CONFOCAL MICROSCOPY

Wolfson ImagingCentre Oxford

Weatherall Institute of Molecular Medicine

Dr. B. Christoffer Lagerholm
Facility Manager

Dr. Jana Koth
Assistant Facility Manager

Lecture Goals

- To develop a brief familiarity with what you can achieve with light microscopy

- To develop a basic understanding of how a confocal microscope works

- To be able to identify ways to optimize your microscopy acquisition

Outline

- Survey of Image Examples from the Wolfson Imaging Centre
- Introduction of Light Microscopy
- Brief Review of Fluorescence
- The importance of understanding the microscope objective
  - Magnification
  - Resolution
  - Abberations
  - Working distance
- Confocal Microscopy
- Two-photon Microscopy
- Spinning disc confocal
- Summary

tools.ndm.ox.ac.uk/newt/imm/wolfson-imaging-centre-oxford

Weatherall Institute of Molecular Medicine

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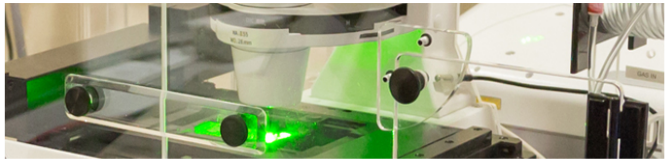
DNA Sequencing/HLA Typing | Flow Cytometry | Wolfson Imaging Centre Oxford



The Wolfson Imaging Centre – Oxford was established in 2012 with funds received from the Wolfson Foundation. The facility contains a diverse range of wide-field and confocal microscopes suitable for imaging specimens ranging in complexity from single cells to small laboratory animals.

The Centre is open for access to internal and external users at modest fees. Click here for pricing.

For access please contact either the Scientific Director of the Centre, Dr. Christian Eggeling, or the Facility Manager, Dr. Christoffer Lagerholm.

Equipment

Wide-field & TIRF

- Delta Vision Elite Live Cell Imaging System
- Olympus IX83 TIRF

Confocal

- Zeiss 510 Inverted Confocal
- Zeiss 780 Inverted Confocal
- Zeiss 780 Upright Confocal
- Zeiss Spinning Disc Confocal

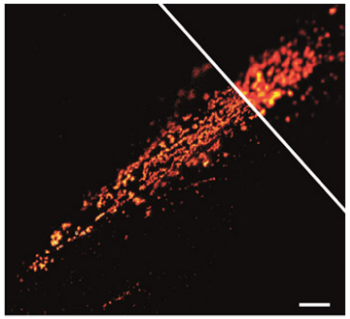
Super-resolution

- Leica SP8 Gated STED

Data Analysis

- Imaris
- Huygens

Data Storage



STED/Confocal image of Acetylcholine Receptor Clusters in Myotubes
Anti-AChR /Alexa488-anti-IgG
Leica SP8 Gated STED Microscope
Dr. Judith Cossins / Prof. David Beeson
Molecular Neurosciences
Dr. B. Christoffer Lagerholm / Dr. Christian Eggeling
Weatherall Institute of Molecular Medicine

Wolfson Imaging Centre Oxford

Wide-field & TIRF

- Delta Vision Elite Live Cell Imaging System
- Olympus IX83 TIRF

Confocal

- Zeiss 510 Inverted Confocal
- Zeiss LSM 780 Confocal Microscope - Inverted Microscope
- Zeiss LSM 780 Multi-Photon / Confocal Microscope
- Zeiss Spinning Disc Confocal

Super-resolution

- Leica SP8 Gated STED

Data Analysis

- Imaris
- Huygens

Data Storage

- Omero

Internal Links

- Image Gallery
- Pricing
- Progress Reports
- Imaging Resources (internal)

External Links

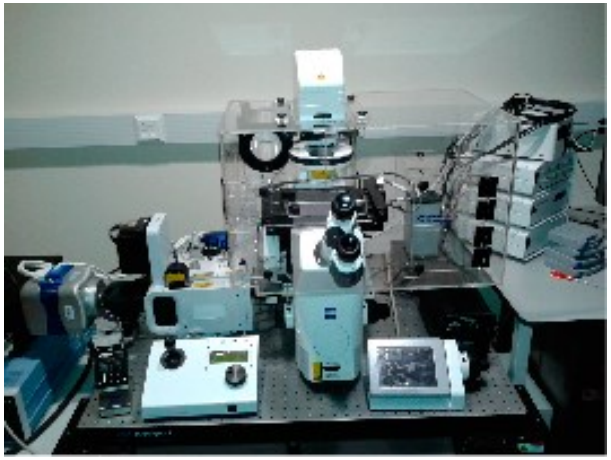
- Micron
- NanO

http://www.imm.ox.ac.uk/wolfson-imaging-centre-oxford

Wolfson Imaging Centre – Microscopes I

Deltavision Elite Wide-field Microscope

Zeiss Observer Spinning-disc Confocal Microscope



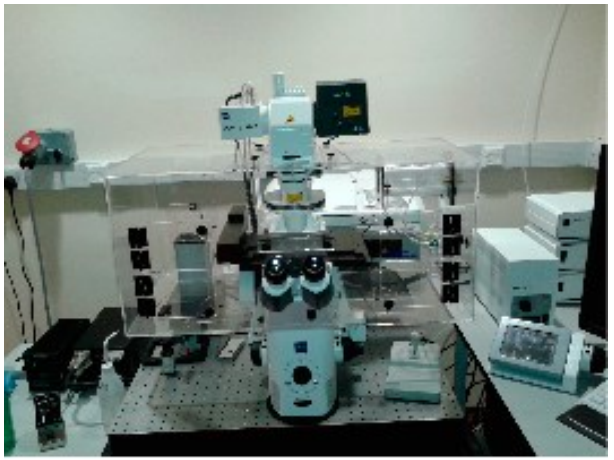
Olympus IX83 Total Internal Reflection Fluorescence Microscope

Leica SP8X Inverted Confocal and gSTED Microscope

Wolfson Imaging Centre – Microscopes I

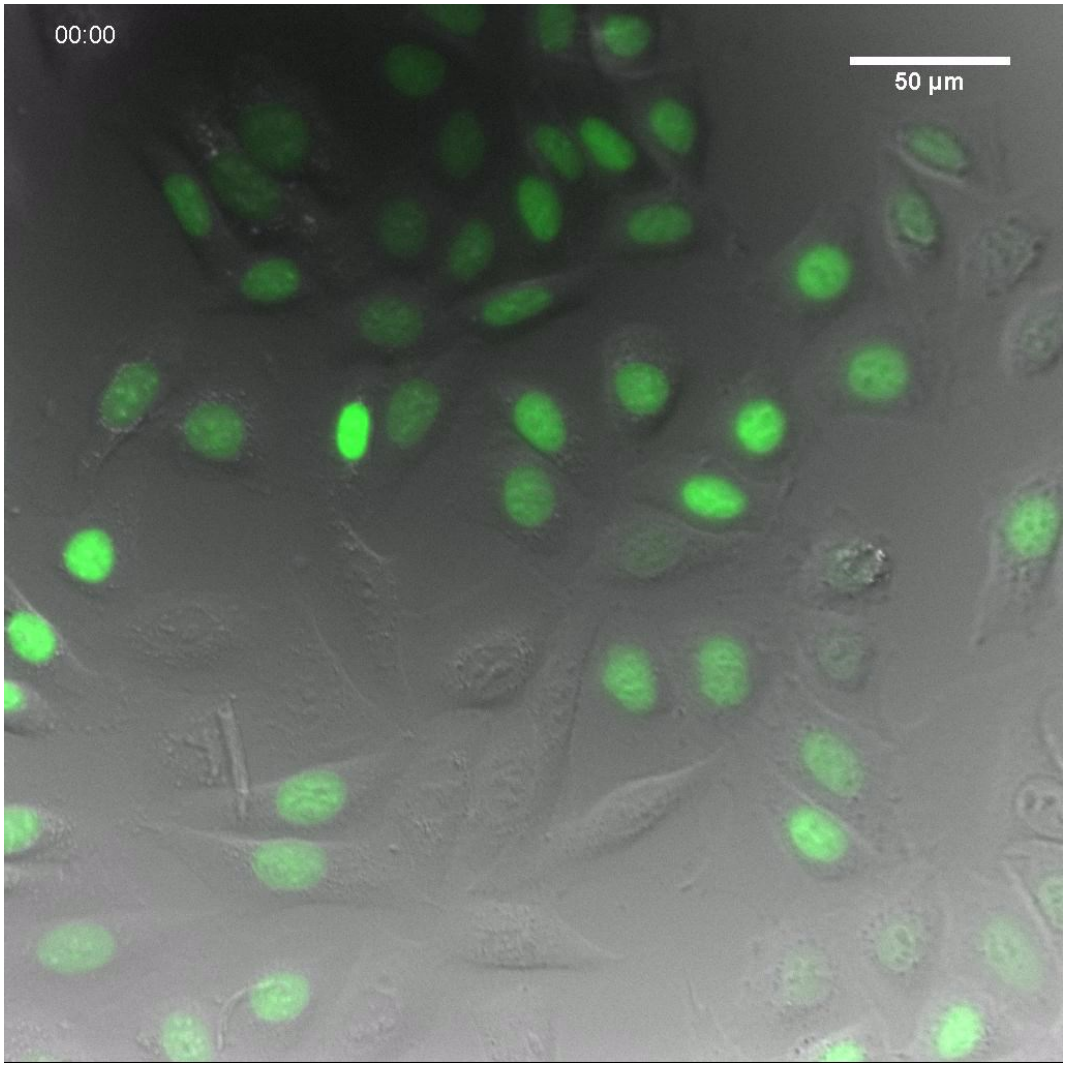
Zeiss 780 Upright Confocal and Multi-photon Microscope

Zeiss 780 Inverted Confocal Microscope



Zeiss 880 Inverted Confocal Microscope

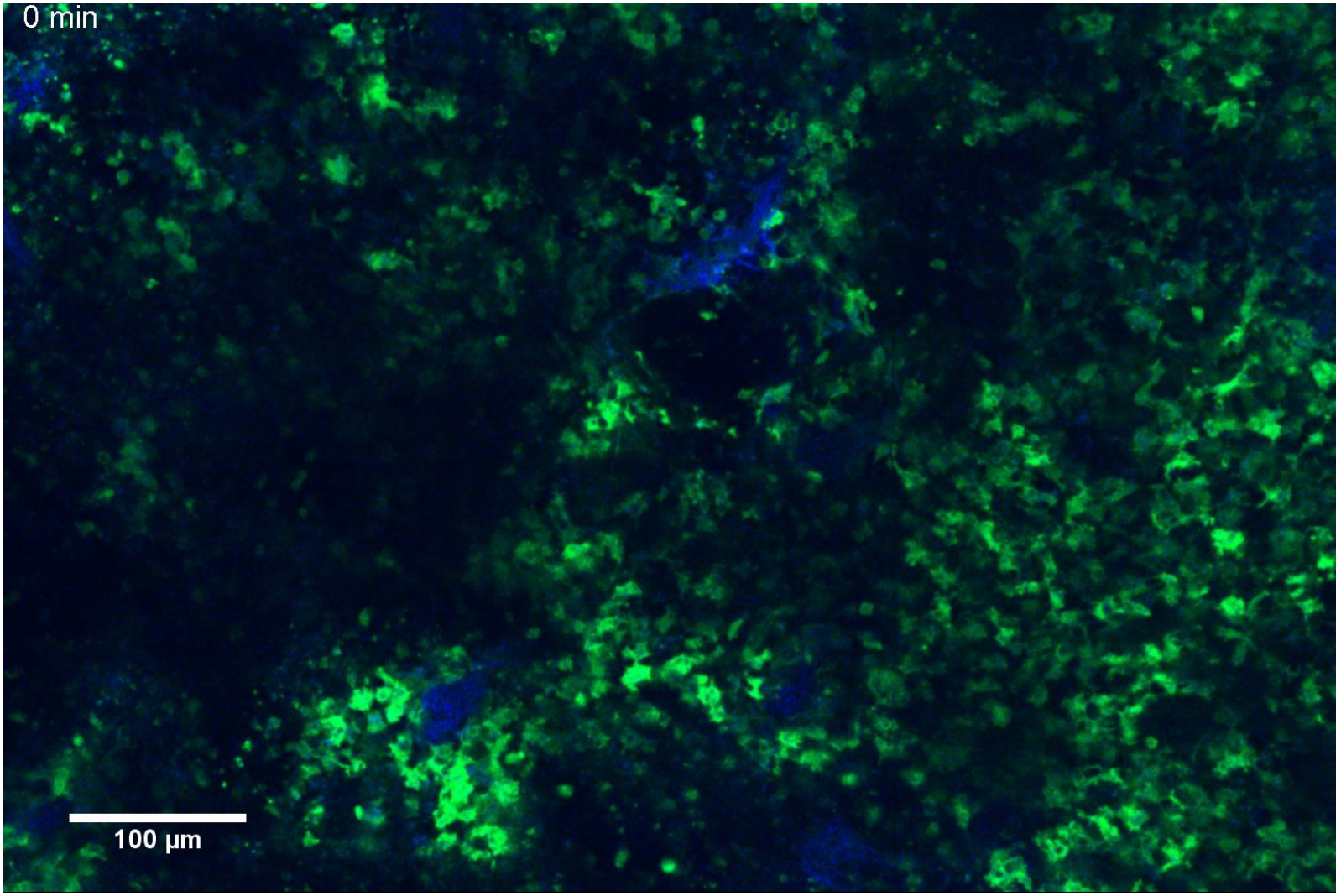
Imaging cell division in HeLa cells (H2B-GFP)
Deltavision Elite Wide-field Microscope



Activation of macrophages in ex vivo mouse spleen (CD68-GFP)

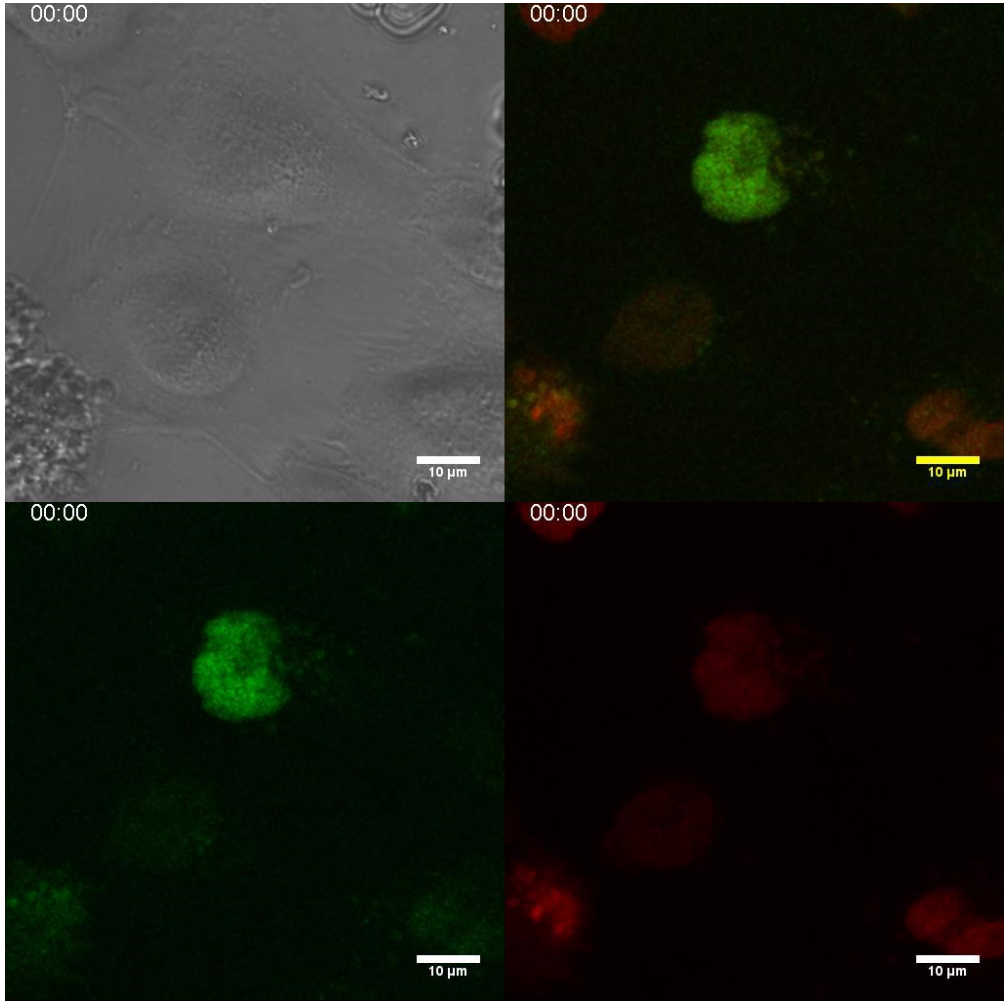
Prof. Robin P Choudhury & Naveed Akbar, Department of Cardiovascular Medicine

Zeiss 780 Upright Multi-photon Microscope



Non-sensitized DNA damage response on Zeiss Multi-photon with λ=720 nm (pixel dwell time=6 μs, 10 iterations)

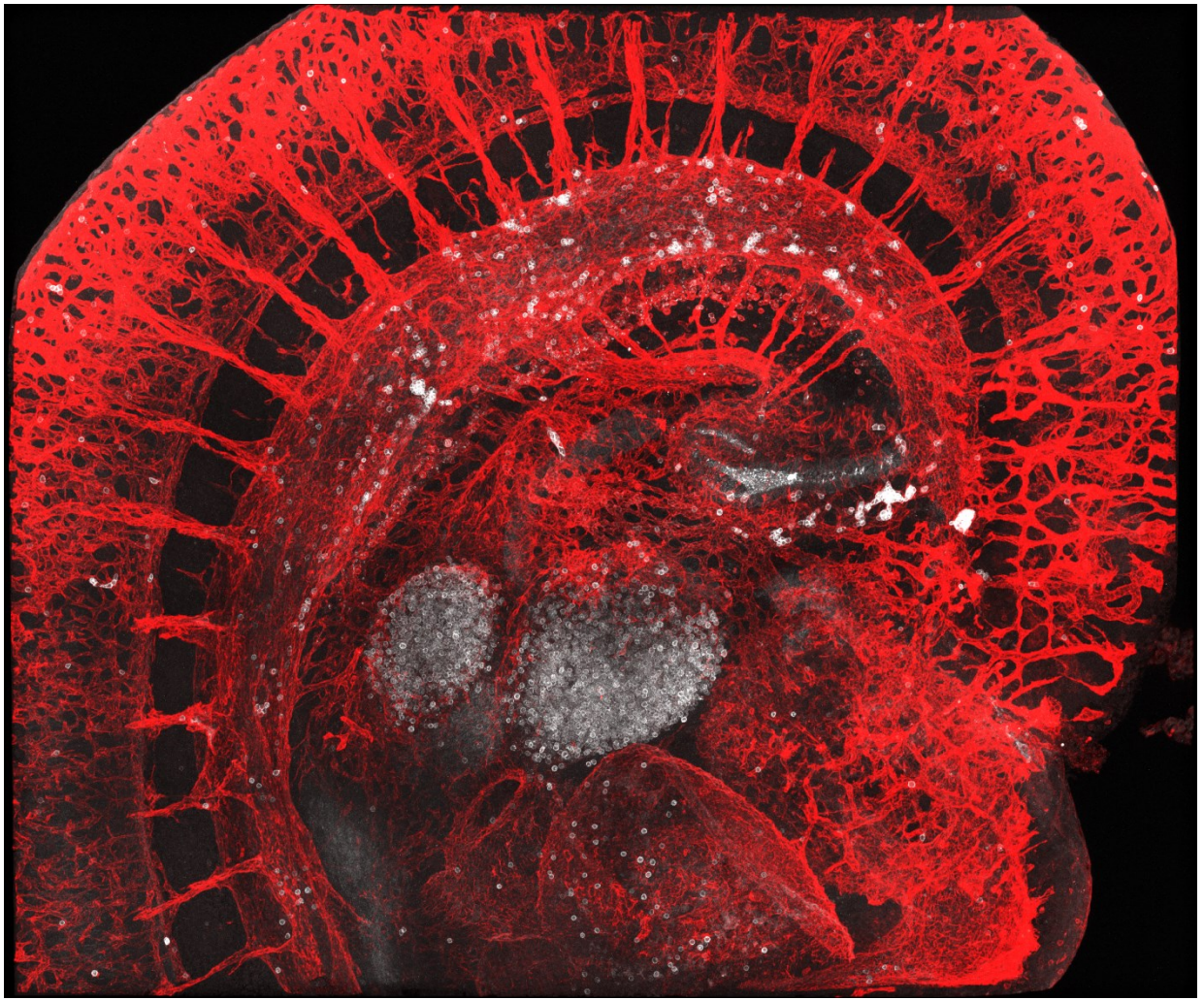
Dr. Martin Cohn, Yasunaga Yoshikawa & Dr. Ian Dobbie, Dept of Biochemistry



3D reconstructions of immunolabeled whole mount mouse embryos (E10.5).

