Objectives, Optical Aberrations, Resolution, Point Spread Function

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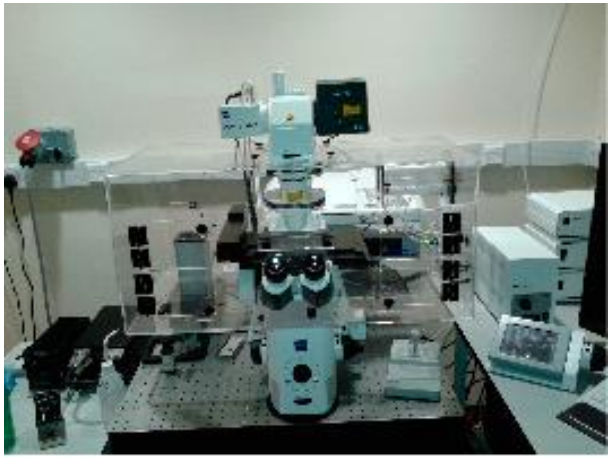
MRC Medical Research Council

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



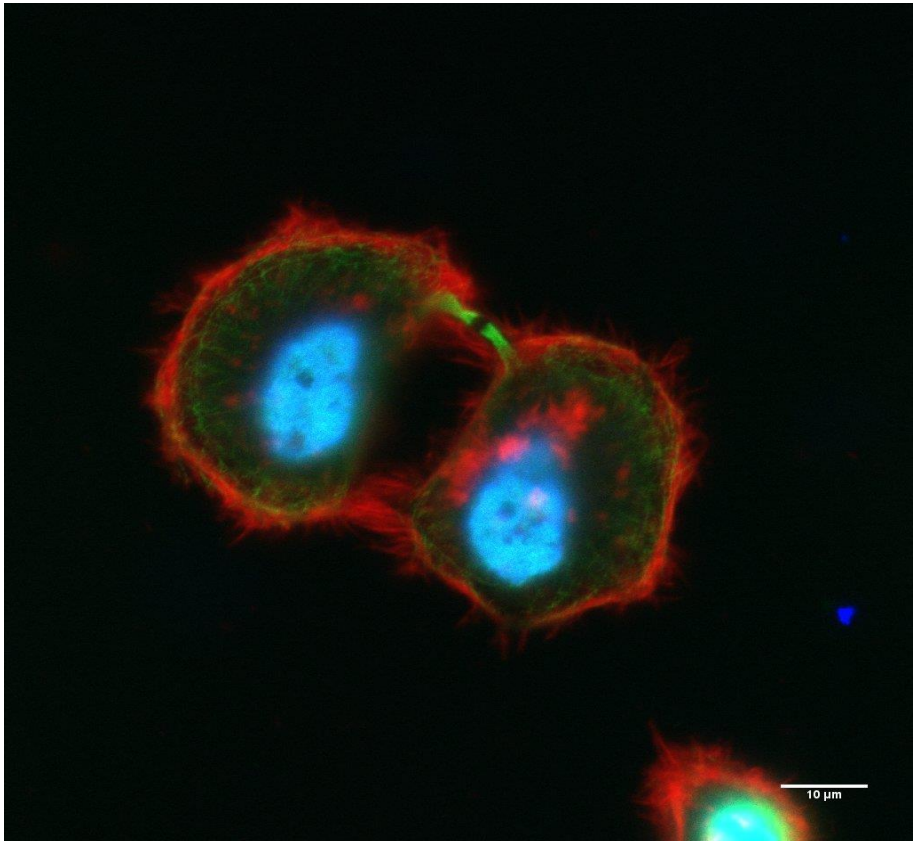
Conventional WIDE-FIELD Microscopy

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 37C and 5% CO_2.

- This system is equipped with fluorescence filters for imaging e.g. DAPI, CFP, GFP, YFP, RFP, mCherry, and

WIDE-FIELD



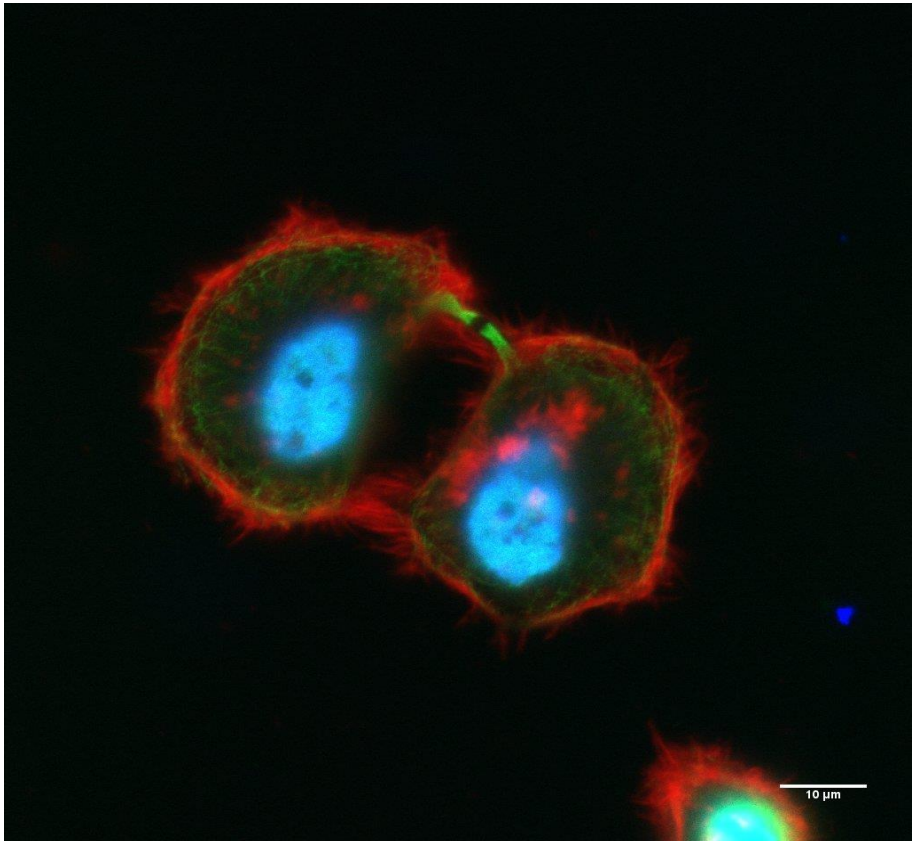
CONFOCAL Microscopy

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) .

WIDE-FIELD



CONFOCAL

SUPER-RESOLUTION Microscopy

7. Leica SP8 gSTED Microscope

- The LEICA SP8 is a conventional inverted laser-scanning confocal microscope that is additionally equipped for 3D super-resolution gated STED imaging (max resolution$_{x,y}$ ~ 50 nm; max resolution$_{z}$ ~ 150 nm)

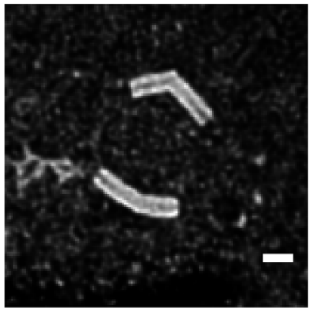
- This system is equipped with continuous wave (CW) lasers (@ 405, 458, 488, and 514 nm), a tuneable pulsed white laser (470-670 nm), a pulsed laser at 440 nm, and high power CW lasers at 592 nm, 660 nm, and soon 775 nm for STED imaging.

Application Example: 2D STED of Centrosomes in Drosophila

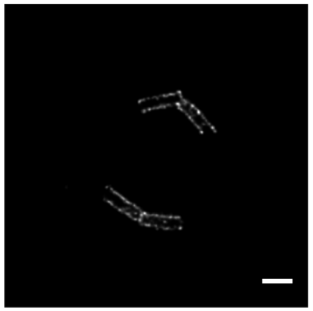
Prof. Jordan Raff / Dr. Alan Wainman, Sir William Dunn School of Pathology

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

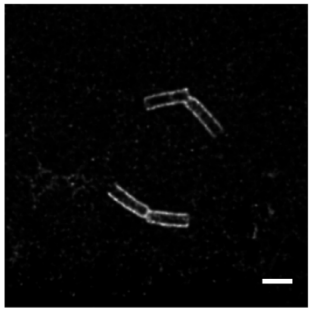
Confocal (70 nm pixels)



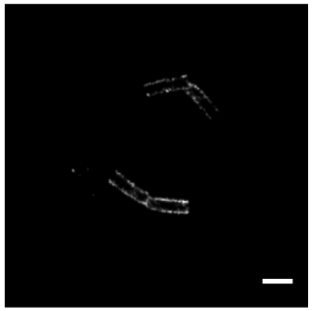
gSTED @ 25% STED Power (20 nm pixels)



gSTED @ 50% STED Power (20 nm pixels)

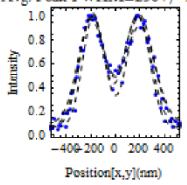


gSTED @ 100% STED Power (20 nm pixels)

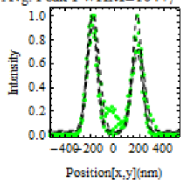


Analysis of Peak Separation and FWHM of each strand (N=4)

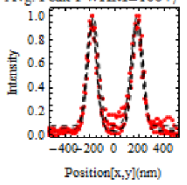
Confocal
Avg. Peak Separation=378+/−16 nm
Avg. Peak FWHM=250+/−22 nm



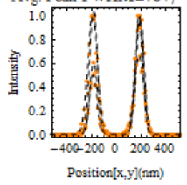
gSTED @ 25% STED Power
Avg. Peak Separation=364+/−14 nm
Avg. Peak FWHM=104+/−10 nm



gSTED @ 50% STED Power
Avg. Peak Separation=364+/−16 nm
Avg. Peak FWHM=100+/−4 nm



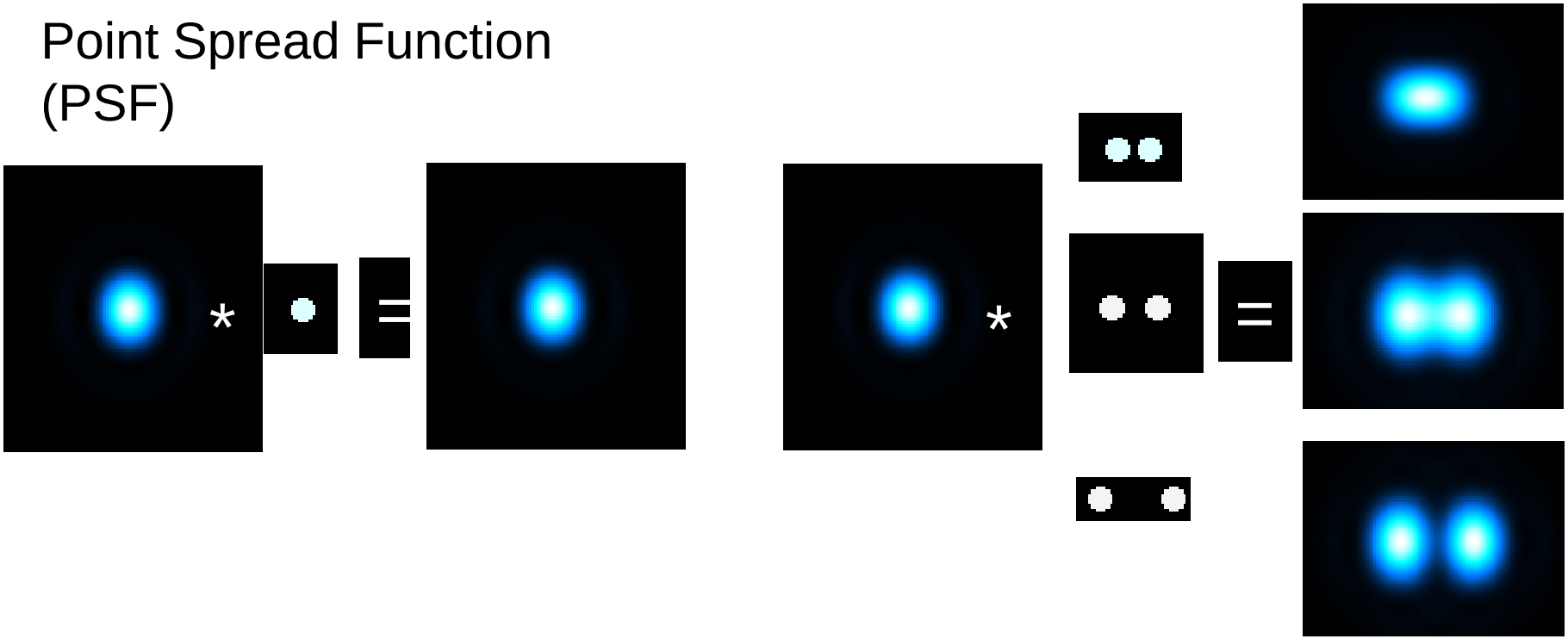
gSTED @ 100% STED Power
Avg. Peak Separation=372+/−8 nm
Avg. Peak FWHM=78+/−6 nm



