Chapter 3:  
Tools of the Laboratory:  
The Methods of Studying Microorganisms

Method of Culturing Microorganisms – The Five I’s

Five Basic Techniques Microbiologists use to manipulate, grow, examine, and characterize MOs.

1. Inoculation
2. Incubation
3. Isolation
4. Inspection
5. Identification

Sterile: complete absence of viable microbes.
Aseptic: prevention of infection or contamination.
Pure culture: growth of a single species of microbe.

Inoculation:

1. **Inoculation:** Producing a culture.
   - Culture or cultivate microbes by introducing a sample into a **sterile medium** that will sustain the growth of the microorganism.

Specimens can be taken from various places:

- Clinical species for determining disease can be taken from body fluids:
  - blood, cerebrospinal fluid
  - discharges – sputum, urine, or feces
  - disease tissues

- Microbiological analysis:
  - soil, water, sewage, foods, air, and inanimate objects.

Incubation:

- Incubated: placed in a temperature-controlled chamber (incubator) to encourage multiplication.
- Usual temperature used in lab (20 to 40° C)
- Different incubation time – varies from days to weeks.

Isolation of Pure Cultures:

**Isolation:** Separating one species from another:

**Axenic:** culture is free of other living things except for the one being studied.
Subculture:
- make a second-level culture from a well isolated colony. A tiny bit of cell is transferred into a separate container of medium and incubated.

In nature, mos usually grow in a complex mixed populations containing several species, therefore it is necessary to isolate the bacteria into single species for study.

Pure Culture:
- a population of cells arising from a single culture cell characterized as an individual species.
- A culture containing a single type of MO.
- 1% prokaryotes can be cultured in the lab.
- In order to obtain and maintain a pure culture, aseptic techniques (sterile) environment is necessary. All glassware, media, and instruments that come in contact with the bacteria must be sterile.

Mixed Culture:
- Two or more identified species of microorganisms.

Contaminated Culture:
- Once pure or mixed, but now contains unwanted microbes of uncertain identity.

Streak Plate:

Streak Plate: The simplest, preferred, and most common used technique to isolate pure colonies. The purpose is to reduce the number of cells being spreaded with each successive series of streaks, diluting the concentration of cells.

- Using an inoculating loop or swab, obtain bacteria, transfer to the edge of the agar plate and then streaked out over the surface in one or several patterns. Single cells drop of the loop at it is rubbed along the surface of the agar and develop into separate colonies.
- Successful isolation depends on spatial separation of single cells in both streak and spread plate.
- Several different methods to use, but we will use the T-streak and Quad Streak
  - T-Streak - 3 Quadrants
  - Quad Streak - 4 Quadrants
Spread Plate Method:

**Spread Plate:** a technique where a mixture of cells is spread out on an agar surface so that every cell grows into a completely separate colony.

**Colony:** a macroscopically visible growth or cluster of MO on a solid medium, where each colony represents a pure colony. About 1 million cells are required to for a colony to be visible with the naked eye.

- A small volume no more than 0.1 ml is transferred to the center of an agar plate and spread evenly over the surface with a sterile bent-rod glass.
- The dispersed cells develop into an isolated colonies.
- The number of colonies should equal the number of viable organisms in the sample.
- Used to count the microbial population.

**Enumeration of bacterial colonies:**
- Viable Plate Count contains 30-300 bacterial colonies.
- TMTC - more than 300 colonies on the plate.
- TFTC – less than 30.

The Pour Plate:

**Pour Plate:** original sample is diluted several times to reduce microbial population to obtain separate colonies when plating.

- Small volume of several diluted samples are mixed with liquid agar (cooled to 45° C)
- Mixtures are poured immediately in sterile culture Petri dishes
- After agar is solidified, each cell is fixed in place and forms an individual colony.
Loop Dilution: same concept using a loop in test tubes…

**Colony Morphology and Growth:**

- **Form of the colony and shape of the edge or margin** can be determined by looking down at the top of the colony.
- **Elevation of the colony** when viewed from the side as the plate is held at eye level.

The most rapid cell growth occurs at the **colony edge:**
- At colony edge, oxygen and nutrients are plentiful.

Slower growth takes place at the **colony center:**
- Much thicker growth than at the edge.
- Cells autolysis takes place in the older central portions of some colonies.
- Oxygen does not diffuse readily into the center, toxic metabolic products cannot be eliminated quickly, and growth in the colony center is slowed or stopped.

