UNIVERSITÉ DE FRIBOURG
UNIVERSITÄT FREIBURG

Advanced Fluorescence Imaging

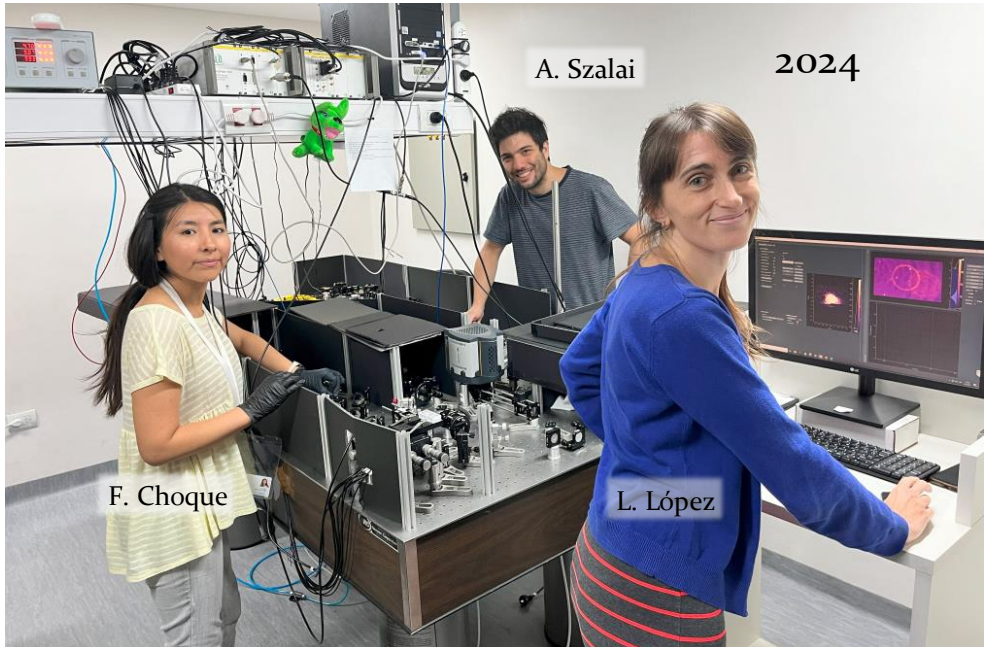
Prof. Dr. Fernando D. Stefani

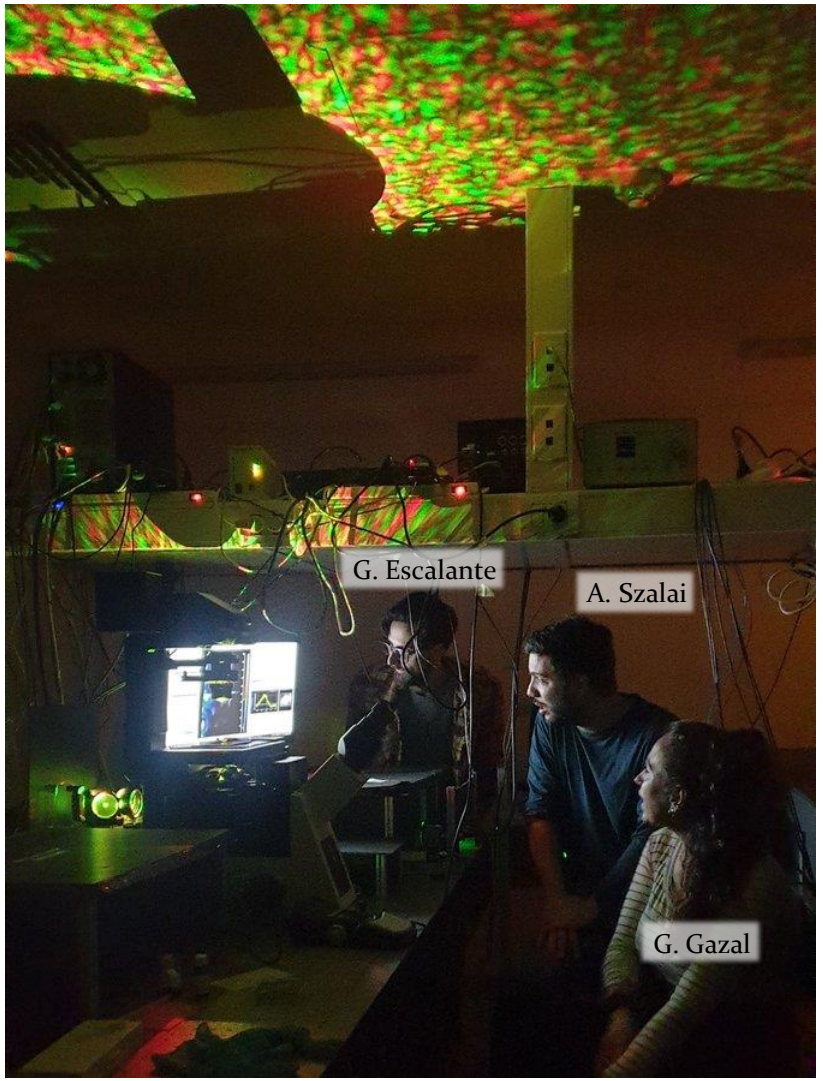
CIBION
CONICET

https://stefani-lab.ar/

https://github.com/Stefani-Lab

2023 / 2024





@FerStefaniLab

https://stefani-lab.ar/

Acknowledgements

MPI-bpc Göttingen
Stefan W. Hell
Tom Jovin

INIMEC Córdoba
Alfredo Cáceres
Nicolás Unsain
Mariano Bisbal

University of Fribourg
Guillermo Acuña

INIFTA La Plata
Omar Azzaroni
Felix Requejo

IBIOBA Buenos Aires
Damián Refojo

LMU Munich
Philip Tinnefeld
Jochen Feldmann
Stefan Maier

UCL London
Sabrina Simoncelli

UNSAM Buenos Aires
Oscar Campetella
Juan Mucci
Marina Simian
Dante Chialvo

OMITEC Brno
Pavel Zemanek

FIL Buenos Aires
Fernando Goldbaum

Monash Uni Melbourne
Stefan Maier

UNC Córdoba
Eduardo Coronado
Rodolfo Acosta

DIPC Donostia
Juan José Sáenz

UTN Campana
Alberto Scarpetini

UBA Buenos Aires
Andrea Bragas
Lía Pietrasanta

Acknowledgements

MPI-bpc Göttingen
Stefan W. Hell
Tom Jovin

INIMEC Córdoba
Alfredo Cáceres
Nicolás Unsain
Mariano Bisbal

University of Fribourg
Guillermo Acuña

INIFTA La Plata
Omar Azzaroni
Felix Requejo

IBIOBA Buenos Aires
Damián Refojo

LMU Munich
Philip Tinnefeld
Jochen Feldmann
Stefan Maier

UCL London
Sabrina Simoncelli

FIL Buenos Aires
Fernando Goldbaum

UNSAM Buenos Aires
Oscar Campetella
Juan Mucci
Marina Simian
Dante Chialvo

OMITEC Brno
Pavel Zemanek

Monash Uni Melbourne
Stefan Maier

UNC Córdoba
Eduardo Coronado
Rodolfo Acosta

DIPC Donostia
Juan José Sáenz

UTN Campana
Alberto Scarpetini

UBA Buenos Aires
Andrea Bragas
Lía Pietrasanta

Advanced Fluorescence Imaging

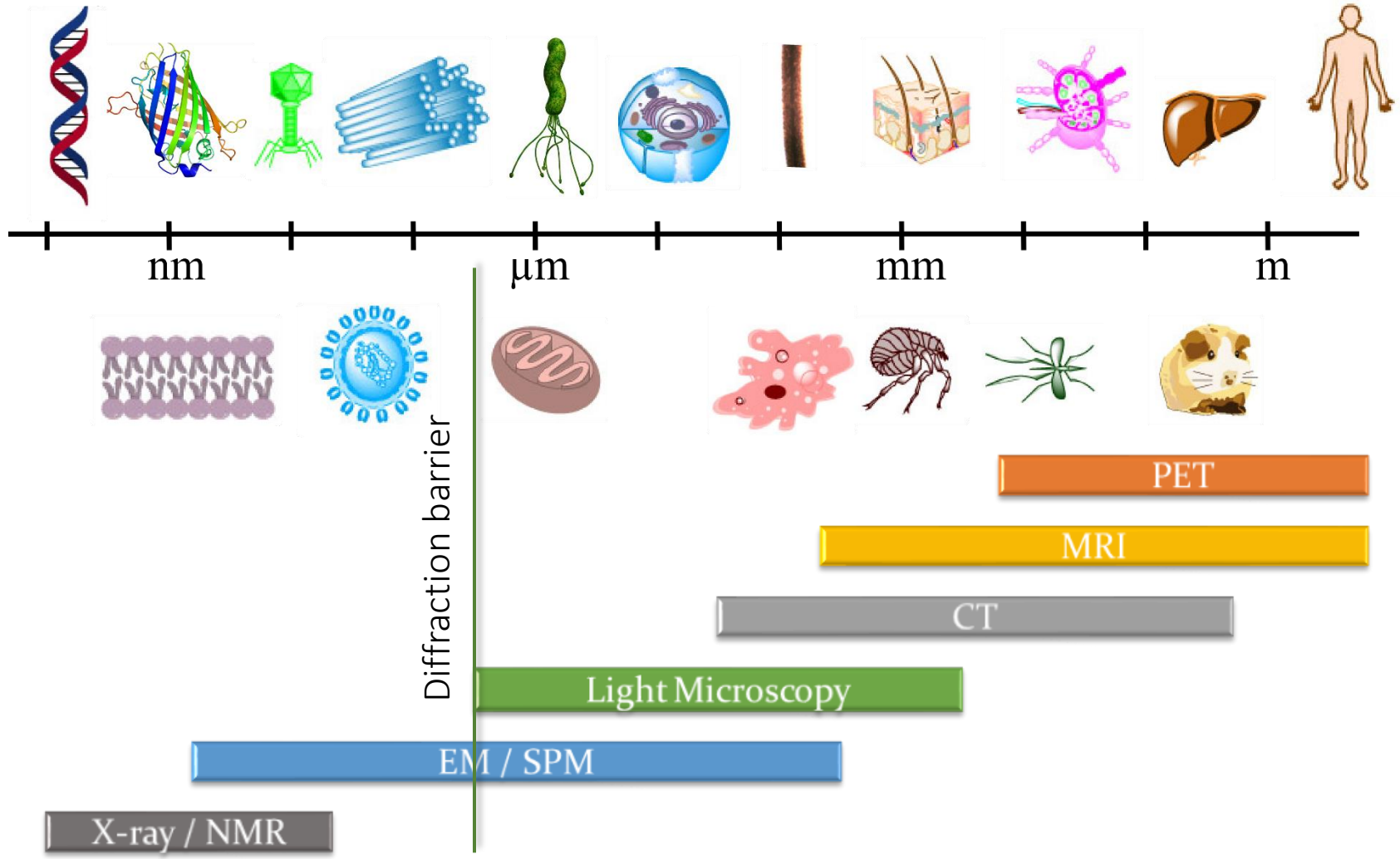
Day 1:	Fluorophores, Fluorescence
	Fluorescence Microscopy
	Intro to Single-Molecule Localization Microscopy
	Lab experience and Q&A
Day 2:	Diffraction-limited imaging
	Super-resolution
	Coordinate-stochastic methods: SMLM
	Coordinate-targetted methods: STED
	New methods for sub-10 nm resolution
	Discussion

Super-resolution Fluorescence Microscopy

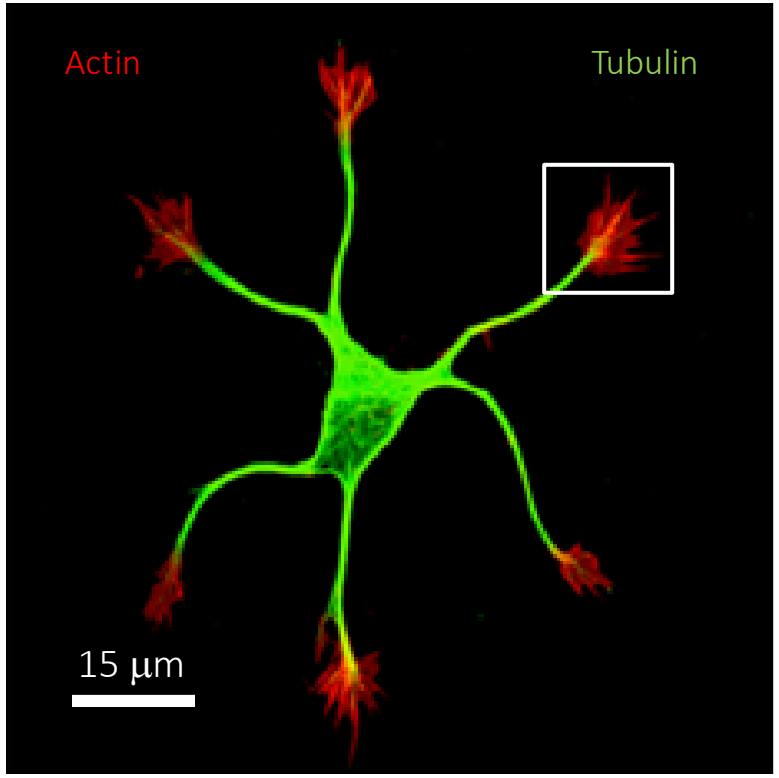
a.k.a.

Fluorescence Nanoscopy

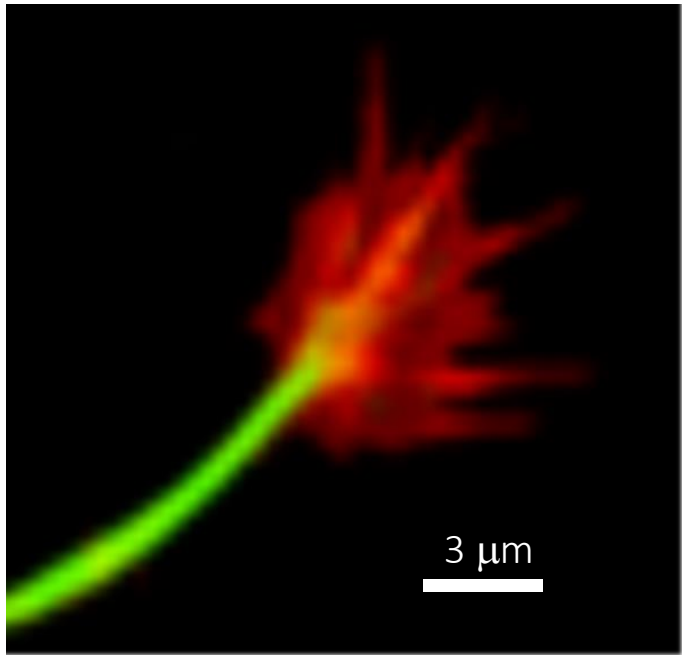
Imaging tools and their spatial resolution



