Physics 250, Fall 2012

Special Topics in Biophysics:
Frontiers in Biophysical Imaging

• **When and where:** TuTh 2-3.30 PM, 9 EVANS
• **Format:** two 1.5-hr class meetings per week (student participation strongly encouraged); discussion on individual basis (by appointment)
• **Instructor:** Ahmet Yildiz
• Office: 474 Stanley
• tel. 666-3792
• e-mail: yildiz@Berkeley.edu
• research group web page: [physics.berkeley.edu/research/yildiz/](http://physics.berkeley.edu/research/yildiz/)
• Lecture notes will be uploaded to the research group page
• **Office hours:** by appointment; in 474 Stanley
Synopsis

• First half of this course will consist of two components:
  1. Lectures on current microscopy techniques in biophysical imaging
  2. Discussion of selected "hot" techniques in Biophysics
• In the second half, students will select several recently published major papers in one of the techniques discussed and present in front of the class (PowerPoint, Q&A, abstract...)
• The course is open for both graduate and advanced undergraduate students in physics, chemistry, biology and engineering.
• Optics background (Physics 7B minimum) is required.
Grading

• Student Presentation (50%)
  • Particular advanced microscopy technique
  • A brief one-page (professionally formatted and edited) summary.
  • Professional style abstract and presentation (as if the talk is for a scientific meeting) sent to the class two weeks before the presentation
  • Discussion of a recent major paper (Advances/Results/Analysis/Limitations)
  • Future directions of the field
  • Q&A

• Take Home Exam (30%), covers the topics discussed in first half of the semester

• Attendance and student participation (20%)
Syllabus

• What is an inverted microscope? Lecture 1
  • Optical Components of the Light Microscope
  • Diffraction, Interference, Spatial Resolution
  • Modern Microscope Design

• Transmission Light Microscopy Lecture 2
  • Bright Field and Dark Field Imaging
  • Phase Contrast/DIC
  • Polarization Microscopy

• Fluorescence Microscopy Lectures 3,4,5
  • Introduction to Fluorescence
  • Live Cell Fluorescence Imaging
  • Confocal Microscopy
  • Video Microscopy
  • Total Internal Reflection Microscopy
  • Fluorescence Resonance Energy Transfer
  • FLIM, FRAP

• Single Molecule Fluorescence Lecture 6
  • Particle Tracking
  • Single Molecule FRET
• Superresolution Microscopy                                            Lecture 7
  • STORM, PALM
  • Structured Illumination
  • STED
• Force Microscopy                                                   Lectures 8-9
  • Optical Trap Microscopy
  • Atomic Force Microscopy
  • Cantilever Probes
• Electron Microscopy                                               Lectures 10, 11
  • Limits of Resolution with an Electron Beam
  • Transmission Electron Microscopy
  • Scanning Electron Microscopy
  • Cryo-Electron Microscopy
  • Electron Tomography
  • Limitations and Disadvantages of Electron Microscopy
• Student Presentations                                             Lectures 12-
• **Required text:** none

• **Recommended texts (general):**
  • Murphy, *Fundamentals of Light Microscopy and Electronic Imaging*, Wiley
  • Hawkes & Spence, *Science of Microscopy v1&2*, Springer

• **Recommended texts and websites:**
  • Pawley, *Handbook of Biological Confocal Microscopy*, Springer
  • Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer
  • Von Ooijen, *Handbook of Single Molecule Biophysics*, Springer
  • Nikon Microscopy Tutorials  http://www.microscopyu.com
  • Olympus Microscopy Resource Center  http://www.olympusmicro.com
  • Zeiss Online Campus
Light Microscopy

Bovine Pulmonary Artery Epithelial Cells

Need for microscopy to measure smaller and fainter objects in cells

Play cell size tutorial!
• Various features of light can be used to observe biological material
• Probe size must be smaller than the structures being examined
• Wavelength of light must be comparable to the size of the object
Effects of Light on Cells

Absorption of Water

Visible light is ideal for live cell imaging!
Quality of Light

- Wave
- Vector
- Phase
- Beam

Monochromatic vs. Polychromatic
- Linearly polarized vs. Nonpolarized
- Coherent vs. Noncoherent
- Collimated vs. Divergent
Reflection
Refraction

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

Light travels more slowly in a more dense medium. Change of speed of light at the interface leads to bending of light.

