Optical Microscopy: Introduction

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Outline

- Brief history

- The three tasks of microscopy:
  - **Magnification**
    - Image formation
    - Optical components of an optical microscope
  - **Resolution**
    - Objectives, NA, Airy disk
  - **Contrast formation**
    - Amplitude contrast (bright field), Dark field, Phase contrast, DIC
    - Fluorescence
      - Confocal microscopy
      - 2PEF microscopy
• *Simple microscopes* - simple single lens devices that are often hand-held, such as a magnifying glass

• *Compound microscopes* - multiple-lens designs with objectives and condensers
Simple Compound

- magnifications up to 200 times
- **Von Leeuwenhoek** was the first to uncover the world of bacteria, observed blood cells and gave the first description of sperm cells

- compound microscopes were invented around 1595, nearly 40 years before Von Leeuwenhoek was born but it took aprox. 150 years of optical development before they were able to provide the same magnification and quality image as Van Leeuwenhoek’s simple microscope.
Microscopy: Brief History

- one of the most elegant microscopes built during the period
- poor images due to significant chromatic and spherical aberration

Achromatic objectives commercialized by Harmanus Van Deyl in 1807

- a famous producer of top quality microscopes
Carl Zeiss Jena produced its first oil immersion objective in 1880, designed by Ernst Abbe, who was the founder of the optical theory of microscope lenses.

- contains accessories for DIC, fluorescence, polarized light, phase contrast and photomicrography using several film formats and digital image capture.
The three tasks of a microscope

➢ produces a magnified image of the specimen (Magnification)

➢ separates the details in the image (Resolution)

➢ renders the details visible to the human eye/detector (Contrast)
Microscopy: Magnification

\[ d \gg \gg \gg 2f \]

\[ d > 2f \]

\[ d = 2f \]

\[ f < d < 2f \]
Because of the limited ability of the eye’s lens to change its shape, objects brought very close to the eye cannot have their images brought to focus on the retina. The accepted minimal conventional viewing distance is 10in (25cm) - the near point of the eye.
Eye accommodation

The eye accommodates for close vision by tightening the ciliary muscles, allowing the pliable crystalline lens to become more rounded.

The relaxed eye is focused at infinity!

http://hyperphysics.phy-astr.gsu.edu/HBASE/vision/accom.html#c1
Image appears on the same side of the lens. It cannot be projected onto a screen → virtual image

Image perceived by eye as if it were at a distance of 10 inches or 25 centimeters (near point)

Light reflected from the rose enters the lens in straight lines, refracted and focused by the lens to produce an image on the retina.
Compound Microscope: Ray Diagram
Compound Microscopes

Optical microscope with the image at infinity (for relaxed eye)

Optical microscope with the image at the near point of the eye

The final image can be formed at infinity or at the standard near point distance of 25cm.

http://hyperphysics.phy-astr.gsu.edu/HBASE/geoopt/micros2.html
Compound Microscope: Components

- Lens closest to the object: **objective**.
- Light from **condenser**, forms light cone concentrated onto the object (**specimen**).
- Light passes through the specimen and into the objective
  - projects a real, inverted, and magnified image of the specimen to a fixed plane within the microscope: **intermediate** image plane
Distance between the back focal plane of the objective and the intermediate image is termed the **optical tube length**.

- **Mechanical tube length**: distance between the nosepiece (where the objective is mounted) to the top edge of the observation tubes where the eyepieces (oculars) are inserted.
Compound Microscope: Components

- **Eyepiece or ocular**: fits into the body tube at the upper end
  - further magnifies the real image projected by the objective.
- Eye of observer sees magnified image as if it were at a distance of 10 inches (25 centimeters) from the eye
  - virtual image appears as if it were near the base of the microscope.
- Photomicrography: secondary enlarged real image projected by the objective.
  - projected on the photographic film in a camera or upon a screen held above the eyepiece.
Compound Microscope: Magnification

\[ MP = M(\text{obj})M(e) = \left(\frac{-L}{fo}\right)\left(\frac{254}{fe}\right) \]
Finite and Infinity Optical Systems

19th century

Modern

Figure 2
Objective Specifications

Fl, Fluar, Fluor, Neofluar, Fluotar – for better spherical and chromatic corrections

Apo (Apochromatic) – for the highest degree of correction for spherical and chromatic aberrations

Plan, Achroplan, Plan Apo, Plano – field curvature corrections

micro.magnet.fsu.edu/primer
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     Optical components of an optical microscope

   ➢ Resolution
     Objectives, NA, Airy disk

   ➢ Contrast formation
     Amplitude contrast (bright field), Dark field, Phase contrast, DIC
     Fluorescence
     - Confocal microscopy
     - 2PEF microscopy
**Numerical Aperture and Resolution**

*NA*: measure of ability to gather light and resolve fine specimen detail

\[
\text{Numerical Aperture (NA)} = n \sin \mu
\]

- \(\mu\) - one-half the angular aperture (\(A\))

- \(n\) - the refractive index of the imaging medium (between 1.00 for air to 1.51 for specialized immersion oils)
Numerical Aperture and Resolution

Resolution: smallest distance between two points on a specimen that can still be distinguished as two separate entities.

\[ R = 0.61 \lambda / NA \]
\[ R = 1.22 \lambda / (NA(\text{objective}) + NA(\text{condenser})) \]

NB. When imaging medium is air, NA max = 1.0 (\(\sin(90^\circ) = 1\))
Light from points of specimen passes through the objective, forms image. Points of the specimen appear in the image as small patterns: *Airy patterns*. -caused by diffraction or scattering of the light passing through specimen. Central maximum of the Airy patterns: *Airy disk*, region enclosed by the first minimum -contains 84 percent of the luminous energy.

