STED & STED-FCS

Dr. B. Christoffer Lagerholm
Facility Manager
Wolfson Imaging Centre - Oxford
Weatherall Institute of Molecular Medicine,
University of Oxford,
John Radcliffe Hospital

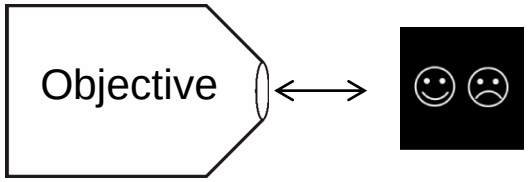
christoffer.lagerholm@imm.ox.ac.uk

Weatherall Institute of Molecular Medicine

Live Cell Microscopy

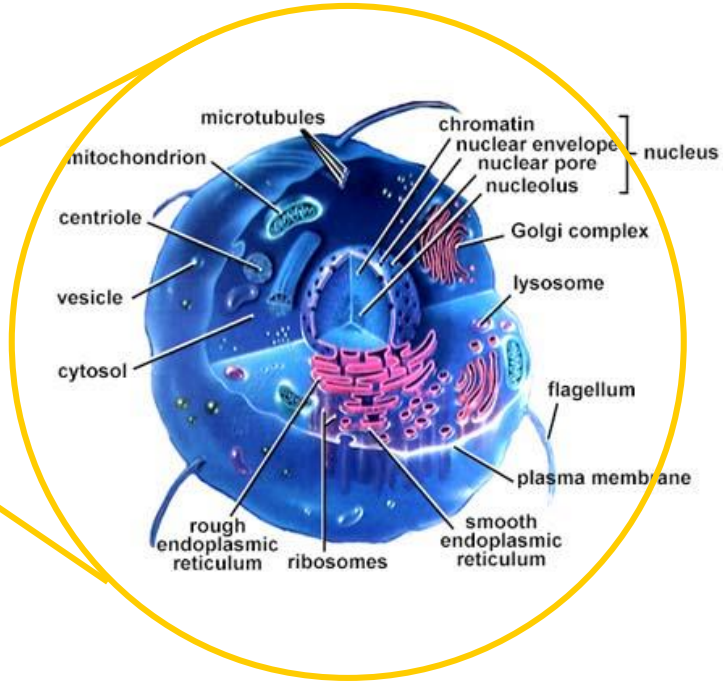
Observation of living cells: Non-Invasive

Light + Far-Field: non-invasive!



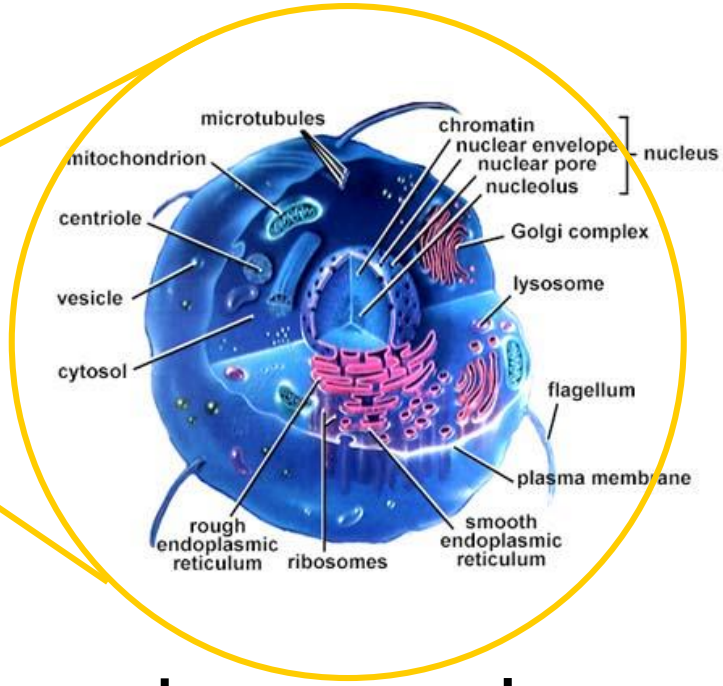
Far-Field

> 1 µm



Far-Field Fluorescence Microscopy

Resolution: Goal

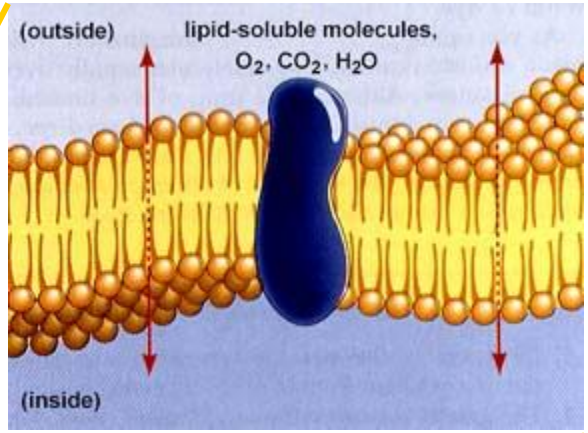


10 µm

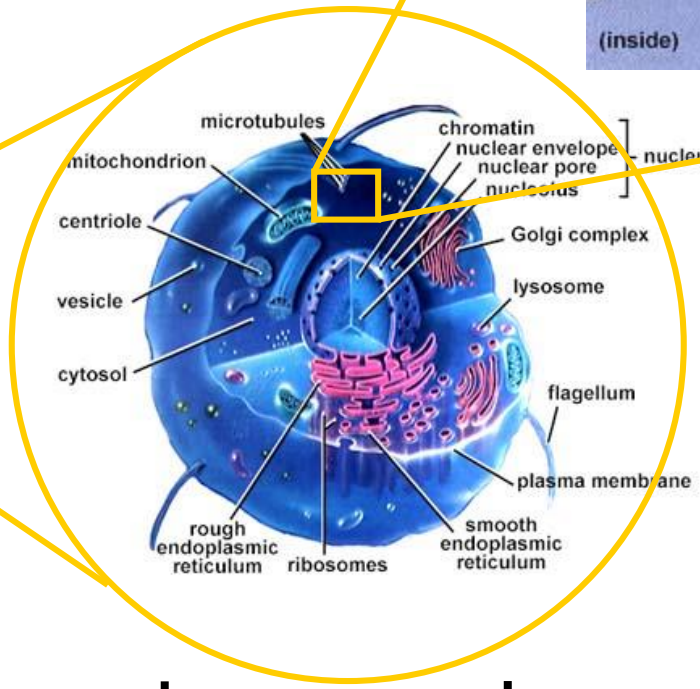
Far-Field Fluorescence Microscopy

Resolution: Goal

⇒ **molecular scale**



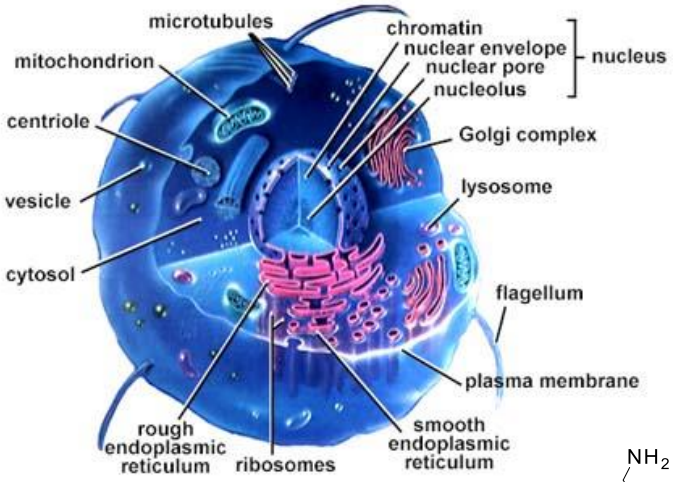
10 nm



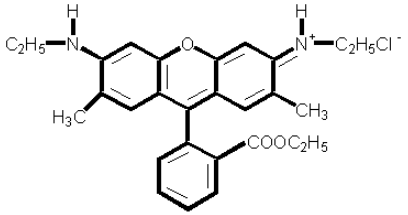
10 µm

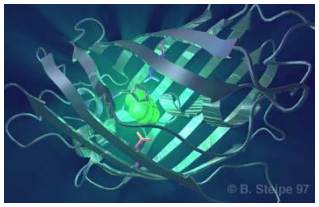
Live Cell Far-Field Microscopy

Fluorescence



Fluorescence-Labeling!

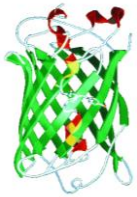


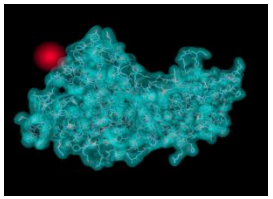


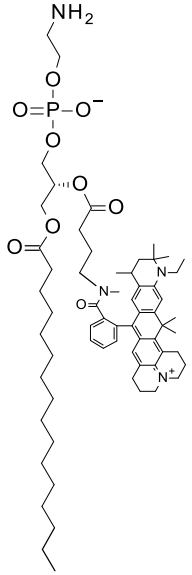
... from chemists

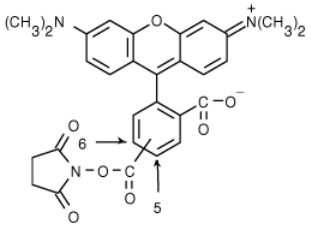
... from biology

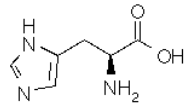
(proteins)



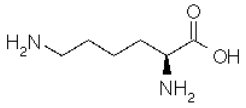




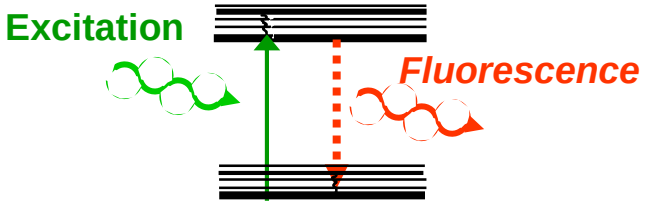


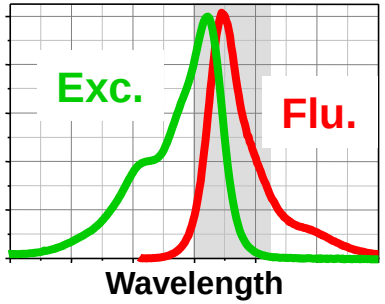


his h Histidin



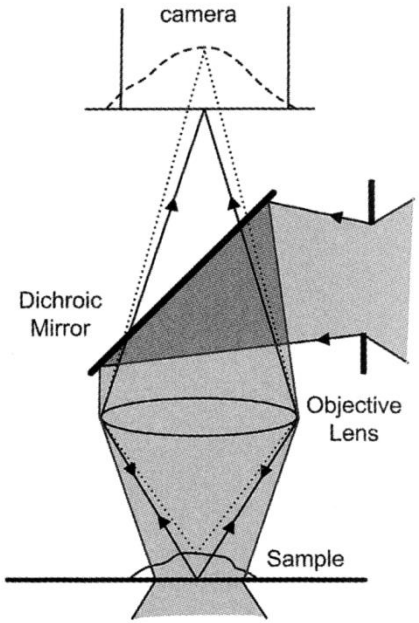
lys k Lysin

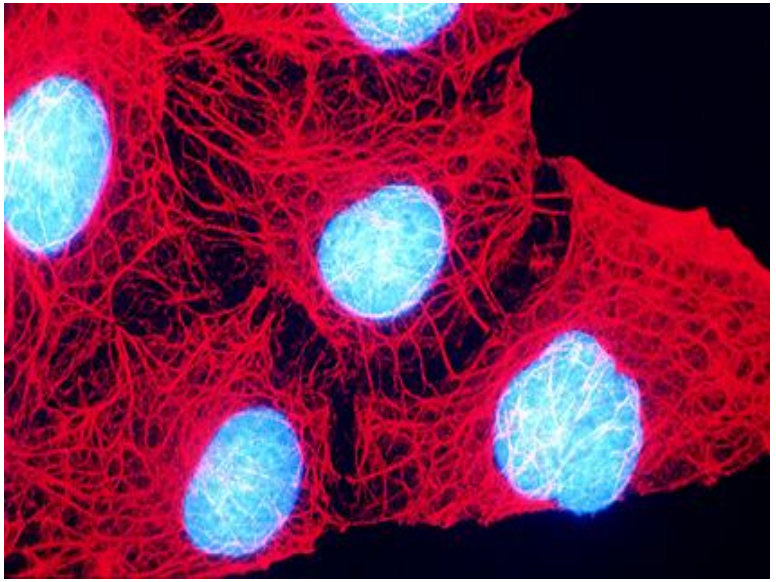




Camera-Based Far-Field Microscopy

Wide-Field Setup



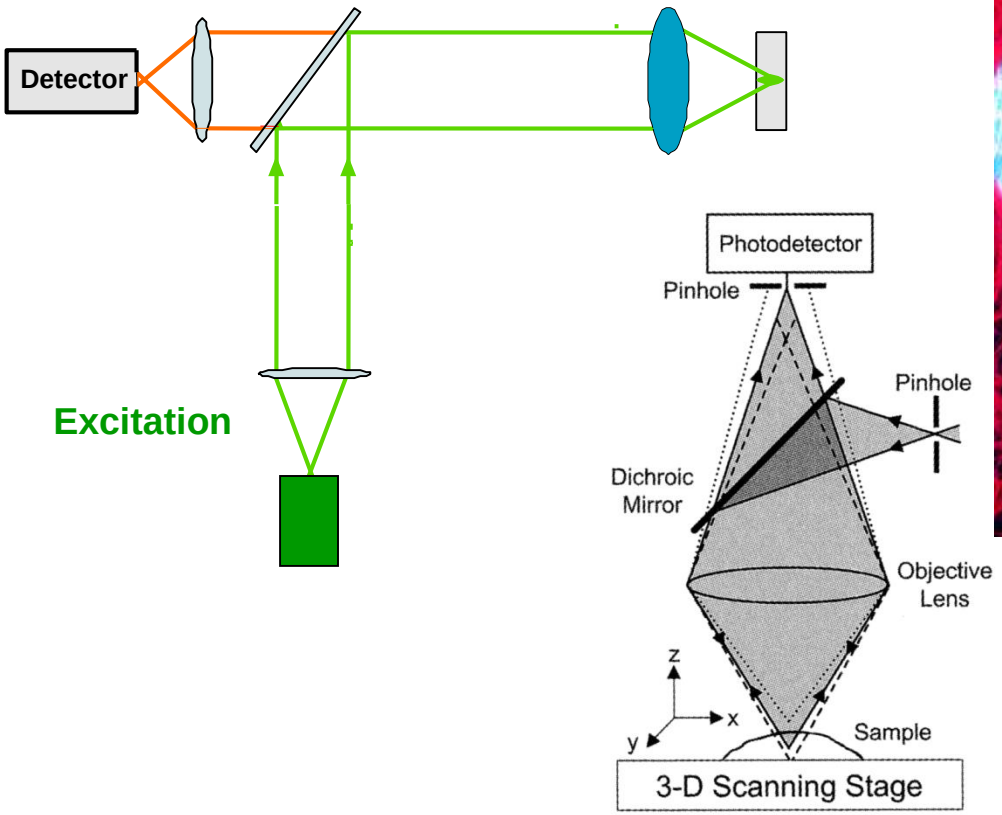


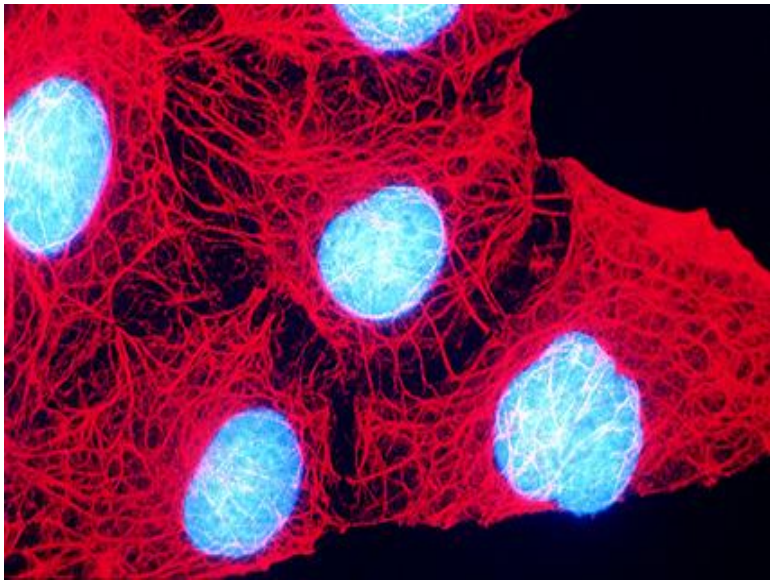
Liver-Cells: Nucleus and **Cell-skeleton**

- Large area illuminated
- **Camera detection**:
  image taken in one step

Far-Field Fluorescence Microscopy

Confocal Setup





Liver-Cells: Nucleus and **Cell-skeleton**

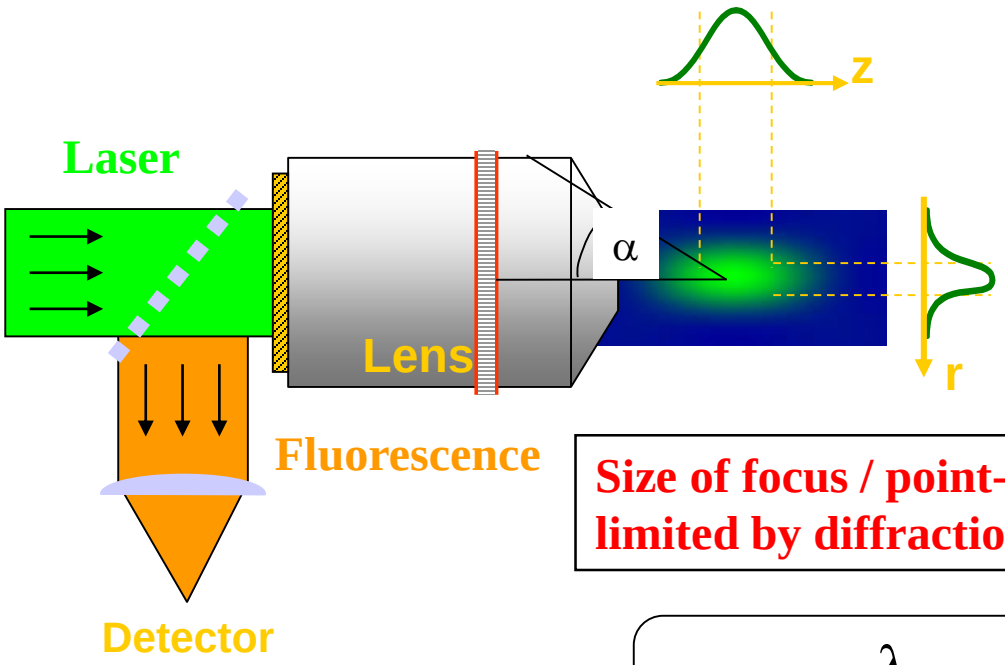
- Small area illuminated
- **Point detection**: **scanning** required to construct image
- **Confinement along z** (pinhole)

Far-Field Microscopy

Resolution Limit: Diffraction Barrier

Far-Field Fluorescence Microscopy: Focussing of light

- away from surfaces – inside cells (3D)



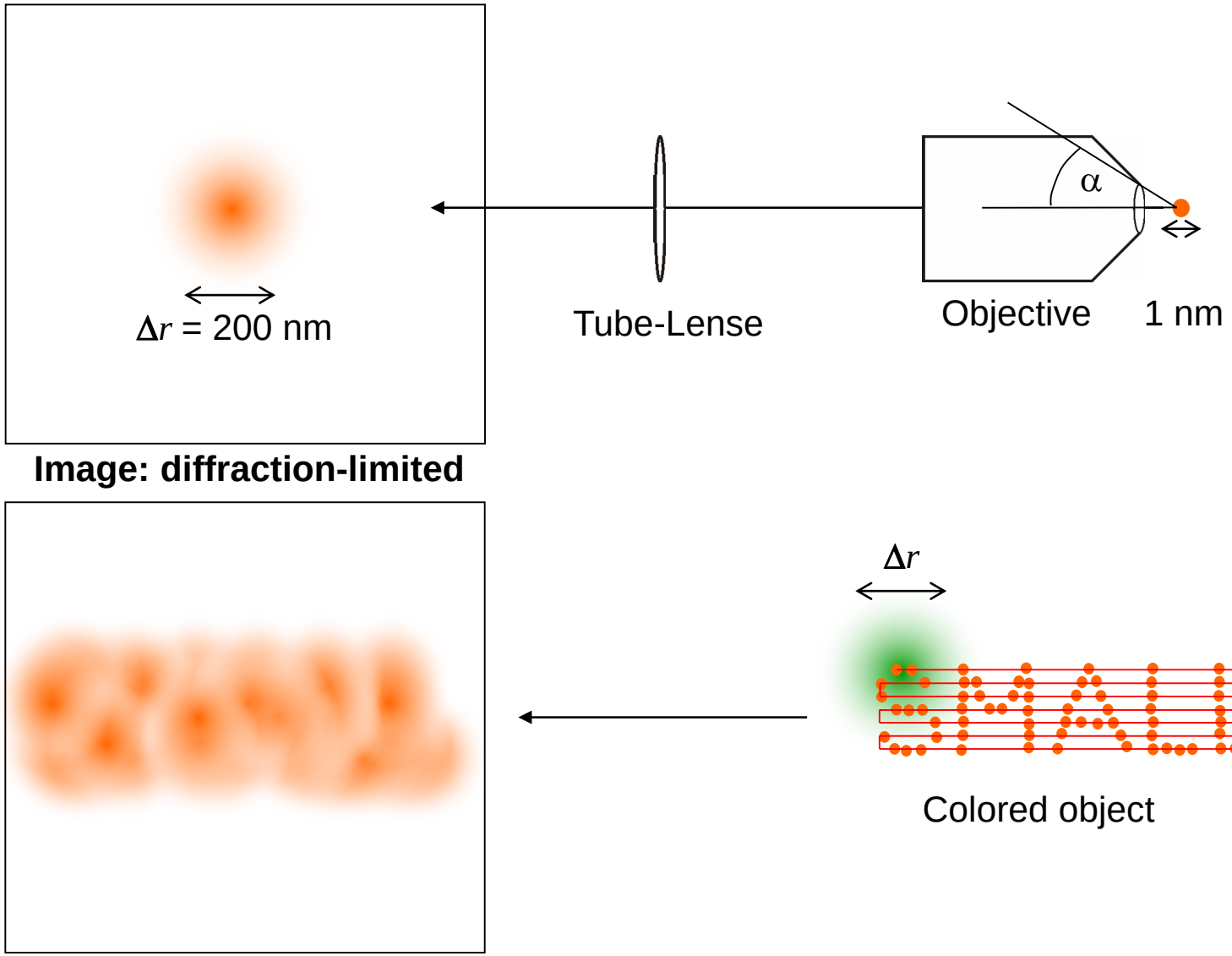
Size of focus / point-spread function limited by diffraction of light!!!

