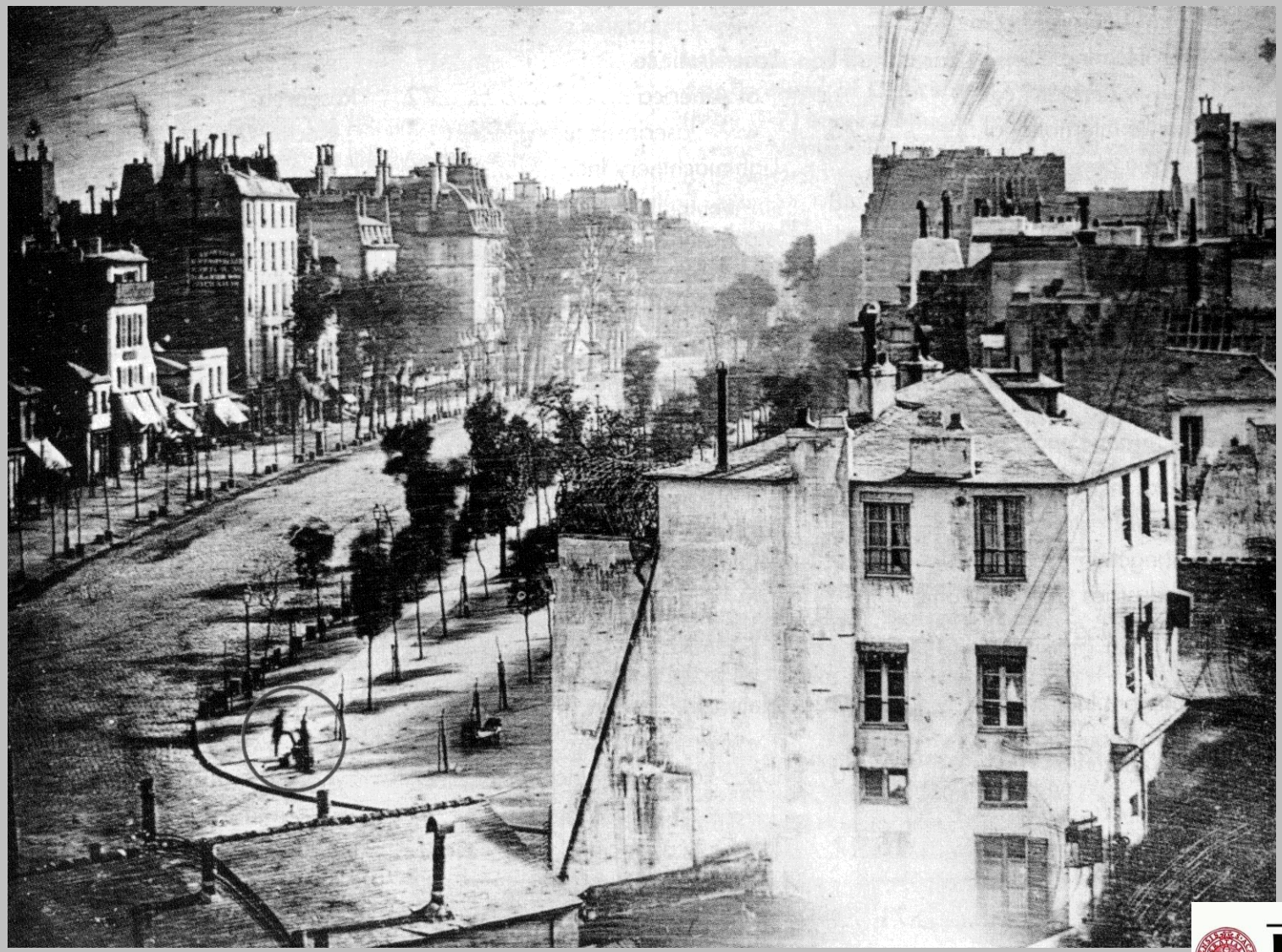
CONFOCAL MICROSCOPY

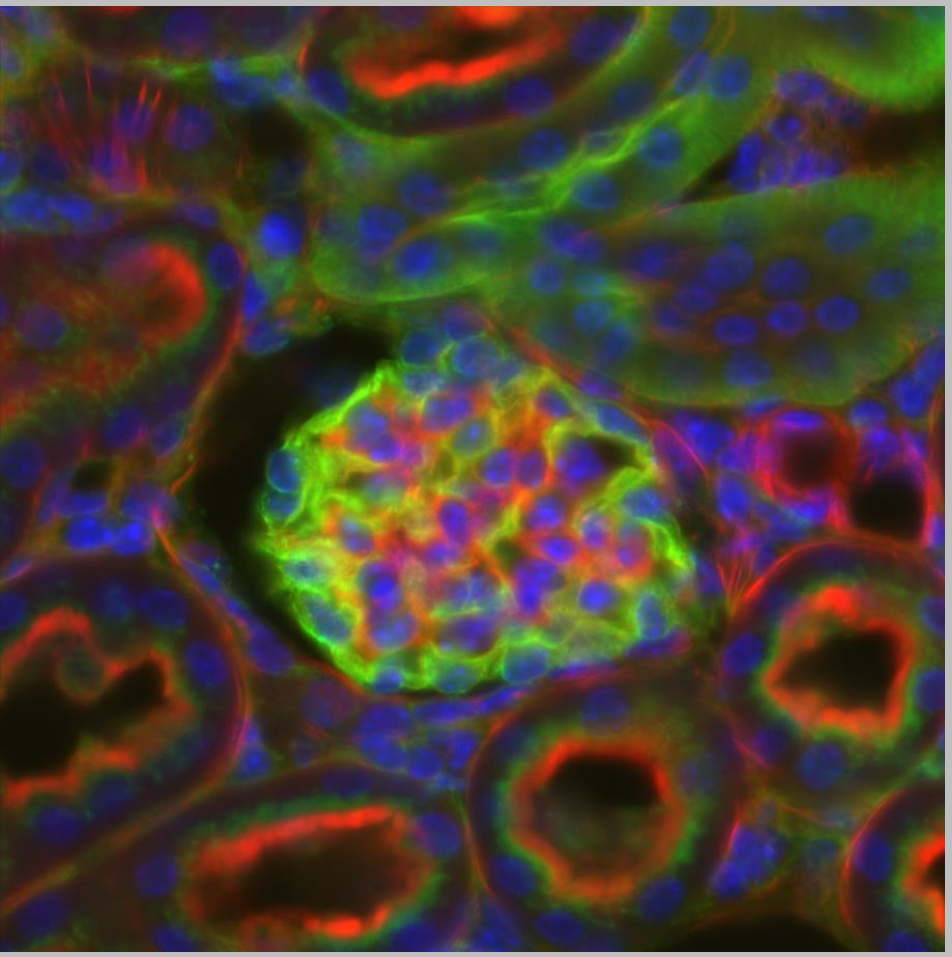
Jeremy Adler

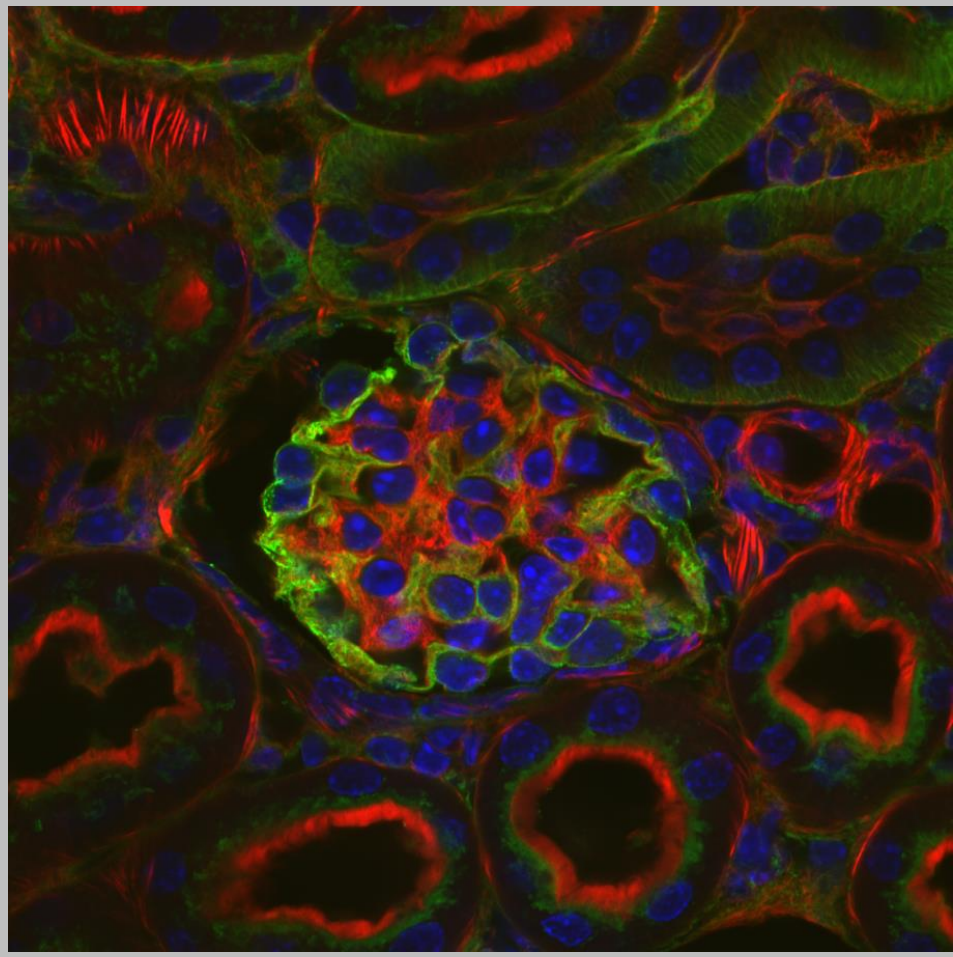
Boulevard du Temple……. a busy street ?



Daguerrotype (Paris,1838)

Widefield & Confocal Microscopy





Widefield

Confocal

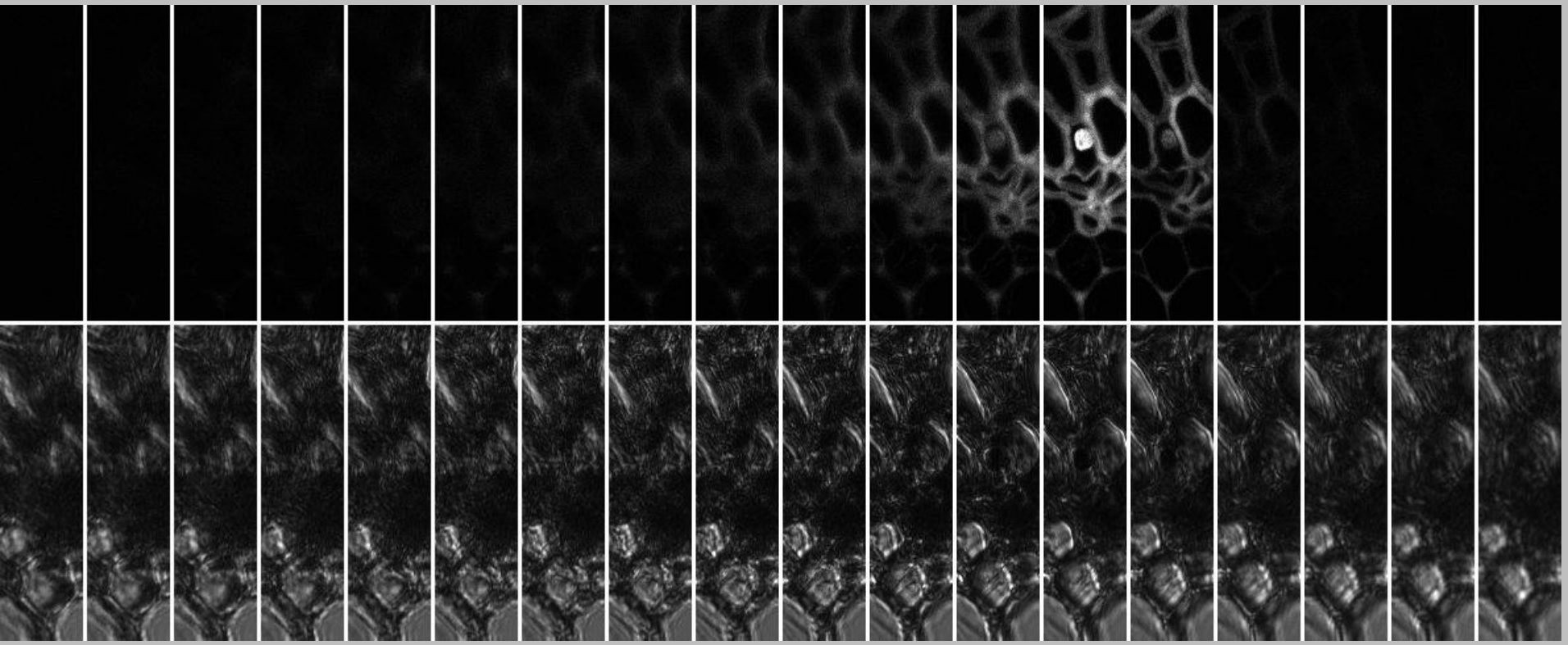
Laser Scanning Microscopy (LSM)

Comparison Confocal & Widefield

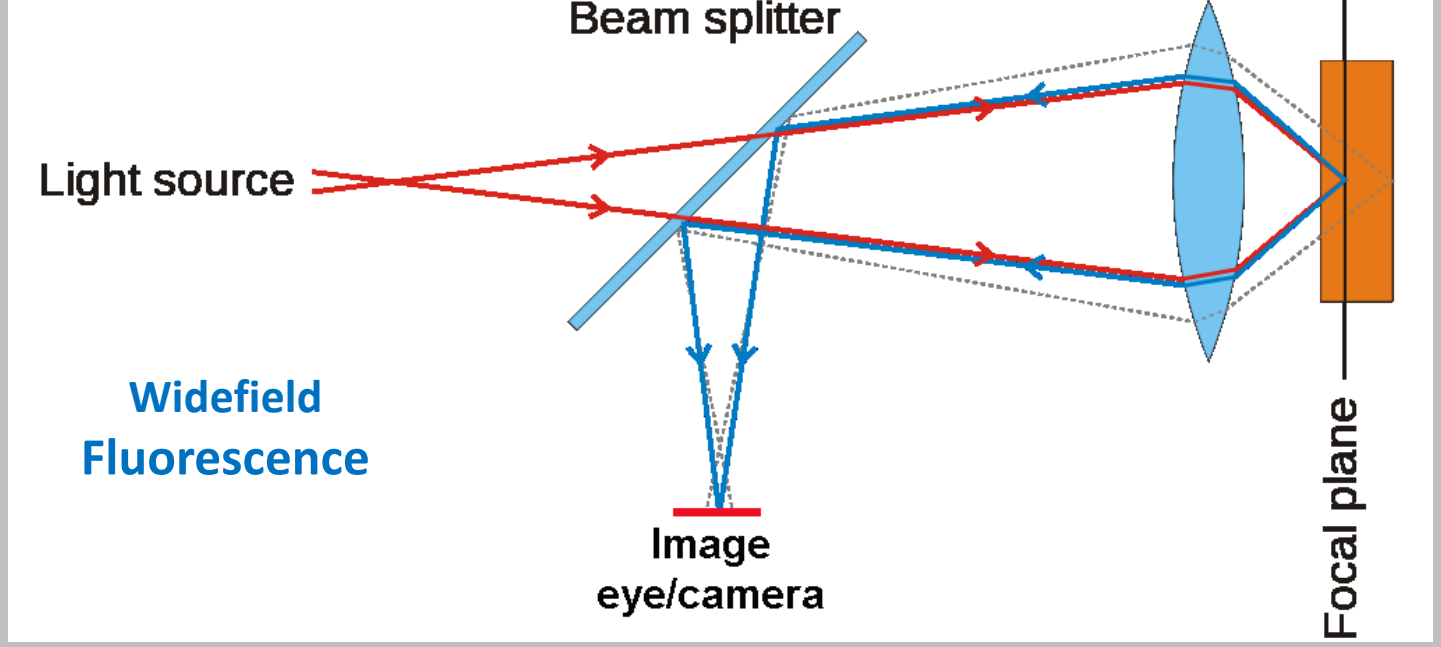
Z series

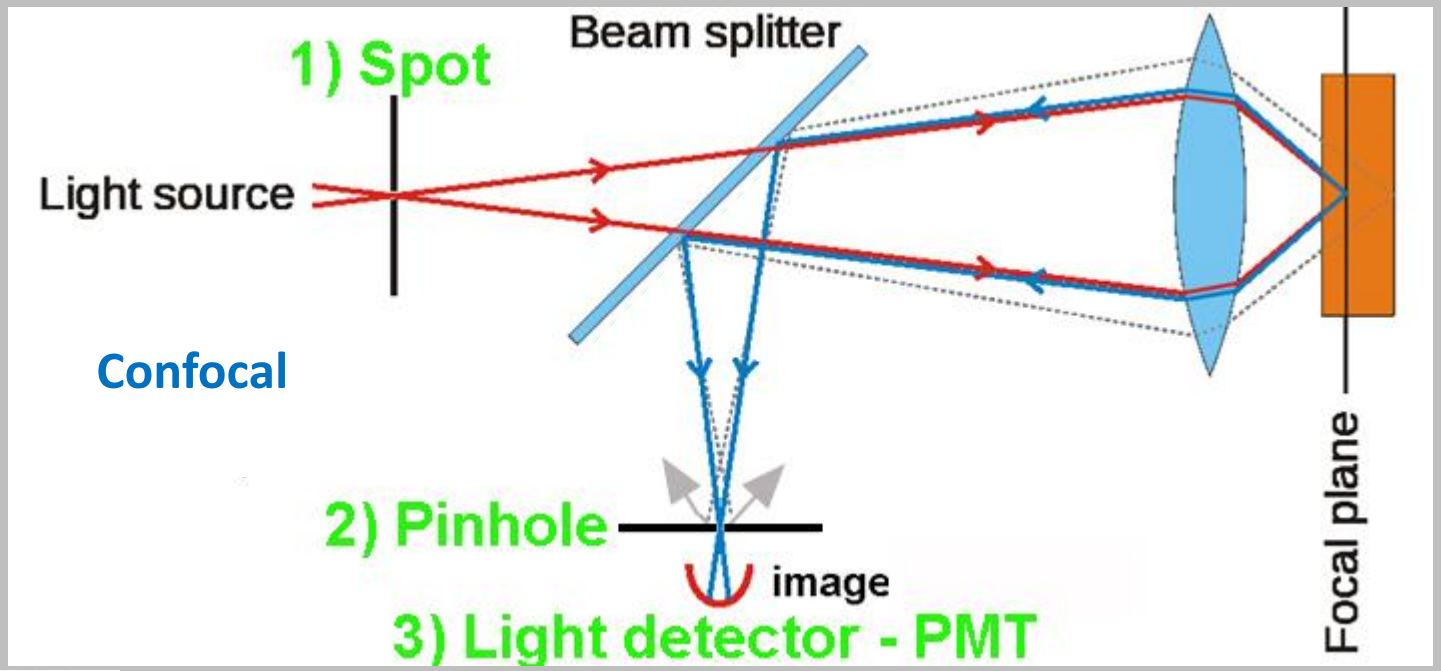
3um steps 20x NA 0.8

Confocal



Widefield

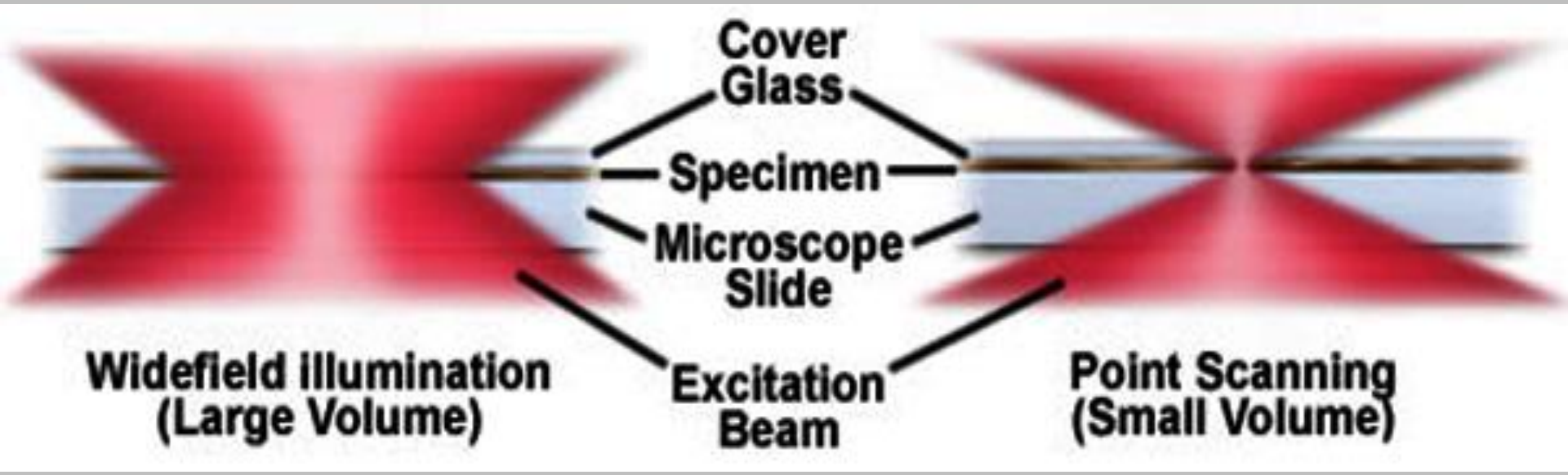

Beam splitter
Light source
Widefield
Fluorescence
Image
eye/camera
Focal plane


1) Spot
Beam splitter
Light source
Confocal
2) Pinhole
image
3) Light detector - PMT
Focal plane

Illumination

Widefield

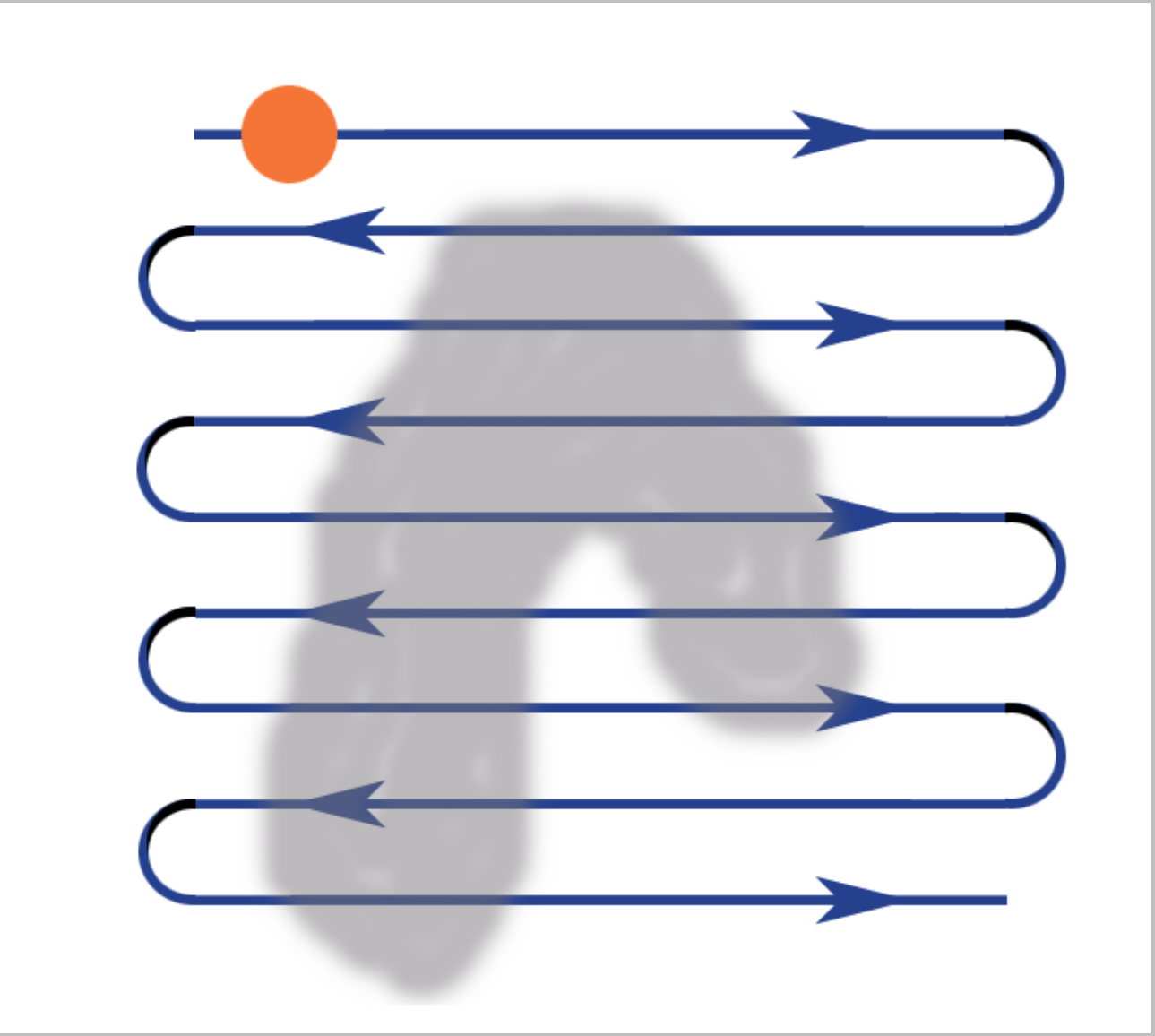
Confocal



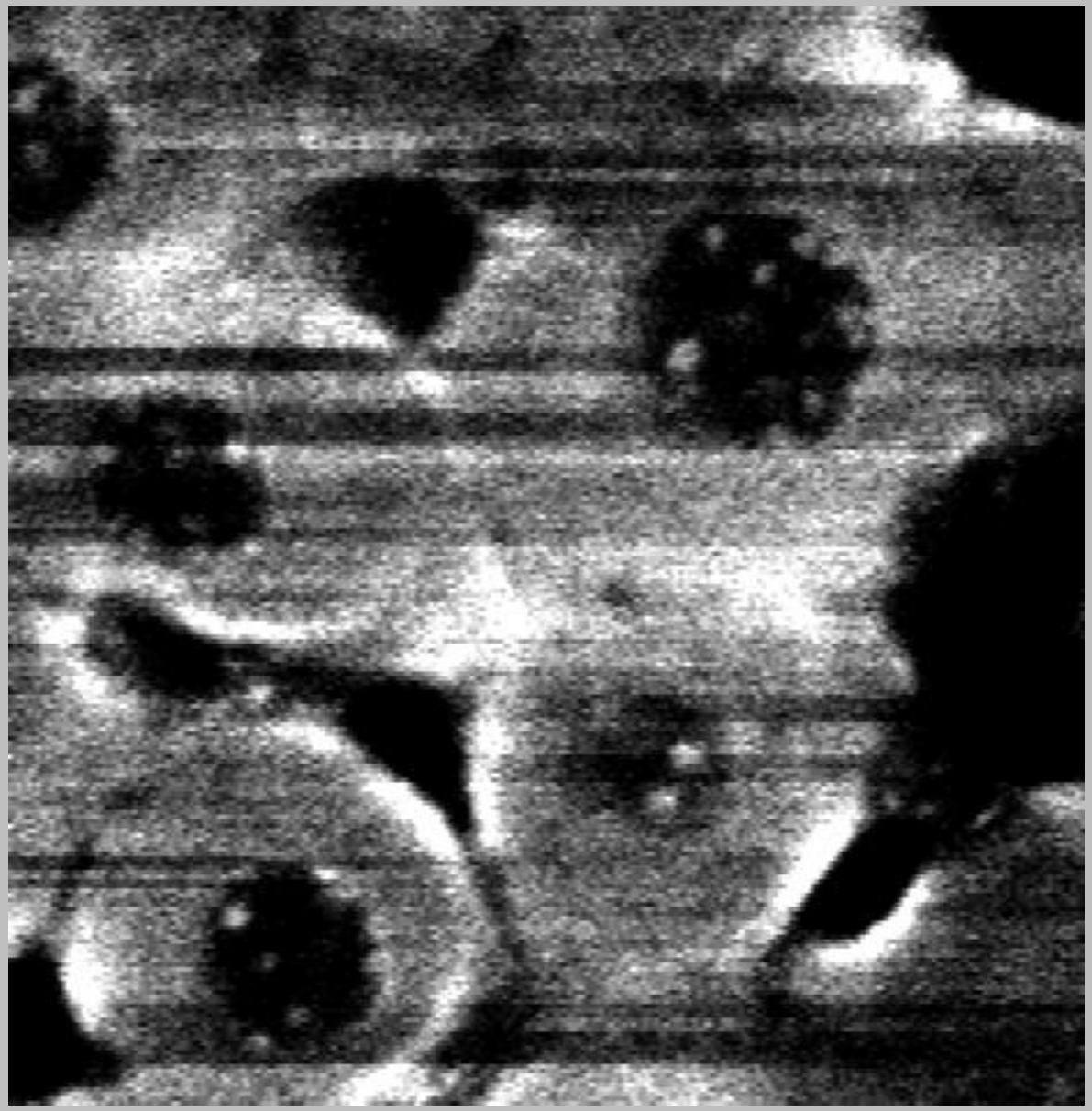
AREA

SPOT

Build an image by scanning the single spot



Scanning optics In the confocal



Note

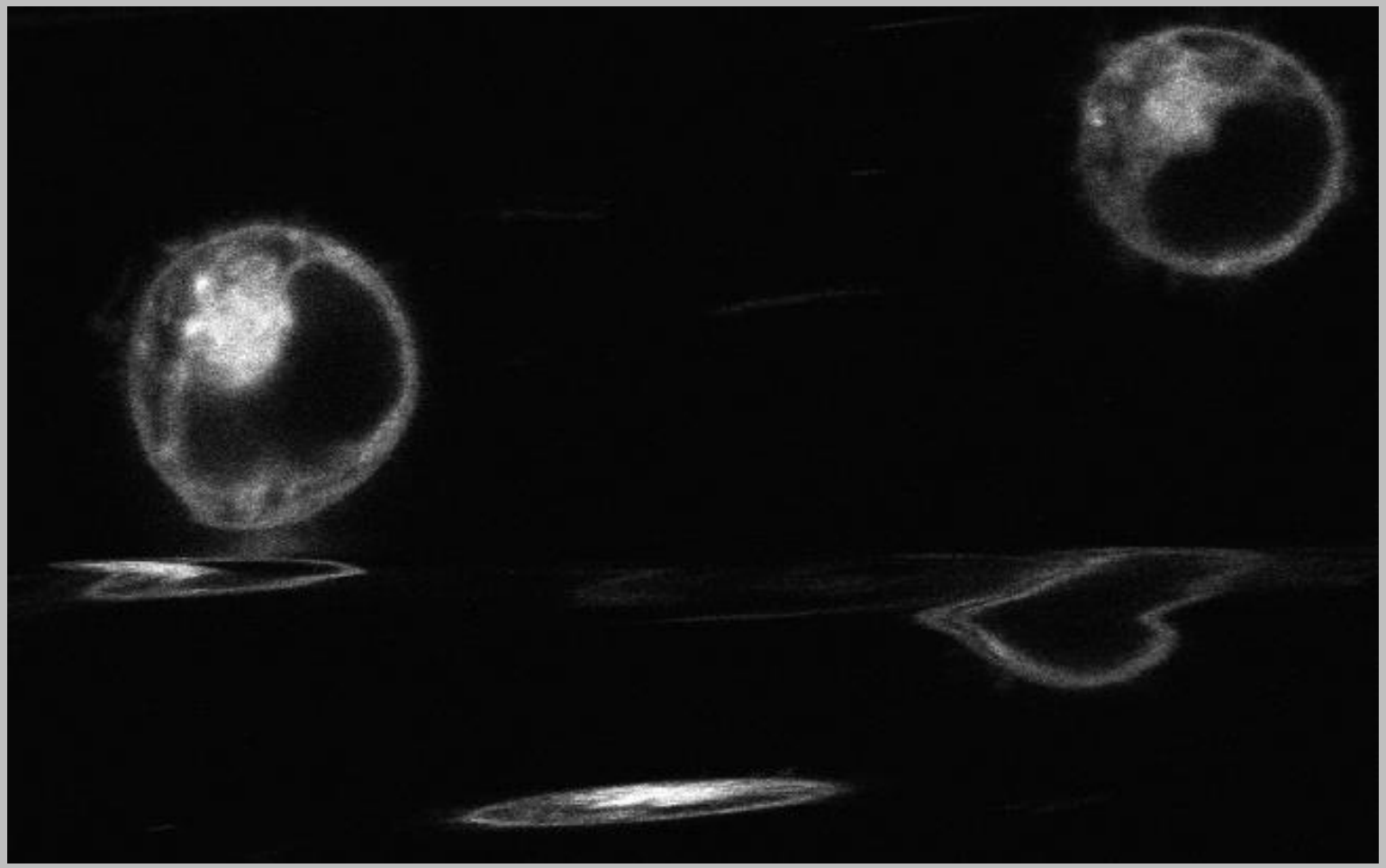
Horizontal lines !