\[ v = \frac{c}{n} \]
Total Internal Reflection

\[ \sin(\theta) = \frac{n(1)}{n(2)} \]
Dispersion

For any substance, as the wavelength of light increases, the refractive index (or the bending of light) decreases.
Light as a Wave: Interference

Interference Pattern is observed when the two beams are out of phase!
Obstacles distort the wavefront
Waves bend around the obstacle which leads to formation of fringes of dark and bright regions due to interference of waves with different phases.
Diffraction of a Coherent Laser Light

• When the wavelength ($\lambda$) is much smaller than the aperture width ($d$), the wave simply travels onward in a straight line.

• However, when the wavelength exceeds the size of the aperture, we experience diffraction of the light according to the equation:

$$\sin \theta = \frac{\lambda}{d}$$

where $\theta$ is the angle between the incident central propagation direction and the first minimum of the diffraction pattern.
Single Slit Diffraction

First minimum, because the two waves are out of phase.

\[ \frac{D}{2} \sin \theta = \frac{\lambda}{2} \]
Double Slit Diffraction

the location of bright fringes:

\[ dsin\phi = \pm m\lambda \ (m = 0,1,2,...) \]
Lenses and Geometric Optics

Positive (converging) lens

Negative (diverging) lens

Object Image Math

Vision

Object

Real image

Virtual image

eye
Oblique Wave Through A Perfect Lens System

Lens (a) Lens (b)

S(1) and S(2) = Conjugate Points

Optical Axis

Focal Points

f(a)  f(b)

Figure 4
Interference between 0th order and higher order diffracted rays in the image plane generates image contrast.

Interference of diffracted rays is the inverse transform of diffraction.
1. Light is diffracted by the specimen
2. Collection of diffracted light by the objective
3. Interference of diffracted and nondiffracted rays
Modern microscopy is limited by the collection of light from a point source through a circular aperture of the objective lens.

Due to diffraction of light, wavefront of the point source cannot be fully reconstructed.

This is often referred to as diffraction limit.

The width of the spot depends on the aperture size, or aperture angle determines the spot size.
Circular Aperture Diffraction

\[ I(\theta) = I_0 \left( \frac{2J_1(ka \sin \theta)}{ka \sin \theta} \right)^2 = I_0 \left( \frac{2J_1(x)}{x} \right)^2 \quad k = \frac{2\pi}{\lambda}, \quad d=2a \]

First minima

\[ \sin \theta = 1.22 \frac{\lambda}{d} \]
Numerical Aperture

$$NA = n \cdot \sin \theta$$

$$n_{\text{air}} \cdot \sin \theta_{c} = n_{\text{glass}} \cdot \sin \theta_{i}$$

$$1 = 1.515 \cdot \sin \theta_{i} \quad \theta_{i} = 40^\circ$$

$NA$ limit is 1.00

$$\theta_{i} = 72^\circ$$

$NA$ limit is 1.49

Resolution Limit of the Light Microscope

Abbe’s Limit

\[ d = \frac{1.22 \lambda}{2 \times NA} \approx 250 \text{ nm in VIS region} \]
On Axis Lens Aberrations

Doublet lenses

Axial Chromatic Aberration

Spherical Aberration

Aspheric biconvex
Off-axis aberrations

(c) Coma

(d) Astigmatism

Distortion

(e) Field curvature

Barrel distortion

Pincushion distortion
Refractive Index Mismatch

Figure 2: Light Paths for Oil and Water Immersion Objectives
- (a) Oil Immersion Objective with Cover Glass (n = 1.515) and Specimen Touching Cover Glass
- (b) Oil Immersion Objective with Specimen Beneath Aqueous Media
- (c) Water Immersion Objective with Specimen Beneath Aqueous Media

Figure 3: Specimen Image Distortion in Aqueous Media
- (a) Fluorosphere in Aqueous Media
- (b) Oil Immersion Objective with Specimen (n = 1.515) and Aqueous Media (n = 1.33)
- (c) Water Immersion Objective with Specimen (n = 1.35) and Aqueous Media (n = 1.33)
The working distance is the distance between the surface of the front lens element of the lens and the surface of the coverslip.

