

Chapter 2

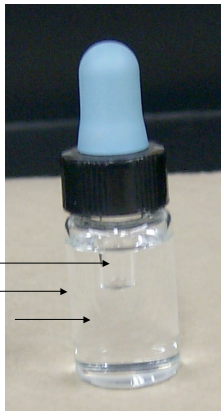
Microscopy and Specimen Preparation

Lenses and the Bending of Light

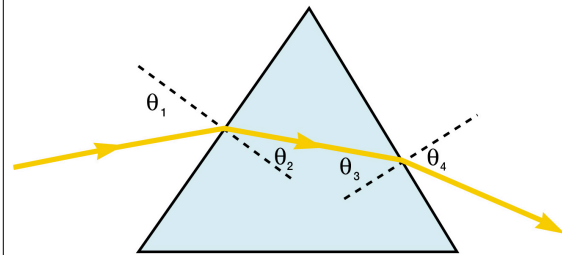
- ✓ light is refracted (bent) when passing from one medium to another
- ✓ refractive index
 - a measure of how greatly a substance slows the velocity of light
- ✓ direction and magnitude of bending is determined by the refractive indexes of the two media forming the interface

Refractive index

- Air with a refractive index of 1.00
- Glass has refractive index of 1.52
- Mineral oil with a refractive index of 1.52



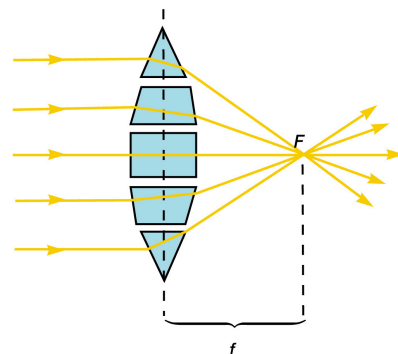
Bending of light by a prism



Lenses

- ✓ focus light rays at a specific place called the **focal point**
- ✓ distance between center of lens and focal point is the **focal length**
- ✓ strength of lens related to focal length
 - short focal length \Rightarrow more magnification

Lens function



The Bright-Field Microscope

- ✓ produces a dark image against a brighter background
- ✓ has several objective lenses
 - **parfocal** microscopes remain in focus when objectives are changed
- ✓ total magnification
 - product of the magnifications of the ocular lens and the objective lens

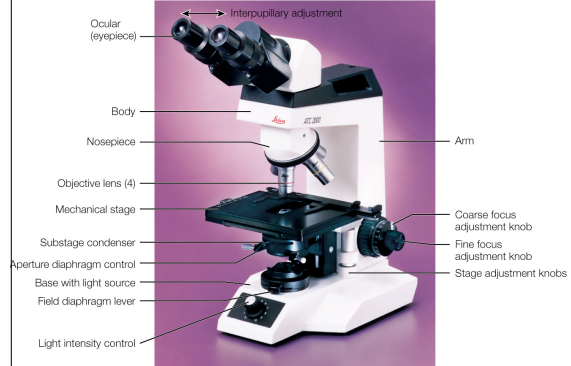
The Light Microscope

- ✓ many types
 - bright-field microscope
 - dark-field microscope
 - phase-contrast microscope
 - fluorescence microscopes
- ✓ Compound microscopes
 - image formed by action of ≥ 2 lenses

Table 2.1 Common Units of Measurement

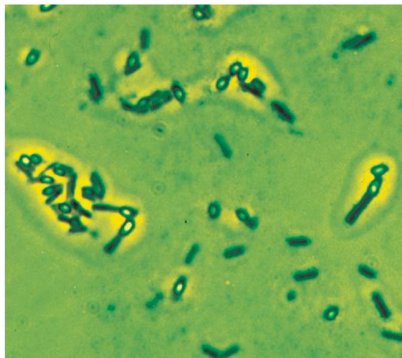
Unit	Abbreviation	Value
1 centimeter	cm	10^{-2} meter or 0.394 inches
1 millimeter	mm	10^{-3} meter
1 micrometer	μm	10^{-6} meter
1 nanometer	nm	10^{-9} meter
1 Angstrom	\AA	10^{-10} meter

