MODULE 4: Introduction to Light Microscopy

LEARNING OUTCOMES

1. Define basic terms and principles of brightfield microscopy.
2. Describe appropriate units of measurement for microorganisms.

INTRODUCTION

The pioneers of microscopy opened a window into the invisible world of microorganisms. Early microscopes, which used visible light to illuminate cells, continued to advance in the centuries that followed. The 20th century saw the development of microscopes that leveraged nonvisible light, such as fluorescence microscopy (which uses an ultraviolet light source) and electron microscopy (which uses short-wavelength electron beams). These advances led to major improvements in magnification, resolution, and contrast.

Brightfield Microscopy

The brightfield microscope is one of the most common types of light microscopes used in microbiology laboratories. It is a compound microscope, meaning that more than one type of lens is used to magnify an image. Visible light is the source of illumination and specimens are observed against a bright field or background. Some brightfield microscopes are equipped with special attachments that change the field to appear darker than the specimens being viewed. This is known as darkfield microscopy and is often helpful when viewing live microorganisms, such as protists, that might otherwise be killed if stained (Figure 4.1).

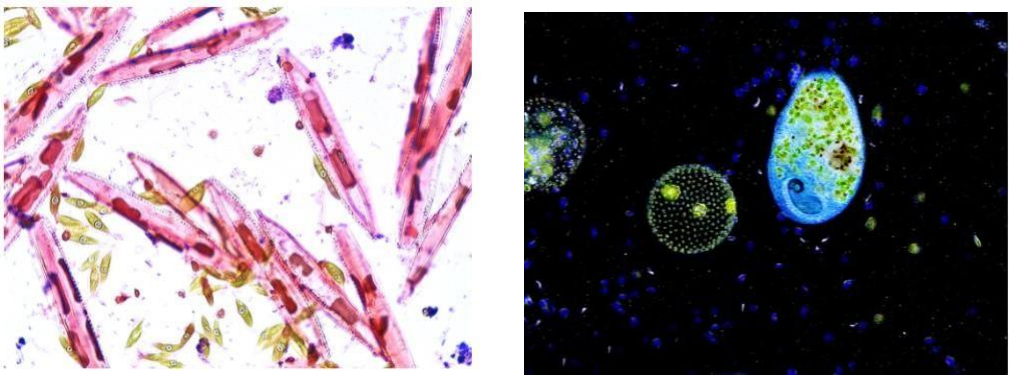

Figure 4.1: Gram stain of freshwater diatoms, euglenoids, and bacteria (left) and darkfield image of liveprotists (right).

The size of microbes can be hard to imagine because they are so small in comparison to what most people see day to day. Even when compared to plant or animal cells, microbes tend to be much smaller. The unit micrometer (μm), also known as a micron, is used when describing the size of bacterial cells. A micrometer is 1/1000 of a millimeter and 1/1,000,000 of a meter. To put it more tangibly, a typical cell of *Staphylococcus* bacteria measures one micrometer, or about 1/400 the size of the period at the end of this sentence.

Viruses, which are too small to be viewed with a light microscope and instead must be observed using a much more powerful electron microscope, are measured in nanometers (nm). One nanometer is 1/1000 of a micrometer. Most viruses range in size from 10 to 100 nanometers. See Figure 4.2 for a comparison of relative cell sizes.

