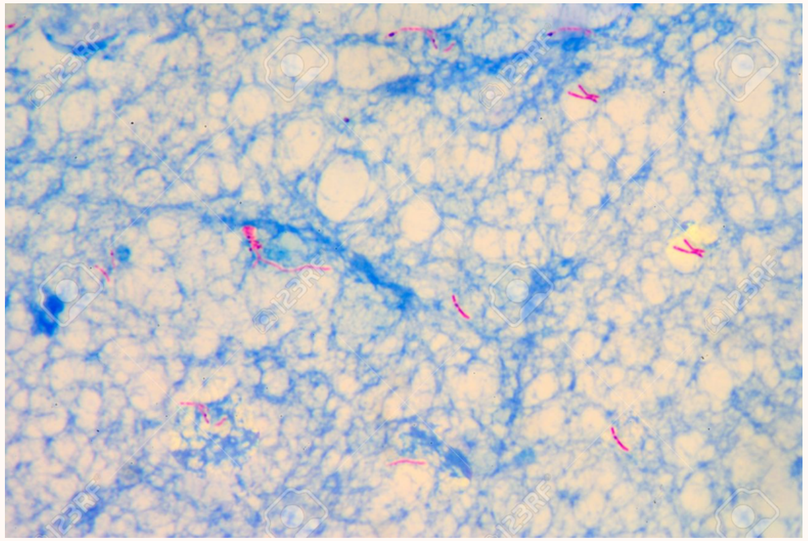
- The organism itself
  Some gram positive bacteria are more able to retain the crystal violate-iodine complex than others

AFB STAIN

OBJECTIVES

1. TO DIFFERENTIATE BETWEEN TWO MAJOR CATEGORIES OF MICROORGANISMS:ACID FAST AND NON-ACID FASTNESS

2. TO UNDERSTAND HOW AFB STAIN REACTION AFFECTS ACID FAST AND NON-ACID FAST BASED ON THE BIOCHEMICAL AND STRUCTURAL DIFFERENCES OF THEIR CELL WALLS