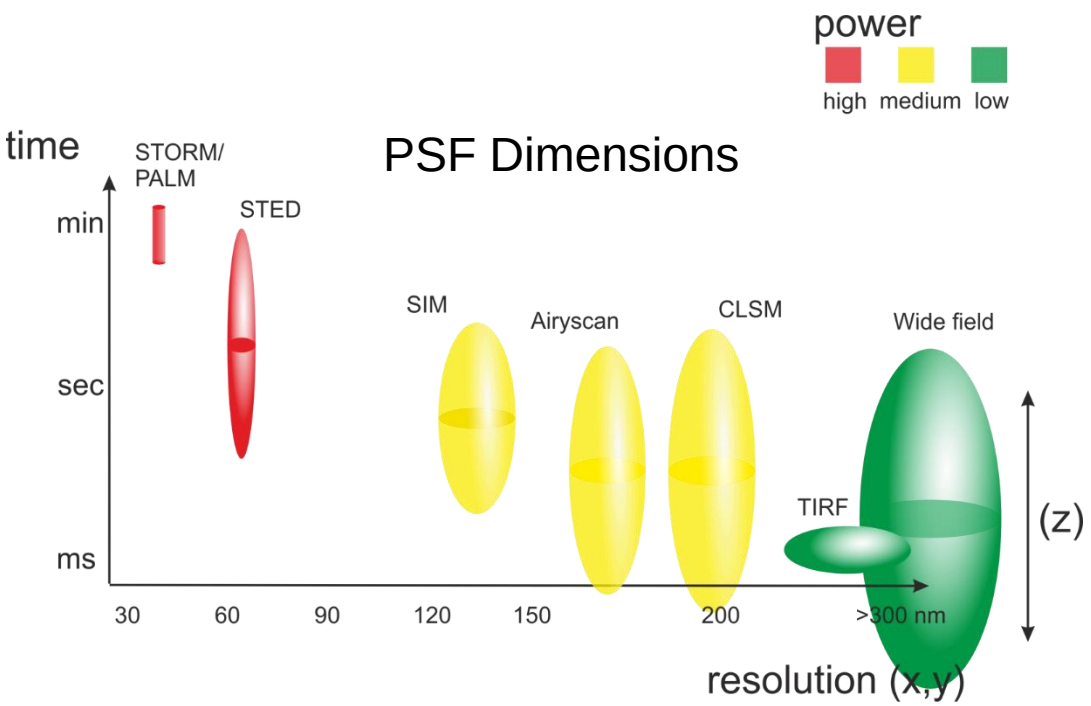
Basics of Image Formation

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

Point Spread Function (PSF)



Optical microscopy methods in numbers



Schermelleh, L. et al. *J. Cell Biol.* **2010**, *190*, 165–175.

Dimensions of the widefield PSF

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

$$D_Z = 2\lambda\eta/(NA_{obj})^2 \quad \cdots\cdots 705$$

(η = refractive index of the object medium)

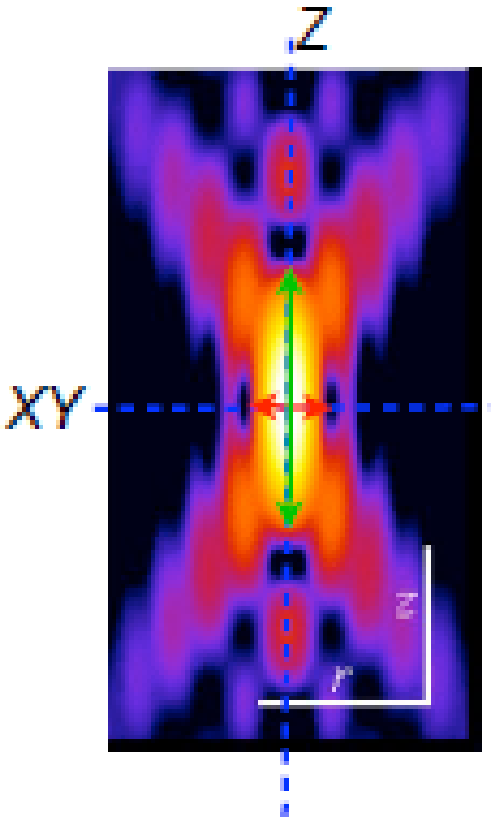
Than it is in the lateral dimension (XY)

$$D_{XY} = 1.22\lambda/2NA_{obj} \quad \cdots\cdots 227$$

FWHM

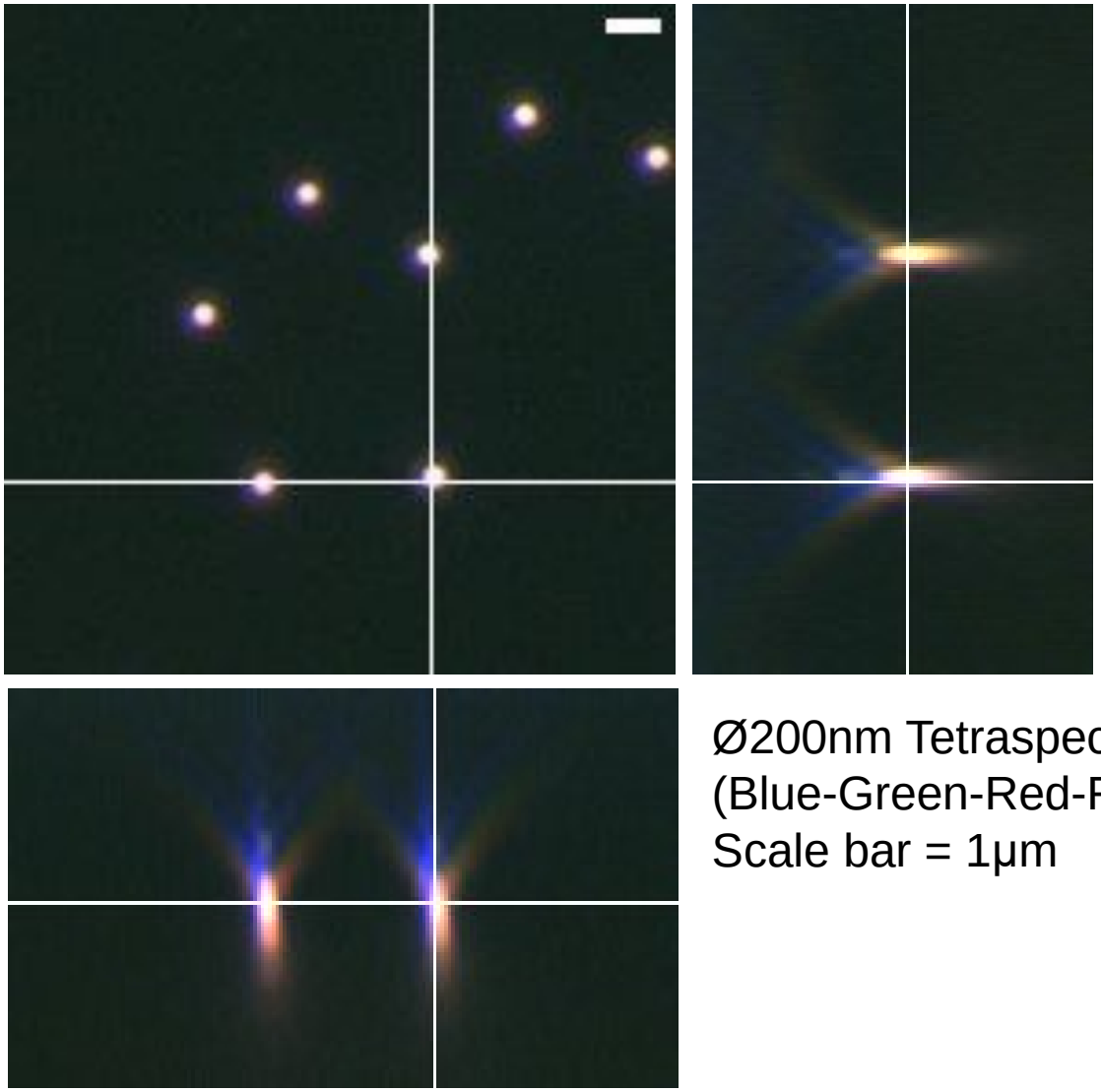
The relationship between the two is:

$$D_Z/D_{XY} = 3.28\eta/NA_{obj} \quad \cdots \approx 3$$



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

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Deltavision Widefield
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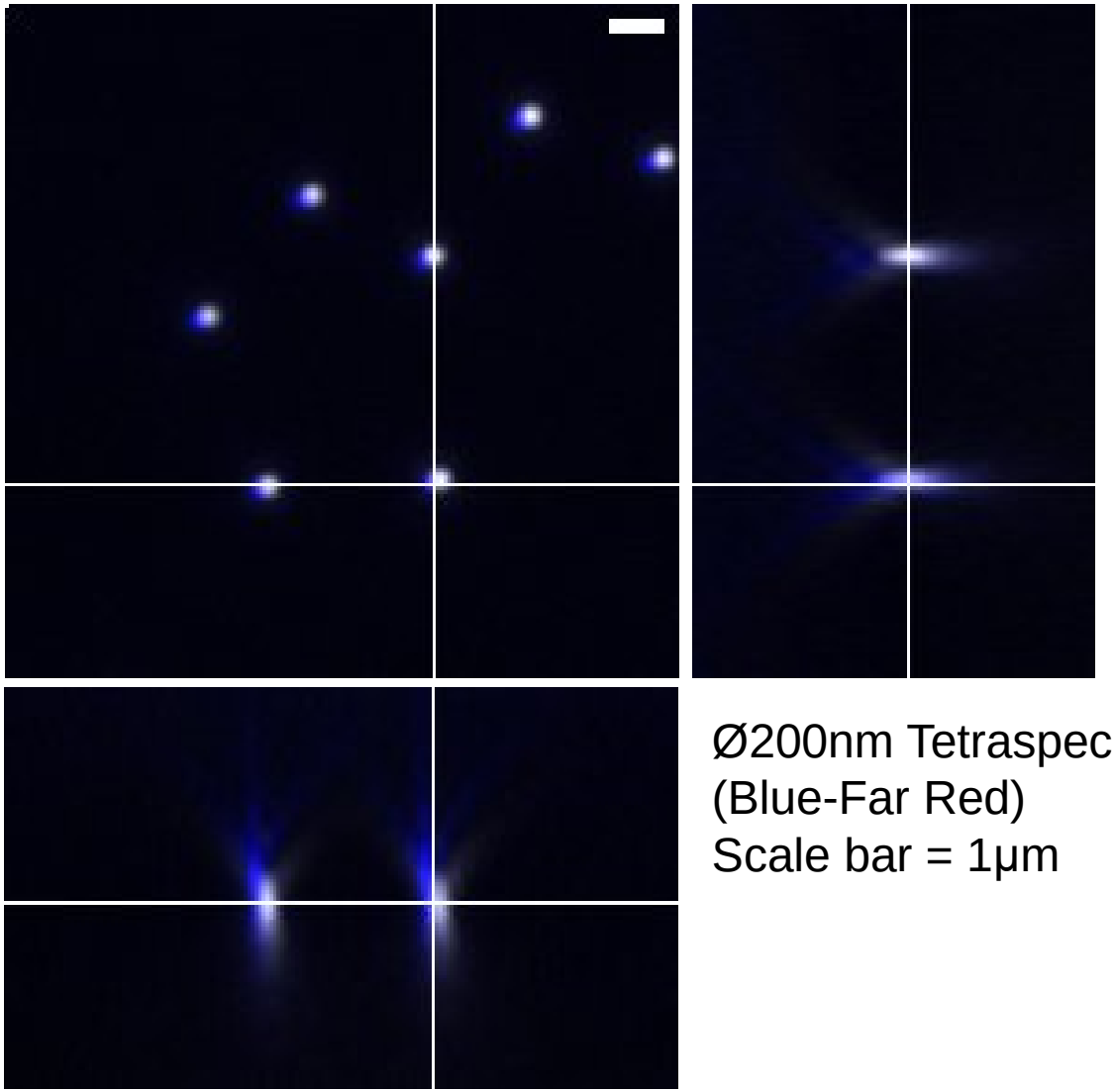

