Optical Microscopy
Principles and Applications

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Self Introduction

BE in Electrical Engineering from Tribhuvan University
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M.Sc. in Electrical Engineering from South Dakota State University
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Major Functions of the Microscope

- Illuminate
- Magnify
- Resolve features
- Generate Contrast
- Capture and display image
Lecture Outline

• Propagation, diffraction, and polarization
• Absorption, and scattering
• Wide-field imaging techniques
  • Bright-field/dark-field imaging,
  • Phase-contrast imaging, and
  • Differential interference contrast imaging
• Scanning imaging techniques
• Confocal detection
• Differential phase-gradient detection
• Non-linear imaging techniques
  • Multi-photon,
  • Second harmonic, and
  • Raman scattering
Light as photons, waves or rays

Light is an electromagnetic (EM) field in space-time. Photon is the smallest, discrete quanta of EM field. Rays are the propagation direction of the EM field.

\[
\begin{align*}
\nabla \cdot E &= 0, \quad \nabla \times E = -\mu \frac{\partial H}{\partial t} \\
\nabla \cdot H &= 0, \quad \nabla \times H = -\varepsilon \frac{\partial E}{\partial t} \\
\n\nabla^2 E - \mu \varepsilon \frac{\partial^2 E}{\partial t^2} &= 0 \\
\frac{n^2}{c^2} &= \mu \varepsilon
\end{align*}
\]

Wave equation in Linear medium
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\[ \frac{n^2}{c^2} = \mu \varepsilon \]

Maxwell equations

Wave equation in Linear medium

Ray Optics: Optical rays travelling between two points A and B follow a path such that the time of travel (or optical path-length) between two points is minimal relative to the neighboring paths.

\[ \delta \int_A^B n(r) ds = 0 \]

Light travels along the path of least time.
Light propagation

Wave equation
\[ \nabla^2 E - \frac{1}{c^2} \frac{\partial^2 E}{\partial t^2} = 0 \]

Helmholtz equation
\[ \nabla^2 U + k^2 U = 0 \]

Solution
\[ E(r, t) = a(r)e^{-ikr}e^{i2\pi vt} \]

Plane wave:
\[ U(r) = A e^{-ikr} \]

Spherical wave:
\[ U(r) = \frac{A}{r} e^{-ikr} \]

Fresnel Approx. of Spherical wave:
\[ U(r) \approx \frac{A}{r} e^{-ikz} e^{-ik\frac{x^2+y^2}{2z}} \]

Paraxial wave:
\[ U(r) \approx A(r)e^{-ikz} \]

Light from stars

Point source

Point source at large distance

\[ \nabla^2 A - i2k A = 0 \]

Gaussian Beam
Light travels more slowly in matter

The speed ratio is the **Index of Refraction**

\( n = \frac{c}{v} \)
Light travels more slowly in matter

The speed ratio is the **Index of Refraction** \((n = \frac{c}{v})\)

\[ n = \begin{cases} 1 & n > 1 \\ 1 & n < 1 \end{cases} \]

The reflected wave:

\[ \theta_r \]

The incident wave:

\[ \theta_1 \]

The refracted wave:

\[ \theta_2 \]

\[ \lambda \]

\[ \frac{\lambda}{n} \]

Snell's law:

\[ n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \]

Mirror law:

\[ \theta_r = \theta_1 \]

\[ \theta_1 \rightarrow \text{Reflected wave} \]

\[ \frac{\lambda}{n} \]

\[ \theta_2 \rightarrow \text{Refracted wave} \]

\[ \theta_1 \rightarrow \text{Incident wave} \]
Lenses work by refraction

Incident light

Focal length $f$

Rays are perpendicular to wave fronts
Single lens Imaging

The lens law: \[ \frac{1}{L_1} + \frac{1}{L_2} = \frac{1}{f} \]

Magnification: \[ M = \frac{d_2}{d_1} = \frac{L_2}{L_1} \]
Finite vs. Infinite Conjugate Imaging

Finite conjugate imaging

Object $f_0$ Image

Field-of-view (FOV) is determined by the size (optics diameter) of the lenses.

