Lab – 5

Bacterial Smear Preparation & Staining

Bacterial Smear Preparation

- It is a preparation that includes a **small amount of biological sample or culture spread in a very thin film on the surface of the slide**.
- Smear preparation is a step before staining.
- A good bacterial smear preparation is the key for good stain.

Materials

- Bacterial culture (whether on plate or broth)
- Glass slide
- Bunsen burner.
- Inoculating loop
- Permanent marker or pencil

Method

Step I: Properly label a new microscope slide

Step II: Sterilize an inoculating loop by burning it and let it cool

Step III: Remove bacteria from plate or broth sample

A: From plate (solid media)

1. **Place a drop of water on the slide**
2. **Using a sterilized and cooled inoculation loop to obtain a very small sample of a bacterial colony from a culture plate**
3. **Gently mix the bacteria with the water drop.**
4. **Spread the mixture in a circular motion, this will avoid cell clumping.**

B. From broth (liquid media)

1. Gently tapping the broth tube by the finger to re- suspense the bacteria

2. Using a sterilized and cooled inoculation loop to obtain broth (one or two loopfuls taken according to the size of the loop)

3. Apply the broth on the center of the slide and spread it evenly.

Step IV: Allow the smear to dry completely by air at room temperature

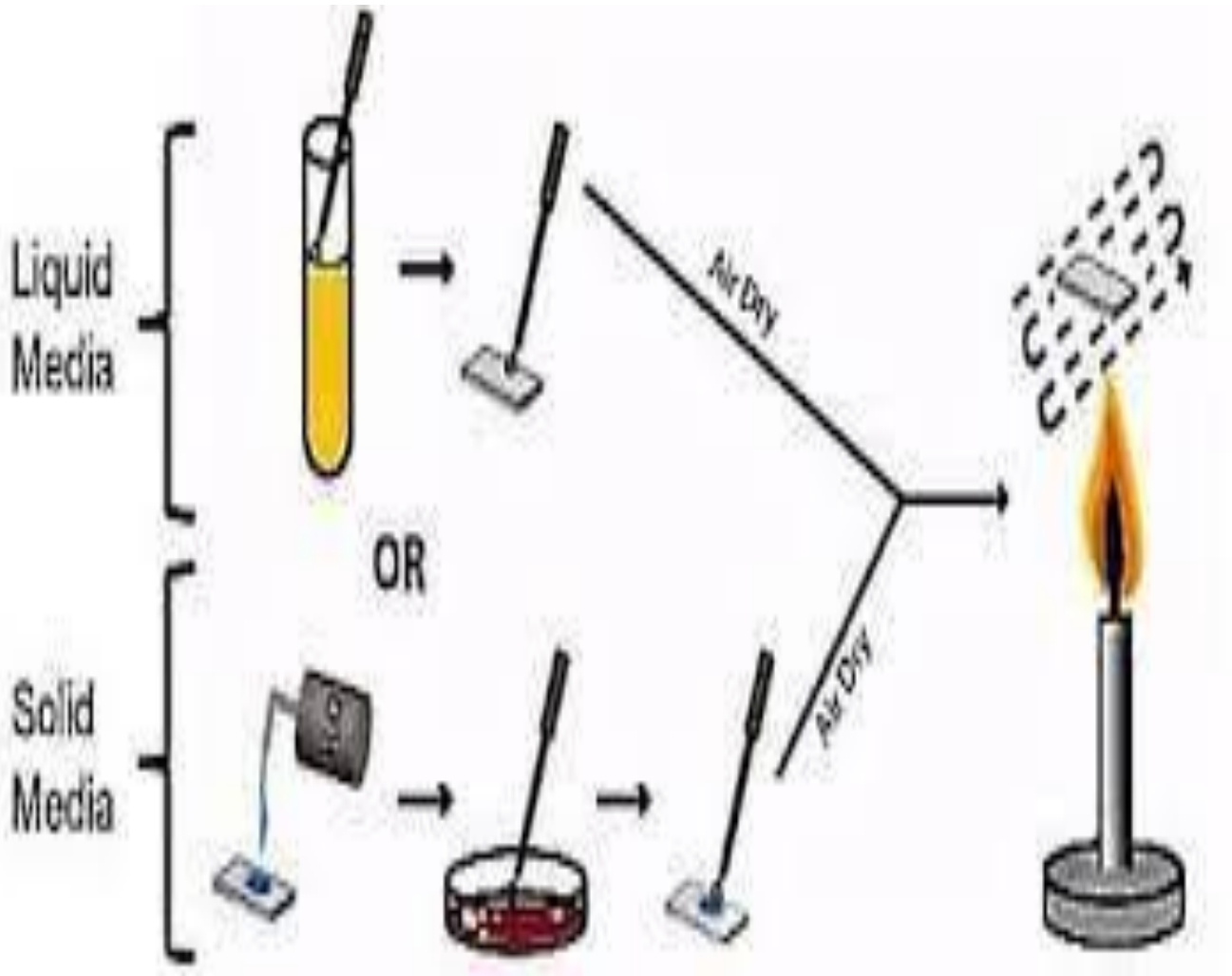
Step V: Heat fixation

i. After smear is dried completely by air, rapidly pass it 3- 4 times through flame of Bunsen burner

ii. Avoid too much heating

iii. Allow smear to cool before staining

Summery of ideal bacterial smear preparation



Smear Preparation

1. Label a clean micro-scope slide.
2. Sterilize the inoculating loop.
3. Remove a bacterial or broth sample.

   Smear on slide area.
4. Air dry.
5. Heat fix.

An ideal bacterial smear should be thin, semi-transparent, whitish layer or film, circular or oval with 2 cm diameter .

The importance of heat fixation is:

1) Kill the M.O.

2) Make the M.O. stuck to the surface of the slide.

3) Make the M.O. more permeable to the stain.

