Lecture 02
Microscopy, Staining, Classification
(Ch3)
Topics
– Methods of Culturing Microorganisms
– Microscope (History, Types, Definitions)
– Staining (Gram’s)
– Dichotomous keys

Classification and Identification of Microorganisms

• **Taxonomic Keys**
  – Dichotomous keys
  • Series of paired statements, usually only one of two choices applies to an organism
  – Key directs you
• Why? To identify the organism!!
• How? Morphology, biochemical results, observations, etc.

Example Dichotomous Taxonomic Key
Microorganism Culturing Methods

(How to grow…)

• Five basic techniques of culturing
• Media
• Microbial growth

Five Basic Techniques of Culturing Bacteria

1. Inoculate
2. Incubate
3. Isolation
4. Inspection
5. Identification

A single visible colony represents a pure culture or single type of bacterium isolated from a mixed culture.

Fig. 3.2 Isolation technique
Media

- Classified according to three properties
  - Physical state
  - Chemical composition
  - Functional types

Media: Physical State

- Liquid media
- Semi-solid media
- Solid media
Semi-solid media contain a low percentage (<1%) of agar, which can be used for motility testing.

Solid media contain a high percent (1-5%) of agar, this enables the formation of discrete colonies.

Media: Chemical content

- Synthetic media
- Nonsynthetic or complex media
Synthetic media contain chemically defined pure organic and inorganic compounds (known molecular formula).

| Chemicals Defined Synthetic Medium for Growth and Maintenance of Pathogens and Bacterial strains |
|---|---|---|
| 0.25 Gram Each of Three Amines | 0.5 Gram Each of Three Amines | 0.12 Gram Each of Three Amines |
| Cystine | Arginine | Citric acid |
| Histidine | L-arginine | Cholic acid |
| Lysine | L-norleucine | |
| Phenylalanine | Methionine | |
| Phenylalanine | Threonine | |
| Tyrosine | Threonine | |

Additional ingredients:
- 0.005 moles of manganese
- 0.005 moles of boron
- 0.5 micrograms of thiamine
- 0.15 grams of magnesium sulfate
- 0.15 grams of ammonium phosphate
- 0.125 grams of iron chloride

Ingredients dissolved in 1000 milliliters of distilled water and bottled for a basic nutrient solution.

Complex or enriched media contain ingredients that are not chemically defined or pure (animal extracts).

Functional types of growth media
- Enriched media
- Selective media
- Differential media
Enriched media grows fastidious bacteria.

Blood hemolysis

- Grows all
- Differential media - shows different reactions, like color.
- Selective media - enables one type of bacteria to grow.

Examples of media that are both selective and differential

- Mannitol salt agar is a selective media.
- MacConkey agar is a differential media.
Example miscellaneous media such as reducing, fermentation and transportation media.

Carbohydrate fermentation broth

Microbial growth

- Incubation
  - Varied temperatures, atmospheric states
- Inspection
  - Mixed culture
  - Pure culture
- Identification
  - Microscopic appearance
- Maintenance and disposal
  - Stock cultures
  - sterilization

Microscopy and Staining - how we see closer up

- Intro to Microscopy
  - Evolutionary Trends
  - Some Concepts of Definition and Relationship
  - Some Microscopy Techniques
- Staining Methods
Evolution of Microscopy

Characterized by:

- A constant search for better resolution
- Constant search for ability to see better detail in smaller objects
- Always waiting on Technology…

General Principles of Microscopy

- Wavelength of radiation
- Magnification
- Resolution
- Contrast
Some Concepts to Consider

Definitions and Relationships
- Resolution
- Magnification
- Depth of Focus
- Field of Vision
- Numerical Aperture

Resolution
- Resolution is the ability to distinguish between two points;
- The closer the two points, the higher the resolution

Resolution distinguishes magnified objects clearly.
Resolution can be increased using immersion oil.

Magnification comparison

Magnification

- Relative enlargement of the specimen
- The power of magnification is calculated by multiplying the power of the eye piece lens by the power of the objective lens.
- Empty Magnification

Specimen magnified when light passes through objective and ocular lenses.

Pathway of light and the two stages of magnification in a compound microscope.
More Concepts...

- Depth of focus - thickness of a specimen that can be seen in focus at one time; as magnification $\uparrow$ the depth of focus $\downarrow$.
- Field of vision - the surface area of view; the area $\downarrow$ as magnification $\uparrow$.
- Numerical aperture (N.A.) – the amount of light reaching the specimen; As N.A. $\uparrow$ resolution $\uparrow$.

Always Improving

- Van Leeuwenhoek -- size $\downarrow$
- Zeiss Brothers – size $\downarrow$ and $\uparrow$ resolution!
- 1930’s Electron Microscope -- SIZE $\downarrow$
- New Scopes: SEM, TEM, TM, etc. etc.- $\uparrow$ fine details!

