STED Nanoscopy and FCS

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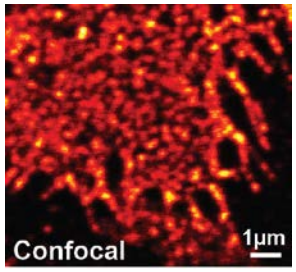
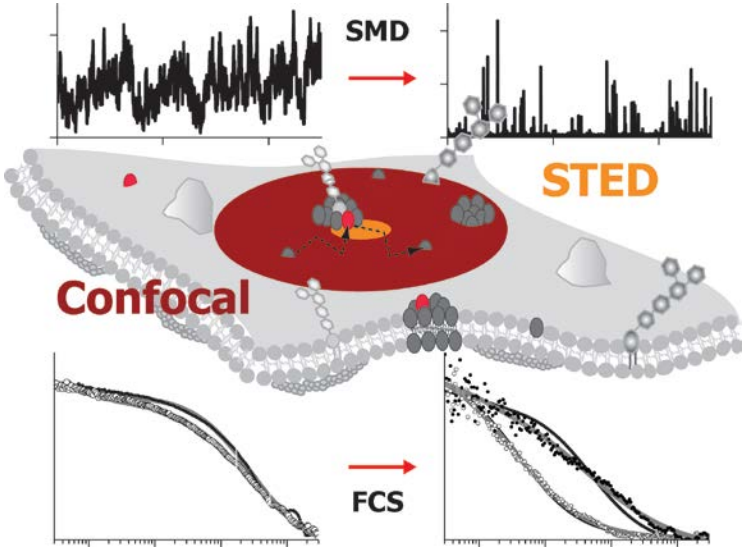
University of Oxford

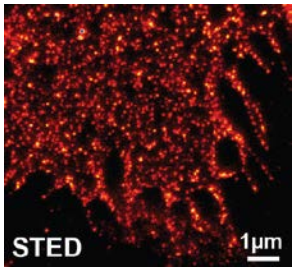
Previously:
Max Planck Institute for biophysical Chemistry
Dep. NanoBiophotonic (Prof. Hell)
Göttingen, Germany

Imaging





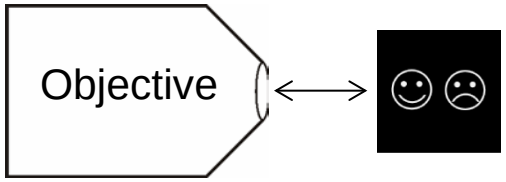
Imaging



Live Cell Microscopy

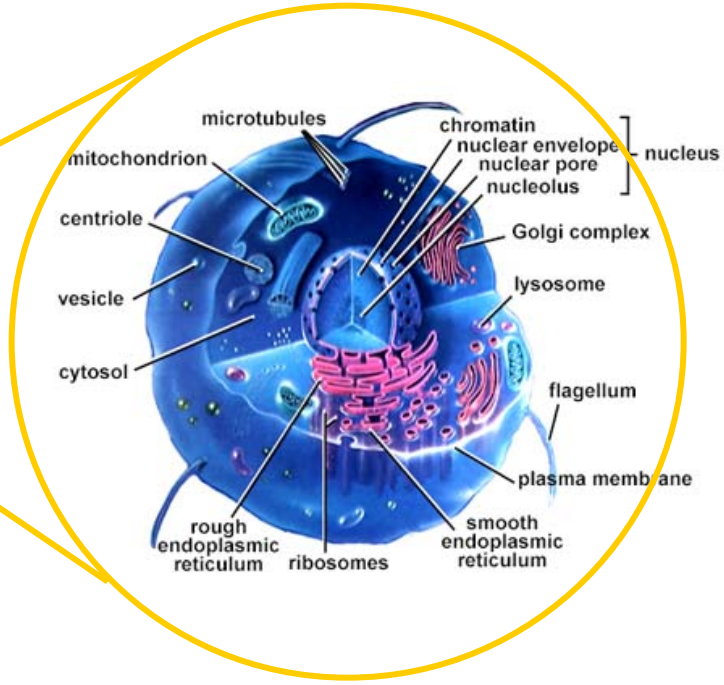
Observation of living cells: Non-Invasive

Light + Far-Field: non-invasive!



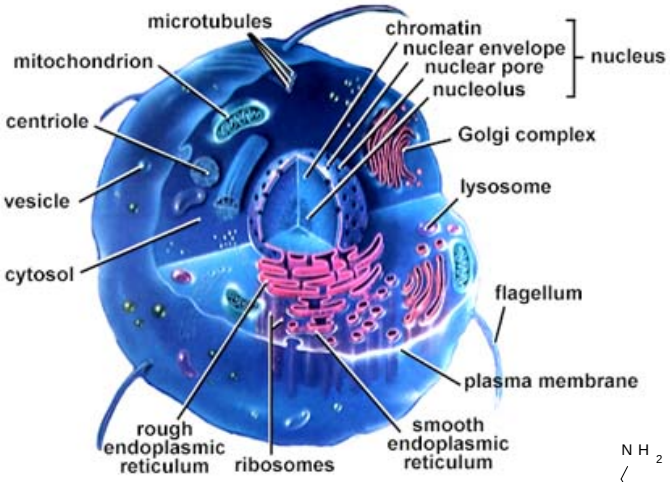
Far-Field

> 1 µm

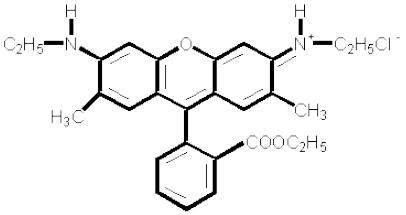


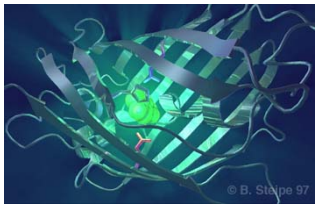
Live Cell Far-Field Microscopy

Fluorescence



Fluorescence-Labeling!



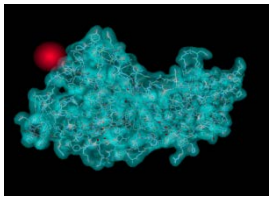


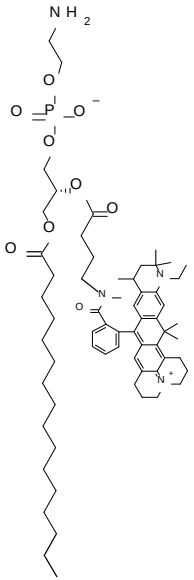
... from chemists

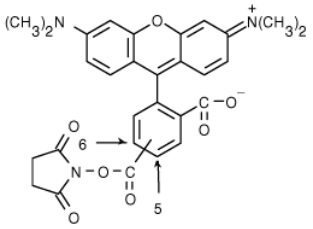
(organic + anorg.)

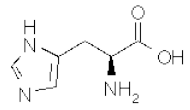
... from biology

(proteins)

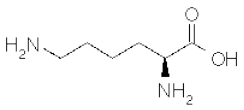




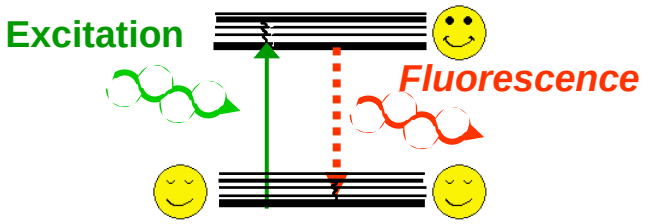


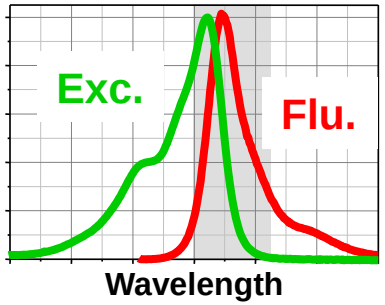


his h Histidin



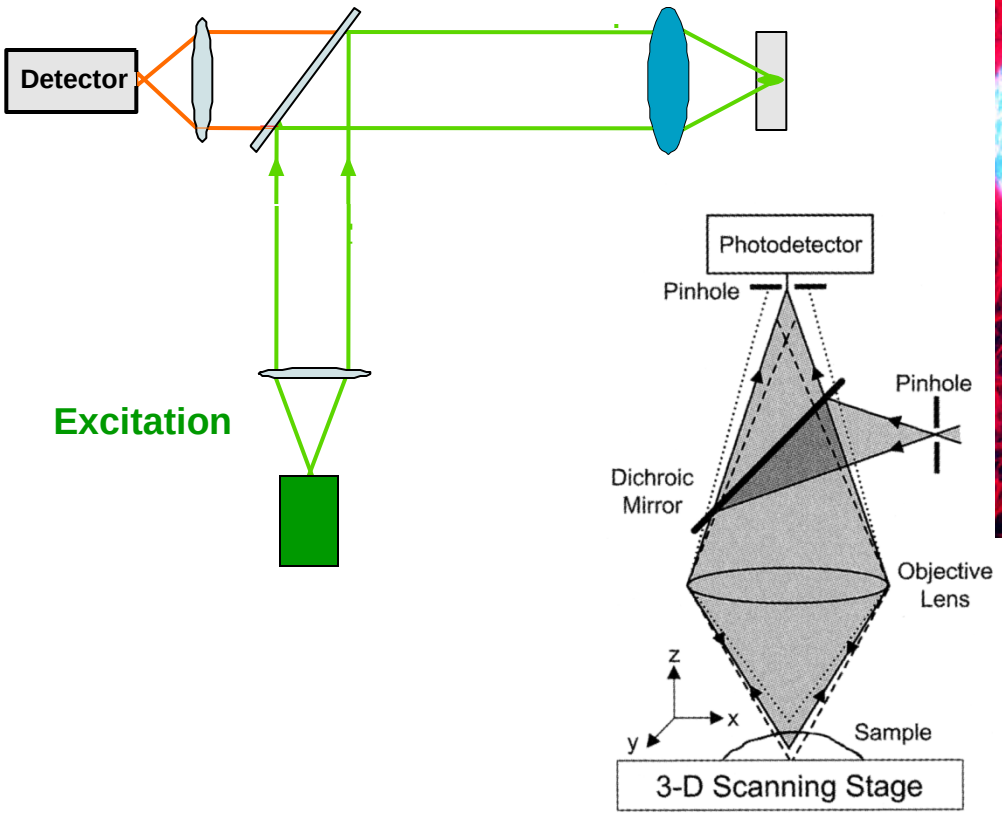
lys k Lysin

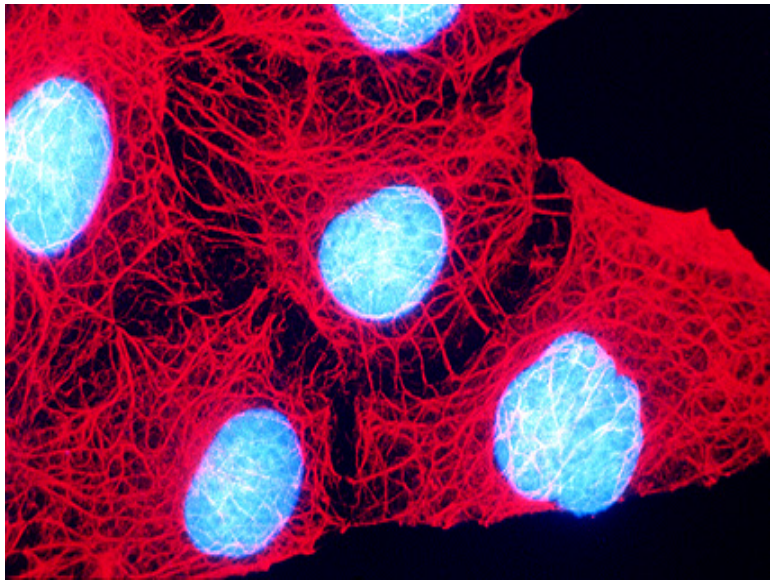




Far-Field Fluorescence Microscopy

Confocal Setup



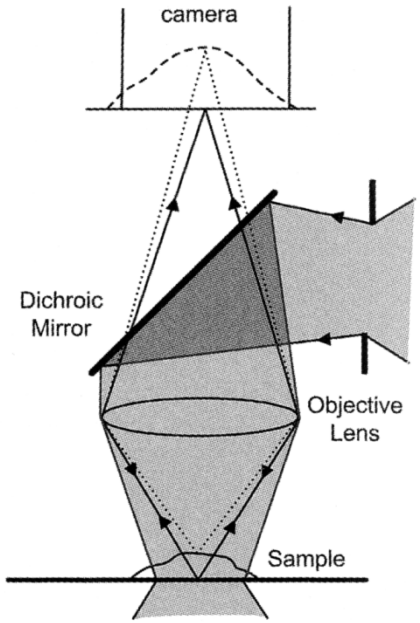


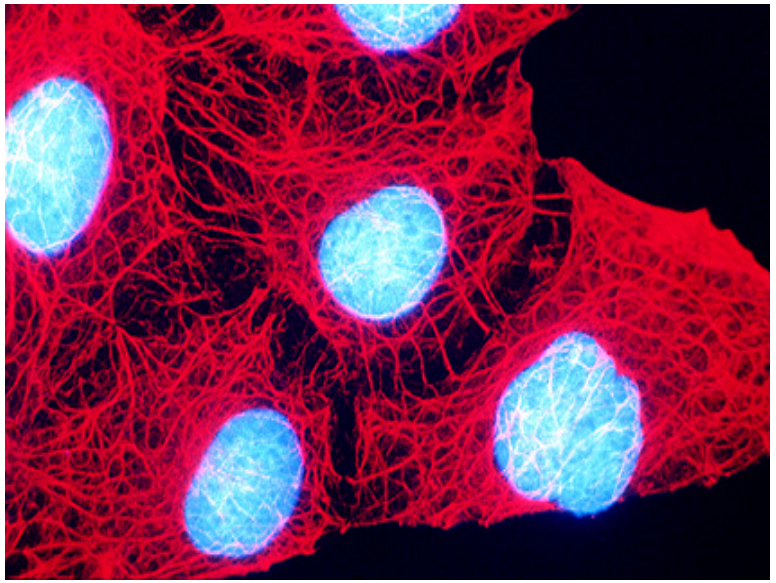
Liver-Cells: Nucleus and **Cell-skeleton**

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

Camera-Based Far-Field Microscopy

Wide-Field Setup



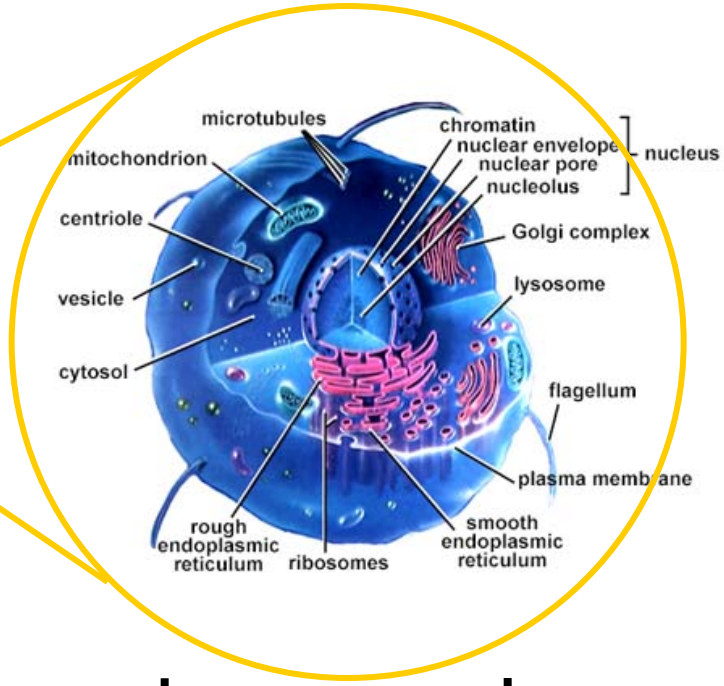


Liver-Cells: Nucleus and **Cell-skeleton**

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

Far-Field Fluorescence Microscopy

Resolution: Goal

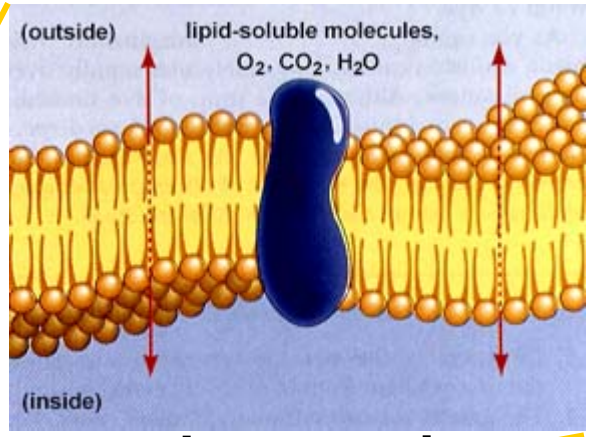


10 µm

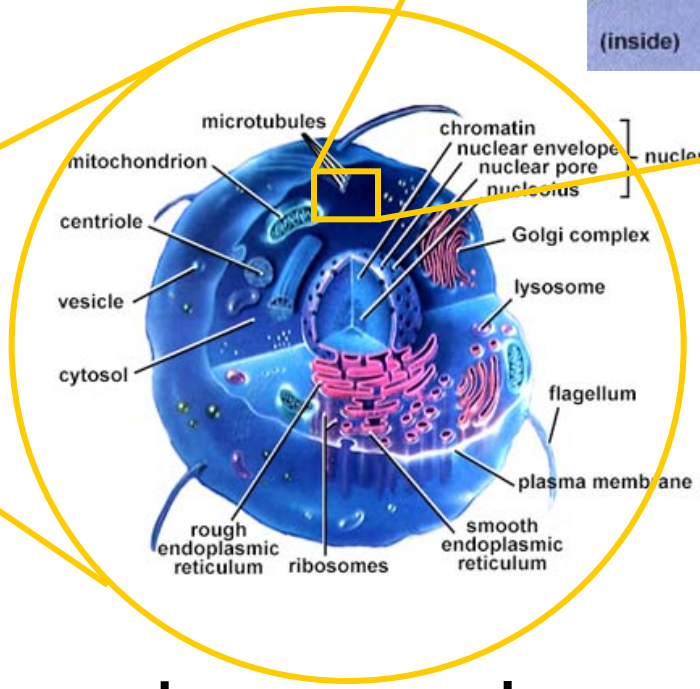
Far-Field Fluorescence Microscopy

Resolution: Goal

⇒ **molecular scale**



10 nm



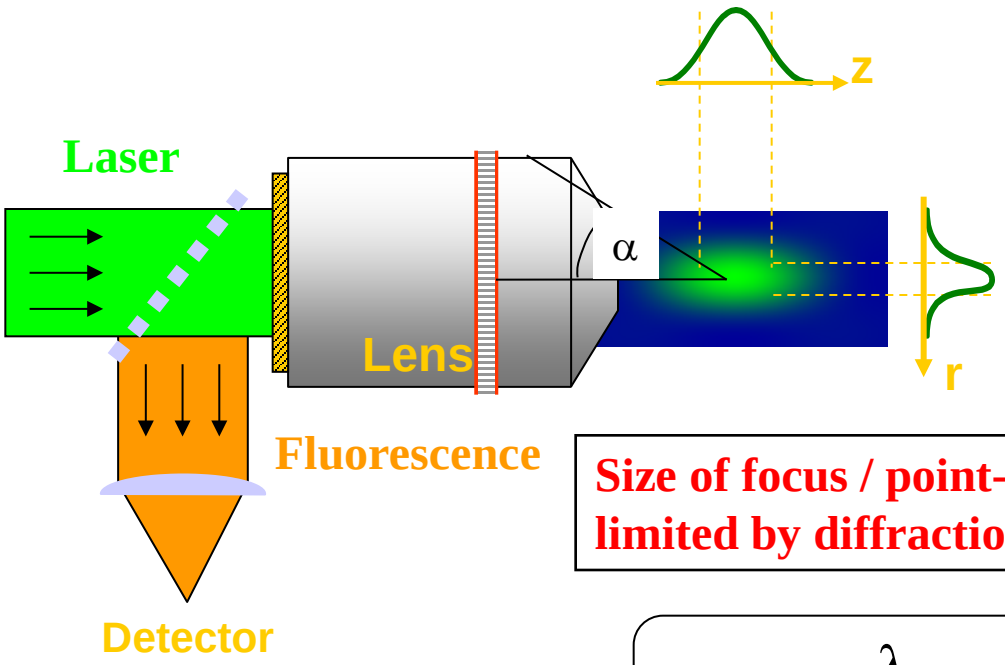
10 µm

Far-Field Microscopy

Resolution Limit: Diffraction Barrier

Far-Field Fluorescence Microscopy: Focussing of light

- away from surfaces – inside cells (3D)



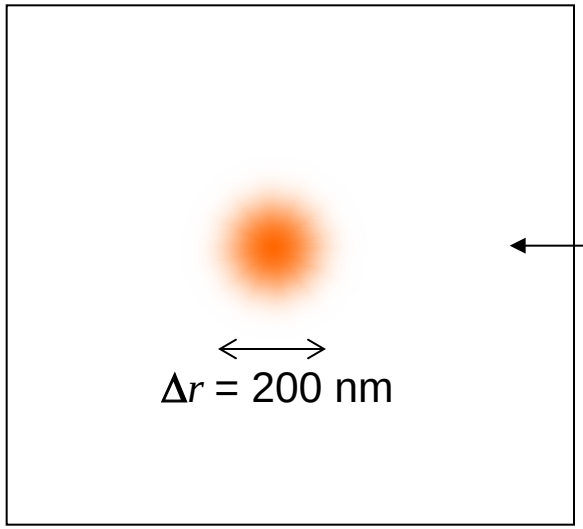
Size of focus / point-spread function limited by diffraction of light!!!