**Numerical Aperture and Resolution**

**zeroth order**
(maximum) surrounded by concentric 1st, 2nd, 3rd, etc., order maxima of sequentially decreasing brightness that make up the intensity distribution.
If separation between the two disks exceeds their radii, they are resolvable (b).

Limit at which two Airy disks can be resolved into separate entities is often called the Rayleigh criterion.
Brief history

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Contrast formation

Contrast is defined as the difference in light intensity between the image and the adjacent background relative to the overall background intensity.

\[
\text{Percent Contrast (C)} = \frac{(I(s) - I(b)) \times 100}{I(b)}
\]

where \( I(b) \) is the intensity of the background and \( I(s) \) is the specimen intensity.

As light travels through a medium, interaction with this medium causes its amplitude and phase to change. Changes in amplitude → absorption of light
Imaging Techniques

**Transmitted Light Microscopy** - light is transmitted through the sample, focused with the objective and then passed into the eyepieces of the microscope. *Diascopic*

**Reflected Light Microscopy** - light passed through the objective and is then reflected from the surface of the sample and into the microscope objective. *Episcopic*

- Amplitude (bright field)
- Phase contrast
- DIC (Nomarski)
- Dark field
- Fluorescence
- Reflectance
Amplitude contrast (bright field)

- Stained specimens
- Histology
- In-vitro

Frog ciliated epithelium
Dark field microscopy

- an illumination technique that capitalizes on oblique illumination to enhance contrast in specimens that are not imaged well under normal brightfield illumination conditions

Specimens (scattering objects):
- Live
- Unstained (stained also respond well)
- Thin (to avoid diffraction artifacts)
Phase Contrast Microscopy

- the zeroth order light passes undeviated
- the diffracted light lags behind $\frac{1}{4}$ $\lambda$
- Zernike’s phase plate speeds up the diffracted light by an additional $\frac{1}{4}$ $\lambda$
  $\rightarrow$ destructive interference between deviated and undeviated light.

Specimens (transparent, phase objects):
- Unstained
- Live
- Thin
Differential Interference Contrast (Nomarski)

Specimens (transparent, phase objects):
- Unstained
- Live
- Thin

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Fluorescence: Principle

Fluorescence - the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength after a brief interval (fluorescence lifetime).

- A-photon absorption
- F-fluorescence (emission)
- P-phosphorescence
- S-singlet state
- T-triplet state
- IC-internal conversion
- ISC-intersystem crossing

Jablonski energy diagram
This overlap of excitation and emission intensities and wavelengths must be eliminated in fluorescence microscopy, by appropriate selection of bandpass filters and dichroics.
Anatomy of a fluorescence microscope

Example of filters-dichroic combination
Fluorescence microscopy studies material that can be made to fluoresce, either in its natural form (endogenous or autofluorescence) or when treated with chemicals capable of fluorescing (exogenous fluorescence).

Autofluorescence - does not require any treatment of fixing and staining of the specimens (+)
- increases the background signal (non-specificity) (-)

Exogenous fluorescence - helps to identify cells and sub-microscopic cellular components and other entities with a high degree of specificity (+)
- cellular staining can be toxic or may interfere with the functioning of the cell (-)

Hundreds of fluorochromes with known intensity curves of absorption and emission and well-understood biological structure targets were developed.
Alexa Fluor dyes are typically used as cell and tissue labels in fluorescence microscopy and cell biology.

Mouse intestine section labeled with four fluorescent dyes. Filamentous actin was labeled with Alexa Fluor 488 phalloidin (green), fibronectin was labeled with anti-fibronectin antibody and visualized using Alexa Fluor 647 goat anti–chicken IgG (pseudocolored purple), goblet cells were labeled with an Alexa Fluor 594 conjugate of wheat germ agglutinin (red), and nuclei were labeled with anti–cdc6 peptide antibody and visualized using Alexa Fluor 405 goat anti–mouse IgG (blue).
Fluorescence from the sample is collected by an objective lens and directed toward a pinhole aperture. The pinhole allows the emitted light from a narrow focal plane (black solid lines) to pass to the detector, while blocking most of the out-of-focus light (green and red lines).

**Confocal system:**
- a point light source for illumination
- a point light focus within the specimen
- a pinhole at the image detecting plane

**Light sources for confocal systems:**
Laser systems are preferred over lamp sources due to their small spot size, low divergence and high radiant flux.
Benefits of confocal microscopy over conventional widefield optical microscopy:

- the ability to control depth of field
- elimination or reduction of background information away from the focal plane (that leads to image degradation)
- the capability to collect serial optical sections from thick specimens.
Two Photon Fluorescence

Absorption and Emission Spectra with Overlap Profile

1 photon excitation (e.g. 400nm)
2 photon excitation (e.g. 800nm)
Two Photon Fluorescence Microscopy

\[ I \propto z^{-2} \]
\[ 2\text{PA} \propto I^2 \]
\[ 2\text{PA} \propto z^4 \]
Optical Microscopy

- To magnify image
- To resolve minute details
- To obtain enough contrast to make details visible

- To record and/or analyze image
  - Archiving
  - Post-processing
  - Quantification
  - 3-D reconstruction