Prof. Marella de Bruijn & Dr. Emanuele Azzoni, MHU

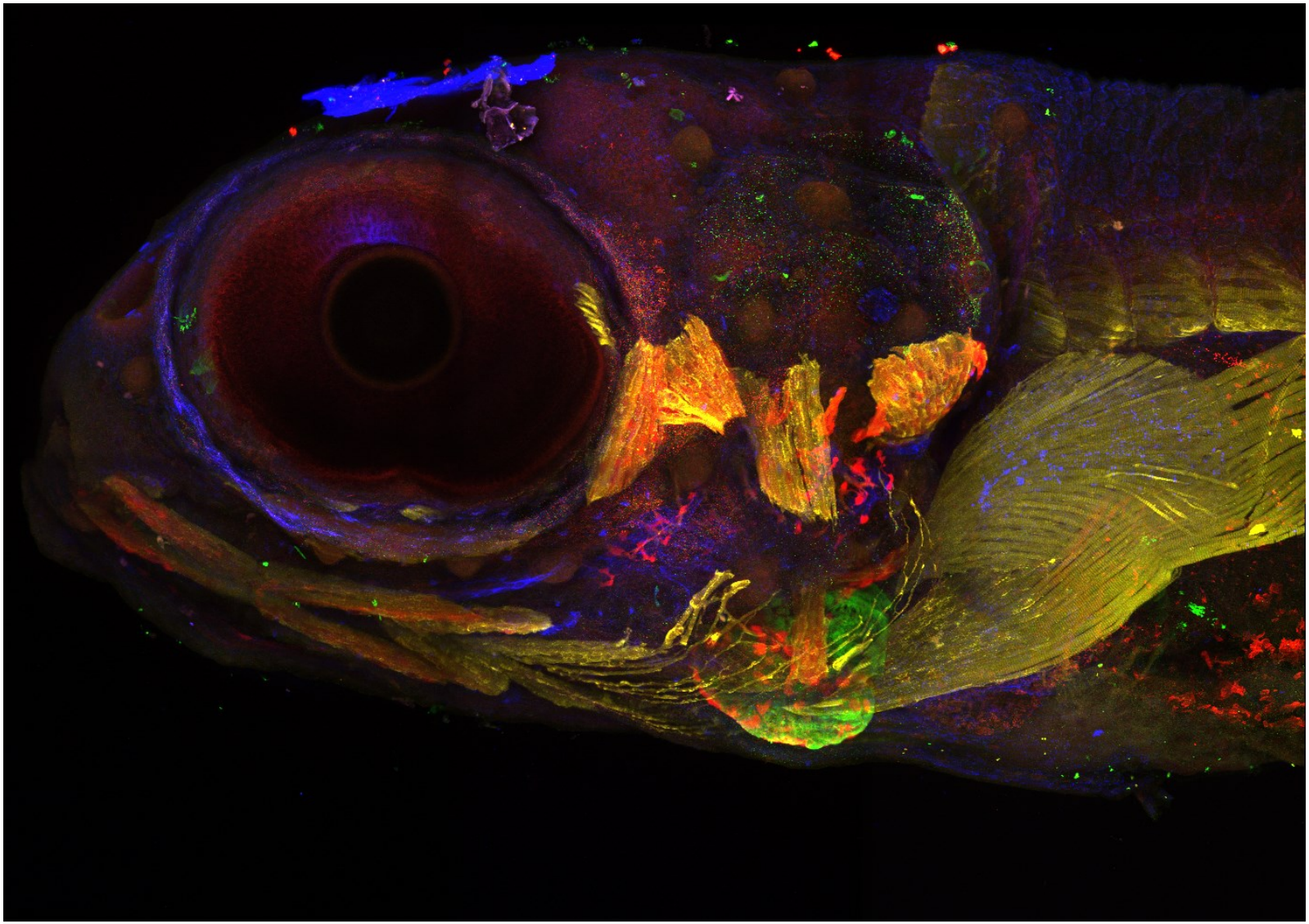
Zeiss 780 Upright Confocal Microscope



Blood vasculature (CD31; red) and hematopoietic clusters and hematopoietic cells (c-Kit; light grey)

Prof. Roger Patient & Dr. Jana Koth, MHU

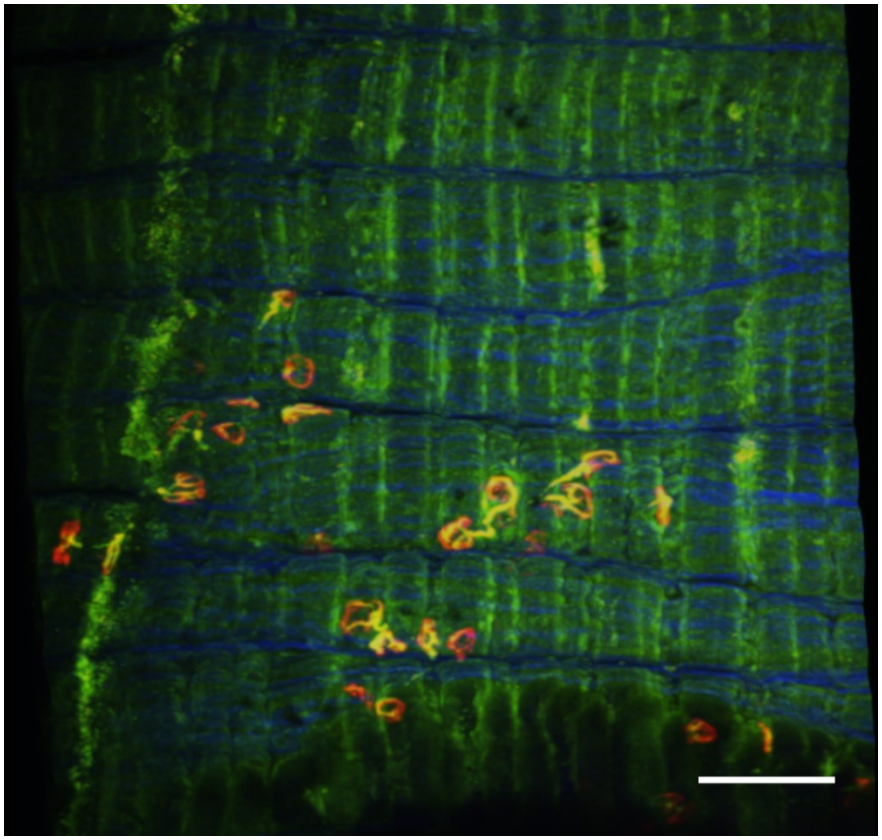
Zeiss 780 Upright Confocal Microscope



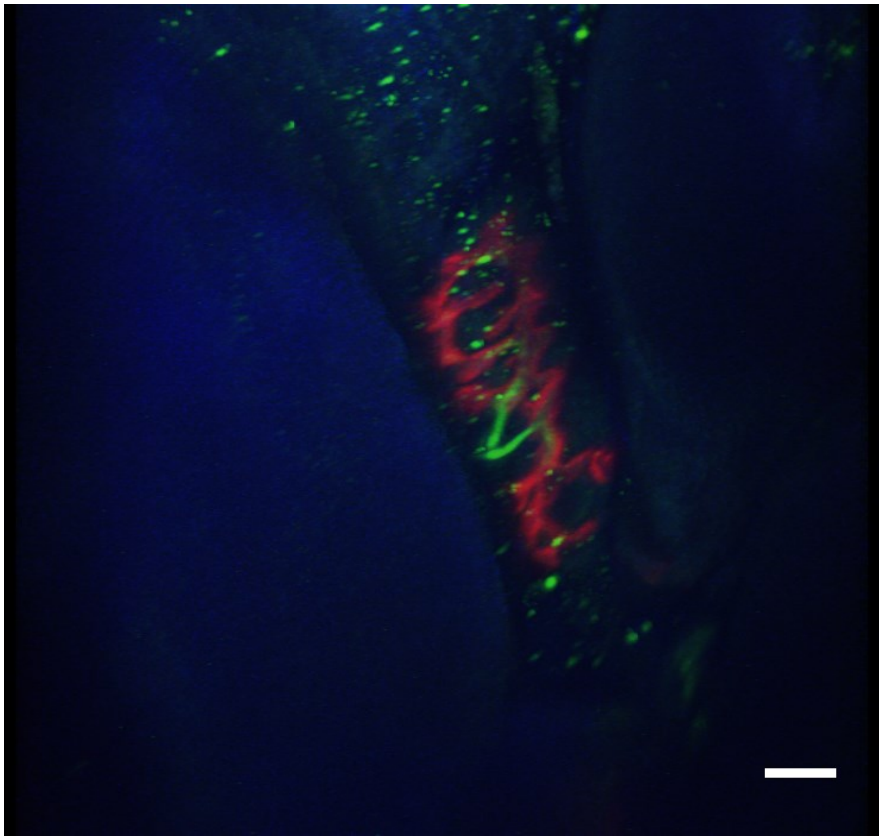
5 days old zebrafish embryo: antibody staining for cardiomyocytes (green), tcf21-expressing epicardial cells (red), muscle myosin (yellow) and raldh2 (blue)

Two-photon Microscopy of Neuromuscular Junction Endplates

Yu Cheung / Richard Webster / Susan Maxwell /Prof. David Beeson



Scale Bar = 100 μm

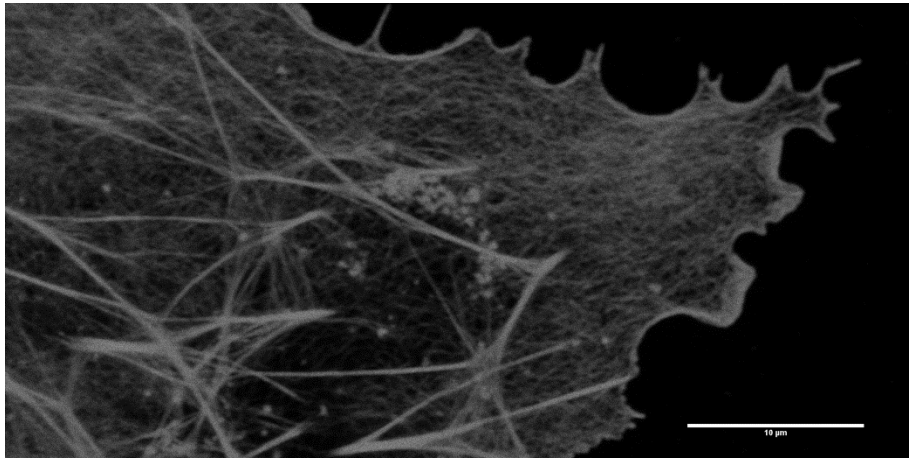


Scale Bar = 10 μm

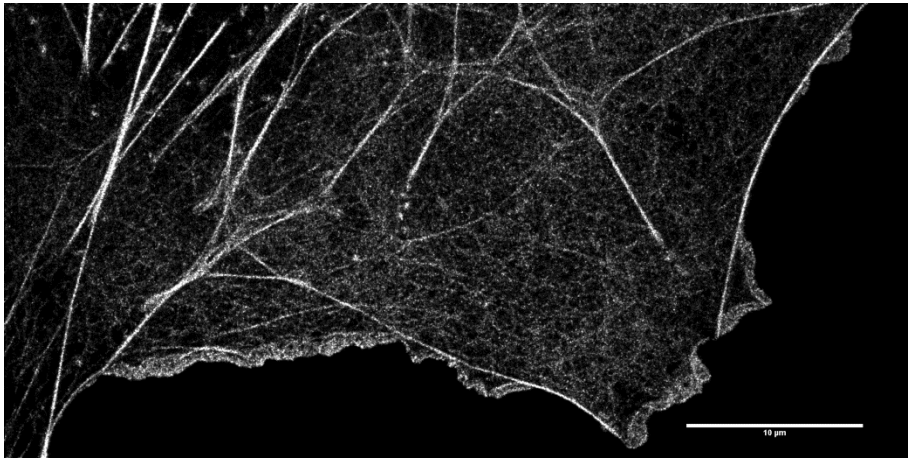
Neurofilament (Green) / Bungarotoxin/AChR (Red) / Second Harmonic Generation (Blue)

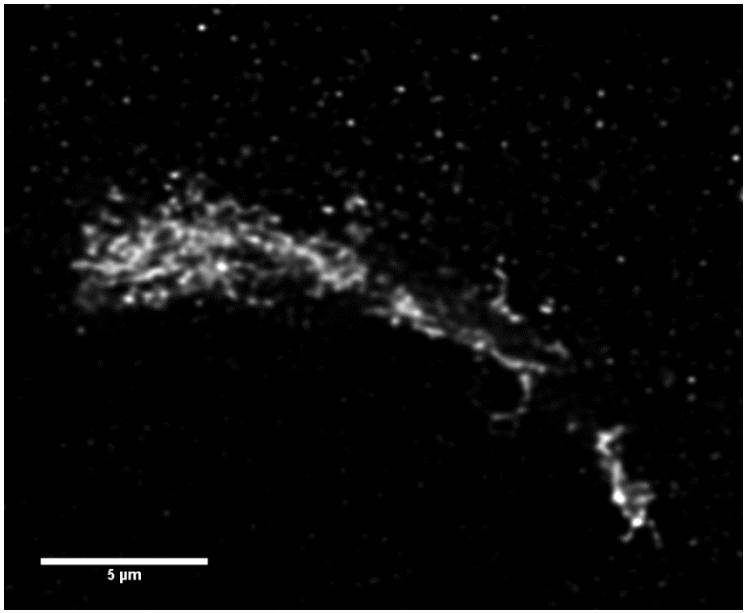
Super-resolution Comparison (STED, SIM, STORM)
Dr. Eva Wegel & Ian Dobbie, Dept. of Biochemistry

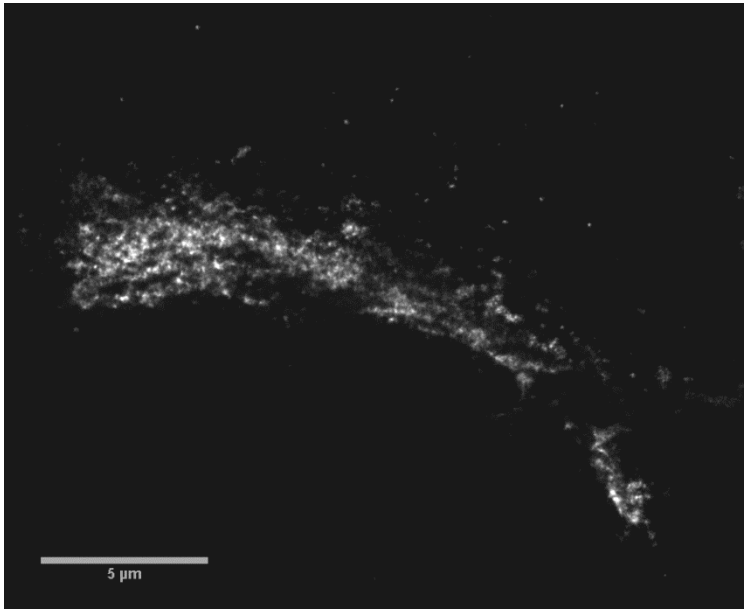
Confocal



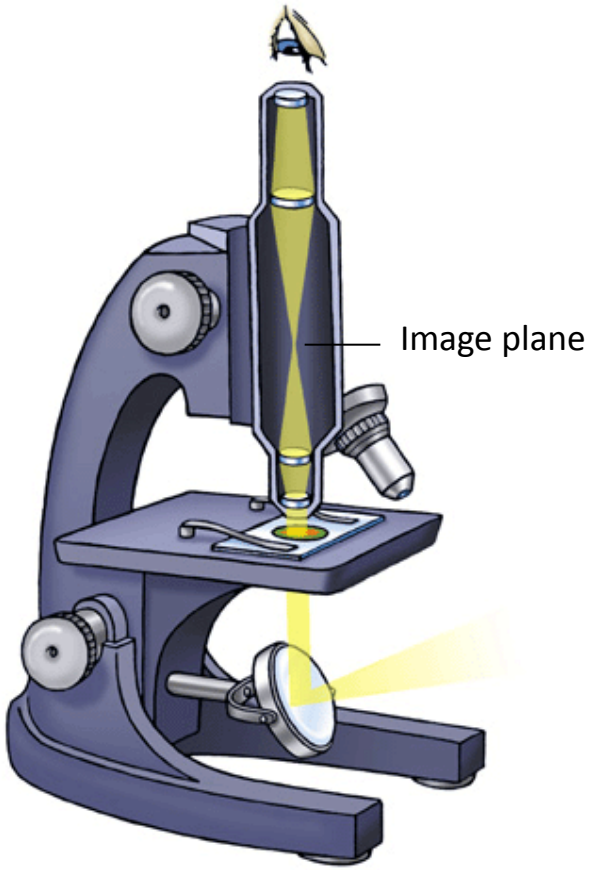
gSTED



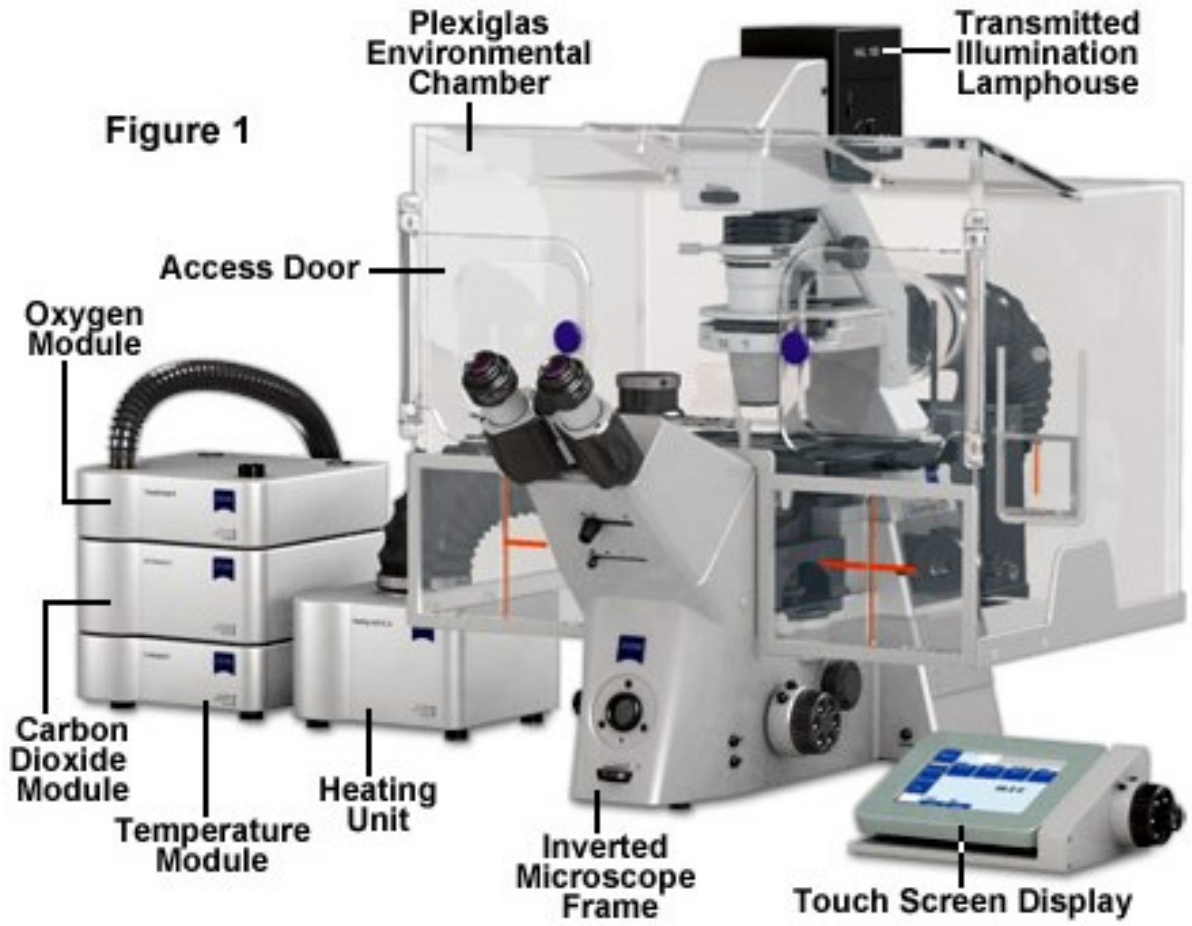




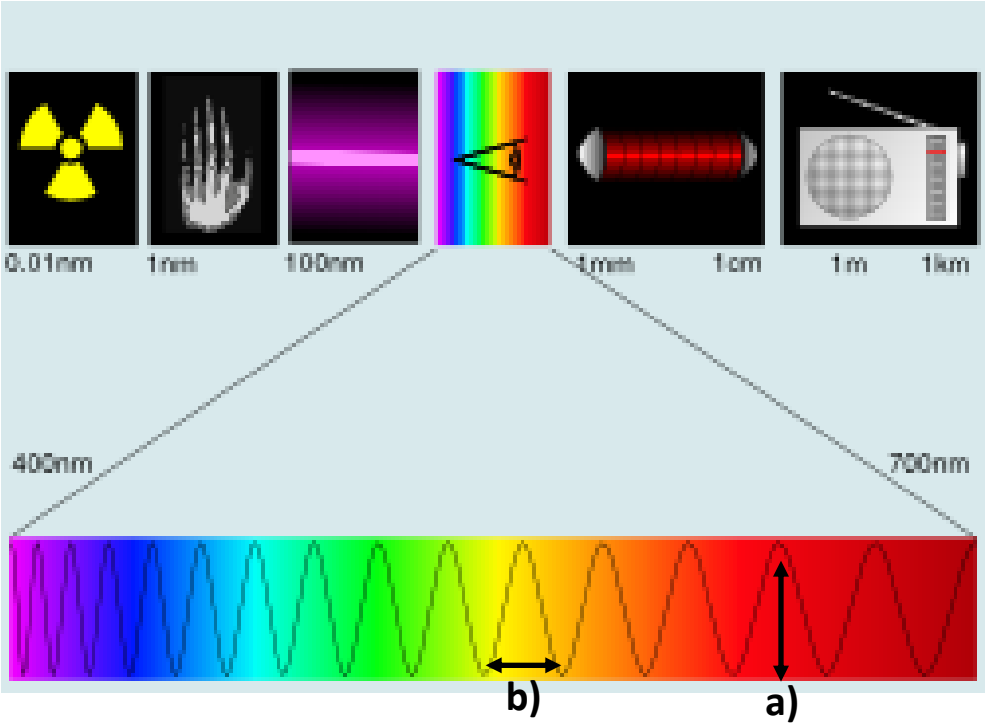
What is a microscope?



Classic compound microscope



Relevant spectra for Microscopy



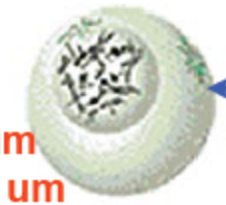
Light is electromagnetic radiation. What we usually describe as light is only the visible spectrum of this radiation with wavelengths between 400nm and 700nm.

