

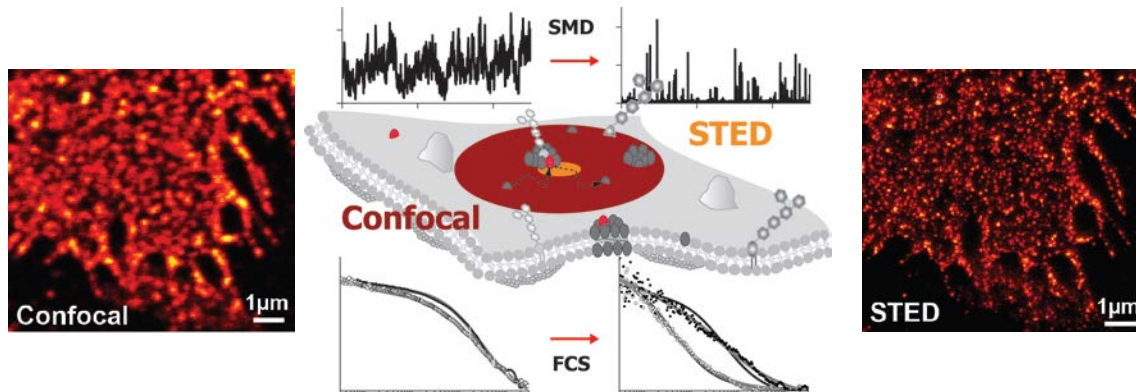
Optical Super-Resolution Microscopy



MRC Human Immunology Unit &
Wolfson Imaging Centre Oxford
Weatherall Institute of Molecular Medicine
University of Oxford

Christian Eggeling

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



Super-Resolution Microscopy

The Nobel Prize in Chemistry 2014



Photo: Matt Staley/HHMI

Eric Betzig

Prize share: 1/3



Photo: Wikimedia
Commons, CC-BY-SA-3.0

Stefan W. Hell

Prize share: 1/3



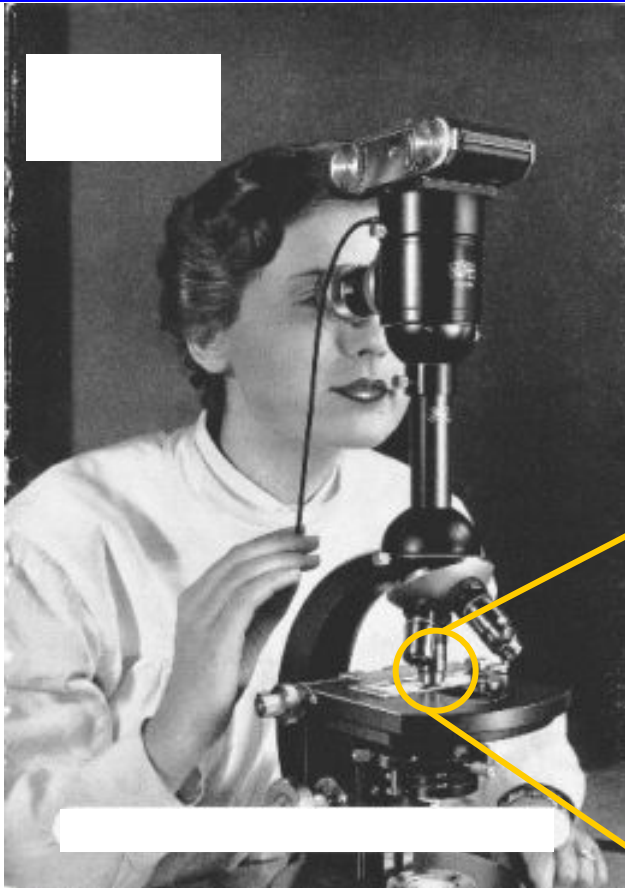
Photo: K. Lowder via
Wikimedia Commons, CC-
BY-SA-3.0

William E. Moerner

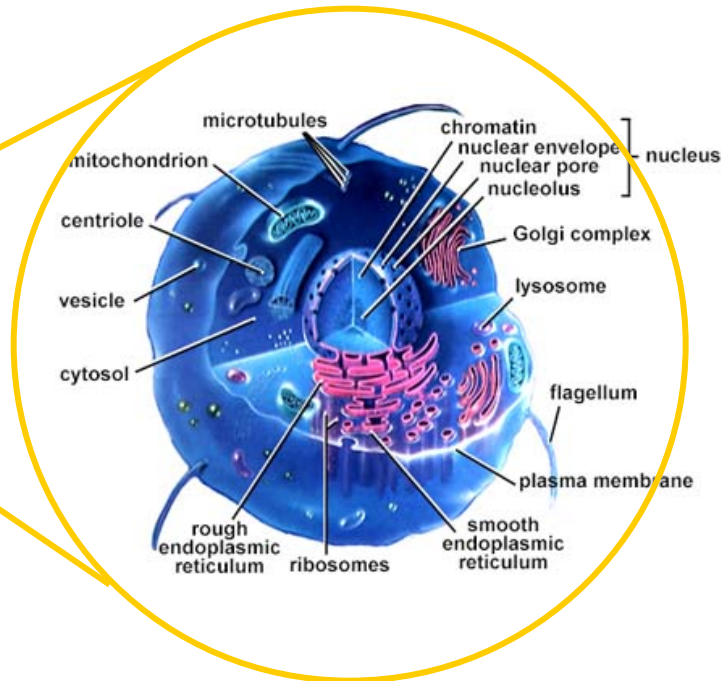
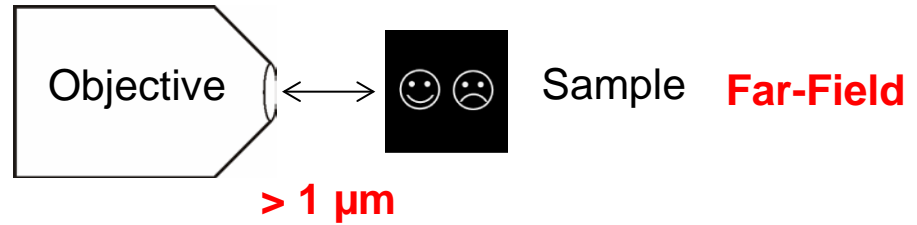
Prize share: 1/3

Live Cell Microscopy

Observation of living cells: Non-Invasive



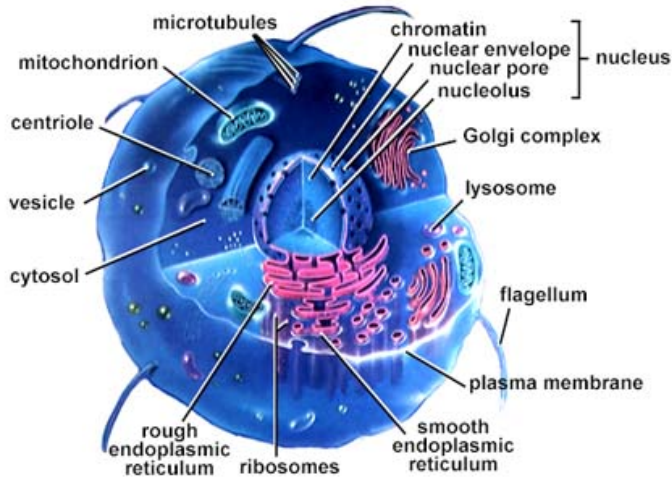
Light + Far-Field: non-invasive!



Live Cell Far-Field Microscopy

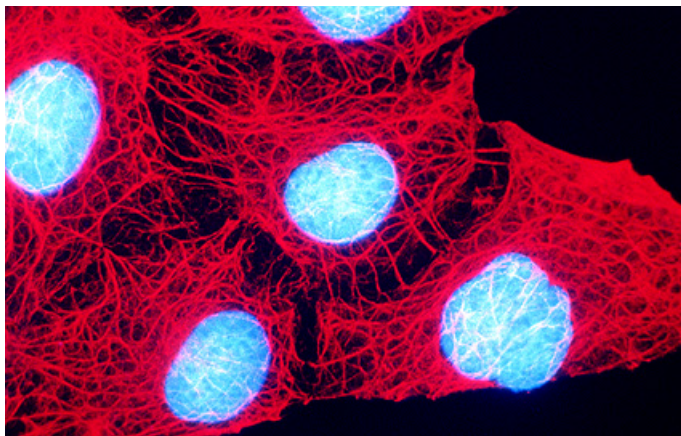
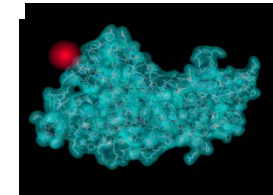
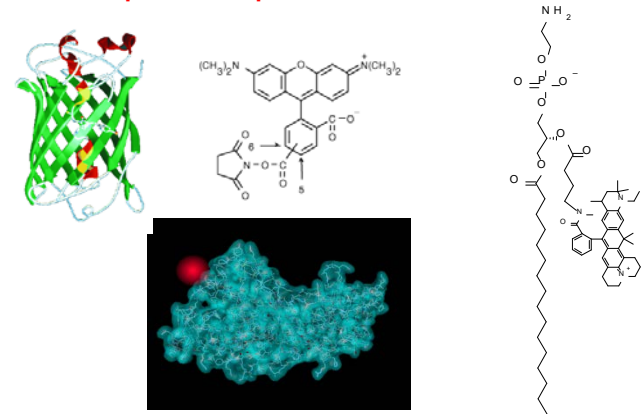
Fluorescence

Study specific molecular processes in the living cell:



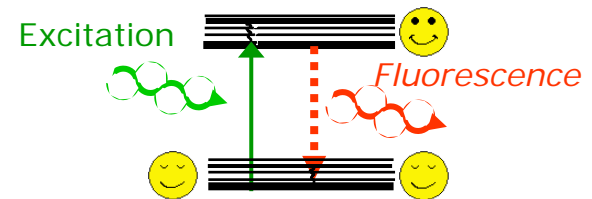
Fluorescence microscopy

Label specific protein/molecule



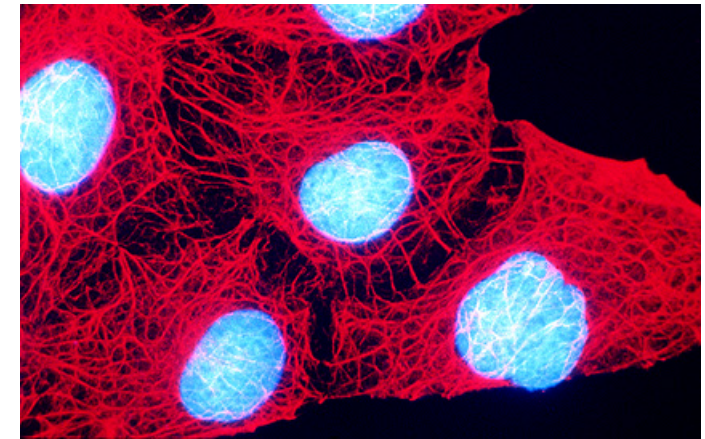
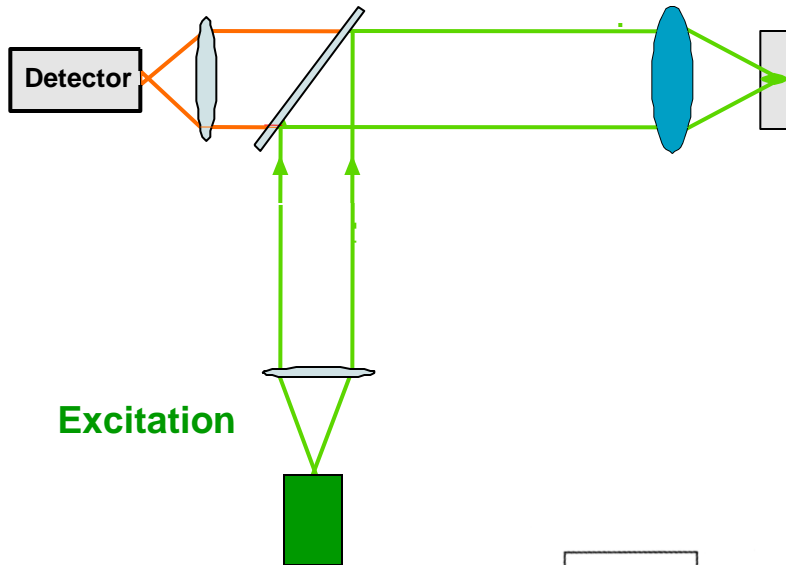
Liver-Cells: Nucleus and Cell-skeleton

Excite fluorescence by laser light



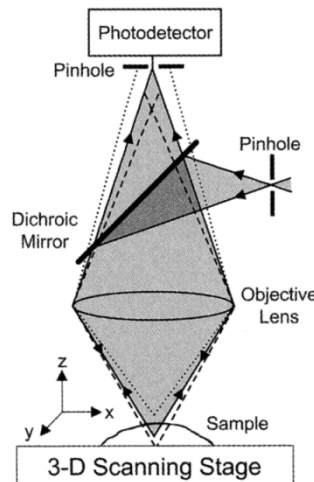
Far-Field Fluorescence Microscopy

Confocal Setup



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

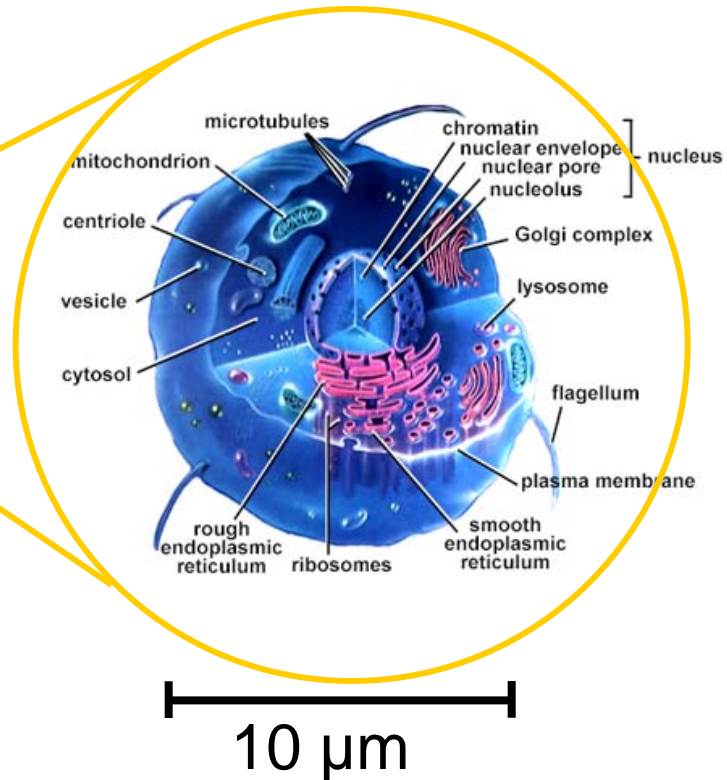
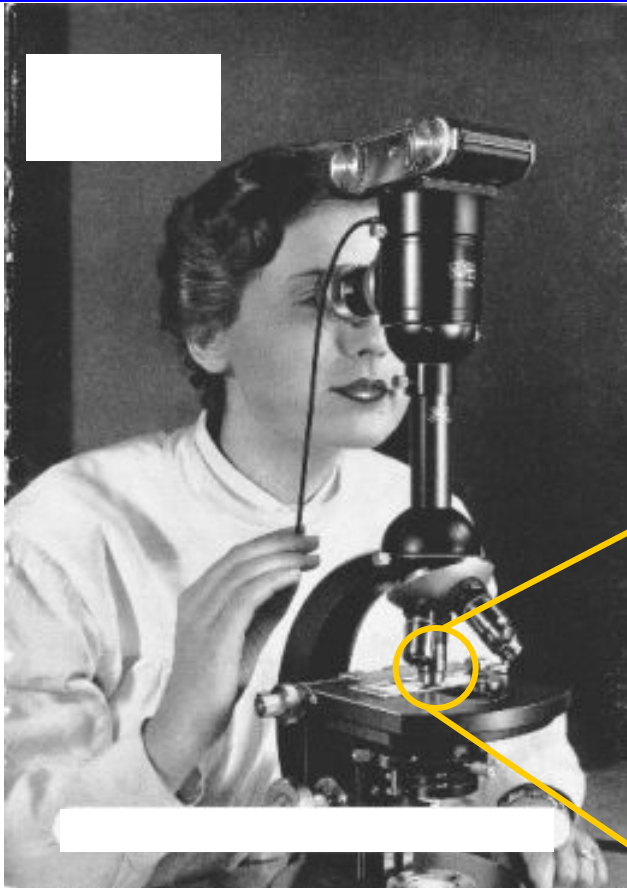
- Small area illuminated
- **Point detection: scanning** required to construct image
- **Confinement along z** (pinhole)



- Large area illuminated
- **Camera detection:** image taken in one step
- No z-confinement

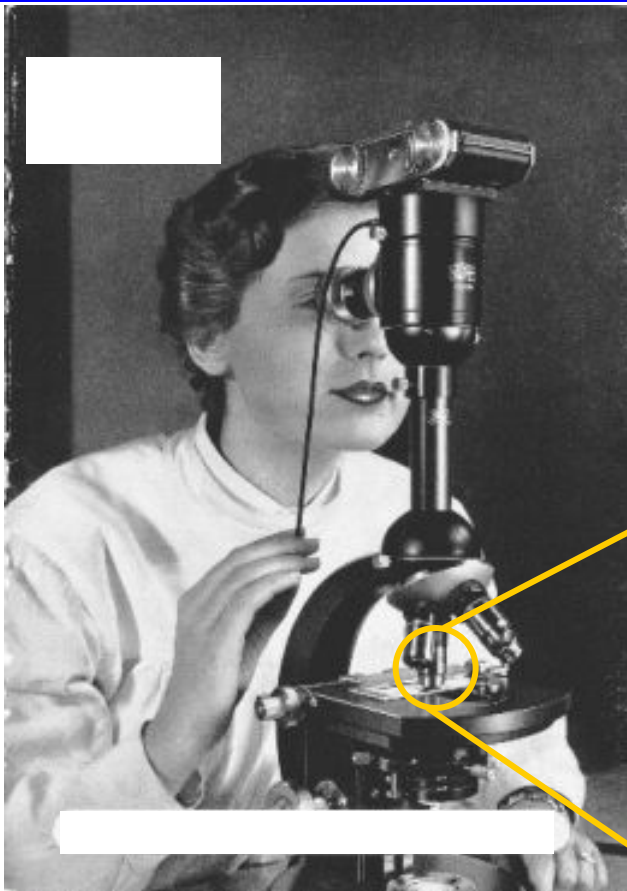
Far-Field Fluorescence Microscopy

Resolution: Goal

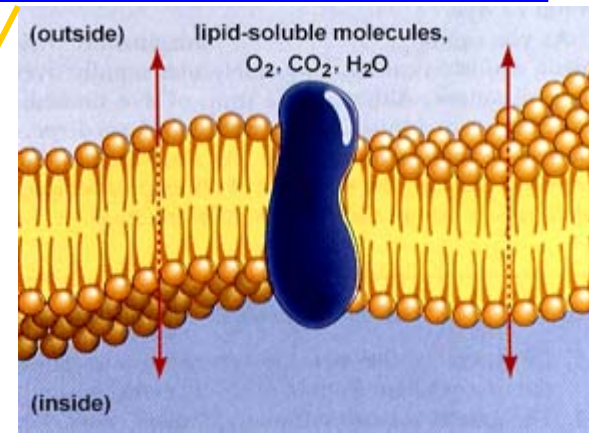


Far-Field Fluorescence Microscopy

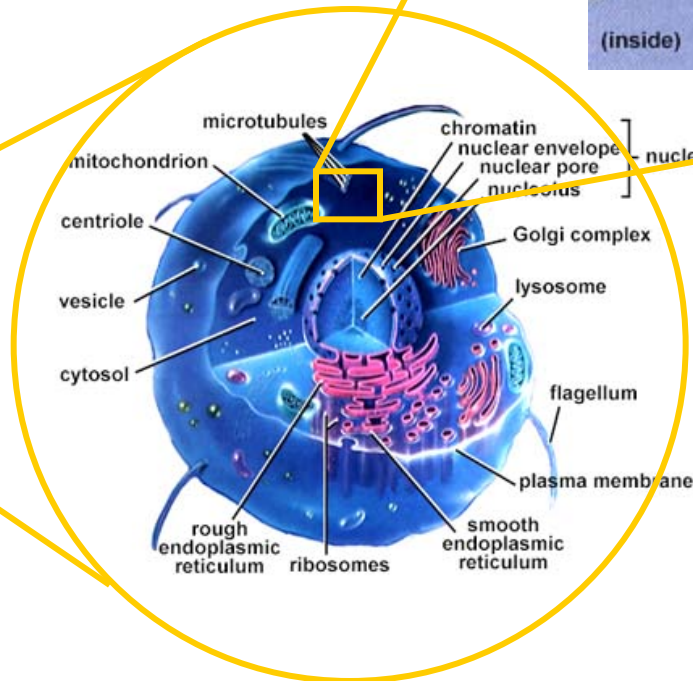
Resolution: Goal



⇒ molecular scale



10 nm



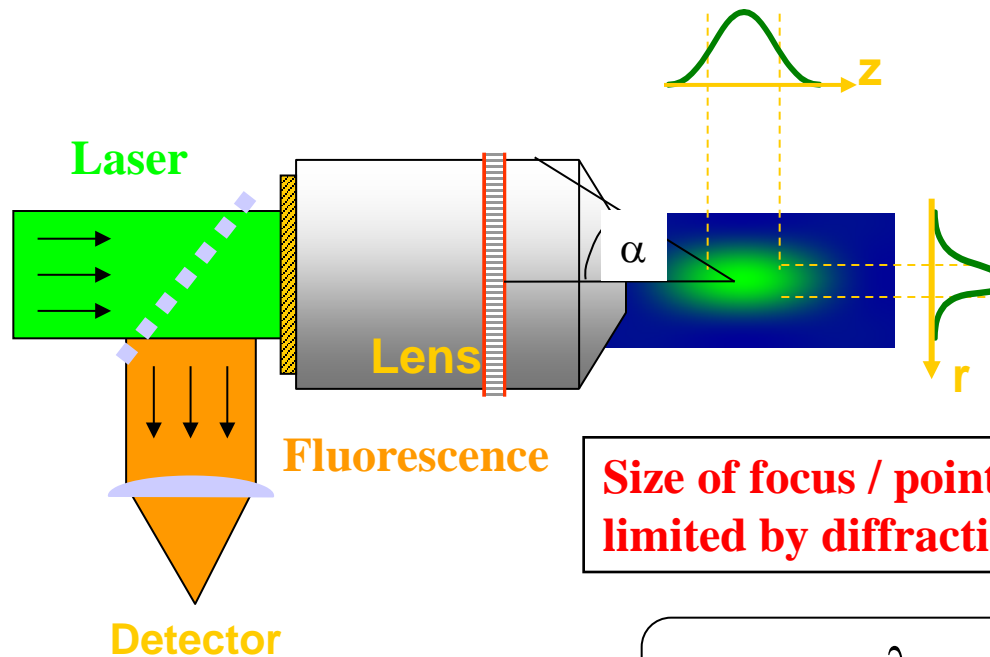
10 μm

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

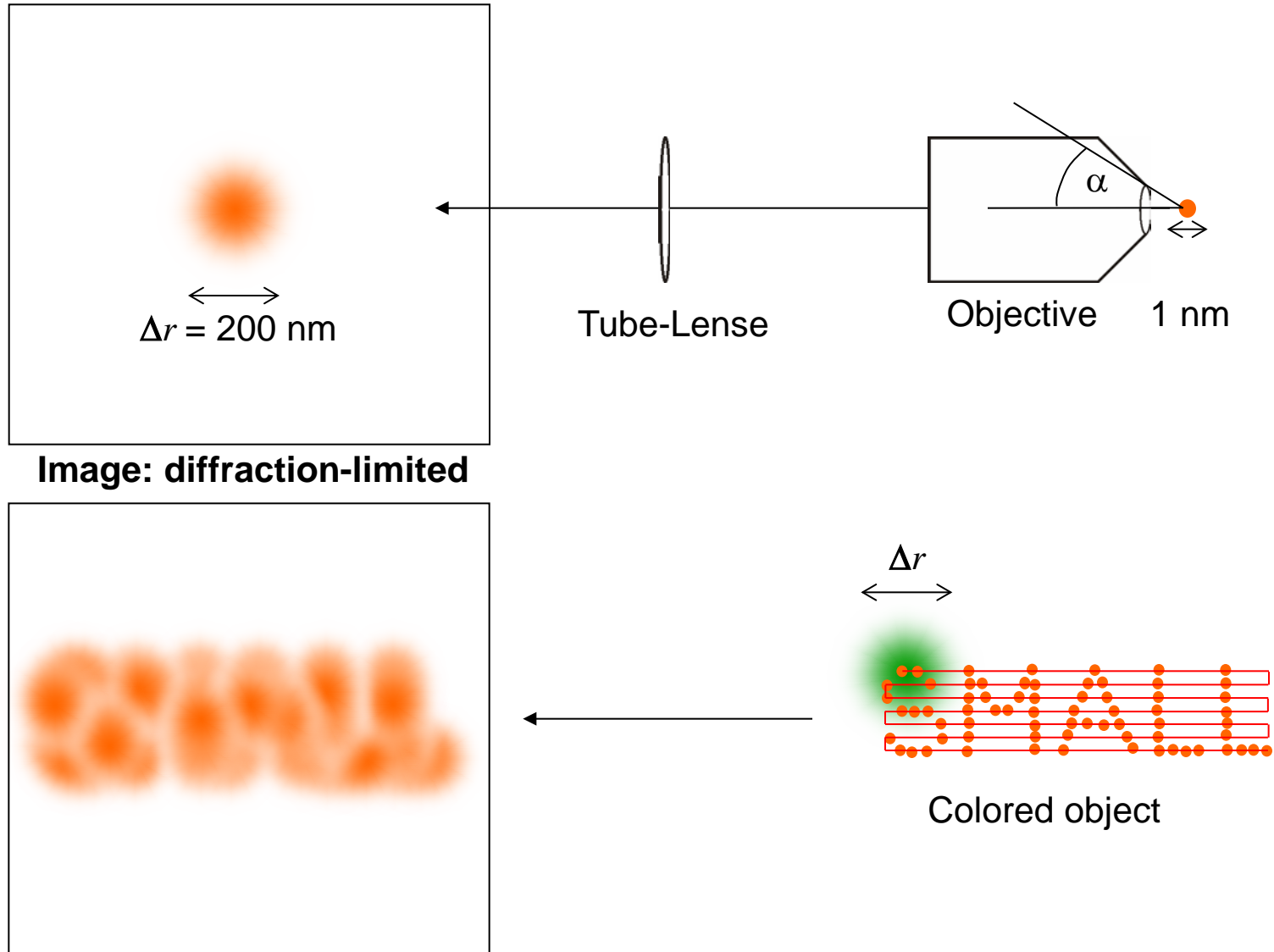
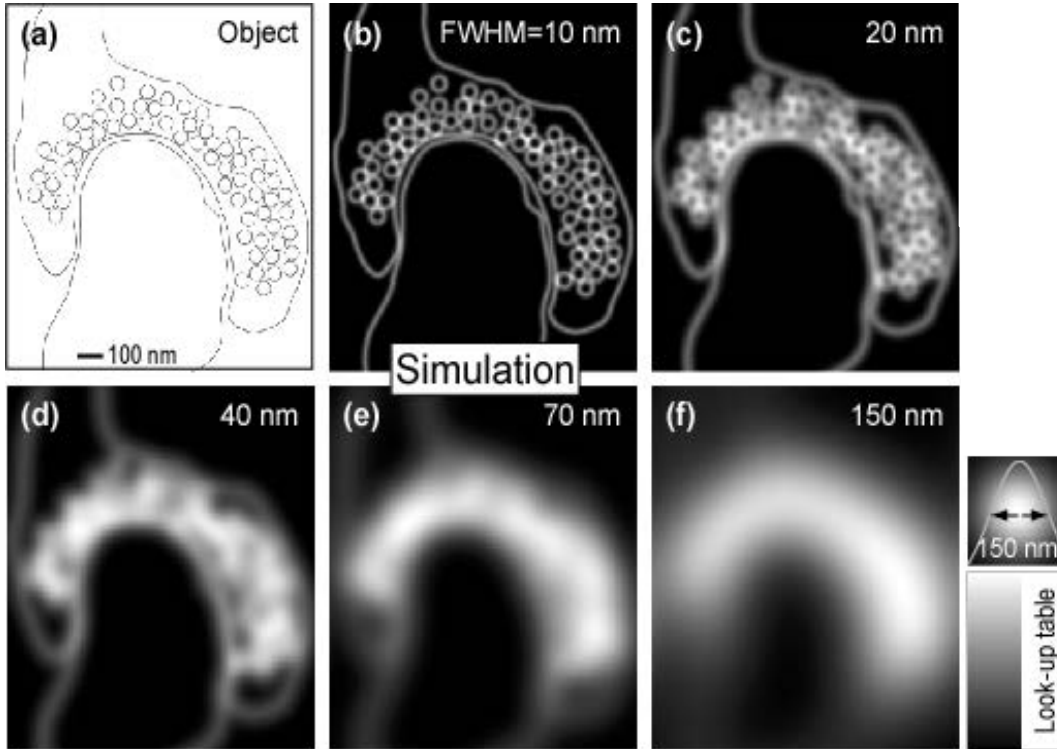


Image: diffraction-limited

Colored object

Resolution of Optical Microscopy Resolving Close-By Objects

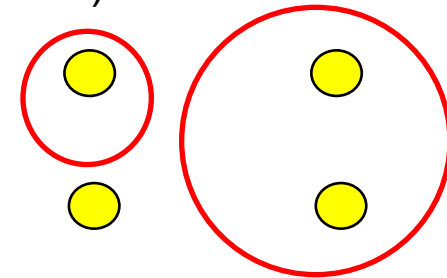


Cartoon of excitatory synapse

(from electron micrograph)

(vesicles + plasma membrane)

(cortex of brain)

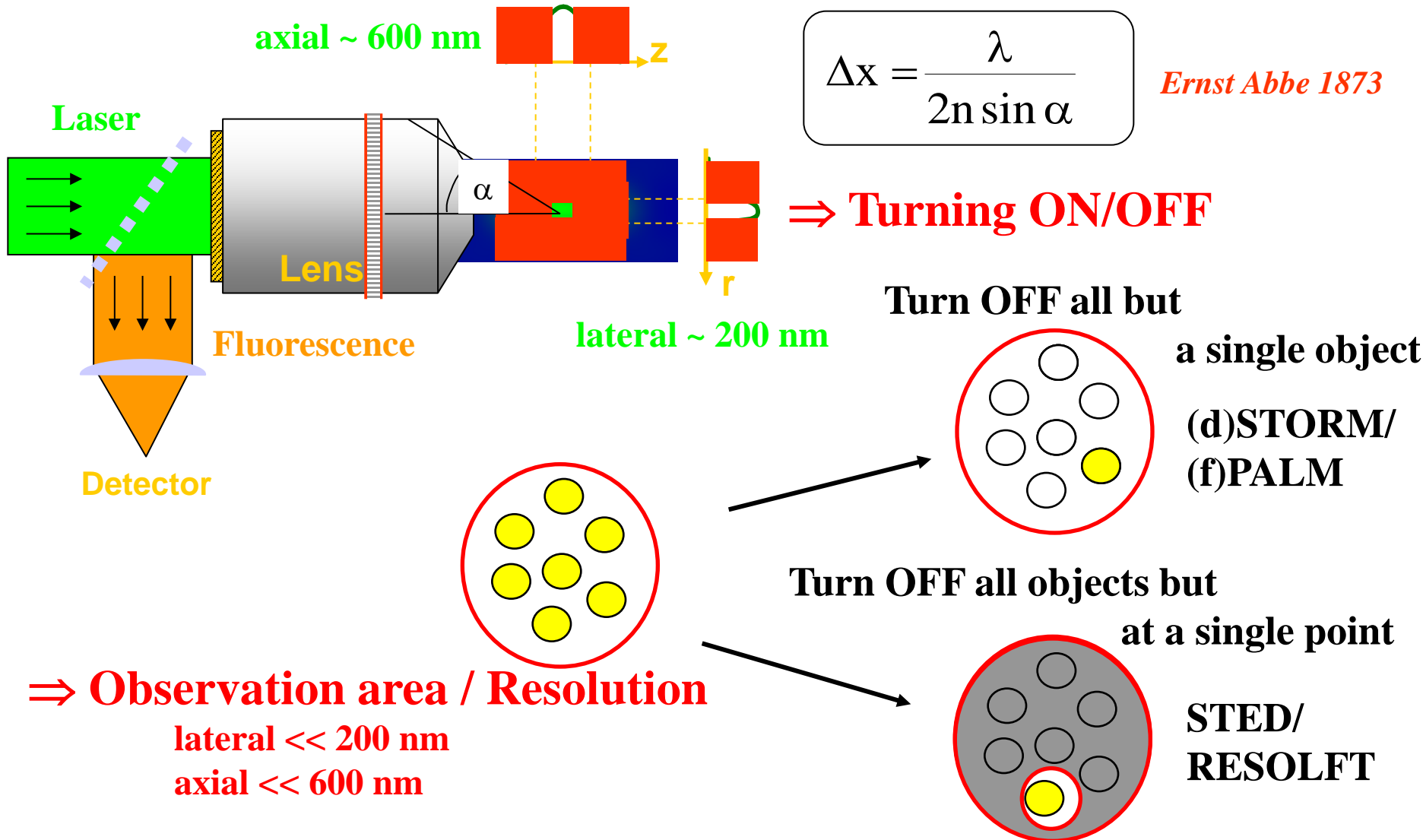


Scanning microscopy:

The smaller the spot, the sharper the image !

