Light microscopy in Cellular Biology

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WHICH IMAGING TECHNIQUE SHOULD I USE?

WHEN TO USE CONFOCAL?

- Confocal is not a magic bullet
  - It is extremely wasteful of photons
  - Laser-scanning confocal is 100 – 200-fold less sensitive than widefield
  - Spinning-disk confocal is ~4-fold less sensitive than widefield

WAYS TO CIRCUMVENT LIMIT

- Near Field Microscopy (NSOM)
- Far Field Microscopy
  - Confocal, 4pi and I^5M, SIM
- Super-Resolution
  - Spatially Patterned Excitation
    - STED
    - RESOLFT
    - SSIM
  - Localization Methods
    - STORM
    - PALM
    - FPALM

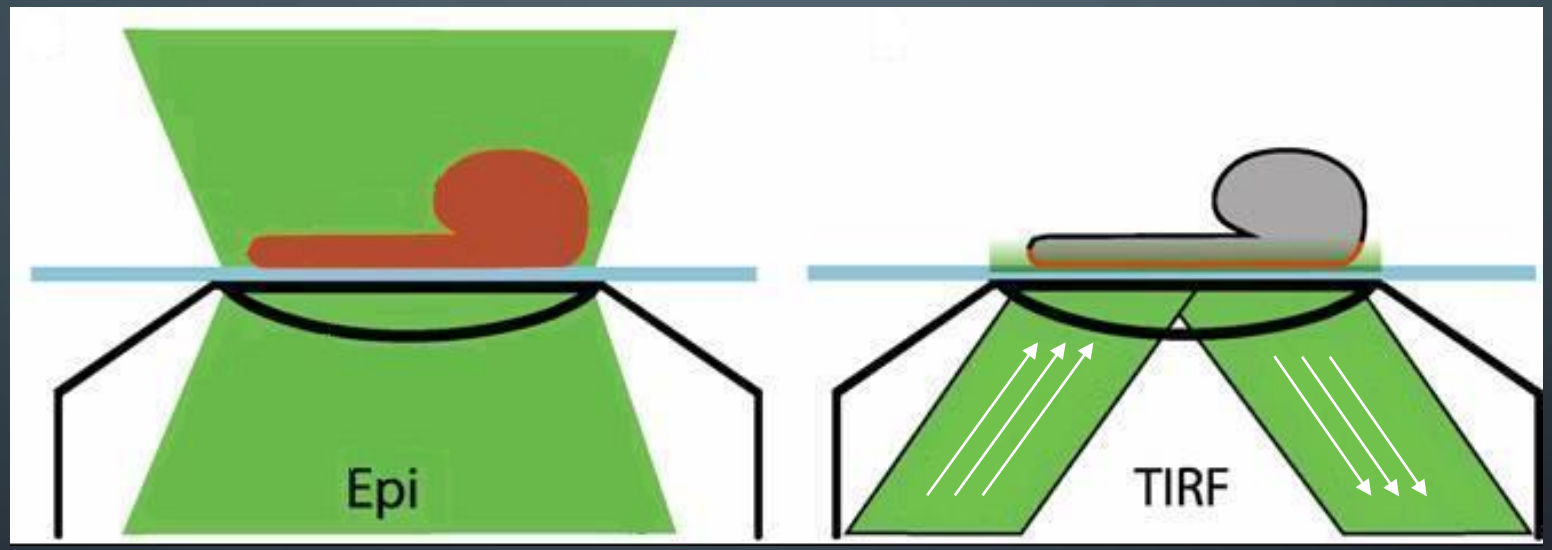
NEAR FIELD IMAGING (NSOM)

-place microscope distance less than 1 wavelength from sample

-20-50 nm resolution

problem: cannot image into sample because of wavelength restriction

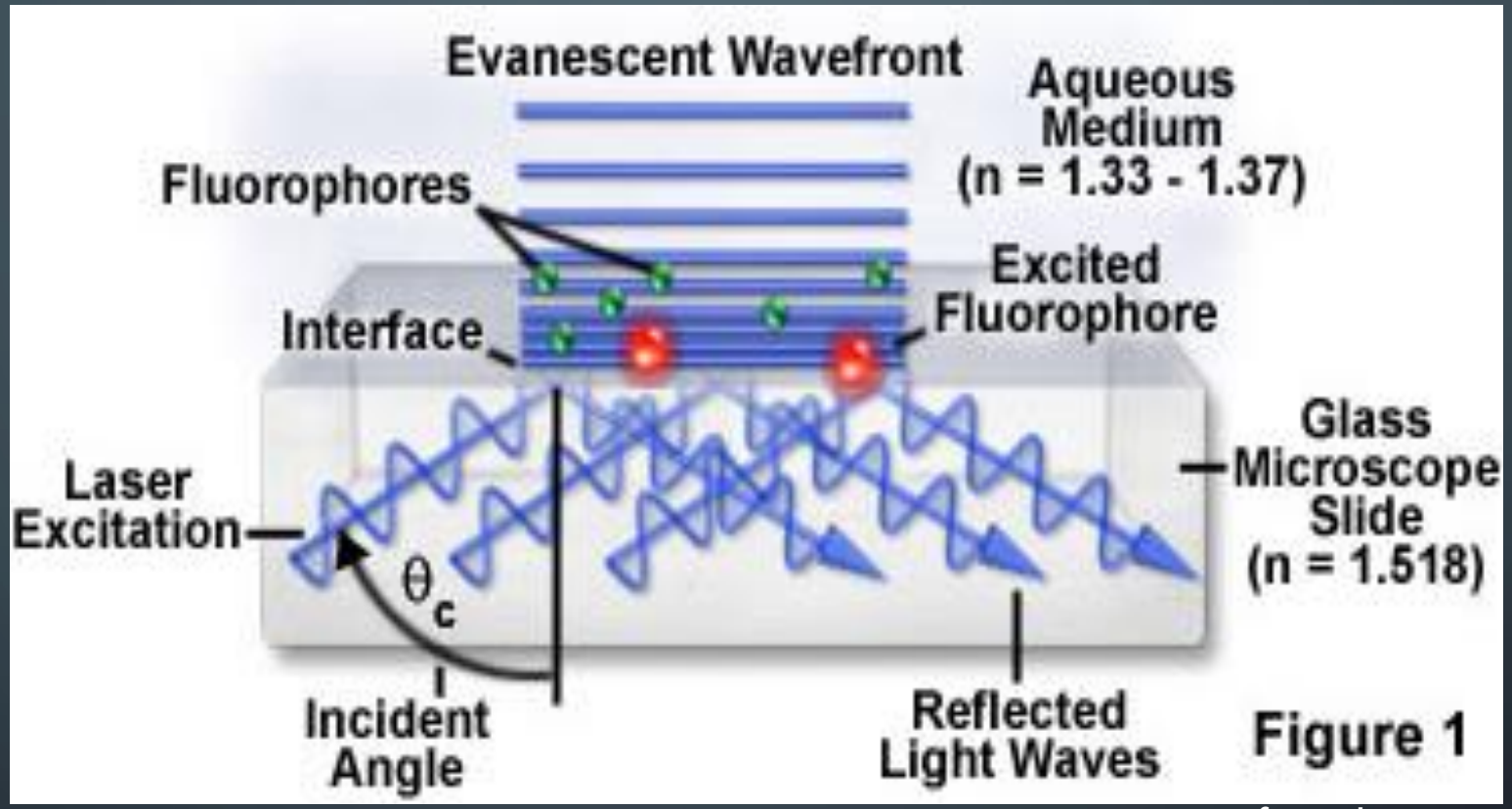
TIRF (TOTAL INTERNAL REFLECTION FLUORESCENCE)



You need:

- TIRF objectives with high NA
- TIRF condensor, where you are able to change the angle of illumination
- Glass coverslips

TIRF



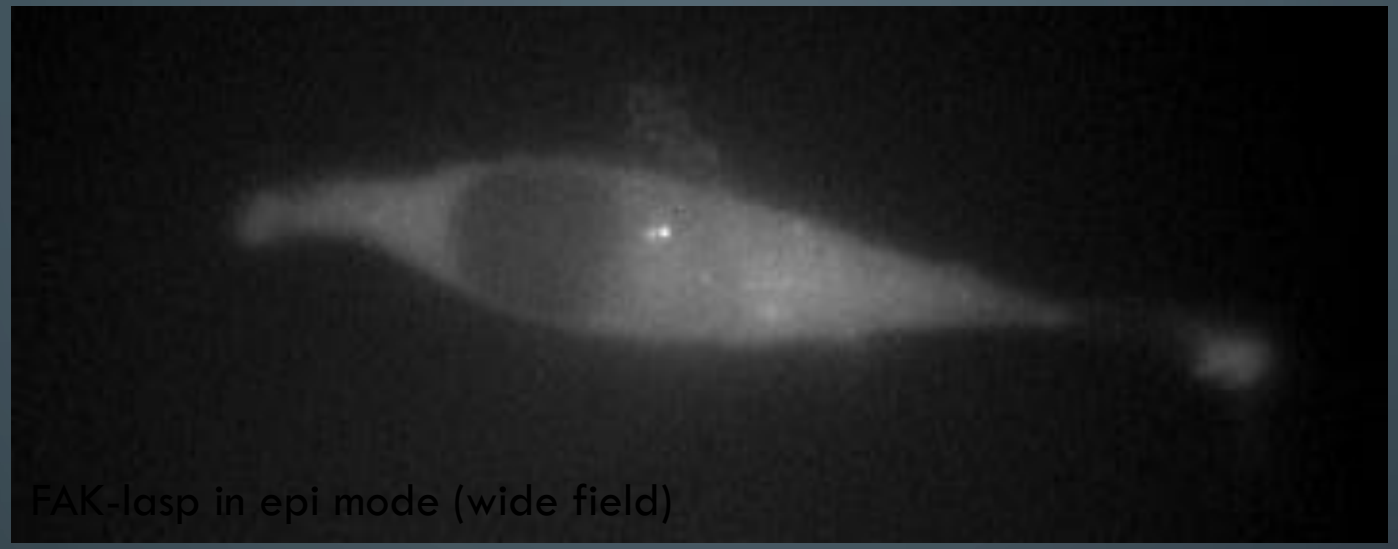
micro.magnet.fsu.edu

Result: very thin section at the bottom of the sample → 150-200nm

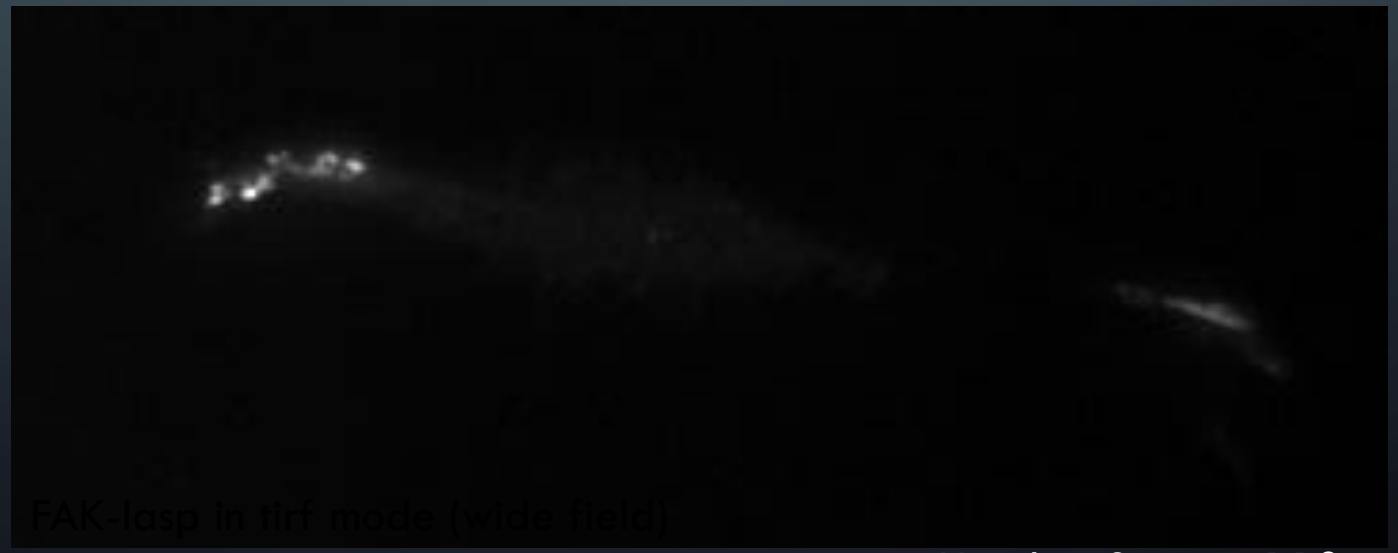
Use: to study membrane dynamics (endocytosis, focal adhesions, receptor binding)

→ *Nikon TE 2000*

TIRF VS EPI



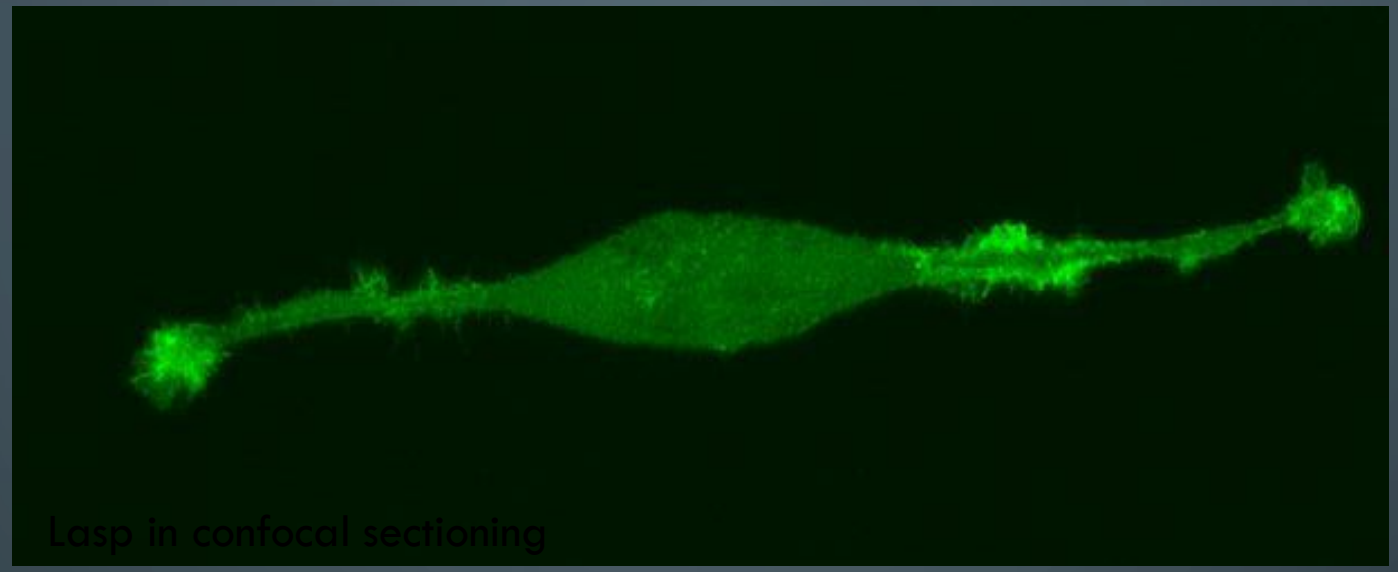
FAK-lasp in epi mode (wide field)

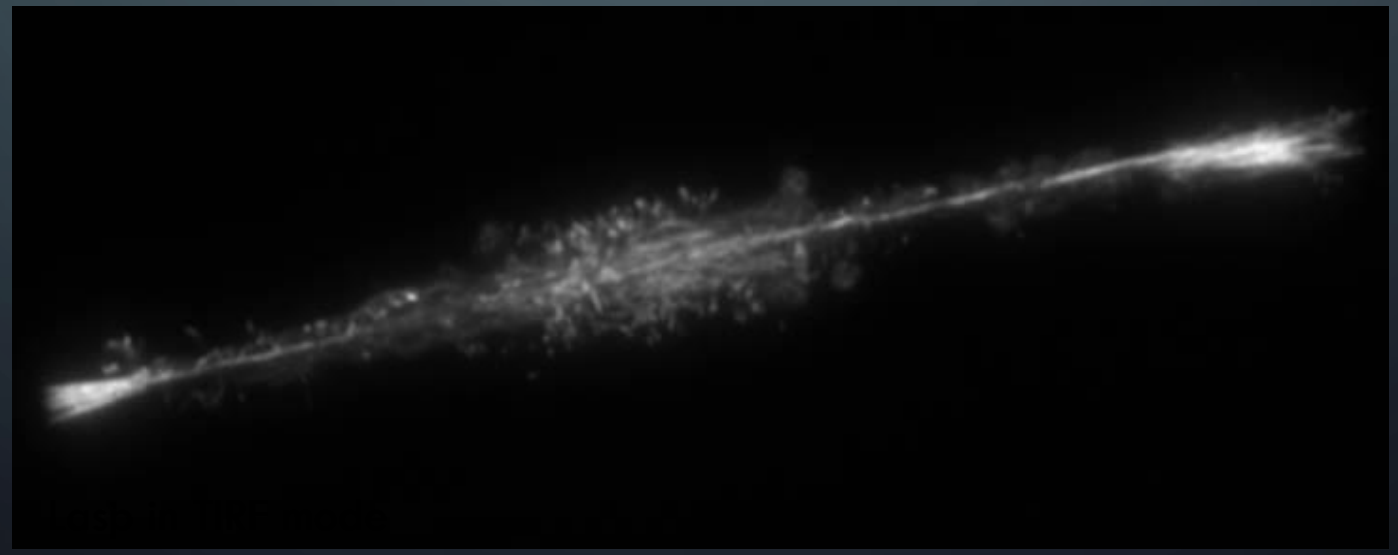


FAK-lasp in tirf mode (wide field)

Heather Spence, R10

TIRF VS EPI



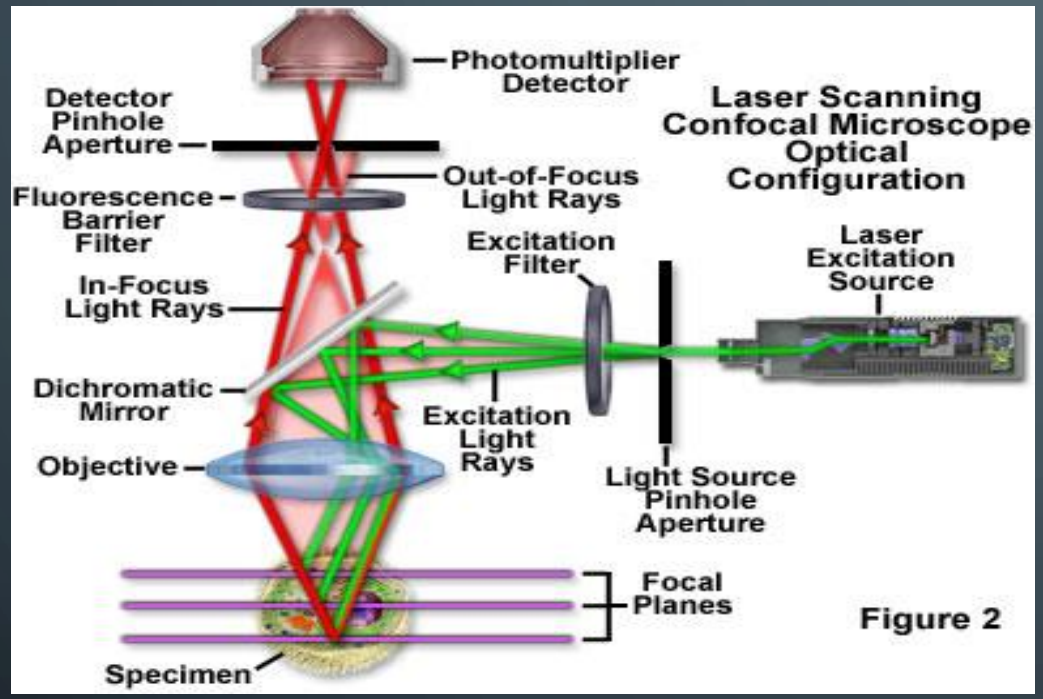


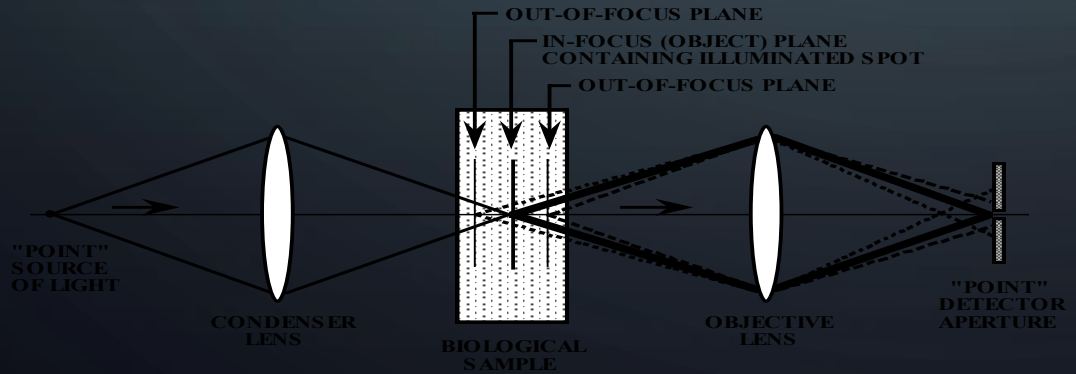
Heather Spence, R10

SUMMARY/COMPARISON

method	excitation	detection	sectioning	use
Wide field	Whole sample	Whole sample	No sectioning	Simple fluorescence samples
confocal	Whole sample	One z-plane	350-500nm	High contrast images, optical sectioning
2-Photon	One z-plane	One z-plane	500-700nm	Deep tissue imaging, optical sectioning
FLIM/FRET				Protein interactions
FRAP + photoactivation	405 laser (UV)			dynamics/mobility
TIRF	Only bottom plane	Only bottom plane	150-200nm	Membrane dynamics

PRINCIPLE OF CONFOCAL MICROSCOPY





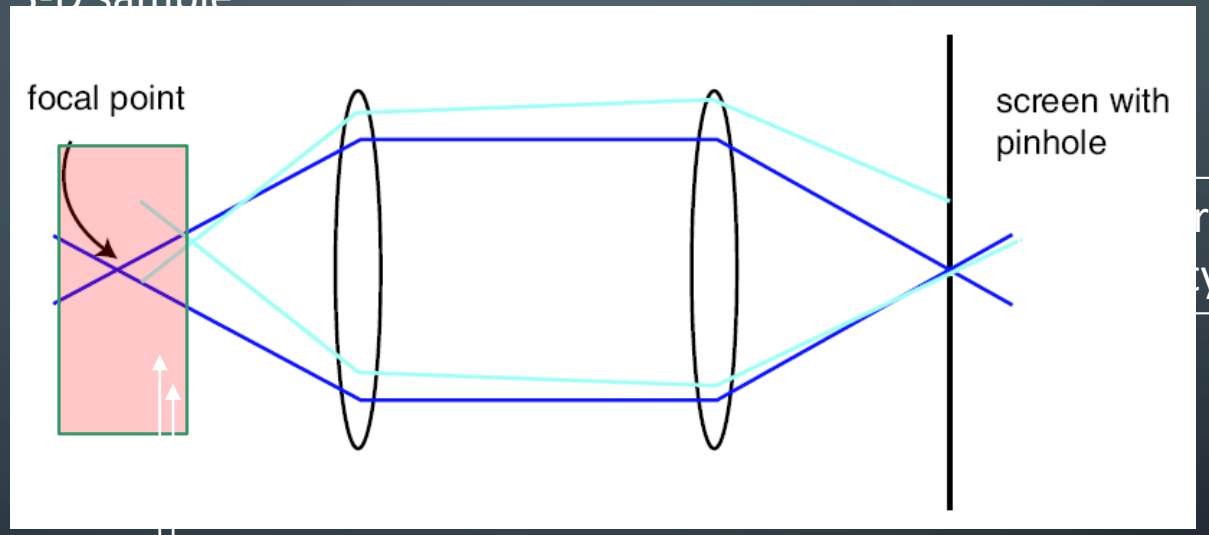
In confocal microscopy two pinholes are typically used:

- A pinhole is placed in front of the illumination source to allow transmission only through a small area
- This illumination pinhole is imaged onto the focal plane of the specimen, i.e. only a point of the specimen is illuminated at one time
- Fluorescence excited in this manner at the focal plane is imaged onto a confocal pinhole placed right in front of the detector
- Only fluorescence excited within the focal plane of the specimen will go through the detector pinhole
- Need to scan point onto the sample

Confocal Detection

SAMPLE IS 3-D. DETECTORS ARE 2-D. HOW DO YOU GET Z-AXIS SECTIONING WITH MICROSCOPY?