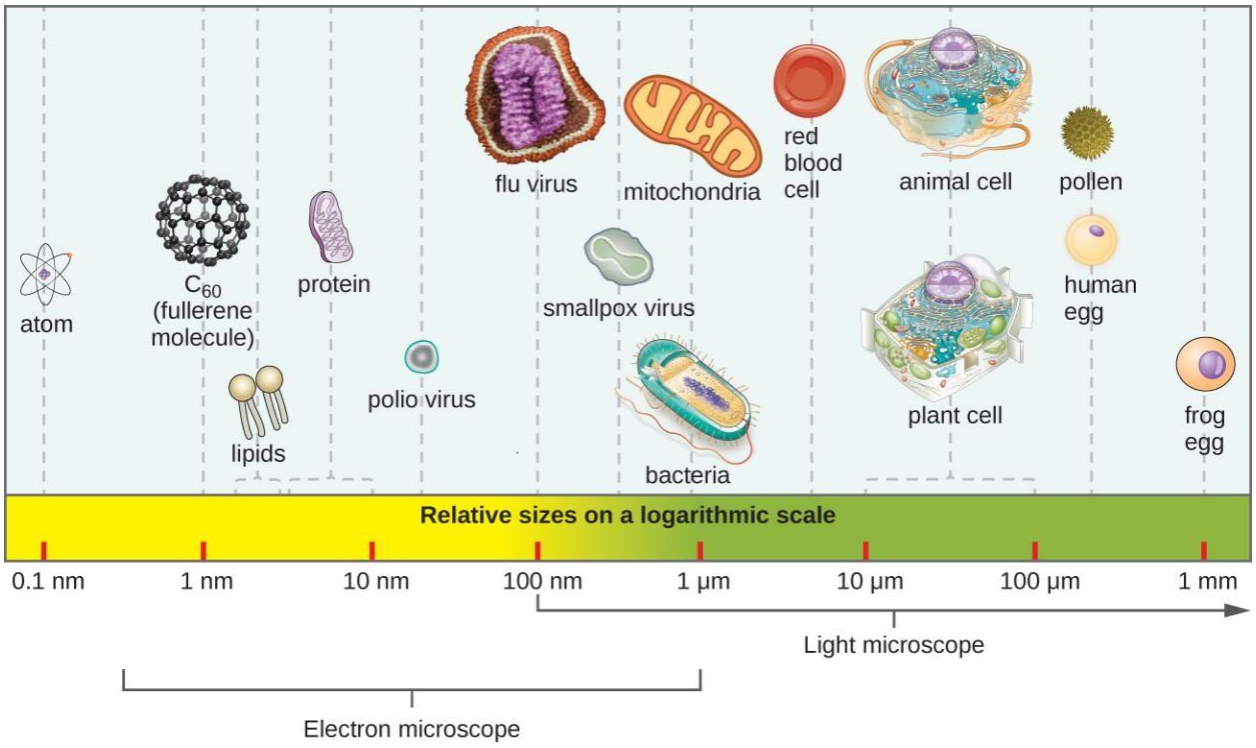

Figure 4.2: Relative sizes of various cellular and non-cellular structures. Bacteria and larger microorganisms such as protists and fungi are visible with a light microscope, while a more powerful electron microscope is required to observe most viruses.

In this module, you will use the light microscope to view bacterial smears that were previously prepared. The maximum magnification of the microscope, 1000X, will be used to observe all cells.

Exercise 4.1 – Using the Light Microscope

LEARNING OUTCOMES

1. List the ways in which a microscope is properly maintained and stored.
2. Identify and give the function of key parts of a compound light microscope.
3. Discuss the principles of magnification and resolution; define key terms.
4. Calculate total magnification.
5. Use the scanning, low power, and high-power objective lenses to focus the letter “e.”

Microscope Care

Even a very powerful microscope cannot deliver high-resolution images if it is not properly cleaned and maintained. Microscopes are rather delicate instruments, and great care must be taken to avoid damaging parts and surfaces.

Each student is assigned a microscope for use during the semester. Be sure to record the number of your microscope and follow the guidelines below when obtaining and storing it.

Care and Storage of the Light Microscope
1. Carry your microscope with two hands, one on the arm and the other under the base.
2. Lift and place the microscope to reposition it on the benchtop; do not drag it.
3. Clean the stage and objective lenses before and after use with lens paper/cleaner.
4. Lower the light intensity before turning off the microscope.
5. Move the stage to its lowest position before storage.
6. Position the 4X objective to point down toward the stage before storage.
7. Return your microscope to the corresponding number compartment in the cabinet.

Parts of the Microscope

Basic components of the light microscope are shown in Figure 4.3.

