Microscopy and Staining

Jacquelyn G. Black, Microbiology, 9th Edition

Chapter 3 – Page 51

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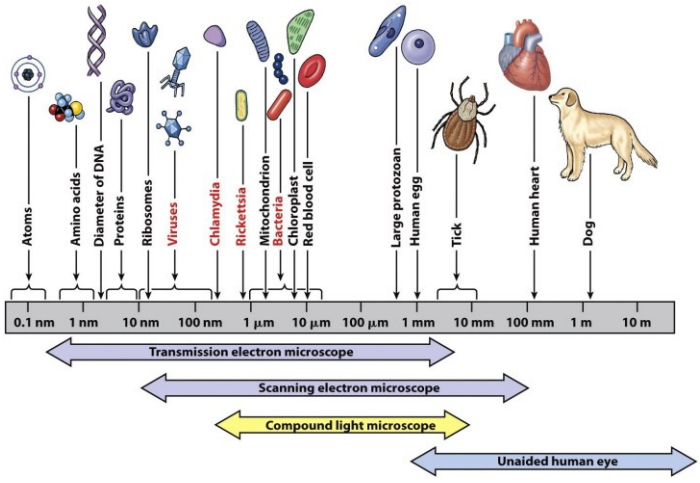
Introduction

- Microscopy is the technique of viewing very small objects that otherwise cannot be seen by the naked eye.



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Resolution

- Resolution is the ability to see and distinguish two adjacent items as separate & discrete structures
- Resolving Power (RP) of a lens is a measure of the resolution that can be obtained with that lens (the smaller the distance between objects that can be distinguished, the greater the resolving power of the lens
- Order in term of resolving power:

  Naked eye < light microscope < scanning electron microscope < transmission electron microscope

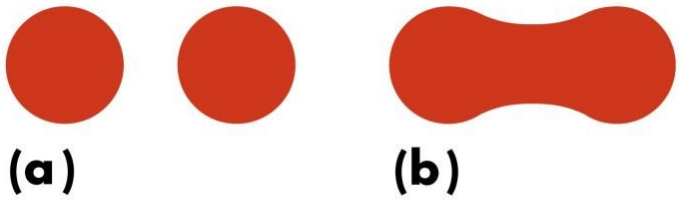

Figure 3-5 Microbiology, 6/e
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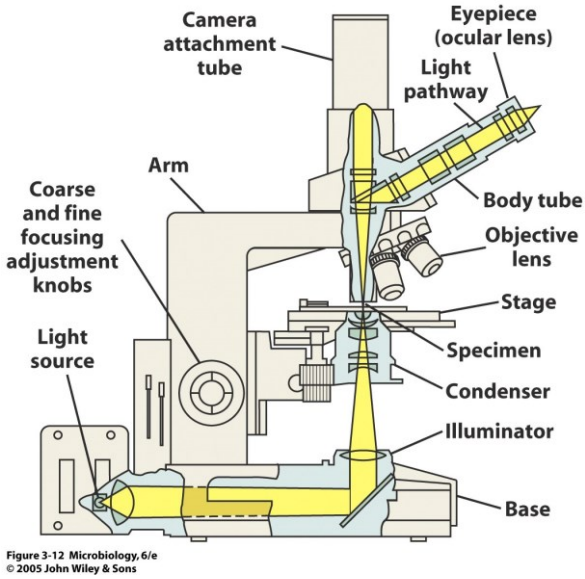
Types of Microscopes

1. Light (optical) microscopes

   any microscope that uses visible light to make specimens observable

1.1. The compound light microscope

- It has more than one lens: eye piece (ocular lens) & objective lens
- It can be monocular or binocular (eye piece)
- Total magnification= magnification of ocular lens x magnification of objective lens
- Light is condensed & transmitted directly through the specimen (bright field illumination)



The compound light microscope

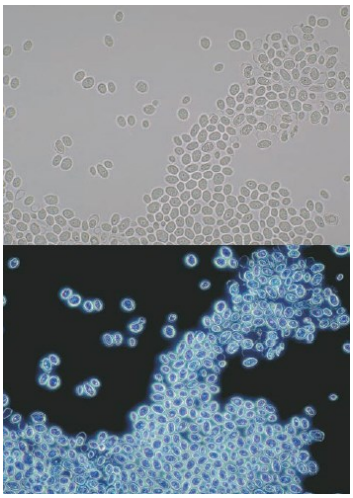
Types of Microscopes

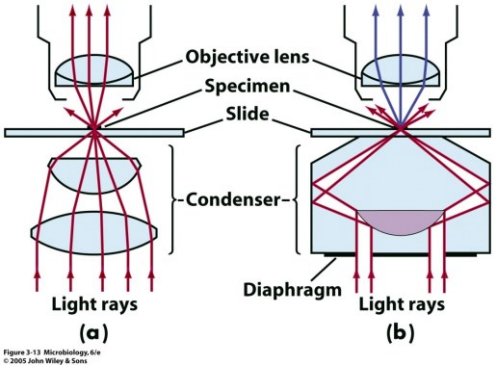
1.2. Dark field microscope

- Used for some light sensitive organisms that lack contrast with their background in a bright field