There are 3 basic dimensions of light

a) Intensity (amplitude) which is related to the perception of brightness
b) Frequency (wavelength), perceived as colour
c) Polarization (angle of vibration) which is not or weakly perceptible to humans

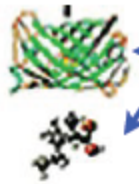
Useful size range for light microscopy

We use the light microscope to image structures and substructures within the range: from about 300 μm down to about 0.3 μm (300 nm)



Eukaryote = 10 um
>50 um

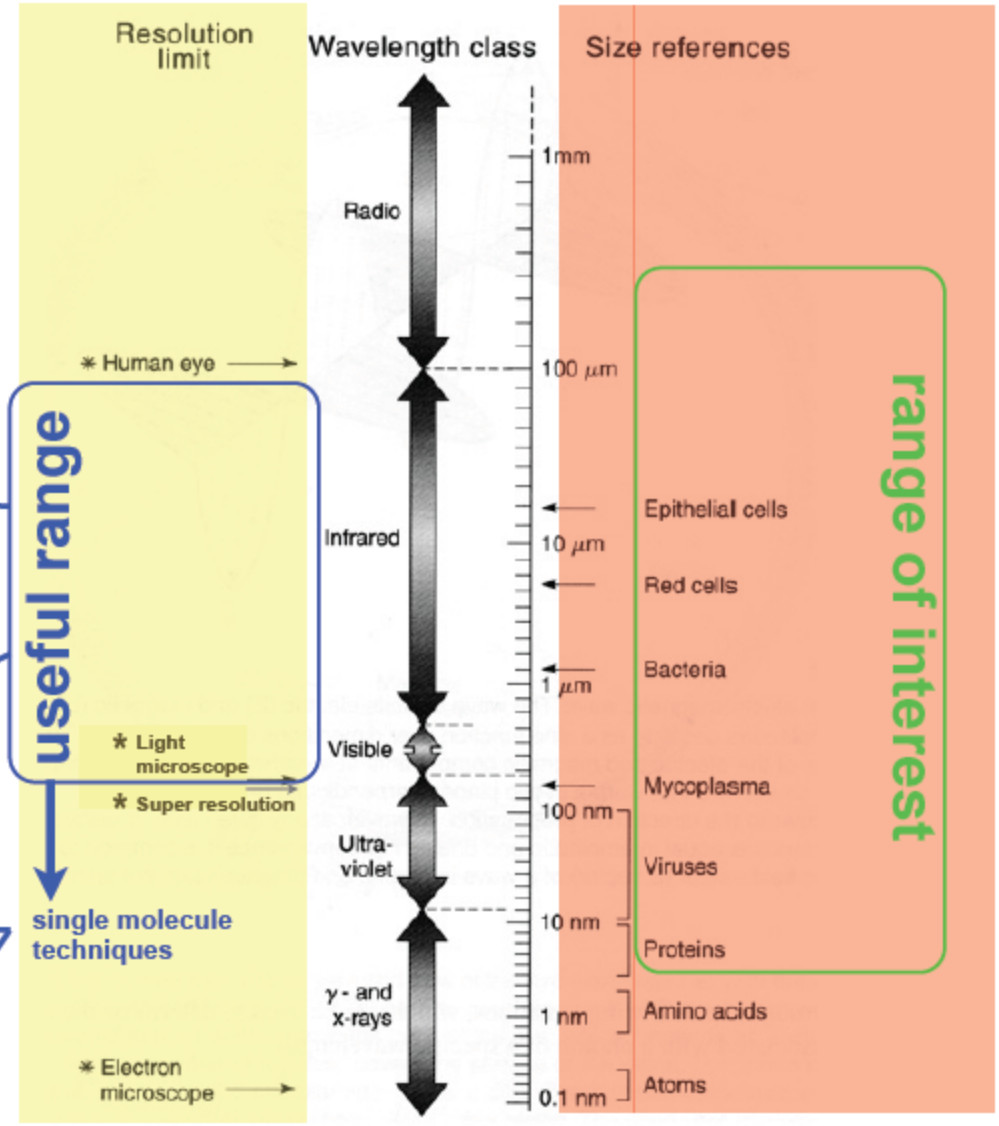
Bacterium = 1 um



Single GFP = 5 nm

Fluorescein = 1 nm

(1 nm = 10 Angstom)



Brief Review of Fluorescence

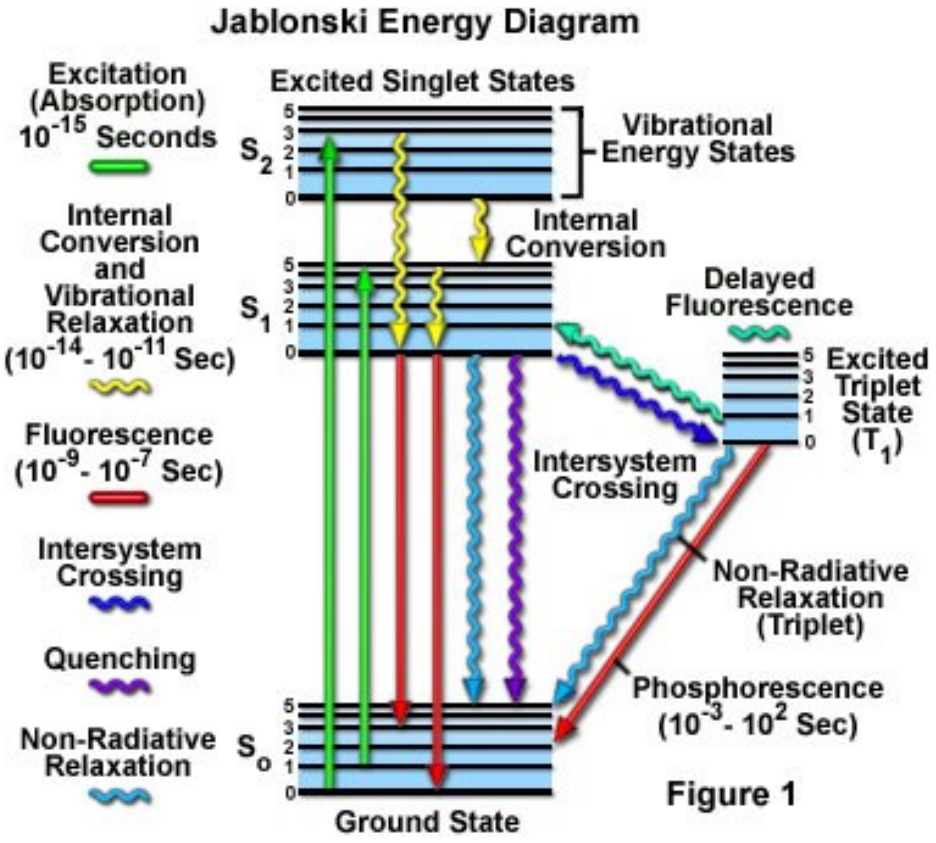
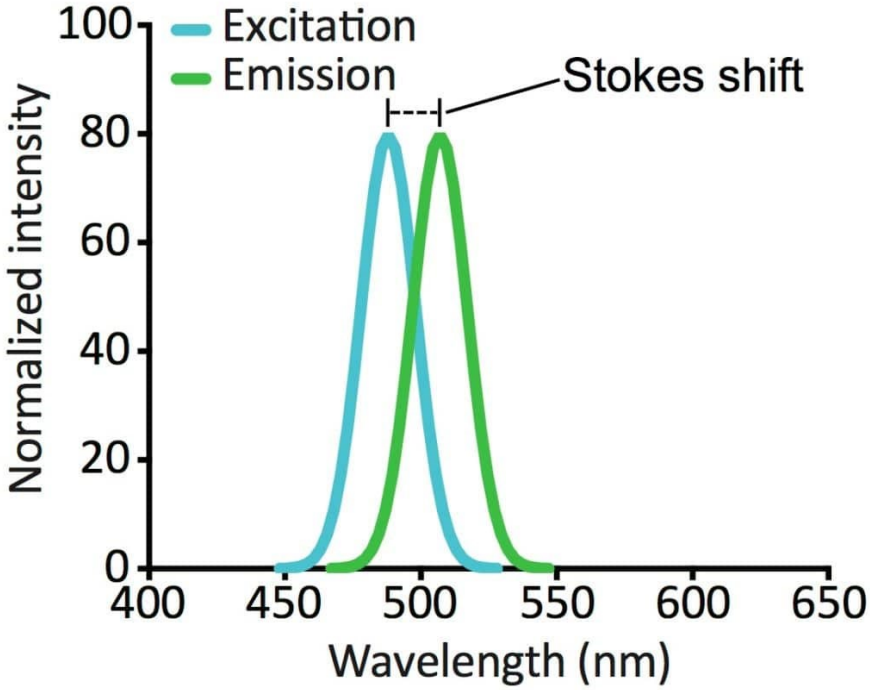
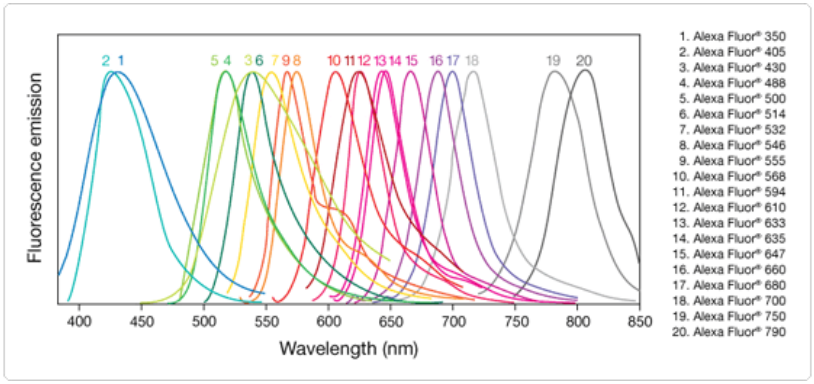


Figure 1



Spectral properties of Molecular Probes Alexa Fluor dyes



Alexa Fluor Dye	Absorption Max (nm) *	Emission Max (nm) *	Emission Color †	Extinction Coefficient ‡
Alexa Fluor 350	346	442	Blue	19,000
Alexa Fluor 405	402	421	Blue	35,000
Alexa Fluor 430	434	539	Yellow-green	15,000
Alexa Fluor 488	495	519	Green	73,000
Alexa Fluor 514	518	540	Green	80,000
Alexa Fluor 532	531	554	Yellow	81,000
Alexa Fluor 546	556	573	Orange	112,000
Alexa Fluor 555	555	565	Orange	155,000
Alexa Fluor 568	578	603	Red-orange	88,000
Alexa Fluor 594	590	617	Red	92,000
Alexa Fluor 610	612	628	Red	144,000
Alexa Fluor 633	632	647	Far-red	159,000
Alexa Fluor 635	633	647	Far-red	140,000
Alexa Fluor 647	650	668	Far-red	270,000
Alexa Fluor 660	663	690	Near-IR §	132,000
Alexa Fluor 680	679	702	Near-IR §	183,000
Alexa Fluor 700	702	723	Near-IR §	205,000
Alexa Fluor 750	749	775	Near-IR §	290,000
Alexa Fluor 790	782	805	Near-IR §	260,000

* Approximate absorption and emission maxima, in nm, for conjugates.
† Typical emission color seen through the eyepiece of a conventional fluorescence microscope with appropriate filters.
‡ Extinction coefficient of the reactive dye at emission maximum in $cm^{-1}M^{-1}$.
§ Human vision is insensitive to light beyond ~650 nm; it is not possible to directly view far-red– and near-IR–fluorescent dyes.

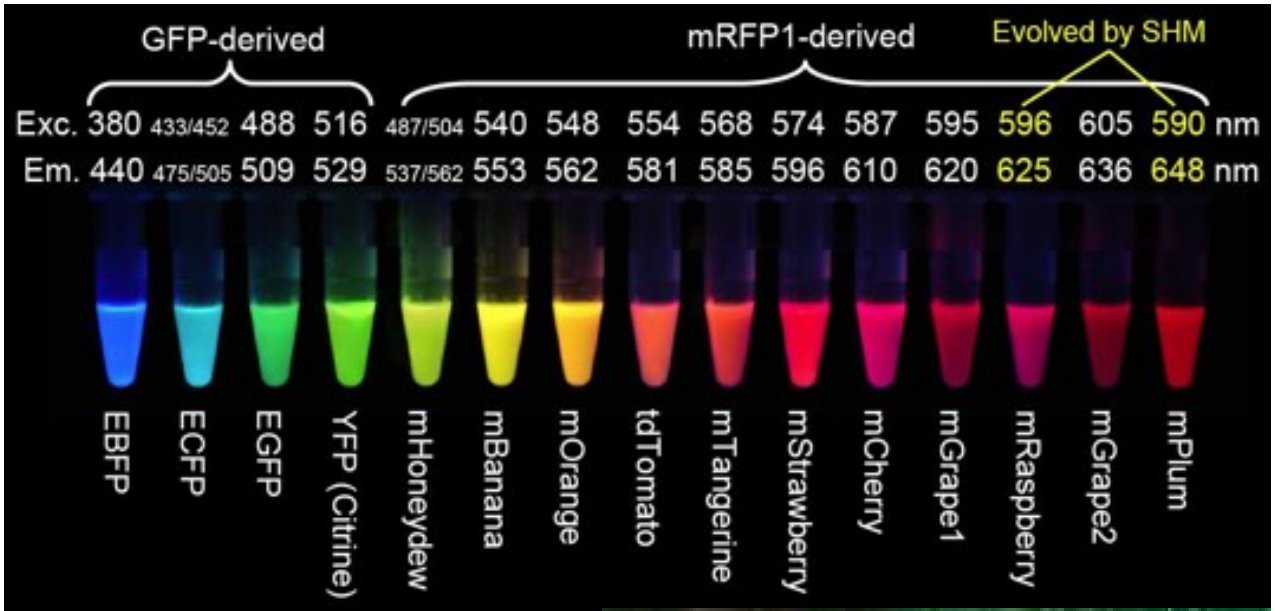
Fluorescence quantum yields (QY) and lifetimes (τ) for Alexa Fluor dyes—Table 1.5

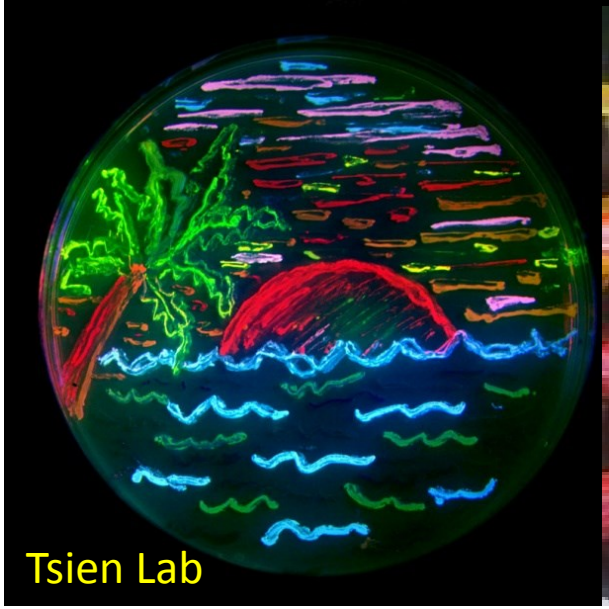
Alexa Fluor Dye *	QY †	τ (ns) ‡
Alexa Fluor 488	0.92	4.1 §
Alexa Fluor 532	0.61	2.5
Alexa Fluor 546	0.79	4.1
Alexa Fluor 555	0.10	0.3
Alexa Fluor 568	0.69	3.6 §
Alexa Fluor 594	0.66	3.9 §
Alexa Fluor 647	0.33	1.0
Alexa Fluor 660	0.37	1.2 **
Alexa Fluor 680	0.36	1.2
Alexa Fluor 700	0.25	1.0
Alexa Fluor 750	0.12	0.7

* Measurements were made on free succinimidyl ester derivatives in aqueous solutiuons. † For Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, Alexa Fluor 594 and Alexa Fluor 647 dyes, QY measurements were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to fluorescein in 0.01 M NaOH (QY = 0.92). For Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 700 and Alexa Fluor 750 dyes, QY measurements were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to Alexa Fluor 647 succinimidyl ester in PBS (QY = 0.33). ‡ Except for the footnoted values, lifetime measurements were made in water at 22°C, data provided by ISS Inc. (Champaign, IL). § Lifetime measurements were provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc. ** Lifetime measurement was made in pH 7.5 buffer at 20°C by Pierre-Alain Muller, Max Planck Institute for Biophysical Chemistry, Göttingen.

Fluorescent proteins

(Nobel Prize in Chemistry in 2008 to Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien)





The US company Yorktown Technologies markets to aquarium shops green fluorescent zebrafish (GloFish) that were initially developed to detect pollution in waterways.(not allowed in the EU)

The importance of understanding the microscope objective

- Magnification
- Resolution
- Abberations
- Working distance

The microscope objective

Labeling of the Objective
Objective class, special designations are used for this, e.g.
LD for Long Working Distance

Magnification / Numerical Aperture
plus additional details on
- immersion medium (Oil /W/ Glyc)
- adjustable cover glass correction (Korr.)
- contrast method

Tube Length / Cover Glass Thickness (mm)
ICS optics: ∞
Infinity Color Corrected System
standard cover glass: 0.17
without cover glass: 0
insensitive: -

Mechanical Correction Collar
- cover glass thickness correction
- different immersion
- different temperature
- adjusting an iris diaphragm

Color of writing
Contrast method

Standard	
Pol / DIC	
Ph 0 1 2 3	

Color Coding of Magnification

1.0/1.25	
2.5	
4/5	
6.3	
10	
16/20/25/32	
40/50	
63	
100/150	

Immersion Fluid

Oil	
Water	
Glycerin	
Oil /Water / Glycerin	

Apo- Aberration correction
Plan- Flat field Correction

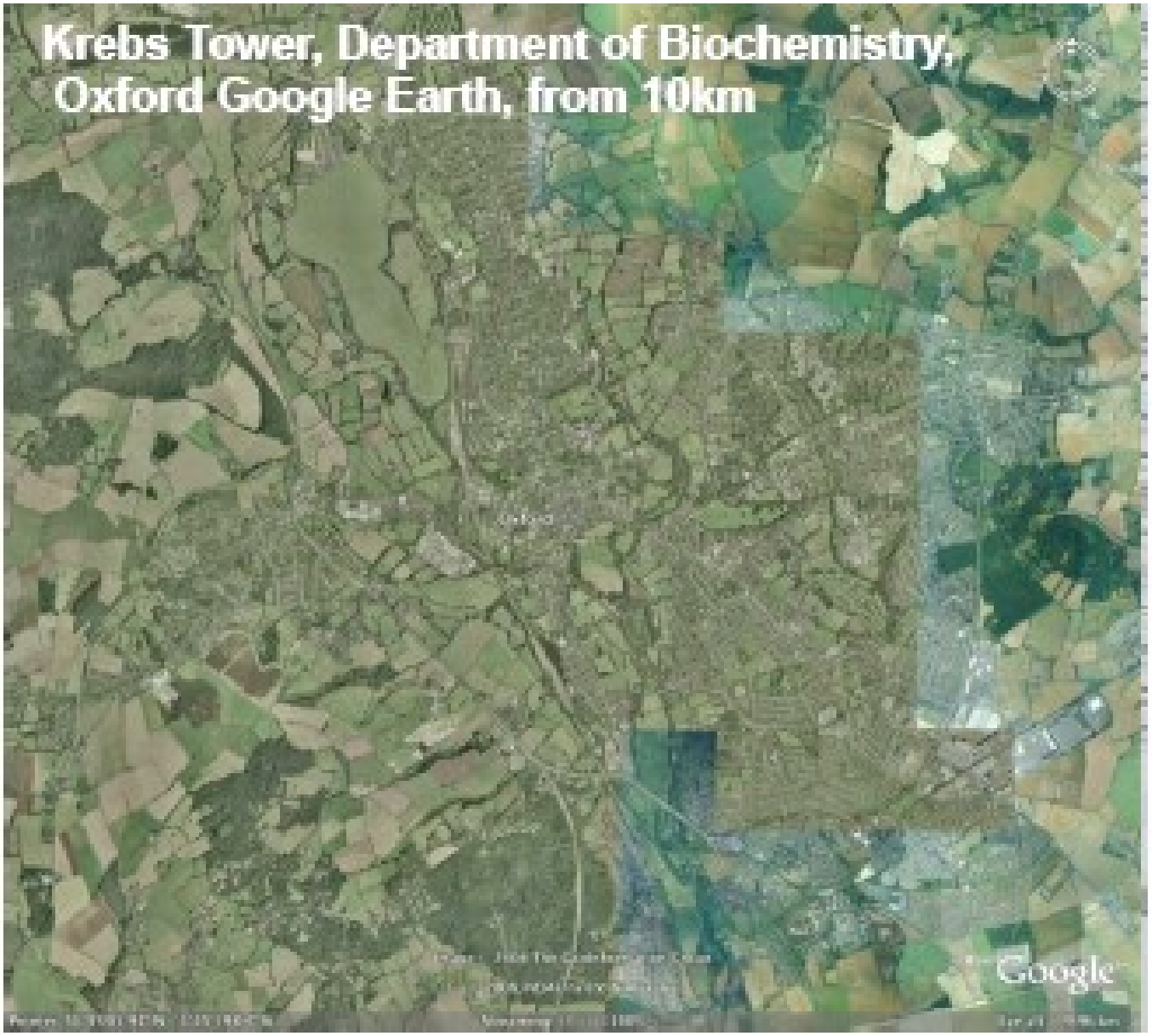
What is magnification?

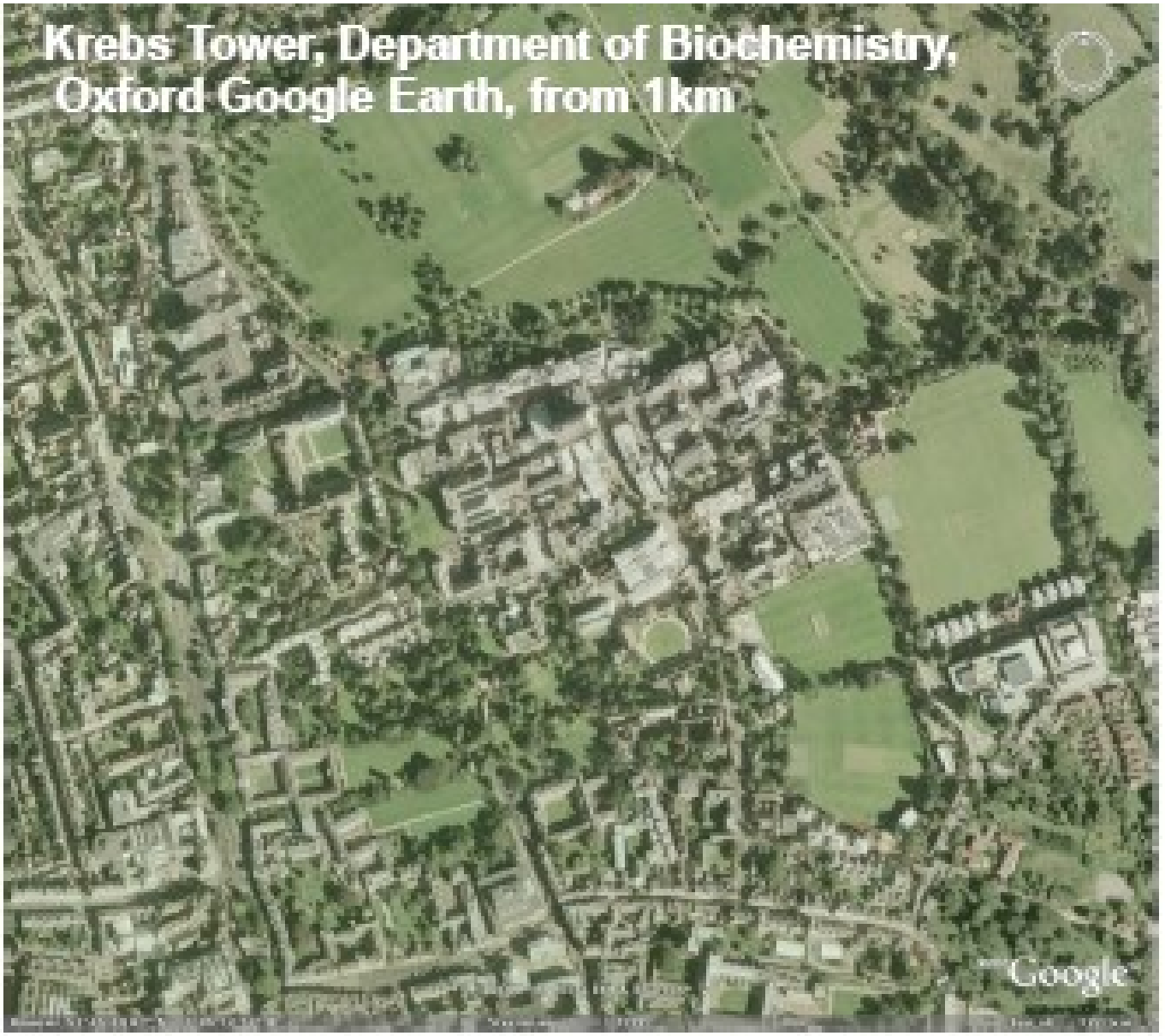
Magnification is defined by the

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

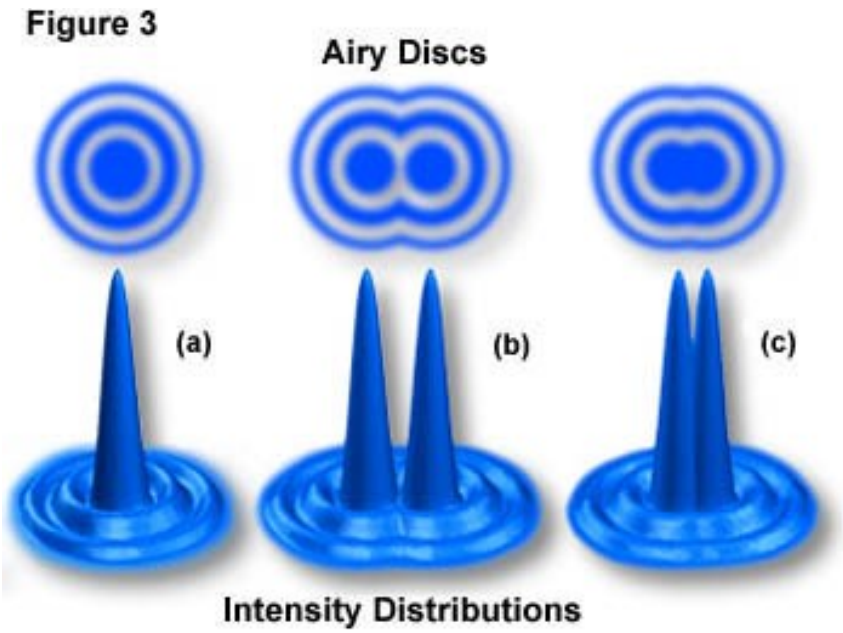

Krebs Tower, Department of Biochemistry, Oxford Google Earth, from 100m
Google


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


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

What is resolution?