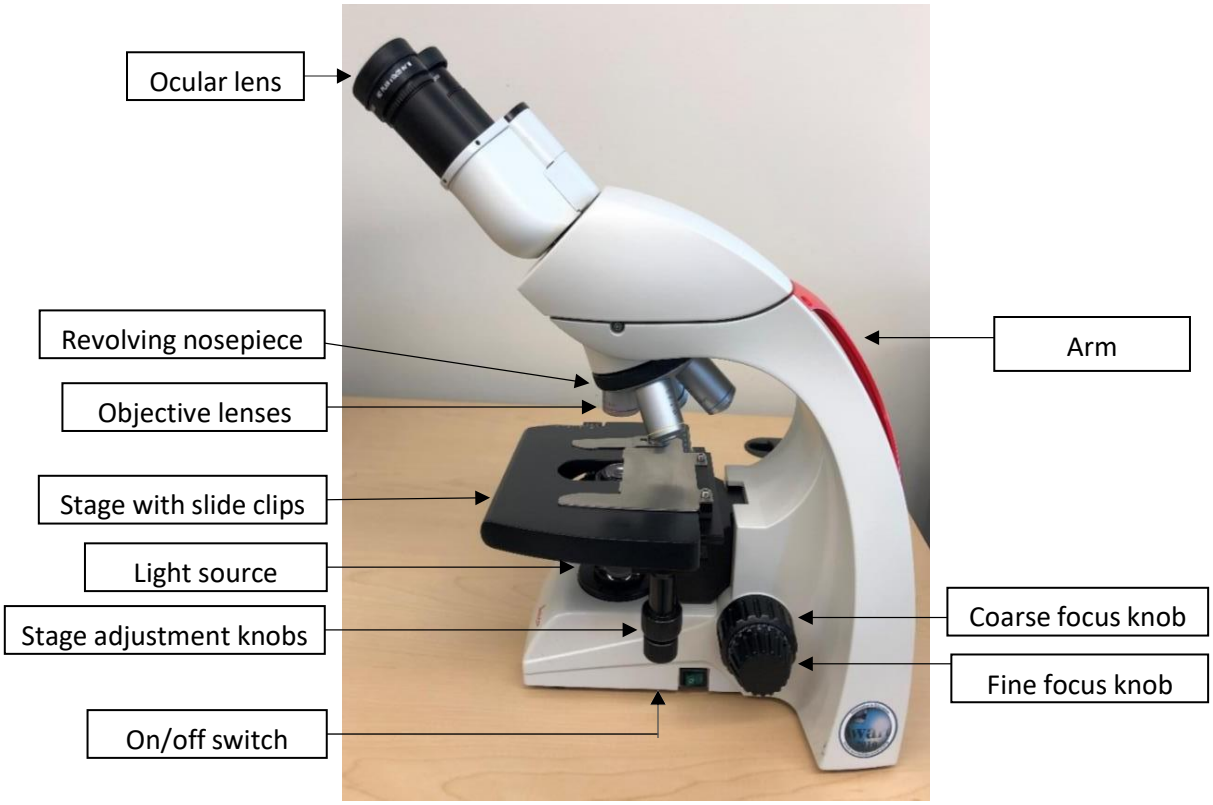

Figure 4.3a: Components of a typical brightfield microscope.

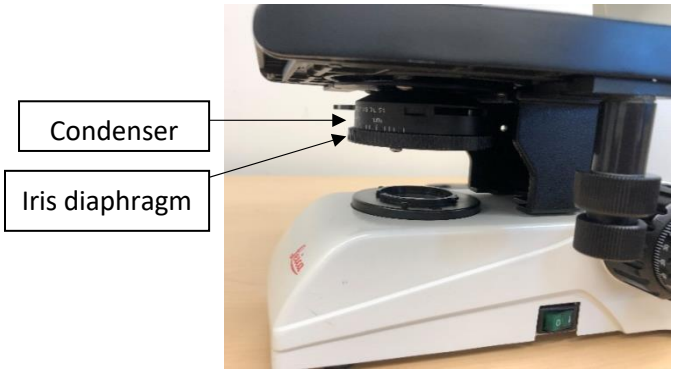

Figure 4.3b: The amount of light traveling through the condenser lens is controlled by turning the adjustment for the iris diaphragm located inside the condenser.

Summary of Microscope Components

- **Ocular lens:** Eyepiece that usually magnifies 10X; binocular microscopes have two oculars that are adjustable for interpupillary distance between the eyes. Oculars may have a pointer and/or a ruler for measuring cells called an ocular micrometer.
- **Revolving nosepiece**: Rotates to allow each objective to align in place with the ocular.
- **Objective lenses**: Seated in the nosepiece. Each objective lens has a different magnifying power: scanning (4X), low power (10X), high power (40X) and oil immersion (100X).
- **Coarse focus knob**: Outer large knob that raises and lowers the stage to bring the specimen into initial focus; used with the scanning objective lens.
- **Fine focus knob:** Smaller inner knob that raises and lowers the stage to bring the specimen into sharp focus; used with low power, high power, and oil immersion lenses.
- **Mechanical stage**: Horizontal surface on which slide is placed and held by stage clips; the stage is moved left and right by turning the x-y mechanical stage knobs.
- **Illuminator**: Light source turned on by a switch on the base and controlled by a rheostat located on the side of the base that adjusts the brightness of the light.
- **Iris diaphragm and condenser**: The iris diaphragm can be adjusted to control the amount of light passing from the illuminator through the bottom of the slide. It is located inside the condenser, which is a lens system that gathers and directs light up from the illuminator.

Magnification and Resolution

Light microscopes use visible light and a series of lenses to view microscopic specimens. The condenser lens focuses the light as it goes through the specimen and can be adjusted for optimization. The objective lenses magnify the specimen, capturing the transmitted and reflected light to create a real image of the specimen. The ocular lens further magnifies the image and creates a virtual image for viewing. This difference can be observed with a slide of the letter “e” or “p” and noting how the image changes when viewed through the ocular.

What is observed through the microscope is the *field of view*. While an entire organism might be visible in the field of view using the scanning lens, only a small portion of it may be seen under high power. Since microorganisms have a wide range of sizes, the most appropriate objective touse for each varies. For example, while a large protist such as *Amoeba* may be viewed under low power, this would not be suitable for viewing bacteria which are much smaller.

Most modern microscopes are *parfocal* in that they remain in relative focus when changing magnifications. This property eliminates the need for extensive re-focusing when switching between objective lenses.

