The Abdus Salam
International Centre for Theoretical Physics

United Nations
Educational, Scientific and
Cultural Organization

IAEA
International Atomic Energy Agency

1932-9

Winter College on Micro and Nano Photonics for Life Sciences

11 - 22 February 2008

Nanoscale Resolution in Far-field Fluorescence Microscopy (part I &II)

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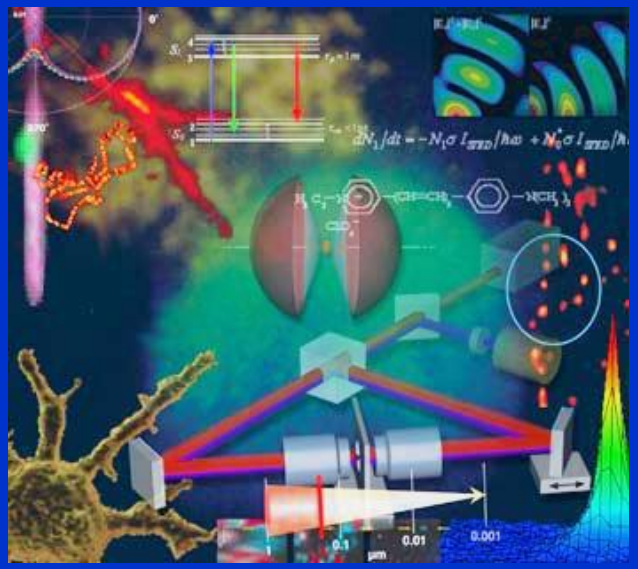
Nanoscale Resolution

in

Far-field Fluorescence Microscopy

Part I

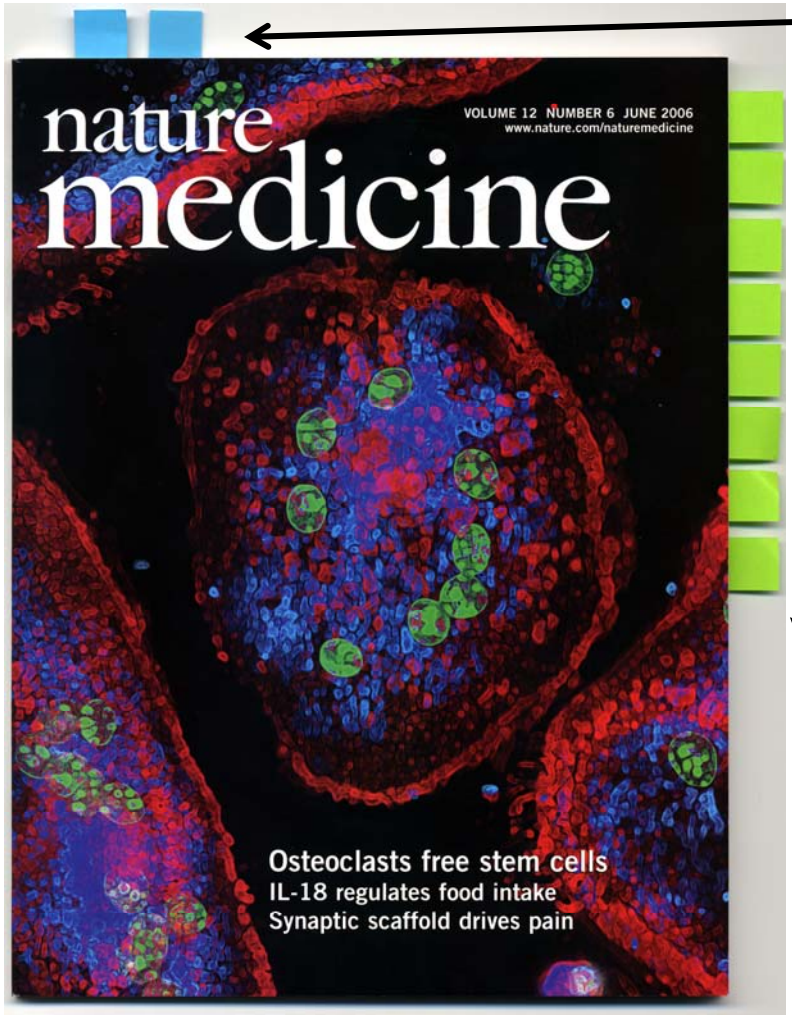
Basics and Axial Resolution Improvement



Stefan W. Hell

Max Planck Institute for Biophysical Chemistry
Department of NanoBiophotonics
Göttingen, Germany

Fluorescence microscopy importance in the life sciences:



Electron microscopy, etc.

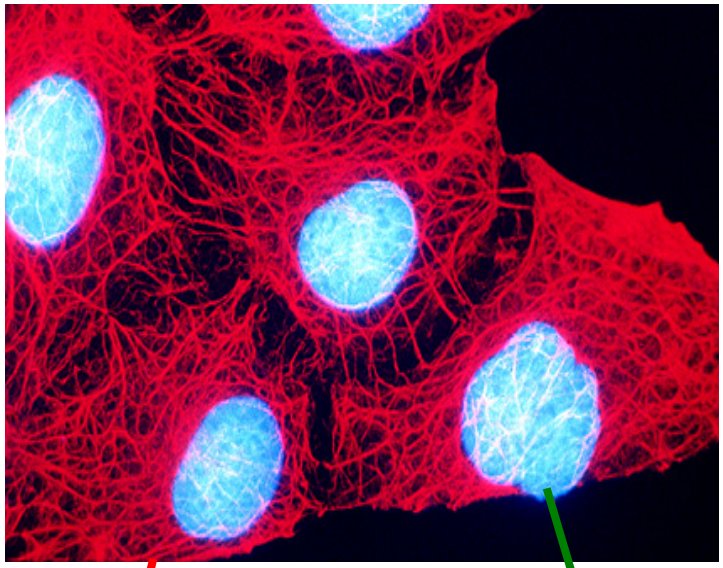
20 %

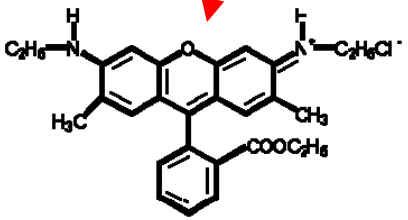
Ratio

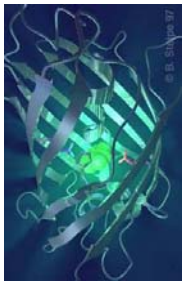
80 %

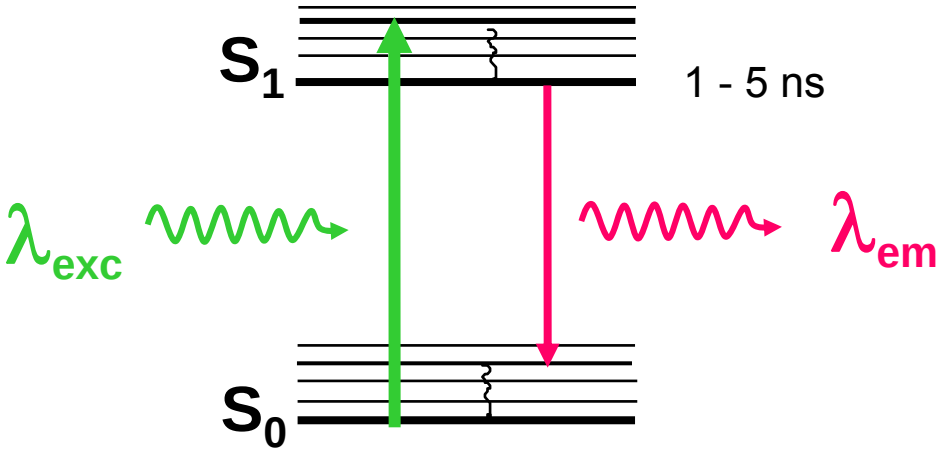
Fluorescence Microscopy

Fluorescence excitation and emission of labels:



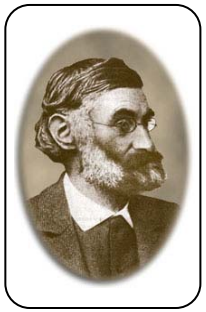




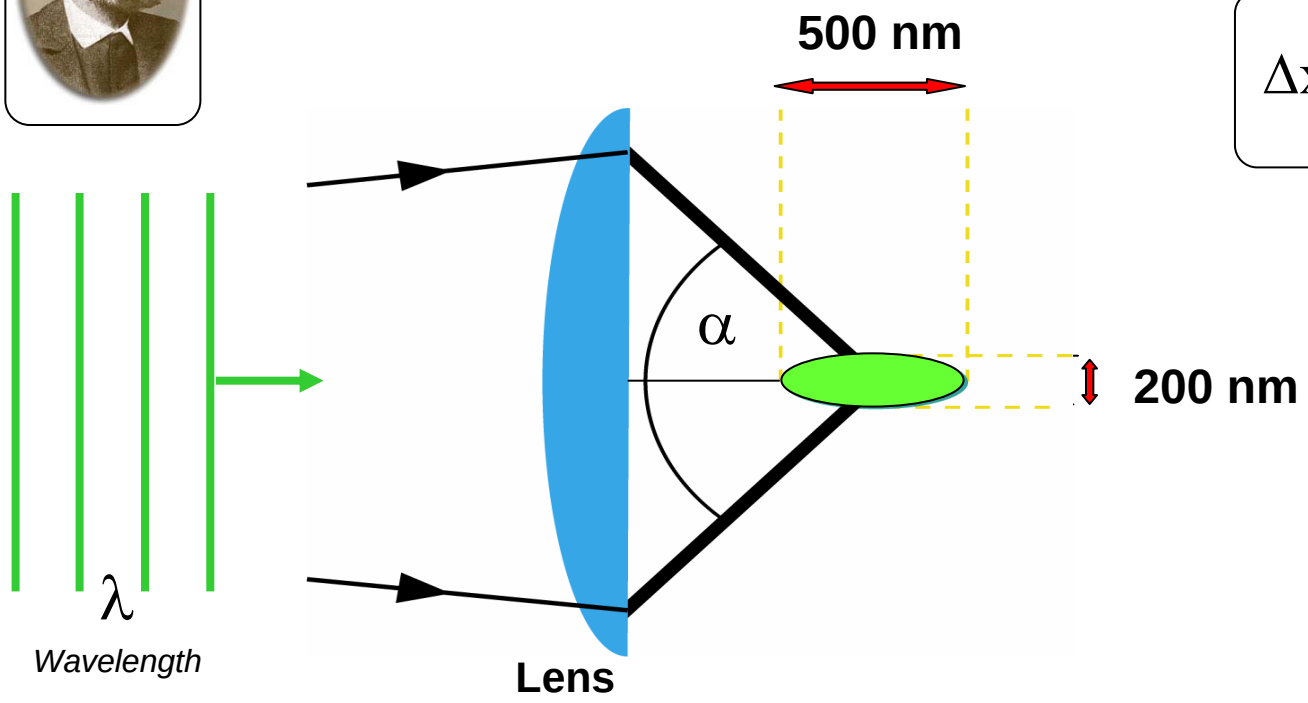


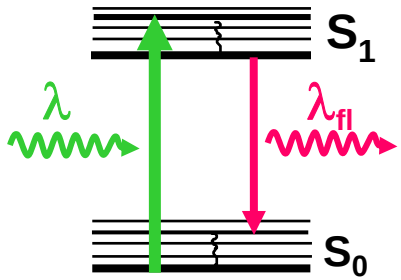
Synth. organic fuorophore

Fluoresc. Proteins (GFP)



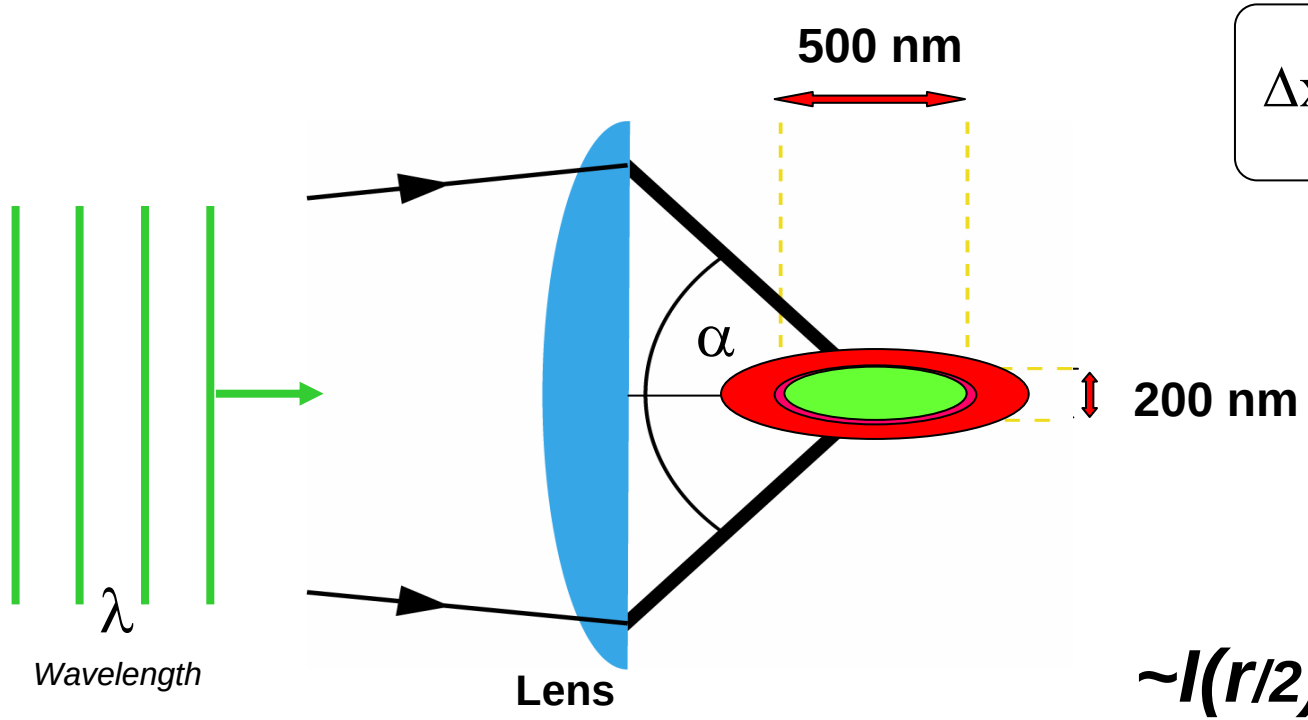
$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$





Sheppard, Kompfner, *Appl Opt.* (1978)

Denk, Strickler, Webb, *Science* (1990)



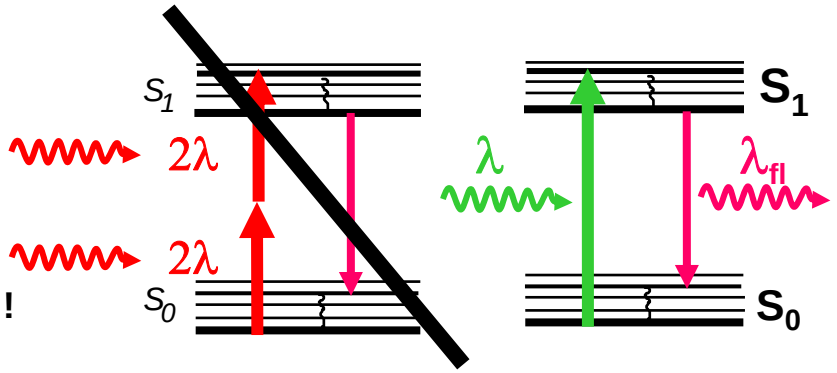
$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$

$\sim I(r/2)^2$ $\sim I(r)$

Multiphoton processes

<u>don't work</u>

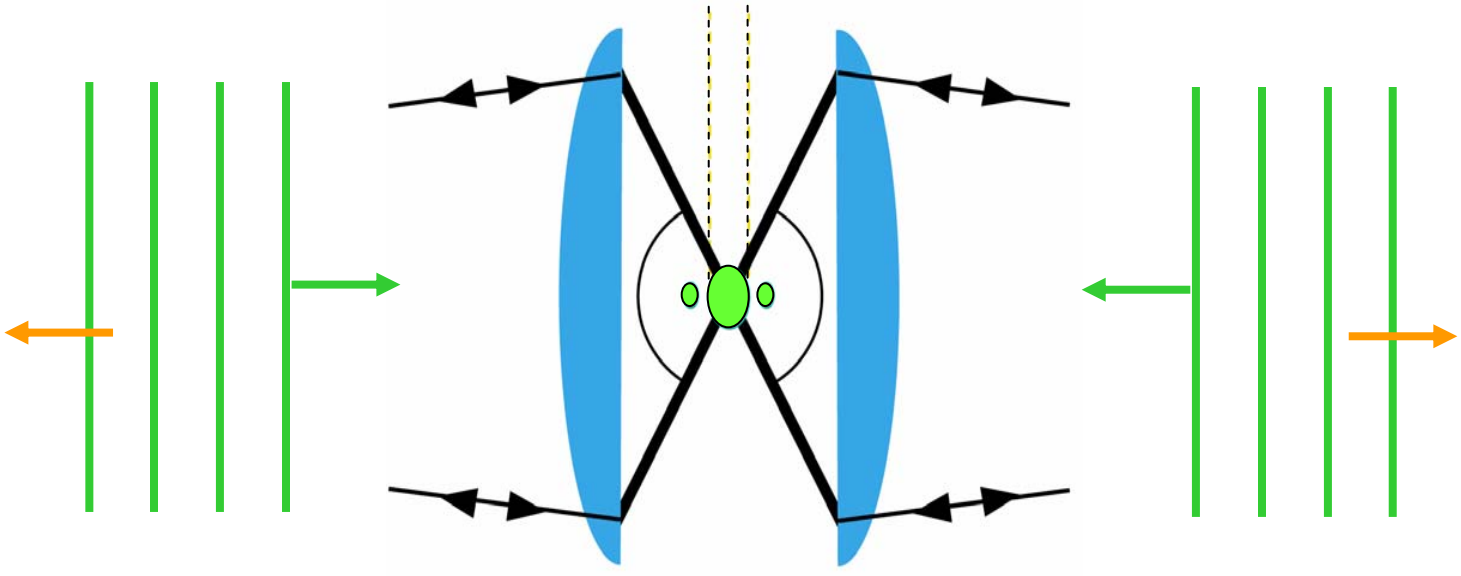
for resolution improvement !



Z- resolution improvement

4Pi- Microscopy:

70 - 140 nm



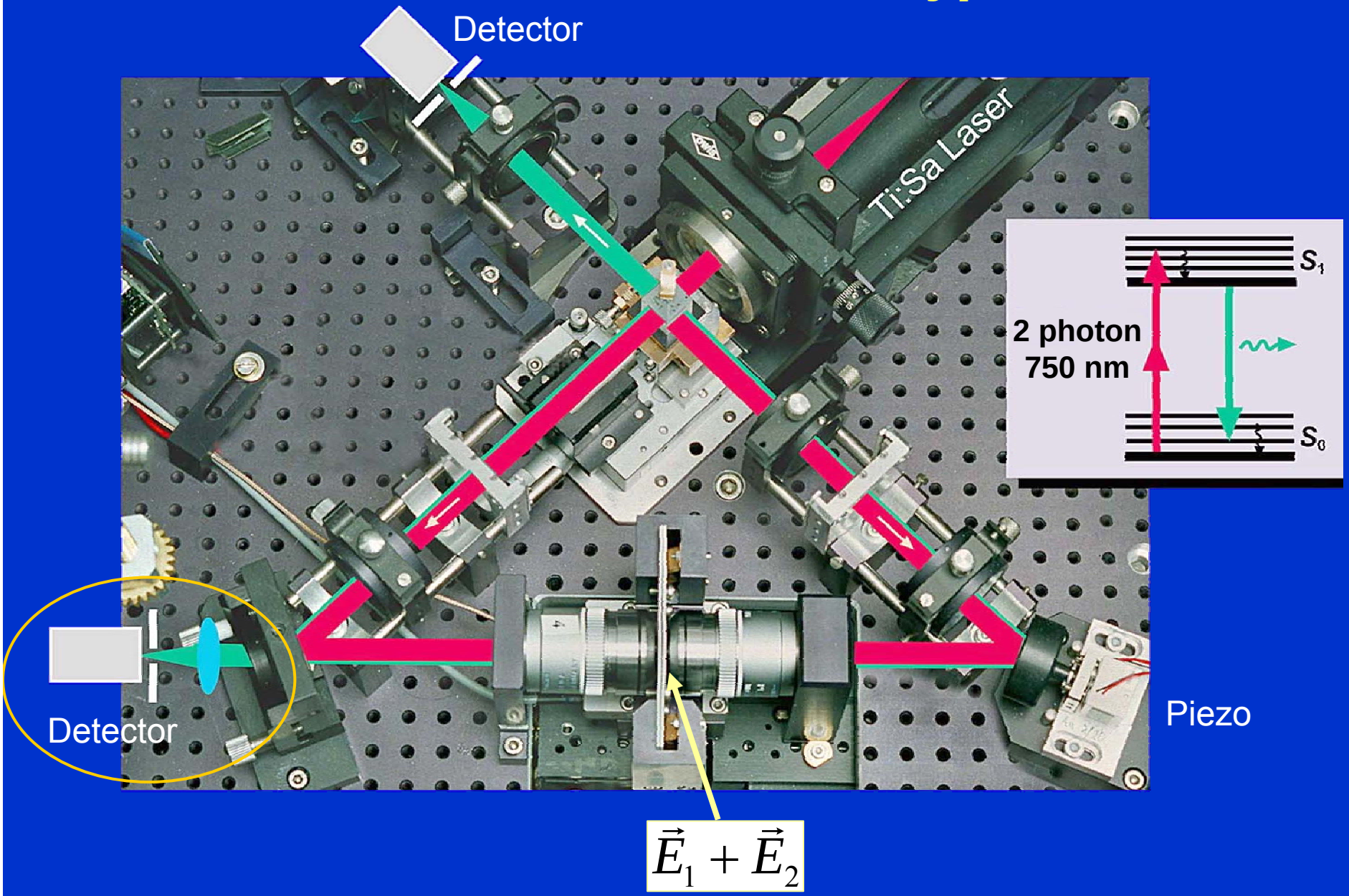
$$\vec{E}^{4Pi}(r,z,\varphi) = \vec{E}_1(r,z,\varphi) + \vec{E}_2(r,-z,\varphi)$$

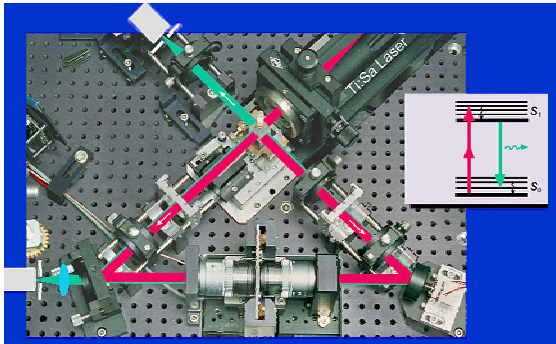
Coherent illumination and/or fluorescence detection

Hell, *Europ Patent* OS 0491289 (1990)
Hell, et al JOSA A (1992)
Hell, et al , *Appl. Phys. Lett*., 64, 11 (1994)

1997

4Pi type A





$\sim I(r)$

1-Photon Exc.

$\sim I(r/2)^2$

2-Photon Exc.

Excitation
488 nm

Detection
510 nm

Effective
PSF

Z

X

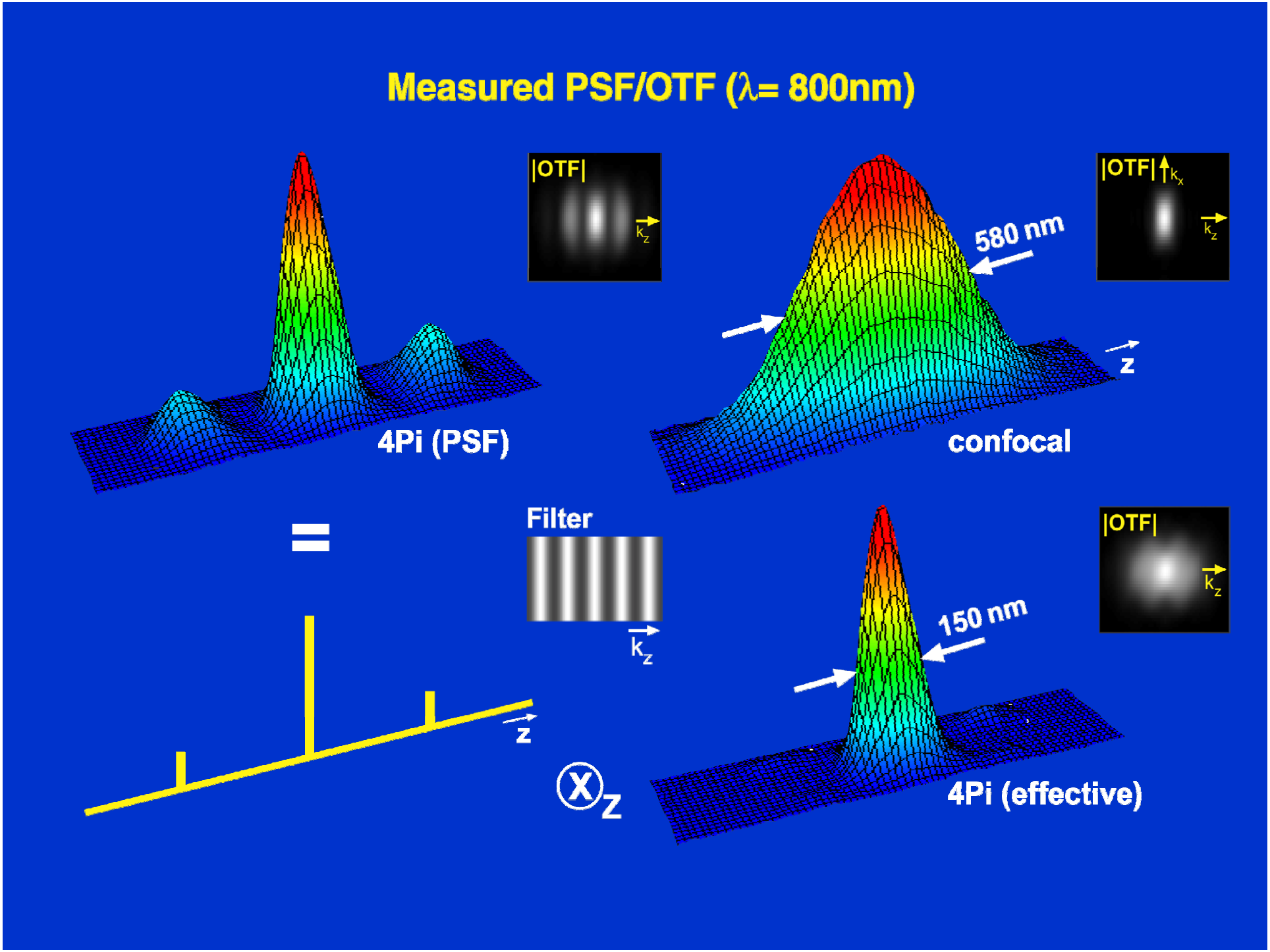
Excitation
800 nm

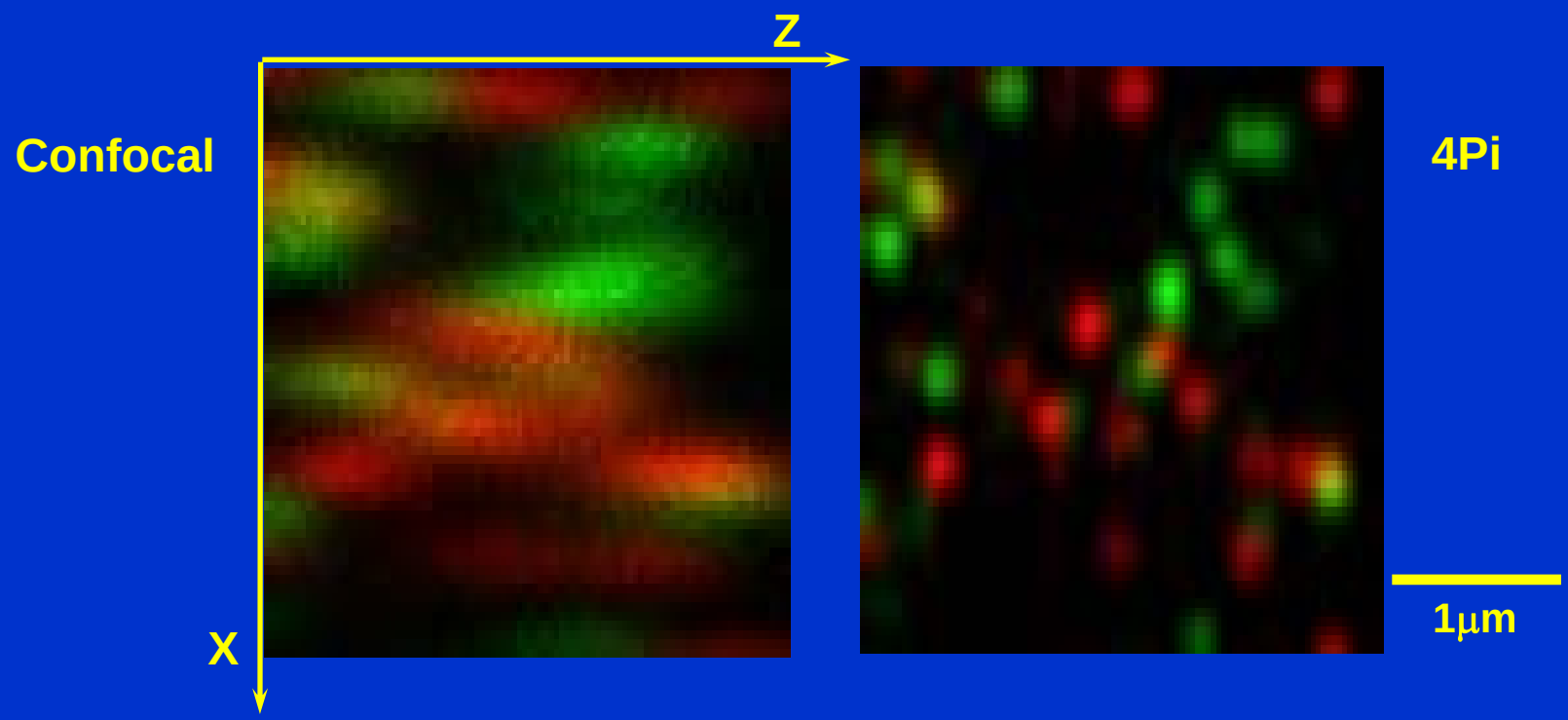
Detection
510 nm

Effective
PSF

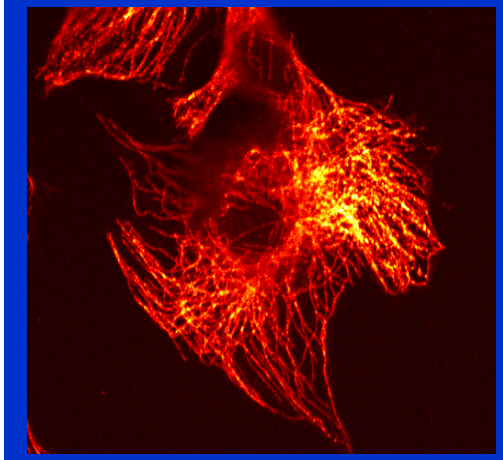
Z

X


Measured PSF/OTF (λ= 800nm)
|OTF|
k_z
4Pi (PSF)
580 nm
|OTF| k_x
k_z
z
confocal
=
Filter
k_z
|OTF|
k_z
150 nm
z
X
Z
4Pi (effective)



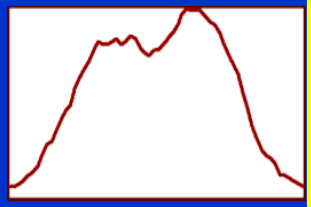
Green and red beads, 100 nm diameter



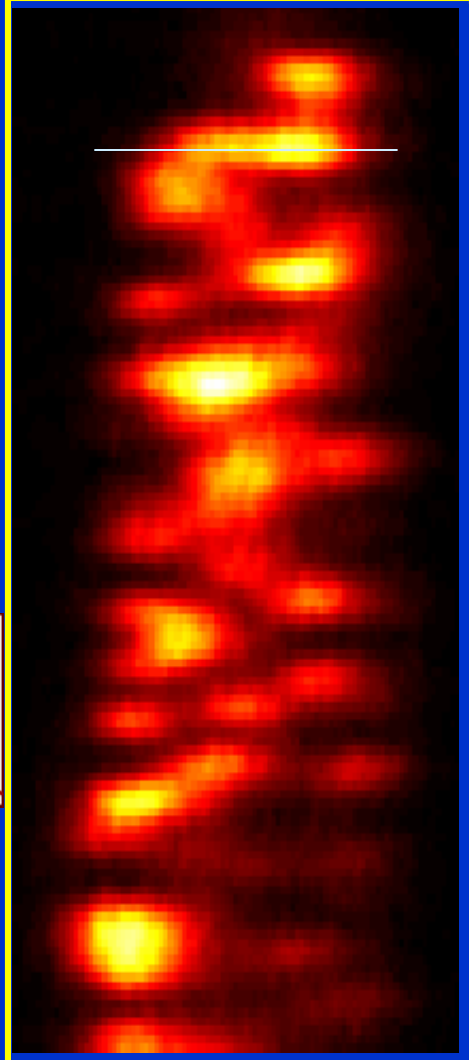
Microtubules, mouse fibroblast
Immunofluor, Oregon Green

Z

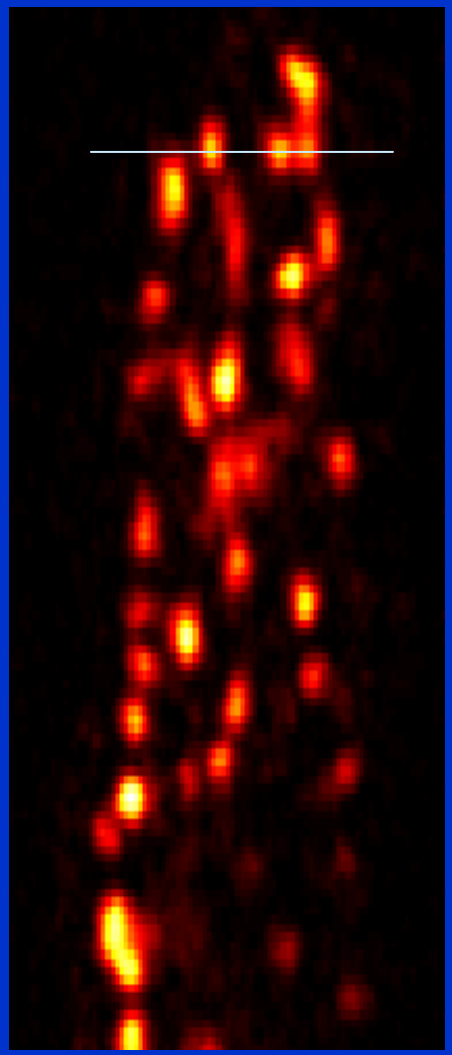
X



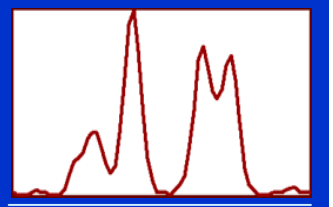
2 µm



confocal



4Pi



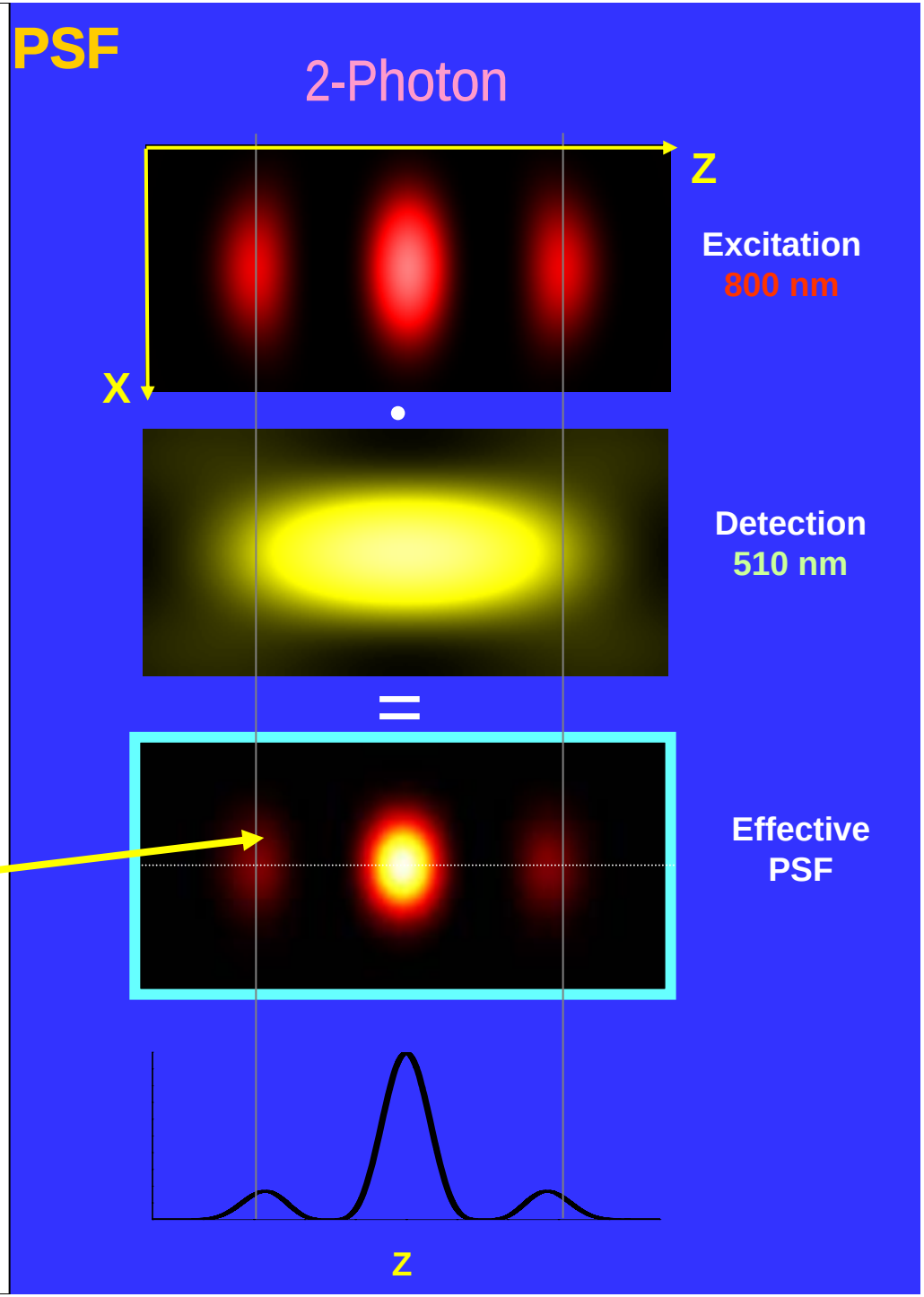
2 µm

How does the linear lobe filter really work ?