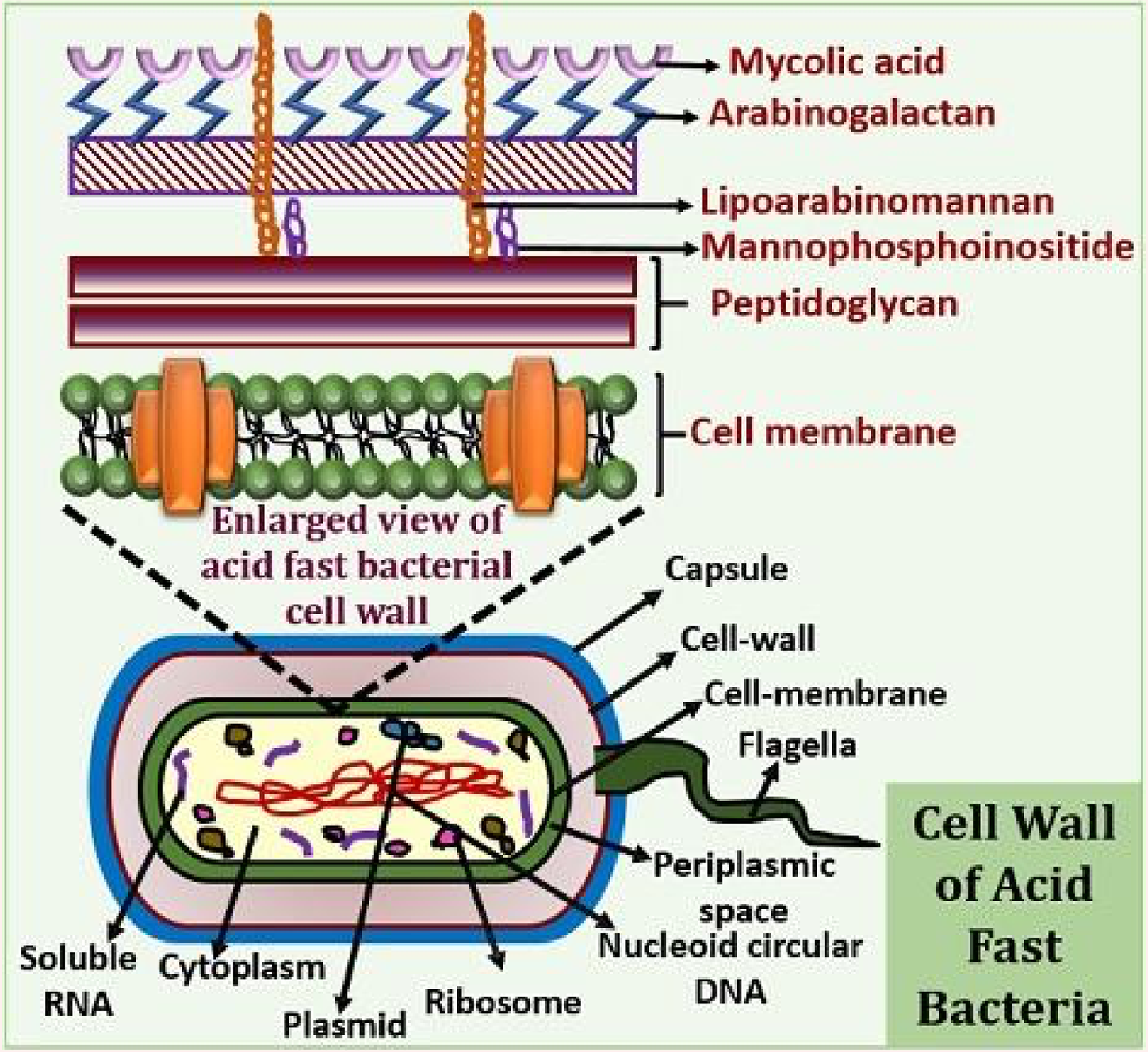


PRINCIPLE

- In the cell wall of acid fast bacteria, a lipid called mycolic acid is present that is a waxy substance
- Mycolic acid resists the staining of the bacterial cell by simple stains or using simple techniques but when carbol fuchsin and heat is applied, the mycolic aicd gets weaken and become porous, due to which the carbol fuchsin enters the cell wall
- During decolorisation, a special decolorizer containing sulfuric acid or hydrochloric acid is used
- Bacterial cells that get decolorized and appear colorless were said to be non acid-fast bacteria and those which do not get decolorized even with this powerful decolorizer were said to be acid fast bacteria
- After decolorisation, the acid fast bacteria appear red in colour whereas the non aicd fast bacteria appear as color bodies
- Methylene blue is applied to stain non-acid fast bacteria blue

PRINCIPLE

- When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates 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 due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration 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.


Mycolic acid
Arabinogalactan
Lipoarabinomannan
Mannophosphoinositide
Peptidoglycan
Cell membrane
Enlarged view of acid fast bacterial cell wall
Capsule
Cell-wall
Cell-membrane
Flagella
Periplasmic space
Nucleoid circular DNA
Ribosome
Plasmid
Cytoplasm
Soluble RNA
Cell Wall of Acid Fast Bacteria

