Microbiology: A Systems Approach,

Chapter 3: Tools of the Laboratory

3.1 Methods of Culturing Microorganisms: The Five I's

- Microbiologists use five basic techniques to manipulate, grow, examine, and characterize microorganisms in the laboratory: inoculation, incubation, isolation, inspection, and identification

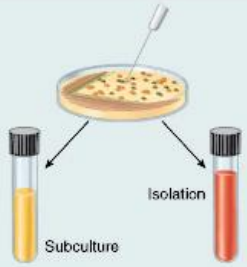
An Overview of Major Techniques Performed by Microbiologists to Locate, Grow, Observe, and Characterize Microorganisms

Specimen Collection:
Nearly any object or material can serve as a source of microbes. Common ones are body fluids and tissues, foods, water, or soil. Specimens are removed by some form of sampling device: a swab, syringe, or a special transport system that holds, maintains, and preserves the microbes in the sample.

A GUIDE TO THE FIVE I's: How the Sample Is Processed and Profiled

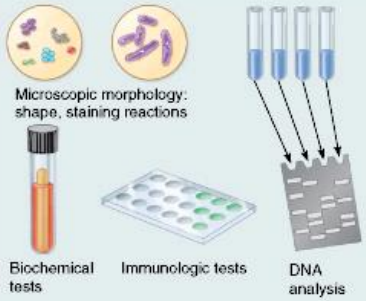
1. Inoculation:
The sample is placed into a container of sterile **medium** containing appropriate nutrients to sustain growth. Inoculation involves spreading the sample on the surface of a solid medium or introducing the sample into a flask or tube. Selection of media with specialized functions can improve later steps of isolation and identification. Some microbes may require a live organism (animal, egg) as the growth medium.

2. Incubation:
An incubator creates the proper growth temperature and other conditions. This promotes multiplication of the microbes over a period of hours, days, and even weeks. Incubation produces a culture—the visible growth of the microbe in or on the medium.



3. Isolation:
One result of inoculation and incubation is **isolation** of the microbe. Isolated microbes may take the form of separate colonies (discrete mounds of cells) on solid media, or turbidity (free-floating cells) in broths. Further isolation by subculturing involves taking a bit of growth from an isolated colony and inoculating a separate medium. This is one way to make a pure culture that contains only a single species of microbe.

4. Inspection:
The colonies or broth cultures are observed macroscopically for growth characteristics (color, texture, size) that could be useful in analyzing the specimen contents. Slides are made to assess microscopic details such as cell shape, size, and motility. Staining techniques may be used to gather specific information on microscopic morphology.



5. Identification:
A major purpose of the Five I's is to determine the type of microbe, usually to the level of species. Information used in identification can include relevant data already taken during initial inspection and additional tests that further describe and differentiate the microbes. Specialized tests include biochemical tests to determine metabolic activities specific to the microbe, immunologic tests, and genetic analysis.

Figure 3.1

Inoculation and Isolation

- **Inoculation**: producing a culture
  - Introduce a tiny sample (the inoculums) into a container of nutrient **medium**
- **Isolation**: separating one species from another
  - Separating a single bacterial cell from other cells and providing it space on a nutrient surface will allow that cell to grow in to a mound of cells (a **colony**).
  - If formed from a single cell, the colony contains cells from just that species.

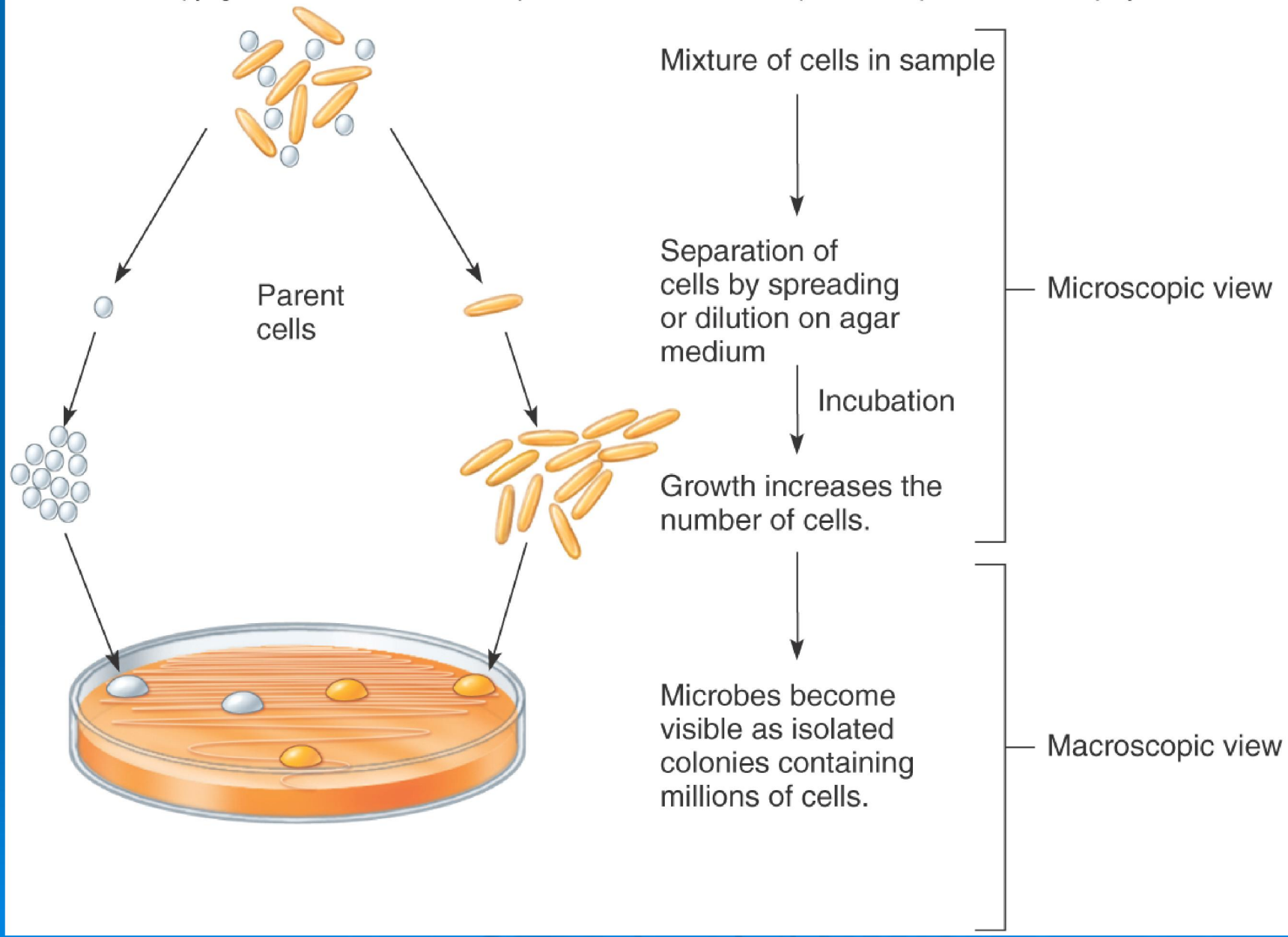
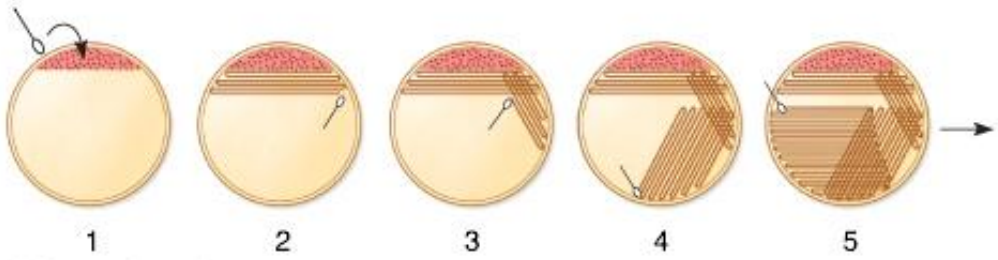


Figure 3.2

Streak Plate Method

- Streak plate method- small droplet of culture or sample spread over surface of the medium with an inoculating loop
  - Uses a pattern that thins out the sample and separates the cells

Note: This method only works if the spreading tool (usually an inoculating loop) is resterilized after each of steps 1–4.



(a) **Steps in a Streak Plate**

(b)

Figure 3.3 a,b

Loop Dilation Method

- Loop dilation, or pour plate, method- sample inoculated serially in to a series of liquid agar tues to dilute the number of cells in each successive tubes
  - Tubes are then poured in to sterile Petri dishes and allowed to solidify