Ø200nm Tetraspeck Beads
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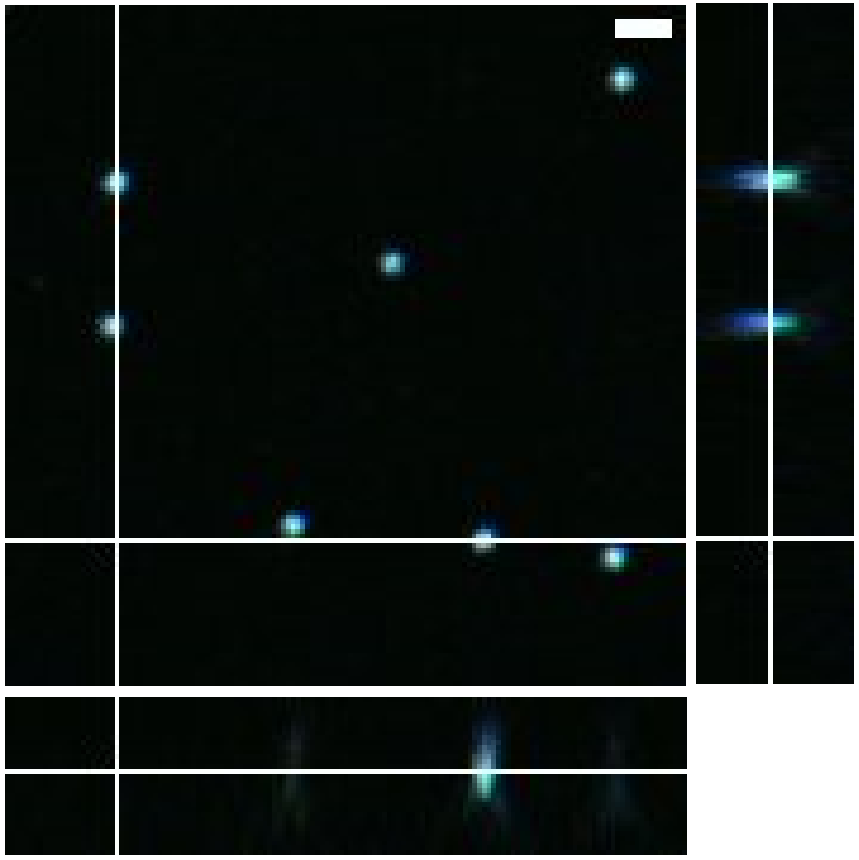
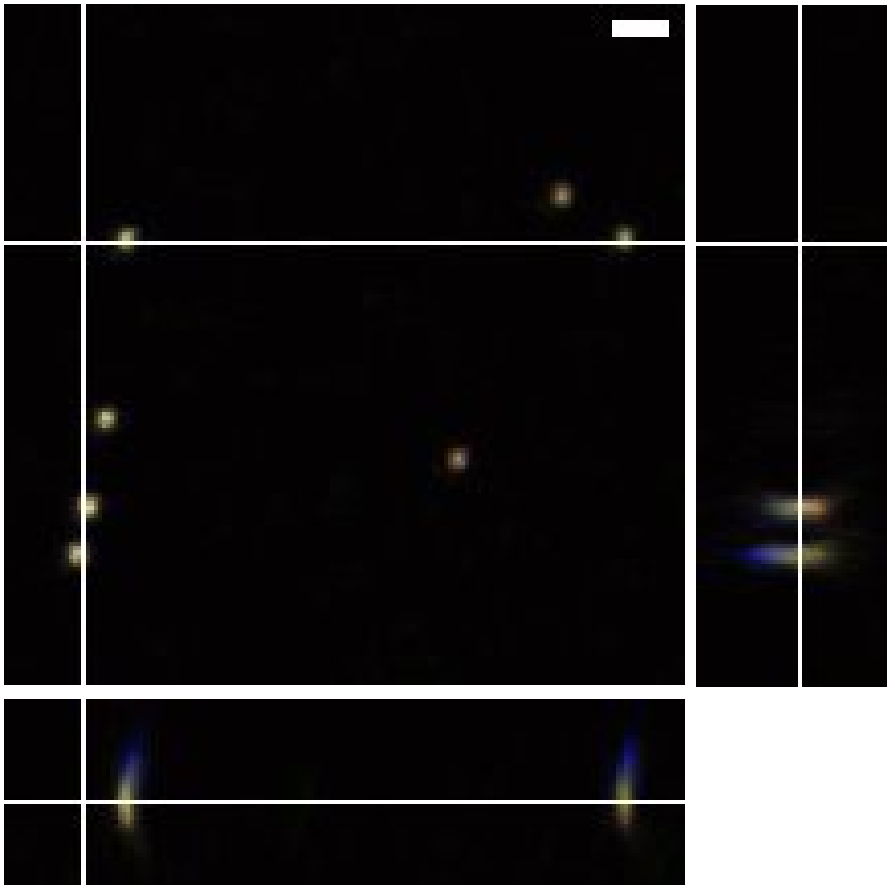
Some Example of Experimental PSFs

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



Zeiss 900 Confocal
Zeiss C-Plan-Apo 63X 1.40NA objective



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Some Example of Experimental PSFs

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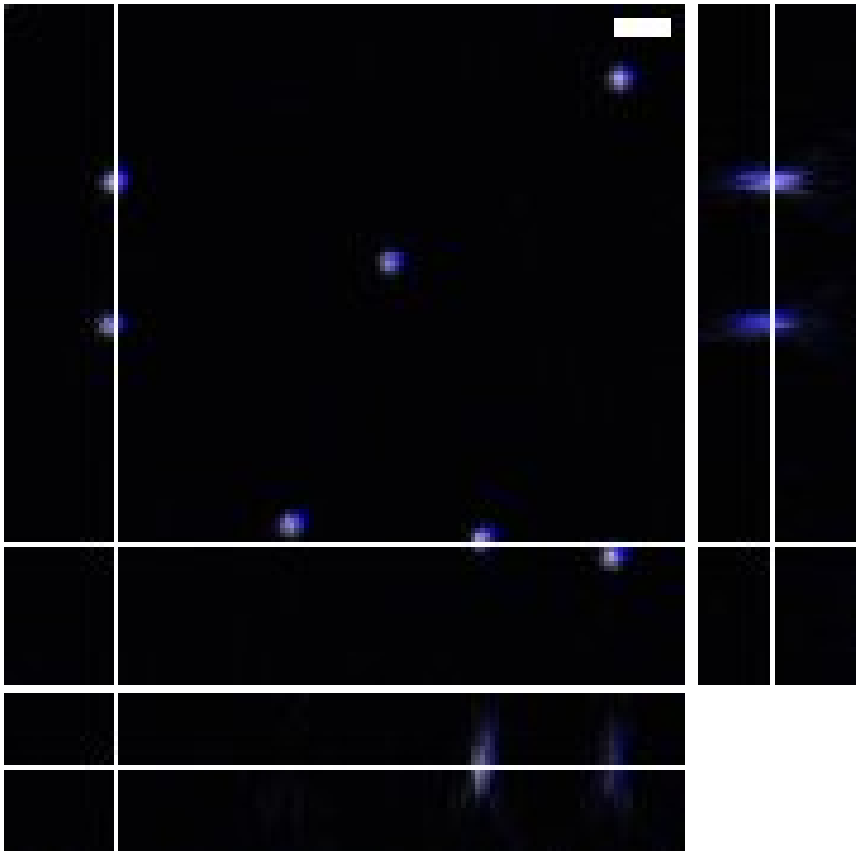

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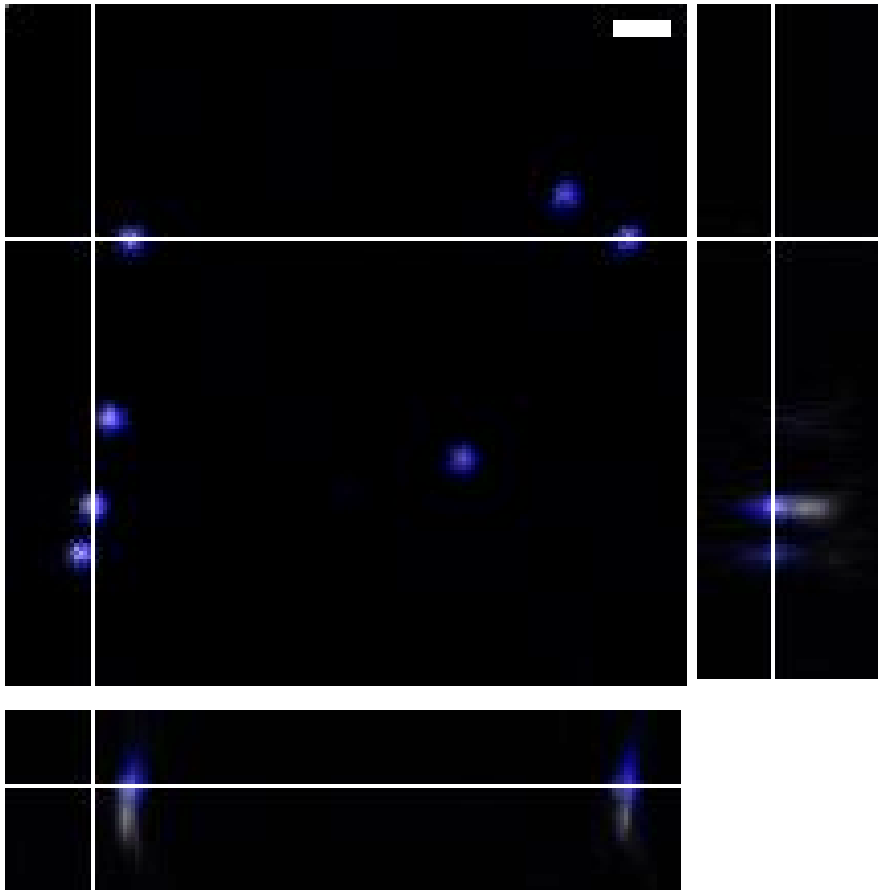
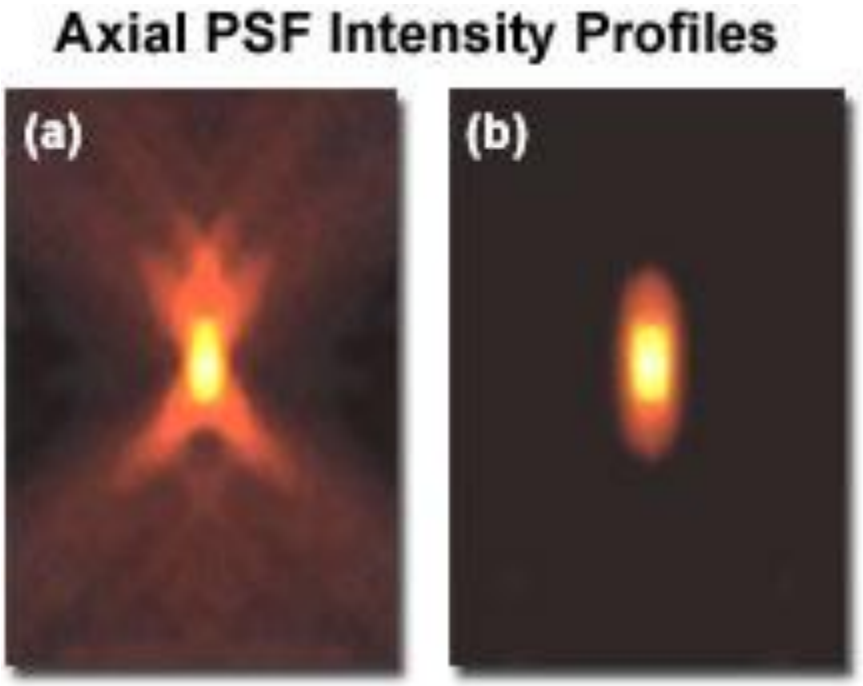


Ø200nm Tetraspeck Beads
(Blue-Far Red)
Scale bar = 1µm

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

Figure 1

Figure 1

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

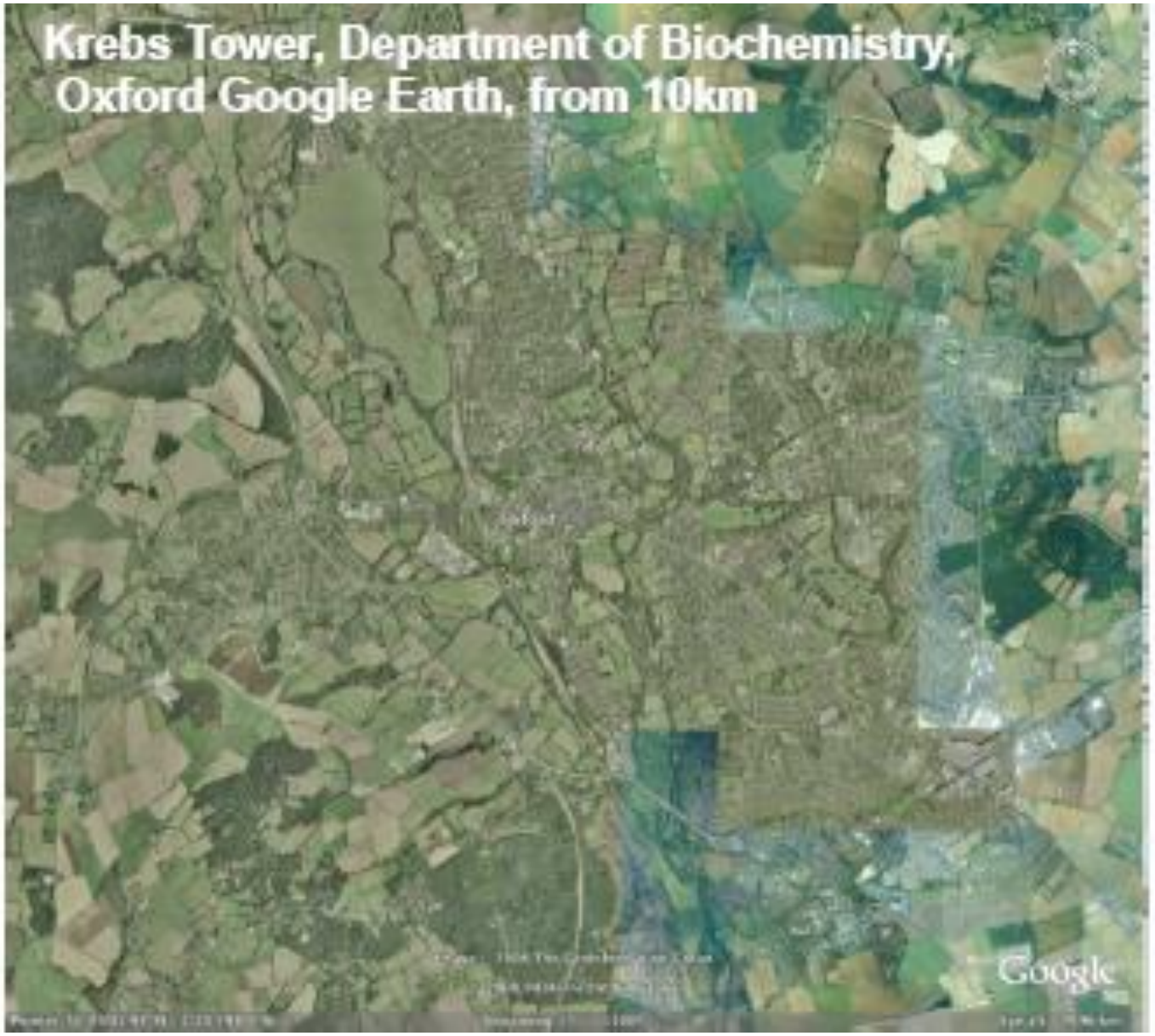
What is magnification?

