Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Fundamentals of Advanced Light Microscopy Part 2

Topics today

- Confocal Microscopy (point scanners)
- How to record a good image
- Deconvolution

Break

- Multi-photon: fluorescence, SHG and THG
- Superresolution microscopy: STED and other approaches

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

BIOMEDICAL CENTER
CORE FACILITY BIOIMAGING

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Confocal Microscopy (point scanners)

Walter Brendel Zentrum München

BIN LMU BioImaging Network Munich

EUROPEAN REFERENCE CENTER FOR

Leica MICROSYSTEMS

Confocal laser scanning microscopy

Clsm has 2 fundamental differences compared to conventional LM:

- Out-of-focus light is blocked physically
- No image is generated in the microscope itself. It is reconstructed in the computer

Since the eye cannot see anything, all commercial confocals have an additional excitation source for conventional fluorescence microscopy, like a mercury arc Lamp or an LED.

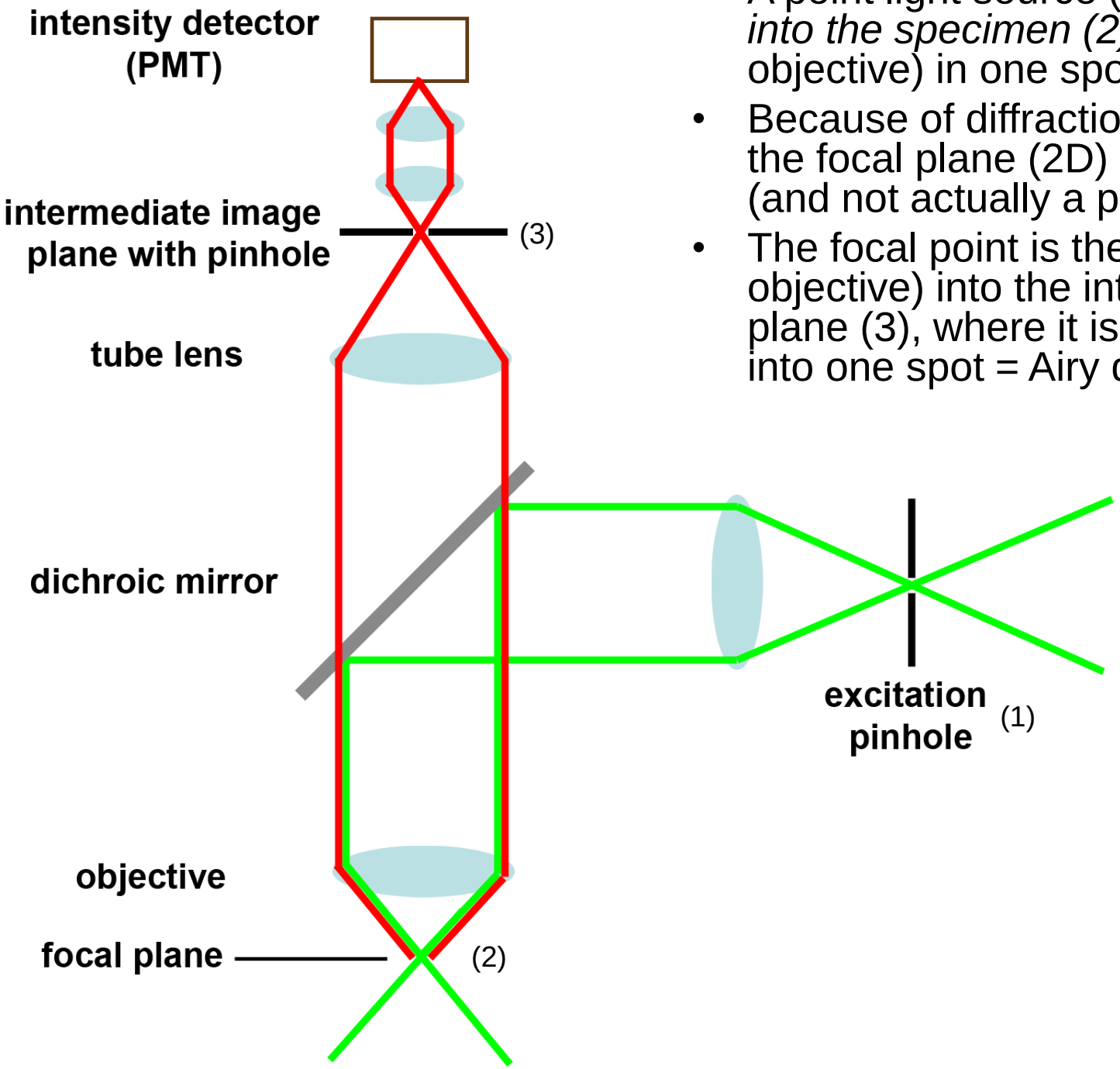
OED | Oxford English Dictionary
The definitive record of the English language

confocal, *adj*.

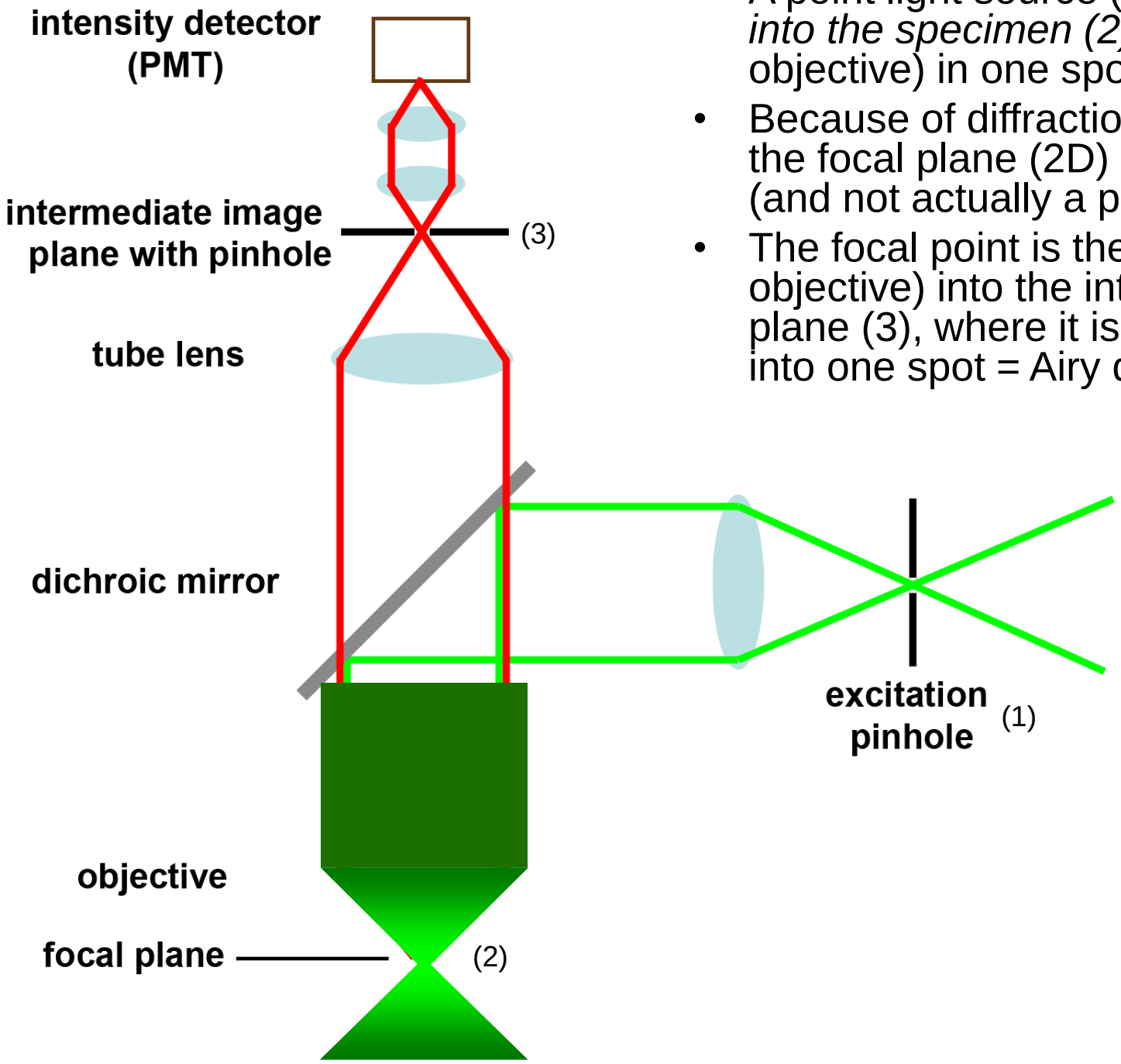
Having the same focus or foci.

1867 W. Thomson & P. G. Tait *Treat. Nat. Philos.* §494 Any two confocal homogeneous solid ellipsoids of equal masses produce equal attraction through all space external to both.

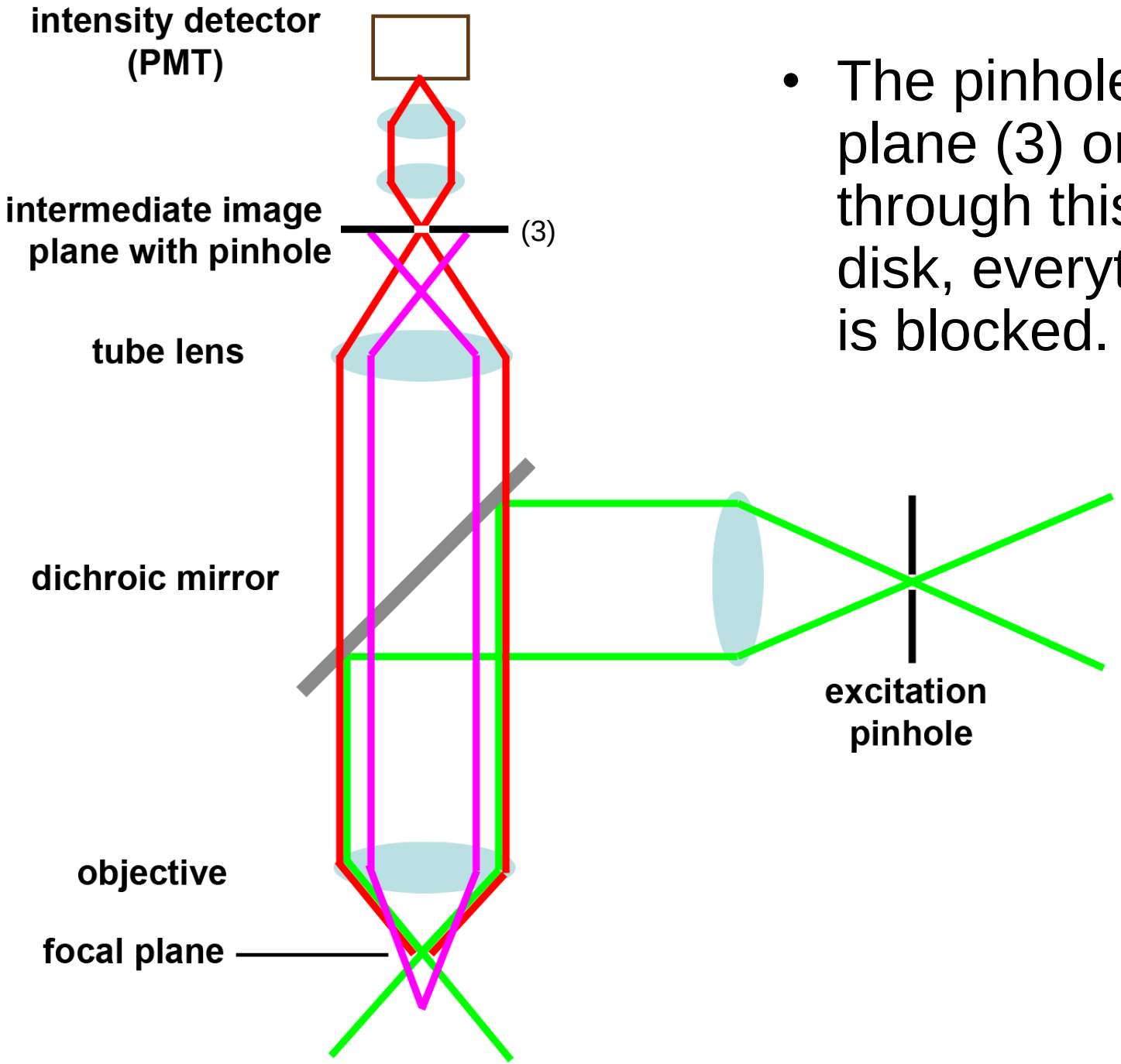
(„Con“ means „with“, as in chili con carne)



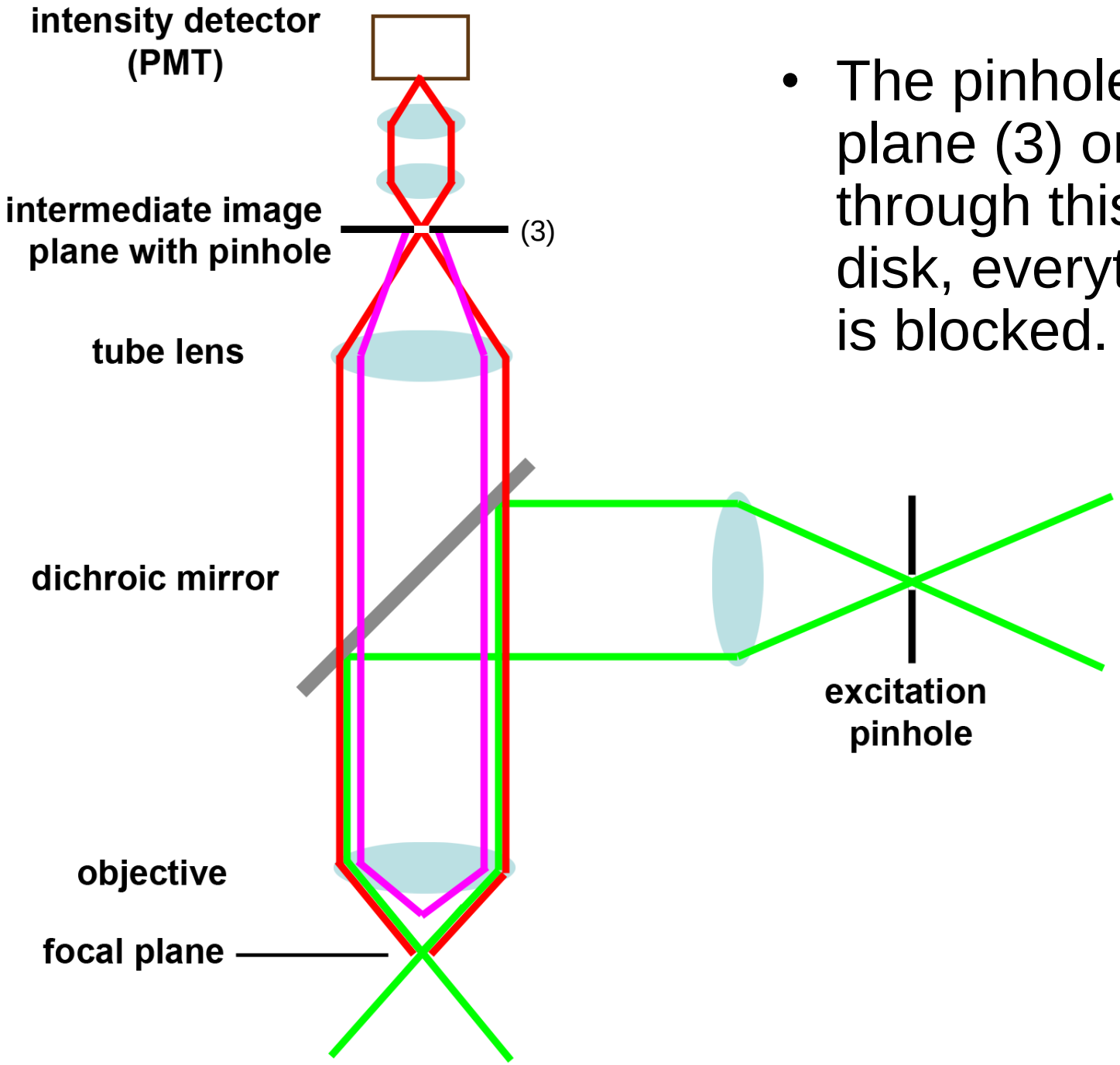
- A point light source (1) is imaged *into the specimen (2)* (by the objective) in one spot, the focal point.
- Because of diffraction, this image in the focal plane (2D) is an Airy disk (and not actually a point).
- The focal point is then imaged (by the objective) into the intermediate image plane (3), where it is again focused into one spot = Airy disk.



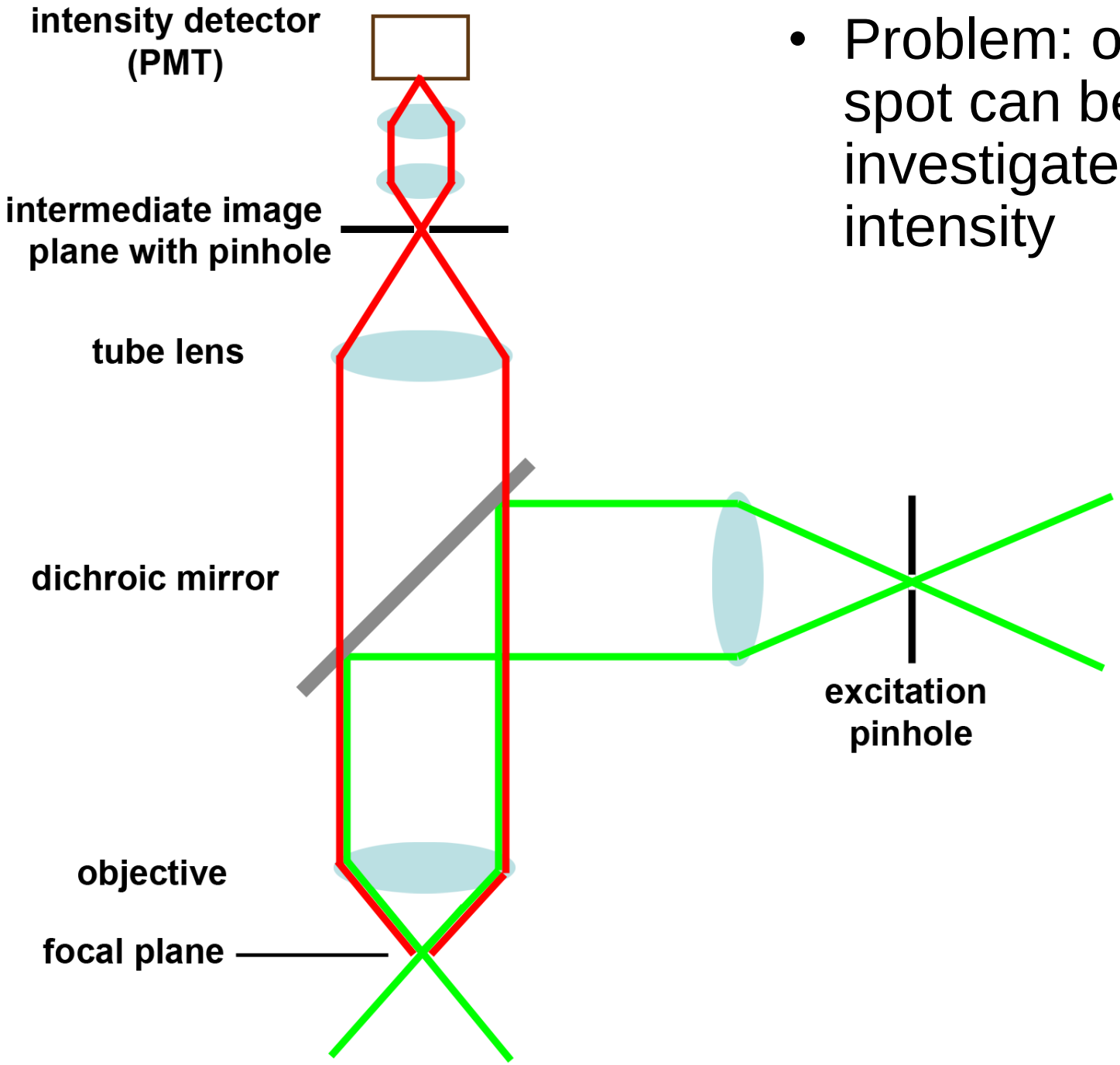
- A point light source (1) is imaged *into the specimen (2)* (by the objective) in one spot, the focal point.
- Because of diffraction, this image in the focal plane (2D) is an Airy disk (and not actually a point).
- The focal point is then imaged (by the objective) into the intermediate image plane (3), where it is again focused into one spot = Airy disk.



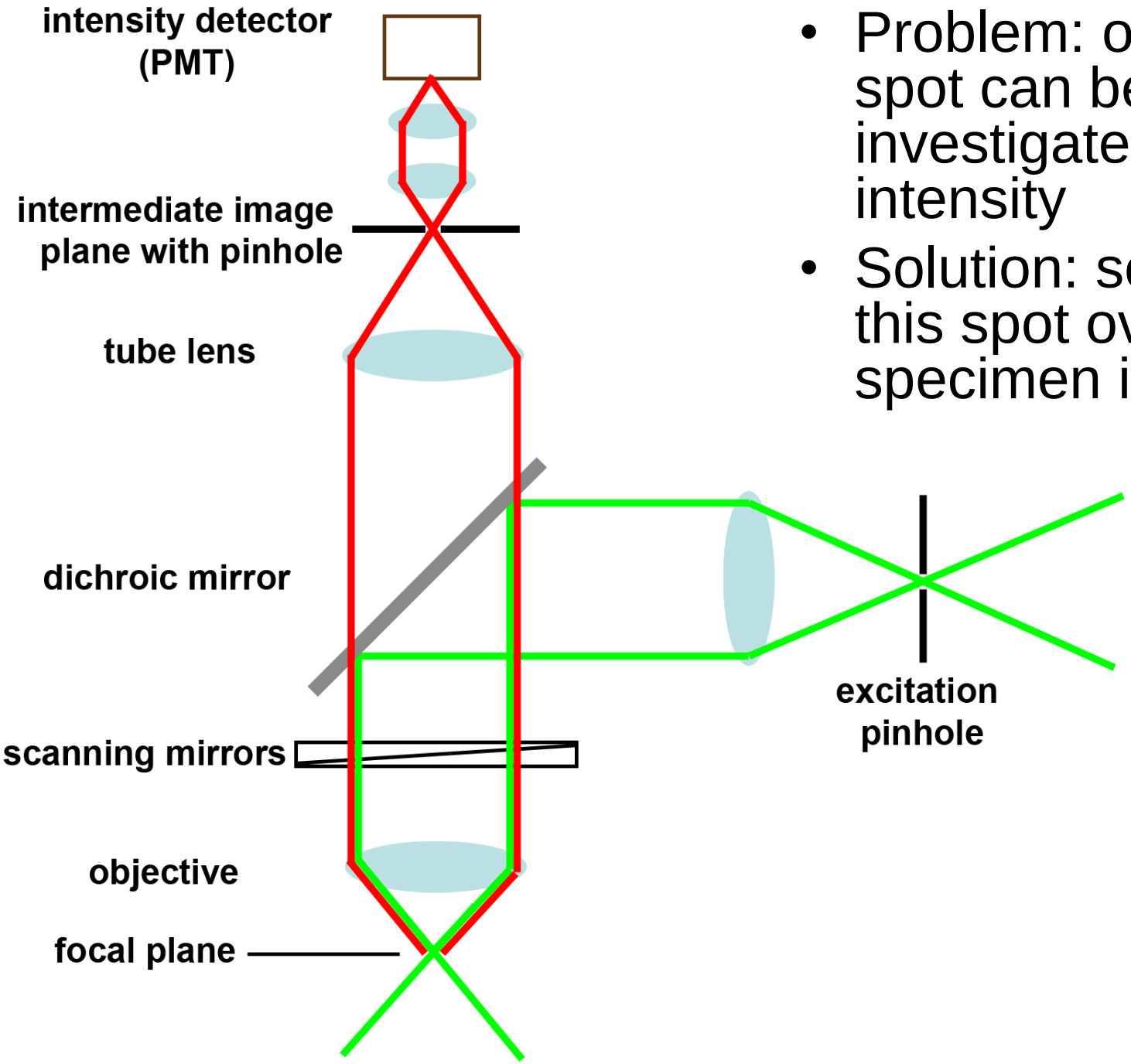
- The pinhole in this plane (3) only lets through this Airy disk, everything else is blocked.



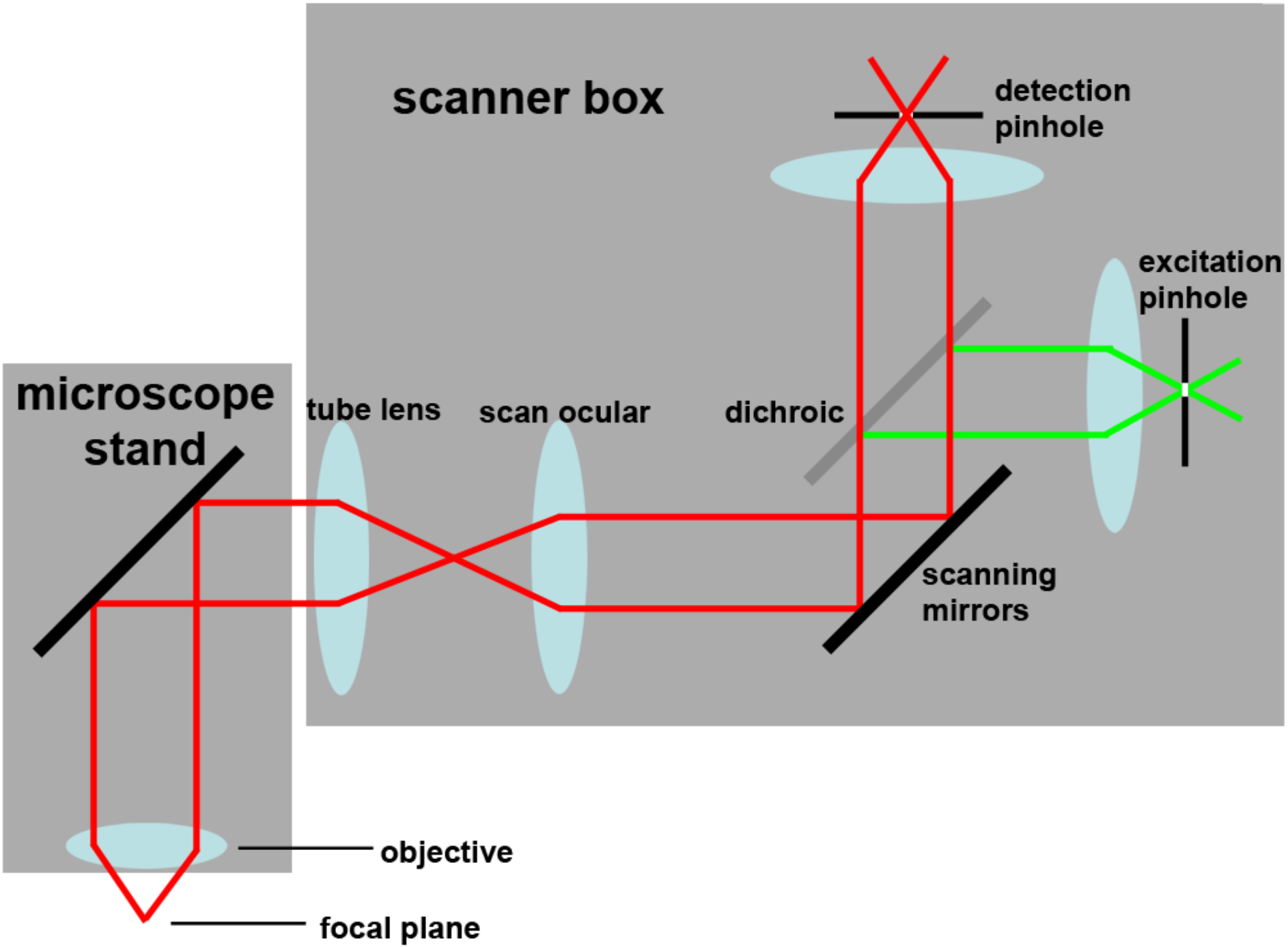
- The pinhole in this plane (3) only lets through this Airy disk, everything else is blocked.



- Problem: only one spot can be investigated for intensity



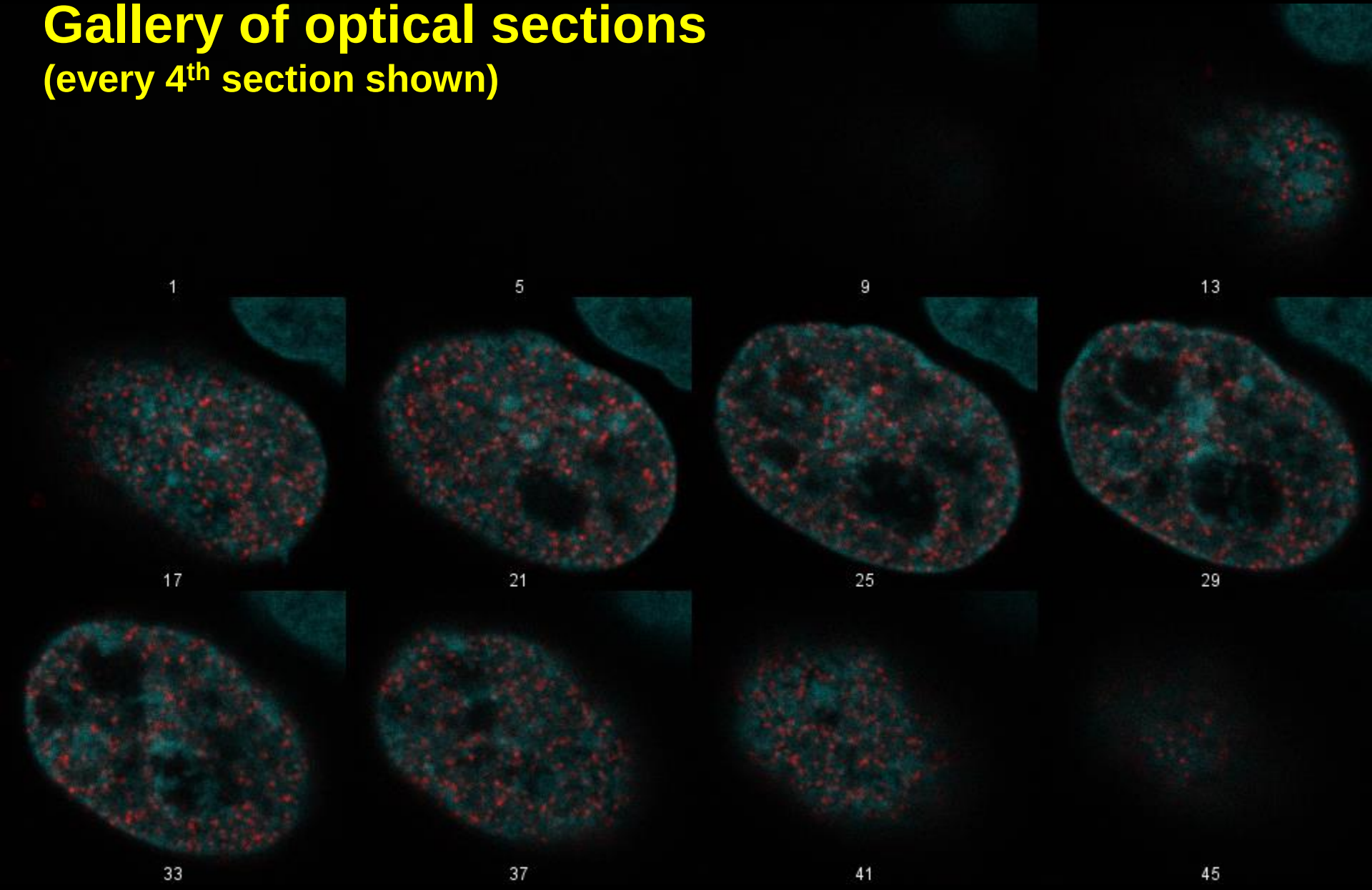
- Problem: only one spot can be investigated for intensity
- Solution: scanning this spot over the specimen in 3D

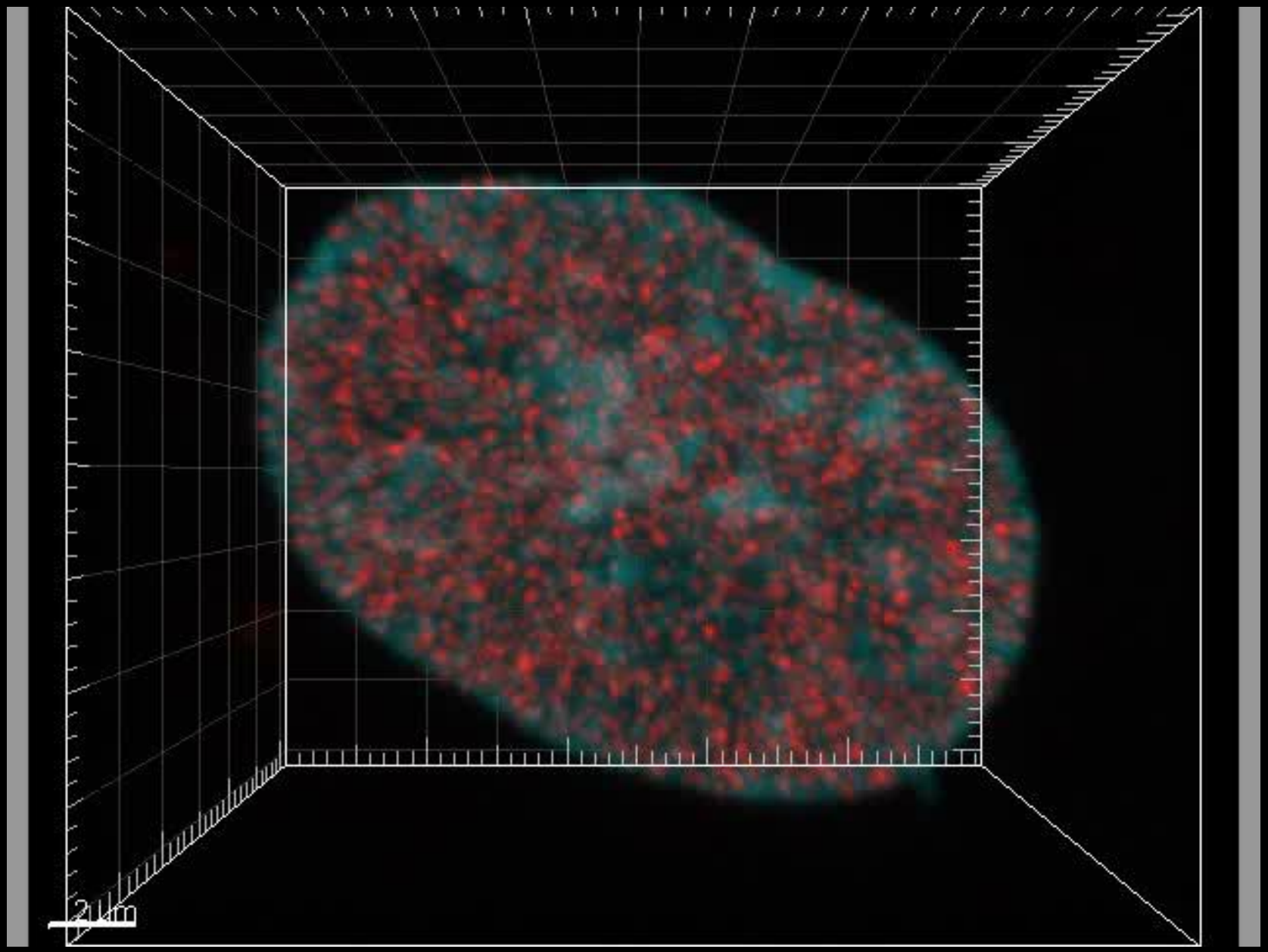

scanner box
detection pinhole
excitation pinhole
microscope stand
tube lens
scan ocular
dichroic
scanning mirrors
objective
focal plane

Clsm and 3D

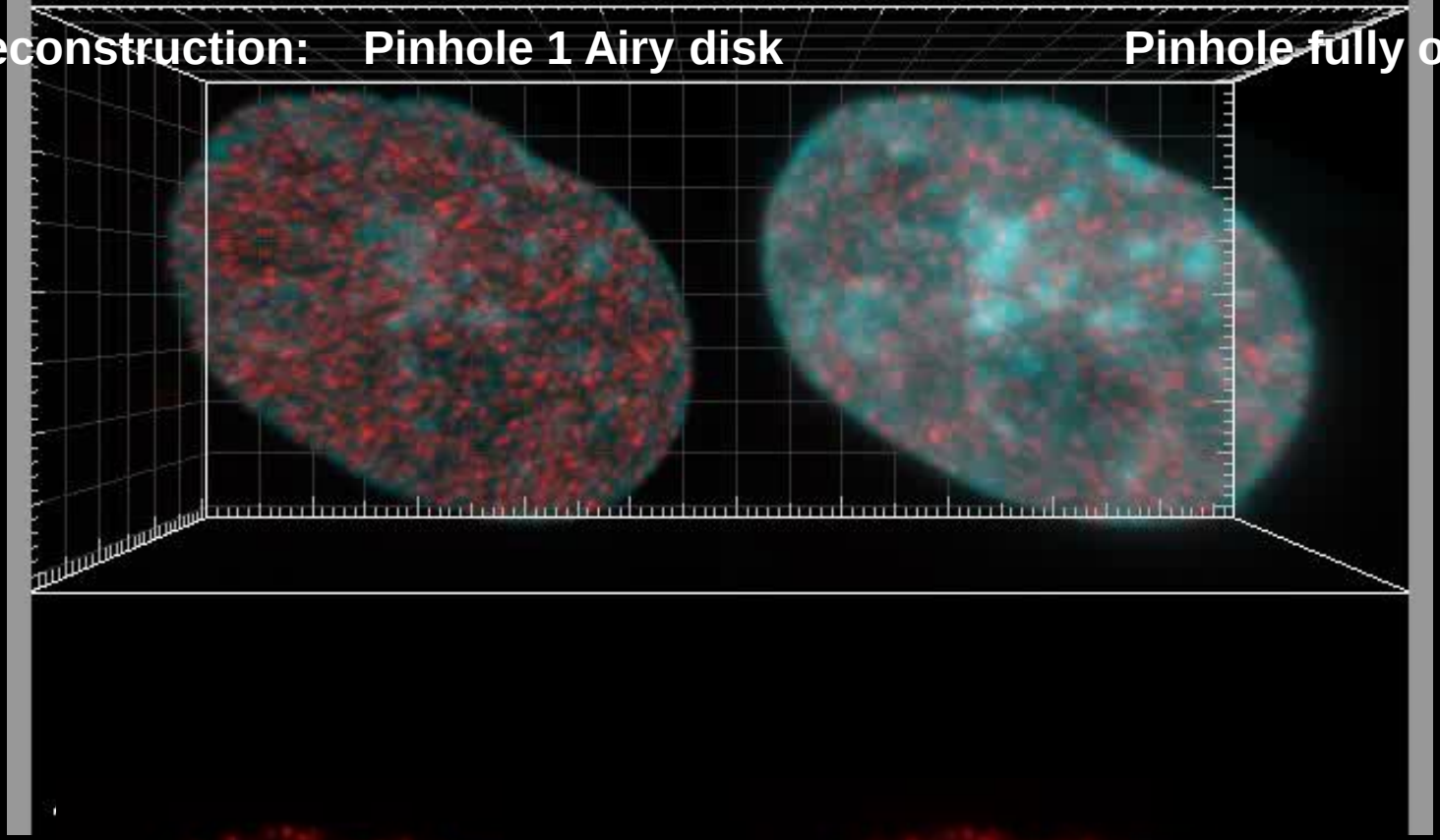
- Technically, „confocal“ has nothing to do with 3D.
- However, all clsm around have the capability of moving the focal plane through the specimen. Thus a series of images can be generated which can be combined to a 3D image stack.