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

Ernst Abbe 1873

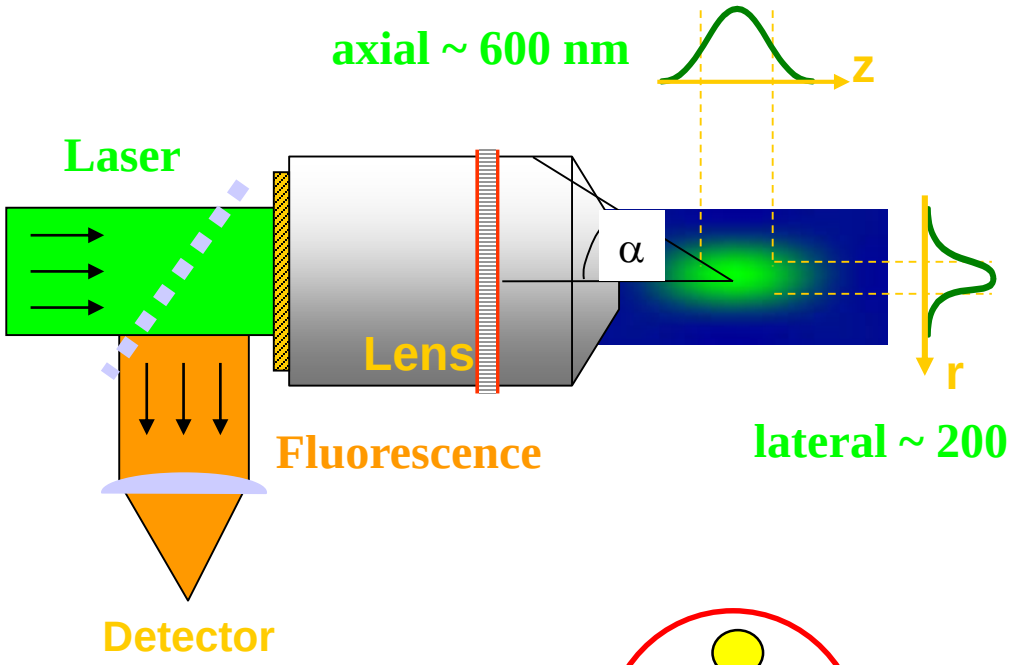
Far-Field Microscopy

Resolution Limit: Diffraction Barrier



Far-Field Microscopy

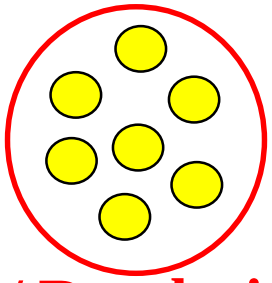
Surpassing the Resolution Limit: Turning ON/OFF



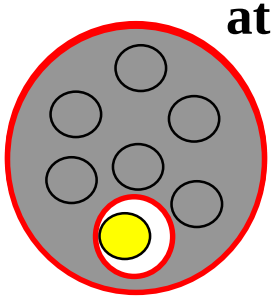
$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$

Ernst Abbe 1873

⇒ **Turning ON/OFF**



Turn OFF all objects but at a single point



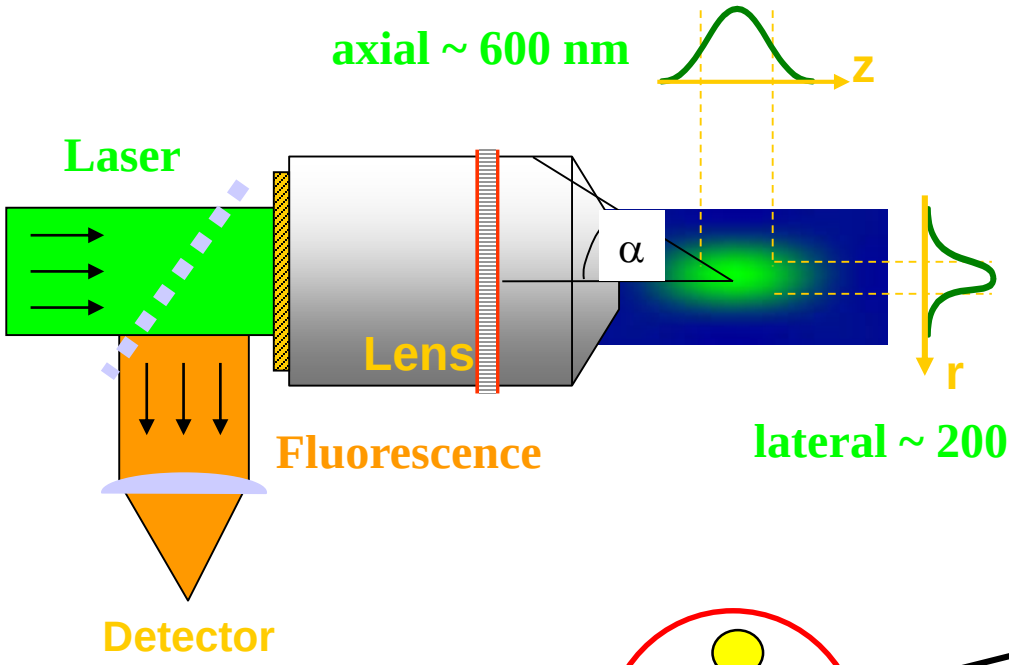
⇒ **Observation area / Resolution**

lateral << 200 nm

axial << 600 nm

Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF

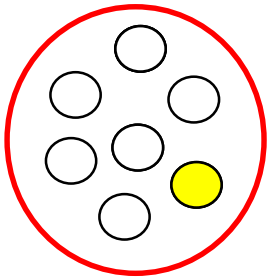


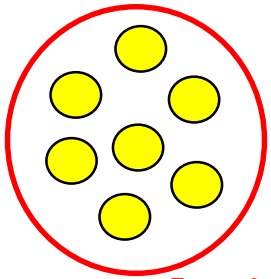
$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$

Ernst Abbe 1873

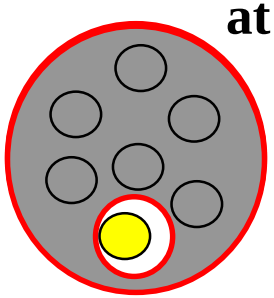
⇒ Turning ON/OFF

Turn OFF all but a single object





Turn OFF all objects but at a single point



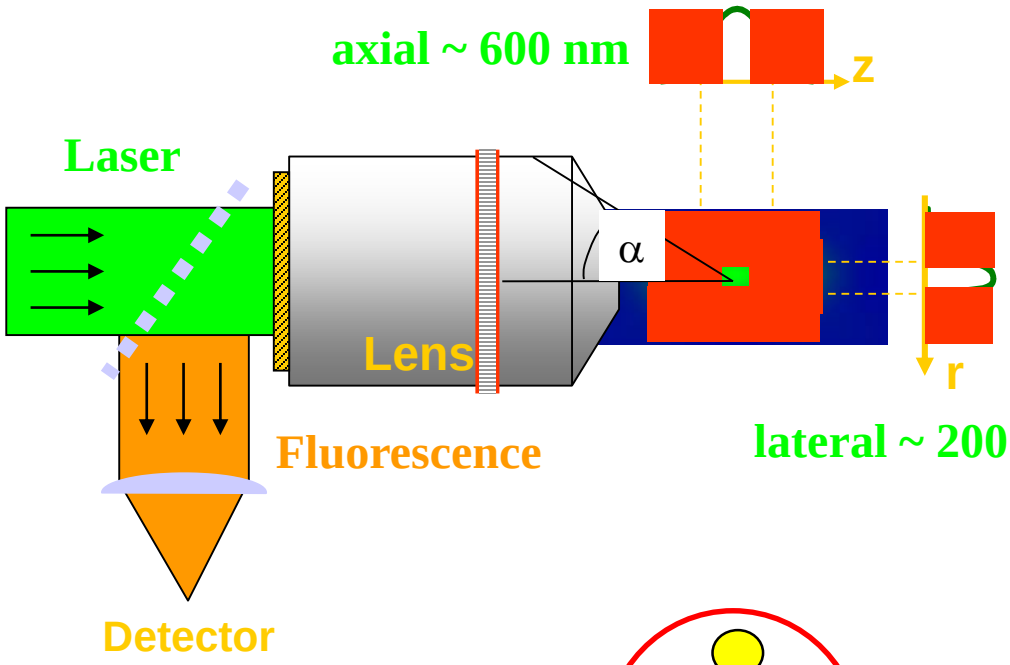
⇒ Observation area / Resolution

lateral << 200 nm

axial << 600 nm

Far-Field Microscopy

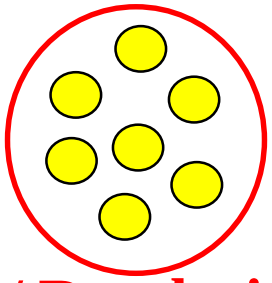
Surpassing the Resolution Limit: Turning ON/OFF



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

Ernst Abbe 1873

⇒ Turning ON/OFF

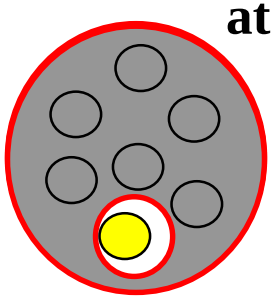


⇒ Observation area / Resolution

lateral << 200 nm

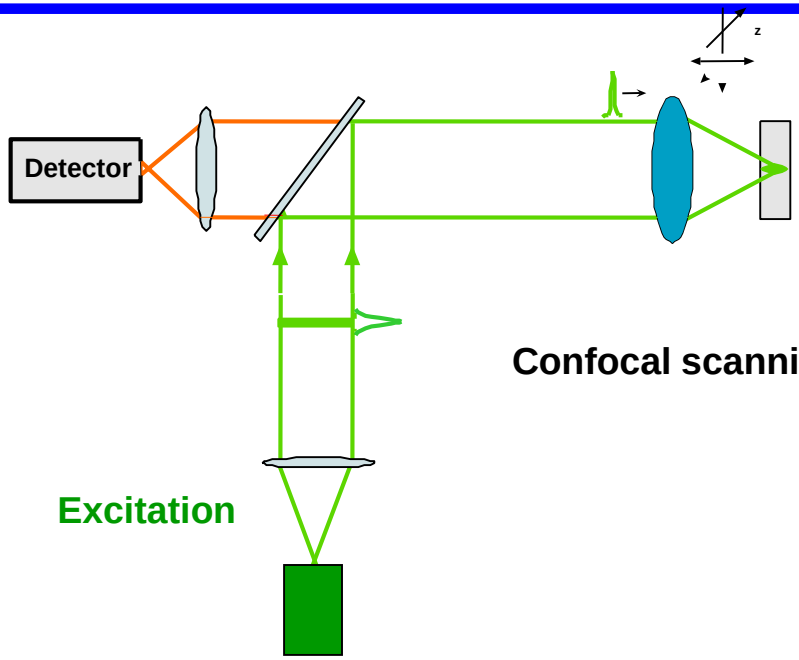
axial << 600 nm

Turn OFF all objects but at a single point

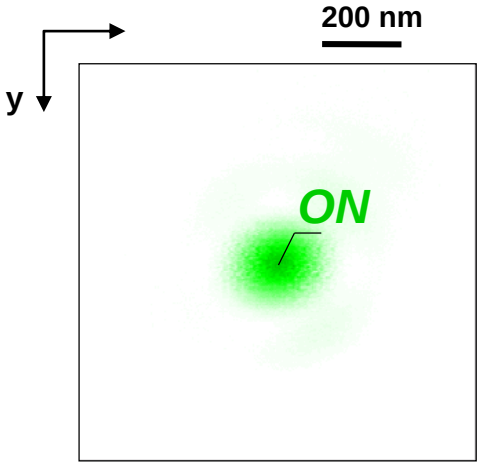


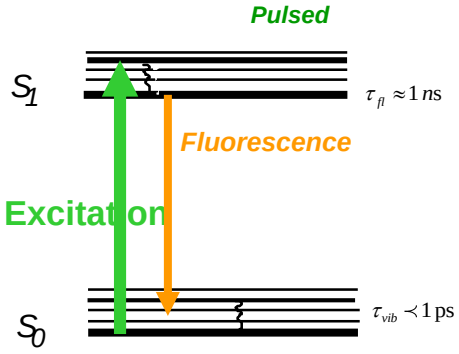
Fluorescence Microscopy

STED Microscopy



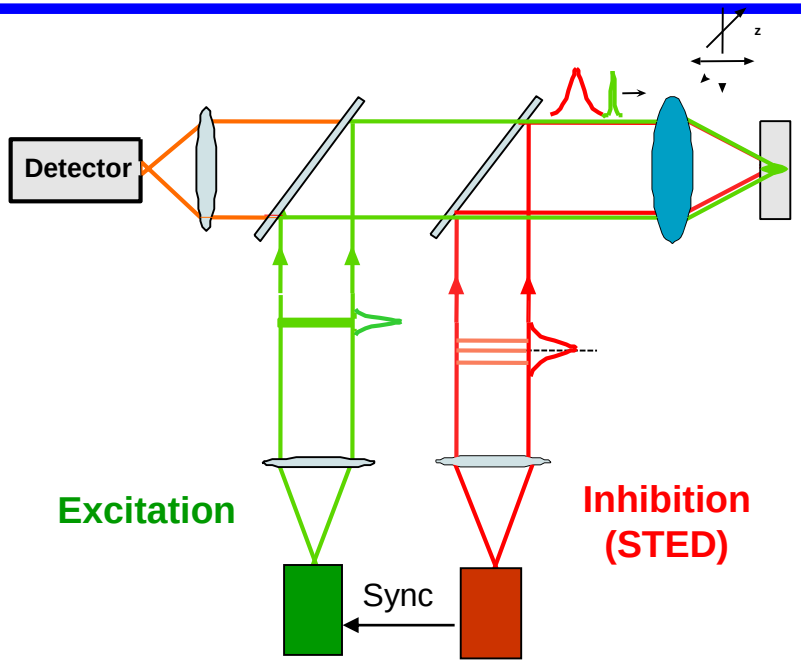
Confocal scanning microscope

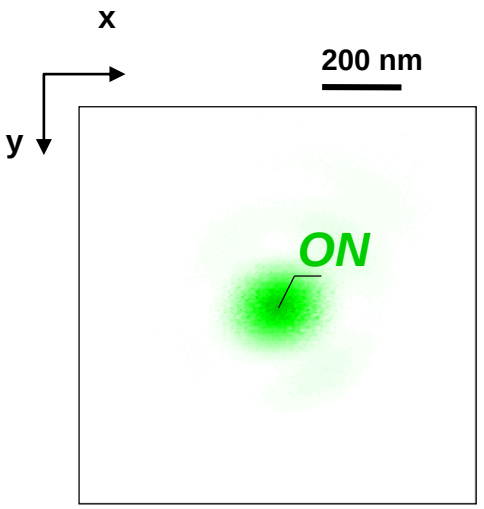


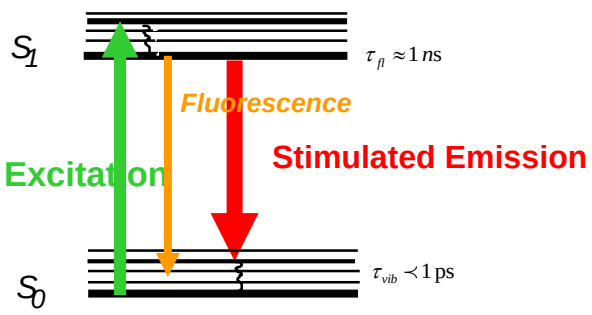


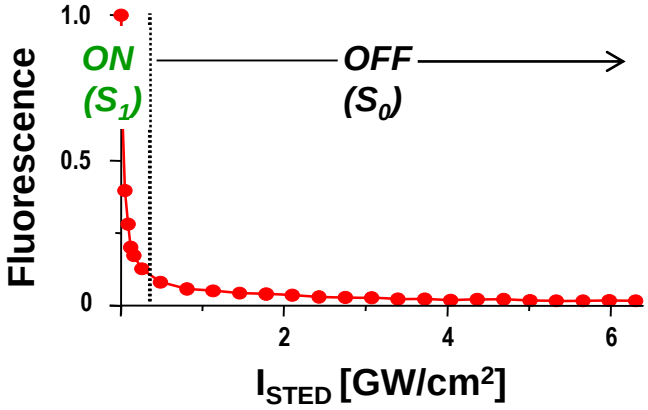
Fluorescence Microscopy

STED Microscopy



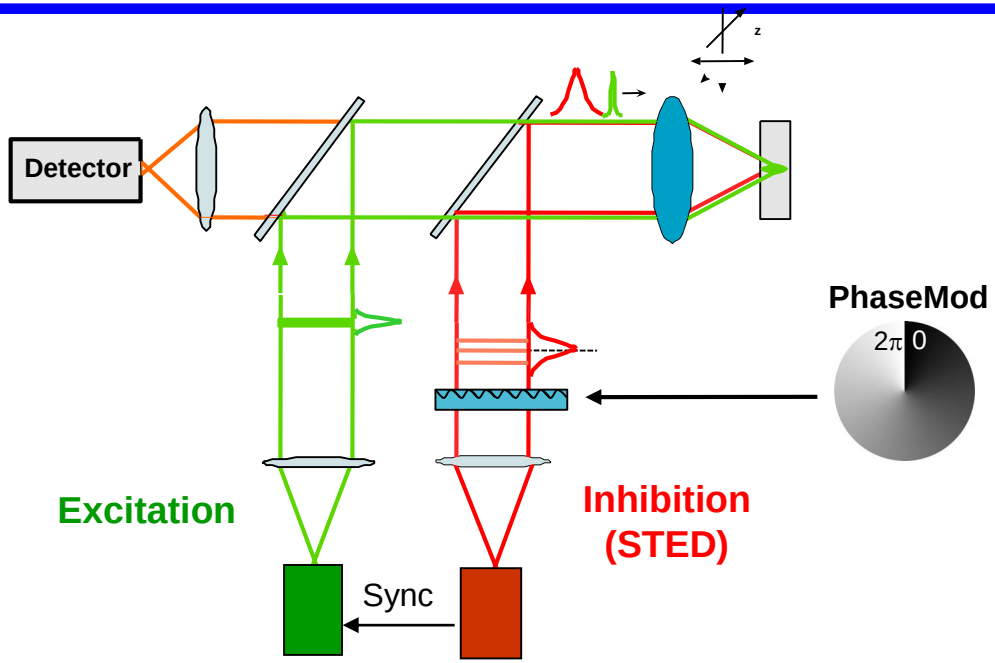


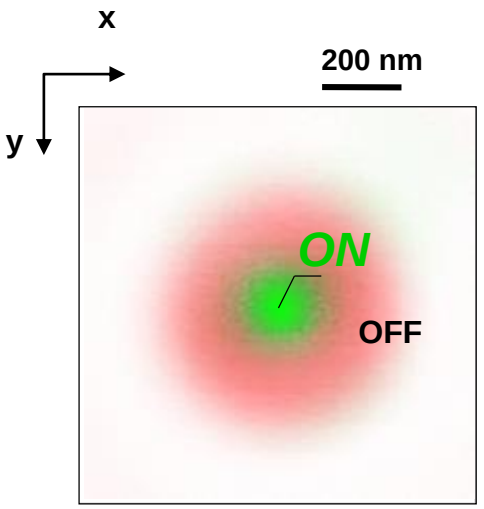


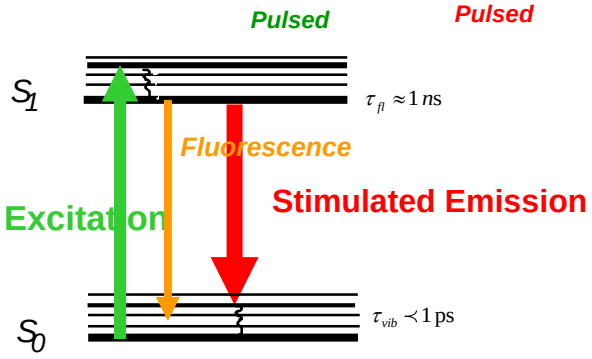


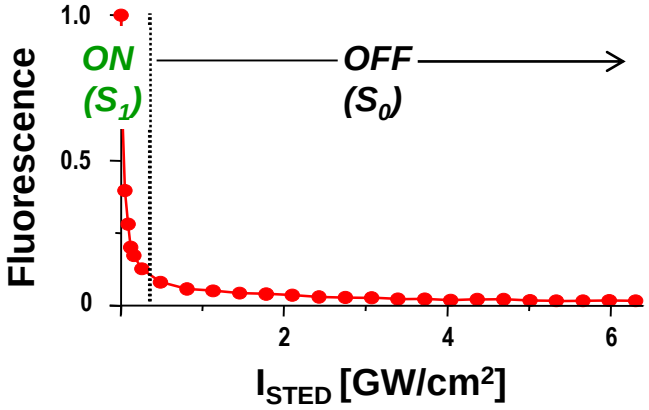
Fluorescence Microscopy

STED Microscopy



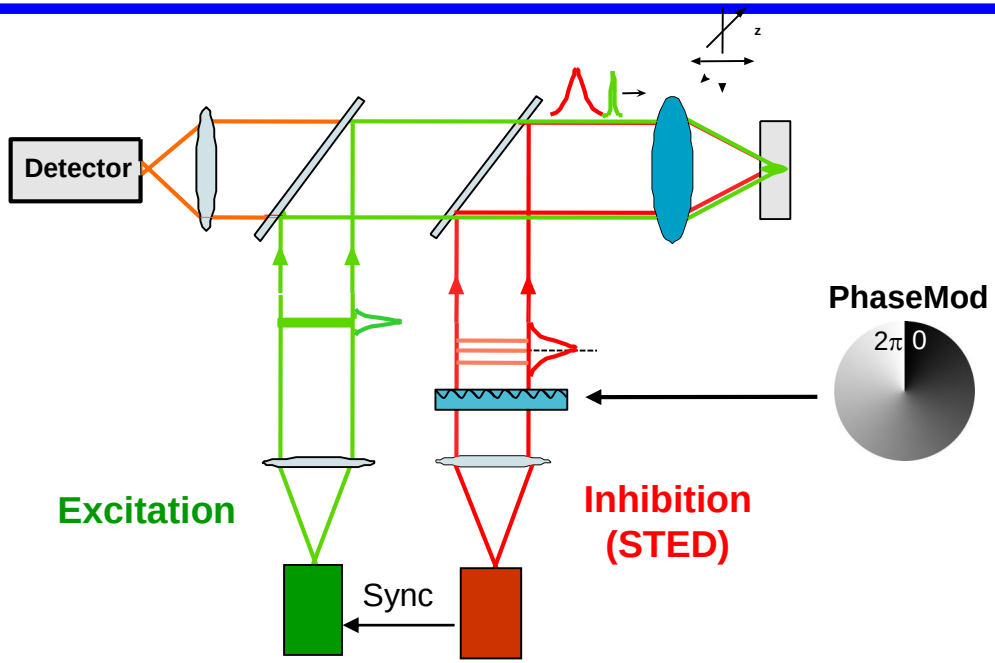


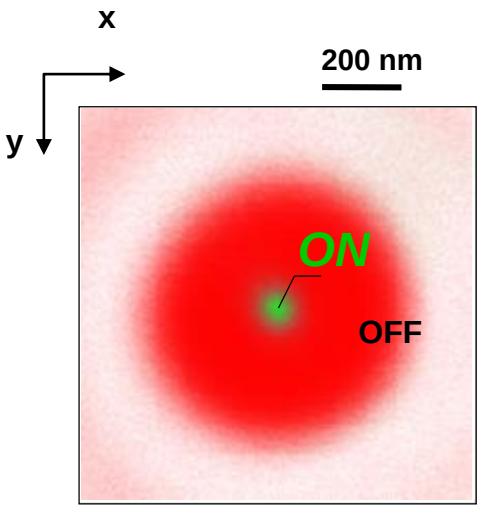


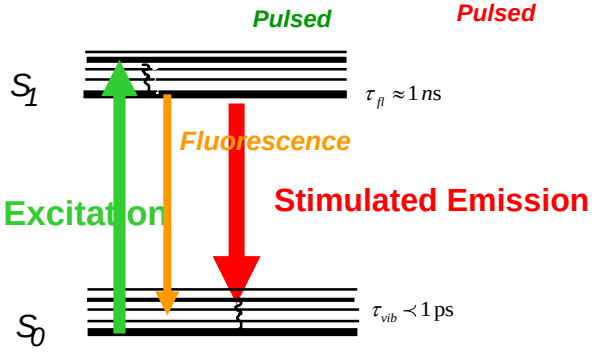


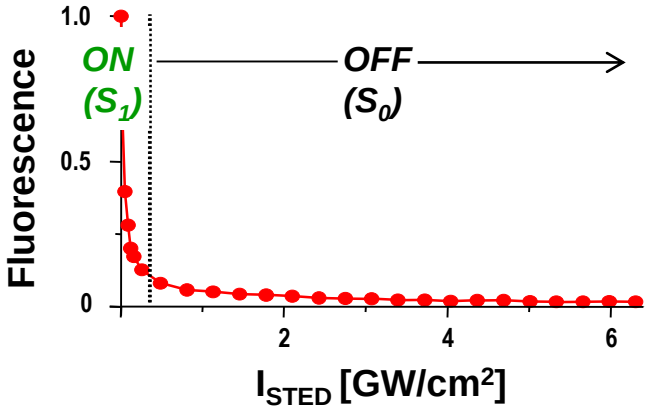
Fluorescence Microscopy

STED Microscopy





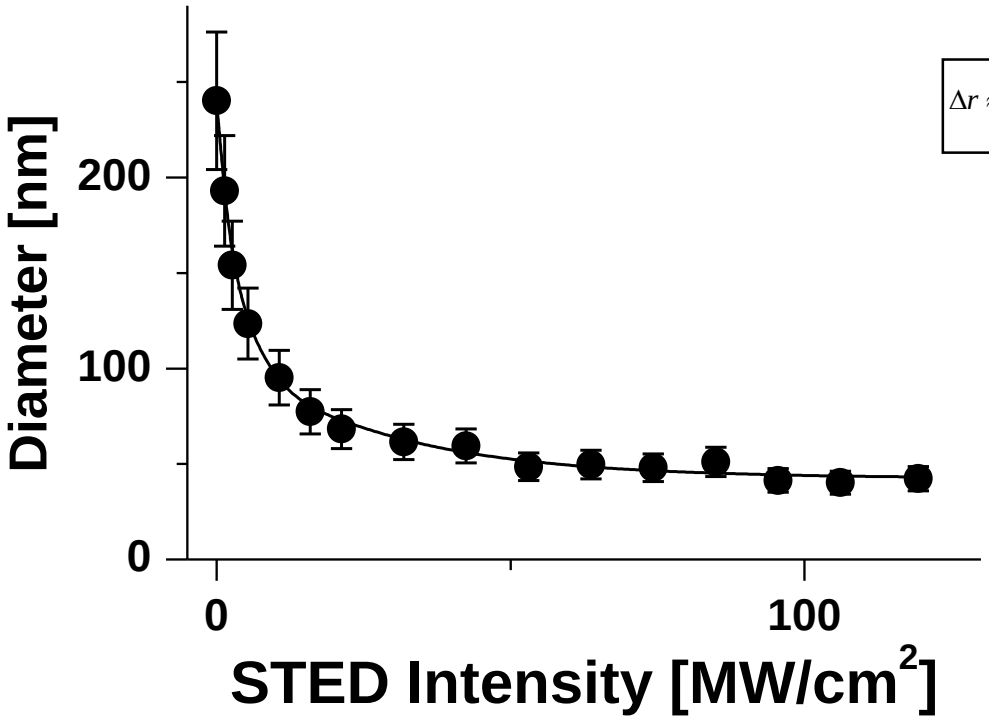




STED Microscopy

Dynamical confinement of resolution

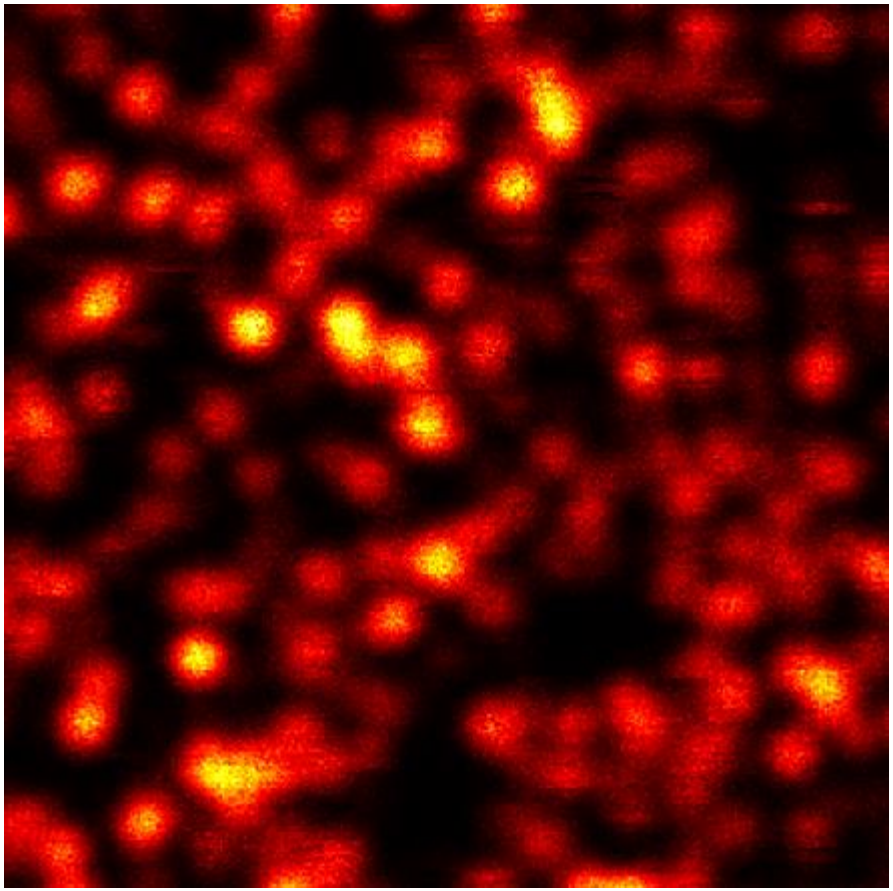
Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!

