

STAINS & STAINING

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OBJECTIVES OF STAINING

- ▶ Improves visibility by greater contrast between the organism and the background,
- ▶ differentiate various morphological types (by shape, size, arrangement, etc.),
- ▶ determine the staining characteristic of organism and, at times, direct diagnosis of disease, and
- ▶ demonstrate the purity of culture.
- ▶ observe certain structures (flagella, capsules, endospores, etc.),

STAINS AND DYES

- A dye is a general-purpose coloring agent, whereas a stain is used for coloring biological material.
- A stain is an organic compound containing a benzene ring plus a chromophore and an auxochrome group.
 - chromophore is a chemical group that imparts color to benzene.
 - auxochrome group is a chemical compound that conveys the property of ionization of chromogen (ability to form salts) and bind to fibers or tissues.

REQUIREMENTS FOR STAINING

Stain – Majority of the stains used for staining bacteria are of the basic type as nucleic acid of bacterial cells attract the positive ions, e.g. methylene blue, crystal violet. Acidic stains are used for background staining.

Mordant – It is a chemical that forms an insoluble complex with the stain and fixes it or causes the stain to penetrate more deeply into the cell. These are used in indirect staining. For example, Gram's iodine in Gram staining and phenol in Ziehl Neelson's staining.

Accentuator – It is a chemical which when added to a stain to make the reaction more selective and intense. For example, potassium hydroxide added in Loeffler's methylene blue.

Decolorizer – It is a chemical used to remove the excess stain in indirect regressive staining. For example, ethanol in Gram's staining.

Types of staining techniques

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graph TD; A[Types of staining techniques] --> B[Simple staining]; A --> C[Differential staining]; B --> D["Direct (Positive)"]; B --> E["Indirect (Negative)"]; C --> F["Separation into groups"]; C --> G["Visualization of structures"]; F --> H["1. Gram stain"]; F --> I["2. Acid fast stain"]; G --> J["1. Flagella stain"]; G --> K["2. Capsule stain"]; G --> L["3. Spore stain"];
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Simple staining

(Use of of single stain)

Direct

Indirect

(Positive)

(Negative)

Differential staining

(Use of two contrasting stains)

Separation

into groups

1. Gram stain

2. Acid fast

stain

Visualization

of structures

1. Flagella stain

2. Capsule stain

3. Spore stain

SIMPLE STAINING

SIMPLE STAINING

- **A staining method that uses only a single dye that which does not differentiate between different types of organisms**
- **There is only a single staining step and everything is stained with the same color.**
- **Simple stains are used to stain whole cells or to stain specific cellular components.**
- **Types of simple staining:**
 - 1. Direct / Positive staining : stain object**
 - 2. Indirect / Negative staining: stain background**

Bacteria have a slight (-) charge.