Bright-field microscope

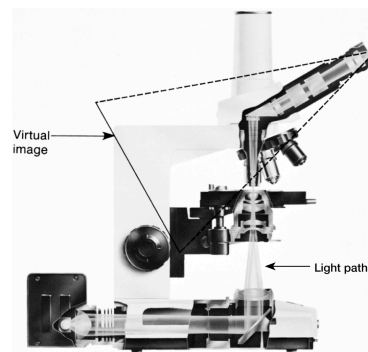


Bright-field Microscope

Clostridium botulinum



Light path



Microscope Resolution

- ✓ ability of a lens to separate or distinguish small objects that are close together
- ✓ wavelength of light used is major factor in resolution
 - ✓ shorter wavelength \Rightarrow greater resolution

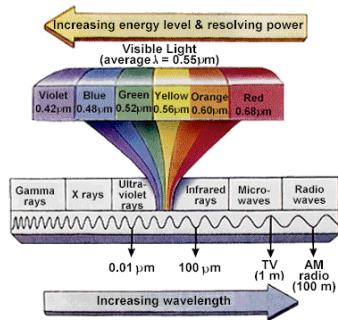
Resolving Power

- Are they two bacteria or just one bacterium?
- ✓ Abbe equation determines resolution
- ✓ d = minimum distance to get to separate objects
- ✓ λ is the wavelength of light
- ✓ $n \sin \theta$ is the numerical aperture
- ✓ Smaller d = better resolution



$$d = \frac{0.5\lambda}{n \sin \theta}$$

Wavelength Spectrum



Resolution

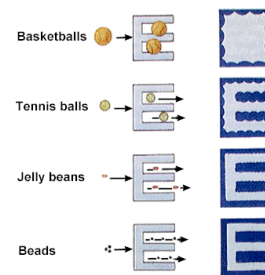
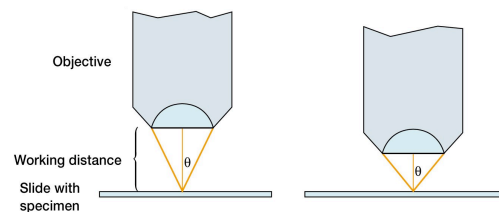


Table 2.2 The Properties of Microscope Objectives

Property	Objective			
	Scanning	Low Power	High Power	Oil Immersion
Magnification	4x	10x	40–45x	90–100x
Numerical aperture	0.10	0.25	0.55–0.65	1.25–1.4
Approximate focal length (f)	40 mm	16 mm	4 mm	1.8–2.0 mm
Working distance	17–20 mm	4–8 mm	0.5–0.7 mm	0.1 mm
Approximate resolving power with light of 450 nm (blue light)	2.3 μ m	0.9 μ m	0.35 μ m	0.18 μ m

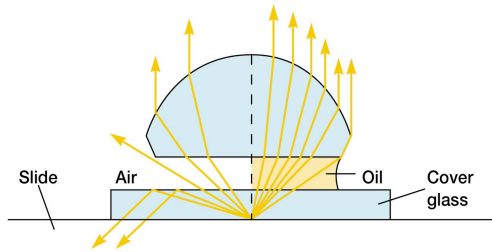
- working distance
 - distance between the front surface of lens and surface of cover glass or specimen

Working distance



- Numerical aperture:
 - θ is 1/2 the angle of the cone of light entering the objective.
 - The angle of light that can enter the lens depends upon the refractive index or n of the media the lens works

Oil immersion objective



Oil increases the numerical aperture
 NA ($n \sin \theta$) = 1.25
 Blue-green light (λ) = 530 nm
 Resolving power (d) = 0.2 nm

$$d = \frac{0.5\lambda}{n \sin \theta}$$

Our Light Microscopes

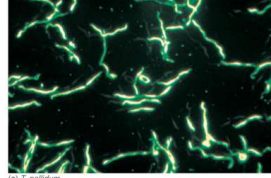
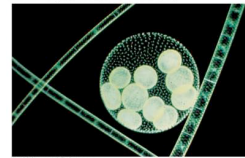
- eye piece or ocular objective (10X)
- Scanning objective (4X)
- low power objective (10X)
- high power objective (40X)
- oil immersion objective (100X)

Magnification

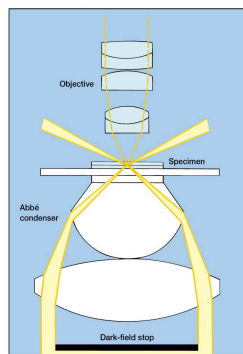
- ocular x objective = magnification
- 10 x Scanning = 40X
- 10 x low power = 100X
- 10 x high power = 400X
- 10 x oil immersion = 1000X

