STED & STED-FCS

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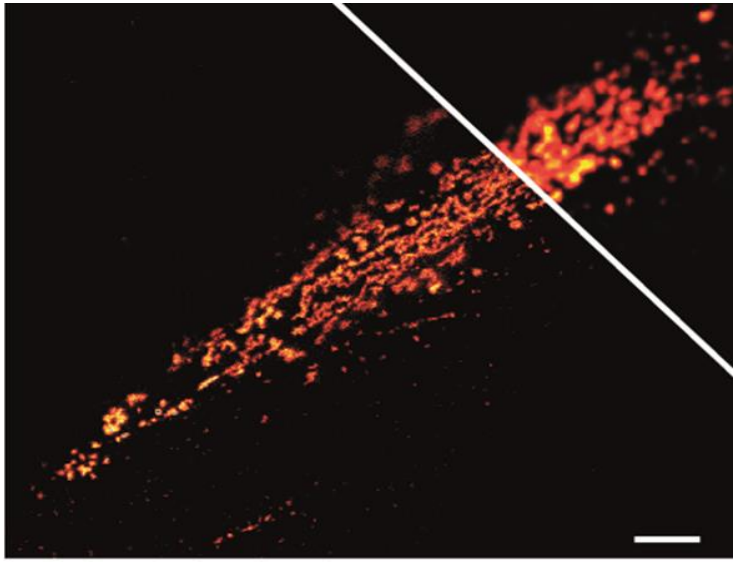
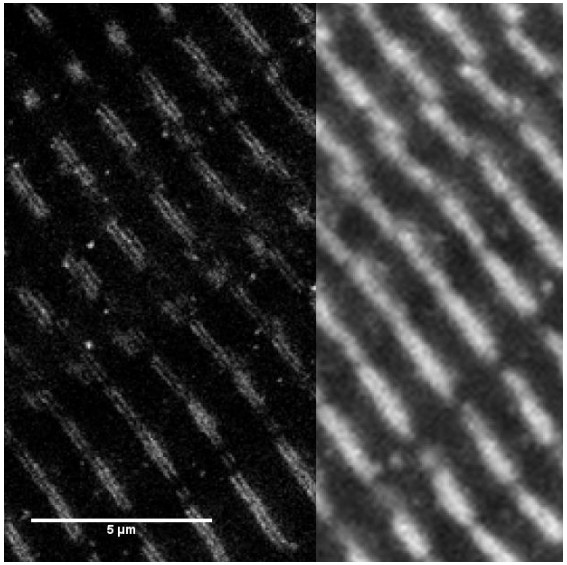
Weatherall Institute of Molecular Medicine

Application Examples – 2D (xy) STED Imaging



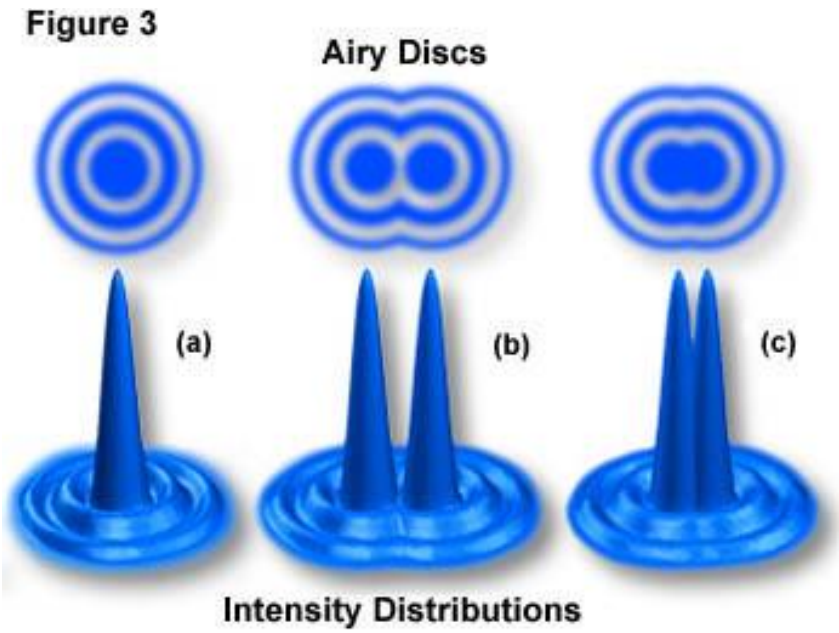
STED/Confocal image of Acetylcholine Receptor Clusters in Myotubes.
Dr. Judith Cossins / Prof. David Beeson
Molecular Neurosciences



STED/Confocal image of Titin Z-disk in cardiomyocytes.
Dr. Katja Gemlich
Department of Cardiovascular Medicine

Resolution in optical microscopy

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

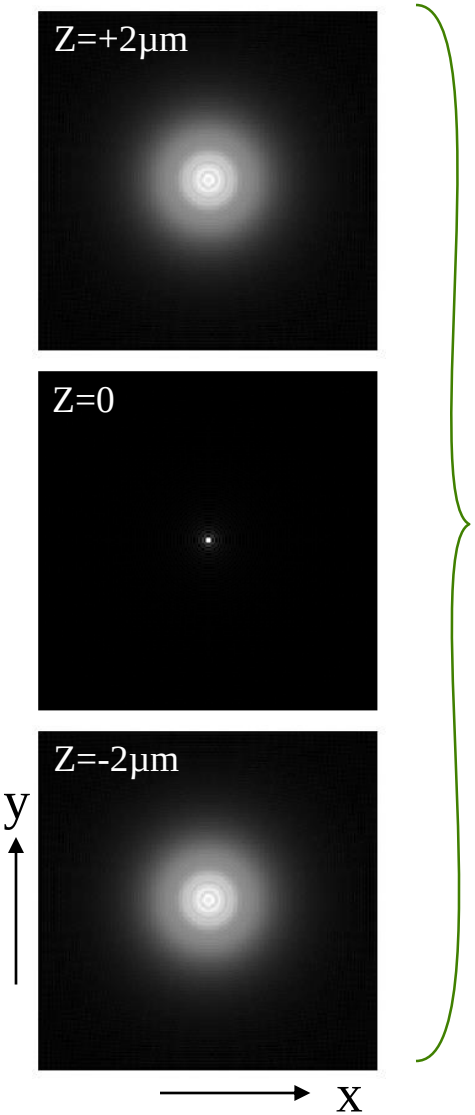


Picture taken from http://microscopy.fsu.edu/primer/anatomy/numaperture.html

The Point Spread Function (PSF)

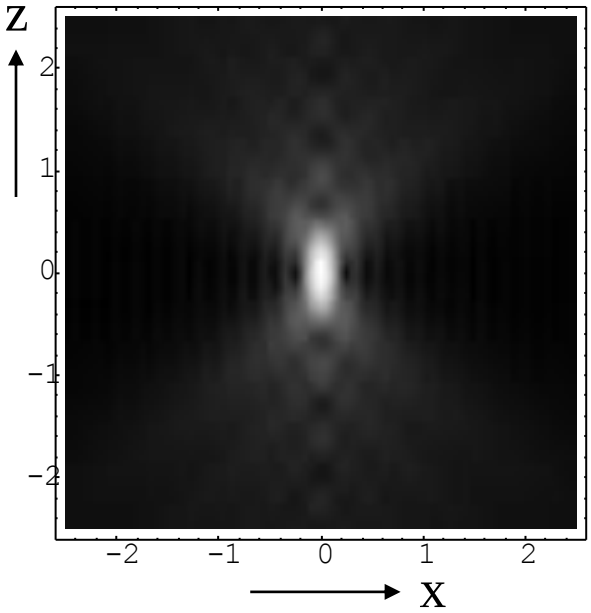
The image of a point object

2D PSF
for different defocus

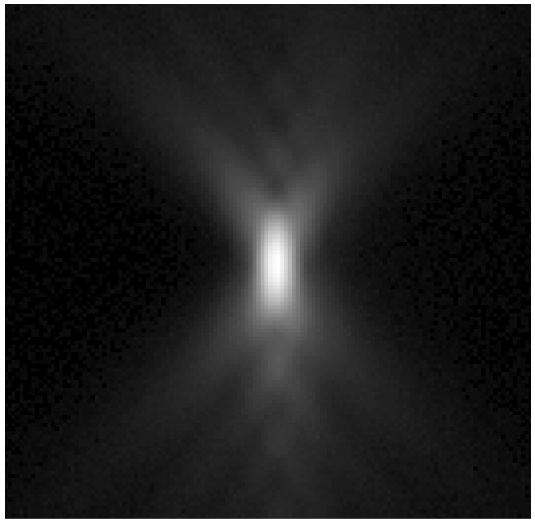


3D PSF

Calculated



Measured



Resolution is worse in the axial dimension (along the optical axis, Z)

$$D_Z = 2\,\lambda\eta/(NA_{obj})^2 \cdots\cdots\cdots\cdots 705$$

(η = refractive index of the object medium)

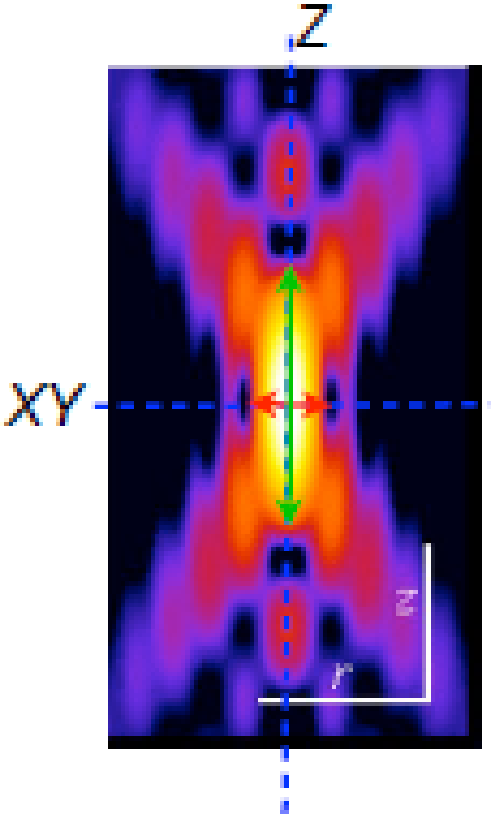
Than it is in the lateral dimension (XY)

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

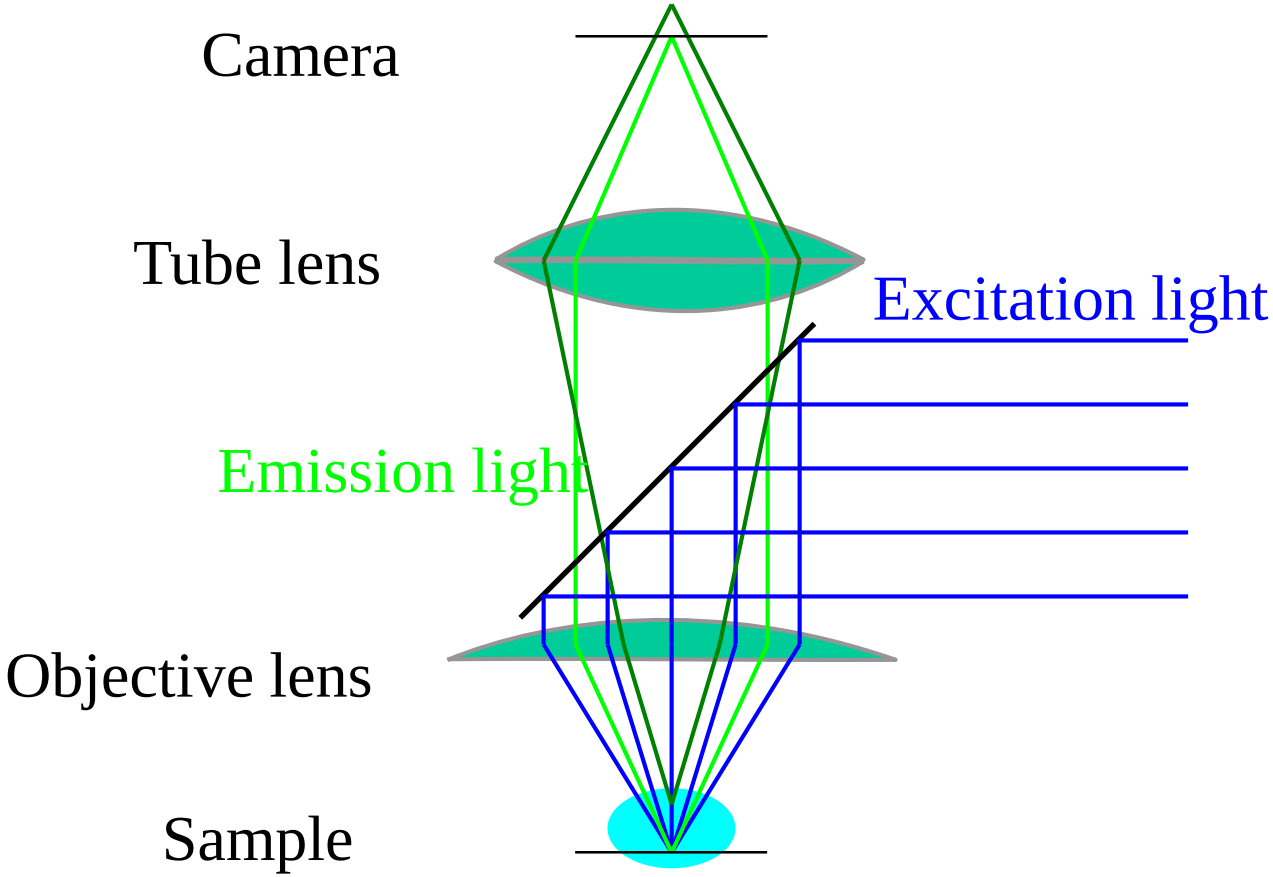
FWHM

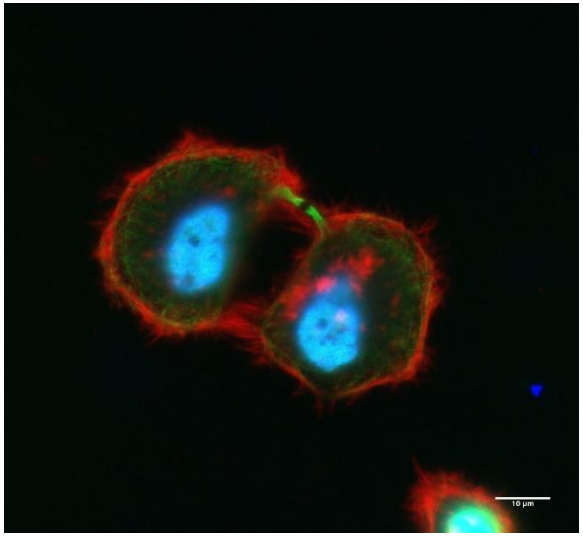
The relationship between the two is:

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



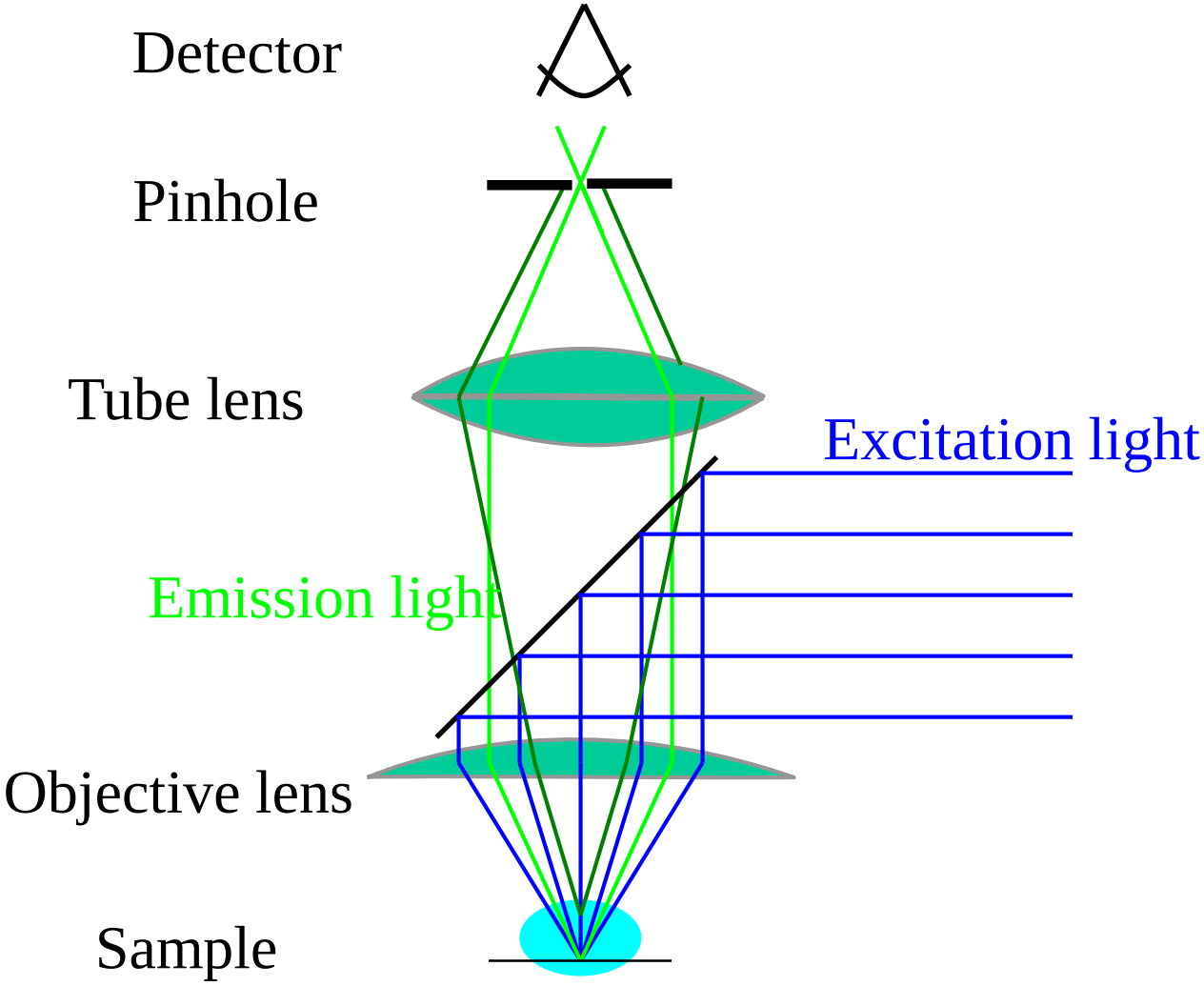
Wide-field Microscopy

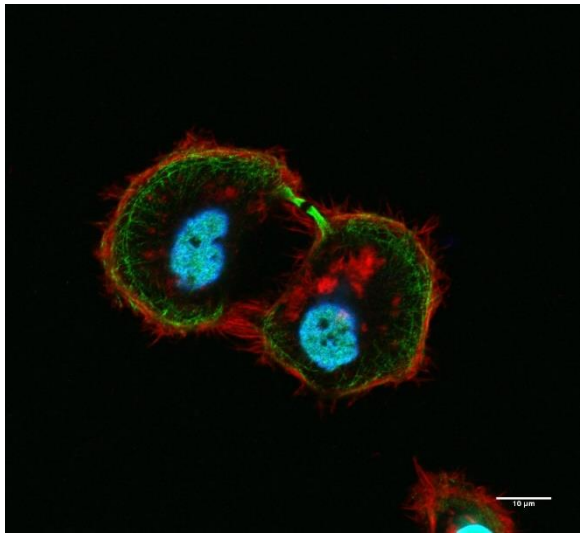




Problem – fluorescence is emitted along entire illuminated cone, not just at focus

