

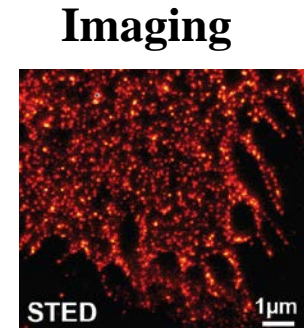
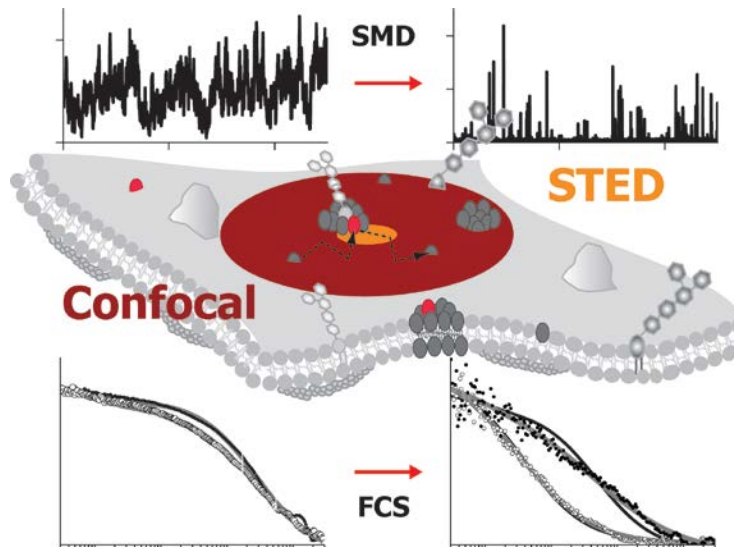
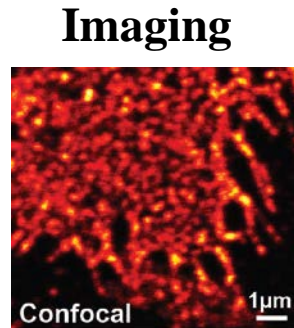
Super-resolution STED microscopy of the living cell



Weatherall Institute of Molecular Medicine, HIU
University of Oxford

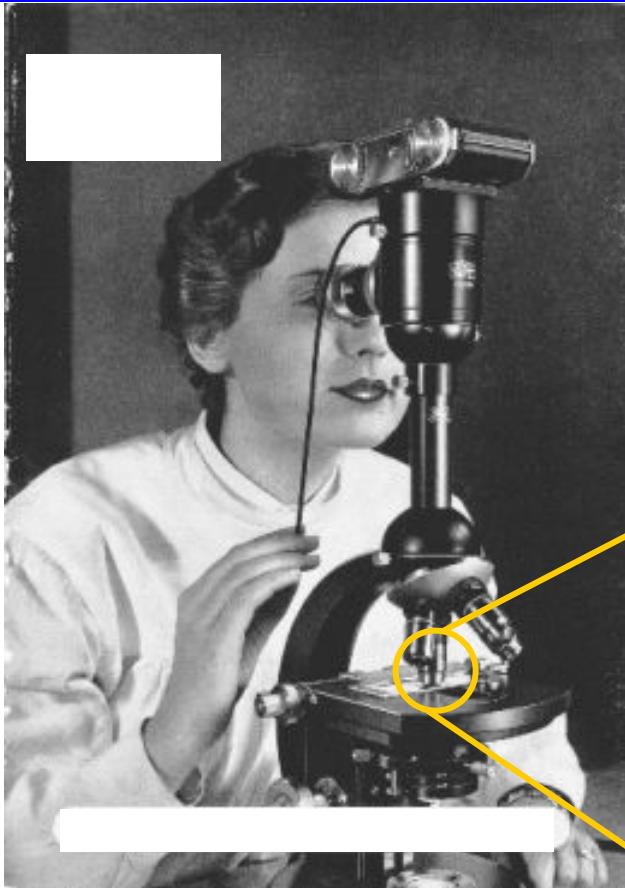
Christian Eggeling

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

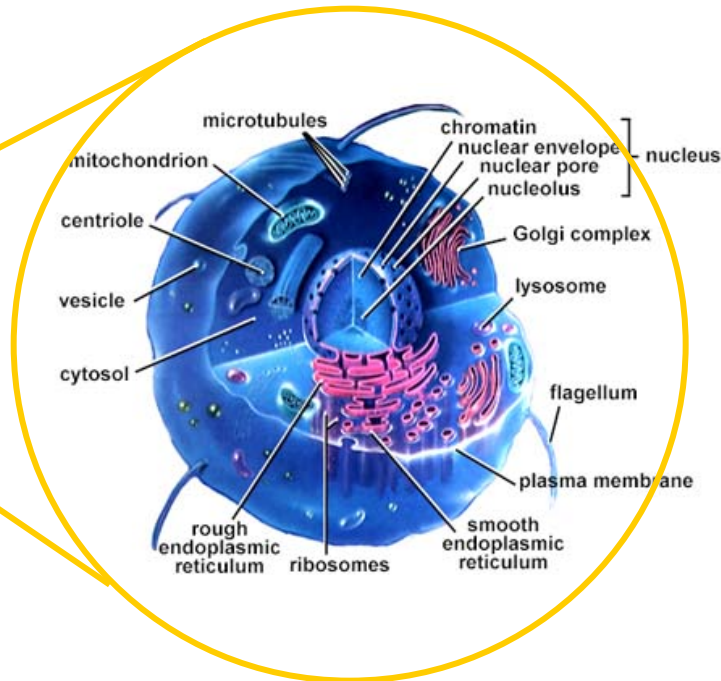
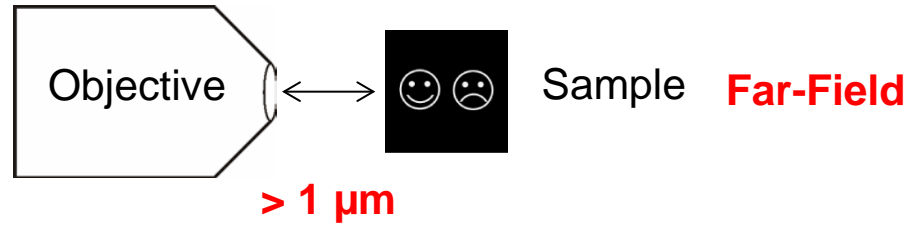


Live Cell Microscopy

Observation of living cells: Non-Invasive



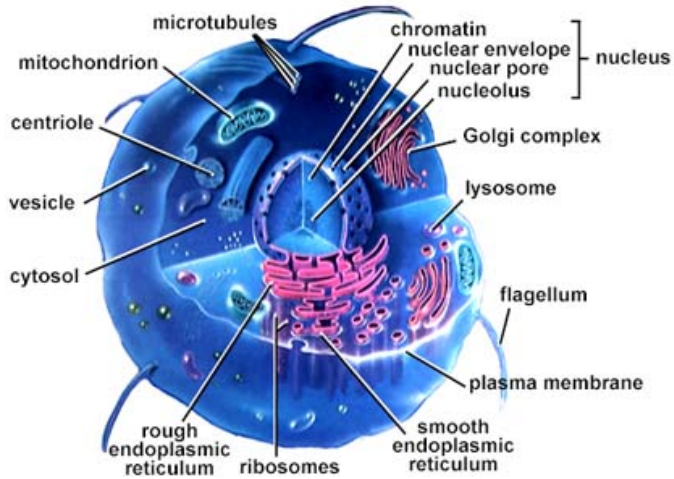
Light + Far-Field: non-invasive!



Live Cell Far-Field Microscopy

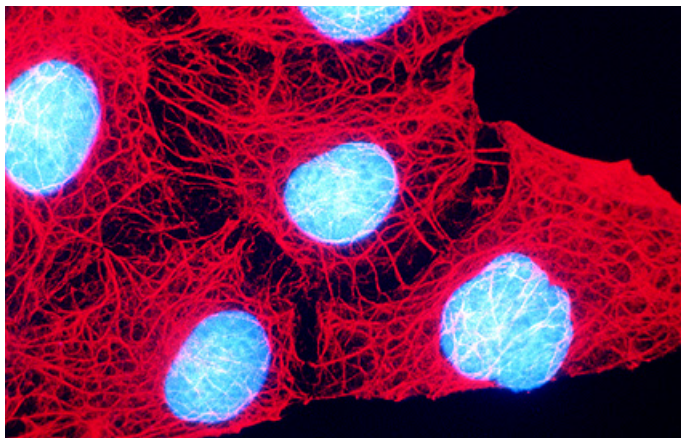
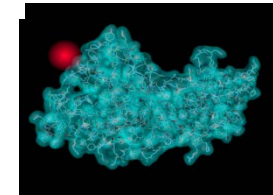
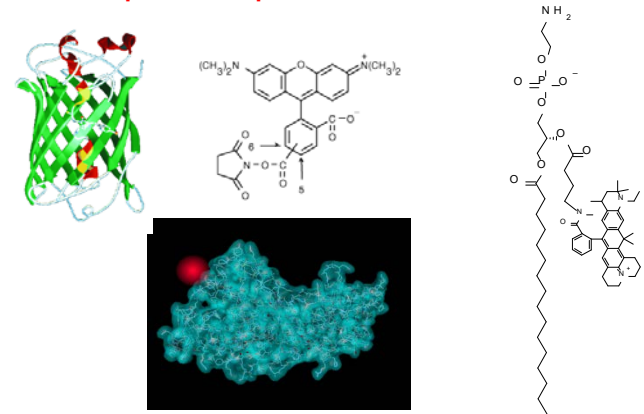
Fluorescence

Study specific molecular processes in the living cell:



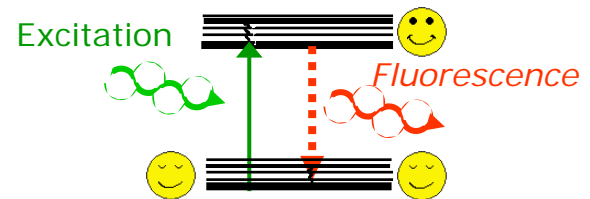
Fluorescence microscopy

Label specific protein/molecule



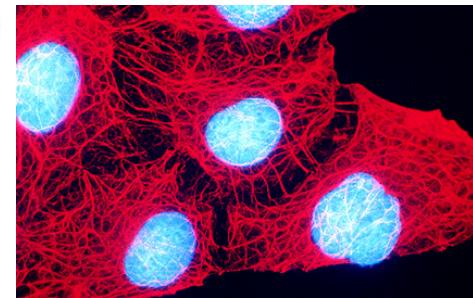
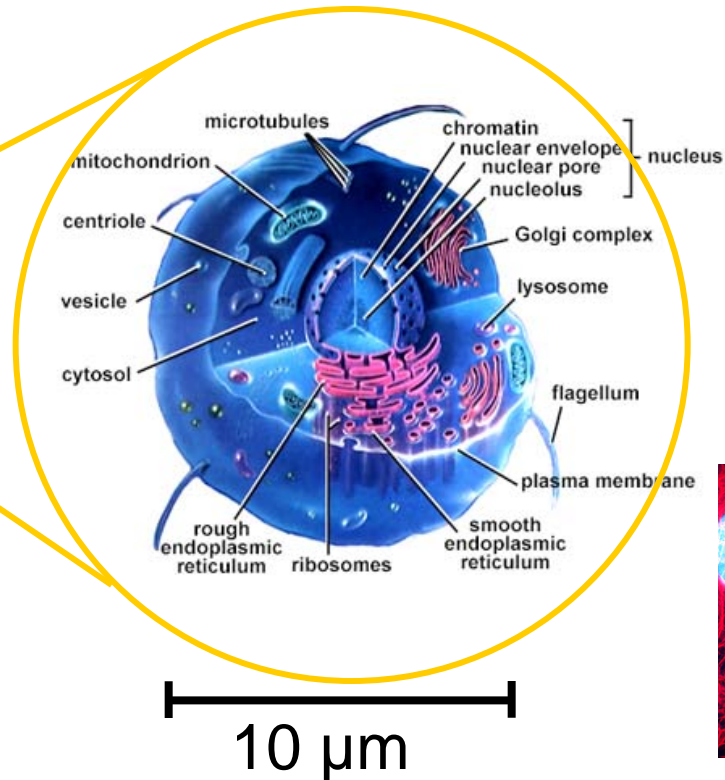
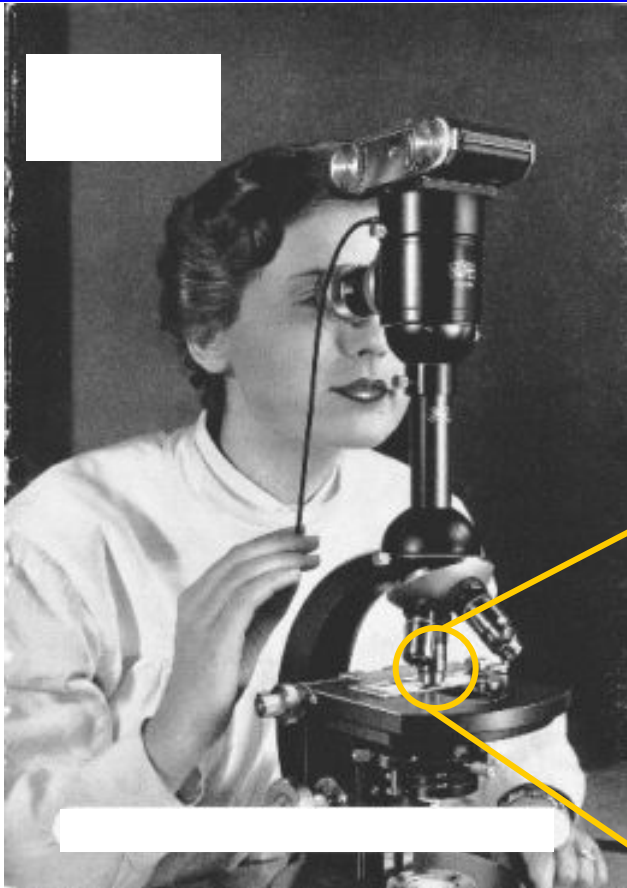
Liver-Cells: Nucleus and Cell-skeleton

Excite fluorescence by laser light



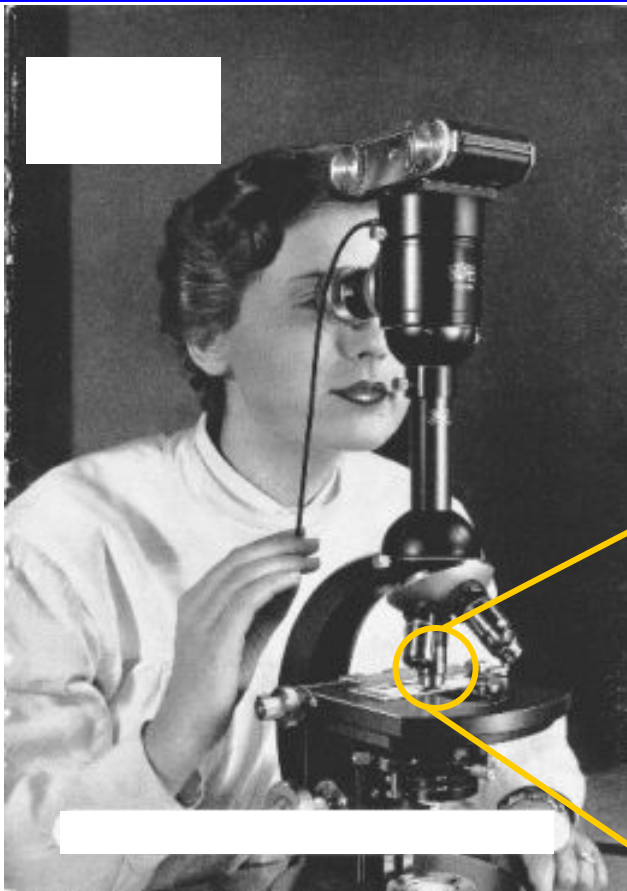
Far-Field Fluorescence Microscopy

Resolution: Goal

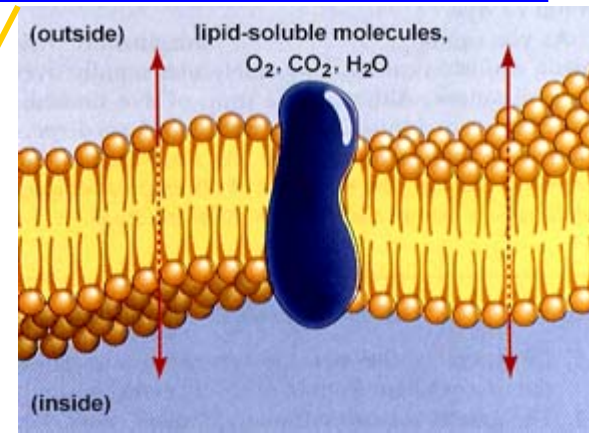


Far-Field Fluorescence Microscopy

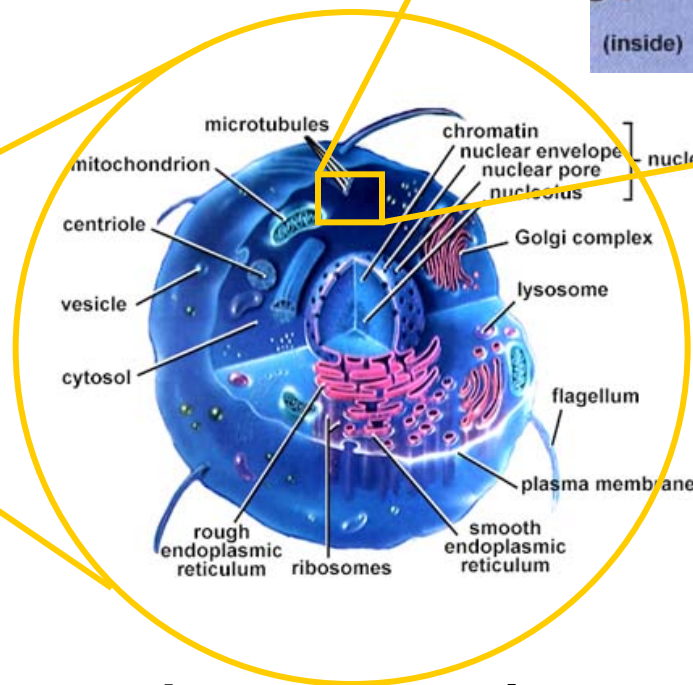
Resolution: Goal



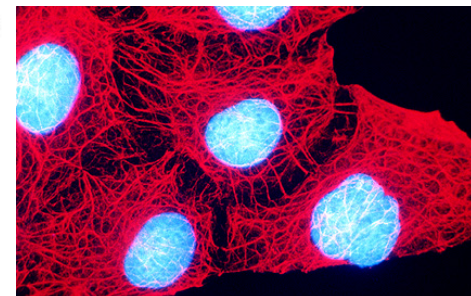
⇒ molecular scale



10 nm



10 μm

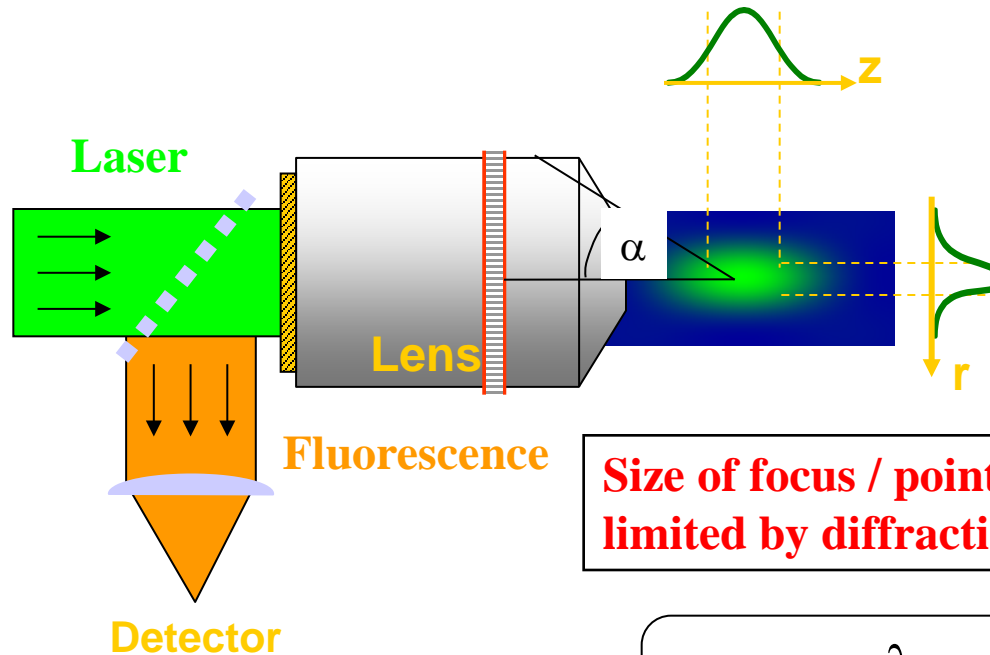


Far-Field Microscopy

Resolution Limit: Diffraction Barrier

Far-Field Fluorescence Microscopy: Focussing of light

- away from surfaces – inside cells (3D)



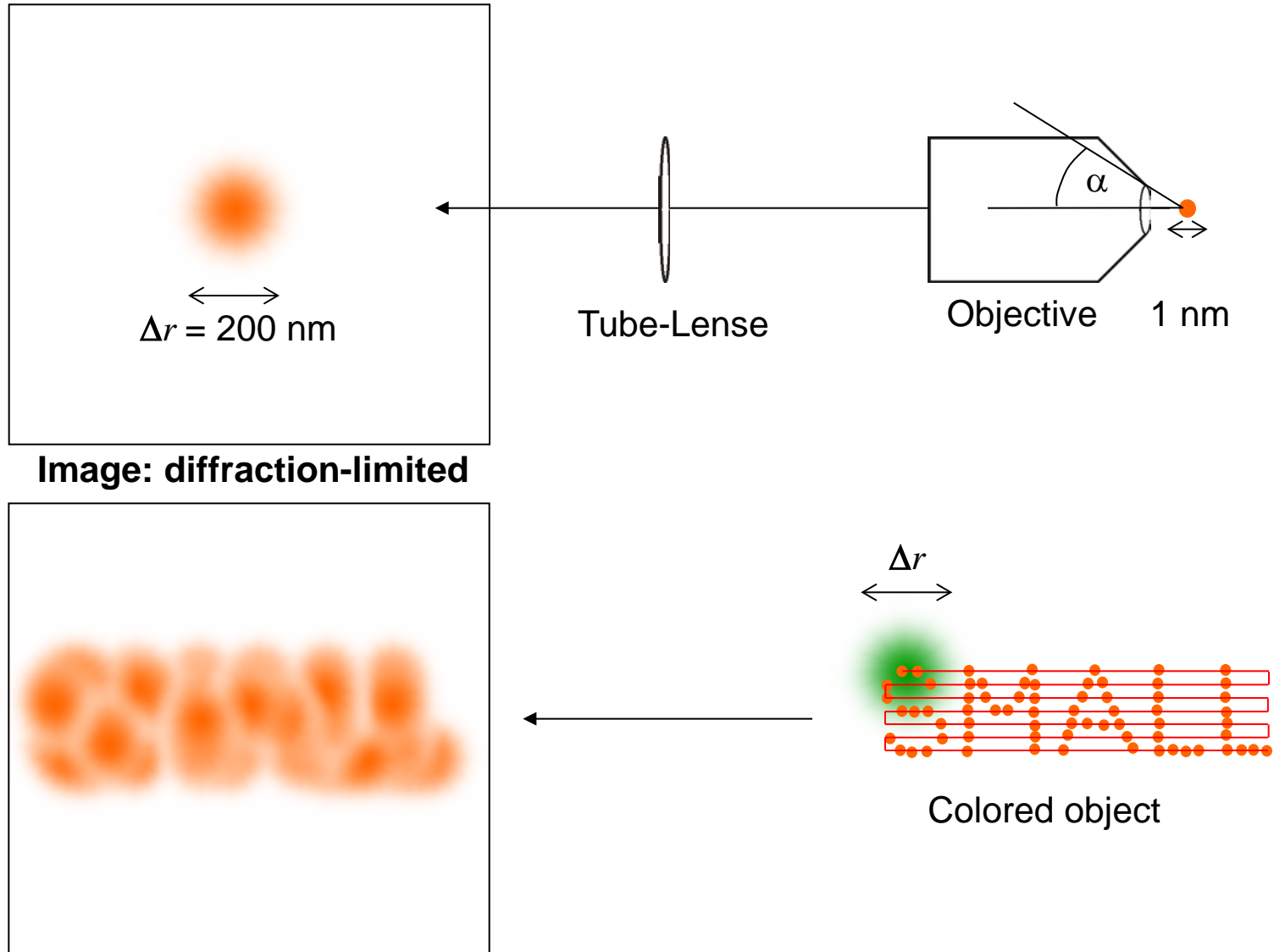
**Size of focus / point-spread function
limited by diffraction of light!!!**