The Dark-Field Microscope

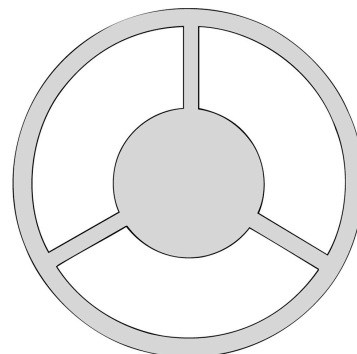
- ✓ produces a bright image of the object against a dark background
- ✓ used to observe living, unstained preparations
- ✓ Used to identify *Treponema pallidum*



Dark-field microscope

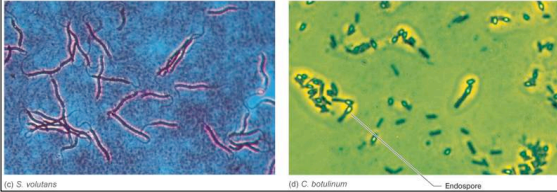


Dark-field stop blocks direct light

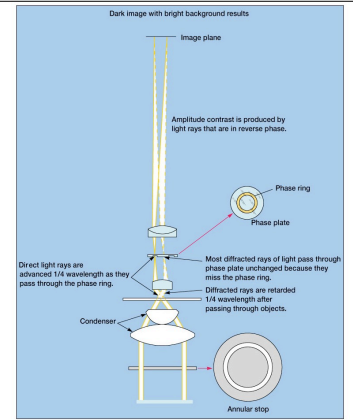


The Phase-Contrast Microscope

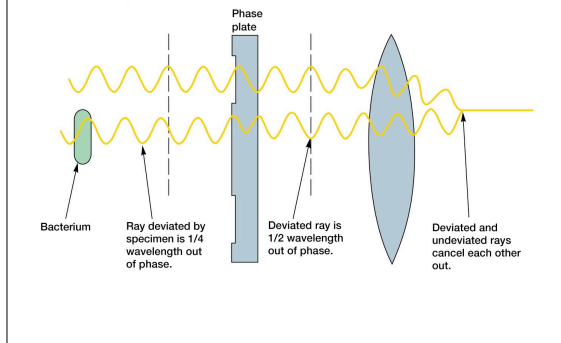
- ✓ enhances the contrast between intracellular structures having slight differences in refractive index
- ✓ excellent way to observe living cells



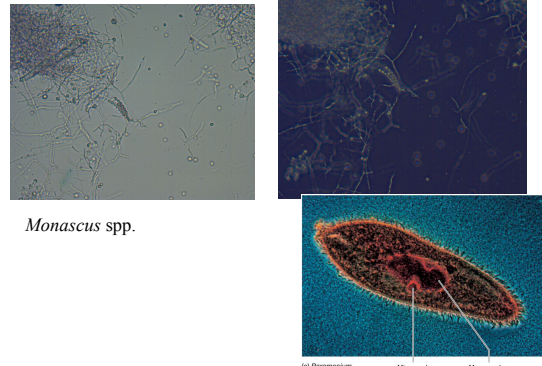
Phase contrast



Deviation of light producing phase



Bright-field verses phase



The Differential Interference Contrast Microscope

- ✓ Two beams of polarized light
- ✓ creates image by detecting differences in refractive indices and thickness of different parts of specimen
- ✓ excellent way to observe living cells

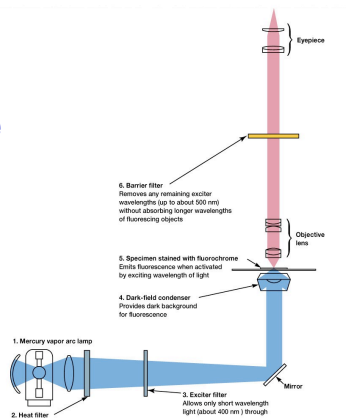
Amoeba proteus



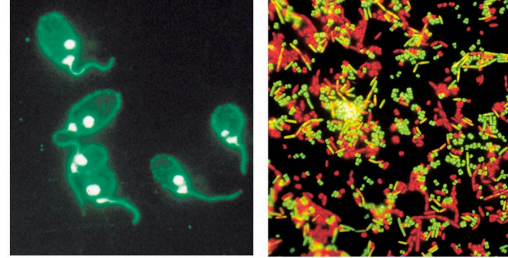
The Fluorescence Microscope

- ✓ exposes specimen to ultraviolet, violet, or blue light
- ✓ specimens usually stained with fluorochromes or emit visible light
- ✓ shows a bright image of the object resulting from the fluorescent light emitted by the specimen