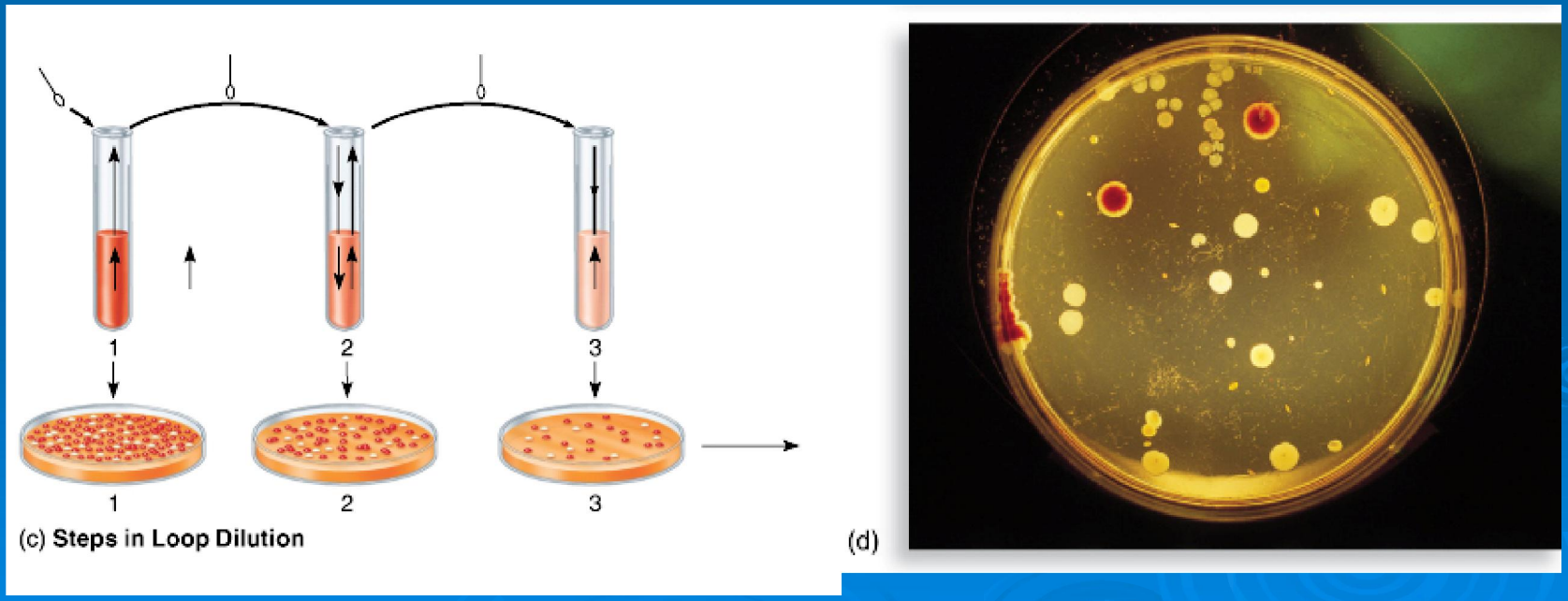


Figure 3.3 c,d

Spread Plate Method

- Spread plate method- small volume of liquid, diluted sample pipette on to surface of the medium and spread around evenly by a sterile spreading tool

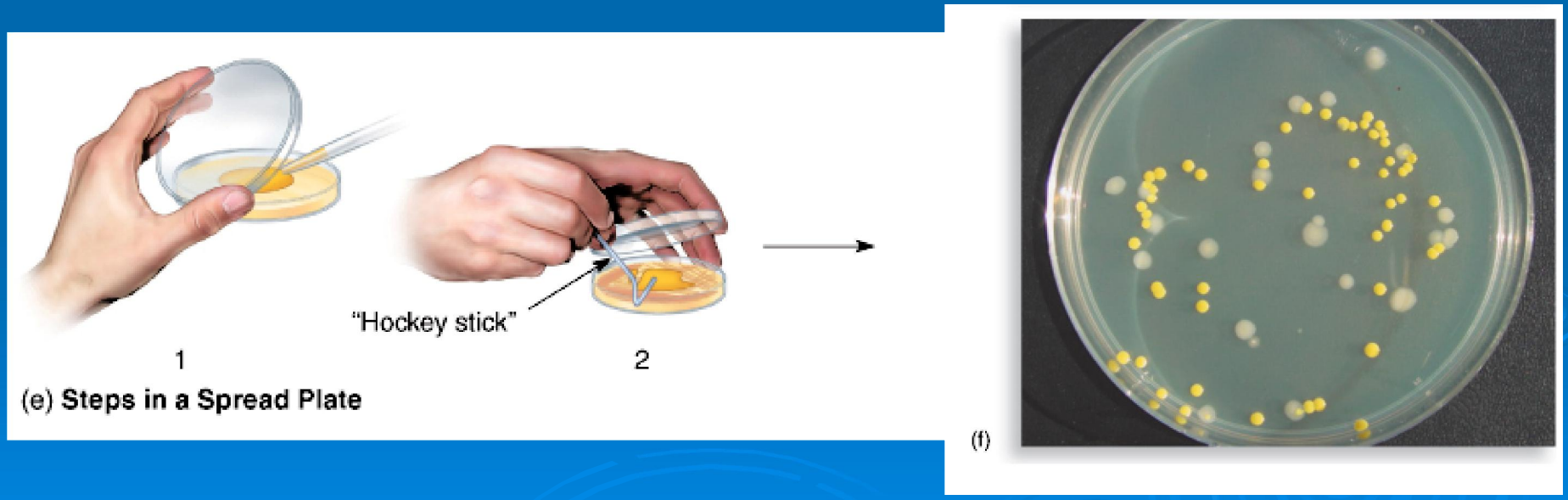


Figure 3.3 e,f

Media: Providing Nutrients in the Laboratory

- At least 500 different types
- Contained in test tubes, flasks, or Petri dishes
- Inoculated by loops, needles, pipettes, and swabs
- Sterile technique necessary
- Classification of media
  - Physical state
  - Chemical composition
  - Functional type

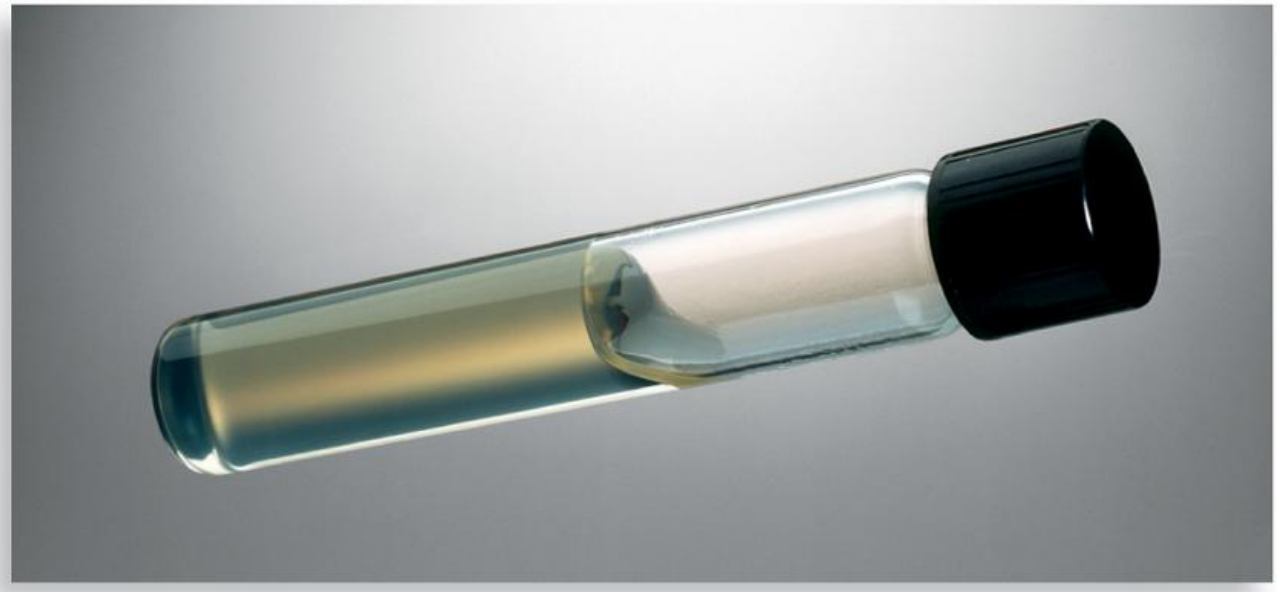
TABLE 3.1 Three Categories of Media Classification

Physical State (Medium's Normal Consistency)	Chemical Composition (Type of Chemicals Medium Contains)	Functional Type (Purpose of Medium)*
1. Liquid	1. Synthetic (chemically defined)	1. General purpose
2. Semisolid	2. Nonsynthetic (complex; not chemically defined)	2. Enriched
3. Solid (can be converted to liquid)		3. Selective
4. Solid (cannot be liquefied)		4. Differential
		5. Anaerobic growth
		6. Specimen transport
		7. Assay
		8. Enumeration

*Some media can serve more than one function. For example, a medium such as brain-heart infusion is general purpose and enriched; mannitol salt agar is both selective and differential; and blood agar is both enriched and differential.

Classification of Media by Physical State

- Liquid media: water-based solutions, do not solidify at temperatures above freezing, flow freely when container is tilted
  - Broths, milks, or infusions
  - Growth seen as cloudiness or particulates
- Semisolid media: clotlike consistency at room temperature
  - Used to determine motility and to localize reactions at a specific site
- Solid media: a firm surface on which cells can form discrete colonies
  - Liquefiable and nonliquefiable
  - Useful for isolating and culturing bacteria and fungi



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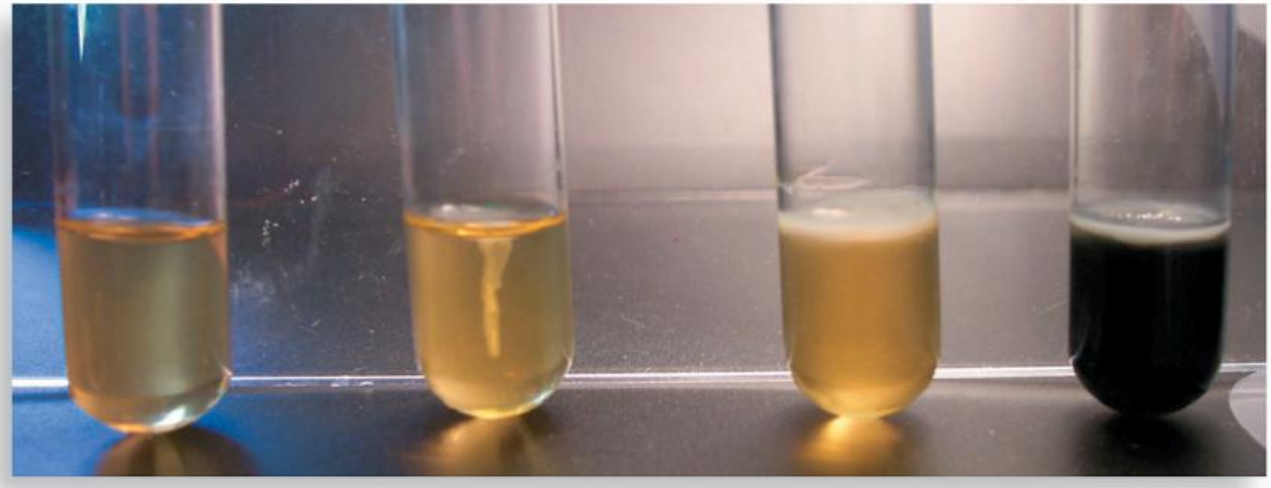