Fluorescence microscopy



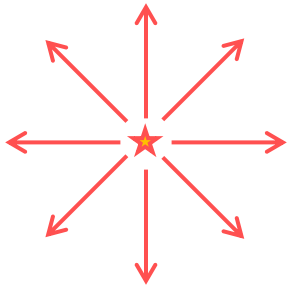
Diffraction-limited imaging



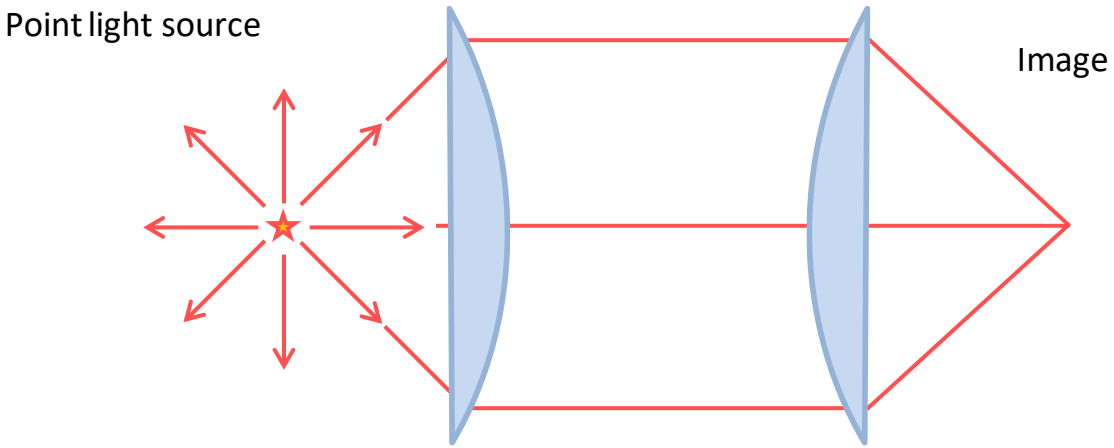
Courtesy of Dr. Alfredo Cáceres

Diffraction-limited imaging

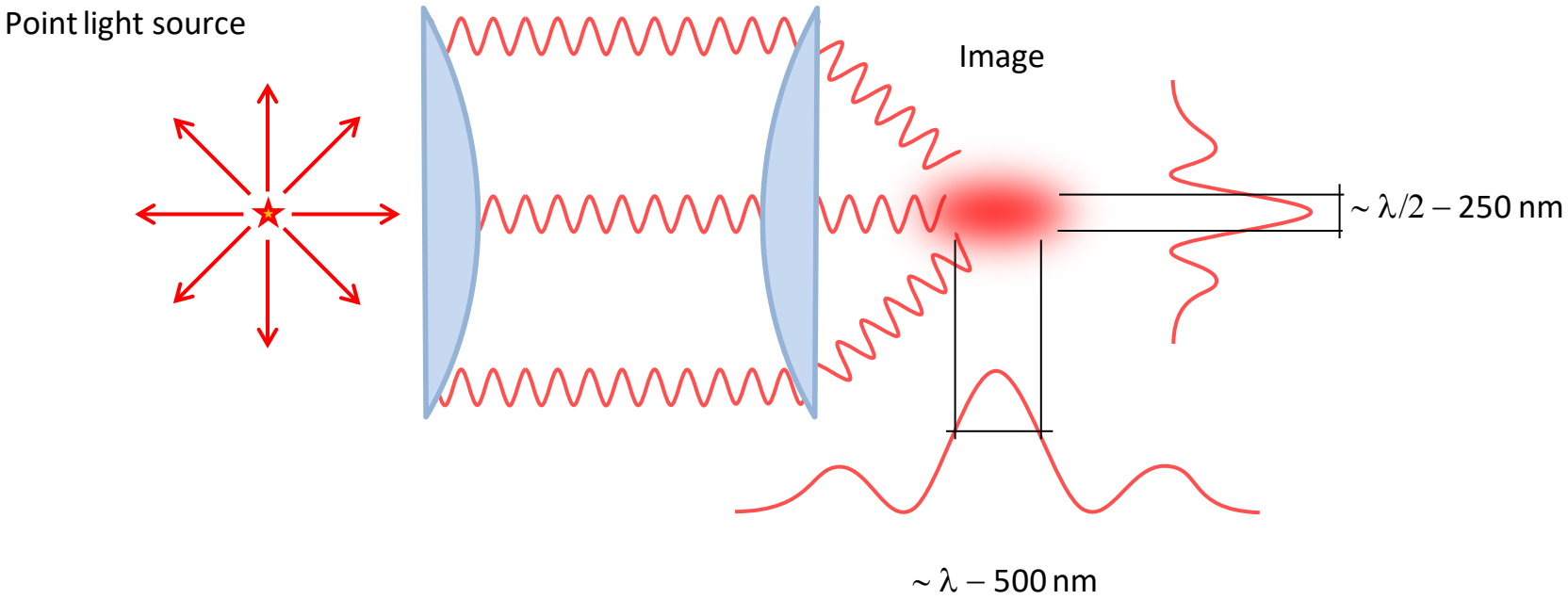
Point light source



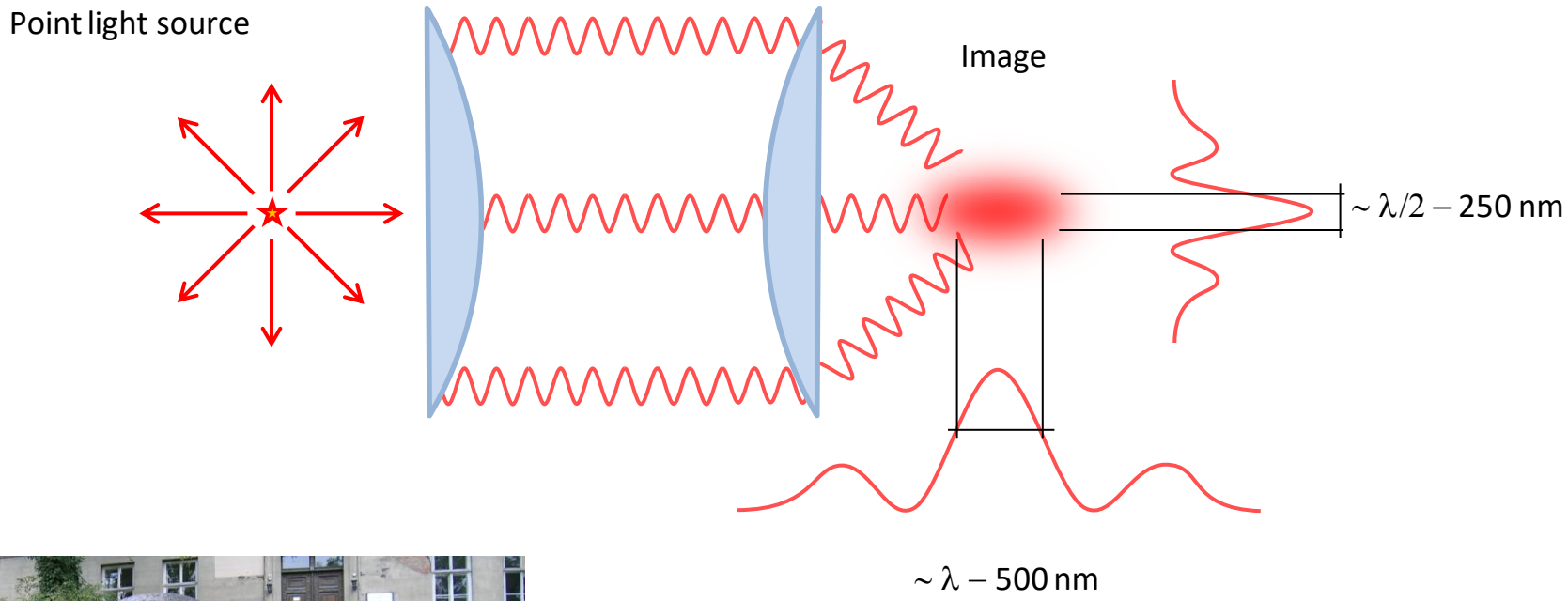
Diffraction-limited imaging



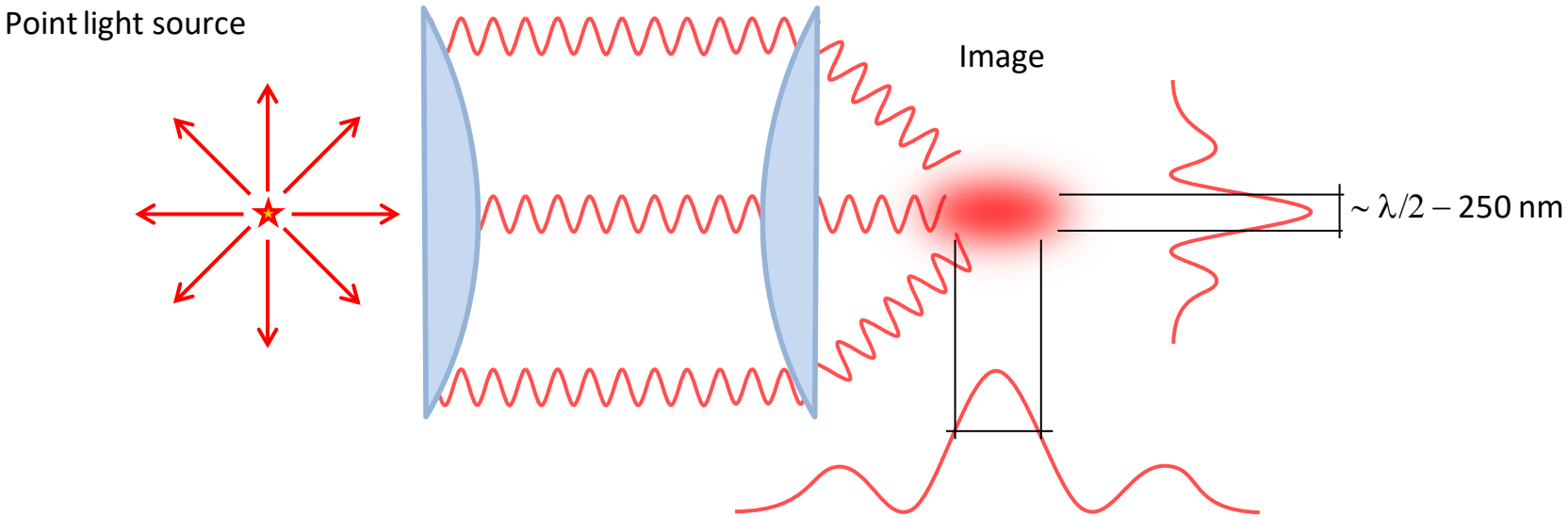
Diffraction-limited imaging

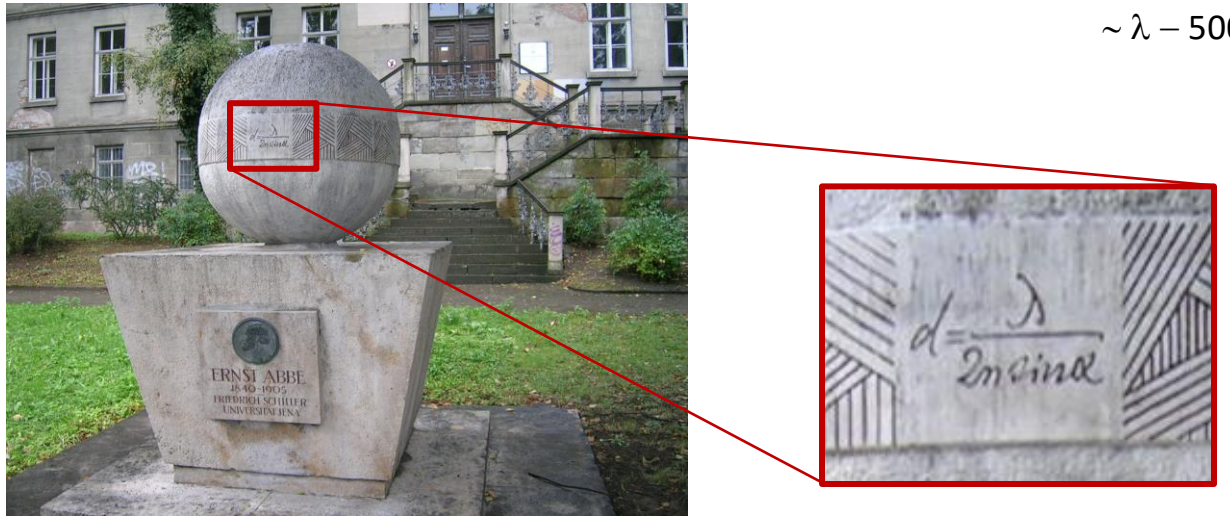


Diffraction-limited imaging



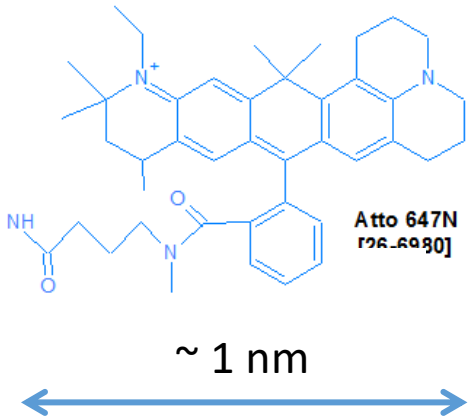
Diffraction-limited imaging



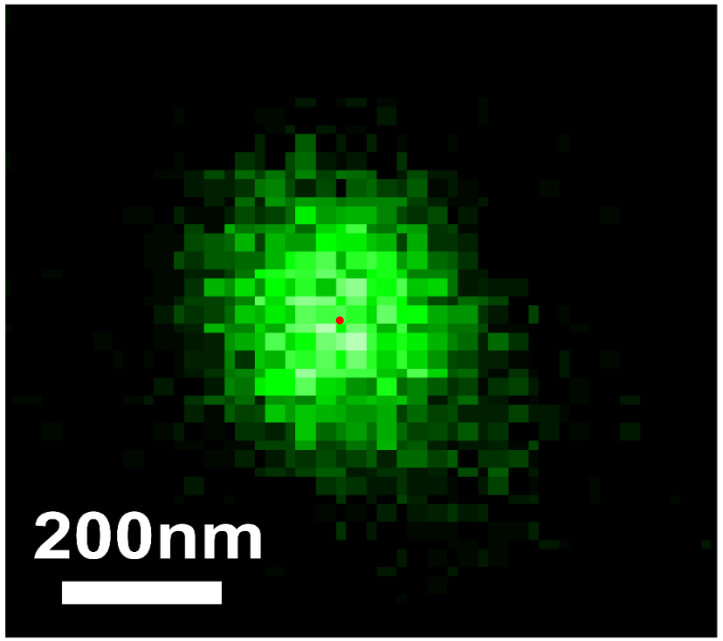


Diffraction-limited imaging

Visible photon source ~ 0.1 - 1 nm



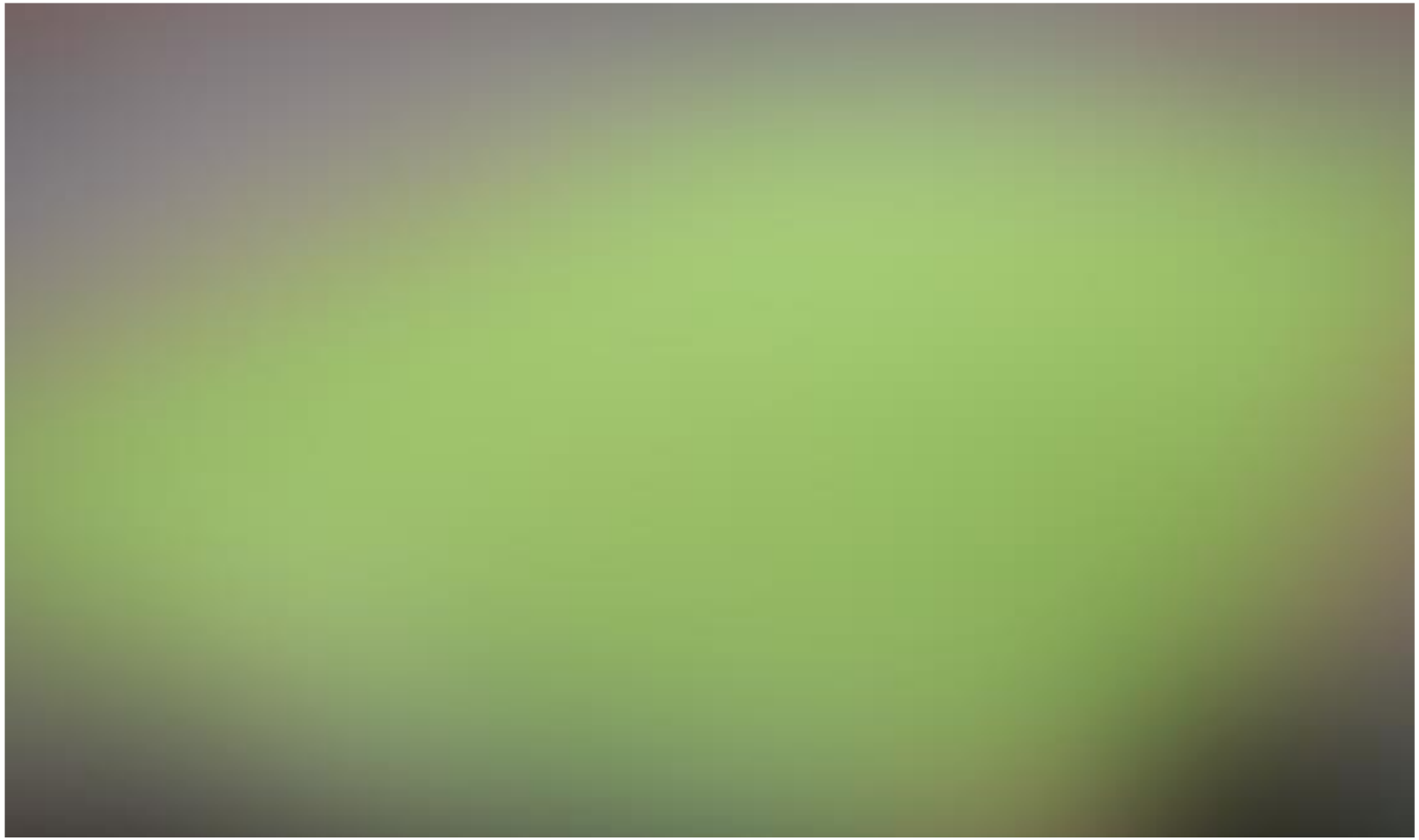
Visible EM wavelength ~ 500 nm



Size mismatch ~ 1:200

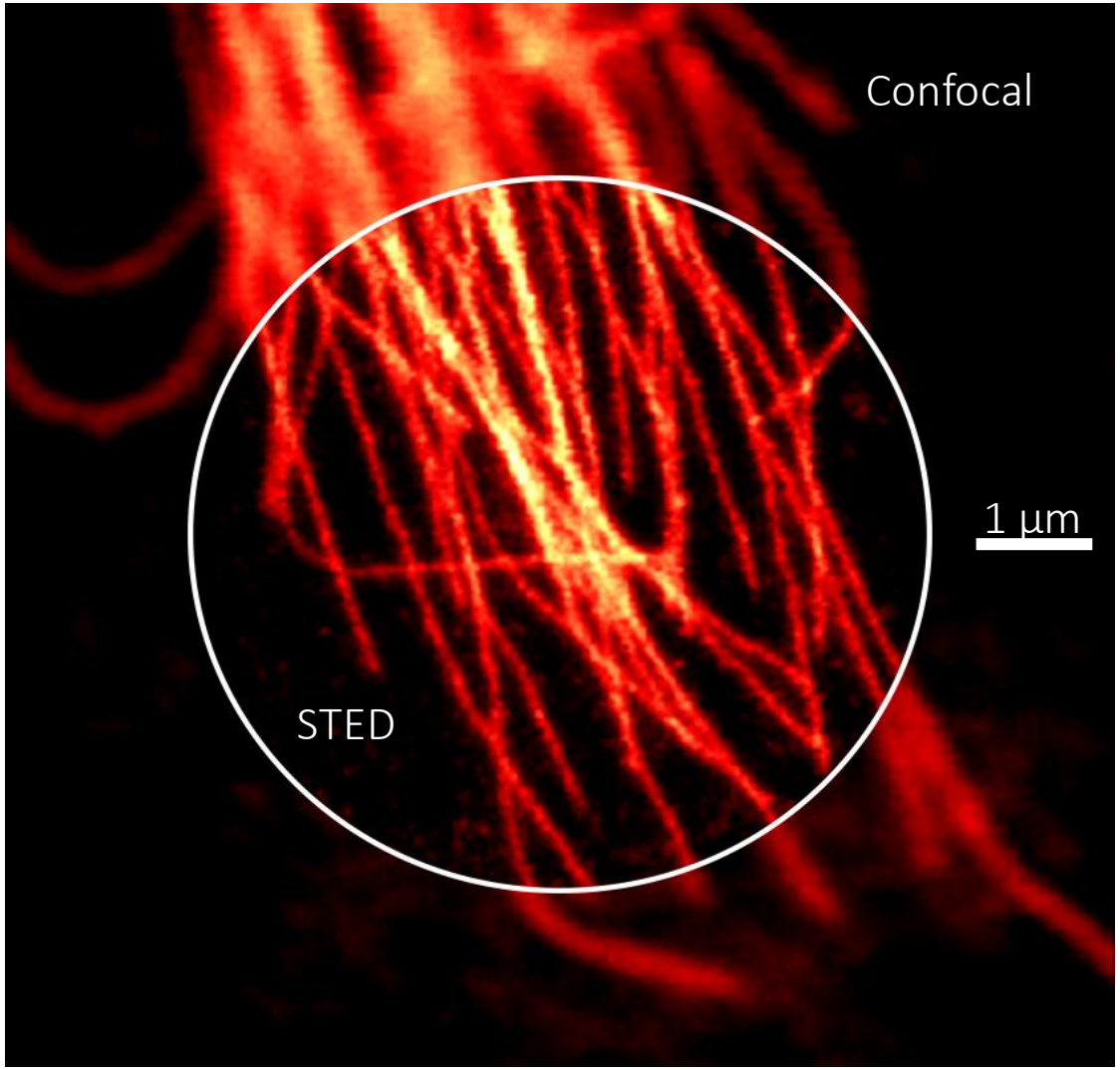
Diffraction-limited imaging

Diffraction-limited imaging

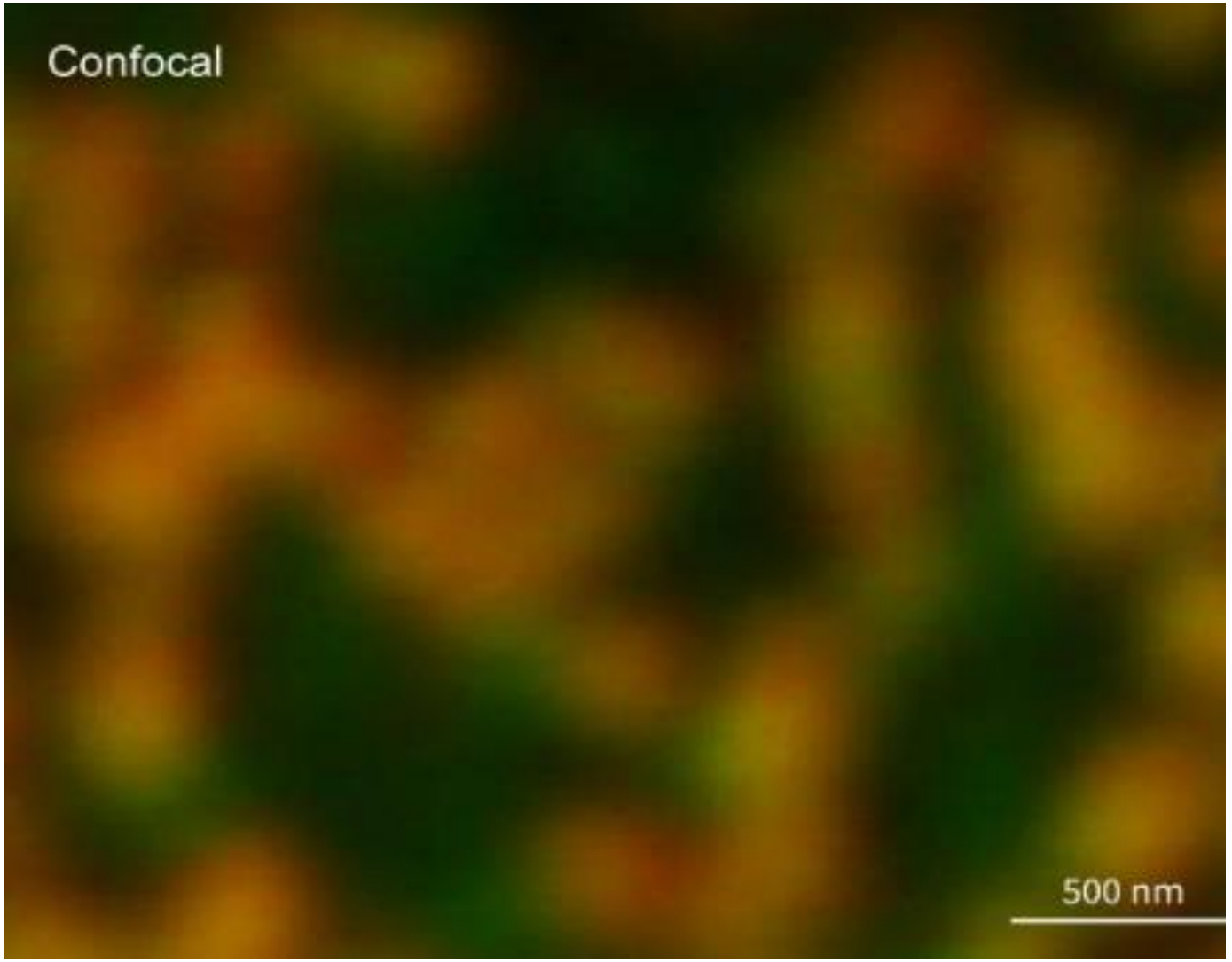


Diffraction-limited imaging

Super-resolution fluorescence microscopy

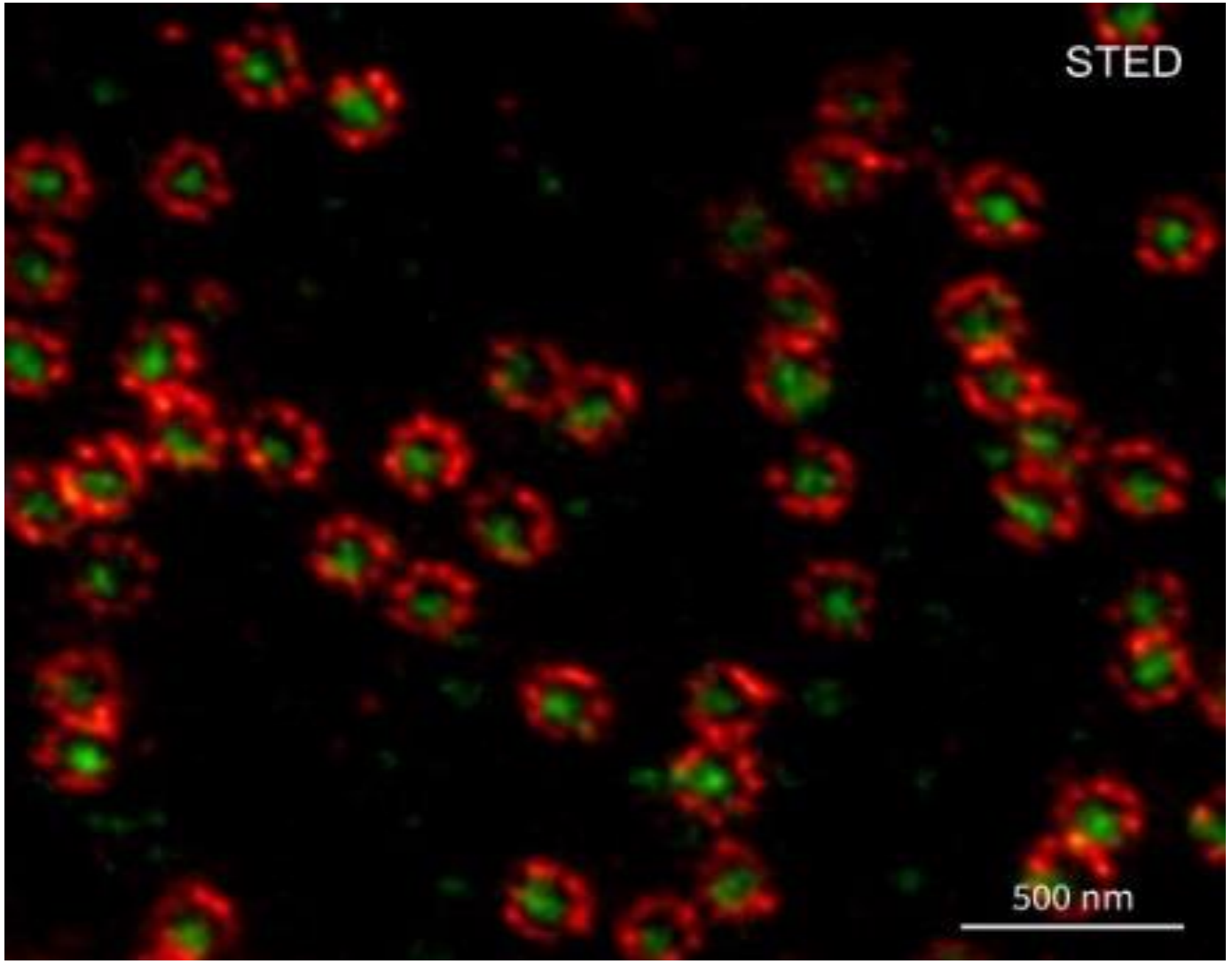


Super-resolution fluorescence microscopy



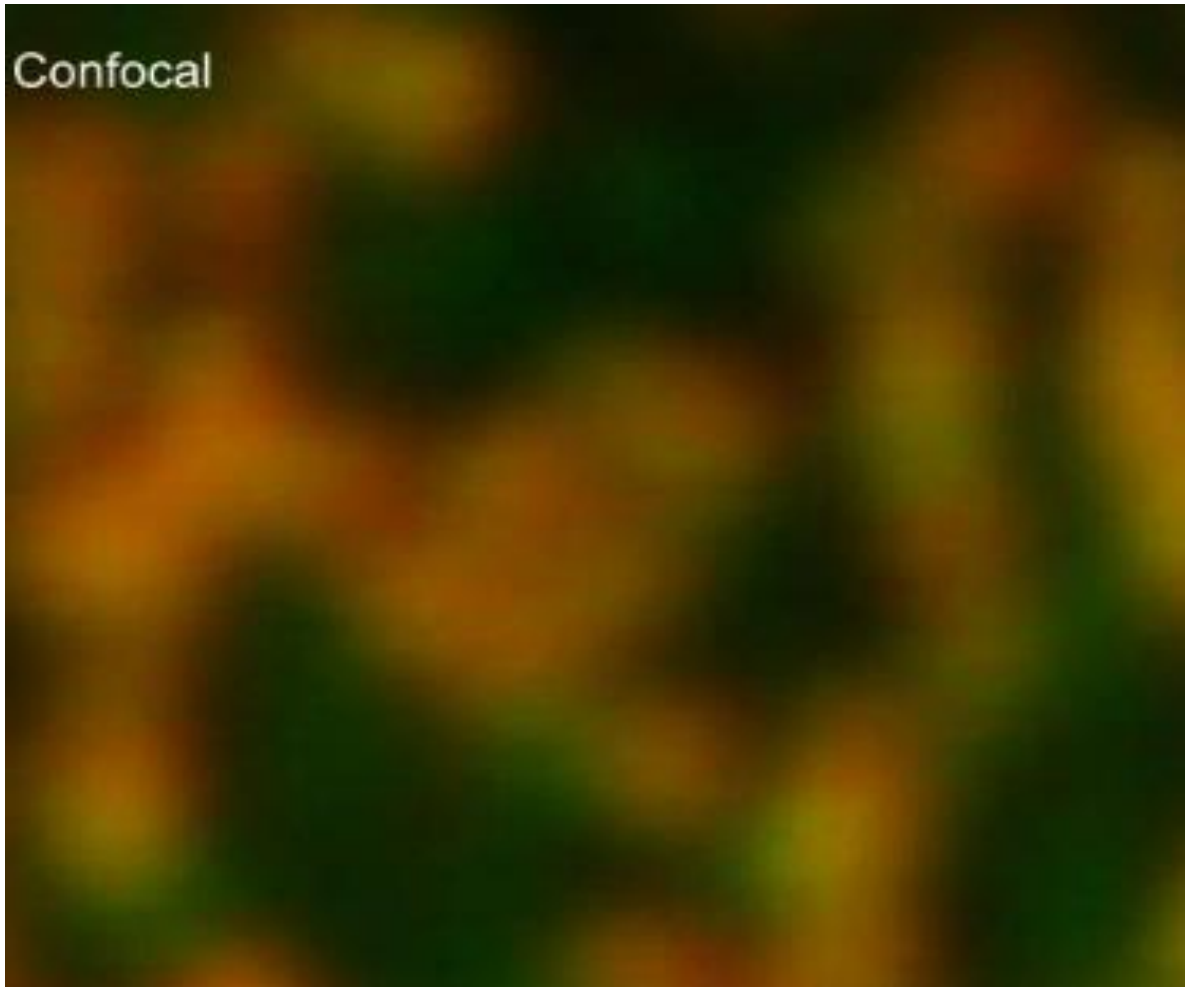
Stefan Hell Lab – Göttfert et al. ***Biophysical Journal*** 105 (2013) L01–L03

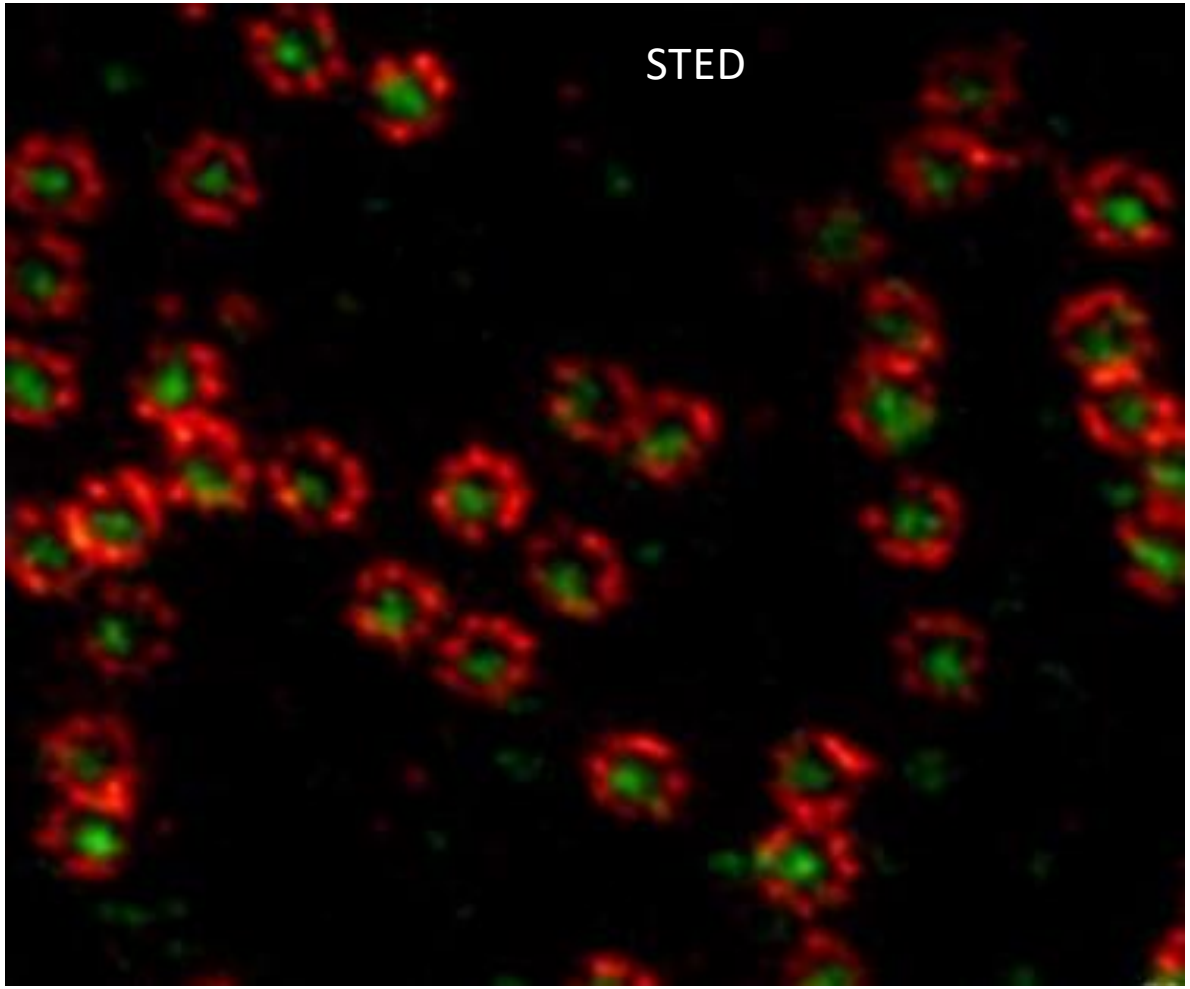
Super-resolution fluorescence microscopy



Stefan Hell Lab – Göttfert et al. ***Biophysical Journal*** 105 (2013) L01–L03

Super-resolution Fluorescence Microscopy

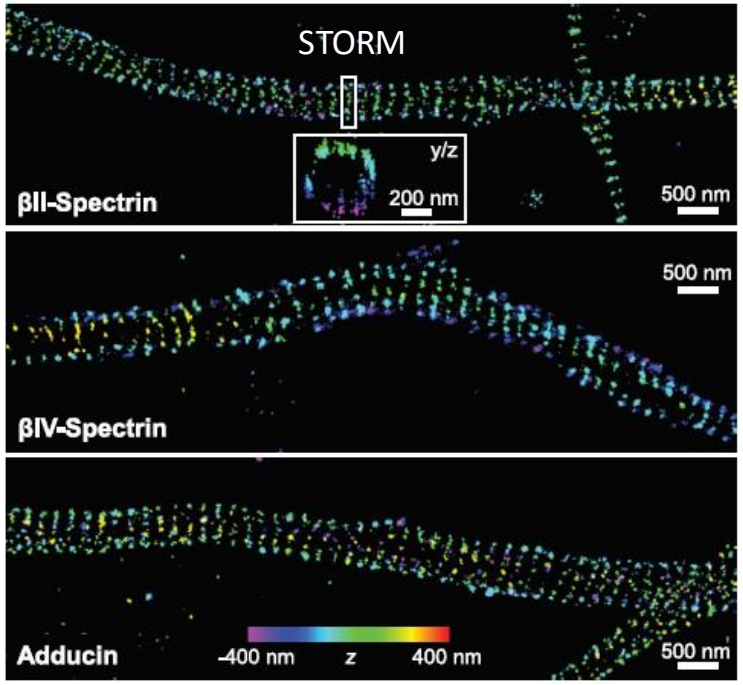


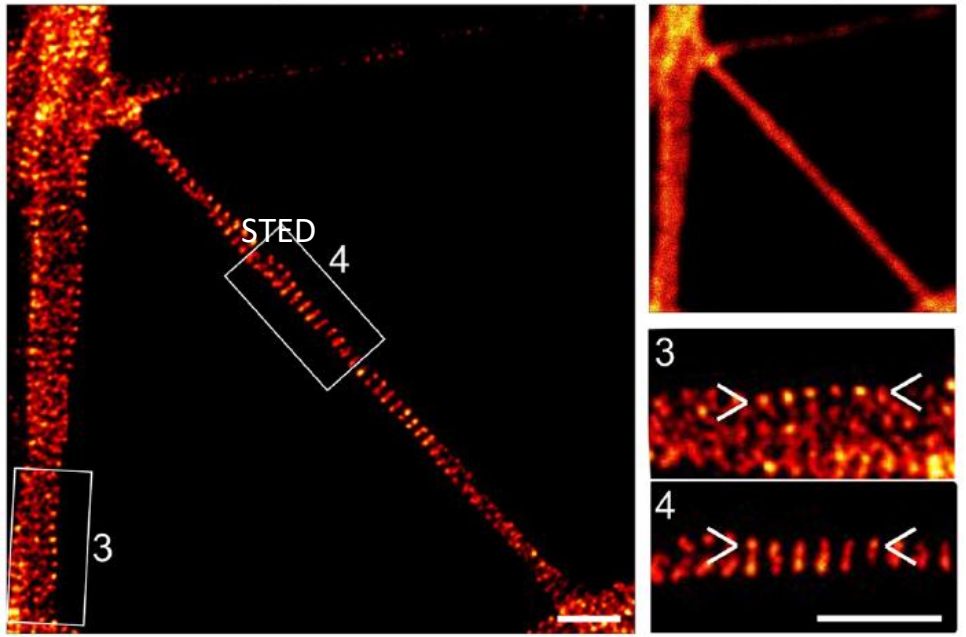


Stefan Hell Lab – Göttfert et al. *Biophysical Journal* 105 (2013) L01–L03

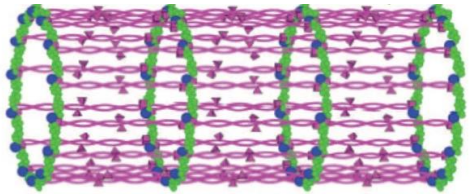
Super-resolution fluorescence microscopy

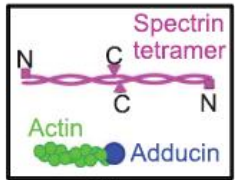
Membrane-associated Periodic Skeleton (MPS) of neurons





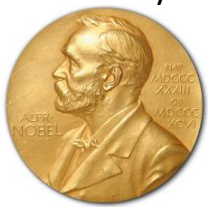
Unsain et al. *Scientific Reports* **2018** 18 3007





Xiaowei Zhuang Lab
Xu et al. *Science* **2013**, *339*, 452–456.

2014 Chemistry



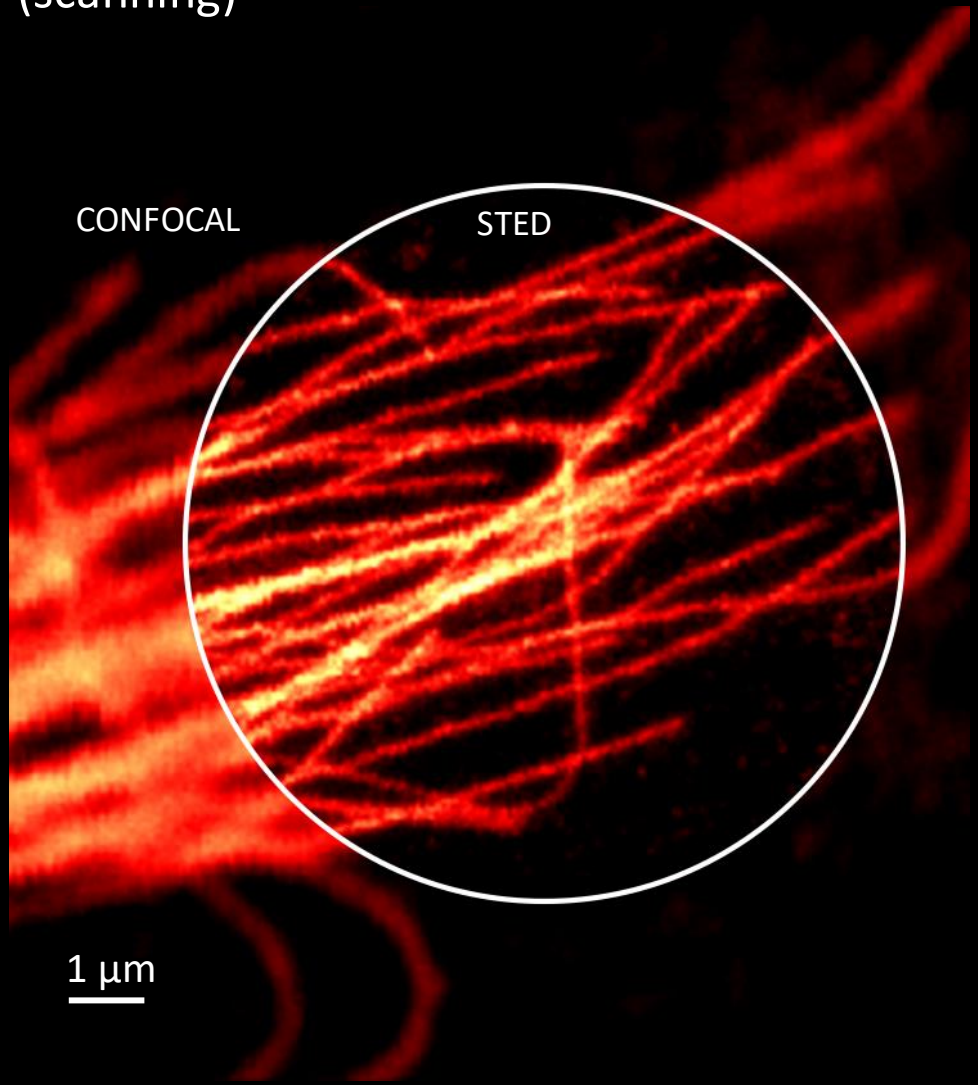
Stefan W. Hell

Eric Betzig

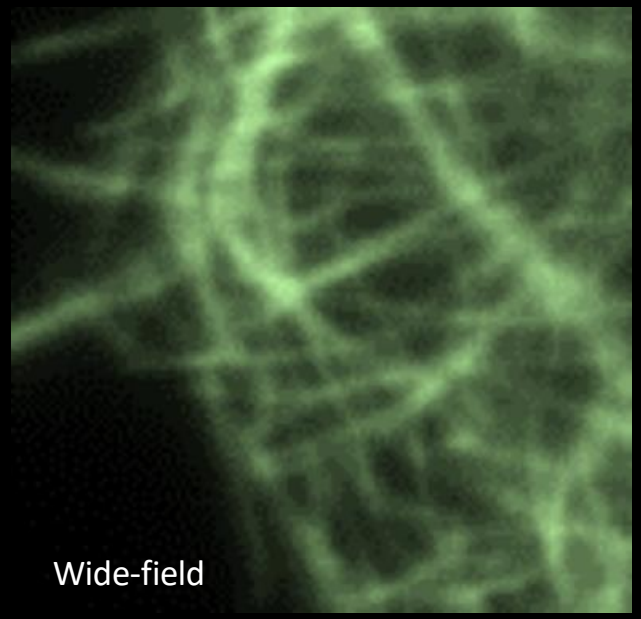
William E. Moerner

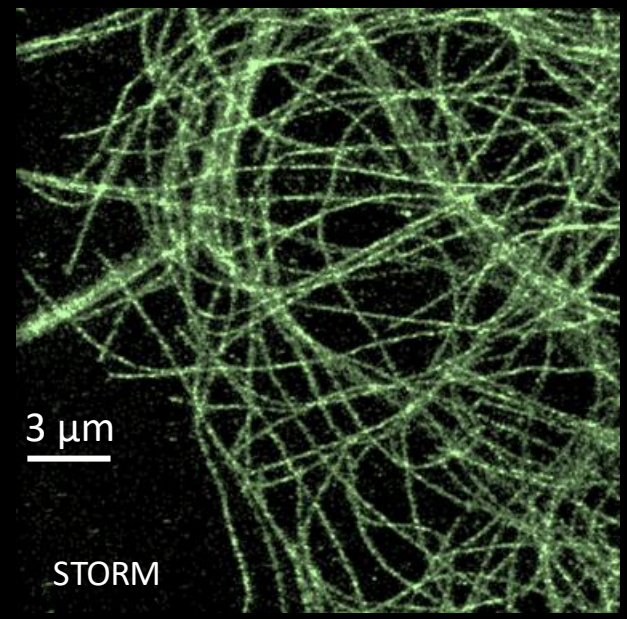
Super-resolution fluorescence microscopy

Coordinate-targeted nanoscopy
(scanning)



Coordinate-stochastic nanoscopy
(wide-field)



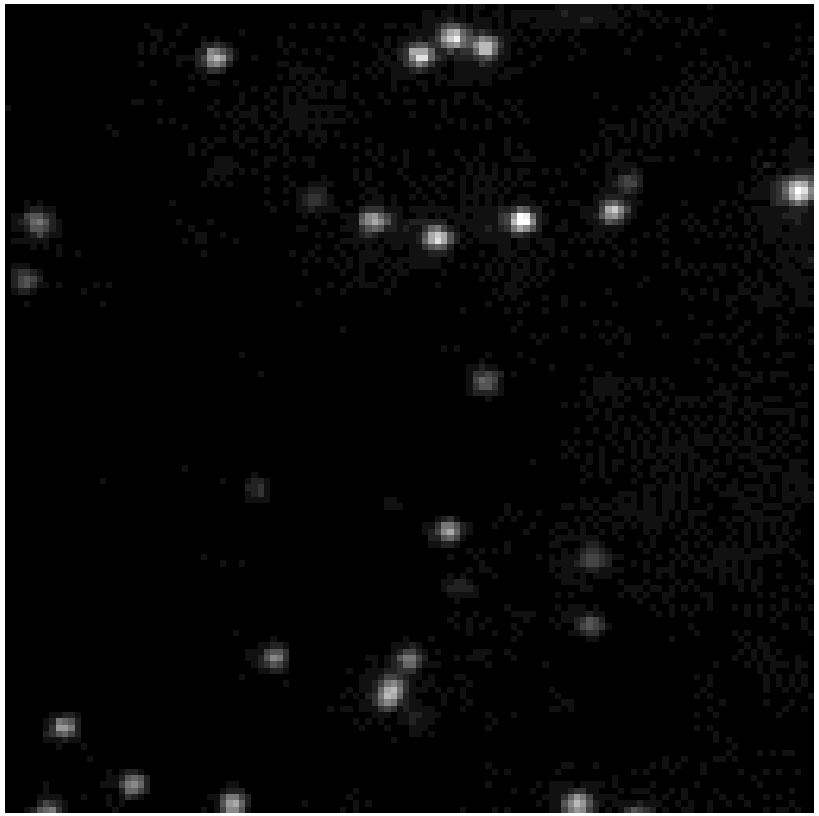


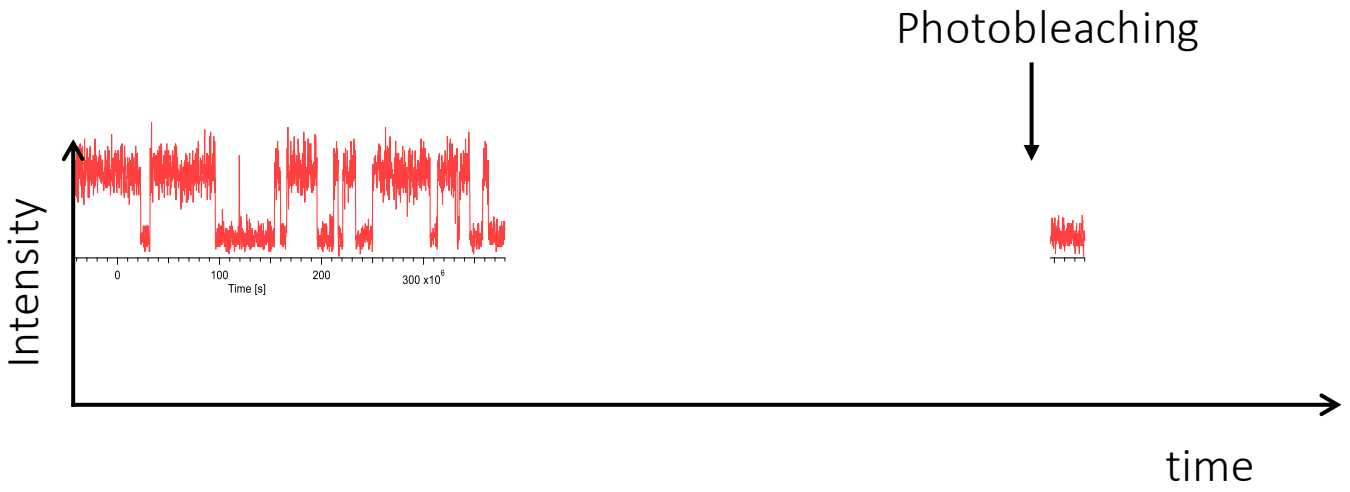
Coordinate-Stochastic Super-resolution Microscopy

a.k.a.

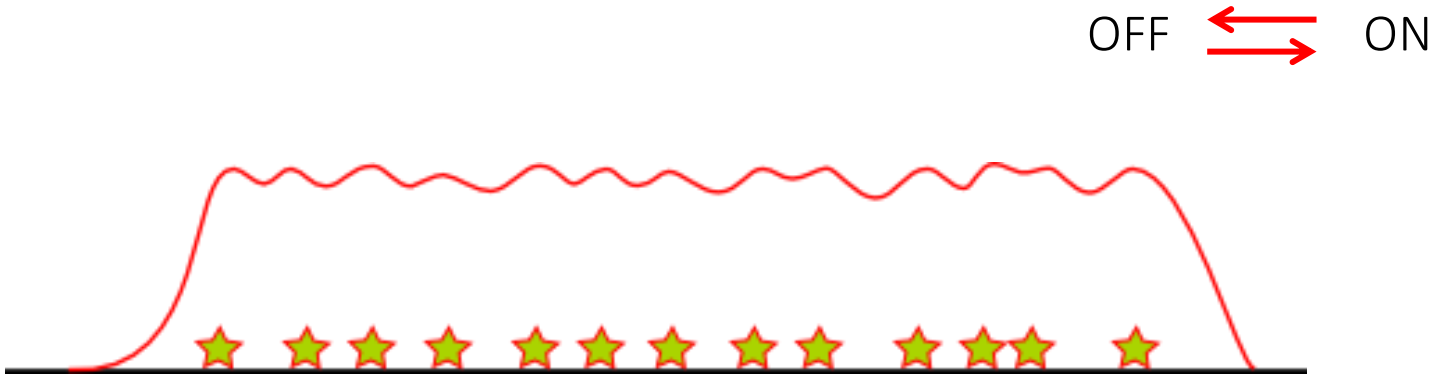
Single-Molecule Localization Microscopy (SMLM)

Single-molecule fluorescence blinking

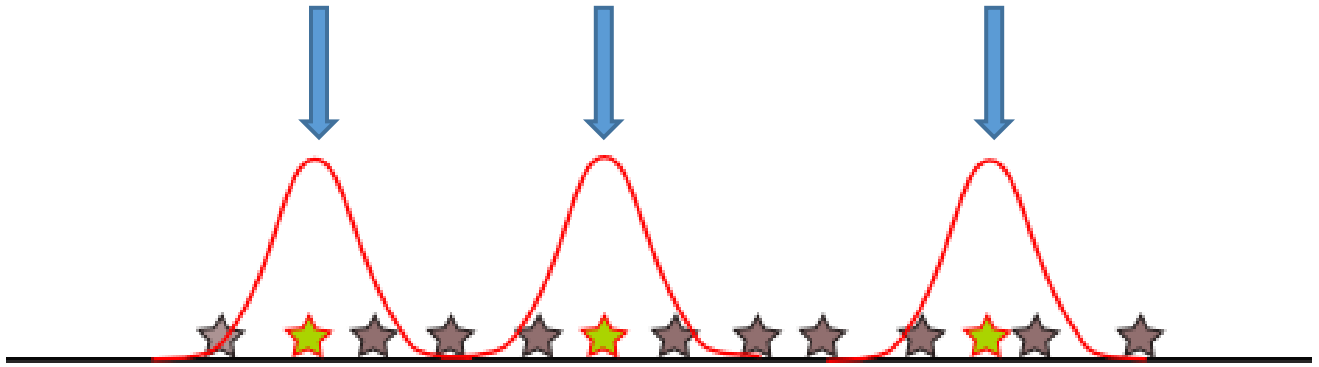




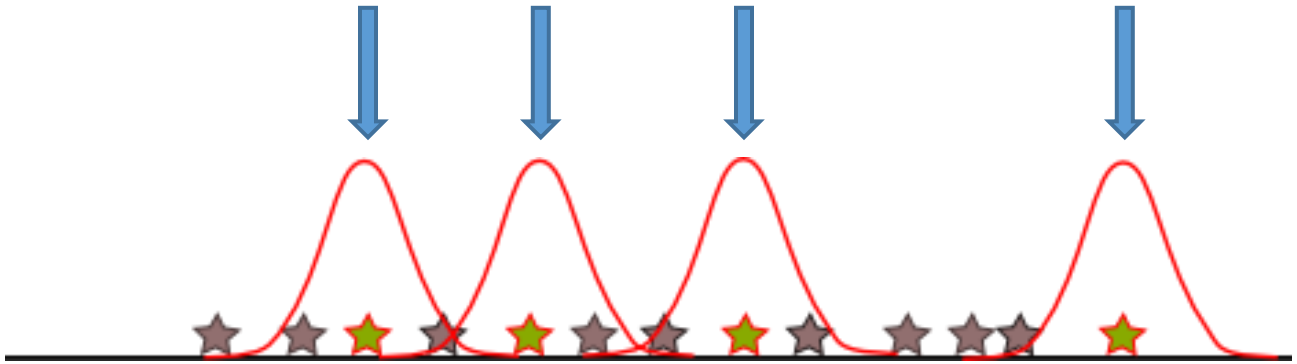
Single-Molecule Localization Microscopy (SMLM)



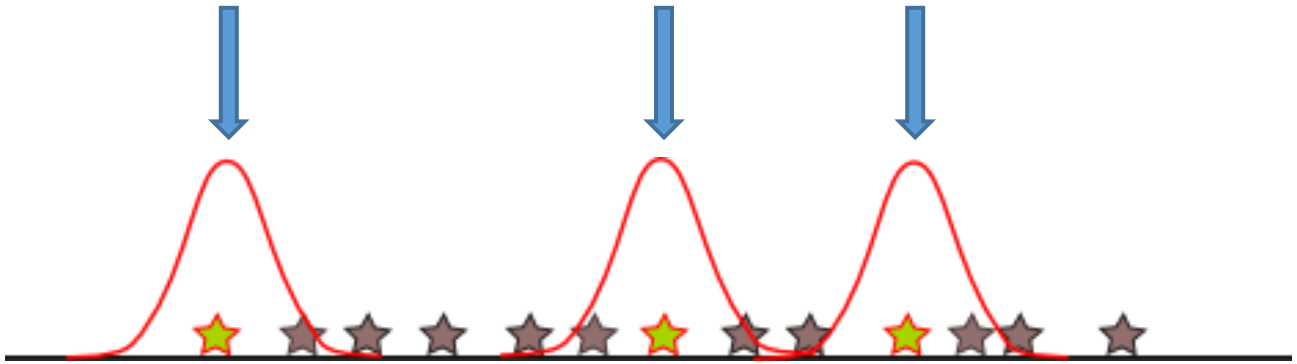
Single-Molecule Localization Microscopy (SMLM)



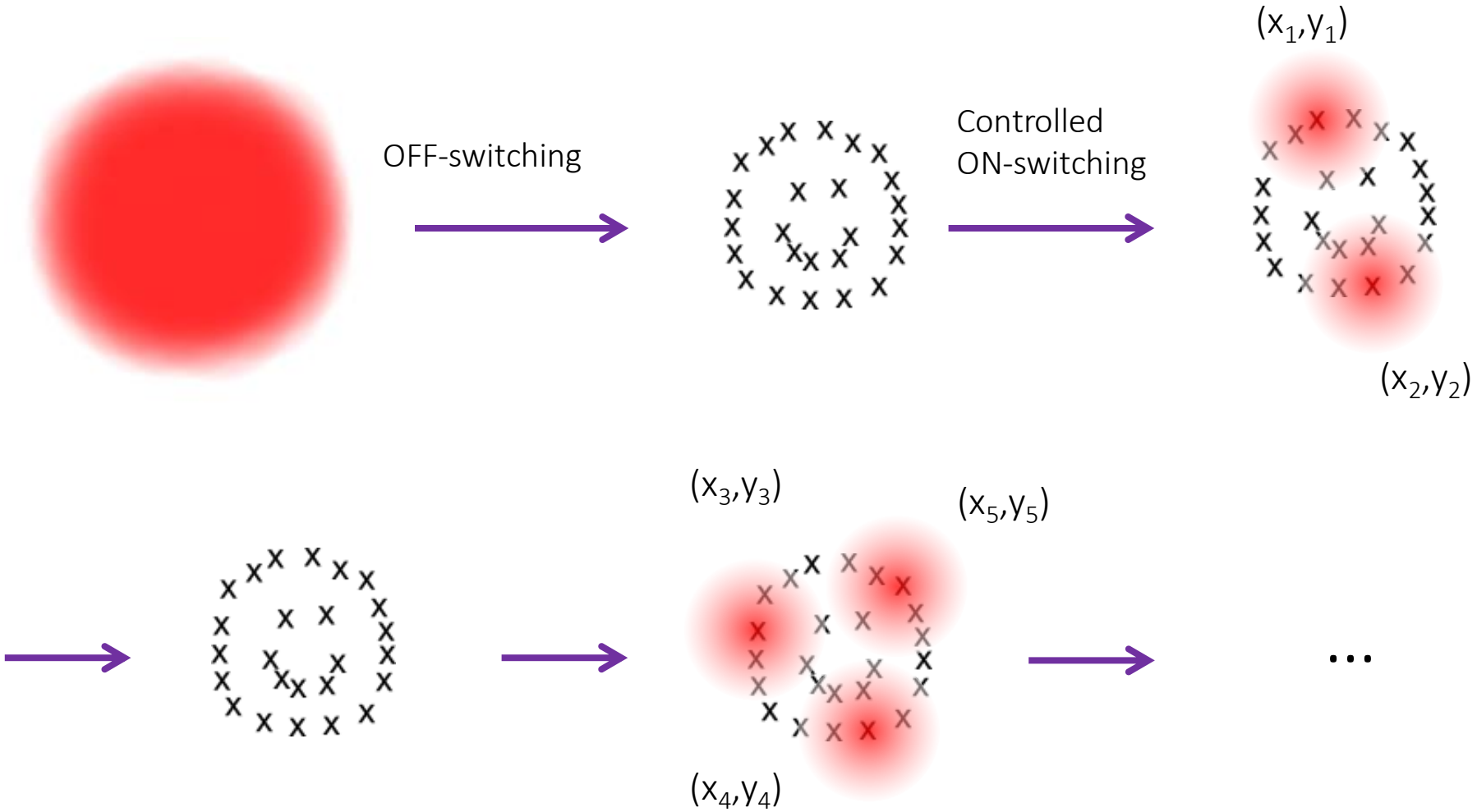
Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)



Single-Molecule Localization Microscopy (SMLM)

(x_1,y_1)

(x_2,y_2)

(x_3,y_3)

(x_4,y_4)

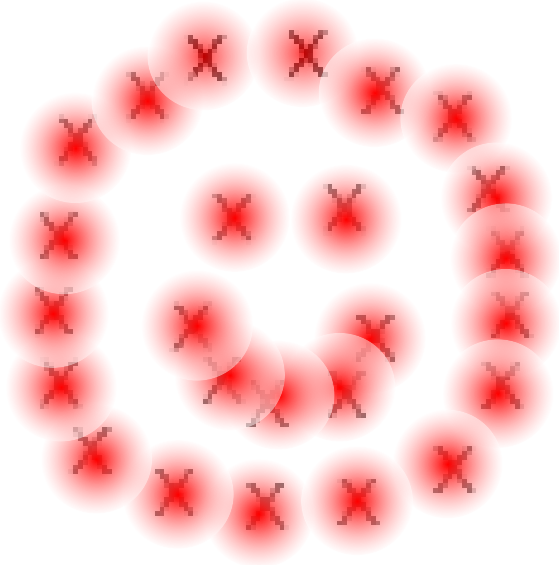
(x_5,y_5)

(x_6,y_6)

(x_7,y_7)

(x_8,y_8)

...