Fluorescence Microscope



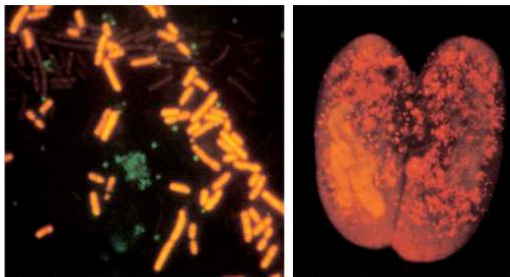
Fluorescence Microscopy



Kinetoplasts stained
Crithidia luciliae

Live versus dead bacteria
Micrococcus luteus & *Bacillus cereus*

Fluorescence Microscopy



(a) *E. coli*

(b) *P. tetraurelia*

Why do we stain microorganisms?

- They are transparent or translucent.
- To show overall structure.
- To identify internal structures.
- To identify and differentiate between types of microorganisms.

Fixation

- ✓ process by which internal and external structures are preserved and fixed in position
- ✓ process by which organism is killed and firmly attached to microscope slide
 - heat fixing
 - preserves overall morphology but not internal structures
 - chemical fixing
 - protects fine cellular substructure and morphology of larger, more delicate organisms

Staining techniques for the light microscope

1. Simple stain -- One dye to reveal basic cell shape & arrangements
2. Differential stain -- two or more dyes to distinguish various properties of the organism
3. Negative stain -- color the background around the cells

Dyes and Simple Staining

✓ dyes

- make internal and external structures of cell more visible by increasing contrast with background
- have two common features
 - chromophore groups
 - chemical groups with conjugated double bonds
 - give dye its color
 - ability to bind cells

Dyes and Simple Staining

✓ simple staining

- a single staining agent is used
- basic dyes are frequently used
 - dyes with positive charges
 - e.g., crystal violet, methylene blue, basic fuchsin, safranin, malachite green,
- Acid dyes
 - Negative charges
 - Eosin, rose bengal, acid fuchsin

Types of simple stains....

- ✓ Methylene blue
- ✓ Crystal violet
- ✓ Carbofuchsin
- ✓ safranin

Differential stains...

- ✓ divides microorganisms into groups based on their staining properties
 - Gram stain
 - Acid fast stain
 - Endospore stain
 - Capsule stain
 - Flagella stain

Differential staining

1. Primary stain
2. Mordant (fixes the primary dye)
3. Decolorizing rinse --critical step
4. Counter stain

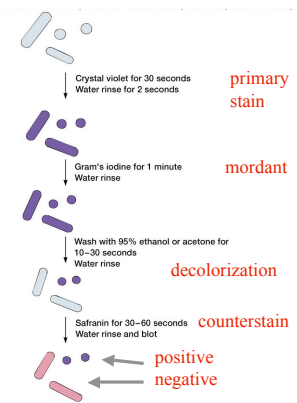
Gram staining

- ✓ most widely used differential staining procedure
- ✓ divides Bacteria into two groups based on differences in cell wall structure

Gram stain

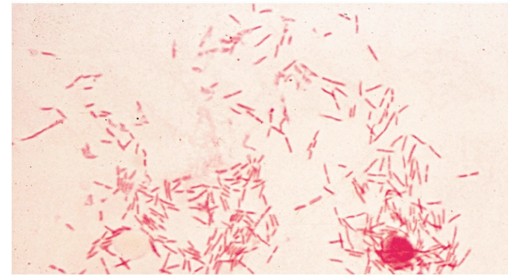
1. Crystal violet
2. Iodine
3. Alcohol rinse
4. Safranin

Gram Stain



Gram reaction

Step	Positive	Negative
Crystal violet		
Iodine		
Alcohol		
Safranin		



Escherichia coli – a gram-negative rod

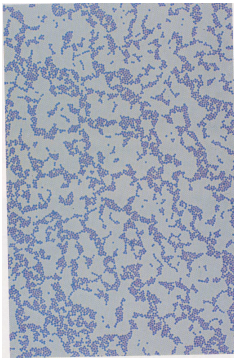


FIGURE 3.7 A Gram stain of a 24-hour culture of *Staphylococcus aureus*, yielding uniform results. All cells are purple, a Gram-positive reaction (3600 \times).