Unstable

laser

or

light detector



Round cells moving during image acquisition

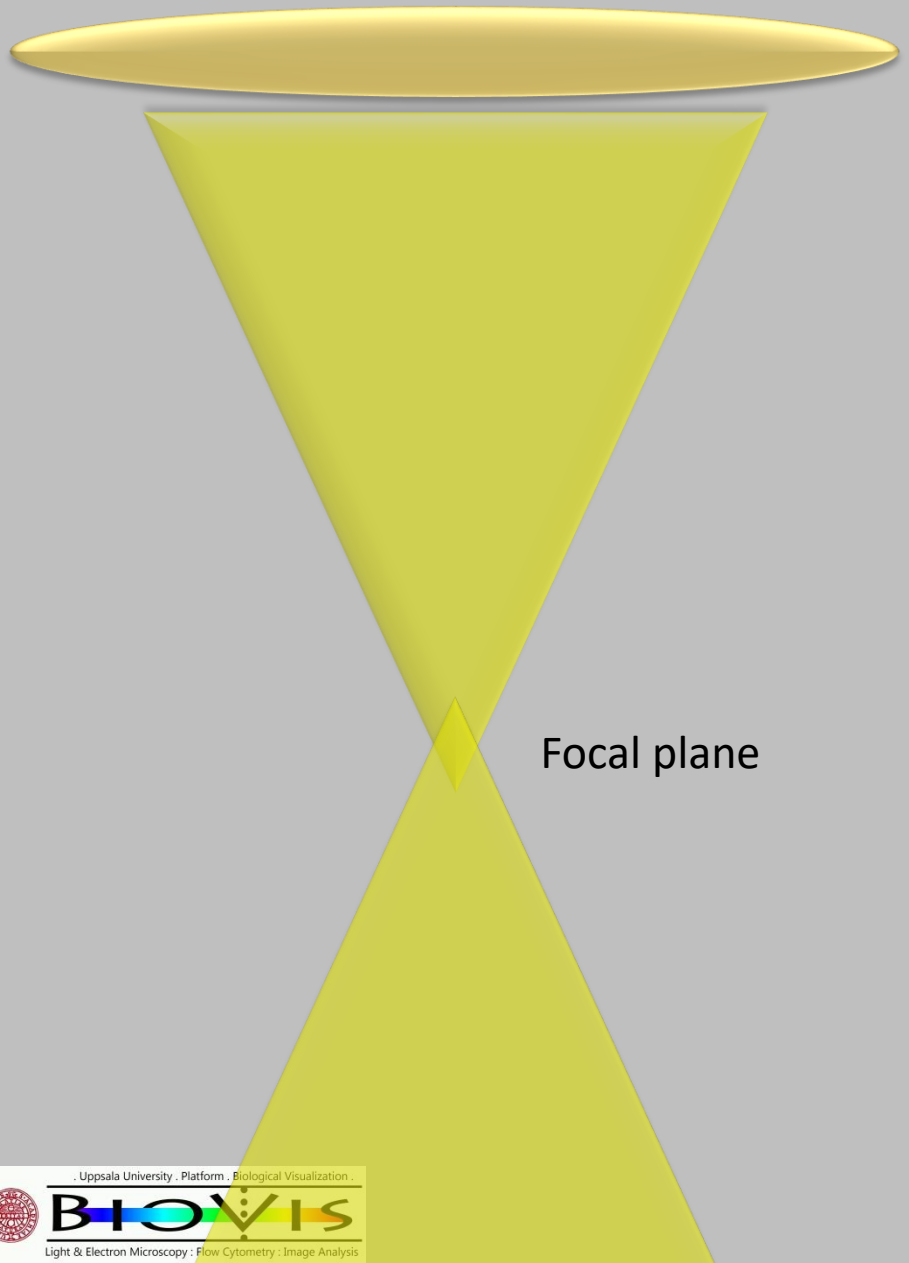
Confocal – Clever Bits

1) spot of light
scanned
to build image

2) A pinhole in front of the detector
Rejects out of focus light

Result – Optical Sectioning

Illumination - fluorescence



Lens

Focal plane

Fluorophores inside the illumination cones are excited

More excitation at the Focal plane

Objectives

Numerical aperture (resolution)

