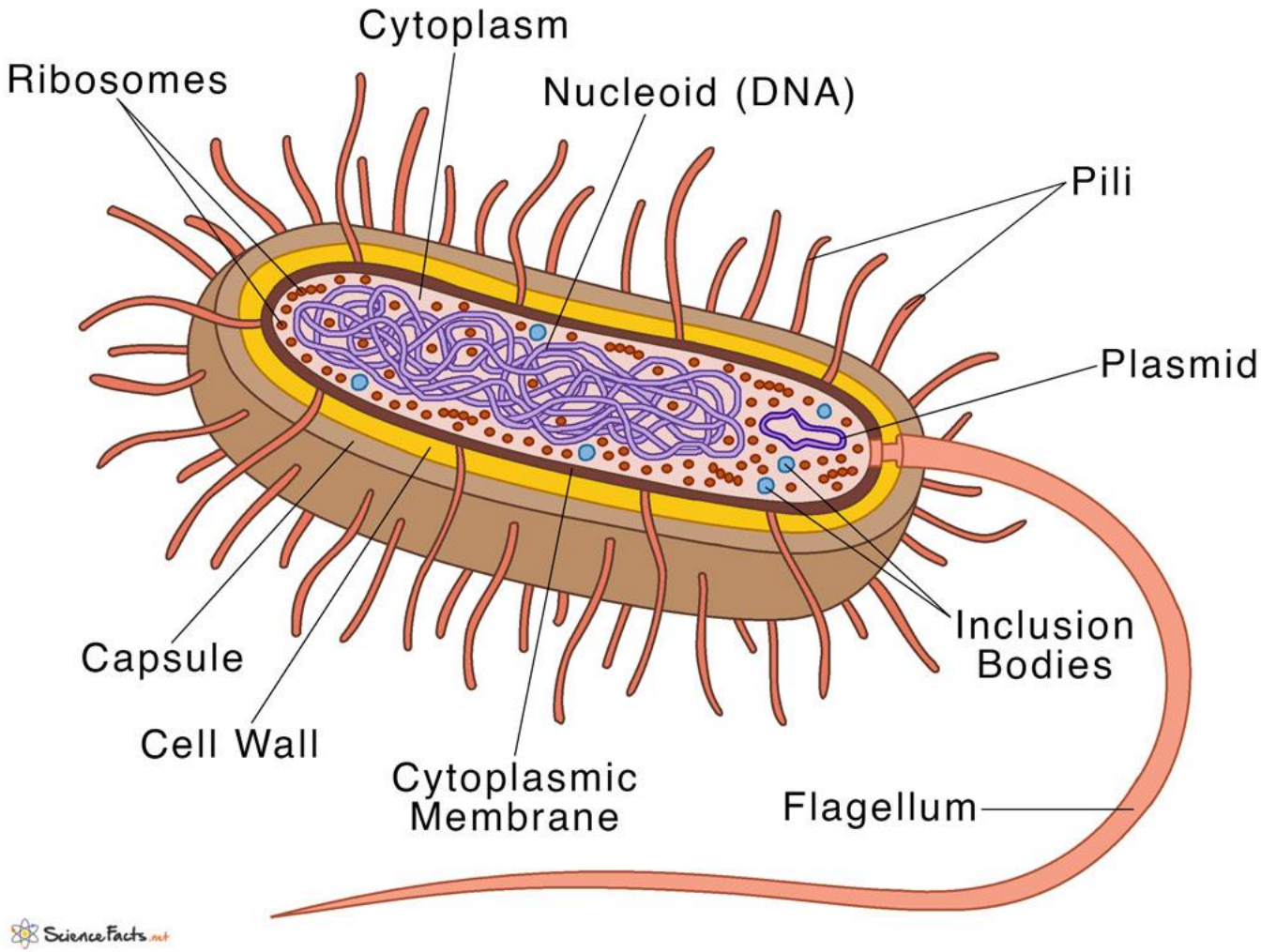
Practical lesson 2

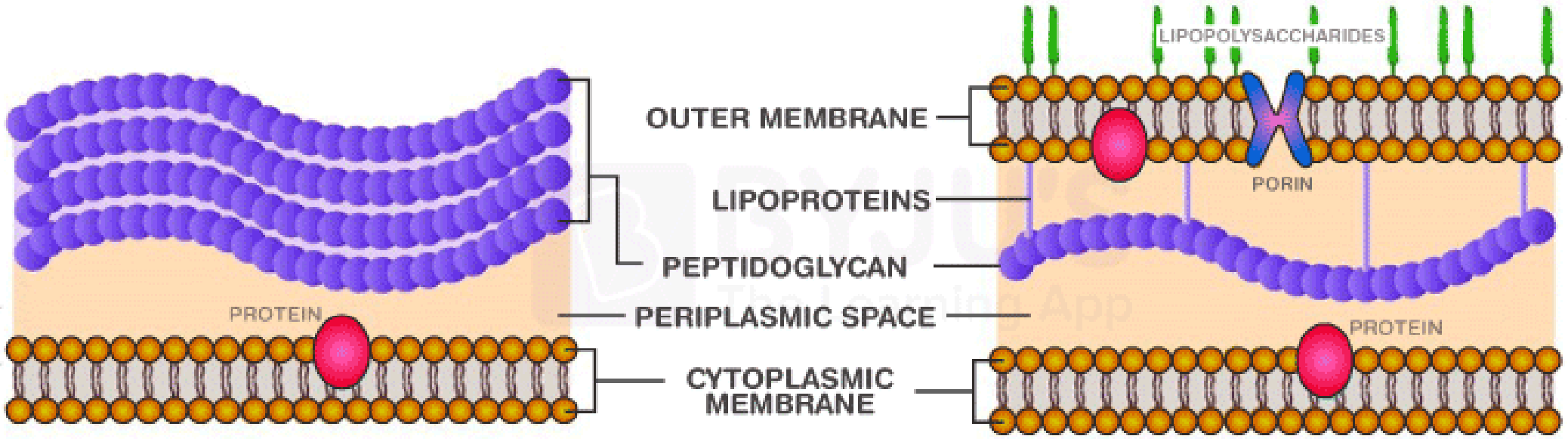
Ultrastructure of a bacterial cell, structure of the cell wall. Gram method. Acid-fast bacteria, their staining by Ziehl-Neelsen method. Spores, detection of spores by spore staining. Intracellular inclusions. Detection of volutin granules by Neisser staining. Flagella. Microbial motility detection methods ("smashed" and "hanging" drop, vital method). Burri's negative staining. Detection of capsule by Gins-Burri staining.

- A procaryotic cell has five essential structural components: a **nucleoid (DNA)**, **ribosomes**, **cell membrane**, **cell wall**, and some sort of **surface layer**, which may or may not be an inherent part of the wall .
- **Cytoplasm** - The cytoplasm, or protoplasm, of bacterial cells is where the functions for cell growth, metabolism, and replication are carried out. It is a gel-like matrix composed of water, enzymes, nutrients, wastes, and gases and contains cell structures such as ribosomes, a chromosome, and plasmids. The cell envelope encases the cytoplasm and all its components. Unlike the eukaryotic (true) cells, bacteria do not have a membrane enclosed nucleus. Bacteria **do not have a nucleus**. They do have two types of DNA –**plasmid** and **chromosomal**. The chromosomal DNA carries most of the genetic information. Plasmid DNA forms small loops and carries extra information.
- **Cytoplasmic Membrane** - A layer of phospholipids and proteins, called the cytoplasmic membrane, encloses the interior of the bacterium, regulating the flow of materials in and out of the cell. This is a structural trait bacteria share with all other living cells; a barrier that allows them to selectively interact with their environment. Membranes are highly organized and asymmetric having two sides, each side with a different surface and different functions. Membranes are also dynamic, constantly adapting to different conditions.
- Bacterial cells are covered by a **cell envelope** that is composed of a cell membrane and a cell wall. The cell membrane is a phospholipid bilayer that regulates the transport of molecules into and out of the cell. This is the weak structure that would burst from the osmotic pressure without reinforcement. The cell wall is the component of the envelope that provides that reinforcement.
- Nearly every genus of bacteria has a **cell wall**, which is a rigid, carbohydrate-containing structure that surrounds the bacterial cell. As is always the case in biology, there are a few oddballs, like the genus *Mycoplasma*, that have lost their cell walls, but since they are a minority, having a cell wall must be a major advantage to the bacteria.