Consequence of outer part suppresion is

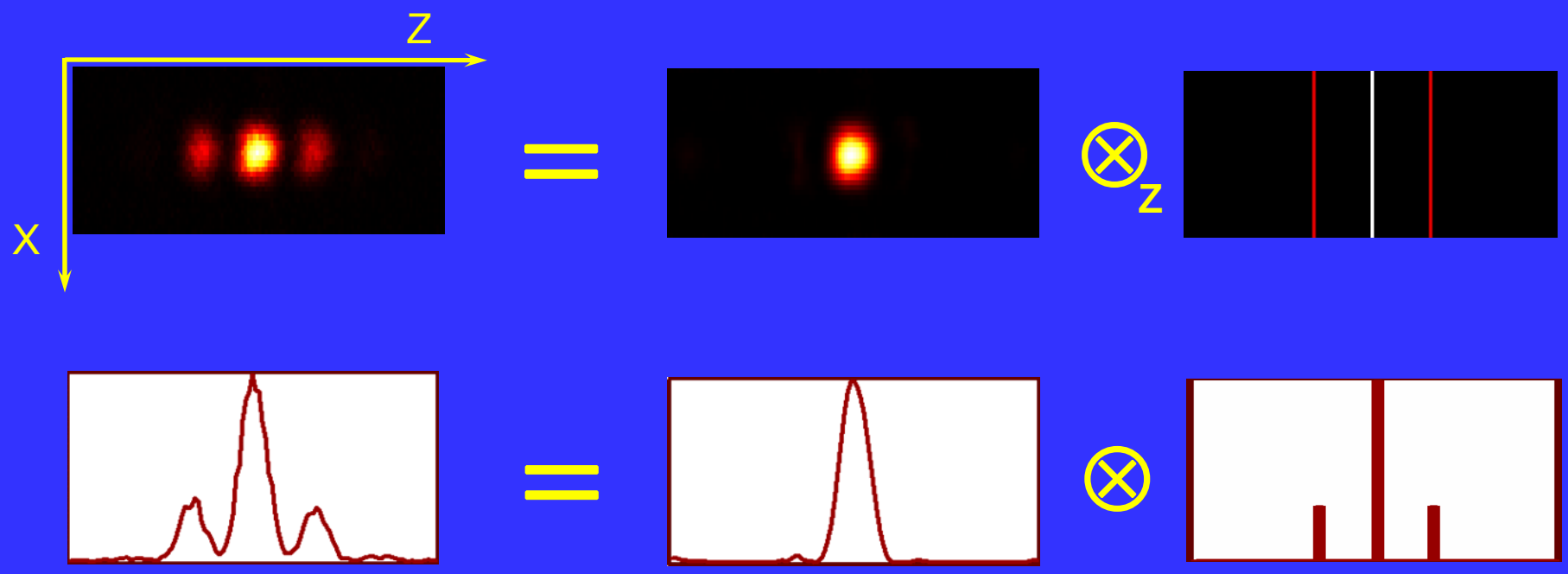
PSF separable in (XY) and (Z)

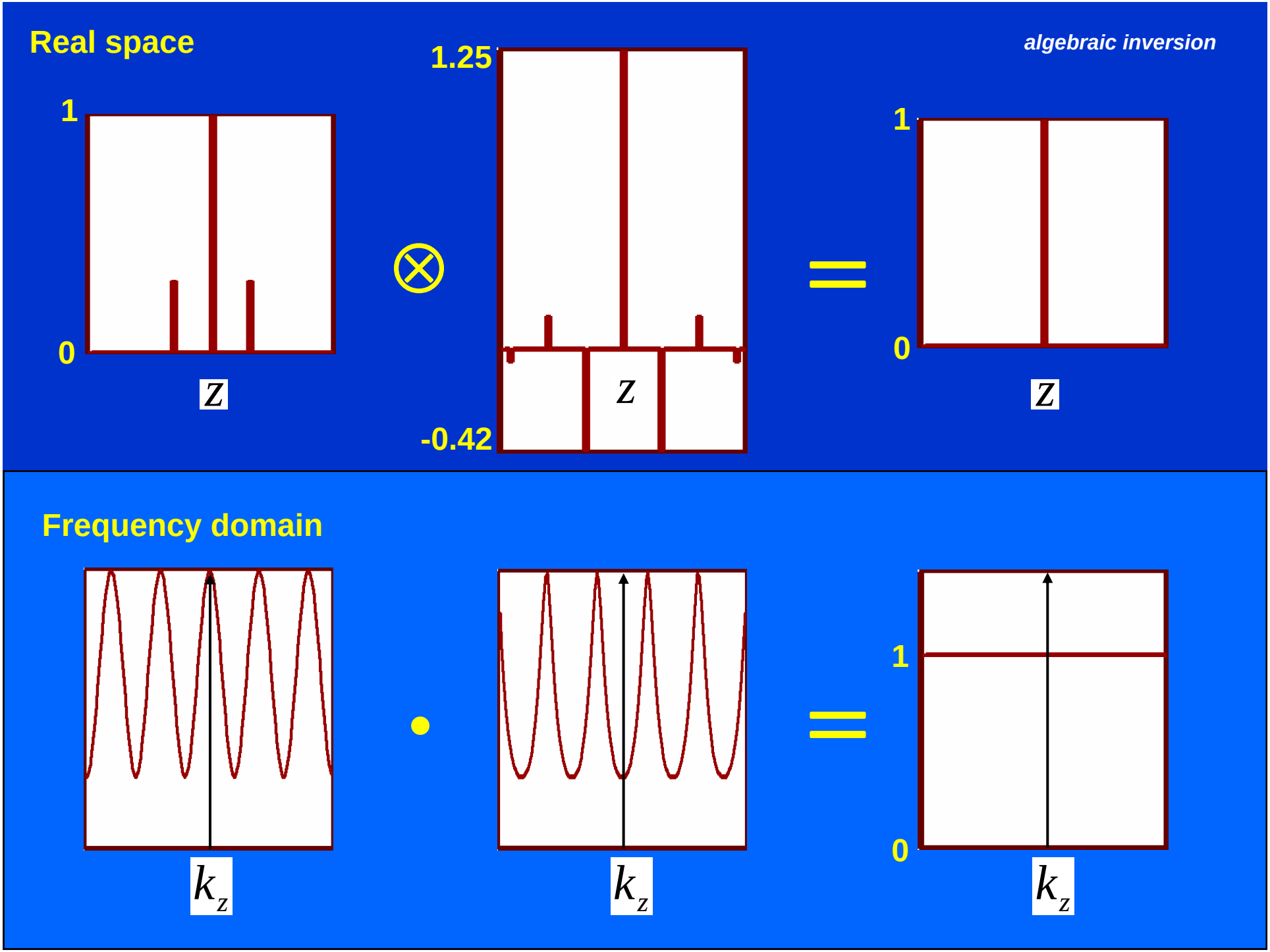
$$h_{4Pi}(x,y,z) \approx c(x,y)h(z)$$



Separability: $h_{4Pi}(x,y,z) \approx c(x,y)h(z)$

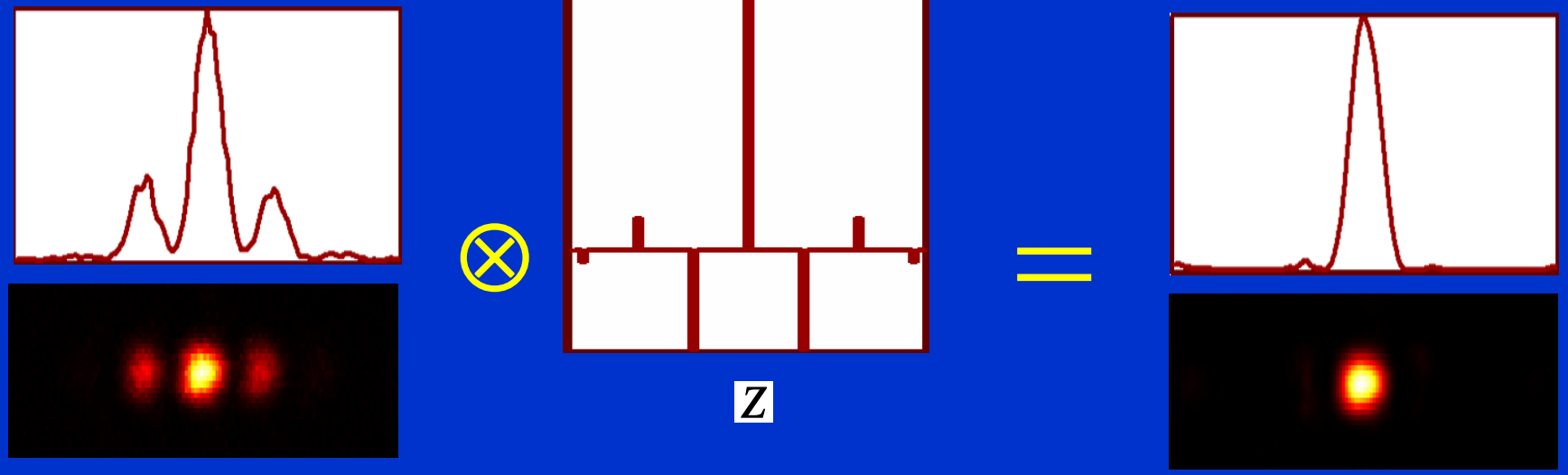
-> treat the lobe problem separately, in the z-direction



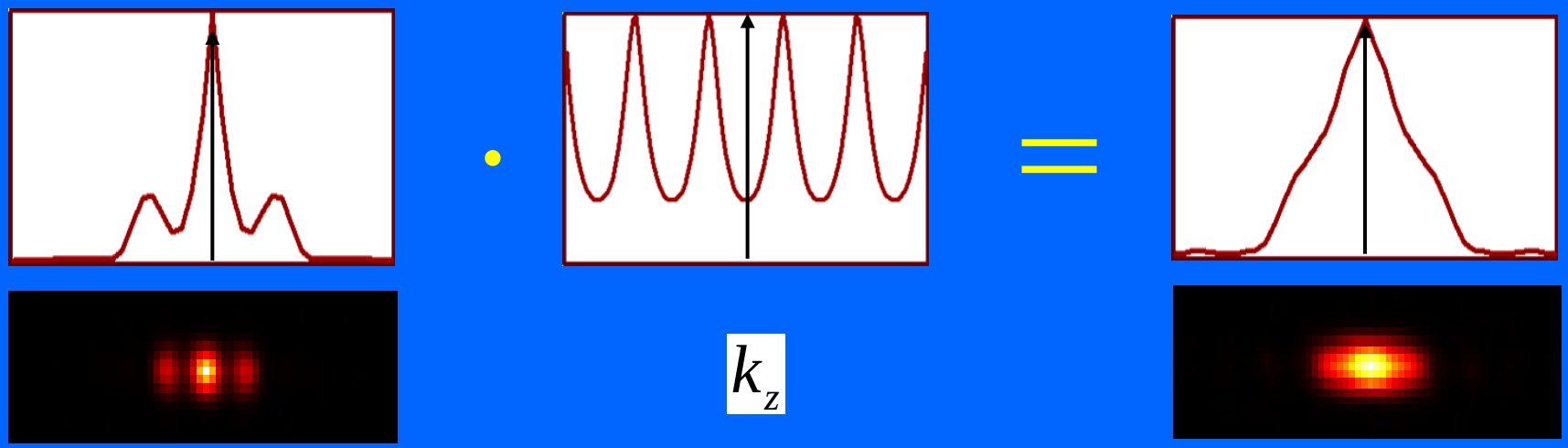

Real space
algebraic inversion
1
0
z
1.25
-0.42
z
1
0
z
Frequency domain
k_z
k_z
1
0
k_z

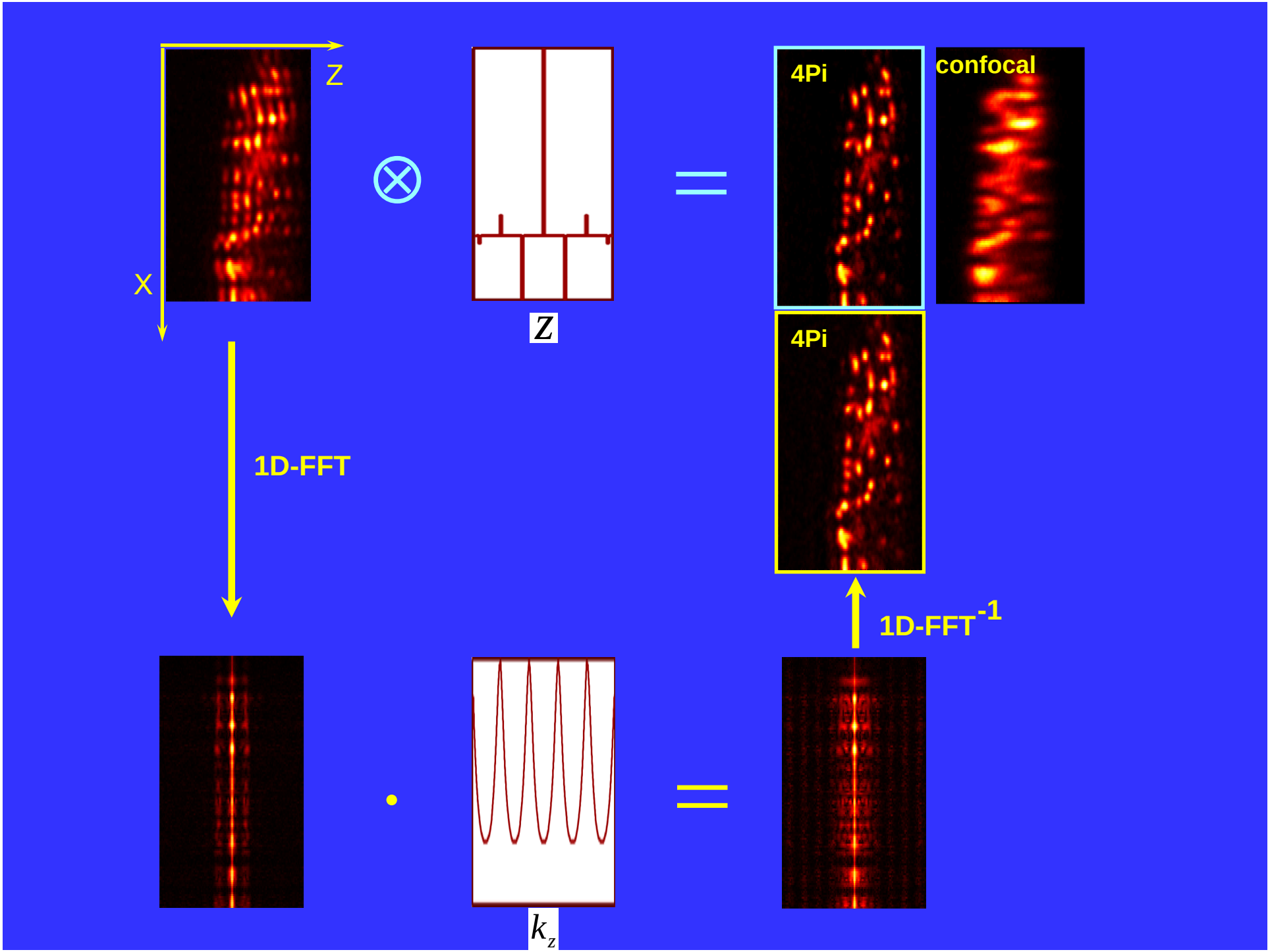
Filtering the PSF

Real space



Frequency domain




Z
X
Z
4Pi
confocal
4Pi
1D-FFT
1D-FFT-1
k_z

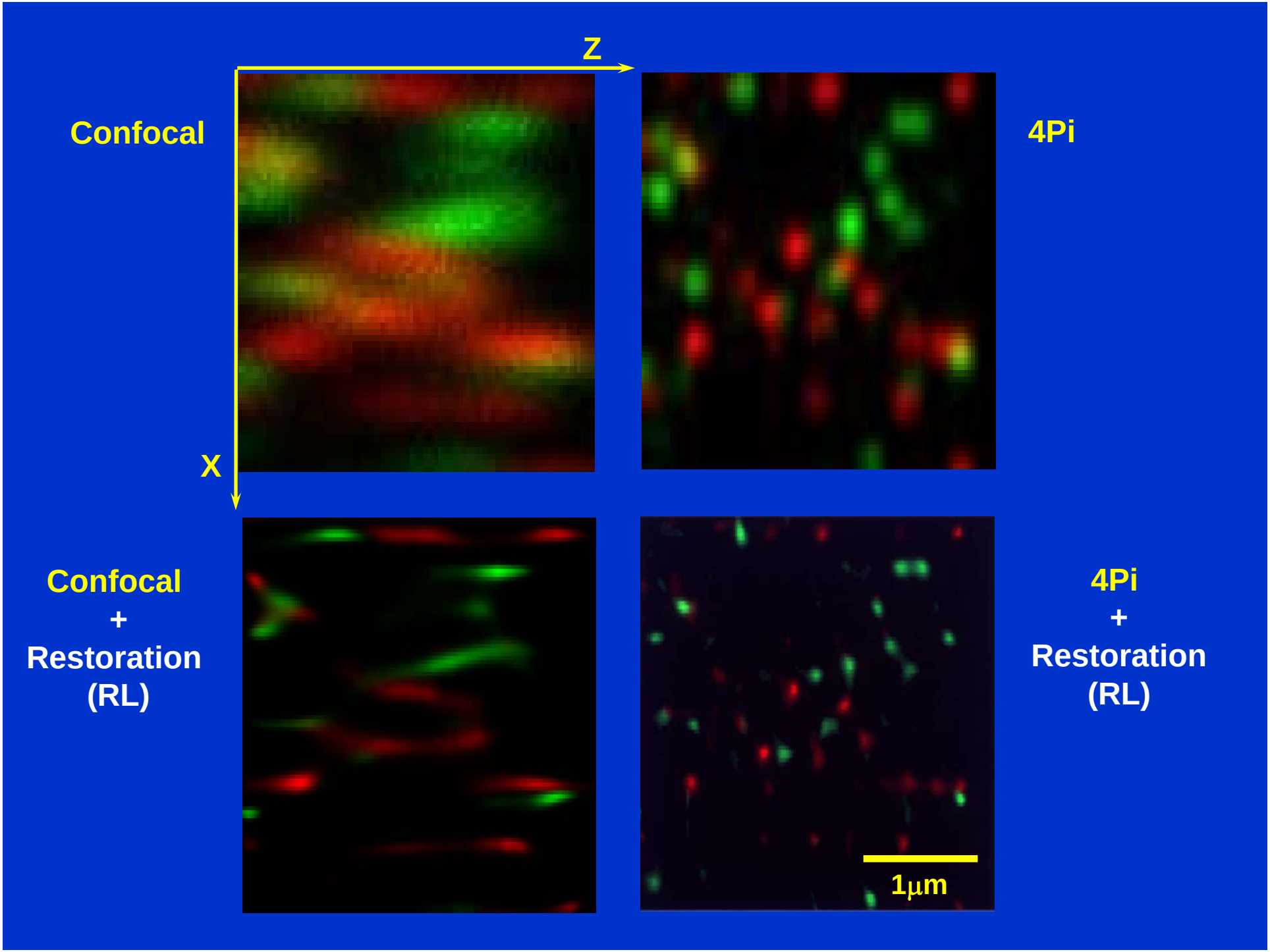
Non-linear image restoration:

- **Know the full PSF (not just height and position of lobes)**

- **<u>Positivity of image</u> (and PSF) assumed**

Examples: ***Richardson-Lucy Algorithm (RL)***

Maximum-Likelihood Estimation Algorithm


Z
X
Confocal
4Pi
Confocal
+
Restoration
(RL)
4Pi
+
Restoration
(RL)
1μm

Microtubules / Mouse fibroblast
Immunofluor. Oregon Green

400 nm

100 nm

400 nm

100 nm

Z

X

Confocal

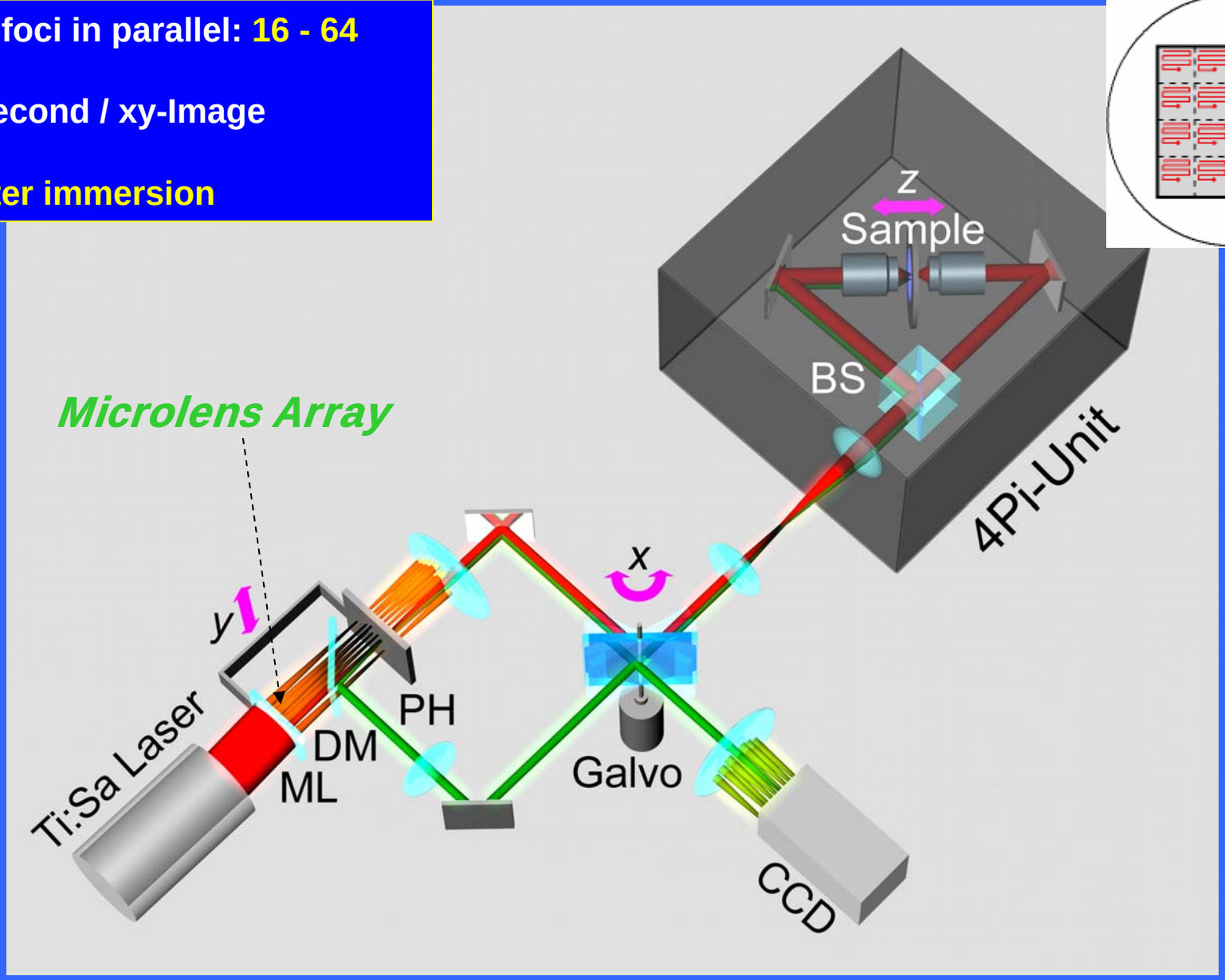
4Pi + restor. (RL)

Confocal

4Pi + restor. (RL)

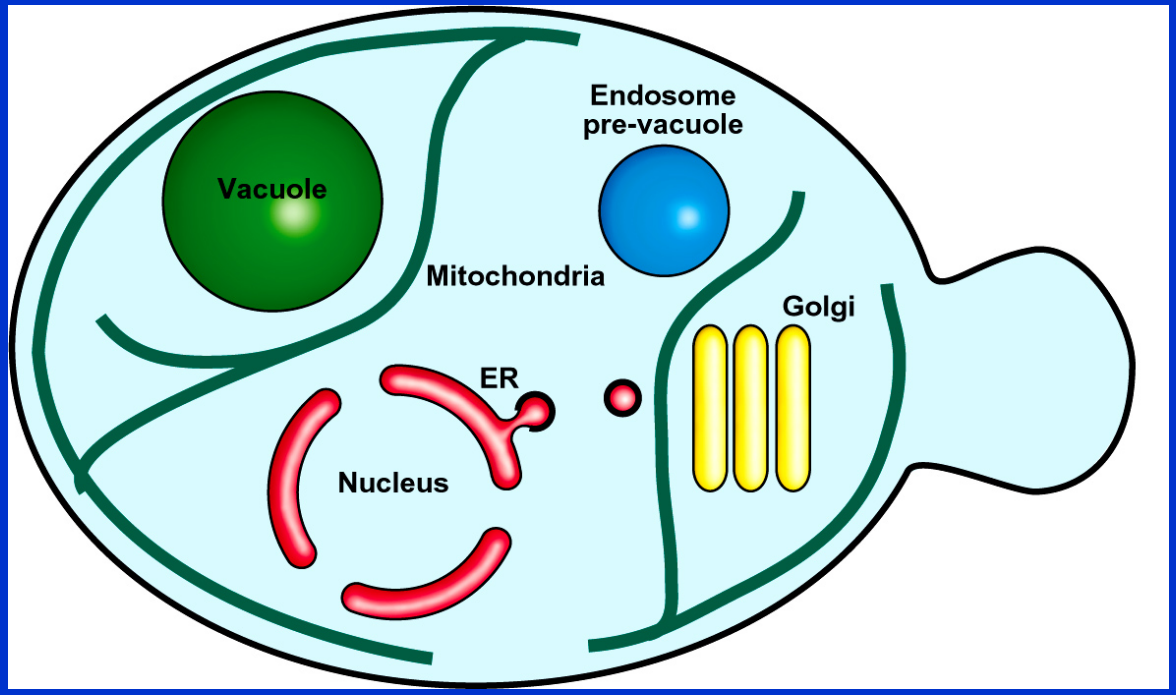
Higher Speed: Multifocal 4Pi - microscopy

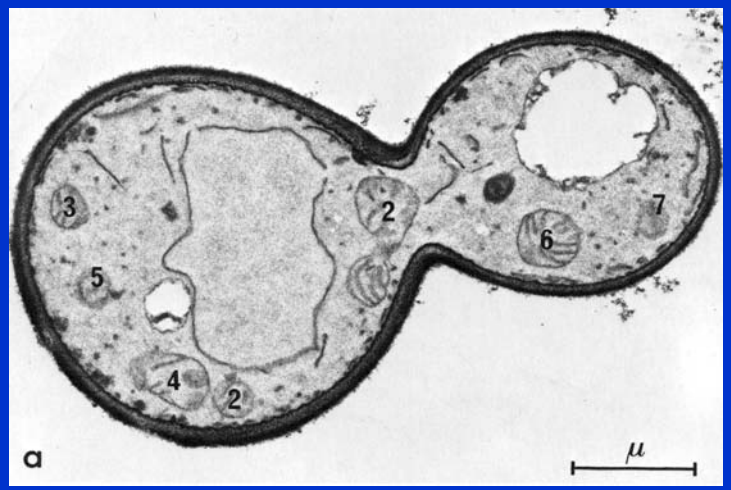
- 4Pi-foci in parallel: 16 - 64
- 1 Second / xy-Image
- Water immersion



Egner,Jakobs & Hell, PNAS, 99, 3370 (2002)

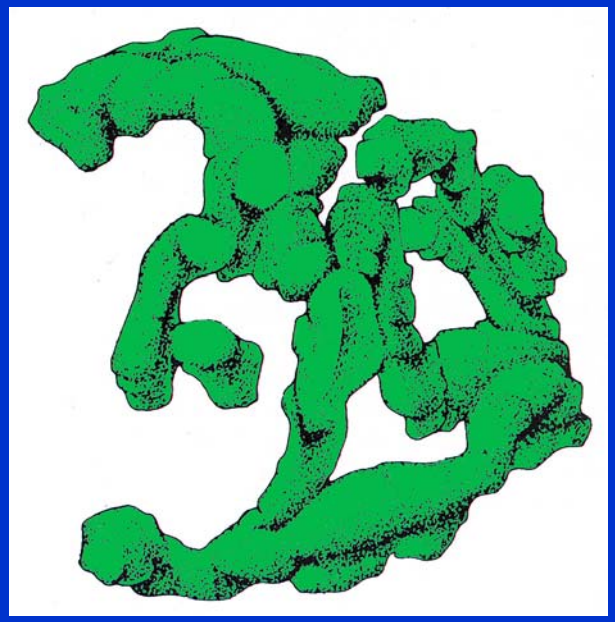
Application: Mitochondrial network in *S. cerevisiae*





3D-reconstruction

Electron Microscopy

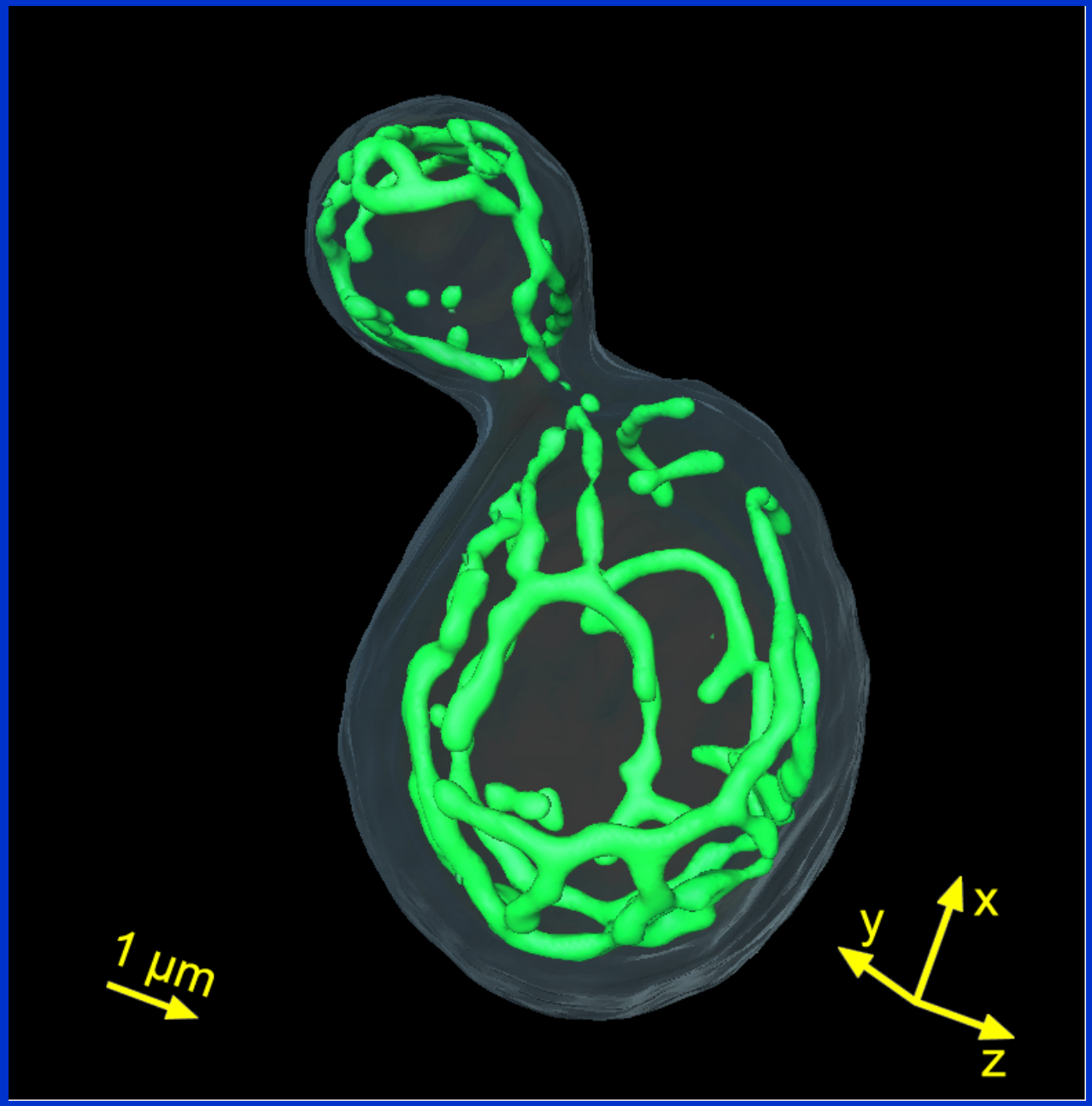


B. J. Stevens & J. G. White, 1975

4Pi microscopy

GFP

Mitochondrial compartment in live yeast @ 100 nm 3D-resolution

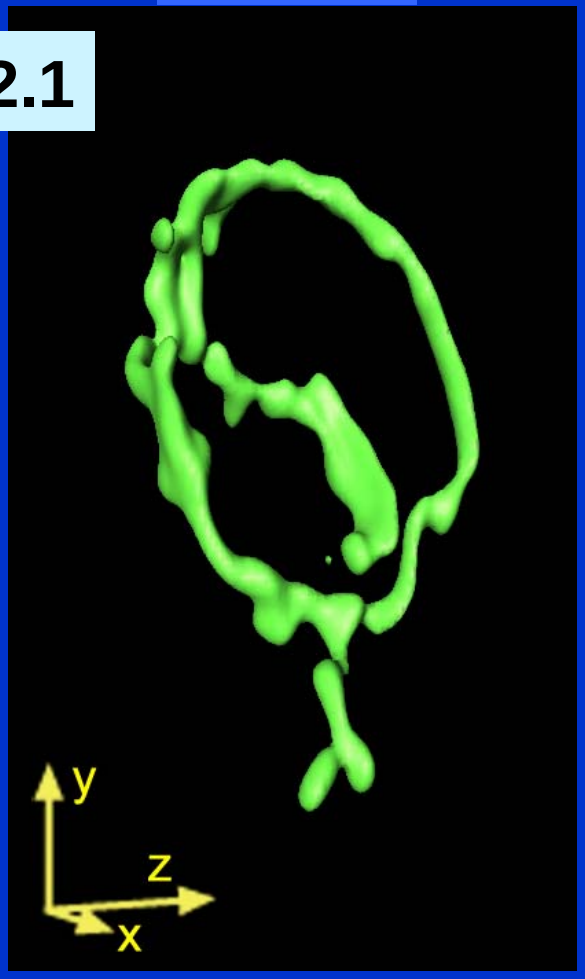


Egner,Jakobs,Hell, PNAS, *(2002)*

→ Number of nodes increases 4-fold

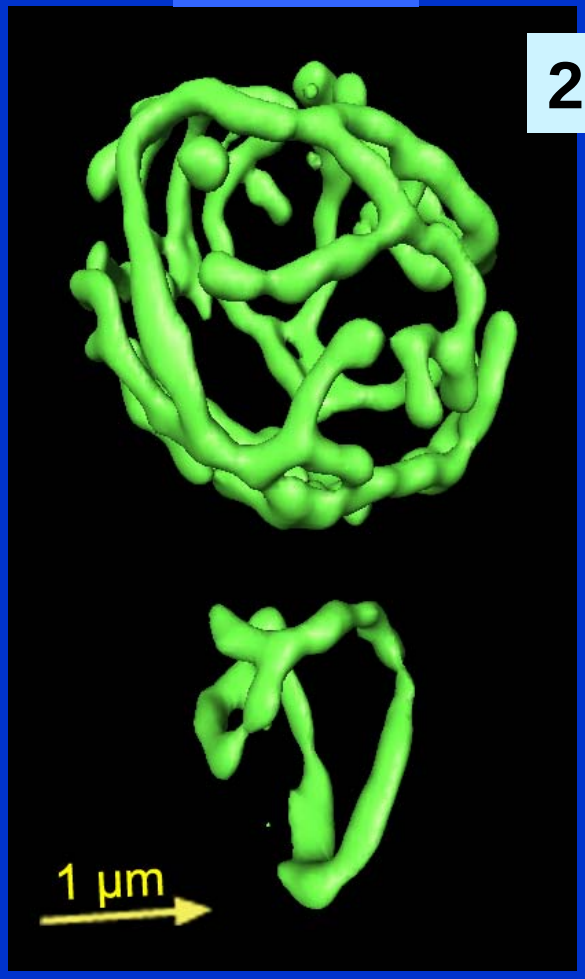
on Glucose

5.8 ± 2.1



on Glycerol

23.0 ± 9.3

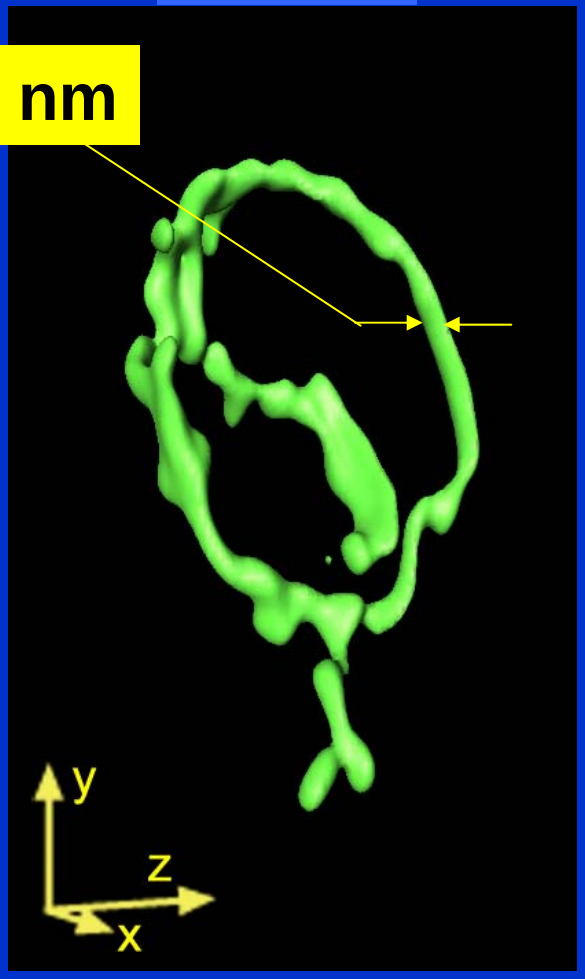


Quantitative live cell 4Pi microscopy

→ **Diameter** increases by 20 nm on average

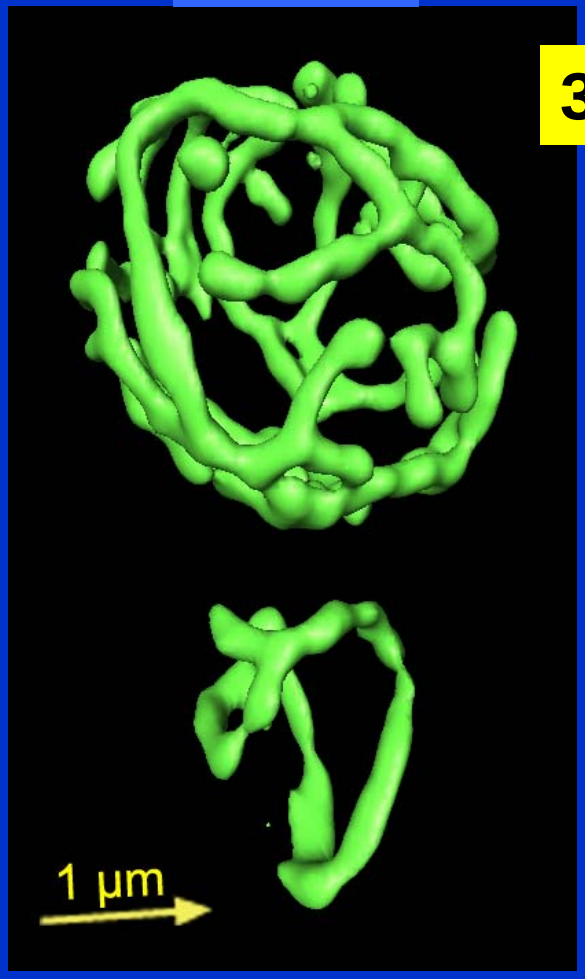
on Glucose

339 ±4 nm



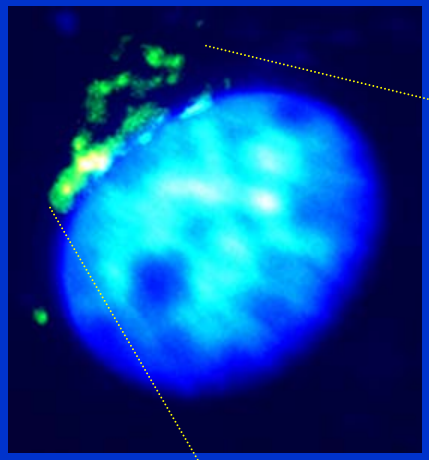
on Glycerol

359 ±5 nm



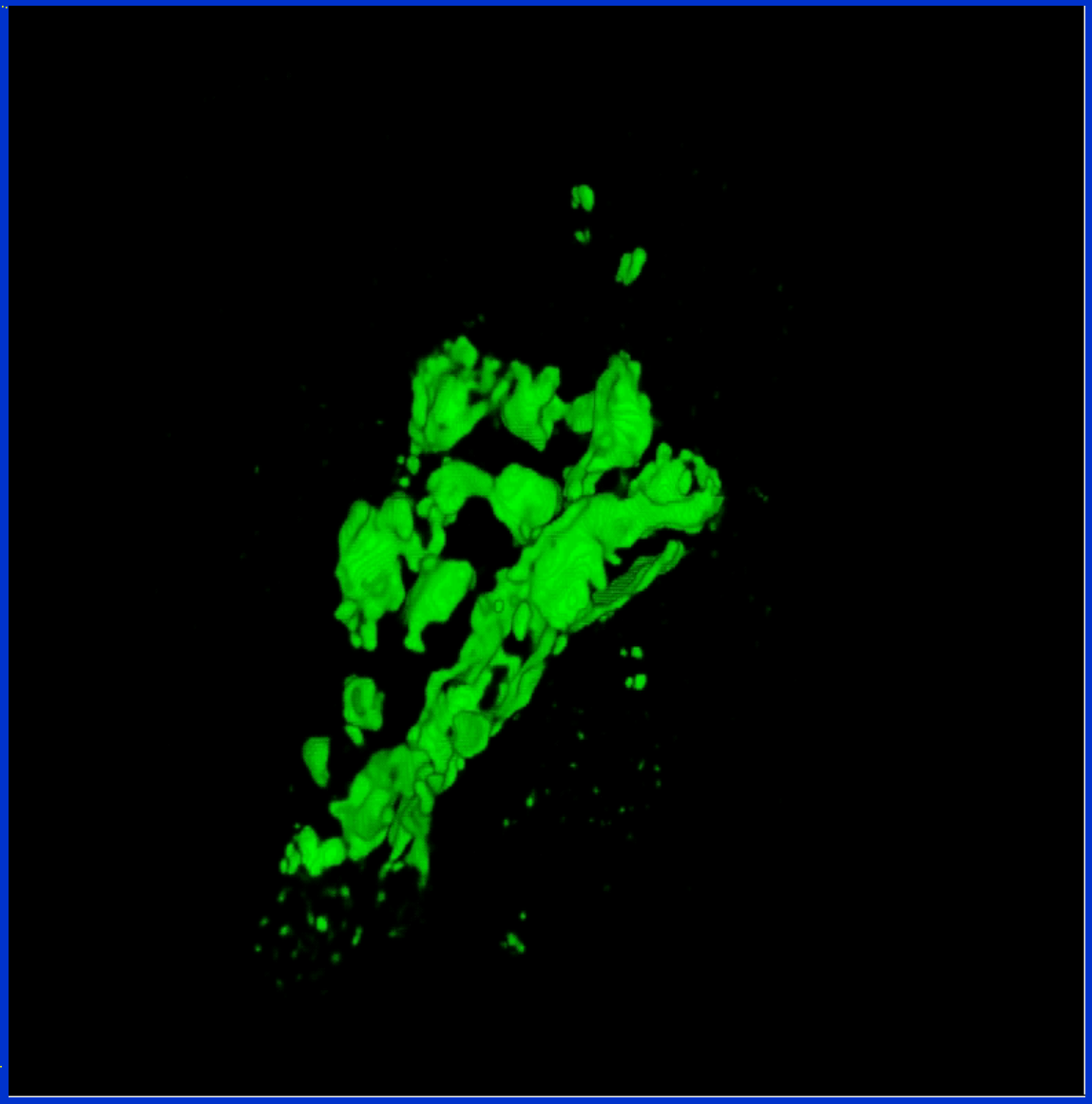
Surface 2.8 x larger

Quantitative live cell 4Pi microscopy

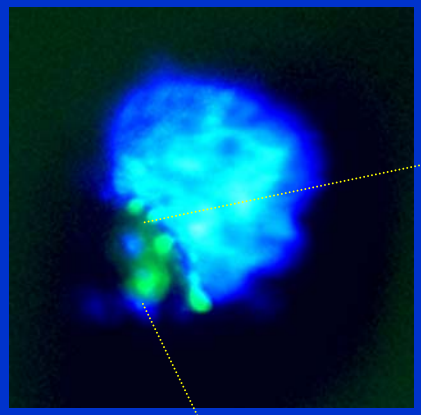


UDP-Galactosyltransferase-
EGFP

4Pi Image
Golgi in *live Vero Cell*



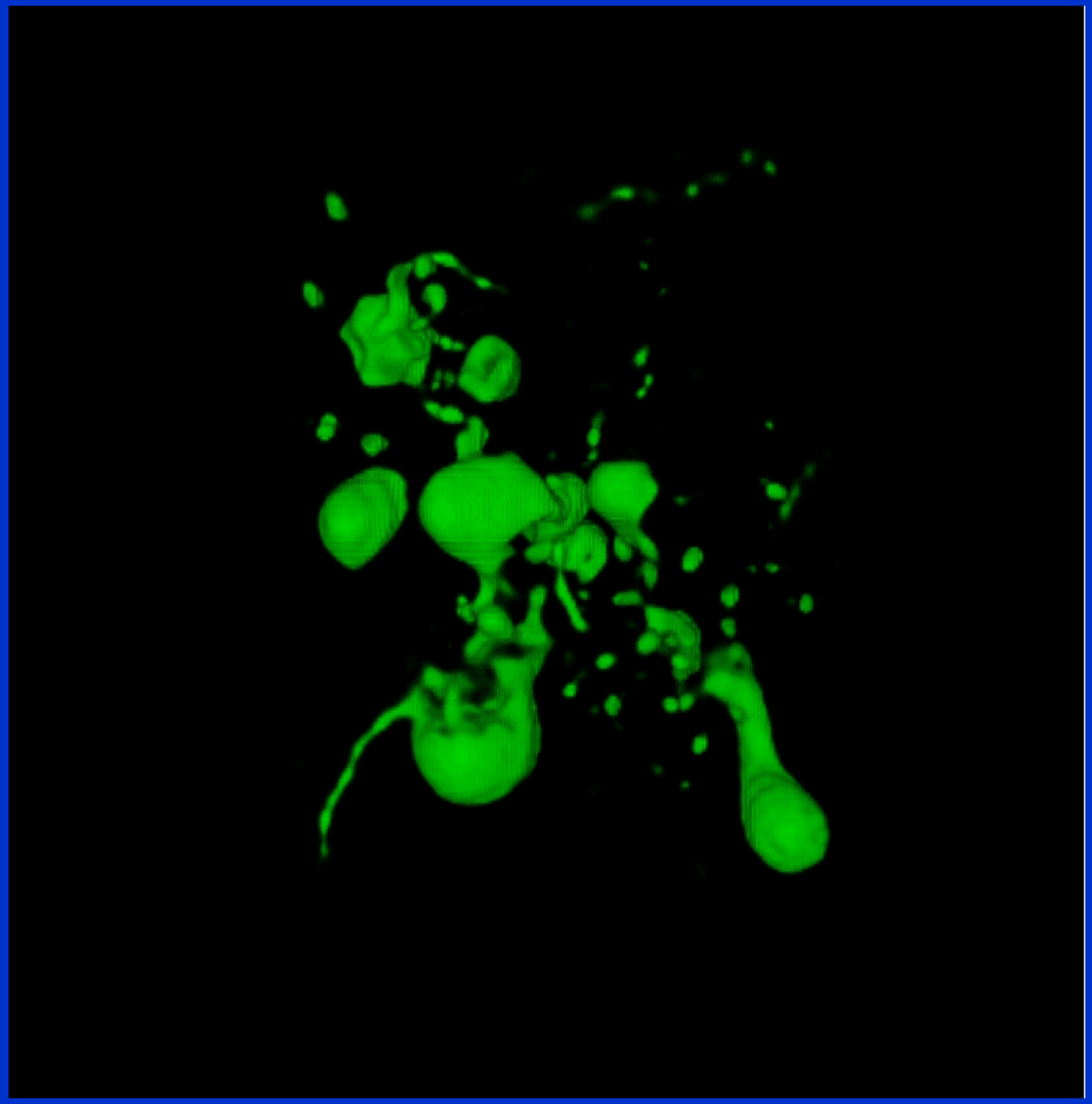
Egner & Hell, TiCB (2004)



