UNIT – IV STAINS AND DYES

Microscopic examination of bacterial cell is usually the first step in the identification of any unknown micro-organism. To observe the micro-organisms in living state is very difficult because they are very small and are transparent. The cells are colourless when suspended in an aqueous medium. If such cells are observed under microscope without staining, then the refractive index of bacterial protoplasm is very similar to that of the medium, in which they are growing; due to which they cannot be easily observed in the unstained condition lacking a contrast. Therefore staining is highly essential step in bacteriological studies. The different purposes of staining are –

1. To observe microscopic, semi-transparent bacteria by producing colour contrast.
2. To study the size, shape and arrangement of micro-organisms.
3. To study various internal and external structures of bacteria, this helps in the identification and classification of bacteria.

Definition of dye and stain

- **Dye**

A dye may be defined as an organic compound containing both chromophore and auxochrome groups linked to benzene ring.

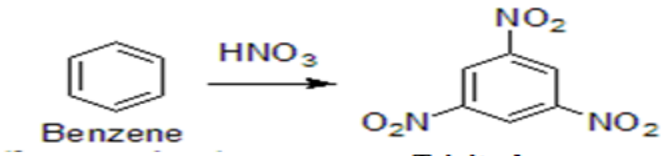
A chromophore group: - It gives colour property to the compound. The compound of benzene with only chromophore radicals is called as 'chromogene'. Such compound itself may be coloured; but one cannot say it as a dye. It possesses no affinity to unite with the objects that has to stain like fiber or cell. The colouring solution applied to the object can be easily removed by a simple mechanical method like shaking. Therefore, any compound to be a dye must possess an auxochrome group along with chromophore group.

Auxochrome group: - It gives the property of electrolytic dissociation or a property of salt formation to the dye. In some cases it may alter the shade of the dye but it is not the property of auxochrome. The function of auxochrome and chromophore can be explained by taking following example.

A yellow coloured compound trinitrobenzene. as in Fig. is a chromogene, but it is not a dye as it lacks the property of electrolytic dissociation.

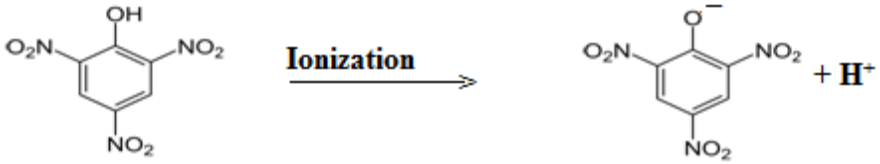
Colouring property of picric acid is due to chromophoric nitro (NO_2) groups but the dying property is due to auxochromic hydroxyl (.-OH) group.)

The terms dyes and stains used synonymously by the biologists, but they are not same. The dye is any colouring agent which is used for general purposes. It may be used to colour buildings, textile, and furniture like inanimate objects. The dyes are not that much pure like a stain.



Trinitrobenzene

Picric acid is a yellow coloured dye in which one H is replaced by –OH group. The –OH group can easily undergo dissociation as shown below.



Picric acid

- **Stain**

Stain is defined as an organic compound containing both chromophore and auxochrome groups linked to benzene ring and which are used for staining / colouring biological objects e.g. microorganisms, cells of plants and animals. Stains are in highly pure form and are manufactured with great care.

A stain is a substance that adheres to a cell, giving the cell color. The presence of color gives the cells significant contrast so they are much more visible. Different stains have different affinities for different organisms, or different parts of organisms. They are used to differentiate different types of organisms or to view specific parts of organisms.

The ability of a stain to bind to cellular component like proteins, nucleic acids depends upon the charge on chromophore group of stain as well as the charge on the cellular components that have to be stained.

- **Natural stains**

Natural stains are stains or colorants derived from plants, invertebrates, or minerals. The majority of natural dyes are vegetable dyes from plant sources, for example:- Indigo. The dyes that are obtained from animal sources are called Cochineal, and those that are got from minerals are Ocher. Natural dyes can provide the much needed alternative to the complex world of chemical dyes. These dyes are environmentally sound and can be grown by organic methods. They are inherently carbon neutral. Vibrant colors can be produced from natural dyes.

The five classic and popular natural dyestuffs are indigo, madder, cochineal, weld, and cutch.

- **Mordant**

Some stains have little or no affinity for the object. In this case an intermediate substance called as 'mordant' is used. Mordant is a substance which increases the affinity between the object and the stain. It helps to fix the stain to the object. Mordants include tannic acid, alum, urine, chrome alum, sodium chloride, and certain salts of aluminium, chromium, copper, iron, iodine, potassium, sodium, and tin. Ex. Gram's iodine used in Gram's staining and tannic acid used in flagella staining.

- **Decolourizer**

A decolorizing agent is an organic solvent that is used to remove the primary stain from the cell. E.g. Ethyl alcohol, Acetone in Gram staining, 20% Sulphuric acid, 3% HCl in 95% Ethyl alcohol in Acid Fast staining.

- **Counter stain**

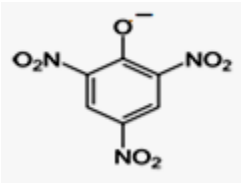
A stain of a contrasting color used to color the components in a microscopic specimen that are not made visible by the primary stain. E.g. Saffranin in Gram staining, Malachite Green or Methylene Blue in Acid Fast staining

- **Classification of stains: Acidic, basic and neutral**

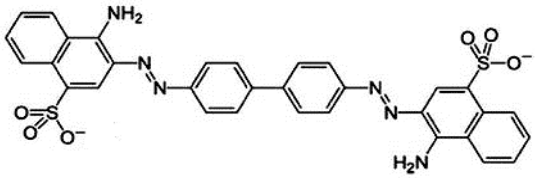
Depending upon the charge present on the chromophore group, the stains are classified into three groups,

1. Acidic stain
2. Basic stain
3. Neutral stain

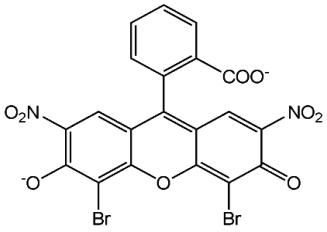
1. Acidic stain: Acidic stains are those stains in which the charge present on chromophore group is negative, after its electrolytic dissociation or ionization. Acidic stains have great affinity towards positively charged cell components like cytoplasmic elements. e.g. Picric acid, Congo red, Eosine, Nigrosine, India ink, Rose Bengal etc.



Picric acid

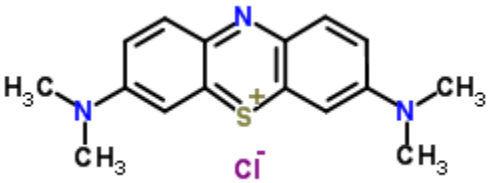


Congo red



Eosin

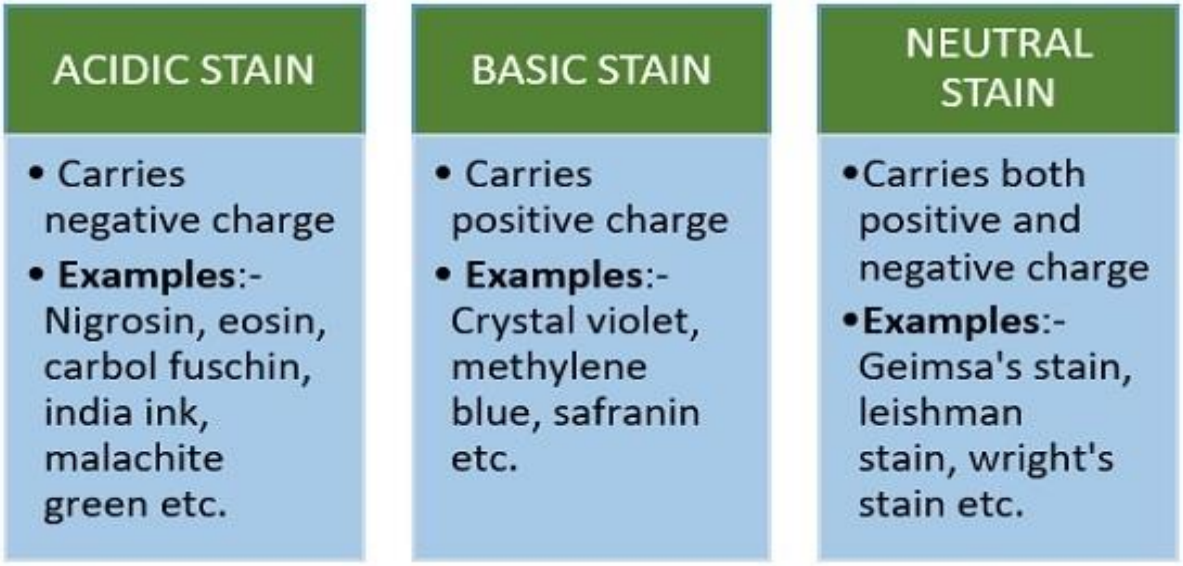
Basic stains: Basic stains are those stains in which the charge present on chromophore group is positive, after its electrolytic dissociation or ionization. Basic stains have great affinity towards negatively charged cell components like nuclei, granules and the bacterial cell surface. E.g. Methylene Blue chloride. It undergoes dissociation to form positively charged Methylene Blue and negatively charged chlorine.