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

Ernst Abbe 1873

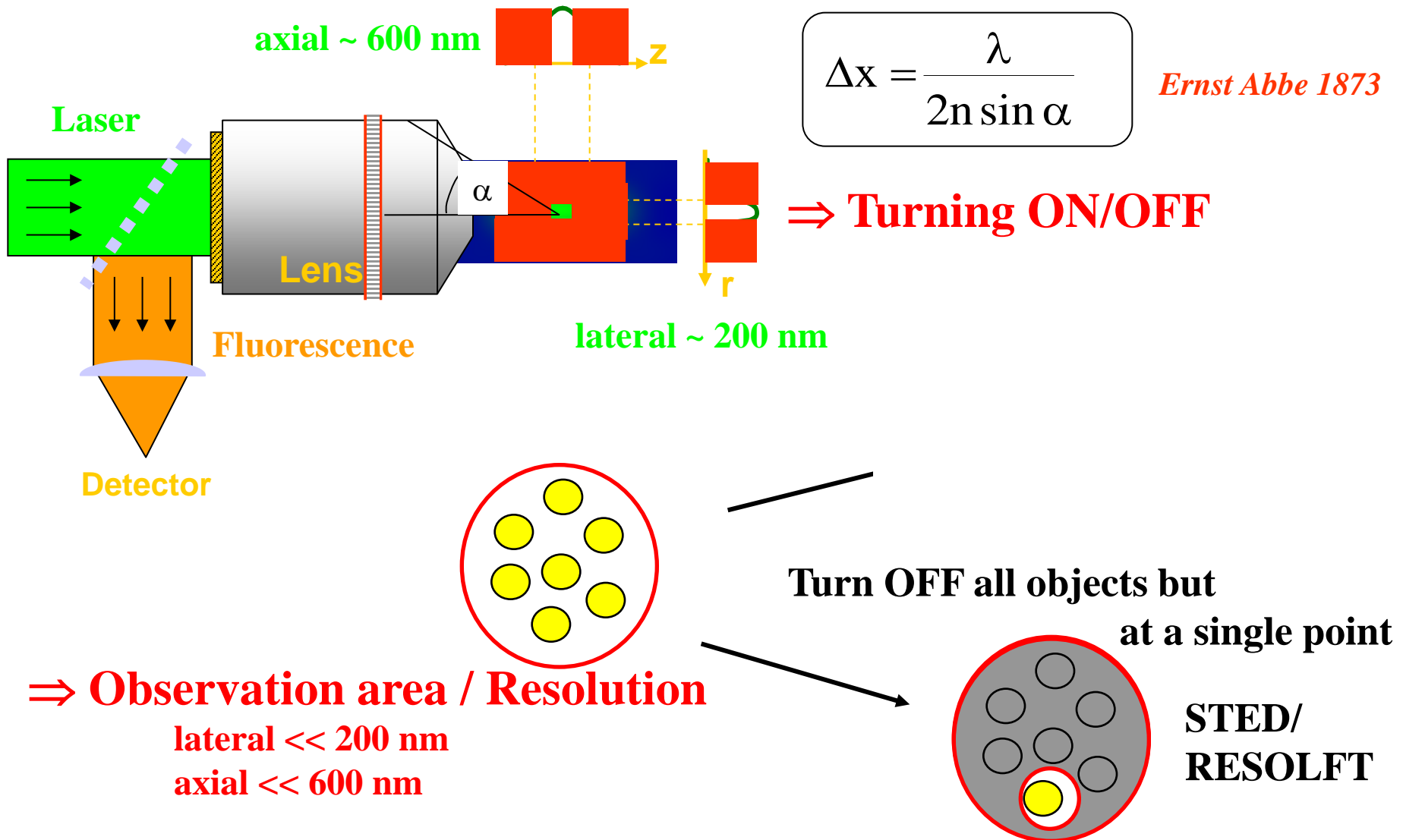
Far-Field Microscopy

Resolution Limit: Diffraction Barrier



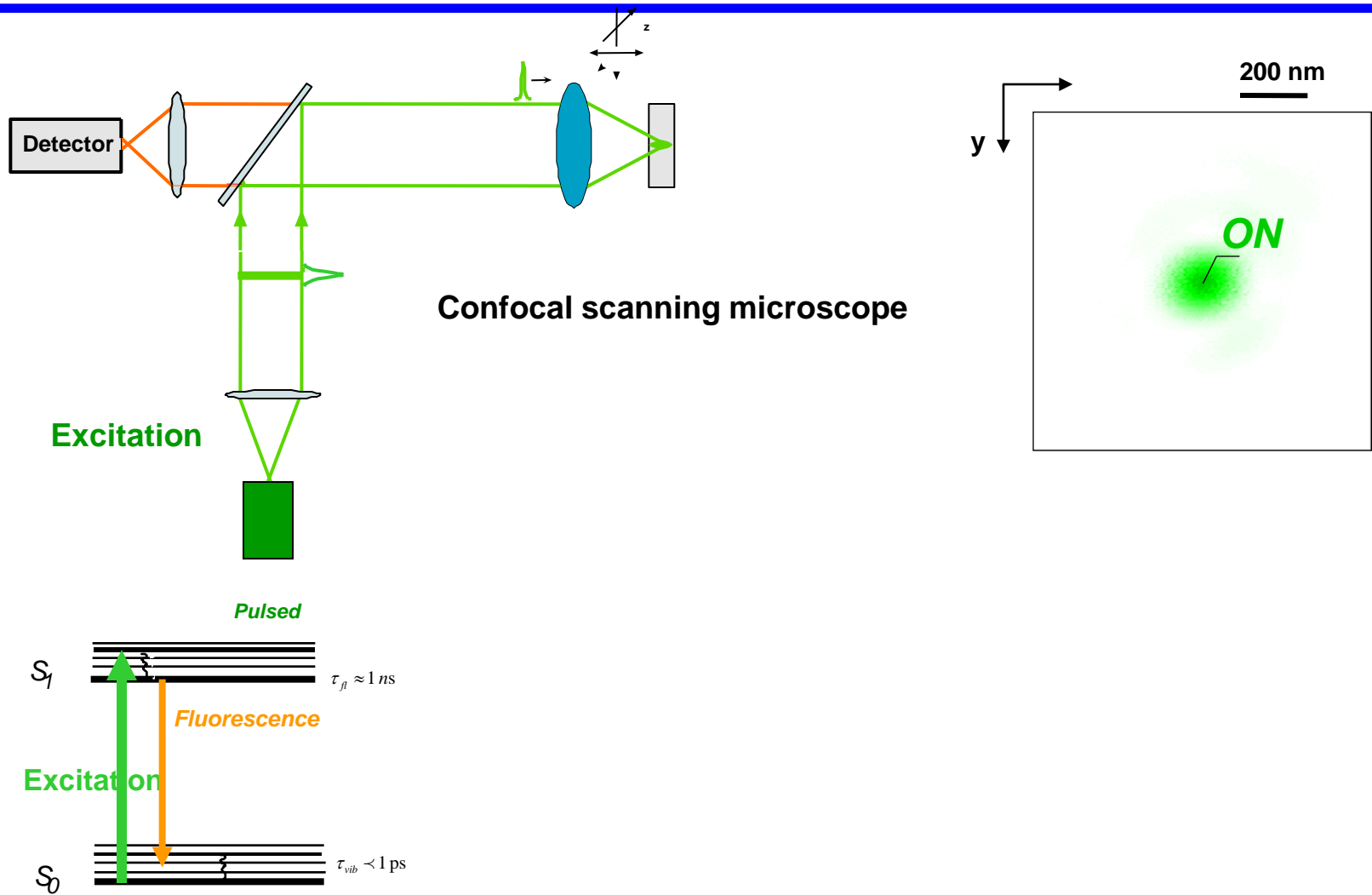
Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF



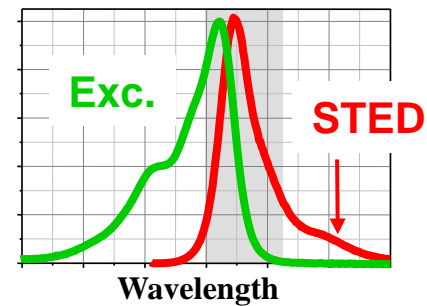
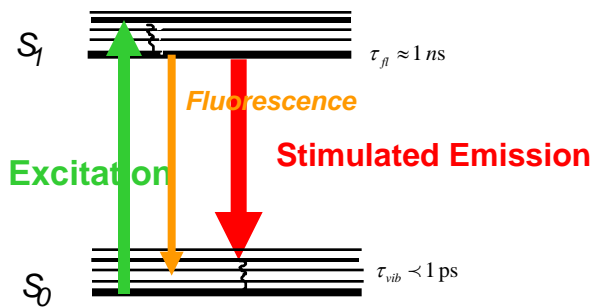
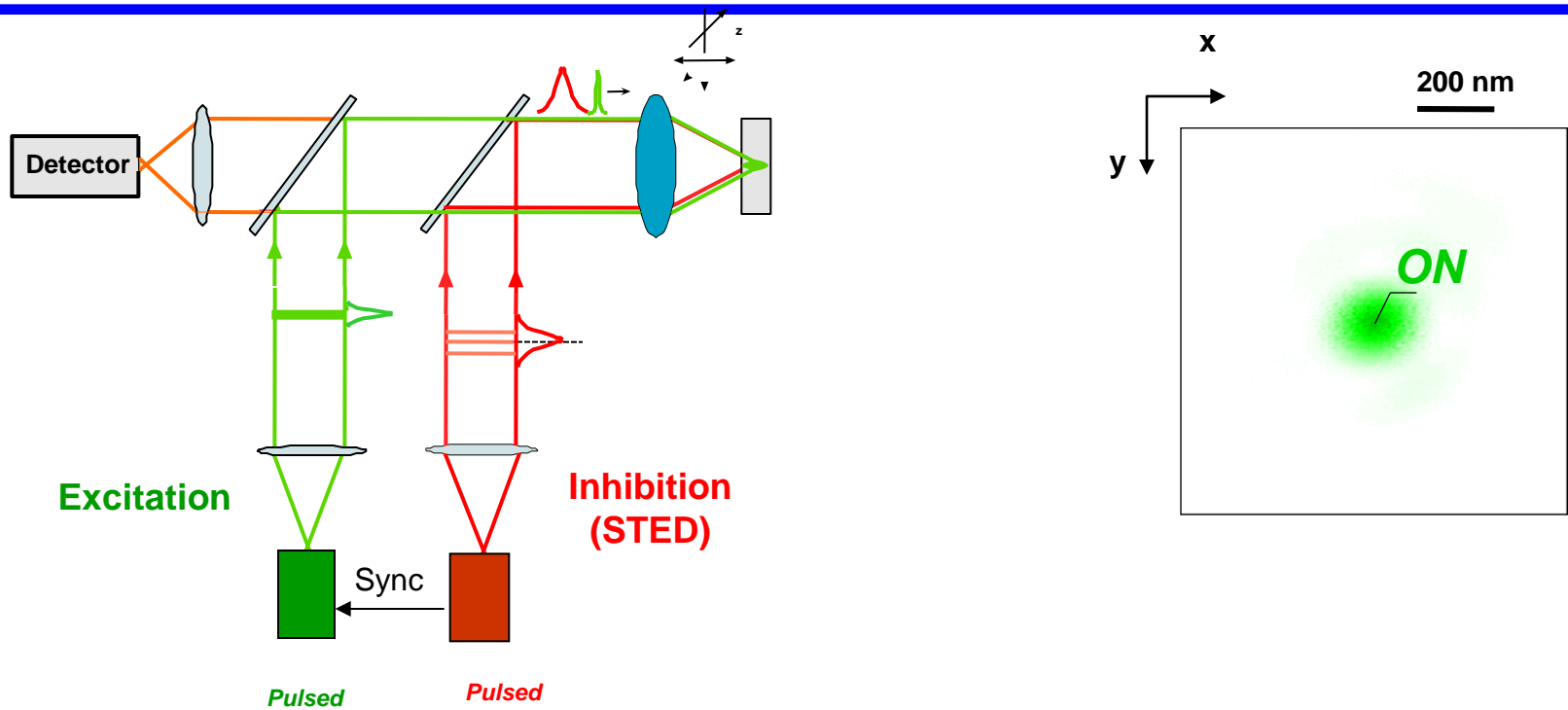
Fluorescence Microscopy

STED Microscopy



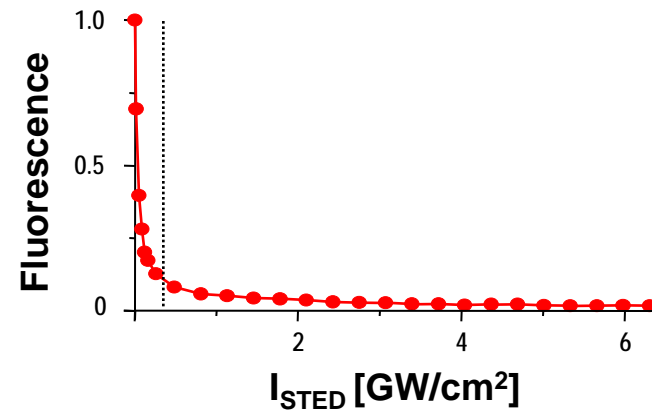
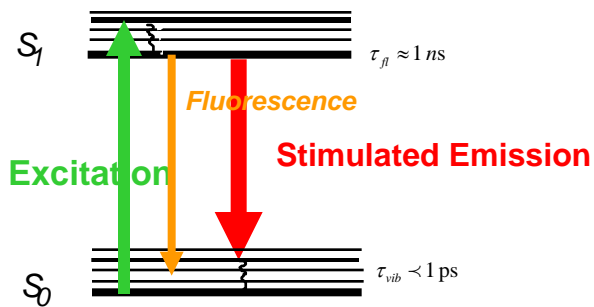
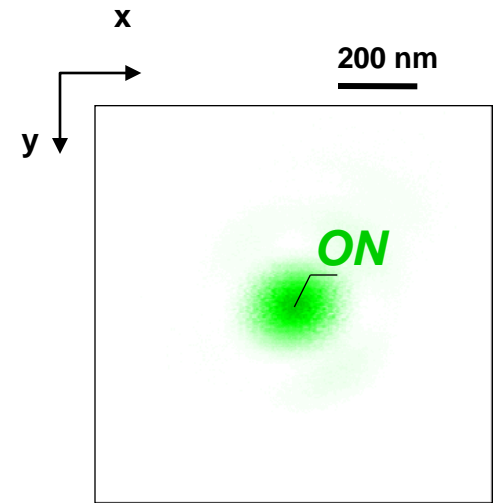
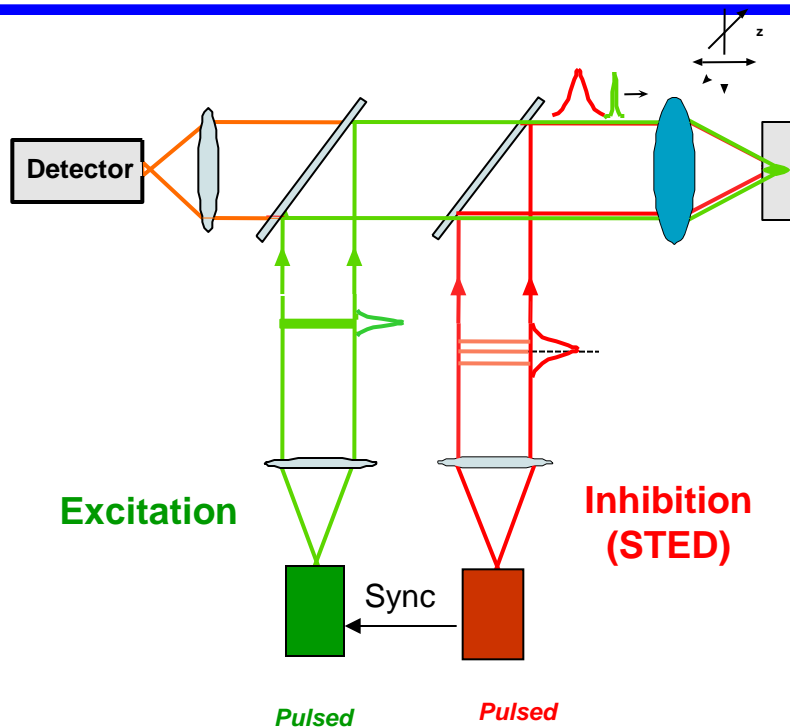
Fluorescence Microscopy

STED Microscopy



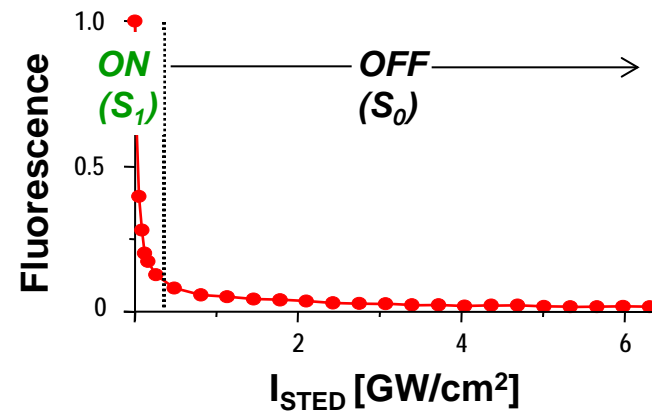
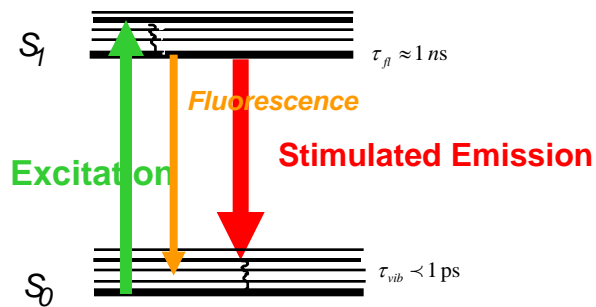
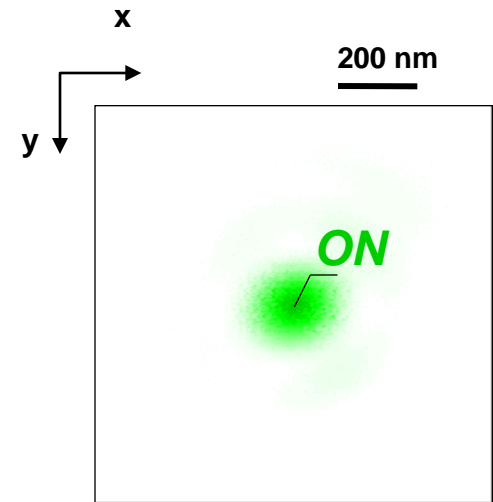
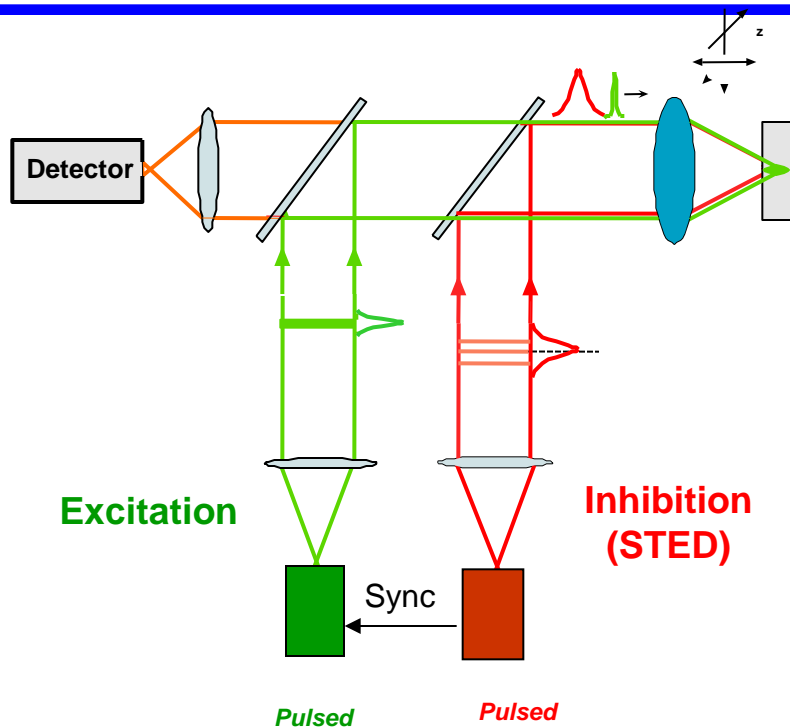
Fluorescence Microscopy