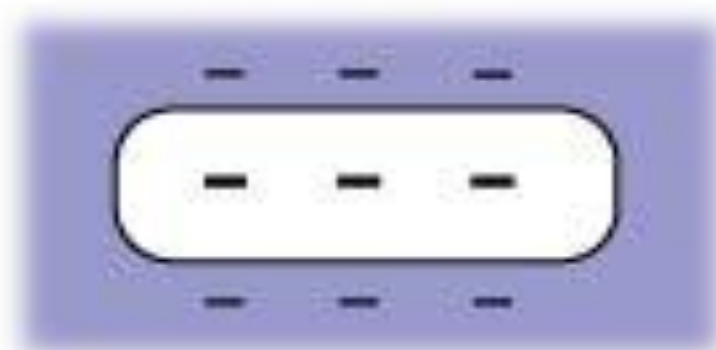


**basic dye
color portion is (+)**

**acidic dye
color portion is (-)**



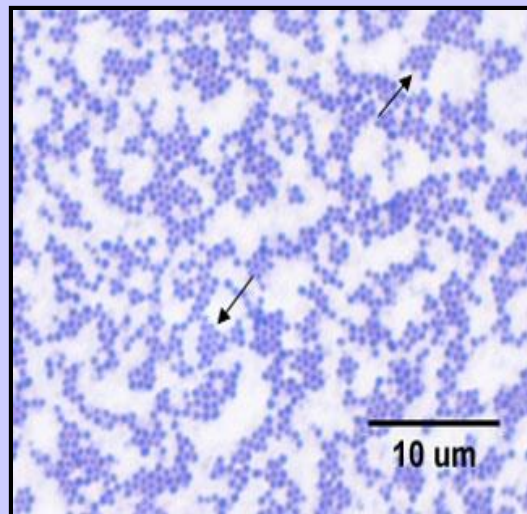
direct stain



indirect stain

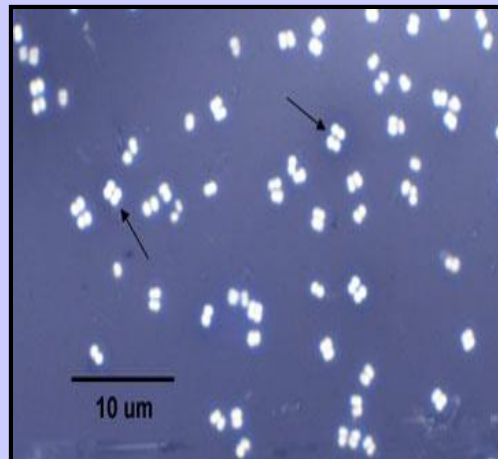
Direct staining (Positive staining)

- A simple staining technique that stains the bacterial cells in a single color.
- Many of the bacterial stains are basic chemicals; these basic dyes react with negatively charged bacterial cytoplasm (opposite charges attract) and the organism becomes directly stained
- Examples are methylene blue, crystal violet, and basic fuchsin.

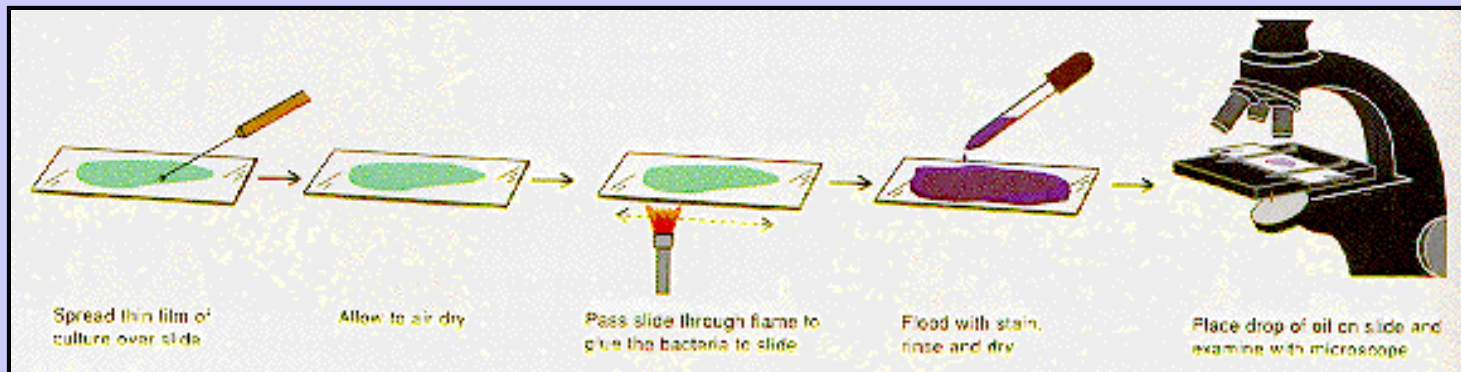


Indirect staining (Negative staining)

- In this staining process, instead of cells background is stained.
- Here, an acidic dye like nigrosin or Indian ink is used. Acidic stain carries a negative charge and repelled by the bacteria, which also carry a negative charge on their surface. Hence, an acidic dye do not stain bacteria,
- Instead, it forms a deposit around the organism, leaving the organism itself colorless or transparent upon examination.