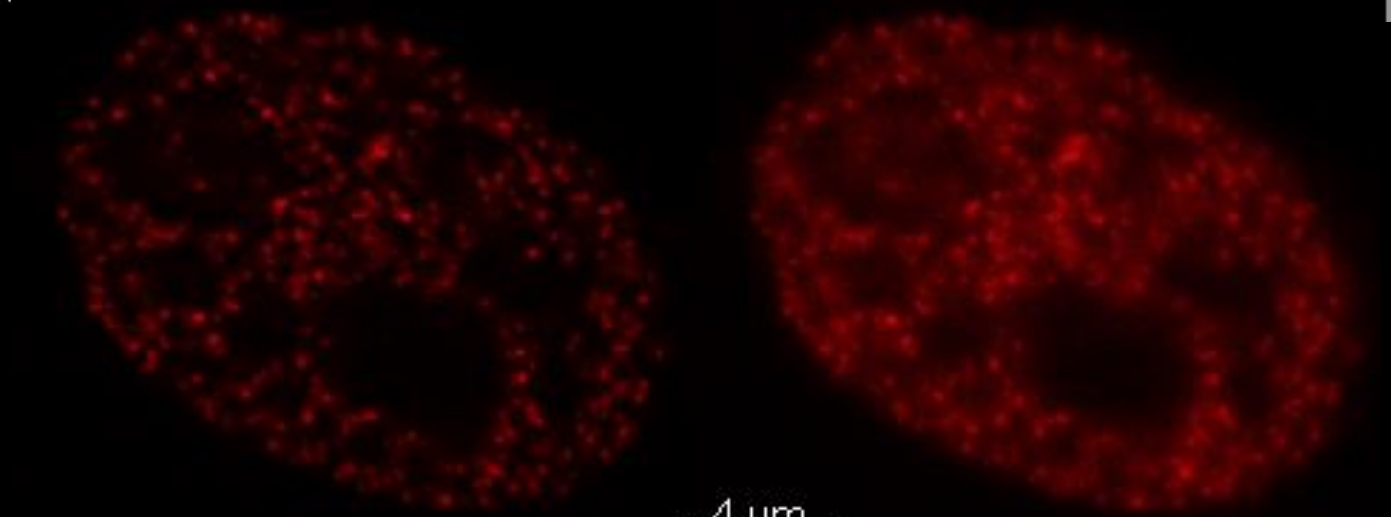
Gallery of optical sections
(every 4th section shown)




2 µm

3D reconstruction: Pinhole 1 Airy disk Pinhole fully open





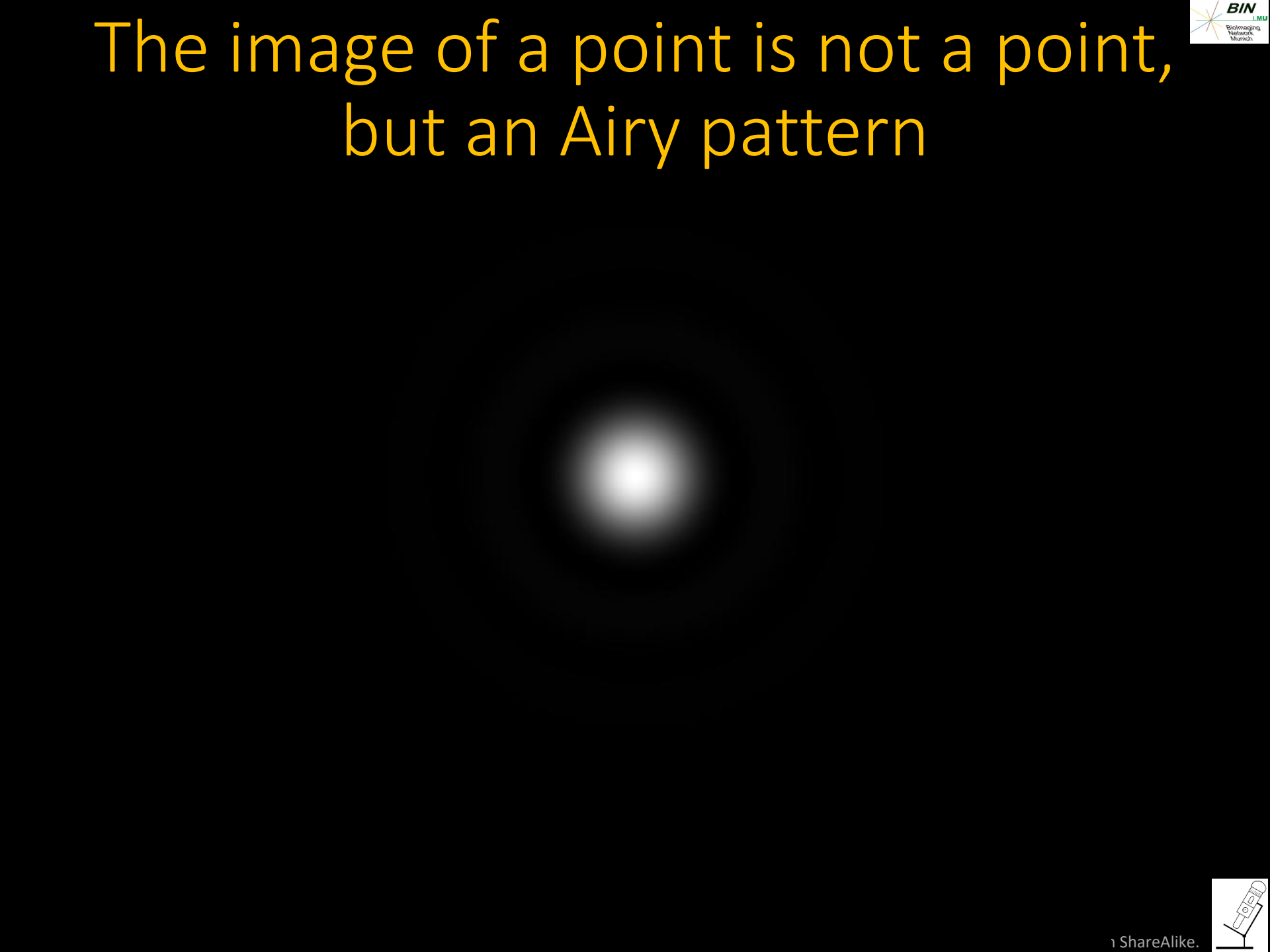
Single sections: Pinhole 1 Airy disk Pinhole fully open

So, a confocal has a much better contrast, due to blocking of out-of-focus fluorescence.

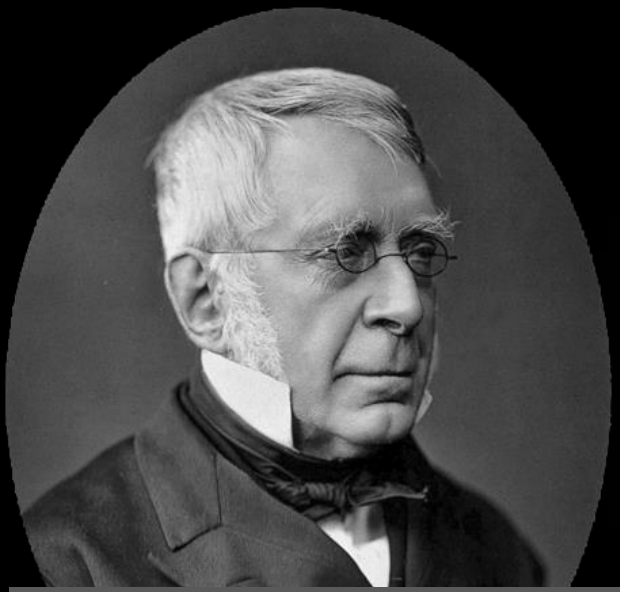
Does it have an improved resolution?

- ❑ No.
- ❑ Yes.
- ❑ It depends.
- ❑ All of the above.

The image of a point is not a point, but an Airy pattern



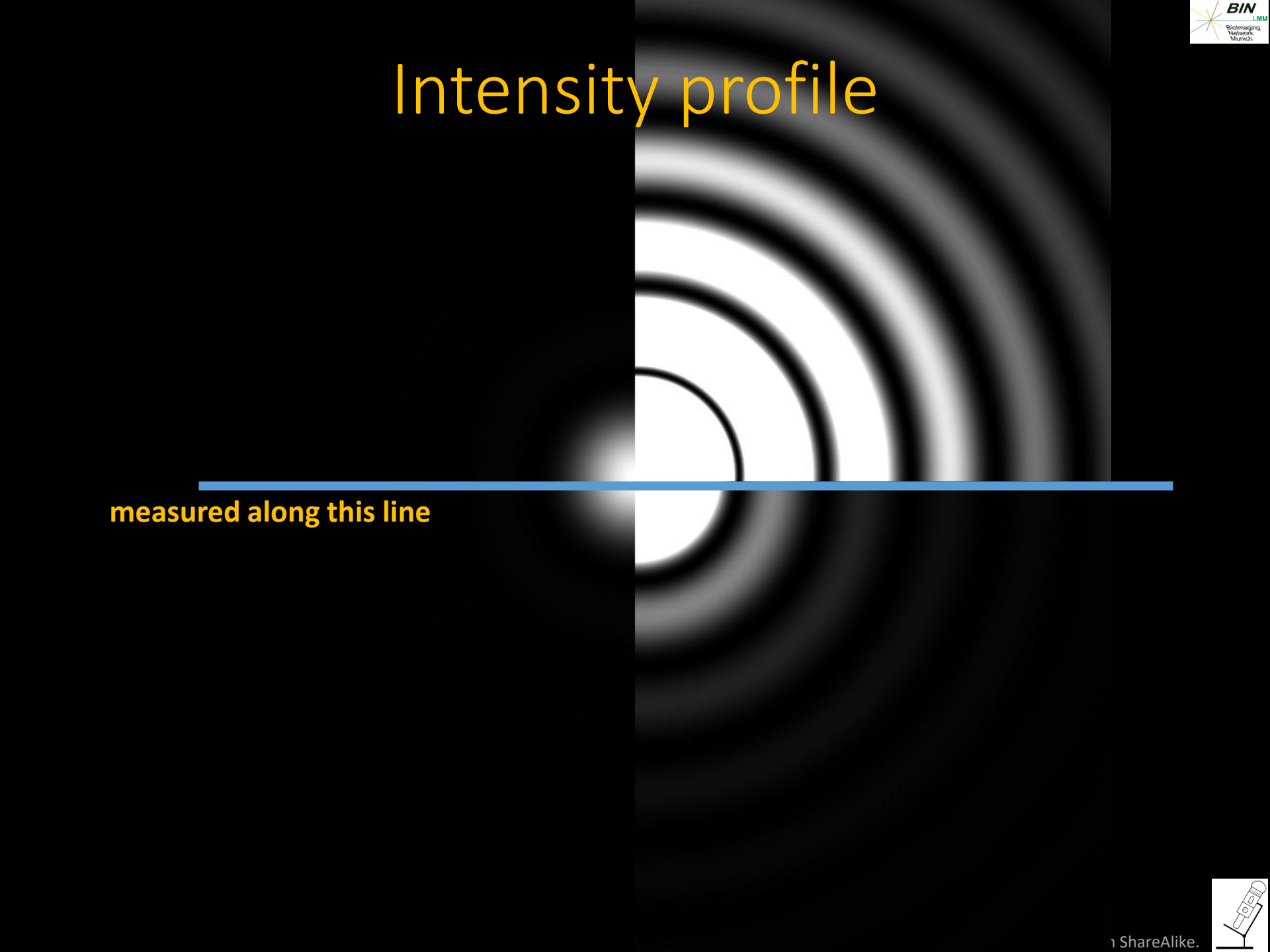
The image of a point is not a point, but an Airy pattern



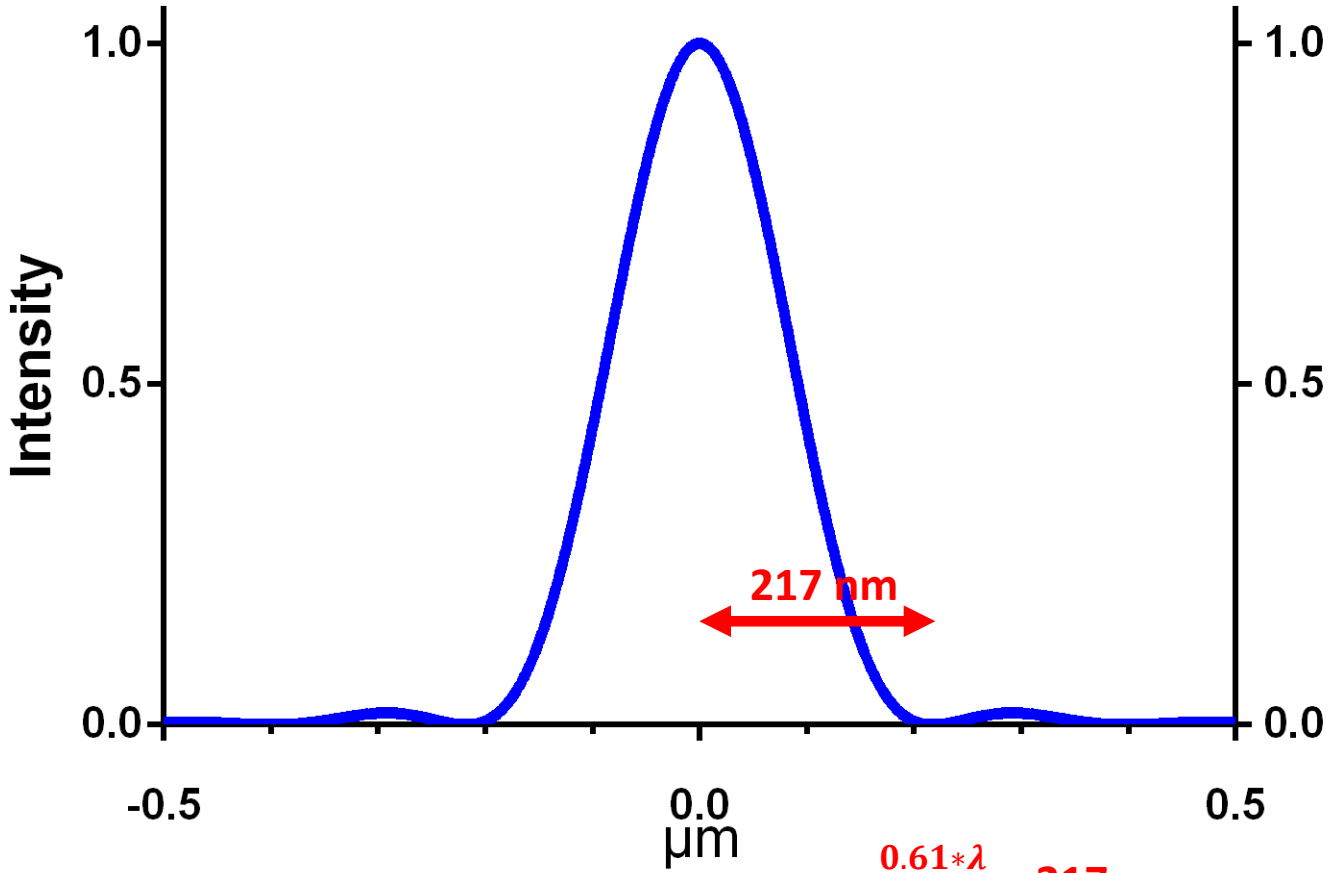
Sir George Biddell Airy,
27.07.1801–02.01.1892
Astronomer Royal 1835-1881

1 AU

Intensity profile

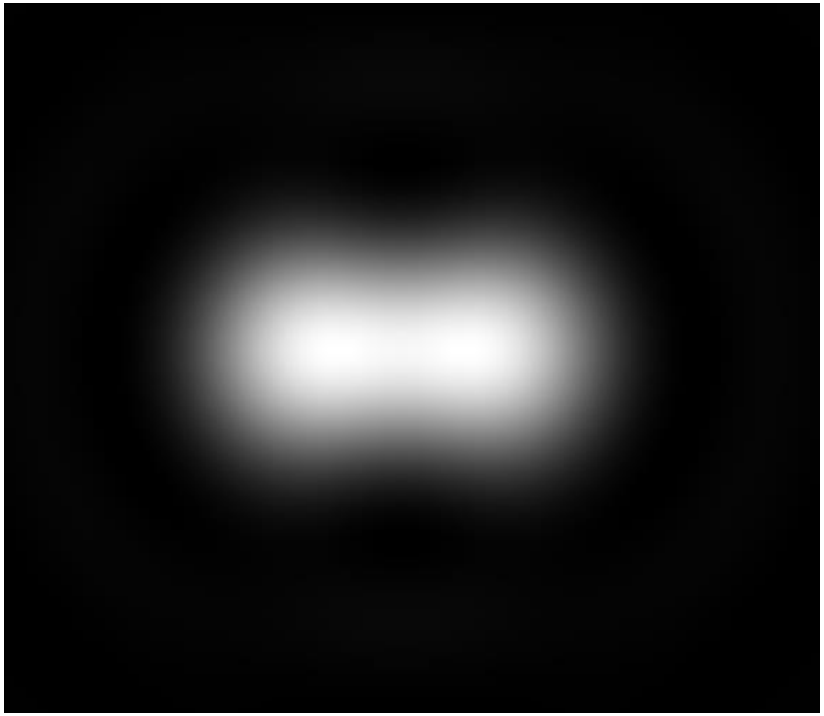


Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



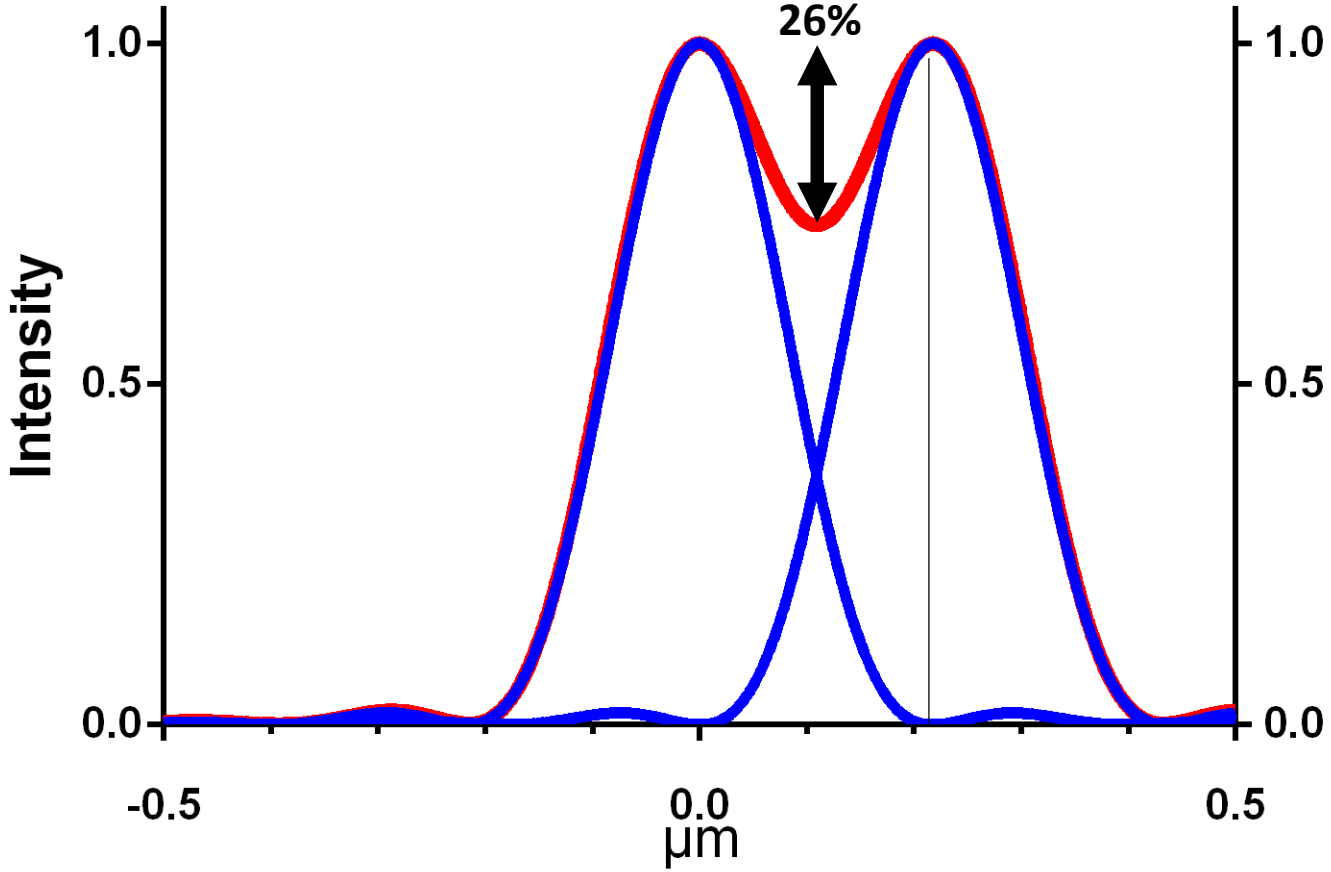
$$r = \frac{0.61 * \lambda}{NA} = 217 \text{ nm}$$

How close can two Airy patterns be together and still be ***resolved*** = recognized as two?



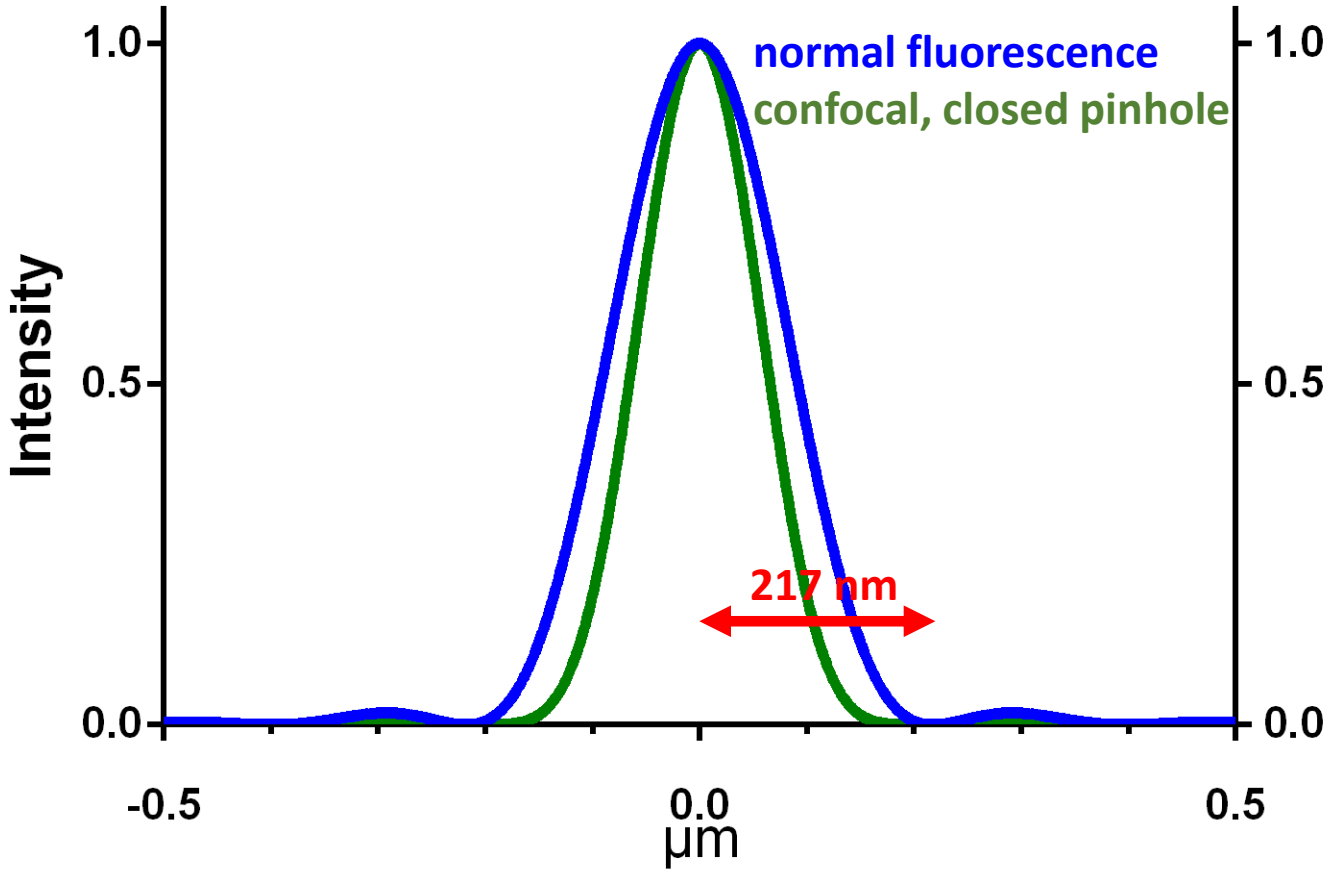
Lord Rayleigh
(John William Strutt)
12.11.1842-30.07.1919

Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm

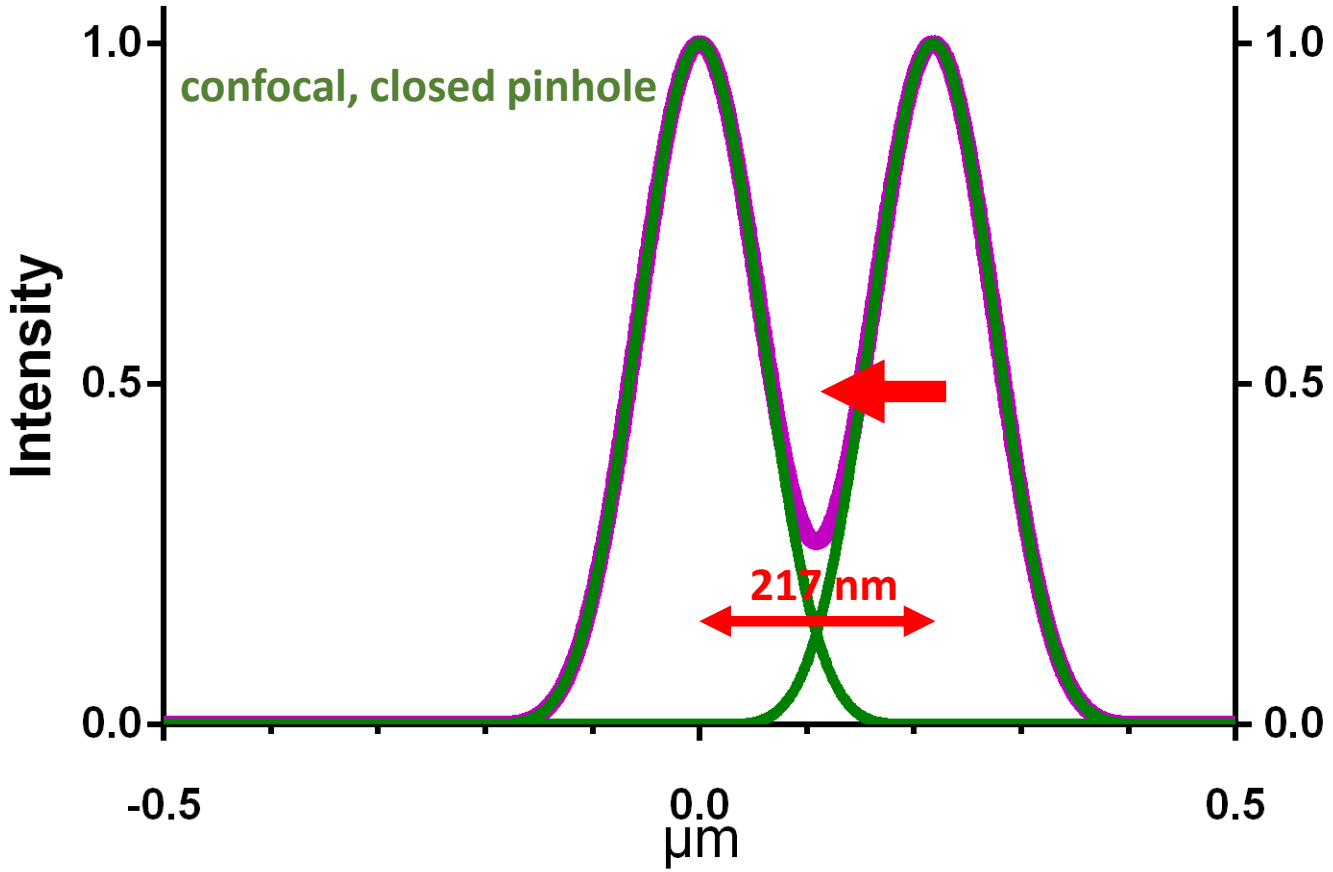


Rayleigh criterion

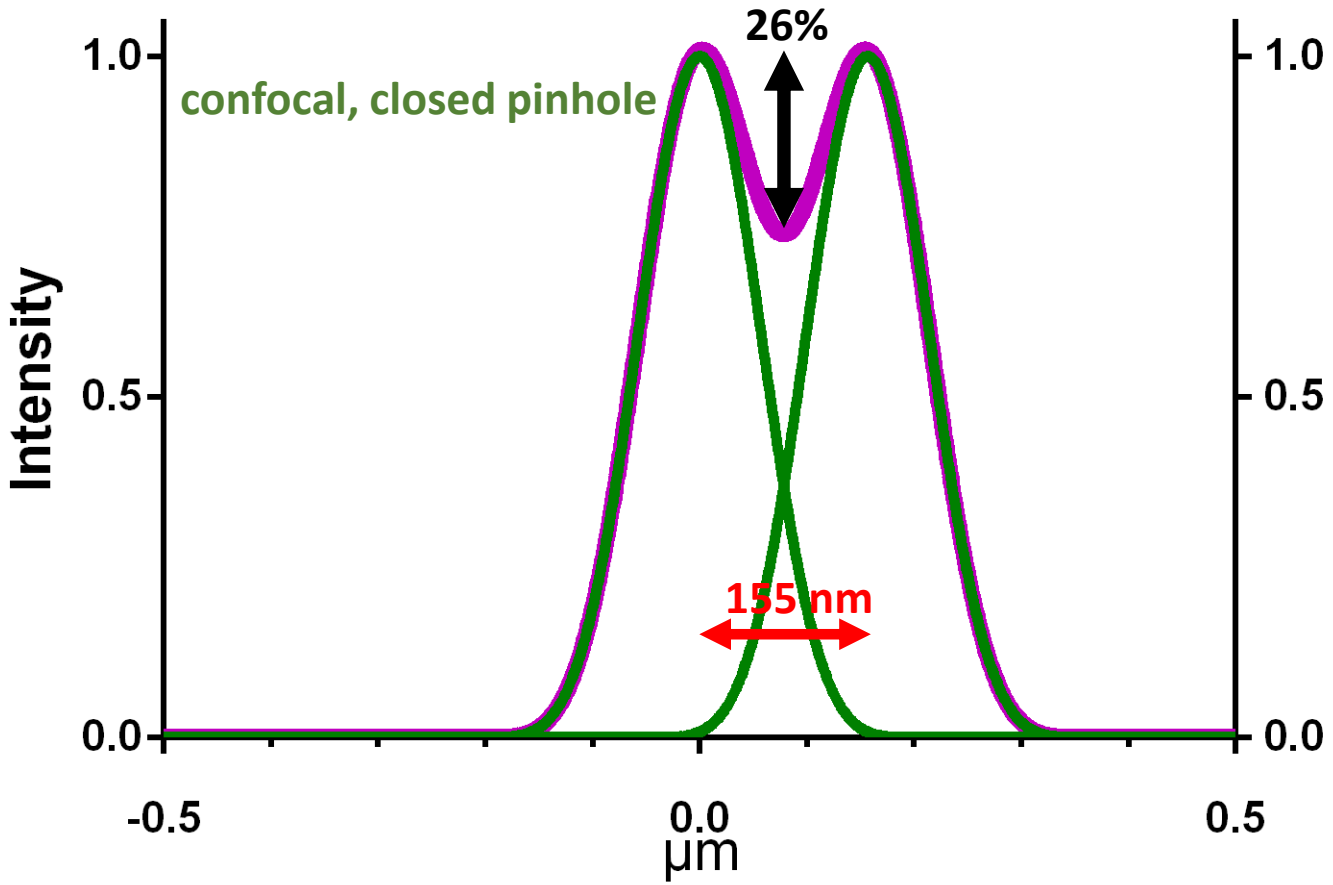
Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm



Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm

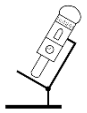


Example: Intensity distribution in the Airy pattern of a point with an NA 1.4 objective with λ=500 nm

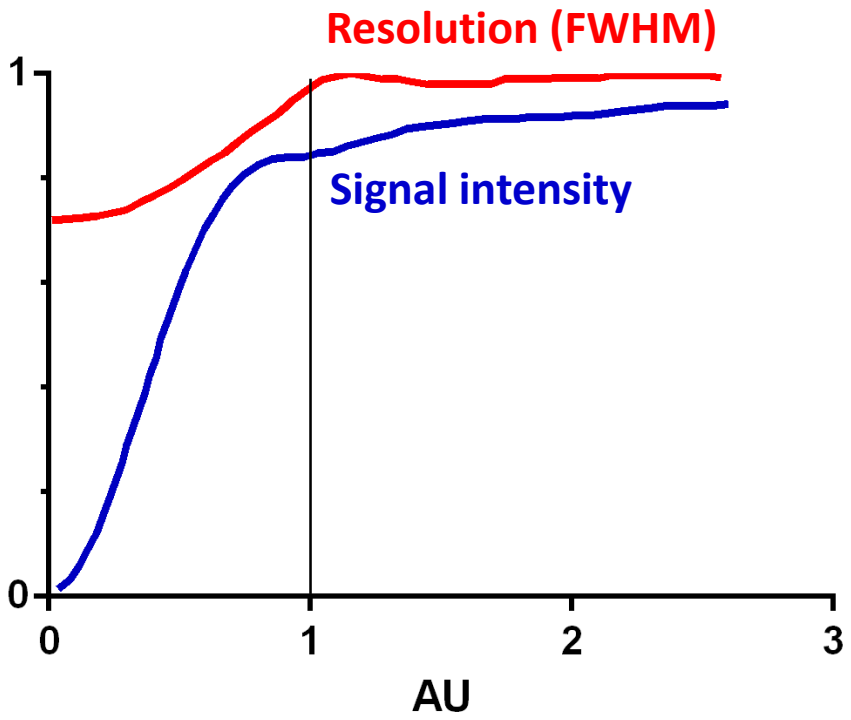


But:

- If we close the pinhole, we don't get a signal on the detector. Therefore, the pinhole is usually set to 0.8 – 1 AU.
- AU = Airy unit, diameter of the first ring-minimum of the Airy pattern

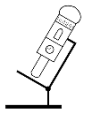


Confocal resolution and intensity vs Pinhole size



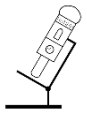
Data source: CJR Sheppard et al. (2006) Signal–to-noise ratio in confocal microscopes. In: JB Pawley (ed) Handbook of biological confocal microscopy (3rd ed) pp 442-452. Numerical data extracted with Engauge Digitizer V.4.1 from Fig 22.2 (circular pinhole) and Fig 22.3 (1/[transverse resolution (point)]).

Concerning the resolution in z, the same principle applies

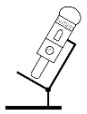


Confocal Microscopy

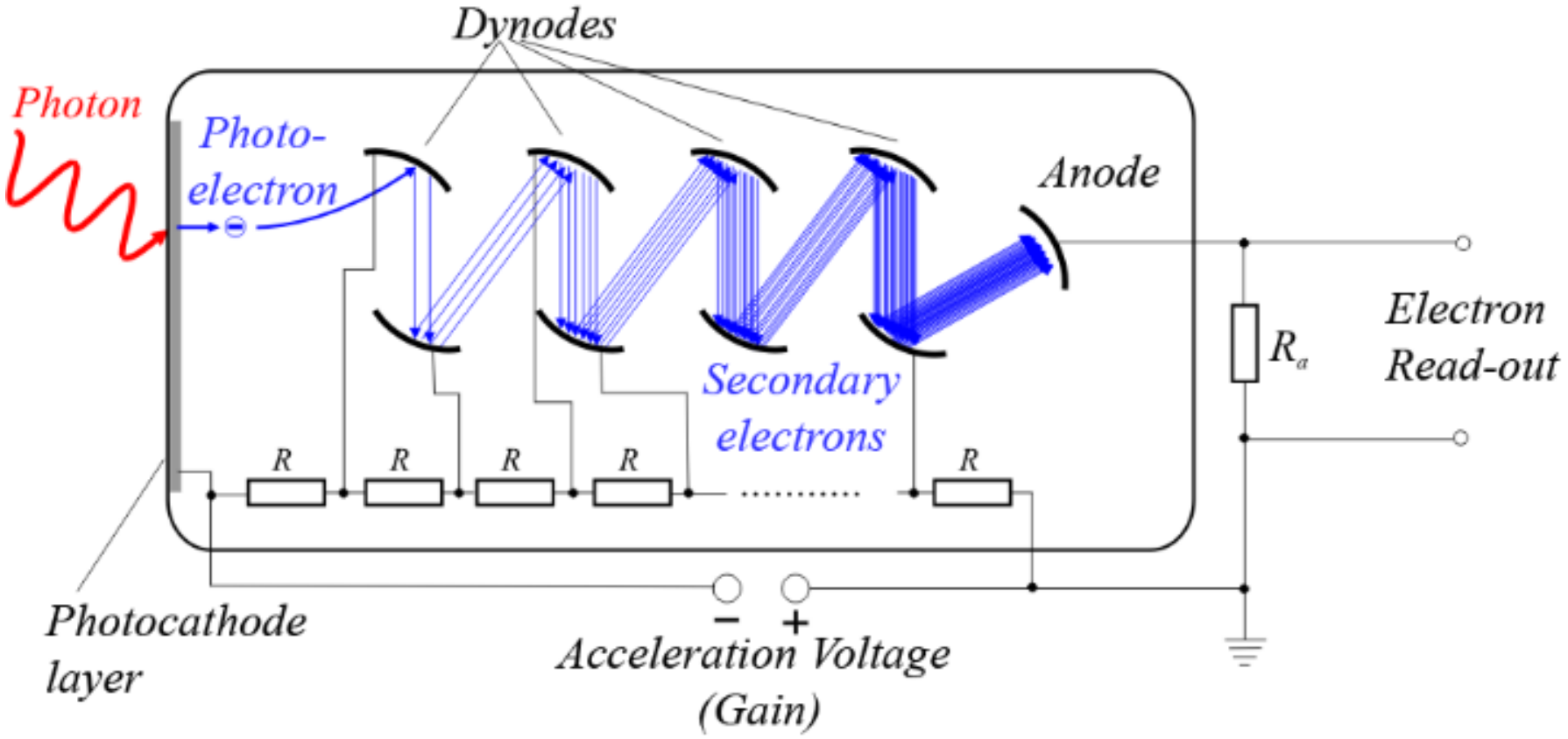
- To produce confocal images, in principle no laser or fluorescence is required:
- Transmission confocal microscopy is possible (e.g. Minsky, 1957) but not commercially available.
- The point is, that only one spot of the preparation is scanned at any one time and only light from that point is detected through the detection pinhole.



- Detectors: PMTs and hybrid detectors



Photomultiplier Tube (PMT)



Side-On Photomultiplier

http://www.olympusmicro.com/primer/java/digitalimaging/photomultiplier/sideonpmt/index.html

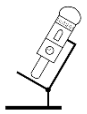
PMT

- Typically, a PMT has 10 Dynodes. If 5 electrons are sputtered for each hitting electron, amplification is 5^{10}, i.e. about 10 million electrons for each photoelectron from the photocathode

- As long as the PMT is not saturated, the total number of secondary electrons is generally proportional to the number of hitting photons, but:

- Amplification is a statistical process: one electron may sputter 5 electrons, the next one maybe 4 or 6.

- This statistical detector noise (Poisson noise from the detector) gets less important when image *contrast* is increased by longer exposure, e.g. image averaging.



PMT

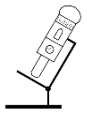
- The analog signal is then digitized, usually with 8 bit (2^8 = 256 values, 0-255).

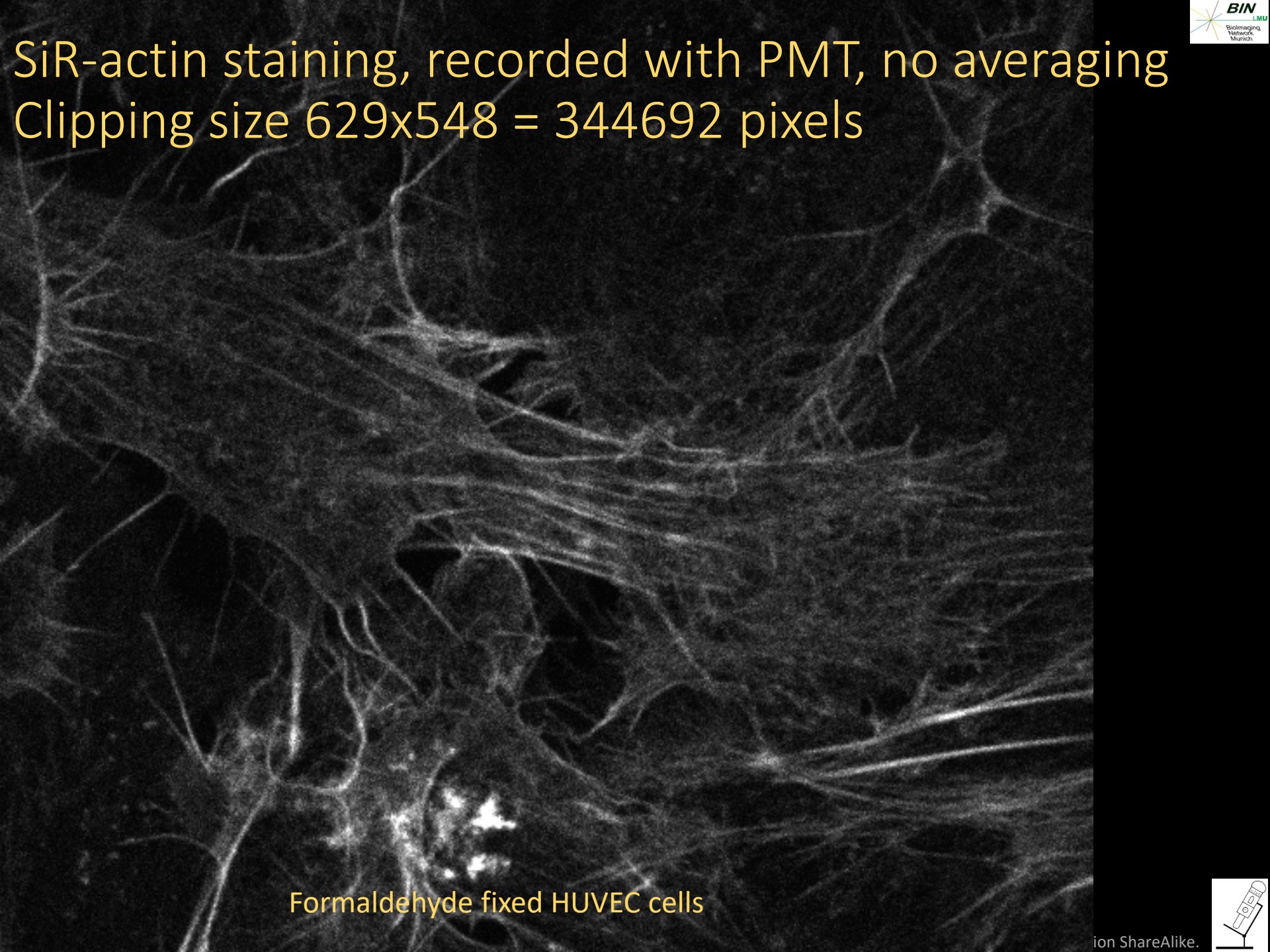
- Higher voltage (= gain) leads to a stronger signal and to more electronic noise.

- the „offset“ defines the zero-point (how many electrons are „zero“).