UDP-
Galactosyl-
transferase-
EGFP

z
x
2.5 mm

4Pi Image
Golgi in *live Vero Cell*

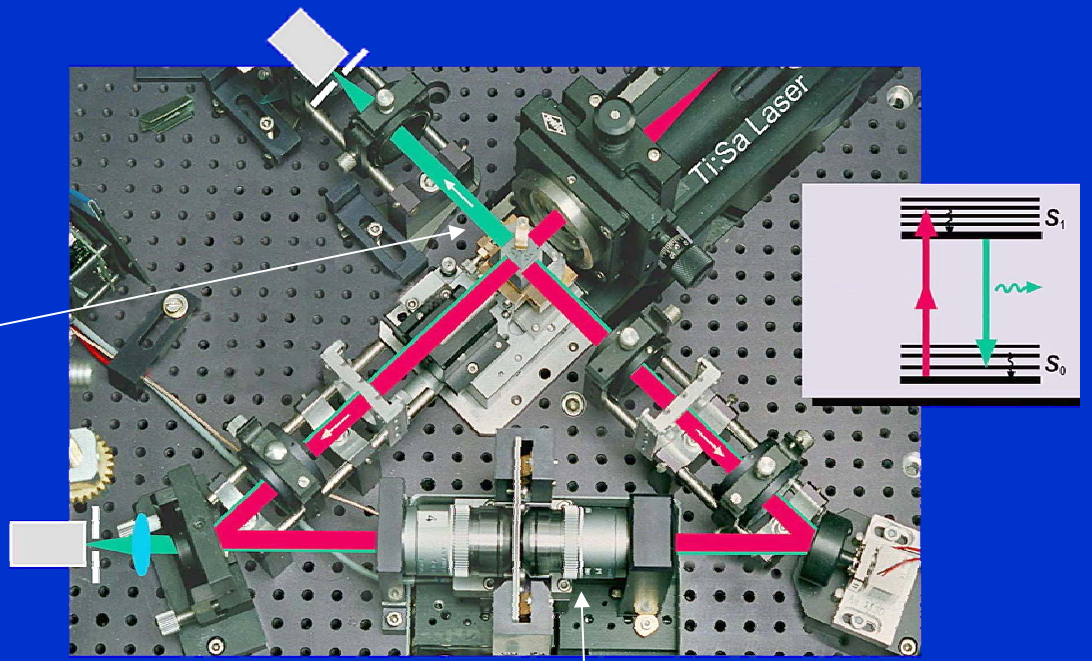


3D-resolution: ~100 nm

Practical issues

Practical issues

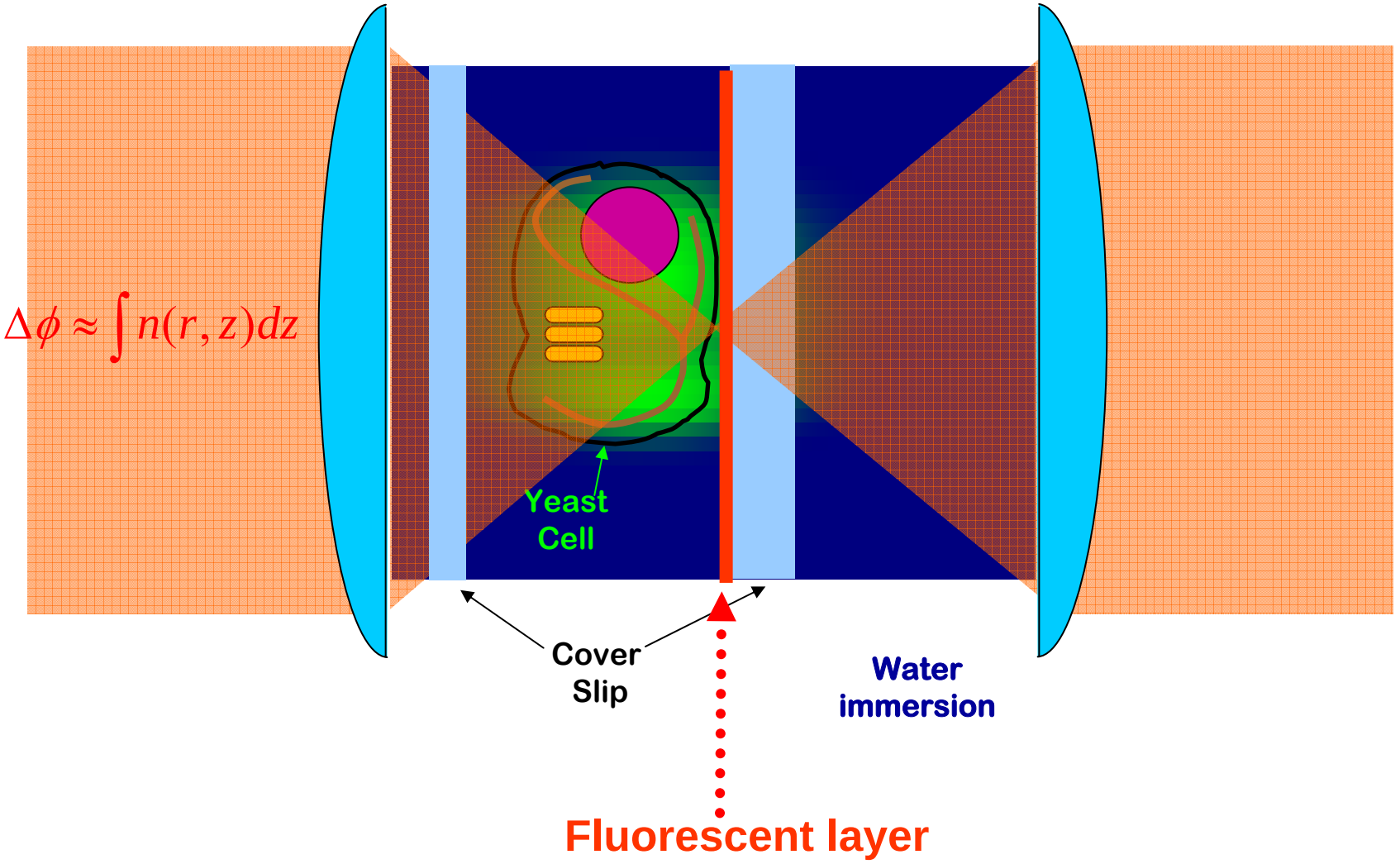
1) Alignment of lenses



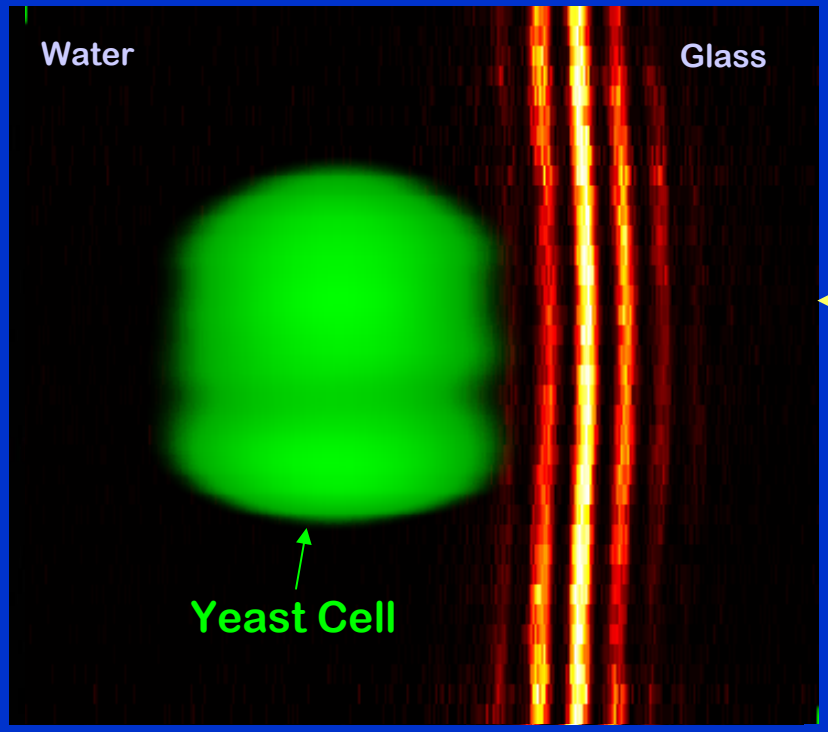
'Dark exit' of
Sagnac interferometer

Piezo closed loop alignment (< 50 nm precision)

Checking out phase changes and aberrations ...induced by live yeast cell



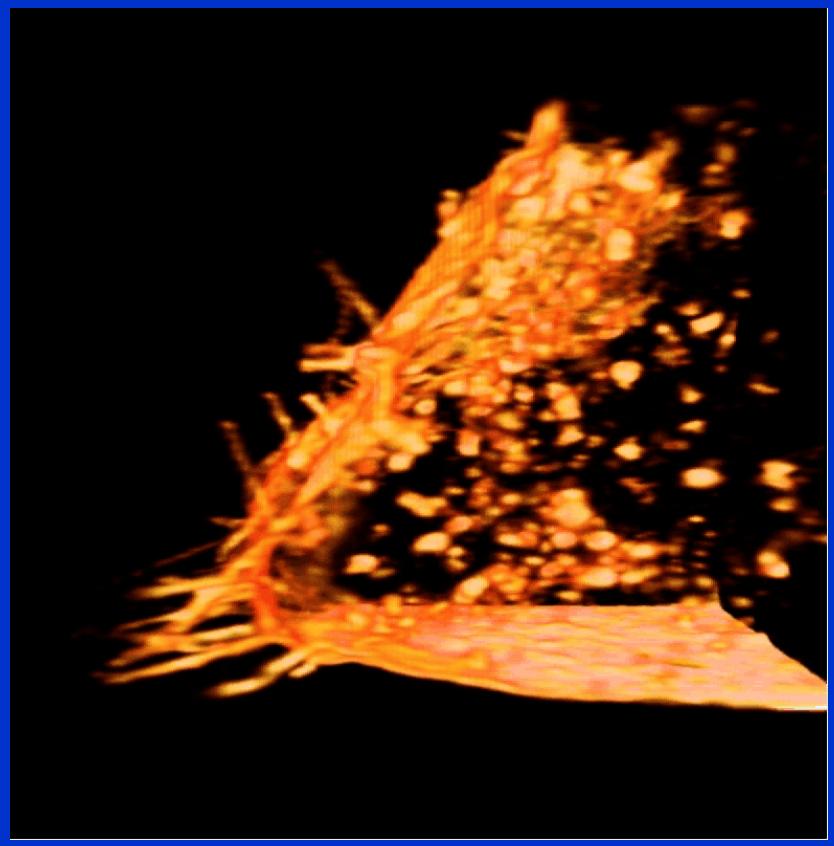
Checking out object induced phase changes and aberrations ...



Shift in absolute position < 100 nm

Practical issues

1) **Alignment of lenses**
2) **Need establish PSF (phase, lobes):** point or planar objects in sample
3) **Refractive index changes:** index matching (water lenses)



erbB1-EGFP

In Chinese Hamster

Ovary (CHO) Cells

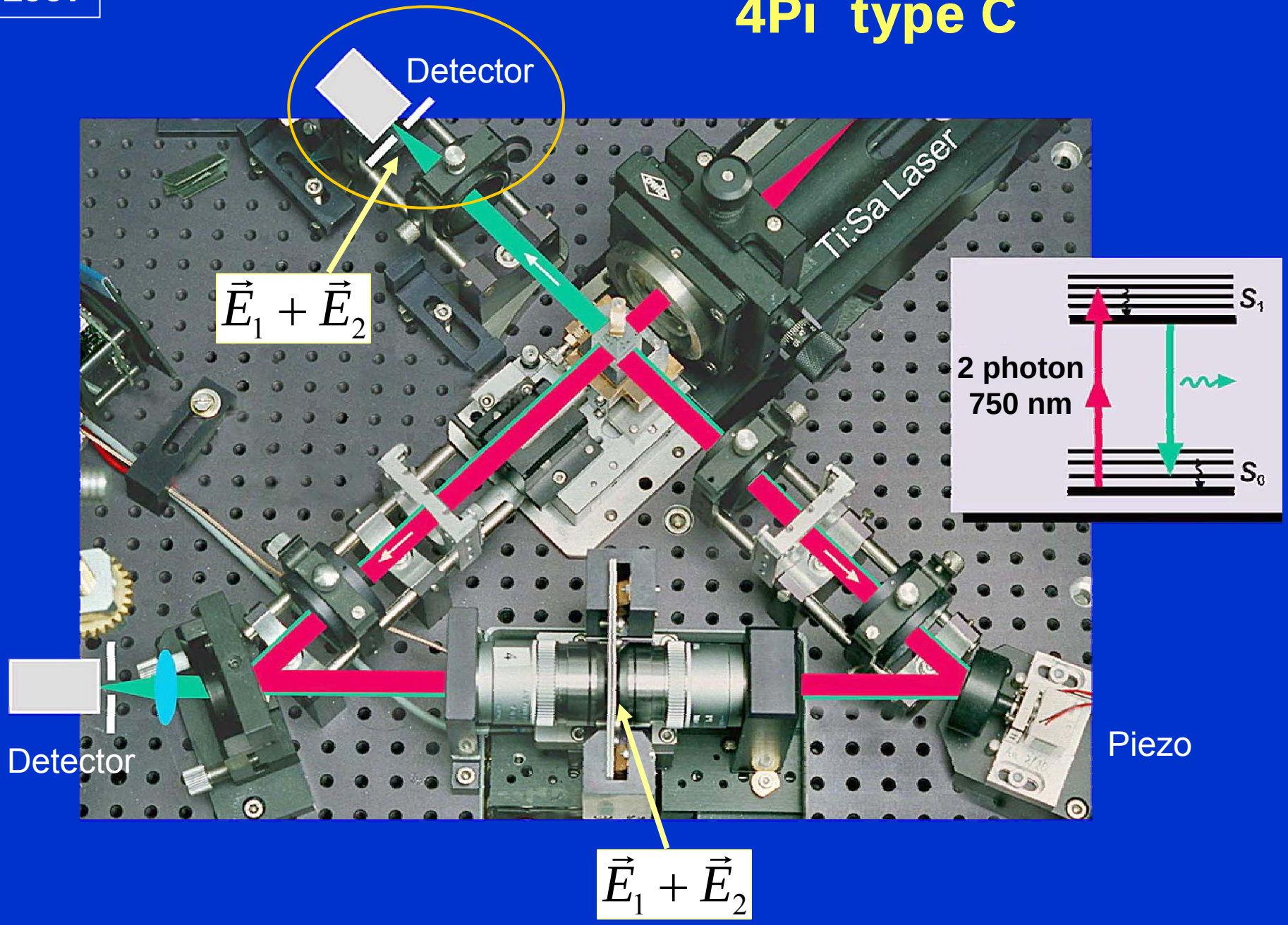
18 x 33 x 16 µm (x,y,z)

4Pi type C

(coherent detection + illumination)

1997

4Pi type C



Z-Response of a layer (Experiment)

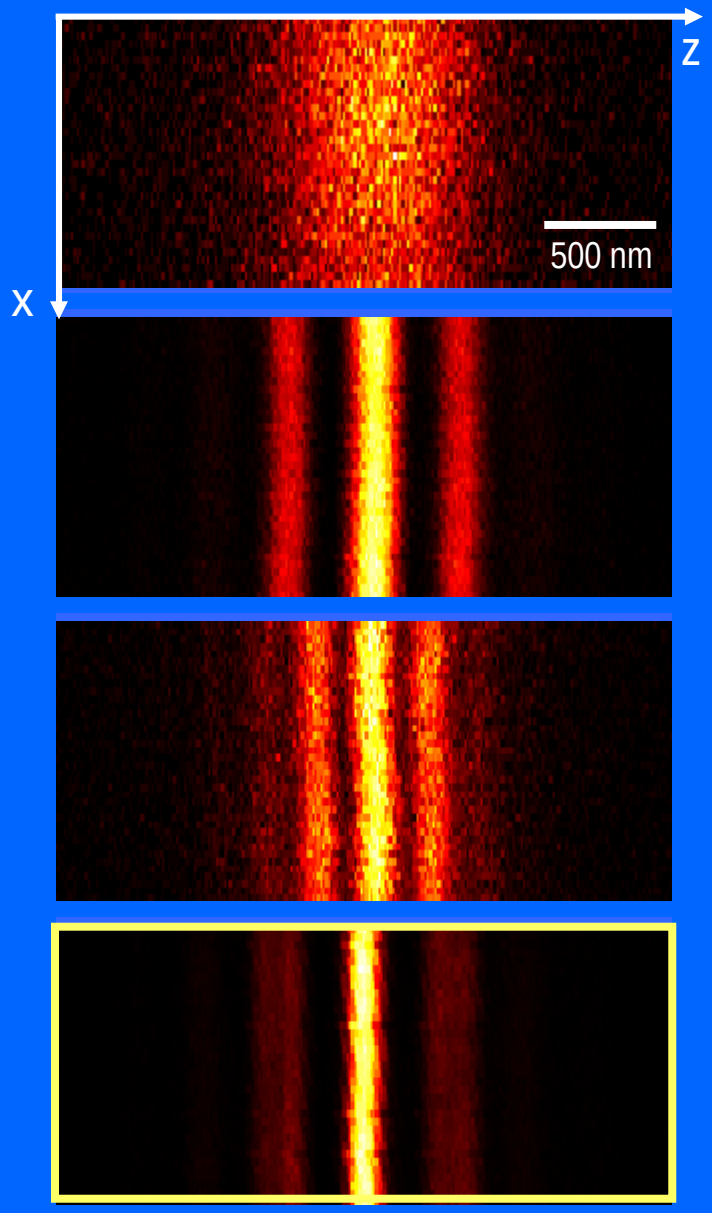
2-Photon / 840 nm
NA = 1.35 Oil

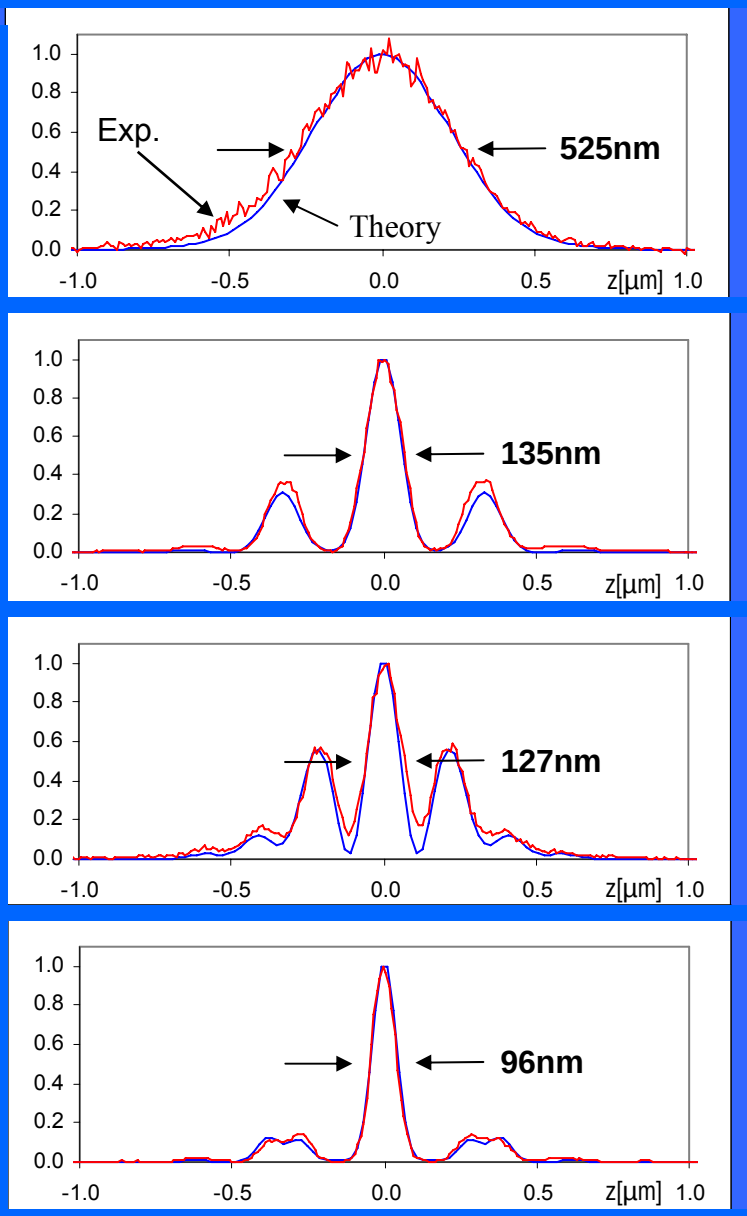
Confocal

4 Pi (A)
Illumination

4 Pi (B)
Detection

4 Pi (C)
Illumination
+
Detection





Z-Response of a layer (Theory)

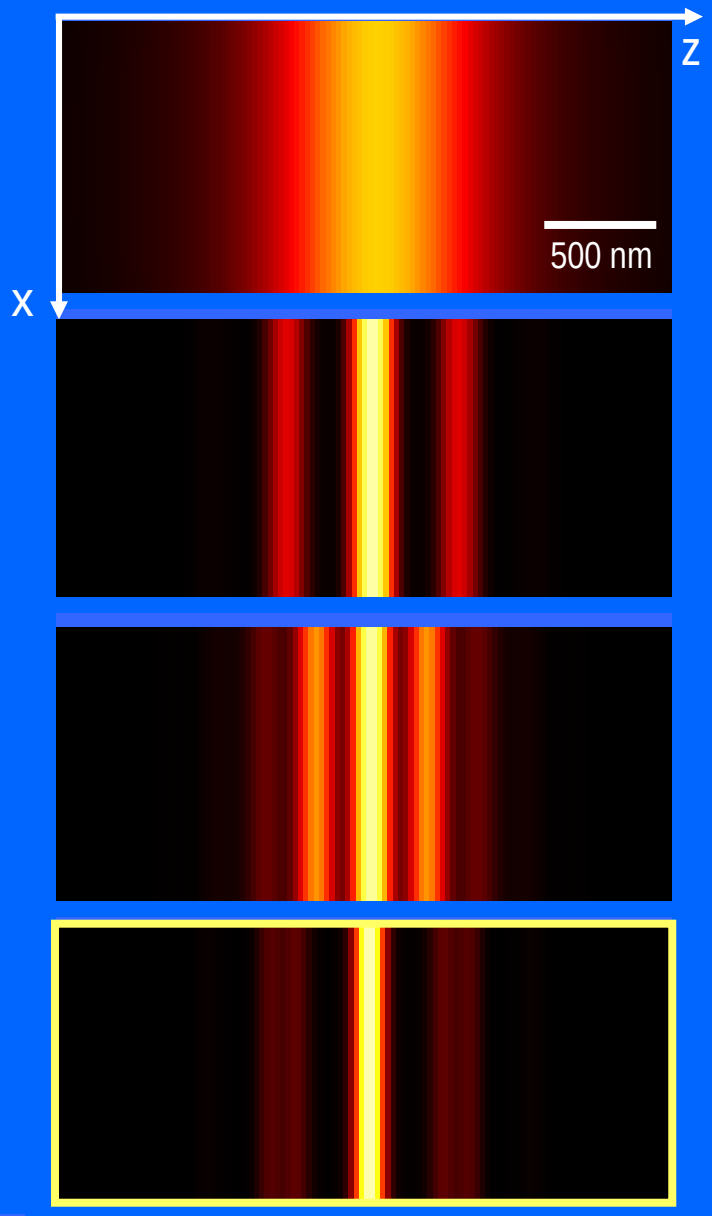
2-Photon / 840 nm
NA = 1.35 Oil

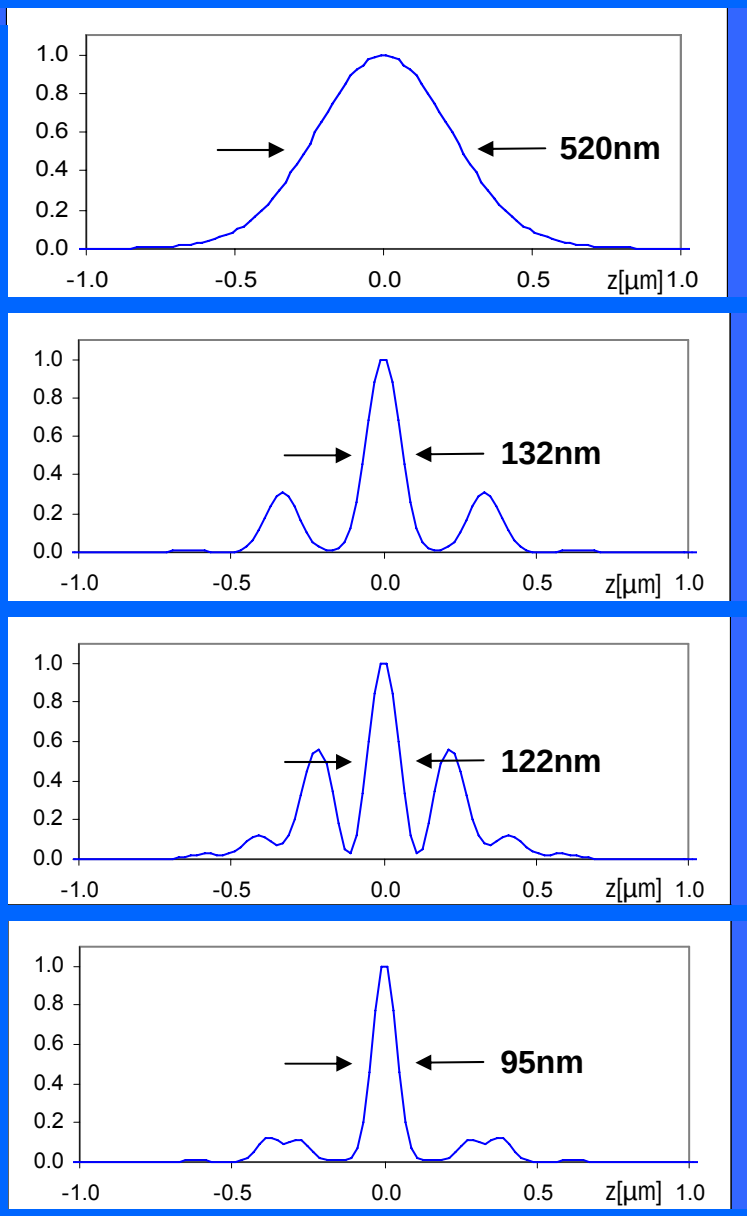
Confocal

4 Pi (A)
Illumination

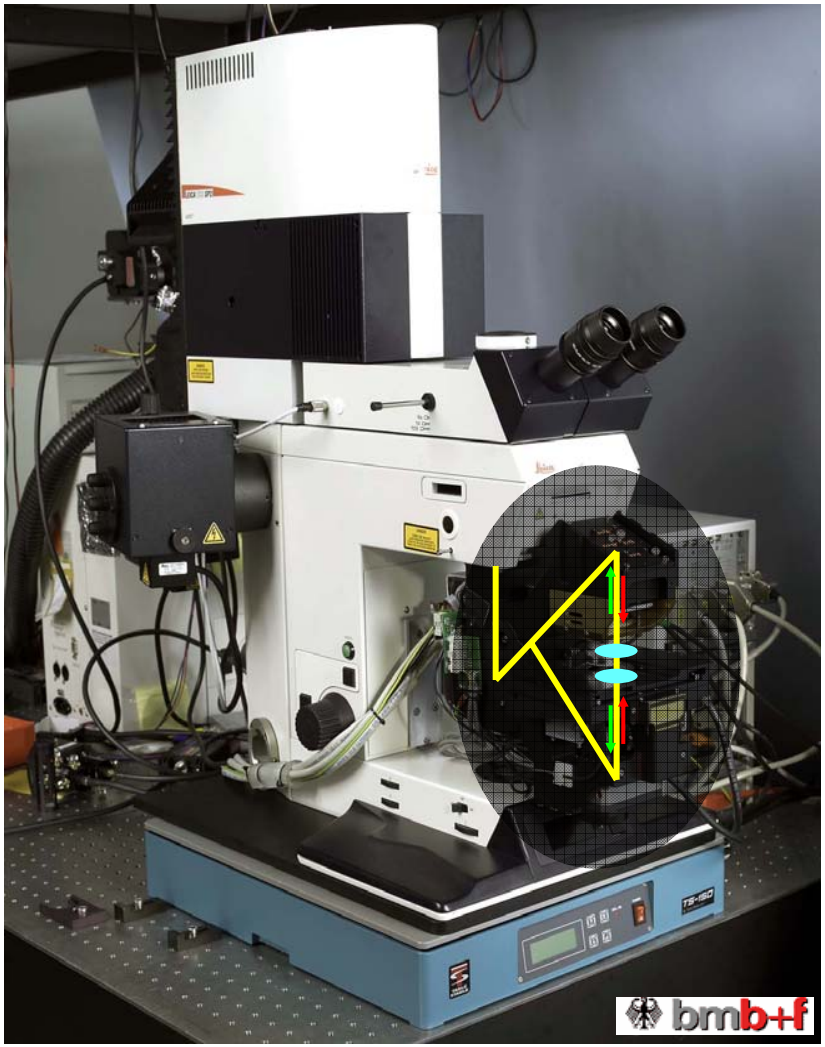
4 Pi (B)
Detection

4 Pi (C)
Illumination
+
Detection



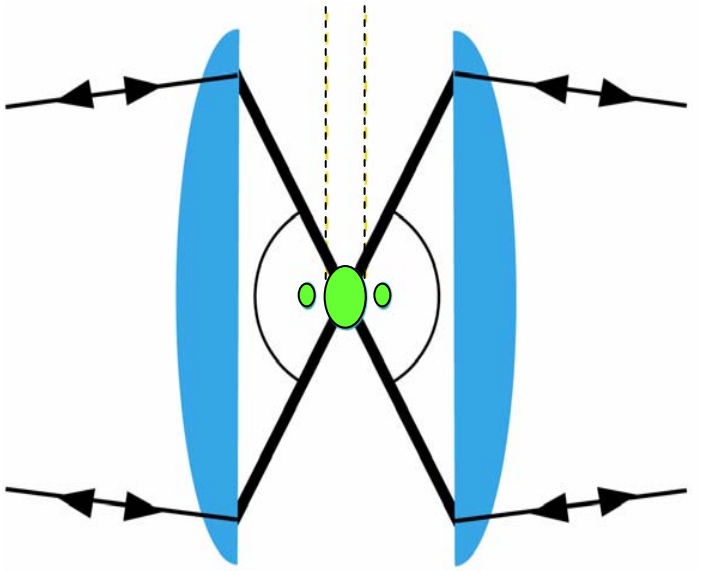


Compact 4Pi-microscope



Z- resol < 100 nm (live cells / aqueous cond.)
1 & 2 photon

How essential is the use of <u>focused</u> (spherical) wavefronts?



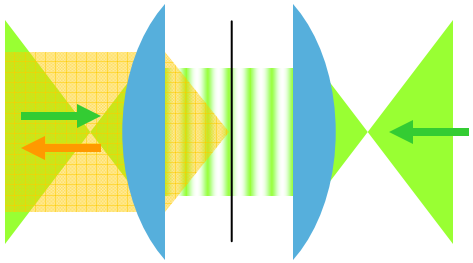
4Pi

Isn’t it just about making a focal “interference standing wave pattern”?

Standing Wave microscopy

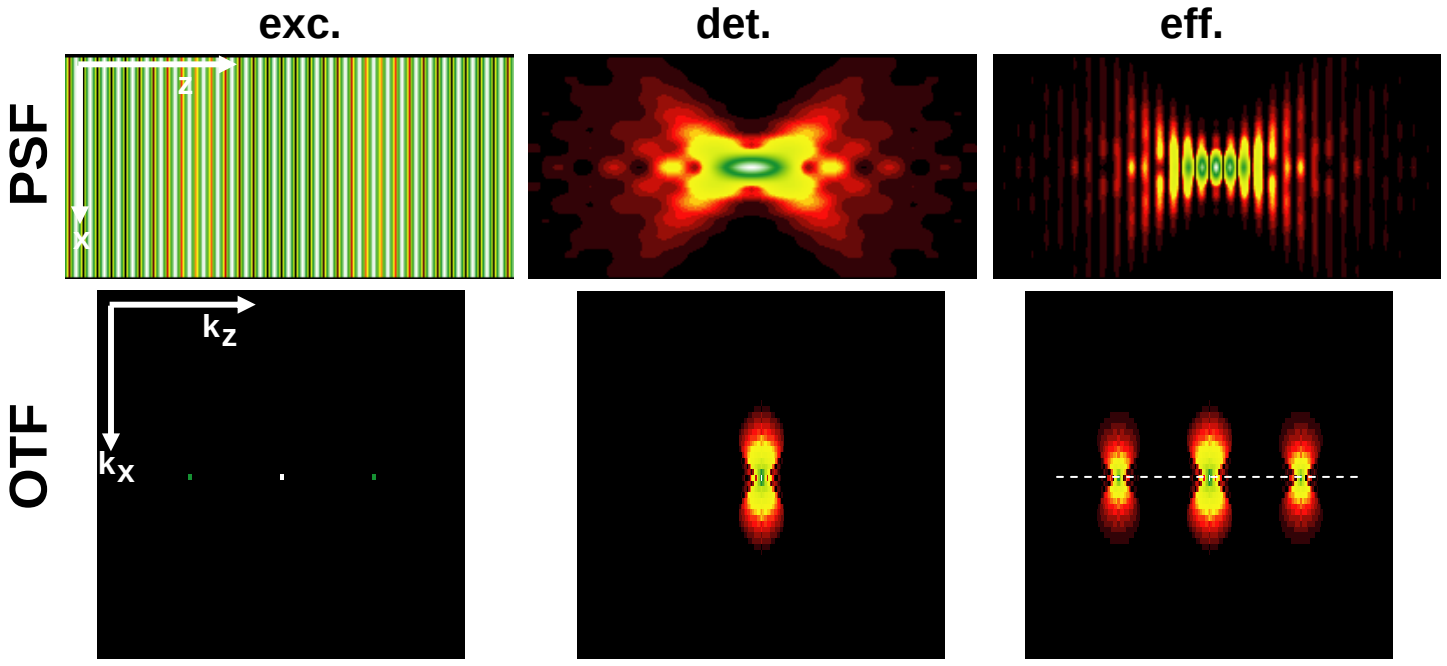
Non-Confocal !

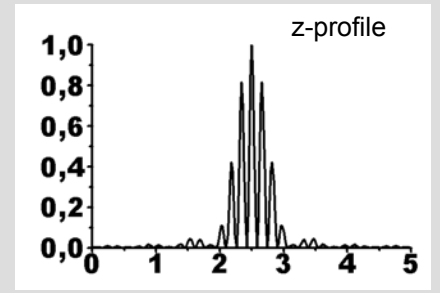
Bailey et al, *Nature* (1993)

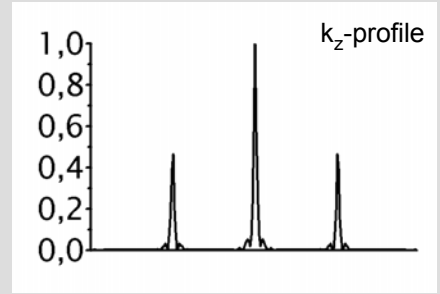


- **Excitation: flat standing wave**
- **Detection: epifluorescence**
- **No ‚4Pi element'**

Does not work for z-resolution improvement !



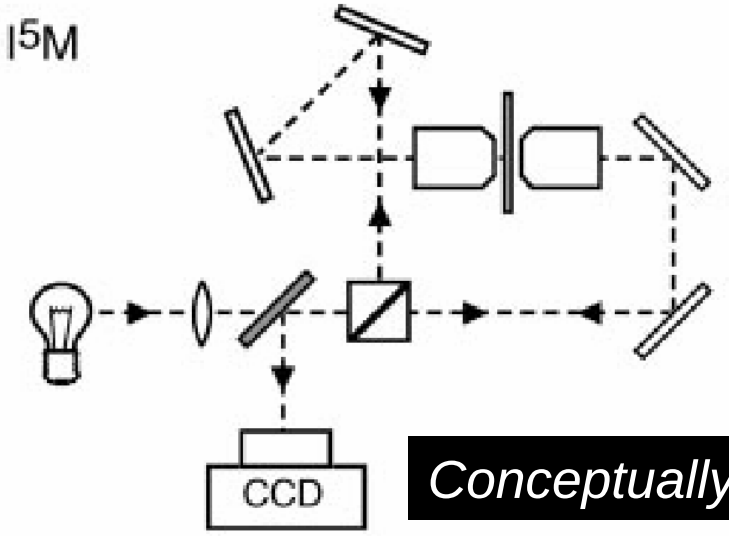


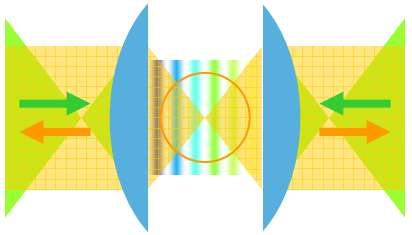


I^{5}M

Gustafsson et al, 1997

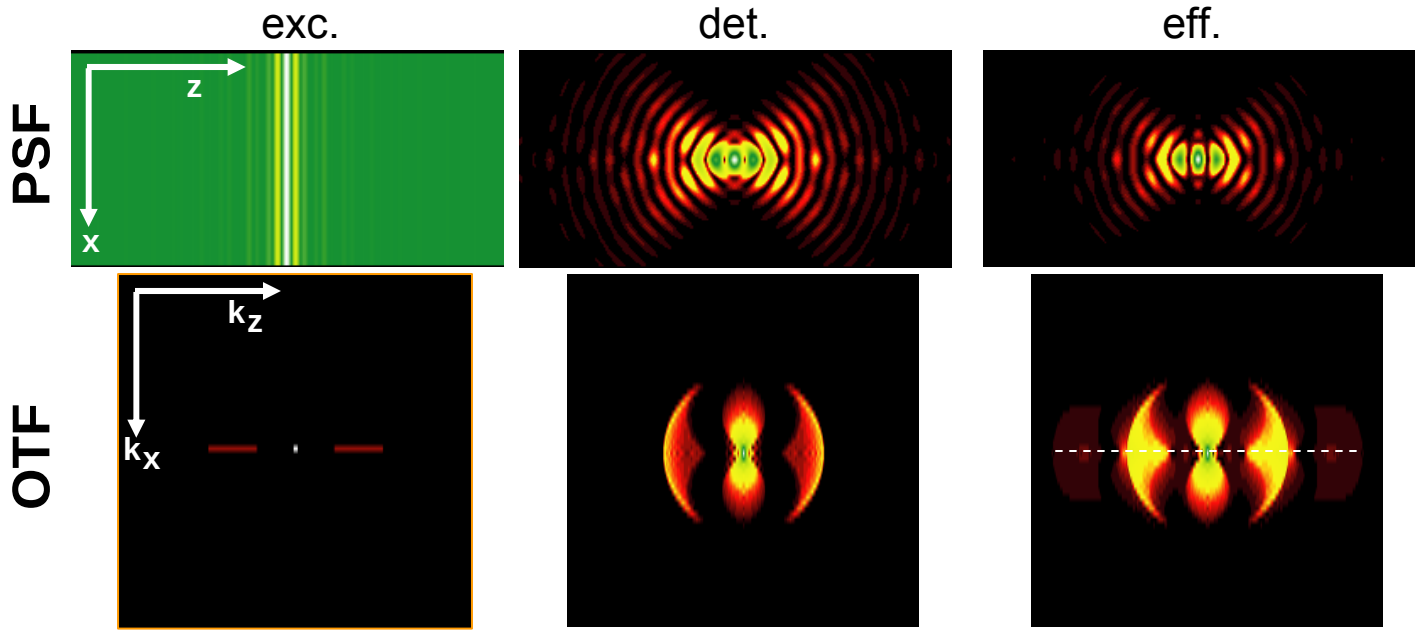
Non-Confocal !

