Microscopy

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BIODIVERSITY I
BIOL1051

MAJOR FUNCTIONS OF MICROSCOPES

• MAGNIFY
• RESOLVE: =>
• INCREASE CONTRAST

Light Microscopy

Eyepiece or ocular

- Magnifies the image produced by the objective
- Usually 5X or 10X
- Different field-of-view (6-28 mm)
- Field Size (mm) = FN / OM
- FN: Field Number
- OM: Objective Magnification
Eyepiece or ocular

- **Most important part**
  - Projects an accurate inverted image of object
  - Numerical Aperture (light-grasping ability) = most important information
  - Permits calculation of:
    - Useful magnification
    - Resolution
    - Depth of field

Objective

- 60x Plan Apochromat Objective
  - Manufacturer
  - Flat-Field Correction
  - Lateral Magnification
  - Specialized Optical Properties
  - Tube Length
  - Cover Glass Thickness Range
  - Cover Glass Adjustment Gauge

Magnification

- **Total Magnification** = Objective magnification \( \times \) eyepiece magnification

- **Useful Magnification** = (500 to 1000) \( \times \) NA (Objective)

Ex: Is it worth using a 20 X eyepiece with this objective?

Useful Magn. = 1000 \( \times \) 0.95 = 950

10 X 60 = 600
20 X 60 = 1200

Resolution

- **Resolution** \( (r) = \frac{\lambda}{2(NA)} \)
- **Resolution** \( (r) = 0.61 \frac{\lambda}{NA} \)
- **Resolution** \( (r) = 1.22 \frac{\lambda}{(NA_{obj} + NA_{cond})} \)
- \( r \) = distance at which two objects will be seen as separated. The smaller this distance, the better is the resolution power. So, the greater the NA, the better.
- N.A. = numerical aperture of the objective
- \( \lambda \) = wavelength

Resolution

- \( r = \frac{\lambda}{2 \text{ N.A.}} \)
- Smaller \( r \) = better resolution
- What light colour will give the better resolution?
- V, B, C, Y, O, R
### Depth of field

- The depth of field means the thickness of the specimen that can be focussed at the same time.
  - \( D_f = R \times n / M \times NA \)
- \( D_f \) = depth of field
- \( R \) = diameter of the “confusion circle” that is a measure of the fuzziness of the image. This value must be lower than 0.2 and a value of 0.145 is used for calculations.
- \( n \) = refractive index at the interface between the objective and the specimen
- \( M \) = magnification of the objective
- \( NA \) = Numerical Aperture of the objective

### Light Microscopy

- Bright field microscopy
- Oil immersion microscopy
- Phase contrast microscopy
- Dark field microscopy
- Differential Interference Contrast or DIC
- Polarised light microscopy
- Ultra violet light microscopy
- Fluorescence microscopy
- Confocal microscopy & Confocal laser scanning microscopy

### Bright field microscopy

- Probably the only one you will ever see.
- Even “student microscopes” can provide spectacular views
- Limitations:
  - Resolution
  - Illumination
  - Contrast
- Improvements:
  - Oil immersion
  - Dark field
  - Phase contrast
  - Differential Interference Contrast
- Best for: stained or naturally pigmented specimens.
- Useful for: living specimens of bacteria
- Inferior for: non-photosynthetic protists, metazoa, unstained cell suspensions, tissue sections

### Oil immersion microscopy

- At higher magnifications, the amount of light passing the object is reduced
- Immersion oil reduces the diffracted light, increasing the amount going through the object.
- Refractive index:
  - Air: 1
  - Immersion oil: 1.515
  - Glass: 1.515

### Phase contrast microscopy

- Increases contrast.
- Translates minute variations in phase into corresponding changes in amplitude, which can be visualised as differences in image contrast. Excellent for living unstained cells.
- For his invention of phase-contrast microscopy, Zernike was awarded the 1953 Nobel Prize in Physics.
**Dark field microscopy**
- Opaque disk in light path
- Only light scattered by objects reaches the eye
- The object seen as white on black background like dust in a sun ray

Cells of the baker's yeast *Saccharomyces cerevisiae* visualized by different types of light microscopy.
(a) Bright-field microscopy.
(b) Phase-contrast microscopy.
(c) Dark-field microscopy. Cells average 8–10 µm wide.

**Fluorescence microscopy**
- Many substances (fluorochromes) emit light when irradiated at a certain wavelength (Auto fluorescence).
- Some can be made fluorescent by treatment with fluorochromes (Secondary fluorescence).
- Preparations can be treated with fluorescent antibodies (Immunofluorescence) or fluorescent genetic probes (FISH).

(a) Bright field illumination
(b) Dark field illumination
(c) Dark field with red filter

Fluorescence microscopy.
(a, b) Cyanobacteria.
(a) Cells observed by bright-field microscopy.
(b) The same cells observed by fluorescence microscopy (cells exposed to light of 546 nm). The cells fluoresce red because they contain chlorophyll a and other pigments.
(c) Fluorescence photomicrograph of cells of *Escherichia coli* made fluorescent by staining with the fluorescent dye, DAPI.
Fluorescence microscopy

- Shallow depth of field
- Elimination of out-of-focus glare
- Ability to collect serial optical sections from thick specimens
- Illumination achieved by scanning one or more focused beams of light (laser) across the specimen
- Stage vs beam scanning

Confocal microscopy

- Images fixed or living cells
- Gives 3-D images
- Specimen has to be labelled with fluorescent probes
- Resolution between light microscopes & TEM

Confocal microscopy

- Principal Light Pathways in Confocal Microscopy
- Stage vs beam scanning

Confocal microscopy

- Neurons
- Wolbachia in red
- Lilly double fertilization

Electron microscopy

\[ r = \frac{\lambda}{2 \text{ N.A.}} \]

- Electron = smaller wavelength than visible light \( \Rightarrow \) better resolution (nm vs \( \mu \)m)
- Modern TEM can reach a resolution power of 0.2-0.3 nm
- Transmission electron microscopy (TEM)
- High resolution electron microscopy (HREM)
- Scanning electron microscopy (SEM)
Transmission electron microscopy (TEM)

- Electron beam produced in vacuum
- Beam focus on sample by magnetic field lenses
- Operates under high voltage (50 to 150 kV)
- Electron beams deflected by object
- Degree deflection permits image formation
- Image formed on fluorescent plate or camera
- Specimens have to be coated with metal

Transmission electron microscopy (TEM)

Herpes virus in nucleus

Bacterium in macrophage

Scanning electron microscopy (SEM)

- Resolution:
  - SEM < TEM
- Depth focus:
  - SEM > TEM
- Surface object scan by electron beams => secondary electrons
- Collected on detector
- Signal increased
- Image on viewing screen
- Preparations have to be coated with metal

Scanning electron microscopy (SEM)

Neutrophile migrating across endothelium

Scanning electron micrograph of M. paratuberculosis

Tunnelling Microscopy

- Piezo-electric scanner position sharp tip above object
- Tunnelling current or z changes recorded
- Transformed into corresponding 3-D image
- AToms can be Visualised!
Tunnelling Microscopy

Oh Where, Oh Where Has My Xenon Gone?
Oh Where, Oh Where Can He Be?
Xenon on Nickel

Atomic Force Microscopy

- Images at atomic level
- Measures forces at nano-Newton scale
- Force between tip and object measured by deflection of µ-cantilever
- Atomically sharp tip scan on surface of object
- Differences in height are converted to 3-D images


AFM topographs of purple membrane from Halobacterium salinarium.

From:
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