Infinite conjugate imaging

Object $f_0$ Pupil

$M = \frac{f_1}{f_0}$

$\Rightarrow$ Need a tube lens

(uncritical)
The Compound Microscope

- Sample
- Objective
- Tube lens
- Eyepiece
- Exit pupil
- Intermediate image plane
- Back focal plane (pupil)
- Object plane
- Final image
- Eye
Köhler Illumination

- Each light source point produces a parallel beam of light at the sample
- Uniform light intensity at the sample even if the light source is not uniform

Critical Illumination

- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform
Trans-illumination

The aperture iris controls the range of illumination angles.
The field iris controls the illuminated field of view.
Interference

In phase

\[ \text{constructive interference} \]

Opposite phase

\[ \text{destructive interference} \]
Diffraction by a periodic structure (grating)

Why is light diffract?

- Light is an EM field.
- Small aperture behaves like point source.
- Light from each point source propagates in all directions.
- Only in-phase field can propagate.

In phase if $d \sin(\theta) = m\lambda$ for some integer $m$. 
Diffraction by an aperture

Light spreads to new angles

Larger aperture ⇔ weaker diffraction

The pure, “far-field” diffraction pattern is formed at $\infty$ distance

It can be formed at a finite distance by a lens

Any aperture produces a diffraction pattern
Point Spread function (PSF)

Diffraction spot on image plane = Point Spread Function

\[ d = \frac{2.44 \lambda f}{D} \]

"Airy disk" diameter

\[ d \]

Sample
Objective
Tube lens

Image plane
Numerical Aperture and Resolution

\[ \text{NA} = n \sin(\alpha) \]

Resolution
\[ \approx 0.61 \frac{\lambda}{\text{NA}} \]

Axial Resolution
\[ \approx 2 \frac{\lambda}{\text{NA}^2} \]

Oil immersion:
\[ n \approx 1.515 \]
\[ \text{max NA} \approx 1.4 \]

Water immersion:
\[ n \approx 1.33 \]
\[ \text{max NA} \approx 1.2 \]

Source: MicroscopyU.com
Polarization

Light is a vector wave: it has not only field strength, but also field direction.

\[ E(z, t) = A e^{-i k z} e^{i 2 \pi v t} \]
\[ A = A_x \hat{x} + A_y \hat{y} \]

Polarizer

Polarizer allows propagation of only one component of electric field.

Linear polarization
\[ \varphi_x = \varphi_y \]

Circular polarization
\[ \varphi_x - \varphi_y = \pi/2 \]
\[ a_x = a_y \]
Polarization

**Birefringent Crystal:** Refractive index depends on the polarization and propagation direction of light.

\[ \n = n_y - n_x \]

**Uniaxial crystal:** There is a single direction governing the optical anisotropy. All other directions perpendicular to it are optically equivalent.

\[ \n = n_e - n_o \]

Where:
- \( n_o \) is ordinary index
- \( n_e \) is extra-ordinary index

**Circular polarization**

\[ \phi_x - \phi_y = \pi/2 \]

\[ a_x = a_y \]

**Linear polarization**

\[ \phi_x = \phi_y \]

\[ a_x = a_y \]

**Quarter Waveplate**

\[ \n \times \n t = \frac{\lambda}{4} \]
Light Scattering and Absorption

Scattering of illumination light by the tissue limits our ability to image deeper.

\[ I_b = I_i \ e^{-\frac{L}{l_e}} \]

Beer – Lambert Law
\( l_e \) attenuation length

\[ \frac{1}{l_e} = \frac{1}{l_s} + \frac{1}{l_a} \]

\( l_s \) scattering mean free path
\( l_a \) absorption length

\[ \frac{1}{l_s} = \mu_s = \rho_s \sigma_s \]

\( \mu_s \) scattering coefficient
\( \rho_s \) volume density
\( Q_s \) scattering efficiency
Wide-field imaging techniques

Wide-field microscopy illuminates whole sample at all times and image is taken by camera.

.....
while in confocal microscopy, only a single focal spot is illuminated and recorded at a time.

Illumination sources: halogen lamp, metal halide lamps, or LED.

Detection: directly by eyes, or with a digital camera.

Contrast methods: Phase Contrast, Differential Interference Contrast (DIC), Fluorescence
Dark-field microscopy

• In Dark-field microscopy, any un-scattered beam is excluded from the image, as a result, the field around the specimen is dark.

• It is well suited for uses involving live and unstained biological samples.

Bright-field

Dark-field
Phase-contrast microscopy

- Phase contrast is an optical contrast technique for making unstained transparent objects visible under the optical microscope.

- An annulus aperture is placed in the front focal plane of the condenser and limits the angle of the penetrating light waves.