(b)

Figure 3.4

Classification of Media by Chemical Content

- Synthetic media- compositions are precisely chemically defined
- Complex (nonsynthetic) media- if even just one component is not chemically definable

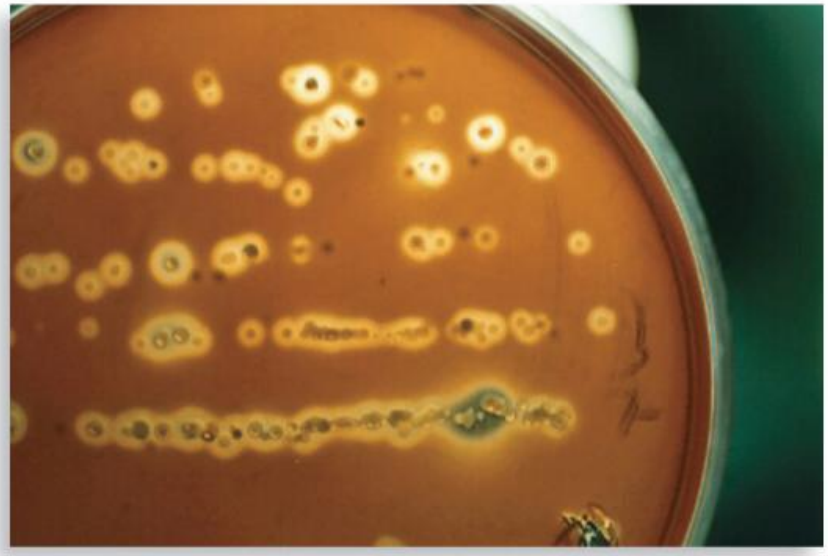
Classification of Media by Function

- General purpose media- to grow as broad a spectrum of microbes as possible
  - Usually nonsynthetic
  - Contain a mixture of nutrients to support a variety of microbes
  - Examples: nutrient agar and broth, brain-heart infusion, trypticase soy agar (TSA).

Enriched Media

- **Enriched media**- contain complex organic substances (for example blood, serum, **growth factors**) to support the growth of **fastidious bacteria**. Examples: blood agar, Thayer-Martin medium (chocolate agar)

(a)



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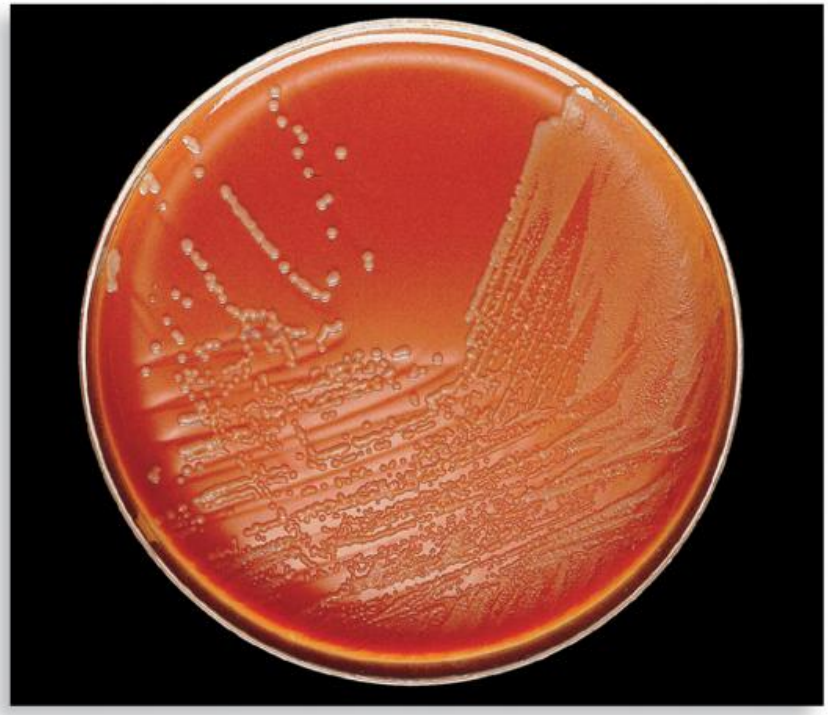

Figure 3.6

Selective and Differential Media

- **Selective media-** contains one or more agents that inhibit the growth of certain microbes but not others. Example: Mannitol salt agar (MSA), MacConkey agar, Hektoen enteric (HE) agar.
- **Differential media-** allow multiple types of microorganisms to grow but display visible differences among those microorganisms. MacConkey agar can be used as a differential medium as well.

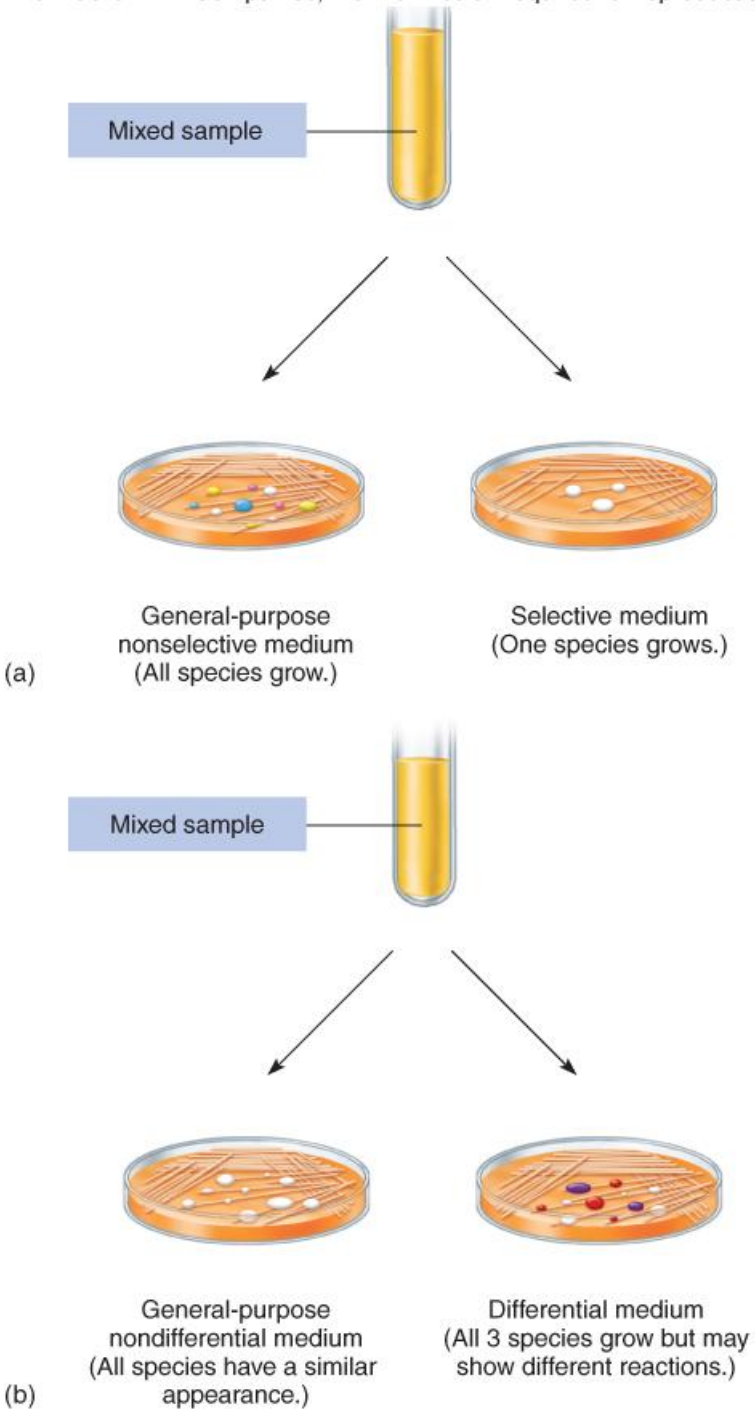

Figure 3.7

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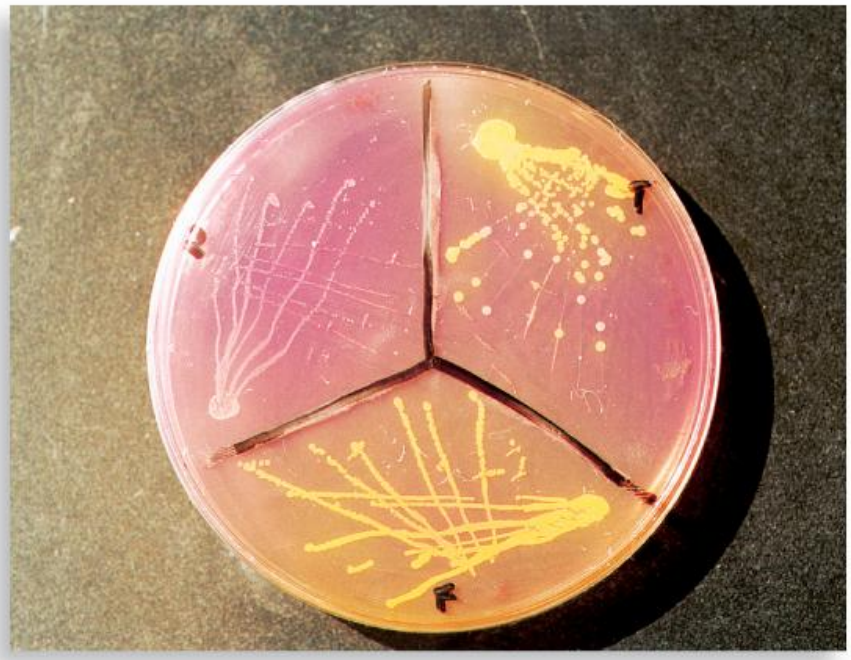