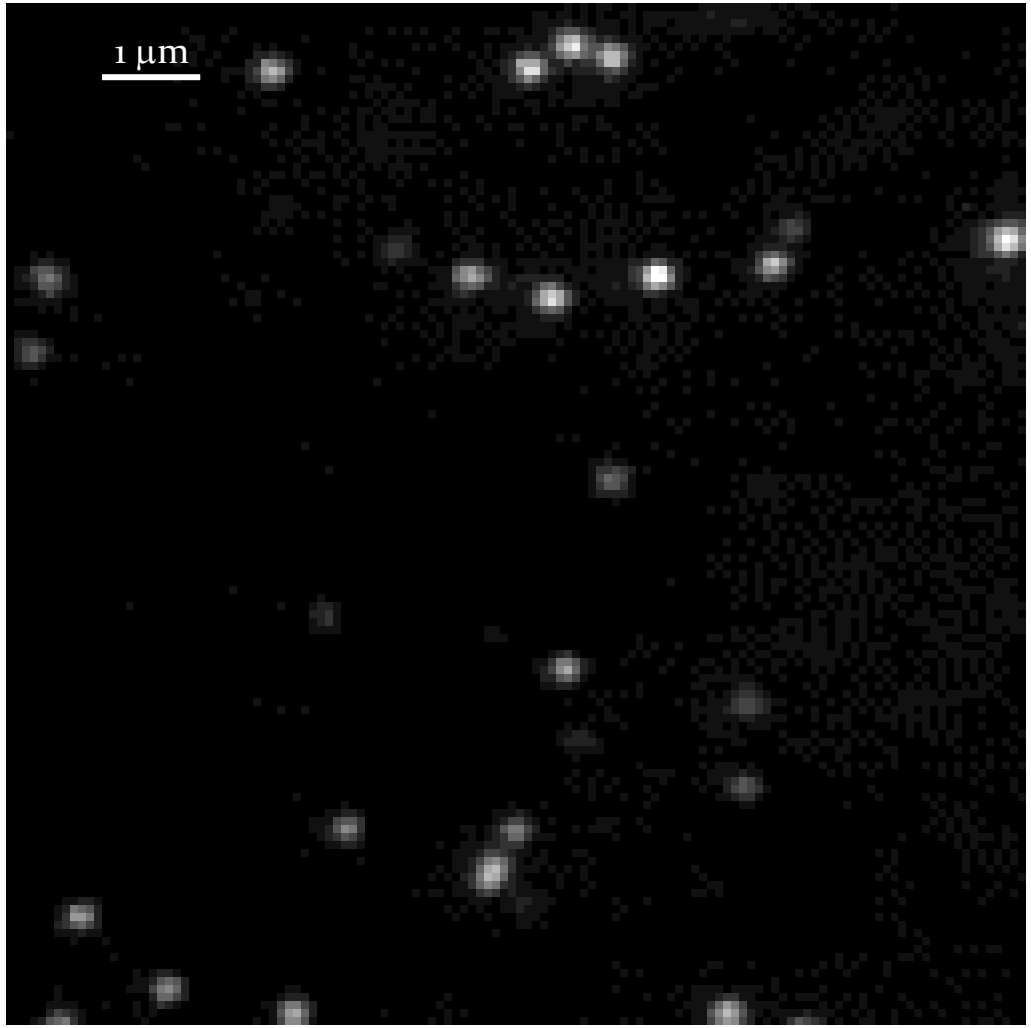


STORM – Stochastic Optical Reconstruction Microscopy

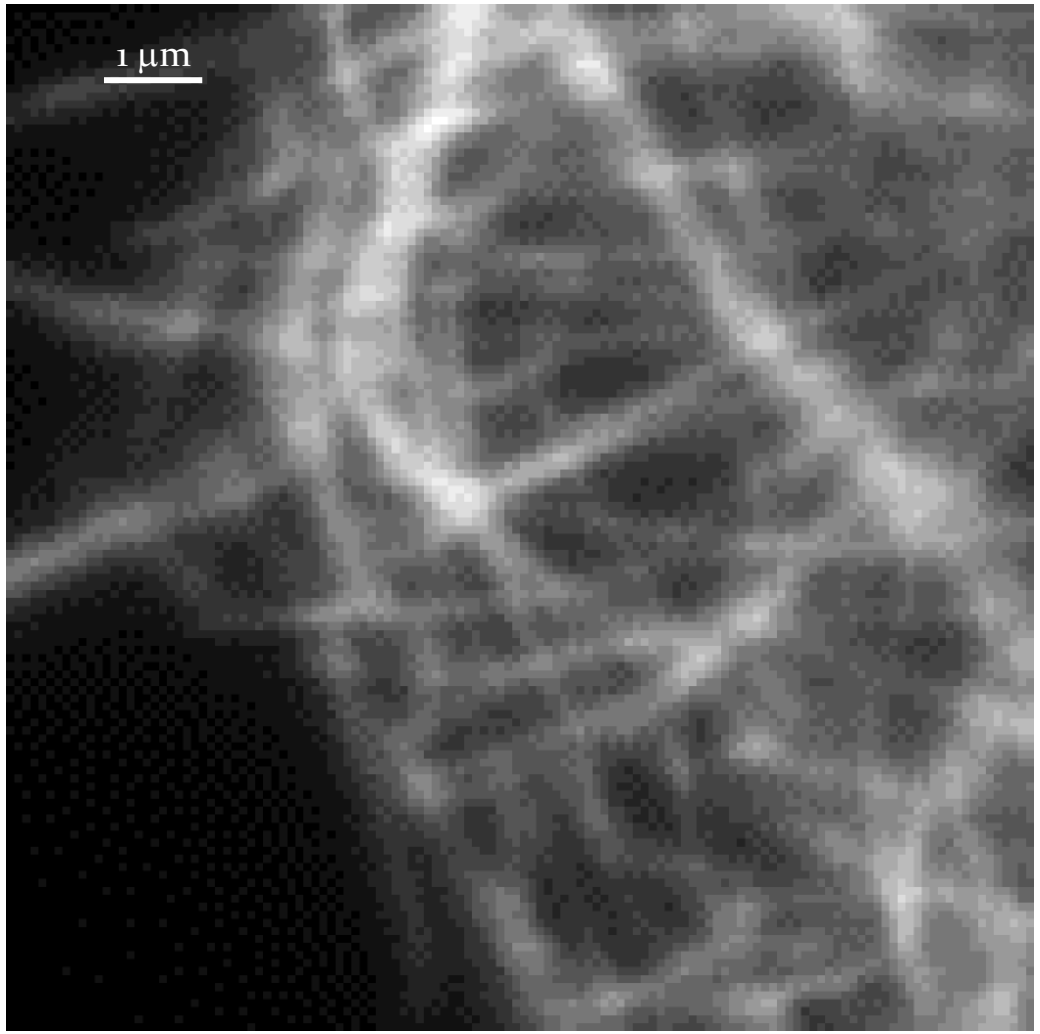
PALM – Photo-Activated Localization Microscopy

PAINT – Points Accumulation for Imaging in Nanoscale Topography

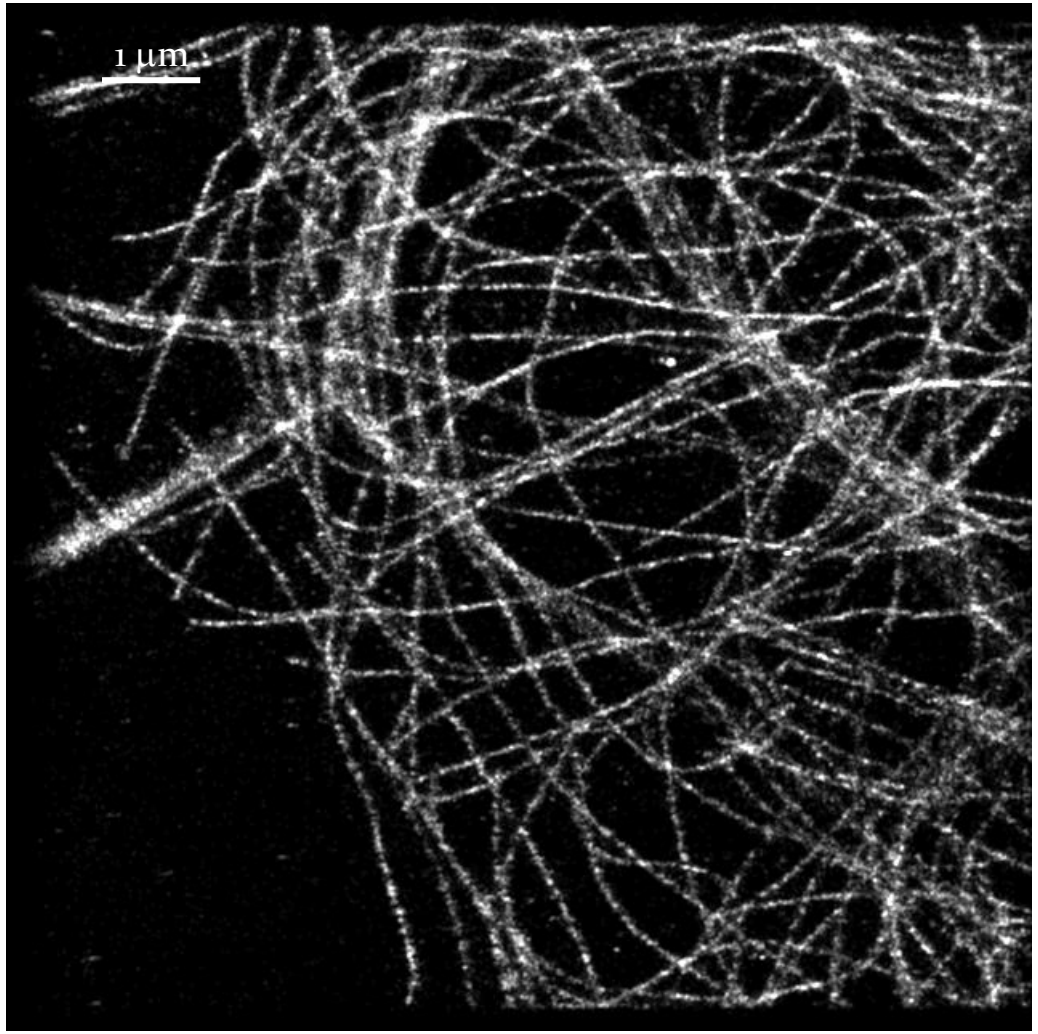
Single-Molecule Localization Microscopy (SMLM)



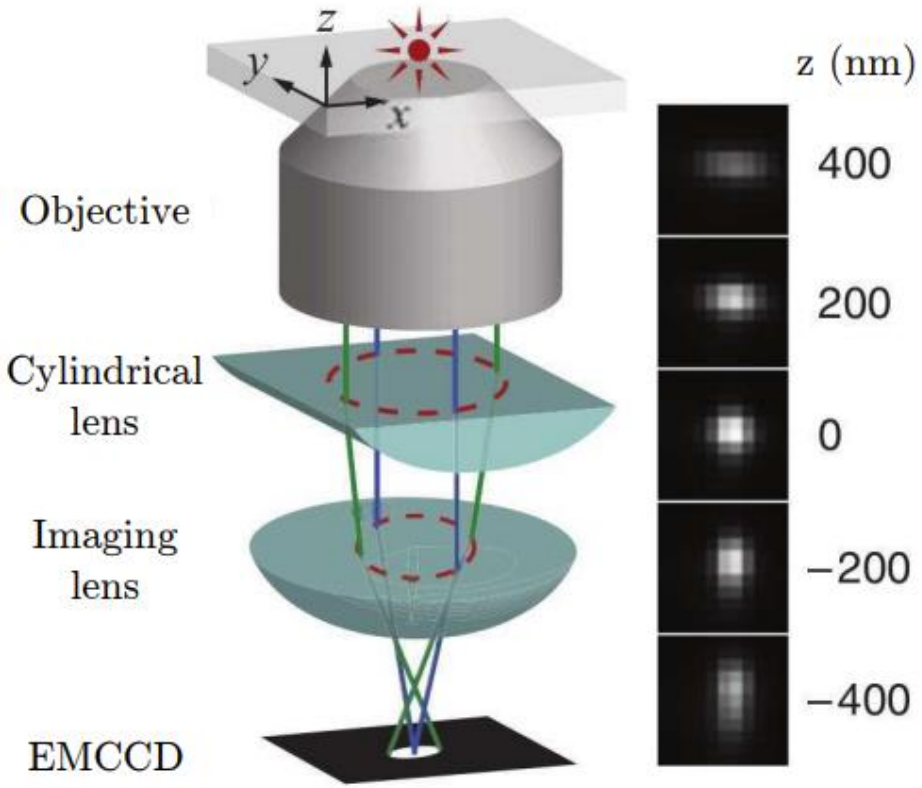
Single-Molecule Localization Microscopy (SMLM)

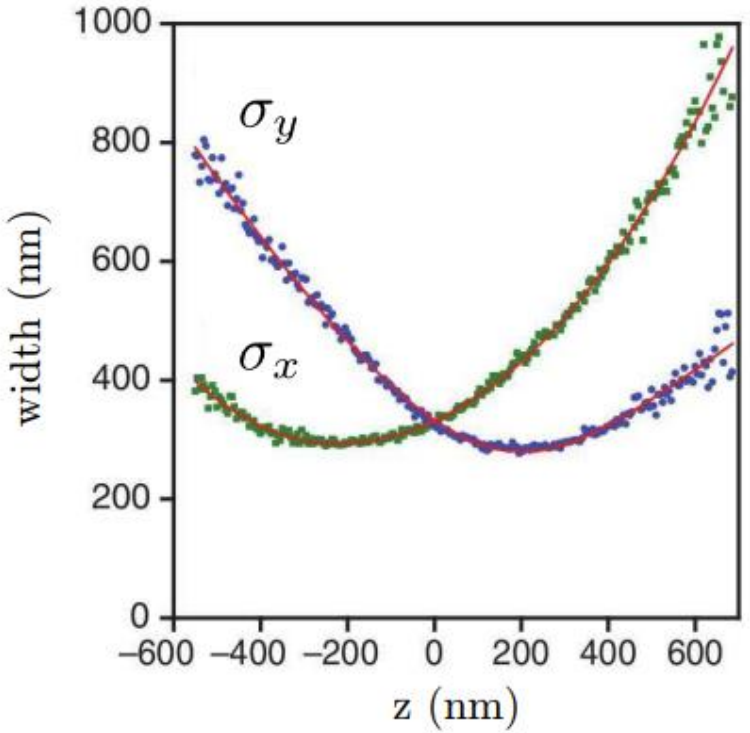


Single-Molecule Localization Microscopy (SMLM)

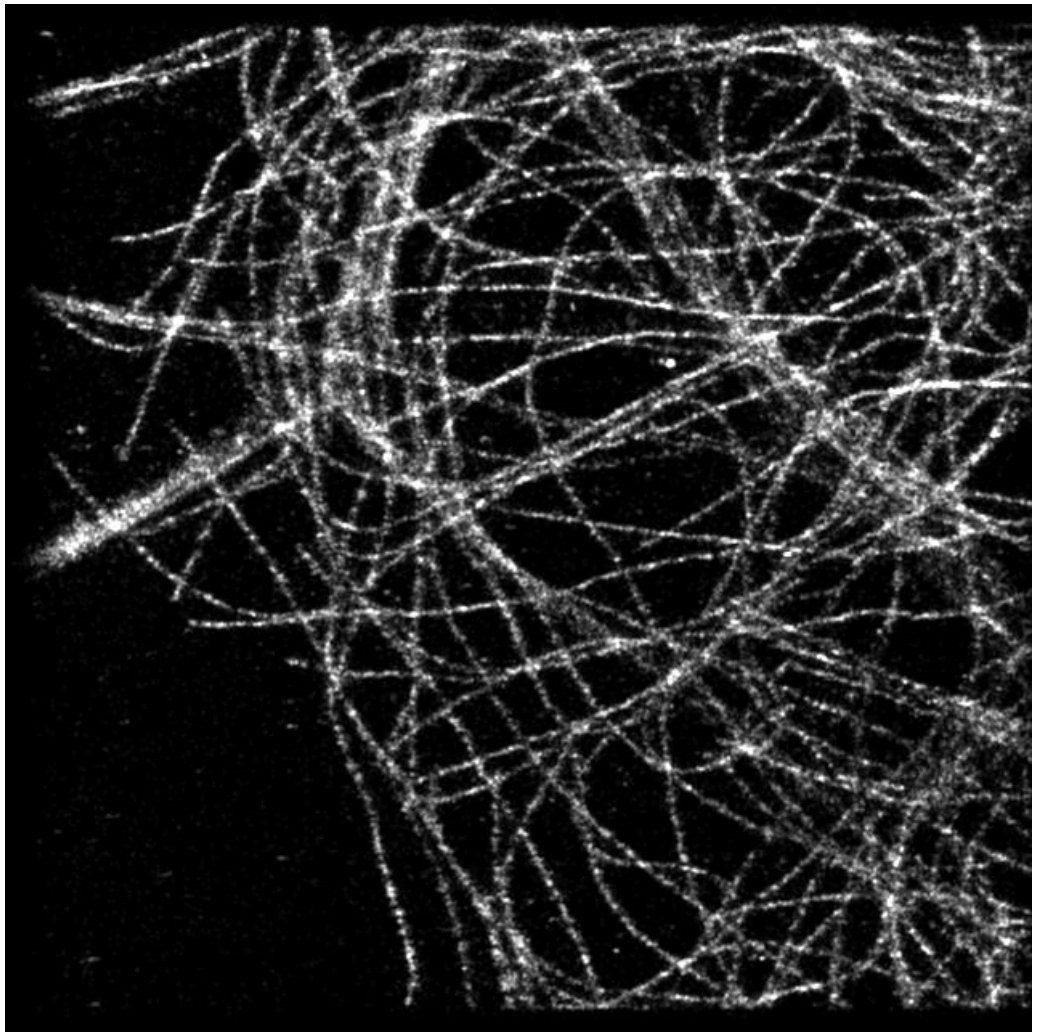


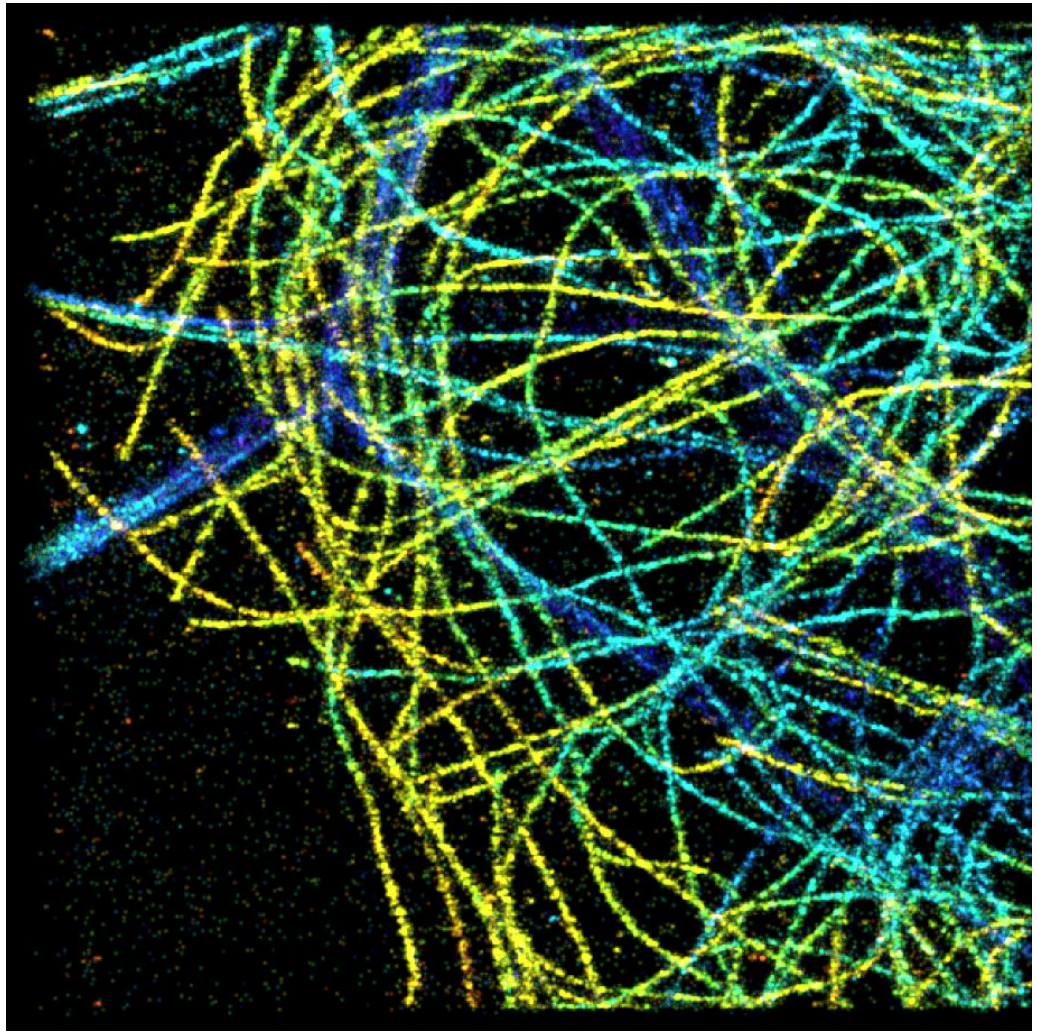
SMLM in 3D


z
y
x
Objective
Cylindrical lens
Imaging lens
EMCCD
z (nm)
400
200
0
−200
−400


1000
800
600
400
200
0
width (nm)
σ_y
σ_x
−600 −400 −200 0 200 400 600
z (nm)

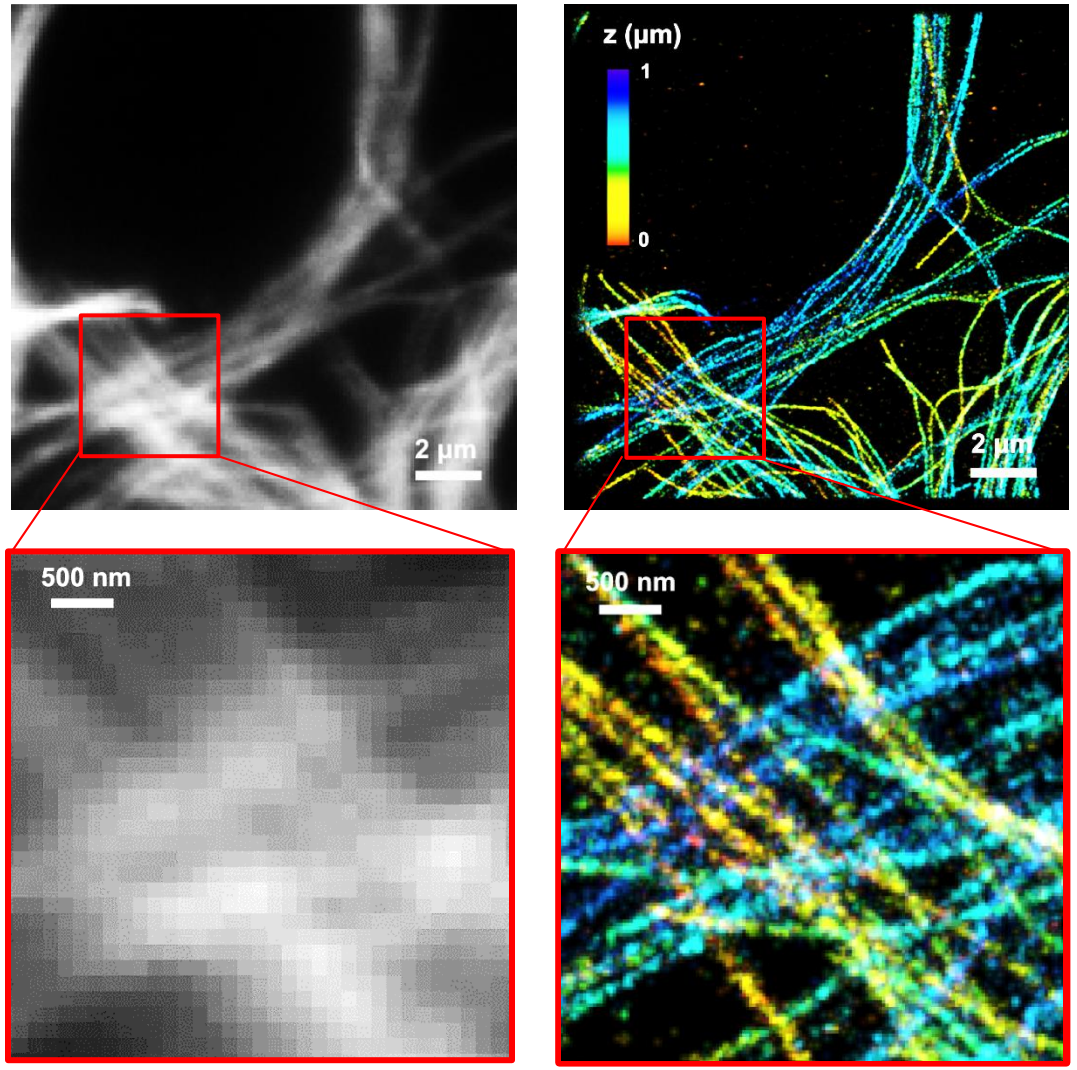
SMLM in 3D





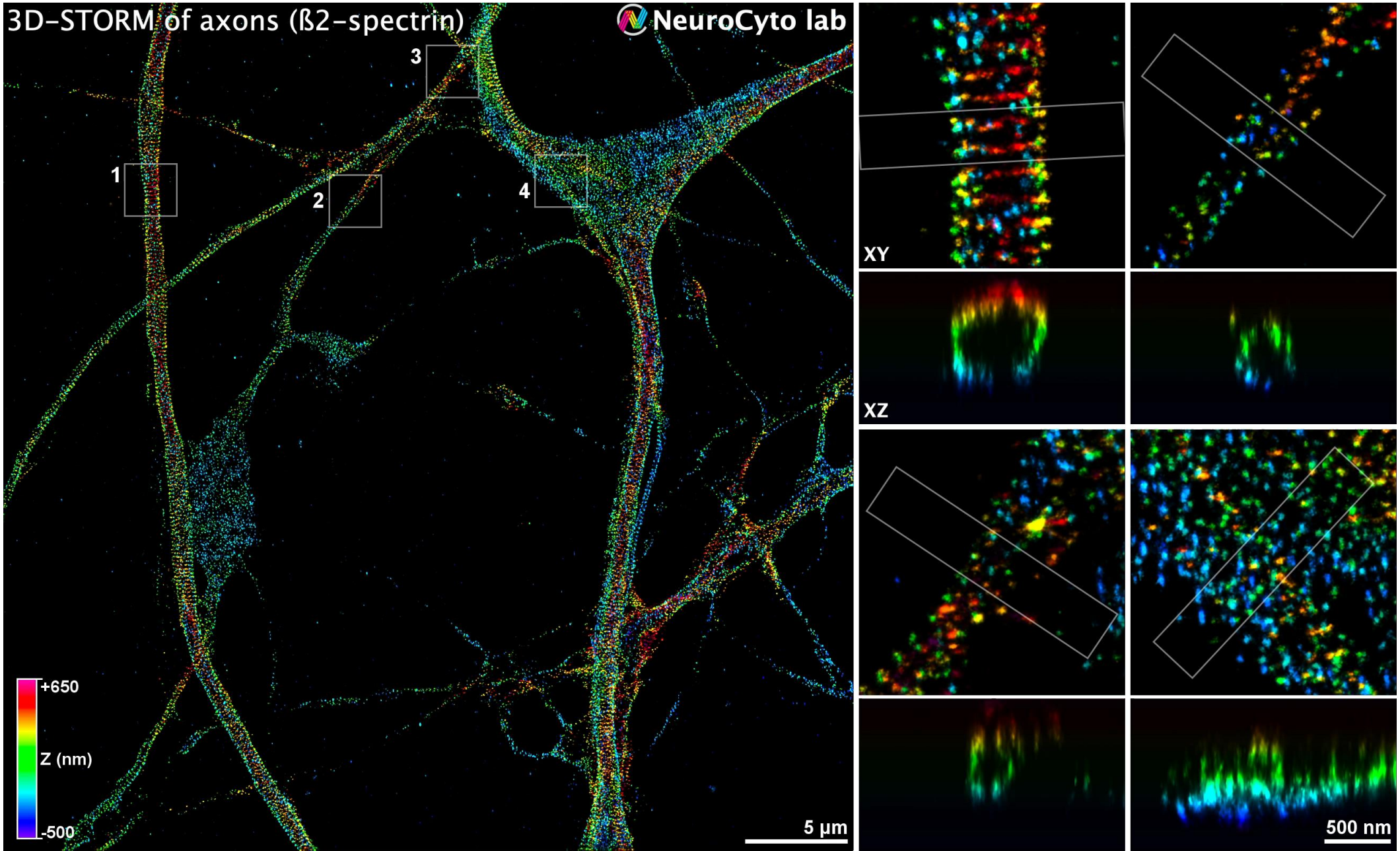
Federico Barabas – PhD thesis

SMLM in 3D



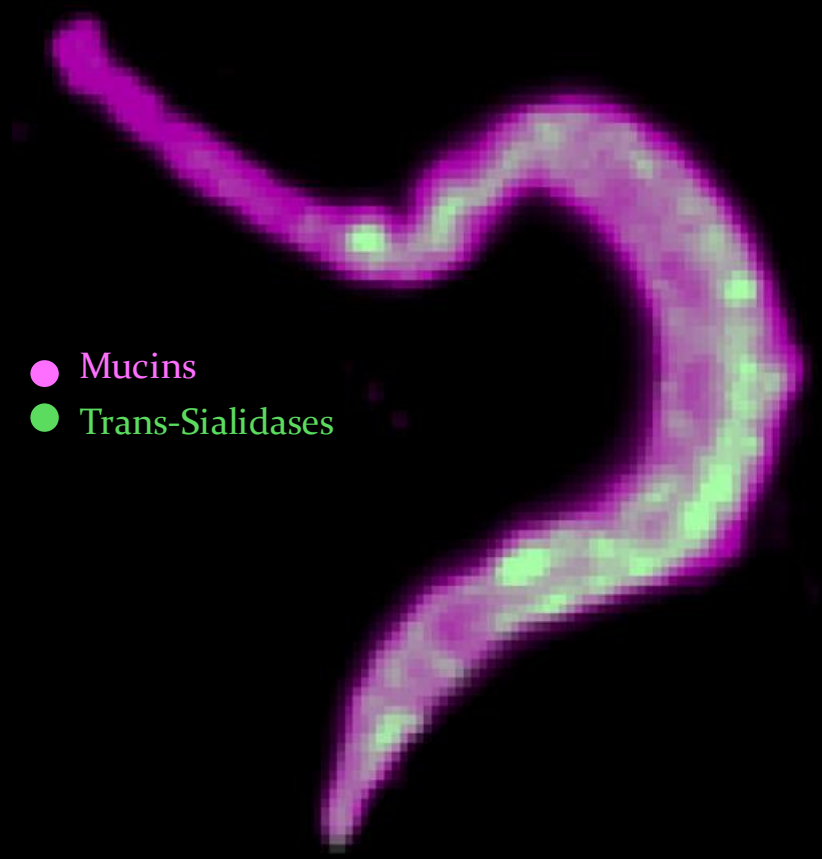
SMLM in 3D

Christophe Leterrier Lab

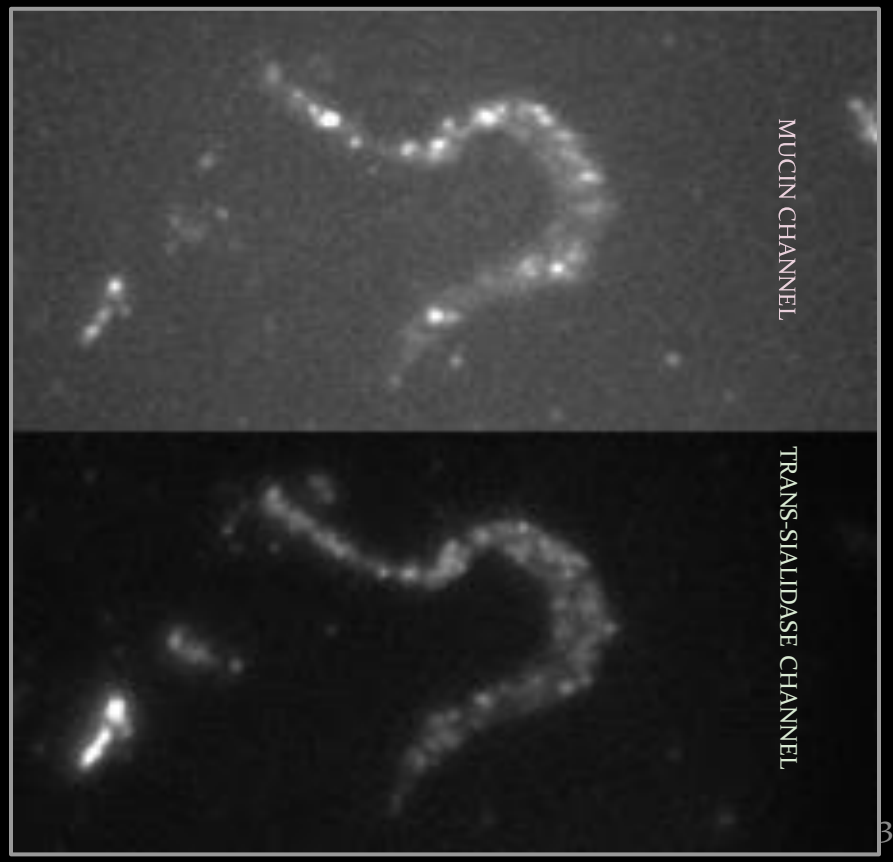


Multicolor SMLM

DIFFRACTION-LIMITED



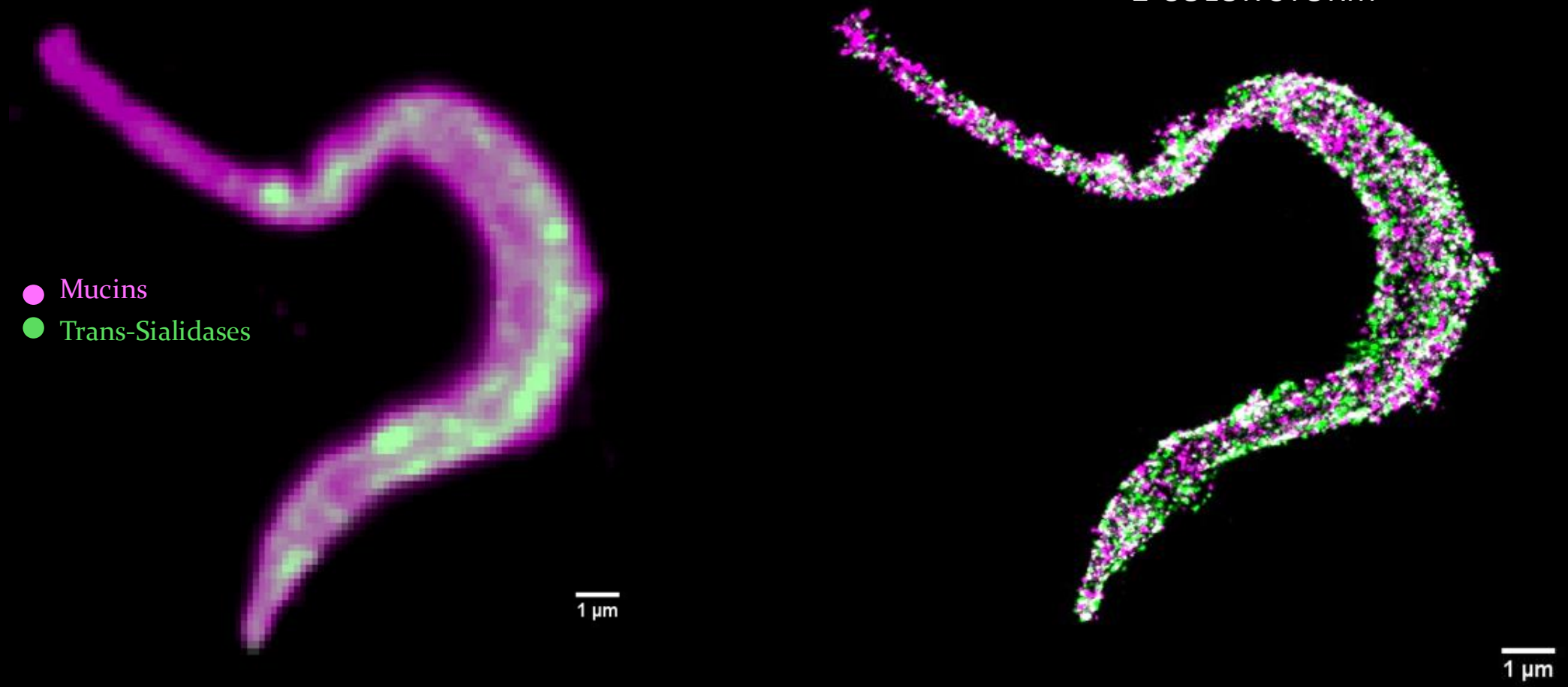
BLINKING SEQUENCE



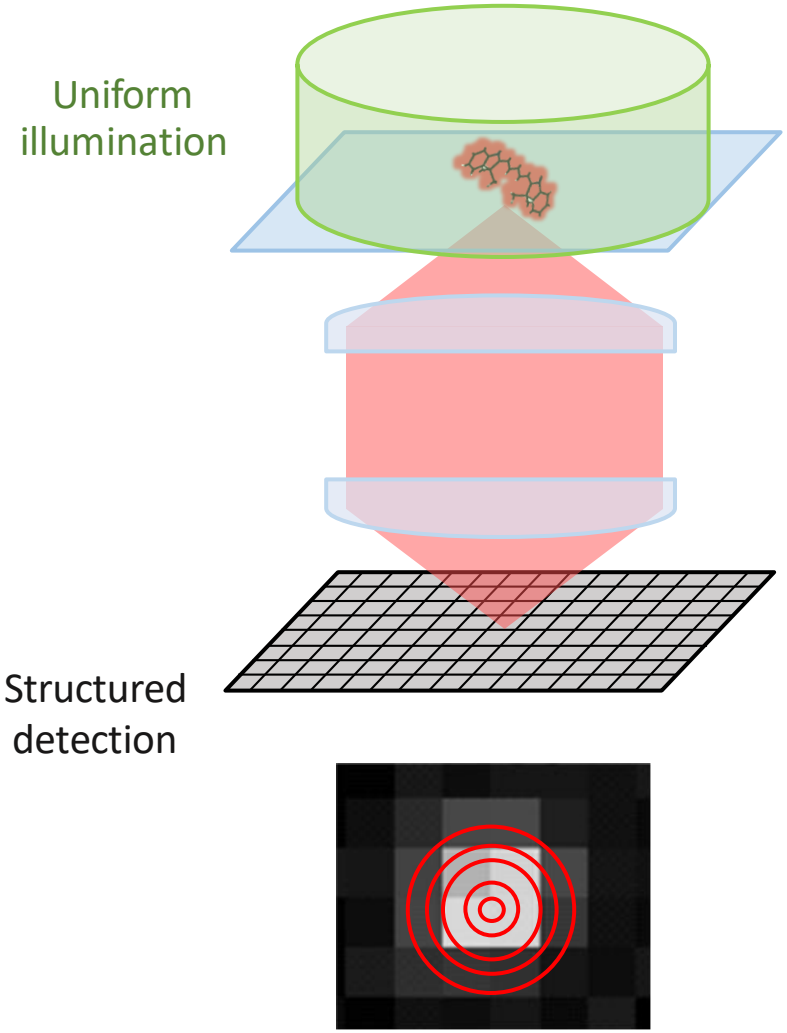
Multicolor SMLM

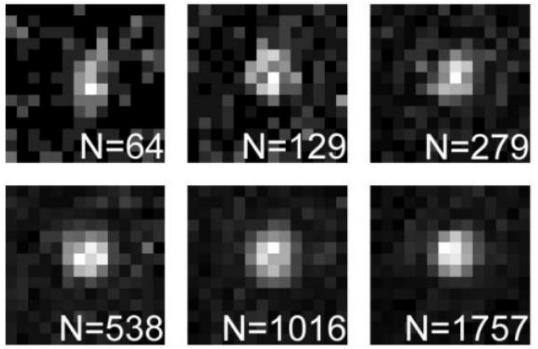
DIFFRACTION-LIMITED

2-COLOR STORM

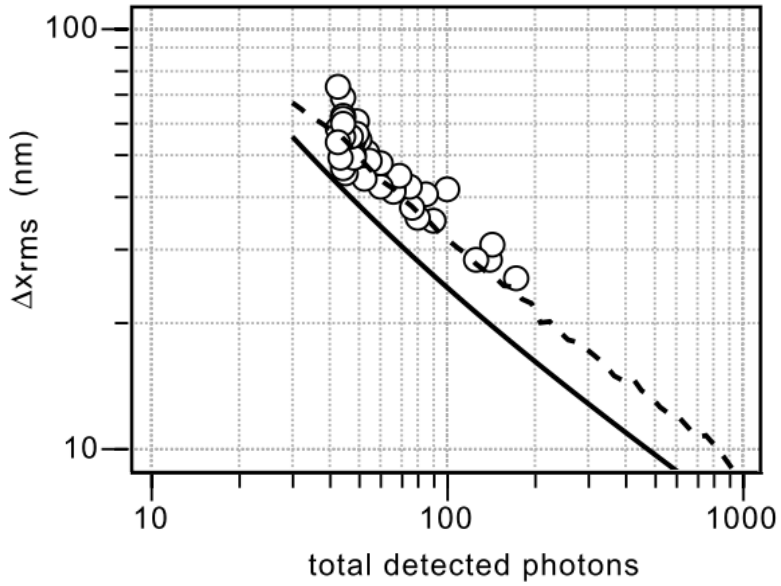


Camera-based single-molecule localization





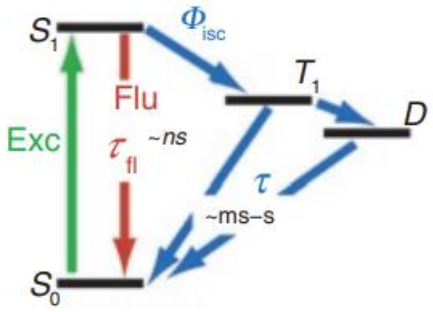
$$\sigma \approx PSF/\sqrt{N}$$

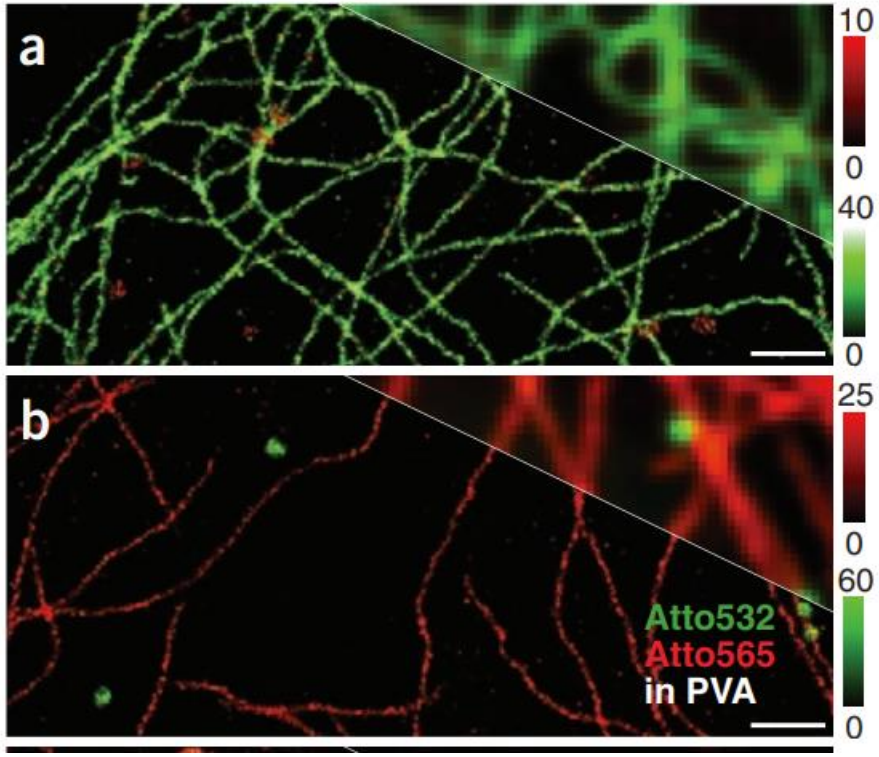


Thompson, R. E.; Larson, D. R.; Webb, W. W.
Biophysical Journal 82 (2002) 2775–2783

