1 **Chapter 3**

Observing Microorganisms Through A Microscope

2 **Observing Microorganisms**

3 **Units of Measurement**

- 1 µm = 10–6 m = 10–3 mm
- 1 nm = 10–9 m = 10–6 mm
- 1000 nm = 1 µm
- 0.001 µm = 1 nm
-

4 **Microscopy: The Instruments**

- A simple microscope has only one lens

5 **Light Microscopy**

- Use of any kind of microscope that uses visible light to observe specimens
- Types of light microscopy
  - Compound light microscopy
  - Darkfield microscopy
  - Phase-contrast microscopy
  - Differential interference contrast microscopy
  - Fluorescence microscopy
  - Confocal microscopy
  -

6 **The Compound Light Microscope**

7 **Compound Light Microscopy**

- In a compound microscope, the image from the objective lens is magnified again by the ocular lens
- Total magnification =
  objective lens × ocular lens

8 **Compound Light Microscopy**

- Resolution is the ability of the lenses to distinguish two points
- A microscope with a resolving power of 0.4 nm can distinguish between two points ≥ 0.4 nm
- Shorter wavelengths of light provide greater resolution
- As does increased numerical aperature. Increased aperature is achieved by increased magnification or the use of higher quality lenses.

9 **Compound Light Microscopy**

- The refractive index is a measure of the light-bending ability of a medium
- The light may bend in air so much that it misses the small high-magnification lens
- Immersion oil is used to keep light from bending, thus reducing refraction and increasing resolution.
- Lost light in this case = poor image quality.

10 **Refraction in the Compound Microscope**

11 **Brightfield Illumination**

- Dark objects are visible against a bright background
- Light reflected off the specimen does not enter the objective lens which is why it appears dark.
- Con: translucent specimens are nearly invisible as light simply passes through them

creating the need for stains.

12 **Darkfield Illumination**

- Light objects are visible against a dark background
- Light reflected off the specimen enters the objective lens
- Background darkens due to lost light

13 **Darkfield Illumination**

- Light enters the microscope for illumination of the sample.
- A specially sized disc, the *"patch stop"* blocks some light from the light source, leaving an outer ring of illumination.
- The condenser lens focuses the light towards the sample.
- The light enters the sample. Most is directly transmitted, while some is scattered from the sample.
- The scattered light enters the objective lens, while the directly transmitted light simply misses the lens and is not collected due to a *direct illumination block*.
- Only the scattered light goes on to produce the image, while the directly transmitted light is omitted.
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14 **Phase-Contrast Microscopy**

- Phase contrast microscopy is a technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image.
- The phase shifts themselves are invisible to the human eye, but become visible when they are shown as brightness changes.
- Accentuates diffraction of the light that passes through a specimen
- Allows in vivo study of cells without staining
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15 **Differential Interference Contrast Microscopy**

- Accentuates diffraction of the light that passes through a specimen; uses two beams of light
- Excellent for use with live, unstained samples.

16 **Differential Interference Contrast Microscopy**

- Differential interference contrast microscopy (DIC), also known as Nomarski Interference Contrast (NIC) or Nomarski microscopy, is an optical microscopy illumination technique used to enhance the contrast in unstained, transparent samples.
- DIC works on the principle of interferometer to gain information about the optical path length of the sample, to see otherwise invisible features.
- A relatively complex lighting scheme produces an image with the object appearing black to white on a grey background.
- This image is similar to that obtained by phase contrast microscopy but without the bright diffraction halo.

17 **Fluorescence Microscopy**

- Uses UV light
- Fluorescent substances absorb UV light and emit visible light
- Cells may be stained with fluorescent dyes (fluorochromes)
- A sample is illuminated with light of a wavelength which causes fluorescence in the sample. The light emitted by fluorescence, which is at a different, longer, wavelength than the illumination, is then detected through a microscope objective.
- Excellent resolution, toxic to live cells

18 **Confocal Microscopy**

- Cells stained with fluorochrome dyes

- Short wavelength (blue) light used to excite the dyes
- The light illuminates each plane in a specimen to produce a three-dimensional image
- Up to 100 μm deep
- Confocal microscopy has incredibly high resolving power compared to conventional forms of light microscopy, but focus can be limited by aberrations.
- Any imperfections along the optical path will cause a reduction in signal intensity and quality of the image.