STED Microscopy



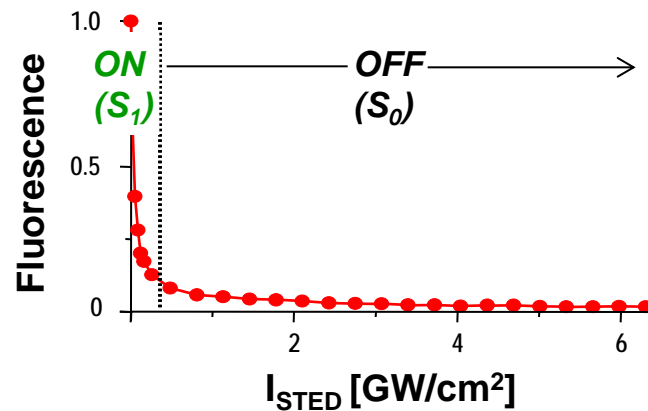
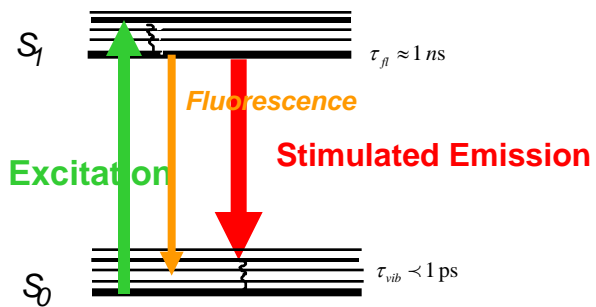
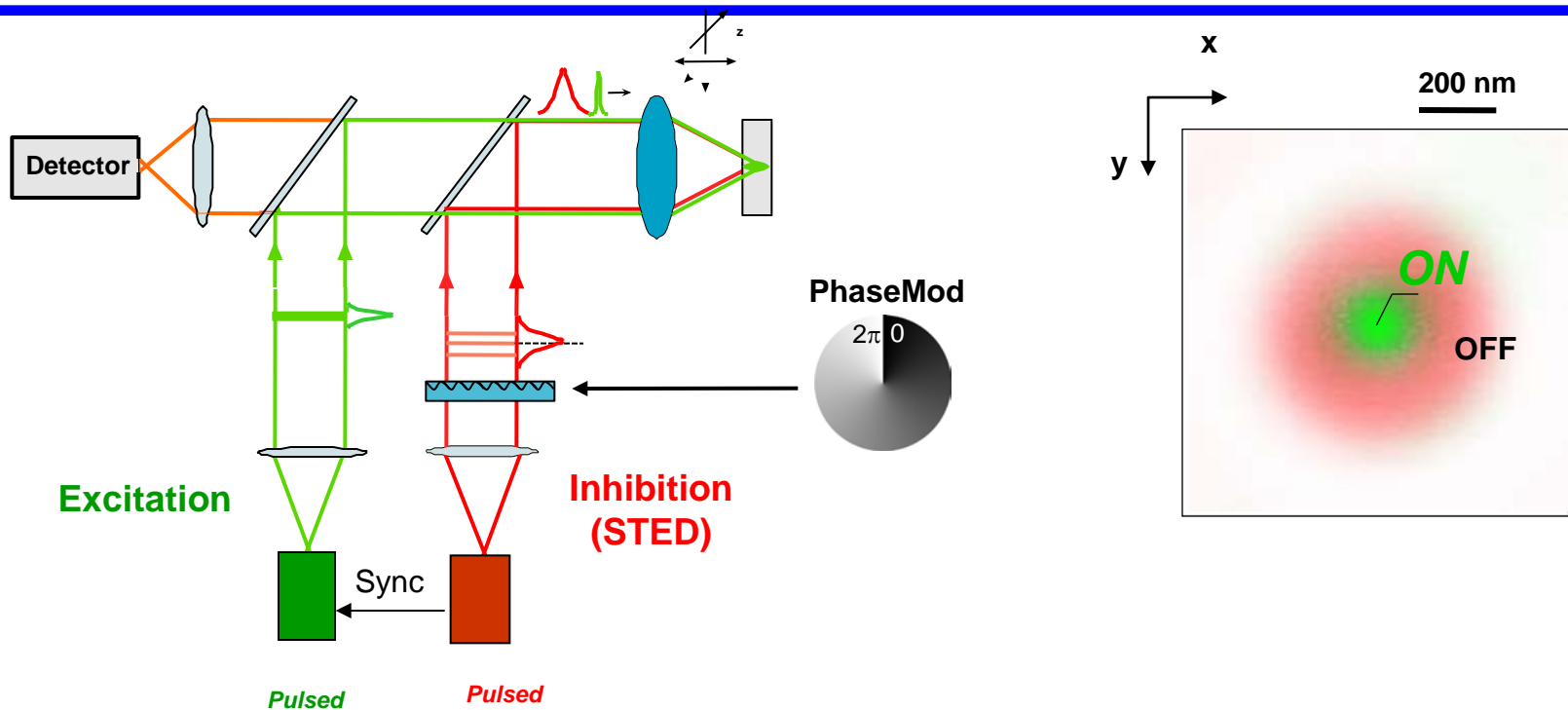
Fluorescence Microscopy

STED Microscopy



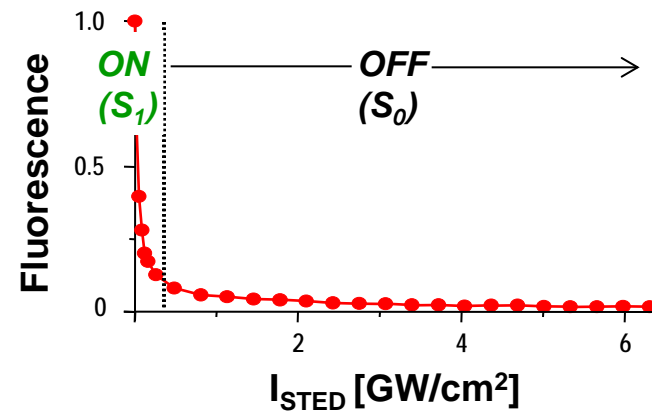
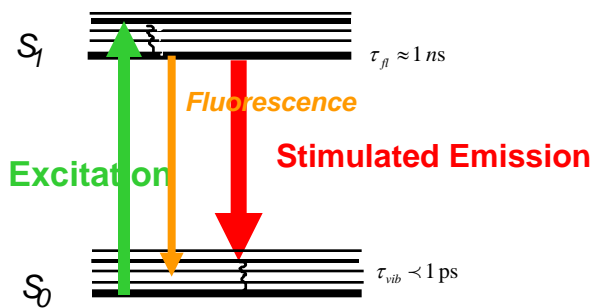
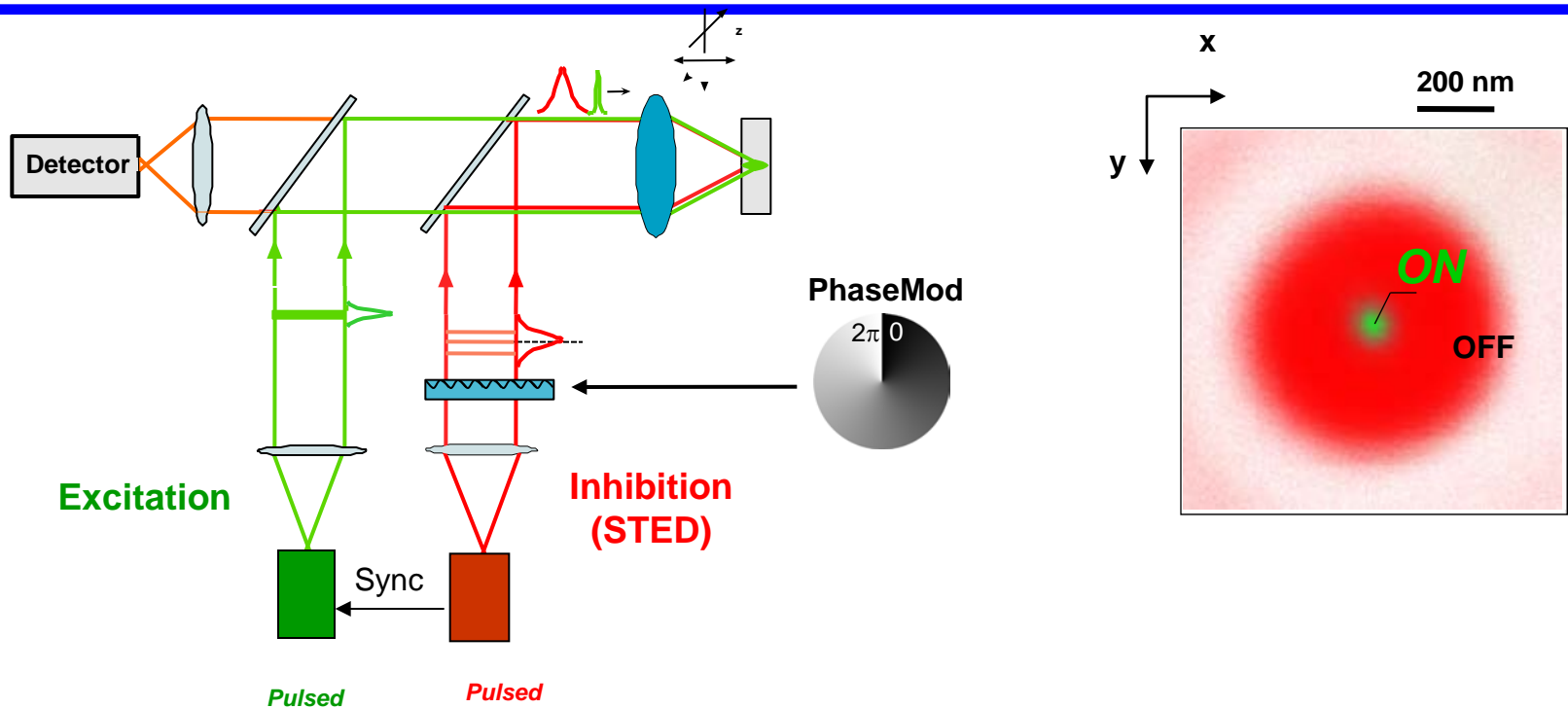
Fluorescence Microscopy

STED Microscopy



Fluorescence Microscopy

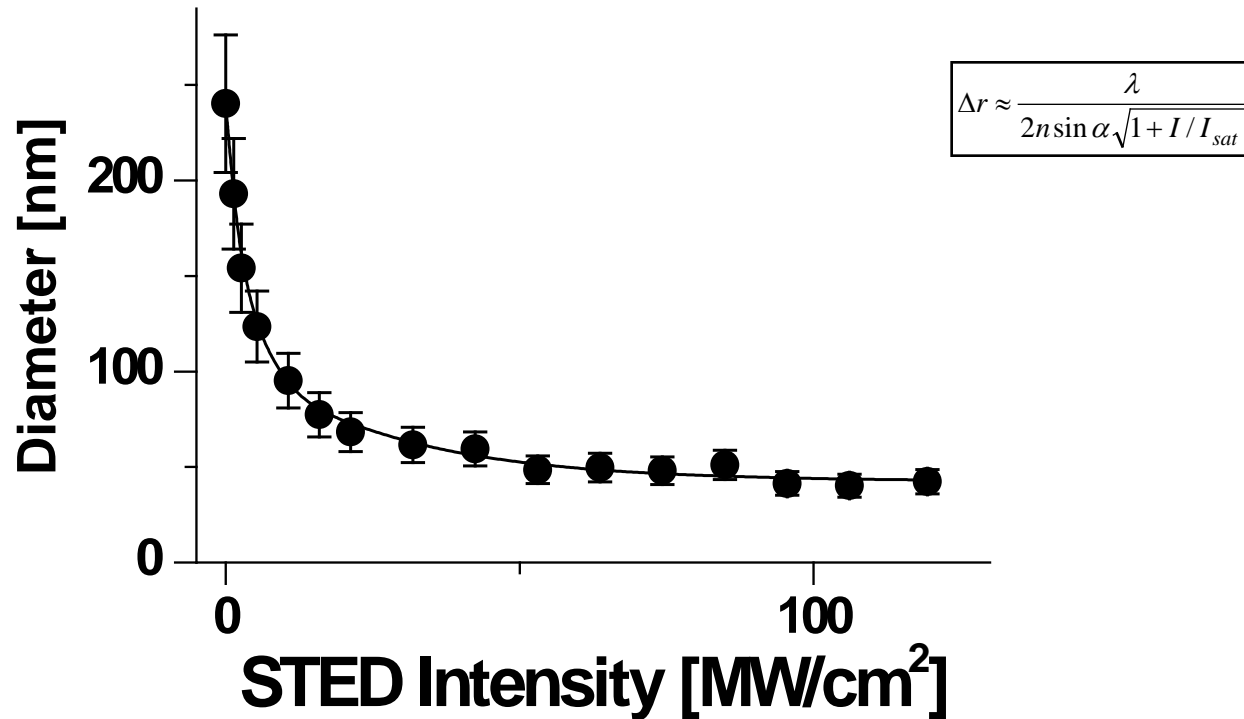
STED Microscopy



STED Microscopy

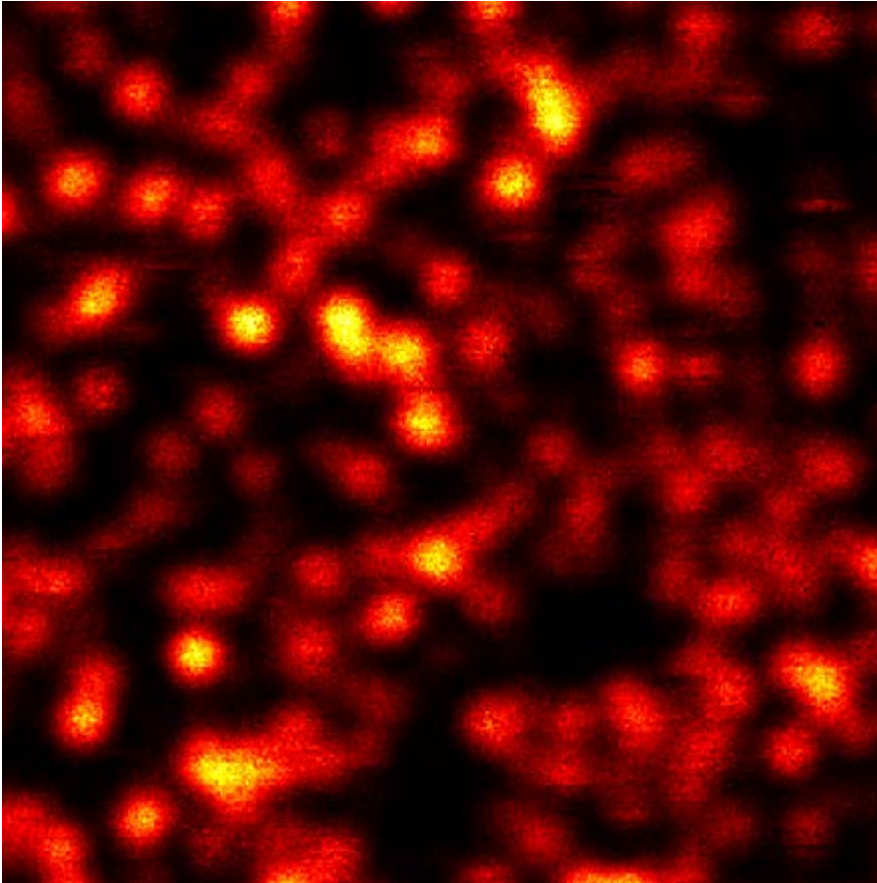
Dynamical confinement of resolution

Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!

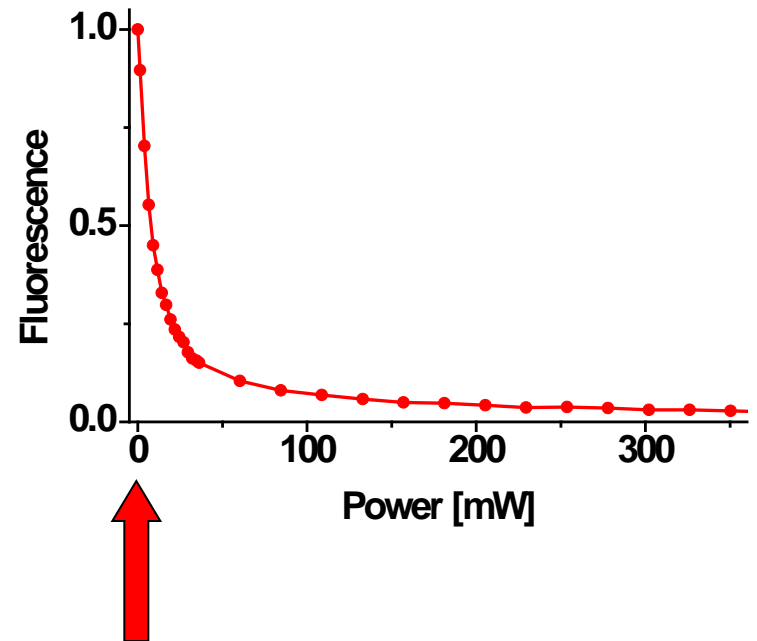
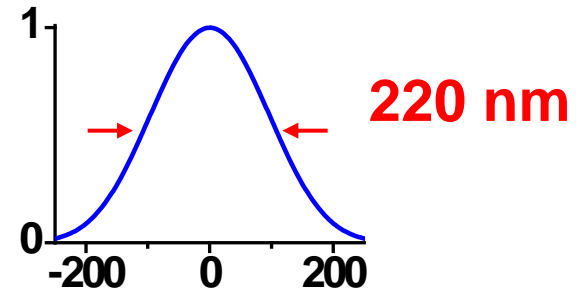


STED-Microscopy

Sub-Diffraction Imaging

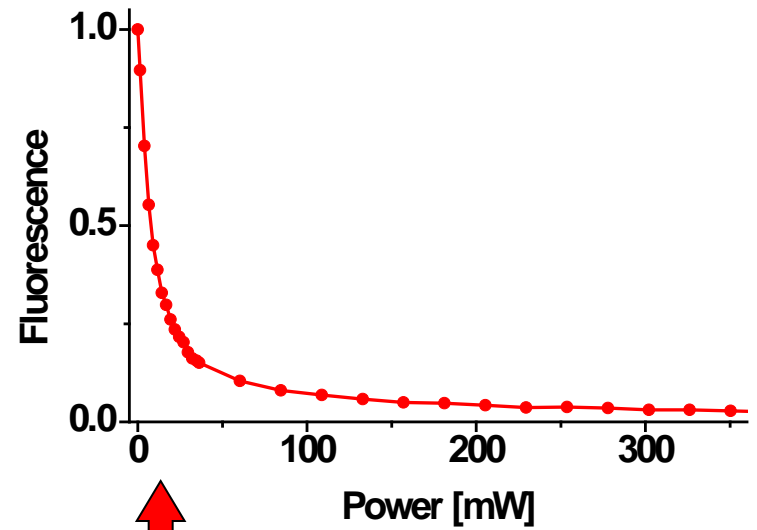
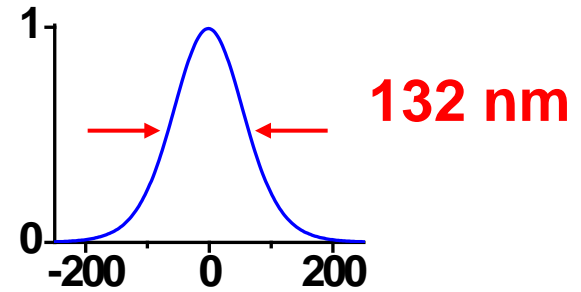
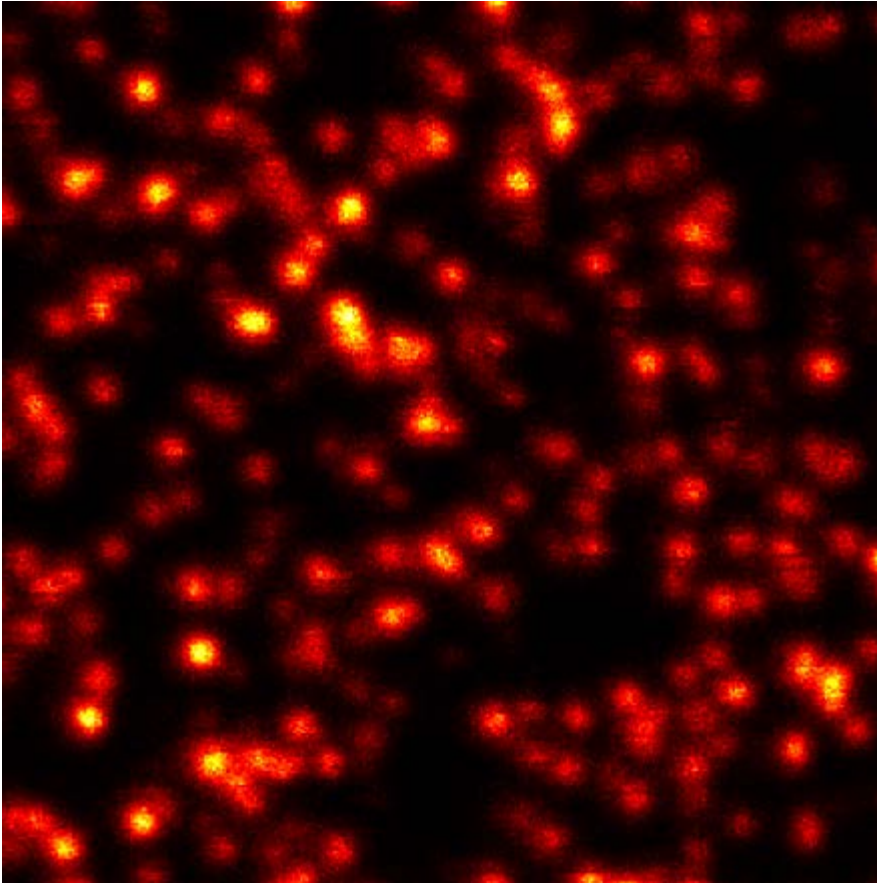


20nm Crimson beads
633nm exc, 90ps, 30kW/cm²
785nm STED 200ps, 76MHz



STED-Microscopy

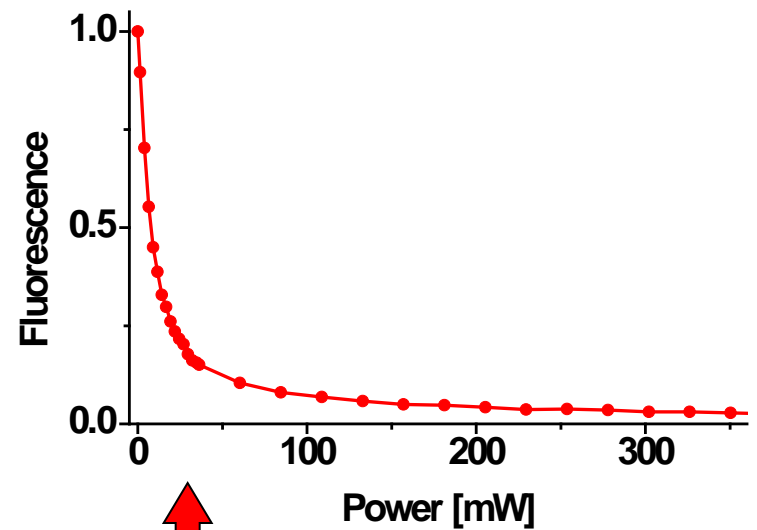
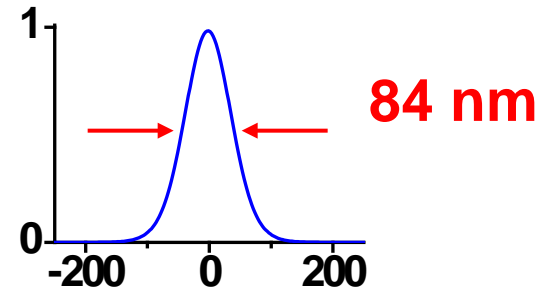
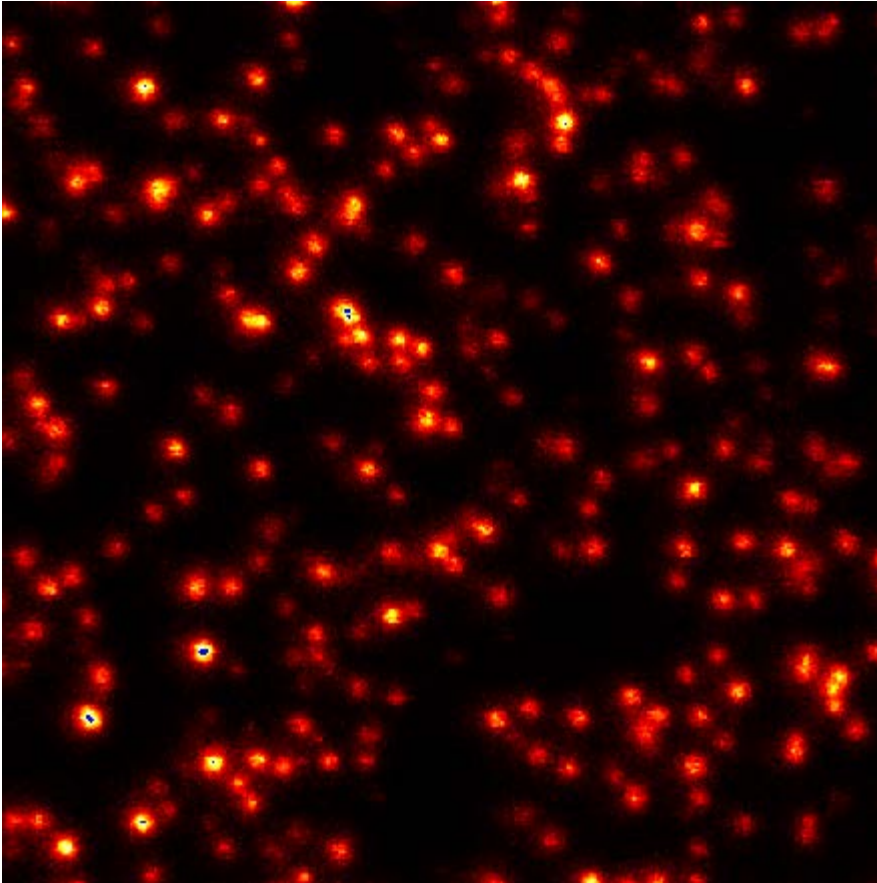
Sub-Diffraction Imaging