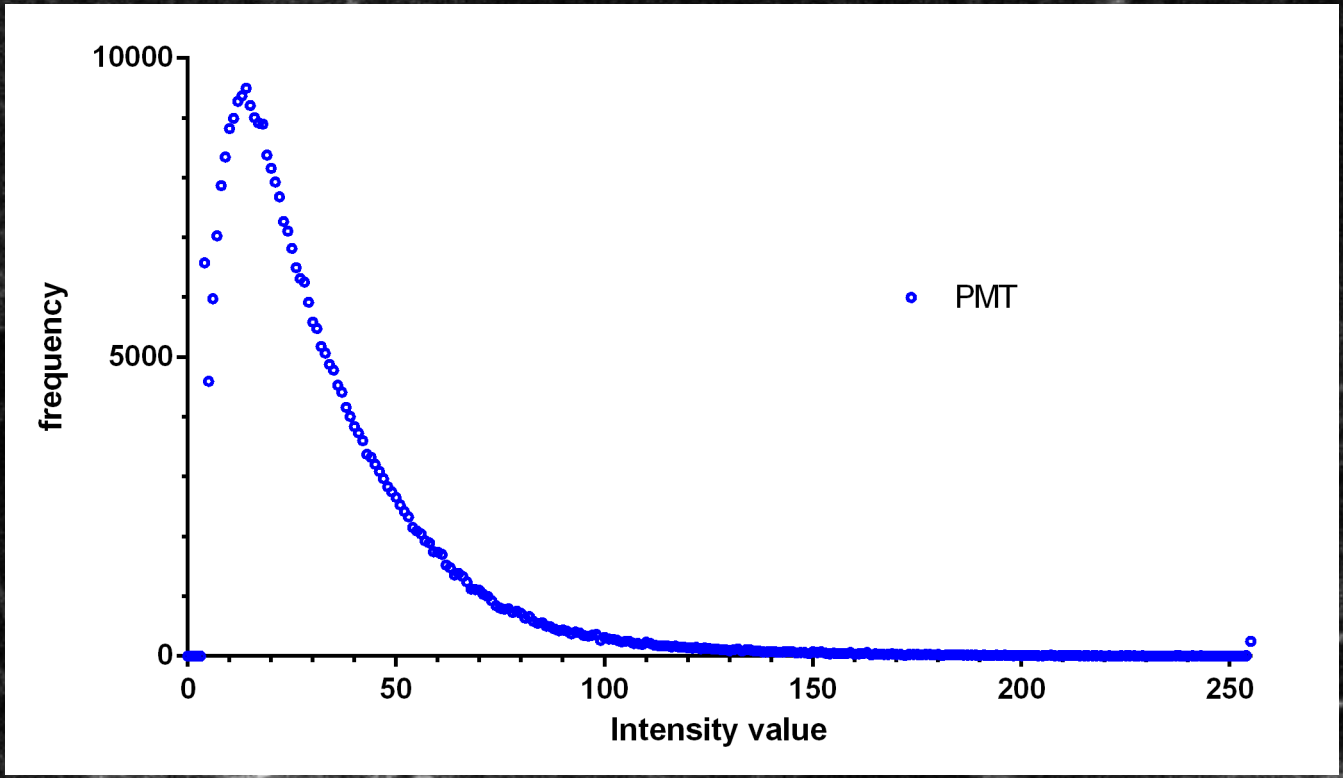
How many photons do we really collect in one pixel?

- Let’s have a look at the grayvalues in an image…



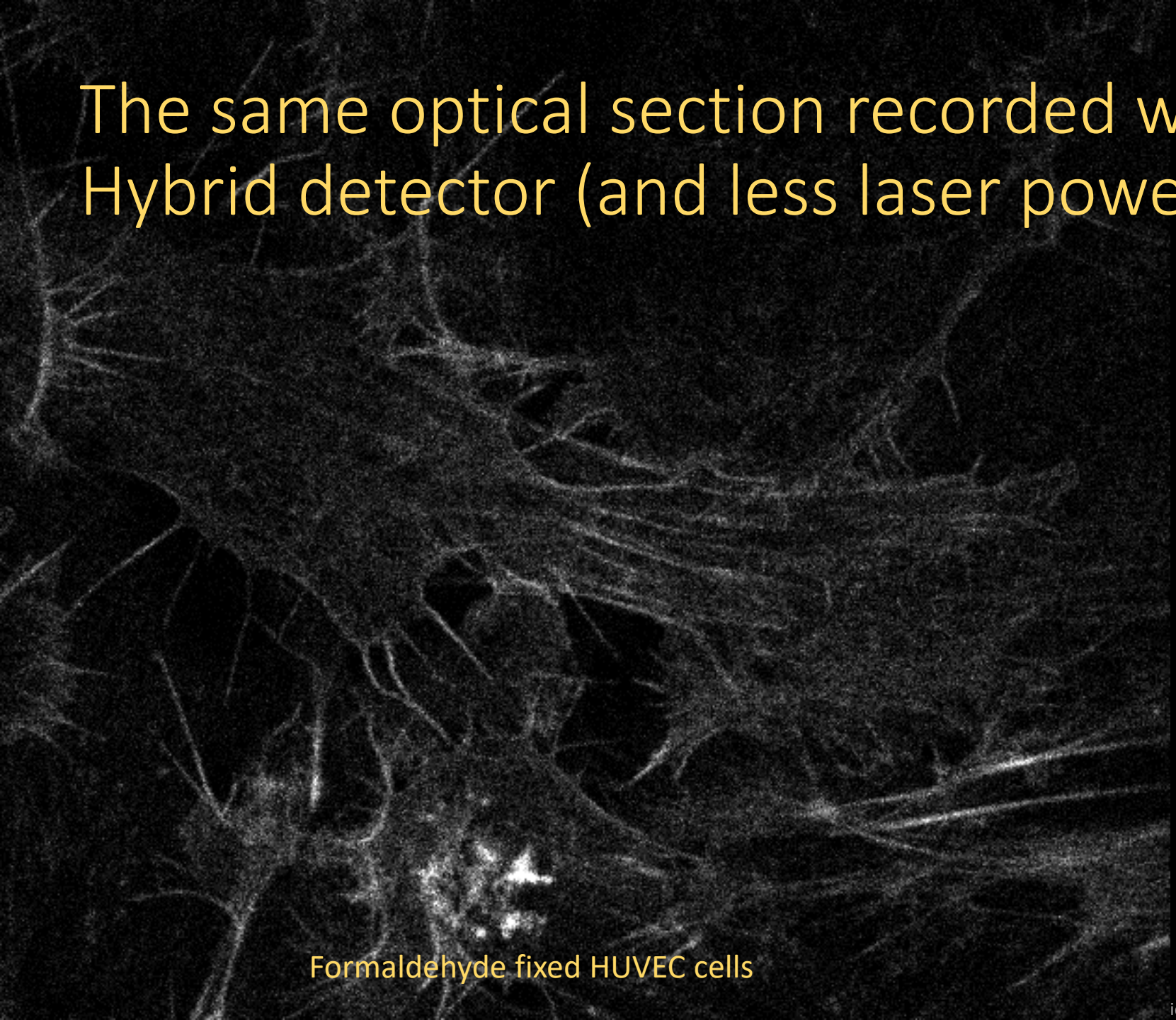


SiR-actin staining, recorded with PMT, no averaging
Clipping size 629x548 = 344692 pixels



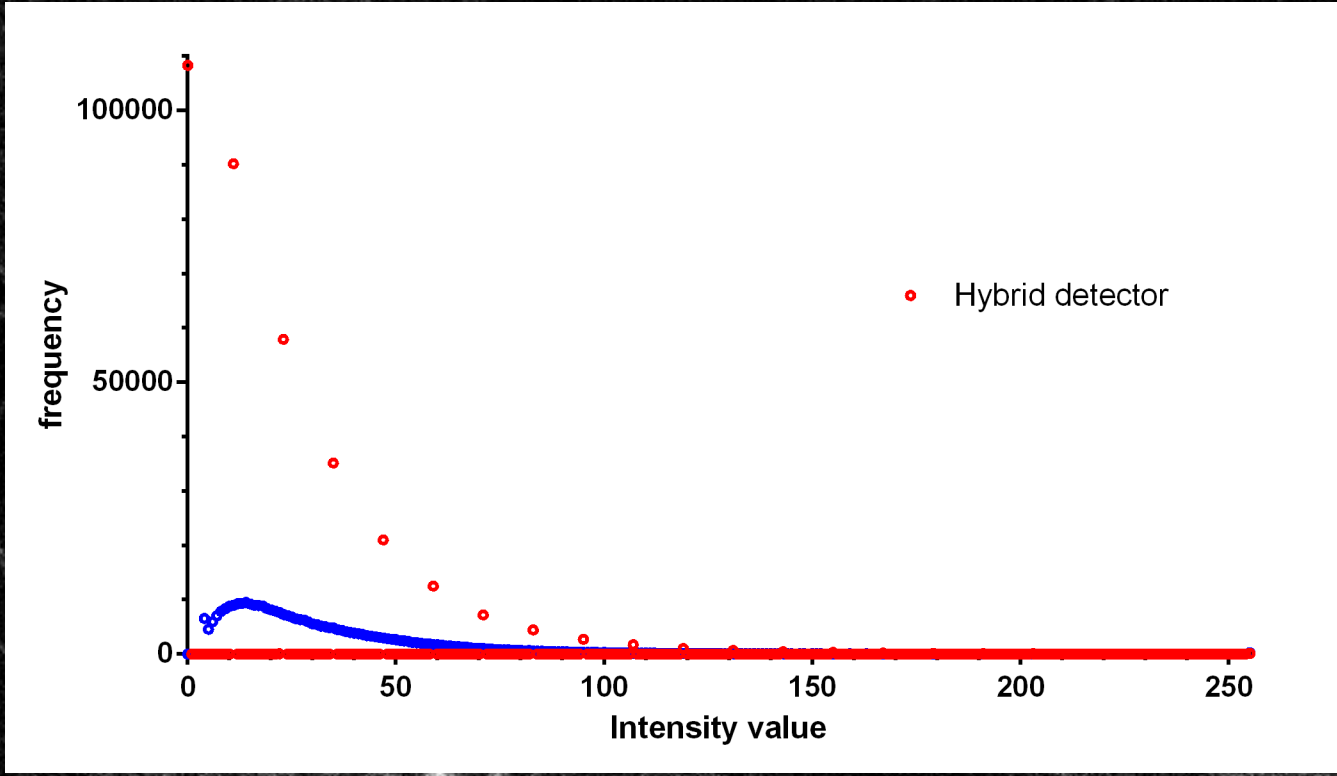
Formaldehyde fixed HUVEC cells

The same optical section recorded with Hybrid detector (and less laser power)



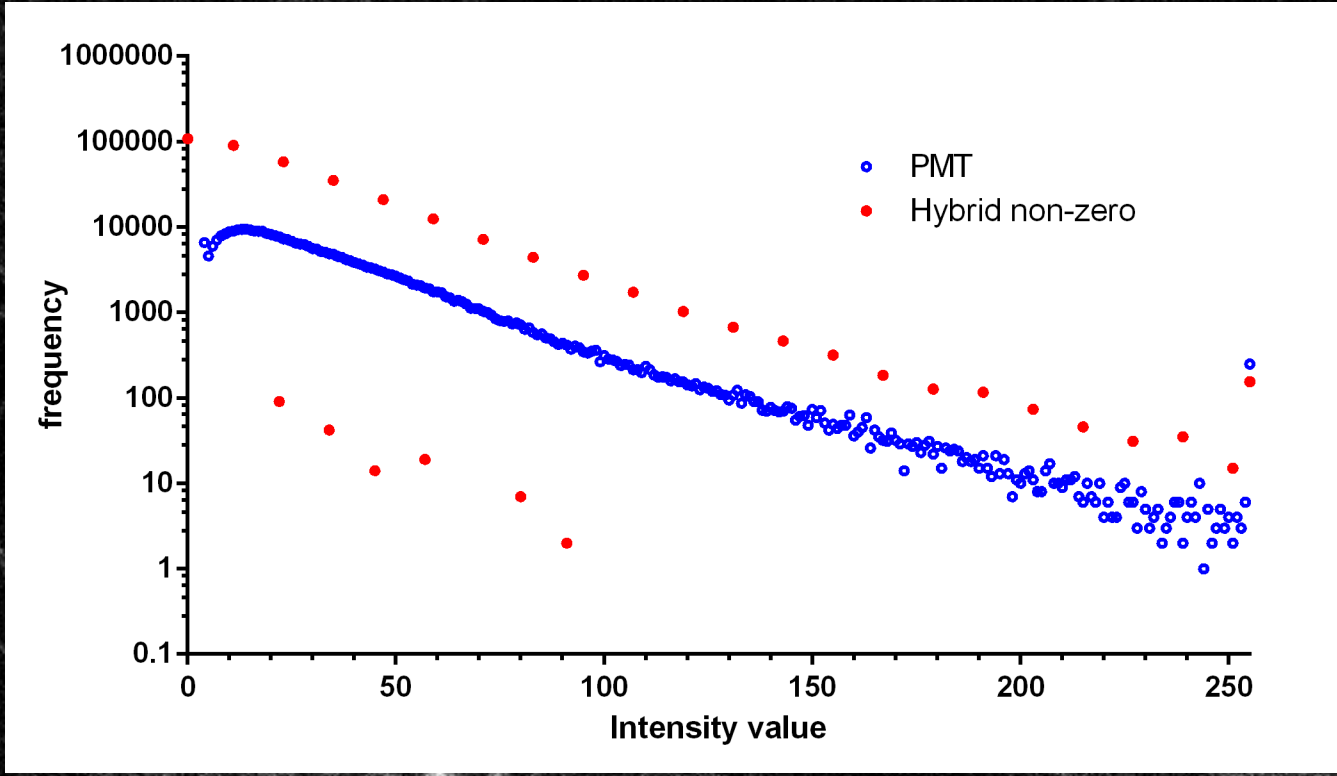
Formaldehyde fixed HUVEC cells

The same optical section recorded with Hybrid detector (and less laser power)



Formaldehyde fixed HUVEC cells

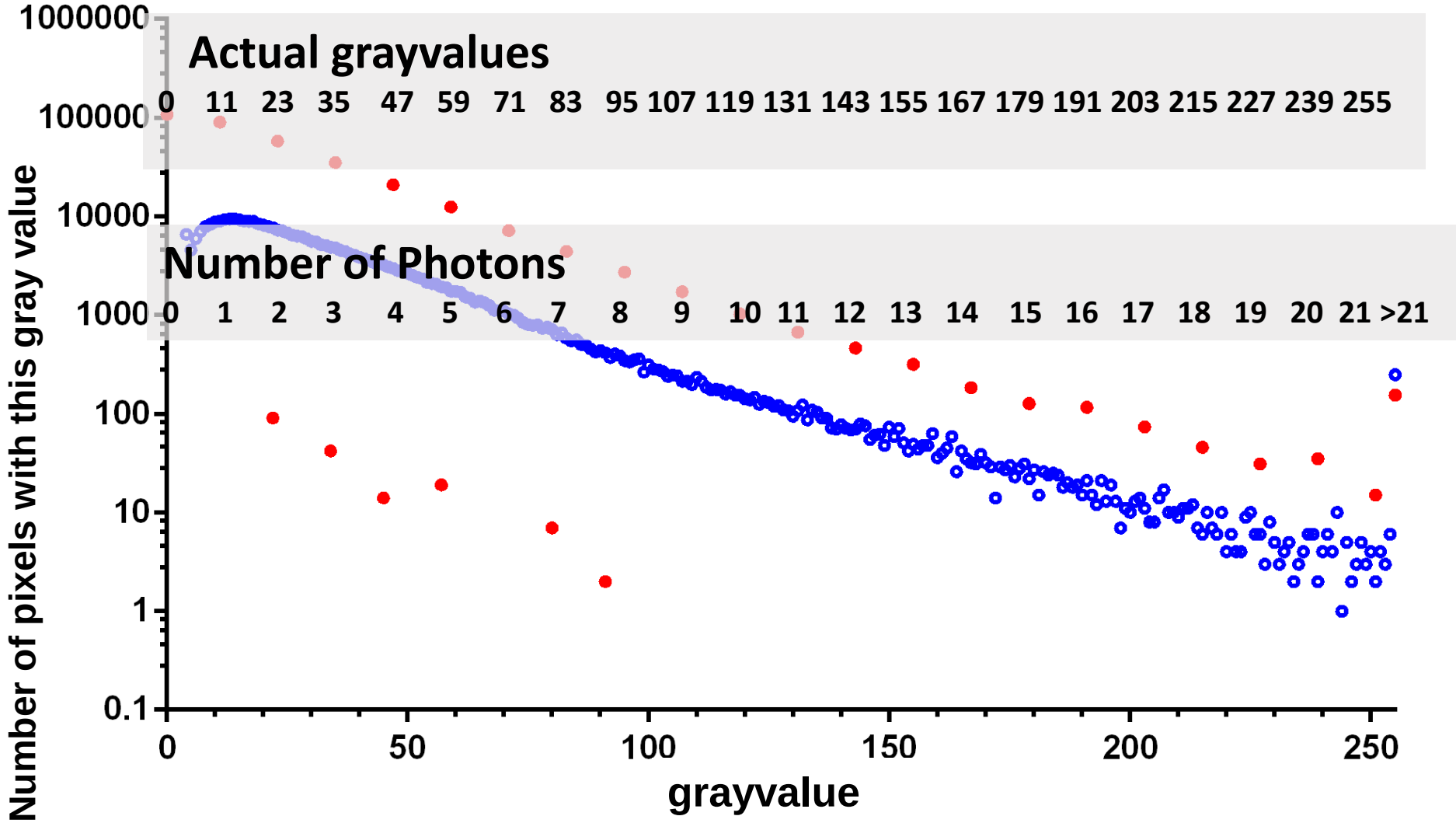
The same optical section recorded with Hybrid detector (and less laser power)



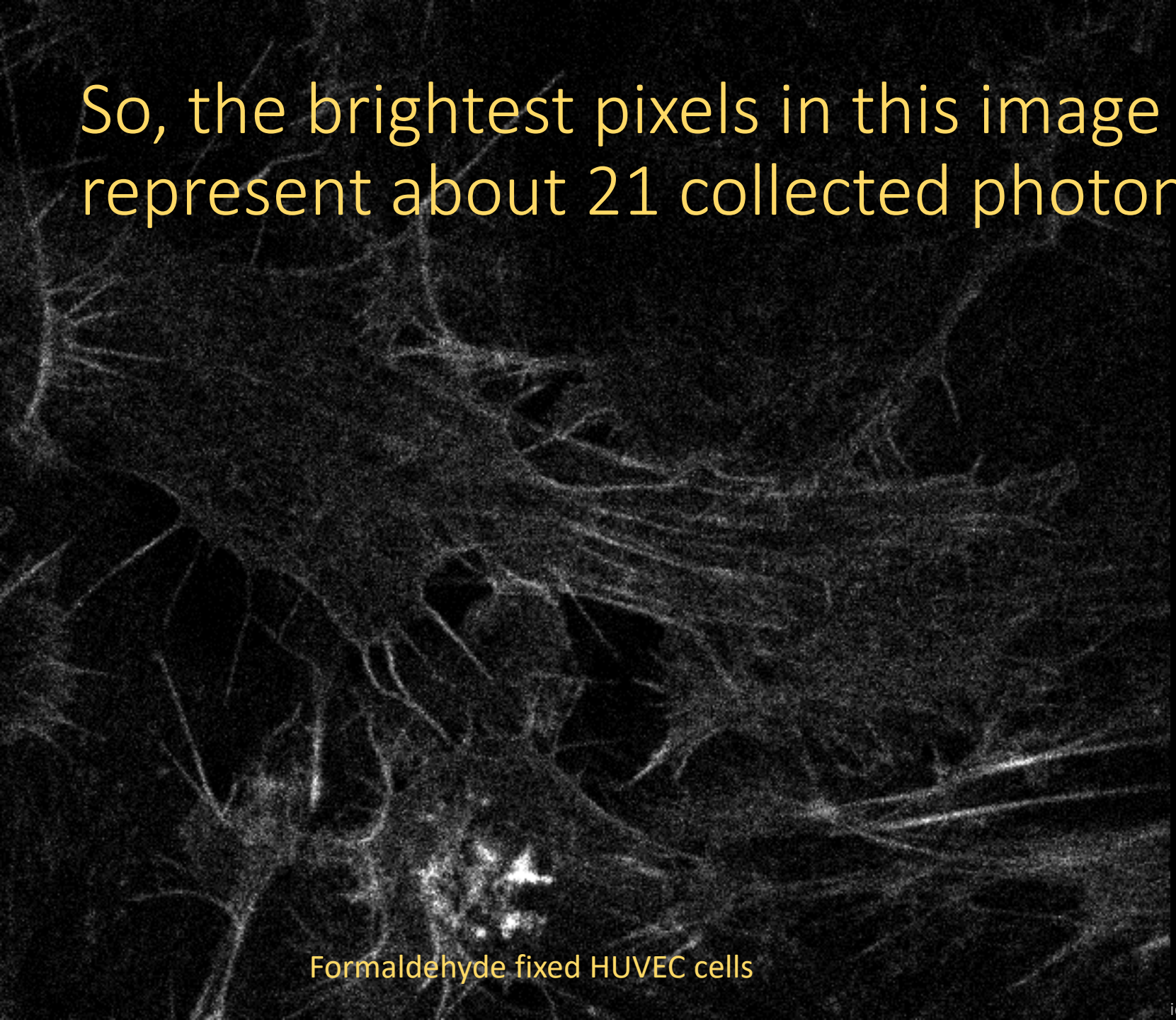
Formaldehyde fixed HUVEC cells

How many photons do we collect per pixel?

Histogram for a confocal image, recorded with PMT and hybrid detector

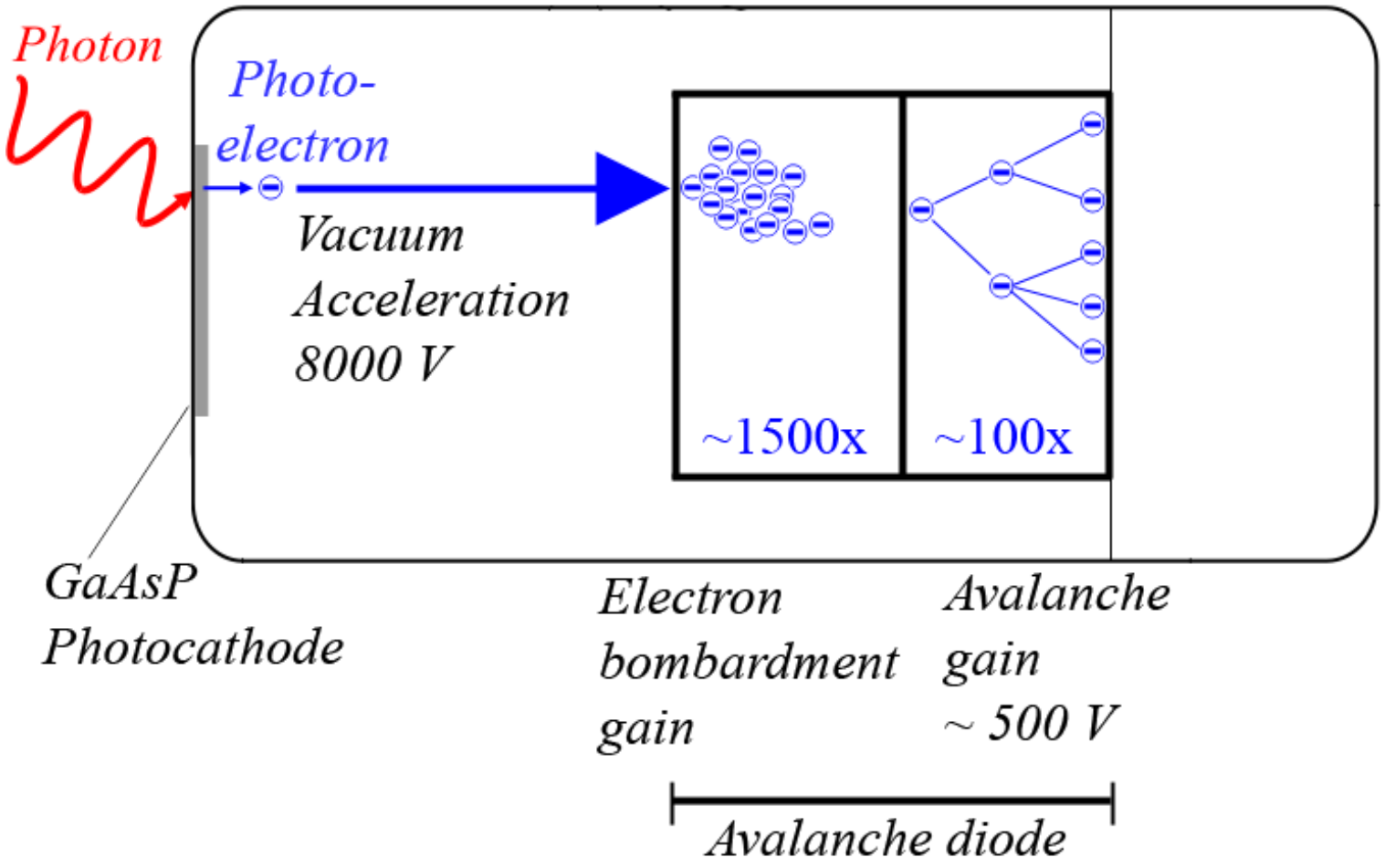


So, the brightest pixels in this image represent about 21 collected photons



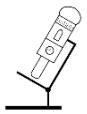
Formaldehyde fixed HUVEC cells

Hybrid detector



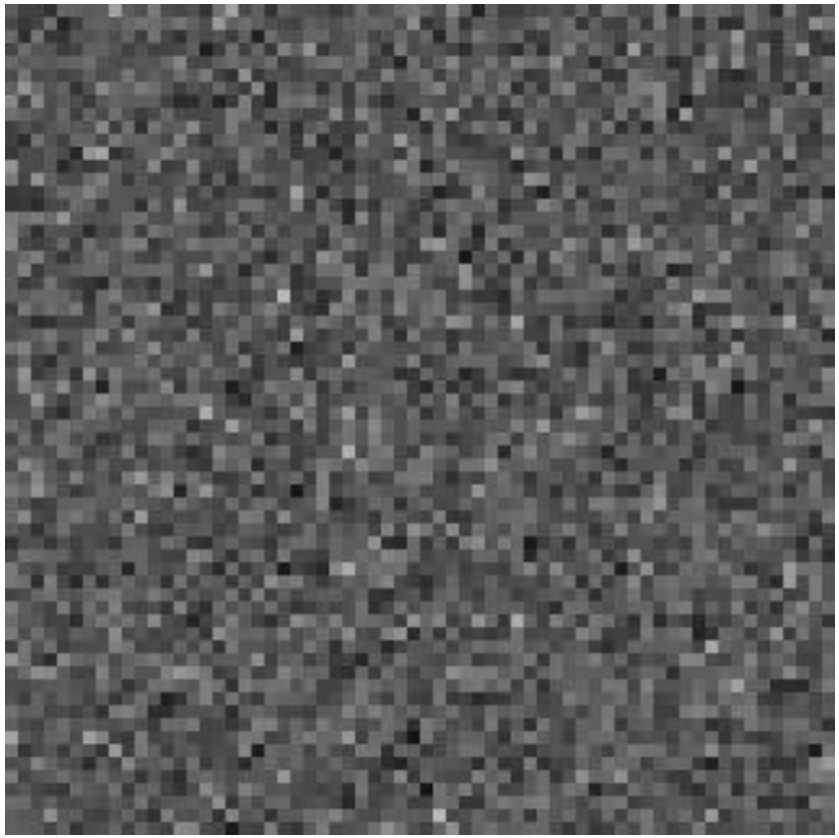
Hybrid detector

- A hybrid between a PMT (Vacuum acceleration) and an avalanche photodiode (semi-conductor amplification)
- The amplification happens in only two steps and the vacuum acceleration is very high (8 kV). Thus it produces less statistical noise than a PMT:
- The number of photons is directly related to the gray level!
- A smaller photocathode results in less dark noise.
- The vacuum voltage is fixed. No adaption to very bright signals is possible.

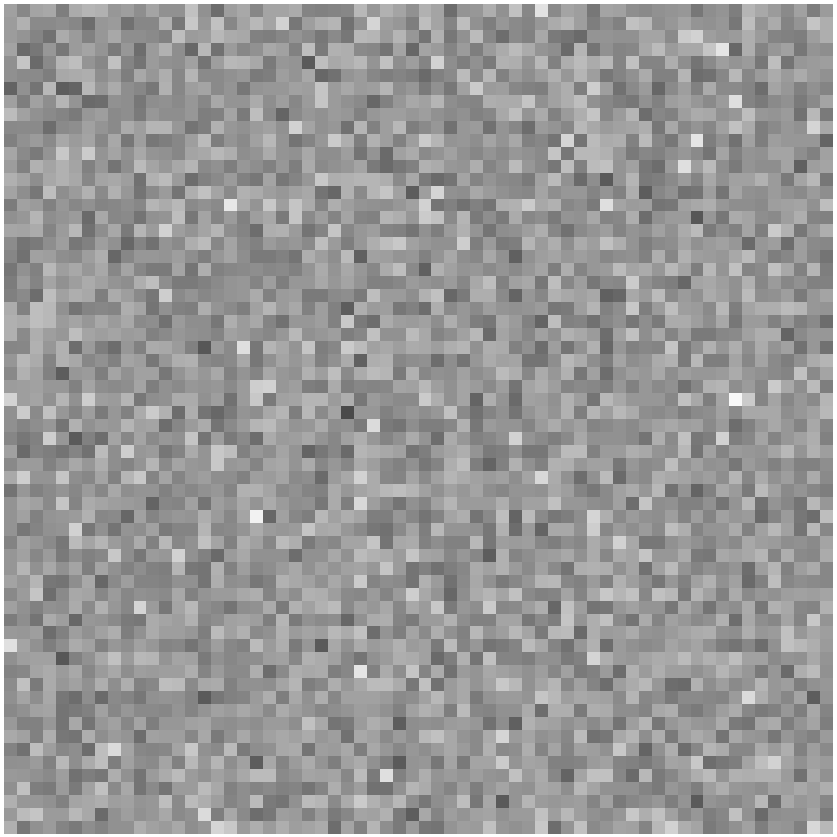


Direct comparison:
Single scan of a fluorescent slide

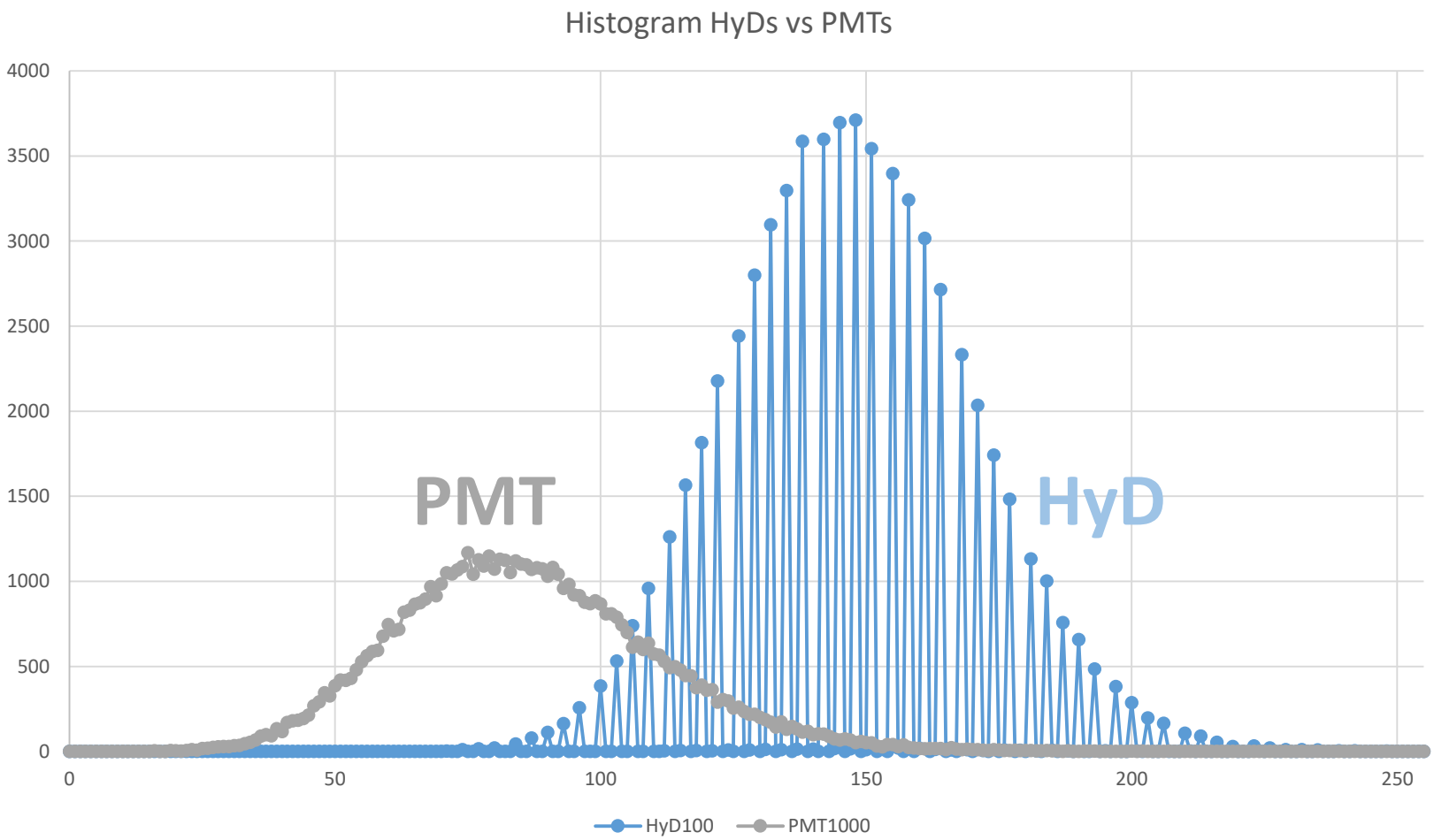
PMT



HybridDetector

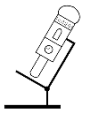


Histogram of a single 256x256 scan of a fluorescent slide



PMTs and hybrid detectors

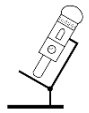
- The spectral response, quantum efficiency, and dark current of a detector depend on the material and size of the photocathode.
- The best classic (alkali) photocathodes for visible light used to have less than 30% quantum efficiency: 70% of the photons impacting on the photocathode do not produce a photoelectron and are therefore not detected.
- New GaAsP photocathodes have a QE of up to 45%. Used in some PMTs (Zeiss, Nikon) and always in hybrid detectors (Leica)



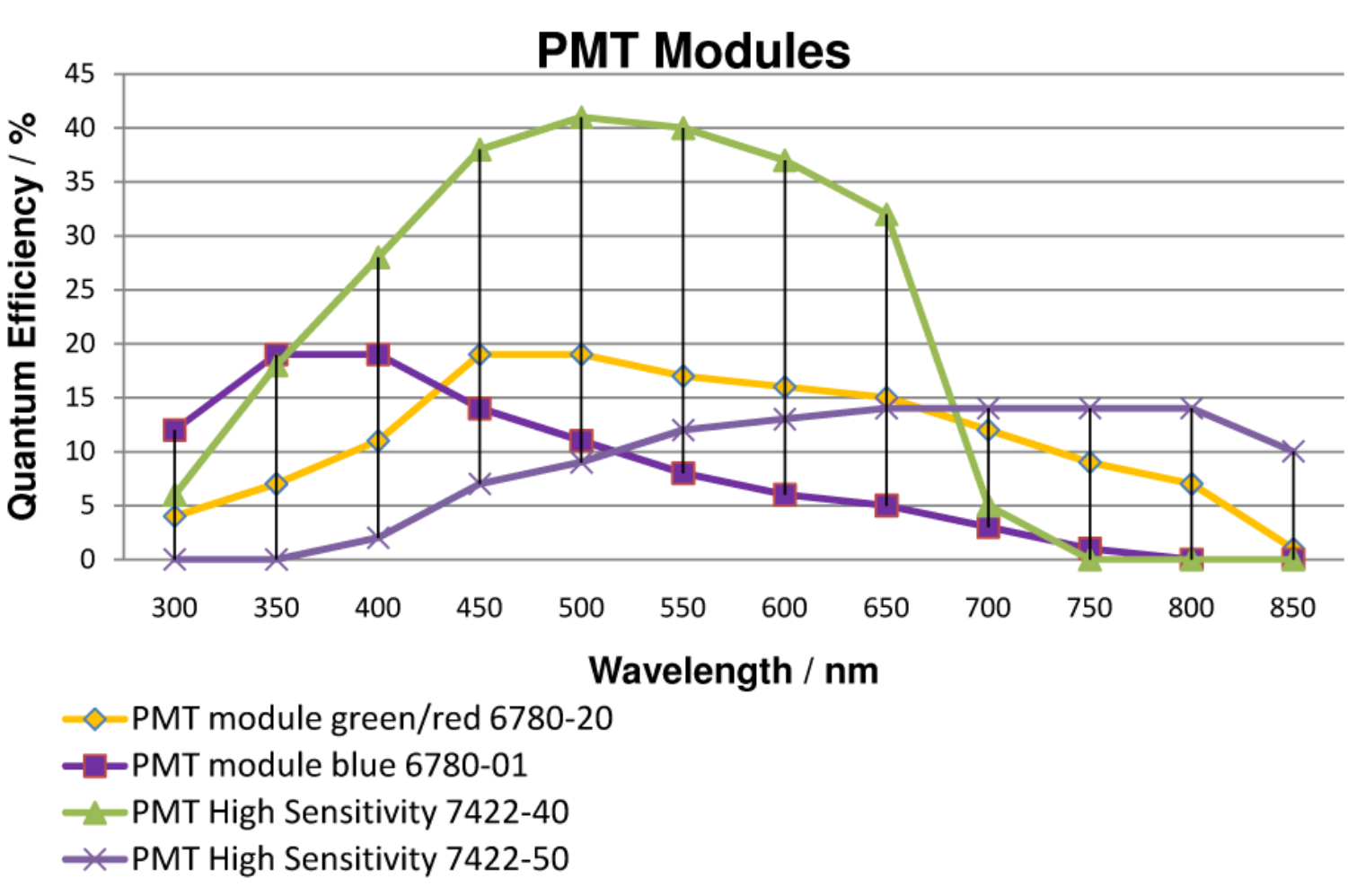
Hybrid vs PMT

- Hybrid detectors have less dark noise due to smaller photocathode
- Less statistical noise due to higher voltage and fewer amplification steps
- Voltage cannot be adapted to varying signal intensity

- PMTs can be easily adapted to very bright signals by lowering the voltage (Gain).
- Photocathode may be GaAsP or Alkali



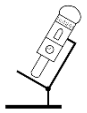
QE of standard vs. GAsP-PMTs



PMTs and hybrid detectors...

- ...are colorblind! All you get is a gray level image.

- The „color“ of the photons arriving at the PMT is determined solely by the filters/optics in front of the PMT.



- The „original data“ is a grid of numbers which encode the intensity.

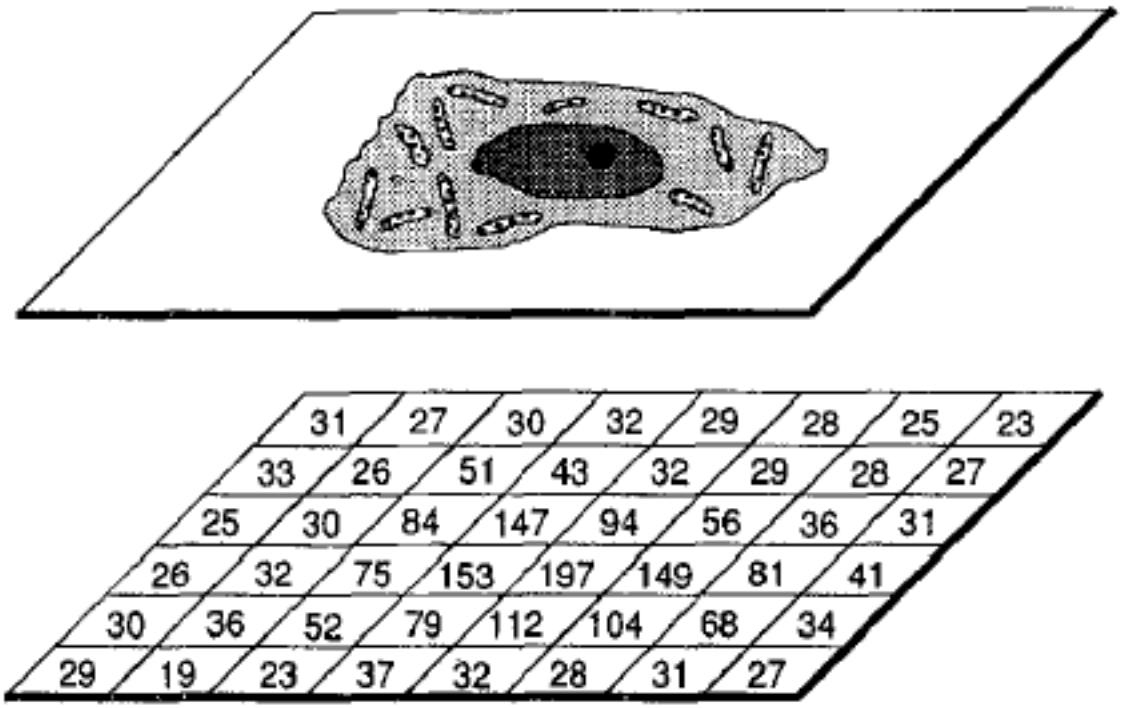
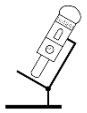


Image source: Shotton DM (1995): Robert Feulgen Prize Lecture 1995. Electronic light microscopy: present capabilities and future prospects. Histochem Cell Biol. 104(2):97-137. DOI: 10.1007/BF01451571

Special Effects in clsm

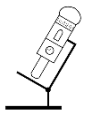
Accusto-Optical Filters

Spectral detection



Saturation of fluorochromes

- Fluorochromes already in an excited state cannot be excited again -> with high light intensities, their response is not linear any more
- Laser excitation (>1mW) can cause this situation, since all excitation is concentrated to one point (higher peak intensity than with widefield excitation!)
- Saturation is a property of the individual fluor molecule: It happens in bright areas as well as in dark ones.
- Solution: lower laser power and average



LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Walter Brendel Zentrum München

How to record a good image

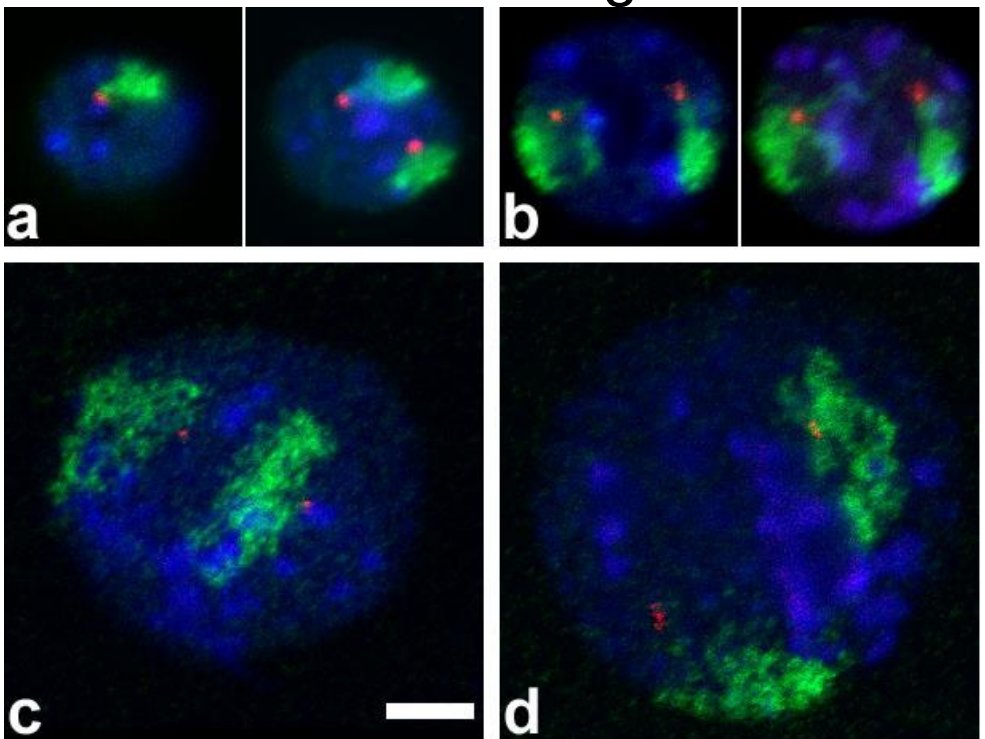
Before you even begin: Check your microscope

- FWHM of the PSF as it should be? (measure with the embedding medium that you plan to use!)

