Chapter 3: Observing Microorganisms Through a Microscope

Microscopy: The technology of making very small things visible to the naked eye.

Units of Measurement: The **metric system** is used to measure microorganisms.

Metric system:

- Basic unit of length: **Meter**.
- All units are related to each other by factors of 10.
- Prefixes are used to indicate the relationship of a unit to the basic unit (e.g.: meter).

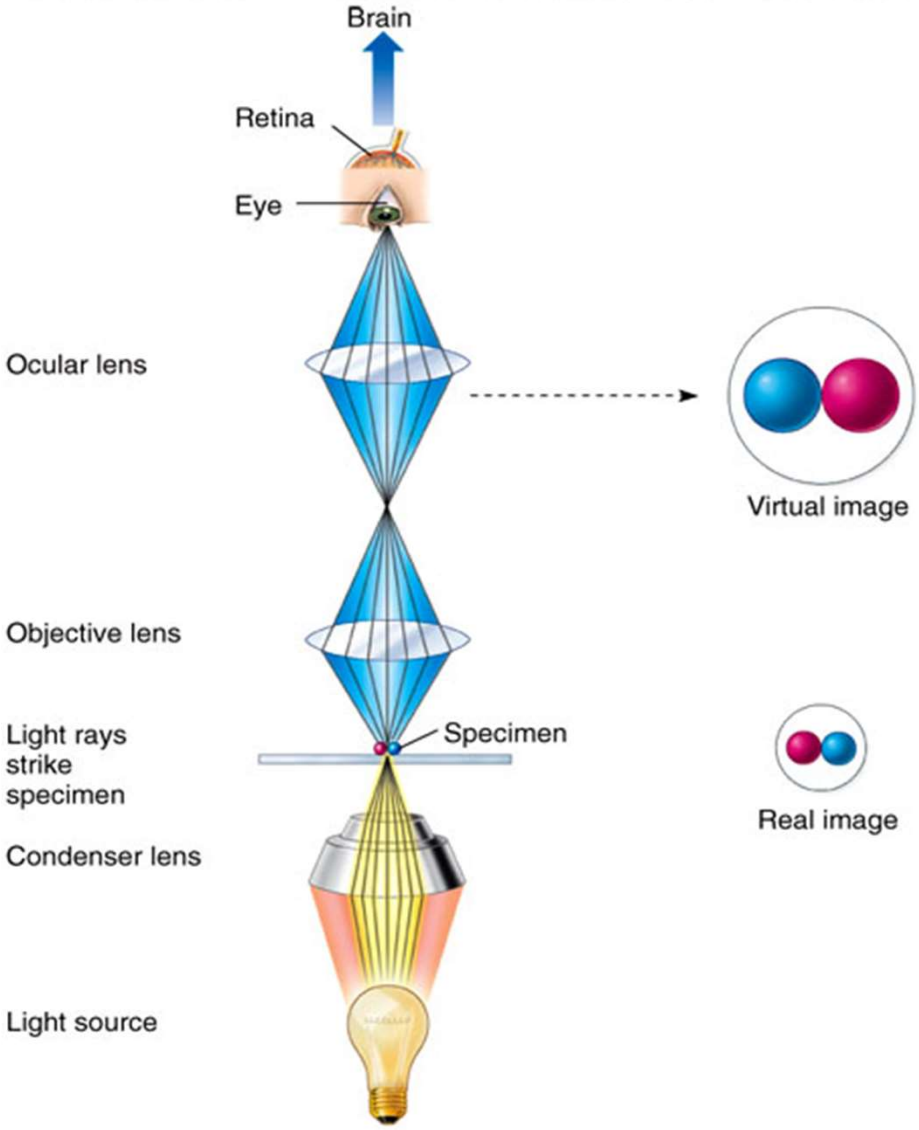
Metric Units of Length and U.S. Equivalents:

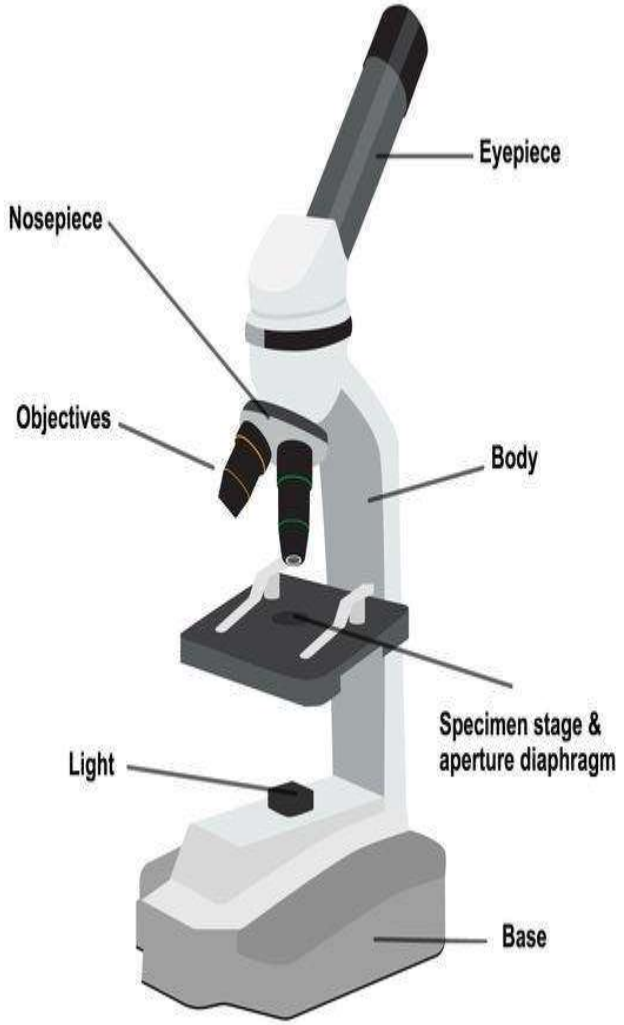
Metric Unit	Relationship to basic unit (meter)	U.S. Equivalent
kilometer (km)	1 km = 1000 m	1 mile = 1.61 km
meter (m)		**1 m = 39.37 in**
decimeter (dm)	1 dm = 0.1 m = 10^{-1}m	1 dm = 3.94 in
centimeter (cm)	1 cm = 0.01 m = 10^{-2}m	2.54 cm = 1 in
millimeter (mm)	1 mm = 0.001 m = 10^{-3}m	
micrometer (um)	1 um = 0.000001 m = 10^{-6}m	
nanometer (nm)	1 nm = 0.000000001 m = 10^{-9}m	
picometer (pm)	1 pm = 0.000000000001 m = 10^{-12}m	

Instruments of Microscopy:

1. Simple Microscopes:

- Only have one lens, similar to a magnifying glass.
- Leeuwenhoeck's simple microscopes allowed him to magnify images from 100 to 300 X.
  - They were so difficult to focus, he built a new one for each specimen, a total of 419.
  - He did not share his techniques with other scientists. Even today, his source of lighting is unknown.
  - His daughter donated 100 of his microscopes to the Royal Society shortly before his death in 1723.





Instruments of Microscopy:

2. Compound Light (CL) Microscopy

History of CL Microscopes:

- First developed by Zaccharias Janssen, Dutch spectacle maker in 1600.
  - Poor quality
  - Could not see bacteria
- Joseph Jackson Lister (Lister's father) developed improved compound light microscope in 1830s.
  - Basis for modern microscopes
- Use **visible light** as a source of illumination.

Instruments of Microscopy:

2. Compound Light Microscopy

- Have **several lenses**:

1. Light originates from an illuminator and passes through **condenser lenses**, which direct light onto the specimen.

2. Light then enters the **objective lenses**, which magnify the image. These are the closest lenses to the specimen:

- **Scanning** objective lens: 4 X
- **Low power** objective lens: 10 X
- **High power** objective lens: 40-45 X
- **Oil immersion** lens: 95-100 X

3. The image of the specimen is magnified once again by the **ocular lens** or **eyepiece** (10 X).

Instruments of Microscopy:

2. Compound Light Microscopy

- **Total magnification**: Obtained by multiplying **objective** lens power by **ocular** lens power. (Condenser lenses do not magnify image).

Lens	Magnification	Ocular Mag.	Total Mag.
Scanning	4 X	10 X	= **40 X**
Low power	10 X	10 X	= **100 X**
High power	45 X	10 X	= **450 X**
Oil immersion	100X	10 X	= **1000 X**

Highest possible magnification with CL microscope is about 2000 X.

Instruments of Microscopy:

2. Compound Light Microscopy

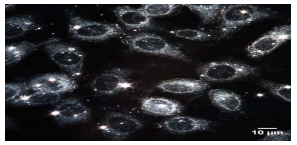
- **Resolution (Resolving power):** Ability of microscope to see two items as separate and discrete units.
  - The smaller the **distance** between objects at which they can be distinguished as separate, the greater the resolving power.
  - Light must pass between two objects in order for them to be seen as separate.
  - Depends on light **wavelength**. If wavelength is too long to pass between objects, they will appear as one.
    - **White light** has a relatively long wavelength (550 nm), and cannot resolve structures less than **220 nm (0.2 um)** apart.
    - **Ultraviolet (UV) light** has a shorter wavelength (100 to 400 nm), and can resolve distances as small as 110 nm.

Instruments of Microscopy:

2. Compound Light Microscopy

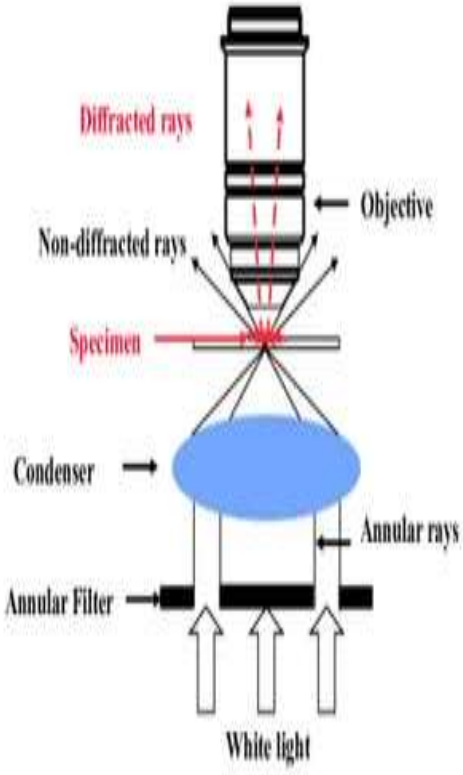
- **Refraction:** Bending of light as it passes from one medium to another of different density.
  - **Index of refraction:** A measure of the speed at which light passes through a material.
    - Can be changed by **staining**, which increases **contrast** between specimen and surrounding medium.
  - When two substances have a different index of refraction, the light will **bend** as it passes from one material to another.
  - As light passes through a glass slide, air, and the objective lens, it bends each time, causing loss of light and a blurred image.
  - **Immersion oil** has the same index of refraction as glass slide, preventing light loss from refraction.

Instruments of Microscopy:



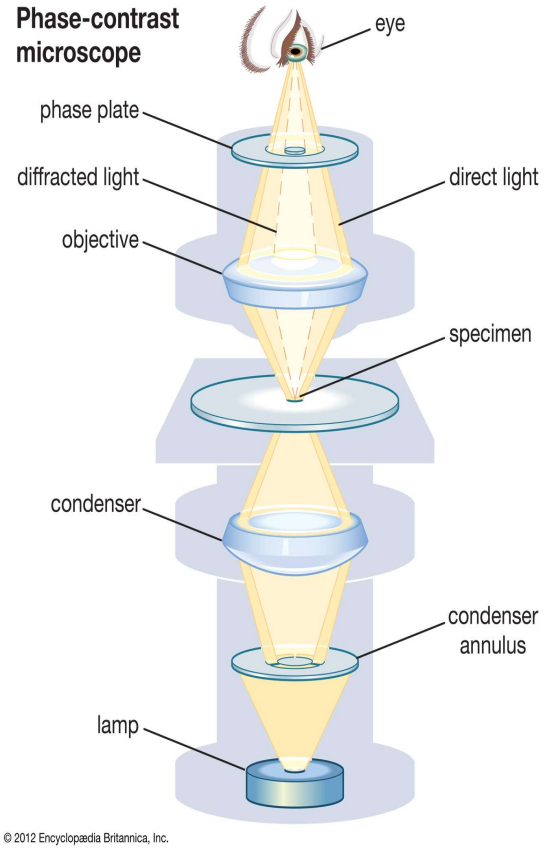
3. Darkfield Microscopy

- Useful to examine **live** or **unstained** specimens.
  - Light sensitive organisms
  - Specimens that lack contrast with their background.
    - Spirochetes which cause syphilis.
- Darkfield condenser with **opaque disc** blocks light that would enter objective lens directly:
  - Light reflects off specimen at an angle.
  - Only light reflected by specimen enters objective lens.
- No direct background light.
- **Image**: **Light specimen** against **dark background**.