STAINING: GENERAL TECHNIQUE



SMEAR

AIR DRY

HEAT FIX

STAIN

LOOK

Importance of fixing the smears

“Fixation accomplishes three things:

- (1) it kills the organisms;
- (2) it causes the organisms to adhere to the slide; and
- (3) it alters the organisms so that they more readily accept stains (dyes).

DIFFERENTIAL STAINING

GRAM'S STAINING

The Gram Stain

In the late 1800's, Christian Gram observed that some genera of bacteria retained a dye-Iodine complex when rinsed with alcohol, while other genera were easily decolorized with alcohol and could be then visualized by a contrasting counter stain.

This staining procedure defines two bacterial groups: those which retain the primary dyes ("Positive by Gram's Method" or "**Gram-Positive**") and those which are easily decolorized ("Negative by Gram's Method" or "**Gram-Negative**"). This is the starting point for bacterial identification procedures.

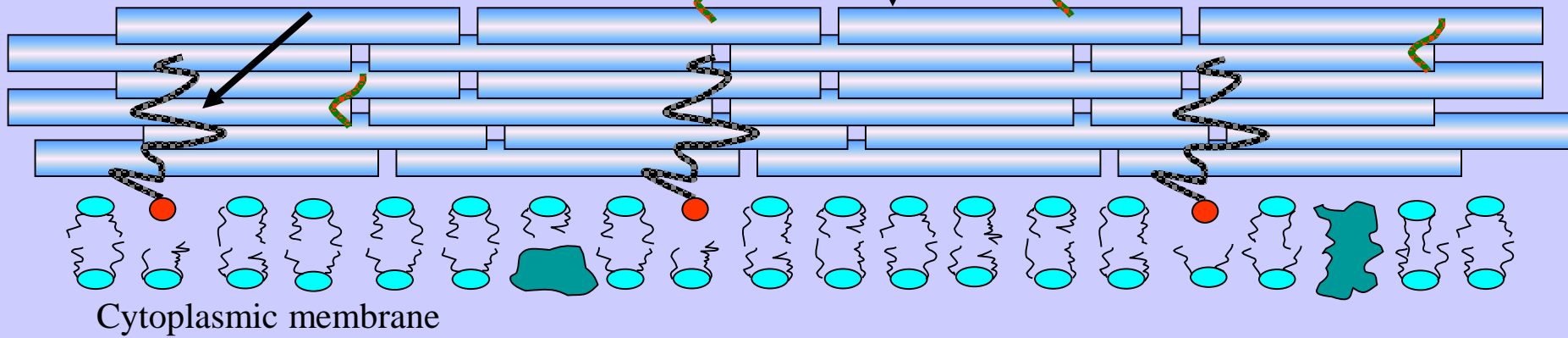
The Gram Stain

- The difference in dye retention is dependent on such physical properties as thickness, density, porosity, and integrity of the bacterial cell wall, as well as its chemical composition.
- **Gram-Positive** bacteria have thick, dense, relatively non-porous walls, while **Gram-Negative** bacteria have thin walls surrounded by lipid-rich membranes.
- Some non-bacterial organisms with thick cell walls (e.g., some yeasts) also stain **Gram-Positive**.
- **Gram-Positive** bacteria which have lost wall integrity through aging or physical or chemical damage may stain **Gram-Negative**.