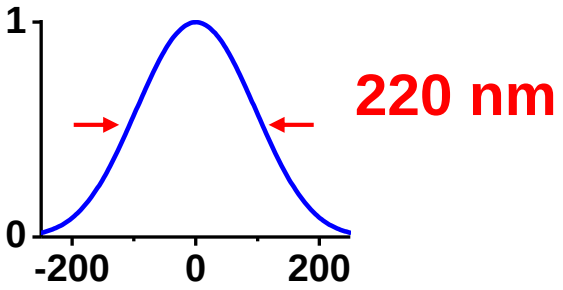


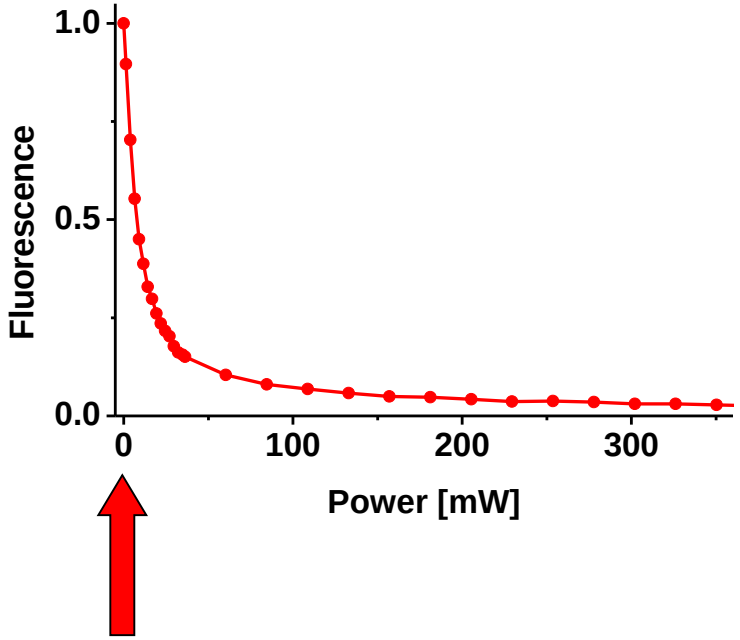
$$\Delta r \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+I/I_{sat}}}$$

STED-Microscopy

Sub-Diffraction Imaging



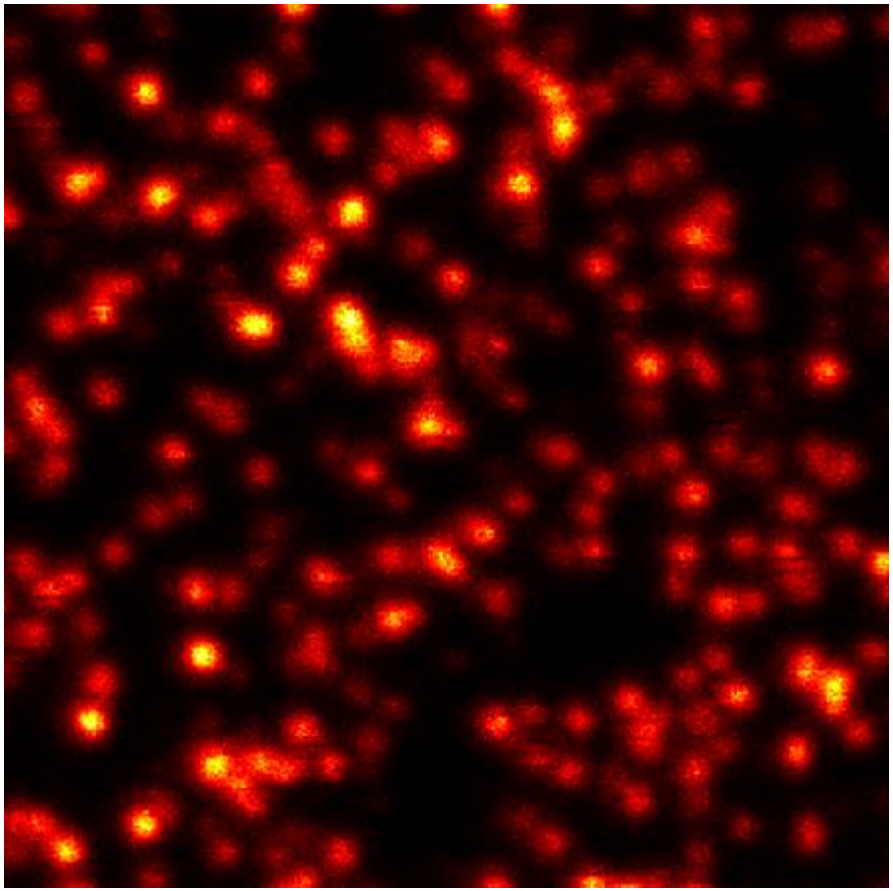


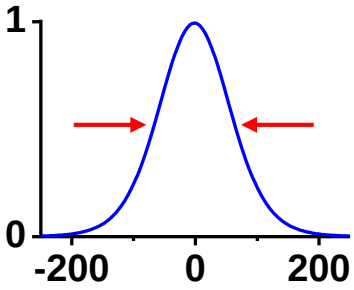


20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

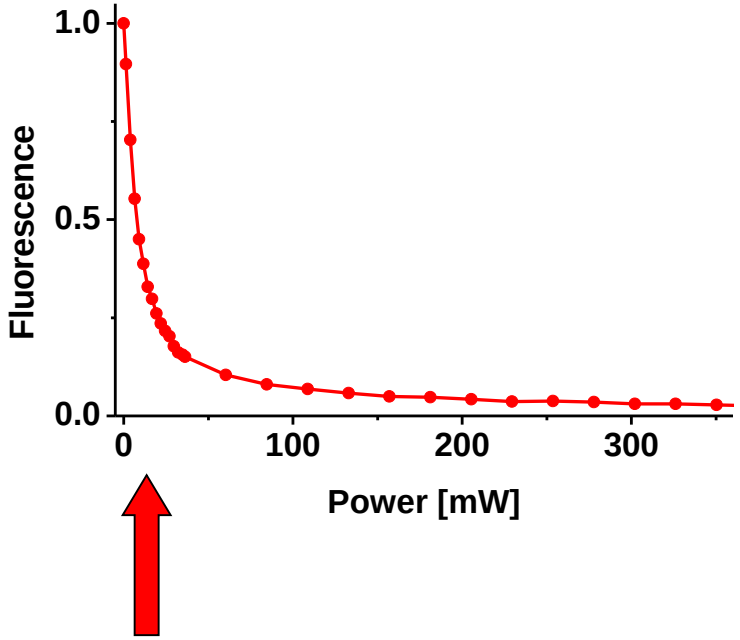
STED-Microscopy

Sub-Diffraction Imaging





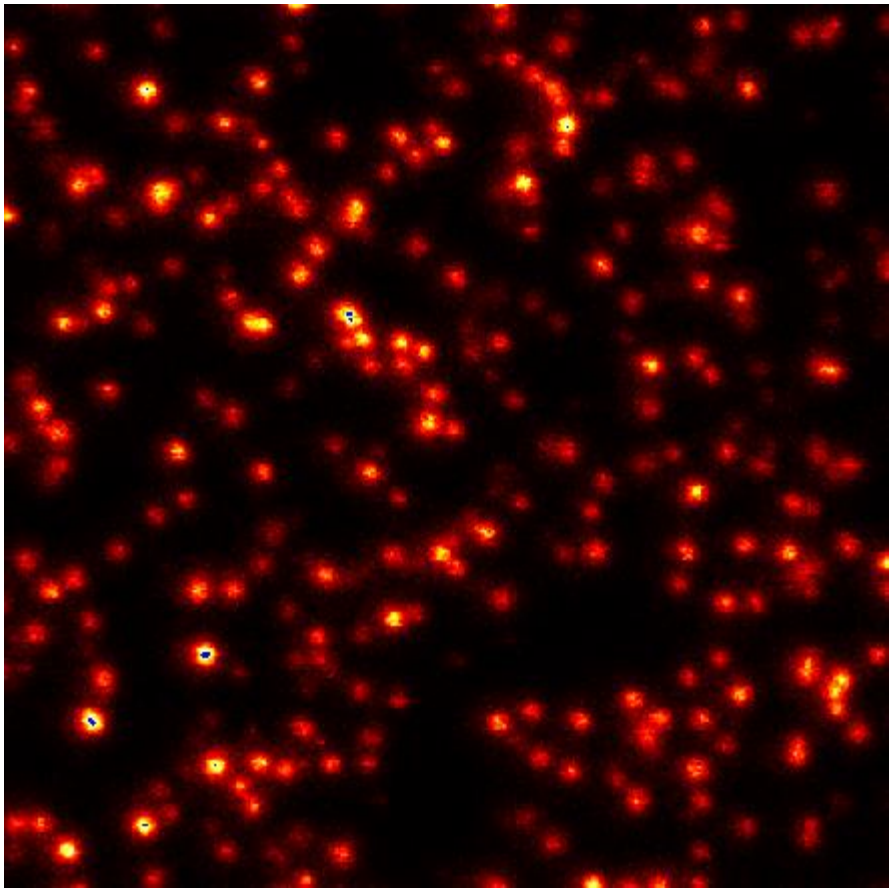
132 nm

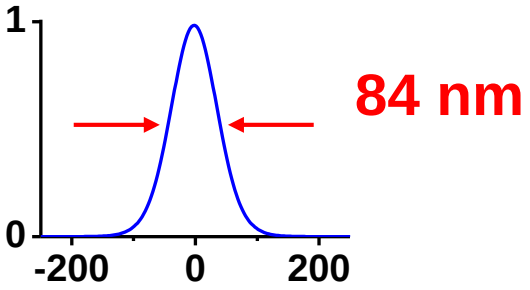


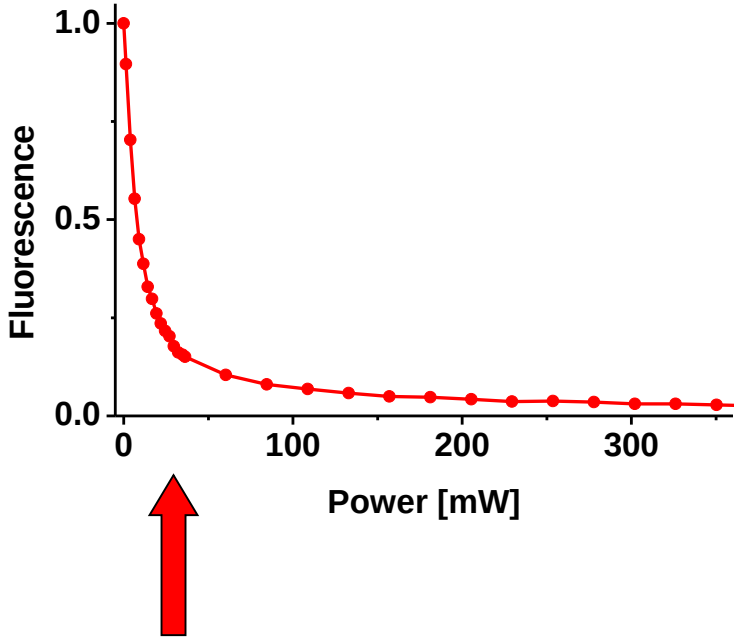
20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging



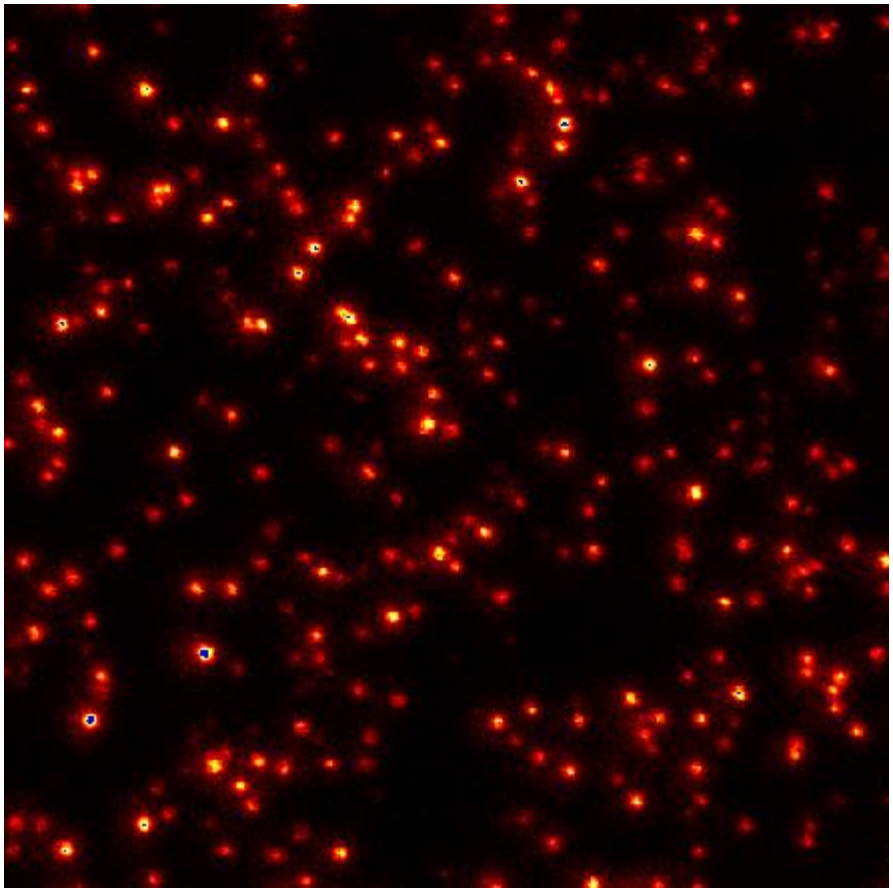


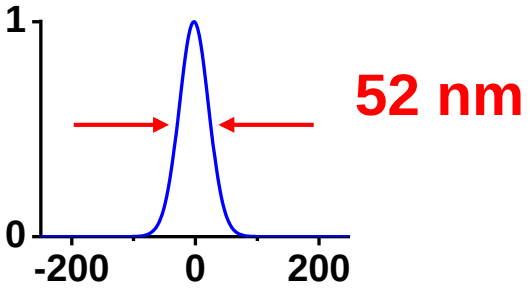


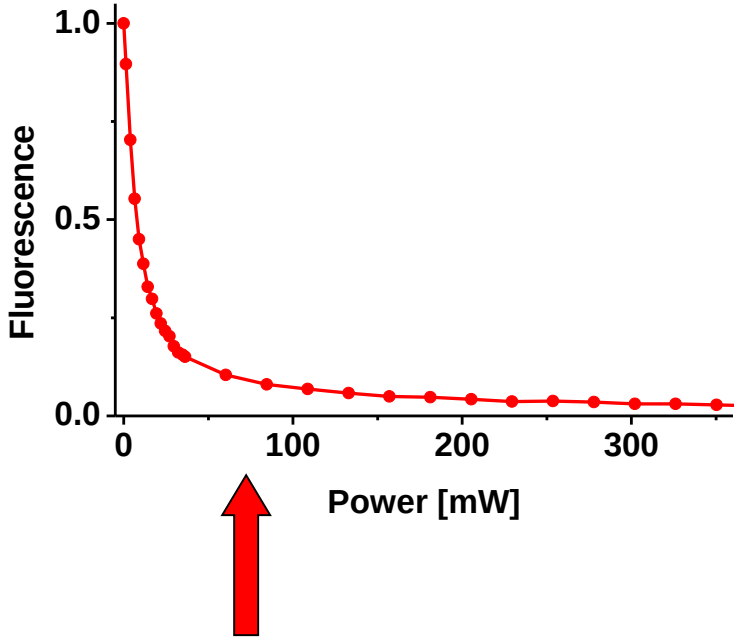
20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging



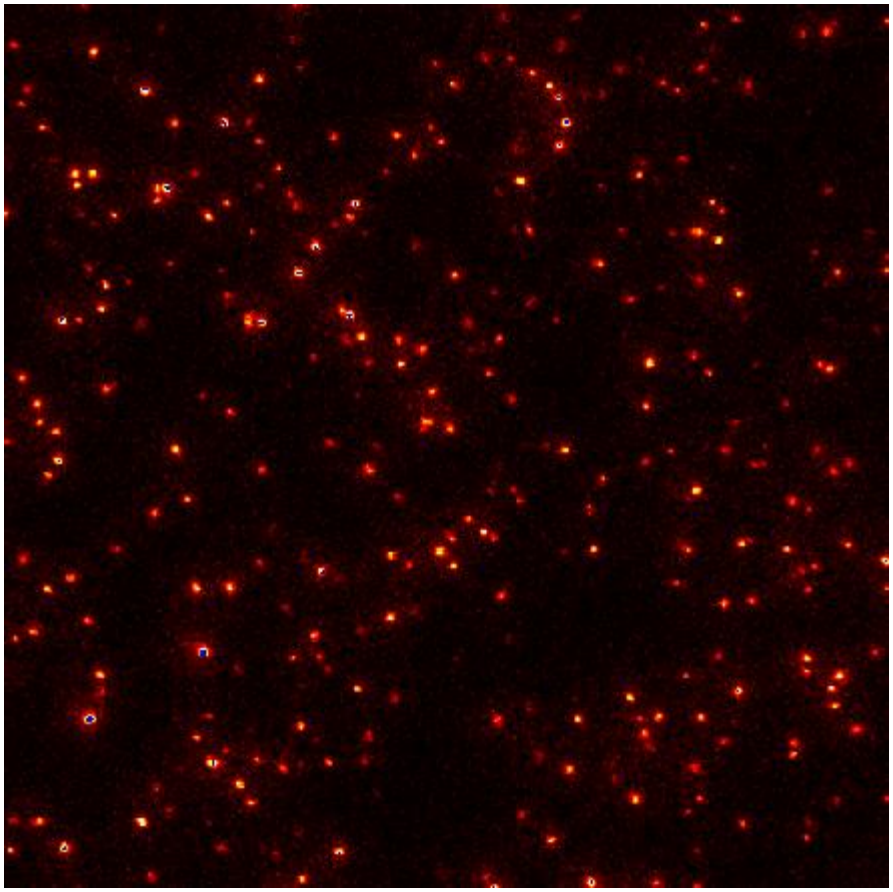


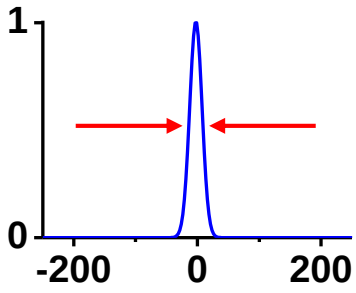


20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

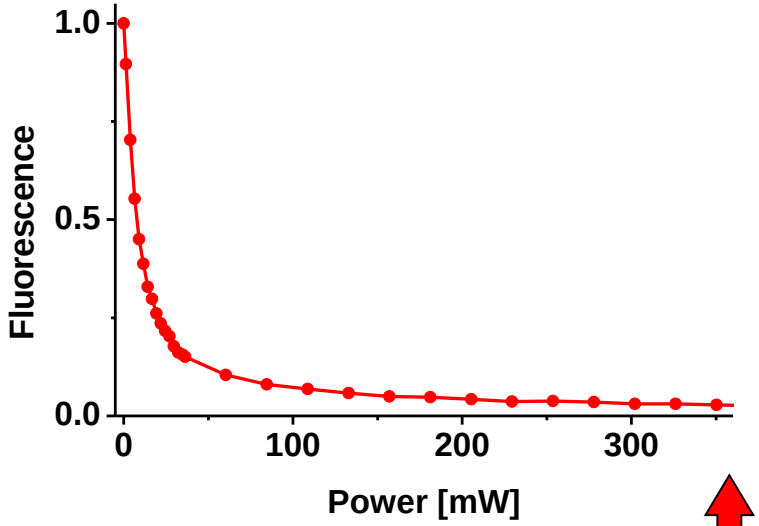
STED-Microscopy

Sub-Diffraction Imaging





22 nm



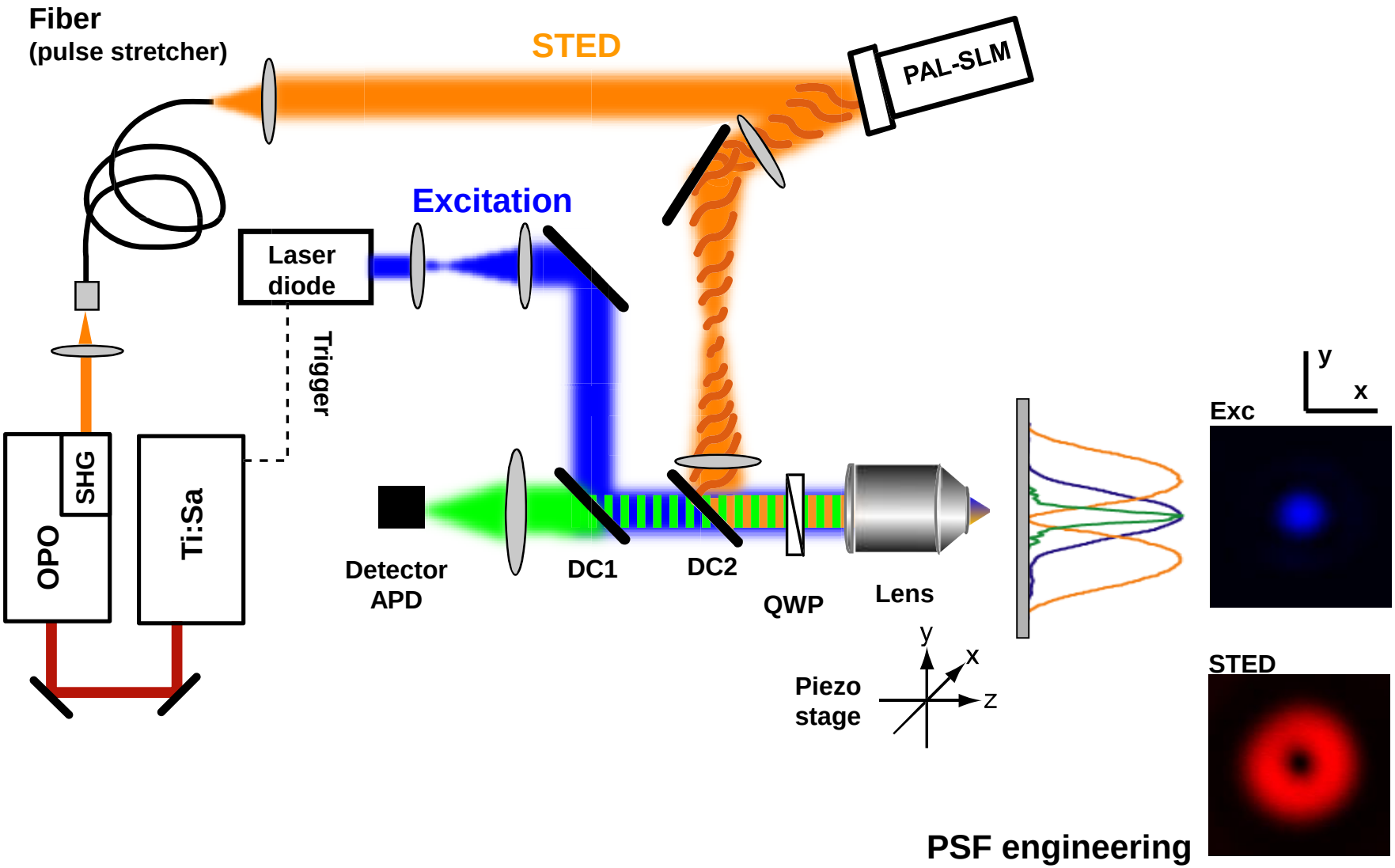
20nm Crimson beads

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

785nm STED 200ps, 76MHz

STED-Microscopy

Setup



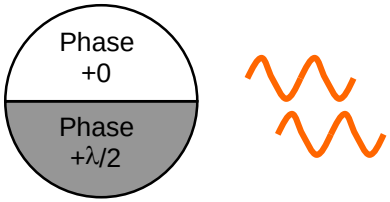
PSF engineering

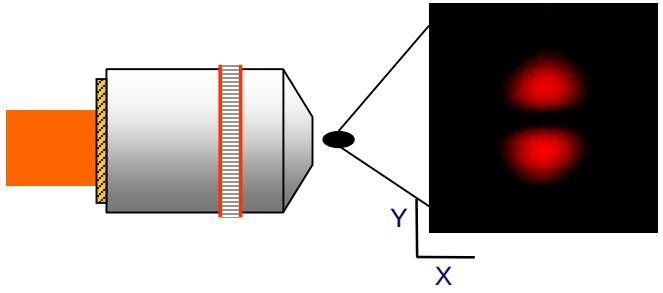
Focal Volume Confinement

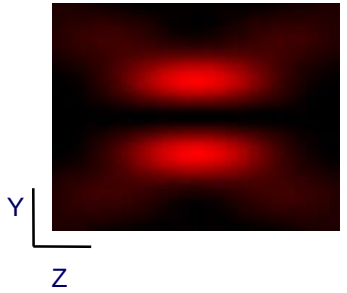
Focal Engineering – Local Zero

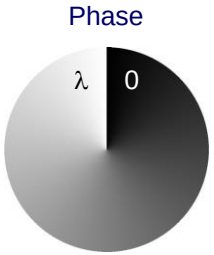
Phase mask:

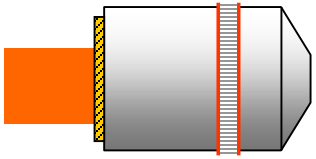
STED PSF:

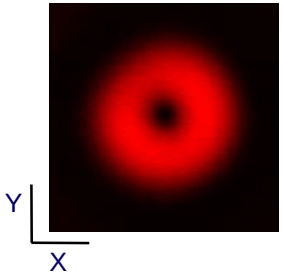


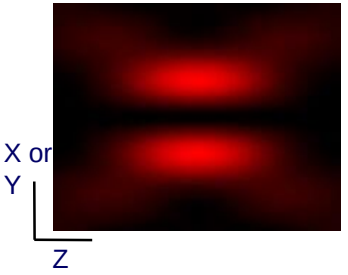


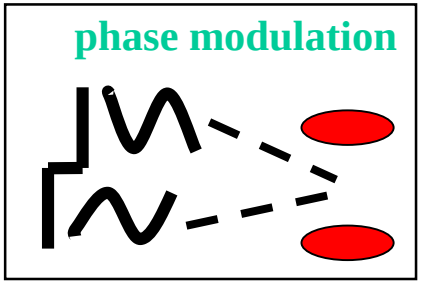










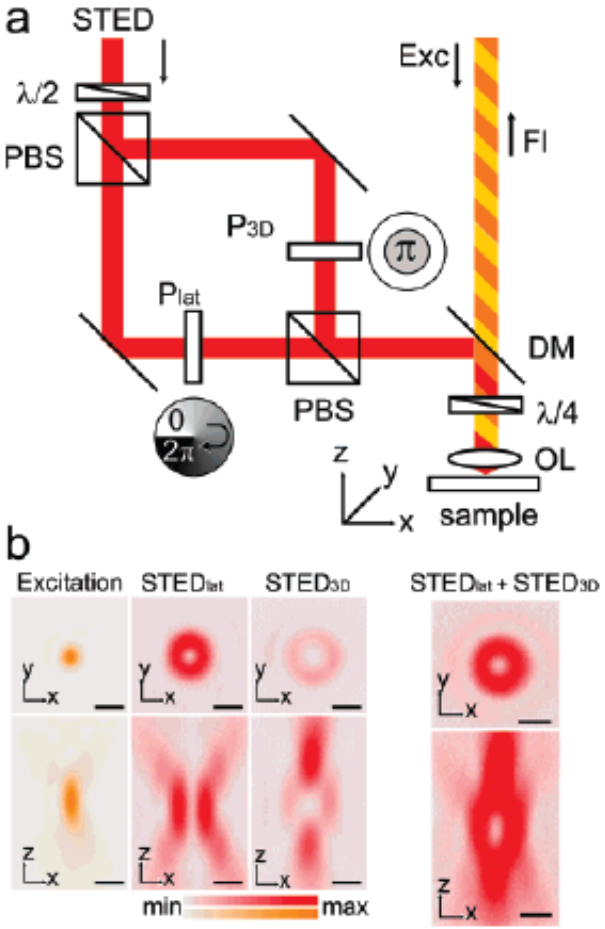


Klar et al, PNAS 2000

STED-Microscopy

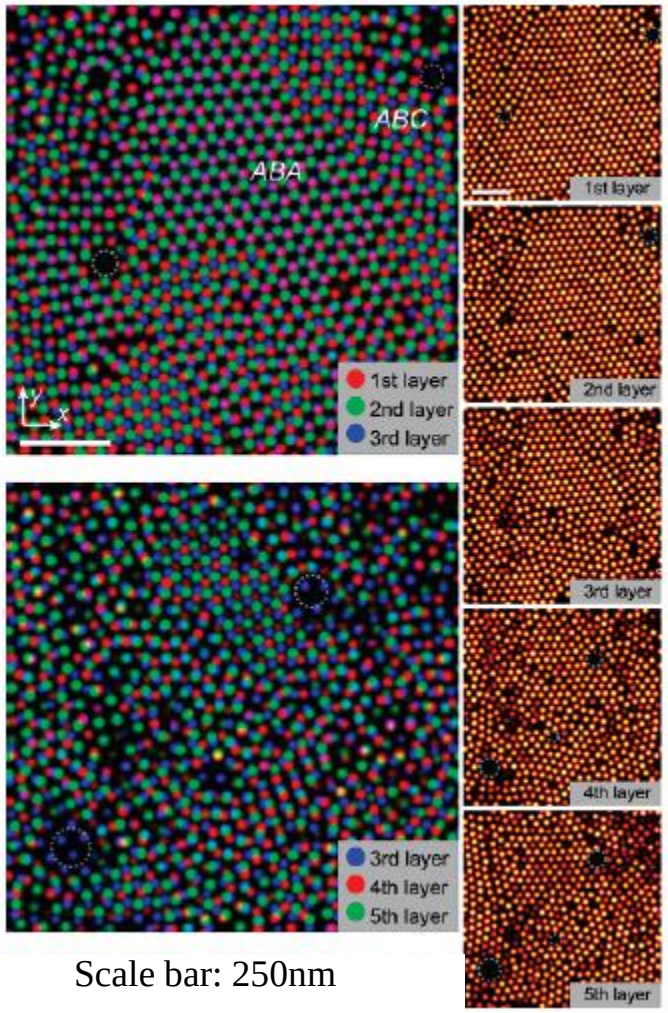
Sub-Diffraction Imaging – 3D

3D STED nanoscopy I



Harke / Ullal et al NanoLett 2008

Fluorescent 100nm Beads – multiple layers on cover glass

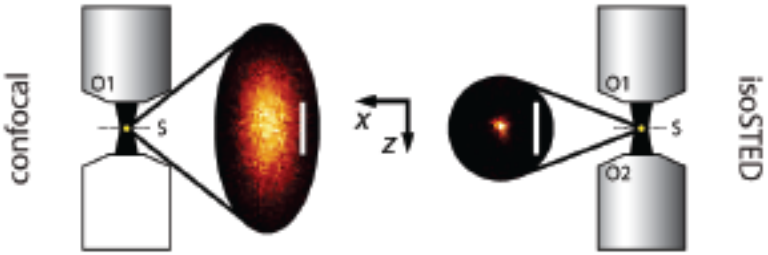


Scale bar: 250nm

STED-Microscopy

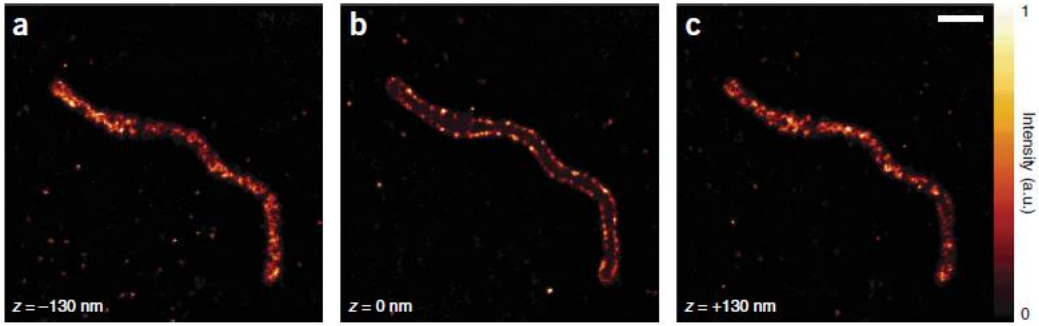
Sub-Diffraction Imaging – 3D

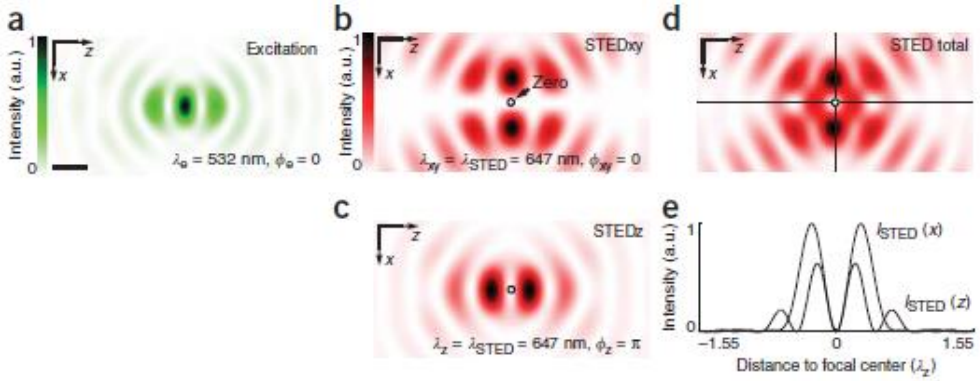
3D STED nanoscopy II – iso STED

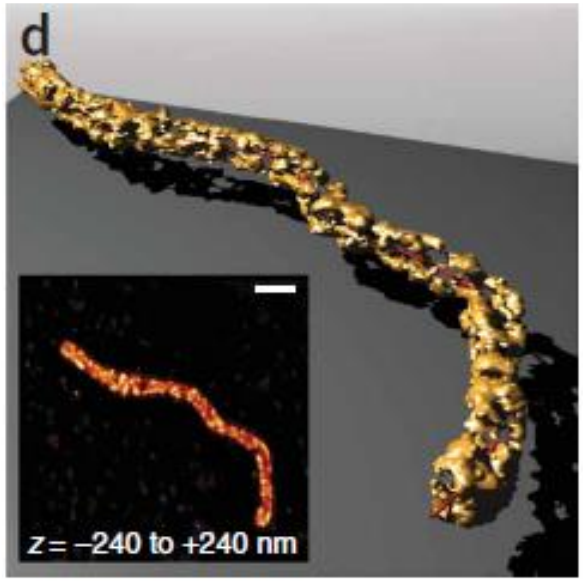


Scale bar: 250nm

Mitochondria in Vero cells:
outer membrane protein Tom20 (NK51, red)





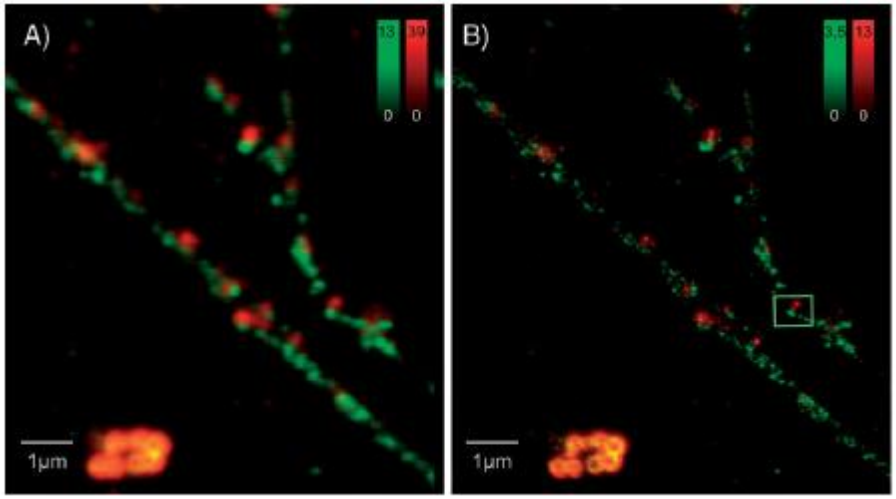


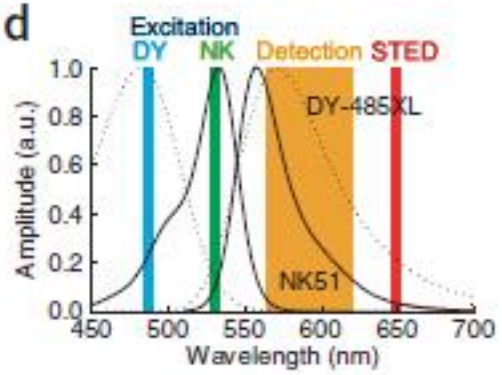
Scale bar: 1µm

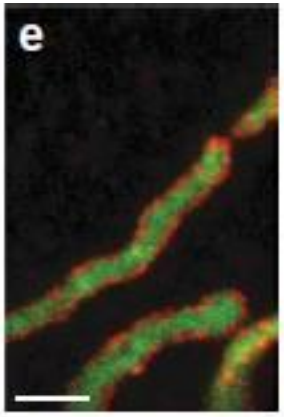
Schmidt et al Nat Methods 2009
Ullal /Schmidt et al NanoLett 2009

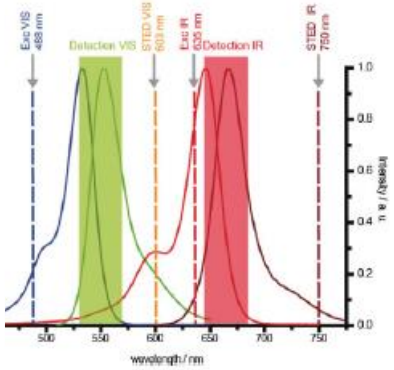
STED-Microscopy

Multi-Color Sub-Diffraction Imaging









4 laser lines:

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

Donnert et al BiophysLett 2006 / Meyer et al Small 2008

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

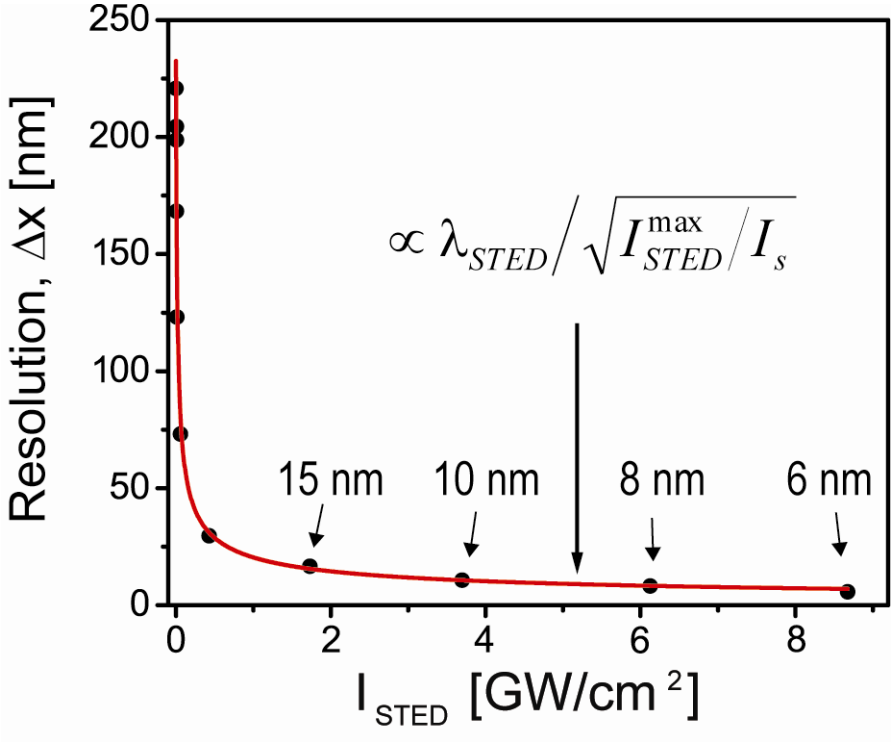
Fluorescence Nanoscopy

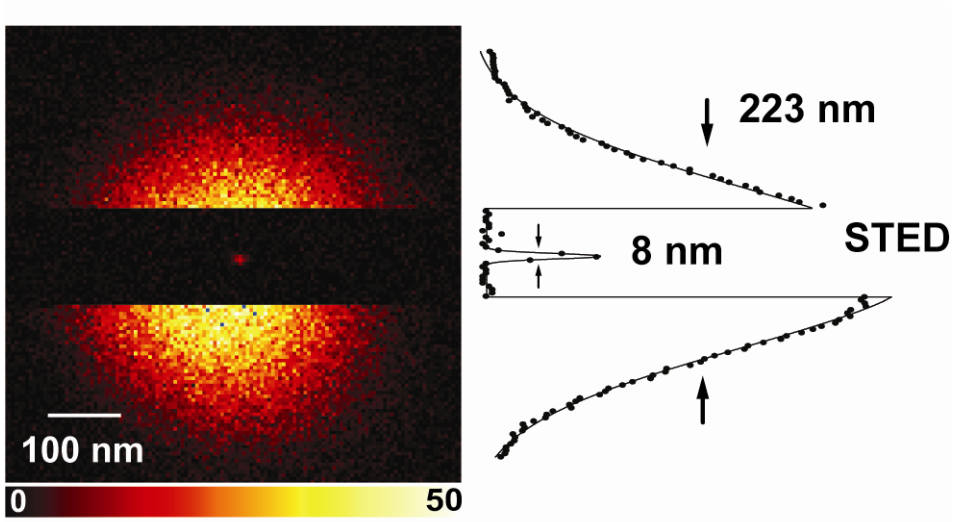
STED imaging on single NV centers

Ultrastable: apply very high STED intensities

→ Reach ultimate spatial resolutions!!!

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





I_{STED} = 3.5 GW/cm²

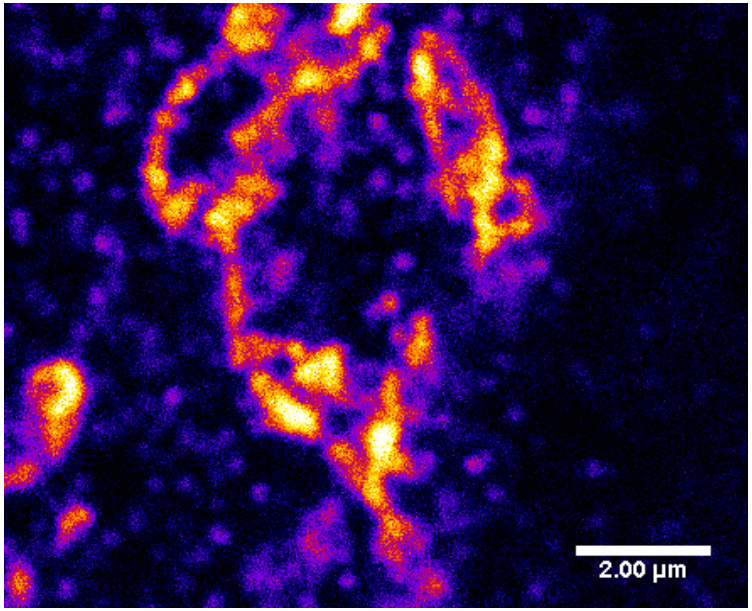
E. Rittweger, K.Y. Han et al, Nature Photonics 2009

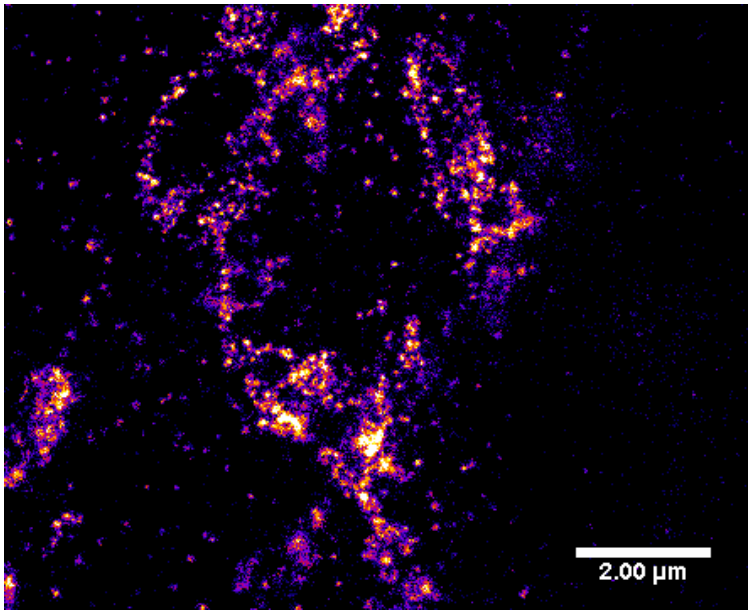
Commercial Leica SP8 gSTED Microscope at WIMM

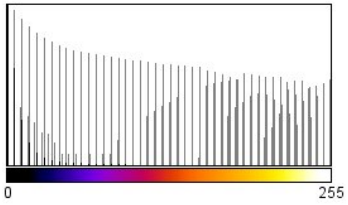
- The LEICA SP8 is a conventional inverted laser-scanning confocal microscope that is additionally equipped for super-resolution STED imaging (resolution$_{x,y}$ ~ 50 nm)

- 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 a high power CW laser at 592 nm for STED imaging

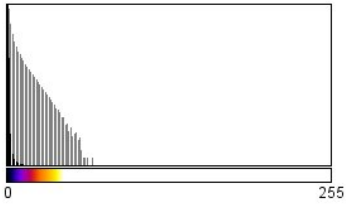
Golgi (TGN46) Dye: Alexa488 with Eva Wegel/Ian Dobbie





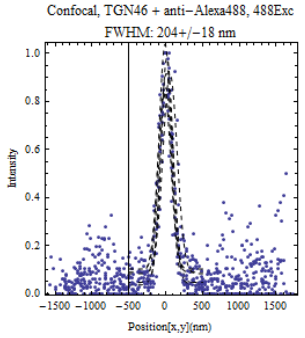


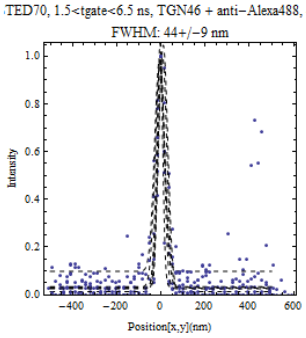
Count: 3348480
Mean: 15.200
StdDev: 33.249
Min: 0
Max: 255
Mode: 0 (1363503)



Count: 3348480
Mean: 0.896
StdDev: 2.529
Min: 0
Max: 73
Mode: 0 (2198878)

anti−TGN46 + 2° Ab−Alexa488

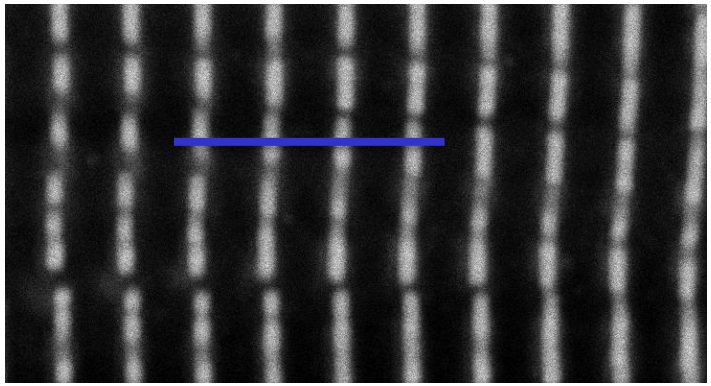


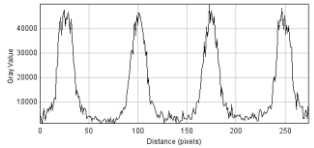


Muscle myocytes/Titin Z-disk/Alexa488 with Katja Gemlich

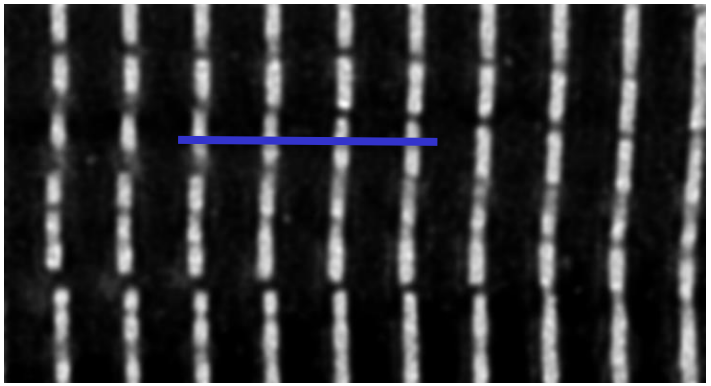
A. Confocal

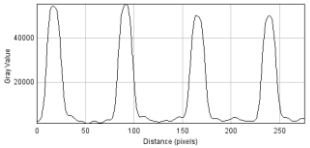
Raw





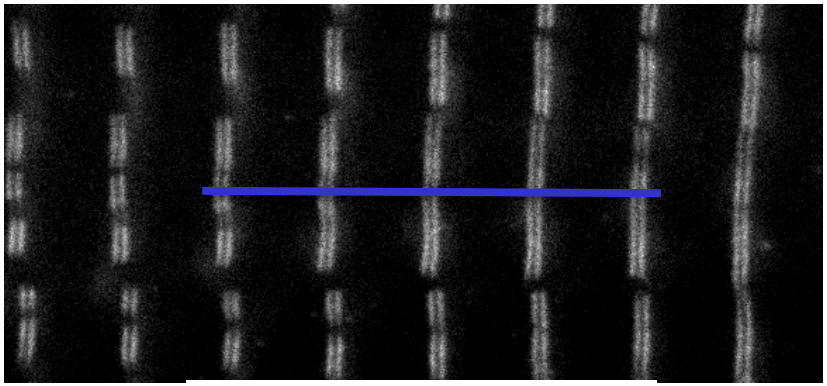
De-convolved (Huygens)

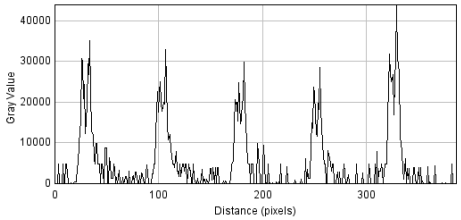




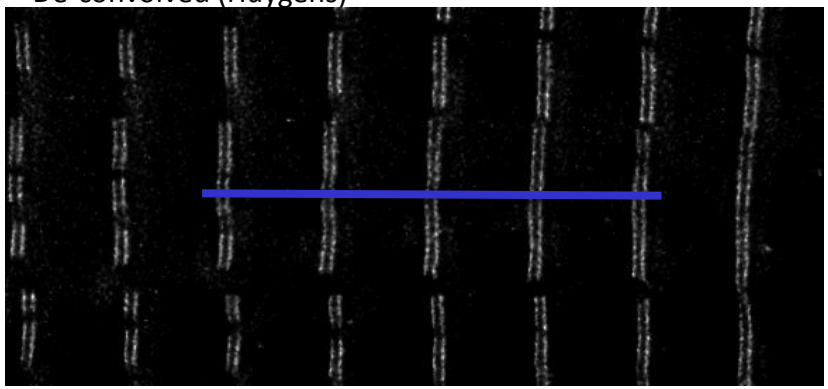
B. Gated STED (50% STED Power)