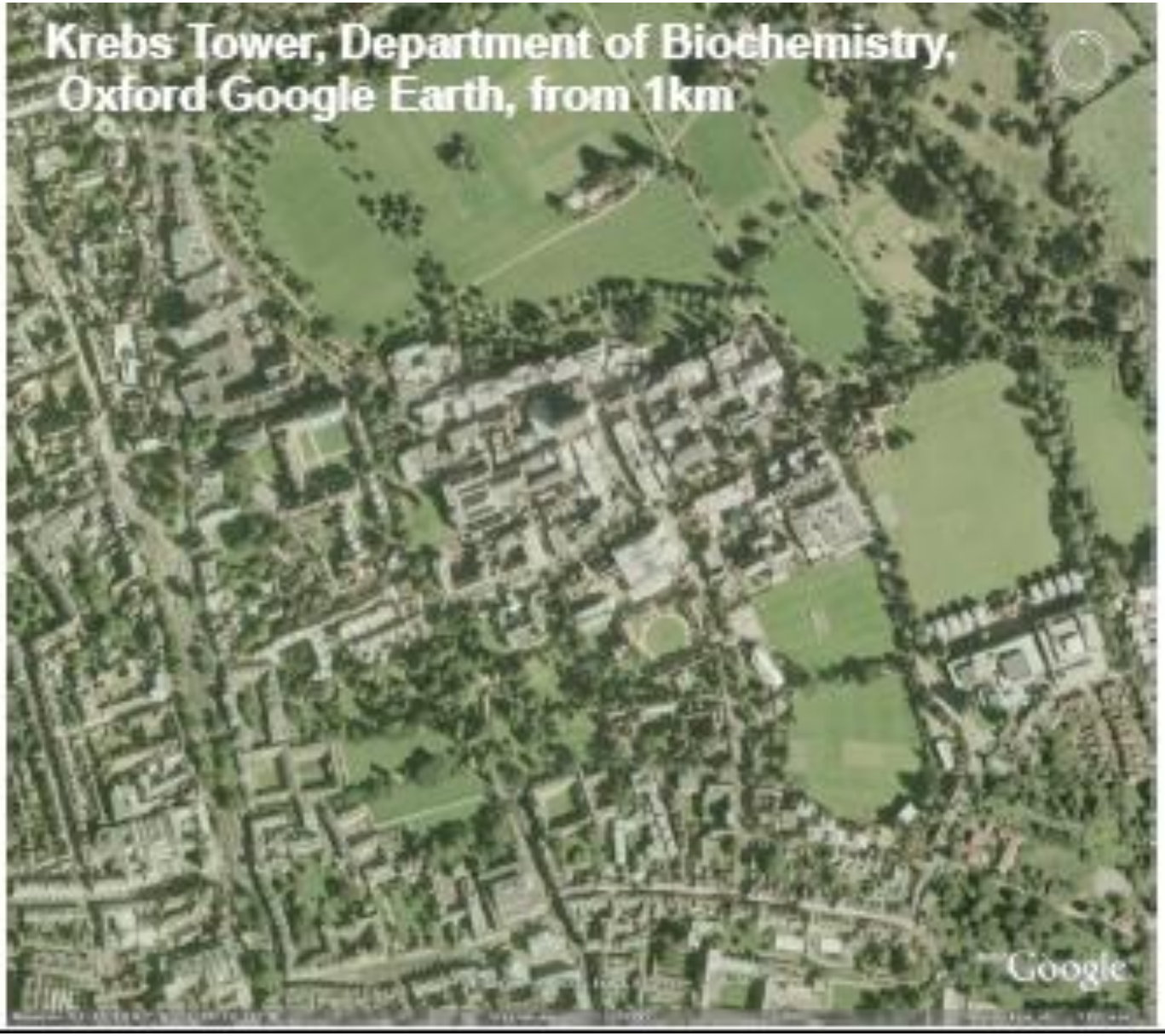
Magnification is defined by the

magnification by the objective
x
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
Google


Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 1km
Google


Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 100m
Google

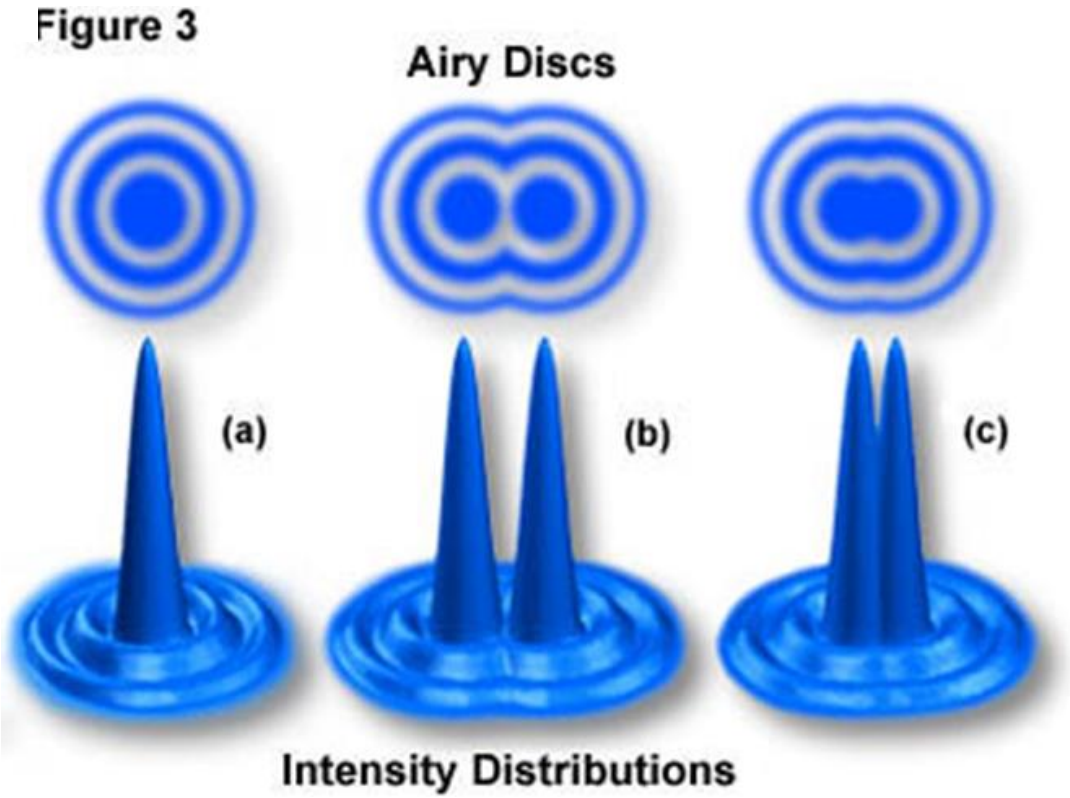
Krebs Tower, Department of Biochemistry,
Oxford Google Earth, from 50m
Google


Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 10m
empty magnification!
Insufficient resolution!
Google

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

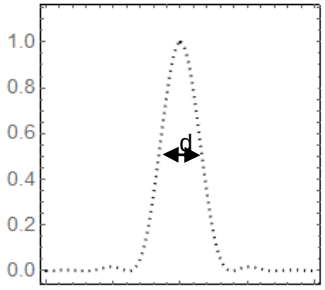
Quantitative Analysis of xy-Resolution – 4 different criteria of resolution