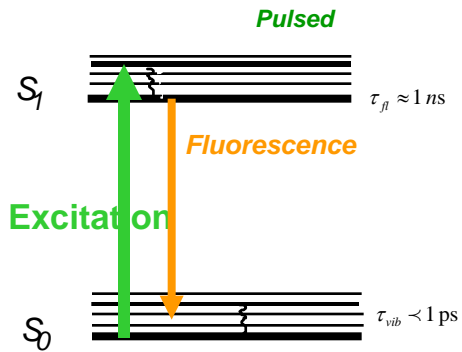
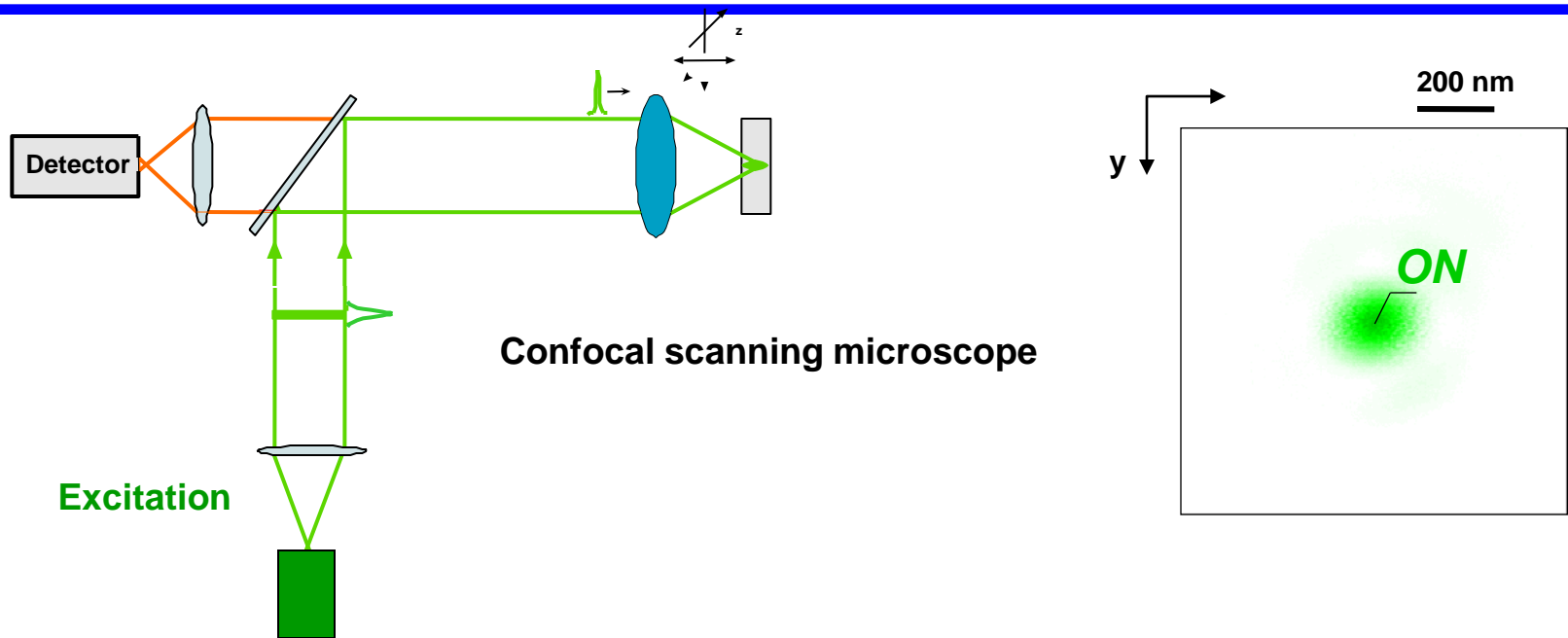
Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF



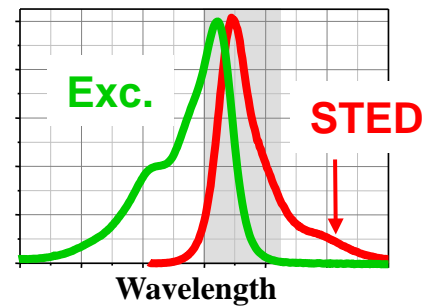
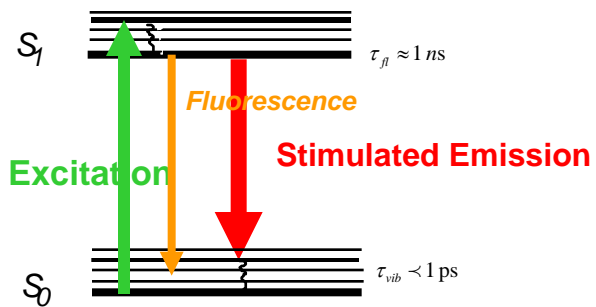
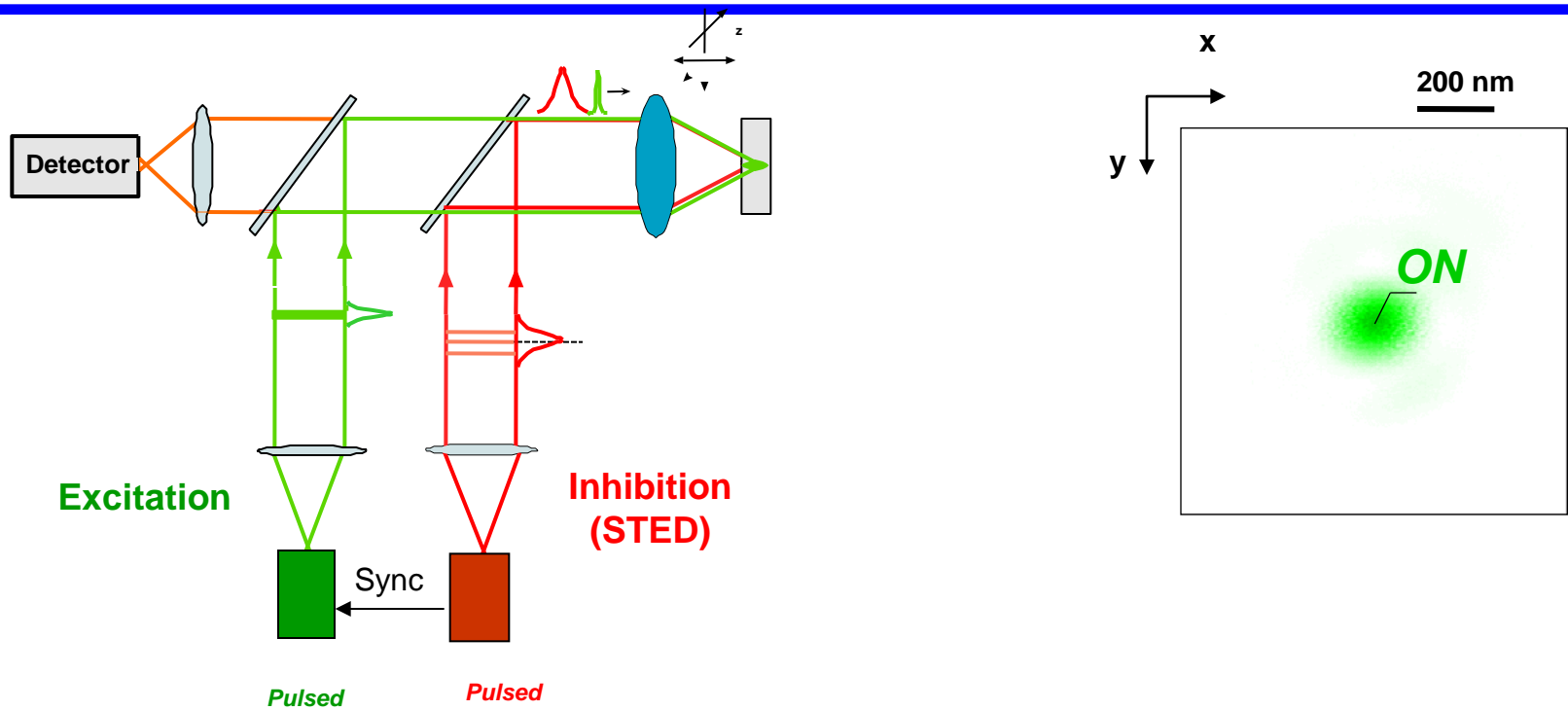
Fluorescence Microscopy

STED Microscopy



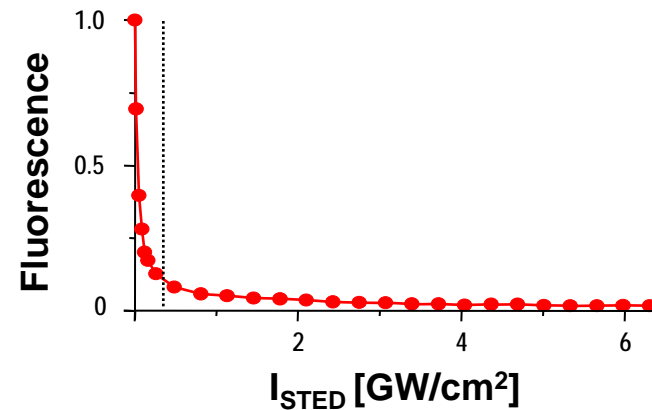
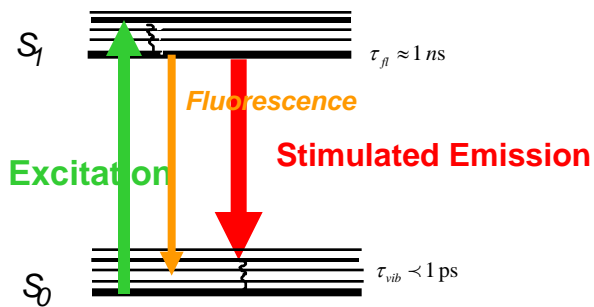
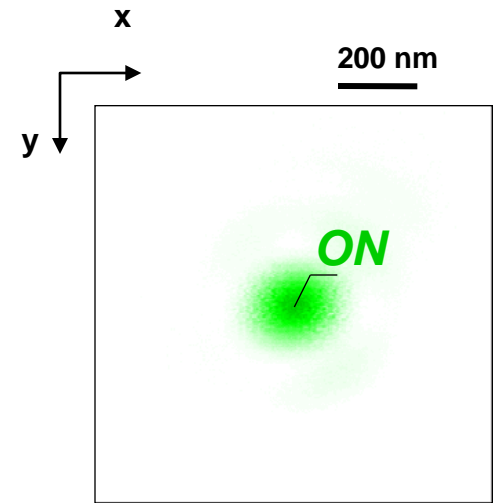
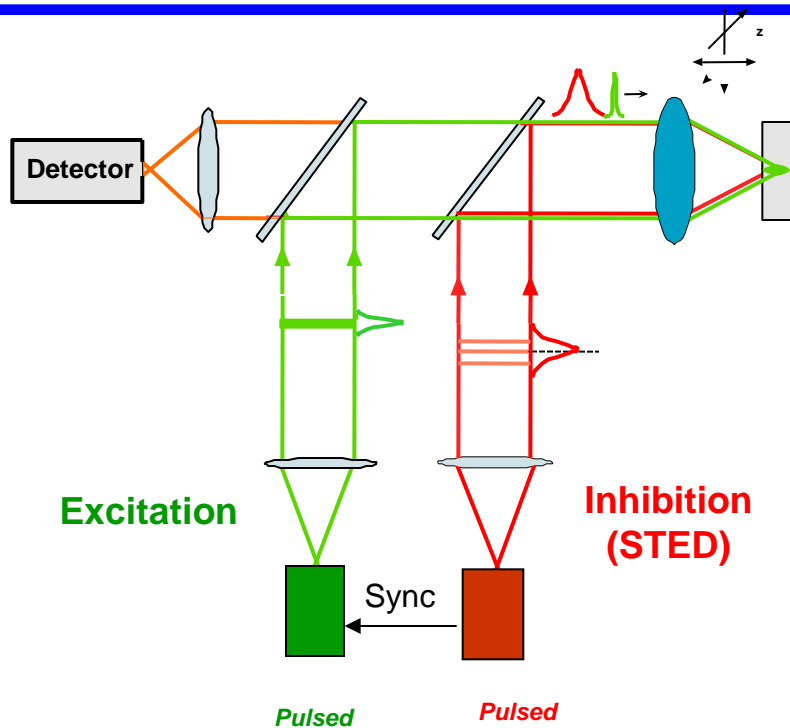
Fluorescence Microscopy

STED Microscopy



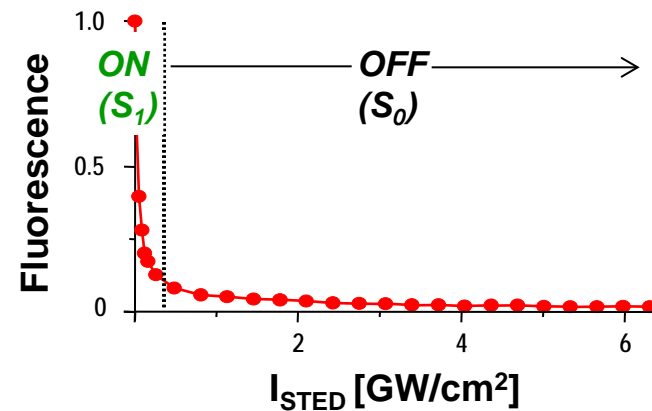
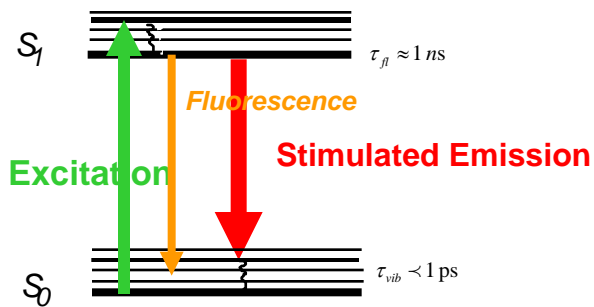
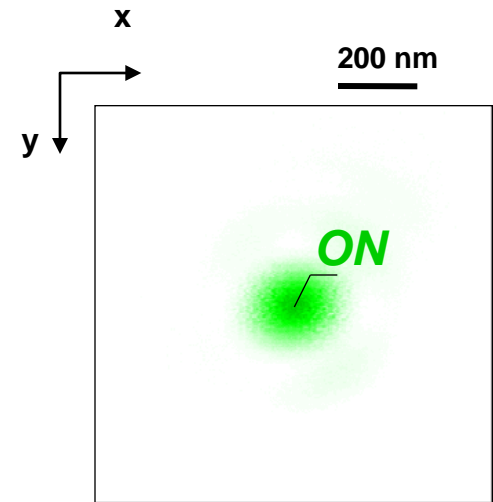
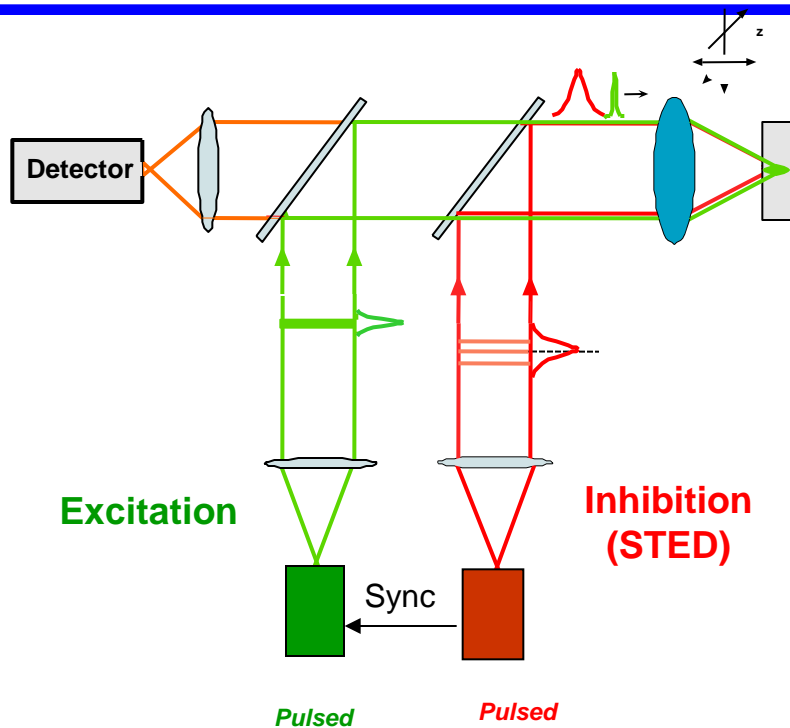
Fluorescence Microscopy

STED Microscopy



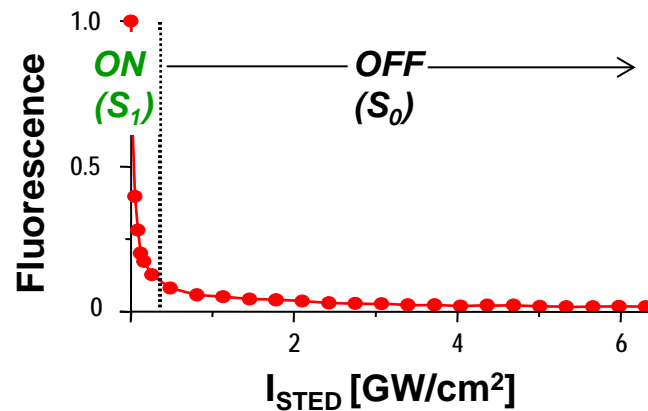
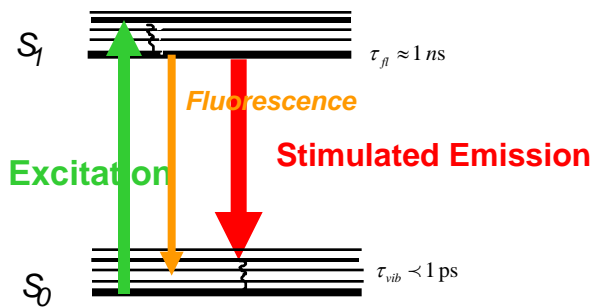
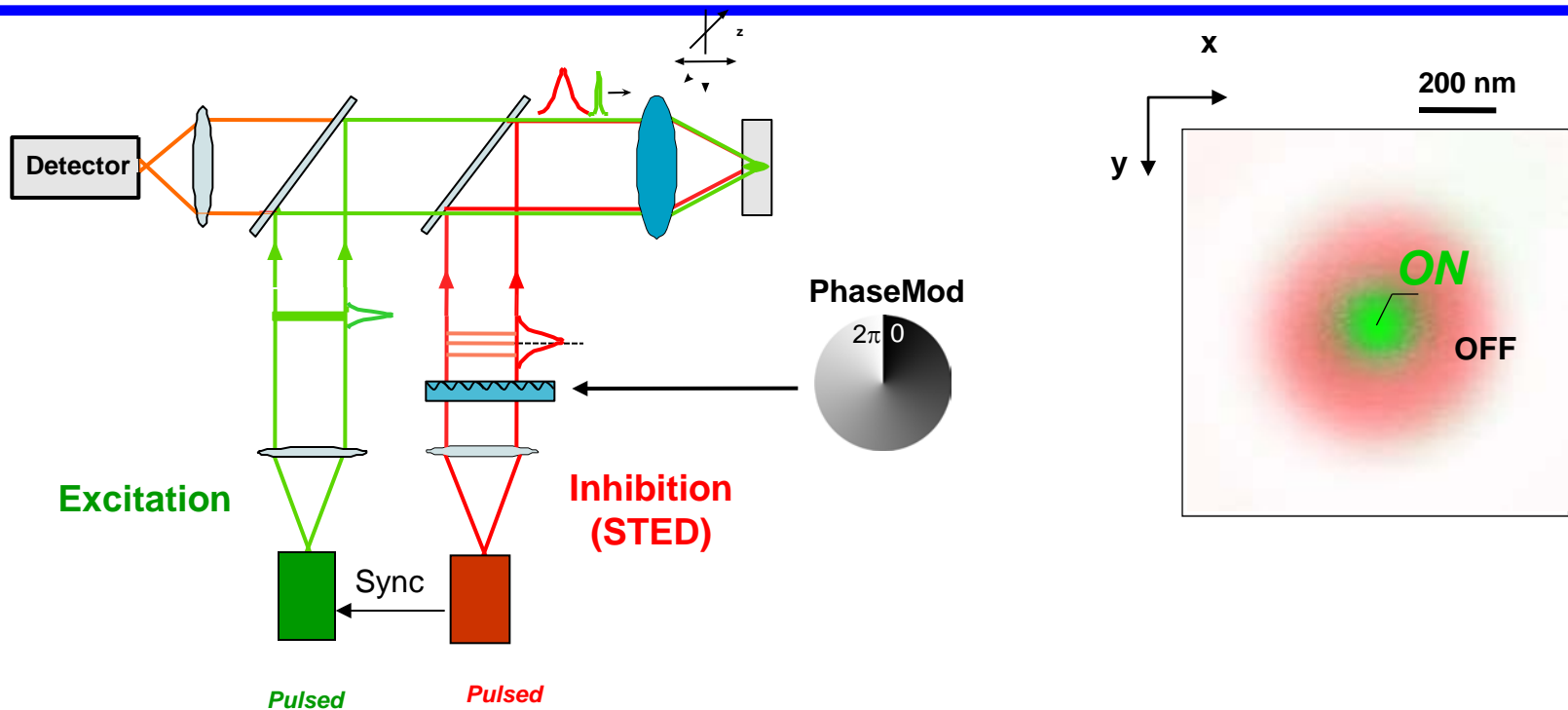
Fluorescence Microscopy

STED Microscopy



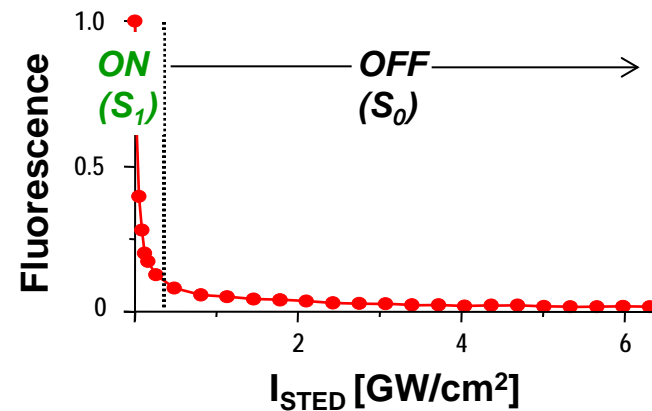
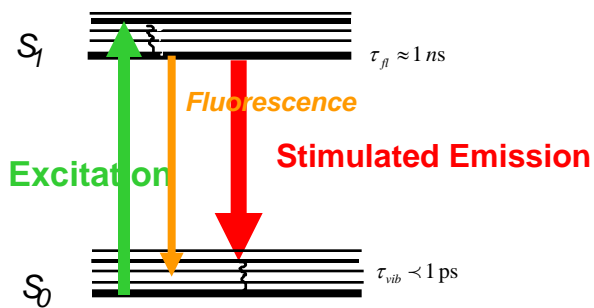
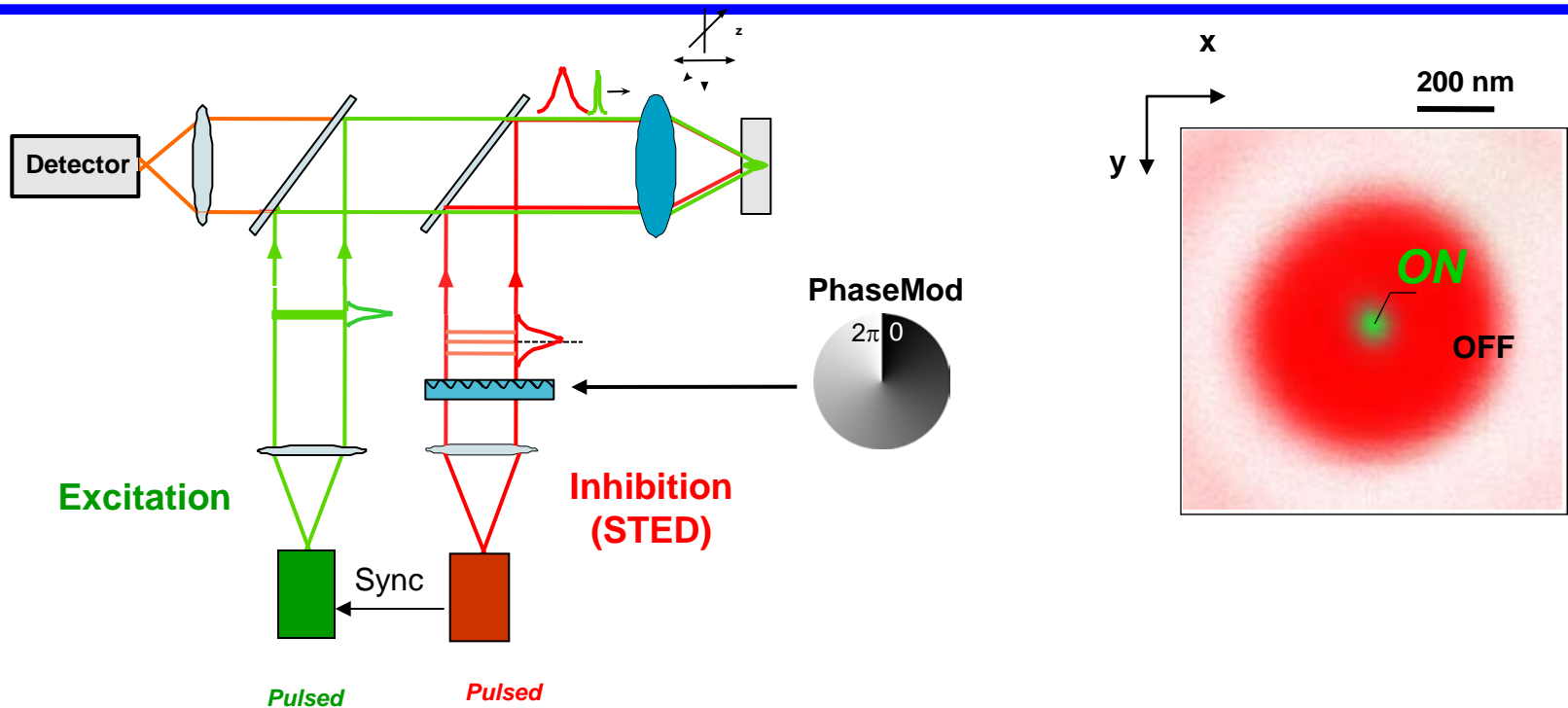
Fluorescence Microscopy