4) Prevent the M.O. from going autolytic changes

Bacterial Staining

- Bacteria are colorless (transparent) MO
- Staining is a process by which we use different dyes to visualize the MO
- By staining we can study size, shape (cocci, bacilli, cocco-bacilli) and arrangment (single, pairs, clusters or chains) of MO

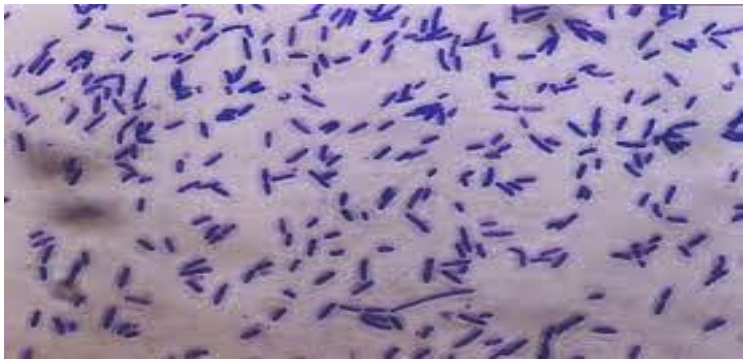
Types of stains:

1. Basic (+ve stains)
2. Acidic (-ve stains)
3. Neutral stains

- **A- Basic stains:**
- Also called +ve stains. They posses (+ve) charge after ionization.
- Basic stains are attracted to negatively charged molecules in the cell including nucleic acids (DNA and RNA) and some proteins.
- Examples: methylene blue, crystal violet, malachite green, basic fuchsin, carbolfuchsin, and safranin.

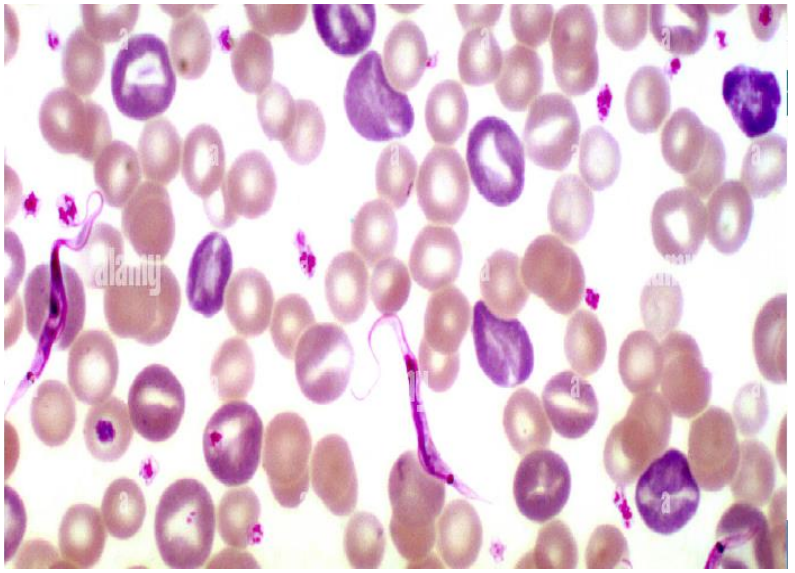
- **B- Acidic stains:**
- Also called –ve stain. these stains posses (-ve) charge after ionization.
- These stains will be repelled from the bacterial cell & only the background is stained i.e. bacteria cells remain unstained and a clear zone around the cell will be seen
- Examples: india ink , acid fuchsin and nigrosin.

- **C. Neutral stains**
- Both +ve and –ve molecules in the smear are stained.
- Neutral stain are actually a salt of acidic and basic stain.
- Examples: Giemsa, Leishman, Wright stain.



Basic stain (crystal violet stain)

Negative stain (India ink) reveals cocci bacteria single, paired and in clusters



Neutral stain (Giemsa stain) usually used to stain blood smears for parasitic infection

Table illustrate Basic, Acidic, and Neutral stains :

Basic (+Ve Charge) Stains	Acidic (-Ve Charge) Stains	Neutral Stains
○ Methylene blue ○ Crystal violet ○ Malachite green ○ Basic fuchsin, ○ Carbolfuchsin ○ Safranin. • Neutral red	• Nigrosin • Acid fuchsin • India ink	• Giemsa • Leishman • Wright

Stains classified according to staining techniques or their function to

1. **Simple stains**
2. **Differential stains**
3. **Special stains**

1. Simple stain:

It is the using of **ONE STAIN (Basic stain)** only to stain microorganisms, so MO that present in the slide appears in **ONE COLOR**.

It used to get information about bacterial size, shape (cocci, bacilli, cocco-bacilli) and arrangment (single, pairs, clusters or chains)

Procedure

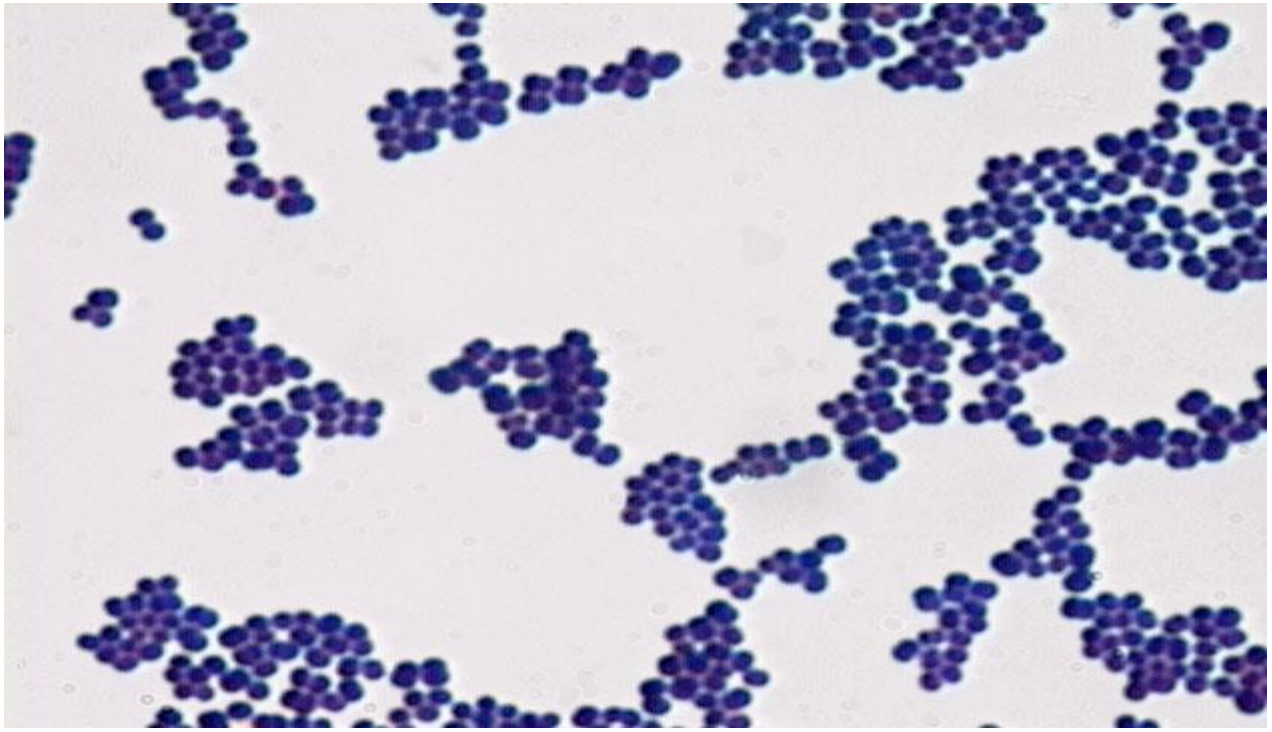
This procedure is simple,easy,time saving and economic. It is applied by using only a single stain and all bacteria in the specimen will be stained by one color.

