EXPERIMENT 2 MICROSCOPIC STAINING TECHNIQUES

Structure

2.1 INTRODUCTION

Visualization of microorganisms in the living state is most difficult, not only because they are minute but also because they are transparent and practically colourless when suspended in an aqueous medium. To study their properties and to differentiate microorganism into specific groups for diagnostic purposes, biological stains and staining procedures in conjunction with light microscopy have become major tools in microbiology. Chemically a stain may be defined as an organic compound containing a benzene ring, a chromophore (chemical group that imparts colour to benzene), and an auxochrome (chemical compound that helps in binding to cells).

Objectives

After studying and performing this experiment, you should be able to:

- learn the practical and theoretical basis of chemical staining;
- describe manipulative technique of smear preparation;
- explain procedures for simple staining and negative staining; and
- perform differential staining procedures such as the Gram's staining, acid fast staining and spore staining.

2.2 EXPERIMENT

2.2.1 Principle

Staining by various dyes provides contrast between microorganisms and their background, permitting differentiation among various morphological types and internal structure such as cell wall, vacuoles or nuclear bodies. It also enables the microbiologist to use higher magnifications.

Numerous staining techniques are available for visualization, differentiation and separation of bacteria in terms of morphological characteristics and cellular structures. A summary of commonly used procedures and their purpose is outlined in Figure 2.1.

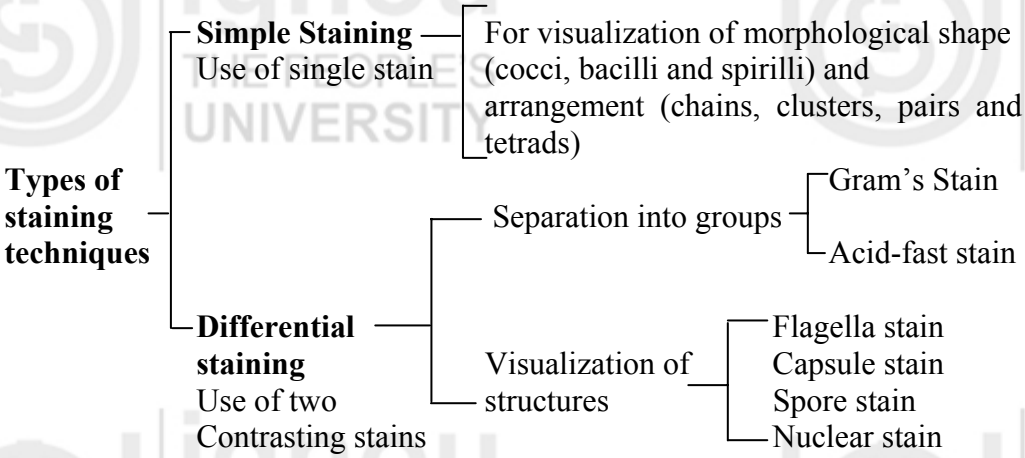


Figure 1: Staining techniques

2.2.2 Requirements (Equipment /Machinery/Instrument and Chemicals/ Material)

- Bunsen Burner
- Microscope
- Test tube shaker
- Inoculating needle
- Cover slips
- Glass slides
- Sterilized test tubes
- Wash bottles
- Microbial cultures
- Distilled water
- Stains
- Immersion oil
- Tissue paper

2.2.3 Procedure

Preparation and fixation of bacteria for staining

Prior to staining, you must "fix" the material to be observed that is make it stick to the glass slide upon which is to be stained. If a preparation is not fixed, the film of cells will wash off during the staining procedure. Purpose of fixation is also to kill the microorganism and coagulate the protoplasm of the cell so as to fix it on glass surface (Figure 2.2).

The fixing technique, although not difficult, requires adequate care in its preparation. Follow these basic rules meticulously:

1. ***Preparation of glass slides:*** Clean slides are essential for preparation of microbial smears. Grease or oil from fingers on slides must be removed by washing the slides with soap and water, followed by a water rinse. After cleaning dry the slides and place them on laboratory towels until ready for use.