  e.g. spirochetes (such as *Treponema pallidum* which causes syphilis)

- Dark field illumination is used in this case to enhance the contrast
- A condenser prevents light from being transmitted through the specimen, rather it causes the light to reflect off the specimen
- The light is then collected and focused into an image where a light object is seen on a dark background





Bright field vs dark field microscopy

Types of Microscopes

1.3. Phase contrast microscope

- Microorganisms are usually killed by staining, so if we want to examine processes in living cells (i.e. replication or movement) we need to use non-stained specimens

  But this will be on the expense of image contrast!

- Phase contrast microscopes utilize special condenser and objective lenses to enhance the contrast of these images by converting 'phase shifts' in light that passes objects with different refractive index to 'brightness change'.

Types of Microscopes

1.4. Fluorescence microscope

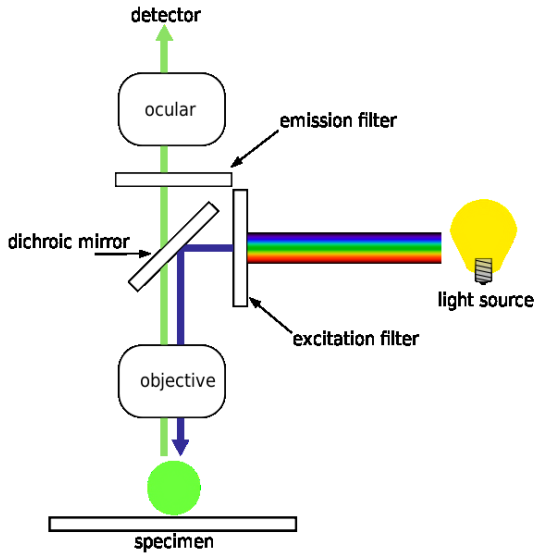
- In this type of microscopy, UV light is usually used (visible light can also be used) to excite molecules so that they emit light **of a longer wavelength** (fluorescence)

  i.e. UV → blue → green → red

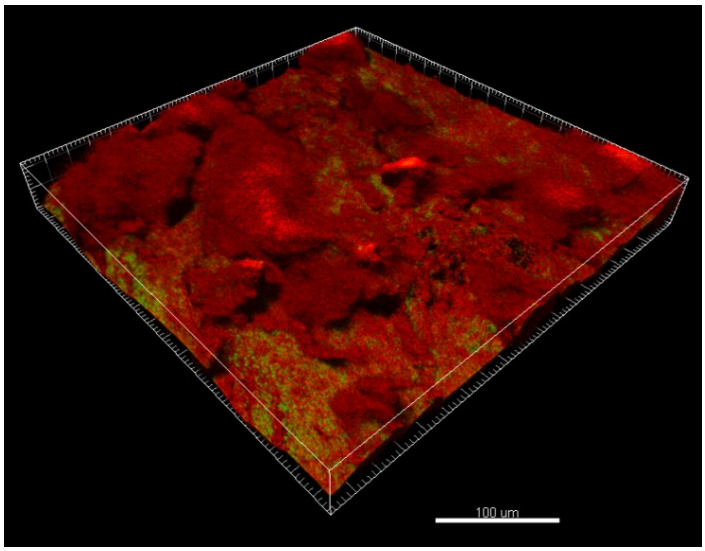
- Only fluorescent objects (i.e. those containing a fluorochrome) can been seen in this technique.

  Some m.o. are naturally fluorescent (e.g. *Pseudomonas*) while others need treatment with fluorescent dyes such as: acridine orange, DAPI, fluorescein and propidium iodide

- Excitation/emission spectra are characteristic to the utilized fluorochrome
- This technique can be used in the viability evaluation of m.o. where live and dead cells are stained with different colors
- Confocal microscope is a special type of fluorescence microscopes that uses 'optical sectioning' to enhance image resolution and construct 3D images, especially for thick samples like biofilms.