1-Use a clean slide, mark it and fellow the steps of smear preparation

2-Flood smear with simple stain,basic stains,such as;crystal violet,methyl violet…

3-Wash with tap water, drain excess water & dry in air.

4-Examine under microscope using the low , high and oil immersion lens.



Picture of simple stain. Using single basic stain (crystal violet) and all bacteria in slide will be stained violet.

2. Differential stain

- This type of stain used to differentiate between two organism
- The mainly used differential stain is **GRAM STAIN**
- This stain first discovered by **HANS CHRISTIAN GRAM 1884**
- By gram stain procedure the bacteria are classified into two main groups,

Gram positive(+ ve):retain the dark blue to violet color of primary stain (crystal violet stain) after using alcohol (decolorizing agent)

Gram negative(-ve): those that can't retain primary stain when the slide washed by alcohol(decolorizing agent)but then take the redcolor of counter stain (safranine)

Procedure for Gram staining :

1. Prepare a fixed smear after marking our slide.
2. Flood the smear with **crystal violet** stain for 1 minute.
3. Wash with tap water .
4. Cover the smear with **Gram iodin (mordant)** for 1 minute:
   The stain now is combined with iodin forming what is called Crystal Violet Iodin complex (CVI complex).This complex will be fixed in bacteria in its cell wall more than the stain alone. At this step all types of bacteria present in the sample smear are colored by one color which is the dark violet color.
5. Wash with tap water and get rid of the excess water on the slide.
6. Decolorize with (which is absolute alcohol 95% or absolute alcohol + acetone) for 5 sec.
7. Wash with running water immediately. Cover the smear with the counter stain which is **safranine** in order to color the bacteria that have lost the stain during the use of alcohol. Leave for 1 minute and wash with tap water.
8. Leave to dry at room temperature & Examine by oile immersion lens.
9. Examine under microscope using the low , high and oil immersion lens.

In summary, the steps of gram stain procedure include:

- 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.

1. **Primary Stain:**
Crystal violet

All bacteria will be stained bluish or bluish-purple

2. **Mordant:**
Gram's Iodine

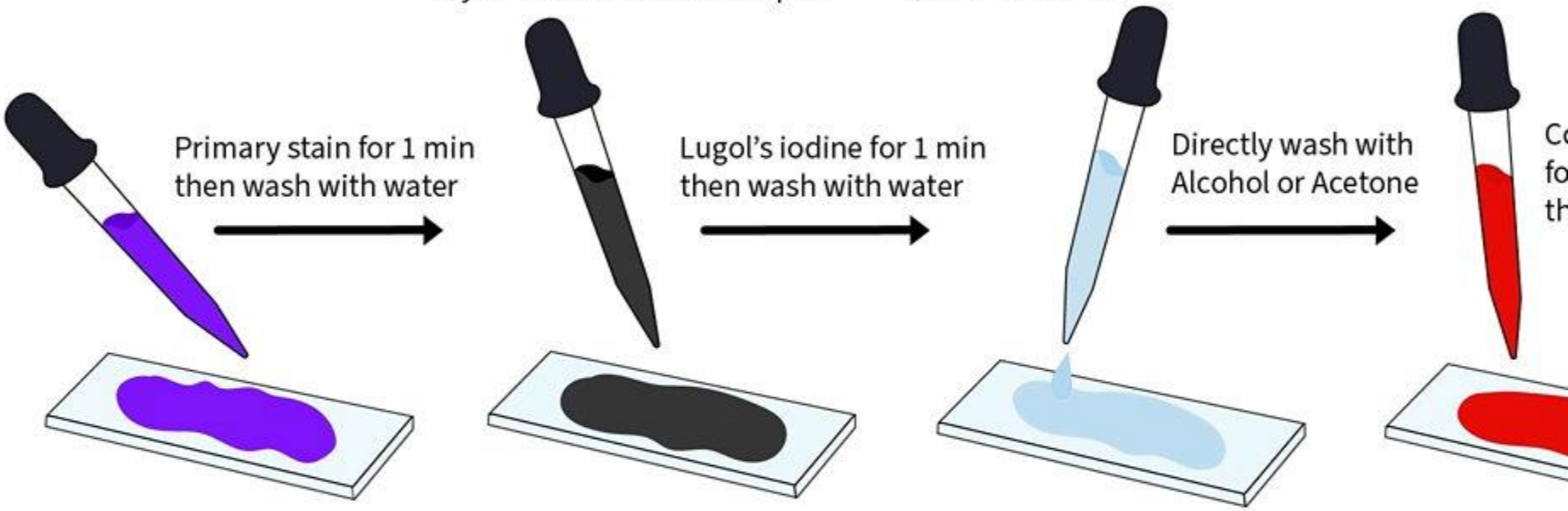
Enhance the Crystal violet staining by forming Crystal violet - Iodine complex