A PINHOLE ALLOWS ONLY IN-FOCUS LIGHT THROUGH

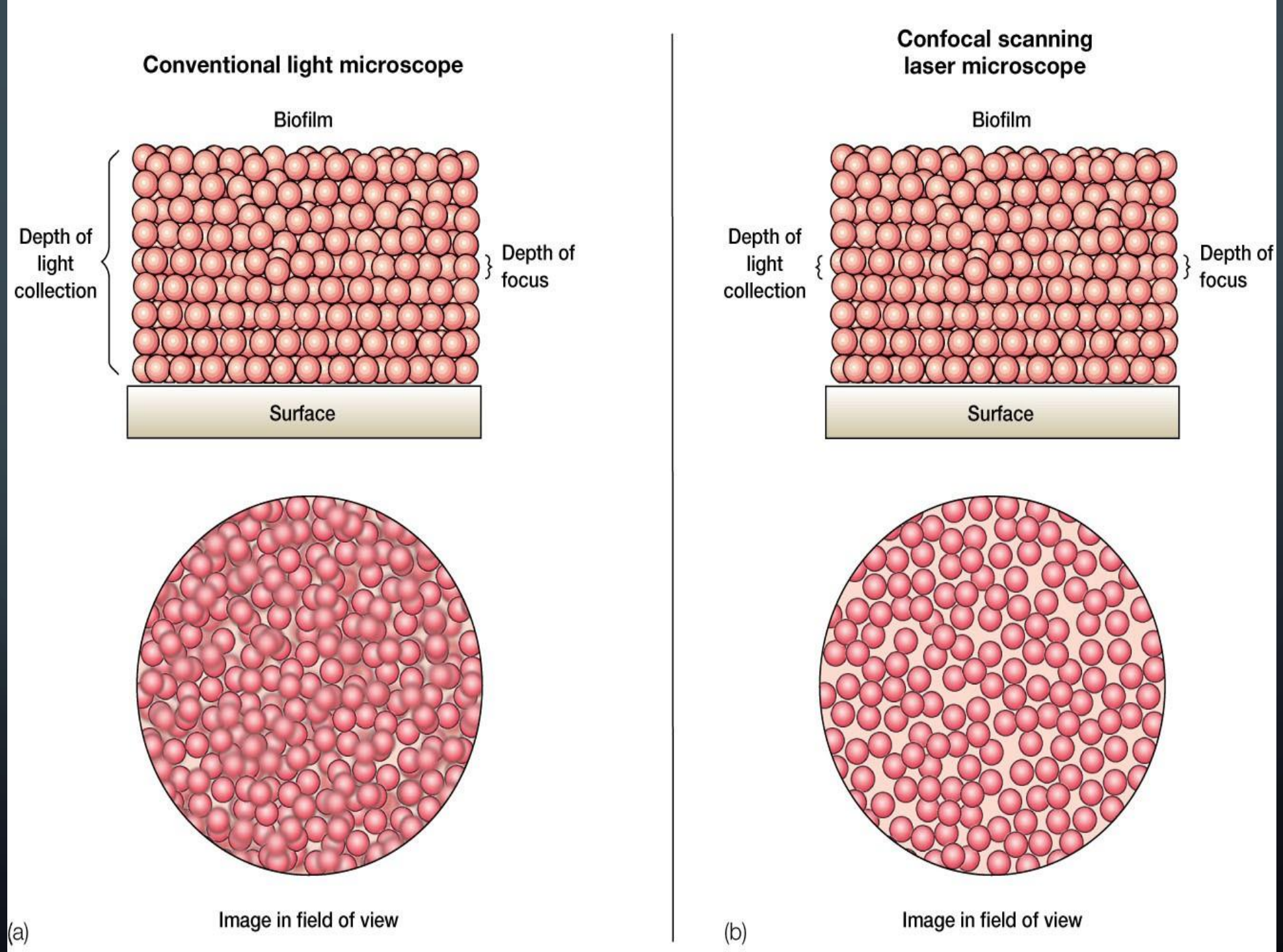


Focused Light creates fluorescence which gets to detector

Light mostly gets rejected

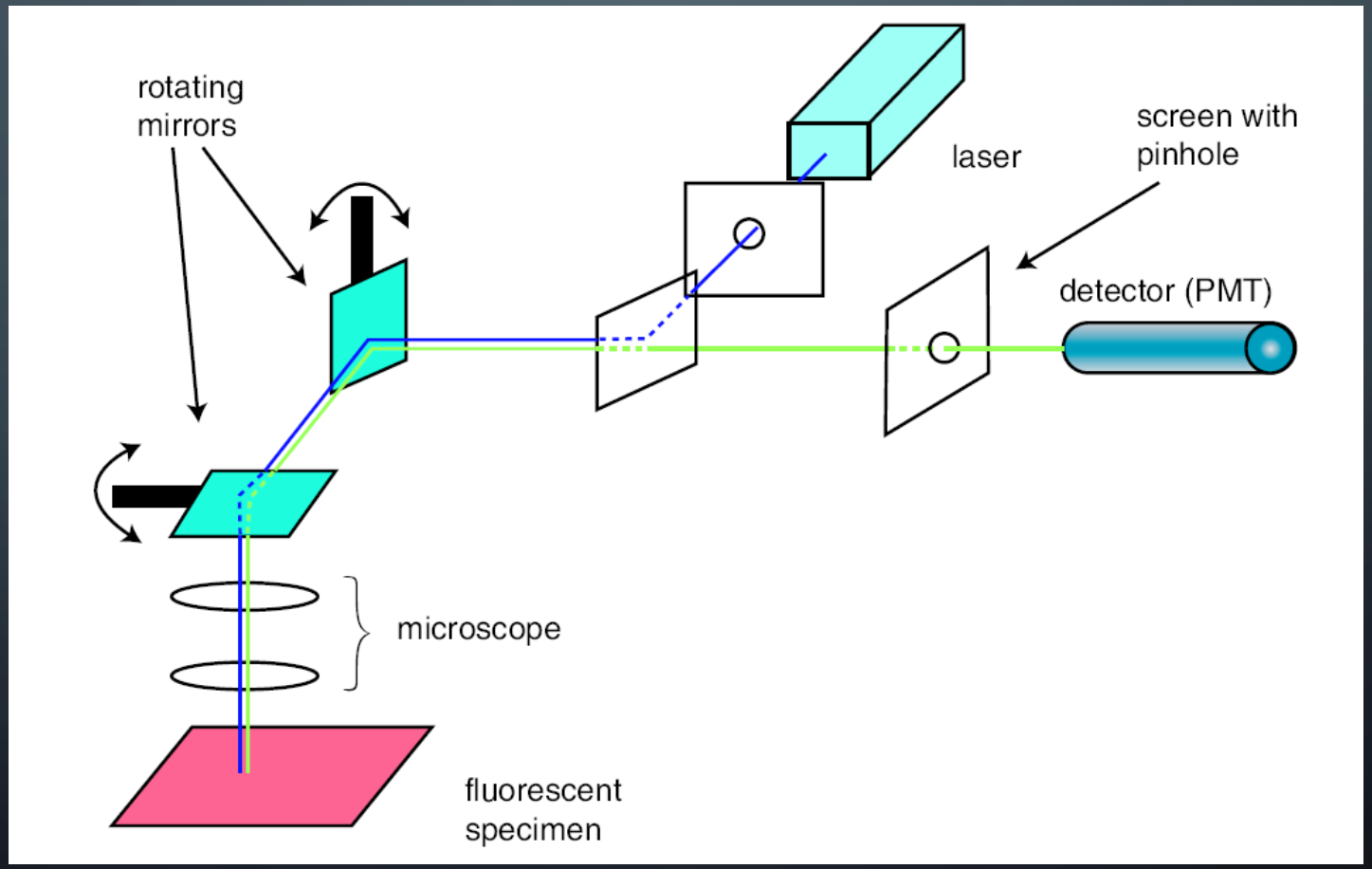
Smaller the pinhole, better out-of-focus discrimination but lose more signal.

Scan sample in x, y, z and reconstruct entire image

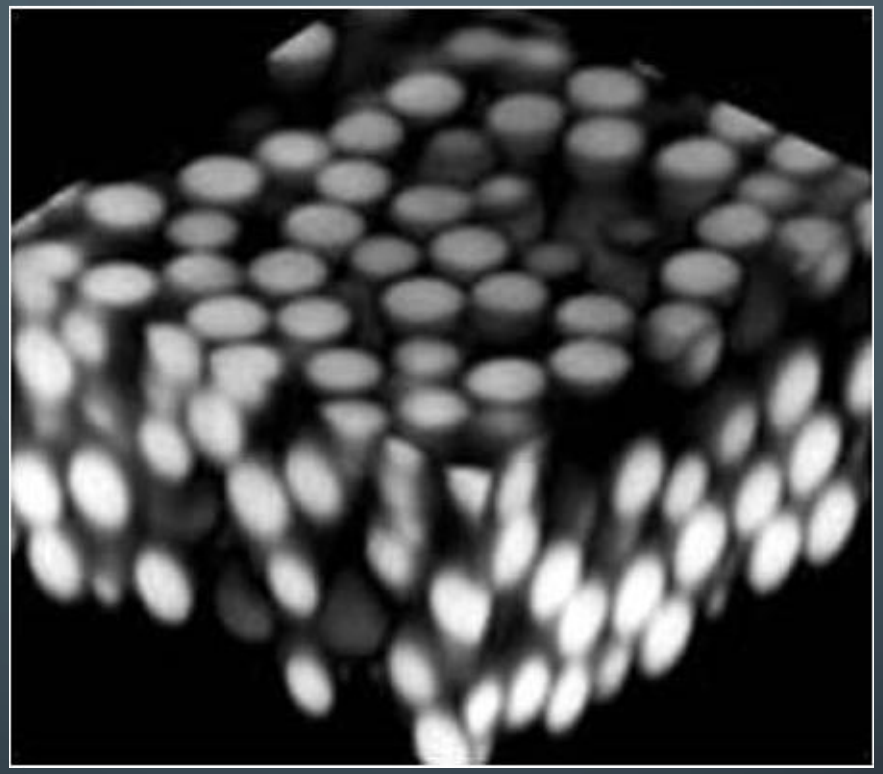

Conventional light microscope
Biofilm
Depth of light collection
Depth of focus
Surface
Image in field of view
(a)
Confocal scanning laser microscope
Biofilm
Depth of light collection
Depth of focus
Surface
Image in field of view
(b)

CONFOCAL MICROSCOPY

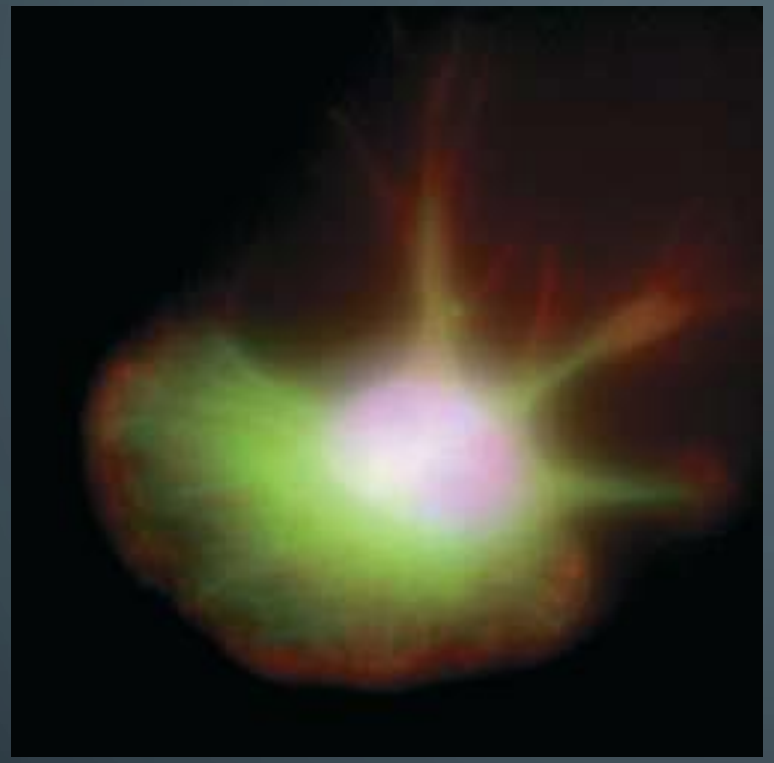
LOTS OF DIFFERENT WAYS OF ARRANGING TO GET FAST SCANNING: MOVEABLE MIRRORS (ONLY HAVE TO MOVE SAMPLE IN Z-DIRECTION, NIPOW DISK….

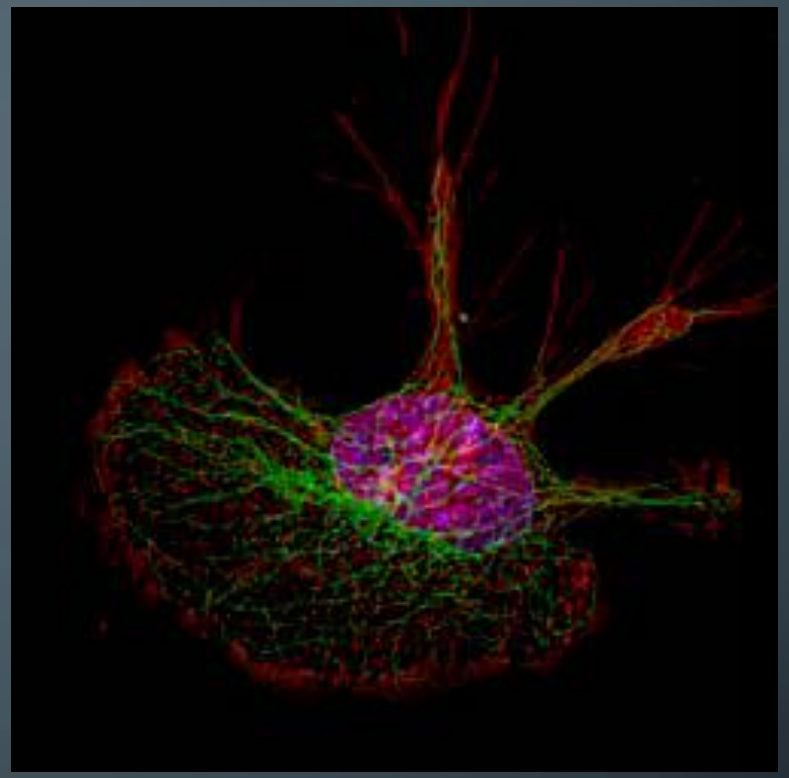


3-D SECTIONING WITH CONFOCAL



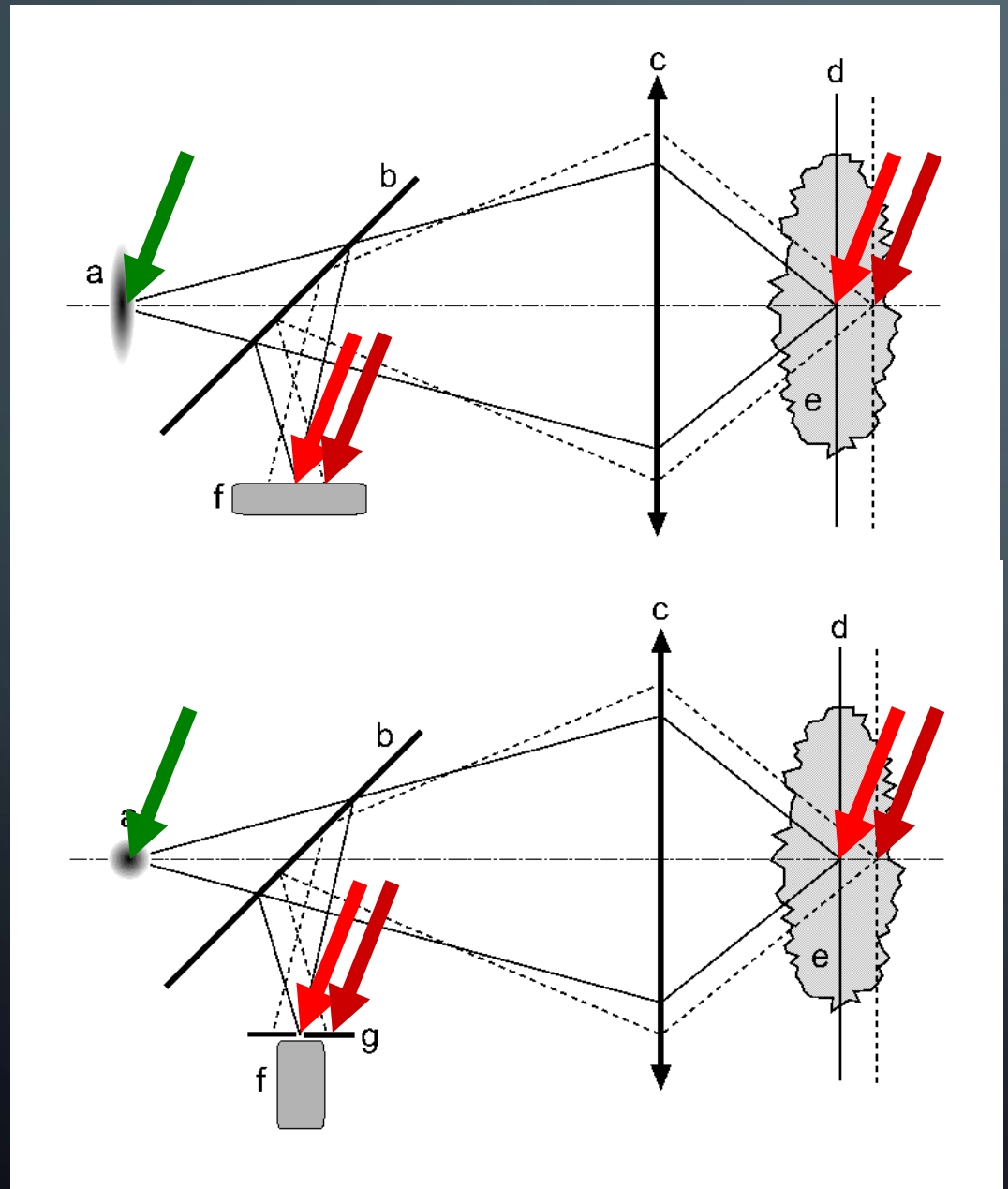
Three-dimensional reconstruction of a series of 2D images of PMMA spheres





A macrophage cell was stained with fluorochrome-labeled reagents specific for DNA (blue), microtubules (green), and actin microfilaments (red). The series of fluorescent images obtained at consecutive focal planes (optical sections) through the cell were recombined in three dimensions.
(a) In this three-dimensional reconstruction of the raw images, the DNA, microtubules, and actin appear as diffuse zones in the cell. (b) After application of the deconvolution algorithm to the
images, the fibrillar organization of microtubules and the localization of actin to adhesions become readily visible in the reconstruction.