19 **Two-Photon Microscopy**

- Cells stained with fluorochrome dyes
- Two photons of long- wavelength (red) light used to excite the dyes
- Allows imaging of living tissue to a depth of 1mm
- Better alternative to Confocal Microscopy due to its deeper penetration.

20 **Scanning Acoustic Microscopy (SAM)**

- Measures sound waves that are reflected back from an object
- Used to study cells attached to a surface
- Can provide information on the elasticity of cells or the physical forces holding structures together.
- Resolution 1 μm

21 **Electron Microscopy**

- Uses electrons instead of light
- The shorter wavelength of electrons gives greater resolution

22 **Transmission Electron Microscopy (TEM)**

- TEM is a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passes through.
- Ultra thin sections of specimens
- Light passes through specimen, then an electromagnetic lens, to a screen or film
- Specimens may be stained with heavy metal salts
- This enables the instrument's user to examine fine detail—even as small as a single column of atoms.

23 **Transmission Electron Microscopy (TEM)**

- 10,000–100,000×; resolution 2.5 nm

24 **Scanning Electron Microscopy (SEM)**

- Type of electron microscopy where electrons fired at a specimen interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition, and other properties such as electrical conductivity.
- An electron gun produces a beam of electrons that scans the surface of a whole specimen
- Secondary electrons emitted from the specimen produce the image

25 **Scanning Electron Microscopy (SEM)**

- 1,000–10,000×; resolution 20 nm

26 **Scanned-Probe Microscopy**

- Scanning tunneling microscopy (STM) uses a metal probe to scan a specimen
- Resolution 1/100 of an atom
- An image of the surface is obtained by mechanically moving the probe over the specimen, line by line, and recording the probe-surface interaction as a function of position.

27 **Scanned-Probe Microscopy**
- Atomic force microscopy (AFM) uses a metal- and-diamond probe inserted into the specimen.
- Produces three-dimensional images with a resolution measured in nm.

28
- Staining: Coloring the microbe with a dye that emphasizes certain structures
- Smear: A thin film of a solution of microbes on a slide
- A smear is usually fixed to attach the microbes to the slide and to kill the microbes

29 **Preparing Smears for Staining**
- Live or unstained cells have little contrast with the surrounding medium. Researchers do make discoveries about cell behavior by observing live specimens.

30 **Preparing Smears for Staining**
- Stains consist of a positive and negative ion
- In a basic dye, the chromophore (color molecule)is a cation (+)
- In an acidic dye, the chromophore is an anion (-)
- Staining the background instead of the cell is called negative staining
- (note: bacteria carry a slight negative charge at pH 7)

31 **Simple Stains**
- Simple stain: Use of a single basic dye
- A mordant may be used to hold the stain or coat the specimen to enlarge it

32 **Differential Stains**
- Used to distinguish between bacteria
  - Gram stain
  - Acid-fast stain

33 **Gram Stain**
- Classifies bacteria into gram-positive or gram-negative
  - Gram-positive bacteria tend to be killed by penicillin and detergents
  - Gram-negative bacteria are more resistant to antibiotics

34 **Gram Stain**

35 **Micrograph of Gram-Stained Bacteria**

36 **Acid-Fast Stain**
- Stained waxy cell wall is not decolorized by acid-alcohol
- *Mycobacterium*
- *Nocardia*

37 **Acid-Fast Stain**

38 **Acid-Fast Bacteria**

39 **Special Stains**
- Used to distinguish parts of cells
  - Capsule stain
  - Endospore stain
  - Flagella stain

40 **Negative Staining for Capsules**
- Cells stained
- Negative stain

41 **Endospore Staining**

- Primary stain: Malachite green, usually with heat
- Decolorize cells: Water
- Counterstain: Safranin
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42 **Flagella Staining**

- Mordant on flagella
- Carbolfuchsin simple stain

43

- ✓ Which stain would be used to identify microbes in the genera *Mycobacterium* and *Nocardia*?
  3-10
- ✓ How do unstained endospores appear?
  Stained endospores? 3-11
- ✓
- ✓
- ✓
- ✓
- ✓
- ✓