GRAM POSITIVE

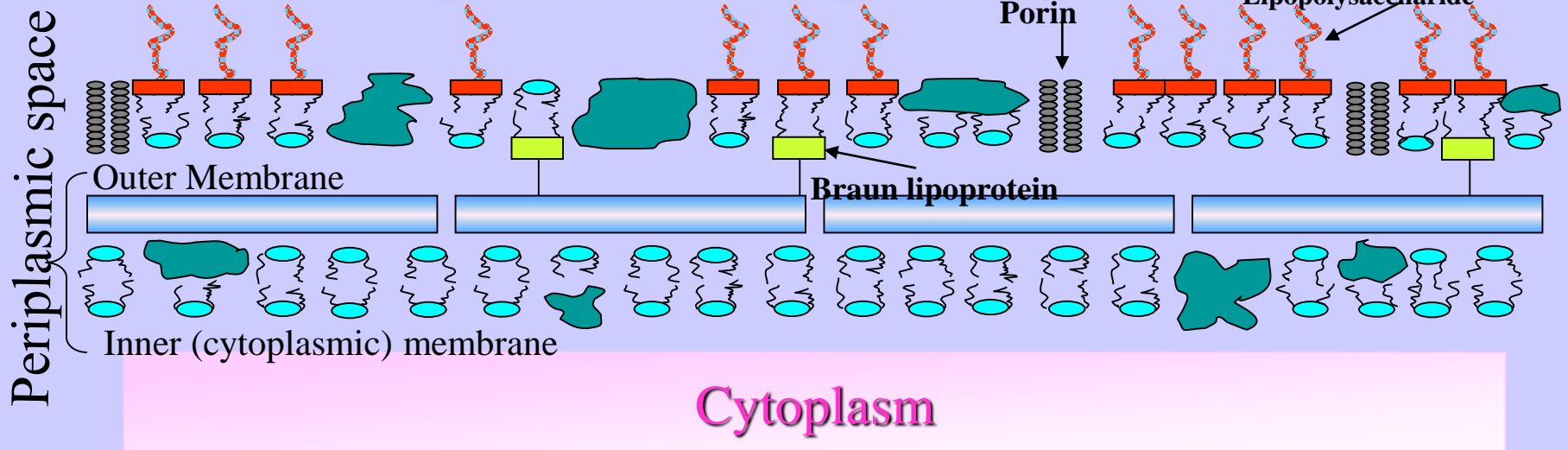
Lipoteichoic acid

Peptidoglycan-teichoic acid



Cytoplasm

GRAM NEGATIVE



Gram staining – Requirements

- Gram-staining is a four part procedure.
- The specimen is mounted and heat fixed on a slide before proceeding to stain it.
- The reagents required are:
 - Crystal Violet (the Primary Stain)
 - Iodine Solution (the Mordant)
 - Decolorizer (ethanol)
 - Safranin (the Counter stain)
 - Water (preferably in a squirt bottle)



Gram staining – Procedure

- 1 The bacteria are first stained with the basic dye crystal violet (primary stain). Both gram-positive and gram-negative bacteria become directly stained and appear purple after this step.
- 2 The bacteria are then treated with gram's iodine solution (mordant). This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex, called as 'iodine lake'. Both gram-positive and gram-negative bacteria remain purple after this step.
- 3 Gram's decolorizer, a mixture of ethyl alcohol and acetone, is then added. **This is the differential step.** Gram-positive bacteria retain the crystal violet-iodine complex while gram-negative are decolorized.
- 4 Finally, the counter stain safranin (also a basic dye) is applied. Since the gram-positive bacteria are already stained purple, they are not affected by the counter stain. Gram-negative bacteria, that are now colorless, become directly stained by the safranin. Thus, gram-positive appear **purple**, and gram-negative appear **pink**.

GRAM-POSITIVE BACTERIA

GRAM-NEGATIVE BACTERIA

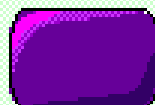
Heat - fix on slide



Crystal violet



Stained purple



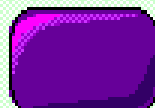
Stained purple



Iodine solution (Mordant)