- Measure chromatic aberration, especially if you want to do colocalization studies (measure with the embedding medium that you plan to use!). Even for high NA objectives, expect to find up to 500 nm in z-direction.

Rule # 1: Start with a good preparation!

- "*Garbage in, garbage out*" (anonymous)
- Use a 170 µm coverslip. Pay attention to Ri mismatch.
- Fixed cells: Don't destroy the structure of your cells if you want to look at structural organization



Hepperger C, Otten S, von Hase J, Dietzel S. (2007) Preservation of large-scale chromatin structure in FISH experiments. Chromosoma 116(2):117-33. DOI: 10.1007/s00412-006-0084-2

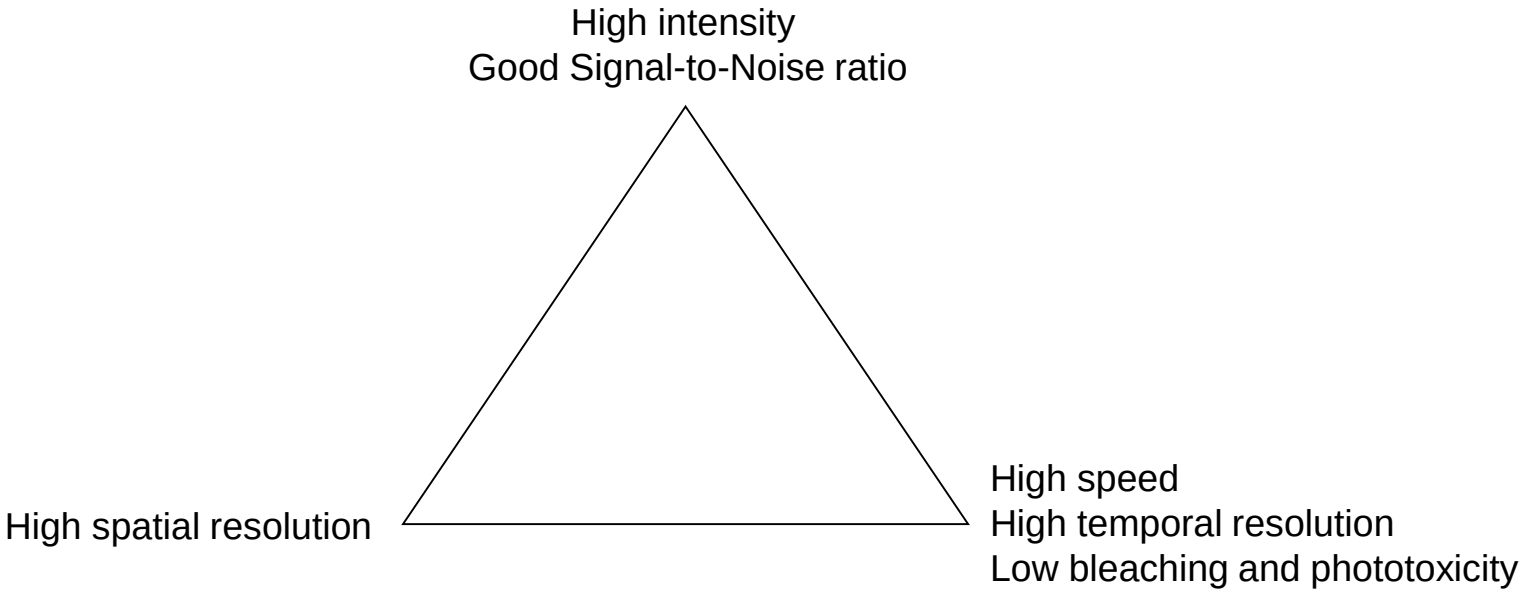
The Uncertainty Principle of fluorescence based microscopy:

The more detailed you look, the more you destroy (bleaching, phototoxicity).

- The highest physical resolution and contrast may require high laser powers and are thus often incompatible with specimen preservation and thus biological reliability

The eternal triangle of compromise

Modified after Shotton DM (1995): Robert Feulgen Prize Lecture 1995. Electronic light microscopy: present capabilities and future prospects. Histochem Cell Biol. 104(2):97-137. DOI: 10.1007/BF01451571



Rule # 2: Find the best possible compromise between image quality and introducing artefacts

- Scan speed: The number of *lines* per sec. Examples: 400 Hz, 1200 Hz
- Averaging: line (each line is scanned x times and the average is immediately computed, good for moving objects) or frame (full frame image is scanned and averaged)
- Accumulation (gray levels of subsequent scans are added up; useful for weak signals and in photon counting mode)
- Voxel size (for good resolution with NA 1.4: ~80 nm in xy, 240 nm in z)

And then: Check for bleaching!

- Record the image stack again with the same settings than before:

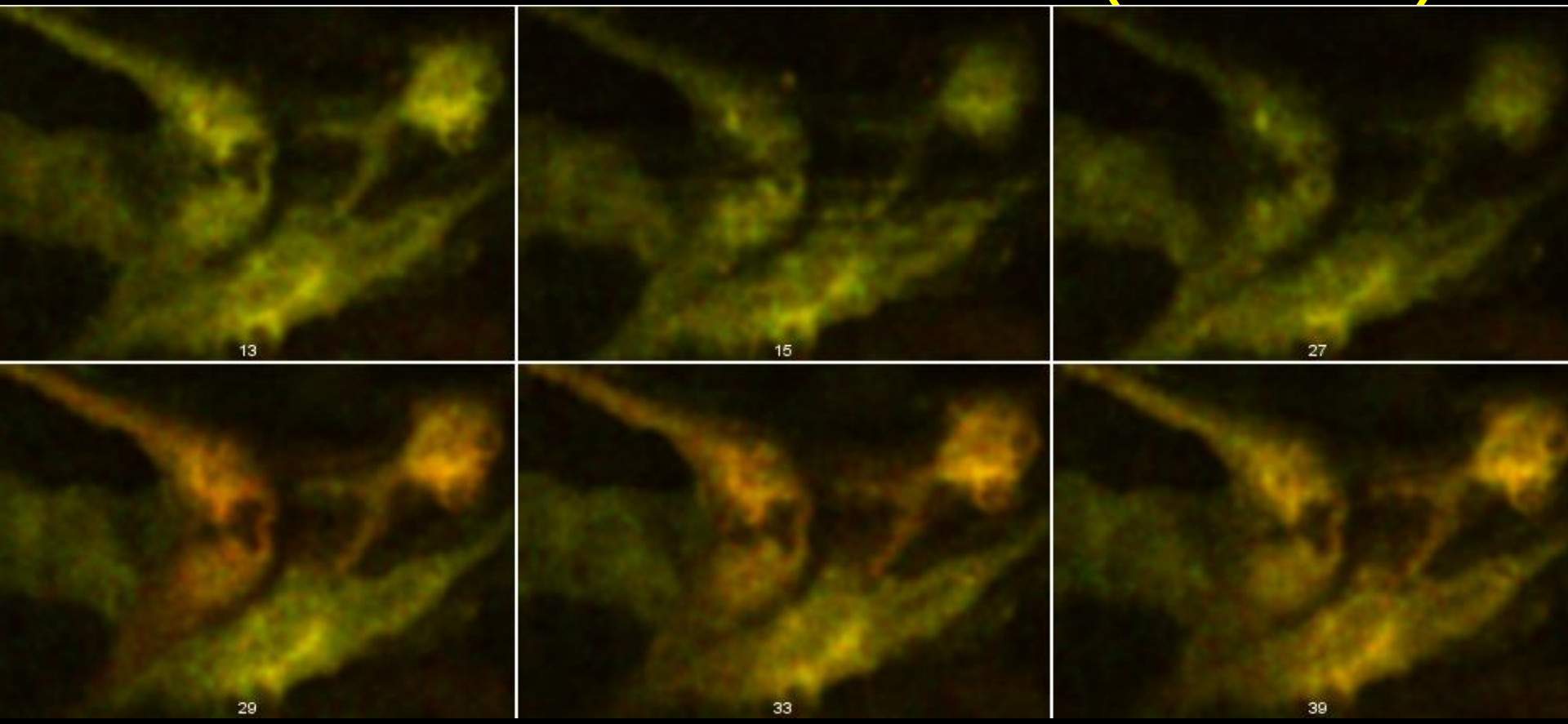
- Is the second stack (nearly) as bright as the first one?

Opening the pinhole for brighter images?

- You don‘t want just ‚bright‘ images, you want a good signal to background ratio.

- What is signal, what is background? Depends on your sample and question.

Example 1: Calcium ratio measurement in isolated blood vessels (t-series)

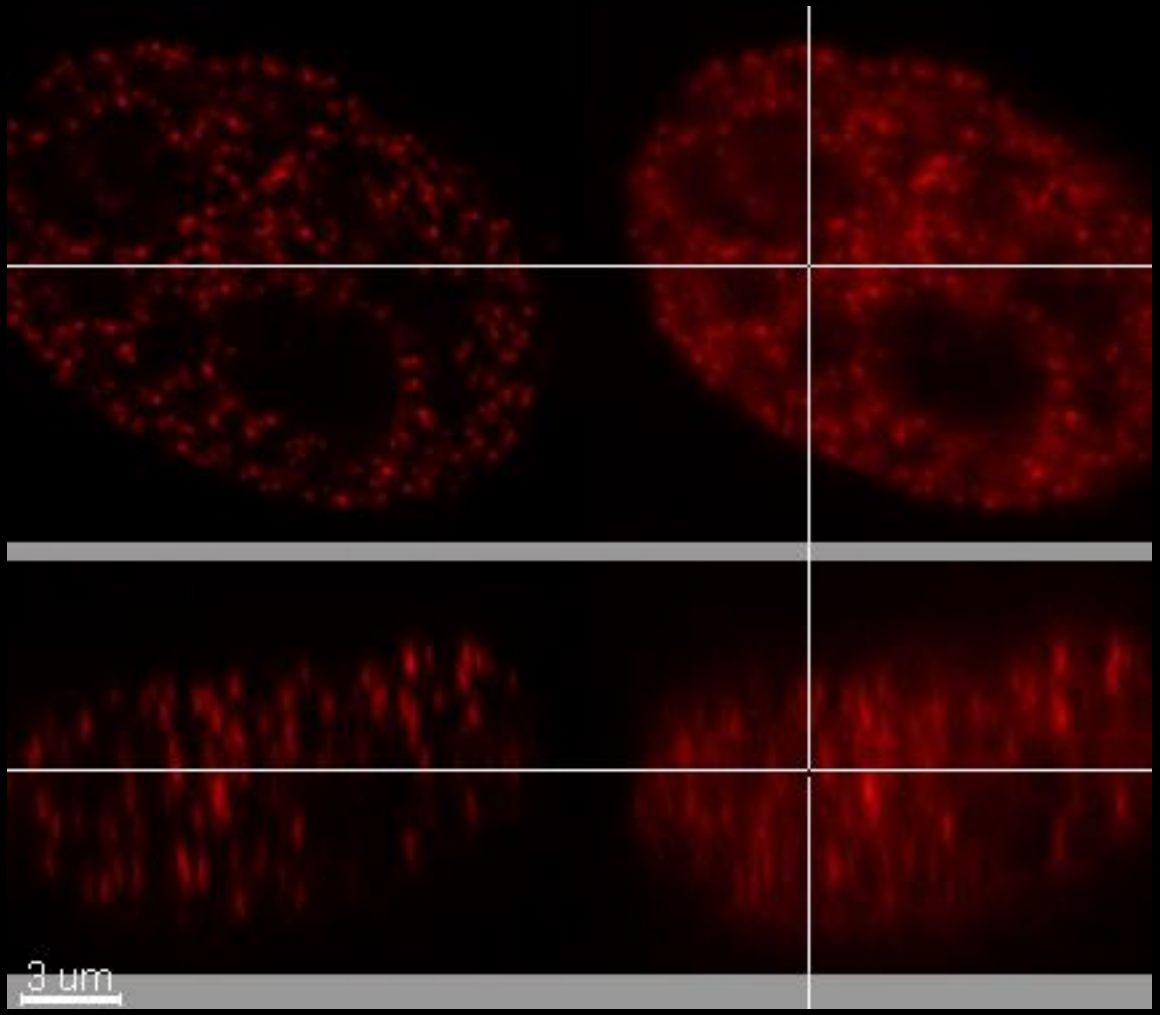


A CFP-YFP Ca-detector (TN-XXL) is distributed in the whole cytoplasm and the signal is weak. Here, opening the pinhole led to an increase in „signal“ because out of focus fluorescence contributes to the „signal“.

For experimental details see: Direnberger, S., M. Mues, V. Micale, C.T. Wotjak, S. Dietzel, M. Schubert, A. Scharr, S. Hassan, C. Wahl-Schott, M. Biel, G. Krishnamoorthy, and O. Griesbeck. 2012. Biocompatibility of a genetically encoded calcium indicator in a transgenic mouse model. Nat Commun. 3:1031.

Example 2:
Small dots (replication labeling) in cell nucleus

Pinhole 1 Airy disk

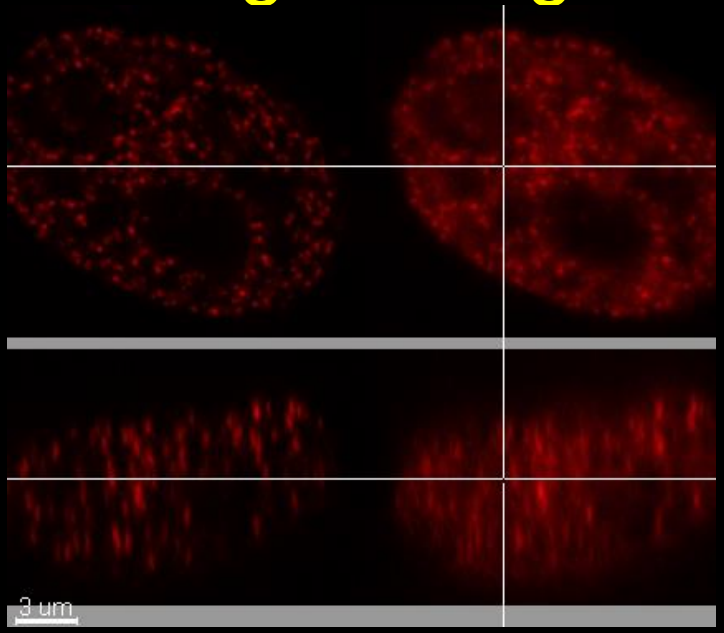


Pinhole open

No signal-to-background improvement by opening the pinhole, just more background! Because this „signal“ is limited to the focal plane anyway.

In most cases, opening the pinhole *decreases* the signal-to-background ratio:

Only the background gets brighter.



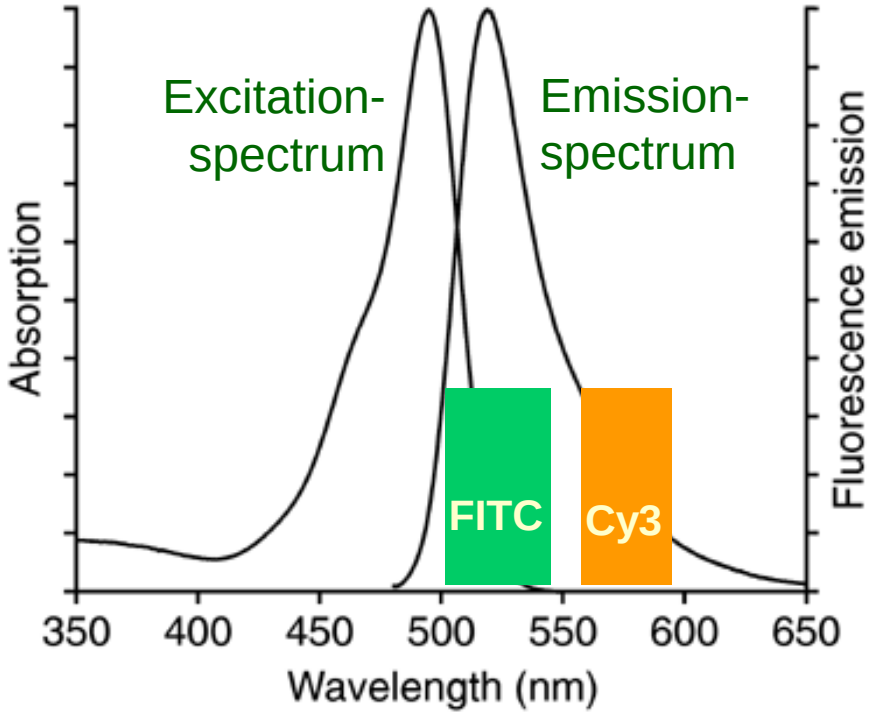
- In most cases, pinhole size of 1 Airy unit or a little less (0.8) is best.

Rule # 3: Make sure the dynamic range covers all structures of interest in your sample

- Do not overexpose!
- For PMTs, with 8-bit images, set offset and PMT-gain such that your images use the 256 available graylevels as good as possible.
- Use a look-up table (LUT) that will show you when you over- or underexpose:

Bleed through = cross talk

- Make sure you don‘t get bleed through!



FITC goat anti–mouse IgG antibody/pH 8.0
http://probes.invitrogen.com/servlets/spectra?fileid=143iggp8

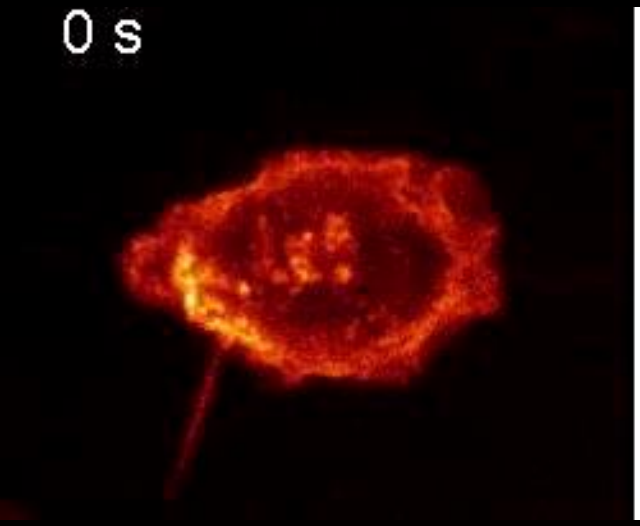
Transmission image

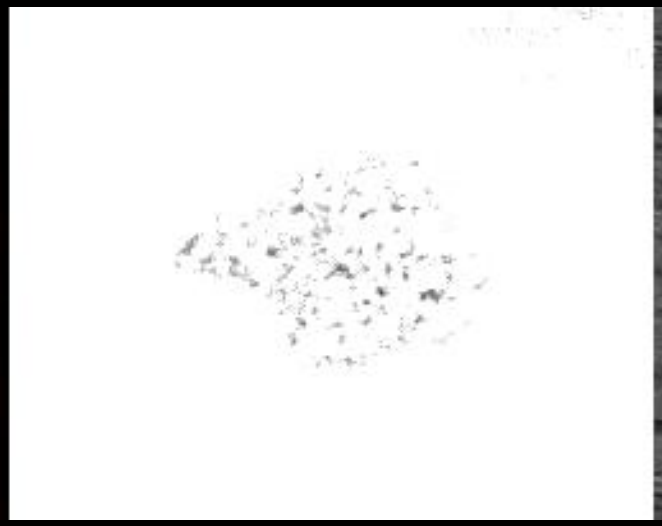
- If your confocal has a transmission laser detector, you can get a transmission image „for free“, i.e. without additional damage to the sample.
- Consider if such an image would help to understand your data.
- If yes, adjust your condenser to Köhler Illumination!!! This can be done by using white light halogen (=normal) transmission light.
- Set your laser power to achieve a good fluorescent image first!
- Then, be prepared to play around with the gain and offset a while before you find the optimal setting for the transmission setting. Try closing and opening the condenser aperture diaphragm for better contrast.

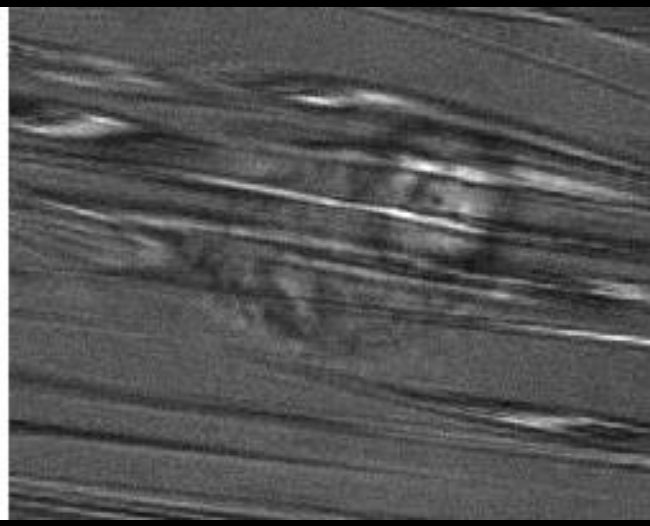
Reflection confocal image

- A reflection confocal image may help to identify some structures, such as pseudopodia of cells.
- Set up a normal PMT such that it collects the excitation light of the laser. In theory, this light should not arrive at the detector, due to the dichroic beam splitter. In practice, the small percentage of reflected light passing the dichroic is sufficient to create an image.
- Be careful! Too intense light may damage the PMT! Start with low laser power and/or low PMT-voltage and work your way up. Don’t use a HyD!!!
- When you have determined the limits of safe operation, set your laser power to achieve a good fluorescent image, before calibrating voltage of the reflection image.
- In contrast to the transmission image, the reflected image is a true confocal image, since the light passes the pinhole. Depending on sample type an Ri-differences in the sample, this may or may not lead to useful results.

Fluorescence Reflection Transmisson







confocal

not confocal

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Deconvolution

Point spread function

- The PSF is the 3D image of a point-like light source, the 3D diffraction pattern (=Airy pattern).

- Point-like in practical terms means: smaller than the resolution of the microscope

Convolution

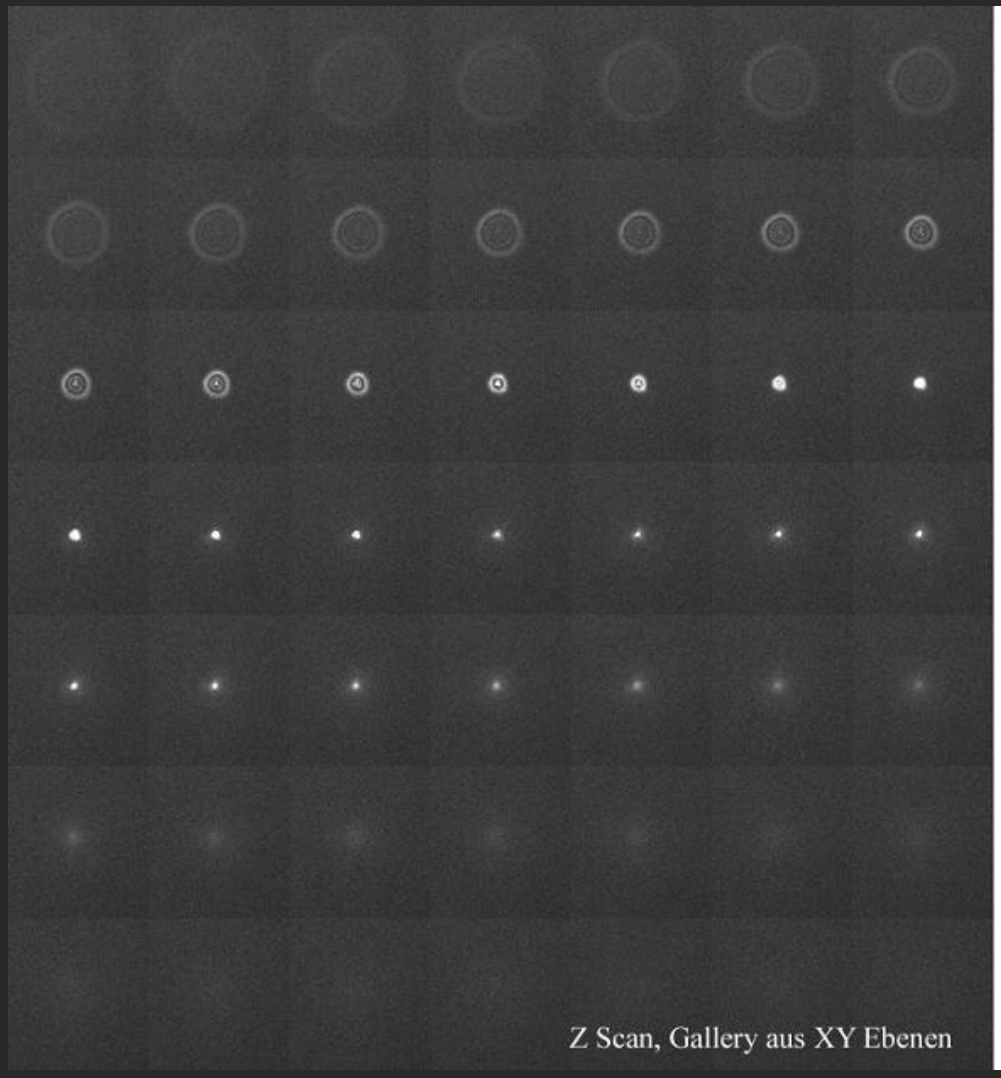
x PSF =

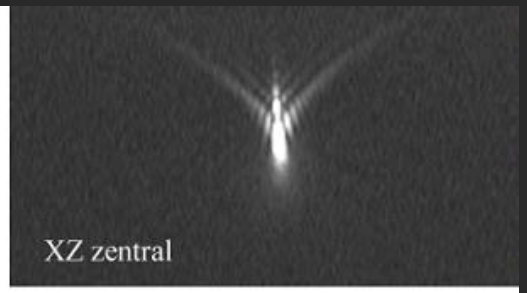
Deconvolution

x 1/PSF =

Point spread function and deconvolution

- PSF recorded with a 175nm bead





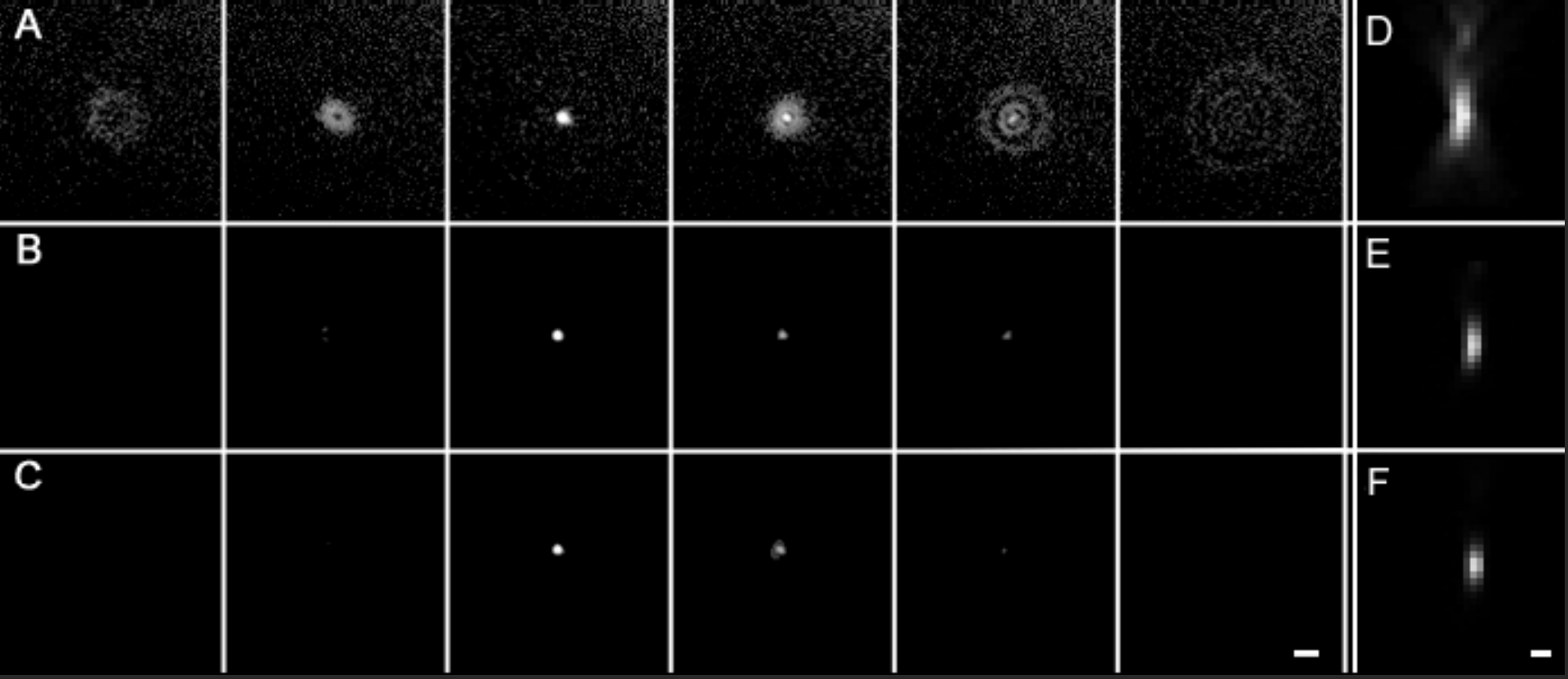
PSF

Weitfeldmikroskop
175nm bead
Cy3

Theoretical and actual PSF

Theoretische und tatsächliche PSF

http://www.olympusmicro.com/primer/digitalimaging/deconvolution/deconintro.html



175 nm Bead (blue channel) in wide field microscope.
Sections in 1 µm z-distance are shown. Scale Bar: 1 µm.

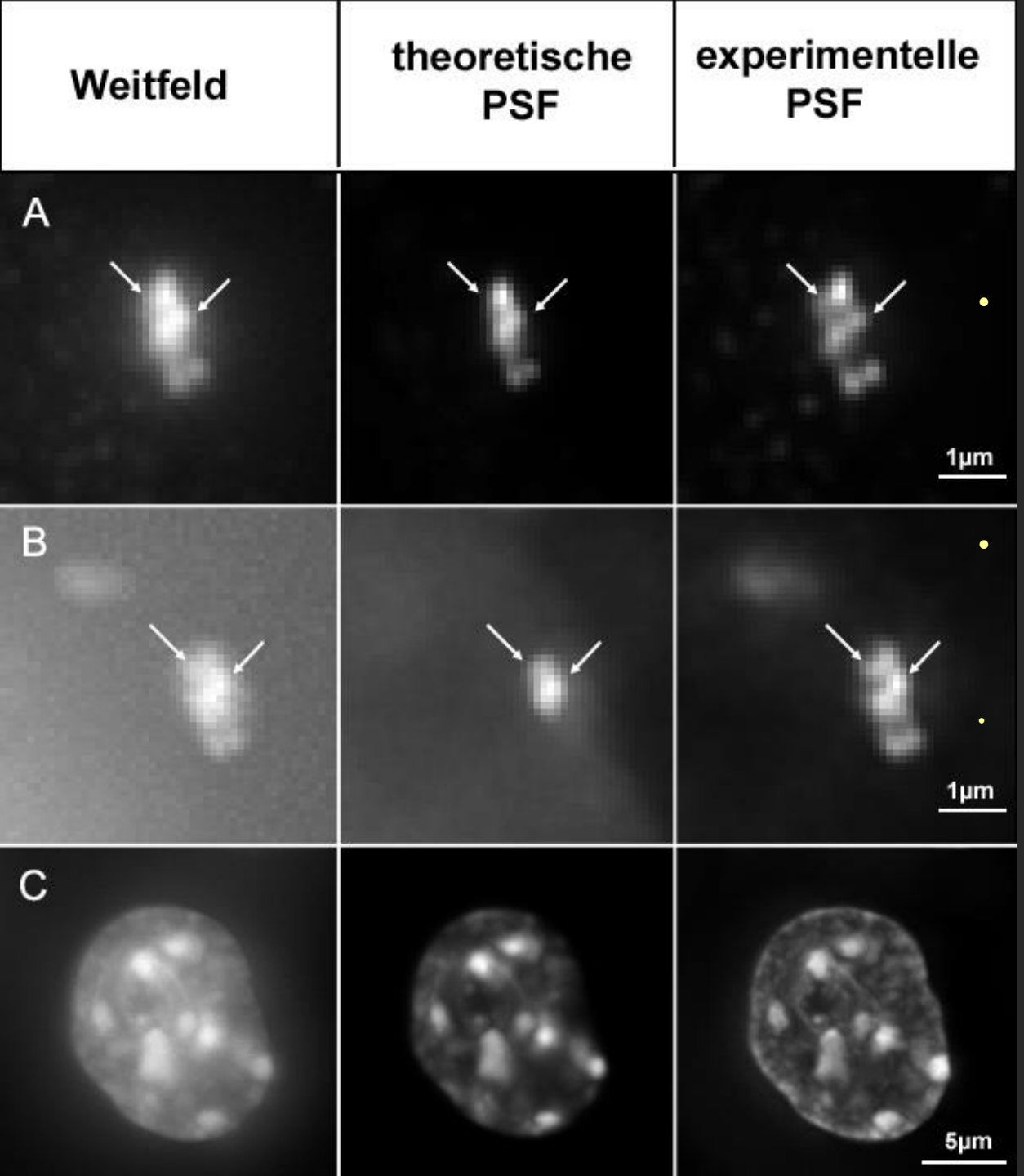
A) Originals

B) After deconvolution with theoretical PSF

C) After deconvolution with experimental PSF which was extracted from this bead

D-F: xz-images

Simone Otten, Diplomarbeit, 2004



Deconvolution results, projections of image stacks, MEL cells with lac op chromatin GFP label.

A) Anti-GFP antibody, Cy5 label.

B) GFP-Signal

C) DAPI

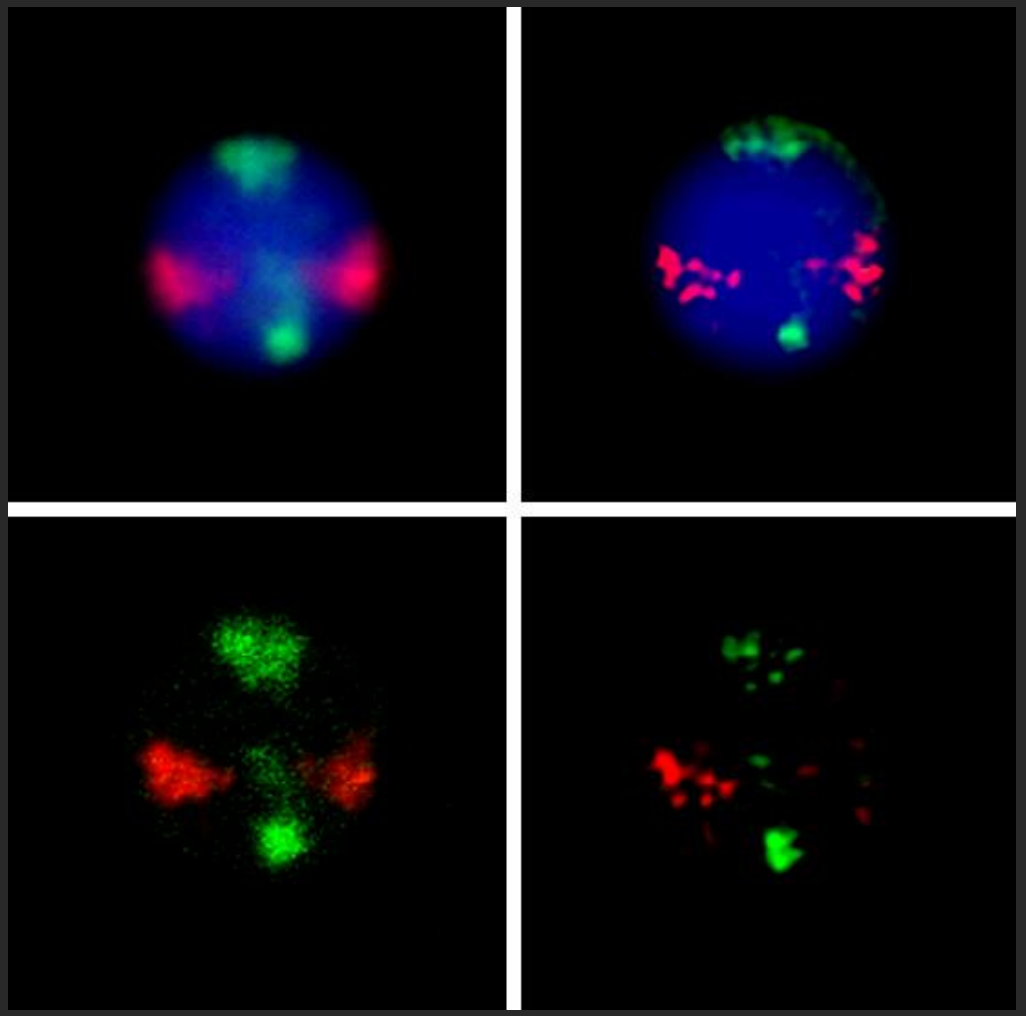
Simone Otten, Diplomarbeit, 2004

Deconvolution

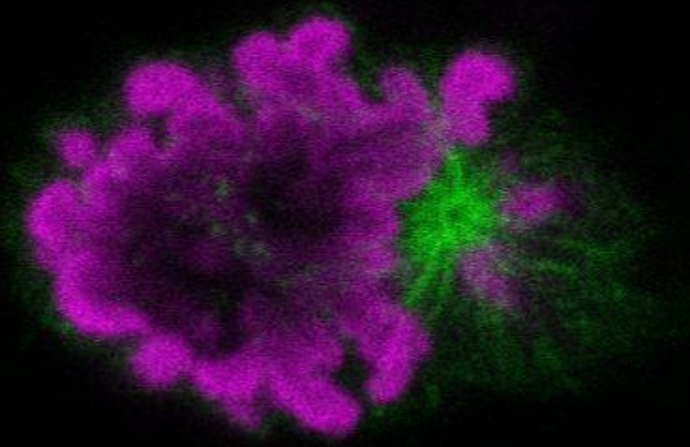
- Can be performed with wide field as well as with confocal images.

Deconvolution artefacts

- Caution,
  don‘t overdo it!