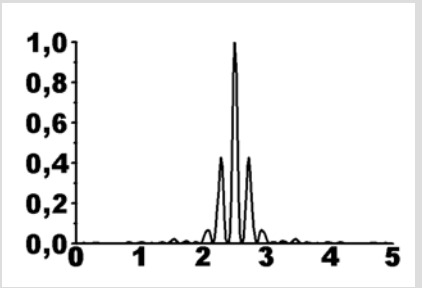


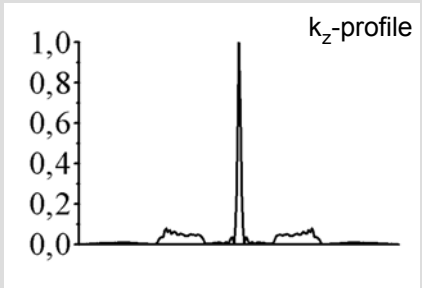


- **Excitation: flat standing wave**
- **Detection: 4Pi, spherical waves**

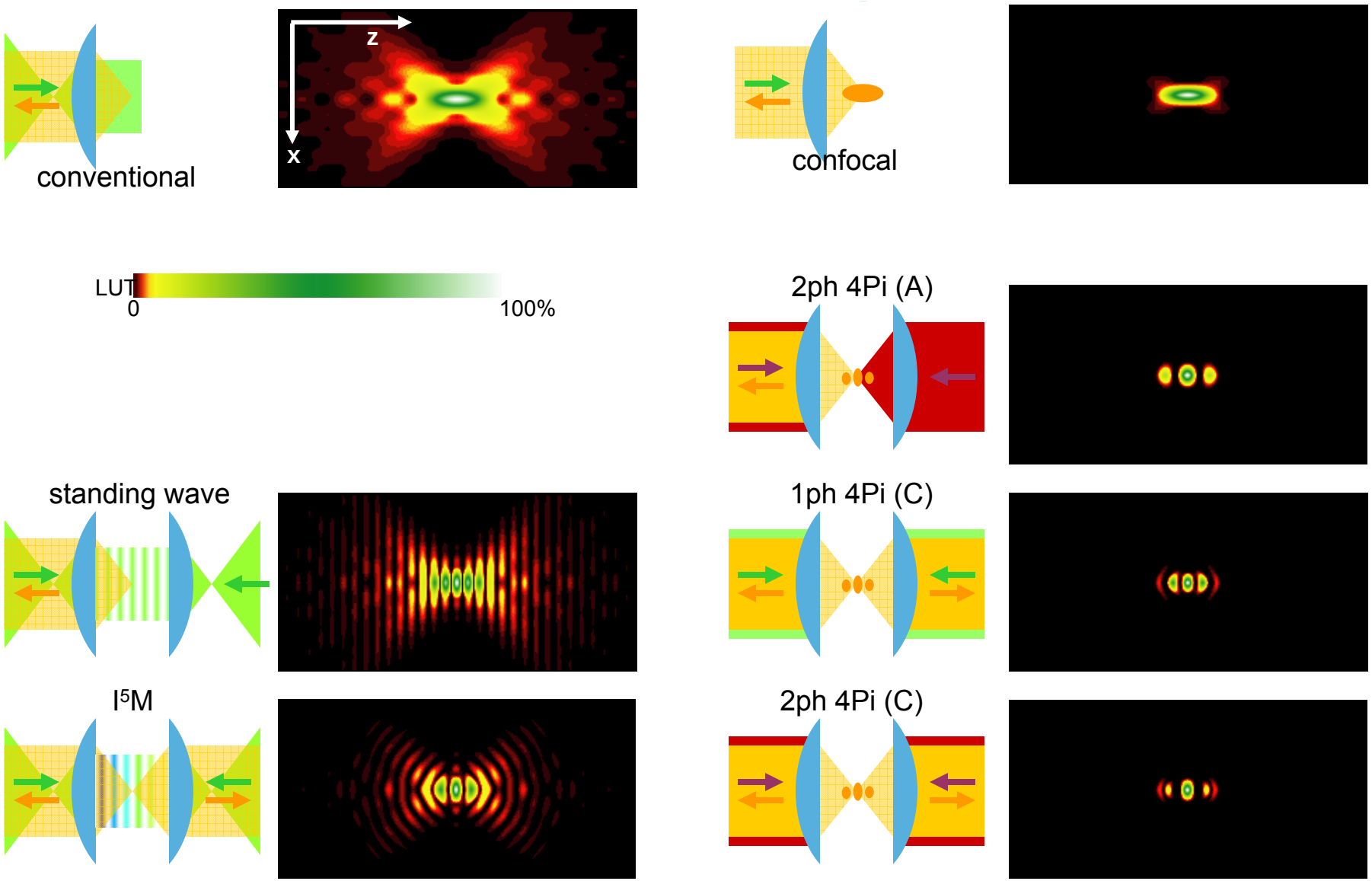
Conceptually fine, but not very robust



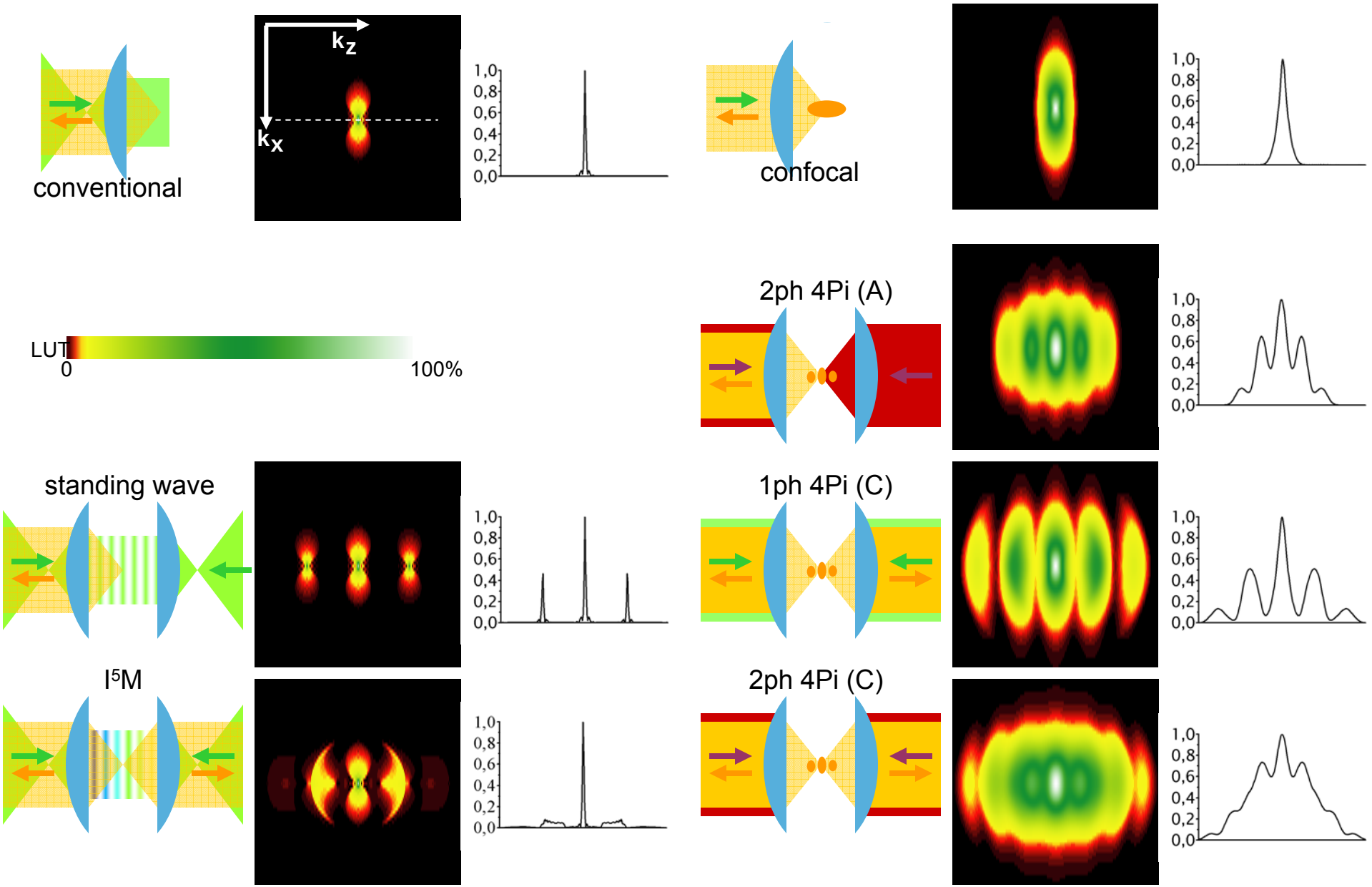




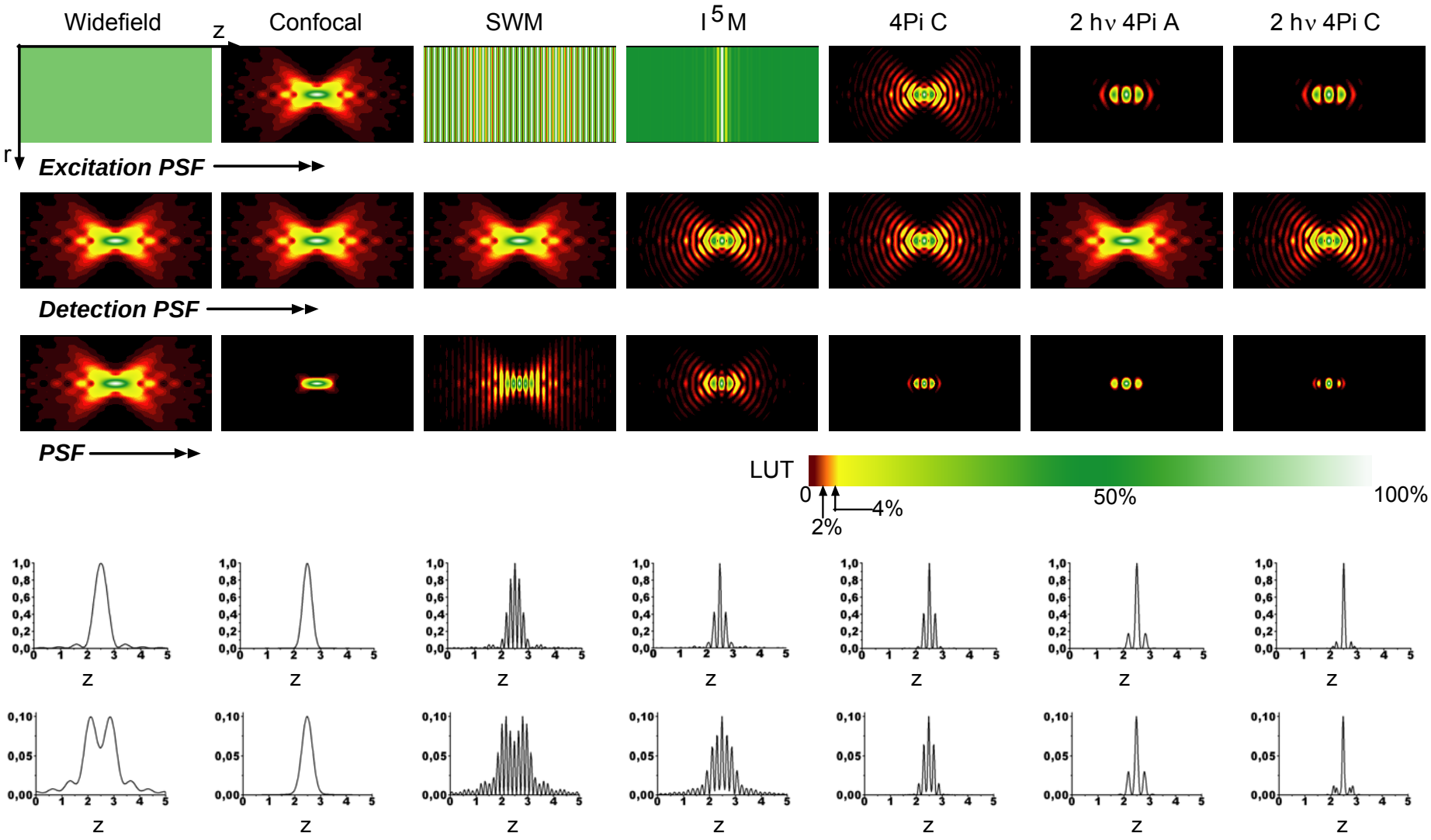
Comparison of the effective PSFs



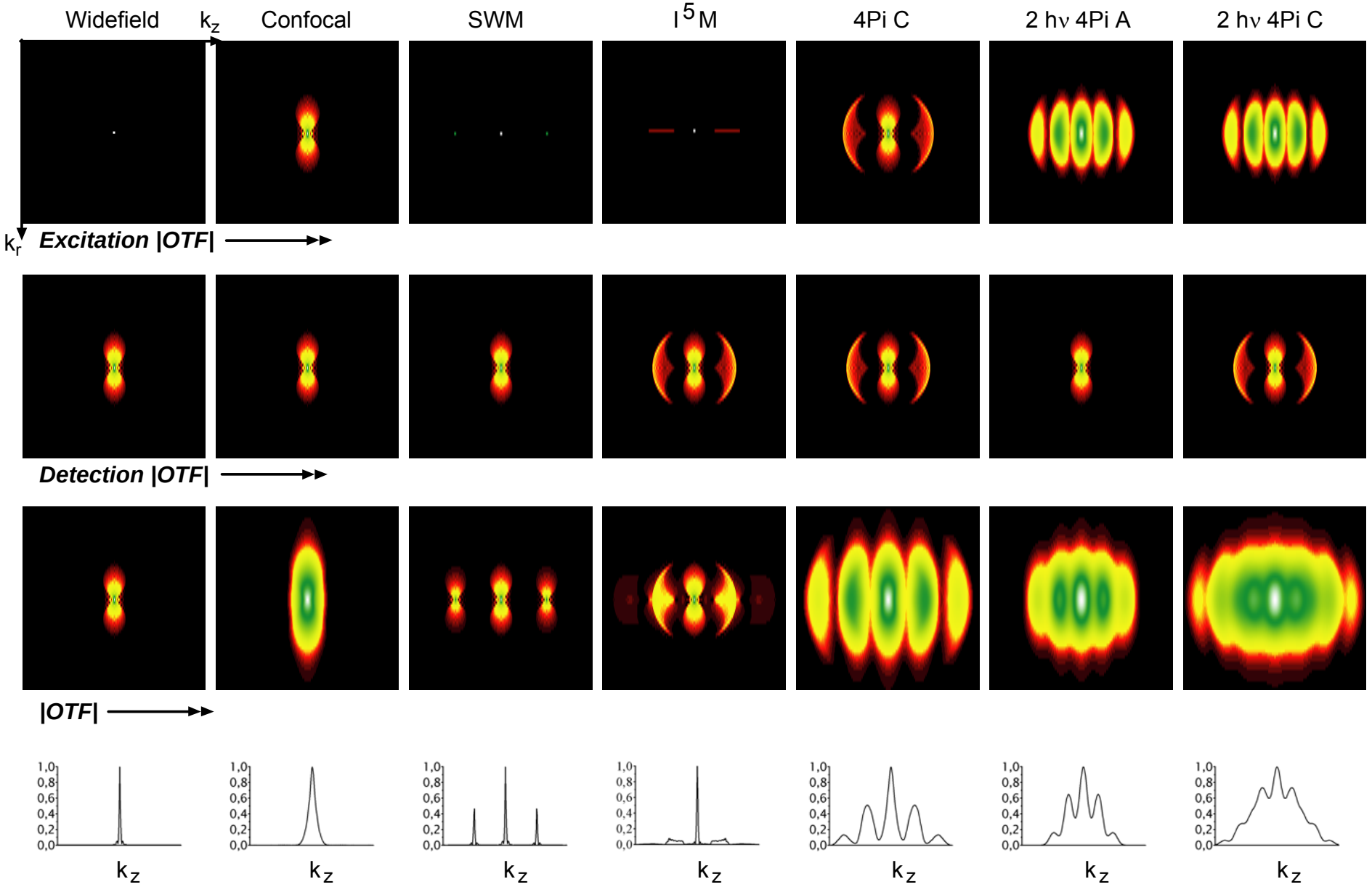
Comparison of the effective OTFs



PSFs: Overview

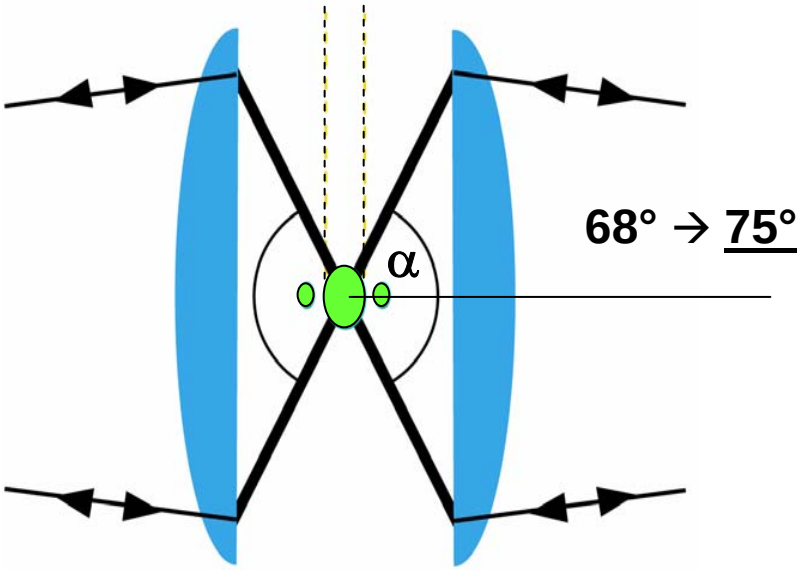


OTFs: Overview



No.

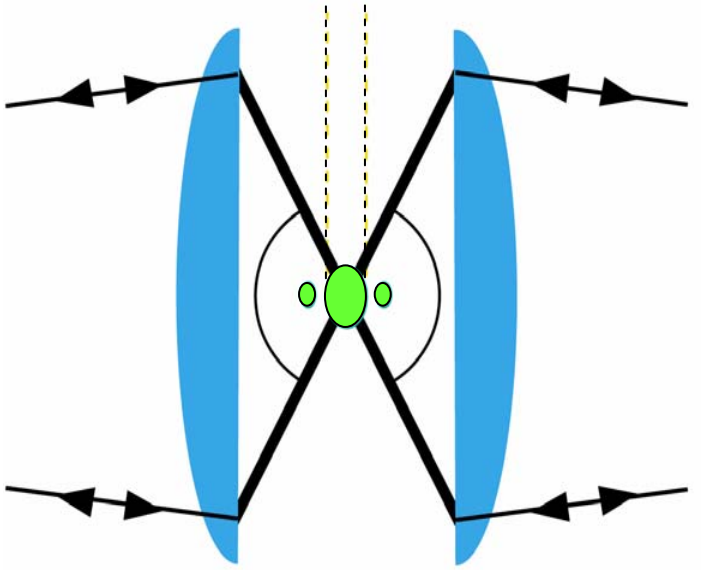
Essential physical element: increase in solid angle (“4π”)



Isn’t it just about making a focal “interference standing wave pattern”?

Two opposing lenses give 3-7 fold increased z-resolution

60- 150 nm



..provided that they increase the aperture angle of the microscope

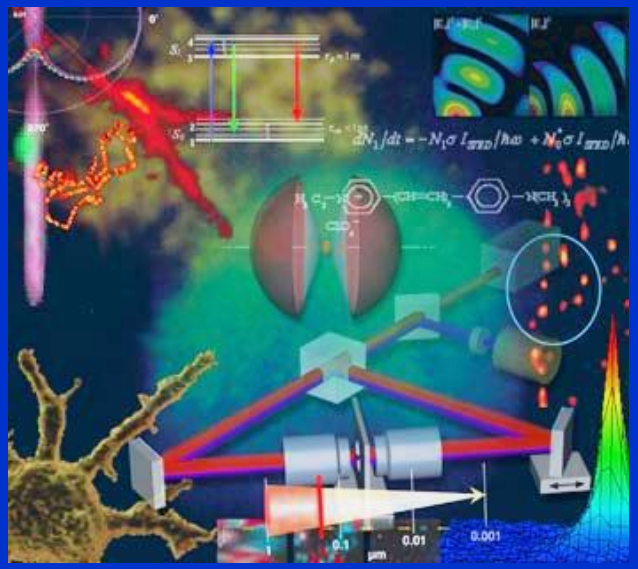
Nanoscale Resolution

in

Far-field Fluorescence Microscopy

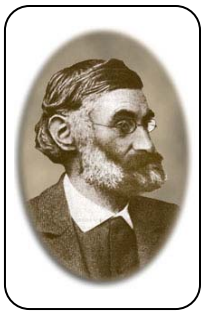
Part II

Breaking the diffraction barrier

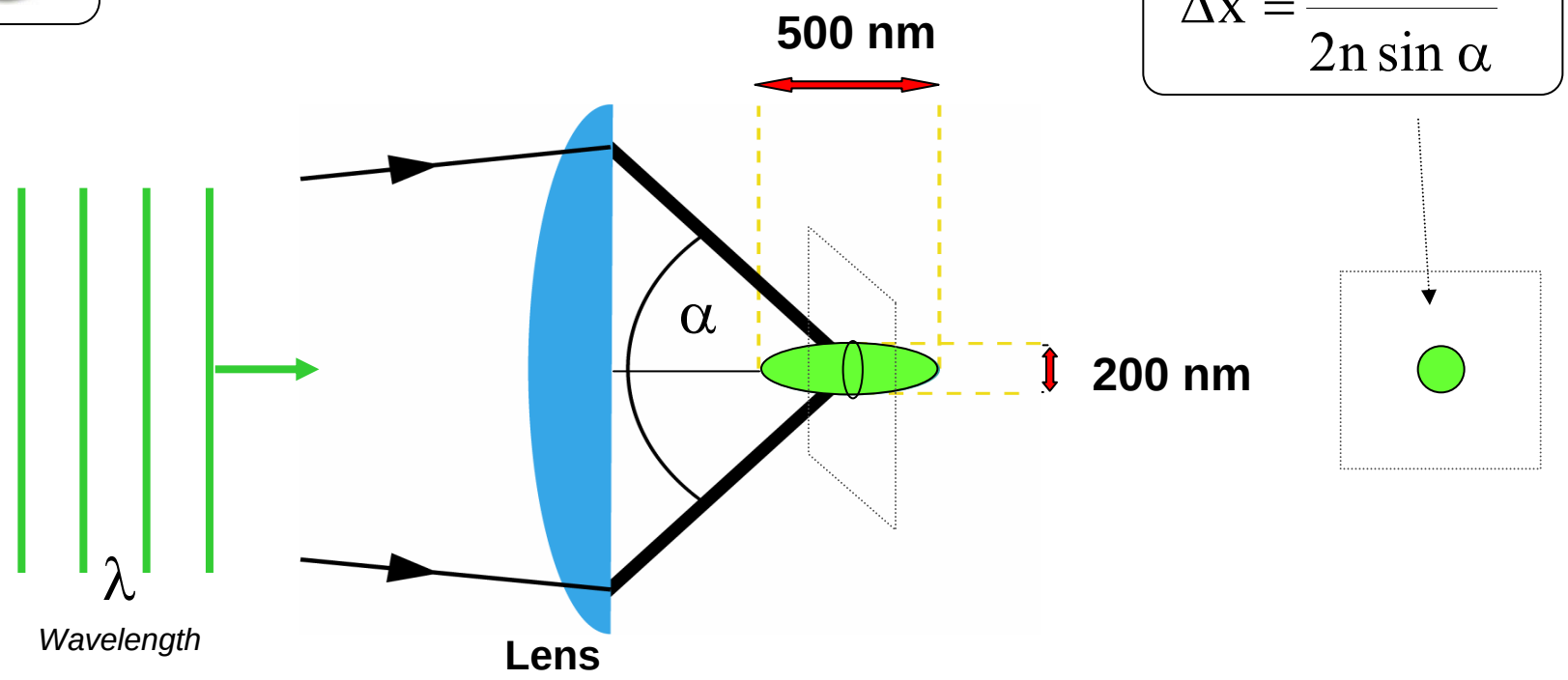


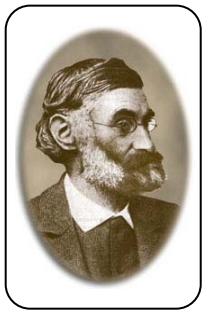
Stefan W. Hell

Max Planck Institute for Biophysical Chemistry
Department of NanoBiophotonics
Göttingen, Germany



$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$



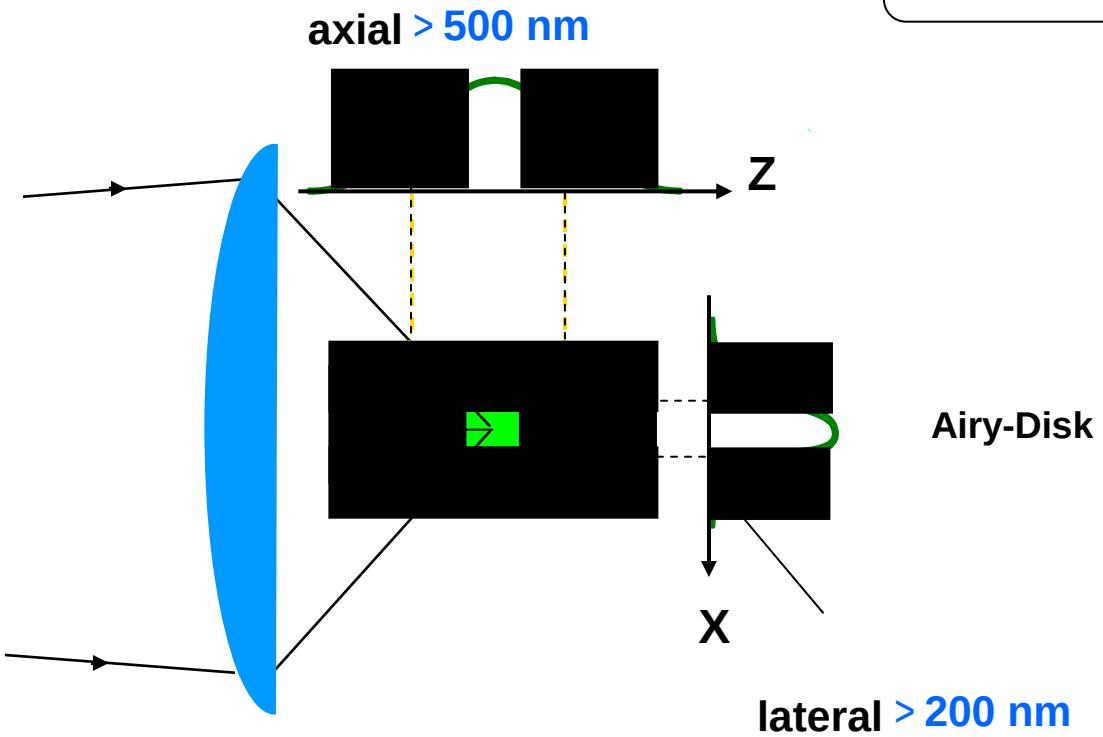


Jena,
Germany

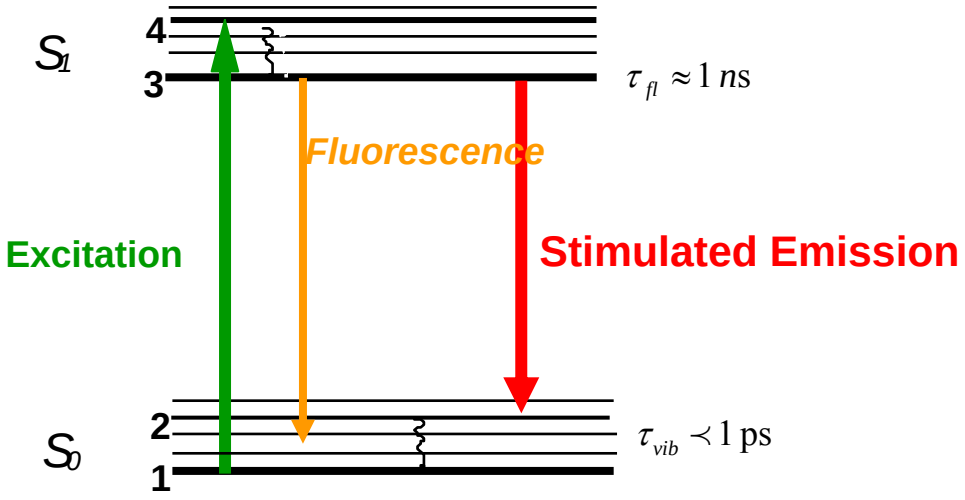
"Breaking the diffraction resolution limit by stimulated emission"

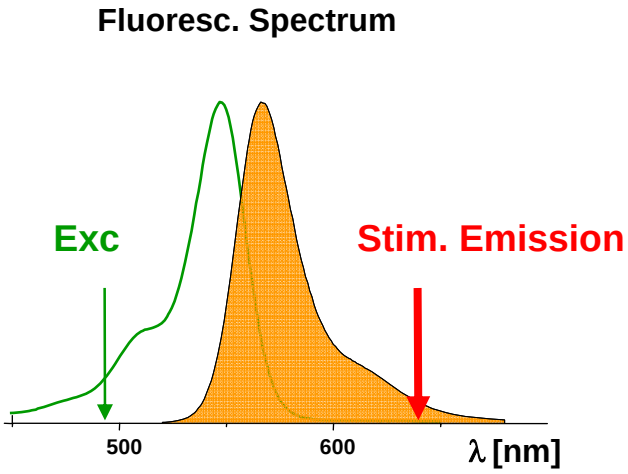
Hell & Wichmann, Opt. Lett. 19, 11, (1994)

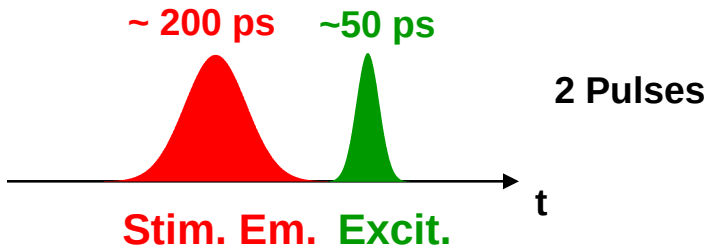
$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$



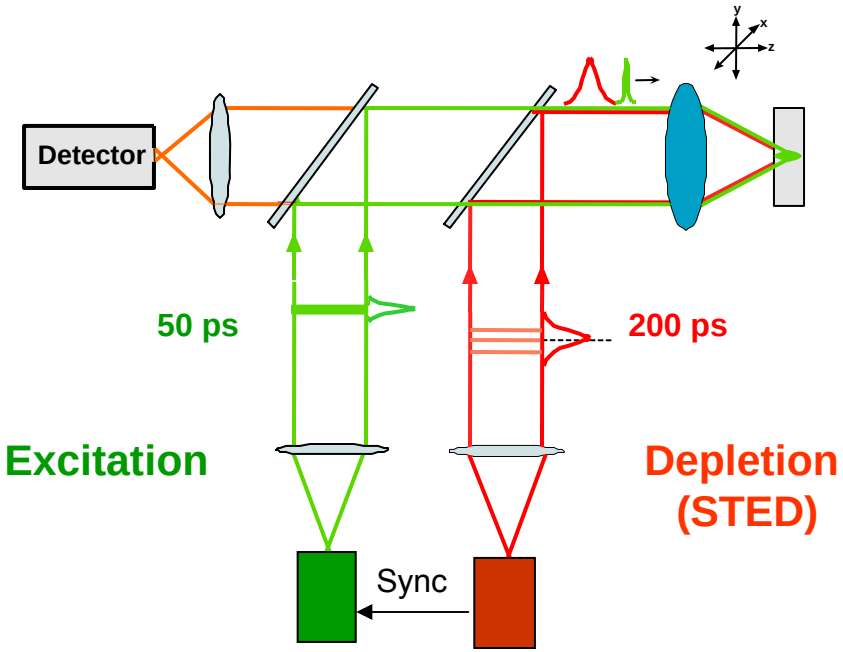
Stimulated Emission Depletion (STED)

