Basic Concepts of Microscopy

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Light: a Bridge between Earth and Space: Preparatory School
- Introduction
- Lens formula, Image formation and Magnification
- Resolution and lens defects
- Basic components and their functions
- Collimators
- Specialized Microscopy Techniques
- Typical examples of applications
Basic concepts of microscopy

Similar to confocal optical (fluorescence, Raman) microscope, and optical tweezers.

Schematic diagram of the TLM, S: sample; SS: sample stage 3-D control; M₁, M₂ and M₃: mirrors; CH: chopper; DM: dichroic mirror; P: linear polarizer; L₁, L₂, L₃; L₄ and L₅: lenses; O: focusing objective lens; PH: pinhole; F: interference filter at 632.8 nm; PD: photodiode; LA: lock-in amplifier; PC: personal computer; EL: excitation laser; PL: probe laser.
Microscope Components

- Ocular
- Objectives
- Condenser
- Numerical Aperture
- Refractive Index
- Aberrations
- Optical Filters
Basic concepts of microscopy

Basic components and their functions

(1) **Eyepiece (ocular lens)**
(2) Revolving nose piece (to hold multiple objective lenses)
(3) **Objective lenses**
(4) And (5) **Focus knobs**
   - (4) Coarse adjustment
   - (5) Fine adjustment
(6) **Stage** (to hold the specimen)
(7) **Light source** (lamp)
(8) **Condenser lens** and diaphragm
(9) Mechanical stage (move the specimen on two horizontal axes for positioning the specimen)
Reflection and Refraction

- **Snell’s Law**: The angle of reflection ($\theta_r$) is equal to the angle of incidence ($\theta_i$) regardless of the surface material.

- The angle of the transmitted beam ($\theta_t$) is dependent upon the composition of the material.

\[ n_1 \sin \theta_i = n_2 \sin \theta_t \]

The velocity of light in a material of refractive index $n$ is $c/n$.
Optics of a thin lens (1)

Thin Lens: \( \frac{d}{F} \to 0 \)

Focus

C

F

C=2F

Basic concepts of microscopy
Optics of a thin lens (2)

- Three different scenarios:

![Diagram showing the three different scenarios involving thin lenses and focal lengths (F and 2F).]
Properties of thin Lenses

\[ \frac{1}{p} + \frac{1}{q} = \frac{1}{f} \]

Magnification = \[ \frac{q}{p} \]
The Concept of Magnification

Magnification of the Microscope

- $M_{Microscope} = M_{Objective} \times M_{Eyepiece} \times M_{Intermediate\ Factor}$

$M = \text{Magnification}$

- Example:  
  - Objective = 60 x  
  - Eyepiece = 10 x  
  - Intermediate Factor = 1 x  

  Overall $M = 600 \times$
The characteristics of objectives

Figure 1

Basic concepts of microscopy
Objectives configurations

**Figure 1**
- Objective
- Intermediate Image Plane
- Eyepiece
- Specimen
- Focal Point

**Figure 2**
- Objective
- Tube Lens

**Equations**
- \( M_0 = \frac{F_t}{F_0} \) (Objective magnification)
- \( F_t \): Tube lens focal length
- \( F_0 \): Objective focal length

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Lens systems and collimators (telescopes)

2 thin lenses separated by distance $d$

$$f_{\text{comb}} = \frac{1}{\frac{1}{f_1} + \frac{1}{f_2} - \frac{d}{f_1 f_2}}$$
If $d$ tends to zero

\[
\frac{1}{f_{\text{comb}}} = \frac{1}{f_1} + \frac{1}{f_2}
\]

Example, if $d=3$ cm, then $f=1.5$ cm for the combined system.
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Transporting system

\[ M_{\text{transportador}} = \frac{h_{\text{img}}}{h_{\text{obj}}} = -\frac{f_2}{f_1} \]
Afocal telescopes or collimators

If \( d = f_1 + f_2 \), then \( f_{\text{comb}} \) is undefined therefore the afocal telescopes can not be represented as a single lens.