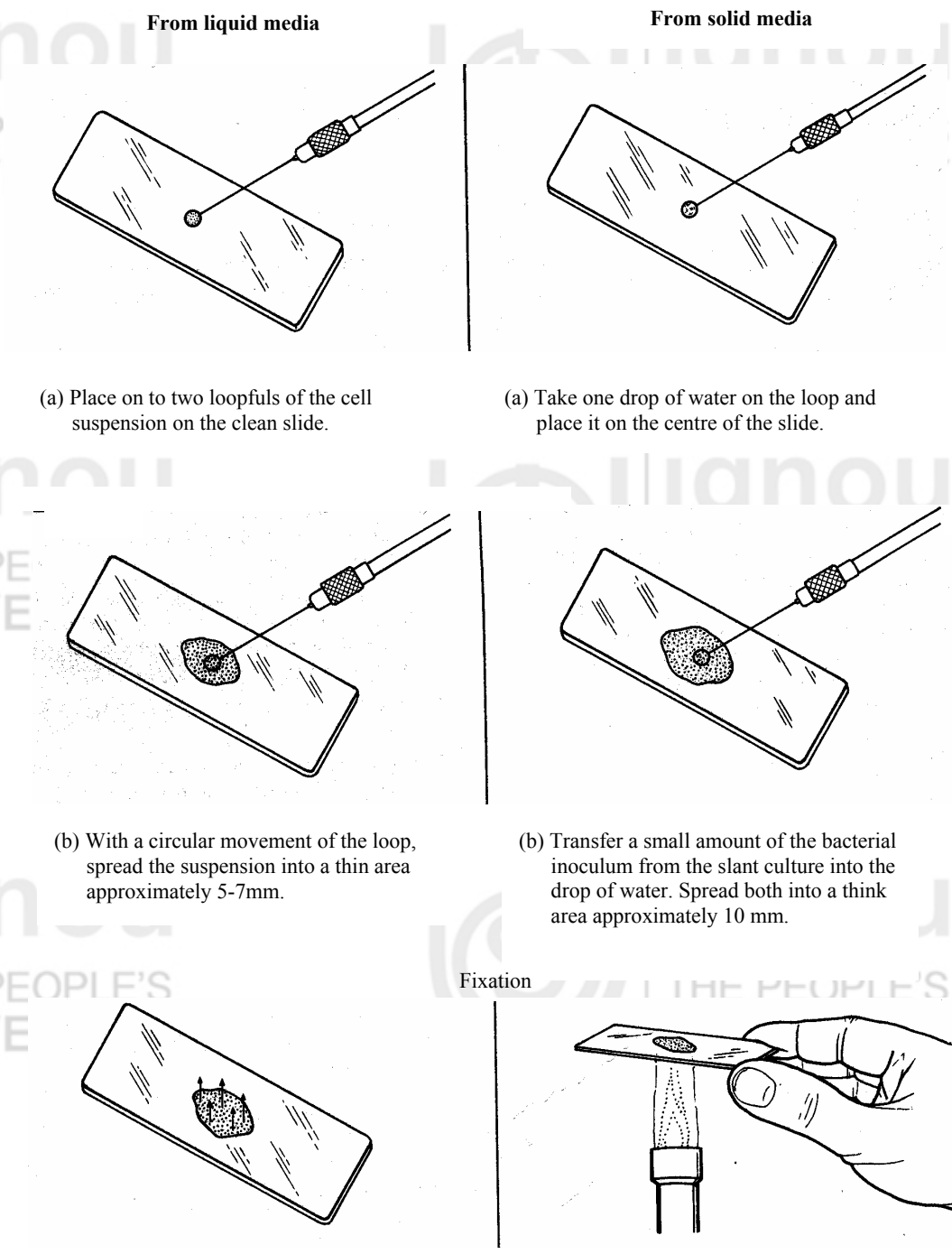


Figure 2.2: Bacterial smear preparation

2. ***Preparation of smear:*** Avoidance of thick, dense smears is absolutely essential. A good smear is one that, when dried, appears as a thin whitish layer or film. Those made from broth cultures or cultures from a solid medium require variations in technique.

   - *Broth cultures:* One or two loopful of suspended cells should be applied directly to the glass slide with a sterile inoculating loop and spread evenly over a small area.

   - *Cultures from a solid media:* Organisms cultured in a solid medium produce thick, dense surface growth and are not amenable to direct transfer to the glass slide. These cultures must be diluted by placing a

loopful of water on the slide in which the cells will then be emulsified. Suspension is accomplished by spreading the cells in a circular motion in the drop of water with the needle tip. At this point, the smear must be allowed to dry completely. **Do not blow or wave it in the air.**

3. ***Heat fixation:*** Unless fixed on the glass slide, the bacterial smear will wash away during the staining procedure. This is avoided by heat fixation, during which the bacterial proteins are coagulated and fixed to the glass surface. Heat fixation is performed by the rapid passage of the air-dried smear two or three times over the flame of the Bunsen burner.

Staining with basic dyes

Herein, the bacterial smear is stained with a single basic stain. The bacterial nucleic acid and certain cell wall components carry a negative charge that strongly attract and bind to the cationic (negatively charged) chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacteria.

The most commonly used basic stains are methylene blue, crystal violet and carbol fuchsin. Note that exposure time for staining cells to these dyes differs for each of these stains; carbol fuchsin requires 15-30 seconds, crystal violet 20-60 seconds and methylene blue 1-2 minutes for fresh cultures. For old cultures more time is required for staining.