Related to the concept of field of view are *depth of field* and *working distance*. Depth of field refers to the nearest and furthest planes of a specimen that are in focus at the same time. Depth of field depends on thickness of the specimen and decreases as magnification increases. The working distance, or space between the slide and objective lens, decreases as magnification increases. To avoid damaging the objective lenses or the slide, the coarse focus knob should only be used for initial focus when the working distance is greatest.

Calculating Total Magnification

Magnification is the process of making an object appear larger than it is. The magnification of each objective is printed on the metal portion of the lens. The *scanning* objective has a magnification of 4X and is used when first bringing an image into focus. The next objective is *low power* which magnifies 10X. The high-power objective, sometimes called the *high dry* objective because it is used without immersion oil, magnifies 40X and is used when fine focusing an image. Finally, the *oil immersion* objective has a magnification of 100X and is used when viewing bacterial cells.

The ocular and objective lenses work together to create a magnified image. Total magnification (TM) is calculated by multiplying the ocular and objective magnifications:

Total magnification = (ocular magnification) x (objective magnification)

For example, if the ocular is 10X and the 40X objective lens is selected, TM is (10X)(40X) = 400X. Total magnification using each objective lens for your microscope is given in Table 4.1.

Table 4.1. Total Magnification

Objective Lens Magnification	Ocular Lens Magnification	Total Magnification
Scanning (4X)	10X	40X
Low power (10X)	10X	100X
High power (40X)	10X	400X
Oil immersion (100X)	10X	1000X

Unlike magnification, *resolution* is the ability to distinguish two objects as separate entities. The resolving power for a light microscope is about 0.2 micrometers, meaning that any two objects which are closer than two tenths of one micrometer will be seen as a single point.

The following exercise is designed to provide practice using the light microscope to view a slide with the letter “e.” Work through the steps slowly and apply the same principles when viewing stained slides in later exercises.

Exercise 4.1 – Using the Light Microscope: Viewing the Letter "e"

OBJECTIVE

Use the light microscope to practice focusing under scanning, low power, and high power.

MATERIALS

- EQUIPMENT: Light microscope, Sta-clear paper, lens paper, lens cleaner
- SLIDE: Letter "e"

PROCEDURE - *Take your time and work through steps in order.*

1. Obtain a microscope from the cabinet. Remember to carry it with two hands and reposition it on the bench by lifting rather than dragging.

2. Place the microscope directly in front of you on the bench. Sit up straight and push in your chair so that you are comfortable. Do not bend over or kneel on your chair to view slides.

   - Record the number that is found on the back of your microscope:_____

3. Verify that the student before you stored the microscope correctly, making sure:

   _____The stage is clean, has no slides, and is free from oil.

   _____The scanning (4X) objective lens is pointing down toward the stage.

   _____The stage is lowered completely.

   _____The rheostat (light intensity dial on the base) is turned down all the way.

4. Clean the oculars and objective lenses with lens paper and lens cleaner, checking that each objective lens is securely screwed into the revolving nosepiece.

   - Record the magnification printed on the oculars:_____X

5. Plug in your microscope and turn it on using the power switch on the base.

6. Raise the light intensity by turning the rheostat to a high number on the base and adjust brightness by closing the iris diaphragm rather than lowering the rheostat.

7. Move the oculars together or apart so that you can use both eyes to view the slide. Note that one ocular will have a pointer and the other will have a micrometer for measuring cells.

   - Record the interpupillary distance between the oculars:_____

8. Obtain a slide of the letter “e” from the slide tray and clean it using Sta-Clear paper and lens cleaner.

9. Place the slide on the stage with the label facing up and to the left, securing corners in the stage clips so that it lies flat and pushing the slide back as far as it will go.

    - Record the appearance of the letter as it appears looking at the stage:_____

10. Using the stage control knobs, position the slide so that the letter is over the light source.

11. Look through the oculars and keep turning the coarse focus knob until the image comes into focus. This may require significant rotation of the focus knob. If you go too far and miss the image, turn the knob slowly in the opposite direction.

    - Record the appearance of the letter as it appears through the oculars:_____
    - Record the total magnification using this objective:_____X
    - Circle the appearance of a “p” as it would be viewed through the oculars: p d b q

12. View the slide under low power by rotating the 10X objective in place and turning the fine focus knob until the image is clear. If necessary, adjust the iris diaphragm to lower the light.

13. If directed to do so, raise your hand for the instructor to verify your observation.

    - Record total magnification using this objective:_____X
    - Which property maintains focus while changing objectives?_____

14. View the slide under high power by rotating the 40X objective in place and turning the fine focus knob until the image is clear. Increase light by opening the diaphragm. If the image is blurry, use lens paper to firmly clean the bottom of the objective.