WIDE-FIELD VS. CONFOCAL MICROSCOPY



a - light source

b - dichroic filter

c - objective lens

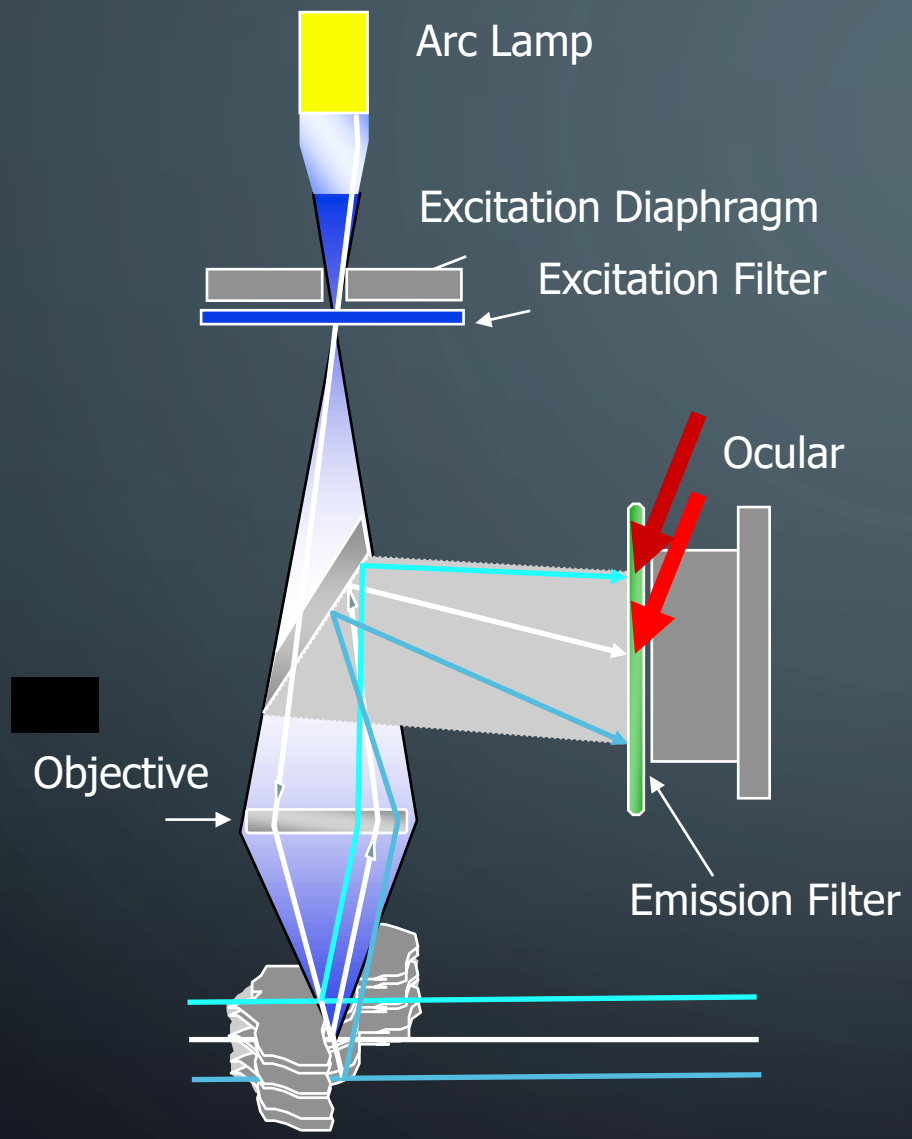
d - focal plane

e - specimen

f - light detector

g - confocal aperture

Fluorescence Microscope



Confocal Fluorescence Microscope

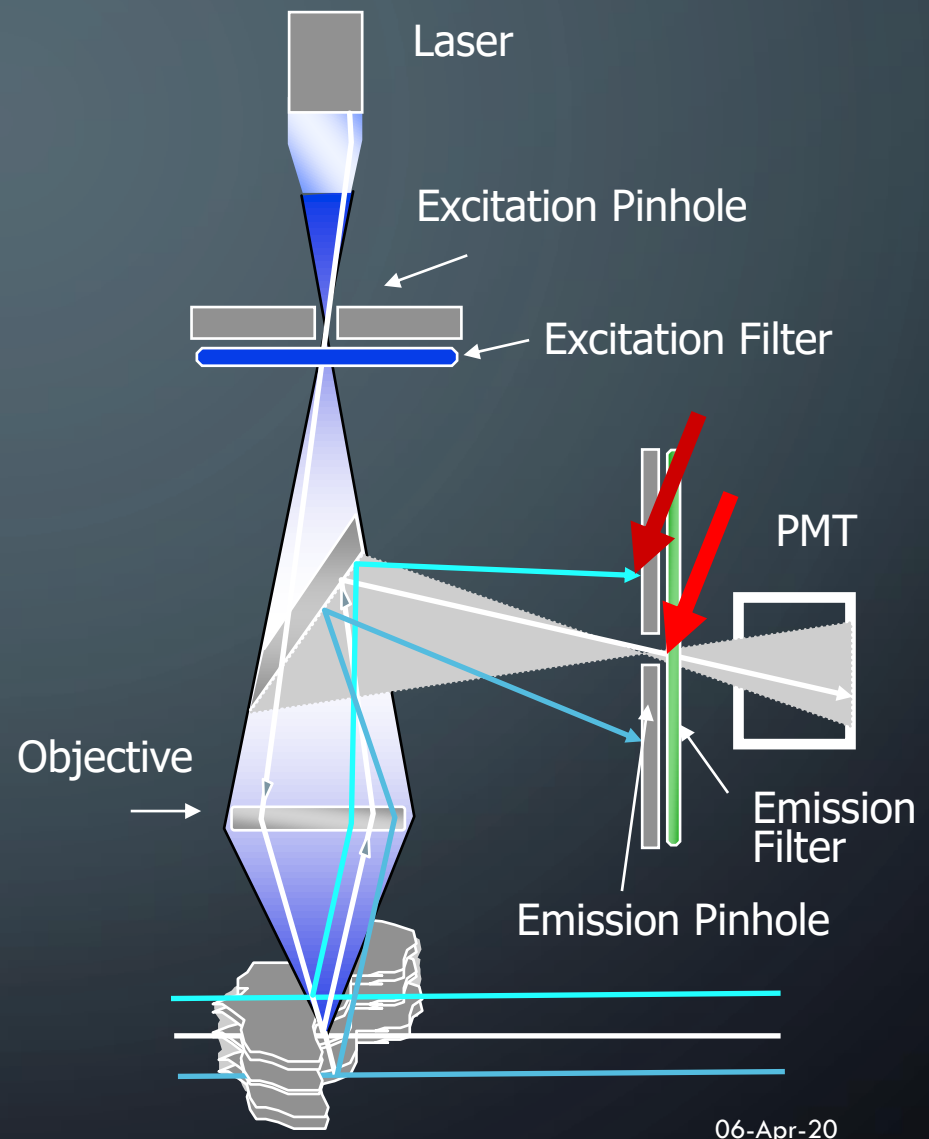
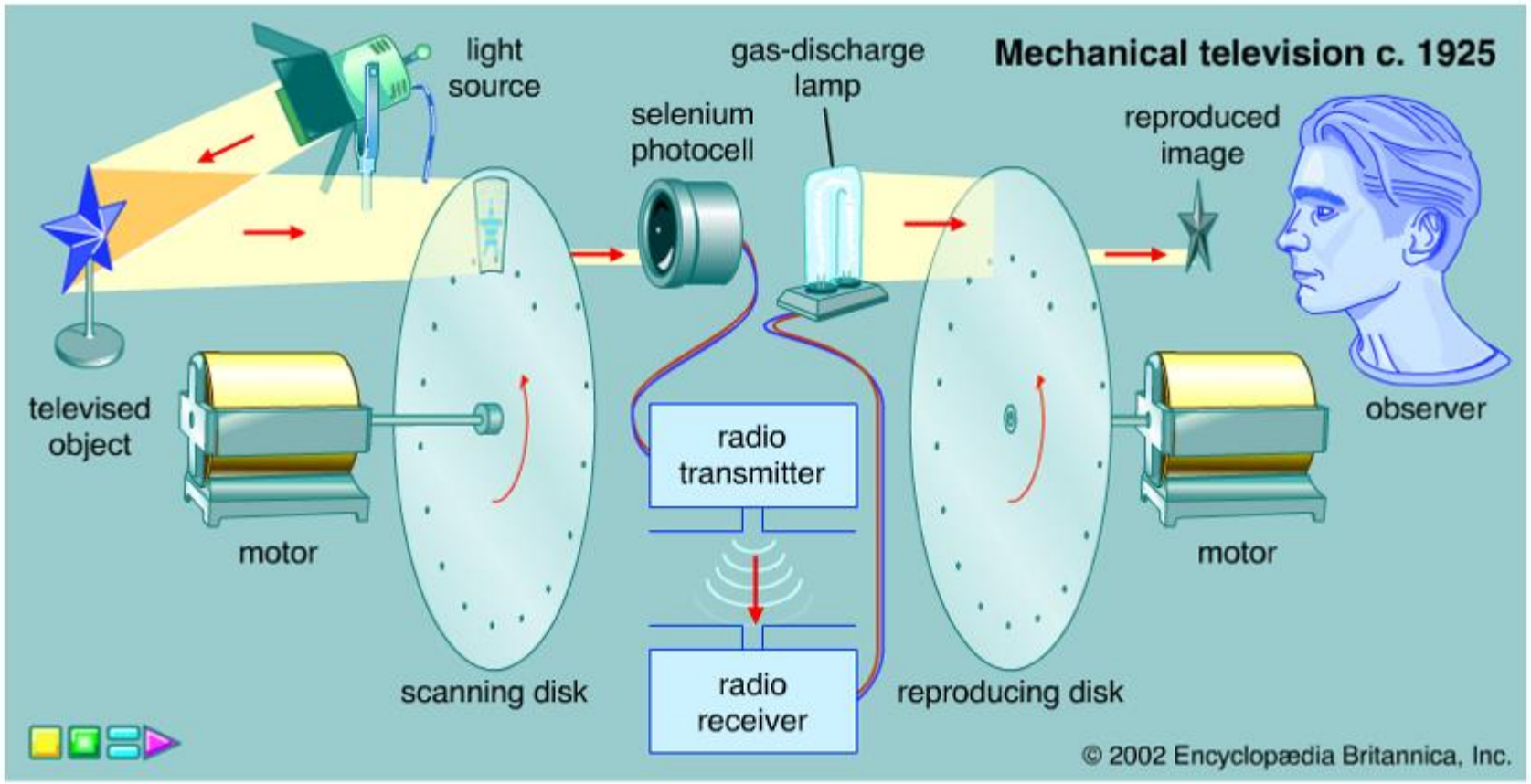
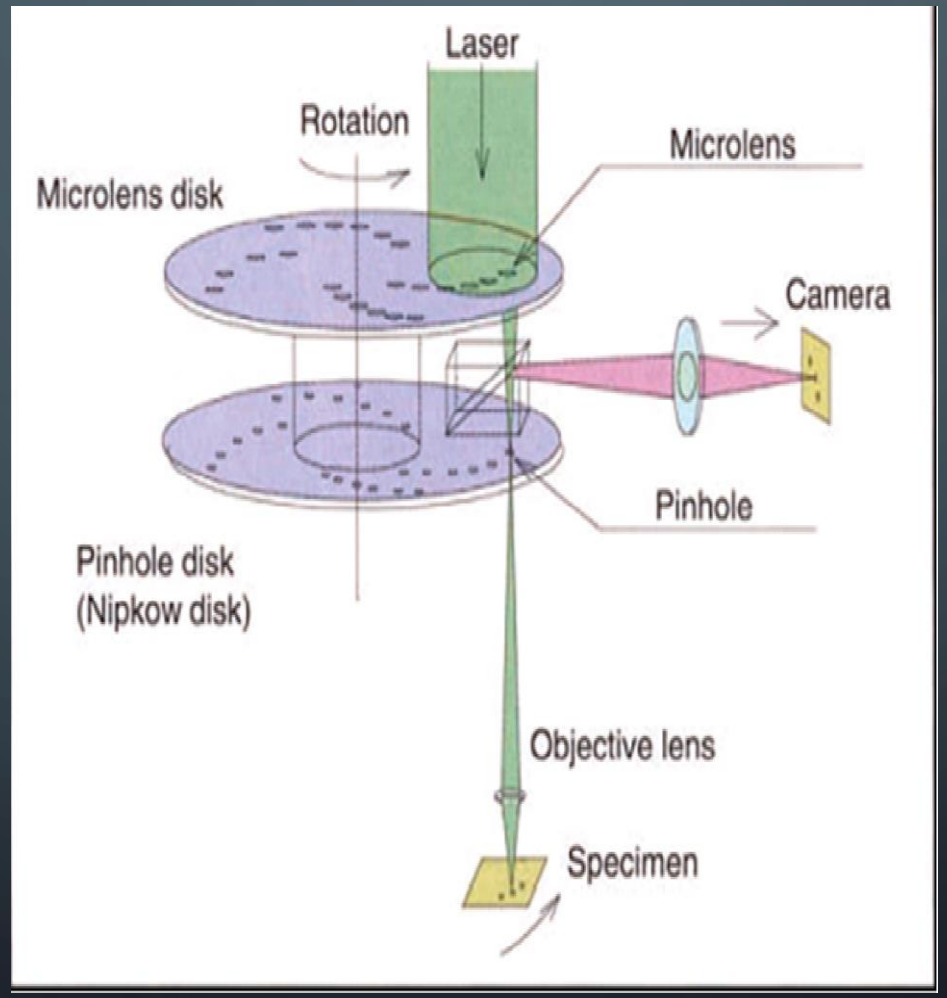


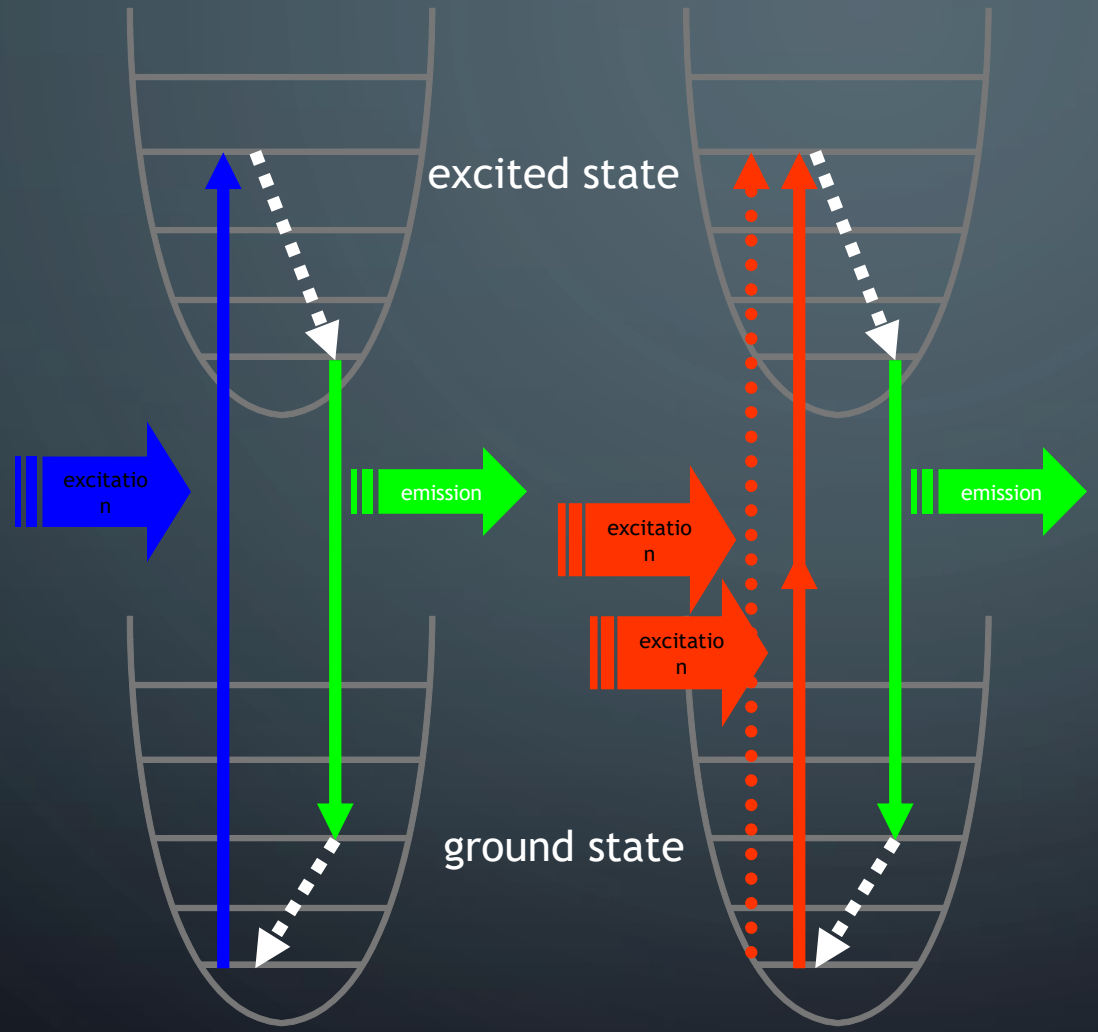
IMAGE FORMATION – NIPKOW DISK (MECHANICAL TV)



Spinning Disk Confocal

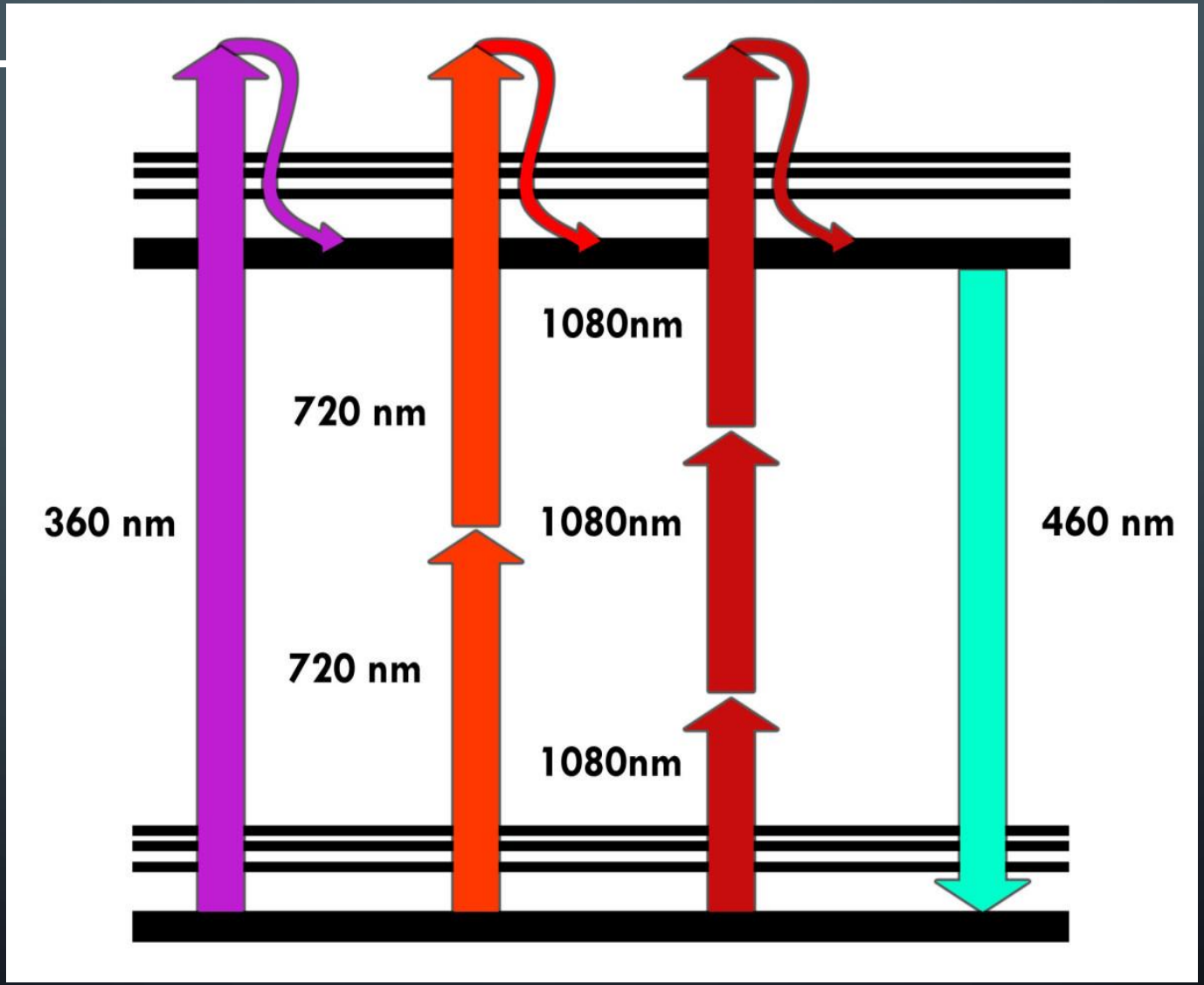


2-PHOTON EXCITATION



- Two-photon excitation occurs through the absorption of two lower energy photons via short-lived intermediate states.
- After either excitation process, the fluorophore relaxes to the lowest energy level of the first excited electronic states via vibrational processes.
- The subsequent fluorescence emission processes for both relaxation modes are the same.

FROM 2-



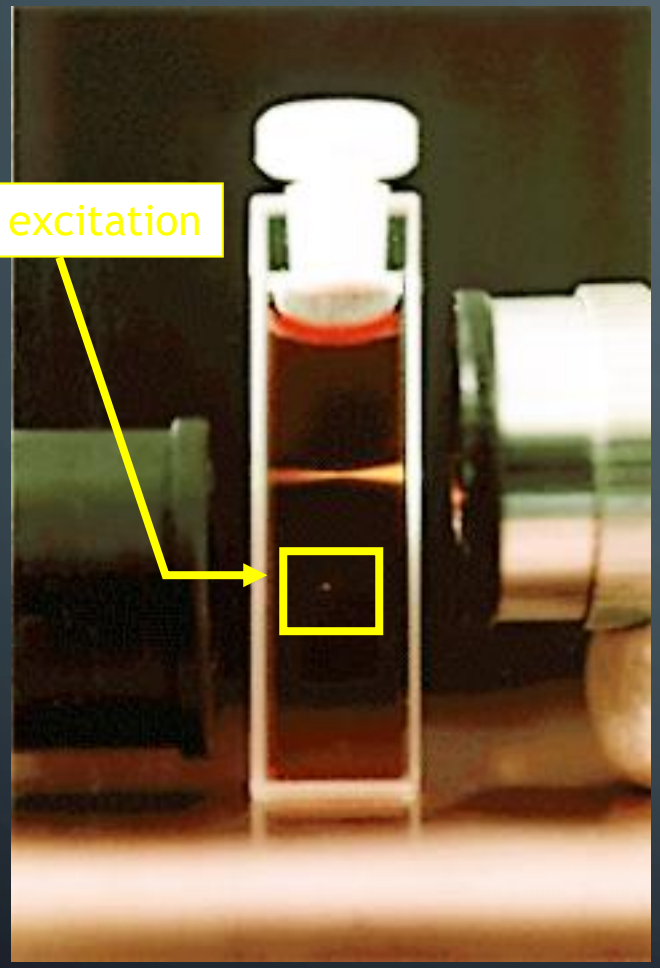
FAR FIELD: CONFOCAL MICROSCOPY

- Non-linear 2-photon excitation and pinhole detection decrease SPF beyond classical limits
- $2^{1/2}$ improvement in resolution

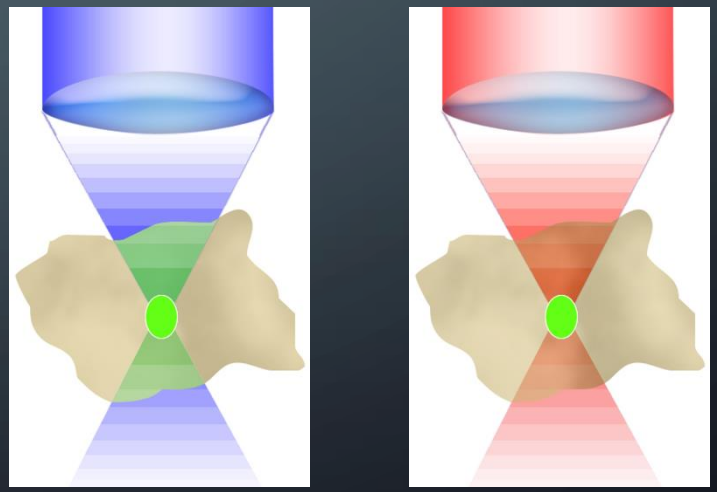
problem: 2-photon excitation uses high wavelengths which increase SPF:

$$\Delta_{x\text{-}y} = (0.61\ \lambda)/(\eta\ \sin(\alpha))$$

DEMONSTRATION OF THE DIFFERENCE BETWEEN SINGLE- AND TWO-PHOTON EXCITATION

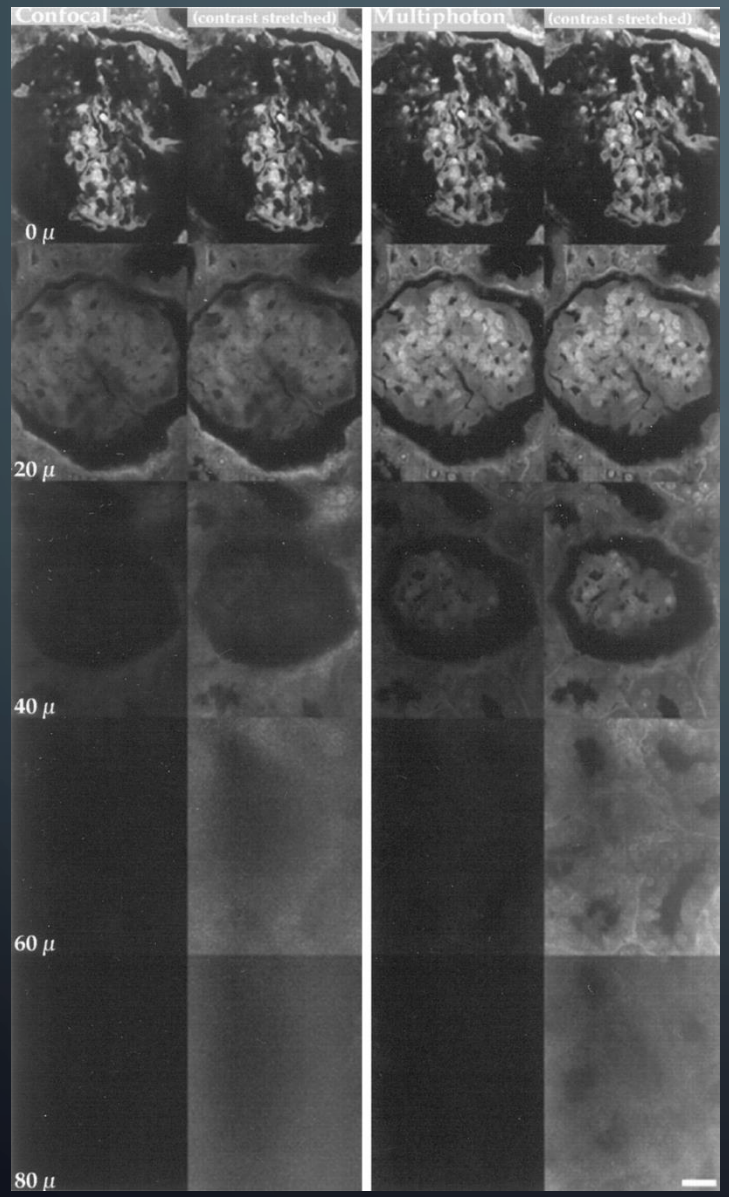


The cuvette is filled with a solution of a dye, safranin O, which normally requires green light for excitation. Green light (543 nm) from a continuous-wave helium-neon laser is focused into the cuvette by the lens at upper right. It shows the expected pattern of a continuous cone, brightest near the focus and attenuated to the left. The lens at the lower left focuses an invisible 1046-nm infrared beam from a mode-locked Nd-doped yttrium lanthanum fluoride laser into the cuvette. Because of the two-photon absorption, excitation is confined to a tiny bright spot in the middle of the cuvette.



Slide credit: Brad Amos, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

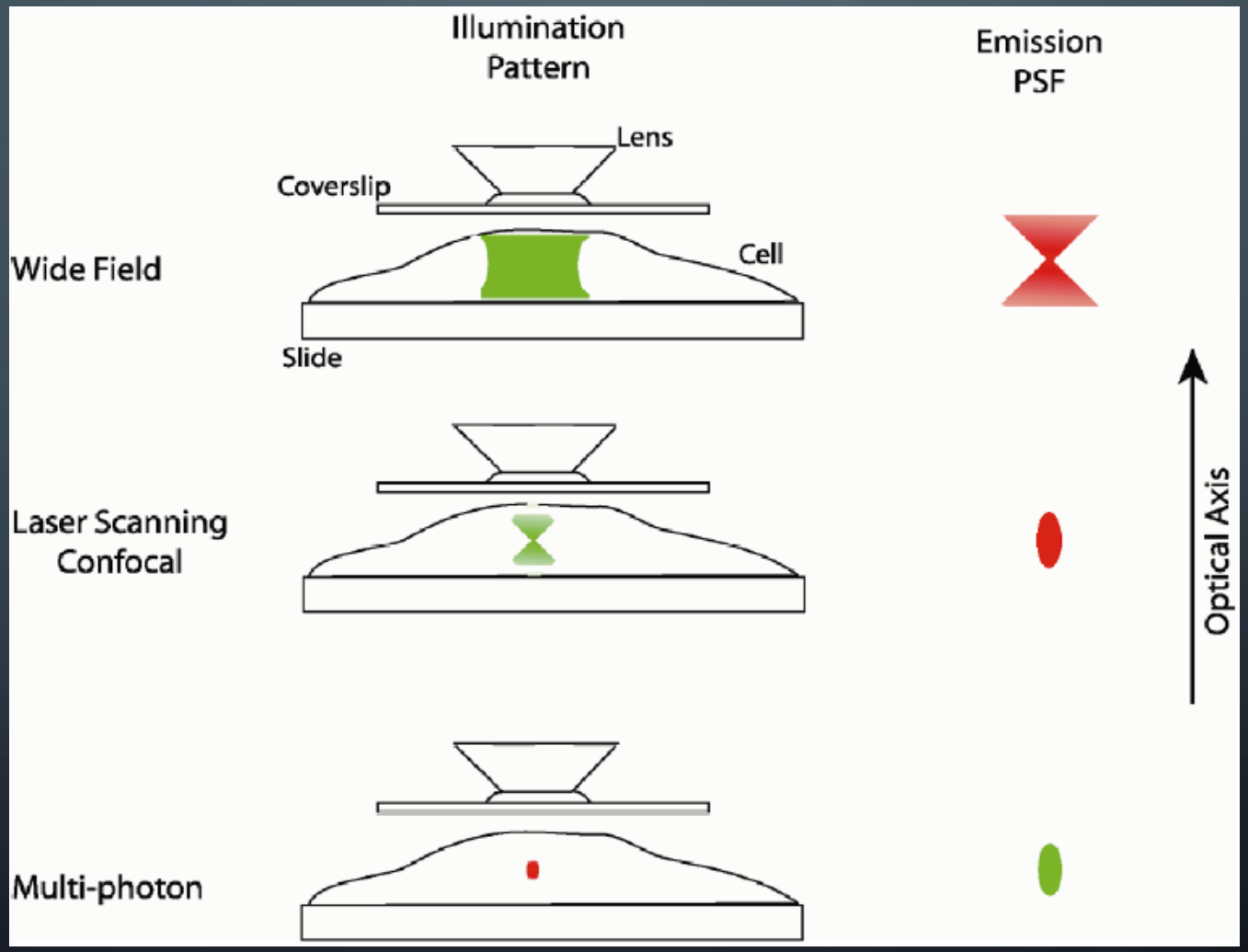
PENETRATION DEPTH



Comparison of imaging penetration depth between confocal and multiphoton microscopy. Optical sections through a glomerulus from an acid-fucsin-stained monkey kidney pathology sample imaged by confocal microscopy with 2 μW of 532-nm light (*left, columns 1 and 2*) and multiphoton microscopy with 4.3 mW of 1047-nm light (descanned; *right, columns 3 and 4*) were compared. At the surface, the image quality and signal intensity are similar; however, at increasing depth into the sample, signal intensity and quality of the confocal image falls off more rapidly than the multiphoton image. Images were collected at a pixel resolution of 0.27 μm with a Kalman 3 collection filter. Scale bar, 20 μm.