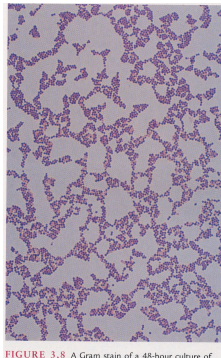


FIGURE 3.8 A Gram stain of a 48-hour culture of *Staphylococcus aureus*. A 48-hour culture does not yield uniform results. Even though most cells are purple, a Gram-positive reaction, some cells are red, a Gram-negative reaction (3600 \times).

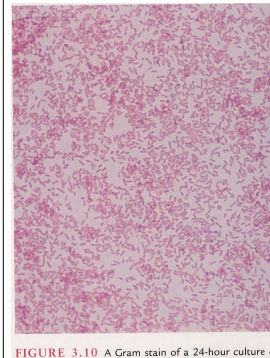


FIGURE 3.10 A Gram stain of a 24-hour culture of *Escherichia coli*, yielding uniform results. All cells are red, a Gram-negative reaction (3600 \times).

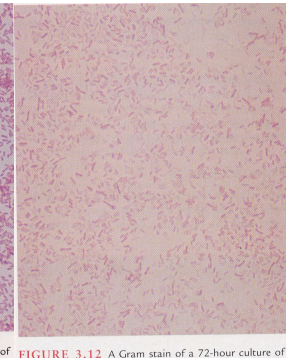
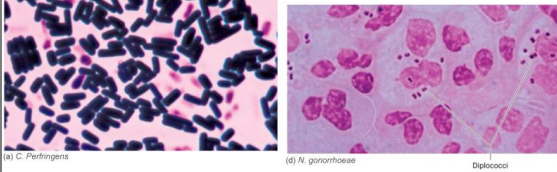


FIGURE 3.12 A Gram stain of a 72-hour culture of *Escherichia coli*. Only a few cells are red, and most cells stain only lightly (3600 \times).

More stained bacteria



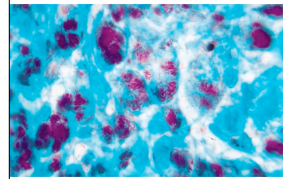
Acid-fast staining

- ✓ particularly useful for staining members of the genus *Mycobacterium*
 - e.g., *Mycobacterium tuberculosis* – causes tuberculosis
 - e.g., *Mycobacterium leprae* – causes leprosy
- high lipid content in cell walls is responsible for their staining characteristics

Acid fast reaction

Step	Positive	Negative
Carbolfuchsin		
Acid alcohol		
Methylene blue		

Acid-fast stain



Mycobacterium lepre

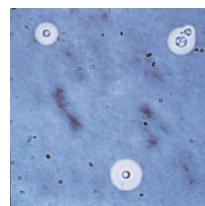


FIGURE 3.23 An acid-fast stain of *Mycobacterium tuberculosis*, an acid-fast rod. Acid-fast rods appear red (3600×).

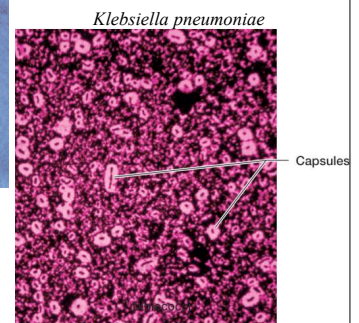
Staining Specific Structures

- ✓ Negative staining
 - often used to visualize capsules surrounding bacteria
 - capsules are colorless against a stained background

Negative stain



Cryptococcus neoformans



Negative stain (bacillus)



FIGURE 3.5 A negative stain of the rod *Bacillus* (3600X).

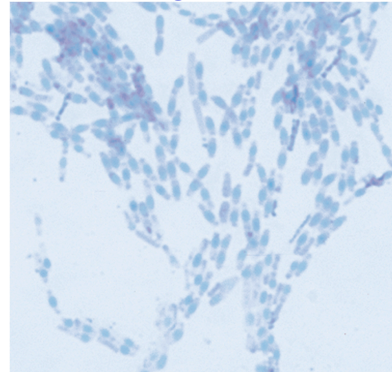
Types of negative stains....