    - Record total magnification using this objective:_____X
    - What happens to the field of view as magnification increases?_____
    - Which objective lens is most appropriate for viewing the letter?_____

15. Return the slide to the corresponding numbered slot on the tray.

16. When you are done using the microscope, prepare it for storage by ensuring:

    _____The stage is clean, has no slides, and is free from oil.

    _____The scanning (4X) objective lens is pointing down toward the stage.

    _____The stage is lowered completely.

    _____The rheostat (light intensity dial on the base) is turned down all the way.

17. Show your microscope to the instructor before returning it to the cabinet.

Exercise 4.2 – Bacterial Cellular Morphology & Arrangement

LEARNING OUTCOMES

1. Use the oil immersion objective to view stained bacterial cells.
2. Identify results from simple, Gram, acid-fast, and negative stains.
3. Name the basic shapes and arrangements of bacterial cells.
4. Measure bacterial cell size with the ocular micrometer when using oil immersion.

Using the Oil Immersion Lens

As light rays move through different media (air, glass, water, etc.), the light bends, or refracts, at a particular angle known as the *refractive index*. This explains why swim goggles are needed to see clearly underwater. As light moves from air to water it refracts, and the angle changes. Wearing goggles creates an air space in front of your eyes so that the light bends again, in essence correcting itself in terms of your ability to see clearly.

At very high magnifications, such as when viewing bacteria with the 100X objective lens, resolution may be compromised when light passes through the small amount of air between the specimen and the lens. This is due to the large difference between the refractive index of air and that of glass; the air scatters the light rays before they can be focused by the lens. To solve this problem, a drop of oil can be used to fill the space between the slide and the objective, thus forming a connection between the two through which light can travel. Since oil and glass have a similar refractive index, the light is collected rather than refracted. Thus, adding immersion oil improves the resolution or clarity of the image (Figure 4.4).

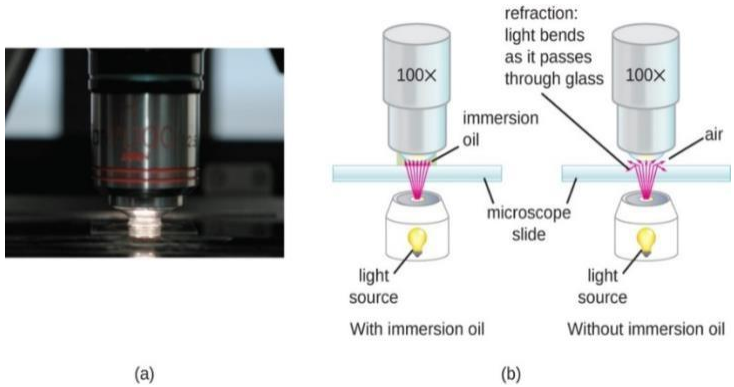

Figure 4.4: (a) Oil immersion lenses like this one are used to improve resolution. (b) Because immersion oil and glass have very similar refractive indices, there is a minimal amount of refraction before the light reaches the lens. Without immersion oil, light scatters as it passes through the air above the slide, degrading the resolution of the image.

Three basic shapes of bacterial cells are spherical *cocci* (singular, coccus), rod-shaped *bacilli* (singular, bacillus), and curved or *helical* bacilli. Cocci are generally less than one micrometer in size, but the length of bacilli can vary. Helical bacilli can be further subtyped based on the degree of cellular curvature. *Vibrio* cells are small, comma-shaped bacilli, while rigid *spirilli* have multiple curves. *Spirochetes* are highly curved flexible bacilli that have a corkscrew-like structure (Figure 4.5).

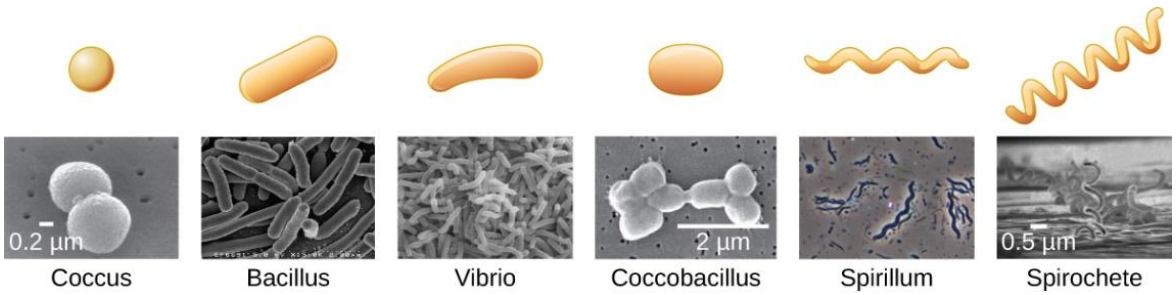

Figure 4.5: Bacterial cellular shapes.

Cellular Arrangements

Bacterial cells divide by an asexual process known as *binary fission*, where one parent cell splits to form two identical new daughter cells. Following division, daughters may separate into individual cells or remain together as a pair, chain, or cluster. The shape and arrangement of cells from stained smears helps microbiologists to preliminarily identify bacteria. In clinical settings, these results provide valuable information upon which initial treatment can be based. Common bacterial arrangements are shown in Figure 4.6.