3. Neutral stain: Neutral stains are those which contain both positively charged and negatively charged chromophore groups, e.g. eosinate of methylene blue (Giemsa's stain)

Examples of neutral stains - Giemsa's stain, Jenner's stain, Wright's stain, Leishman's stain. These stains are generally used for staining of nuclear material or nuclei.

Some parts of the cell are acidic *(e.g.* nuclei) while some are basic (cytoplasmic elements). The acidic constituents of the cell react with the basic stains and basic constituents with acidic stains, when neutral stains are applied to cell bacterial cell.



- **Simple, differential and special staining**

A simple staining consists of use of a solution containing single stain. Staining can be performed with basic dyes such as crystal violet, methylene blue or basic fuchsin, positively charged dyes that are attracted to the negatively charged materials of the microbial cytoplasm. Such a procedure is the **simple stain procedure.** An alternative is to use a acidic dyes such as nigrosin or Congo red, negatively charged dyes. They are repelled by the negatively charged cytoplasm and gather around the cells, leaving the cells clear and unstained. This technique is called the **negative stain technique.** Simple stains allow one to distinguish the shape (morphology) and arrangement of the bacteria. E.g. Monochrome staining, Negative staining.

Differential staining is more complex than simple ones and use more than one stain to differentiate microorganisms e.g bacteria can be differentiated into Gram positive and Gram negative bacteria. They are used to examine structural differences between bacterial groups or to provide contrast to different structures within the same organism. E.g. Gram staining, Acid Fast staining etc.

Special staining techniques are used to observe various bacterial structures like flagella, capsule, cell wall, endospores, granules, nuclear material etc.

- **Physicochemical basis of staining (Theories of staining)**

Phenomenon of staining is explained on the physicochemical basis.

Physical basis (Surface coating theory): -

Physical process is reaction between two substances without the formation of new compound. When bacteria stained by physical method there is no chemical change in stain. When immersed in water, alcohol or other solvents for sufficient period of time, physically stained organisms loose stains. There are three factors which explain physical basis of staining

i) Capillarity and Osmosis
ii) Adsorption
iii) Absorption

i) Capillarity and Osmosis: Nearly all substances stained are more or less porous, simple physical forces such as capillarity and osmosis can account for penetration of the dye into the interior of the tissue.

ii) Adsorption: It is the property possessed by a solid body of attracting to itself minute particles of matter from a surrounding fluid; these particles may be

compounds suspended in the fluid, or they may be ions incapable of existing independently except in solution.

iii) Absorption: A dye may penetrate some cellular element by absorption, remaining there in a state of solid solution.

Chemical basis of staining

Following are the mechanisms of chemical basis of staining

i) Ionic bonding

ii) Hydrogen bonding

iii) Covalent bonding

iv) Van der Waals' forces

i) Ionic bonding

Ionic bonding involves electrostatic attraction between oppositely charged ions. One ion is a fixed ion in the tissue section and the other is the dye ion. Ionic bonding to dyes and therefore staining is pH sensitive since the ionization of tissue groups is affected by pH. Some parts of cells are acidic while some parts are basic. The stains are either acidic or basic. According to chemical theory of staining, acidic stains react with basic parts of cells while basic stains react with acidic parts of cells. The process involves an exchange reaction between stain and active site of cells. For example the colour ions of dye replace ions on cellular components. Certain chemical grouping of cell proteins or nucleic acids may be involved in salt formation with positively charged ions such as Na^+ or K^+.

The bacterial cell surface carries negative charge and combines with positively charged ions. The ion exchange during staining with methylene blue is as follows –

$$(\text{Bacterial cell}^-)\ (Na^+) + (MB^+)\ (Cl^-) \longrightarrow (\text{Bacterial cell}^-\ MB^+) + NaCl$$

An acid dye is the salt (usually the sodium salt) of a color acid, that is, the chromophoric groups are located in the anionic part of the molecule. Basic dye is a salt (usually a chloride, sulfate, or acetate) of a color base, that is, the chromophoric groups are located in the cationic part of the molecule. Anionic (negatively charged) dyes will bind to cations (positively charged) in the tissue, and cationic dyes will bind to tissue anions.

Anionic dyes are also called acid dyes, because they are derived from coloured acids (e.g. eosinic acid) and not because of the pH of the solution. Anything that will stain with an acid dye is called Acidophilic.

Eosin is an example of an anionic dye and is attracted to protein groups that are positively charged (cations) such as amino groups.

Cationic dyes are commonly called basic dyes and so substances staining with such dyes are called basophilic. Methylene blue is an example of a cationic dye and will bind to tissue anions such as carboxylic acid, sulphuric acid and phosphoric acid groups. These groups need to be ionized to bind the dyes. Positively charged methylene blue ions will stain negatively charged tissue ions. Substances that bind basic dyes include nucleic acids.

ii) Hydrogen bonding

Hydrogen bonding is a force of attraction between a hydrogen atom in one molecule and a small atom of high electro-negativity in another molecule. These are formed by sharing of hydrogen atoms between two electron donors. In the staining of elastin fibres hydrogen bonds are probably more important than ionic forces. E. g. staining of protein by Congo red.

iii) Covalent bonding

These are very strong bonds and are not easily broken once formed. These are chemical bonds which involves the sharing of electron pairs between atoms.

E. g. Attachment of dyes to antibodies in immunofluorescence.

iv) Van der Waals' forces

These are weak force of attraction between electrically neutral molecules that are very close to each other. Van der Walls' force is caused by the attraction between electron rich regions of one molecule and electron poor regions of another. The attraction is much weaker than a chemical bond. The adhesion of the section to the slide involves Van der Waals' interactions between section and glass.

❖ Fixatives and fixation of smears

Most initial observations of microorganisms are made with stained preparations. Staining simply means colouring the microorganisms with a dye that emphasizes certain structures. Before the microorganisms can be stained, however, they must be fixed (attached) to the microscope slide. Fixing simultaneously kills the microorganisms and fixes them to the slide. It also preserves various parts of microbes in their natural state with only minimal distortion.

When a specimen is to be fixed, a thin film of material containing the microorganisms is spread over the surface of the slide. This film, called a smear, is allowed to air dry. In most staining procedures the slide is then fixed by passing it through the flame of a Bunsen burner several times, smear side up, or by covering the slide with methyl alcohol for I minute. Stain is applied and then washed off with water; then the slide is blotted with absorbent paper. Without fixing, the stain might wash the microbes off the slide. The stained microorganisms are now ready for microscopic examination.

Fixation may itself consist of several steps–aims to preserve the shape of the cells or tissue involved as much as possible. Sometimes heat fixation is used to kill, adhere, and alter the specimen so it will accept stains. Most chemical

fixatives (chemicals causing fixation) generate chemical bonds between proteins and other substances within the sample, increasing their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid.

- **Principles, Methodology, Mechanism and applications of staining procedures**

A) Simple staining

i) Monochrome staining

ii) Negative staining (Indirect Staining, Background Staining, Relief Staining)

i) Monochrome staining

The colouration of bacteria by applying a single staining solution to a fixed smear is termed as simple staining. It is also called as *monochrome staining method.* (Mono-single and chrome-stain) as only one stain is applied. The staining is also called as *'positive staining'.* Any one of the basic dyes can be used for simple staining. These include methylene blue, safranin, basic fuchsin, malachite green etc. The cells take characteristic colour depending upon the stain used. For example, the cells will appear red if stained with safranin and will appear green if stained with malachite green. The cells are usually stained uniformly.

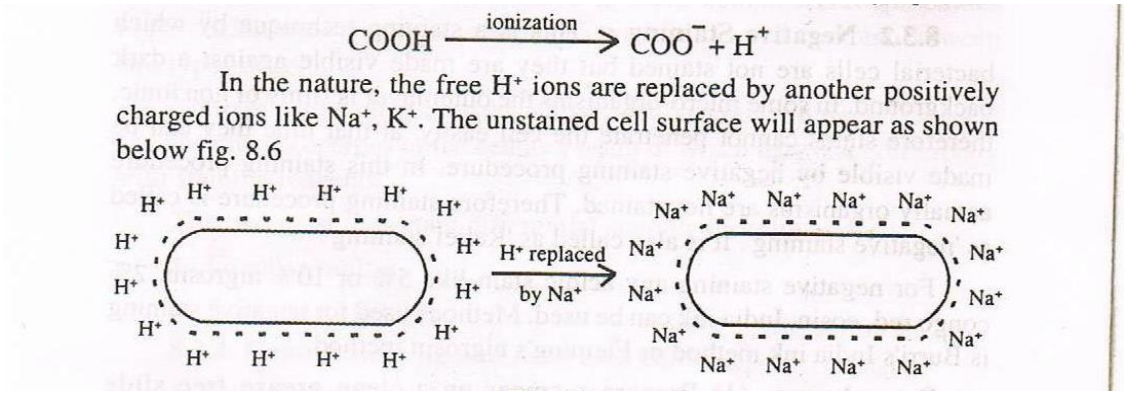
Procedure:

1. Take a clean grease free slide.
2. Prepare a smear on the slide by removing a loop full of suspension with a sterile wire loop. Allow it to air dry.

3. Heat fixes the smear by passing the slide rapidly through flame for three to four times.
4. Place the slide on staining rack and flood the smear with a staining solution (say methylene blue). Then allow it to react for 2-3 minutes.
5. Wash the slide with running water. Air dry or blot dry the slide.
6. Observe the slide under oil immersion lens.