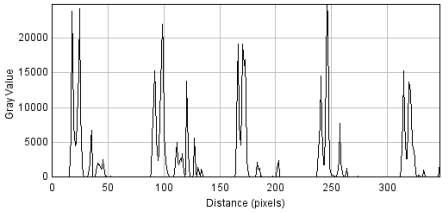
Raw





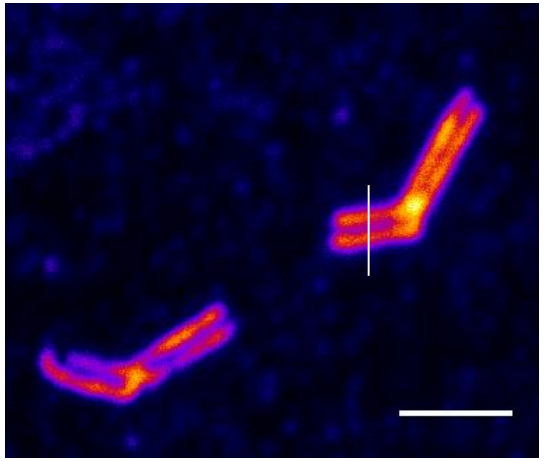
De-convolved (Huygens)



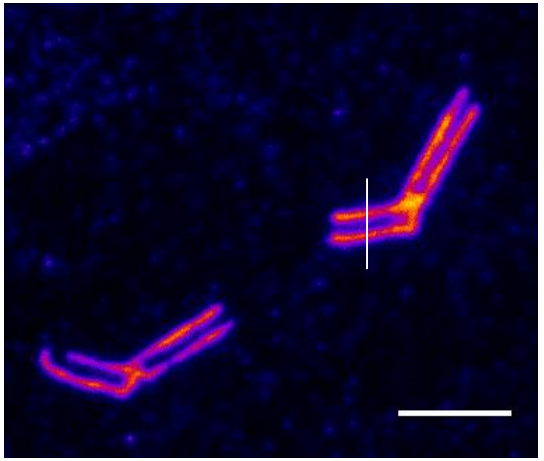


Centrosomes – with Alan Wainman/Jordan Raff - 130513

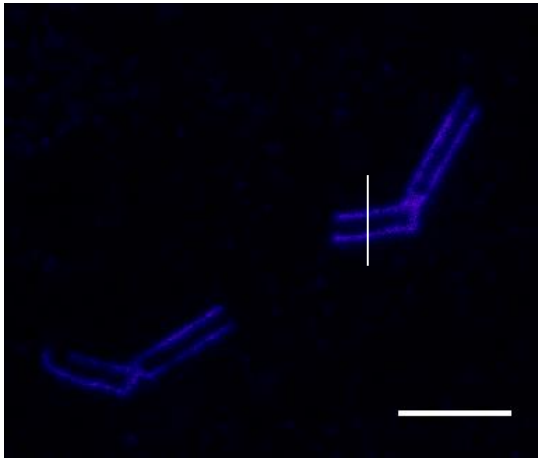
Confocal

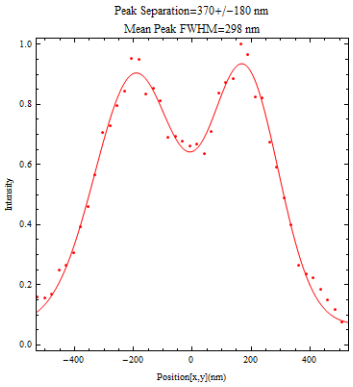


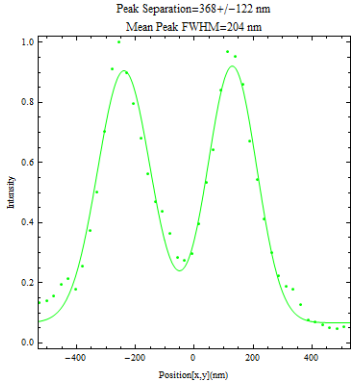
gSTED50

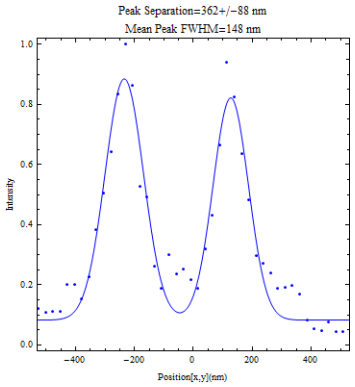


gSTED100







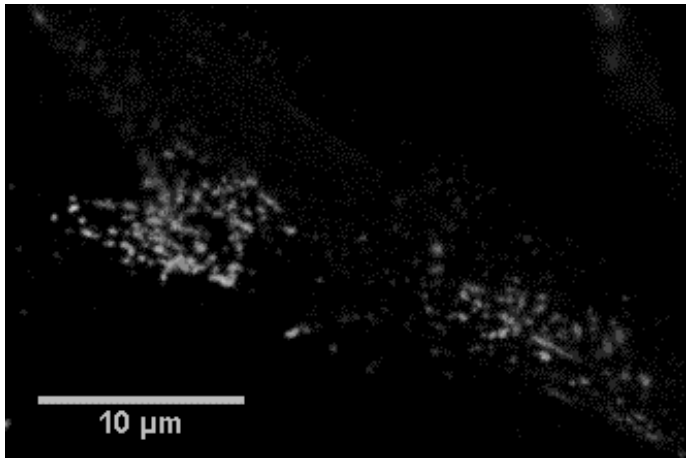


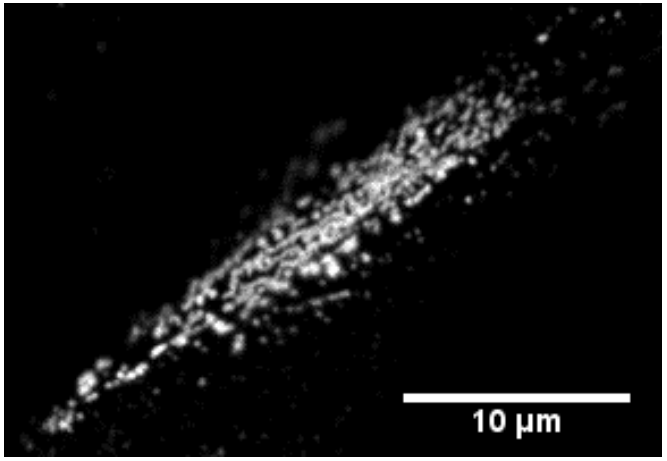
Line profiles fit were fit to Sums of Two Spatial Gaussians; FWHM= $2\ (2\ \mathrm{Ln}[2])^{0.5}\ w$)

Scale Bar = 1 µm

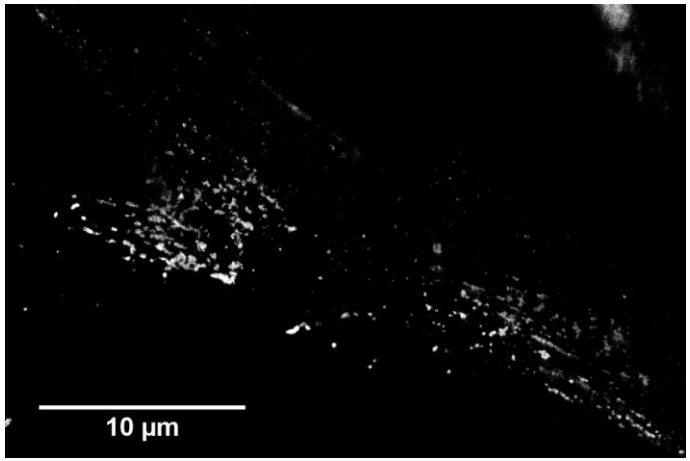
AchR Clusters– with Judy Cossins / David Beeson - 230113

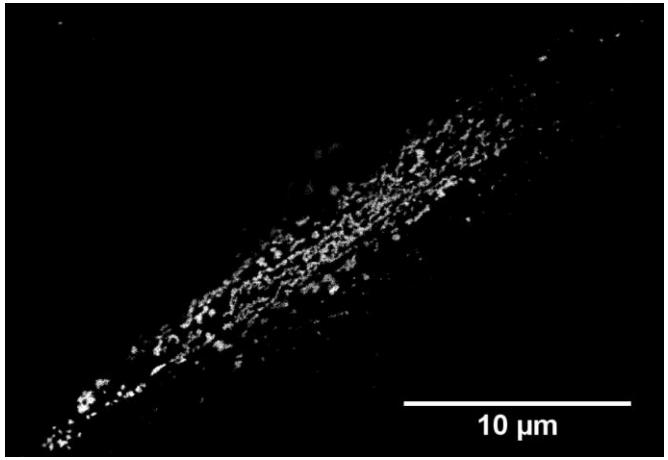
Confocal Images



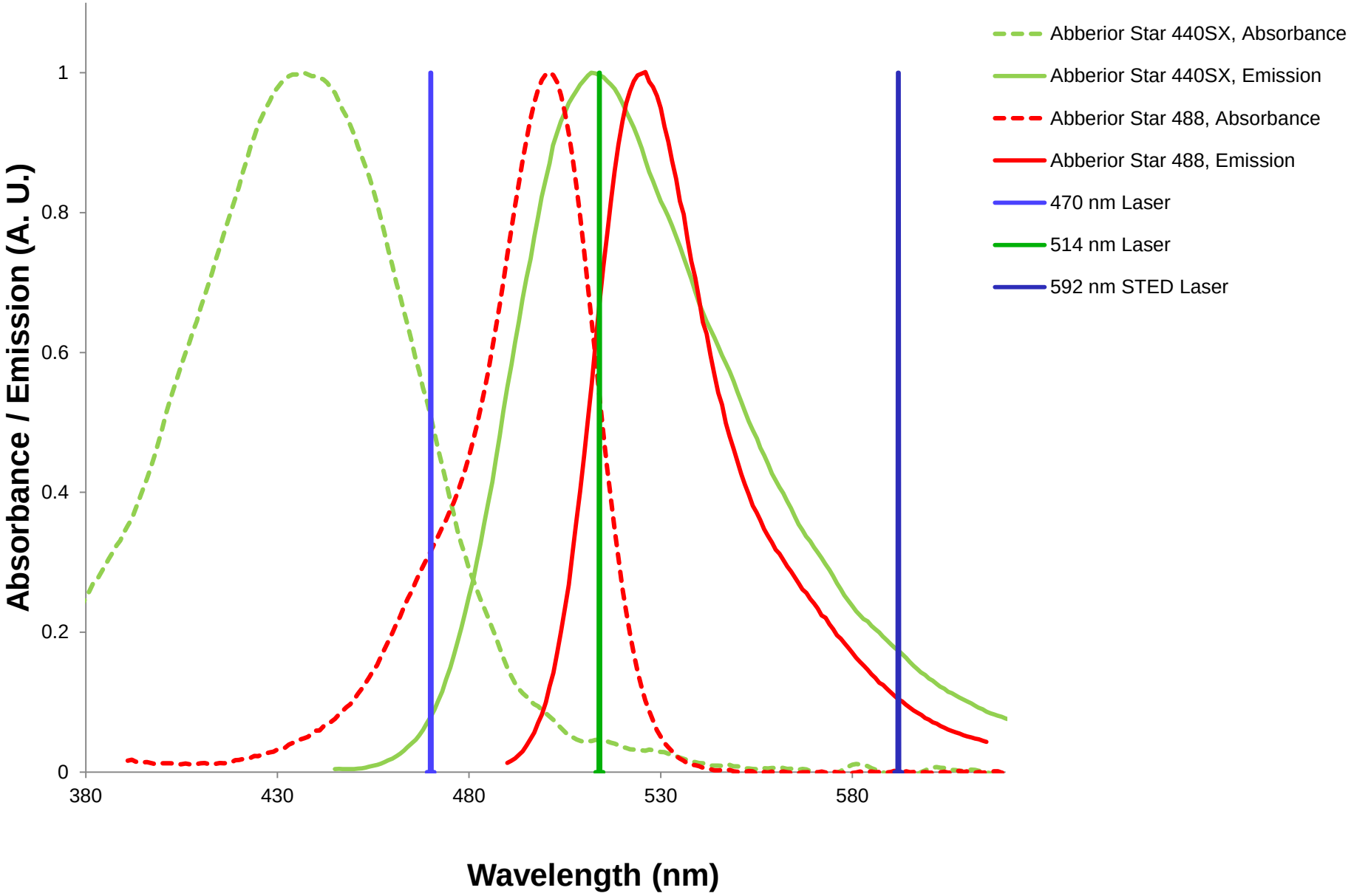


STED Images



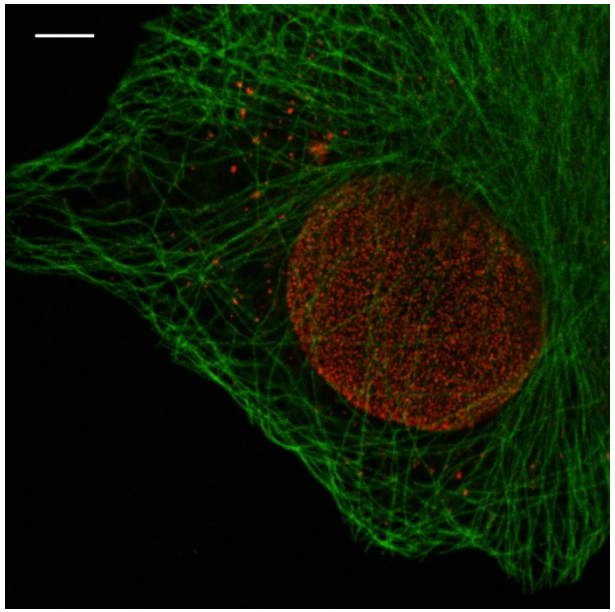


Two-color STED Imaging Example

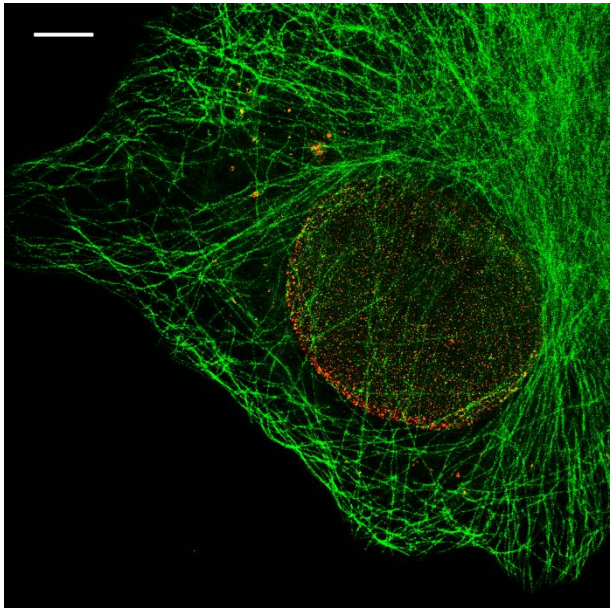


α-Tubulin (Abberior Star 440SX) / Nucler Pores (Atto 488)

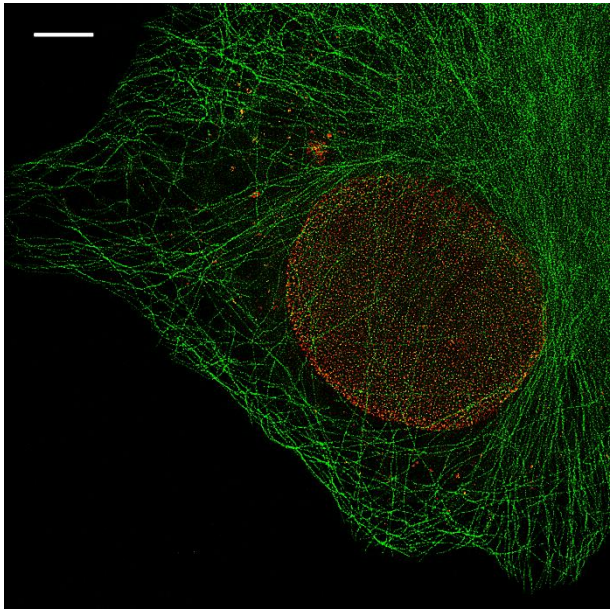
Confocal 440/502 nm



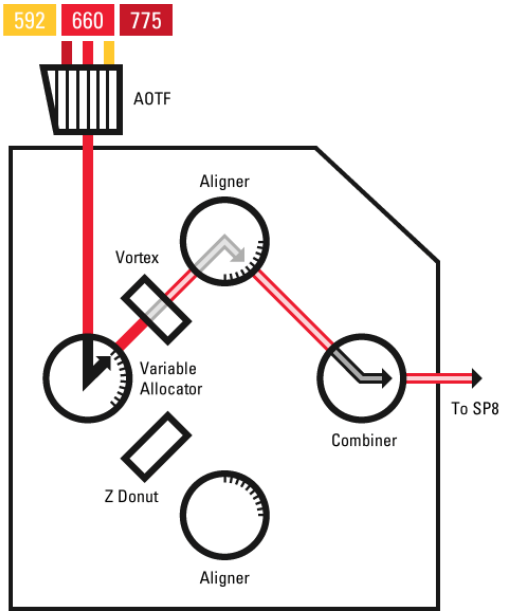
gSTED 50% (440/502 nm)

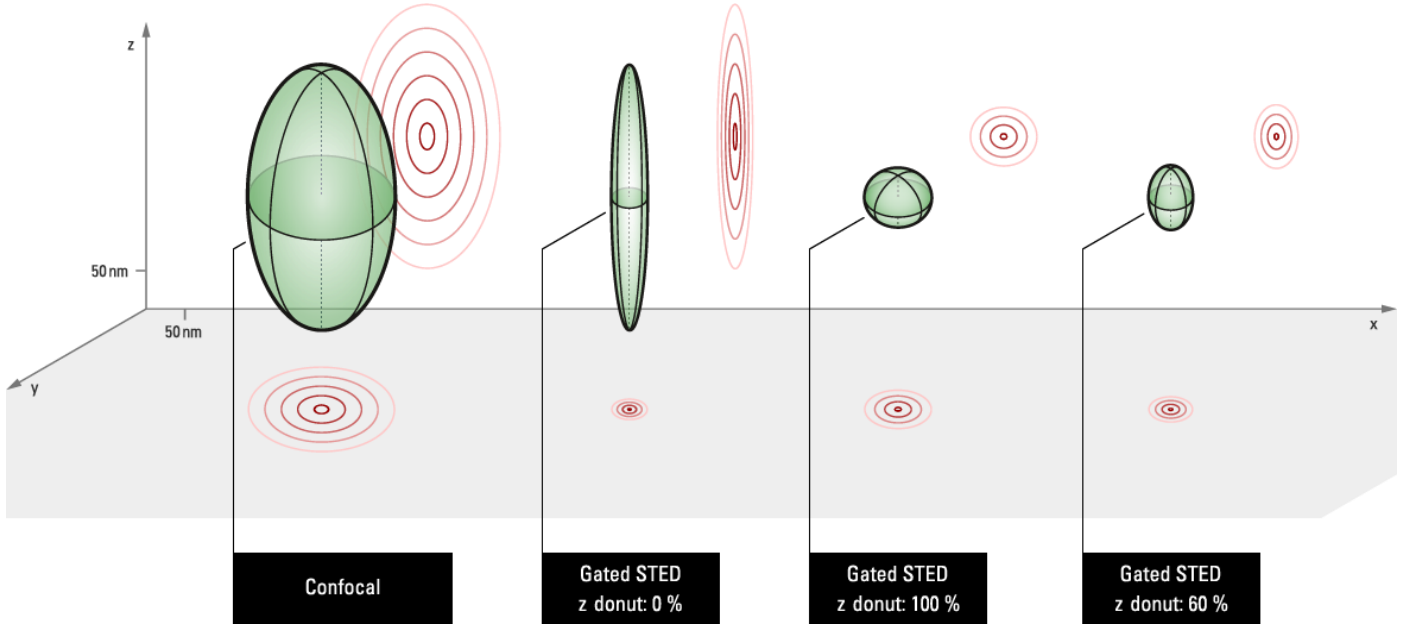


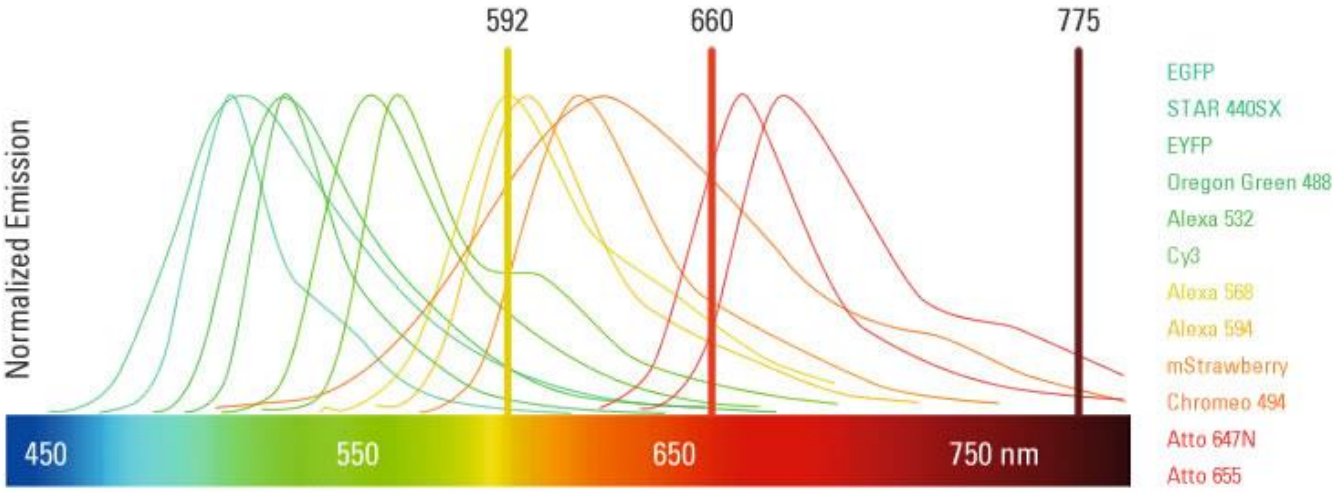
De-convolved (Huygens)
gSTED 50% (440/502 nm)



Commercial STED Microscope – Leica SP8X (soon 3X)



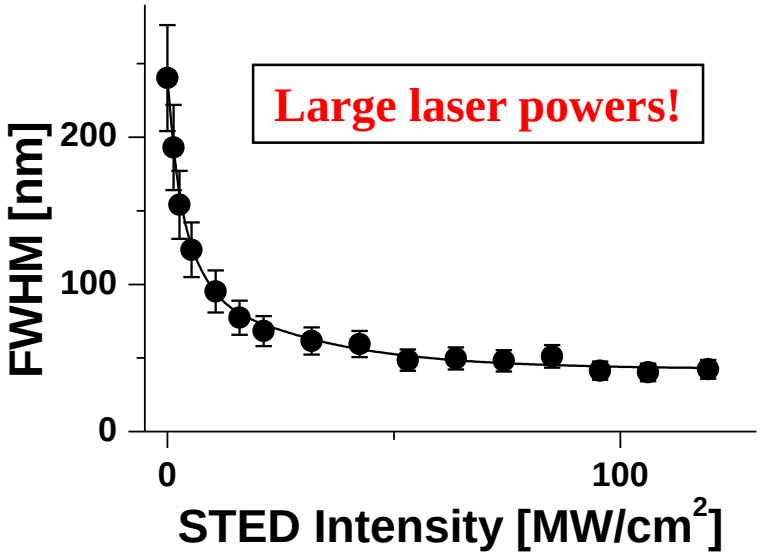




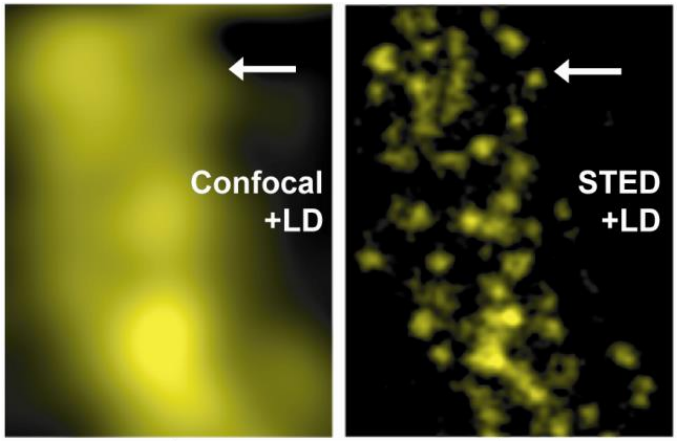
	592 GATED/CW	660 GATED/CW	775 PULSED
Strength	GFP/YFP	Multicolor	Most established spectral range
Colocalization studies	+	++	+
Photostability	+	++	++
Live cell	++	+	(+)