Various SMLM : excursion to the triplet state

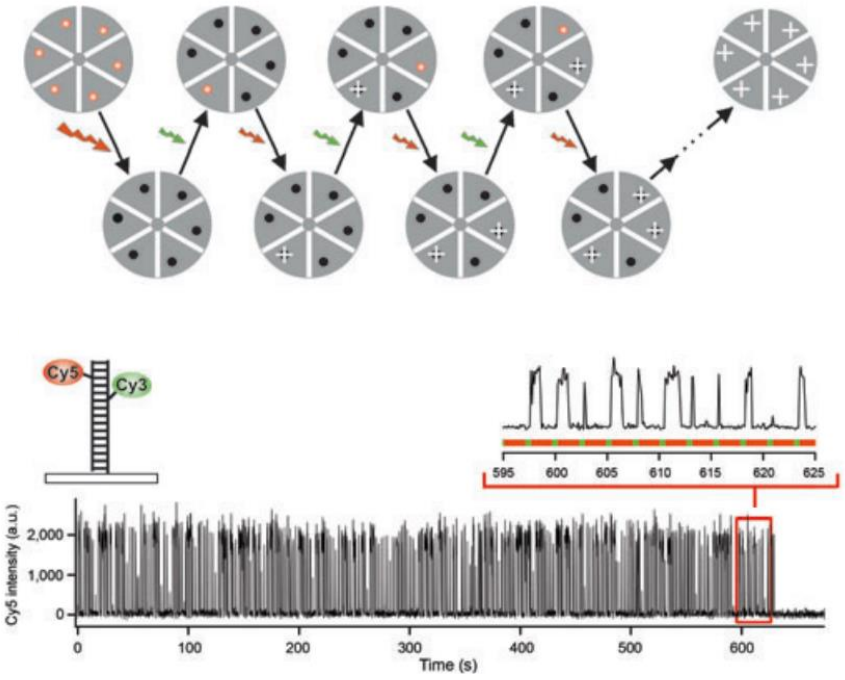
ON-OFF by excursion to triplelt states

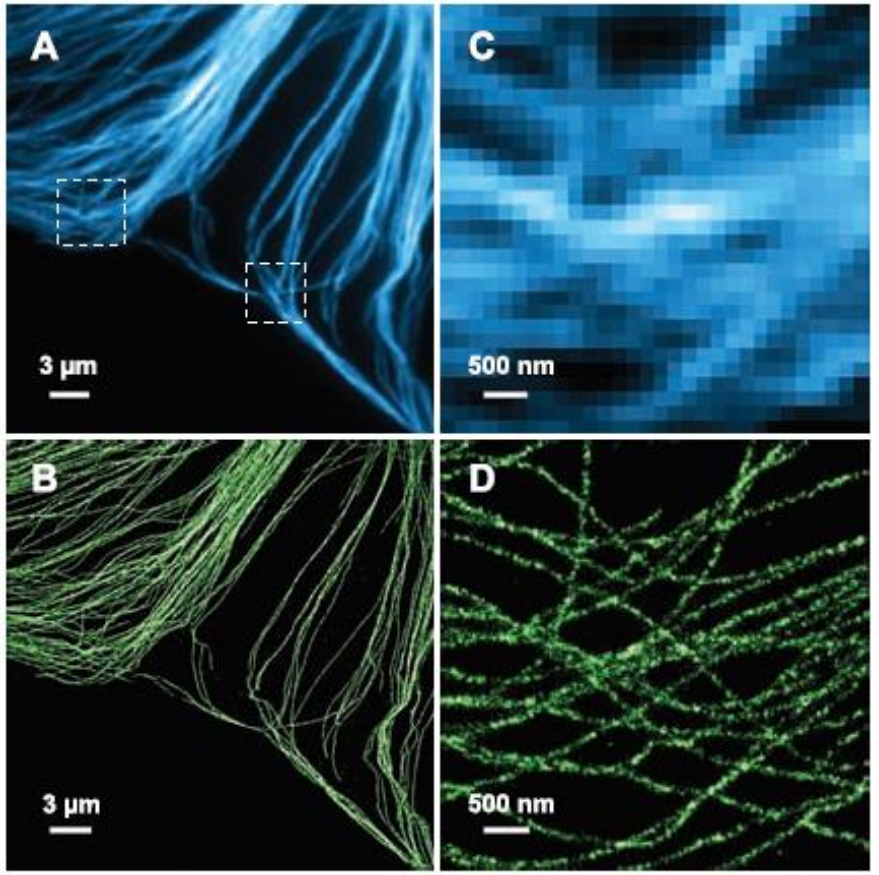




Stefan Hell Lab - Fölling et al. ***Nature Methods*** *5* (2008), 943–945

Various SMLM : photochromic fluorophores



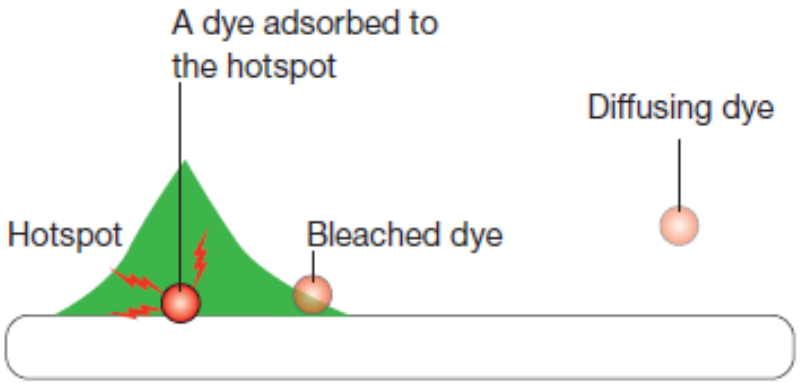


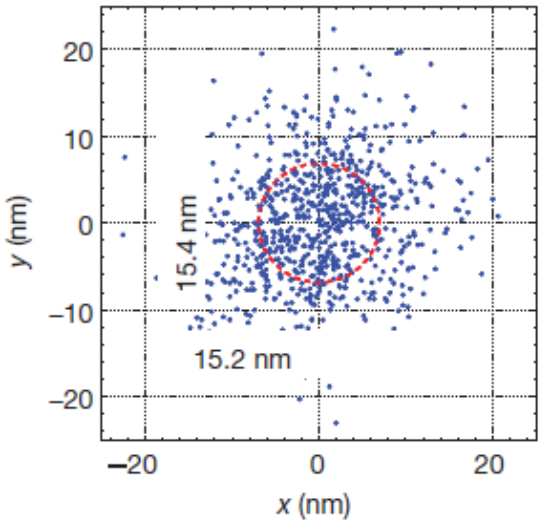
Xiaowei Zhuang Lab

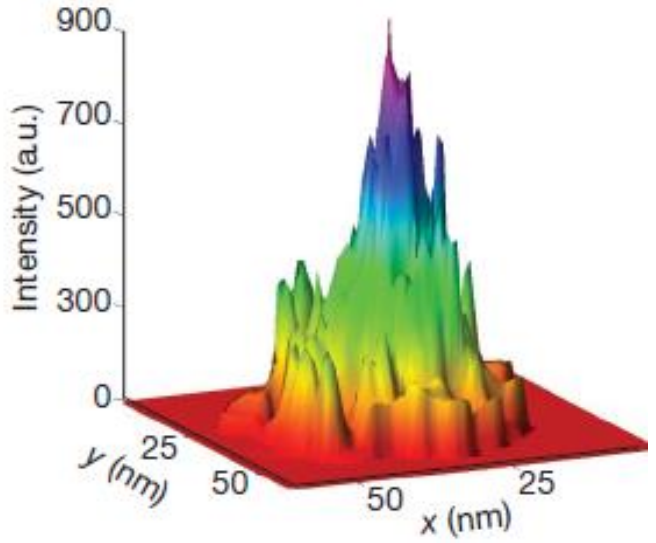
- Rust, M. J.et al. ***Nat. Methods*** 3 (2006) 793–795 (2006) / Bates, M. et al. ***Science*** 317 (2007) 1749–53

Various SMLM : random adsorption PAINT

ON-OFF by adsorption





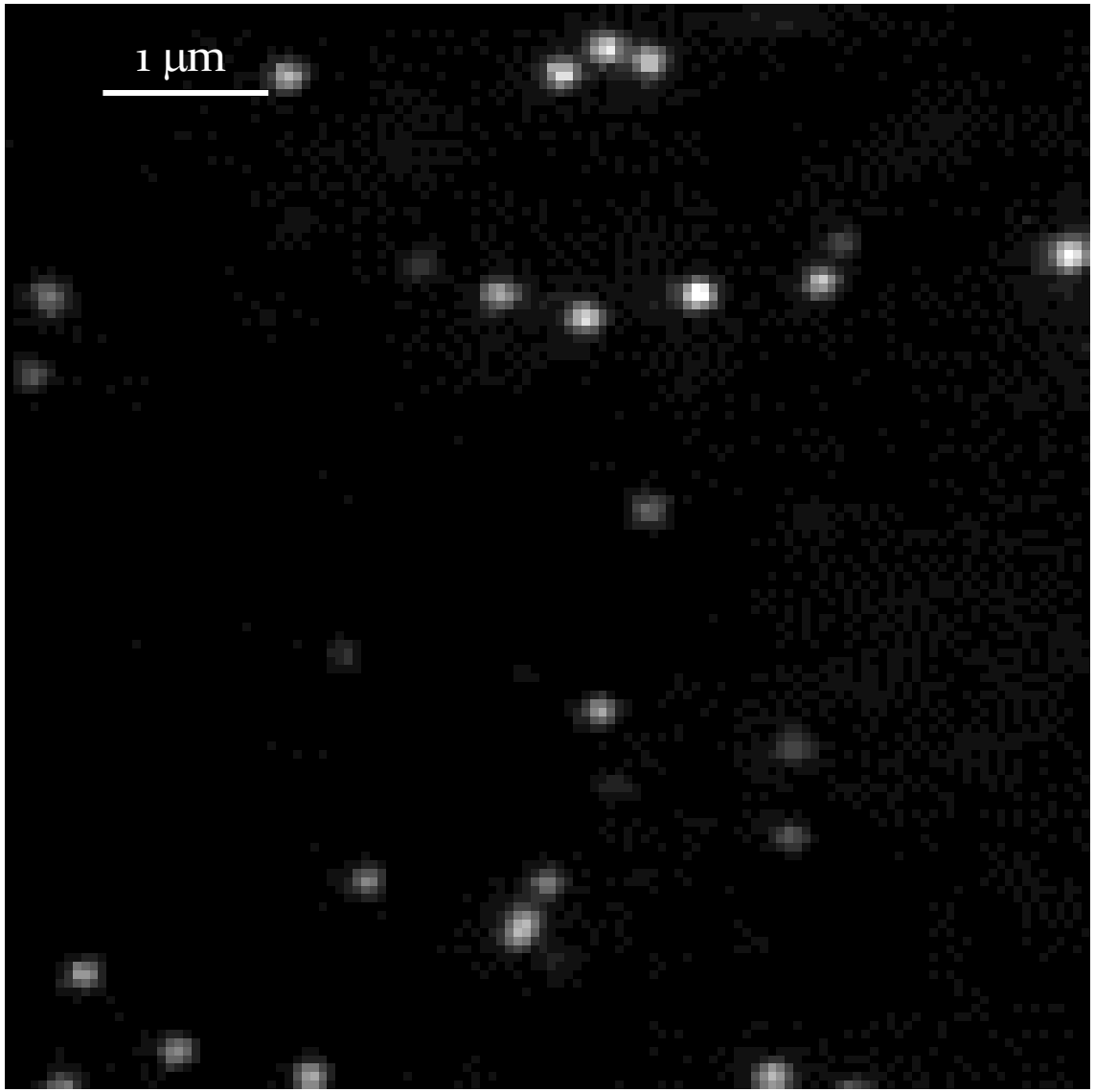


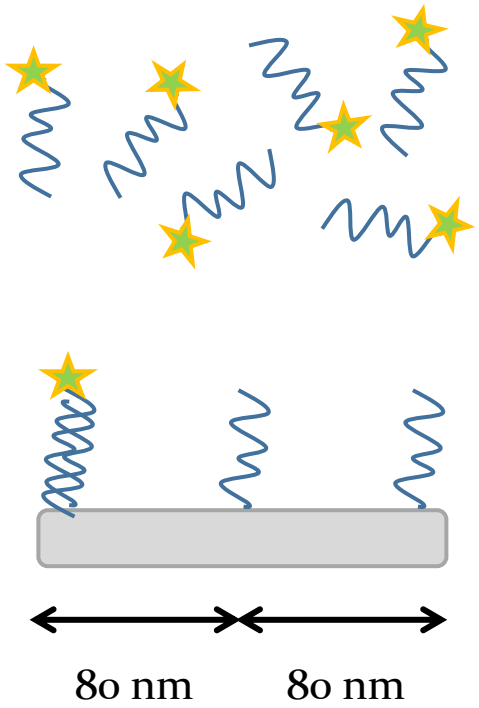
Xiang Zhang Lab - Cang, H. et al. ***Nature*** 469 (2011) 385-388

PAINT: Points Accumulation for Imaging in Nanoscale Topography

A. Sharonov & R. M. Hochstrasser ***PNAS*** 103 (2006) 18911-18916

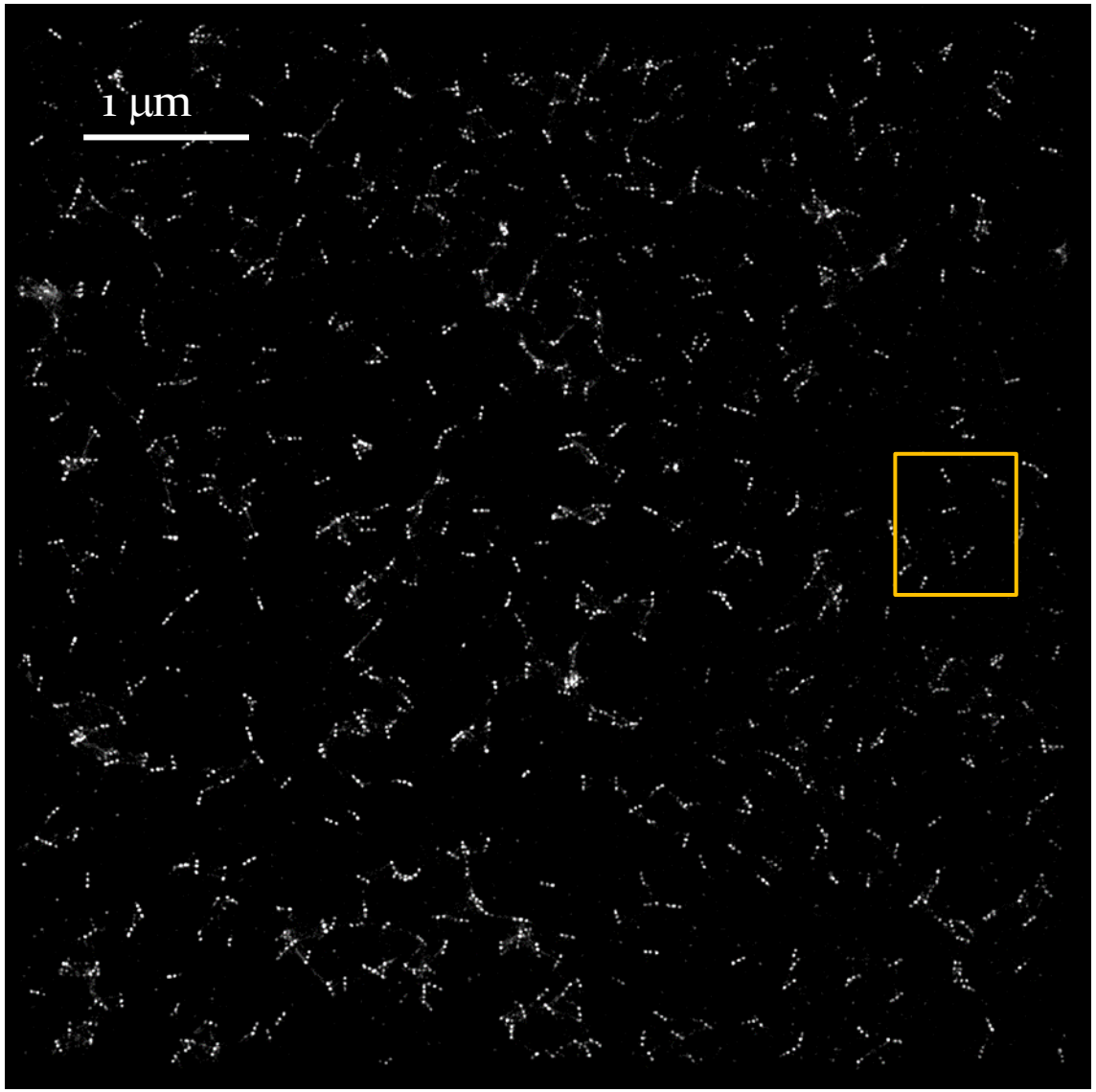
Various SMLM : DNA-PAINT

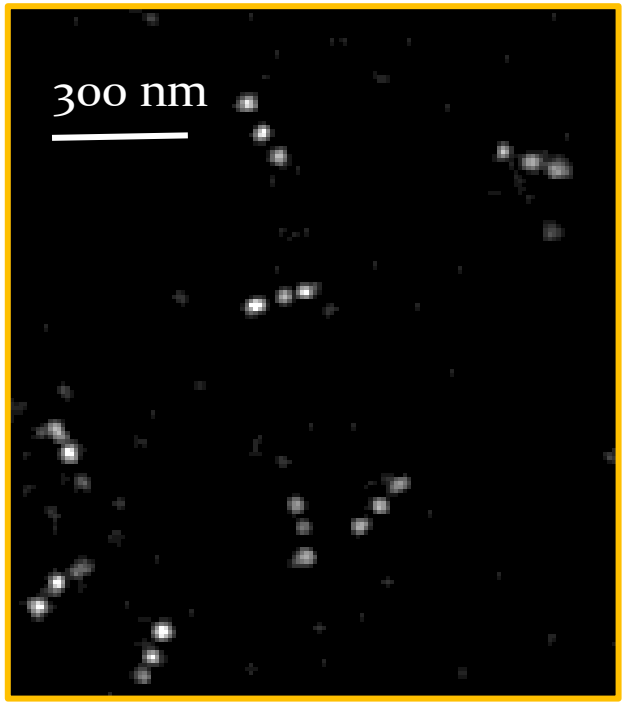




GATTAquant
DNA NANOTECHNOLOGIES

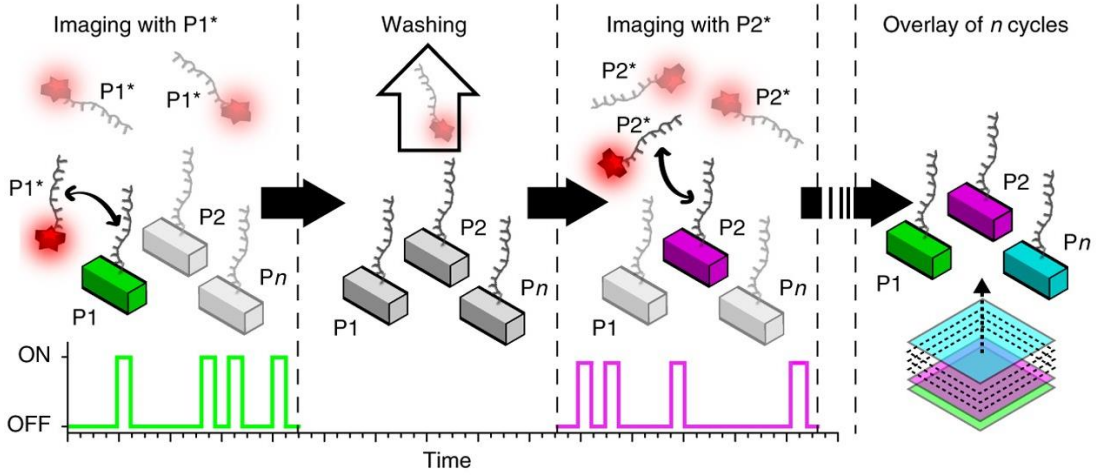
Various SMLM : DNA-PAINT

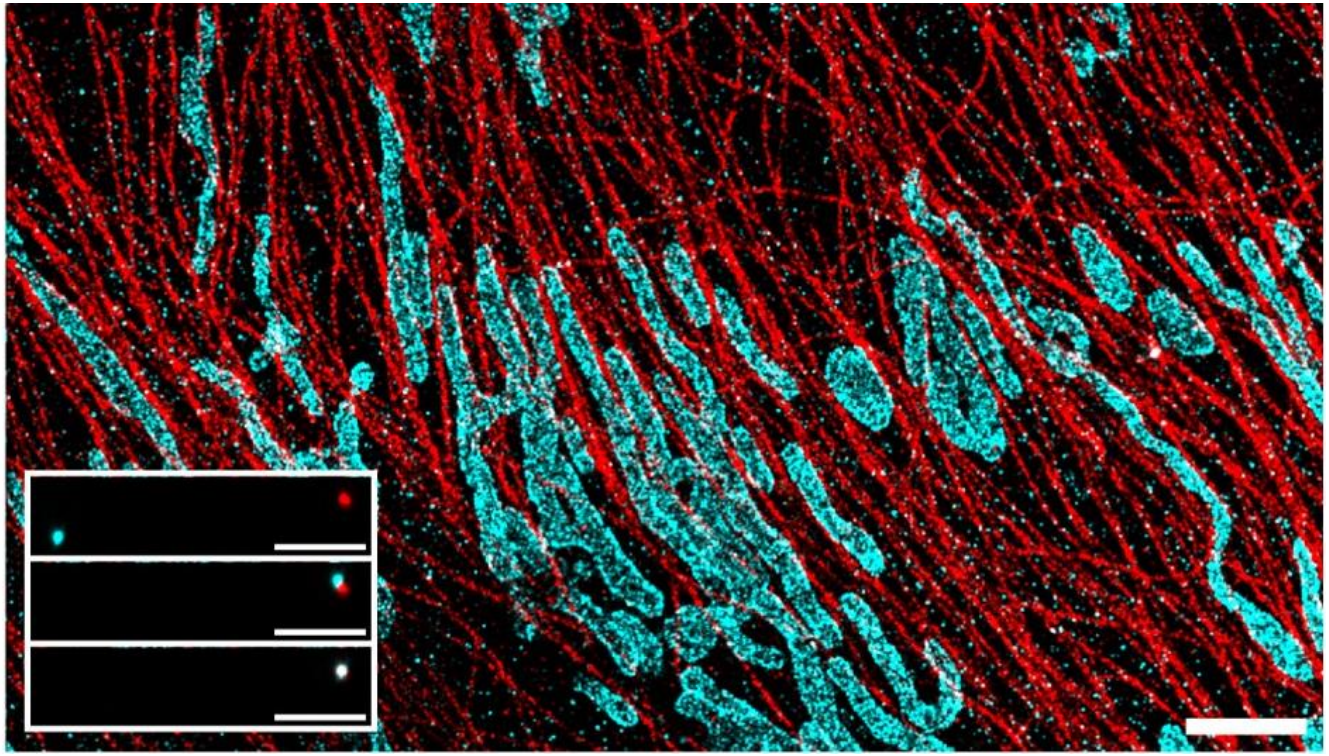




GATTAquant
DNA NANOTECHNOLOGIES

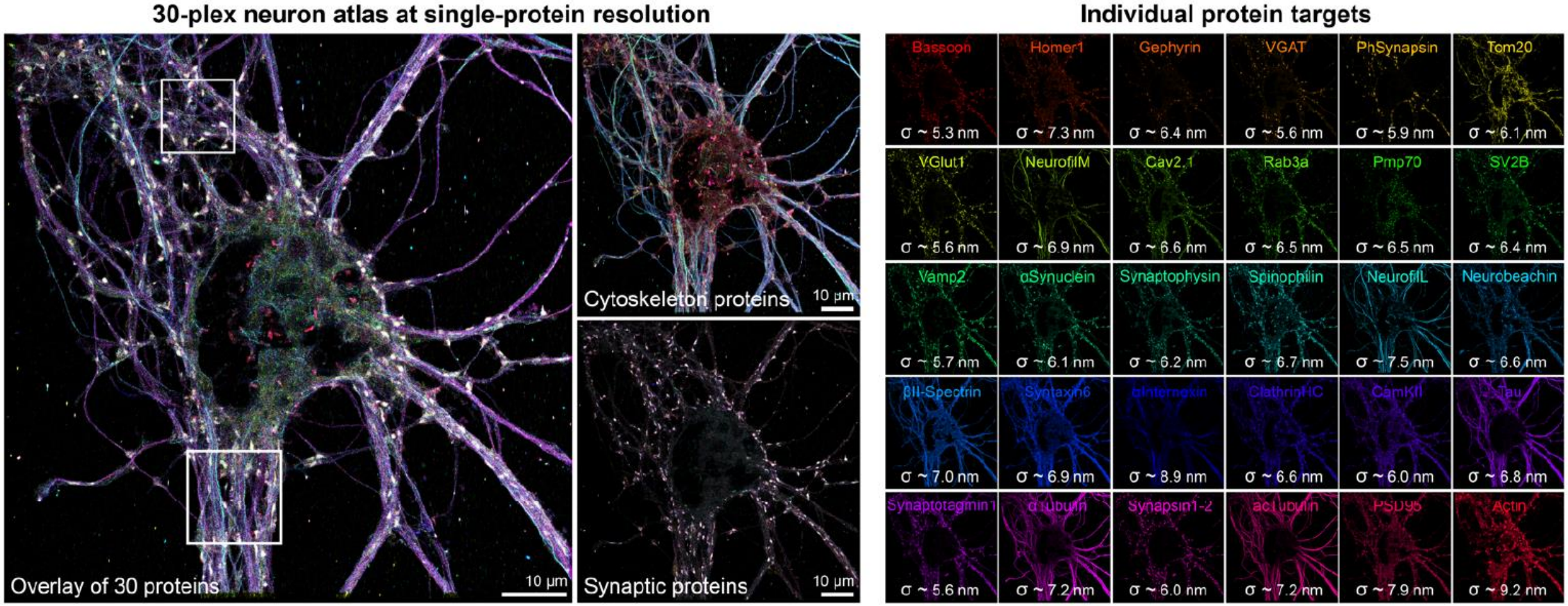
Multicolor DNA-PAINT by sequential multiplexing





Ralf Jungmann Lab - Schnitzbauer, J. ***Nature Protocols*** 12 (2017) 1198 - 1228

Multicolor DNA-PAINT by sequential multiplexing



Ralf Jungmann Lab - Unterauer et al. bioRxiv (2023)

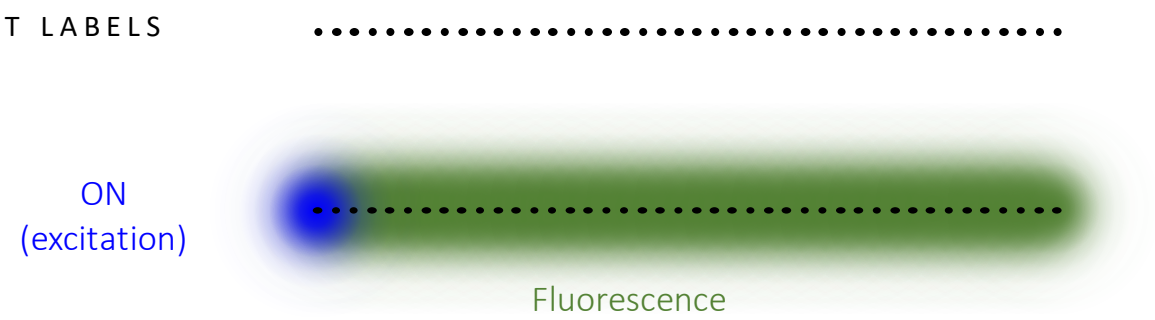
Coordinate-targeted super-resolution microscopy

Coordinate-targeted super-resolution microscopy

FLUORESCENT LABELS .....

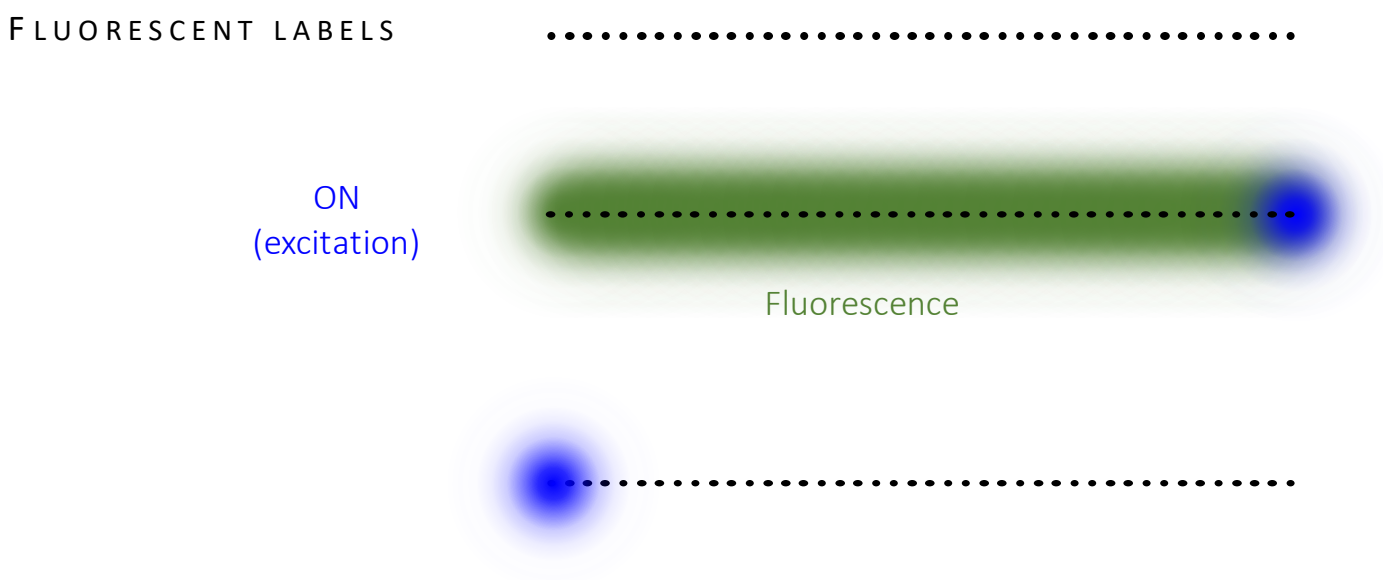
Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

Coordinate-targeted super-resolution microscopy



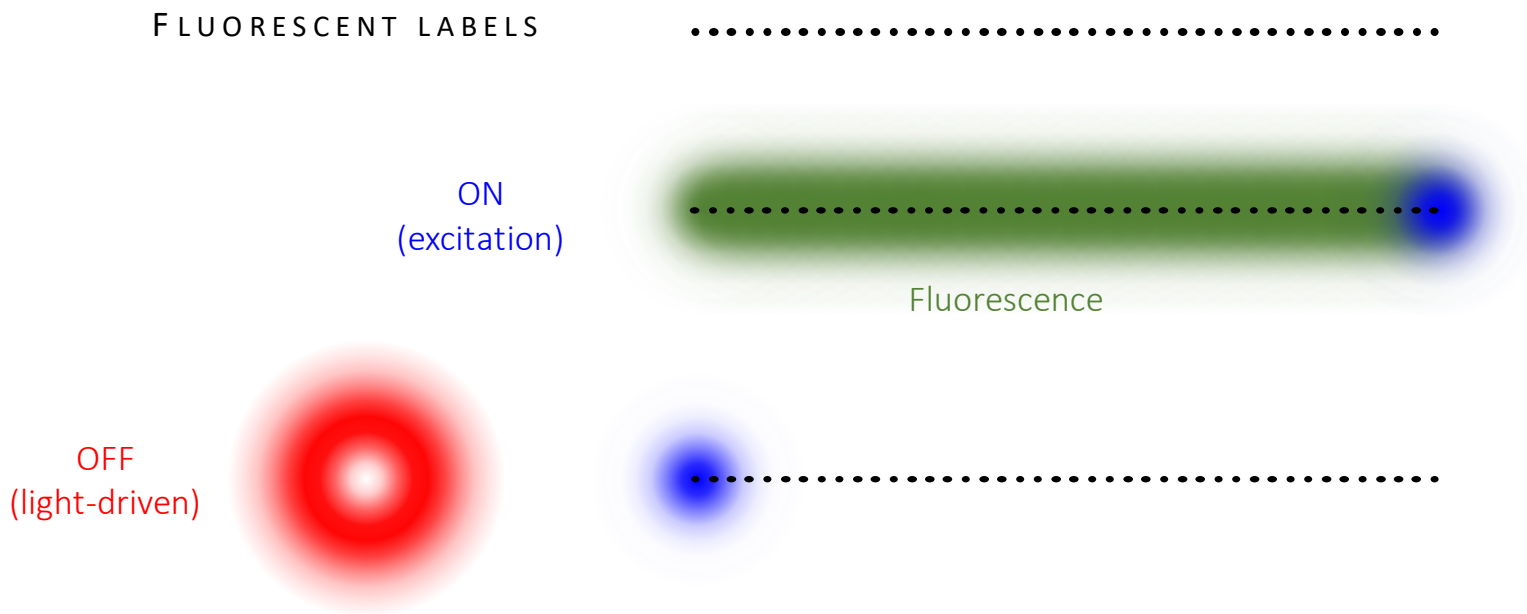
Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

Coordinate-targeted super-resolution microscopy



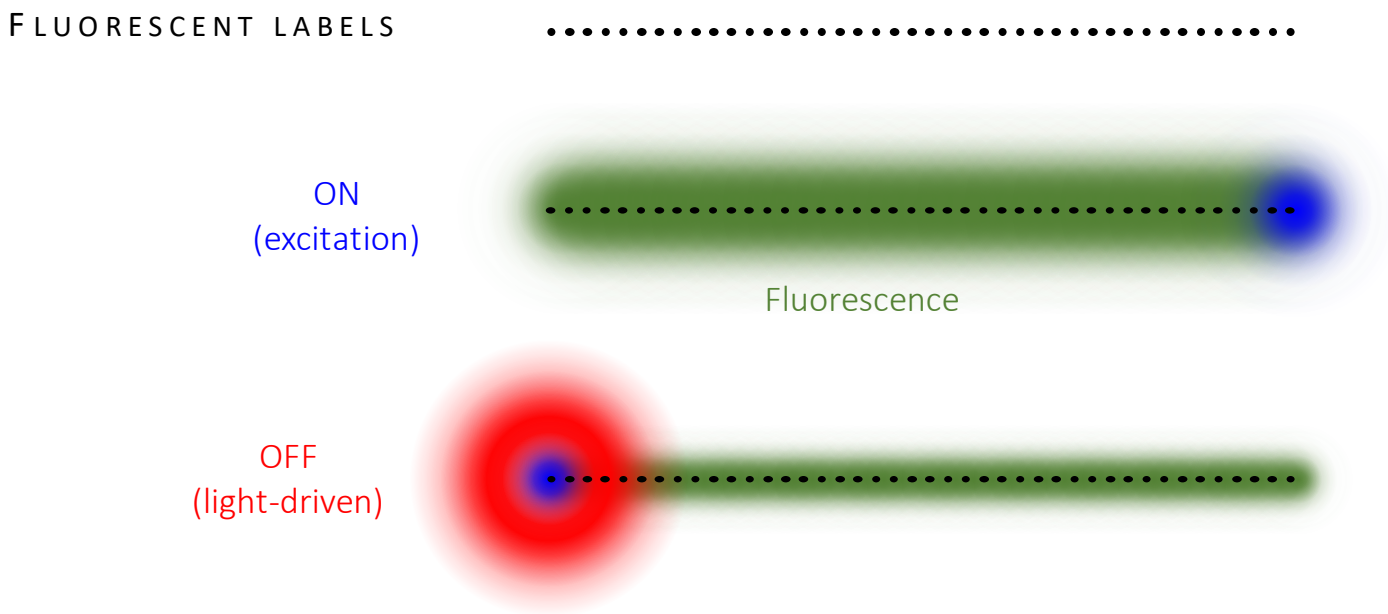
Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

Coordinate-targeted super-resolution microscopy



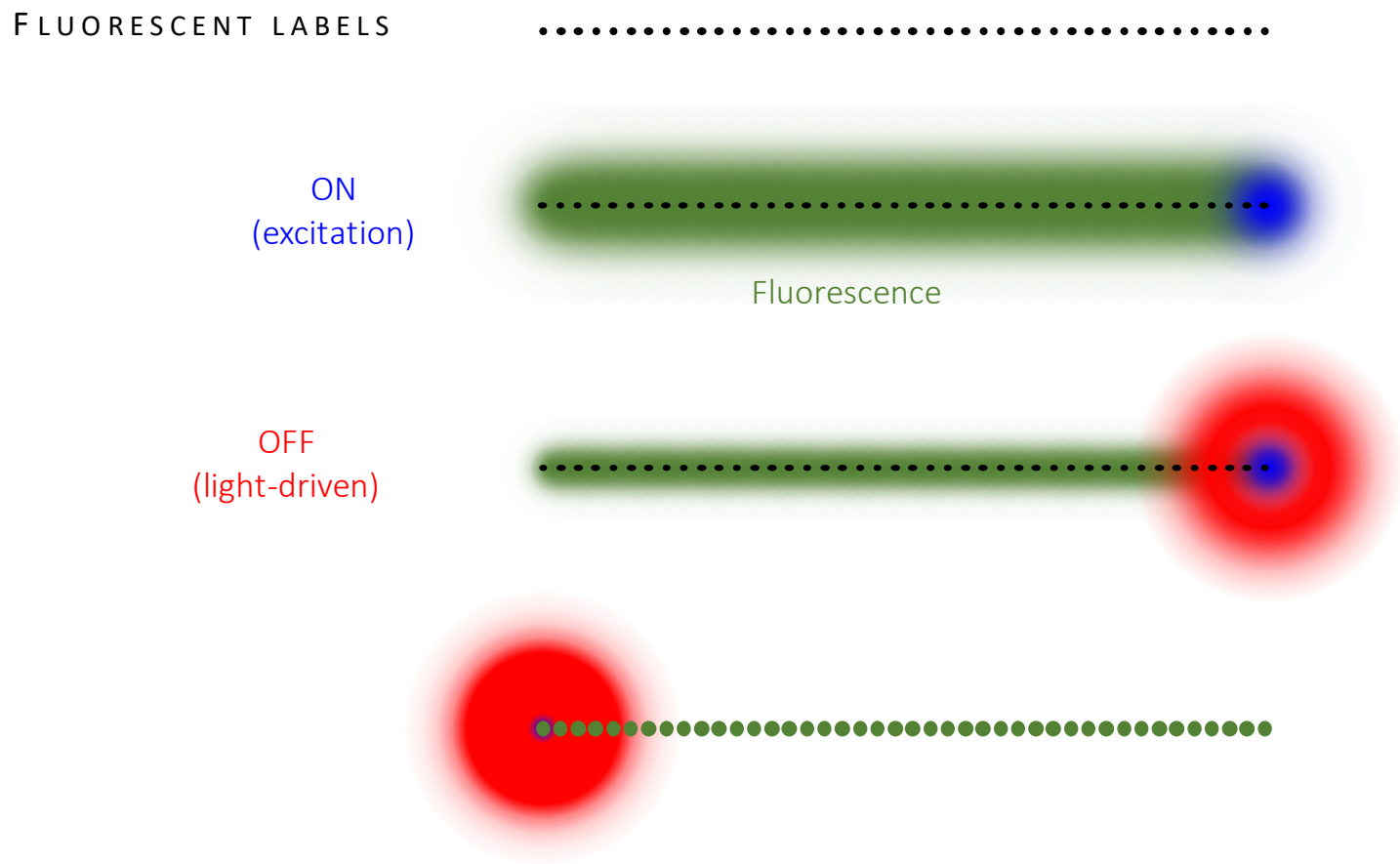
Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