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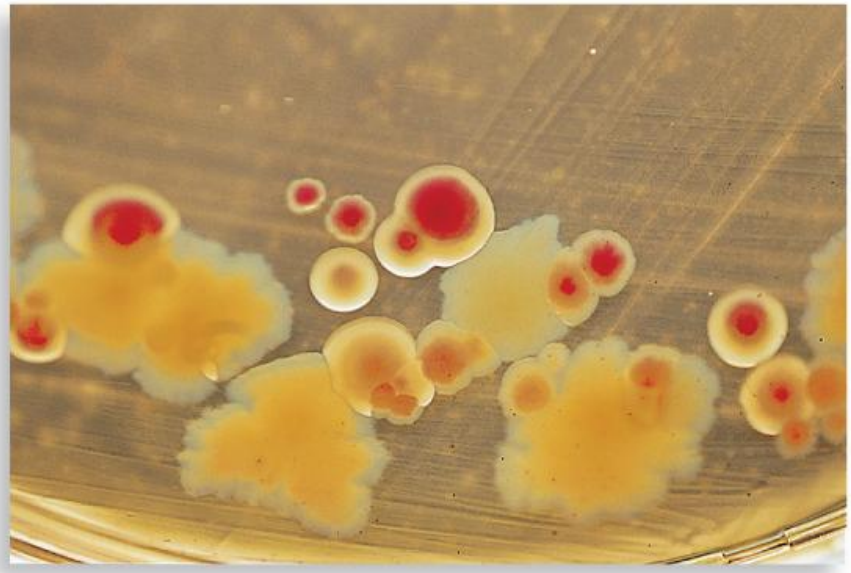
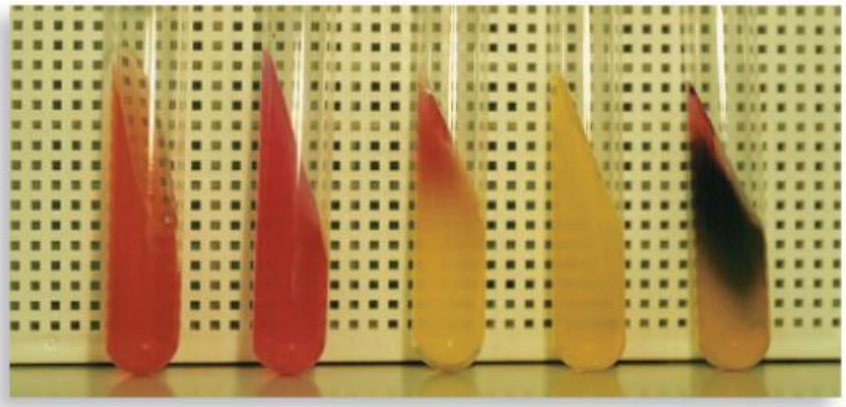


Figure 3.8



(a)

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Figure 3.9

Miscellaneous Media

- Reducing media- absorbs oxygen or slows its penetration in the medium; used for growing anaerobes or for determining oxygen requirements
- Carbohydrate fermentation media- contain sugars that can be fermented and a pH indicator; useful for identification of microorganisms
- Transport media- used to maintain and preserve specimens that need to be held for a period of time
- Assay media- used to test the effectiveness of antibiotics, disinfectants, antiseptics, etc.
- Enumeration media- used to count the numbers of organisms in a sample.

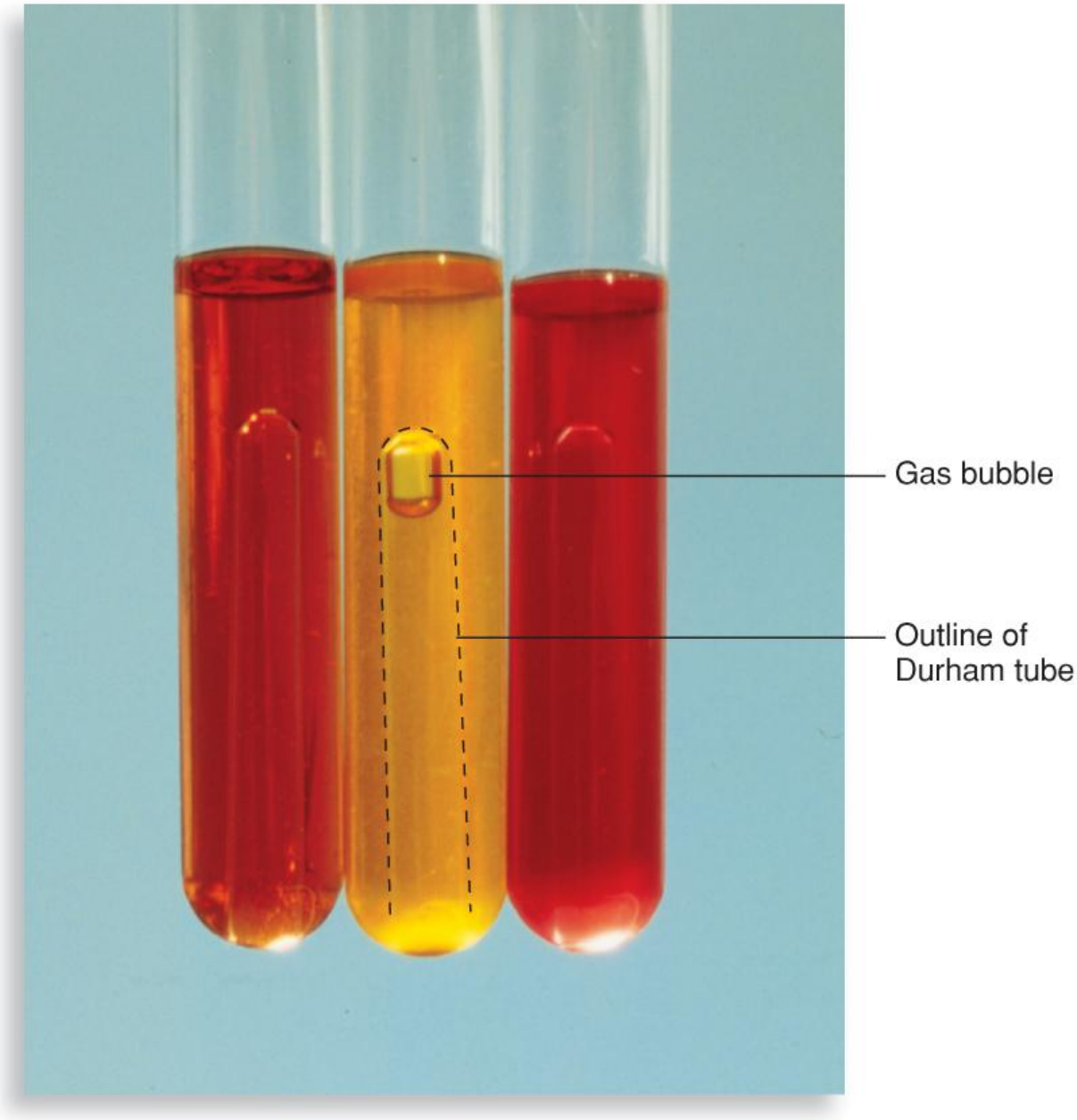

Figure 3.10

Incubation

- **Incubation**: an inoculated sample is placed in an incubator to encourage growth.
  - Usually in laboratories, between 20° and 40°C.
  - Can control atmospheric gases as well.
  - Can visually recognize growth as cloudiness in liquid media and colonies on solid media.
  - **Pure culture**- growth of only a single known species (also called **axenic**)
    - Usually created by **subculture**
  - **Mixed culture-** holds two or more identified species
  - **Contaminated culture-** includes unwanted microorganisms of uncertain identity, or **contaminants**.

Inspection and Identification

- Inspection and identification: Using appearance as well as metabolism (biochemical tests) and sometimes genetic analysis or immunologic testing to identify the organisms in a culture.
- Cultures can be maintained using stock cultures
- Once cultures are no longer being used, they must be sterilized and destroyed properly.

3.2 The Microscope: Window on an Invisible Realm

- Two key characteristics of microscopes: magnification and resolving power
- Magnification
  - Results when visible light waves pass through a curved lens
  - The light experiences **refraction**
  - An image is formed by the refracted light when an object is placed a certain distance from the lens and is illuminated with light
  - The image is enlarged to a particular degree- the power of magnification