Bacteria Cell



GRAM POSITIVE VS. NEGATIVE CELL WALL



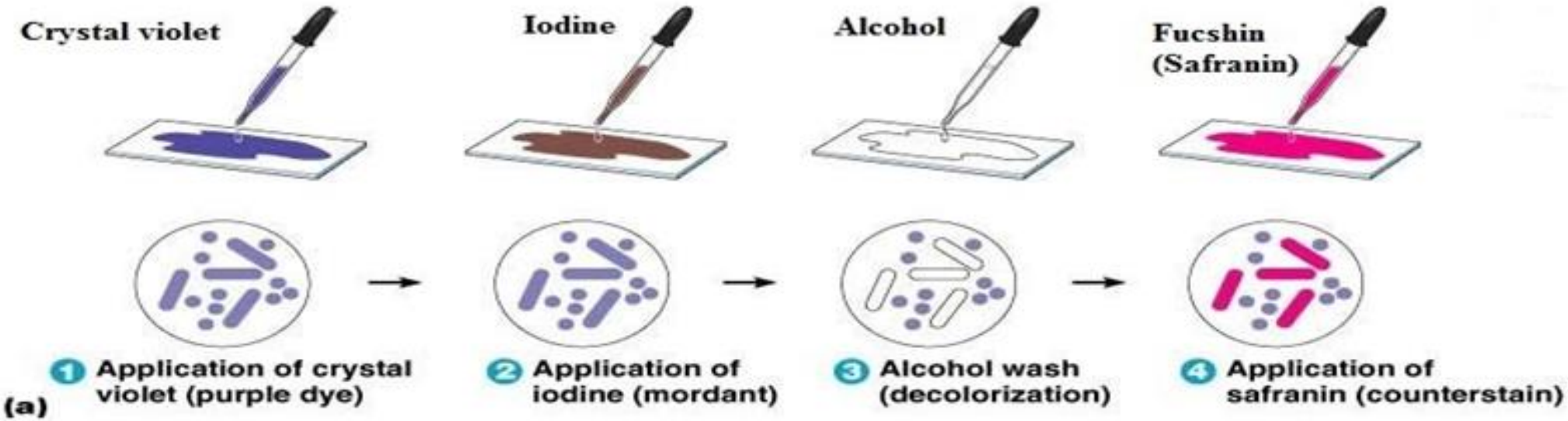
Gram positive

Gram negative

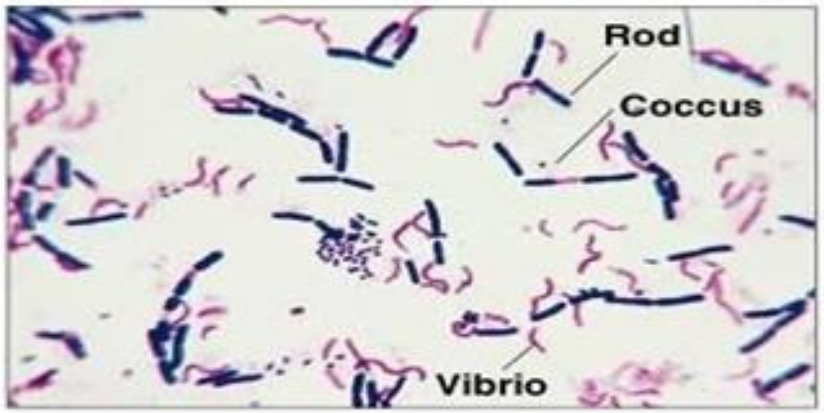
Gram staining

Slides with prepared smears gram-staining kit and wash bottle bibulous paper.

- Cover the smear with **crystal violet**. Let stand for **1-2 minutes .**
- Briefly wash off the stain, using a wash bottle of distilled water. Drain off excess water.
- Cover the smear with **Gram's iodine (Lugol's solution)** solution and let stand for **one minute**. Pour off the Gram's iodine and flood the smear with **95 percent ethyl alcohol** for **10 to 20 seconds.** This step is critical. Thick smears will require more time than thin ones. Decolorization has occurred when the solvent flows colorlessly, from the slide.
- Stop the action of the alcohol by rinsing the slide with wash water for a few seconds.
- Cover the smear with **fuchsin (safranin**) for **3-4 minutes**.
- Wash gently for a few seconds, blot with bibulous paper or paper toweling and let dry at room temperature.
- The slide may be examined under oil immersion immediately.



(a)



(b)

a) stage of procedure

b) after staning

Neisser staining

- Microorganism have a special characteristic these organism store reserve food material in the form of granules. These granules are utilized by these organism when they are in starvation mode. These reserve food granules are also called Volutin granules, Metachromatic granules or Babes Ernst granules. These granules are mainly of four types.
- Volutin granules
- Sulphur granules
- Nitrogenous reserve food material
- Non-nitrogenous reserve food material

Staining Procedure

- 1. Prepare a smear on clean grease free slide.
- 2. Air dry and heat fix the smear.
- 3. Treat the smear with Neisser's stain (methylen blue) and allow it to
- react for about 3 min.
- 4. Drain of the excess stain do not water wash the slide.
- 5. Flood the smear with iodine (lugol) for 10-30 sec.
- 6. Restain the smear with vezuven or chrysoidin for 30 sec.
- 7. Wash the slide with water, air dry and observe under oil immersion
- lens.
- After staning cytoplasm appears light brown, but granules blue-black