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

STED-Microscopy

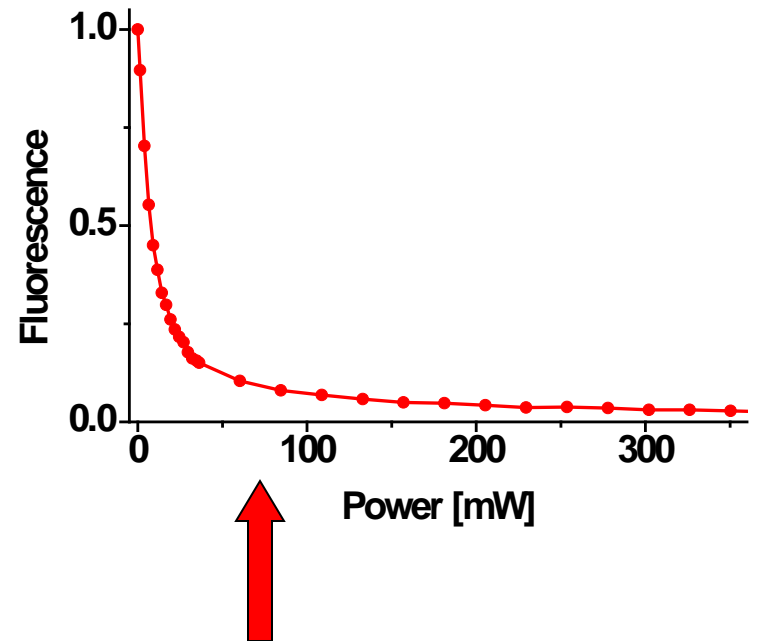
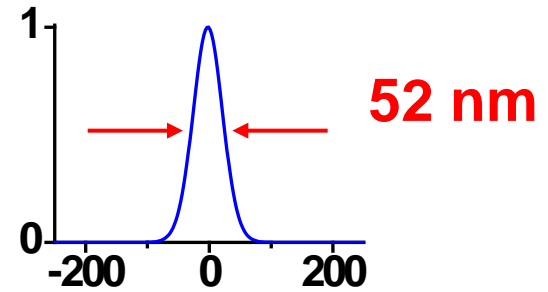
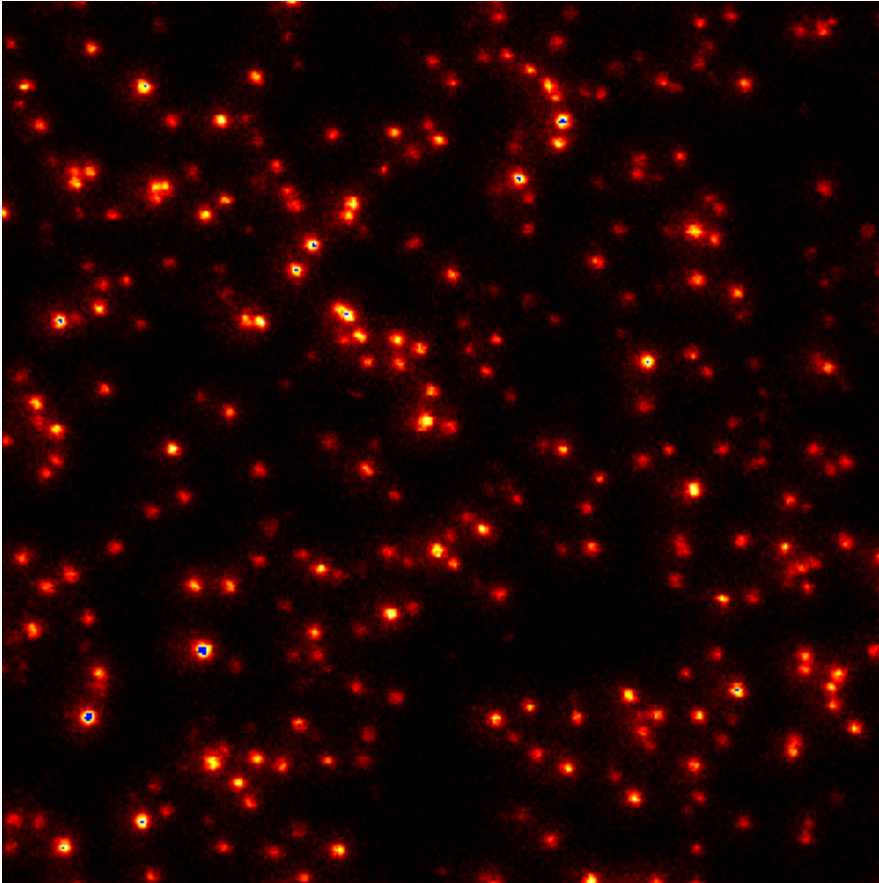
Sub-Diffraction Imaging



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

STED-Microscopy

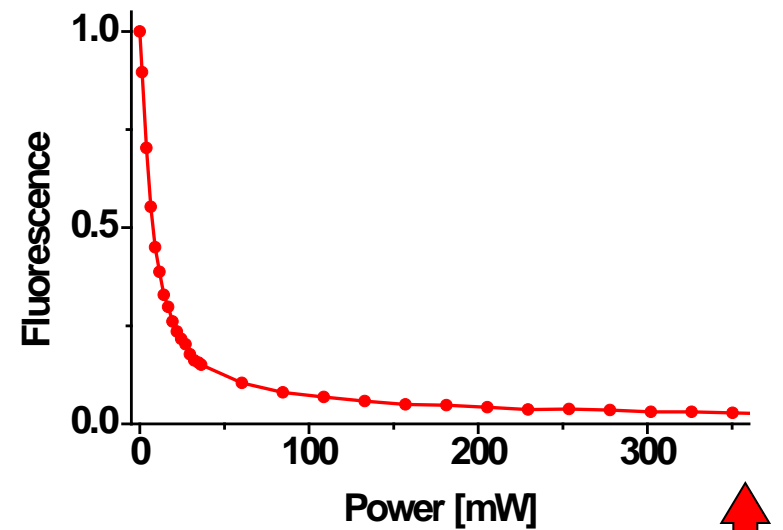
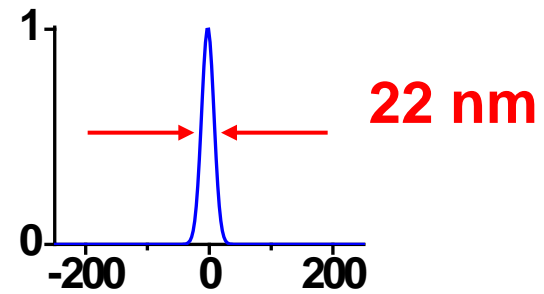
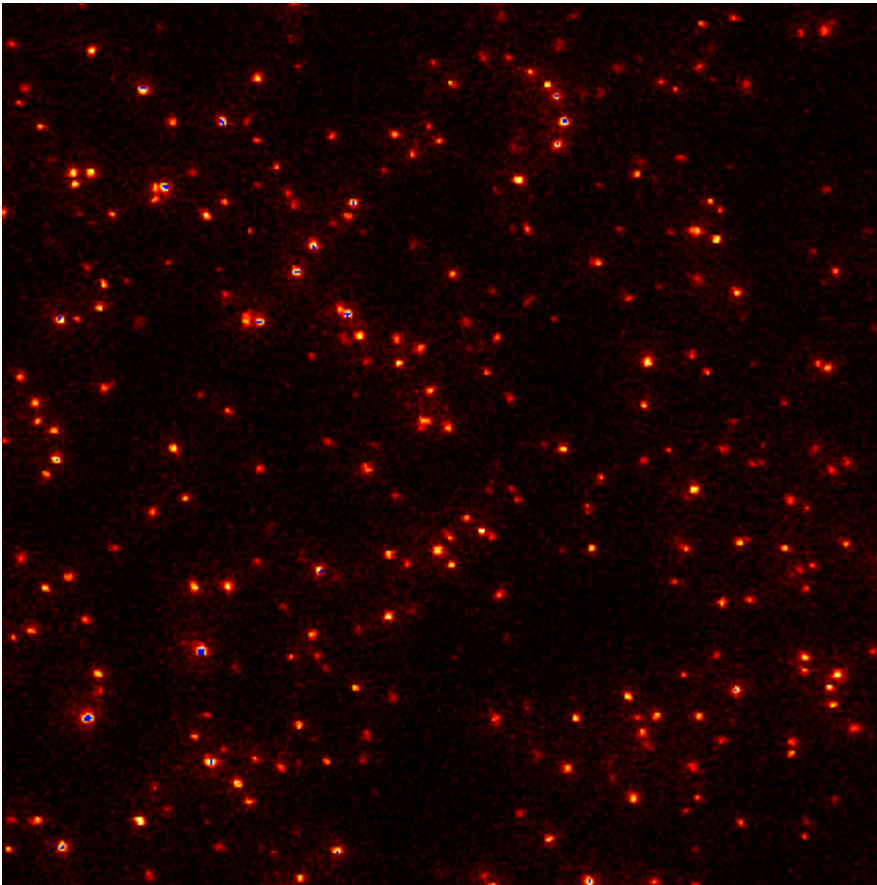
Sub-Diffraction Imaging



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

STED-Microscopy

Sub-Diffraction Imaging

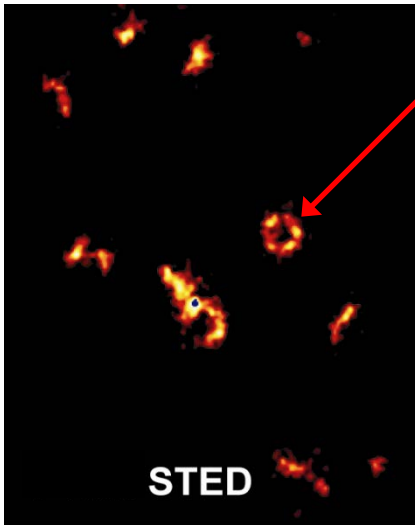
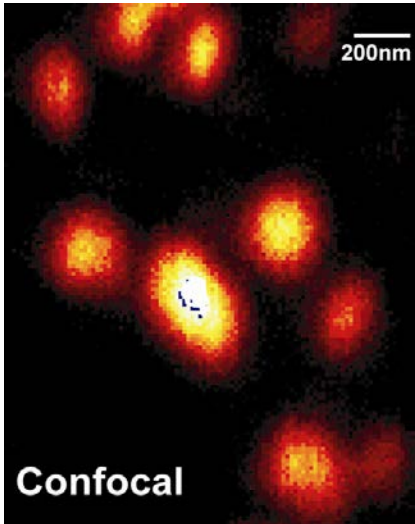


20nm Crimson beads
633nm exc, 90ps, 30kW/cm²
785nm STED 200ps, 76MHz

STED Microscopy

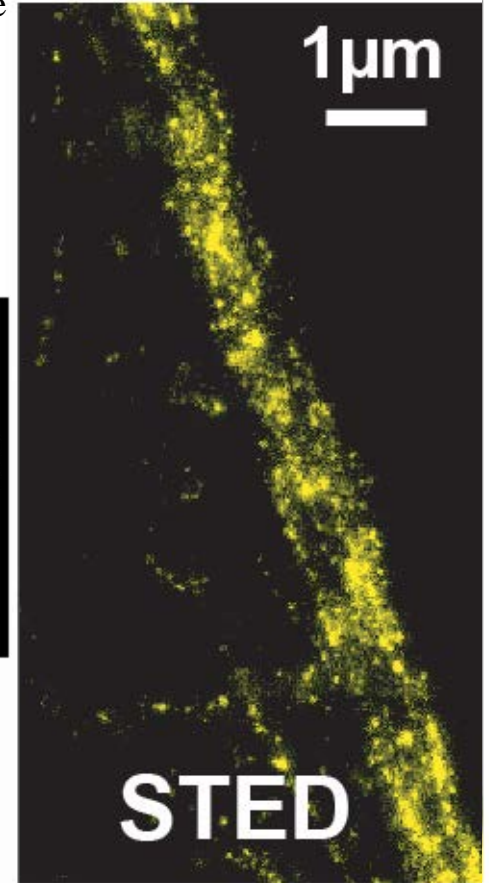
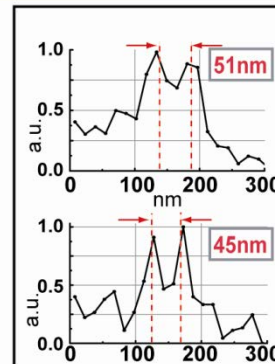
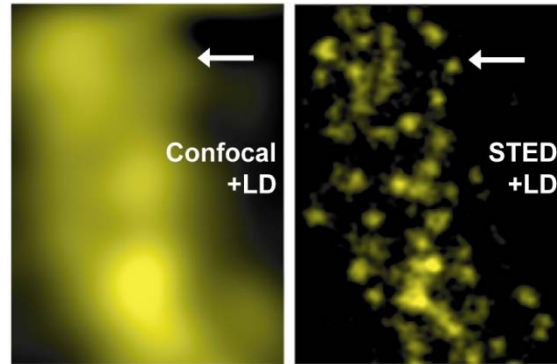
Cellular Imaging

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

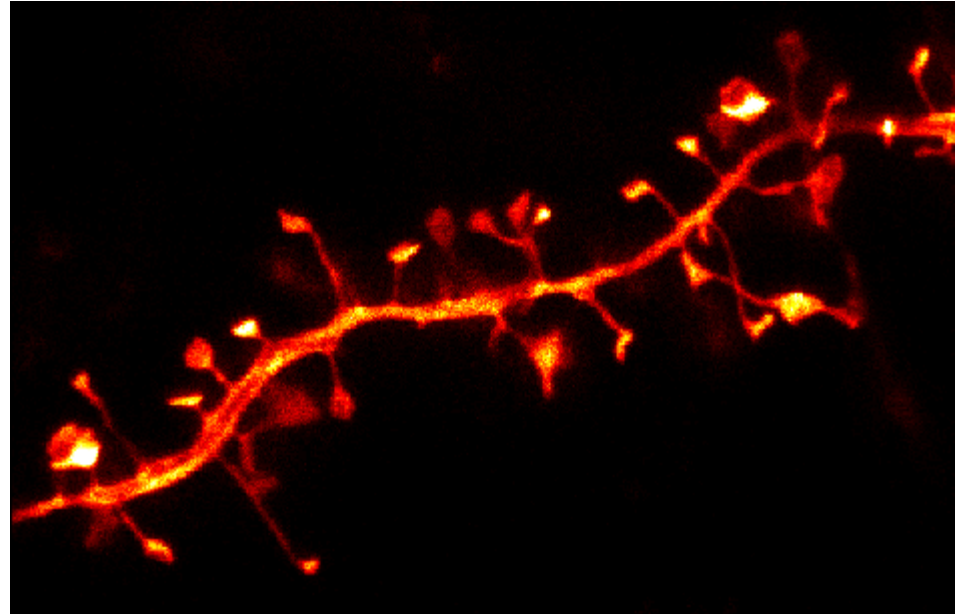
synaptic proteins on endosomes
of PC 12 cells
(neuroendocrine activity;
generate synaptic vesicles)
Atto532-synaptophysin
LD



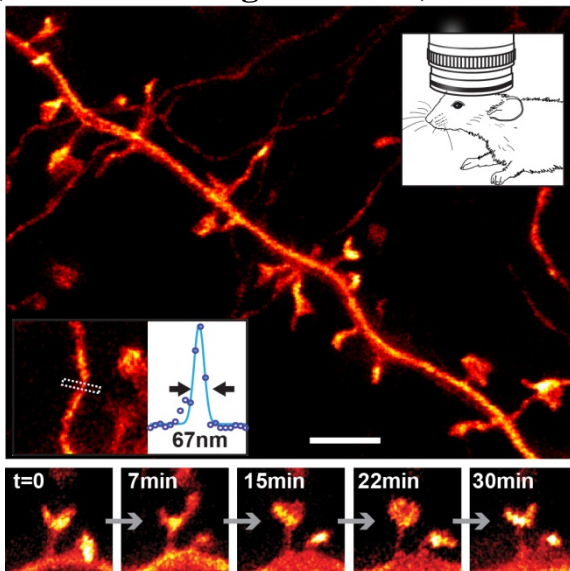
STED Microscopy

Inside Living Cells - Dynamics

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



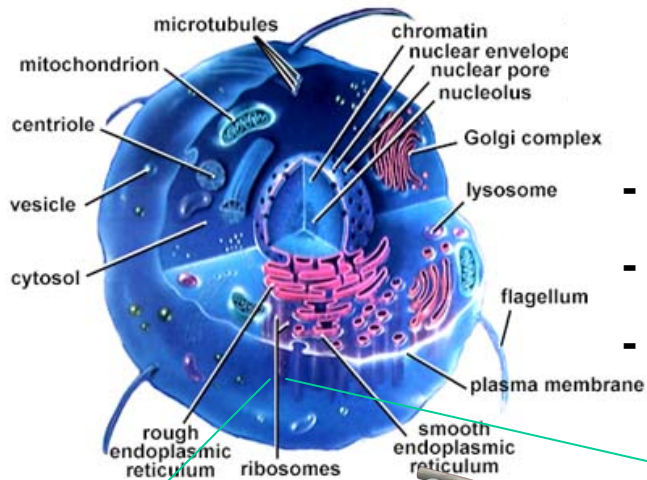
Live Mouse
YFP
(Science Berning et al 2012)



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

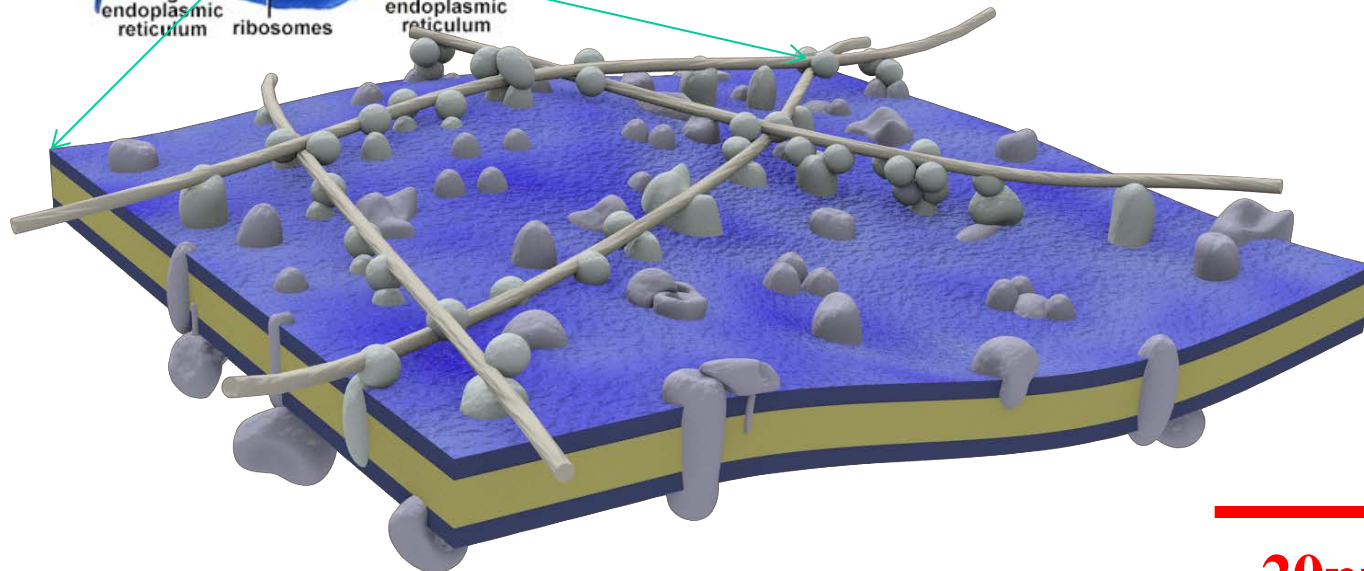
Lipid Plasma Membrane Organization

Nanoscale



Lipid Plasma Membrane Organization:

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



20nm

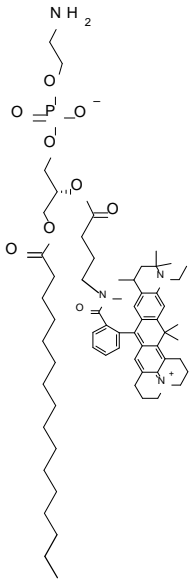
Small spatial
scales!!!!