STED Microscopy



Fluorescence Microscopy

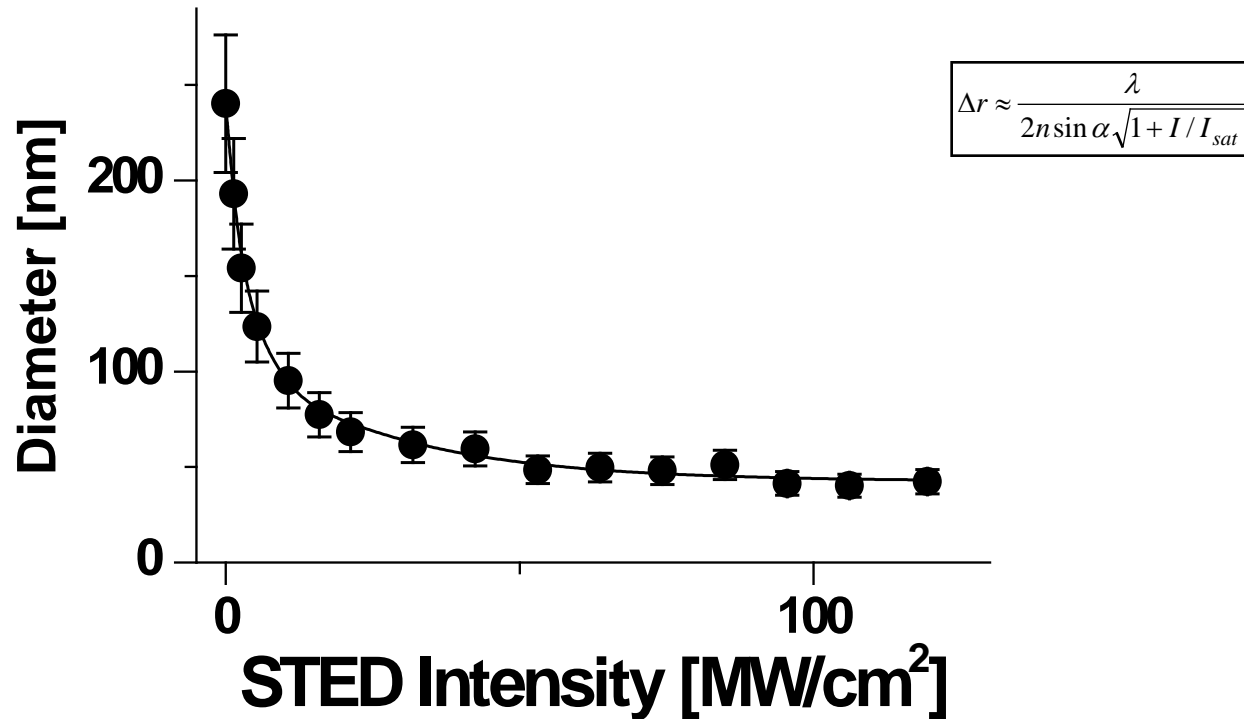
STED Microscopy



STED Microscopy

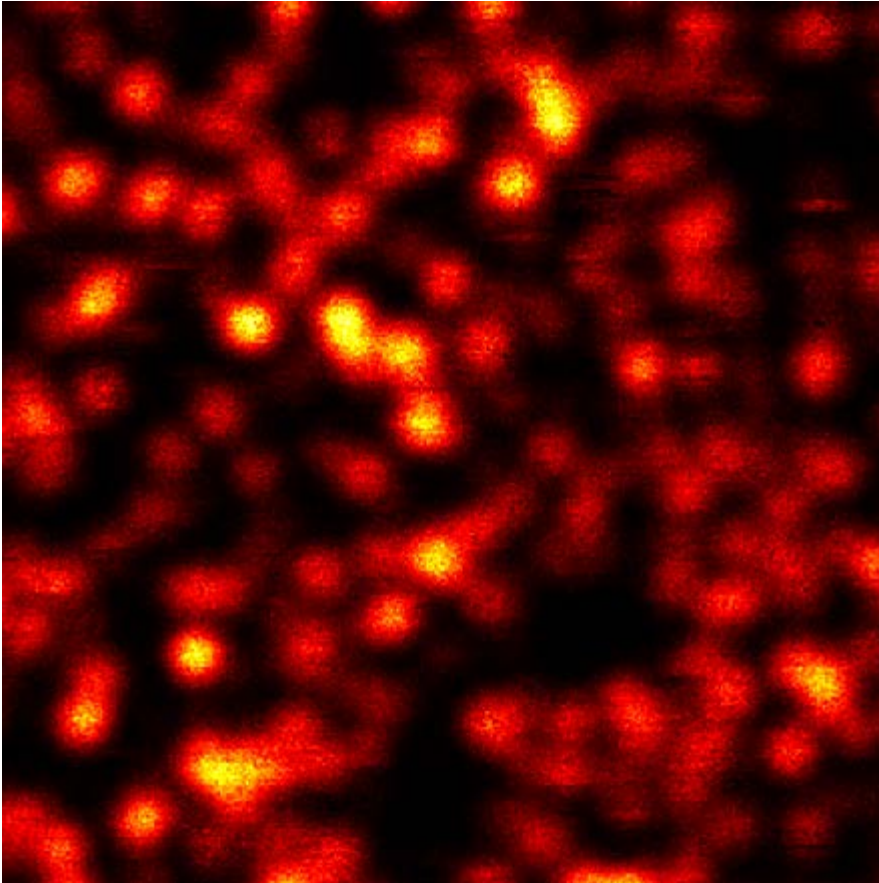
Dynamical confinement of resolution

Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!

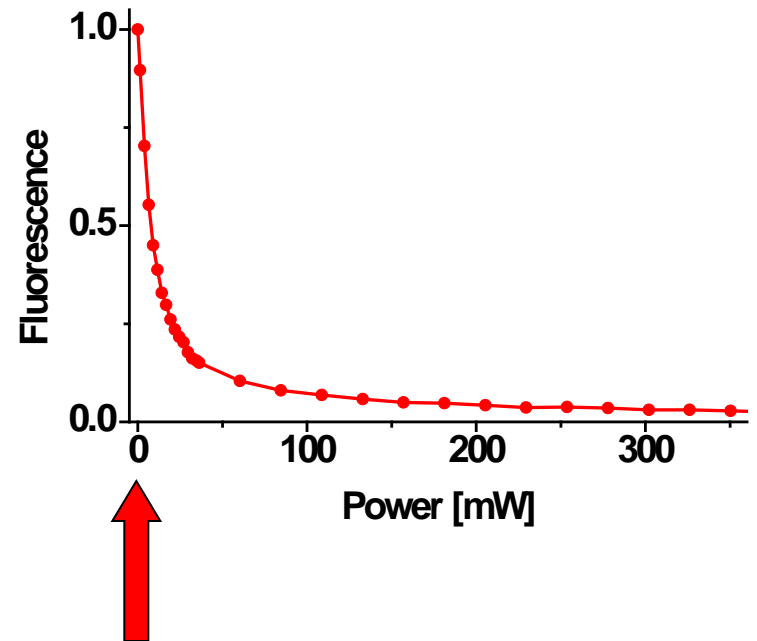
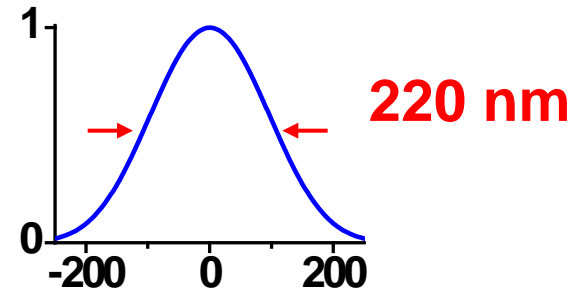


STED-Microscopy

Sub-Diffraction Imaging

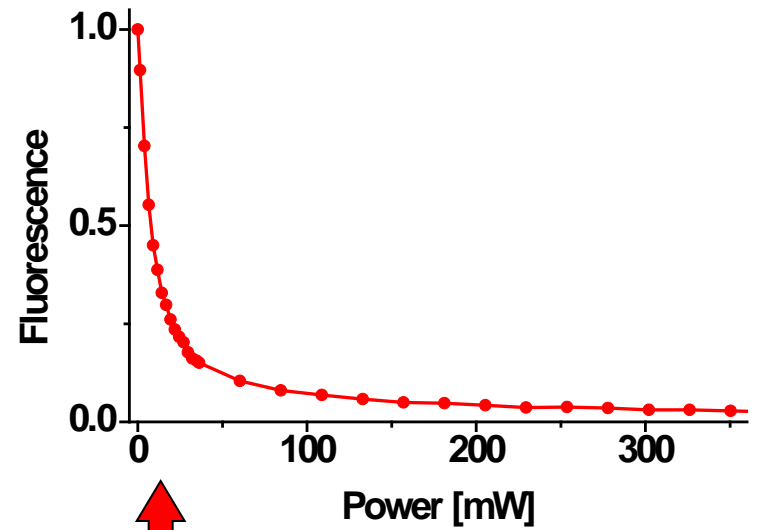
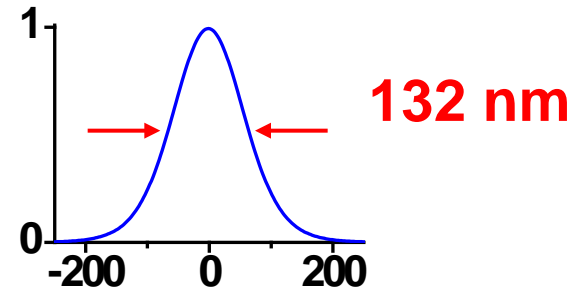
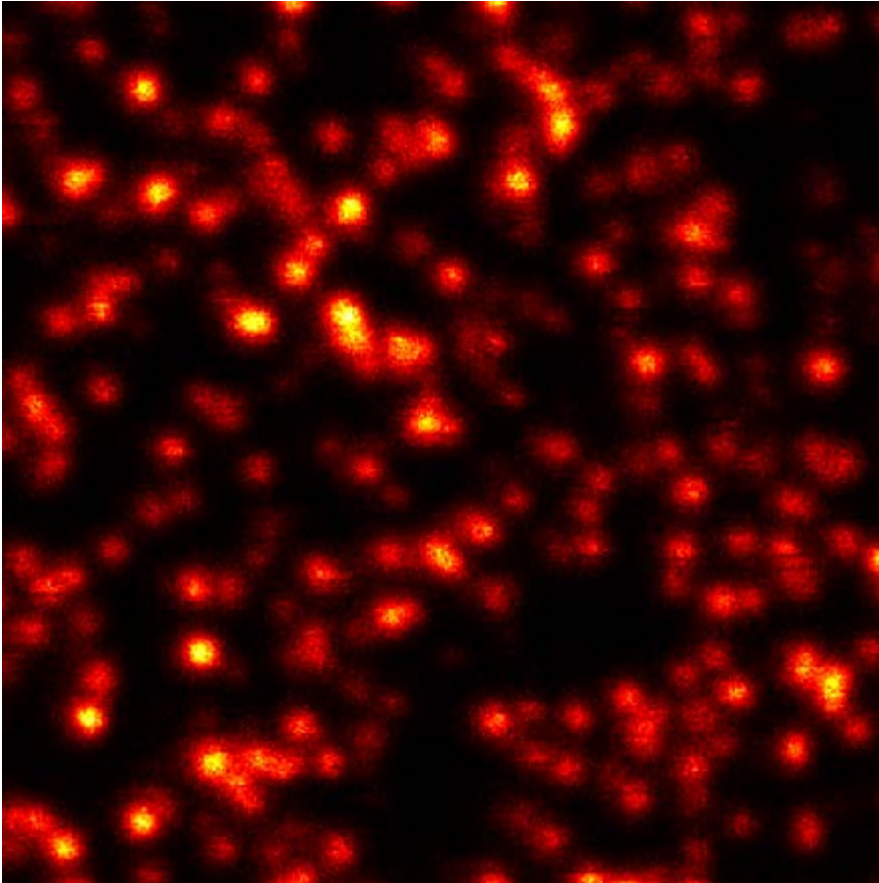


20nm Crimson beads
633nm exc, 90ps, 30kW/cm²
785nm STED 200ps, 76MHz



STED-Microscopy

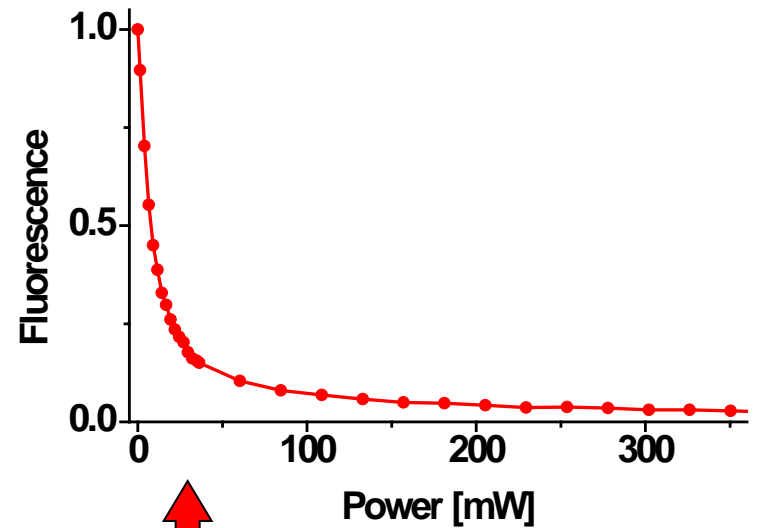
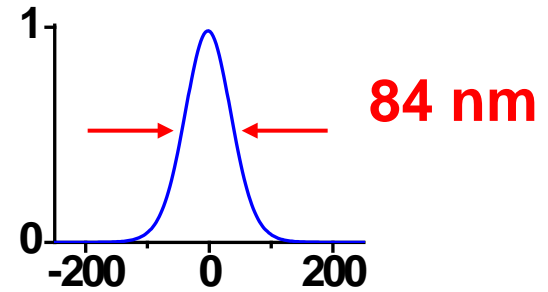
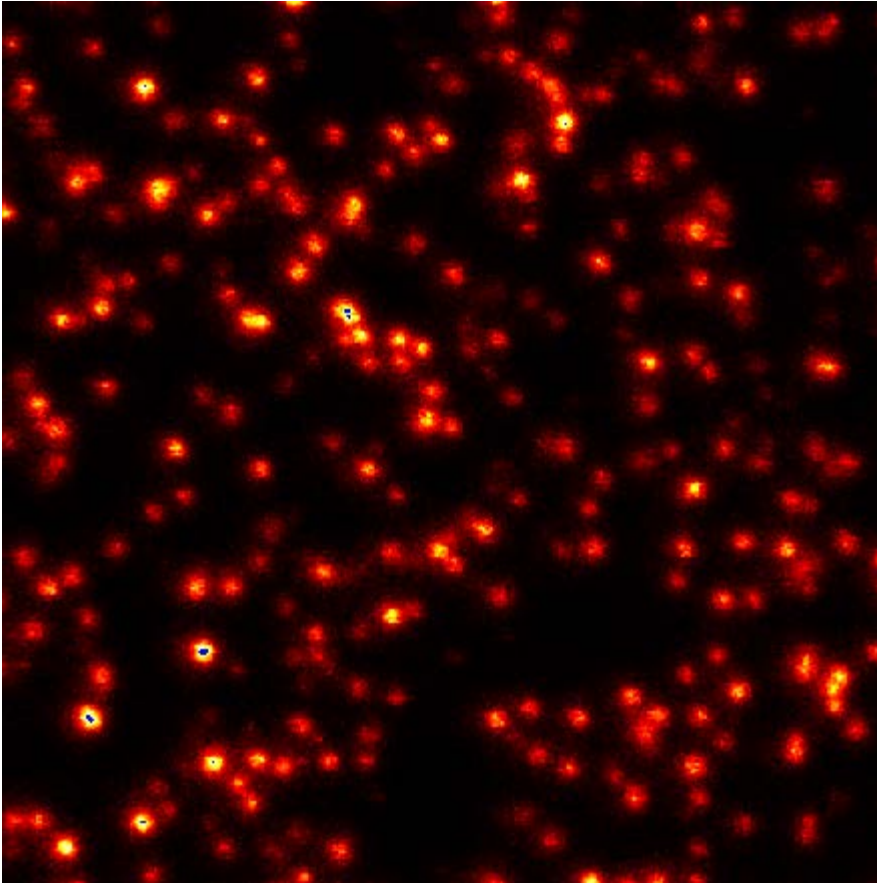
Sub-Diffraction Imaging



20nm Crimson beads
633nm exc, 90ps, 30kW/cm²
785nm STED 200ps, 76MHz

STED-Microscopy

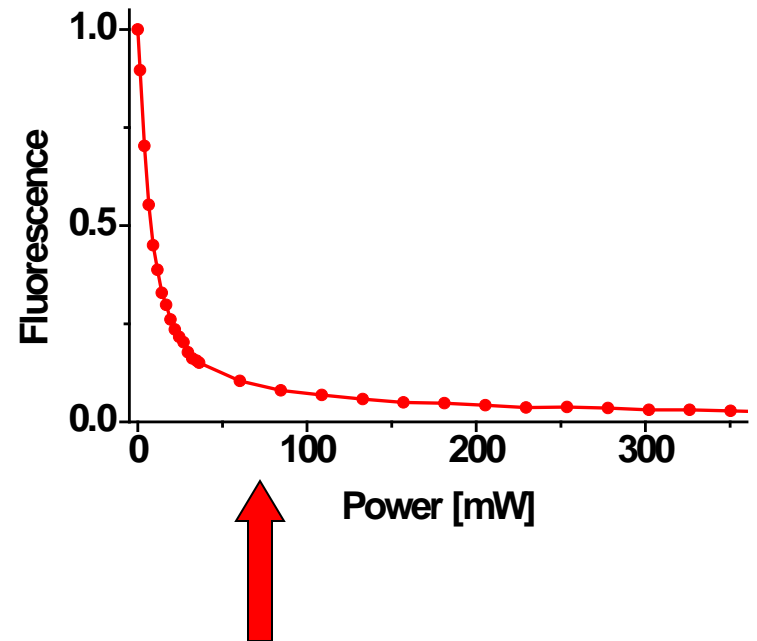
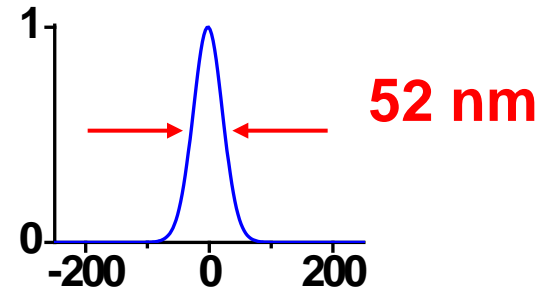
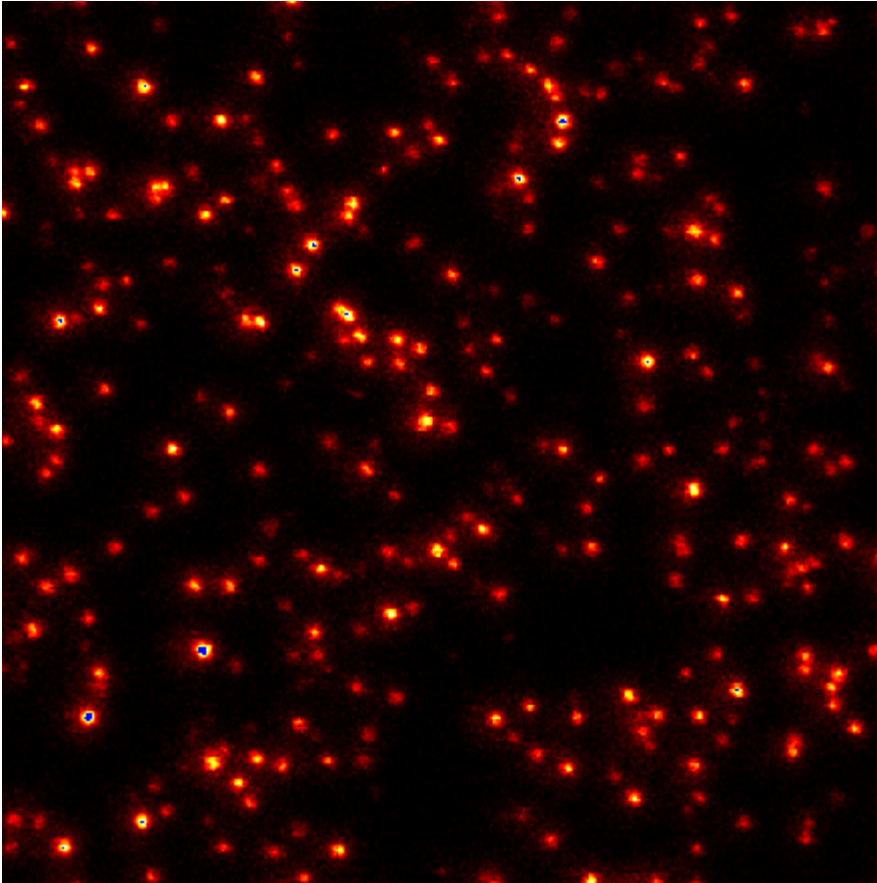
Sub-Diffraction Imaging



20nm Crimson beads
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785nm STED 200ps, 76MHz

STED-Microscopy

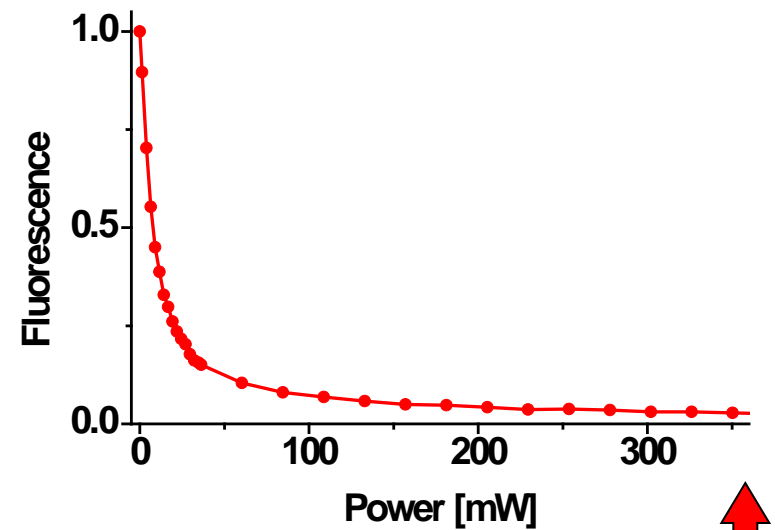
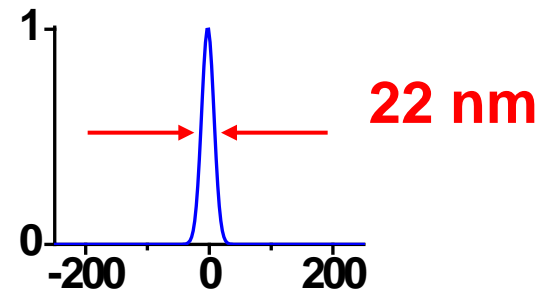
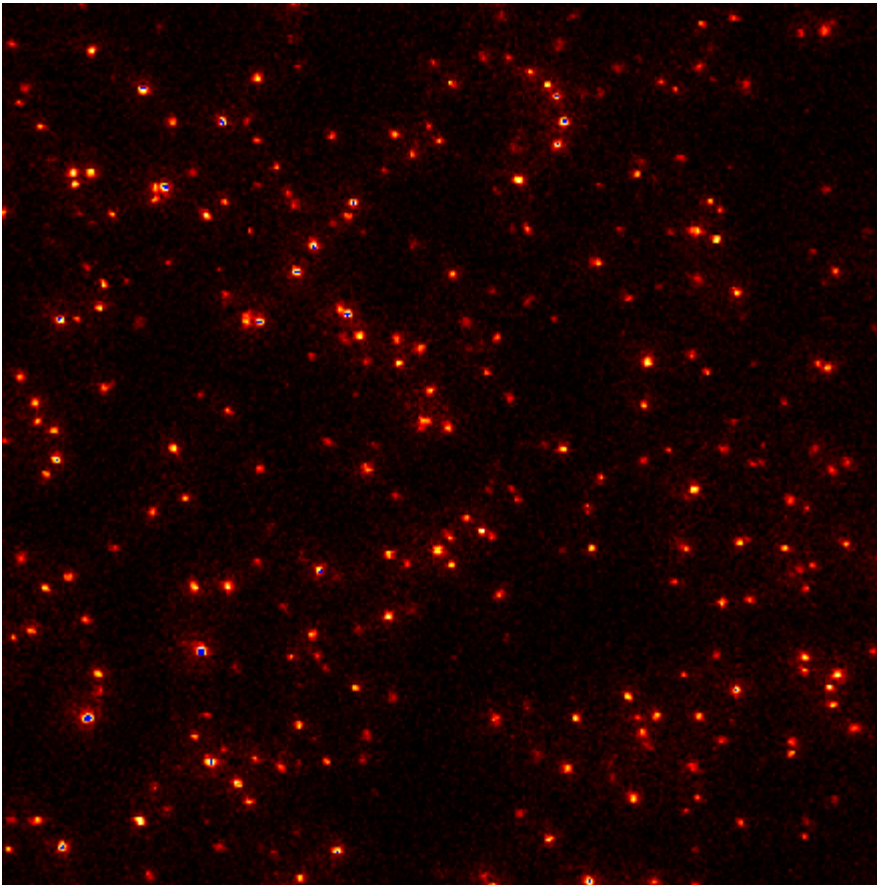
Sub-Diffraction Imaging



20nm Crimson beads
633nm exc, 90ps, 30kW/cm²
785nm STED 200ps, 76MHz

STED-Microscopy

Sub-Diffraction Imaging

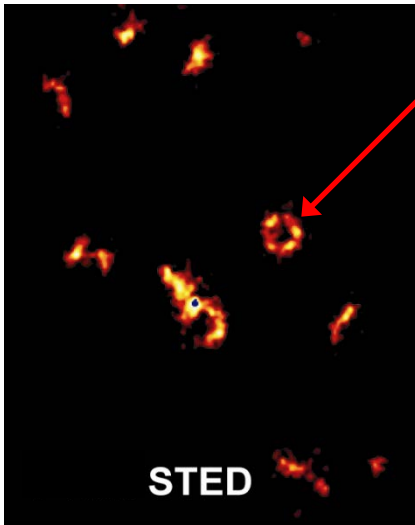
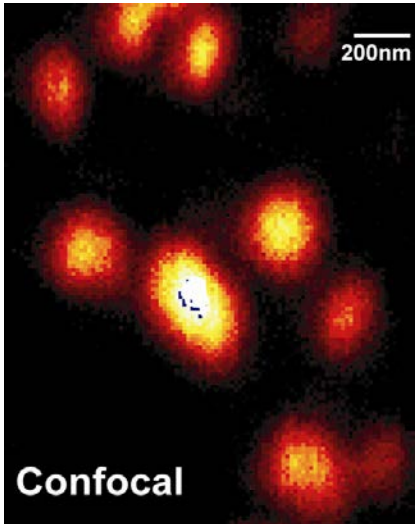


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STED Microscopy

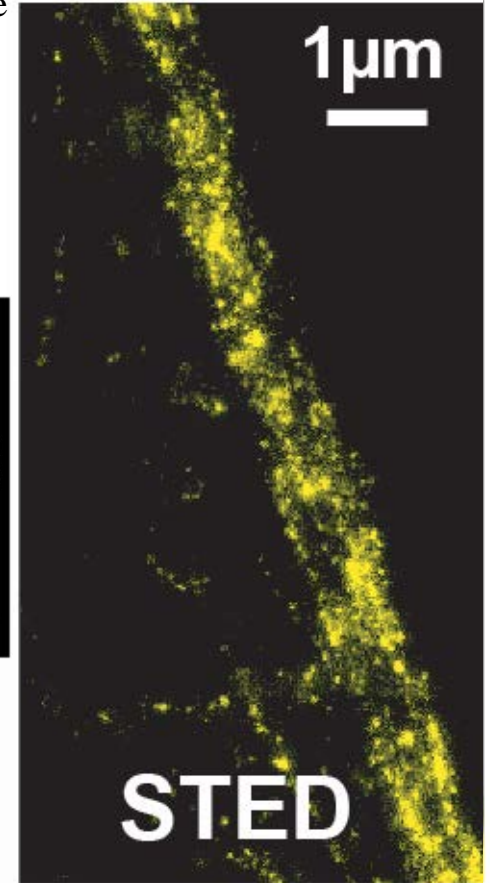
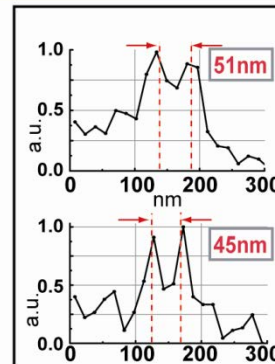
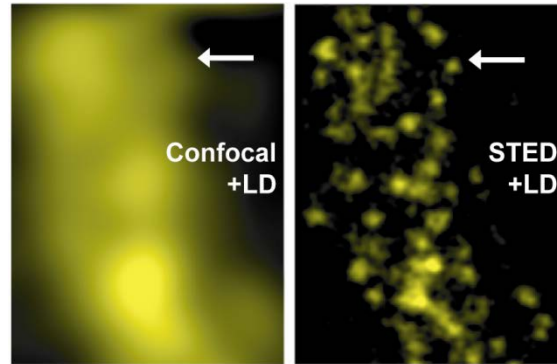
Cellular Imaging