Schematic diagram of a fluorescence microscope



3D confocal image of *P. aeruginosa* biofilm where green color indicates live cells and red color indicates dead cells.

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Types of Microscopes

2. Electron microscopes
   - The resolution of light microscopes is limited by a diffraction limit to about 200 nm

     That means they can only distinguish whole cells and few large subcellular structures.
   - The diffraction limit, and hence the resolving power, is dependent on the light wavelength in addition to the lenses 'refractive properties'
   - Electron microscopes utilize a beam of electrons instead of light & electromagnets (electromagnetic lenses) instead of glass lenses which greatly enhances their resolving power

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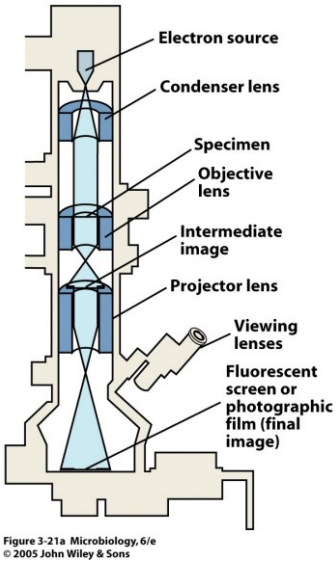

Diagram of an electron microscope

Types of Microscopes

2.1. Transmission electron microscope (TEM)

- Best one for viewing internal structures of microbes
- It can give sub-nanometer resolution and magnifications up to several million times.
- It examines very thin sections from the specimen (70-90nm), which are made with a diamond knife

  Sometimes treatment with heavy metals (gold or platinum) is required to enhance the contrast

Types of Microscopes

2.2. Scanning electron microscope (SEM)

- It has lower resolving power then TEM
- Used to create images of the **surface** of specimen
- It gives 3D view of the exterior of the cell
- Specimen is coated with gold or platinum but there is no need for specimen cutting

2.3. Scanning tunneling microscope (STM)

2.4. Atomic force microscope (AFM)

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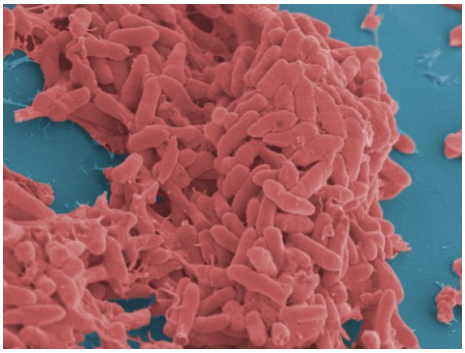

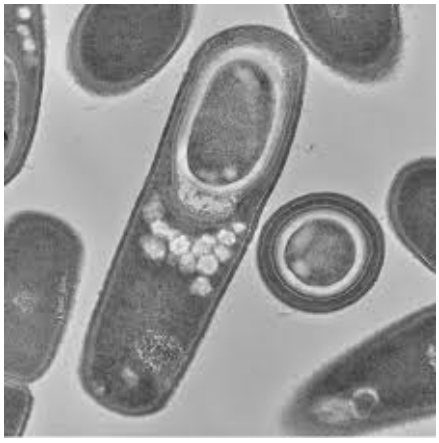

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Specimen Preparation for Light Microscopy

- Wet mount slide
  - A drop of medium containing m.o. is placed on the slide
  - Usually used to view living microorganisms
  - 2% carboxymethylcellulose (thick solution) can be added to slow fast moving m.o.
  - Dark field or phase contrast microscope is usually used for specimens prepared by wet mount
  - Special version of wet mount is **hanging drop** which is used to study microbial motility

    Hanging drop is done by placing a drop of microbial culture on a cover slip surrounded by petroleum jelly to prevent evaporation. The cover slip is then inverted over the well of a depression slide

Specimen Preparation for Light Microscopy

- Smear slide
  - Loopful of microbial culture is spread on a slide, allowed to air dry then passed quickly over a flame 3-4 times (heat fixation)

    Heat fixation causes the cells to adhere to the slide and alters them so they accept the stain

    If the sample is not dry before heat fixation, the m.o. will boil & becomes destroyed
  - The microbial cells are usually killed (by heat) in this technique