Remains purple



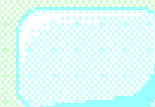
Remains purple



Remains purple



Becomes colourless



Safranin counterstain

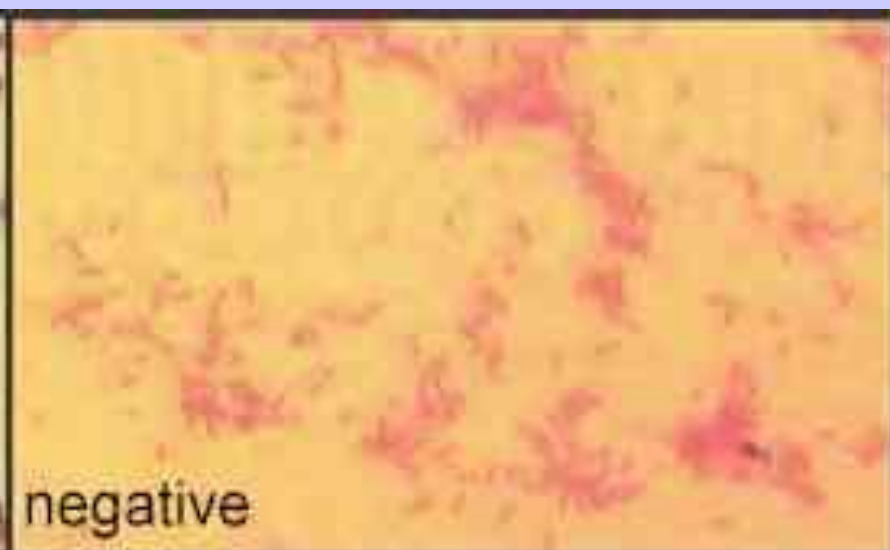
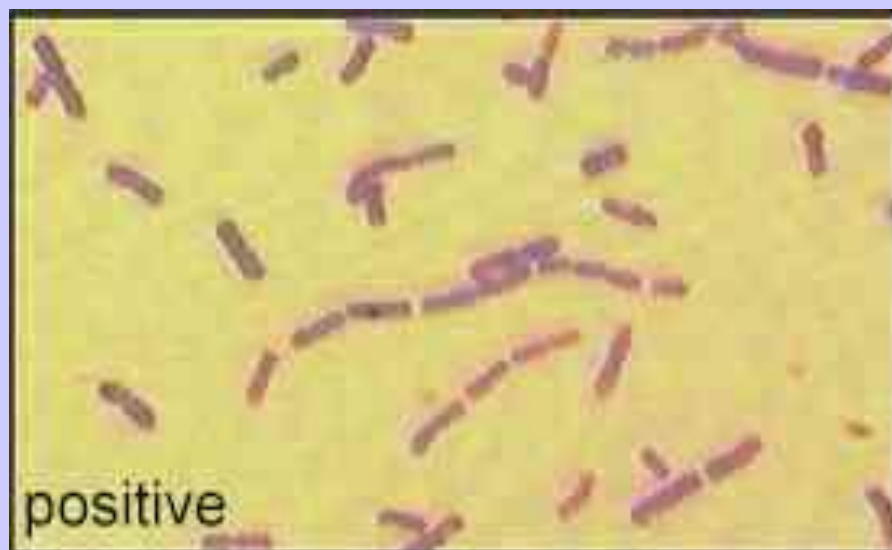


