# Methods of Microbiology

Staining

Media

Microscopy

# Staining

- Increase contrast of microorganisms
- Classified into types of stains
  - Simple stain: one dye, one step
    - Negative stain
    - Positive stain
  - Differential stain: distinguish one group from another
  - Structural or special stains

# Dyes

- Colorizing agents
- Organic salts with positive and negative charges
- One ion is colored -chromophore
- Basic dye: positive ion is colored
  - $MeBlue^{+}$ $Cl^{-}$
- Acidic dye: negative ion is chromphore

# Basic Dye

- Works best at neutral or alkaline pH
- Bacterial cell wall has slight negative charge at pH 7
- Basic dye (positive) attracted to cell wall ( negative)
- Combines with negatively charged molecules
- Crystal violet, methylene blue, safranin

# Acidic Dye

- Chromophore repelled by negative cell wall
- Background stained, bacteria colorless
- Negative stain-look at size, shape
  - Less distortion since heat isn't used
- Acidic dyes stain bacteria if grown at lower pH
- Eosin, India ink

# Simple Stains

- One dye, one step
- Direct  (positive) stain using basic dye
  - Shape and arrangement of cells
  - Stains cells

- Negative stain using acidic dye
  - Less distortion of size and shape
  - No heat used
  - Stains background

# Differential Stains

- More than one dye
- Gram stain, acid fast
  - Distinguish and classify bacteria according to cell wall
- Primary dye
- Decolorizing step
  - Removes dye from certain cells
- Counter stain

# Special/ Structural Stains

- Identify structures within or on cells
  - Capsule stain
  - Endospore stain
  - Flagellar stain

- Different parts of cell are stained different colors

# Media

- Culture media-nutrients for growth of microbes
- Inoculum-organism put on medium

- Pure culture-colony resulting from growth of one cell
  - Streak plates

# Living vs Nonliving

- Viruses, few bacteria
- Living host-eggs, tissue cells
- *Mycobacterium leprae* –armadillos
- Most microbes grow on nonliving media

# Chemically Defined

- Exact chemical composition known

- Chemoheterotrophs
  - Glucose
    - carbon source
    - energy source

## TABLE 6.2 A Chemically Defined Medium for Growing a Typical Chemoheterotroph, Such as *E. coli*

| Constituent | Amount |
|---|---|
| Glucose | 5.0 g |
| Ammonium phosphate, monobasic ($NH_4H_2PO_4$) | 1.0 g |
| Sodium chloride (NaCl) | 5.0 g |
| Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) | 0.2 g |
| Potassium phosphate, dibasic ($K_2HPO_4$) | 1.0 g |
| Water | 1 liter |

TABLE 6.3 **A Chemically Defined Medium for Growing a Fastidious Chemoheterotrophic Bacterium, Such as *Neisseria gonorrhoeae***

| Constituent | Amount | Constituent | Amount |
|---|---|---|---|
| Carbon and energy sources | | Amino acids | |
| Glucose | 9.1 g | Cysteine | 1.5 g |
| Starch | 9.1 g | Arginine, proline (each) | 0.3 g |
| Sodium acetate | 1.8 g | Glutamic acid, methionine (each) | 0.2 g |
| Sodium citrate | 1.4 g | Asparagine, isoleucine, serine (each) | 0.2 g |
| Oxaloacetate | 0.3 g | Cystine | 0.06 g |
| Salts | | Organic growth factors | |
| Potassium phosphate, dibasic ($K_2HPO_4$) | 12.7 g | Calcium pantothenate | 0.02 g |
| Sodium chloride (NaCl) | 6.4 g | Thiamine | 0.02 g |
| Potassium phosphate, monobasic ($KH_2PO_4$) | 5.5 g | Nicotinamide adenine dinucleotide | 0.01 g |
| Sodium bicarbonate ($NaHCO_3$) | 1.2 g | Uracil | 0.006 g |
| Potassium sulfate ($K_2SO_4$) | 1.1 g | Biotin | 0.005 g |
| Sodium sulfate ($Na_2SO_4$) | 0.9 g | Hypoxanthine | 0.003 g |
| Magnesium chloride ($MgCl_2$) | 0.5 g | Reducing agent | |
| Ammonium chloride ($NH_4Cl$) | 0.4 g | Sodium thioglycolate | 0.00003 g |
| Potassium chloride (KCl) | 0.4 g | Water | 1 liter |
| Calcium chloride ($CaCl_2$) | 0.006 g | | |
| Ferric nitrate [$Fe(NO_3)_3$] | 0.006 g | | |

SOURCE: R. M. Atlas, *Handbook of Microbiological Media*, Ann Arbor, MI: CRC Press, 1993.

# Complex Media

- Used for most microorganisms
- Cannot write formula for each ingredient
- C,N,energy, S requirements
  - Peptones

- Vitamins, other growth factors
  - Extracts-yeast or beef
  - Supplement N & C sources