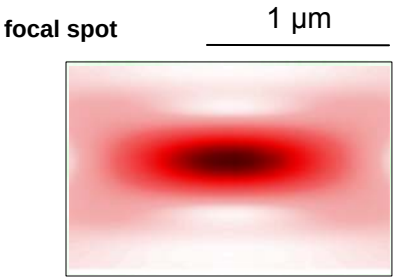


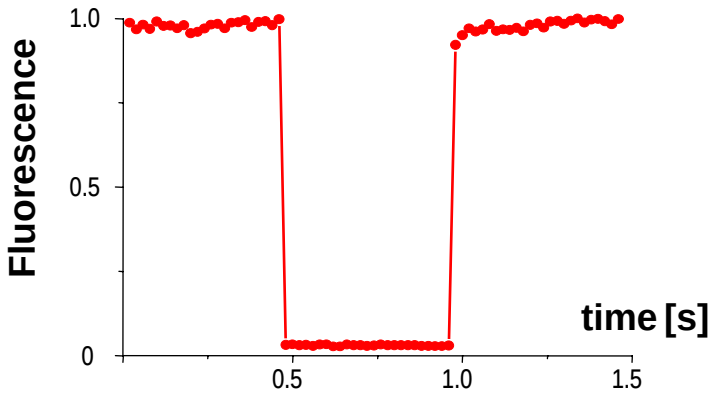


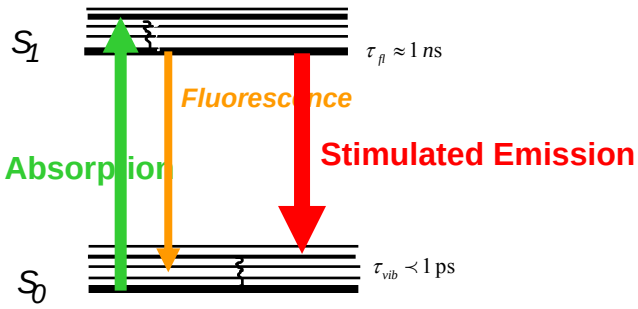


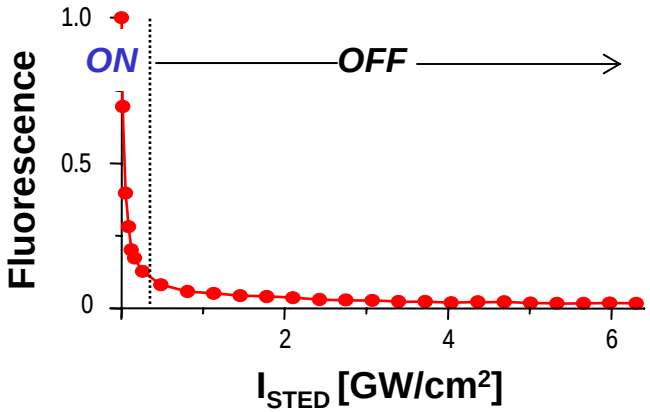
STED microscope



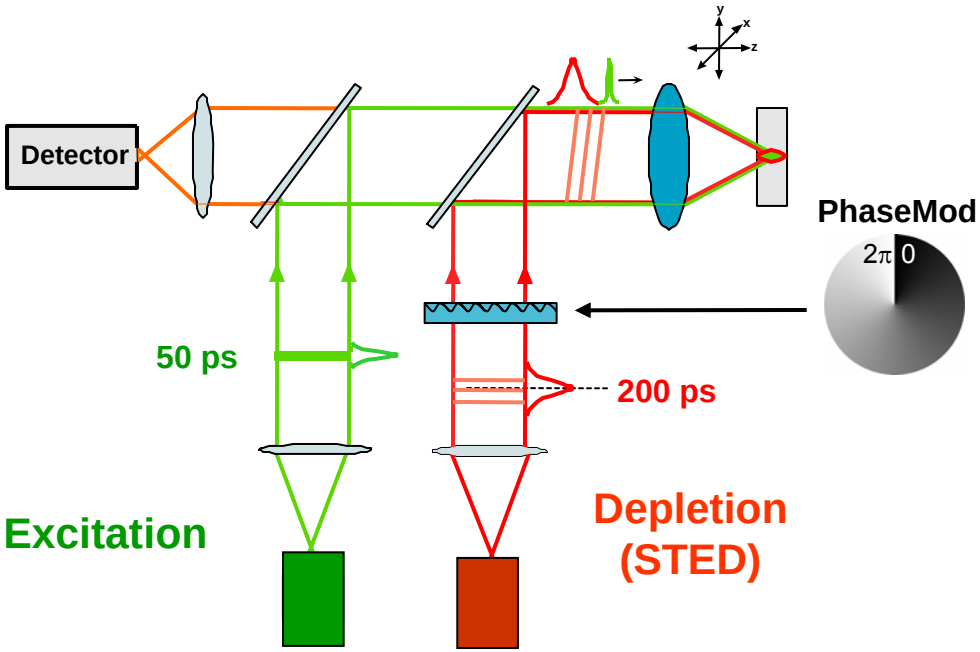


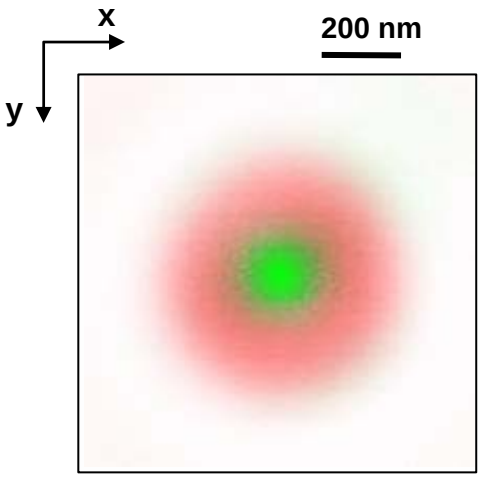


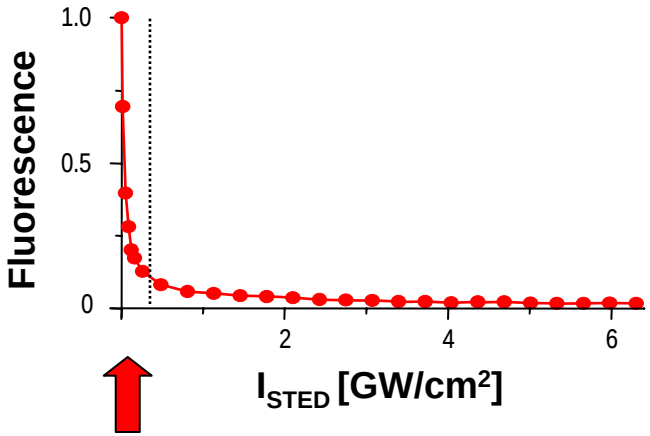




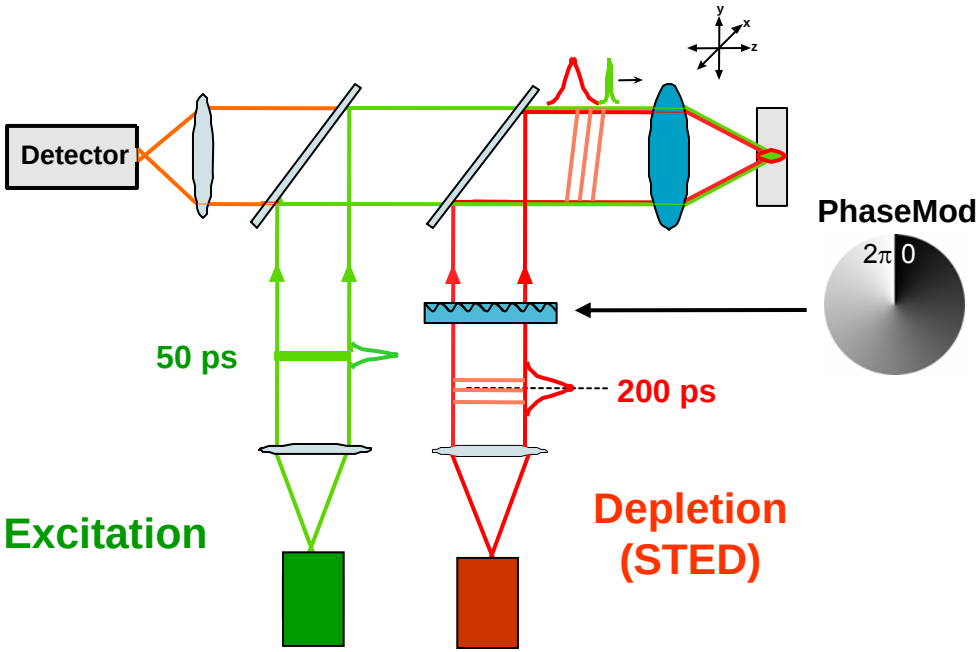
STED microscope

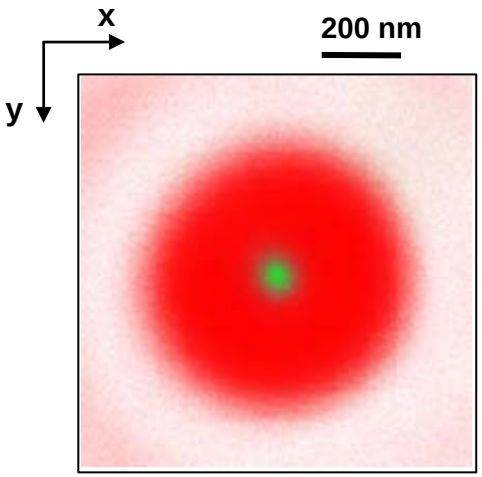


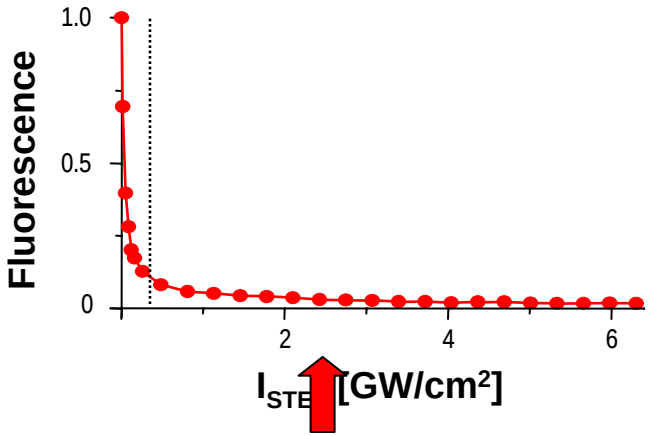




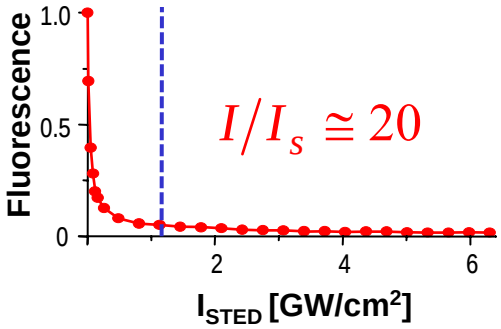
STED microscope

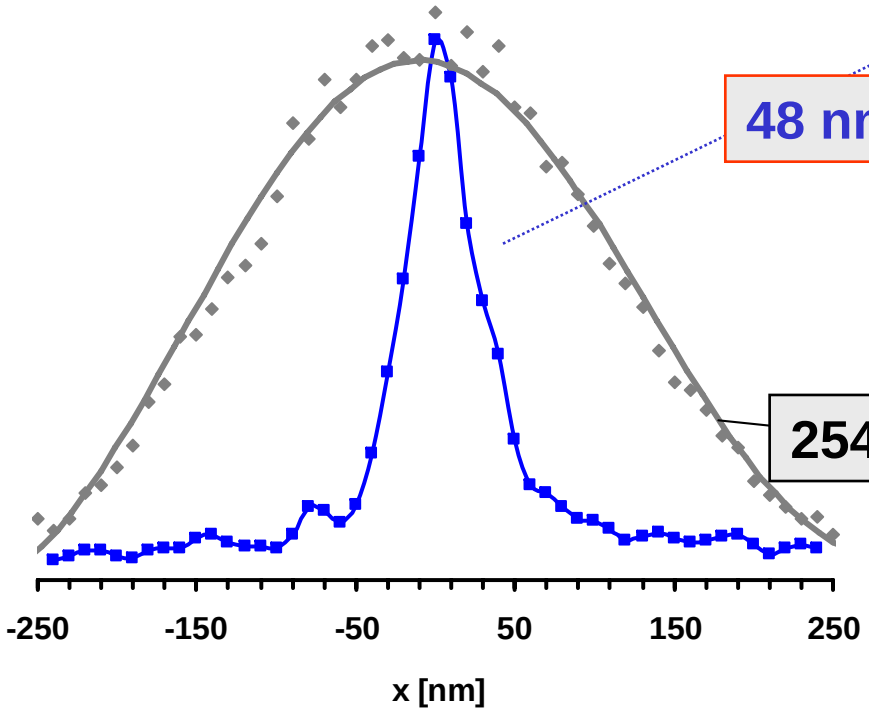


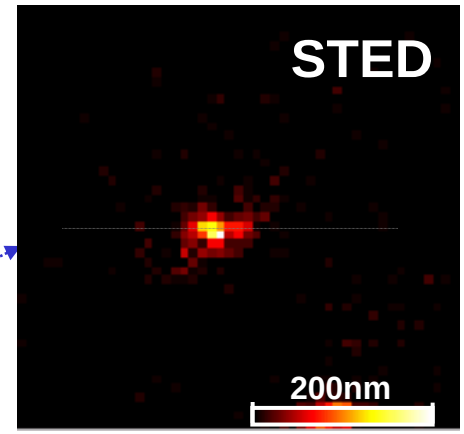


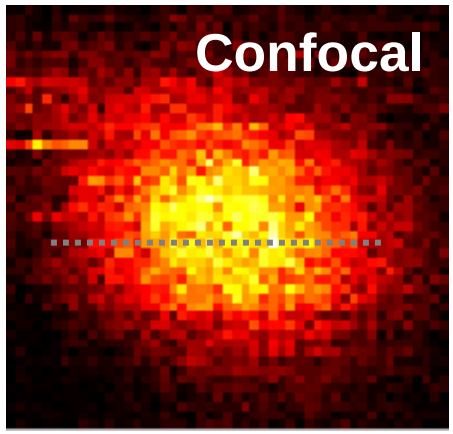


Focal spot *... probed with 1 molecule*







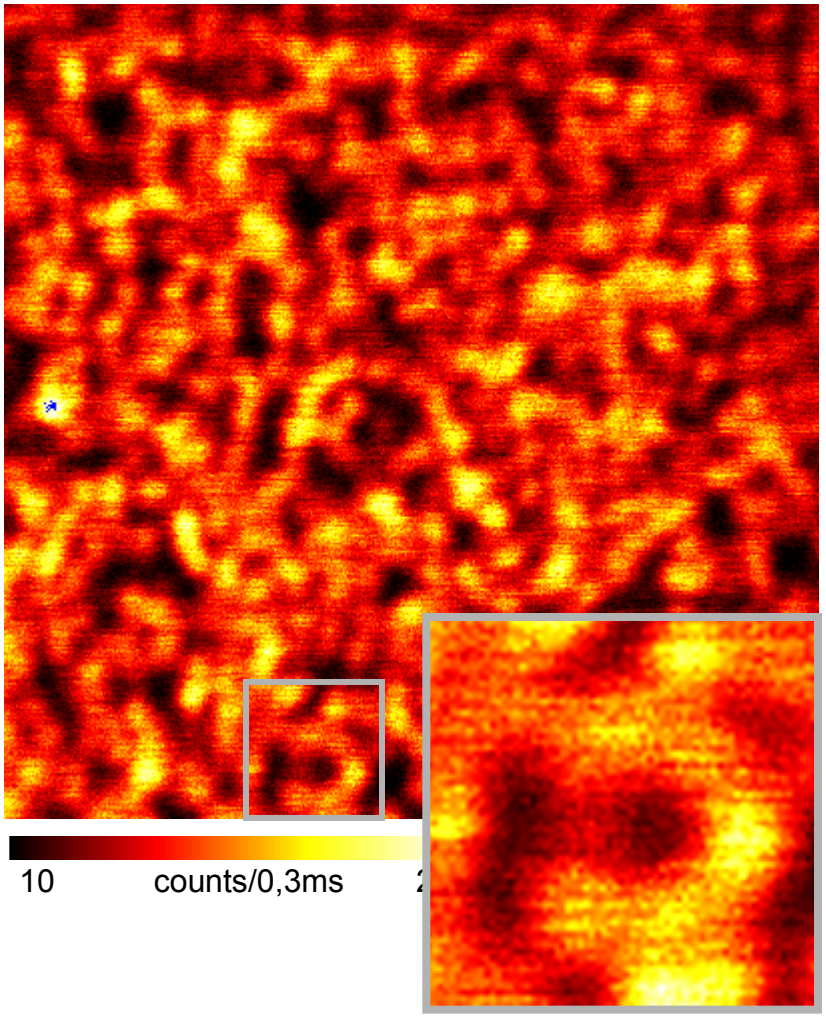


λ_{STED}= 770 nm

Westphal, Hell, *PRL* (2005)

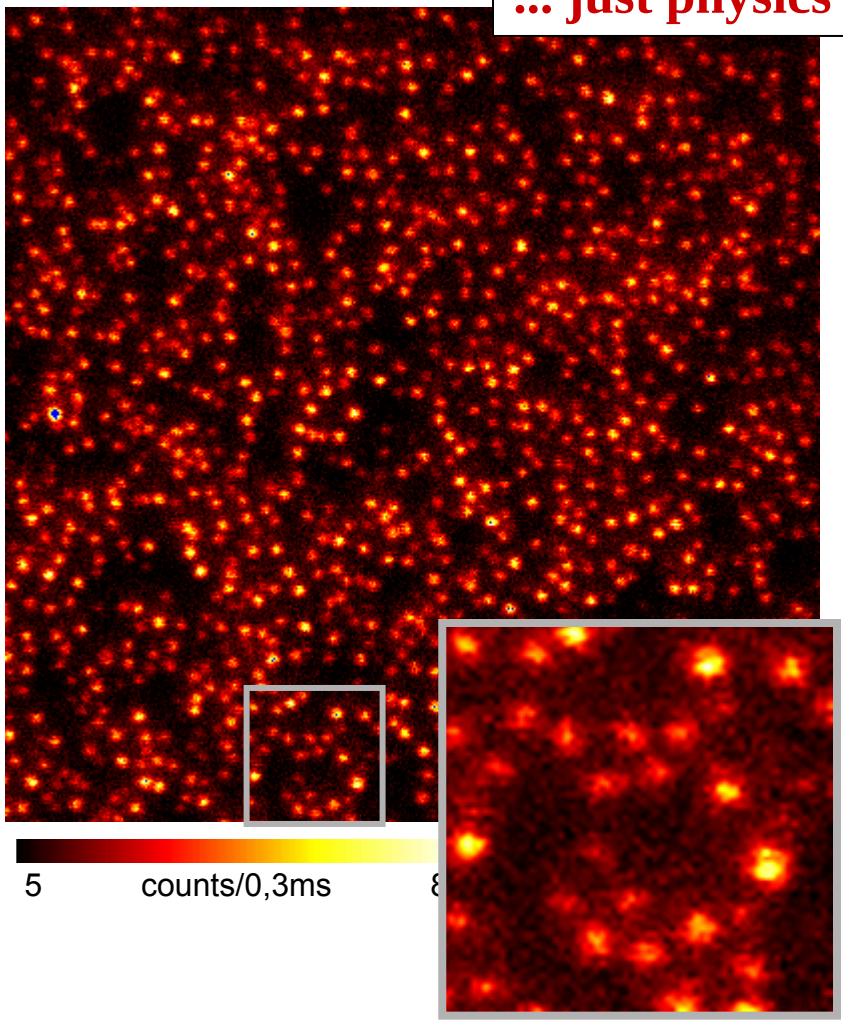
40 nm beads (Molecular Probes)

Confocal



STED

... just physics !



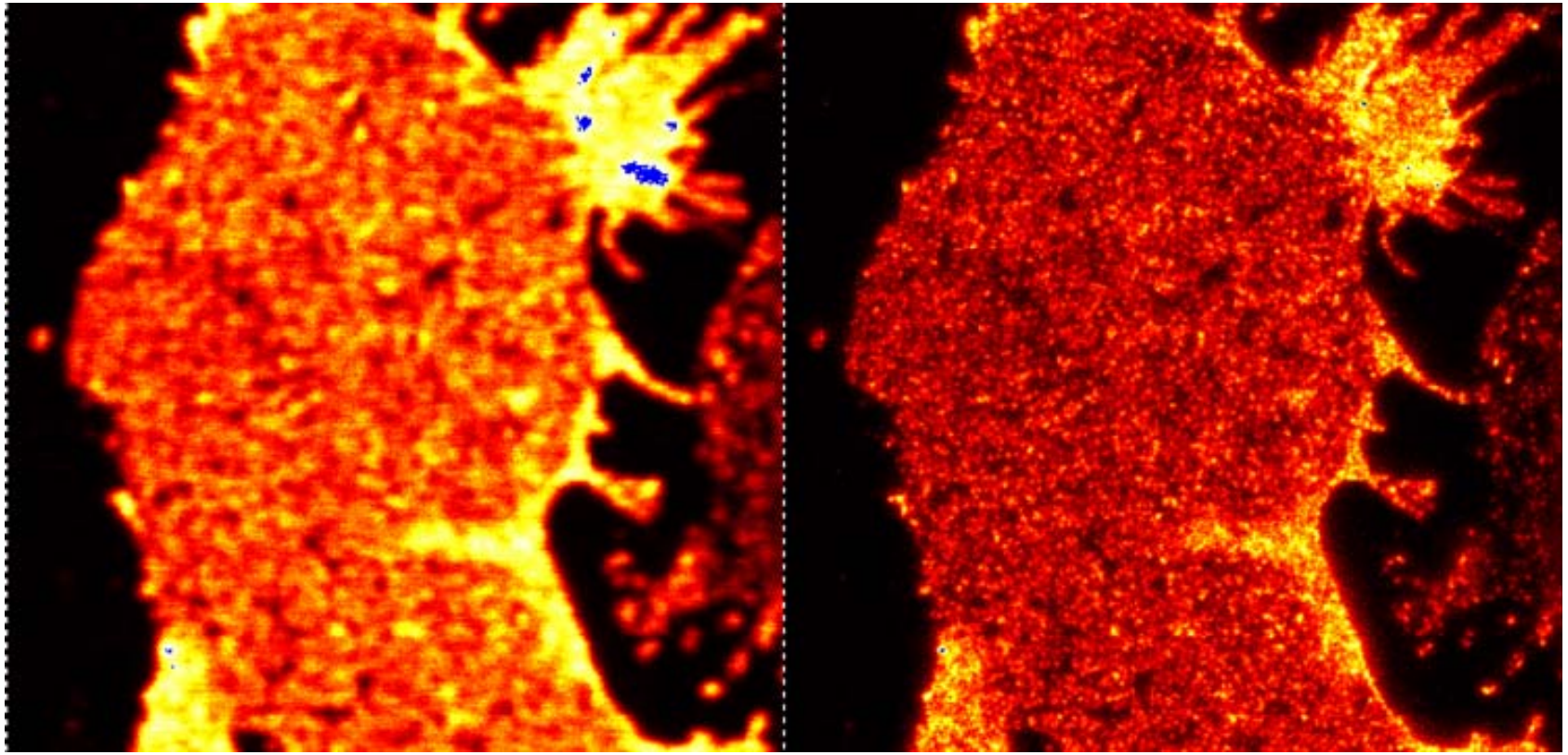
Biological imaging….

(Immunofluorescence)

SNAP- 25 / plasma membrane

...just physics !

Confocal: **STED:**



K. Willig et al, *NJP* (2006)

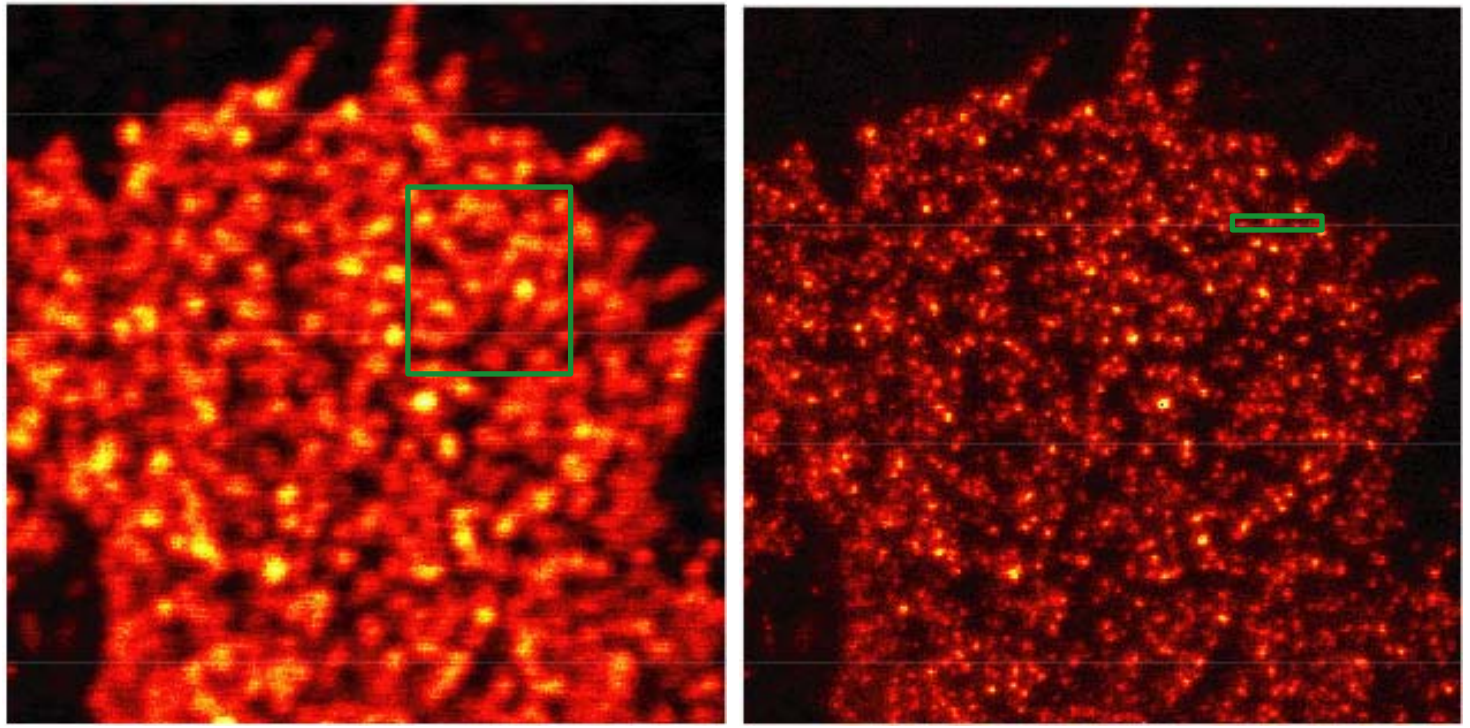
Syntaxin 1A/ plasma membrane

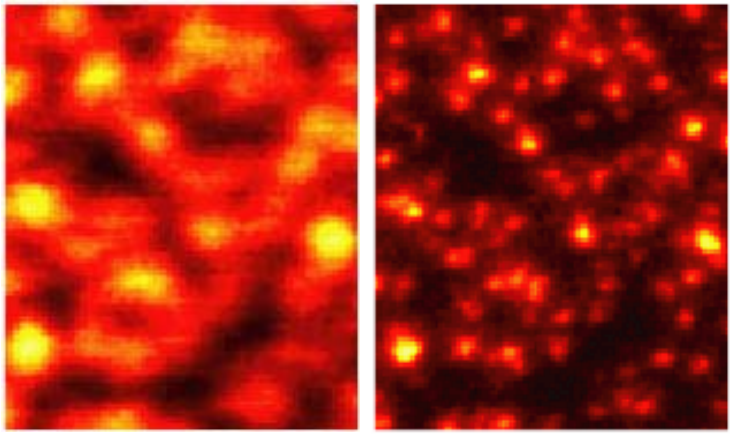
...just physics !

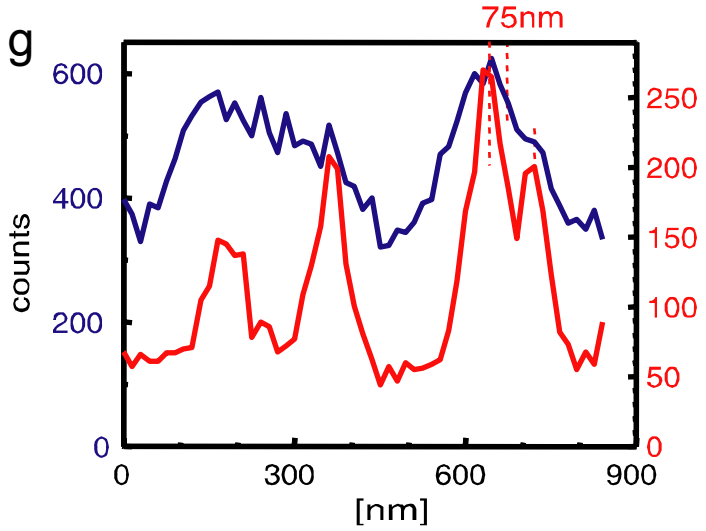
Confocal

STED

9 x 9 µm



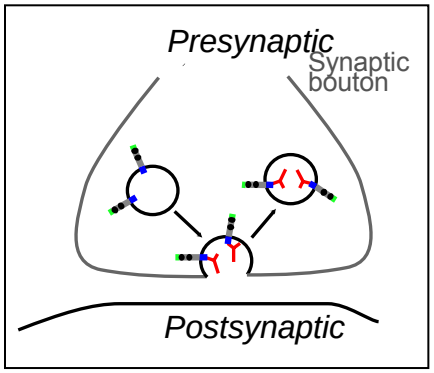


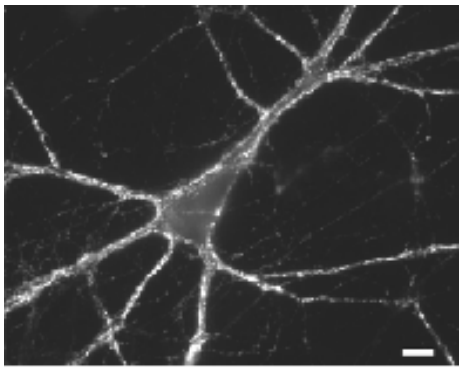


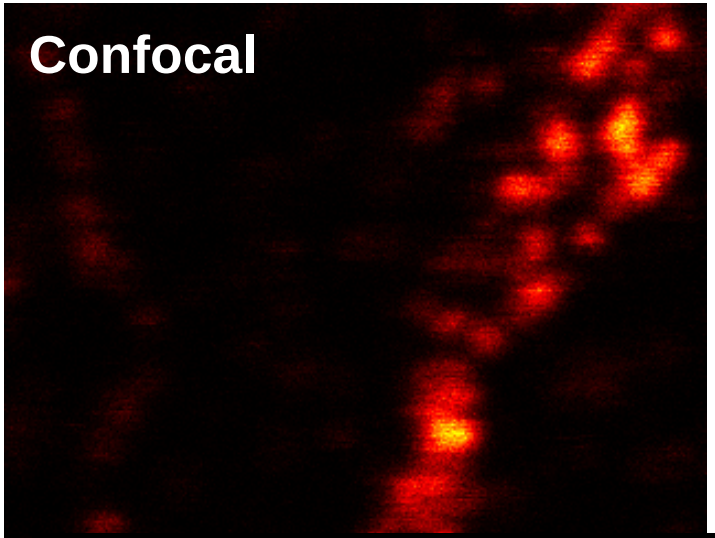
Sample: T. Lang, J. Sieber, R. Jahn

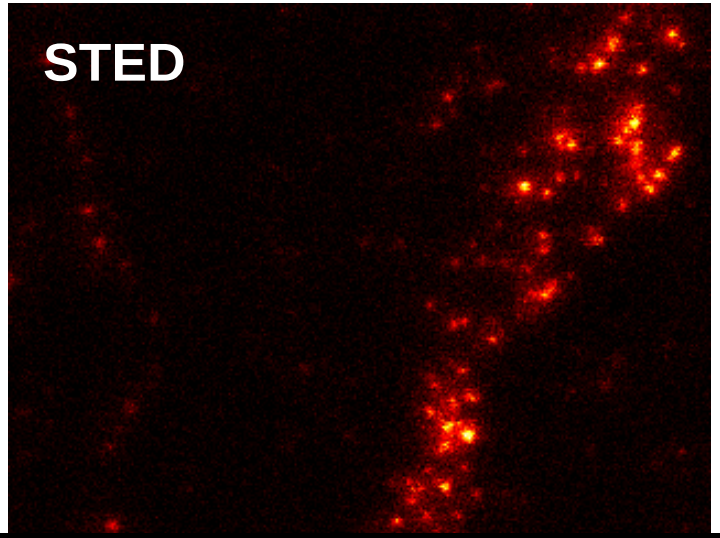
Neuronal communication

Synaptotagmin I antibody labeled



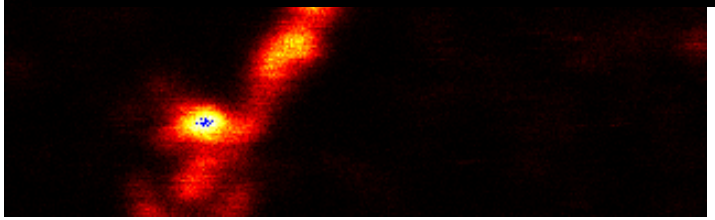


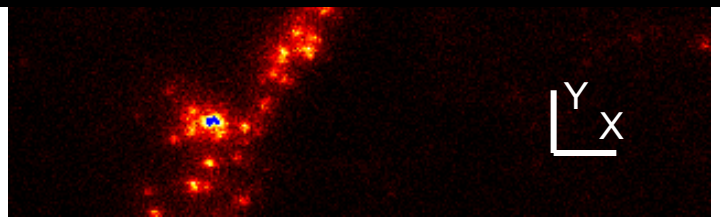




New biology:

Synaptic vesicle protein synaptotagmin I is clustered after exocytosis

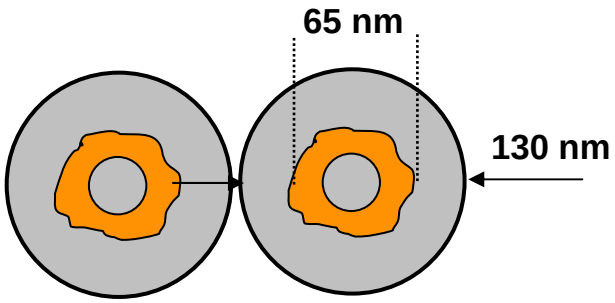




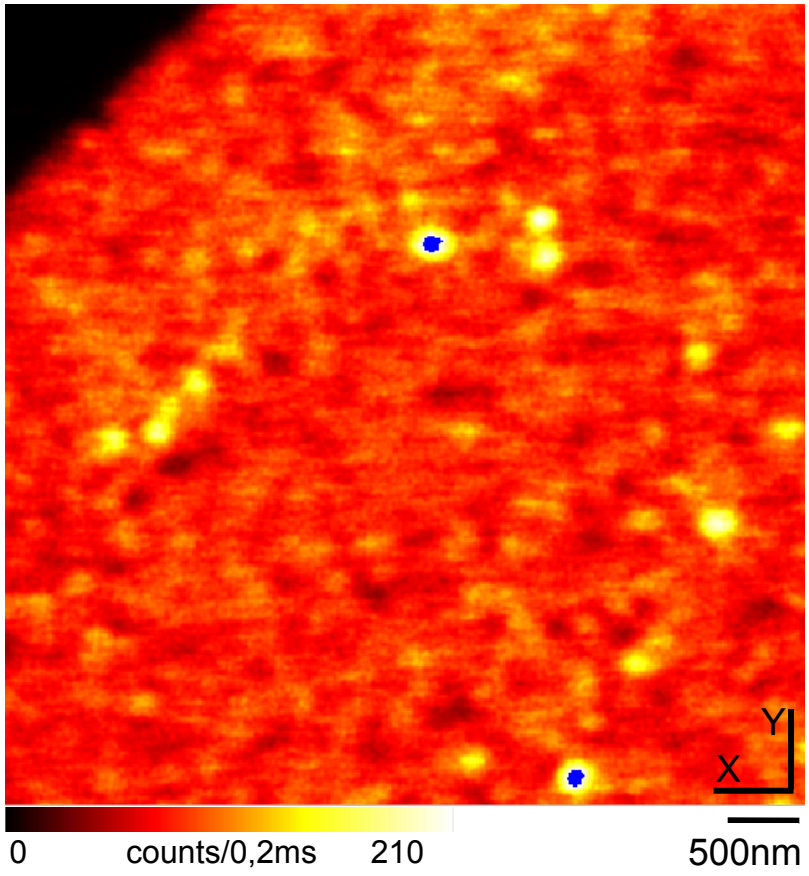
Willig, Rizzoli, Jahn, Hell, *Nature* (2006)

Nonbiological imaging….

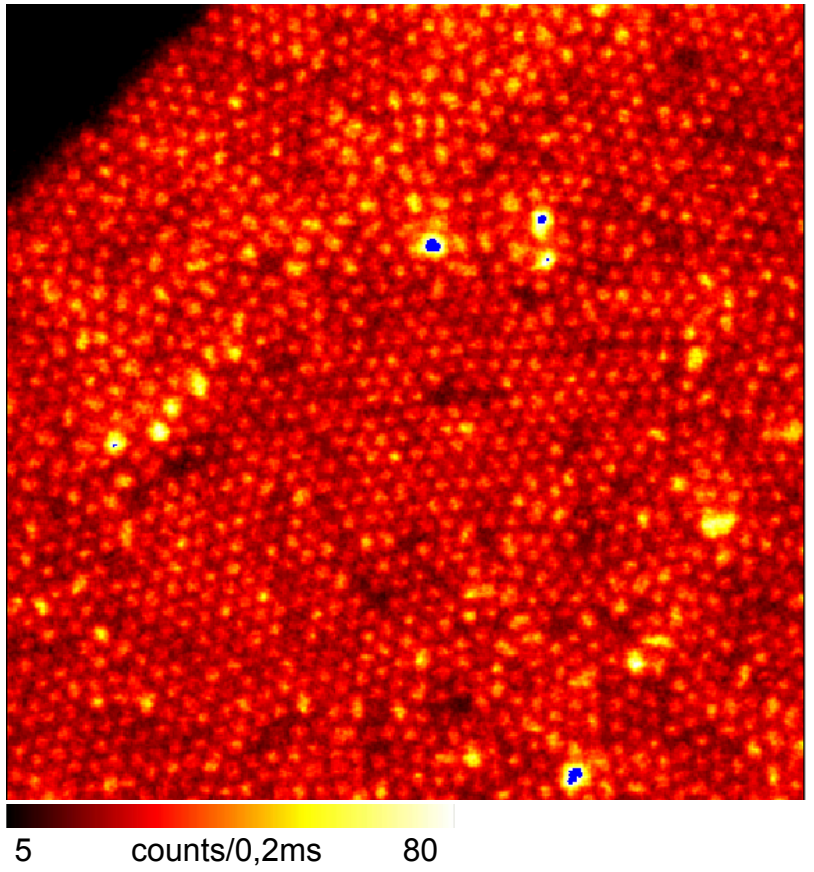
Colloids:
Silica beads with fluorescent core



Confocal



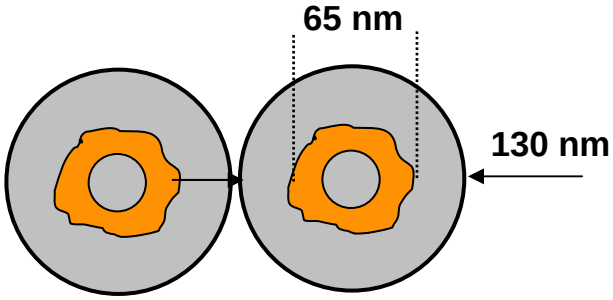
STED



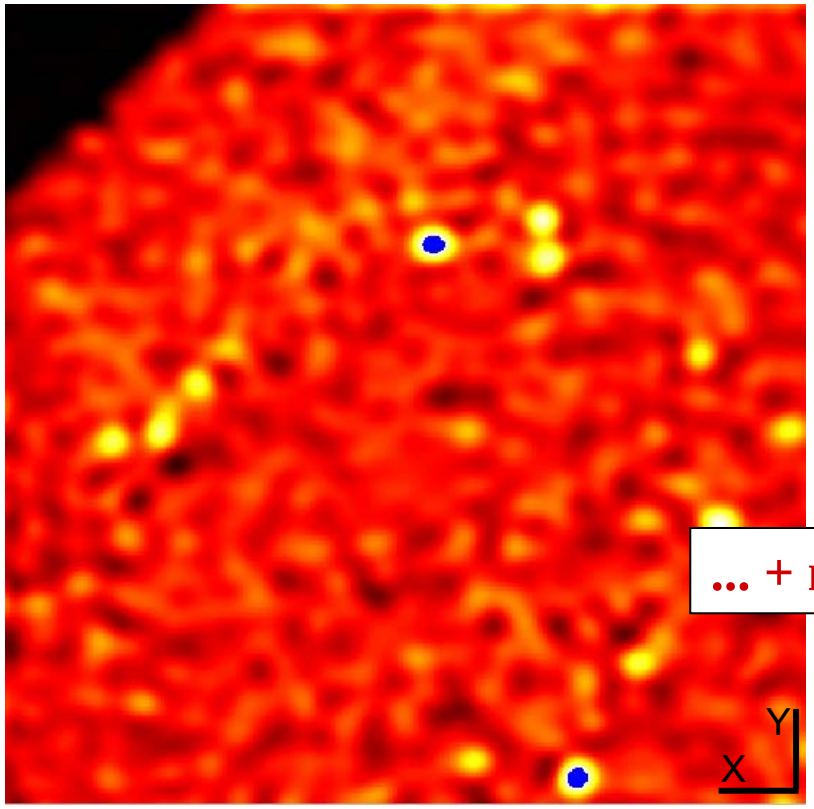
Sample by A van Blaaderen

Willig et al, *New J Phys* (2006)

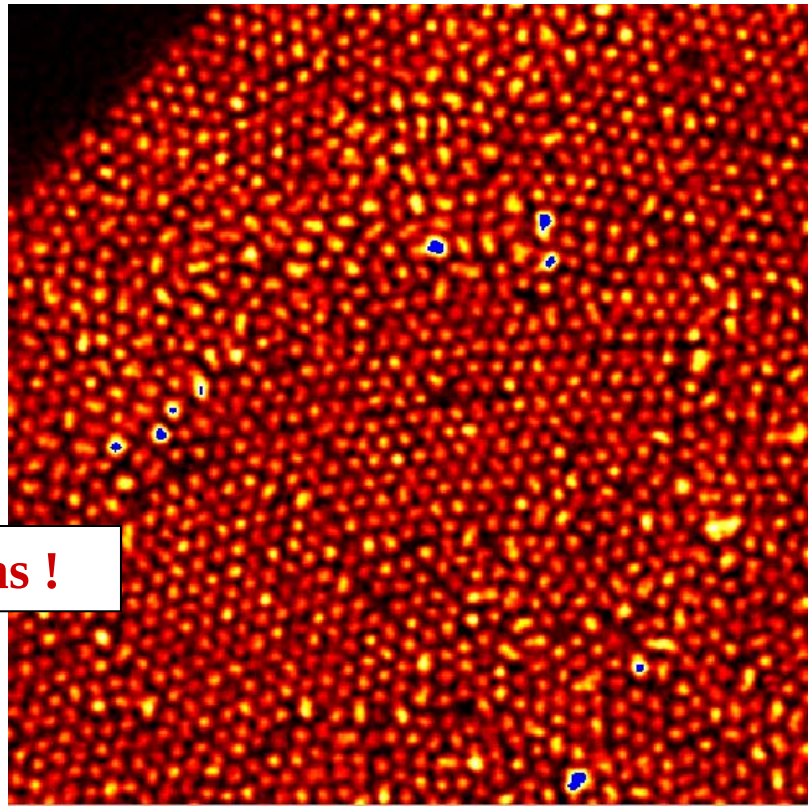
Colloids:
Silica beads with fluorescent core



Confocal + Lin Deconv.



STED + Lin Deconv.

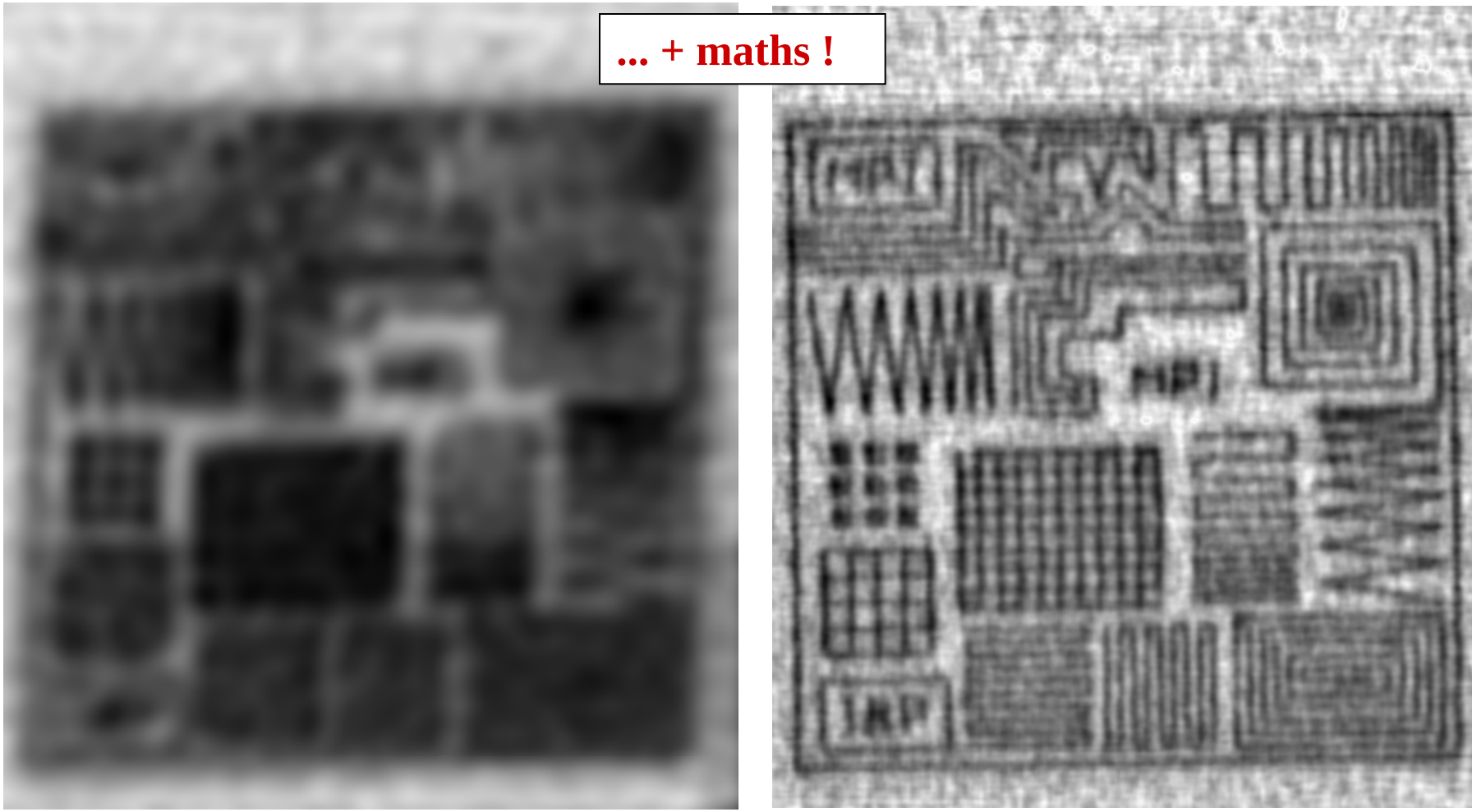


... + maths !

Willig et al, ***New J Phys*** **(2006)**

Confocal + Lin. Deconv.

STED + Lin. Deconv.