Mechanism: As already described the ability of a stain to bind to cellular components depends upon the charge present on chromophore group of a stain and charges present on the cell components.

• The bacterial cell surface has overall negative charges. It is due to presence of large number of free carboxylic groups (-COOH group) of acidic amino acids, located at the cell surface. When ionization of carboxylic group occurs, it gives negative charges to cell surface as per following equation.



While for simple staining basic dyes are used, say for example methylene blue is used. Methylene blue stain is present as its salt methylene blue chloride. When methylene blue chloride undergoes ionization, it results in formation of positively charged chromophore group and negatively charged chloride ions.

$$MBCl \xrightarrow{\text{ionization}} MB^{+} + Cl^{-}$$

Methylene blue chloride → positively charged methylene blue group + negatively charged chloride ion

- When methylene blue stain is applied on the cell surfaces, the positively charged methylene blue ions replace the Na^{+} ions present at the cell surface. Thus ionic bond formation takes place between negatively charged cell surface and positively charged MB^{+} chromophore group. It results in staining of cell as shown below in fig. 8.7

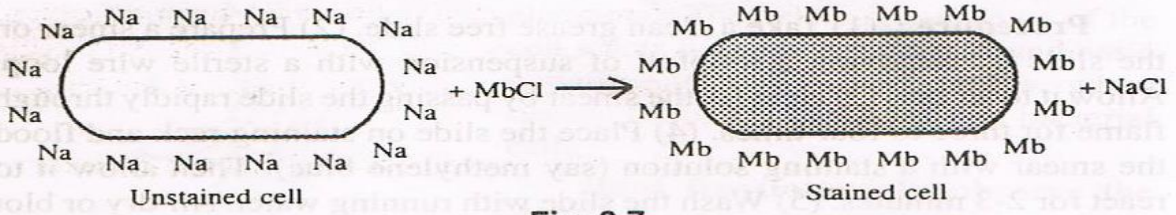
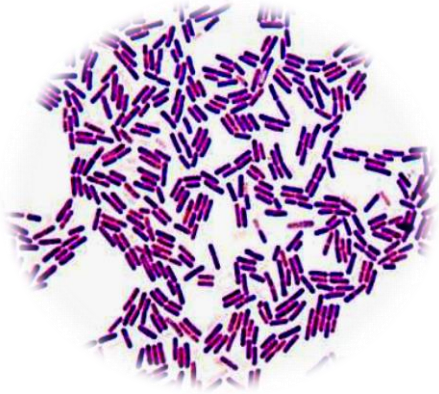


Fig. 8.7

Applications of Staining

1. The main application is to colour the bacteria for easy observation under microscope.
2. It may be used to study the morphological characters of micro-organisms like their size, shape and arrangements.
3. It gives an idea about the unknown bacterias in the specimens under examination.



ii) Negative staining (Indirect Staining, Background Staining, Relief Staining)

Negative staining is a technique by which bacterial cells are not stained but are made visible by staining the background dark. Acidic stains like Nigrosin, Eosin, Congo red, and India ink etc. are used for this method. Acidic stains do not have affinity towards bacterial cells because bacteria are also

acidic in nature (negatively charged) hence; there is repulsion between acidic stains and negatively charged bacteria.

The size and shape of the bacteria can be much more accurately determined by this technique but internal structures cannot be observed.

This technique is advantageous over positive staining technique because it is very easy for the study of cell morphology and cell do not receive vigorous physical and chemical treatments.

In this technique heat fixation is avoided because it may disturb the cells or shrinkage of the bacterial cells occurs. This staining is called as background staining because background is stained, relief staining because bacteria get relief from physical and chemical treatments and indirect staining because bacteria are not stained directly.

Mechanism: -

The chromophore group of acidic stain has negative charge. Hence it cannot combine with negatively charged bacterial cell. On the other hand, acidic stain forms background of bacterial cells, thus resulting into colourless appearance of bacterial cells against the dark background.

Precaution: -

Thick smear of stain and suspension may result cracking hence very thick smear should be avoided. Very thin smear will not give dark background hence very thin smear should be avoided.

Requirements: -

1. Suspension of bacterial cells
2. 10 % aqueous Nigrosin solution

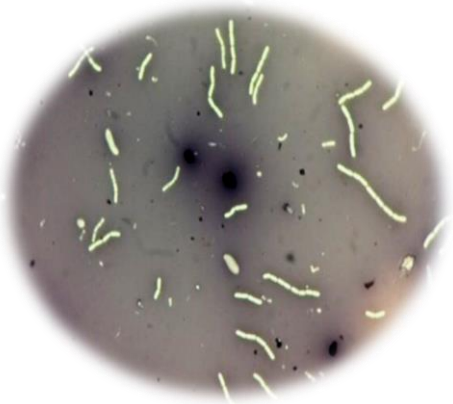
Procedure: -

1. The drop of suspension and the drop of Nigrosin is taken on a clear grease free slide.

2. These two drops are mixed with nichrome wire loop or with another slide.

3. This drop is then spread with the help of nichrome wire loop or with another slide so as to form thin film.

4. This film is air dried and observed under oil immersion lens.



Key Points

- The smear of negative staining should not be too thick as there may be difficulty in passing light through objects.
- Do not over-dry the smear, which forms the cracks on the smear and shows false-positive results.
- Negative Staining does not require heat fixation treatment.
- Cultures used should be 24 hours old.
- Always keep a small drop of stain close to the corner of the slide**.**

Applications

1. This staining method is used to study the exact morphological characters like size, shape and arrangements of bacteria; because in this technique heat fixation is avoided and thus original shape of bacteria is maintained.
2. This method is also useful to observe the bacteria that are difficult to stain e.g. spirilli.
3) It is also used to observe Spirochetes and Capsules of bacteria.

B) Differential staining :

This is a staining procedure which differentiates or distinguishes between bacterial cells or parts of bacterial cells. These are the procedures which do not stain all kinds of cells equally. In simple staining method, only one stain is used and all bacteria get same stain. But in differential staining method two different coloured dyes, primary and counter stains are used, leads to stain two types of organisms in two different colours. The method is more complex and sometimes requires additional chemical reagents to produce desired contrast between two types of cells.

The basic principle behind this differentiation is due to different physical and chemical properties of the cell as a result of which they react differently with staining reagents.

The two most important differential staining techniques are,

1. Gram's staining

2. Acid - fast staining. (Ziehl-Neelsen's method)

1. Gram's staining:

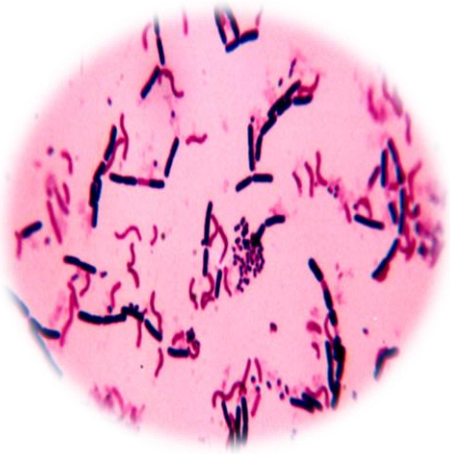
More than century old, first differential staining technique was discovered by Christian Gram in 1884, which is a universal basis of bacterial classification and identification. This staining method is known as Gram's staining method. In

this staining method micro- organisms are classified into two groups as Gram positive bacteria and Gram negative bacteria, depending upon the property whether they retain or loose the primary stain, when subjected to the treatment of decolourizing agent. It is the most important and widely used staining procedure.

Procedure: -

1. Prepare a smear on a clean grease free slide. Allow to air dry and heat fix.
2. Flood the smear with crystal violet and allow to react for 1 minute. Then wash the slide with water.
3. Apply Gram's iodine and allow to react for 1 minute. Then wash the slide with water.
4. Apply' 95% alcohol on the smear until no more colour appear to flow from the preparation. Then wash the slide properly with water.
5. Finally, apply safranin for 1 minute and then wash it with water.
6. Air dry or bolt dry with papers.
7. Observe the slide under oil immersion lens.

Observation: Gram positive bacteria are stained violet in colour and Gram negative organisms are stained pink in colour.



Gram positive bacteria: The bacteria which retain crystal violet and appear deep violet in colour are called Gram positive. For example *Bacillus, Clostridium , Staphylococcus, Streptococcus, Micrococcus, Sarcina.*

Gram negative bacteria: The bacteria which loose crystal violet but take a counter stain and appear pink in colour are called as Gram negative. For example - *Pseudomonas, Xanthomonas, Azotobacter, Rhizobium, Neisseria, Escherichia, Salmonella, Shigella, Proteus, etc.*

Mechanism of Gram's staining: To explain mechanism of Gram's staining, four different theories are proposed. The theories are based on the differences in the structure and composition of cell wall also on some other chemical differences. Recent work suggests that the basis of Gram's staining lies in the permeability differences between two groups of micro-organisms.

i) Lipid content Theory: The cell wall of Gram negative bacteria contain a higher percentage of lipid approximately 11 to 20% than the cell wall of Gram positive bacteria, which is approximately 1 to 4%.