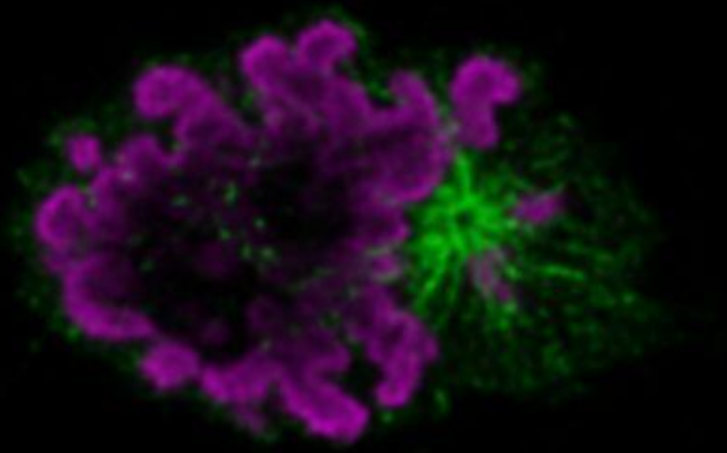


Example: HeLa cell in Mitosis



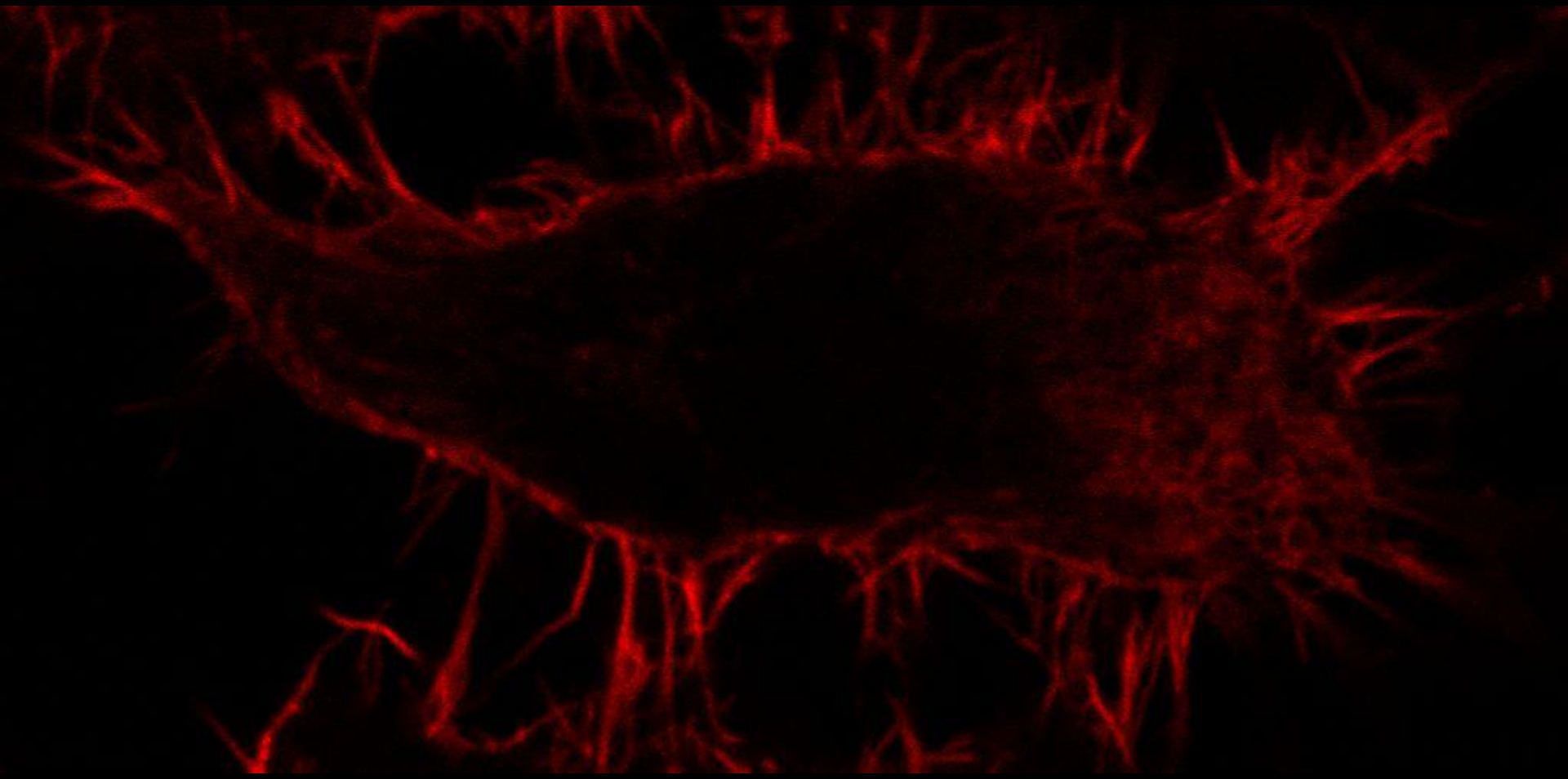
Tubulin, Actin, H3-Serin-10-Phosphorylierung

Example: HeLa cell in Mitosis



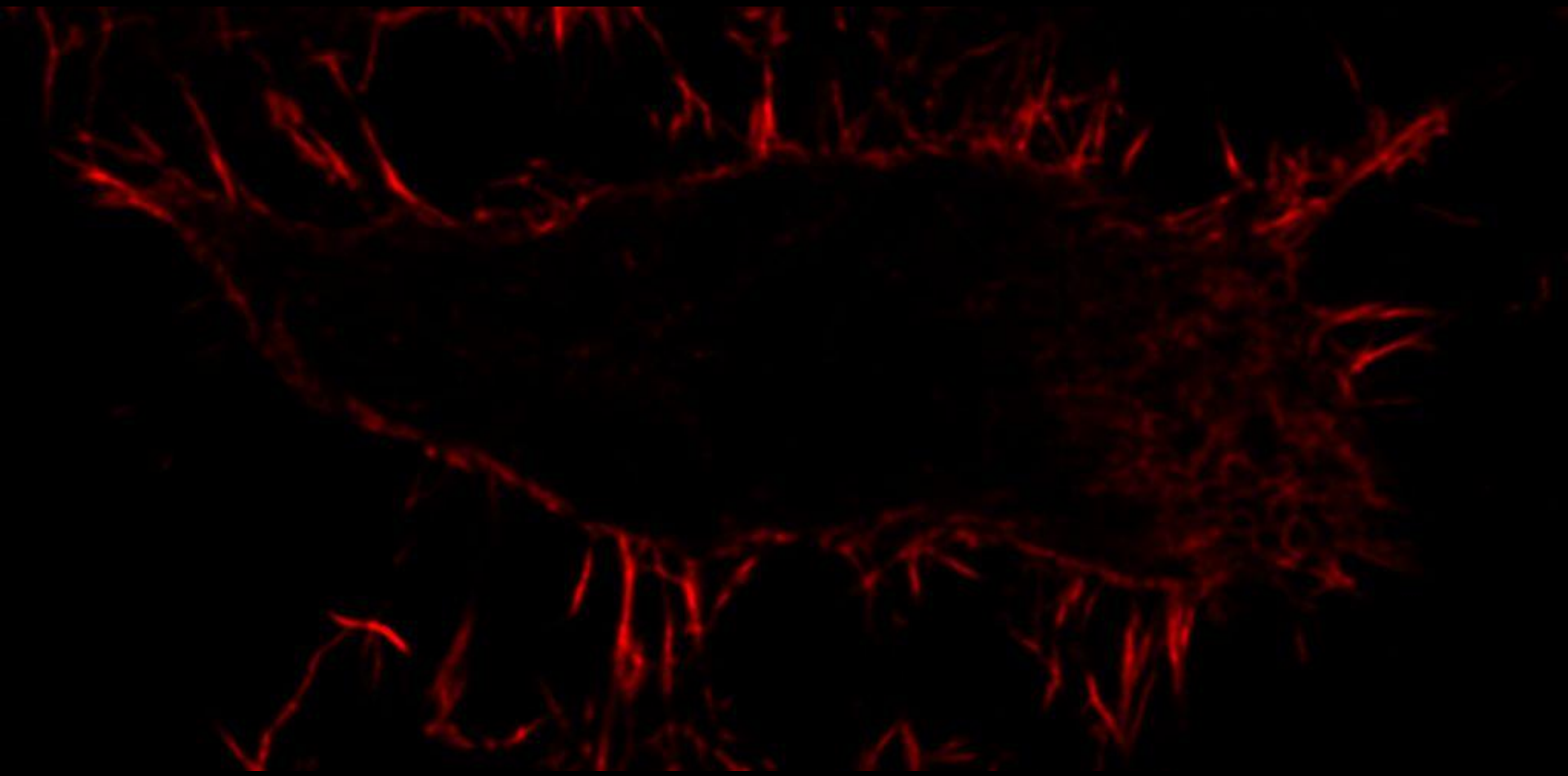
Tubulin, Actin, H3-Serin-10-Phosphorylierung

Example: HeLa cell in Mitosis



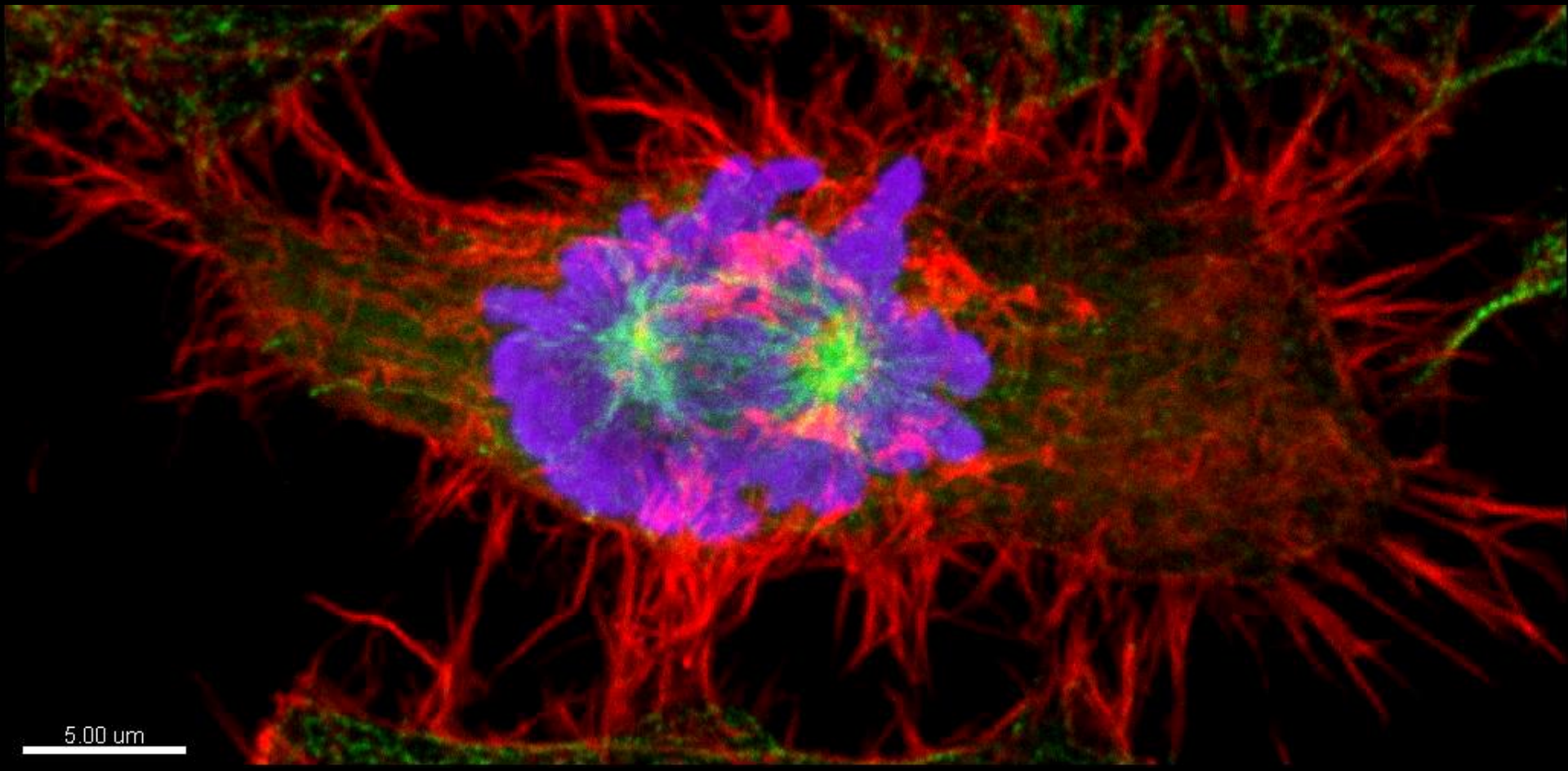
Tubulin, Actin, H3-Serin-10-Phosphorylierung

Example: HeLa cell in Mitosis



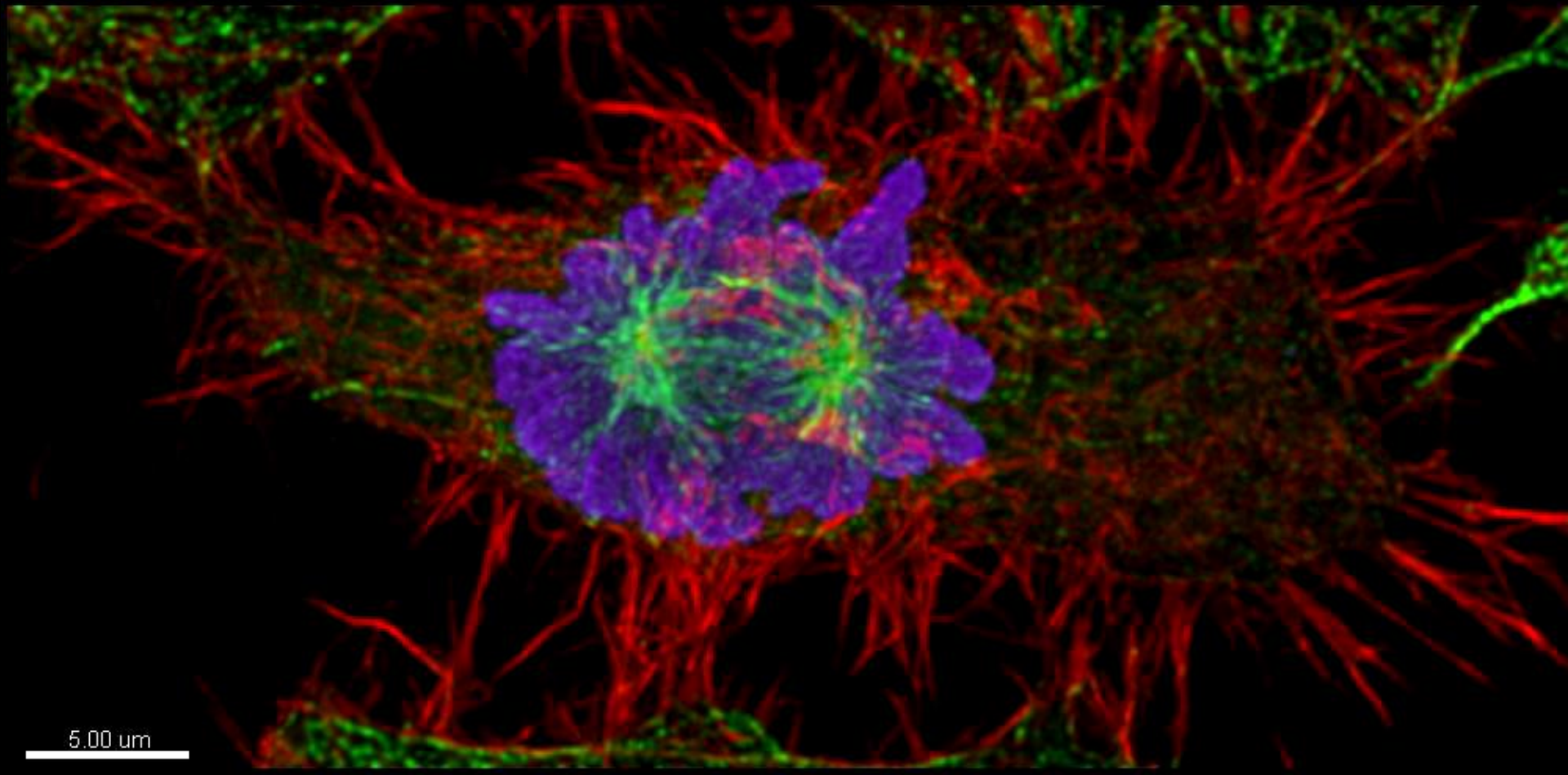
Tubulin, Actin, H3-Serin-10-Phosphorylierung

Example: HeLa cell in Mitosis



Tubulin, Actin, H3-Serin-10-Phosphorylierung

Example: HeLa cell in Mitosis

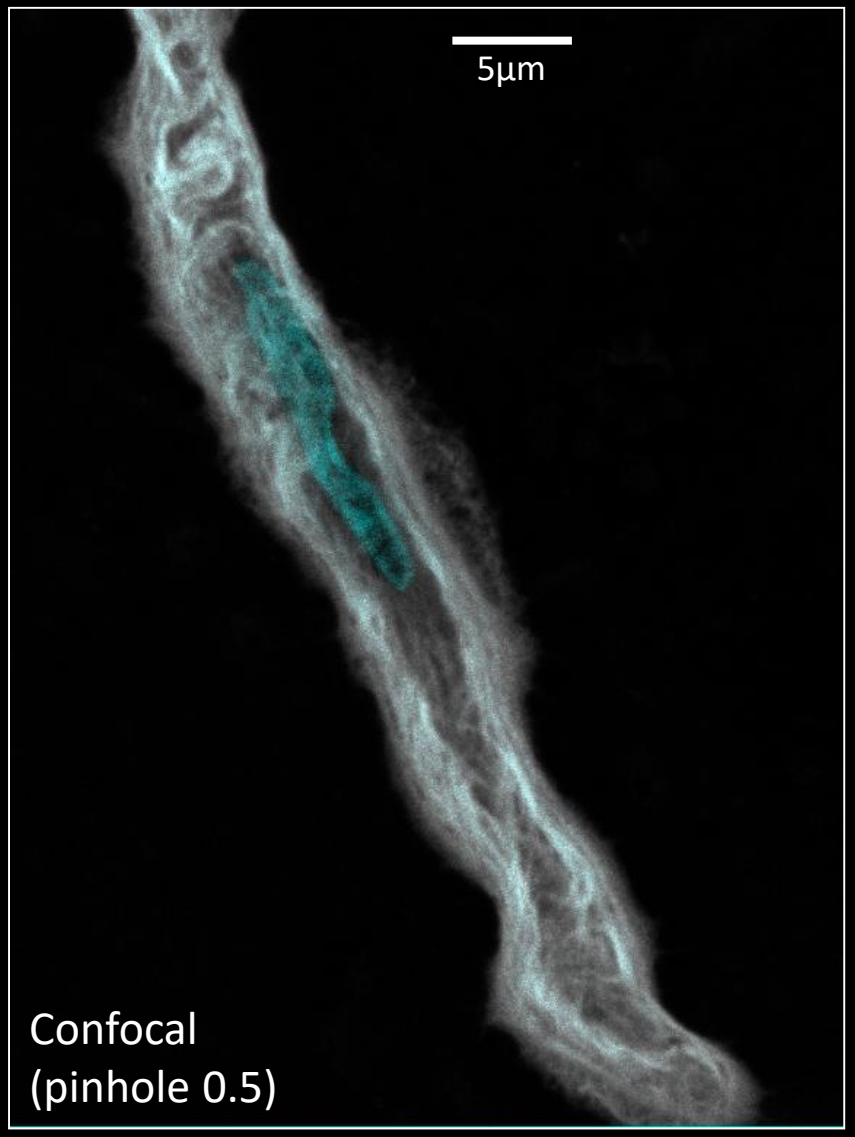


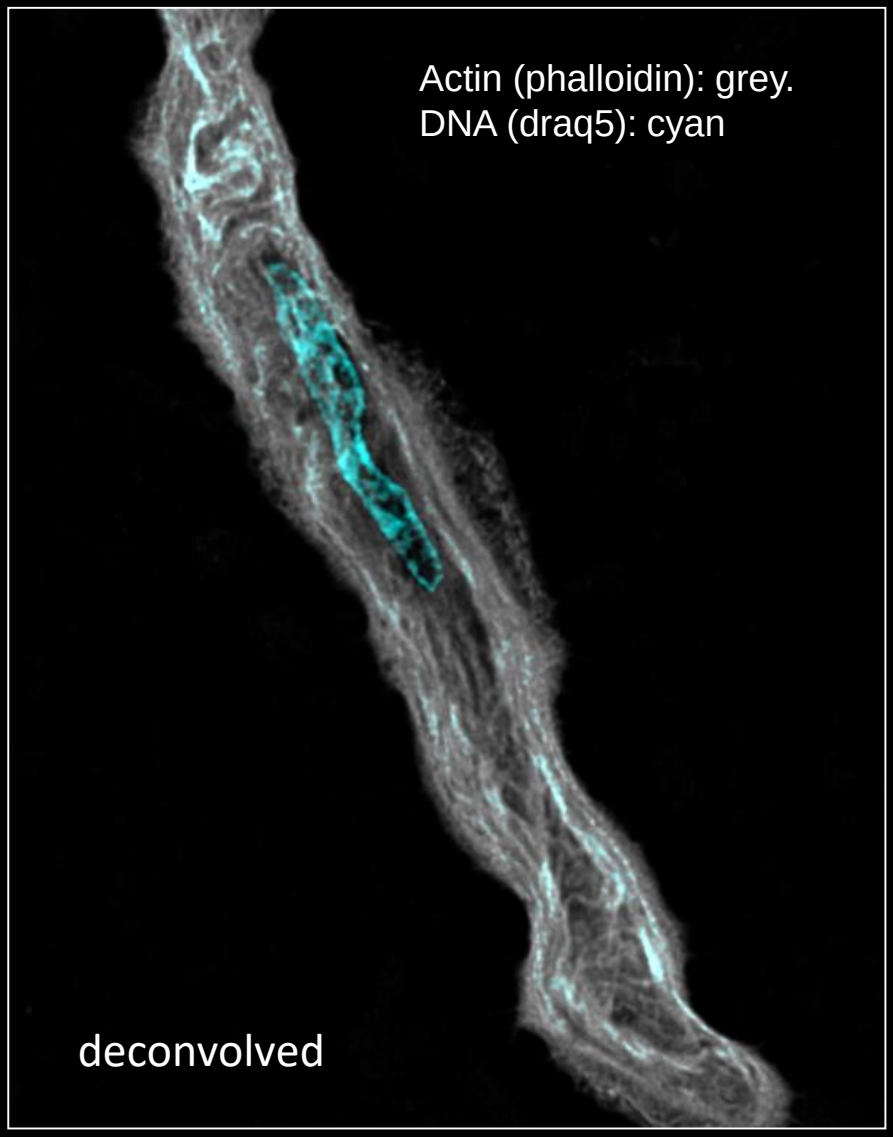
Tubulin, Actin, H3-Serin-10-Phosphorylierung

“Hyvolution”

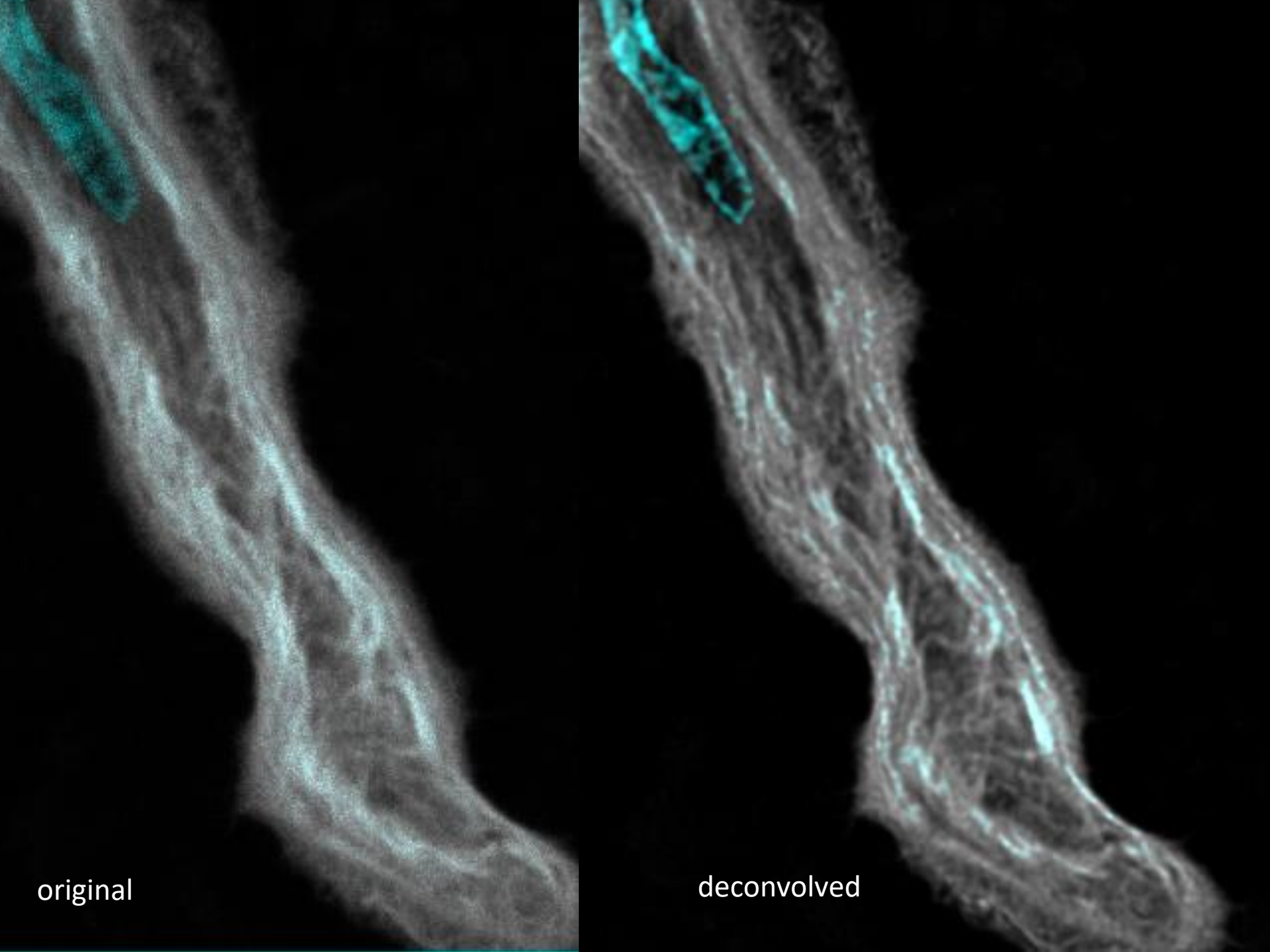
- Closing the pinhole to 0.5 AU
- Then deconvolved
- According to the manufacturer, 140 nm resolution can be reached.

Hyvolution: Isolated Murine Vascular Smooth Muscle Cell





Holger Schneider, Michael Schubert


original
deconvolved

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Session 4

- Multi-photon: fluorescence, SHG and THG
- Superresolution microscopy: STED and other approaches

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Multi-photon Microscopy: Principles and Applications

Flavors of Multi-Photon-Microscopy

- Two-photon excitation fluorescence (TPEF)
- Second harmonic generation (SHG)
- Third harmonic generation (THG)
- Resonance enhanced THG
- Coherent Anti-Stokes Raman Scattering (CARS)

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Walter Brendel Zentrum München

Two-photon excitation fluorescence

Visible light

wavelength

400 nm

800 nm

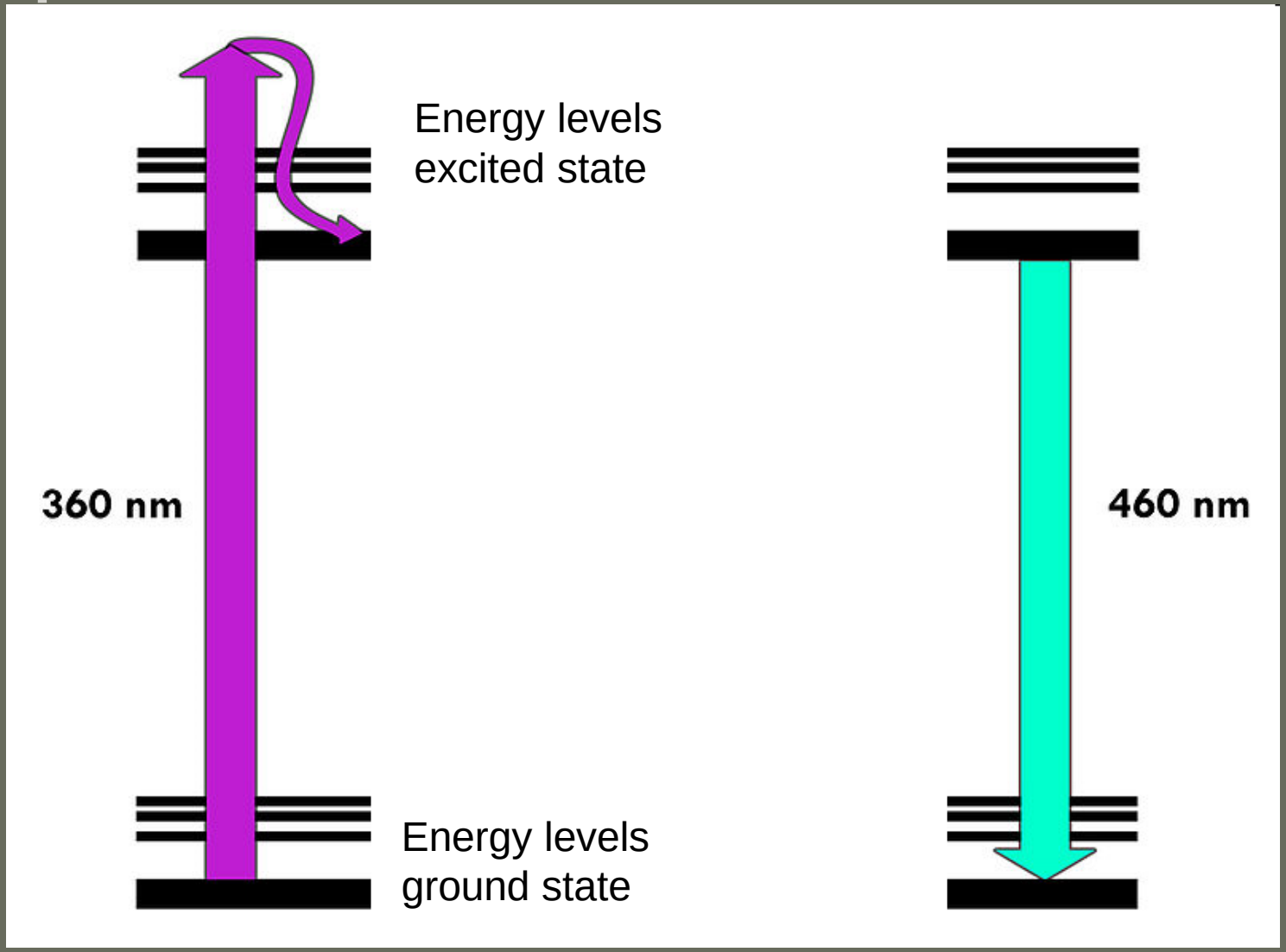
3.27 eV

1.65 eV

Energy

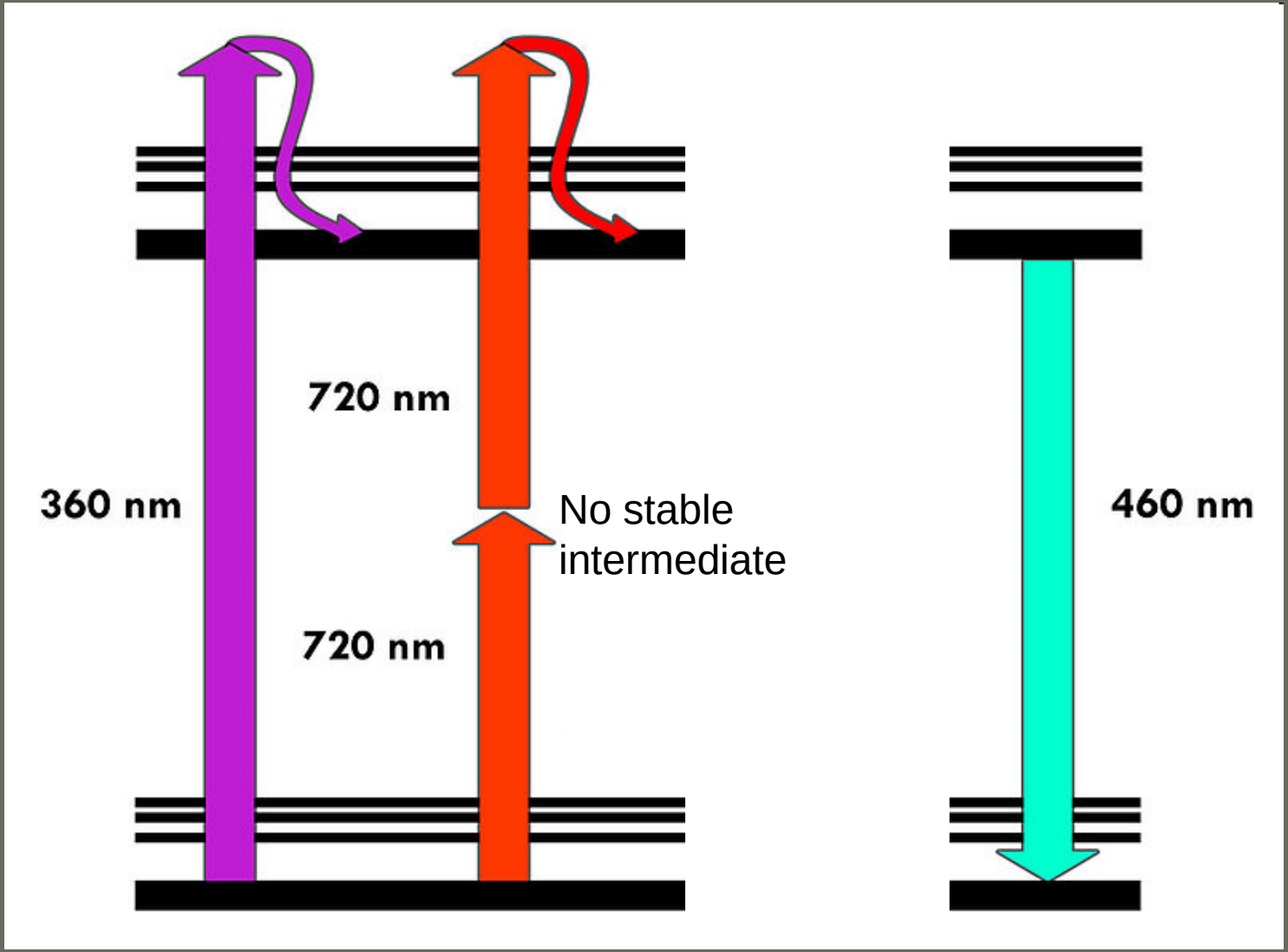
http://commons.wikimedia.org/wiki/File:Spectrum-sRGB-low.svg, Creator: Phrood

1 photon excitation fluorescence



Bildquelle: Multi-photon excitation microscopy. BioMedical Engineering OnLine, 2006, 5:36. DOI:10.1186/1475-925X-5-36.

1+2 photon excitation fluorescence



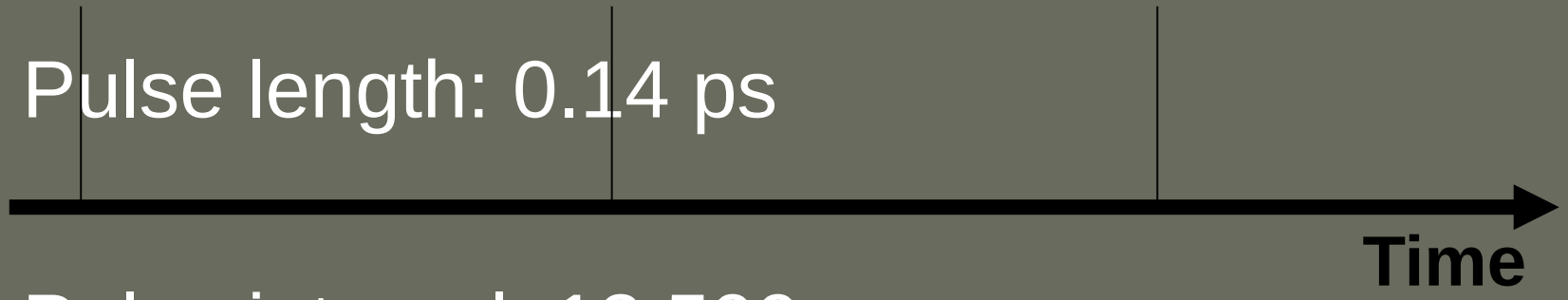
Bildquelle: Multi-photon excitation microscopy. BioMedical Engineering OnLine, 2006, 5:36. DOI:10.1186/1475-925X-5-36.

Two-photon excitation...

- ... only works if two photons arrive at the absorbing electron within 1 attosecond, $1*10^{-18}$s (nano, pico, femto, atto).
- This is statistically very unlikely to happen under normal conditions.
- To generate a high enough photon density, a focused, pulsed laser is required.

Pulsed laser (Chameleon Ultra II)

- Pulse length: 0.14 ps



- Pulse interval: 12 500 ps (= 12.5 ns = 80 MHz)

- So, most of the time, the laser is off, only 0.00112 % on.
- About same relation as a 1 second pulse in one day.

Image formation
(similar to confocal microscope)

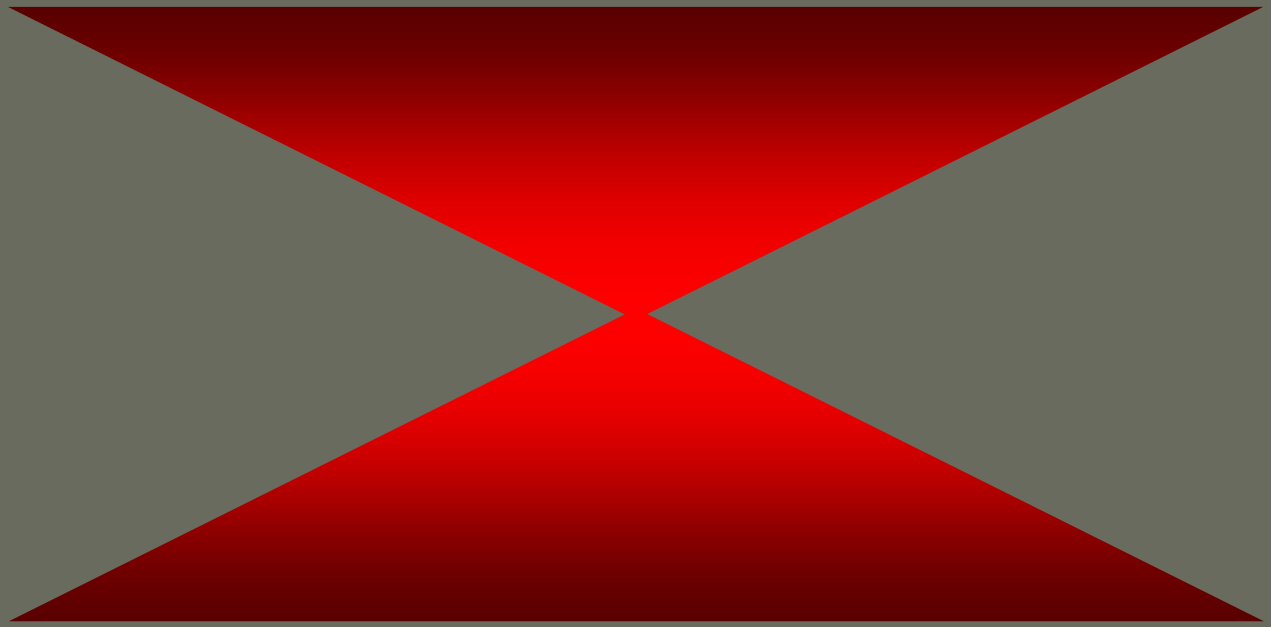
- At any given time, fluorescence comes from only one point.
- This point is scanned over the specimen.
- Detectors (PMTs or HPD) record emitted photons from each point, one by one, the computer constructs the image.

If it‘s so complicated and expensive, why bother?

- Advantage 1: Excitation and bleaching only at the focal point. Not above and not below.

It‘s complicated and expensive, so why bother?

- Advantage 1: Excitation and bleaching only at the focal point. Not above and not below.



Why bother?

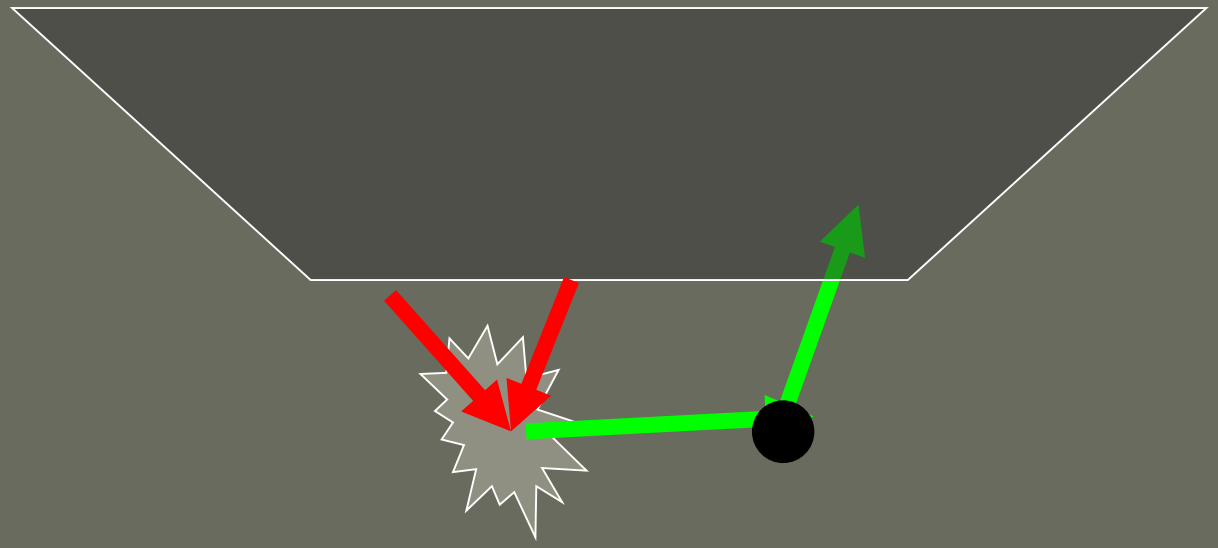
- Advantage 2: Deeper penetration, because
- a) Less absorbtion of tissue in NIR-range

Why bother?

- Advantage 2: Deeper penetration, because
- a) Less absorbtion of tissue in NIR-range
- b) scattering decreases with longer wavelengths:
  $1/(nm)^4$ => factor 16 with doubling of λ

Why bother?