SAMPLE REQUIREMENT

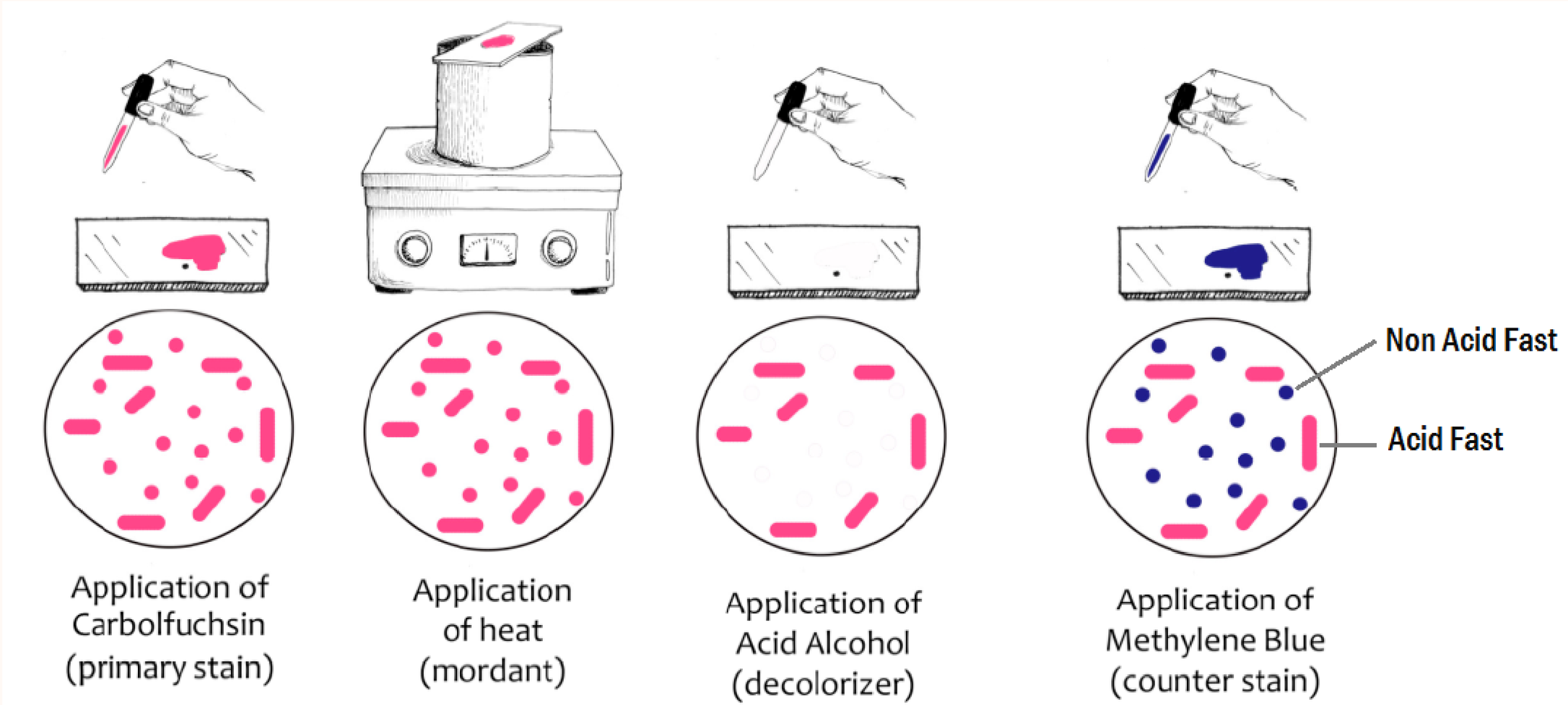
AFB stain can be performed on several types of sample, eg: sputum, tissue, aspirates and urine.

Samples such as urine and aspirates are required to be spun down and smear is done using pellet.

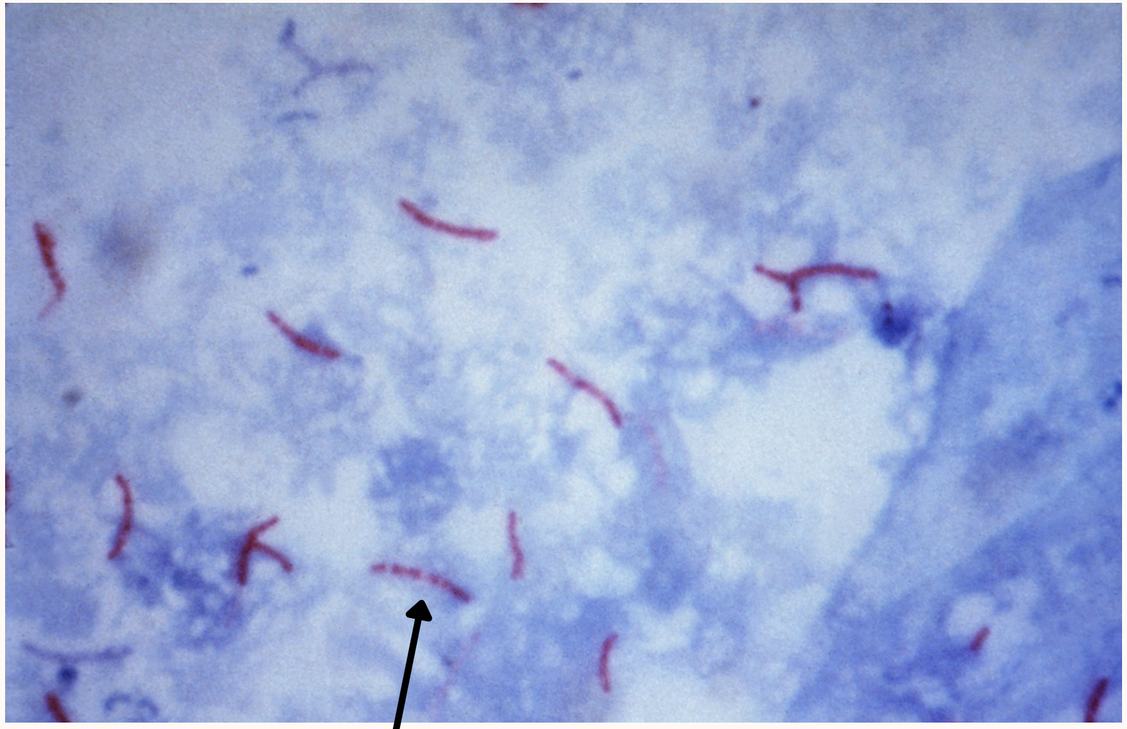
Watery sputum with saliva should be noted to doctor in case recollection of second sample is necessary.