There is no single lens with this behavior.

\[
\frac{1}{f_{\text{comb}}} = \frac{1}{f_1} + \frac{1}{f_2} - \frac{d}{f_1 f_2}
\]

\[
M_{\text{angular}} = \frac{\theta_{\text{salida}}}{\theta_{\text{entrada}}} = -\frac{f_2}{f_1}
\]

\[
|M_{\text{angular}}| = \frac{D_{\text{entrada}}}{D_{\text{salida}}} = \left|\frac{f_2}{f_1}\right|
\]

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Kepler Telescope

Basic concepts of microscopy
Galileo Telescope

Basic concepts of microscopy
Reflective Galileo Telescope

Cassegrain telescope

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T are used to modify the eye field

\[ f_{\text{comb}} = \frac{f_1 \times f_3}{f_2} = |M_{\text{angular}}| \times f_3 \]

\[ f_{\text{comb}} = \frac{f_1 \times f_3}{f_2} = f_1 \times |M_{\text{transportador}}| \]
The characteristics of objectives

Figure 1

Basic concepts of microscopy
Numerical Aperture (N.A.)

**Numerical Aperture** = N.A. = \( n \cdot \sin \alpha \)

\( \alpha \) is half the opening angle of the objective.

\( n \) is the refractive index of the immersion medium used between the objective and the object.

\((n = 1 \text{ for air; } n = 1.51 \text{ for oil or glass})\)
Aperture diaphragm (stop) and number of diaphragm

(Number of diaphragm defined in image space by the margin ray)

\[(F/\#)_{imagen} = \frac{f}{D_{DA}}\]
And for conjugate points in object and image space

\[ (F/\#)_{\text{objeto}} = \frac{p}{D_{DA}} \]

\[ (F/\#)_{\text{imagen}} = \frac{q}{D_{DA}} \]

The number of diaphragm (ND) is inverse to the diameter of the aperture diaphragm. Then increasing ND is an slow system which need more exposure time.
Field diaphragm and field of view (FV)

The maximal size of the object and the image is determined by the FV. Without FV there will be an extended infinite region outside in the object plane forming image in image plane.

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If the object is in infinity we can relate the FV with the magnification $M = -q/p$, $q \approx f$, then larger focal lens five higher magnification.

Small ND and high FV give good flux of light but low quality image due to aberrations and the contrary high ND and low FV give quality images with low brightness.

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Depth of Focus

- We also need to consider the **depth of focus** (vertical resolution). This is the ability to produce a sharp image from a non-flat surface.

\[
DOF \approx \frac{\lambda}{N.A.}
\]

- Depth of Focus is increased by inserting the **objective aperture** (just an iris that cuts down on light entering the objective lens). However, this decreases resolution.
Resolution

Resolving power, the limit up to which two small objects are still seen separately.

\[ d_0 = \frac{1.22\lambda}{N.A_{\text{obj}} + N.A_{\text{Cond}}} \]

or more simply \[ d_0 = \frac{\lambda}{2N.A} \]

\( \lambda = \) wavelength of light, e.g. 550 nm (green)
## Factors Affecting Resolution

- Resolution \( (d_{\text{min}}) \) improves (smaller \( d_{\text{min}} \)) if \( \lambda \downarrow \) or \( n \uparrow \) or \( \alpha \uparrow \)
- Assuming that \( \sin \alpha = 0.95 \) (\( \alpha = 71.8^\circ \))

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Air ((n=1))</th>
<th>Oil ((n=1.515))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red 650 nm</td>
<td>0.42 (\mu) m</td>
<td>0.28 (\mu) m</td>
</tr>
<tr>
<td>Yellow 600 nm</td>
<td>0.39 (\mu) m</td>
<td>0.25 (\mu) m</td>
</tr>
<tr>
<td>Green 550 nm</td>
<td>0.35 (\mu) m</td>
<td>0.23 (\mu) m</td>
</tr>
<tr>
<td>Blue 475 nm</td>
<td>0.31 (\mu) m</td>
<td>0.20 (\mu) m</td>
</tr>
<tr>
<td>Violet 400 nm</td>
<td>0.27 (\mu) m</td>
<td>0.17 (\mu) m</td>
</tr>
</tbody>
</table>