- Advantage 3:
- No loss of emitted photons due to pinhole, non-balistic photons contribute to image

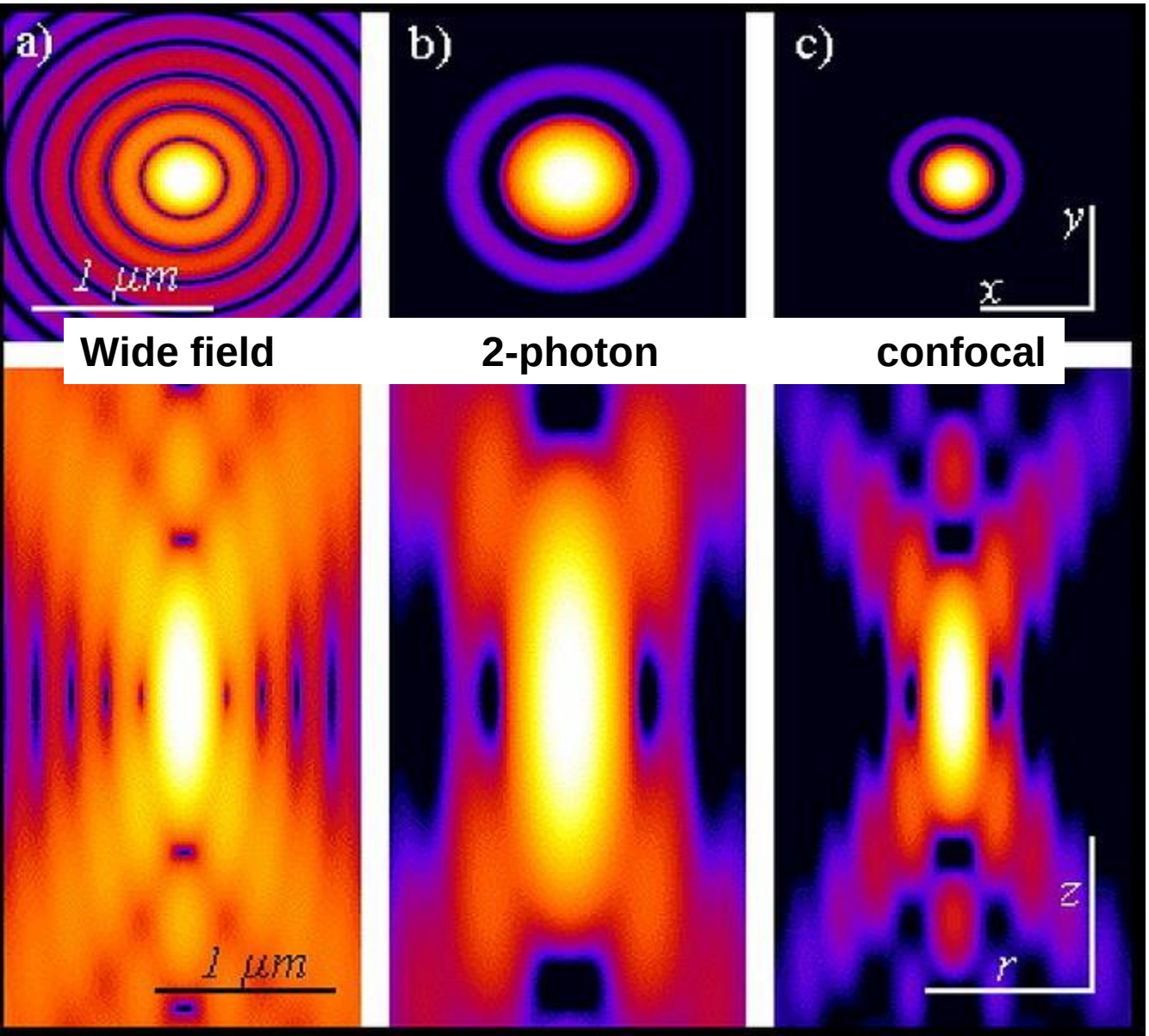


Disadvantages

- Pulsed laser needed, thus
- Expensive
- Light path cannot use no glass fiber

- Separation of neighboring fluorochromes more difficult

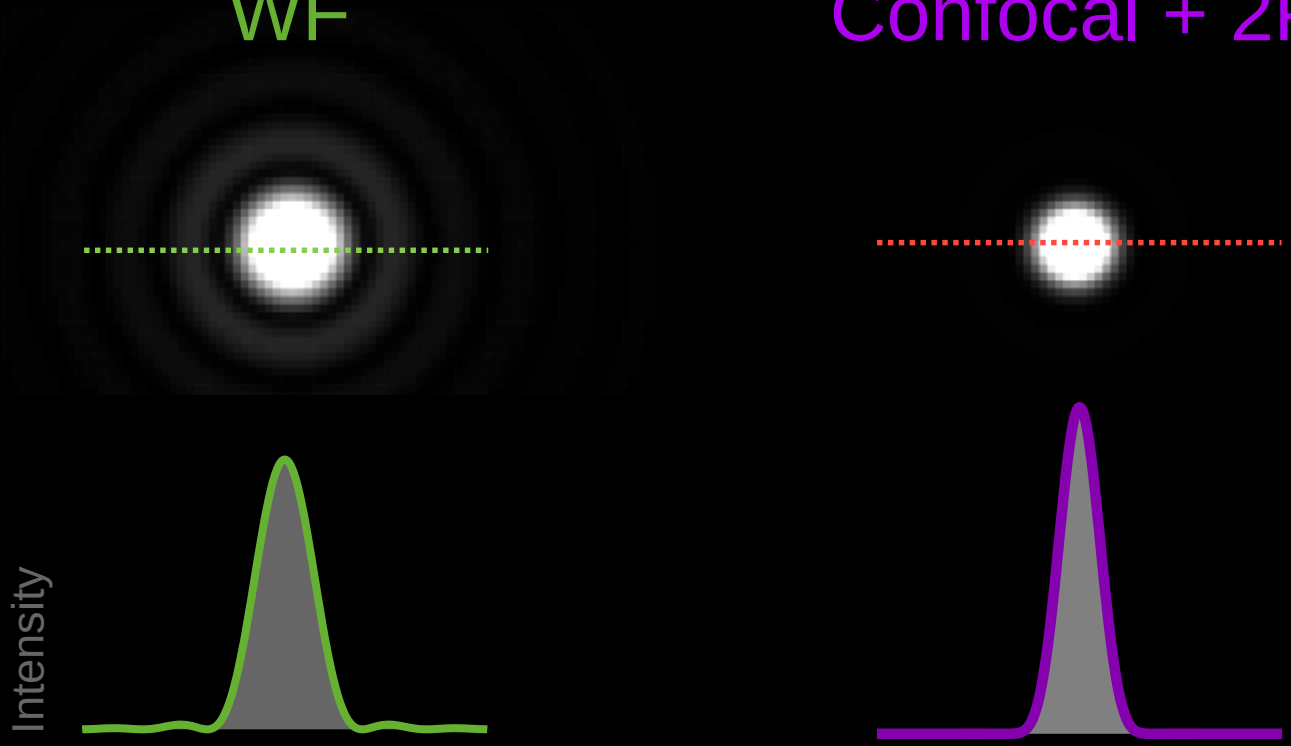
Due to the longer wavelength, multi-photon excitation has a larger PSF



*Pointlike emitter optical response. From left to right: calculated x-y (above) and r-z (below) intensity distributions, in logarithmic scale, for a point like source imaged by means of **wide-field**, **2PE** and **confocal microscopy**. Both 2PE and confocal shapes exhibit a better signal to noise ratio than widefield case. 2PE distribution is larger due the fact that a wavelength twice than in the wide-field and confocal case is responsible for the intensity distribution. Such intensity distributions are also known as point spread functions of the related microscopes. Optical conditions: excitation wavelengths are 488 nm and 900 nm for 1PE and 2PE, respectively; emission wavelength is 520 nm; numerical aperture is 1.3 for an oil immersion objective with oil refractive index value set at 1.515.*

WF

Confocal + 2P



Pinhole size	>1 AU	<0.25
$FWHM_{lateral}$	$\frac{0.51\,\lambda}{NA}$	$\frac{0.51\,\lambda}{\sqrt{2}\,NA} = \frac{0.37\,\lambda}{NA}$
$FWHM_{axial}$	$\frac{0.88\,\lambda}{n-\sqrt{n^2-NA^2}}$	$\frac{0.88\,\lambda}{\sqrt{2}(n-\sqrt{n^2-NA^2})} = \frac{0.64\,\lambda}{n-\sqrt{n^2-NA^2}}$

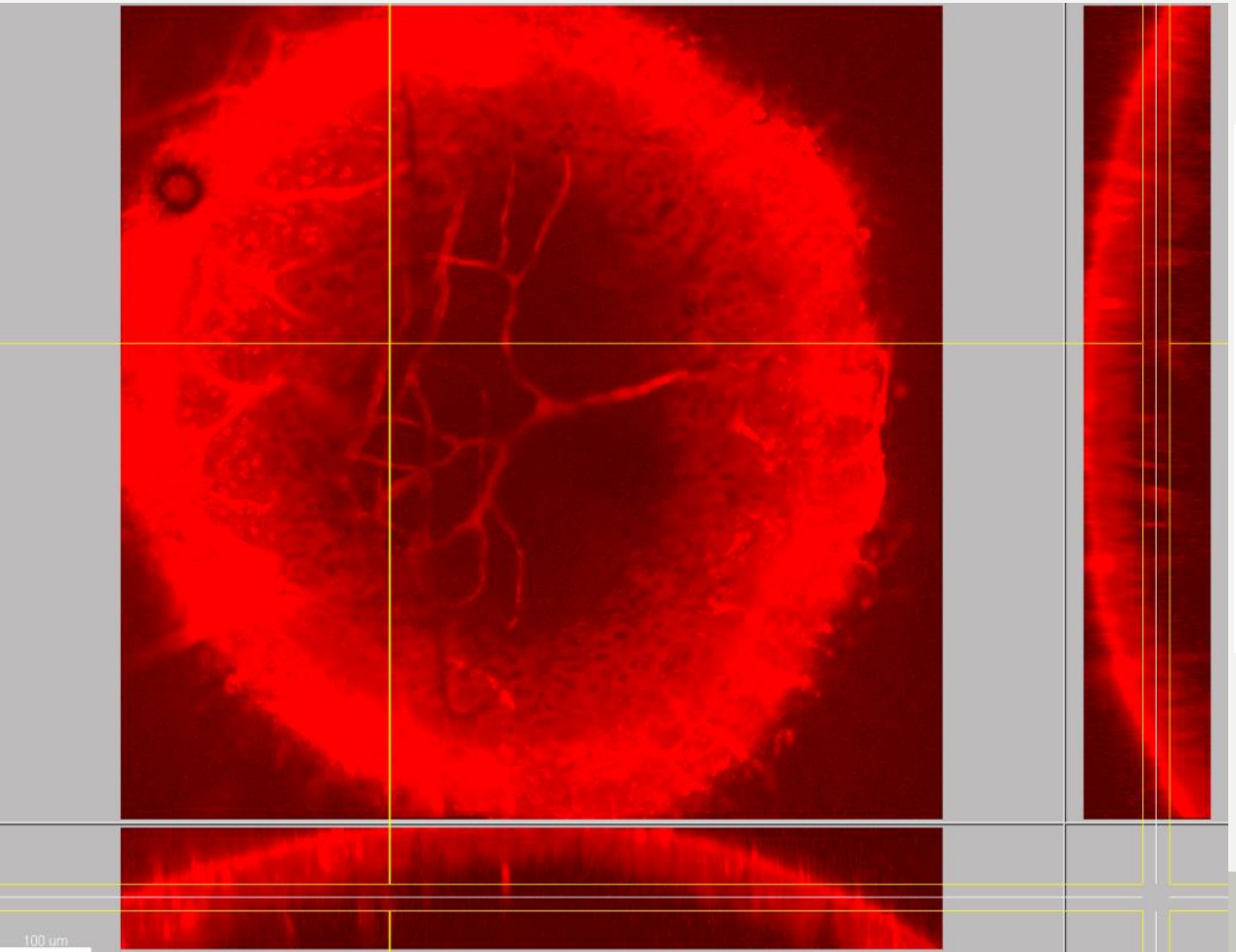
Example for water immersion, NA=1:

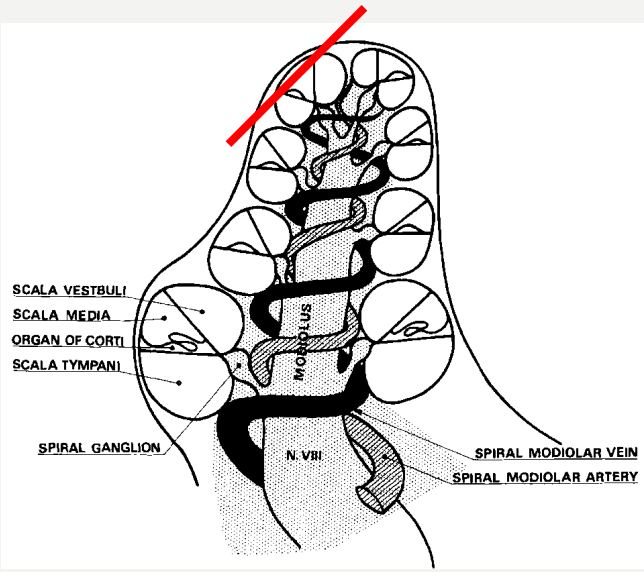
For resolution in multi-photon, only the excitation wavelength is important, not the emission

	widefield (and confocal with AU>1)	two-photon
$FWHM_{lateral}$	$\frac{0.51 * 500\ nm}{1.0}$ =255nm	$\frac{0.51 * 900\ nm}{\sqrt{2} * 1}$ = 324 nm
$FWHM_{axial}$	$\frac{0.88 * 500\ nm}{1.33 - \sqrt{1.33^2 - 1^2}}$ =971nm	$\frac{0.88 * 900\ nm}{\sqrt{2}(1.33 - \sqrt{1.33^2 - 1^2})} = 1236\text{nm}$

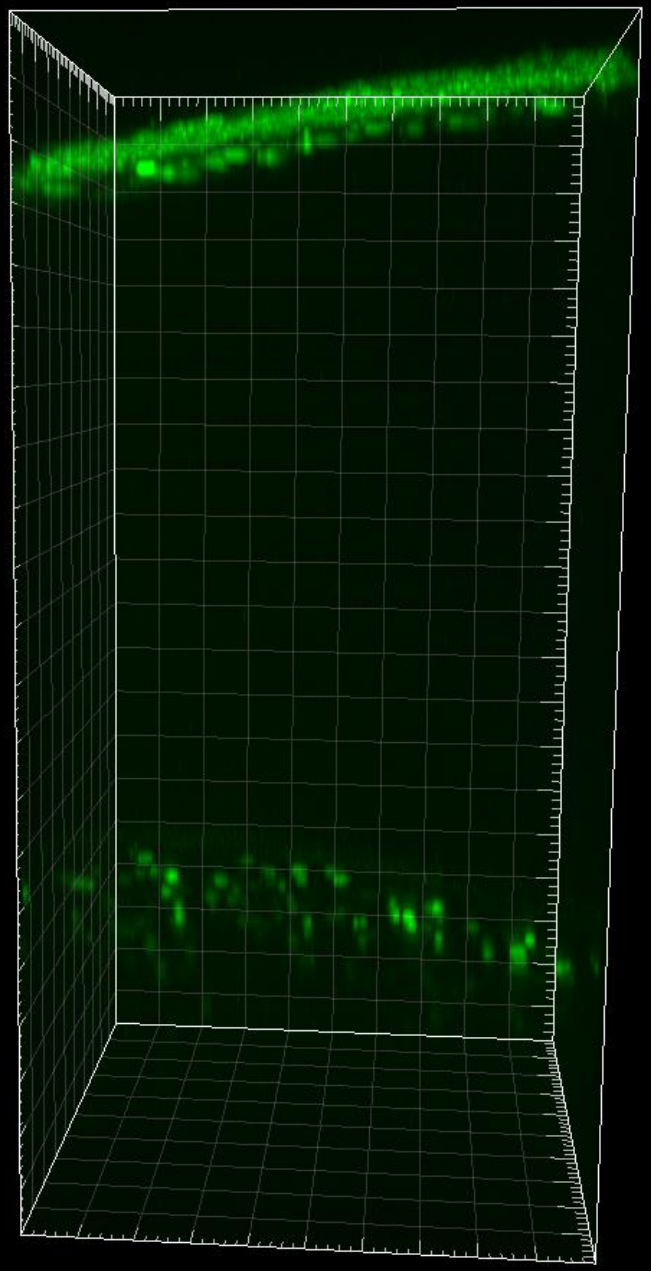
Application examples

Non-invasive visualization of cochlear microcirculation, Fritz Ihler, Martin Canis



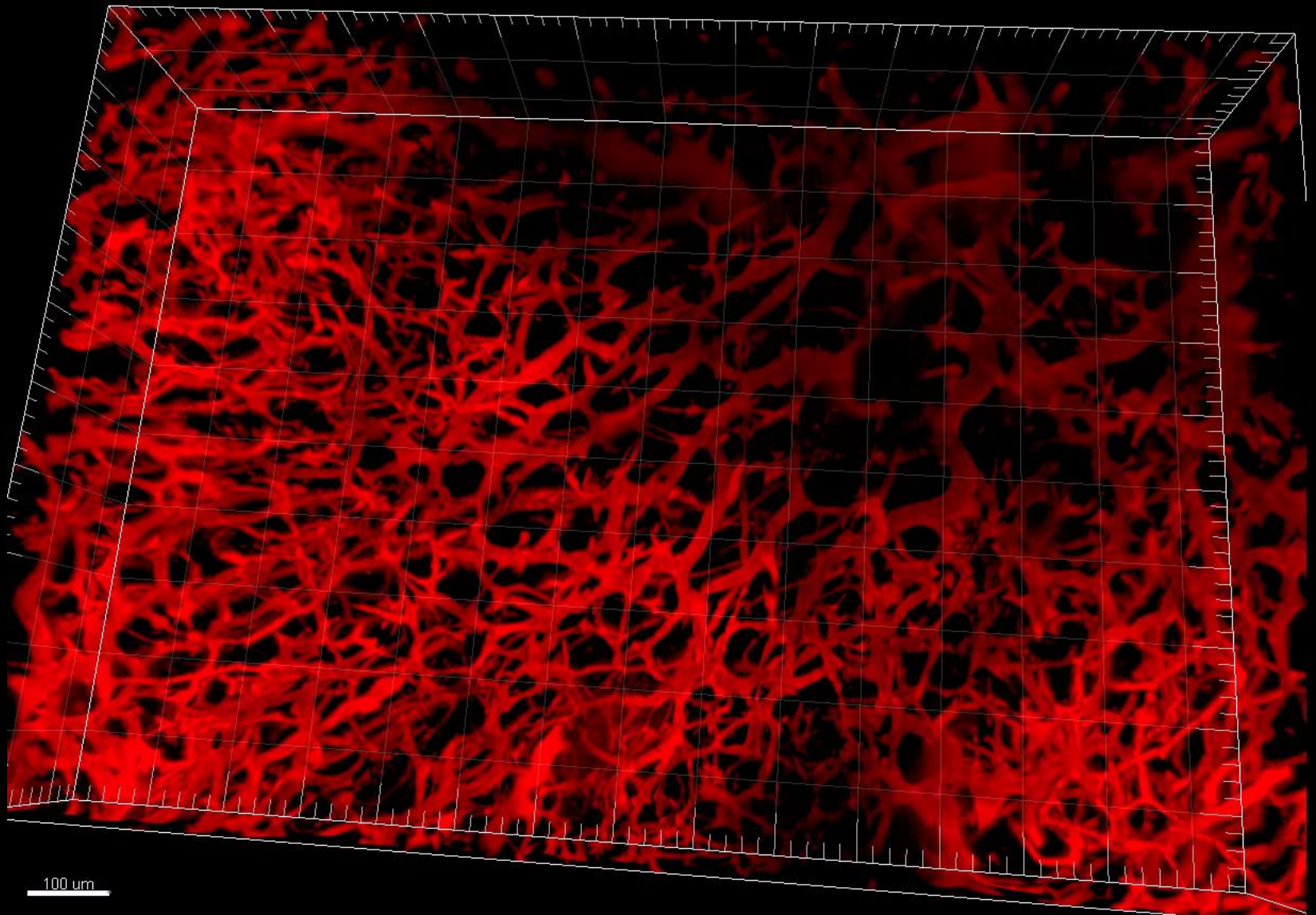


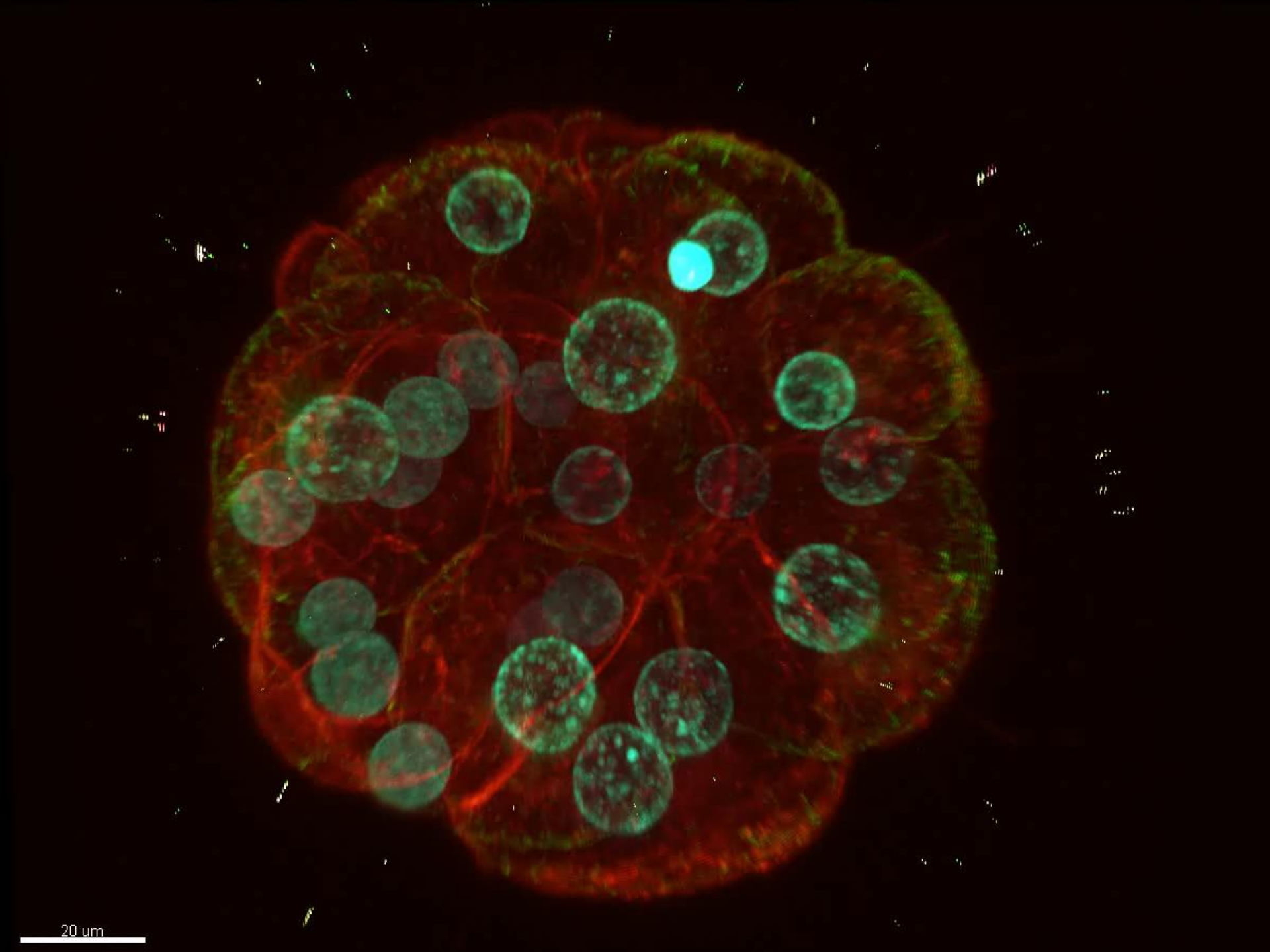
Ly-EGFP mouse embryo, imaged through yolk sac

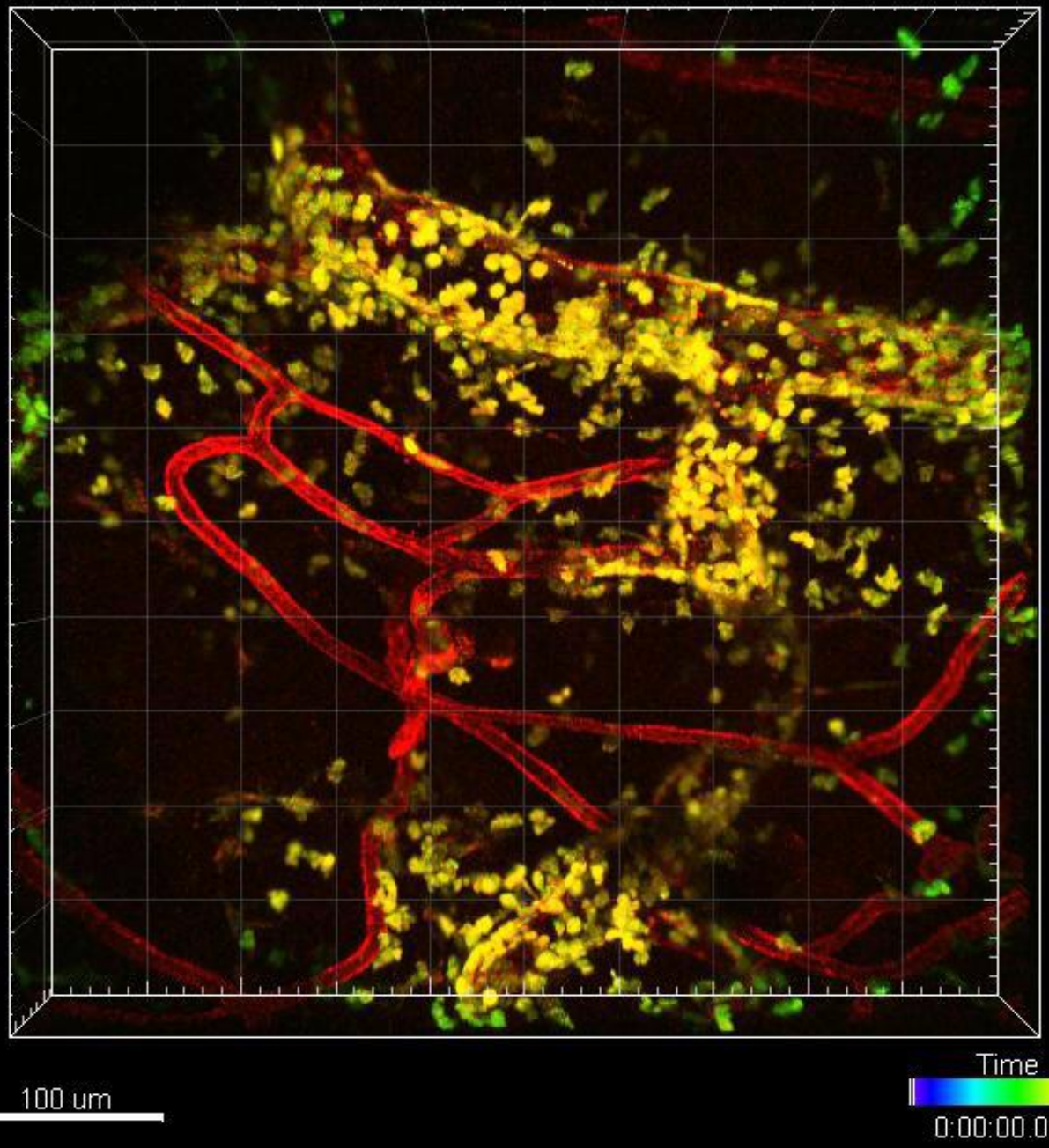


150 um

Tumor Angiogenesis, day 10, Markus Wortmann, Heike Beck

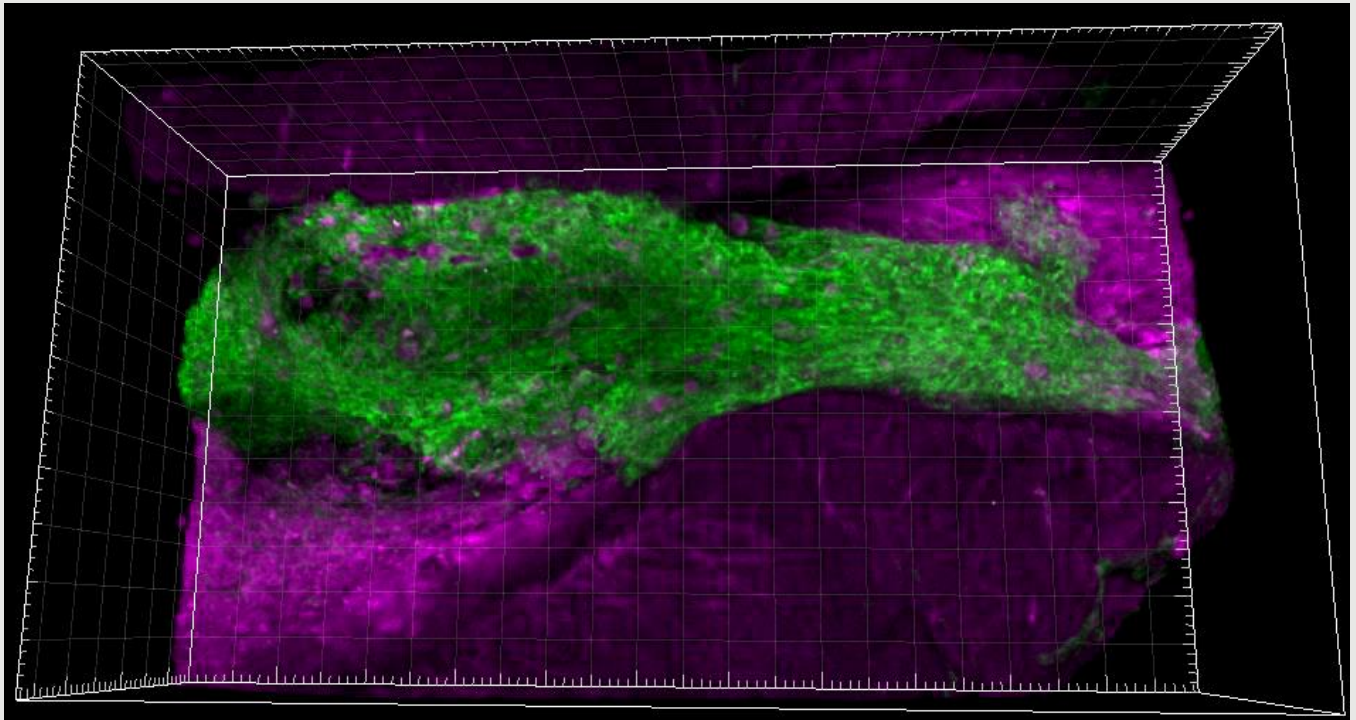



20 um


100 um
Time
0:00:00.000

Multi-photon time laps tile scanning

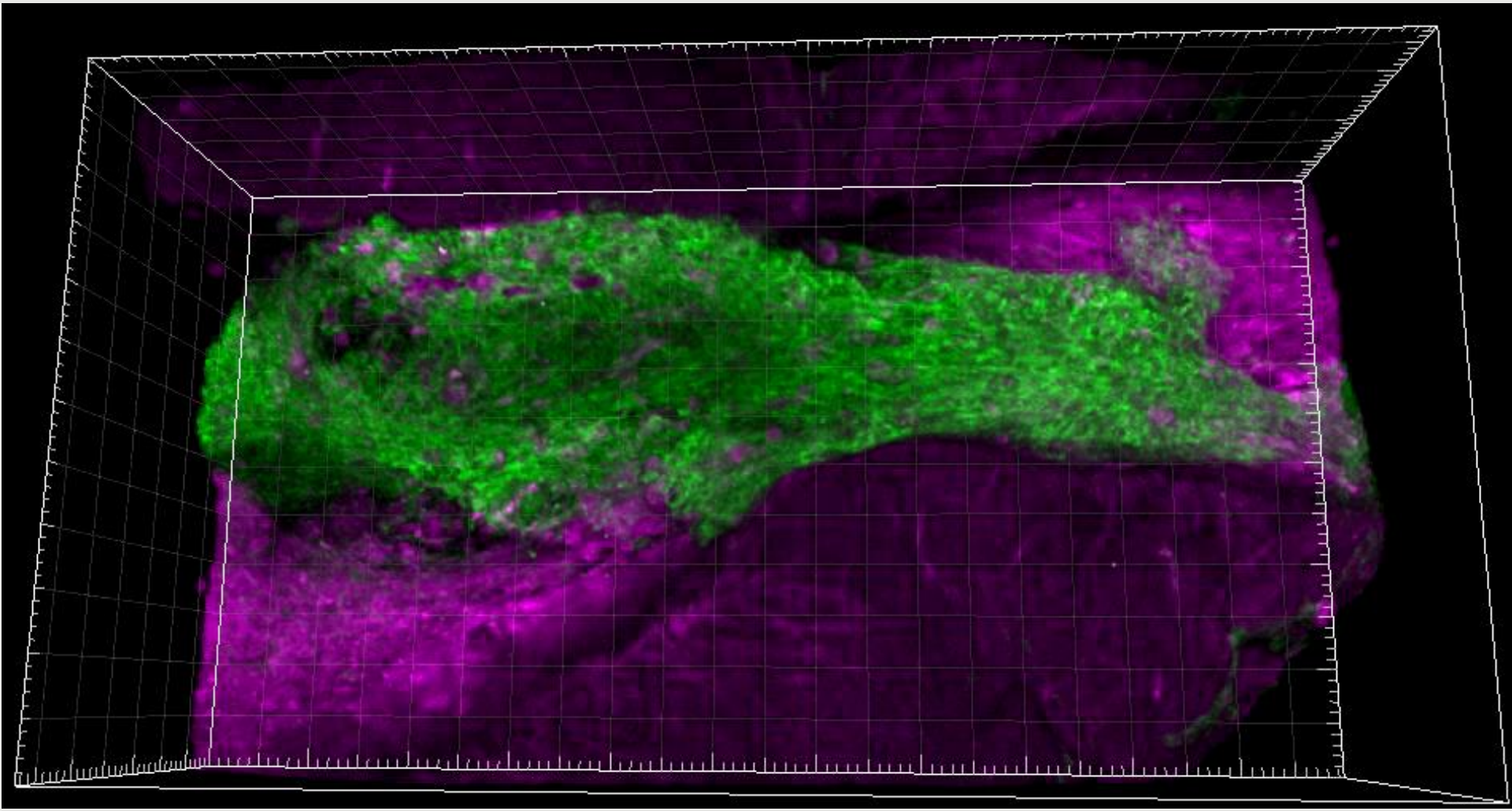
- 2 volumes, each with 0.6x0.6x0.2 mm³. Merged: 1.1x0.6 mm
- 900 nm and 1050 nm excitation for green and red FP
- 24 hours, every 20 min
- Original data size: 16.3 GB



LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

BIOMEDICAL CENTER

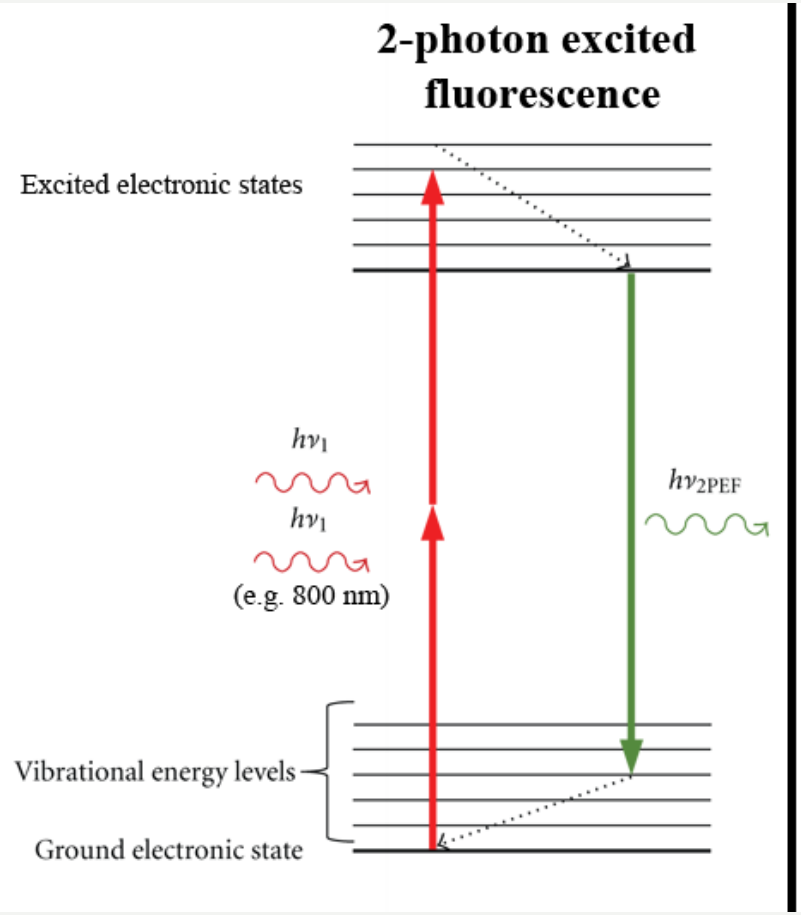
CORE FACILITY BIOIMAGING

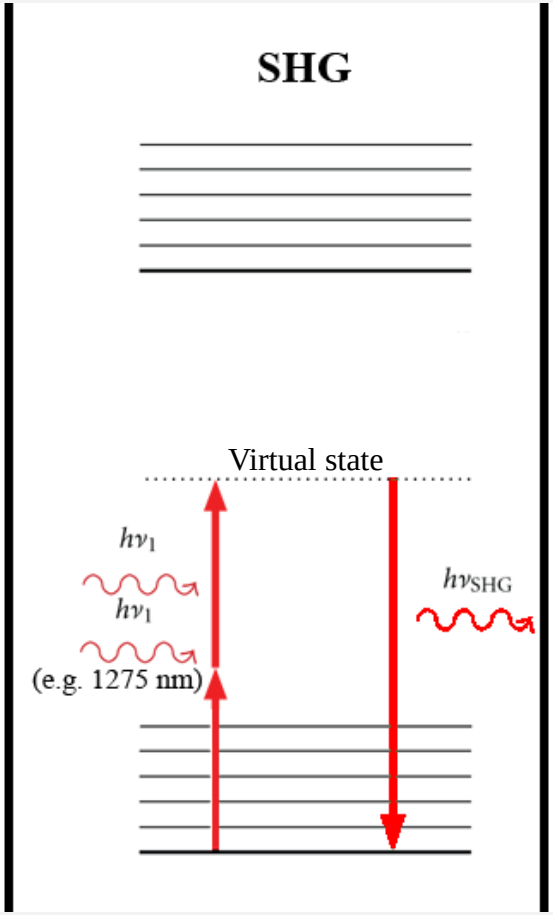


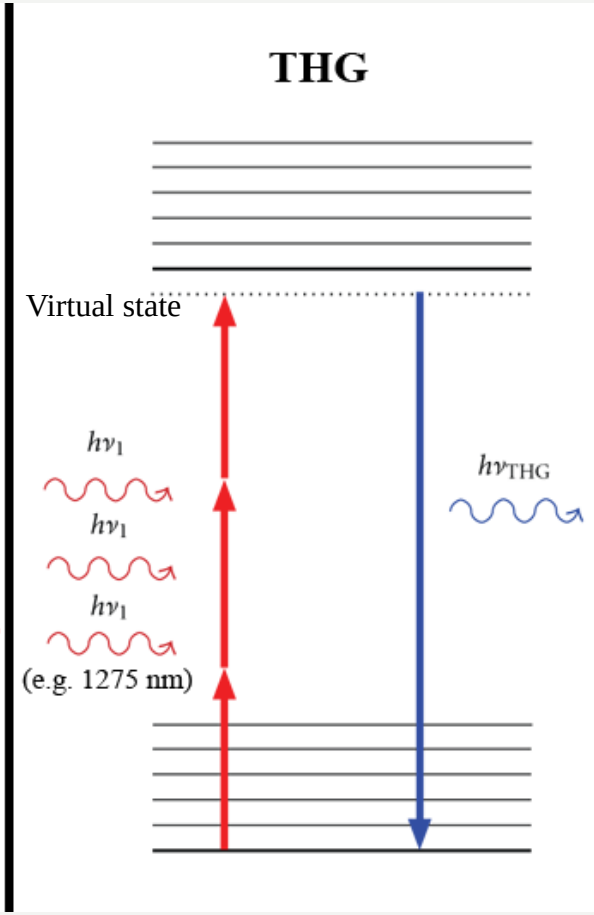
LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

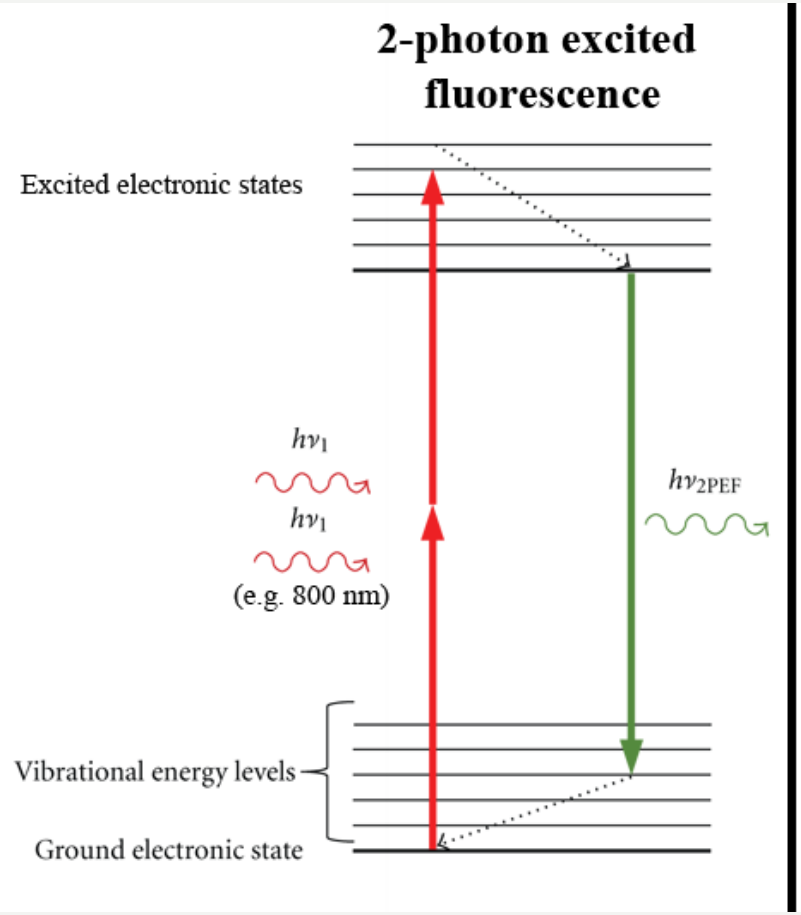
Walter Brendel Zentrum München

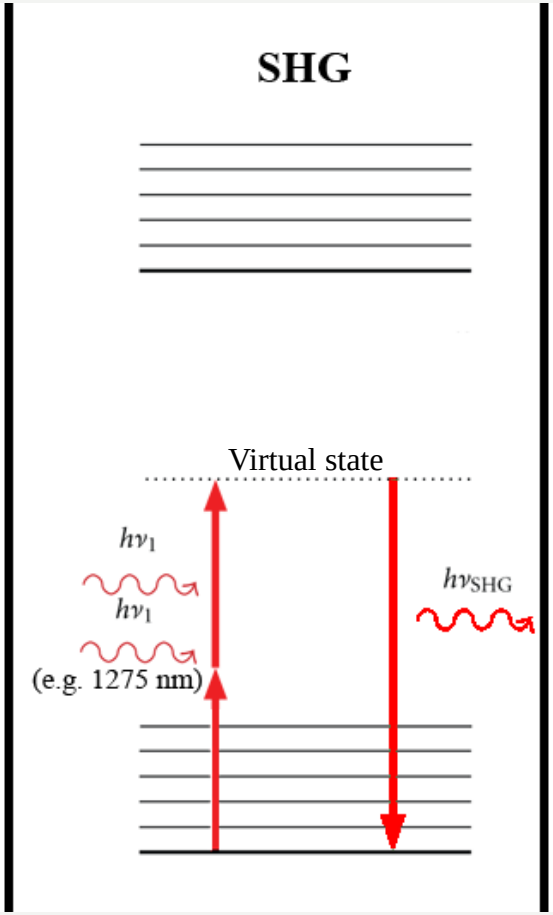
Second and Third Harmonic Generation (SHG + THG)