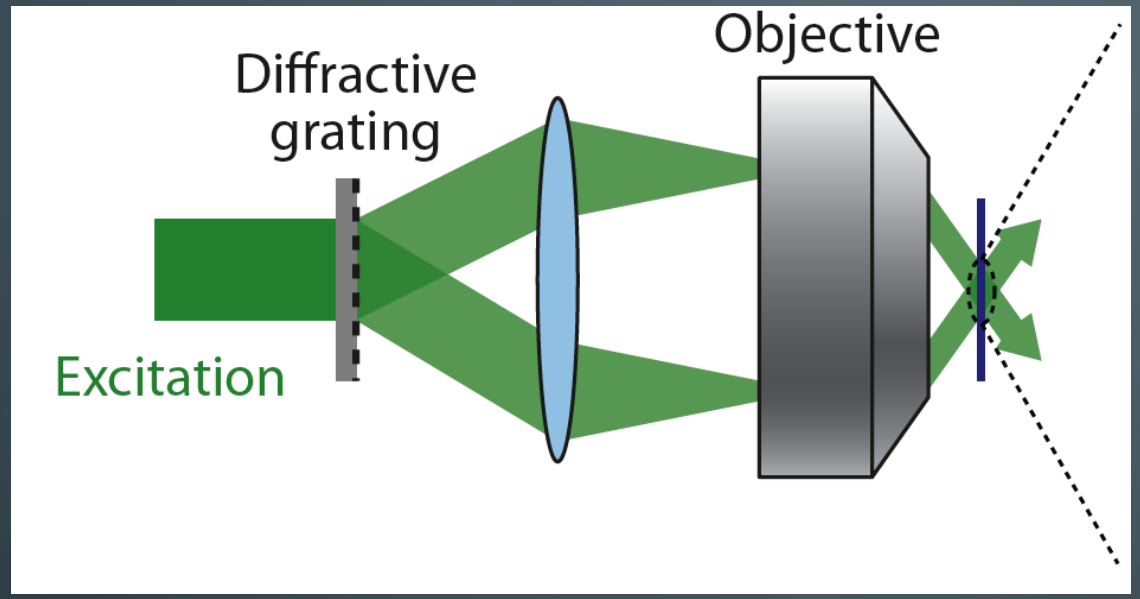
Centonze VE, White JG. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. Biophys J. 1998 Oct;75(4):2015-24.

WIDE-FIELD VS. CONFOCAL VS. 2-PHOTON



Drawing by P. D. Andrews, I. S. Harper and J. R. Swedlow

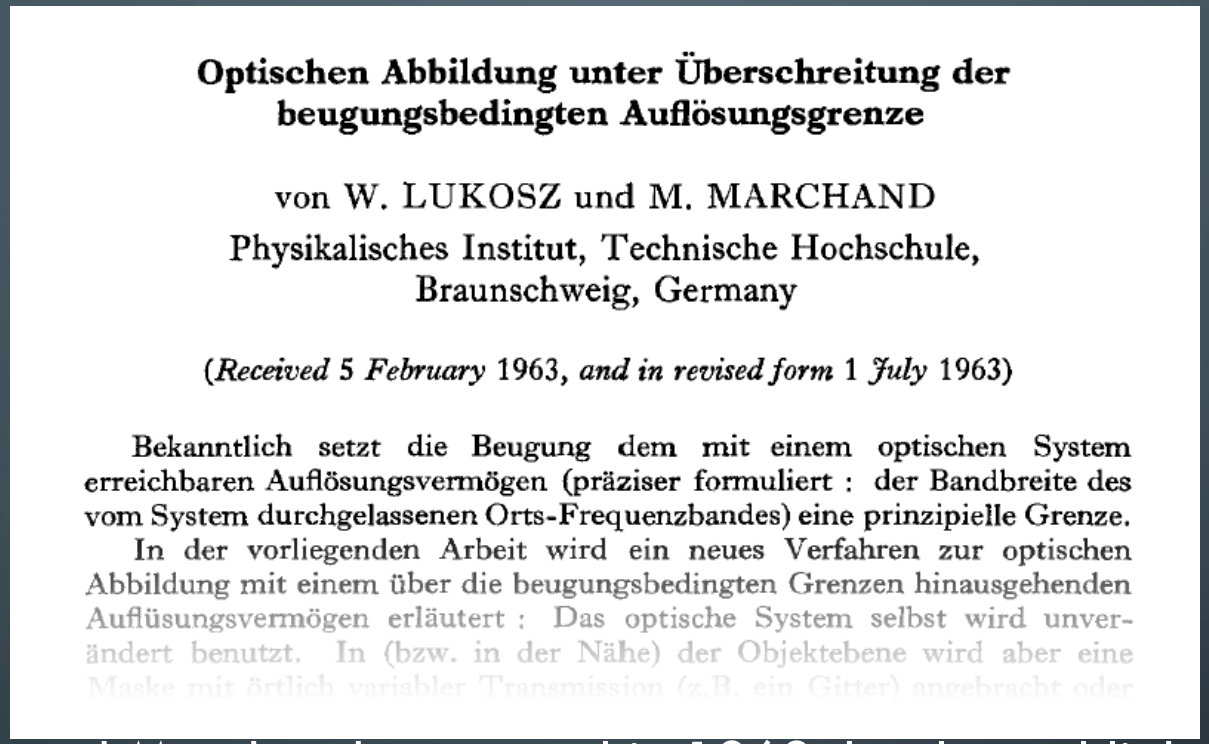
STRUCTURED-ILLUMINATION MICROPSCOPY (SIM)



100 nm resolution possible

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

STRUCTURED ILLUMINATION – HISTORY



Optischen Abbildung unter Überschreitung der beugungsbedingten Auflösungsgrenze

von W. LUKOSZ und M. MARCHAND
Physikalisches Institut, Technische Hochschule, Braunschweig, Germany

(*Received* 5 *February* 1963, *and in revised form* 1 *July* 1963)

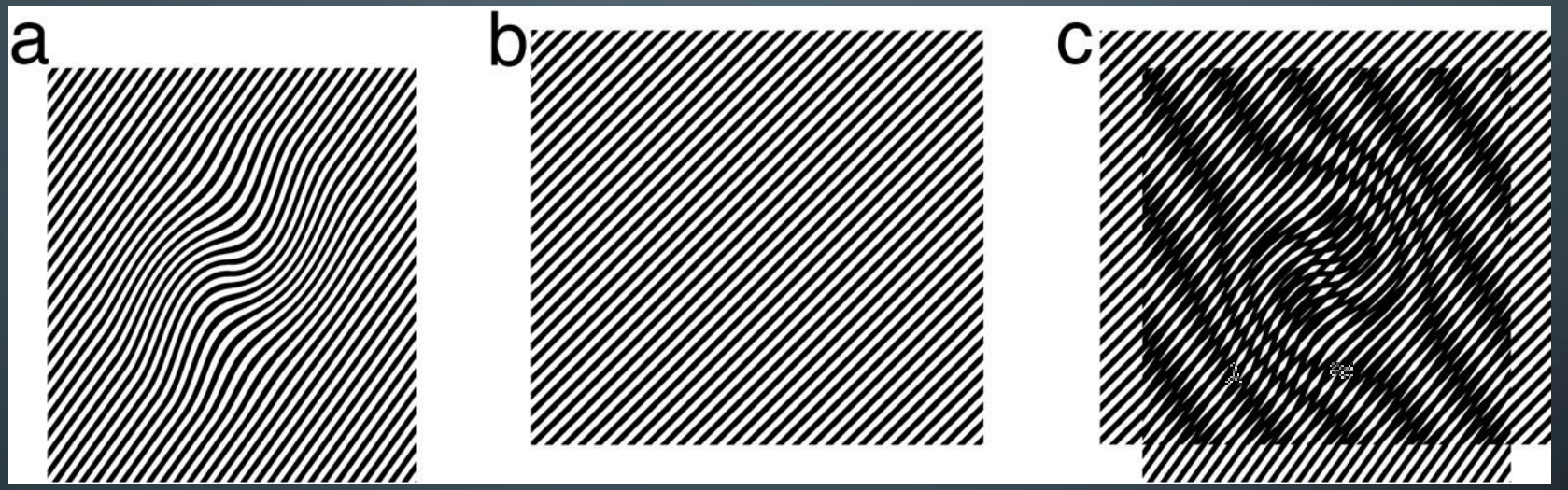
Bekanntlich setzt die Beugung dem mit einem optischen System erreichbaren Auflösungsvermögen (präziser formuliert : der Bandbreite des vom System durchgelassenen Orts-Frequenzbandes) eine prinzipielle Grenze.

In der vorliegenden Arbeit wird ein neues Verfahren zur optischen Abbildung mit einem über die beugungsbedingten Grenzen hinausgehenden Auflüsungsvermögen erläutert : Das optische System selbst wird unverändert benutzt. In (bzw. in der Nähe) der Objektebene wird aber eine Maske mit örtlich variabler Transmission (z.B. ein Gitter) angebracht oder

- Lukosz and Marchand suggested in 1963 that lateral light patterns could be used to enhance resolution
- Practical implementation was reported by T. Wilson et al. in 1997. (Neil, M. A. A., Wilson, T. & Juskaitis, R. (1997) *Opt. Lett.* **22**, 1905–1907.)

RESOLUTION EXTENSION THROUGH THE MOIRÉ EFFECT

If the illumination contains a spatial frequency $\mathbf{k}_1$, then each sample frequency $\mathbf{k}$ gives rise to moiré fringes at the difference frequency $\mathbf{k} - \mathbf{k}_1$. Those fringes will be observable in the microscope if $|\mathbf{k} - \mathbf{k}_1| < k_0$

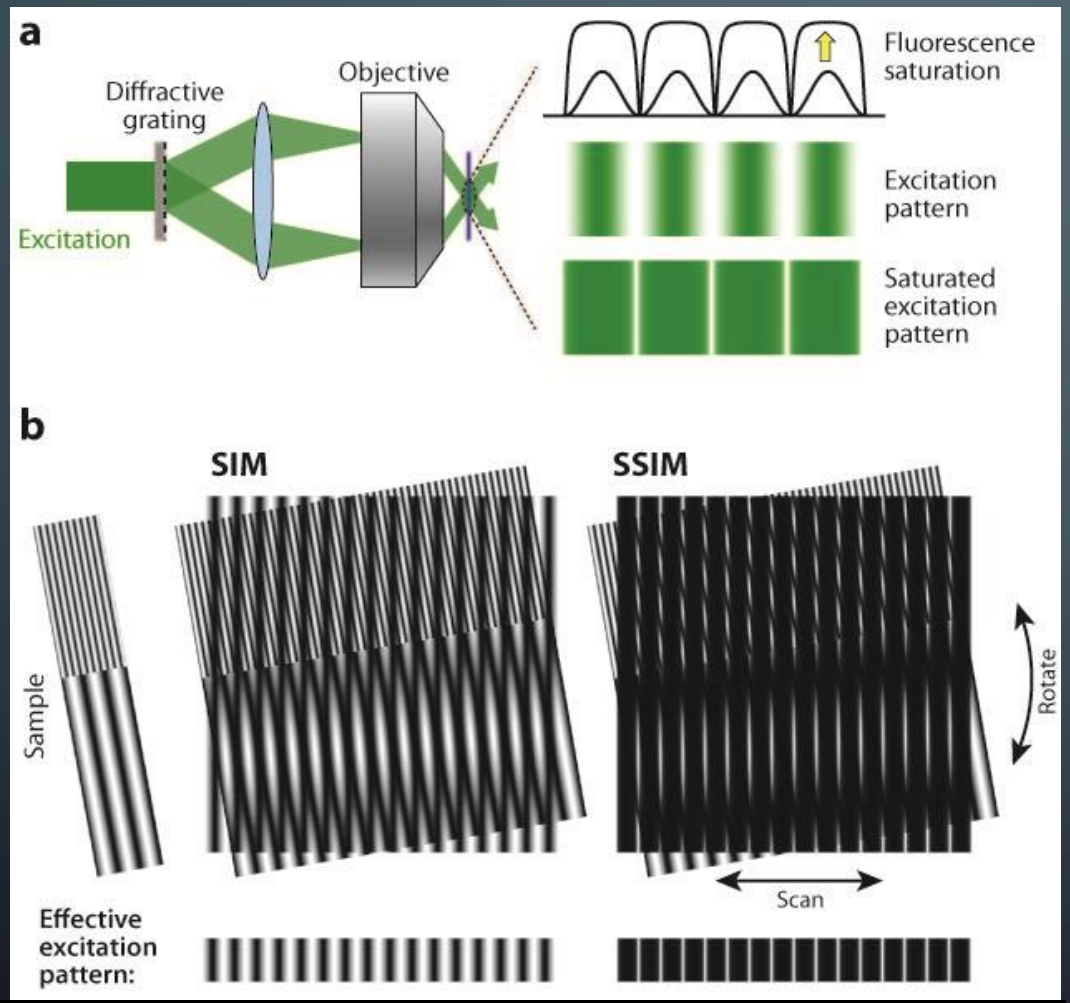


If an unknown sample structure (*a*) is multiplied by a known regular illumination pattern (*b*), moiré fringes will appear (*c*). The Moiré fringes occur at the spatial difference frequencies between the pattern frequency and each spatial frequency component of the sample structure and can be coarse enough to observe through the microscope even if the original unknown pattern is unresolvable. Otherwise-unobservable sample information can be deduced from the fringes and computationally restored.

Gustafsson, M.G.L. (2005) Proc. Natl. Acad. Sci. USA 102, 13081-13086

The word moiré is French (from the past participle of the verb moirer, meaning to water).

SATURATED STRUCTURED ILLUMINATION



Huang, Bo, Mark Bates, and Xiaowei Zhuang. 2009. "Super-Resolution Fluorescence Microscopy." *Annual Review of Biochemistry* 78 (1): 993-1016. doi:10.1146/annurev.biochem.77.061906.092014.

(*a*) A diffractive grating in the excitation path splits the light into two beams. Their interference after emerging from the objective and reaching the sample creates a sinusoidal illumination pattern with alternating peaks and zero points. Strong excitation light saturates the fluorescence emission at the peaks without exciting fluorophores at the zero points, leading to sharp dark regions in the excitation pattern. (*b*) When a sinusoidal illumination pattern is applied to a sample, a moiré pattern at a significantly lower spatial frequency than that of the sample can be generated and imaged by the microscope (SIM panel, *lower part*). Multiple images that resulted from scanning and rotating the excitation pattern are then used to reconstruct the sample structure. SSIM introduces a high-frequency component into the excitation pattern, allowing features far below the diffraction limit to be resolved

CONCLUSIONS

- methods use common dyes (good)
- confocal is easiest, most widely used

- best resolution obtainable only 100 nm (SIM)
- single molecule is problematic

SUPER-RESOLUTION MICROSCOPY

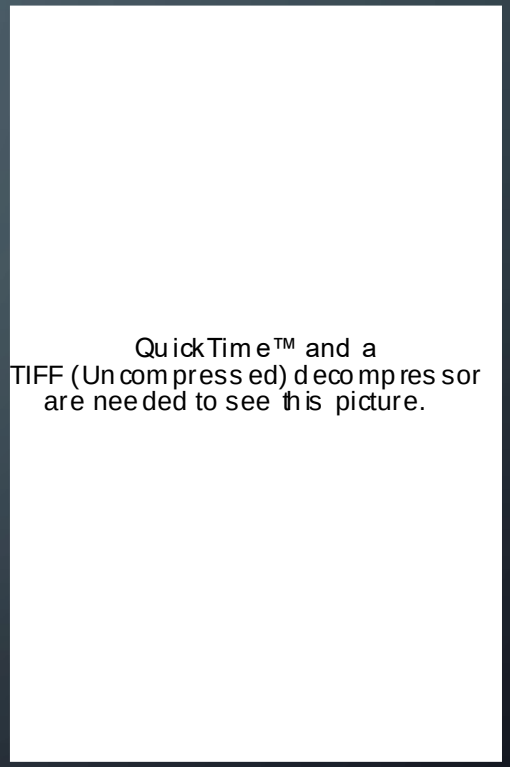
Goal: obtain sub-100 nm resolution

pioneered by Stefan Hell in mid-1990s
Max Plank Institute (Germany)

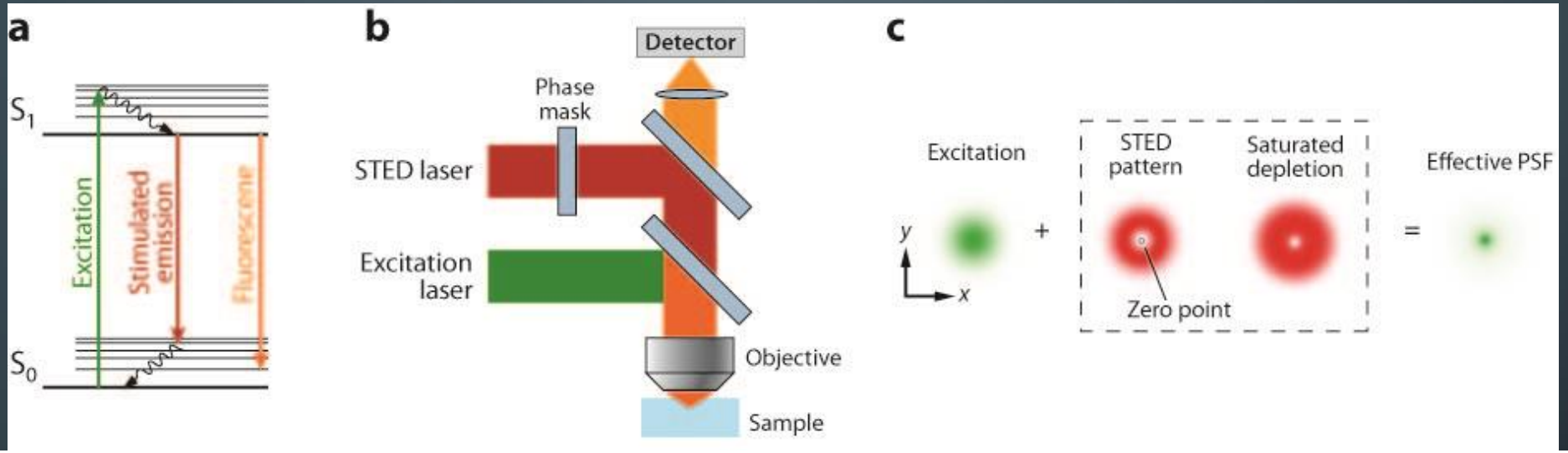
two methods:

(i) Spatially Patterned Excitation
STED, RESOLFT, SSIM

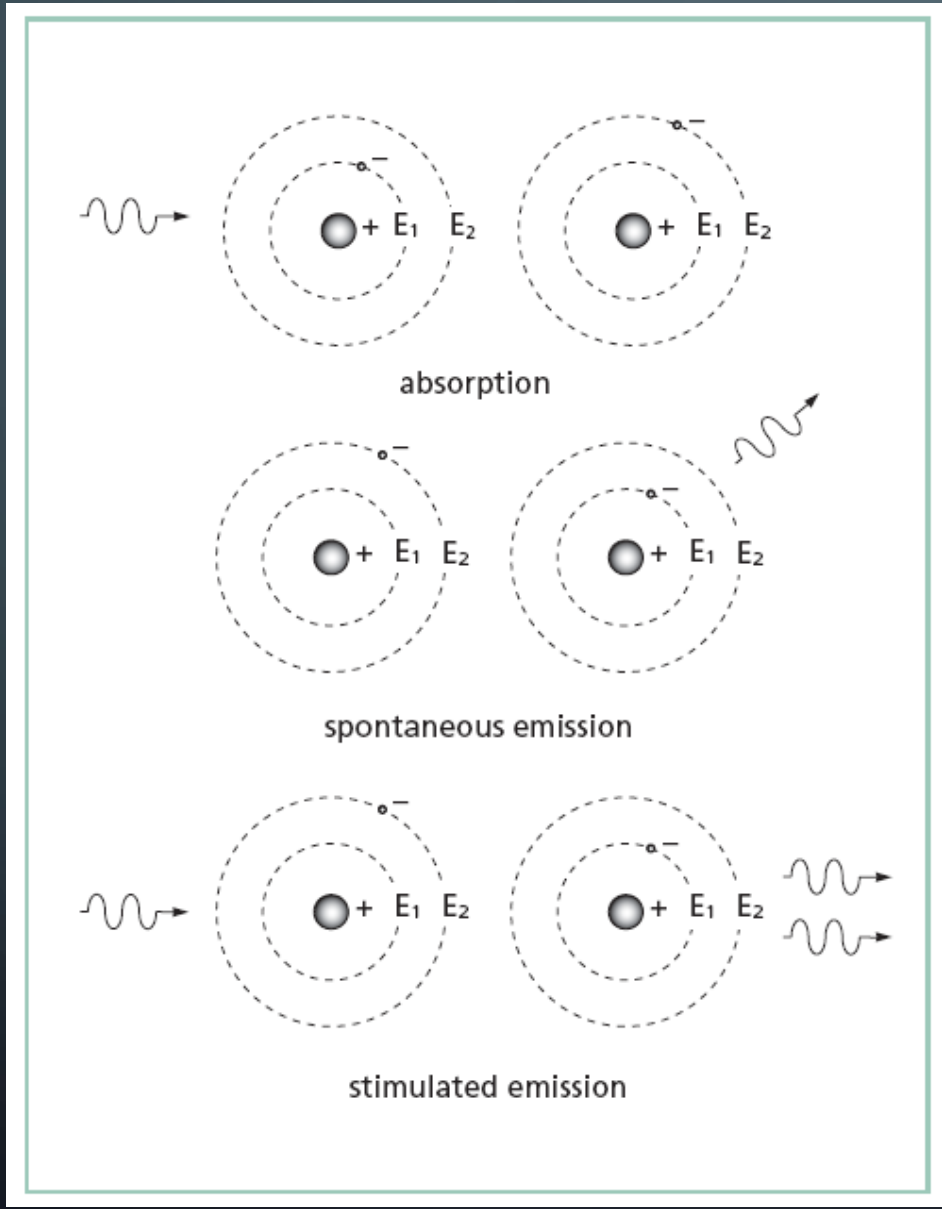
(ii) Localization Methods
STORM, PALM, FPALM



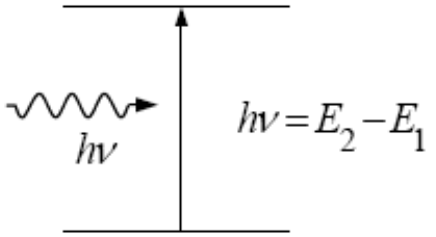
THE PRINCIPLE OF STIMULATED EMISSION DEPLETION (STED) MICROSCOPY



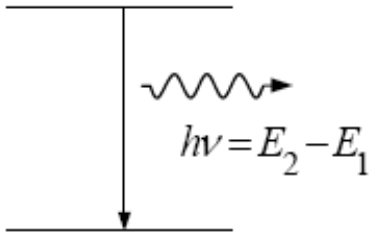
(*a*) The process of stimulated emission. A ground state (S_0) fluorophore can absorb a photon from the excitation light and jump to the excited state (S_1). Spontaneous fluorescence emission brings the fluorophore back to the ground state. Stimulated emission happens when the excited-state fluorophore encounters another photon with a wavelength comparable to the energy difference between the ground and excited state. (*b*) The excitation laser and STED laser are combined and focused into the sample through the objective. A phase mask is placed in the light path of the STED laser to create a specific pattern at the objective focal point. (c) In the *xy* mode, a donut-shaped STED laser is applied with the zero point overlapped with the maximum of the excitation laser focus. With saturated depletion, fluorescence from regions near the zero point is suppressed, leading to a decreased size of the effective point spread function (PSF).