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

Ernst Abbe 1873

Far-Field Microscopy

Resolution Limit: Diffraction Barrier



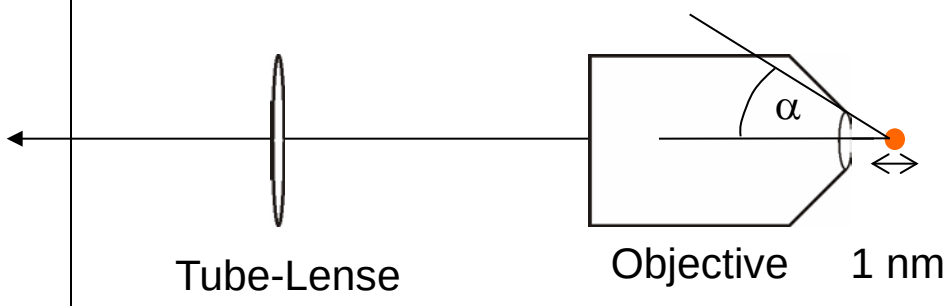
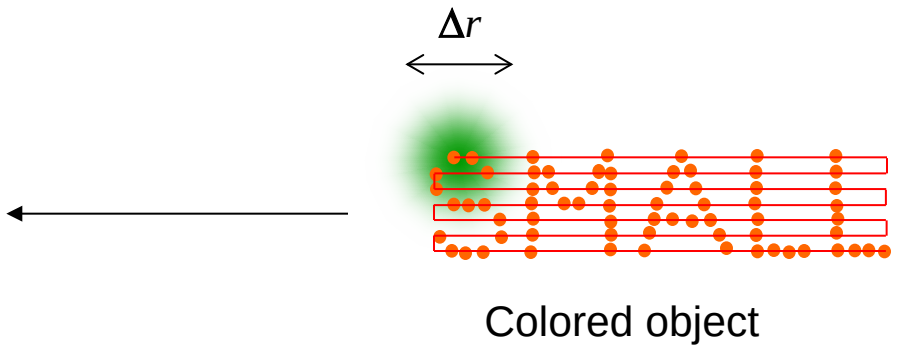
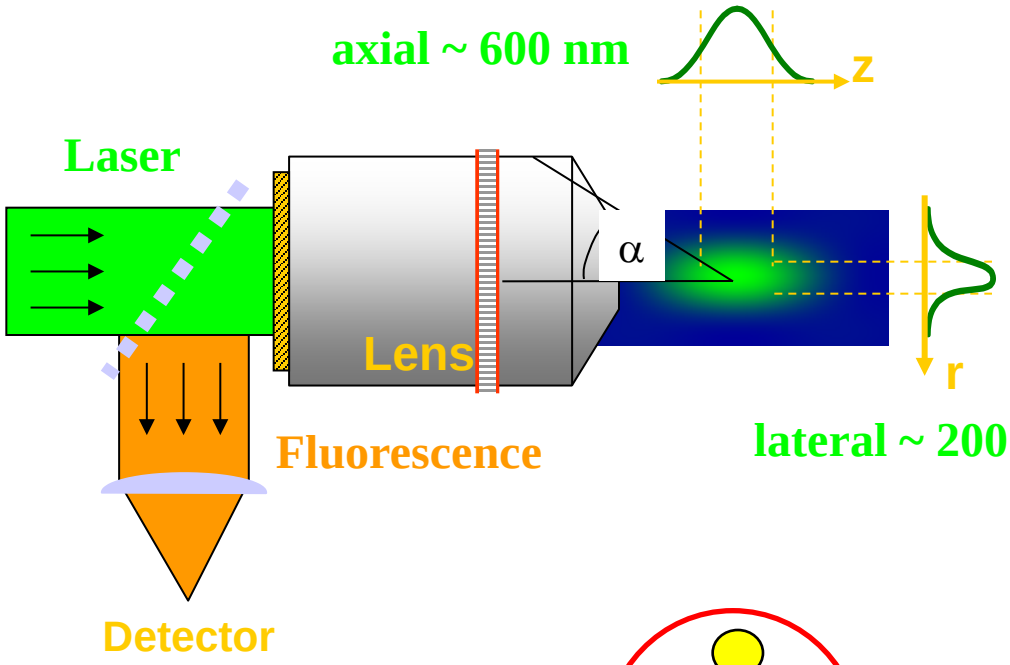


Image: diffraction-limited



Far-Field Microscopy

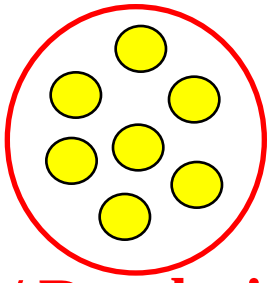
Surpassing the Resolution Limit: Turning ON/OFF



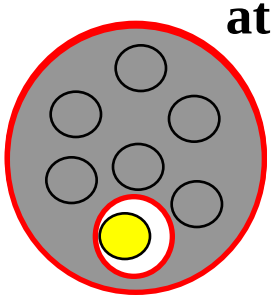
$$\Delta x = \frac{\lambda}{2n \sin \alpha}$$

Ernst Abbe 1873

⇒ **Turning ON/OFF**



Turn OFF all objects but at a single point



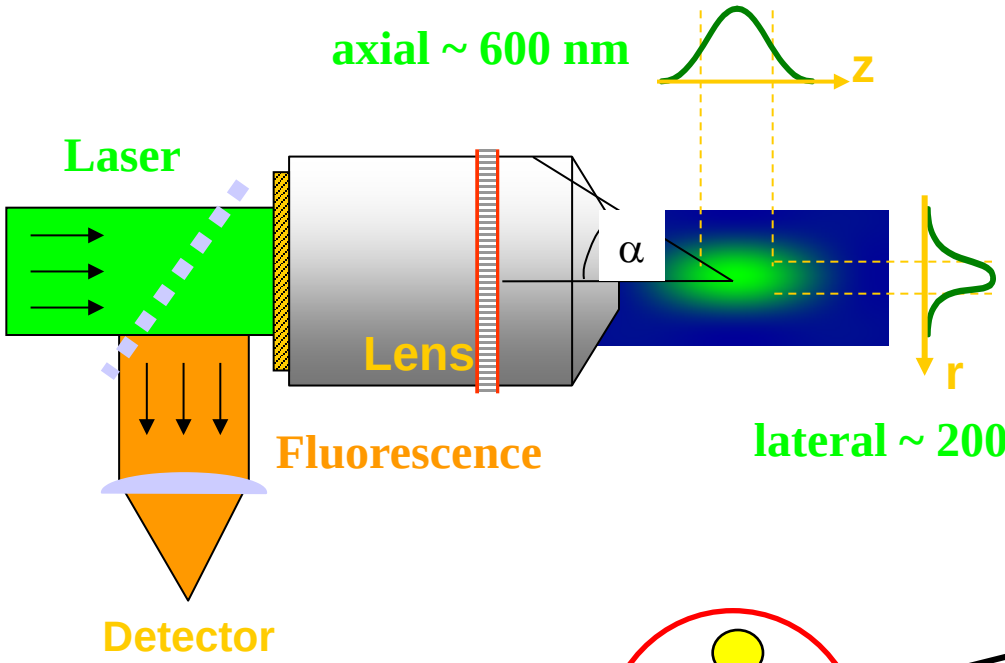
⇒ **Observation area / Resolution**

lateral << 200 nm

axial << 600 nm

Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF



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Ernst Abbe 1873

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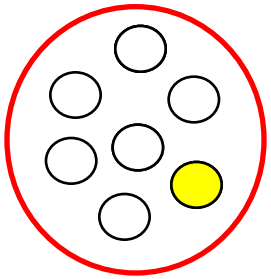
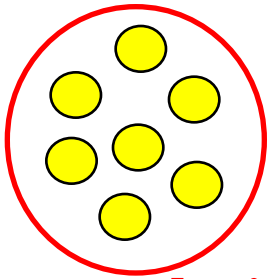
Turn OFF all but a single object





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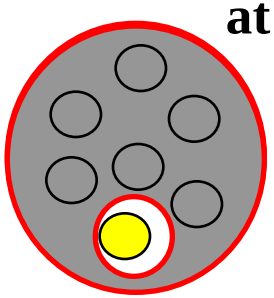

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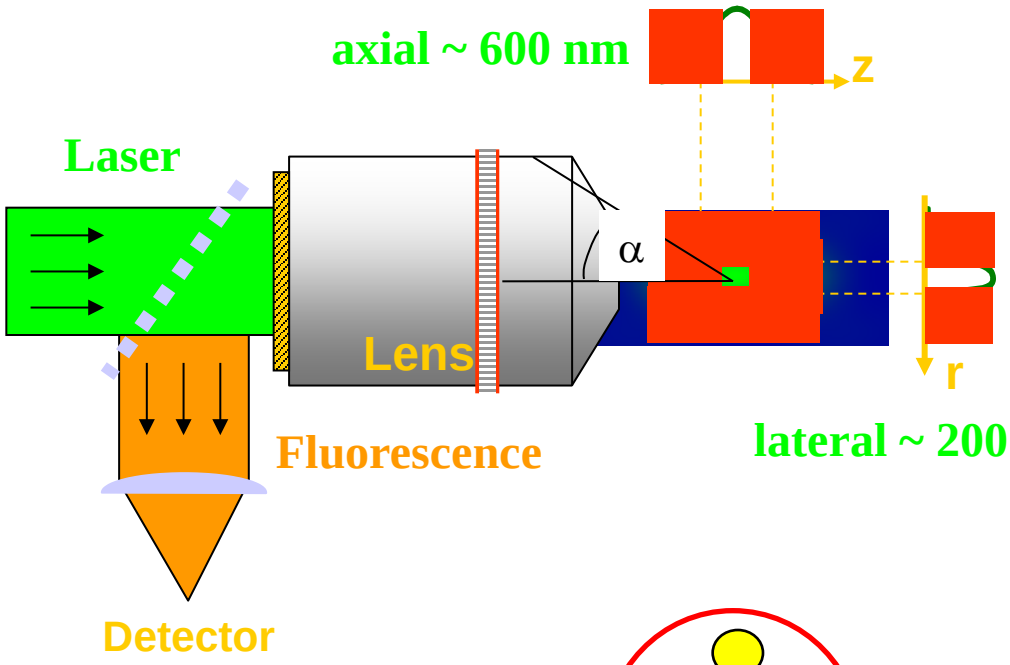
axial << 600 nm

Far-Field Microscopy

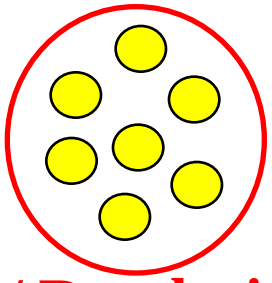
Surpassing the Resolution Limit: Turning ON/OFF



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

Ernst Abbe 1873

⇒ **Turning ON/OFF**



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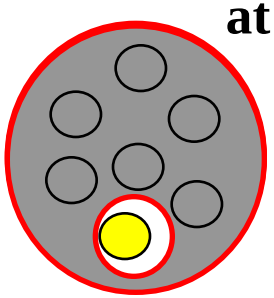

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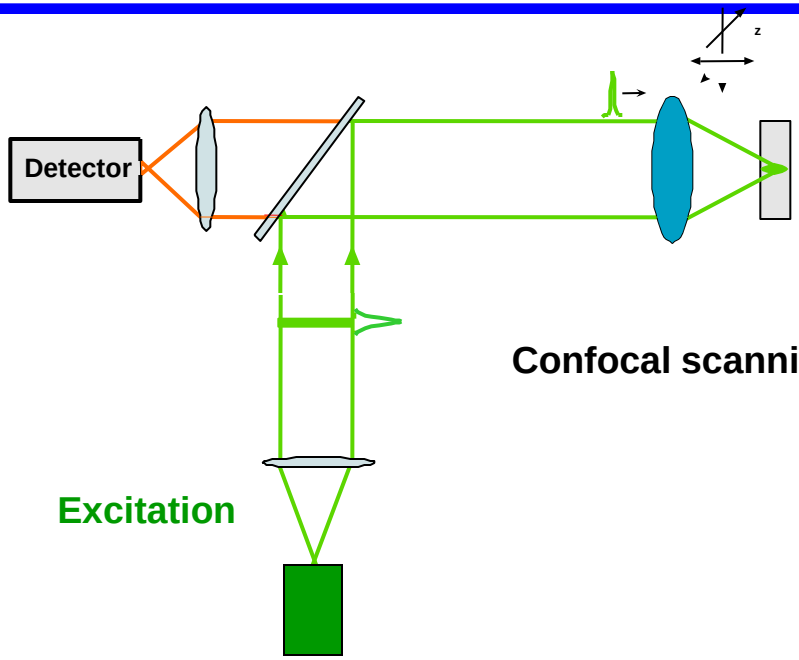
lateral << 200 nm

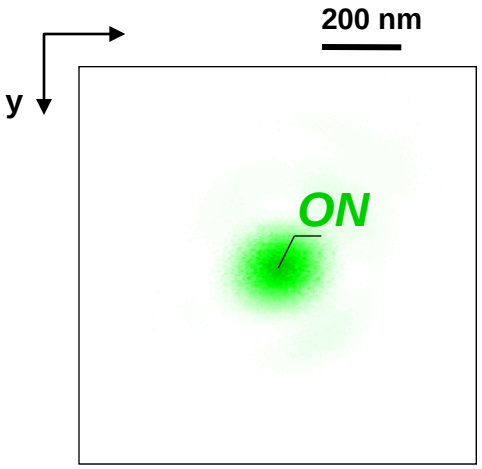
axial << 600 nm

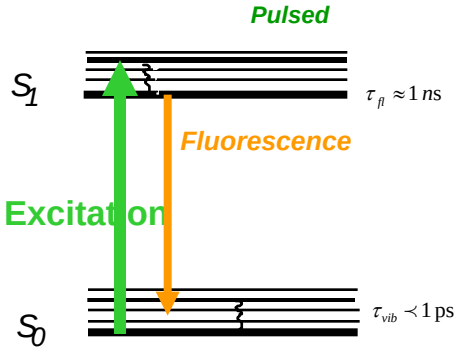
Fluorescence Microscopy

STED Microscopy



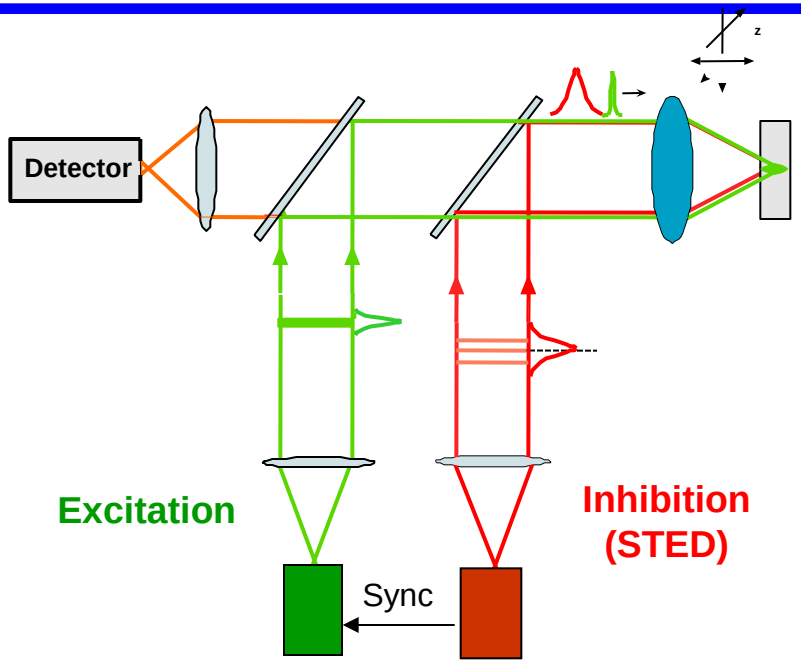
Confocal scanning microscope

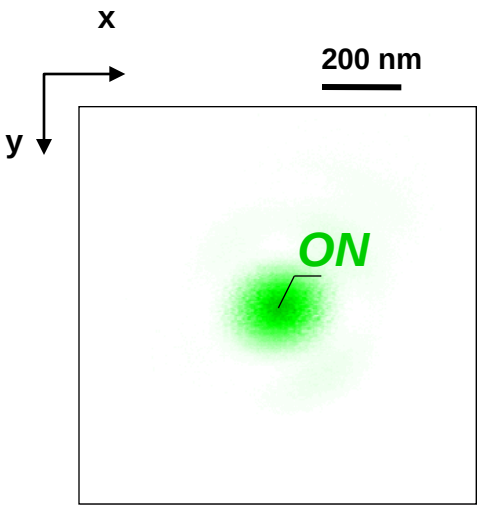


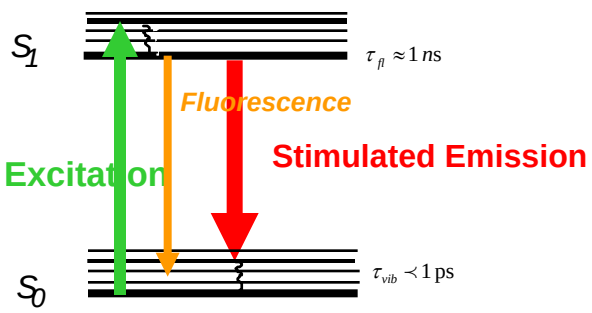


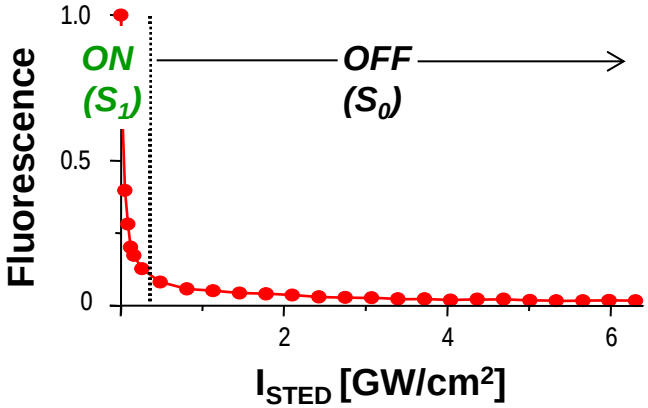
Fluorescence Microscopy

STED Microscopy



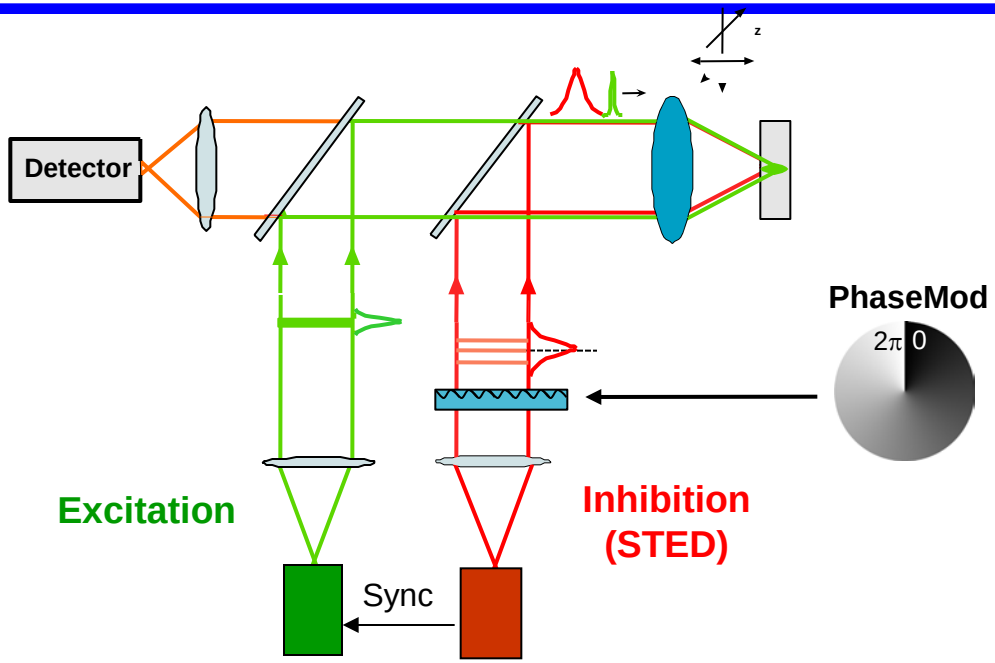


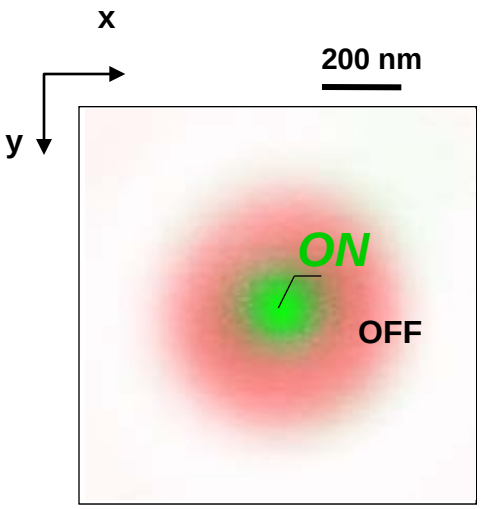


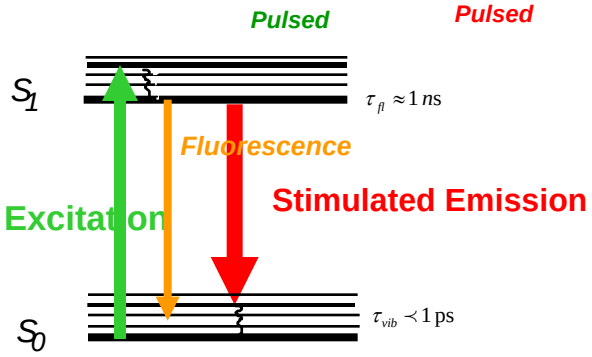


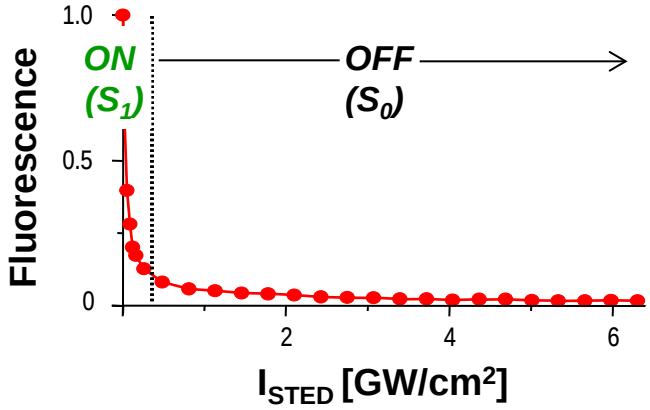
Fluorescence Microscopy

STED Microscopy



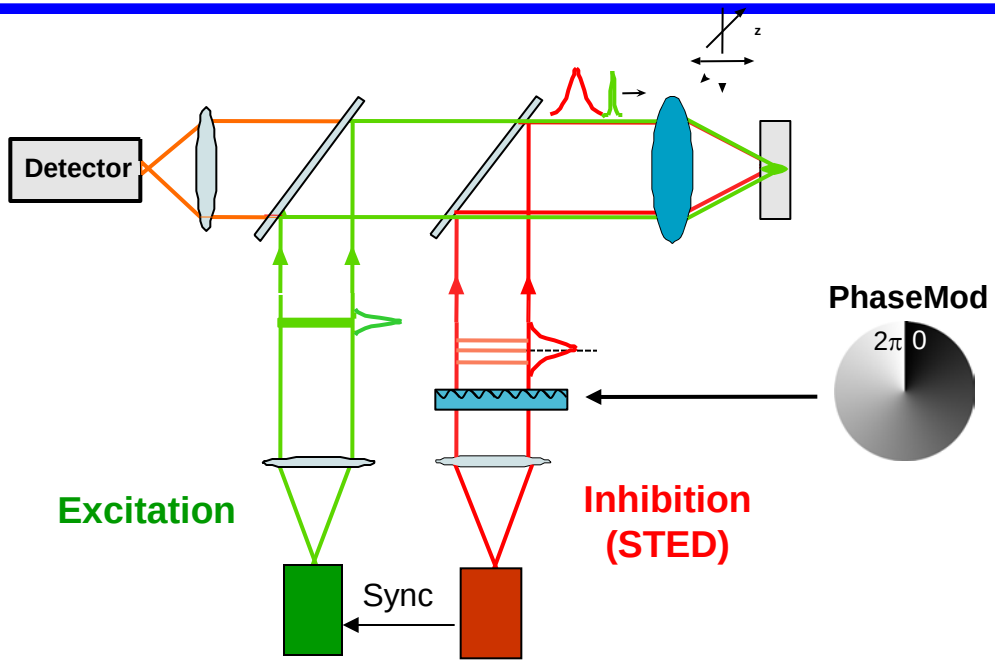


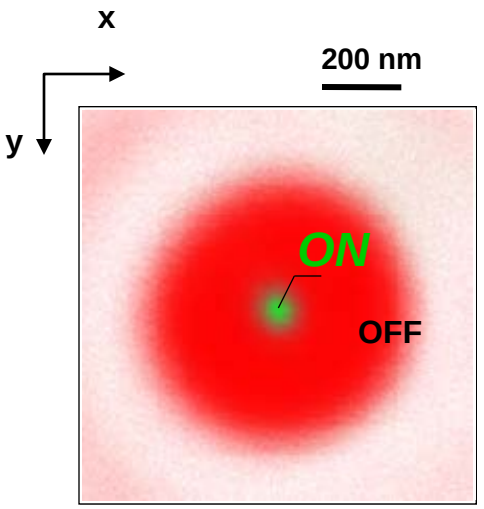


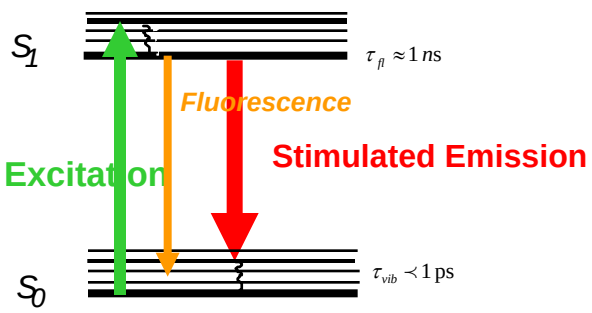


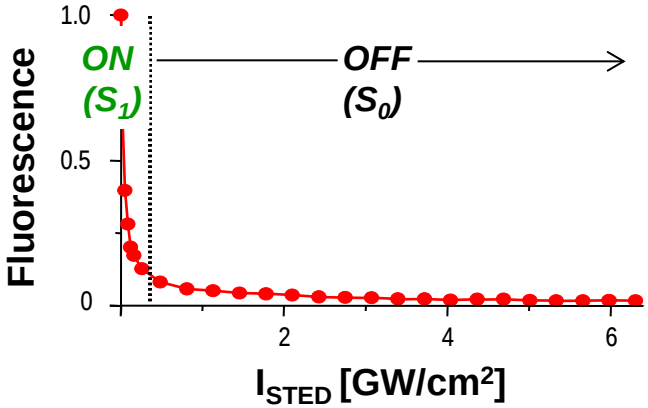
Fluorescence Microscopy

STED Microscopy





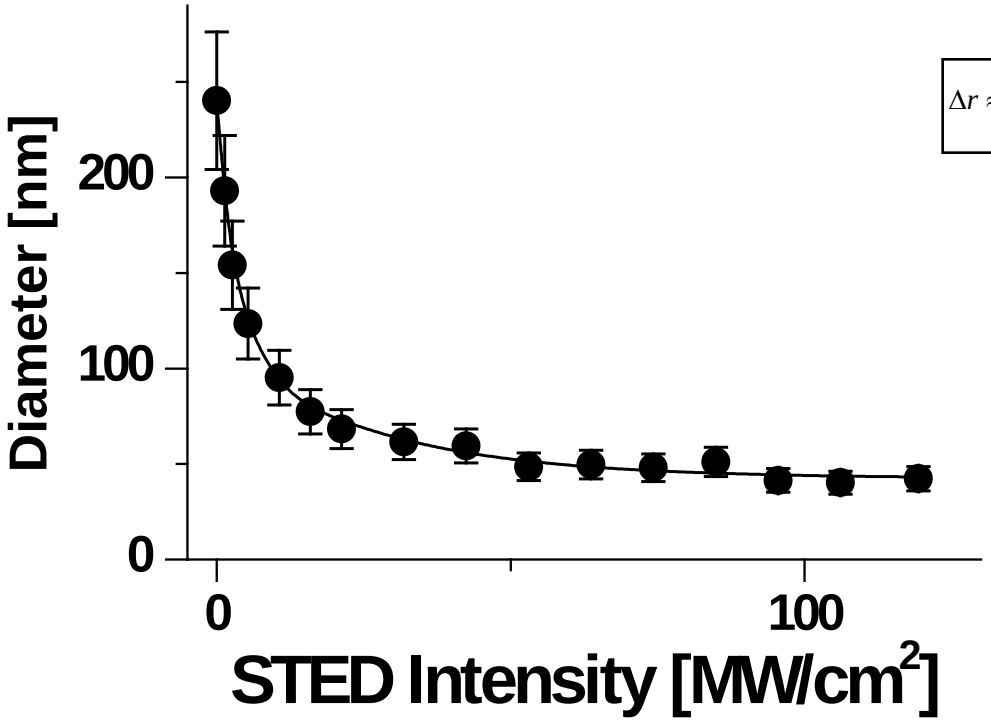




STED Microscopy

Dynamical confinement of resolution

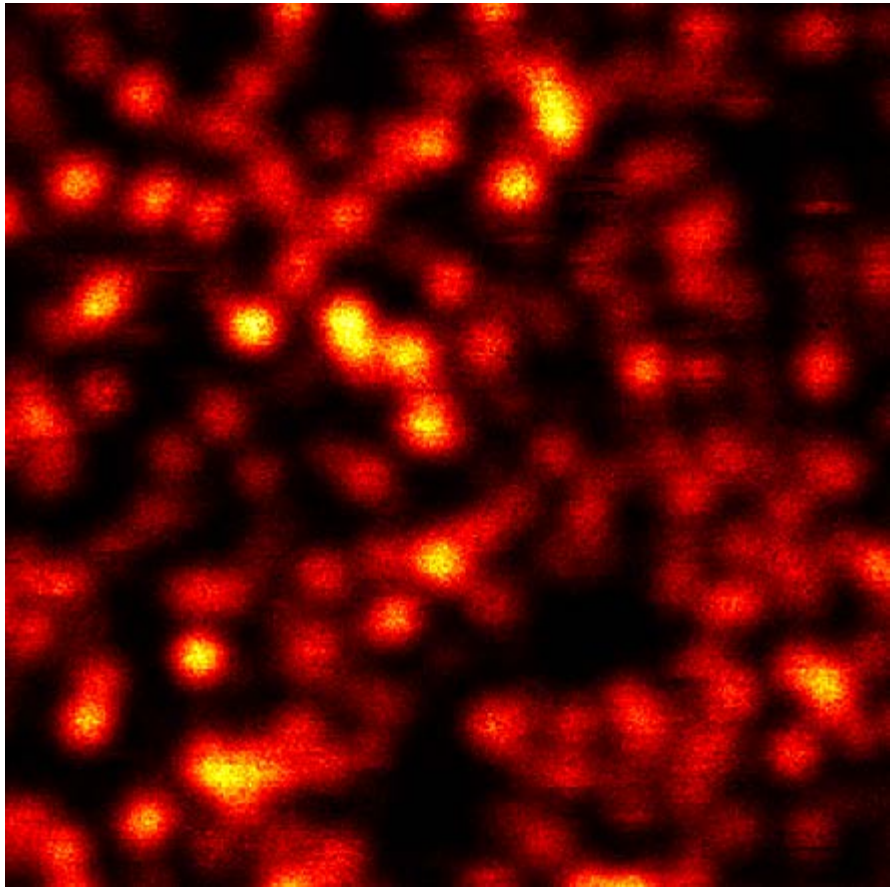
Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!

