BIODIVERSITY I
BIOL1051
Microscopy

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MAJOR FUNCTIONS OF MICROSCOPES

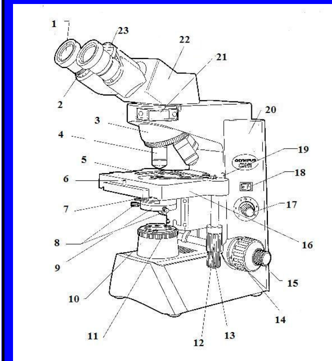
- **MAGNIFY**
- **RESOLVE:**

- **INCREASE CONTRAST**

MICROSCOPY

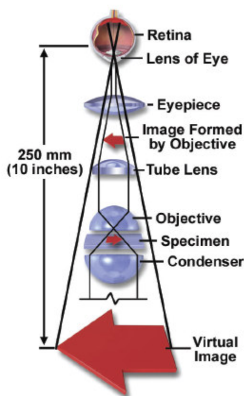
Light
Electron
Tunnelling
Atomic Force

Light Microscopy



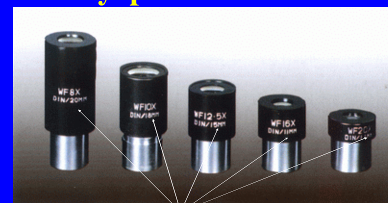
1. Eyepieces	13. X-axis knob
2. Diopter adjustment ring	14. Coarse focus adjustment knob
3. Revolving nosepiece	15. Fine focus adjustment knob
4. Objective	16. Stage
5. Specimen holder	17. Ligh intensity control knob
6. Transport lock pin	18. Main switch
7. Aperture iris diaphragm knob	19. Transport lock pin
8. Condenser centering screws	20. Microscope frame
9. Condenser	21. Dummy slider
10. Filter holder	22. Observation tube
11. Field iris diaphragm ring	23. Interpupillary distance scale
12. Y-axis knob	

Principle of light microscopy



- The **objective** produces an **amplified inverted** image of the specimen
- The **eyepiece** amplifies the image produced by the objective
- The eye sees a virtual image of the object at about 10 inches away.

Eyepiece or ocular



Field number

- 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

Field Size Variations with Field Number

Field Number = 20

Figure 2

Field Number = 26

Objective

- 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

60x Plan Apochromat Objective

Manufacturer, Flat-Field Correction, Lateral Magnification, Specialized Optical Properties, Tube Length, Cover Glass Thickness Range, Cover Glass Adjustment Guage, Nosepiece Mounting Thread, Aberration Correction, Numerical Aperture, Working Distance, Magnification Color Code, Correction Collar, Front Lens Element Assembly

Nikon Plan Apo 60X / 0.95 DIC M ∞/0.11-0.23 WD 0.15

Figure 1

Magnification

- TOTAL MAGNIFICATION = Objective magnification X eyepiece magnification
- Useful Magnification = (500 to 1000) x NA (Objective)

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

Useful Magn. = 1000 X 0.95 = 950

10 X 60 = 600

20 X 60 = 1200

Magnification

50 µm

Flagella, Canal, Eye spot, Reservoir, Chloroplast, Mitochondrion, Nucleus, Paramylon granule

Organism: *Euglena gracilis*
Sample: *Pond water*
Magnification: *1000 X*

Resolution

- Resolution (r) = λ/(2NA)
- Resolution (r) = 0.61 λ /NA
- Resolution (r) = 1.22 λ /(NAobj + NAcond)
- 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
- λ = wavelength

Resolution

Nanometers (1 Nanometer = 0.001 Microns)

Ultraviolet (UV - Invisible) | Visible Wavelengths | Infrared (IR - Invisible)

V B G Y O R

300 350 400 450 500 550 600 650 700 750 800 850

Spectrum of "White" Light (Diagrammatic)

- r = λ/2 N.A.
- Smaller r = better resolution
- What light colour will give the better resolution?
- V, B, G, Y, O, R

Depth of field

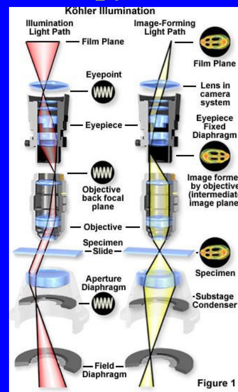
- The depth of field means the thickness of the specimen that can be focussed at the same time.
- **Df = R x n / M x NA**
- Df = depth of field
- R = diameter of the "confusion circle" that is a measure of the fuzziness of the image. This value must be lower than 0.2 and a value of 0.145 is used for 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