# Complex Media

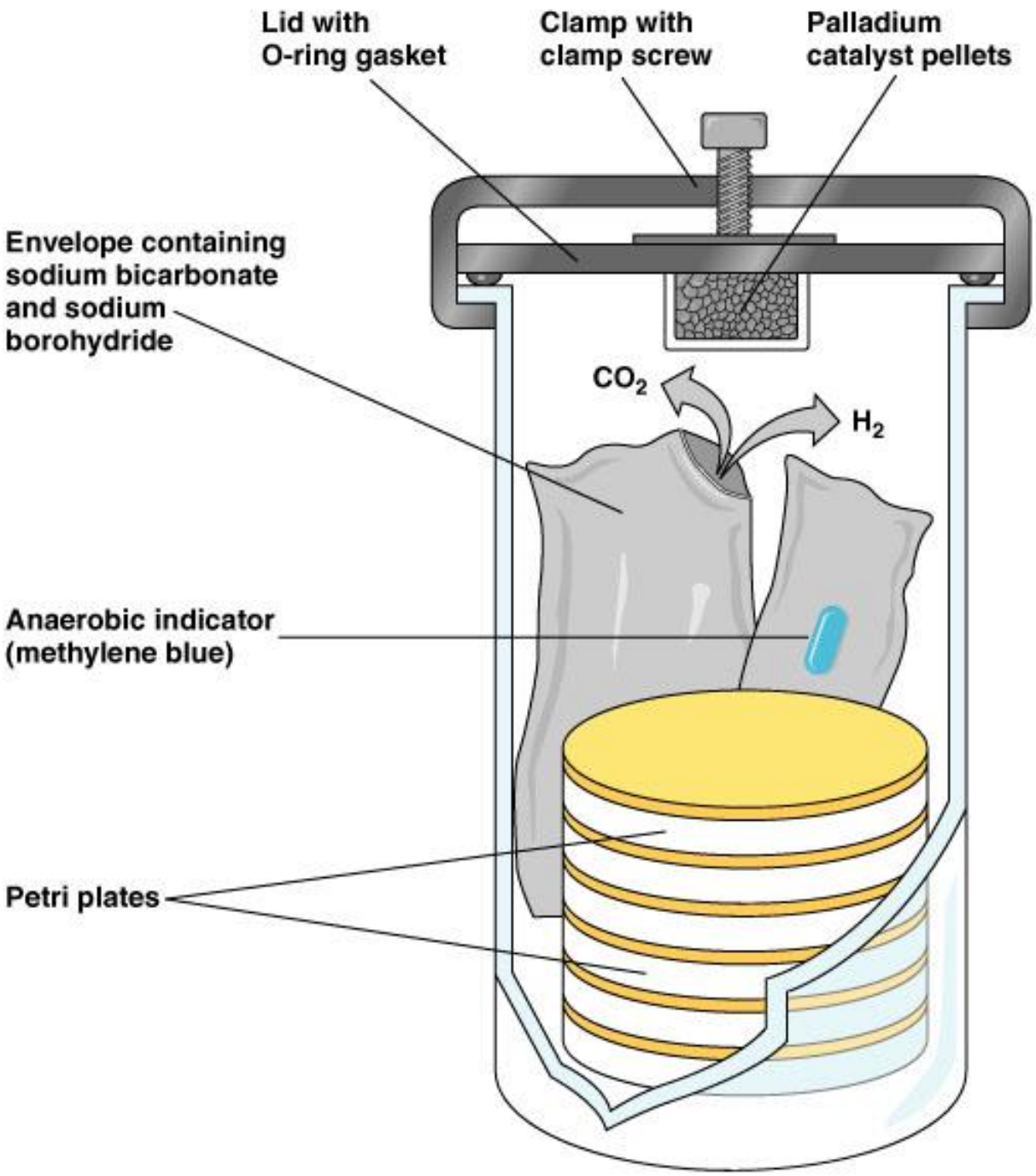
- Nutrient broth –liquid form
- Nutrient agar –solid form
  - Plate (Petri dish)
    - Lid fits over bottom
    - Excludes airborne contaminants
  - Deep
  - Slant in a tube
    - Stock cultures
    - Larger surface area

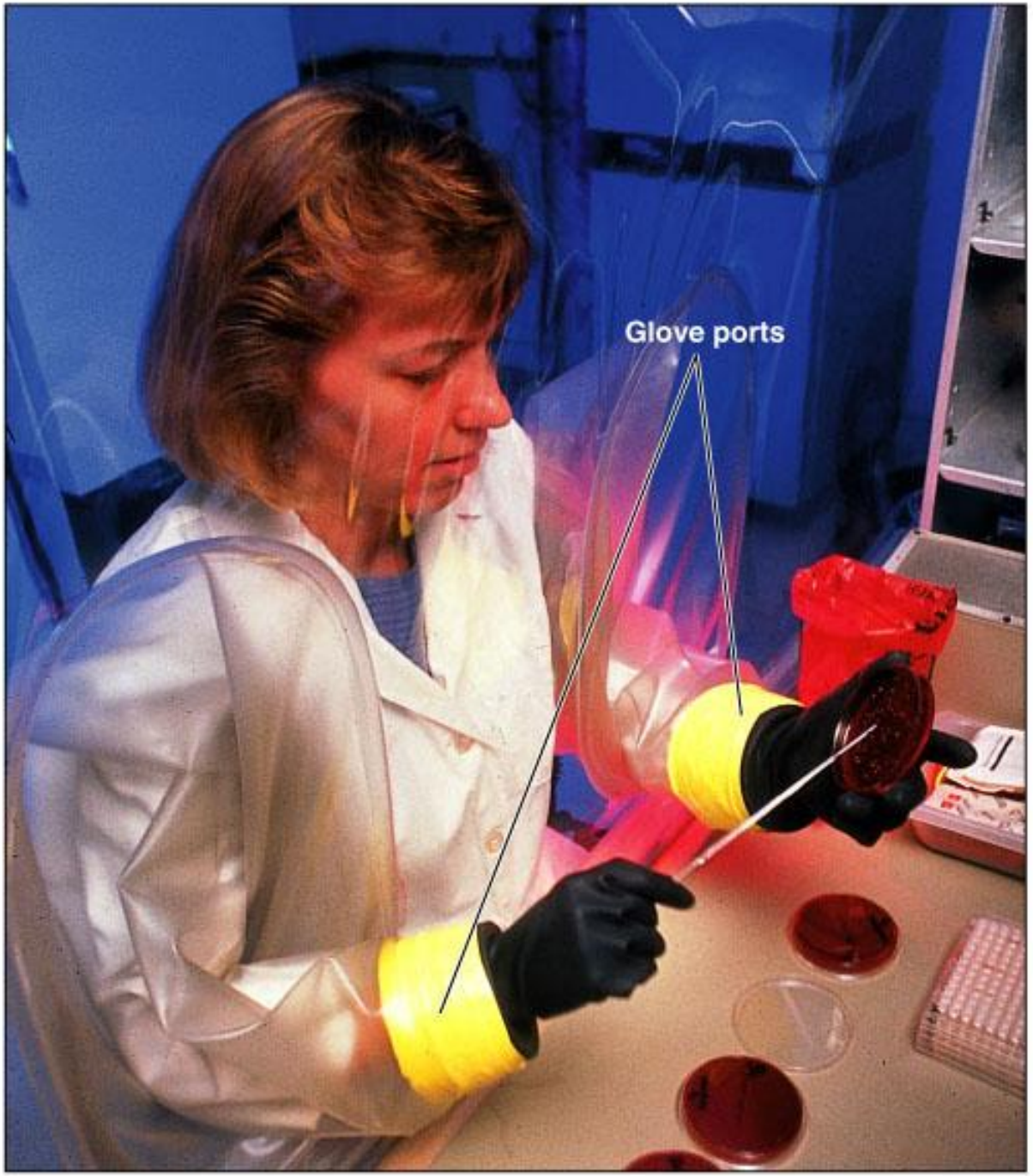
## TABLE 6.4 Composition of Nutrient Agar, a Complex Medium for the Growth of Heterotrophic Bacteria

| Constituent | Amount |
|---|---|
| Peptone (partially digested protein) | 5.0 g |
| Beef extract | 3.0 g |
| Sodium chloride | 8.0 g |
| Agar | 15.0 g |
| Water | 1 liter |