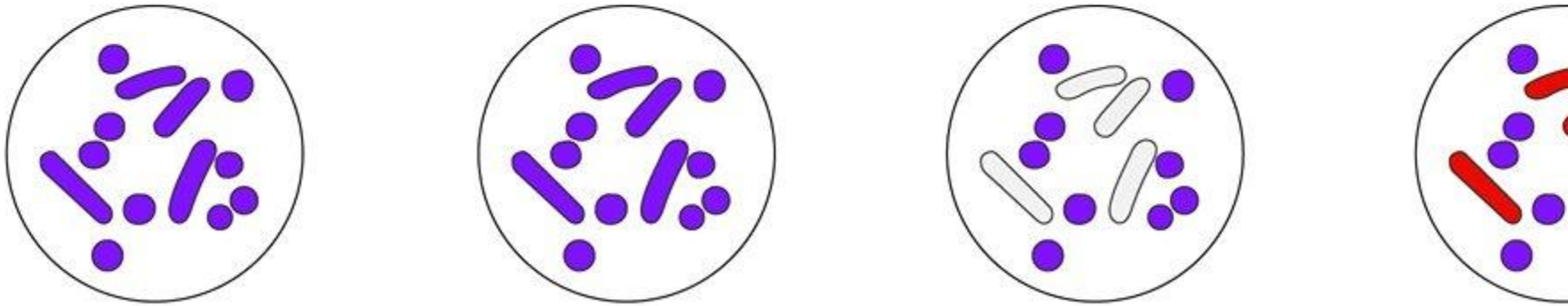
3. **Decolorizer:**
Alcohol/Acetone

Decolorizer washes away Primary stain from gram -ve bacteria

4. **Counter Stain:**
Safranin/Carbol f

Counterstain stai gram -ve bacteria





Gram +ve: Purple
Gram - ve: Purple

Gram +ve: Purple
Gram - ve: Purple

Gram +ve: Purple
Gram - ve: Colorless

Gram +ve
Gram - ve

Principle

1. Crystal violet and iodine combined to form a dye-iodine complex
2. Dye-iodine complex is dissociated by alcohol (decolorizing agent)
3. The cell wall of Gram-negative organisms have relatively little peptidoglycan and mainly consist of lipoproteins and polysaccharides. While in Gram positive organisms the peptidoglycan comprises the major part of the cell wall so Gram +ve more rigid than Gram negative cells and less permeable for the dye-iodine complex to diffuse freely out of the cell during the process of decolorization.
4. The more acid charater of the protoplasm of Gram positive bacteria which is also enhanced by treatment with iodine may partly explain their strong retention of the basic dye.

3. Special stain:

This procedure is used when a certain bacteria or some of their structures could not be stained

Special stain include:

A. **Acid fast stain (Zeihl Neelsen stain)**
B. **Capsular stain**
C. **Spore stain**
D. **Flaggellar stain**

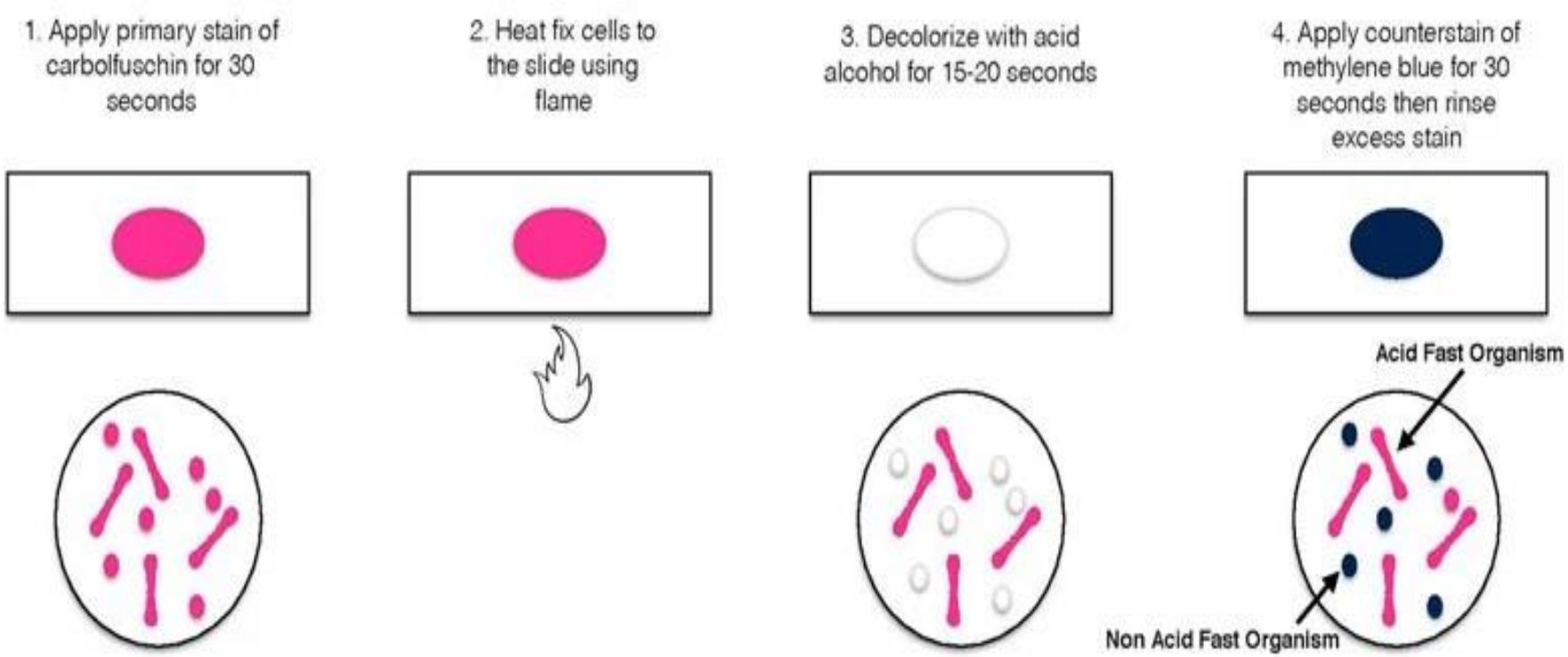
A. Acid fast stain (Zeihl Neelsen stain)

- This technique is used for staining of **M. tuberculosis & M. leprae** (the causative agents of tuberculosis and leprosy respectively) and using for staining of **Nocardia** or **Actinomyces**.
- The above bacteria possess a complex lipid in their cell wall which is called **wax D**
- **Wax D** composed of two molecules of mycolic acid and one molecule of the sugar trehalose, which mainly gives the bacteria resistance to be stained by ordinary stains and also lead to the formation of what is called serpentine cords when a smear is taken from a colony on **Lownstein Jensen medium**.