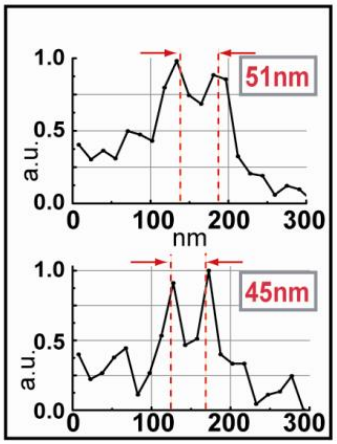
STED Live Cell Microscopy

Problems

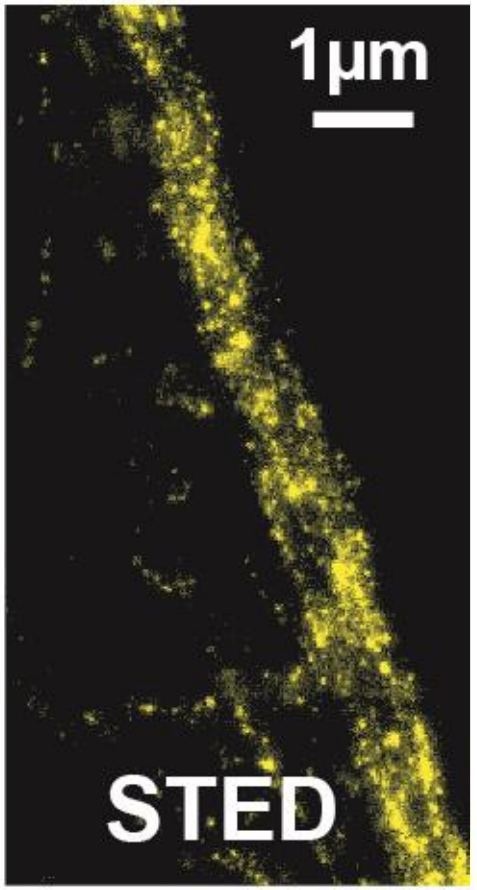


STED Microscopy
Cellular Imaging



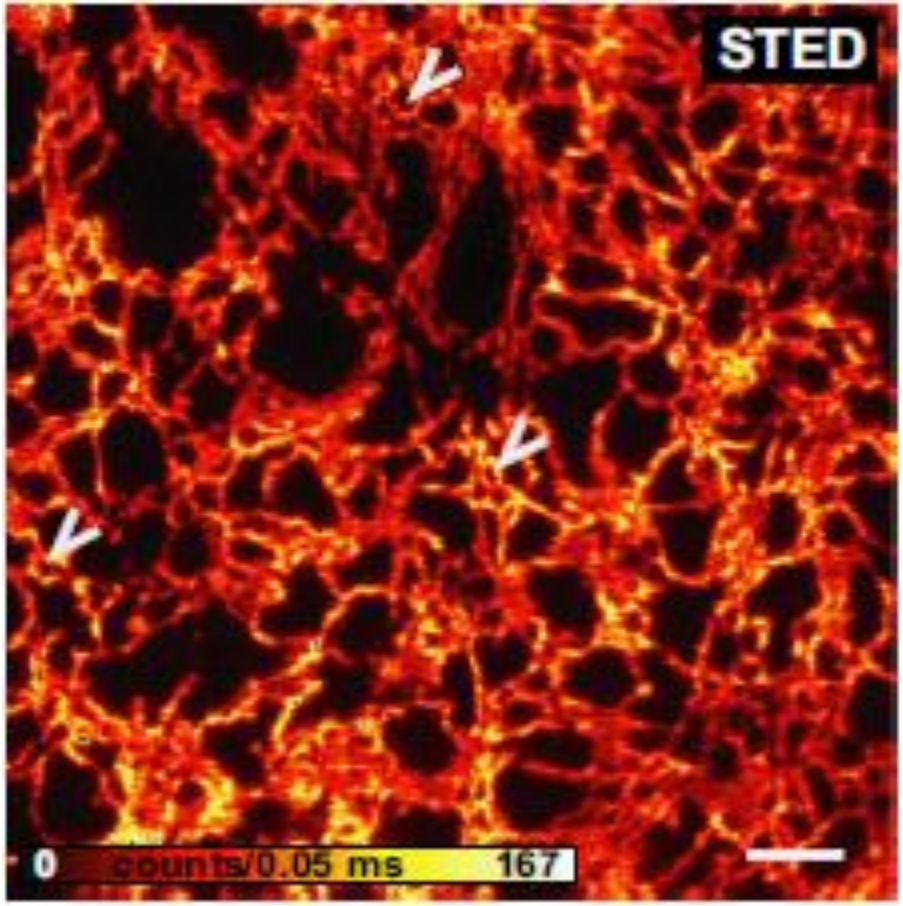


protein-heavy subunit of **<u>neurofilaments in the human neuroblastoma</u>** cell line SH-SY5Y (retinoic acid–BDNF-differentiated);
establishes cross-links to organize and stabilize neurofilaments in axons



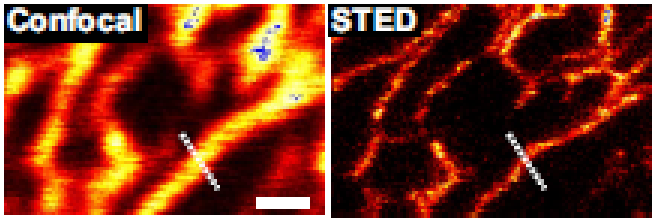
Donnert et al, PNAS 2006

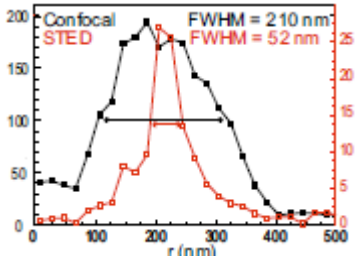
STED-Microscopy
Inside Living Cells



Living Cells:

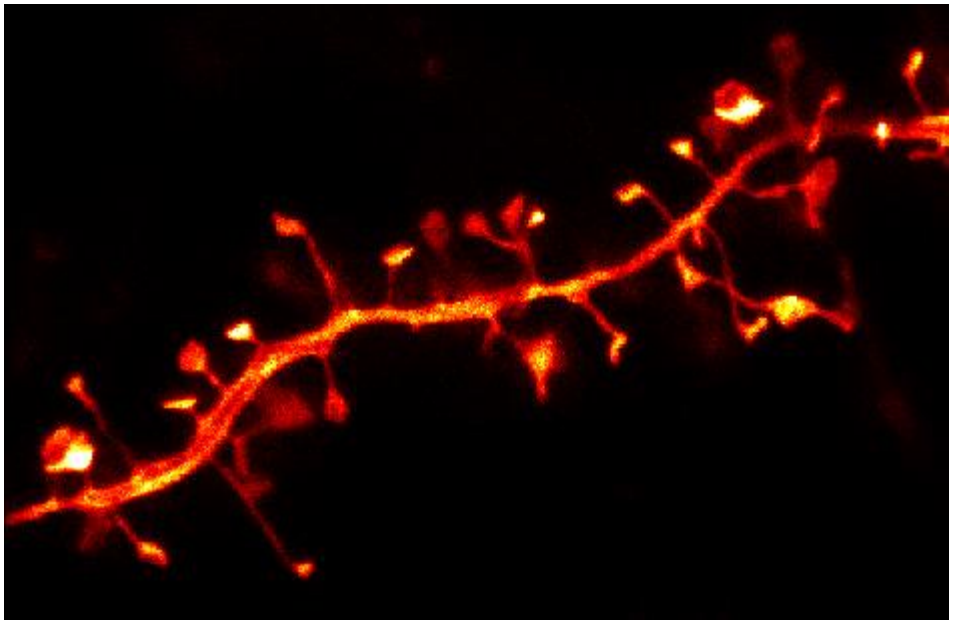
Citrine, Endoplasmatic Reticulum (ER)
Live PtK2 cells
Hein, Willig, Hell PNAS 2008



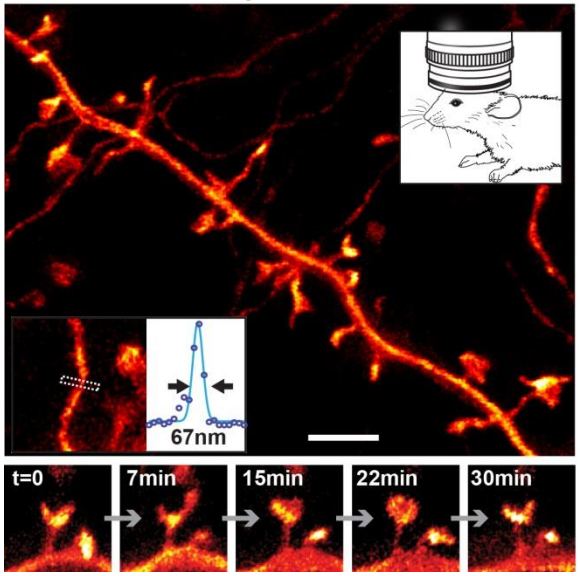


STED Microscopy
Inside Living Cells - Dynamics

YFP-transgenic mouse
Hippocampal slice
CA1 neuron
(PNAS Nägerl et al 2008)
(BiophysJ 2011)

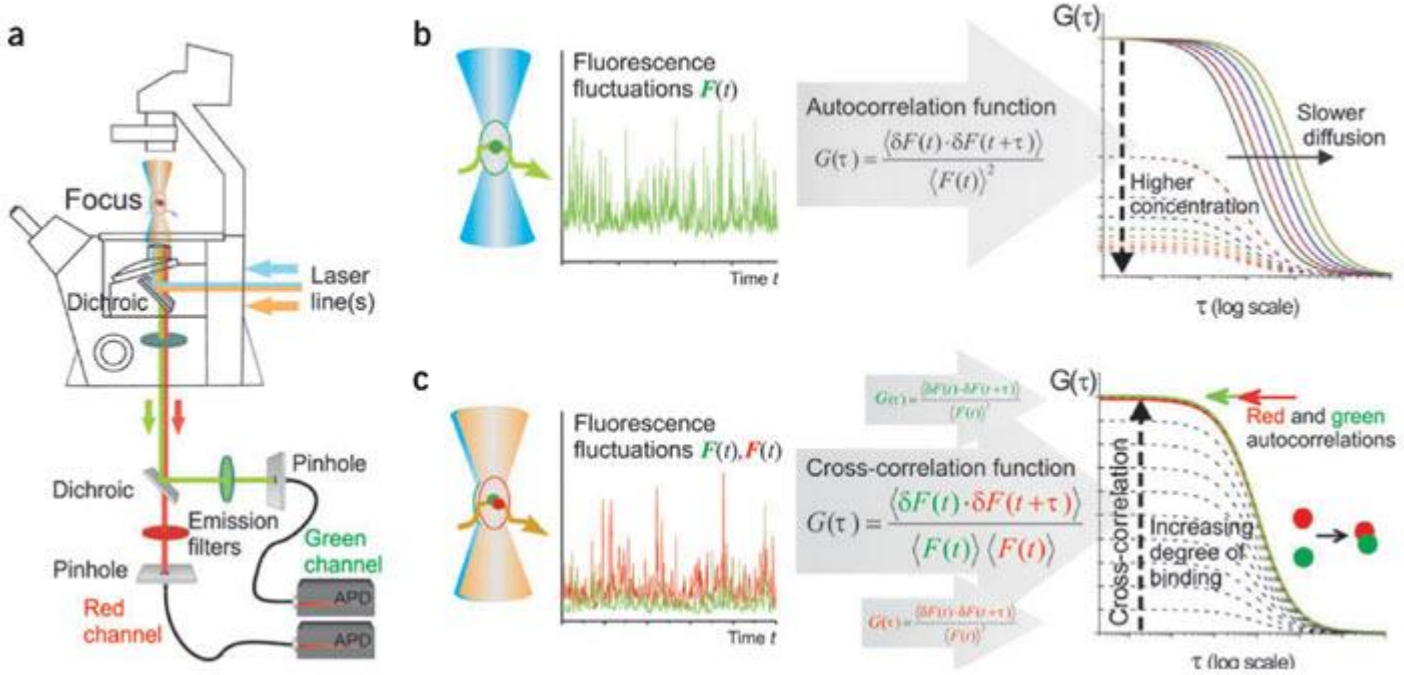


Live Mouse
YFP
(Science Berning et al 2012)



Live-Cell (inside)
Conventional dyes, GFP, …
Two-Photon excitation

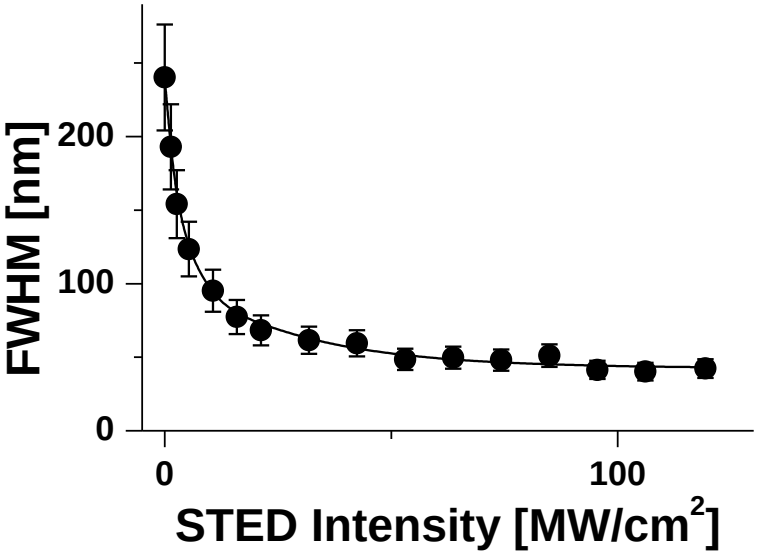
STED can also be combined with Fluorescence Correlation Spectroscopy (STED-FCS)



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



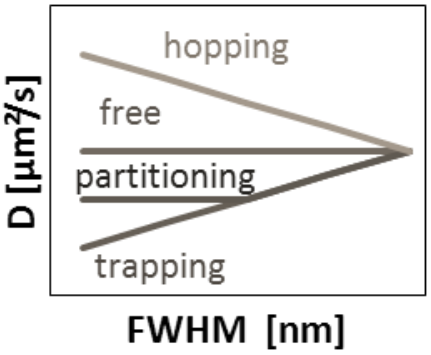
STED-FCS
Determine transit time
for different sizes of observation areas
(different STED intensities)

Calculate
apparent diffusion coefficient:

D ~ area / transit time

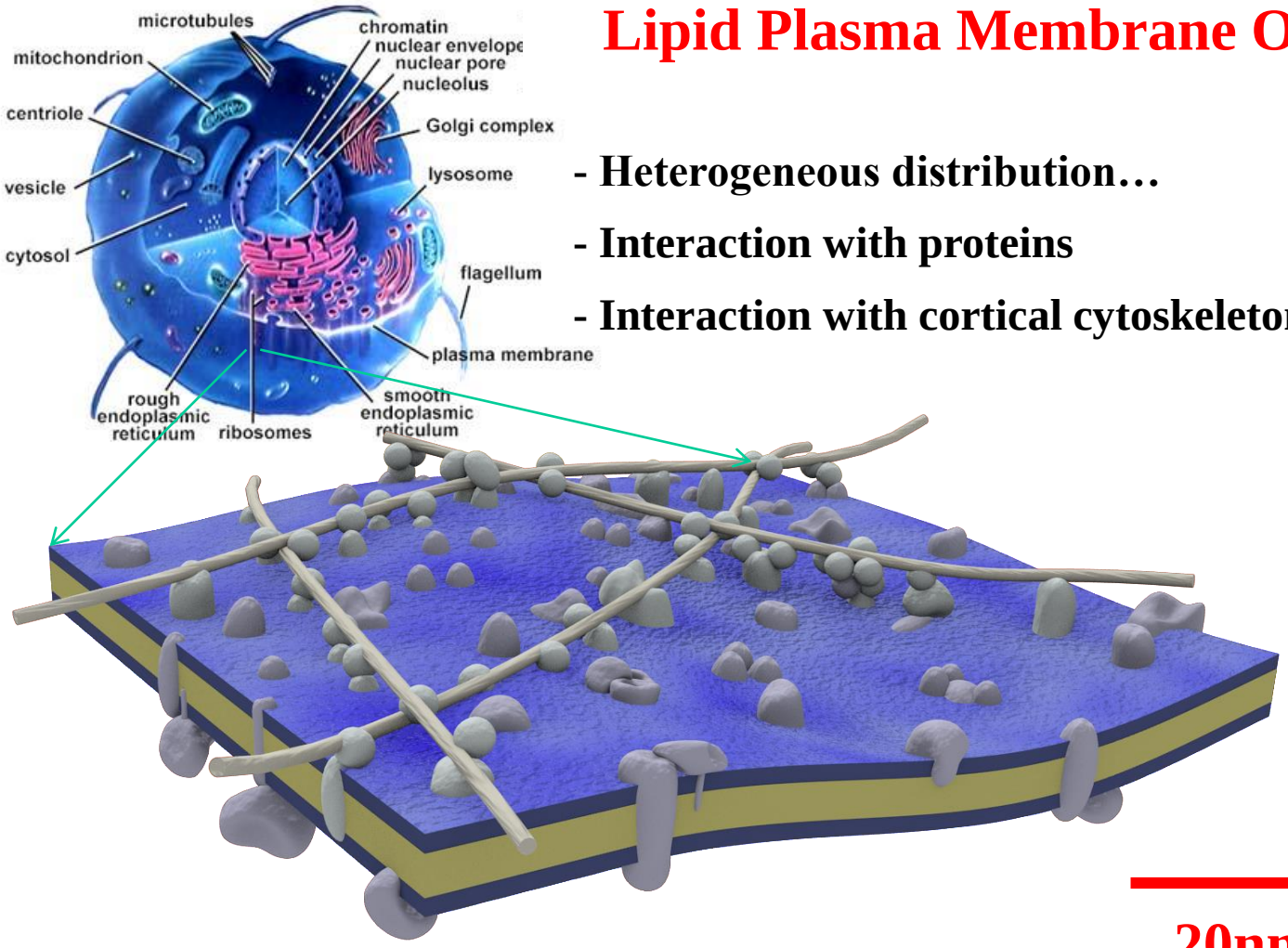
Dependencies: D(diameter)
240nm → 30/40nm

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

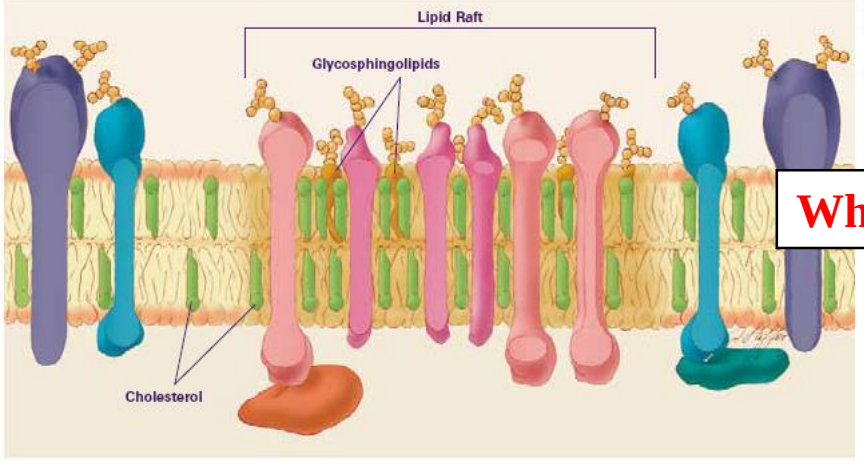
Lipid rafts/nanodomains?

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

Cytoskeleton

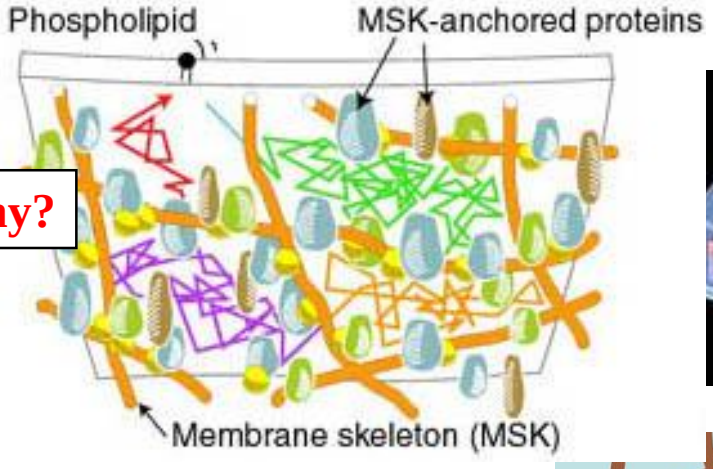
- Membrane divided in compartments
- Proteins: fence/hindrance in diffusion path
- Hopping diffusion

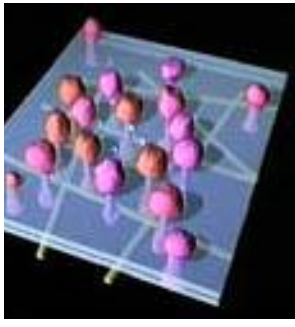
Kusumi

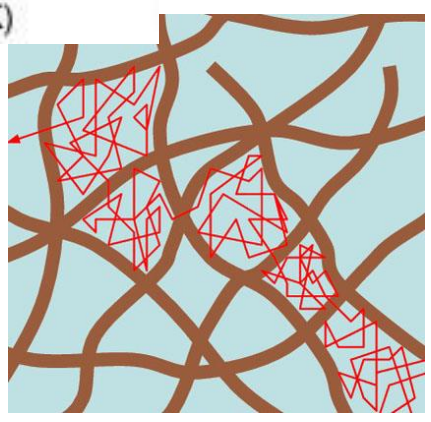


Pike, J.Lipid Res., Keystone meeting 2006

Why?







Problem:

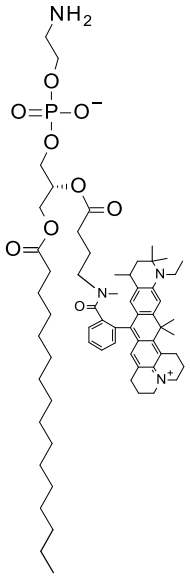
- heterogeneous
  + highly dynamic
- small (<200 nm)

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

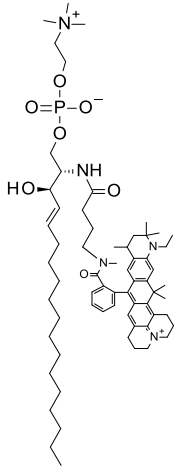
Lipid Plasma Membrane Dynamics

Fluorescence Recordings: Lipids

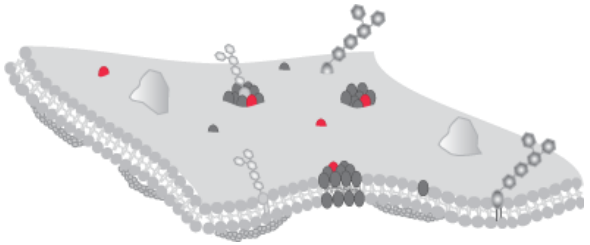
Phosphoglycerolipid:
Atto647N-phosphoethanolamine (PE)

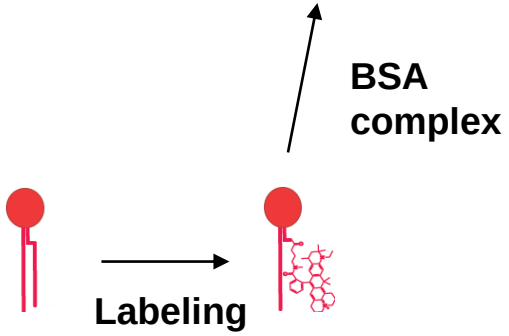


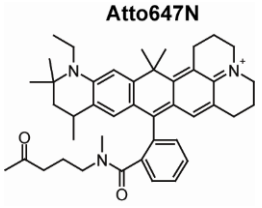
Sphingolipid:
Atto647N-sphingomyelin (SM)



Live PtK2 cells:
physiological conditions
incorporation in plasma membrane

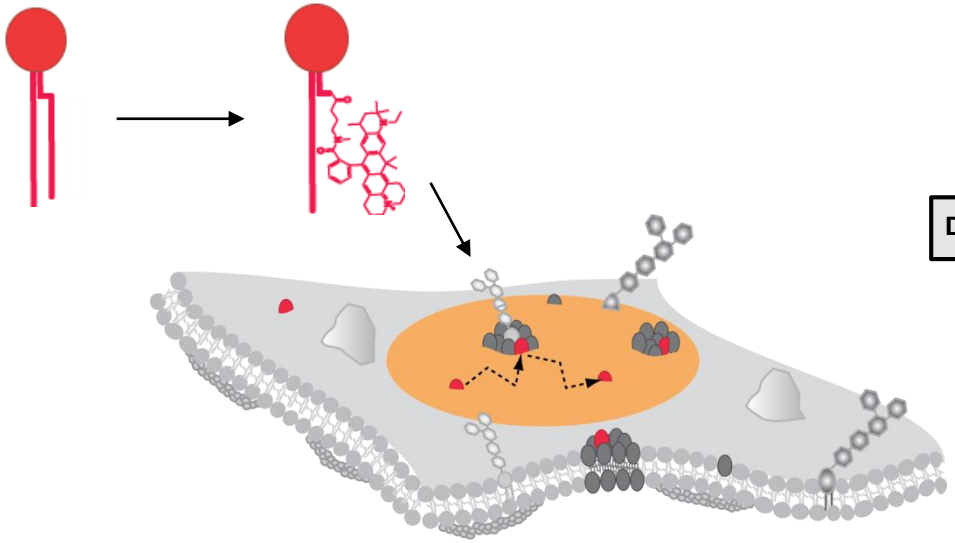


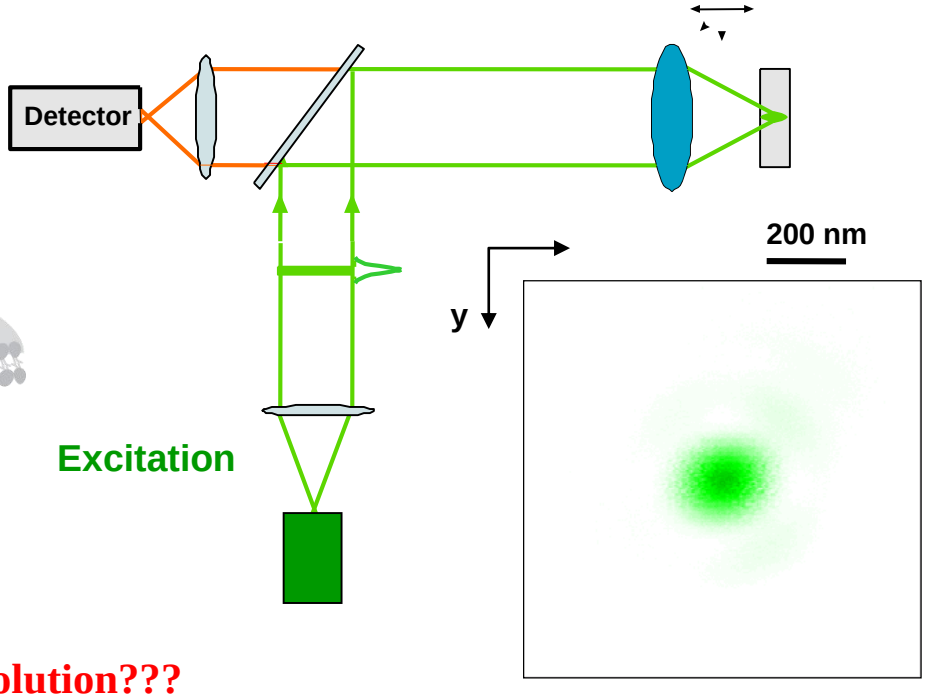


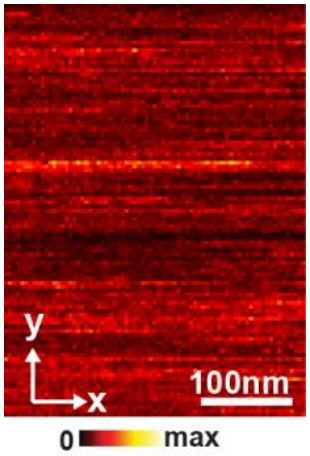


Lipid Plasma Membrane Dynamics

Confocal Recordings







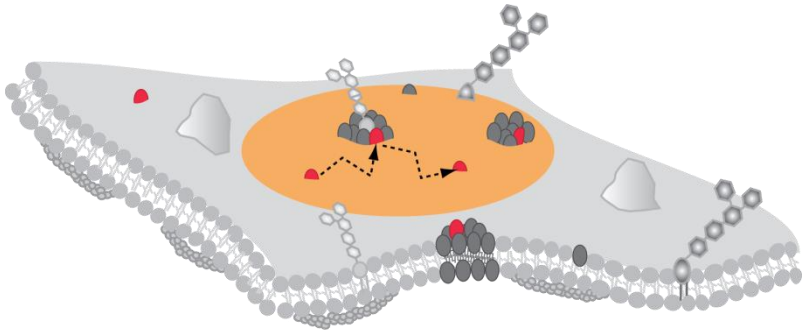
Limited spatial resolution???