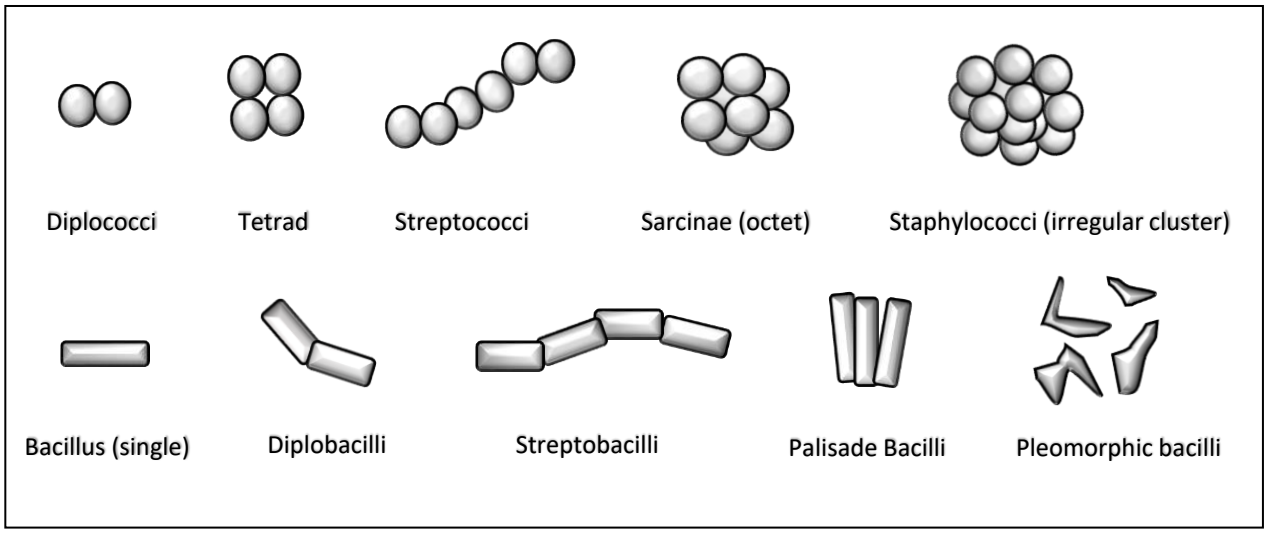

Figure 4.6: Bacterial cellular arrangements.

Exercise 4.2 – Bacterial Cellular Morphology & Arrangement: Protocol

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OBJECTIVE

Use the light microscope to observe and identify the shape and arrangement of bacterial cells.

MATERIALS

- EQUIPMENT: Light microscope, lens paper, lens cleaner, immersion oil, bacterial smears prepared in prior lab

PROCEDURE - *Take your time and work through steps in order.*

1. Place a stained slide of bacteria on the stage and secure it in the stage clips.
2. Follow steps from Exercise 4.1 to bring cells into focus under high power, raising the light.
3. Once the image is in focus under high power with high light, without adjusting the focus knobs, rotate the 40X objective to the side and place a large drop of immersion oil (several taps of the glass wand) directly on the slide.
4. Rotate the 100X objective lens without passing the 40X objective through the oil until it clicks into place. The oil should connect the bottom of the objective and the slide.
5. Focus the image by turning the fine focus (inner) knob **only**; using coarse focus under higher magnifications may crack the lens. Raise the rheostat light control on the base and fully open the diaphragm. You should observe pigmented cells against a white background.

   If you have trouble:

   - Make certain that the objective lens is clicked in place.
   - Check that the slide is flat on the stage and not over/under the clips.
   - Add additional oil.
   - Increase the amount of light.
   - Return to 10X and try again (no need to remove oil from the slide).

6. Once cells are in focus, use the slide adjustment knobs to observe an area near the edge of the smear that is less dense to determine the shape and arrangement of cells.

7. Cell size: Use the ocular micrometer (in one of the eyepieces) to measure cell length. Rotate the ocular to position the micrometer over a single cell. When using the oil immersion objective, each division of the micrometer is equivalent to approximately one micrometer.