(Differences in growth are due to gradients of oxygen, nutrients, and toxic products within the colony).
Inspection:

Inspection: cultures are examined and evaluated macroscopically and microscopically
- Color, texture, and size.
- Microscopic details: cell shape, size, and motility.
- Staining techniques.

Identification:

Microorganisms are identified in terms of their:
- Macroscopic or immunological morphology
- Microscopic morphology
- Biochemical reactions
- Genetic characteristics
Types of Culture Media

Media:
- 500 different types of media are used to culture and identify mos.
- Culture media: test tubes, flasks, Petri plates
- Inoculated: inoculating loop, inoculating needle, pipettes, swabs.
- Some microbes and all viruses require cell cultures or host animal.
- Solid, semisolid, or liquid preparation used to grow, transport, and store MOs.
- Medium must contain all necessary nutrients the MOs requires for growth.
- Medium is used to select and grow specific MOs to help ID a particular species.

Media can be classified:
- Physical state
- Chemical composition
- Functional type

Synthetic or Defined Media
- Media whose compositions are chemically defined.
- Contain pure organic and inorganic cmpds.

Complex Media or Nonsynthetic
- Media that contain at least one ingredient that is not chemically definable.
  (some ingredients of unknown chemical components).
  - Rich and complete to meet the nutritional requirements of many MOs
  - Used b/c nutritional requirements are unknown (used for fastidious MOs)
  - Contain components like peptones, meat extracts and yeast extract:
    - Peptones: protein hydrolysates prepared by partial digestion of meat, casein, soy, meal, gelatin, and other protein sources, serving as C, energy and N sources.
      - Beef extract contain AA, peptides, nucleotides, organic acids, vitamins, and minerals.
      - Yeast Extract: source of B vitamins, nitrogen, and carbon compounds.
      - Three commonly complex media: Nutrient broth/agar, Tryptic broth/agar, and MacConkey agar

Broth can be solidified with the addition of agar (1.5%)
  - Agar is a polysaccharide.
  - Agar: extracted from red algae, *Gelidium*.
  - Solidifying agent b/c most bacteria cannot degrade it.
  - Melt at temp above 95° C, can solidify at temp below 45° C.
  - Translucent
Types of Media:

**General Purpose Media:**
- Non-synthetic and contain a mixture of nutrients that support the growth of many microorganisms.

**Examples:**
- Tryptic soy broth (TSB) or Tryptic soy agar (TSA)
- Nutrient agar or Nutrient broth
- Brain heart infusion

**Enriched Media:**
- Blood or other special growth factors (vitamins, AA) nutrients are added to general purpose media to encourage the growth of fastidious mos.

**Examples:**
- Blood agar – used for growing bacteria from human throat – *Streptococcus pyogenes* and other pathogens.
- Chocolate agar – *Nisseria gonorrhoeae*

**Selective Media:**
- A medium that is specific and favors the growth or isolation of a particular MO.
- Contains one or more agents that inhibit the growth of a certain microbe or microbes but encourages or selecting the growth of others.

**Examples:**
**MSA – Mannitol Salt Agar (MSA)**

- Contains a high concentration of NaCl (7.5%) that is inhibitory to most human pathogens. Staphylococcus grows well and it can be used as an ID medium.

**Bile Salts:**
- Bile salts or dyes like basic fuchsin and crystal violet
  *favors the growth of gram – by inhibiting the growth of gram* +.

- Endo agar, eosin methylene blue agar, and MacConkey agar used for the detection of E.coli

**Differential Media:**
- Media that distinguishes between different groups of bacteria.
- Media that incorporates different ingredients that cause certain organisms to develop a different appearance from other microbes growing on the same medium.
- Differentiation in colony size or color, media color changes, formation of gas bubbles or precipitates…resulting from types of chemicals in media and the ways that microbes react to them.

**Example:** Blood agar is both a differential and enriched medium.
- It distinguished between hemolytic and nonhemolytic bacteria.
It allows you to determine if a bacterial colony has produced the enzyme hemolysin and whether the resulting hemolysis is partial (alpha), complete (beta), or gamma (no hemolysis).

**Microscopy**

- Because microbiology deals with organisms too small they cannot be seen distinctly with the unaided eye, the microscope is essential.

- The *light microscope* is the *single most important research tool* that microbiologists have ever had.

- The *light microscope* is an optical instrument which operates on the principal that light energy will pass through and around a suitably thin object, and with the aid of lenses, *form a magnified impression on the visual sensory layer of the eye*.