Coordinate-targeted super-resolution microscopy



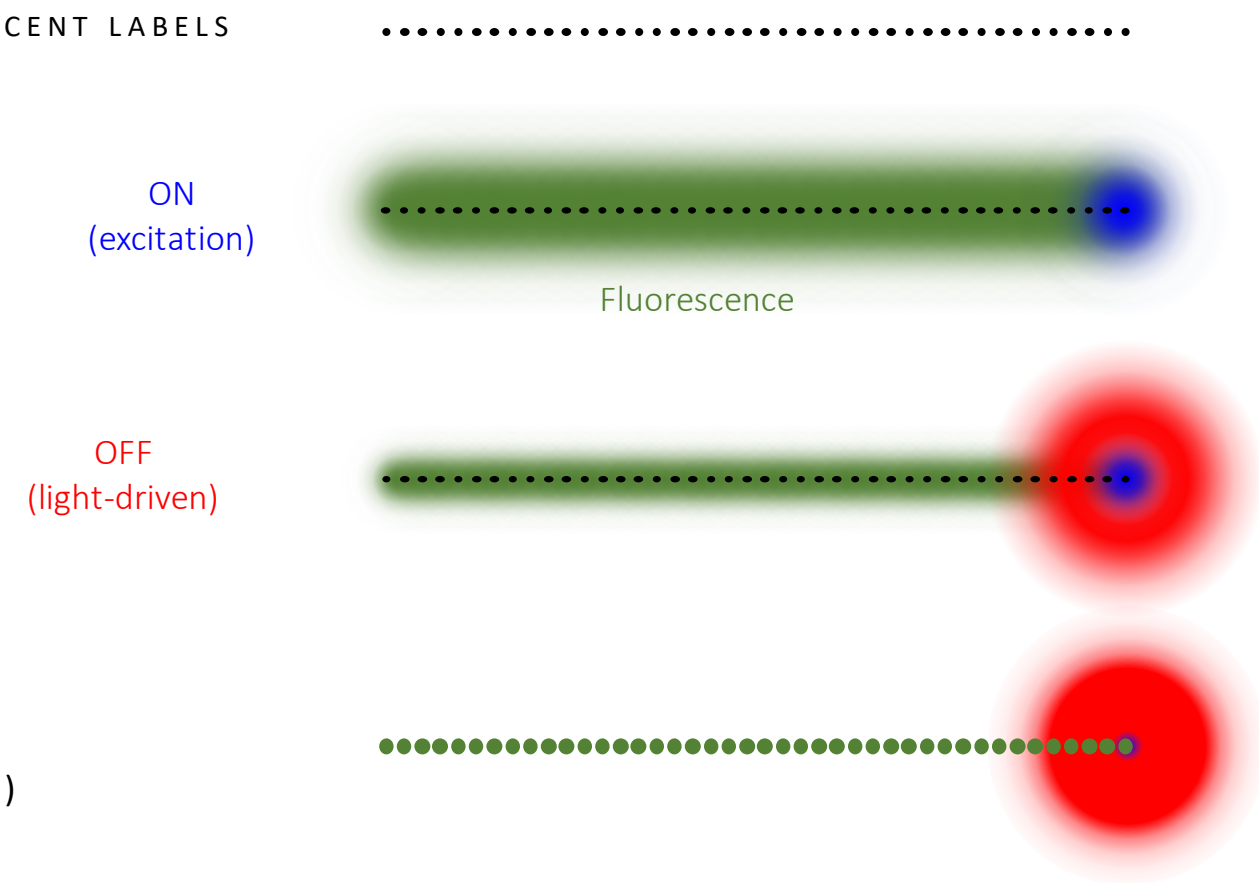
Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

Coordinate-targeted super-resolution microscopy



Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

Coordinate-targeted super-resolution microscopy

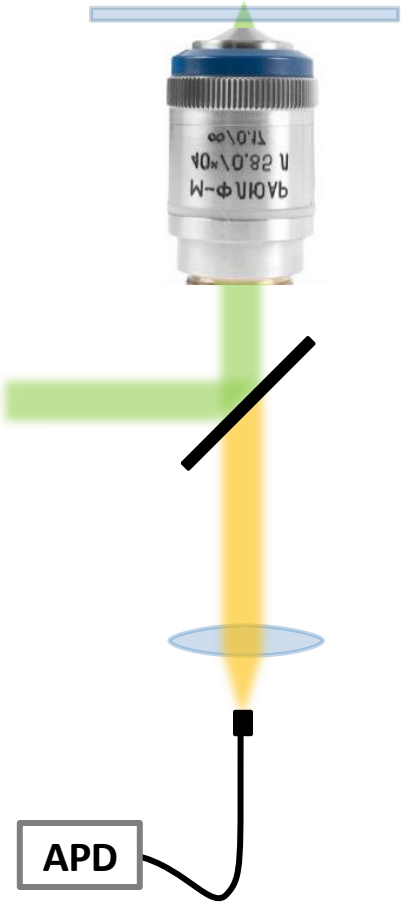


- STIMULATED EMISSION (STED)
- GROUND-STATE DEPLETION (GSDIM)
- PHOTOCHROMISM
- …

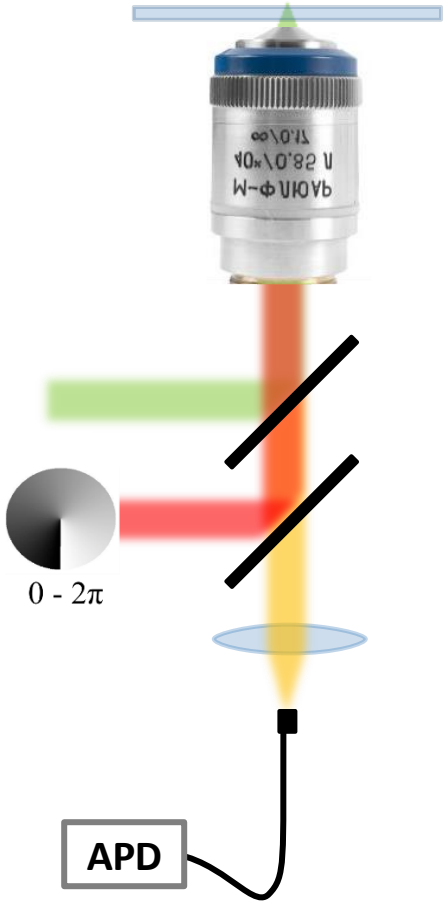
Stefan Hell Lab - Hell and Wichmann. ***Optics Lett***. 19 (1994) 780-782 // Klar and Hell. ***Optics Lett***. 24 (1999) 954-956

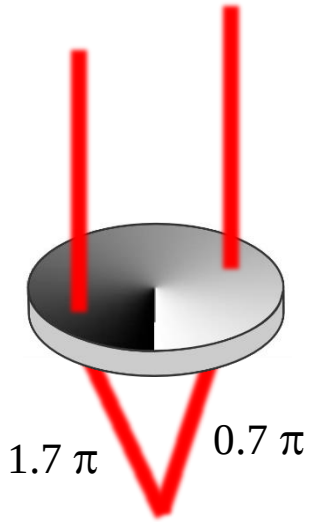
Coordinate-targeted super-resolution microscopy

Confocal Fluorescence Microscope



Coordinate Targetted Nanoscope

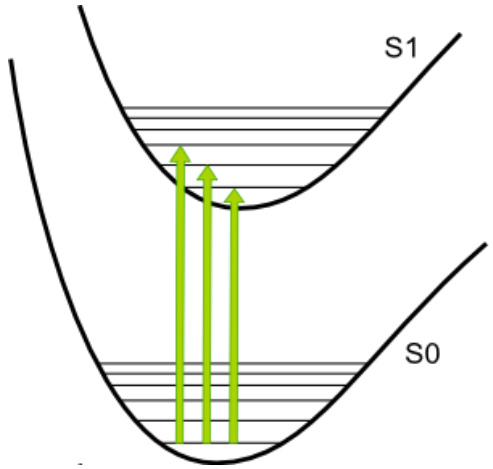


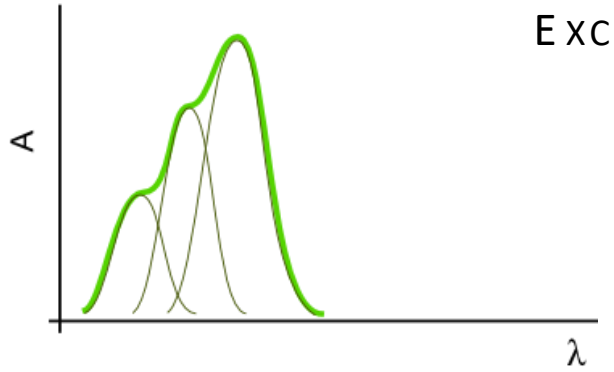


Vortex phase plate

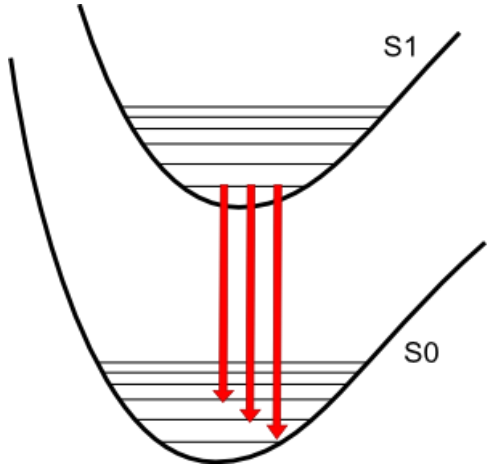
scanning, pulse sync, high-power, positional and focus stability, …

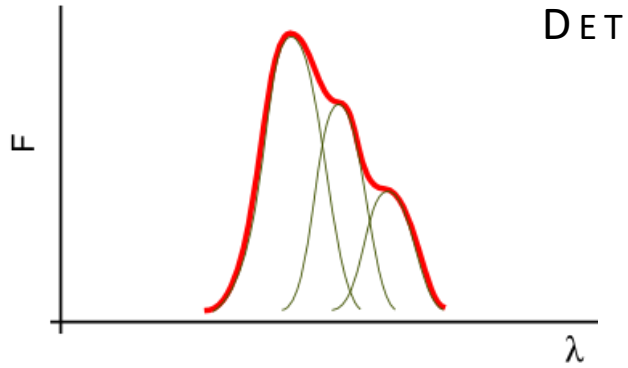
STED nanoscopy





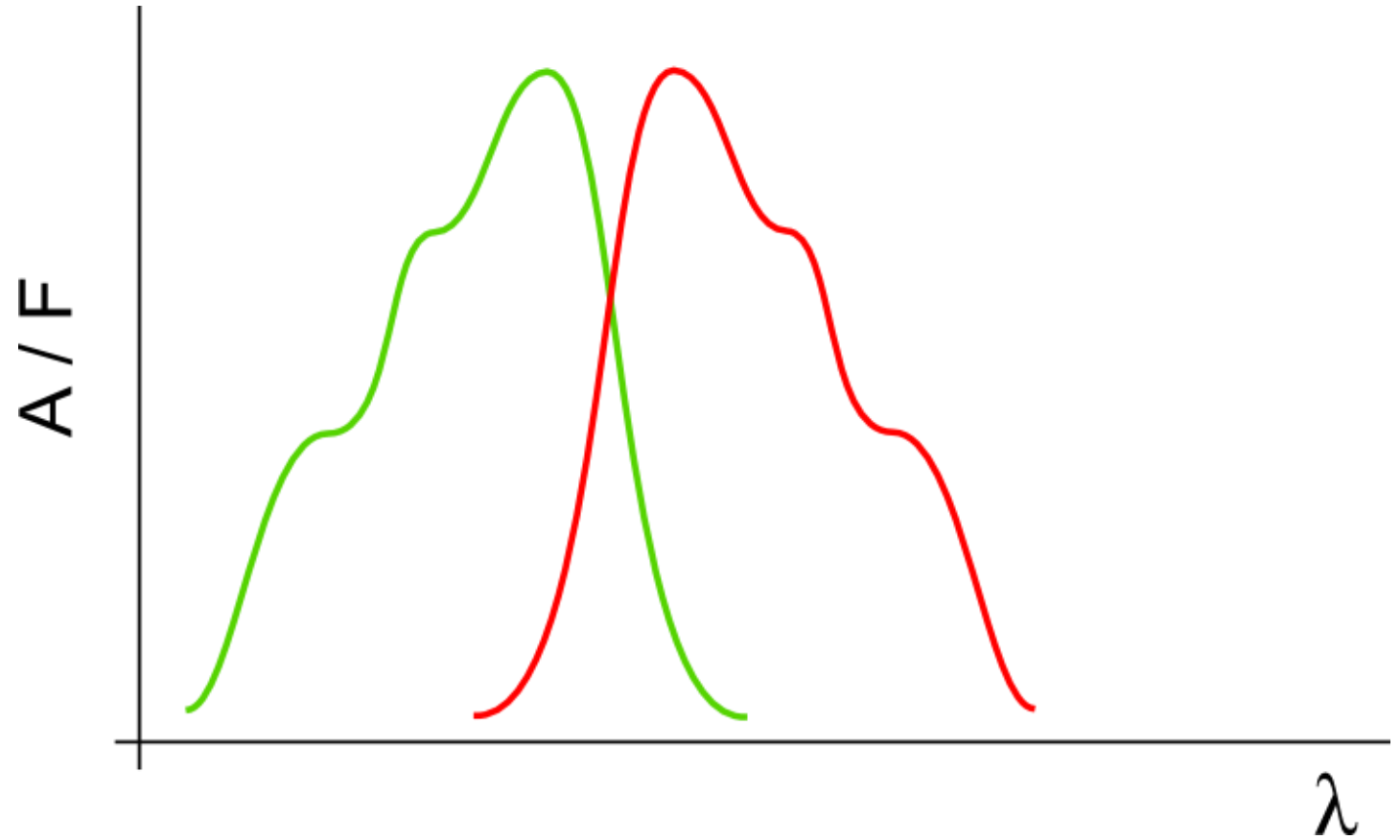
EXCITATION



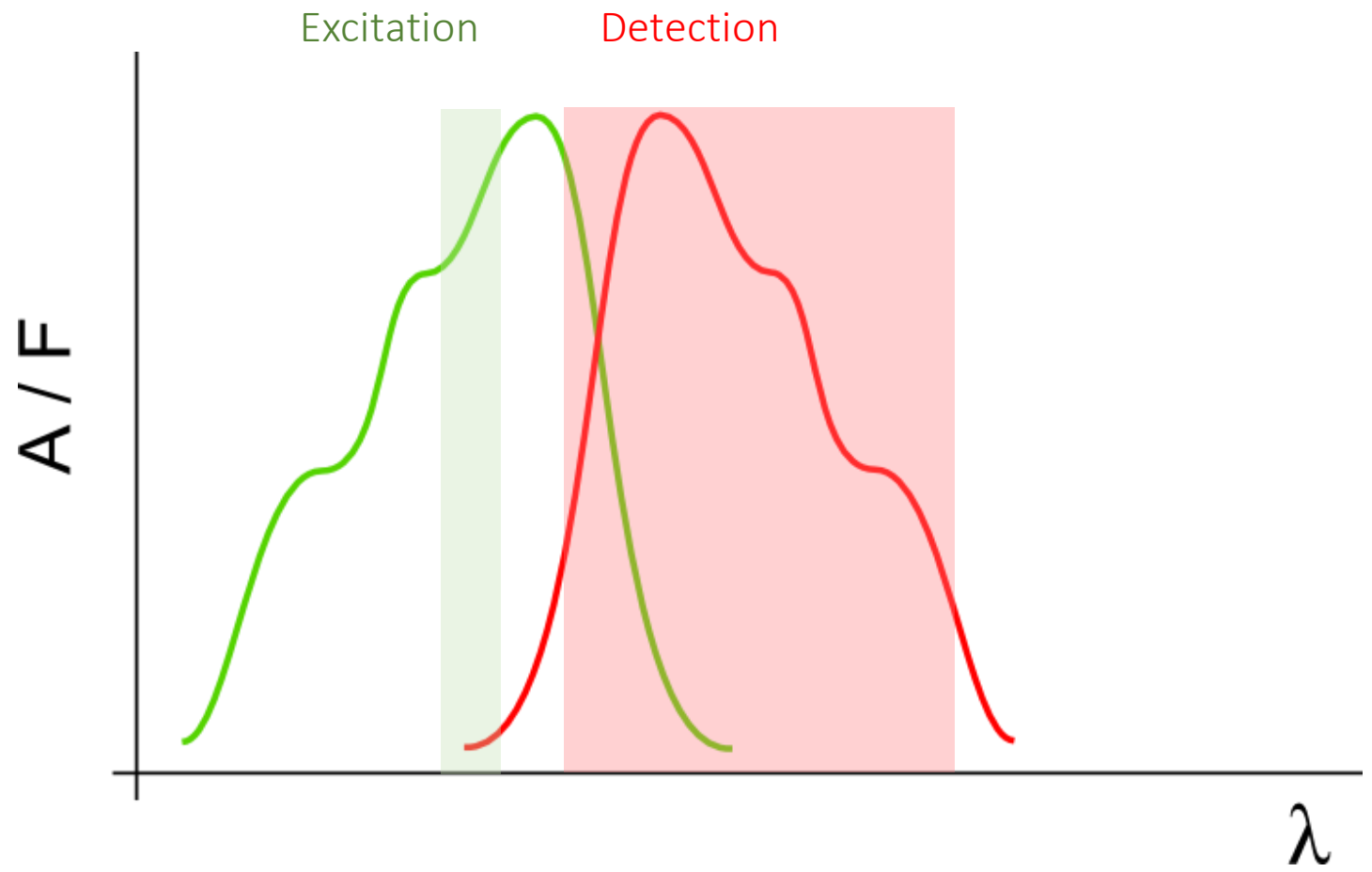


DETECTION

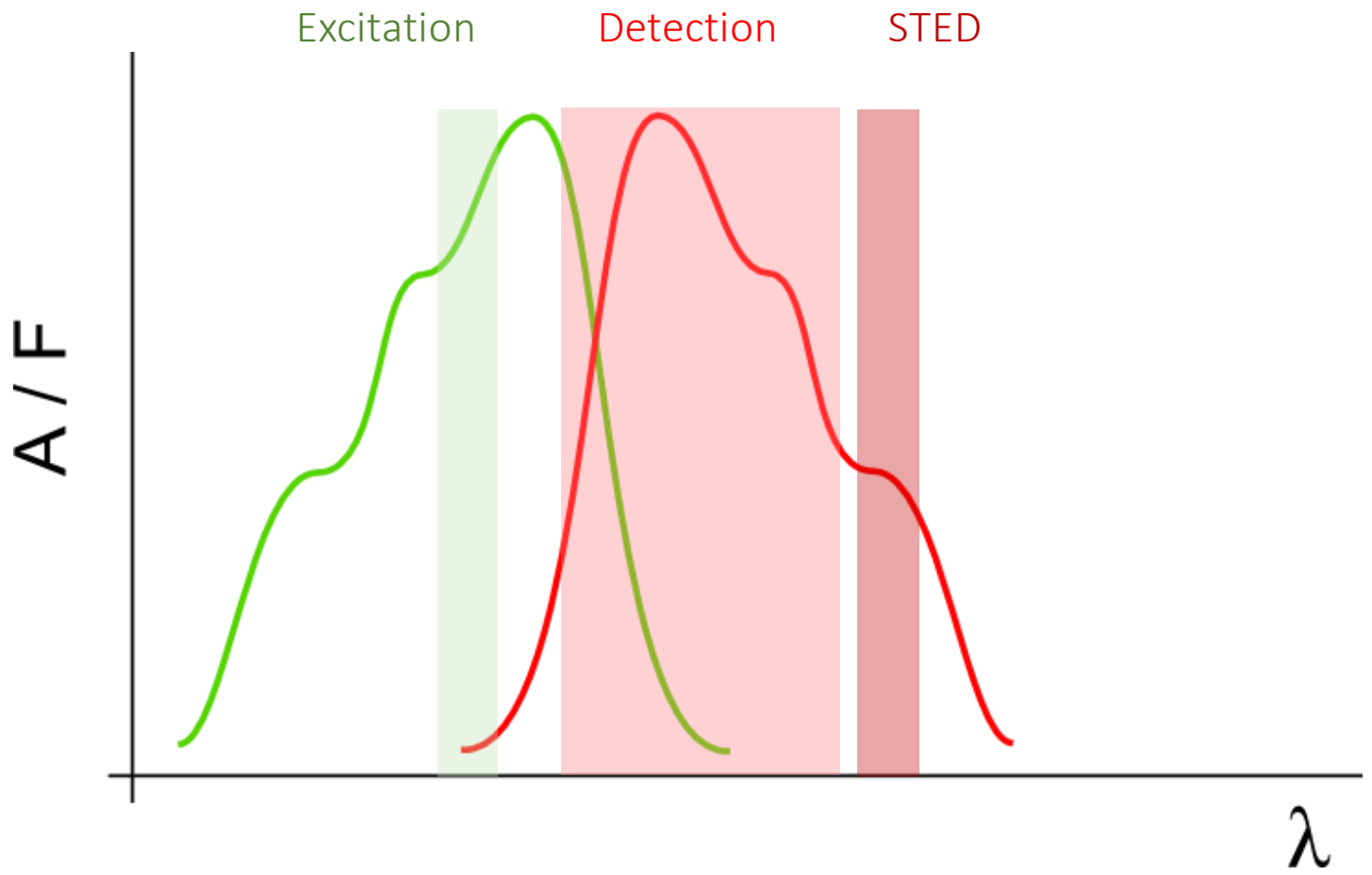
STED nanoscopy



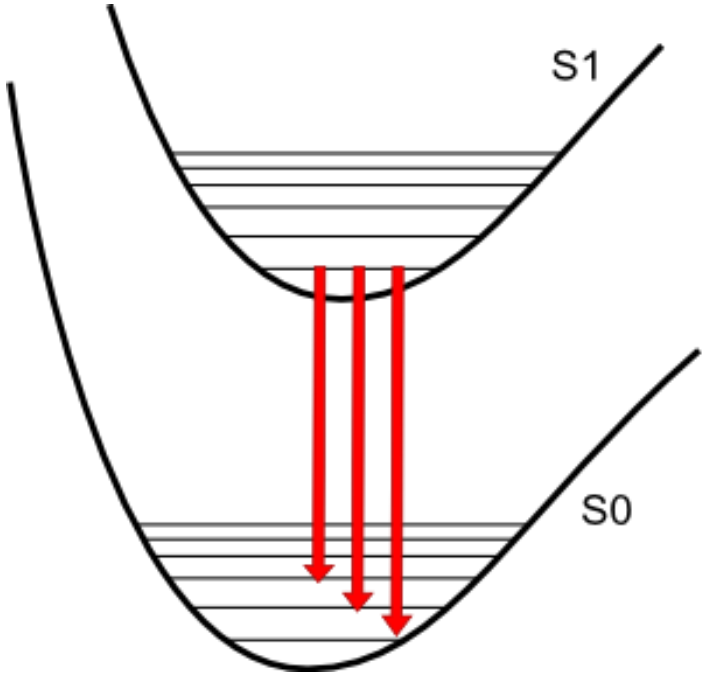
STED nanoscopy

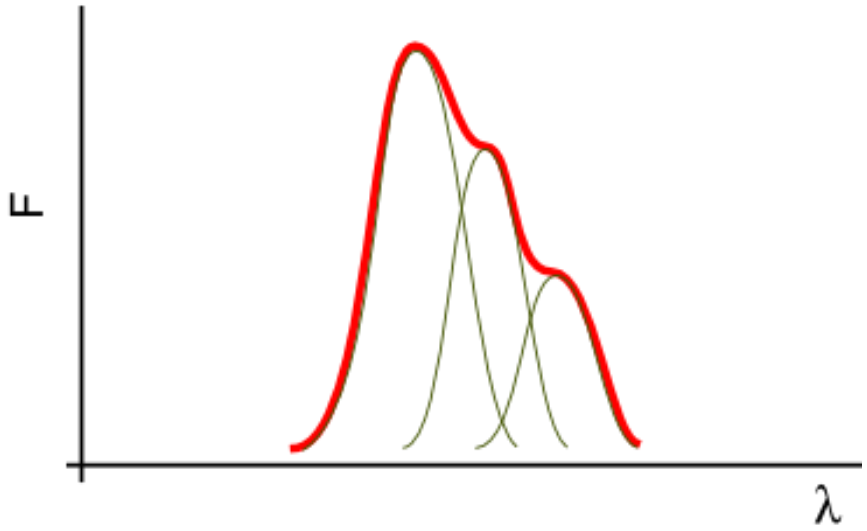


STED nanoscopy

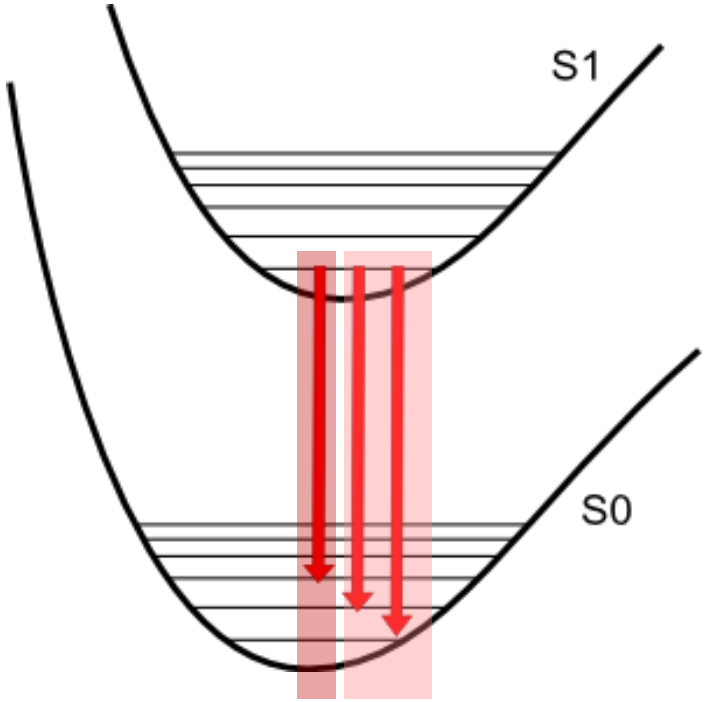


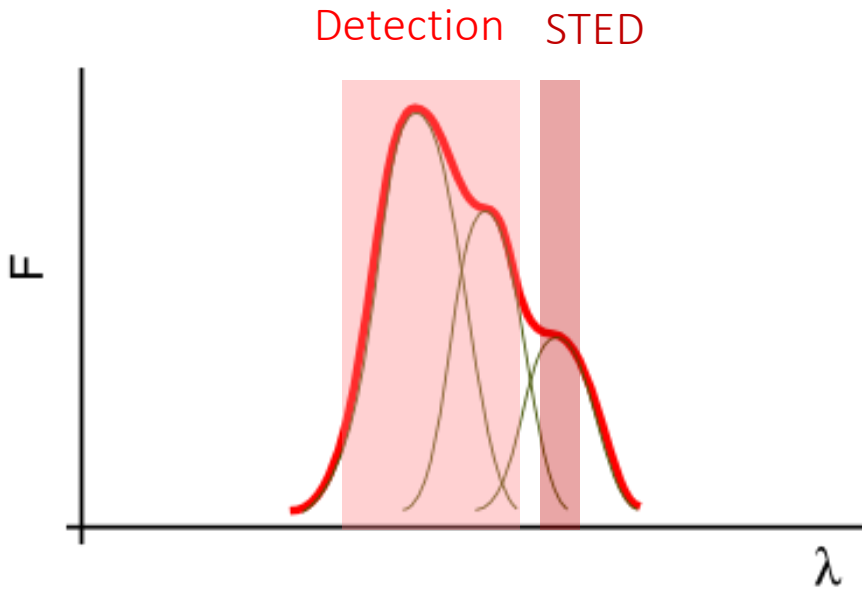
STED nanoscopy





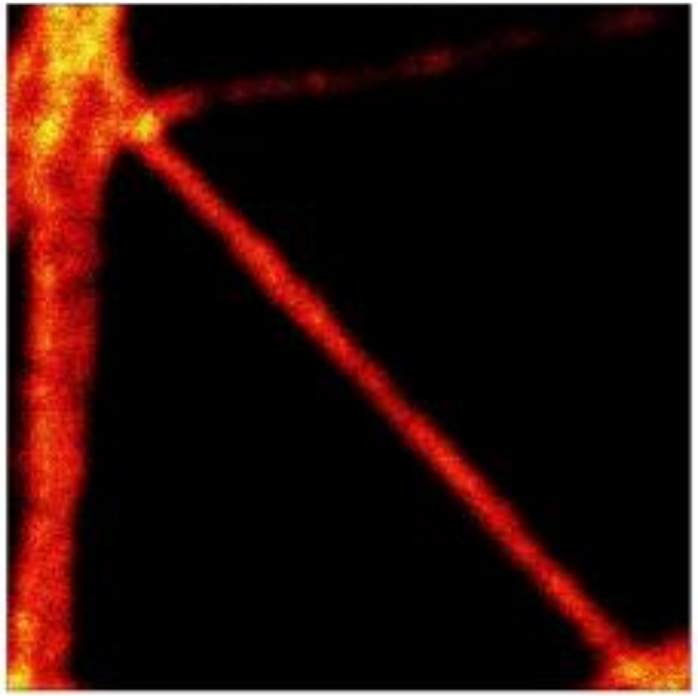
STED nanoscopy



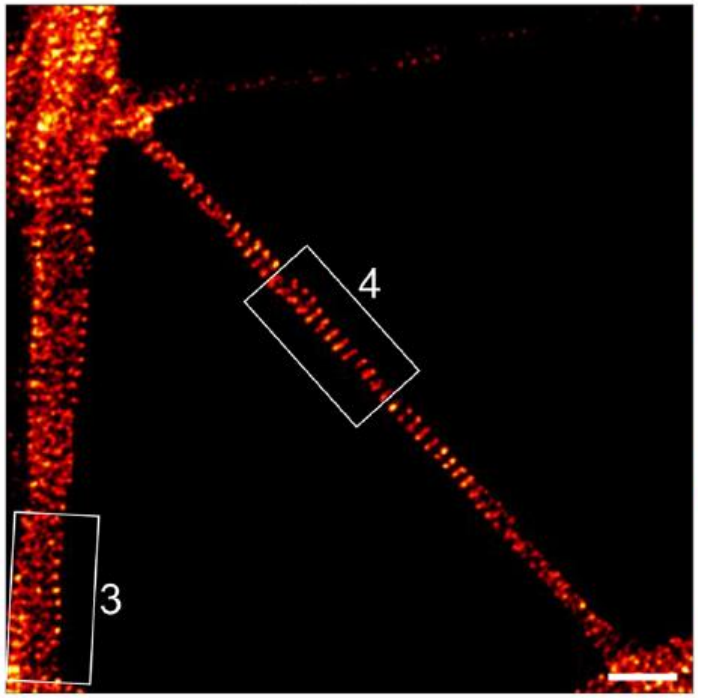


STED nanoscopy

CONFOCAL



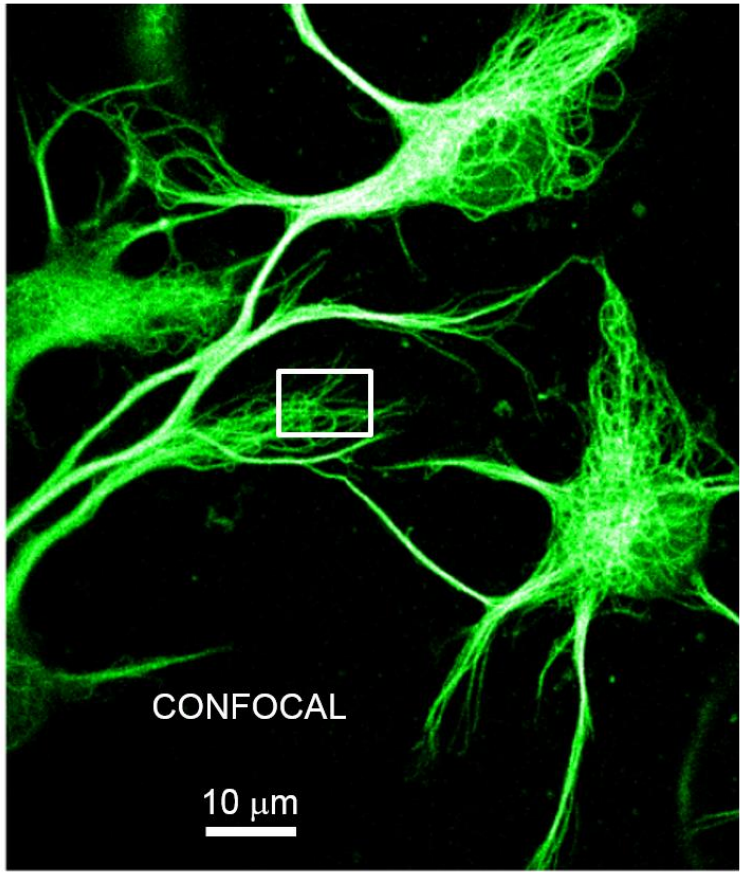
STED

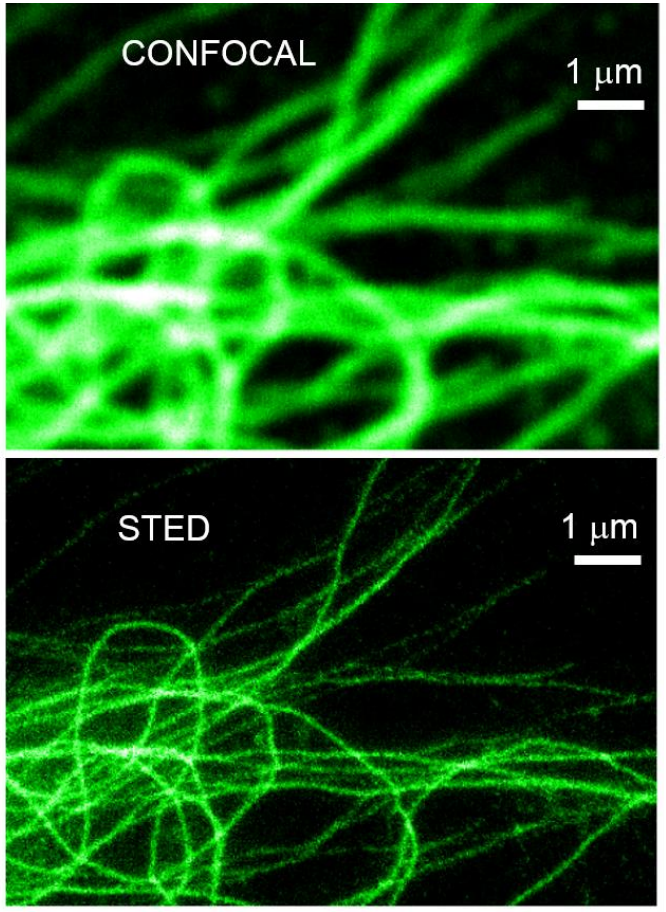


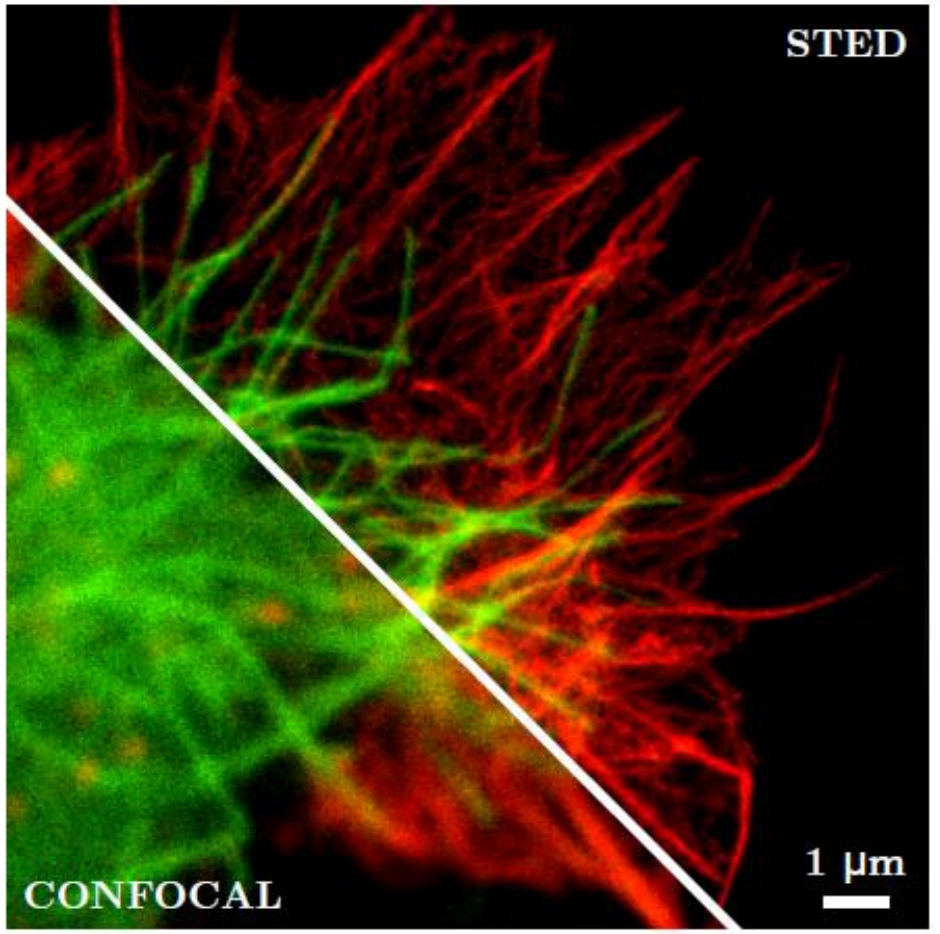
Membrane associated periodic skeleton (MPS) of neurons

Unsain et al. ***Scientific Reports*** 8 (2018) 3007

STED nanoscopy



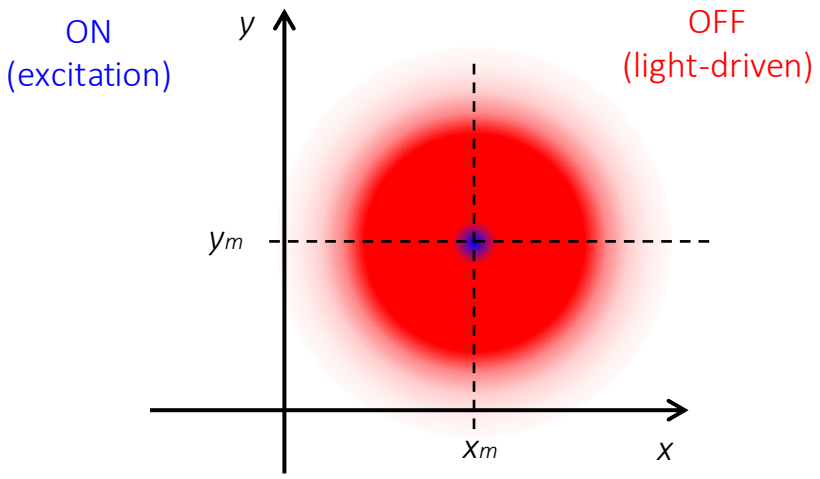




Fluorescence Nanoscopy 2nd Generation

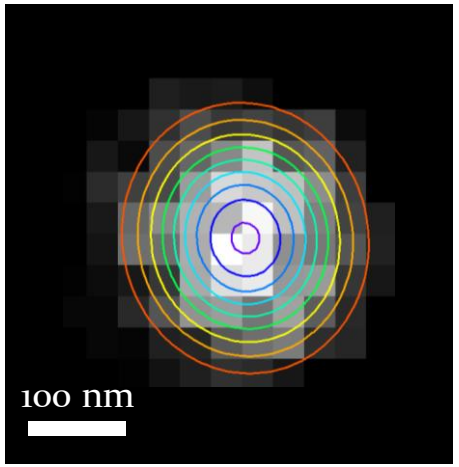
Fluorescence nanoscopy resolution limits

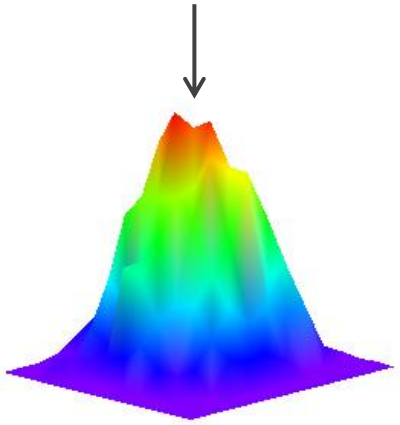
Position information is injected by the light pattern