Immersion medium: air, water, oil

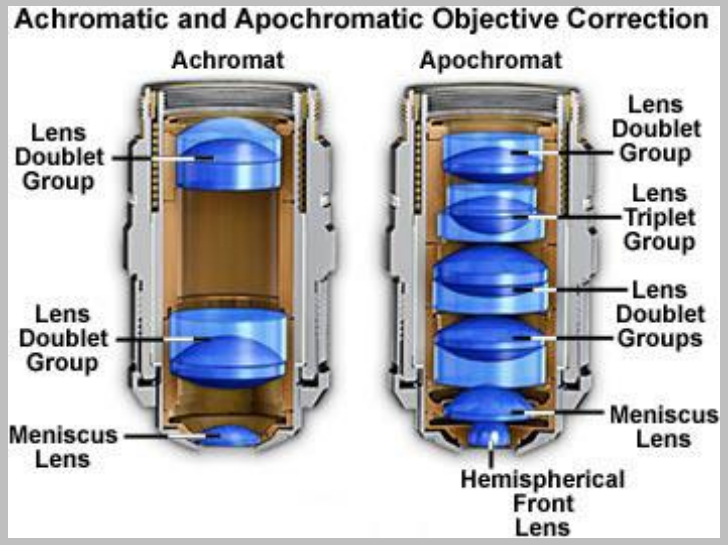
Corrections: spherical, chromatic

Working distance

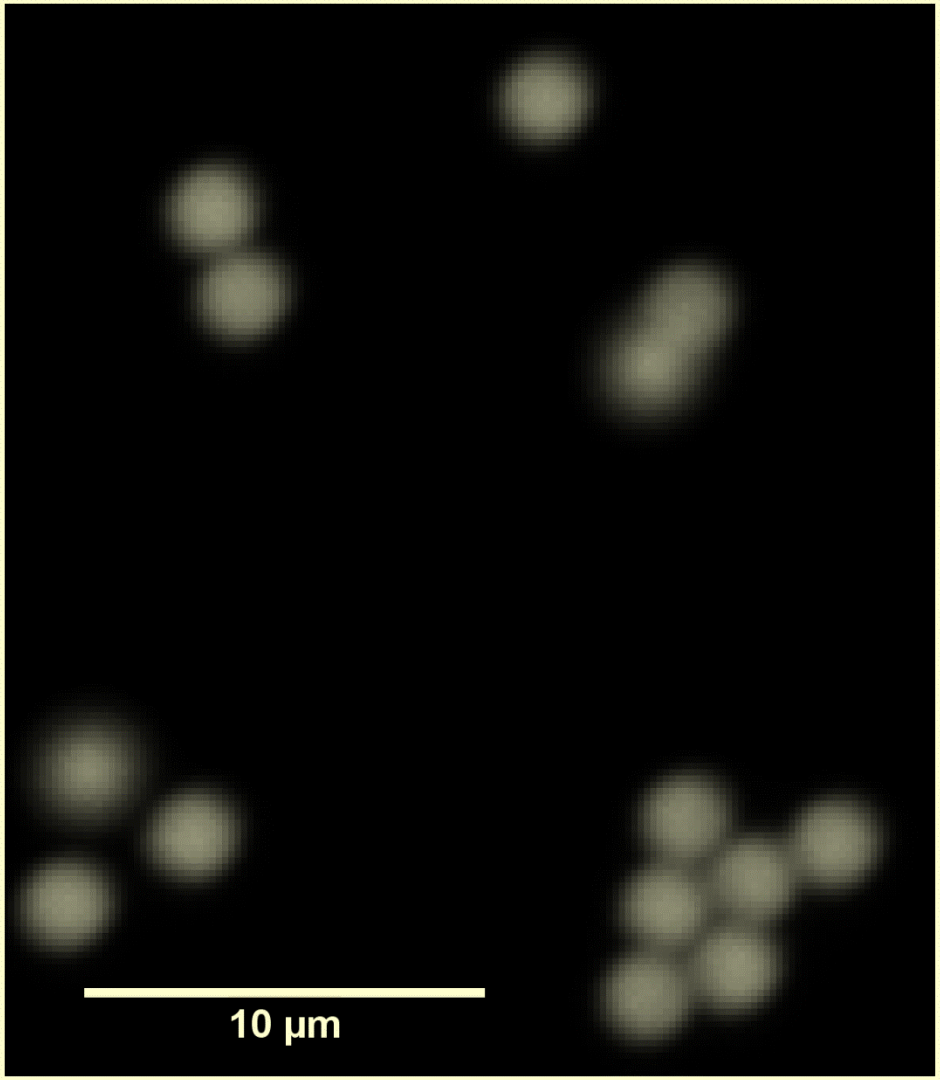
Coverslip thickness

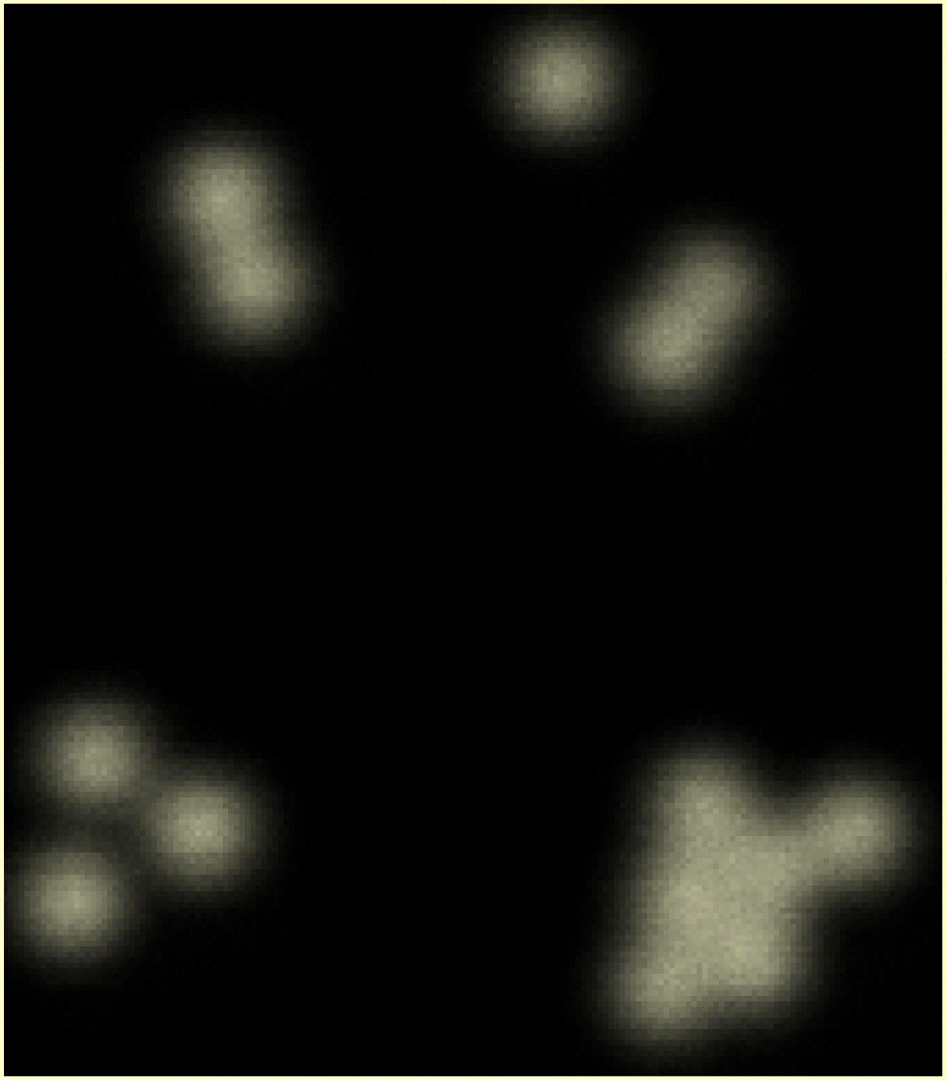
Transmission

Magnification – not very important



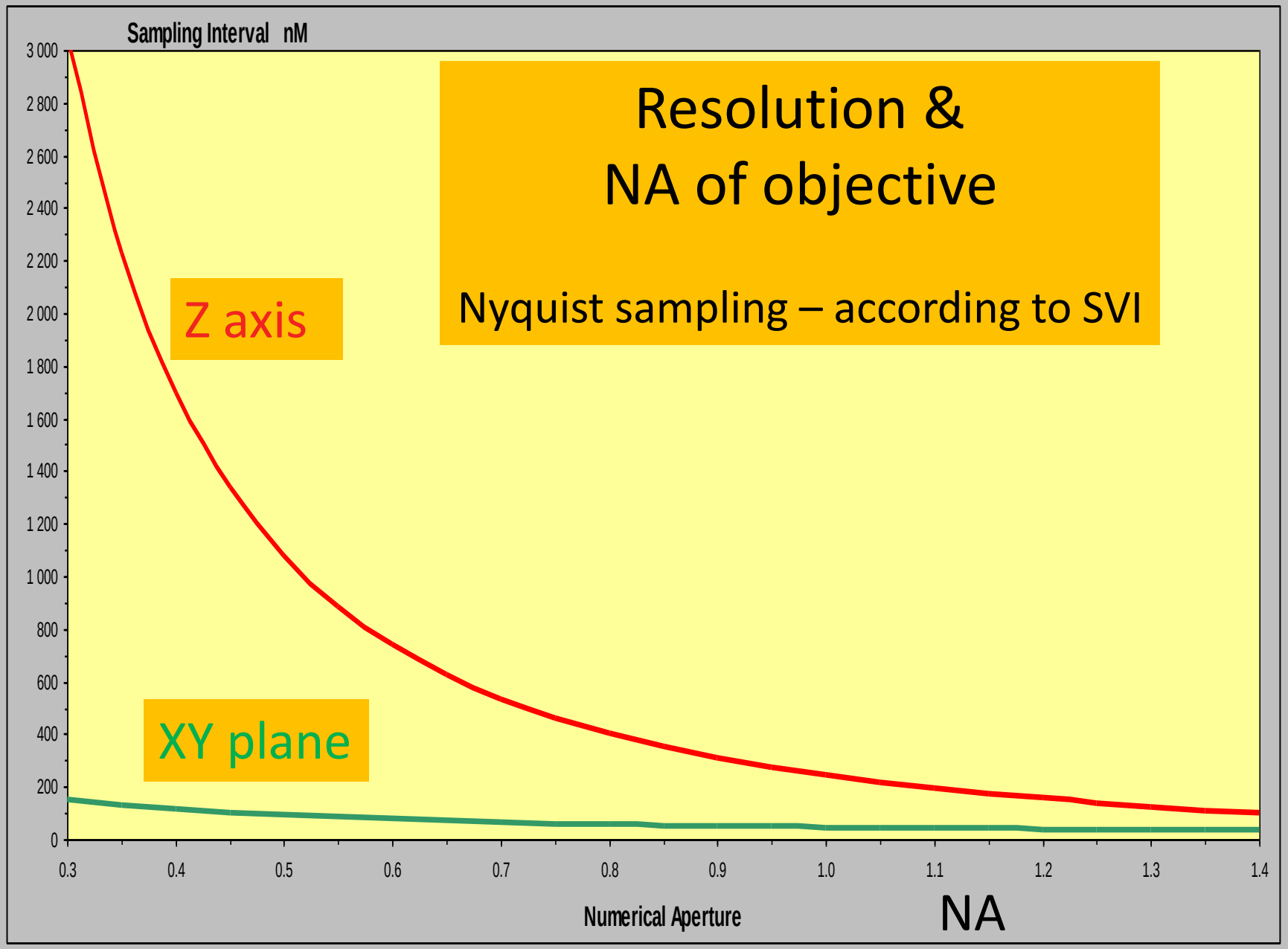
Same magnification - different NA





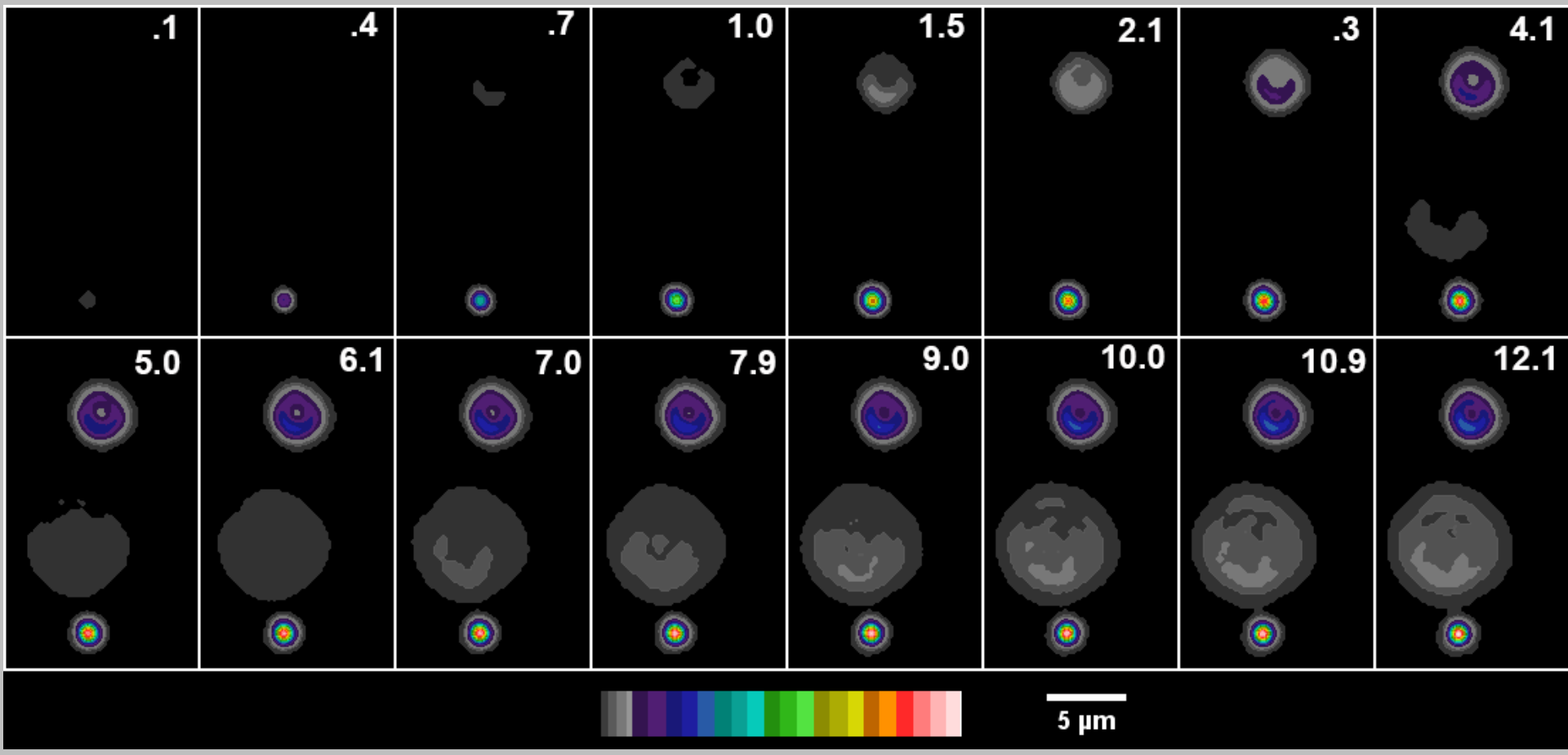
20x NA 0.8

5x NA 0.16



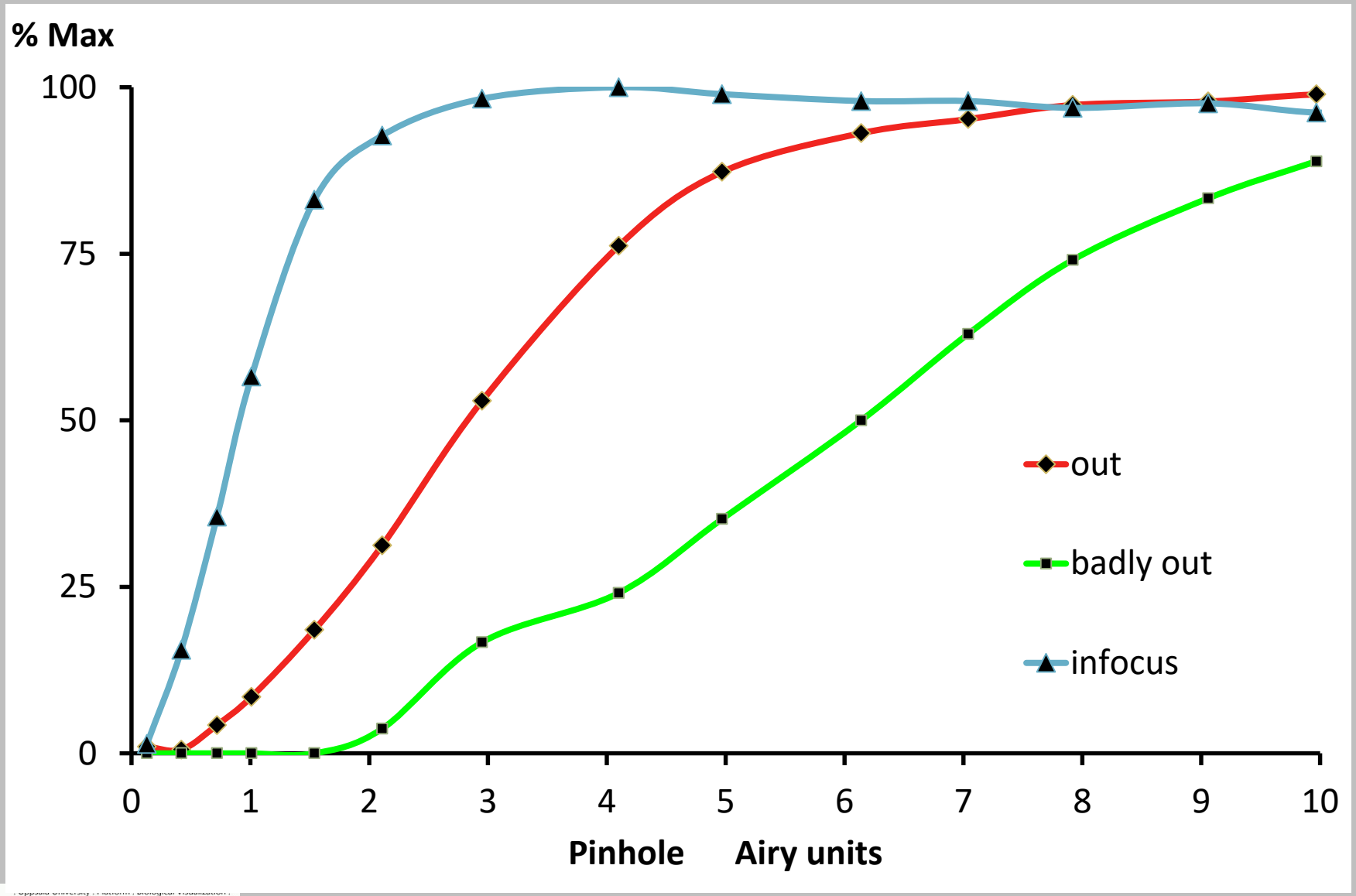
Progressively open the pinhole

More light, but out of focus light

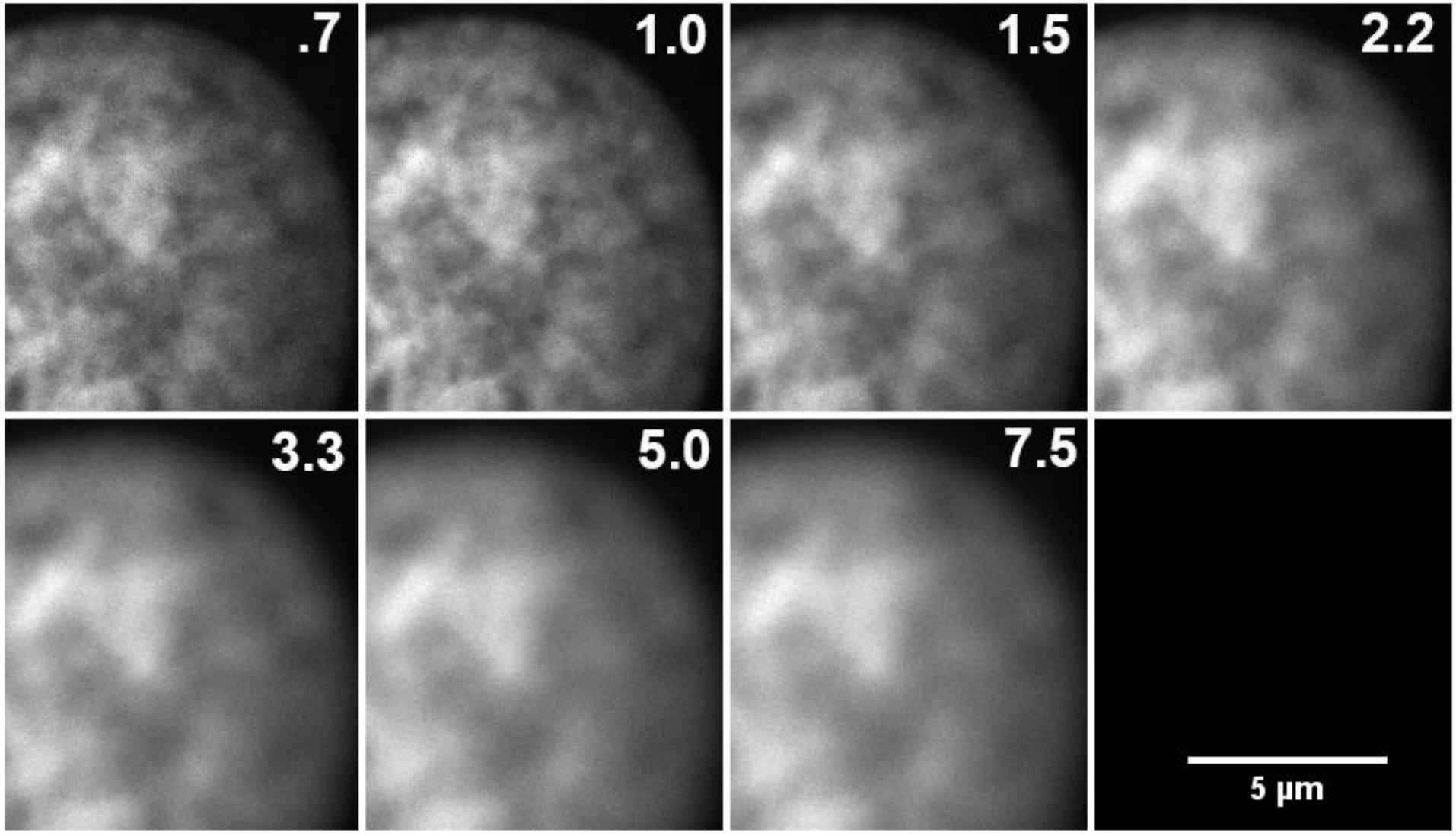


Fluorescent microspheres NA 1.4 oil objective pixels 45nm

Pinhole size and intensity

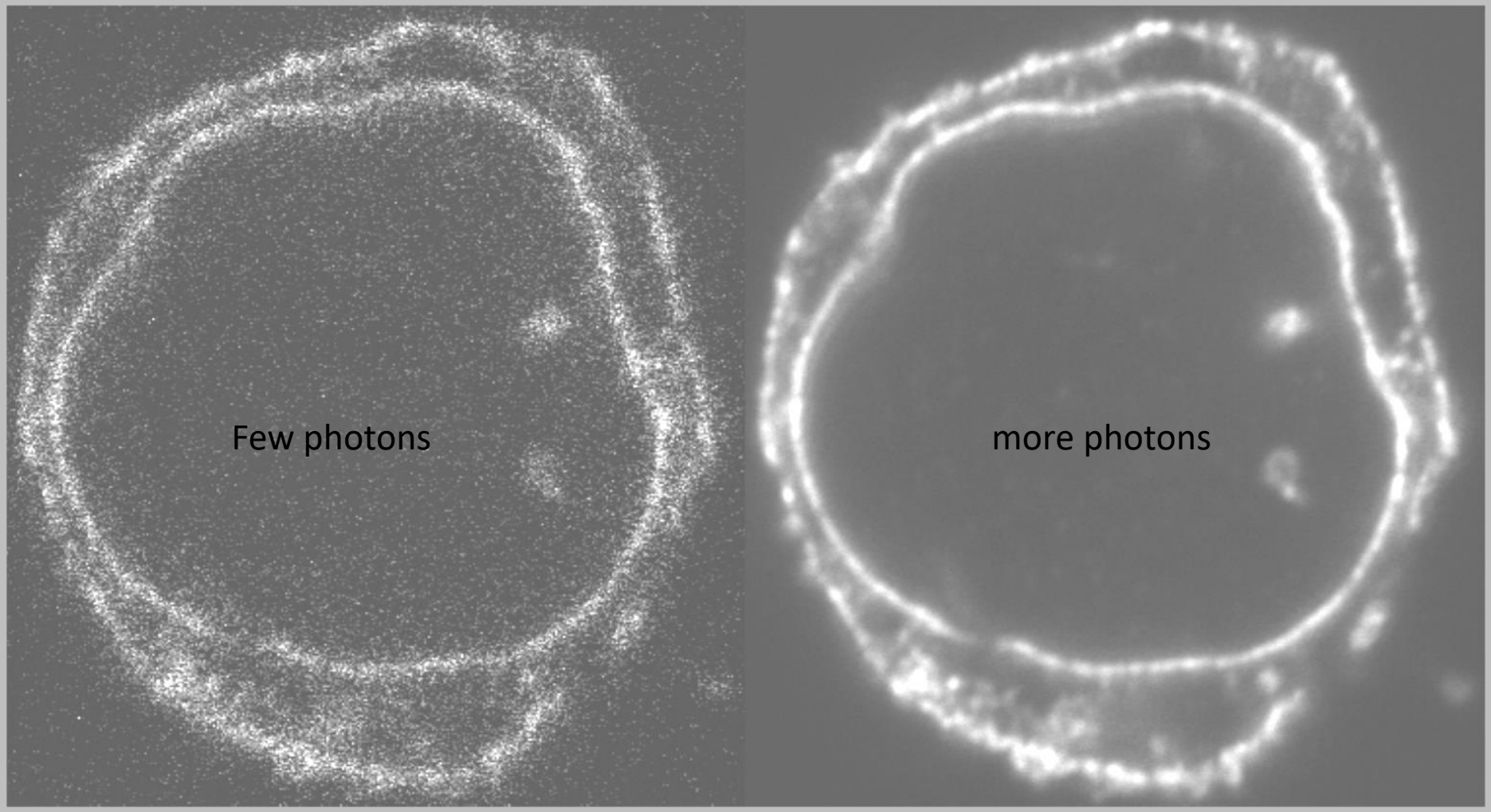


Pinhole Size: nucleus



LSM700 NA 1.4 oil adjusted for equal maximum intensity

Poisson Noise (confocal images)

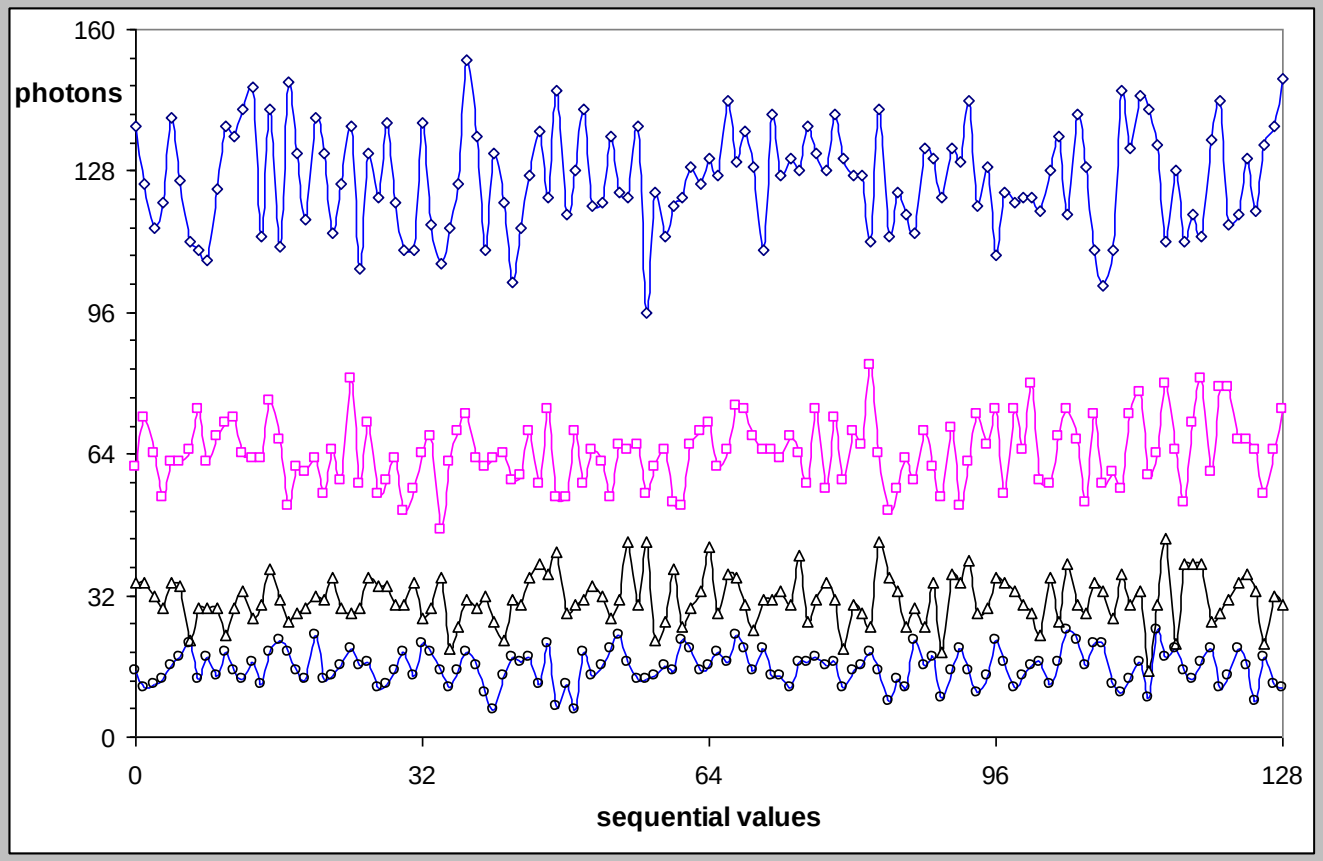


photon count is usually unknown

Limited by (i) time (ii) photobleaching & photodamage

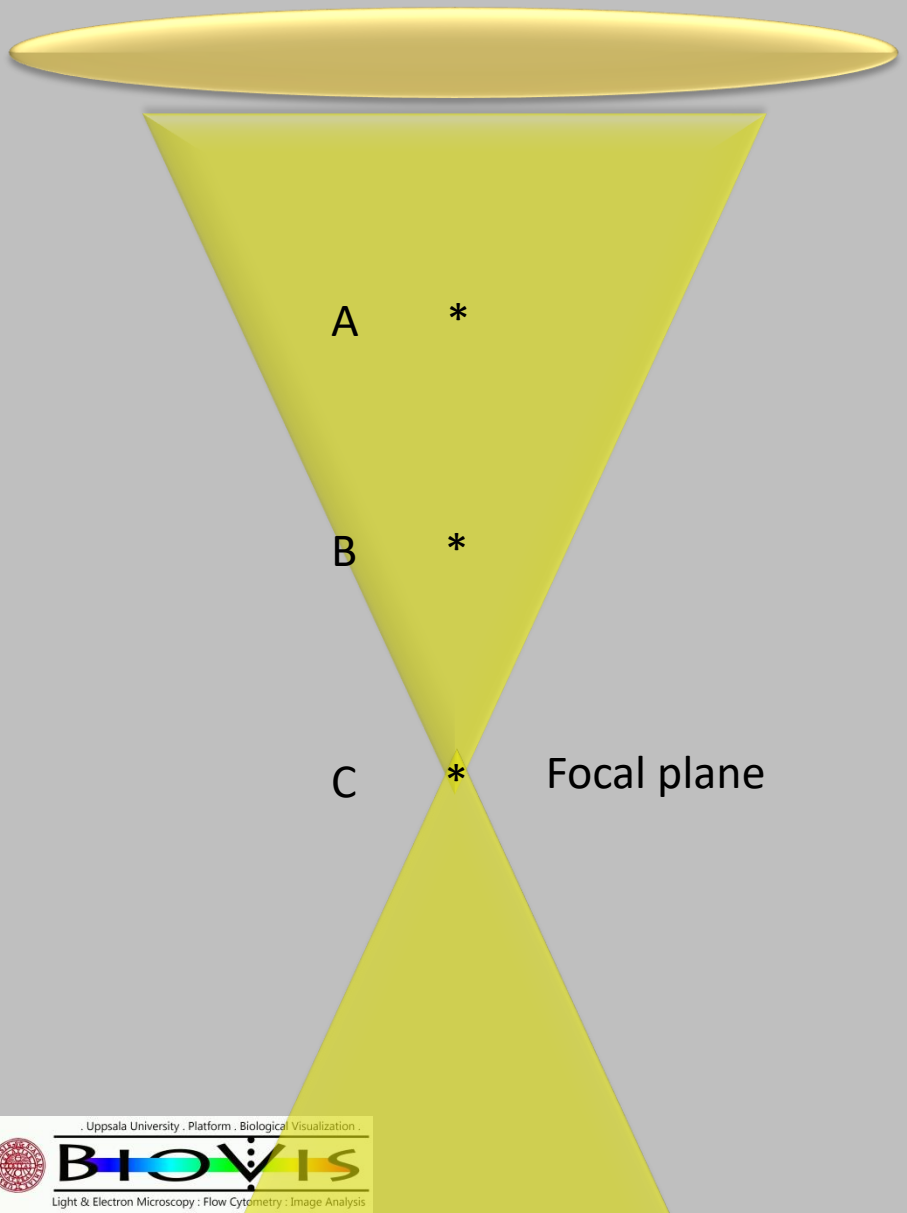
Poisson Noise – how many photons ?

Single pixels in a timeseries



photons	128	64	32	16
mean	126.31	64.26	31.22	15.72
SD	11.81	7.66	5.57	3.91
sqrt	11.31	8.00	5.66	4.00

More Photons – increase laser power ?



Lens

But

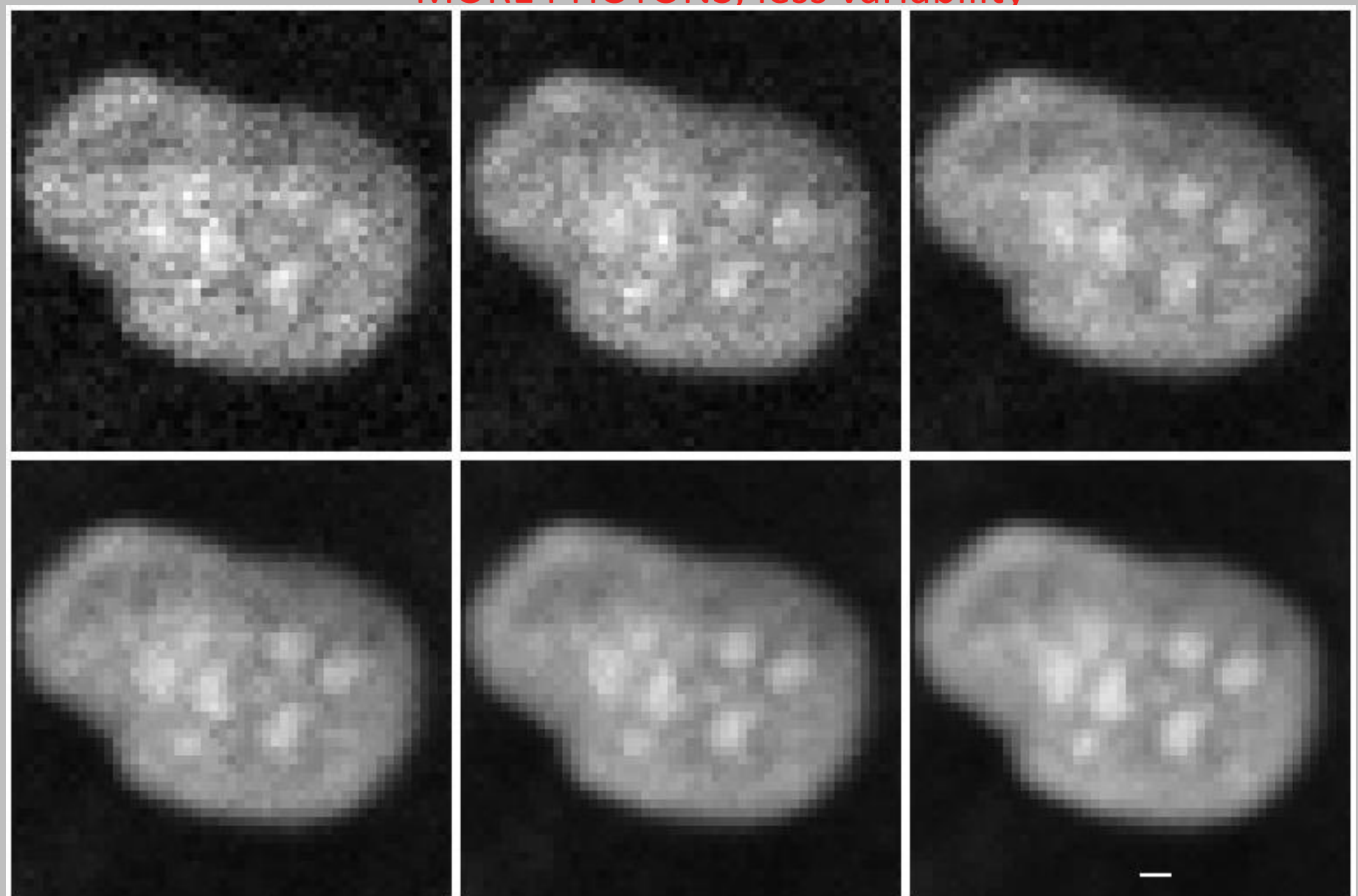
Fluorophore saturation

More photons from fluorophores outside the point of focus

Improving Image Quality

Doubling the image acquisition time:

MORE PHOTONS, less variability



Confocal Illumination

Lasers

monochromatic coherent

types: gas, solid, diode

Argon Ion	353-361, 488, 514 nm
Krypton -Argon	488, 568, 647 nm
Helium Neon	543 nm, 633 nm
Helium Cadmium	543 nm, 633 nm
Diode lasers	405, 488, 635 nm etc

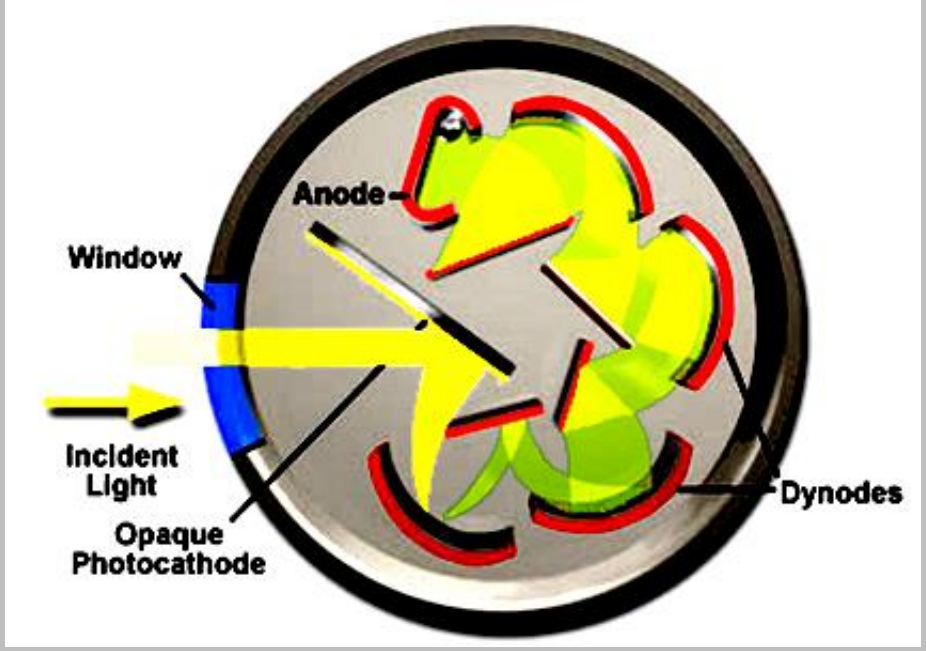
individual lines

The Photomultiplier tube (PMT) 1930s

One Photon hits photocathode

emits photoelectron

which cascades along the application chain



Photon counting possible

Adjust:

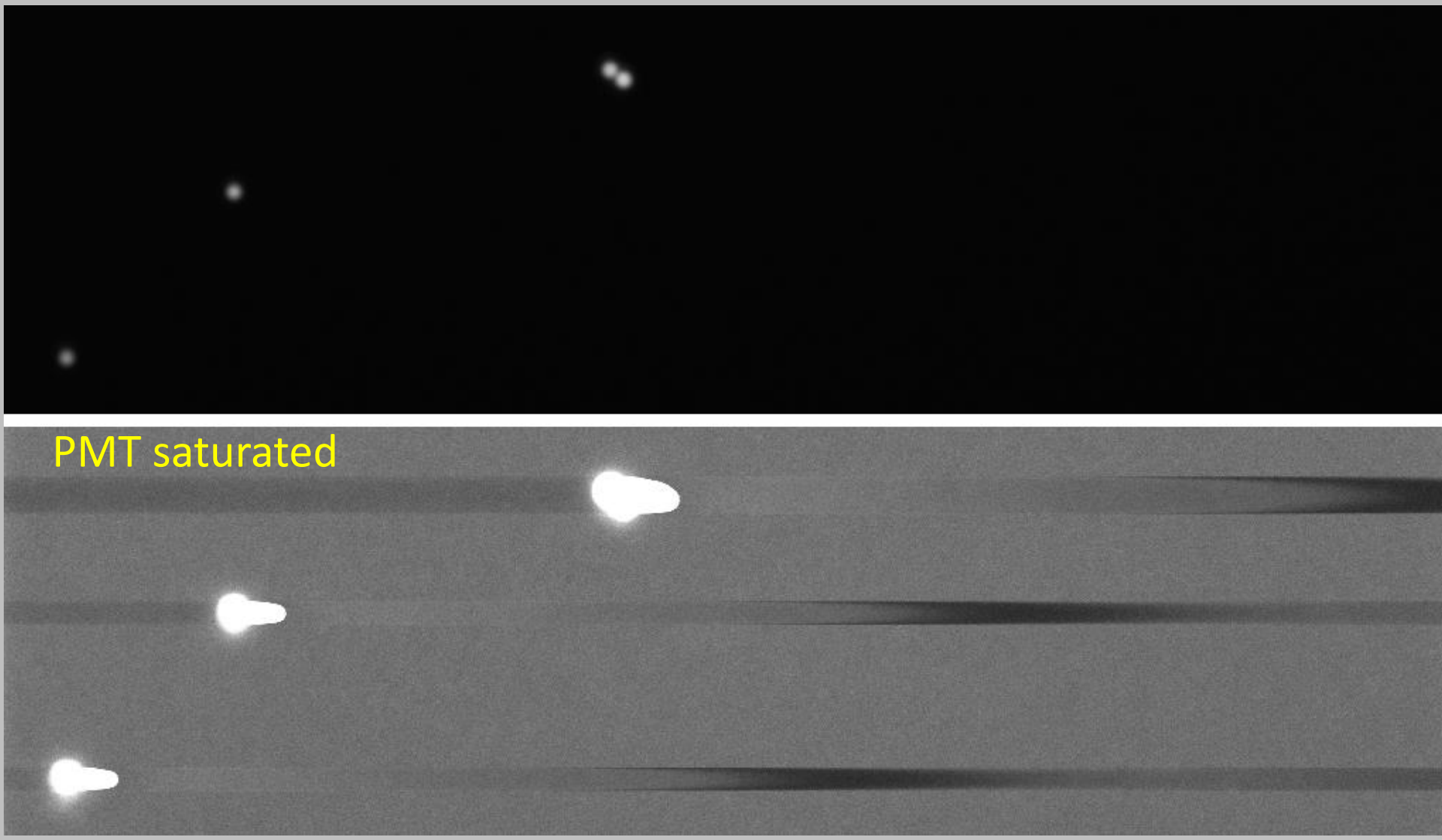
1) Gain (voltage)
2) Zero - offset

PMTs reach 30% quantum efficiency

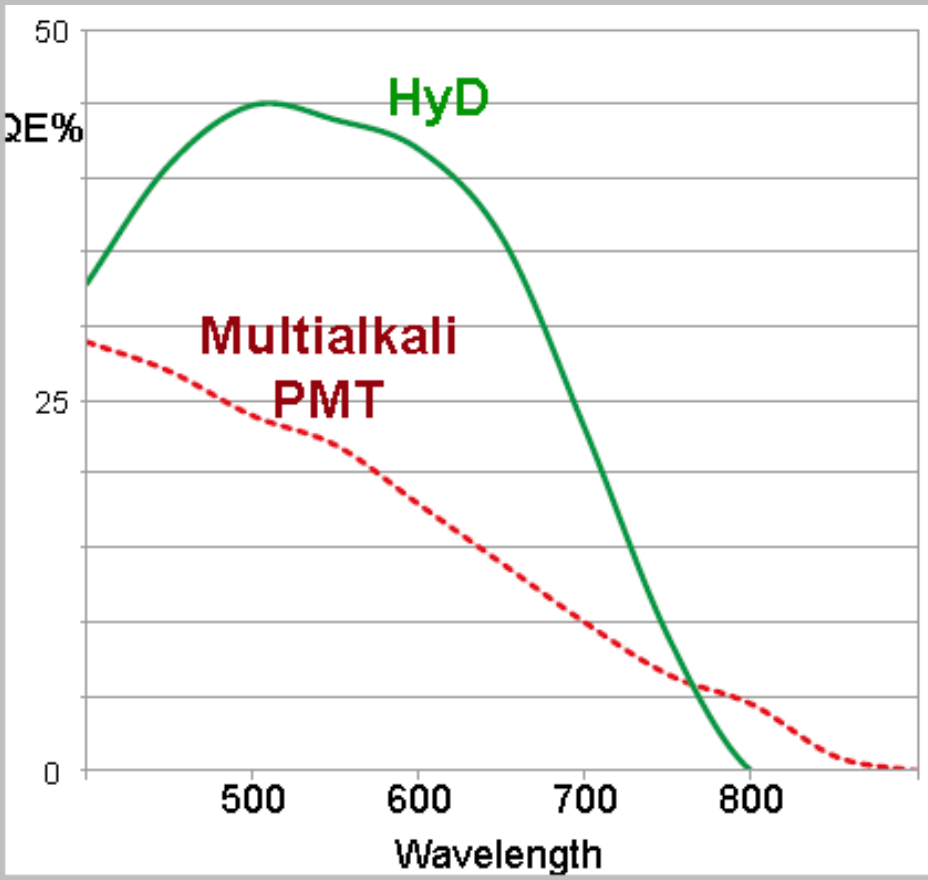
How good are cameras ?