# Anaerobic Methods

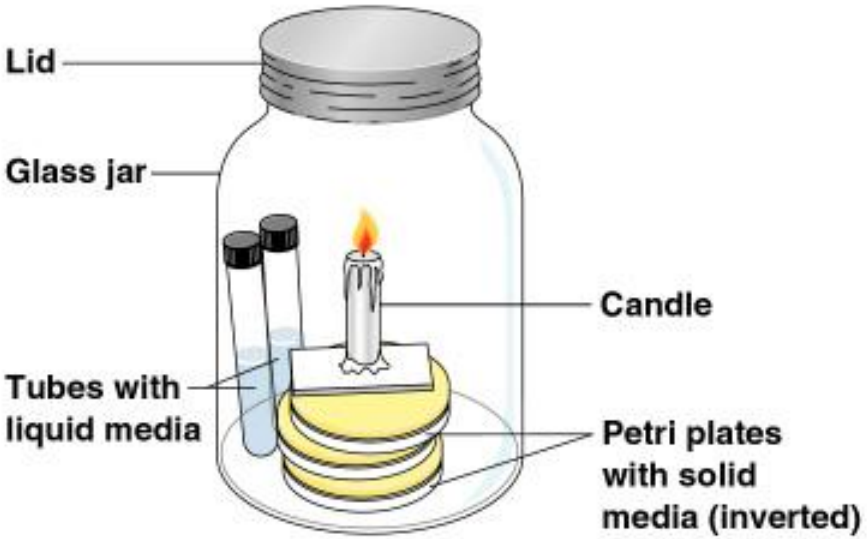
- Reducing media
  - Substance combines with oxygen
  - Ties it up
- Anaerobic jar
  - Use a packet that creates anaerobic environment
- Use both in lab





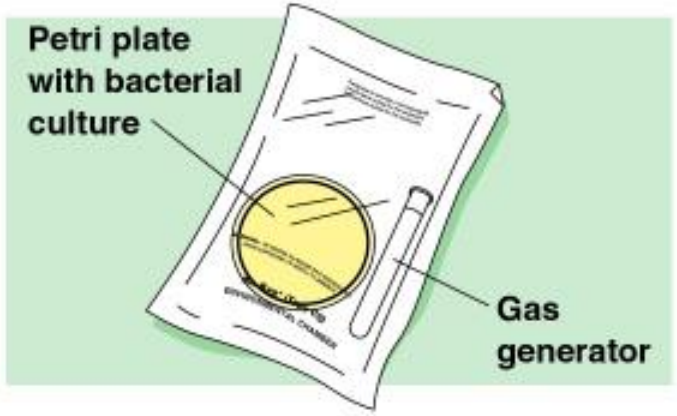
# Candle Jar

- Reduce oxygen levels
- Provides more $CO_2$
- Microaerophilics




**(a) Candle jar**

**Plates and tubes inoculated with, for example, *Neisseria meningitidis* are placed in a jar with a lighted candle, and the jar is sealed. This will provide a $CO_2$ atmosphere of approximately 3%.**




**(b) $CO_2$-generating packet**

**The packet consists of a bag containing a Petri plate and a $CO_2$ gas generator. The gas generator is crushed to mix the chemicals it contains and start the reaction that produces $CO_2$. This gasreduces the oxygen concentration in the bag to about 5% and provides a $CO_2$ concentration of about 10%.**

# Selective and Differential Media

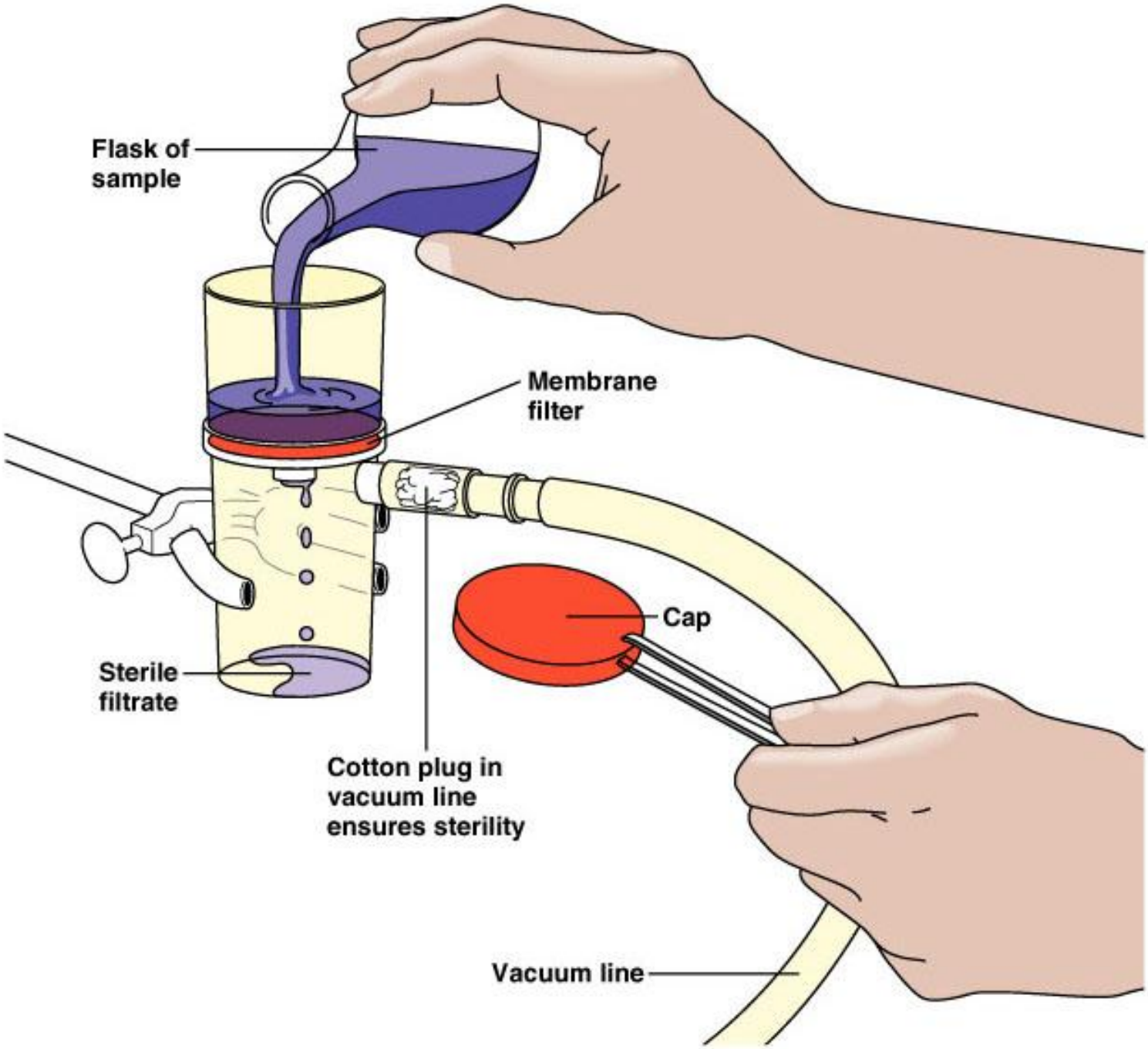
- Selective
  - Suppresses growth of unwanted bacteria
  - Encourages growth of desired bacteria