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



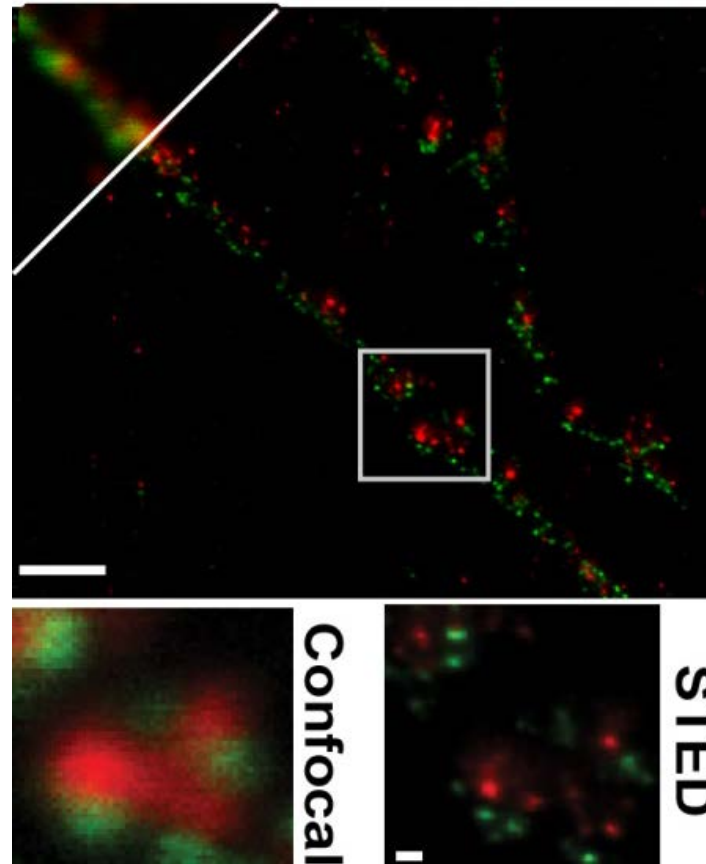
Nanopatterns

synaptic proteins on endosomes
of PC 12 cells
(neuroendocrine activity;
generate synaptic vesicles)
Atto532-synaptophysin
LD



STED Microscopy

Cellular Imaging

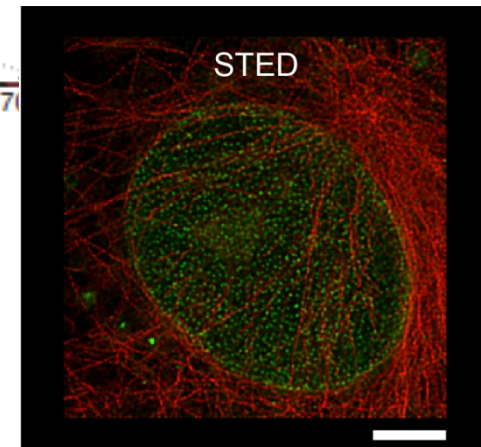
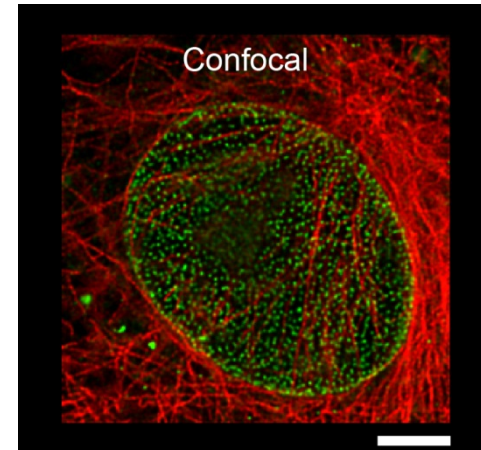
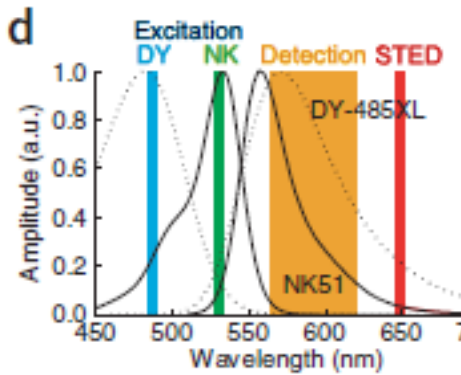
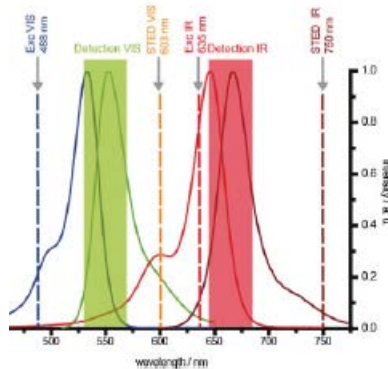
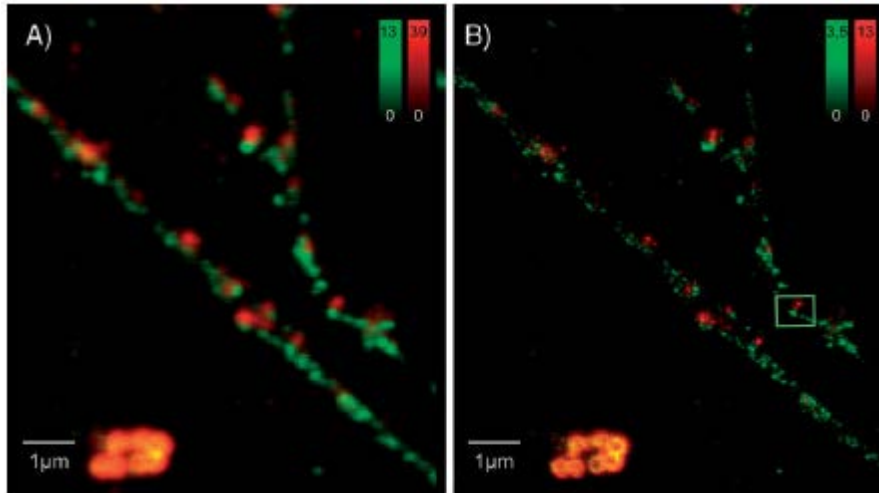


Synaptophysin (red, Atto647N) + syntaxin1 (green, Atto532)
in neurons

Donnert et al BiophysLett 2006 / Meyer et al Small 2008

STED-Microscopy

Multi-Color Sub-Diffraction Imaging



3 laser lines:

Large Stokes shift dye
– only one excitation laser

Abberior STAR 488 (green):

nuclear pore complexes

Abberior STAR 440Sx:

alpha-tubulin in microtubules (red)

fixed HeLa cells

Schmidt et al NatMethods 2008

Clausen et al Nanobioimaging 2013

4 laser lines:

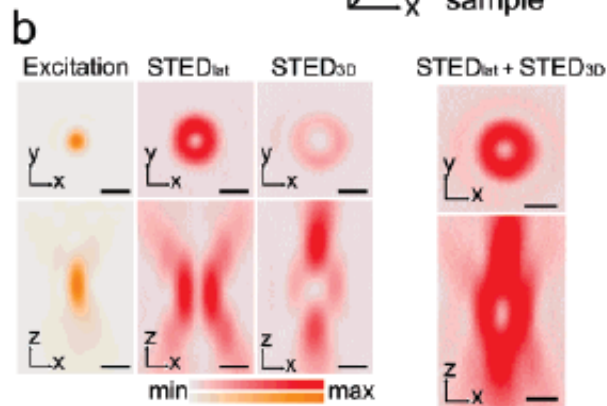
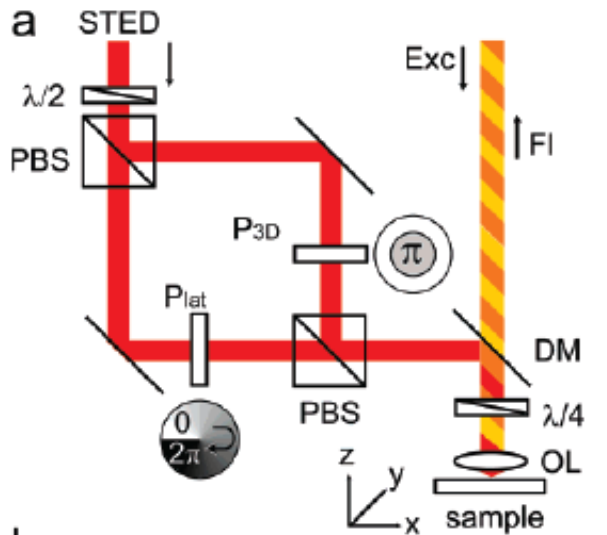
Synaptophysin (red, Atto647N) + syntaxin1 (green, Atto532)
in neurons

Donnert et al BiophysLett 2006 / Meyer et al Small 2008

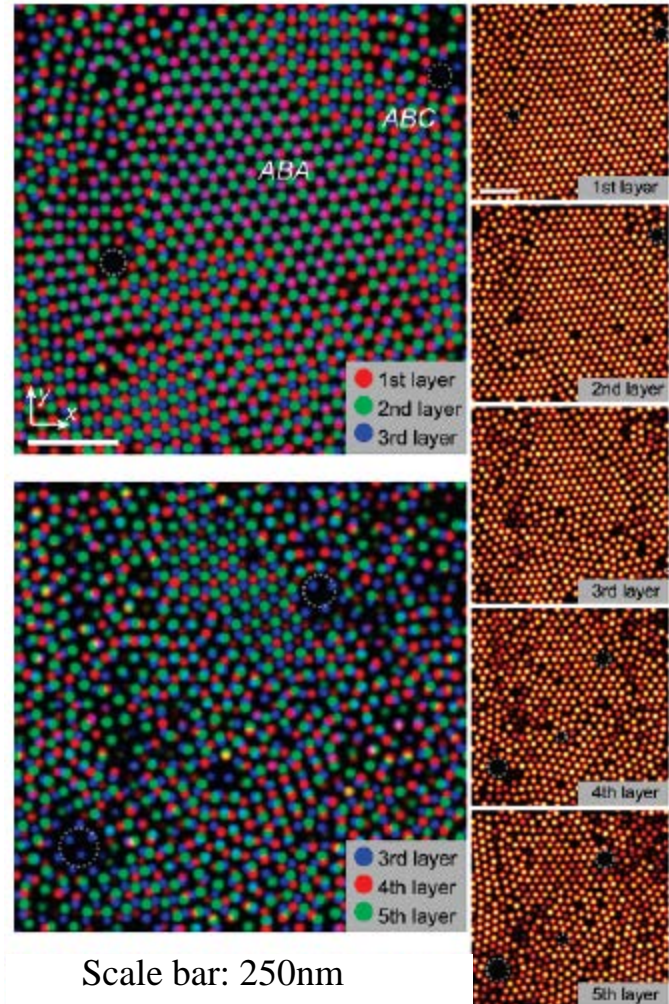
STED-Microscopy

Sub-Diffraction Imaging – 3D

3D STED nanoscopy I



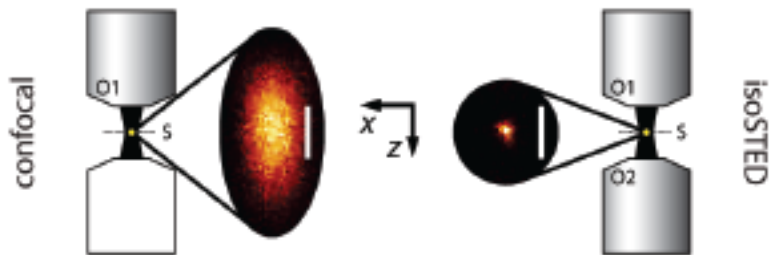
Fluorescent 100nm Beads – multiple layers on cover glass



STED-Microscopy

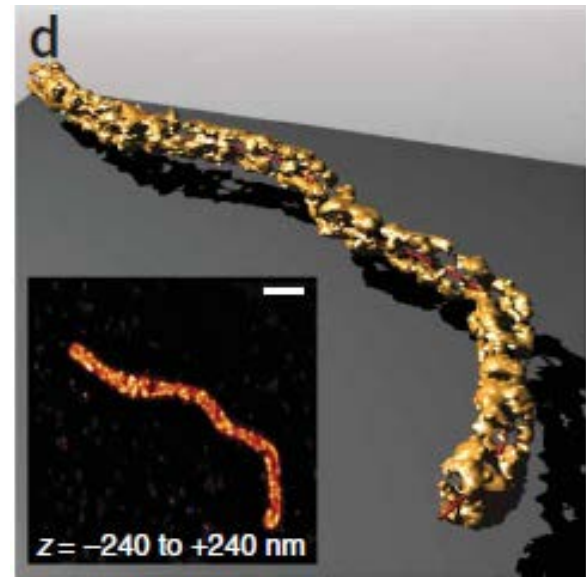
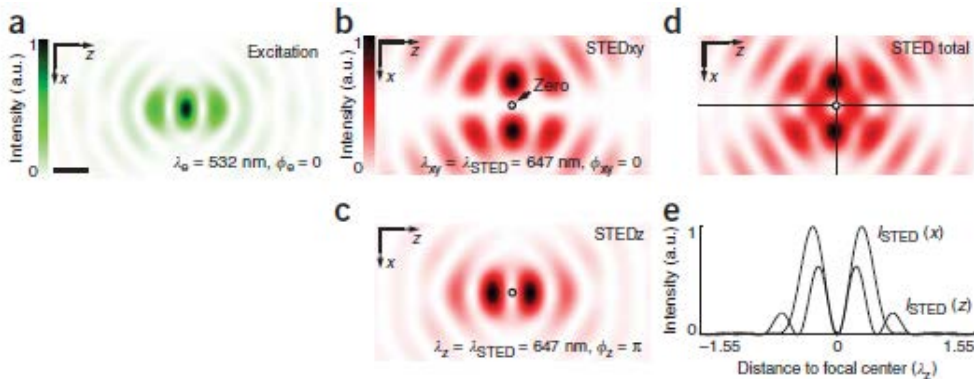
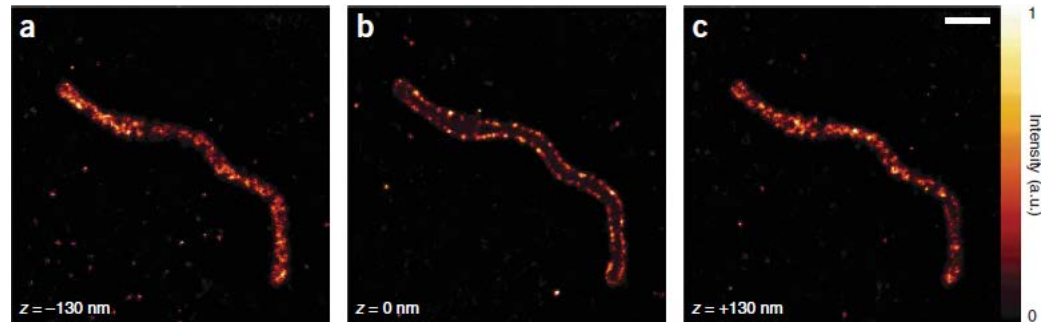
Sub-Diffraction Imaging – 3D

3D STED nanoscopy II – iso STED



Scale bar: 250nm

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

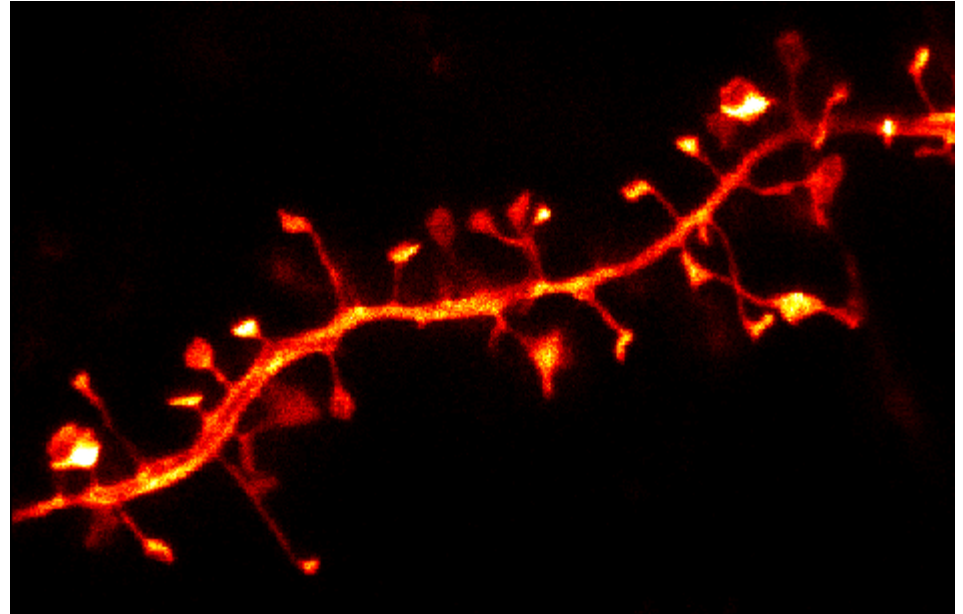


Scale bar: 1µm

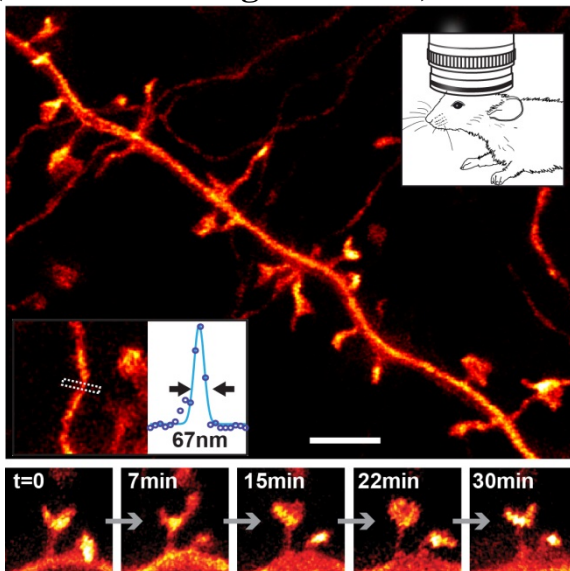
STED Microscopy

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)



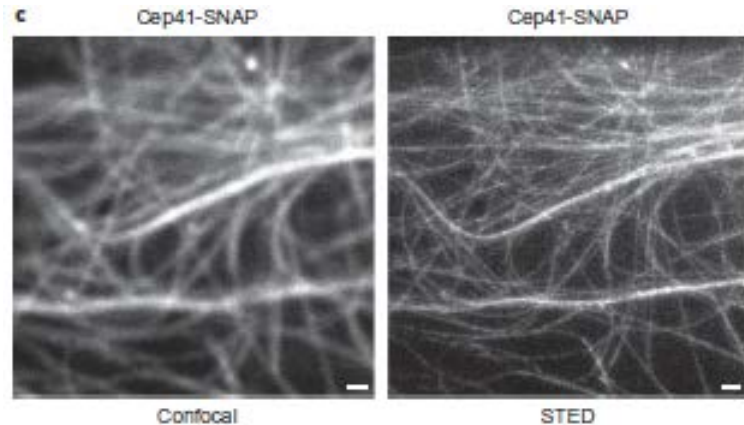
Live-Cell (inside)
Multi-Color (more complex)
Two-Photon excitation
3D possible
Conventional dyes, GFP, ...

STED Live Cell Microscopy

Proteins

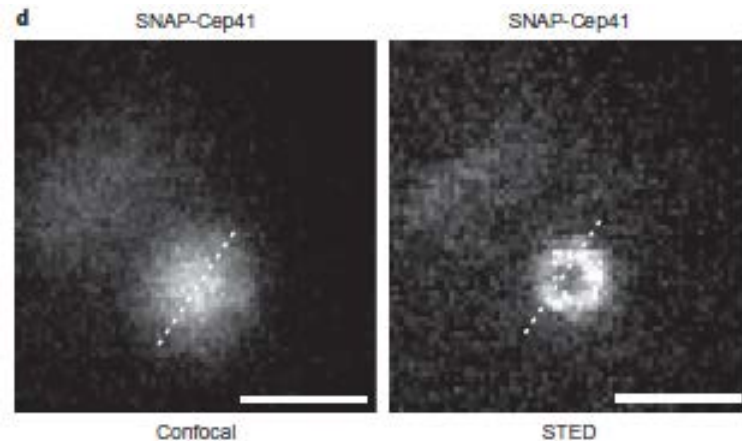
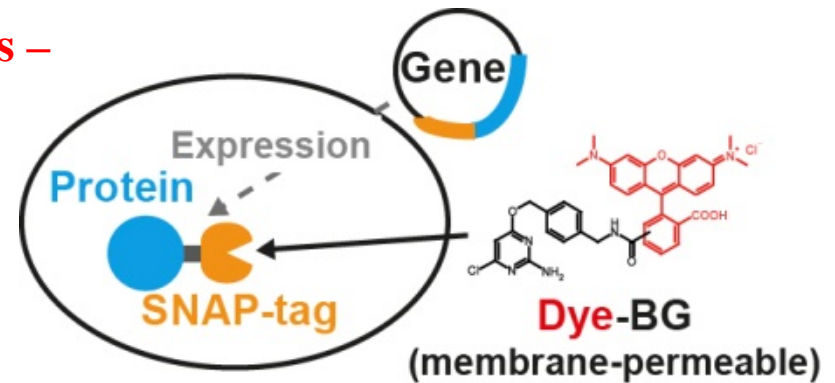
Live-cell labeling of proteins with organic dyes – intracellular:

SNAP-/CLIP-/HALO-tag technology



Confocal and STED imaging of Cep41 protein localization in living U2OS cells:

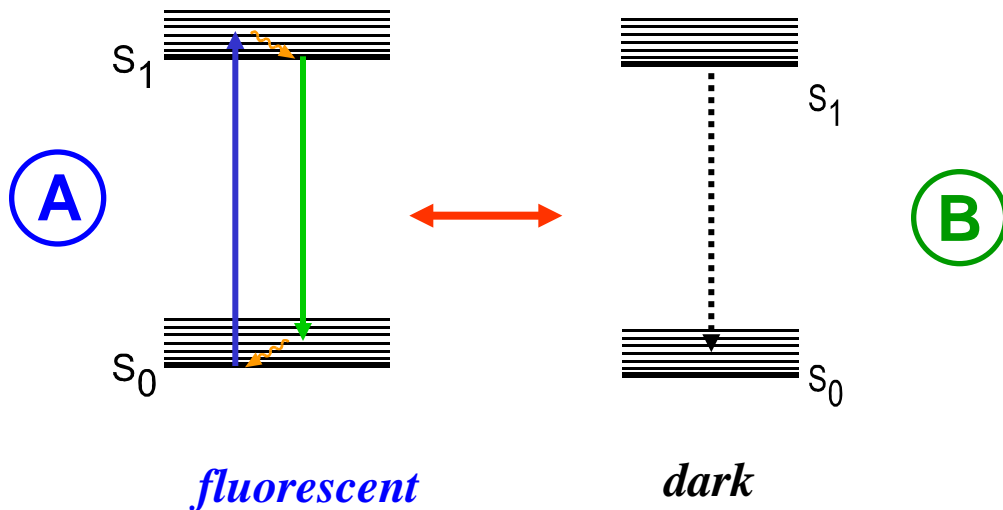
Cep41-SNAP bound to microtubules, scale bar 500 nm
SNAP-Cep41 localized at the centrosome



Far-Field Nanoscopy

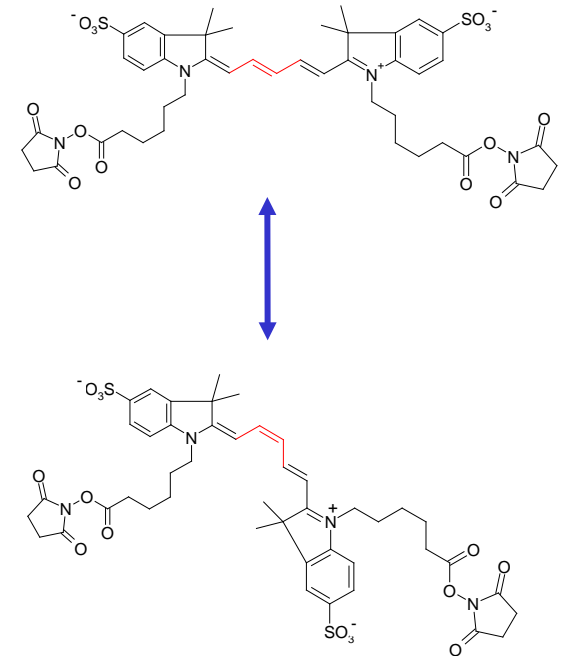
Alternative ON/OFF

Optically Bistable Marker



Reversible control by light
⇒ Fluorescence Turn On-Off

Cis-trans Photoisomerisation



Far-Field Nanoscopy

ON/OFF - *asFP595*

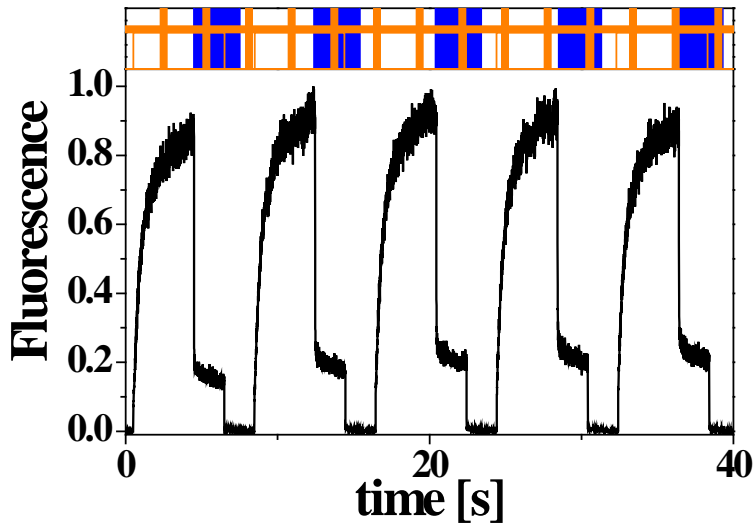
Fluorescent protein *asFP595*

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

cis-trans photoisomerisation

dark (trans)- bright (cis)

Andresen et al. (2005) *PNAS*

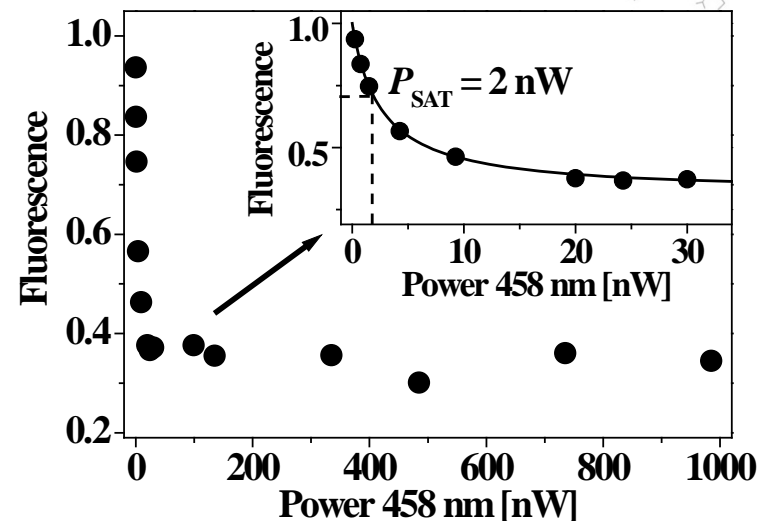
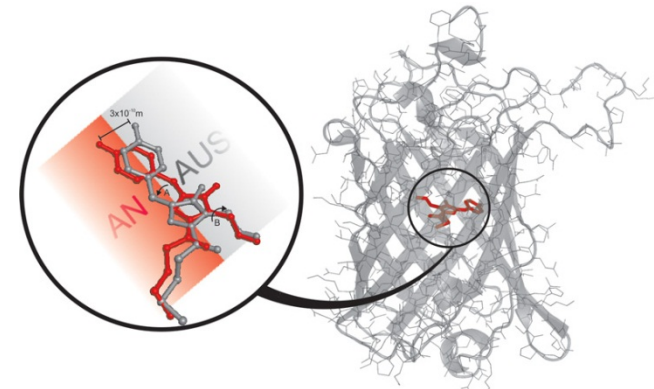


ON/OFF at low CW powers

nW - μ W (\sim kW/cm²) High saturation!