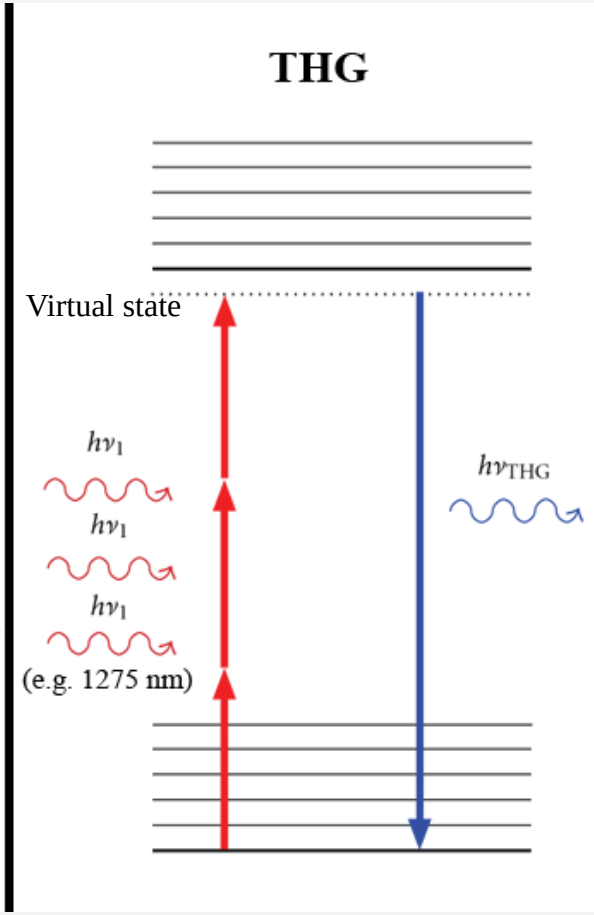

2-photon excited fluorescence
Excited electronic states
$h\nu_1$
$h\nu_1$
(e.g. 800 nm)
$h\nu_{2PEF}$
Vibrational energy levels
Ground electronic state

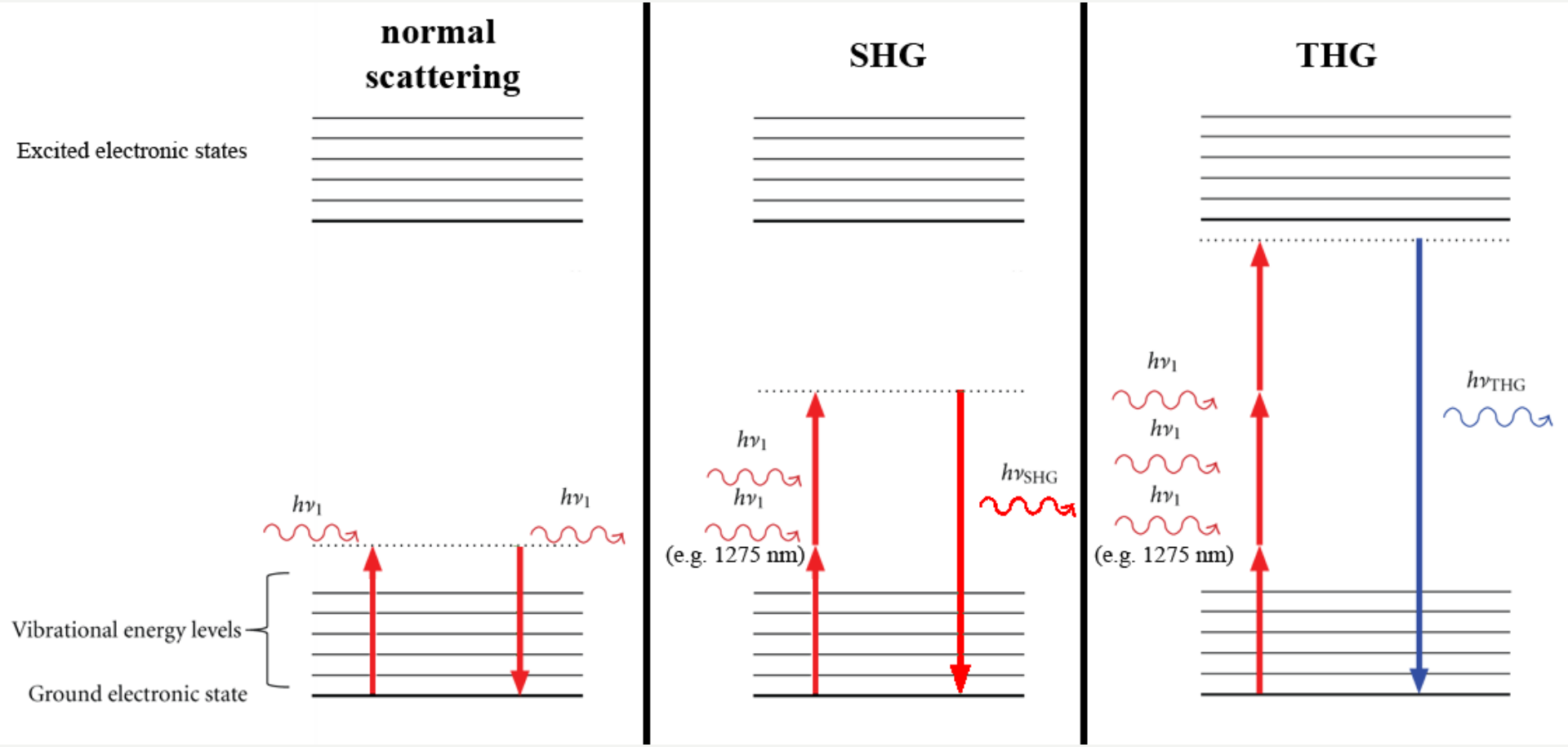

SHG
Virtual state
$h\nu_1$
$h\nu_1$
(e.g. 1275 nm)
$h\nu_{SHG}$


THG
Virtual state
$h\nu_1$
$h\nu_1$
$h\nu_1$
(e.g. 1275 nm)
$h\nu_{THG}$


2-photon excited fluorescence
Excited electronic states
$h\nu_1$
$h\nu_1$
(e.g. 800 nm)
$h\nu_{2PEF}$
Vibrational energy levels
Ground electronic state


SHG
Virtual state
$h\nu_1$
$h\nu_1$
(e.g. 1275 nm)
$h\nu_{SHG}$


THG
Virtual state
$h\nu_1$
$h\nu_1$
$h\nu_1$
(e.g. 1275 nm)
$h\nu_{THG}$


normal scattering
SHG
THG
Excited electronic states
Vibrational energy levels
Ground electronic state
hν1
hν1
hν1
hν1
(e.g. 1275 nm)
hνSHG
hν1
hν1
hν1
(e.g. 1275 nm)
hνTHG

WIKIPEDIA
The Free Encyclopedia

navigation
- Main page
- Contents
- Featured content
- Current events
- Random article

search
Go Search

interaction
- About Wikipedia
- Community portal
- Recent changes
- Contact Wikipedia
- Donate to Wikipedia
- Help

toolbox
- What links here
- Related changes
- Upload file
- Special pages
- Permanent link
- Cite this page

print/export
- Create a book
- Download as PDF
- Printable version

languages
- Български
- Deutsch
- Français
- Lietuvių
- 日本語
- Polski

Second-harmonic generation

From Wikipedia, the free encyclopedia

Second harmonic generation (**SHG**; also called **frequency doubling**) is a nonlinear optical process, in which photons interacting with a nonlinear material are effectively "combined" to form new photons with twice the energy, and therefore twice the frequency and half the wavelength of the initial photons. It is a special case of Sum frequency generation.

Second harmonic generation was first demonstrated by P. A. Franken, A. E. Hill, C. W. Peters, and G. Weinreich at the University of Michigan, Ann Arbor, in 1961. The demonstration was made possible by the invention of the laser, which created the required high intensity monochromatic light. They focused a ruby laser with a wavelength of 694 nm into a quartz sample. They sent the output light through a spectrometer, recording the spectrum on photographic paper, which indicated the production of light at 347 nm. Famously, when published in the journal *Physical Review Letters* (citation below), the copy editor mistook the dim spot (at 347 nm) on the photographic paper as a speck of dirt and removed it from the publication.

In recent years, SHG has been extended to biological applications. Researchers Leslie Loew and Paul Campagnola at the University of Connecticut have applied SHG to imaging of molecules that are intrinsically second-harmonic-active in live cells, such as collagen, while Joshua Salafsky [1] is pioneering the technique's use for studying biological molecules by labeling them with second-harmonic-active tags, in particular as a means to detect conformational change at any site and in real time. SH-active unnatural amino acids can also be used as probes.

Derivation of second harmonic generation

[edit]

The simplest case for analysis of second harmonic generation is a plane wave of amplitude $E(\omega)$ traveling in a nonlinear medium in the direction of its k vector. A polarization is generated at the second harmonic frequency

$$P(2\omega) = 2\epsilon_0 d_{eff}(2\omega;\omega,\omega)E^2(\omega),$$

where $2d_{eff} = \chi^{(2)}$.

The wave equation at 2ω (assuming negligible loss and asserting the slowly varying envelope approximation) is

$$\frac{\partial E(2\omega)}{\partial z} = -\frac{i\omega}{n_{2\omega}c} d_{eff} E^2(\omega) e^{i\Delta k z}$$

where $\Delta k = k(2\omega) - 2k(\omega)$.

At low conversion efficiency ($E(2\omega) << E(\omega)$ the amplitude $E(\omega)$ remains essentially constant over the interaction length, l. Then, with the boundary condition $E(2\omega, z = 0) = 0$ we get

$$E(2\omega, z = l) = -\frac{i\omega d_{eff}}{n_{2\omega}c} E^2(\omega) \int_0^l e^{i\Delta k z} dz = -\frac{i\omega d_{eff}}{n_{2\omega}c} E^2(\omega) l \frac{\sin \Delta k l/2}{\Delta k l/2} e^{i\Delta k l/2}$$

In terms of the optical intensity, $I = n/2\sqrt{\epsilon_0/\mu_0}|E|^2$, this is,

$$I(2\omega, l) = \frac{2\omega^2 d_{eff}^2 l^2}{n_{2\omega} n_\omega^2 c^3 \epsilon_0} \left(\frac{\sin(\Delta k l/2)}{\Delta k l/2}\right)^2 I^2(\omega)$$

SHG

Two photons in, one out: energy is constant, thus wavelength is exactly halved: 860 nm→ 430 nm or 1275 nm→ 638 nm

In mammalian soft tissues generated in collagen fibers and striated muscle myosin (non-centrosymetric, dense substances)

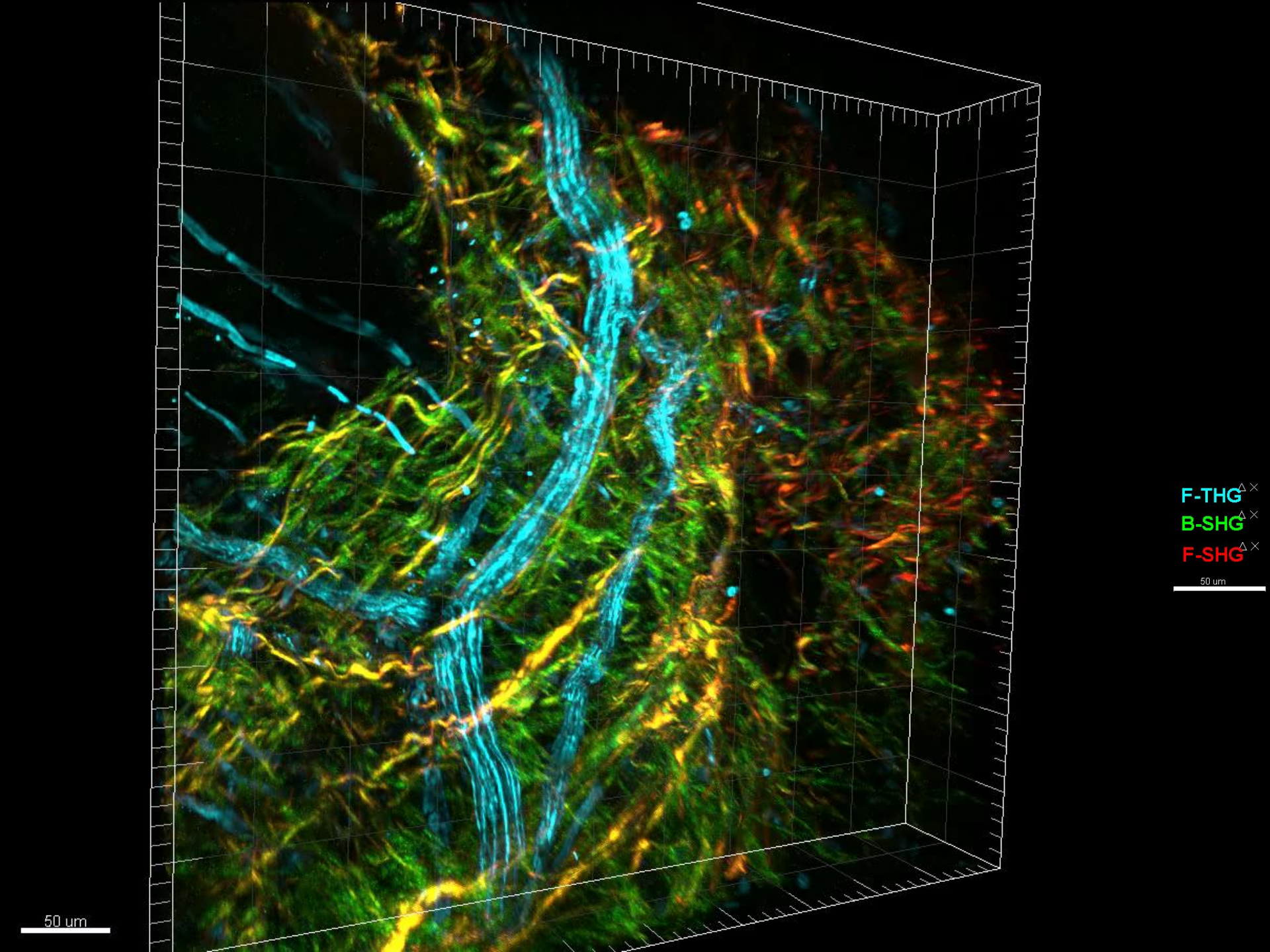
Label-free, 3D,
deep tissue penetration

THG

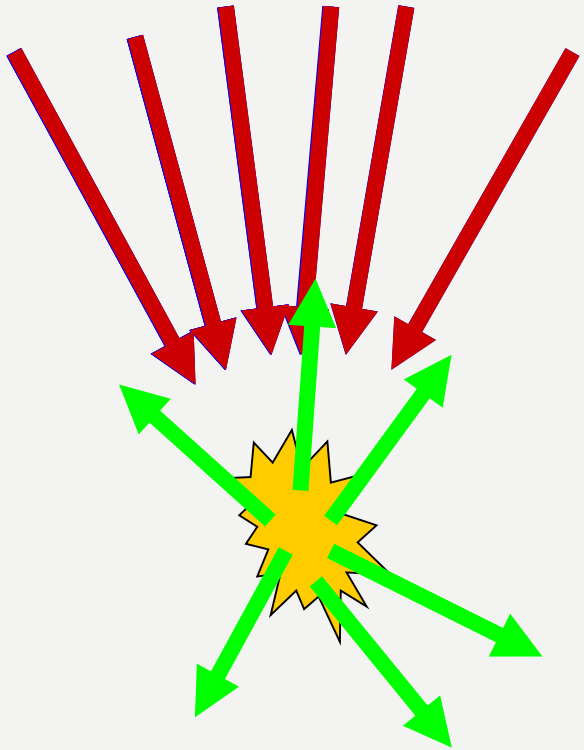
Three photons in, one out: energy is constant, thus wavelength is exactly 1/3: 1275 nm→ 425 nm.

Generated at interfaces, e.g. at membranes or refraction index mismatches

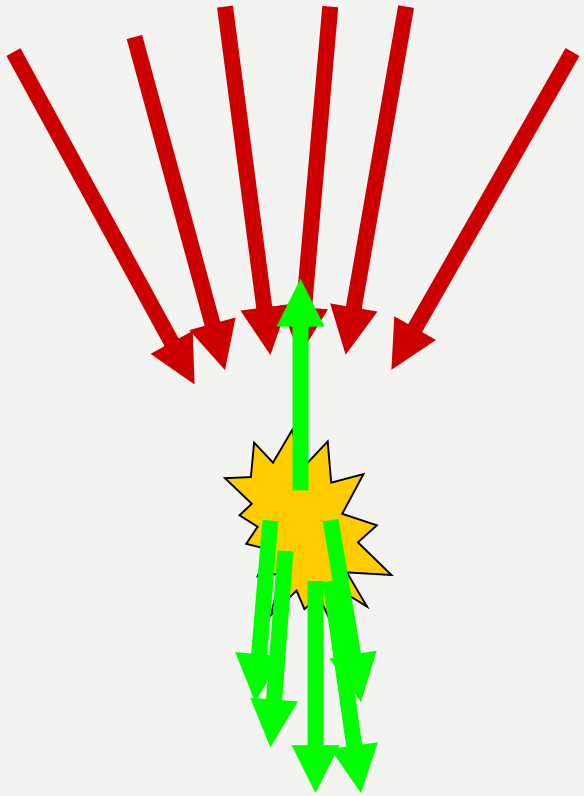
Label-free, 3D,
deep tissue penetration


F-THG
B-SHG
F-SHG
50 um
50 um

Fluorescence



Higher harmonic generation



Mouse cremaster with THG and SHG

Collaboration with Markus Rehberg, AG Krombach

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

BIOMEDICAL CENTER
CORE FACILITY BIOIMAGING

Steffen Dietzel

Walter-Brendel-Zentrum für Experimentelle Medizin

Core Facility Bioimaging at the Biomedical Center

Superresolution microscopy: STED and other approaches

Ernst Abbe, 1873,

Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung.

- Resolution is diffraction limited: Abbe-Limit

„durch kein Mikroskop können Theile getrennt (oder die Merkmale einer real vorhandenen Structur wahrgenommen) werden, wenn dieselben einander so nahe stehen, dass auch der erste durch Beugung erzeugte Lichtbüschel nicht mehr gleichzeitig mit dem ungebeugten Lichtkegel in das Objectiv eintreten kann“.

No microscope can separate parts (or properties of an existing structure), if they are so close to each other, that even the first ring maximum generated by diffraction will not enter the objective together with the undiffracted light.

Abbe

- Abbes insight did not lead to a significant improvement of microscopic resolution. The maximal resolution was reached already shortly before:
- ~200 nm = 0,2 µm = 0,0002 mm.
- For over 100 years the Abbe limit was regarded as an insurmountable barrier in light microscopy.

Ernst Abbe, 1873,

Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung.

- Beugungsbegrenzung der Auflösung, Abbe-Limit:

„Die eigentliche Capacität des Mikroskops im strengeren Sinne aber muss ich - so lange nicht Momente geltend gemacht werden, die ganz ausserhalb der Tragweite der aufgestellten Theorie liegen - schon bei der oben bezeichneten früheren Grenze als vollständig erschöpft ansehen;“

The actual capacity of the microscope in the strict sense I have to regard as completely exhausted already at the limit described above, as long as no elements are invoked which are entirely outside of the theory presented here.

Ernst Abbe, 1873,

Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung.

- Beugungsbegrenzung der Auflösung, Abbe-Limit:

„Die eigentliche Capacität des Mikroskops im strengeren Sinne aber muss ich - so lange nicht Momente geltend gemacht werden, die ganz ausserhalb der Tragweite der aufgestellten Theorie liegen - schon bei der oben bezeichneten früheren Grenze als vollständig erschöpft ansehen;“

The actual capacity of the microscope in the strict sense I have to regard as completely exhausted already at the limit described above, as long as no elements are invoked which are entirely outside of the theory presented here.

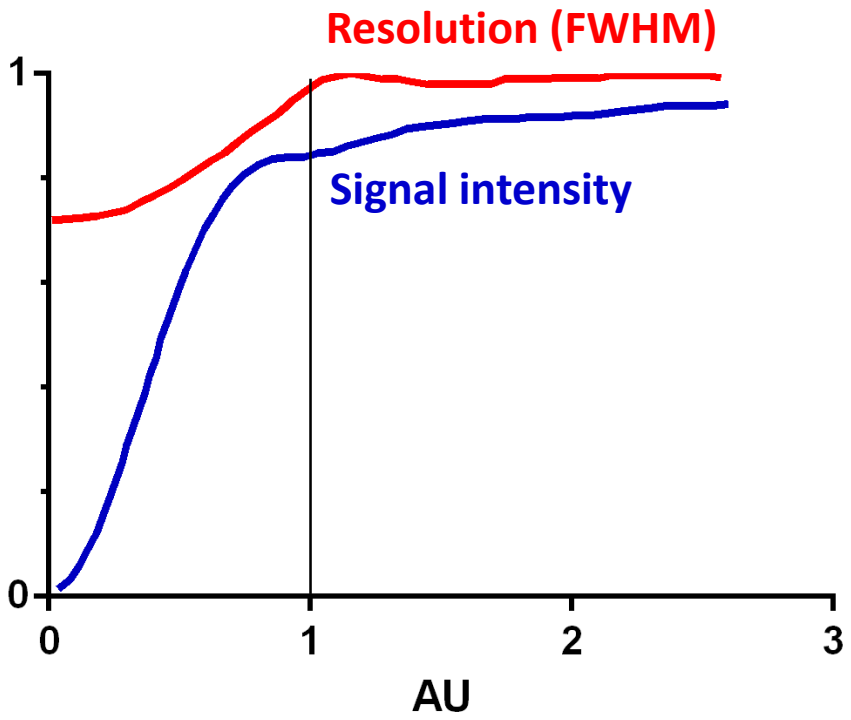
„Momente, die ganz ausserhalb der Tragweite der aufgestellten Theorie liegen“

Most popular superresolution methods:

- Localization microscopy
- Structured illumination (3D-SIM)
- STED
- …

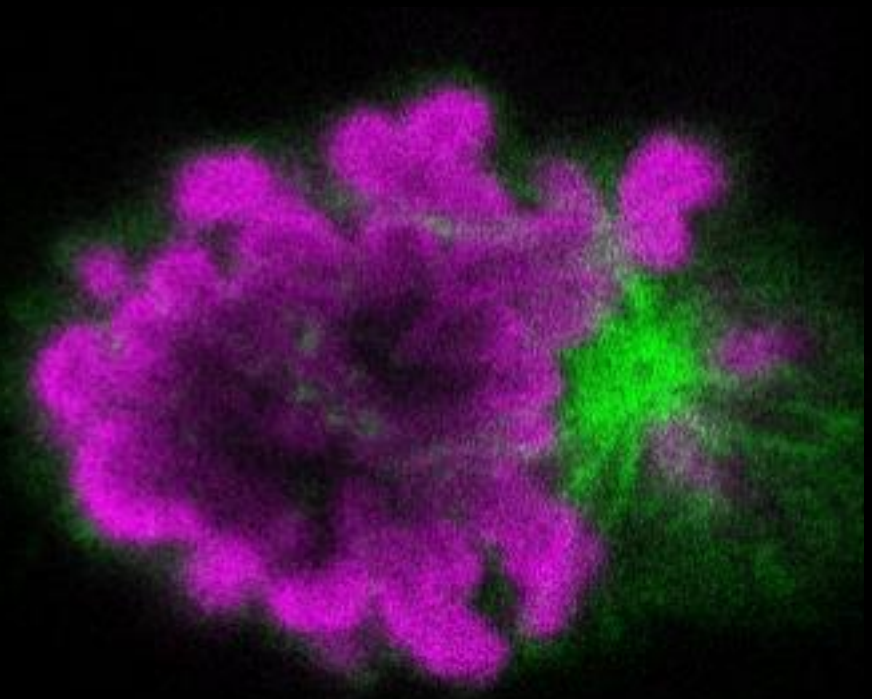
- All these methods work only with fluorescence

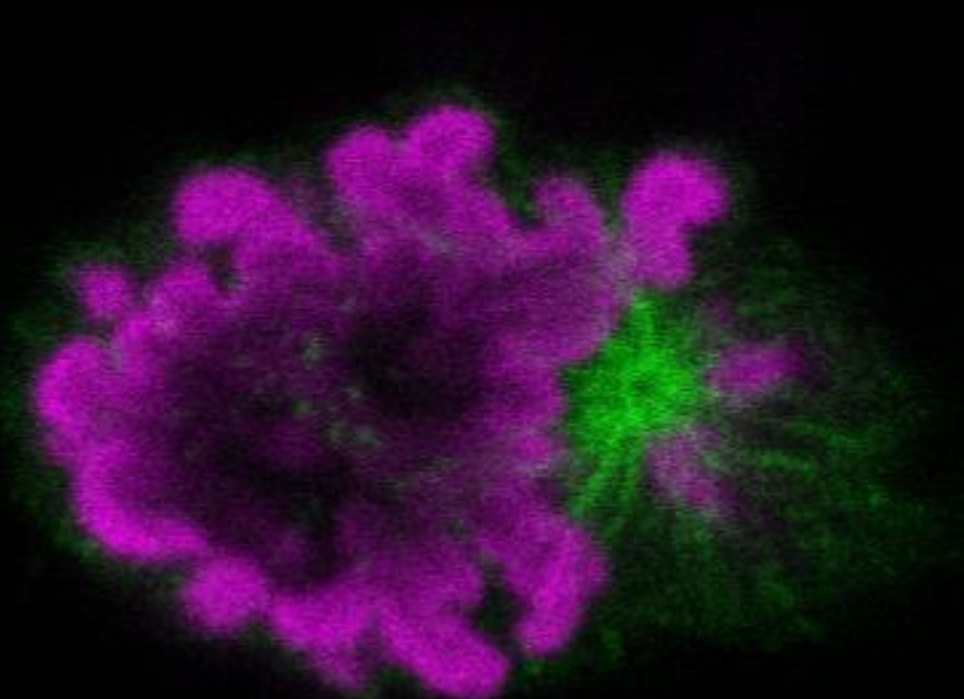
Confocal resolution and intensity vs Pinhole size



Data source: CJR Sheppard et al. (2006) Signal–to-noise ratio in confocal microscopes. In: JB Pawley (ed) Handbook of biological confocal microscopy (3rd ed) pp 442-452. Numerical data extracted with Engauge Digitizer V.4.1 from Fig 22.2 (circular pinhole) and Fig 22.3 (1/[transverse resolution (point)]).

Example: HeLa cell in Mitosis





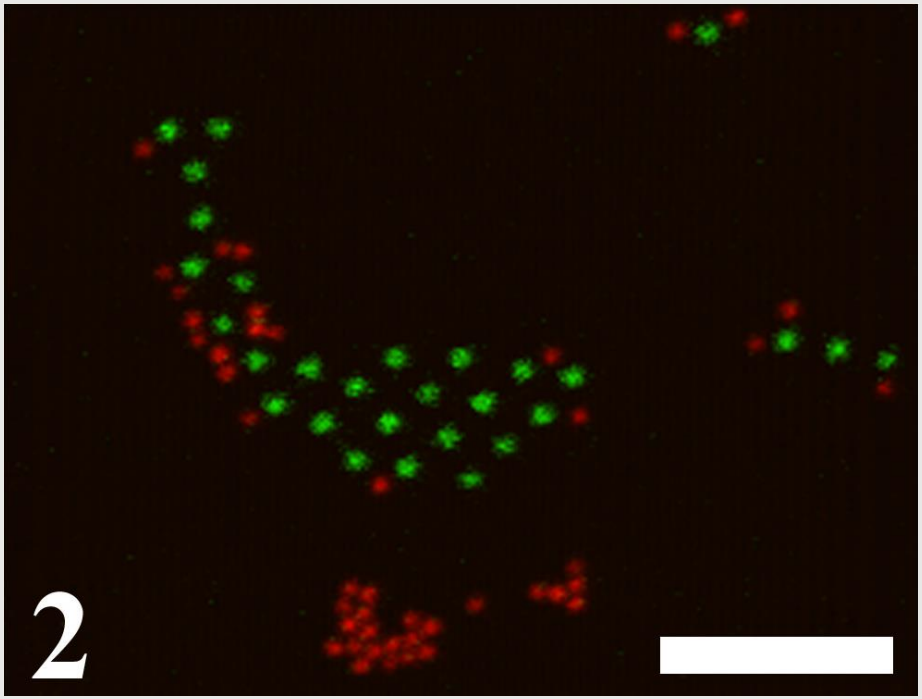
Pinhole: 1 AU 0.5 AU
(both without deconvolution)

Tubulin, Actin, H3-Serin-10-Phosphorylierung

Cheating:
Using normal microscopy to achieve a better resolution

- Spectral separation of spots

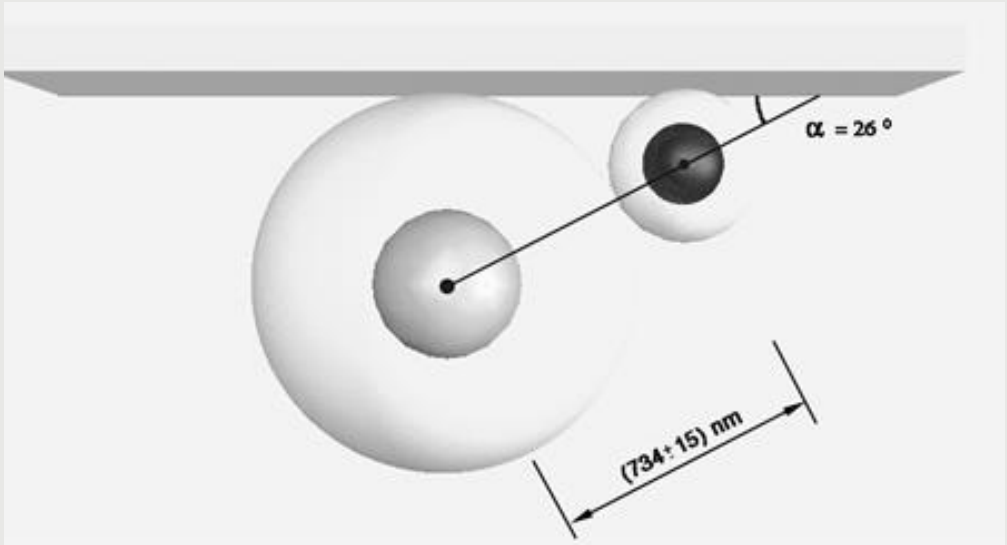
Spectral precision distance measurements



- Clusters of tightly packed FITC- and RITC-labeled glass spheres

Dietzel S, Eils R, Sätzler K, Bornfleth H, Jauch A, Cremer C, Cremer T: Evidence against a looped structure of the inactive human X-chromosome territory. *Exp Cell Res* 1998, 240(2):187-196.

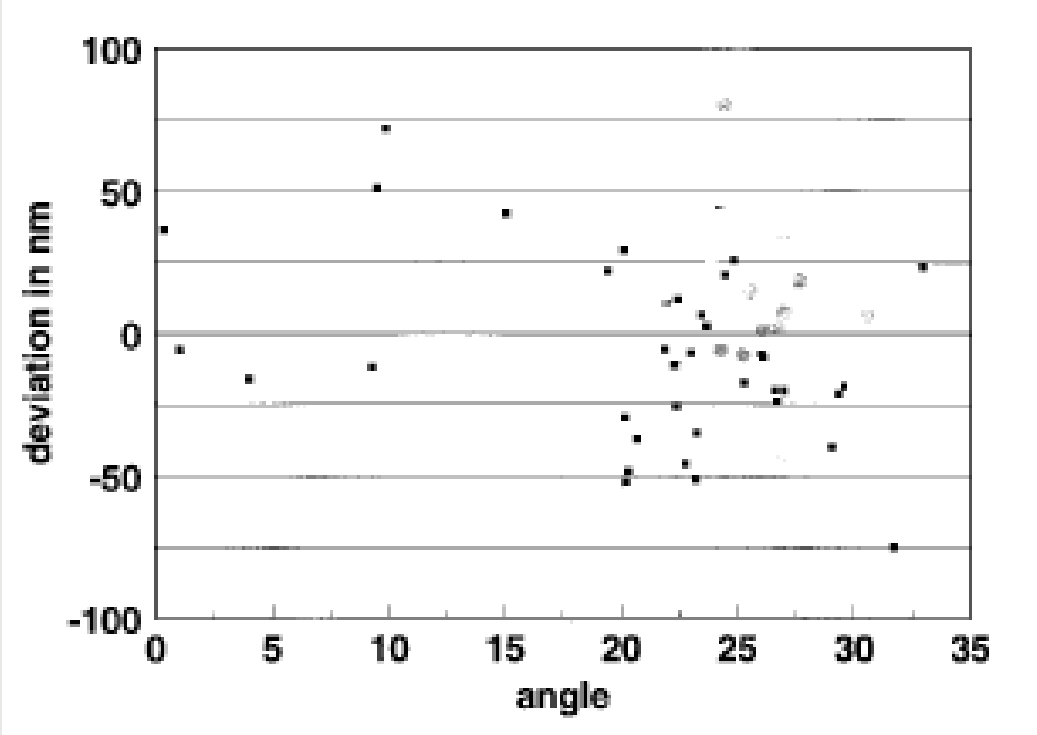
Spectral precision distance measurements



- Clusters of tightly packed FITC- and RITC-labeled glass spheres

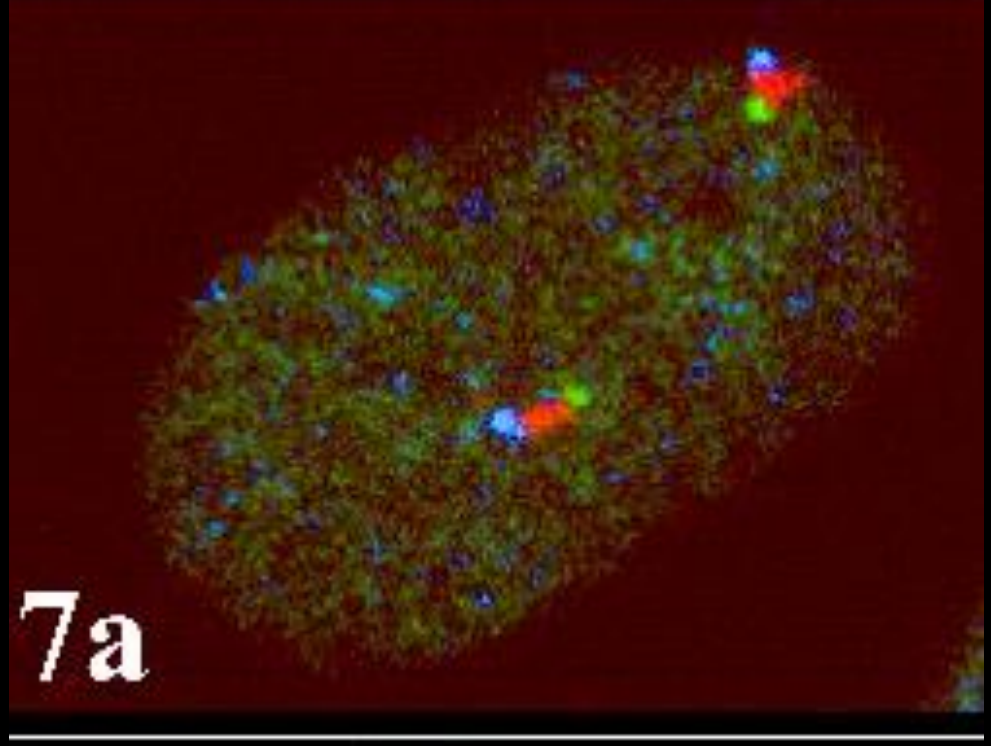
Dietzel S, Eils R, Sätzler K, Bornfleth H, Jauch A, Cremer C, Cremer T: Evidence against a looped structure of the inactive human X-chromosome territory. *Exp Cell Res* 1998, 240(2):187-196.

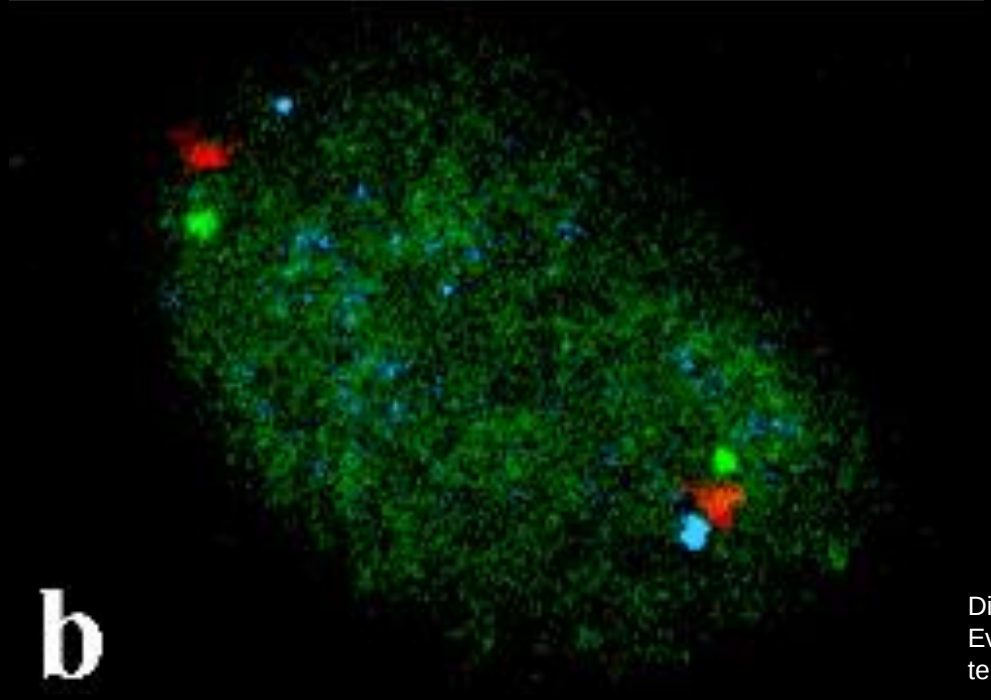
Spectral precision distance measurements



Clusters of tightly packed FITC- and RITC-labeled glass spheres

Dietzel S, Eils R, Sätzler K, Bornfleth H, Jauch A, Cremer C, Cremer T: Evidence against a looped structure of the inactive human X-chromosome territory. *Exp Cell Res* 1998, 240(2):187-196.



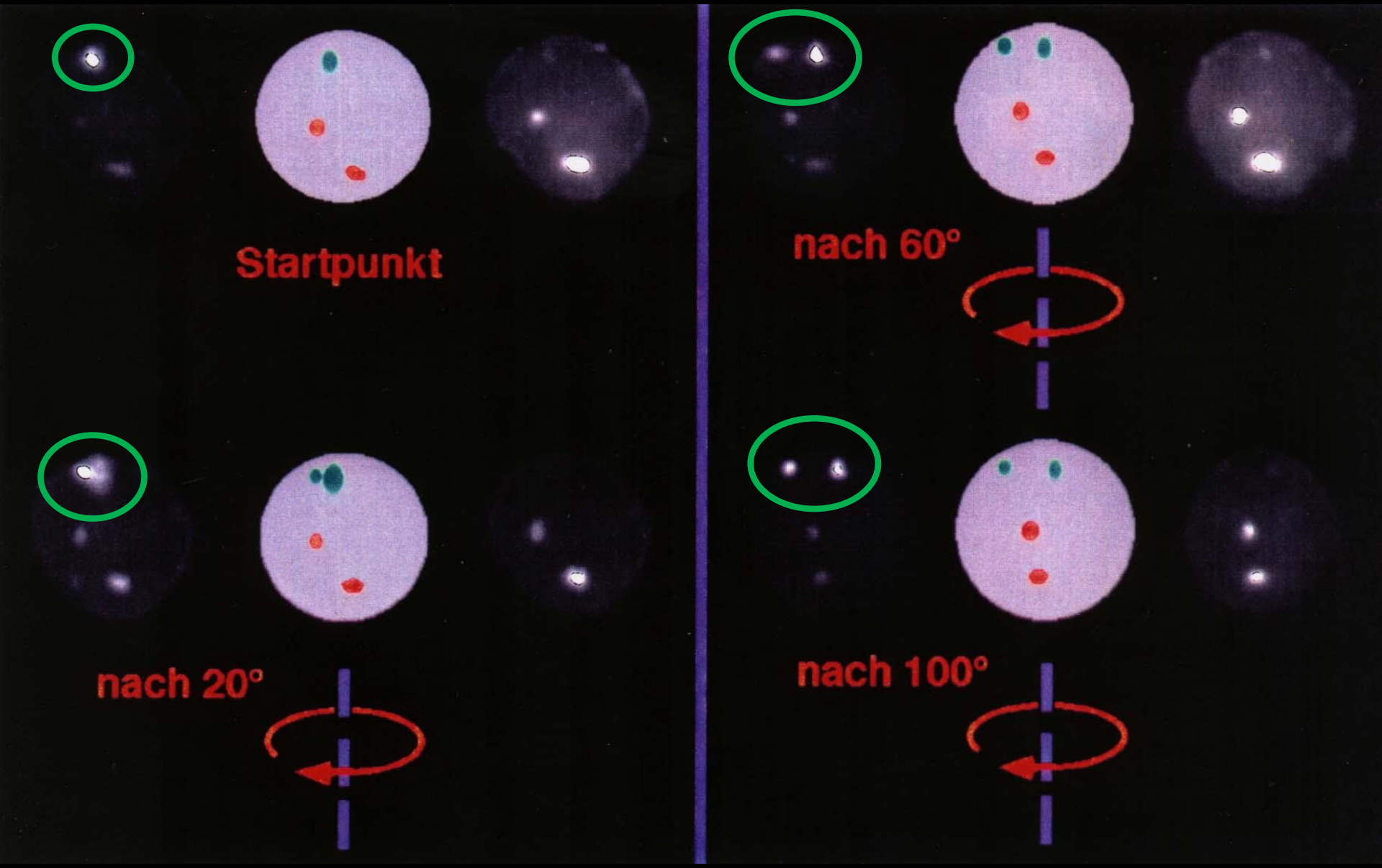


Dietzel S, Eils R, Sätzler K, Bornfleth H, Jauch A, Cremer C, Cremer T: Evidence against a looped structure of the inactive human X-chromosome territory. *Exp Cell Res* 1998, 240(2):187-196.