- Differential
  - Most grow
  - Can distinguish  desired organisms from others

| TABLE 6.5 | **Culture Media** |
|---|---|
| **Type** | **Purpose** |
| Chemically defined | Growth of chemoautotrophs and photoautotrophs, and microbiological assays. |
| Complex | Growth of most chemoheterotrophic organisms. |
| Reducing | Growth of obligate anaerobes. |
| Selective | Suppression of unwanted microbes; encouraging desired microbes. |
| Differential | Differentiation of colonies of desired microbes from others. |
| Enrichment | Similar to selective media but designed to increase numbers of desired microbes to detectable levels. |

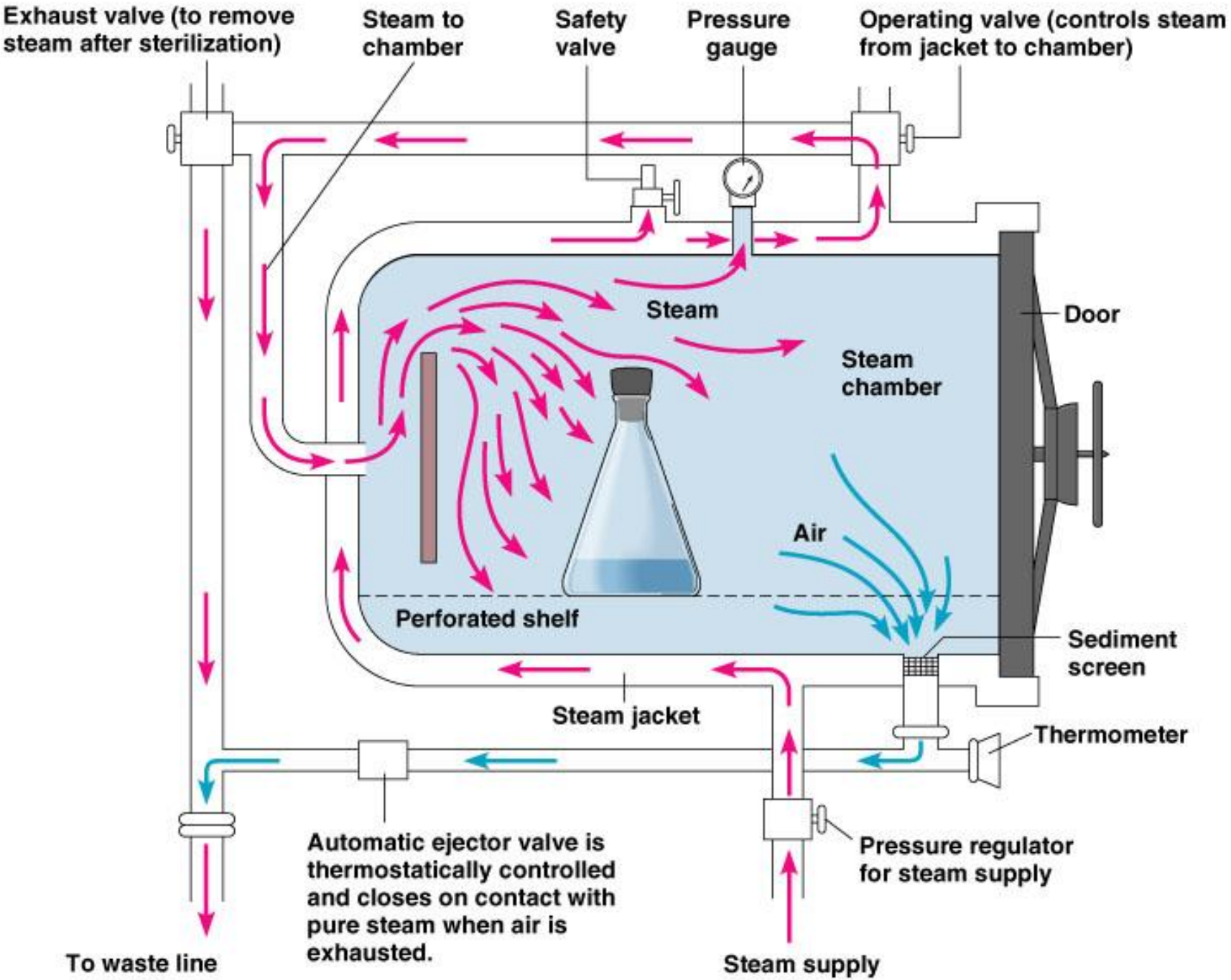
# Filtration

- Passage of liquid through screen device
- Pores small enough to retain microbes
- Sterilize heat sensitive materials
  - Culture media
  - Enzymes
  - Vaccines
  - Antibiotics
- Negative-uses vacuum
- Positive uses pressure



# Autoclave

- Uses temperature above boiling water
- Steam under pressure
- Preferred method unless material is damaged
- Higher the pressure, higher the temperature
- Need direct contact with steam
- 15 psi at 121 C for 15 mins



# Identifying Microorganisms

- Important for treatment of disease
- Lab quickly IDs specific organism
  - PCR Tests
- Cell wall composition, morphology, differential staining, biochemical testing