During Gram's staining, when crystal violet is applied, this is a basic stain, both types of cells stain violet in colour. While when Gram's iodine is applied, there is formation of CV-I complex in both types of cells. When the cells are treated with 95% alcohol as a decolourizing agent, then the higher percentage of lipids of cell wall of Gram negative bacteria get dissolved in it. It results in increase in the porosity or permeability of cell wall. The CV-I complex formed earlier is extracted and cells become colourless after alcohol treatment. Such cells then take counter stain applied and appear pink in colour. On the other hand, due to less percentage of lipids, the cells are dehydrated by ethanol treatment, the pore size decreases and the permeability is reduced. Thus the CV-I complex cannot be extracted and cells remain violet in colour.

ii) Peptidoglycan Theory: In the cell wall of Gram negative bacteria, very small amount of peptidoglycan is present. It is approximately 5 to 10% of dry weight of cell. While in case of Gram positive bacteria it is 40 to 90% of dry weight of the cell. In case of Gram negative bacteria, only one sheet of peptidoglycan is present, while in Gram positive bacteria about 40 sheets of peptidoglycan are present.

The peptidoglycan of cell wall of Gram positive bacteria is extensively cross-linked, therefore the CV-I complex is trapped in such a tough network. Thus they cannot be decolourized by alcohol treatment and remain violet in colour. But in case of Gram negative bacteria percentage of cross linkages in the peptidoglycan of cell wall is very less, therefore network or meshwork is loose and pores are large enough through which CV-I complex can be extracted easily during alcohol treatment. These cells subsequently take colour of counter stain and appear pink in colour.

iii) Magnesium ribonucleate Theory: Magnesium ribonucleate is the compound which is present only in Gram positive bacteria but this compound is absent in Gram negative bacteria. In case of Gram positive bacteria, the already formed CV-I complex, forms a covalent bond with Magnesium ribonucleate. Therefore the CV-I complex can't be extracted during alcohol treatment and cells remain violet in colour. While in case of Gram negative organisms, the CV-I complex can be easily extracted by alcohol treatment as lacking magnesium ribonucleate. These decolourized cells then take counter stain and appear pink in colour.

iv) Stearn and Steam theory: According to them, when the lipodial material is extracted from both Gram positve and Gram negative bacteria, then it is found that the lipodial material from Gram positive bacteria contain larger amount of

unsaturated fatty acids. Such unsaturated fatty acids have more affinity for oxidising agents. A mordant (Gram's iodine) used in Gram's staining is an oxidizing agent. The oxidized product of lipoidal material is acidic in nature. Therefore in Gram positive cells more acidic condition is produced, this increases affinity of the cell for basic dye, than **Gram** negative bacteria. Thus the crystal violet iodine complex is not extracted from Gram positive bacteria but extracted from Gram negative bacteria. And thus Gram positive organisms appear violet in colour while Gram negative organisms appear pink by taking counter stain.

Applications: Gram's staining is the basis of several bacteriological units.

1. Taxonomy: It is the universal basis of bacterial classification and identification.
2. Identification and diagnosis of infection: It is an important and unbeatable first tool in the diagnosis of causative agent of clinical infections.
3. It not only differentiates the organisms but also pin point about other morphological characters like size, shape, arrangement.
4. By performing the staining, one can get a rough idea about the structure of cell wall.
5. By differentiation of micro-organisms in two groups, one can get, useful information about the selection of drugs.

2. Acid-Fast staining (Ziehl-Neelsen's method)

It is another, widely used differential staining procedure in bacteriology. This technique was developed by Paul Ehrlich in 1883, during his work on tuberculosis. By this method, organisms are grouped into two groups as acid fast organisms and non-acid fast organisms.

Most of the micro-organisms are easily stained by usual staining procedures like simple staining or Gram's staining, but there are some bacteria which cannot be stained by usual staining procedures and if any how they are stained, can't be decolourized even by strong decolourizing agents like acid. Therefore such organisms are called as acid-fast organisms while all other micro-organisms are easily decolourized by acid. Therefore they are called as non-acid fast organisms. The acid fastness property is observed in *Mycobacterium tuberculosis, Mycobacterium leprae* and also in *Nocordia* species. The acid fastness is due to presence of covering made up of waxy substances.

Procedure: -

1. Prepare a smear on a clean grease free slide. Allow it to air dry and heat fix.
2. Flood the smear with carbol fuchsin stain. Then place the slide on water bath and steaming is done for 3 to 5 minutes. Apply the stain repeatedly during steaming to avoid evaporation and drying.
3. Wash the slide with water.
4. Decolourize the smear with acid alcohol until no more stain appear in washing.
5. Wash the slide properly with water.
6. Flood the smear with counter stain as 0.3% methylene blue or 1% malachite green. Allow to react for one minute.
7. Wash the slide with water.
8. Blot dry the slide and observe under oil immersion objective.

Requirements: -

1. Primary stain-ZNCF stain: Most of the strains of Mycobacterium are not stained by common stains like carbol fuchsin prepared as aqueous solution. But-ZNCF is a phenolic stain that is stain is prepared in phenol. The phenolic stain is soluble in the waxy covering of *Mycobacterium.*

2. **Decolourizing agent (3% HC1 +95% alcohol):** Acid-fast organisms are resistant to the action of decolourizing agent, retains the carbol fuchsin and appear pink in colour. But non-acid fast organisms are easily decolourized and become colourless then subsequently take counter stain.

3. Counter stain (0.3% methylene blue or 1% malachite green**)**

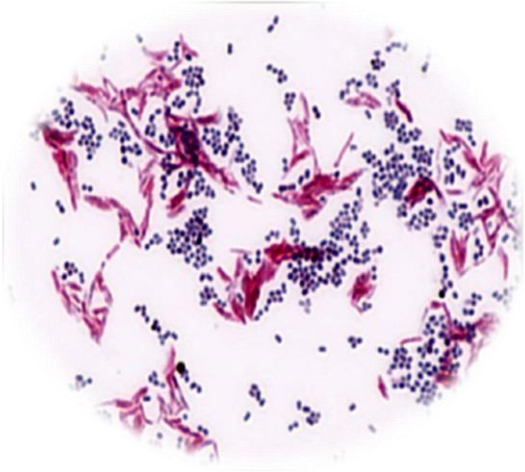
Counter stain stains already decolourized non-acid fast organisms appear blue or green depending upon the stain.

Mechanism: The acid fastness of *Mycobacterium* and *Nocardia* is due to presence of high lipid content of the cell wall (may be up to 60% w/w). The major lipids include mycolic acid and glycolipids. Due to high lipid content in cell wall, the cells are less permeable to stain. The penetration of primary stain can be increased by two ways -

(1) Chemical way - use of phenolic stain with high affinity for waxy covering of organisms. (2) Physical way - use of steaming during staining.

The real mechanism of acid fast staining can be explained on the basis of relative solubilities. The primary stain fuchsin is more soluble in phenol than in water or acid-alcohol, while the phenol is more soluble in the waxy layers of cell wall of organisms. Therefore when phenol containing acid fuchsin is applied as a primary stain, then it enters the lipids of the cell wall. Heating or steaming allows the easy penetration of stain by softening the waxy material. Now once the waxy layer is stained, it will not be decolourized by strong decolourizing agent like acid-alcohol. The reason is that the phenolic stains are

more soluble in lipids than acid-alcohol. Thus acid-fast organisms retain the primary stain and appear pink in colour. But non-acid fast organisms are easily decolourized by acid-alcohol. Such organisms are stained by a counter stain appear blue or green.



Applications

1. The diagnosis of tuberculosis in patients with positive skin test and x-rays can be done by acid-fast staining of sputum or other samples.
2. The diagnosis of leprosy is done by the detection of acid-fast bacteria in the smear of skin lesions or nasal discharges or tissue samples.

- **Cell wall staining**

All true bacteria possess a rigid cell wall; it gives rigidity and shape to bacteria. The cell wall is the structure that immediately surrounds the cell membrane.

The chemical composition of the cell wall may vary in different species of bacteria.Peptidoglycan (also known as murein and mucopeptide) substance found only inprokaryotes cell wall. According to chemical nature of cell wall

there are two classes of bacteria, Gram positive bacteria and Gram negative bacteria.

Principle:-

In this method two stains are used, one is basic fuchsin [basic stain] it stains cell wall and cytoplasm of the cell. The second stain is Congo red [acidic stain] it reacts with basic fuchsin from cytoplasm and neutralizes it therefore cytoplasm appears colorless but can't decolorize cell wall because it strongly stained with basic stain.

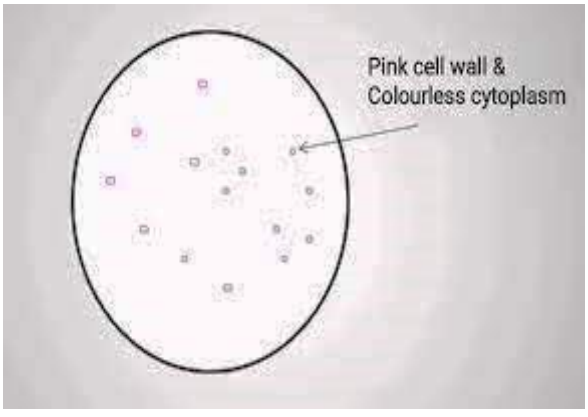
Requirement:-

1. 24 hrs, old culture of bacteria
2. 0.5 % Basic Fuchsin
3. 0.5 % Congo red

Procedure

1. Prepare a smear and heat fix.
2. Flood the smear with 0.5% Basic fuchsin for 1 minute.
3. Wash with water
4. Flood the smear with 0.5 % Congo red for 5 minute Wash with water
5. Air dry and observe under oil immersion lens.

Observation: - Cell wall appears pink, cytoplasm appears colorless.



