

Microscopy and Microscopical Examination

By:

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- **What is a Microscope?**

A microscope is an optical instrument consisting of a combination of lenses which magnifies the image of the object seen through it. It is used for the morphological study of very small organisms which are not visible to the naked eye. Micro means small and scope means to view.

- The morphological study of bacteria requires the use of microscopes. Microscopy has come a long way since Leeuwenhoek first observed bacteria using hand ground lenses.
- The types of Microscopes:
 - (i) Light or optical microscope
 - (ii) Phase contrast microscope
 - (iii) Dark field/ Dark ground microscope
 - (iv) Electron microscope
 - (v) Fluorescence Microscope

- **Priniciples of an ordinary light or optical microscope:**

The specimen/sample is kept on a glass slide. Light falls from the light source on the mirror and is focussed on the specimen. The specimen throws a shadow in the beam of light which is magnified in two stages as it passes up the microscope into the eye. The objective lens starts the magnifying process. It operates at a distance that is roughly equivalent to its focal length and a real, inverted and enlarged primary image is formed in the upper part of the tube. Here, the field lens collects the diverging rays of the primary image. These rays then pass through the eye piece lens which magnifies it still further.

- When the rays leave the eye piece lens to reach the lens of the observer's eye they are divergent and the image seen by the retina is virtual and enlarged.
- Discussion : structures of light microscope.

Phase contrast microscope

- This imposes the contrast and makes evident the structure within the cells that differ in thickness or refractive index. The difference in the refractive index between bacteria cells and the surrounding medium makes them clearly visible.
- Uses of Phase Contrast Microscope:
It is used to study flagellar movement, living blood cells, internal structure of bacteria, the cytopathic effect of viruses on tissue cell cultures and the growth and subdivision of bacteria.

Dark field / Dark ground microscope

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- Another method of improving the contrast is the dark field microscope in which reflected light is used instead of the transmitted light used in the ordinal microscope. The contrast gives an illusion of increased resolution, so that very slender organisms such as spirochete, not visible under ordinary illumination, can be clearly seen under the dark field microscope.
- Use:
To view delicate organisms like spirochaetes and vibrio which become clearer due to increased contrast.

Electron Microscope

- Beams of electron are used instead of beam of light, used in light microscope. The object which is held in the path of beam scatters the electrons and produces an image which is focused on a fluorescent viewing screen.
- Use:
To study very small organisms like viruses or to see structural details within cells.

Fluorescence Microscope

- When certain materials e.g. oil or fat droplets or certain dyes are exposed to ultraviolet radiation, they convert the invisible short wave radiation into longer wave length of visible light and so become luminous and are said to fluoresce. So if microorganisms or tissue cells are stained with a fluorescent dye and are examined under the microscope with UV radiation, (instead of ordinary visible light) they are seen as bright objects against a dark background.

Use:

- Some of these fluorescent dyes have a special affinity for microorganisms or cell components and can be used to directly stain them and so make them easily visible with ultraviolet light i.e.: Auramine O: Stains tubercle bacilli with yellow fluorescence.

Acridine orange R: It has affinity for nucleic acid. It fluoresces orange red with RNA and yellow green with DNA. It can be used to stain viral nucleic acids in infected cells and tissues.

INTRODUCTION:

- **Microbial Staining: giving colour to microbes.**
- **Why we used stains? Because microbes are colourless and highly transparent structures.**
- **Staining: process in which microbes are stained.**

INTRODUCTION - STAINS

- **Stains/dyes: Organic compounds which the coloring part either positive charges or negative charges or both.**
- **Based on the charges:**
 - Basic stain/dyes: stain with +ve charge.**
 - Acidic stain/dyes: stain with –ve charge.**
 - Neutral stain/dyes: stain with both charges.**

- **Based on function of stain:**

1. **Simple staining:** only one dye is used. Differentiation among bacteria is impossible.
2. **Differential staining:** more than one dye is used. Differentiation among bacteria is possible, e.g. Gram's staining, Acid-fast staining.
3. **Special staining:** more than one dye used. Special structures are seen. e.g. Capsule staining, Spore staining.

Principle of staining:

- Each staining methods have own principles but the following steps may be common:
- **Basic stain(+ve charge):**
To stain **-ve charged** molecules of bacteria and mostly used because bacterial **cell surface is -ve charge**.
- **Acidic Stain(-ve charge):**
To stain **+ve charged** molecules of bacteria used to stain the **bacterial capsules**.

Basic requirements for smear preparation and staining:

- **Clean grease-free slide.**
- **Bacteria to be stained.**
- **Inoculating loops: to transfer bacterial suspension to slide.**
- **Bunsen burner: to sterilize inoculating loops before and after smear preparation.**
- **Pencil marker: to mark the slide where bacterial smear is applied.**

Basic initial steps before staining:

- **Smear preparation:**

Putting of bacterial suspension (bacteria in liquid) to be stained on the central portion of slide in a circular fashion, air-dried, heat-**fixed** by passing the slide three times through the flame or using chemical fixatives, the resultant preparation called *bacterial smear*-appears dull white.

Purposes of Fixation

- 1- To stuck material to the slide.
- 2- Kill non sporing microorganisms.
- 3- To preserve bacteria by destroy proteolytic enzymes.
- 4- To help penetration of stain to the bacteria.