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

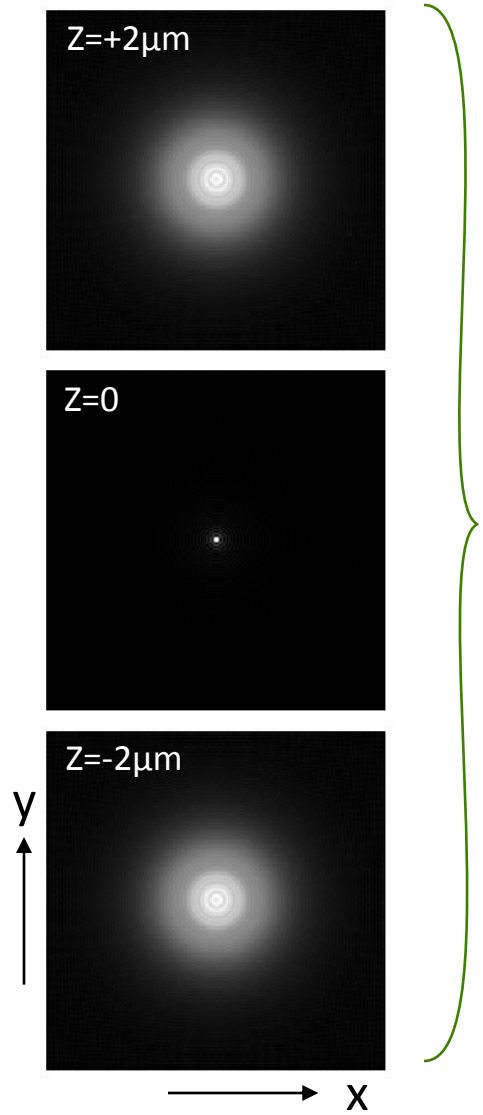


Picture taken from http://microscopy.fsu.edu/primer/anatomy/numaperture.html

The Point Spread Function (PSF)

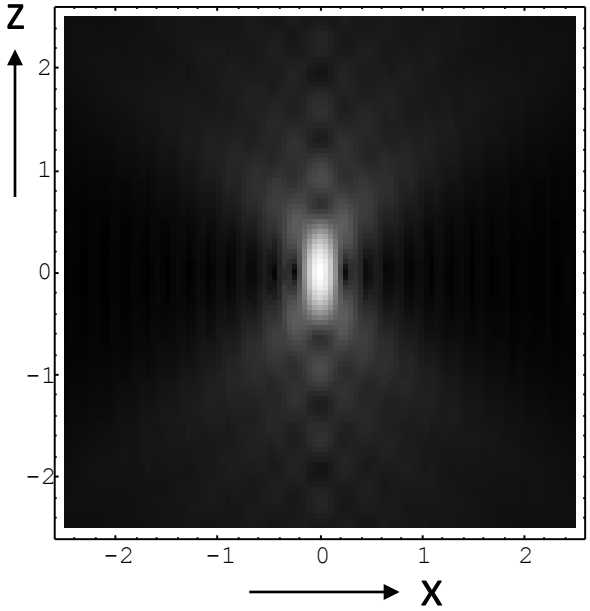
The image of a point object

2D PSF
for different defocus

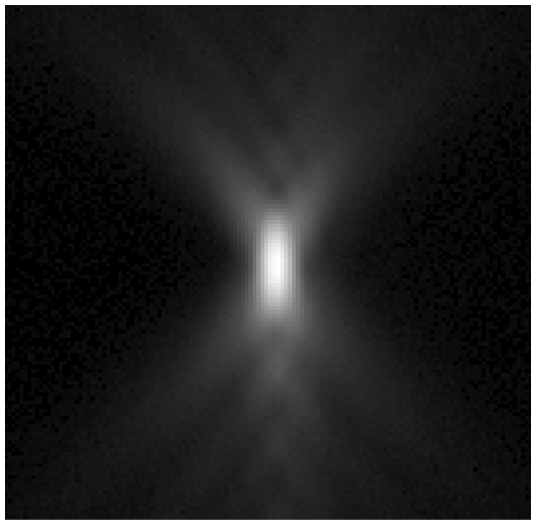


3D PSF

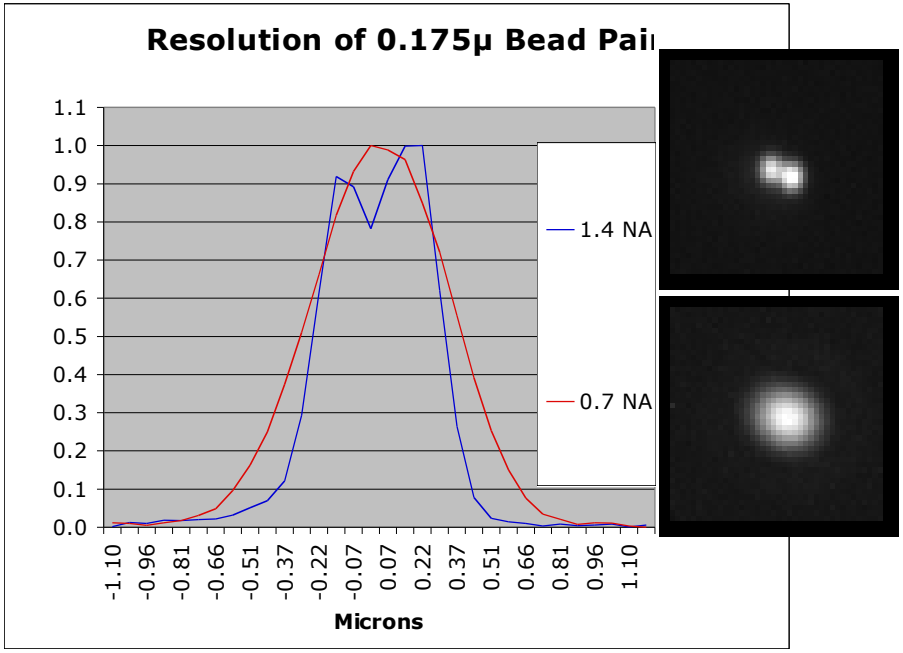
Calculated



Measured



Numerical aperture, NOT magnification limits resolution!



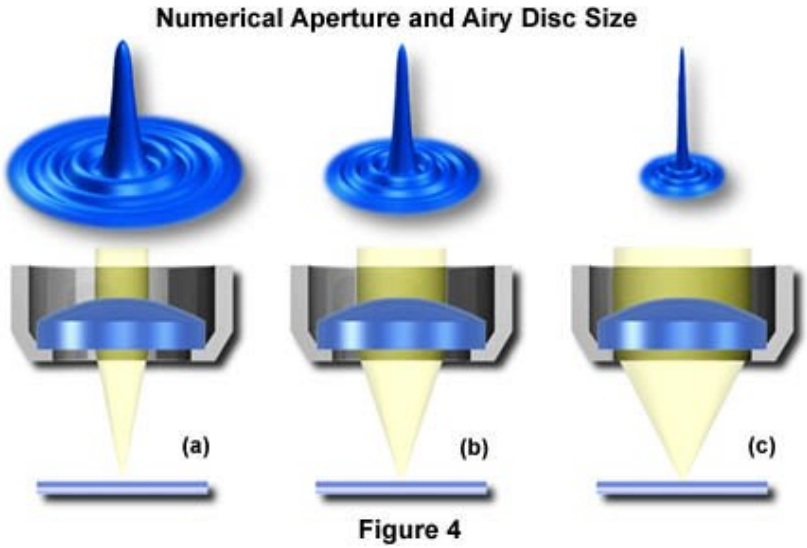
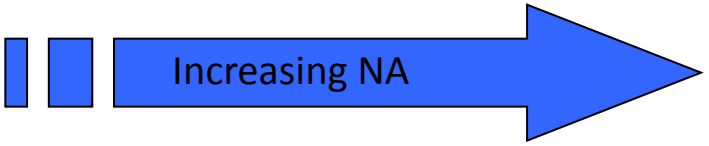


Figure 4



The numerical aperture (NA) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light. The term is used in microscopy to describe the acceptance cone of an objective (and hence its light-gathering ability and resolution)

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.)

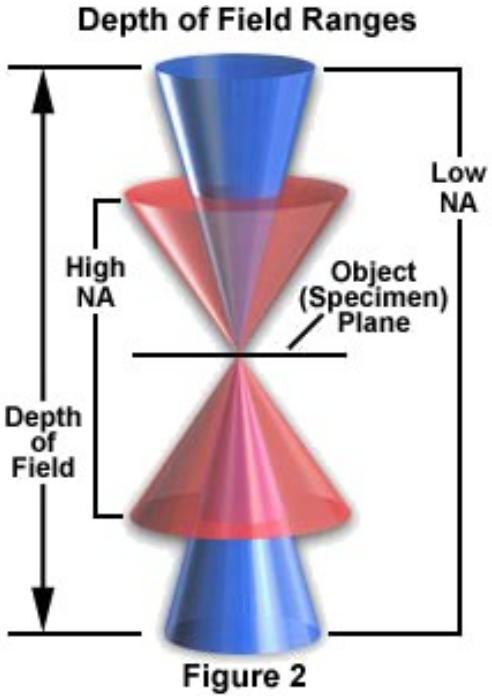


Figure 2

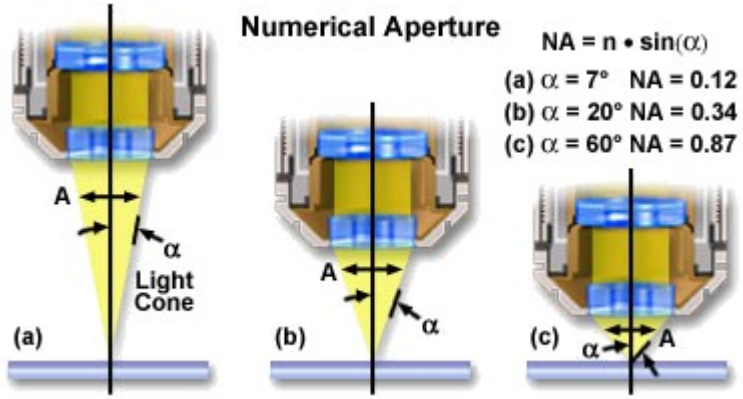


Figure 1

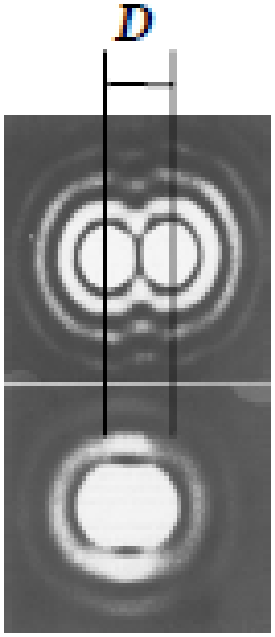
Numerical Aperture = N.A. = n · sin α

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)

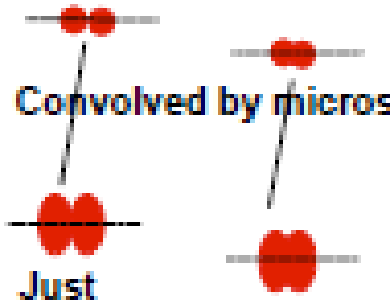
Calculating Lateral Resolution: The Rayleigh Criterion



D = the distance between the two closest points that can still be distinguished

$$D = 1.22\,\lambda/(NA_{obj}+NA_{cond})$$

Epi-Fluorescence: $NA_{cond} = Na_{obj}$



Just resolved

Not resolved
Airy patterns must
Overlap by less
Than 42% of the
Maximal intensities

Limit of resolution $\approx \lambda_{em}/2$

Axial Resolution:

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)

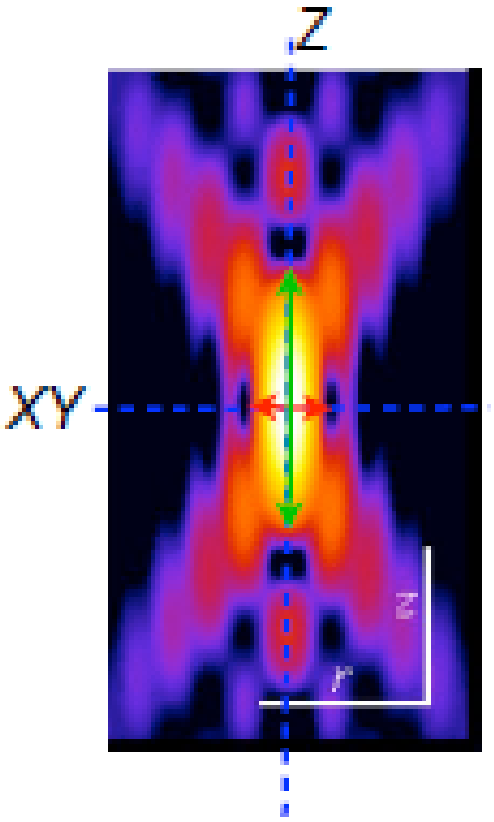
Than it is in the lateral dimension (XY)

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

FWHM

The relationship between the two is:

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



More from the objective

Objectives can be classified into transmitted light and reflected-light (Epi) versions.

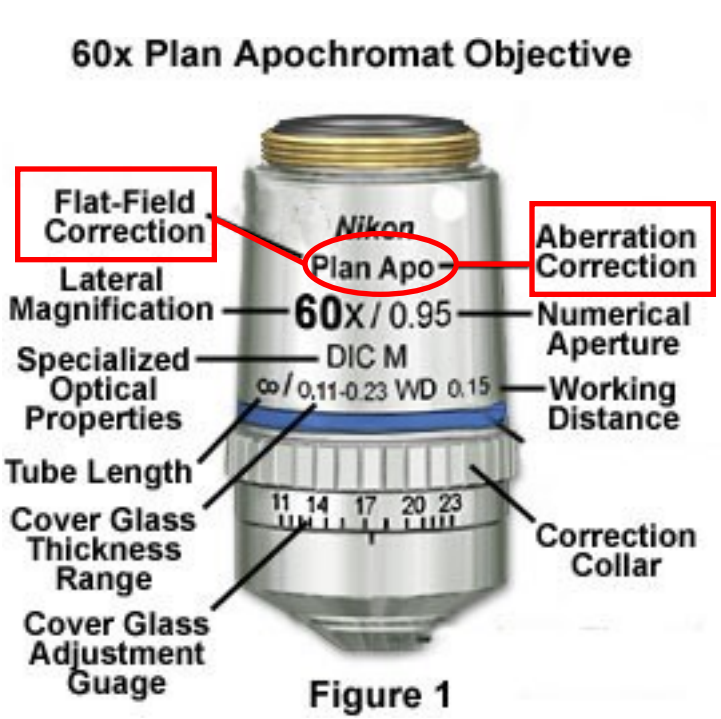


Figure 1

Figure 1

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

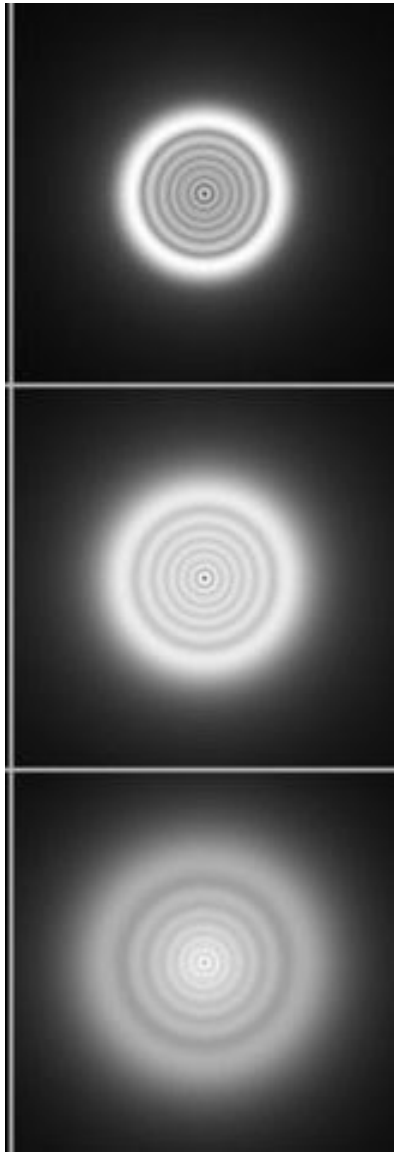
  Improved Achromat objectives with good image flatness for fields of view with dia. 20 or even 23 mm. Achroplan for transmitted light and Achroplan Ph for phase contrast.

- ## 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.

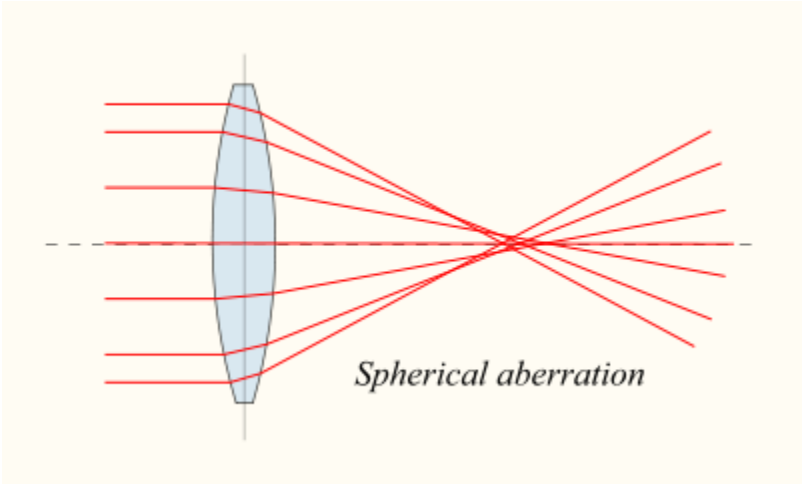
- ## Plan-Apochromat

  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.

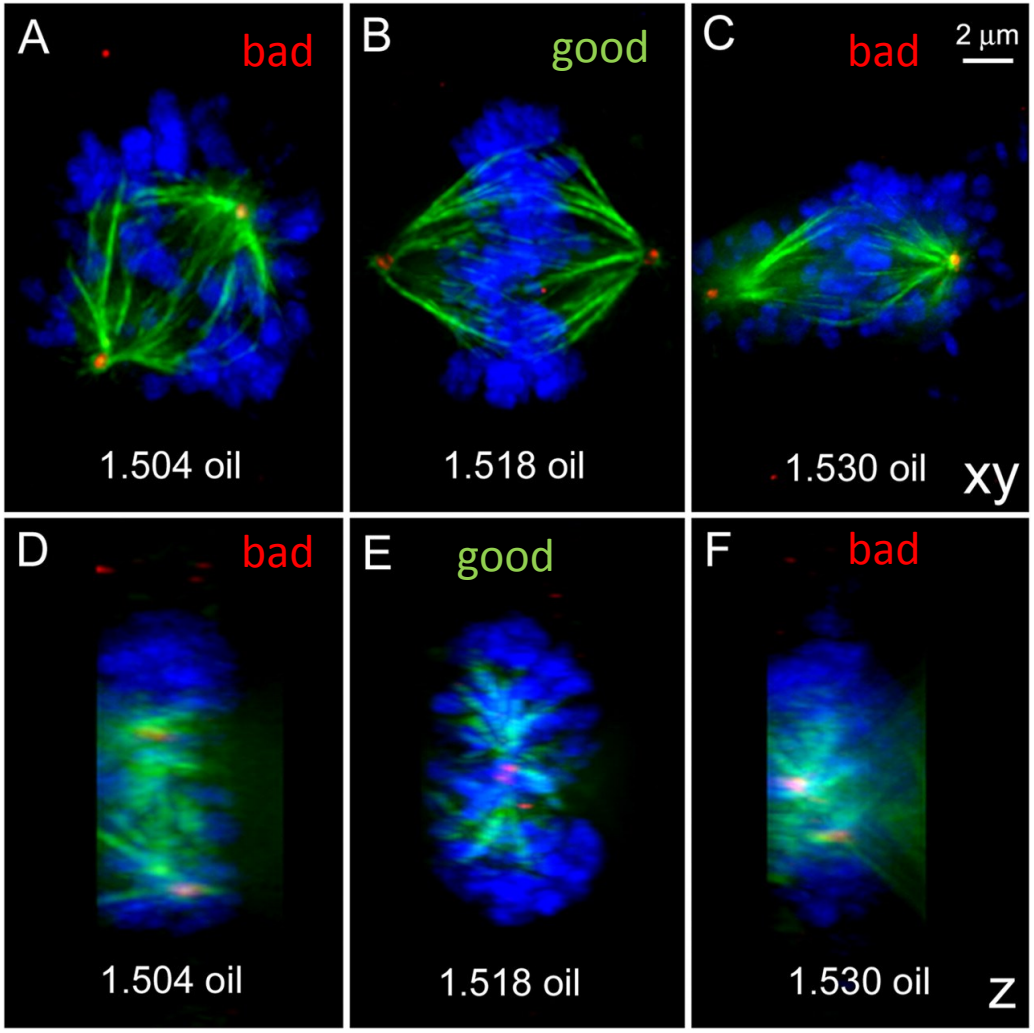


Spherical aberration

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.