Abbe Criterion

$$d \approx \frac{\lambda}{2\,NA}$$

$d \approx$ FWHM

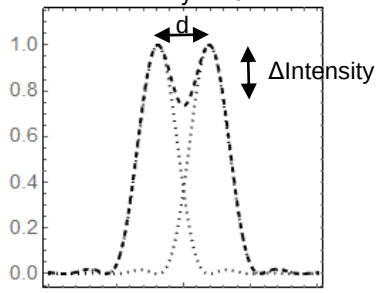
$$FWHM = 2\sqrt{2\,Log[2]\sigma}$$



Rayleigh Criterion

$$d = \frac{0.61\lambda}{NA}$$

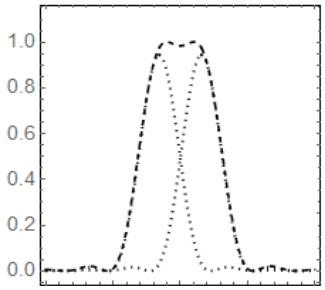
Δintensity ≈ 26.7%



Abbe Criterion

$$d \approx \frac{\lambda}{2\,NA}$$

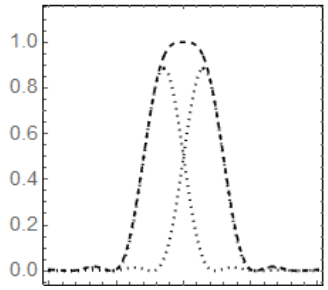
Δintensity ≈ 1.6%



Sparrow Criterion

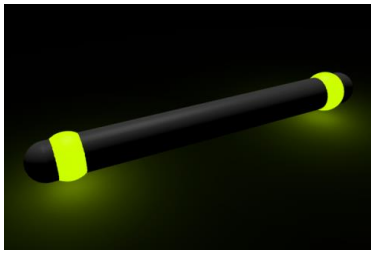
$$d \approx \frac{0.47\,\lambda}{NA}$$

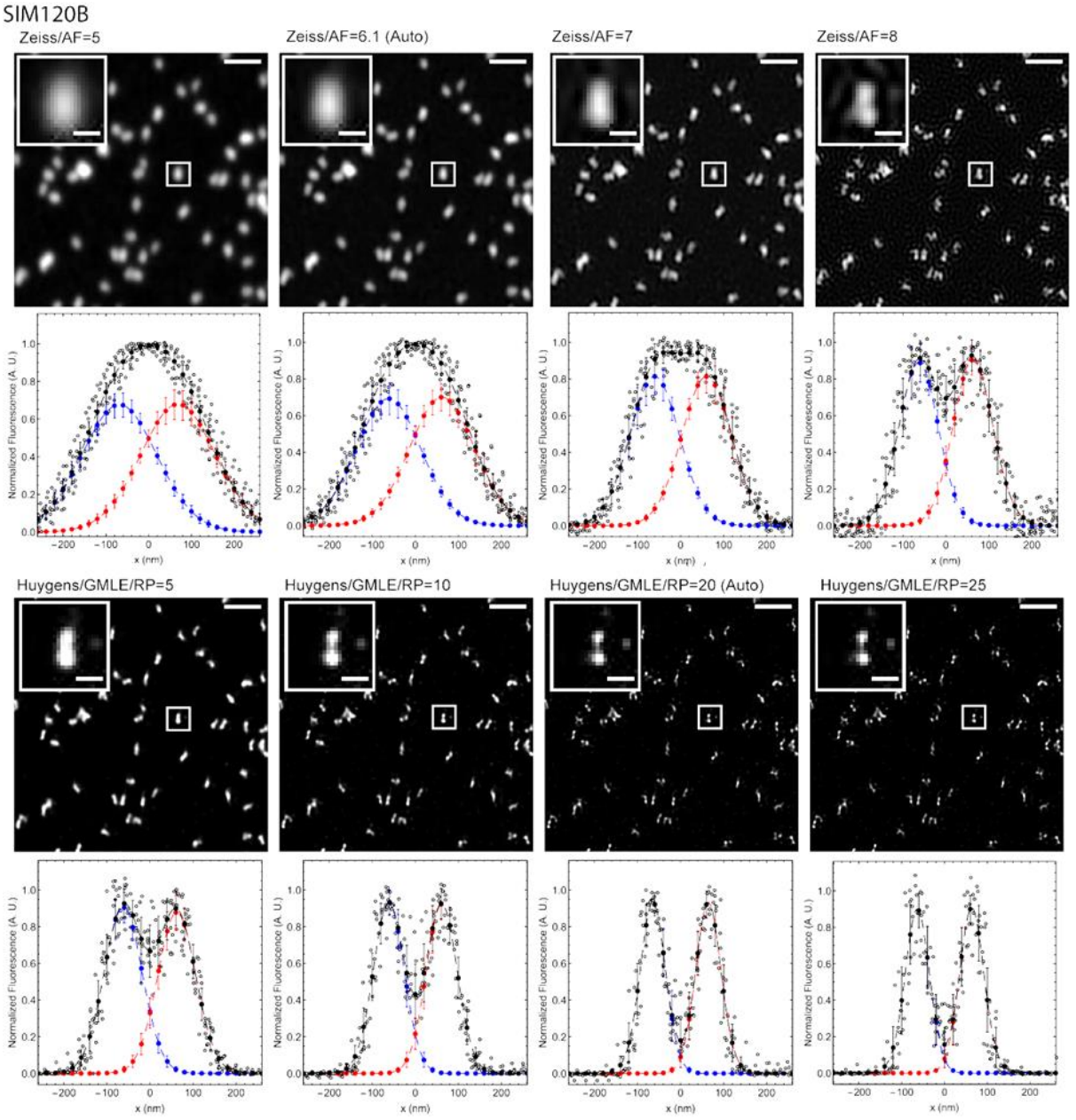
Δintensity = 0%



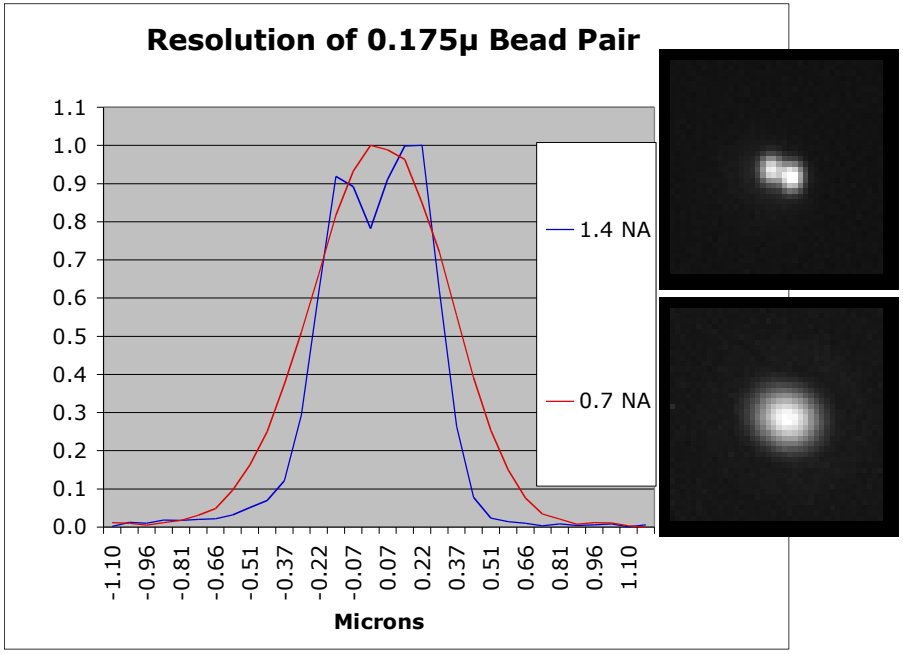
Example – Comparison of image reconstruction algorithms for Airyscan data

Fluorescent DNA origami Nanorulers from GattaQuant





Numerical aperture, NOT magnification determines resolution!



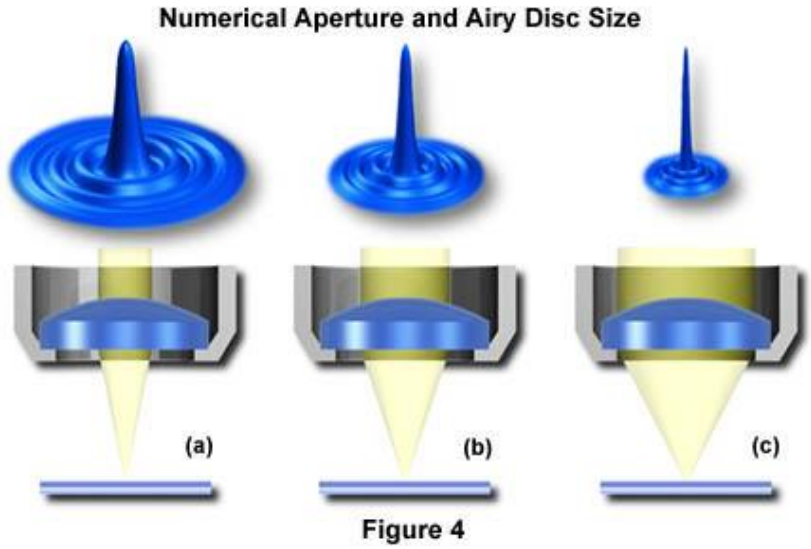


Figure 4

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.)

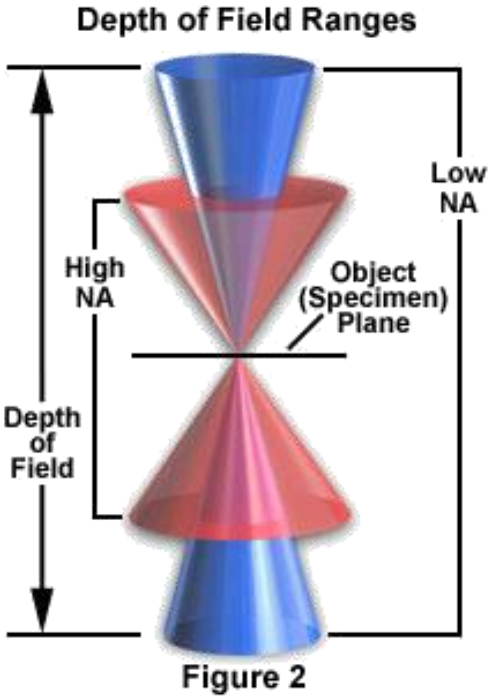


Figure 2

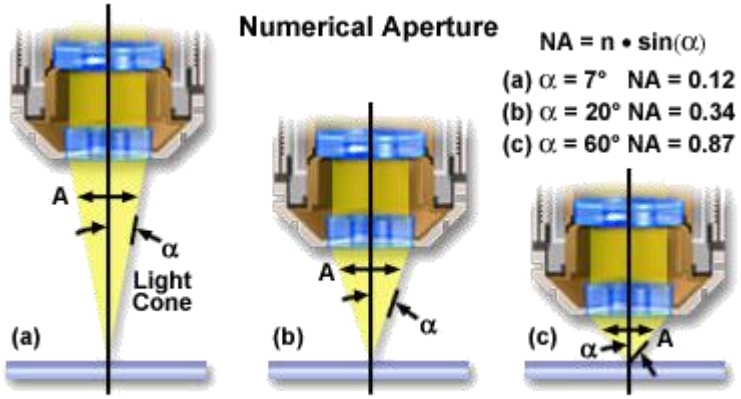


Figure 1

Numerical Aperture = N.A. = n · sin α

α 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 for air; n = 1.51 for oil or glass)

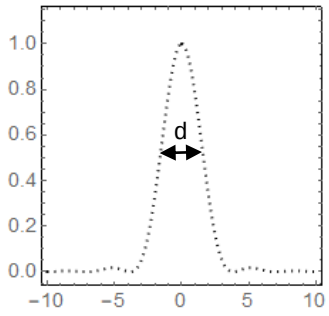
Summary - What is resolution?

Abbe limit $d_{Abbe} = \frac{0.5\ \lambda}{NA_{objective}}$

Abbe Criterion

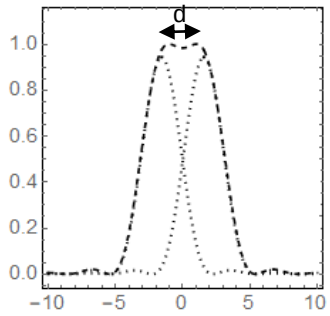
$d \approx \frac{\lambda}{2\ NA}$

Δintensity ≈ 1.6%



$d \approx$ FWHM

$FWHM = 2\sqrt{2\ Log[2]}\sigma$



Rayleigh limit

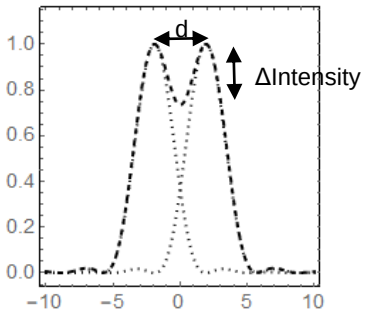
$d_{Raleigh} = \frac{0.61\ \lambda}{NA_{objective}}$ (reflected light)

$d_{Raleigh} = \frac{1.22\ \lambda}{NA_{objective} + NA_{condenser}}$ (transmitted light)