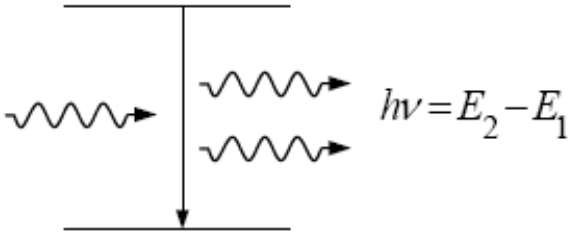
Absorption

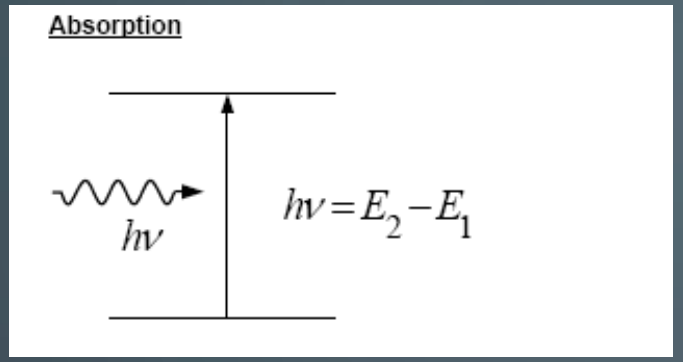


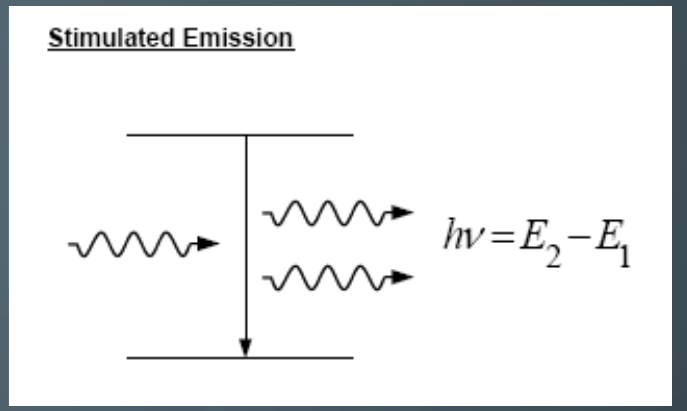
Spontaneous Emission



Stimulated Emission







Absorption Rate:

$-\sigma_{12} F N_1$

- N_1: Number of atoms or molecules in lower energy level (Unit: per cm^3)
- σ_{12}: Absorption Cross-Section, Units → cm2
- F: Photon Flux, Units → $\#/cm^2 sec$

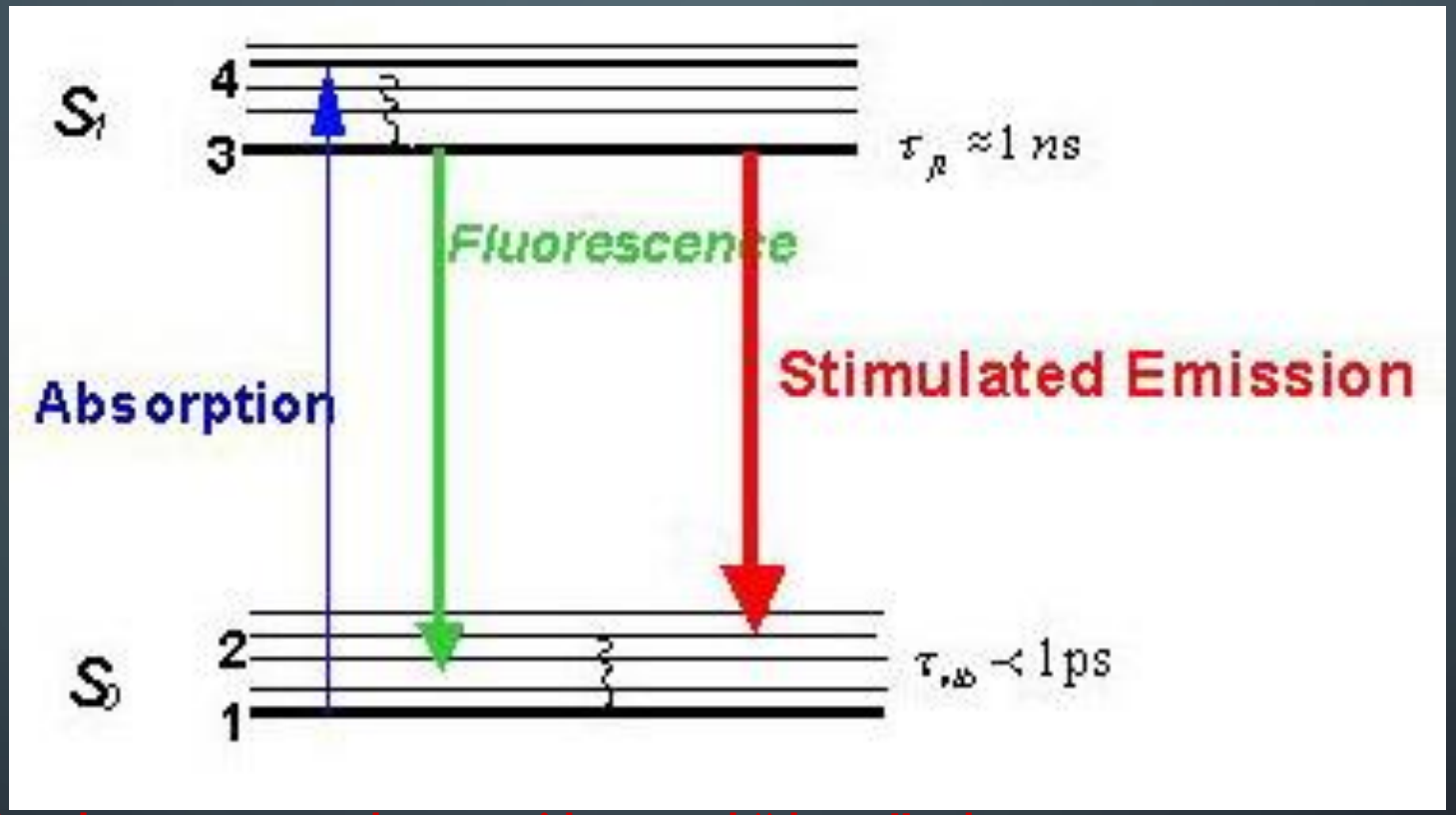
Stimulated Emission Rate:

$-\sigma_{21} F N_2$

- N_2: Number of atoms or molecules in lower energy level (Unit: per cm^3)
- σ_{21}: Stimulated emission Cross-Section, Units → cm2 (typical value ~ 10^{-19} to 10^{-18} cm^2)
- F: Photon Flux, Units → $\#/cm^2 sec$

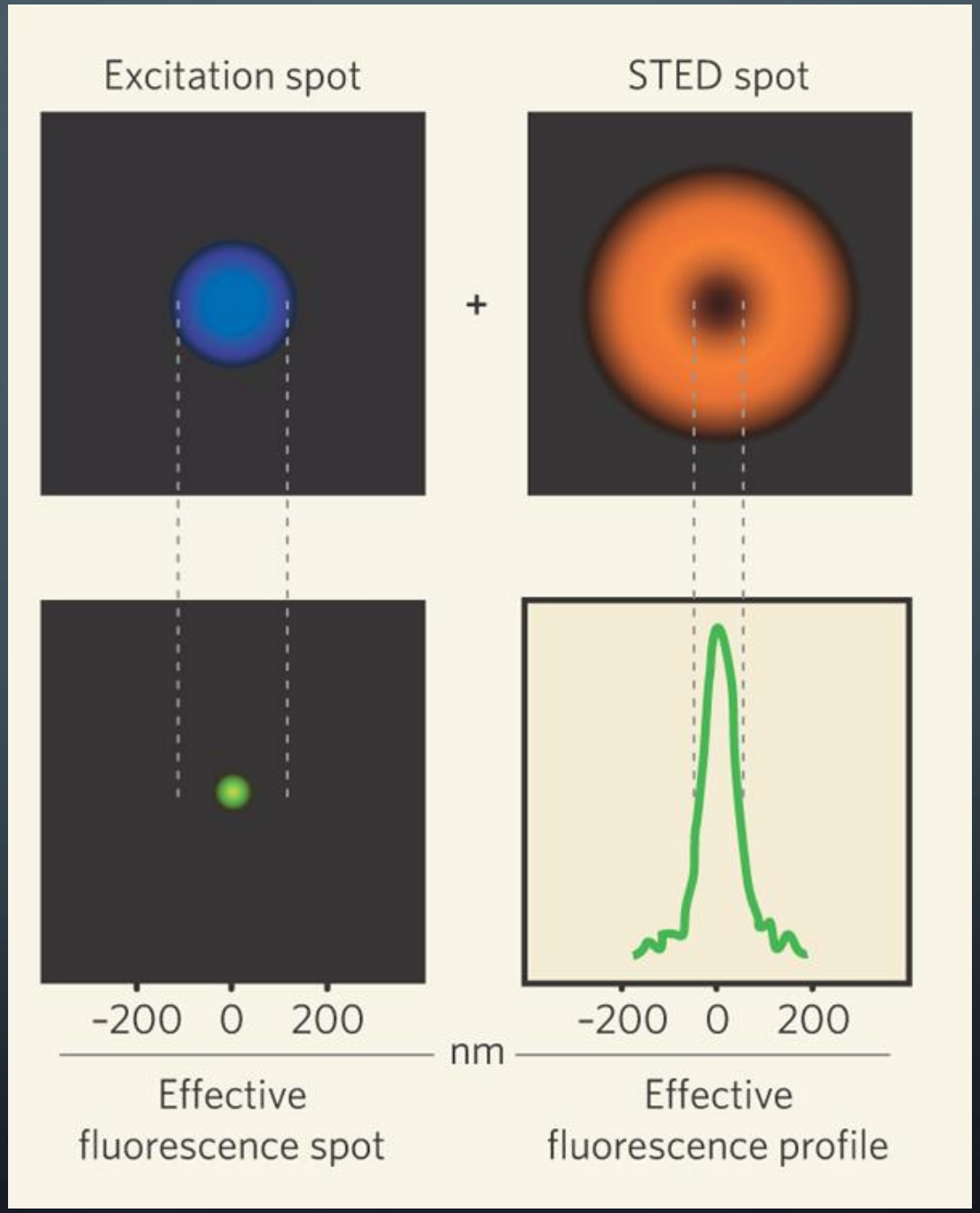
$$\sigma 12 = \sigma 21$$

Stimulated Emission Depletion (STED)

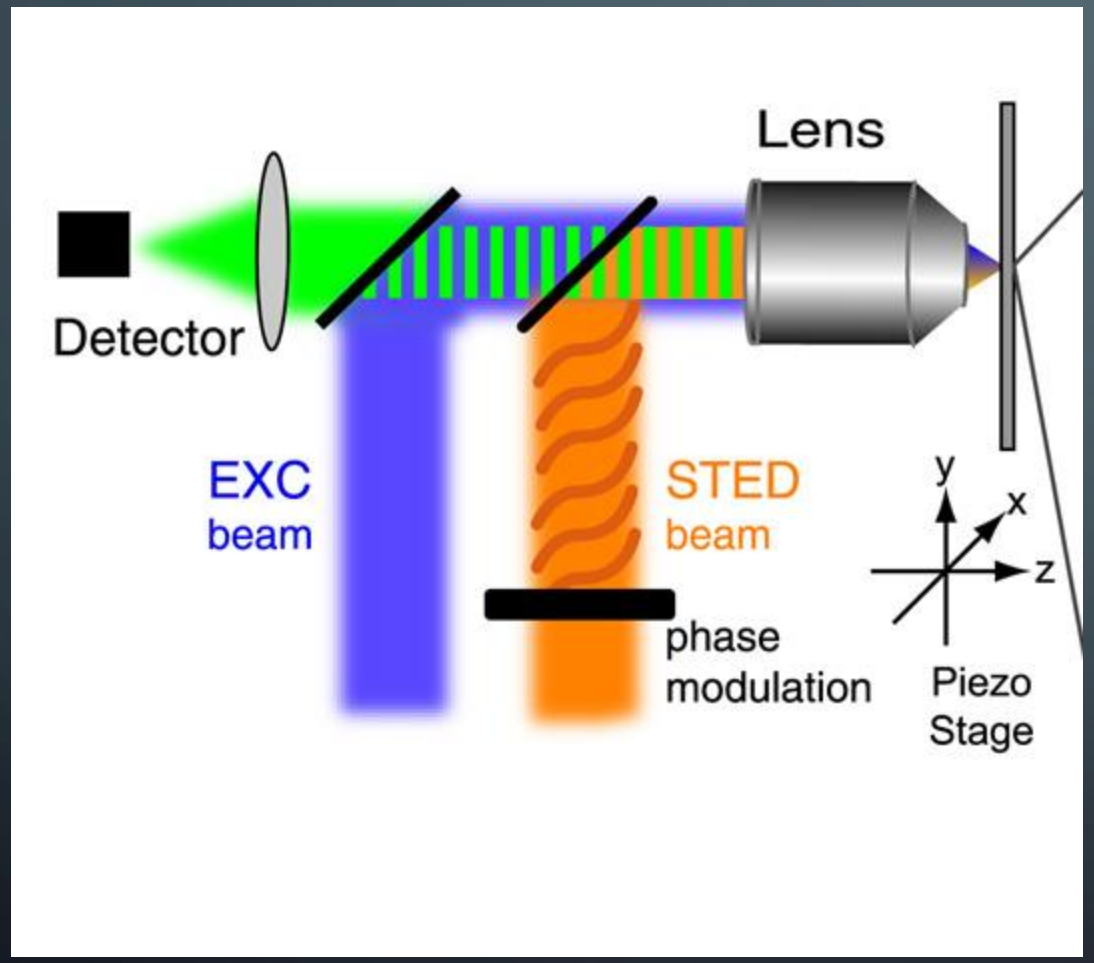


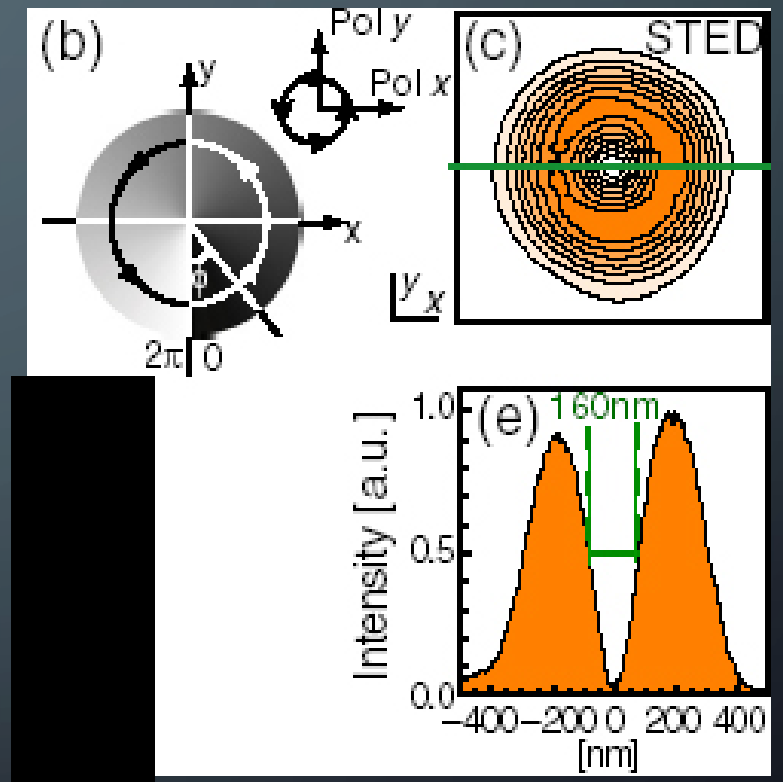
Drive down to ground state with second "dump"pulse,
Before molecule can fluoresce

Quench fluorescence and Combine with spatial control
to make "donut", achieve super-resolution in 3D (unlike NSOM)

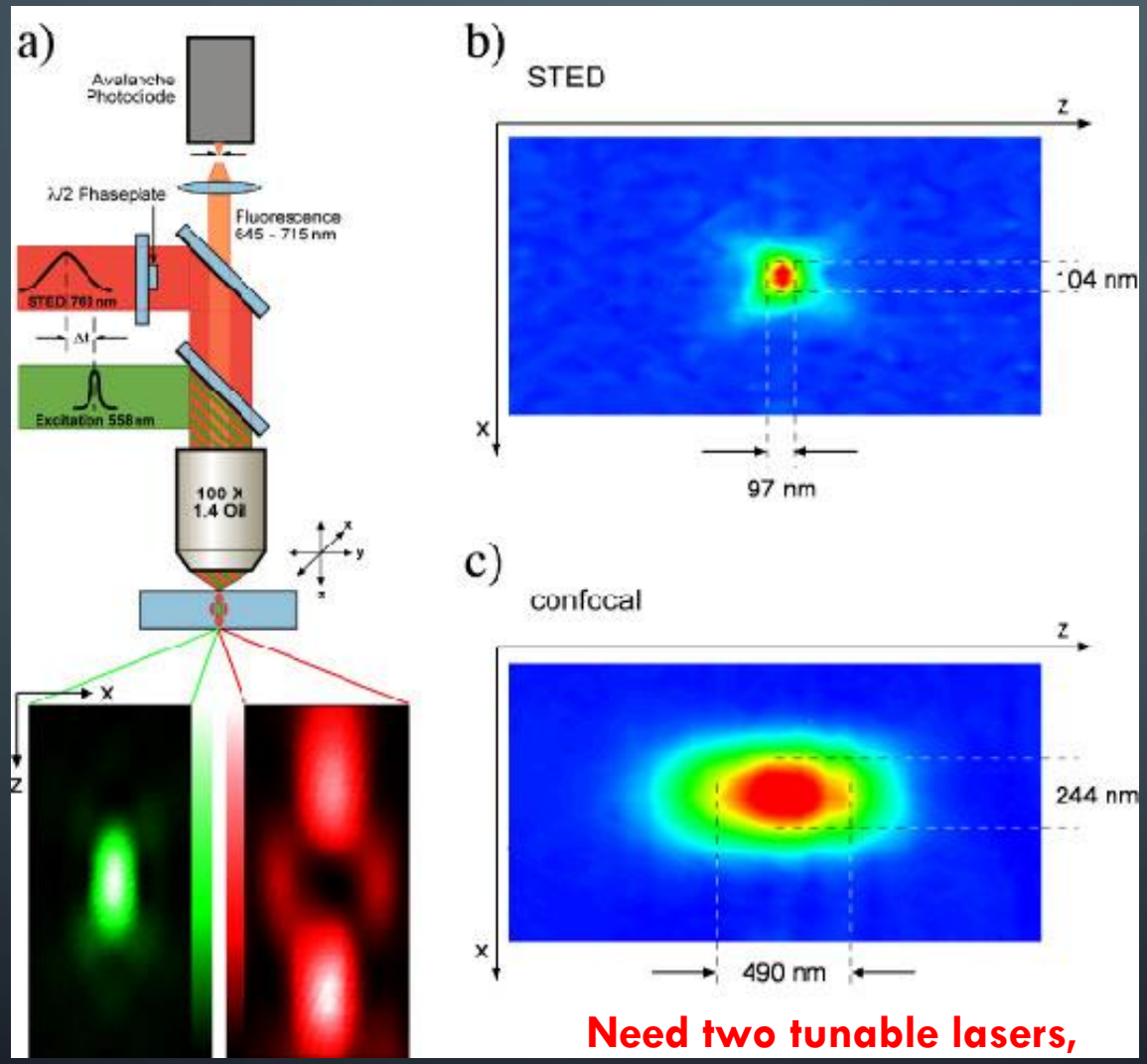

Excitation spot
STED spot
+
-200
0
200
nm
-200
0
200
Effective
fluorescence spot
Effective
fluorescence profile

SETUP


Lens
Detector
EXC
beam
STED
beam
phase
modulation
Piezo
Stage
y
x
z


(b)
Pol y
Pol x
y
x
2π
0
(c)
STED
y
x
(e)
160nm
1.0
0.5
0.0
Intensity [a.u.]
−400 −200 0 200 400
[nm]

STED Experimental Setup and PSF's

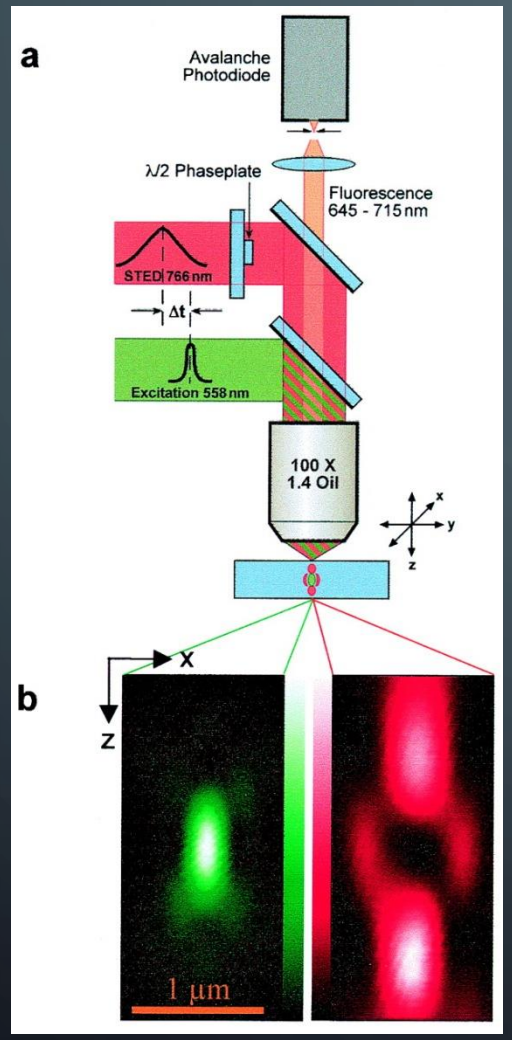


100 nm
Axial and lateral
PSFs

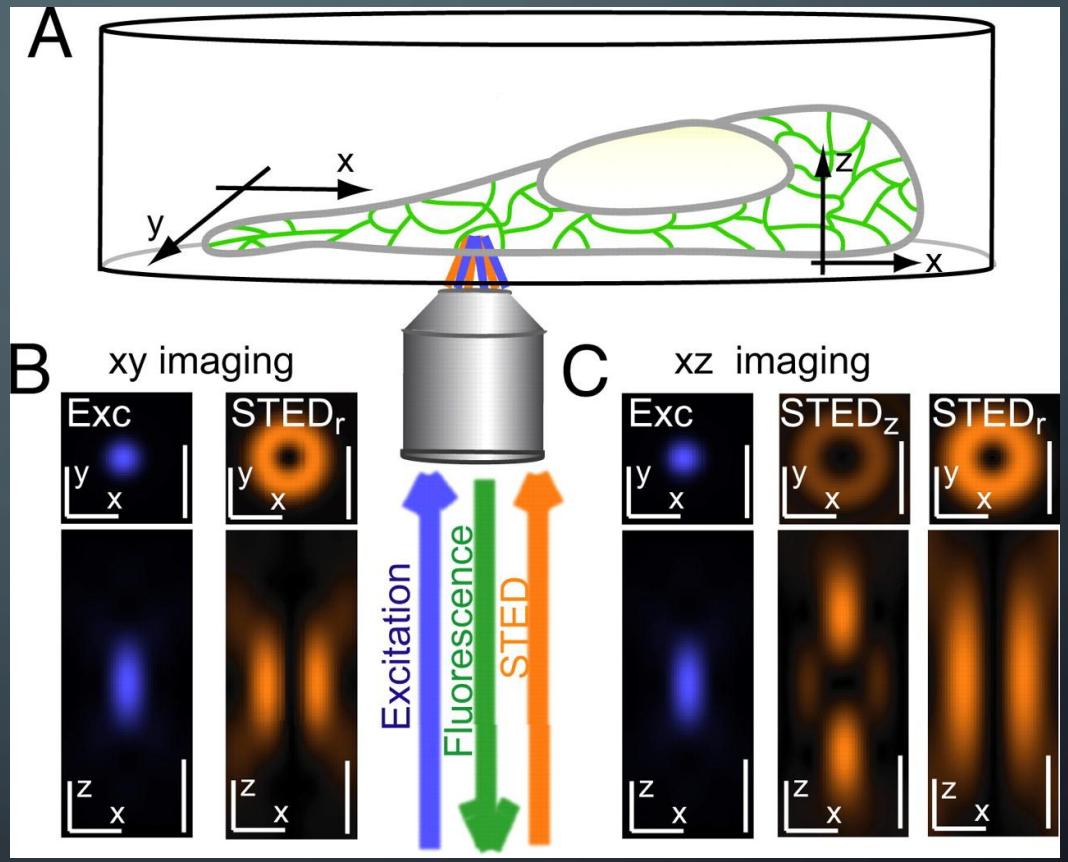
Need two tunable lasers,
Overlapped spatially, temporally
And synchronized