Mitotic spindles of HeLa cells were stained for microtubules (green), centrosomes (red), and chromosomes (blue)

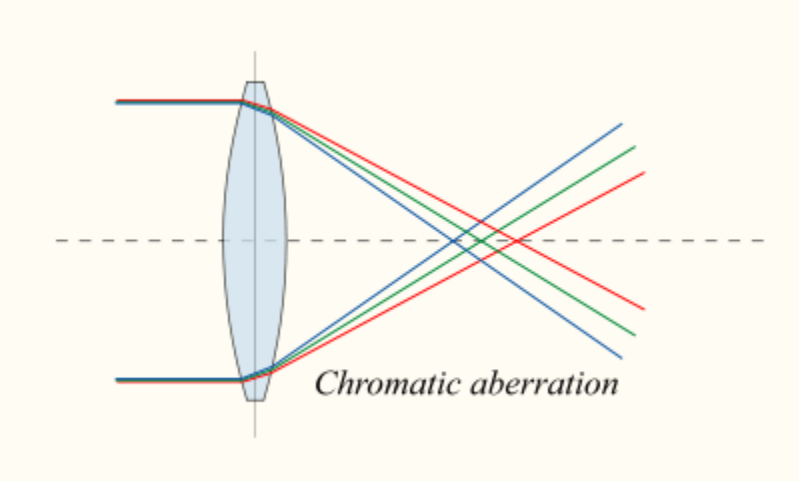
North A. JCB 2006;172:9-18

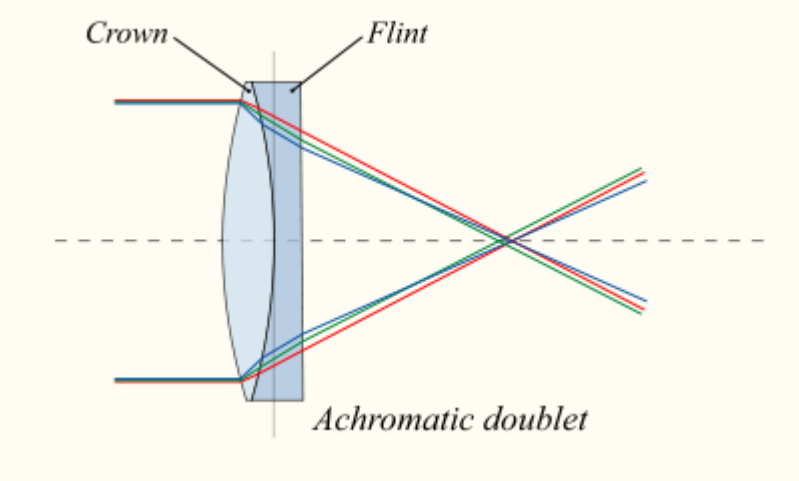
Oils of different refractive index were used to demonstrate the spherical aberrations caused by mismatch between lens immersion medium and sample.

JCB

Chromatic aberration

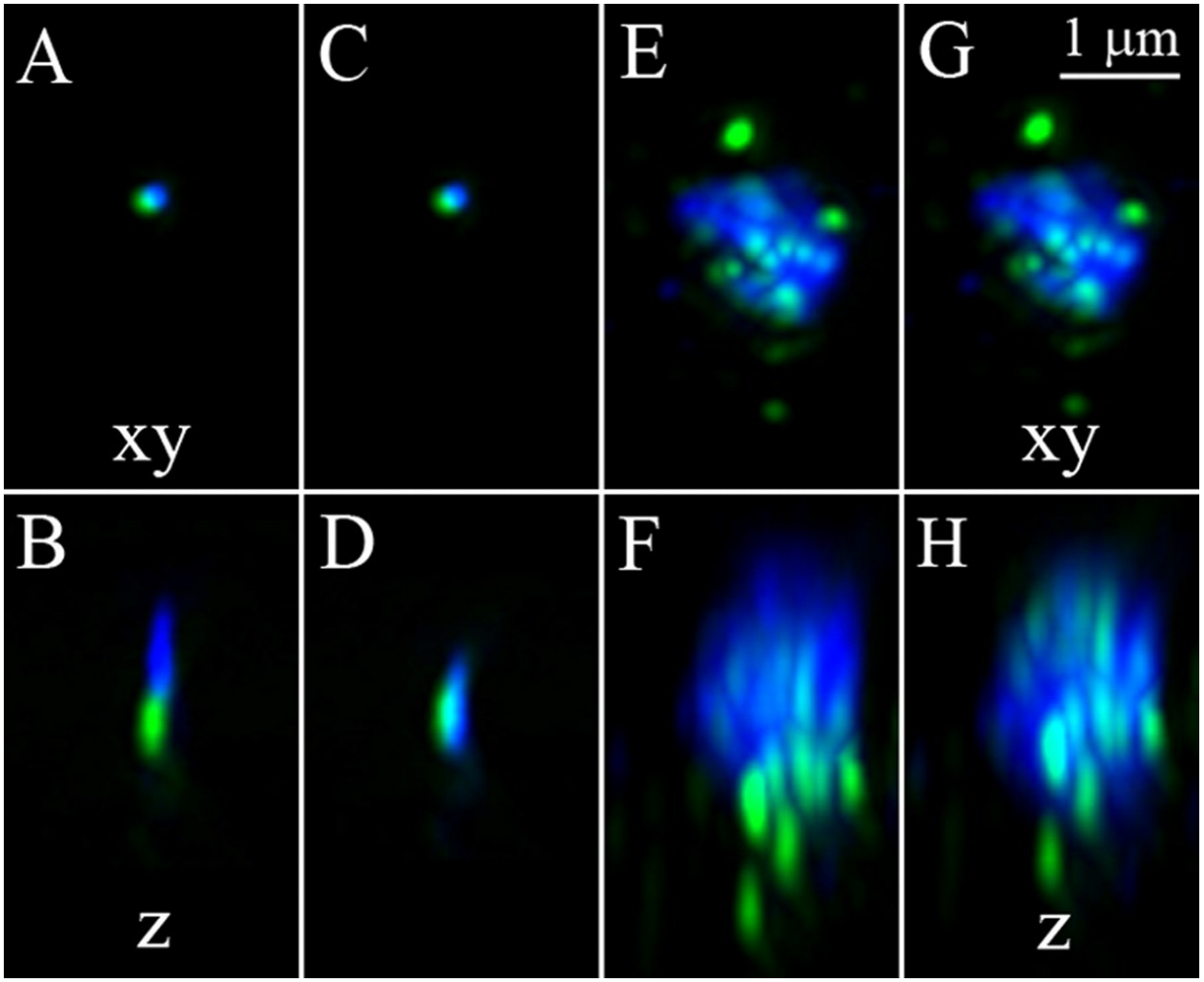
Chromatic aberration is caused by a lens having different refractive indexes for different wavelengths. Since the focal length of a lens is dependent on the refractive index, different wavelengths will be focused on different positions in the focal plane. Chromatic aberration is seen as fringes of colour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

60x Plan Apochromat Objective

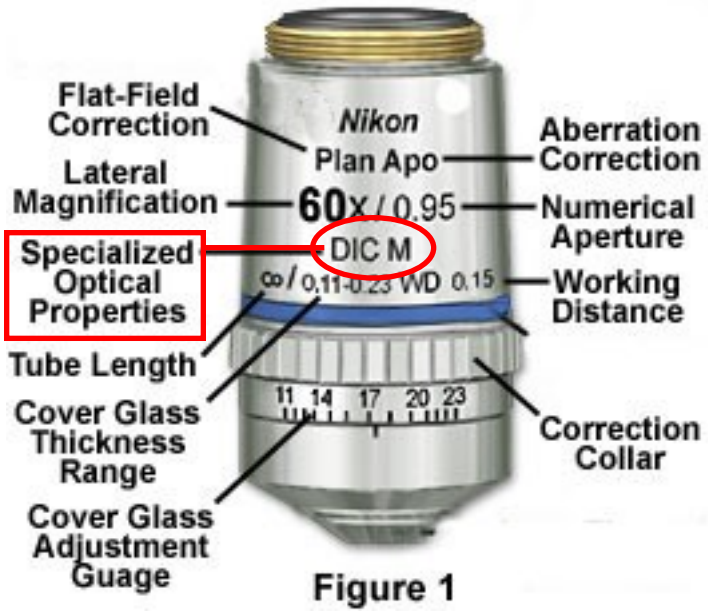


Figure 1

60x Plan Apochromat Objective

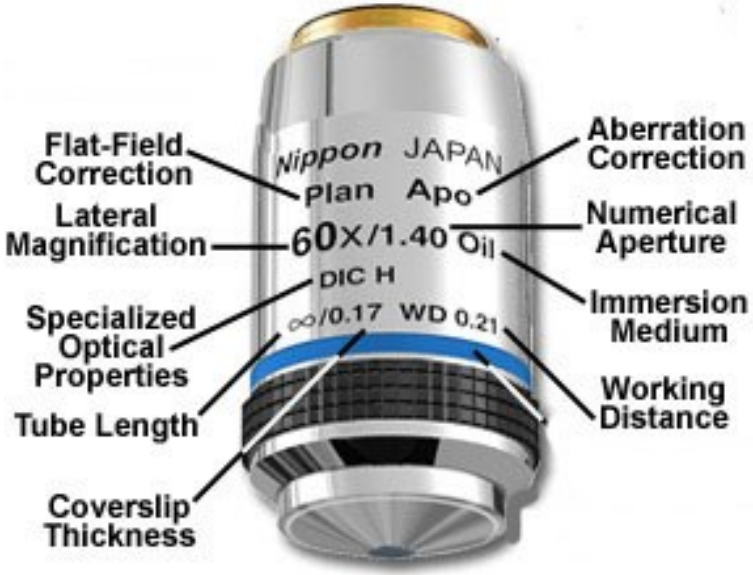


Figure 1

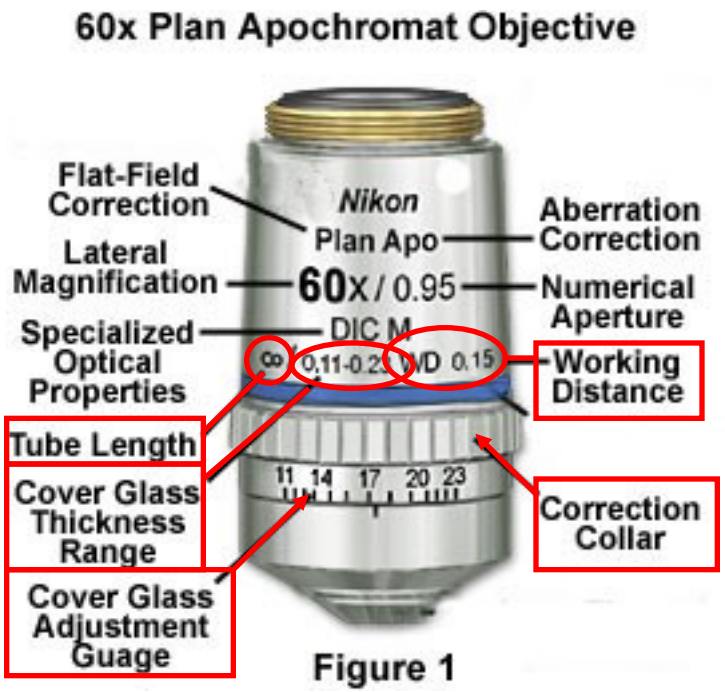
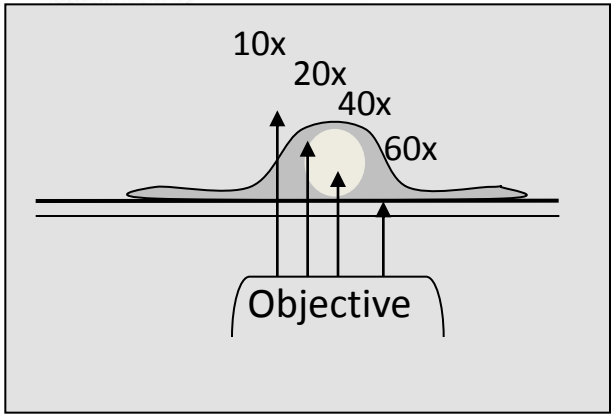


Figure 1

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



Summary - Objectives

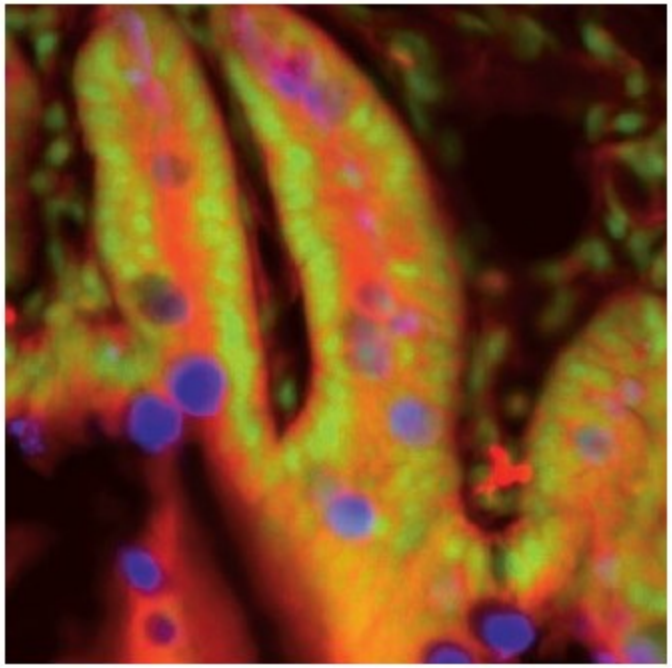
- Know your objectives (Magnification, NA, Immersion media)

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

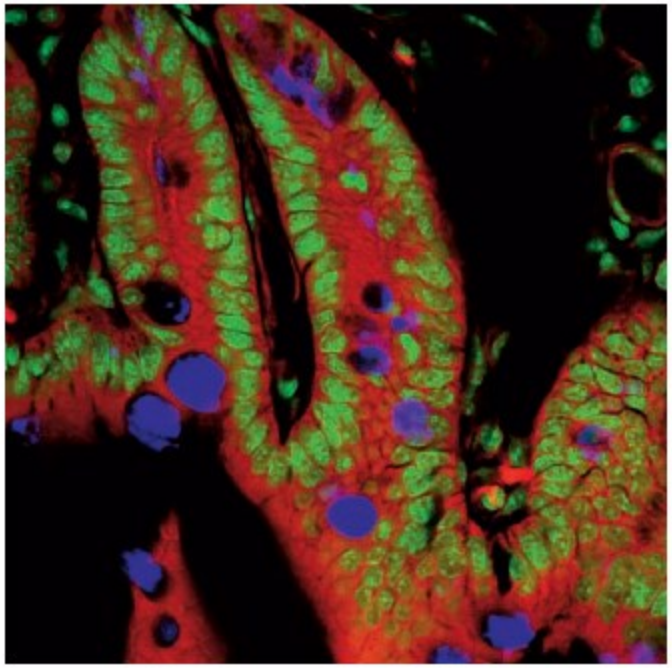
- Be very careful with the objectives!

Confocal Microscopy

Wide-field Microscopy

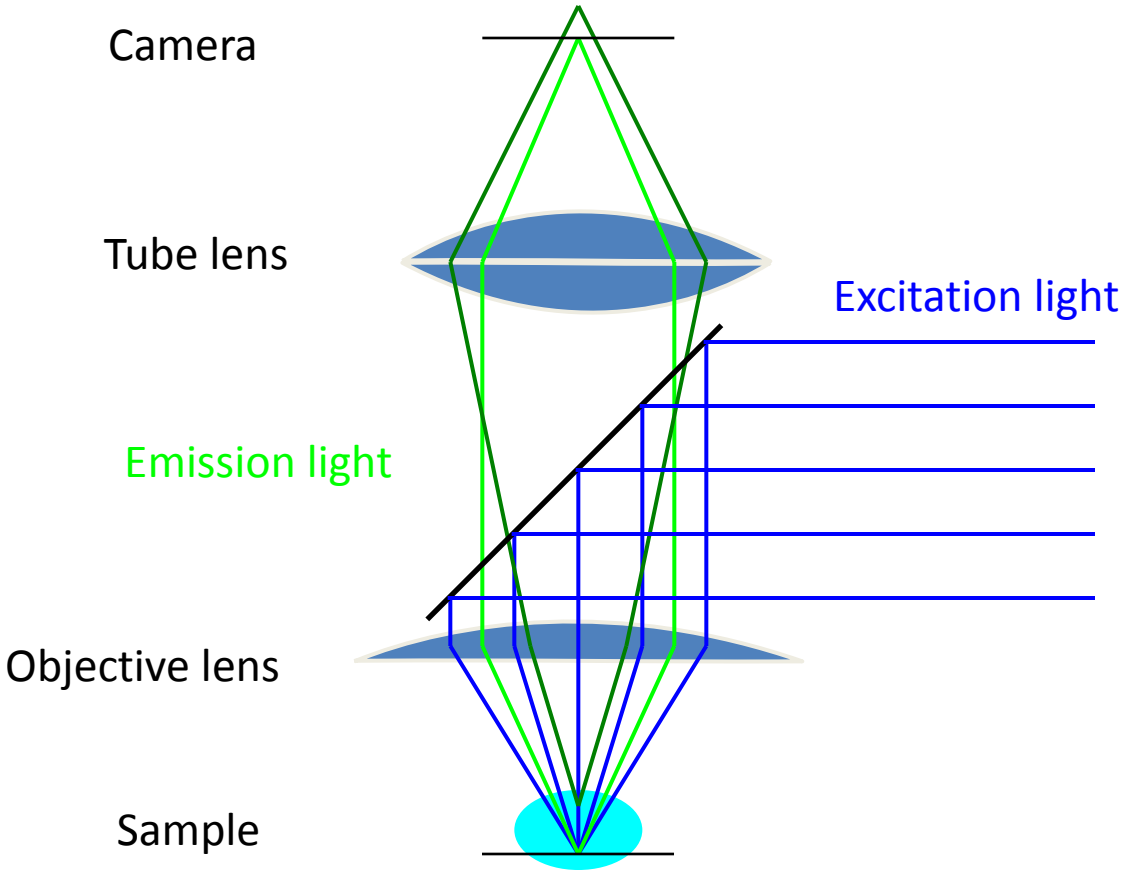


Laser Scanning Confocal Microscopy



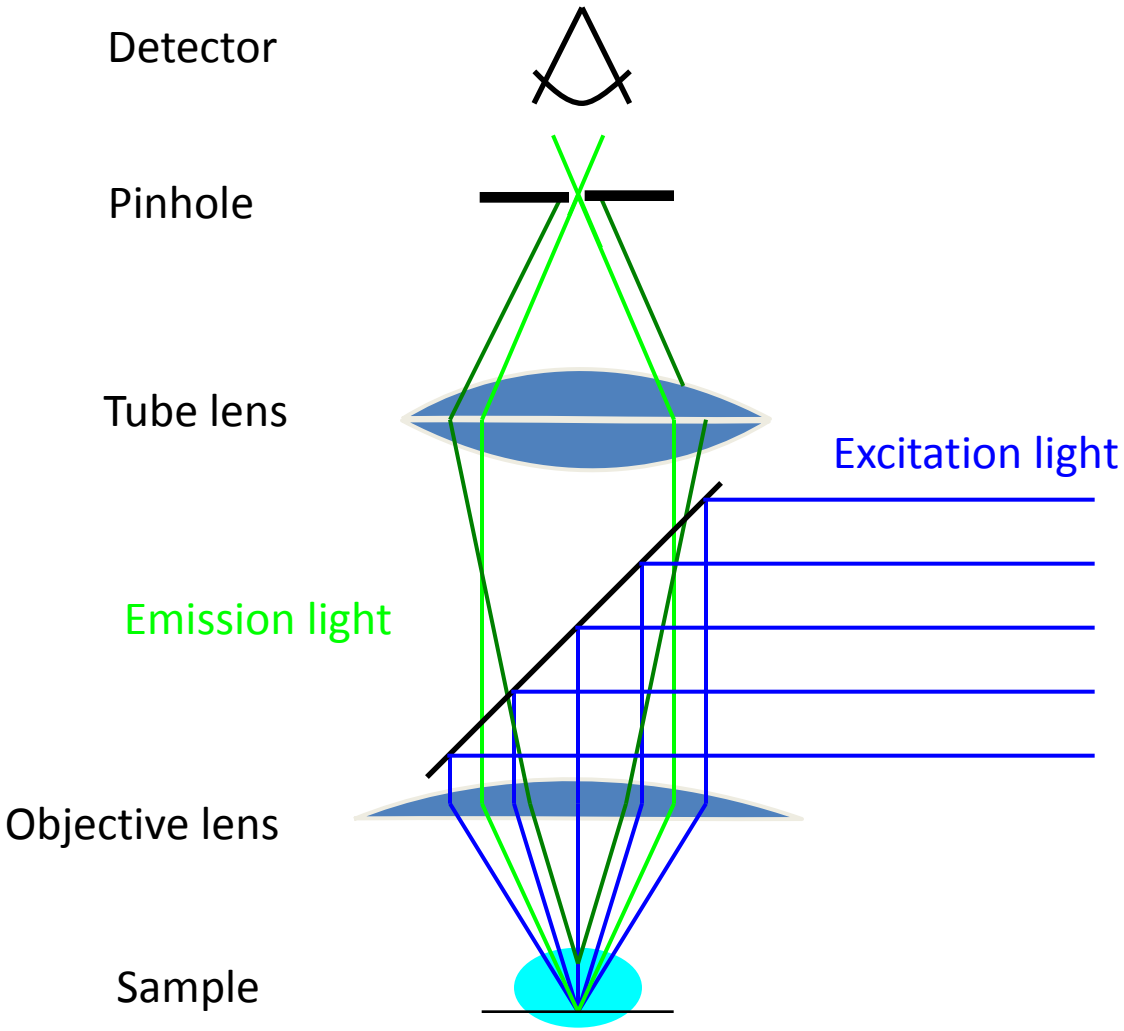
Triple-labeled cell aggregate in mouse intestine section
(Carl Zeiss. Confocal Laser Scanning Microscopy)