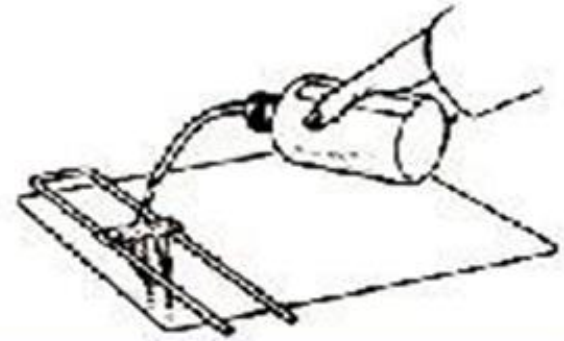
Ziehl-Neelsen staining

- Bacteria which are not readily decolorized with acid-alcohol after staining with hot carbol-fuchsin are said to be **acid-fast**. These microorganisms contain considerable quantities of wax-like lipoidal material which combines tenaciously with this red dye.
- **Ziehl-Neelsen method for acid-fast staining**

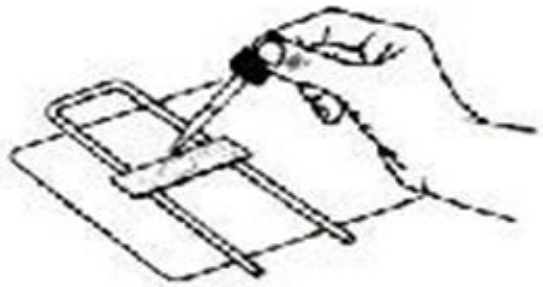
1. Heat fix an air dried smear at 80ºC for at least 15 minutes or for 2 hours
- on an electric hot plate at 65ºC – 70ºC
- 2. Place a slide with an air-dried and heat-fixed smear on suitable staining
- device. Cut a piece of absorbent paper to fit the slide and saturate the paper
- with the carbolfuchsin stain.
- 3. Carefully heat the underside of the slide by passing a flame under the rack
- or by placing the slide on a hot plate until steam rises (without boiling!).
- Keep the preparation moist with stain and steaming for 5 minutes,
- repeating the heating as needed (Fig.29).
- 4. Wash the film in a gentle and indirect stream of tap water until no color
- appears in the effluent.
- 5. Holding the slide with forceps, wash the slide with the decolorizing
- solvent. Immediately wash with tap water, as above. Repeat the
- decolorizing and the washing until the stained smear appears faintly pink
- and the fluid washing off the slide runs clear.
- 6. Flood the smear with the methylene blue counterstain for 20 to 30 seconds,
- and wash with tap water, as above.
- 7. Gently blot, or air dry the smear.
- 8. Examine under oil immersion.

1 Cover smear with carbolfuchsin.Steam over boiling water for 8 minutes. Add additional stain if stain boils off.

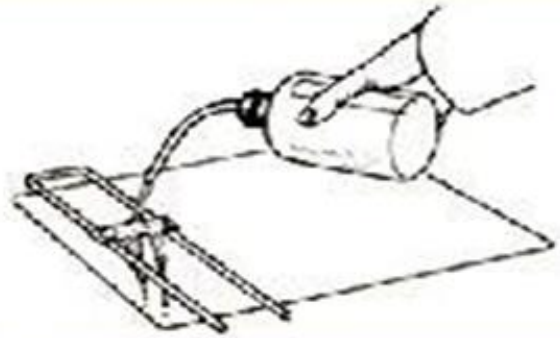
2 After slide has cooled decolorize with acid-alcohol for 15 to 20 seconds.



