

STAINING OF BACTERIAL CELLS

Objective

- To learn the techniques of smear preparation, Gram staining, nigrosin staining and correlating the results of Gram staining with KOH test.

Introduction

Observation of bacteria with conventional bright field microscopy yields relatively little useful information. This is because of the lack of contrast coupled with the small size of the bacterial cell. The use of stains that react chemically with cell material will enhance the contrast between the cell and the background. A stain is a dye consisting of a colored ion (a chromophore) and a counter ion to balance the charge. Attachment of the chromophore part of the dye complex to a cellular component represents the staining reaction. Depending upon the dye, the chromophore can be either positively charged (cationic) and have an affinity for negative ions or negatively charged (anionic) with an affinity for positive ions. Bacteria carry a net negative charge at pH 7. Therefore, cationic dyes such as methylene blue, basic fuchsin, or crystal violet are useful for the direct staining of cells, whereas anionic stains, such as eosin and nigrosin, will not directly stain bacterial cells. However, negatively charged stains, are useful for revealing the outlines of bacterial cells; anionic dyes stain the background, leaving the bacterial cells clear and bright against a dark background.

Simple staining implies the use of only a single stain, which is usually sufficient to reveal the morphological features of most microbial cells, including relative size, shape, and characteristic arrangements for groups of cells.

To obtain additional information, various specialized differential staining procedures have been developed. Stains that react differently with different cell types are known as differential stains, and they have an important role in the identification of taxonomic groups. The most important and widely used differential stain for bacteria is the Gram stain. On the basis of their reaction to the Gram stain, bacteria can be divided into two groups: Gram positive and Gram negative. The differential response to the Gram stain is based on fundamental differences in the cell wall structure and composition of cells. In a manner quite similar to the Gram stain, the acid-fast stain differentiates an important group of bacteria, the mycobacteria, on the basis of lipid content of their cell wall.

In the Gram stain, cells from a culture are spread in a thin film over a small area of a slide, dried, and then fixed by heating or with a chemical fixative to make the cells adhere to the glass slide. A good smear preparation (1) will be of an appropriate thickness to view individual cells, (2) will withstand repeated washing during staining; and (3) will retain the original cell morphology after fixation and staining. The slide is then stained with crystal violet dye, which is rinsed off and replaced with an iodine solution. The smear is then decolorized with a solution of ethyl alcohol and acetone and counterstained with safranin. In Gram positive organisms the purple crystal violet stain, treated with iodine solution, is not removed by the decolorizer solution and thus the organisms remain purple. On the other hand, the purple stain is removed from Gram negative organisms by the decolorizer and so the colorless cells take up the red color of the safranin counterstain.

After you have stained your bacterial smears, you will examine them with the oil immersion lens, noting the morphological and staining characteristics of each species. The usual bacteria which you will see range in size from 0.5 to nearly 10 microns (A few species are many times that long.) The bacteria may show the following shapes: spherical (coccus), rod-shaped (bacillus), curved (comma), or helical (spiral). After cell division, the cells may assume a characteristic arrangement: some occur singly, as do most Gram negative rods; others frequently appear in pairs, as in *Klebsiella pneumonia*, *Streptococcus pneumoniae*, and in the *Neisseria* genus. Still others occur in chains, as in the *Bacillus* and *Streptococcus* genera; in irregular clumps, as in *Staphylococcus*; in regular cubical packets of four or eight cells as in some *Micrococcus* species; and lying side by side or at sharp angles as in a palisade or a Chinese character as in *Corynebacterium* species.

The differential Gram stain gives additional information about the bacterial cell wall, which may be Gram positive (the deep purple color of crystal violet), or Gram negative (the pink color of the counterstain safranin). This difference in staining reflects a fundamental difference in chemical composition of the cell wall and in the ultrastructure of the wall as revealed in electron microphotographs. The Gram negative organisms lose the primary stain after decolorization, and after counterstaining with safranin they appear pink. If Gram positive cells are not treated with the iodine mordant after primary staining, they too will lose their primary stain at the decolorizing step and appear as Gram negative cells. Several factors may affect the results of Gram staining:

- If the smear is too thick, proper decolorizing will not be possible.
- If the smear is overheated during heat fixing, the cell walls will rupture.
- Concentration and freshness of reagents may affect the quality of the stain.
- Washing and drying of the smear between steps should be consistent. Excess water left on the slide will dilute reagents, particularly Gram's iodine.
- Gram stain is reliable only on cells from cultures that are in the exponential phase of growth. Older cultures contain more ruptured and dead cells. Cells from old cultures may stain Gram negative even if the bacteria are Gram positive.