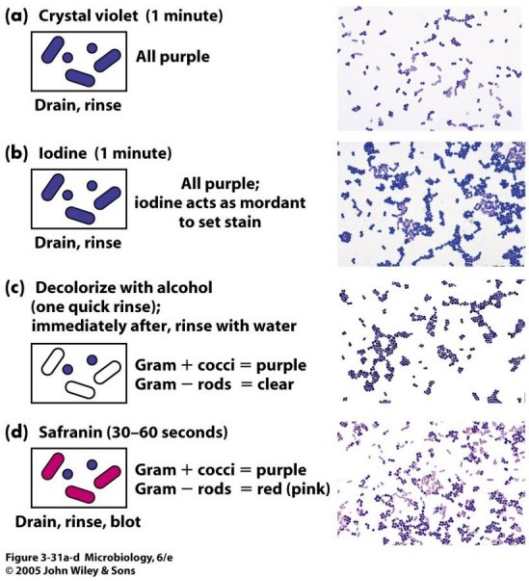
Principles of Staining

- A stain (dye) is a molecule that can bind to a cellular structure & give it a colour
- Staining helps investigators to group major categories of m.o., and to examine structural & chemical differences in cellular structures
- Dyes can be **cationic** (basic) such as crystal violet, methylene blue and safranine; or **anionic** (acidic) such as eosin and nigrosin

  The cell membranes of most bacteria have negatively charged surfaces and thus attract the positively charged basic dyes so they are more commonly used to stain cells, whereas anionic dyes are usually used to stain the background
- Staining can be done using **simple** or **differential** stain
  - Simple stain: contains single dye and used to reveal basic cell shape, size & cell arrangement.
  - Differential stain: contains two or more dyes and used to distinguish between different kinds of m.o. or different cell parts

Principles of Staining

- The Gram stain
  - A differential stain developed by Hans Gram which depends on the cell wall structure
  - Procedures:
    1. Bacterial cells are first stained with **crystal violet** (purple)
    2. Iodine is then added as a **mordant** (i.e. a chemical that helps retain the stain in certain cells)
    3. Ethanol (95%) or ethanol/acetone is used to decolorize the cells that can not retain crystal violet; this is followed by rinsing with water
    4. The cells are then counterstained with **safranine** (red/pink)



Gram staining

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Principles of Staining

- 4 groups of organisms can be distinguished based on the Gram stain:
    1. Gram positive: the cells retain crystal violet stain
    2. Gram negative: the cells don't retain crystal violet but rather counterstain with safranine
    3. Gram non-reactive: the cells don't (or poorly) stain
    4. Gram variable: the cells stain unevenly
- The differentiation between Gram-positive and Gram-negative organisms reveals a fundamental difference in the nature of the **cell walls** of bacteria
- Cells from cultures that are older than 24-48hr may become Gram variable due to changes in cell wall with aging

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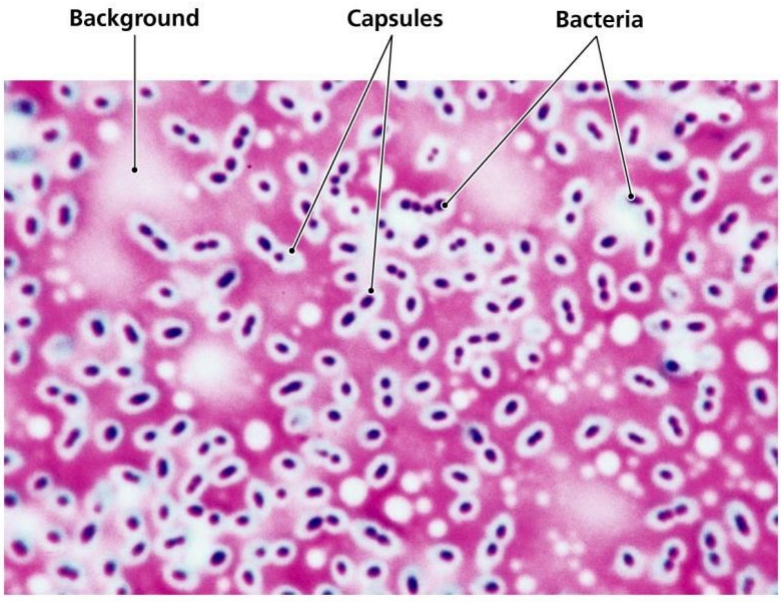
Principles of Staining

- The Ziehl-Neelsen acid-fast stain
  - A differential stain that also depends on the cell wall structure
  - Used to detect Mycobacteria (TB, leprosy)
  - Procedures:
    1. First stain with carbolfuchsin (red)
    2. Heat and rinse
    3. Decolorize with 3% HCl in ethanol and rinse
    4. Counterstain with methylene blue (blue)
  - Most bacterial will lose the red stain when decolorized & stained with blue counterstain, but acid fast bacteria (i.e. Mycobacteria) will retain the red colour.