3 Stop decolorization action of acid-rinsing briefly with water.



4 Counterstain with methylene blue for 30 seconds.

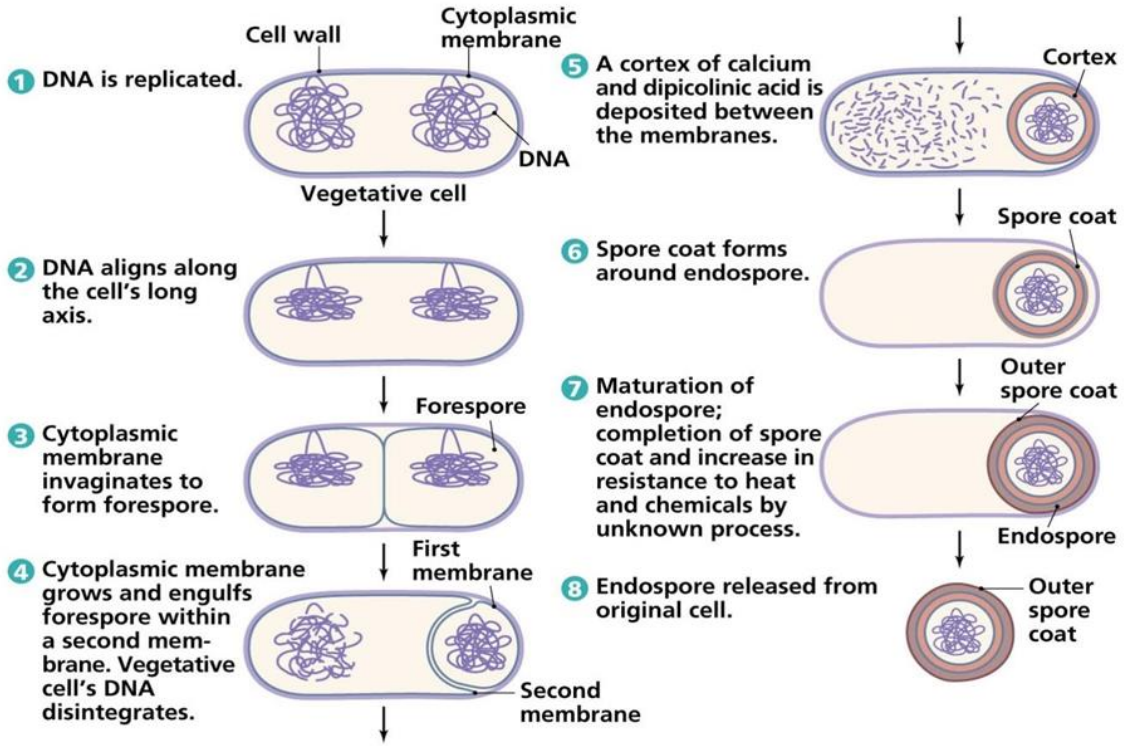


5 Rinse briefly with water to remove excess methylene blue.

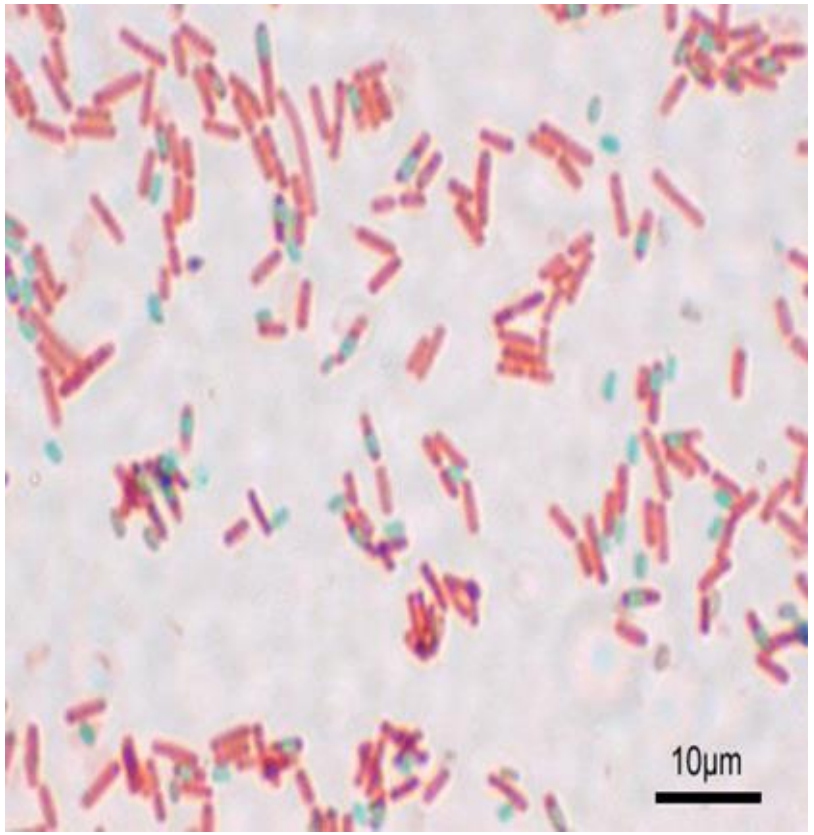
6 Blot dry with bibulous paper. Examine directly under oil immersion.

