Principles and Practice of Light Microscopy

- Lectures Mondays 10–12 in BH212
  Mats Gustafsson: mats@msg.ucsf.edu, 514-4385
- Labs Wed or Th 4–6 in BH309, starting April 18 (pick one of the two lab groups)
  Orion Weiner: orion.weiner@ucsf.edu, 514-4508
  John Sedat: sedat@msg.ucsf.edu, 476-4156
  Kurt Thorn: kurt.thorn@ucsf.edu, 514-9709
- Optional reading materials:
  - Douglas Murphy, Fundamentals of Light Microscopy and Digital Imaging ($85 at Amazon - sorry!)
  - micro.magnet.fsu.edu
  - www.microscopyu.com

Lecture course (draft)

Apr 2: Light, refraction, diffraction, ray optics, lenses, images. The light microscope, numerical aperture, Köhler illumination.

Apr 9: No lecture

Apr 16: Resolution and contrast, aberrations, spatial frequencies and the Fourier transform, the point spread function, the optical transfer function.

Apr 23: Phase contrast, DIC, darkfield, polarization microscopy.

Apr 30: Fluorescence, probes, photobleaching, filters and dichroics, fluorescent proteins.

May 7: TIRF, FRET, FRAP, FLIP, FLIM, photo-activation, image fluorescence correlation spectroscopy, optical tweezers, single molecule microscopy, live cell techniques.

May 14: Confocal, spinning disk, multi-photon, second/third harmonic generation, coherent anti-Stokes Raman microscopy (CARS)

May 21: Detectors, light sources, noise. Image analysis and filtering: scaling, gamma, filtering, filtering artifacts, image arithmetic, ratioing, linear unmixing, segmentation.

May 28: Deconvolution, advanced techniques: 4Pi, structured illumination, SPIM, PALM/FPALM/STORM...

The Light Microscope

- Four centuries of history
- Vibrant current development
- One of the most widely used research tools
Electromagnetic Waves

<table>
<thead>
<tr>
<th>gamma rays</th>
<th>X-rays</th>
<th>ultraviolet rays</th>
<th>infrared rays</th>
<th>radar</th>
<th>FM</th>
<th>TV</th>
<th>shortwave</th>
<th>AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-3</td>
<td>10^-2</td>
<td>10^-1</td>
<td>10^-0</td>
<td>1</td>
<td>10^1</td>
<td>10^2</td>
<td>10^3</td>
<td></td>
</tr>
</tbody>
</table>

Visible Light

Wavelength (nanometers)

Most matter interacts mostly with the electric field

$\Rightarrow$ We will ignore the magnetic field

Polarization = direction of electric field

Waves vs. Photons vs. Rays

- Quantum wave-particle duality
- EM field = collective wave function for the photons
- Light intensity $\propto$ photon flux $\propto |\text{field}|^2$
- Rays: photon trajectories
- Rays: propagation direction of waves

Rays are perpendicular to wavefronts

Light as an Electromagnetic Wave
Light travels more slowly in matter.

The speed ratio is the *Index of Refraction, $n$*

\[ v = \frac{c}{n} \]

**Refractive Index Examples**

- Vacuum: 1
- Air: 1.0003
- Water: 1.333
- Cytoplasm: 1.35–1.38
- Glycerol: 1.475 (anhydrous)
- Immersion oil: 1.515
- Fused silica: 1.46
- Optical glasses: 1.5–1.9
- Diamond: 2.417

Depends on wavelength and temperature.

**Snell’s law**:

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

**Mirror law**:

\[ \theta_r = \theta_1 \]
Total Internal Reflection

\[ n_2 < n_1 \]

Snell’s Law: \[ n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \]

If \( n_1 \sin(\theta_1) > n_2 \), then \( \sin(\theta_2) \) would have to exceed 1.
Impossible \( \Rightarrow \) No light can be transmitted
\( \Rightarrow \) All is reflected: Total internal reflection

Happens only when going to a lower-index medium

Lenses work by refraction

Ray Tracing Rules of Thumb
(for thin ideal lenses)

Parallel rays converge at the focal plane
Rays that cross in the focal plane end up parallel

Rays through the lens center are unaffected

Imaging

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

Magnification:
\[ M = \frac{d_2}{d_1} = \frac{L_2}{L_1} \]
Real and virtual images

- **Real image**: Object is between lens and focal length $f > 0$.
- **Virtual image**: Object is beyond the focal length $f > 0$.
- **Real object**: Object is before the lens $f < 0$.
- **Virtual object**: Object is after the lens $f < 0$.

The same lens law applies: Negative lenses have negative $f$.
Virtual objects or images have negative values of $L_1$ or $L_2$.