Wide-field Microscopy



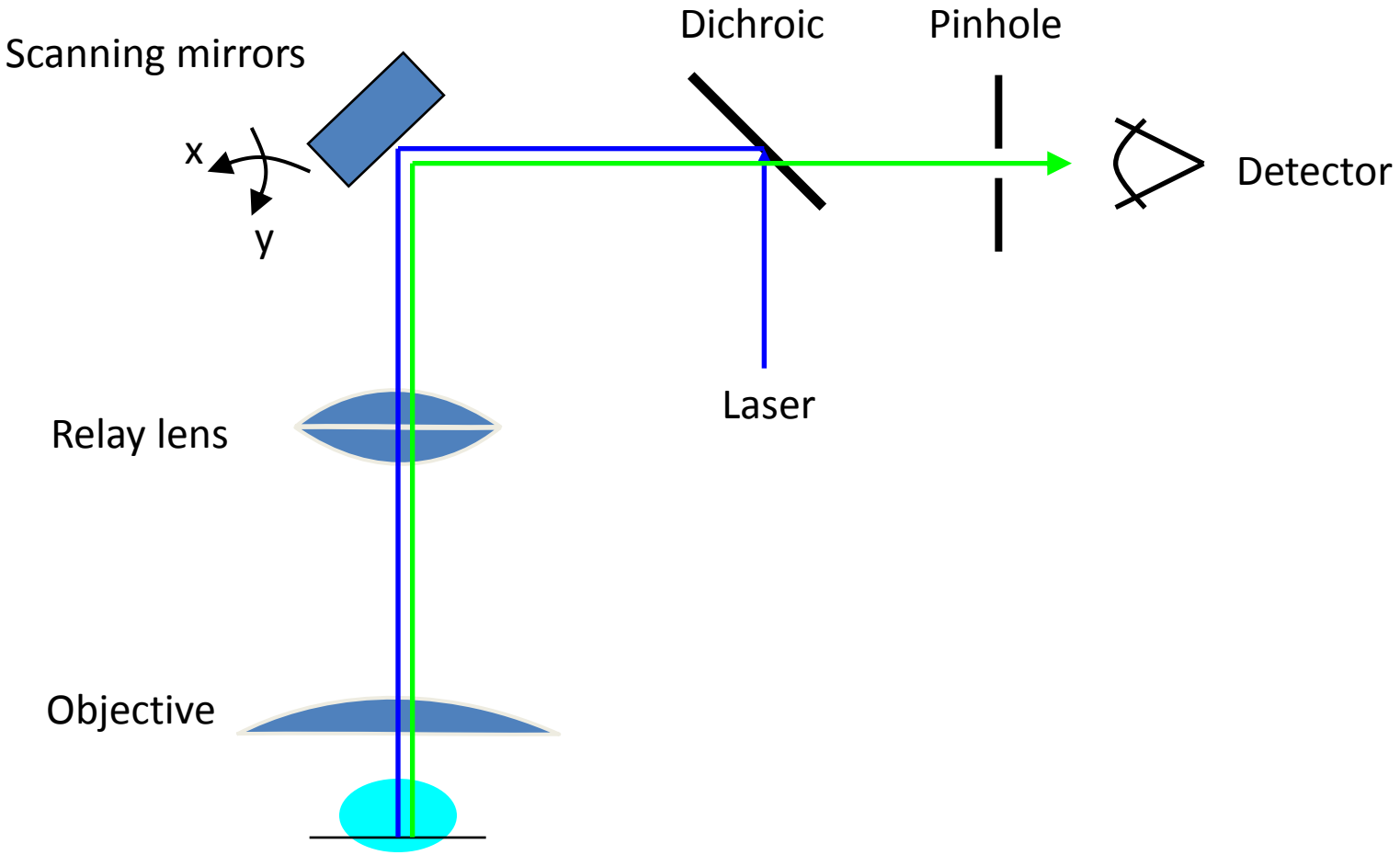
Problem – fluorescence is emitted along entire illuminated cone, not just at focus

The confocal microscope



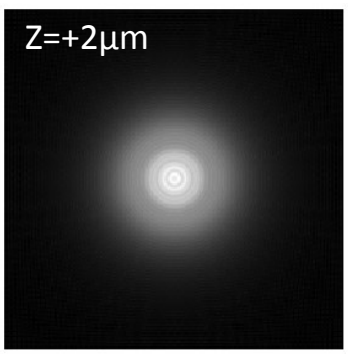
Scan excitation spot point-by-point to build up image

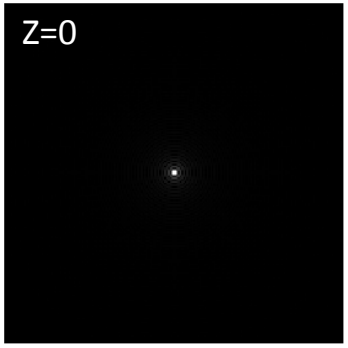
Confocal optical path

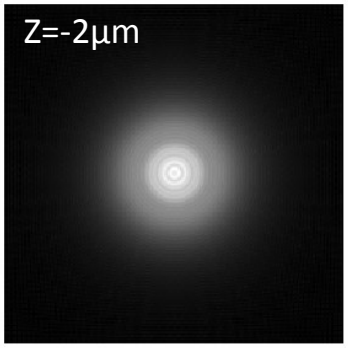


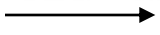
The Point Spread Function (PSF)

2D PSF
for different defocus





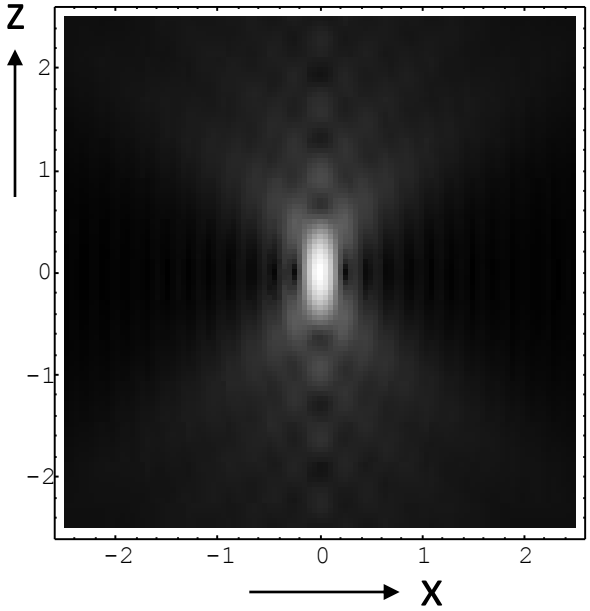




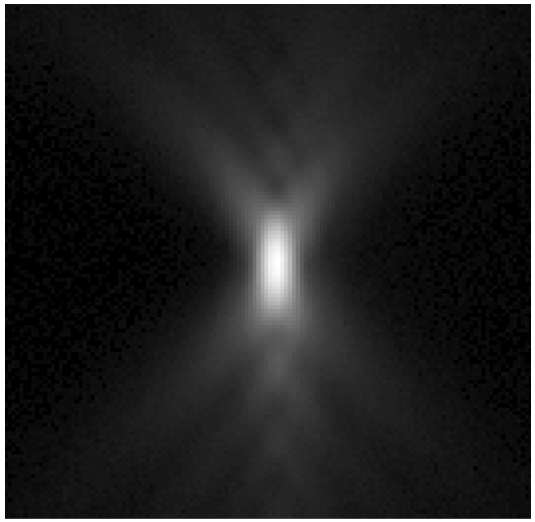
The image of a point object

3D PSF

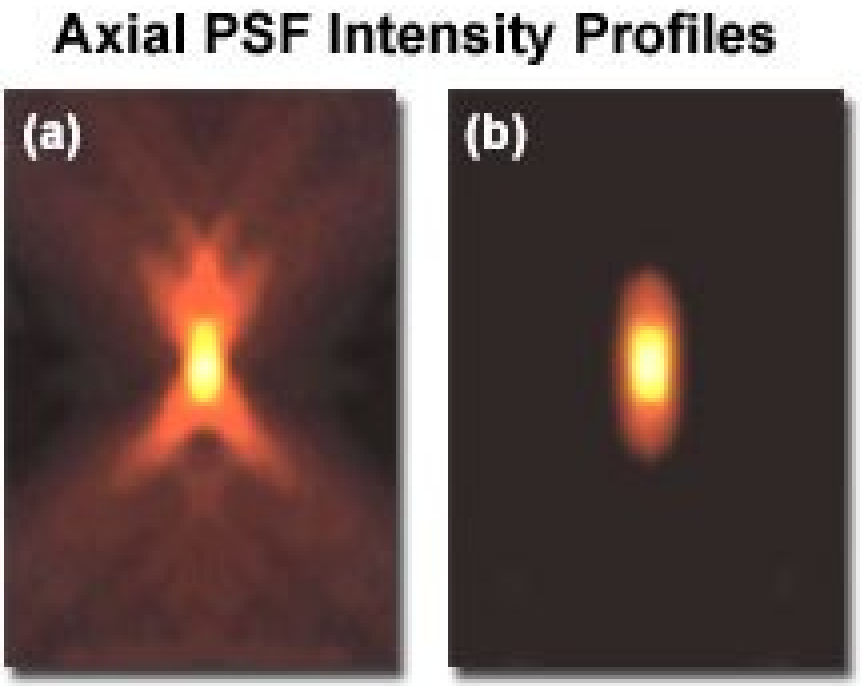
Calculated



Measured



What do you get?

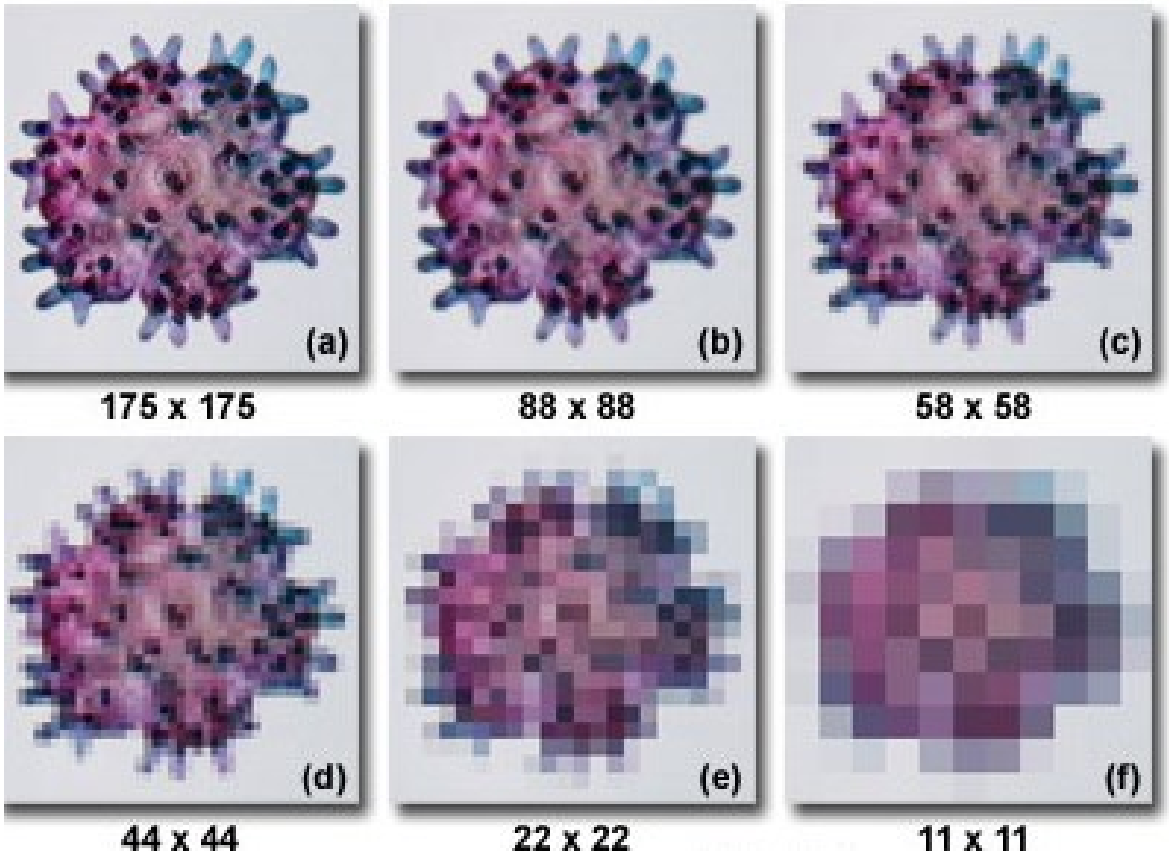


Importance of proper adjustment of Zoom in Confocal

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

A **bandlimited continuous**-time signal can be sampled and perfectly **reconstructed** from its samples if the waveform is sampled over twice as fast as it's highest frequency component.



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 - interference patterns)

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

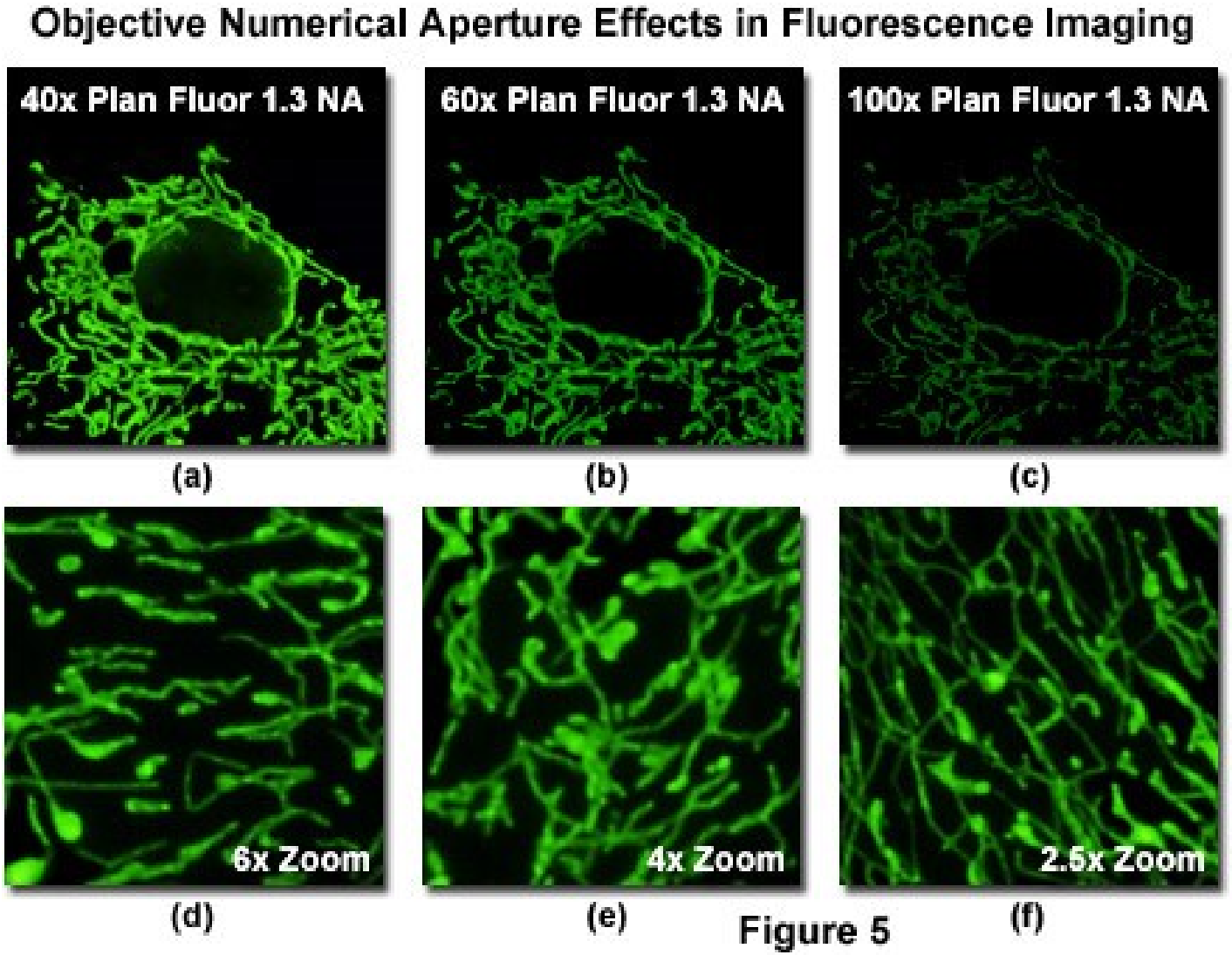
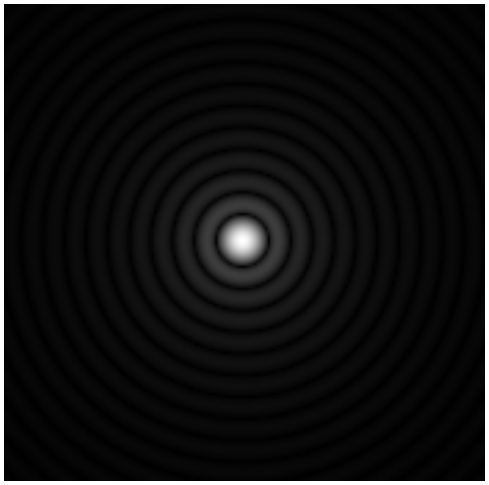


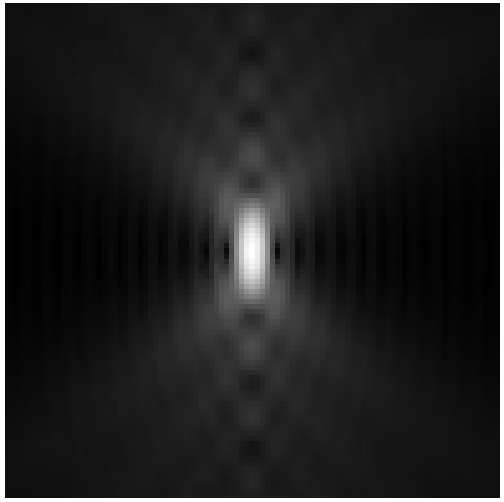
Figure 5

Adjustment of pin hole size for image optimization



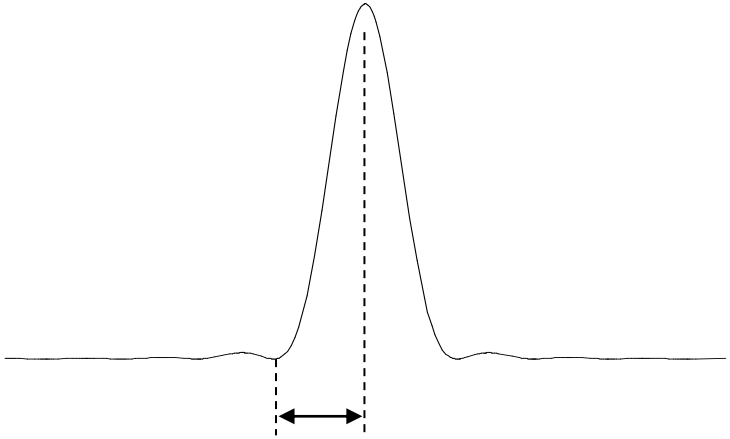
Y

X



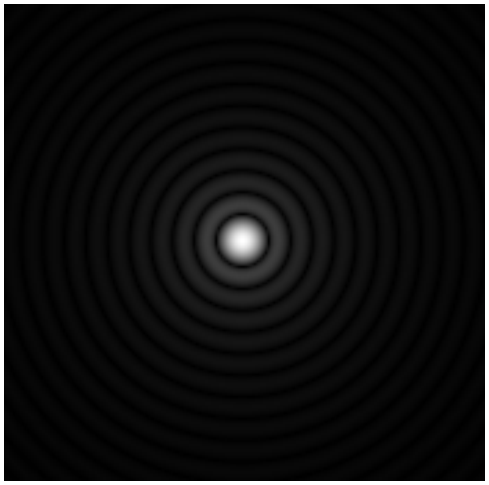
Z

Resolution is limited by the point-spread function



Airy disk radius
≈ 0.61 λ /*NA*

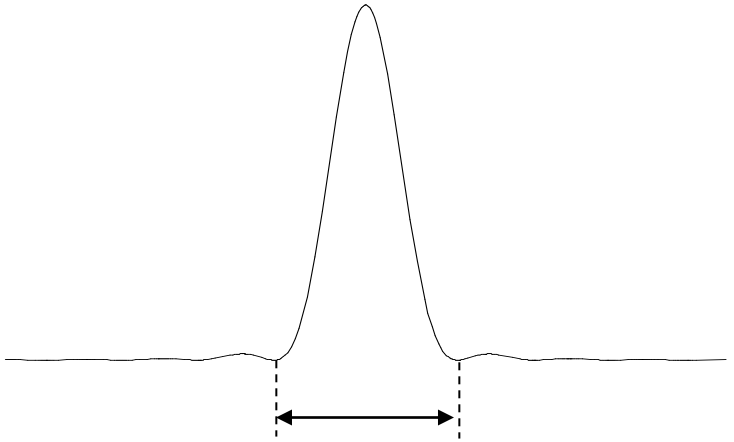
How big should your pinhole be?



Y

X

Want pinhole to pass entire Airy disk



Airy disk diameter
≈ 1.22 λ /*NA*

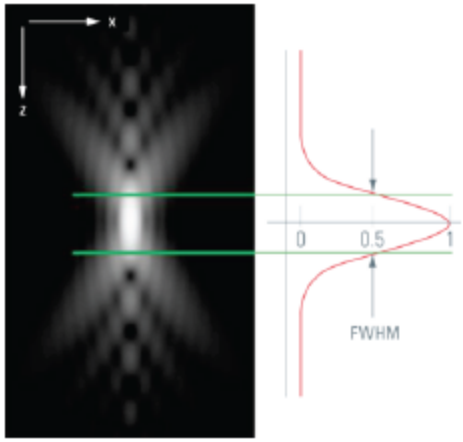
Width of point spread function at pinhole:
Airy disk diameter × magnification of lens

How big should your pinhole be?