- ✓ Nigrosin
- ✓ India ink

Staining Specific Structures

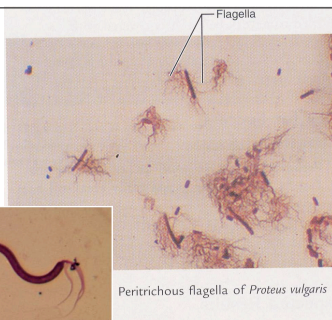
- ✓ Spore staining
 - double staining technique
 - bacterial endospore is one color and vegetative cell is a different color
- ✓ Flagella staining
 - mordant applied to increase thickness of flagella
- ✓ Capsule

Spore Staining



Flagellum stain

Spirillum volutans



Peritrichous flagella of *Proteus vulgaris*

Diplobacilli

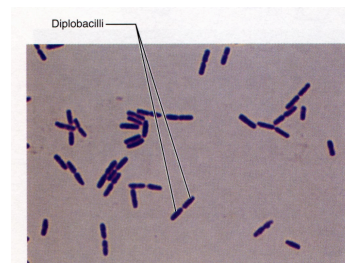


FIGURE 2.14 A 24-hour culture of *Bacillus cereus*. Many cells are undergoing binary fission and appear as diplobacilli (4000X).

Diplococci

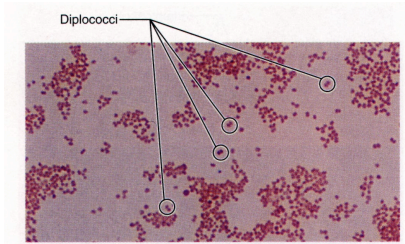


FIGURE 2.18 *Neisseria gonorrhoeae*, a diplococcus, is the causative agent of gonorrhea. The paired cocci have flattened sides that lie adjacent to each other (3600 \times).

Staphylococci

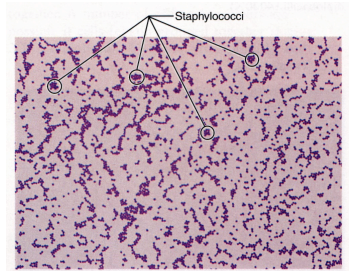
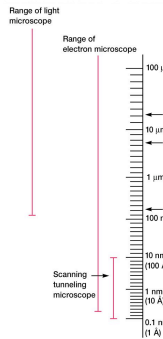


FIGURE 2.22 Cocci in clusters (staphylococci) in *Staphylococcus aureus*. These clusters are due to cell division occurring in more than three planes (2500 \times).

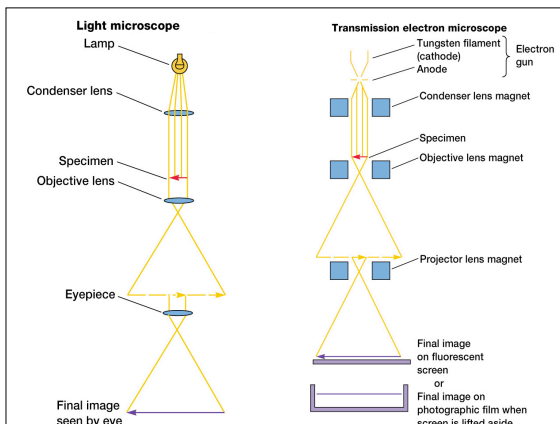
Electron Microscopy

- ✓ beams of electrons are used to produce images
- ✓ wavelength of electron beam is much shorter than light, resulting in much higher resolution



The Transmission Electron Microscope

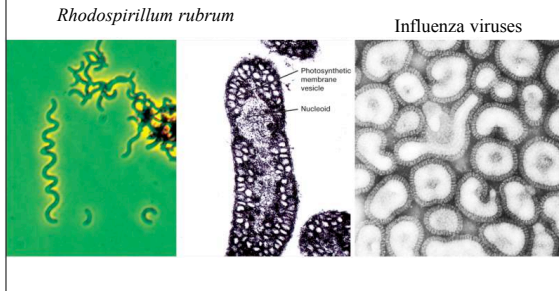
- ✓ electrons scatter when they pass through thin sections of a specimen
- ✓ transmitted electrons (those that do not scatter) are used to produce image
- ✓ denser regions in specimen, scatter more electrons and appear darker



Transmission electron microscope



Light versus electron microscopy



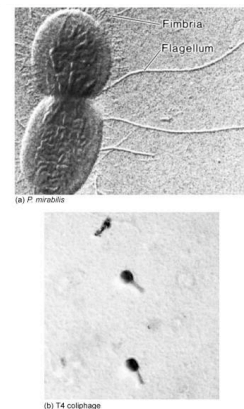
Specimen Preparation

- ✓ analogous to procedures used for light microscopy
- ✓ for transmission electron microscopy, specimens must be cut very thin
- ✓ specimens are chemically fixed and stained with electron dense material