Finite vs. Infinite Conjugate Imaging

- **Finite conjugate imaging (older objectives)**
  - Object is at finite distance $f_0$.
  - Image is at a finite distance $f_0$.
  - Magnification: $M = \frac{f_1}{f_0}$

- **Infinite conjugate imaging (modern objectives)**
  - Object is at infinity $f_0$.
  - Image is at infinity $f_0$.
  - Back focal plane is at $f_0$.
  - Need a tube lens.

Back focal plane

- Rays that leave the object with the same angle meet in the objective’s back focal plane.

The Compound Microscope

- Sample
- Objective
- Tube lens
- Primary or intermediate image plane
- Back focal plane (pupil)
- Exit pupil
- Eyepiece
- Object plane
The Compound Microscope

- Eye
- Exit pupil
- Eyepiece
- Intermediate image plane
- Tube lens
- Back focal plane (pupil)
- Objective
- Sample
- Object plane
- Final image
**The Compound Microscope**

- Camera
- Final image
- Secondary pupil
- Projection Eyepiece
- Intermediate image plane
- Tube lens
- Back focal plane (pupil)
- Objective
- Sample
- Object plane

**Eyepieces (Oculars)**

**Features**
- Magnification (10x typical)
- "High eye point" (exit pupil high enough to allow eyeglasses)
- Diopter adjust (at least one must have this)
- Reticle or fitting for one
- Eye cups

**Trans-illumination Microscope**

**Imaging path**
- Camera
- Final image plane
- Secondary pupil plane
- Projection Eyepiece
- Intermediate image plane
- Tube lens
- Back focal plane (pupil)
- Objective
- Sample
- Object plane
- Condenser lens
- Aperture iris
- Field lens
- Field iris
- Collector
- Light source
- (pupil plane)

**Illumination path**
- Aperture iris
- (pupil plane)
- Field iris
- (image plane)
- Light source
- (pupil plane)

**Köhler Illumination**

- Sample
- Object plane
- Aperture iris
- (pupil plane)
- Field iris
- (image plane)
- Light source
- (pupil plane)

**Critical Illumination**

- Sample
- Object plane
- Aperture iris
- (pupil plane)
- Field iris
- (image plane)
- Light source
- (pupil plane)

- 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 "ugly" (e.g. a filament)
- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform
A Simple Microscope

A Research Microscope

How view the pupil planes?

Two ways:

• “Eyepiece telescope”
• “Bertrand lens”

By far the most important part: the Objective Lens

Each major manufacturer sells 20-30 different categories of objectives. What are the important distinctions?
The focal length of a lens depends on the refractive index...

\[ f \propto \frac{1}{(n-1)} \]

...and the refractive index depends on the wavelength ("dispersion")

⇒ Chromatic aberration

- Different colors get focused to different planes
- Not good...

Dispersion vs. refractive index of different glass types

<table>
<thead>
<tr>
<th>Glass types</th>
<th>Refractive index</th>
<th>Wavelength (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanthanum dense flint LaSF9</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Dense flint SF10</td>
<td>1.85</td>
<td>0.4</td>
</tr>
<tr>
<td>Flint F2</td>
<td>1.75</td>
<td>0.6</td>
</tr>
<tr>
<td>Barium crown BaK4</td>
<td>1.65</td>
<td>0.8</td>
</tr>
<tr>
<td>Borosilicate crown BK7</td>
<td>1.55</td>
<td>1.0</td>
</tr>
<tr>
<td>Fluorite crown FK51A</td>
<td>1.45</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Abbe dispersion number

<table>
<thead>
<tr>
<th>Glass types</th>
<th>Abbe dispersion number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni-Glasses</td>
<td>High dispersion</td>
</tr>
<tr>
<td>AP-Glasses</td>
<td>Low dispersion</td>
</tr>
<tr>
<td>Classical Glasses</td>
<td>Moderate dispersion</td>
</tr>
<tr>
<td>Series T1</td>
<td>Moderate dispersion</td>
</tr>
</tbody>
</table>

Hence, different types of glass have varying dispersion properties, affecting the focal length and color focus in lenses.
Achromatic Lenses

- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element.

Achromats and Apochromats

- Achromat (2 glass types)
- Apochromat (3 glass types)

Correction classes of objectives

- Achromat (cheap)
- Fluor "semi-apo" (good correction, high UV transmission)
- Apochromat (best correction)

Curvature of Field

- Focal plane
- Focal surface
- Sample
- Tube lens
- Objective

Correction for other (i.e. monochromatic) aberrations also improves in the same order.
Plan objectives

- Corrected for field curvature
- More complex design
- Needed for most photomicrography

- Plan-Apochromats have the highest performance (and highest complexity and price)

Putting one brand of objectives onto another brand of microscope?