SIMPLE STAINING:

- Simple to perform- only one basic stain used.
eg. **Crystal violet**, **Methylene blue**, **Basic fuschin**, **Malachite green 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:

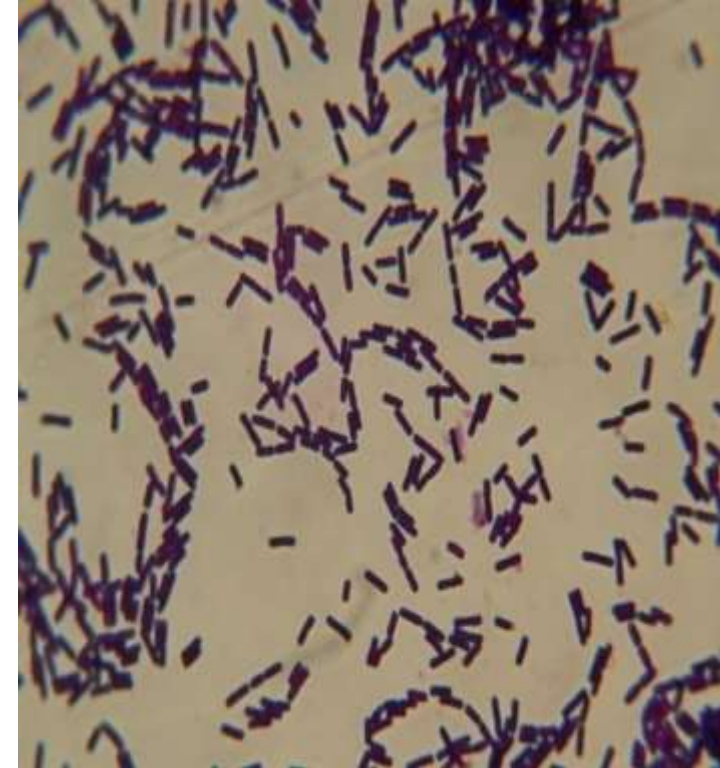
To study morphology and arrangement of bacteria.

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 focussed with 100x (Oil immersion lens).

Results:

- **Morphology:** spherical or cocci / rod.
- Arrangement:** clusters/chains.



GRAM STAINING :

- **DANISH BACTERIOLOGIST HANS CHRISTIAN GRAM**
- **Based on this reaction, bacteria classified into Gram positive and Gram negative bacteria.**
- **The cell wall composition differences makes difference.**

REQUIREMENTS – STAINING REAGENTS:

- 1. Crystal violet – Primary stain**
- 2. Gram's iodine- mordant/fixitive.**
- 3. Acetone Alcohol (95%)- decoloriser, highly flammable.**
- 4. Safranin/dilute carbol fuchsin/Neutral red – counter stain**

PRINCIPLE:

1. **Crystal violet:** all bacteria take crystal violet- so all appears violet.
2. **Iodine** – Crystal Violet-iodine(CV-I) complex is formed.
3. **Acetone Alcohol-** bacteria with high lipid content loose CV-I complex (appear colourless) but bacteria with less lipid content retains CV-I complex (appear violet). Also Gram positive bacteria have thick layer of peptidoglycan and Teichoic acid which make them less permeable.
4. **Safranin/ dilute carbol fuchsin/Neutral red** – only colourless bacteria takes – appear pink.

RESULT:

Colour:

Purple colored bacteria – Gram positive

Pink colored bacteria – Gram negative

Shape:

Spherical – cocci

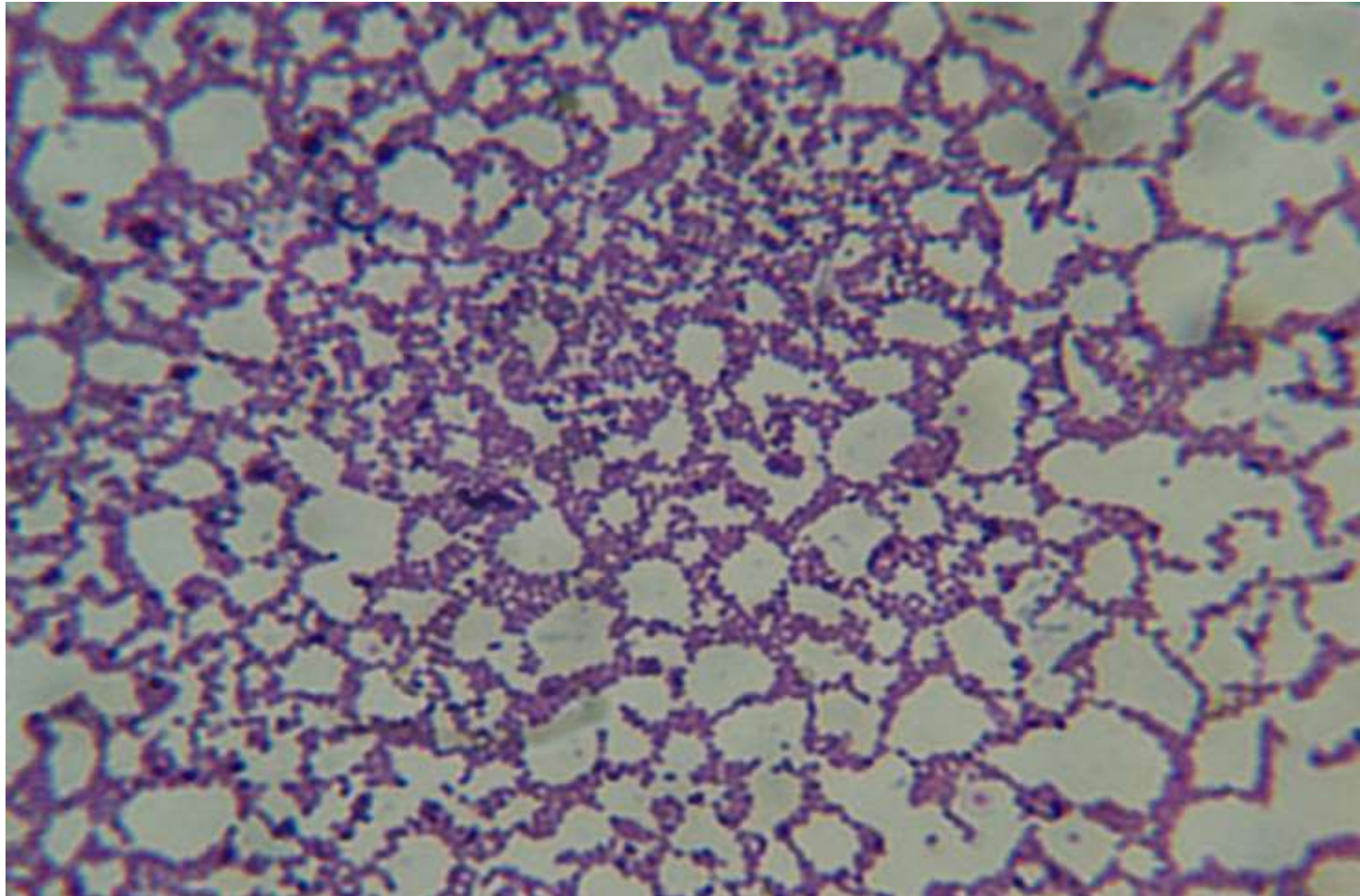
Rod – bacilli

Arrangement

Cocci in clusters – staphylococci

Cocci in chains - streptococci

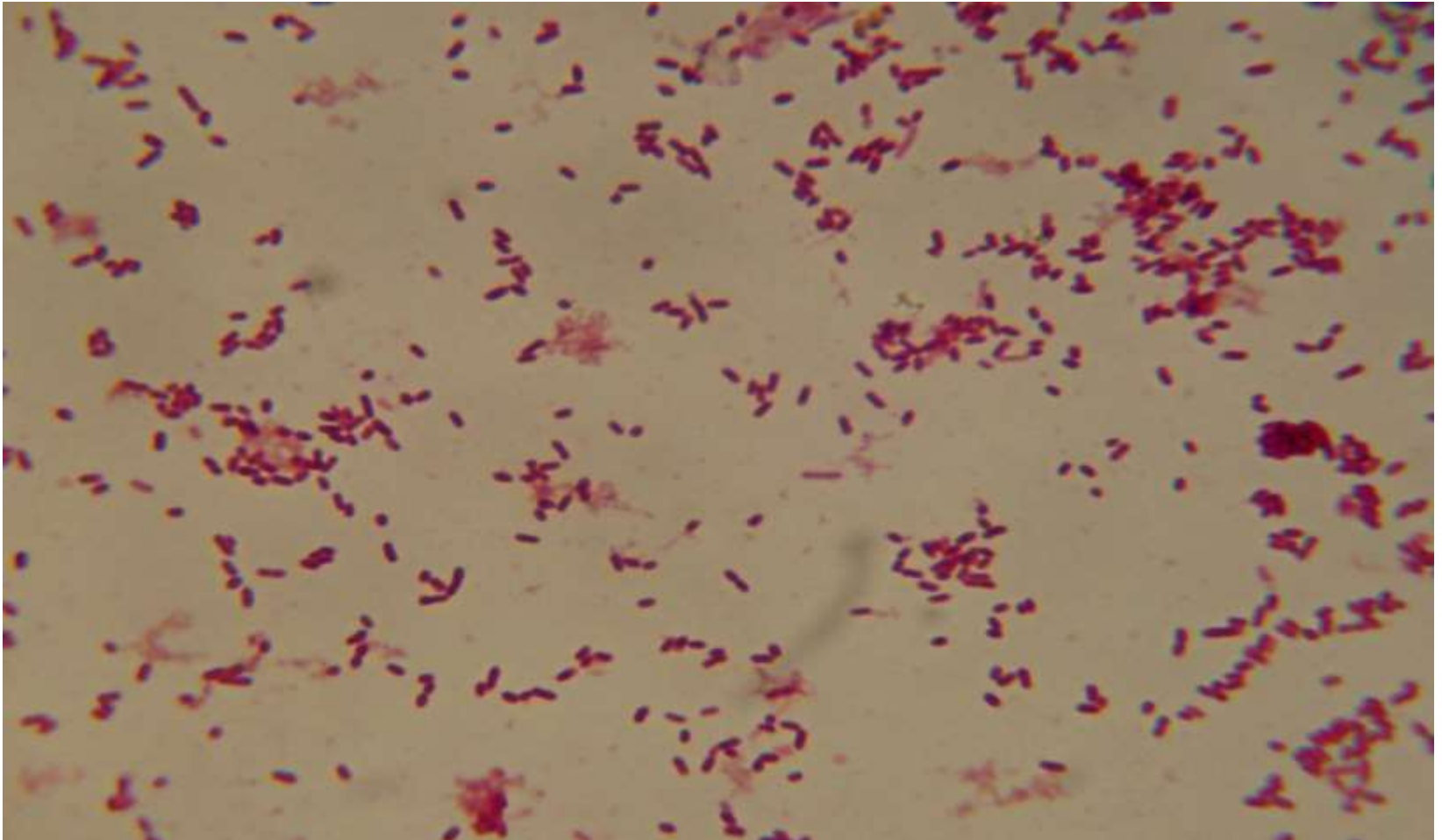
GRAM POSITIVE COCCI IN CLUSTERS



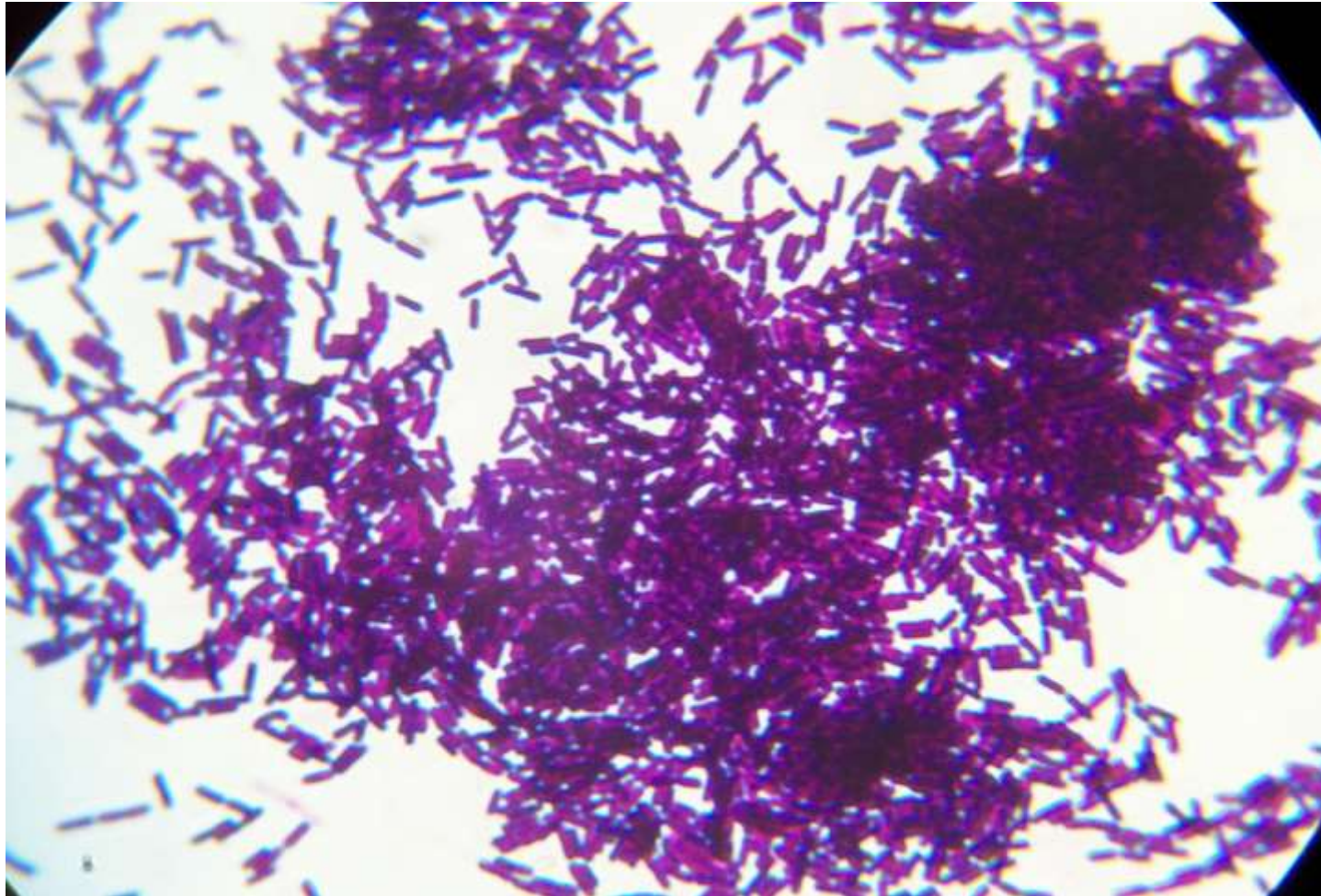
GRAM POSITIVE COCCI IN CHAINS



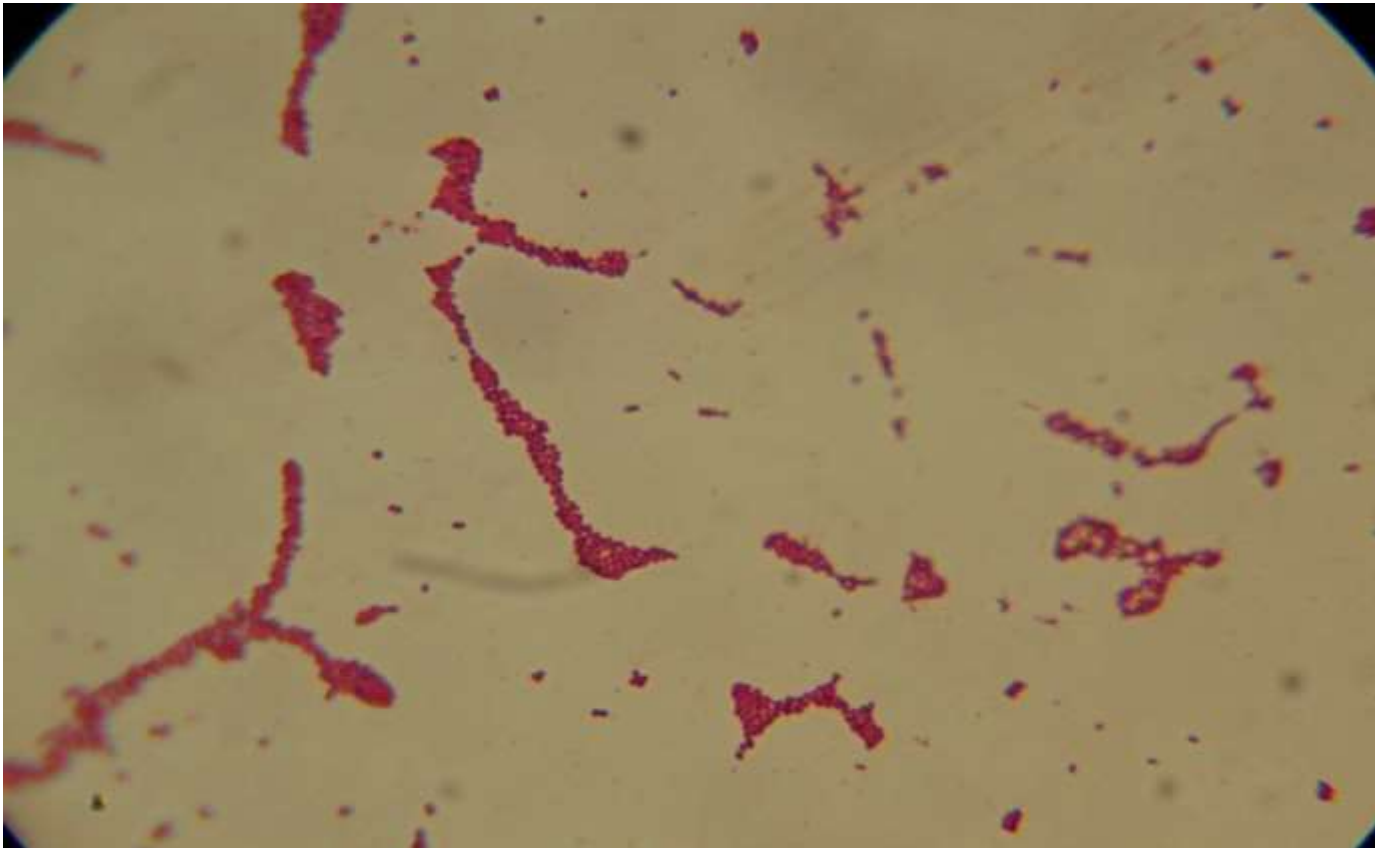
GRAM NEGATIVE BACILLI



GRAM POSITIVE BACILLI



GRAM NEGATIVE COCCI