- **Bacterial Spore Staining by Schaefer and Fulton's method**

The German botanist Ferdinand Cohn discovered the existence of endospores in bacteria; spore production is a very important characteristic of some bacteria such as members of anaerobic genera *clostridium* and aerobic genus *Bacillus*. They are highly resistant and metabolically inactive forms. They occur when environmental conditions become unfavorable for continuing vegetative cellular activities, particularly with the exhaustion of nutritional carbon sources. Because of chemical composition of spore layers, the spore is resistant to heat, freezing radiation, desiccation and chemical agents. The heat resistance of spores has been linked to their high content of calcium and dipicolinic acid.

In1922, **Dorner** published a method for staining endospores. **Shaeffer and Fulton** modified Dorner's method in 1933 to make the process faster The endospore stain is a differential stain which selectively stains bacterial endospores. The main **purpose** of endospore staining is to differentiate bacterial spores from other vegetative cells and to differentiate spore formers from non-spore formers.

A single bacterium forms a single spore by a process called sporulation. **Principle:** -In the Schaeffer-Fulton`s method, a primary stain-malachite green is forced into the spore by steaming the bacterial emulsion. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells may be decolorized with water. Safranin is then applied to counterstain any cells which have been decolorized. At the end of the staining process, vegetative cells will be pink, and endospores will be dark green.

Spores may be located in the middle of the cell, at the end of the cell, or between the end and middle of the cell. Spore shape may also be of diagnostic use. Spores may be spherical or elliptical.

The application of heat facilitates penetration of primary stain, malachite green. Water act as decolorizing agent it removes stain from vegetative cell but not from endospore and spore remains green. Red coloured safranin as counter stain is used as the second reagent to colour the decolorized vegetative calls. The spores look green and vegetative cell red.

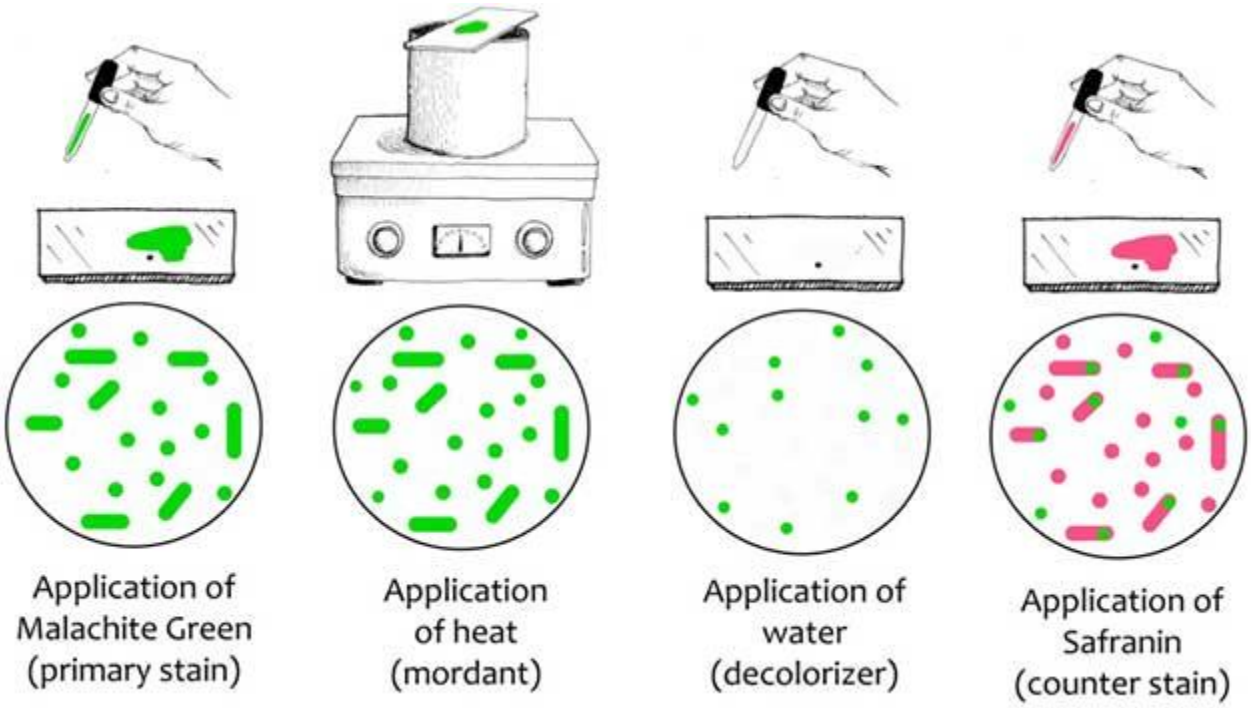
Requirements:-

1. Bunsen burner, beaker of boiling water, staining tray, glass slides, inoculating loop
2. Malachite green and safranin.
    - Preparation of malachite green: -The stain is prepared by dissolving 5gm of malachite green in 100ml of distilled water.
    - Preparation of safranin stain:-

      The stain is prepared by dissolving 0.5 grams of safranin in 100ml of distilled water.

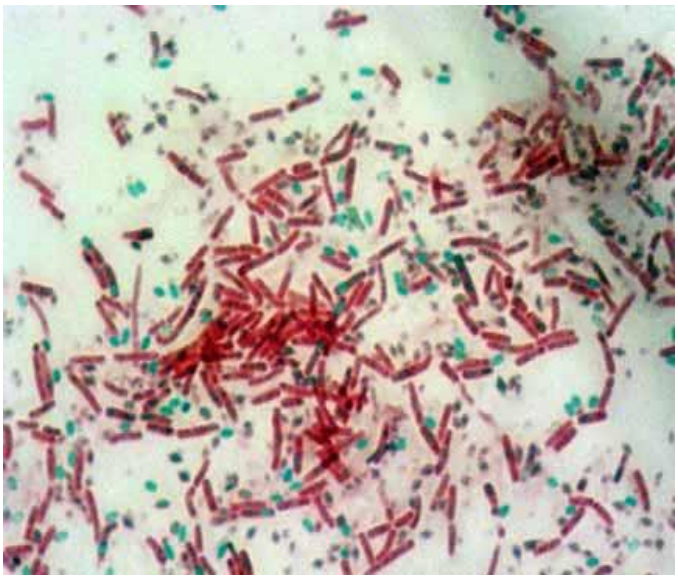
Procedure:-

1. Prepare a smear and air dry. Heat fix by passing the slide 2-3 time gently over the flame with the smear slides up.
2. Put the slide with the smear over a beaker of boiling water resting it on the run with the bacterial film upper most.
3. Flood the smear with 5% aqueous solution of malachite green for 1 minute while water continues to boil.
4. Wash the smear with water.
5. Then cover the smear with 0.5% safranin or 0.05% basic fuchsine. Allow it to act for 30 seconds.
6. Rinse the smear again under tap water and air dry.
7. Observe the smear under oil immersion objective.



Observation: -

Red coloured vegetative cells with green coloured spores are observed.



Endospores are bright green and Vegetative cells are brownish red to pink.

Spores may be located in the middle of the cell, at the end of the cell, or between the end and middle of the cell. Spore shape may also be of diagnostic use. Spores may be spherical or elliptical.

- **METACHROMATIC (VOLUTIN) GRANULE STAINING BY ALBERT AND NEUSSER'S METHOD**

The diphtheria bacillus, *Corynebacterium diptheriae* has well developed granules with the bacterial cytoplasm. These granules are known as volutin granules. These granules are made up of polymetaphosphate and are seen in unstained wet preparations as round, refractile bodies within the bacterial cytoplasm. With basic dyes, granules tend to stain more strongly than the rest of the bacterium, with toludine blue or methylene blue, they stain metachromatically, and appear reddish purple in colour. These granules are demonstrated best by special stains such as Albert's, Neusser's stain. With Albert's staining, the bacilli appear green with bluish black metachromatic granules.

Principle:-

The granules present in the diphtheria bacilli exhibit metachromasia property and hence appear bluish-black coloured when stained with the toludine blue present in Albert reagent and diphtheria bacillus appears green due to malachite green present in Albert reagent.

Metachromasis is a phenomenon when a substance is stained with particular coloured dye, and if a change in original colour is observed, this is called Metachromasis.

Requirements

1. Albert's stain I and II

Preparation of Albert's Stain I

Composition of stain

Toluidine blue	- 1.5 grams.
Malachite green	- 2 grams
Glacial acetic acid	- 10 ml.
Alcohol (95% ethanol)	- 10 ml
Distilled water `	- 1000 ml

Toluidine blue and malachite green are dissolved in the alcohol and then added to the water and acetic acid. The stain is then allowed to stand for one day and then filtered.

Preparation of Albert's stain II: (Albert's iodine)

Iodine	–	1 gram
Potassium iodide	-	2 grams
Distilled water	-	300 ml.