General staining

Procedure for staining with different dyes:

1. Prepare bacterial smear of the organisms. Note: All smears must be heat fixed prior to staining.
2. Flood the smear with any one of the stains, using the appropriate exposure time.
3. Wash the stained preparation with tap water to remove excess stain. During this step, hold the slide parallel to the stream of water; in this way you can reduce the loss of organisms from the preparation.
4. Dry the slide using blotting paper.
5. Examine the stained preparation under the oil-immersion objective of the microscope.

Observe closely for significant difference in cell size, shape and arrangements.

Negative or indirect staining

1. Place a small drop of nigrosin close to one end of a clean slide.
2. Using sterile technique, place a loopful of inoculum from the mixed culture in the drop of nigrosin and mix.
3. With the edge of the second slide held at above 30° angle and placed in front of the bacterial surface, push the mixture to from a thin smear.
4. Air dry. Do not heat fixed slide.
5. Examine the slide under oil-immersion objective of the microscope.

Differential staining

Gram's stain

1. Prepare smear of the bacterial culture. Air-dry and fix these preparations with heat.
2. Flood smear with crystal violet and let stained for 30 seconds.
3. Rinse with water.
4. Cover the film with Gram's Iodine instantly and let stained for 1 min.
5. Wash with tap water.
6. Decolorize with 95% alcohol. For a thin smear, 10-20 second is long enough.
   Caution: Do not over-decolorize. Add reagent drop by drop until crystal violet fails to wash from smear.
7. Rinse with water.
8. Counter stain with safranine for 20-30 seconds.
9. Rinse with water and blot dry.
10. Examine under the oil-immersion objective.

Table 1: Steps in the gram's stain

Step	Procedure	Results	
		Gram +	Gram -
Initial Stain	Crystal Stain for 30 seconds	Stains purple	Stains purple
Mordant	Iodine for 30 seconds	Remains purple	Remains purple
Decolourization	95% ethanol for 10-20 seconds	Remains purple	Becomes colourless
Counterstain	Safranine for 20-30 seconds	Remains purple	Stains pink

Acid fast stain

1. Prepare a smear of bacterial culture.
2. Allow to air dry and heat fix in usual manner.
3. Flood smear with carbol fuchsin and place on a warm hot plate, allowing the preparation to steam for 5 minutes. **Caution**: Do not allow stain to evaporate, replenish stain as needed. Also prevent stain from boiling by adjusting the hot plate to a proper temperature.
4. Wash with tap water. Heated slides must be cooled prior to washing.
5. Decolorize with acidic alcohol (95% ethyl alcohol containing 2.5% HNO_3) for 10-30 seconds, a carbol fuchsin fails to wash from smear.
6. Wash with water.

7. Counter stain with methylene blue for 2 min.
8. Wash smear with tap water and blot dry.
9. Examine under the oil immersion objective.

Structural stain

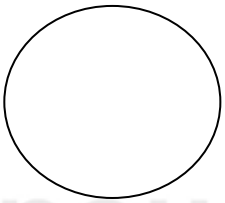
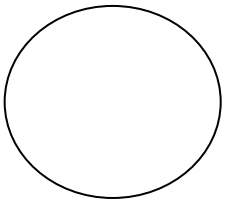
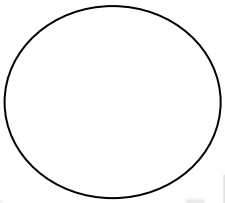
Endospore stain

1. Prepare smear, air dry and fix with heat.
2. Flood smear with malachite green and place on a warm hot plate, allowing the preparation to steam for 2-3 minute.
   Caution: Do not allow stain to evaporate; replenish stain as needed. Prevent the stain from boiling by adjusting the hot plate at a proper temperature.
3. Cool slide and wash with water.
4. Counter stain with safranine for 30 min.
5. Wash with water and blot dry.
6. Examine under oil immersion objective.

2.2.4 Observations

In the space provided:

1. Draw a representative field for each organism
2. Describe the morphology of the organism with reference to their shape (bacilli, cocci, spirilli) and arrangements (chains, clusters, pairs)

Stain	Methylene blue	Gram Stain	Carbol Fuschin
Drawing of a representative field			
Cell morphology			
Arrangement			
Cell colour			

2.2.5 Results

Staining the microorganisms makes them contrast in colour with their surroundings so that they are more readily visible. Certain stains can also be used to identify certain structures of the cell which would otherwise be unseen.

2.3 PRECAUTIONS

- Clean, dry glass slide must be taken to prepare a smear.
- Thick dense smears should be avoided.
- The smear should be properly heat fixed on the slide to avoid its washing off during staining procedure.
- Do not heat fix in case of negative staining.
- Do not over decolorize in case of Gram's staining.
- Do not allow stain to evaporate while acid staining technique. Replenish stain as needed.