4. Phase Contrast Microscopy

- Useful to examine **live** specimens:
  - **Doesn't require fixing** or **staining,** which usually kill and/or distort microorganisms.
- Permits **detailed** examination of **internal structures**.
- Special objective lenses and condenser with ring shaped diaphragm accentuate small differences in refractive indexes of internal structures.
- **Image:** Direct rays and reflected light rays come together, forming an image with many shades of gray to black.



Instruments of Microscopy:

5. Fluorescence Microscopy

- **Fluorescence:** Ability of substances to absorb short wavelengths of light (ultraviolet light) and emit them at a longer wavelength.
  - **Natural Fluorescence**: Some microorganisms fluoresce naturally under UV light (*Pseudomonas*).
  - **Fluorochrome**: Fluorescent dye.
    - Acridine orange: Binds to nucleic acids, colors cells orange, green, or yellow depending on light source.
  - **Immunofluorescence**: Fluorescent antibodies can be used to detect specific antigens. Very useful for the rapid diagnosis of specific diseases (e.g.: syphilis).
- **Image:** Luminescent bright object against a dark background.

Instruments of Microscopy:

Limitations of light microscopy:

- **Magnification**: Up to 2000 X.
- **Resolving Power**: Up to 0.2 um.

Because of the limits of magnification and resolving power**, viruses and most internal structures of cells cannot be seen with a light microscope**.

Instruments of Microscopy:

6. Electron Microscopy

- Electron microscopes were first developed in 1932, and became widely available in 1940s.
- Use a **beam of electrons** instead of a beam of light.
  - Wavelength of electron beam is about 100,000 times **smaller** than visible light.
  - Used to examine structures too small to be resolved with a light microscope.
- Two types of electron microscope:

A. Transmission Electron Microscope (TEM)

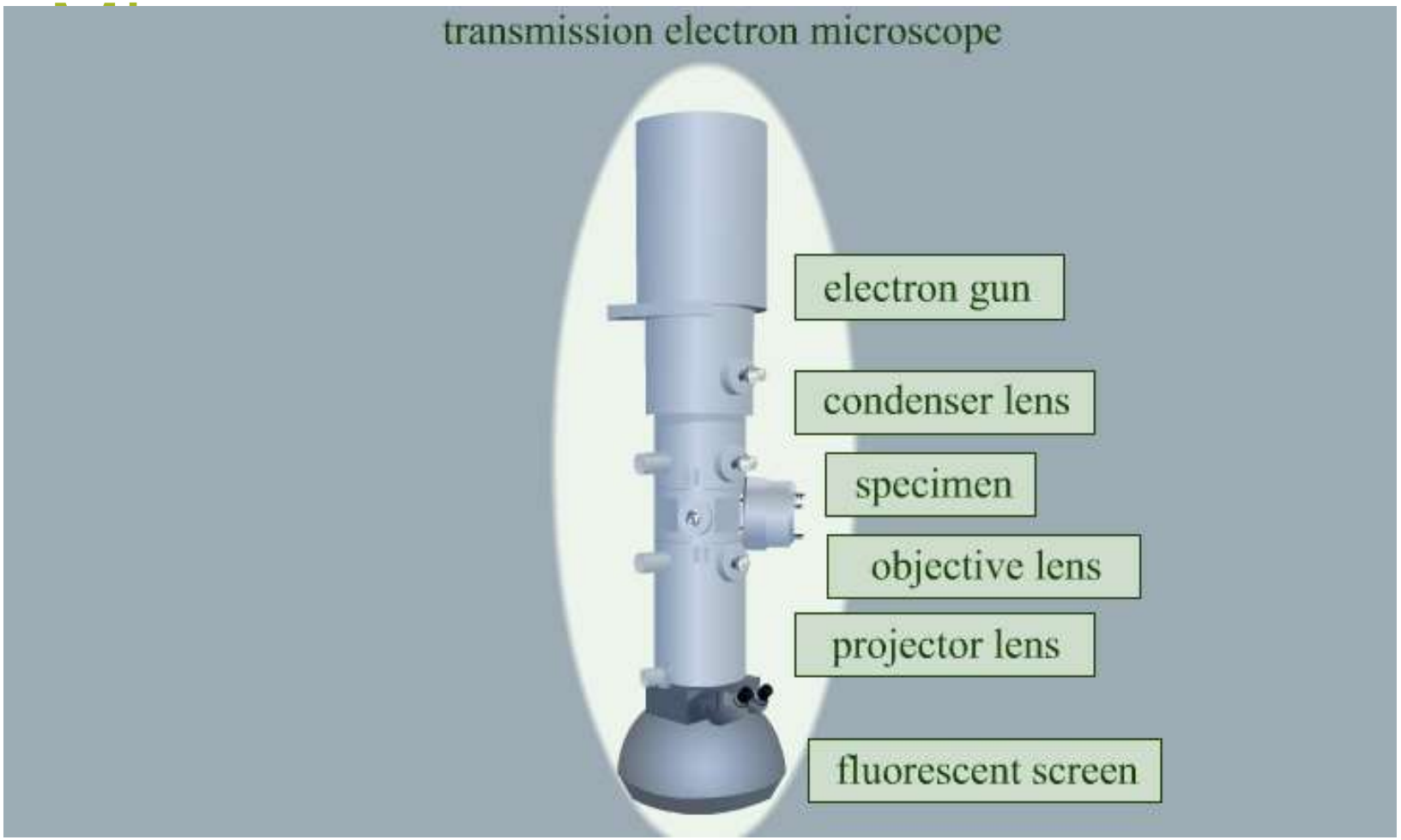
B. Scanning Electron Microscope (SEM)

Instruments of Microscopy:

6. Electron Microscopy

A. Transmission Electron Microscope (TEM)

- Gives excellent view of **internal structures.**
  - **Magnification:** 100,000 X or more.
  - **Resolving power:** 2.5 nm or better.
  - **Two dimensional** image.
- **Drawbacks of TEM:**
  - Due to limited penetrating power of electrons, can only view very thin slices (70-90 nm) of specimen.
  - Must slice, fix, dehydrate, and view specimen under a vacuum. Staining may be used to enhance image contrast.
  - Treatments kill specimen and may cause shrinkage and distortion of cells (**artifacts**).



PLAY Animation: Electron Microscopy

A transmission electron microscope (TEM)

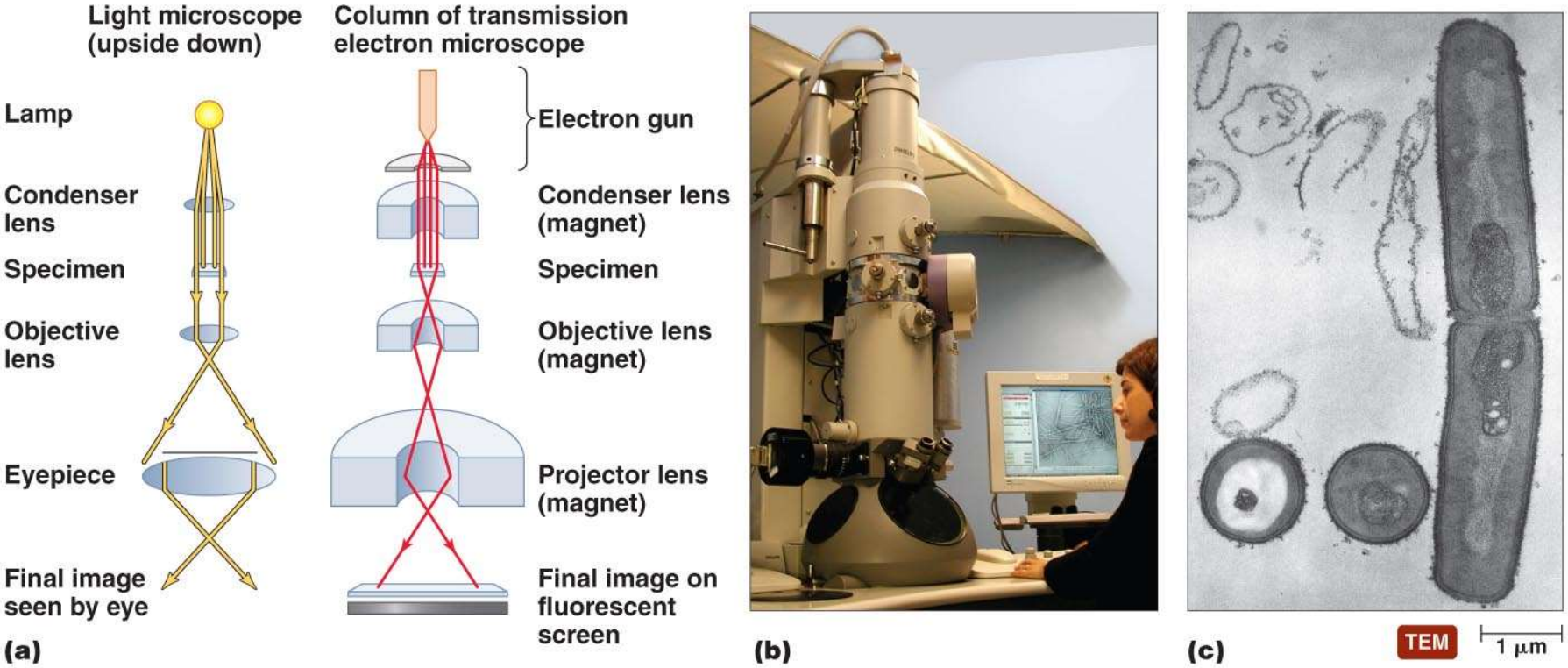


Figure 4.11

Instruments of Microscopy:

6. Electron Microscopy

B. Scanning Electron Microscope (SEM)

- Gives excellent view of **external surface.**
  - **Magnification:** 10,000 X or more.
  - **Resolving power:** 20 nm or better.
  - **Three dimensional** image.
- More recent invention than TEM. Used mainly to observe the **surfaces of cells and viruses**.
  - Specimens are covered with a layer of heavy metal (gold or palladium).
  - A narrow beam of electrons (**primary electron beam**) is swept across specimen surface.
  - Electrons on the specimen surface are knocked out, creating a **secondary electron beam** which is collected and amplified to produce an image.

7. Scanning Tunneling Microscopy and Atomic Force Microscopy (AFM)

- Developed in the 1980s.
- Used to observe structure and surface of biological molecules and silicon computer chips.

A. Scanning Tunneling Microscope (STM)

Uses a thin **metal probe** that scans the **surface** of a specimen.

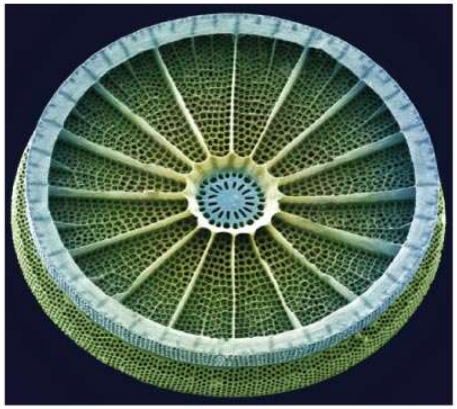
B. Atomic Force Microscopy (AFM)

Uses a **diamond and metal probe** that scans surface of specimen.