- A phase plate is placed in the back focal plane of the objective.

- The light waves which are not interacting with the specimen are focused as a bright ring in the back focal plane of the objective.
Phase-contrast microscopy

• Phase plate changes the phase by $\lambda/4$ and dim the light.
• Scattered light is phase shifted by $-\lambda/4$.
• Phase shift in scattered light is caused by the differences in optical path length in the specimen.
• Phase contrast is generated via interference.

Human Blood cells

Source: MicroscopyU.com
Differential interference contrast (DIC)

DIC microscopy is a technique which uses gradients in the optical path length or phase shifts to make phase objects visible under the light microscope. In this way it is possible to observe living cells and organisms with adequate contrast and resolution.

The polarized light is dispersed into two distinct light rays with an orthogonal plane of polarization using a Wollaston prism.

These two light rays are extremely near to each other.
Differential interference contrast (DIC)

The two rays experience different phase shifts from the specimen. After recombination they interfere with each other producing interference contrast. Images are relief-like, have a shadow cast, and no halo artifacts. Relatively thick specimens can be imaged due to the possibility of optical sectioning.

Source: microscopyU.com
micro.magnet.fsu.edu
Fluorescence imaging

Fluorescence microscopy uses fluorescence and phosphorescence of biochemical compounds as a contrast mechanism.

Fluorescent proteins and dyes have been powerful tools to visualize cellular components of cells.

Rat Heart Tissue Labeled with Alexa Fluor 568 (for F-actin), Oregon Green 488 (for N-acetylglucosamine and N-acetyleneuraminic), and Hoechst 342 (nucleus)

Source: MicroscopyU.com
Fluorescence imaging

- Excitation filter selects specific wavelengths for illumination.
- Fluorophore emits light of longer wavelengths.
- Fluorescence light can be collected by the illumination objective lens (epifluorescence).
- Fluorescence light is separated from the strong illumination light by spectral emission filters.

Rat Heart Tissue Labeled with Alexa Fluor 568 (for F-actin), Oregon Green 488 (for N-acetylglucosamine and N-acetylneuraminic), and Hoechst 342 (nucleus)

Source: MicroscopyU.com
Scanning Imaging Microscopy

• In scanning microscopy one focal point is illuminated at a time.
• Illumination point is raster scanned using beam scanner.
• Image is formed serially (pixel by pixel) by a single pixel detector.

It provides better background rejection and optical sectioning.

Optical sectioning:
• Confocal pinhole
• Differential detection
• Non-linear techniques
Scanning Confocal Microscopy

- Confocal scanning microscopy has a pinhole in its return path after scanners.
- Pinhole is placed at image plane.
- The Pinhole **rejects background light** and provides **optical sectioning**.
- Avalanche Photodiode (APD) or Photomultiplier Tube (PMT) are used for light detection.

Confocal Scanning → Confocal reflectance

Confocal Scanning → Confocal fluorescence
Confocal Fluorescence Microscopy

- Fluorescence light is separated from the illumination light by a **dichroic filter**.
- **Fluorescence filter** provides further rejection of out of band light.
- Pinhole rejects background fluorescence and provides optical sectioning.
Confocal Fluorescence Microscopy

• Fluorescence light is separated from the illumination light by a **dichroic filter**.

• **Fluorescence filter** provides further rejection of out of band light.

• Pinhole rejects background fluorescence and provides optical sectioning.

*Pig kidney epithelial cells labeled with EGFP and mCherry (source: microscopyu.com)*
Confocal Reflectance Microscopy

• Confocal reflectance detects a sharp index variation in tissue.
• Non-confocal light is rejected using a $\lambda/4$ plate and polarization beam splitter (PBS).
Confocal Reflectance Microscopy

- Confocal reflectance detects a sharp refractive index variation in tissue.
- Non-confocal light is rejected using a $\lambda/4$ plate and polarization beam splitter (PBS).
Differential phase-gradient detection

Label-free contrast enhancement.
It is important for in-vivo imaging in humans.
Differential phase-gradient detection
Non-linear microscopy

Optical sectioning is provided by the higher probability of multiphoton absorption or harmonic generation.

Multiphoton absorption or higher order harmonic generation is proportional to the square (or third power) of intensity.

At focal plane, intensity of light is highest.

\[
\text{Signal } \propto I \quad \text{Signal } \propto I^2
\]
Multi-photon microscopy

Two-photon absorption cross-section is very low, therefore, it needs very high photon density.