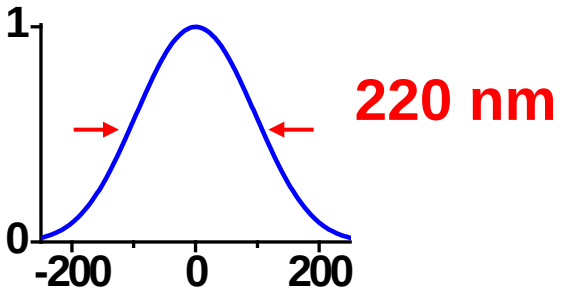


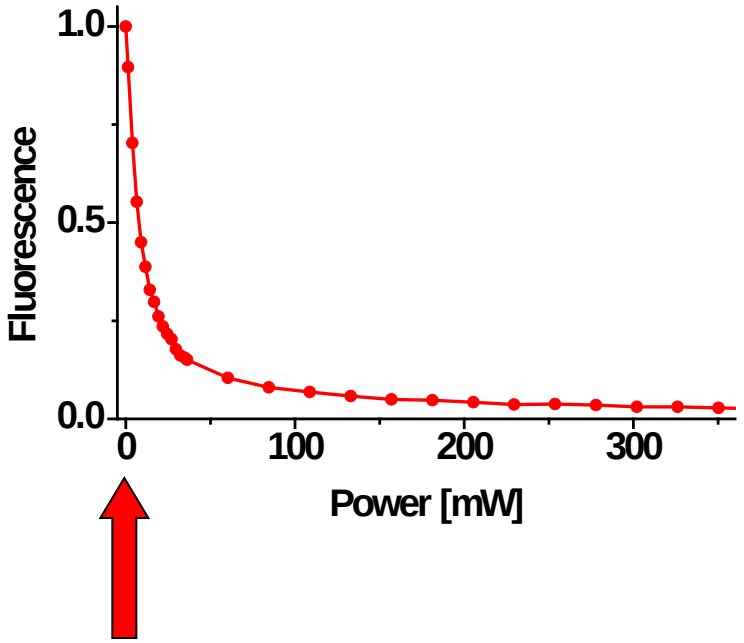
$$\Delta r \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+I/I_{sat}}}$$

STED-Microscopy

Sub-Diffraction Imaging



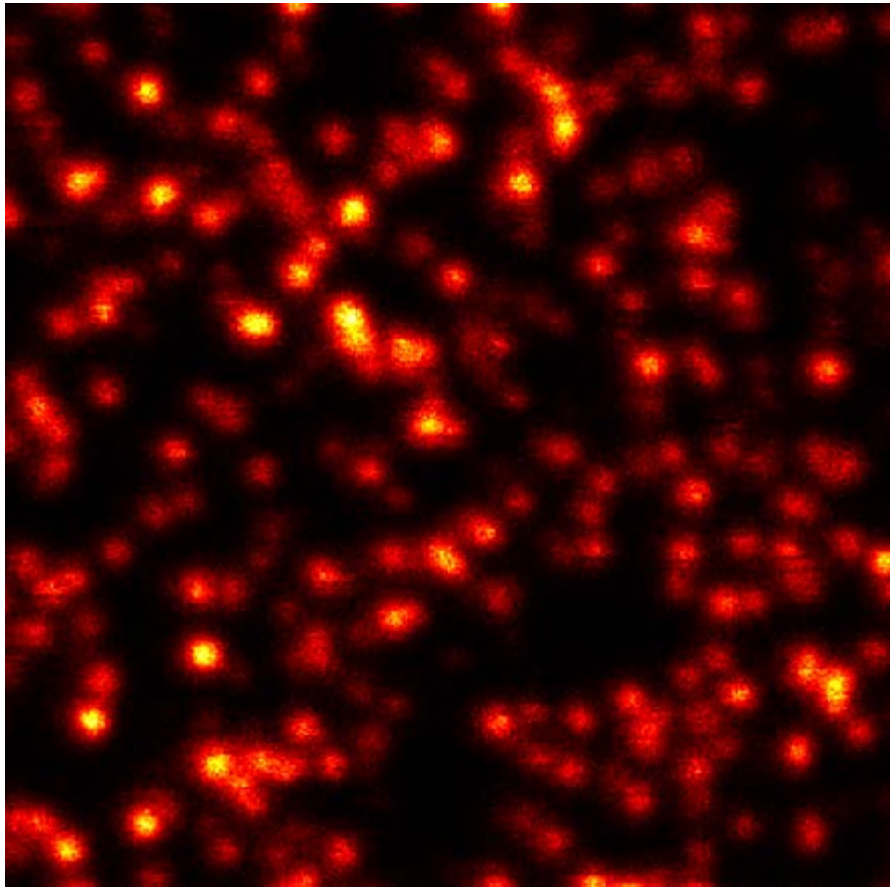


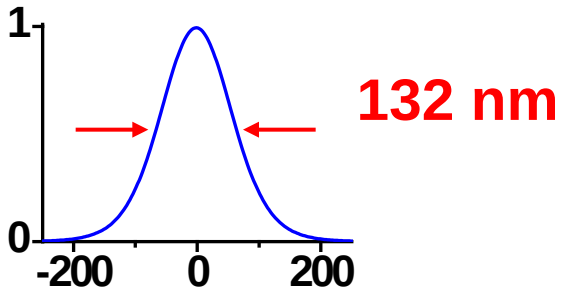


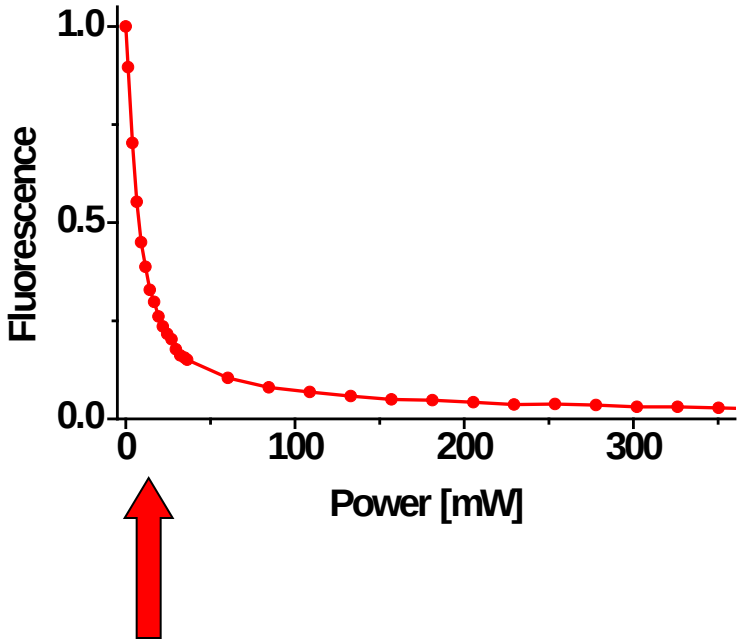
20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging



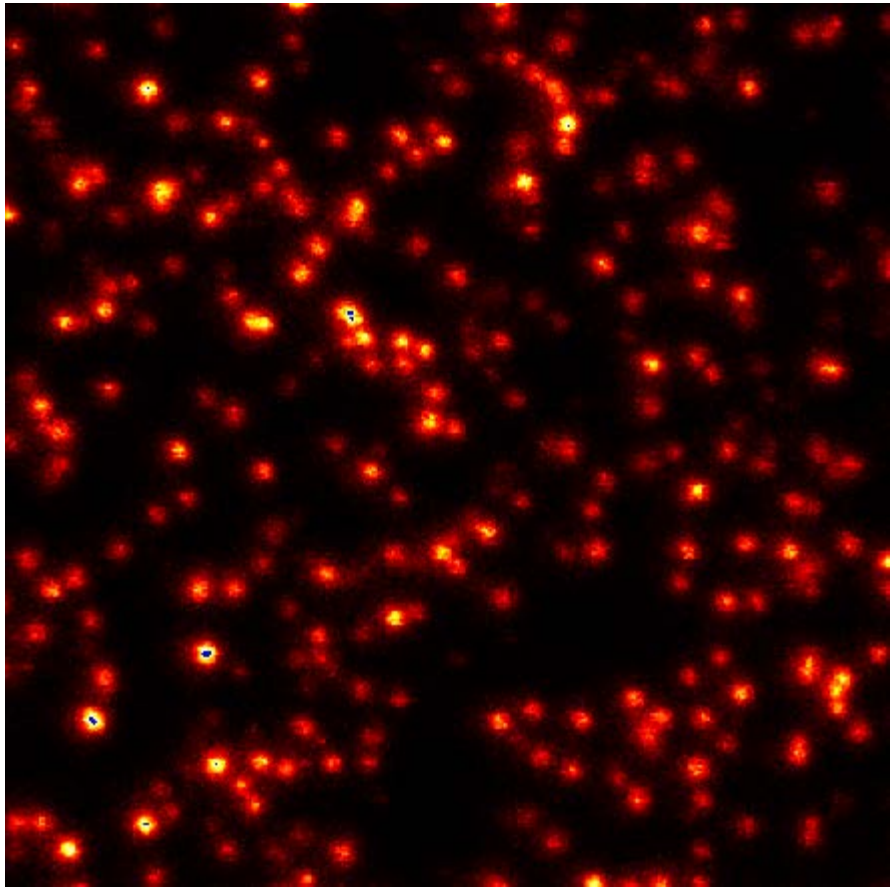


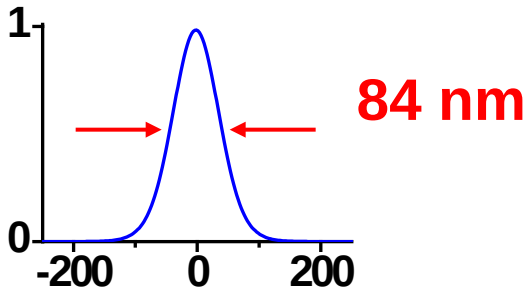


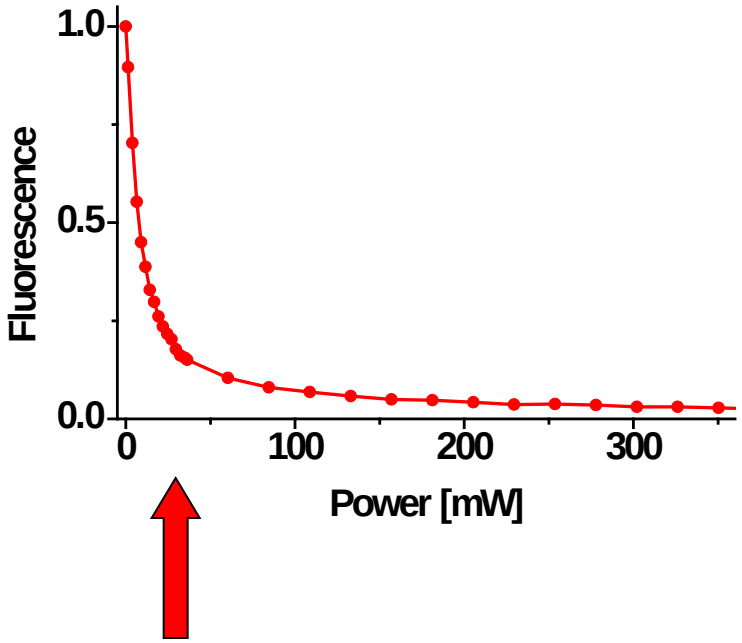
20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging



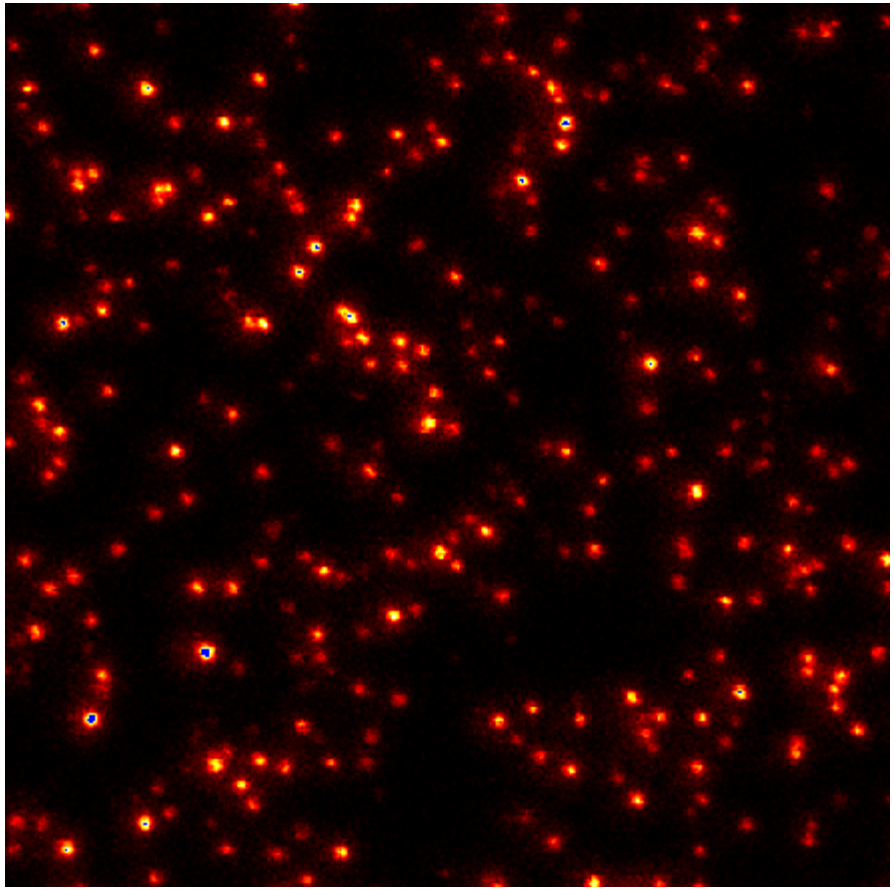


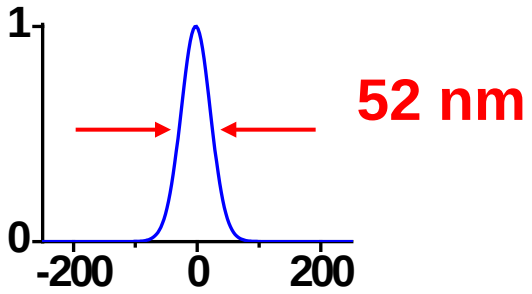


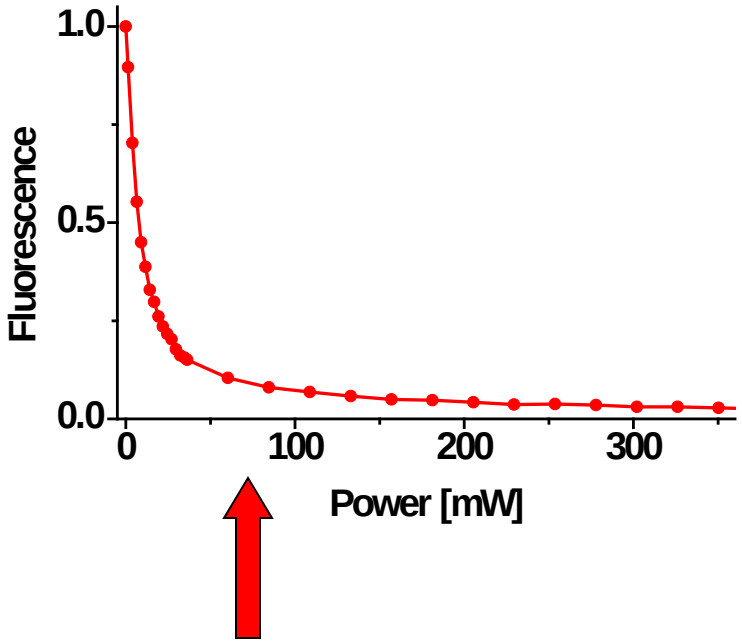
20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging



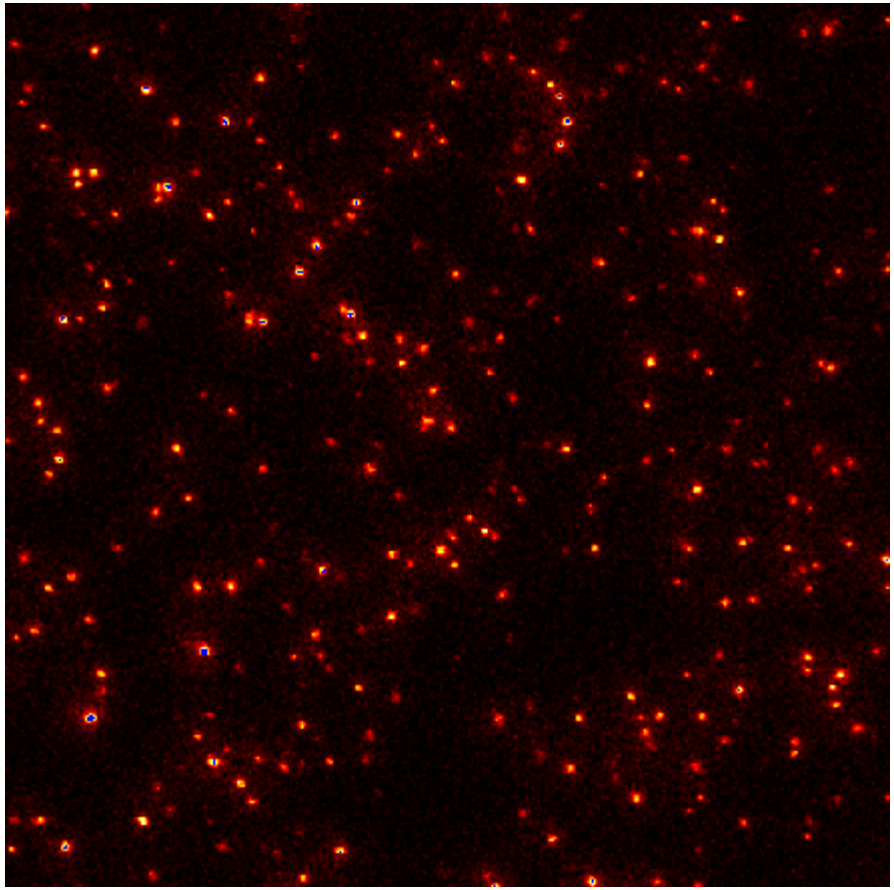


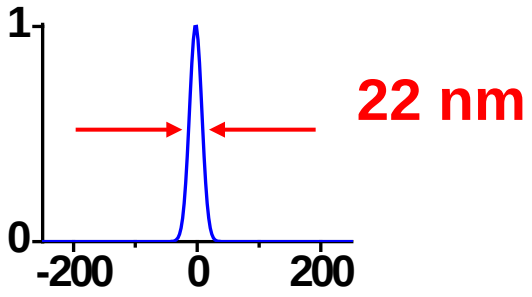


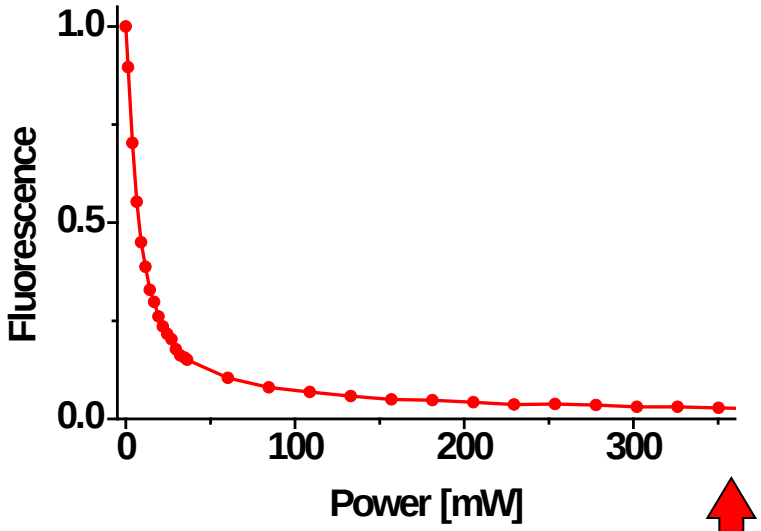
20nm Crimson beads
633nm exc, 90ps, 30kW/cm^2
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging





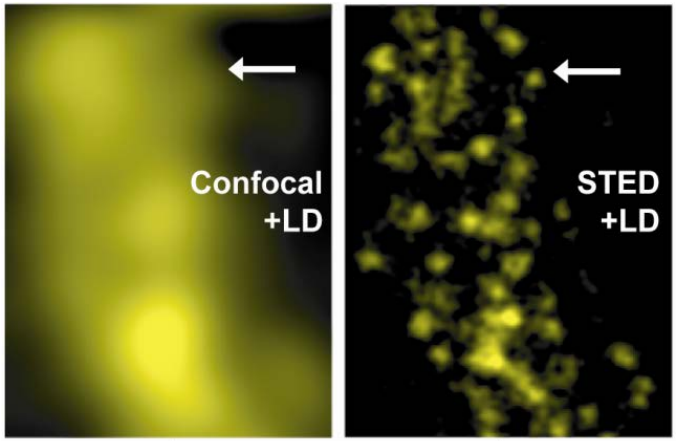


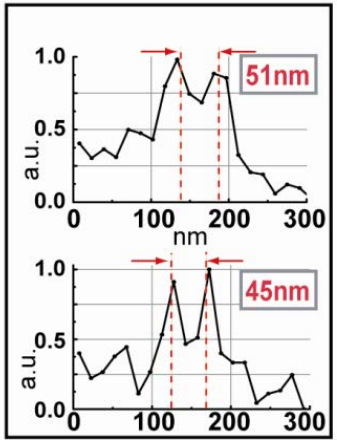
20nm Crimson beads

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

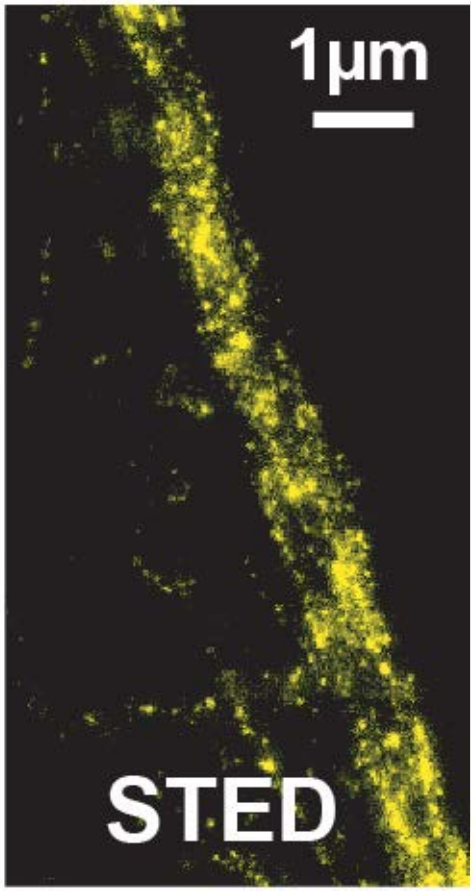
785nm STED 200ps, 76MHz

STED Microscopy
Cellular Imaging



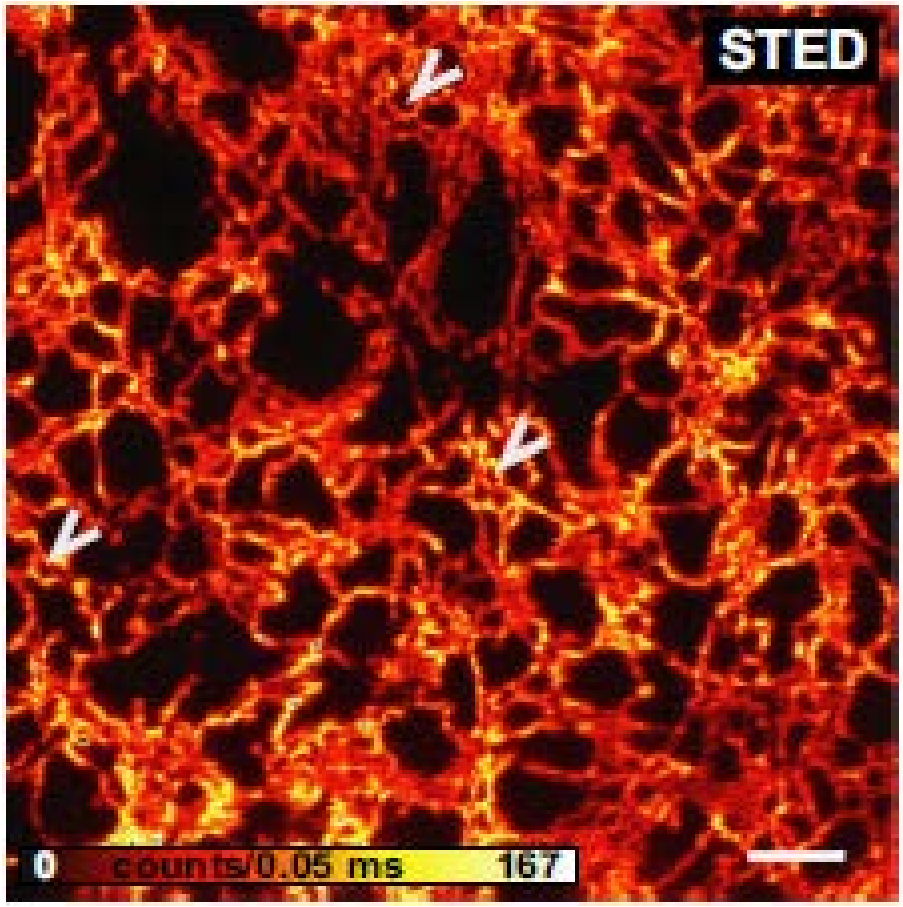


protein-heavy subunit of **<u>neurofilaments in the human neuroblastoma</u>** cell line SH-SY5Y (retinoic acid–BDNF-differentiated);
establishes cross-links to organize and stabilize neurofilaments in axons



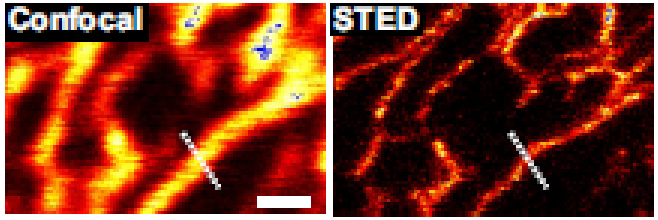
Donnert et al, PNAS 2006

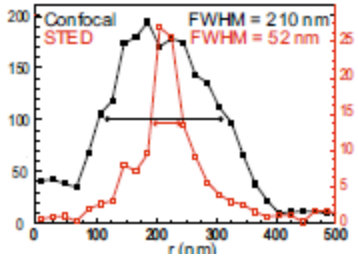
STED-Microscopy
Inside Living Cells



Living Cells:

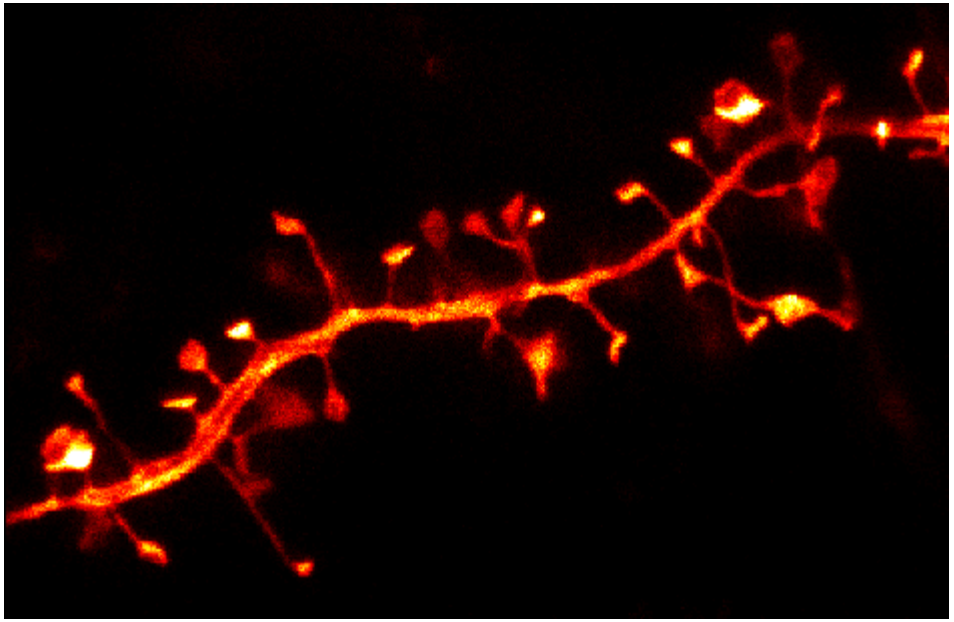
Citrine, Endoplasmatic Reticulum (ER)
Live PtK2 cells
Hein, Willig, Hell PNAS 2008



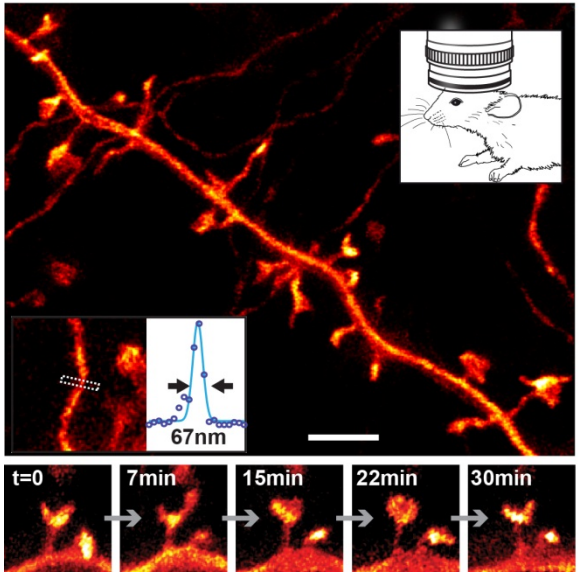


STED Microscopy
Inside Living Cells - Dynamics

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



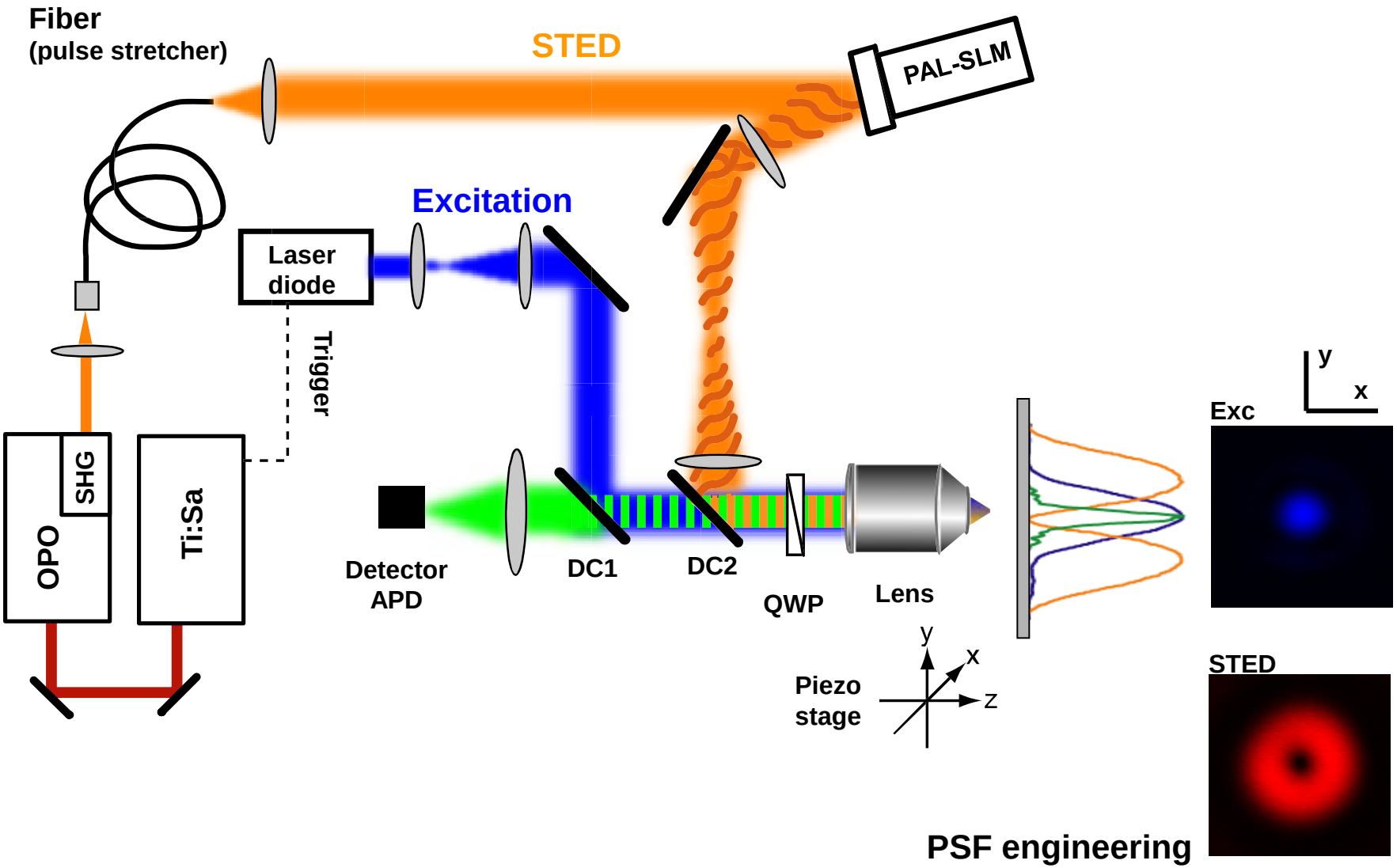
Live Mouse
YFP
(Science Berning et al 2012)



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

STED-Microscopy

Setup

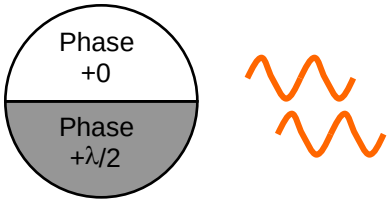


PSF engineering

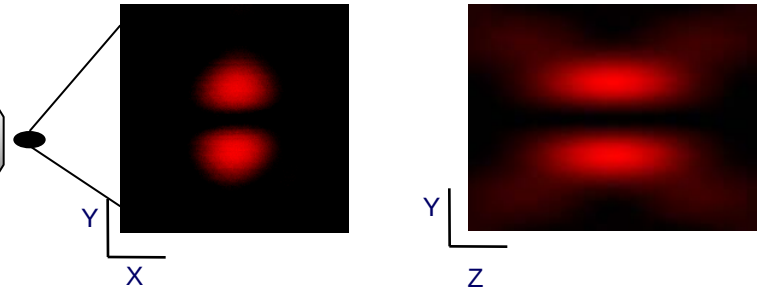
Focal Volume Confinement

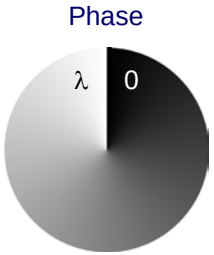
Focal Engineering – Local Zero

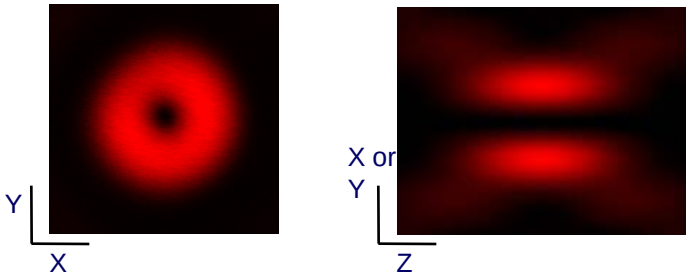
Phase mask:

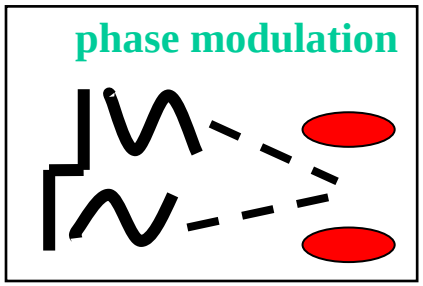


STED PSF:







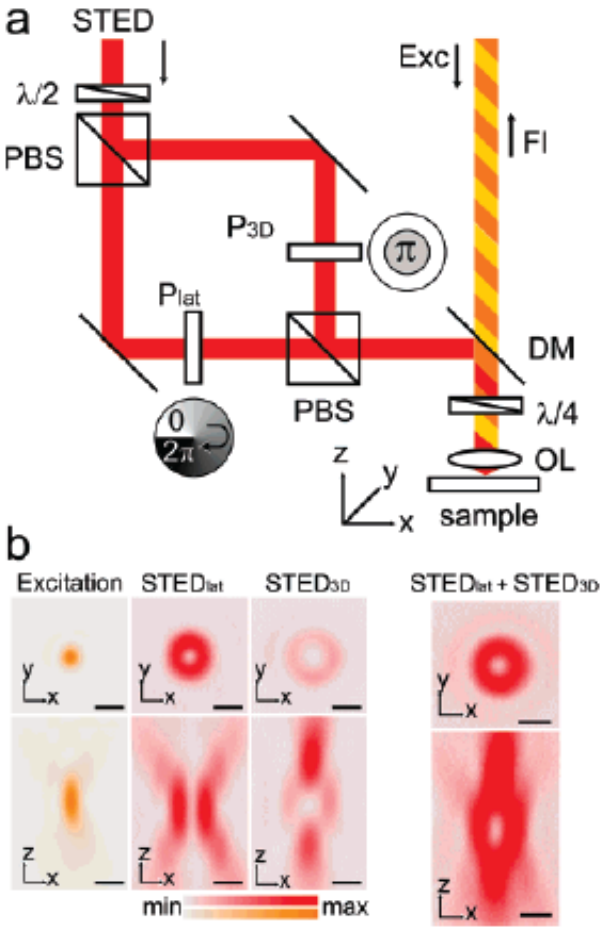


Klar et al, PNAS 2000

STED-Microscopy

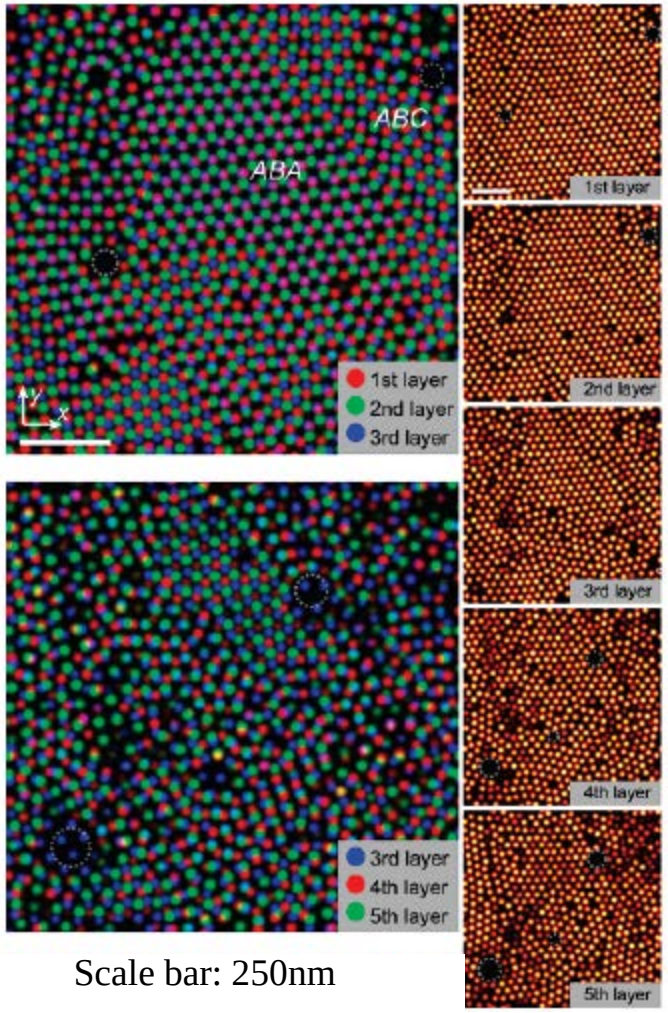
Sub-Diffraction Imaging – 3D

3D STED nanoscopy I



Harke / Ullal et al NanoLett 2008

Fluorescent 100nm Beads – multiple layers on cover glass

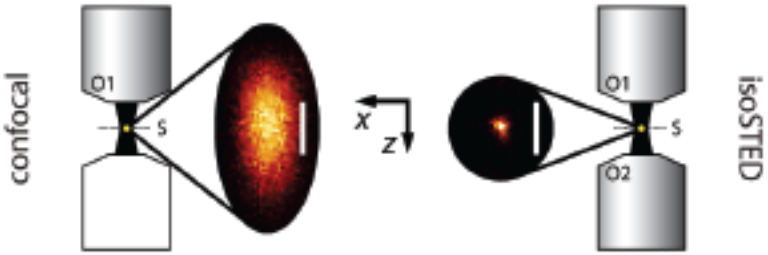


Scale bar: 250nm

STED-Microscopy

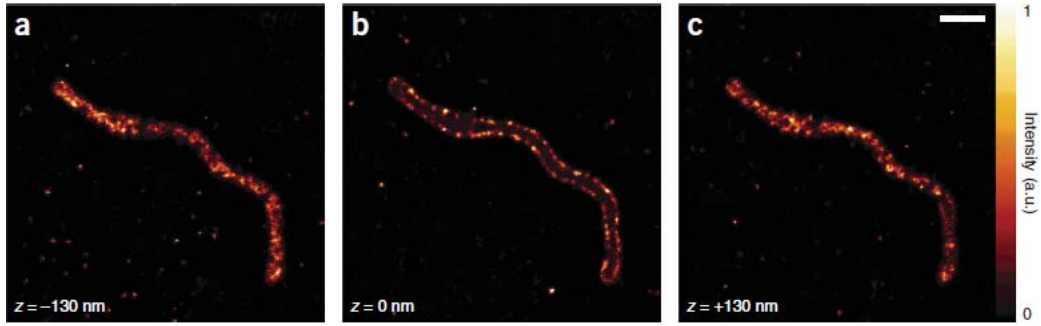
Sub-Diffraction Imaging – 3D

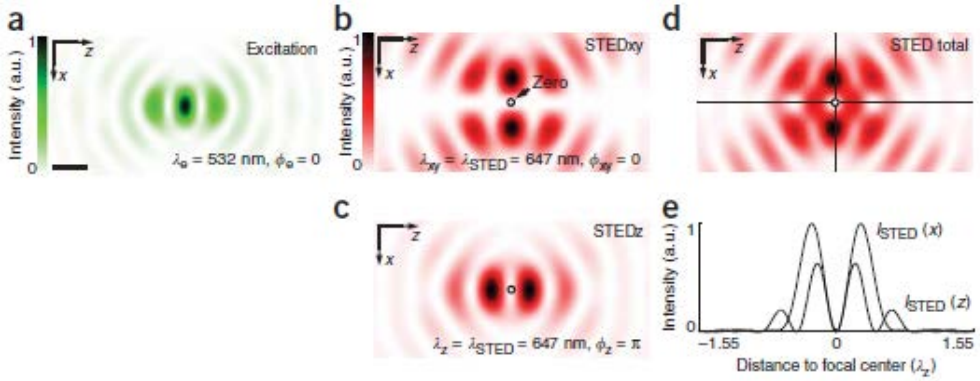
3D STED nanoscopy II – iso STED

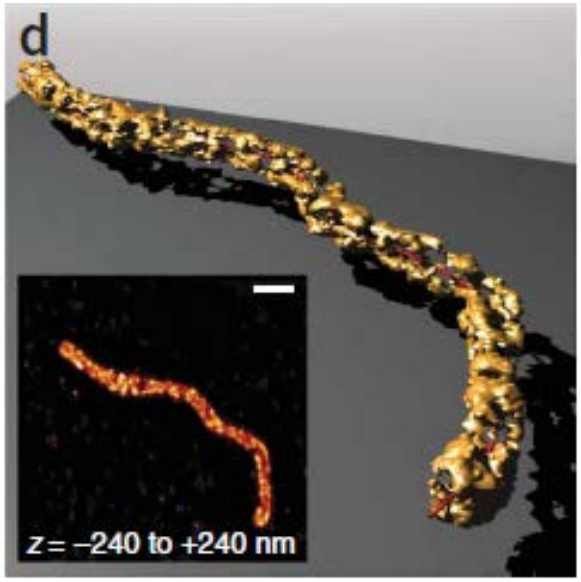


Scale bar: 250nm

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





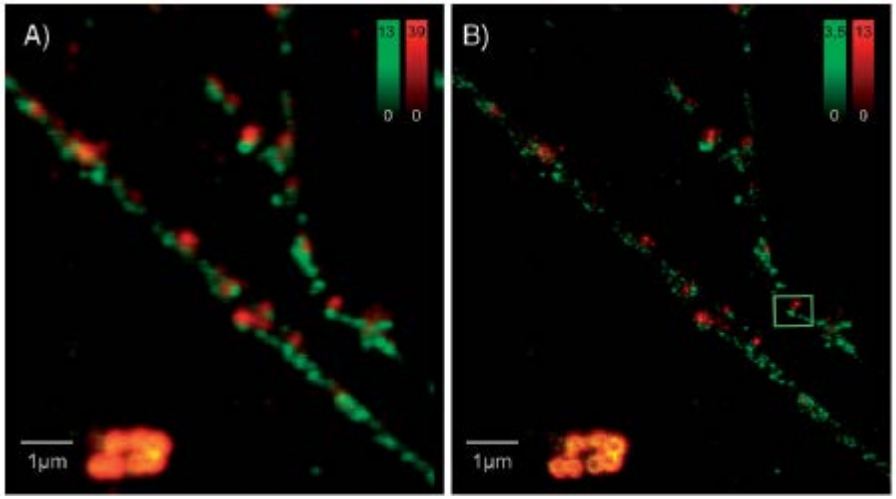


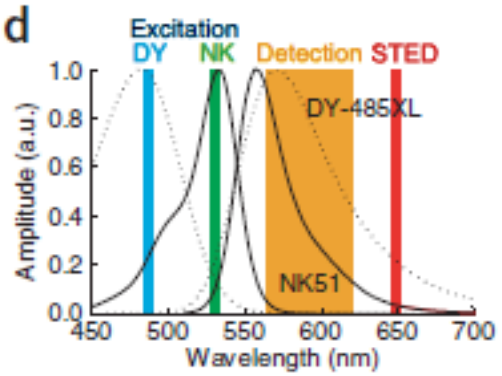
Scale bar: 1µm

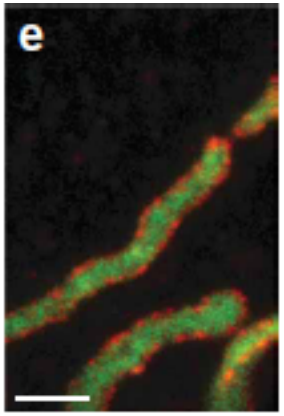
Schmidt et al Nat Methods 2009
Ullal /Schmidt et al NanoLett 2009

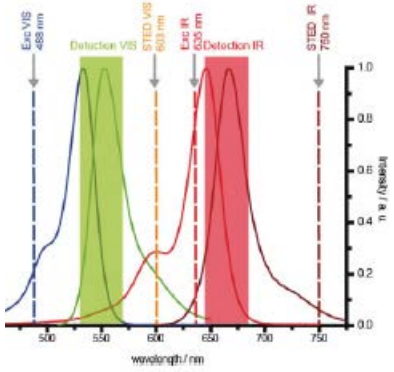
STED-Microscopy

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

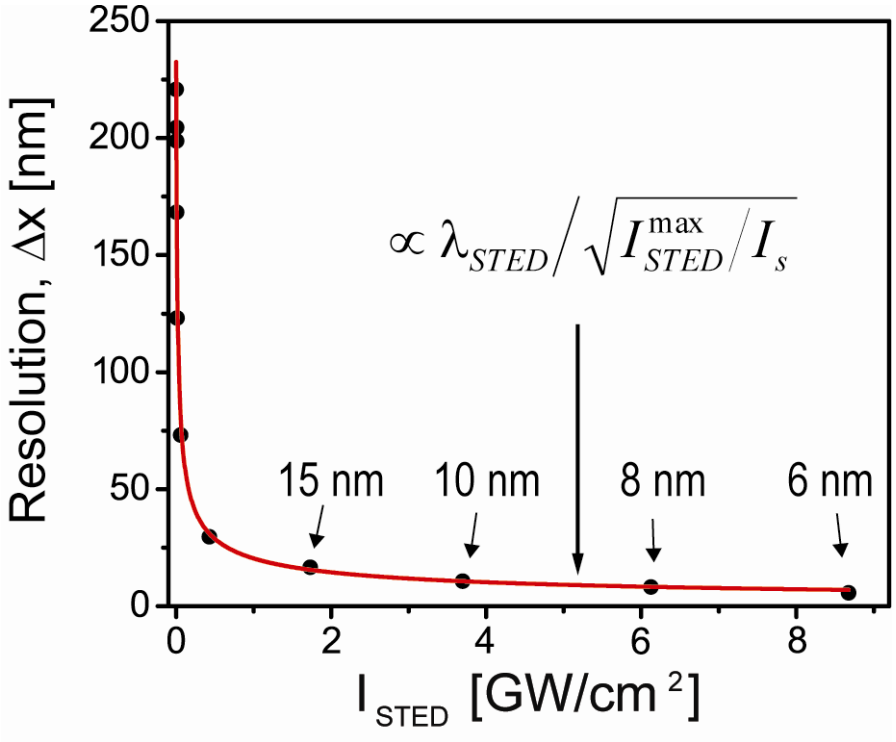
Fluorescence Nanoscopy

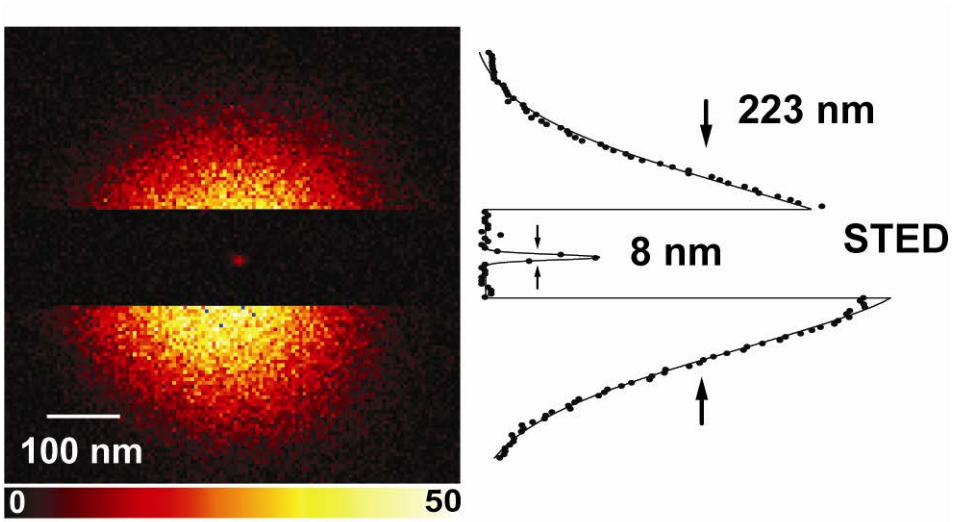
STED imaging on single NV centers

Ultrastable: apply very high STED intensities

→ Reach ultimate spatial resolutions!!!