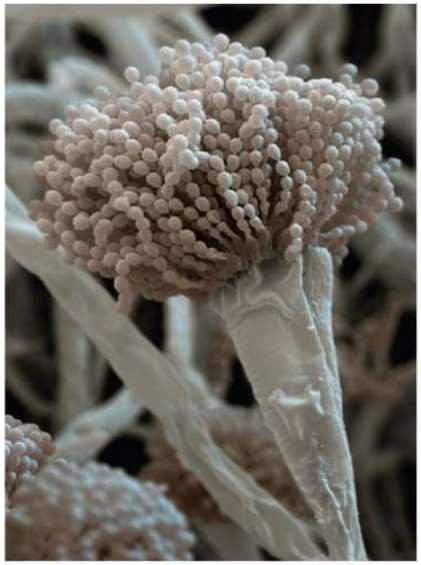
Advantages of both microscopes:

- **Higher resolving power** than electron microscopes
- **No special specimen preparation required**

SEM images

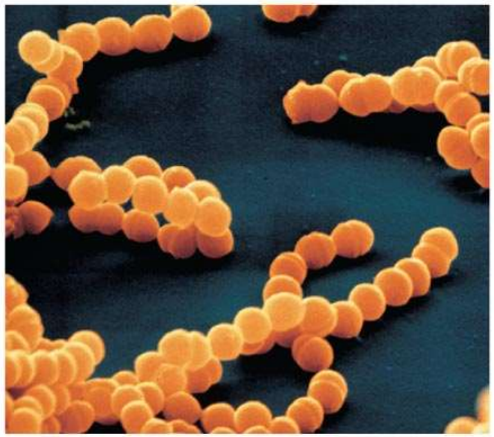


(a) *Arachnoidiscus* SEM 15 μm



(b) *Aspergillus* SEM 50 μm

(c) *Paramecium* SEM 15 μm



(d) *Streptococcus* SEM 2 μm

Figure 4.13

Probe microscopy

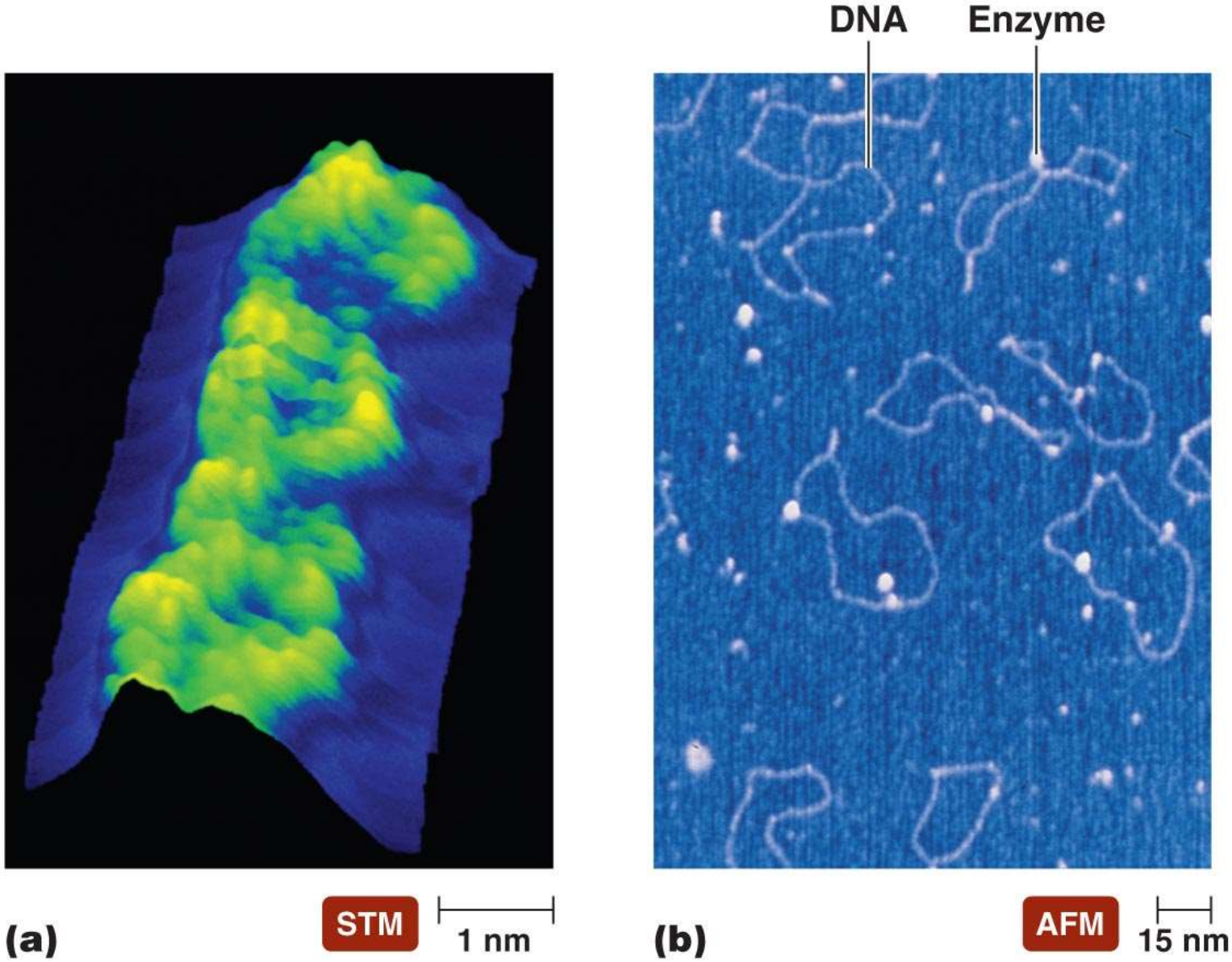


Figure 4.14

Preparation of Specimens for Light Microscopy

1. **Smear:** Spread a thin film of material containing microorganisms over slide surface. Allow to air dry.
2. **Fixing:** Process that kills microorganisms and attaches them to a microscope slide. Fixing preserves and minimizes distortion of cells.

Two main methods of fixation:

- **Heat fixation**: Pass over Bunsen burner flame several times.
- **Chemical fixation**: Cover with methanol for 1 minute.

Preparation of Specimens for Light Microscopy

3. **Staining:** Coloring microorganisms with a dye that emphasizes certain structures. Before staining a sample, it must be **fixed**.

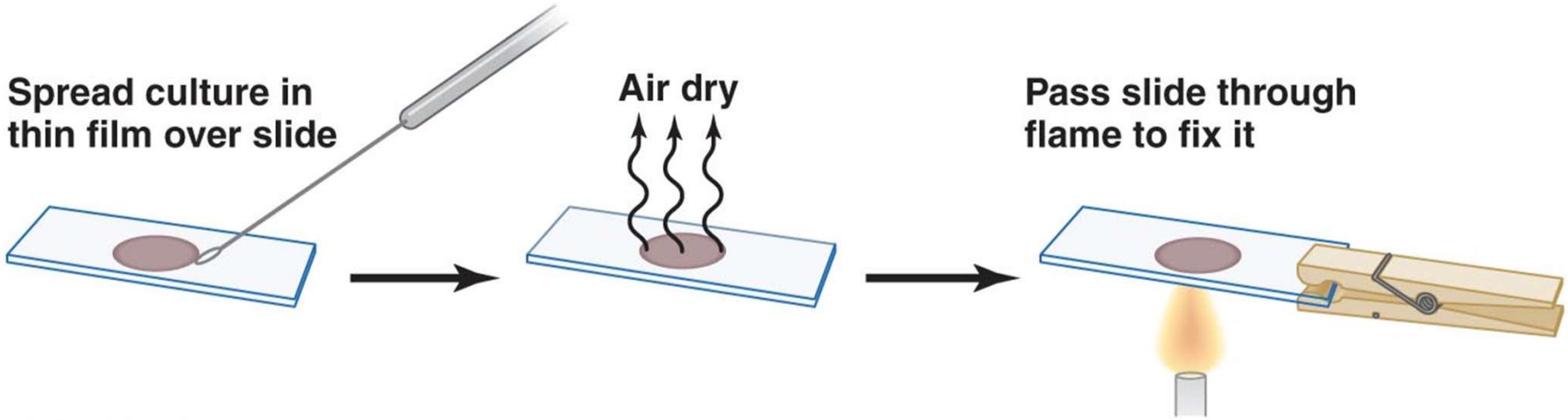
Stains are **salts** composed of a positive ion (cation) and a negative ion (anion).

The colored ion is called the **chromophore**.

Two types of dyes:

A. Basic dyes

B. Acidic dyes



Preparation of Specimens for Light Microscopy

A. Basic dyes:

- Chromophor is in **positive** ions.
- Most commonly used dyes.
- Bacteria are slightly negatively charged at pH 7, therefore they stain with basic dyes.
- **Examples**:
  - Crystal violet
  - Methylene blue
  - Saffranin

Preparation of Specimens for Light Microscopy

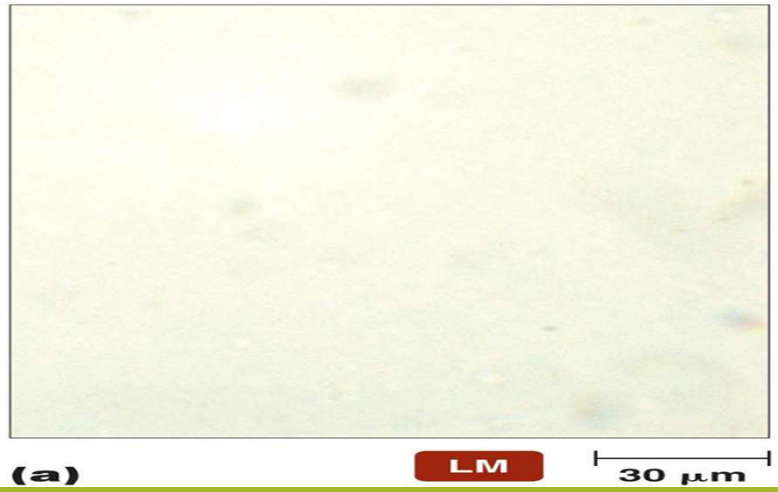
B. **Acidic dyes**:

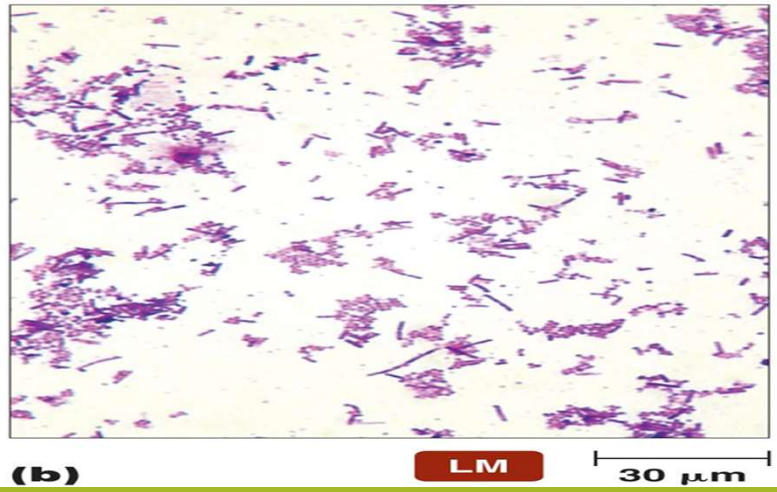
- Color is in **negative** ions.
- Stain the background: **negative staining**.
- Bacteria do not stain with acidic dyes.
- Used to observe cell shape, size, and capsules.
- Minimal distortion because heat fixing is not necessary an dye is not taken up by cells.
- Examples:
  - Eosin
  - Nigrosin
  - India ink.

Preparation of Specimens for Microscopy

1. Simple Stains

- Aqueous or alcohol solution of a single basic dye.
- Primary purpose is to stain entire microorganism to view cell shape and basic structures.
- Procedure:
  - Stain is applied for a certain time, and then washed off.
  - Slide is dried and examined.
  - **Mordant:** May be used to increase stain intensity. Increases affinity of stain for specimen.
- Examples: Safranin, methylene blue, crystal violet, and carbolfuchsin.





Preparation of Specimens for Microscopy

2. Differential Stains

- React differently to different types of bacteria.
- Can be used to distinguish among different groups of bacteria.
- There are two important differential stains used in microbiology:

A. Gram stain

B. Acid-Fast stain

Preparation of Specimens for Microscopy

2. Differential Stains

A. Gram Stain

- Developed in 1884 by Hans Gram, a Danish microbiologist.
- The most useful staining procedure in medical microbiology.
- Distinguishes bacteria of two large and medically important groups:
  - **Gram-positive bacteria**
  - **Gram-negative bacteria**
- Provides useful information for disease treatment.