The confocal microscope





Far-Field Microscopy

Resolution Limit: Diffraction Barrier

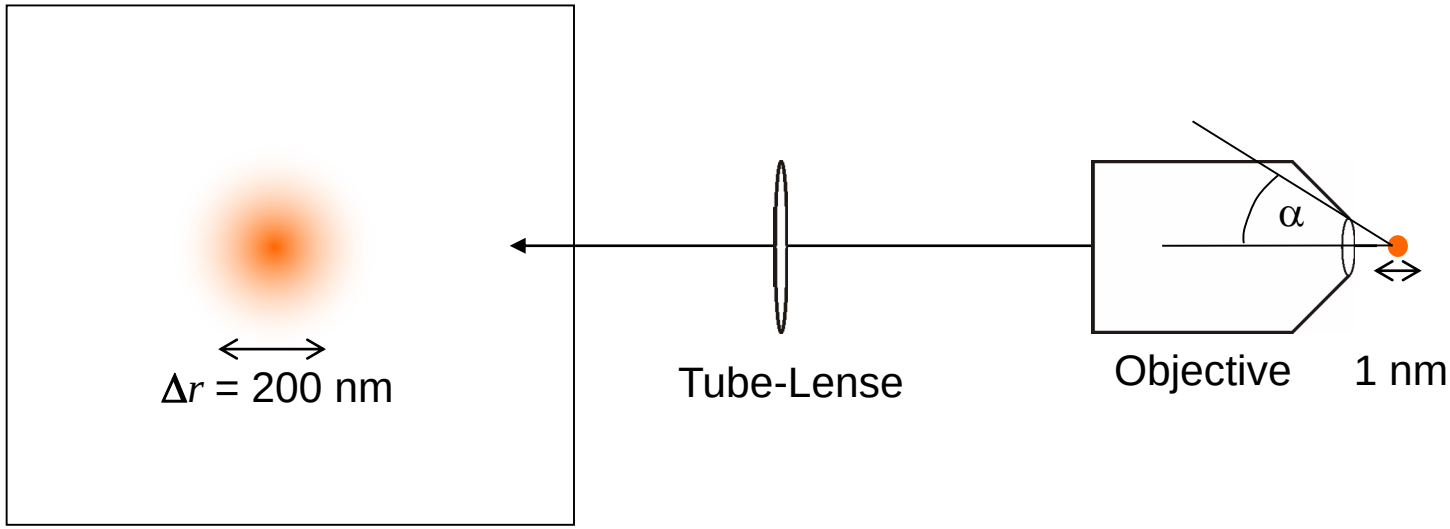
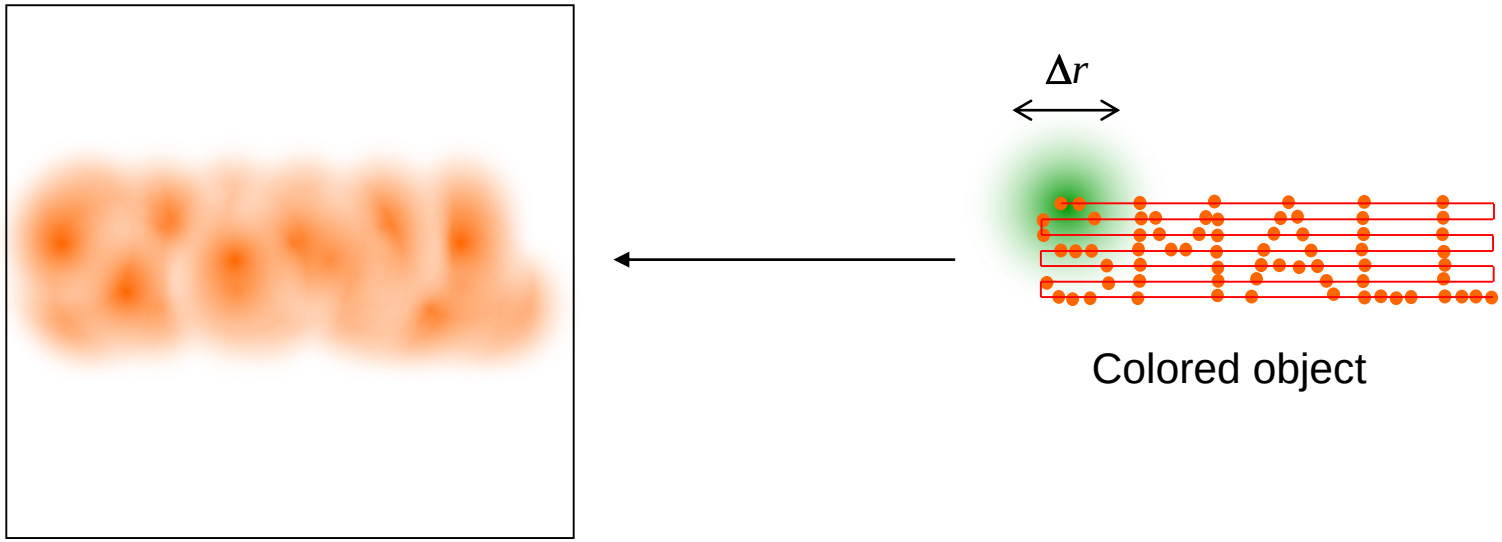


Image: diffraction-limited

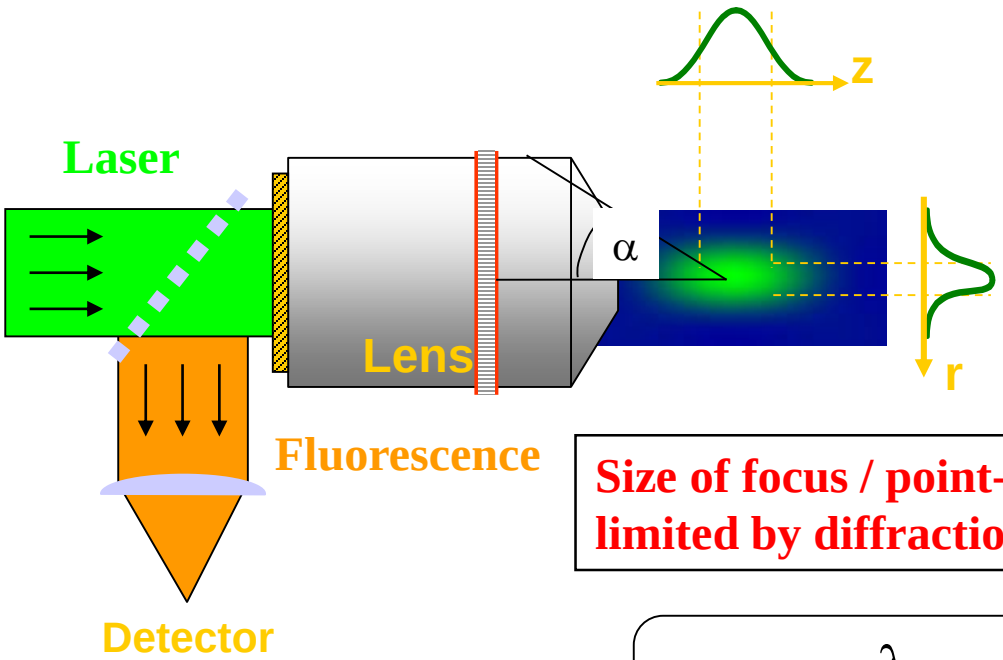


Far-Field Microscopy

Resolution Limit: Diffraction Barrier

Far-Field Fluorescence Microscopy: Focussing of light

- away from surfaces – inside cells (3D)



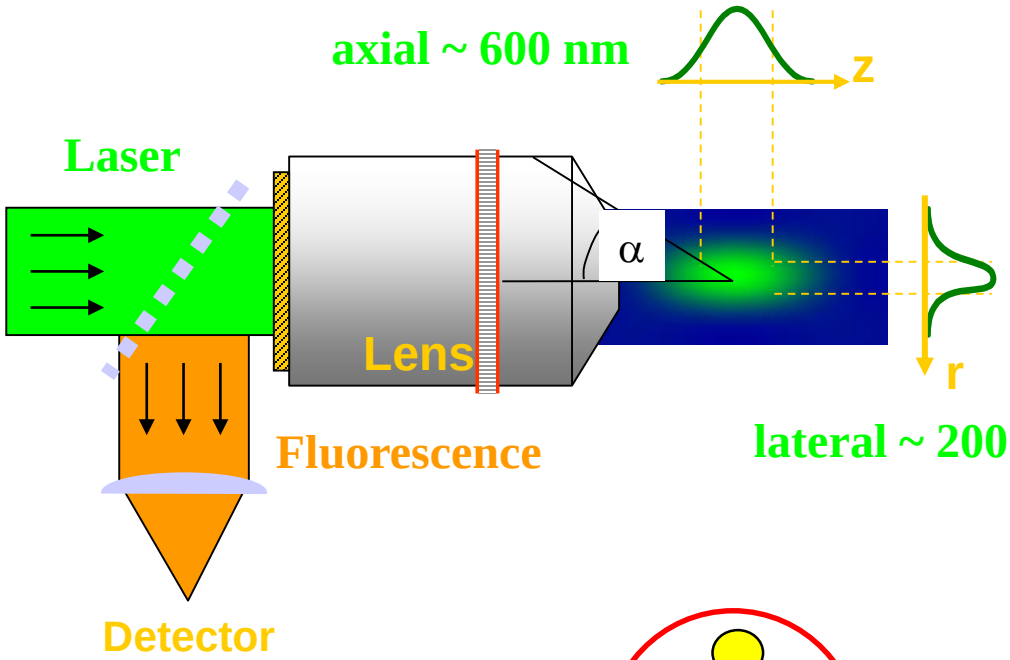
Size of focus / point-spread function limited by diffraction of light!!!

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

Ernst Abbe 1873

Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF



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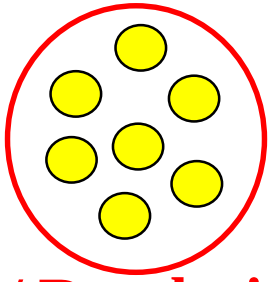
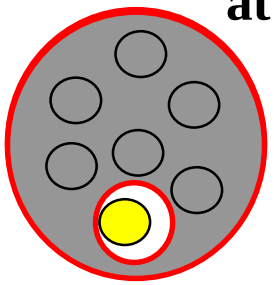
Ernst Abbe 1873

⇒ Turning ON/OFF



Turn OFF all objects but at a single point



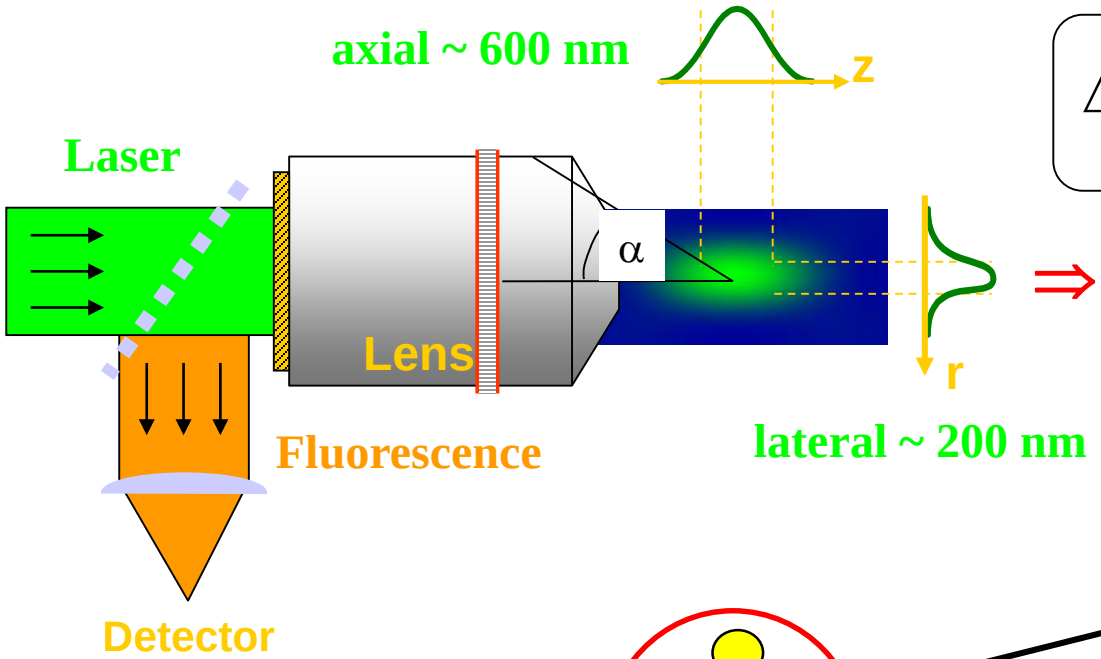
⇒ Observation area / Resolution

lateral << 200 nm

axial << 600 nm

Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF



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Ernst Abbe 1873

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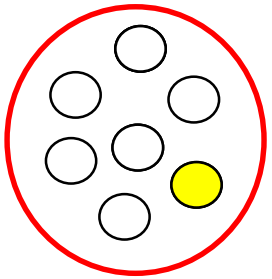

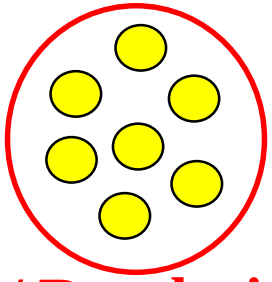

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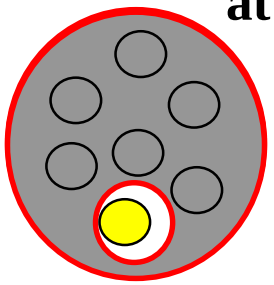

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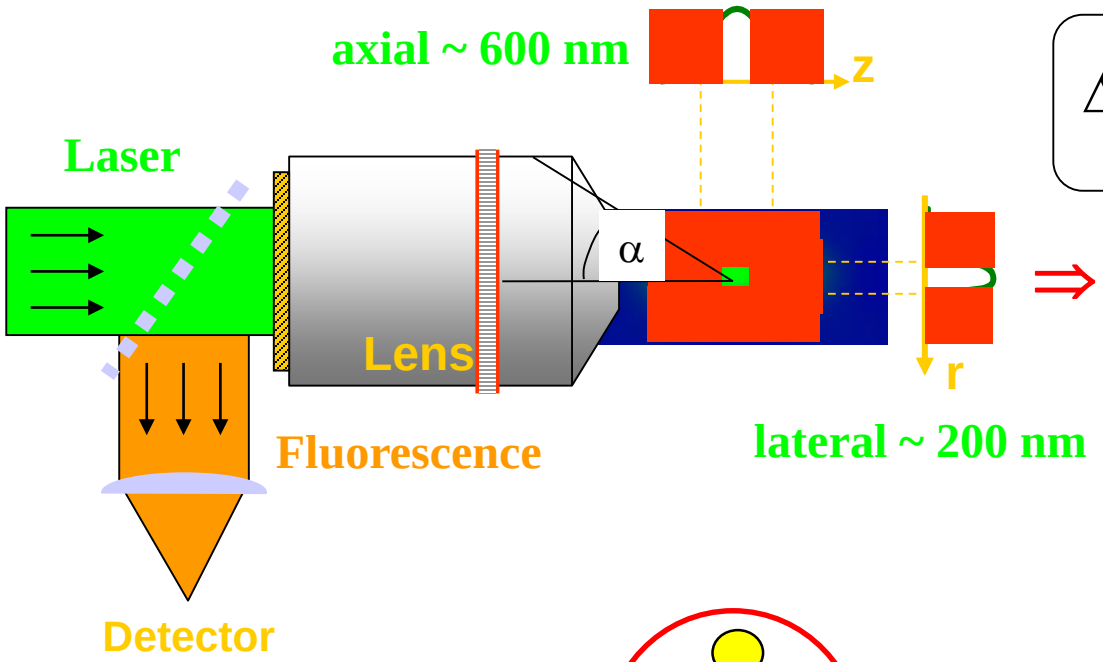

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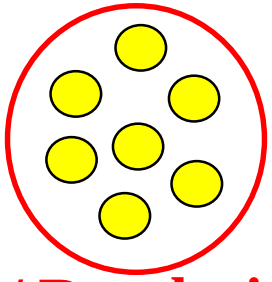
⇒ Turning ON/OFF



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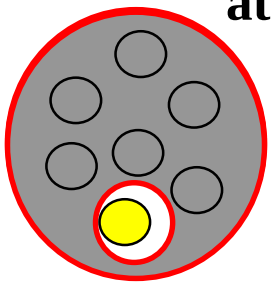

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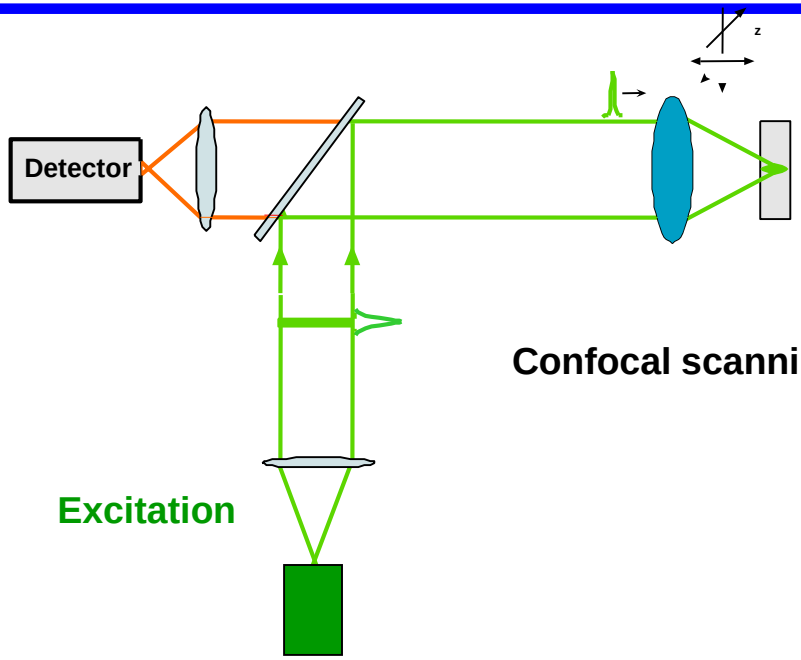
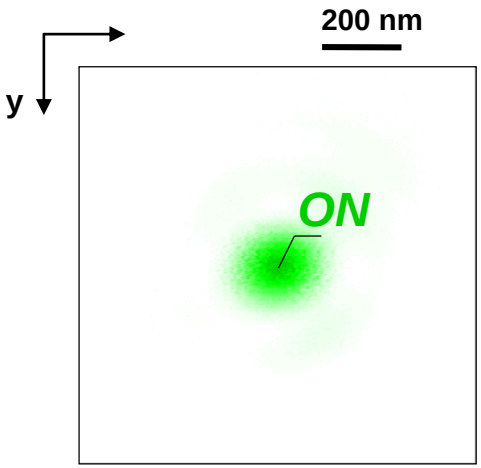
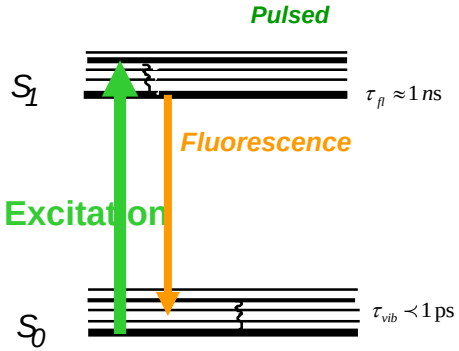
Fluorescence Microscopy