8. Complete the Module 4 report.

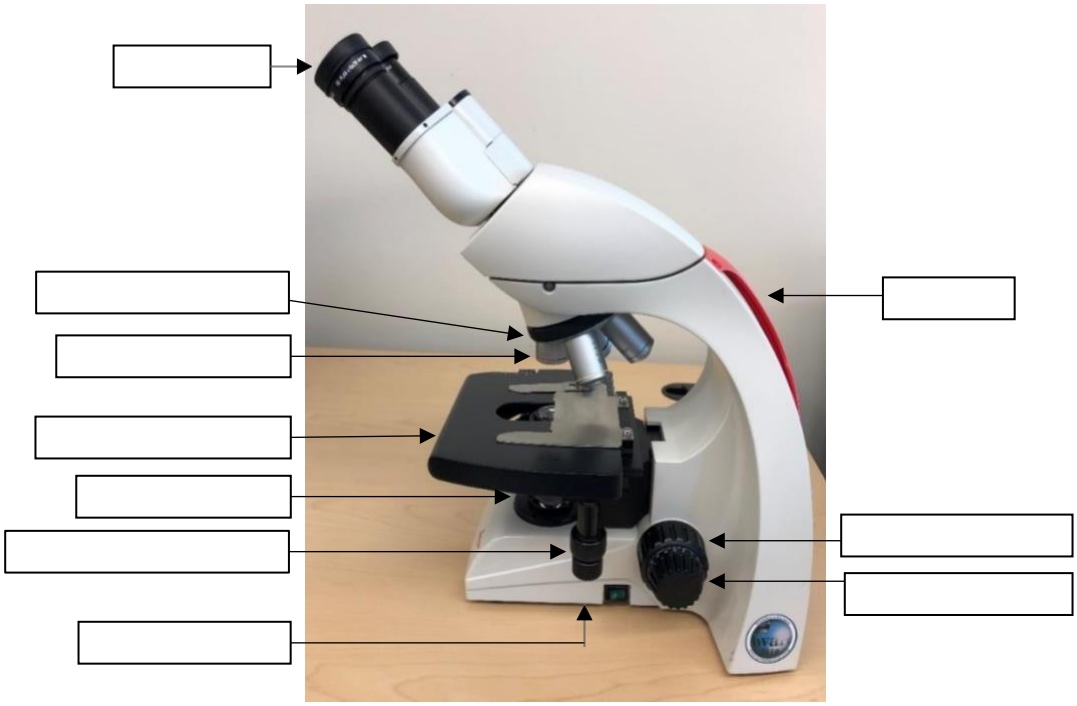
9. When you are finished, dispose of all smears directly in the disinfectant beaker.

BIO 211 MODULE 4 REPORT **NAME:**_____

MICROSCOPY **REPORT DATE:**_____ **MICROSCOPE #:**_____

EXERCISE 4.1 – USING THE LIGHT MICROSCOPE

Label the parts of the microscope in the image below:



Complete the table:

Objective Lens	Magnification of Objective Lens	Magnification of Ocular Lens	Total Magnification
Scanning			
Low power			
High power			
Oil immersion			

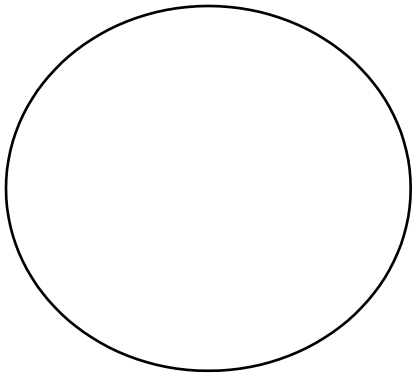
Name five important things that you should do to properly store your microscope:

1. _____
2. _____
3. _____
4. _____
5. _____

BIO 211 MODULE 4 REPORT **NAME:**_____

EXERCISE 4.2 – BACTERIAL CELL MORPHOLOGY

View all cells using oil and 1000X total magnification. Draw several representative cells with colored pencils to depict bacterial shape and arrangement.