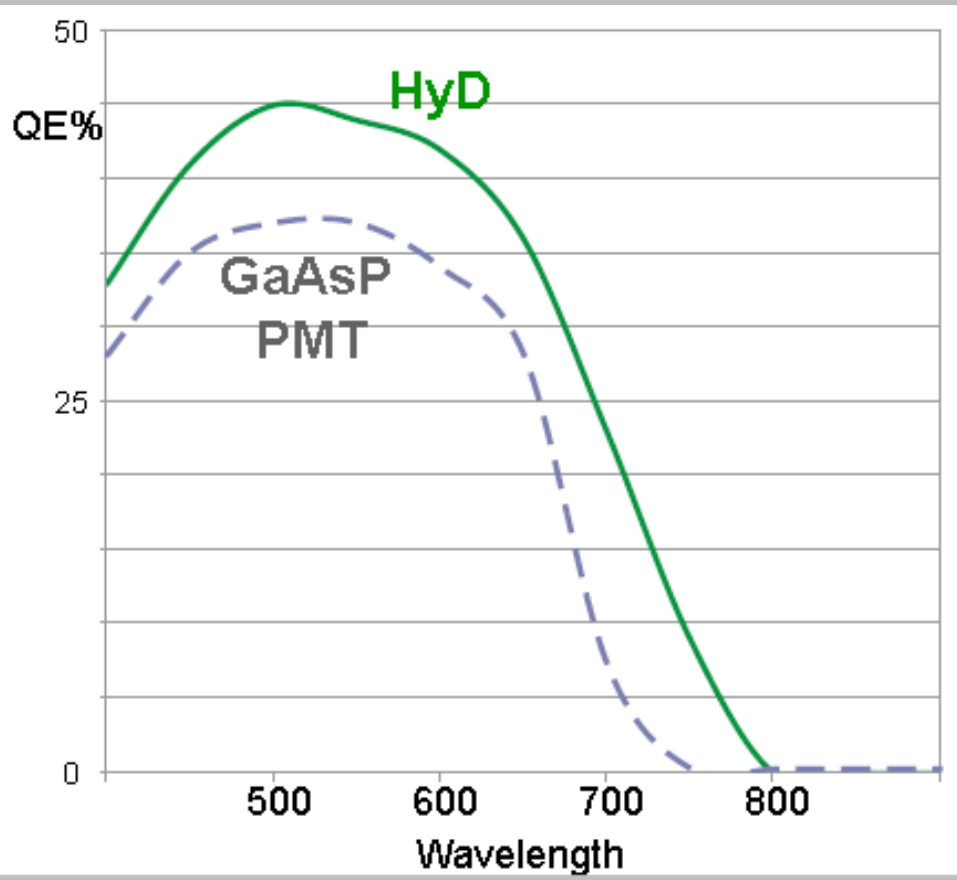
PMT saturation

Point scanning



Confocal; detectors

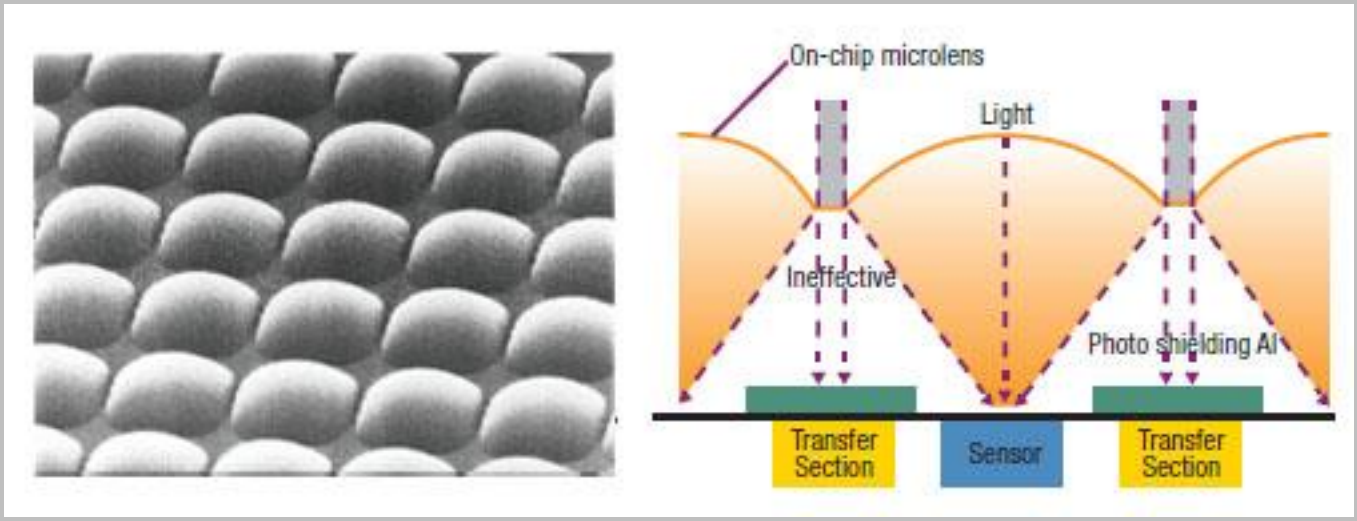




from Leica

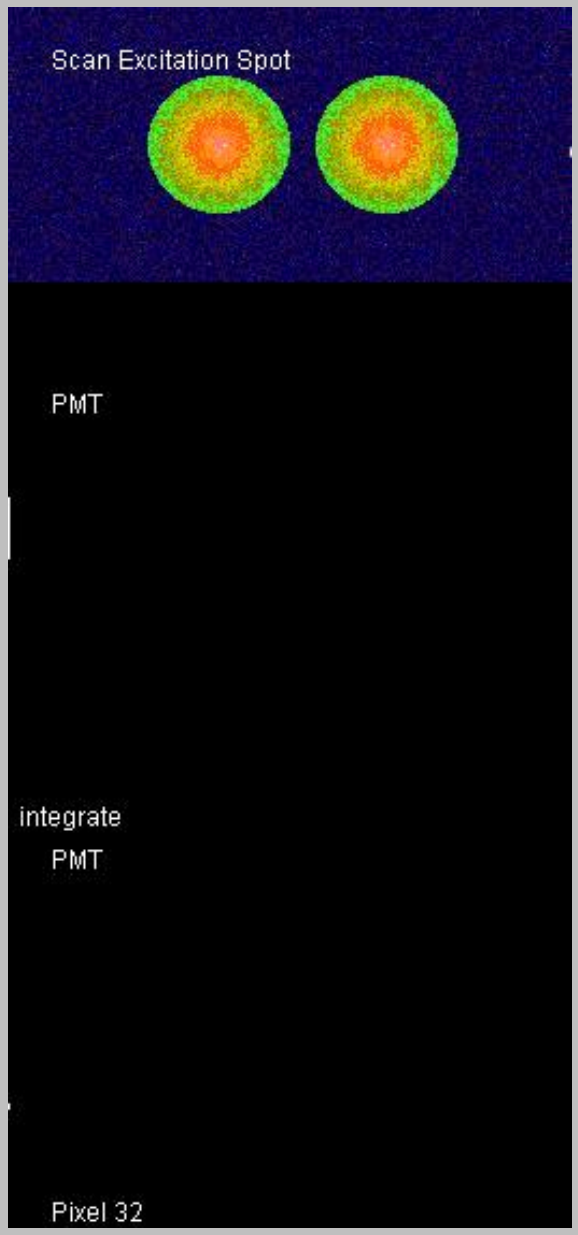
Where do pixels come from ?

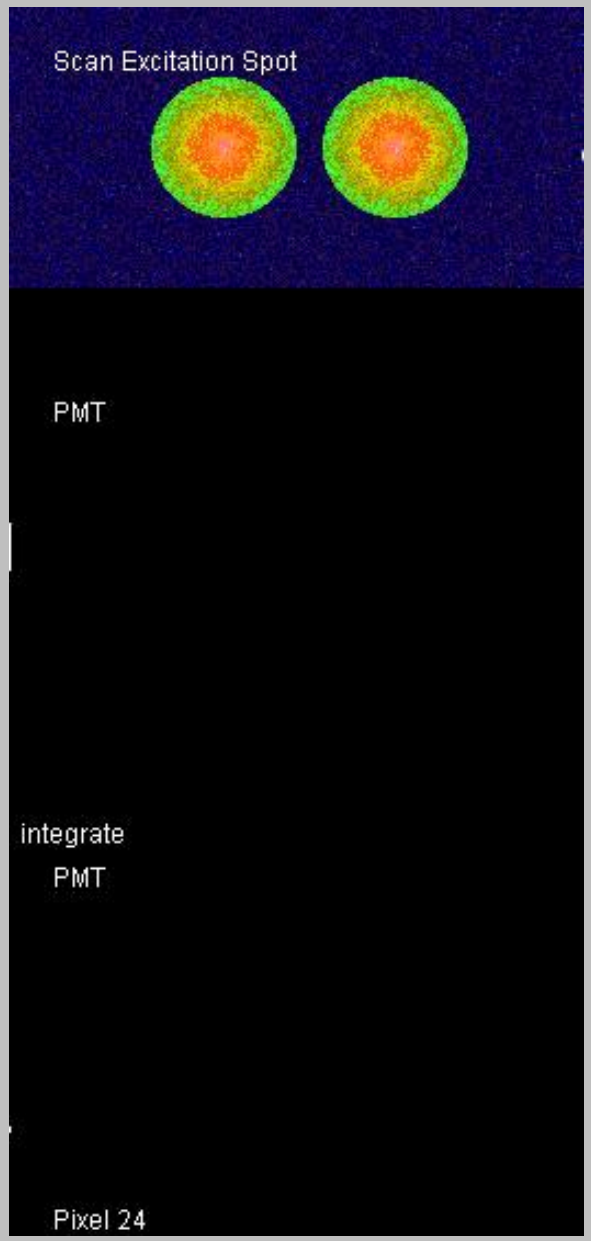
Camera

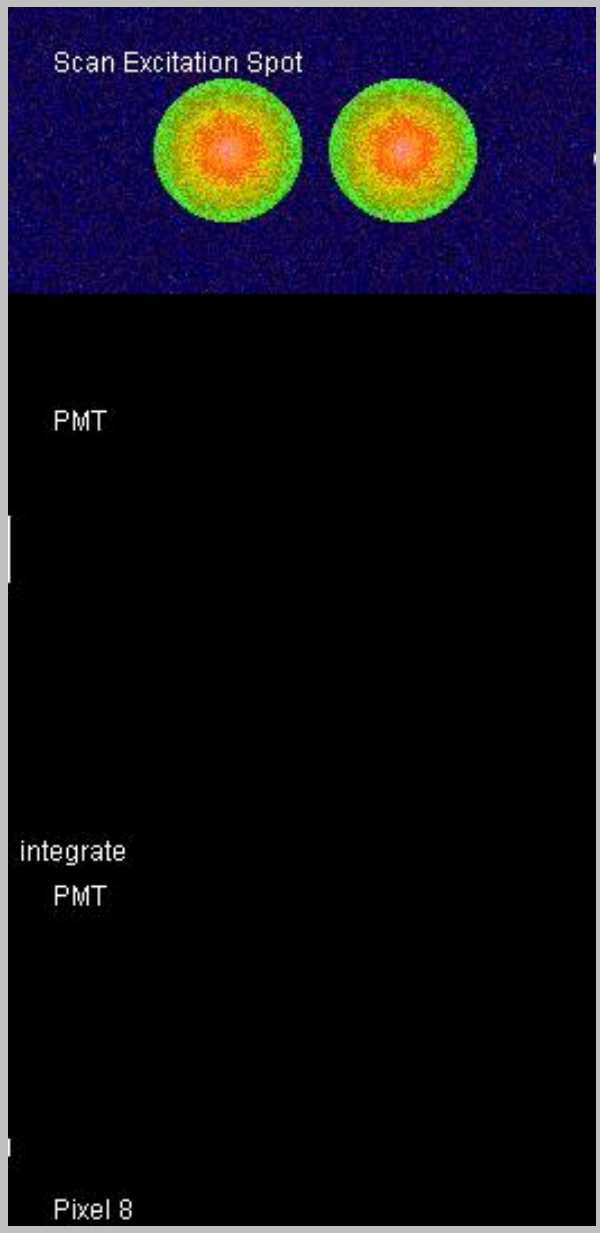


Confocal ?

Pixels in Confocal Microscope







Pixels

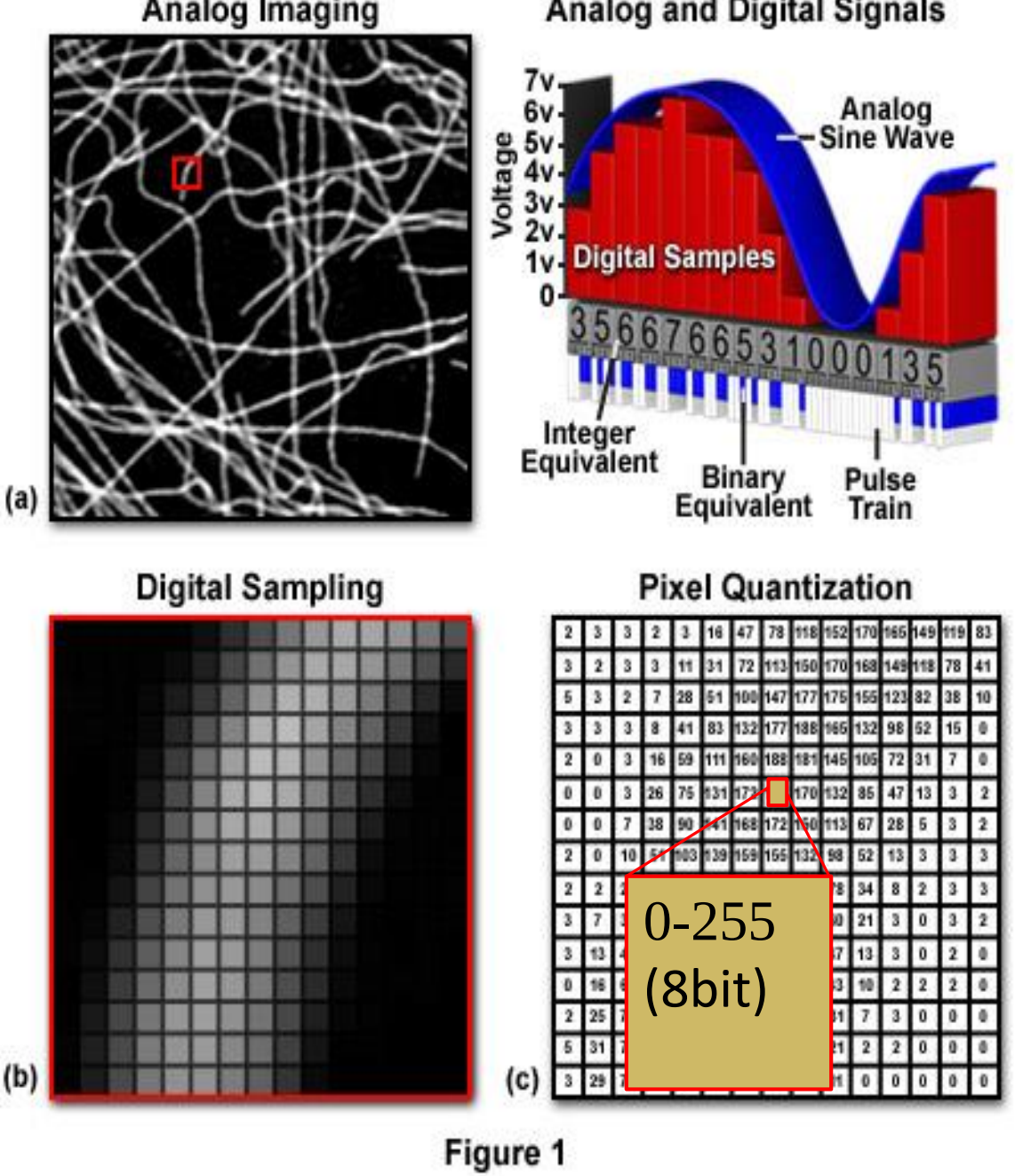


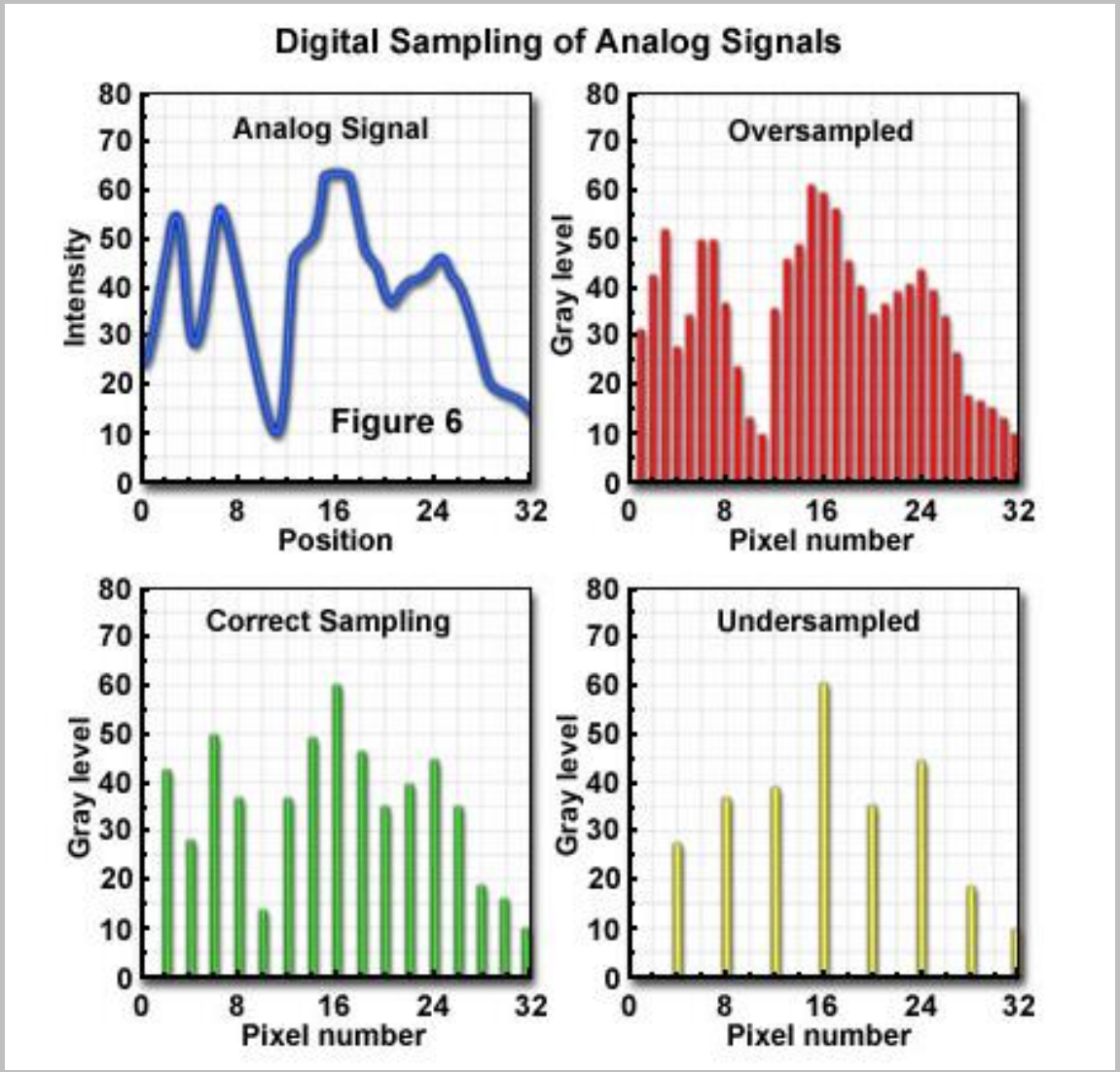
Figure 1

The signal from the PMT is digitized

How large should pixels be ?

Nyquist

sampling theory



to reconstruct a pure sine wave, sample at least twice in each cycle, 2x the frequency.

Nyquist Pixel size: fluorophore and NA

an OPTIMAL button in confocal software
calculates the (Zeiss) Nyquist pixel size

LSM700		Widefield	Nyquist	Confocal	Nyquist
	NA	x,y nm	Z nm	x,y nm	Z nm
5x	0.16	812	20181	381	9469
10x	0.5	260	1940	122	910
20x	0.8	162	650	76	305
40x	0.95	136	378	64	177
63x oil	1.4	92	277	43	130
63x water	1.2	108	348	50	163

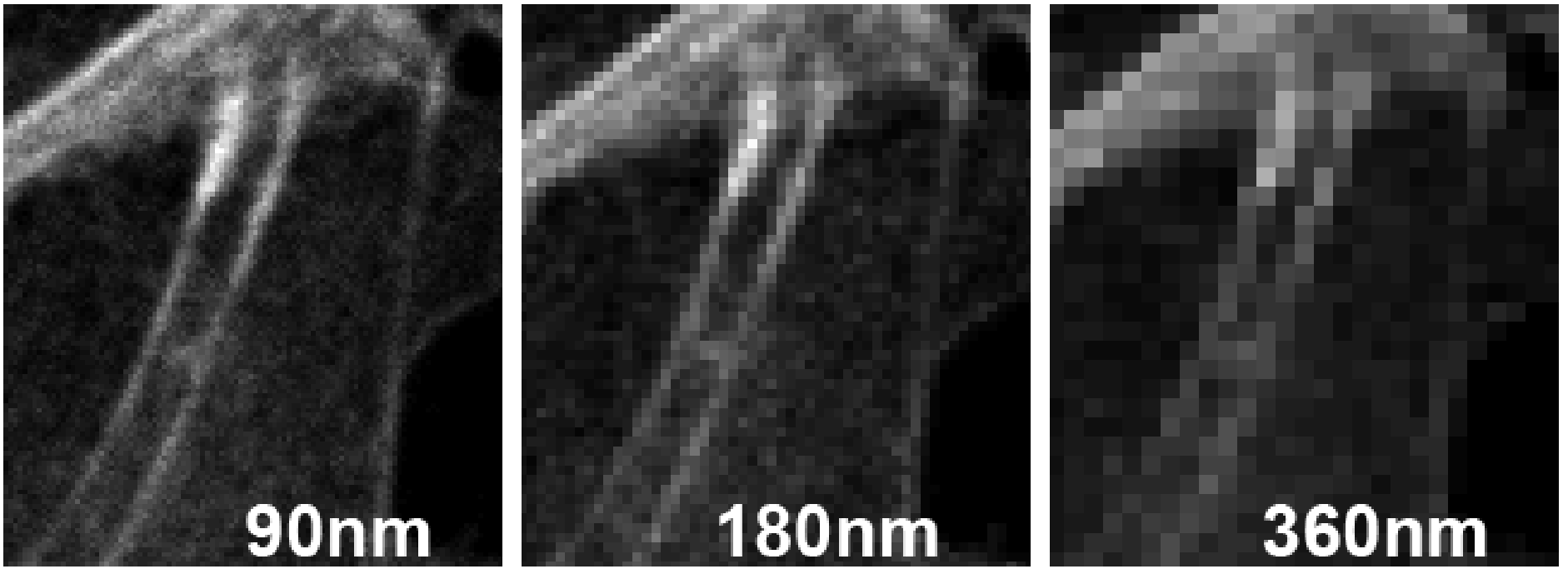
In Practice: Compromise between

Image quality: noise & resolution

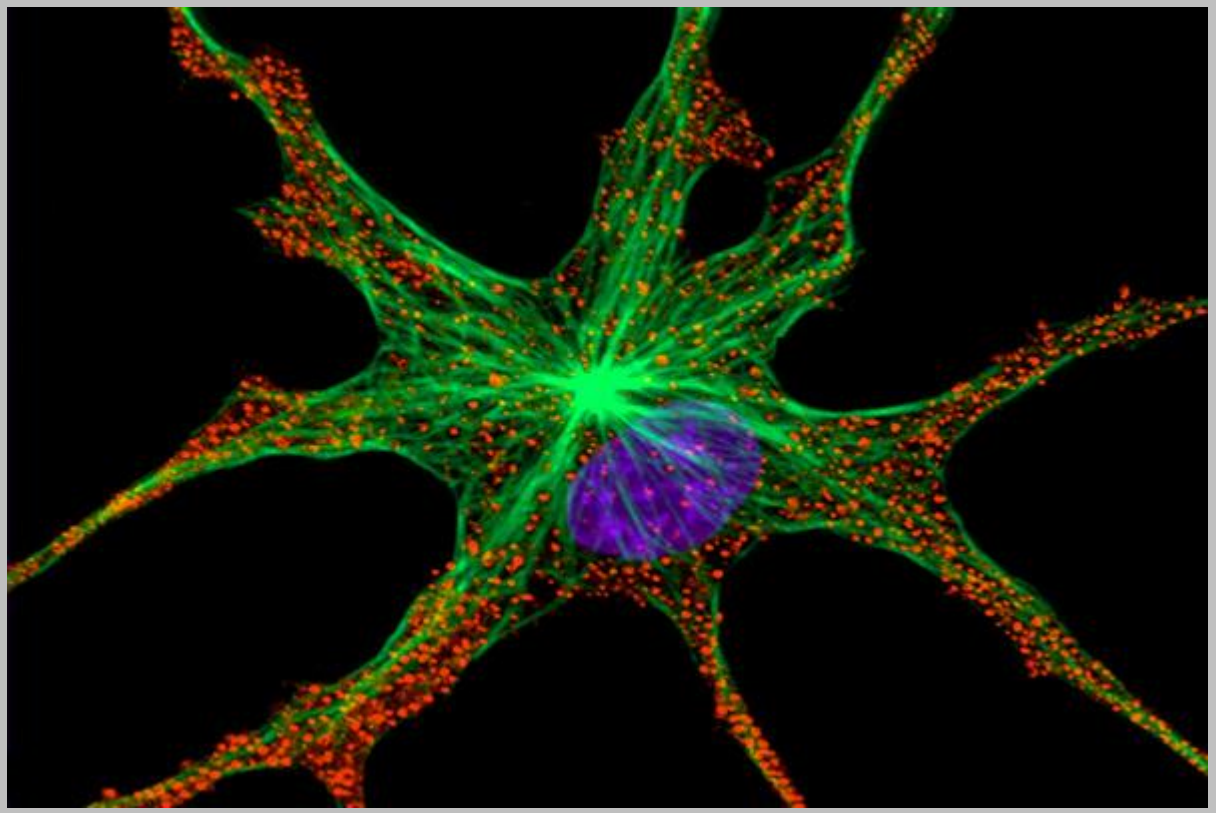
V

Acquisition time, bleaching, photodamage

Pixels – how large ?



IMAGING WITH LSM



MRC 500 confocal microscope

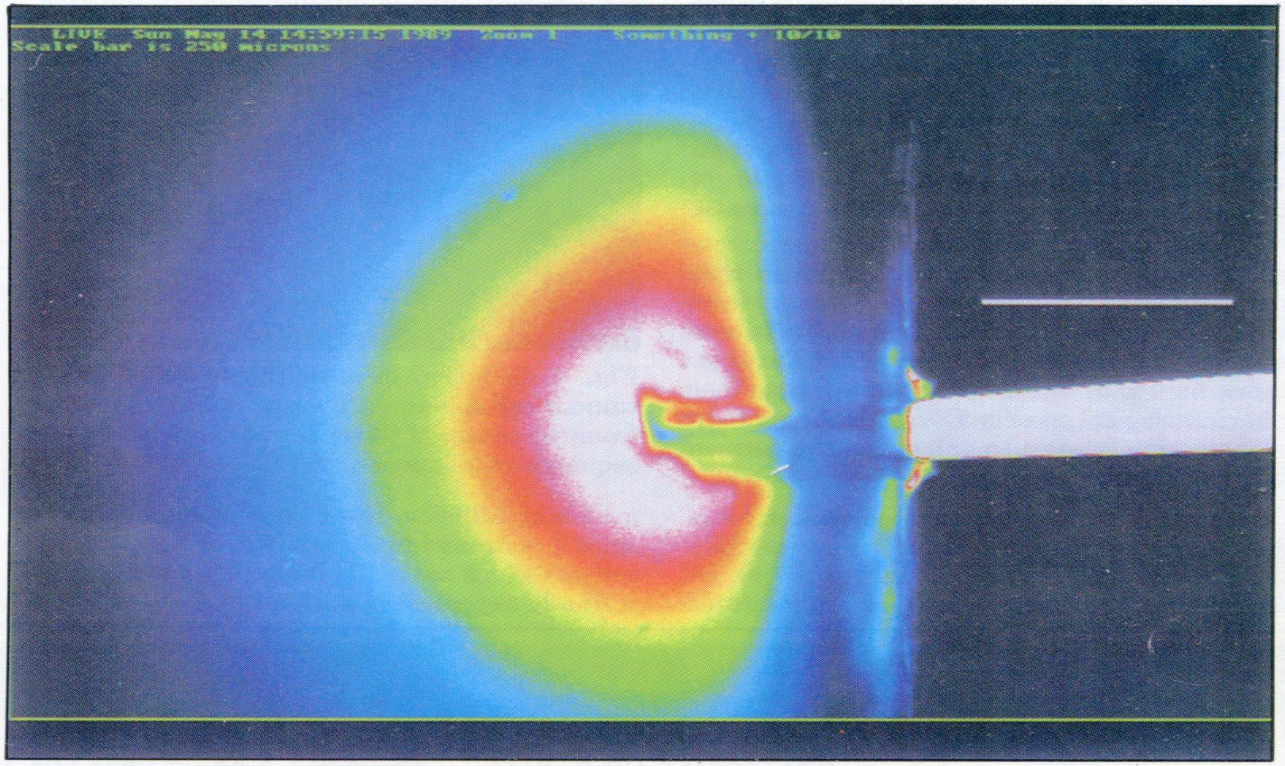


Fig 1. Diffusion of fluorescein from a micropipette into an agar block.