*In order to know how the microscope works we must first have a basic understanding about how lenses bend and focus light to form images.*

- When light energy passes from one medium to another (i.e. AIR and GLASS) the light rays are bent at the point of interface. This process is called *refraction*.

- The measure of how greatly a substance slows the velocity of light is called the *refractive index*.

- Our eyes cannot focus on objects nearer than about 25 cm or 10 inches. We overcome this limitation by using a convex lens as a simple magnifier (or microscope) and holding it close to the object.

**The Light Microscope**

There are a variety of *light microscopes* mostly employed in Microbiology:

- Bright-field
- Dark-field
- Phase-contrast
- Fluorescence

*The modern scopes are all compound scopes, meaning the magnified image formed by the objective lens is further enlarged by one or more additional lenses.*

**The Bright-field Microscope**

- Called the ordinary microscope because it forms a dark image against a brighter background.
- Common multi purpose microscope for live and preserved stained specimens.
- Provides fair cellular detail.
- **Parafocal**: image should remain in focus when objectives are changed.

**Path of Light:**
- The objective lens forms an enlarged real image within the microscope and the eyepiece lens further magnifies this primarily image.
- Upon looking in the microscope, the enlarged specimen image – *Virtual image* appears to lie just beyond the stage about 25 cm away.
- Total magnification is calculated by multiplying the objective and eyepiece magnification together.

**Three factors determine the quality of an optical image:**

a. Magnification  
   b. Resolution  
   c. Contrast

**Magnification:**

*Magnification* is the apparent increase in size affected by a convex lens

- A **compound microscope** uses two sets of lenses, with differing focal lengths, to facilitate magnification – objective lens and ocular lens.
- The total magnification achieved by the lens array is the product of each individual lens.

\[
\text{Magnification (total)} = \text{magnification (obj. lens)} \times \text{magnification (ocu. lens)}
\]

- Example: \(\text{Mag (obj)} = 40X\) and \(\text{Mag (ocular)} = 10X\)
- Then \(\text{Mag (total)} = (40X) \times (10X) = 400X\).

It is much easier to make two lenses with average magnifying powers and put them together in a compound microscope than to make a single lens with a very high magnifying power.
- Compound microscopes are usually designed to give a highest possible magnification of only 1,000-1,500X.

**Resolution**

The most important part of the microscope is the **objective**, which must produce *a clear image, not just a magnified one*. 
Resolution:
- is the ability to separate points - to observe fine detail. The ability of a lens to distinguish between small objects that are close together.
- Resolve: clearly separate.

Example: car headlights.

Resolution is not the same thing as magnification.

One way to increase the resolution of an image is to increase the amount of light that enters the objective lens by using immersion oil.
- Immersion oil (with a density closer to glass) can be used to reduce the refraction of light rays (compared to air) and allows more light to enter the objective. This improves resolution.

Only the highest power (100X) objective on the microscope is designed for use with immersion oil. DO NOT USE OIL WITH THE LOWER POWER (10X AND 40X) OBJECTIVES!
When light energy passes from one medium to another (i.e. AIR and GLASS) the light rays are bent at the point of interface. This process is called \textit{refraction}.

The measure of how greatly a substance slows the velocity of light is called the \textit{refractive index}.

Another way to increase resolution of an image is to decrease the wavelength of light that is used to illuminate the specimen (use blue light instead of white light).

\textbf{Contrast}

- \textit{Reflects the number of visible shades in a specimen.}

Microbes are composed of \textit{water, nucleic acids, proteins, and lipids} (transparent). Most \textit{appear colorless against a colorless background} when observed using bright field microscopy. Therefore in order to see them, we must \textit{devise a way to increase the contrast.}

Brightfield microscopy:
- Direct staining of the microorganisms
- Indirect (negative) staining of the background

In order to stain a specimen, it must first be fixed to the slide and chemically altered. This results in the death of a specimen.

\textit{Additional microscopic techniques have been developed to increase contrast of living microorganisms.}
Types of Microscopy

The Bright-field Microscope
- Called the ordinary microscope because it forms a dark image against a brighter background.
- Common multi purpose microscope for live and preserved stained specimens.
- Provides fair cellular detail.

Dark-field microscopy is one such technique that is often used to observe living, unstained cells and organisms.
- Organisms viewed through a dark field microscope stand out as bright objects against a dark background; provides outline of specimen with reduced internal cellular detail.
- Dark field microscopes illuminate the sample in such a way that unreflected and unrefracted light does not enter the objective, only light that has been reflected/refracted by the specimen passes through the objective and forms the image.