Observation area on plasma membrane

Lipid Plasma Membrane Dynamics

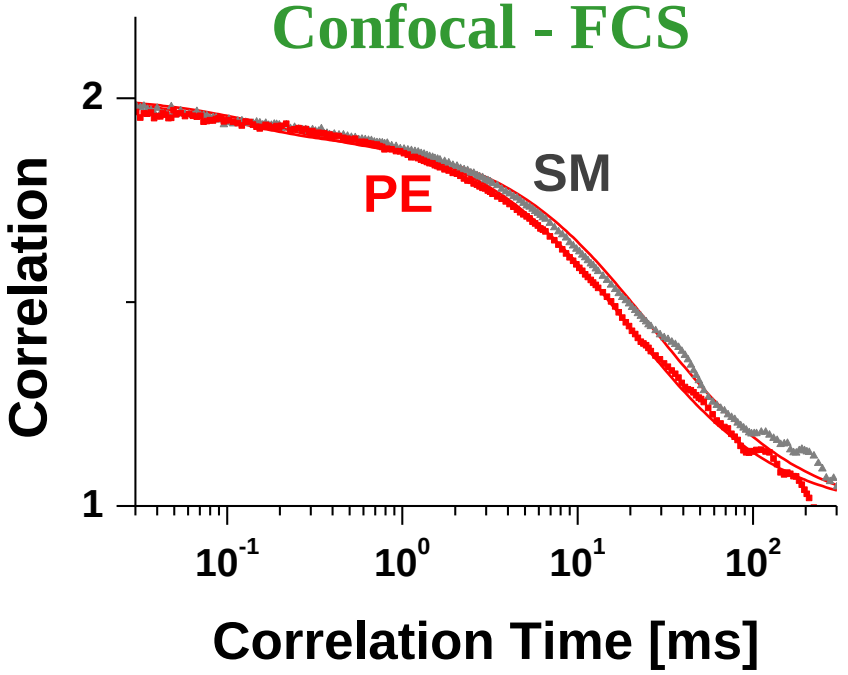
Confocal Recordings

Confocal: Limited spatial resolution !!!



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

Confocal - FCS



SM diffusion slightly prolonged but still normal

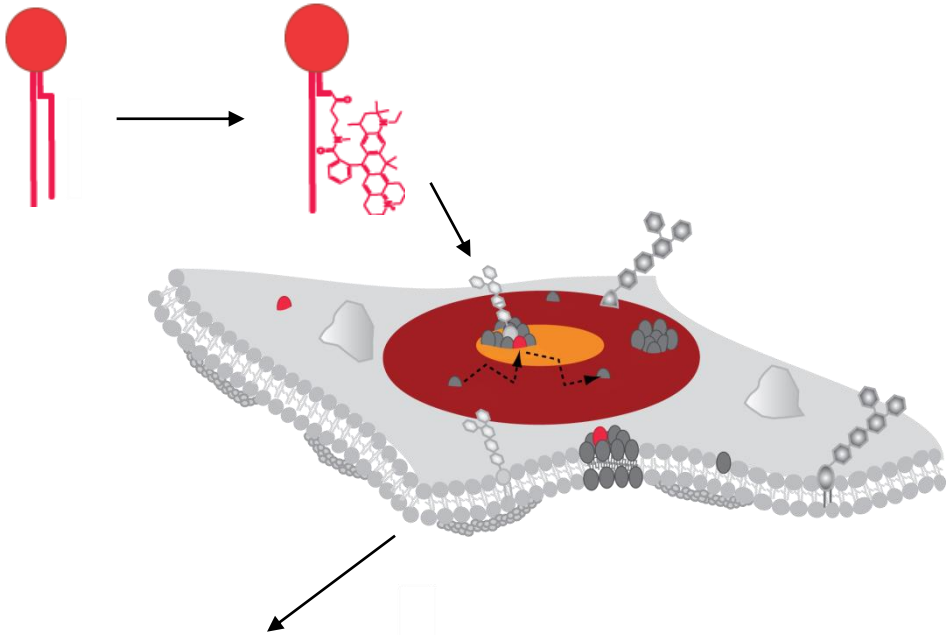
$\tau_d \approx$ 20 ms (PE) / 30ms (SM)

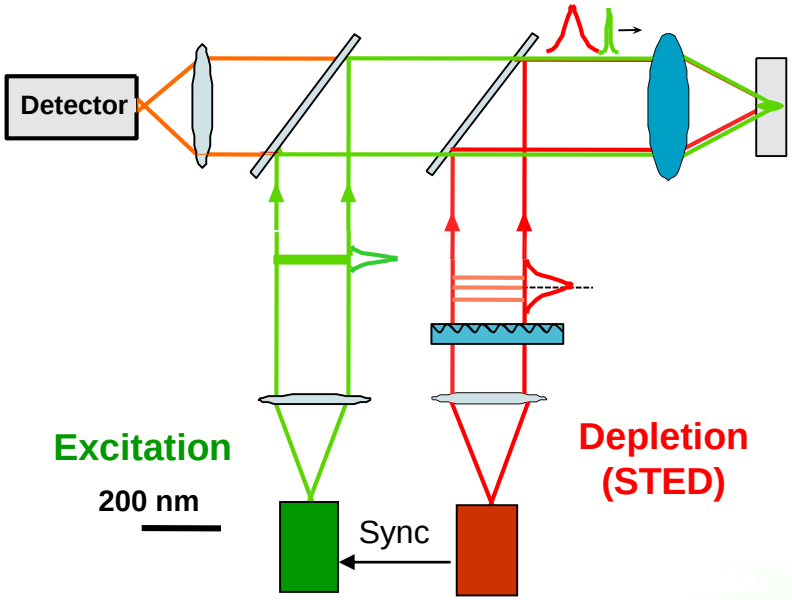
$(1/\alpha) \approx 1$ (PE / SM)

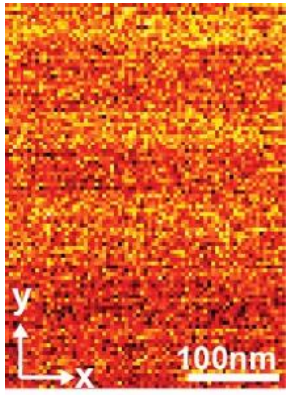
Slower normal diffusion
but no anomalous diffusion???

Lipid Plasma Membrane Dynamics

STED Nanoscopy Measurement

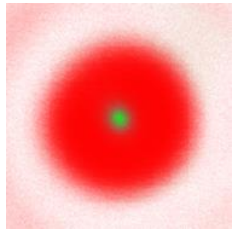






No heterogeneities:

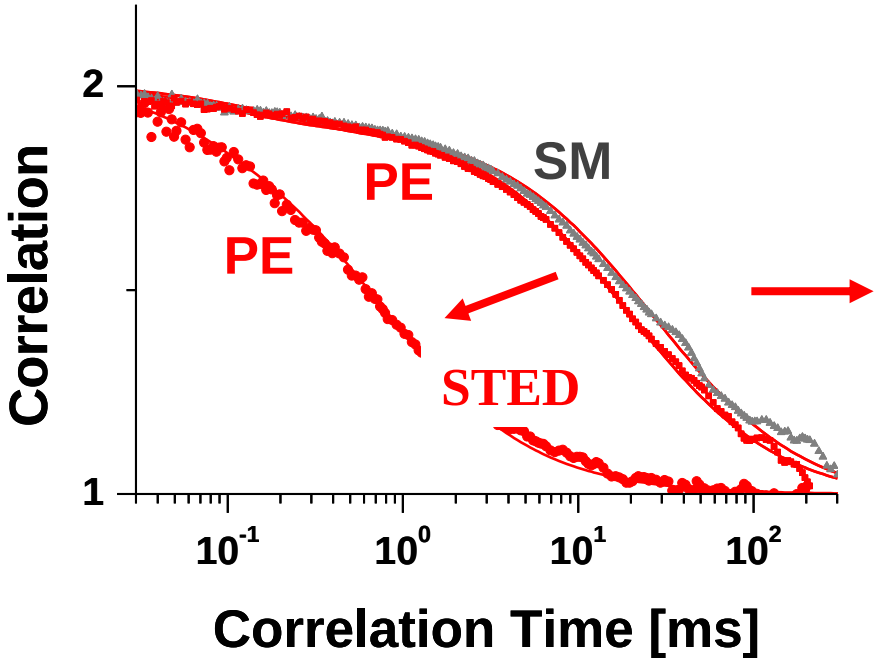
Fast diffusion → Limited temporal resolution!



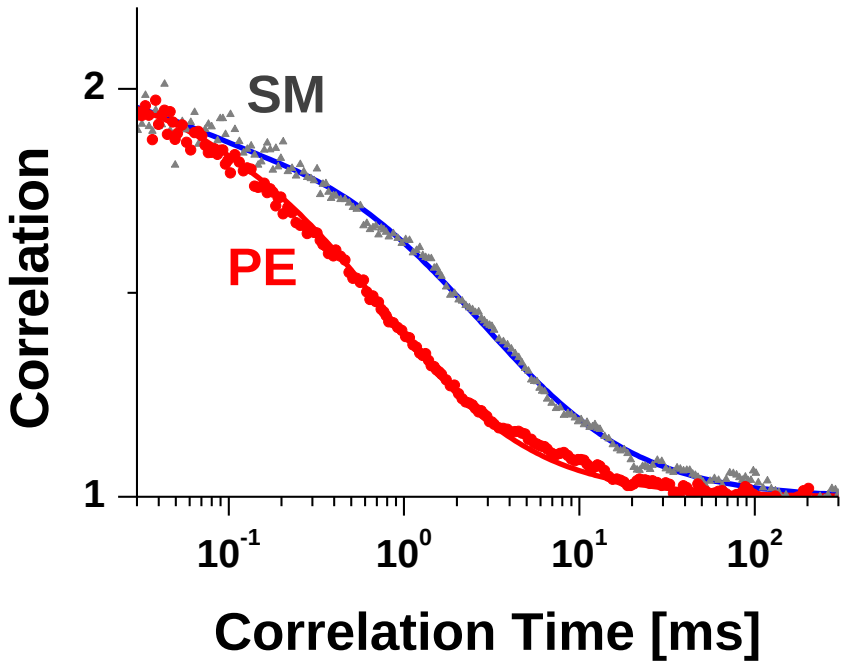
STED Live Cell Spectroscopy

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

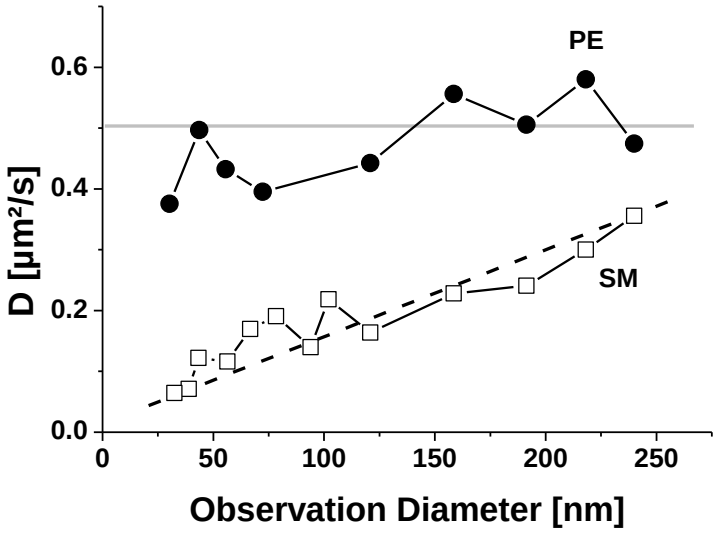
τ_d: 30 -> 3ms (10-fold)

and anomalous

$(1/\alpha) \approx 1.5$

STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM



→ **Complex on molecular scale**

(proteins, lipid-shells, …)

~10 ms, no movement during trapping

Cholesterol-assisted

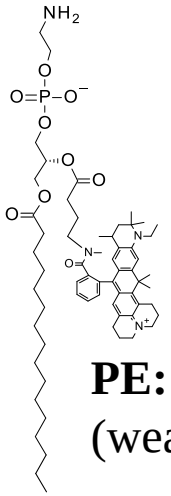
(COase/ß-Cyclo-Dextrin/Zaragozic acid…)

Binding partner bound to cytoskeleton

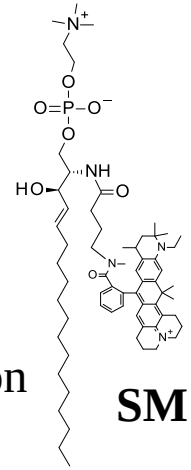
(Latrunculin/Jasplakinolide/Nocodazole…)

Slight dependence on endogenous SM level

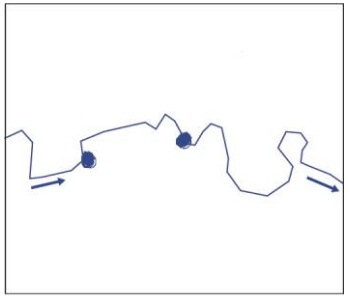
(Myriocin)

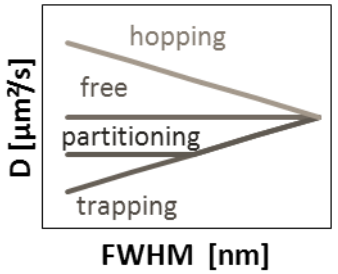


PE: free diffusion
(weak trapping)



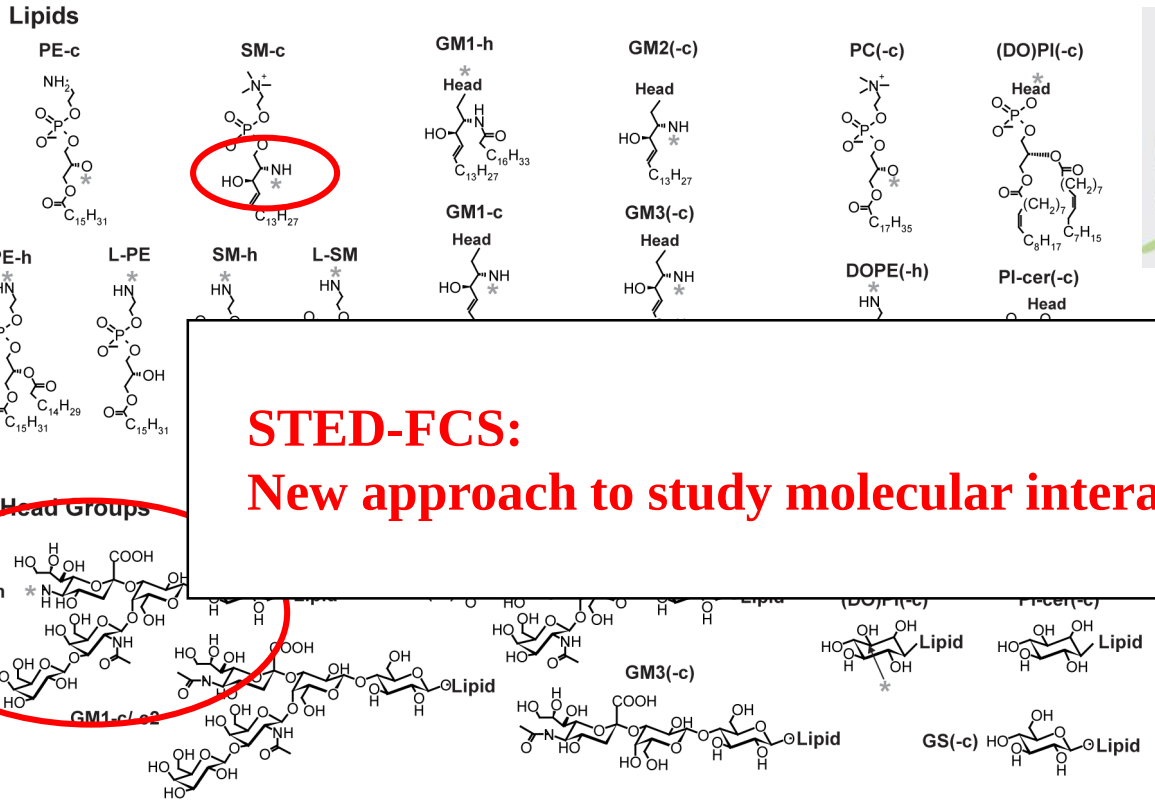
SM: trapping

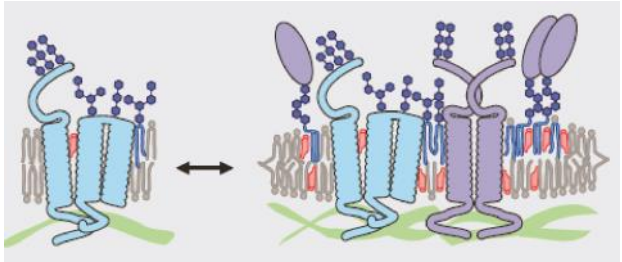




STED-FCS

Membrane Dynamic – Lipid Structure





STED-FCS:
New approach to study molecular interactions!

Specific lipid-protein interactions!

Binding partners?

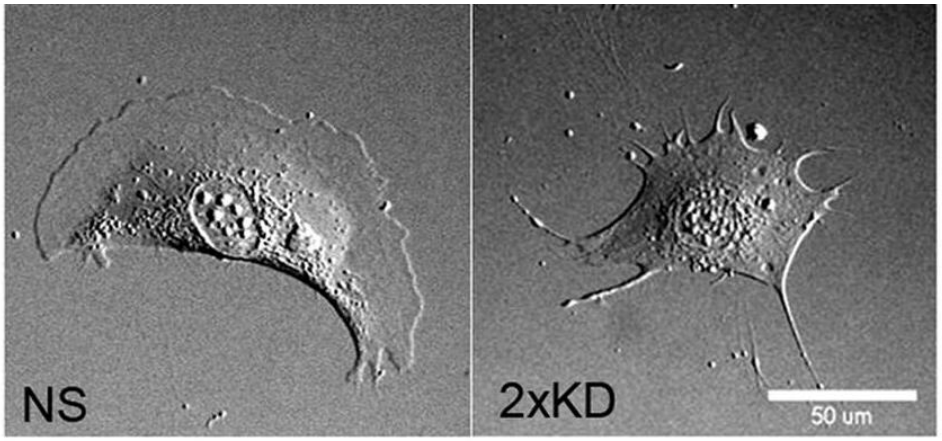
2nd color!

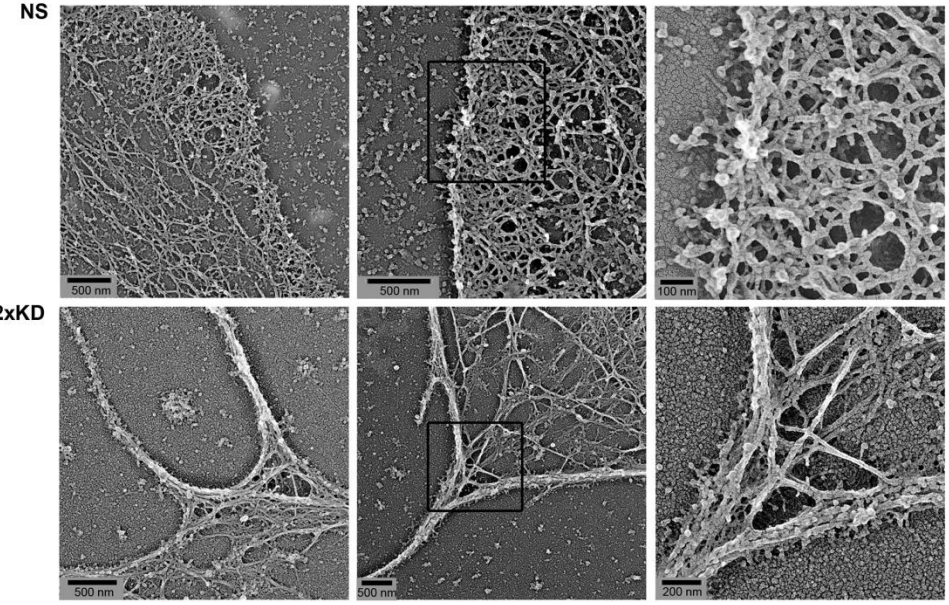
Interactions differ for different lipids!
(trapping strength, Coase+Latrunculin dependence)

But not on dye and label position!

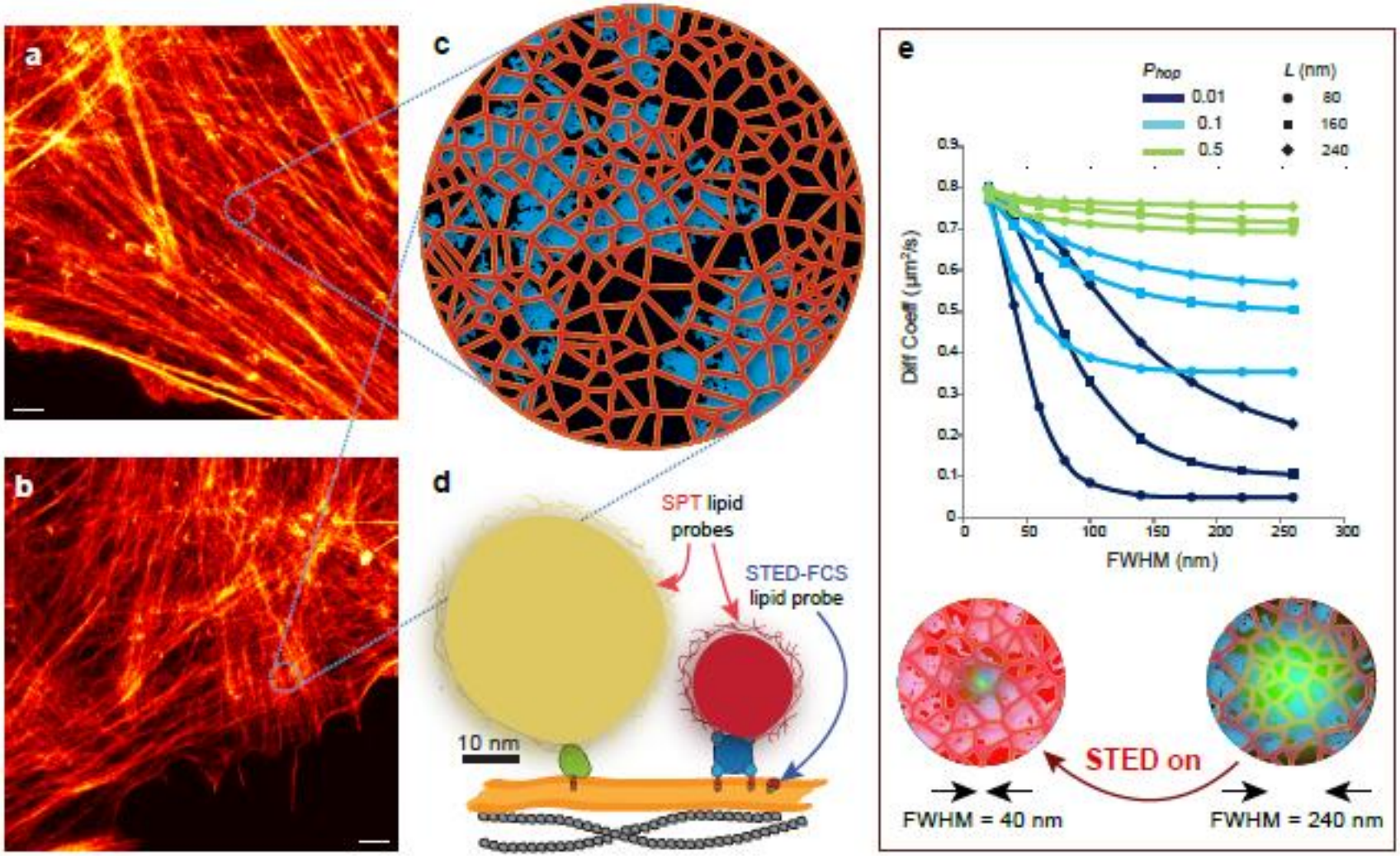
Arp2/3 Is Critical for Lamellipodia and Response to Extracellular Matrix Cues but Is Dispensable for Chemotaxis

