Introduction to Light Microscopy

(Image: T. Wittman, Scripps)
The Light Microscope

- Four centuries of history
- Vibrant current development
- One of the most widely used research tools
**a**  
14000 dsRNA (96-well plate x 146)  
S2 cell + Cdc27 dsRNA  
96-well, glass-bottom plate (ConA-coated)  
Immuno-fluorescence  
DNA  
γ-tubulin  
α-tubulin  
phospho-HistoneH3  
Automated microscopy

**b**
Merged images showing cellular structures stained with various markers.
Major Imaging Functions of the Microscope

- Magnify
- Resolve features
- Generate Contrast
- Capture and Display Images
An Upright Epifluorescence Microscope

Figure 1
Waves vs. Photons vs. Rays

- Quantum wave-particle duality
- Rays: photon trajectories
- Rays: propagation direction of waves
Rays are perpendicular to wavefronts
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
Refraction by an Interface

Refraction by an Interface

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

Mirror law:
\[ \theta_r = \theta_1 \]
Which Direction?

Refraction goes *towards* the normal in the *higher*-index medium.

$n_1$

$n_2 > n_1$
Lenses work by refraction

Incident light

Focal length $f$

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

- **Finite conjugate imaging (older objectives)**

  \[ M = \frac{f_1}{f_0} \]

- **Infinite conjugate imaging (modern objectives).**
  - Image at infinity
  - ⇒ Need a *tube lens*

\[ f_0 > f_0 \]

\[ f_0 = f_0 \]

\[ (uncritical) \]
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)
- Object plane
The Compound Microscope

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

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

Diagram showing the components of a compound microscope and their respective planes.
The Compound Microscope

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

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

Illumination path

- The aperture iris controls the range of illumination angles.
- The field iris controls the illuminated field of view.
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 “ugly” (e.g. a filament)

Critical Illumination

- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform
Conjugate Planes in 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?
In general, high NA lenses have short working distances.

However, extra-long working distance objectives do exist.

Some examples:
- 10x/0.3   WD = 15.2mm
- 20x/0.75  WD = 1.0mm
- 100x/1.4  WD = 0.13mm
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

Abbe-Diagram

- N-Glasses
- KZFS-Glasses
- Classical Glasses
- Series 11

Higher dispersion (→)

Abbe dispersion number

Refractive index

Bergen K7

Calcium Fluoride

Fused Silica

Acrylic

Styrene
Polycarbonate
Achromatic Lenses

- Use a weak negative flint glass element to compensate the dispersion of a positive crown glass element
Achromats and Apochromats

Wavelength vs. Focal length error

- Achromat (2 glass types)
- Apochromat (≥3 glass types)
- Simple lens
Correction classes of objectives

Achromat (cheap)

Fluor “semi-apo” (good correction, high UV transmission)

Apochromat (best correction)

Correction for other (i.e. monochromatic) aberrations also improves in the same order
Curvature of Field

Focal plane
Focal surface

Tube lens

Objective

Sample

Focal surface
Plan objectives

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

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

In phase

Constructive interference

Opposite phase

Destructive interference
Diffraction by a periodic structure (grating)
In phase if:

\[ d \sin(\theta) = m \lambda \]

for some integer \( m \).
Diffraction by an aperture

Larger aperture \implies weaker diffraction

Light spreads to new angles

Larger aperture $\iff$ weaker diffraction
Diffraction by an aperture

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

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

(for small angles \( d/f \))

Height of first ring

\[ \approx 1.75\% \]
Aperture and Resolution

Diffraction spot on image plane = Point Spread Function

Sample

Objective

Tube lens

Intermediate image plane

Back focal plane aperture
Aperture and Resolution

Diffraction spot on image plane = Point Spread Function
Aperture and Resolution

Diffraction spot on image plane = \textit{Point Spread Function}

Sample

Objective

Tube lens

Intermediate image plane

Back focal plane aperture
Aperture and Resolution

• Image resolution improves with aperture size

Numerical Aperture (NA)

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

where:

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

4X / 0.20 NA
\[ \alpha = 11.5^\circ \]

100X / 0.95 NA
\[ \alpha = 71.8^\circ \]
Immersion Objectives

Oil immersion:
- $n \approx 1.515$
- max NA $\approx 1.4$ (1.45–1.49 for TIRF)

Glycerol immersion:
- $n \approx 1.45$ (85%)
- max NA $\approx 1.35$ (Leica)

Water immersion:
- $n \approx 1.33$
- max NA $\approx 1.2$

$\Rightarrow$ NA cannot exceed the *lowest* $n$ between the sample and the objective lens

$\Rightarrow$ NA $>1$ requires *fluid immersion*

NA can approach the index of the immersion fluid
Resolution
Ernst Abbe’s argument (1873)

Consider a striped sample ≈ a diffraction grating

- Back focal plane
- Objective lens
- Sample
- Condenser
- Light source

Diffracted beams
\[ d \sin(\beta) = \lambda \]

Consider first a point light source

If \( \beta > \alpha \), only one spot makes it through
⇒ no interference
⇒ no image formed

Resolution (smallest resolvable \( d \)):
\[ d_{\text{min}} = \frac{\lambda_{\text{sample}}}{\sin(\alpha)} = \frac{\lambda}{n \sin(\alpha)} = \frac{\lambda}{\text{NA}} \]
(Abbe’s argument, continued)

Now consider oblique illumination (an off-axis source point):

\[ d \left[ \sin(\beta_{\text{in}}) + \sin(\beta_{\text{out}}) \right] = \lambda \]

One spot hopelessly lost, but two spots get through \( \rightarrow \) interference \( \rightarrow \) image formed!

Resolution (smallest resolvable \( d \)) with incoherent illumination (all possible illumination directions):

\[ d_{\text{min}} = \frac{\lambda}{(NA_{\text{obj}} + NA_{\text{condenser}})} \]

\[ \rightarrow \frac{\lambda}{2} NA \quad \text{if} \quad NA_{\text{condenser}} \geq NA_{\text{obj}} \quad (\text{“Filling the back focal plane”}) \]
Aperture, Resolution & Contrast

Can adjust the condenser NA with the **aperture iris**

Q: Don’t we always want it full open??

A: **No**

Why? Tradeoff: resolution vs. **contrast**
NA and Resolution

High NA Objective

Low NA Objective
Alternate Definitions of Resolution

As the Full Width at Half Max (FWHM) of the PSF

As the diameter of the Airy disk (first dark ring of the PSF) = “Rayleigh criterion”

(Probably most common definition)
Objective Types

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

Correction class
- Achromat
- Fluor
- Apochromat

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
Further reading

www.microscopyu.com
micro.magnet.fsu.edu

Douglas B. Murphy “Fundamentals of Light Microscopy and Electronic Imaging”


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