Objectives, Optical Aberrations, Resolution, Point Spread Function

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Outline

• Examples of Modern Microscopes

• Point Spread Function (PSF)

• The importance of understanding the microscope objective
  – Magnification
  – Resolution
  – Sampling
  – Abberations
  – Working distance
Some examples of modern research microscopes
Conventional WIDE-FIELD Microscopy
1. DeltaVision Elite–Live Cell Imaging System

- The DeltaVision Elite is a conventional wide-field fluorescence microscope that has been optimized for: 1) high-sensitivity imaging in 3D, and 2) long-term time-lapse imaging of live cells at 37°C and 5% CO₂.

- This system is equipped with fluorescence filters for imaging e.g. DAPI, CFP, GFP, YFP, RFP, mCherry, and
WIDE-FIELD
CONFOCAL Microscopy
1. Zeiss 780 Inverted Confocal

- The Zeiss 780 Inverted Confocal is a conventional laser-scanning confocal microscope that has been optimized for high-sensitivity, multi-color 3D imaging.

- This system is equipped with lasers (405, 458, 488, 514, 561, 594, and 633 nm) and detectors for imaging in the visible spectrum (400 < λ < 700 nm).
SUPER-RESOLUTION Microscopy
The LEICA SP8 is a conventional inverted laser-scanning confocal microscope that is additionally equipped for 3D super-resolution gated STED imaging (max resolution$_{x,y}$ $\sim$ 50 nm; max resolution$_z$ $\sim$ 150 nm).

This system is equipped with continuous wave (CW) lasers (@ 405, 458, 488, and 514 nm), a tuneable pulsed white laser (470-670 nm), a pulsed laser at 440 nm, and high power CW lasers at 592 nm, 660 nm, and soon 775 nm for STED imaging.
Application Example: 2D STED of Centrosomes in Drosophila

Prof. Jordan Raff / Dr. Alan Wainman, Sir William Dunn School of Pathology

Huygens Deconvolved Image Examples - Act / Alexa 488
All images are a single plane from a de-convolved z-stack acquired with dz=130 nm

Confocal (70 nm pixels)  gSTED @ 25% STED Power (20 nm pixels)

gSTED @ 50% STED Power (20 nm pixels)  gSTED @ 100% STED Power (20 nm pixels)

Analysis of Peak Separation and FWHM of each strand (N=4)
Basics of Image Formation

Convolution of microscope optics (PSF) with object = Airy Pattern

Point Spread Function (PSF)
Optical microscopy methods in numbers

Resolution is worse in the axial dimension (along the optical axis, Z):

$$D_Z = \frac{2 \lambda \eta}{(NA_{\text{obj}})^2} \quad \approx 705$$

($\eta$ = refractive index of the object medium)

Than it is in the lateral dimension (XY):

$$D_{XY} = \frac{1.22 \lambda}{2NA_{\text{obj}}} \quad \approx 227$$

FWHM

The relationship between the two is:

$$\frac{D_z}{D_{xy}} = \frac{3.28 \eta}{NA_{\text{obj}}} \quad \approx 3$$
Some Example of Experimental PSFs

Deltavision Widefield
Olympus Plan-Apo 100X 1.40NA objective

Ø200nm Tetraspeck Beads (Blue-Green-Red-Far Red)
Scale bar = 1μm
Some Example of Experimental PSFs

Deltavision Widefield
Olympus Plan-Apo 100X 1.40NA objective

Ø200nm Tetraspeck Beads (Blue-Far Red)
Scale bar = 1µm
Some Example of Experimental PSFs

Zeiss 880 Confocal
Zeiss C-Plan-Apo 63X 1.40NA objective

Ø200nm Tetraspeck Beads
(Blue-Green-Red-Far Red)
Scale bar = 1μm

Zeiss 900 Confocal
Zeiss C-Plan-Apo 63X 1.40NA objective
Some Example of Experimental PSFs

Zeiss 880 Confocal
Zeiss C-Plan-Apo 63X 1.40NA objective

Ø200nm Tetraspeck Beads
(Blue-Far Red)
Scale bar = 1μm

Zeiss 900 Confocal
Zeiss C-Plan-Apo 63X 1.40NA objective
What do you get?

Wide-field = No pinhole  Confocal = Pinhole
What do we need to acquire a high quality image?

- Magnification
- Resolution
- Contrast
- Sampling
The microscope objective
What is magnification?

Magnification is the process of enlarging the apparent size, not physical size, of something.
What is resolution?

**Resolution** describes the minimal distance of two points that can be distinguished.
What is sampling?

**Sampling** is the process of taking measurements in time or space.
What is magnification?

Magnification is defined by the

magnification by the objective

×

the magnification by eyepiece

BUT maximum magnification does not mean maximum resolution!
Example from Richard Parton, Dept. Of Biochemistry, Oxford
Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 10km
Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 50m
Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 10m

empty magnification!

Insufficient resolution!
What is resolution?