Remains purple



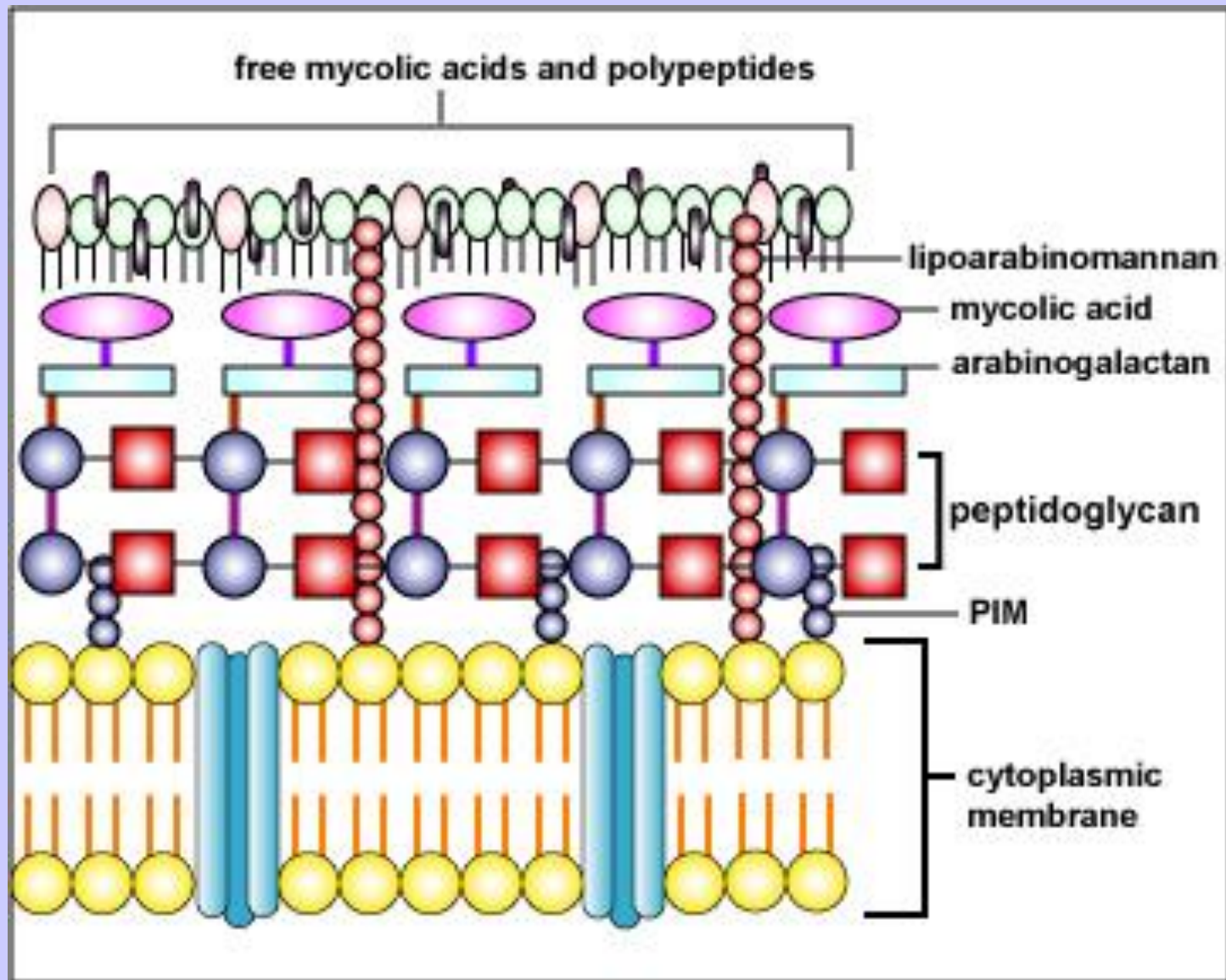
Stained red





ACID FAST STAINING

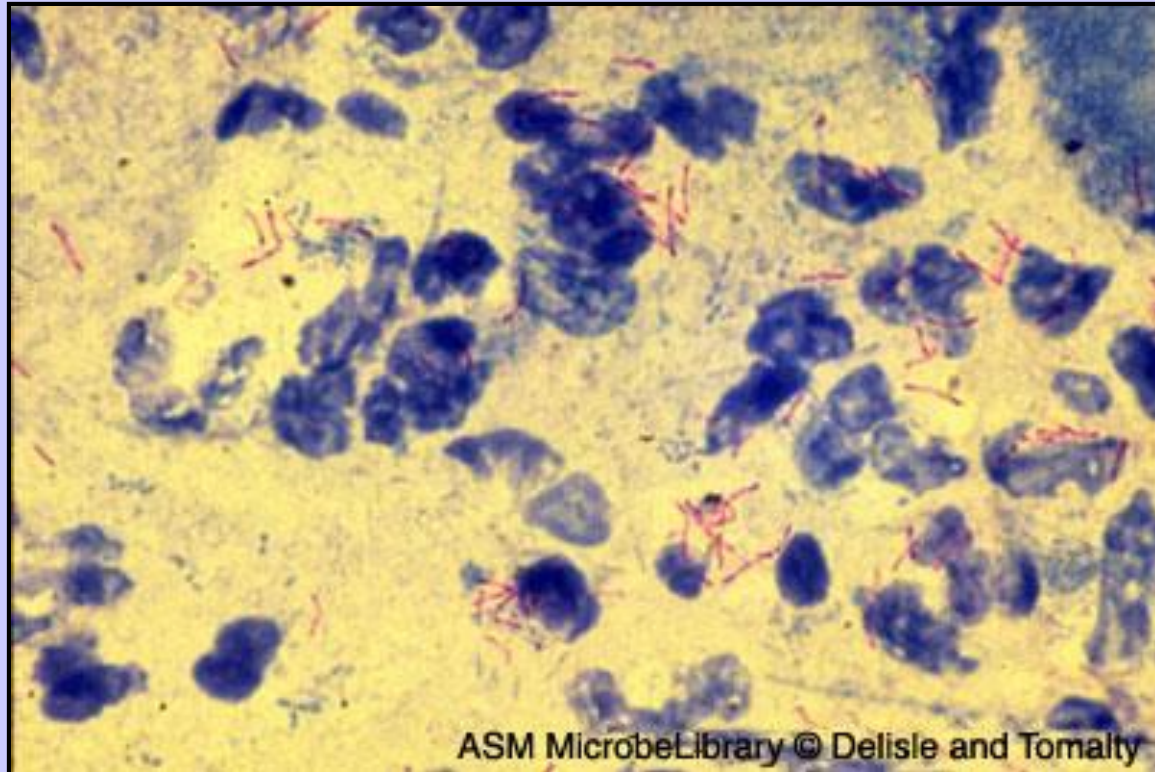
Structure of an Acid-Fast Cell Wall



Acid fast staining - theory

- Once stained the acid fast bacterial cells resist decolorization with acidified organic solvents, e.g acid alcohol and are therefore called **ACID FAST**.
- Acid fast staining property of the genus, *Mycobacteria*, depends upon their lipid-rich cell walls which are relatively impermeable to various basic dyes unless the dyes are combined with phenol.
- The exact method by which the stain is retained is unclear but it is thought that some of the stain becomes trapped within the cell and some forms a complex with the mycolic acids. This is supported by the finding that shorter chain mycolic acids or mycobacterial cells with disrupted cell walls stain weakly acid-fast, e.g. *Nocardia*

Acid-Fast Stain of *Mycobacterium tuberculosis* in Sputum



Note the reddish acid-fast bacilli among the blue normal flora and white blood cells in the sputum that are not acid-fast.

SPECIAL STAINING

CAPSULE STAINING

- Capsule staining is diagnostically useful since it is a virulent factor(e.g. pneumococci).
- Bacterial capsules are non-ionic, so neither acidic nor basic stains will adhere to their surfaces.
- Capsules are demonstrated either by negative staining (Nigrosin or India ink) or by special staining, e.g. Hiss' method, Anthony's method

Hiss Method

- The capsule is non-ionic in nature so it doesn't get stain by a acidic stain but a basic stain, such as crystal violet, stains the cell as well as the capsule.
- This is followed by treatment with hypertonic solution - 20% Copper sulphate solution, which **serves dual role of both the decolorizing agent and counter stain.**
- Copper sulphate solution, being hypertonic, causes diffusion of stain towards outer surface of cell.
- After drying of slide, the stain which is not passed from the capsular layer during diffusion retains in the capsular layer. Copper sulphate then decolorizes the capsule.
- Capsule appears as a faint blue halo around a purple cell.