Hell et al

EXCITATION AND DEEXCITATION BEAMS FOR 3D STED

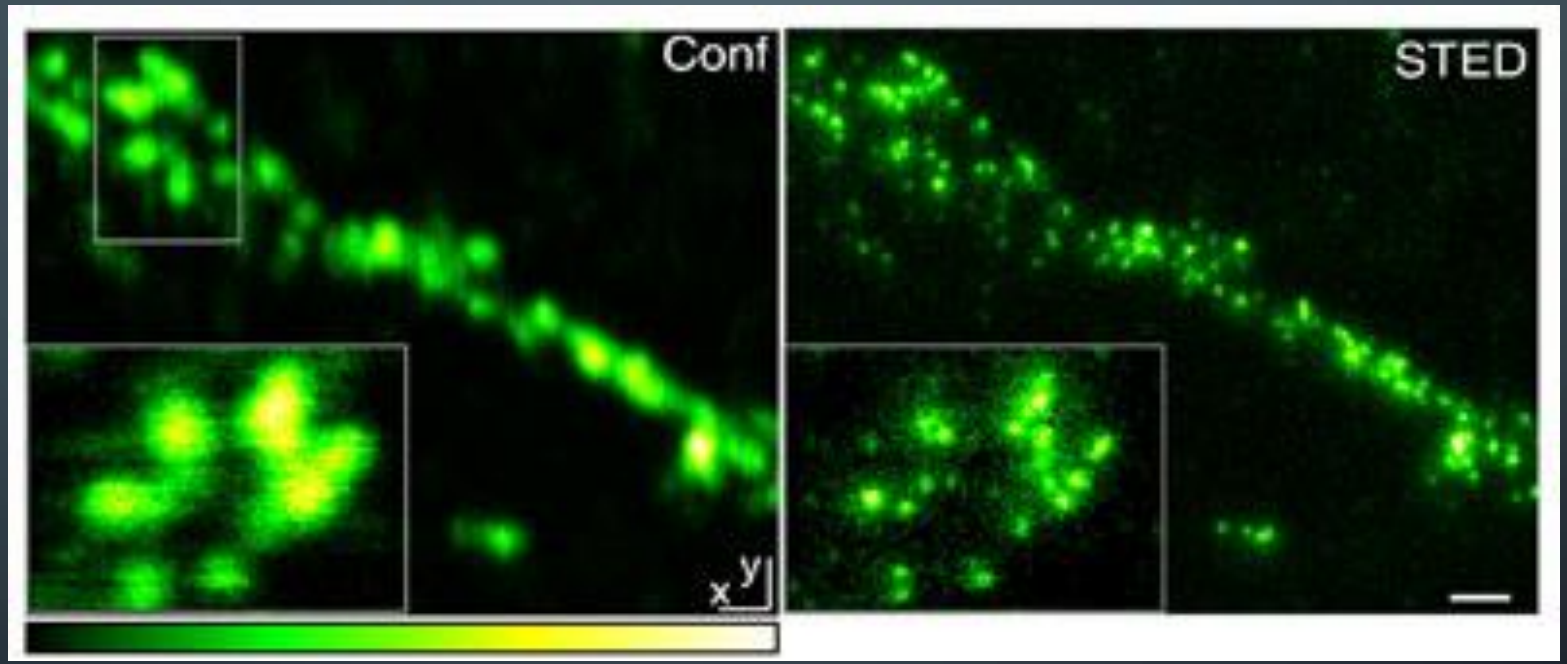


Klar T A et al. PNAS 2000;97:8206-8210

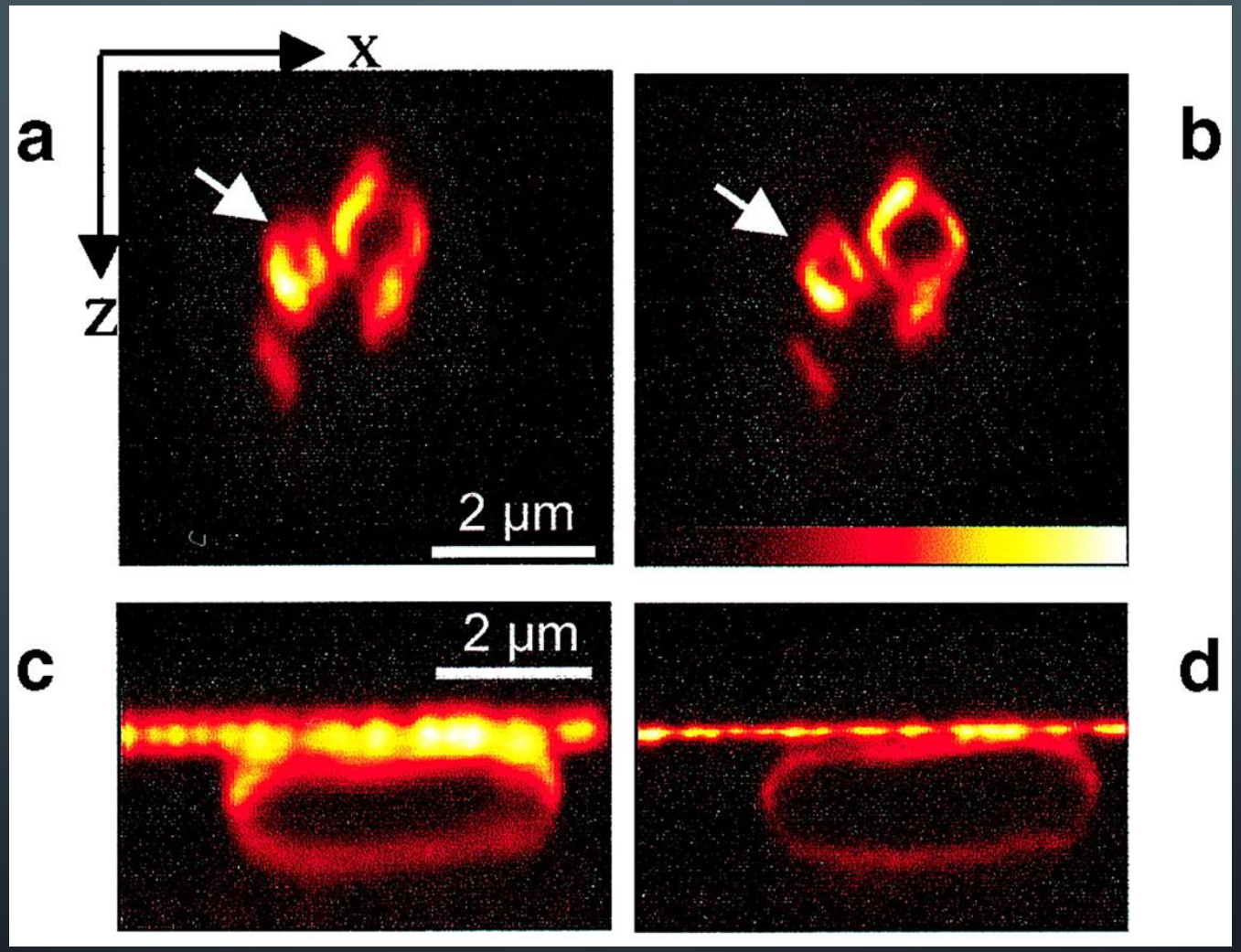


Hein B et al. PNAS 2008;105:14271-14276

RESOLUTION IMPROVEMENT IN STED

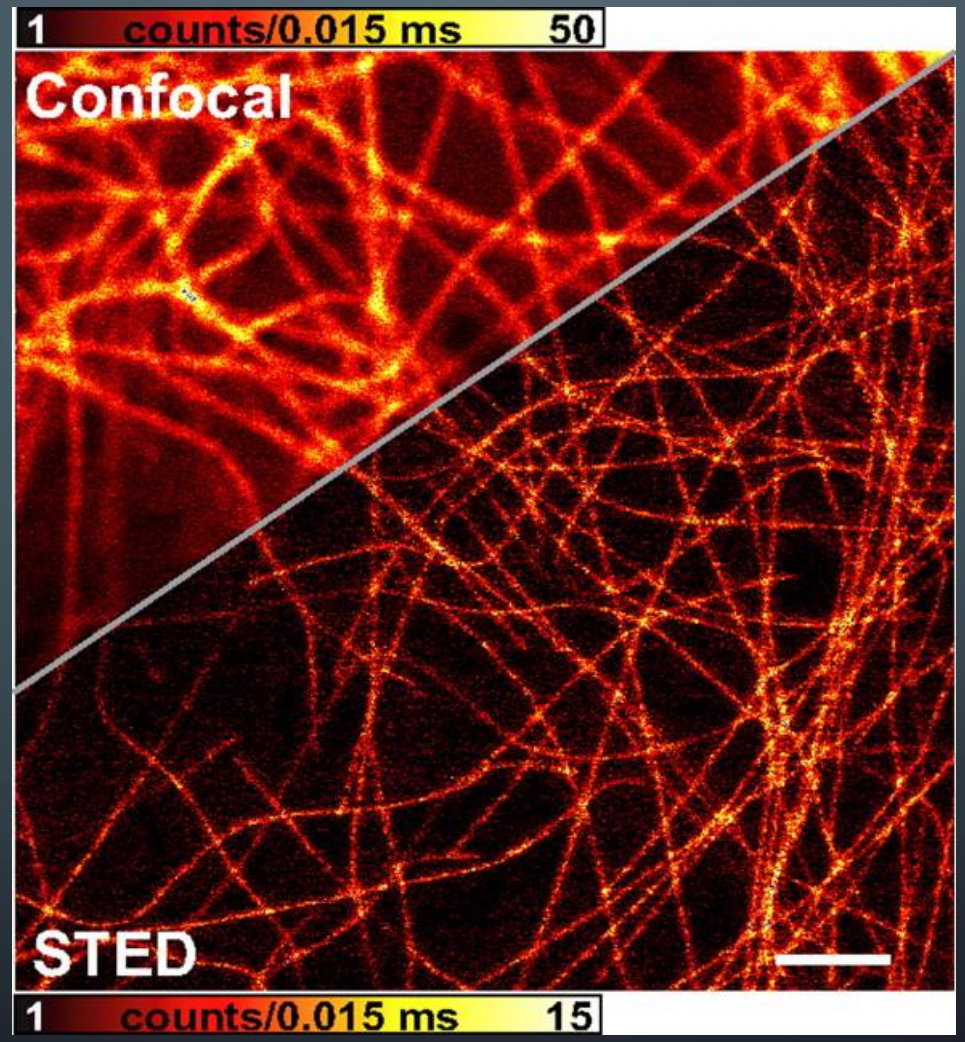


RESOLUTION IMPROVEMENT IN STED



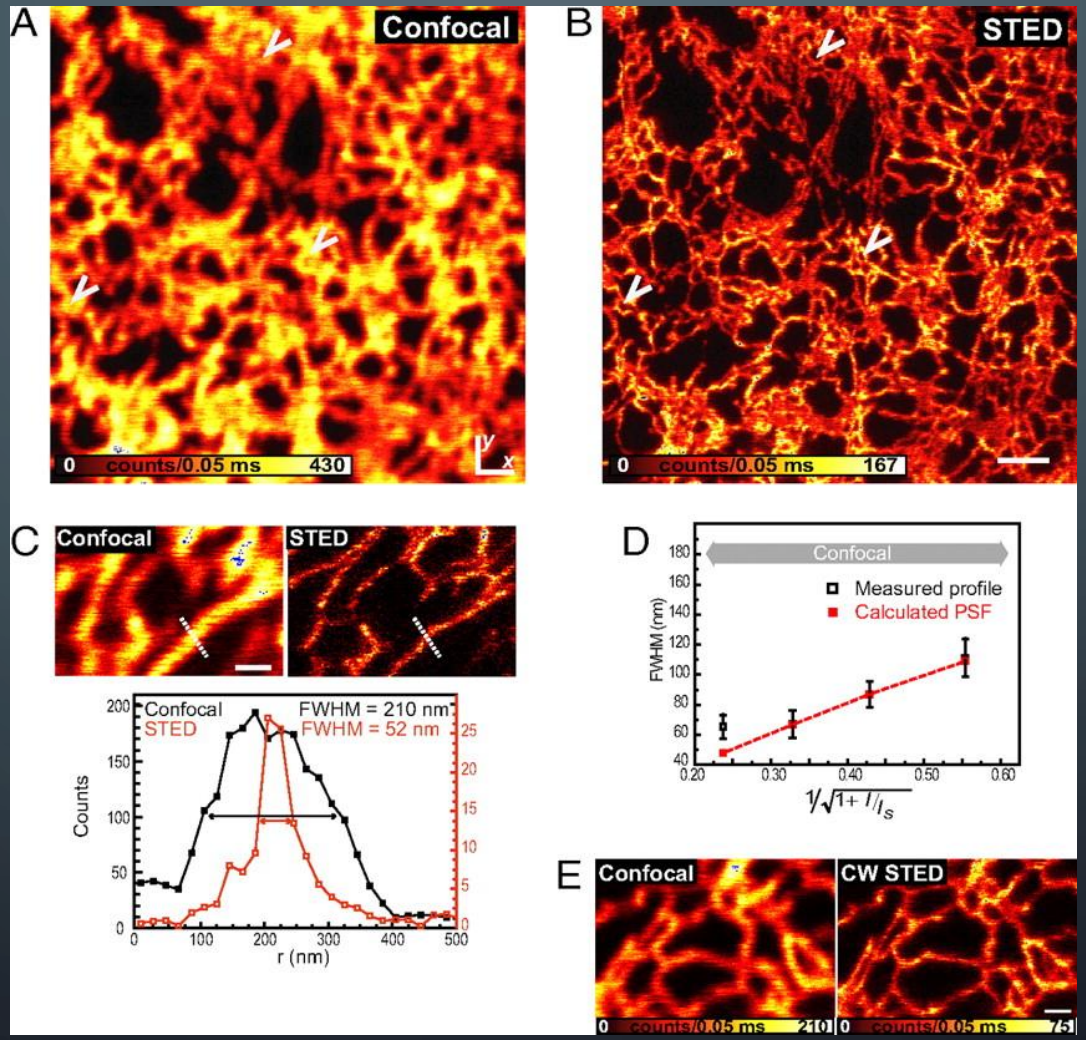
Klar T A et al. PNAS 2000;97:8206-8210

EXAMPLE: SUBDIFFRACTION RESOLUTION FLUORESCENCE IMAGING OF MICROTUBULES



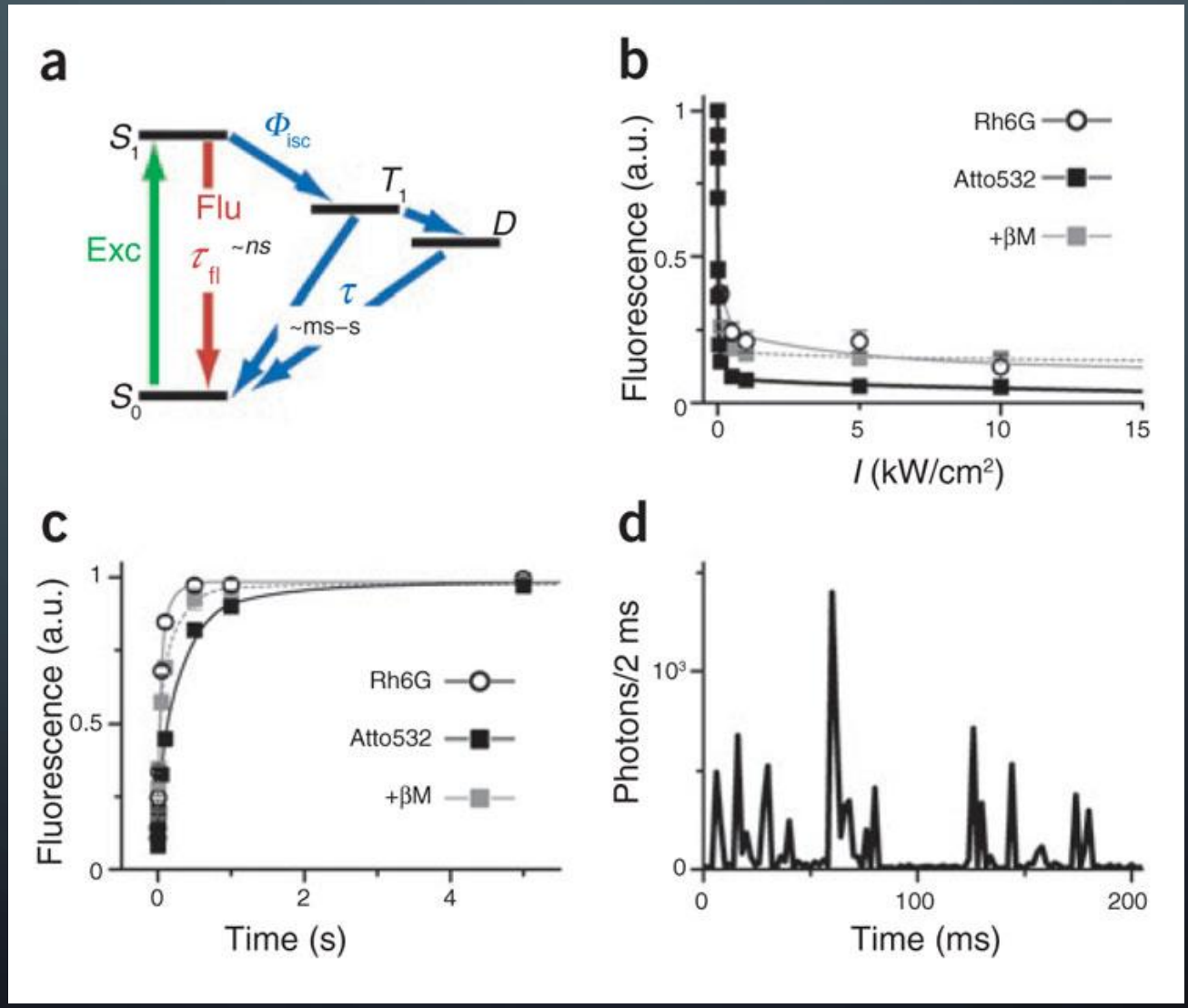
Hein B et al. PNAS 2008;105:14271-14276

EXAMPLE: SUBDIFFRACTION-RESOLUTION IMAGING OF THE ER IN A LIVING MAMMALIAN CELL.



Hein B et al. PNAS 2008;105:14271-14276

GROUND-STATE DEPLETION (GSDIM)



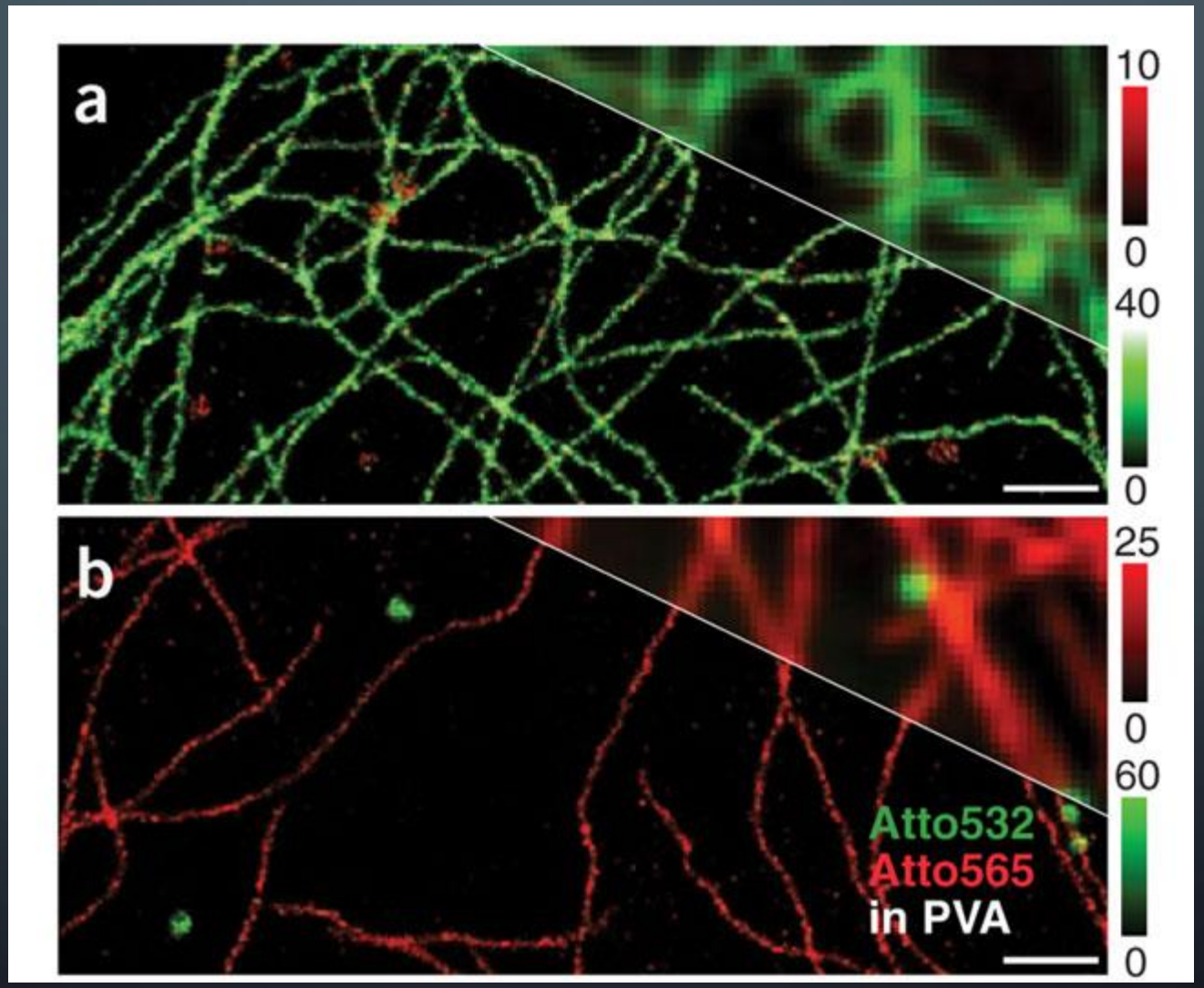
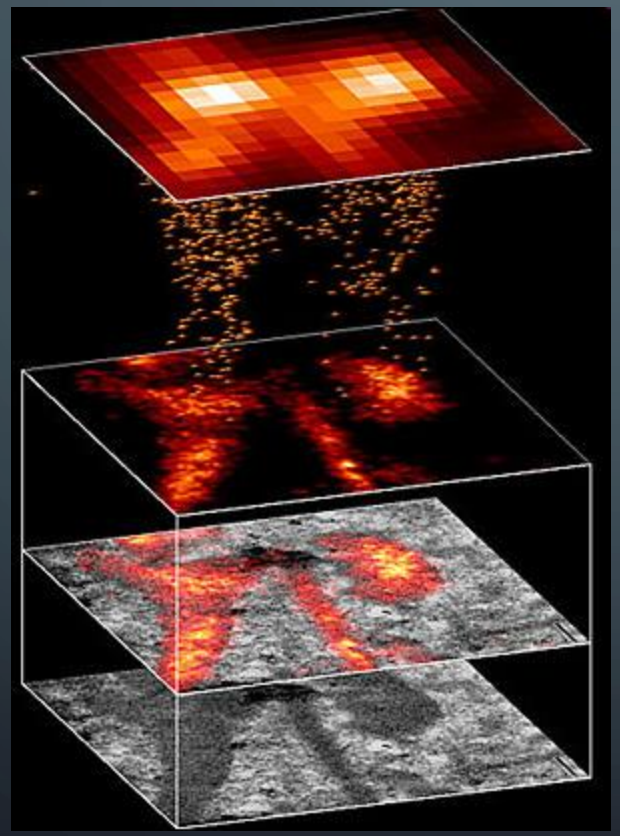
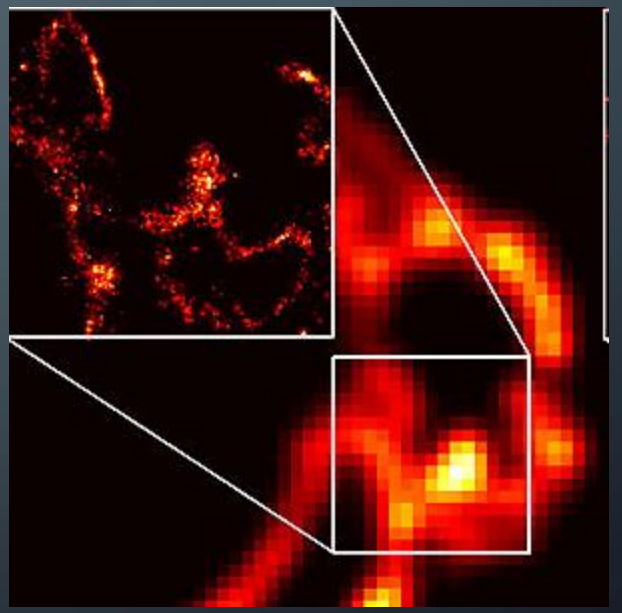
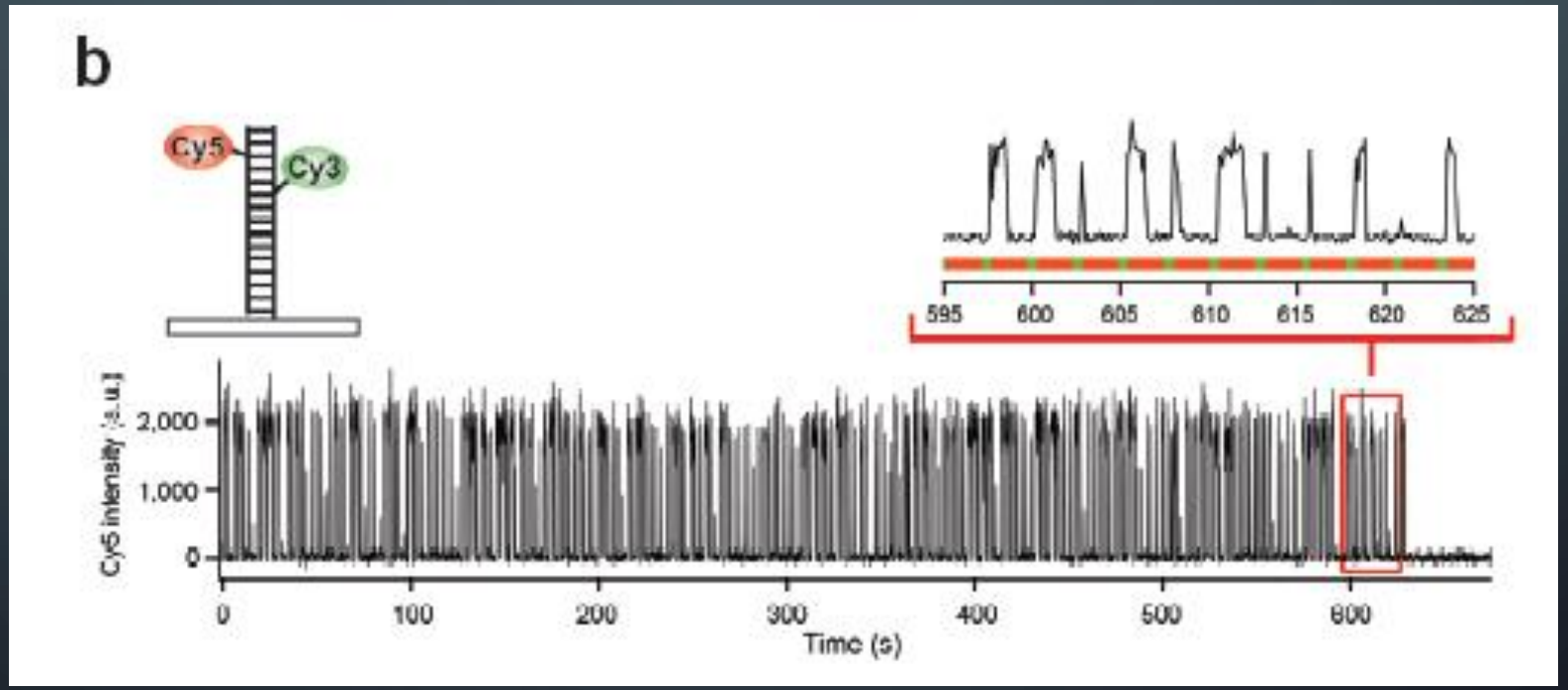

a
10
0
40
0
b
25
0
60
0
Atto532
Atto565
in PVA

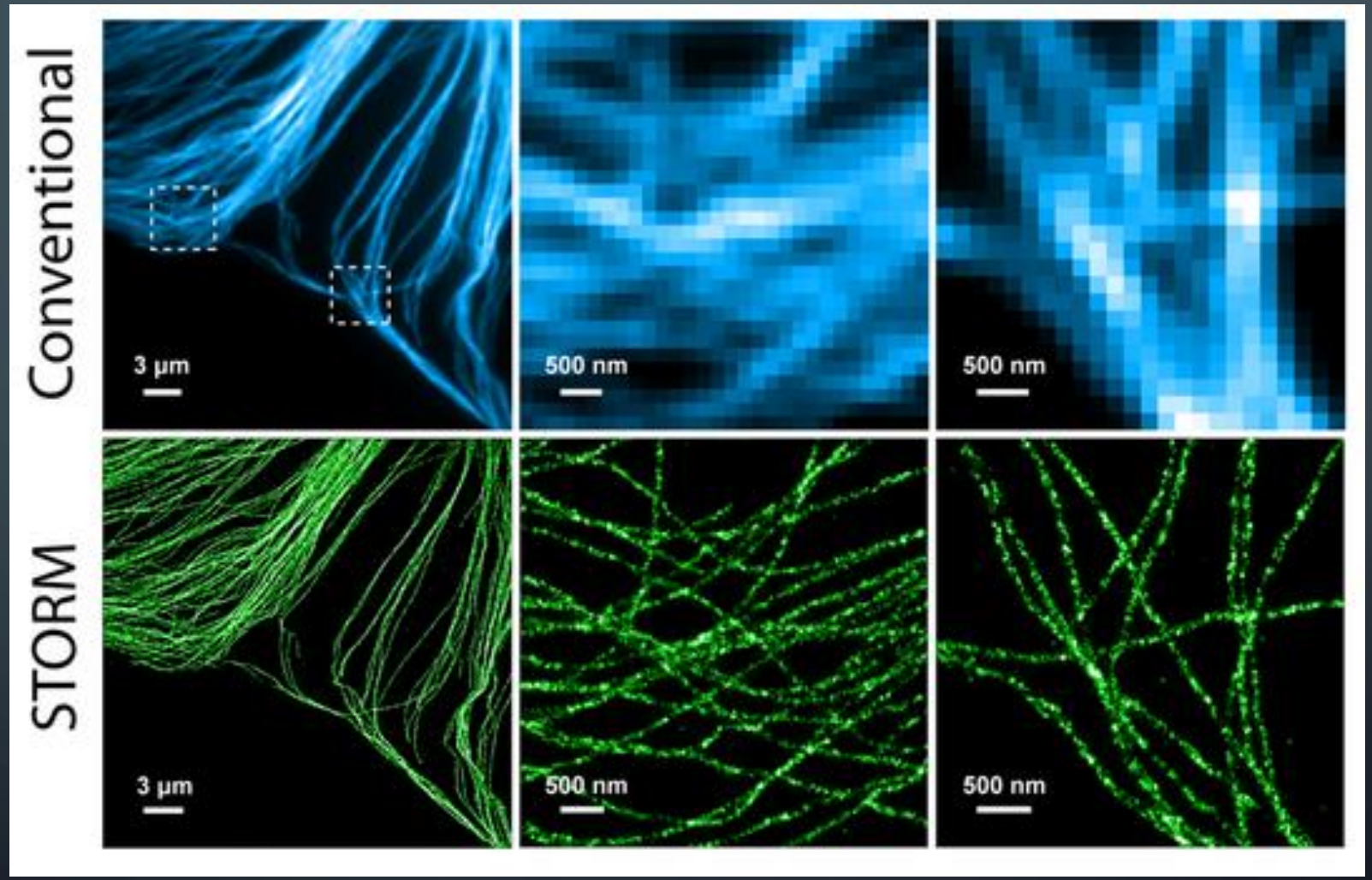
PHOTO-ACTIVATION LOCALIZATION MICROSCOPY (PALM)