Position information obtained from the emission (image)

$$\sigma_x = \frac{PSF}{\sqrt{N}}$$



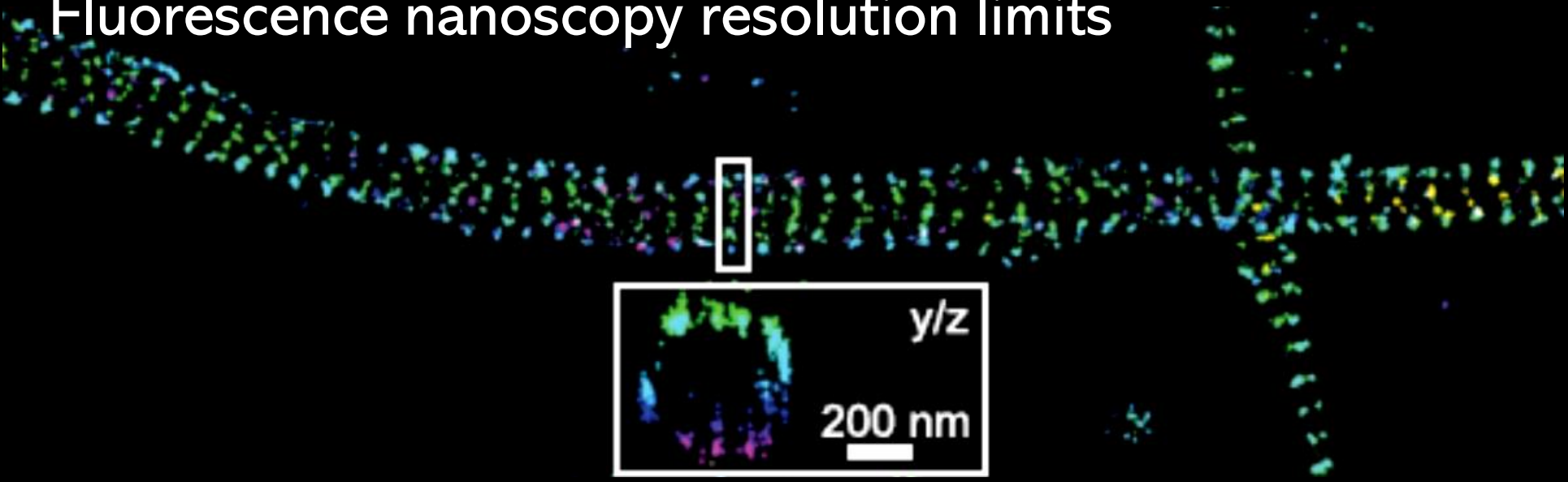


Ideally (no background, perfect off-switching) one photon locates the emitter

In practice, high spatial resolution requires N

High localization precision require shigh N

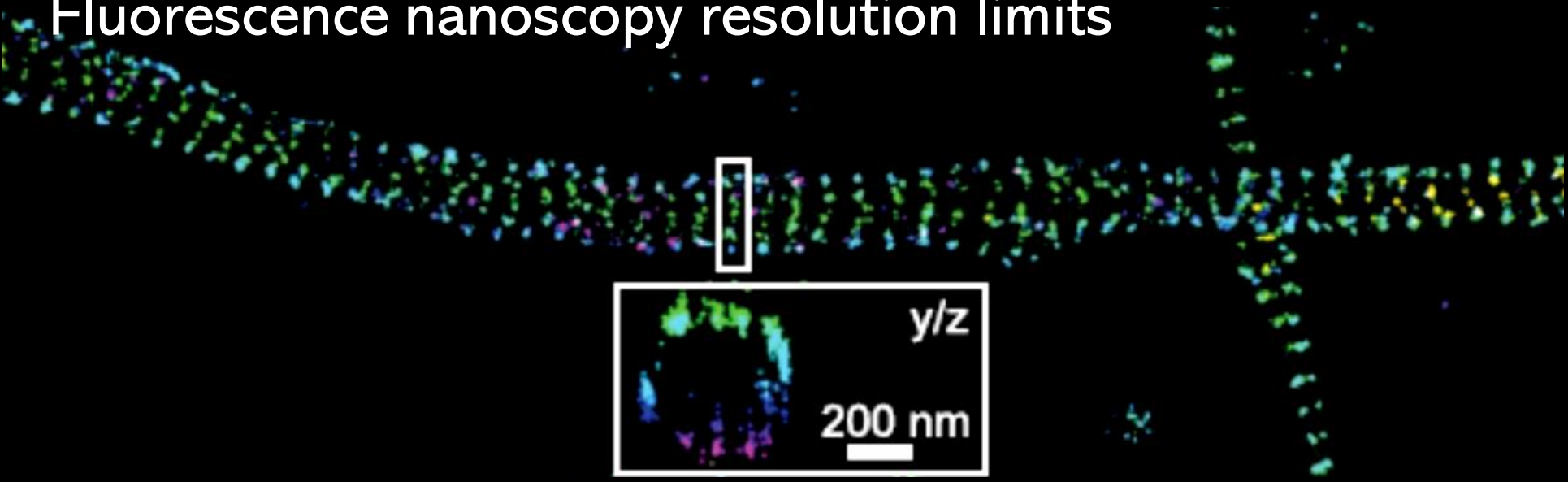
Fluorescence nanoscopy resolution limits



$$\sigma_x = \sigma_y = 15 - 60 \text{ nm}$$

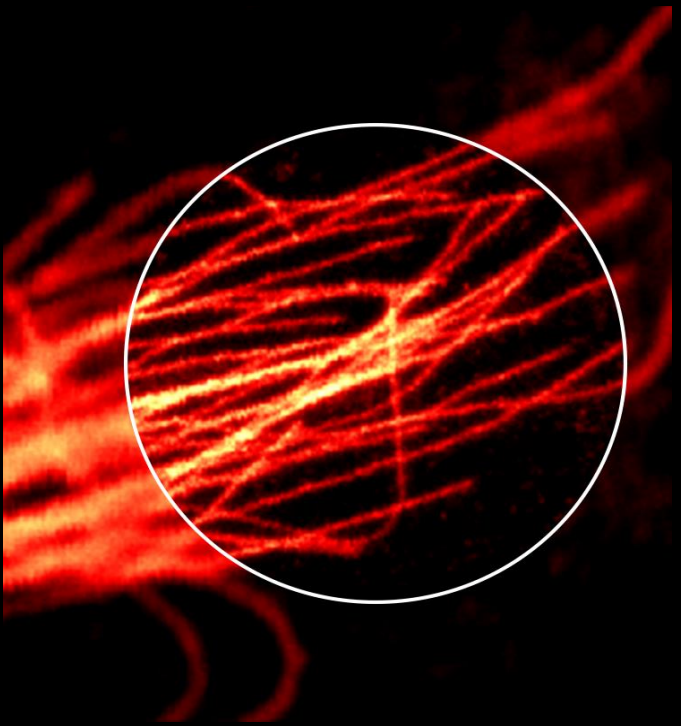
$$\sigma_z = 30 - 120 \text{ nm}$$

Fluorescence nanoscopy resolution limits

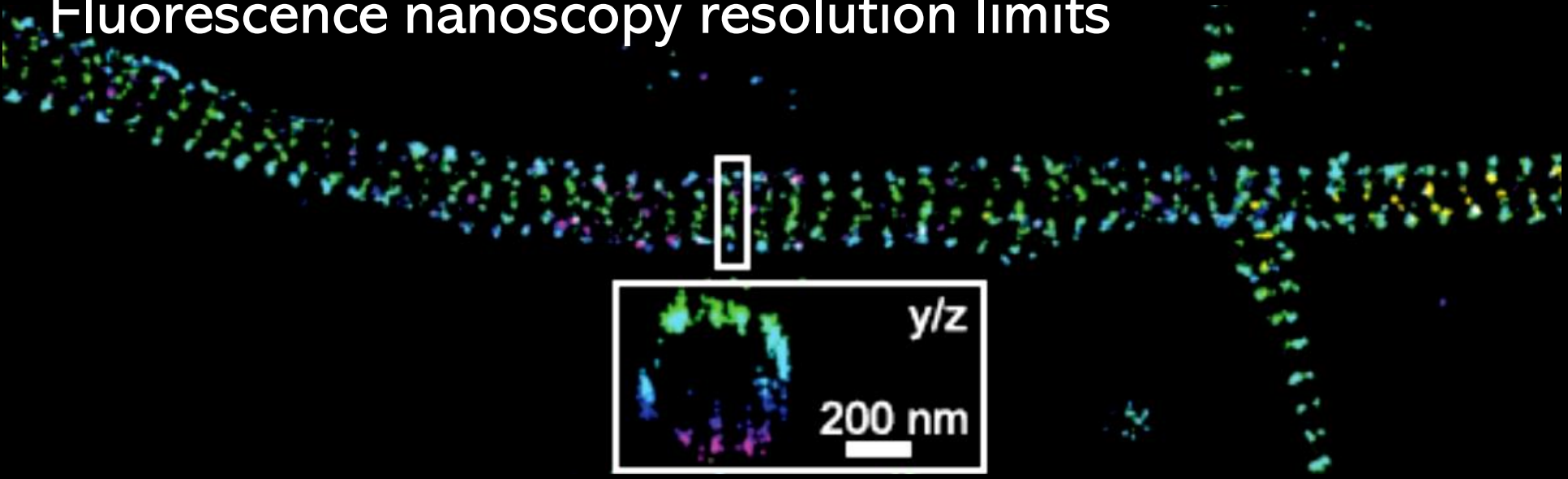


$$\sigma_x = \sigma_y = 15 - 60 \text{ nm}$$

$$\sigma_z = 30 - 120 \text{ nm}$$

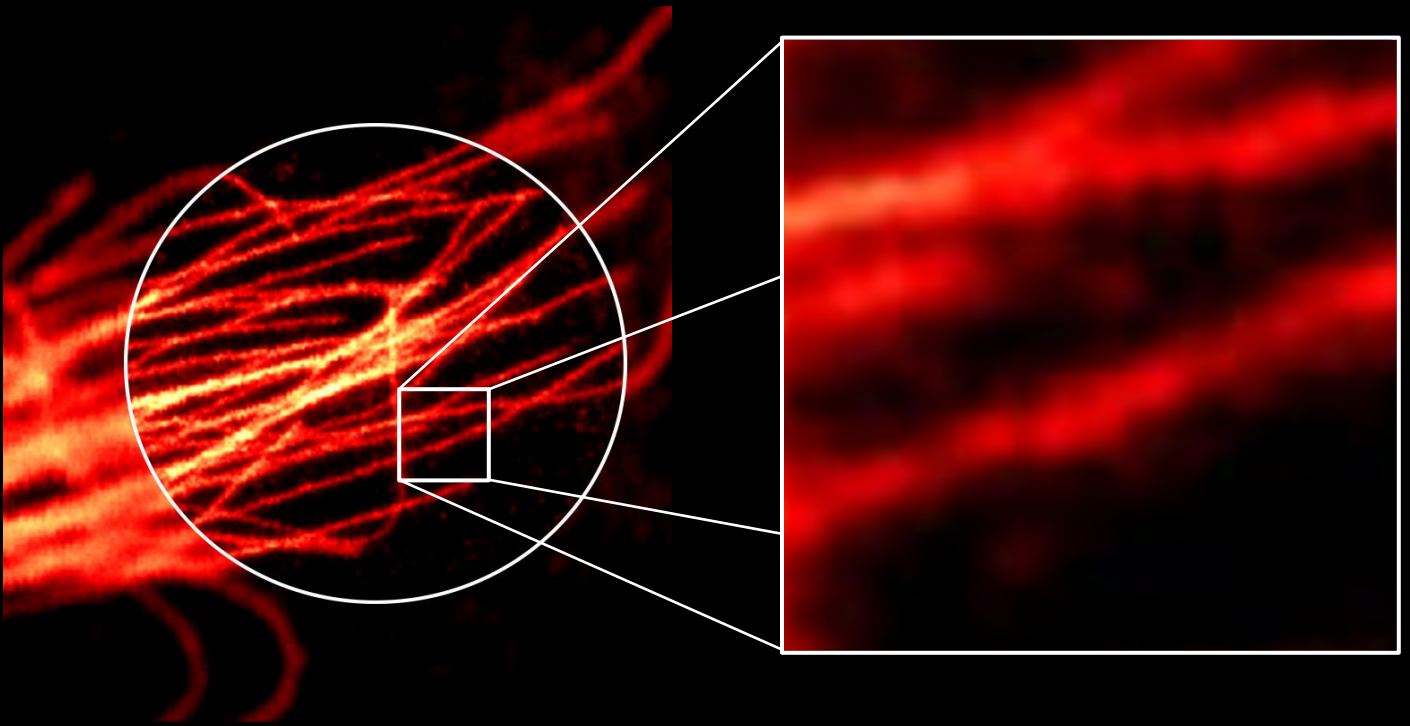


Fluorescence nanoscopy resolution limits

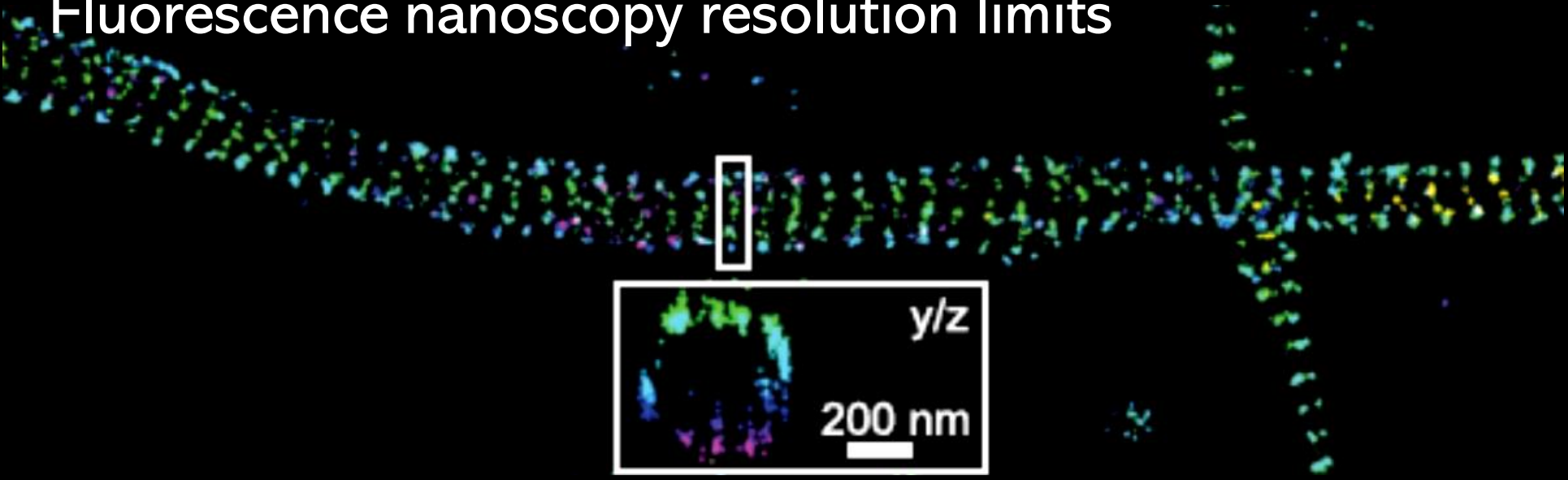


$$\sigma_x = \sigma_y = 15 - 60 \text{ nm}$$

$$\sigma_z = 30 - 120 \text{ nm}$$

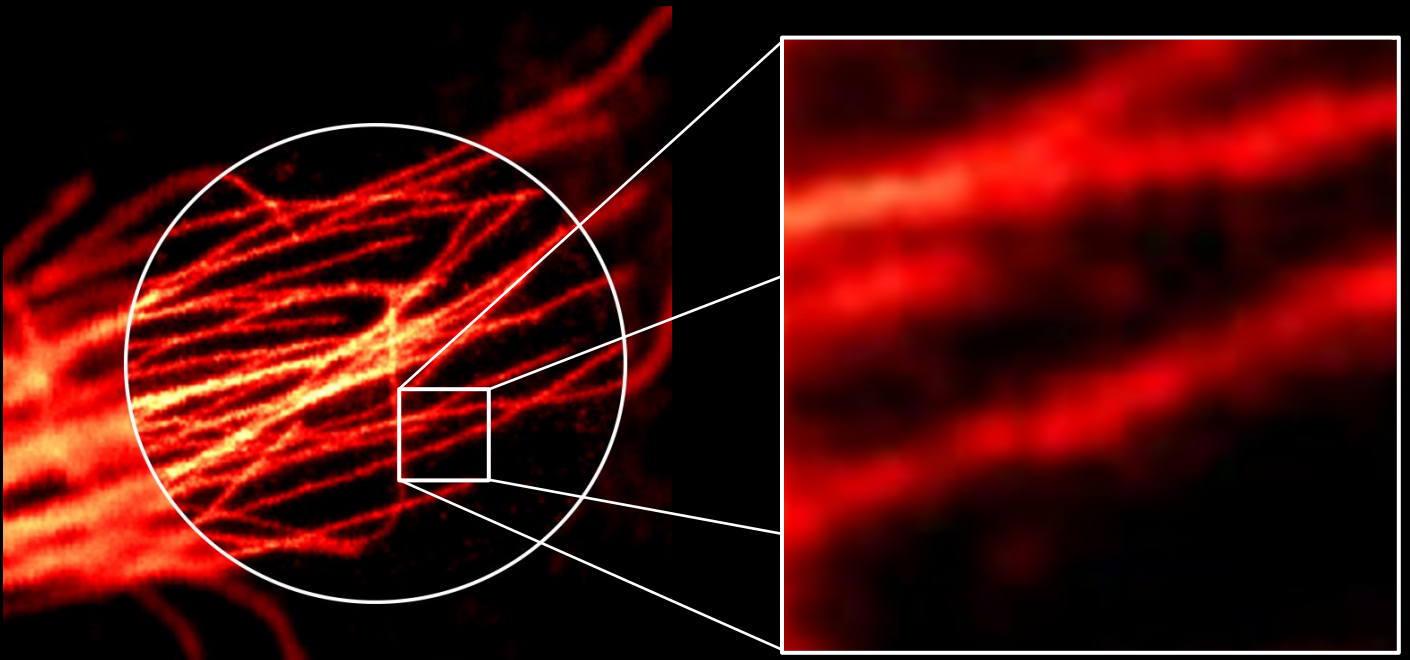


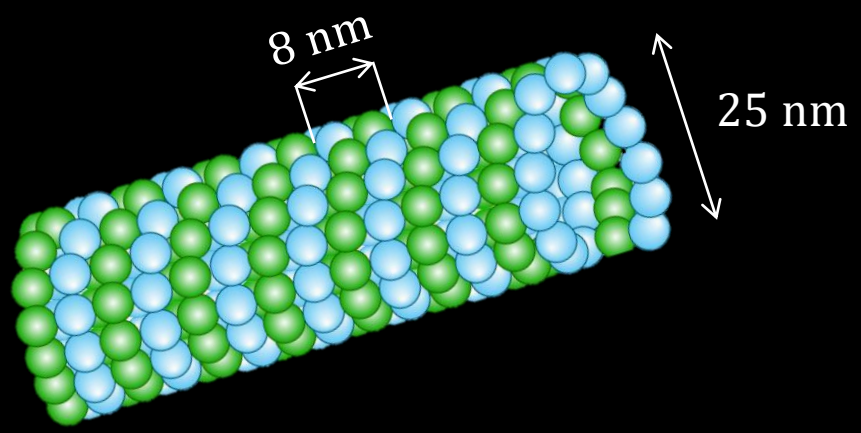
Fluorescence nanoscopy resolution limits



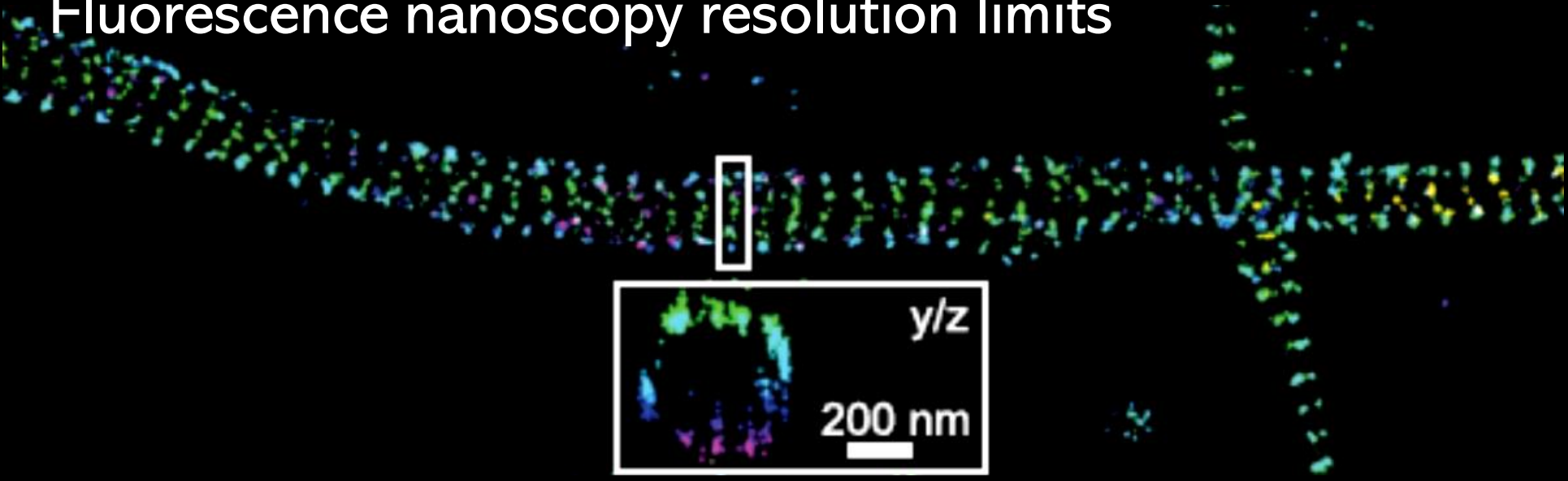
$$\sigma_x = \sigma_y = 15 - 60 \text{ nm}$$

$$\sigma_z = 30 - 120 \text{ nm}$$



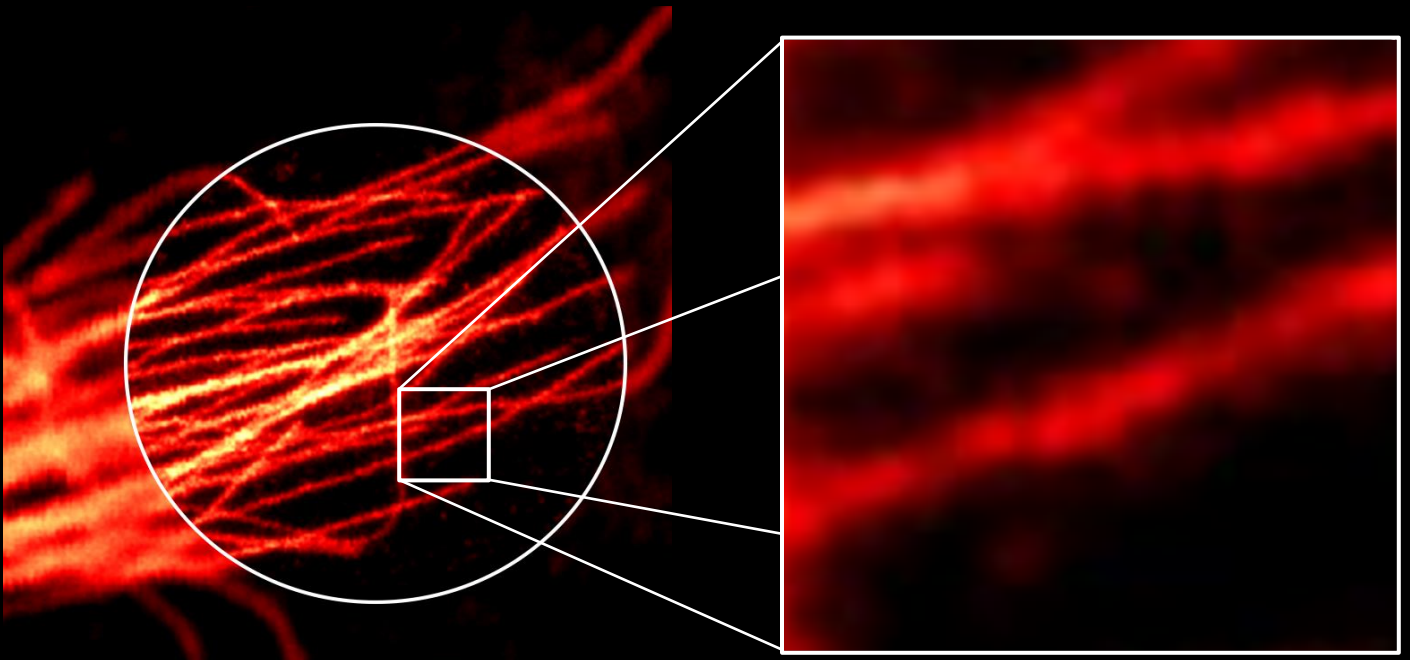


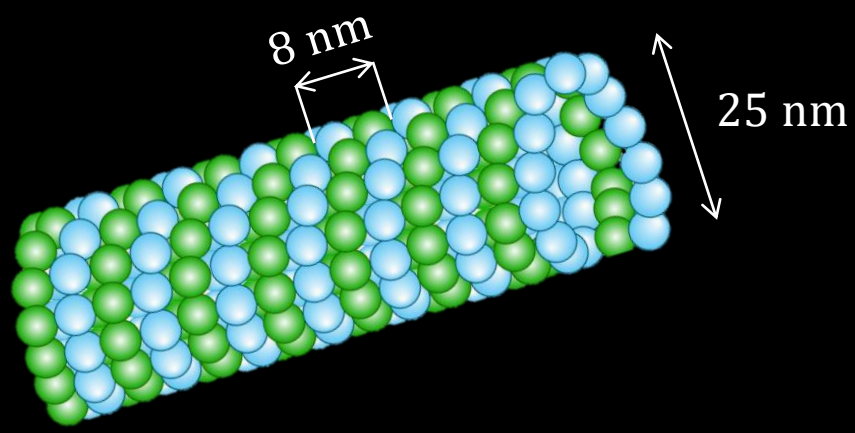
Fluorescence nanoscopy resolution limits



$$\sigma_x = \sigma_y = 15 - 60 \text{ nm}$$

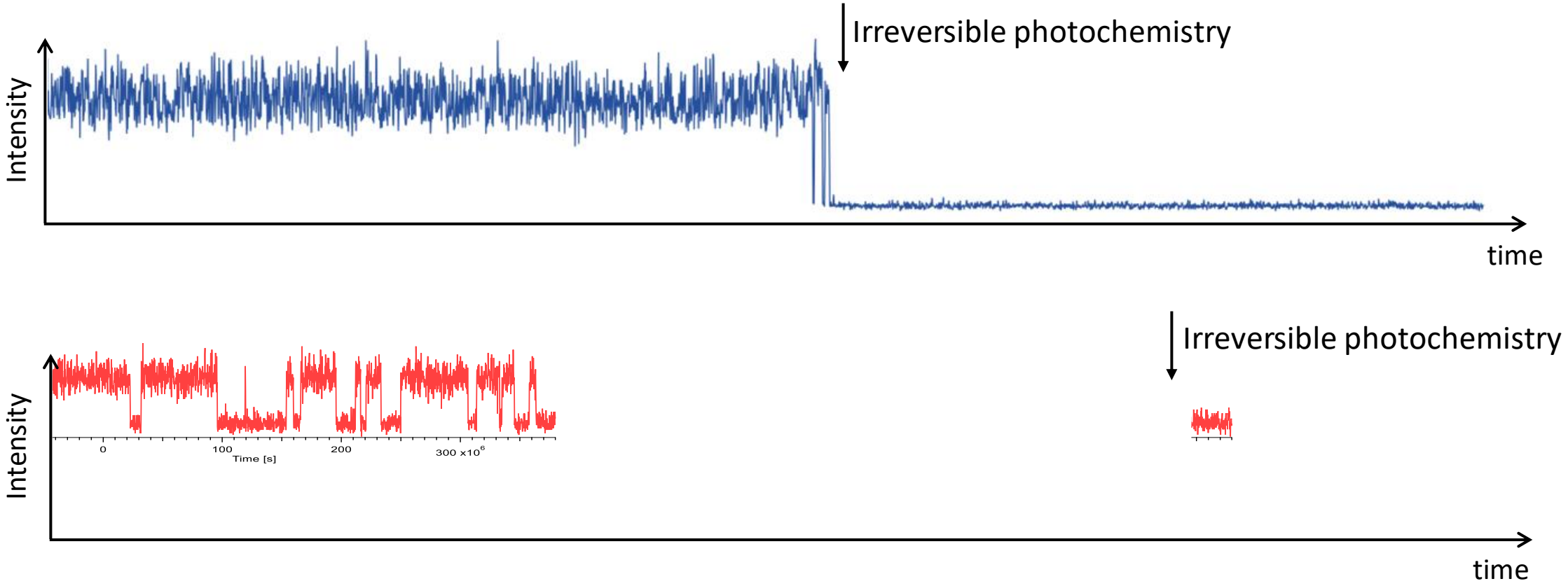
$$\sigma_z = 30 - 120 \text{ nm}$$





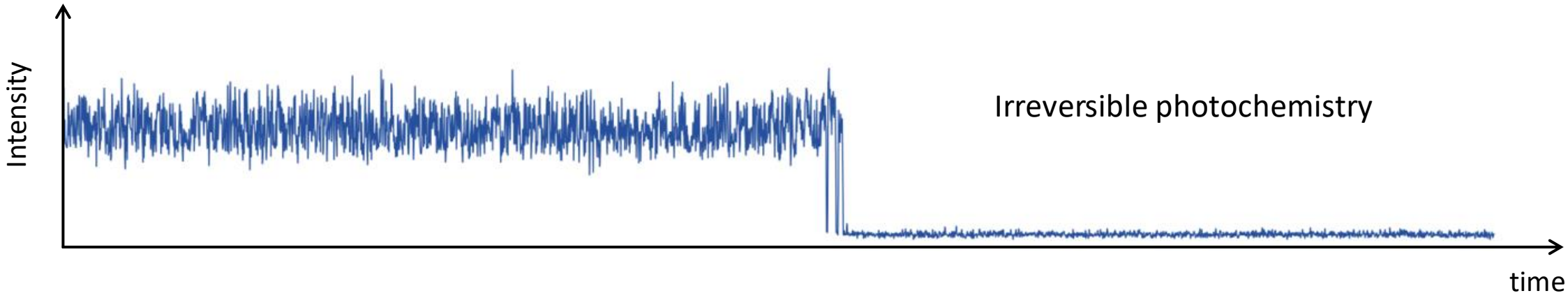
We need an extra push into the sub-10 nm regime

Fluorescence nanoscopy resolution limits: photobleaching



- Imaging resolution limited to a few tens of nm
- Single-molecule tracking limited time/length

Nanoscopy reslolution limit: photon budget



Solutions

Get more photons:

DNA-PAINT

Stabilizing buffers

Self-healing dyes

Get more information:

SML-SSI (MINFLUX, RASTMIN)

SIMPLER

STED-FRET

Nanoscopy with sub-10 nm resolution

SML-SSI Single-Molecule Localization with Sequential Structured Illumination

Balzzarotti et al. ***Science*** 355 (2017) 606-612

Masullo et al. ***Nano Letters*** 21 (2021) 840-846

Masullo et al. ***Biophysical Reports*** 2 (2022) 100036

Masullo et al. ***Light: Science & Applications*** 11 (2022) 70

Masullo et al. ***Light: Science & Applications*** 11 (2022) 199

Zdańkowski et al. ***ACS Photonics*** 9 (2022) 3777–3785

SIMPLER Supercritical Illumination Microscopy Photometric z-Localization w/ Enhanced Resolution

Szalai et al. ***Nature Communications*** 12 (2021) 517

STED-FRET Super-resolved energy transfer imaging

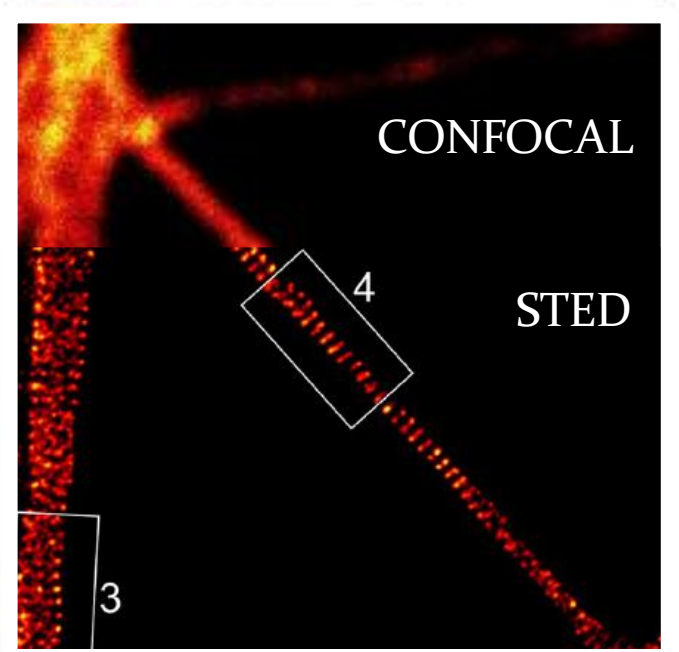
Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

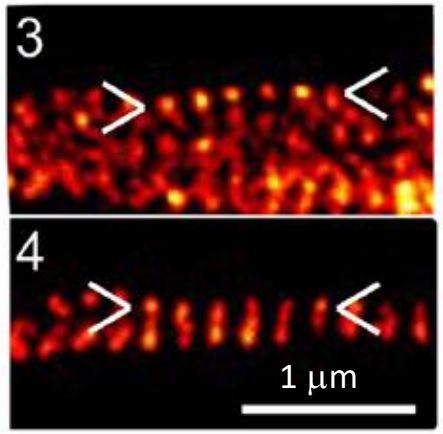
Szalai et al. ***Nanoscale*** 13 (2021) 18421-18433

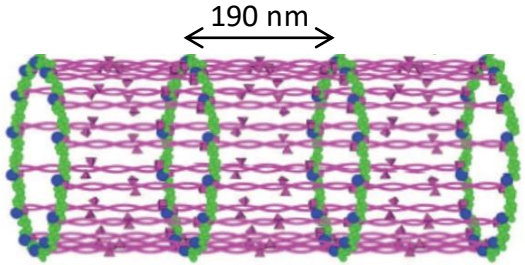
REVIEW: Masullo, et al. ”Fluorescence nanoscopy at the sub-10 nm scale” ***Biophysical Reviews*** 13 (2022) 1101-1112