Preparation of Specimens for Microscopy

2. Differential Stains

Steps of Gram Stain

1. Primary stain: Cover a heat fixed smear with a basic dye (crystal violet).
   - **All cells,** gram-positive and gram-negative, are **stained with crystal violet** (appear **purple**).
2. Mordant: After smear is rinsed with water, an **iodine mordant** solution is applied.
   - Crystal violet-iodine [CV-I] complex forms

2. Differential Stains

Steps of Gram Stain

3. Decolorizing: Slide is washed with alcohol, which will remove stain from Gram-negative cells but not from Gram-positive cells.
   - **Gram-negative** cells will be **decolorized**.
   - **Gram-positive** cells will remain **purple**.
4. Counterstain: Alcohol is rinsed off. Safranin is applied, which will stain cells that were decolorized.
   - **Gram-negative** cells are stained **pink**.
   - **Gram-positive** cells remain **purple**.

2. Differential Stain

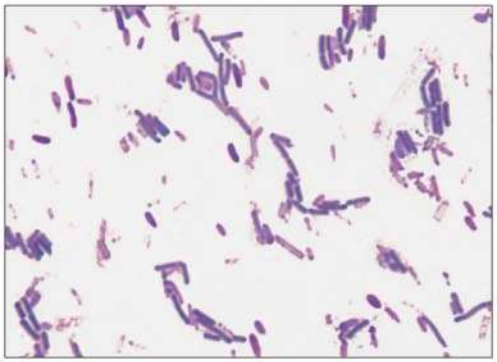
What accounts for the differential staining between Gram-positive and Gram-negative cells?

- Gram-positive cells have very thick peptidoglycan cell walls, whereas gram-negative cells have very thin cell walls. Crystal violet easily penetrates both cell types.
- Because of its larger size, the crystal violet-iodine complex [CV-I] is not easily removed from gram-positive cells, due to their thick cell wall. The CV-I complex is readily washed out of gram-negative cells with alcohol.
- Counterstain only colors gram-negative cells.

The Gram staining procedure

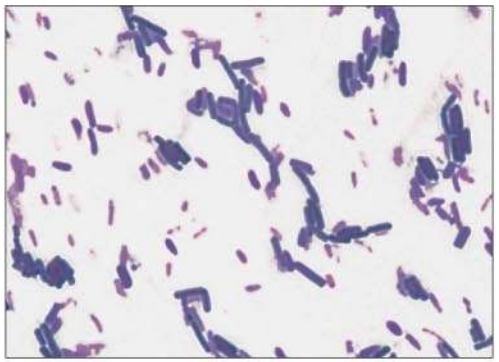
1 Slide is flooded with crystal violet for 1 min, then rinsed with water.

Result: All cells are stained purple.



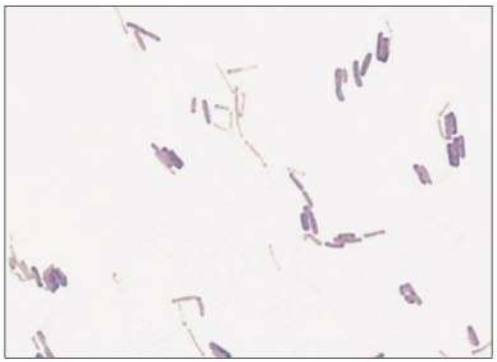
2 Slide is flooded with iodine for 1 min, then rinsed with water.

Result: Iodine acts as a mordant; all cells remain purple.



3 Slide is flooded with solution of ethanol and acetone for 10–30 sec, then rinsed with water.

Result: Smear is decolorized; Gram-positive cells remain purple, but Gram-negative cells are now colorless.



4 Slide is flooded with safranin for 1 min, then rinsed with water and blotted dry.

Result: Gram-positive cells remain purple, Gram-negative cells are pink.

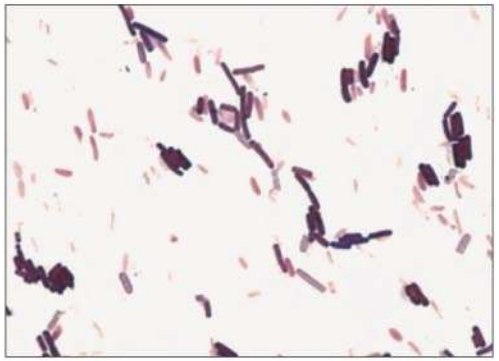
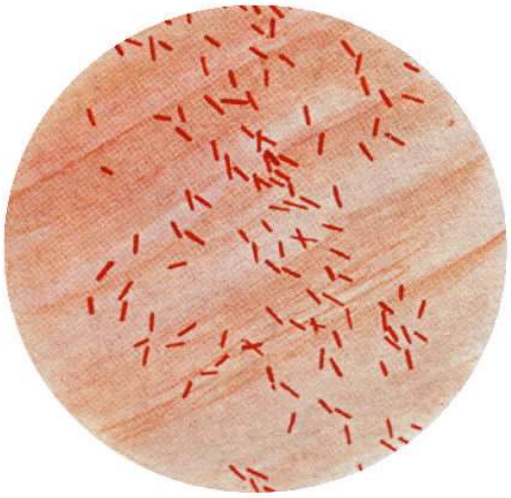
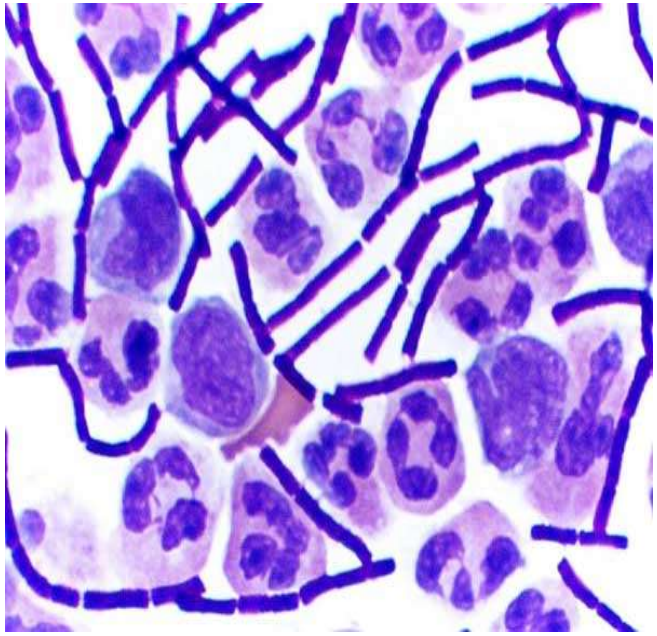


Figure 4.17

Microbiology Chapter 3, part 2

- Grams





2. Differential Stain

Applications and Limitations of the Gram stain

Chemotherapy:

- Gram-positive cells with their very thick peptidoglycan cell walls, are susceptible to penicillins and cephalosporins.
- Gram-negative cells with their thin cell walls and lipopolysaccharide layer are resistant to these antibiotics.

- **Limitations**:
  - Not all bacterial cells stain well with the Gram-stain.
  - Gram-stain only works well on young bacterial cultures, that are actively growing. Therefore it is best to use cultures that are 18 to 24 hours old.
  - Older cultures (over 24-48 hours), are often gram-variable.

Preparation of Specimens for Microscopy

2. Differential Stains

B. Acid-Fast Stain (Ziehl-Nielsen Stain)

- Modification of a method developed in 1882 by Paul Ehrlich.
- Used to detect tuberculosis and leprosy causing organisms of the genus *Mycobacterium* and pathogens of the genus *Nocardia*.
- These bacteria have waxy cell walls, which makes them difficult to stain.

Preparation of Specimens for Microscopy

2. Differential Stains

Steps of Acid-Fast Stain

1. **Primary stain**:

- Cover a heat fixed smear with carbolfuchsin, a red basic dye.
- Gently heat for several minutes to increase penetration and retention of dye.
- Allow to cool and rinse with water.

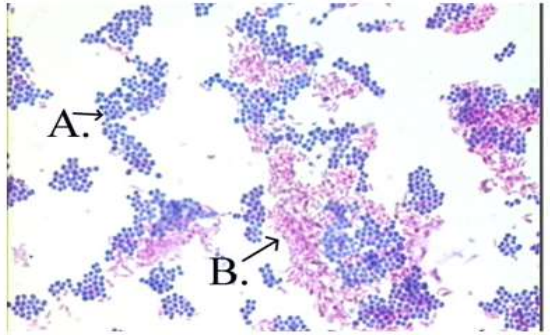
Preparation of Specimens for Microscopy

2. Differential Stains

Steps of Acid-Fast Stain

2. **Decolorizing:** Slide is washed with **acid-alcohol**.
   - **Non acid-fast** cells will be **decolorized**.
   - **Acid-fast** cells will remain **red**.
3. **Counterstain**: Acid-alcohol is rinsed off. Methylene blue is applied, which will stain cells that were decolorized.
   - **Non acid-fast** cells are stained **blue**.
   - **Acid-fast** cells remain **red**.

- Acid fast (for tb)



Preparation of Specimens for Microscopy

3. Special Stains

Used to color and isolate specific parts of microorganisms such as:

- Endospores
- Capsules
- Flagella

Preparation of Specimens for Microscopy

3. Special Stains

A. Endospore Stain

- Endospores are extremely resistant, dormant structures that are formed by some gram-positive bacteria to protect them from harsh environmental conditions: heat, drought, chemicals, radiation, etc.
- Ordinary staining methods cannot penetrate the thick endospore wall.
- Most commonly used method is **Schaeffer-Fulton** endospore stain.

Preparation of Specimens for Microscopy

3. Special Stains

A. Endospore Stain

Steps for Schaeffer-Fulton Endospore Stain

1. **Primary stain:** Malachite green is applied to heat fixed smear and steamed for about 5 minutes.
   - Malachite green will penetrate endospore.
2. **Wash**: Rinse with water for 30 seconds.
   - Removes green dye from rest of the cell, except for endospore
3. **Counterstain**: Safranin will stain rest of the cell.

Appearance of cell with endospore:

Pink cell with green endospore.

Schaeffer-Fulton endospore stain

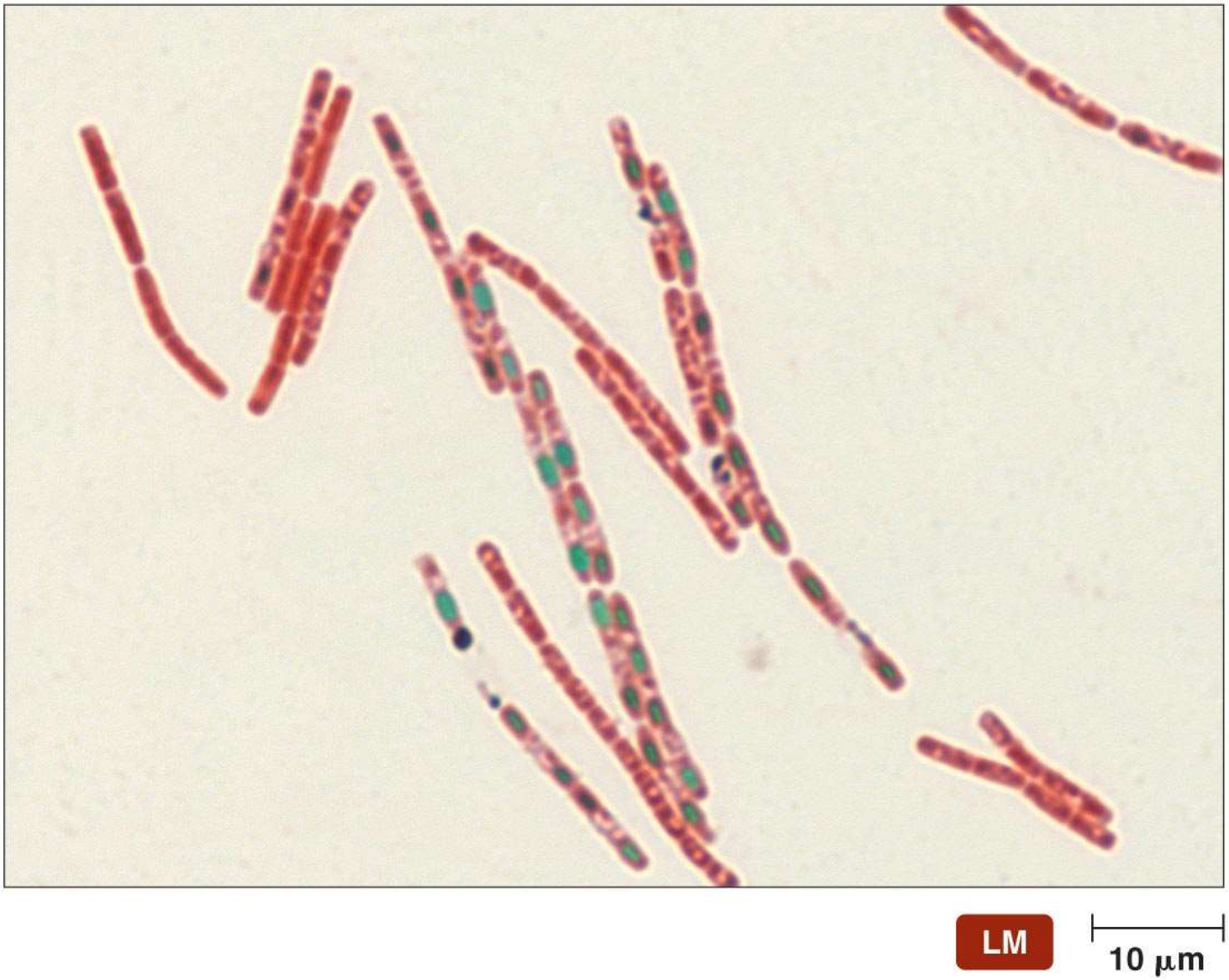
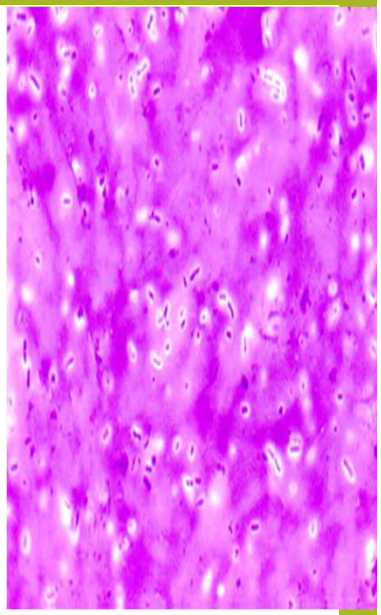