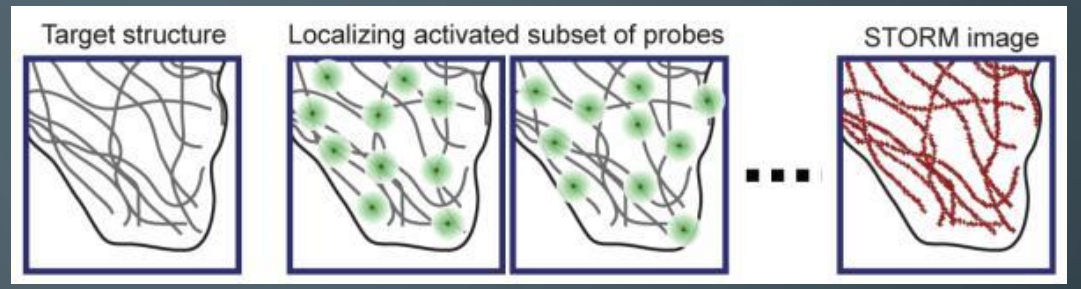
STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY
STORM



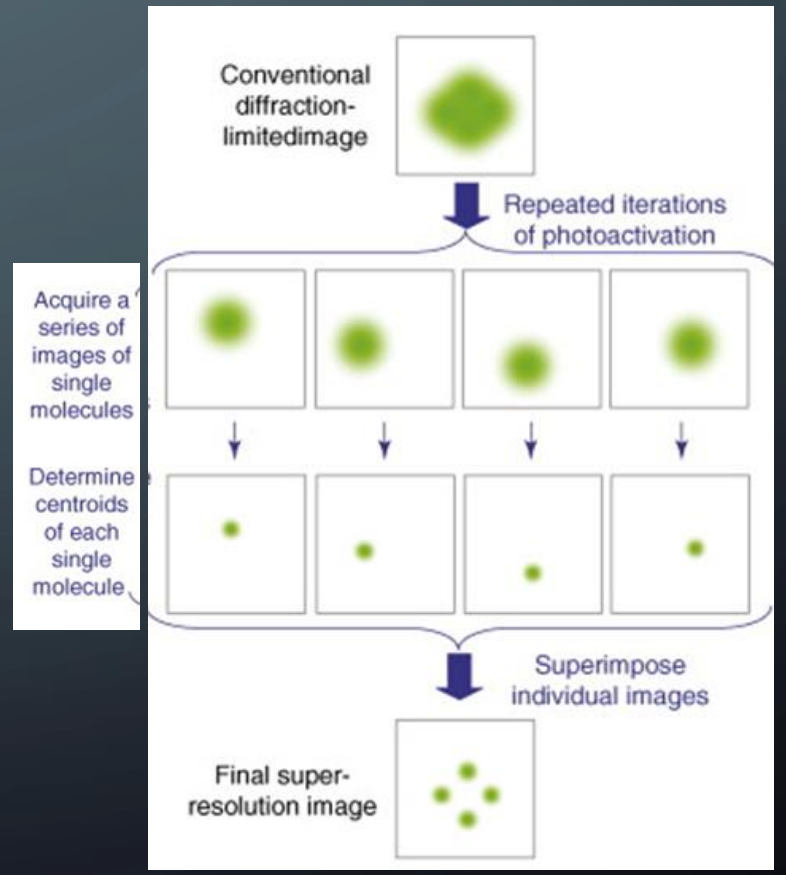

Conventional
STORM
3 µm
500 nm
500 nm
3 µm
500 nm
500 nm

STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY (STORM) OR (FLUORESCENCE) PHOTOACTIVATION LOCALIZATION MICROSCOPY ((F)PALM)

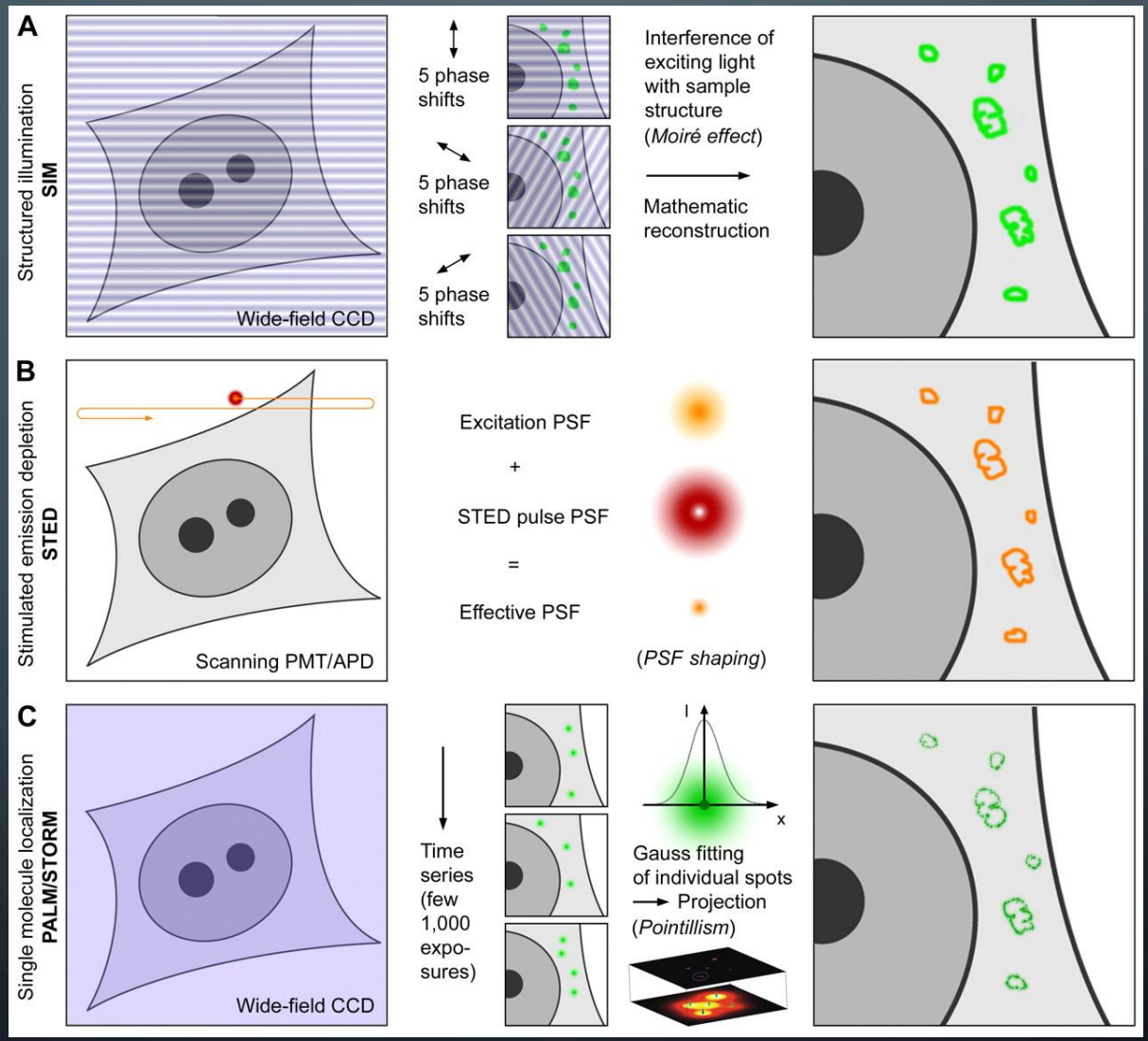
Zhuang, Xiaowei. 2009. “Nano-imaging with Storm.” *Nature photonics* 3 (7): 365-367. doi:10.1038/nphoton.2009.101.



Different fluorescent probes marking the sample structure are activated at different time points, allowing subsets of fluorophores to be imaged without spatial overlap and to be localized to high precision. Iterating the activation and imaging process allows the position of many fluorescent probes to be determined and a super-resolution image is then reconstructed from the positions of a large number of localized probe molecules.



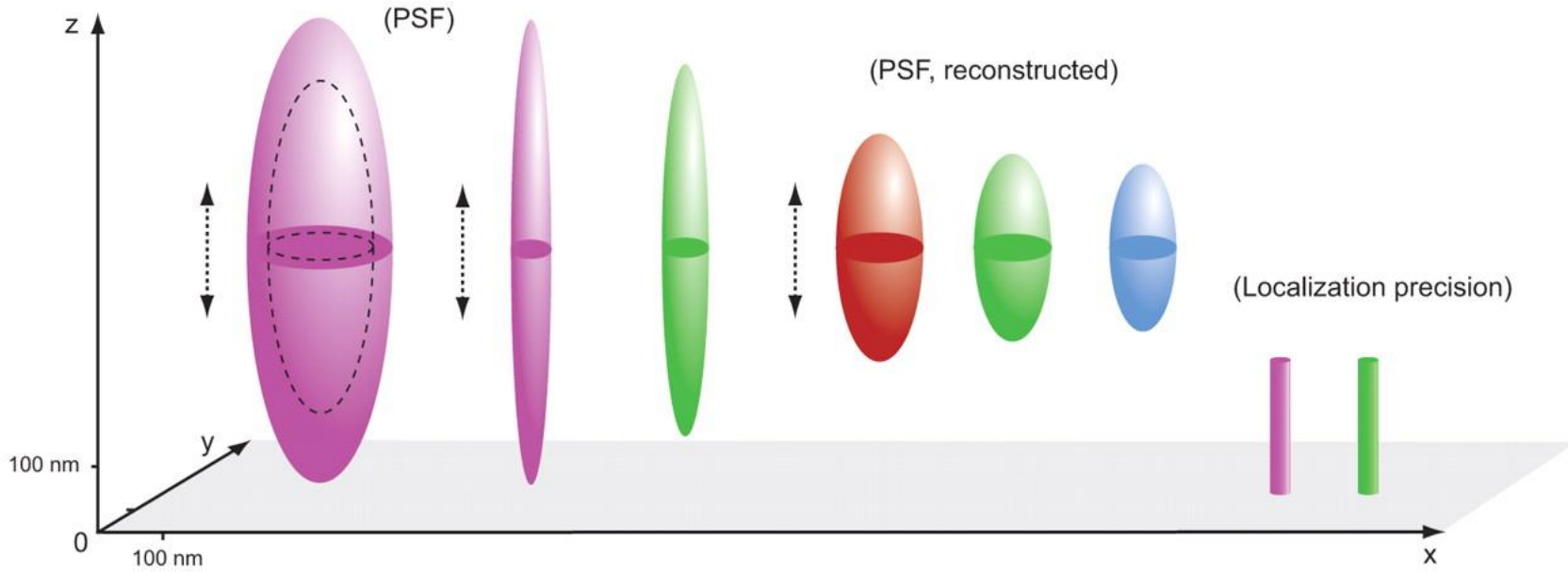
SUPER-RESOLUTION IMAGING PRINCIPLES.



Schermelleh L et al. J Cell Biol 2010;190:165-175

RESOLVABLE VOLUMES OBTAINED WITH CURRENT COMMERCIAL SUPER-RESOLUTION MICROSCOPES.

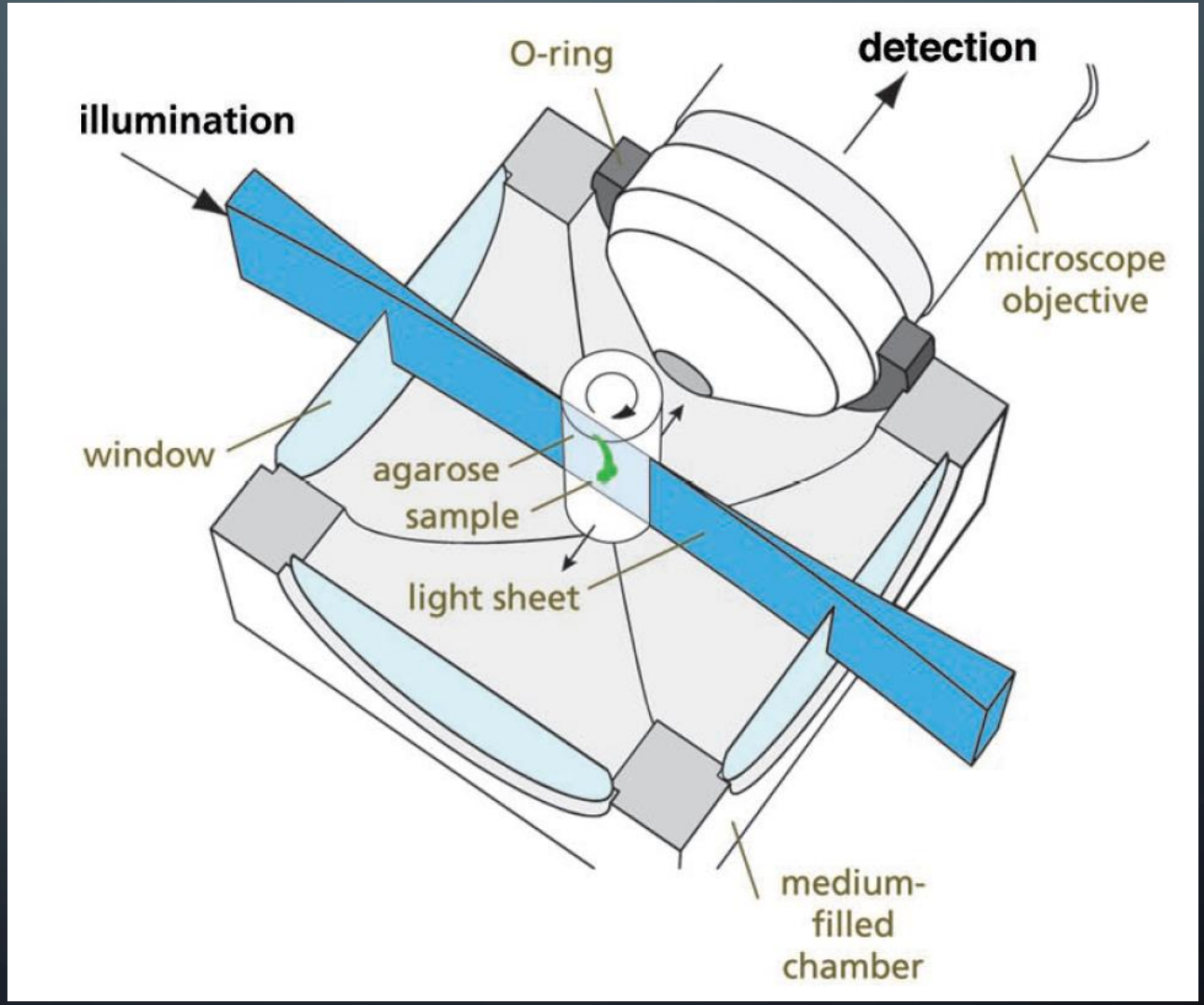
	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520



~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

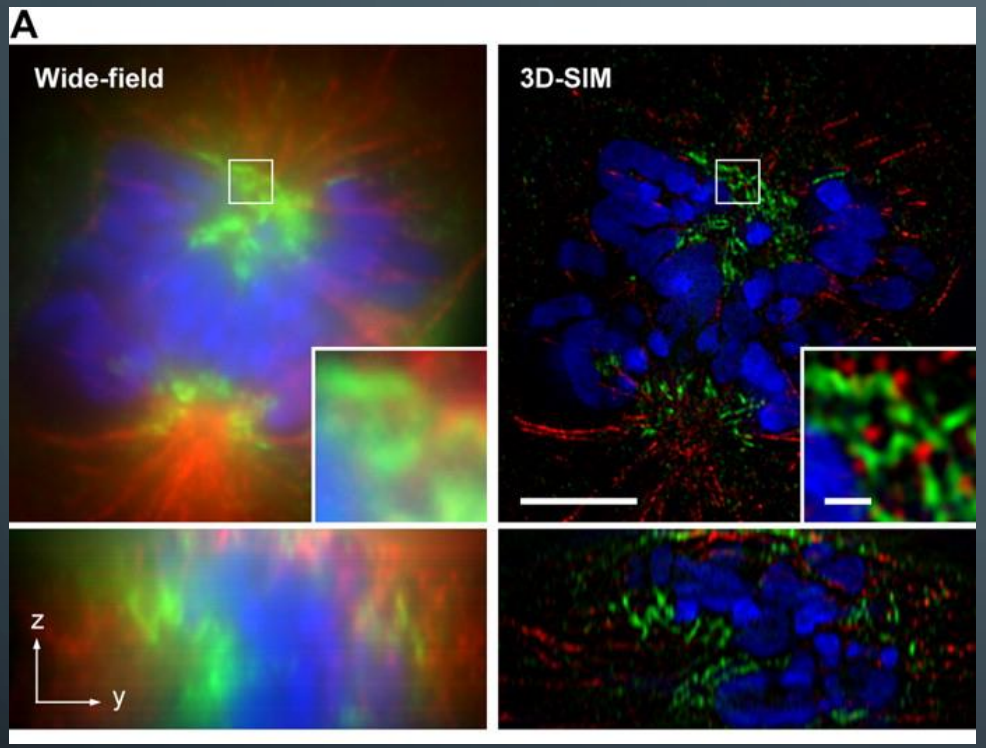
Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. “A guide to super-resolution fluorescence microscopy.” *The Journal of Cell Biology* 190 (2) (July 26): 165 -175. doi:10.1083/jcb.201002020.

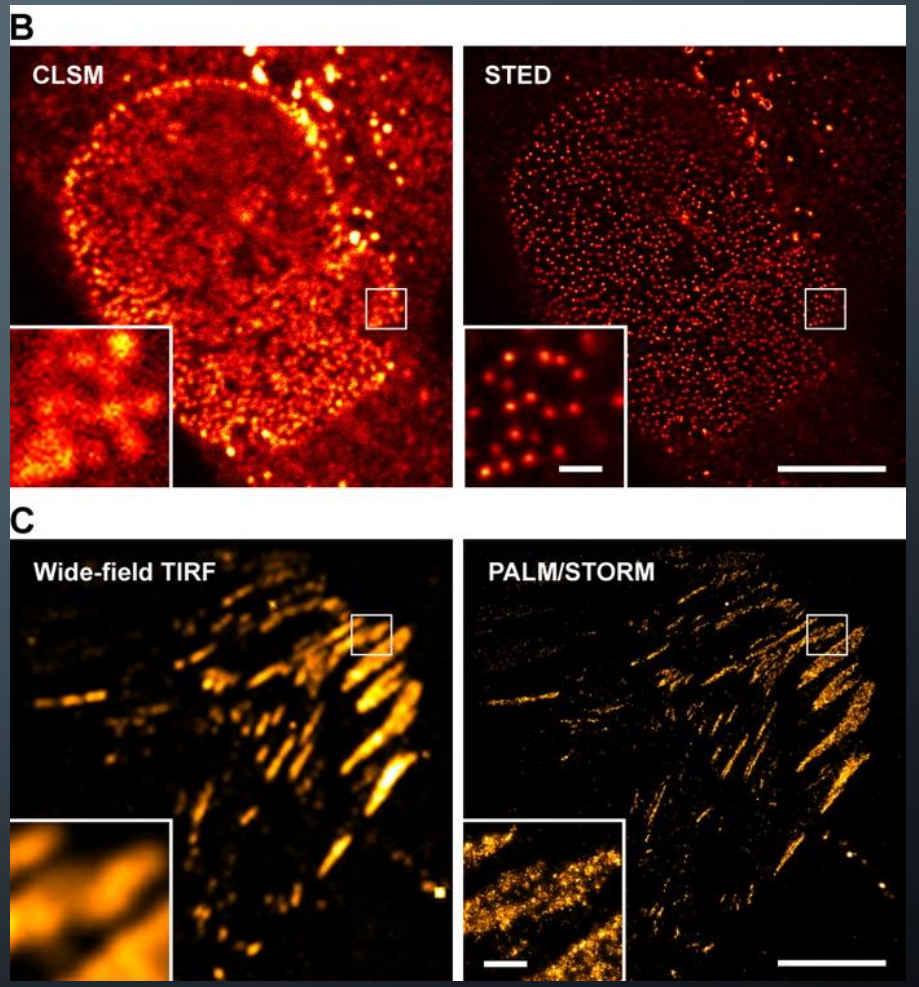
SELECTIVE PLANE ILLUMINATION MICROSCOPY (SPIM)



Jan Huisken,* Jim Swoger, Filippo Del Bene, Joachim Wittbrodt, Ernst H. K. Stelzer*, **Optical Sectioning Deep Inside Live Embryos by Selective Plane Illumination Microscopy,** Science, Vol. 103, p. 1007-1009, 2004

SUPER-RESOLUTION MICROSCOPY OF BIOLOGICAL SAMPLES.