2. Specimen: - Culture of *Corynebacterium diphtheria*

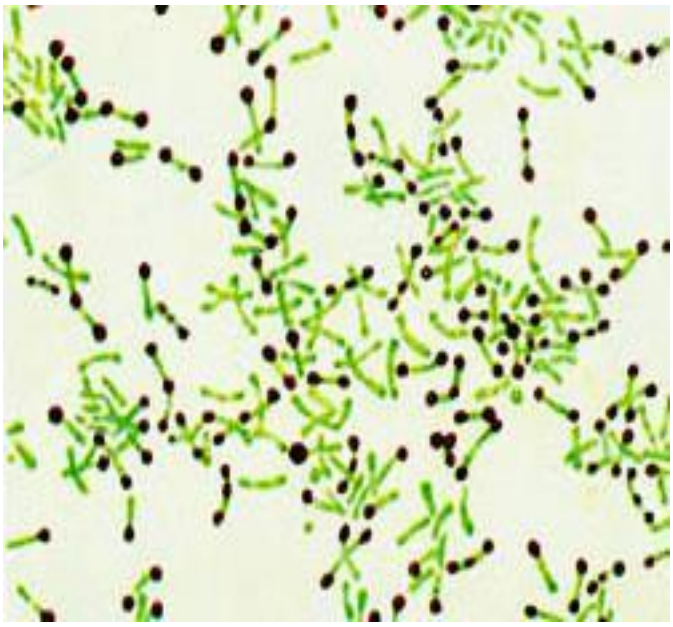
Procedure:-

1. Prepare a smear and air dry. Heat fix the smears by passing the slide 2-3 times gently over the flame with the Smear side up.
2. Put the smear on a slide rack and cover the smears with Albert's stain I, Allow it Stain for 3-5 minutes.
3. Rinse the smear gently under tap water and dry.
4. Then cover the smears with Albert's stain II, Allow it to act for 1 minute.
5. Rinse the smears again under tap water and dry.
6. Observe the smear oil immersion (100 X) objective.

Observation-Bluish-black metachromatic granules are observed

Albert stain is a type of differential stain used for staining high-molecular-weight polymers of polyphosphate known as metachromatic granules or volutin granules found in *Corynebacterium diphtheriae*. Metachromatic granules are also found in *Yersinia pestis*, and Mycobacterium species.

It is named metachromatic because of its property of changing colour i.e. when stained with blue stain they appear red in colour. When grown in Loffler's slopes, *C. diphtheriae* produces a large number of granules.



Principle of Albert Staining:

Albert stain is basically made up of two stains that are toluidine blue' O' and malachite green both of which are basic dyes with high affinity for acidic tissue components like cytoplasm. The pH of Albert stain is adjusted to 2.8 by using acetic acid which becomes basic for volutin granules as the pH of volutin granule is highly acidic.

Therefore on applying Albert's stain to the smear, toluidine blue' O' stains volutin granules i. e the most acidic part of cell and malachite green stains

the cytoplasm blue-green. On adding Albert's iodine due to effect of iodine, the metachromatic property is not observed and granules appear blue in colour.

Composition of Albert Stain

Albert stain is composed of two reagents:

Albert's A solution consist of

Toludine blue	0.15 gm
Malachite green	0.20 gm
Glacial acetic acid	1 ml
Alcohol (95% ethanol)	2ml

Dissolve the dyes in alcohol and add to the distilled water and acetic acid. Allow the stain to stand for one day and then filter.

Add Distilled water to make the final volume 100ml

Albert's B solution consist of

Iodine	2gm
Potassium iodide (KI)	3 gm

Dissolve KI in water and then add iodine. Dissolve iodine in potassium iodide solution

Requirements:

1. Smear on a glass slide
2. Staining rack
3. Albert's A solution
4. Albert's B solution

Procedure of Albert Staining

1. Prepare a smear on clean grease free slide.
2. Air dry and heat fix the smear.
3. Treat the smear with Albert's stain and allow it to react for about 7 mins.
4. Drain of the excess stain do not water wash the slide with water.

5. Flood the smear with Albert's iodine for 2 minutes.
6. Wash the slide with water, air dry and observe under oil immersion lens.

Result

If *Corynebacterium diphtheriae* is present in the sample it appears green colored rod-shaped bacteria arranged at an angle to each other, resembling English letter 'L', 'V', or Chinese letter pattern along with bluish-black metachromatic granules at the poles.

Uses

Albert stain helps to distinguish *Corynebacterium diphtheriae* from most of the short nonpathogenic diphtheroid which lack granules.

- **Flagella Staining**

Most motile bacteria possess flagella, the shape, number, and position of which are important characteristics in the differentiation of genera and species identification. Staining bacterial flagella differs from staining other bacterial structures because it usually requires extraordinary care for the slides, stain, and cells. Flagellar stains are painstakingly prepared to coat the surface of the flagella with dye or a metal such as silver.

The number and arrangements of flagella are critical in identifying species of motile bacteria.

Flagellum is a filamentous, unbranched, thread-like structure which is emerging from the bacterial cell wall. Bacterial cell may contain single polar flagellum (monotrichous), tufts of flagella at each pole (lophotrichous), one or more flagella at both poles (amphitrichous), or flagella surrounding the perimeter of the cell (peritrichous).

Bacterial flagella are made of a protein subunit termed flagellin. In eukaryotic flagella, flagellin has a distinct structure.

In 1930, Leifson introduced a simple flagella stain, using this stain he observed that mutations leading to nonflagellated from flagellated bacteria were common,

as well as mutation leading to loss of motility without loss of the flagellum itself.

Principle of Flagella Staining

The Leifson flagella stain method uses tannic acid and a dye. When bacterial flagella absorbs this tannic acid and a dye, it forms a colloidal precipitate as a result the flagella is colorized and as well as increase in diameter, thus amenable to viewing by light microscopy.

The tannic acid-dye complex is more solvable in alcohol as compared to water, and furthermore soluble with reduced pH. The alcohol concentration in the Leifson solution is enough to maintain the solubility of the ingredients.

When the processed specimen is stained, the alcohol dries faster than the water, and the intensity of the tannic acid and dye rises to create precipitation, leading to staining of the flagella.

Salt concentration also influences the staining, probably by changing charge of the tannic acid-dye complex and the flagellum itself.

The flagella stain is finicky because numerous variables affect the outcome: age of the bacterial culture, thickness of the culture on the microscope slide, age of the staining solutions, pH, temperature, alcohol concentration, dye concentration, heat, and/or airflow

Leifson flagella staining Method

Flagella Staining Requirement

1. 16 and 20 hours old bacterial culture, because older cultures tend to lose flagella.
2. microscope slides
3. 95% ethanol and Kimwipes to clean slides
4. Heat—provided by a Bunsen burner, alcohol lamp, ceramic heater, or other appropriate heat source
5. Micropipettors to deliver 5 to 200 ml
6. Sterile disposable tips for micropipettors

7. Distilled water
8. Bibulous paper

Reagents

1. Leifson flagella stain
2. Solution A: Can be prepared by mixing 1.5 g Sodium chloride with 100 ml Distilled water.
3. Solution B: Can be prepared by mixing 3.0 g Tannic acid with 100ml Distilled water.
4. Solution C: Can be prepared by mixing 0.9 g Pararosaniline acetate and 0.3 g Paraosaniline hydrochloride with 100 ml Ethanol, 95% (vol/vol).

Mix equal volumes of solutions A and B; then add 2 volumes of the mixture to 1 volume of solution C. The resulting solution may be kept refrigerated for 1 to 2 months.

Flagella Staining Procedure

Preparation of culture

From Solid Culture

1. From an agar plate or slant cultures, prepare a suspension by removing a small amount of growth, approximately one-fourth of the colony, with an inoculating loop using proper aseptic technique.
2. Emulsify in 100 ml of distilled water in a microcentrifuge tube by gently vortexing.
3. The emulsion should be only slightly cloudy. Using too much inoculum results in the inability to visualize the flagella.

From Liquid Culture

For staining from liquid cultures, Leifson recommends two rounds of centrifugation and final suspension in distilled water to remove any medium components.

1. Place 100 ml of the liquid culture in a microcentrifuge tube, centrifuge, and remove spent medium.
2. Re-suspend in 100 ml of distilled water by gently vortexing, again centrifuge, and remove supernatant.
3. Form a slightly cloudy emulsion by resuspending in ~200 ml of distilled water. Gently vortex. Again, emulsions should be only slightly cloudy prior to proceeding to staining.
4. Optimization of the washing procedure will most likely be necessary to maximize quality of flagella stain.

Slide Preparation

1. Wipe clean a new microscope slide with 95% ethanol and a Kimwipe. Flame to dry thoroughly. Use slides immediately.
2. When the slide is cool enough to handle, label it using tape with the name of the organism you will be staining.
3. Place 5 to 10 ml of the culture emulsion on one end of the slide using a micropipettor and spread the emulsion using the same pipette tip held parallel to the microscope slide.
4. Allow the sample to dry at room temperature. Do not heat fix as this will destroy the proteinaceous flagella structure.

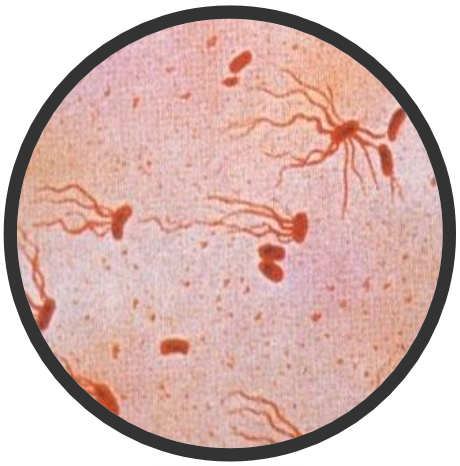
Staining

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 a 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.

Flagella Staining Result

Bacterial bodies and flagella will stain red.



- **STAINING OF FUNGI**

The identification of molds is based on the shape, method of production, and arrangement of spores.