**TABLE 4-1. Characteristics of Selected Objective Lenses**

<table>
<thead>
<tr>
<th>M</th>
<th>Type</th>
<th>Medium (n)</th>
<th>WD (mm)</th>
<th>NA</th>
<th>$d_{\text{min}}$ (µm)</th>
<th>DOF (µm)</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Achromat</td>
<td>1</td>
<td>9.9</td>
<td>0.12</td>
<td>2.80</td>
<td>38.19</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>Achromat</td>
<td>1</td>
<td>4.4</td>
<td>0.25</td>
<td>1.34</td>
<td>8.80</td>
<td>0.4</td>
</tr>
<tr>
<td>20</td>
<td>Achromat</td>
<td>1</td>
<td>0.53</td>
<td>0.45</td>
<td>0.75</td>
<td>2.72</td>
<td>1.0</td>
</tr>
<tr>
<td>25</td>
<td>Fluorite</td>
<td>1.515</td>
<td>0.21</td>
<td>0.8</td>
<td>0.42</td>
<td>1.30</td>
<td>6.6</td>
</tr>
<tr>
<td>40</td>
<td>Fluorite</td>
<td>1</td>
<td>0.5</td>
<td>0.75</td>
<td>0.45</td>
<td>0.98</td>
<td>2.0</td>
</tr>
<tr>
<td>40</td>
<td>Fluorite</td>
<td>1.515</td>
<td>0.2</td>
<td>1.3</td>
<td>0.26</td>
<td>0.49</td>
<td>17.9</td>
</tr>
<tr>
<td>60</td>
<td>Apochromat</td>
<td>1</td>
<td>0.15</td>
<td>0.95</td>
<td>0.35</td>
<td>0.61</td>
<td>2.3</td>
</tr>
<tr>
<td>60</td>
<td>Apochromat</td>
<td>1.515</td>
<td>0.09</td>
<td>1.4</td>
<td>0.24</td>
<td>0.43</td>
<td>10.7</td>
</tr>
<tr>
<td>100</td>
<td>Apochromat</td>
<td>1.515</td>
<td>0.09</td>
<td>1.4</td>
<td>0.24</td>
<td>0.43</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*aThe magnification (M), type of lens design, refractive index (n) of the intervening medium (air or immersion oil), working distance (WD), numerical aperture (NA), minimum resolvable distance ($d_{\text{min}}$), depth of field (DOF), and brightness (B) are indicated. Terms are calculated as: wave-optical depth of field, $nA/NA^2$; brightness in epi-illumination mode, $10^4 NA^2M^2$. Resolution and depth of field are discussed in Chapter 6.*
Depth of Field

*Depth of field Z in the object plane refers to the* thickness of the optical section along the z-axis within which objects in the specimen are in focus; *Depth of focus is the thickness of the image plane itself.*

\[ Z = \frac{n\lambda}{NA^2} \]

the larger the aperture angle (the higher the NA), the shallower will be the depth of field
Light Sources for Biological Microscopy

1. Lamps

Point source
Slightly coherent
Polychromatic
Unpolarized
2. LASERs

Collimated
Highly coherent
Monochromatic
Linearly polarized
Stimulated Emission

Electrons in excited state will drop to the ground state spontaneously ($10^{-10}$ sec).

Stimulated emission is not usually possible due to the short lifetime of the excited state. Incoming photons will be absorbed and spontaneous emission will dominate.

A critical requirement for laser action is a longer-lived upper energy level (metastable state).

Stimulated emission can be made to dominate if there are more atoms in the excited state than in the lower energy state, so that emitted photons are more likely to stimulate emission than to be absorbed. Because this condition is the inverse of the normal equilibrium situation, it is termed a population inversion.

$$N_2/N_1 = \exp[-(E_2 - E_1) / kT]$$
How Lasers Generate Coherent Light

RESONANCE \[ N \cdot \lambda = 2 \cdot (\text{Cavity Length}) \] where \( N \) is an integer, and \( \lambda \) is the wavelength.

