10. STAINING METHODS

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Introduction

Staining is technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells. Bacteria have nearly the same refractive index as water, therefore, when they are observed under a microscope they are opaque or nearly invisible to the naked eye. Different types of staining methods are used to make the cells and their internal structures more visible under the light microscope. Microscopes are of little use unless the specimens for viewing are prepared properly. Microorganisms must be fixed & stained to increase visibility, accentuate specific morphological features, and preserve them for future use

Stain

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

Staining techniques

Direct staining - The organism is stained and background is left unstained Negative staining - The background is stained and the organism is left unaltered Stains are classified as

- Simple stain
- Differential stain
- Structural or special stains

Fixing Before staining it is essential to fix the bacterial sample on to the slide. Smear is prepared in the following way:

(i) With a wire loop place a small drop of the broth culture or a loop full of bacteria on a clean slide.

(ii) Place a drop of water over it.

(iii) Spread the culture so as to form a thin film.

(iv) Allow slide to dry in the air or by holding it above a bunsen flame.

(v) Avoid excess heating.

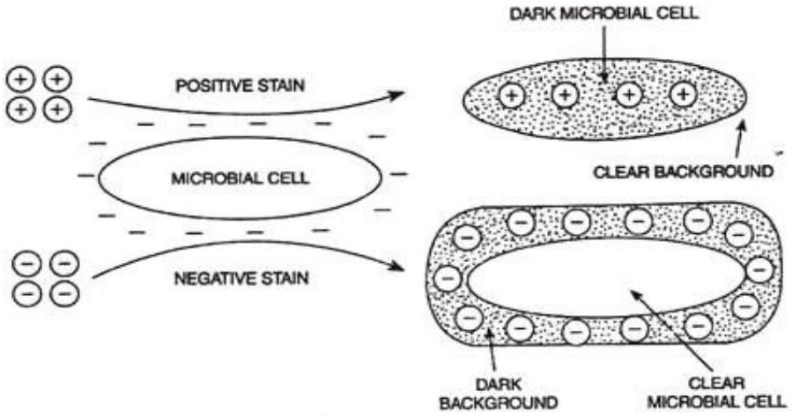
The purpose of fixation is to kill the microorganisms, coagulate the protoplasm of the cell and cause it to adhere to the slide Simple Staining The staining process involves immersing the sample (before or after fixation and mounting) in dye solution, followed by rinsing and observation. Many dyes, however, require the use of a mordant, a chemical compound that reacts with the stain to form an insoluble, coloured precipitate. When excess dye solution is washed away, the mordant stain remains. Simple staining is one step method using only one dye. Basic dyes are used in direct stain and acidic dye is used in negative stain. Simple staining techniques is used to study the morphology better, to show the nature of the cellular contents of the exudates and also to study the intracellular location of the bacteria.

Commonly used simple stains are

- Methylene blue
- Dilute carbol fuchsin
- Polychrome methylene blue

Simple Staining Procedure:

When a single staining-reagent is used and all cells and their structures stain in the same manner, the procedure is called simple staining procedure. This procedure is of two types – positive and negative. In positive staining, the stain (e.g., methylene blue) is basic (cationic) having positive charge and attaches to the surface of object that is negatively charged. In negative staining, the stain (e.g., India ink, nigrosin) is acidic (anionic) having negative charge and is repelled by the object that is negatively charged, and thus fills the spaces between the objects resulting in indirect staining of the object.



Simple staining of microbial cell.

Differential Staining

Differential Stains use two or more stains and allow the cells to be categorized into various groups or types. Both the techniques allow the observation of cell morphology, or shape, but differential staining usually provides more information about the characteristics of the cell wall (Thickness). Gram staining (or Gram's method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell wall. The Gram stain is almost always the first step in the identification of a bacterial organism, While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming Gram variable and Gram indeterminate groups as well.

Gram staining

Gram Staining is the common, important, and most used differential staining techniques in microbiology, which was introduced by Danish Bacteriologist Hans Christian Gram in 1884. This test differentiates the bacteria into Gram Positive and Gram Negative Bacteria, which helps in the classification and differentiations of microorganisms.

Principle of Gram Staining

When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears blue or purple in colour. In case of gram negative bacteria, cell wall also takes up the CV-Iodine complex but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with saffranin, they take the stain and appear red in color.

Materials Required:

Clean glass slides, inoculating loop, Bunsen burner, Bibulous paper ,Microscope ,Lens paper and lens cleaner, Immersion oil, Distilled water , 18 to 24 hour cultures of organisms

Reagents:

1. Primary Stain - Crystal Violet
2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Saffranin

Gram Stain Procedure

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.
8. Gently flood with saffranin to counter counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion.

Interpretation

Gram Positive: **Blue/Purple Color**

Gram Negative: **Red Color**

Gram Positive Bacteria:*Actinomyces, Bacillus, Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactobacillus, Listeria, Mycoplasma, Nocardia, Staphylococcus, Streptococcus, Streptomyces* ,etc.

Gram Negative Bacteria: *Escherichia coli* (*E. coli*), *Salmonella*, *Shigella*, and other Enterobacteriaceae, *Pseudomonas*,*Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella* etc.

Acid-fast staining

The Ziehl–Neelsen stain, also known as the acid-fast stain, widely used differential staining procedure. The Ziehl – Neelsen stain was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894) a pathologist. In this type some bacteria resist decolourization by both acid and alcohol and hence they are referred as acid-fast organisms. This staining technique divides bacteria into two groups namely acid-fast and non acid-fast. This procedure is extensively used in the diagnosis of tuberculosis and leprosy. Mycobacterium tuberculosis is the most important of this group, as it is responsible for the disease called tuberculosis (TB) along with some others of this genus

Principle

Mycobacterial cell walls contain a waxy substance composed of mycolic acids. These are β-hydroxy carboxylic acids with chain lengths of up to 90 carbon atoms. The property of acid fastness is related to the carbon chain length of the mycolic acid found in any particular species.