- Width of point spread function at pinhole =
  Airy disk diameter × magnification of lens = 1 Airy unit
  = resolution of lens × magnification of lens × 2

  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 44 μm
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 19 μm
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 16 μm
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 14 μm

The relationship between pinhole diameter, Numerical aperture and light wave length



FWHM=Full Width Half-Maximum

Shorter the wavelength the thinner the optical section

$$FWHM_{tot,axial} = \frac{0.64\,\overline{\lambda}}{\left(n - \sqrt{n^2 - NA^2}\right)}$$

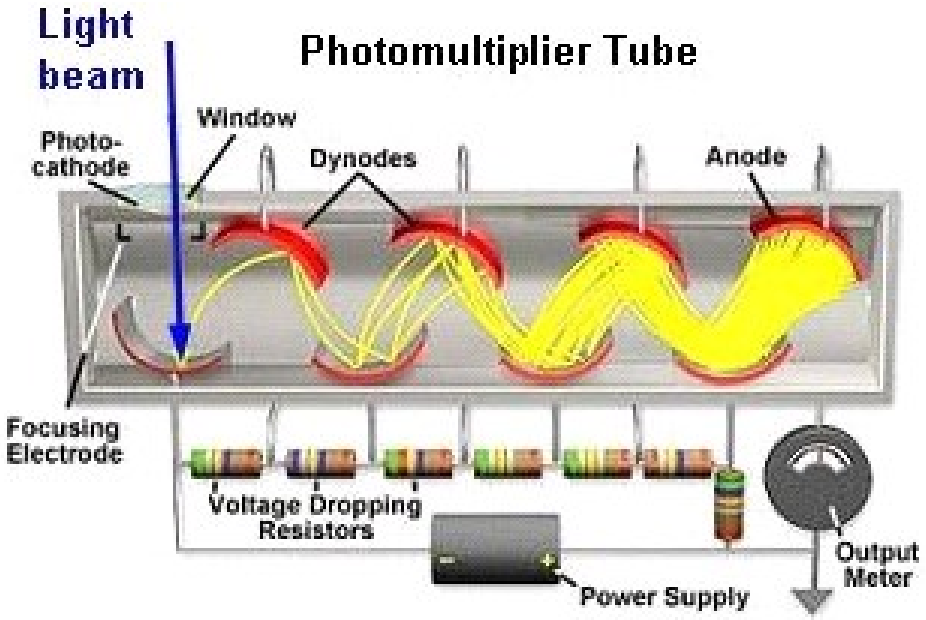
Diameter of the pinhole
Larger pinhole thicker section

The higher the NA.
the thinner the section

Weak signal > open pinhole > more light but thicker section

Detectors - PMTs

Must be fast – confocal beam spends only a few μs on each pixel



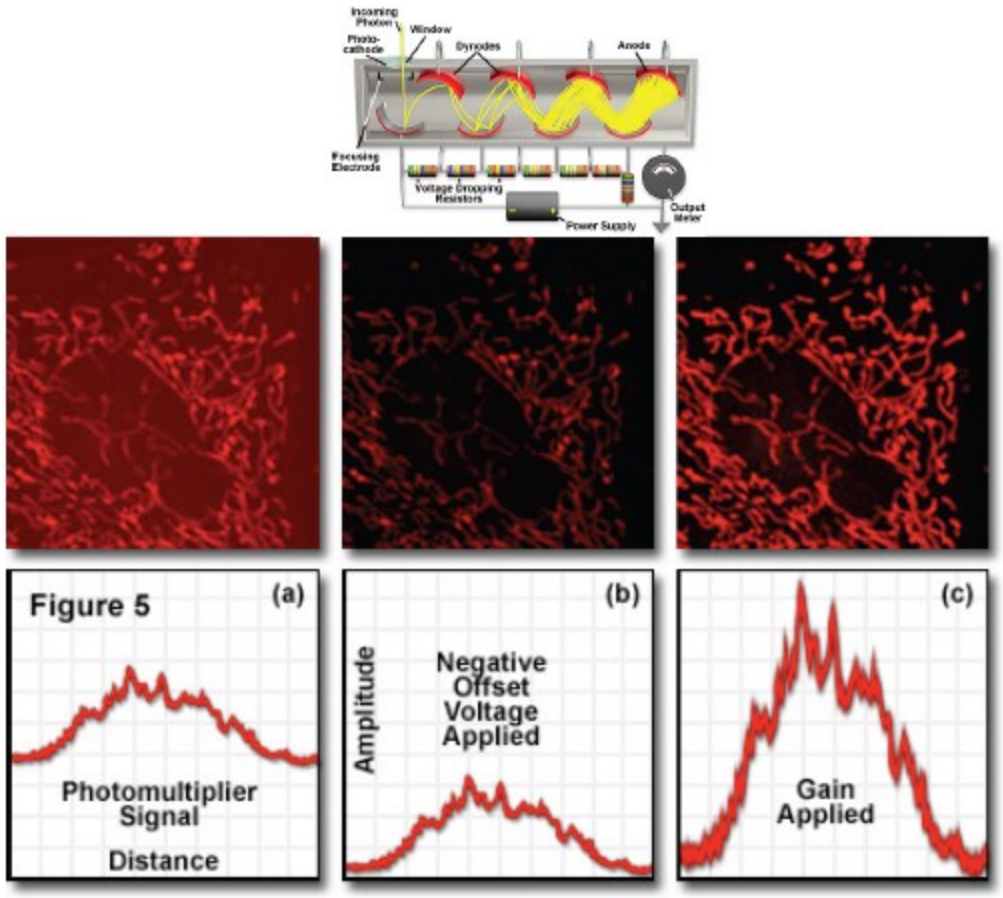
Pulse width for single photon
~ 10-100ns

Very linear

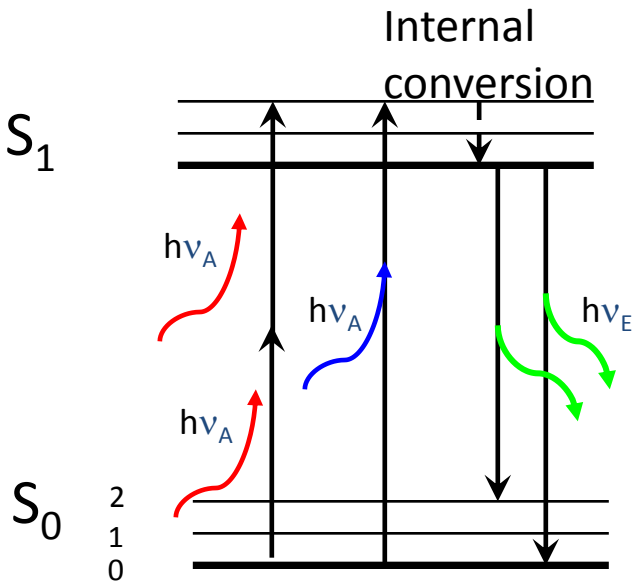
Very high gain

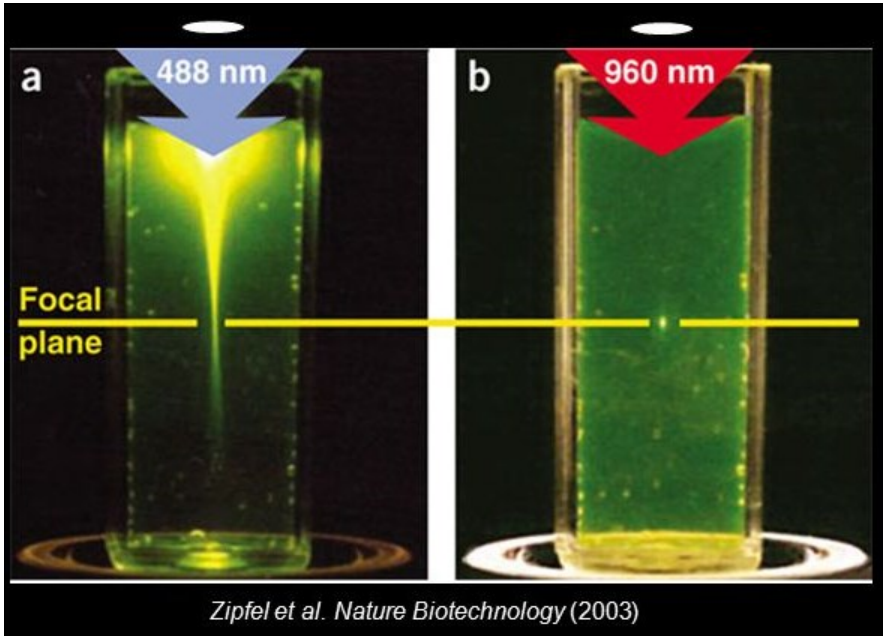
~ 0 read noise

Adjustment of detector gain/offset for image optimization

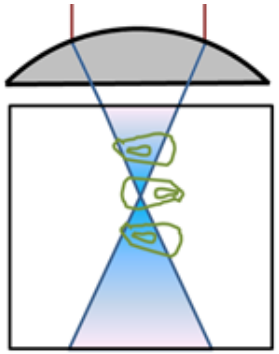


Multi-photon excitation

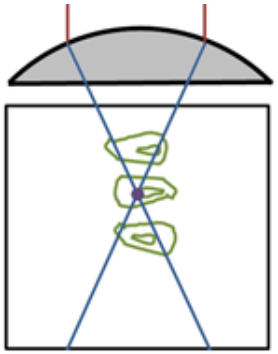




Multi-photon excitation does not excite out-of-focus light, so you can get rid of the pinhole

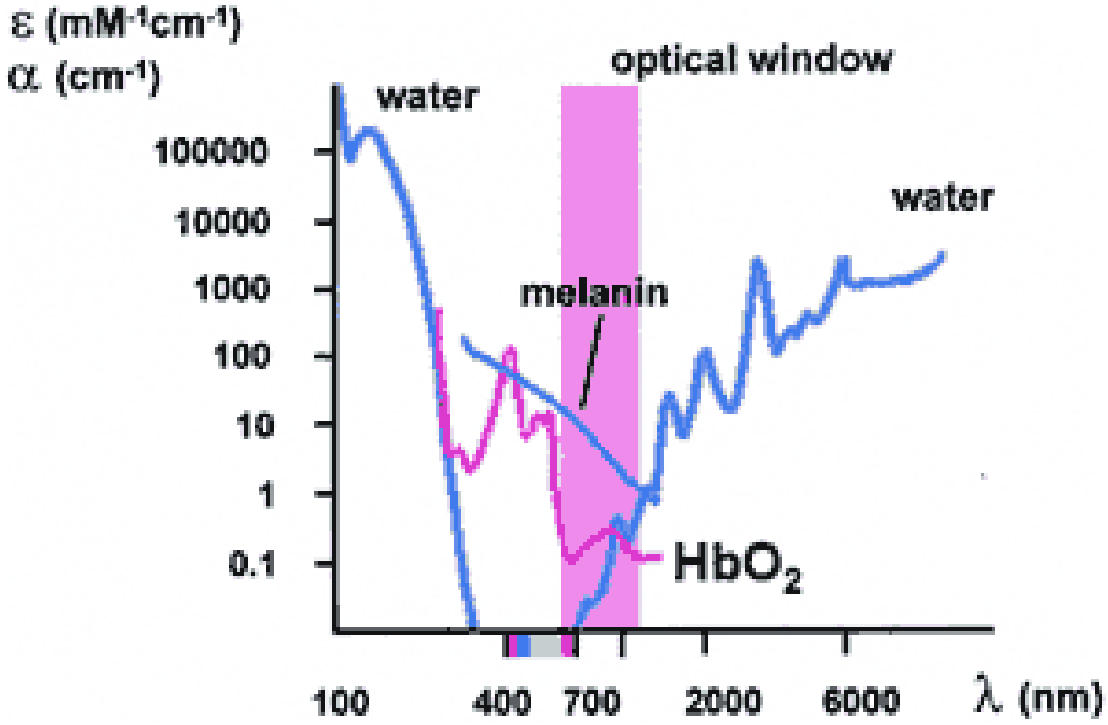


Confocal Laser
Scanning Microscope



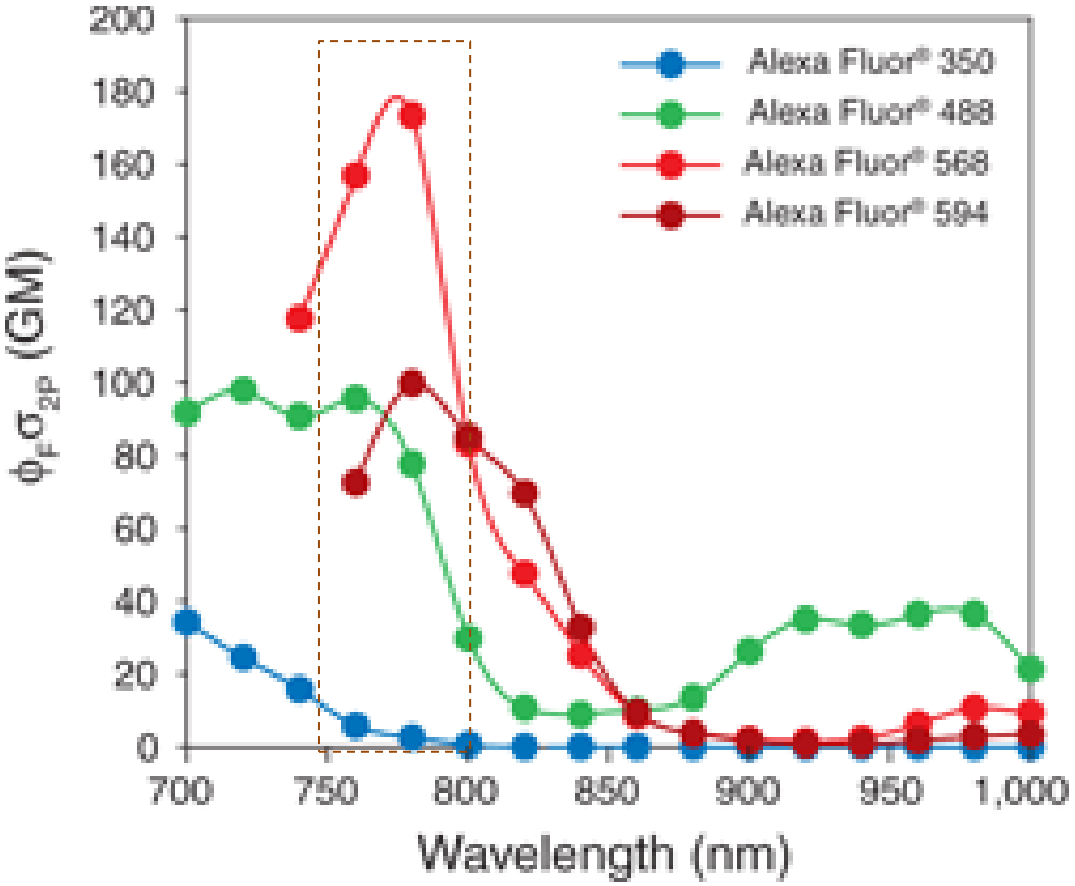
Two-photon Laser
Scanning Microscope

2π Microscopy is superior for thick specimen



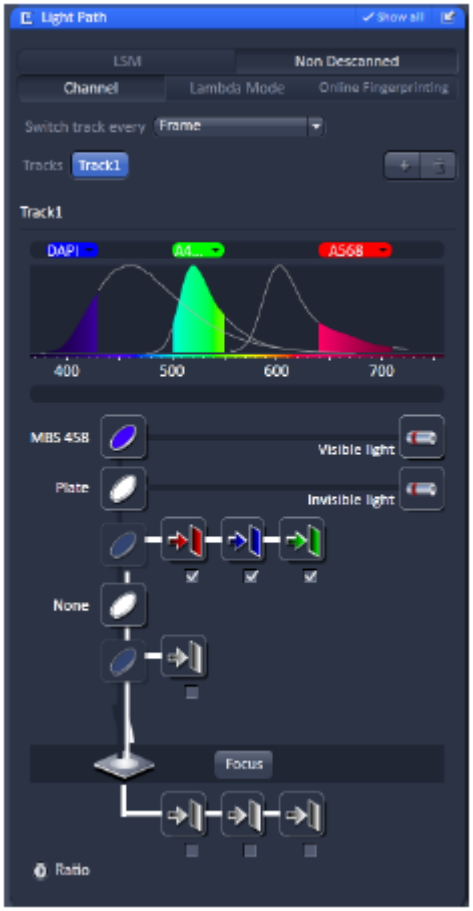
- Less susceptible to loss of signal due to scattering
- Low tissue absorption at 700-1000 nm

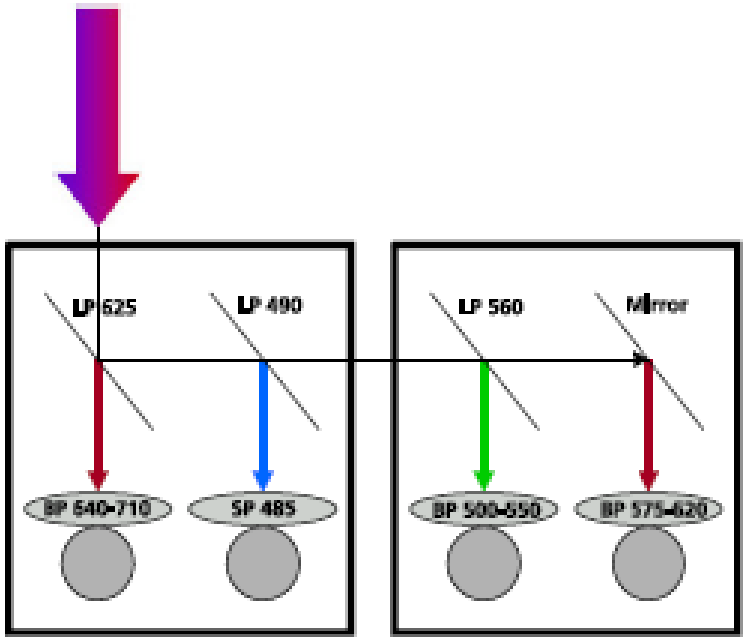
Fluorescence quantum yields (Φ_F) of two-photon absorption cross-sections (σ)



https://www.thermofisher.com/uk/en/home/references/molecular-probes-the-handbook/technical-notes-and-product-highlights/fluorescent-probes-for-two-photon-microscopy.html

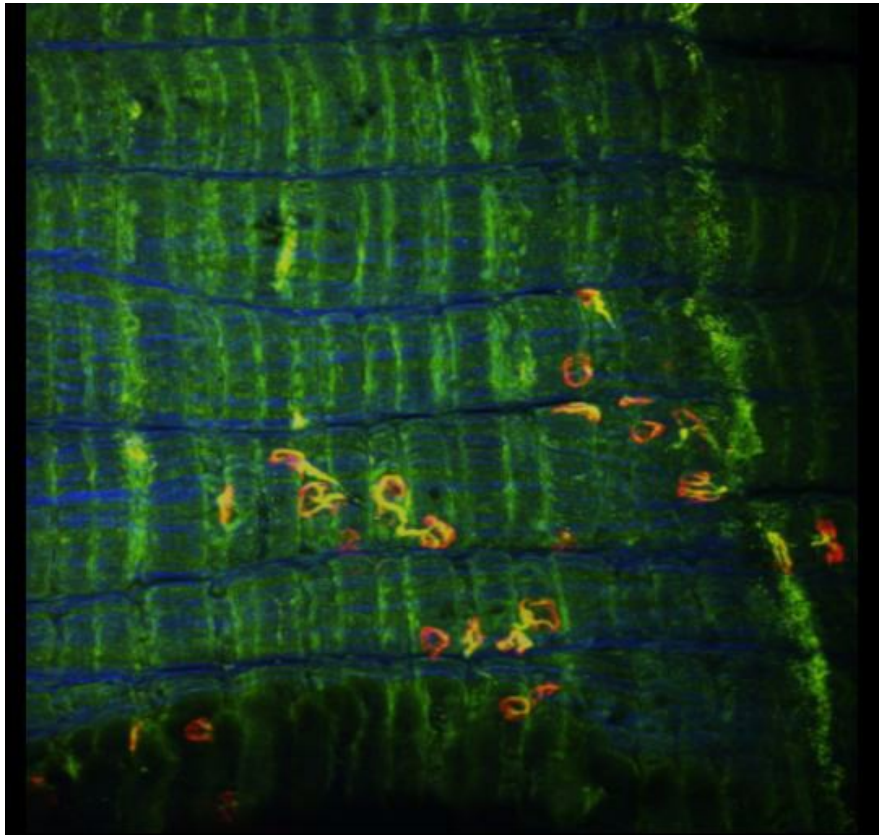
Non-Descanned Detectors (NDD)

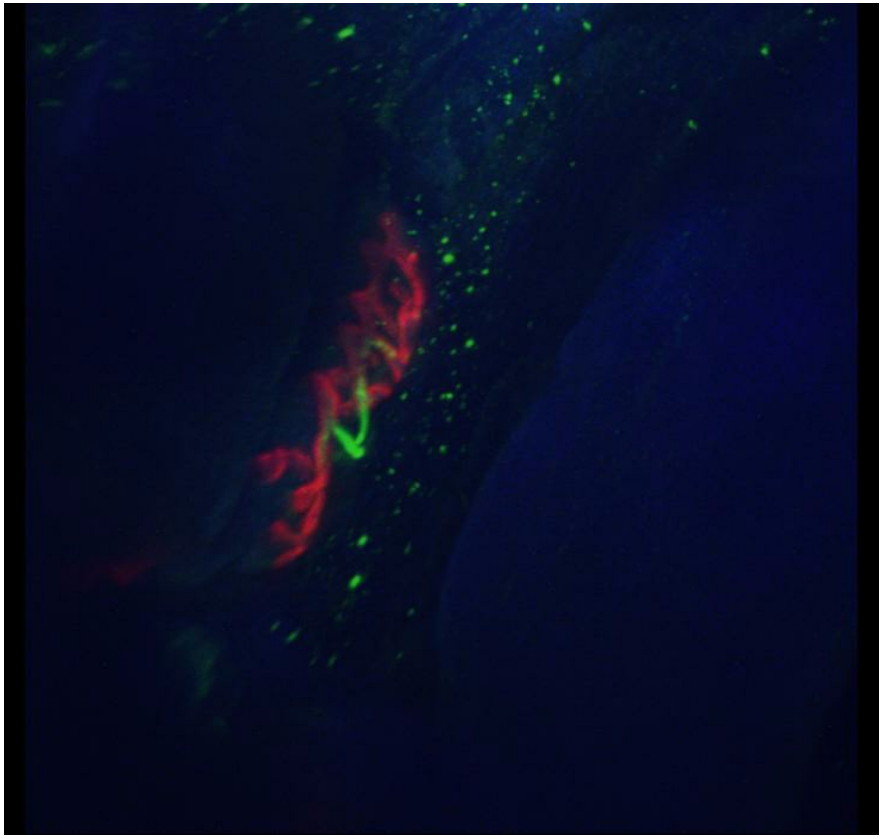




NDD module type A, 2 channels for upright microscopes, BIG NDD module as channel 3+4

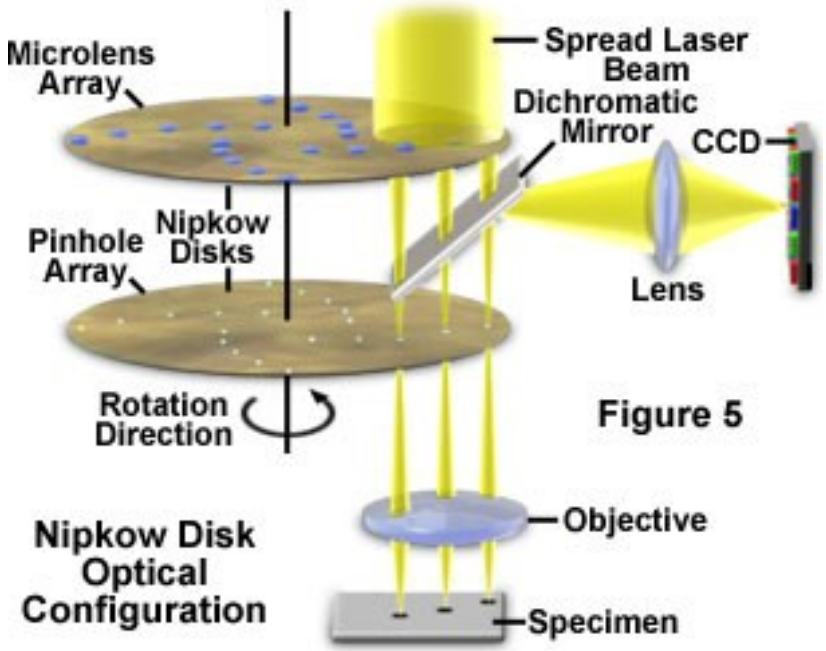
Two-photon Microscopy of Neuromuscular Junction Endplates
Yu Cheung / Richard Webster / Susan Maxwell /Prof. David Beeson