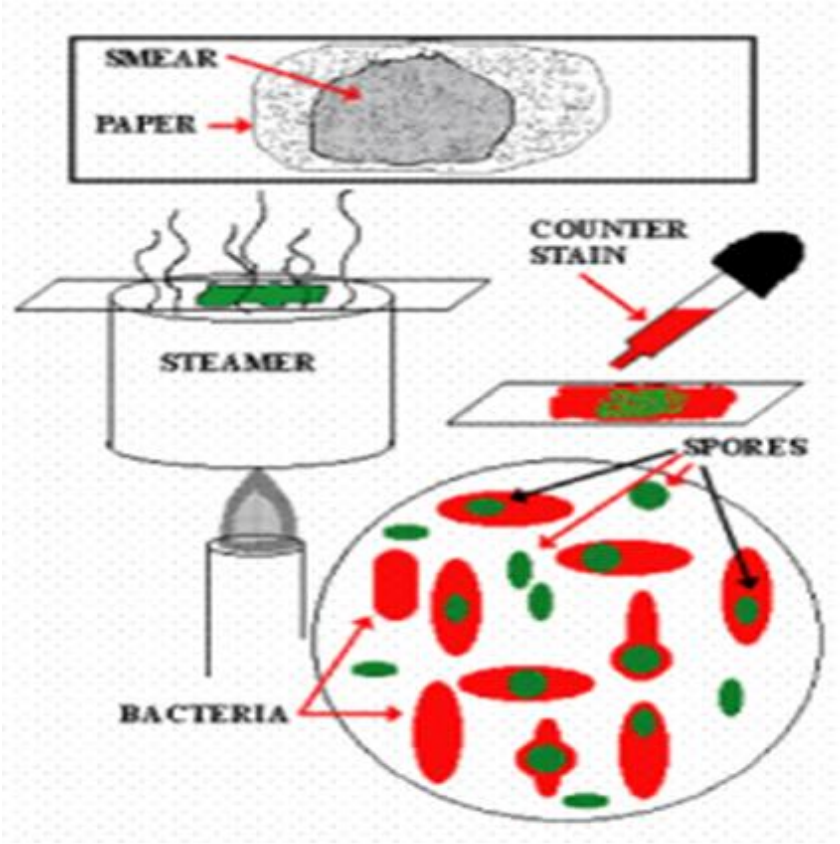
Spores and spore stain

- Bacterial spores are highly resistant, dormant structures formed in response to adverse environmental conditions. They help in the survival of the organisms during adverse environmental conditions; they do not have a role in reproduction. Spore formation (sporulation) occurs when nutrients, such as sources of carbon and nitrogen are depleted. Bacterial spores are highly resistance to Heat, Dehydration, Radiation and Chemicals.



- 1. Place the heat-fixed bacterial slide over screened water bath and then apply the primary stain malachite green.
- 2. Allow the slide to sit over the steaming water bath for 5 minutes, reapplying stain if it begins to dry out.
- 3. Remove the slide from the water bath and rinse the slide with water until water runs clear.
- 4. Flood slide with the counterstain safranin for 1 minute, then rinse.
- 5. View specimen under oil immersion
- When visualized under microscopy (Fig.34) the cells should have three characteristics: the vegetative cells should appear pink, the vegetative cells that contain endospores should stain pink while the spores should be seen as green ellipses within the cells (Fig.35). Mature, free endospores should not be associated with the vegetative bacteria and should be seen as green ellipses.