Phase-contrast microscopes exploits the differences between refractive index of the cells and the surrounding medium, resulting in a darker appearance of the denser material.
- Used for live organisms.
- Specimen is contrasted against gray background
- Excellent for internal cellular detail

The Interference Microscope
- Brightly colored and highly contrasting 3D images of live specimens.

Fluorescence Microscopy. Specimens are treated with dye molecules called fluorochromes which brightly fluoresce when exposed to light of a specific wavelength.
- The color that the cell will appear depends on the type of dye used.

Confocal Microscopy:
- Confocal Scanning Laser Microscope is used to construct a 3D image of a thick structure such as a community of microorganisms and provides a detail sectional view of the interior of an intact cell.
- Eliminates a murky, fuzzy, and crowded image.
- Fluorescently stained specimen
- A focused laser beam strikes a point in the specimen
- Light from the illuminated spot is focused by an objective lens onto a plane above the objective.
- The laser is scanned over a plane in the specimen (beam scanning) or the stage is moved (stage scanning) and a detector measures the illumination from each point to produce an image of the optical section.
Many sections are scanned, a computer combines them to form a three-dimensional image from the digitized signals. Image can then be measured and analyzed quantitatively.

ELECTRON MICROSCOPY

A physical relationship exists between resolution and light: resolution of an image increases as the wavelength of light used to illuminate it decreases.

Since bacteria are generally 1000 nm in diameter, the most we can resolve using light microscopy is their general shape and some of the major morphological features (is spores, flagella, capsules)

Electron microscopes are used to achieve up to more than 100,000 greater magnification and more than 1000 times greater resolution than the light microscope.

Sub-cellular structures can be easily observed – Much greater details can be seen.

The technique of electron microscopy is based on the principal that electron beams behave like waves and can be focused much like light in a compound microscope.

Electron microscopes do not use lenses to focus the electron beams, instead, they use magnetic fields.

Used only for preserved material.

There are two types of electron microscopes SEM and TEM

1. Transmission Electron Microscope

Used to observe fine details of cell structure, such as the number of layers that envelope a cell.
Requires elaborate and painstaking specimen preparation.
Transmission electron microscope (TEM) projects a beam of electrons through a specimen.
A TEM uses a series of electromagnets to focus an intense electron beam onto/ and through the sample.
Phosphorescent image is then recorded on photographic film.
100,000X maximum magnification.
2. **Scanning Electron Microscope**

- Used for observing surface details, but not internal structures of cell.
- The electron beam is focused (via electron magnets) and projected onto (not through) the specimen.
- To improve contrast, the specimen is coated with an *ultra thin layer of heavy metal (such as gold) to improve electron scattering*.
- Image is formed as the scattered electrons collide with a phosphorescent detector screen.
- This technique can be used to observe specimens that are *several millimeters thick*, and produces *spectacular three dimensional images*.
- 650,000X magnification

3. **Scanning Probe Microscopy**

  - *Scanning Probe Microscope* measure surface features by moving a sharp probe over the object’s surface and making it possible to view images at an atomic scale.

  - *Scanning Tunneling Microscope*
    - Invented in 1980
    - Can achieve magnification of 100 million and allow the viewing of the atom on the surface of a solid.

  - *Atomic Fore Microscope*
    - Moves a sharp probe over the specimen surface while keeping the distance between the probe tip and the surface constant.
    - Accomplished by exerting a very small amount of force on the tip.

**Preparation and Staining of Specimens**

- *Microorganisms must be fixed and stained to increase visibility, accentuate specific morphological features, and preserve them for future study.*

**Fixation:**

- Process by which internal and external structures of the cells and microorganisms are preserved and fixed in position.

- This process inactivates enzymes that might disrupt cell morphology and toughens cell structures so that they do not change during staining and observation. A microorganism usually is killed and attached firmly to the microscope slide during fixation.
Two types of fixation:

- **Heat-fixed** bacterial smears by gently flame heating an air-dried film of bacteria, preserving the overall morphology but not structures within cells.

- **Chemical Fixation** is used to protect fine cellular substructures and the morphology of larger, more delicate microorganisms.
  - Chemical fixatives penetrate cells and react with cellular components, usually proteins and lipids, to render them inactive, insoluble, and immobile.