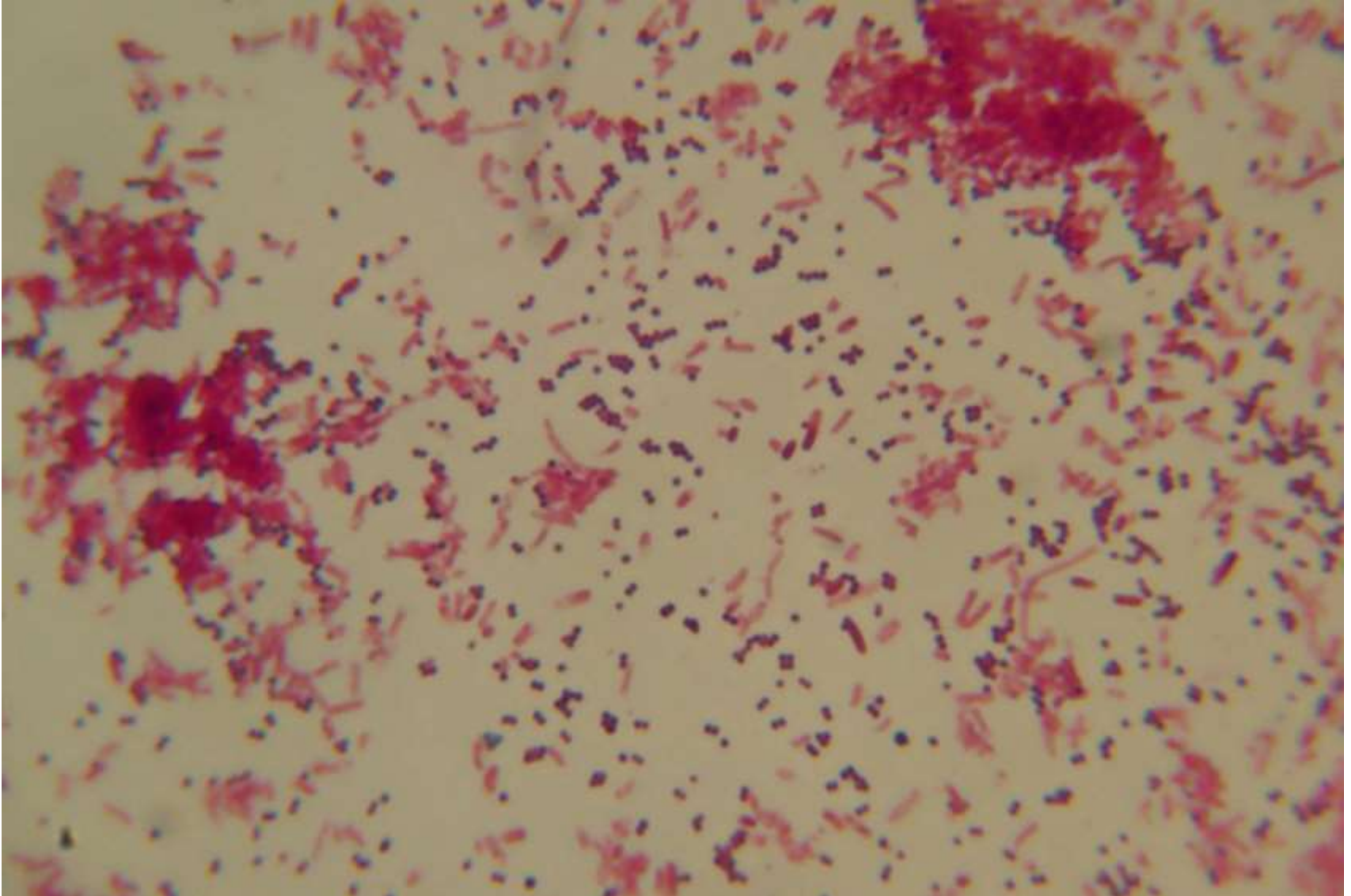
PROCEDURE:

- Crystal violet – 1 min - wash.
- Iodine – 1 min – wash.
- Acetone Alcohol-add drop by drop (about 10 seconds) and watch out colour comes out – wash immediately.
- Safarnin/dilute carbol fuchsin/Neutral red – 1 min- wash.
- Allow to dry – examine under microscope.

Note: Results should be confirmed only with 100x.

Gram stain (Mixed)

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EXAMPLES:

- **Gram positive cocci in clusters:**

Staphylococci species.

- **Gram negative cocci in chains:**

Streptococci species.

- **Gram negative cocci:**

Neisseria species.

- **Gram negative bacilli:**

1. *Escherichia coli*

2. *Klebsiella pneumoniae*

- **Gram positive bacilli:**
 1. *Clostridium species.*
 2. *Corynebacterium species.*
 3. *Bacillus anthracis.*

Staining Procedure

Click Step 1 button.



Step 1

Flood the slide with crystal violet for 1 minute. Rinse with water.

Step 2

Flood the slide with Gram's iodine for 1 minute. Rinse with water.

Step 3

Decolorize the slide by gently rinsing with an acetone-alcohol solution for 10 seconds. Rinse with water.

Step 4

Flood the slide with safranin for 1 minute. Rinse with water and air dry.

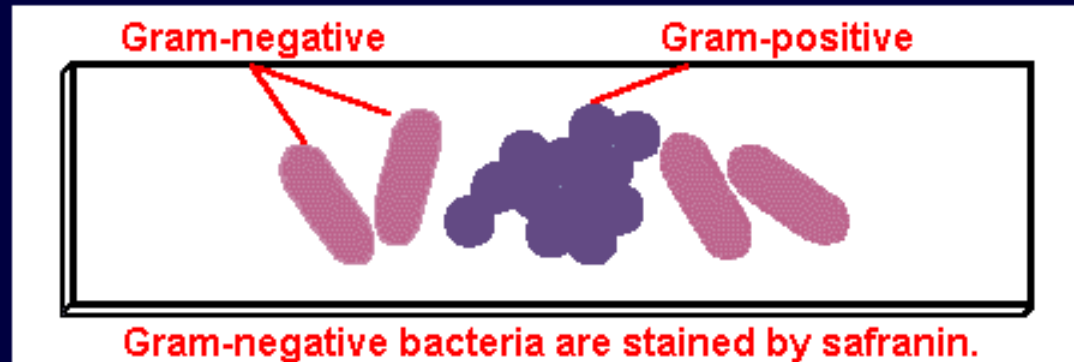
[Menu](#)

[Back](#)

[End](#)