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



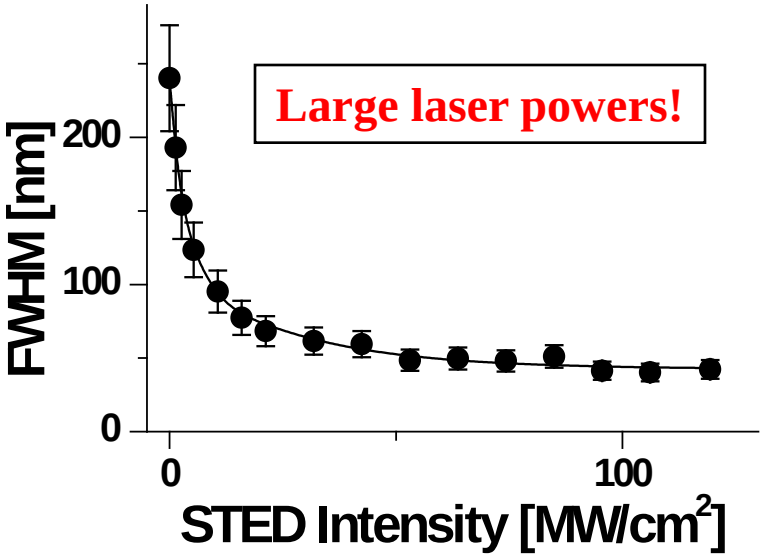


I_{STED} = 3.5 GW/cm²

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

STED Live Cell Microscopy

Problems

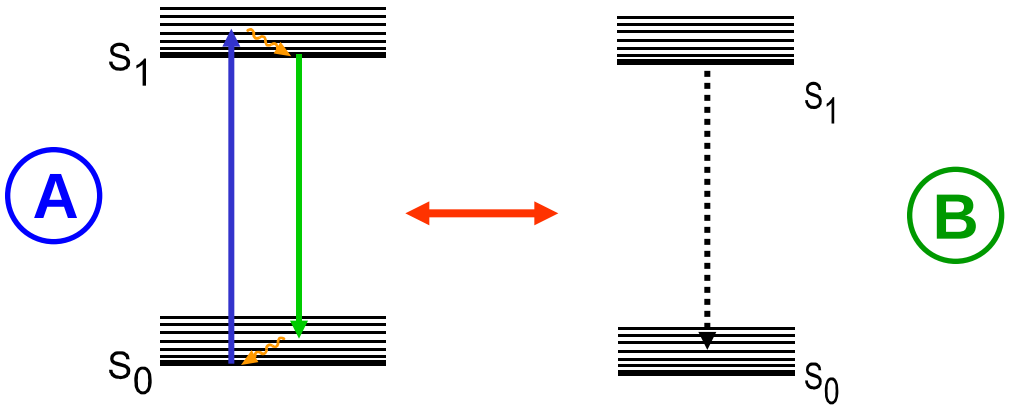


Far-Field Nanoscopy

Alternative ON/OFF

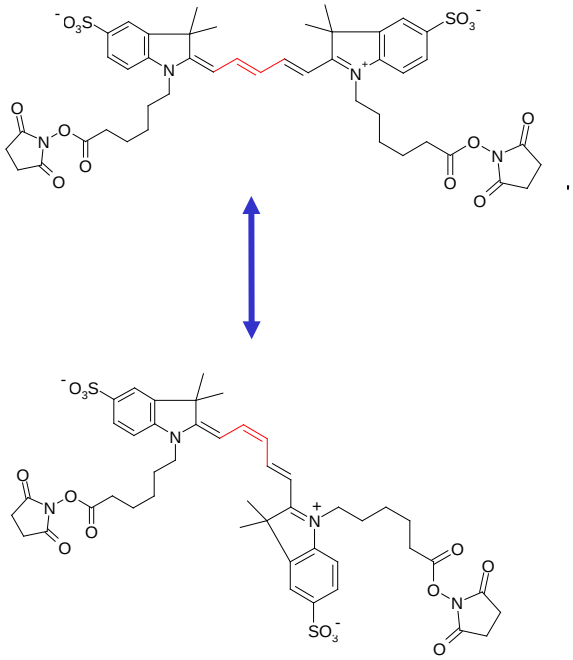
ON $\xleftrightarrow{\text{light}}$ OFF

Optically Bistable Marker



fluorescent *dark*

Cis-trans
Photoisomerisation



Reversible control by light

⇒ Fluorescence Turn On-Off

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

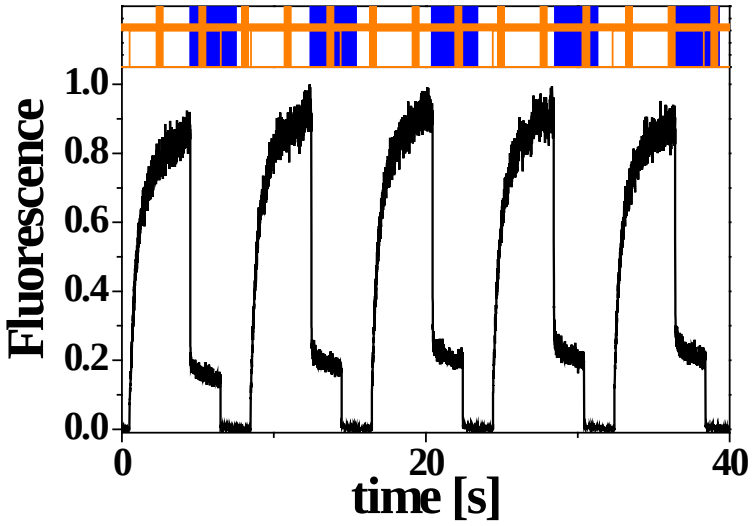
Hell, Jakobs, Kastrup, *Appl. Phys. B 77* (2003)

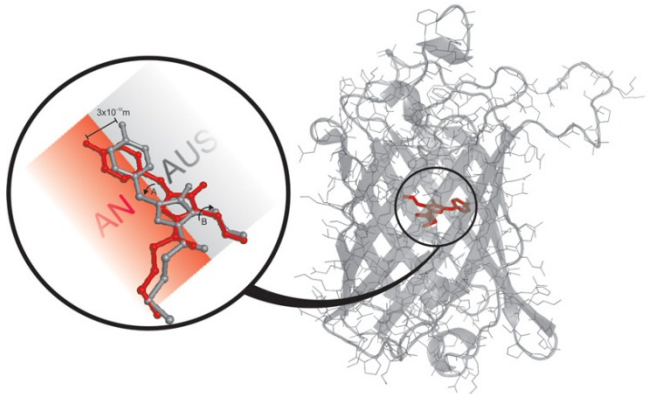
Far-Field Nanoscopy
ON/OFF - asFP595

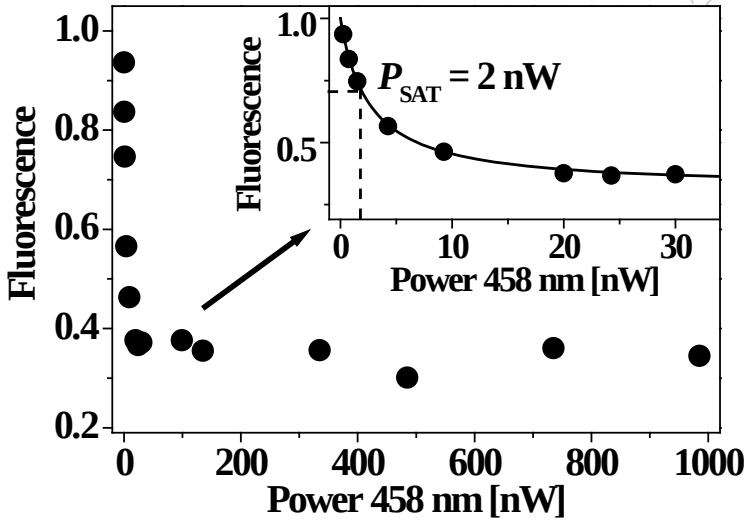
Fluorescent protein asFP595

sea anemone *Anemonia sulcata*, Lukyanov et al. (2000) *J. Biol. Chem.*

cis-trans photoisomerisation
dark (trans)- bright (cis)
Andresen et al. (2005) *PNAS*







ON/OFF at low CW powers

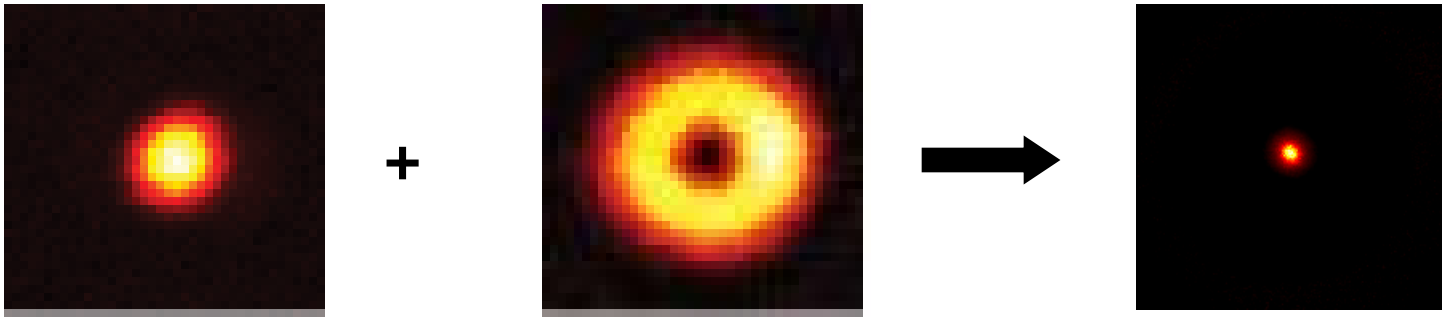
nW - µW (~ kW/cm^2) High saturation!

ON: 560nm

OFF: 400-450nm (local intensity zero)

Sub-Diffraction Microscopy
with asFP595 – RESOLFT-Microscopy

Focal engineering



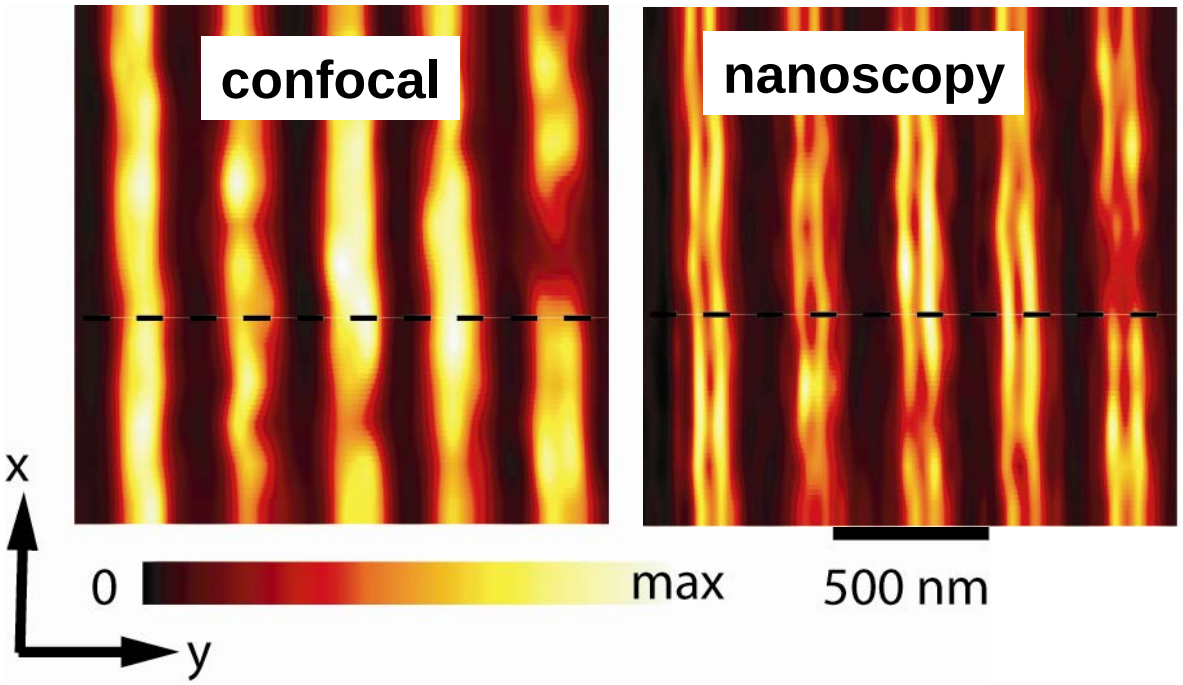
yellow (568 nm) **blue** (458 nm) **Sub-diffraction**

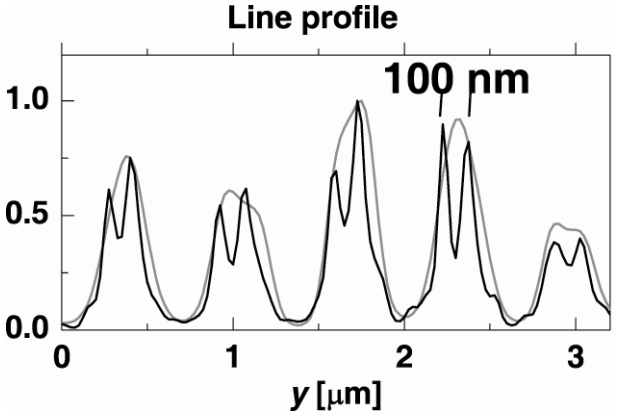
RESOLFT = *R*eversible *S*aturable *O*ptical *F*luorescence *T*ransition

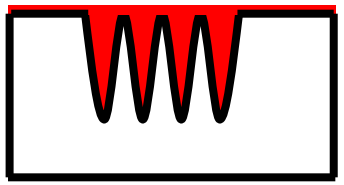
Hofmann et al, PNAS 2006

Far-Field Nanoscopy

ON/OFF - asFP595







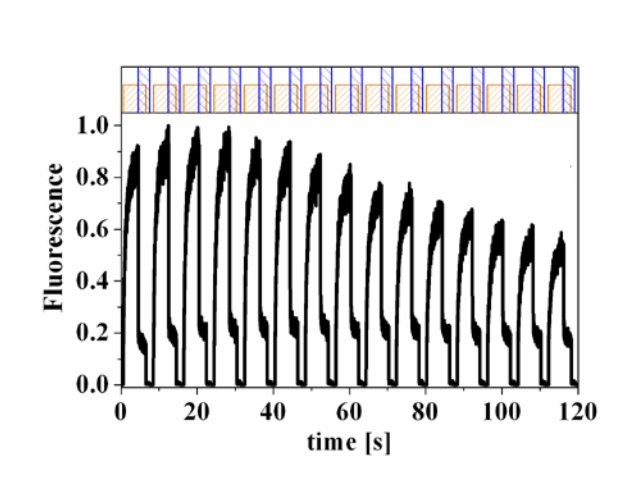
custom-prepared glass slides - parallel grooves
focused ion beam milling (Fraunhofer Institute IISB, Erlangen, Germany)
10 µm long, 0.5–1 µm deep, 100 nm wide, distance 500 nm
images RL-deconvolved

Hofmann et al, PNAS 2006

Far-Field Nanoscopy

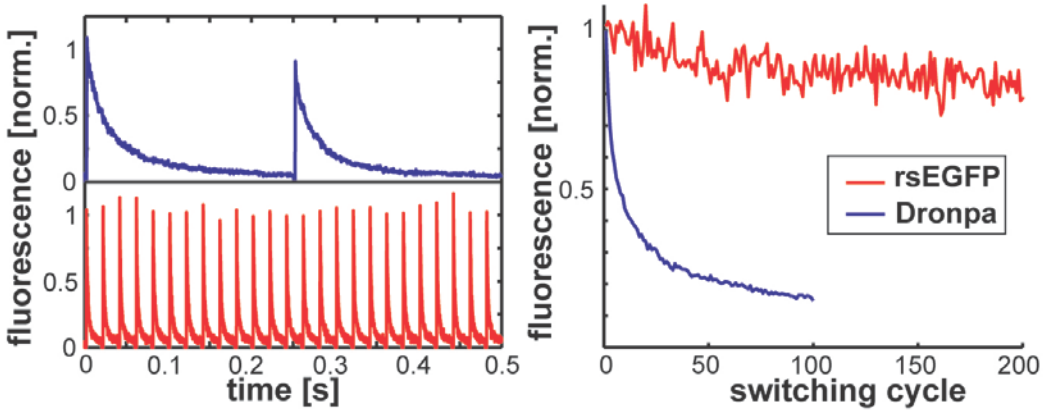
ON/OFF – limits + advances

Switching fatigue - photobleaching



⇒ improve ON/OFF cycling

- less cross-talk
- faster switching



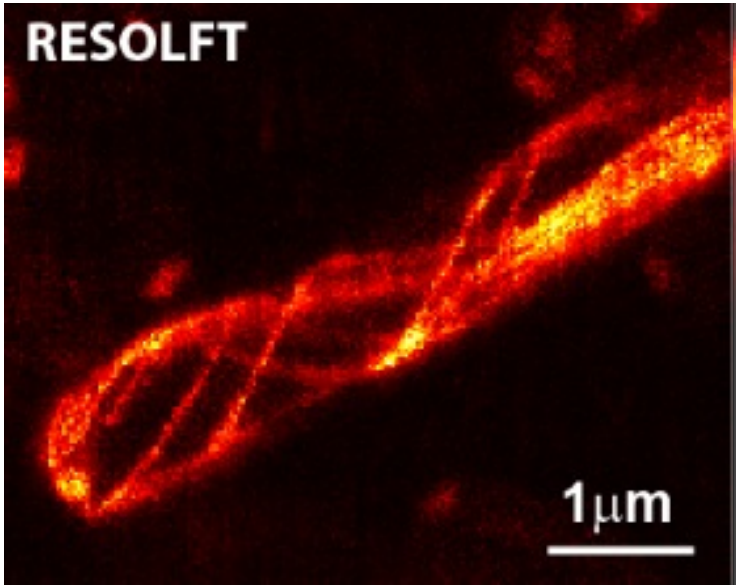
Dronpa → rsFastLime
Stiel et al, Biochem J 2007

GFP → photoswitching (rsEGFP)
Grotjohann et al, Nature 2011

Switch-off + Readout: 488nm
Switch-on: 405nm

Far-Field Nanoscopy

RESOLFT – Advance Photoswitchers



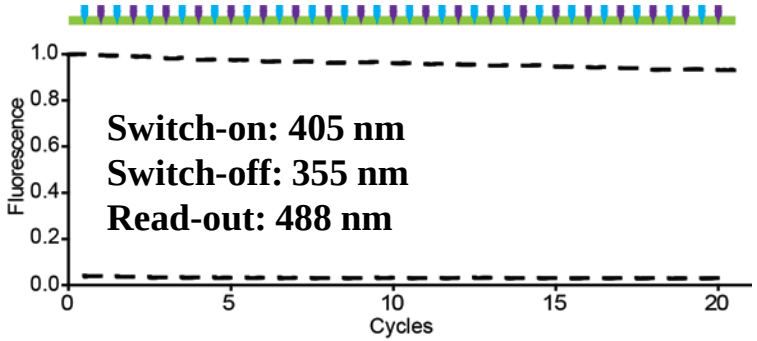
GFP → photoswitching (rsEGFP)
Grotjohann et al, Nature 2011

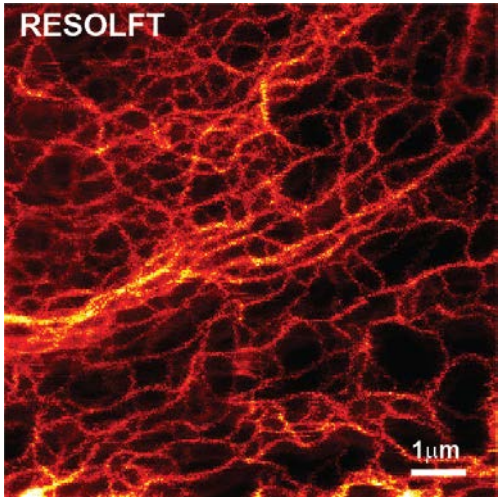
Switch-off + Readout: 488nm
Switch-on: 405nm

E. coli bacterium expressing rsEGFP–MreB
bacterial actin homologue MreB

Intensity ≈ 1 kW/cm^2

Citrine → Dreiklang
Brakemann et al, Nature Biotechnol. 2011

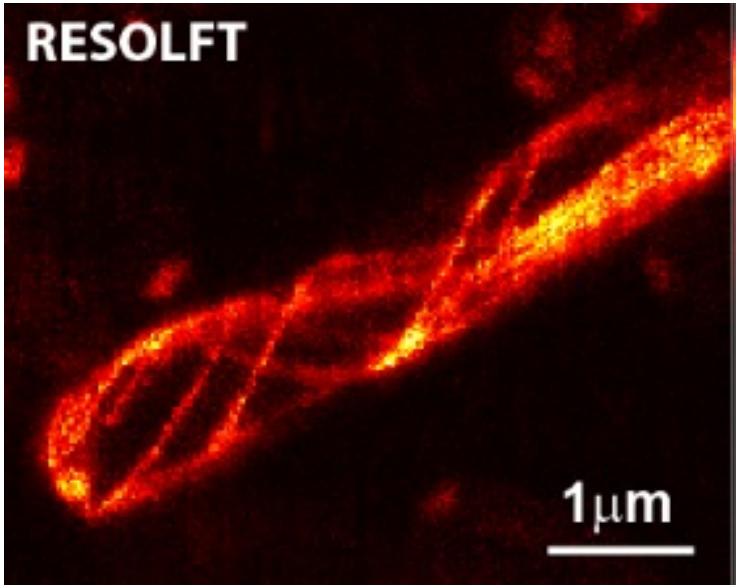




Keratin19-Dreiklang
expressed
in living PtK2 cells

Far-Field Nanoscopy

RESOLFT – Advance Photoswitchers

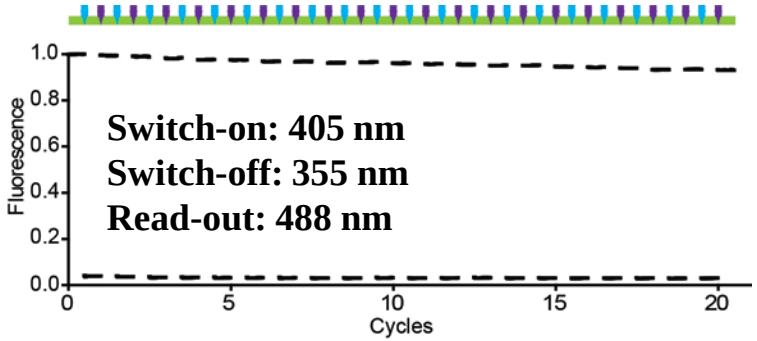


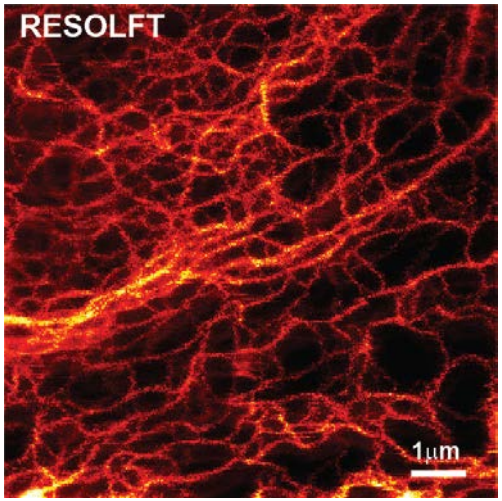
Excellent for Live-Cell (low light levels)