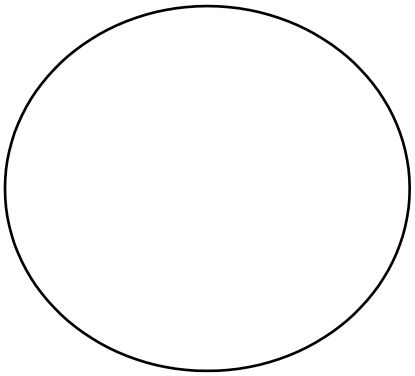
SIMPLE STAIN 1

Organism:_____

Cell shape:_____ Size:_____μm

Arrangement:_____

Endospores (circle): Present / Absent



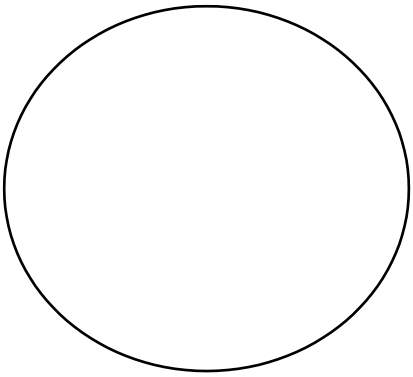
SIMPLE STAIN 2

Organism:_____

Cell shape:_____ Size:_____μm

Arrangement:_____

Endospores (circle): Present / Absent



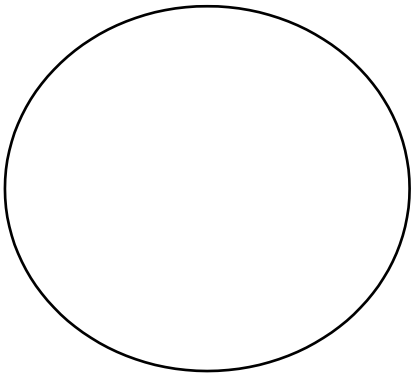
GRAM STAIN 1

Organism:_____

Cell shape:_____ Size:_____μm

Arrangement:_____

Gram reaction (circle): Positive / Negative



GRAM STAIN 2

Organism:_____

Cell shape:_____ Size:_____μm

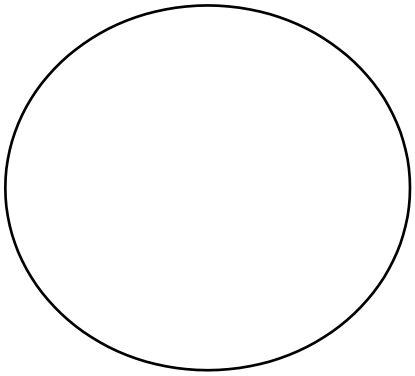
Arrangement:_____

Gram reaction (circle): Positive / Negative

BIO 211 MODULE 4 REPORT **NAME:**_____

EXERCISE 4.2 (CONT'D) – BACTERIAL CELL MORPHOLOGY

Your instructor may ask you to raise your hand when you have an image in focus to verify that you are viewing the image accurately. Dispose of used slides in the disinfectant beaker.

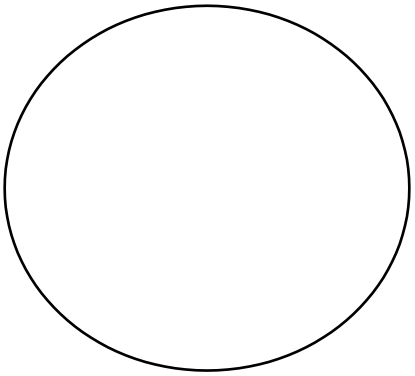


UNKNOWN SAMPLE

Gram reaction (circle): Positive / Negative

Cell shape:_____ Size:_____μm

Arrangement:_____

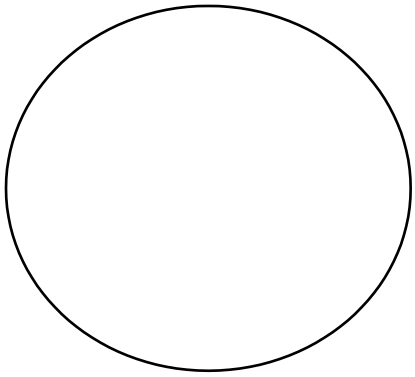


NEGATIVE STAIN

Organism:_____

Cell shape:_____ Size:_____μm

Arrangement:_____



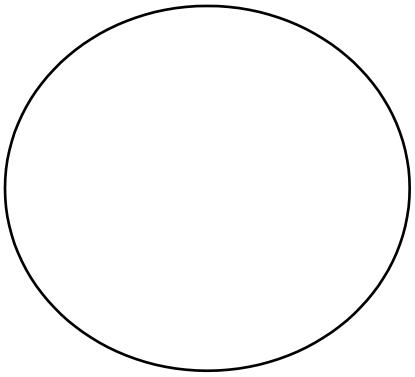
ACID FAST STAIN 1

Organism:_____

Cell shape:_____ Size:_____μm

Arrangement:_____

Acid-fast result (circle): Positive / Negative



ACID FAST STAIN 2

Organism:_____

Cell shape:_____ Size:_____μm

Arrangement:_____

Acid-fast result (circle): Positive / Negative

QUESTIONS FOR REVIEW

1. If a Gram-stained slide of *Staphylococcus* was over-decolorized, what color might cells appear when the slide was viewed microscopically?_____

2. Based on the Gram stain, could the unknown organism be *Escherichia coli*?_____
   Why or why not?_____

3. If safranin and crystal violet were switched for a Gram stain on a smear of Gram-negative bacteria, what color would cells appear when viewed microscopically?_____

4. Two students used the same culture of *Mycobacterium* to prepare and stain slides by the Kinyoun method. When viewing their slides microscopically, one student observed cells, but the other student saw nothing at all on the slide. What was this student's most likely error?

5. Drainage from a post-surgical wound is sent to the micro lab for analysis. The physician suspects MRSA (methicillin-resistant *Staphylococcus aureus*), a prevalent healthcare-associated pathogen, is the cause of the infection. The initial lab report indicates that "heavy Gram-negative bacilli" are observed on the initial smear.
   Based on these results, is the physician correct?_____Explain._____

6. Using your knowledge from this lab, complete table regarding color of cells for each stain.

GENUS	GRAM STAIN	ACID-FAST STAIN	NEGATIVE STAIN
Staphylococcus		Blue	
Bacillus	Purple		
Escherichia			Colorless
Mycobacterium	Purple (weak)		