AFB STAIN STEPS

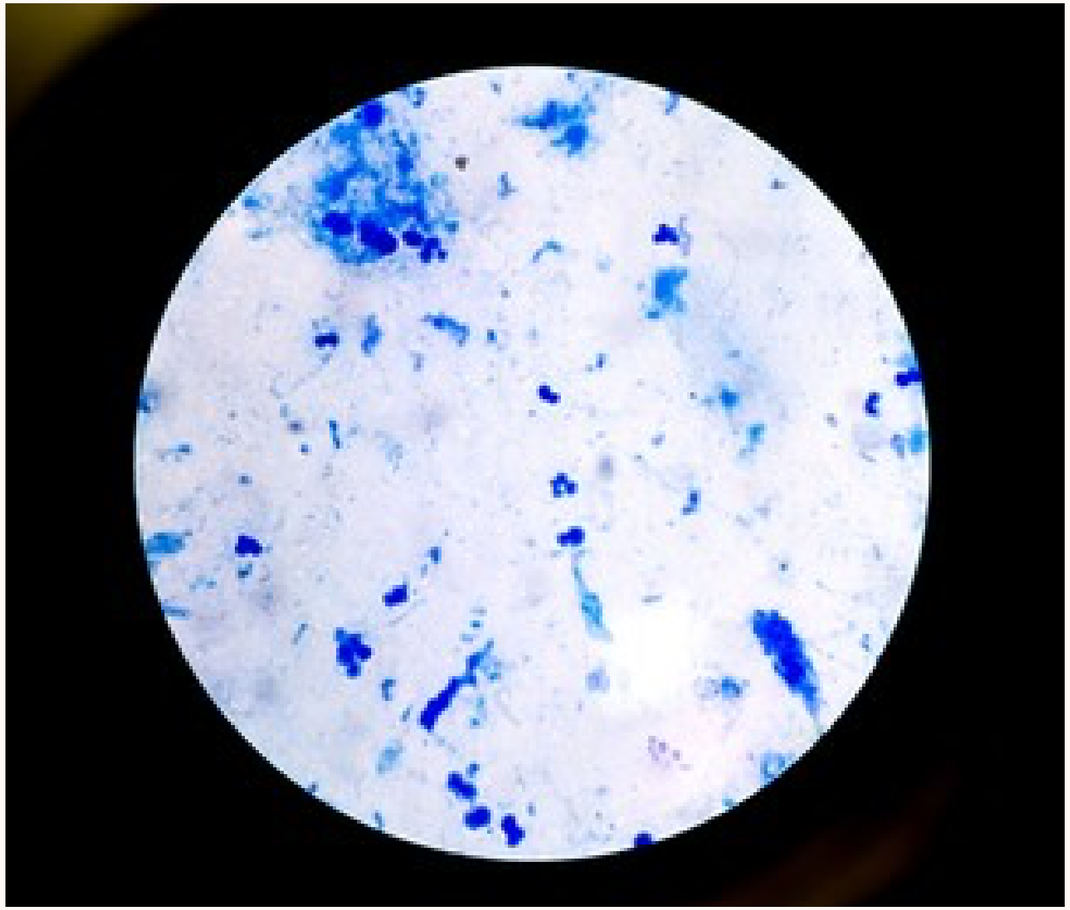
1. Perform a 50 cent size smear on a clean glass slide in biosafety cabinet
2. Heat fix the slide for at least 45 seconds
3. Flood the slide using carbol fuschin
4. Flame the slide with Bunsen burner until white fume just begins to rise *(do not overheat or until boiling)*
5. Leave smear to stain for 5 minutes
6. Wash the smear with running tap water
7. Cover the smear with 3% v/v of alcohol for 5 minutes
8. Wash the smear with running tap water
9. Stain smear with methylene blue for 2 minutes
10. Wash the smear with running tap water
11. Dry the slide and read under x100 with immersion oil



Acid Fast Bacilli Staining Procedure



Acid fast bacilli seen



THANK YOU