\( \text{Resolution}_{\text{air}} \) \quad \text{Resolution}_{\text{oil}}

(The eye is more sensitive to blue than violet)
Two sets of conjugate planes in the light microscope

Understanding the reciprocal relationship between the two sets of conjugate planes is crucial for properly understanding:

- Image formation
- Image resolution
- How phase-contrast and DIC work

Conjugate planes are "parfocal" with each other

When something is in focus in one set of conjugate planes, it is "maximally out-of-focus" in the other set of planes

These two sets are often called "reciprocal" or "transform" planes (with respect to each other)
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- Laser Scanning Microscope (Confocal System)

- Light emitted from the focal plane
- Light emitted from the out-of-focus region
Confocal Aperture

Decreasing the pinhole size rejects more out of focus light, therefore improving contrast and effective z resolution.

Decreasing the pinhole will increase x,y resolution (1.3x wide field)

Decreasing pinhole size decreases the amount of the Airy disk that reaches the detector. This results in less light from each point being collected.

Generally, collecting the diameter of 1 Airy disk is considered optimal. This collects about 85% of light from a sub-resolution point.

**Limits:**
- **Open pinhole:** nearly wide field resolution (still some confocality)
- **Closed:** no image
ALIGNMENT OF APERTURES IS CRITICAL

X, Y alignment: Different wavelengths focus at different lateral position. Lateral color aberrations can be important for multi-color imaging (multiple dyes with multiple lasers)

Z alignment: Different wavelengths focus at different depths in image plane. Chromatic aberrations can be important. Need well-corrected lenses
Wide field versus confocal scanning
WF vs C - Fluorescence Imaging

Confocal
Greatly reduces
Out of focus blur

Wide-field
Brighter but
No sectioning
More examples

Confocal and Widefield Fluorescence Microscopy

(a) widefield medulla
(b) widefield muscle
(c) widefield pollen
(d) confocal medulla
(e) confocal muscle
(f) confocal pollen

Basic concepts of microscopy
Thermal lens microscopy set up

Schematic diagram of the TLM, S: sample; SS: sample stage 3-D control; M₁, M₂ and M₃: mirrors; CH: chopper; DM: dichroic mirror; P: linear polarizer; L₁, L₂, L₃, L₄ and L₅: lenses; O: focusing objective lens; PH: pinhole; F: interference filter at 632.8 nm; PD: photodiode; LA: lock-in amplifier; PC: personal computer; EL: excitation laser; PL: probe laser.

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Thermal lens effect and signal

\[ I(r) = \frac{2P_e}{\pi w_e^2} e^{-2r^2/w_e^2} \]
Physical mathematical model

\[ z_p >> L >> z_e ; \quad t \to \infty ; \quad z_p \to \infty ; \quad z = 0 \]

\[
S(z, t) = \Phi_0 \arctan \left( \frac{4m(z)\nu(z)t / t_C(z)}{\nu^2(z) + [1 + 2m(z)]^2 + [1 + 2m(z) + \nu^2(z)]2t / t_C(z)} \right)
\]

\[
I(r) = \frac{2P_e}{\pi w_e^2} e^{-2r^2/w_e^2}
\]

\[ D = \kappa / \rho C_p \]

\[ t_c(z) = \omega_e^2(z) / 4D \]

\[ S_{max} = \pi \Phi_0 / 2 \]

\[ \Phi_0 = \frac{P_e \alpha \lambda d n}{\lambda_p k dT} \]


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Applications

Calibration curves for Cr(III) solutions in 80% water with the addition of 20% of acetonitrile in 0.5 mm cell at 407 nm for 4 and 17 mW of excitation powers.
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Lumidots: Quantum Dot Nanocrystals

CdSe/ZnS quantum dot nanocrystals

Emission spectra of Lumidot™ CdSe/ZnS nanocrystals
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For 5 persons

For 10 persons

For 2 persons (side by side)

For 2 persons (face to face)

For 3 persons

Thanks for your attention!