STED Microscopy



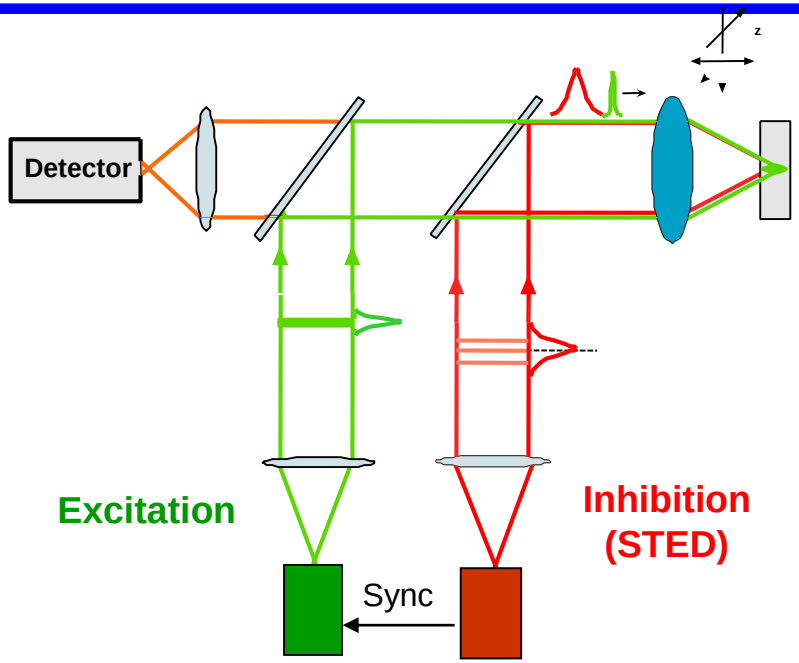
Confocal scanning microscope

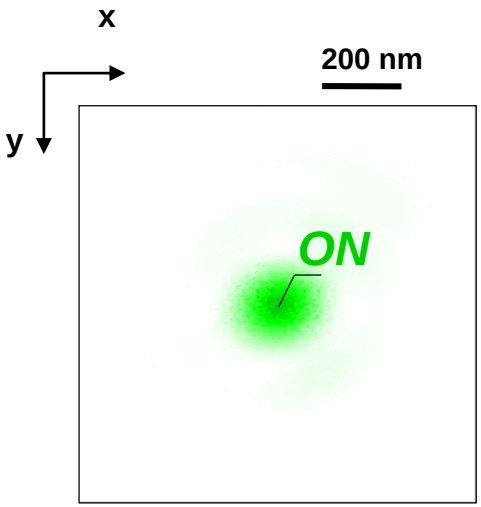


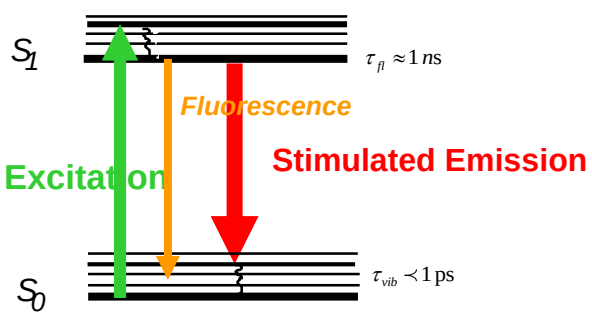


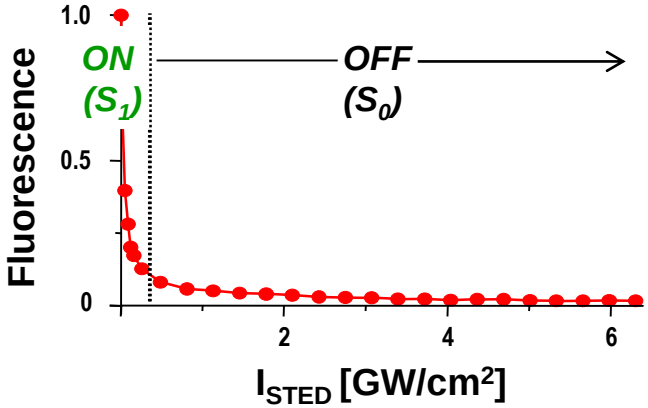
Fluorescence Microscopy

STED Microscopy









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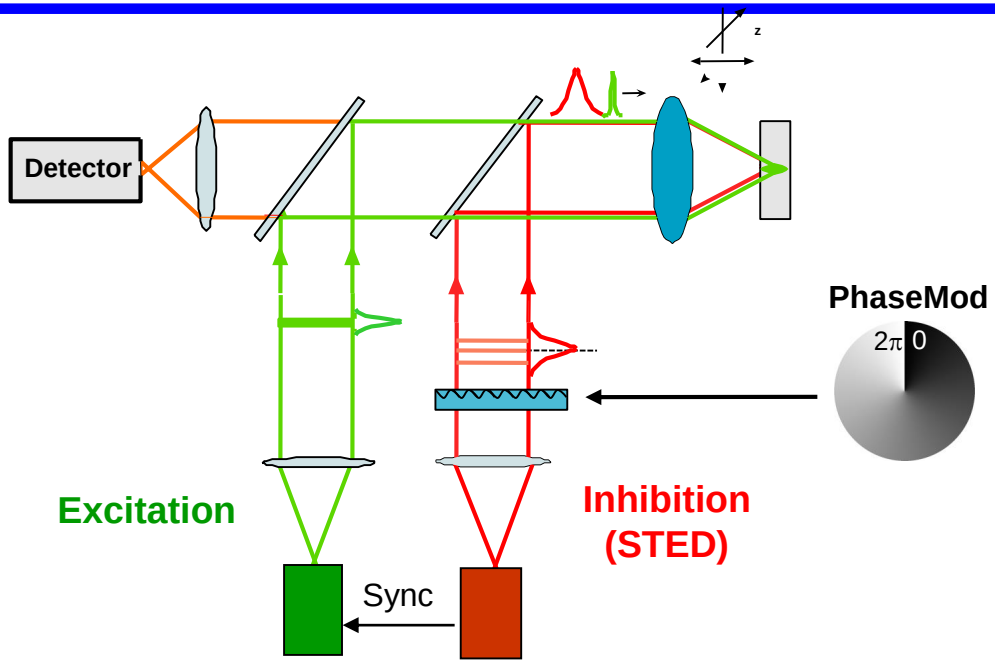

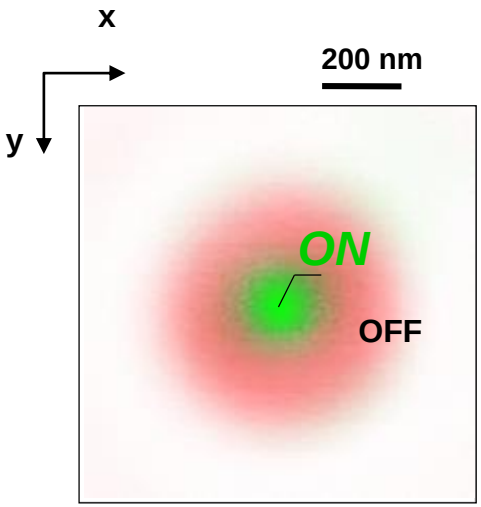

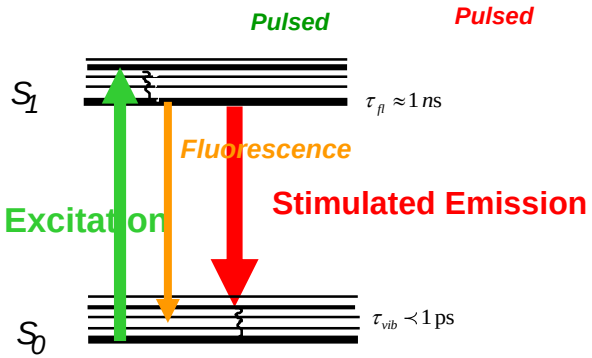

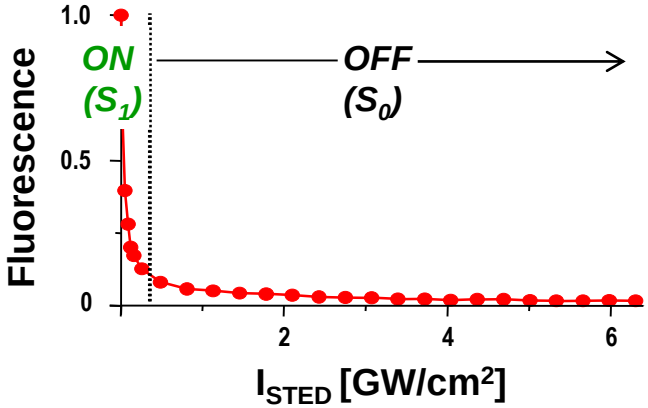

Fluorescence Microscopy

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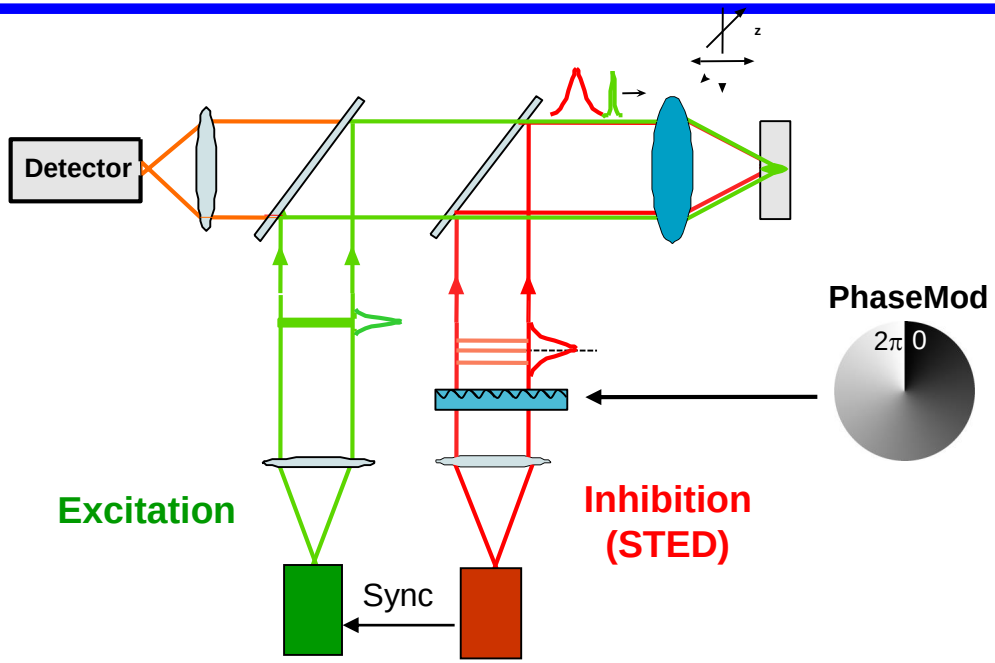
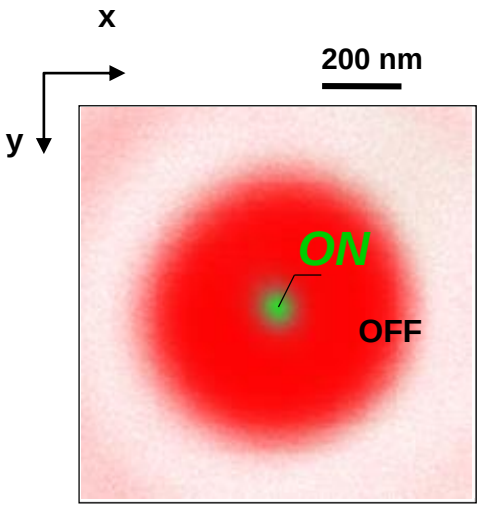
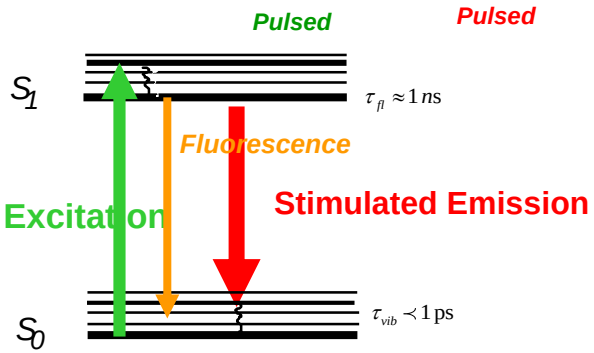
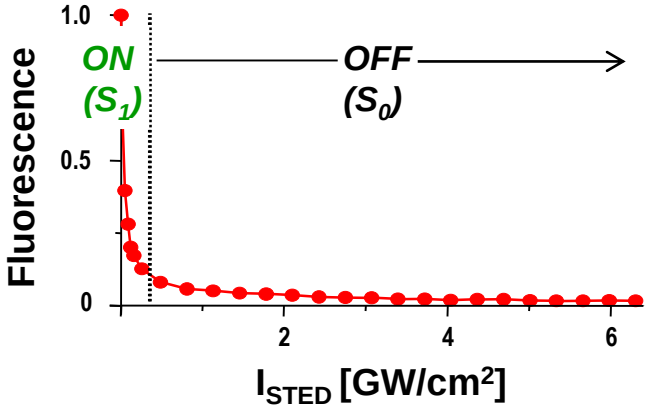




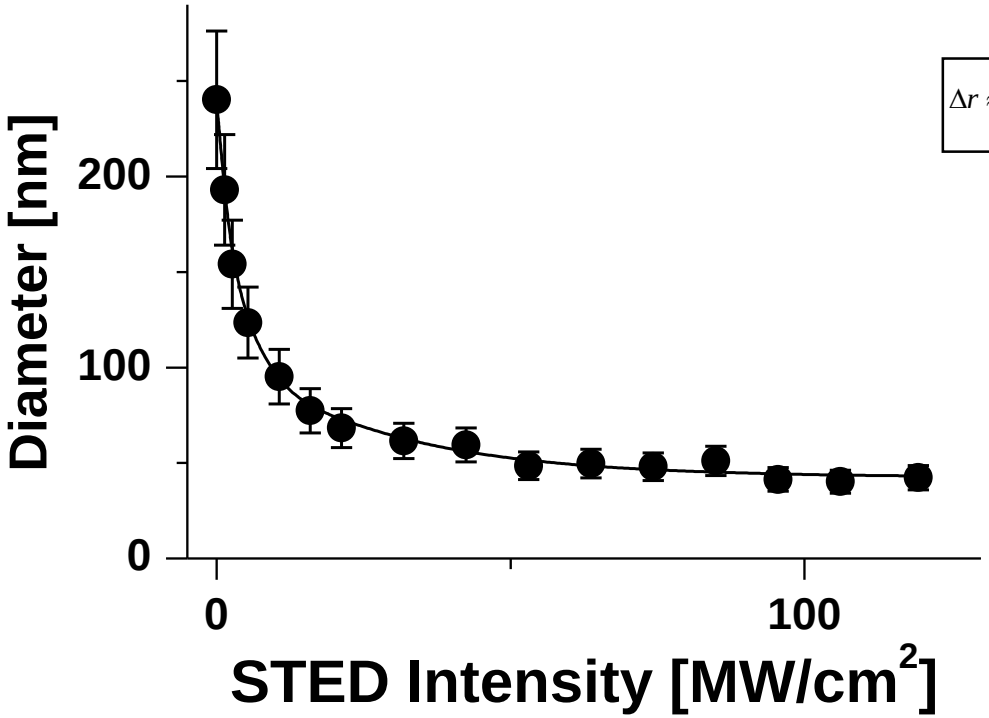




STED Microscopy

Dynamical confinement of resolution

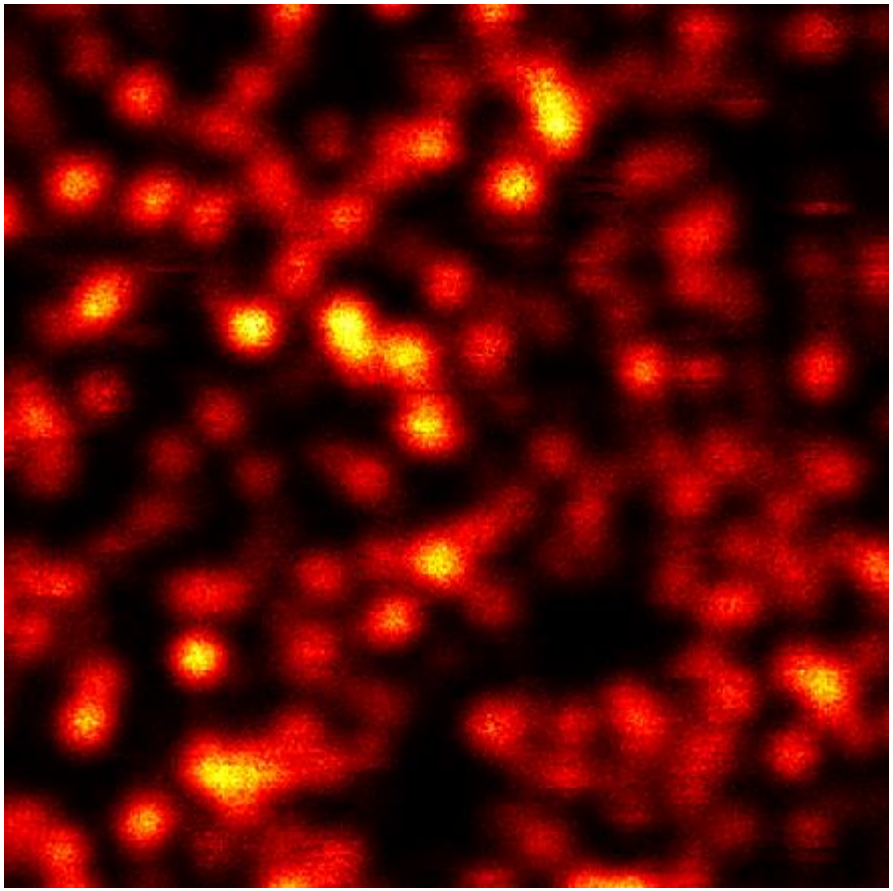
Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!