Resolution describes the minimal distance of two points that can be distinguished.
Resolution describes the minimal distance of two points that can be distinguished.

Picture taken from http://microscopy.fsu.edu/primer/anatomy/numaperture.html
Quantitative Analysis of xy-Resolution – 4 different criteria of resolution

**Abbe Criterion**

\[ d \approx \frac{\lambda}{2 \cdot NA} \]

\[ d \approx \text{FWHM} \]

\[ \text{FWHM} = 2 \sqrt{2 \cdot \log(2)} \sigma \]

\[ \Delta \text{intensity} \approx 1.6\% \]

**Rayleigh Criterion**

\[ d = \frac{0.61 \lambda}{NA} \]

\[ \Delta \text{intensity} = 26.7\% \]

**Sparrow Criterion**

\[ d \approx \frac{0.47 \lambda}{NA} \]

\[ \Delta \text{intensity} = 0\% \]
Fluorescent DNA origami Nanorulers from GattaQuant
Numerical aperture, NOT magnification determines resolution!

Increasing NA

A lens with a larger NA will be able to visualize finer details and will also collect more light and give a brighter image than a lens with lower NA.
**Numerical Aperture (N.A.)**

Numerical Aperture = N.A. = \( n \cdot \sin \alpha \)

\( \alpha \) is half the opening angle of the objective.

\( n \) is the refractive index of the immersion medium used between the objective and the object.

\( (n = 1 \text{ for air; } n = 1.51 \text{ for oil or glass}) \)
Summary - What is resolution?

Abbe limit

\[ d_{\text{Abbe}} = \frac{0.5 \lambda}{\text{NA}_{\text{objective}}} \]

Rayleigh limit

\[ d_{\text{Raleigh}} = \frac{0.61 \lambda}{\text{NA}_{\text{objective}}} \]  
(Reflected light)

\[ d_{\text{Raleigh}} = \frac{1.22 \lambda}{\text{NA}_{\text{objective}} + \text{NA}_{\text{condenser}}} \]  
(Transmitted light)

Abbe Criterion

\[ d \approx \frac{\lambda}{2 \, \text{NA}_{\text{objective}}} \]
\[ \Delta \text{intensity} \approx 1.6\% \]

Rayleigh Criterion

\[ d \approx \text{FWHM} \]
\[ \text{FWHM} = 2 \sqrt{2 \log(2)} \sigma \]
\[ \Delta \text{intensity} \approx 26.7\% \]
Demonstrating resolution and contrast
### Microscope resolution in numbers

<table>
<thead>
<tr>
<th>Objective NA</th>
<th>NA=0.8</th>
<th>NA=1.2</th>
<th>NA=1.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\lambda)</td>
<td>(xy)</td>
<td>(z)</td>
<td>(xy)</td>
</tr>
<tr>
<td>405</td>
<td>310</td>
<td>1010</td>
<td>205</td>
</tr>
<tr>
<td>488</td>
<td>370</td>
<td>1220</td>
<td>250</td>
</tr>
<tr>
<td>561</td>
<td>430</td>
<td>1400</td>
<td>285</td>
</tr>
<tr>
<td>594</td>
<td>450</td>
<td>1490</td>
<td>300</td>
</tr>
<tr>
<td>633</td>
<td>480</td>
<td>1580</td>
<td>320</td>
</tr>
</tbody>
</table>

\[
D_{xy} = 1.22 \frac{\lambda}{2N_{\text{A}_{\text{obj}}}} \quad FWHM
\]

\[
D_{z} = 2 \frac{\lambda \eta}{(N_{\text{A}_{\text{obj}}})^{2}} \quad (\eta = \text{refractive index of the object medium})
\]
….. So which objective would result in best resolution?
What is contrast?

**Contrast** is defined as the difference in light intensity between points in the image relative to the overall background intensity.
What is contrast?

Intensity drop ~26%

Rayleigh criterion (between two objects)

NOTE! Resolution requires Contrast
What is sampling?

Sampling is the process of taking measurements in time or space.
Nyquist Sampling Theorem = Sample at ~2-3x frequency
But imaging at highest resolution also requires proper sampling

Nyquist sampling theorem - lateral

- the size of the pixel should be 2-2.5x smaller than the lateral optical resolution to realize maximum optical resolution
### Sampling in numbers

<table>
<thead>
<tr>
<th>Objective NA</th>
<th>NA=1.4</th>
<th>Sampling @ 3x Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ</td>
<td>xy</td>
</tr>
<tr>
<td>405</td>
<td>180</td>
<td>580</td>
</tr>
<tr>
<td>488</td>
<td>210</td>
<td>700</td>
</tr>
<tr>
<td>561</td>
<td>240</td>
<td>800</td>
</tr>
<tr>
<td>594</td>
<td>260</td>
<td>850</td>
</tr>
<tr>
<td>633</td>
<td>280</td>
<td>900</td>
</tr>
</tbody>
</table>

\[
D_{xy} = 1.22 \frac{\lambda}{2NA_{obj}}
\]

\[
D_{z} = 2 \frac{\lambda \eta}{(NA_{obj})^2}
\]