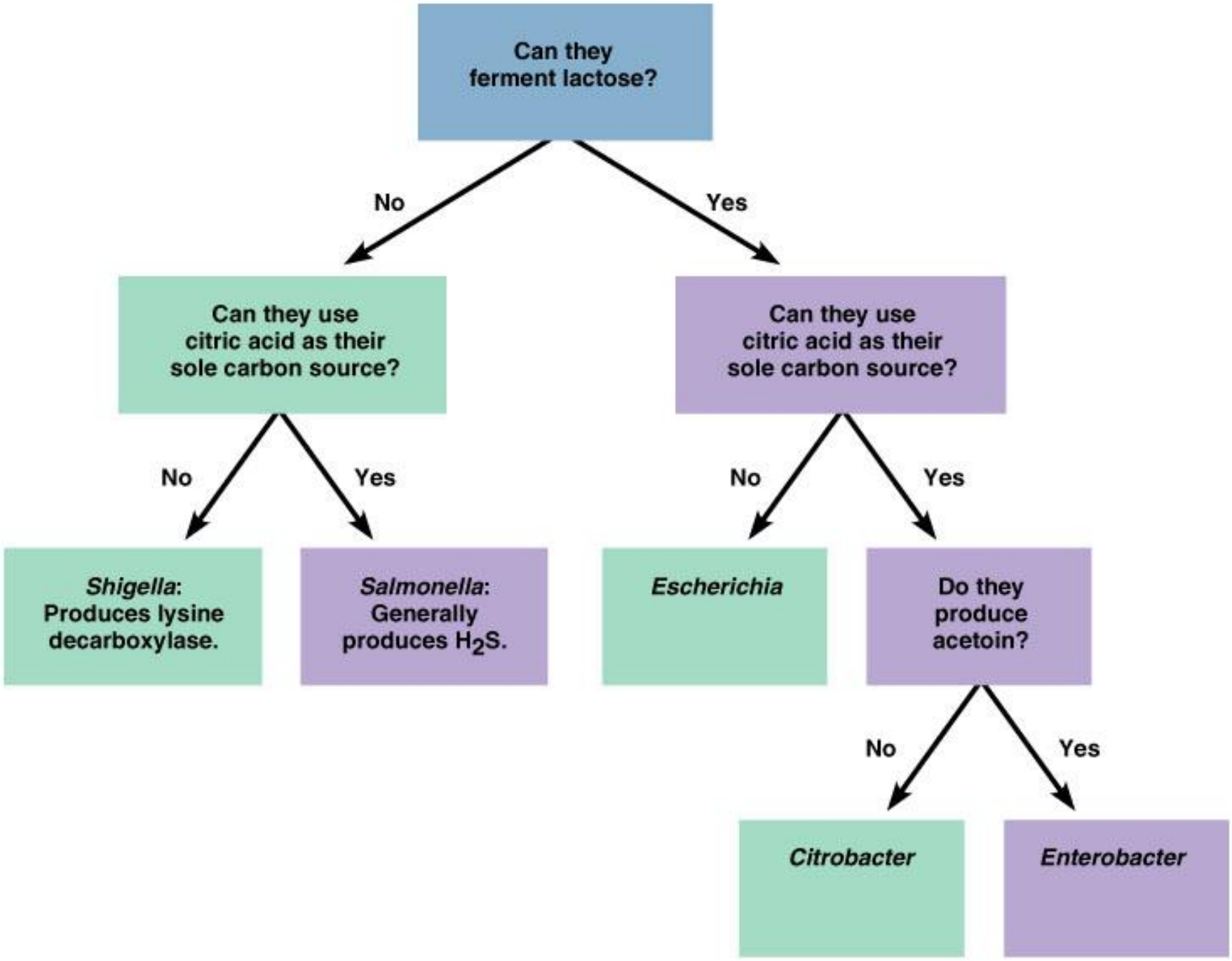
# ID in Laboratory

- Staining
  - Morphology and arrangement of cells
  - Presence of endospores, capsules etc.
  - Gram stain
  - Acid fast stain

# ID Organisms

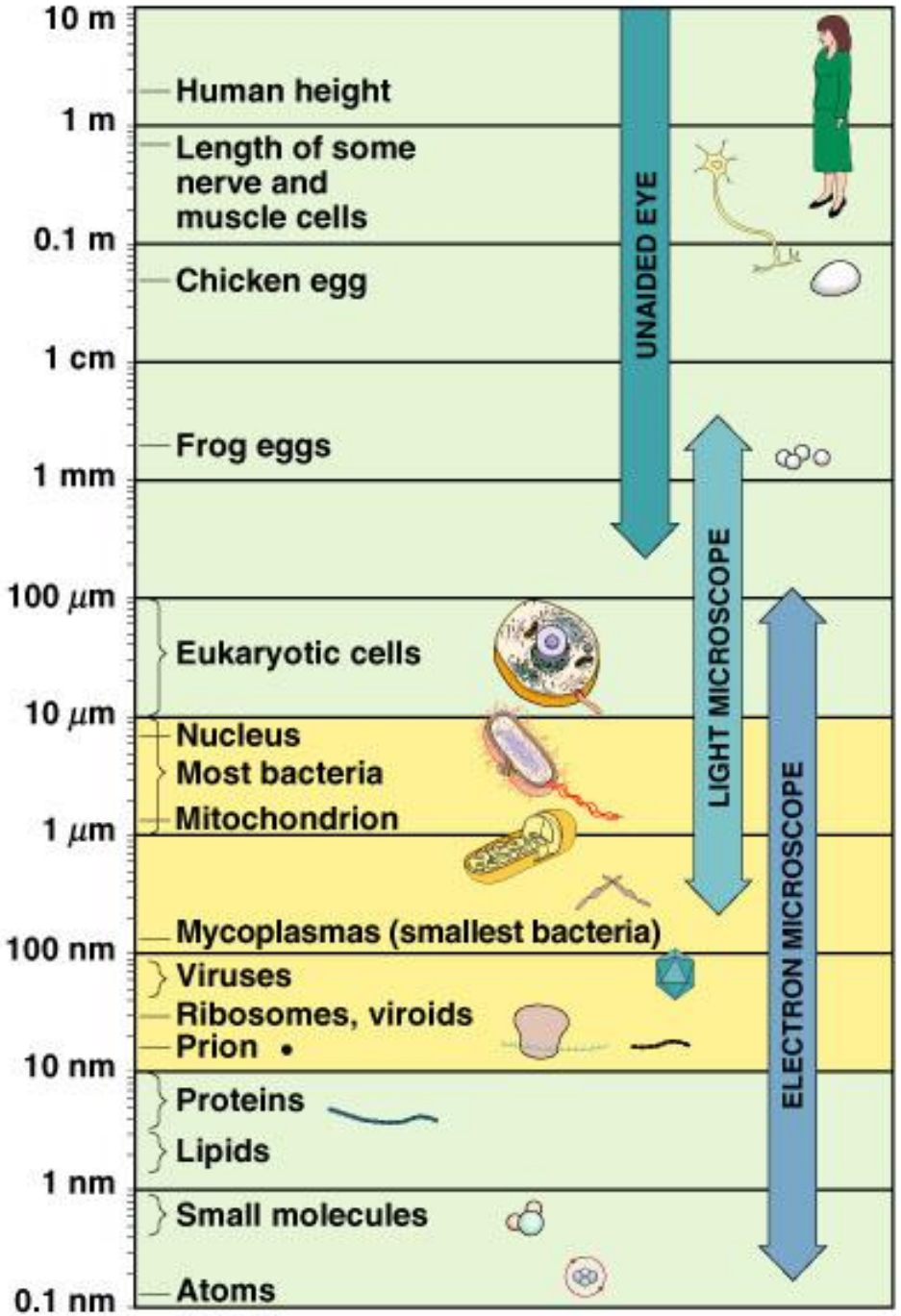
- Biochemical tests
  - Fermentation of selected nutrients
  - Rapid ID –several tests at same time

- Dichotomous key



# Microscope

- Simple vs compound
- Assigned scope
- Know parts & functions
- Proper use & care of scope



# Compound Microscope

- Light or electron microscope
  - Light for intact cells
  - Electron for details & internal structures
- Light scopes uses visible or UV light
- Both use lenses to magnify objects

# Lenses

- **Total magnification** of compound scope
  - Product of objective lens X ocular lens
  - 1500 X upper limit for light scope
  - Above this resolution does not improve