# Wavelengths of Common Light Sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Ultraviolet</th>
<th>Violet</th>
<th>Blue</th>
<th>Green</th>
<th>Yellow</th>
<th>Orange</th>
<th>Red</th>
<th>Near-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon-Ion</td>
<td>351, 364</td>
<td>-</td>
<td>457, 477, 488</td>
<td>514</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diode</td>
<td>-</td>
<td>405, 440</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>635, 640</td>
<td>650, 685</td>
</tr>
<tr>
<td>DPSS</td>
<td>355</td>
<td>430, 442</td>
<td>457, 473</td>
<td>532</td>
<td>561</td>
<td>593</td>
<td>638</td>
<td>660, 671</td>
</tr>
<tr>
<td>He-Cd</td>
<td>322, 354</td>
<td>442</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kr-Ar</td>
<td>-</td>
<td>-</td>
<td>488</td>
<td>-</td>
<td>568</td>
<td>-</td>
<td>647</td>
<td>676</td>
</tr>
<tr>
<td>Krypton-Ion</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>647</td>
<td>676, 752</td>
</tr>
<tr>
<td>Helium-Neon</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>543</td>
<td>594</td>
<td>612</td>
<td>633</td>
<td>-</td>
</tr>
<tr>
<td>Mercury Arc</td>
<td>365</td>
<td>405, 436</td>
<td>-</td>
<td>546</td>
<td>579</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xenon Arc</td>
<td>-</td>
<td>-</td>
<td>467</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Metal Halide</td>
<td>365</td>
<td>435</td>
<td>495</td>
<td>520, 545</td>
<td>575</td>
<td>-</td>
<td>625</td>
<td>685</td>
</tr>
</tbody>
</table>
3. LEDs

The first semiconductor material is doped with atoms containing excess electrons (n-type). A second semiconductor, containing atoms having too few electrons (a p-type), is then deposited onto the first semiconductor to form the diode. The region between the semiconductor materials is known as the active layer.

When a voltage is applied, holes from the p-type and electrons from the n-type meet in the active layer to produce light. The wavelength of light is dependent upon relative energy levels of the doped semiconductor materials. By varying the composition of the doped semiconductors, a wide range of emitted wavelengths can be generated.

4. Diode Lasers

- LEDs operate with spontaneous emission.
- To dominate stimulated emission in a diode, the electron population of an upper energy level is induced to grow larger than that of a lower level (population inversion).
- Stimulated emission occurs when a passing photon triggers the recombination of an electron and hole, with emission of a second photon with the same frequency.
- Emission is confined in optical cavity by reflective coatings.
- Emission undergoes gain before being emitted from the edge of the semiconductor material.
- Emitted light is Gaussian, but highly elliptical.
- Anamorphic expansion prisms can be used to obtain more circular beam shape.
- As a result, diode lasers has narrow emission spectrum and highly directed laser output relative to LEDs.
5. Diode Pumped Solid State Lasers

- A powerful (>200 mW) 808 nm wavelength infrared GaAlAs laser diode pumps Nd:YAG or Nd:YVO4 crystal which produces 1064 nm wavelength light from the main spectral transition of neodymium ion.
- This light is then frequency doubled using a nonlinear optical process in a KTP crystal, producing **Green 532 nm** light. Up to 35% efficiency.
- DPSS lasers can be combined with an acousto-optical modulator to obtain a Q-switch (pulse lasers).

- **Blue 473 nm**: 808 nm light is being converted by an Nd:YAG crystal to 946 nm light (selecting this non-principal spectral line of neodymium in the same Nd-doped crystals), which is 3-5% efficient.
- **Violet 404 nm**: 808 nm GaAlAs pump diode frequency doubled, for a violet light output of 120 mW (12% efficiency). **gallium nitride** (GaN) is a direct 405 nm diode lasers.
- **Yellow 593.5 nm**: A 808 nm pump diode is used to generate 1,064 nm and 1,342 nm light, which are summed in parallel to become 593.5 nm. Only around 1% efficient.

Comparison of Light Sources

Incandescent Lamps
Large, energy inefficient, low beam quality (coherence, phase, color, divergence)
Robust (wide range of excitation wavelengths can be generated by a prism or diffraction grating coupled to a monochromator, or by using excitation filter turret).

Gas Lasers
Very good beam quality (divergence, coherence, TEM00 mode)
Low power due to low gain, long beam cavity, energy inefficient, noisy, bulky.

Diode lasers
lower cost
more energy efficient
more stable (DPSS lasers have a tendency to fluctuate or mode-hop)
no residual IR
less complex construction

DPSS lasers
better beam specs
can reach very high powers while maintaining a low divergence and small beam diameter