Figure 4.19

Preparation of Specimens for Microscopy

3. Special Stains

B. Capsule Stain



- Capsules are gelatinous covers on top of the cell wall, which are important virulence (disease) factors.
- **Capsules are difficult to stain** because they repel most stains, are water soluble, and are easily disrupted with harsh treatment.
- Negative stain is used to obtain a dark background (E.g.: India ink or nigrosin).
- Cell is stained with a basic dye (E.g.: safranin).

Capsule appearance: Light halo around stained cell, dark background.

Negative (capsule) stain

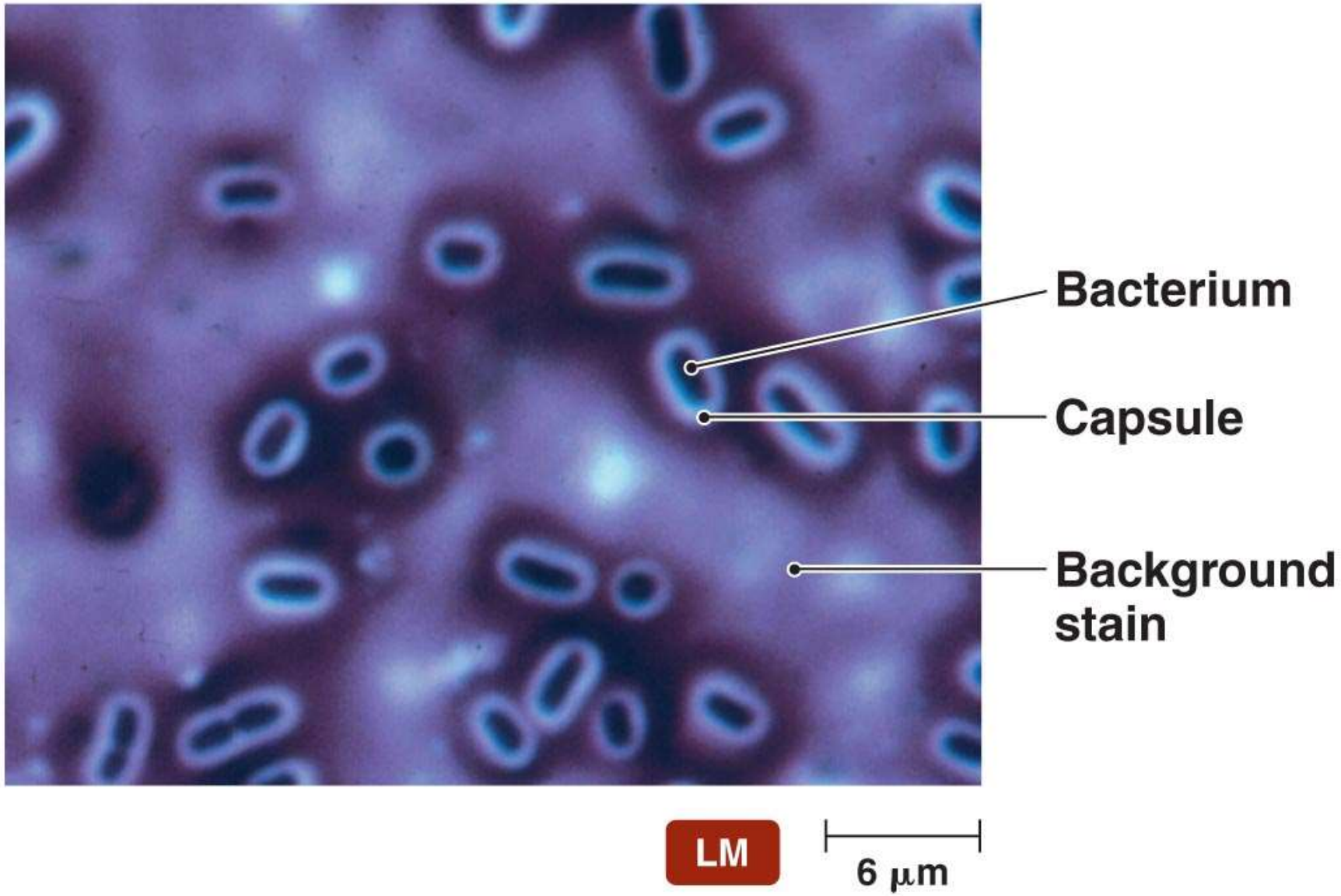


Figure 4.20

Preparation of Specimens for Microscopy

3. Special Stains

C. Flagella Stain

- Flagella are appendages used for locomotion that are too thin to be seen easily with a light microscope.
- Staining procedures are difficult. Usually involve using a mordant and coating the flagellar surface with a dye or metal (e.g.: silver).
- The number and arrangement of flagella can be used as diagnostic aids.

Flagellar stain

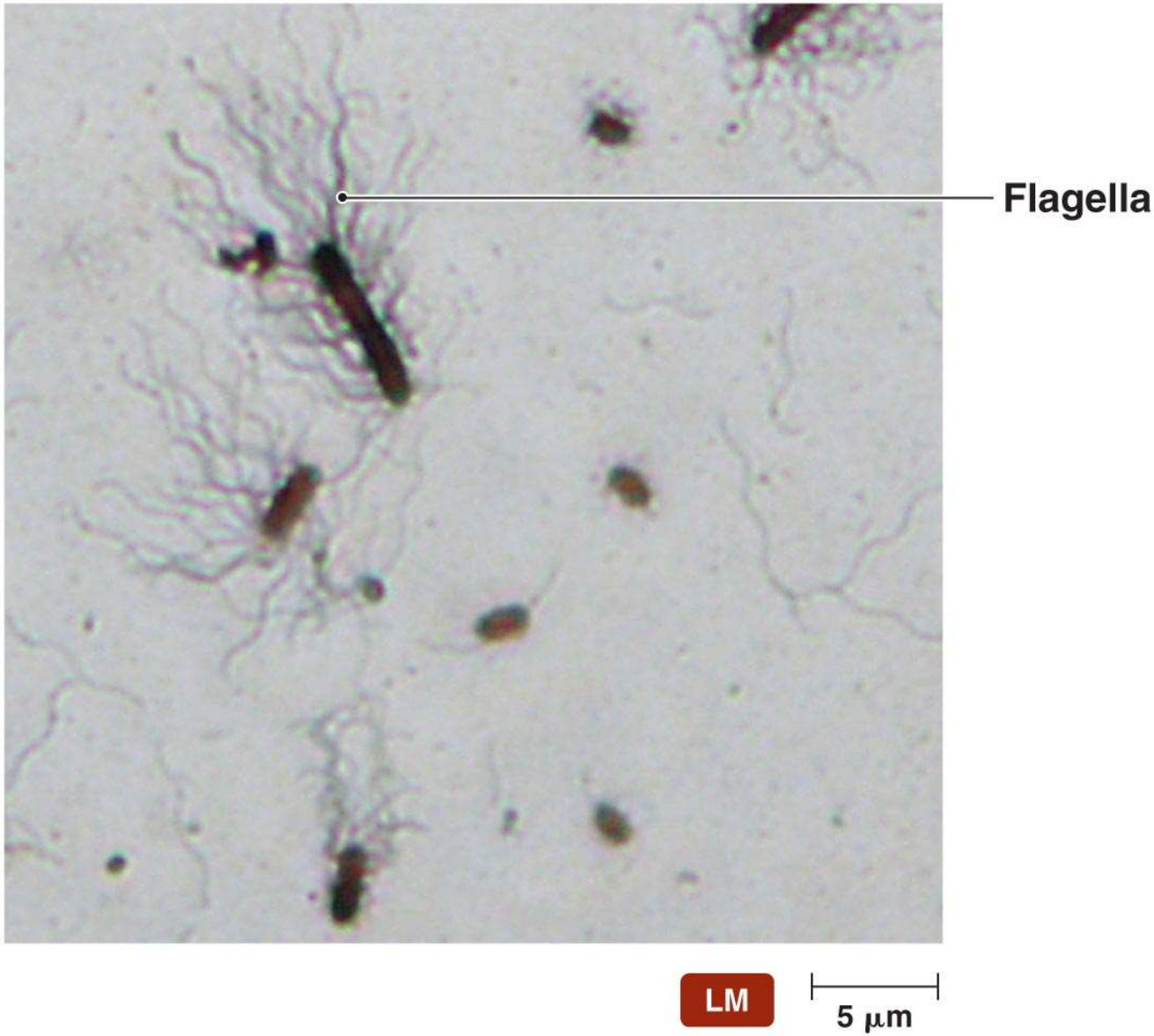
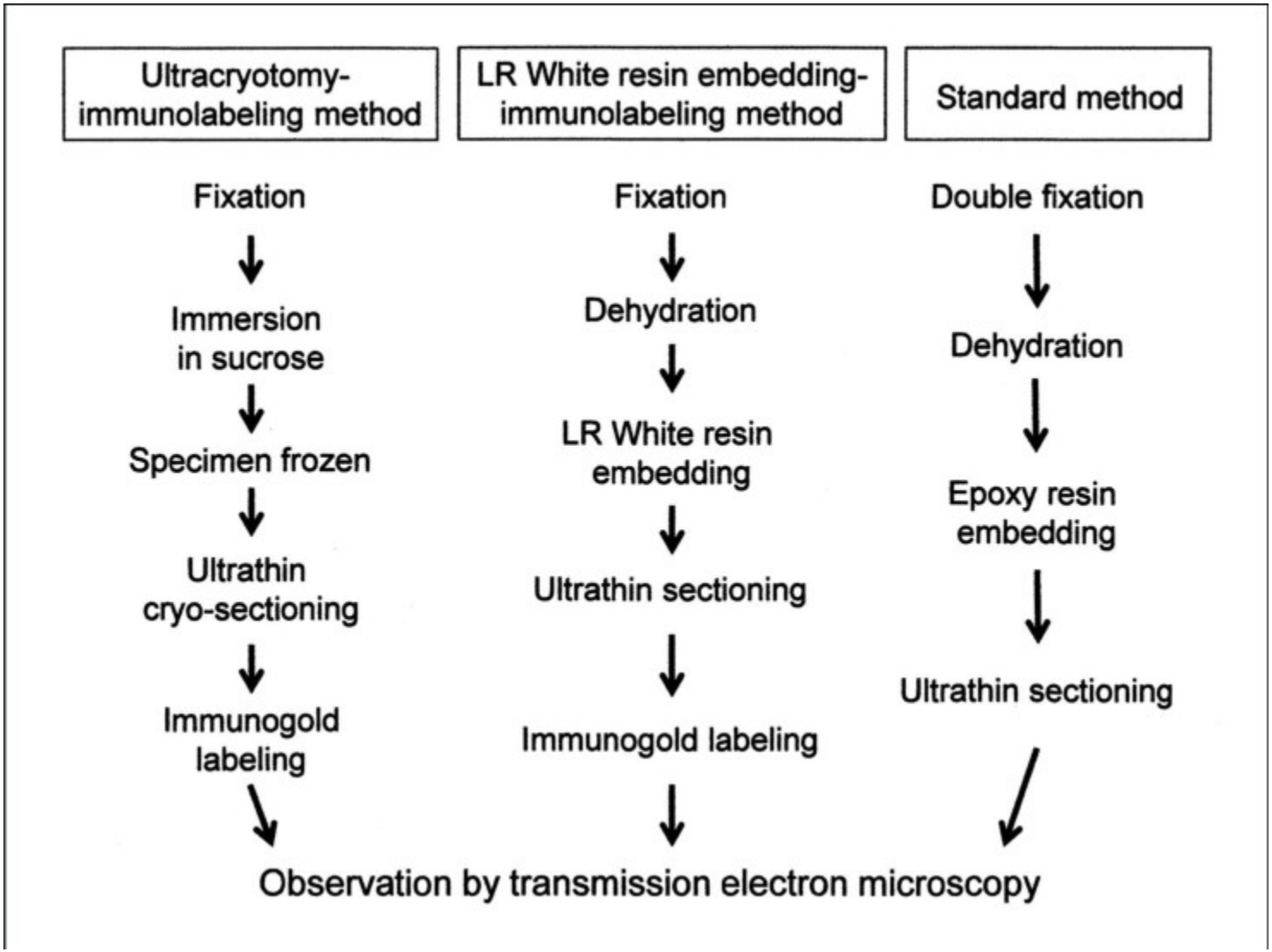
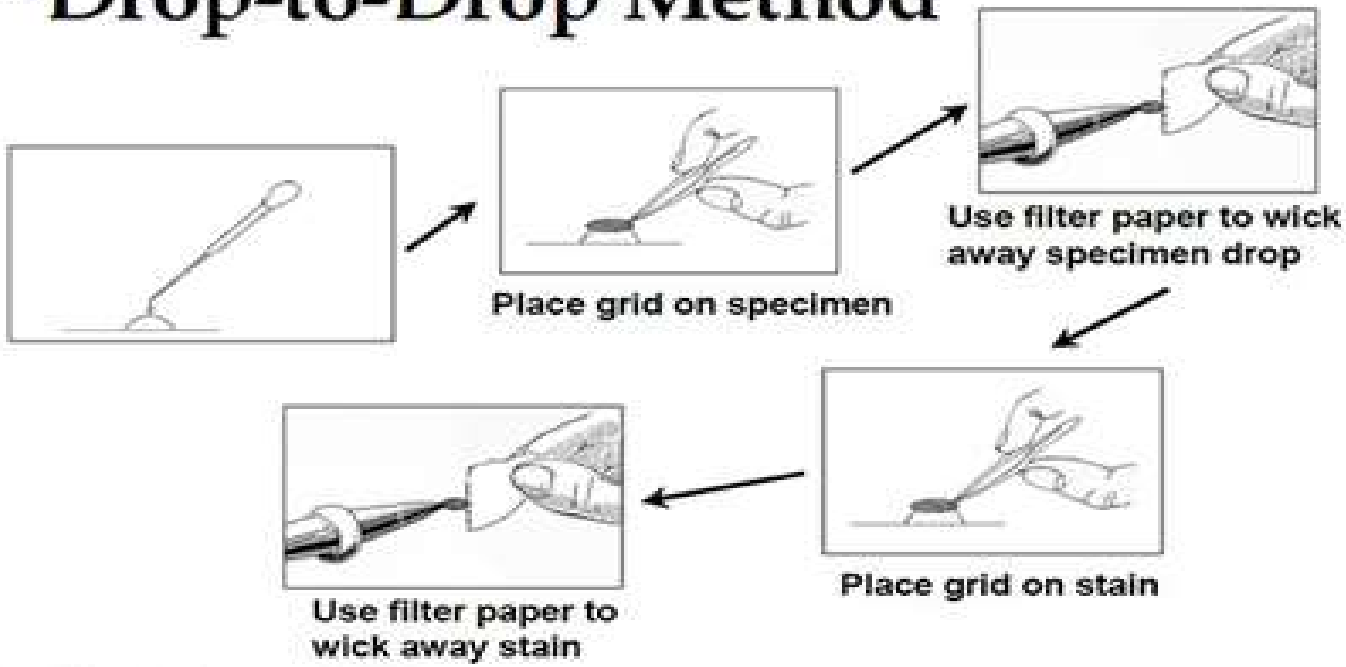