- Parfocal lenses
- Working distance

# Resolution

- Ability to distinguish 2 adjacent objects as separate and distinct
- Dictated by the physical properties of light
  - Determines what we are able to see distinctly with scope
- Limit is 0.2 um for our light scope

# Light Microscope

- Visible light, where?
- Average wavelength of 0.55um
  - Enters condenser lens
  - Light focused into a cone on slide

- Aperture diaphragm
  - Varies diameter of cone
  - Need more light with 100x lens


***Ocular lens*** *(eyepiece)*
Remagnifies the image formed by the objective lens

***Body tube***
Transmits the image from the objective lens to the ocular lens

***Arm***

***Objective lenses***
Primary lenses that magnify the specimen

***Stage***
Holds the microscope slide in position

***Condenser***
Focuses light through specimen

***Diaphragm*** Controls the amount of light entering the condenser

***Coarse focusing knob***

***Illuminator*** Light source

***Base***

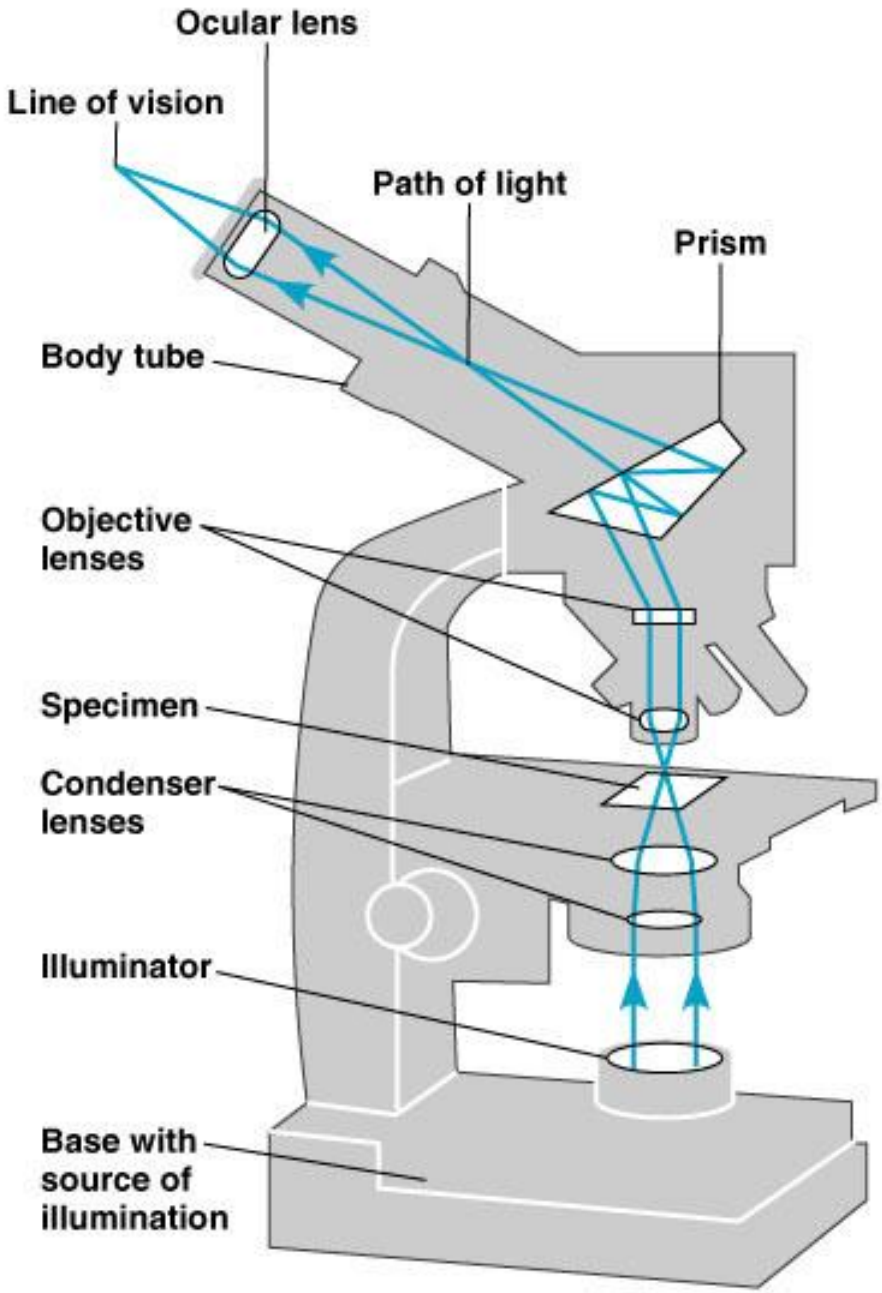
***Fine focusing knob***

**(a) Principal parts and functions**

# Light Path

- Light enters objective lens
  - Collect light from specimen
  - Forms a  magnified inverted image
  - Image magnified by ocular lens & passed to eye
- Total magnification (40x X 10x = 400x)

- Parfocal




**(b) The path of light (bottom to top)**

# Contrast

- Density between object & background
- Difference in light intensity
  - Absorption of light & scattering of light
  - Improves image detail

- Bacteria are colorless
  - Need to increase artificially by staining
- Contrast is property of specimen

# Resolution

- Distinguish detail within image
  - TV with clear picture-high resolution
- Property of lens system, measured as **resolving power**
- Closest that 2 points can be together and still seen as separate
- RP = $\frac{\text{wavelength of light}}{2 \text{ X NA}}$

# Resolving Power

- Function of numerical aperture: NA
  - Measure of light gathering ability
  - Stamped on side of lens
  - Generally lenses with higher magnification have higher NA

# Resolving Power

- Function of wavelength of light
  - Shorter wavelength increases resolution
- Refractive index of material between objective lens & specimen