Lipid Plasma Membrane Organization

Fluorescence Recordings: Lipids

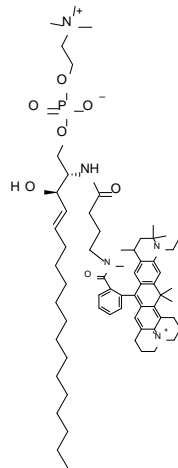
Phosphoglycerolipid:

Atto647N-phosphoethanolamine (PE)



Sphingolipid:

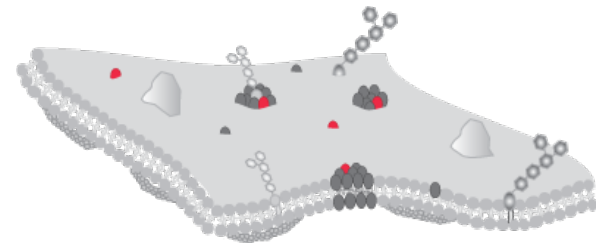
Atto647N-sphingomyelin (SM)



Live PtK2 cells:

physiological conditions

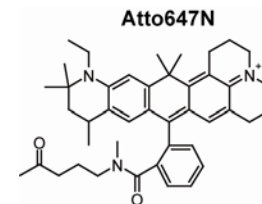
incorporation in plasma membrane



**BSA
complex**

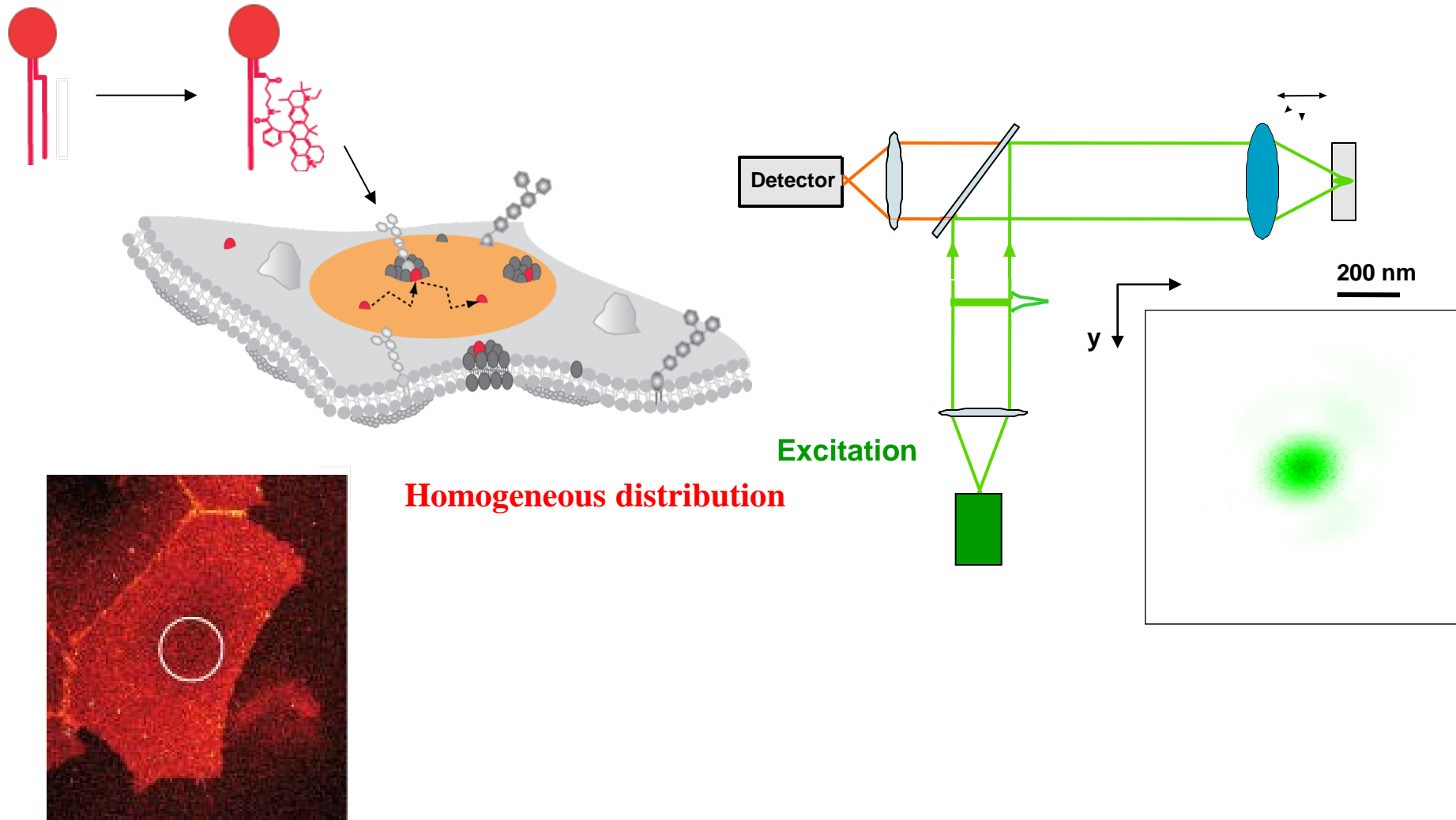


Labeling



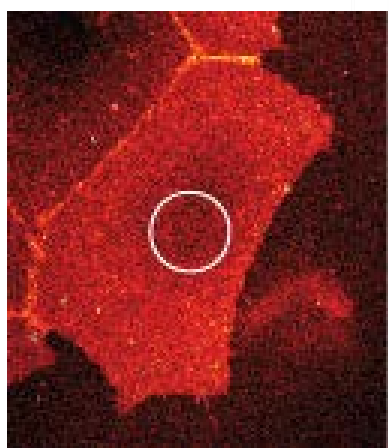
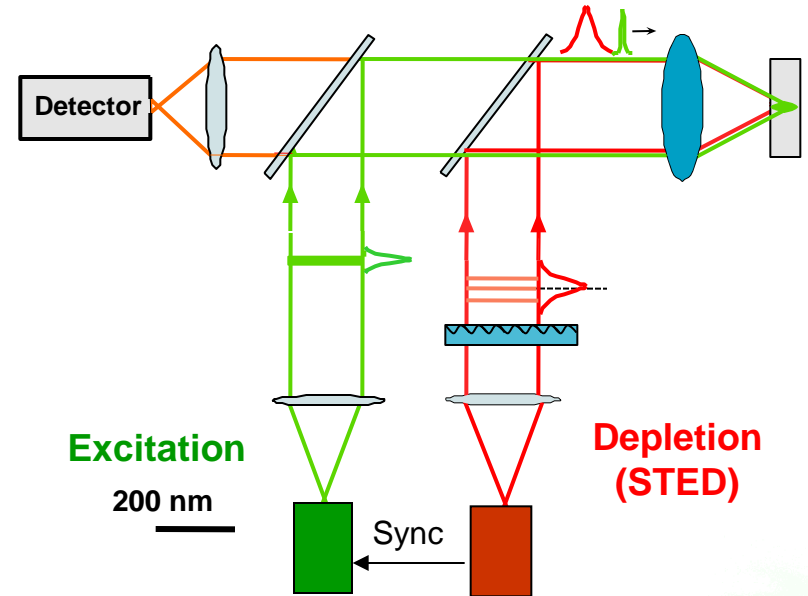
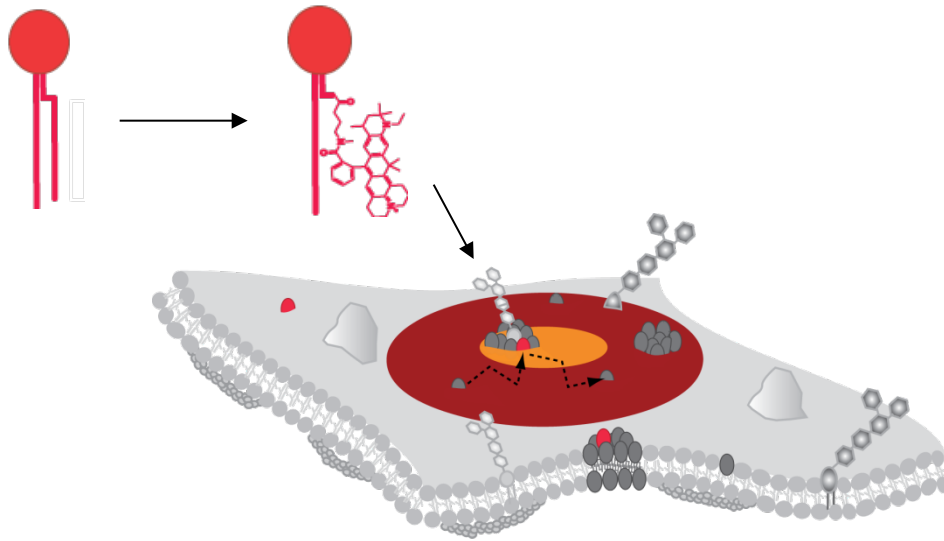
Lipid Plasma Membrane Organization

Confocal Recordings

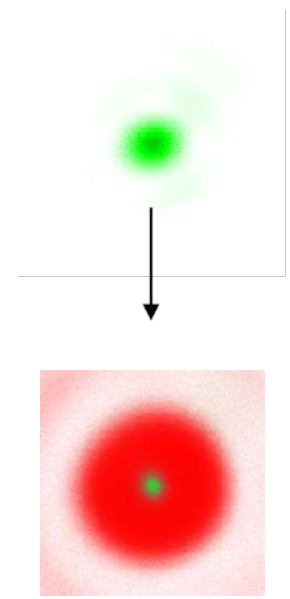


Lipid Plasma Membrane Organization

STED Nanoscopy Measurement

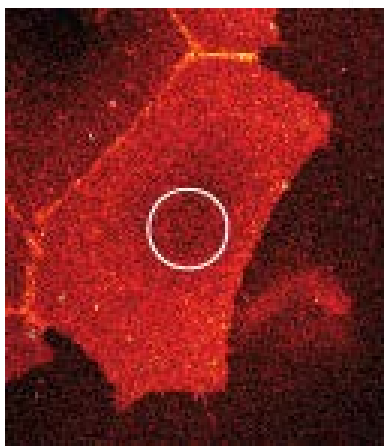
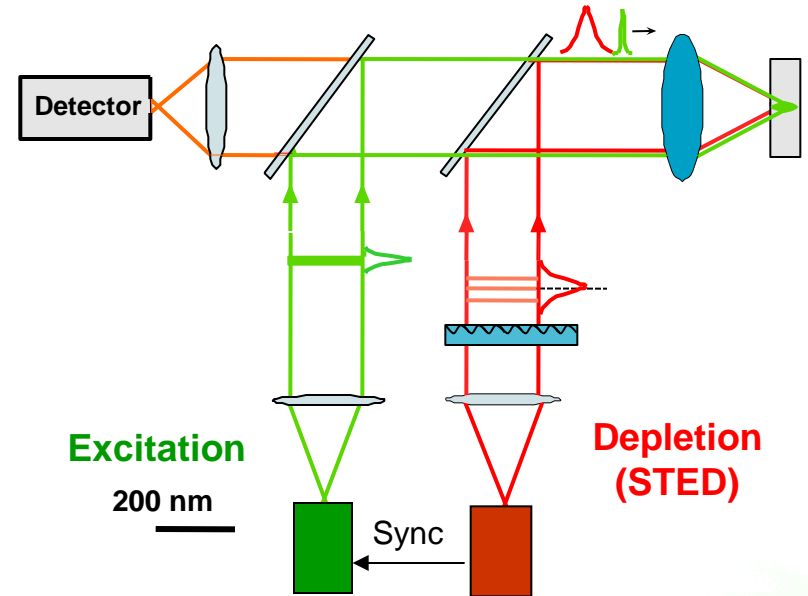
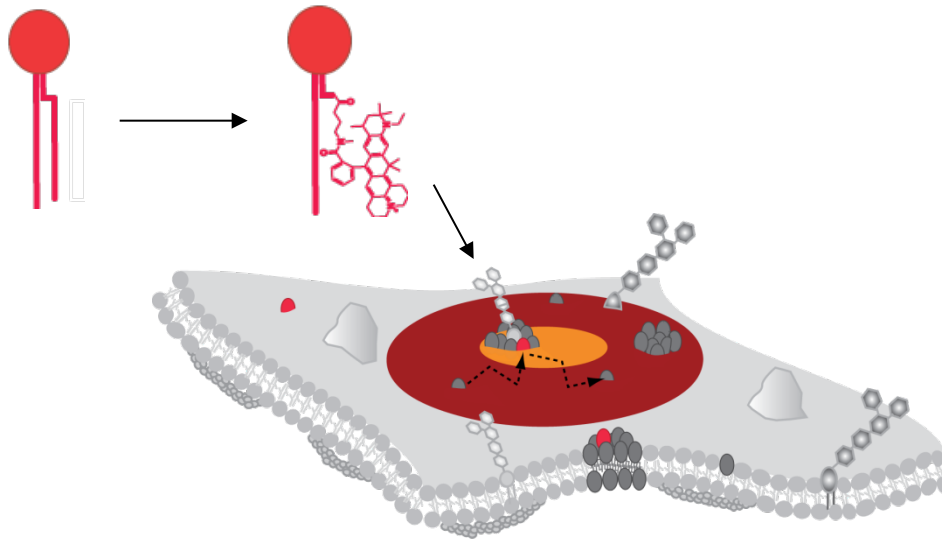


Homogeneous distribution



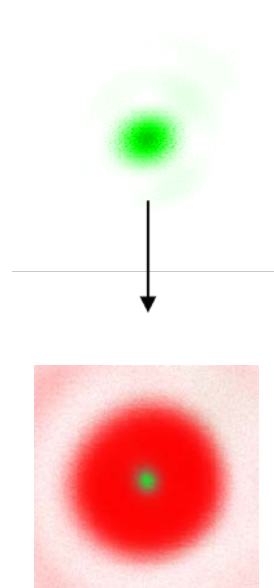
Lipid Plasma Membrane Organization

STED Nanoscopy Measurement



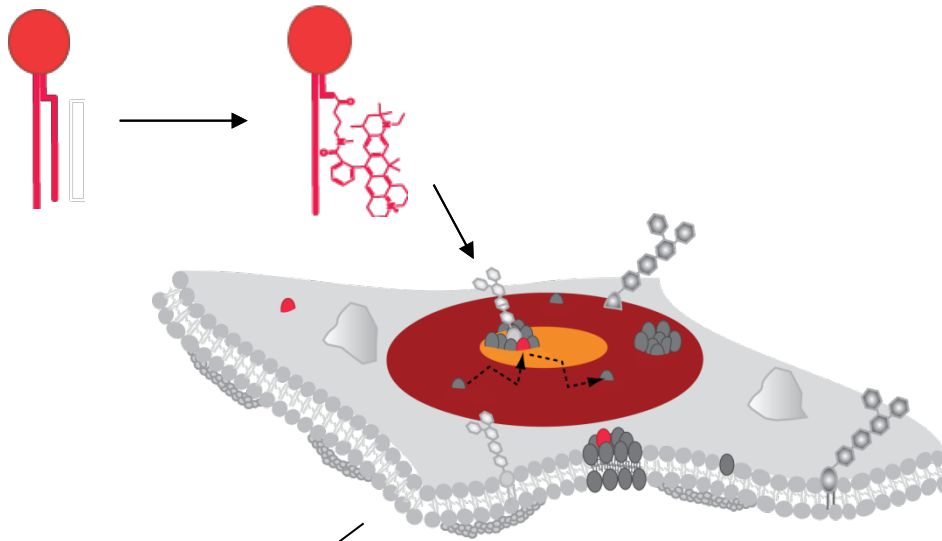
Homogeneous distribution

Fast diffusion → Limited temporal resolution!

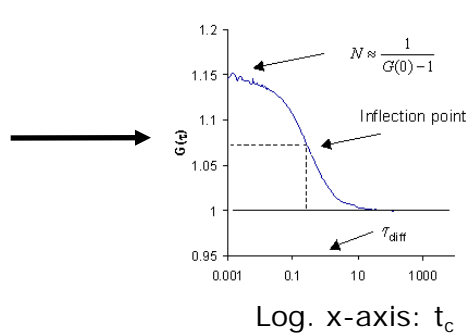
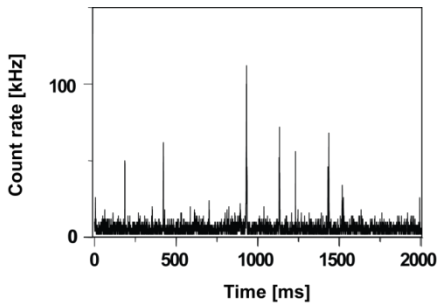
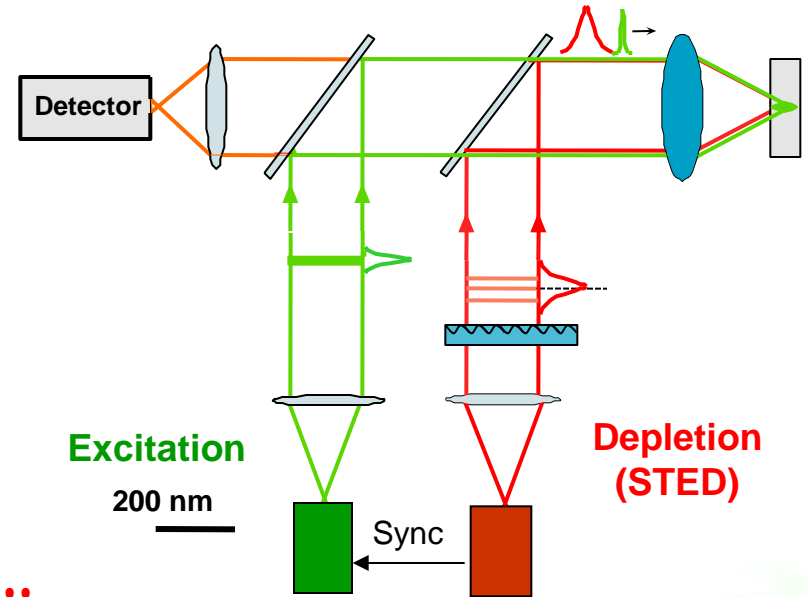


Lipid Plasma Membrane Dynamics

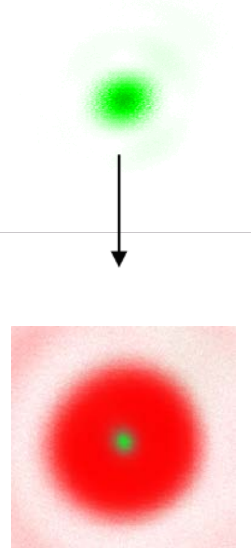
STED Nanoscopy Measurement



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



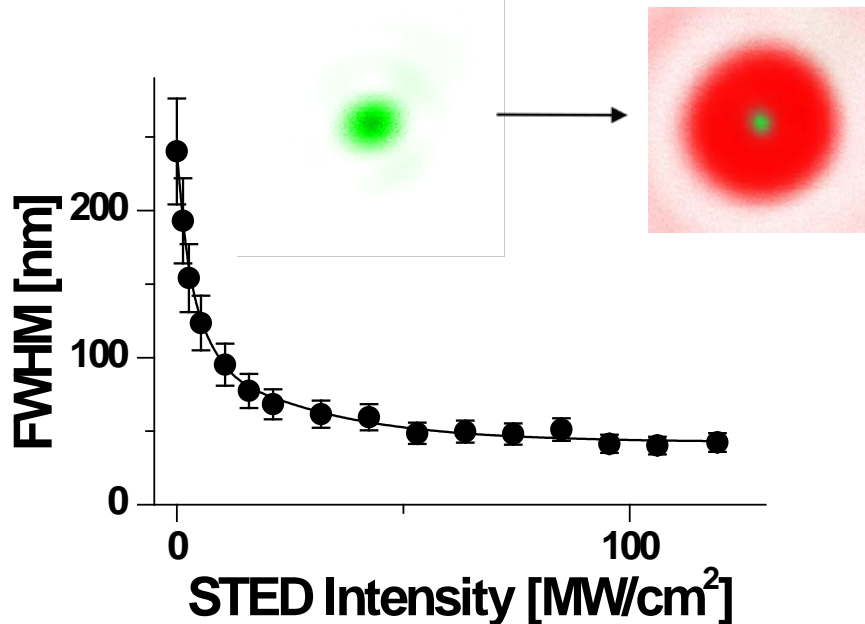
**molecular
diffusion coefficient
=
molecular
mobility**



Live Cell Nanoscopy

STED-FCS

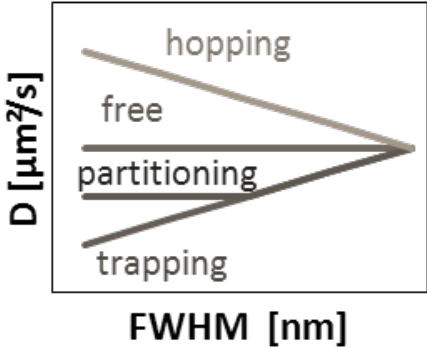
STED-Microscopy: Tuning of observation area



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

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

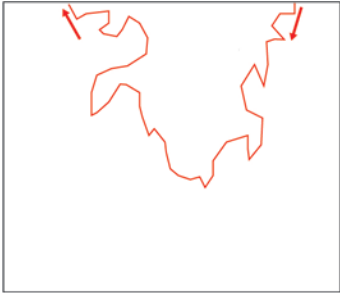
Dependencies: $D(\text{diameter})$
 $240\text{nm} \rightarrow 30/40\text{nm}$
Varies for different diffusion modes



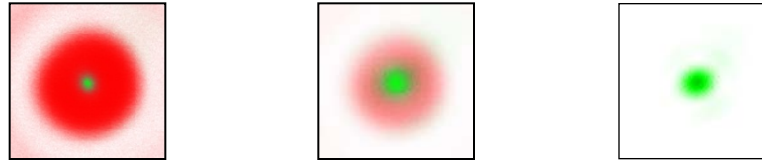
Live Cell Nanoscopy

STED-FCS - Diffusion Models

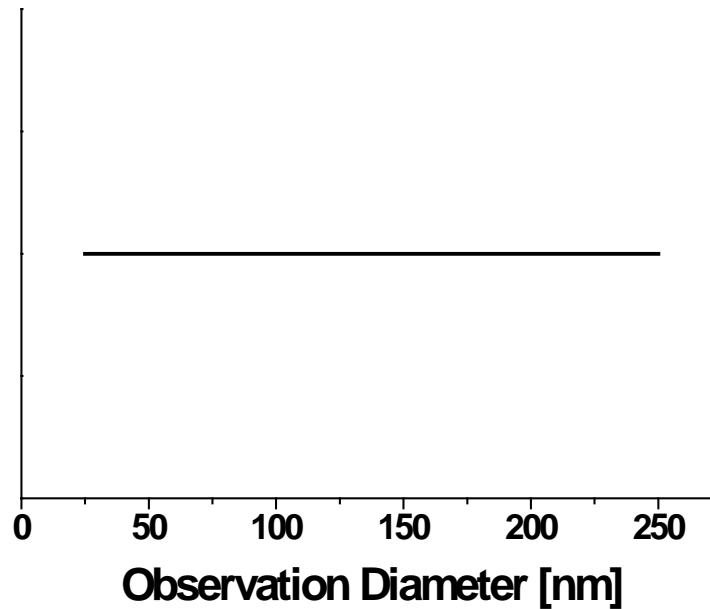
Free diffusion



← **STED Intensity**



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



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:

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

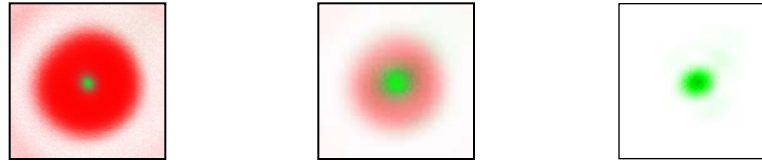
Live Cell Nanoscopy

STED-FCS - Diffusion Models

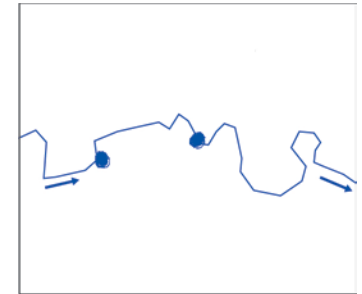
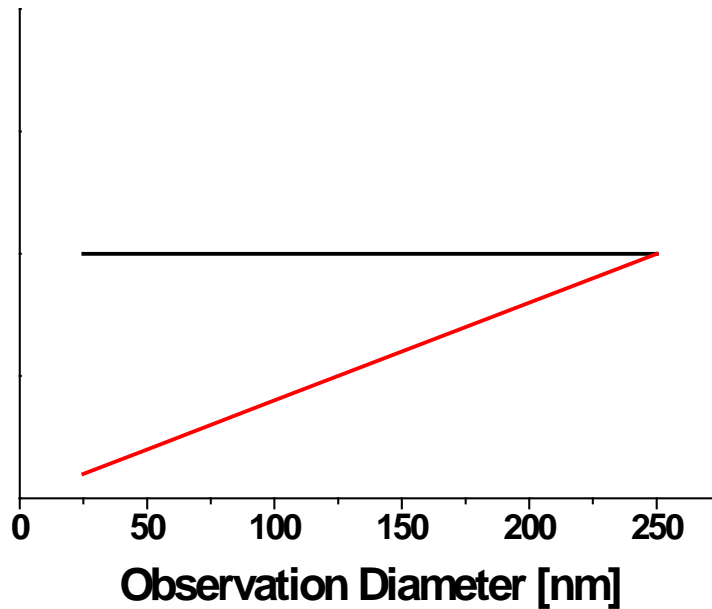
Free diffusion



← **STED Intensity**



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

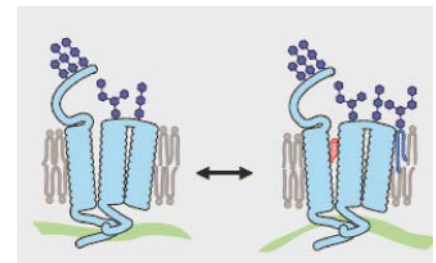


Trapping

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:

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



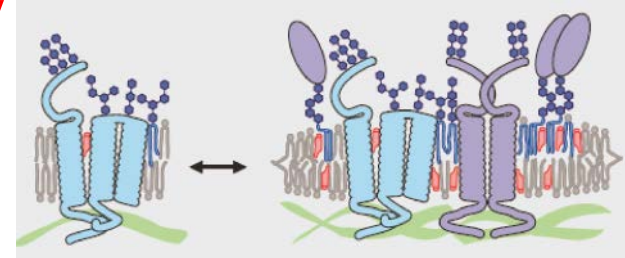
Live Cell Nanoscopy

STED-FCS - Diffusion Models

Free diffusion

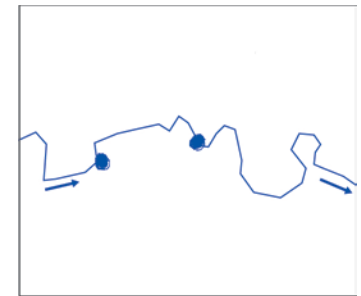
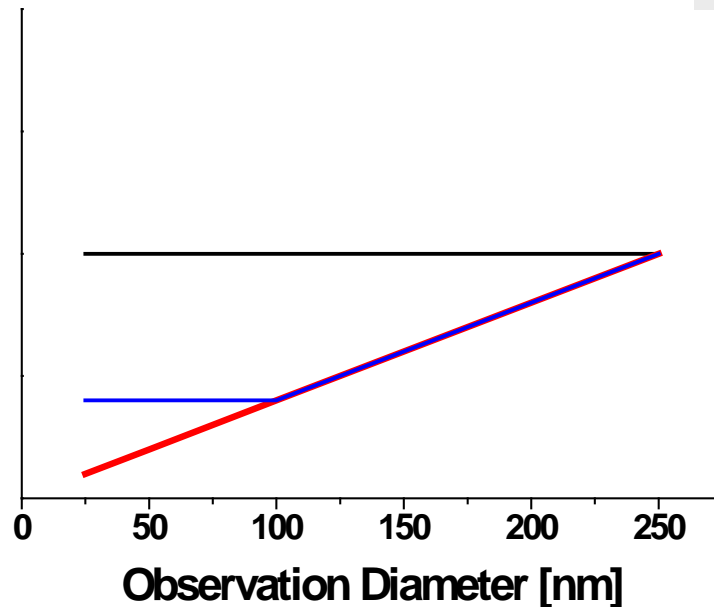


← STED Intensity

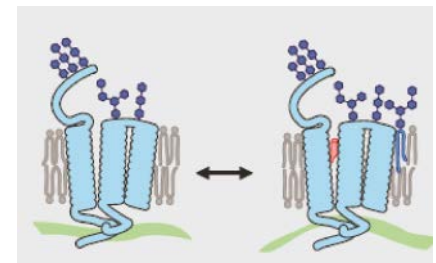


Domain incorporation

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



Trapping



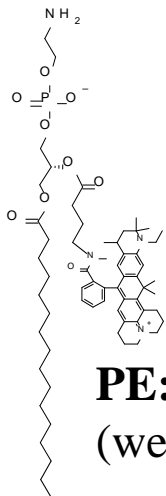
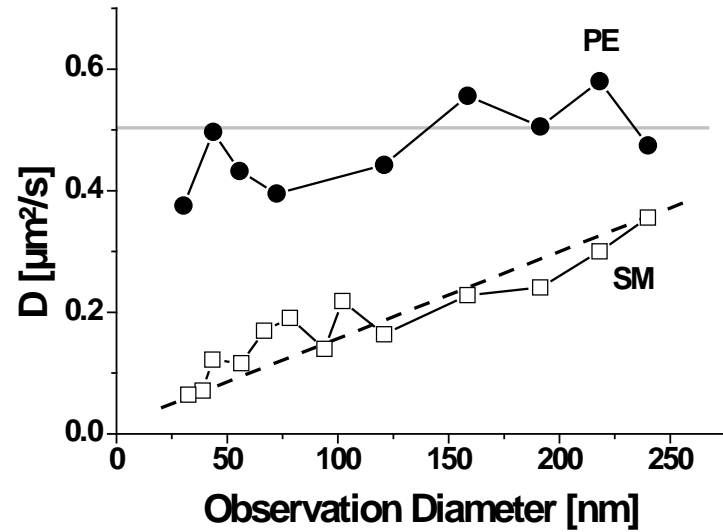
Wawrezynieck et al. *Biophys J.*
2005 December; 89(6)
Eggeling et al. *Nature* 457,
1159-1162, 2009
Mueller et al. *Biophys J* 2011

Apparent diffusion coefficient:

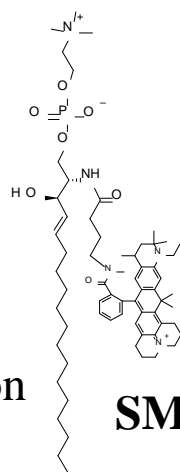
$D \sim \text{area} / \text{transit time}$

STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM

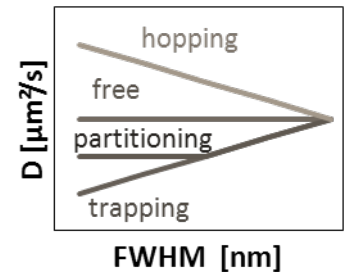
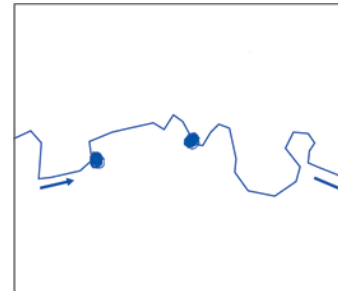


PE: free diffusion
(weak trapping)



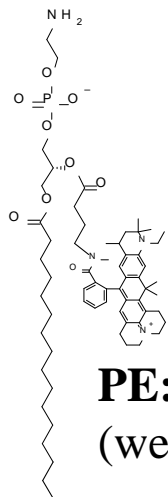
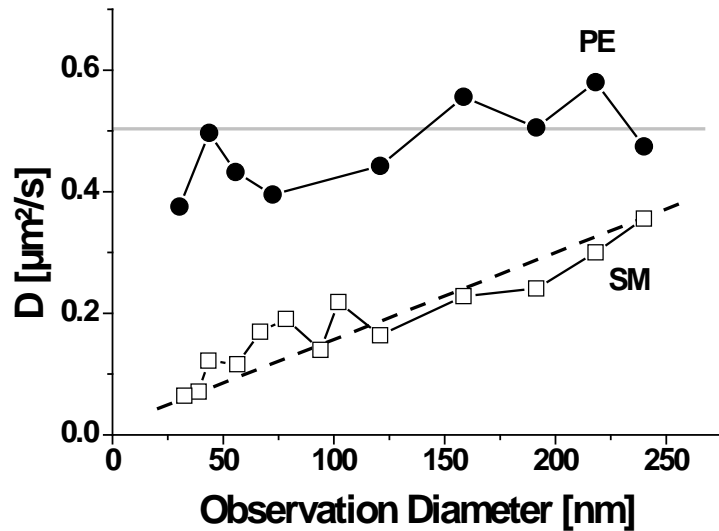
SM: trapping

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

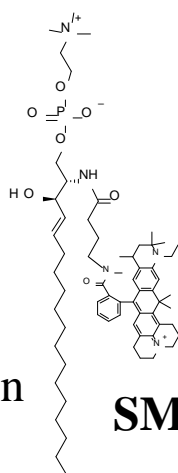


STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM



PE: free diffusion
(weak trapping)



SM: trapping

→ **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

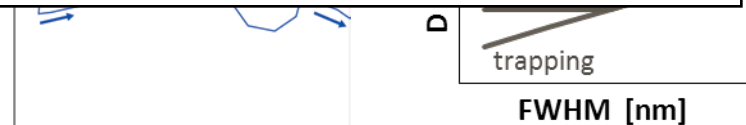
(Latrunculin/Jasplakinolide/Nocodazole...)

Slight dependence on endogenous SM level

(Myriocin)

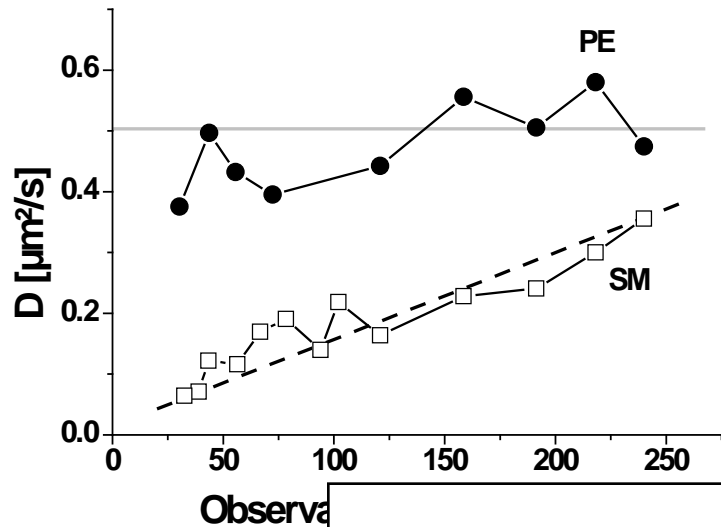
Dependence on lipid structure

(but not dye)



STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM



→ **Complex on molecular scale**

(proteins, lipid-shells, ...)

~10 ms, no movement during trapping

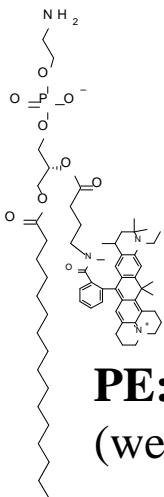
Cholesterol-assisted

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

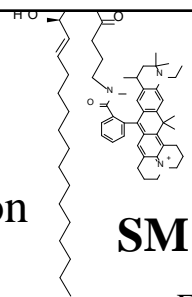
STED-FCS:
New approach to study molecular interactions!

cytoskeleton
 (Nocodazole...)

SM level

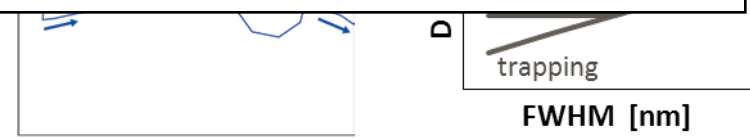


PE: free diffusion
 (weak trapping)



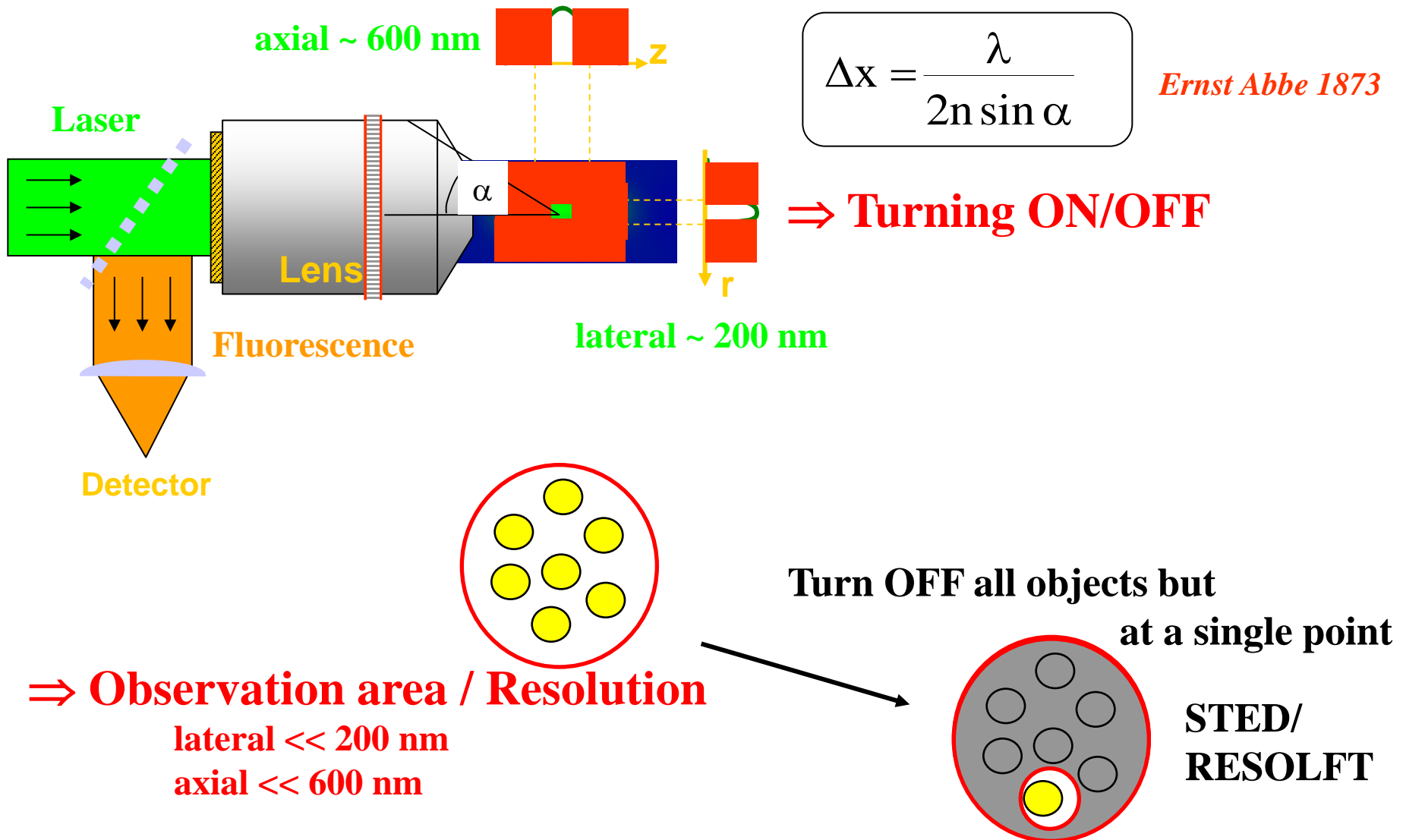
SM: trapping

Dependence on lipid structure
 (but not dye)



Far-Field Microscopy

Surpassing the Resolution Limit: Turning ON/OFF

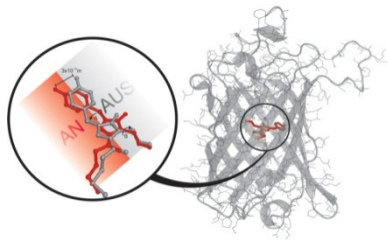


Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins

Photoswitchable Proteins

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

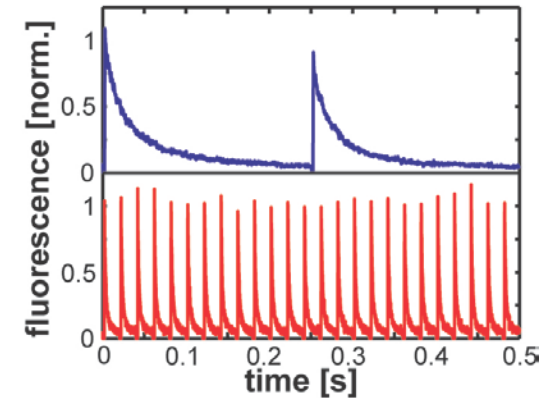


Photoisomerisation
cis-trans conformational states
dark (trans)- bright (cis)
Andresen et al. (2005) *PNAS*

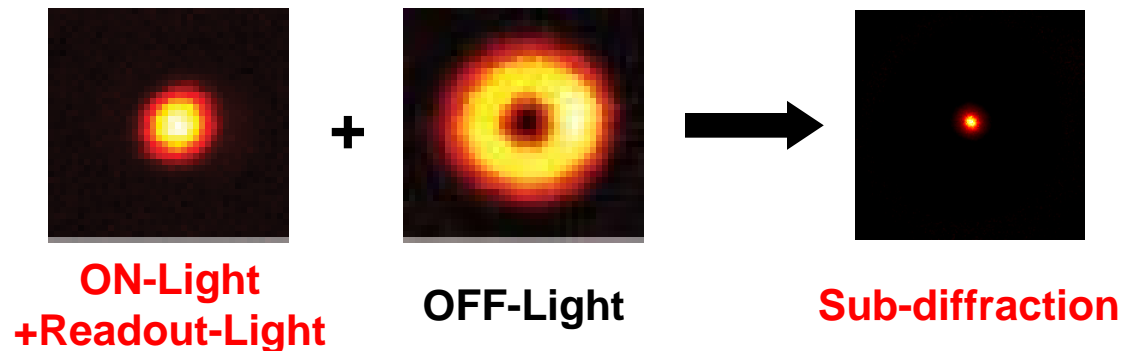
Dronpa \rightarrow **rsFastLime**
Stiel et al, *Biochem J* 2007

GFP \rightarrow **photoswitching (rsEGFP)**
Grotjohann et al, *Nature* 2011

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



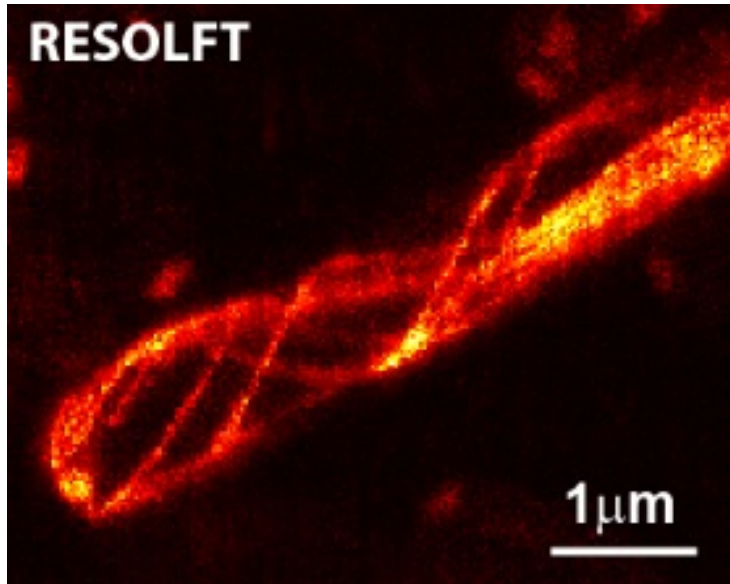
ON/OFF at low CW powers
nW - μ W (\sim kW/cm²)



RESOLFT = Reversible Saturable Optical Fluorescence Transition

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

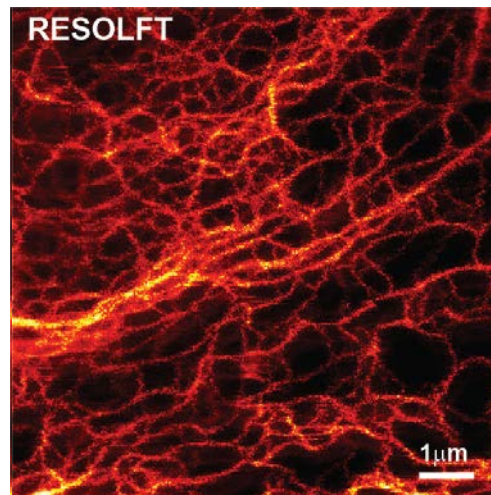
Brakemann et al, Nature Biotechnol. 2011

Switch-on: 405 nm

Switch-off: 355 nm

Read-out: 488 nm

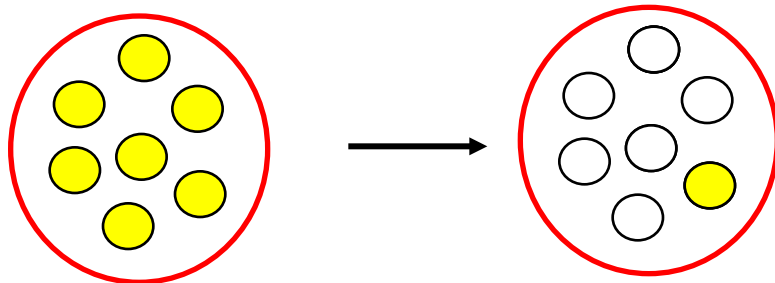
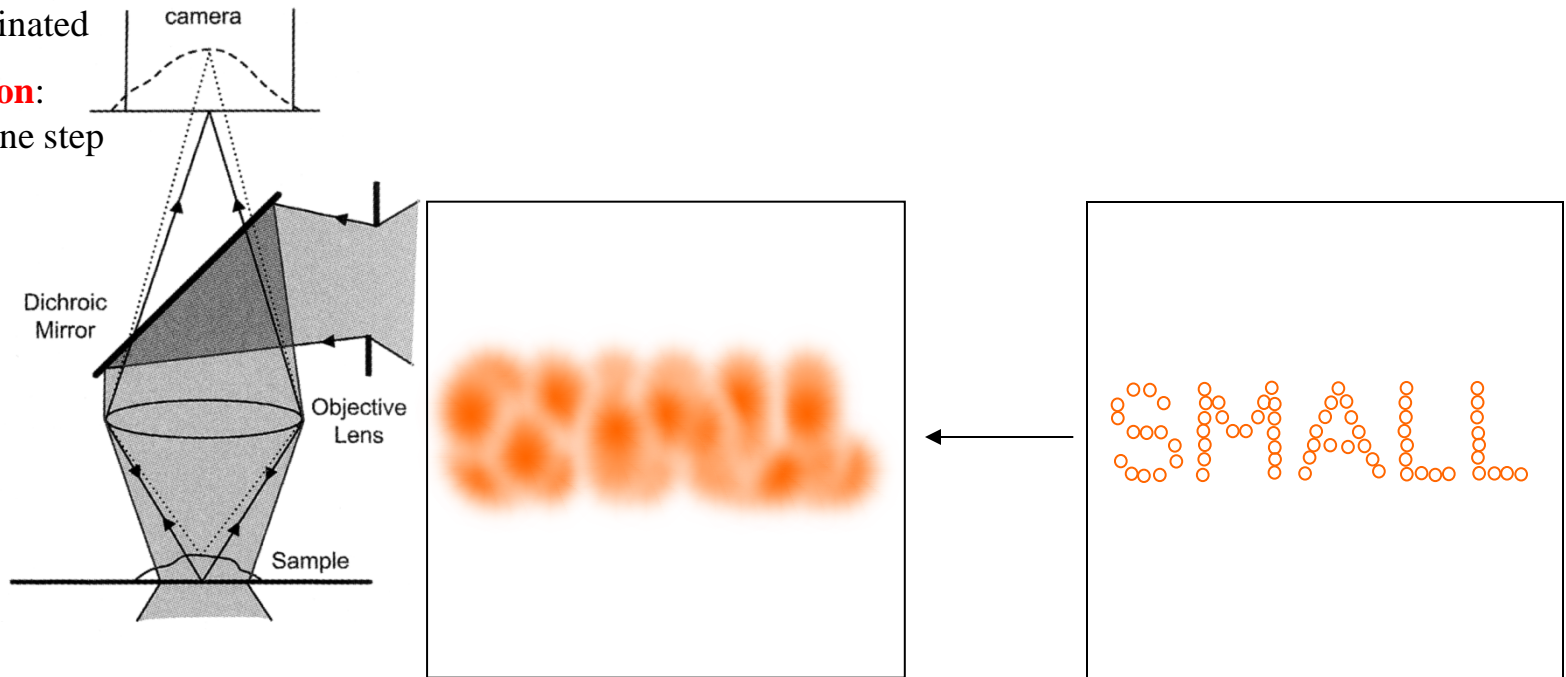
Keratin19-Dreiklang
expressed
in living PtK2 cells