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

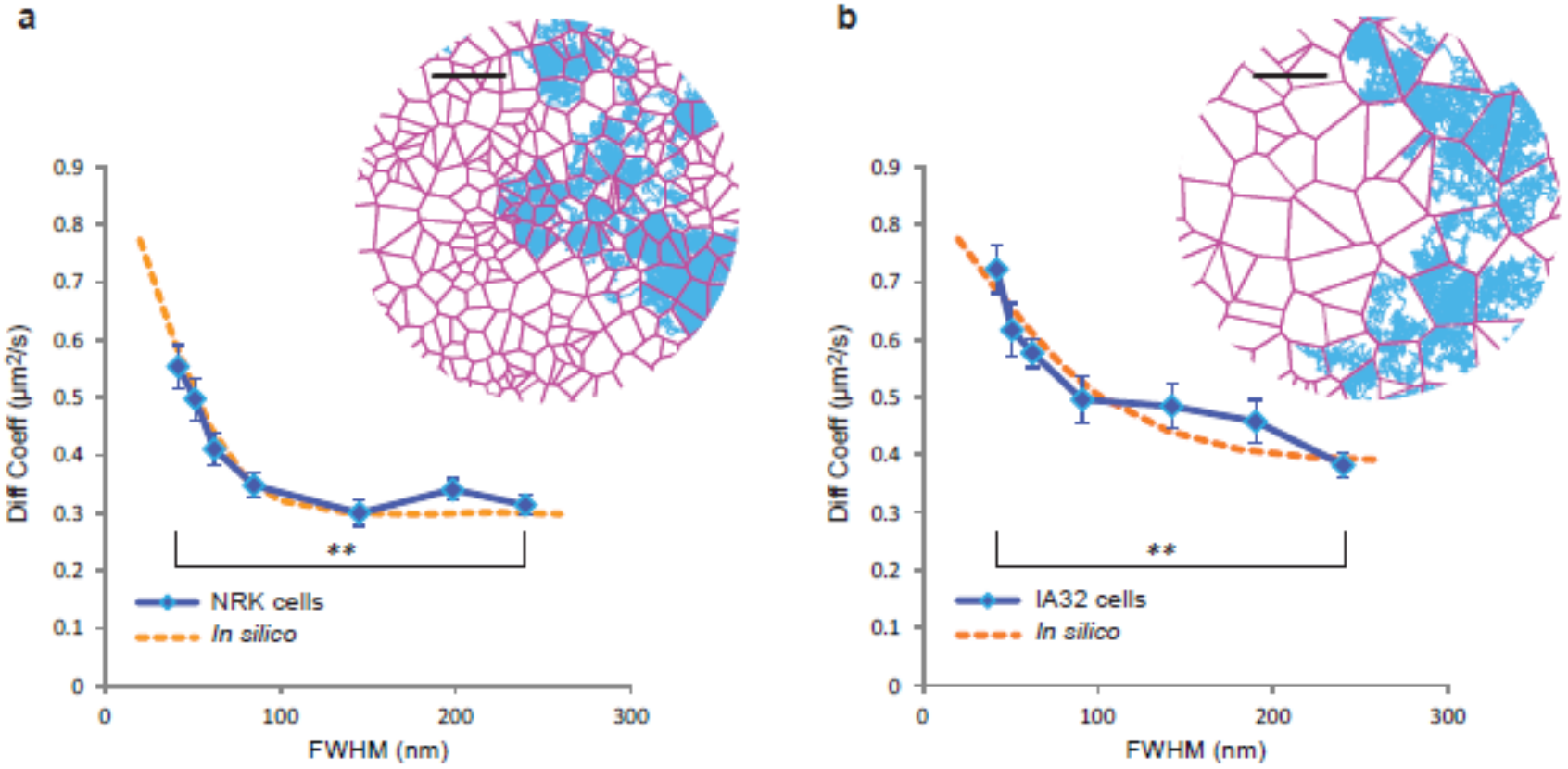




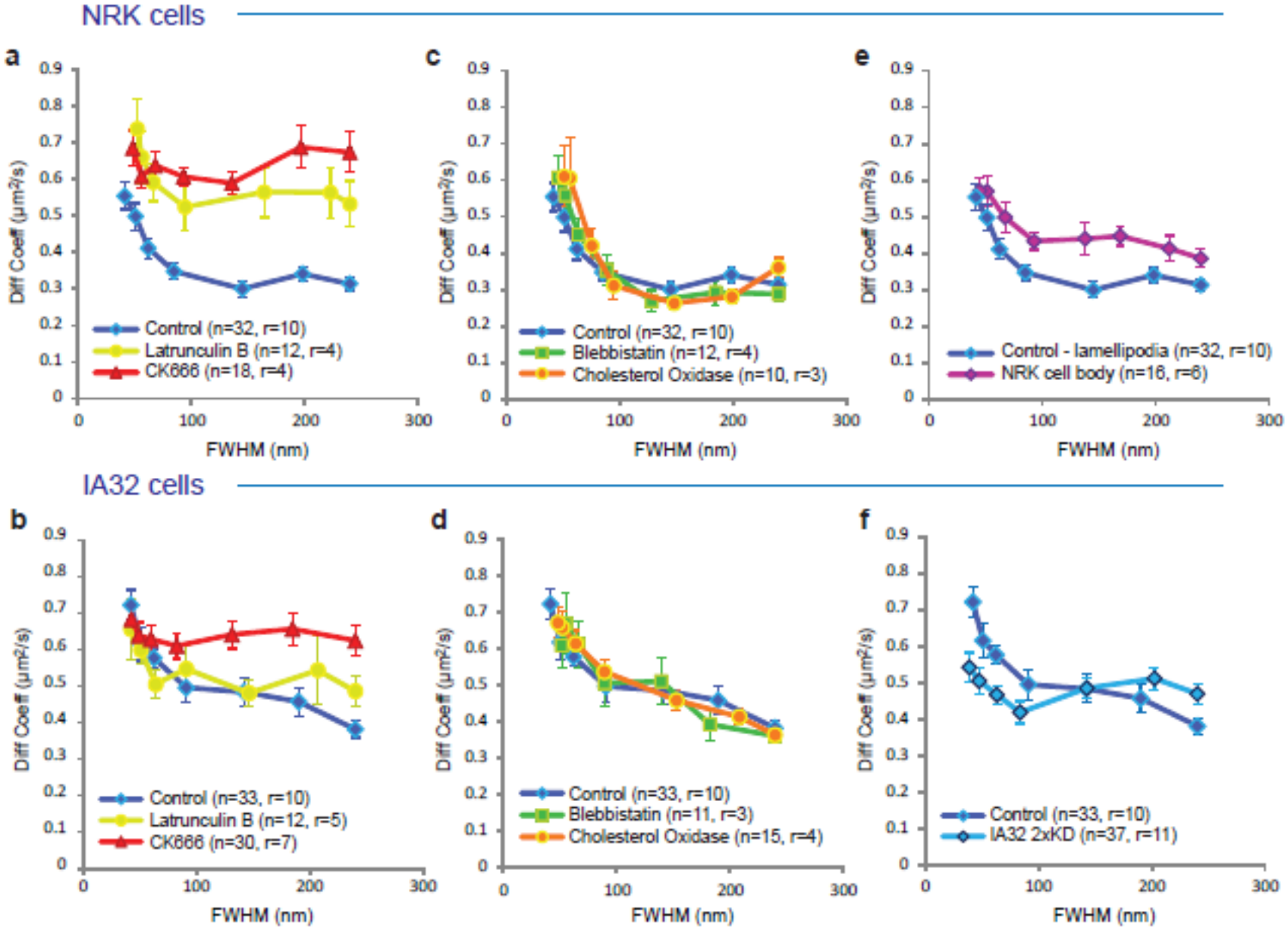
Measuring Hop-Diffusion with STED-FCS



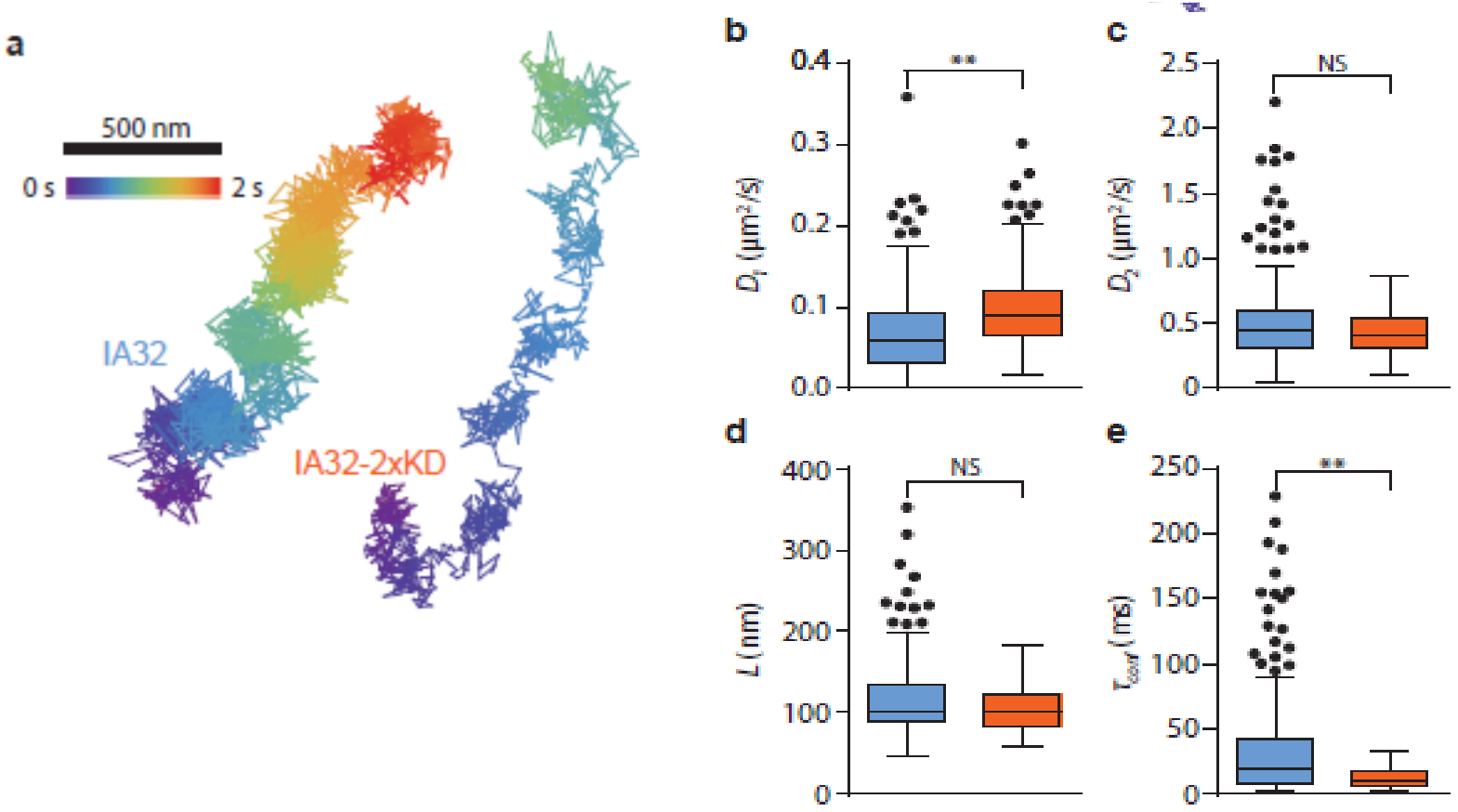
Measuring Hop-Diffusion with STED-FCS



Measuring Hop-Diffusion with STED-FCS

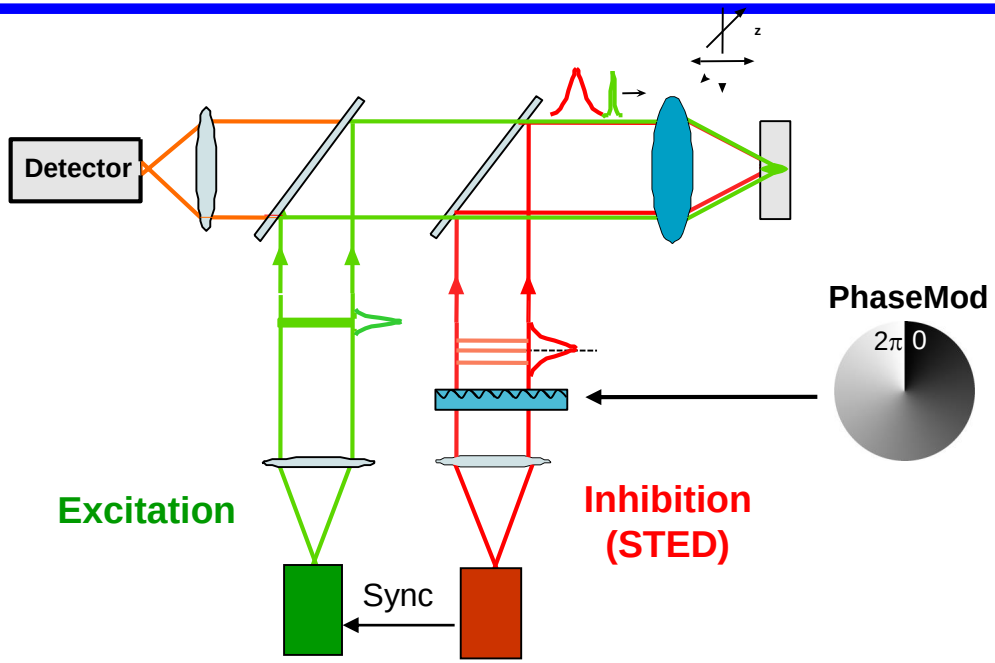


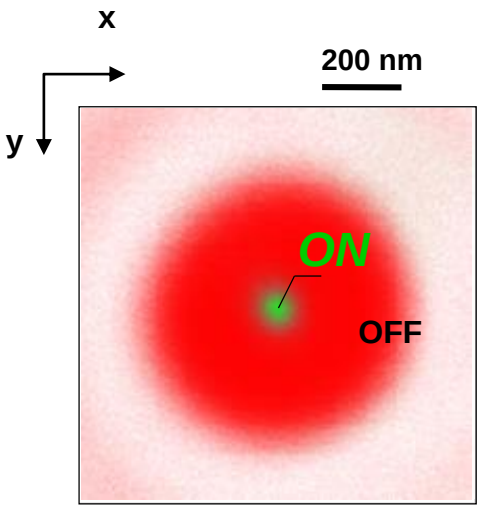
Agreement of SPT with Qdots and STED-FCS

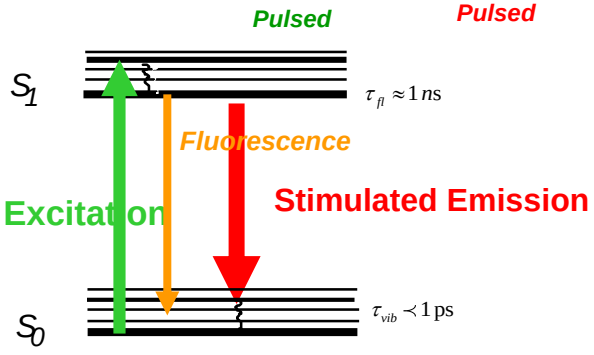


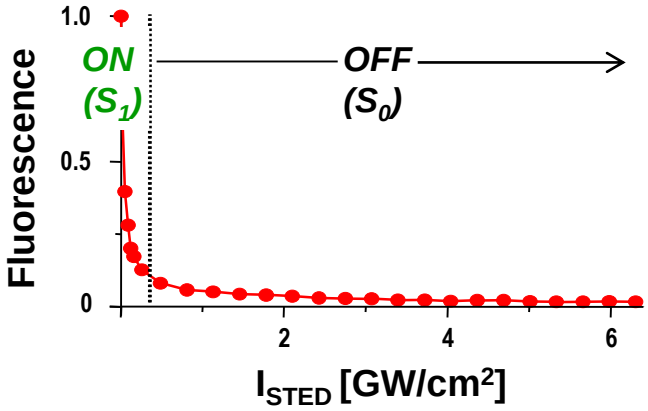
Fluorescence Microscopy

STED Microscopy









Acknowledgement

MPI, Göttingen
Stefan Hell
+ whole group

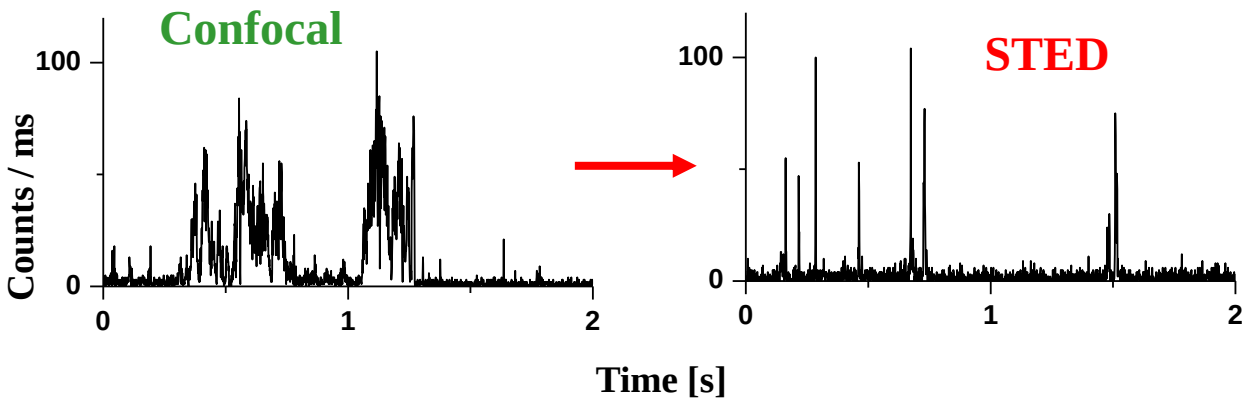
Many Slides
Christian Eggeling

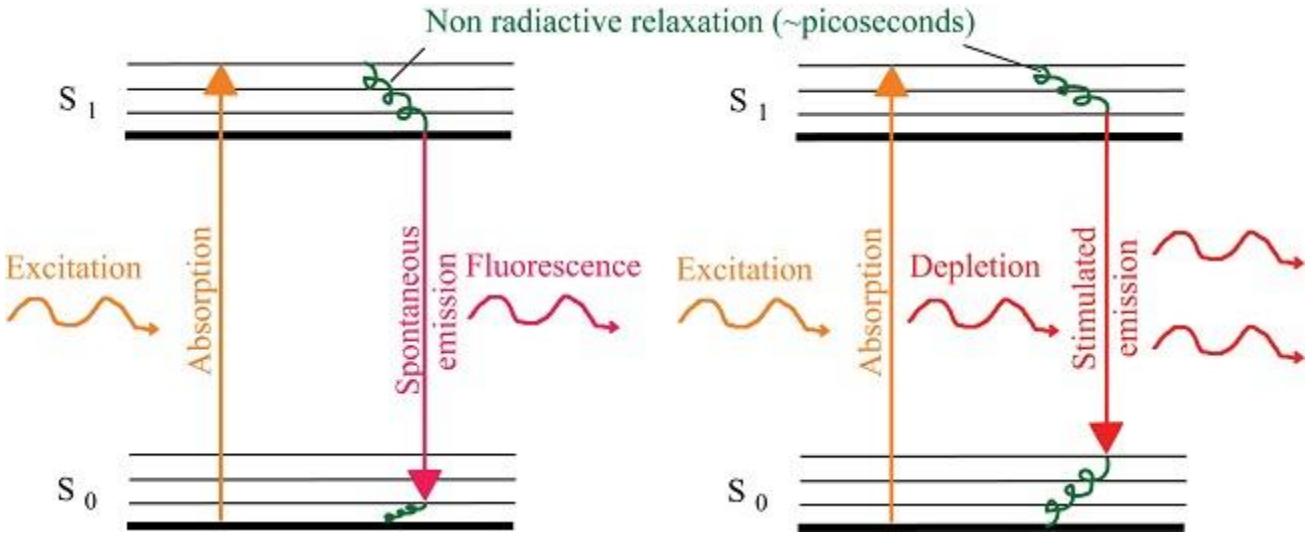
Lipid Experiments
Veronika Mueller
Alf Honigmann
Christian Ringemann
Rebecca Medda
Birka Lalkens
Giuseppe Viccidomini
Haisen Ta
Andreas Schönle

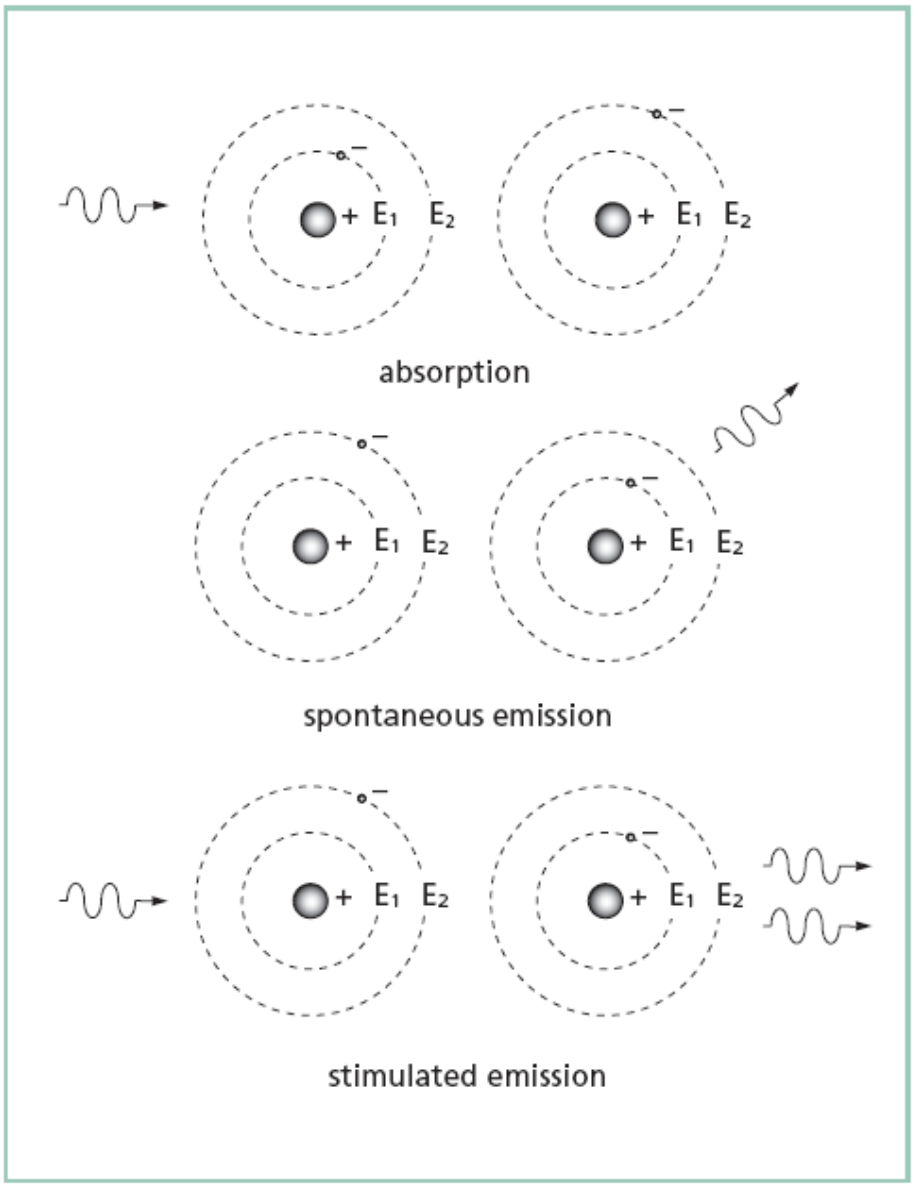
Lipid labeling
Dr. V. Belov
S. Polyakova

Lipid labeling
Dr. G. Schwarzmann
Prof. K. Sandhoff
(Kekule-Inst. Bonn)

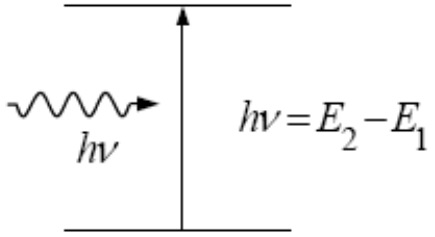
Hop diffusion
Christian Eggeling
Debora Machado-Andrade
Mathias Clausen



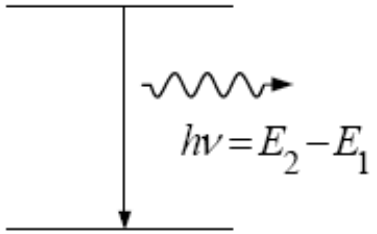

Non radiactive relaxation (~picoseconds)
S 1
S 0
Excitation
Absorption
Spontaneous emission
Fluorescence
S 1
S 0
Excitation
Absorption
Depletion
Stimulated emission



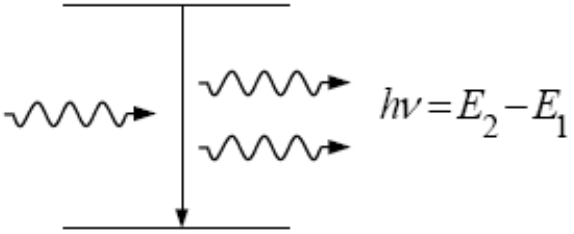
Absorption



Spontaneous Emission



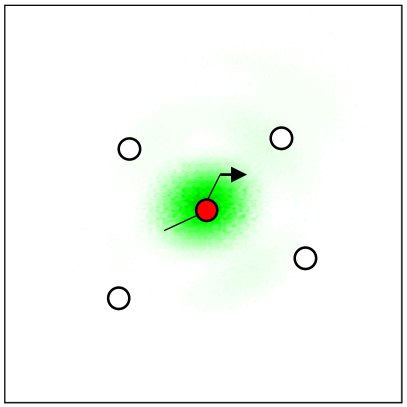
Stimulated Emission

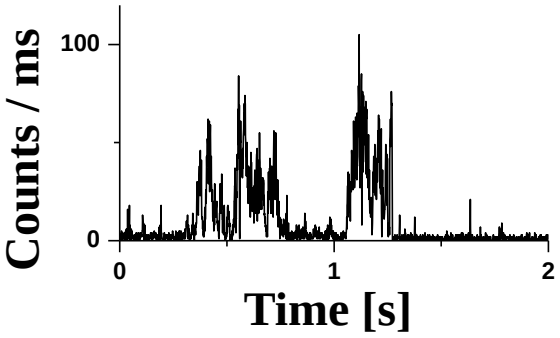


Fluorescence Correlation Spectroscopy
FCS

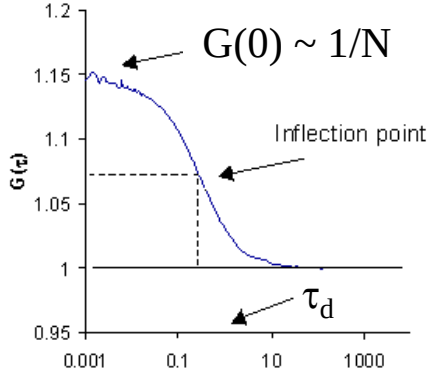
Fluorescence intensity over time

Low fluorescent concentration
⇒ **diffusion of single-molecules**
= fluorescence bursts





Statistics in Time



Log. x-axis: $\mathbf{t_c}$

Fluorescence Correlation Spectroscopy (FCS)
data acquisition - calculation of correlation function
data analysis – length and density of fluctuations

Fitting: anomalous sub-diffusion: $G(t_c) \sim 1/(1 + (t_c/\tau_d)^{\alpha})$
⇒ **transit time** τ_d **(~ mass, obs. area) = decay time**
$\sim d^2 / D$

⇒ **anomaly 1/α:**
(1/α) = 1: normal free diffusion
(1/α) > 1: anomalous diffusion (e.g. trapping)