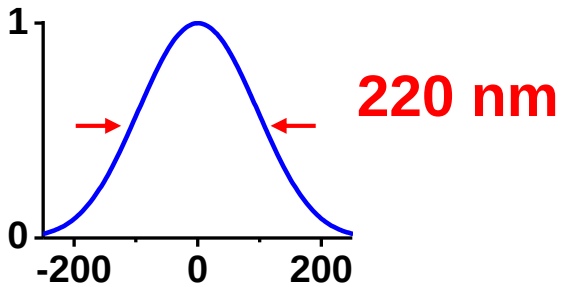


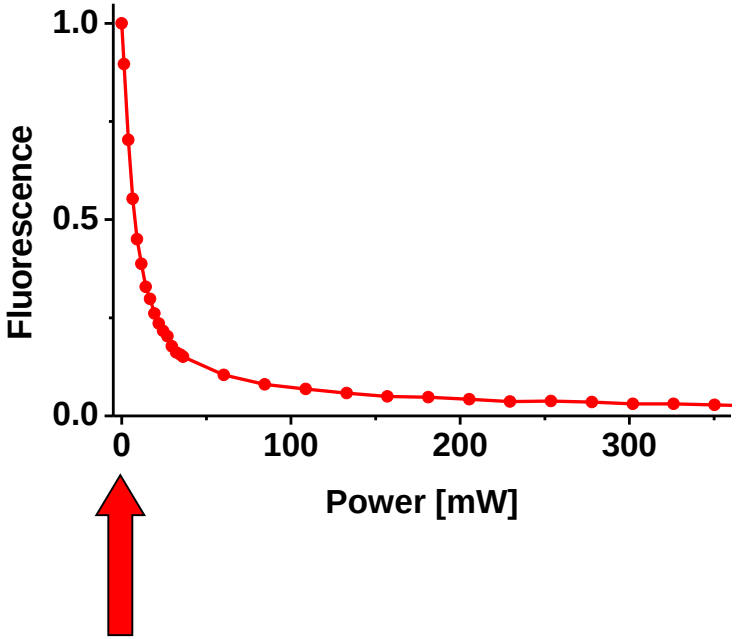
$$\Delta r \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+I/I_{sat}}}$$

STED-Microscopy

Sub-Diffraction Imaging







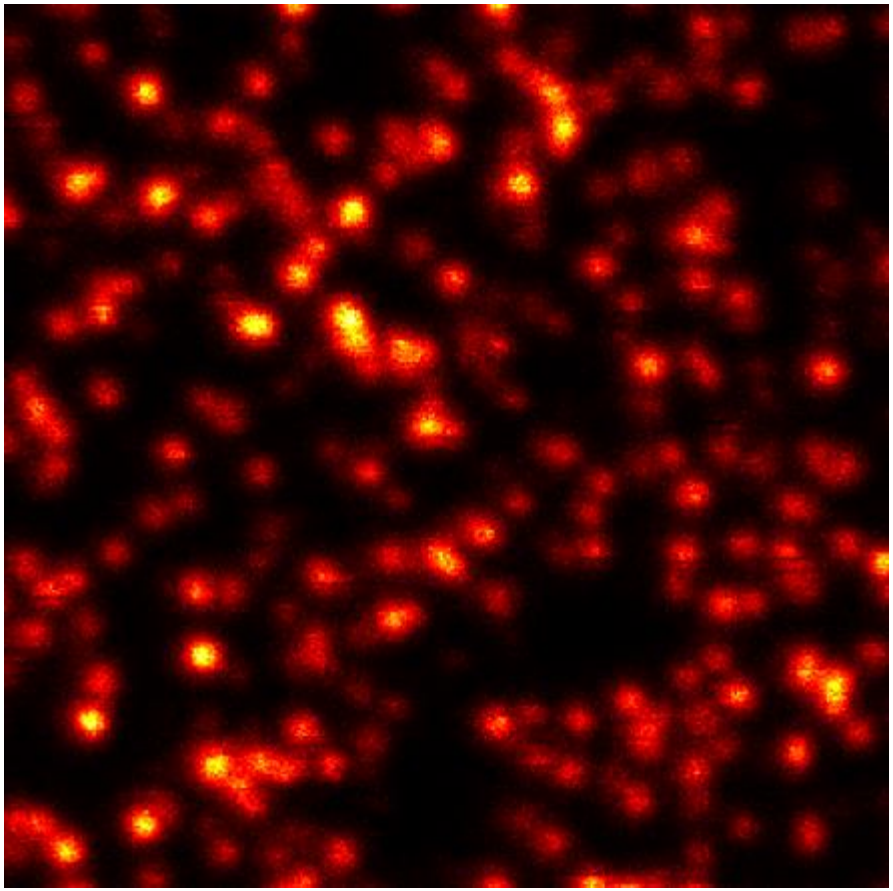
20nm Crimson beads

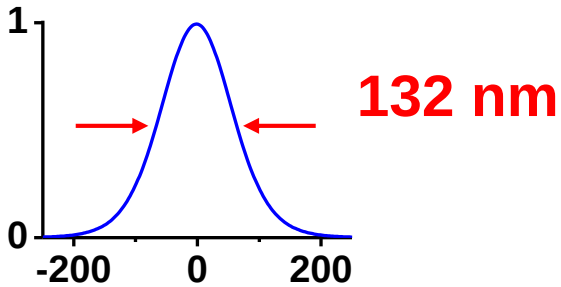
633nm exc, 90ps, 30kW/cm^2

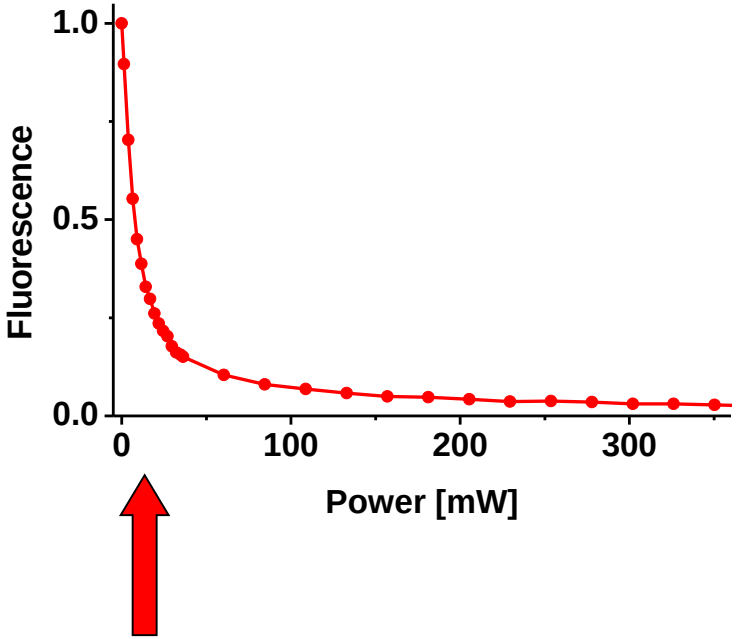
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging







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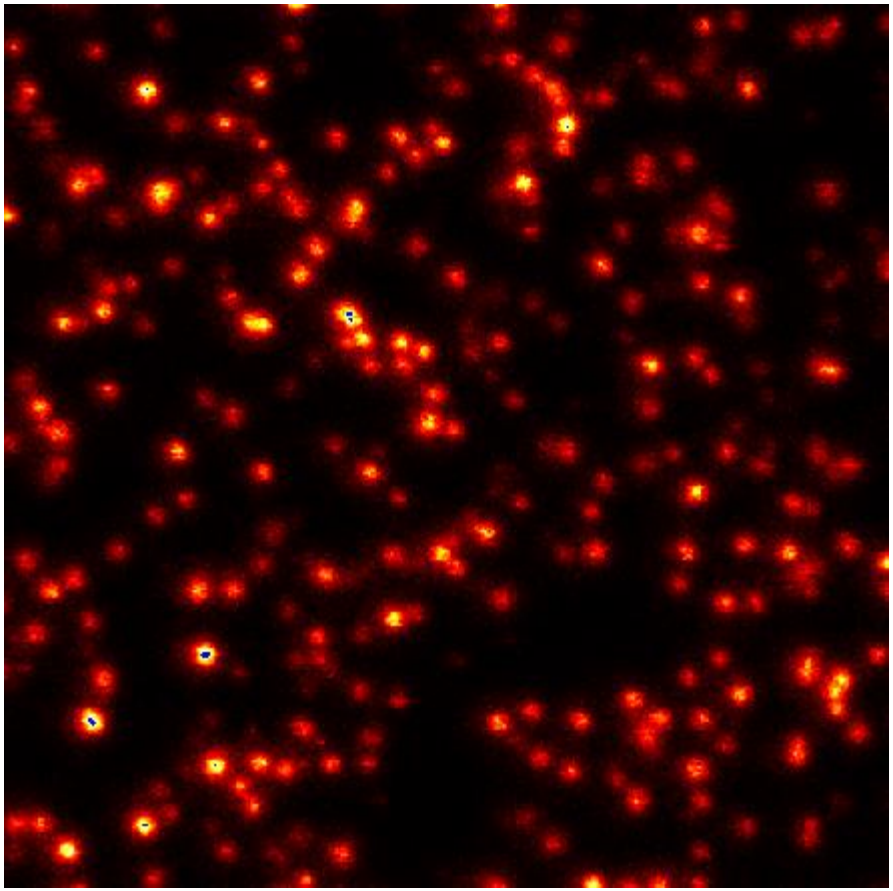

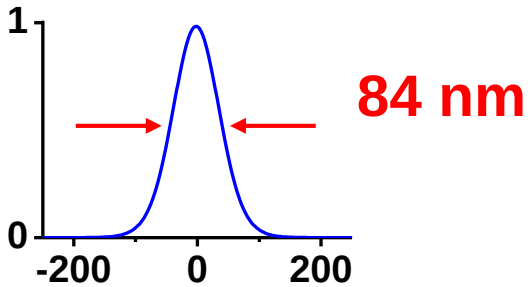

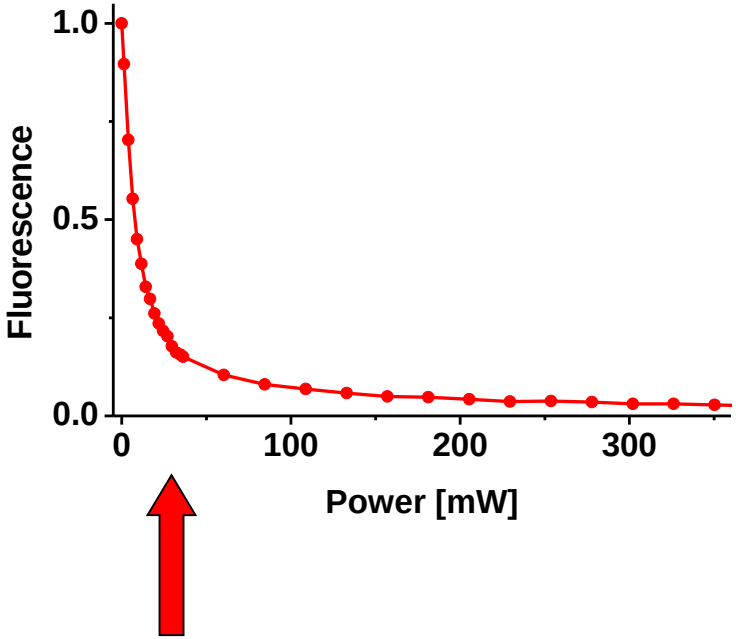

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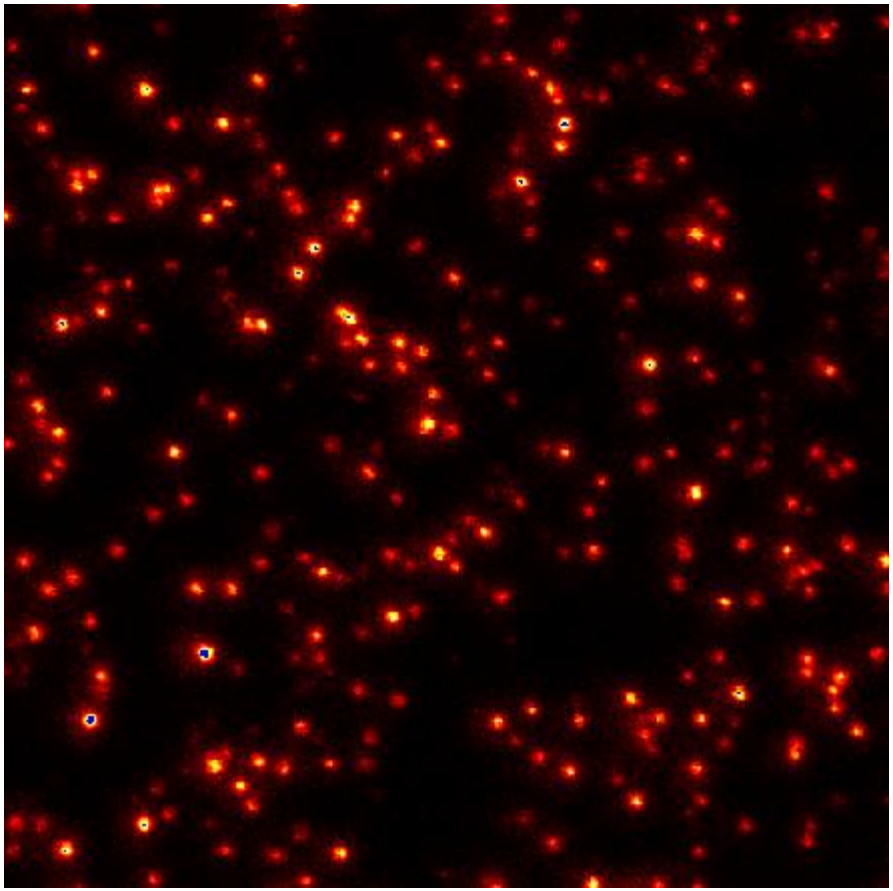

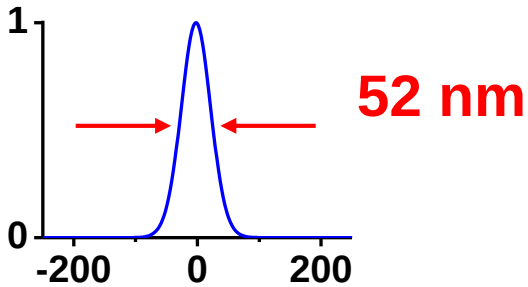

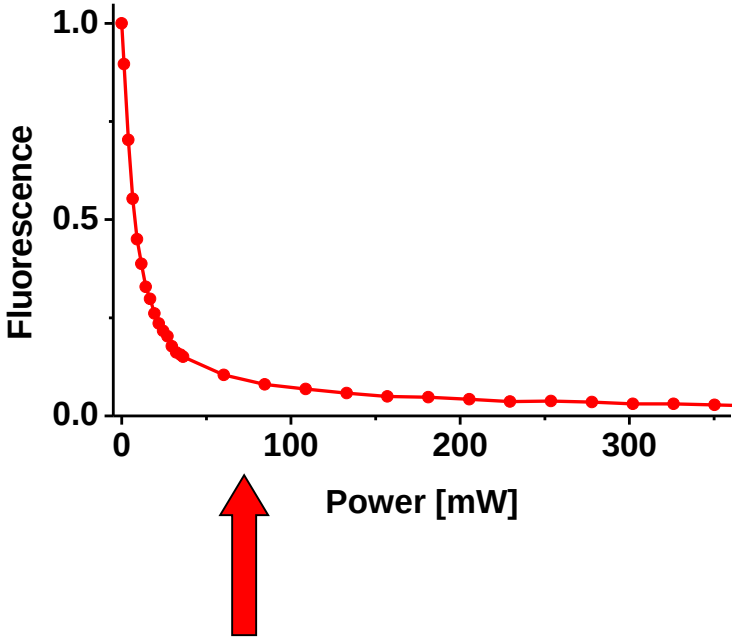

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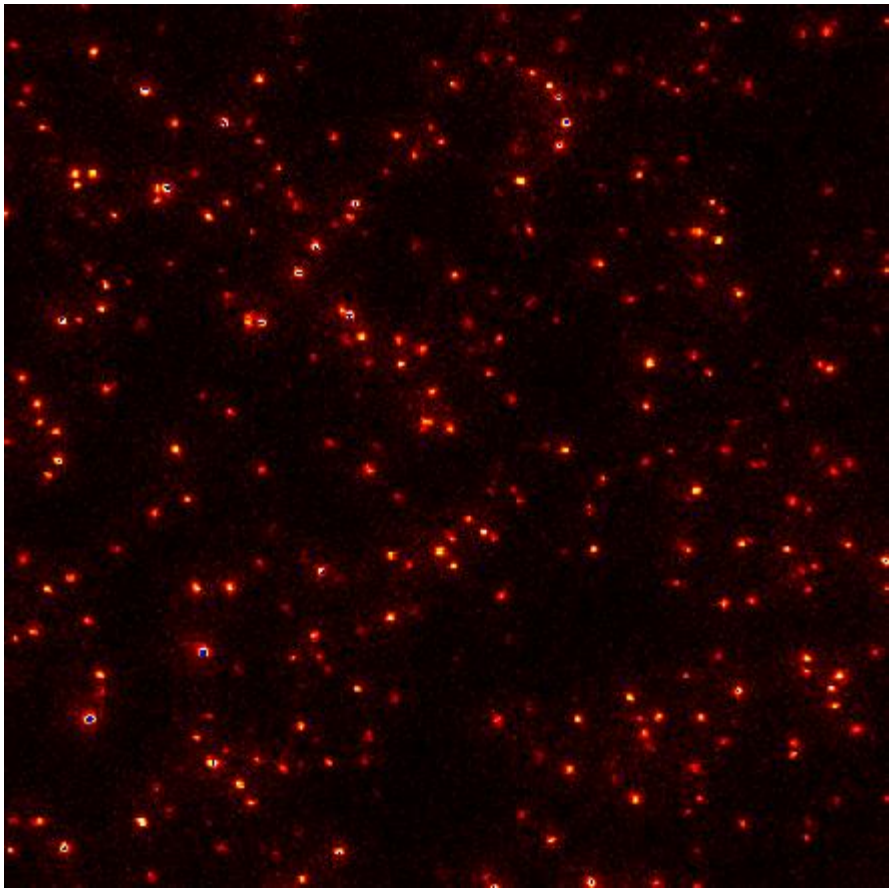
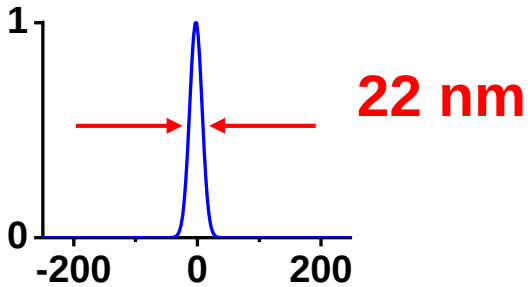
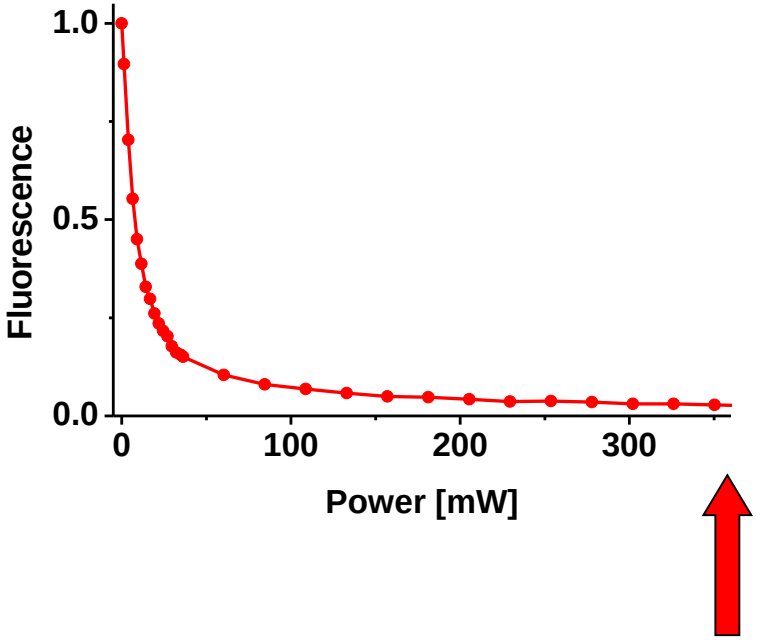