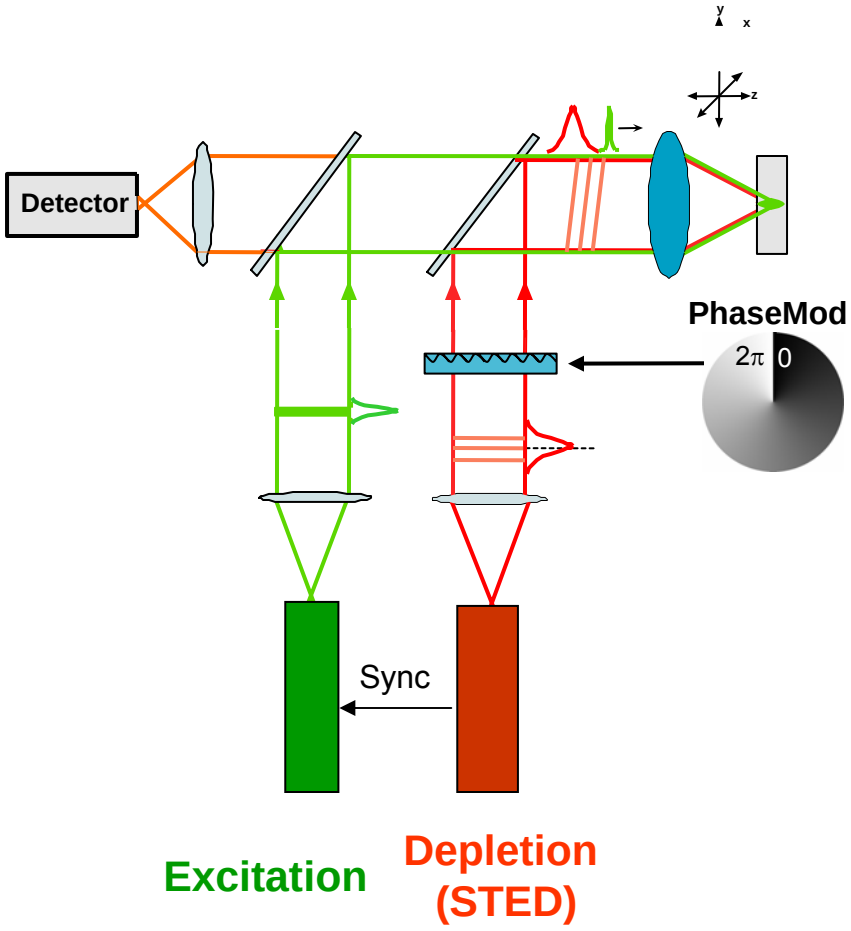


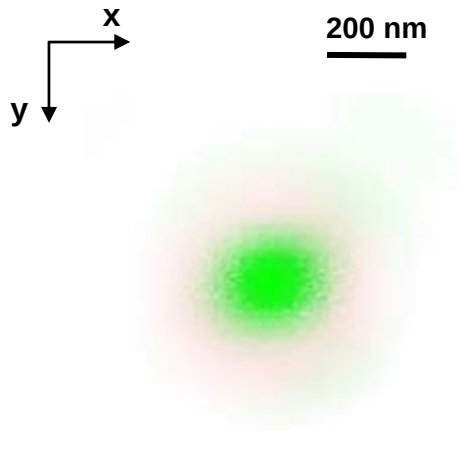
Field: (6x6) µm; Pitch 120 nm; Line width: *60 nm*

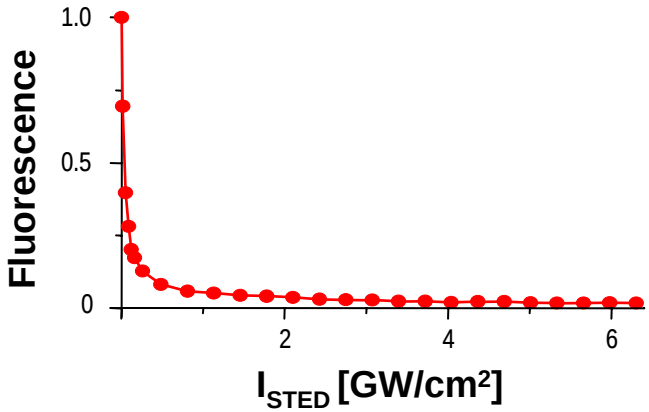
E-beam lithography on dyed PMMA

Westphal et al, *J Phys B* (2005)

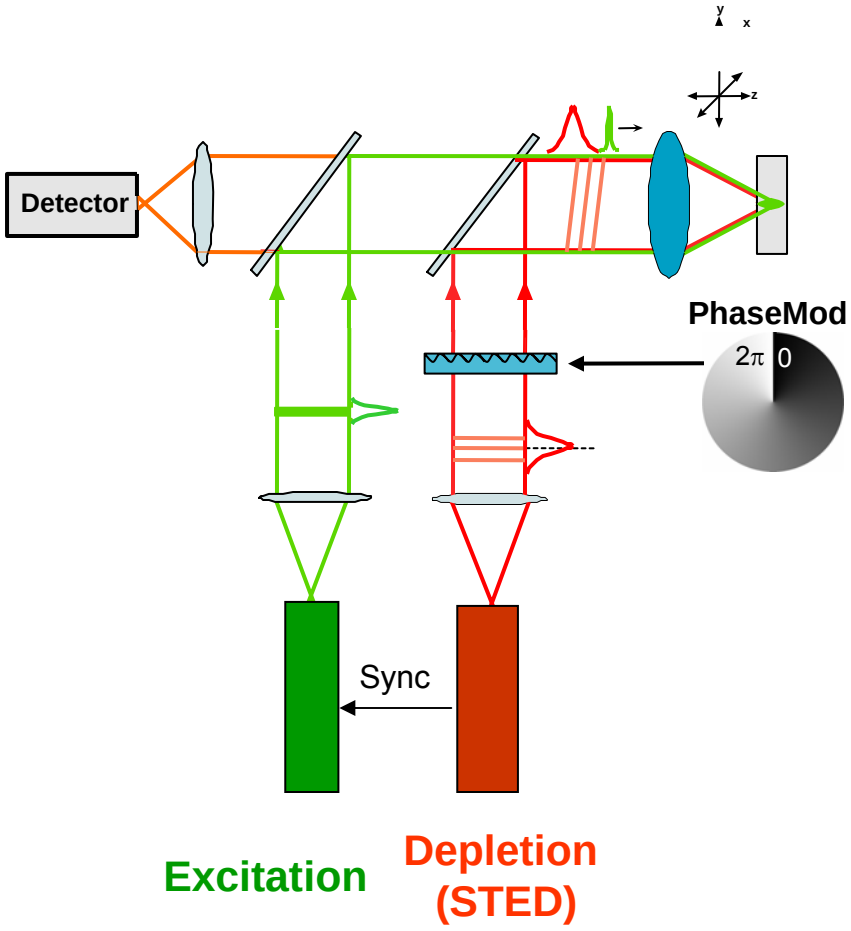
STED microscope

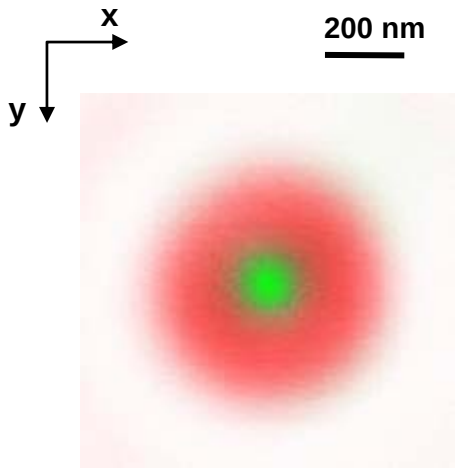


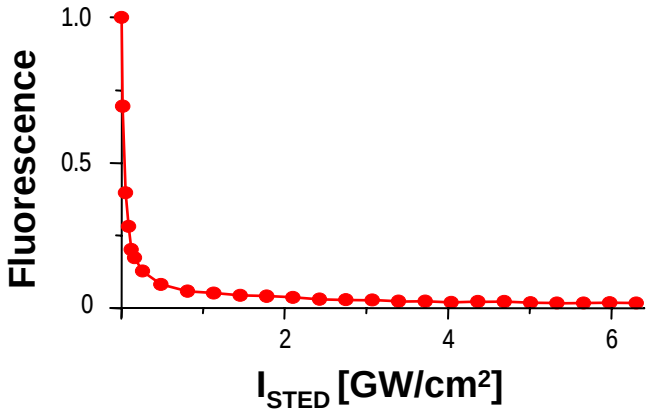




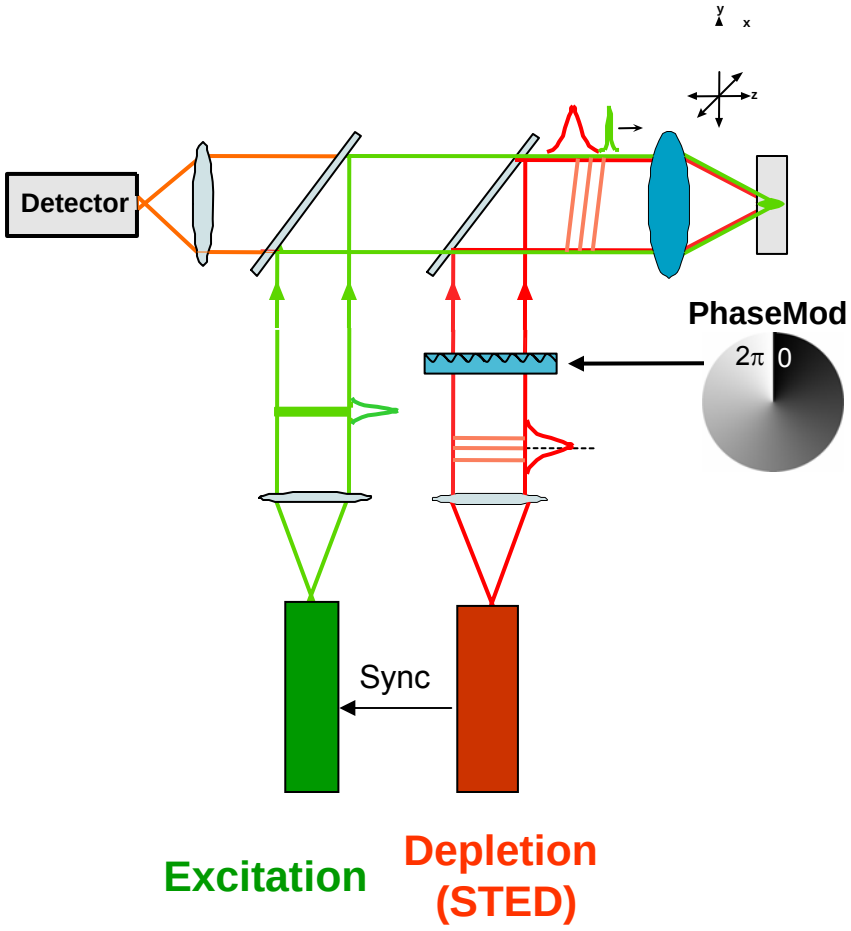
STED microscope

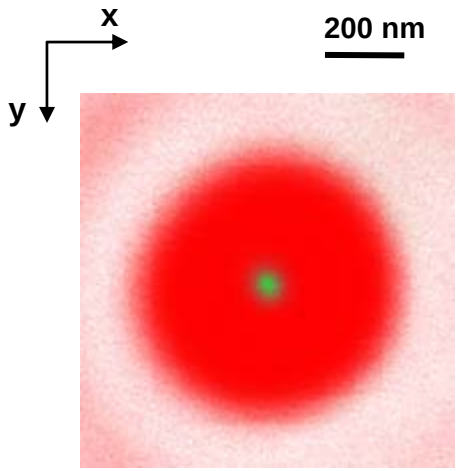


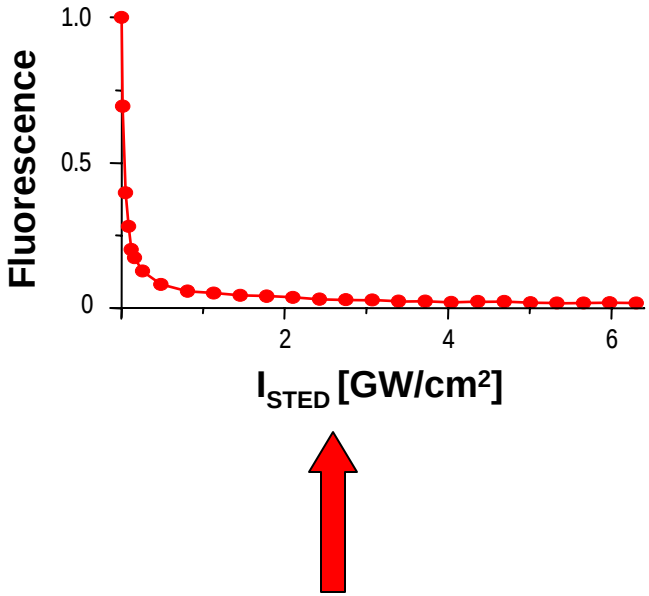




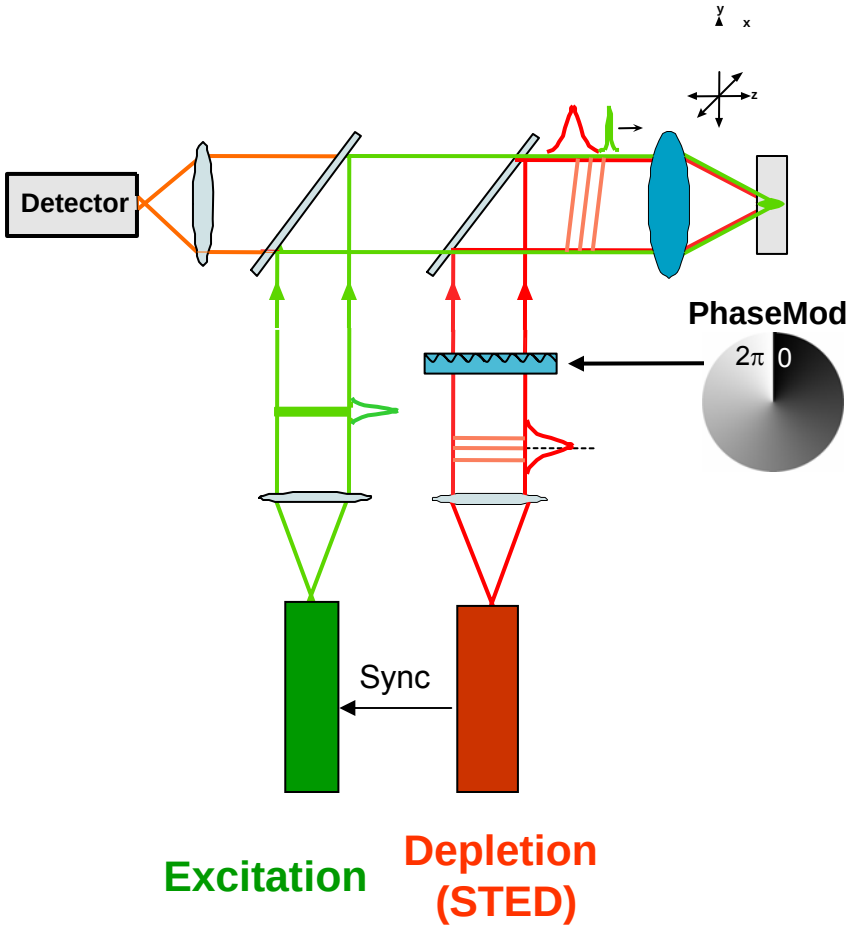
STED microscope

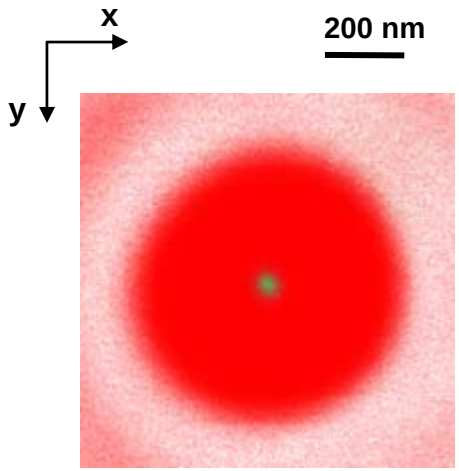


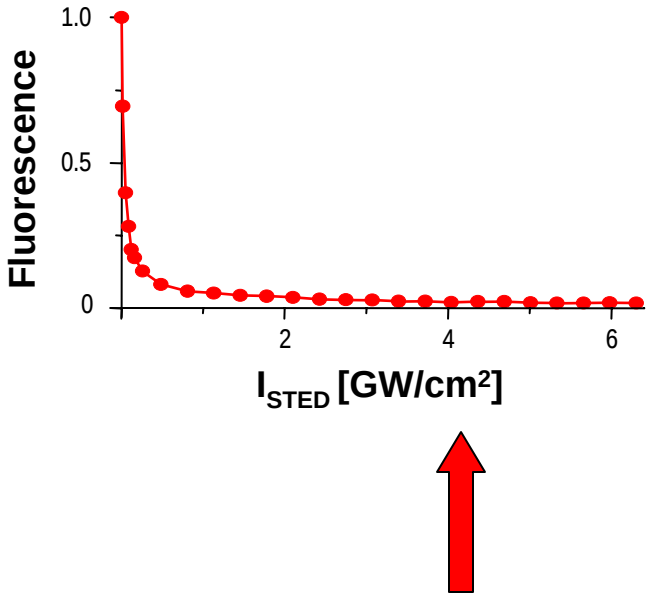




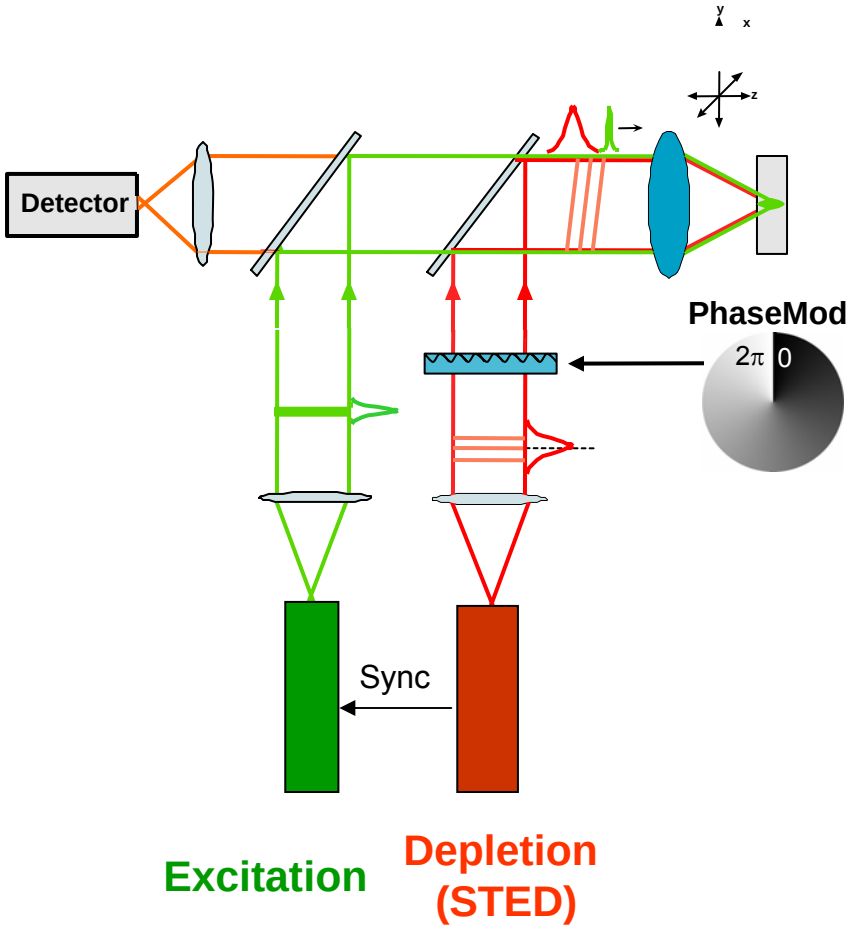
STED microscope

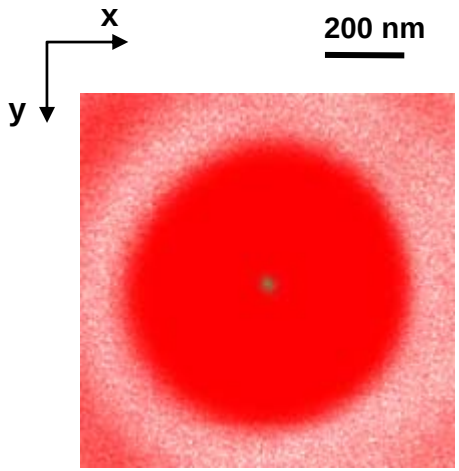


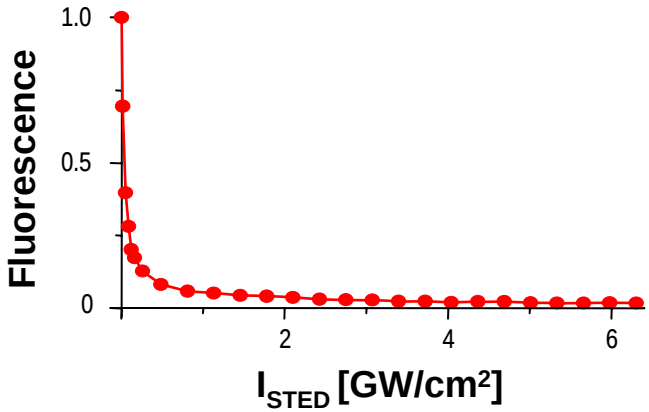




STED microscope







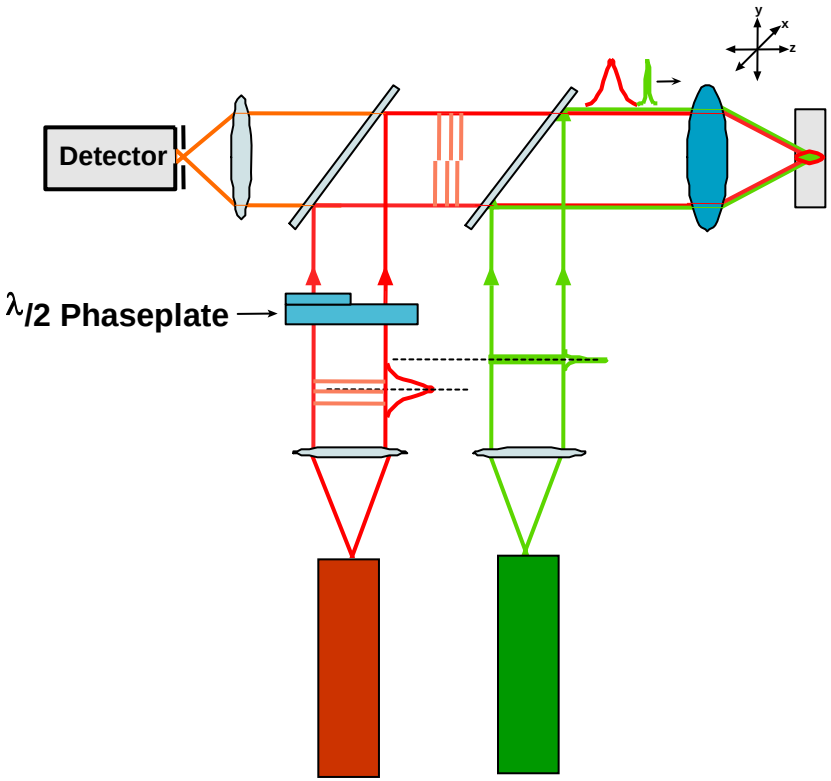
In far-field fluorescence microscopy:

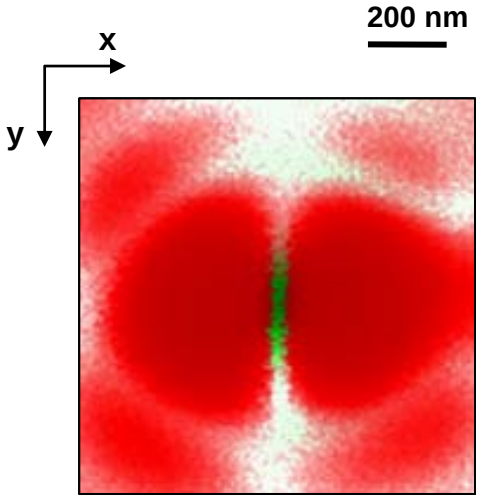
Diffraction resolution barrier does no longer exist !

Molecular scale resolution is possible with visible light and regular lenses.

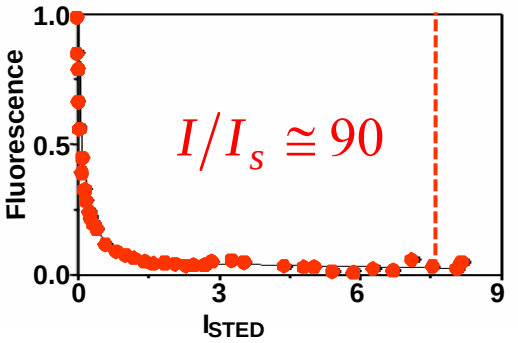
"With a rectangular depletion curve, the resolution could be increased to infinity."
Hell & Wichmann, *Opt. Lett.* 19 (1994)

STED Microscopy



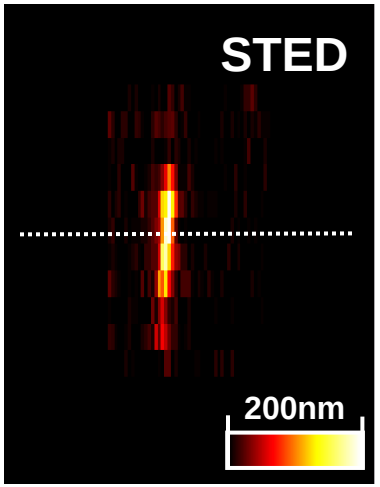


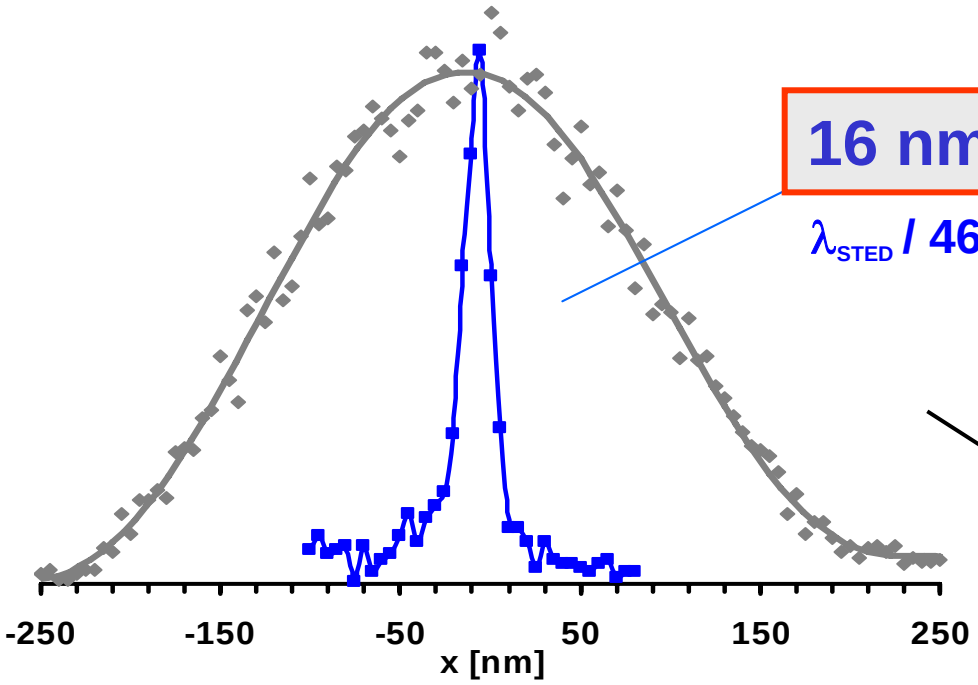
Westphal & Hell, *PRL* 94 (2005)



... 14 x below Abbe

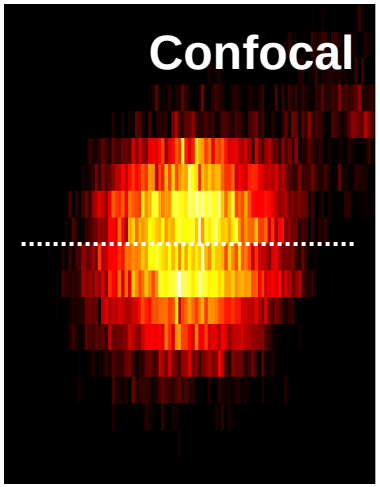
λ_{STED}= 770 nm





16 nm

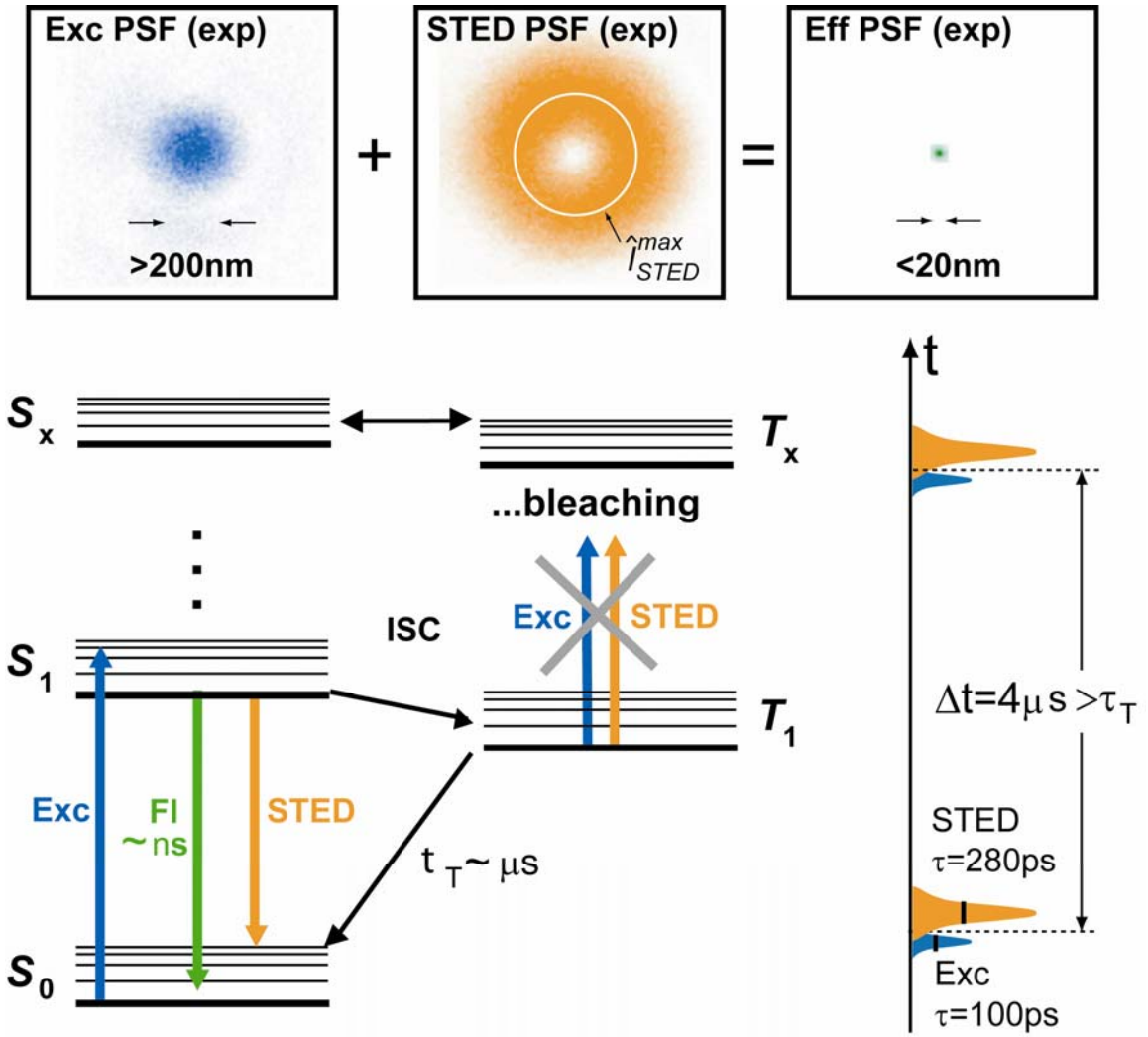
λ_{STED} / 46



Westphal & Hell, *PRL* 94 (2005)

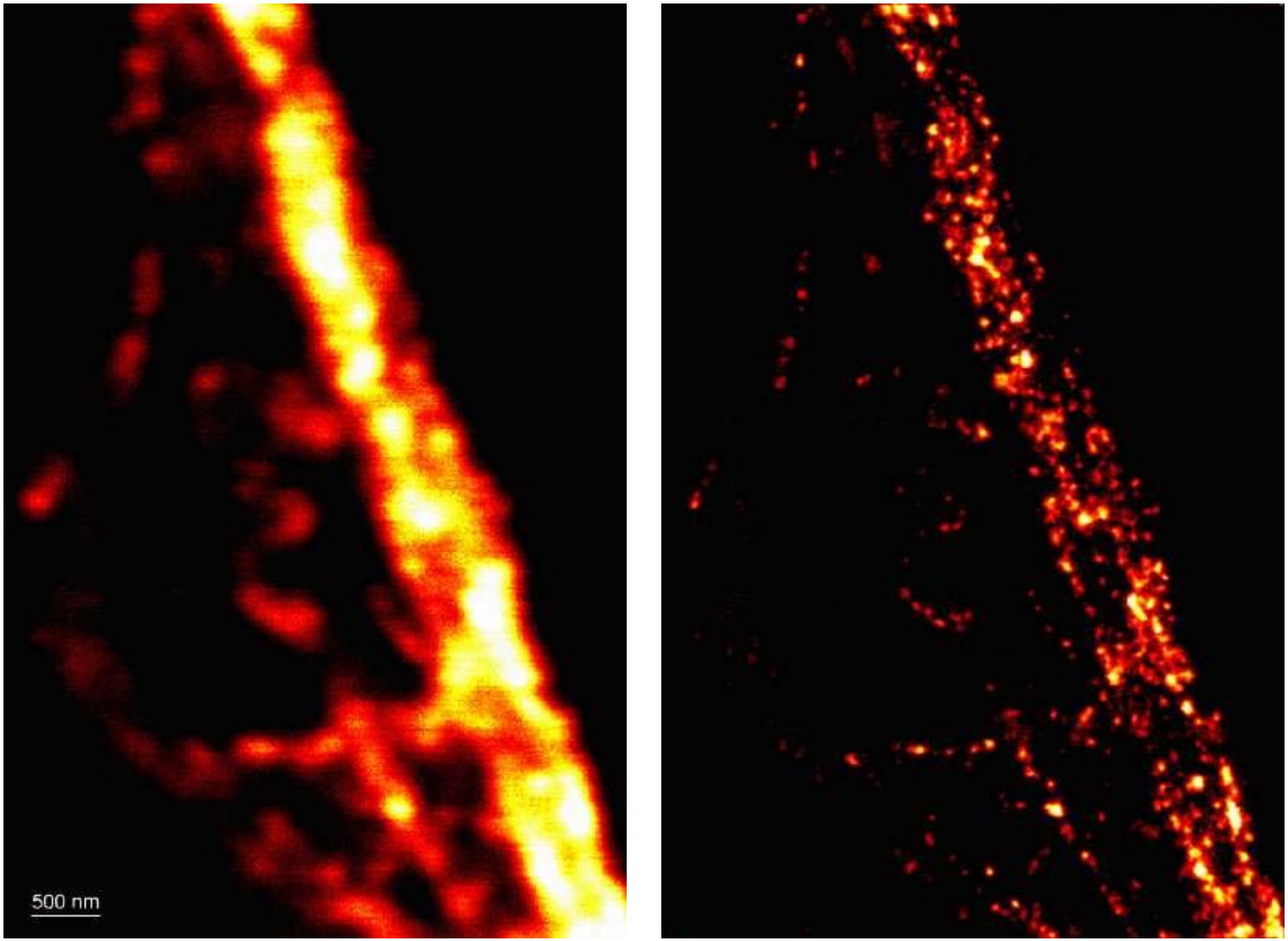
Triplet Relaxation STED microscopy

..provides <20 nm resolution (x,y)



Donnert et al, *PNAS* (2006)

Heavy subunit of neurofilaments in neuroblastoma

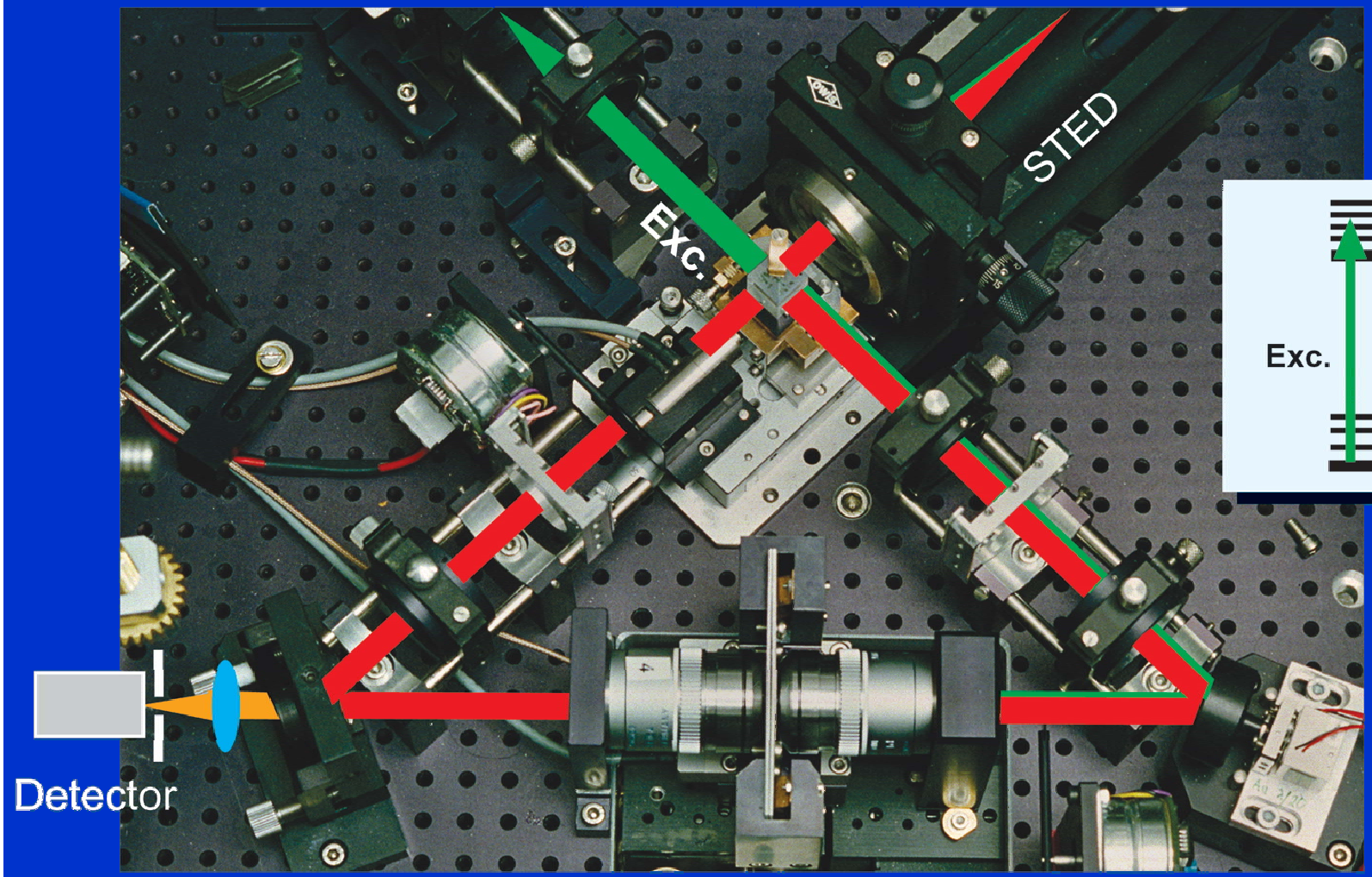


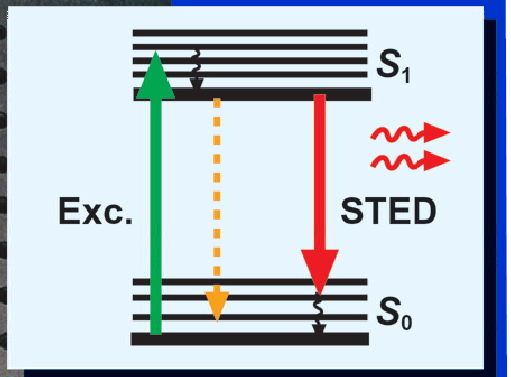
Donnert et al, *PNAS* (2006)

Axial resolution improvement

by

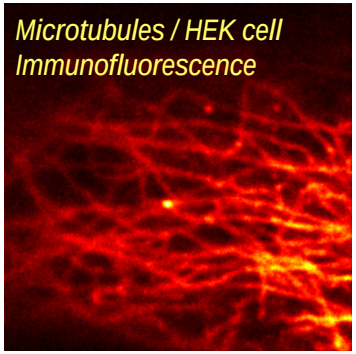
STED - 4Pi microscopy



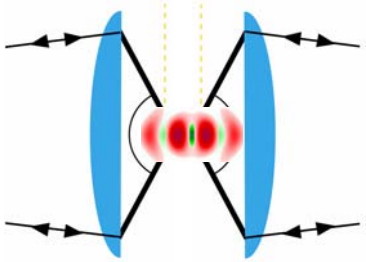


STED-4Pi

S.W. Hell, *in Topics Fluoresc Spectr V*, Plenum Press (1997)