At this time the exact mechanism of the Gram stain reaction is not yet known. The most prominent current theories are as follows:

(1) The cell wall of a Gram positive bacterium is composed of a heteropolymer of amino acids and sugars called peptidoglycan. This wall provides a barrier through which the crystal violet-iodine complex cannot pass during decolorization. When this wall is removed enzymatically with lysozyme, Gram positive cells no longer retain the stain complex and become Gram negative. A Gram negative bacterium contains less peptidoglycan and more lipid than a Gram positive organism. These chemical characteristics cause more effective and rapid removal of dye complex when decolorizer is applied.

(2) There may exist a specific Gram positive substrate within the cell. All major cellular macromolecules have been implicated, including lipids, carbohydrates, proteins, and nucleic acids. This theory is hard to prove because removal of any of these components from the cell vastly alters the chemistry of the cell wall. However, there is some evidence for a crystal violet-ribonucleic acid-iodine complex in Gram positive cells.

(3) The real situation may be the combination of above two theories suggesting that a Gram positive bacterium is not only less permeable but also contains a compound that reacts with and holds the stain complex tightly.

A new and easy technique (KOH test) has recently been developed by Ryu that can also characterize the Gram stain reaction to a large extent. We use this new test in our lab to complement the results of the Gram test and not as a replacement for the commonly used Gram stain. In some rare cases, Gram stain and KOH test results differ.

Laboratory Supplies

Stock cultures:

<i>Bacillus cereus</i> , Gram positive rod	1 plate/table
<i>Escherichia coli</i> , Gram negative rod	1 plate/table
<i>Staphylococcus aureus</i> , Gram positive coccus	1 plate/table
<i>Neisseria flava</i> , Gram negative coccus	1 plate/table
Bright field microscope	1/student
Acid/alcohol in Coplin jar	3/table
Lens paper	3/table
Immersion oil	3/table
Microscopic slides	1 box/table
Dropper	3/table
Inoculating loop	3/table
Glass marking pencil	3/table
KOH, 3% solution	1 bottle/table
Wooden toothpicks	20/table
Crystal violet	3 bottles/table
Gram's iodine	3 bottles/table
Gram's decolorizer	3 bottles/table
Safranin	3 bottles/table
Staining bowls	3/table
Glass staining rack	3/table
Slide forceps	3/table
Water squeeze bottle	3/table
Nigrosin bottle	3/table
Methanol bottle	1/table

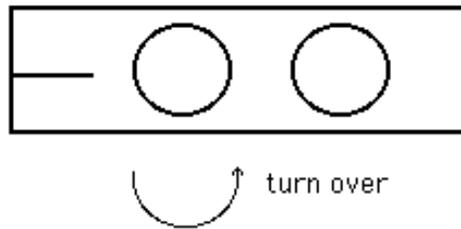
Procedures

We suggest two students to team up for doing these exercises.

Smear Preparation

1. The stock plates you will be using are to be shared with other students in your section and other sections. Obtain 2 glass slides and dip them momentarily into the acid alcohol jar. Dry them with paper towel and do not touch the surfaces with your fingers. Acid alcohol removes oil and dirt particles from the slide.
2. Using a glass-marking pencil, draw a single line to the left and then two round large circles, leaving a margin on the right side of the slide. The left-hand line indicates where numbering begins. Turn the slide over, so that the marks you made are on the underside. Repeat this procedure with the other slide.
3. Place a drop of water in the middle of each circle with a dropper.

4. Sterilize your loop (from the hub of the handle along the entire length) in the Bunsen burner flame by holding it almost perpendicularly in the flame until it glows. Remove the loop from the flame and let it cool.



5. Remove a very small amount of inoculum by gently touching a single representative colony on the plate with your loop.

NOTE: If too much inoculum is taken, you will not obtain a good smear due to flaking of cell aggregates upon drying. If you see a clump of cells on your loop, you are taking too much.
6. Spread the inoculum in the first circle, filling the circle and mixing with the water.
7. Flame the loop again until the wire glows. Cool the loop and draw a sample from another plate and place in the second circle. With circular motion of the loop, dissolve the cells in the water.
8. Repeat the above steps for the second glass slide, making sure you label the slides so that you know which organisms are on each slide.
9. Let the slides dry in the air. This process can be speeded up by placing the slide in a warm spot near a lamp or the Bunsen burner.