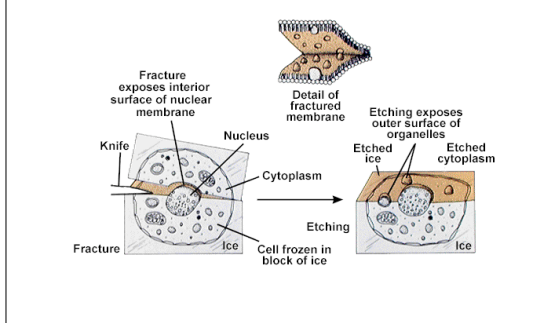
Other preparation methods

- ✓ shadowing
 - coating specimen with a thin film of a heavy metal
- ✓ freeze-etching
 - freeze specimen then fracture along lines of greatest weakness (e.g., membranes)

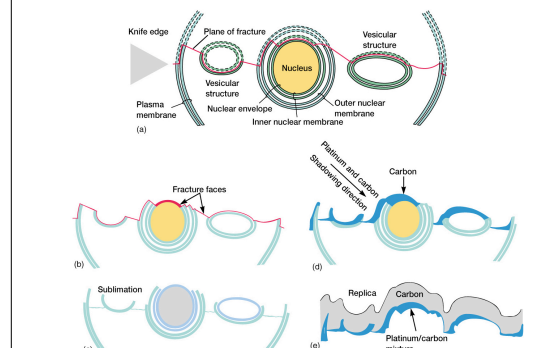
TEM after shadowing with uranium metal



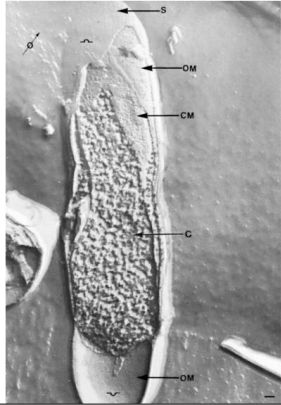
Freeze-etch



Freeze-etching



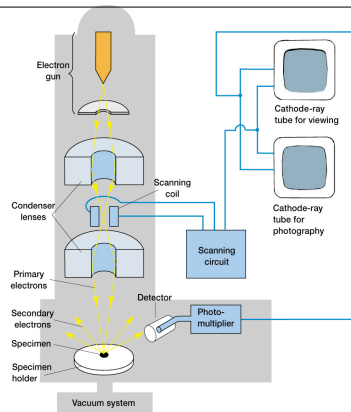
Freeze-Etching



The Scanning Electron Microscope

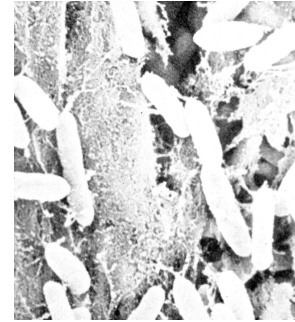
- ✓ uses electrons reflected from the surface of a specimen to create image
- ✓ produces a 3-dimensional image of specimen's surface features

Scanning Electron

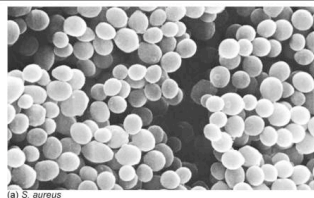


Pseudomonas syringae

- Gram negative bacteria
- Aerial surfaces of plants
- Produces antifungal molecules

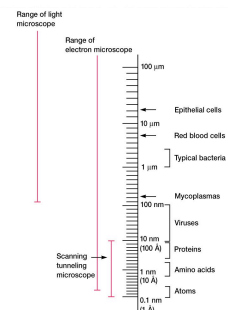


Scanning electron micrographs



Newer Techniques in Microscopy

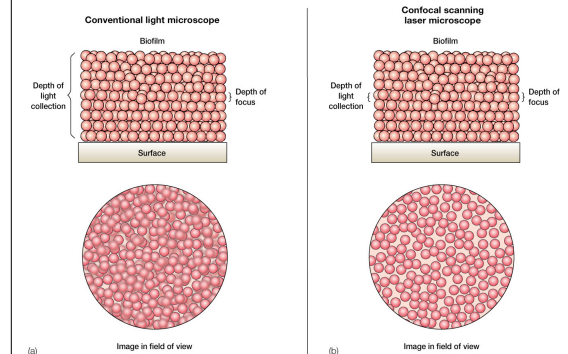
- ✓ confocal microscopy and scanning probe microscopy
- ✓ have extremely high resolution
- ✓ can be used to observe individual atoms