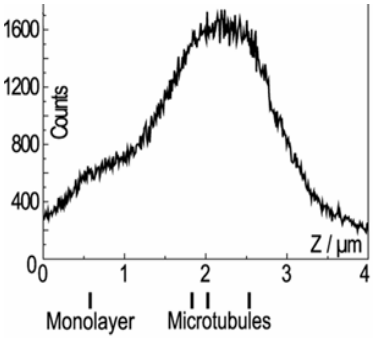


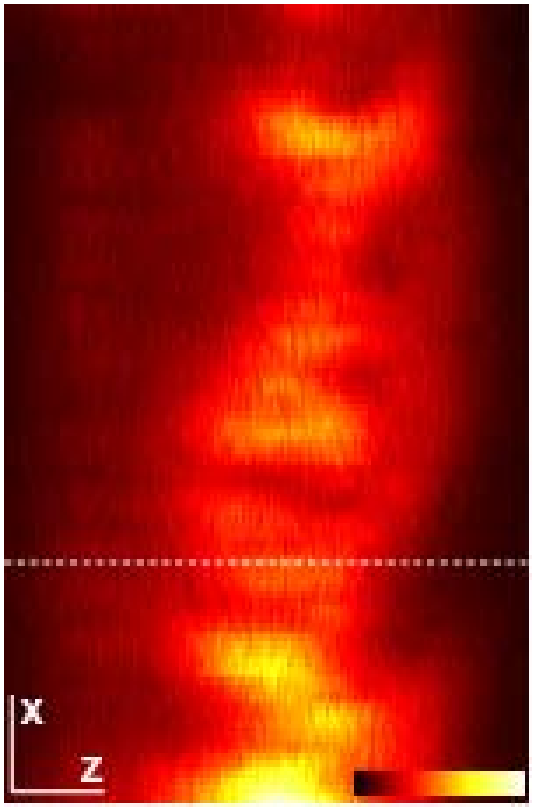
XY-Overview

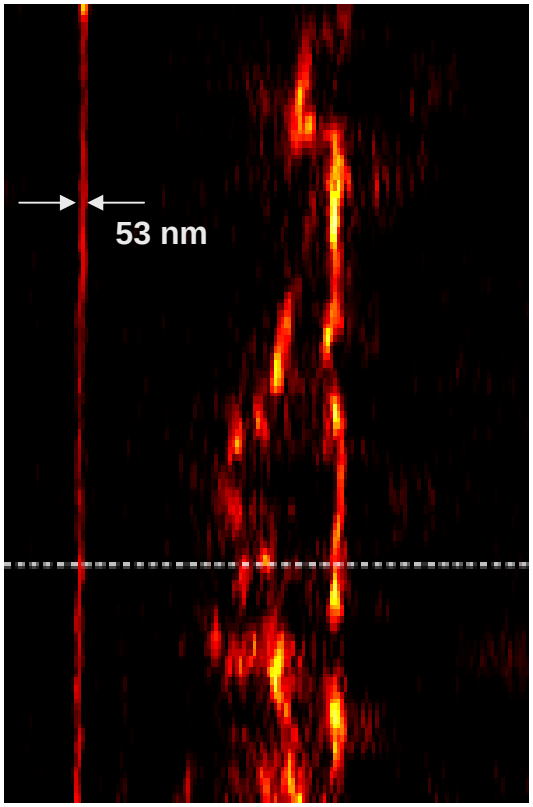


Confocal

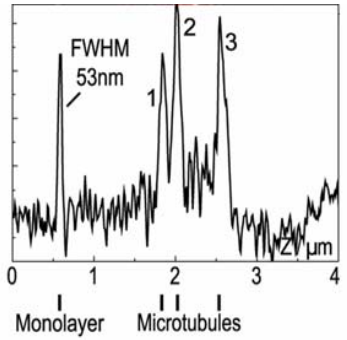
STED-4Pi







... 15 x improved

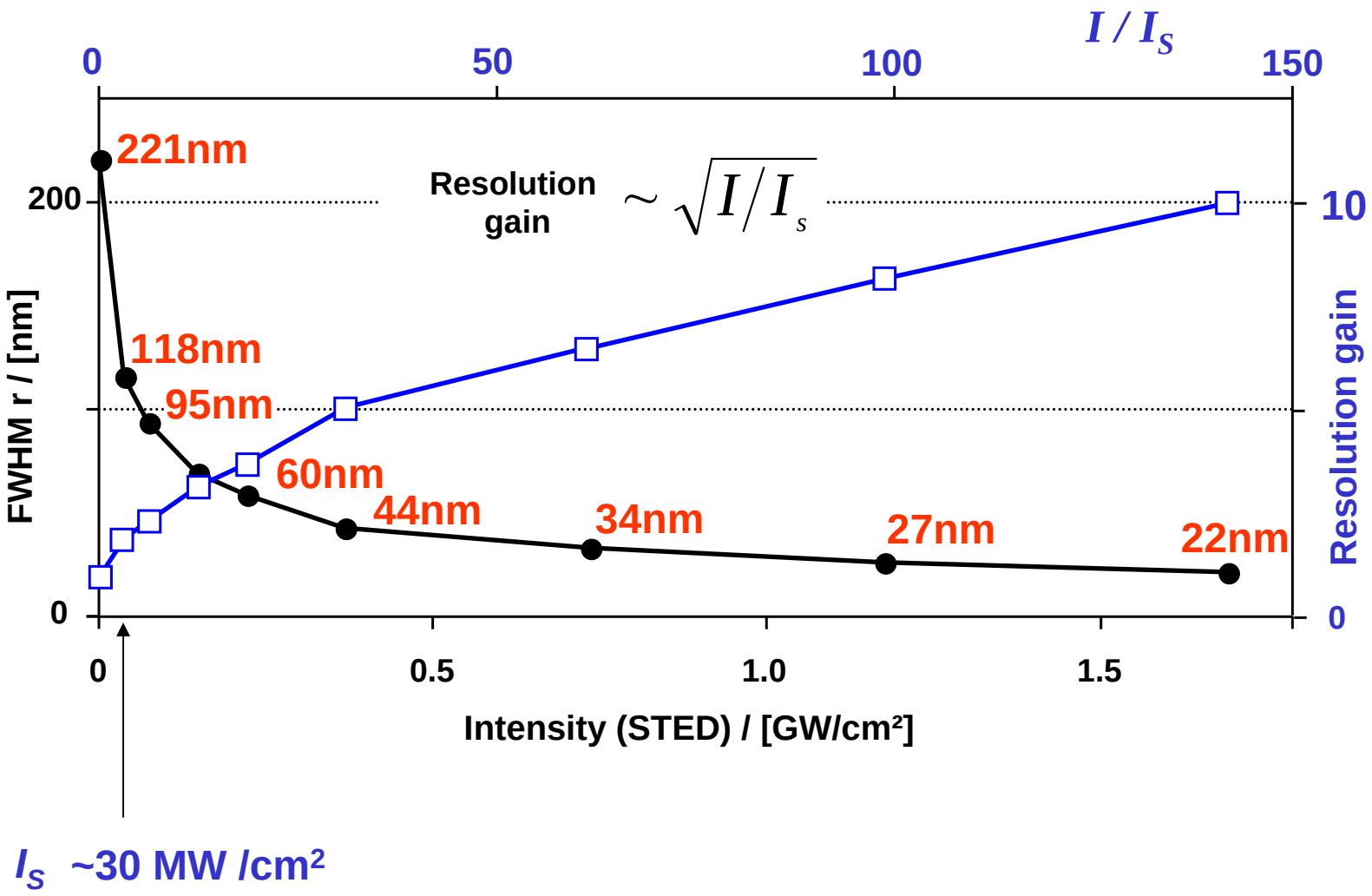


Monolayer

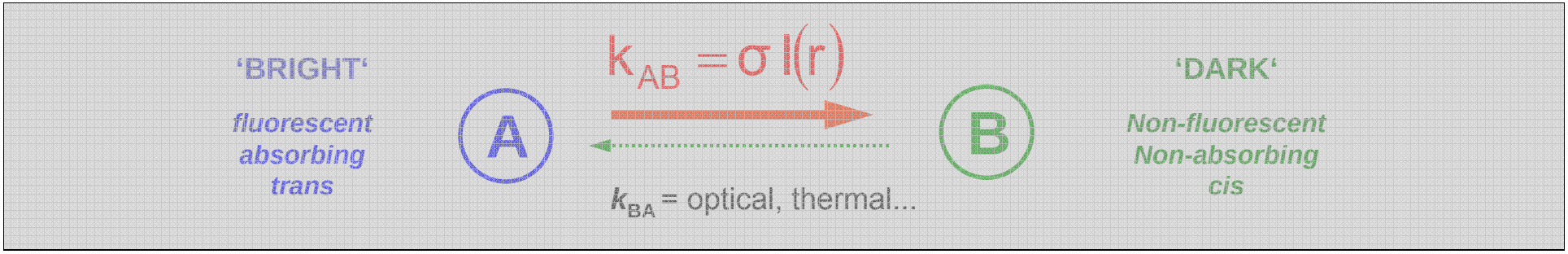
M. Dyba &SWH, *Nature Biotech* 21, (2003)

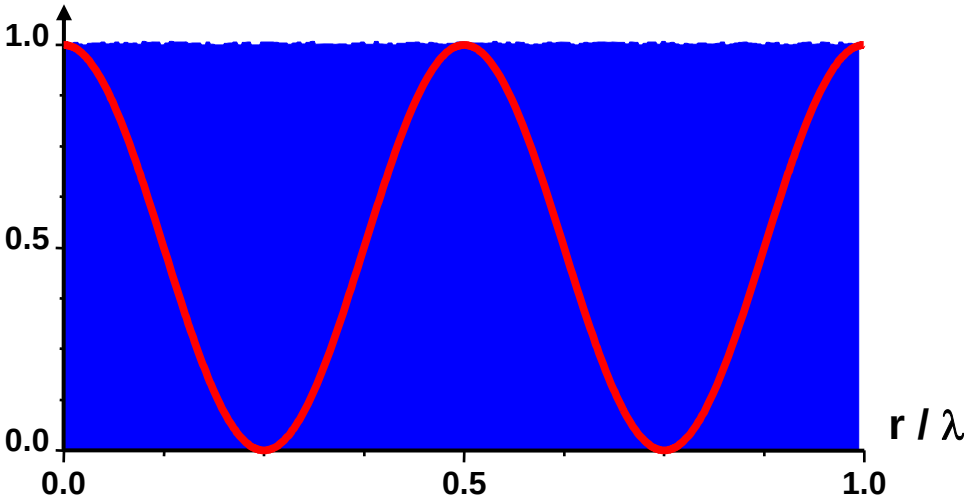
How does resolution scale with intensity ?

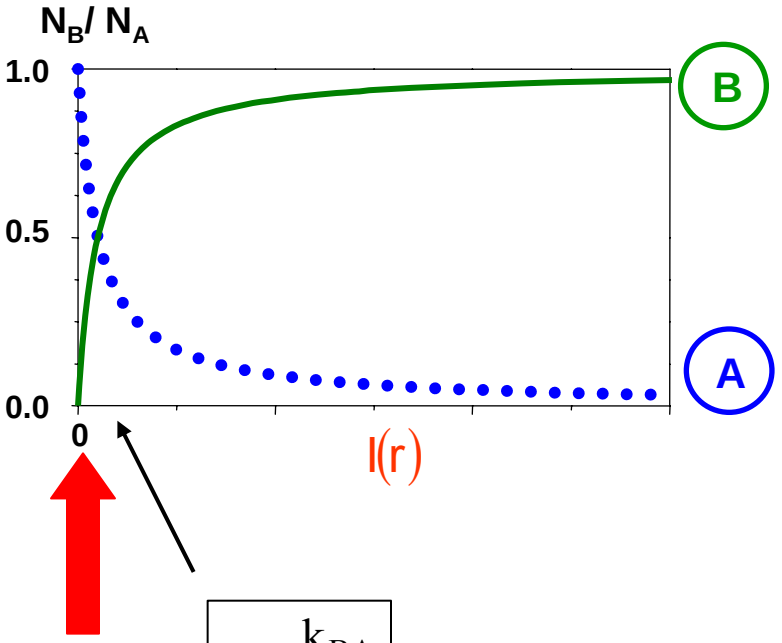
Resolution gain with intensity



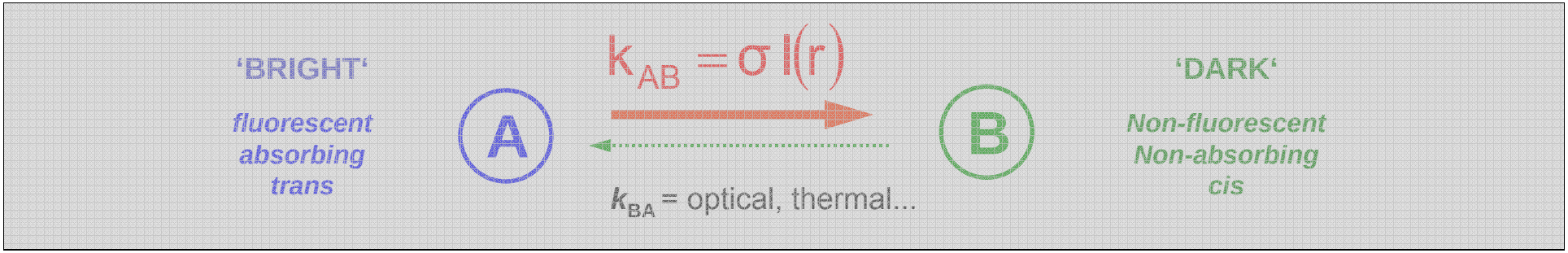
The basic principle ....

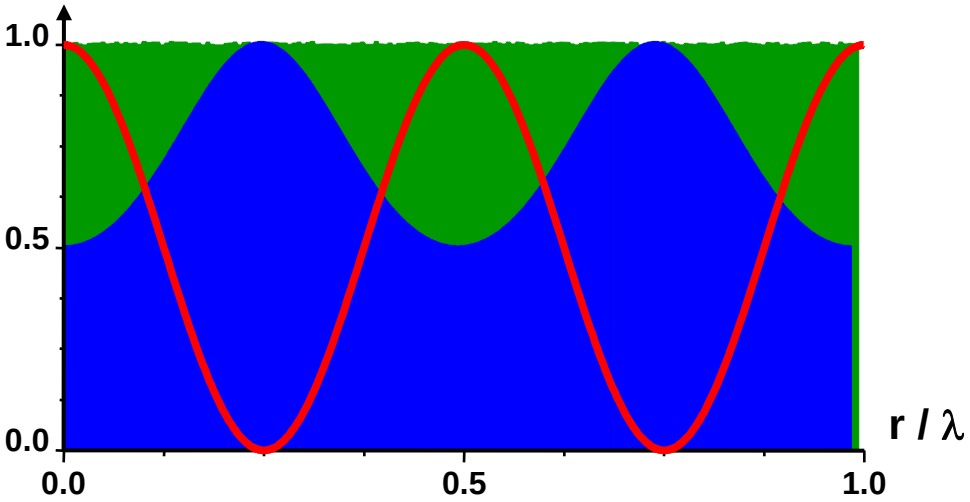


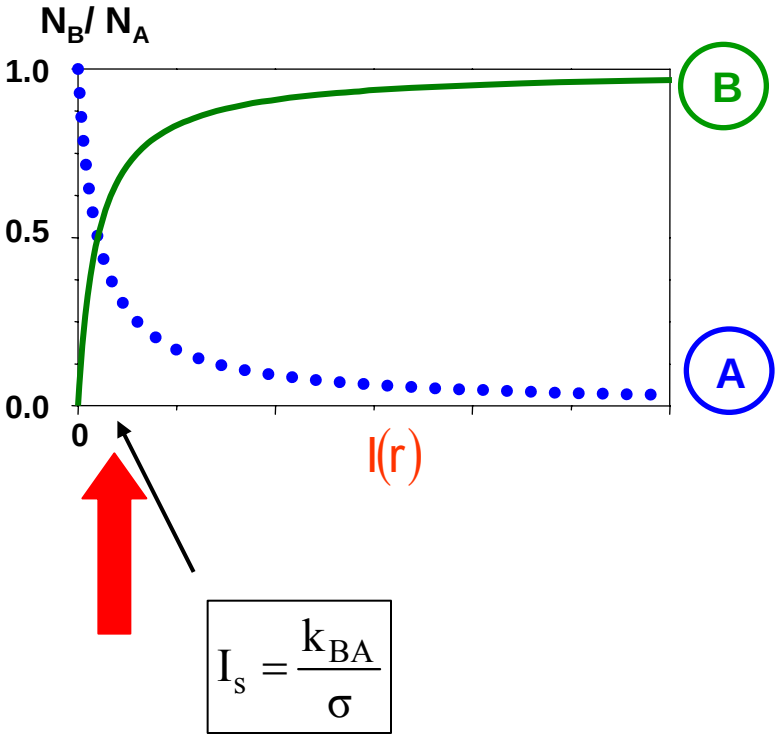


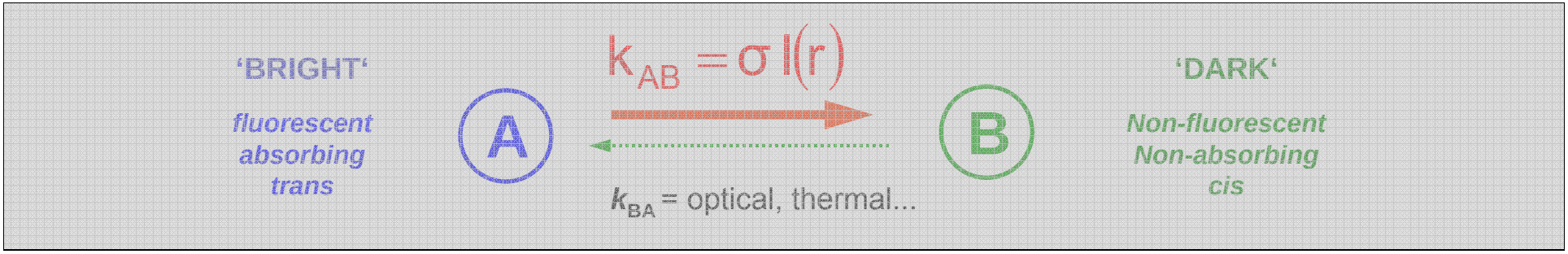


$$I_s = \frac{k_{BA}}{\sigma}$$


'BRIGHT'
fluorescent
absorbing
trans
A
$k_{AB} = \sigma I(r)$
k_{BA} = optical, thermal...
B
'DARK'
Non-fluorescent
Non-absorbing
cis


1.0
0.5
0.0
0.0
0.5
1.0
r / λ


N_B / N_A
1.0
0.5
0.0
0
B
A
I(r)
$I_s = \frac{k_{BA}}{\sigma}$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fluorescent
absorbing
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A
$k_{AB} = \sigma I(r)$
k_{BA} = optical, thermal...
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Non-fluorescent
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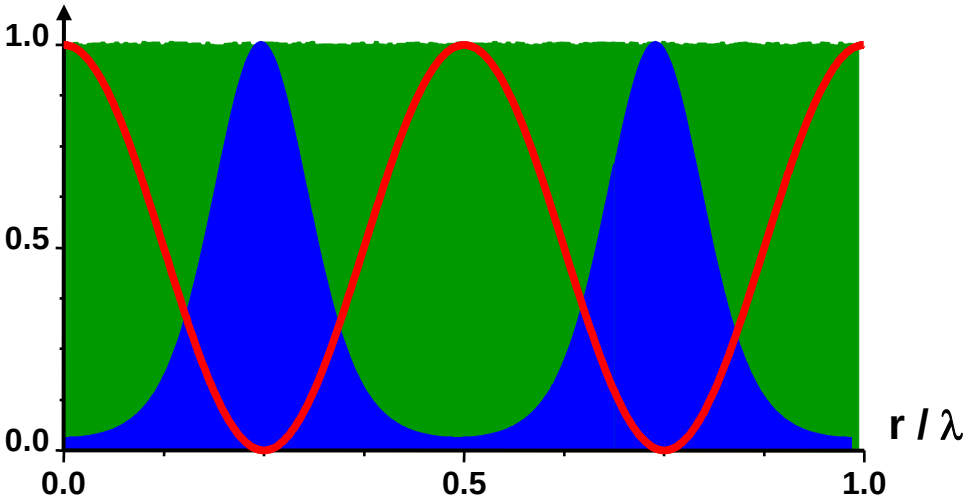
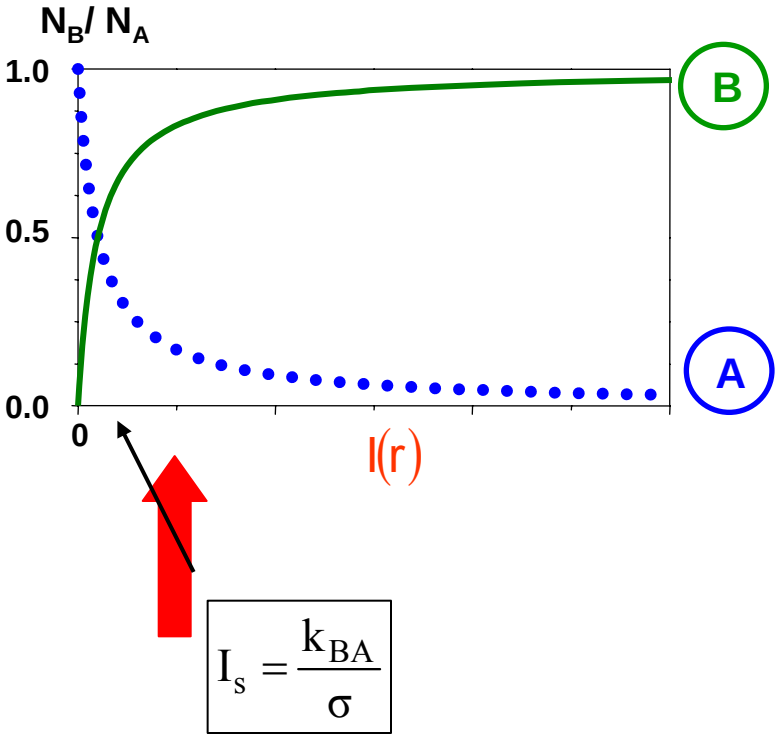
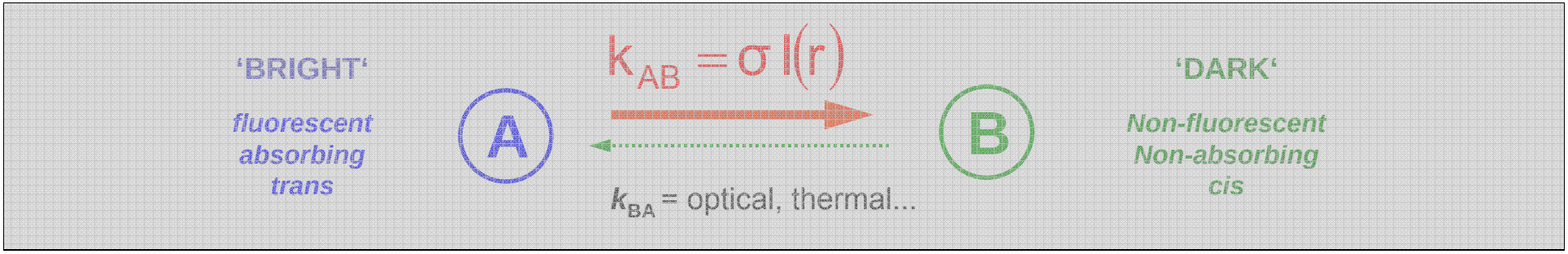
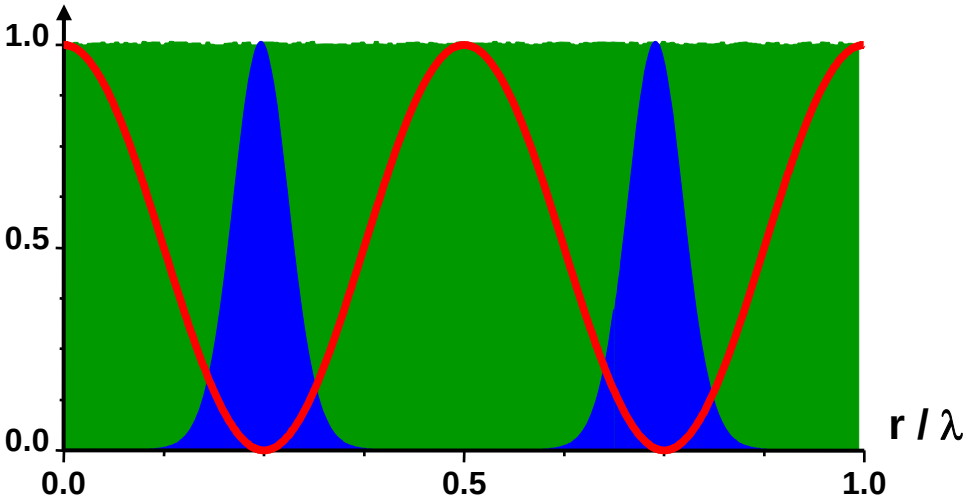
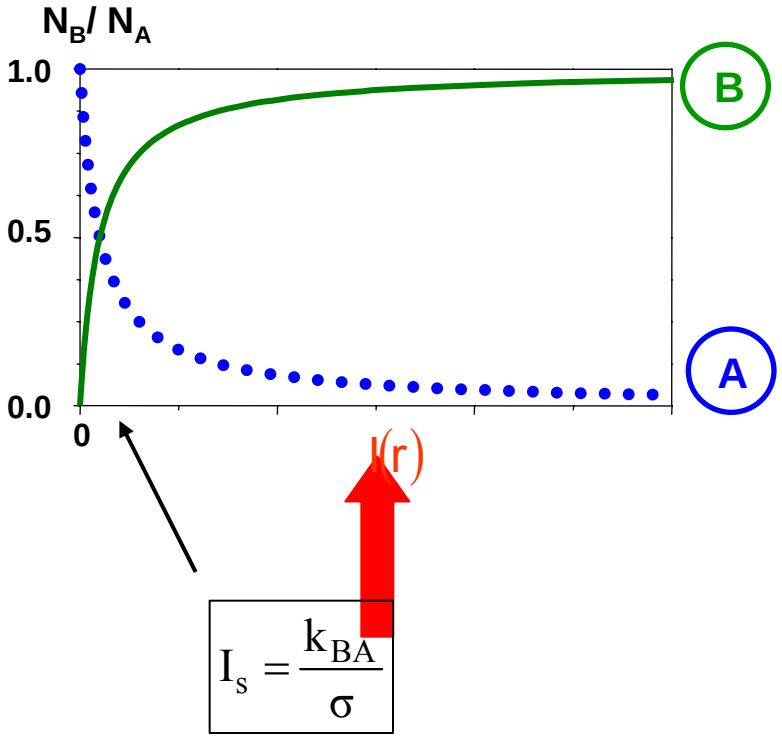
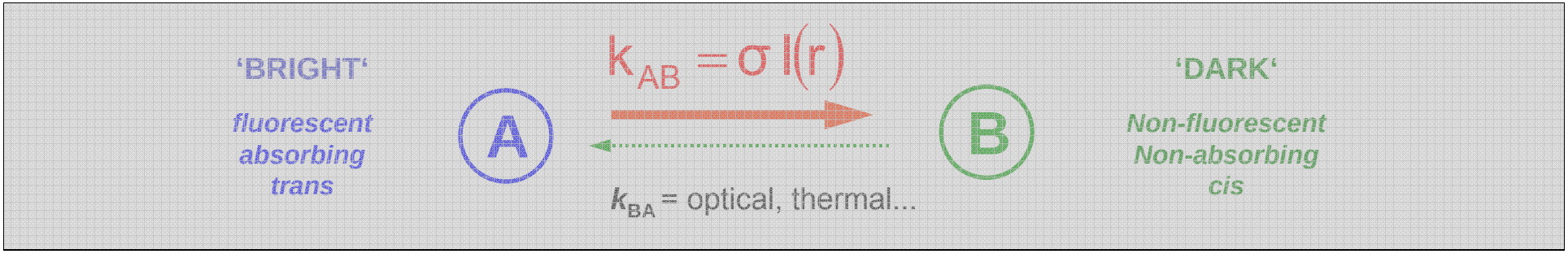

1.0
0.5
0.0
0.0
0.5
1.0
r / λ

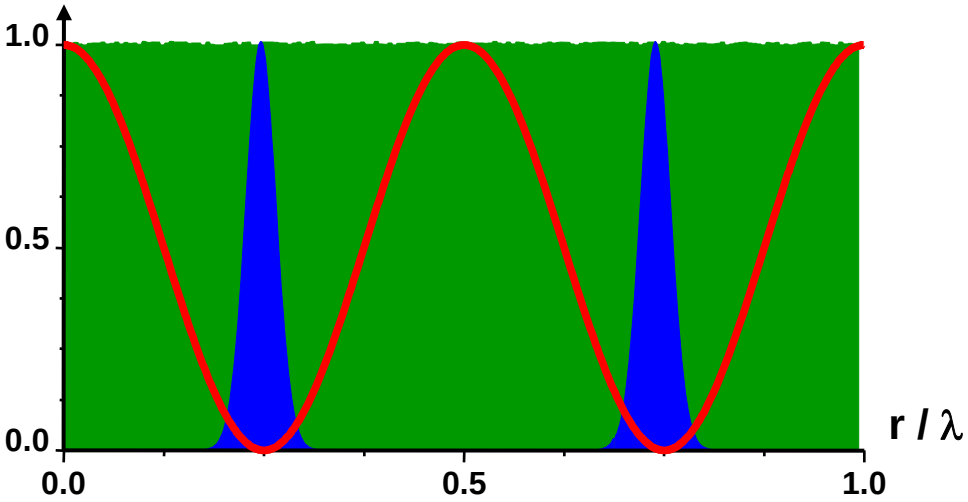

N_B / N_A
1.0
0.5
0.0
0
B
A
I(r)
$I_s = \frac{k_{BA}}{\sigma}$

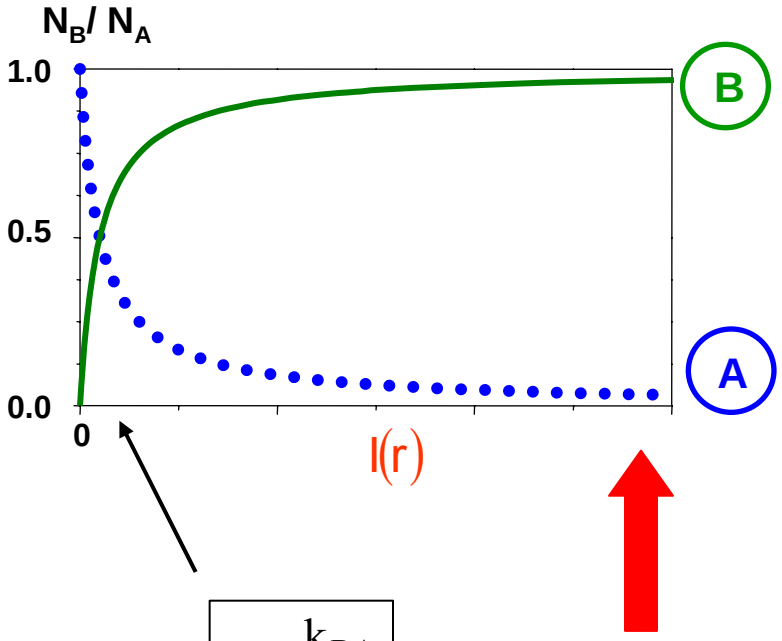

'BRIGHT'
fluorescent
absorbing
trans
A
$k_{AB} = \sigma I(r)$
k_{BA} = optical, thermal...
B
'DARK'
Non-fluorescent
Non-absorbing
cis


1.0
0.5
0.0
0.0
0.5
1.0
r / λ

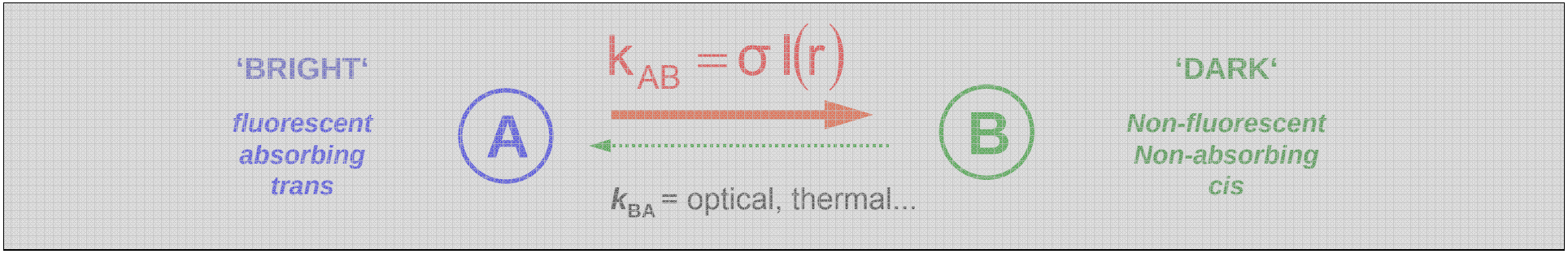

N_B / N_A
1.0
0.5
0.0
0
B
A
I(r)
$I_s = \frac{k_{BA}}{\sigma}$

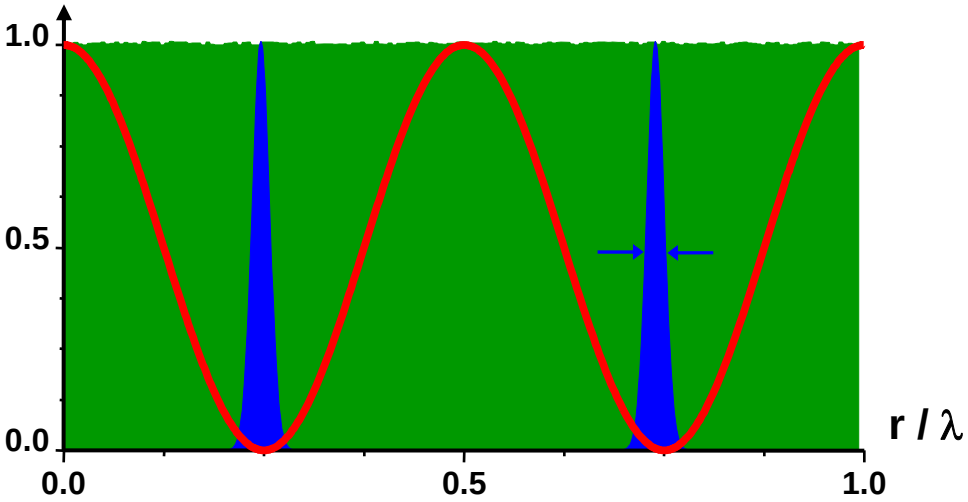


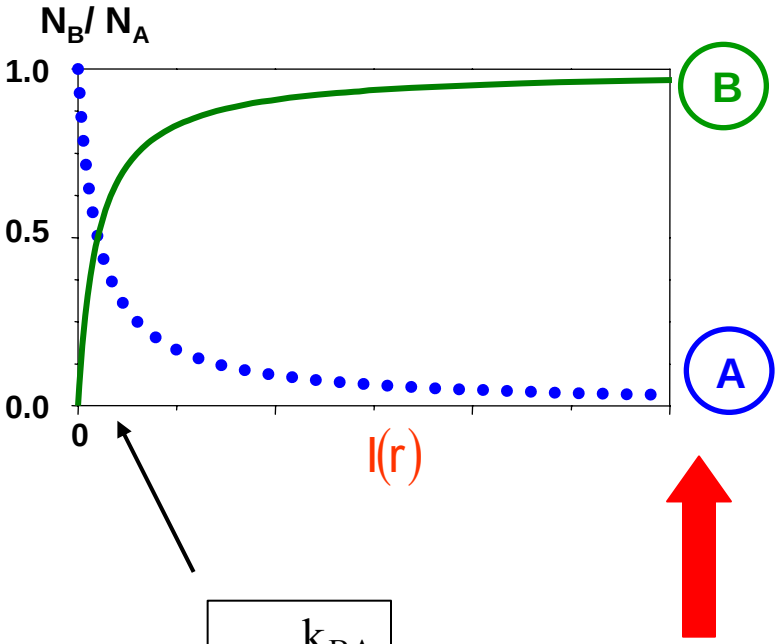




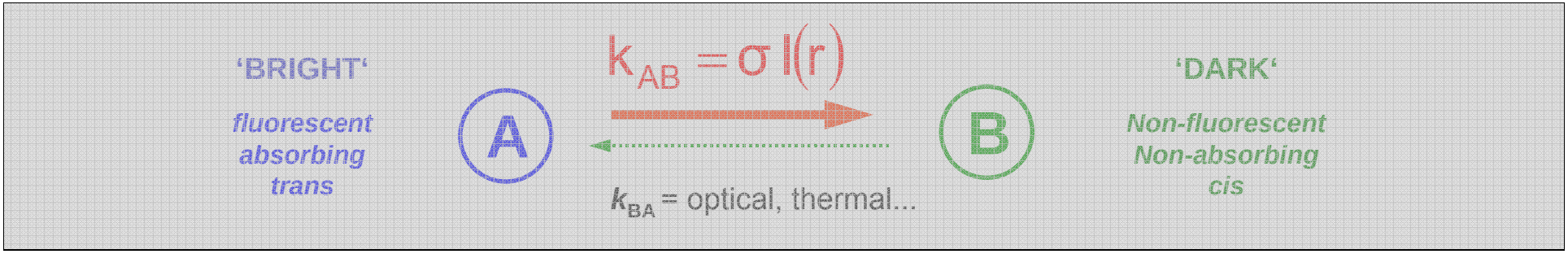
$$I_s = \frac{k_{BA}}{\sigma}$$

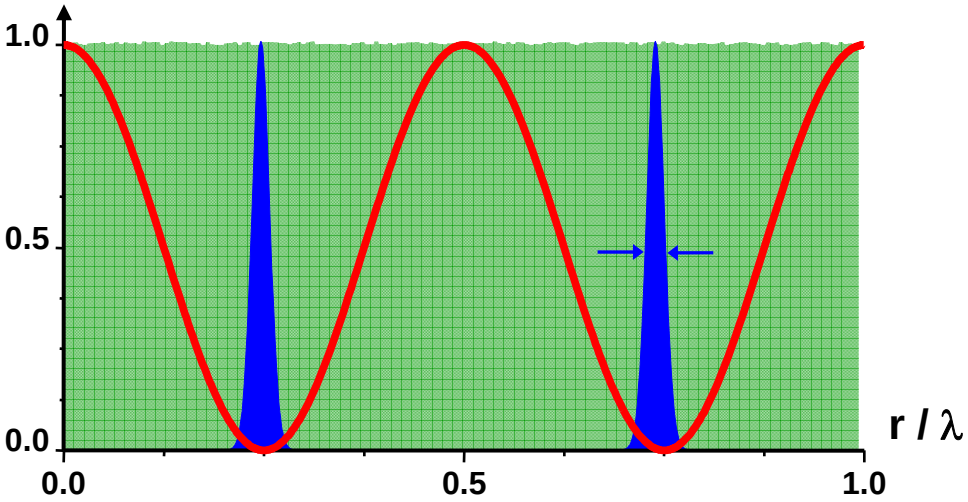


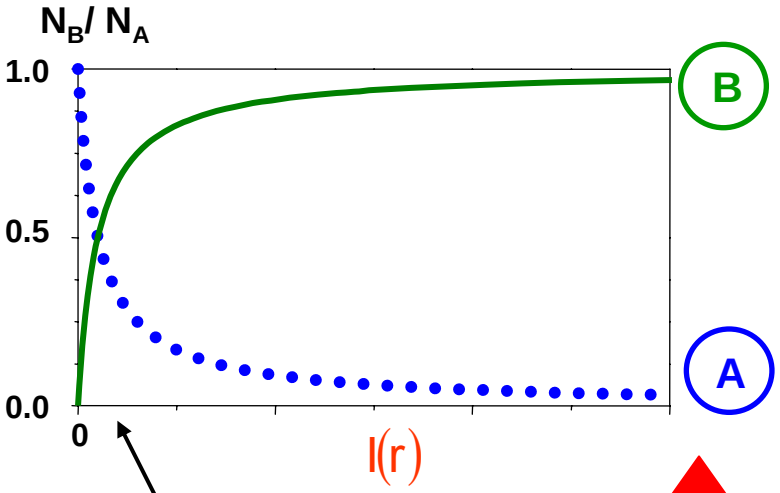




$$I_s = \frac{k_{BA}}{\sigma}$$

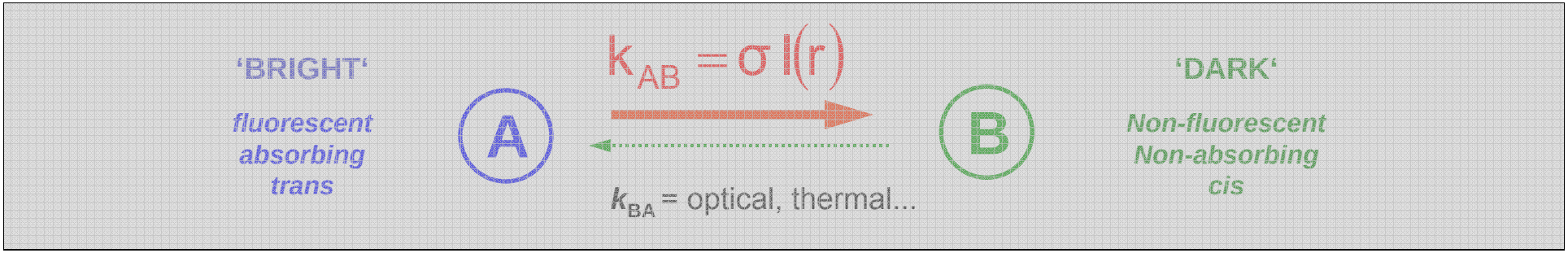


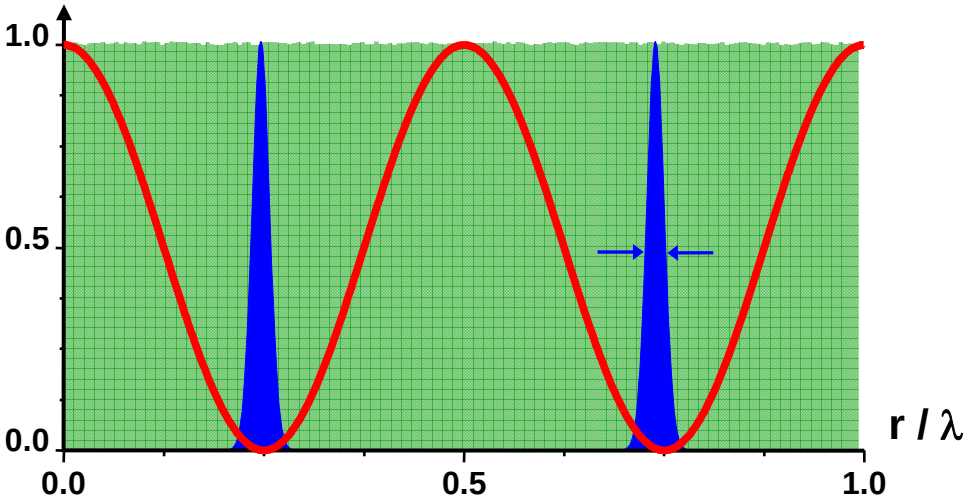




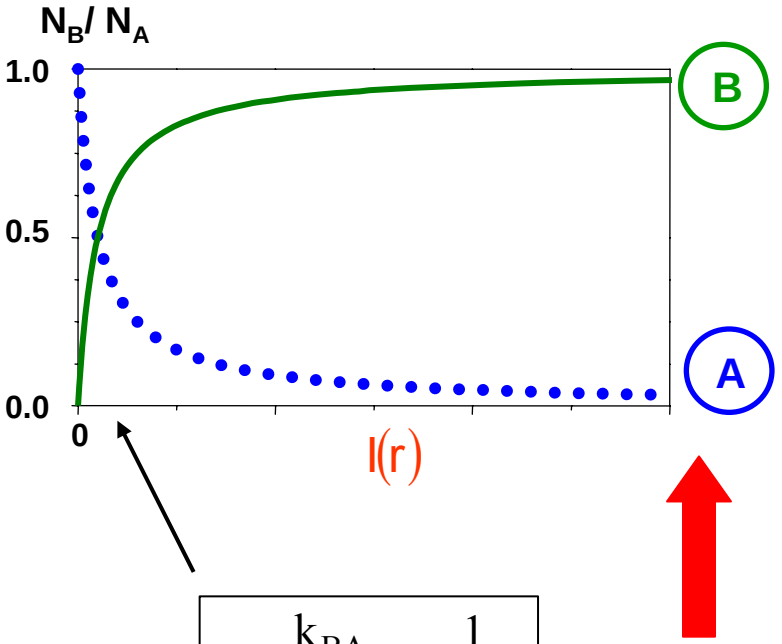
$$\Delta x \approx \frac{\lambda}{\pi n \sqrt{I/I_s}}$$