Femto-second pulsed laser provides the photon density without damaging tissue.

Imaging depth is limited by scattering. Longer wavelengths have lower scattering.

For a 150fs, 80MHz pulsed laser the intensity at focus is 100,000 times higher than the same power CW laser.

Ti-sapphire tunable lasers are the most common source of two-photon excitation. 680nm-1080nm, 150fs, 3W
Three-photon microscopy

Three-photon absorption cross-section is even lower, however, due to the third order non-linearity **SNR is much higher.**

Imaging depth is greater due to longer wavelength of excitation light.

**Source:** Horton 2013 Nat. Phot.

Needs a long wavelength femto-second laser source.

**Source:** Xu 2013, Nat. Phot.
Soliton is a locally stable solution of nonlinear differential equation

Electric field propagating in non-linear medium shows **optical Kerr** effect, that is, the refractive index changes due to the electric field intensity.

\[
n(I) = n_o + n_2 I,
\]

\[
E(z,t) = A_m e^{i\varphi(t,z)}
\]

\[
\varphi(t,z) = \omega_o t - k_o n(t)z
\]

\[
\omega(t) = \frac{\partial \varphi(t)}{\partial t} = \omega_o - k_o n_2 z \frac{\partial I(t)}{\partial t}
\]

Large mode area (LMA) photonic crystal fiber (PCF) fibers can have soliton and single mode even at large power.

Supercontinuum from 1550nm laser
Soliton Self frequency shifting (SSFS)

Due to intrapulse **Raman scattering**, the blue portion of the soliton spectrum pumps the red portion of the spectrum, causing a continuous redshift in the soliton spectrum.

Rayleigh Scattering is an **elastic scattering**: no loss or gain.

Raman Scattering (Stokes and Anti-Stokes) are **inelastic scattering**.

Rayleigh Scattering

\[ \frac{\partial v}{\partial z} \propto \frac{h(\tau)}{\tau^4} \]

- \( h(\tau) \): Raman gain function
- \( \tau \): temporal width of pulse

**Ground state**

**Virtual state**

- **Stokes**
- **Rayleigh**
- **Anti-Stokes**

**Vibrational or rotational Modes**

1550nm fiber laser
180fs, 1-10 MHz

- \( \lambda/2 \) plate
- PBS
- LMA, PCF
- LP filter 1650nm
- \( \lambda=1700\text{nm} \)

1500 1700 1900 2100 2300
Wavelength (nm)

1,600 1,680 1,760
Wavelength (nm)

LMA 15

SHG Crystal

HP filter 1100nm

\( \lambda=850\text{nm} \)
Second harmonics generation

Second harmonic generation (SHG) is a non-linear optical process in which two photons with same wavelength interact with a non-linear material and generate a new photon with twice the energy.

\[ D = \varepsilon_0 E + P, \quad P = \varepsilon_0 \chi E, \]

\[ P = \varepsilon_0 \chi^{(1)} E^1 + \varepsilon_0 \chi^{(2)} E^2 + \varepsilon_0 \chi^{(3)} E^3 \ldots \]

- \( P \): polarization density
- \( \varepsilon_0 \): electric permittivity
- \( \chi \): electric susceptibility

Non-centrosymmetric (polar or chiral) molecules, e.g., Collagen, Bone

Molecules like lipid

Must match both the frequency and the phase

Mouse skin collagen fiber

BBO crystals on glass slide
Raman scattering

Bose-Einstein Statistics
If $N$ photons occupy a given state, the transition rates into that state are proportional to $(N+1)$.

$$\langle n + 1 | a^+ | n \rangle = \sqrt{n + 1}$$
Stimulated Raman scattering (SRS)

\[
\text{rate}_{\text{stim.}} = n_{\text{Stokes}} + 1
\]

Source: Freudiger Science (2008)
Coherent Anti-Stokes Raman Scattering (CARS)

CARS is a third-order nonlinear process that involves a pump beam and a Stokes beam.

In vivo imaging of a larvae of a fruit fly (Drosophila melanogaster). Fat cells shown in red (816 nm) and auto fluorescence in green.

CARS anti-Stokes frequency: $\omega_{AS} = 2\omega_p - \omega_S$

Vibrational contrast created at frequency: $\nu = \omega_p - \omega_S$
Thank you!

Questions?