Prepare a smear
air dry and don't
heat fix

Flood the smear
with Crystal violet
for 4-5 mins

Rinse the slide with
20 % Copper
sulphate solution

Air dry the slide
and observe under
oil immersion lens

Hiss method of Capsule staining

ENDOSPORE STAINING

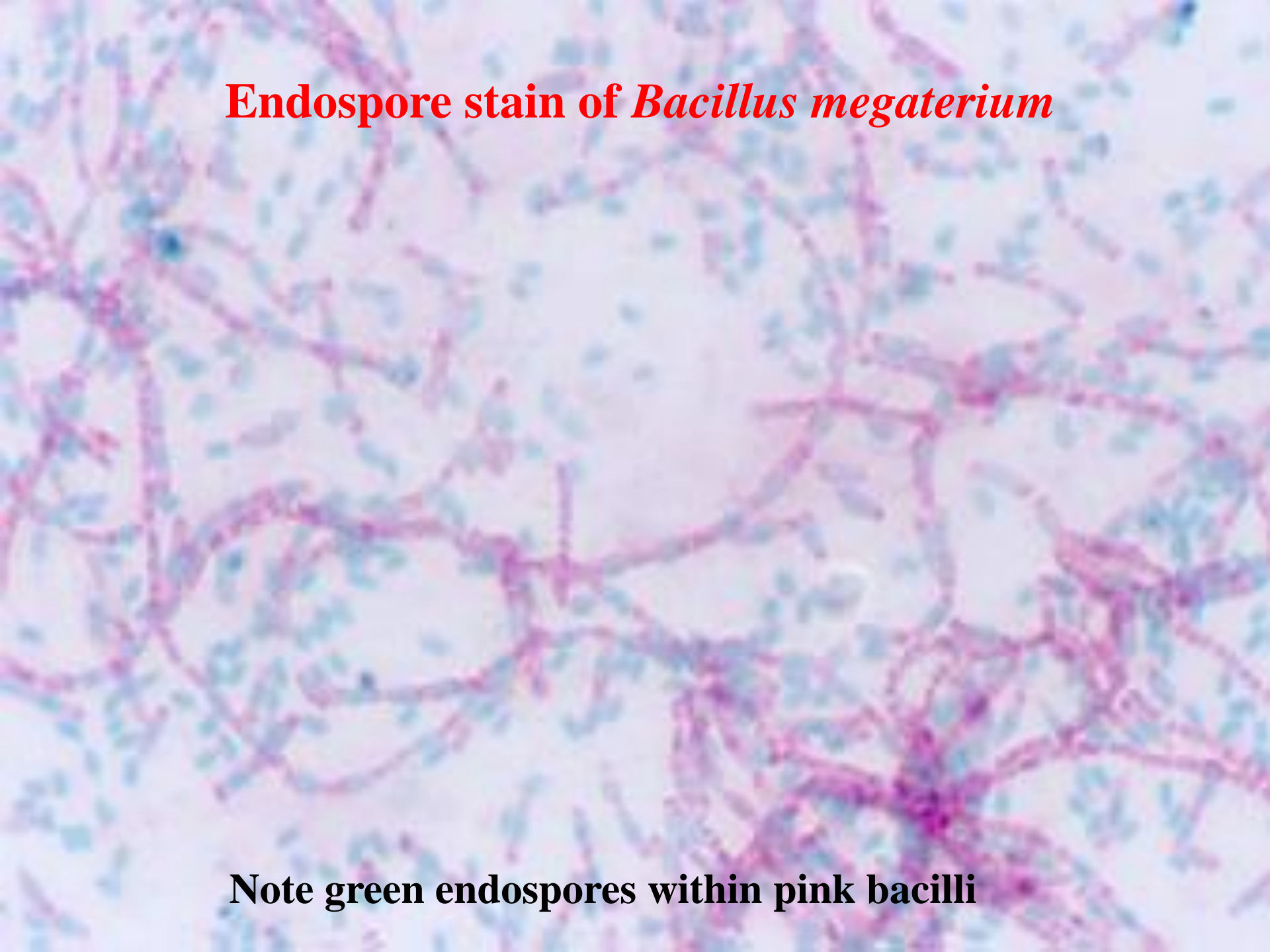
Endospore staining

- **Spores are normally impervious to stains.**
- **Under the light microscope endospores have a high light refractivity indicative of high protein content.**
- **Endospores can be stained by:**
 - **Modified Zeihl-Nelson's method using 0.25-0.5% sulphuric acid as decolorizing agent,**
 - **Barthelomew-Mittwar's method**
 - **Schaeffer-Fulton stain technique.**

Schaeffer-Fulton method

- **Malachite green** is used to stain the endospores (**primary stain**)
- The malachite green is **forced** to permeate the spore wall by **heating (mordant)**.
- Washing with water remove stain from vegetative cells, but not from spore wall.
- The endospores thus retain the primary dye while the vegetative cells lose the primary stain and take the red color of **secondary stain (safranin)**.

Endospore stain of *Bacillus megaterium*



Note green endospores within pink bacilli