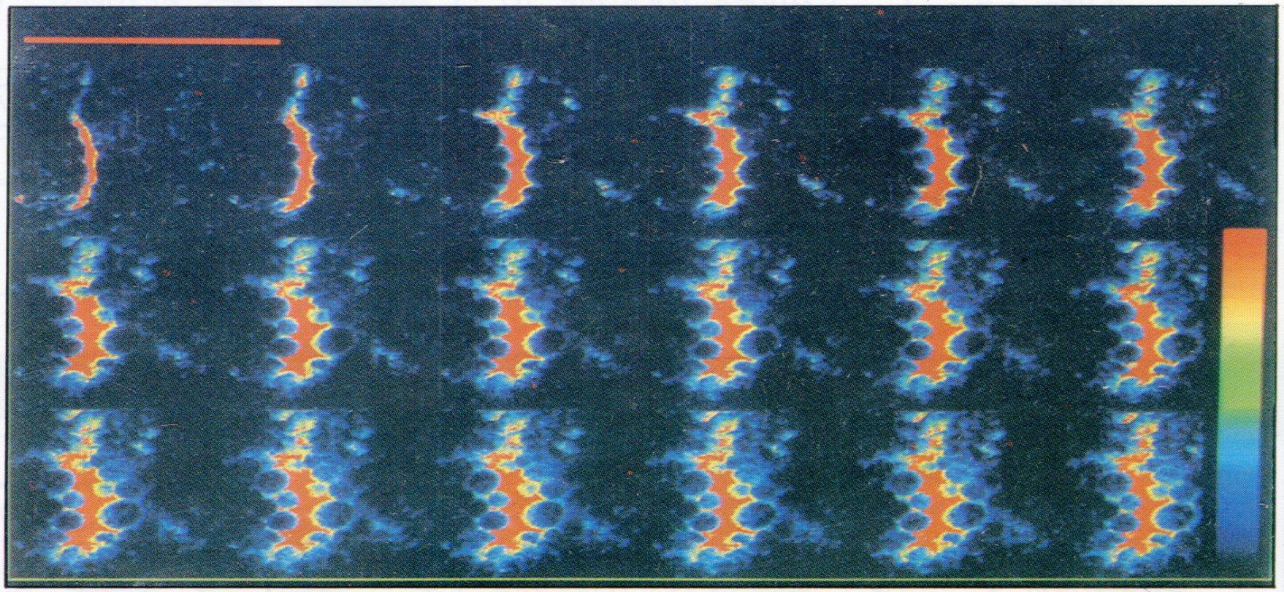
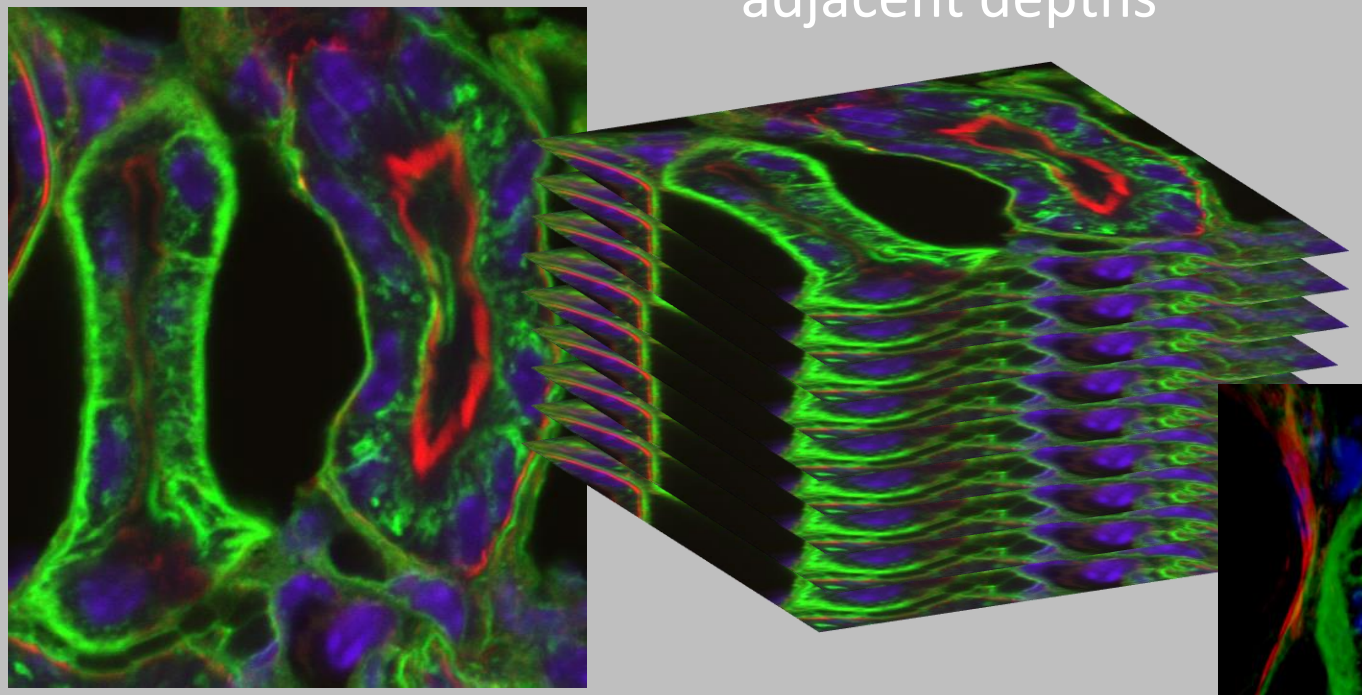


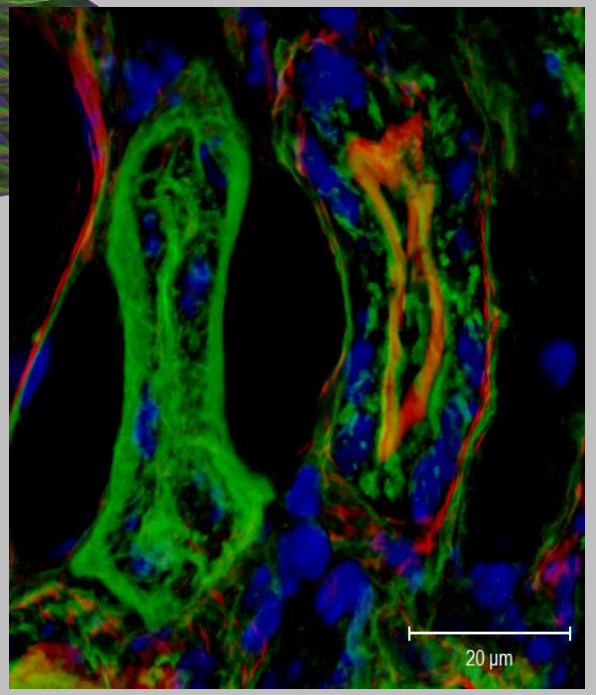
Fig. 2. Sequence (2 second intervals) of fluorescein diffusing from a capillary into adipose tissue.

Optical sectioning: Z series

Optical slice from certain depth in sample

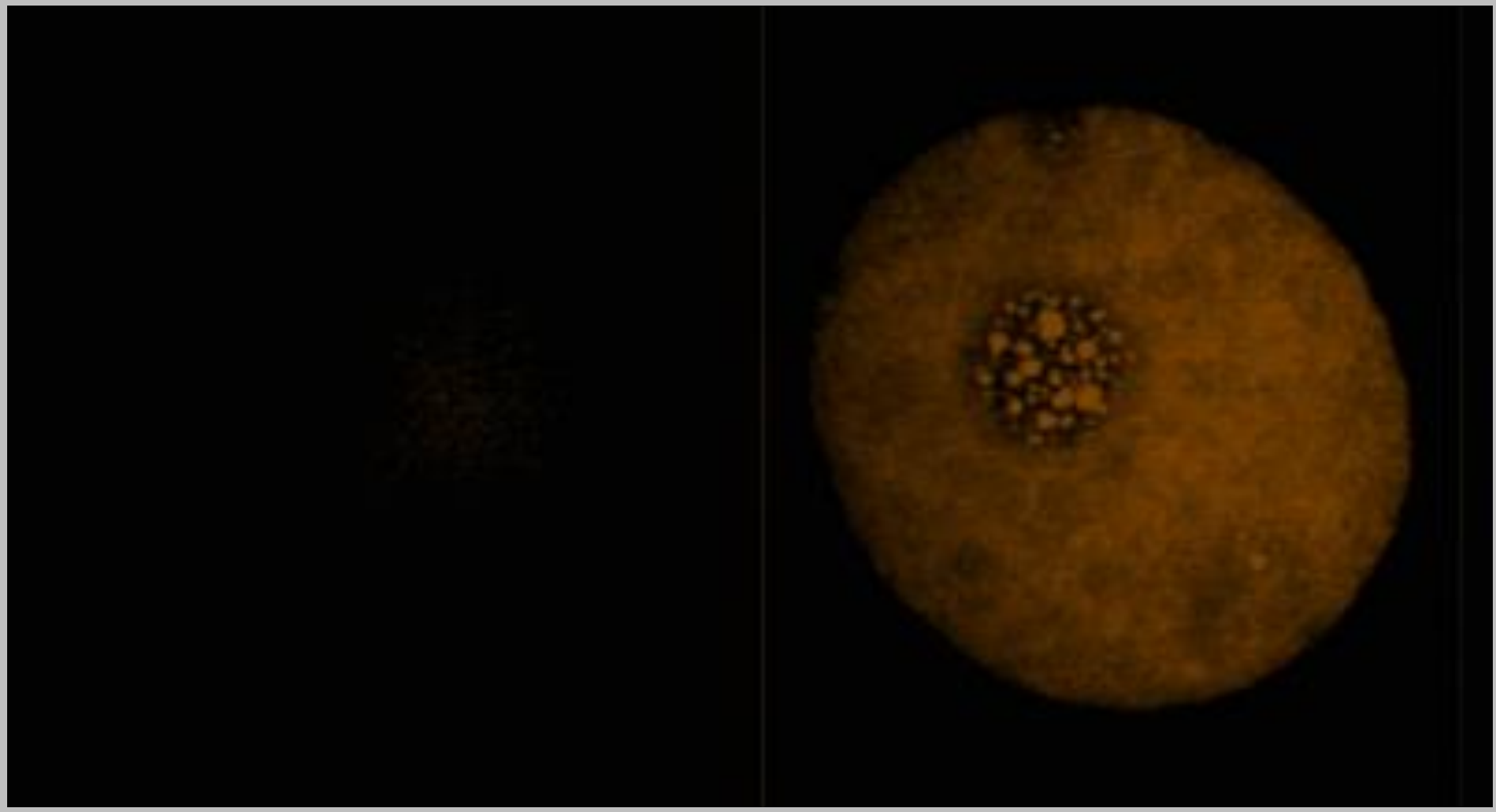
Many slices from adjacent depths



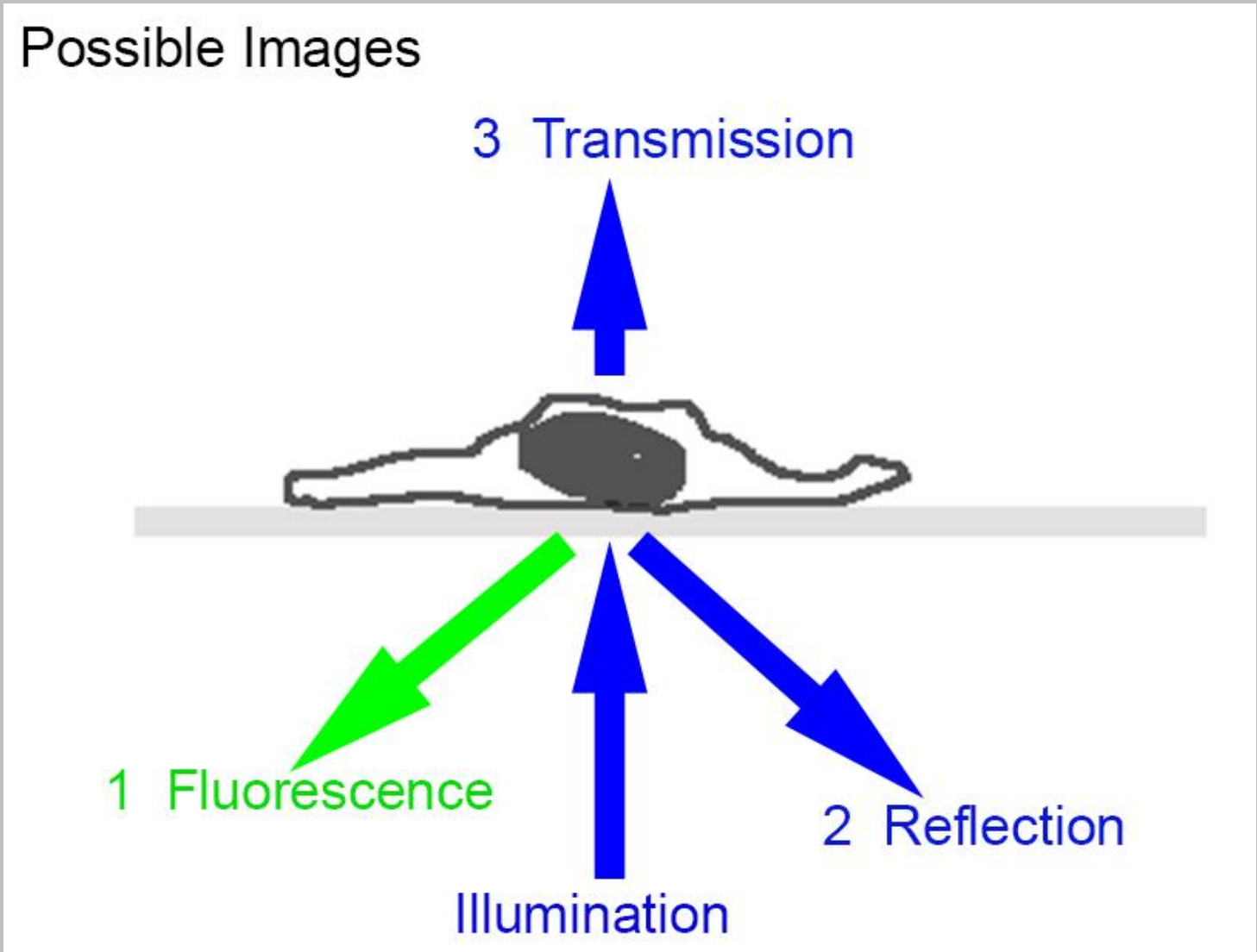


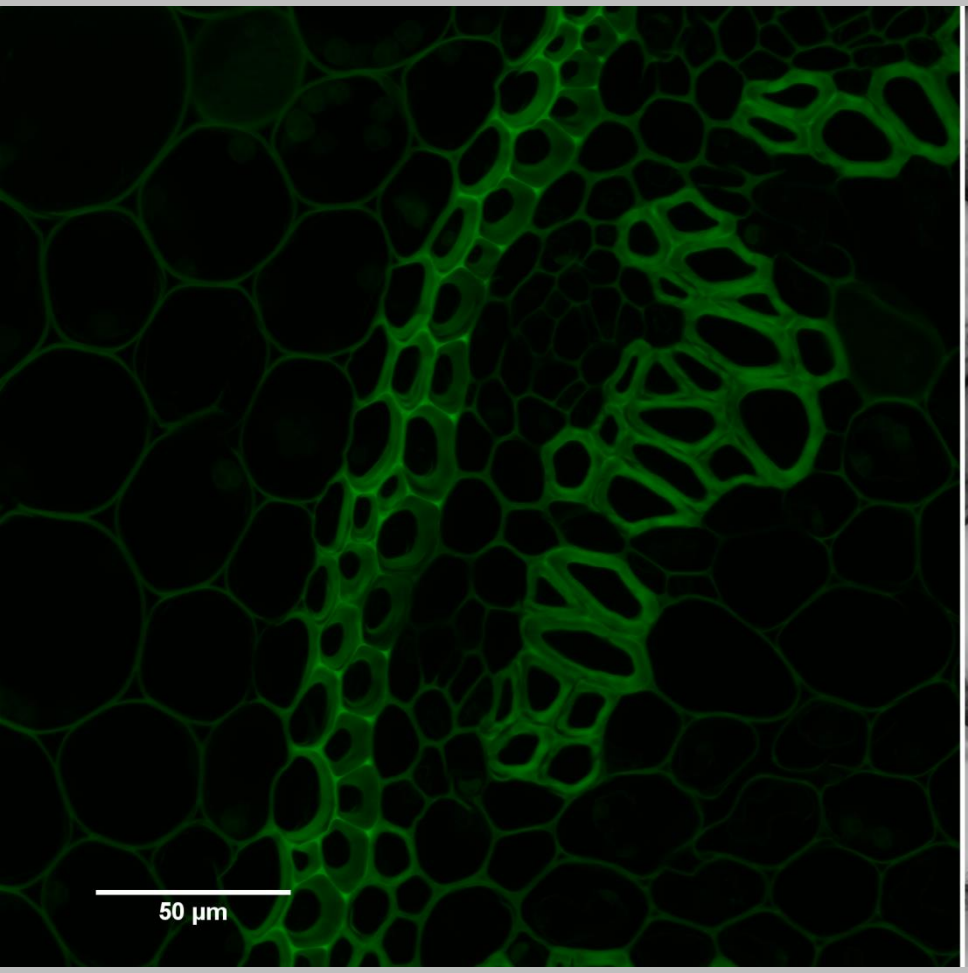
reconstruction from slices

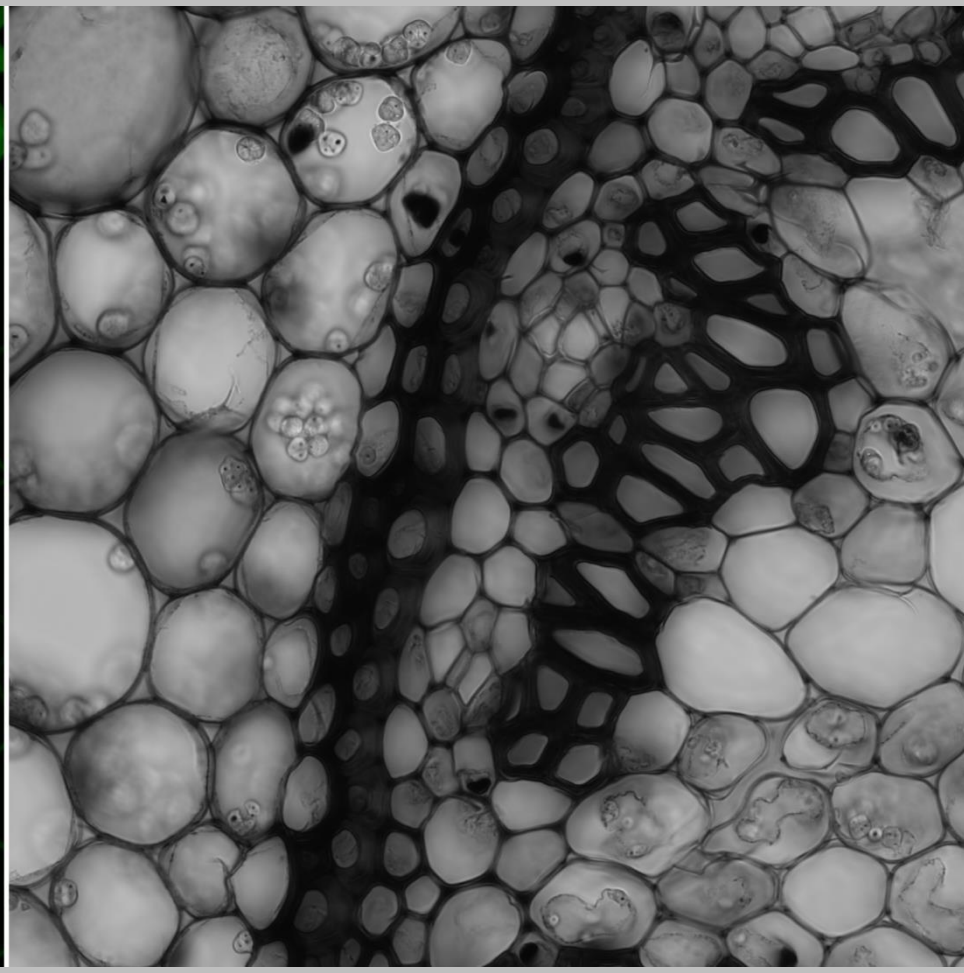
Z series - 3D rendered



Imaging with a Confocal







Fluorescence (excite 555nm)

Transmission (555nm)

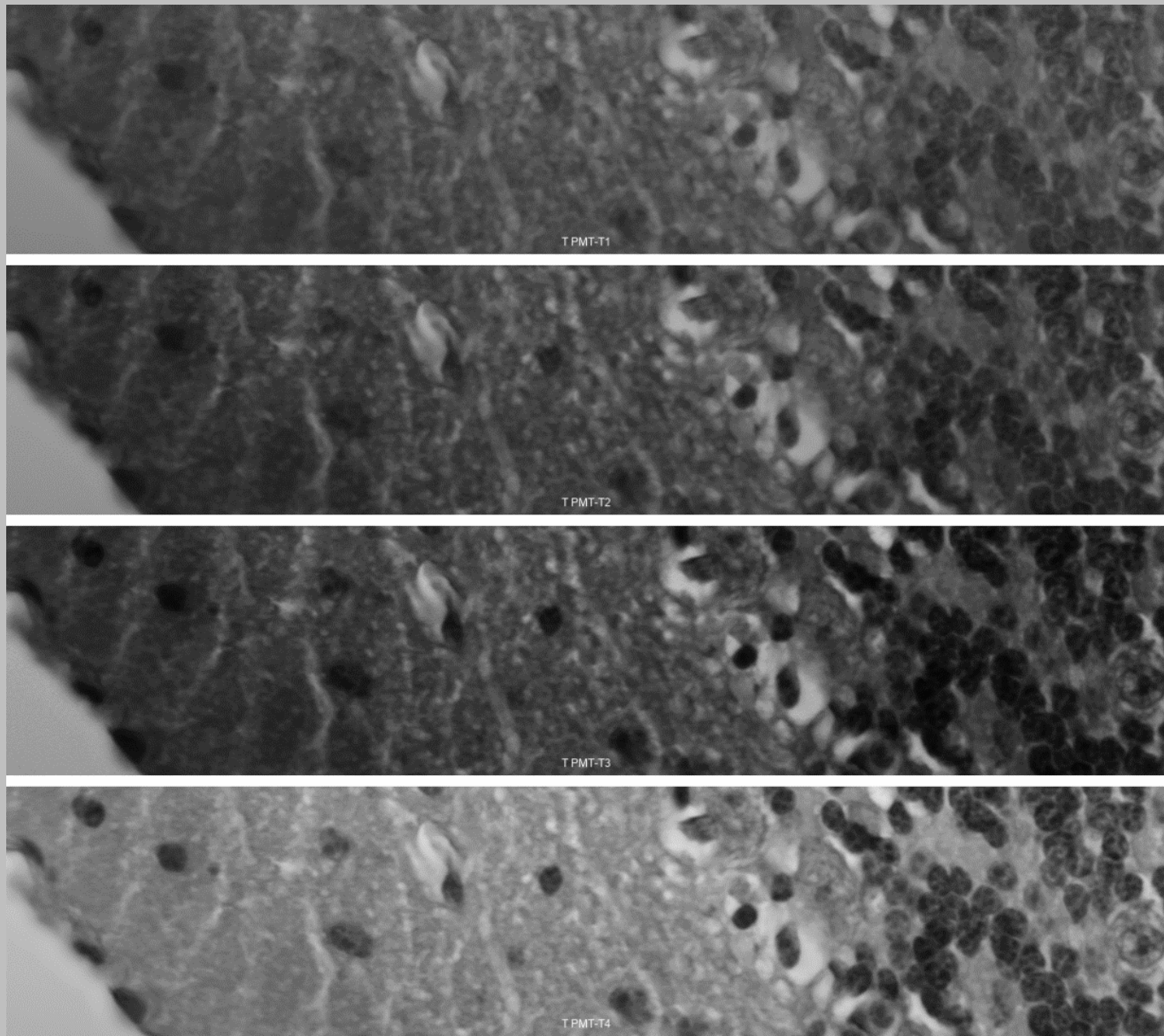
Transmitted light: 4 laser lines (H & E staining)