Procedure for Acid Fast staining :

1. Specimen:taken from either a suspected tuberculosis patients Or formalin killed culture.
2. Prepare a heat fixed smear after marking our slide.
3. Flood the smear with Strong **carbol fuchin** stain and steam for 5 minutes.
4. Leave to cool down, hold the slide with forceps in an angle position and add 20% H_2SO_4 or acid alcohol until no more color runs off.
5. Wash with tap water and add the counter stain which is **Methylene blue**. .
6. Wash with tap water,Leave to dray in an angel position .
7. Examine by oil immersion lens for Red bacilli.

Procedure for Ziehl-Neelsen Staining




Acid Fast Bacilli
Acid-Fast Stained Smear of Sputum
Mag. 4000X

B. Capsule stain :

- Capsule is a gelatinous ,non ionic structure, spherical or oval shape surrounding certain of bacteria as a protective extracellular structure, protect the cell from phagocytosis by phagocytic cell,
- It is considered as virulence factor & Antigenic structure .
- The capsule is consisting of 97-98% water and only 2-3% of dissolved solid (polysaccharides,glycoproteins ,
- polysaccharides with certain lipids)
- Since the capsule consisting of large amount of water, it requires a special procedure for staining . One of the procedures is called **Hiss method** :

Procedure for Capsule staining (Hiss method) :

1. Prepare a smear for bacteria that possess a capsule, such as; Klebsiella or Pneumococci and heat fix very gently on the flame to avoid damaging of the capsule by excess heat.
2. Flood the smear with 1% aqueous solution of **crystal violet** stain.
3. Steam very gently for 2-3 minutes.
4. Leave to cool and rinse with 20% **copper sulfate (CuSo4).**
5. Leave to dray and examine under the oil immersion lens. capsule appear faint surrounding dark blue cell.

Other methods to view capsule include :

A. Capsule-swelling test (Quelling reaction).

B. Negative stain : India ink , Nigrosin (-ve stain).

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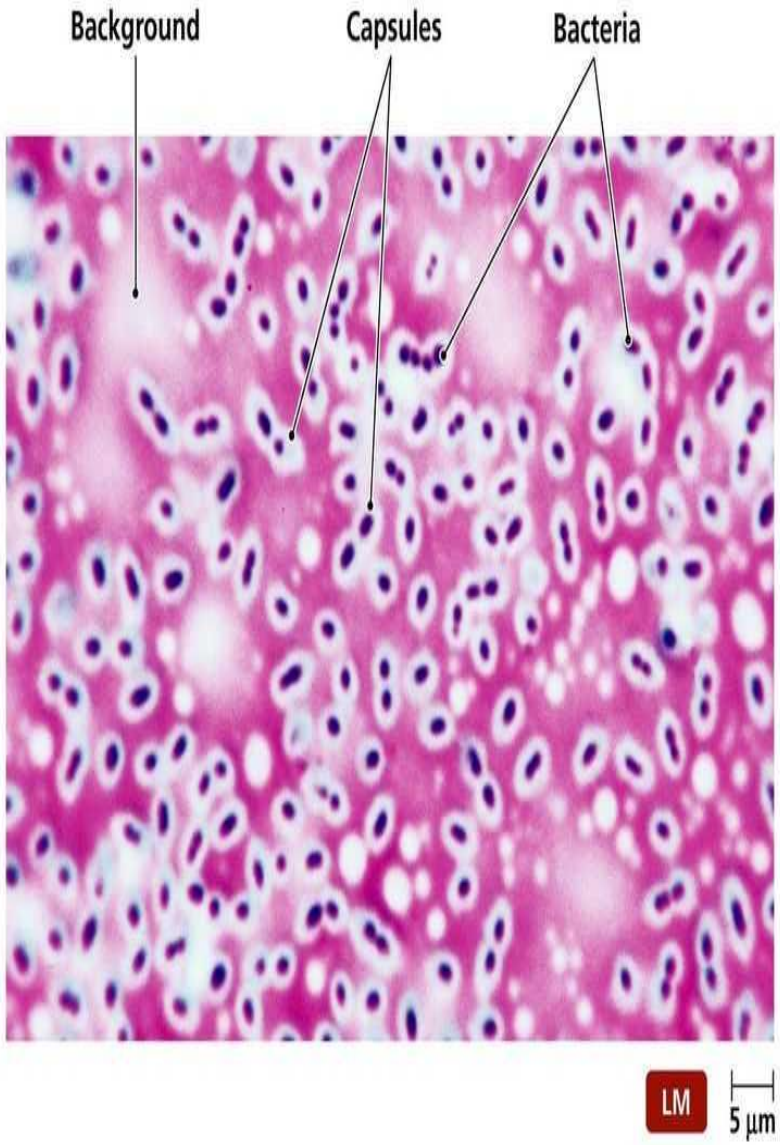