Multi-Color (new fluorescent proteins)
3D possible
Photoswitchable proteins / dyes

Intensity ≈ 1 kW/cm²

Citrine → Dreiklang
Brakemann et al, Nature Biotechnol. 2011



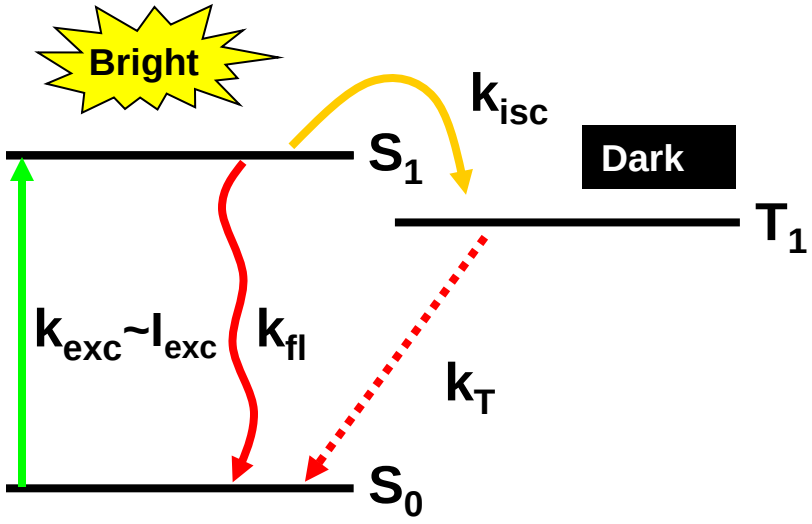


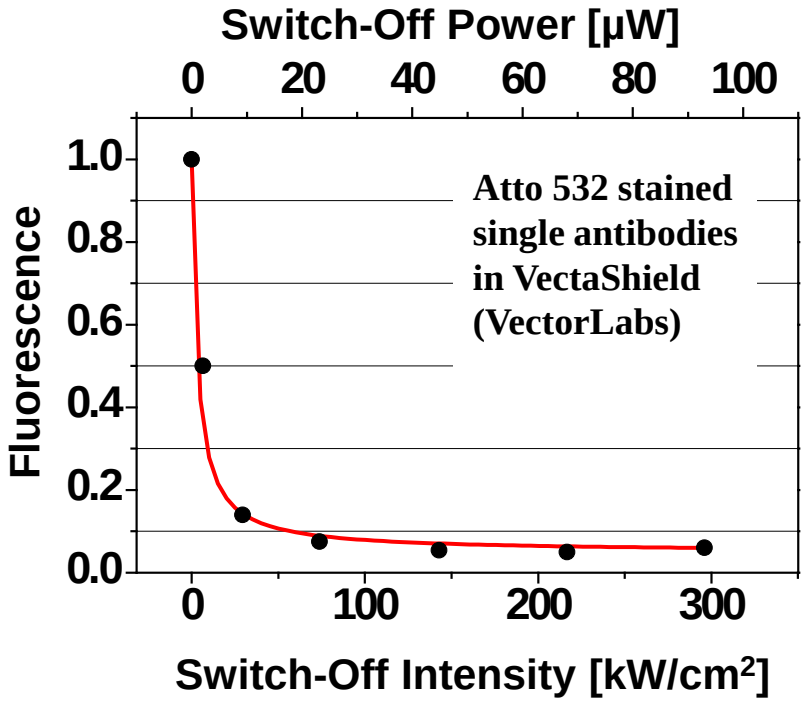
Keratin19-Dreiklang
expressed
in living PtK2 cells

Far-Field Nanoscopy

ON/OFF via Triplet/Dark States

GSD (Ground State Depletion)





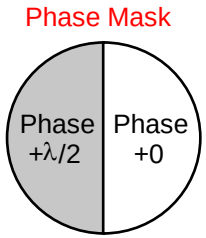
Turn-off fluorescence by pumping into a long-living dark (triplet) state

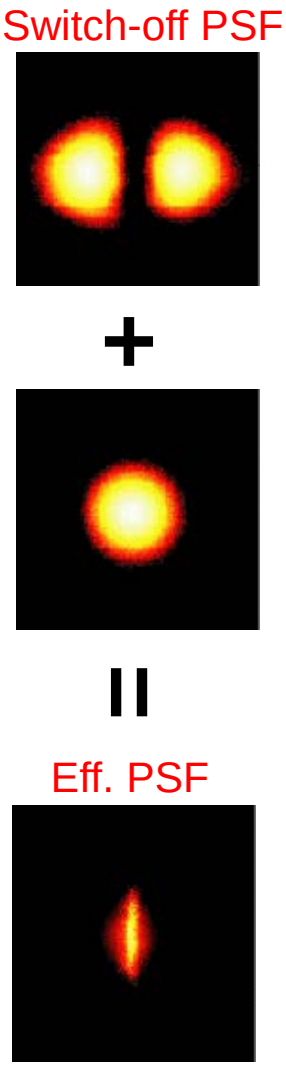
Low CW powers (µW – kW/cm²)

Appl. Phys. B 60: 495-497 (1995) , Hell et al.

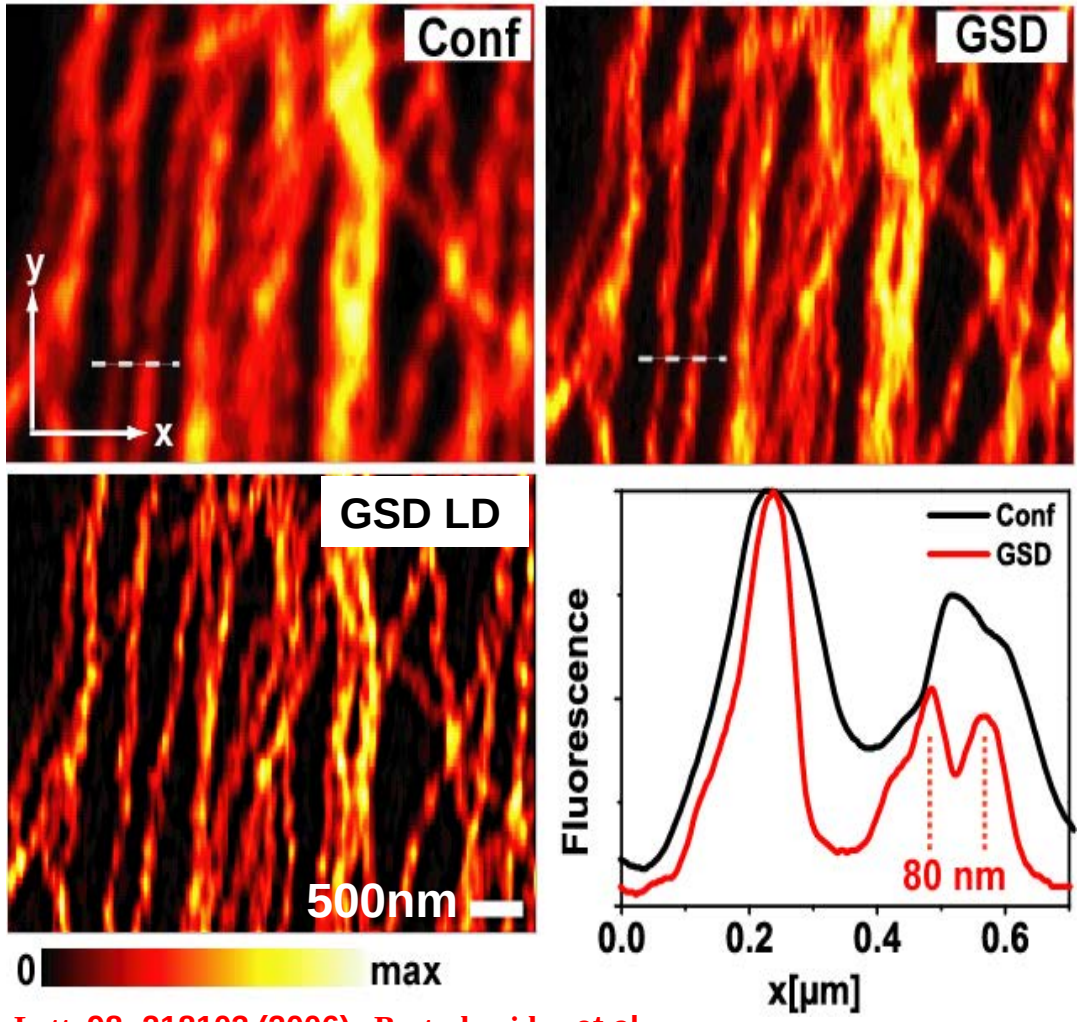
GSD-Microscopy

Far-Field Nanoscopy using the triplet state





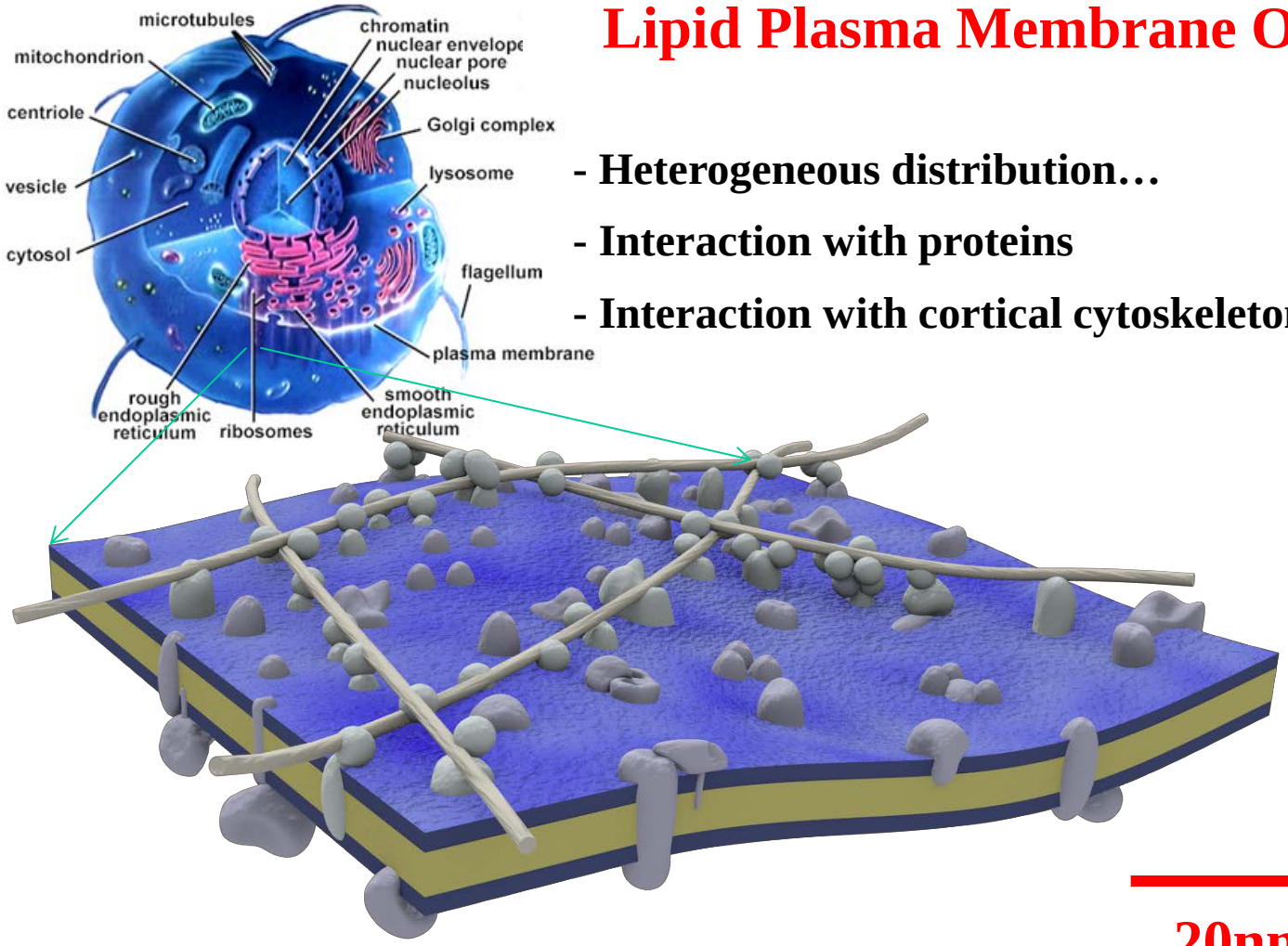
Atto 532 stained microtubuli



Phys. Rev. Lett. 98: 218103 (2006) , Bretschneider et al.

Lipid Plasma Membrane Dynamics

Nanoscale



Lipid Plasma Membrane Organization:

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

20nm

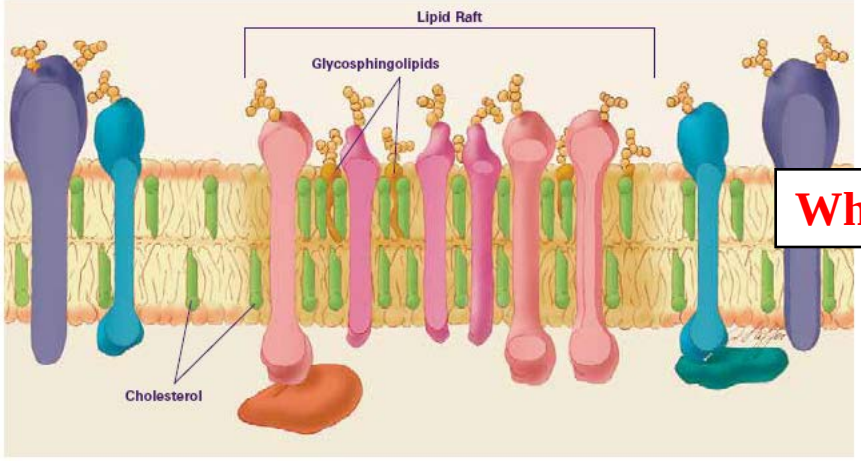
Small spatial scales!!!!

Lipid Plasma Membrane Dynamics

Interactions on the Nanoscale: Nanodomains

Lipid rafts/nanodomains?

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

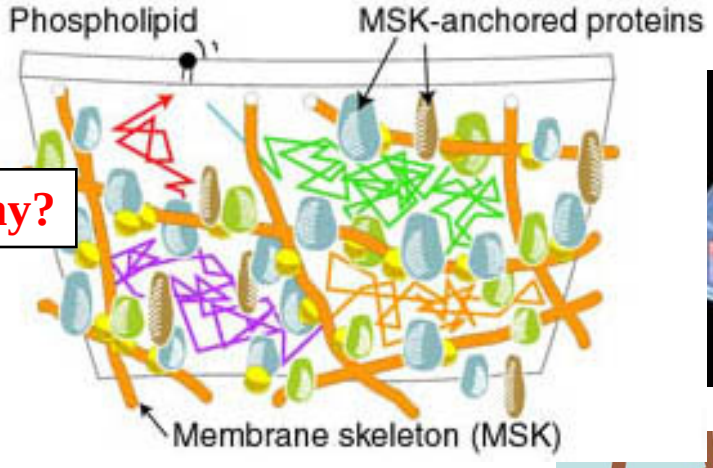


Pike, J.Lipid Res., Keystone meeting 2006

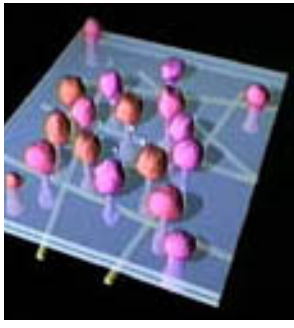
Cytokeleton

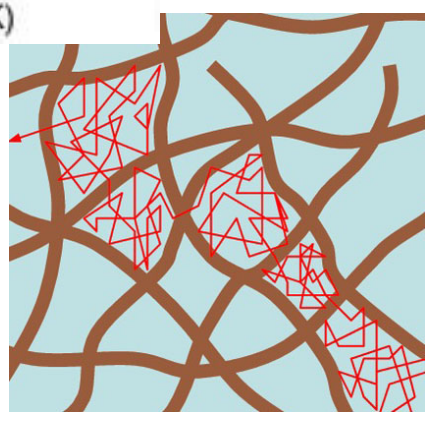
- Membrane divided in compartments
- Proteins: fence/hindrance in diffusion path
- Hopping diffusion

Kusumi



Why?





Problem:

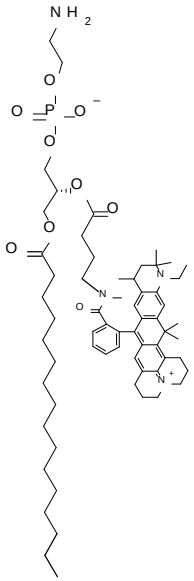
- heterogeneous
  + highly dynamic
- small (<200 nm)

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

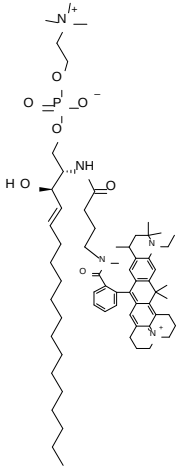
Lipid Plasma Membrane Dynamics

Fluorescence Recordings: Lipids

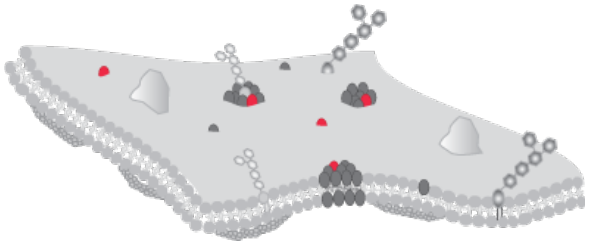
Phosphoglycerolipid:
Atto647N-phosphoethanolamine (PE)

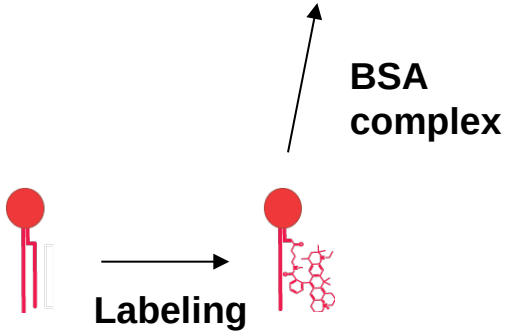


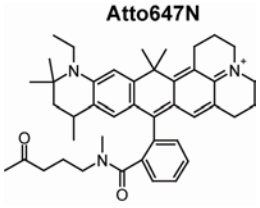
Sphingolipid:
Atto647N-sphingomyelin (SM)



Live PtK2 cells:
physiological conditions
incorporation in plasma membrane

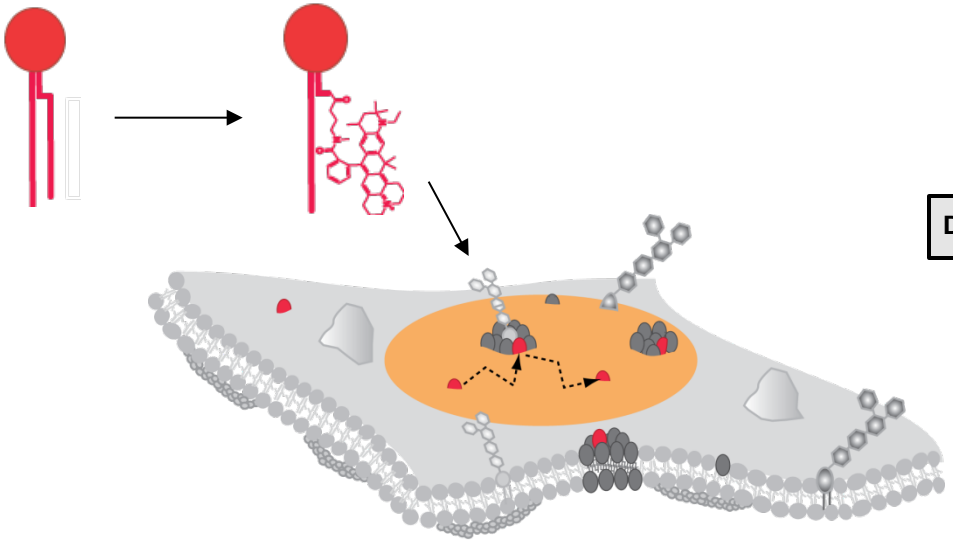


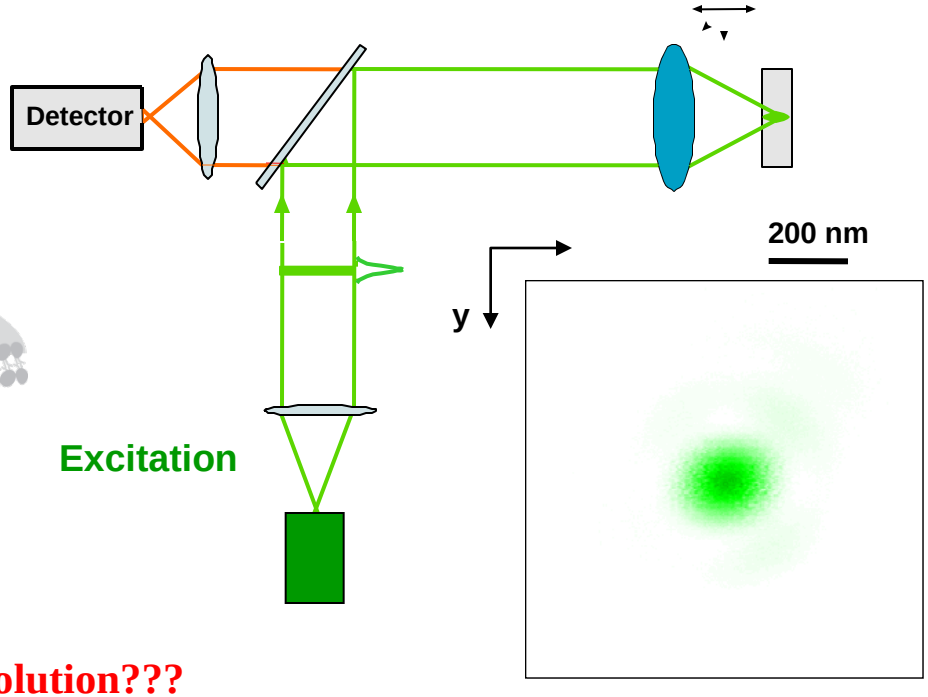


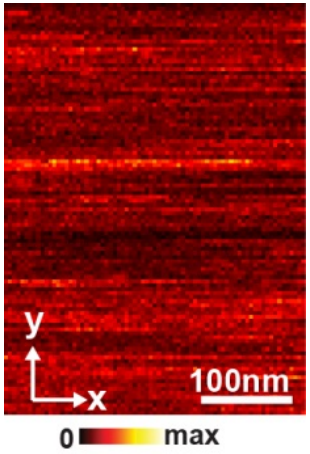


Lipid Plasma Membrane Dynamics

Confocal Recordings





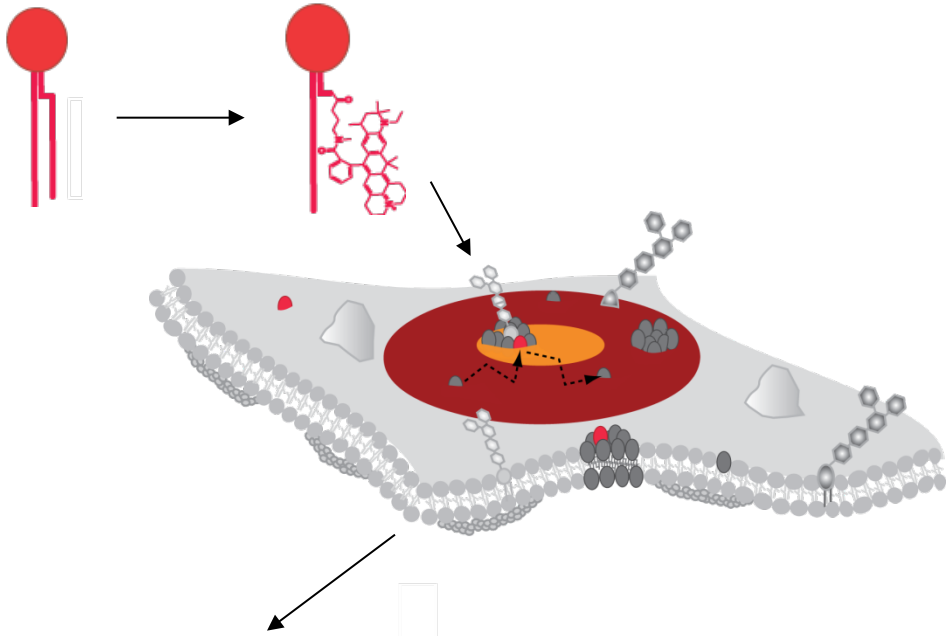


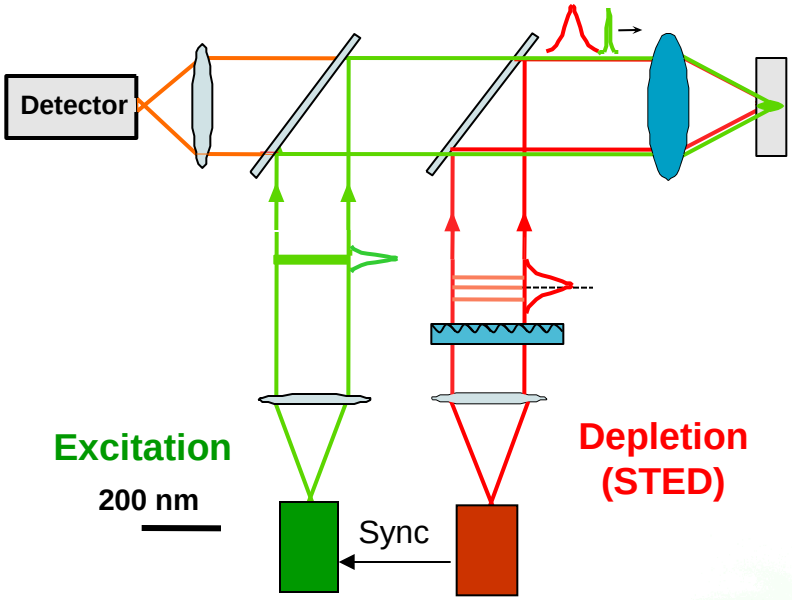
Limited spatial resolution???