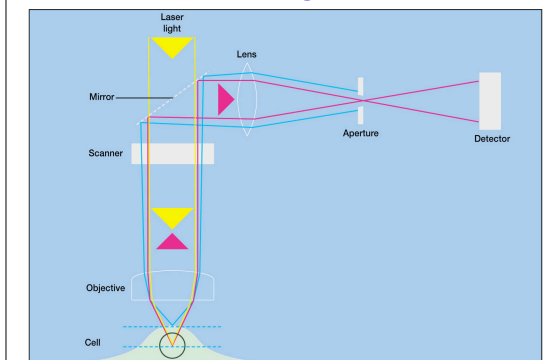
Confocal Microscopy

- ✓ confocal scanning laser microscope
- ✓ laser beam used to illuminate spots on specimen
- ✓ computer compiles images created from each point to generate a 3-dimensional image

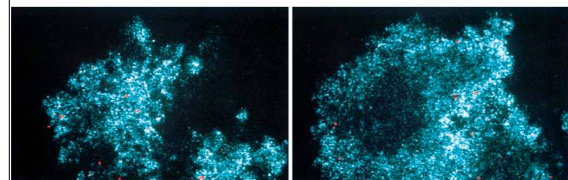
Confocal Scanning Laser



Confocal Scanning Laser



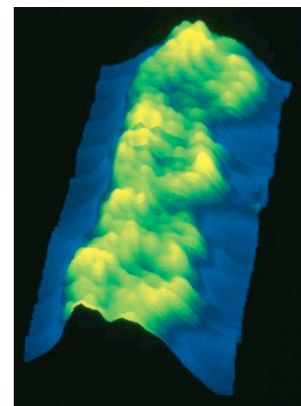
Confocal images of a biofilm



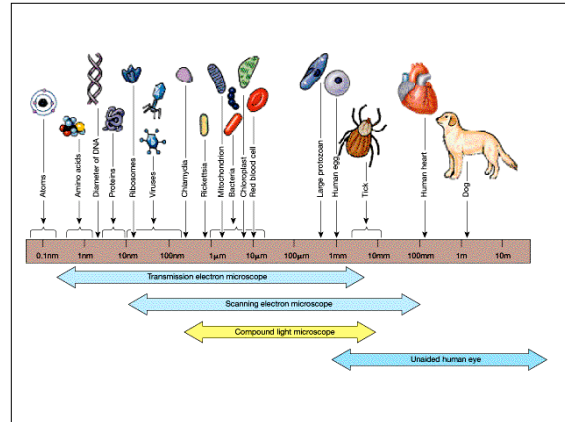
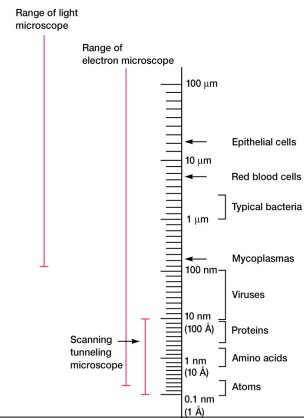
Scanning Probe Microscopy

- ✓ scanning tunneling microscope
 - steady current (tunneling current) maintained between microscope probe and specimen
 - up and down movement of probe as it maintains current is detected and used to create image of surface of specimen

Scanning Tunneling Microscopy



Microscopic resolution



Magnification (EM)

Light microscopy: 10-2000X

Electron microscopy:
10X-200,000-400,000X

Comparison of Light and Electron

Table 2.3 Characteristics of Light and Transmission Electron Microscopes

Feature	Light Microscope	Electron Microscope
Highest practical magnification	About 1,000-1,500	Over 100,000
Best resolution ^a	0.2 μm	0.5 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Type of lens	Glass	Electromagnet
Source of contrast	Differential light absorption	Scattering of electrons
Focusing mechanism	Adjust lens position mechanically	Adjust current to the magnetic lens
Method of changing magnification	Switch the objective lens or eyepiece	Adjust current to the magnetic lens
Specimen mount	Glass slide	Metal grid (usually copper)

^aThe resolution limit of a human eye is about 0.2 mm.

The End.