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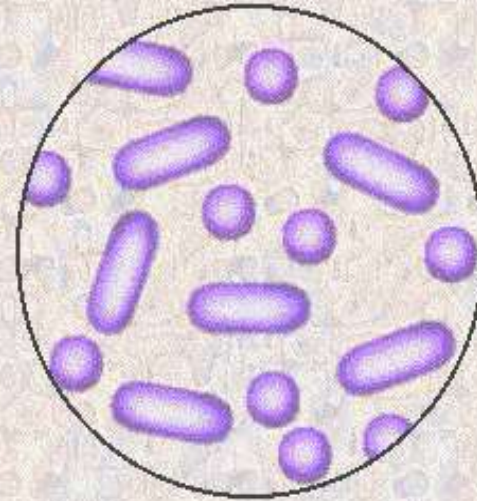
[Back](#)

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Gram Stain

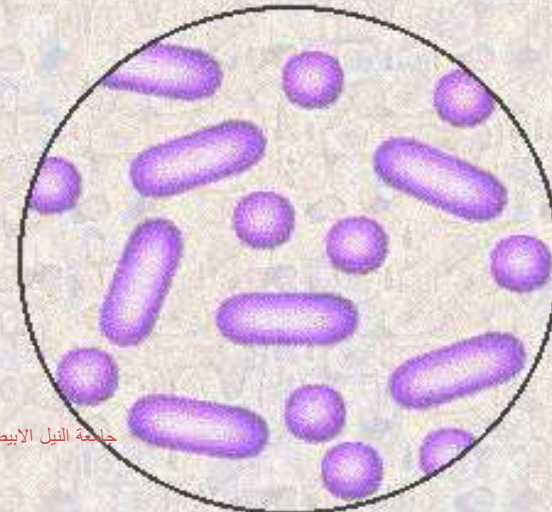
The Gram Stain

Step 1: Stain with crystal violet (primary stain), which turns both Gram-positive and Gram-negative cells purple.



The Gram Stain

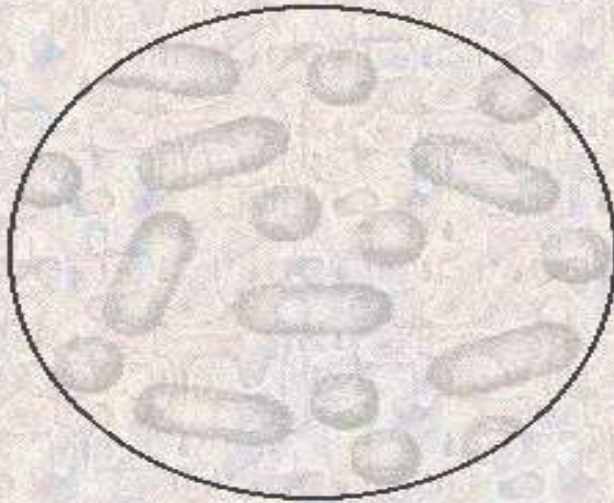
Step 2: Add iodine, which acts as a mordant to set the stain.



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The Gram Stain

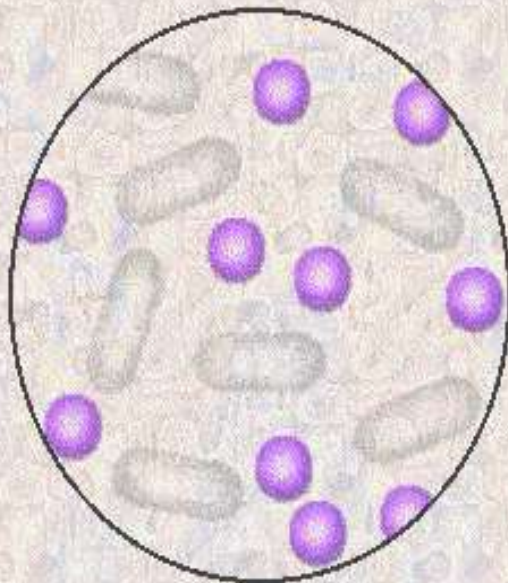


Gram Positive Cocci

Gram Negative Bacilli

The Gram Stain

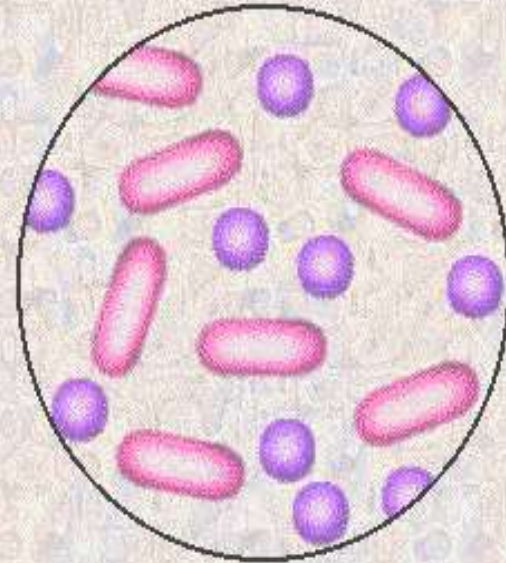
Step 3: Decolorize with acetone or alcohol, which causes the Gram-negative bacteria to lose their purple stain.