ON: 560nm

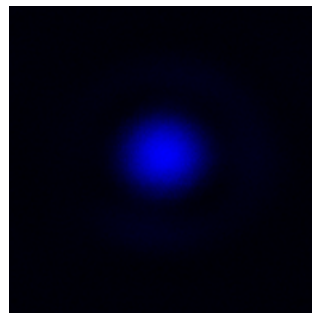
OFF: 400-450nm (local intensity zero)



Sub-Diffraction Microscopy

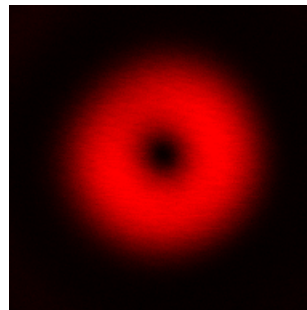
with asFP595 – RESOLFT-Microscopy

Effective observation spot



yellow (568 nm)

+



blue (458 nm)

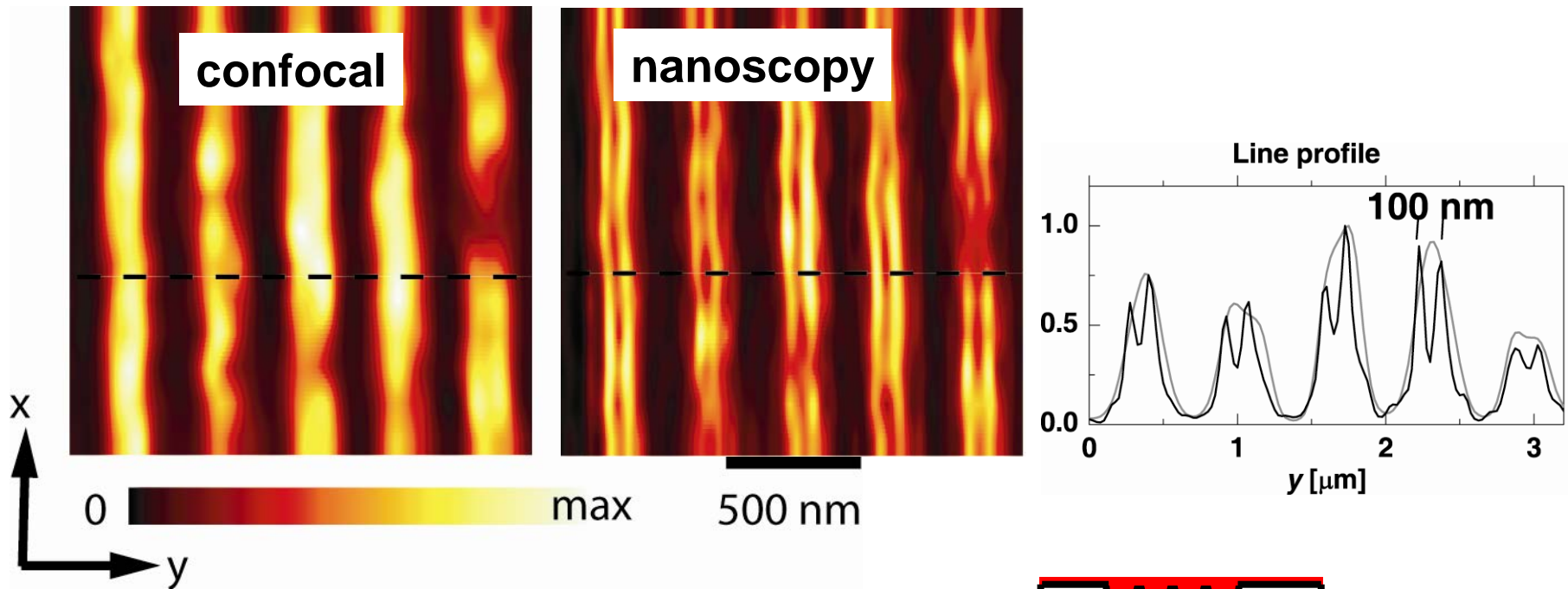


Sub-diffraction

RESOLFT = Reversible Saturable Optical Fluorescence Transition

Far-Field Nanoscopy

ON/OFF - asFP595



custom-prepared glass slides - parallel grooves

focused ion beam milling (Fraunhofer Institute IISB, Erlangen, Germany)

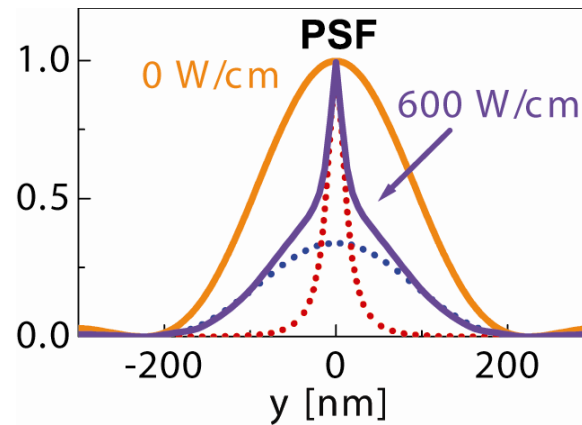
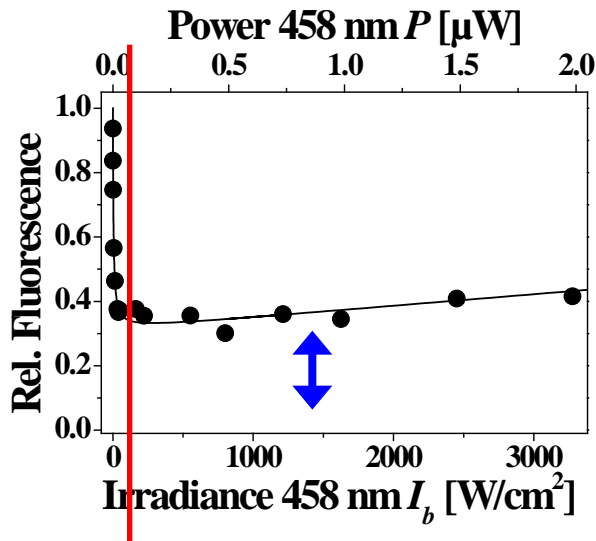
10 μm long, 0.5–1 μm deep, 100 nm wide, distance 500 nm

images RL-deconvolved

Sub-Diffraction Microscopy *asFP595 - Limitations*

Cross-talk by blue light - incomplete depletion

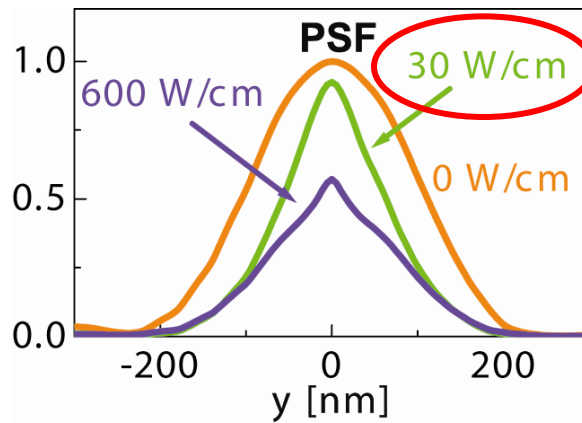
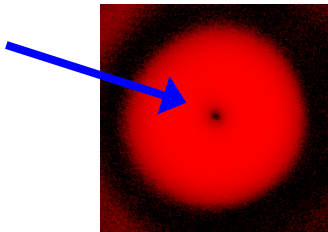
Hofmann et al, PNAS 2006



\Rightarrow only apply
30 W/cm²

\Rightarrow low
saturation =
low resolution

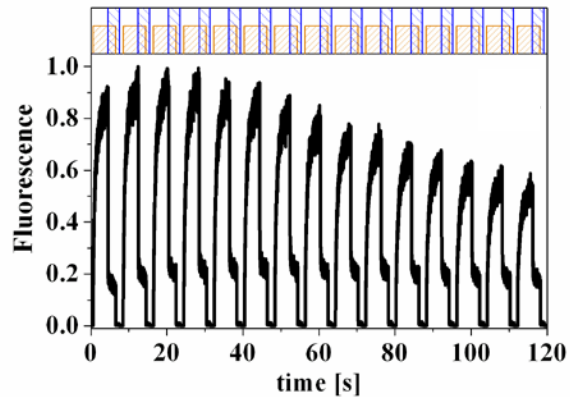
Incomplete zero - Depletion in central zero



Far-Field Nanoscopy

ON/OFF – limits + advances

Switching fatigue - photobleaching



⇒ improve ON/OFF cycling

- less cross-talk
- tetramer!!!
- more on/off cycles
- high brightness
- faster switching

Dronpa → rsFastLime

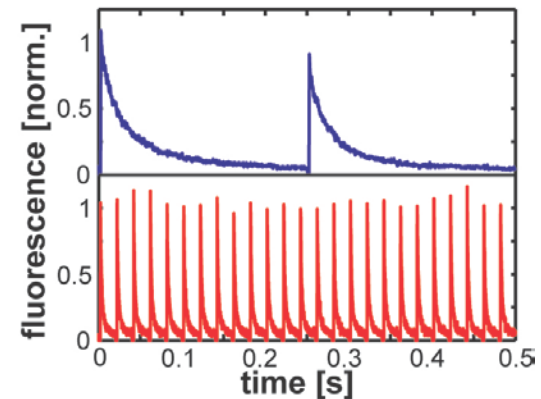
Stiel et al, Biochem J 2007

GFP → photoswitching (rsEGFP)

Grotjohann et al, Nature 2011

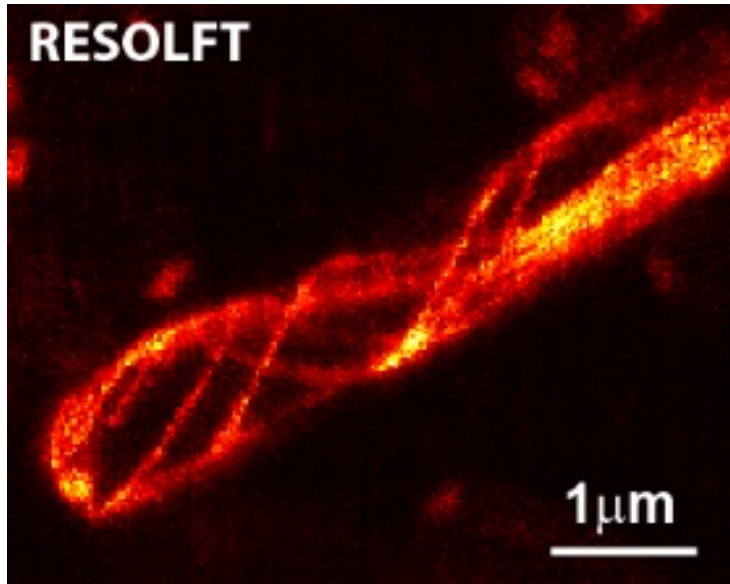
Switch-off + Readout: 488nm

Switch-on: 405nm



Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins



Excellent for Live-Cell (low light levels)

Multi-Color (new fluorescent proteins)

3D possible

Photoswitchable proteins / dyes

Intensity $\approx 1 \text{ kW/cm}^2$

Citrine \rightarrow Dreiklang

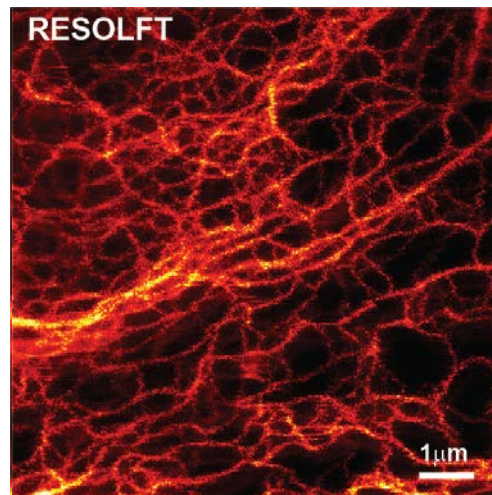
Brakemann et al, Nature Biotechnol. 2011

Switch-on: 405 nm

Switch-off: 355 nm

Read-out: 488 nm

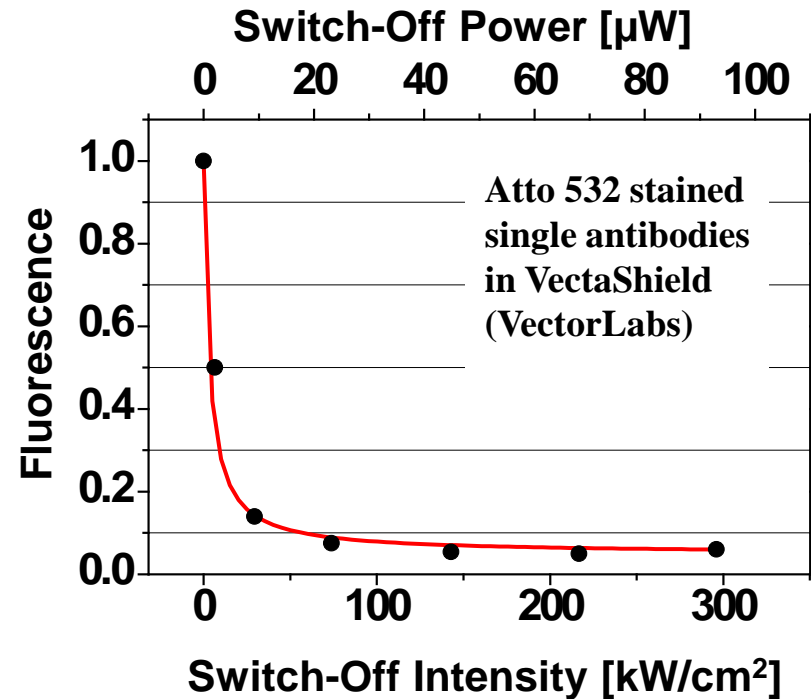
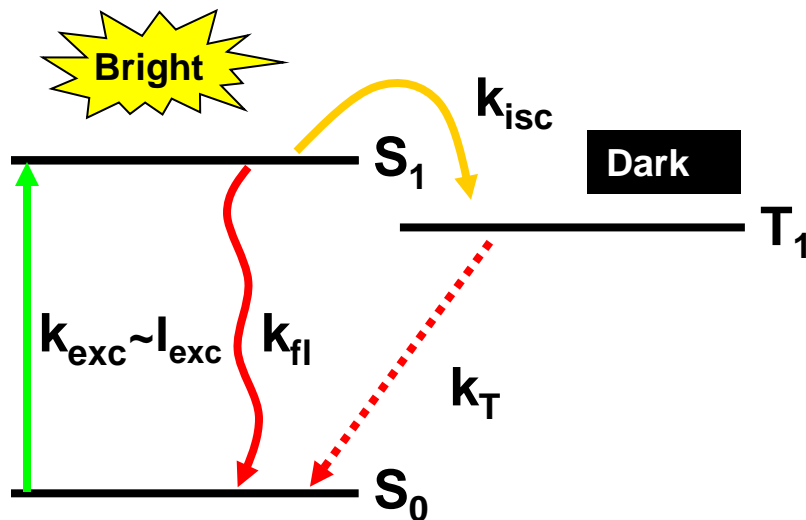
Keratin19-Dreiklang
expressed
in living PtK2 cells



Far-Field Nanoscopy

ON/OFF via Triplet/Dark States

GSD (Ground State Depletion)



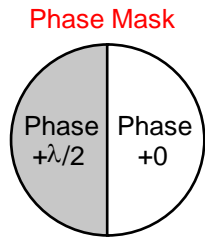
Turn-off fluorescence by pumping into a long-living dark (triplet) state

Low CW powers (μW – kW/cm^2)

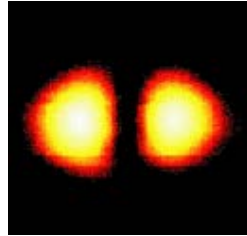
GSD-Microscopy

Far-Field Nanoscopy using the triplet state

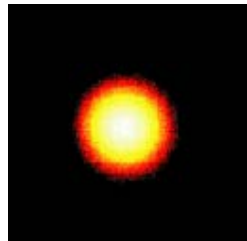
Atto 532 stained microtubuli



Switch-off PSF

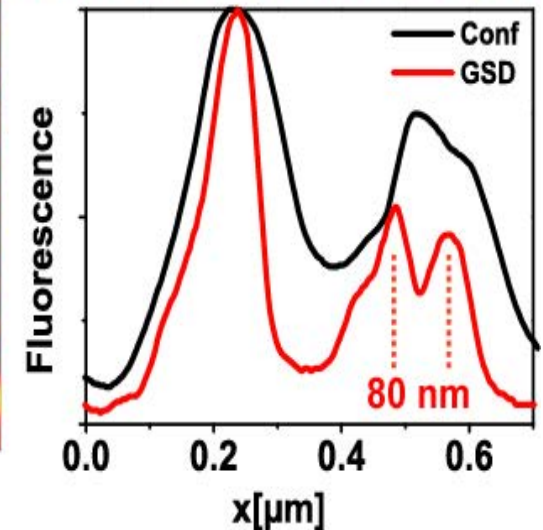
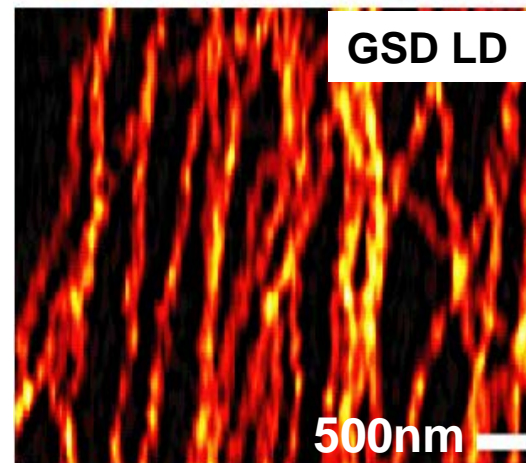
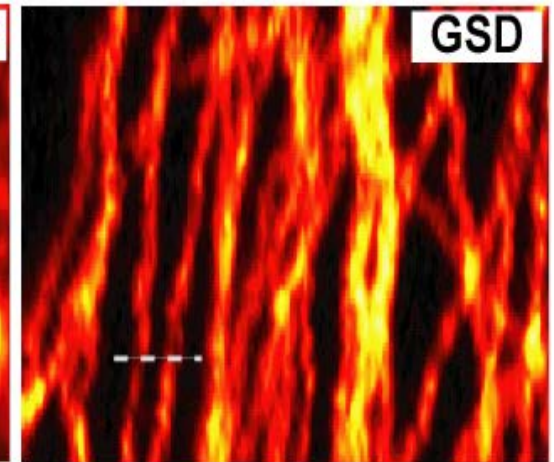
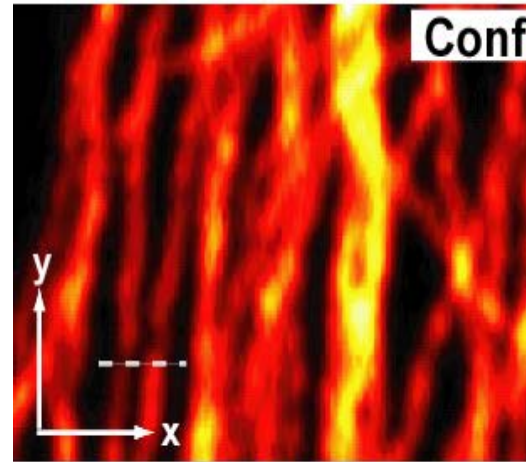
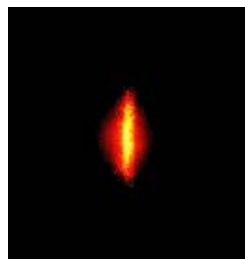


+



||

Eff. PSF



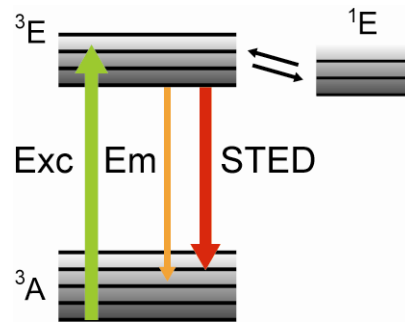
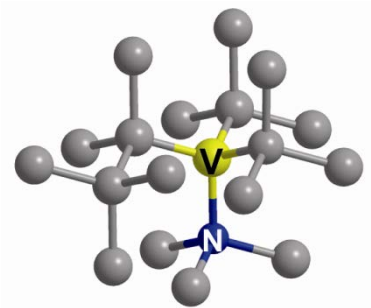
0 max

Fluorescence Nanoscopy

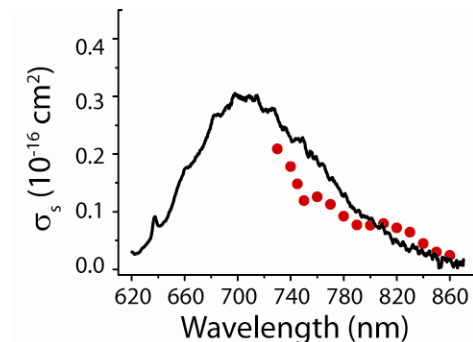
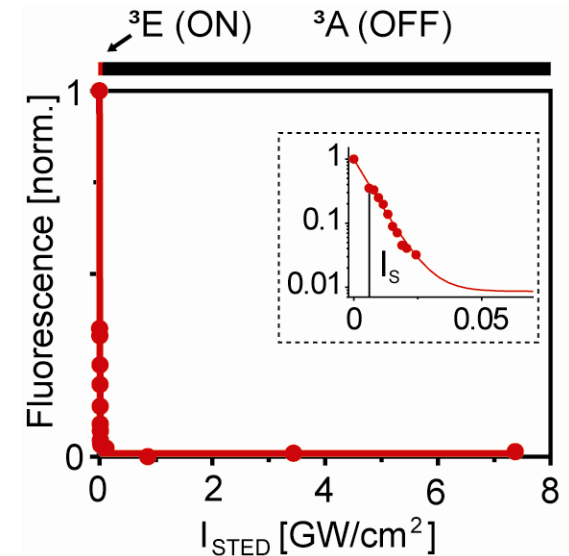
NV centers in diamond

Nitrogen vacancies (NV) centers in diamond

Ultrastable luminescence sources (Gruber et al. Science 1996)



Excitation 532nm – Luminescence ~700nm



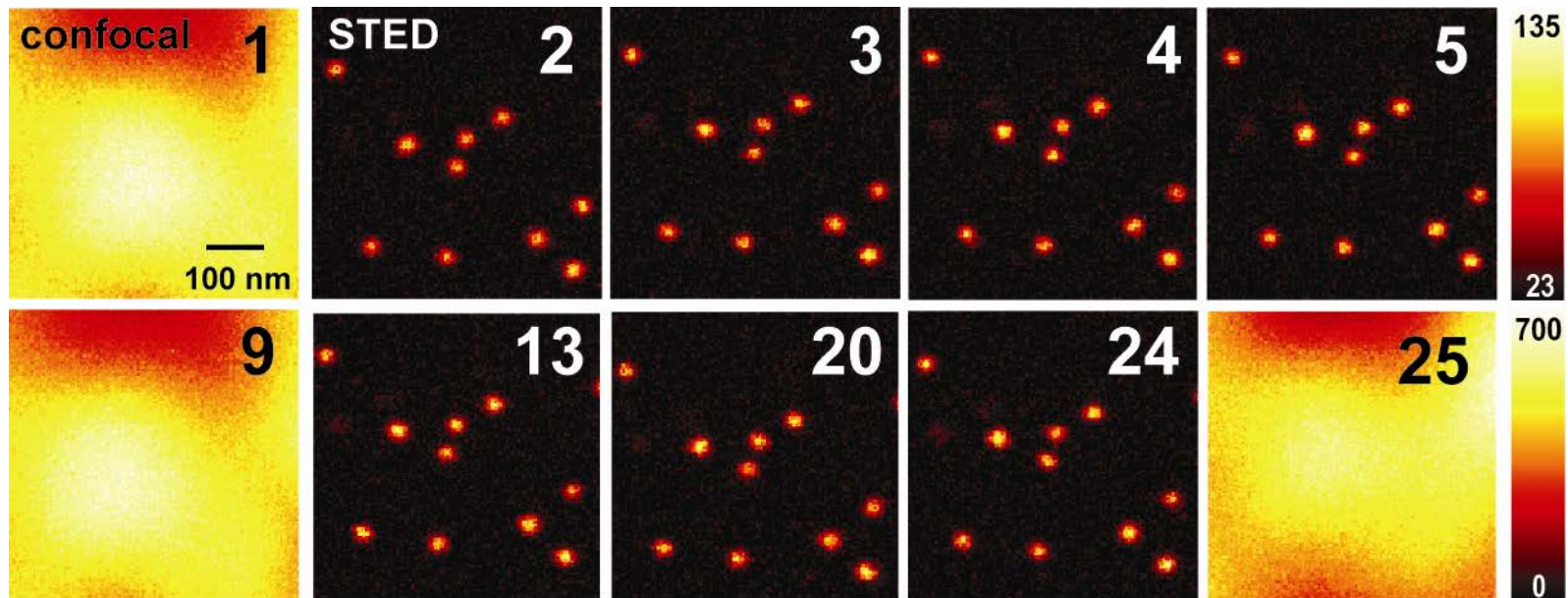
Fluorescence Nanoscopy

STED Imaging on single NV centers

STED on “isolated“ NV centers

in diamond of type IIa grown by chemical vapour deposition (Jelezko, Wrachtrup (Stuttgart))

exc: 532nm - STED: 775nm 8MHz



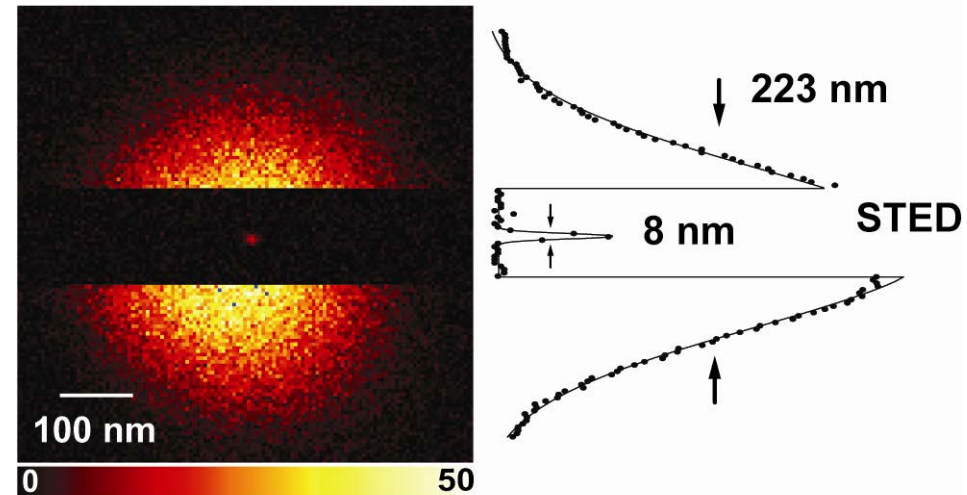
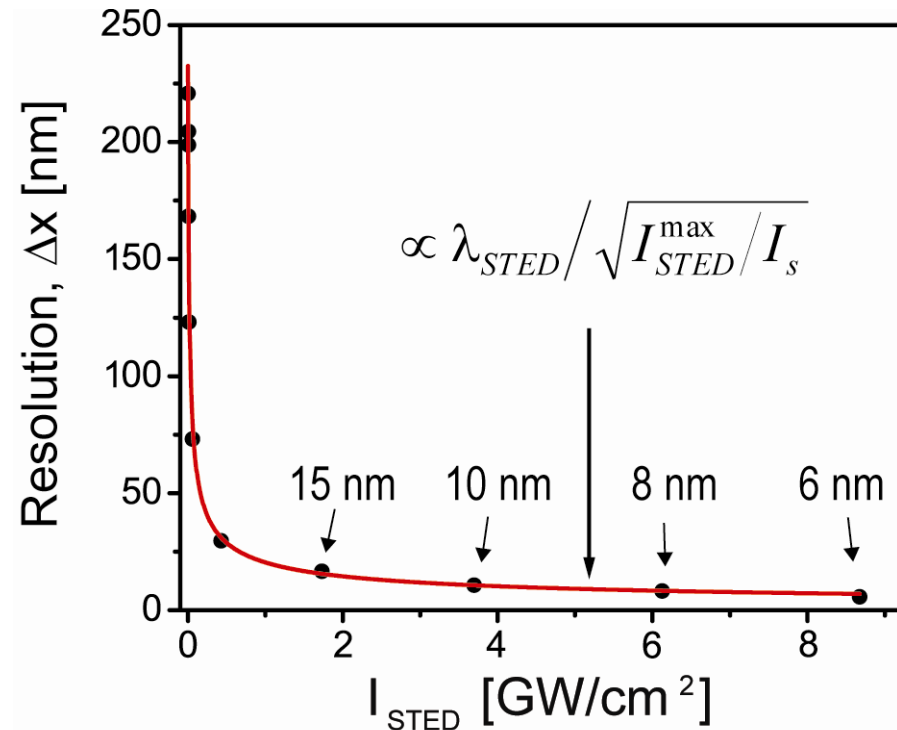
Fluorescence Nanoscopy

STED imaging on single NV centers

Ultrastable: apply very high STED intensities

→ Reach ultimate spatial resolutions!!!

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



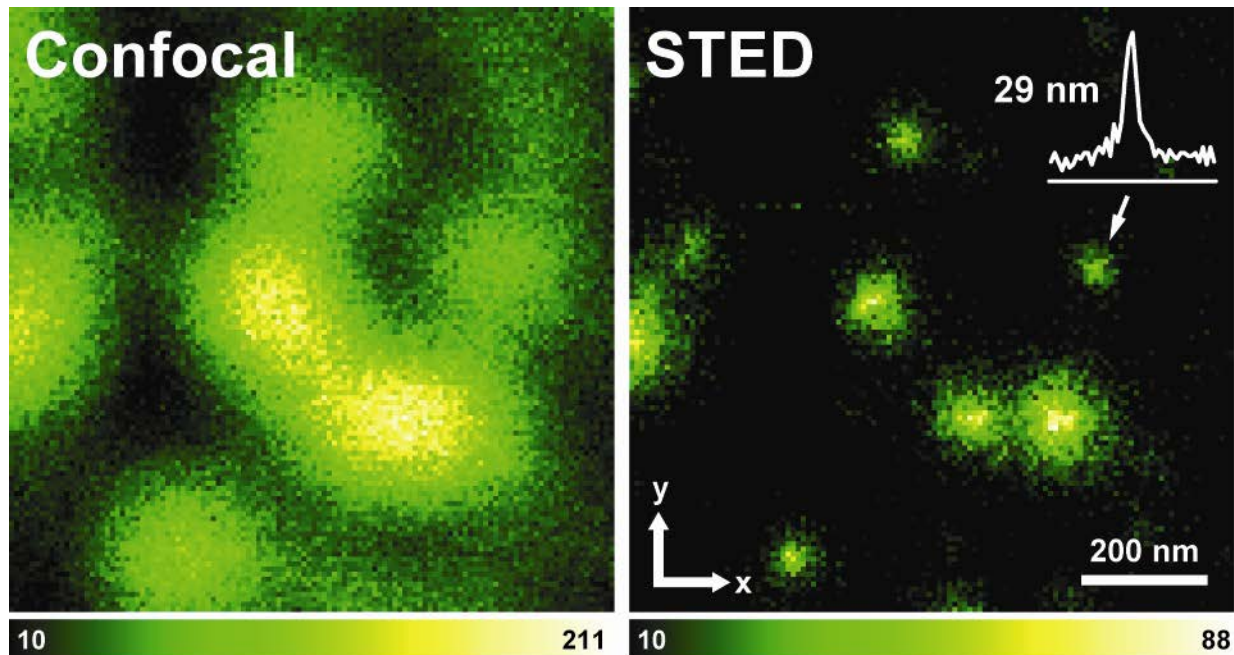
$$I_{STED} = 3.5 \text{ GW/cm}^2$$

STED Microscopy

NV centers as labels for STED microscopy?