(d)STORM/(f)PALM Far-Field Nanoscopy

Resolution Limit – Camera Detection

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

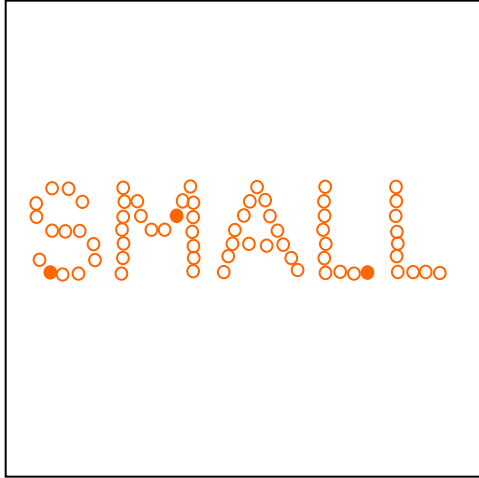


**Turn OFF all but
a single object**

**(d)STORM/
(f)PALM**

Far-Field Nanoscopy

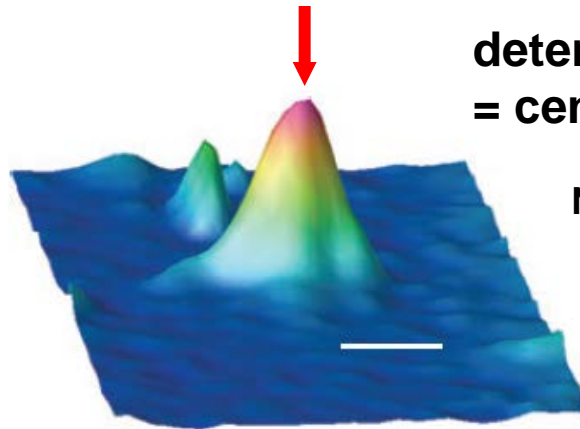
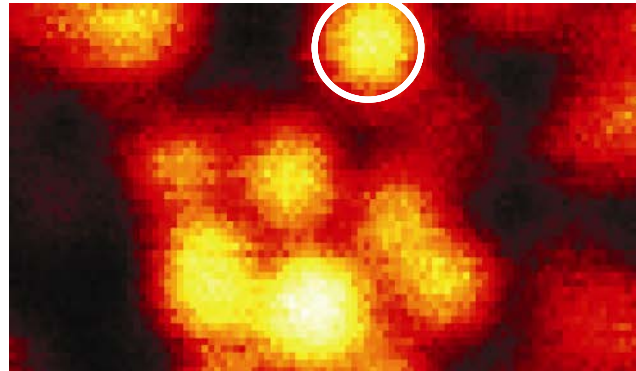
ON/OFF: Single-Molecule



Localization of single molecules

- accuracy down to the molecular scale

Diffraction-limited



determination of exact position
= center of diffr.-lim. peak

N_{photons} = number of photons detected
per single molecule

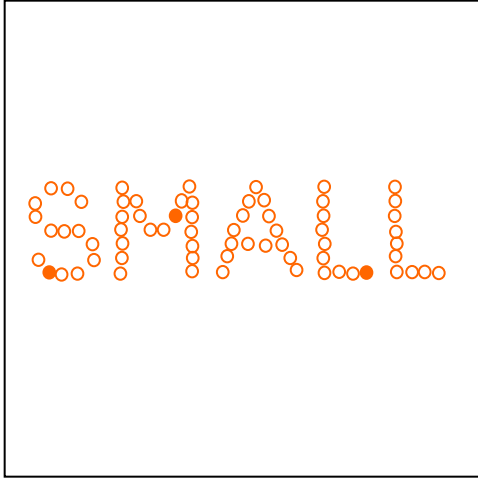
$$\Delta x = \Delta x_{\text{diffr.}} N_{\text{photons}}^{0.5}$$

1000 photons: ~ 10nm

Moerner, Nat. Meth. 2006

Far-Field Nanoscopy

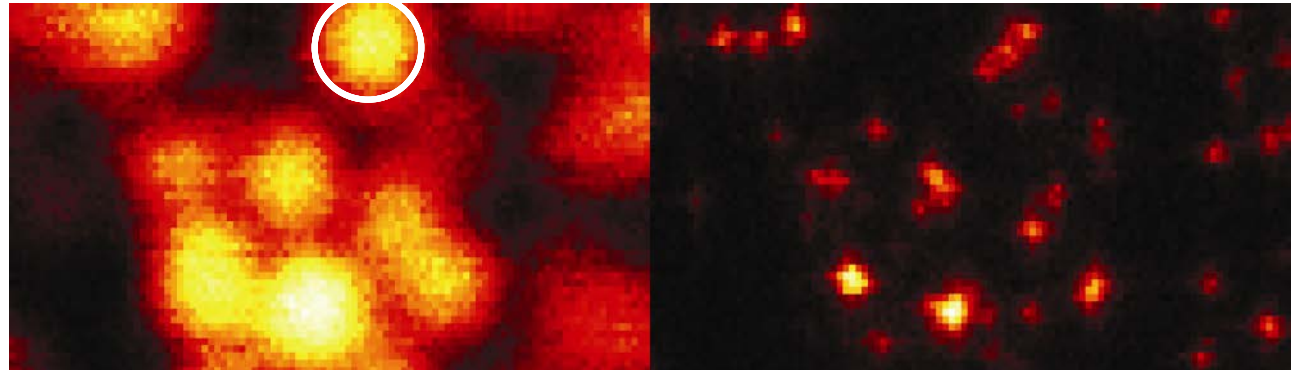
ON/OFF: Single-Molecule



Localization of single molecules

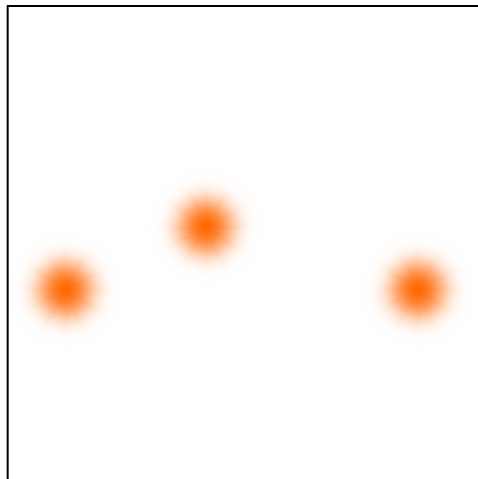
- accuracy down to the molecular scale

Diffraction-limited



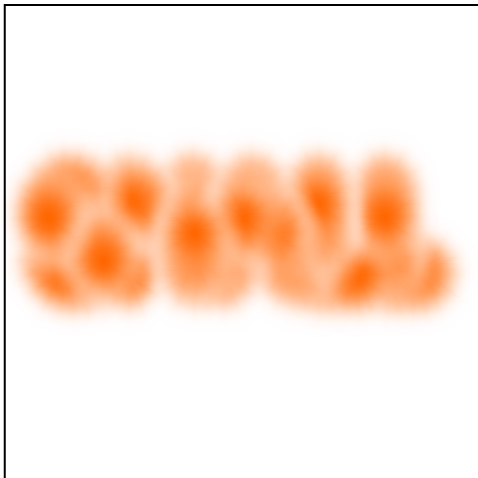
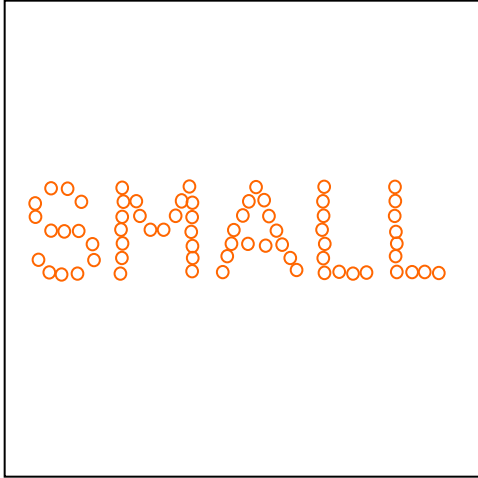
⇒ really single molecule?

(localization not resolution)

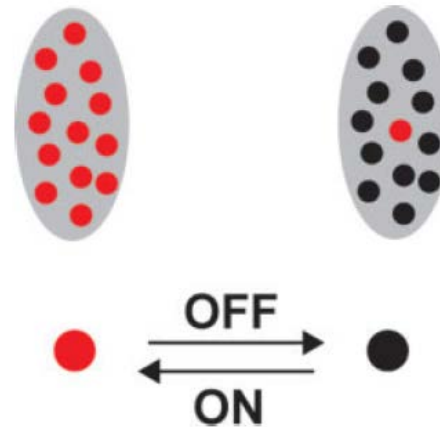


Far-Field Nanoscopy

Single-Molecule Switching



**Downscale ensemble to
single isolated molecules**



⇒ **Single molecules separated by more
than the diffraction limit**

⇒ **Localization of real single molecule**

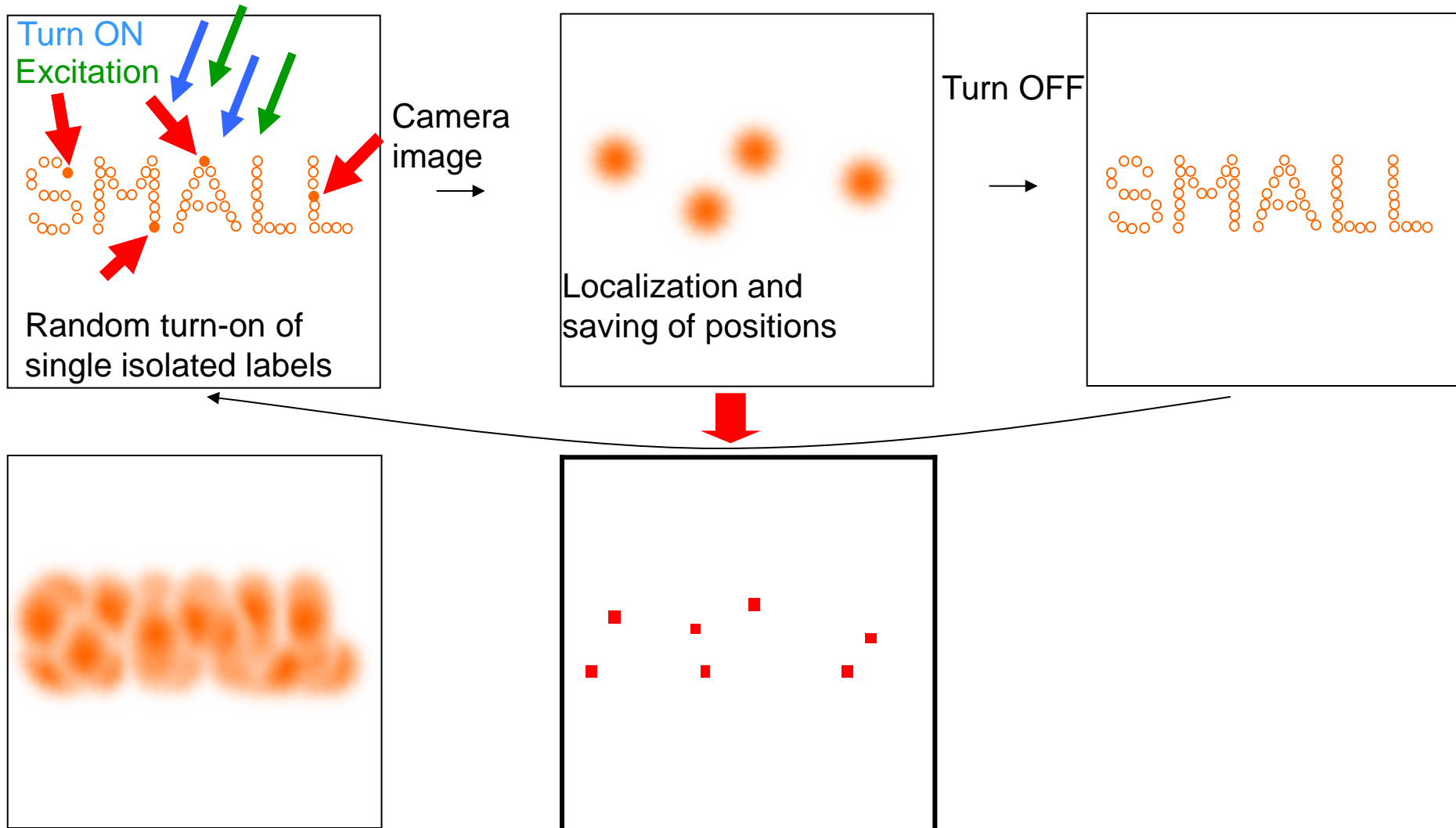
Far-Field Nanoscopy

Single-Molecule Switching + Localization



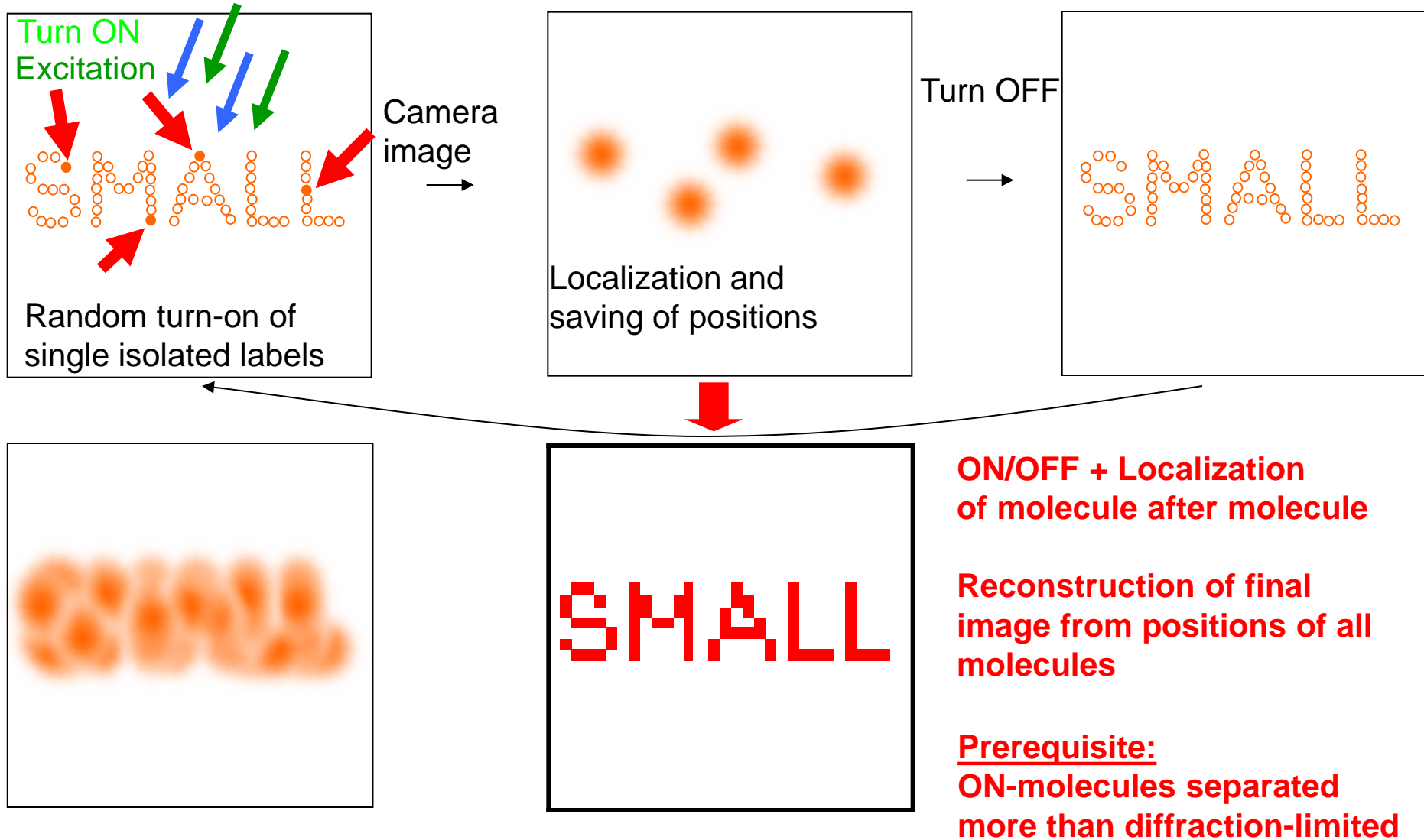
Far-Field Nanoscopy

Single-Molecule Switching + Localization



Far-Field Nanoscopy

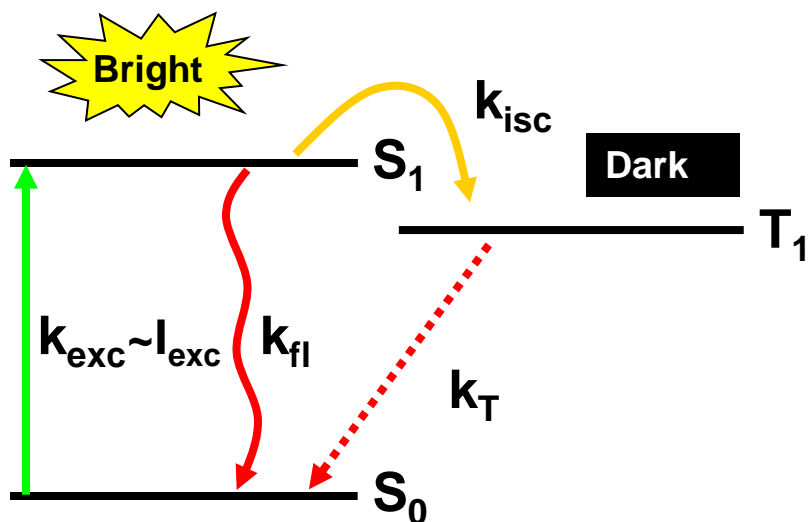
Single-Molecule Switching + Localization



Far-Field Nanoscopy

*GSDIM – Ground State Depletion microscopy
followed by Individual Molecule return*

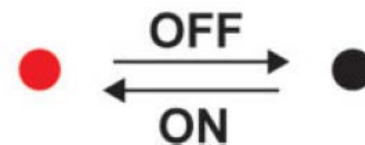
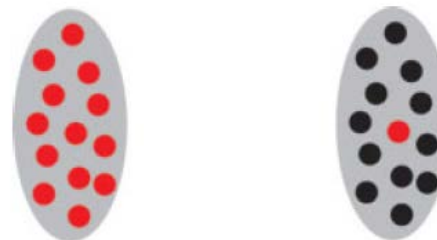
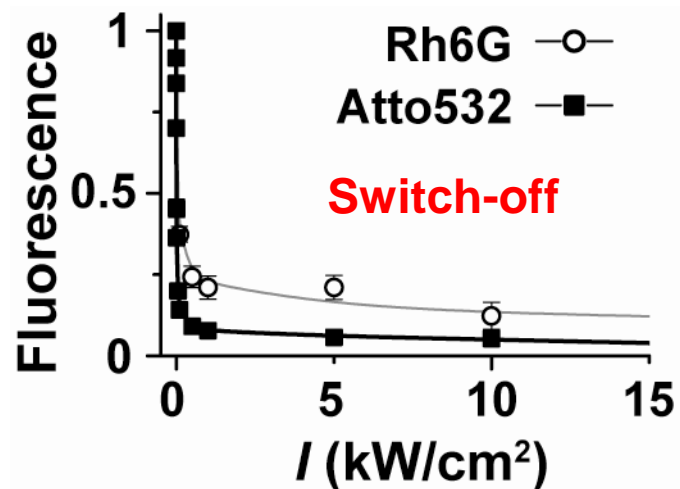
Turn OFF:
Shelve into long-lived dark triplet state



Conventional fluorophores have dark state!!