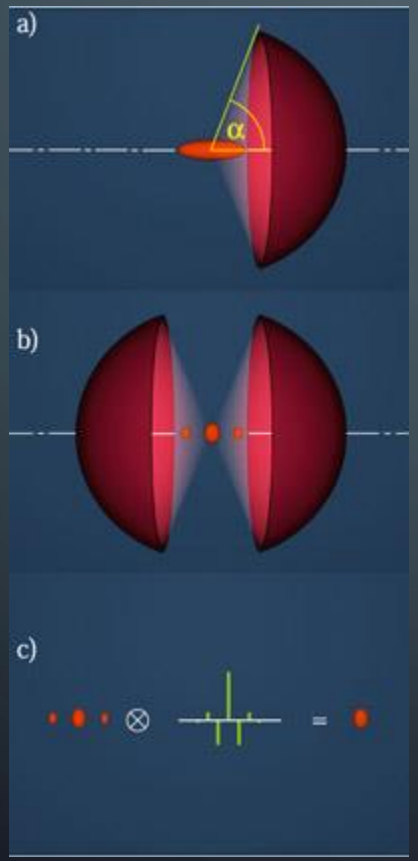


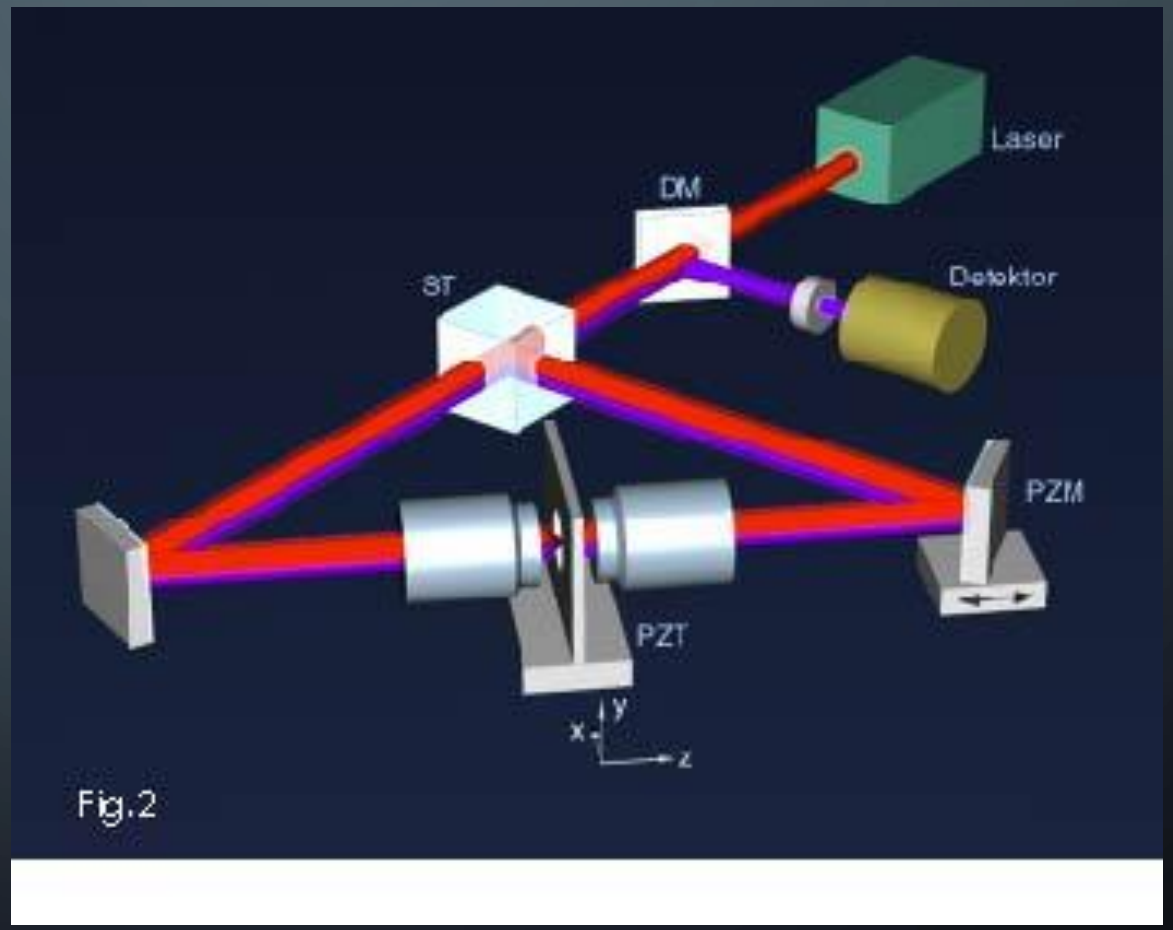
Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. “A guide to super-resolution fluorescence microscopy.” *The Journal of Cell Biology* 190 (2) (July 26): 165 - 175. doi:10.1083/jcb.201002020.

4-PI MICROSCOPY

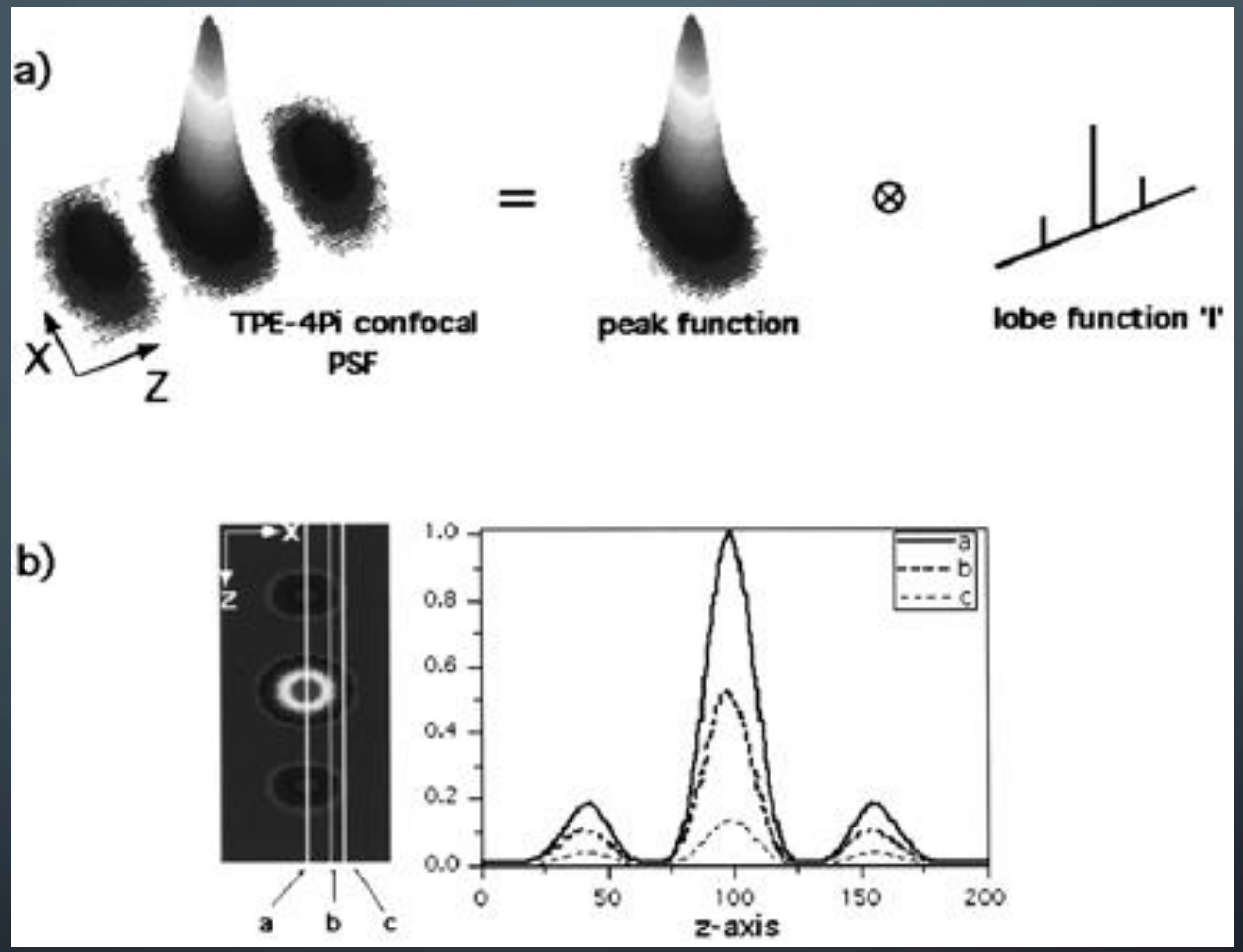
4pi Microscopy: Improves Axial Resolution

Excite high NA top and bottom

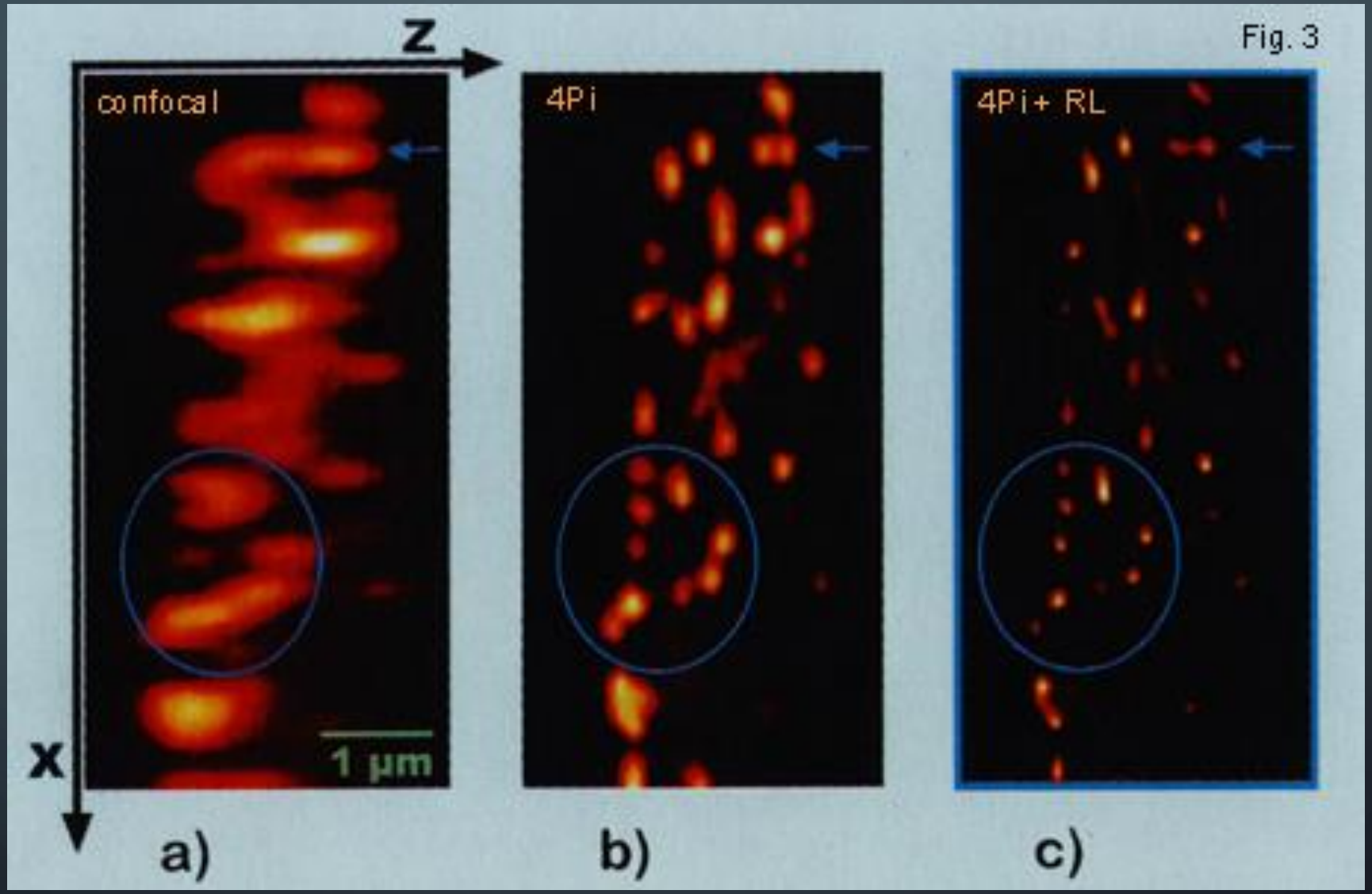




Standing Wave interference makes sidelobes

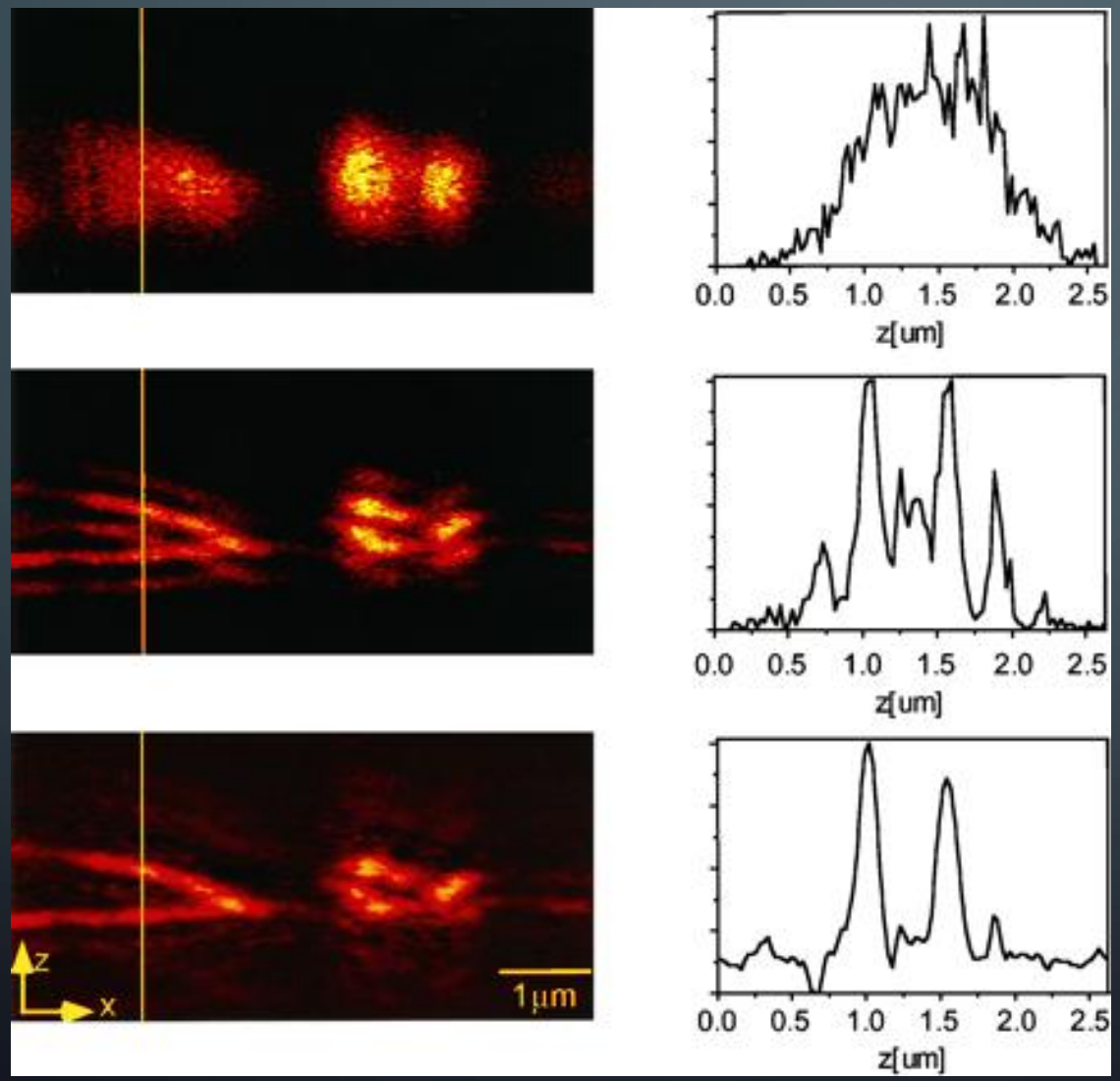


Need deconvolution to remove sidelobes from image



The resolution is largely given by the extent of the effective 4Pi-spot, which is 3-5 times sharper than the spot of a regular confocal microscope

~100 nm Axial Resolution

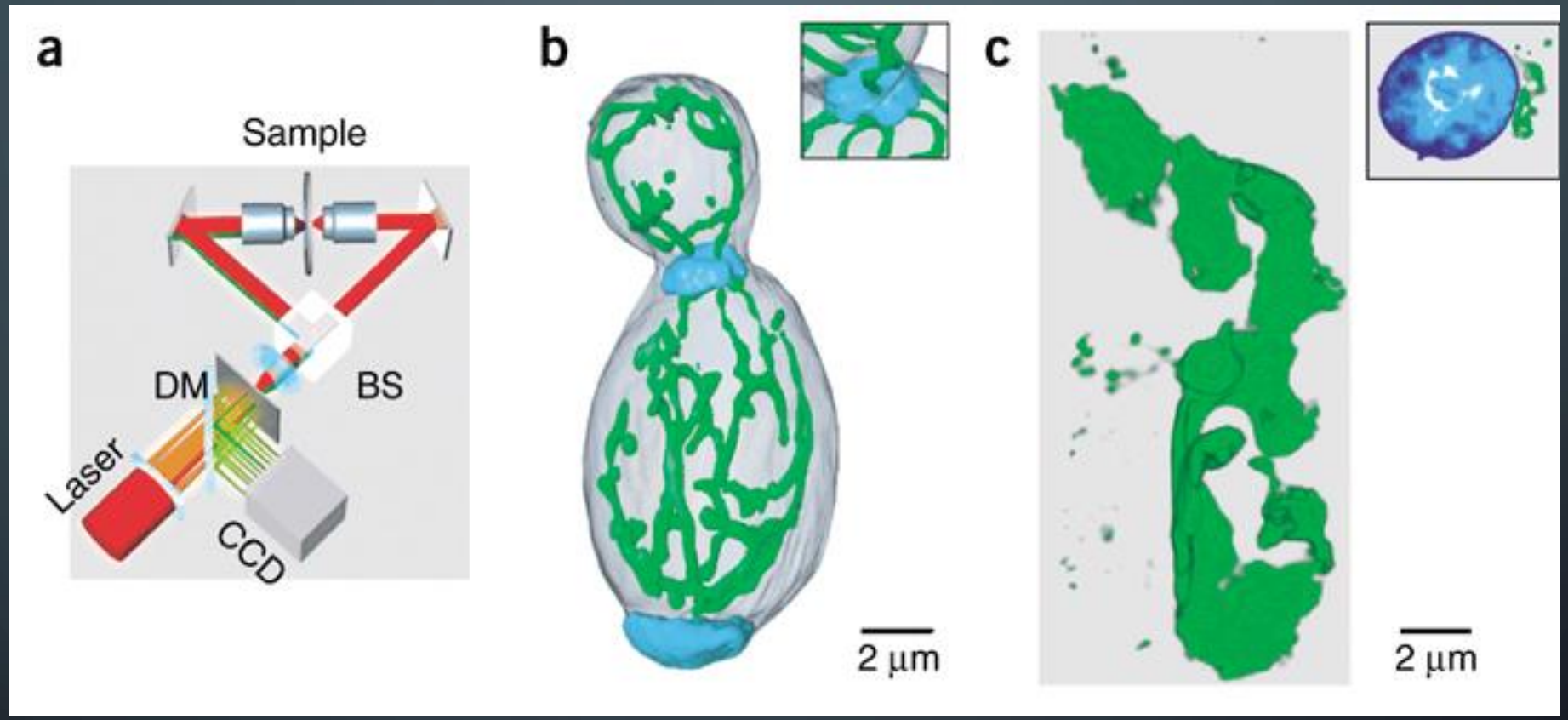


2-photon confocal

2-photon 4pi

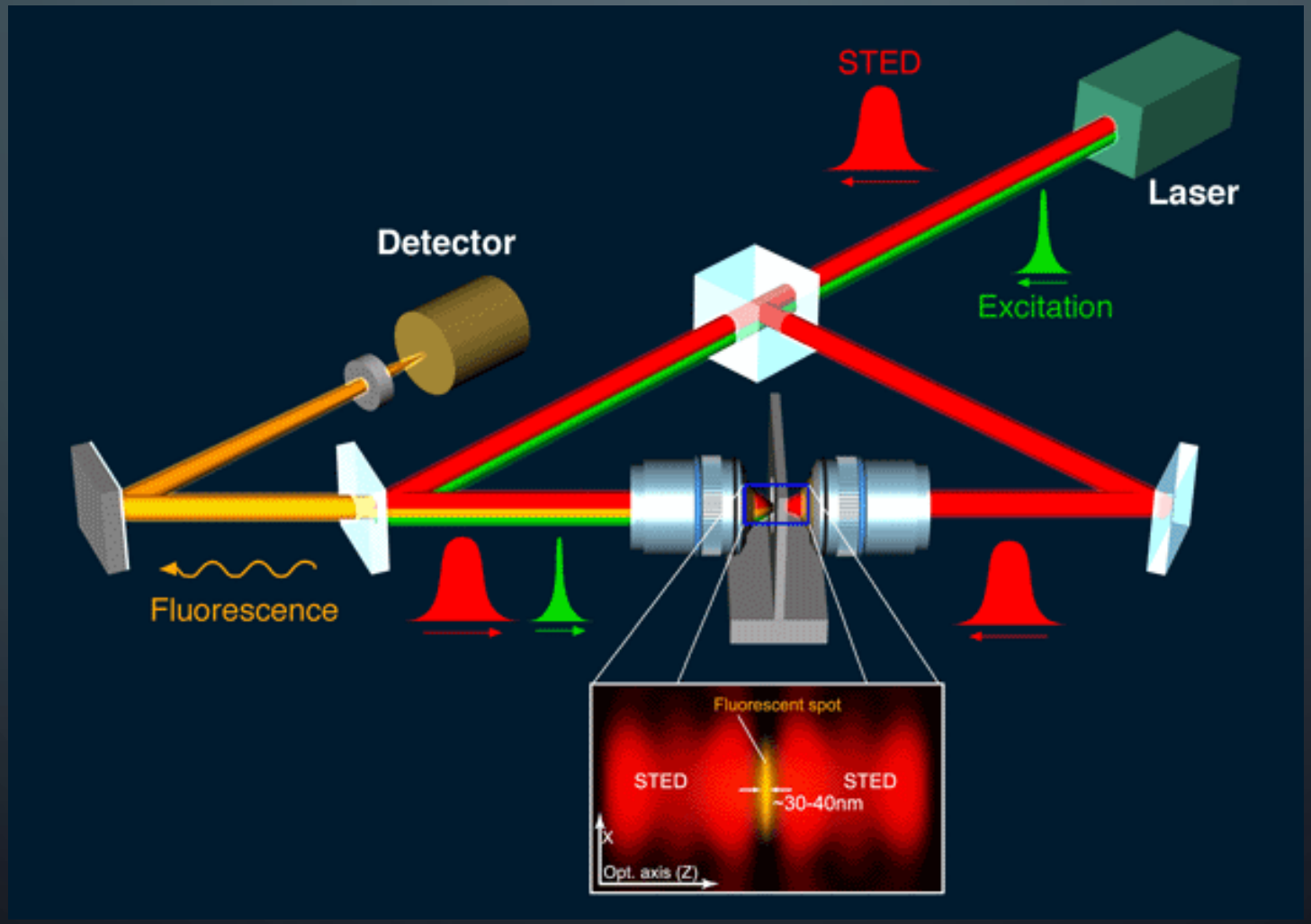
2-photon 4pi
With sidelobes gone

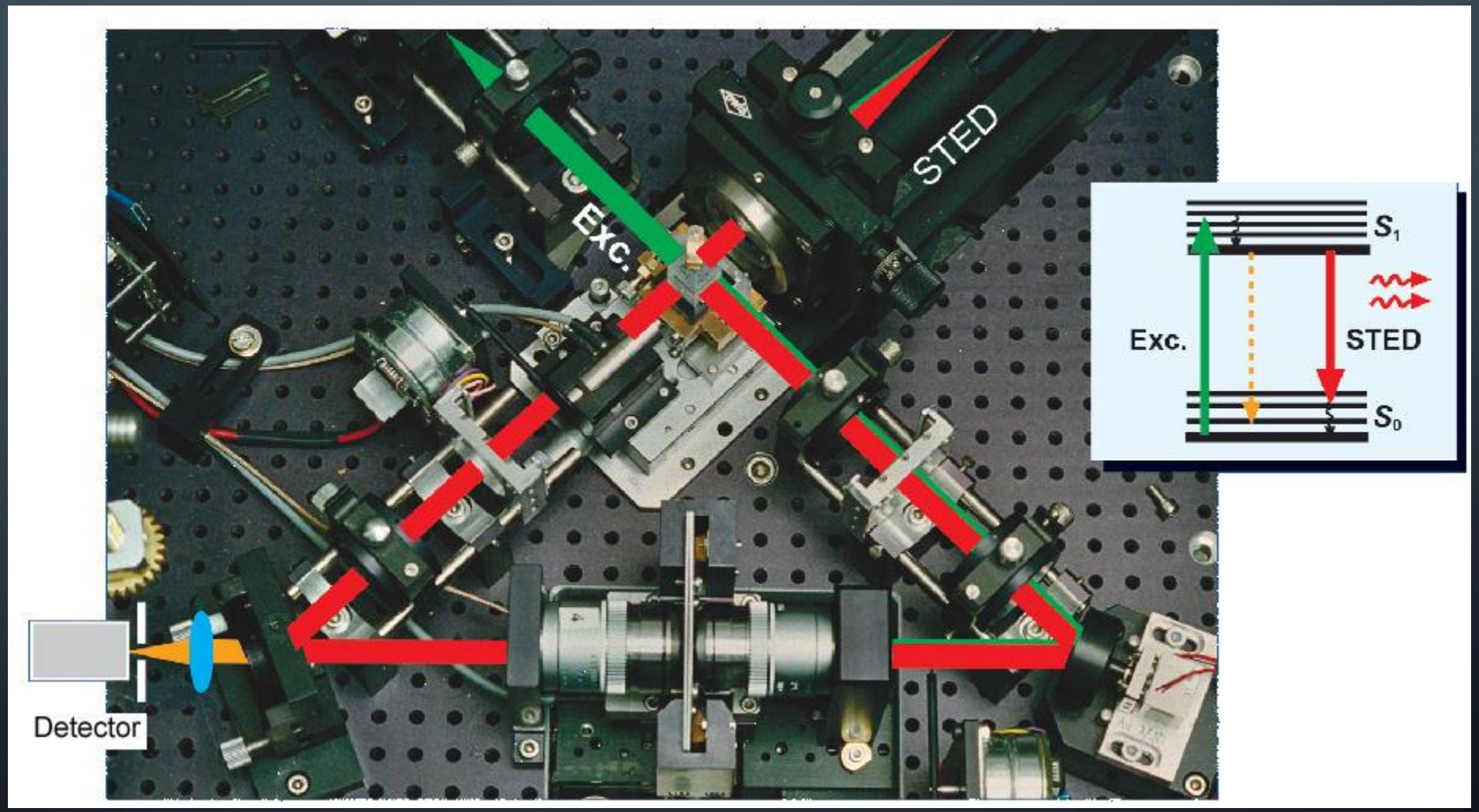
4-pi scope readily works for cell imaging



GFP-labeled mitochondrial compartment of live *Saccharomyces cerevisiae*.

Combine STED with 4 pi for improved 3D resolution
Over STED or 4Pi alone




Exc.
STED
Detector
S_1
Exc.
STED
S_0