Optical microscopes

- All have a theoretical maximum magnification of 2000X
  - Bright-field
  - Dark-field
  - Phase-contrast
  - Differential interference
  - Fluorescent
  - Confocal
**Bright-field microscopy**

- Most commonly used
- Can observe live or preserved
- Stained or unstained specimens

![Bright-field microscopy images](http://micro.magnet.fsu.edu/)

**Dark-field microscopy**

- Observe live unstained specimens
- View an outline of the specimens

![Dark-field microscopy image](http://www.microbiological-garden.net)
Phase-contrast microscopy

- Observe live specimens
- View internal cellular detail

Image from: http://biology.kenyon.edu

Principles of phase microscopy

- Rays in phase
- Rays out of phase

Examples of dark-field, bright-field and phase-contrast microscopy.

Three views of a cell
Example of phase-contrast and differential interference.

Visualizing internal structures.

Fluorescent Microscopy

- Direct UV light source at specimen
- Specimen radiates energy back as a longer, visible wavelength
- UV light increases resolution and contrast
- Some cells are naturally fluorescent; others must be stained
- Used in immunofluorescence to identify pathogens and to make visible a variety of proteins

Fluorescent microscopy

- Fluorescence stain or dye
- UV radiation causes emission of visible light from dye
- Diagnostic tool
Immunofluorescence

Example fluorescent microscopy- stained cheek scrapings specimen

Confocal Microscopy

- fluorescent dyes
- UV lasers to illuminate fluorescent chemicals in a single plane
- Resolution increased because emitted light passes through pinhole aperture
- Computer constructs 3-D image from digitized images
Confocal microscopy

• Fluorescence or unstained specimen images combined to form a three-dimensional image.

Electron Microscopy

• Very high magnification (100,000X)
• Transmission electron microscope (TEM)
  – View internal structures of cells
• Scanning electron microscope (SEM)
  – Three-dimensional images

Electron Microscopy

– Light microscopes cannot resolve structures closer than 200 nm
– Electron microscopes → greater resolving power and magnification
– Magnifies objects 10,000X to 100,000+X
– Detailed views of bacteria, viruses, internal cellular structures, molecules, and large atoms
– Two major types
  • Transmission electron microscopes
  • Scanning electron microscopes
False-color scanning electron micrograph…
ha! No colors with electrons!

Ant in S.E.M.

Ant portrait
Insect eye at 250x

Insect eye at 2500x

Hydrothermal Worm

[Image: FEI and Philippe Crassous](http://www.fei.com/resourcemanager/gallery/hydro-worm-2908.aspx)

Micro for Health Sciences
**Probe Microscopy**

- Magnifies more than 100,000,000 times
- Two scope types
  - Scanning tunneling
  - Atomic force

**Manipulating Atoms w/ STM!**

- Carbon dioxide man, individual carbon dioxide atoms on platinum
- The word atom, in kanji, written with individual iron atoms on copper


**Staining – why?**

- Increases contrast and resolution by coloring specimens with stains/dyes
- 1st make smear
- Microbiological stains contain chromophore
- Acidic dyes stain alkaline structures; more commonly, basic dyes stain acidic structures
Simple stain results

![Simple stain results](image)

Stain Types

- **Positive stains**
  - Dye binds to the specimen
- **Negative stains**
  - Dye does not bind to the specimen, but rather around the specimen.

Positive stains are basic dyes (+ charge) that bind negatively charged cells.

Negative stains are acidic dyes (- charge) that bind the background.

Comparison of positive and negative stains

![Comparison of positive and negative stains](image)
Positive stains

- Simple
  - One dye
- Differential
  - Two different colored dyes
    - Ex. Gram stain
- Special
  - Emphasize certain cell parts
    - Ex. Capsule stain

Staining Technique Example

- Gram Stain
  - Fix
  - 20 secs Crystal Violet, H2O rinse
  - 15 secs Iodine Mordant, H2O Rinse
  - Alcohol rinse
  - 20 secs Safranin Counter stain, H2O Rinse

Preparing a specimen for staining

- Spread culture in thin film over slide
- Air dry
- Pass slide through flame to fix it
Staining

- Simple stains
- Differential stains
  - Gram stain
  - Acid-fast stain
  - Endospore stain
- Special stains
  - Negative (capsule) stain
  - Flagella stain

Examples of simple, differential and special stains.

Ziehl-Neelsen acid-fast stain

for mycolic acid
Staining for EM

- Chemicals containing heavy metals used for transmission electron microscopy
- Stains can bind molecules in specimens or background

Classification and Identification of Microorganisms

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

Two biochemical tests for identifying bacteria

- Inverted tubes to trap gas
- Gas bubble
- Acid with no gas
- Acid with gas
- Hydrogen sulfide present
- No hydrogen sulfide

Micro for Health Sciences
One tool for the rapid identification of bacteria

An agglutination test, one type of serological test

Phage typing