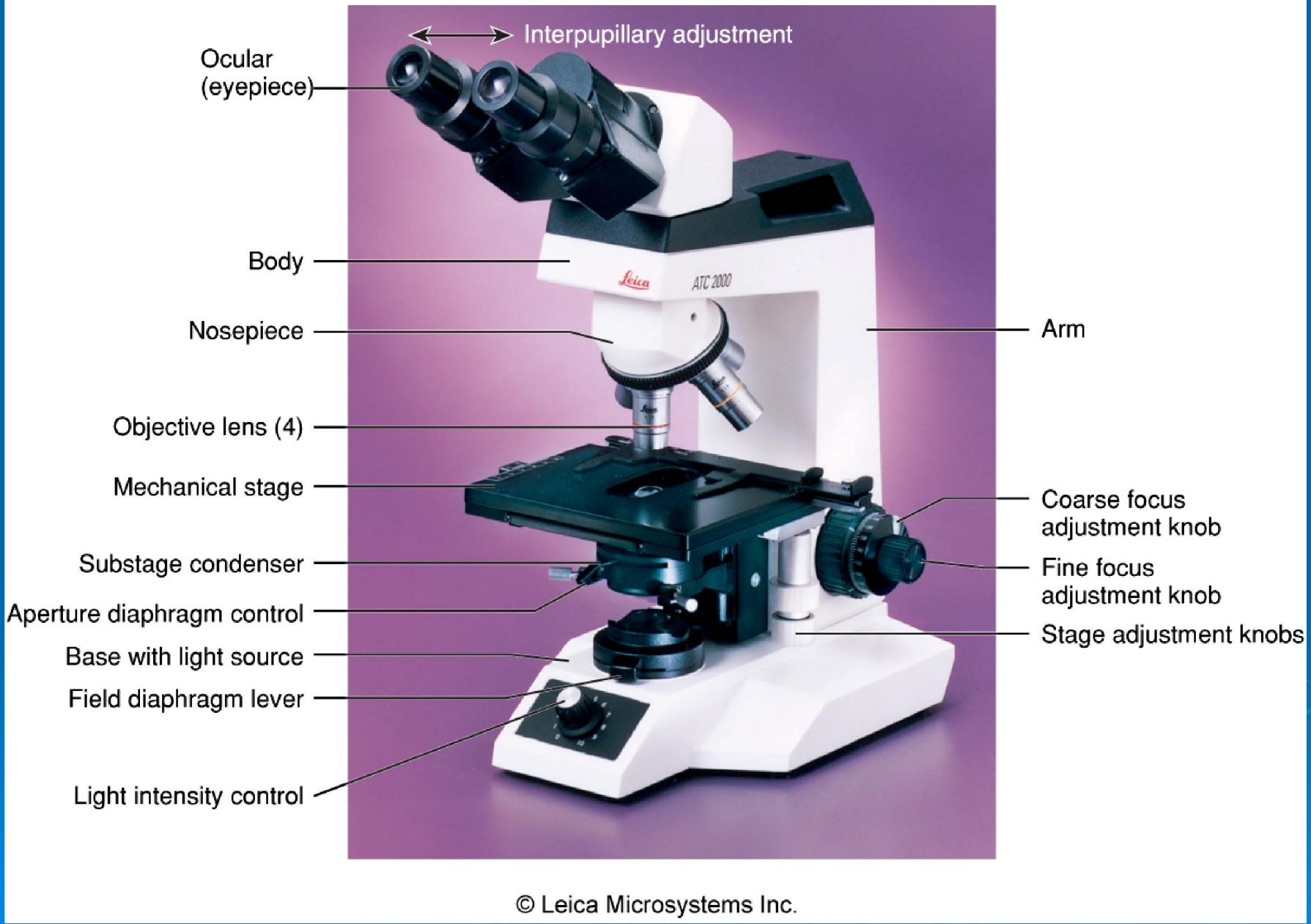

Figure 3.13

Principles of Light Microscopy

- Magnification- occurs in two phases
  - Objective lens- forms the **real image**
  - Ocular lens- forms the **virtual image**
  - Total power of magnification- the product of the power of the objective and the power of the ocular

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Administrator; 24.01.2008

Resolution

- Resolution- the ability to distinguish two adjacent objects or points from one another
- Also known as **resolving power**
  - Resolving power (RP) = $\frac{\text{Wavelength of light in nm}}{2 \times \text{Numerical aperture of objective lens}}$
  - Resolution distance= $\frac{0.61 \times \text{wavelength of light in nm}}{\text{Numerical aperture of objective lens}}$
  - Shorter wavelengths provide a better resolution
  - **Numerical aperture**- describes the relative efficiency

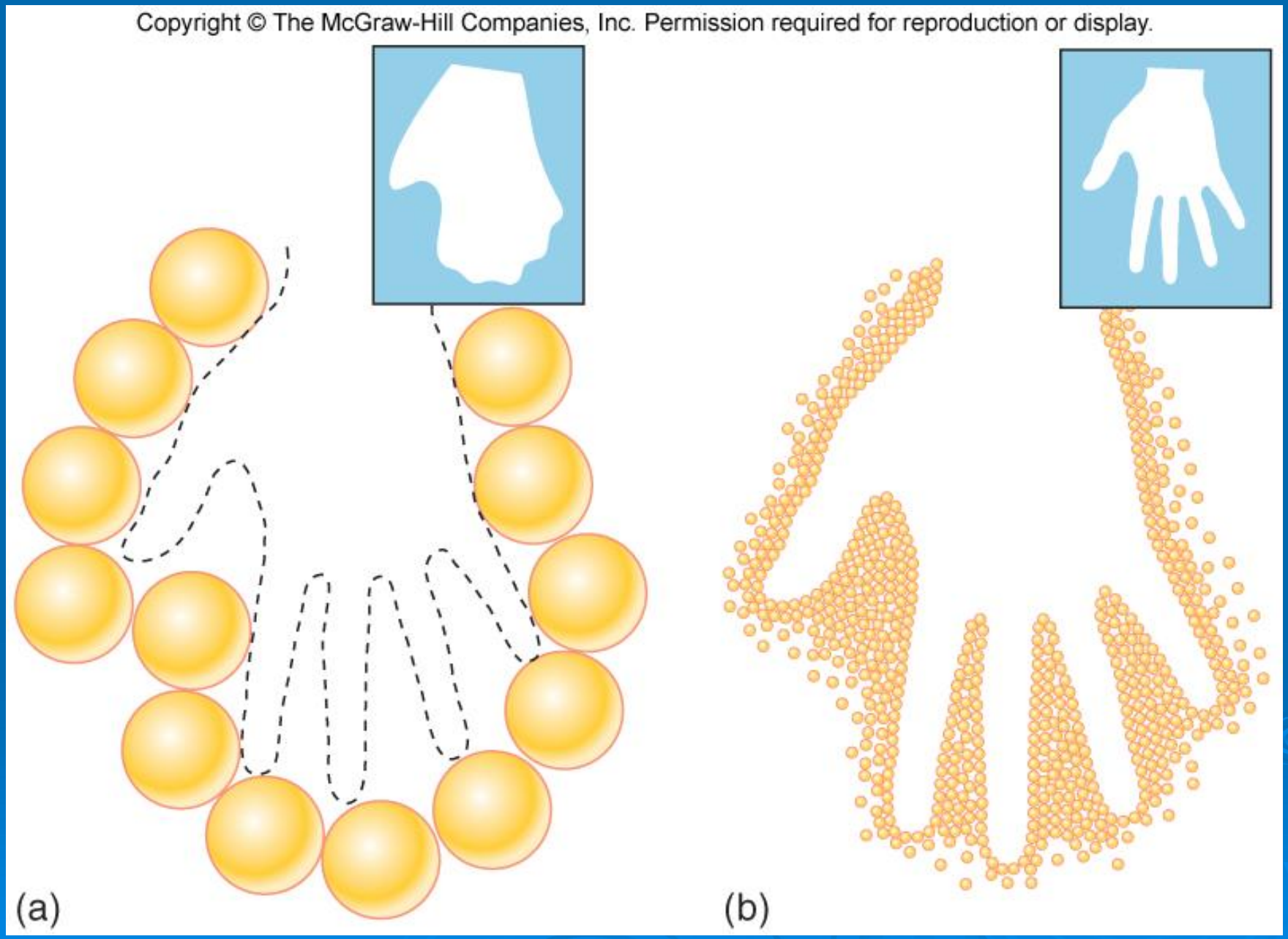


Figure 3.15

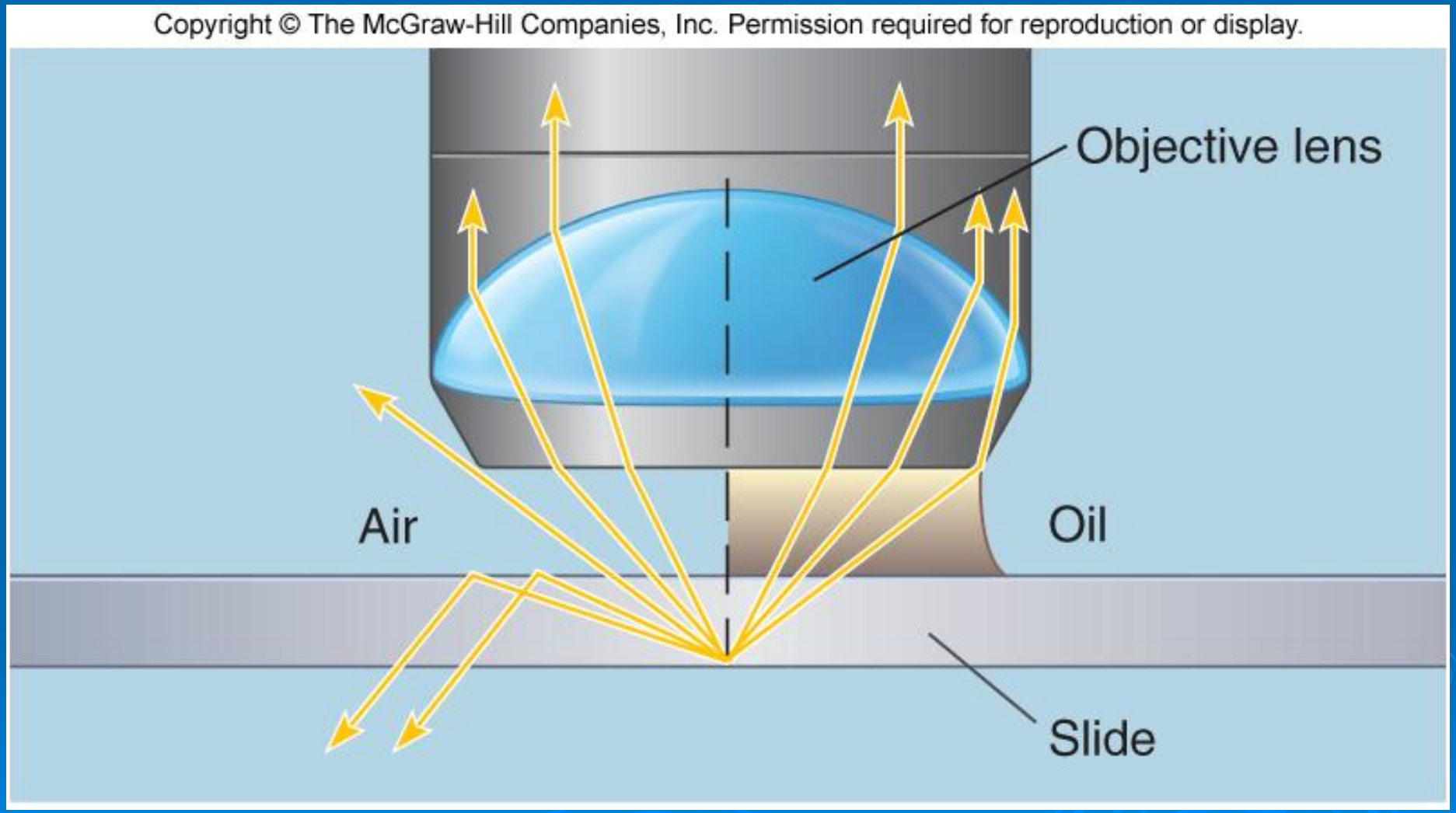


Figure 3.16

Magnification and Resolution

- Increased magnification decreases the resolution
- Adjusting the amount of light entering the condenser using an adjustable iris diaphragm or using special dyes help increase resolution at higher magnifications