Figure 4.21

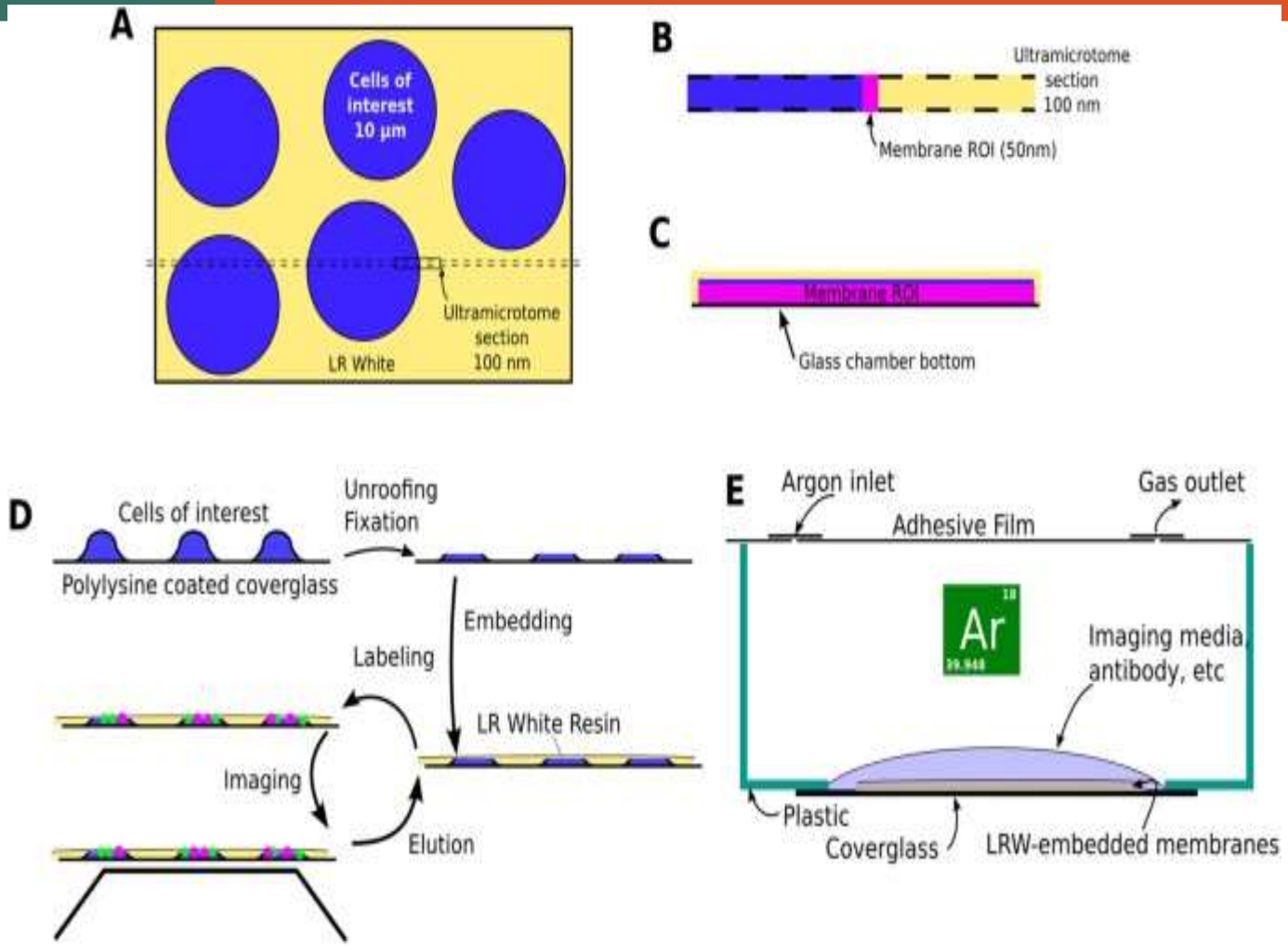
Staining techniques electron microscopy

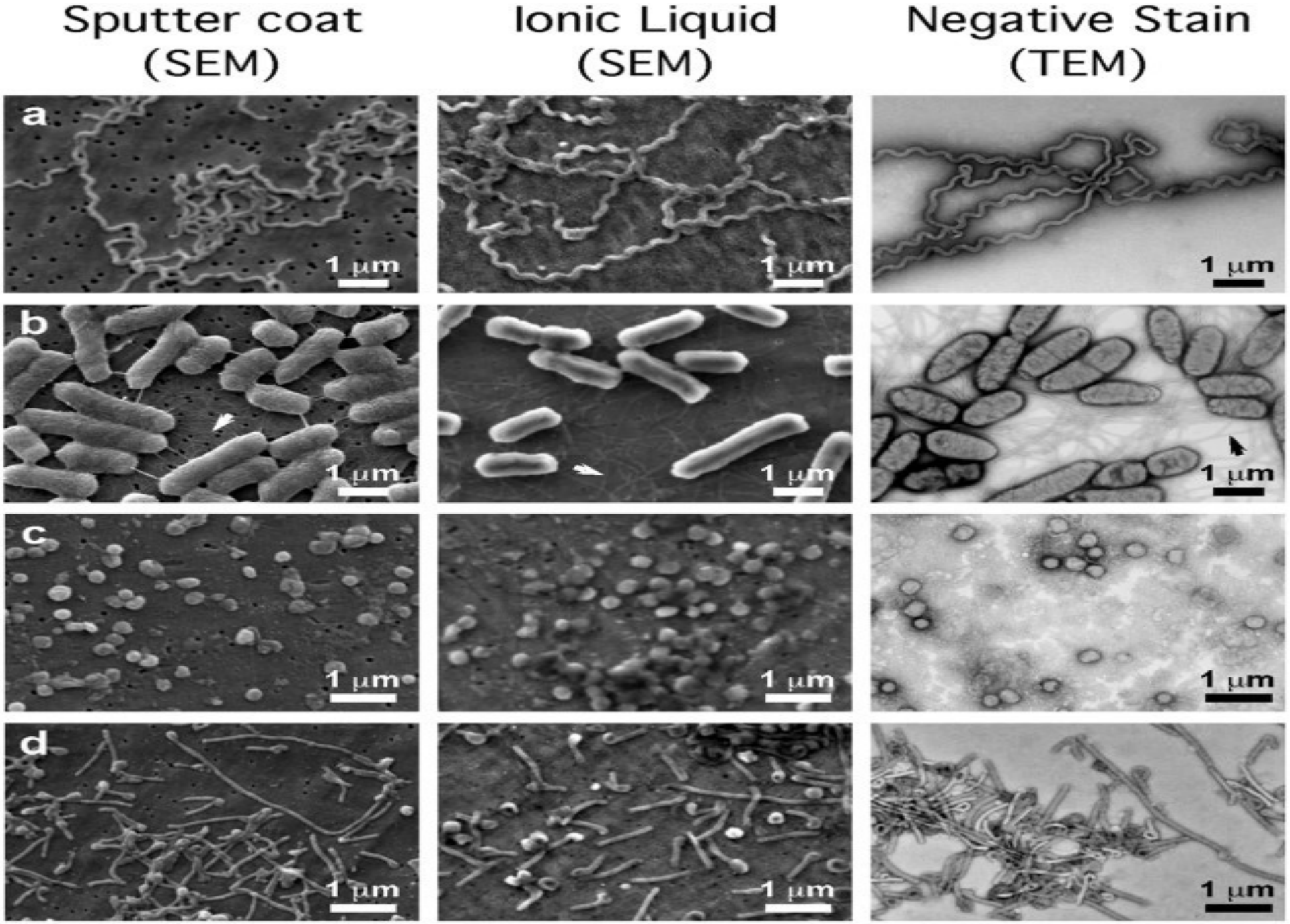


**Drop-to-Drop Method



Adapted from FW Doane and N Anderson, "Electron Microscopy in Diagnostic Virology"