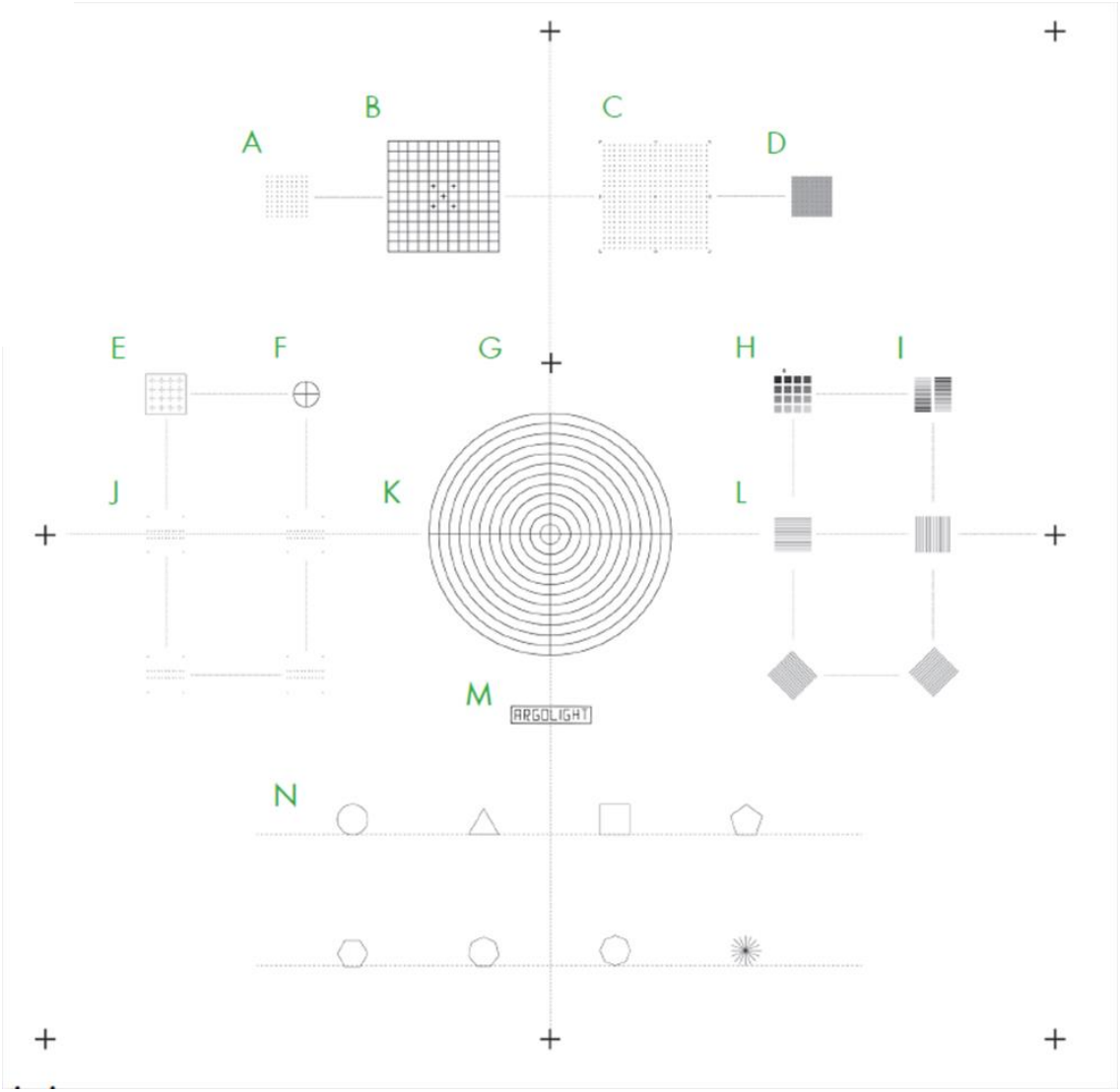
Rayleigh Criterion

$d = \frac{0.61\lambda}{NA}$

Δintensity ≈ 26.7%

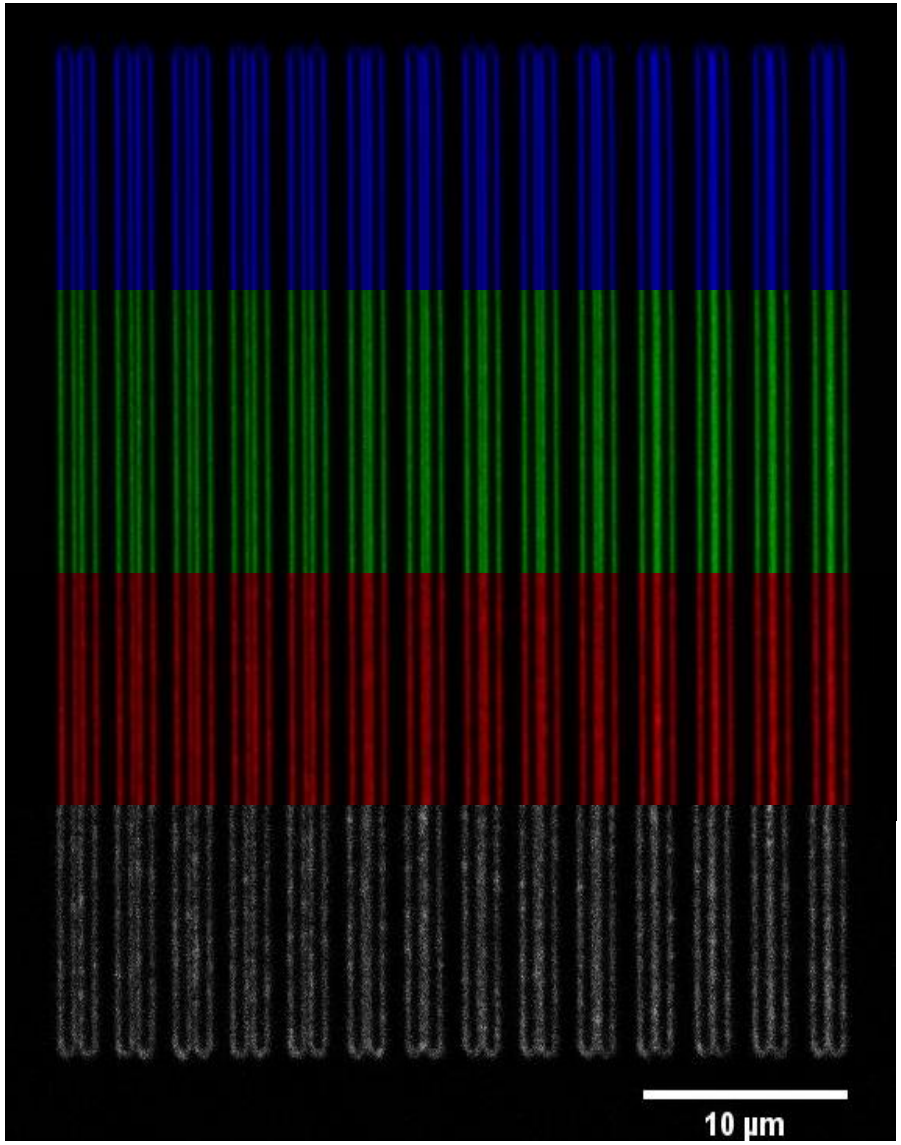


Demonstrating resolution and contrast

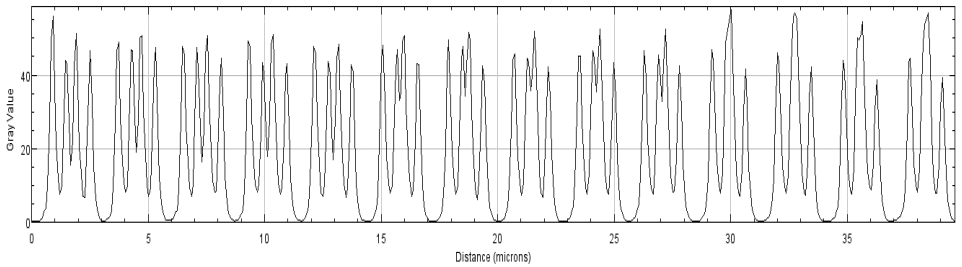


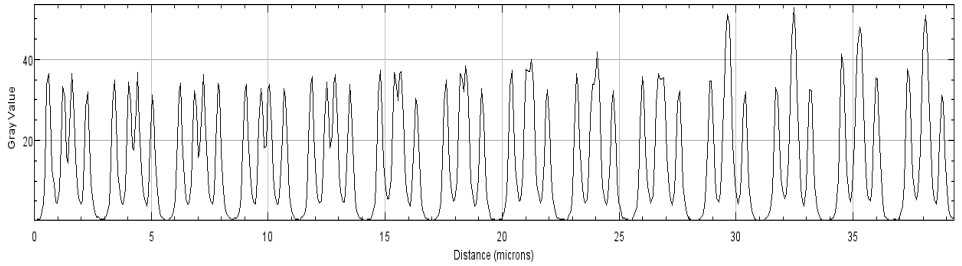
Pattern L

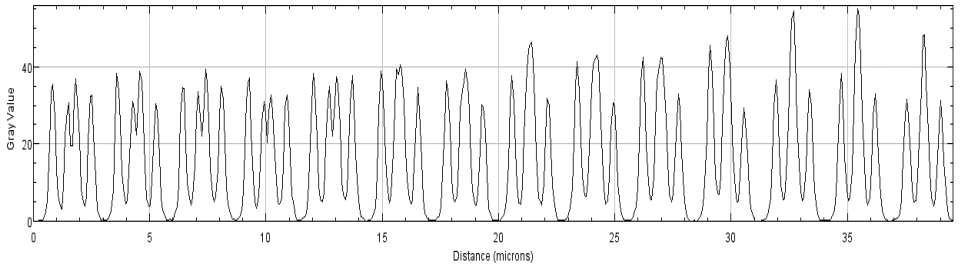
340 320 300 280 260 240 220 200 180 160 140 120 100 80

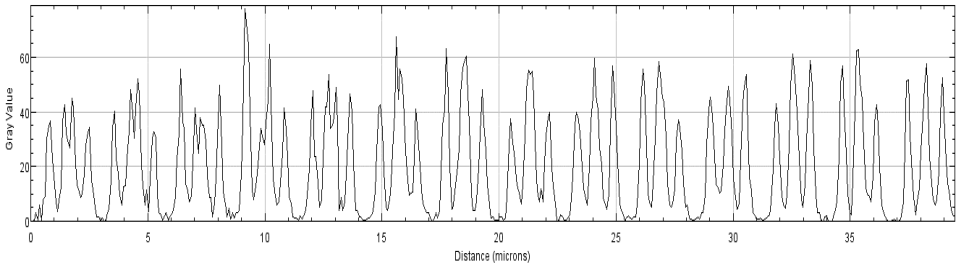


340 320 300 280 260 240 220 200 180 160 140 120 100 80









Microscope resolution in numbers

Objective NA	NA=0.8		NA=1.2		NA=1.4	
λ	xy	z	xy	z	xy	z
405	310	1010	205	675	180	580
488	370	1220	250	810	210	700
561	430	1400	285	935	240	800
594	450	1490	300	990	260	850
633	480	1580	320	1060	280	900

$$D_{XY} = 1.22\,\lambda / 2NA_{obj} \quad \text{.....}$$

FWHM

$$D_Z = 2\,\lambda\eta / (NA_{obj})^2 \quad \text{.....}$$

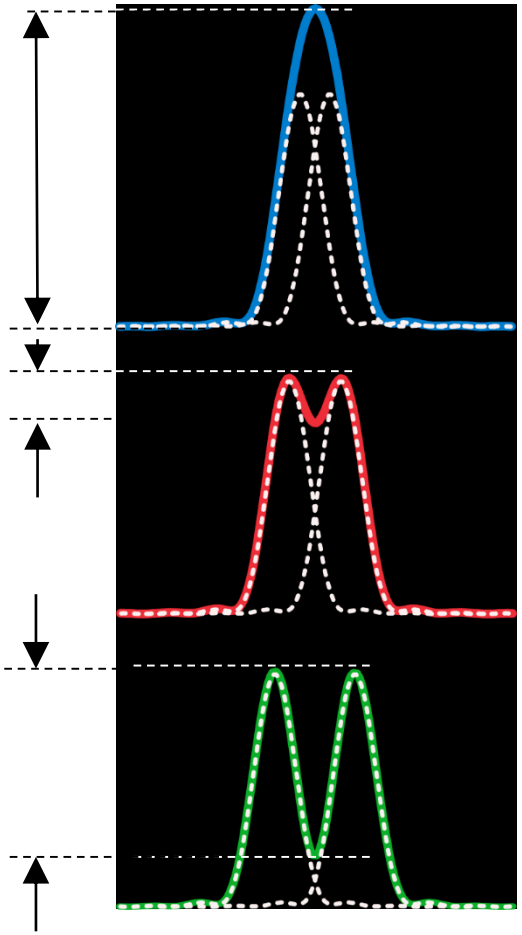
(η = refractive index of the object medium)

….. So which objective would result in best resolution?

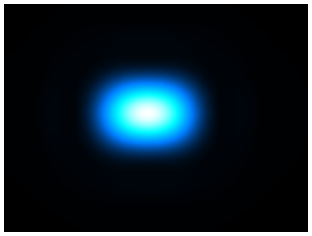
What is contrast?

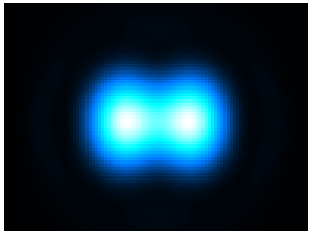
Contrast is defined as the difference in light intensity between points in the image relative to the overall background intensity.

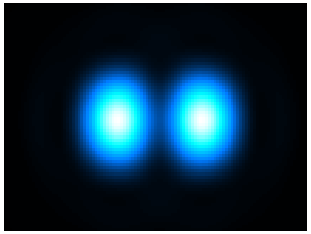
What is contrast?



Intensity drop ~26%







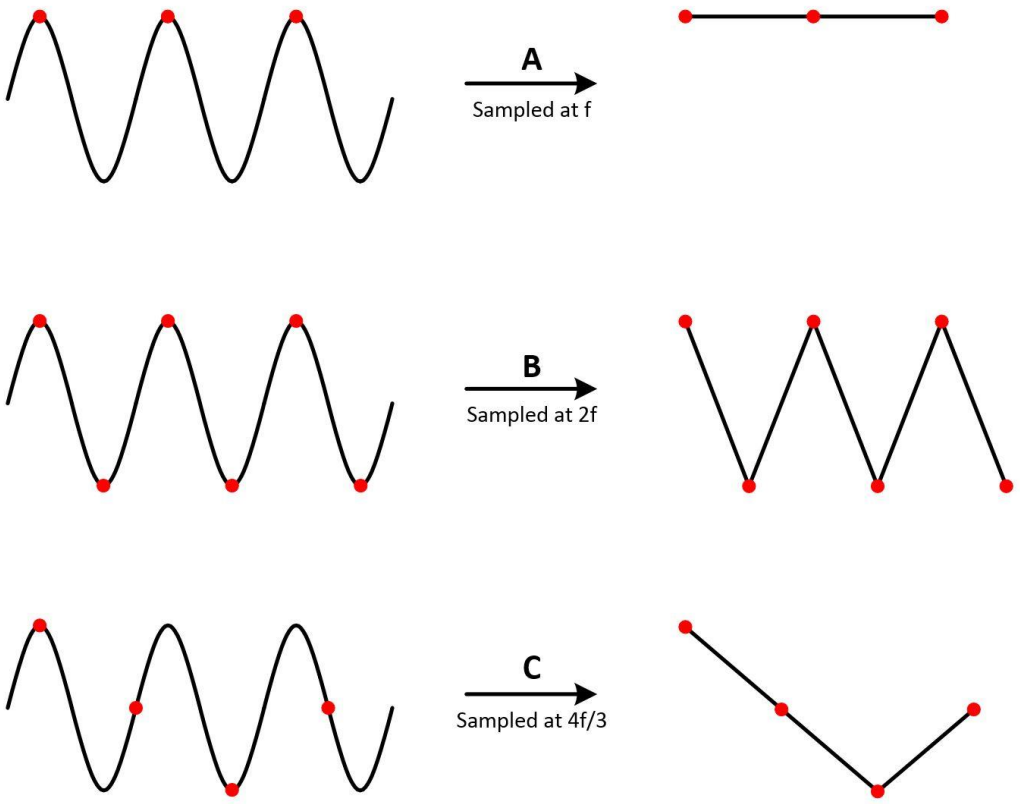
Rayleigh criterion
(between two objects)

NOTE! Resolution requires Contrast

What is sampling?

Sampling is the process of taking measurements in time or space

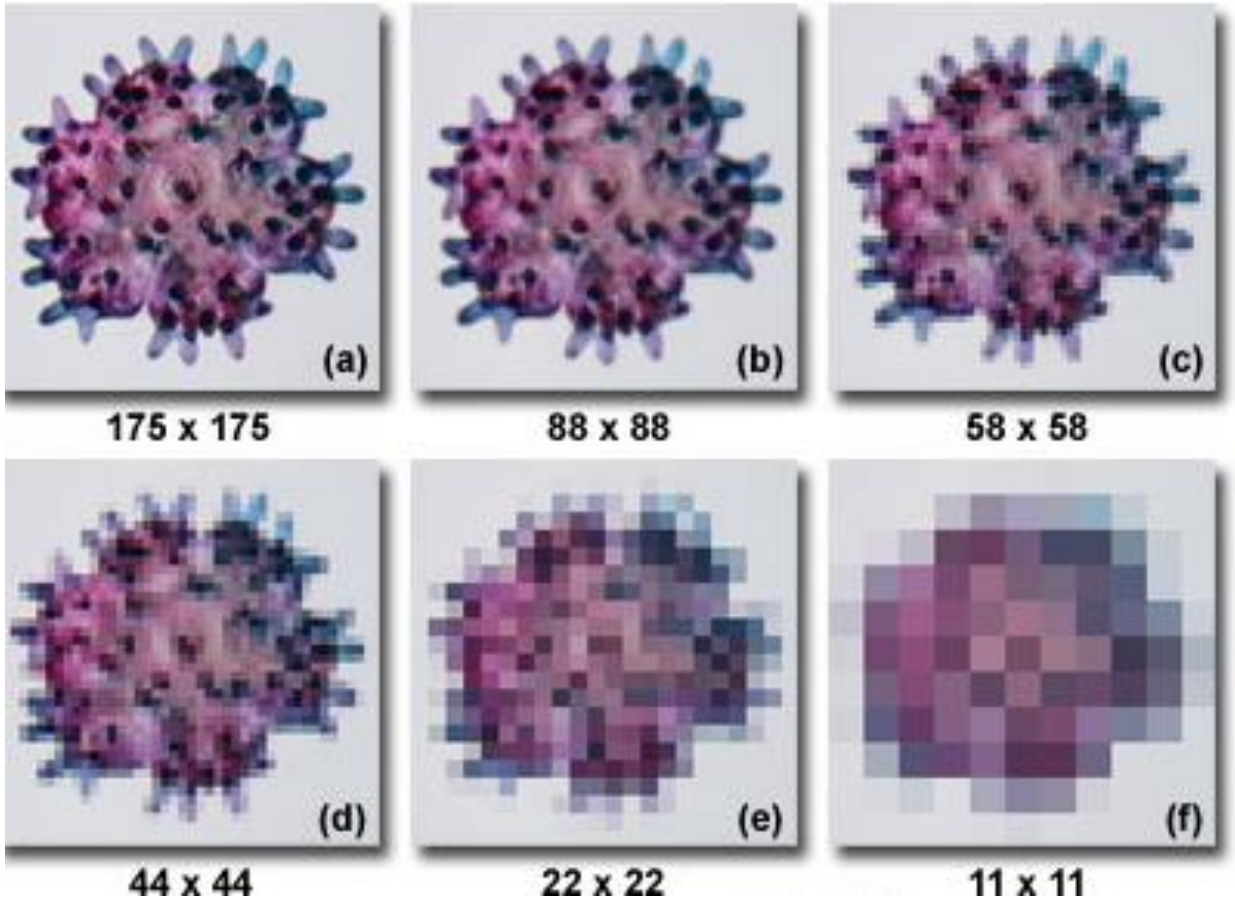
Nyquist Sampling Theorem = Sample at ~2-3x frequency



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

Objective NA	NA=1.4		Sampling @ 3x Frequency	
λ	xy	z	xy	z
405	180	580	60	190
488	210	700	70	230
561	240	800	80	270
594	260	850	85	280
633	280	900	90	300

$$D_{XY} = 1.22\,\lambda / 2NA_{obj} \quad \text{FWHM}$$

$$D_Z = 2\,\lambda\eta / (NA_{obj})^2$$

(η = 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.