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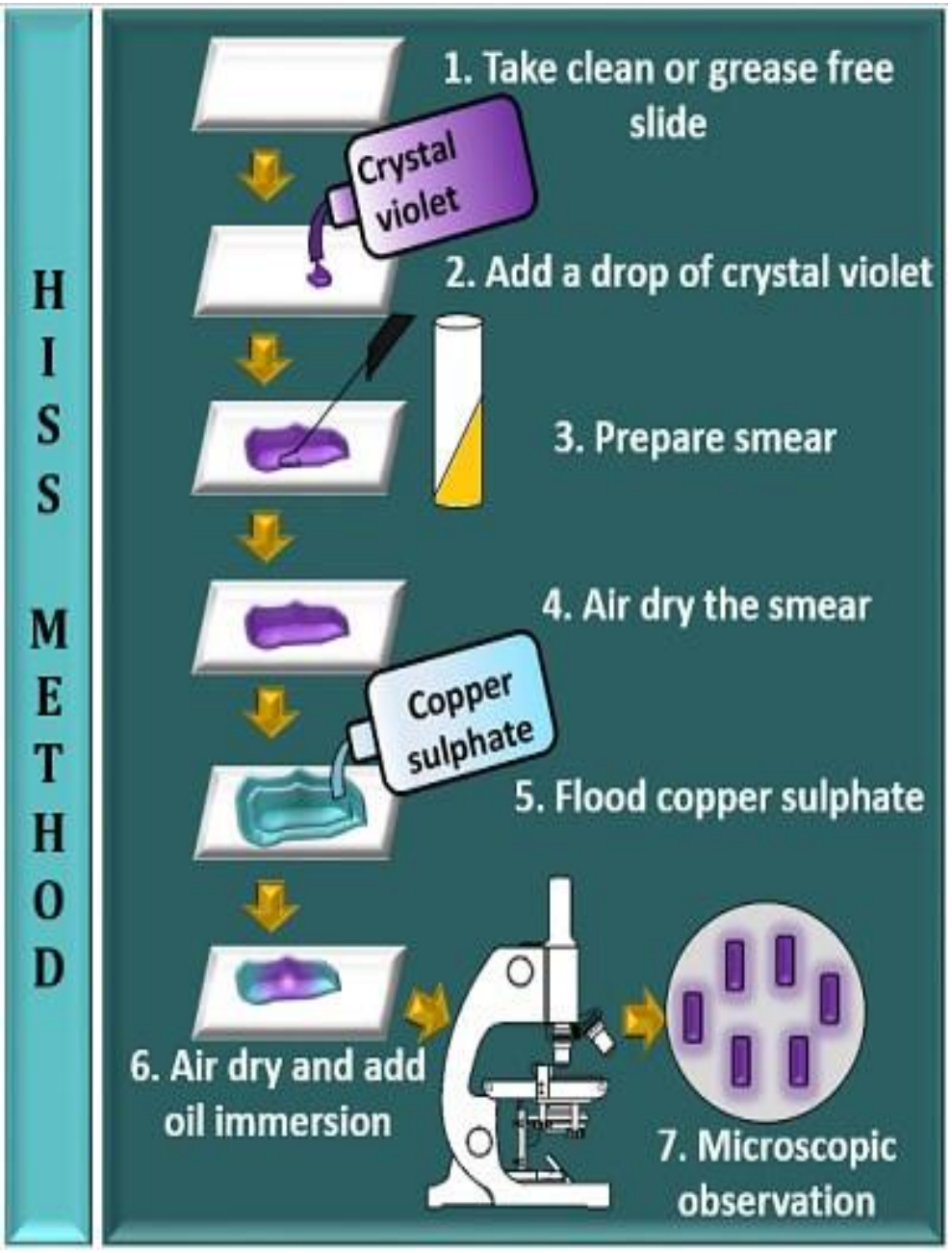
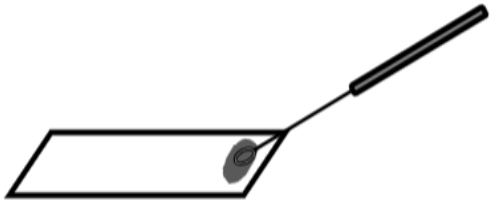


Negative stain
India ink, Nigrosin stain

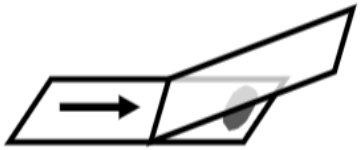
- Used to visualize MO that not easily stained e.g. cryptococcus neoformans or visualize capsule of encapsulated bacteria (eg streptococcal pneumonia).
- Negative stain repelled by negatively charged bacteria cell wall so only background will be stained

Negative stain procedure

Step #1: Begin with a SMALL drop of acidic stain (nigrosin) at one end of a clean slide. Be sure to wear gloves.



Step #2: Aseptically add organisms and emulsify with a loop. Do not over-inoculate.
Avoid splattering the mixture. Sterilize the loop after



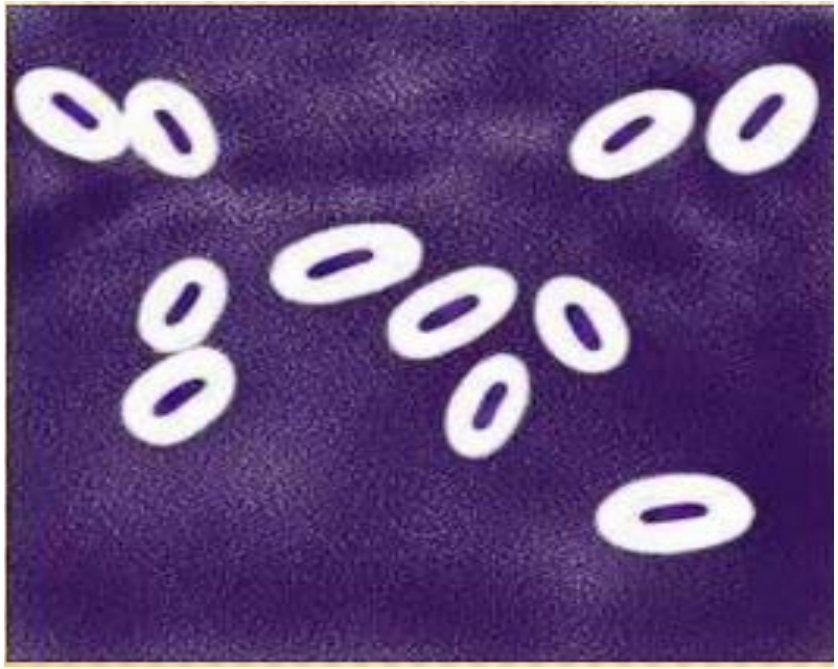
Step #3: Take a second clean slide, place it on the surface of the first slide, and draw it back into the drop.