Lactophenol Blue Solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal elements are stained intensely blue.

Composition:

Distilled water	100 ml
Methyl Blue (Cotton Blue, Aniline Blue)	50 mg
Phenol	25 g
Lactic Acid	25 ml
Glycerol	50 mol

Procedure:

1. Place a drop of Lactophenol Blue Solution on a slide.

2. Using an inoculating needle carefully spread the fungal culture into a thin preparation.

3. Place a coverslip edge on the drop and slowly lower it. Avoid trapping air bubbles under the coverslip. Wait for about 5 minutes.

4. If desired, seal the edges of the coverslip with nail polish to preserve the mount as a reference slide.

5. Observe under a microscope with low power for screening in low intensity.

- **Micrometry**

Micrometry is the art of measurement of the size that any visible microscopic microorganism. Micrometry actually is built on the principles of physical science. In the event that we can determine the dimensions of a given scale, the size of an unknown scale can be determined. Similar to this is the case with micrometry. The primary components utilized in micrometry include the stage micrometer and the ocular micrometer.

Ocular Micrometer

The Ocular Micrometer is a round disc that is placed inside the eyepiece (ocular). It is divided into several smaller pieces i.e. divisions marked from 0 to 100. The Ocular Micrometer is located in the eyepiece of a microscope. The distance of the lens varies with the focus that the lens has. It is easy to determine the distance by using a stage microscope.

Stage Micrometer

Stage Micrometers are glass slides that has been graduated by 1 mm i.e. the scale is only 1 millimeter (1000 millimeters). Also, 1 mm is broken down into 10 major divisions and 100 smaller divisions. One large division is split into 10 smaller divisions (hence 10×10 =100 divisions). The divisions are equally spaced. Thus, the smallest number is 0.01 millimeters This means that every smaller micrometer of stage is equivalent to 10 micrometer. The measurement of microorganisms is completed in the two steps that follow:

Calibration and Standardization of Microscope by using Ocular Micrometer and Stage micrometer

Micrometer Principle

A microscope comes with an objective of 10X (low power) 45X (high power) and 100X (oil immersion). and the ocular lens with 5X, 10X and 15X. In general, an 10-X ocular lens is used in most cases. If an animal can be clearly seen with 10X ocular lens and 45X objective, this means that the size is 10×45, which is 450 times larger than the standard one. It could be determined using an calibrated scale. Thus the calibration of all lenses (eyepieces) is performed.

Requirements

- A microscope
- Ocular micrometer
- Stage micrometer

Procedure

Calibration of low power lens (10X): Use a 10X lens inside the objective, and then follow the steps below.

1. Remove your eyepiece (ocular) installed with a microscope. Open the screw.
2. Insert the ocular micrometer into the ocular. Close the screw, and then put it back in the tube of the microscope.
3. Place the stage microscope on the microscope's stage.
4. Adjust the mirror, condensor, ocular, and the objective (10X, 45X, or 100X) that are being calibrated.

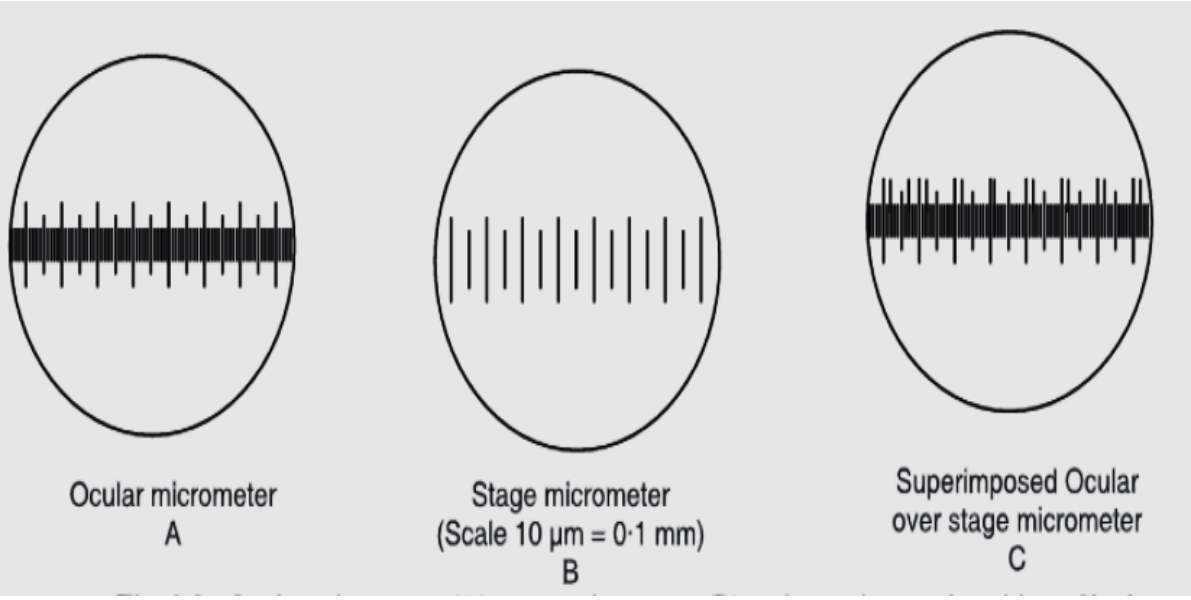
5. Examine the microscope under the lens and then focus the microscope properly using precise adjustments. Two different scales for the stage and ocular micrometers are visible.
6. The ocular should be turned so it is parallel to the lines on both micrometers can be made parallel.
7. Now, you can focus on the superimposed scales, and then coincide an arc on the left and observe the line that is coincided to the right.
8. Find how many ocular divisions which coincide with the lines from both the ocular micrometers and stage micrometers.
9. Take at least 5-6 readings, following the steps from 6 to 8.

Results

Record the reading as given in Table 2, and calculate average value of 1 division of ocular

No. of divisions of ocular micrometer	No. of divisions of stage micrometer	Value of one division of ocular micrometer (um)
10	15	15

Suppose, 10 divisions of ocular micrometer = 15 divisions of stage micrometer
1 division of ocular micrometer = 15/10 = 1.5 divisions of stage micrometer
Since, 1 division of stage micrometer = 10 mm
1.5 division of stage micrometer = 1.5 x 10 = 15 micrometer
However, it may be calculated by using the following formula:



(a) Ocular Micrometer, (b) Stage Micrometer, (c) Superimposed ocular over stage micrometer

- **Calibration of high power lens (45X):** Use a high power lens (45X) of the objective. Follow the instructions above to calibrate a low-power lens.
- **Calibration of oil immersion (100X):** Use an ounce of oil for immersion in the stage micrometer. Turn the lens's mouth over it, and then focus it by using the fine adjustments. Calculate the value of one division on the Ocular Micrometer, as previously described for lenses with low power.

Measurement of Dimension (size) of the Given Microorganism (bacilli)

After the ocular micrometer has been calibrated in the manner described above the dimensions of any microorganism can be determined easily by measuring the area occupied by microorganisms' cells or filament and then multiplying that number with the calibrator factor for the ocular micrometer.

Procedure

1. Be careful, place the ocular micrometer calibrated inside the eyepiece, and then put it back into the microscope's tube.

2. Place one drop of oil into the middle of the slide prepared with bacteria it will be measured and focused in a way that is correct.

3. The total number of Ocular Divisions which is contained in one cell of Bacillus. The size of the cells is 5-10 and then takes an average of it.

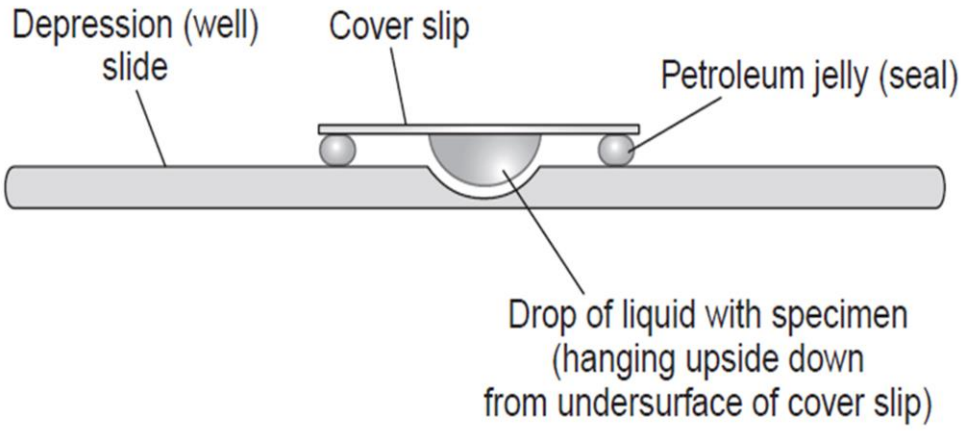
4. Find out the precise size of the bacteria by multiplying it by the calibration value of the ocular micrometer.