    The cell wall of acid fast bacteria is very hydrophobic that resist staining , so we use heating to force staining , once it's stained it resists de-staining even with the strongest decolorizing agents (such as HCL with ethanol)

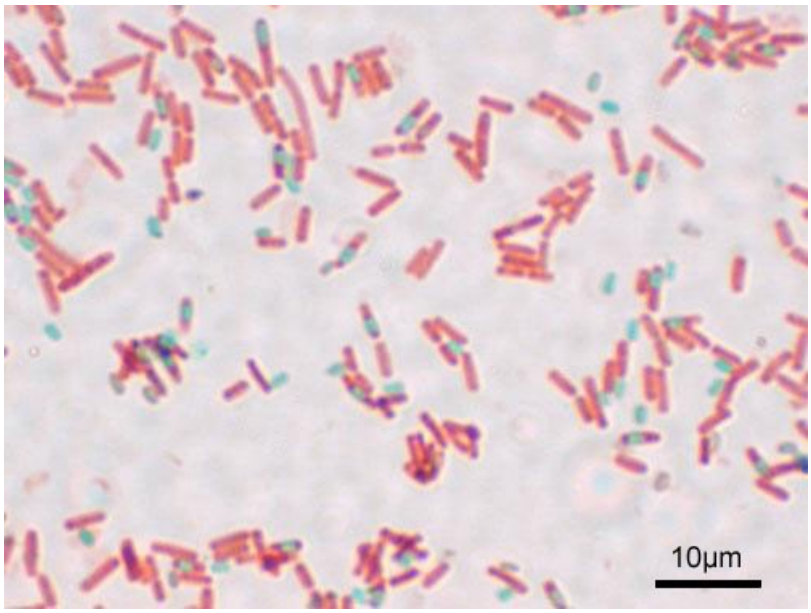
Principles of Staining

- **Special Staining**
- Negative staining:
  - Used when a specimen or part of it (e.g. capsule) resist taking up the stain
  - The background around the m.o. is filled with stain (usually acidic such as nigrosin) while the organism (or part of it) is clear (unstained)
  - For **capsule stain**: the background is dark, cells are purple (stained with crystal violet) & the capsules are unstained
- Flagellar staining:
  - Flagella are appendages that some cells have and use for locomotion. But they are too thin to be seen easily with the light microscope
  - Flagellar stains coat the surface of the flagella with a dye or a metal such as silver by building up layers of stain on flagella surfaces



Principles of Staining

- **Special Staining**
- Endospore staining (Schaeffer-Fulton spore stain):
  - The wall of endospores resists penetration of ordinary stains, so they appear as clear areas within bacterial cells
  - Schaeffer-Fulton spore stain can make spores more easily visualized
  - Procedures:
    1. Heat-fixed smear is covered with malachite green
    2. Heated until steaming; to increase spore permeability to the dye
    3. Rinse with water
    4. Counterstain with safranine (red)
  - Vegetative cells appear red, spores appear green



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Comparison of Staining Techniques

Type	Examples		Result	Uses
Simple Stains				
Use a single dye; do not distinguish organisms or structures by different staining reactions	Methylene blue Safranin Crystal violet ⟶	a	Uniform blue stain Uniform red stain Uniform purple stain	Shows sizes, shapes, and arrangements of cells
Differential Stains				
Use two or more dyes that react differently with various kinds or parts of bacteria, allowing them to be distinguished	Gram stain	b	Gram +: purple with crystal violet Gram −: red with safranin counterstain Gram-variable: intermediate or mixed colors (some stain + and some − on same slide) Gram-nonreactive: stain poorly or not at all	Distinguishes Gram +, Gram −, Gram-variable, and Gram nonreactive organisms
	Ziehl-Neelsen acid-fast stain	c	Acid-fast bacteria retain carbolfuchsin and appear red. Non–acid-fast bacteria accept the methylene blue counterstain and appear blue	Distinguishes members of the genera *Mycobacterium* and *Nocardia* from other bacteria
	Negative stain	d	Capsules appear clear against a dark background	Allows visualization of organisms with structures that will not accept most stains, such as capsules
Special Stains				
Identify various specialized structures	Flagellar stain		Flagella appear as dark lines with silver, or red with carbolfuchsin	Indicates presence of flagella by building up layers of stain on their surface
	Schaeffer-Fulton spore stain	e	Endospores retain malachite green stain. Vegetative cells accept safranin counterstain and appear red	Allows visualization of hard-to-stain bacterial endospores such as members of genera *Clostridium* and *Bacillus*