Step #4: When the drop flows across the width of the spreader slide…

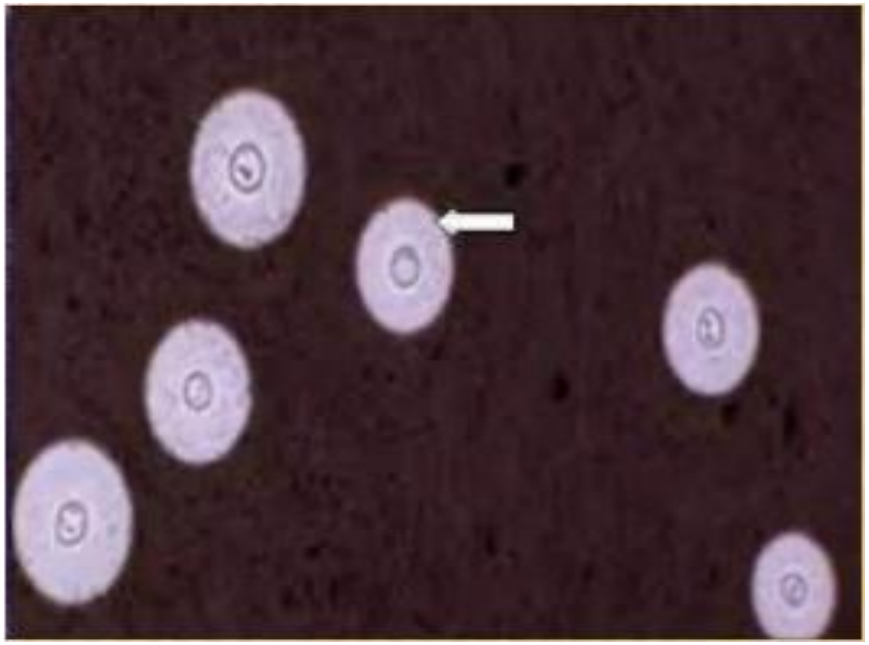
Step #5: …push the spreader slide to the other end. Dispose of the spreader slide in a jar of disinfectant or sharps container.

Step #6: Air dry COMPLETELY and observe under the microscope. Do NOT heat fix.

Negative stain



Negative stain (india ink)
Capsule of bacteria



Negative stain (India Ink)
Cryptococcus neoformans

C. Spore stain :

During sporulation process,the cell forms several layers :

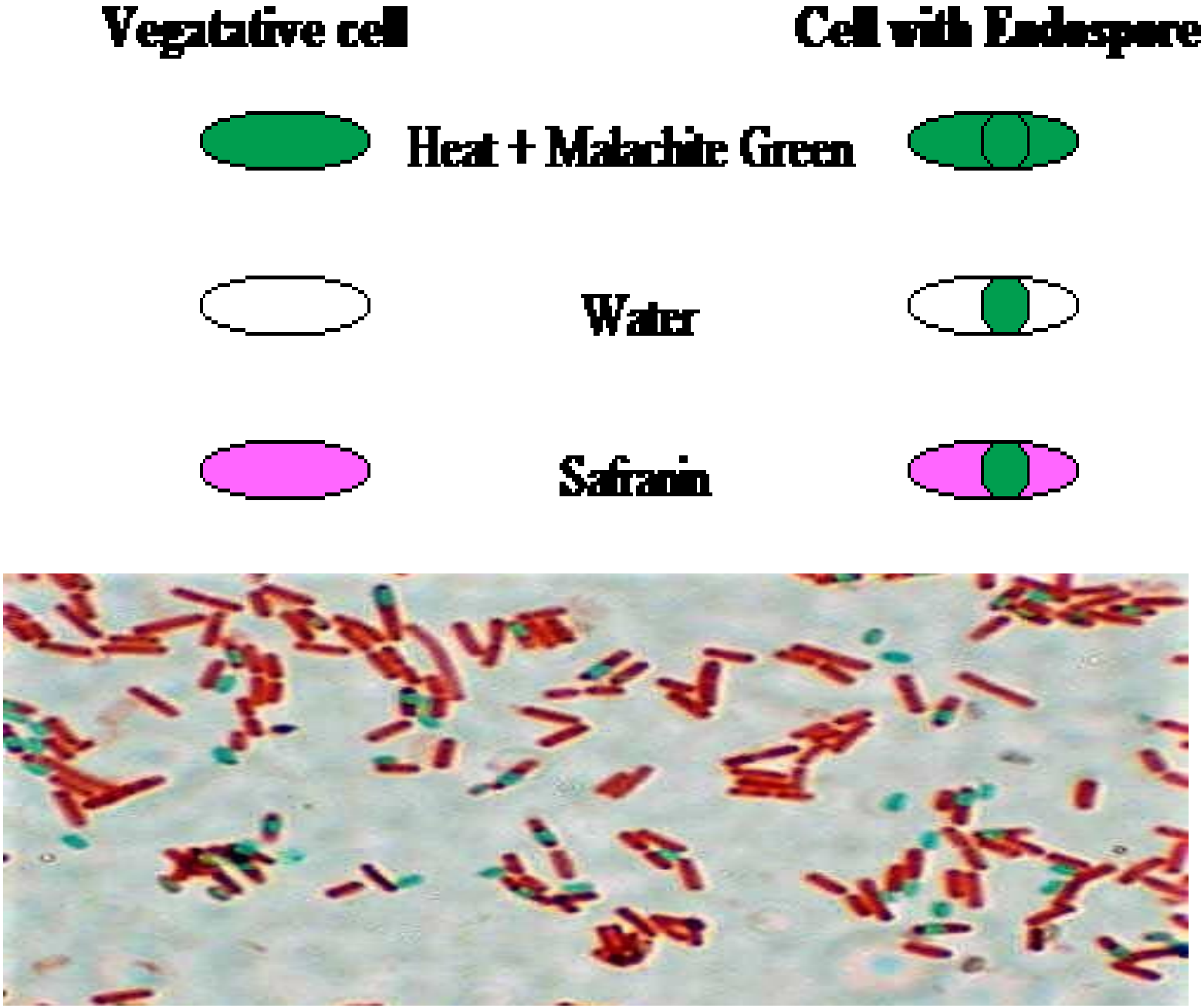
- **Core:** which contains its DNA and some ribosomes and dipicolinic acid and some other most important factors that keep the cell resistant to most adverse conditions.
- **Cortex:** consist of double layer of peptidoglycan, differs from that of the cell wall.
- **Exosporium:** consist of keratinized protein.

The above layers give the spore protection against several hard environmental conditions, at the same time, become difficult to stain the spore with the ordinary staining techniques; therefore its staining requires a special procedure such as steaming in order to allow the stain to penetrate into the spore.

Procedure for Spore staining (Schaeffer-fulton method) :

1. Prepare a heat fixed smear of a spore forming bacteria such as Bacillus subtilis or any species of Clostridium.
2. Flood the smear with **Malachite green** stain and steam for 5 minutes.
3. Leave to cool down and wash with tap water.
4. Add **Safranine** as a counter stain ,for 1 minutes.
5. Wash with tap water, Leave to dray in upright position .
6. Examine by oil immersion lens .
7. Location and the size of endospores vary with species, thus they are of value in identify bacteria.
8. Endospores position within the cell is characteristic. It can be either :

   1.Central. 2. Subterminal. 3. Terminal.