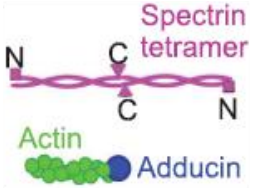
https://stefani-lab.ar/

STED-FRET

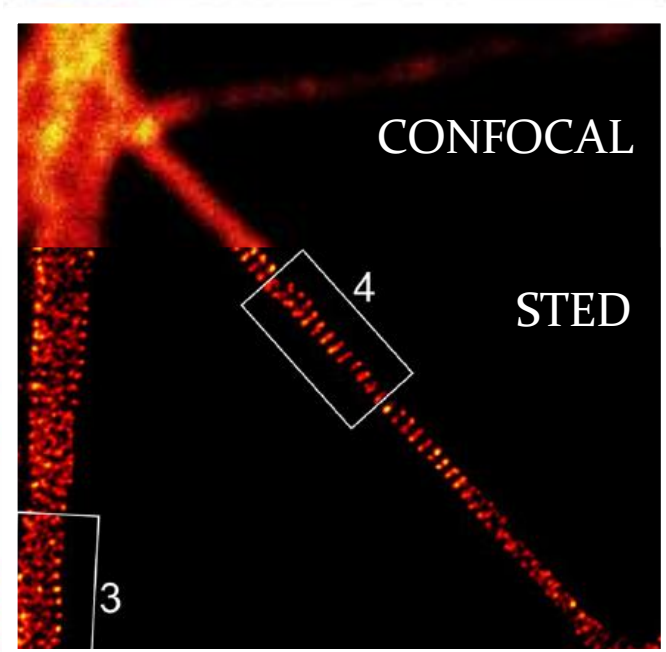

CONFOCAL
STED
4
3

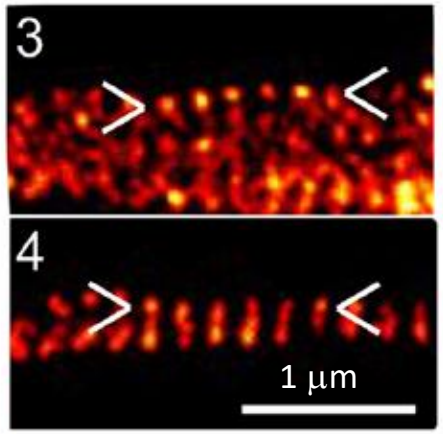

3
4
1 µm

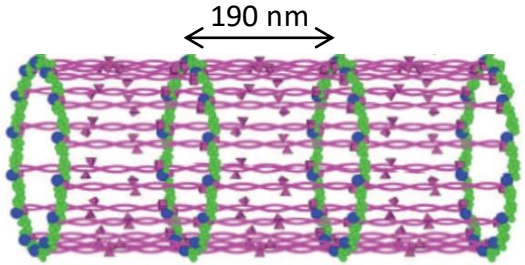

190 nm

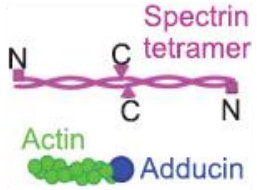

Spectrin
tetramer
N
C
C
N
Actin
Adducin

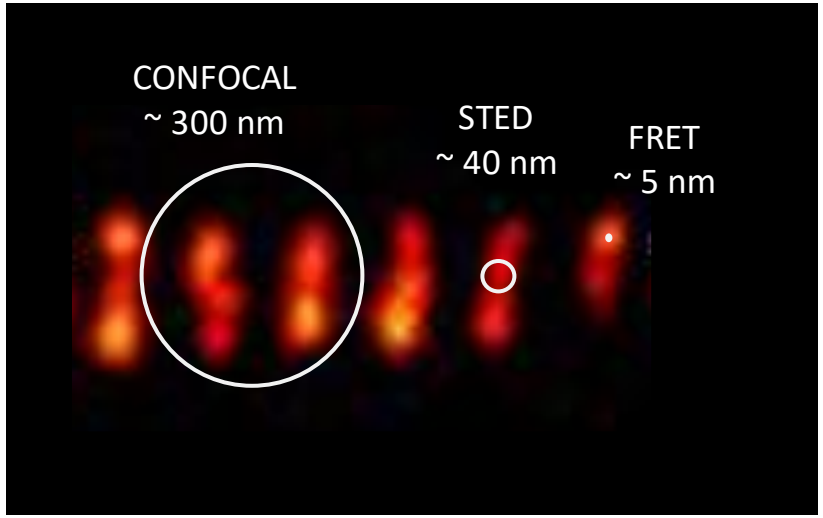
STED-FRET


CONFOCAL
STED
4
3

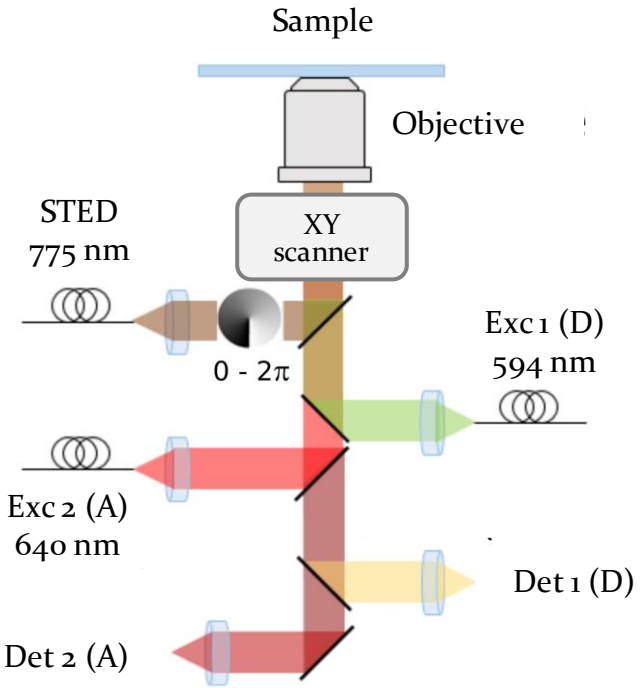

3
4
1 µm


190 nm


Spectrin
tetramer
N
C
C
N
Actin
Adducin

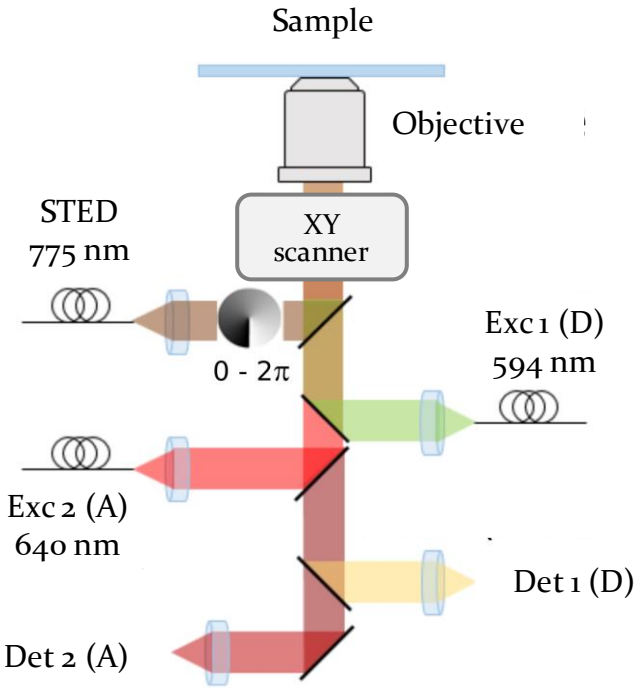

CONFOCAL
~ 300 nm
STED
~ 40 nm
FRET
~ 5 nm

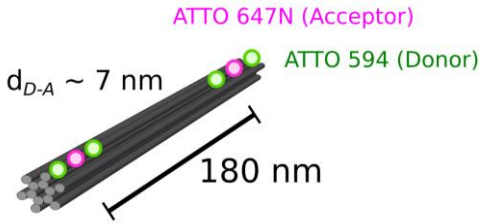
STED-FRET



Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

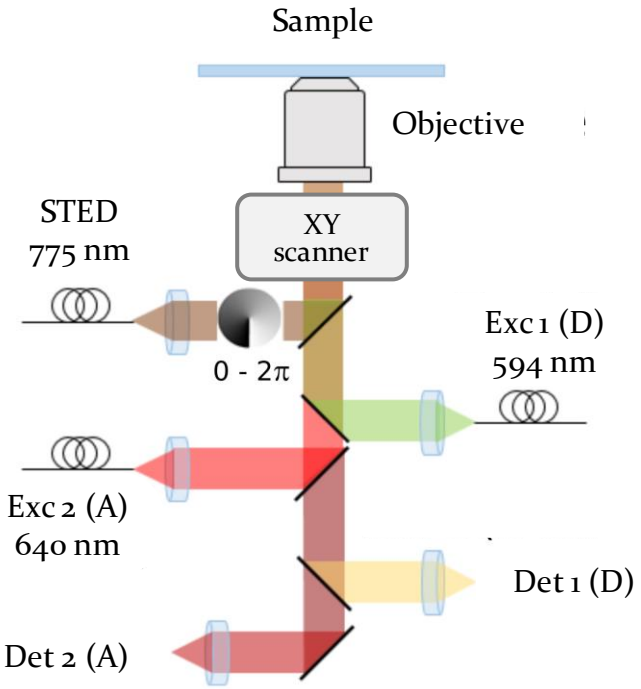
STED-FRET

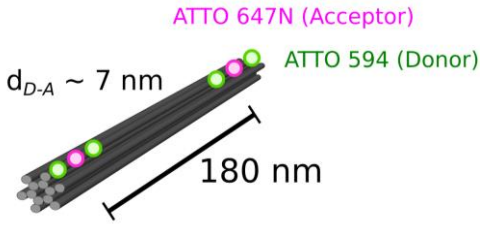


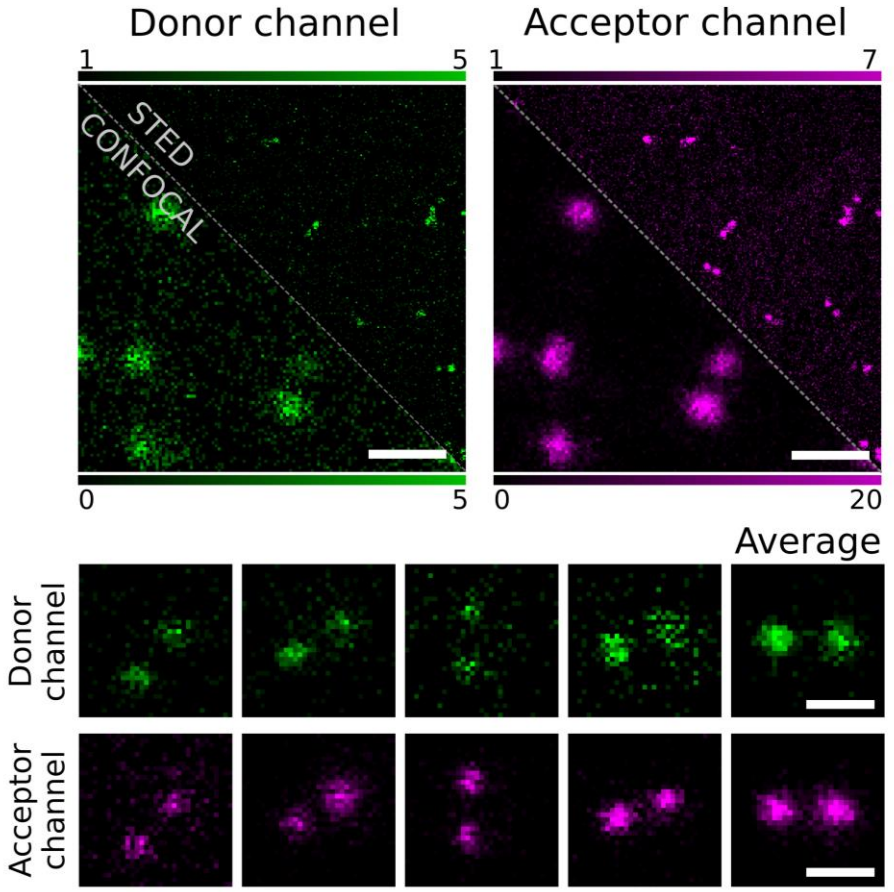


Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

STED-FRET

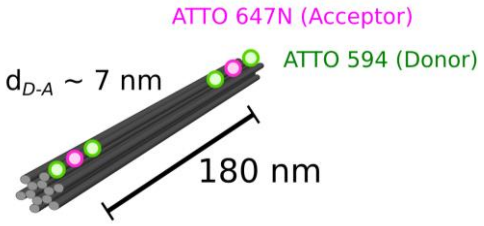




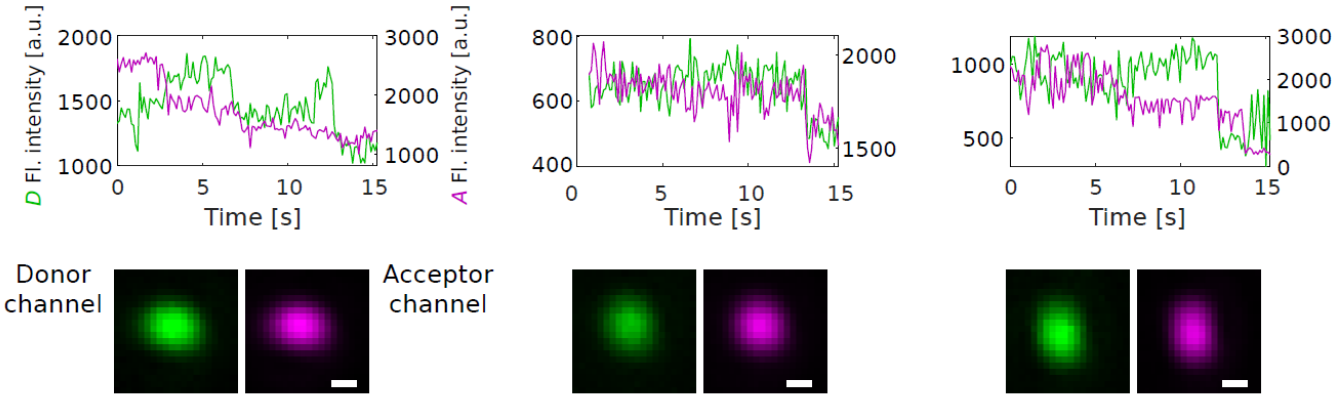


Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

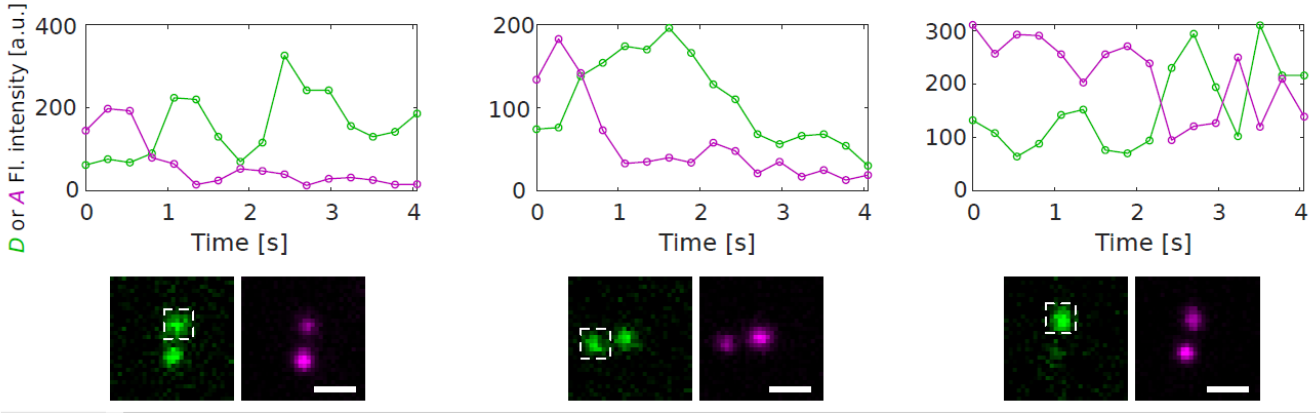
STED-FRET



Confocal FRET



STED-FRET



Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

STED-FRET

$$\frac{k_t}{k_{f,D}} = \left[\frac{\Gamma_0}{r_{DA}}\right]^6 = \frac{1}{\phi_A}\frac{d_D^D}{d_A^A}\boldsymbol{F} \qquad \Gamma_0^6 = \frac{J\kappa^2}{n^4}$$

$$\boldsymbol{F} = \frac{F_A^D}{F_D^D} - \frac{I^D\,\sigma_A^D\,f_{bl}\;F_A^A}{I^A\,\sigma_A^A\;F_D^D} - \frac{d_D^A}{d_D^D}$$

Work-flow:

1. Confocal F_A^A
2. STED F_A^A
3. Confocal F_A^A
4. STED F_D^D+F_A^D
5. Denoising (optional)
6. Background subtraction
7. Masking
8. f_{bl} from 1. and 3.
9. $\boldsymbol{F}$ image $\propto k_t$

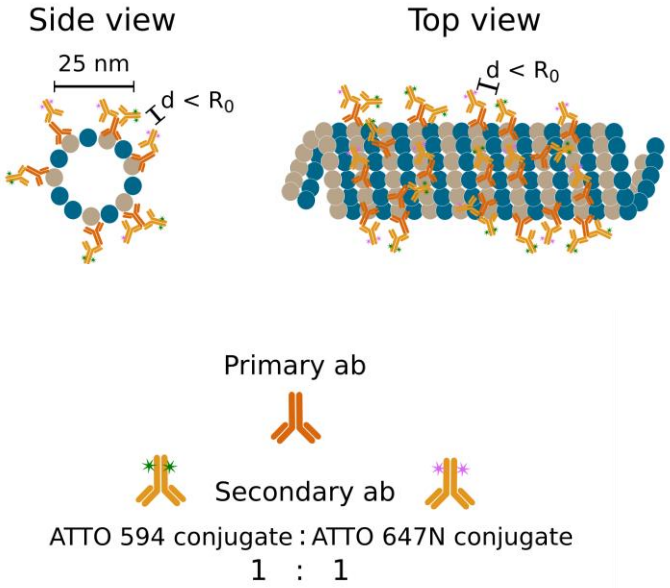
F_D^D and F_A^D of singly-labeled samples → $\frac{d_D^A}{d_D^D}$ →

T. Jovin, E. Jares-Erijman, et al.

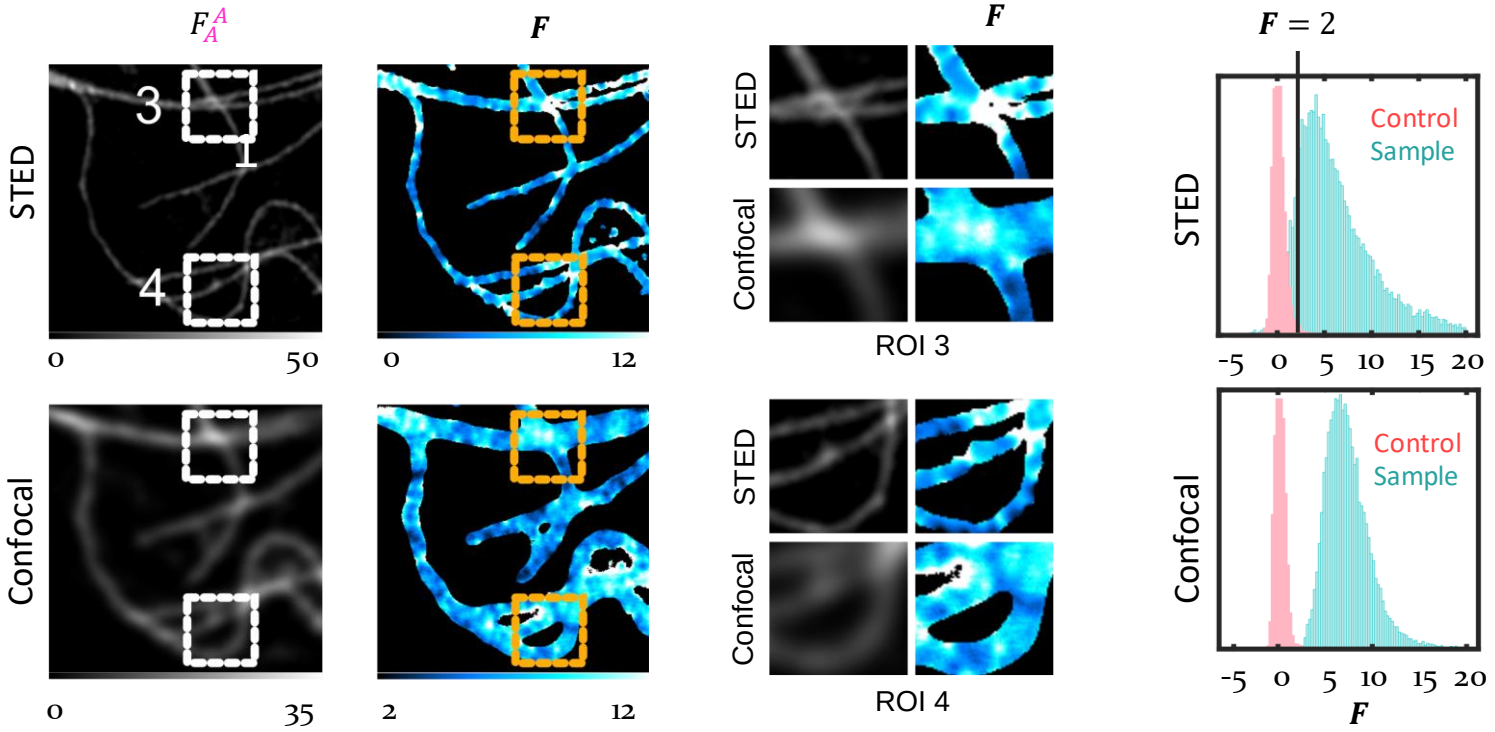
- Chapter 12 in "FRET and FLIM Imaging"
- ***ChemPhysChem*** 2011, 12 (3), 563–566.

Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

STED-FRET

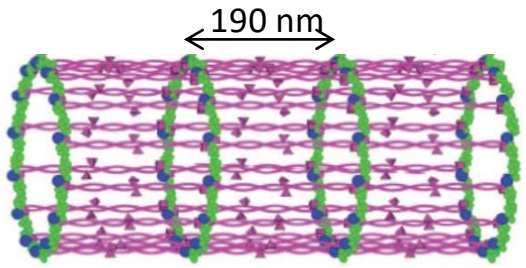


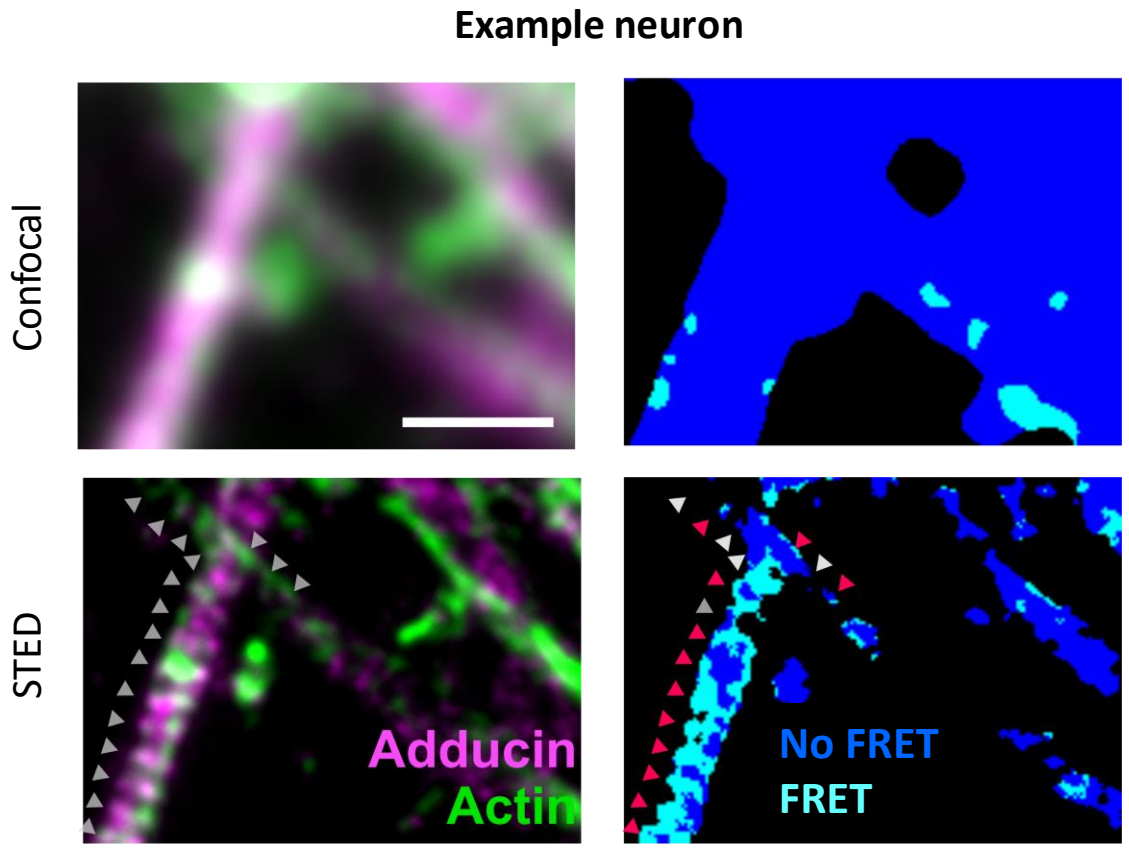
CONTROL to account for local variations of *D* and *A* concentrations



Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

Super-resolving biomolecular interactions with STED-FRET





Szalai et al. ***Nano Letters*** 21 (2021) 2296–2303

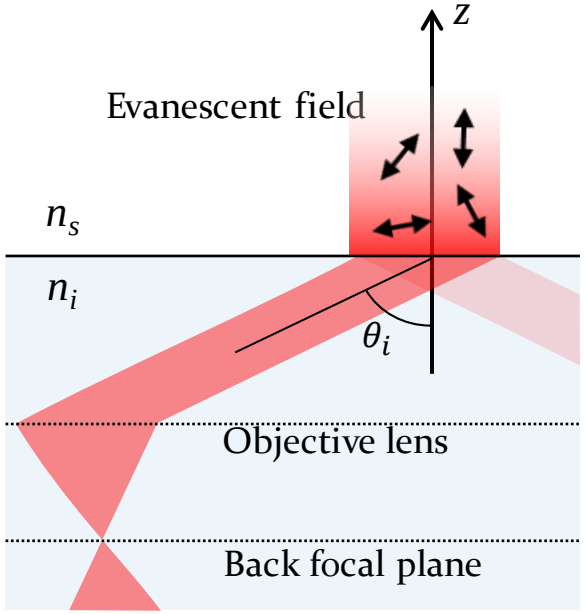
SIMPLER 3D TIRF Nanoscopy

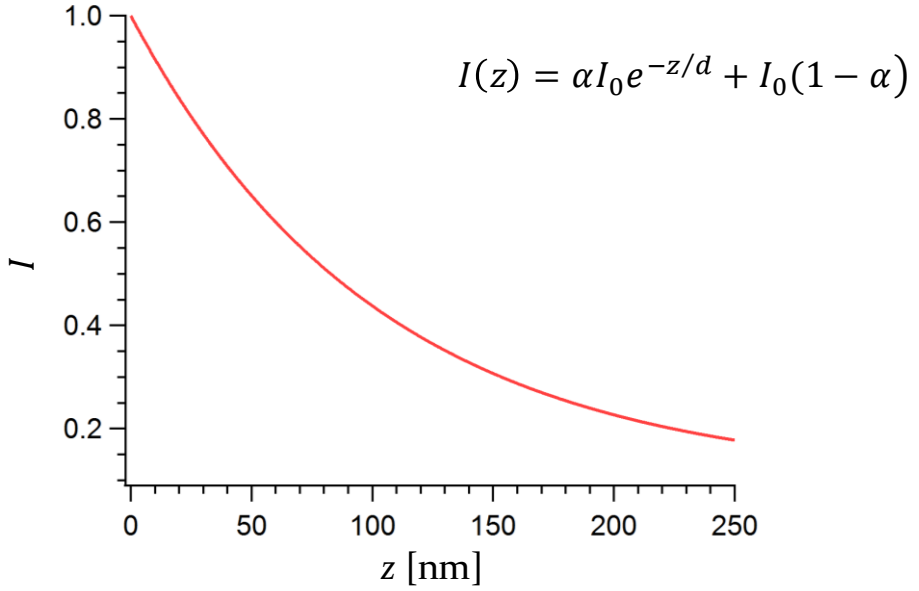
Supercritical Illumination Microscopy Photometric z-Localization w/ Enhanced Resolution

Szalai et al. ***Nature Communications*** 12 (2021) 517

https://stefani-lab.ar/

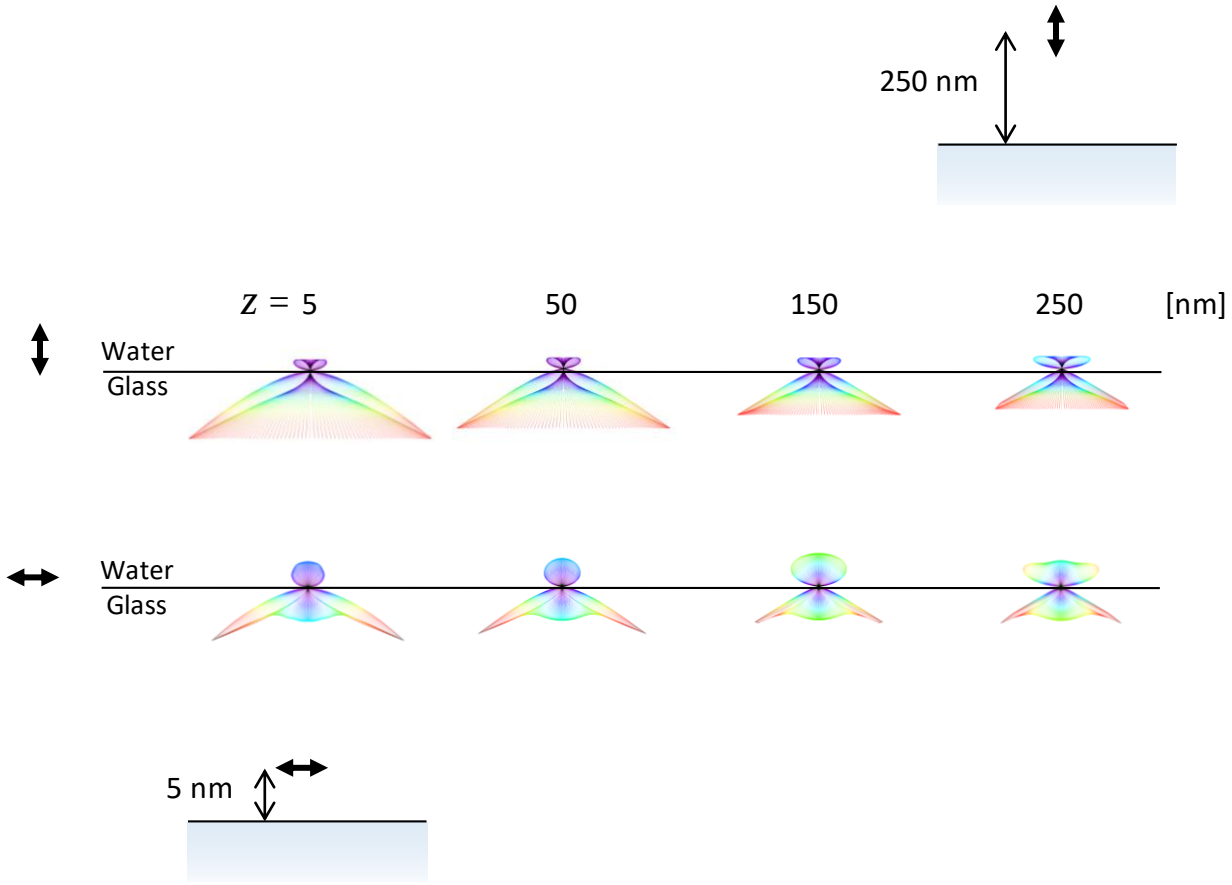
SIMPLER 3D TIRF nanoscopy

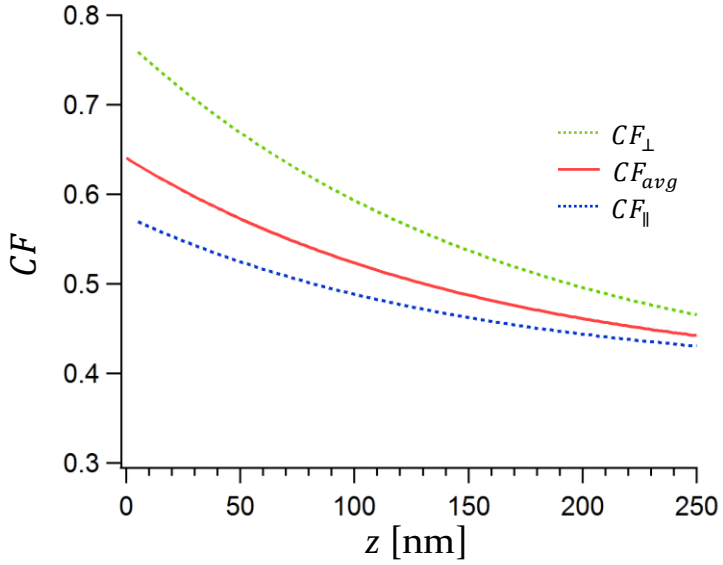




Szalai et al. ***Nature Communications*** 12 (2021) 517

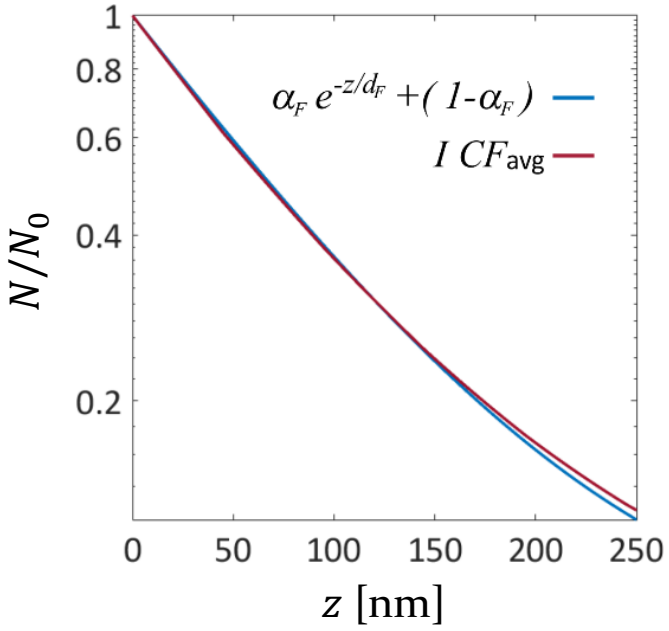
SIMPLER 3D TIRF nanoscopy

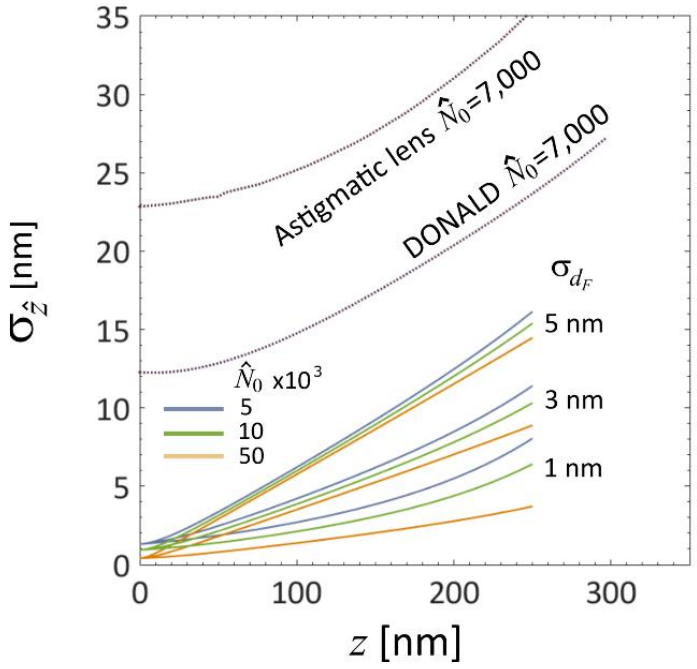




Szalai et al. ***Nature Communications*** 12 (2021) 517

SIMPLER 3D TIRF nanoscopy

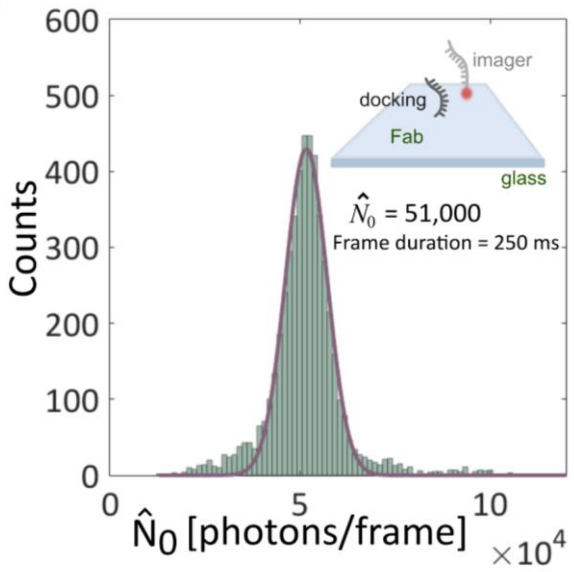


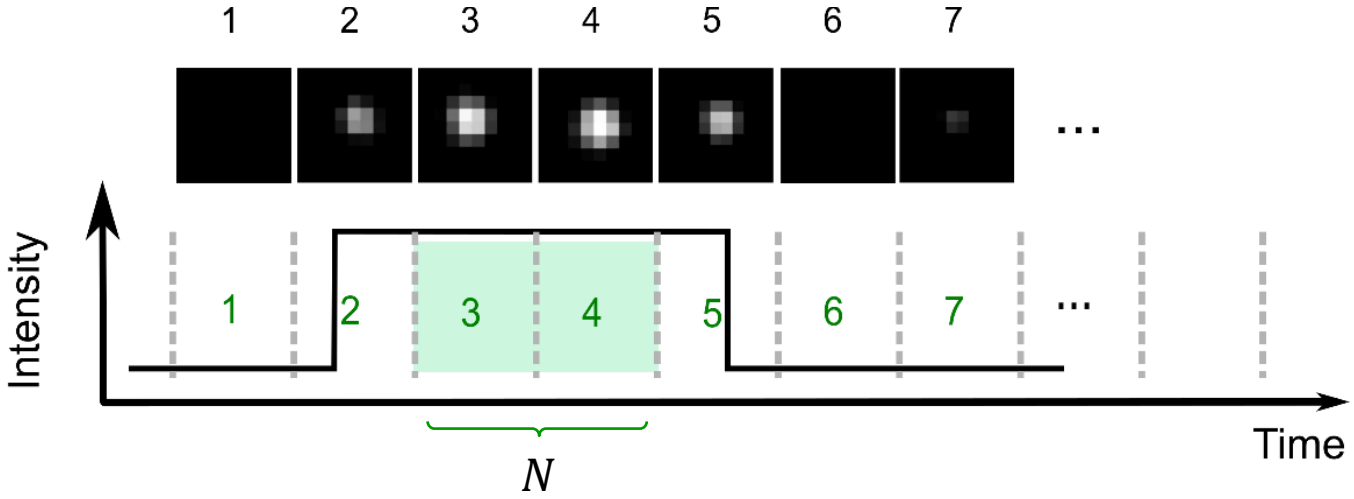


$$\frac{N(z)}{N_0} = \alpha_F\, e^{-z/d_F} + (1-\alpha_F)$$

Szalai et al. ***Nature Communications*** 12 (2021) 517

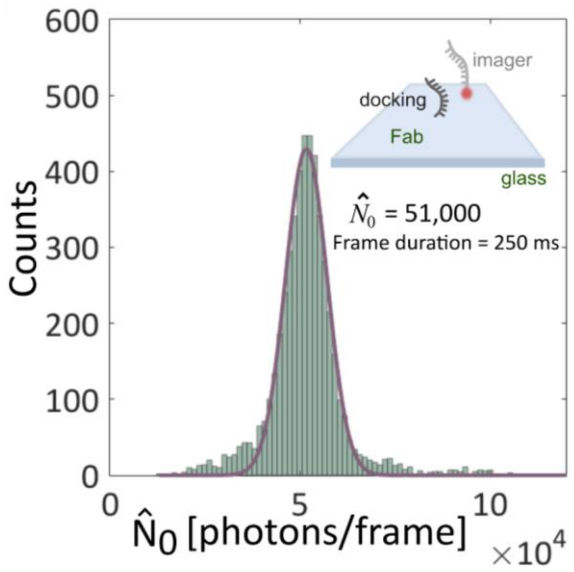
SIMPLER 3D TIRF nanoscopy

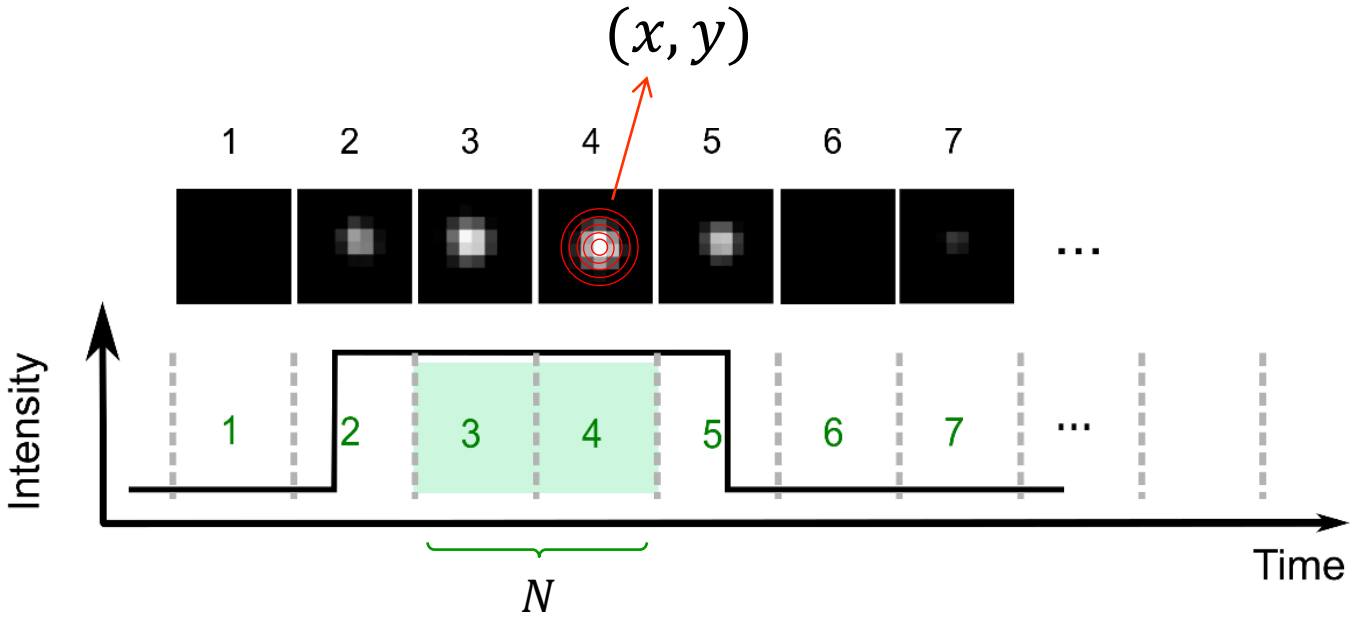




Szalai et al. ***Nature Communications*** 12 (2021) 517

SIMPLER 3D TIRF nanoscopy

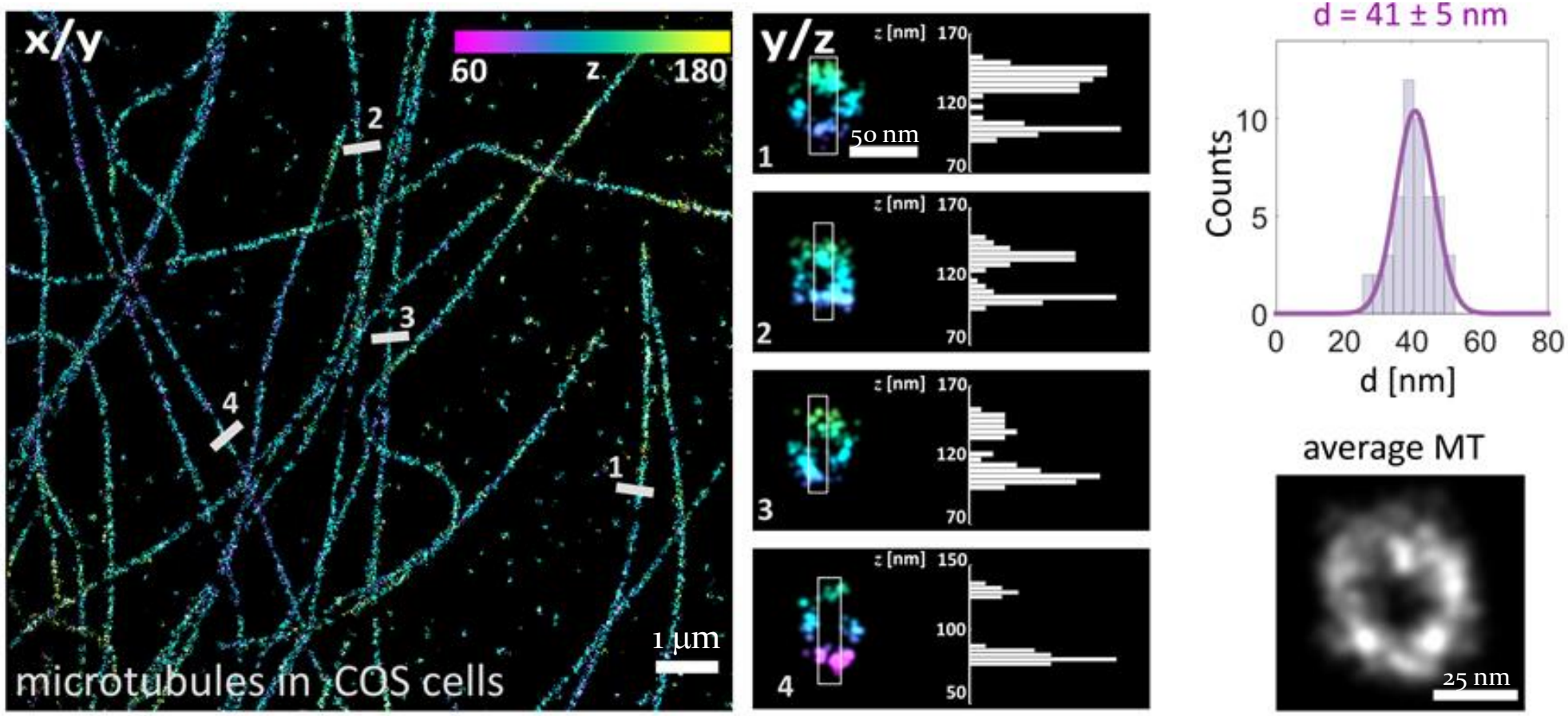




$$\frac{N}{N_0} \rightarrow z$$

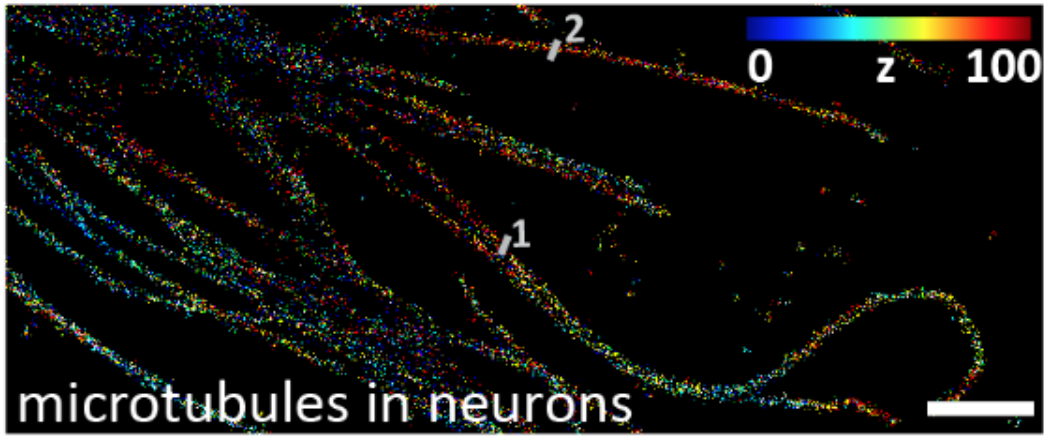
Szalai et al. ***Nature Communications*** 12 (2021) 517