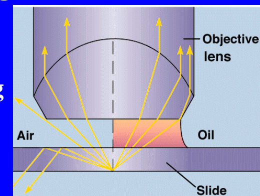
Bright field microscopy

- 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.
- Useless for: living specimens of bacteria
- Inferior for: non-photosynthetic protists, metazoans, unstained cell suspensions, tissue sections

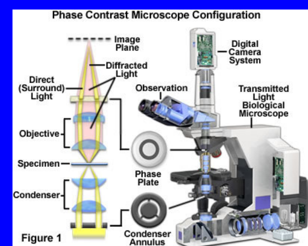


Oil immersion microscopy

- 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.

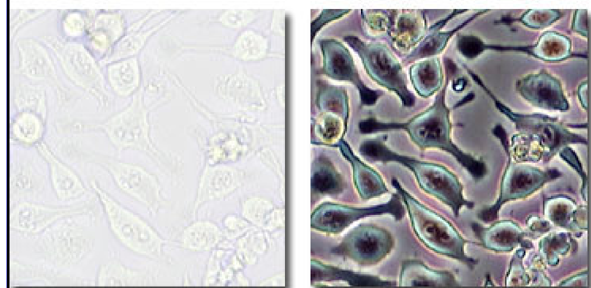


Figure 2

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

Radiolarian in Brightfield and Darkfield Illumination

Figure 2

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

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)

Fluorescence microscopy

Fluorescence microscopy.

(a, b) Cyanobacteria.

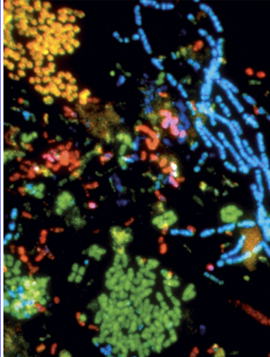
(a) Cells observed by bright-field microscopy.

(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.

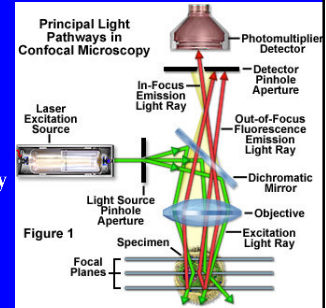
Fluorescence microscopy

Fluorescence microscopy

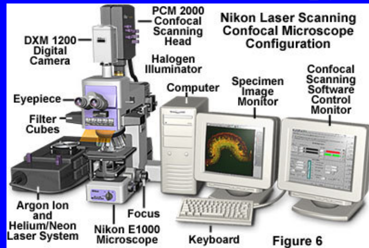


Confocal microscopy

- 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

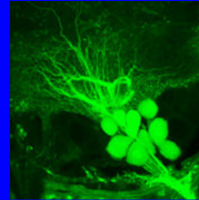


Confocal microscopy

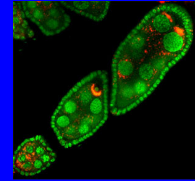


- Images fixed or living cells
- Gives 3-D images
- Specimen has to be labelled with fluorescent probes
- Resolution between light microscopes & TEM

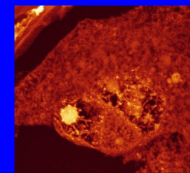
Confocal microscopy



Neurons



Wolbachia in red



Lilly double fecondation

MICROSCOPY

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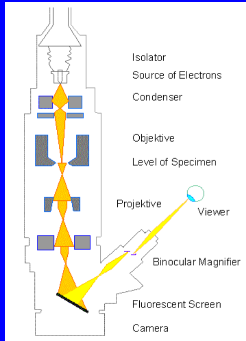
Electron microscopy

$$r = \lambda/2 \text{ N.A.}$$

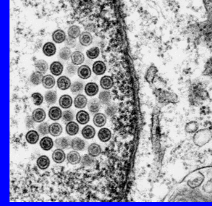
- Electron = smaller wavelength than visible light => better resolution (nm vs µm)
- Modern TEM can reach a resolution power of 0.2-0.3 nm
- Transmission electron microscopy (TEM)
- High resolution electron microscopy (HREM)
- Scanning electron microscopy (SEM)

Transmission electron microscopy (TEM)