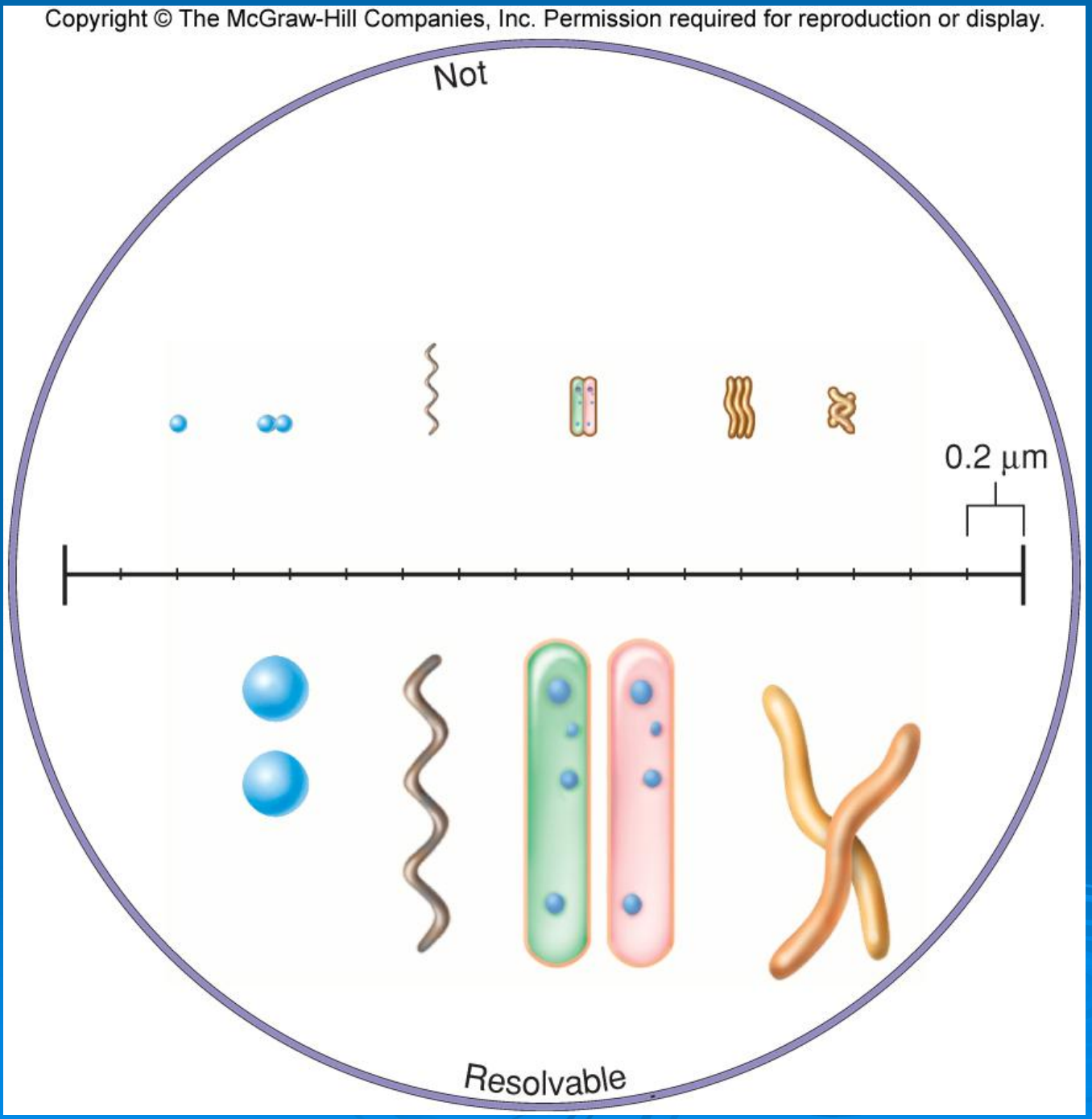


Figure 3.17

Variations on the Optical Microscope

- Visible light microscopes- optical microscopes that use visible light. Described by their field.
  - Four types: bright-field, dark-field, phase-contrast, and interference
- Other light microscopes include fluorescence microscopes and confocal microscopes

TABLE 3.5 Comparisons of Types of Microscopy

Microscope	Maximum Practical Magnification	Resolution	Important Features
Visible light as source of illumination			
Bright-field	2,000×	0.2 µm (200 nm)	Common multipurpose microscope for live and preserved stained specimens; specimen is dark, field is white; provides fair cellular detail
Dark-field	2,000×	0.2 µm	Best for observing live, unstained specimens; specimen is bright, field is black; provides outline of specimen with reduced internal cellular detail
Phase-contrast	2,000×	0.2 µm	Used for live specimens; specimen is contrasted against gray background; excellent for internal cellular detail
Differential interference	2,000×	0.2 µm	Provides brightly colored, highly contrasting, three-dimensional images of live specimens
Ultraviolet rays as source of illumination			
Fluorescent	2,000×	0.2 µm	Specimens stained with fluorescent dyes or combined with fluorescent antibodies emit visible light; specificity makes this microscope an excellent diagnostic tool
Confocal	2,000×	0.2 µm	Specimens stained with fluorescent dyes are scanned by laser beam; multiple images (optical sections) are combined into three-dimensional image by a computer; unstained specimens can be viewed using light reflected from specimen
Electron beam forms image of specimen			
Transmission electron microscope (TEM)	100,000×	0.5 nm	Sections of specimen are viewed under very high magnification; finest detailed structure of cells and viruses is shown
Scanning electron microscope (SEM)	650,000×	10 nm	Scans and magnifies external surface of specimen; produces striking three-dimensional image
Atomically sharp tip probes surface of specimen			
Atomic force microscope (AFM)	100,000,000×	0.01 Angstroms	Tip scans specimen and moves up and down with contour of surface; movement of tip is measured with laser and translated to image
Scanning tunneling microscope (STM)	100,000,000×	0.01 Angstroms	Tip moves over specimen while voltage is applied, generating current that is dependent on distance between tip and surface; atoms can be moved with tip

Bright-Field Microscopy

- Most widely used
- Forms its image when light is transmitted through the specimen
- The specimen produces an image that is darker than the surrounding illuminated field
- Can be used with live, unstained and preserved, stain specimens

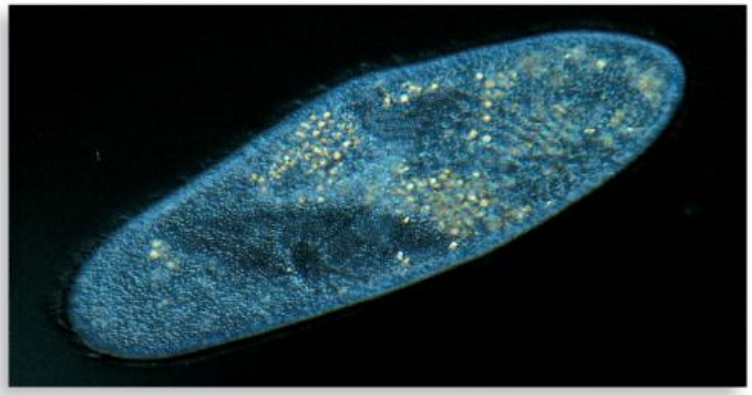
Dark-Field Microscopy

- A bright-field microscope can be adapted to a dark-field microscope by adding a stop to the condenser
- The stop blocks all light from entering the objective lens except for peripheral light
- The specimen produces an image that is brightly illuminated against a dark field
- Effective for visualizing living cells that would be distorted by drying or heat or that can’t be stained with usual methods
- Does not allow for visualization of fine internal details of cells

Phase-Contrast Microscopy

- Transforms subtle changes in light waves passing through a specimen into differences in light intensity
- Allows differentiation of internal components of live, unstained cells
- Useful for viewing intracellular structures such as bacterial spores, granules, and organelles

(a)



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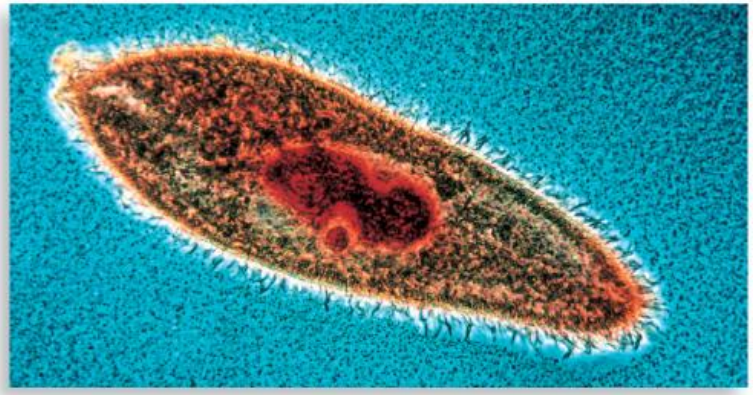

(c)

Figure 3.18

Interference Microscopy

- Interference Microscopy
  - Uses a differential-interference contrast (DIC) microscope
  - Allows for detailed view of live, unstained specimens
  - Includes two prisms that add contrasting colors to the image
  - The image is colorful and three-dimensional