Downscale ensemble
to isolated
single- molecule

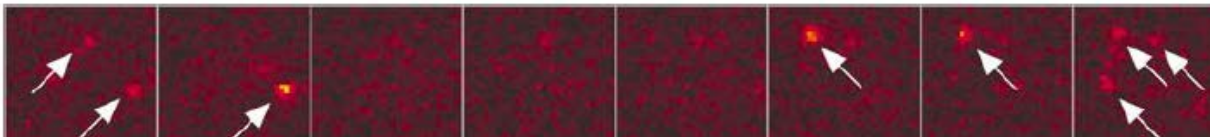
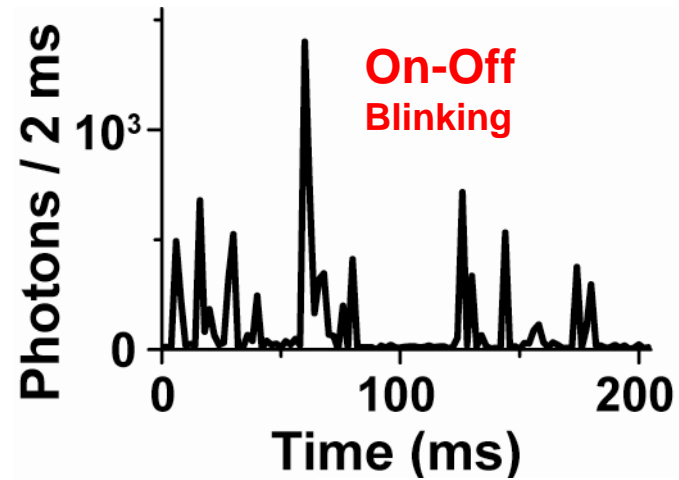
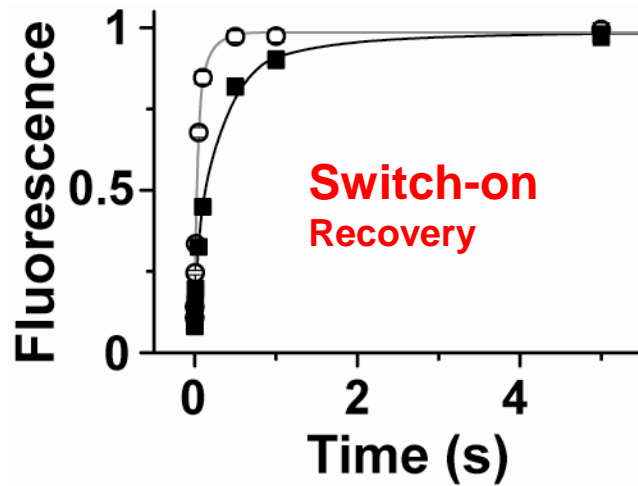
Rh6G, Atto532 in PVA



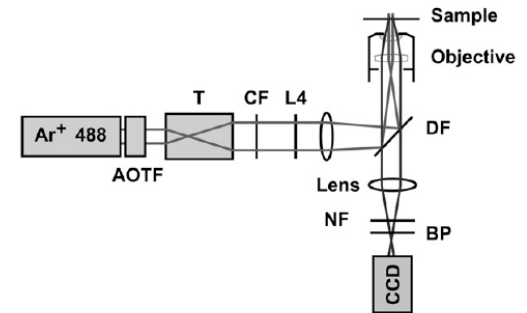
Far-Field Nanoscopy

GSDIM

Reversible

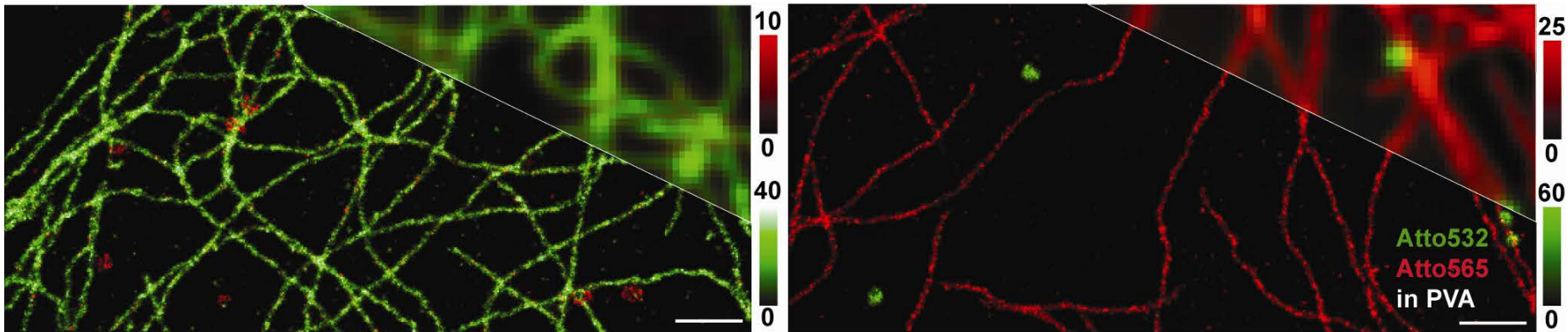


$\Delta x < 20\text{nm}$ ($N \sim 1000$)



Far-Field Nanoscopy

GSDIM

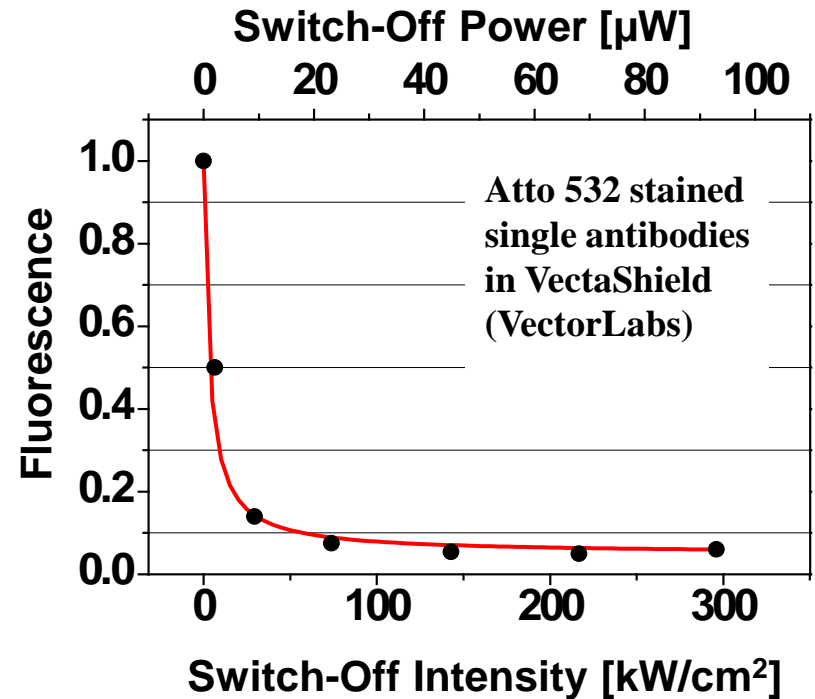
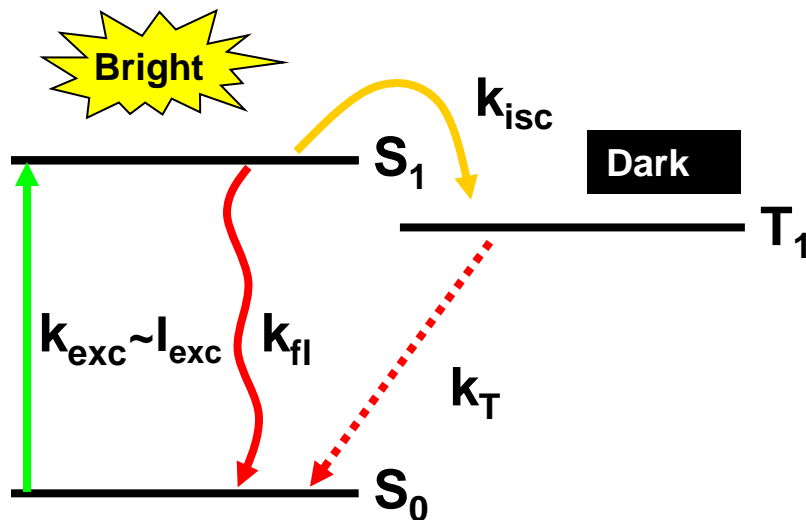


Microtubulin-Peroxisomes, Fölling et al, Nature Methods 2008

Far-Field Nanoscopy

ON/OFF via Triplet/Dark States

GSD (Ground State Depletion)



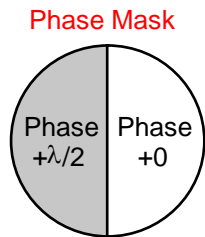
Turn-off fluorescence by pumping into a long-living dark (triplet) state

Low CW powers (μW – kW/cm^2)

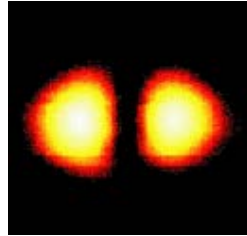
GSD-Microscopy

Far-Field Nanoscopy using the triplet state

Atto 532 stained microtubuli



Switch-off PSF

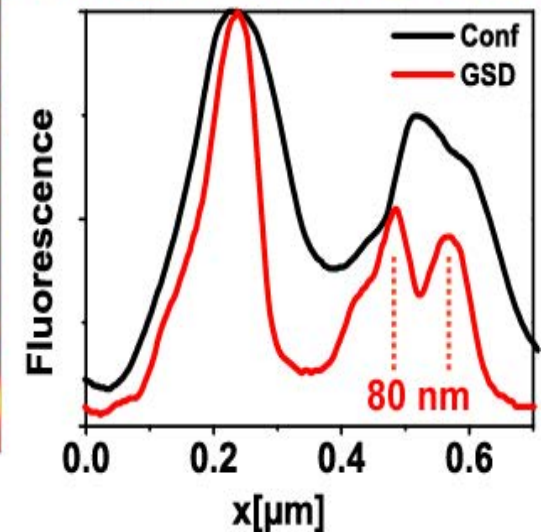
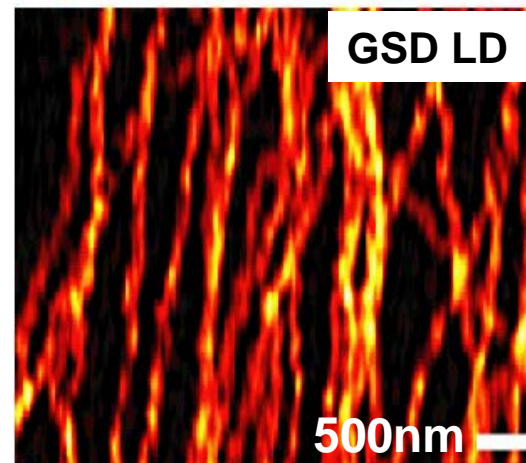
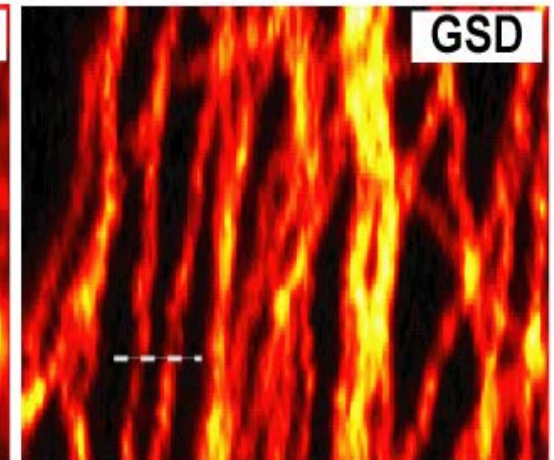
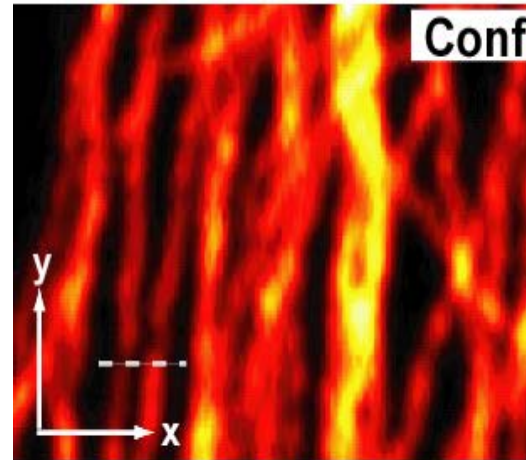
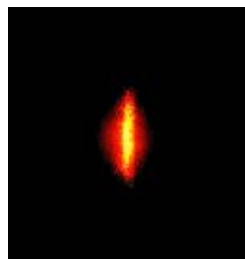


+



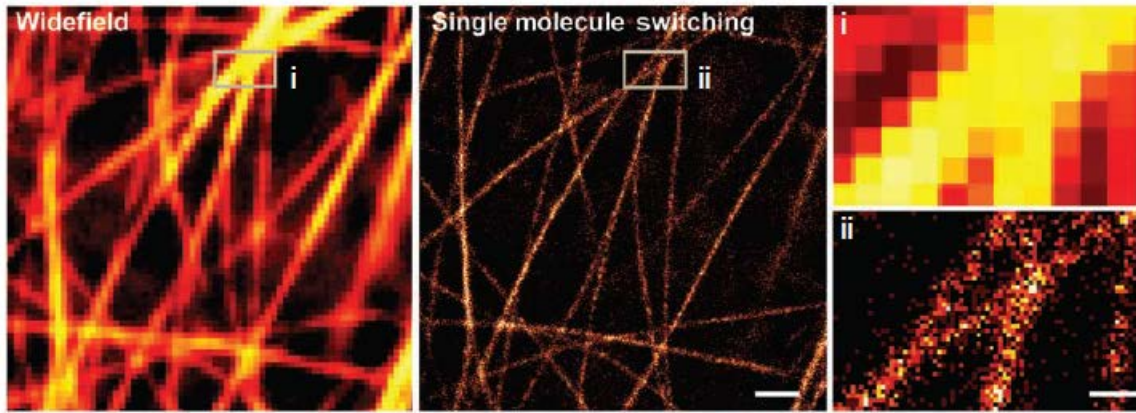
||

Eff. PSF

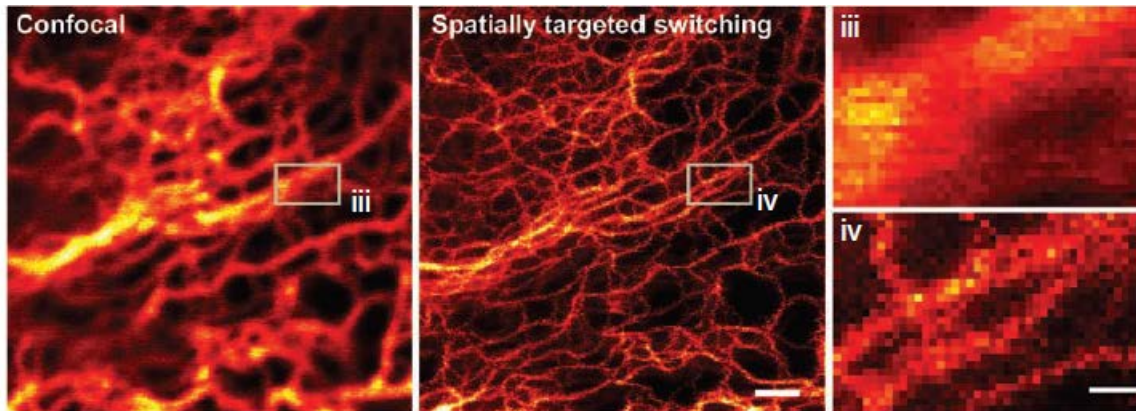


Far-Field Nanoscopy

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!**

Far-Field Nanoscopy

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

Issues super-resolution:

Increased sensitivity =
increased potential for bias

Labeling (size, labeling degree, influence,...)

Photo-toxicity

Speed

Biological applicability

STED/RESOLFT vs. PALM/...

Same principle: ON/OFF

**Similar techniques:
Own advantages/disadvantages**

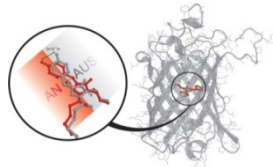
**Same labels / samples
- New control!**

STED/RESOLFT

Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins

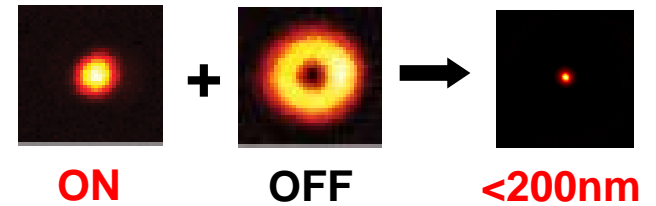
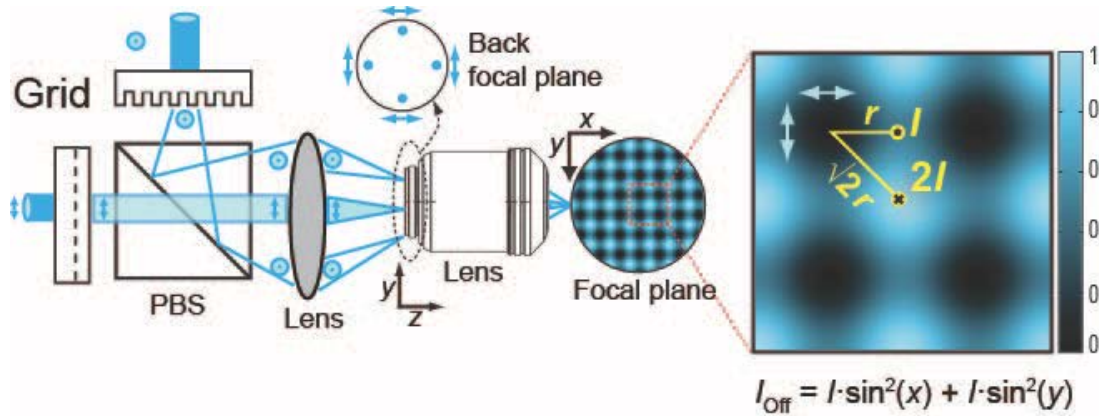
RESOLFT



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

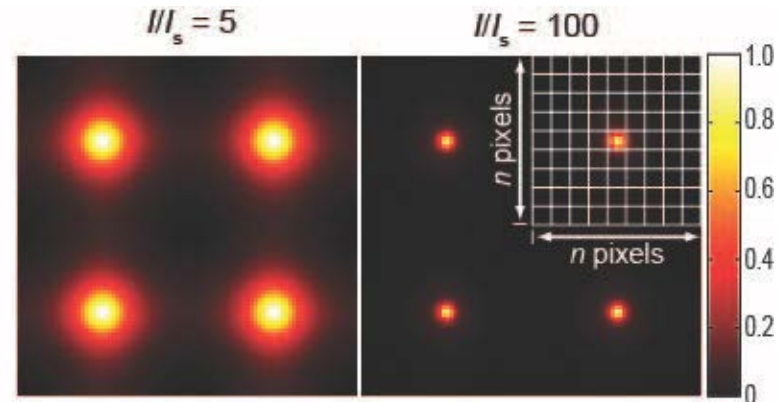
Intensity $\approx 1 \text{ kW/cm}^2$

Parallelization



Thousand doughnuts (CCD detection)

Chmyrov et al, Nature Meth. 2013



RESOLFT: on-state regions

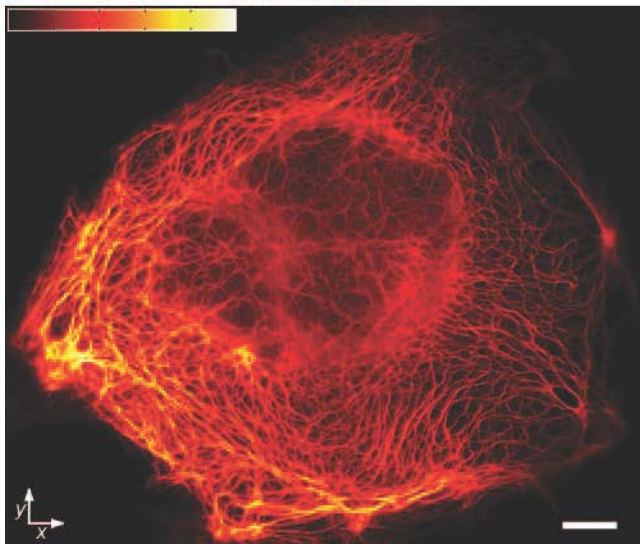
Far-Field RESOLFT Nanoscopy

Reversibly Photoswitchable Fluorescent Proteins

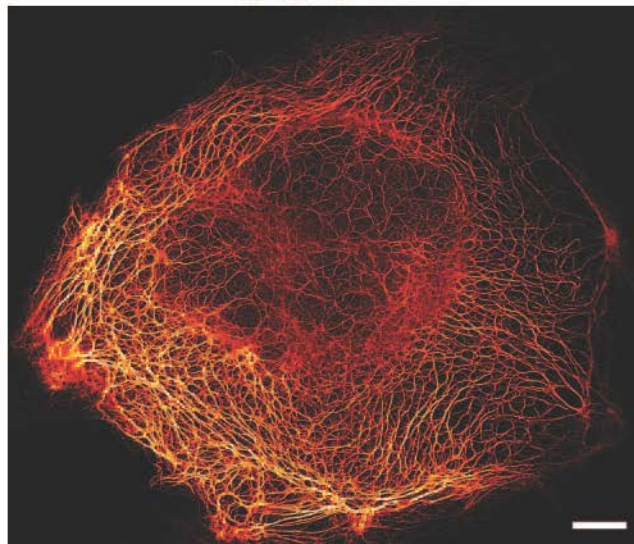
Parallelization

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

Wide field



RESOLFT



Keratin19-rsEGFP
expressed
in living PtK2 cells

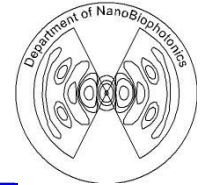
Scale 10 μm

120x100 μm – 1s

Chmyrov et al,
Nature Meth. 2013



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

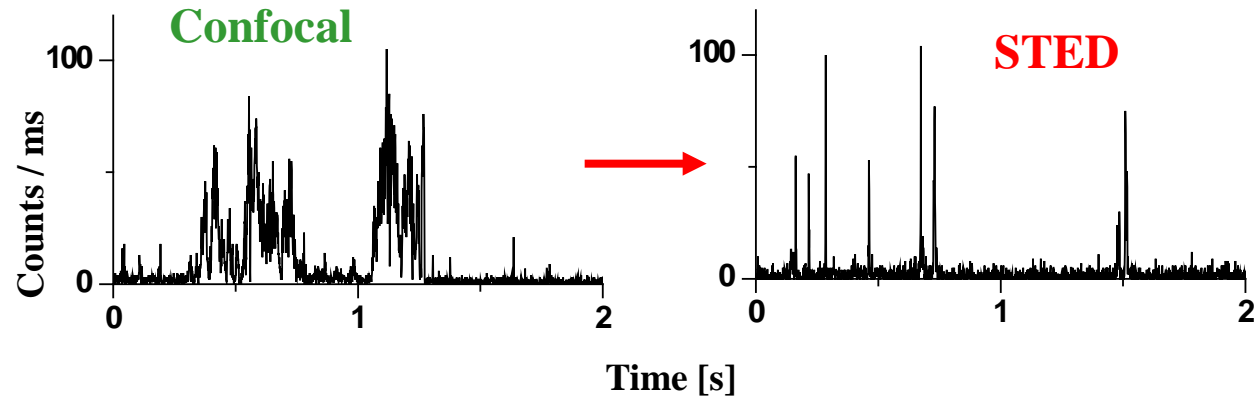
+ whole group

Lipid labeling

Dr. G. Schwarzmann

Prof. K. Sandhoff

(Kekule-Inst. Bonn)



SLB/Phase Sep.

Erdinc Sezgin

Jens Ehrig

Petra Schwille

(Dresden)

Proteins/Discussions

Ilya Leventhal

Uenal Coskun

Michal Gryzbeck

Daniel Lingwood

Kai Simons

(Dresden)

Membranes

Alf Honigmann

Richard Wagner

(Osnabrück)

Hopping

Aki Kusumi

(Kyoto)

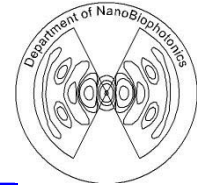
Fruitful discussion

Herve Rigneault

(Marseille)



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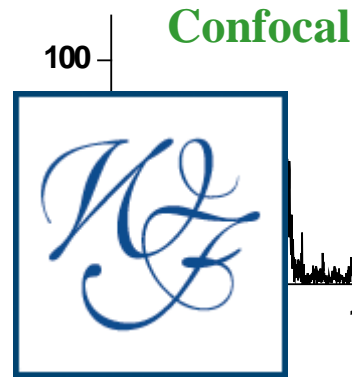
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Lipid labeling
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 (Kekule-Inst. Bonn)



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 Mathias Clausen (Biophysics - membrane)
 Silvia Galiani (Physics – nanoscope setup/organelles)
 Marco Fritzsche (Physics - cytoskeleton)
 Erdinc Sezgin (Biophysics – membrane)
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 Huw Colin York (Physics – microscopy/force)
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 Martin Booth, Achillefs Kapanidis,
 Philipp Kukura...