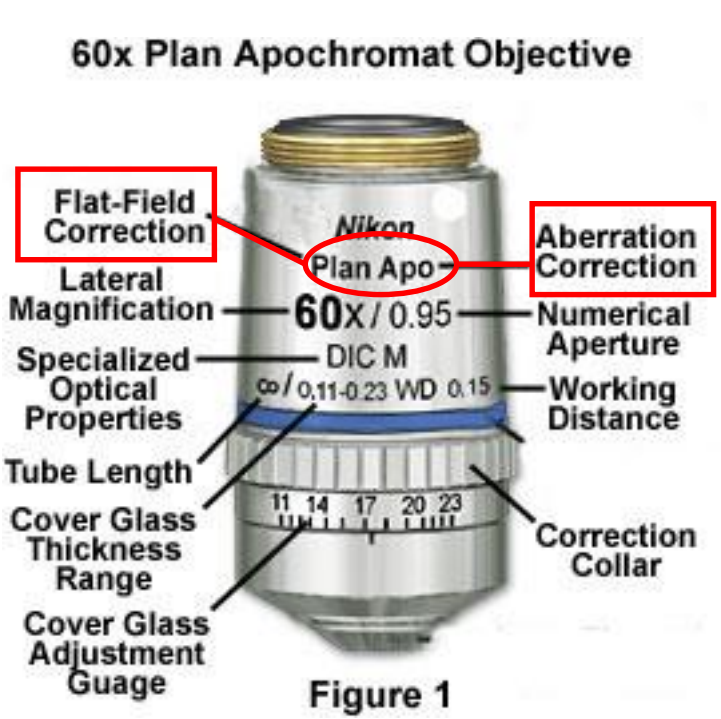


Figure 1

Figure 1

Flat-field correction and aberration correction

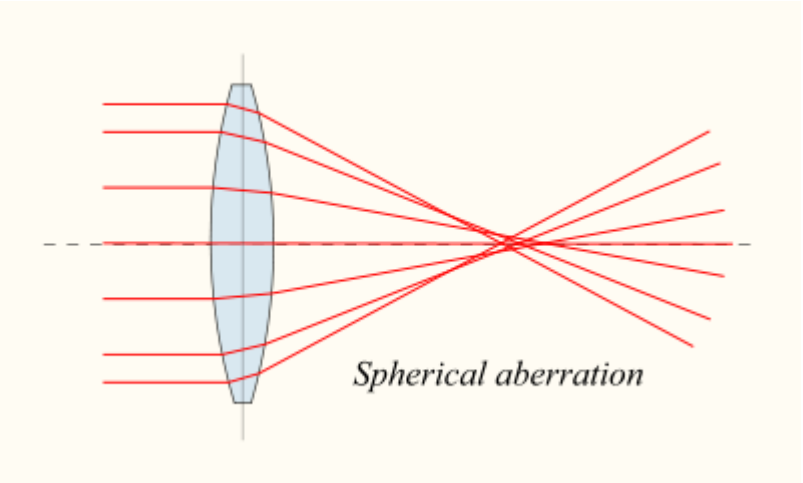
Describe two main criteria for the quality of an objective:

Flatness of the intermediate image

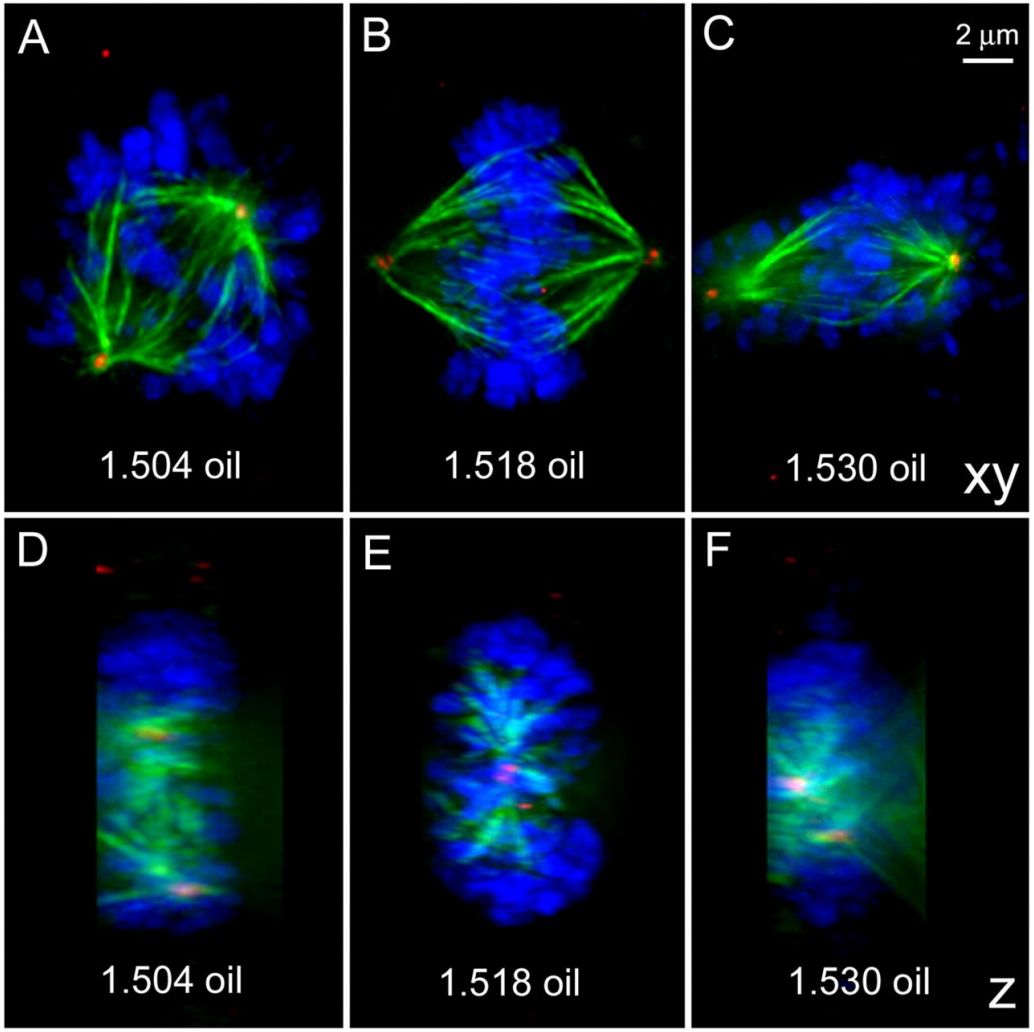
Elimination of chromatic errors

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.

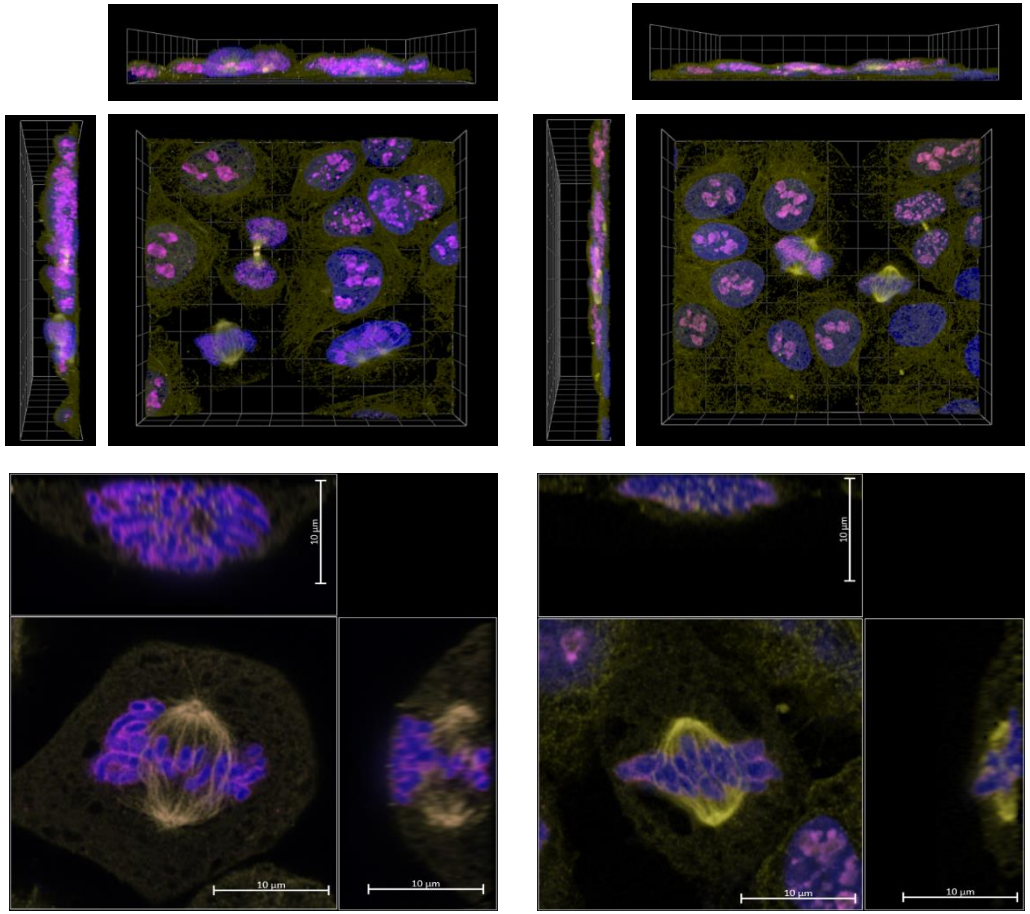


The effect of spherical aberrations on images.



North A. JCB 2006;172:9-18

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

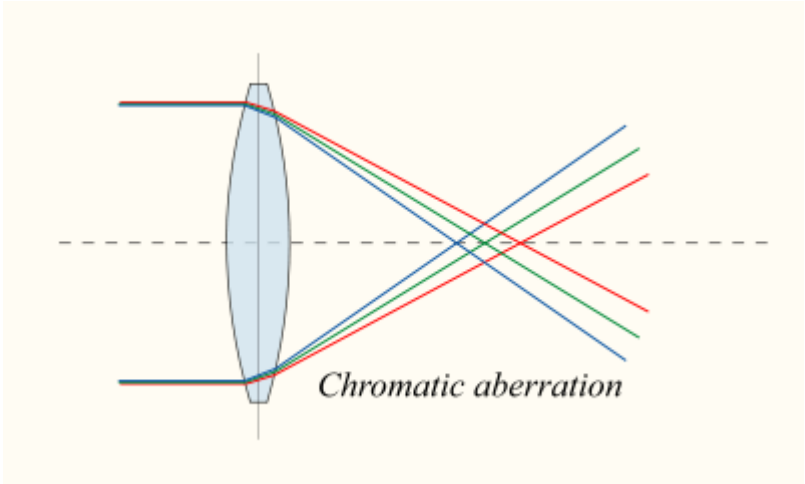
Describe two main criteria for the quality of an objective:

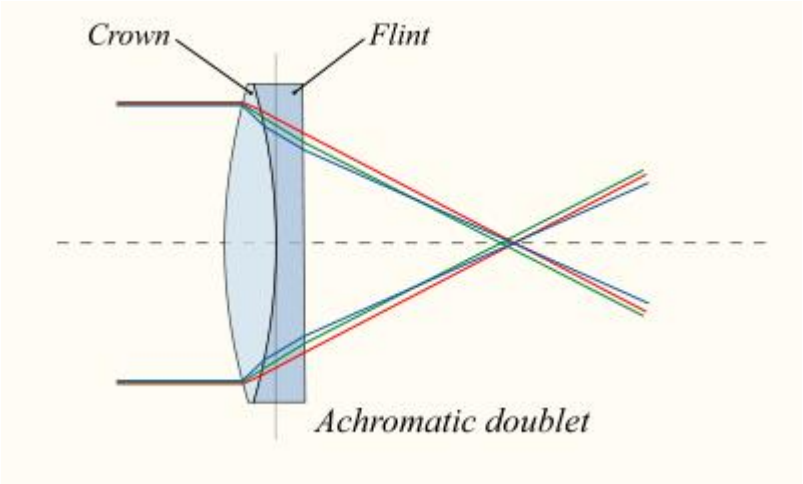
Flatness of the intermediate image

Elimination of chromatic errors

Chromatic aberration

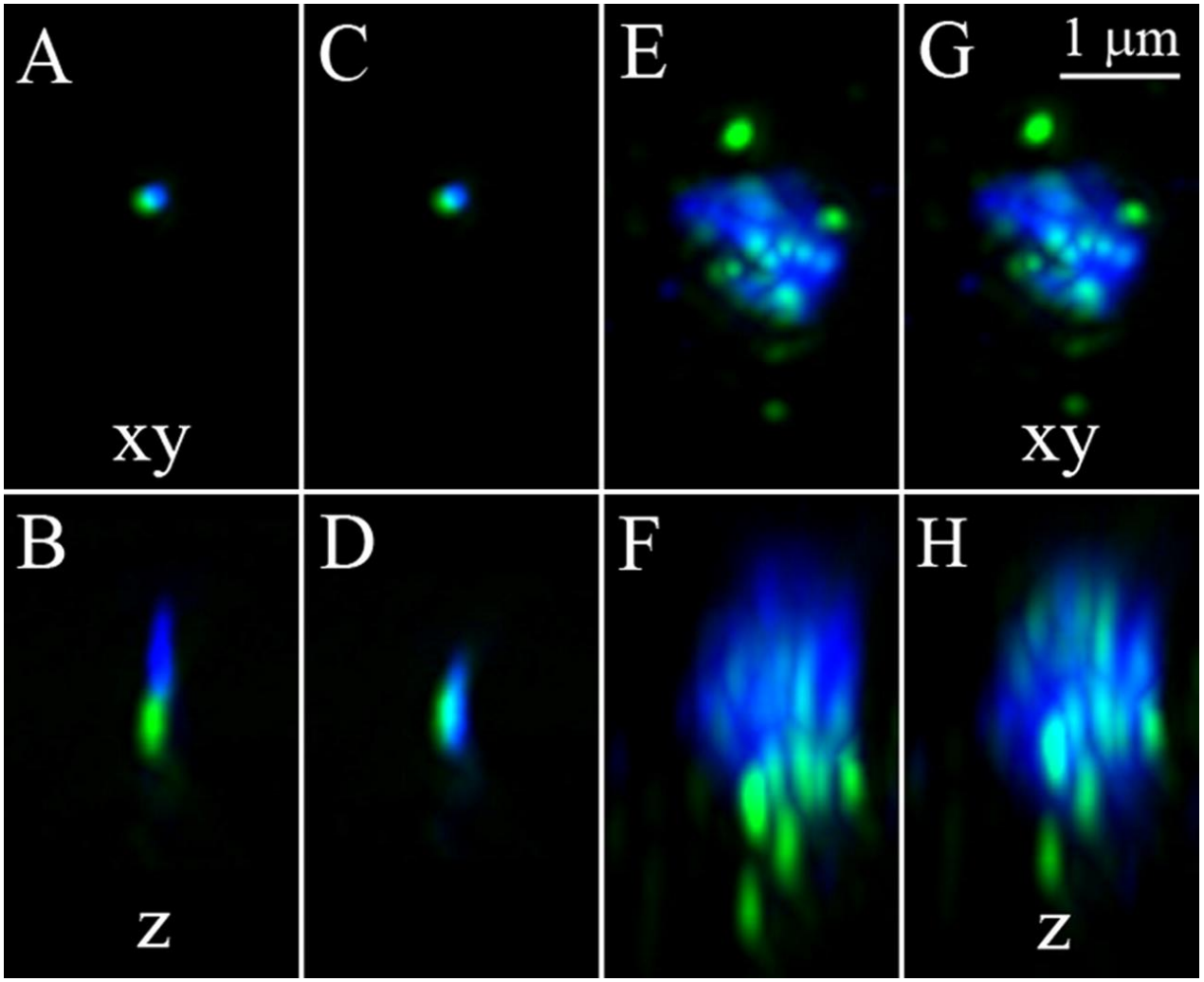
Chromatic aberration is caused by a lens having different refractive indexes for different wavelengths. Since the focal length of a lens is dependent on the refractive index, different wavelengths will be focused on different positions in the focal plane. Chromatic aberration is seen as fringes of colour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



North A. JCB 2006;172:9-18

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.

60x Plan Apochromat Objective

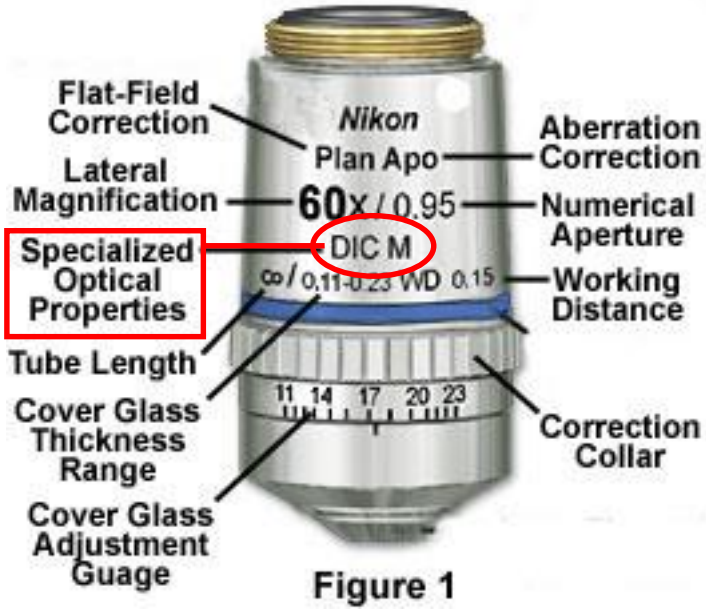


Figure 1

60x Plan Apochromat Objective

Figure 1

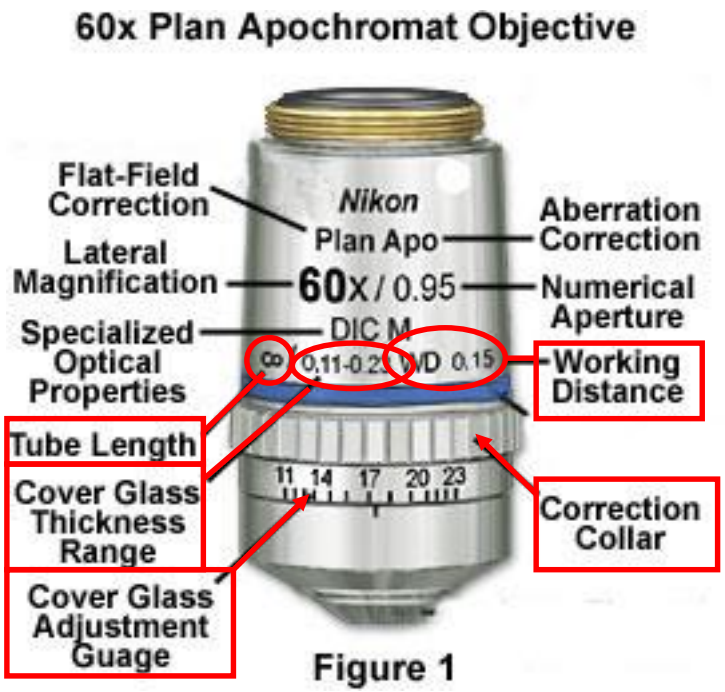


Figure 1

Coverslip-types:

1:	0.13 - 0.17 mm
1.5:	0.16 - 0.19 mm
2.0:	0.19 - 0.23 mm

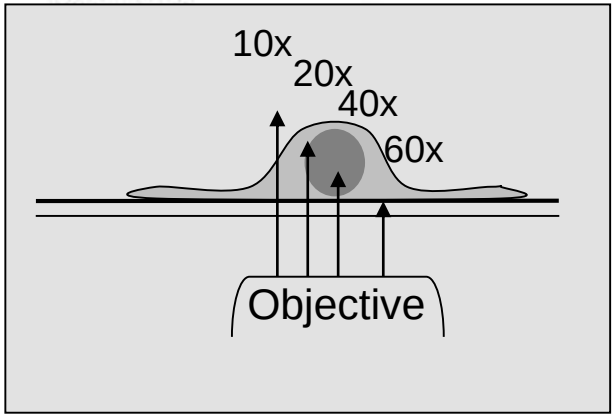


Table 1 - Common Objective Working Distances

Manufacturer	Correction	Magnification	Numerical Aperture	Working Distance
Nikon	PlanApo	10x	0.45	4.0 mm
Nikon	PlanFluor	20x	0.75	0.35 mm
Nikon	PlanFluor (oil)	40x	1.30	0.20 mm
Nikon	PlanApo (oil)	60x	1.40	0.21 mm
Nikon	PlanApo (oil)	100x	1.40	0.13 mm

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/

- *Alison J. North.* **Seeing is believing? A beginners' guide to practical pitfalls in image acquisition.** *JCB Volume 172(1):9-18 January 2, 2006*
- *RW Cole, T Jindasa, CM Brown.* ***Measuring and interpreting point spread functions to determine confocal microscope resolution and ensure quality control.*** *doi:10.1038/nprot.2011.407http://www.microscopist.co.uk/wp-content/uploads/2017/04/Cole_Brown-Nature-Protocols-PSF-fitting.pdf*

But beware because image brightness is dependent on both the magnification and the NA where $I \sim NA^4 / M^2$

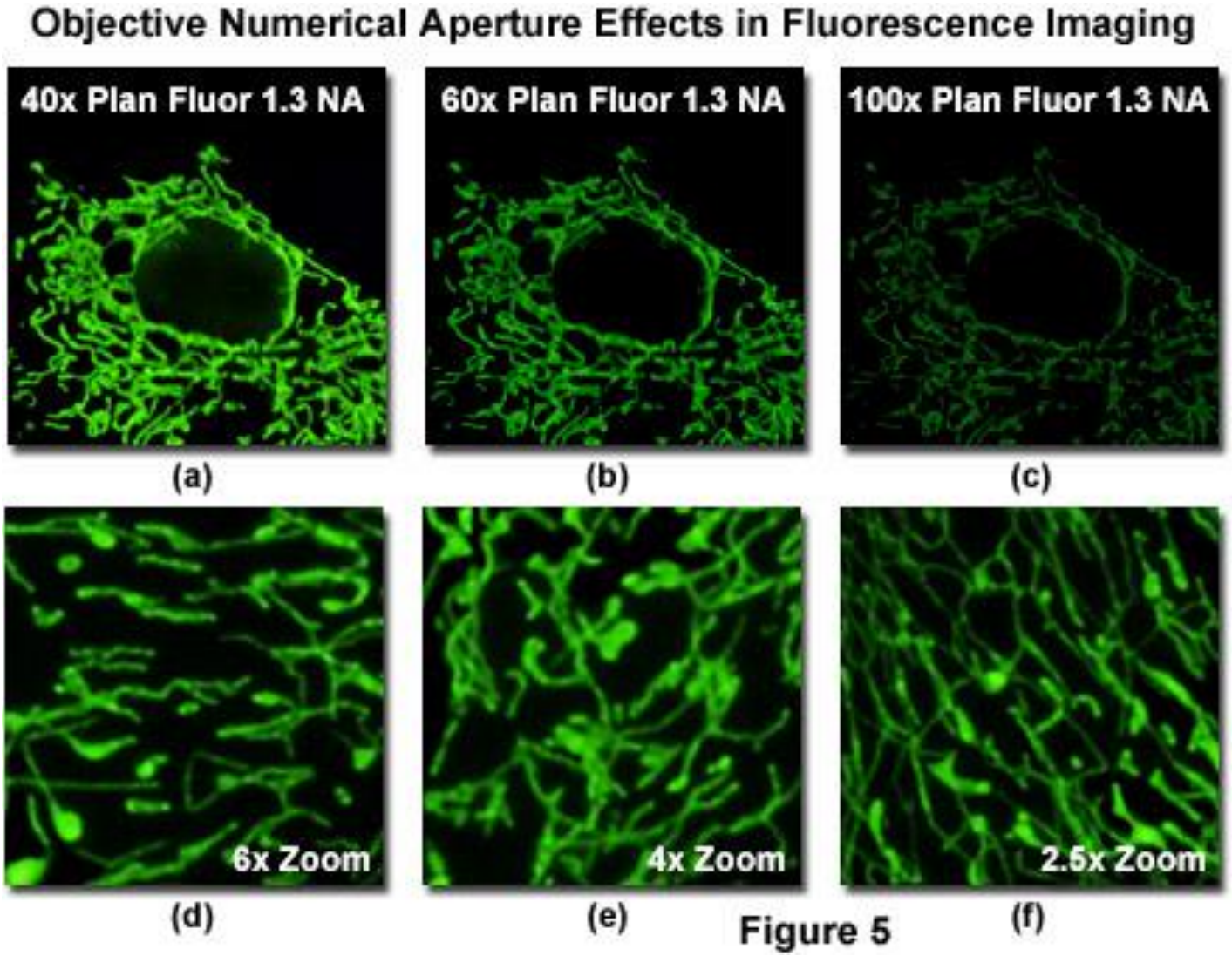


Figure 5