Vegitative cell
Cell with Endospore
Heat + Malachite Green
Water
Safranin

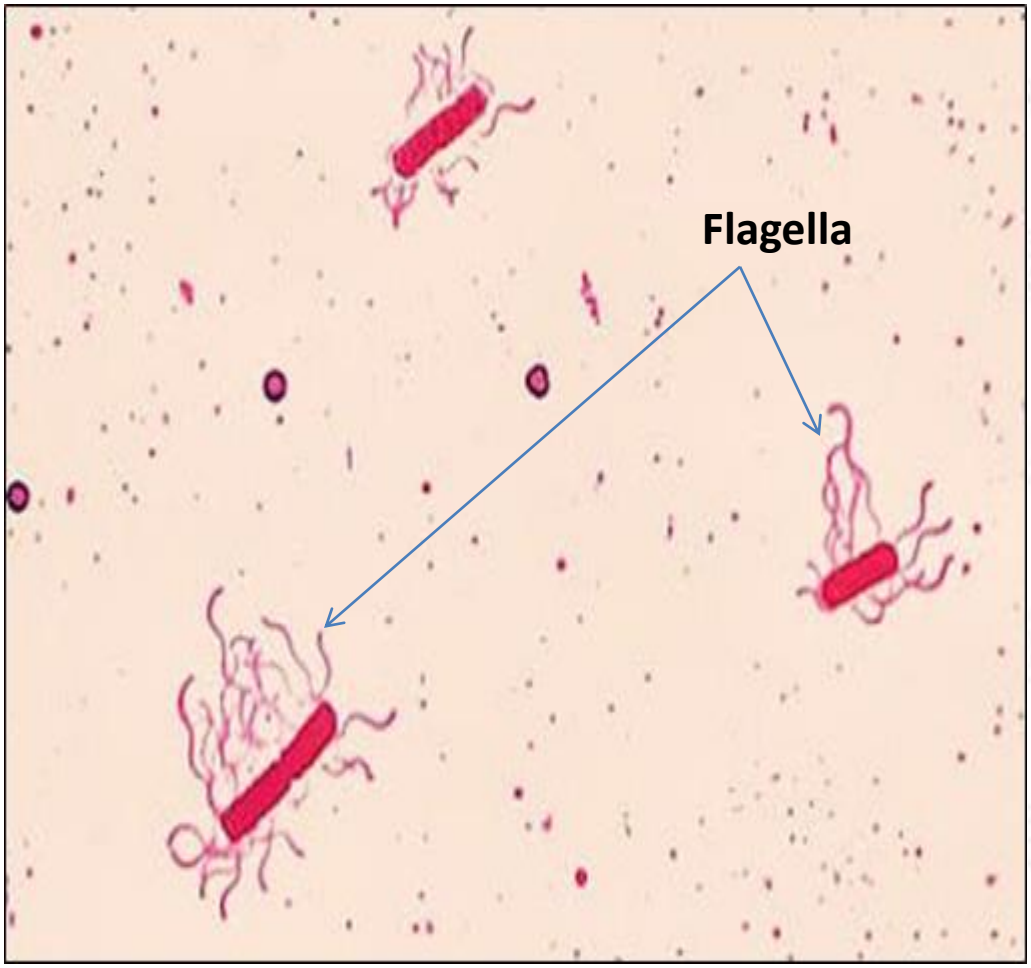
D. Flagella stain :

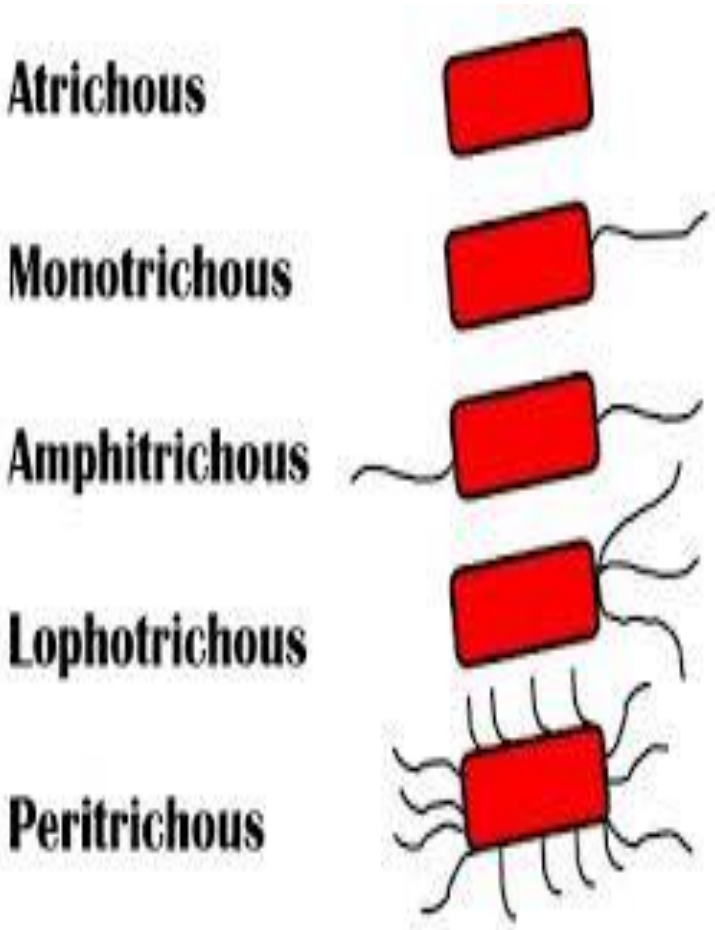
- Flagella is tiny hair like structure of locomotion . Their numbers and patterns of arrangement provide clues to identify species.
- Flagella can be viewed by:

1. E. M.
2. D.F.M (Dark field).
3. Flagellar staining

- Flagellar stain contains **Rosaniline dyes (basic fuchsin),** and a mordant, **tannic acid,** applied to bacterial suspension fixed in formalin and spread across a glass slide .
- The dye and mordant precipitate around the formalin fixed flagella surface, enlarging their diameter and making then visible.

Flagella arrangement






THANK YOU