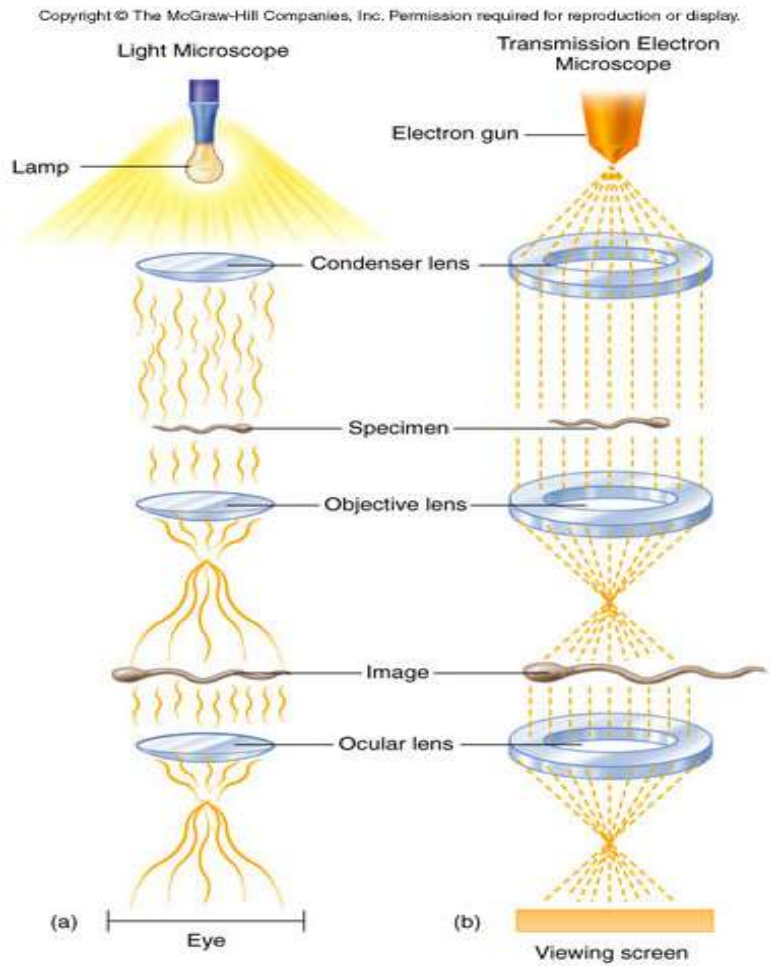
Microbiology Chapter 3, part 2

- Compare and contrast Light and Electron Microscope

Light	Electron
Energy – light	Energy – electron beam
Cost - $1200	Cost – $120,000
Simple to use	Complex processes. trained technician
Magnification – 1200X	Magnification – 100,000X
Viewed by eye, camera	Viewed with CRT, photos

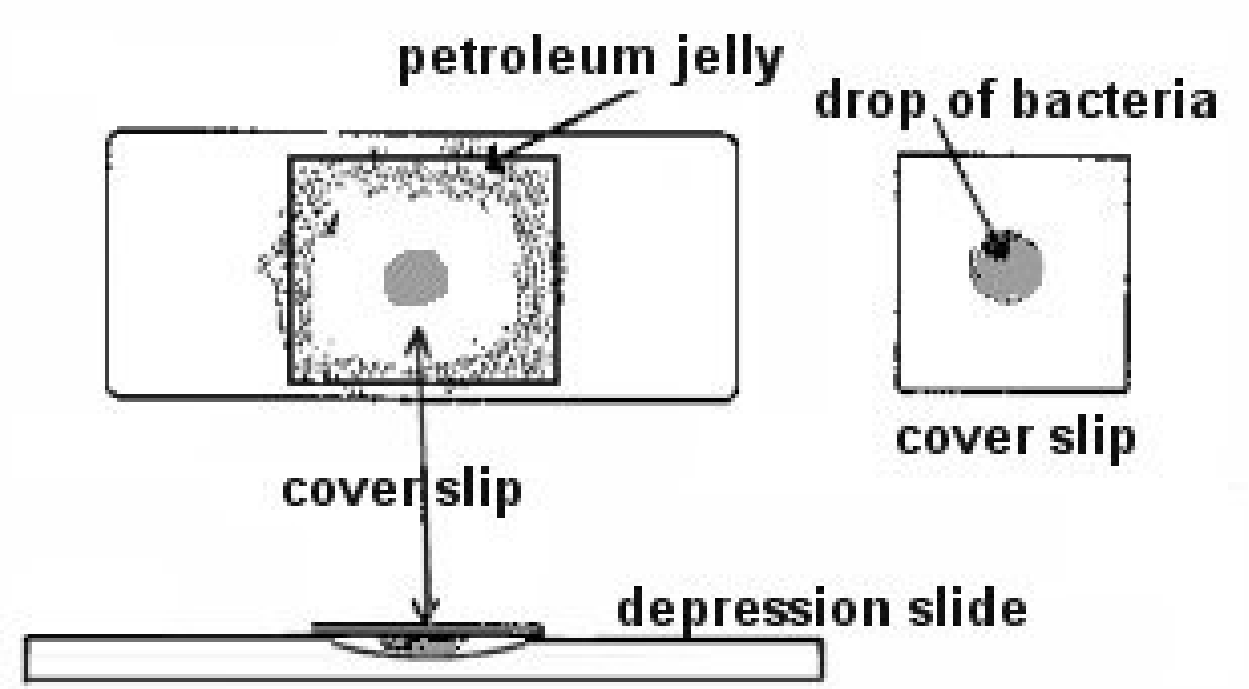
Microbiology Chapter 3, part 2

- Compare and contrast Light and Electron Microscope



Microbiology Chapter 3, part 2

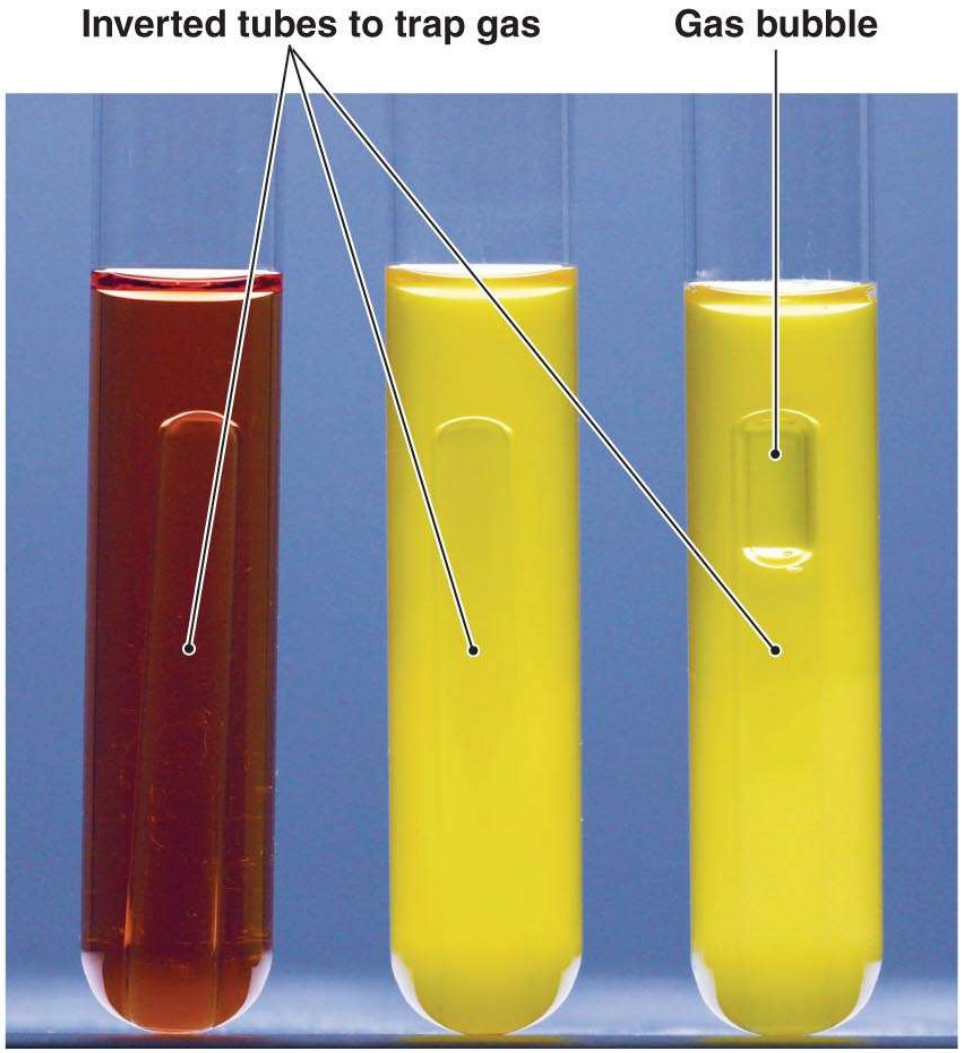
- Preparation of samples for light microscope
- Wet mounts (ex. Hanging drop) for live observation



Classification and Identification of Microorganisms

- **Taxonomic and Identifying Characteristics**
  - Physical characteristics
  - Biochemical tests
  - Serological tests
  - Phage typing
  - Analysis of nucleic acids

Two biochemical tests for identifying bacteria



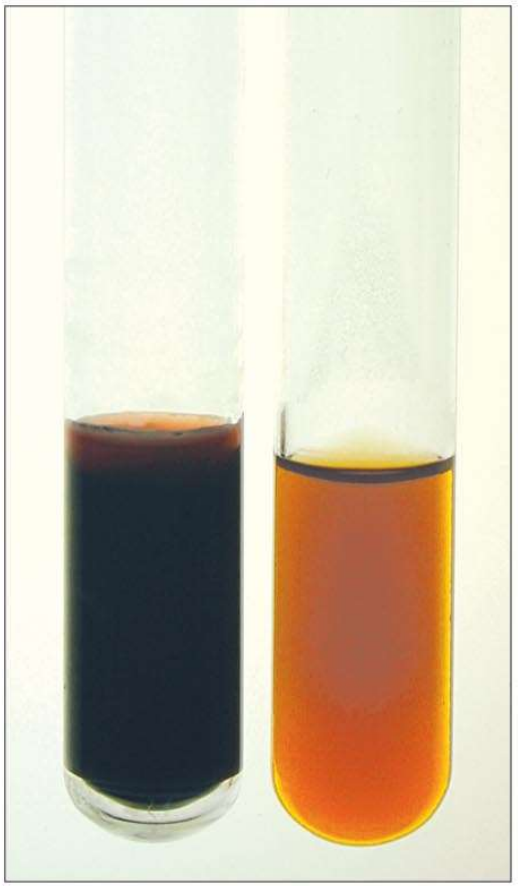
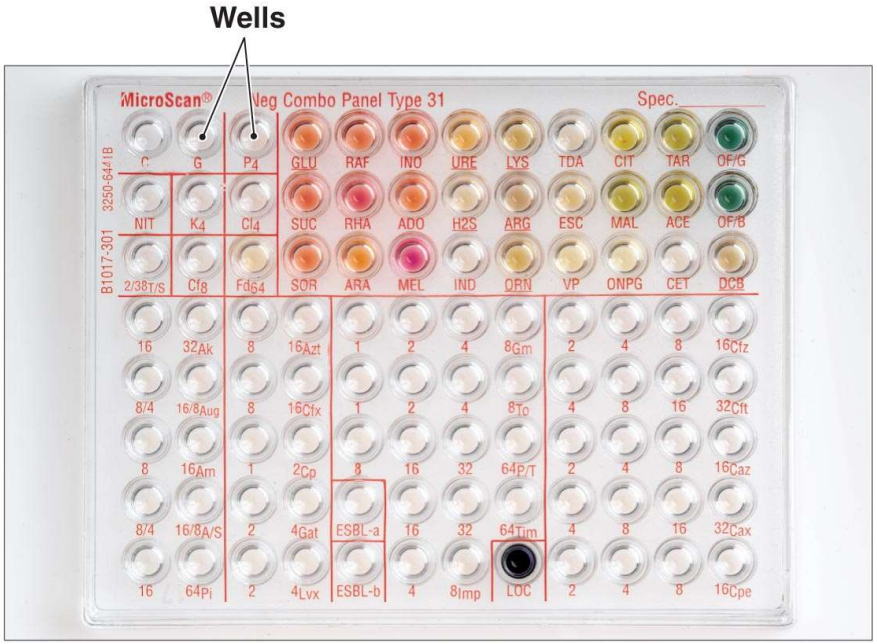