405nm

488nm

555nm

639nm

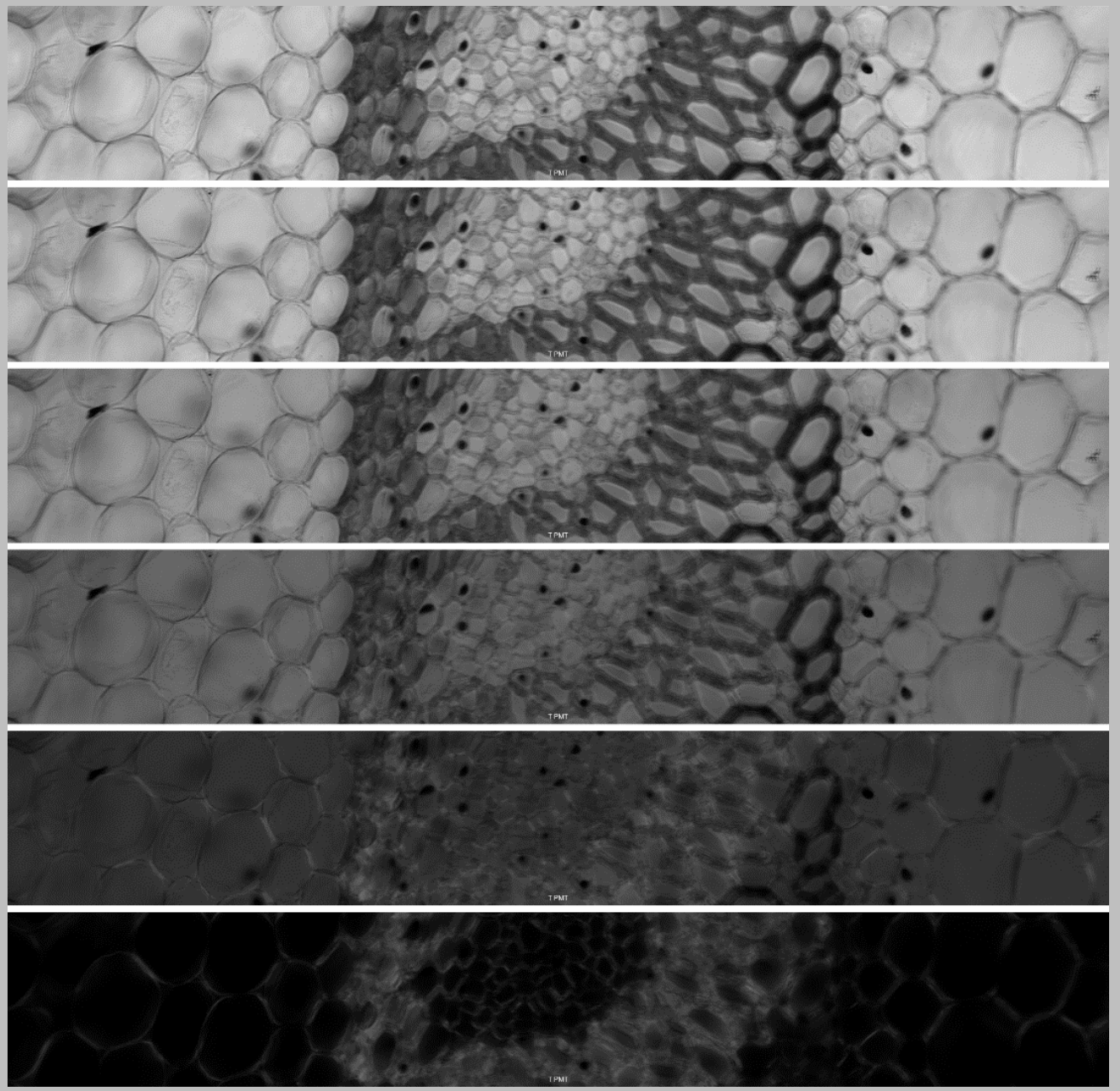


Laser light is polarized

Transmitted: specimen can change polarization

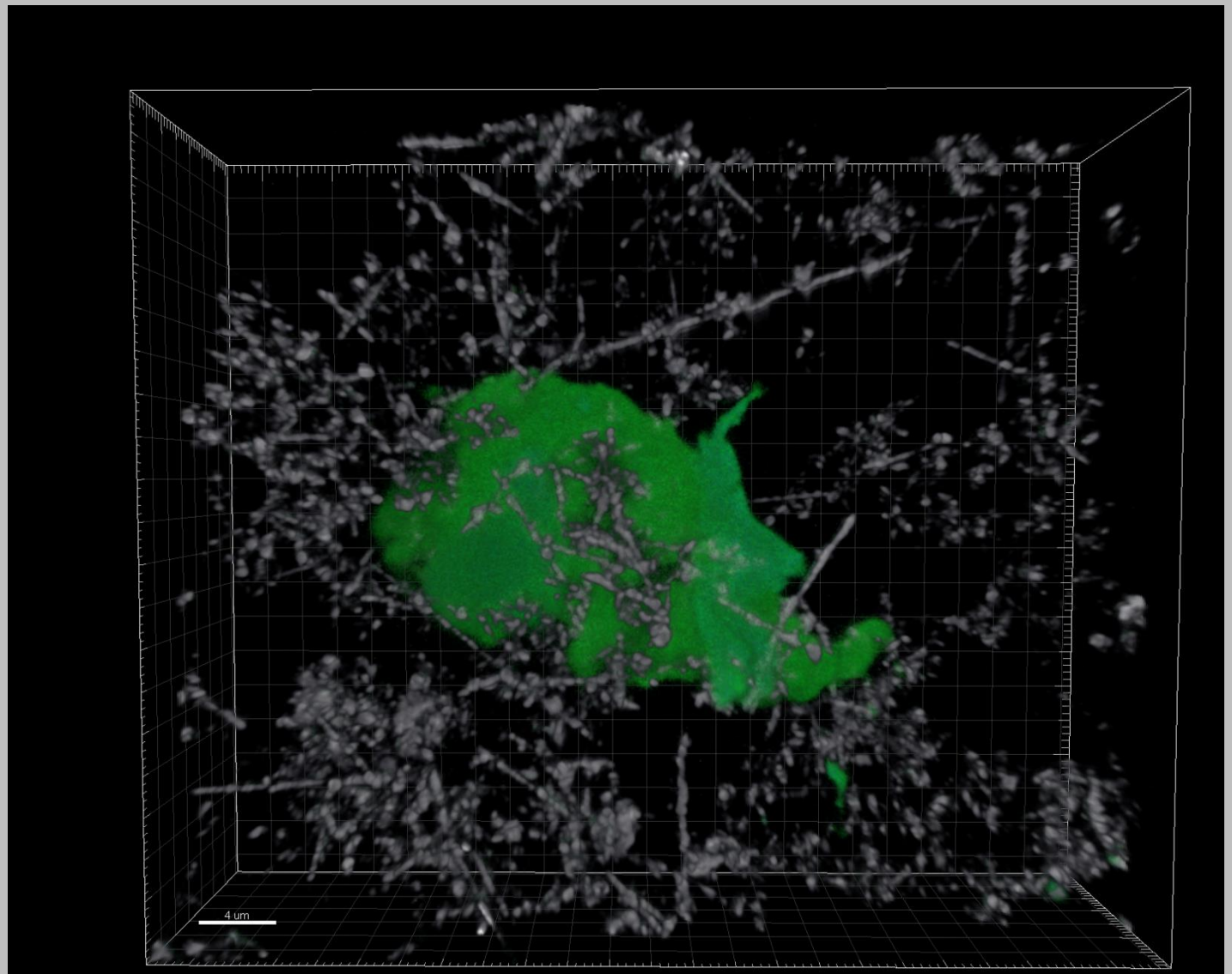
Aligned

Adjustable Polarizing filter in the Transmission Light path



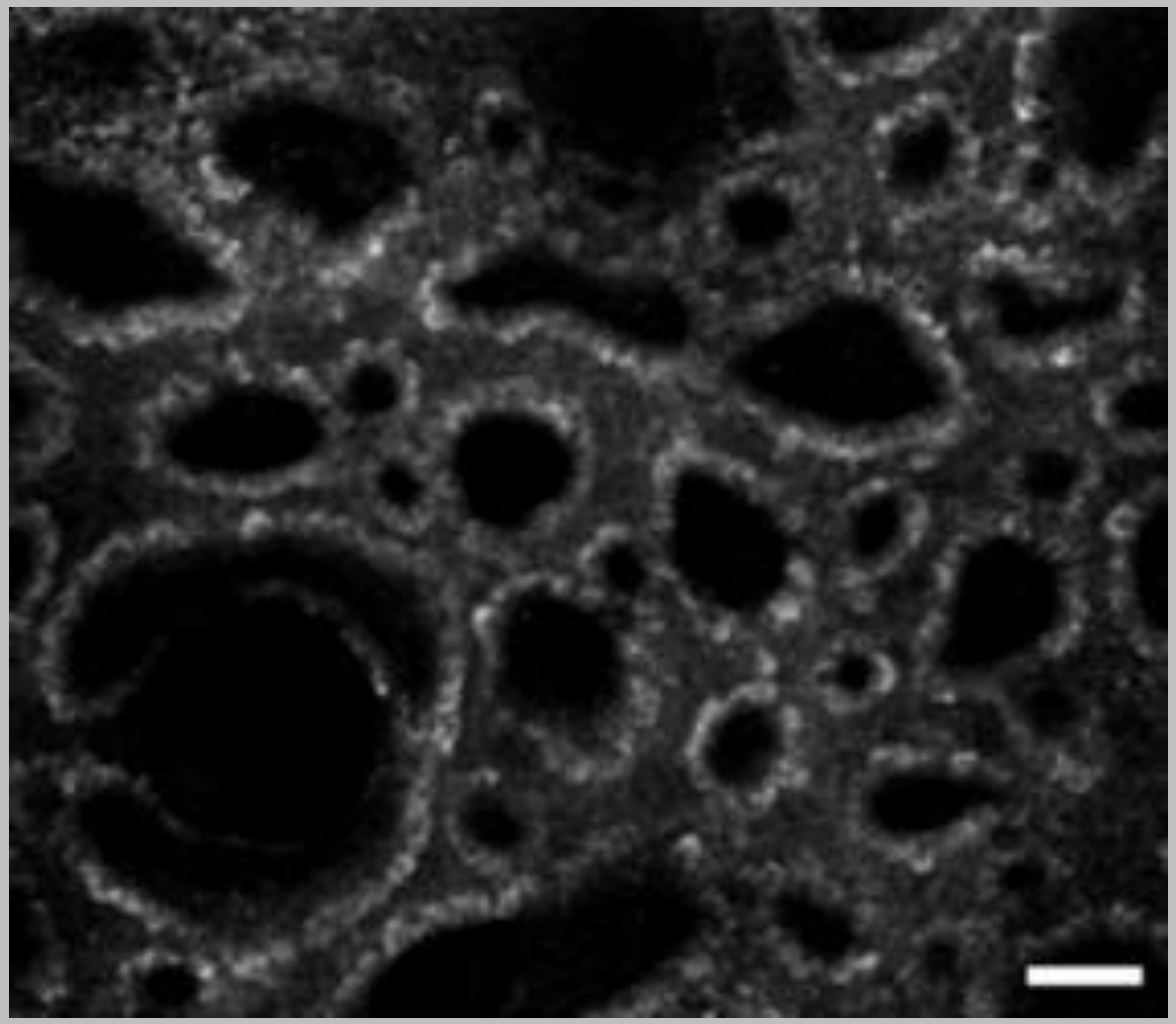
Misaligned (crossed)

Fluorescence (488nm) & Reflection (405nm)



Single cell –GFP(green) in a nanowire matrix

Skin Reflection confocal microscopy; 830nm laser

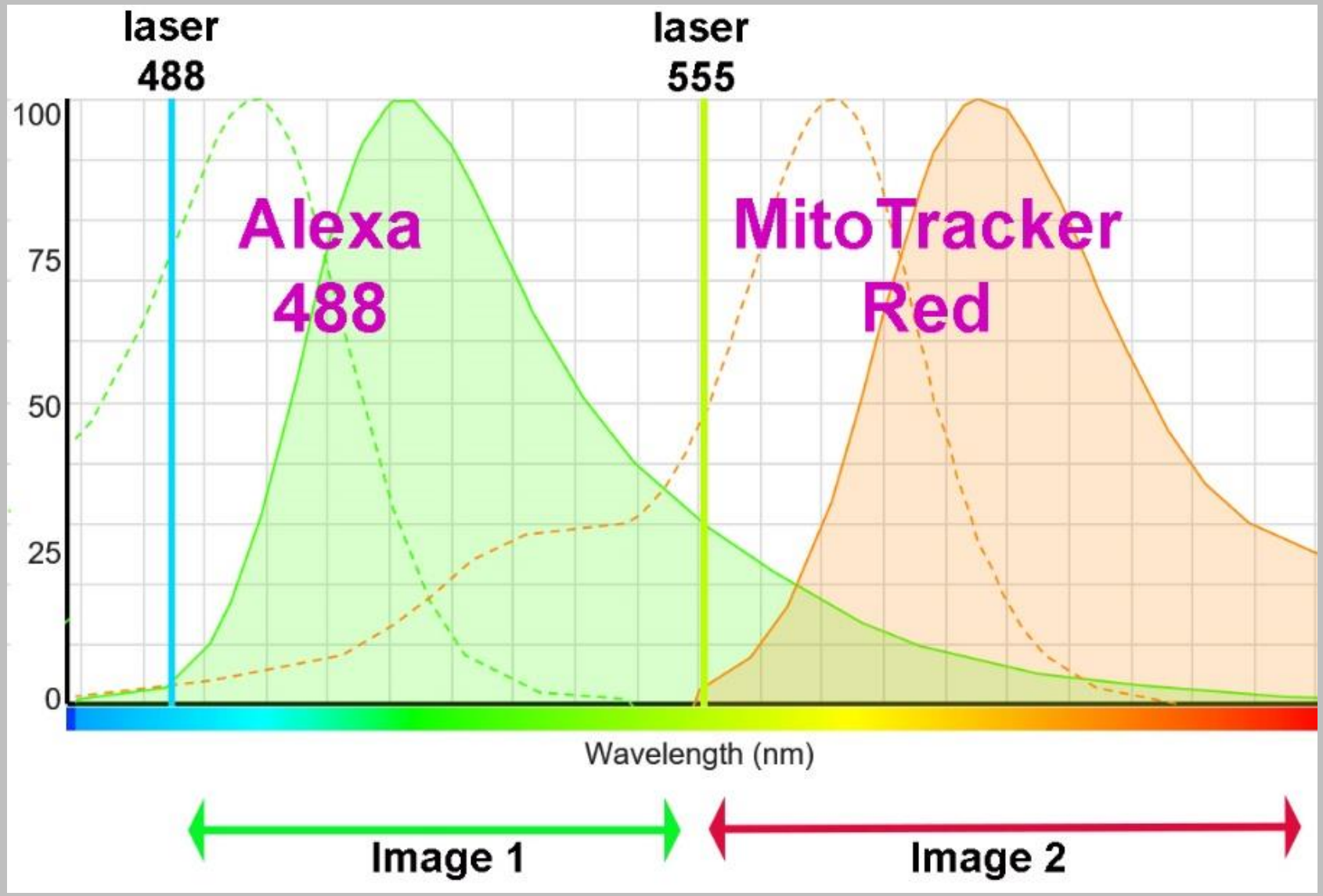


Experimental Dermatology, Volume: 19, Issue: 3, Pages: 233-239, First published: 11 February 2010, DOI: (10.1111/j.1600-0625.2009.00983.x)

Acquisition: Simultaneously or Sequentially

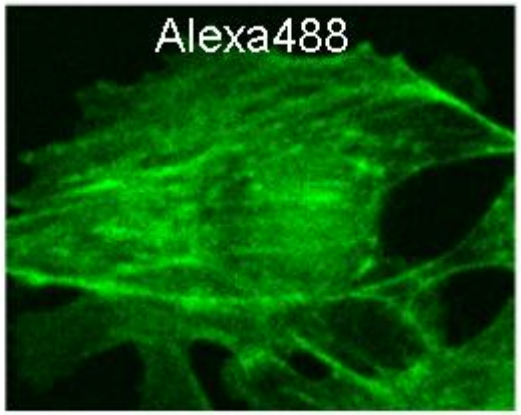
2 or more PMTs

can acquire 2 fluorescence images simultaneously **Faster**



Acquisition:

Simultaneous (both lasers) 488 & 555



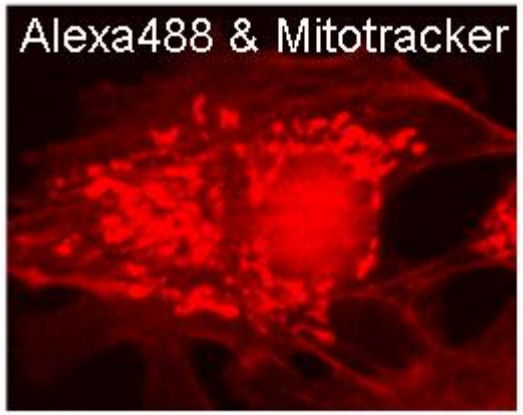
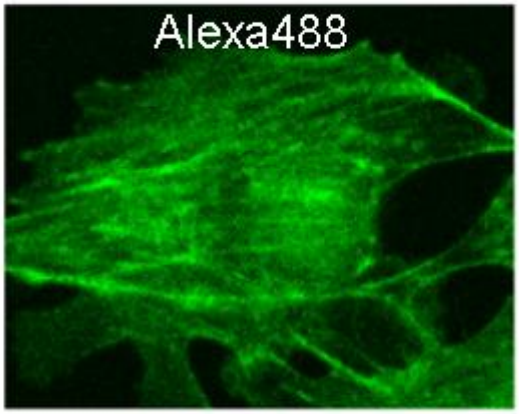


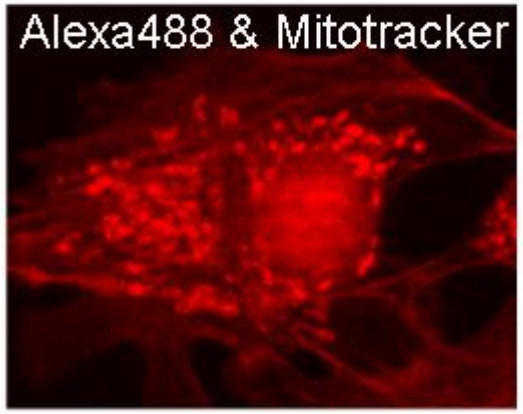
Image 1

Image 2

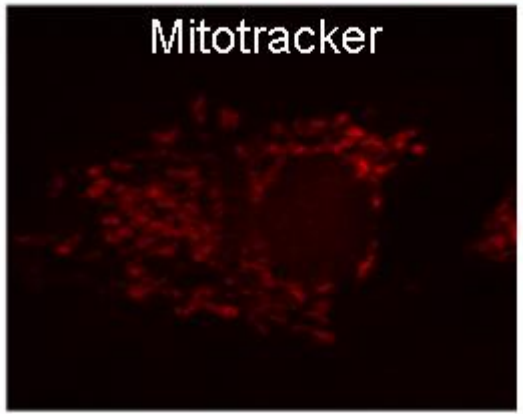
Sequential (single laser)

488

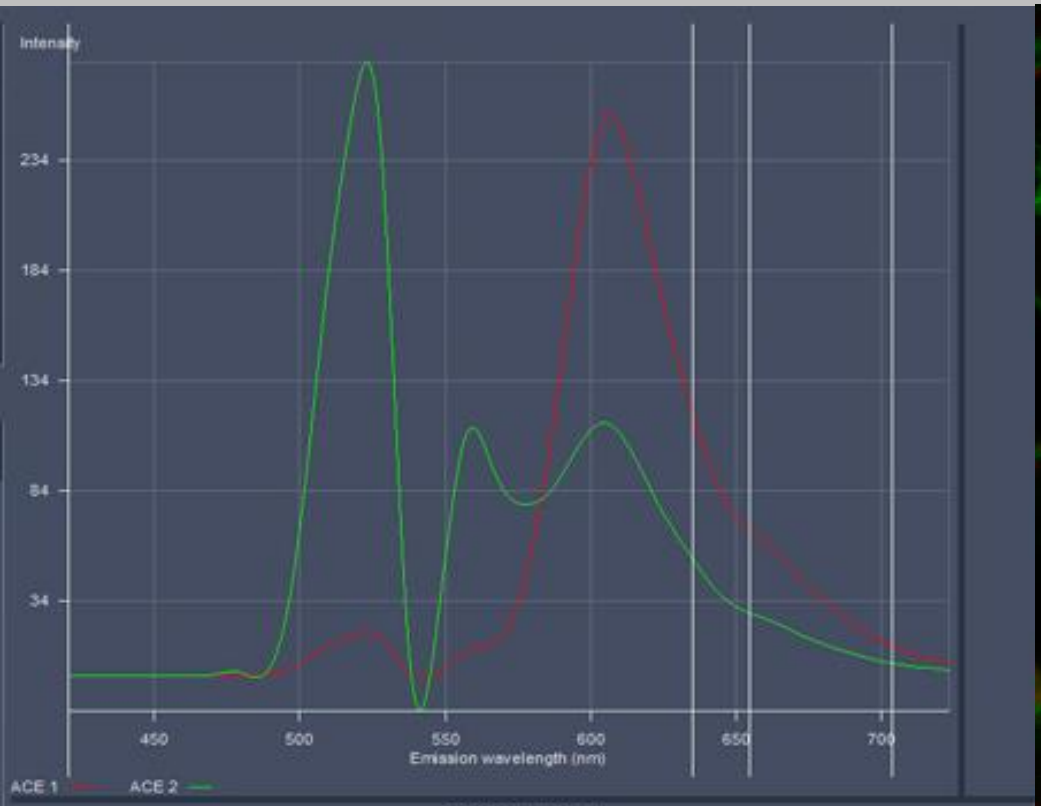


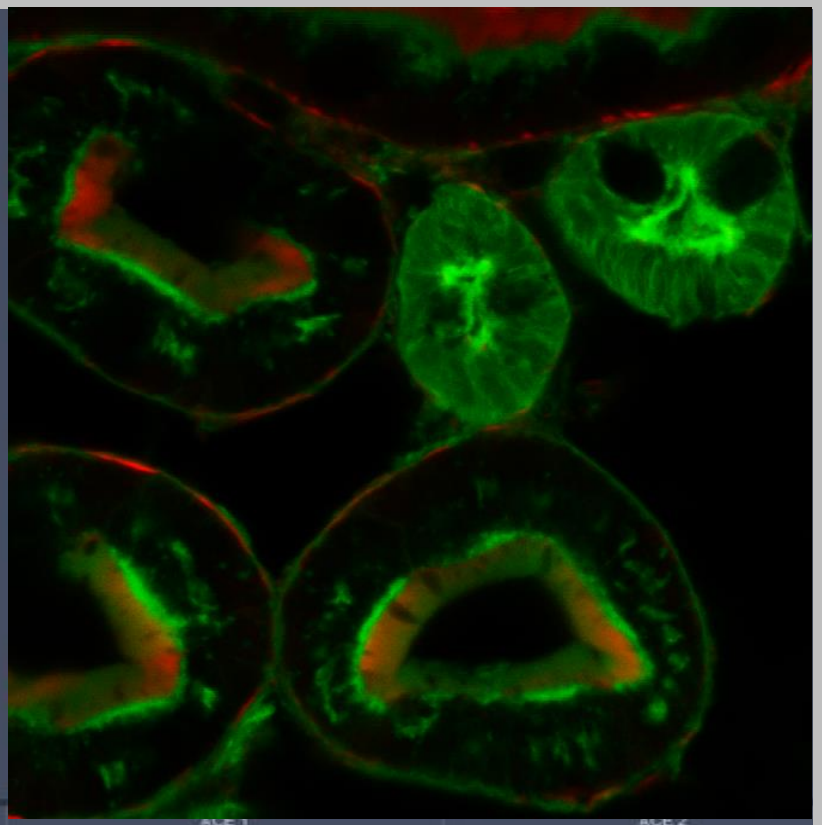


555



Lambda Scan– linear unmixing – separate overlapping fluorophores

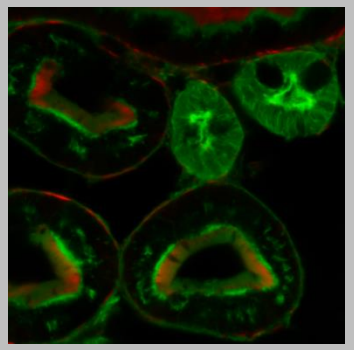




Emission wavelength [nm]	ACE 1	ACE 2
420	0.0	0.2
430	0.0	0.2
440	0.0	0.1
[illegible]	0.0	0.1
[illegible]	0.0	0.1
469	0.0	0.2
[illegible]	0.0	1.7
[illegible]	0.0	1.5
498	4.2	50.5
[illegible]	12.7	163.9
[illegible]	18.6	255.0
528	17.1	243.7
[illegible]	0.8	10.6
[illegible]	1.5	25.0
557	11.0	109.3
567	14.8	92.4
576	39.0	77.9
586	105.1	83.5
596	200.1	104.4

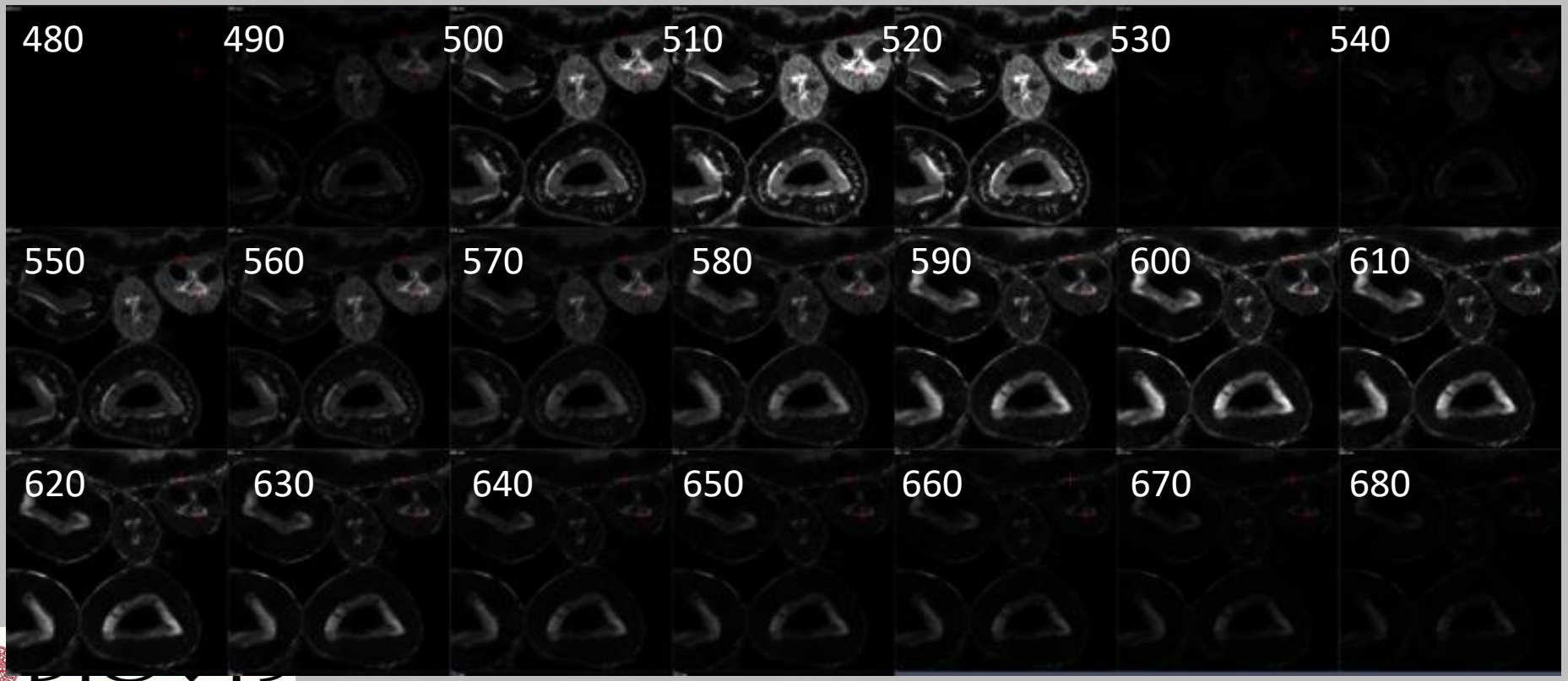
Linear Unmixing determines the relative contribution from each fluorophore for every pixel of the image.

Spectral (lambda) scan with LSM (Zeiss)

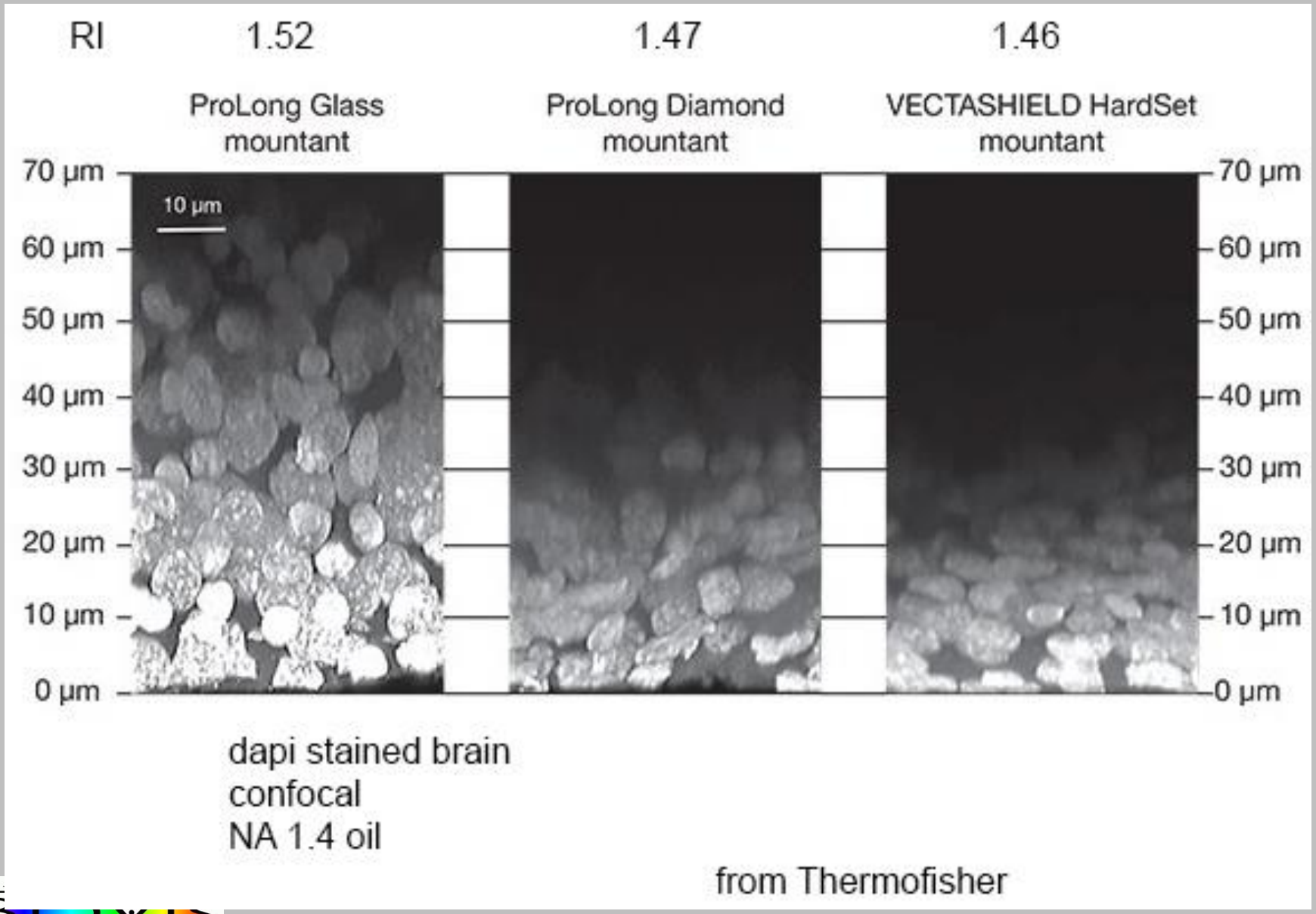


Emission spectra for fluorophore or autofluorescence

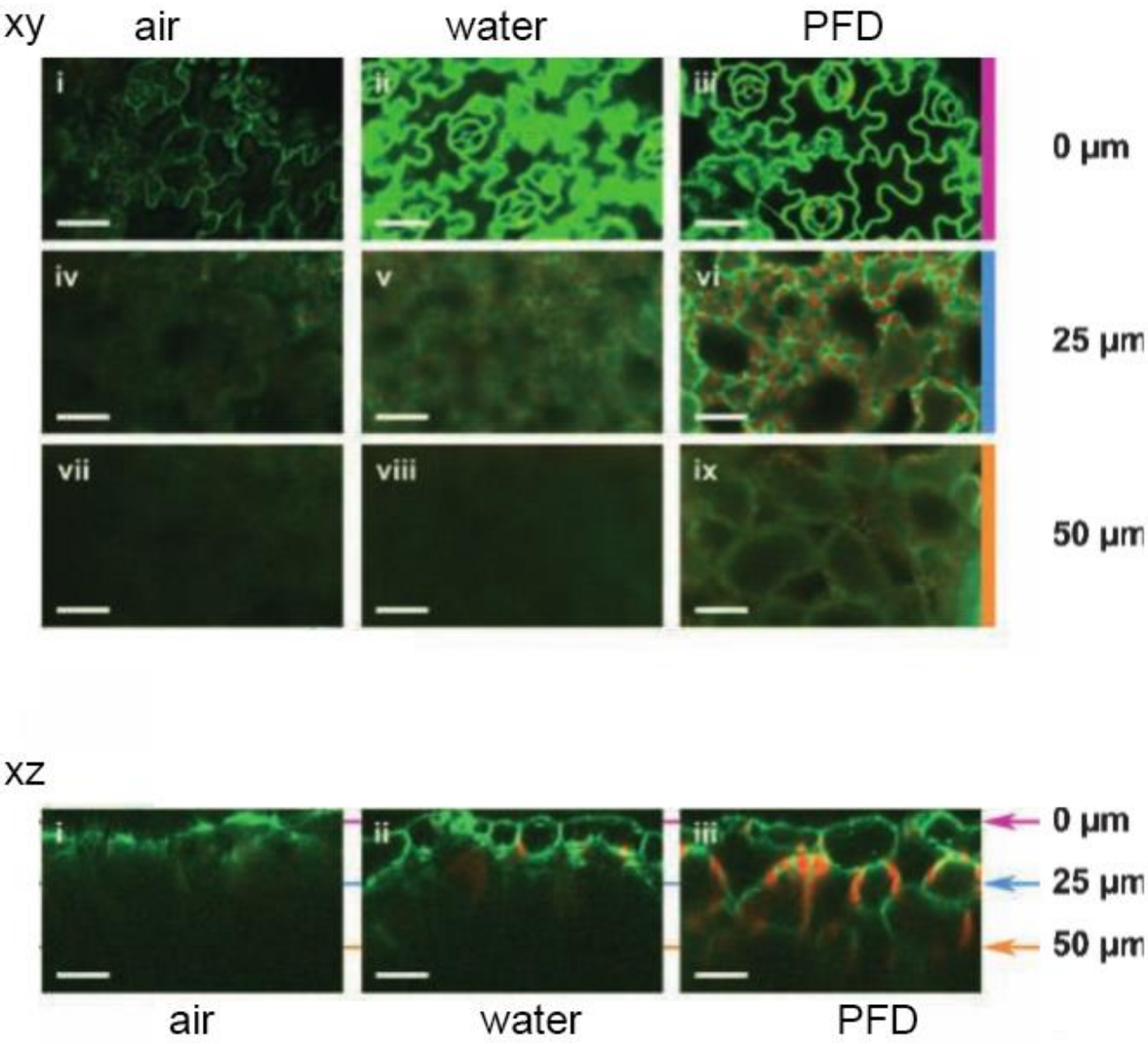
QUASAR detector of LSM710 with 32 detectors