30-35nm-sized nanodiamonds (by milling)

exc: 532nm - STED: 775nm 80MHz or CW



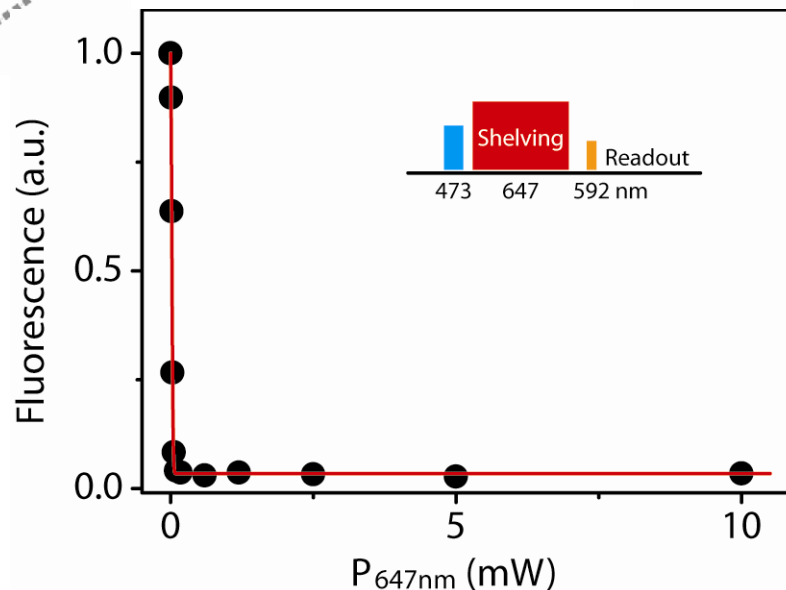
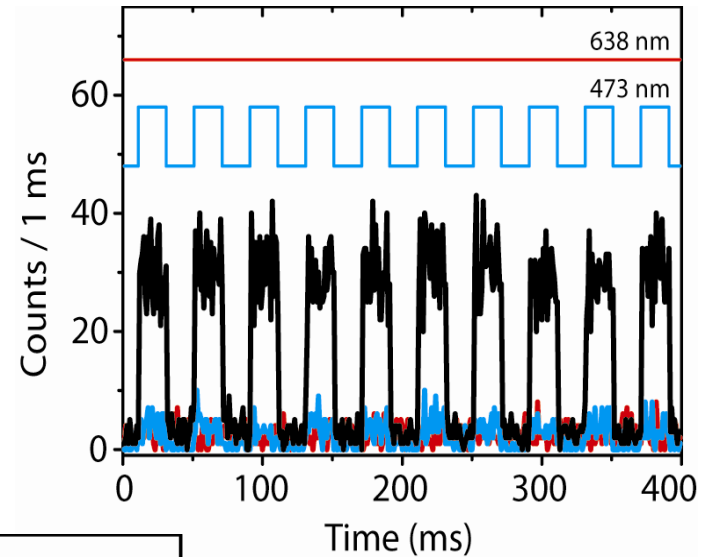
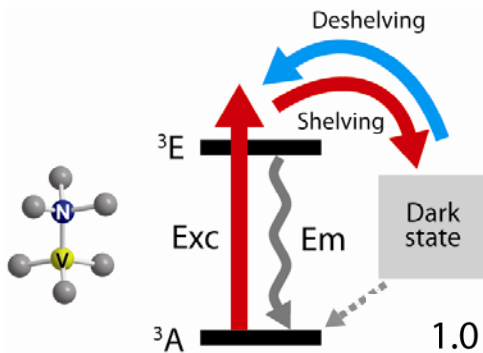
Functionalization for biological labeling!

Fluorescence Nanoscopy

GSD imaging by optical dark state shelving

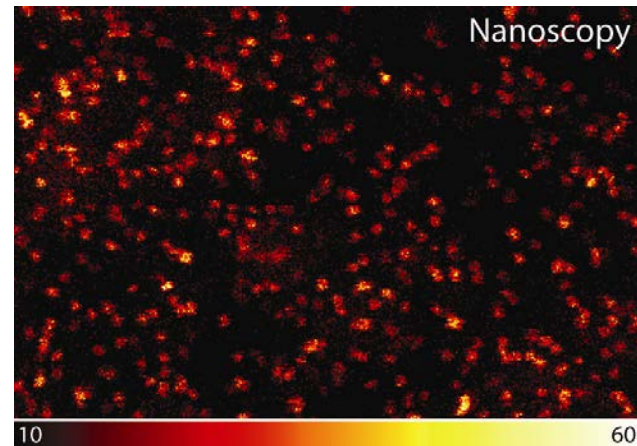
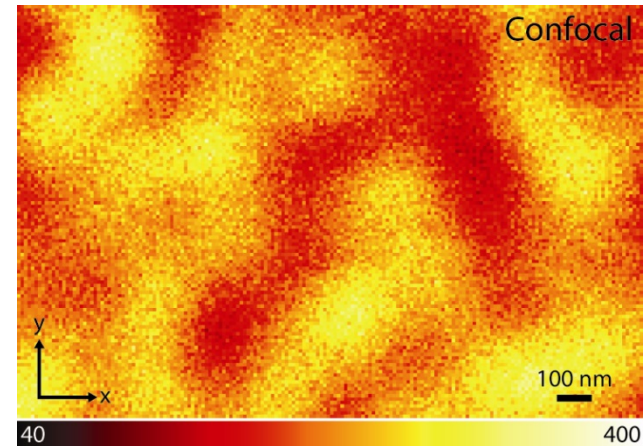
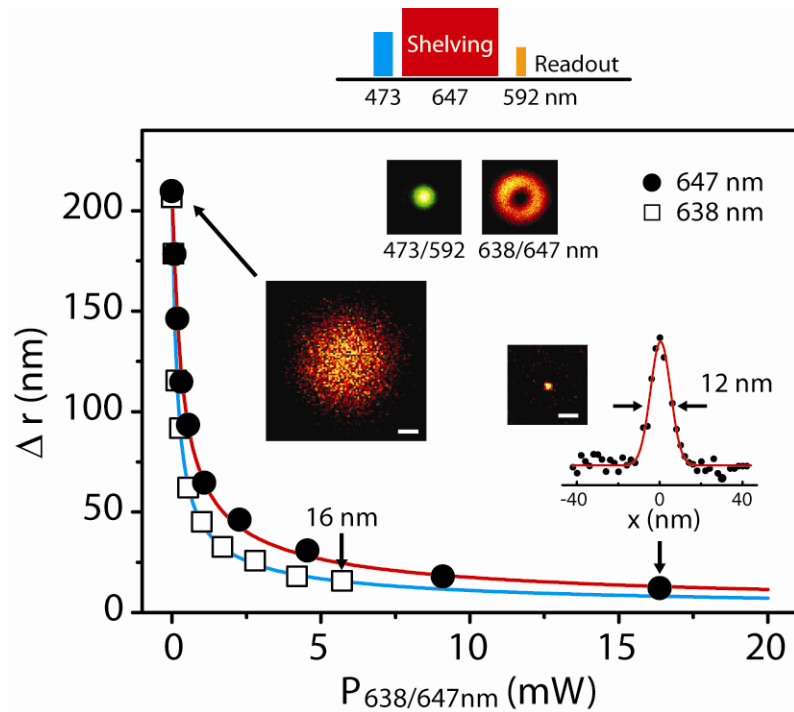
Fluorescence of NVs can be photoswitched via dark state

GSD: Ground-State-Depletion



Fluorescence Nanoscopy

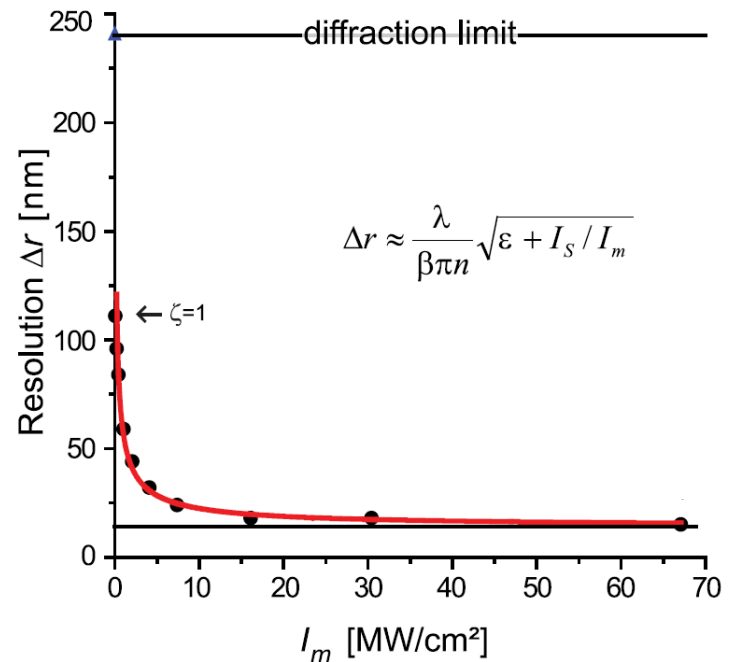
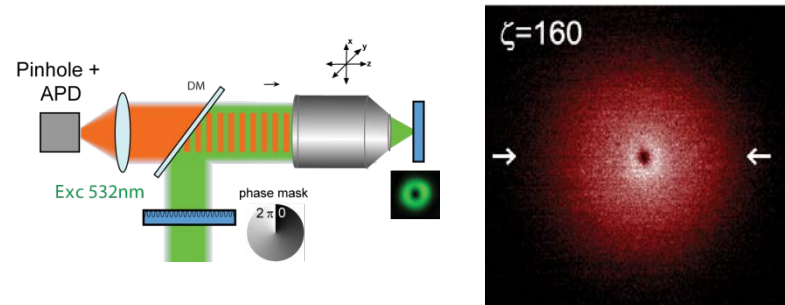
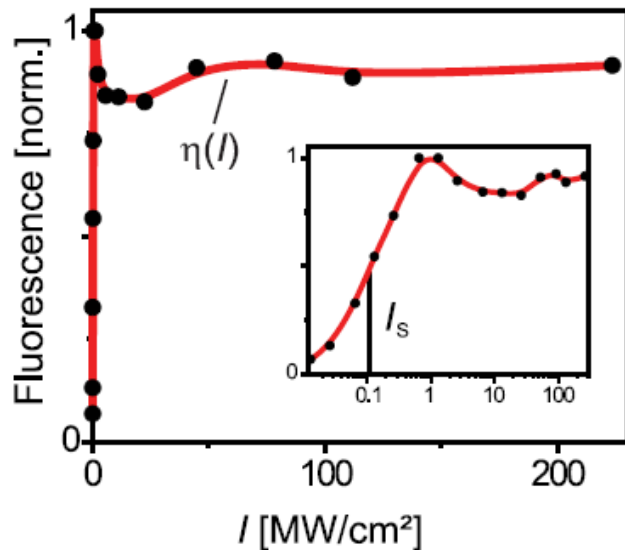
GSD imaging by optical dark state shelving



Fluorescence Nanoscopy

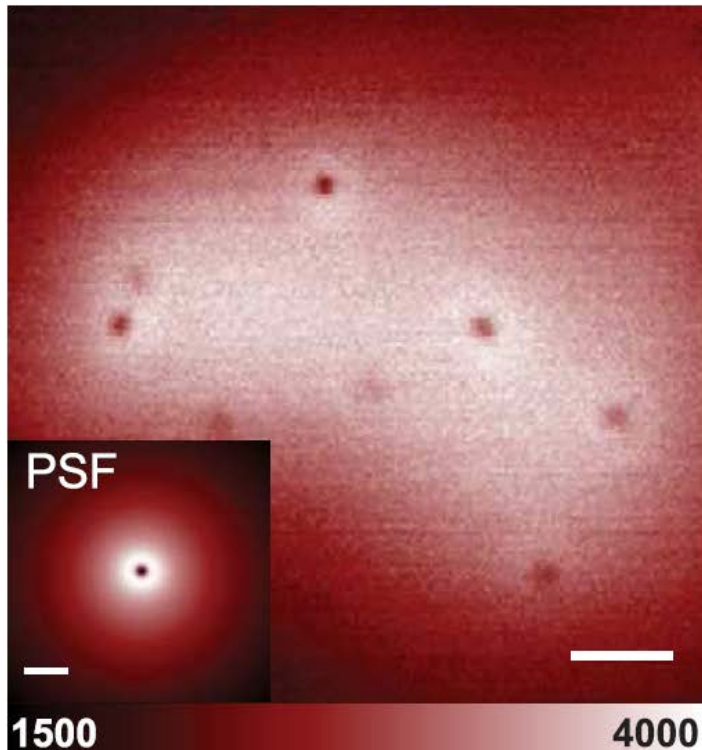
GSD imaging on single NV centers

Fluorescence of NVCs
can be saturated



Fluorescence Nanoscopy

GSD imaging on single NV centers



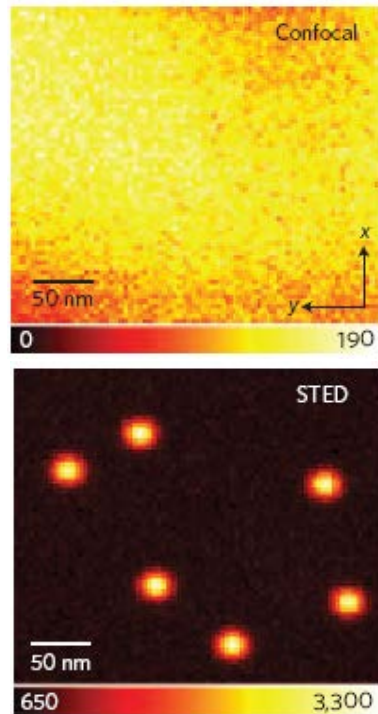
Fluorescence Nanoscopy

Photoswitching is key

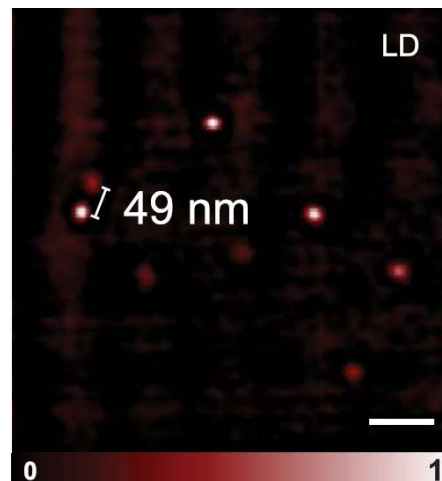
Same nanoscopy of NVs: via 3 different switching mechanisms

PHOTOSWITCHING is key to nanoscale!!!

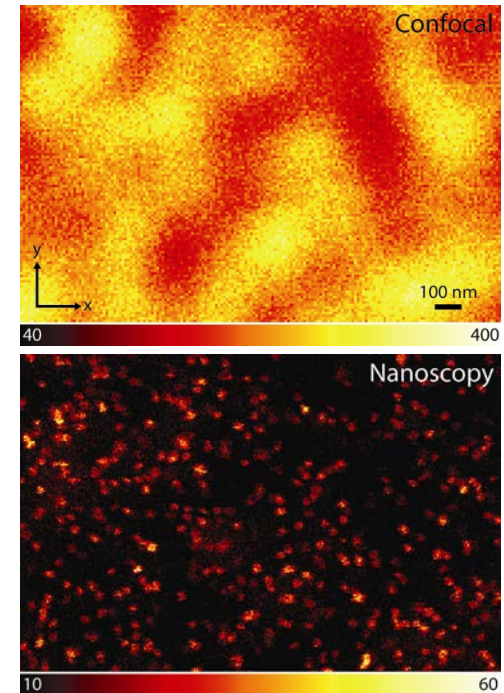
STED



GSD saturation

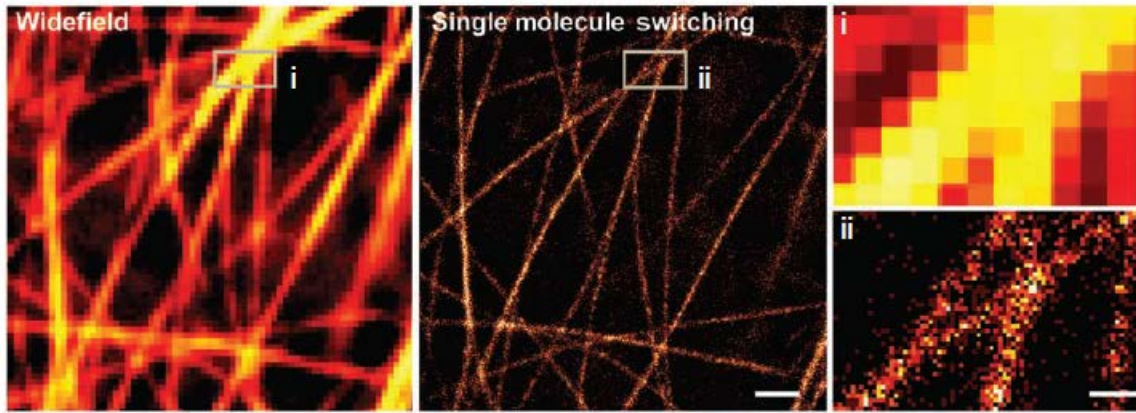


GSD dark state shelving

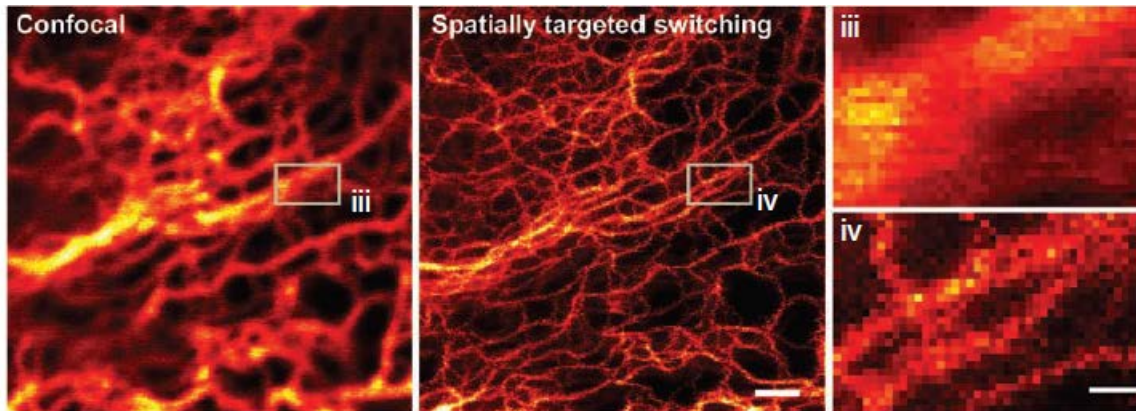


Far-Field Nanoscopy

STED/RESOLFT vs. PALM/STORM/...



PALM/STORM ...



STED/RESOLFT

STED/RESOLFT vs. PALM/...

Same principle: ON/OFF

**Similar techniques:
Own advantages/disadvantages**

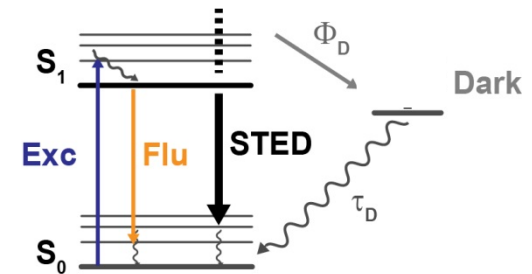
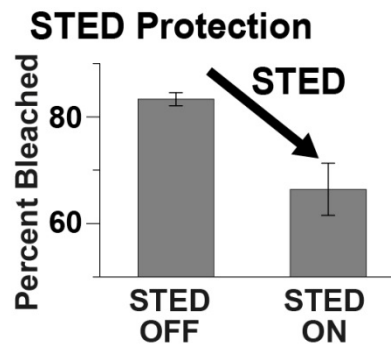
**Same labels / samples
- New control!**

Optimization of Fluorescence Signal *STED*

Simultaneous excitation with Excitation and STED light

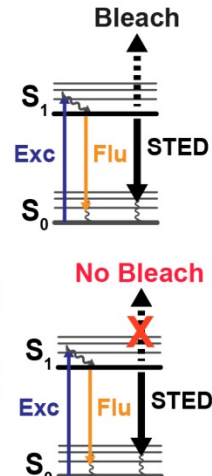
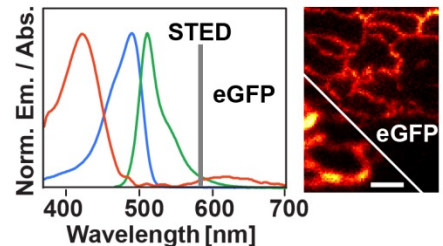
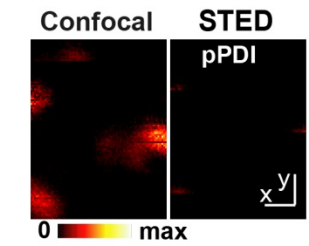
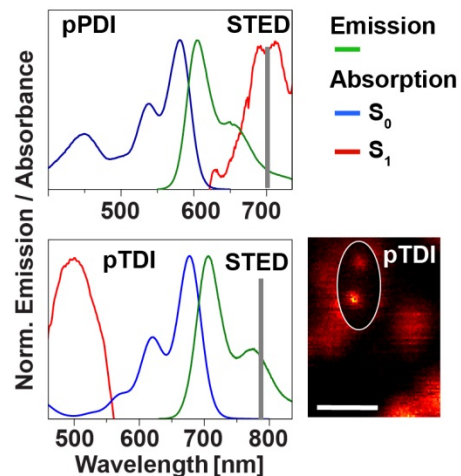
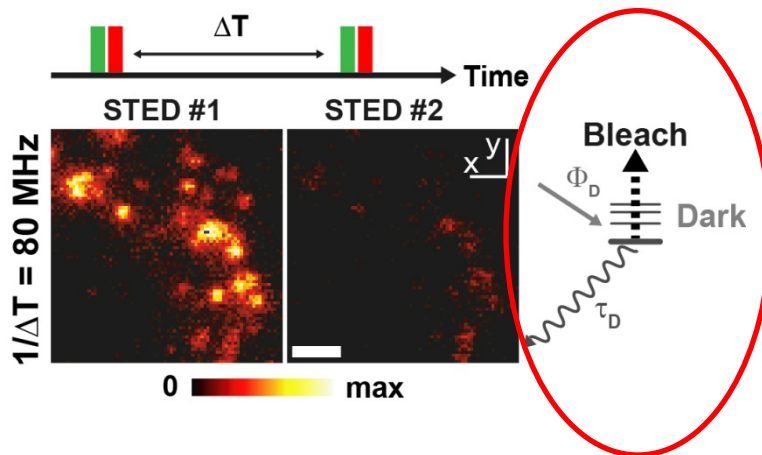
Two effects:

- ⇒ Shortening of excited state lifetime
- ⇒ Reduction of photobleaching



⇒ Excited state absorption - photobleaching

Singlet State: Excited State Absorption

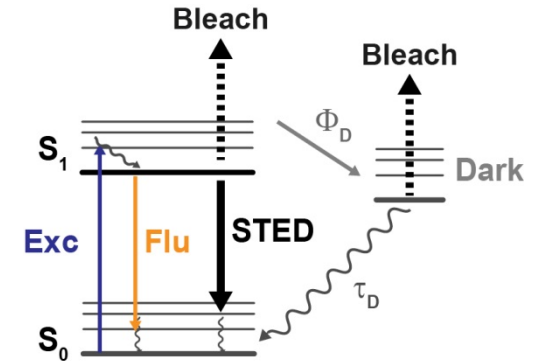
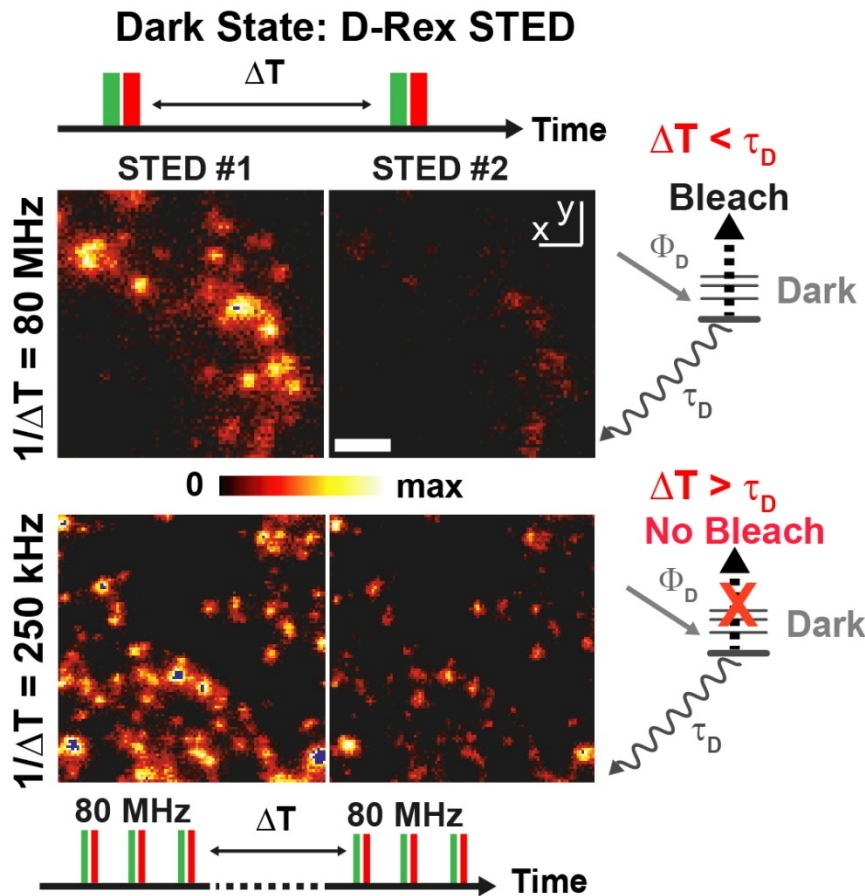


Optimization of Fluorescence Signal

STED – D-Rex

Simultaneous excitation with Excitation and STED light

⇒ Excited state absorption - photobleaching

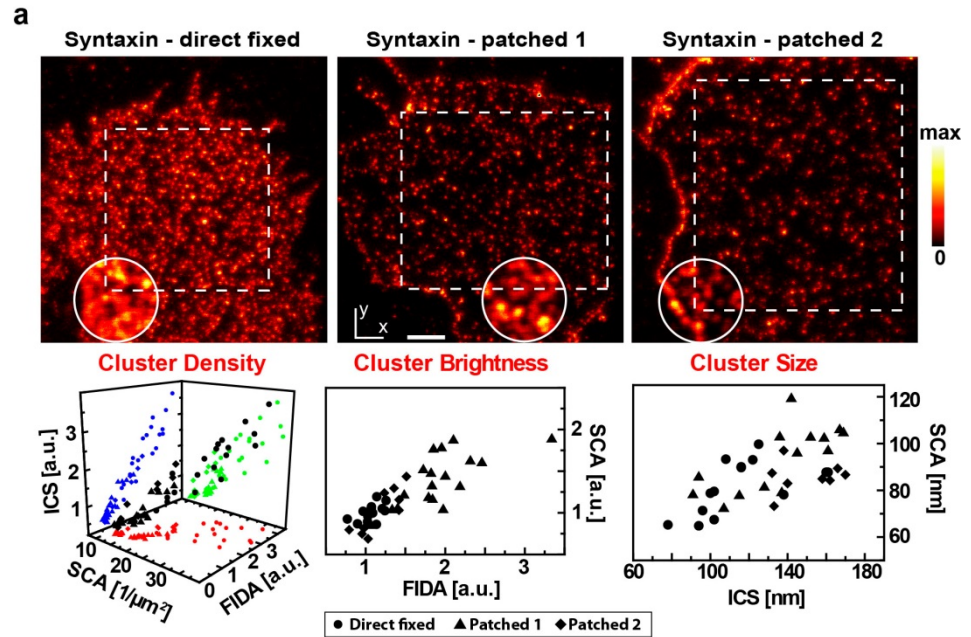


STED Microscopy

Example

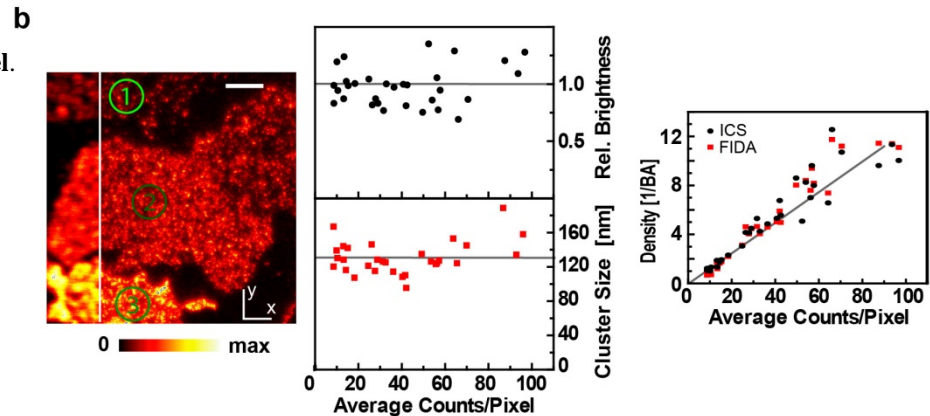
Syntaxin clusters

membrane sheets of PC12 cells immunolabelled for syntaxin for three different preparations
 directly fixed (left)
 patched 1/2 (right): clustering reinforced/density decreased
 scale bar: 500 nm



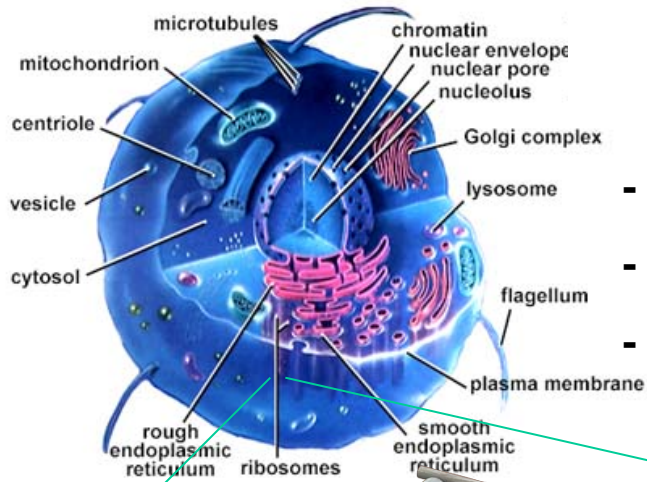
Syntaxin clusters

Syntaxin cluster morphology is independent of its expression level.
 membrane sheets generated from PC12 cells expressing different levels of immunolabeled myc syntaxin 1
 scale bar: 500 nm



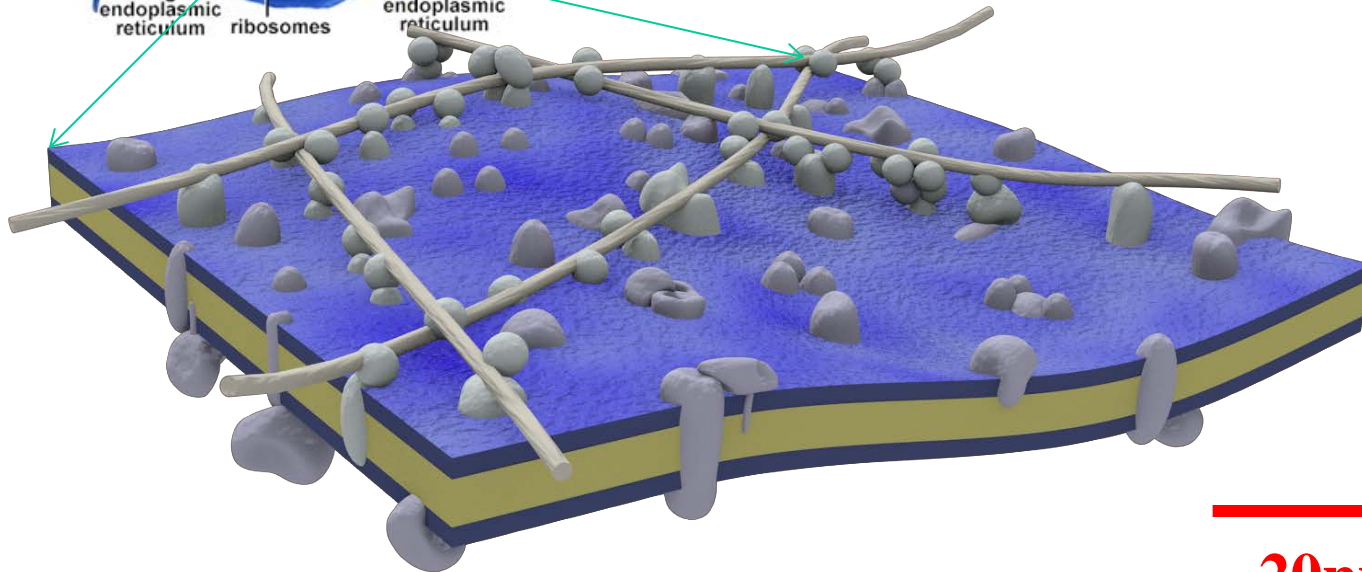
Lipid Plasma Membrane Organization

Nanoscale



Lipid Plasma Membrane Organization:

- Heterogeneous distribution (viscosity, curvature, ...)
- Interaction with proteins
- Interaction with cortical cytoskeleton



20nm

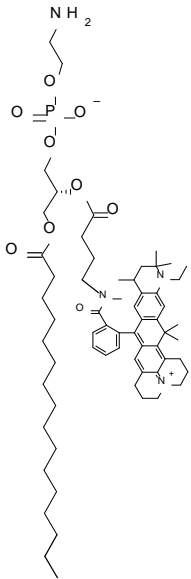
Small spatial
scales!!!!