Figure 4.24

One tool for the rapid identification of bacteria

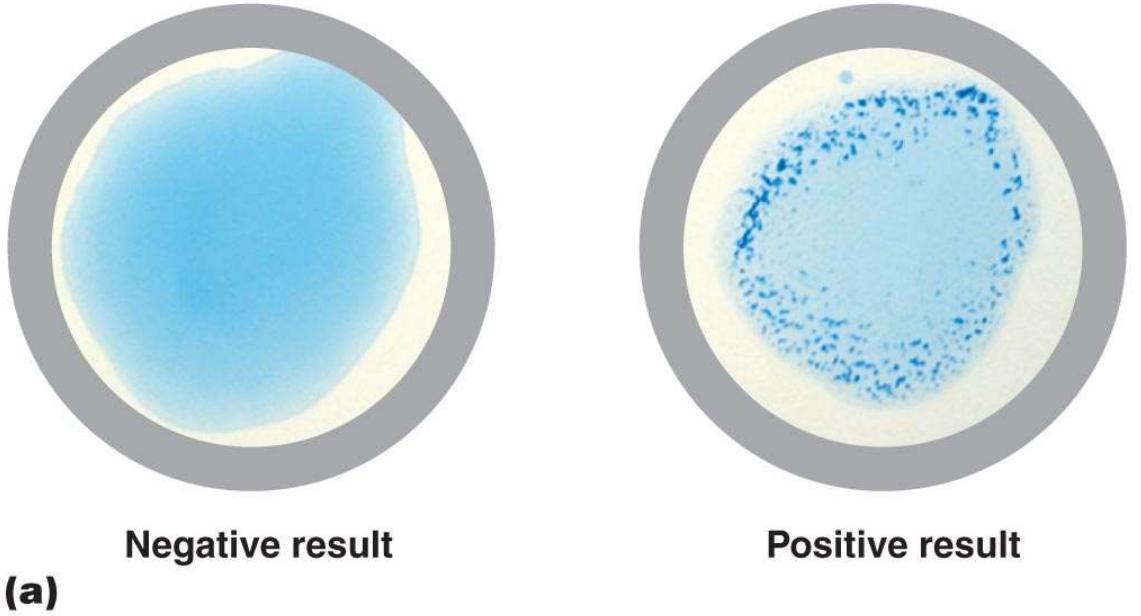
(a) MicroScan instrument



(b) MicroScan panel

Figure 4.25

An agglutination test, one type of serological test



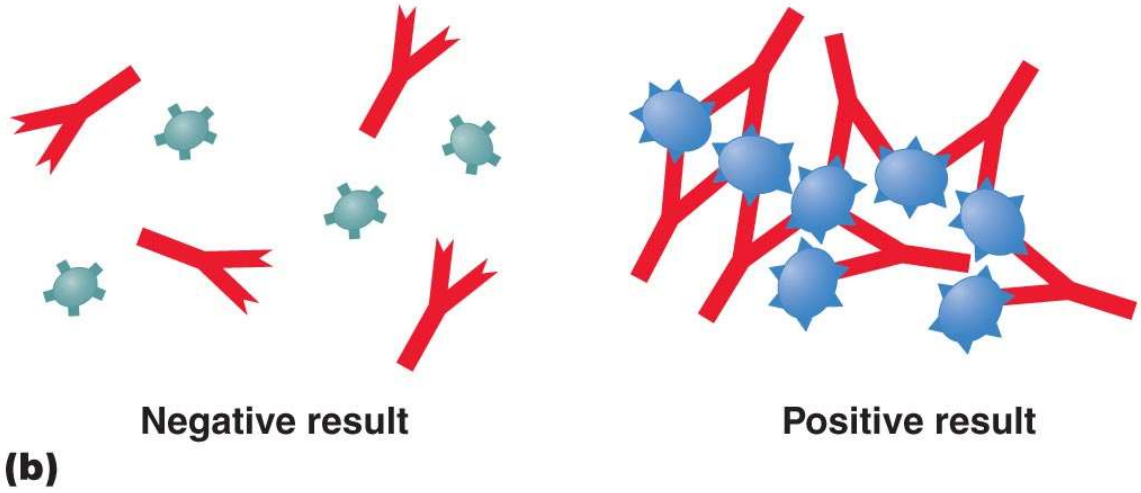


Figure 4.26

Phage typing

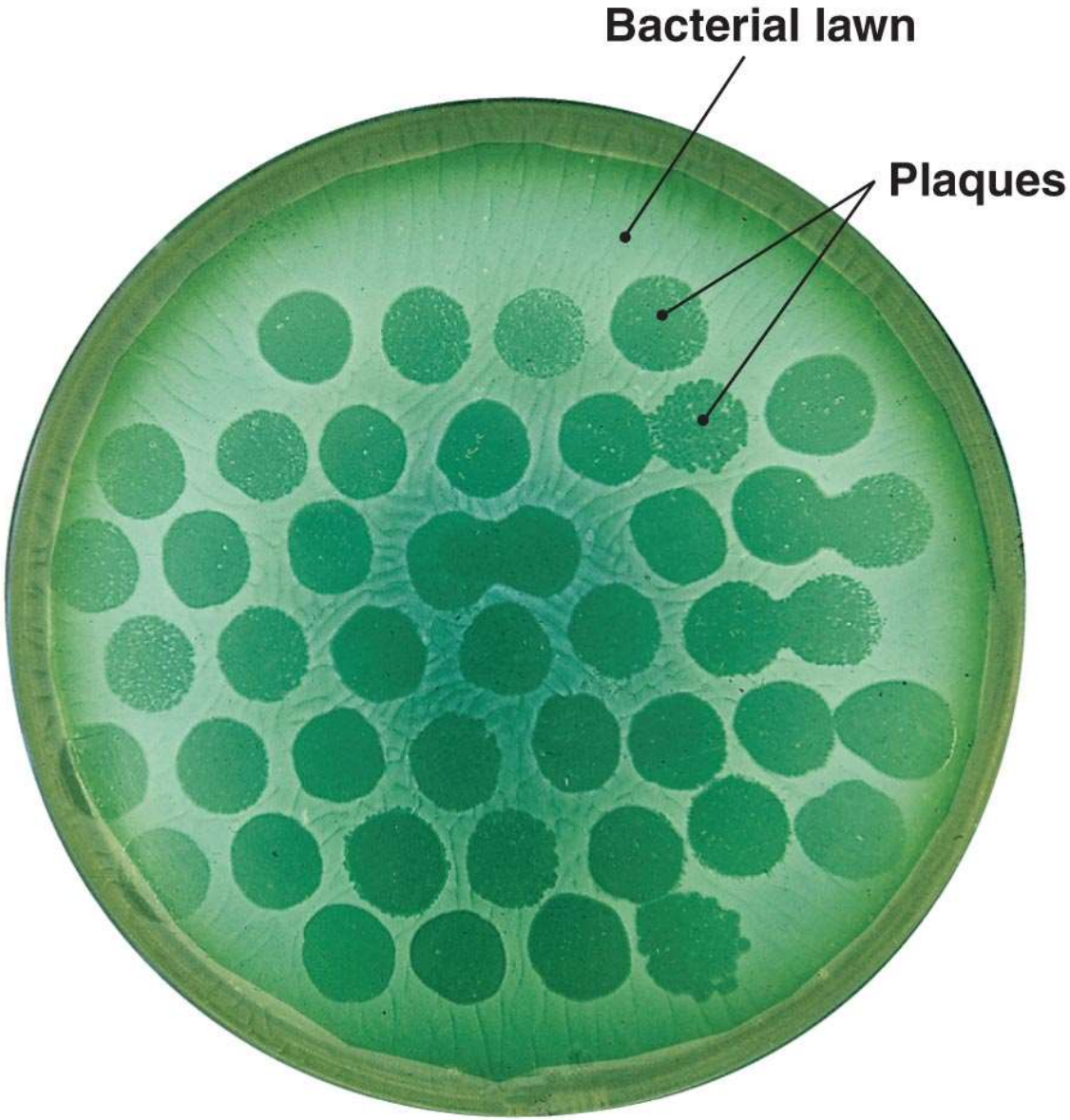


Figure 4.27

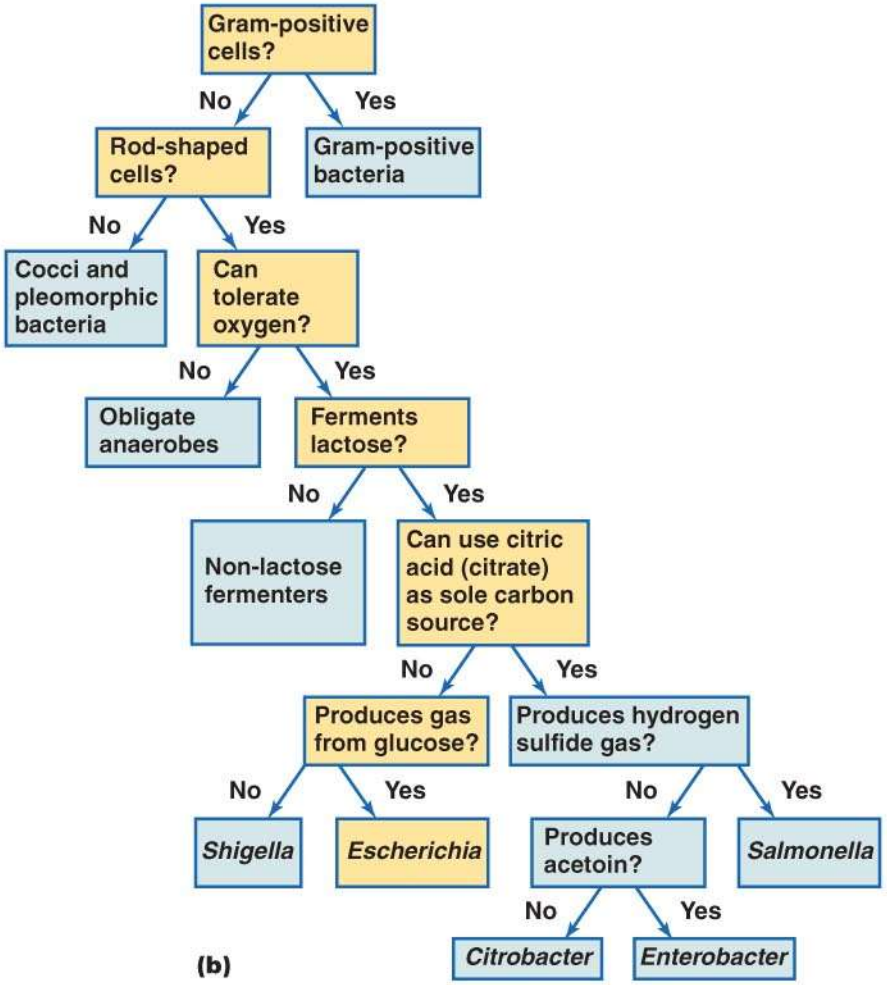
Classification and Identification of Microorganisms

- **Taxonomic Keys**
  - Dichotomous keys
    - Series of paired statements where only one of two "either/or" choices applies to any particular organism
  - Key directs user to another pair of statements, or provides name of organism

Use of dichotomous taxonomic key

1a. Gram-positive cells.....	Gram-positive bacteria
1b. Gram-negative cells.....	2
2a. Rod-shaped cells.....	3
2b. Non-rod-shaped cells.....	Cocci and pleomorphic bacteria
3a. Can tolerate oxygen.....	4
3b. Cannot tolerate oxygen.....	Obligate anaerobes
4a. Ferments lactose.....	5
4b. Cannot ferment lactose.....	Non-lactose fermenters
5a. Can use citric acid as a sole carbon source.....	6
5b. Cannot use citric acid alone.....	8
6a. Produces hydrogen sulfide gas.....	*Salmonella*
6b. Does not produce hydrogen sulfide gas..	7
7a. Produces acetoin.....	*Enterobacter*
7b. Does not produce acetoin.....	*Citrobacter*
8a. Produces gas from glucose.....	*Escherichia*
8b. Does not produce gas from glucose.....	*Shigella*

(a)



(b)

Figure 4.28