NOTE: When the slide is dry, the specimen may be hardly visible; however, the surface of the slide will be dull and not shiny.
10. Fix the bacteria onto the slide by passing the slide, smear side up, quickly through the flame of the burner three times. Avoid getting the slide too hot. This fixation process coagulates the proteins and fixes the bacteria onto the slide so they will not wash off during staining.

Gram Staining

1. Place the slides on a staining rack, smear side up and crayon markings down. Do not allow the slides to touch each other.
2. Add a few drops of crystal violet stain on the smears. Stain for 1 min.
3. Rinse the slide with a gentle jet of water from a wash bottle. At this stage all bacteria will be stained purple by the crystal violet.
4. Drain off the rinse water, and add a few drops of Gram's iodine solution and let the slide stand for 1 min.

5. Rinse the slide with a gentle jet of water as described above.
6. Decolorize the stain by letting the decolorizer (ethyl alcohol: acetone) run down over the slide, which should be held at an angle, until the stain no longer is being removed from the slide.
7. Quickly rinse the slide with water.

NOTE: A thick stain takes longer to decolorize than a thin one, so the exact time cannot be specified. It is usually not more than 20 seconds. Too little or too much decolorization can affect your results. (At this stage, the Gram positive organisms will remain purple. If the smear is thick, they may appear so visually, but a thin smear may not be visible. Gram negative bacteria will be colorless at this stage.)

8. Add a few drops of Safranin and let the smears stain for 30 seconds.
9. Rinse the slides with water.
10. Let the slides air-dry. The slides should be completely dry before adding oil for microscopic examination.

Examination of Stained Slides

1. Examine the stained slides with low power objectives and finally use the oil immersion lens. For this you will need to give maximum light by opening the diaphragm, raising the condenser as much as possible, and adjusting the light source intensity.
2. Examine the bacteria in each circle and observe the size, shape (rod, spherical or curved), Gram staining (positive: purple; negative: pink), and arrangement (singly, in pairs, in chains, irregular clusters, or regular packets of four or eight). Compare the Gram negative organism(s) with the Gram positive and note the difference in color. If not clearly evident, check your procedure with your TA.

NOTE: Achieving a good focus with oil immersion is frequently difficult and time-consuming for the beginner; be patient and get help!

3. Sketch a few of the organisms found in each circle, enlarging your drawing enough so that the arrangement, shape and relative size of the organisms can be shown. Do not try to sketch the entire field.

Nigrosin Staining

1. Place a small drop of nigrosin on one end of a slide. Sterilize your loop and aseptically transfer some cells to the nigrosin drop, and spread with a circular motion to make an even suspension.
2. Use the end of a fresh slide to spread the mixture to a thin film, as per your TA's demonstration. Allow the slide to air dry.
3. Examine the stained slide microscopically. You can go to the oil lens but make sure your slide is completely dry.

4. Repeat the above steps for all the bacterial samples.

Use of KOH as an Indicator of Gram Stain Reaction

1. Place two drops of 3% potassium hydroxide (KOH) solution on a clean slide.
2. Transfer bacterial cells from the culture medium with a wooden toothpick. You need to get a rather good amount of inoculum for this test to work. Mix bacteria into the solution on the slide rapidly and in a circular motion.
3. After 5-8 seconds, raise and lower the toothpick just off the slide surface to determine the stringing effect. If there is stringing (i.e. increased viscosity) within 15 seconds, the KOH test is positive, while the bacteria are considered to be Gram negative. If no stringiness is observed, the KOH test is negative and the bacteria are considered to be Gram positive. See if your results from this test agree with those from the Gram stain and record your observations.

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Results of Bacterial Staining Lab Exercise

Name _____ Partner's name _____ Date _____ Section _____

1. In the table below, determine the species name based on cell shape and Gram reaction. Note down the cell shape, sketch enough cells to show some detail and spatial arrangements (nigrosin staining). Record the color taken up by the cell and the Gram stain reaction (Gram positive: purple; Gram negative: pink). Try the KOH method (stringiness=positive) and see if the results agree with those of the Gram stain.

<u>Species Name</u>	<u>Cell Shape</u>	<u>Sketch of Cells</u>	<u>Gram Stain</u>	<u>KOH Test</u>
#1.				

#2.

#3.

#4.

2. Consult textbooks and websites to make neat and detailed drawings to show the differences between the cell wall components of Gram positive and Gram negative bacteria and label all parts.