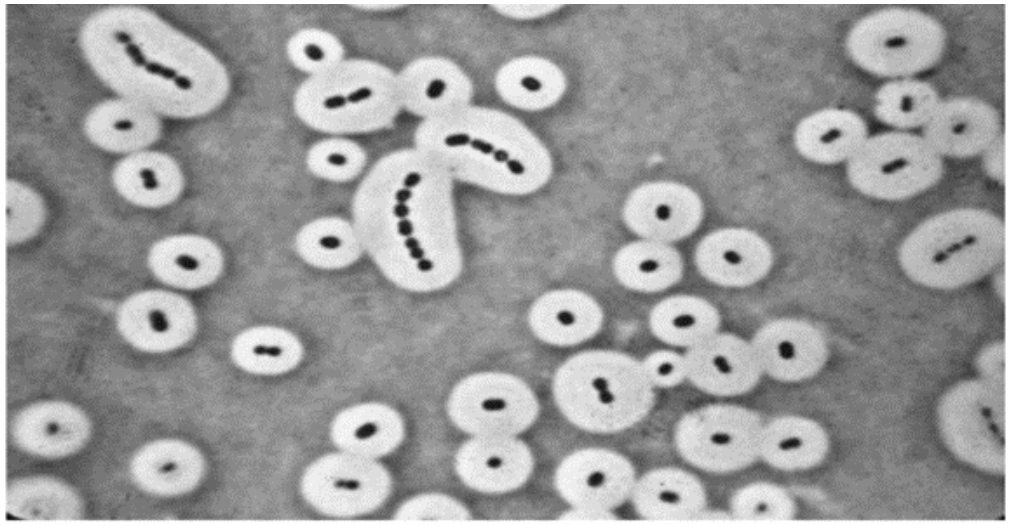


Capsule

- Some species of bacteria have a third protective covering, a capsule made up of polysaccharides (Exception: The capsule of *Bacillus anthracis* is composed of polymerized D-glutamic acid). **Capsule is g**elatinous layer covering the entire bacterium. Capsule is located immediately exterior to the murein layer of gram-positive bacteria and the outer membrane of gram-negative bacteria.
- Negative staining is an excellent way to determine a bacterial capsule. Since the capsule themselves are not stained, their morphology is not distorted in any way. The nigrosin or Indian ink provides a dark background against which the shapes of the unstained cells are clearly visible. This method provides a high degree of contrast not available in most other staining procedures.
- **Procedure**
- 1. Place a very small drop of Indian Ink near one end of a well-cleaned and flamed slide.
- 2. Remove a small amount of the culture from the slant with an inoculating loop and disperse it in the drop of stain without spreading the drop.
- 3. Use another clean slide to spread the drop of stain containing the organism using the following technique (thin blood smear).
- 4. Rest one end of the clean slide on the center of the slide with the stain. Tilt the clean slide toward the drop forming an acute angle and draw that slide toward the drop until it touches the drop and causes it to spread along the edge of the spreader slide. Maintaining a small acute angle between the slides, push the spreader slide toward the clean end of the slide being stained dragging the drop behind the spreader slide and producing a broad, even, thin smear.
  5. Allow the smear to dry without heating. DON'T HEAT FIX!
- 6. Focus a thin area under oil immersion and observe the unstained cells surrounded by the gray stain

Burry-Gins method

- 1. Then a smear is uniformly distributed to a thin layer with the help of the second glass.
- 2. Remove a small amount of the culture from the slant with an inoculating loop and disperse it in the drop of stain without spreading the drop.
- 3. Use another clean slide to spread the drop of stain containing the organism(as thin blood smear)
- 4. A preparation is dried up, flooded in 1% solution of muriatic alcohol for some seconds.
- 5. After exsiccation or the burning of alcohol above the flame
- 6. A smear is counterstained with water fuchsine for 1 minute.
- 7. A preparation is washed with water, dried up and microscoped.
- The red microbes surrounded by colourless capsules are visible against the dark background