RI matching: objective-specimen

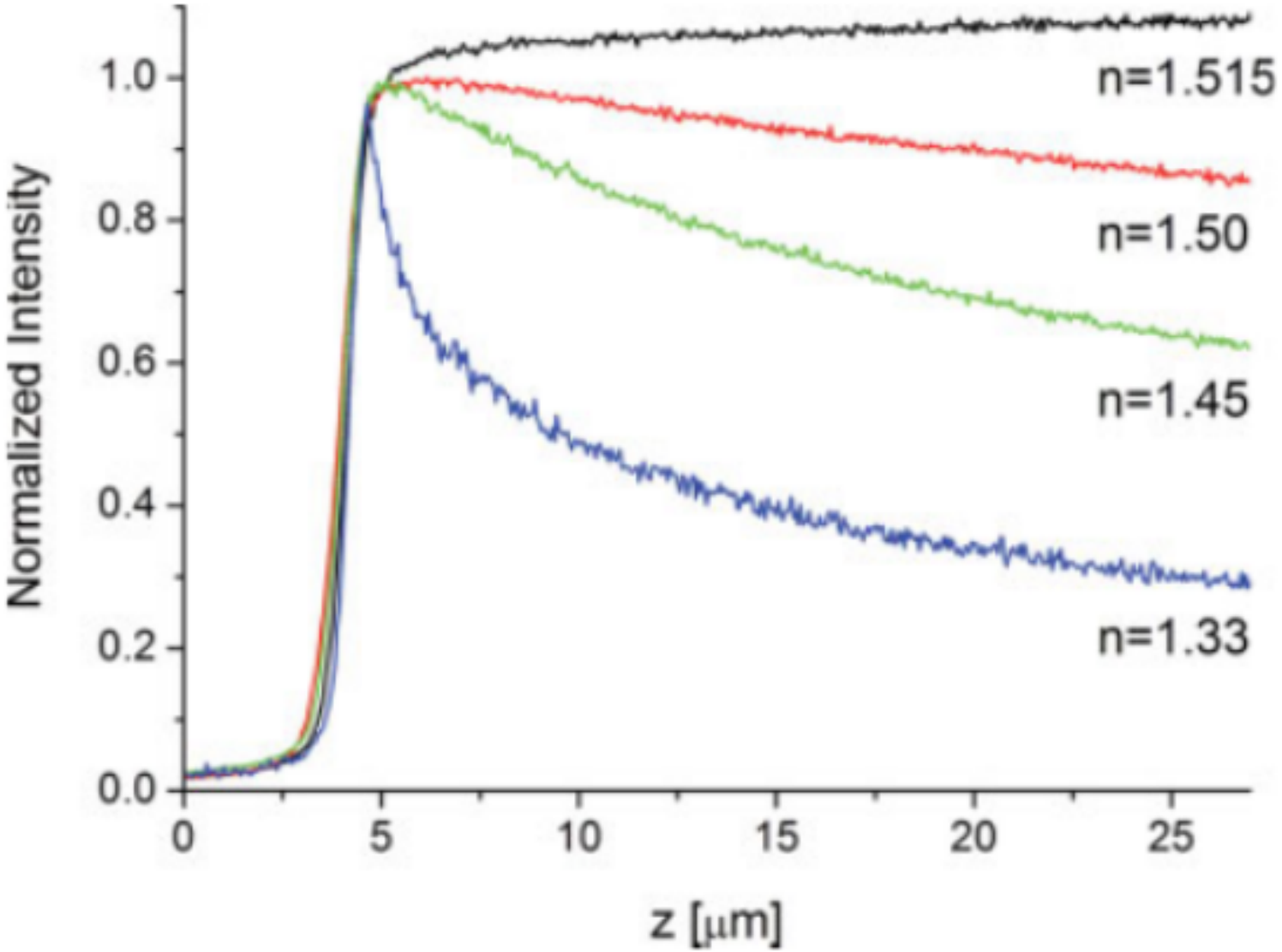


RI matching



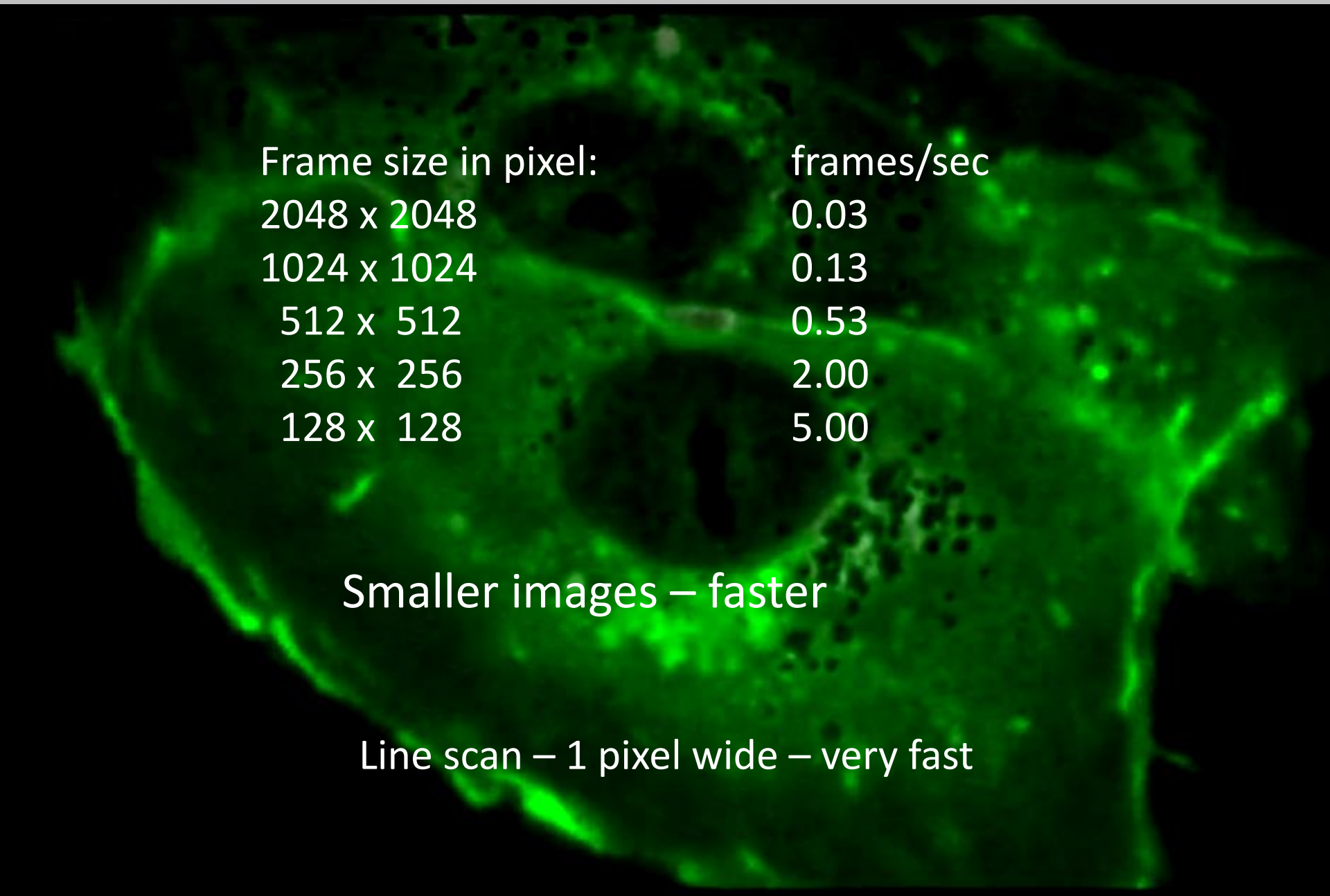
New Phytologist (2010) **186**: 1018–1025
doi: 10.1111/j.1469-8137.2010.03244.x

Confocal: Refractive index matching



Microsc Res Tech 2006 70(1) 1-9, Hell et al

Live imaging (time lapse)

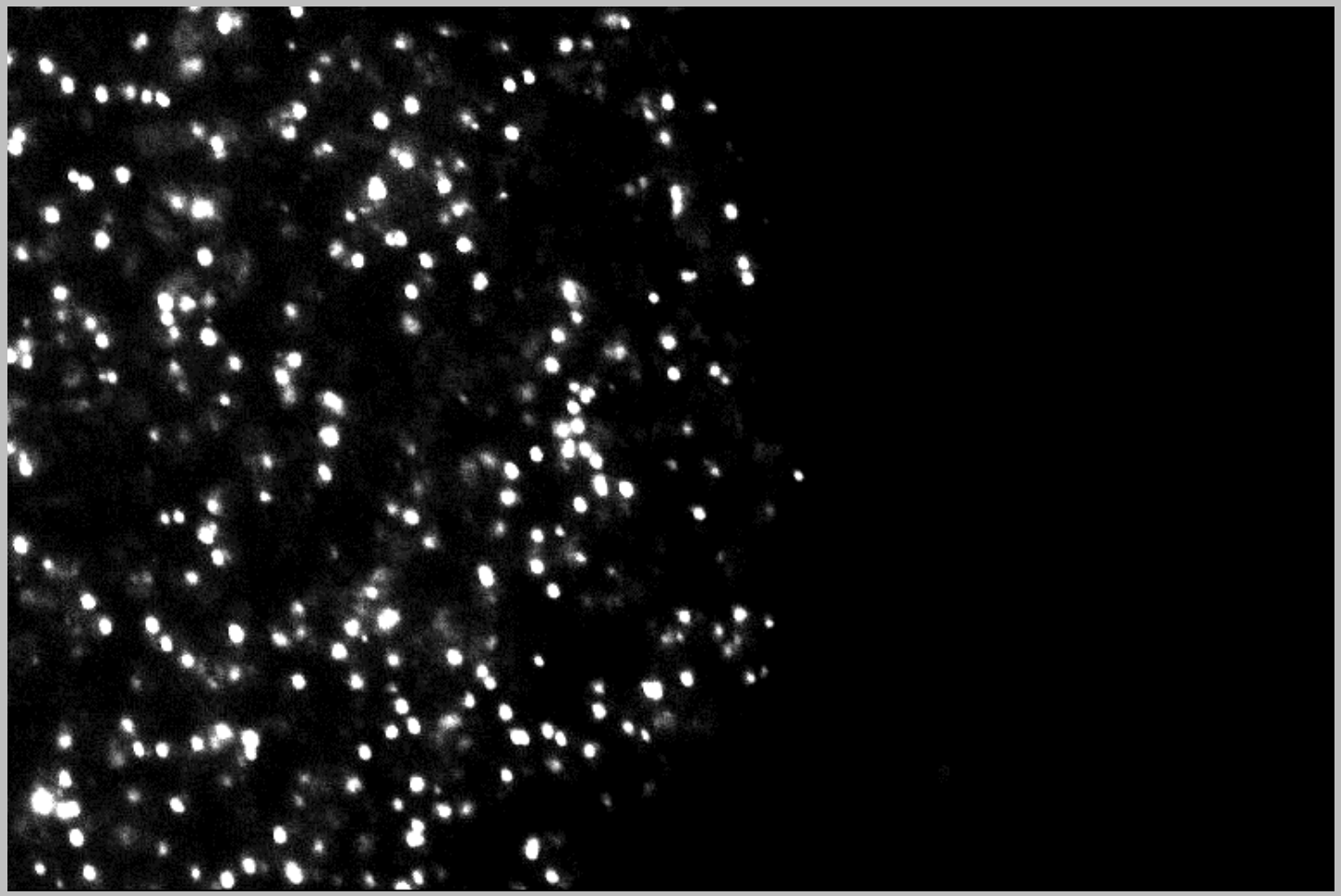


Frame size in pixel:	frames/sec
2048 x 2048	0.03
1024 x 1024	0.13
512 x 512	0.53
256 x 256	2.00
128 x 128	5.00

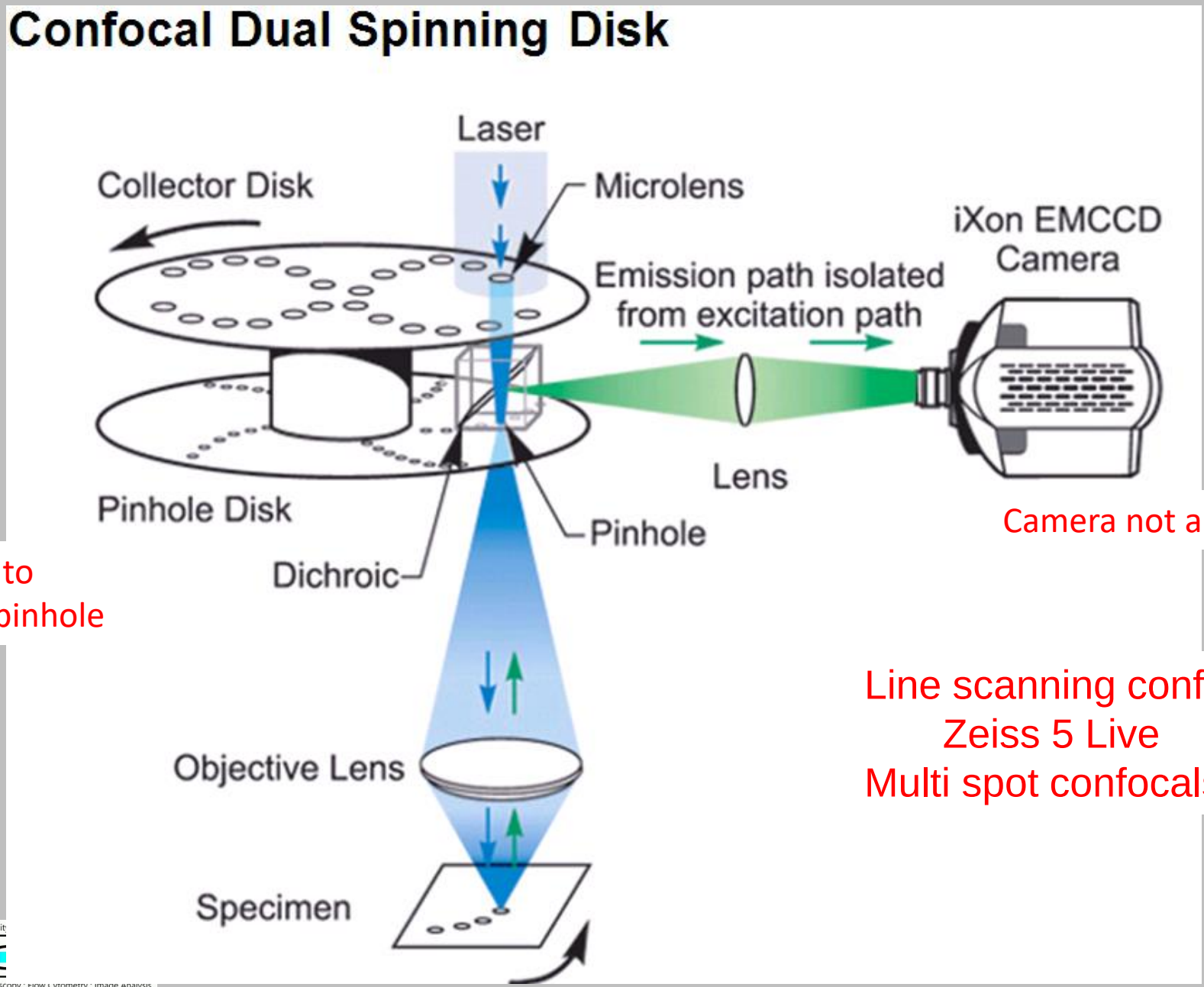
Smaller images – faster

Line scan – 1 pixel wide – very fast

Fluorescent microspheres in a matrix confocal images



Faster Confocal Imaging many points

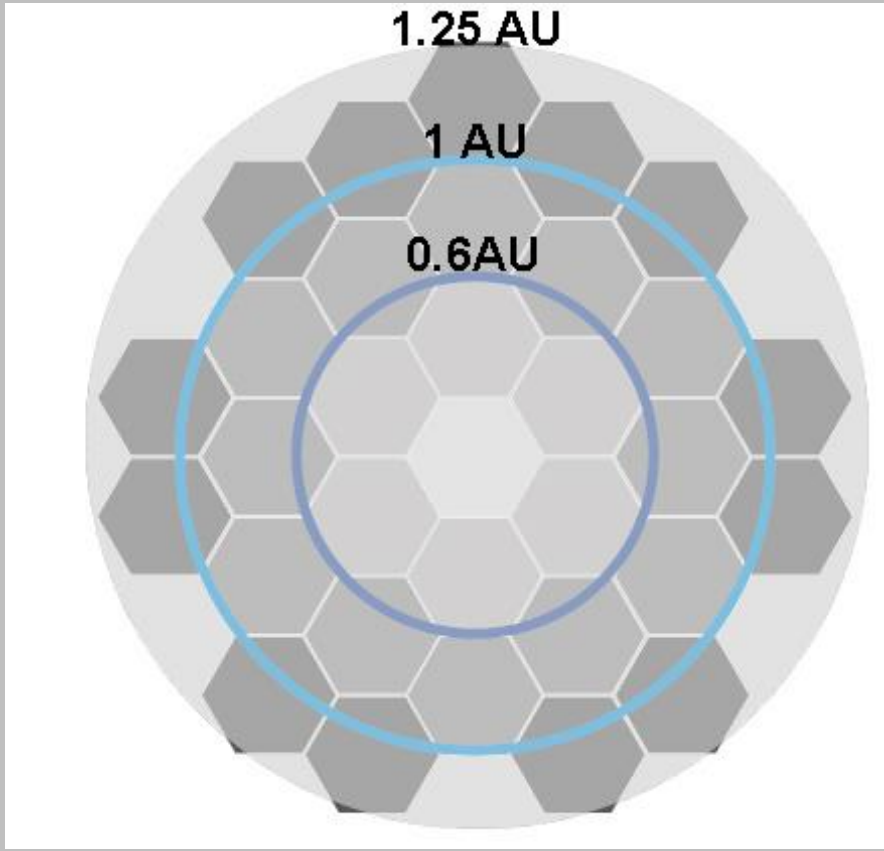


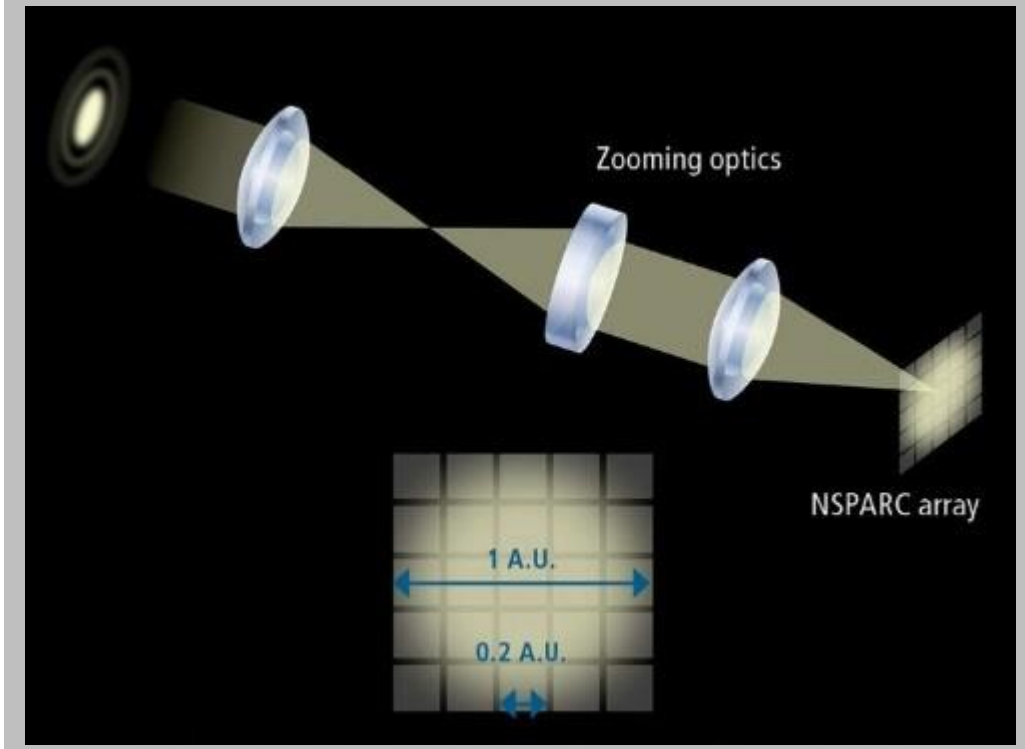
Camera not a PMT

Difficult to change pinhole

Line scanning confocals
Zeiss 5 Live
Multi spot confocals

replace conventional pinhole & single detector
With an array of detectors



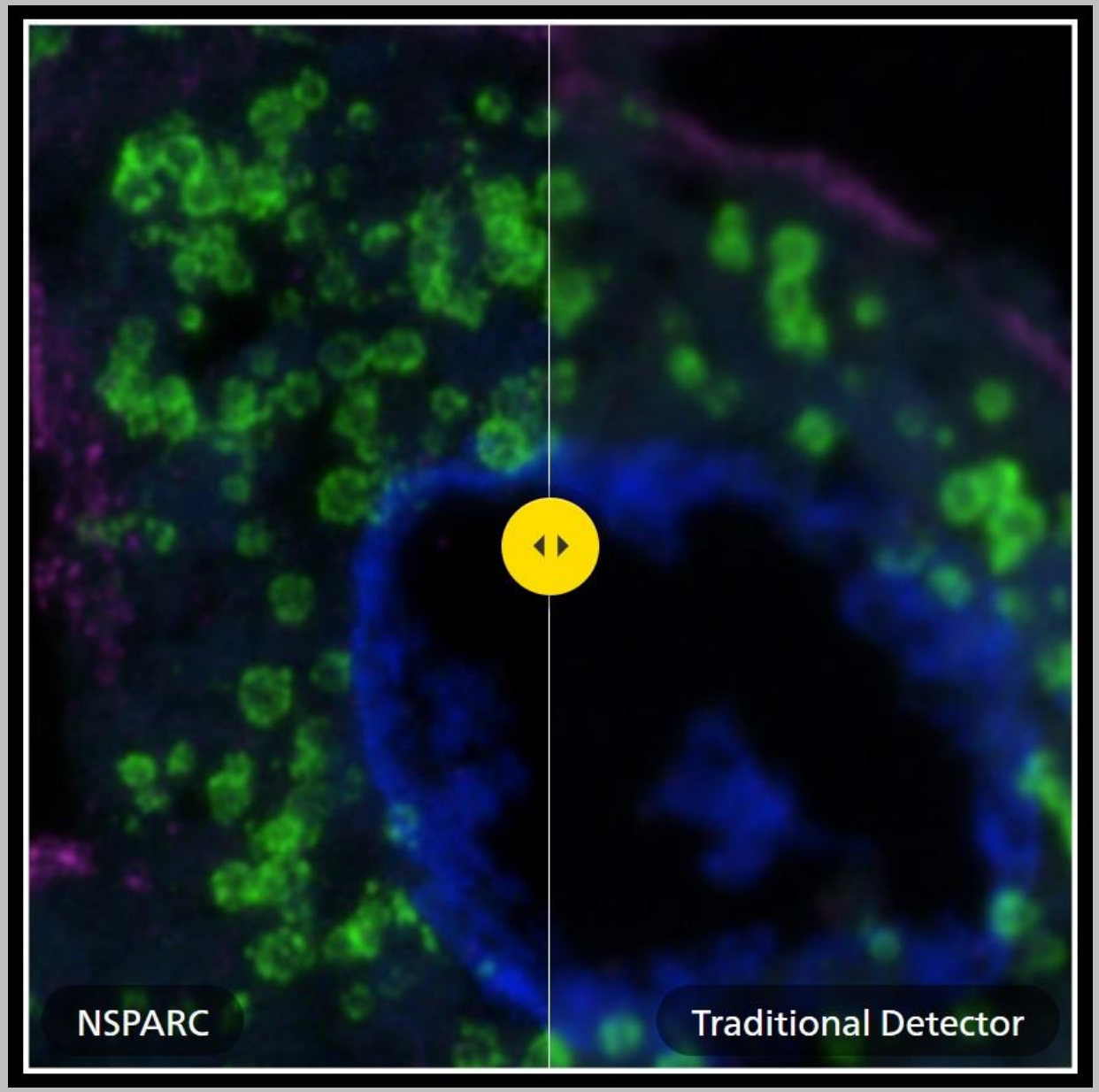


Zeiss Airyscan –array of 32 detectors

Nikon NSPARC 5x% array
Photon sensitive

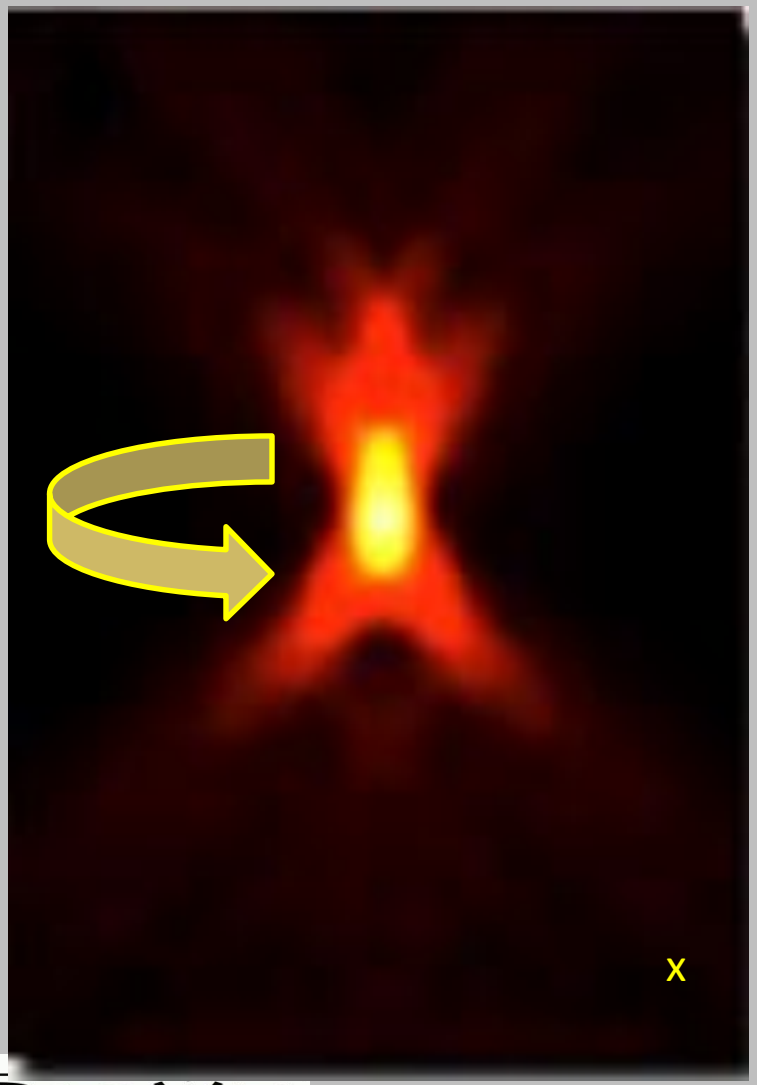
Confocal.nl have a rescan confocal – fast, higher resolution