20nm Crimson beads

633nm exc, 90ps, 30kW/cm^2

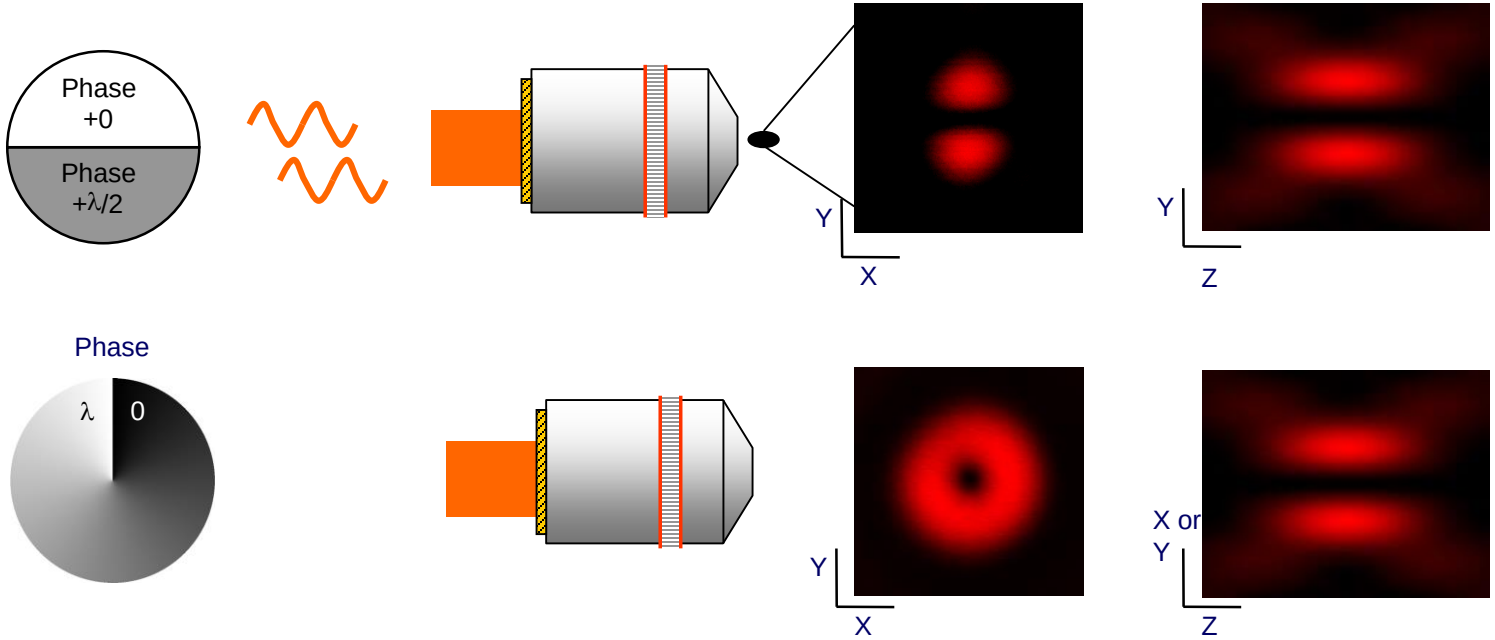
785nm STED 200ps, 76MHz

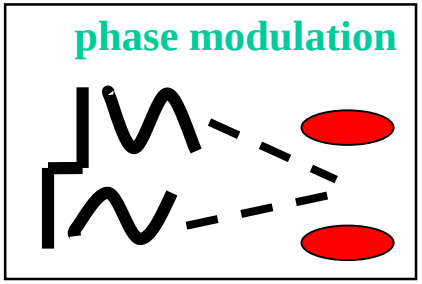
Focal Volume Confinement

Focal Engineering – Local Zero

Phase mask:

STED PSF:

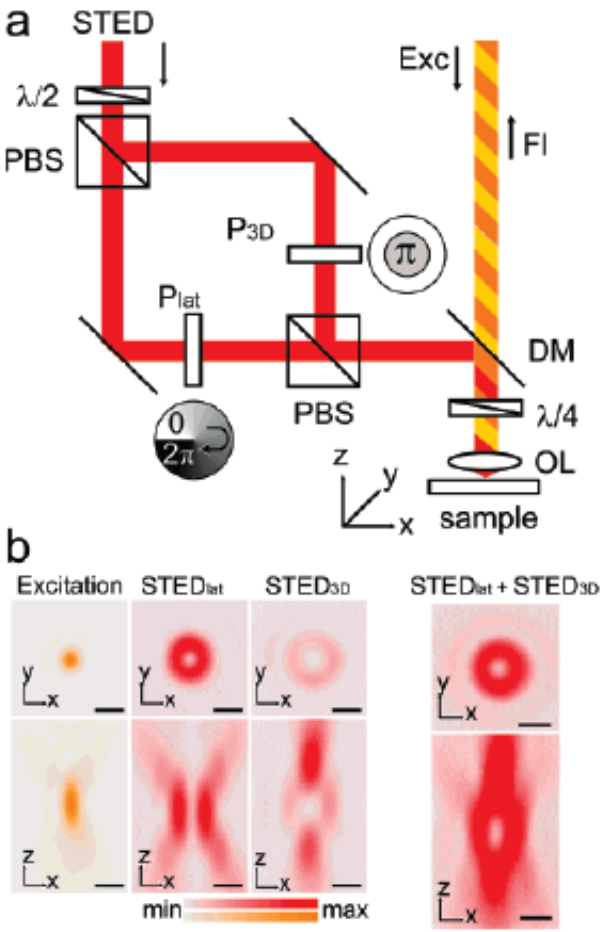




Klar et al, PNAS 2000

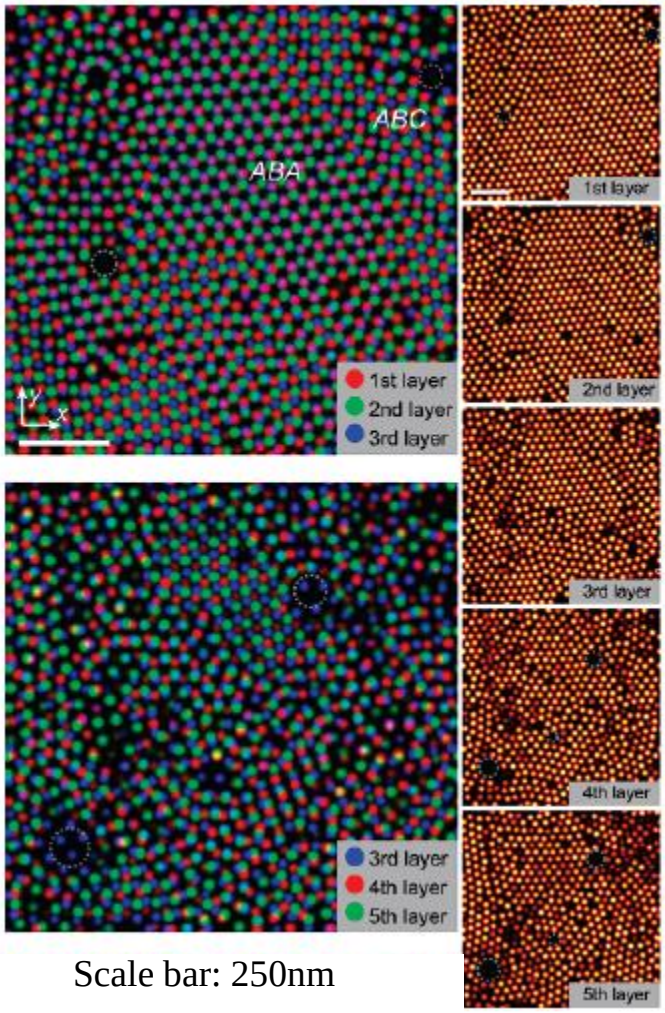
STED-Microscopy
Sub-Diffraction Imaging – 3D

3D STED nanoscopy I



Harke / Ullal et al NanoLett 2008

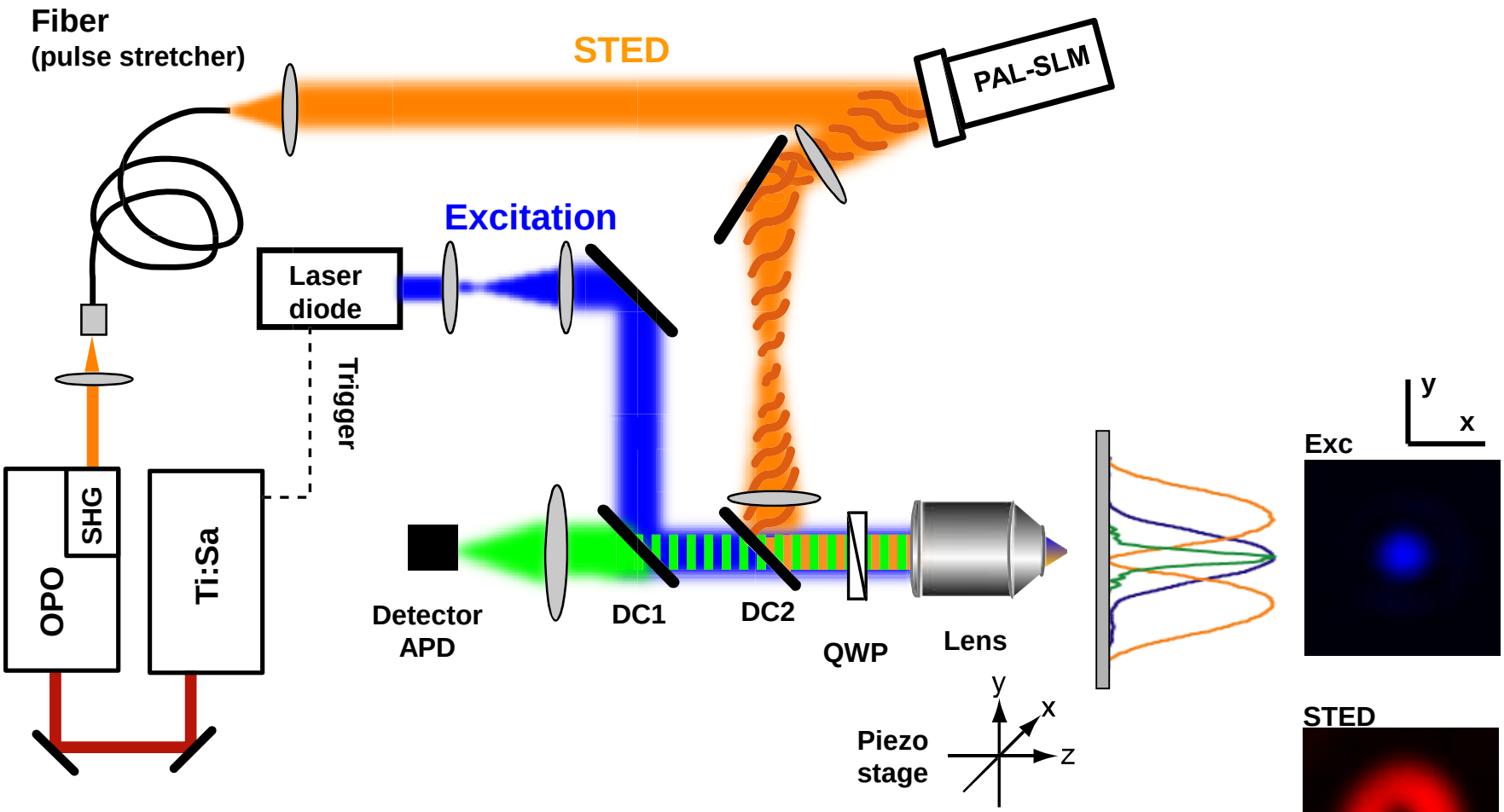
Fluorescent 100nm Beads – multiple layers on cover glass



Scale bar: 250nm

STED-Microscopy

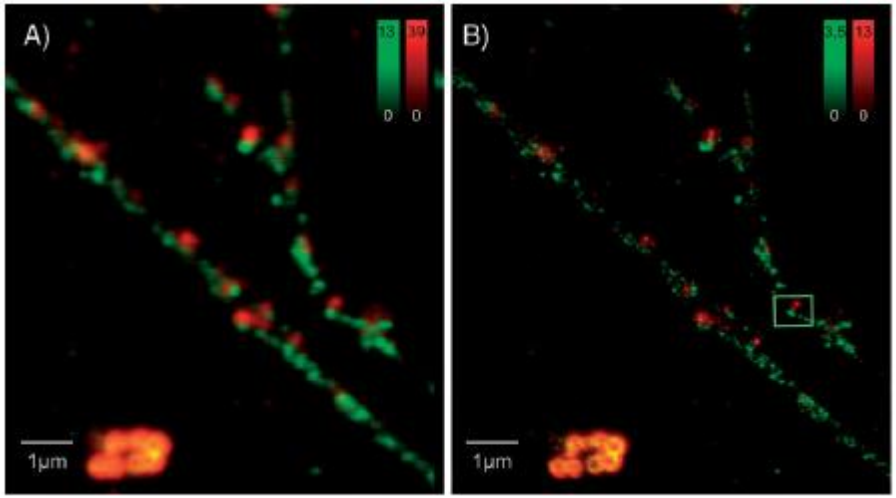
Setup

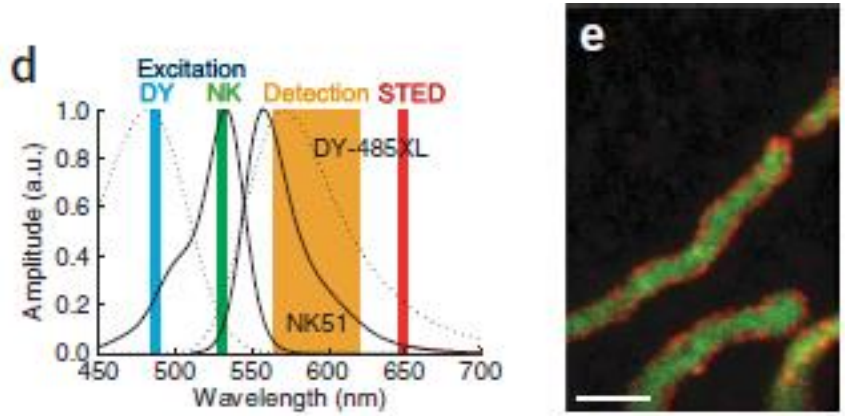


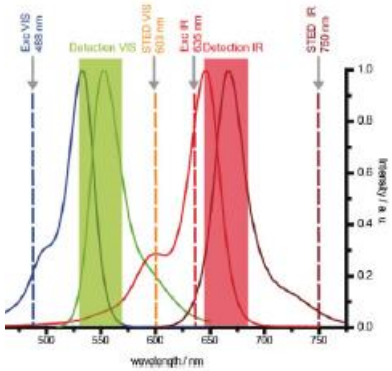
PSF engineering

STED-Microscopy

Multi-Color Sub-Diffraction Imaging







3 laser lines:

Large Stokes shift dye – only one excitation laser
Mitochondria in Vero cells:
outer membrane protein Tom20 (NK51, red)
matrix protein Hsp70 (Dy-485XL, green)

Schmidt et al NatMethods 2008

4 laser lines:

Synaptophysin (red, Atto647N) + syntaxin1 (green, Atto532) in neurons

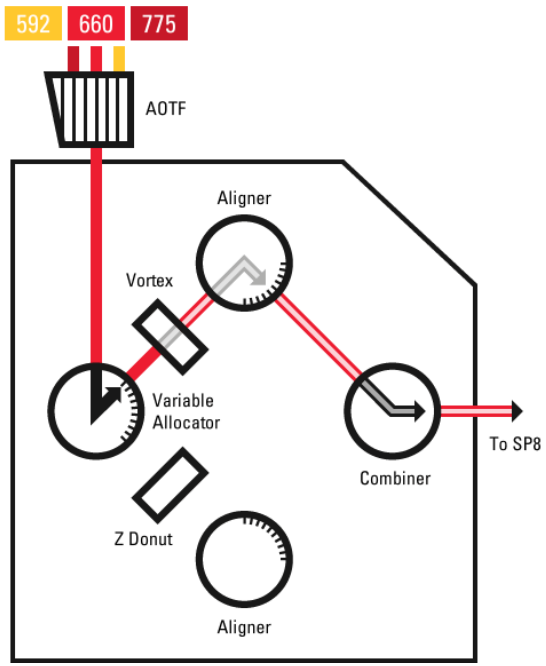
Donnert et al BiophysLett 2006 / Meyer et al Small 2008

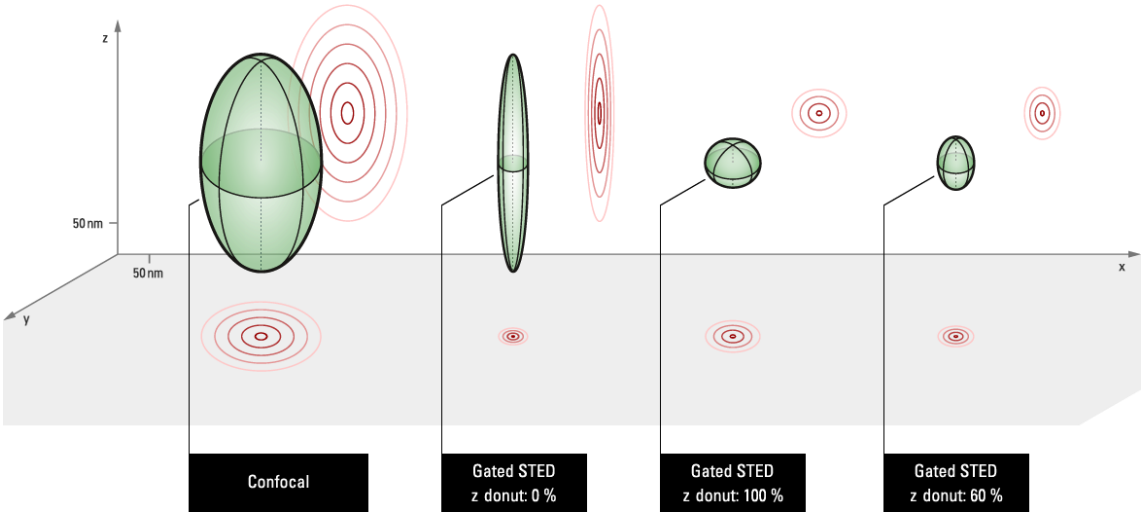
Commercial Leica SP8 gSTED Microscope at WIMM

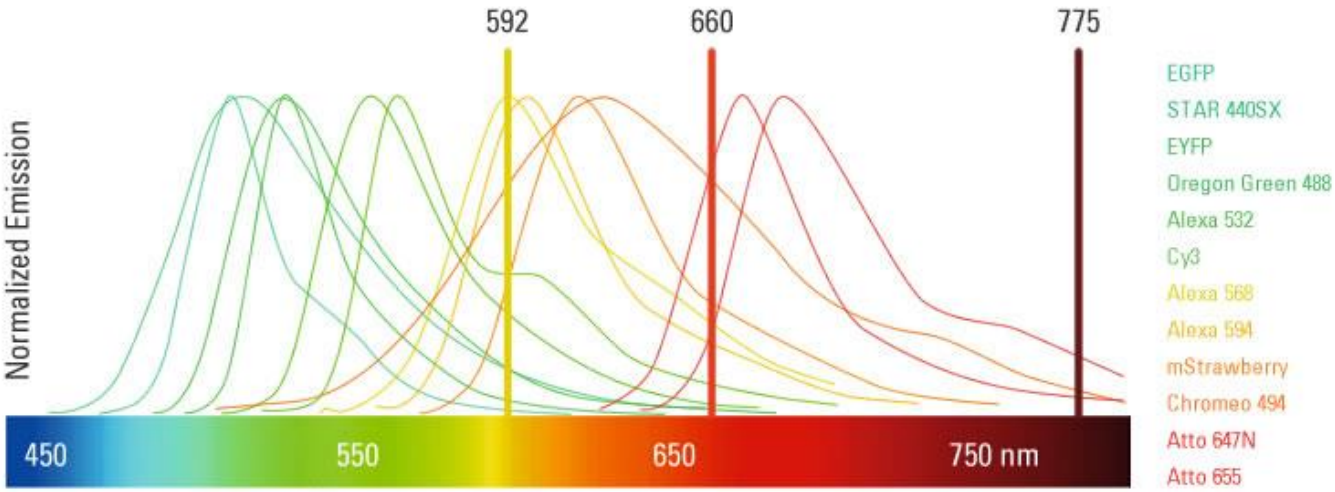
- The LEICA SP8 is a conventional inverted laser-scanning confocal microscope that is additionally equipped for 3D super-resolution gated STED imaging (max resolution$_{x,y}$ ~ 50 nm; max resolution$_{z}$ ~ 150 nm)

- This system is equipped with continuous wave (CW) lasers (@ 405, 458, 488, and 514 nm), a tuneable pulsed white laser (470-670 nm), a pulsed laser at 440 nm, and high power CW lasers at 592 nm, 660 nm, and soon 775 nm for STED imaging.