Figure 3.19

Fluorescence Microscopy

- Includes a UV radiation source and a filter that protects the viewer's eyes
- Used with dyes that show **fluorescence** under UV rays
- Forms a colored image against a black field
- Used in diagnosing infections caused by specific bacteria, protozoans, and viruses using fluorescent antibodies

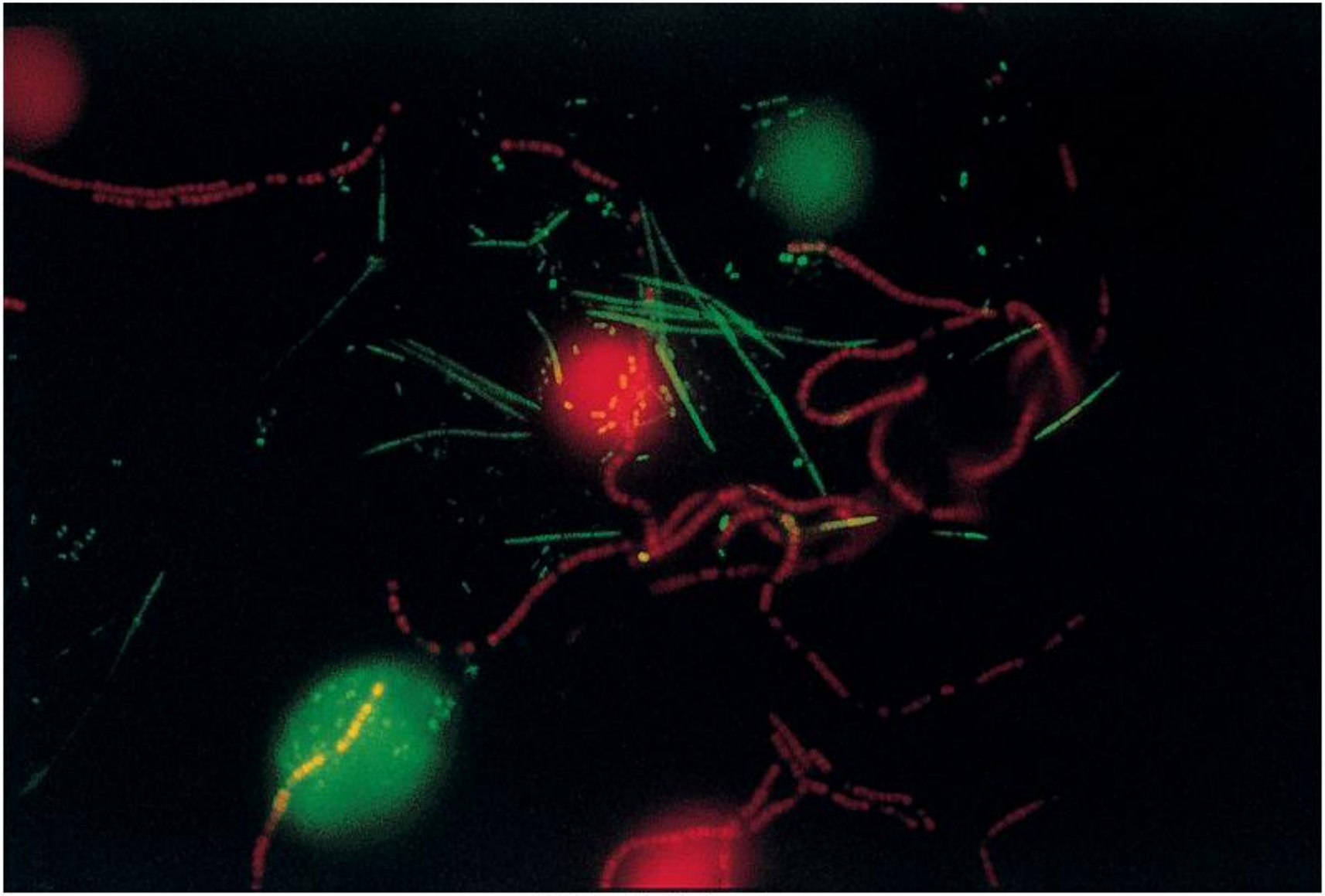

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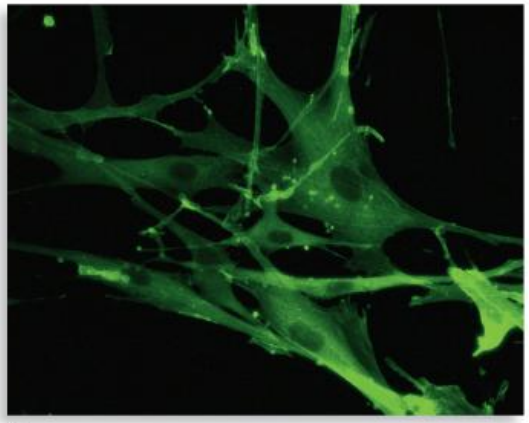
Figure 3.20

Confocal Microscopy

- Allows for viewing cells at higher magnifications using a laser beam of light to scan various depths in the specimen
- Most often used on fluorescently stained specimens

(a)



(b)

(c)

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Figure 3.21

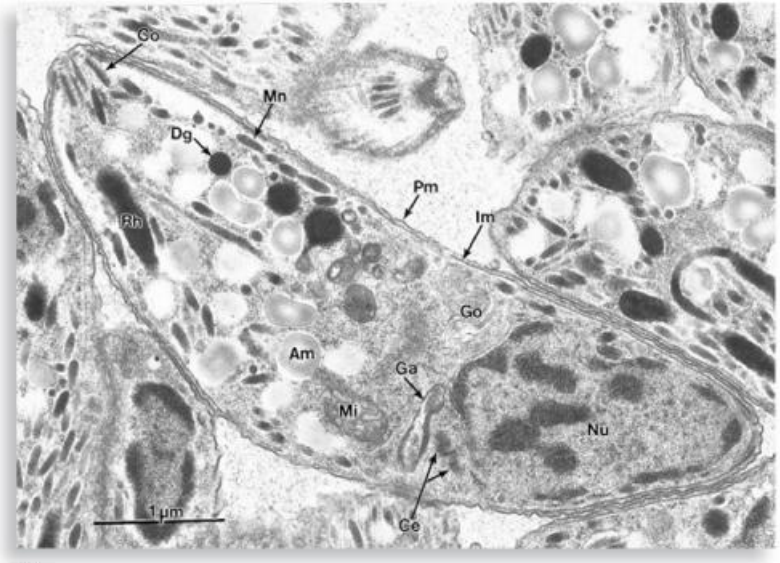
Electron Microscopy

- Originally developed for studying nonbiological materials
- Biologists began using it in the early 1930s
- Forms an image with a beam of electrons
  - Electrons travel in wavelike patterns 1,000 times shorter than visible light waves
  - This increases the resolving power tremendously
- Magnification can be extremely high (between 5,000X and 1,000,000X for biological specimens)
- Allows scientists to view the finest structure of cells
- Two forms: transmission electron microscope (TEM) and scanning electron microscope (SEM)

TEM

- Often used to view structures of cells and viruses
- Electrons are transmitted through the specimen
- The specimen must be very thin (20-100 nm thick) and stained to increase image contrast
- Dark areas of a TEM image represent thicker or denser parts

(a)



(b)

Figure 3.22

SEM

- Creates an extremely detailed three-dimensional view of all kinds of objects
- Electrons bombard the surface of a whole metal-coated specimen
- Electrons deflected from the surface are picked up by a sophisticated detector
- The electron pattern is displayed as an image on a television screen
- Contours of specimens resolved with SEM are very revealing and surprising

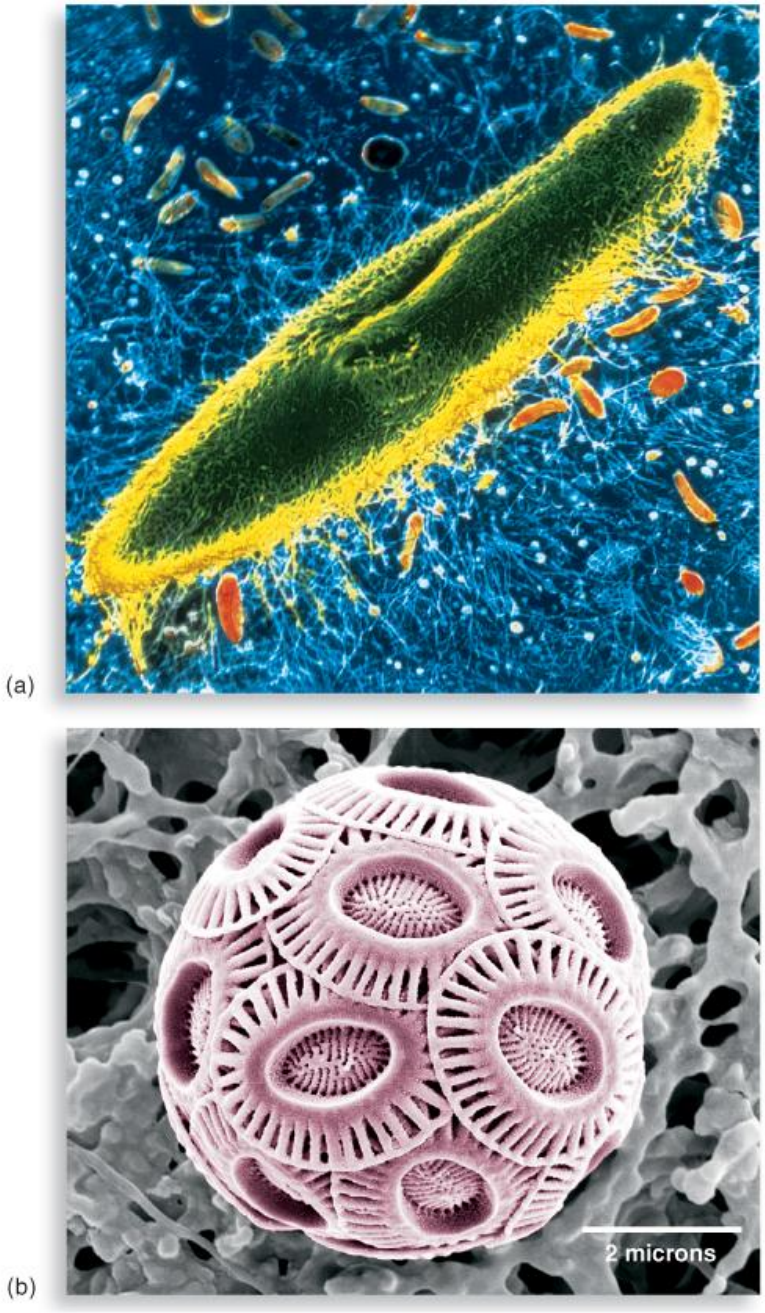


Figure 3.23

Preparing Specimens for Optical Microscopes

- Generally prepared by mounting a sample on a glass slide
- How the slide is prepared depends on
  - The condition of the specimen (living or preserved)
  - The aims of the examiner (to observe overall structure, identify microorganisms, or see movement)
  - The type of microscopy available