Higher resolution from array of detectors



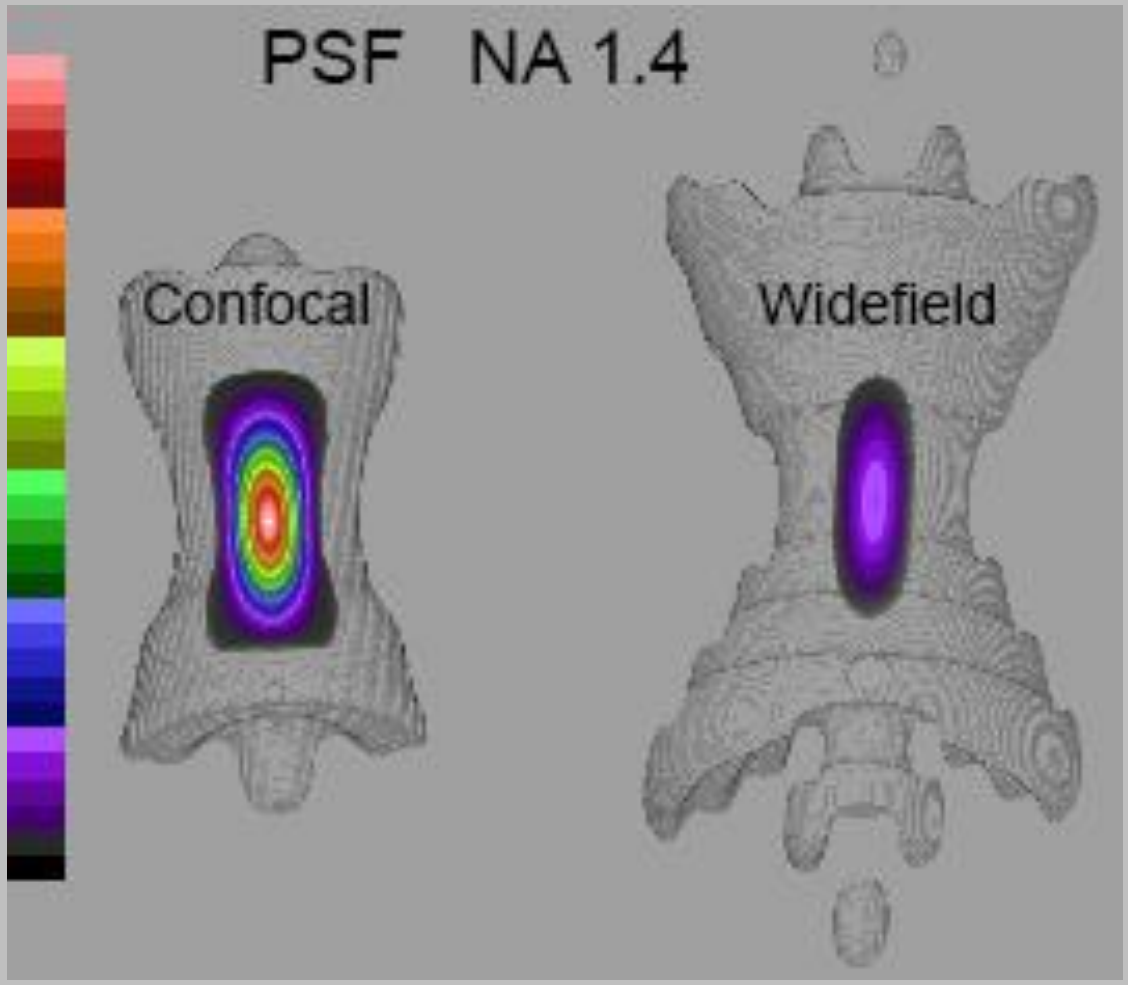
POINT SPREAD FUNCTION- resolution

A sub resolution object becomes a blob in the image



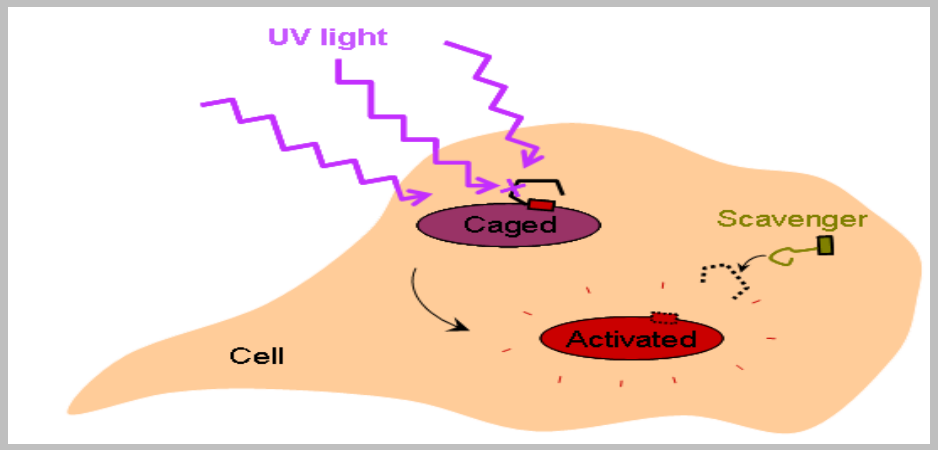
1. NA of the objective
2. Size of confocal pinhole
3. Wavelength of emission
4. Refractive index matching

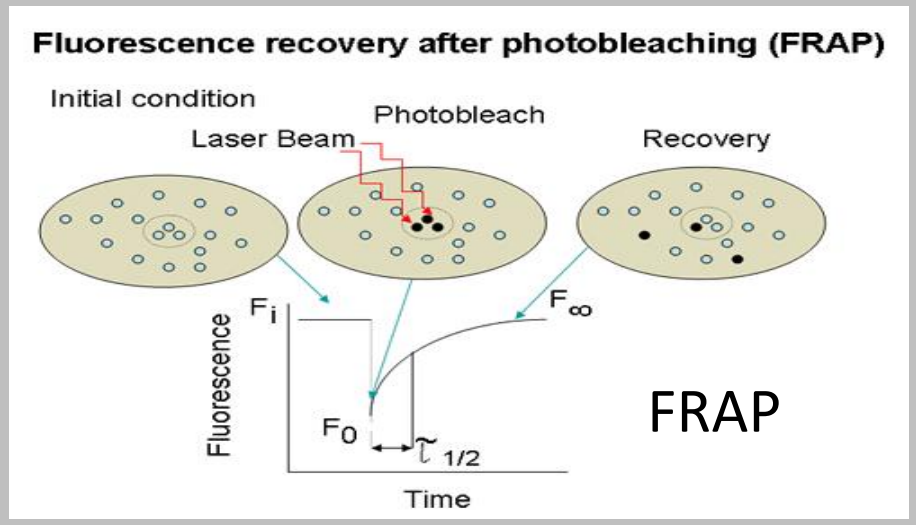
Point Spread Function (PSF) widefield and confocal



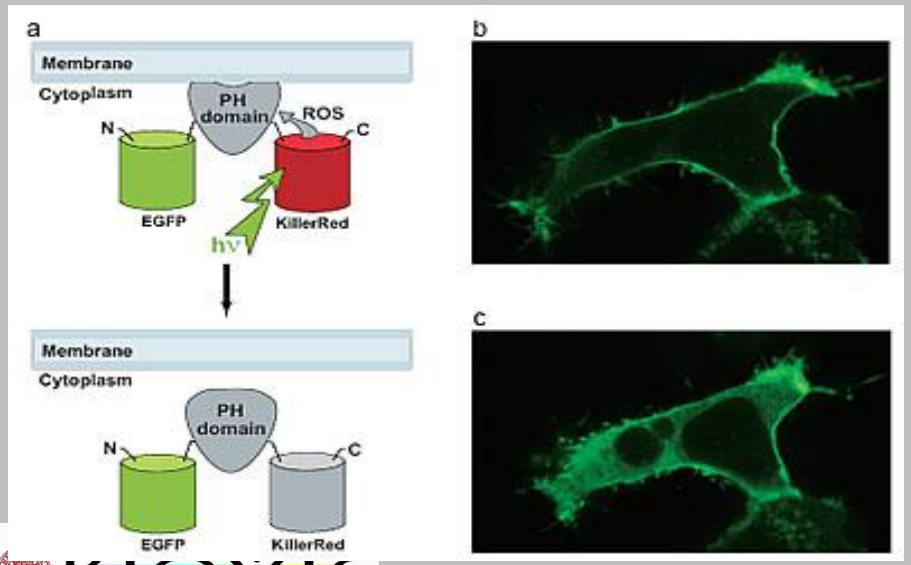
Techniques for the LSM and Live Cell Imaging

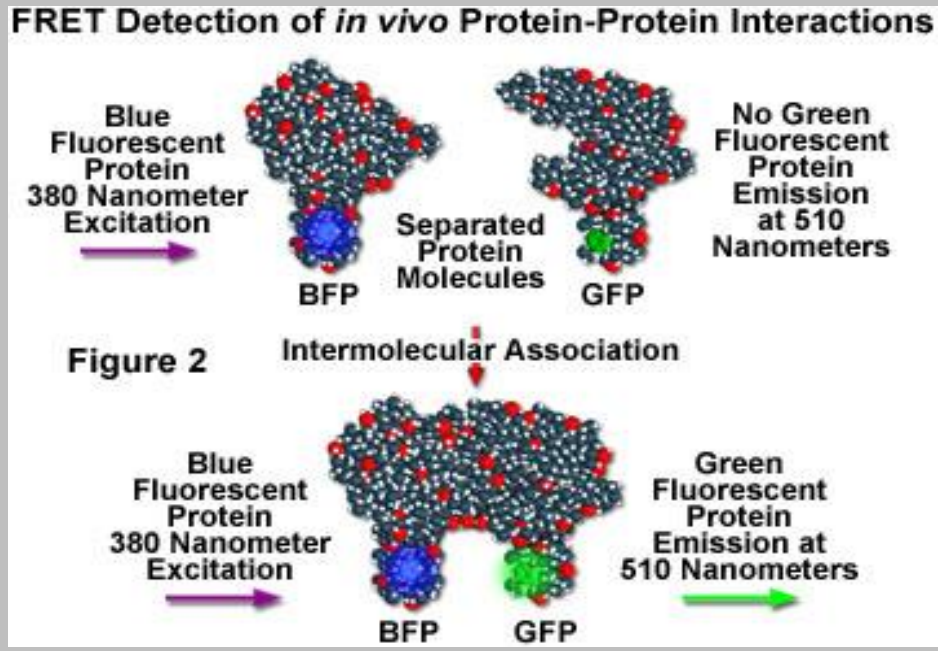
Photoactivation /uncaging





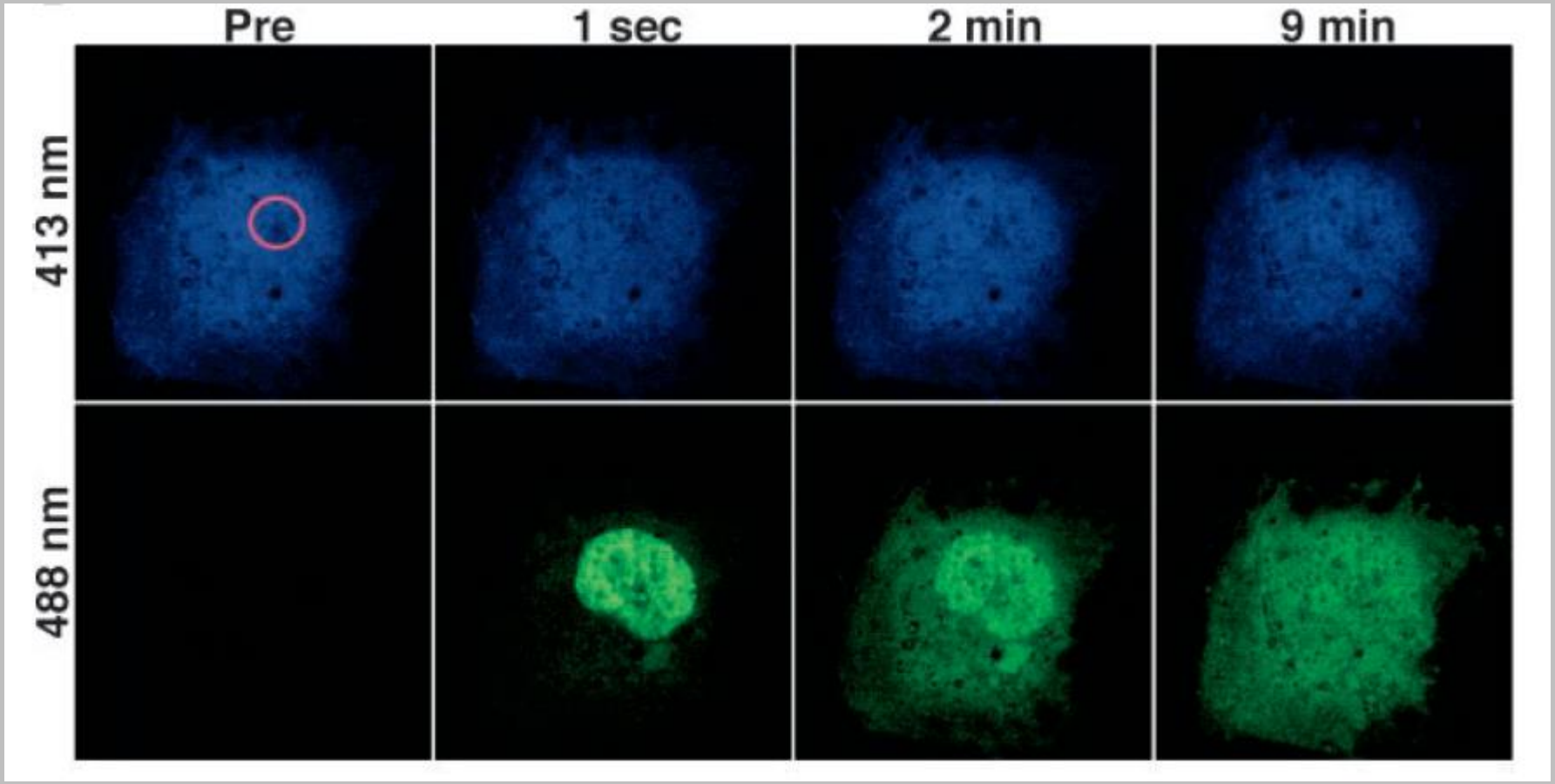
CALI Chromophore Assisted Light inactivation



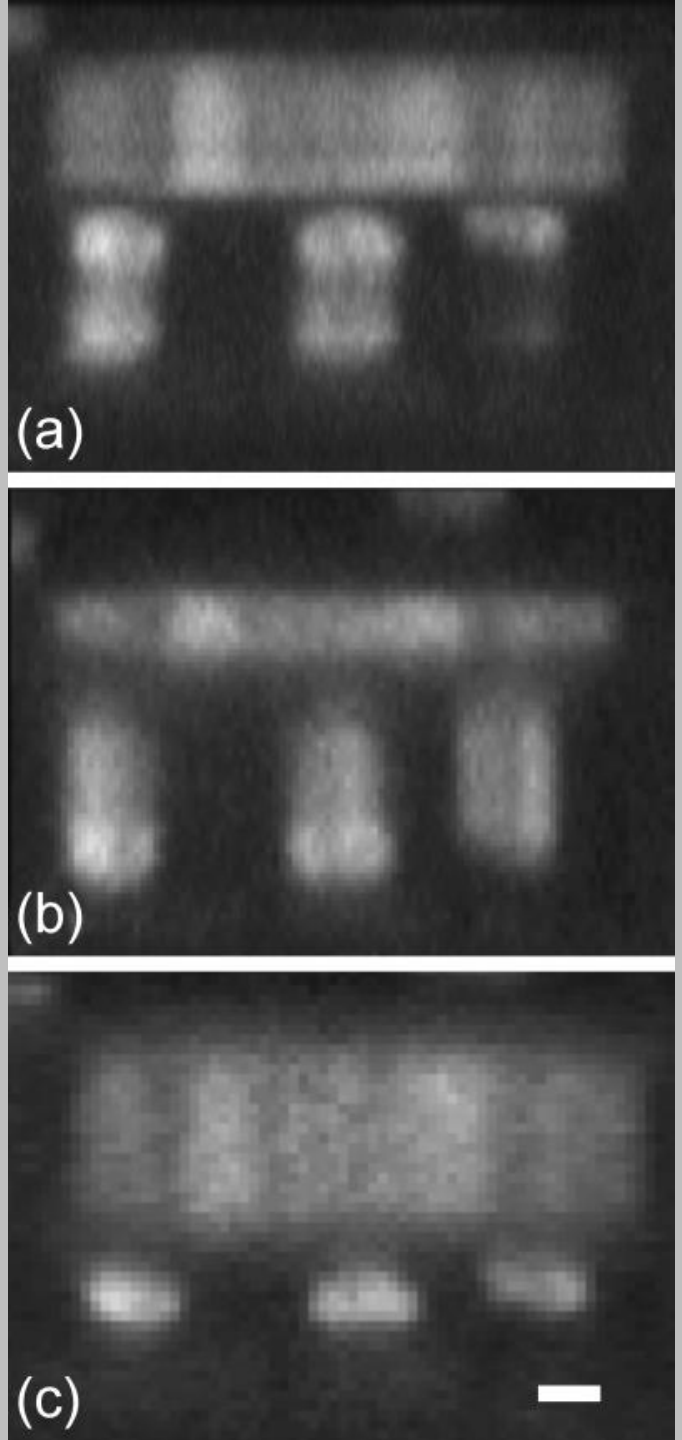


Photoactivatable Fluorophores

- GFP activated by 413nm – observe with 488nm excitation
- Pulse chase experiments



Science (2002), 297, 1873-1877



3 Replicate XZ Images

Nuclei in smooth muscle
3 round
1 elongated

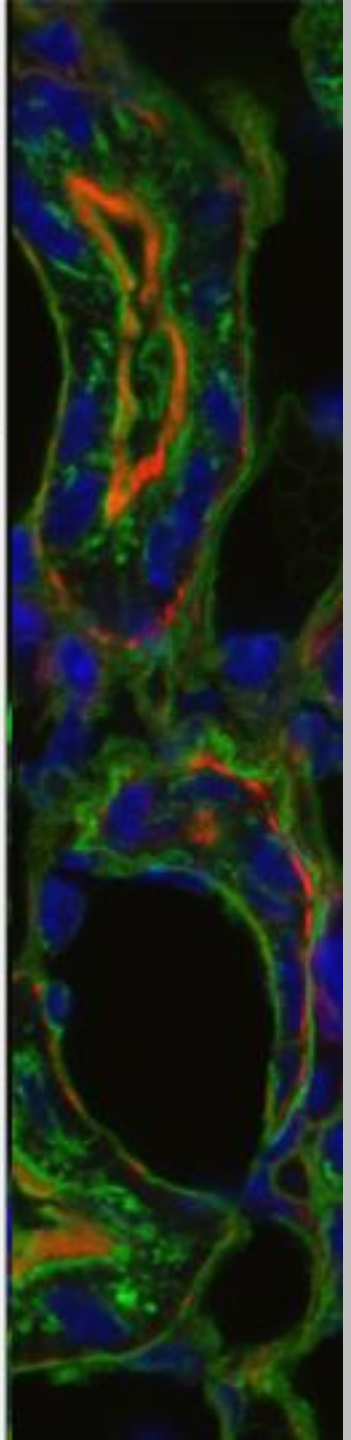
XZ image made from Z series

Cause ?

Solution

protect the microscope from temperature changes

User :	pachocore
Scan Mode :	Stack
Scaling :	X: 0.13 µm Y: 0.13 µm Z: 0.37 µm
Stack Size :	X: 135.86 µm Y: 135.86 µm Z: 13.85 µm
Scan Zoom :	1.7
Objective :	Plan-Neofluar 40x/1.3 Oil
Average :	Line 2
Pinhole :	Ch2-1 : 56 µm Ch2-2 : 65 µm Ch3-3 : 73 µm
Filters :	Ch2-1 : BP 420-480 Ch2-2 : BP 505-530 Ch3-3 : LP 560
Beam Splitters :	MBS-1 : HFT 405/514 DBS1-1 : Mirror DBS2-1 : NFT 490 DBS3-1 : None FW1-1 : None MBS-2 : HFT 488 DBS1-2 : Mirror DBS2-2 : NFT 545 DBS3-2 : None FW1-2 : None MBS-3 : HFT 488/543 DBS1-3 : Mirror DBS2-3 : NFT 545 DBS3-3 : None FW1-3 : None
Wavelength :	405 nm T1 30.7 % 488 nm T2 30.7 % 543 nm T3 29.7 %



Acquisition information:
metadata

recorded with the image

Reuse

same setup

measurements

Pixel size:

Objective's magnification

Area scanned – ZOOM

Number of pixels

In Practice

- 1) slide with every fluorophore - acquisition settings
- 2) slides with single fluorophores - bleed through
- 3) slide with no added fluorophores - autofluorescence

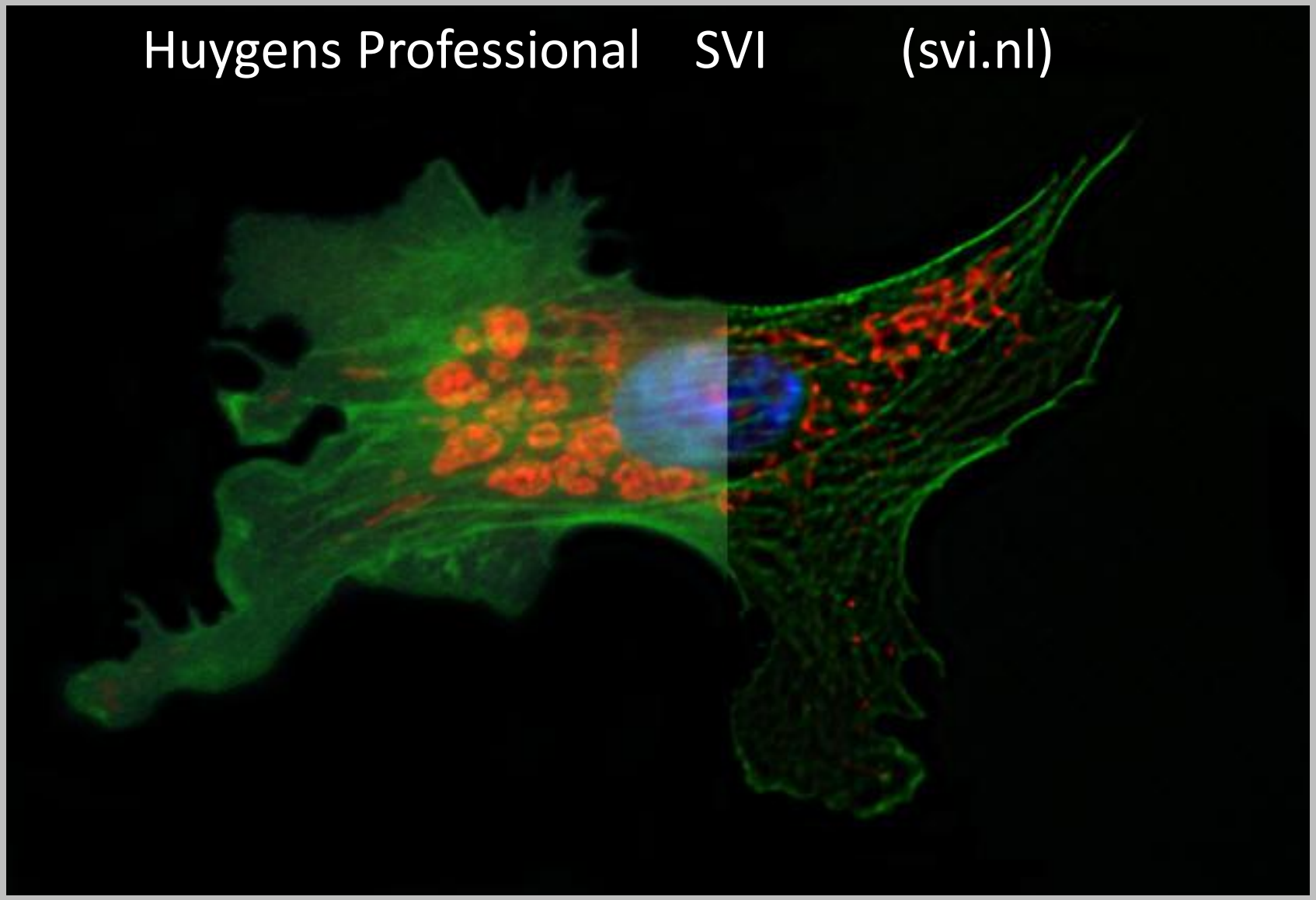
Confocal v Widefield (Deconvolution)

- Optical sectioning – pinhole, Z series — Z series
- Variable magnification – zooming
- Timeseries – live imaging — faster
- Motorized XY stage – tiling — also
- Spectral scanning

Problems

- Slow — faster
- Pinhole throws away photons — Capture more photons
- Poor detectors — Higher quantum efficiency
- Photobleaching — reduced

Image Deconvolution both confocal & widefield



Analyse your Images

Introduction to Image Analysis (2 days) February

February 21st (Wednesday) and 23rd(Friday) 2024 8 places

9.30-4.30pm

"That which is not measurable is not science" Rutherford

Aim

Appreciate the potential of image analysis & how to use the Fiji version of ImageJ – the most widely used freeware.

To register

Send

Name, email address, PI, billing code – fee 1600sek for 2 days.

To jeremy.adler@igp.uu.se

Introduction to Image Analysis Software May 2024

IAS 5 day course - no fee, 1.5 credits

http://www2.medfarm.uu.se/utbildning/forskarniva/for_doktorander/kurslista2024.html

Methods for Cell Analysis (MCA Autumn 2024)

http://www2.medfarm.uu.se/utbildning/forskarniva/for_doktorander/kurslista2024.html

References etc

Tutorial: guidance for quantitative confocal microscopy.

Jonkman, J., Brown, C.M., Wright, G.D. *et al.*

Nat Protoc (2020 April).

https://doi-org.ezproxy.its.uu.se/10.1038/s41596-020-0313-9

Seeing is believing – beginners guide to practical pitfalls in image acquisition

Pawley J Cell Biol, 2006, 172, 9-18

How to reduce background autofluorescence and improve confocal microscopy

Ben Libberton, Leica Microsystems

Learn how to Remove Autofluorescence from your Confocal Images | Learn & Share | Leica Microsystems (leica-microsystems.com)

Websites

Microscopy: Zeiss, Leica, Nikon, Olympus, BioRad

Fluorophores: Thermo Fisher Scientific

Filters: Chroma, Omega, Semrock

Spectra Etc

https://public.brain.mpg.de/shiny/apps/SpectraViewer/ additional information : https://brain.mpg.de/326043/spectra-viewer

https://www.thermofisher.com/order/fluorescence-spectraviewer

END

Confocal Components

Miniature objectives

Temperature Control

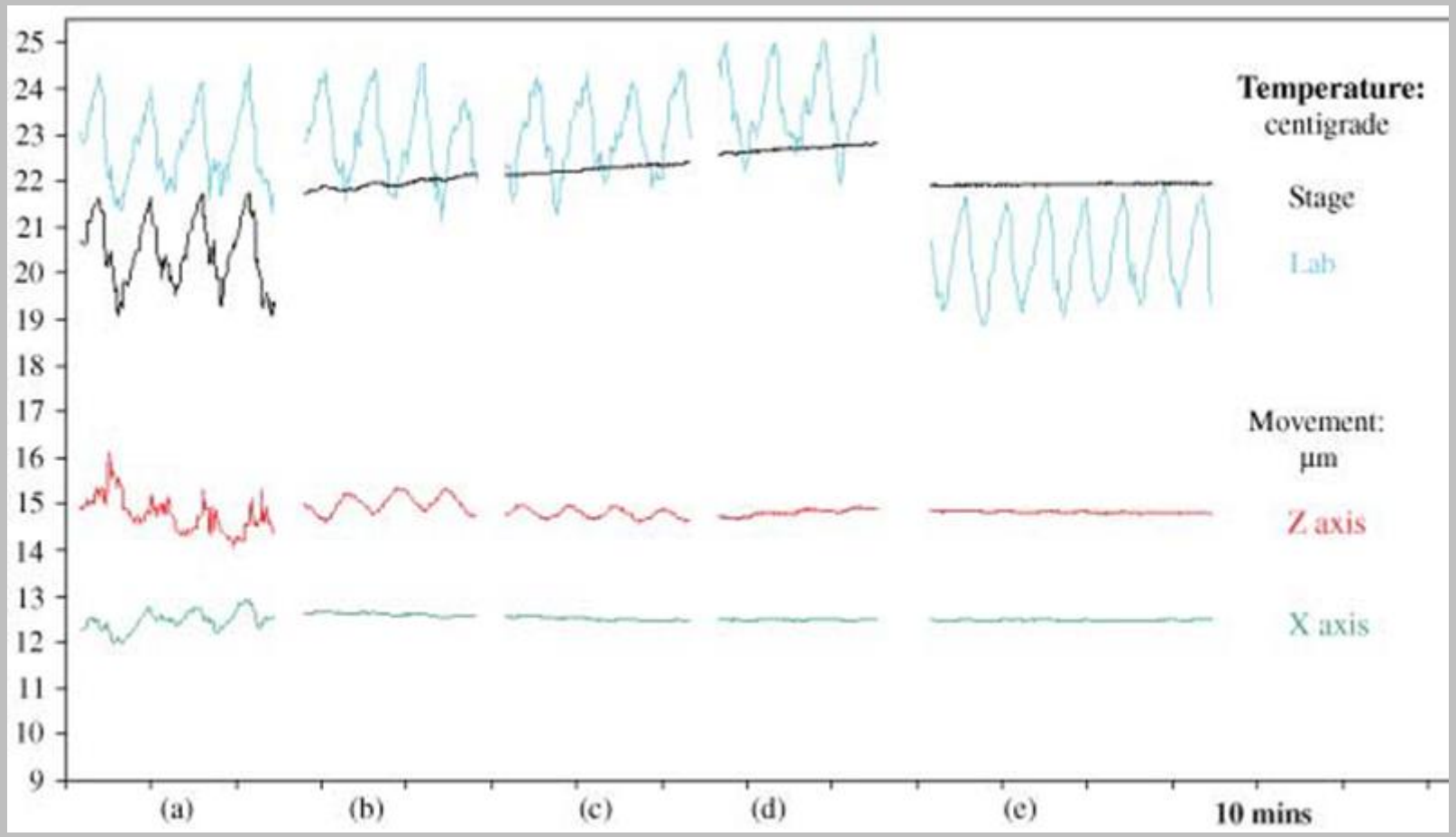


Image Quality

Photon Noise /Poisson Noise/Shot Noise

Compare 2 sequential mages