Results

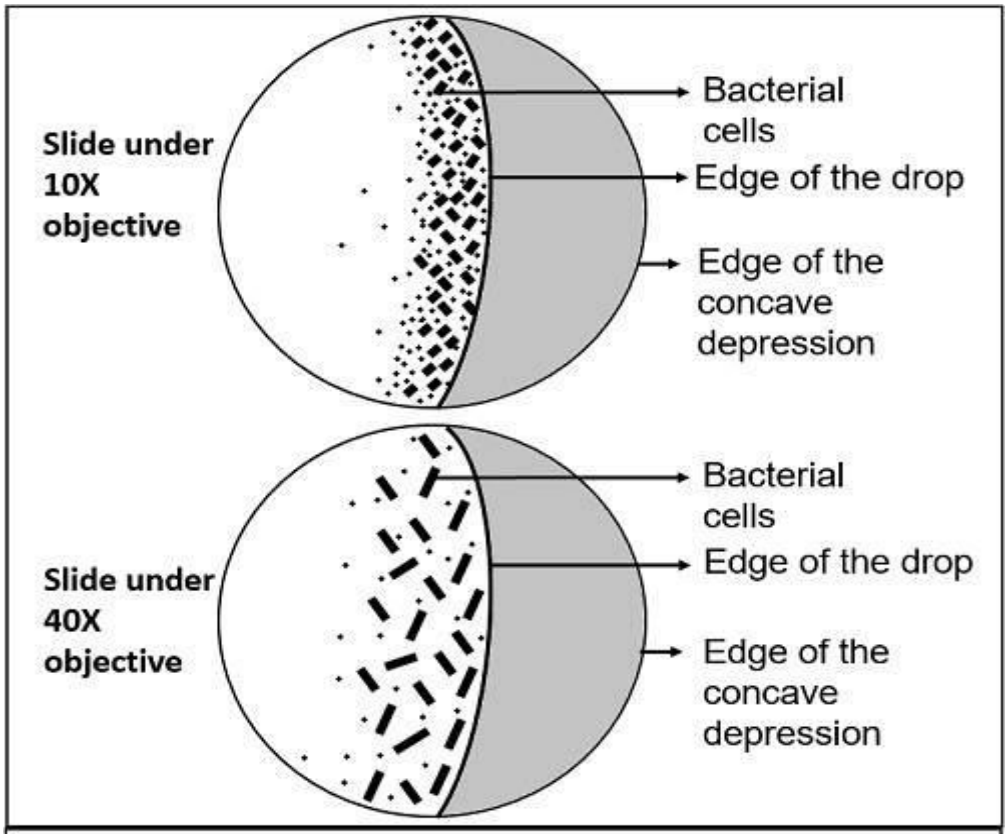
For example, if Bacillus cells are located within four divisions of an ocular micrometer and the calibrated value of the micrometer in the ocular is 2.2micrometer the length of the bacillus will measure 4 x 2.2 = 8.8 micrometer Measurement of Dimension (size) of the Given Microorganism (bacilli)

- **Hanging drop technique**

Hanging drop preparation is a special type of wet mount in which a drop of medium containing the organisms is placed on a microscope slide, often used in dark illumination to observe the motility of bacteria**.**



1. Take grease-free or clean **coverslip** and then carefully dab **petroleum jelly** towards the corner of it by using a toothpick or ear bud.
2. After that, place a loopful of the **microbial suspension** on the centre of the prepared coverslip.
3. Then, take a clean or grease-free cavity glass slide and turn it **upside down**. Place the cavity slide in a manner that the circular concavity should be towards the drop of fluid over the coverslip.
4. Again turn the cavity glass slide in such a manner that the microbial specimen will found **inverted** over the concave depression or observed hanging from the coverslip.
5. Then put the prepared culture slide on the **slide holder** of the light microscope and adjust the just in a way that the edge of drop must be visible through the eyepiece.
6. Use **10X objective** lens and close the diaphragm. Then, slowly adjust the **coarse focus** knob accordingly until you observe a curve that crossing the field of view, in which one half shows the edge of the drop and the other half shows the edge of concave depression.
7. Adjust the **fine focus** knob by looking to the specimen over the slide through the eyepiece, until the edge of the drop appears as a thick dark line.
8. Then, look onto each side of the line and focus onto the particulate matter in the fluid suspension. The cells may vary in colour, size and shape.
9. After that, use a **40X objective** lens for higher resolution and also turn on the diaphragm lever. Then, adjust the light in order to maximize the visibility of the microorganisms.
10. After all this set-up, carefully observe the specimen and note down the **microscopic properties** like the shape, colour, motility of the specimen placed on the glass slide. All the cells will show Brownian movement, but few may possess true motility.
11. At last, thoroughly **clean** the depression slide by putting into a Lysol solution for at least 15 minutes, as the microorganisms remain alive in such slide preparation.



HANGING DROP METHOD: OBSERVATION

Applications

The morphology of spiral bacteria can be explicitly studied in hanging drop method, as its shape becomes distorted in the heat fixing method. Spiral bacteria like spirochetes need to be examined in a living state, and there shape and arrangement can be well studied under a dark field microscope.

Bacterial motility or mobility can be well studied by employing a hanging drop method, in which a bacterial cell can freely move in the liquid medium

Advantages

- It is an important tool to study the bacterial motility along with the shape, size and arrangement of the bacteria.
- It does not distort the cell shape and arrangement.
- Besides, the hanging drop method provides a better view of motility in bacteria than the wet mount method.

- It is also helpful in the classification of the bacteria, whether they are motile or immotile.
- The Brownian movement can be studied through this method, which causes erratic movement of the bacterial cells in the field of view due to the bombardment of water molecules.
- The petroleum jelly used in this technique seals the coverslip to the cavity slide that helps in the study of the tested specimen for many times.

Disadvantages

- It is risky for the study of pathogenic bacteria in a living condition.
- The depression slide is cost-effective, and the coverslip is fragile to work with.

- **Microscopic Photograph**

A micrograph or photomicrograph is a photograph or digital image taken through a microscope or similar device to show a magnified image of an object. This is opposed to a macrograph or photo macrograph, an image which is also taken on a microscope but is only slightly magnified, usually less than 10 times. Micrography is the practice or art of using microscopes to make photographs.

A micrograph contains extensive details of microstructure. A wealth of information can be obtained from a simple micrograph like behaviour of the material under different conditions, the phases found in the system, failure analysis, grain size estimation, elemental analysis and so on. Micrographs are widely used in all fields of microscopy.

Photomicrograph

A light micrograph or photomicrograph is a micrograph prepared using an optical microscope, a process referred to as *photomicroscopy*. At a basic

level, photomicroscopy may be performed simply by connecting a camera to a microscope, thereby enabling the user to take photographs at reasonably high magnification.

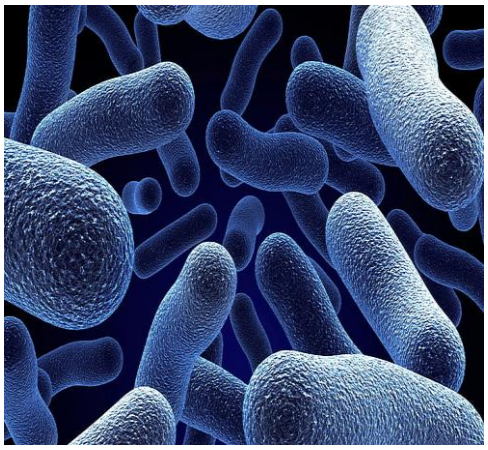
Scientific use began in England in 1850 by Prof Richard Hill Norris FRSE for his studies of blood cells.

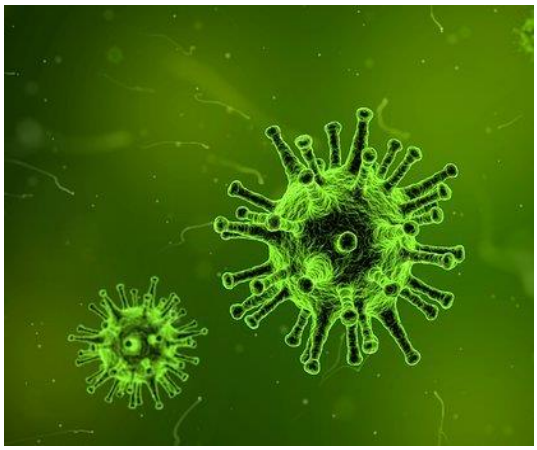
Roman Vishniac was a pioneer in the field of photomicroscopy, specializing in the photography of living creatures in full motion. He also made major developments in light-interruption photography and **colour photomicroscopy**.

Photomicrographs may also be obtained using a USB microscope attached directly to a home computer or laptop.

Electron micrograph

An electron micrograph is a micrograph prepared using an electron microscope.





Magnification and micron bars

Micrographs usually have micron bars, or magnification ratios, or both. Magnification is a ratio between the size of an object on a picture and its real size. Unfortunately, magnification can be a misleading parameter as it depends on the final size of a printed picture and therefore varies with picture size. A *scale bar*, or *micron bar*, is a line of known length displayed on a picture. The bar can be used for measurements on a picture. When the picture is resized the bar is also resized making it possible to recalculate the magnification.

Ideally, all pictures destined for publication/presentation should be supplied with a scale bar; the magnification ratio is optional. All but one (limestone) of the micrographs presented on this page do not have a micron bar; supplied magnification ratios are likely incorrect, as they were not calculated for pictures at the present size.

Micrography as art

The microscope has been mainly used for scientific discovery. It has also been linked to the arts since its invention in the 17th century. Early adopters of the microscope, such as Robert Hooke and Antonie van Leeuwenhoek were excellent illustrators. Cornelius Varley's graphic microscope made sketching from a microscope easier with a camera-lucida-like mechanism. After the invention of photography in the 1820s the microscope was later combined with the camera to take pictures instead of relying on an artistic rendering.