Schematics of Leica SP8 3D STED microscope







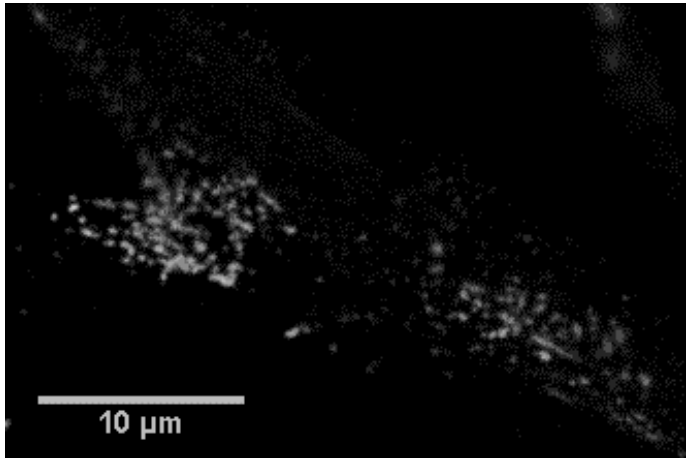
	592 GATED/CW	660 GATED/CW	775 PULSED
Strength	GFP/YFP	Multicolor	Most established spectral range
Colocalization studies	+	++	+
Photostability	+	++	++
Live cell	++	+	(+)

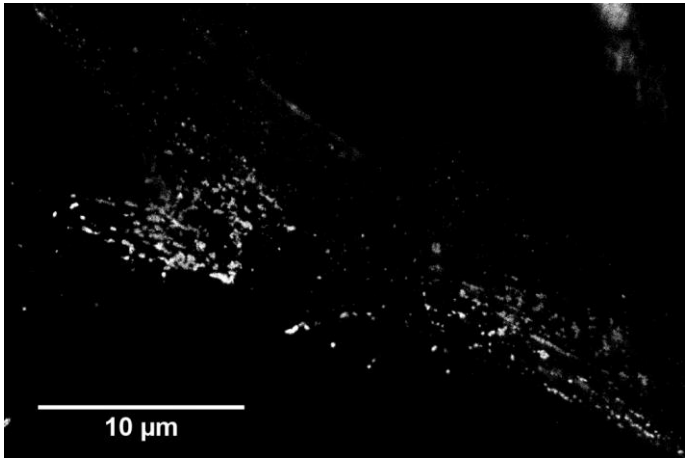
Application Example: 2D (xy) STED of AchR Clusters

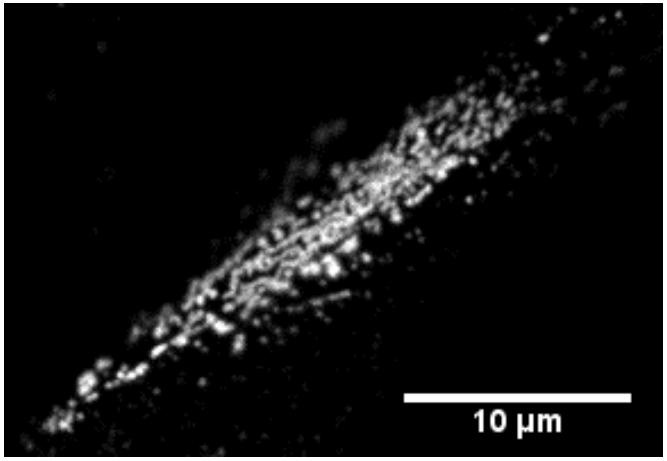
Dr. Judy Cossins / Prof. David Beeson, Neurosciences, WIMM

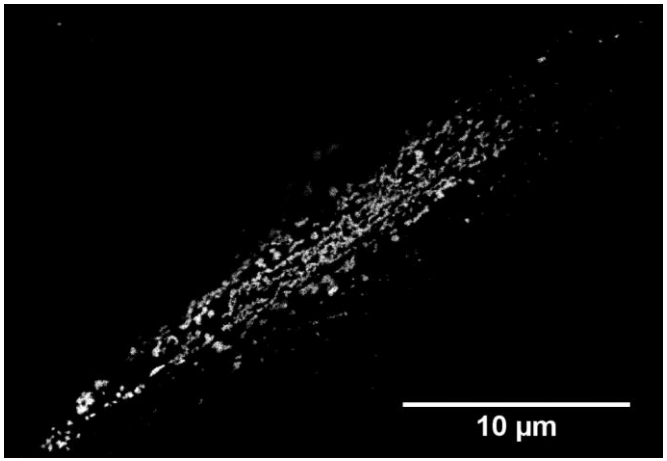
Confocal Images

STED Images





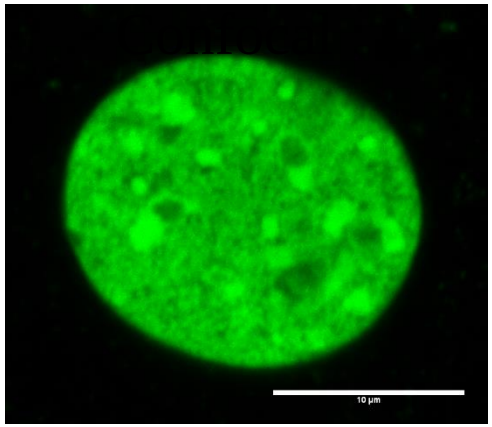




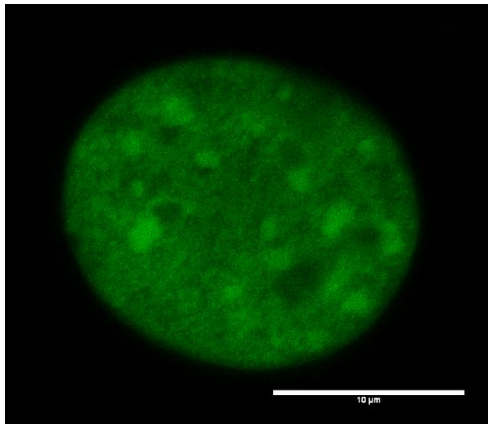
Application Example: 3D (xyz) STED of Sytox Green stained cell nuclei in HeLa cells

Dr. Lothar Schermelleh, Biochemistry & Micron

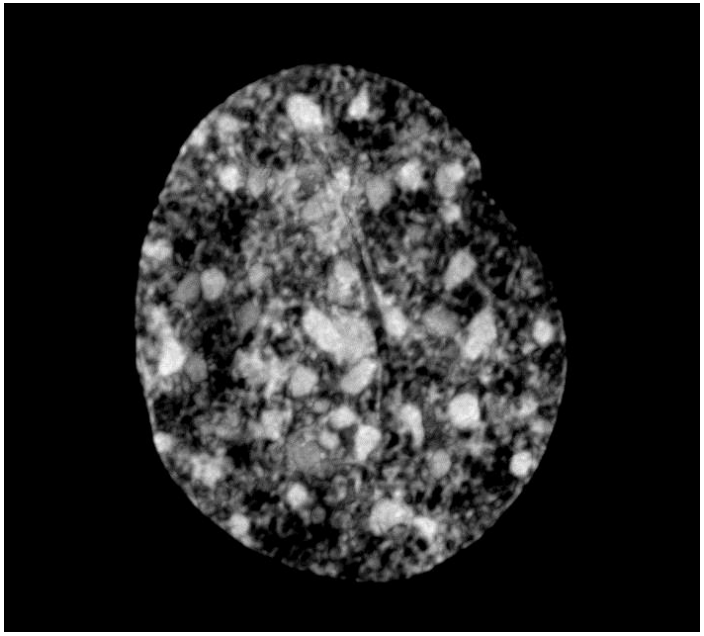
Confocal



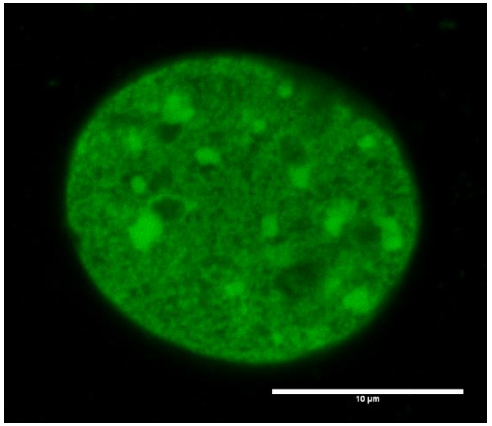
gSTED at 25% Power (xy:z=100:0)



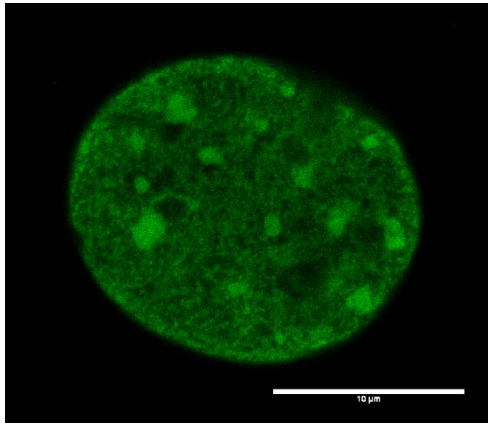
gSTED at 100% Power (xy:z=50:50)



gSTED at 25% Power (xy:z=0:100)

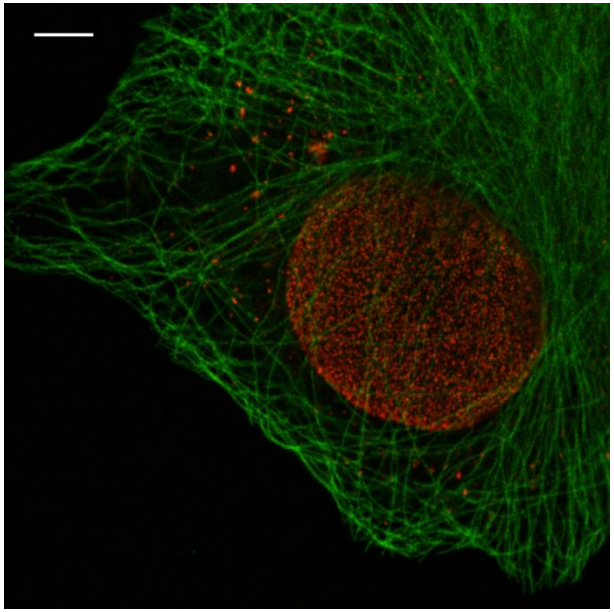


gSTED at 100% Power (xy:z=50:50)

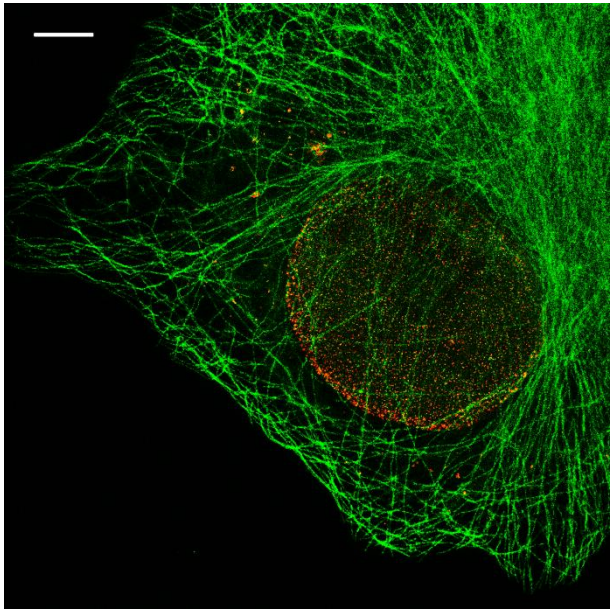


Application Example: Two color 2D (xy) STED of α-Tubulin (Abberior Star 440SX) / Nucler Pores (Atto 488)

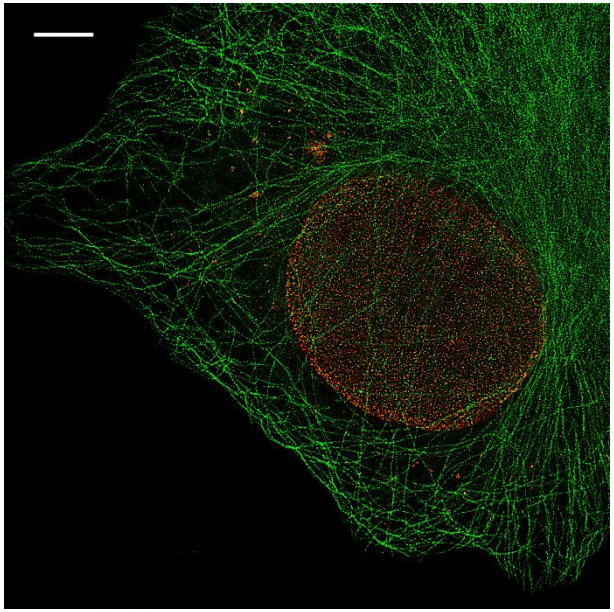
Confocal 440/502 nm



gSTED 50% (440/502 nm)

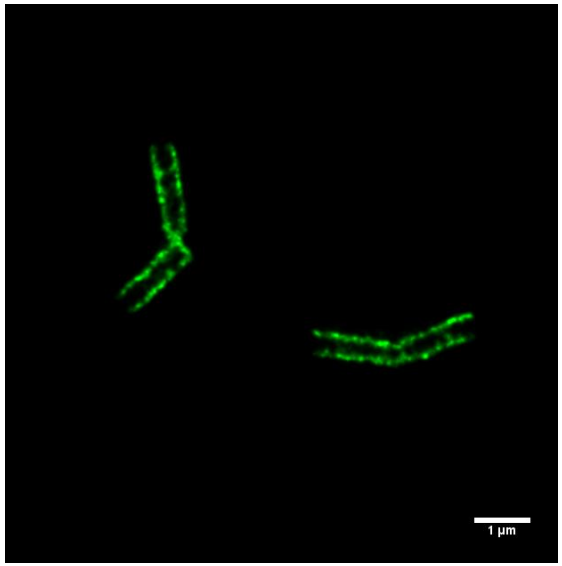


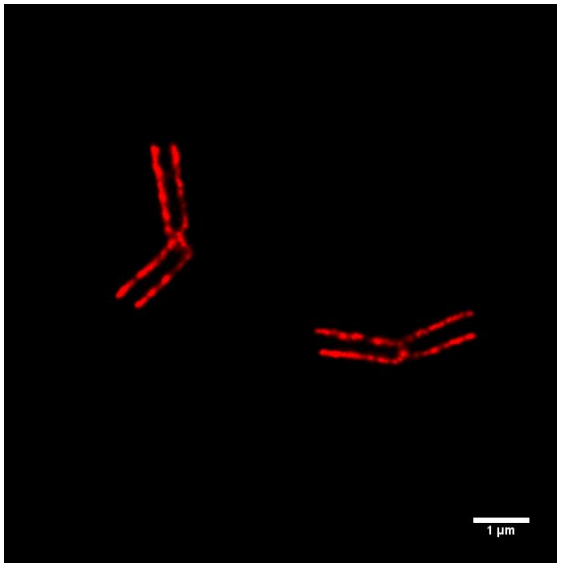
De-convolved (Huygens)
gSTED 50% (440/502 nm)

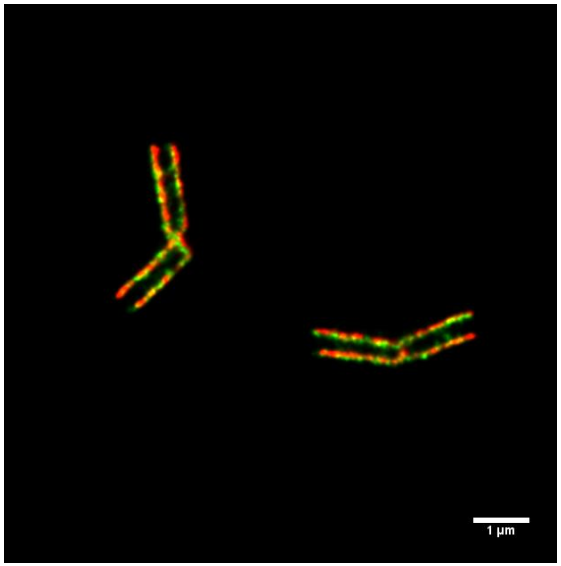


Application Example: Two color 2D STED of Centrosomes in Drosophila with GFP/Atto488 and TMR

Prof. Jordan Raff / Dr. Alan Wainman, Sir William Dunn School of Pathology



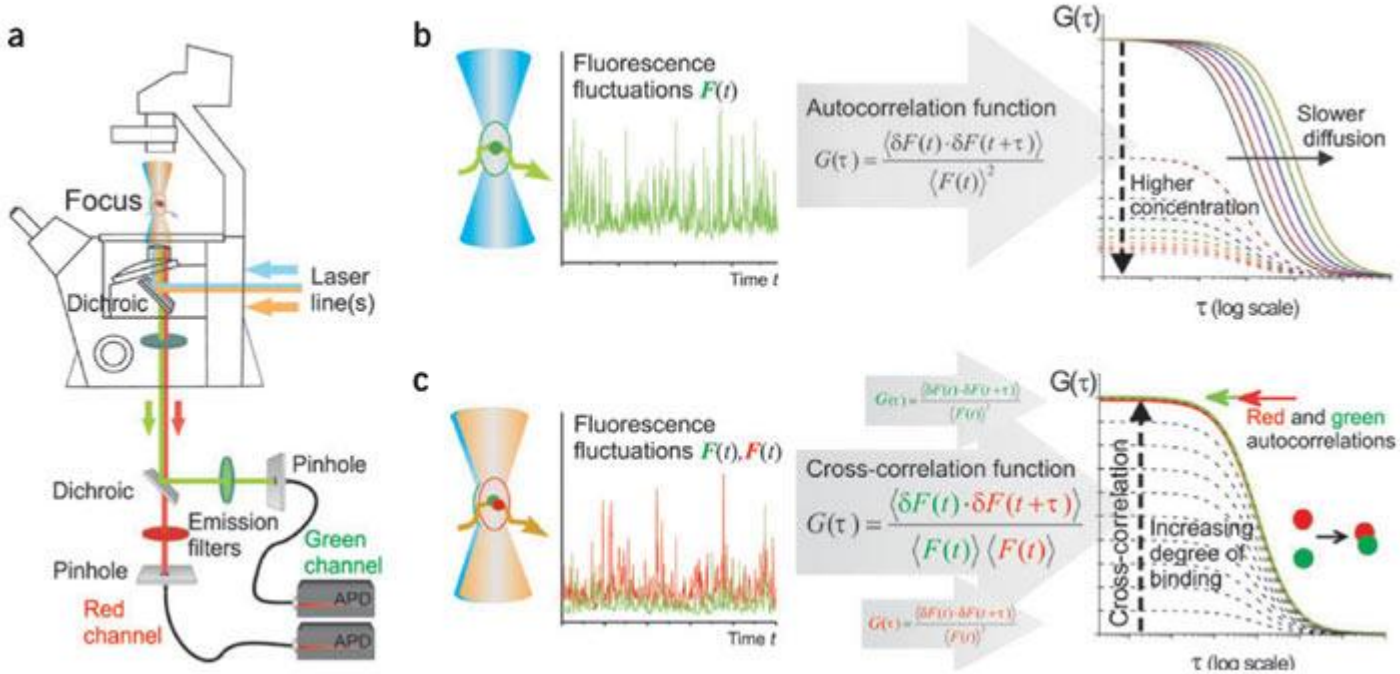




Spd2-GFP/GFP-Booster-Atto488 Asl/TMR

GFP signal amplified with GFP-Booster from ChromoTek
(http://www.chromotek.com/products/nano-boosters/)

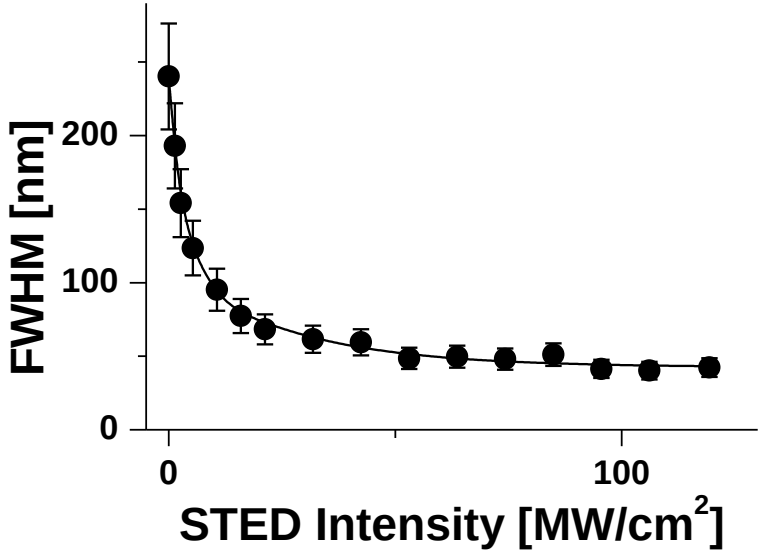
STED can also be combined with Fluorescence Correlation Spectroscopy (STED-FCS)



Bacia, K; Kim, SA; Schwille, P. (2006) Fluorescence cross-correlation spectroscopy in living cells. Nature Methods 3: 83 – 89.