Lipid Plasma Membrane Organization

Fluorescence Recordings: Lipids

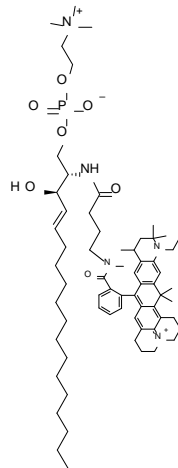
Phosphoglycerolipid:

Atto647N-phosphoethanolamine (PE)



Sphingolipid:

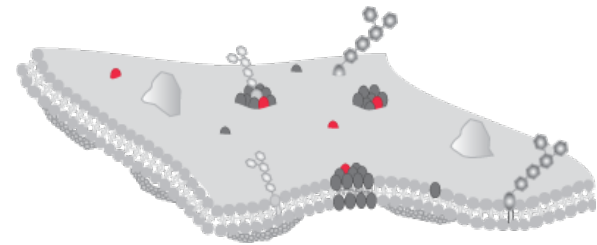
Atto647N-sphingomyelin (SM)



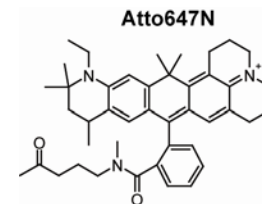
Live PtK2 cells:

physiological conditions

incorporation in plasma membrane

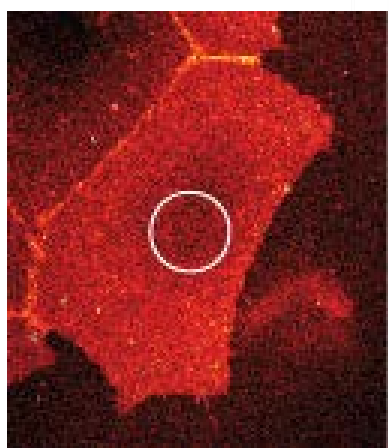
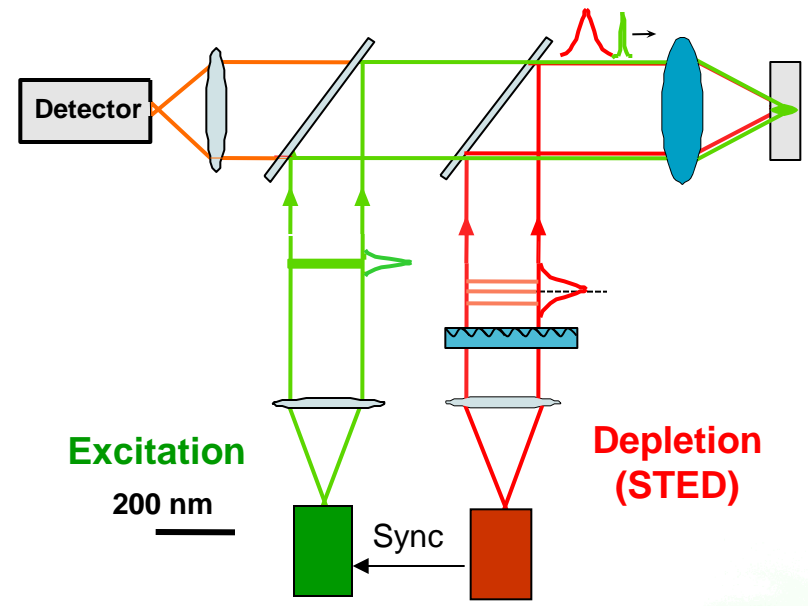
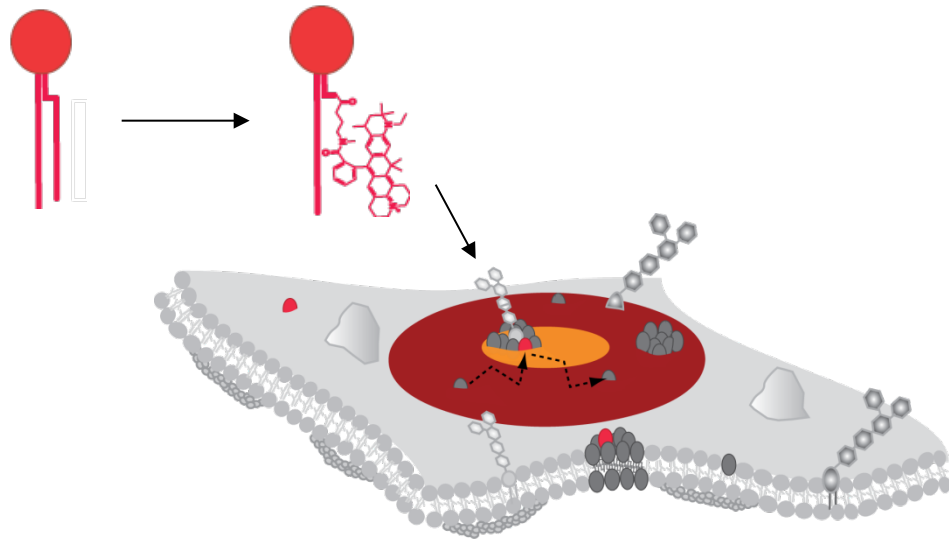


**BSA
complex**

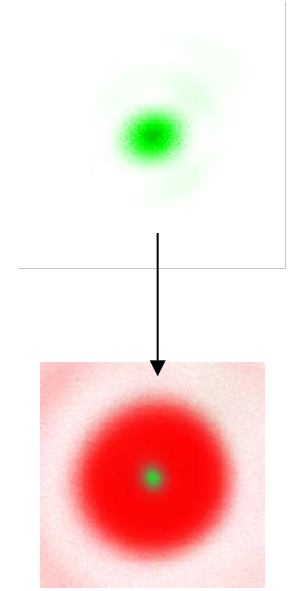


Lipid Plasma Membrane Organization

STED Nanoscopy Measurement

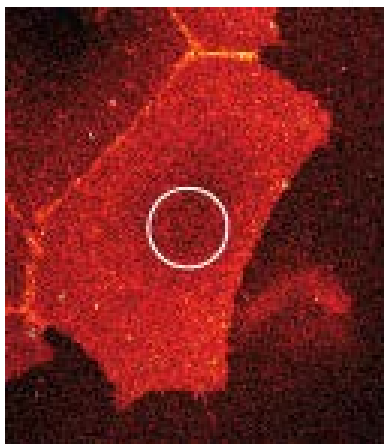
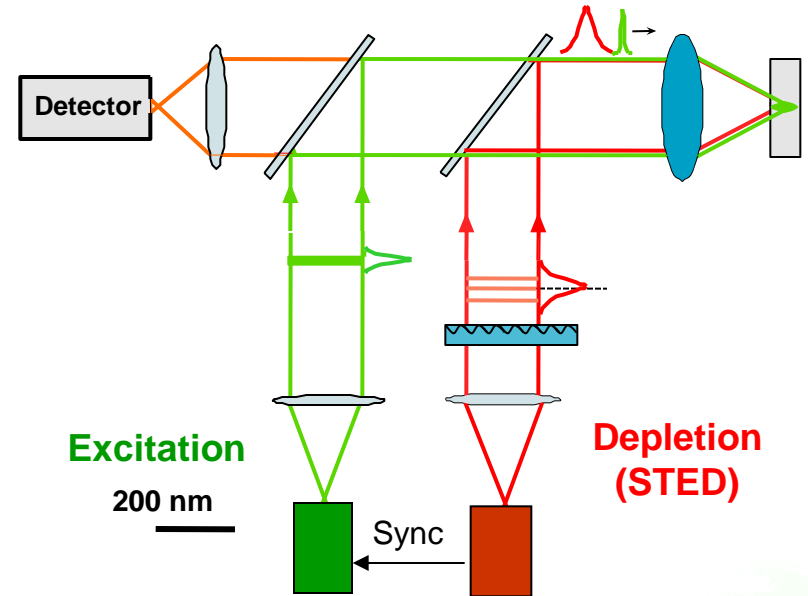
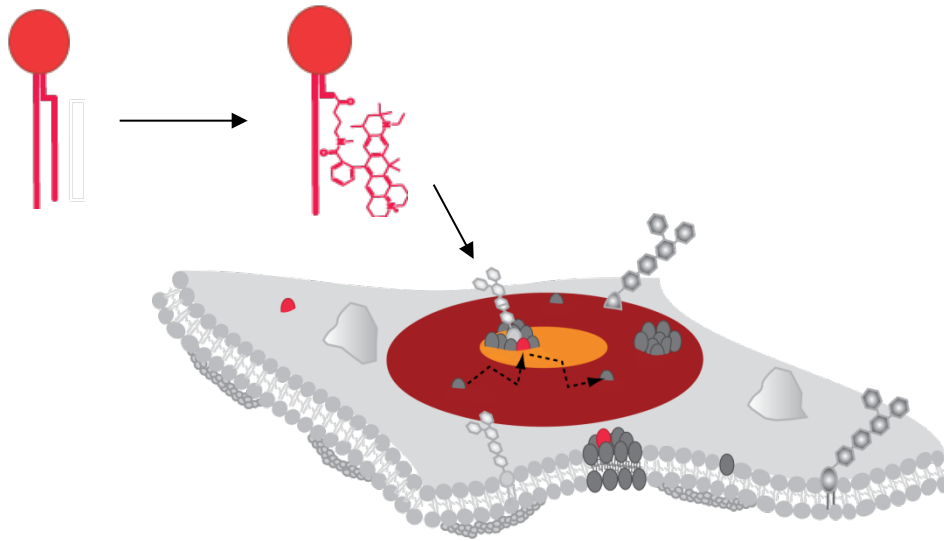


Homogeneous distribution



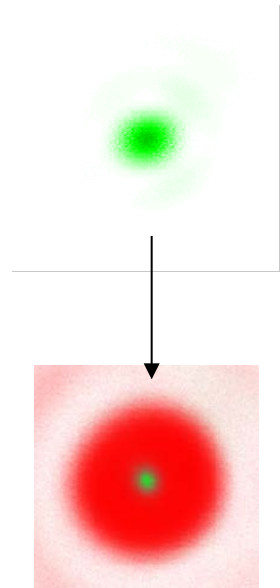
Lipid Plasma Membrane Organization

STED Nanoscopy Measurement



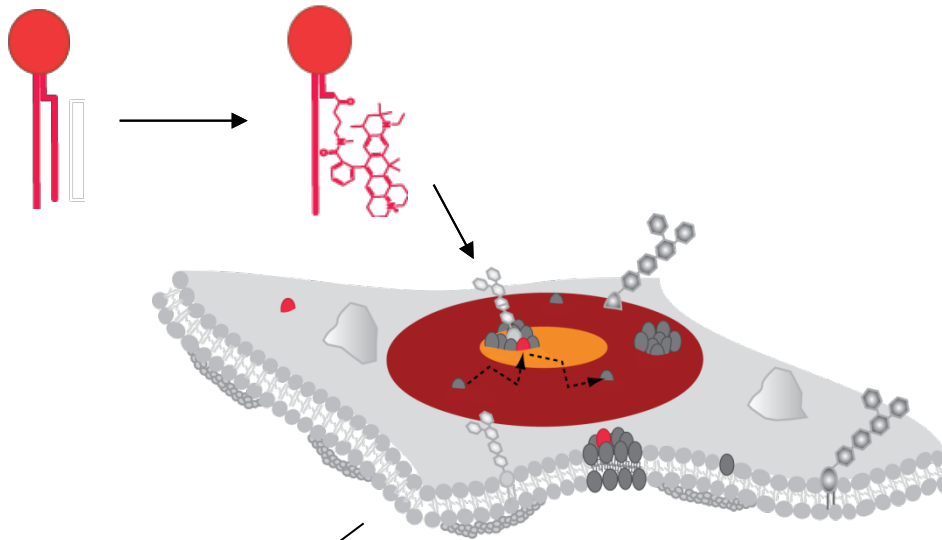
Homogeneous distribution

Fast diffusion → Limited temporal resolution!

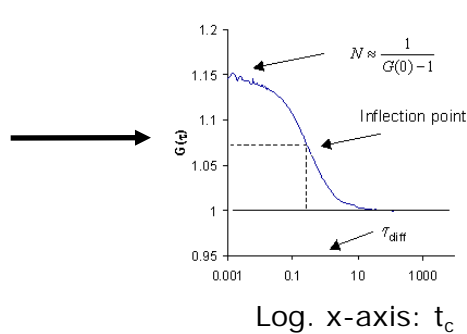
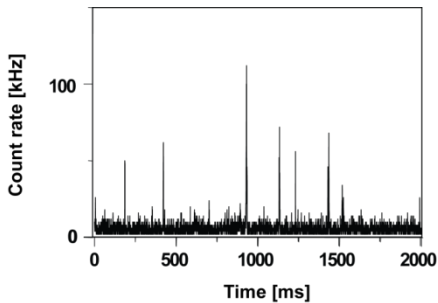
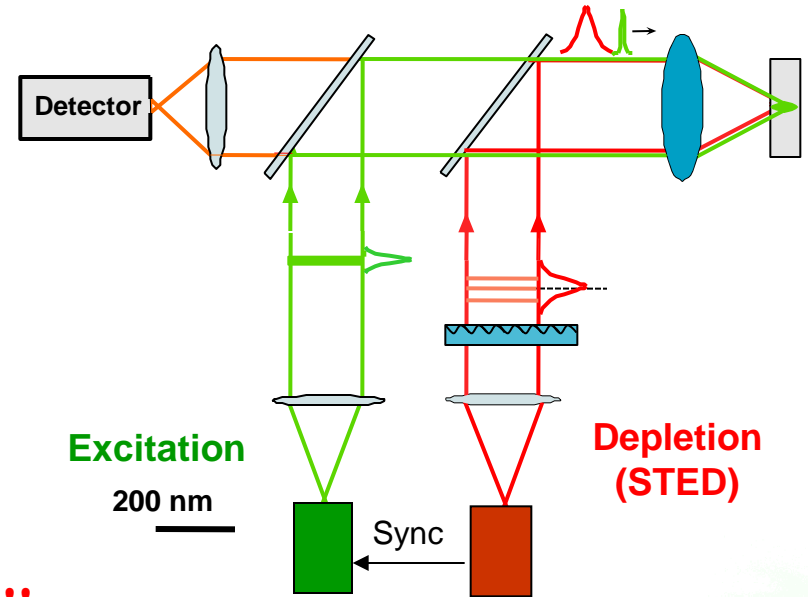


Lipid Plasma Membrane Dynamics

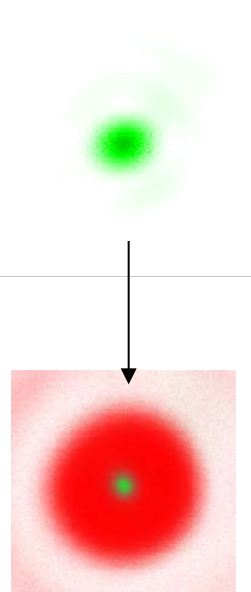
STED Nanoscopy Measurement



Discover diffusion dynamics!!!
Fluorescence Correlation Spectroscopy (FCS)



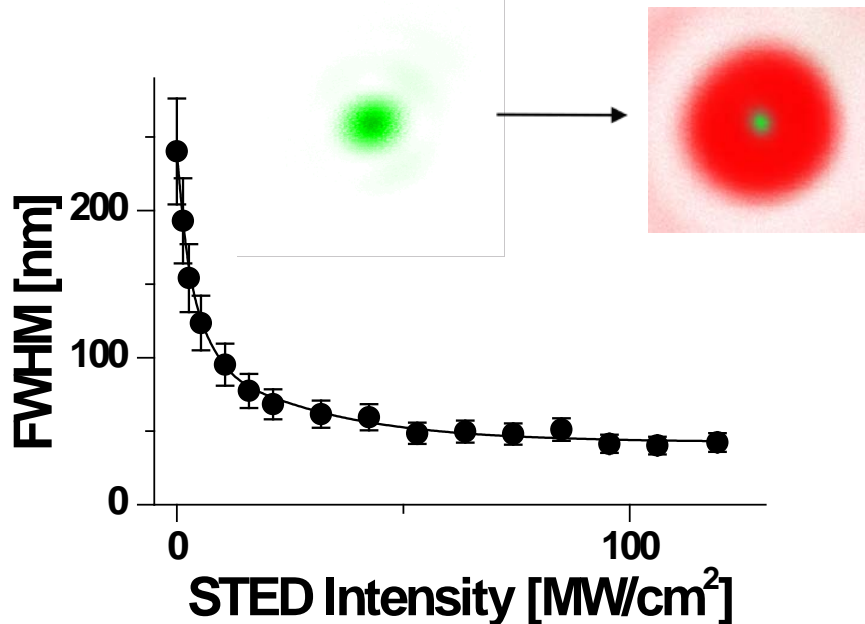
**molecular
diffusion coefficient
=
molecular
mobility**



Live Cell Nanoscopy

STED-FCS

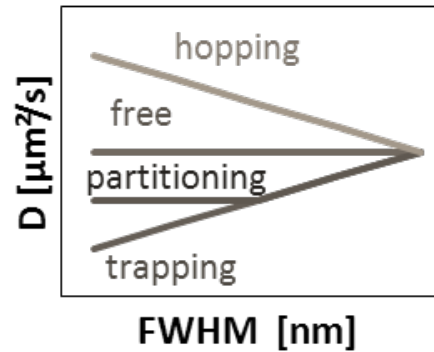
STED-Microscopy: Tuning of observation area



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

Calculate
apparent diffusion coefficient:
 $D \sim \text{area} / \text{transit time}$

Dependencies: $D(\text{diameter})$
 $240\text{nm} \rightarrow 30/40\text{nm}$
Varies for different diffusion modes



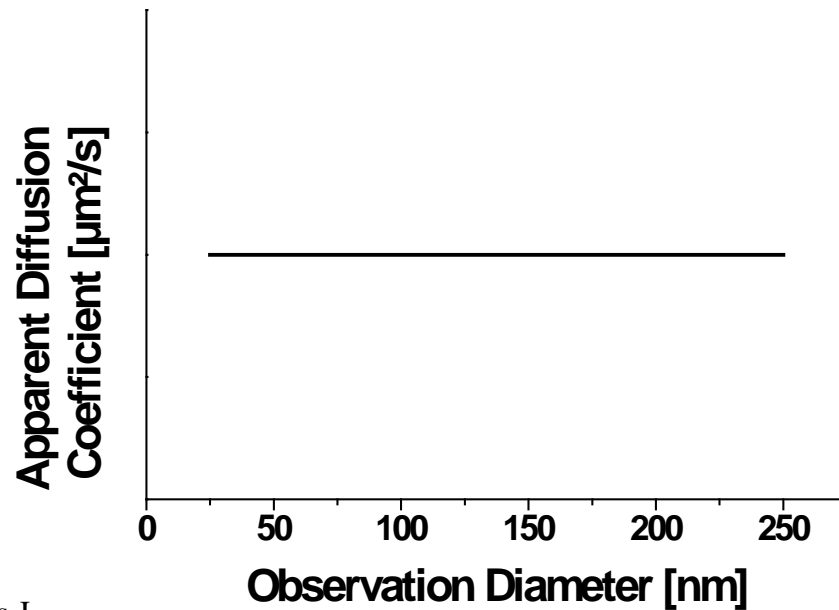
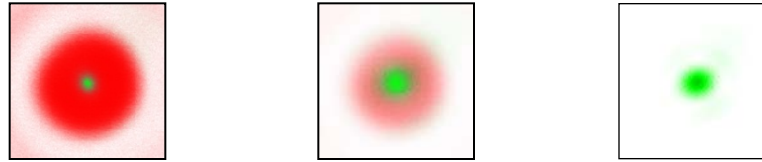
Live Cell Nanoscopy

STED-FCS - Diffusion Models

Free diffusion



← **STED Intensity**



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

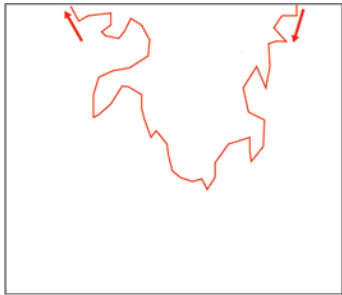
Apparent diffusion coefficient:

$D \sim \text{area} / \text{transit time}$

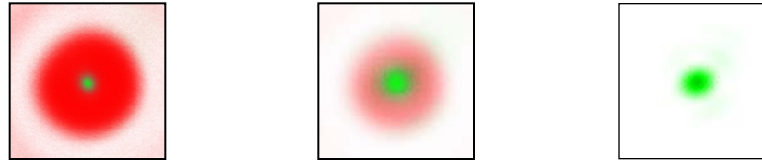
Live Cell Nanoscopy

STED-FCS - Diffusion Models

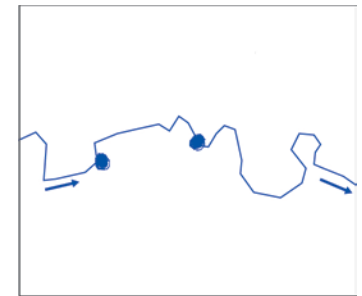
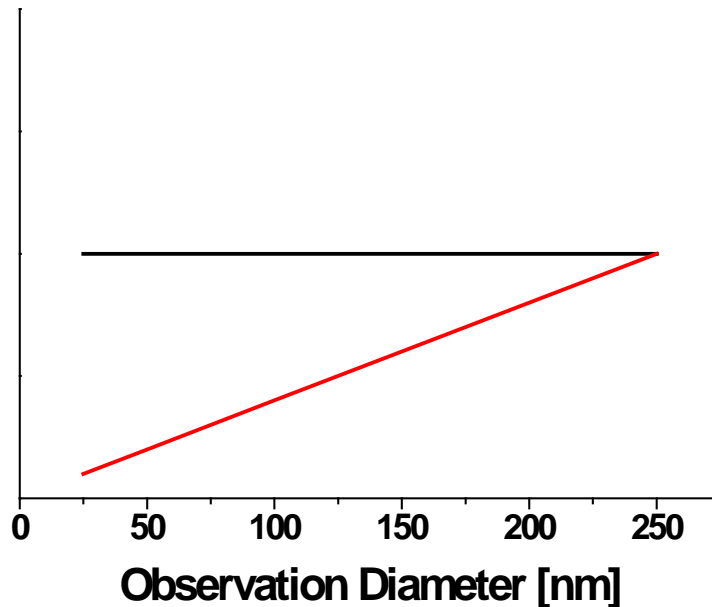
Free diffusion



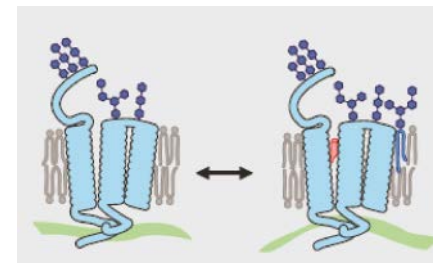
← **STED Intensity**



Apparent Diffusion Coefficient [$\mu\text{m}^2/\text{s}$]



Trapping



Wawrezynieck 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:

$D \sim \text{area} / \text{transit time}$

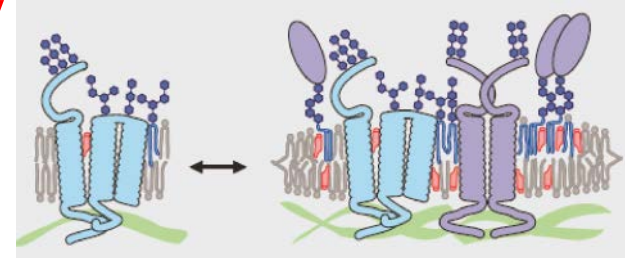
Live Cell Nanoscopy

STED-FCS - Diffusion Models

Free diffusion

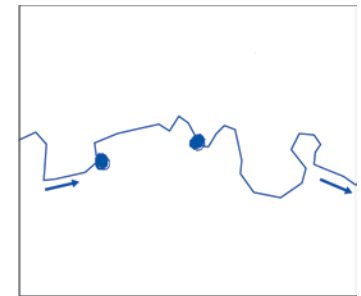
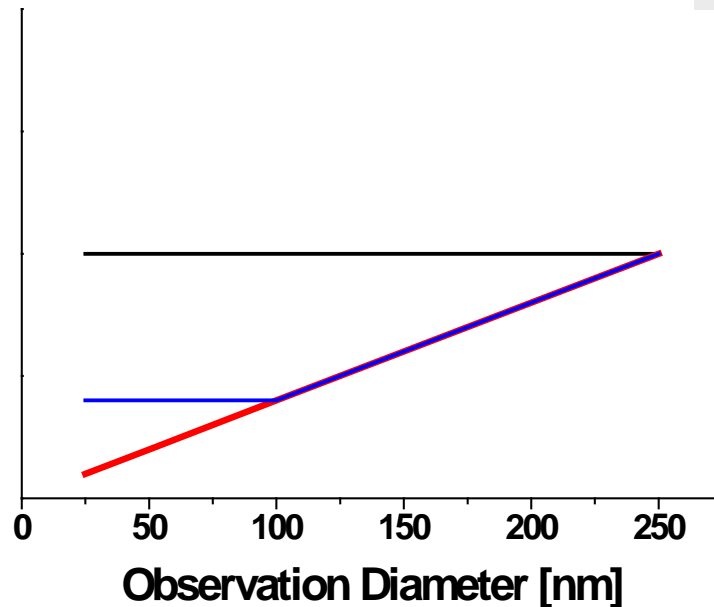


← **STED Intensity**

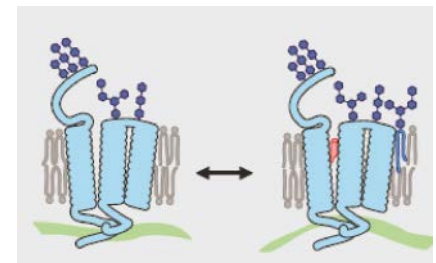


Domain incorporation

Apparent Diffusion Coefficient [$\mu\text{m}^2/\text{s}$]



Trapping



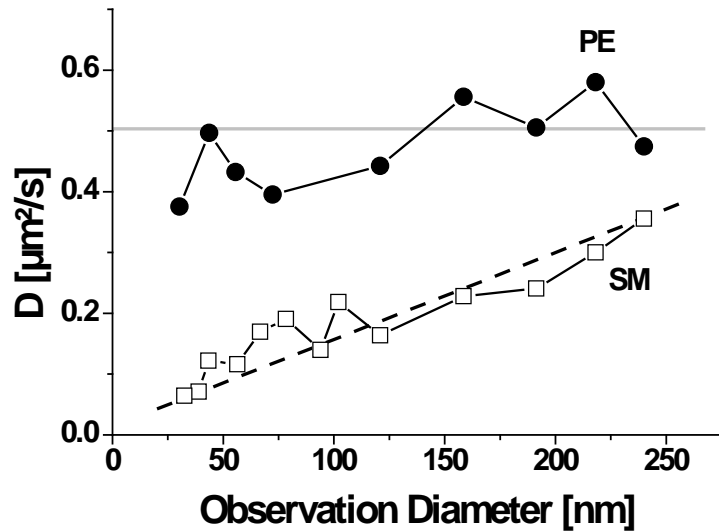
Wawrezynieck et al. *Biophys J.*
2005 December; 89(6)
Eggeling et al. *Nature* 457,
1159-1162, 2009
Mueller et al. *Biophys J* 2011

Apparent diffusion coefficient:

$D \sim \text{area} / \text{transit time}$

STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM



→ **Complex on molecular scale**

(proteins, lipid-shells, ...)

~10 ms, no movement during trapping

Cholesterol-assisted

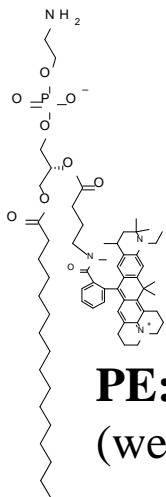
(COase/ β -Cyclo-Dextrin/Zaragozic acid...)