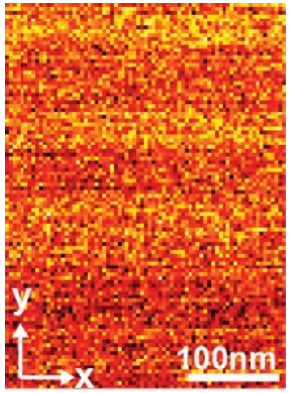
Observation area on plasma membrane

Lipid Plasma Membrane Dynamics

STED Nanoscopy Measurement

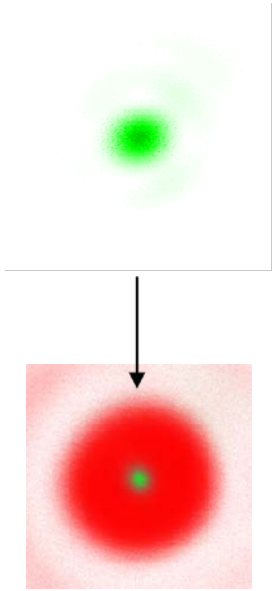






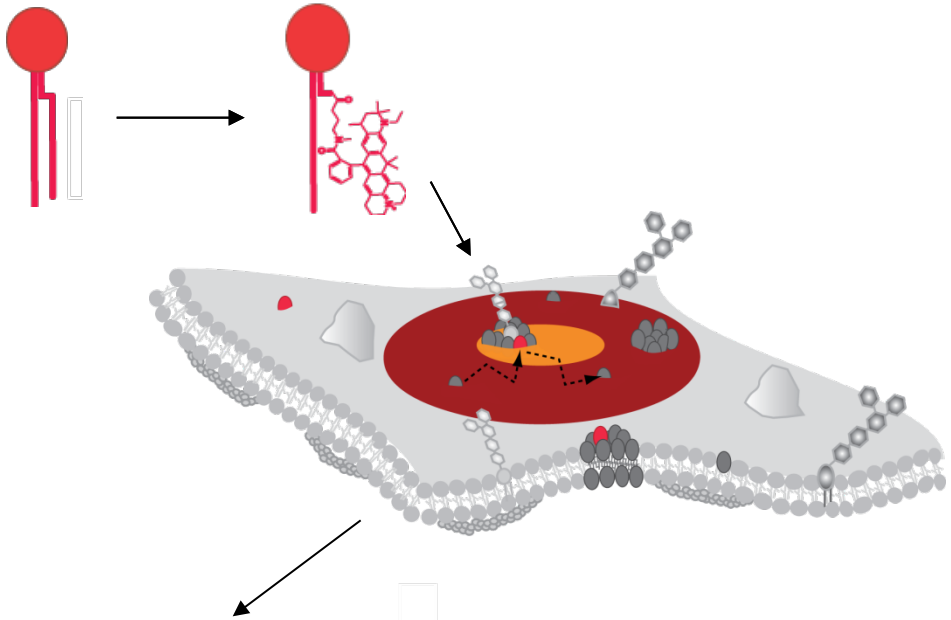
No heterogeneities:

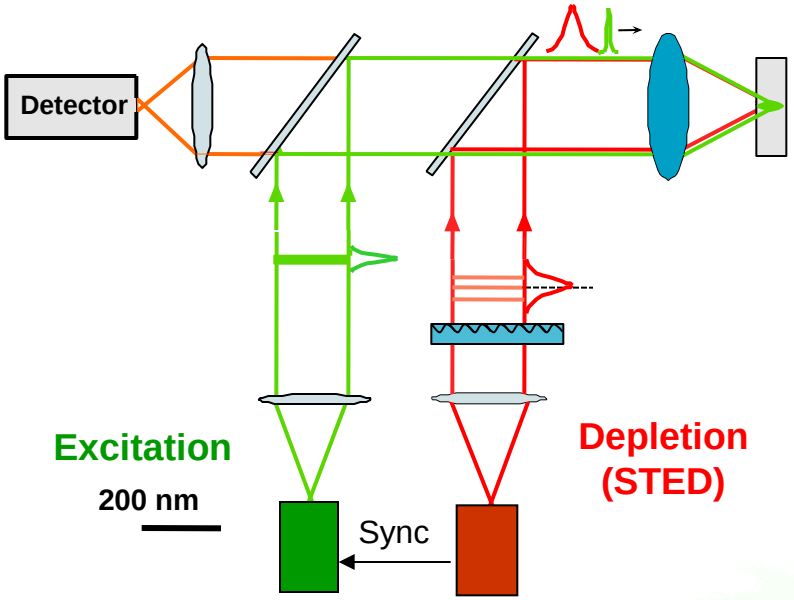
Fast diffusion → Limited temporal resolution!

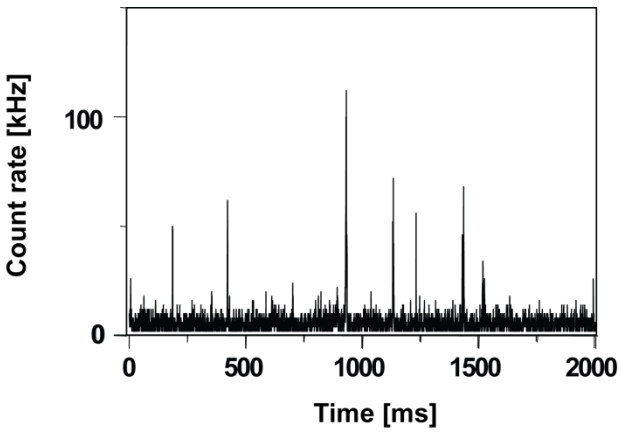


Lipid Plasma Membrane Dynamics

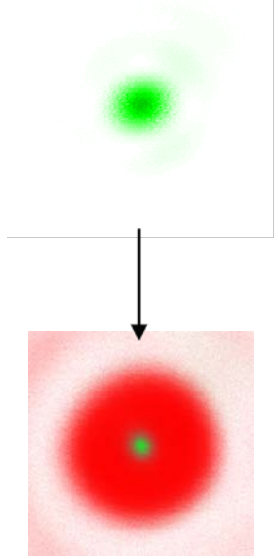
STED Nanoscopy Measurement







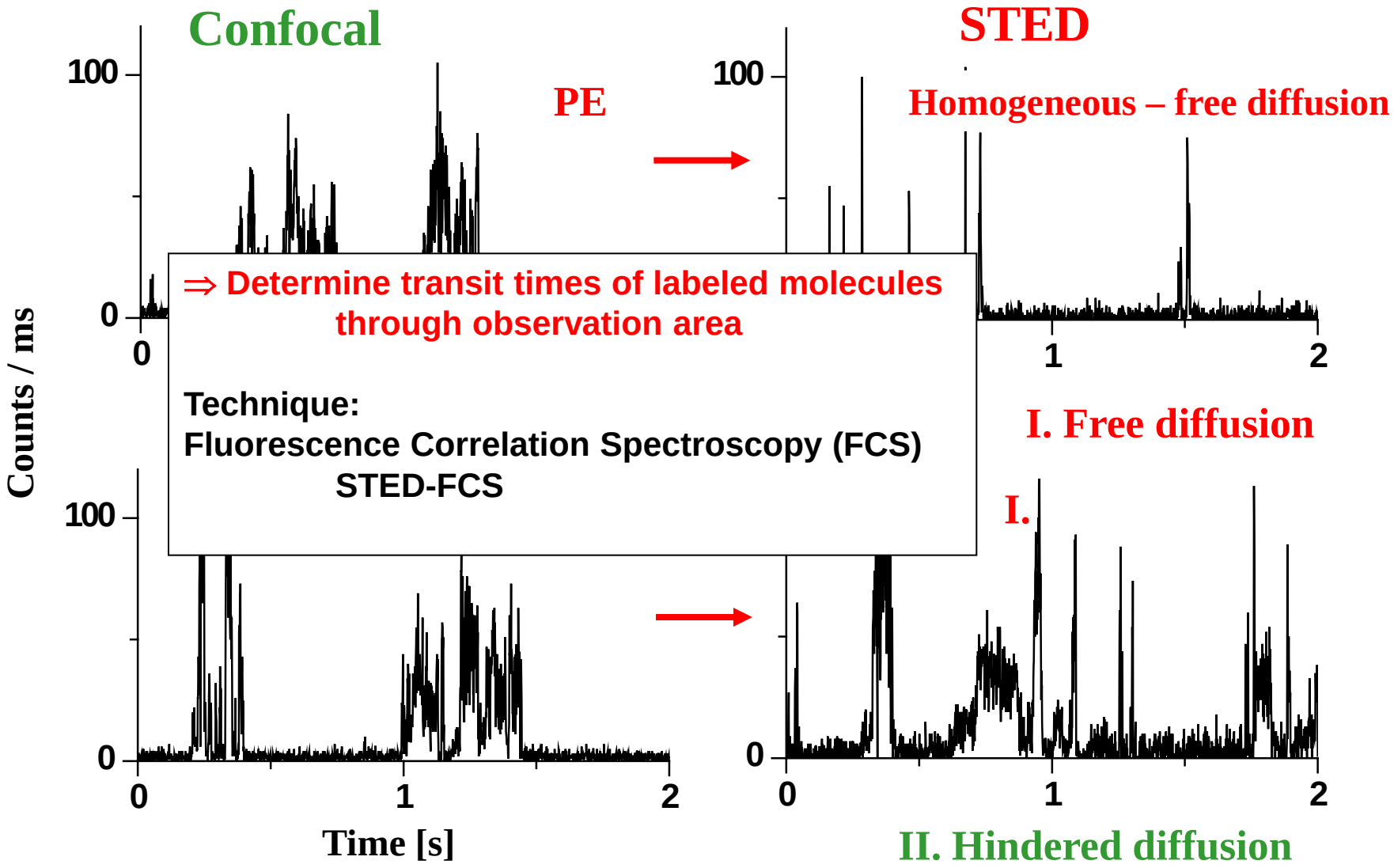
Discover diffusion dynamics!!!



Eggeling et al Nature 2009

STED Live Cell Spectroscopy

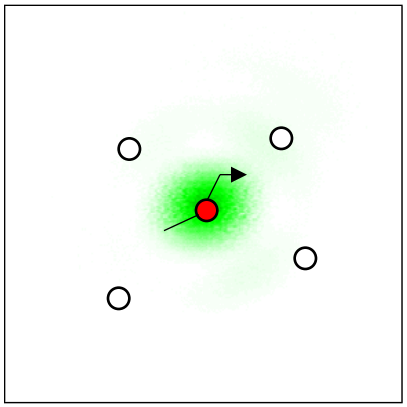
Single Lipid Dynamics

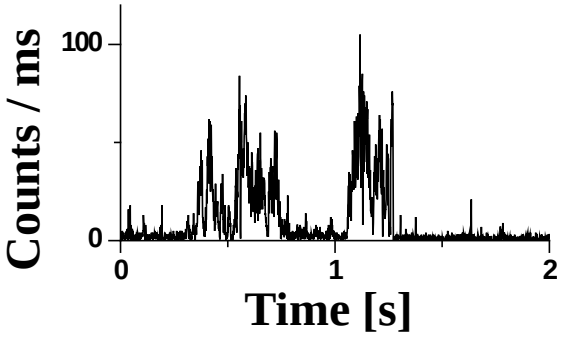


Fluorescence Correlation Spectroscopy
FCS

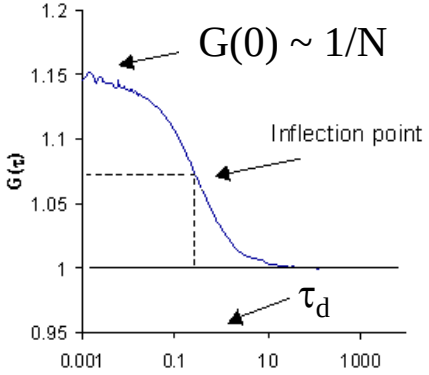
Fluorescence intensity over time

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

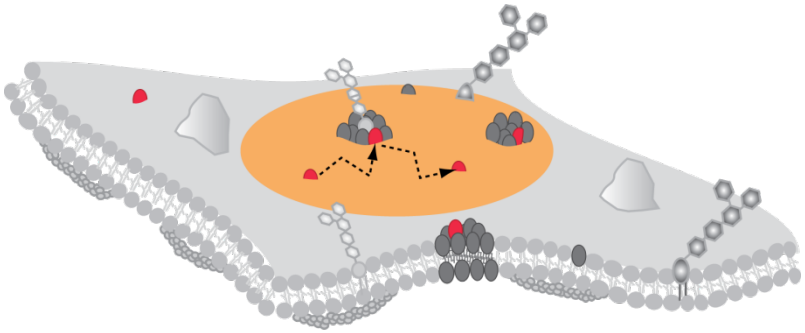
Fitting: anomalous sub-diffusion: $G(t_c) \sim 1/(1 + (t_c/\tau_d)^{\alpha})$
⇒ **transit time** τ_d **(~ mass, obs. area) = decay time**
$\sim d^2 / D$

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

Lipid Plasma Membrane Dynamics

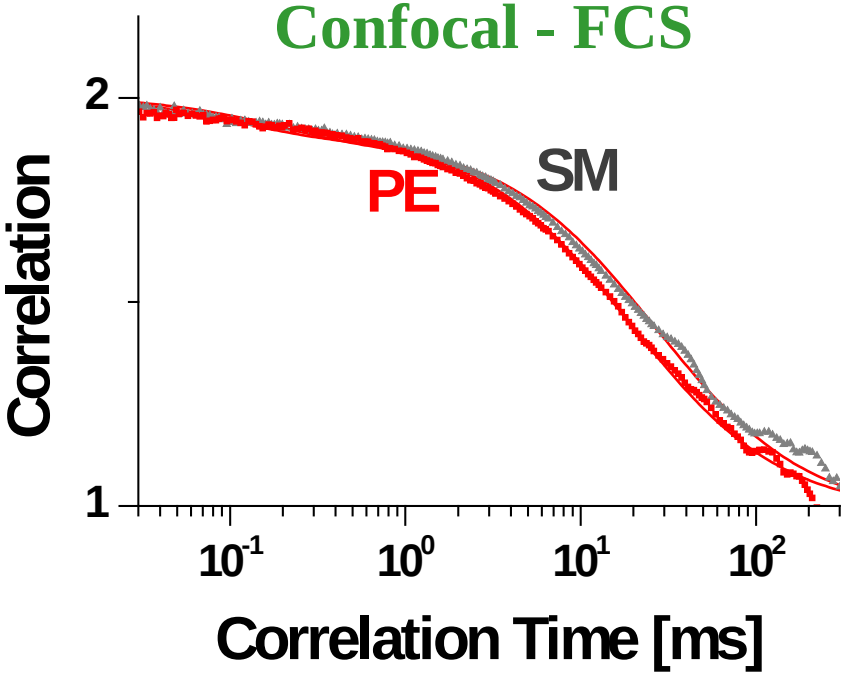
Confocal Recordings

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)

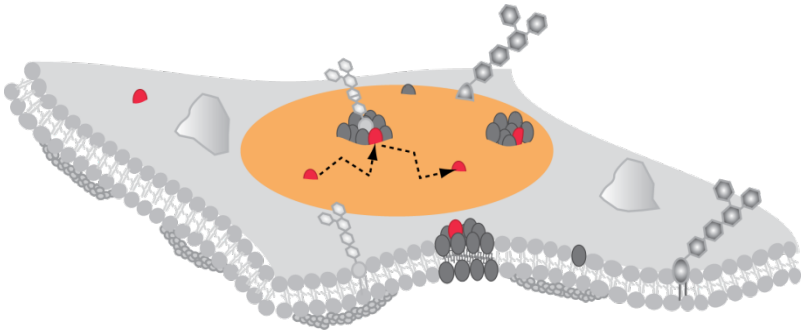
$(1/\alpha) \approx 1$ (PE / SM)

Slower normal diffusion
but no anomalous diffusion???

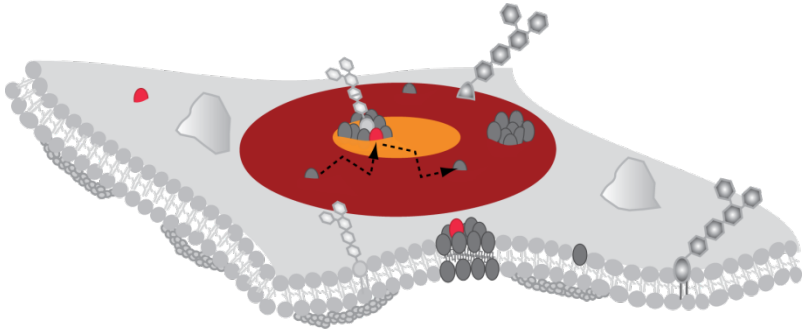
Lipid Plasma Membrane Dynamics

Move to STED

Confocal: Limited spatial resolution !!!



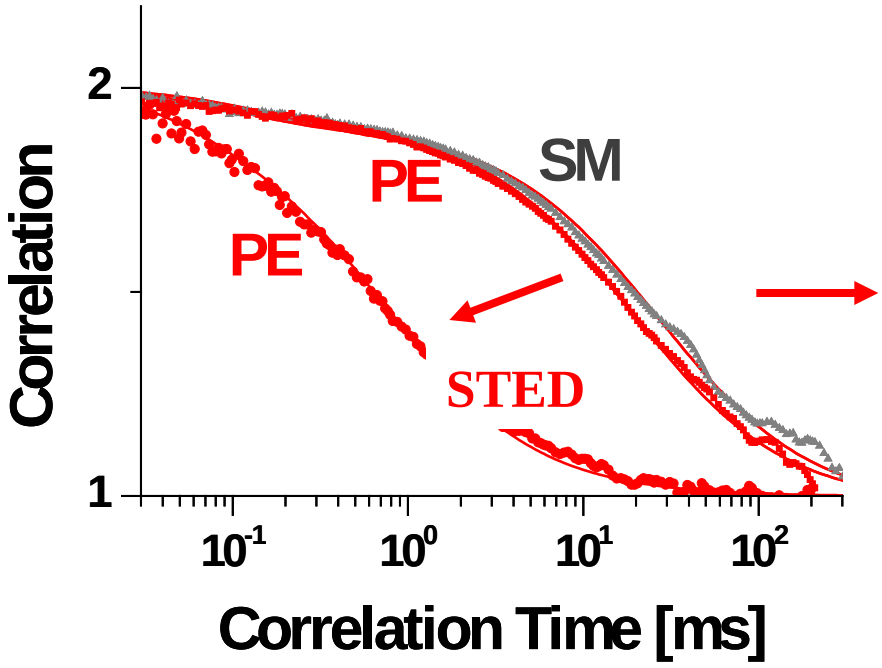
STED!!!!



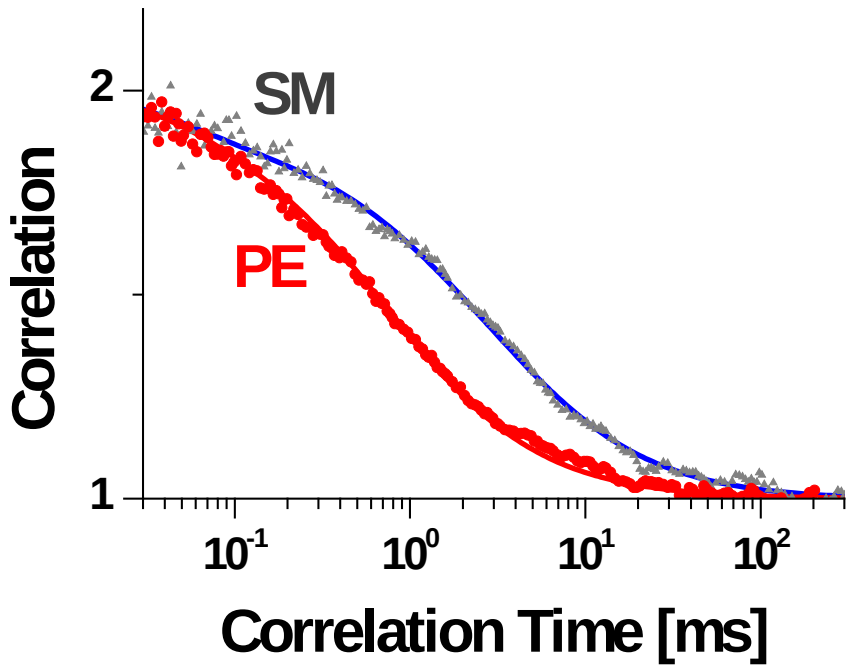
STED Live Cell Spectroscopy

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

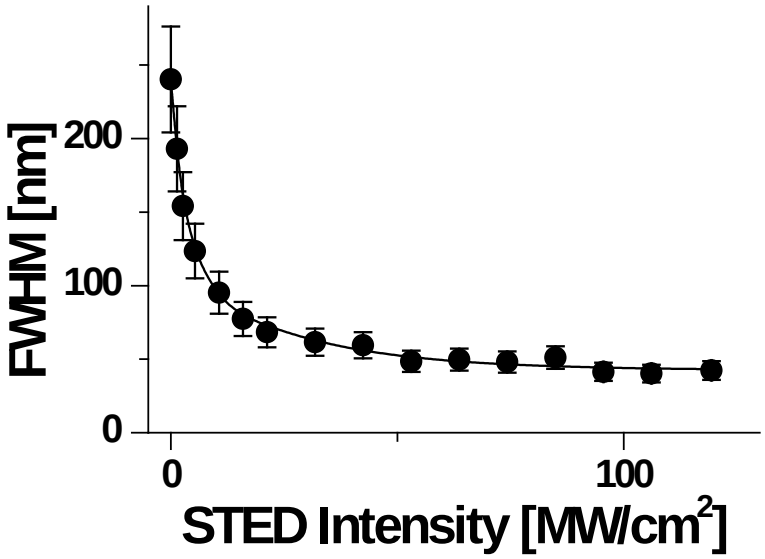
τ_d: 30 -> 3ms (10-fold)

and anomalous

$(1/\alpha) \approx 1.5$

Live Cell Nanoscopy
STED-FCS

STED-Microscopy: Tuning of observation area



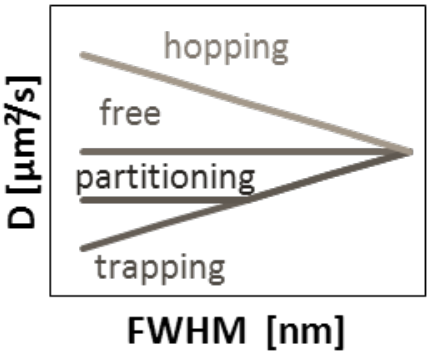
STED-FCS
Determine transit time
for different sizes of observation areas
(different STED intensities)

Calculate
apparent diffusion coefficient:

D ~ area / transit time

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

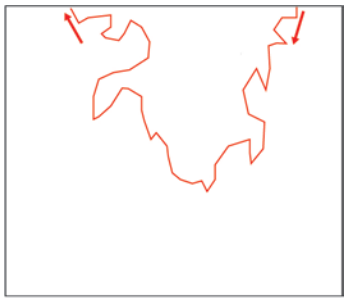
Varies for different diffusion modes



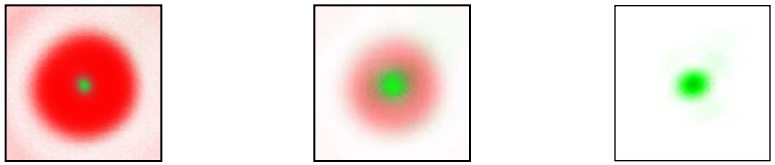
Live Cell Nanoscopy

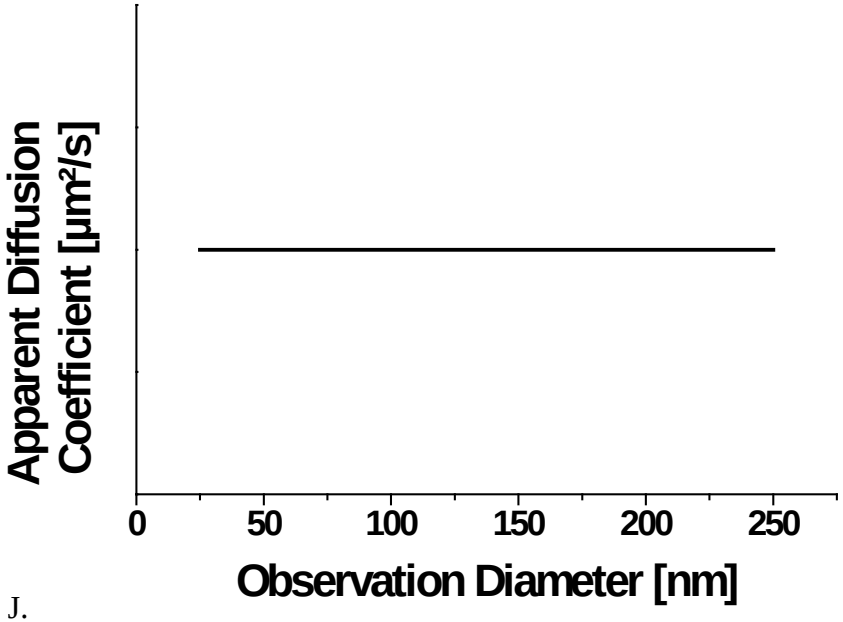
STED-FCS - Diffusion Models

Free diffusion



← STED Intensity





Wawrezinieck et al. Biophys J. 2005 December; 89(6)
Eggeling et al. *Nature* 457, 1159-1162 ,2009
Mueller et al. *Biophys J* 2011

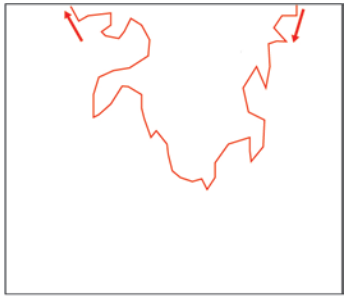
Apparent diffusion coefficient:

D ~ area / transit time

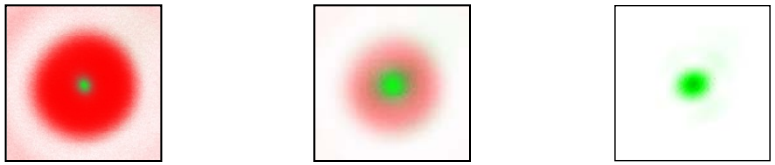
Live Cell Nanoscopy

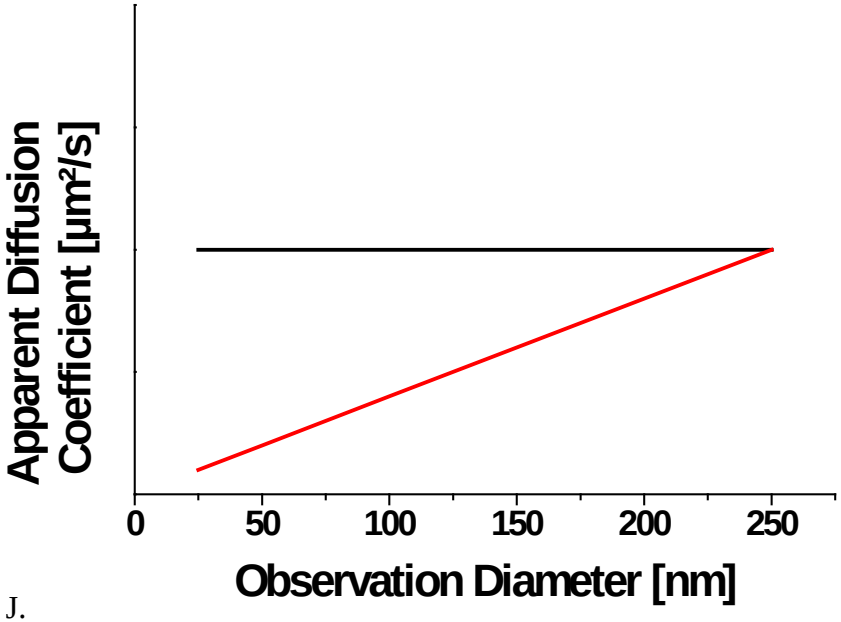
STED-FCS - Diffusion Models

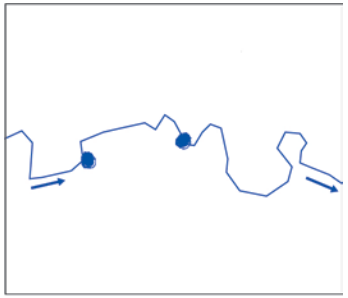
Free diffusion



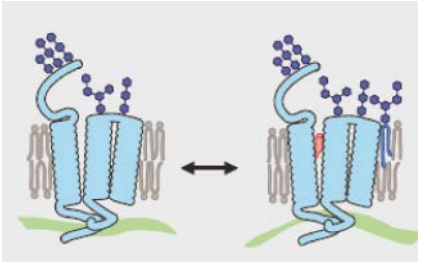
← **STED Intensity**







Trapping



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Mueller et al. *Biophys J* 2011

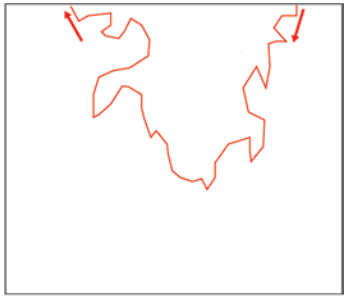
Apparent diffusion coefficient:

D ~ area / transit time

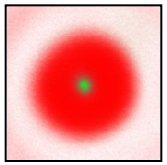
Live Cell Nanoscopy

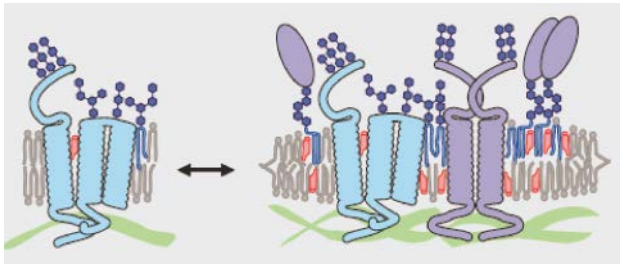
STED-FCS - Diffusion Models

Free diffusion

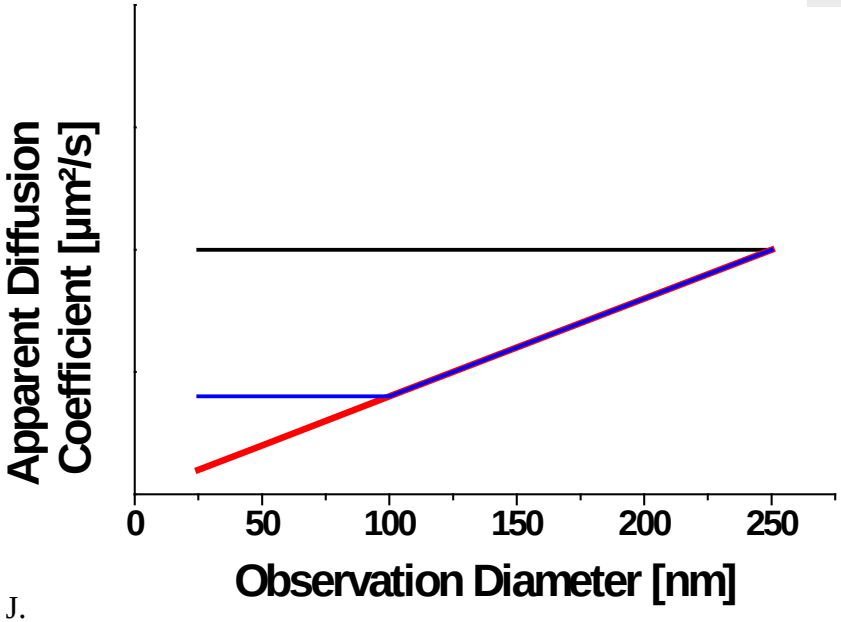


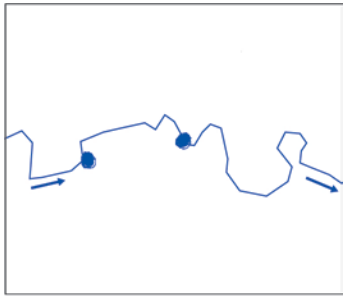
← STED Intensity



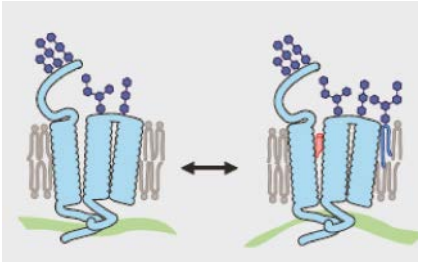


Domain incorporation





Trapping



Wawrezinieck et al. Biophys J. 2005 December; 89(6)
Eggeling et al. *Nature* 457, 1159-1162 ,2009
Mueller et al. *Biophys J* 2011

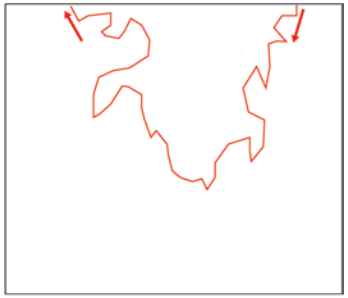
Apparent diffusion coefficient:

D ~ area / transit time

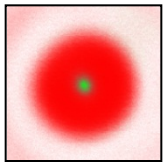
Live Cell Nanoscopy

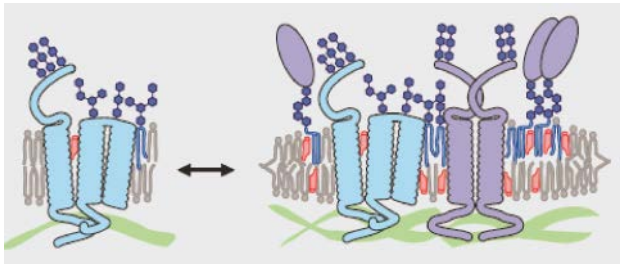
STED-FCS - Diffusion Models

Free diffusion

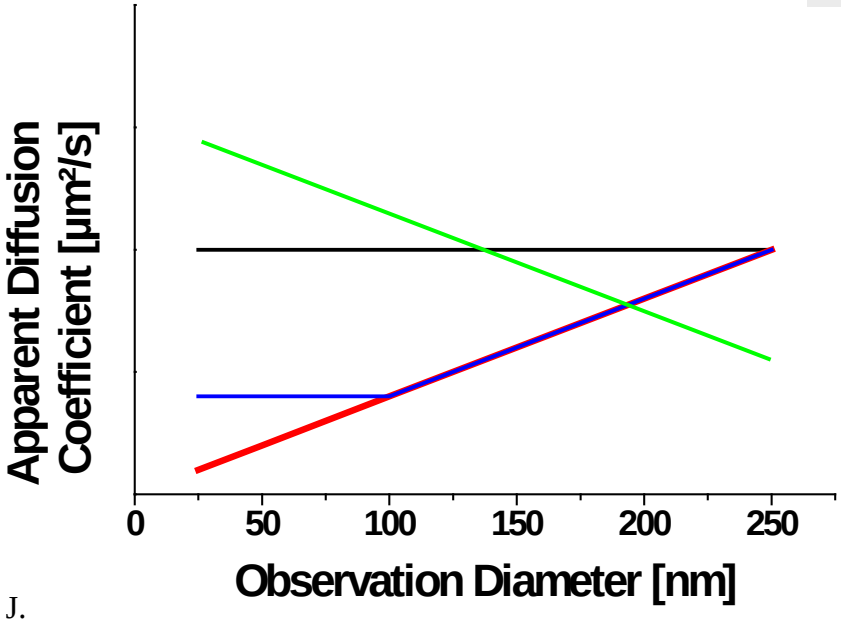


← STED Intensity

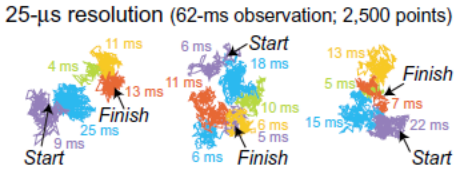


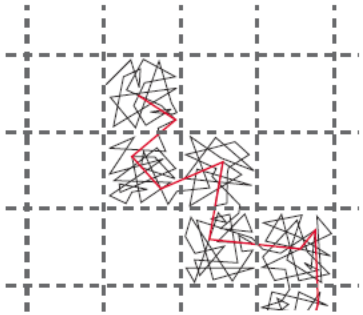


Domain incorporation

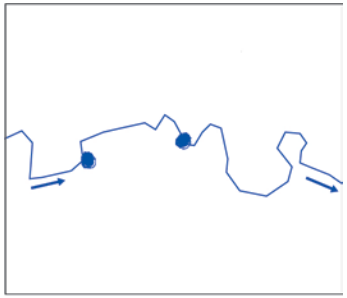


Hopping (Kusumi)

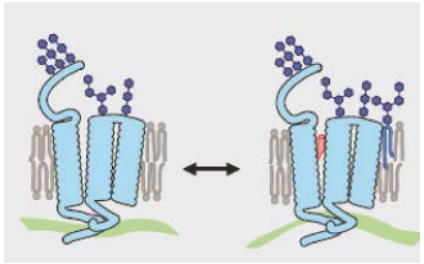




J.



Trapping

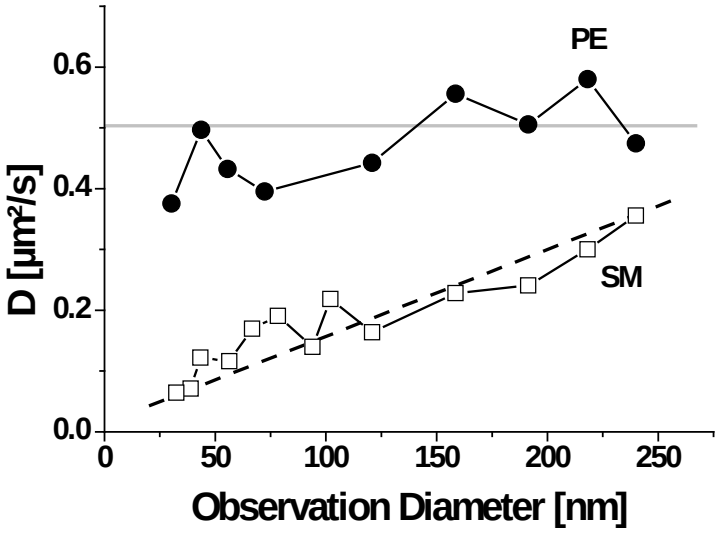


Apparent diffusion coefficient:

D ~ area / transit time

STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM



→ **Complex on molecular scale**

(proteins, lipid-shells, …)

~10 ms, no movement during trapping

Cholesterol-assisted

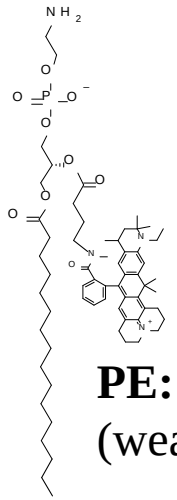
(COase/ß-Cyclo-Dextrin/Zaragozic acid…)

Binding partner bound to cytoskeleton

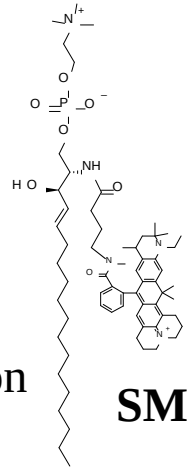
(Latrunculin/Jasplakinolide/Nocodazole…)

Slight dependence on endogenous SM level

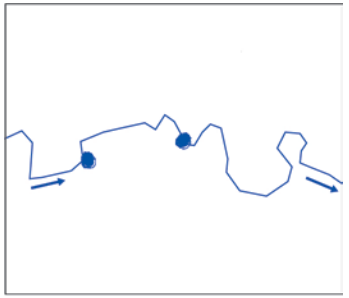
(Myriocin)

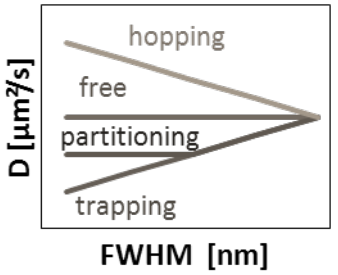


PE: free diffusion
(weak trapping)



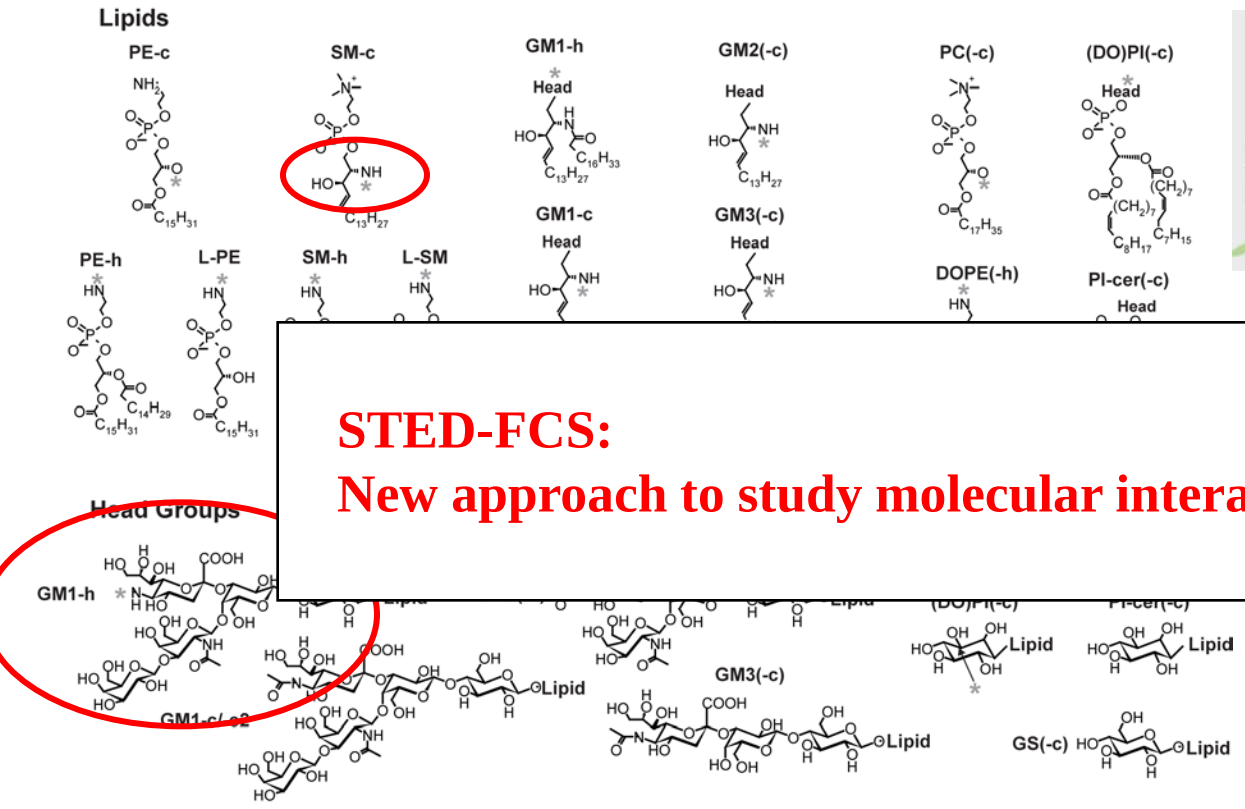
SM: trapping

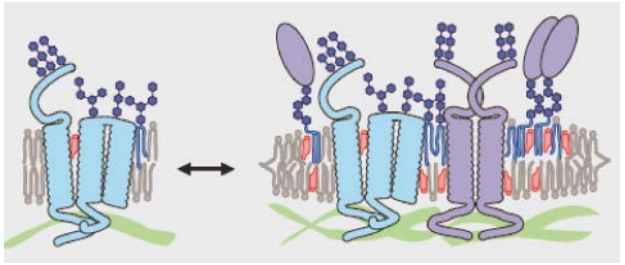




STED-FCS

Membrane Dynamic – Lipid Structure





STED-FCS:
New approach to study molecular interactions!

Specific lipid-protein ions!

Binding partners?

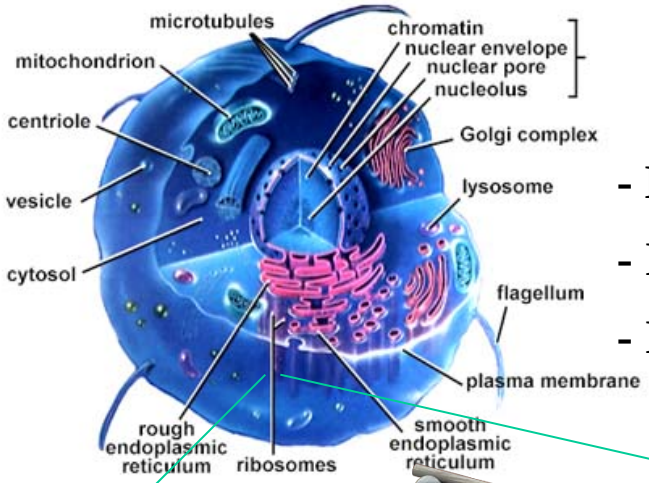
2nd color!

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

But not on dye and label position!

Lipid Plasma Membrane Dynamics

Nanoscale Diffusion



Molecular Membrane Dynamics:

- **Heterogeneous diffusion**
- **Interaction with proteins / lipids**
- **Interaction with cortical cytoskeleton**

Highly dynamic!

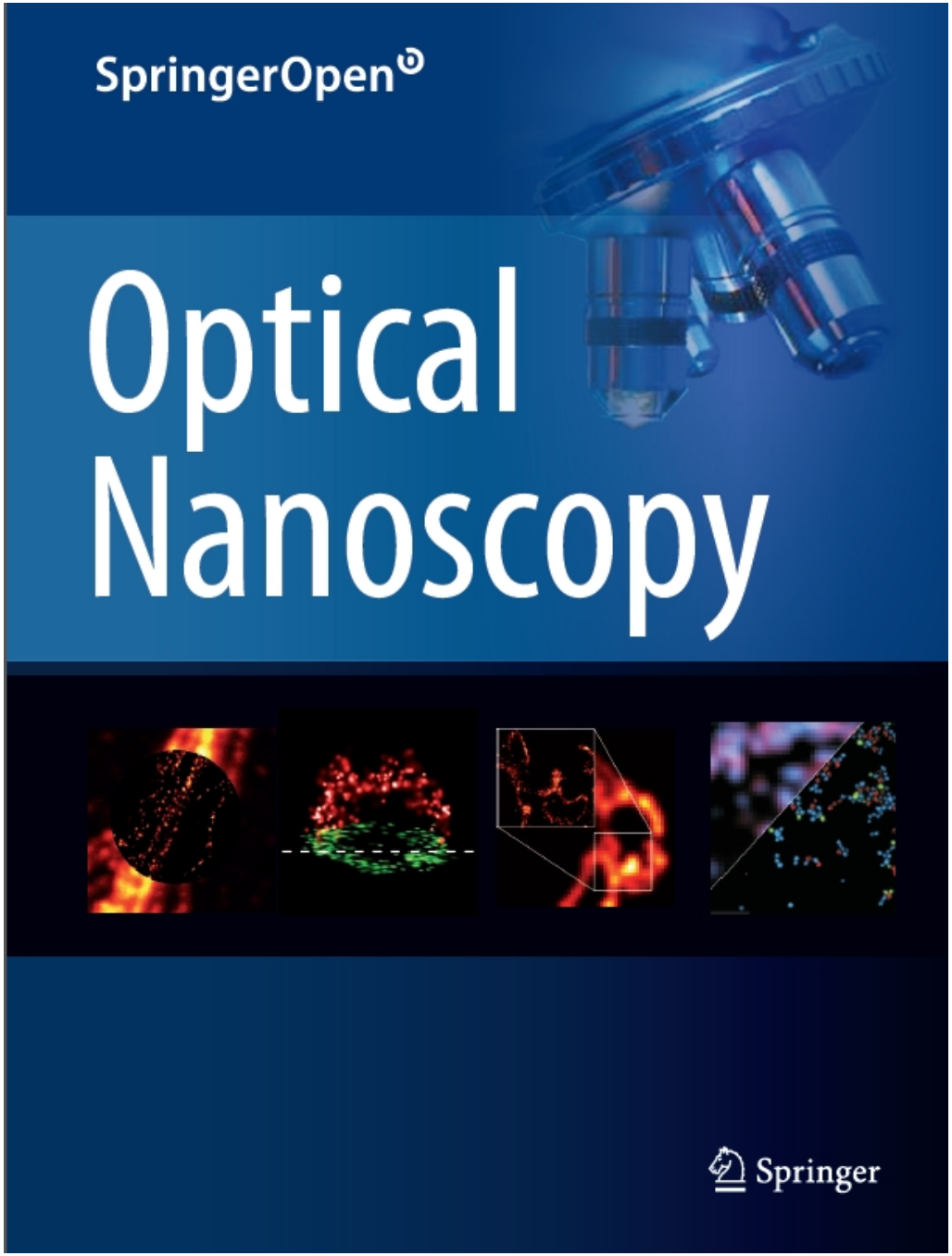
Very molecule-specific!!!!

Link to functionality!?

Purpose
→ increase probability of interactions of less abundant molecules
→ trigger cellular signaling

20nm

Small spatial scales!!!!



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