Live Cell Nanoscopy
STED-FCS

STED-Microscopy: Tuning of observation area



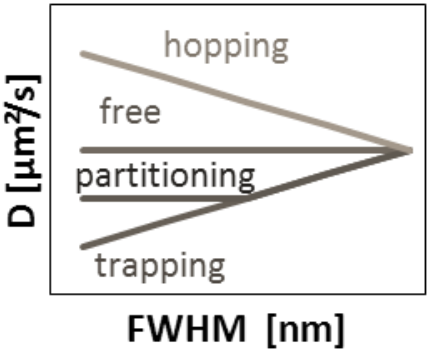
STED-FCS
Determine transit time
for different sizes of observation areas
(different STED intensities)

Calculate
apparent diffusion coefficient:

D ~ area / transit time

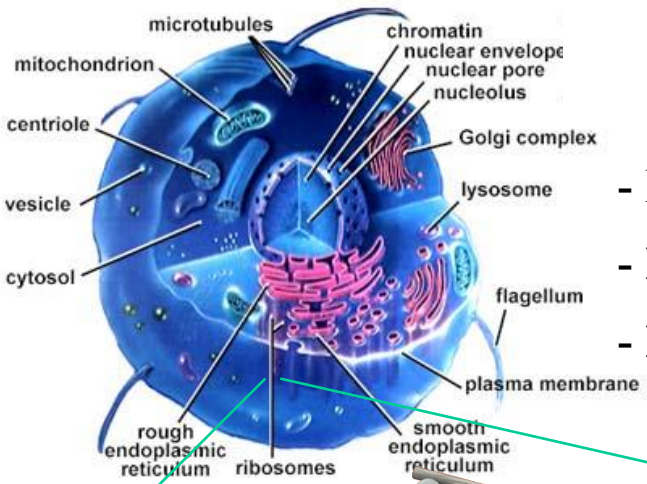
Dependencies: D(diameter)
240nm → 30/40nm

Varies for different diffusion modes



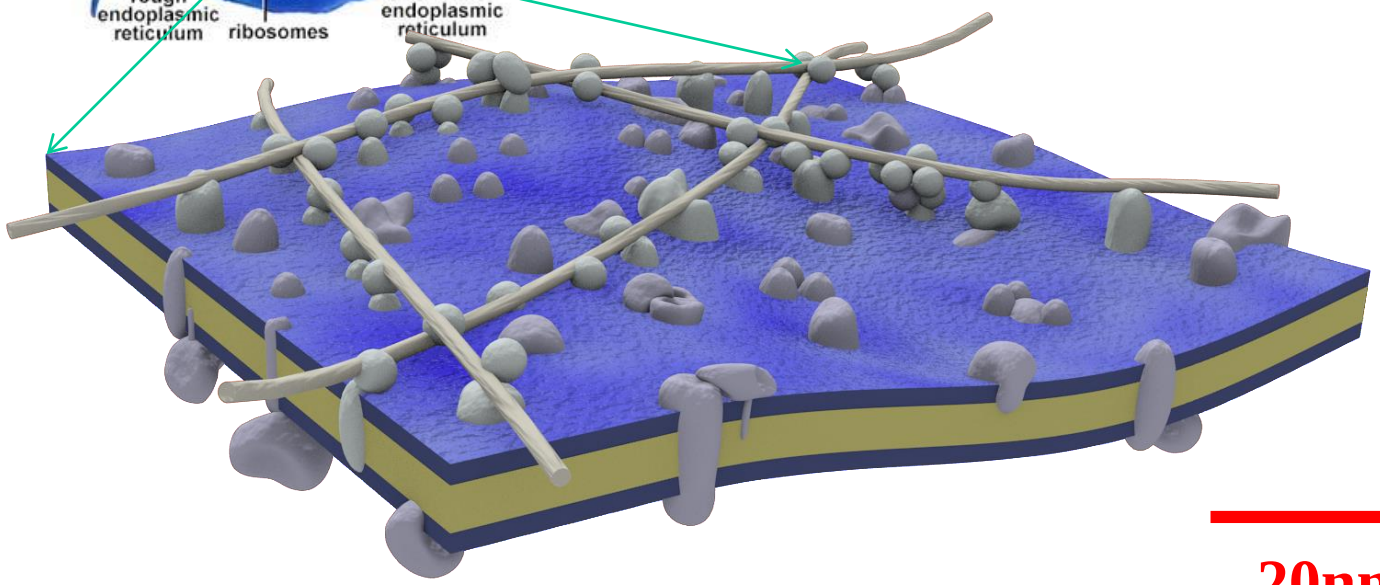
Lipid Plasma Membrane Dynamics

Nanoscale



Lipid Plasma Membrane Organization:

- Heterogeneous distribution…
- Interaction with proteins
- Interaction with cortical cytoskeleton



20nm

Small spatial scales!!!!

Lipid Plasma Membrane Dynamics

Interactions on the Nanoscale: Nanodomains

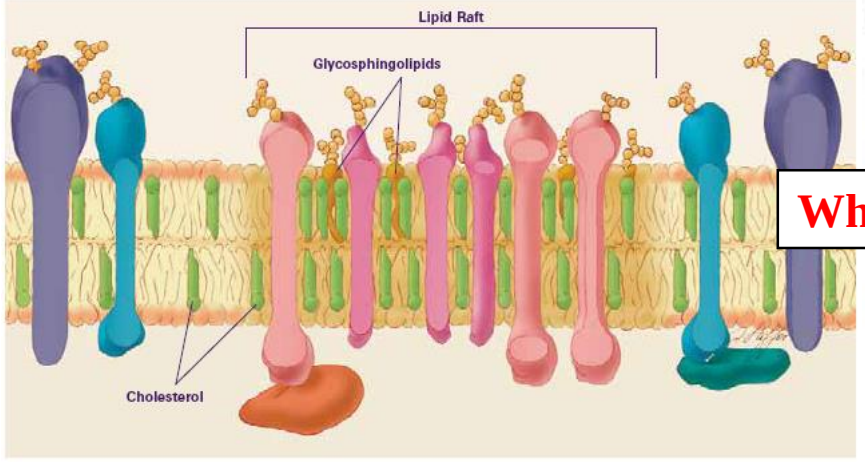
Lipid rafts/nanodomains?

- (Transient) cholesterol/sphingolipid-enriched
- Dense molecular packing (ordered)
- Compartmentalize cellular processes

Cytoskeleton

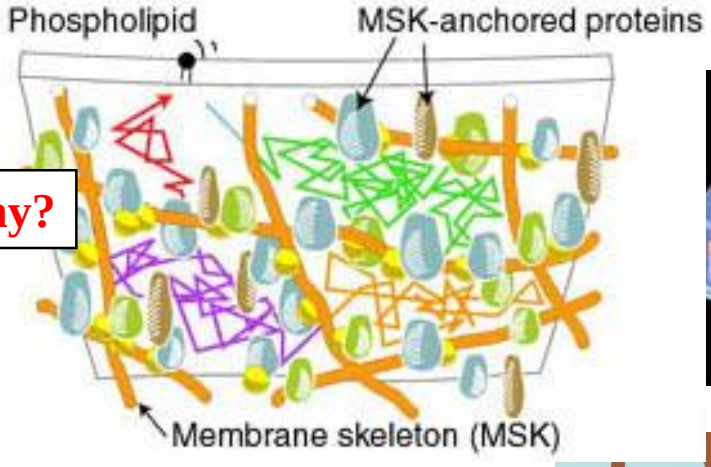
- Membrane divided in compartments
- Proteins: fence/hindrance in diffusion path
- Hopping diffusion

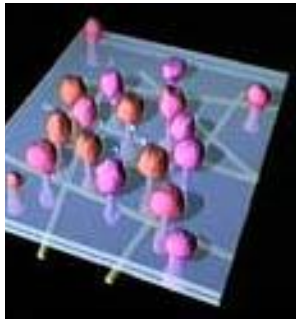
Kusumi

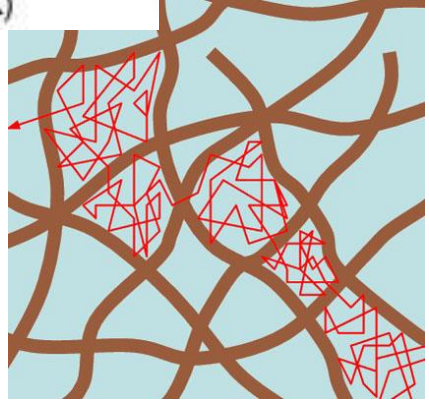


Pike, J.Lipid Res., Keystone meeting 2006

Why?







<u>Problem:</u>

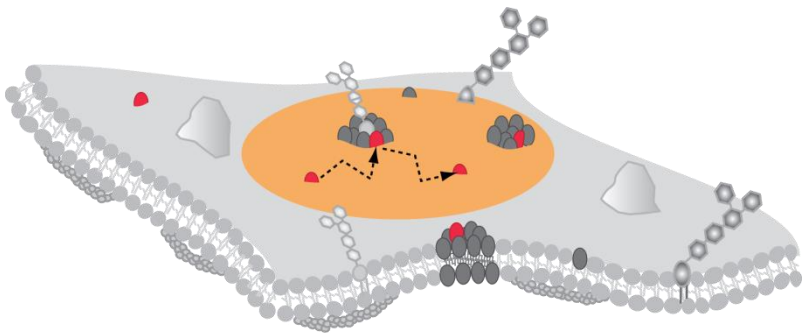
- heterogeneous
  + highly dynamic
- small (<200 nm)

Missing temporal/spatial resolution
→ hardly any direct observation method
→ highly debated

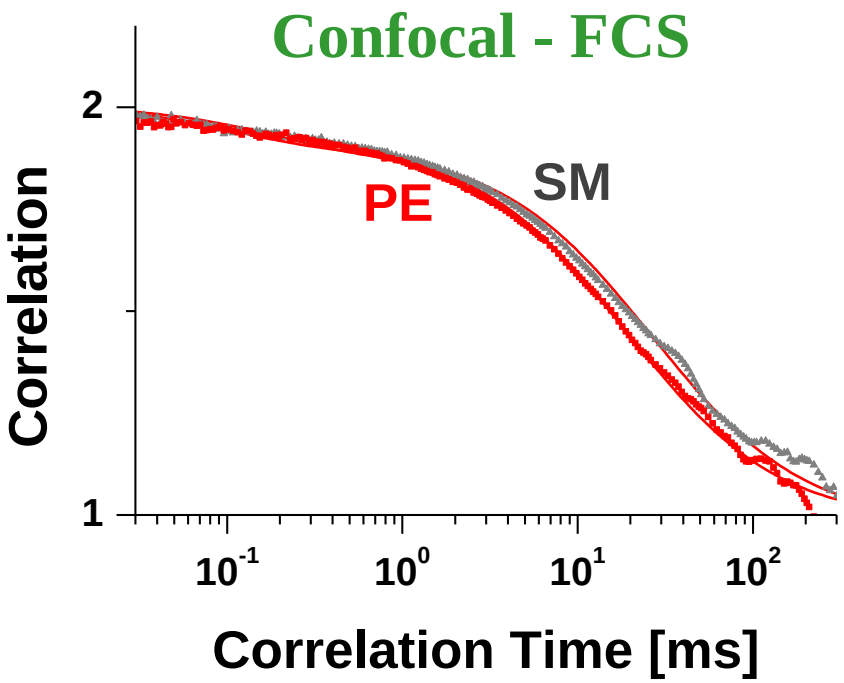
Lipid Plasma Membrane Dynamics

Confocal Recordings

Confocal: Limited spatial resolution !!!



Relative large confocal observation area:
averages over details on nanoscale
cannot distinguish normal diffusion
from nanoscale hindered diffusion



SM diffusion slightly prolonged but still normal

$\tau_d \approx$ 20 ms (PE) / 30ms (SM)

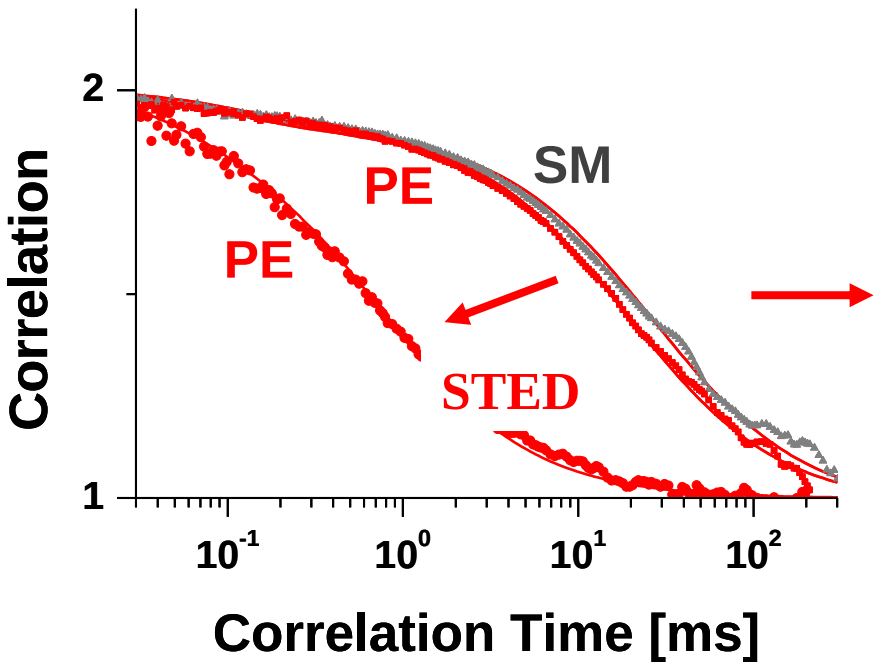
$(1/\alpha) \approx 1$ (PE / SM)

Slower normal diffusion
but no anomalous diffusion???

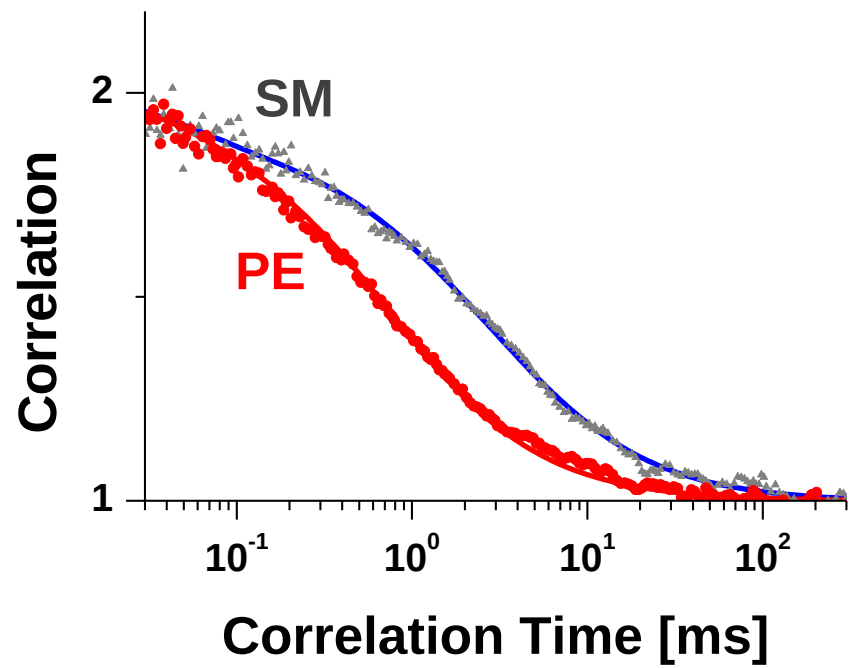
STED Live Cell Spectroscopy

Single Lipid Dynamics - FCS

Confocal



STED – 40nm



STED (240 -> 40nm):

PE diffusion scales with area reduction

τ_d: 20 -> 0.6ms (35-fold)

and still normal

$(1/\alpha) \approx 1$

STED:

SM diffusion much longer than PE

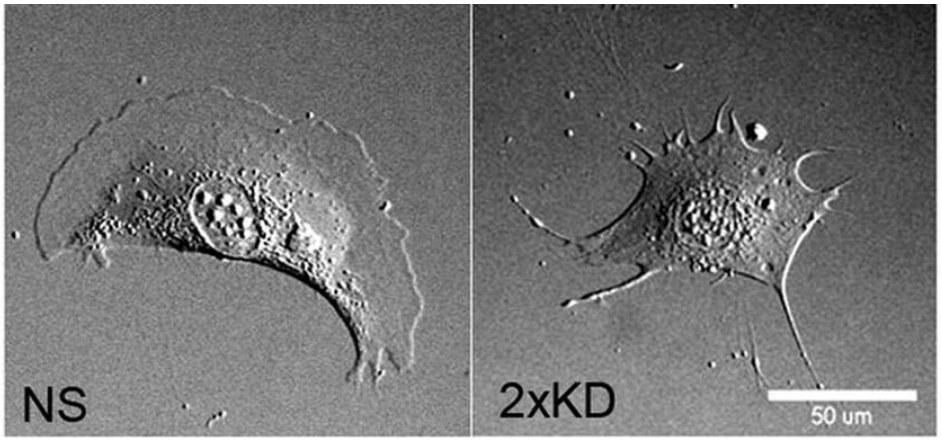
τ_d: 30 -> 3ms (10-fold)