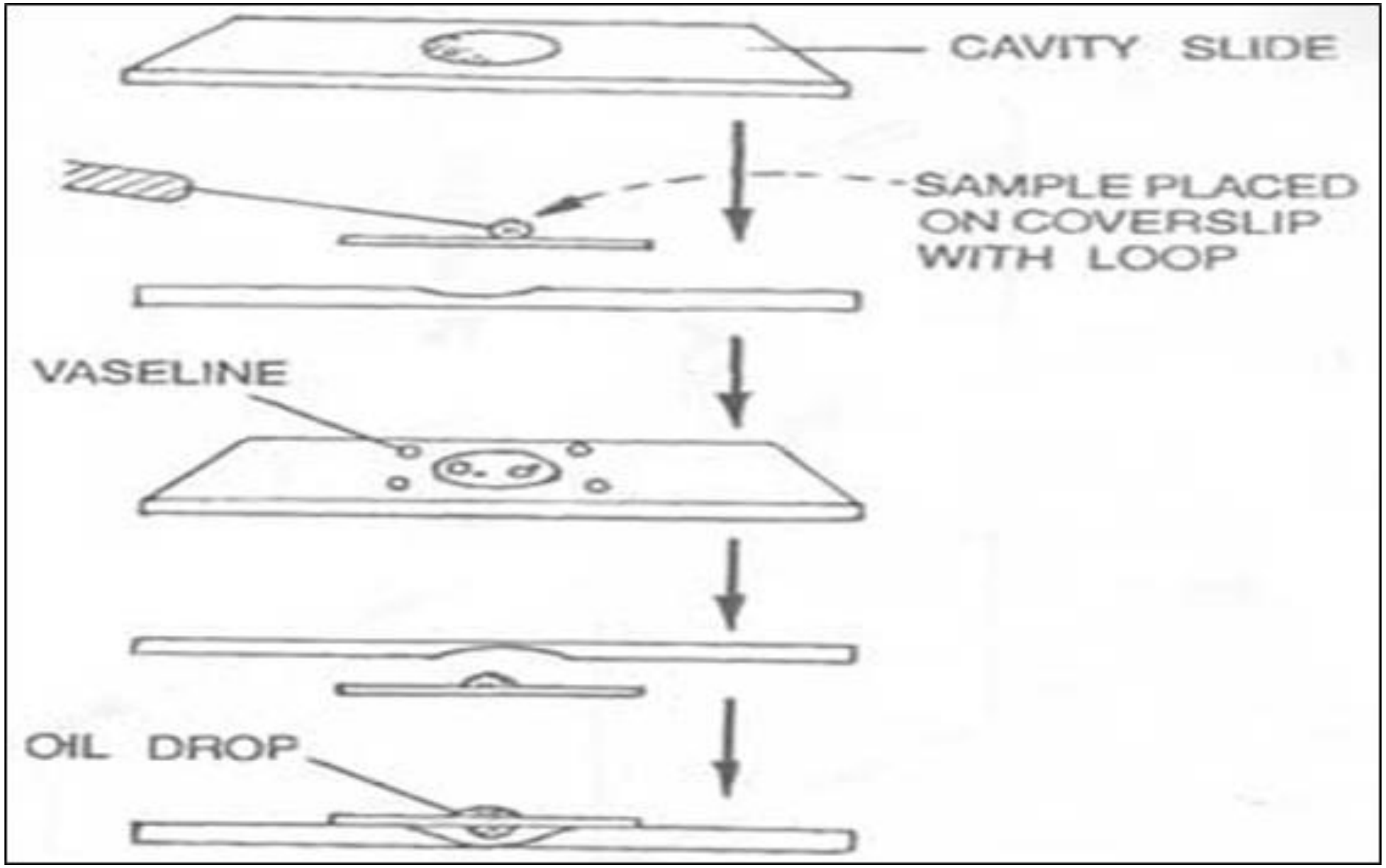


Flagella. Vital staining

- Motile bacteria are subdivided into creeping and swimming bacteria. Creeping bacteria move slowly (creep) on a supporting surface as a result of wave-like contractions of their bodies, which cause periodic alterations in the shape of the cell. Swimming bacteria move freely in a liquid medium. Flagella (singular: **flagellum**) are long, thin, whip-like appendages attached to a bacterial cell that allow for bacterial movement (also known as motility). Different bacterial species have different flagella arrangements, from a single flagellum to one on each end to tufts of many.
- **Staining:**
- 1. Take a prepared slide and using a wax pencil draw a rectangle around the dried sample. Place slide on staining rack.
- 2. Flood Leifson dye solution on the slide within the confines of the wax lines. Incubate at room temperature for 7 to 15 minutes. The best time for particular preparation will require trial and error.
- 3. As soon as a golden film develops on the dye surface and a precipitate appears throughout the sample, as determined by illumination under the slide, remove the stain by floating off the film with gently flowing tap water. Air dry.
- 4. View using oil immersion, at 1,000x magnification, by bright-field microscopy. Bacterial bodies and flagella will stain red.

Motility Determination

- **The Hanging Drop Method**
- When it is necessary to study viable organisms on a microscope slide for a longer period of time than is possible with a wet mount, one must resort to a hanging drop slide. Organisms are observed in a drop that is suspended under a cover glass in a concave slide. Since the drop lies within an enclosed glass chamber, drying out occurs very slowly.
- In general, hanging drop slides usually are observed with a brightfield microscope, either with high-dry or oil immersion. Phase-contrast optics can be used, but not as satisfactory as for wet mounts. Hanging drop slides yield the same information as wet mount slides: motility information, cell shape and cell arrangement. The procedure is as follows:
- **Materials:**
- Depression slides and cover glasses, inoculating loop, Vaseline, toothpicks, Bunsen burner, Nutrient broth cultures of organisms (young cultures)
- **Procedure:**
- 1. Prepare a clean depression slide and cover glass by washing with soap and warm water. All grease must be removed.
- 2. With a china marking pencil label the slide with the name of the organism.
- 3. Place a very small amount of vaseline near each corner of a cover glass. The vaseline will provide adhesion of the cover glass to the depression slide .
- 4. Shake the culture tube and transfer two loopfuls of the culture onto the cover glass.**Be sure to flame the loop prior to removing the second loopful from the culture.**


CAVITY SLIDE
SAMPLE PLACED ON COVERSLIP WITH LOOP
VASELINE
OIL DROP