Binding partner bound to cytoskeleton

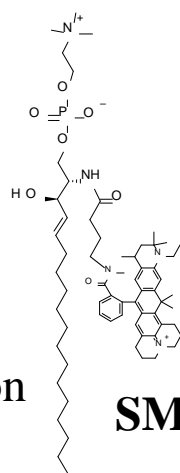
(Latrunculin/Jasplakinolide/Nocodazole...)

Dependence on lipid structure – proteins as well

(not label)

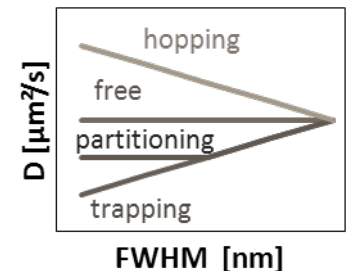
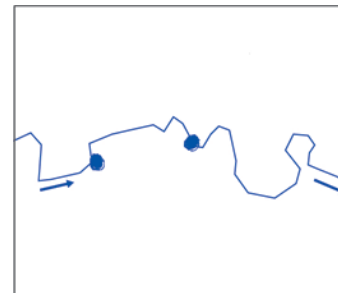


PE: free diffusion
(weak trapping)



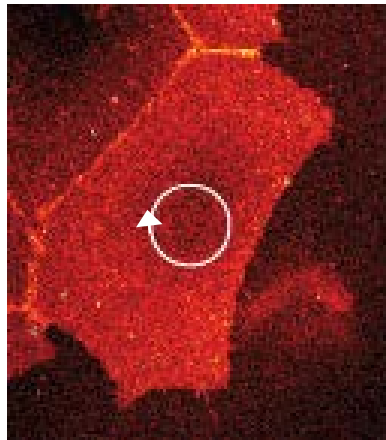
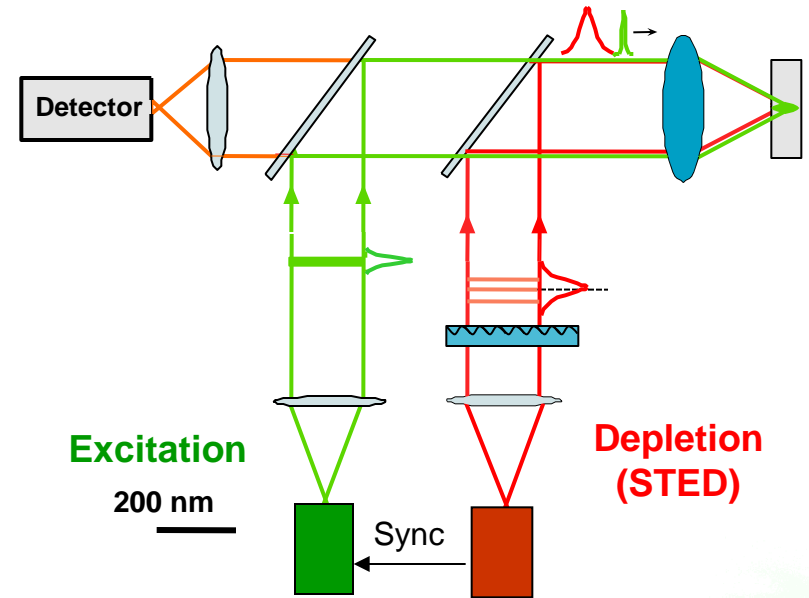
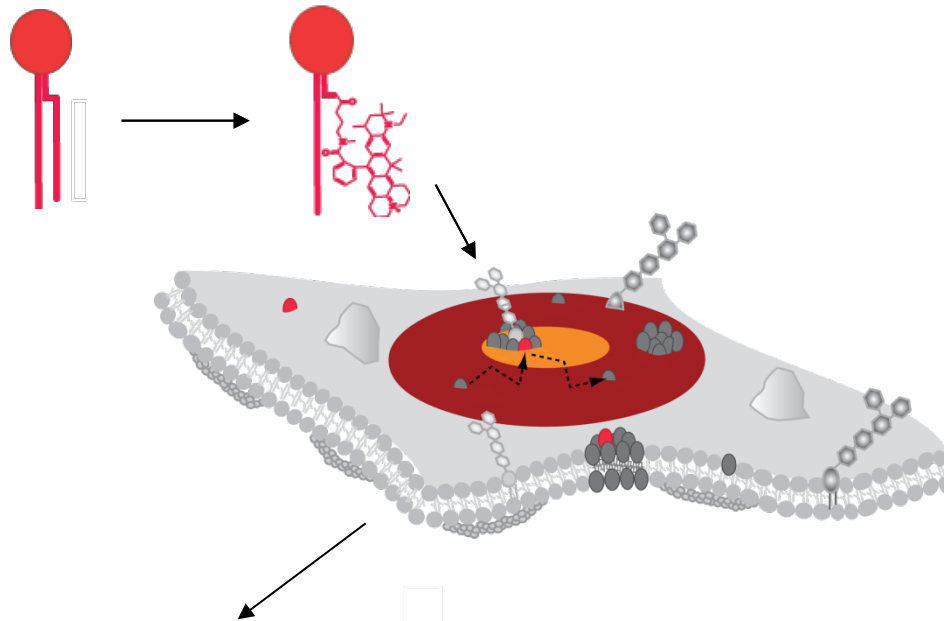
SM: trapping

Eggeling et al. *Nature* 2009
Mueller et al. *Biophys J* 2011

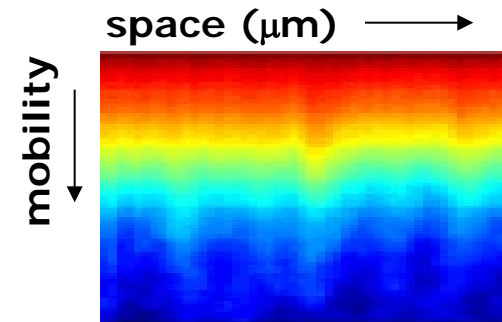
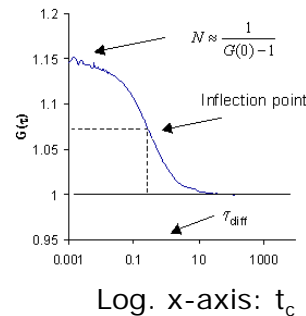


Lipid Plasma Membrane Dynamics

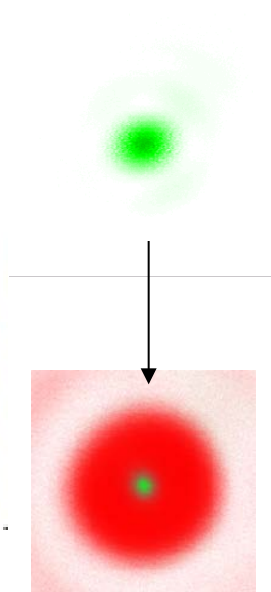
STED Nanoscopy Measurement



Diffusion map along circle

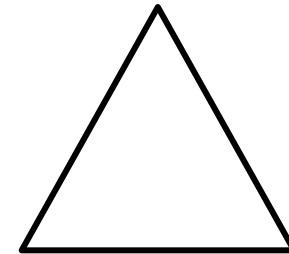
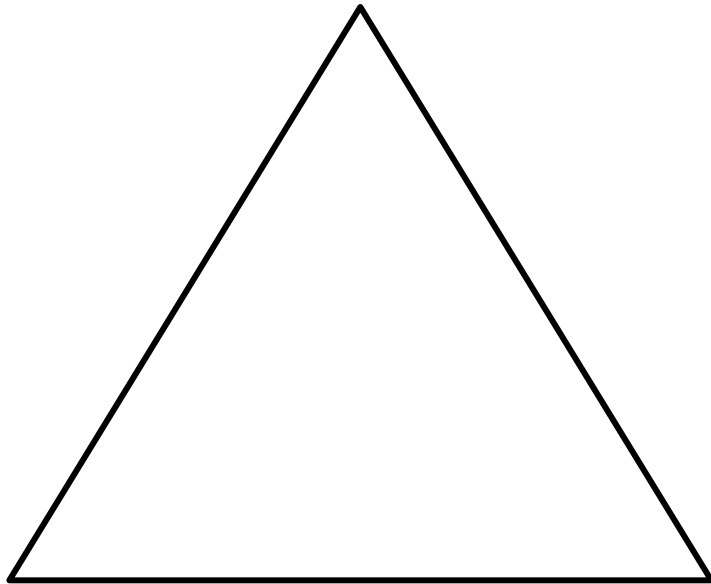


Mueller/Honigmann 2014



Advanced Microscopy *Trade-offs*

Phototoxicity
(Live-Cell)

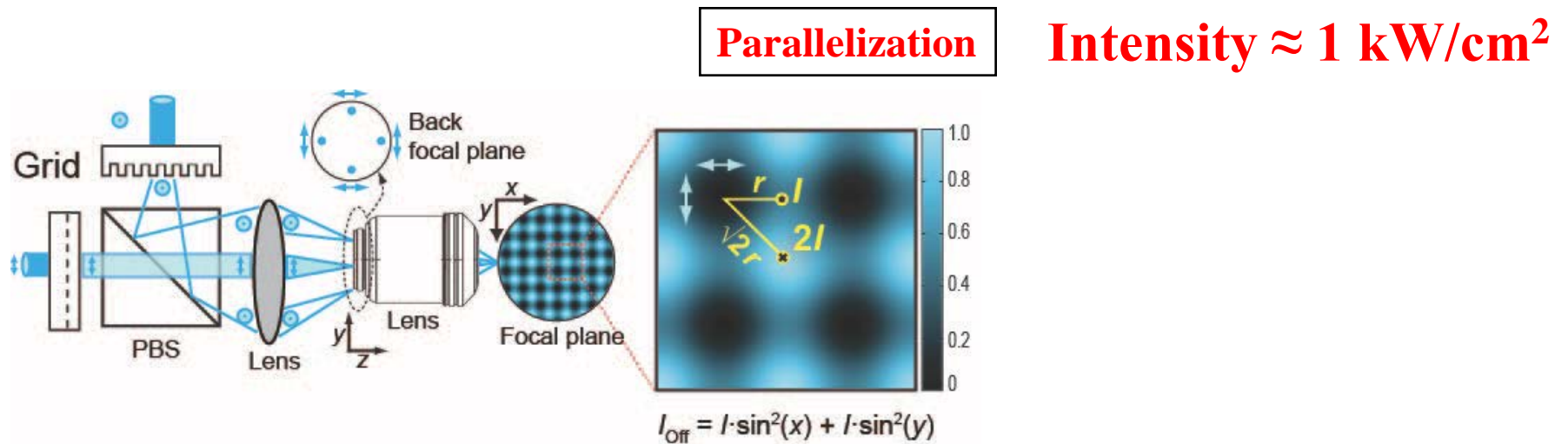


Temporal Resolution
(Dynamics)

Spatial Resolution
(Molecular details)

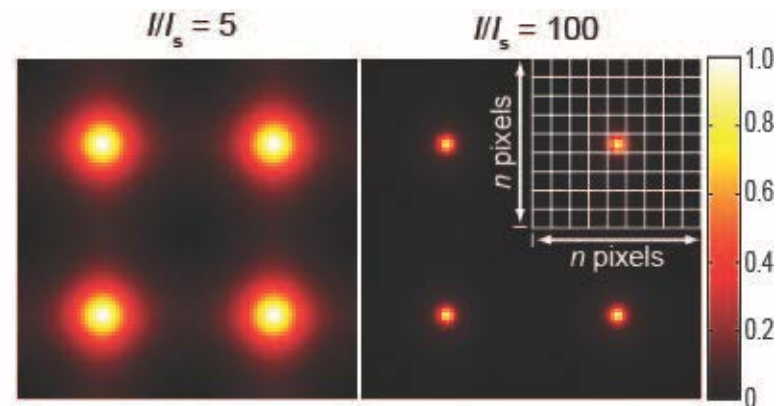
Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins



Thousand doughnuts (CCD detection)

Chmyrov et al, Nature Meth. 2013



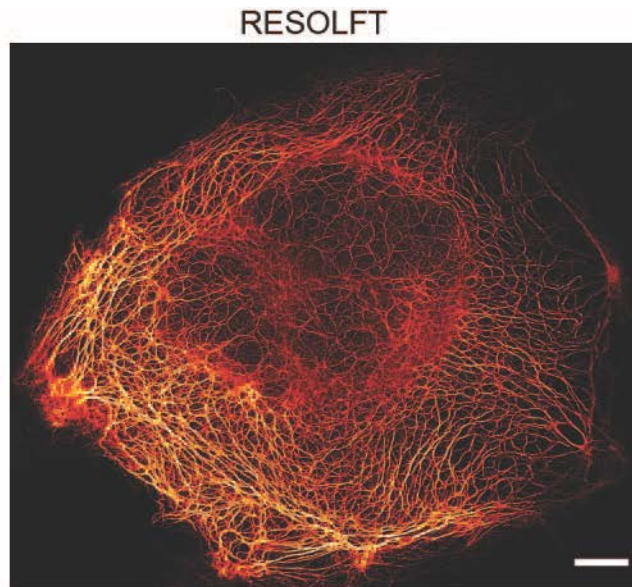
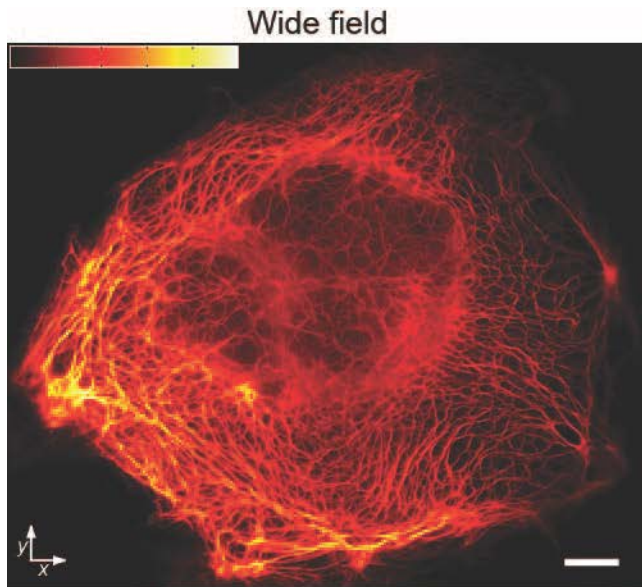
RESOLFT: on-state regions

Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins

Parallelization

Intensity $\approx 1 \text{ kW/cm}^2$

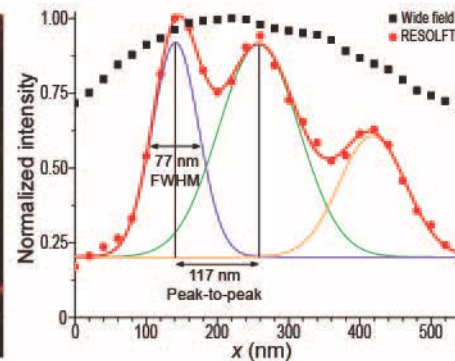
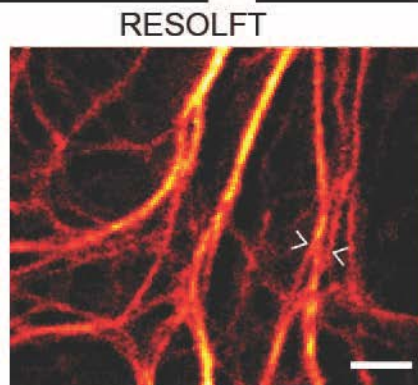
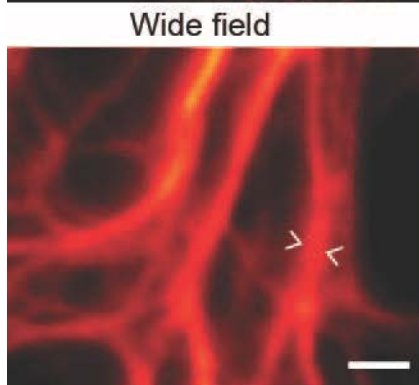


Keratin19-rsEGFP
expressed
in living PtK2 cells

Scale $10 \mu\text{m}$

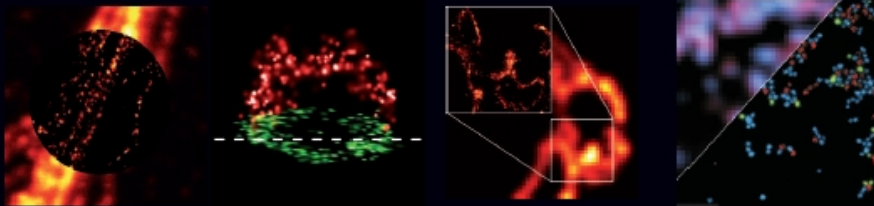
$120 \times 100 \mu\text{m} - 1 \text{ s}$

Chmyrov et al,
Nature Meth. 2013



SpringerOpen[®]

Optical Nanoscopy



 Springer

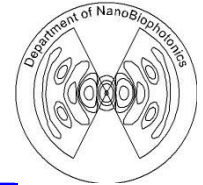
 Optical Nanoscopy
a SpringerOpen Journal

www.optnano.com

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Acknowledgement



MPI, Göttingen

Lipid Experiments

Veronika Mueller

Alf Honigmann

Debora Machado Andrade

Christian Ringemann

Rebecca Medda

Birka Lalkens

Giuseppe Viccidomini

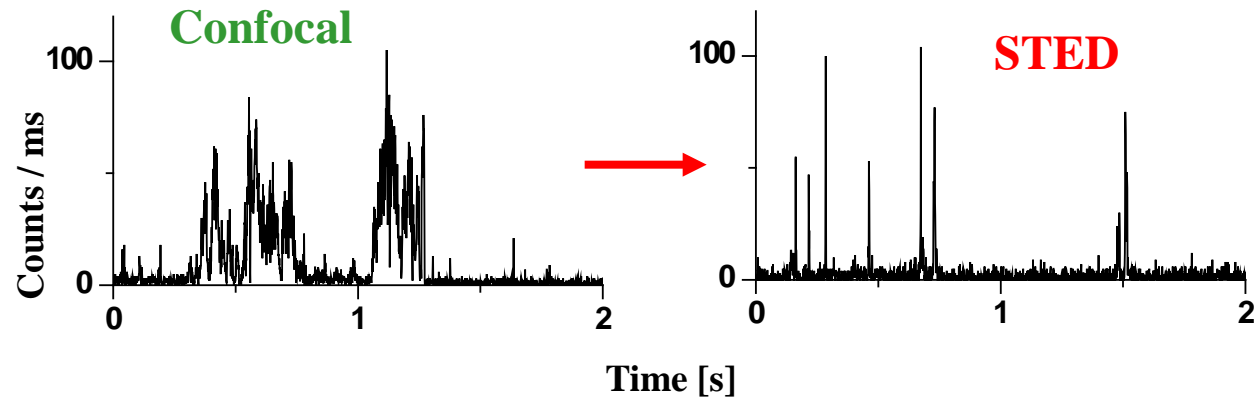
Haisen Ta

Andreas Schönle

Lipid labeling

Dr. V. Belov

S. Polyakova



Stefan Hell
+ whole group



Team – HIU/WIMM:

Jorge Bernardino de la Serna (Biophysics – membrane)

Mathias Clausen (Biophysics - membrane)

Silvia Galiani (Physics – nanoscope setup/organelles)

Marco Fritzsche (Physics - cytoskeleton)

Erdinc Sezgin (Biophysics – membrane)

Jakub Chojnacki (Biochemistry - virus)

Huw Colin York (Physics – microscopy/force)

Tess Sanley (Biology – receptor)

Antonio Gregorio Dias (Biology – virus)

Sumita Ganglui (Biology)

Wolfson Imaging Centre Oxford

Christoffer Lagerholm (manager)

Veronica Buckle

WIMM

Vincenzo Cerundolo

Doug Higgs

Simon Davis

David Jackson , Graham Ogg ...

Micron/Oxford

Ilan Davis, Lothar Schermelleh, ...

Martin Booth, Achillefs Kapanidis,

Philipp Kukura...

Mike Dustin...

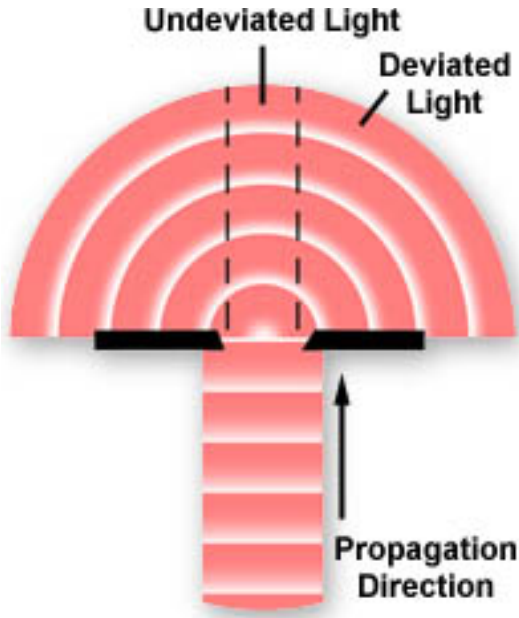
Labeling

Harry Anderson (Chem. Oxford)

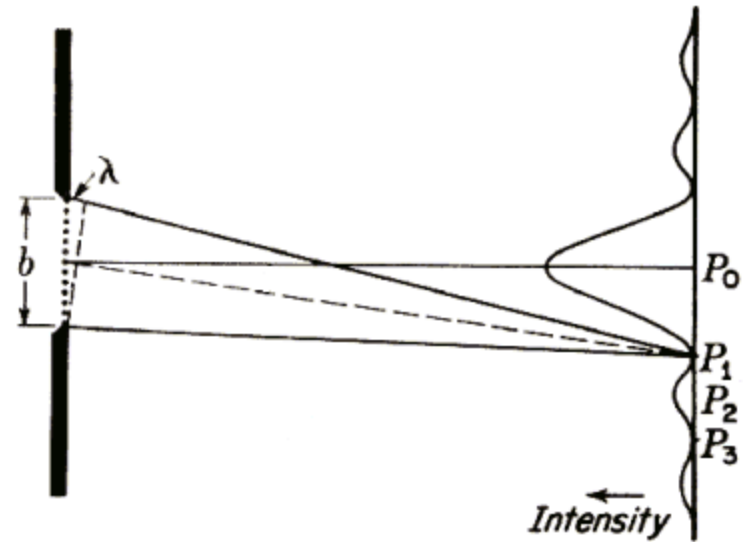
Del Besra (Birmingham)

Resolution of Optical Microscopy - Diffraction

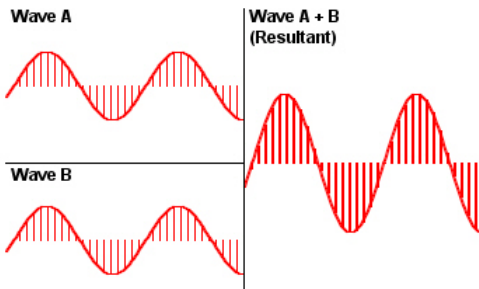
Light rays bend around edges – new wavefronts are generated at sharp edges



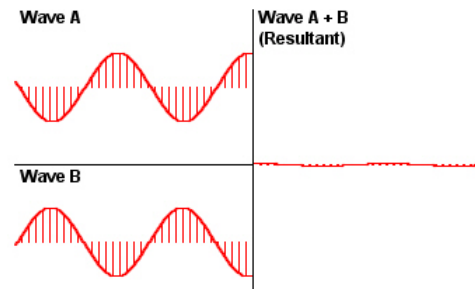
and interfere



Constructive interference



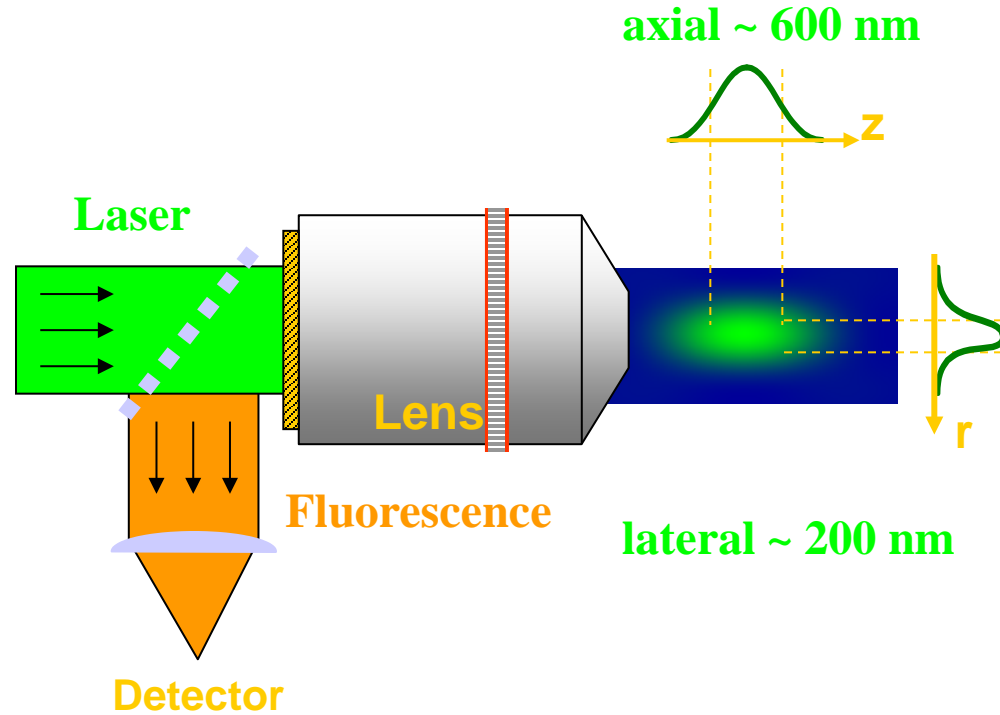
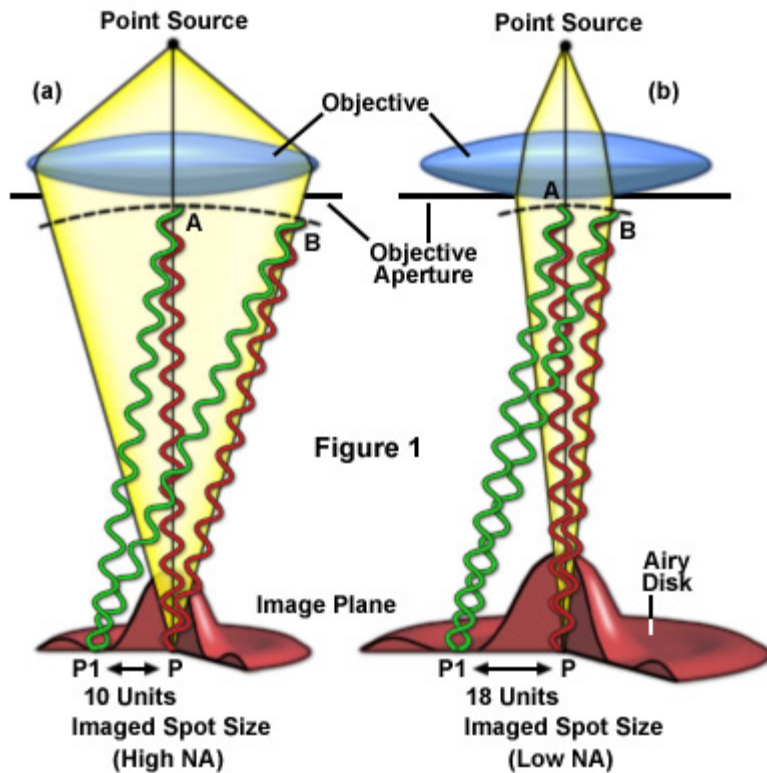
Destructive interference



Resolution of Optical Microscopy

Airy pattern formation by microscope objective

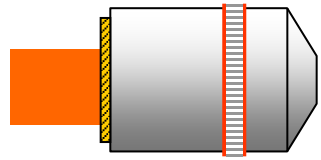
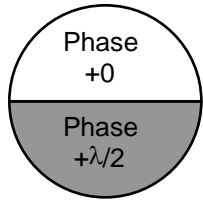
Resolution Limit Imposed by Wave Nature of Light



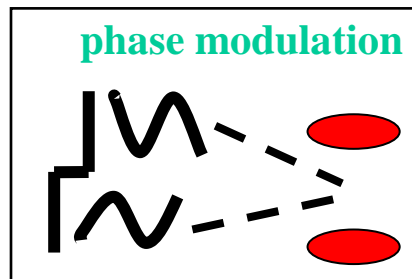
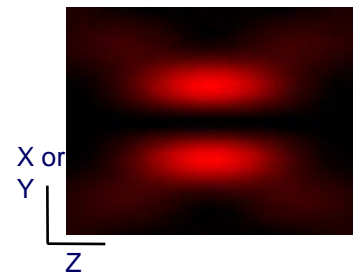
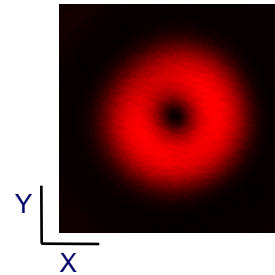
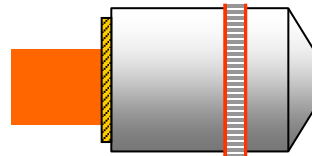
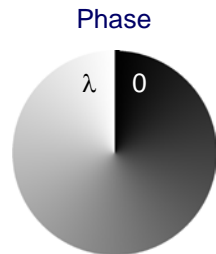
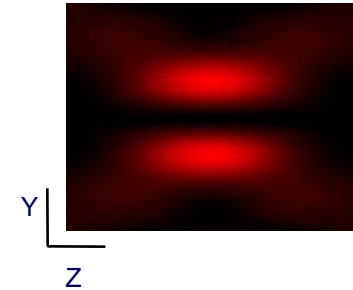
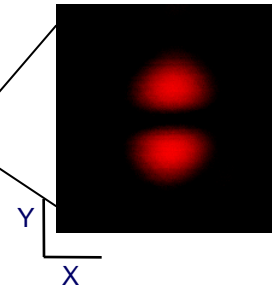
Focal Volume Confinement

Focal Engineering – Local Zero

Phase mask:



STED PSF:



STED-Microscopy Setup