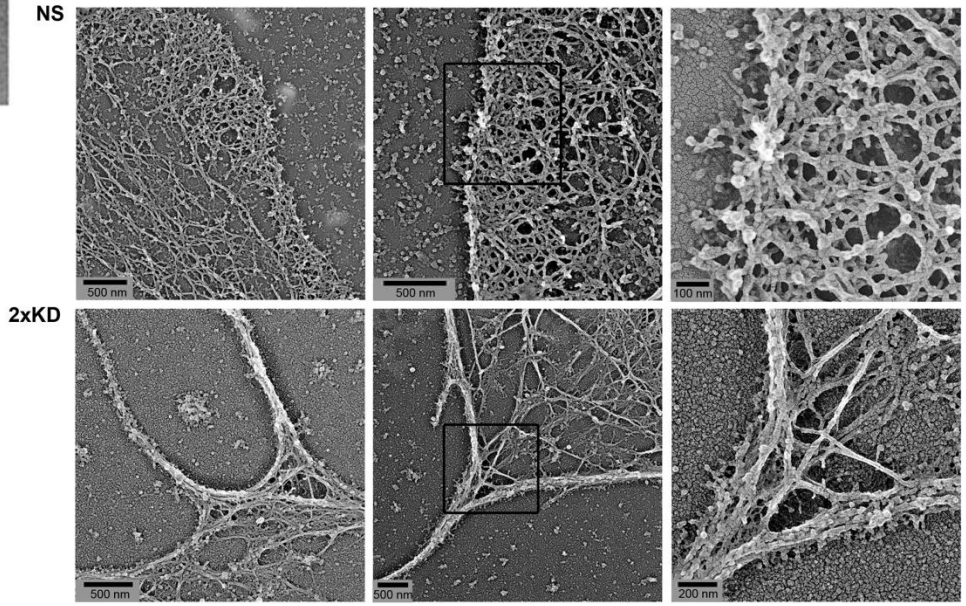
and anomalous

$(1/\alpha) \approx 1.5$

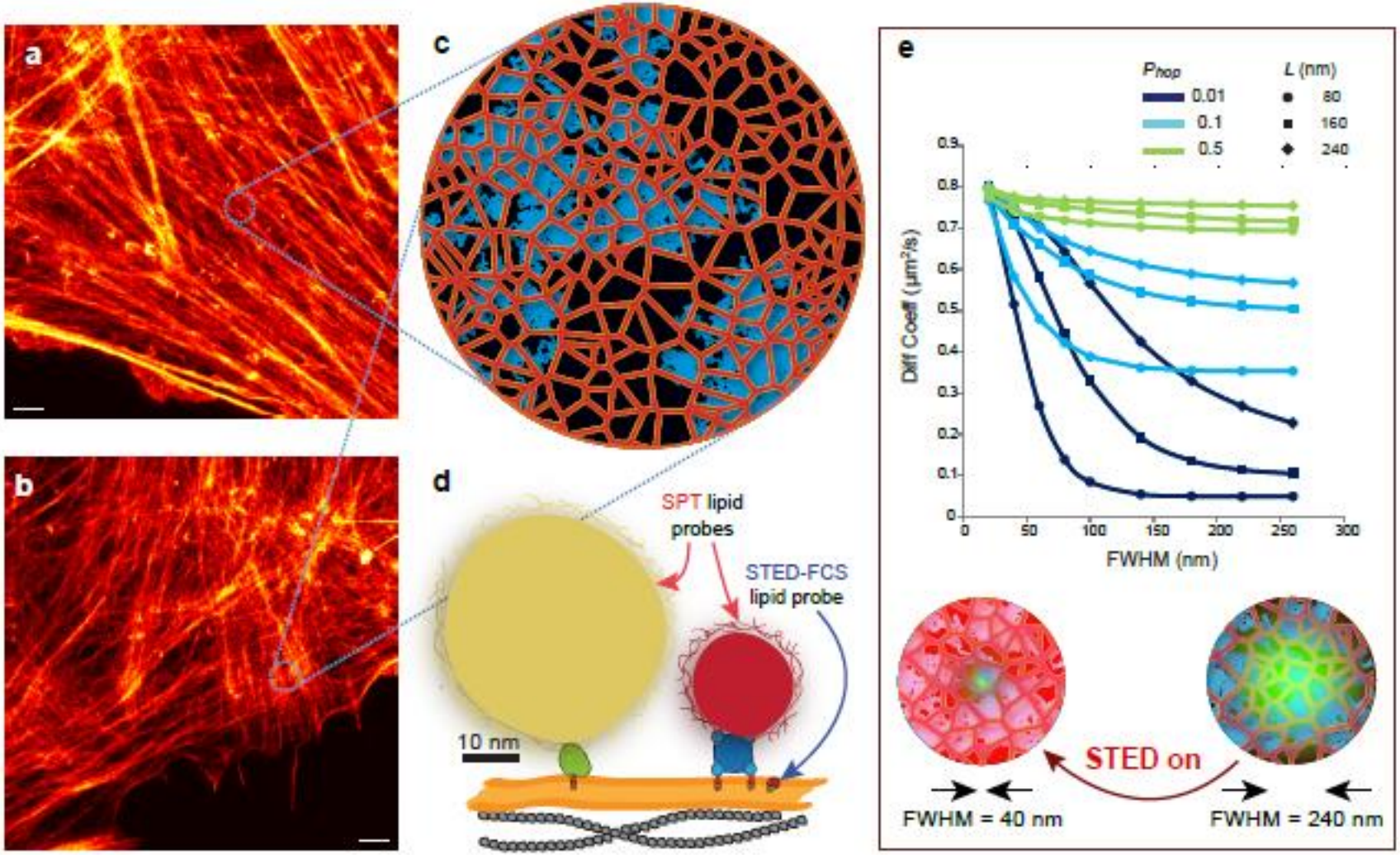
Arp2/3 Is Critical for Lamellipodia and Response to Extracellular Matrix Cues but Is Dispensable for Chemotaxis

Congying Wu,[1,4,7] Sreeja B. Asokan,[1,4,7] Matthew E. Berginski,[2] Elizabeth M. Haynes,[1,4] Norman E. Sharpless,[3,4] Jack D. Griffith,[4,5] Shawn M. Gomez,[2] and James E. Bear[1,4,6,*]
[1]Department of Cell and Developmental Biology
[2]Departments of Biomedical Engineering, Computer Science, and Pharmacology
[3]Department of Genetics
[4]Lineberger Comprehensive Cancer Center
[5]Department of Biochemistry
[6]Howard Hughes Medical Institute
University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA
[7]These authors contributed equally to this work
*Correspondence: jbear@email.unc.edu
DOI 10.1016/j.cell.2011.12.034

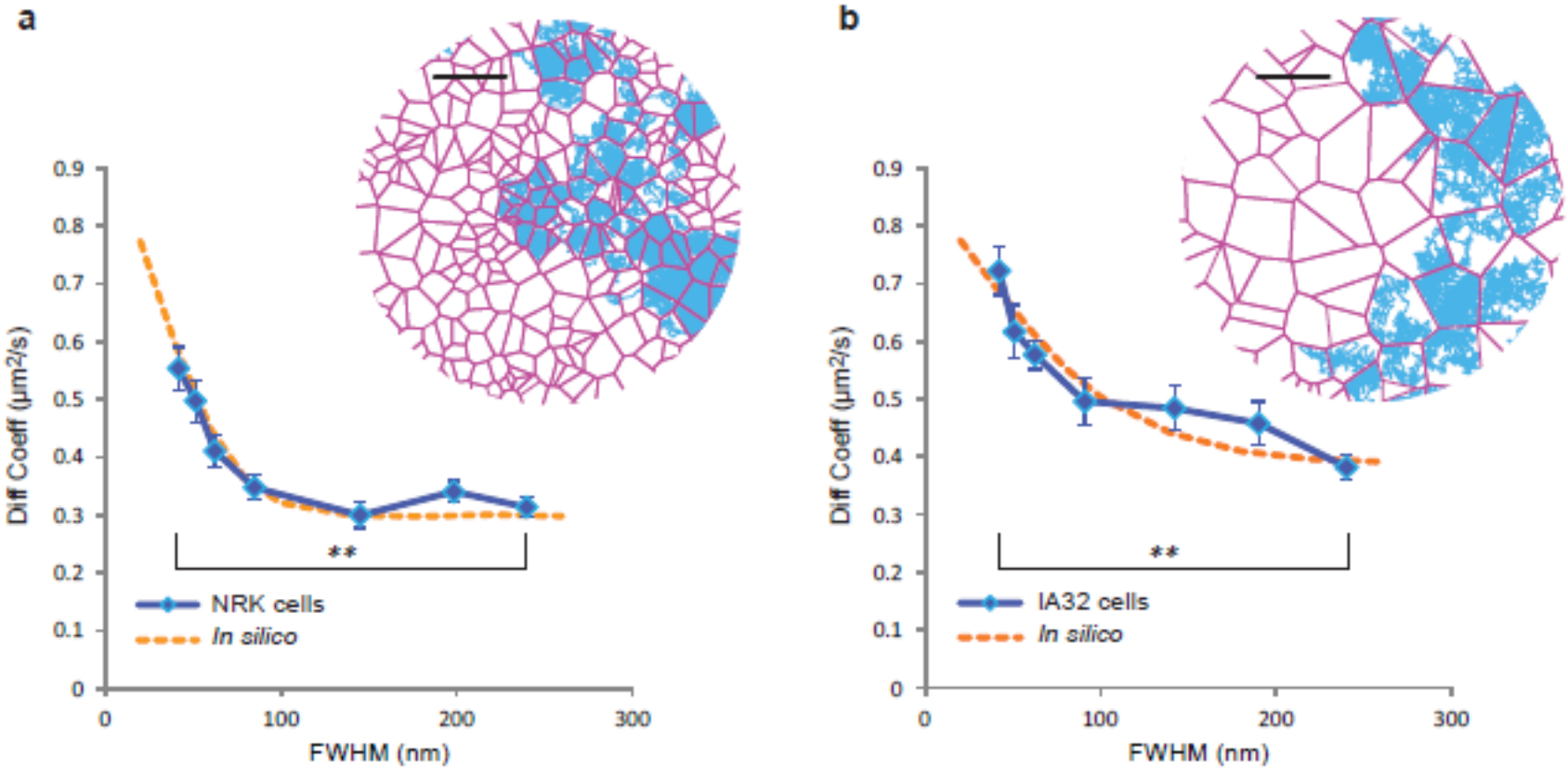




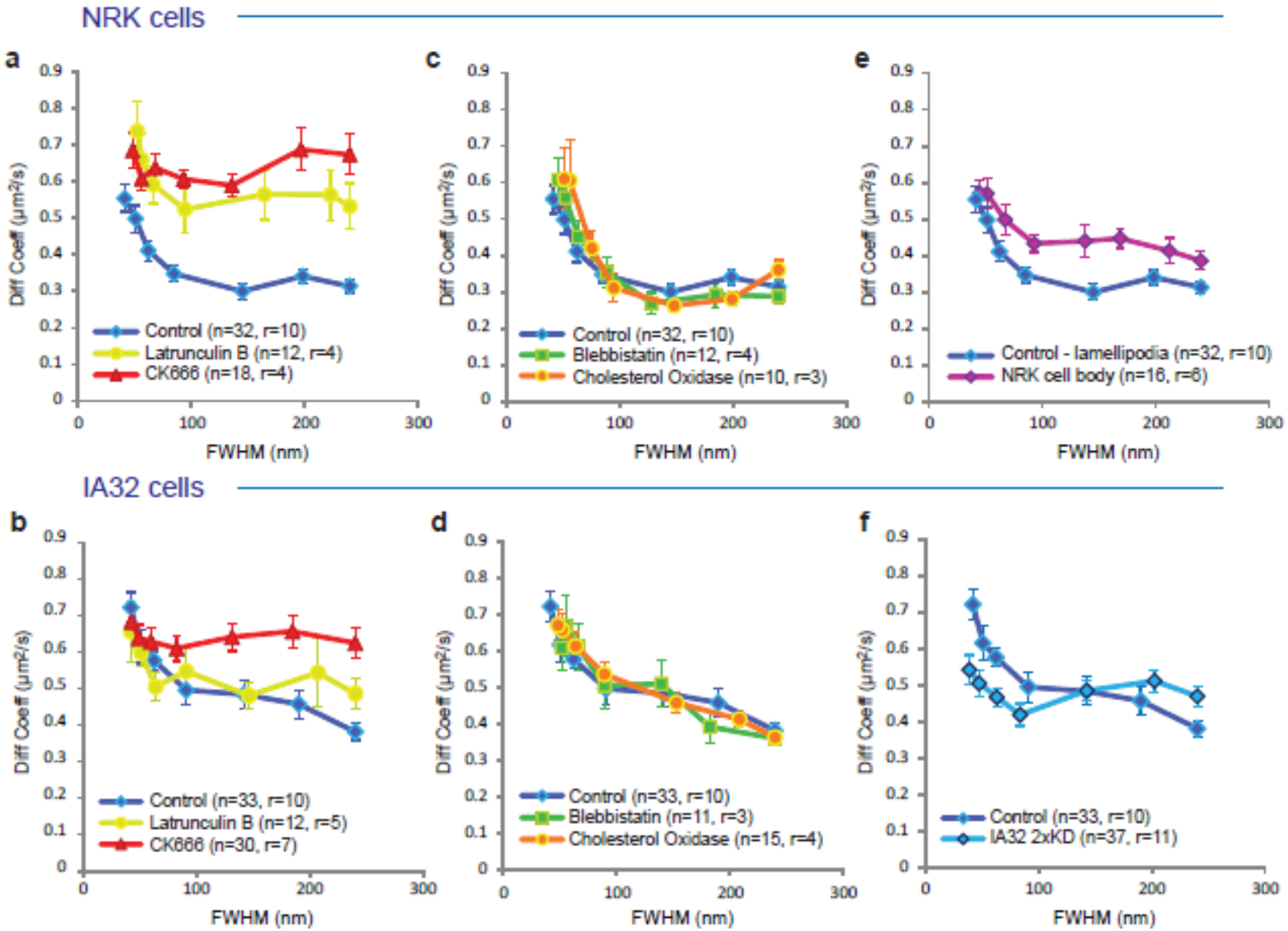
Measuring Hop-Diffusion with STED-FCS



Measuring Hop-Diffusion with STED-FCS



Measuring Hop-Diffusion with STED-FCS



Summary

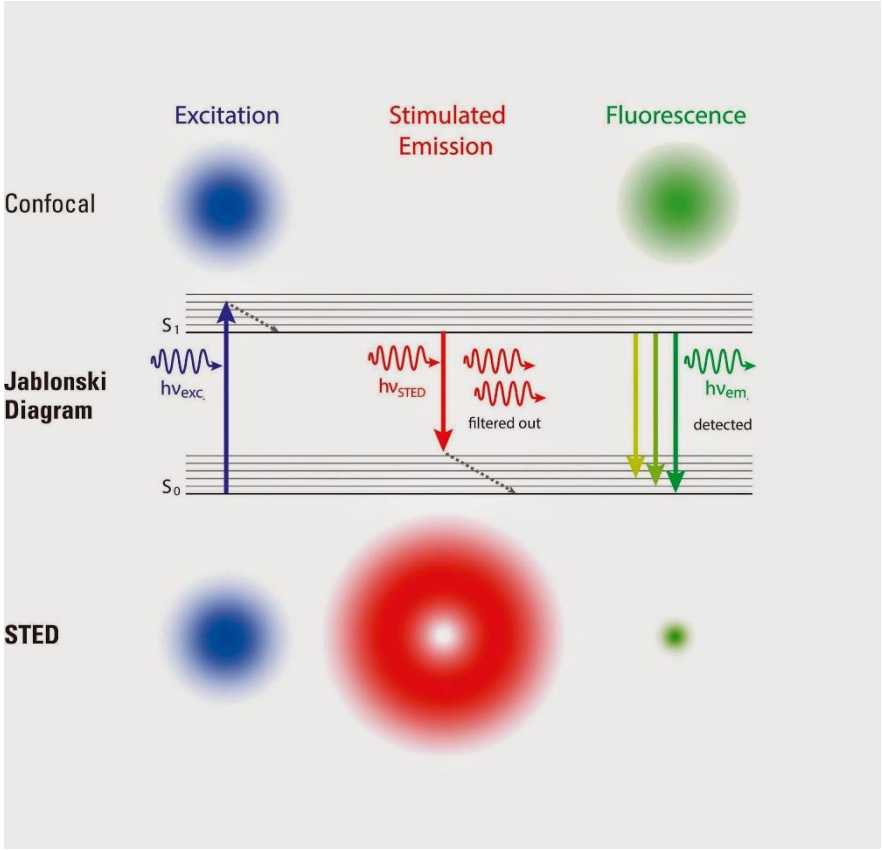

Excitation
Stimulated Emission
Fluorescence
Confocal
Jablonski Diagram
S1
S0
hvexc.
hvSTED
filtered out
hvem.
detected
STED

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Image Examples: Mentioned users

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Veronica Buckle

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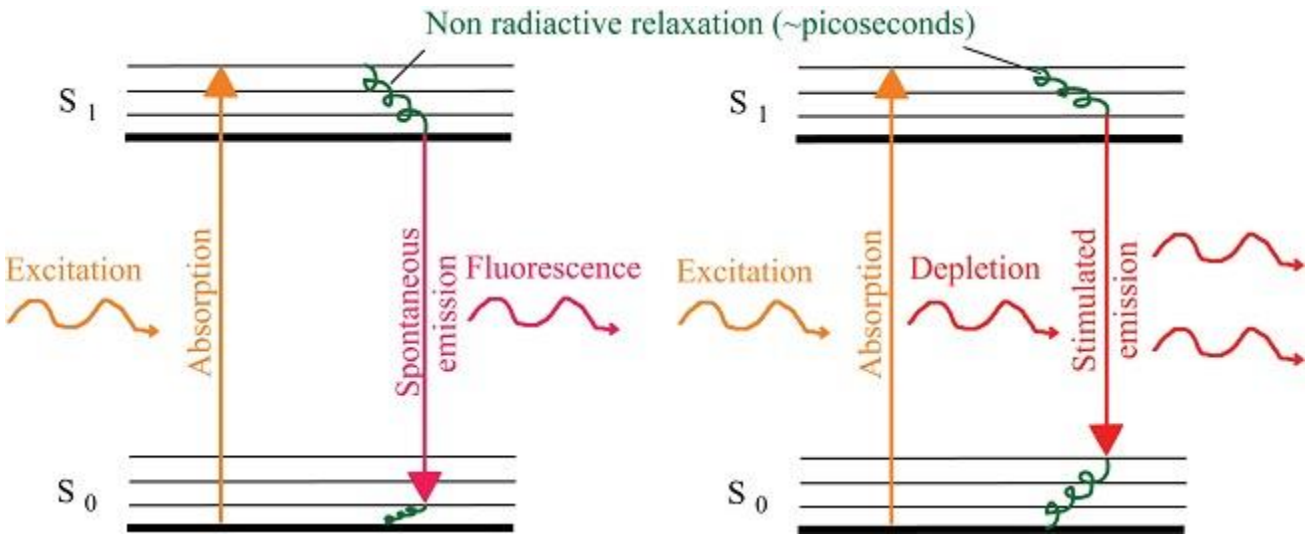
Web-site: www.imm.ox.ac.uk/wolfson-imaging-centre-oxford


Non radiactive relaxation (~picoseconds)
S 1
S 0
Excitation
Absorption
Spontaneous emission
Fluorescence
S 1
S 0
Excitation
Absorption
Depletion
Stimulated emission