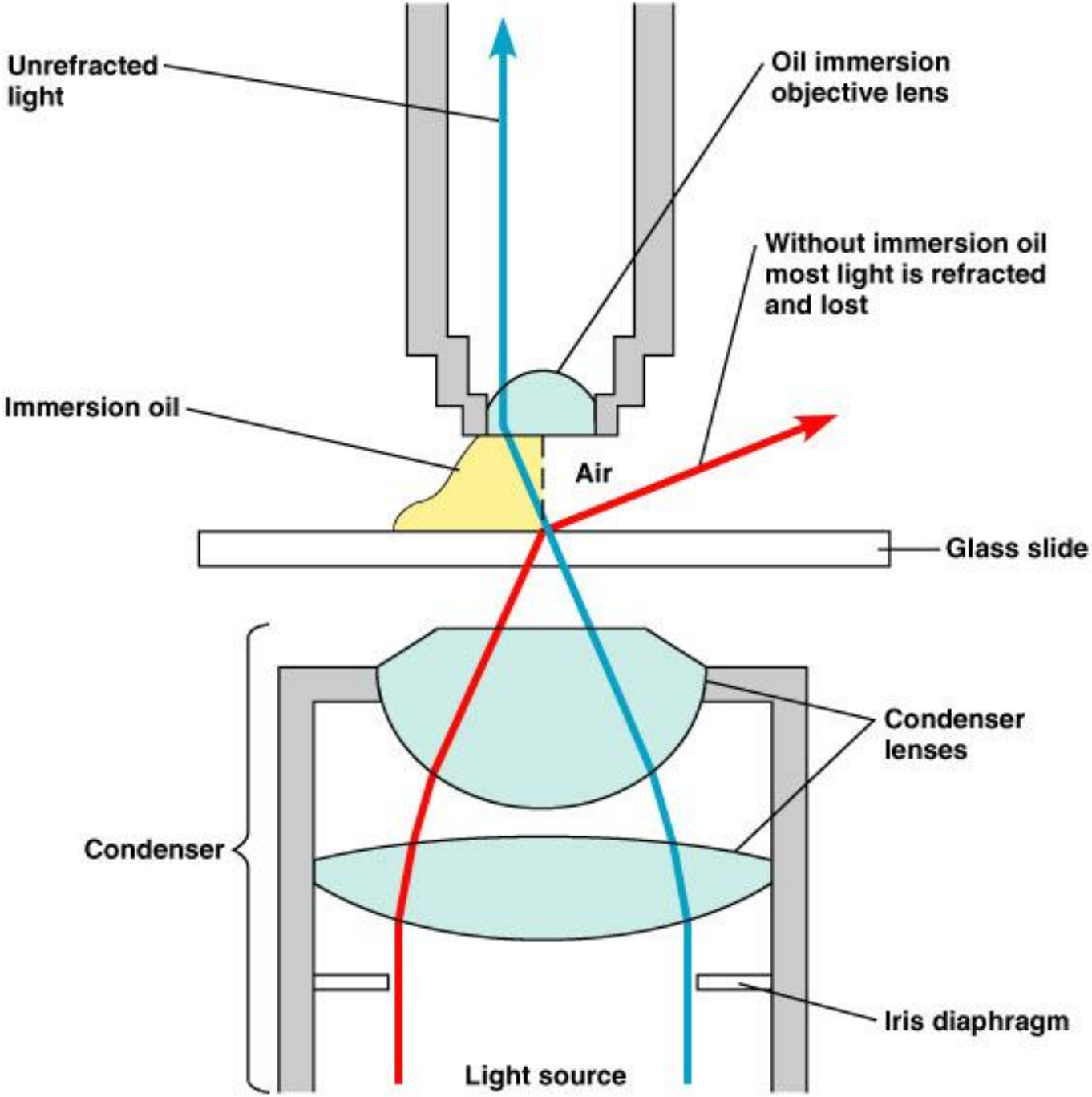
# Oil Immersion Lens

- Light bends (refracts) as it passes from glass into air
  - Some light does not enter this smaller objective lens
- Use oil between slide and 100x lens
  - Displaces air between lens and specimen
  - Glass and oil have same RI so less bending
  - Oil becomes part of the optics of glass
- Increases resolution

# Oil Immersion Lens

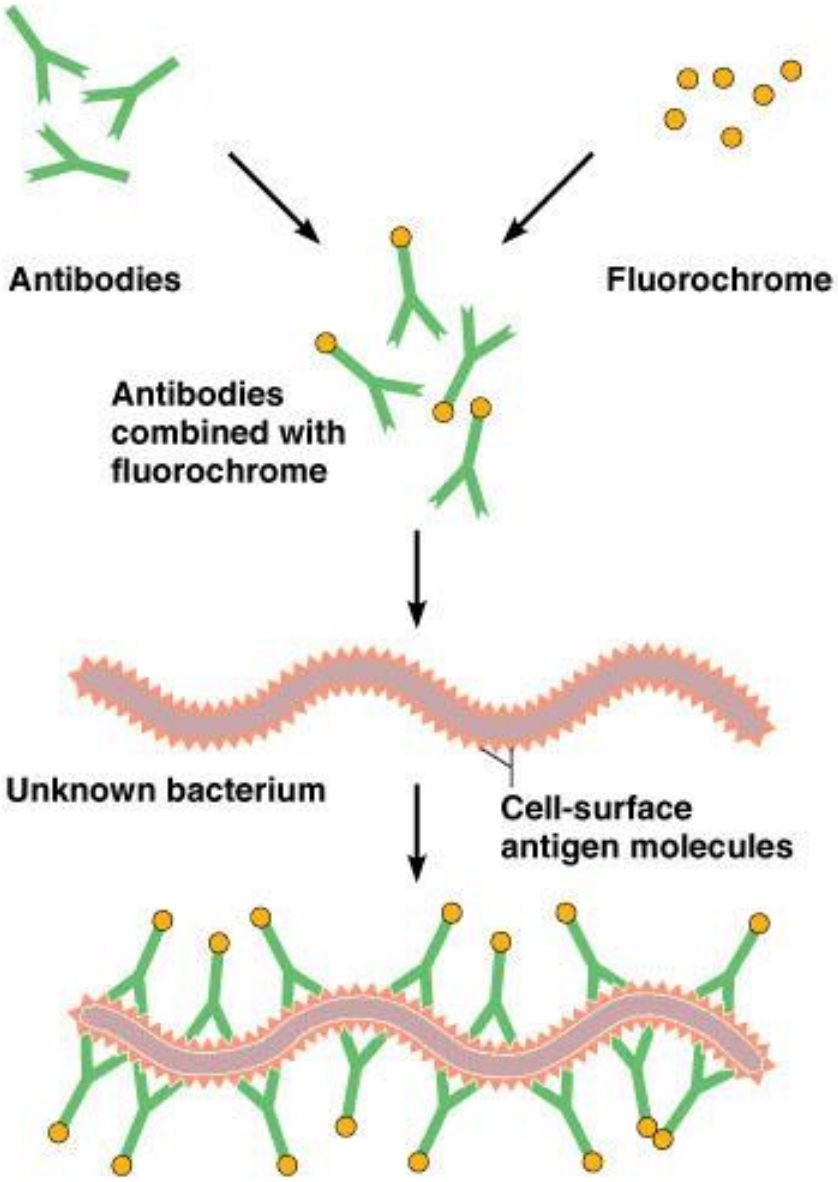
- Lens captures more light since light travels at same speed through oil as glass
  - Less refraction of light
  - Increase in NA (ability to capture light) of the 100x lens which increases resolution

- Summary: increased resolution
  - Increases illumination by decreasing refraction of light