(\(\eta\) = refractive index of the object medium)
Consequences of not sampling at Nyquist

Oversampling
- pixels small compared to the optical resolution
- specimen needlessly exposed to light
- image needlessly large

Undersampling
- degraded spatial resolution
- photobleaching reduced
- image artefacts (eg. aliasing)
More from the objective

Objectives can be classified into transmitted light and reflected-light (Epi) versions.
Flat-field correction and aberration correction

Describe two main criteria for the quality of an objective:

- Flatness of the intermediate image
- Elimination of chromatic errors
Spherical aberration

Spherical aberration causes beams parallel to but away from the lens axis to be focussed in a slightly different place than beams close to the axis. This manifests itself as a blurring of the image.
The effect of spherical aberrations on images.
Specimen Preparation Considerations – Effect of Mounting Media (e.g. Slowfade Diamond vs. Prolong Gold)

Esther Garcia & Christoffer Lagerholm, Wolfson Imaging Centre - Oxford
Flat-field correction and aberration correction

Describe two main criteria for the quality of an objective:

- Flatness of the intermediate image
- Elimination of chromatic errors
Chromatic aberration

Chromatic aberration is caused by a lens having different refractive indexes for different wavelengths. Since the focal length of a lens is dependent on the refractive index, different wavelengths will be focused on different positions in the focal plane. Chromatic aberration is seen as fringes of colour around the image.

It can be minimised by using an achromatic doublet (= achromat) in which two materials with differing dispersion are bonded together to form a single lens.
The effect of chromatic aberrations on images.
Objective types

elimination of chromatic errors
flatness of the intermediate image

• CP-Achromat
  Good colour correction – exactly for two wavelengths. Field flatness in the image center, refocusing also covers the peripheral areas. For fields of view up to dia. 18 mm. Versions for phase contrast.

• Achroplan
  Improved Achromat objectives with good image flatness for fields of view with dia. 20 or even 23 mm. Achroplan for transmitted light and Achroplan Ph for phase contrast.

• Plan-Neofluar
  Excellent colour correction for at least three wavelengths. Field flattening for the field of view with dia. 25 mm. Highly transmitting for UV excitation at 365 nm in fluorescence. All methods possible, special high-quality variants are available for Pol and DIC.

• Plan-Apochromat
  Perfect colour rendition (correction for four wavelengths!). Flawless image flatness for fields of view with dia. 25 mm. Highest numerical apertures for a resolving power at the very limits of the physically possible.
Figure 1

60x Plan Apochromat Objective

- Flat-Field Correction
- Lateral Magnification
- Aberration Correction
- Numerical Aperture
- Working Distance
- Tube Length
- Cover Glass Thickness Range
- Cover Glass Adjustment Gauge
- Correction Collar

Specialized Optical Properties
DIC M

60x/0.95

Figure 1

60x Plan Apochromat Objective

- Flat-Field Correction
- Lateral Magnification
- Aberration Correction
- Numerical Aperture
- Specialized Optical Properties
- Tube Length
- Coverslip Thickness
- Working Distance

Figure 1
Coverslip types:
1: 0.13 - 0.17 mm
1.5: 0.16 - 0.19 mm
2.0: 0.19 - 0.23 mm
Table 1 - Common Objective Working Distances

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Correction</th>
<th>Magnification</th>
<th>Numerical Aperture</th>
<th>Working Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nikon</td>
<td>PlanApo</td>
<td>10x</td>
<td>0.45</td>
<td>4.0 mm</td>
</tr>
<tr>
<td>Nikon</td>
<td>PlanFluor</td>
<td>20x</td>
<td>0.75</td>
<td>0.35 mm</td>
</tr>
<tr>
<td>Nikon</td>
<td>PlanFluor (oil)</td>
<td>40x</td>
<td>1.30</td>
<td>0.20 mm</td>
</tr>
<tr>
<td>Nikon</td>
<td>PlanApo (oil)</td>
<td>60x</td>
<td>1.40</td>
<td>0.21 mm</td>
</tr>
<tr>
<td>Nikon</td>
<td>PlanApo (oil)</td>
<td>100x</td>
<td>1.40</td>
<td>0.13 mm</td>
</tr>
</tbody>
</table>
Summary - Objectives

• Know your objectives (Magnification, NA, Immersion media)

• Match your objective to your sample (Immersion media, Working distance)

• Be very careful with the objectives!
Useful online links

• Zeiss – Microscopy from the very beginning
  http://zeiss-campus.magnet.fsu.edu/index.html

• Molecular Expressions homepage
  http://micro.magnet.fsu.edu/


• RW Cole, T Jindasa, CM Brown. Measuring and interpreting point spread functions to determine confocal microscope resolution and ensure quality control.
But beware because image brightness is dependent on both the magnification and the NA where \( I \sim \text{NA}^4 / M^2 \).