**Usually a bad idea:**
- May not even fit
- May get different magnification than is printed on the objective
- Incompatible ways of correcting lateral chromatic aberration (LCA)

\[ \Rightarrow \text{mixing brands can produce severe LCA} \]

### Lateral chromatic aberration

(= LCA, lateral color, chromatic difference of magnification)

= Different magnification for different colors

---

**Interference**

- **In phase**
  \[ + \]

\[ \Rightarrow \text{constructive interference} \]

- **Opposite phase**
  \[ + \]

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

In phase if:
\[ d \sin(\theta) = m \lambda \]
for some integer \( m \)

Diffraction by an aperture

Light spreads to new angles

Larger aperture \( \Rightarrow \) weaker diffraction

The pure, "far-field" diffraction pattern is formed at \( \infty \) distance...

...or can be formed at a finite distance by a lens...

...as happens in a microscope
The Airy Pattern

= the far-field diffraction pattern from a round aperture

"Airy disk" diameter
\[ d = 2.44 \times \frac{\lambda f}{d} \]
(for small angles \( d/f \))

Height of first ring
\[ 1.75\% \]

Aperture and Resolution

Numerical Aperture (NA)

\[ NA = n \sin(\alpha) \]

where:
- \( \alpha \) = light gathering angle
- \( n \) = refractive index of sample
Numerical Aperture

**4X / 0.20 NA** = 11.5°

**100X / 0.95 NA** = 71.8°

**Numerical Aperture**

\[ NA = n \sin(\alpha) \]

**Snell’s law:**

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

- \( n \sin(\theta) \) doesn’t change at horizontal interfaces
- \( \sin(\text{anything}) \leq 1 \)

⇒ NA cannot exceed the lowest \( n \) between the sample and the objective lens

**Numerical Aperture**

**Oil immersion:**

\( n \approx 1.515 \)

- max NA = 1.4 (1.45–1.49 for TIRF)

**Glycerol immersion:**

\( n \approx 1.45 \) (85%)

- max NA = 1.35 (Leica)

**Water immersion:**

\( n \approx 1.33 \)

- max NA = 1.2

NA can approach the index of the immersion fluid

**Immersion Objectives**

Immersion Fluid

Cover glass

Sample

• NA >1 requires **fluid immersion**
**Objective Types**

**Field flatness**
- Plan or not

**Phase rings for phase contrast**
- Positive or negative
- Diameter of ring (number)

**Special Properties**
- Strain free for Polarization or DIC

**Features**
- Correction collar for spherical aberration
- Iris
- Spring-loaded front end
- Lockable front end

**Basic properties**
- Magnification
- Numerical Aperture (NA)
- Infinite or finite conjugate
- Cover slip thickness if any
- Immersion fluid if any

**Correction class**
- Achromat
- Fluor
- Apochromat

**Objective Designations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achi, Achromat</td>
<td>Achromatic aberration correction</td>
</tr>
<tr>
<td>Apo, Aplan, Nuementum, Planar</td>
<td>Achromatic aberration correction correction</td>
</tr>
<tr>
<td>Neu, Achromator</td>
<td>Planar, Achromatic aberration correction</td>
</tr>
<tr>
<td>OTF, Achromat</td>
<td>Planar, Achromatic aberration correction</td>
</tr>
<tr>
<td>BD, NA</td>
<td>Numerical Aperture of objective</td>
</tr>
<tr>
<td>ELWD</td>
<td>Extra Long Working Distance</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorite</td>
</tr>
<tr>
<td>PL, PM, PL, PM, DI, SI</td>
<td>Diaphanous, Diaphanous, Immersion</td>
</tr>
<tr>
<td>UI, UI</td>
<td>Ultra-long Working Distance</td>
</tr>
<tr>
<td>ELWD</td>
<td>Extra Long Working Distance</td>
</tr>
<tr>
<td>FL, NA</td>
<td>Fluorite, Numerical Aperture</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorite</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>U, UT</td>
<td>Universal, Universal</td>
</tr>
<tr>
<td>DI</td>
<td>Diaphanous</td>
</tr>
<tr>
<td>MI</td>
<td>Microscopic</td>
</tr>
<tr>
<td>TI</td>
<td>Transmitted</td>
</tr>
<tr>
<td>DI, MI, TI</td>
<td>Interferometry, Interferometry, Interferometry</td>
</tr>
<tr>
<td>DI, MI, TI</td>
<td>Interferometry, Interferometry, Interferometry</td>
</tr>
<tr>
<td>DI, MI, TI</td>
<td>Interferometry, Interferometry, Interferometry</td>
</tr>
</tbody>
</table>

**Refractive Index**
- Water, Oil, HI, Corr
- ULWD, SLWD, ELWD
- L, LU, Plan

**Other Designations**
- DI, MI, TI
- UPLAN, Plan
- N, EF, Plan, Pl, Apo
- Achro, Achromat
- Abbreviation

**Features**
- Lockable front end
- Spring-loaded front end
- Iris
- Correction collar for spherical aberration