Microscopy: Fundamental Principles and Practical Approaches

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Online Resource:
http://micro.magnet.fsu.edu/primer/index.html

Overview

• Image Formation: Diffraction and Interference
• Limits to Resolution: Numerical Aperture and Immersion Objectives
• Light Path and Köhler Illumination
• Getting Contrast: Phase Contrast
• Getting Contrast: DIC
• Bothersome Aberrations
What can you see in an optical microscope?
You can’t resolve objects smaller than ~ 300 nm
(larger than most cellular organelles)
Lenses

Figure 2: Simple Thin Lens Geometrical Optics

Figure 4: Oblique Wave Through A Simple Twin Lens System
James Carville Says:
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“It’s the **RESOLUTION**, stupid!!!”
Diffraction
Diffraction, Interference and Image Formation
Diffraction and Spacing in the Specimen

• Abbe: The details of a specimen will be resolved if the objective capture the 0th and 1st diffracted orders (or any two orders).
Resolution Between Two Objects

Different criteria specify different spacings between the images to achieve “resolution”
Resolution is Dictated by Numerical Aperture

The smaller the NA, the bigger the focal spot, 
And the less resolution obtained
Numerical Aperture

• A measure of the angle of the cone of illumination captured by the objective

• $NA = n\sin(\theta)$

• $\theta$ is the angular aperture

• $n$ is the refractive index of the immersion medium
• In practice it is difficult to achieve N.A.s above 0.95 with dry objectives.

• The refractive index of the medium between the objective and the specimen is increased by using oils (n=1.51) or water (n=1.33)
Immersion Medium

Figure 7
• Oil immersion objectives can have higher NA, and hence resolution
• Spherical aberrations at micron distances from the coverglass can be problematic
Criteria for Maximum Resolution

R=\lambda/2NA

R=0.61\lambda/NA (Rayleigh Criterion)

R=1.22 \lambda/(NA(obj) + NA(cond))

So at NA=0.95

360 nm \quad R=0.19\text{ micrometers}

450 nm \quad R=0.24

550 nm \quad R=0.29

700 nm \quad R=0.37
Resolution of light microscopy

**Horizontal**

\[ 1.22 \times \frac{\lambda}{(N.A_{\text{objective}} + N.A_{\text{condenser}})} \]

e.g. 488 nm light, N.A. 1.4 = 213 nm

**Vertical**

\[ 2 \times \lambda \times n \times \frac{1}{(N.A_{\text{objective}})^2} \]

e.g. 488 nm light, oil, N.A. 1.4 = 754 nm

*where:*

- \( \lambda \) is the wavelength of light
- N.A. is numerical aperture
- \( n \) is the refractive index of the sample medium
Markings on the Objective

Figure 1
Aberrations/Corrections

• Chromatic aberrations:
• Achro, Achromat, Apochromat (wider range of wavelengths), Fluor
Aberrations/Corrections

• Spherical Aberrations
• Light passing through the periphery of lens not brought to focus with light through center
• Lenses are well corrected for standard 17 mm cover glass, or have adjustment collar
Aberrations/Corrections

• Flat Field Corrections
• Plan
Mechanical Tube Length

160 mm fixed

Infinity
Köhler Illumination is Absolutely Required for Good Transmitted Light Contrast.

There are two sets of conjugate Optical planes in the microscope:

1. Aperture or Illumination Plane
2. Focus (Object), Image Plane

These two are Fourier transforms of each other -- This means that they are related in specific ways.
Proper Alignment of the Condensor

Focus and Center the Illumination

1. Close diaphragm
2. Focus diaphragm in image field
3. Center diaphragm in field
4. Open the diaphragm to fill the field
Contrast

- Unstained biological specimens usually have low contrast in bright field images.
- Phase contrast and differential interference contrast use different optical tricks to introduce contrast based on changes in the refractive index across the specimen.
Biological Specimens as Phase Objects

- Visibility of light after interference is a function of coherence
- Can be maximized by decreasing the size of the condenser diaphragm, but at cost to resolution (decrease NA)
Contrast and Resolution Vary with Illumination

Condenser Aperture Size and Image Quality

Figure 4

NA = 100%  NA = 75%  NA = 25%

Note! For many contrast methods, including DIC, Hoffman and Fluorescence, resolution is given by the smallest NA in the system.
Optical Path Difference

- OPD = t(n(s) - n(m))
- Phase Difference
  \[ \delta = \left(\frac{2\pi}{\lambda}\right)(OPD) \]

- Optical path differences in unstained specimens are small but give phase differences that are exploited in the phase contrast microscope.
Constructive and Destructive Interference

Two waves

Result when waves are combined

Constructive → White

Gray

Black

Destructive
Phase Contrast

- Unstained specimens that do not absorb light retard its phase by ~1/4 wavelength compared to undeviated light.
- Direct zeroth order light passes through specimen undeviated, diffracted light lags behind by ~1/4 wavelength, but in interference this is not sufficient to observably reduce intensity.
- Phase microscope speeds up direct light by ¼ wavelength, so that it ends up ½ wavelength out of phase with the diffracted light, giving destructive interference (black).
Phase Contrast illuminates a ring, but in this case the ring is in the aperture plane. Unscattered light is “phase delayed” for maximum interference.
Limitations

• Halos
• Phase annuli limit working NA, hence resolution
• Poor for thick specimens due to phase shifts from planes above and below focus
Phase and DIC

Figure 1

Transparent Specimens in Phase Contrast and DIC

(a)  (c)  (e)
(b)  (d)  (f)
Phase and DIC

- **Phase**: intensity based on optical path variation- high OPD=dark, low OPD=light
- **DIC**: intensity variation based on magnitude of gradients in OPD. Sharp gradients give pseudo relief shading. Shallow gradients appear with similar intensity to background
DIC Image Plane Wavefront Interference

(a) No Bias

(b) Specimen Amplitude at Extinction

(c)

(d)

(e) Specimen Amplitude with Bias

(f)

Figure 8
DIC Allows Optical Sectioning

Angles in back aperture correspond to positions in the object/image

Wollaston Prisms
Separate Two Polarizations
To Different Angles

θ

d

Large Path Difference

Similar Paths
Advantages and Disadvantages of DIC

- Capable of high resolution, no halos, optical sectioning is possible.
- Cannot image through tissue culture plastics, harder to set up, requires well-corrected objectives.