Cheating:
Using normal microscopy to achieve a better resolution

- Spectral separation of spots
- Placing a distance in the x,y-plane instead of z.

Bildquelle: Dissertation Steffen Dietzel, 1996, Universität Heidelberg



Bildquelle: Dissertation Steffen Dietzel, 1996, Universität Heidelberg

Cheating:
Using normal microscopy to achieve a better resolution

- Spectral separation of spots
- Placing a distance in the x,y-plane instead of z.

Both approaches work for distance measurements, but not for general improvement of resolution

Clever physical tricks to achieve superresolution

- 4 Pi Microscopy
- TIRF (total internal reflection fluorescence)

- Localization Microscopy (PALM, STORM,…)
- 3D Structured illumination microscopy (3D-SIM)
- STED

Most popular

Commercial superresolution microscopes

Localization Microscopes

- Leica GSD (dSTORM)
- Zeiss Elyra P (PALM)
- Brucker Vutara

3D Structured illumination microscopes (3D-SIM):

- OMX from General Electric (formerly Applied Precision)
- Elyra S from Zeiss
- Nikon N-SIM

STED

- Leica STED
- Abberior Instruments STED

Localization Microscopy

- PALM (Photo Activated Localization Microscopy)
- STORM (Stochastic Optical Reconstruction Microscopy)
- dSTORM (direct STORM)
- GSD (Ground state depletion)
- DNA-PAINT

Localization-Microscopy

Advantages:

- Theoretically unlimited resolution. In practice dependent on localization precision and number of exposures. 10 nm have been reported.
- Relatively cheap.

Disadvantages:

- Limited to switchable dyes.
- Multi-color is difficult
- Several thousand exposures may be needed to calculate one image.
- 3D stacks are difficult to obtain.

3D-SIM

3D structured illumination microscopy

Advantages:

- Multi-color is no problem.
- Increase in resolution for (at best) ~2x, ~120 nm

Disadvantages:

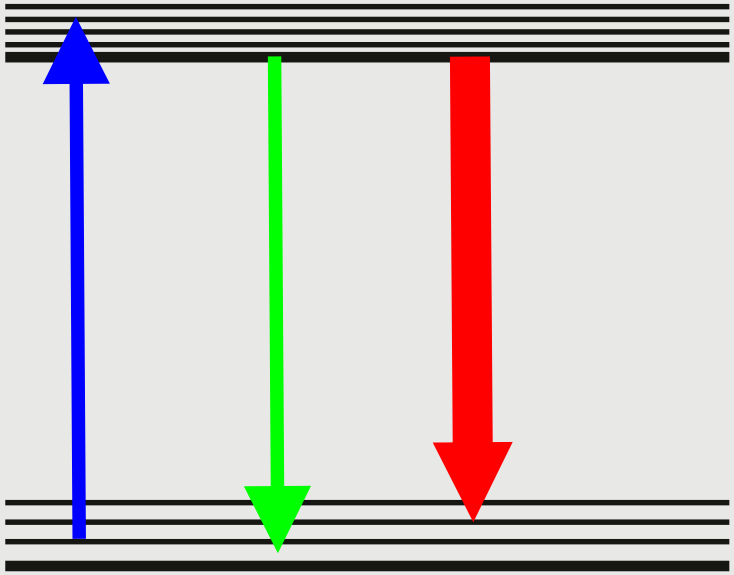
- Many images needed for one calculated image, therefore time consuming (10-15 min for 3D-stack with 64 slices one color) and large data sets (~760 Mbyte).
- The calculations may introduce artefacts.
- Expensive, ~1 Mio Euro for multicolor.

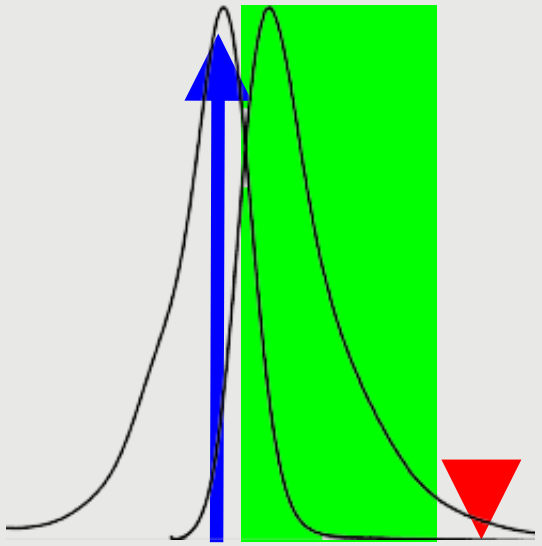
STED

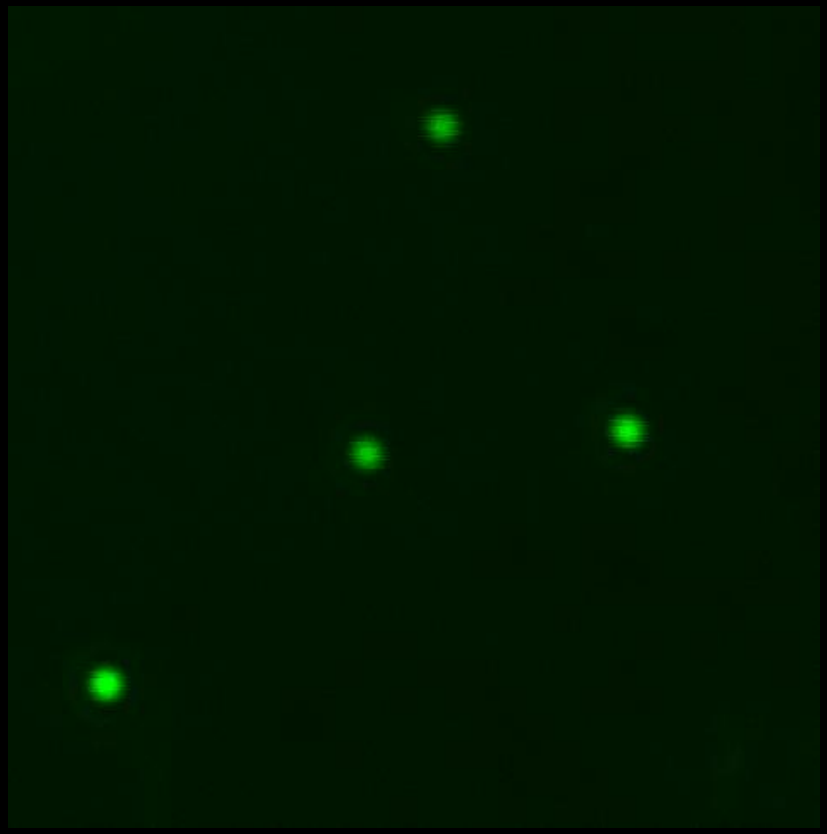
Stimulated Emission Depletion

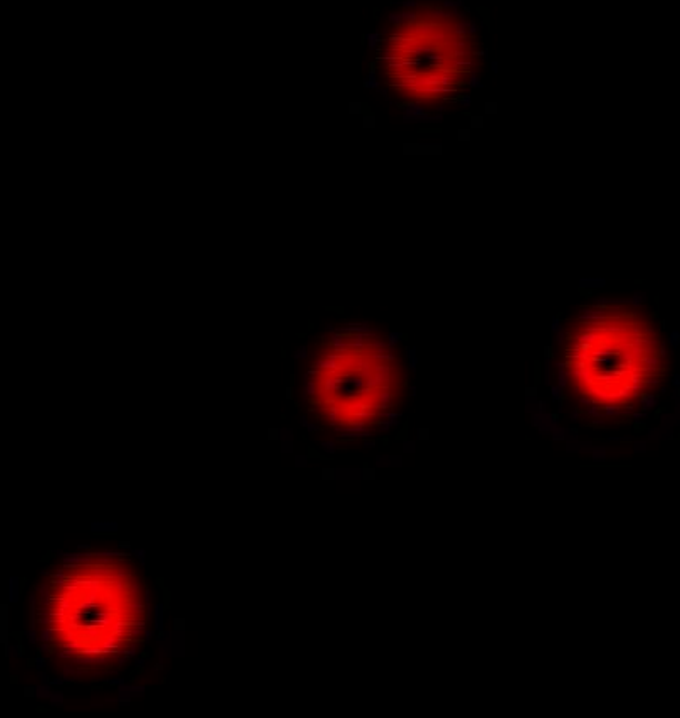
- STED is based on a point scanner clsm.
- It breaks the Abbe Limit by exciting only a very small volume
- Resolution limit is set only by photon statistic, theoretically.

STED

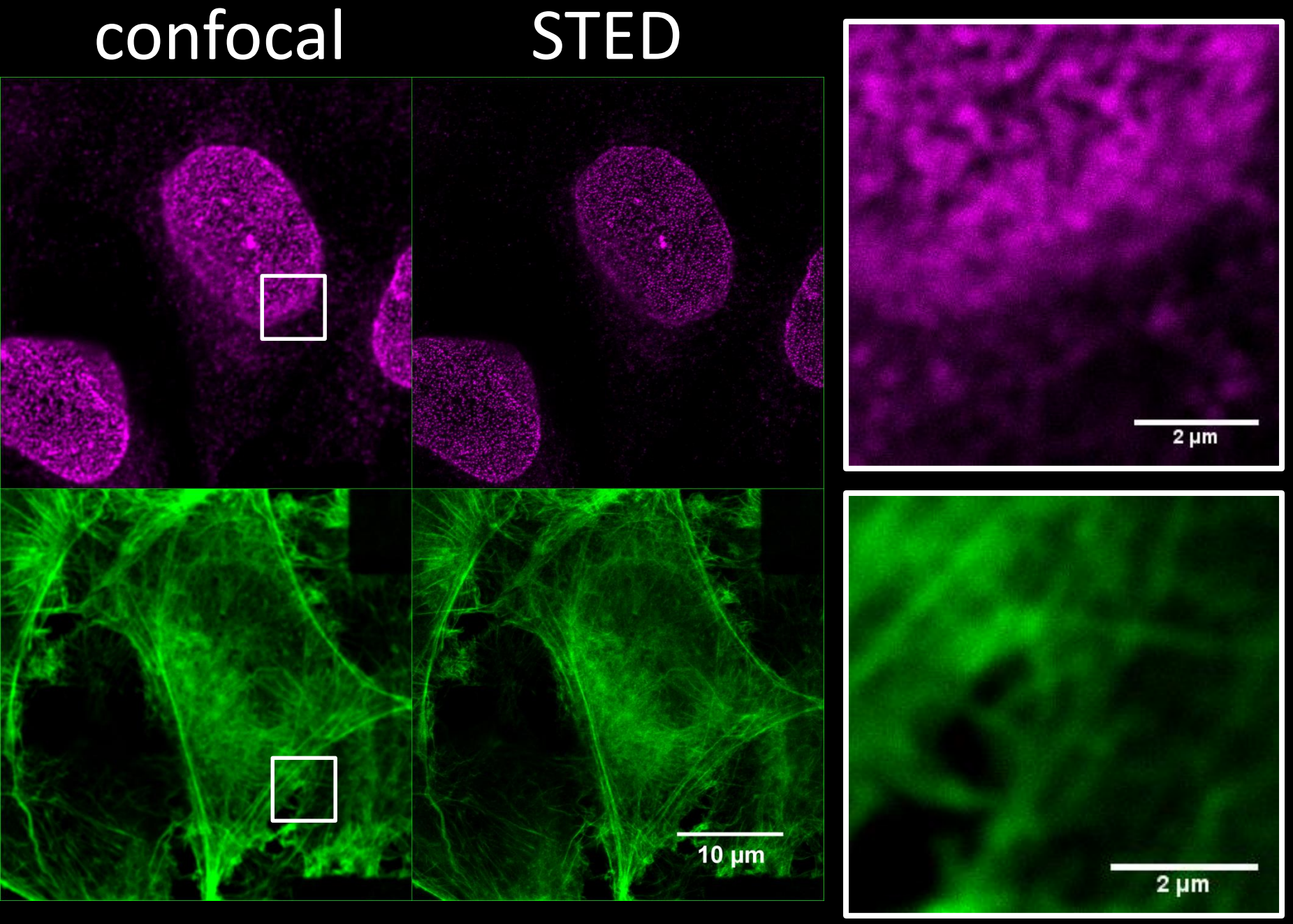




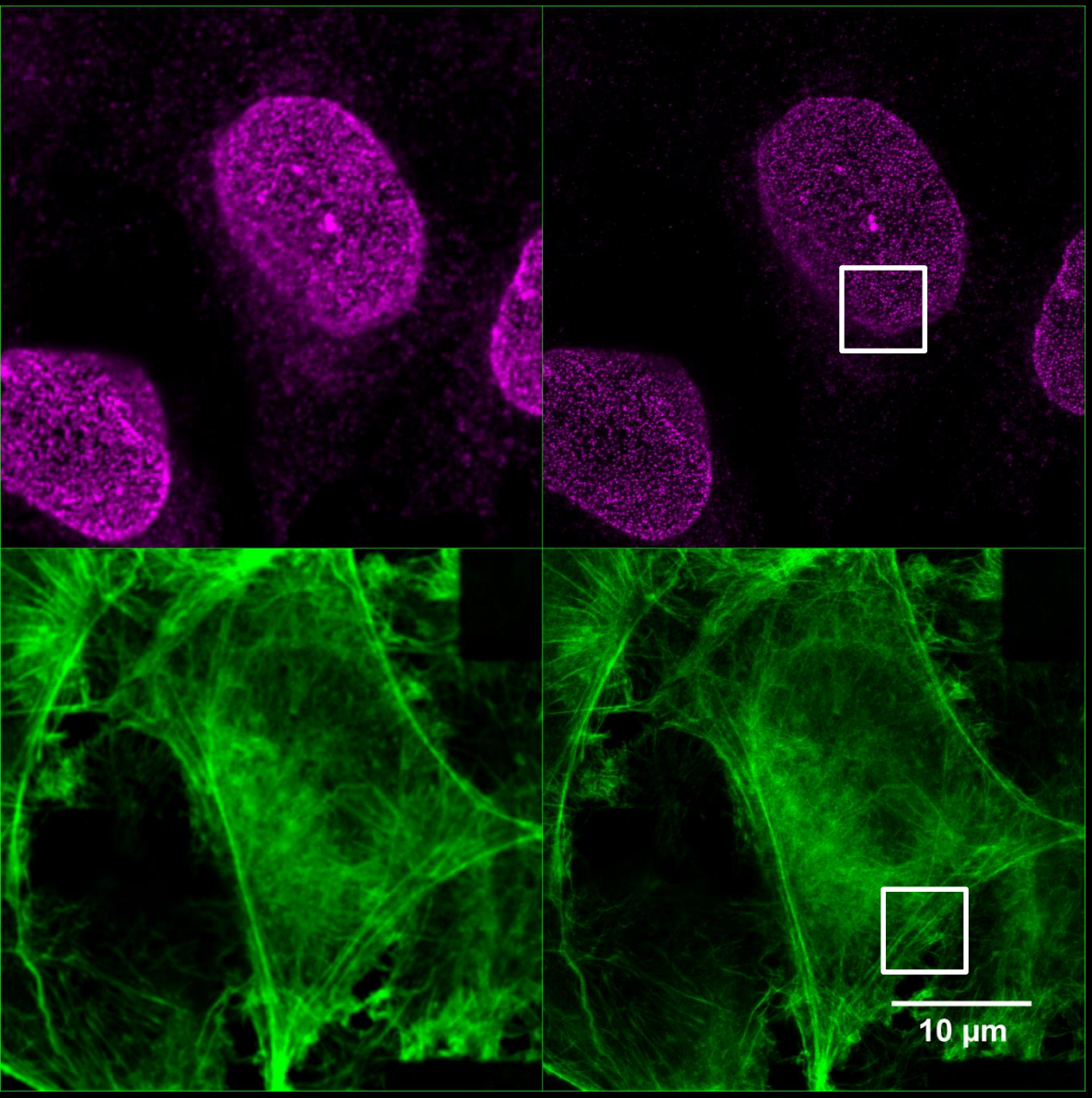


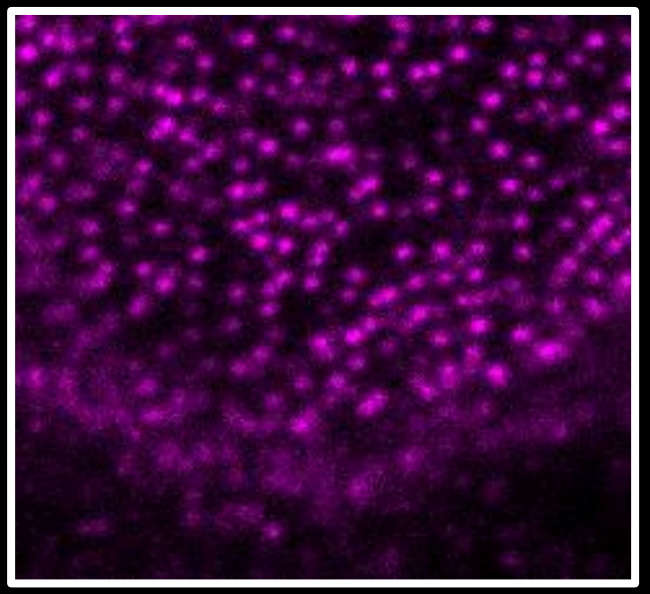


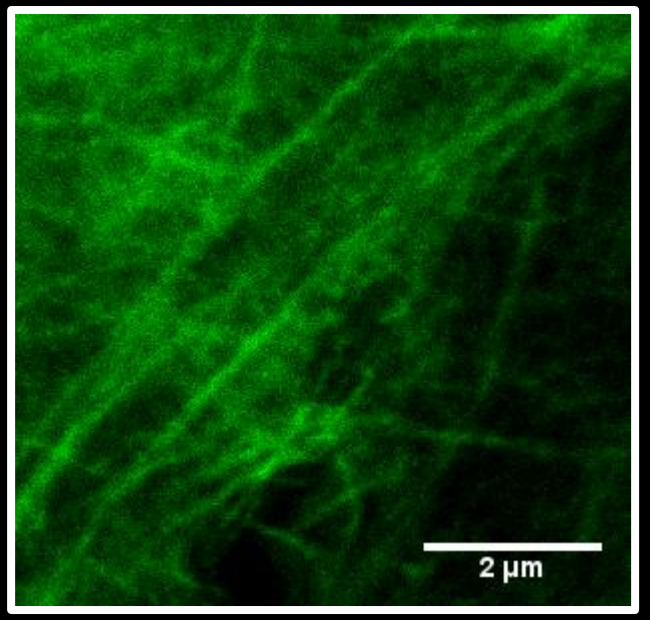
STED

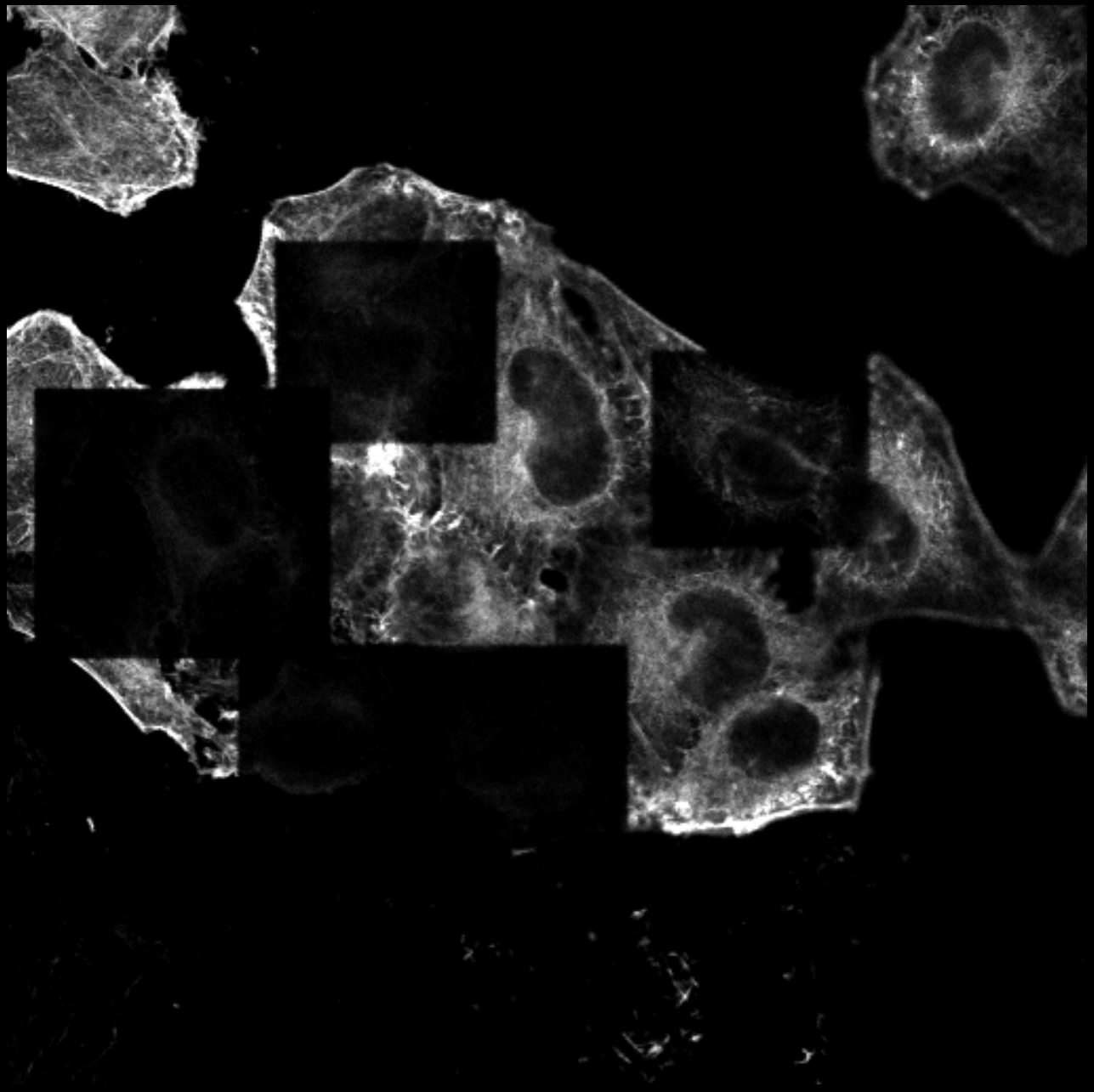

confocal
STED
2 µm
10 µm
2 µm

confocal STED









STED Bleaching

STED-Microscopy

Advantages:

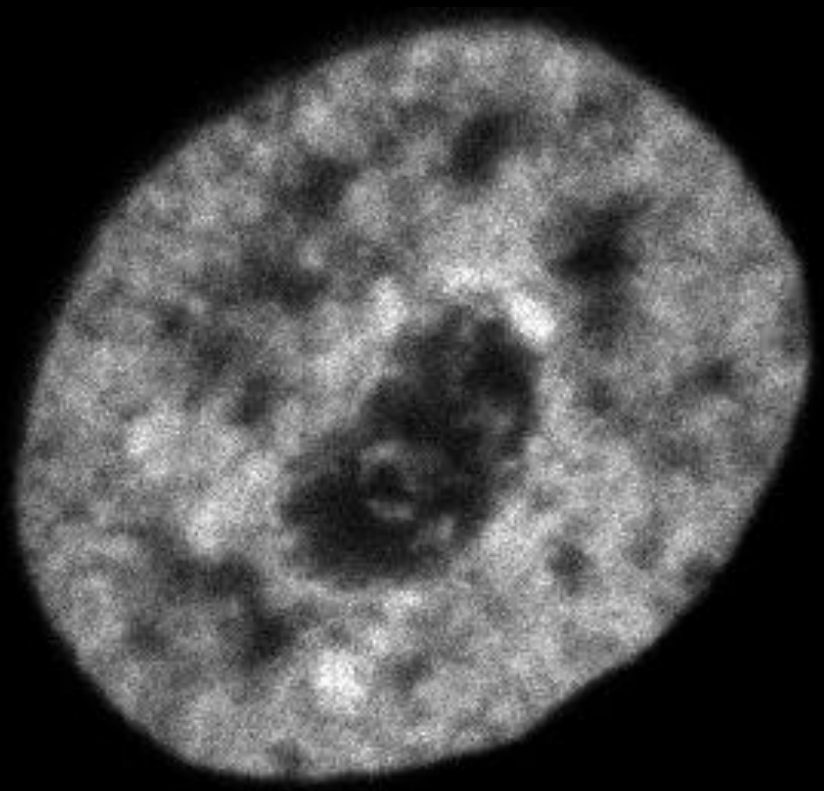
- Theoretically unlimited resolution (in practice limited by technical problems, but 50 nm work).
- Multi-color is possible.
- Not much more complicated than a confocal to use

Disadvantages:

- Depletion laser has very high intensity. This can destroy dyes or living cells.
- Expensive, 1 Mio Euro or more

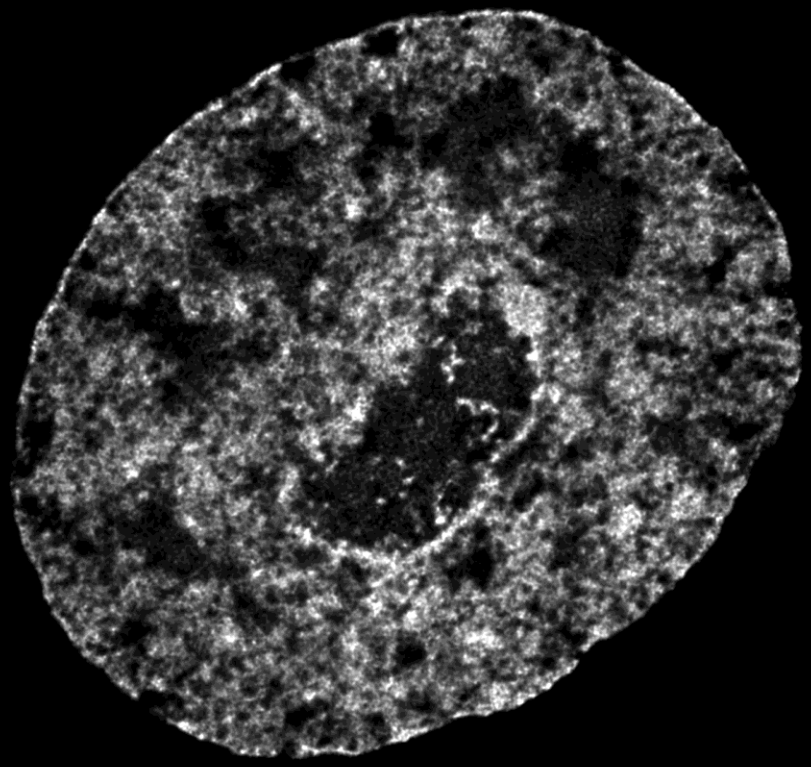
STED-Microscope

HeLa cells - 24hrs 10 µM EdU incorporation



Confocal

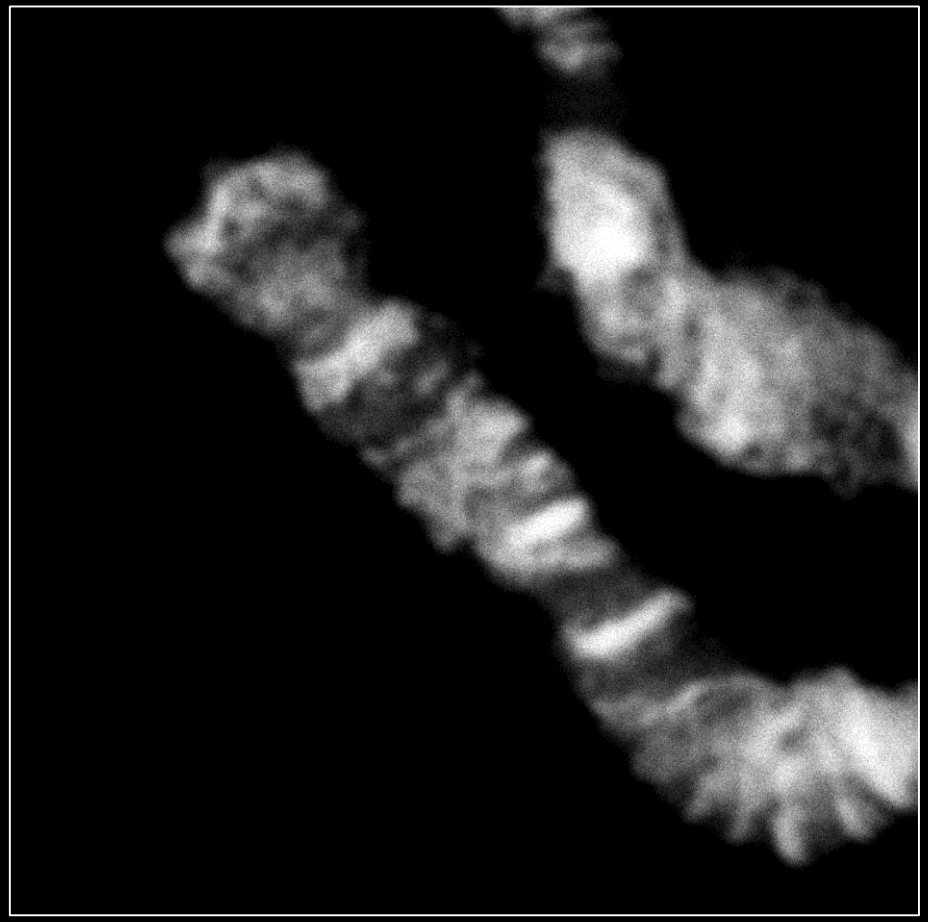
no deconvolution



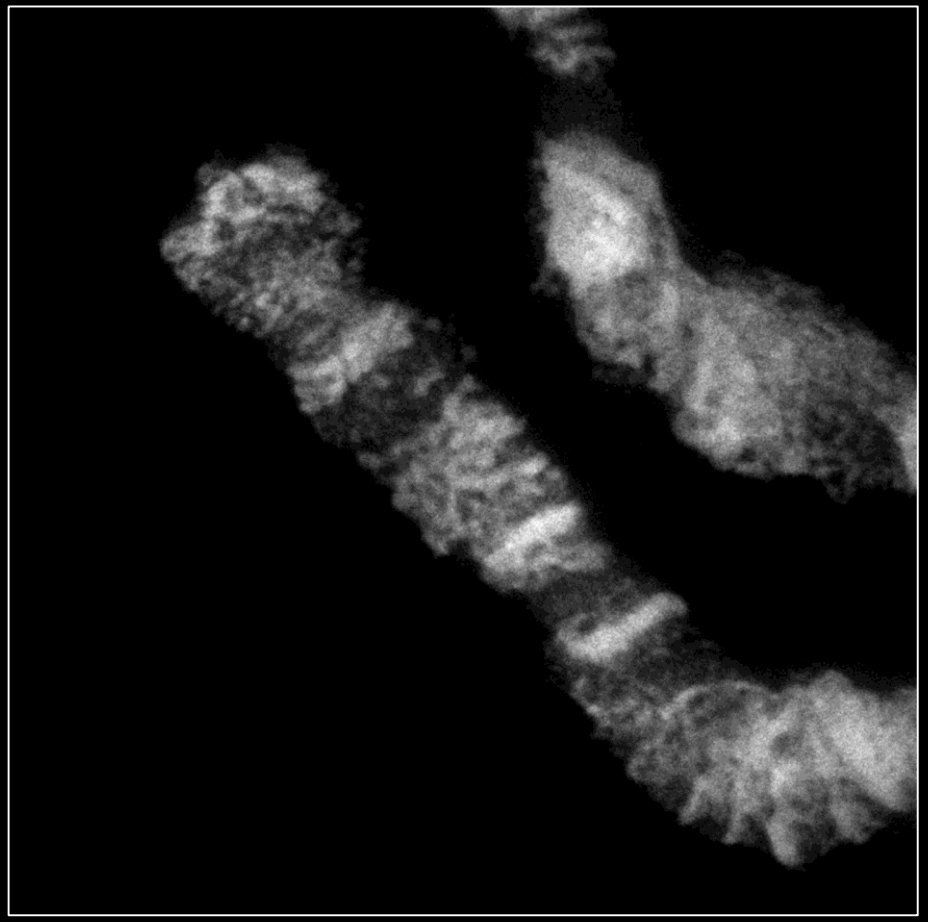
STED

deconvolved

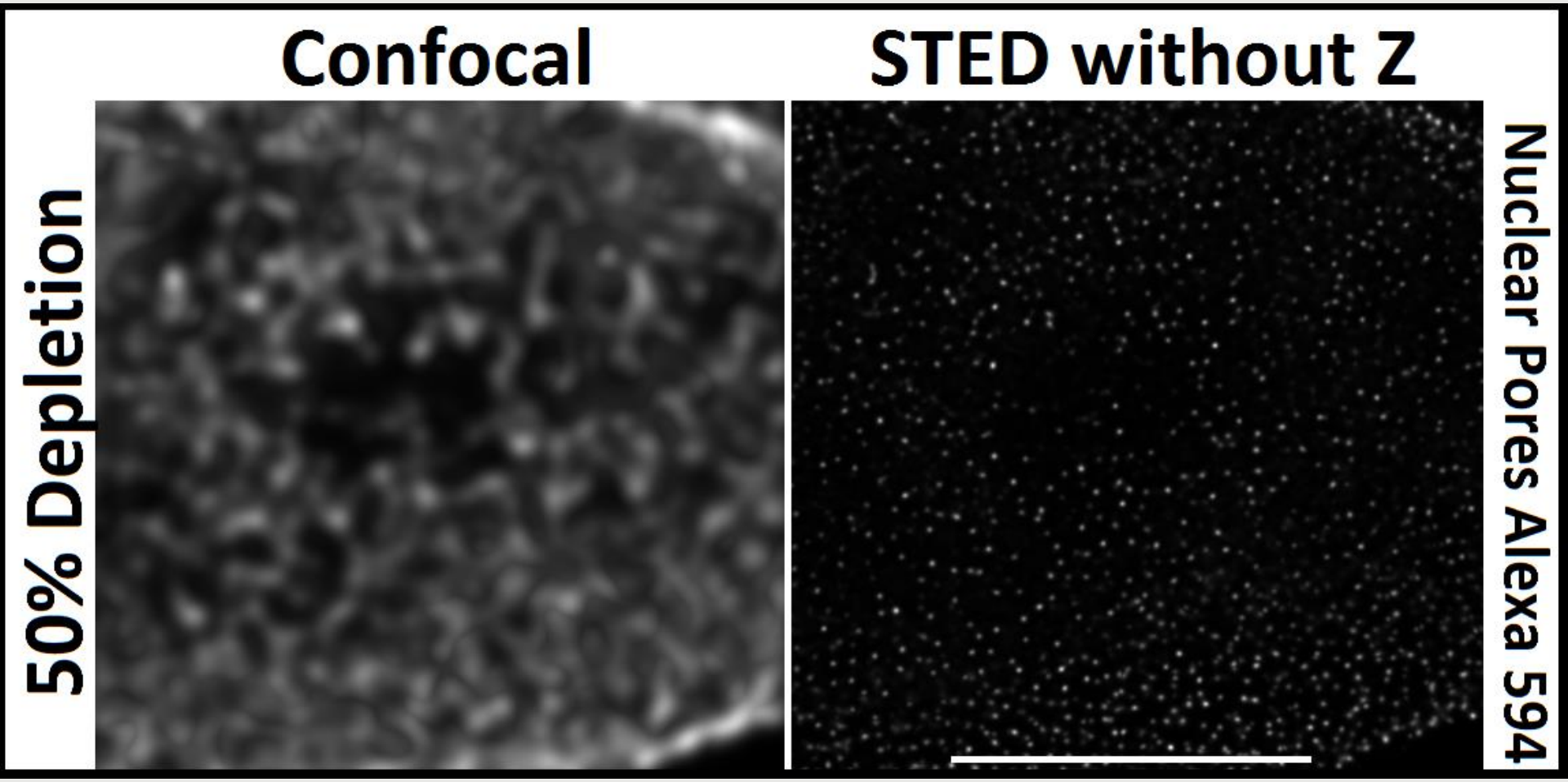
SiR-DNA staining of *Drosophila* Polytene Chromosomes



Confocal

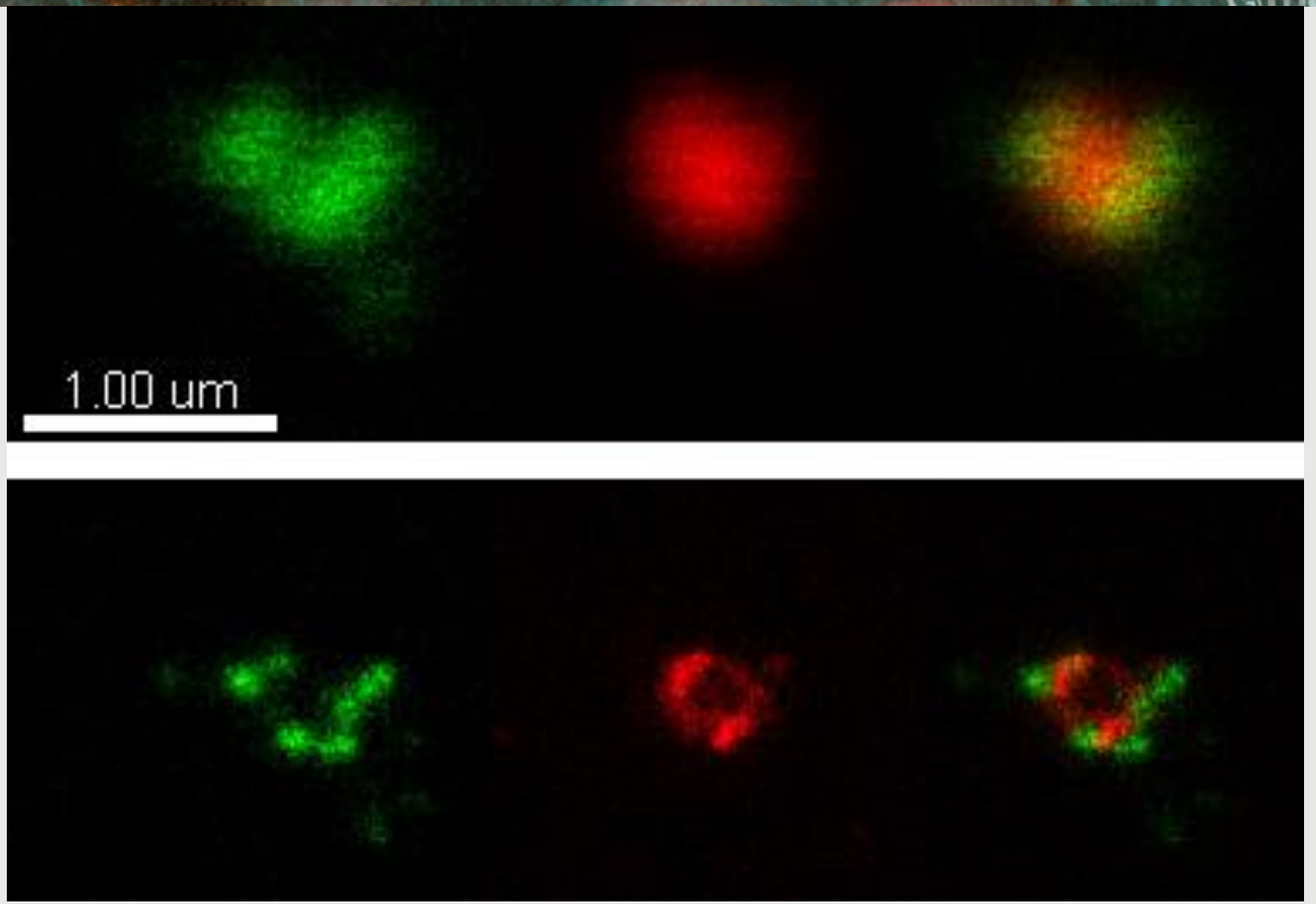


STED


Confocal
STED without Z
50% Depletion
Nuclear Pores Alexa 594

Confocal

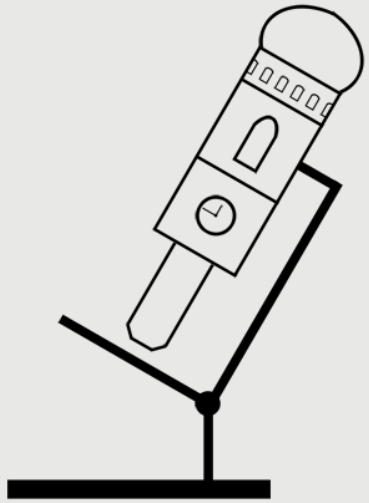
STED



Sample by Germán Camargo Ortega, AG Götz. Centrosomes.
Green: Akna Aberrior 580. Red: ODF3 Aberrior 635P
Single optical sections. No deconvolution.

The team

- Andreas Thomae
- Anna Klemm
- Katarina Wachal



www.bioimaging.bmc.med.lmu.de

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

BIOMEDICAL CENTER
CORE FACILITY BIOIMAGING

Thank you

for your attention. dietzel@lmu.de

Please send any further comments to

dietzel@lmu.de