- Common fixative mixtures:
  - Ethanol, acetic acid, mercuric chloride, and formaldehyde

**Biological Stains**

- One way to increase contrast of an image is to **stain** colorless microorganisms.
- Stains are very pure dyes which first came into common use in biology around 1850.
- Stains are typically salts, composed of positive and negative ions (e.g., Na\(^+\) Cl\(^-\)). Only one of the ions carries the **chromogen**, or colored part of the molecule.
- Many dyes used to stain M.O. have two common features:
  1. They have **chromophore groups**, groups with conjugated double bonds that gives dyes its color.
  2. They can bind with cells by ionic, covalent, or hydrophobic bonding.

**Positive and Negative Staining:**

**Positive Stain - Basic Stain:**

- dye sticks to the specimen and gives it color.
- If the positive ion (cationic) contains the chromogen, then the stain is referred to as a basic stain.

**Examples:** methylene blue is a chloride salt (MB\(^+\)Cl\(^-\))

- Basic fuchsin, crystal violet, safranin, malachite green.
- Basic dyes bind to negatively charged molecules like nucleic acids, proteins, and cell wall components, and the surface of the cells themselves.
- Because the surfaces of bacterial cells are negatively charged, basic dyes are most often used.
Negative Stain -- Acidic Stain:
- The dye does not stick to the specimen but settles around its outer boundary.
- Stains the glass slide to produce a dark background around the cells.
- **The cells themselves do not stain because these dyes are negatively charged and are repelled by the negatively charged surface of the cells.**
- **If the negative ion (anionic) contains the chromogen, the stain is an acidic stain.**

Example: Nigrosin and India Ink

Would you expect a basic stain or an acidic stain to react with the bacterial cell itself?
- Acidic stains do not react with the negatively-charged bacterial cells and therefore become deposited in the background.
- This results in a preparation of colorless cells within a stained background (indirect or negative staining).

Staining a Bacterial Smear

How to make a smear…*Too thick vs. Too thin*
- Too thick can cause dye to accumulate between cells
- Too thin, too hard to see
- Ideal smear is one cell layer thick

**Simple stains …Requires only one (1) dye.**

- Cover the fixed smear with stain
- Wash excess stain off with water
- Blot slide dry
- Frequently used basic dyes: CV, MB, CF are used to determined size, shape and arrangement of BACTERIA.

Differential Stains

- *In contrast, we can have Differential stains make use of more than one dye to identify two or more types of bacteria.*

- Primary and secondary or counter stain to distinguish between cell types and parts.

- Different bacteria can be stained different colors (to help distinguish between them).
The Gram stain

The most important differential stain is the Gram stain, which was devised by Christian Gram in 1884.

- Almost all bacteria can be divided into two groups (Gram-positive and Gram-negative) by this stain.
- Results of the Gram stain are correlated with cell wall composition.
- The Gram stain is usually the first step taken to identify an unknown bacterium.

Procedure

a. The primary stain is crystal violet (purple color). 30 sec - HOH rinse

b. The mordant is a solution of iodine. 1 min - HOH rinse
   - The mordant forms a complex with crystal violet and helps it adhere to the cell. (iodine increases the interaction between CV and cell).

c. The decolorizing agent is alcohol (95% ethanol). 10-30 sec - HOH rinse
   - This is the Differential step.
   - Gram positive bacteria retain the CV, whereas Gram negative lose CV and become pink upon the addition of the counterstain.

d. The secondary stain or counterstain is safranin (pink color). 30-60 sec - HOH rinse
Gram-positive cells retain the crystal violet-iodine complex and stain purple. Gram-negative cells lose this complex during the decolorizing step, react with safranin, and stain pink.

Acid Fast Staining

Some bacteria have an unusually high lipid content in their cell walls which will not react with simple stains.

- Bacteria from the genus Mycobacterium

  High concentrations of mycolic acids (branched chain hydroxy lipids) in their cell walls. These fatty acids give the colonies an overall, waxy, appearance, and will not stain by conventional methods.

- To stain mycobacteria, you must use a combination of heat and phenol to drive the basic fuchsin stain into the cell wall.

- Once the stain has penetrated, it is not easily removed, or decolorized with acid alcohol.

- Thus the cells are said to be ACID FAST

- Bacteria without the waxy cell wall are easily decolorized with the acid alcohol, and must be counterstained with methylene blue before they can be visualized.

  Therefore acid fast=red
- Non acid fast = blue

- Other examples of Differential stains
  - Spore stain: used to identify endospores
  - Capsule stain, also an example of negative stain: used to identify capsules.