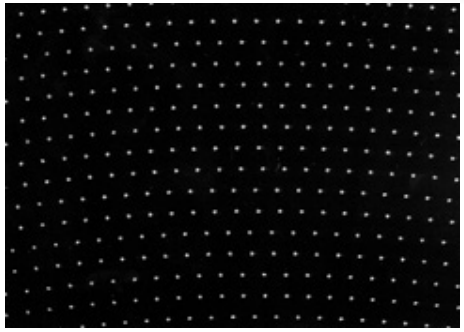




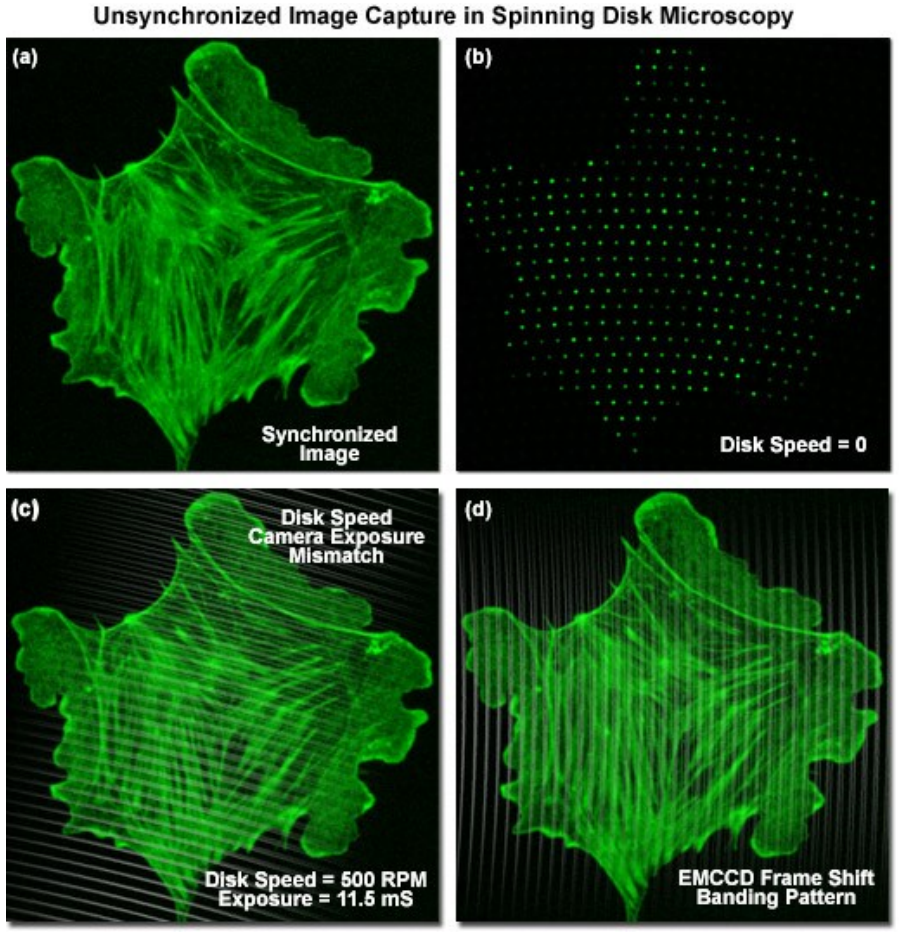
Neurofilament (Green) / Bungarotoxin/AChR (Red) / Second Harmonic Generation (Blue)

Spinning Disk Confocal





approximately 20,000 pinholes arranged in a series of nested spirals.

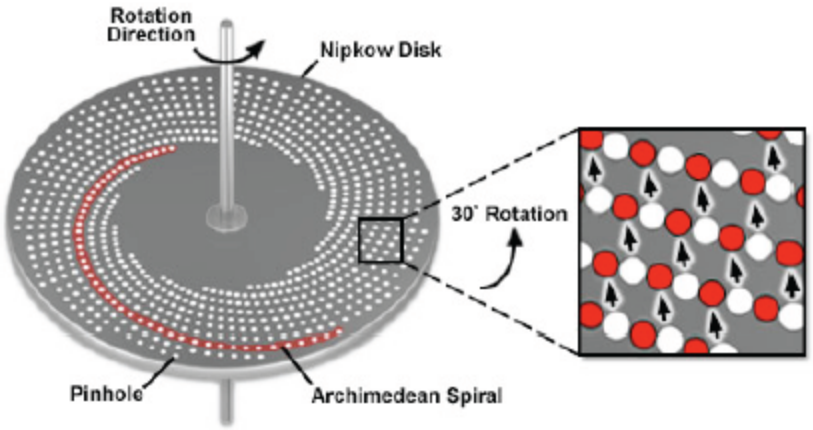


http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/introduction.html

Image with many pinholes at once > fast

Use CCD as detector (not detector) > so much higher Quantum Efficency

The Spinning Disk



Larger pinholes - brighter image, but less “confocal”

Pinholes fixed size: Typically = 50um

(optimised for biology)

How big should your pinhole be?

- Width of point spread function at pinhole =
  Airy disk diameter × magnification of lens = 1 Airy unit
  = resolution of lens × magnification of lens × 2

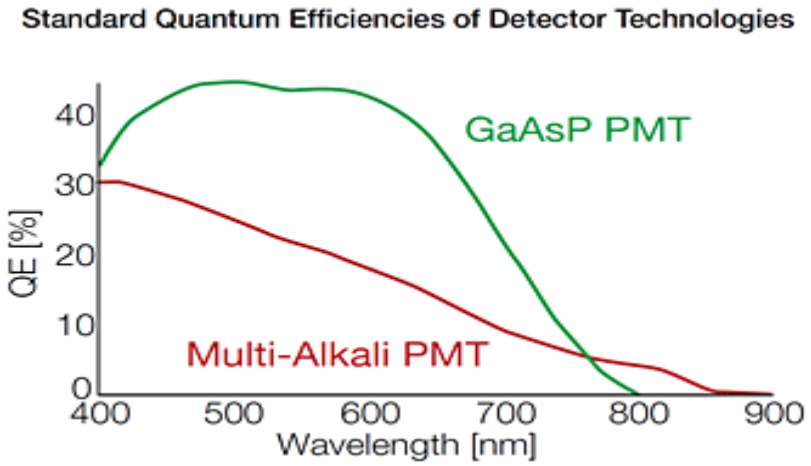
  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 44 μm
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 19 μm
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 16 μm
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 14 μm

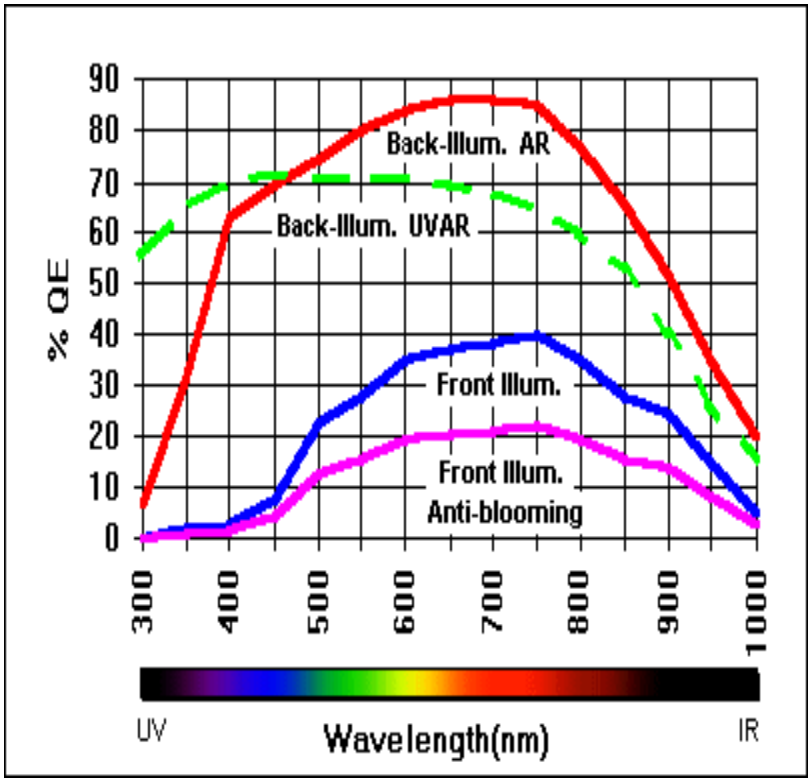
Pros/Cons of spinning disk

- Fast – multiple points are illuminated at once
- Photon efficient – high QE of CCD
- Gentler on live samples – usually lower laser power

- Fixed pinhole – except in swept-field
- Small field of view (usually)
- Crosstalk through adjacent pinholes limits sample thickness

Detectors - PMTs vs CCDs





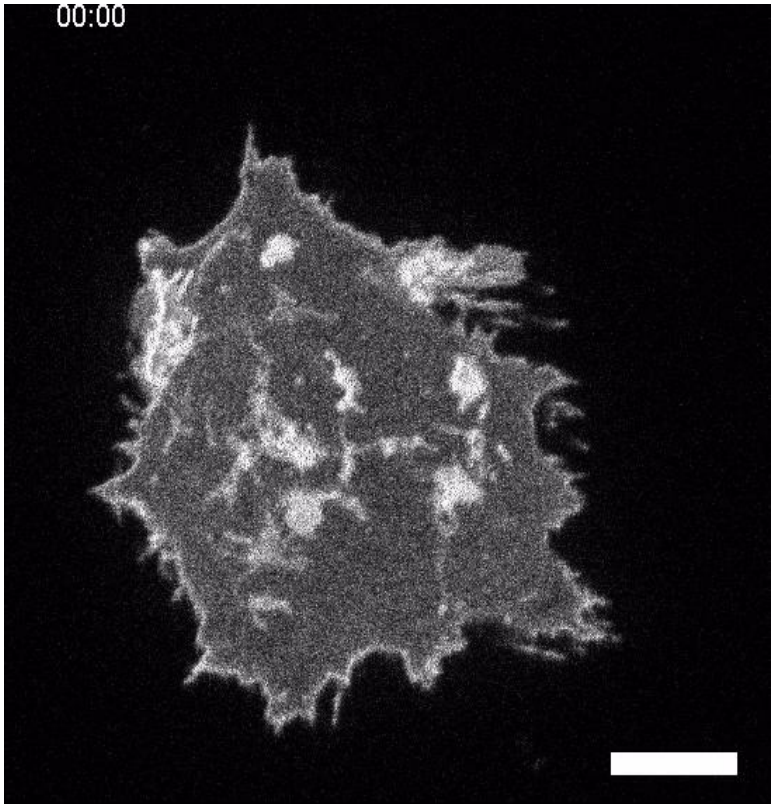
Scanning Confocal vs Spinning Disc Confocal

Point Scanning

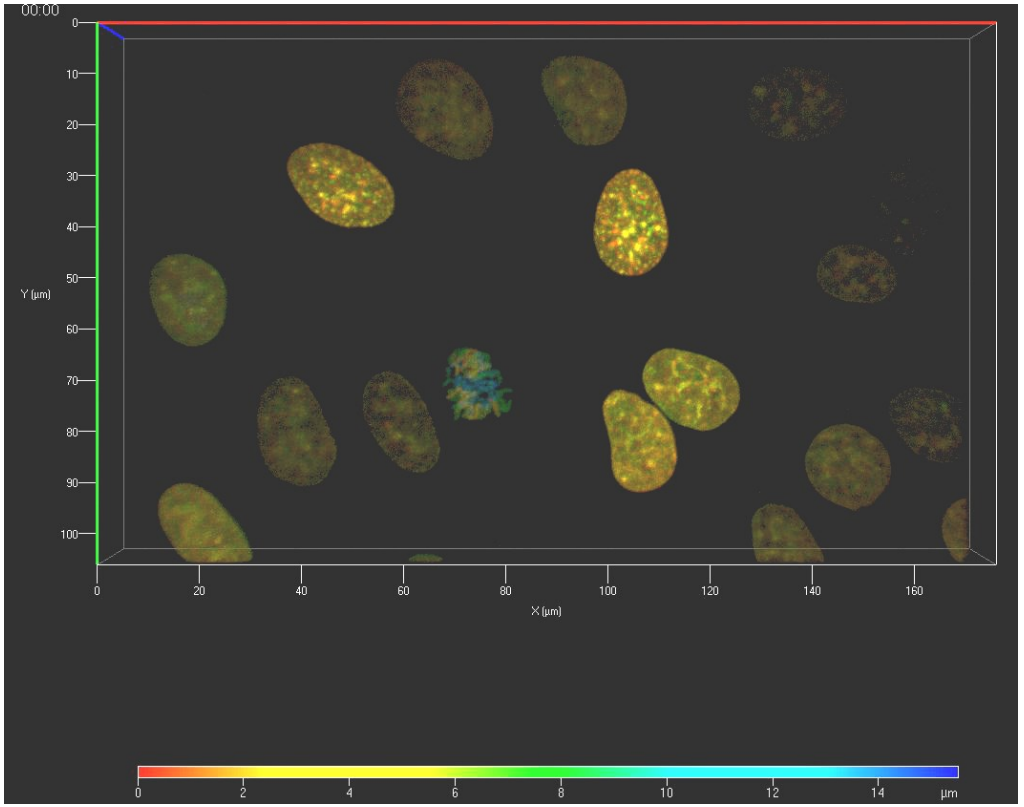
Spinning Disc

Speed	Slow (secs)	Fast (msecs)
Sensitivity	OK	OK
Flexibility	Good	Poor
Bleaching	Poor	Good
Pretty Pictures	Unbeatable!	Pretty damn good!
Pretty Movies	Good – if process slow	Unbeatable!

Spinning disc examples

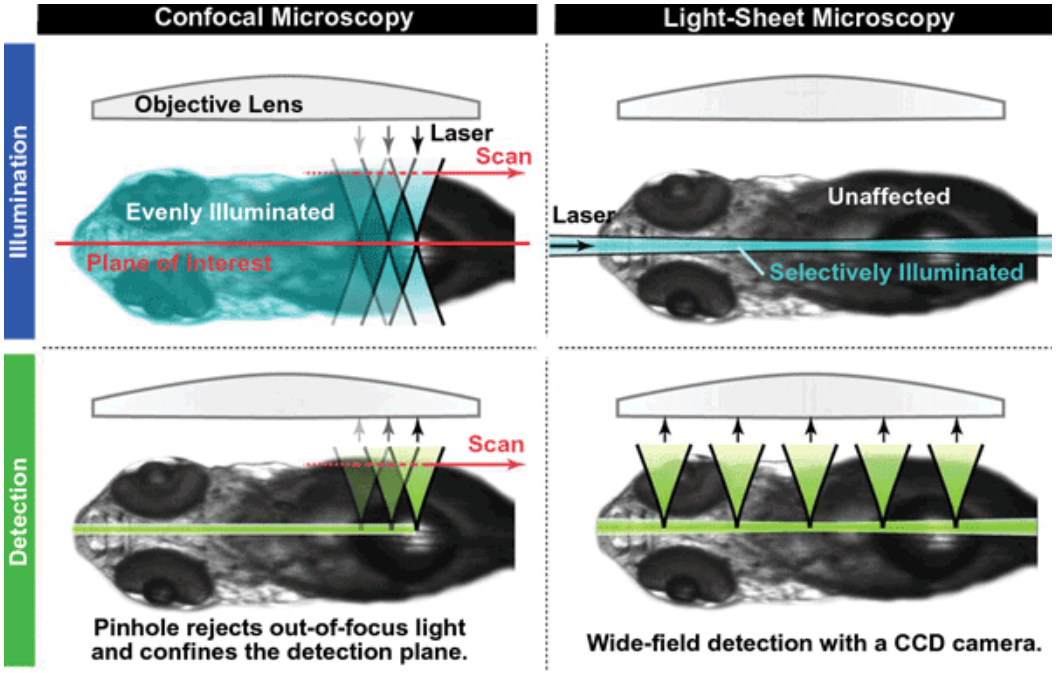


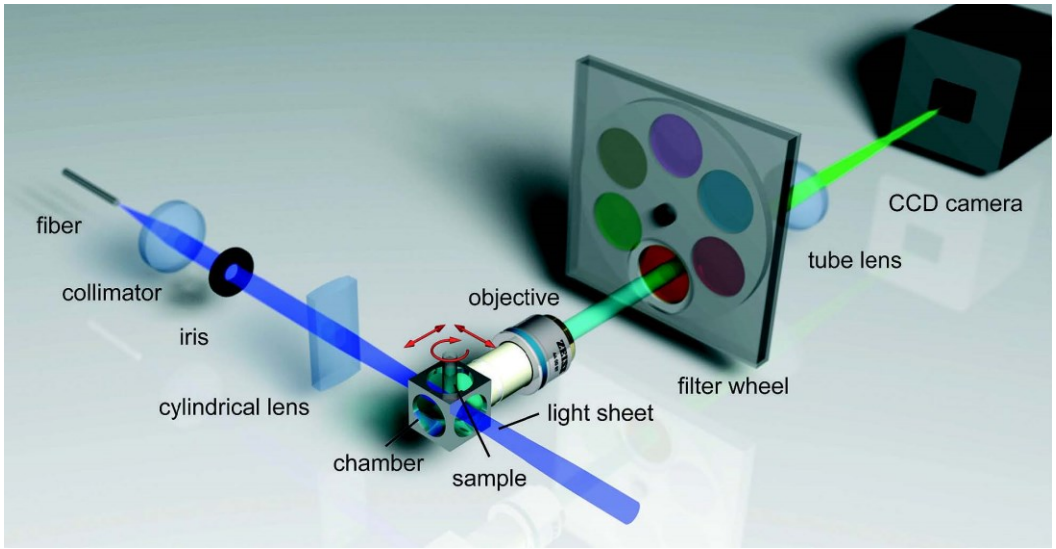
Plasma membrane dynamics in a mouse embryo fibroblast (MEF) that expresses a fluorescent fusion protein that localizes to the plasma membrane (KRas2-YFP)



Dividing HeLa cells

Light sheet microscope





Advantages:

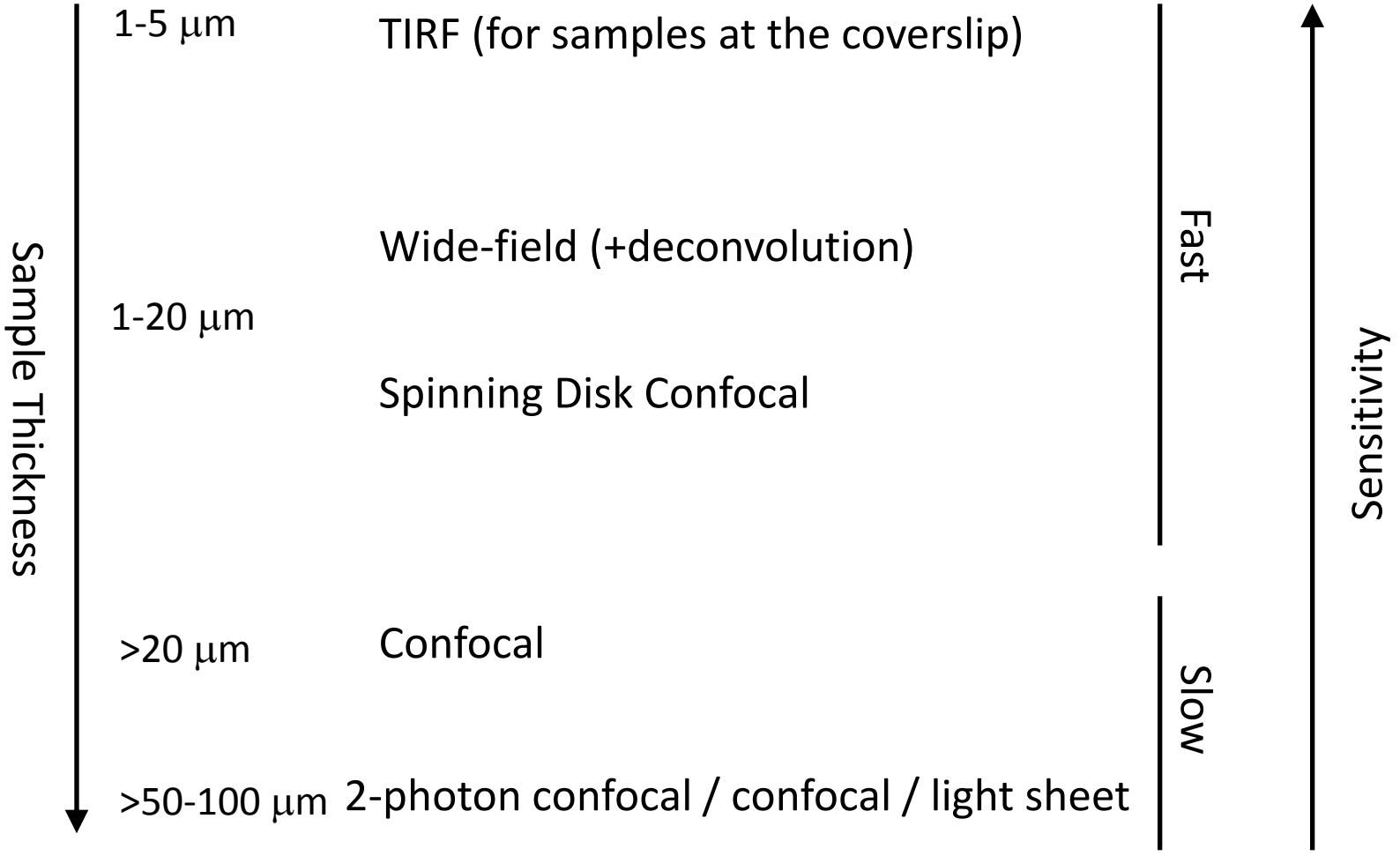
- ✓ Fast
- ✓ High resolution
- ✓ Low light required
- ✓ Uses cameras instead of detectors

Disadvantages:

- ✓ More difficult sample mounting
- ✓ Immense data volume
- ✓ Requires ultrafast hardware [PC discs, transmitting cables and storage devices]
- ✓ Requires dedicated processing computers

Great for in vivo 3D time-lapse data at single cell resolution

Which imaging technique should I use?



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*