$$I_s = \frac{k_{BA}}{\sigma} = \frac{1}{\sigma \tau_{BA}}$$

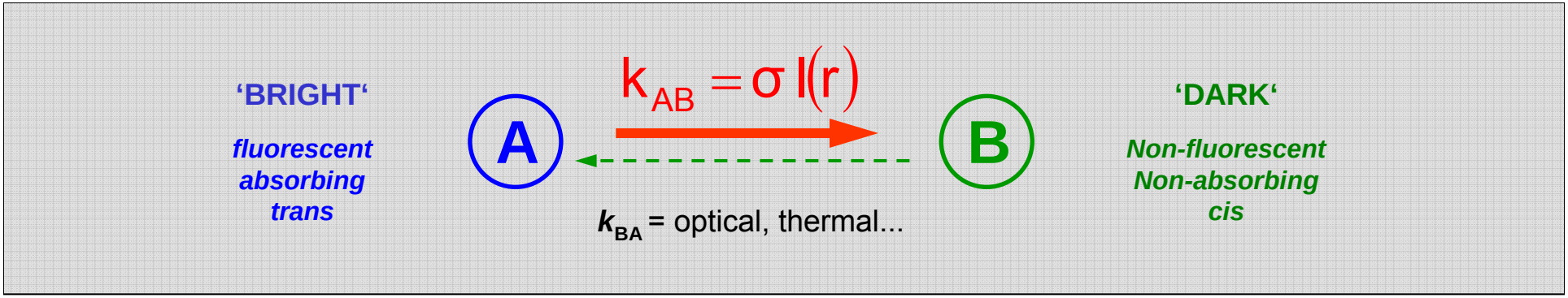




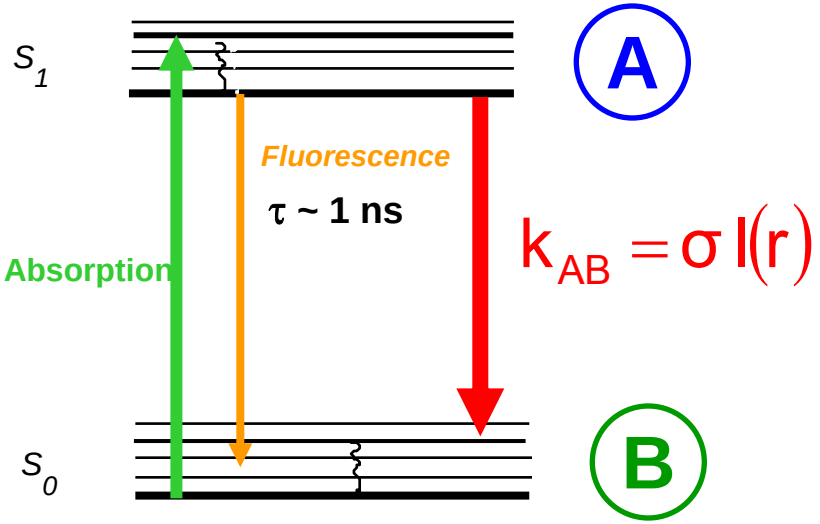
$$\Delta x \approx \frac{\lambda}{\pi n \sqrt{I \sigma \tau_{BA}}}$$



$$I_s = \frac{k_{BA}}{\sigma} = \frac{1}{\sigma \tau_{BA}}$$

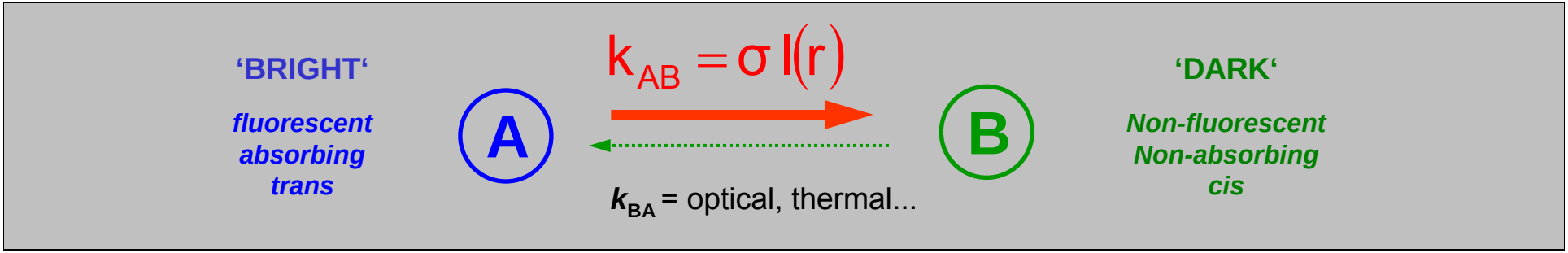


STED microscopy



$$I_s = \frac{1}{\sigma \tau} \approx 10^{-16} cm^2 \, 10^{-9} s \approx$$

$$I_s \approx$$ **30 MW/cm²**



There must also be

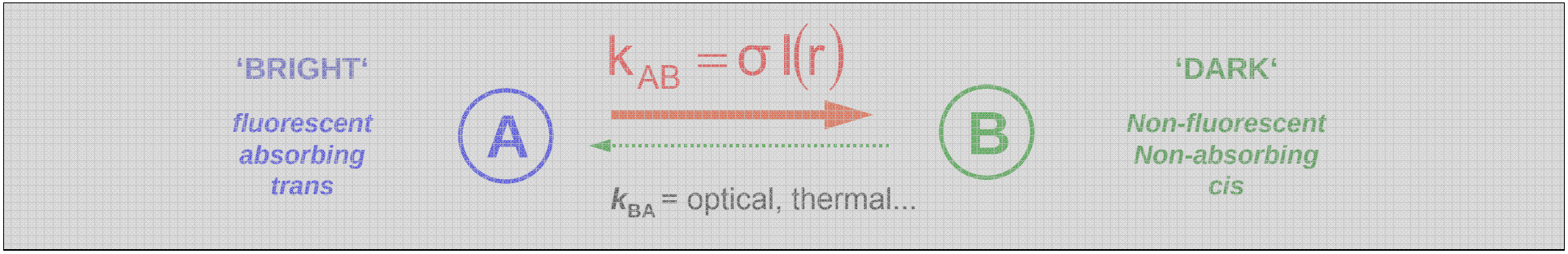
Other Molecular States

&

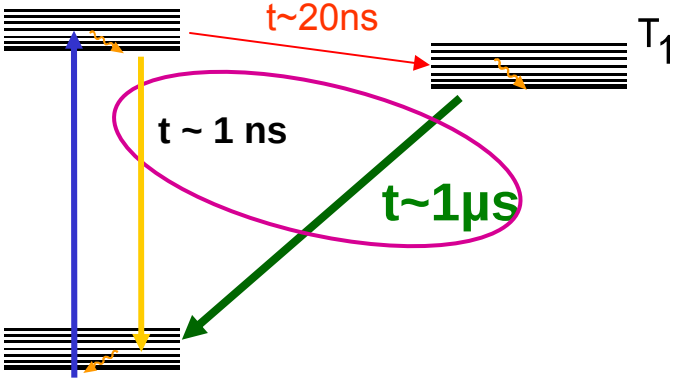
transitions

to

break the diffraction barrier



Ground State Depletion Microscopy



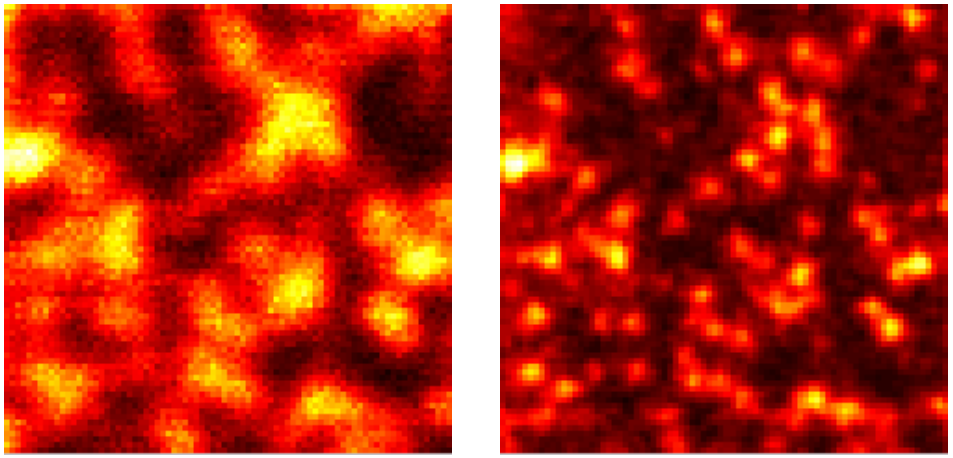
A *Singlet*

B *Triplet*

Hell & Kroug, *Appl. Phys. B* 60 (1995)

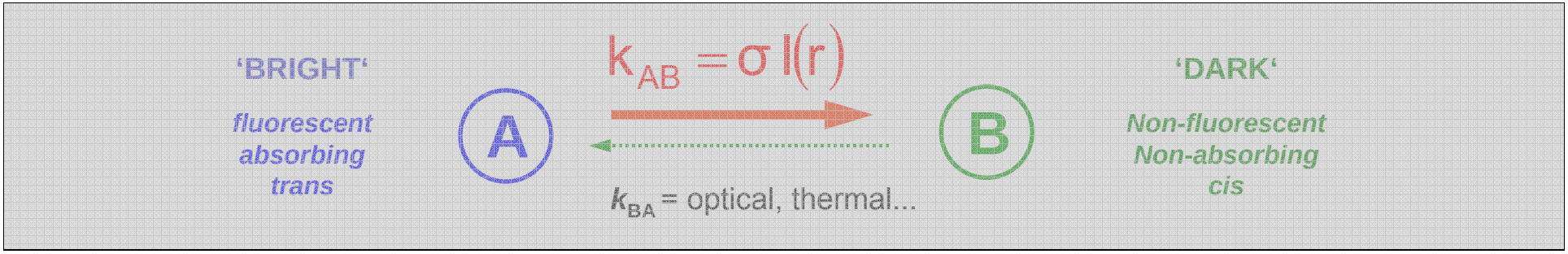
40 nm beads

Confocal **Ground State Depletion Micr.**

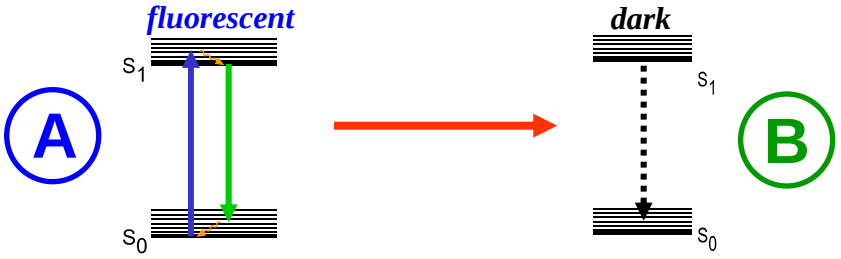


S. Bretschneider, C. Eggeling, SWH *Phys Rev Lett* (2007)

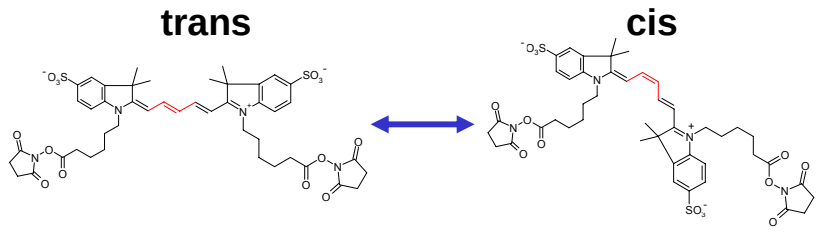
I_S ~ kW/cm²



Optical Bistability (Synthetic compounds)



$$\Delta x \approx \frac{\lambda}{\pi n \sqrt{I \sigma \tau_{BA}}}$$



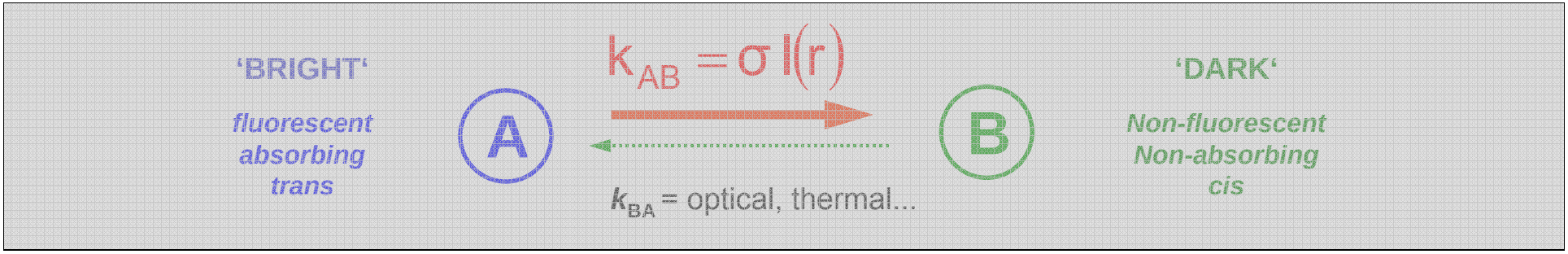
I_S ~ W/cm²

Photoisomerisation

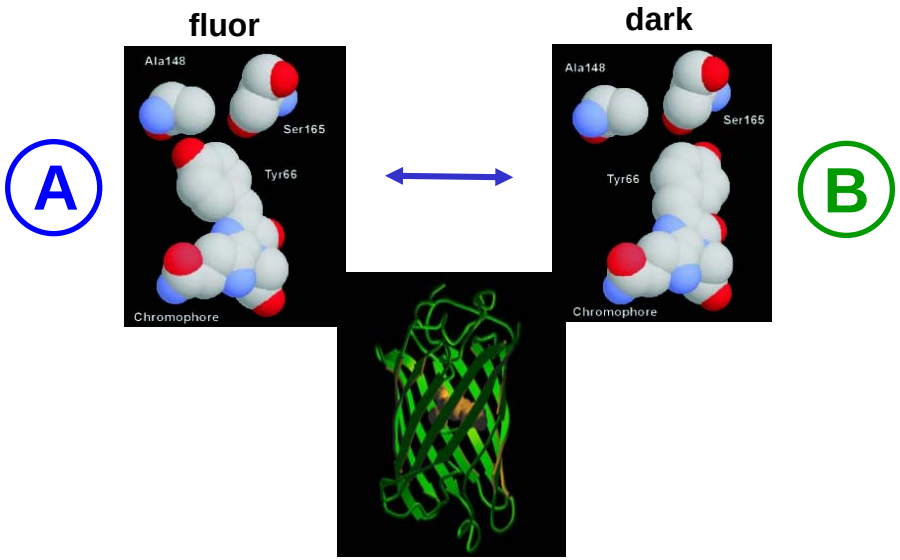
Hell, *in Topics Fluoresc Spectr V*, Plenum Press (1997)

Dyba & Hell, *Phys. Rev. Lett.* 88 (2002)

Hell, Jakobs, Kastrup, *Appl. Phys. B 77* (2003)



Optical Bistability (Fluorescent proteins)



$$\Delta x \approx \frac{\lambda}{\pi n \sqrt{I \sigma \tau_{BA}}}$$

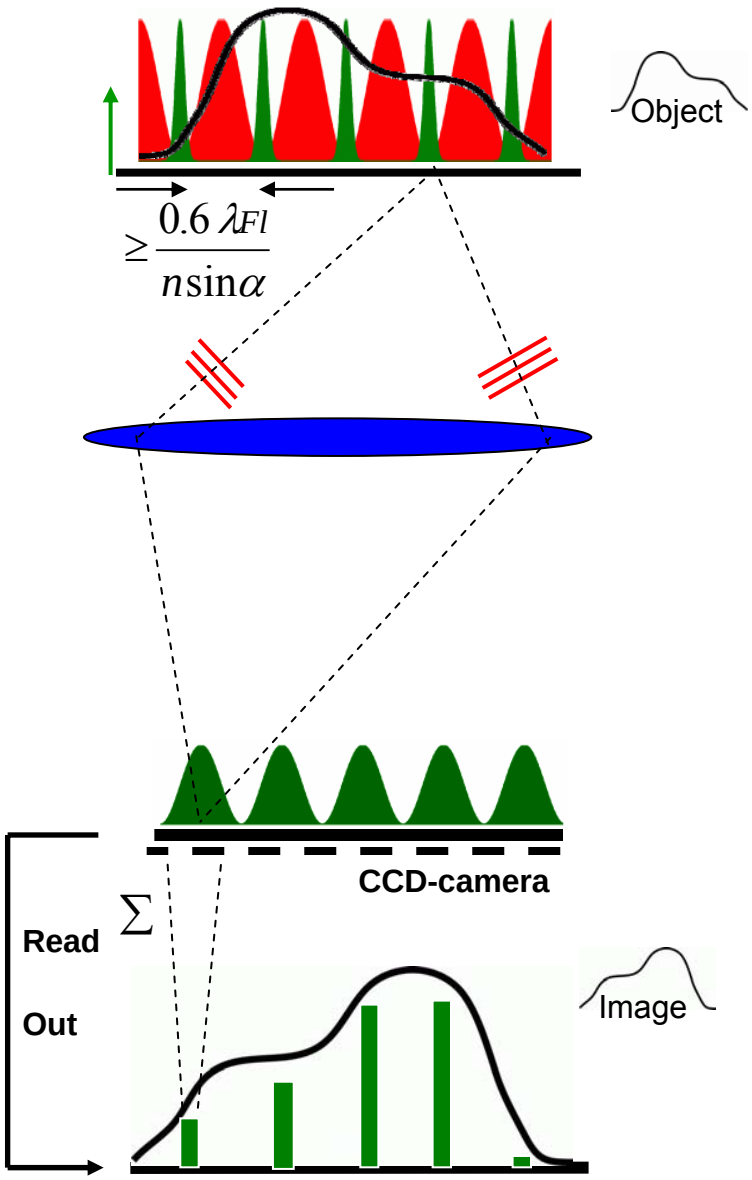
I_S ~ W/cm²

Photoswitchable fluorescent proteins

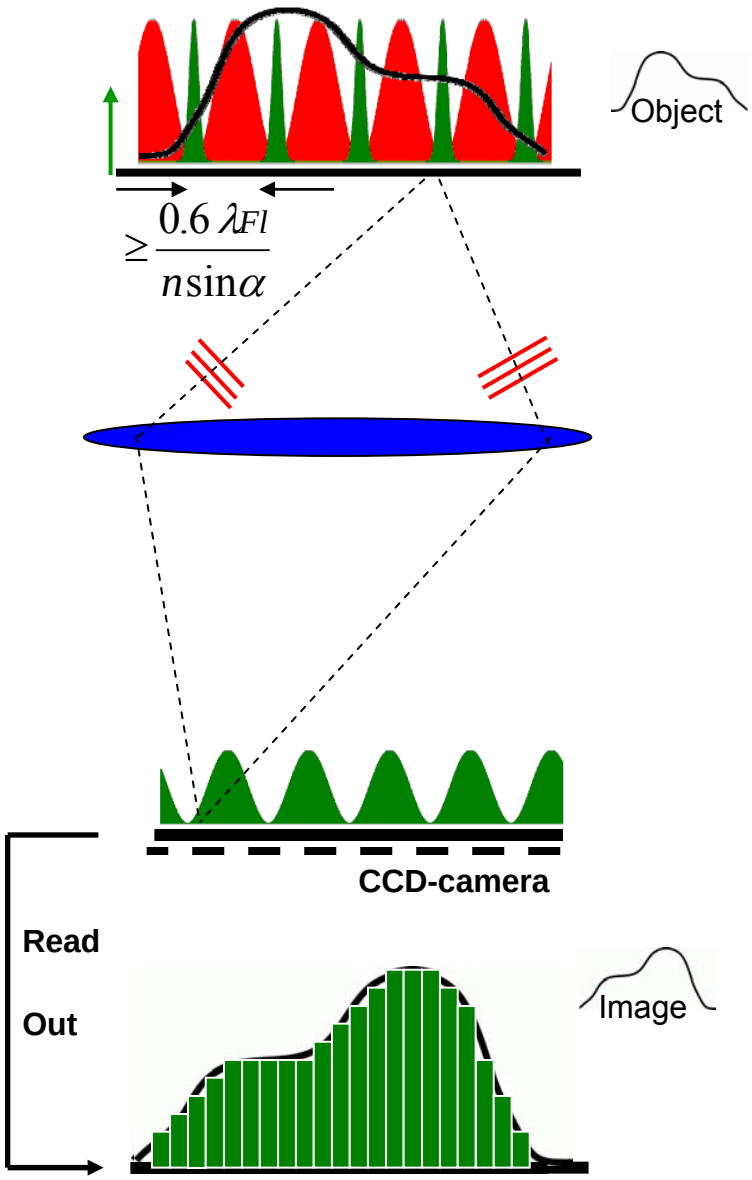
Hell, Jakobs, Kastrup, *Appl. Phys. B 77* (2003)

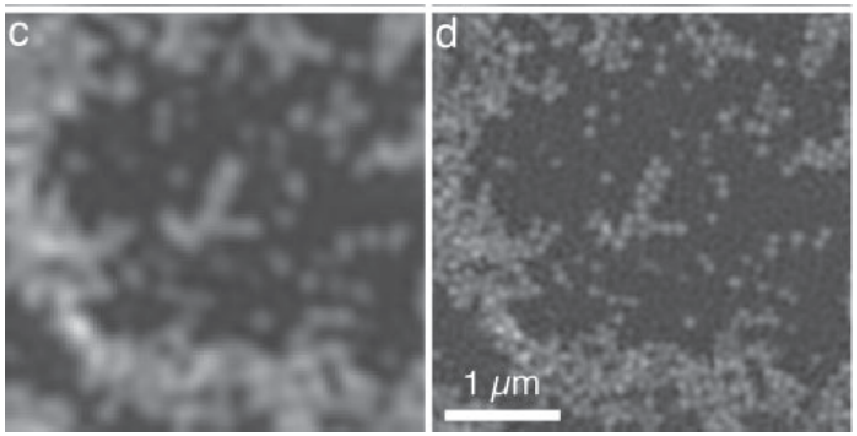
Hofmann, et al PNAS (2005)

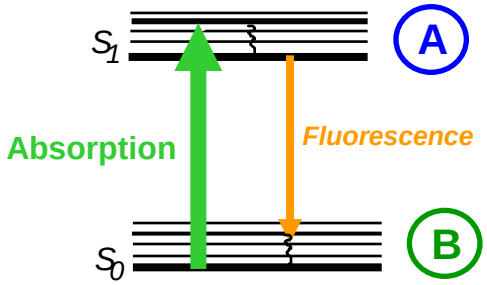
***Fluor.* On**
***Fluor.* Off**



***Fluor.* On**
***Fluor.* Off**







Camera detection possible.

Zeros & scanning are required !

MGL Gustafsson, PNAS (2005)

Sequential read-out with at least one…

Intensity 'Zero'

+

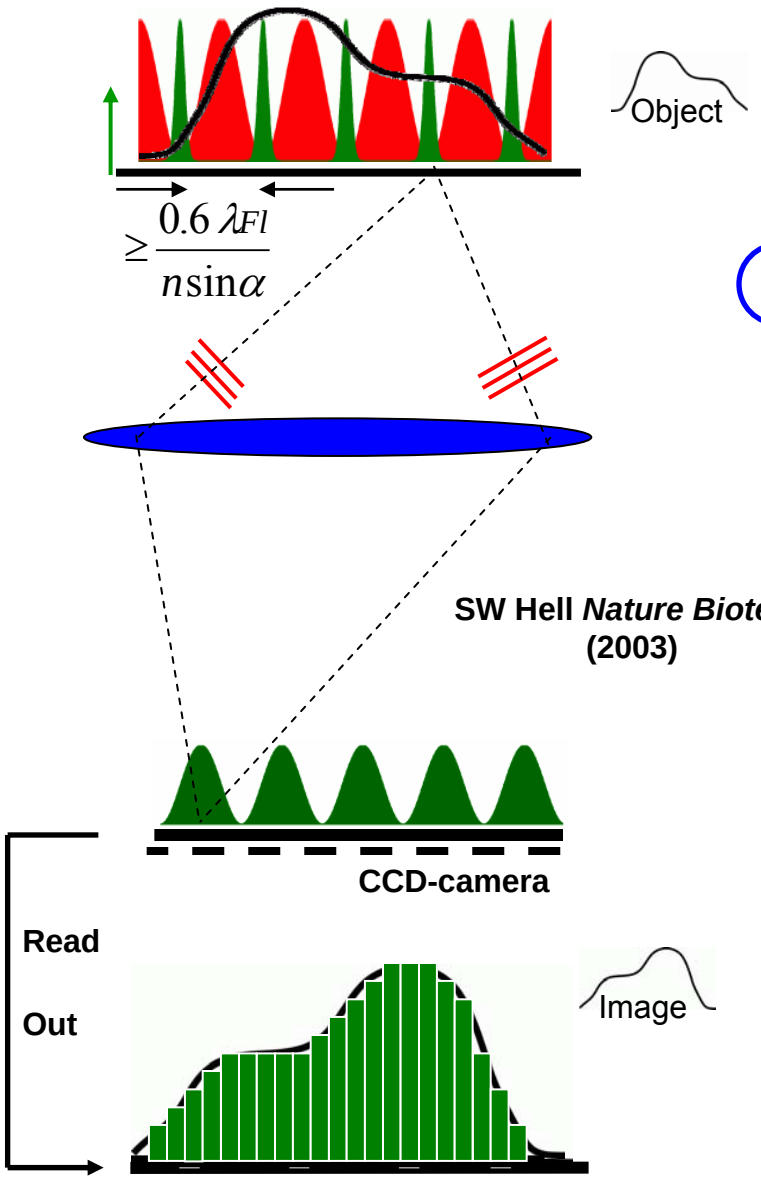
Reversible Saturable / Switchable Optical Linear (Fluorescence) **Transition**

=

IZ RESOLFT

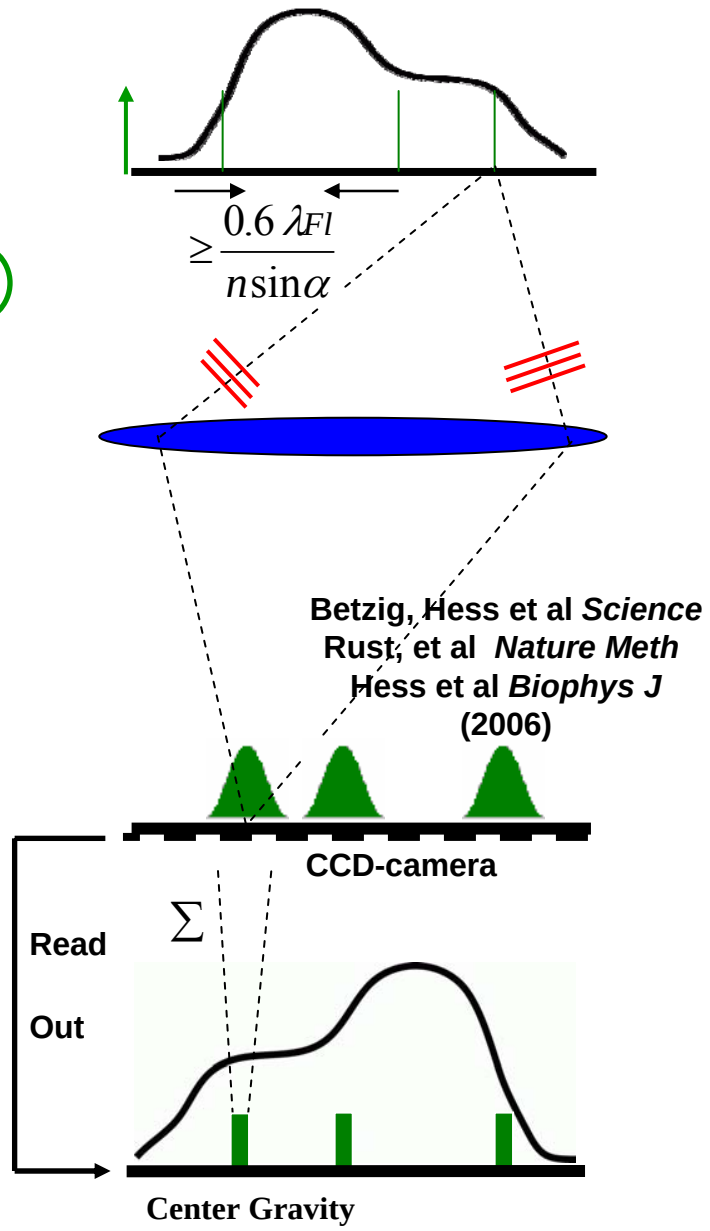
RESOLFT

Switching molecule ensembles (confinement by zeros)



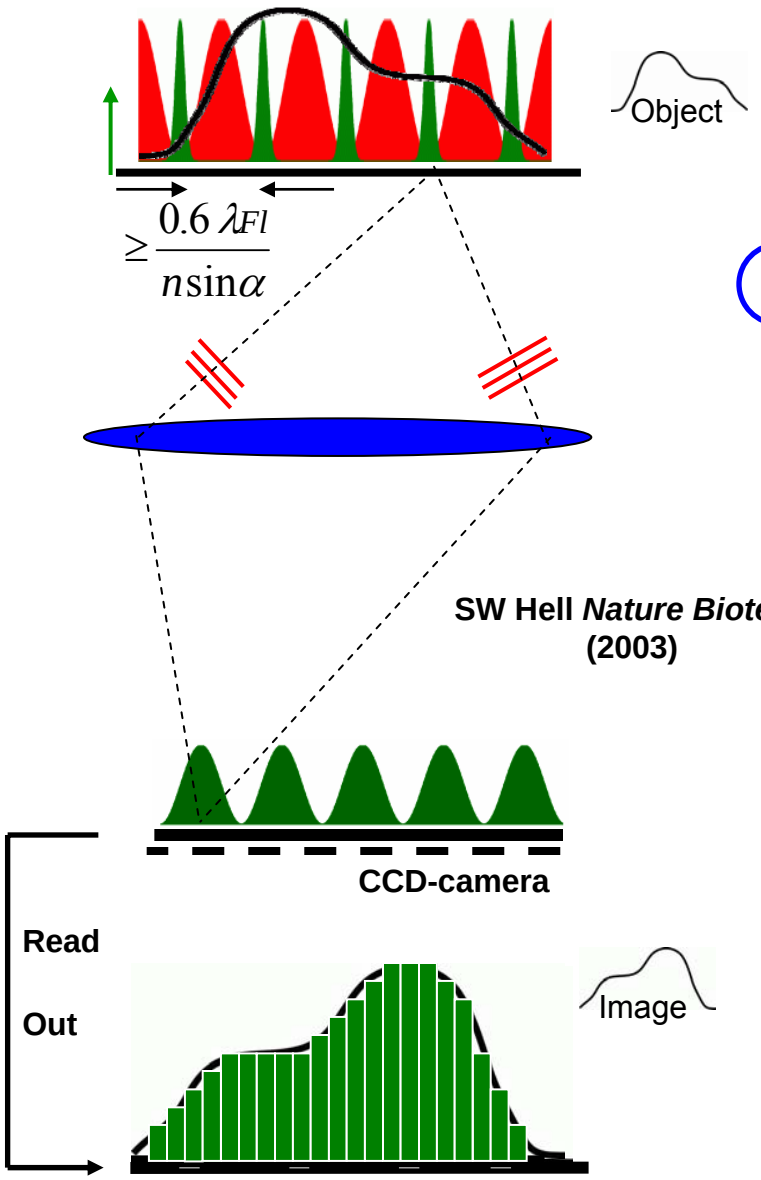
STORM / PALM

Switching molecules individually!



RESOLFT

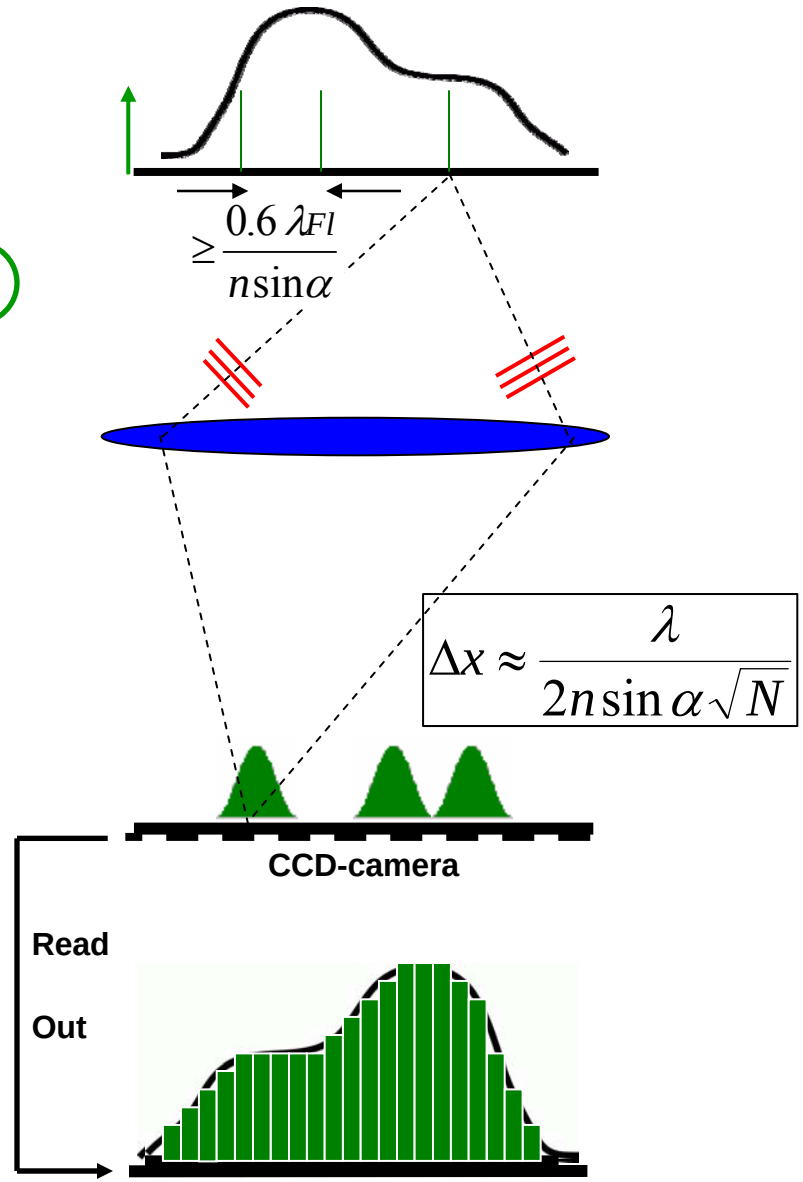
Switching molecule ensembles (confinement by zeros)



SW Hell *Nature Biotech* (2003)

STORM / PALM

Switching molecules individually!



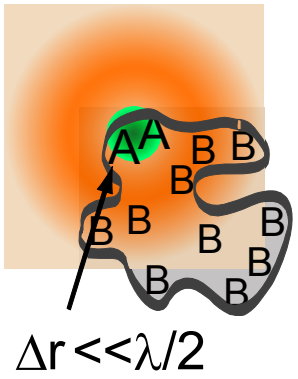
What is the common enabling physical element

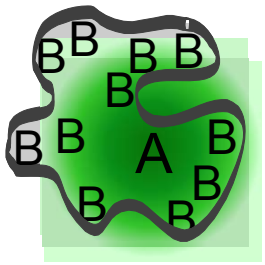
behind all these approaches ?

Establishing a

state (A) contrasted with (B) in a sub-$\lambda/2$ region

moved through the object and detected sequentially



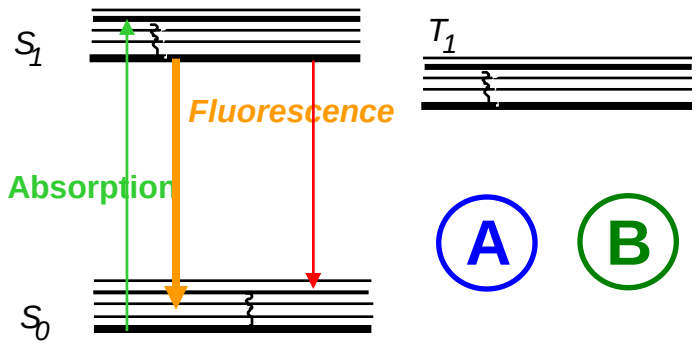


Energy state: S_0, S_1, T_1, ..

(Photophysics)

Conformational states: ***cis, trans, binding***

(Photochemistry)

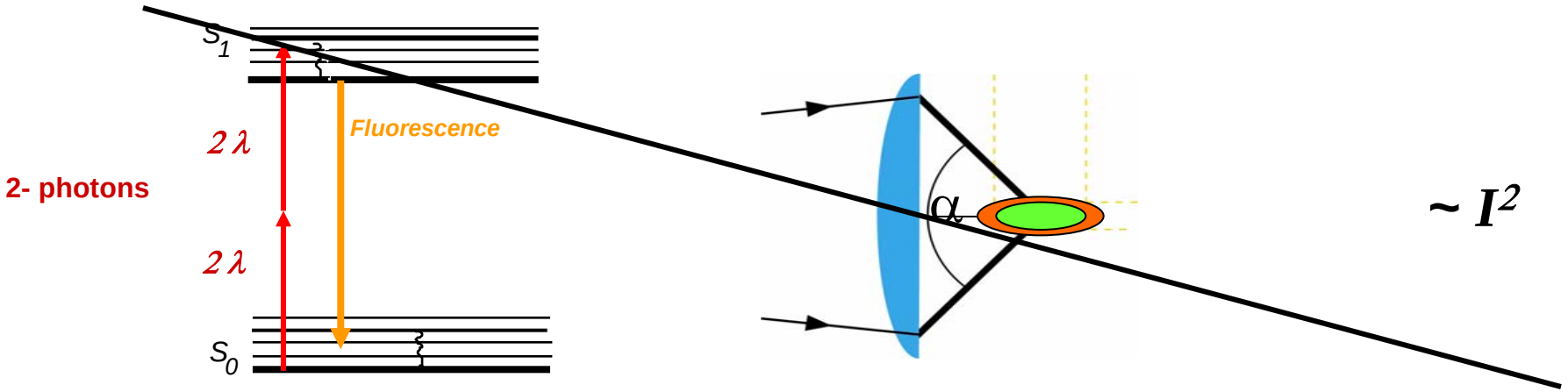


A need not be fluorescent, but just detectable (e.g. heat).

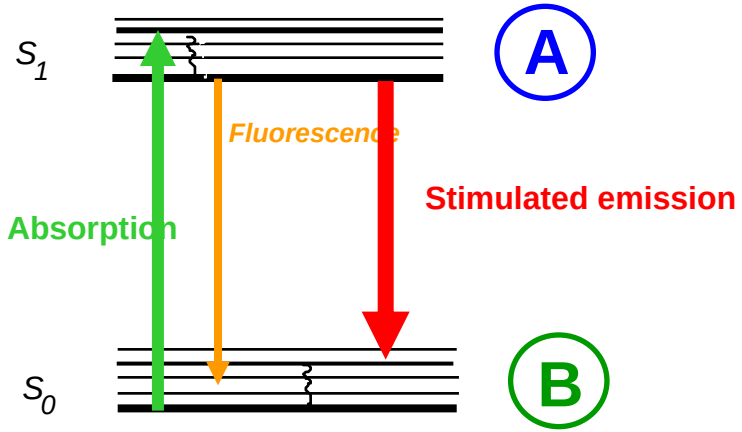
Long lifetimes of states τ are advantageous

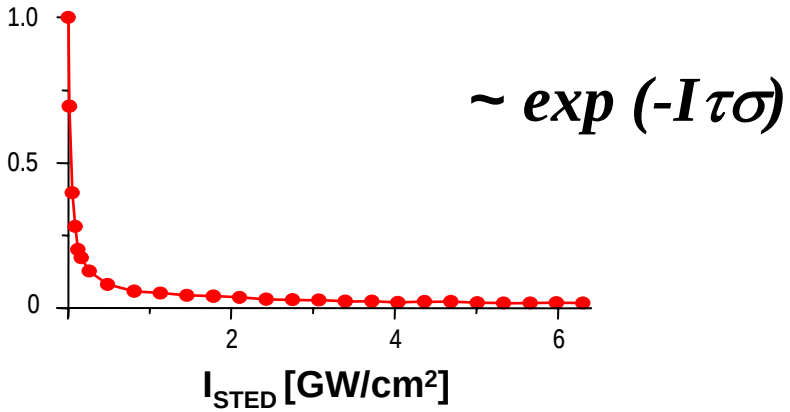
Key is the states, not the density of photons !

m-photon absorption does not really work for resolution!

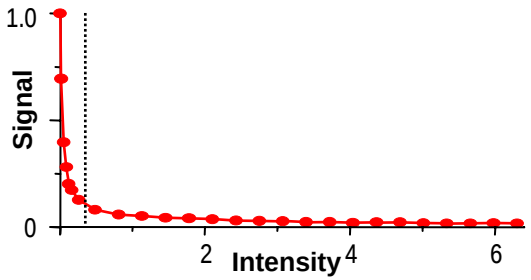


STED





... Abbe's equation ?



$$d = \frac{\lambda}{2n\sin\alpha\sqrt{1+J/J_s}}$$

Concepts using zeros

...expanded.

SWH, Nature Biotech 21, 1347 (2003)

SWH, Phys. Lett. A 326 , 140 (2004)

Westphal, SWH, Phys. Rev. Lett. (2005)

MAX-PLANCK-GESELLSCHAFT

Acknowledgements
References

Pictures/Movies

www.nanoscopy.de

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Physics:

G. Donnert et al — ***PNAS*, *July 24 (2006)***

K Willig, J Keller, M Bossi, SWH — ***New J Phys*, *(2006)***

V Westphal, SWH — ***Phys Rev Lett*, 94 *(2005)***

L Kastrup, H Blom, C Eggeling, SWH — ***Phys Rev Lett*, 94 *(2005)***

Applications:

K Willig, S Rizzoli, R Jahn, SWH — ***Nature*, *April 13, (2006)***

R Kittel, et al — ***Science*, *May 19, (2006)***