Purple: Gram-positive cocci
Clear: Gram-negative rods

The Gram Stain

Step 4: Counterstain with safranin, which turns the Gram-negative bacteria pink.



Purple: Gram-positive cocci
Pink: Gram-negative rods





**Gram-Stain
step 1**

Here crystal-violet dye is applied to the slide specimen beginning the Gram staining procedure.



**Gram-Stain
step 2**

The slide is washed with water for 10 sec. after having stained it with crystal-violet.



**Gram-Stain
step 3**

Gram's iodine is applied, and after one minute it is then washed off with tap water.



**Gram-Stain
step 4**

After the slide has been treated with Gram's iodine, it is washed off under tap water.



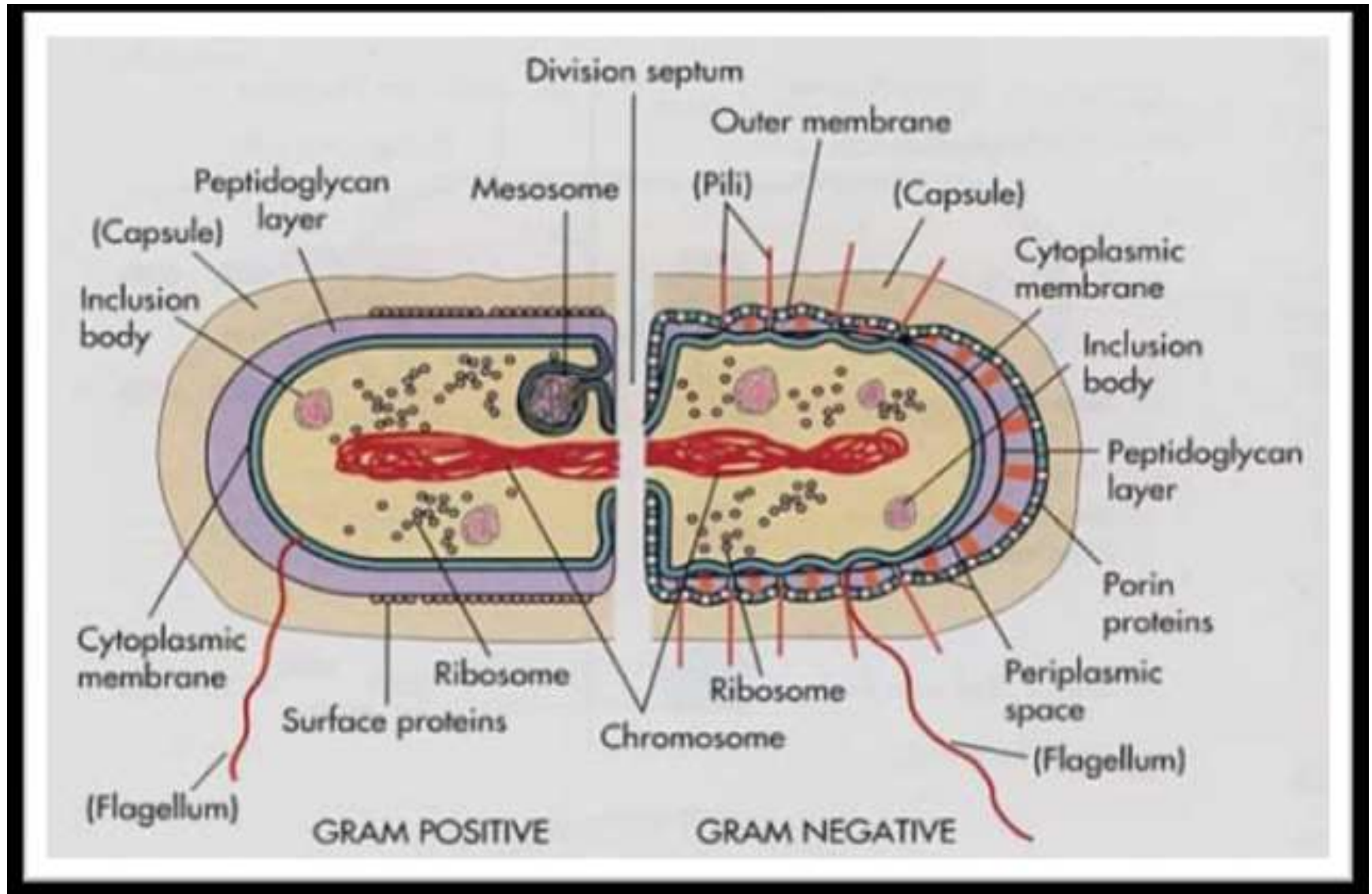
**Gram-Stain
step 5**

Here the slide is being "decolorized" using a mixture of ethanol and acetone.



**Gram-Stain
step 6**

The counterstain Safranin is applied to the slide, imparting a pink color to Gram-negative bacteria.



- Variation of Gram stain:
 - G+ve bacteria can lose its colour and appear as G-ve due to:
 - 1- Damage of cell wall due to antibiotic or excessive heat fixation.
 - 2-Over decolourization.
 - 3-Old Iodine (yellow)
 - 4-Old culture
 - G-ve appear as G+ve in case of thick smear.
- Differences between Gram positive and Gram negative Bacteria (Discuss).