Living Preparations

- Wet mounts or hanging drop mounts
- Wet mount:
  - Cells suspended in fluid, a drop or two of the culture is then placed on a slide and overlaid with a cover glass
  - Cover glass can damage larger cells and might dry or contaminate the observer's fingers
- Hanging drop mount:
  - Uses a depression slide, Vaseline, and coverslip
  - The sample is suspended from the coverslip

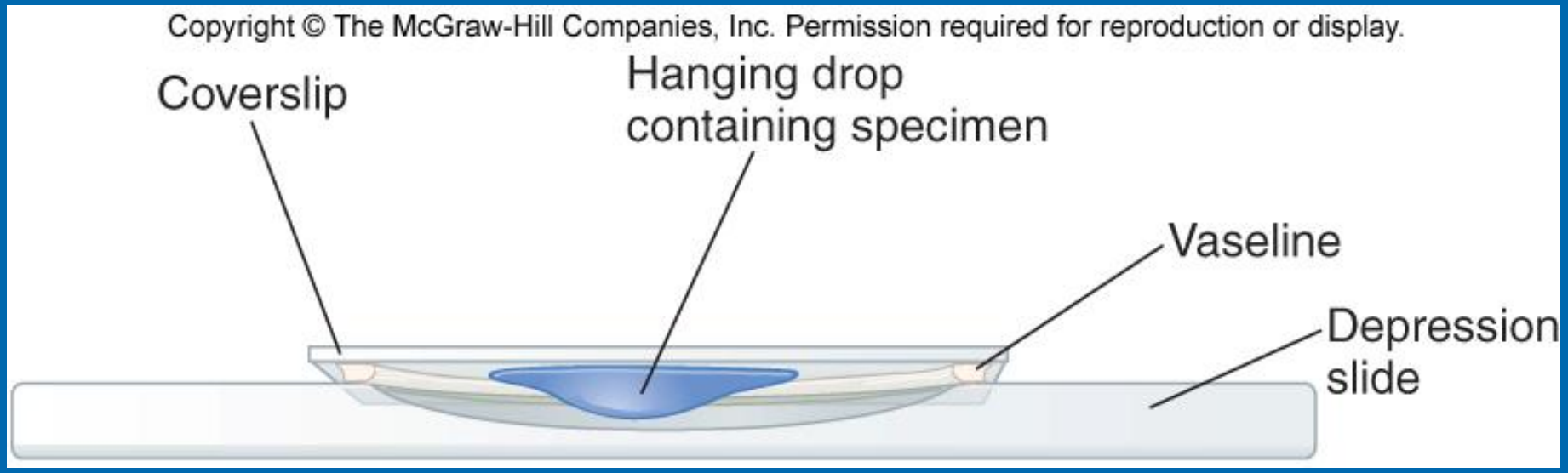
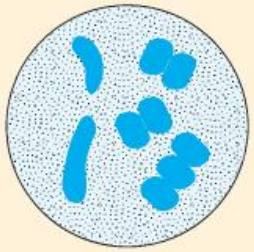


Figure 3.24

Fixed, Stained Smears

- Smear technique developed by Robert Koch
  - Spread a thin film made from a liquid suspension of cells and air-drying it
  - Heat the dried smear by a process called heat fixation
  - Some cells are fixed using chemicals
- Staining creates contrast and allows features of the cells to stand out
  - Applies colored chemicals to specimens
  - Dyes become affixed to the cells through a chemical reaction
  - Dyes are classified as basic (cationic) dyes, or acidic (anionic) dyes.

TABLE 3.7 Comparison of Positive and Negative Stains

	Positive Staining	Negative Staining
Appearance of cell	Colored by dye	Clear and colorless
		
Background	Not stained (generally white)	Stained (dark gray or black)
Dyes employed	Basic dyes: Crystal violet Methylene blue Safranin Malachite green	Acidic dyes: Nigrosin India ink
Subtypes of stains	Several types: Simple stain Differential stains Gram stain Acid-fast stain Spore stain Special stains Capsule Flagella Spore Granules Nucleic acid	Few types: Capsule Spore

Positive and Negative Staining

- **Positive staining**: the dye sticks to the specimen to give it color
- **Negative staining**: The dye does not stick to the specimen, instead settles around its boundaries, creating a silhouette.
  - Nigrosin and India ink commonly used
  - Heat fixation not required, so there is less shrinkage or distortion of cells
  - Also used to accentuate the capsule surrounding certain bacteria and yeasts

Simple Stains

- Require only a single dye
  - Examples include malachite green, crystal violet, basic fuchsin, and safranin
  - All cells appear the same color but can reveal shape, size, and arrangement

Differential Stains

- Use two differently colored dyes, the primary dye and the counterstain
  - Distinguishes between cell types or parts
  - Examples include Gram, acid-fast, and endospore stains

Gram Staining

- The most universal diagnostic staining technique for bacteria
- Differentiation of microbes as gram positive(purple) or gram negative (red)

Acid-Fast Staining

- Important diagnostic stain
- Differentiates acid-fast bacteria (pink) from non-acid-fast bacteria (blue)
- Important in medical microbiology

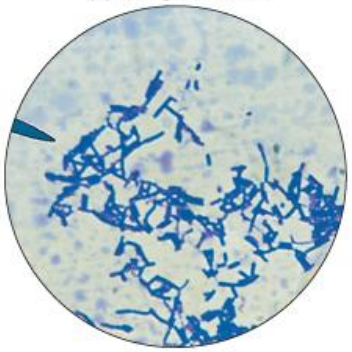
Endospore Stain

- Dye is forced by heat into resistant bodies called spores or endospores
- Distinguishes between the stores and the cells they come from (the **vegetative** cells)
- Significant in medical microbiology

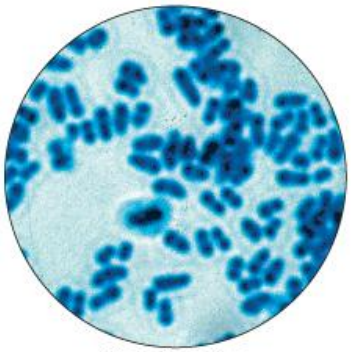
Special Stains

- Used to emphasize certain cell parts that aren't revealed by conventional staining methods
- Examples: capsule staining, flagellar staining

(a) **Simple Stains**

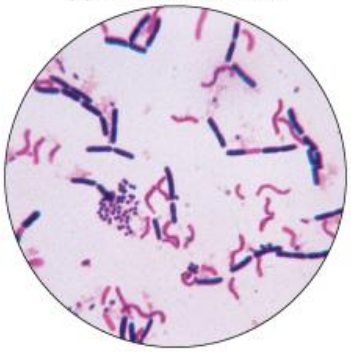


Crystal violet
stain of *Escherichia coli*

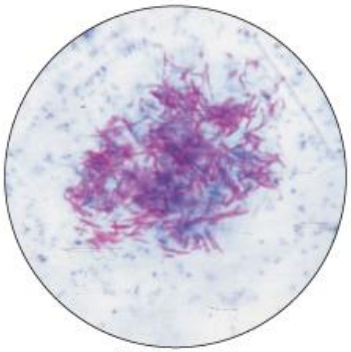


Methylene blue
stain of *Corynebacterium*

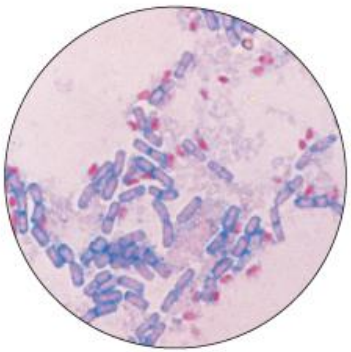
(b) **Differential Stains**



Gram stain
Purple cells are gram-positive.
Red cells are gram-negative.

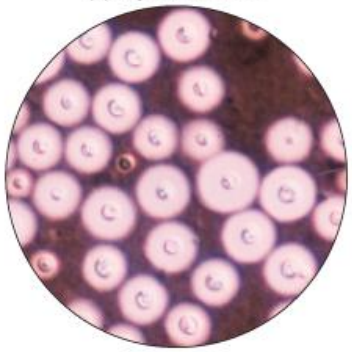


Acid-fast stain
Red cells are acid-fast.
Blue cells are non-acid-fast.

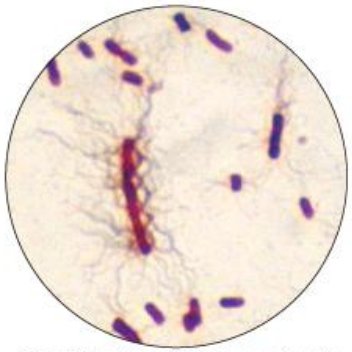


Spore stain, showing spores (red)
and vegetative cells (blue)

(c) **Special Stains**



India ink capsule stain of
Cryptococcus neoformans



Flagellar stain of *Proteus vulgaris*
A basic stain was used to
build up the flagella.

Figure 3.25