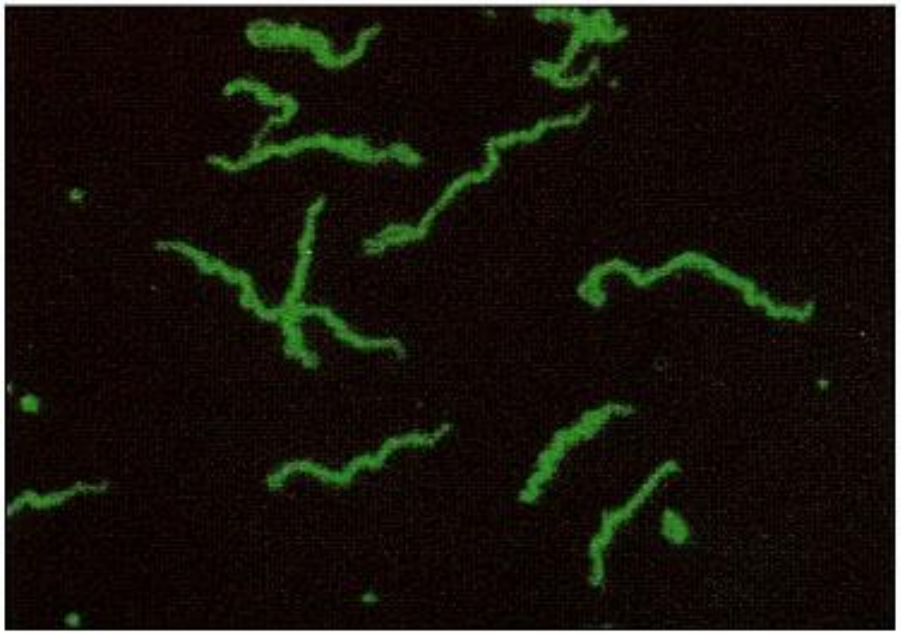
# Fluorescent Microscope

- Used to view antigen antibody reactions
- Specimen tagged with fluorescent dye
  - Molecules absorb light at one wavelength (usually UV)
  - Emit light of a longer wavelength- green or orange color
- Ocular lens fitted with filter that permits longer wavelengths & blocks shorter ones
- UV radiation (0.23-0.35um) so better resolution




Bacterial cell with bound antibodies combined with fluorochrome

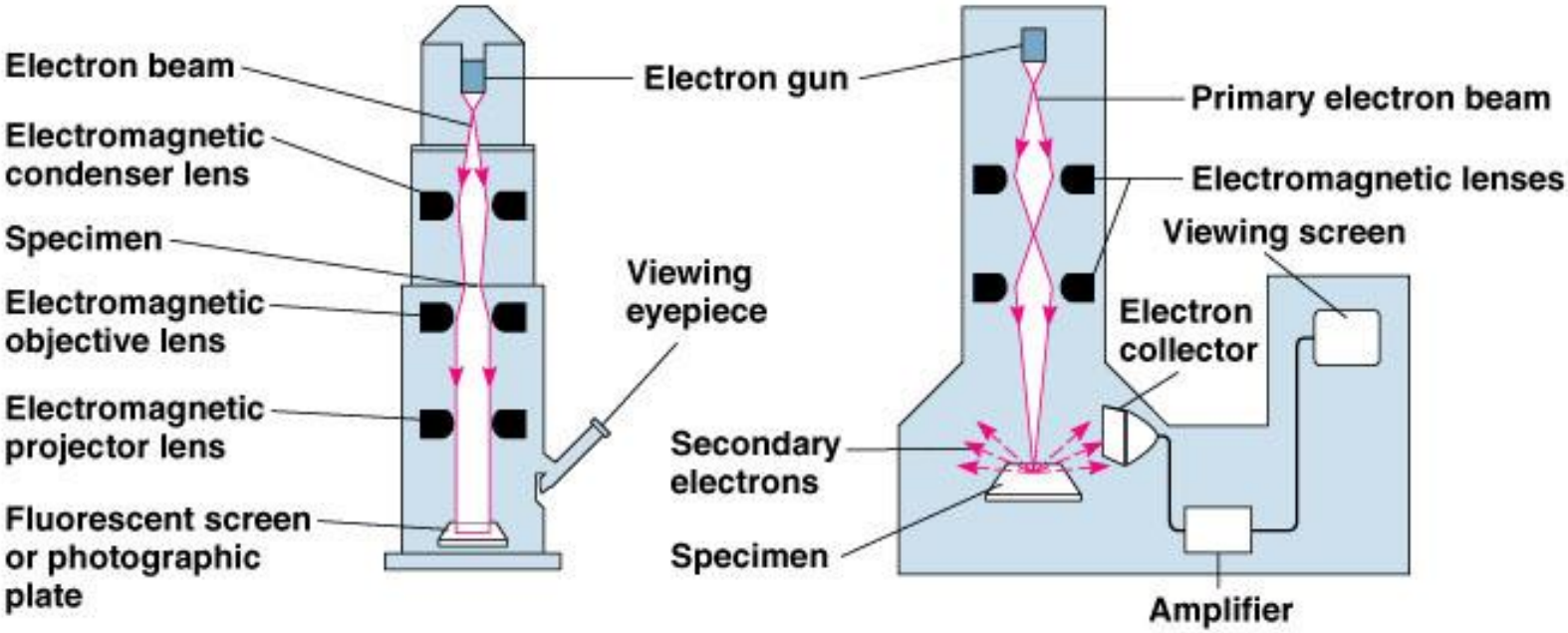
(a)

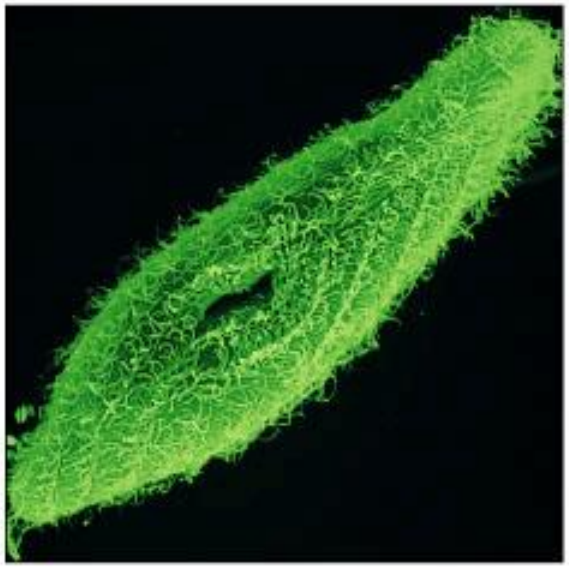


(b)

# Electron Microscopy

- Uses electrons as source of illumination
  - 1000x shorter than visible light
  - Use electromagnetic lenses
  - Image formed by electrons projected upon film
  - Magnification is up to to $10^6$
- Wavelength of electrons is dependent upon voltage of electron beam
  - 0.01nm to 0.001nm



**(a) Transmission** **(b) Scanning**