Ziehl- Neelsen Procedure

1. Make a smear. Air Dry. Heat Fix.
2. Flood smear with Carbol Fuchsin stain
3. Carbol Fuchsin is a lipid soluble, phenolic compound, which is able to penetrate the cell wall
4. Cover flooded smear with filter paper
5. Steam for 10 minutes. Add more Carbol Fuchsin stain as needed
6. Cool slide
7. Rinse with Distilled water
8. Flood slide with acid alcohol (leave 15 seconds). The acid alcohol contains 3% HCl and 95% ethanol, or you can decolorize with 20% H_2SO_4
9. Tilt slide 45 degrees over the sink and add acid alcohol drop wise (drop by drop) until the red color stops streaming from the smear
10. Rinse with Distilled water
11. Add Loeffler's Methylene Blue stain (counter stain). This stain adds blue color to non-acid fast cells. Leave Loeffler's Blue stain on smear for 1 minute
12. Rinse slide. Blot dry.
13. Use oil immersion objective to view.

Capsule staining

The purpose of the capsule stain is to reveal the presence of the bacterial capsule, the water-soluble capsule of some bacterial cells is often difficult to see by standard simple staining procedures or after the Gram stain. The capsule staining methods were developed to visualize capsules and yield consistent and reliable results Capsule may appear as clear halo when a fresh sample is stained by Grams or Leishman stain, Negative staining- using - India ink, Nigrosin.

India ink

Commercially available India ink is used undiluted

Procedure

1. Place a loop full of India ink on the slide
2. A small portion of the culture is emulsified in the drop of ink
3. Place a clean cover slip over the preparation without bubbles. Press down gently
4. Examine under dry objective

Uses

India ink is used to demonstrate capsule which is seen as unstained halo around the organisms distributed in a black background eg. *Cryptococcus*

Endospore Staining (Bartholomew and Mittwer's Method):

Requirement

1. Cell suspension of endospore producing bacteria.
2. Malachite green stain.
3. Saffranin stain.

Procedure

1. Take a clean grease free slide and prepare a thick smear on a slide.
2. The smear is heat fixed by passing the slide from the flame for about 25 times.
3. The slide is allowed to cool.
4. Further the slide is treated with Malachite green stain and allowed it to react for about 10 minutes.
5. After 10 minutes slide is given a water wash treatment.
6. Further the slide is treated with counter stain that is saffranin for about 30 seconds.
7. After 30 seconds the slide is water washed, air dried and observed under oil immersion.

Mechanism

1. In this staining technique a longer heat treatment and prolonged staining technique.
2. Endospore gets stained due to longer heat treatment, prolonged staining and heavy concentration of stain.
3. Here we pass he slide from flame for about 25 times in addition we use concentrated stain that is 7.6 % Malachite green for about 10 minutes.
4. This technique stains the cell as well as the endospore.
5. When we give water wash treatment the water acts as a weak decolorizing agent and decolorizes cytoplasm and not endospore.

6. So here further we apply a counter stain that is Saffranin.
7. Due to application of saffranin the cytoplasm gets stain in pink colour.

Observation

The endospore appears green in colour as well as cytoplasm appears pink in colour.

Flagella Staining (Liefson's Method):

- Bacteria have two types of locomotory organs and that are Flagella and pili.
- Flagella are a thin, hair like structure made up protein called as flagellin.
- It sizes ranges from 20 µ to 200 µ in length.
- Flagella is one of the most important locomotory organ. It is mainly made up of three parts- 1) Basal body 2) Filament 3) Hook.
- Flagella are generally present in rod shape bacteria and very few cocci shape bacteria possess flagella.
- As flagella are very thin and hair like they cannot be easily observed under microscope.
- So a special technique is design to increase thickness of flagella as well as stain it.
- Due to this technique we can observe structure of flagella easily under microscope.

Requirement : Flagellated cell culture slant, Leifson's stain, 1 % Methylene blue, Distilled water.

Procedure:

- Take two hours old flagellated cell culture slant and add two to three drops of sterile distill water in the slant with the help of sterile pipette.
- The distill water is added slowly without disturbing the growth of cells.
- After addition of distill water incubated the slant for 20 minutes.
- Then take a drop of suspension from the slant and place the drop on a clean slide which is kept in slanting position.
- The drop should flow slowly from one end of slide to other end to avoid folding of flagella on cell.
- Allow smear to air dry.
- After air drying the slide is flooded with Leifson's stain till a thin film of shinny surface appear.
- After this give a gentle stream of water wash treatment to a slide.
- Treat the slide with 1 % methylene blue treatment for 1 minute.

- Give the slide water wash treatment, air dry and observe under oil immersion lens.

Mechanism

- First of all, in this procedure thickness of flagella is increase so it can be visible.
- The Leifson's stain is made up of tannic acid, basic fuschin stain prepared in alcohol base.
- When we treat Leifson's stain with cell the tannic acid get attach to the flagella and alcohol get evaporated.
- After evaporation of alcohol the thickness of flagella is increased due to deposition of tannic acid.
- Whereas Basic fuschin stain the Flagella.
- After Leifson's stain treatment cells are treated with Methylene blue stain.
- This Methylene blue stains the cell.

Result

Flagella appear red in colour and bacterial cell appears blue in colour.