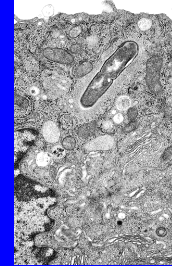


- Electron beam produced in vacuum
- Beam focus on sample by magnetic field lenses
- Operates under high voltage (50 to 150 kV)
- Electron beams deflected by object
- Degree deflection permits image formation
- Image formed on fluorescent plate or camera
- Specimens have to be coated with metal

Transmission electron microscopy (TEM)

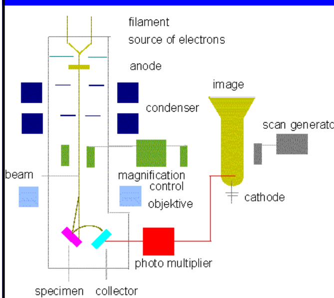


Herpes virus in nucleus



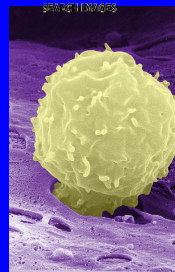
Bacterium in macrophage

Scanning electron microscopy (SEM)

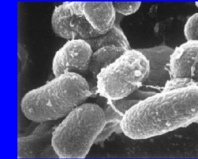


- Resolution:
  - SEM < TEM
- Depth focus:
  - SEM > TEM
- Surface object scan by electron beams => secondary electrons
- Collected on detector
- Signal increased
- Image on viewing screen
- Preparations have to be coated with metal

Scanning electron microscopy (SEM)



Neutrophile migrating across endothelium



Scanning electron micrograph of *M. paratuberculosis*

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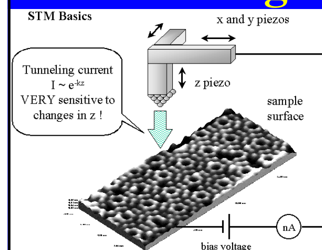
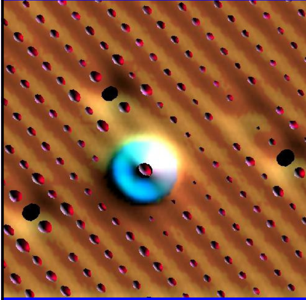
Tunnelling Microscopy



- 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

MICROSCOPY

Light

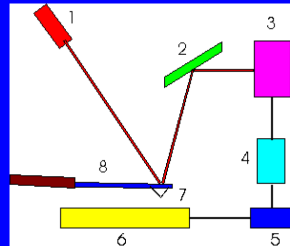
Electron

Tunnelling

Atomic Force

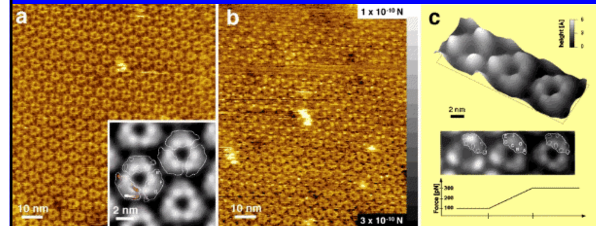
Atomic Force Microscopy

- 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 => 3-D images



1. Laser, 2. Mirror, 3. Photodetector, 4. Amplifier, 5. Register, 6. Sample, 7. Probe, 8. Cantilever.

Atomic Force Microscopy



AFM topographs of purple membrane from *Halobacterium salinarium*.

From:
http://www.mih.unibas.ch/Booklet/Booklet96/Chapter3/Chapter3.html

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http://www.cas.muohio.edu/~mbi-ws/microscopes/microscopeparts.html
http://www.microimaging.ca/parts.htm
http://shs.westport.k12.ct.us/mjvl/biology/microscope/microscope.htm#parts
http://www.biologycorner.com/microquiz/#
http://www.borg.com/~lubehawk/mscope.htm
http://www.usoe.k12.ut.us/curr/science/sciber00/7th/cells/sciber/micrpart.htm
http://www.southwestschools.org/jsfaculty/Microscopes/index.html

More specialised sites on microscopy:
http://micro.magnet.fsu.edu/primer/anatomy/introduction.html
http://www.ruf.rice.edu/~bioslabs/methods/microscopy/microscopy.html
http://www.microscope-microscope.org/microscope-home.htm
http://science.howstuffworks.com/light-microscope.htm/printable

Virtual Microscope:
http://www.udel.edu/Biology/ketcham/microscope/