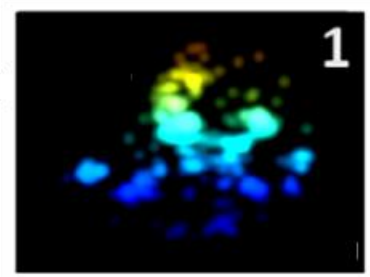
SIMPLER 3D TIRF nanoscopy

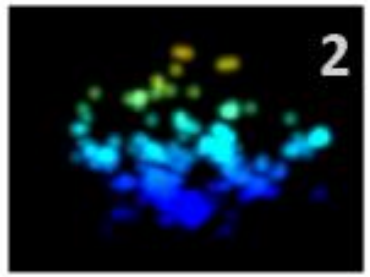


Szalai et al. ***Nature Communications*** 12 (2021) 517

SIMPLER 3D TIRF nanoscopy

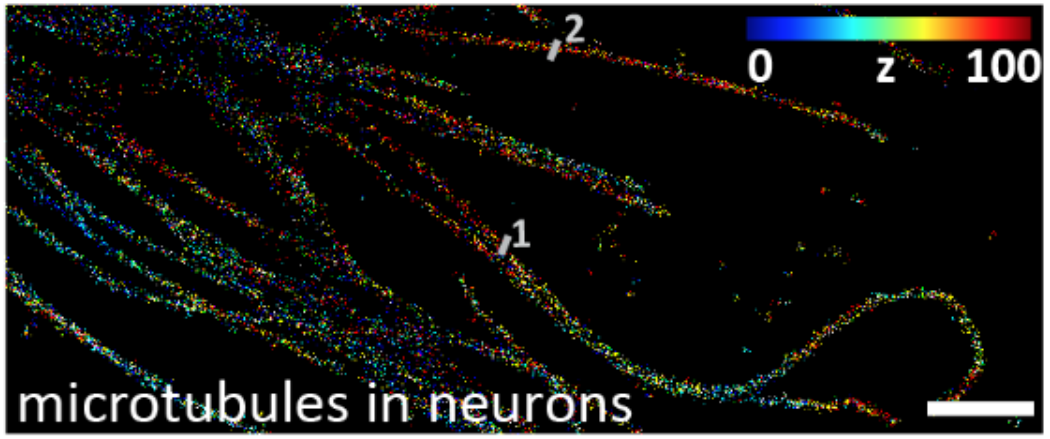


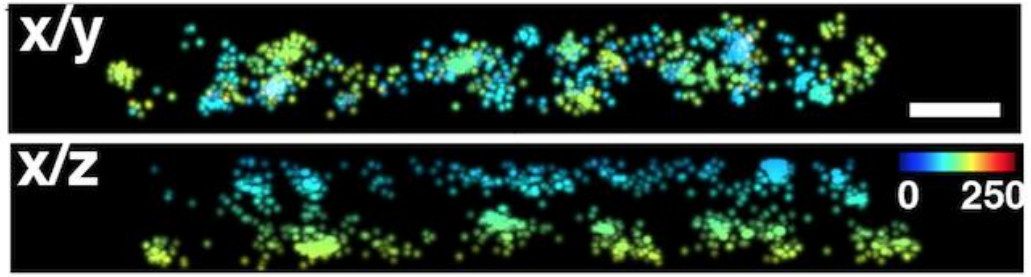


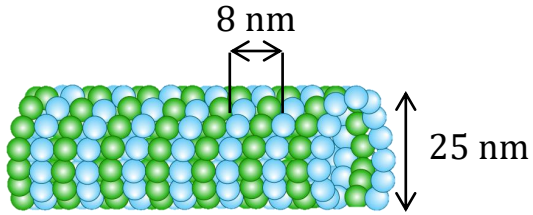


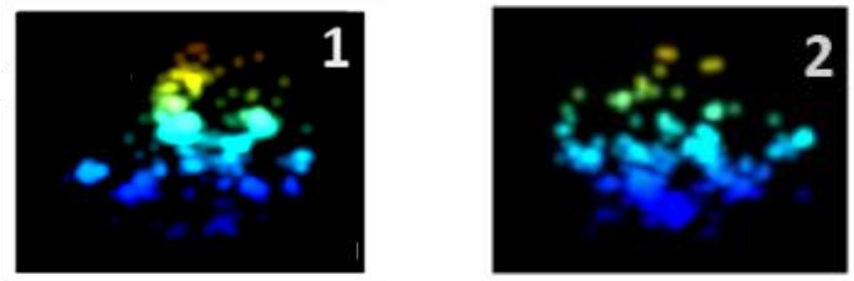
Szalai et al. ***Nature Communications*** 12 (2021) 517

SIMPLER 3D TIRF nanoscopy



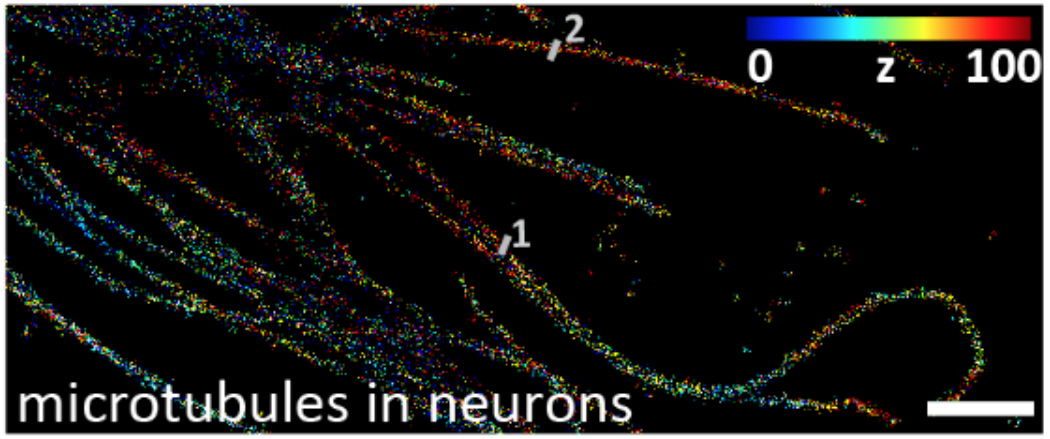


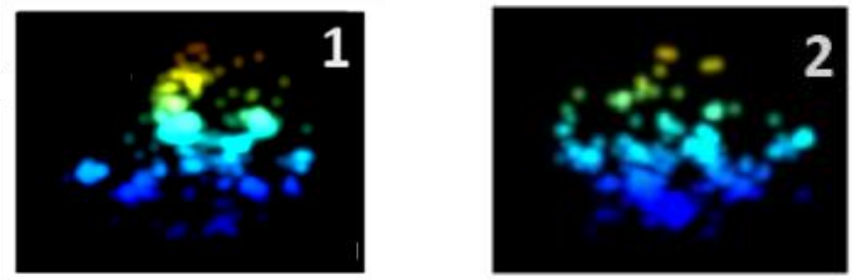


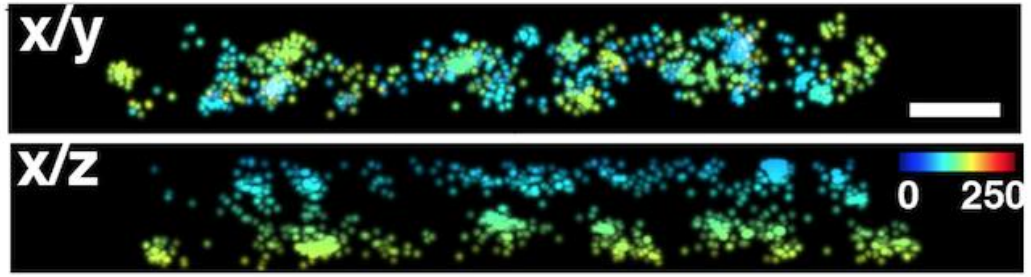


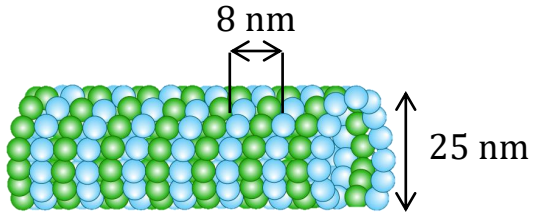
Szalai et al. ***Nature Communications*** 12 (2021) 517

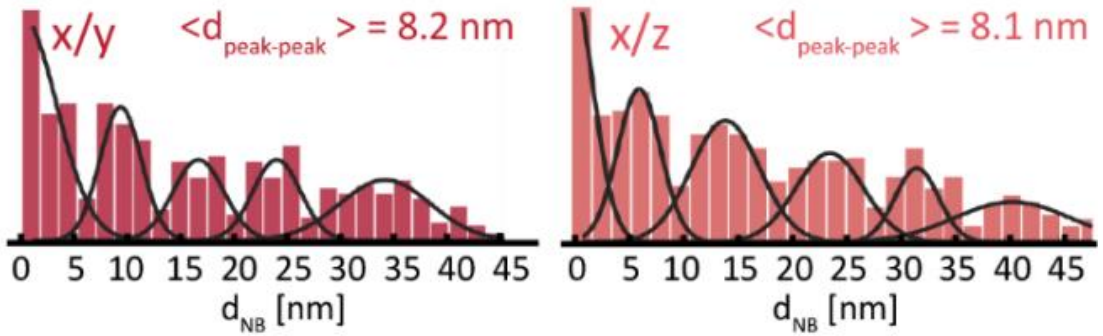
SIMPLER 3D TIRF nanoscopy











Szalai et al. ***Nature Communications*** 12 (2021) 517

SIMPLER 3D TIRF nanoscopy

Open-source Python software

https://github.com/Stefani-Lab

Szalai et al. ***Nature Communications*** 12 (2021) 517

SML-SSI Single-Molecule Localization with Sequential Structured Illumination

Balzzarotti et al. ***Science*** 355 (2017) 606-612

Masullo et al. ***Nano Letters*** 21 (2021) 840-846

Masullo et al. ***Biophysical Reports*** 2 (2022) 100036

Stefani, F. D. ***Nature Photonics*** 17 (2023) 552-553

Masullo et al. Light: Science & Applications 11 (2022) 70

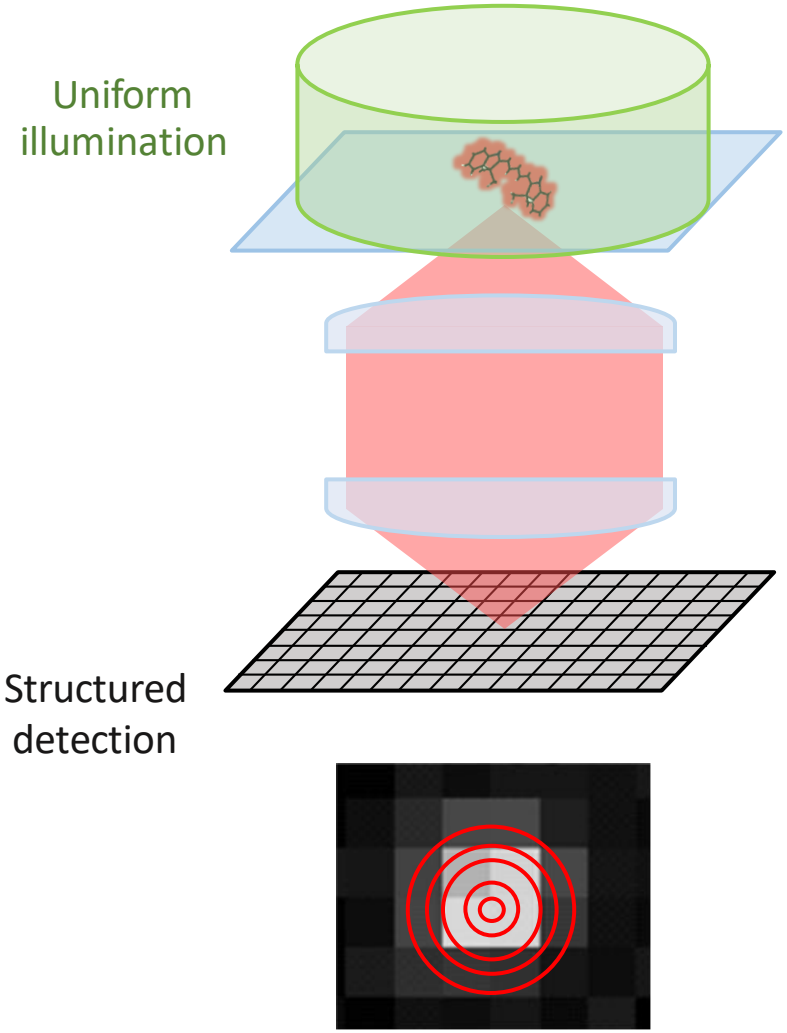
Masullo et al. Light: Science & Applications 11 (2022) 199

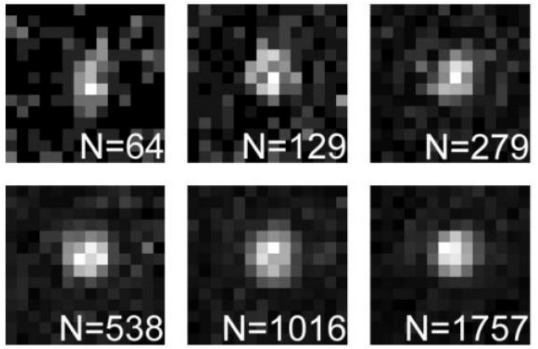
Zdańkowski et al. ***ACS Photonics*** 9 (2022) 3777–3785

Cole et al. ***Nature Photonics*** (2024) – published online

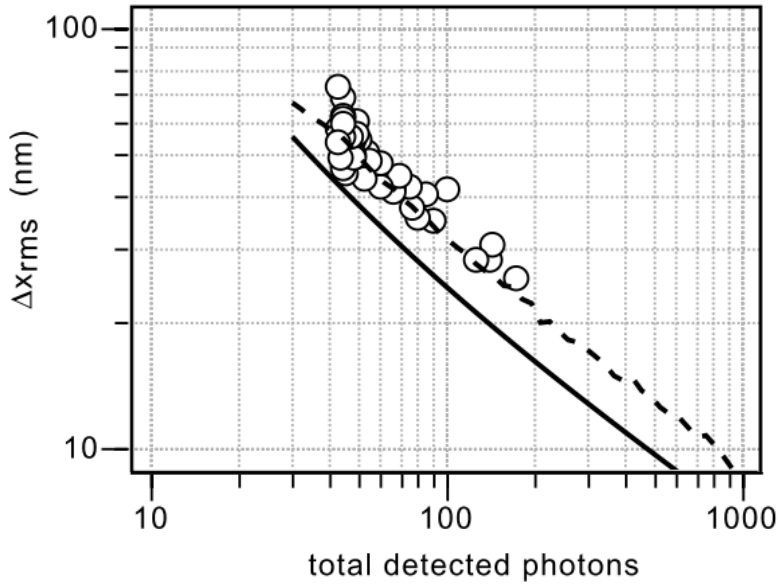
https://stefani-lab.ar/

Camera-based single molecule localization



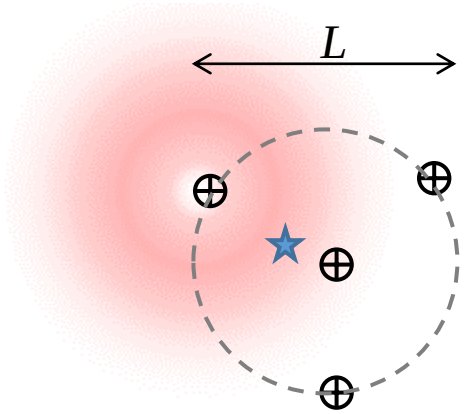


$$\sigma \approx PSF/\sqrt{N}$$

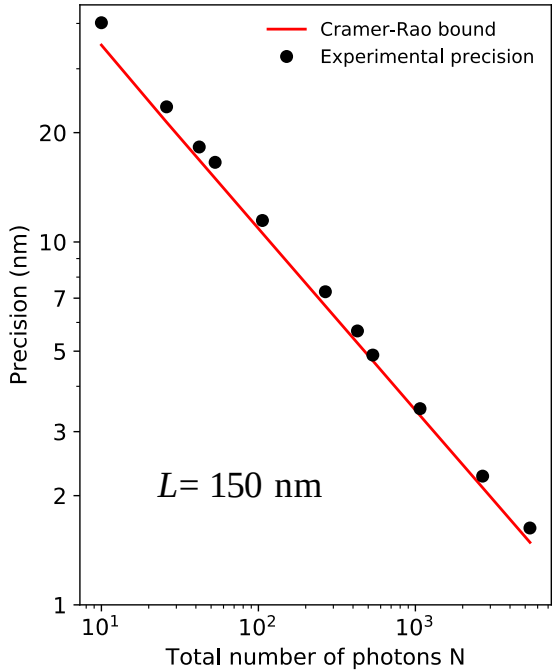


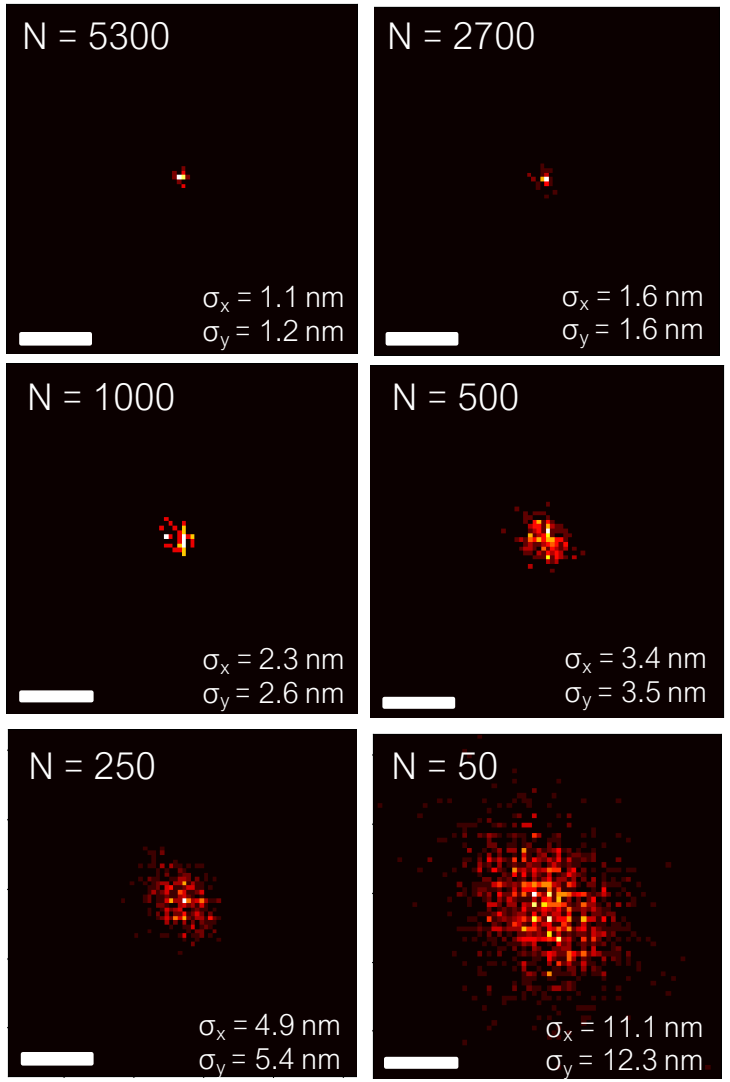
Thompson, R. E.; Larson, D. R.; Webb, W. W.
Biophysical Journal 82 (2002) 2775–2783

MINFLUX



$$\sigma_{CRB} \propto \frac{L}{\sqrt{N}}$$

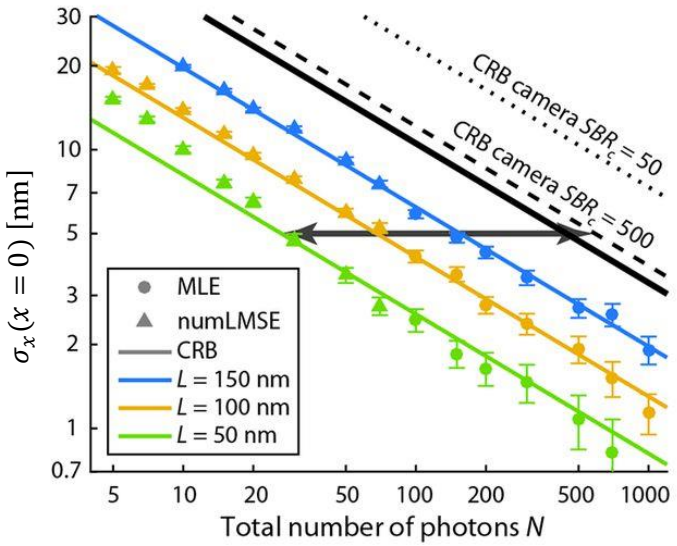




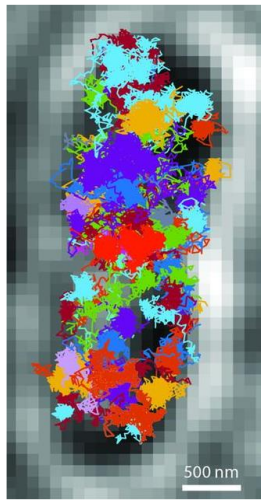
Scale bar: 20 nm

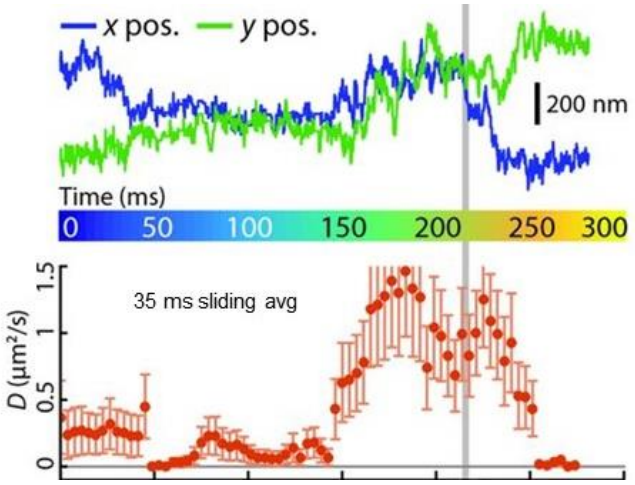
MINFLUX

Tunable nanometer resolution

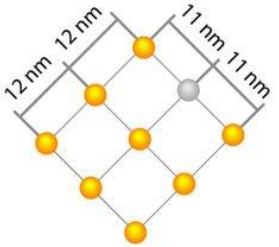


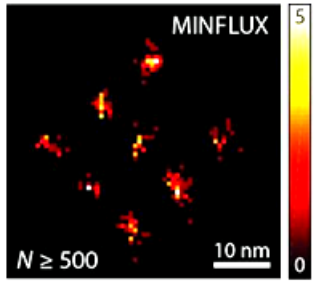
Superfast tracking





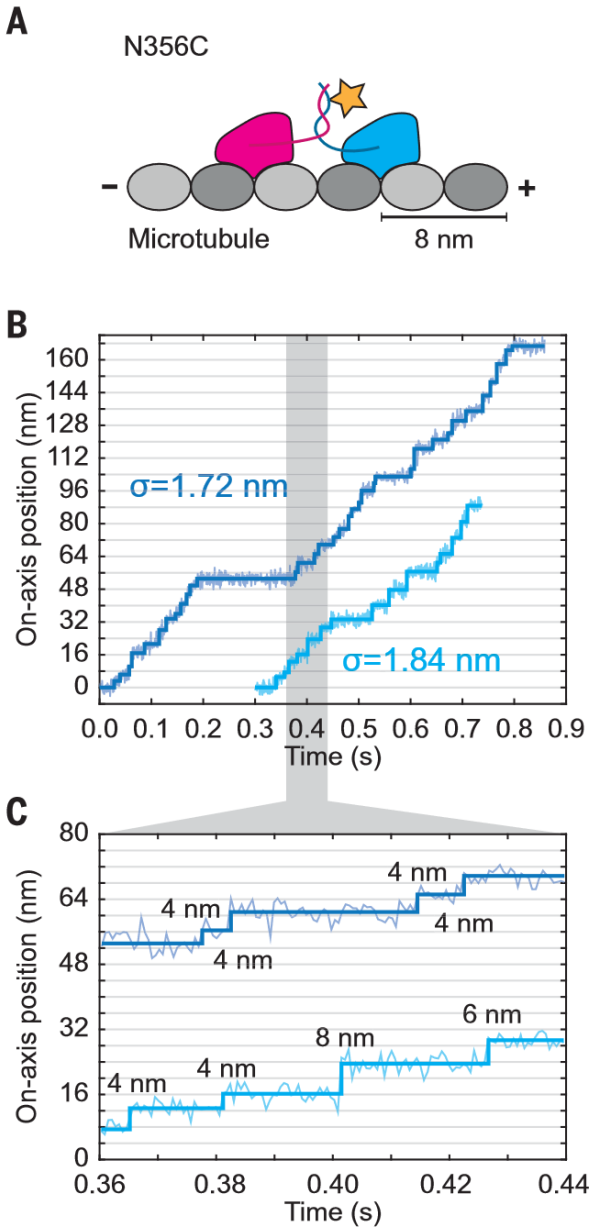
Nanoscopy

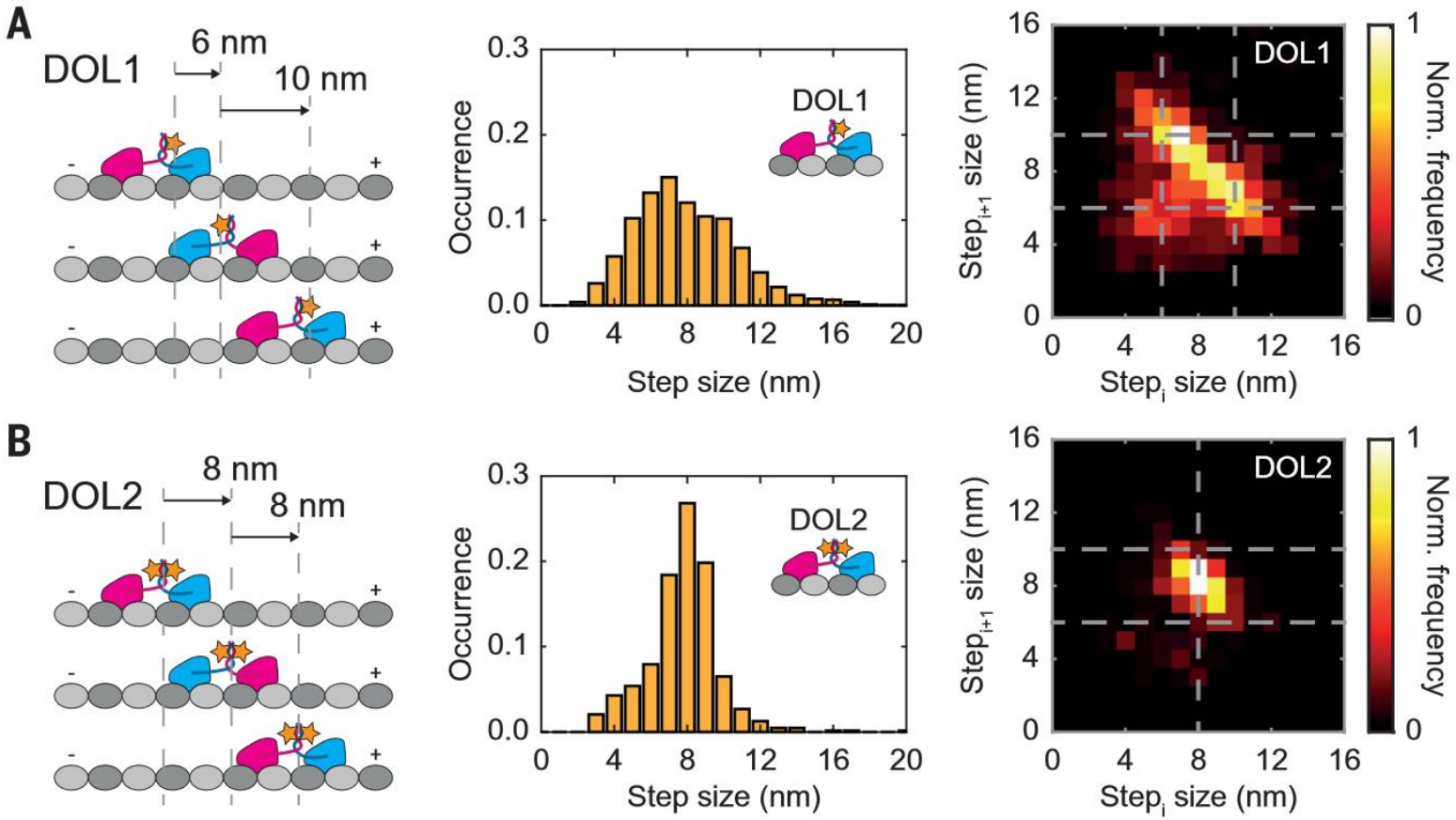




Stefan Hell & Fernando Stefani Labs - Balzarotti et al. ***Science*** 355 (2017) 606-612

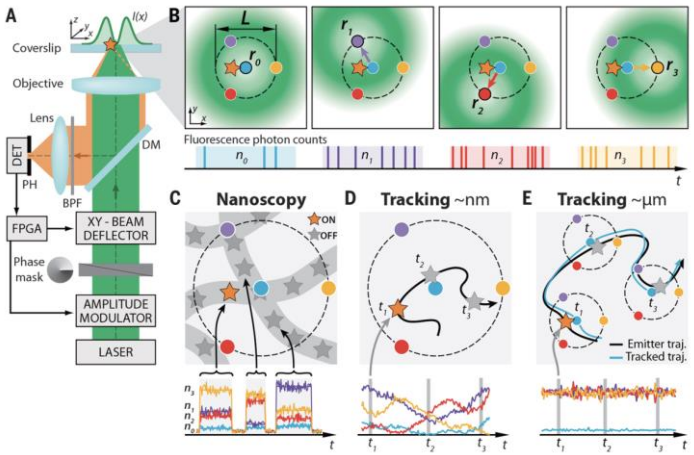
MINFLUX



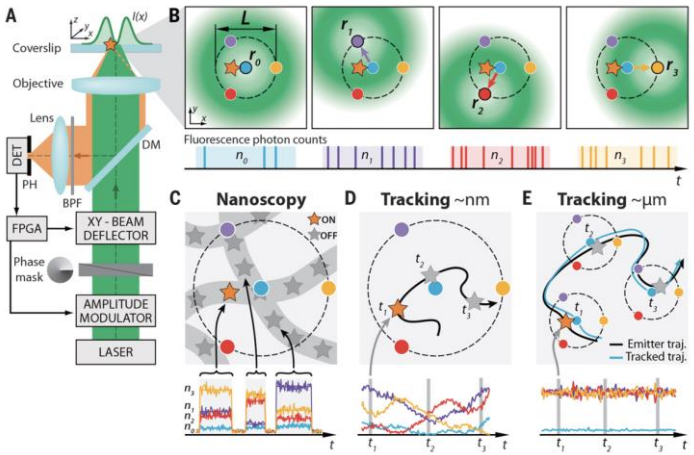


Stefan Hell Lab - Wirth et al. ***Science*** 379 (2023) 1004-1010

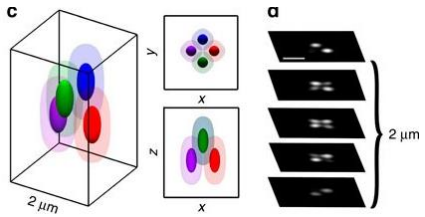
MINFLUX Balzarotti et al, *Science* (2017)

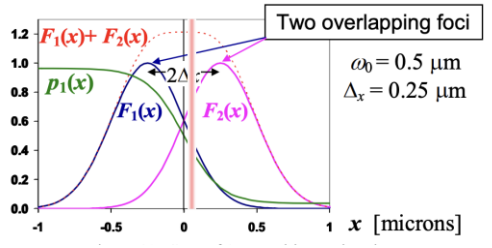


MINFLUX Balzarotti et al, *Science* (2017)

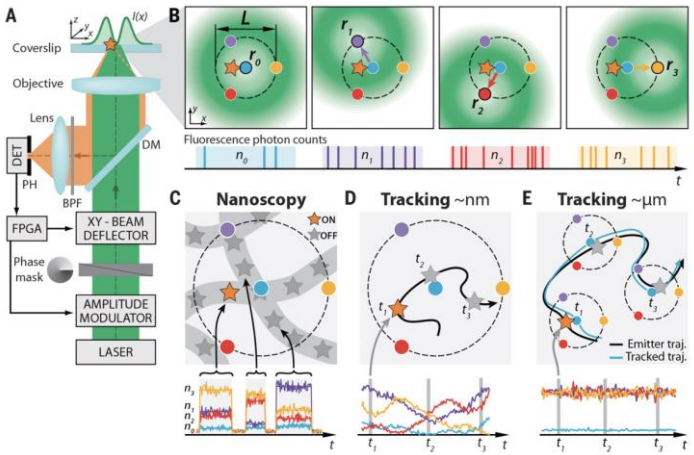


Four-focus particle tracking Perillo et al, ***Nat. Comm.*** (**2015**) Davis et al, ***Opt. Express*** (**2014**)



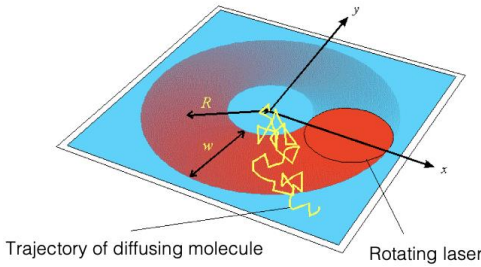


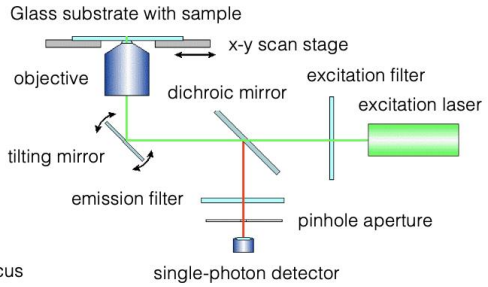
MINFLUX Balzarotti et al, *Science* (2017)



Orbital tracking

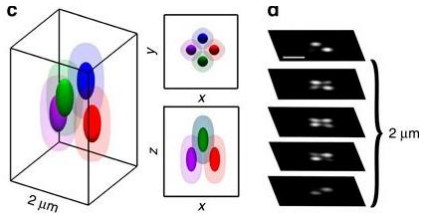
Enderlein, ***Appl. Phys. B Lasers Opt.*** (**2000**) Levi et al, ***Biochem. Soc. Trans.*** (**2003**)

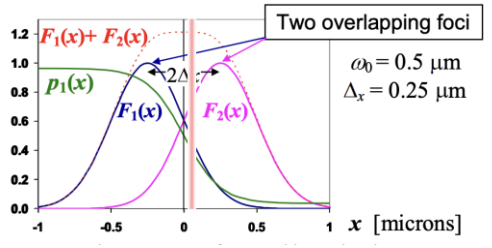




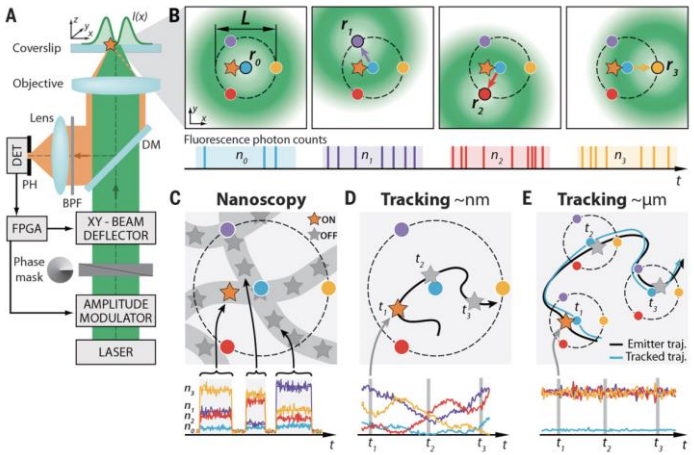
Four-focus particle tracking

Perillo et al, ***Nat. Comm.*** (**2015**) Davis et al, ***Opt. Express*** (**2014**)



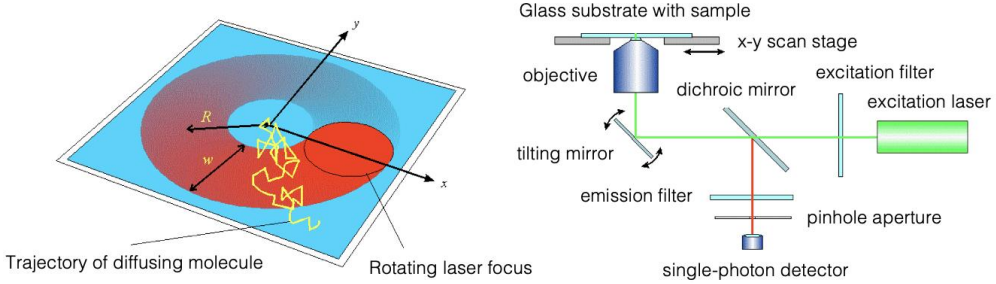


MINFLUX Balzarotti et al, *Science* (2017)



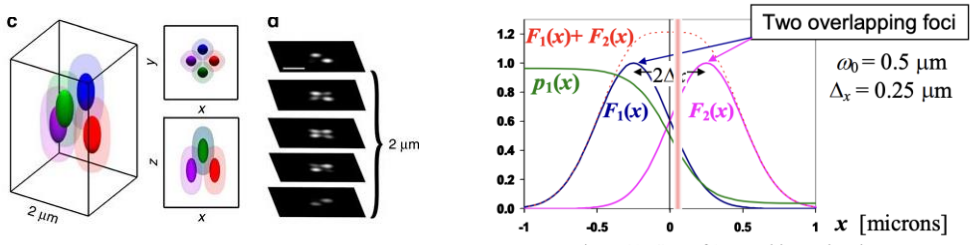
Orbital tracking

Enderlein, ***Appl. Phys. B Lasers Opt.*** (**2000**) Levi et al, ***Biochem. Soc. Trans.*** (**2003**)



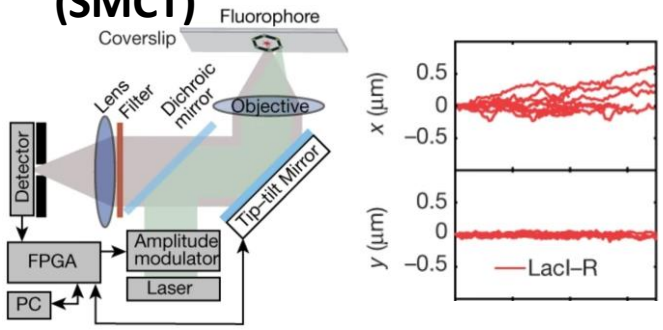
Four-focus particle tracking

Perillo et al, ***Nat. Comm.*** (**2015**) Davis et al, ***Opt. Express*** (**2014**)

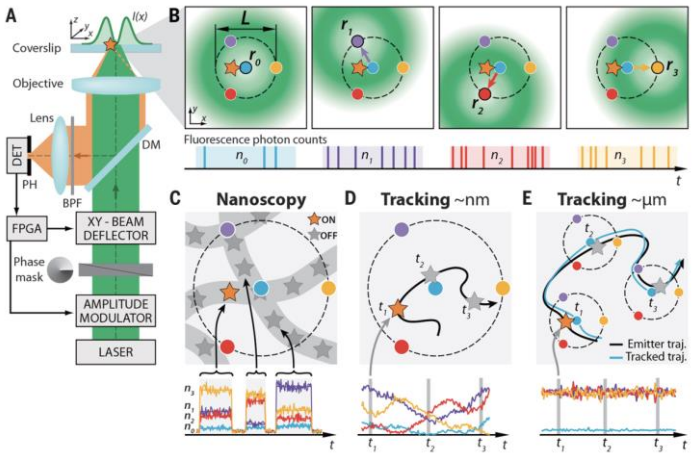


Single-molecule confocal tracking (SMCT)

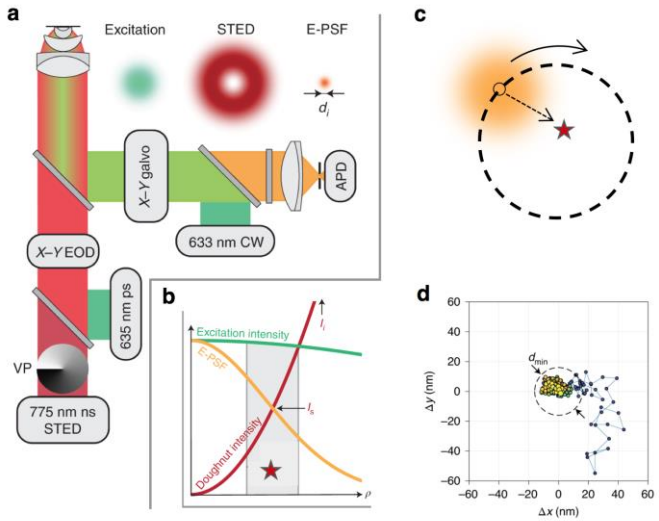
Marklund et al, ***Nature*** (**2020**)



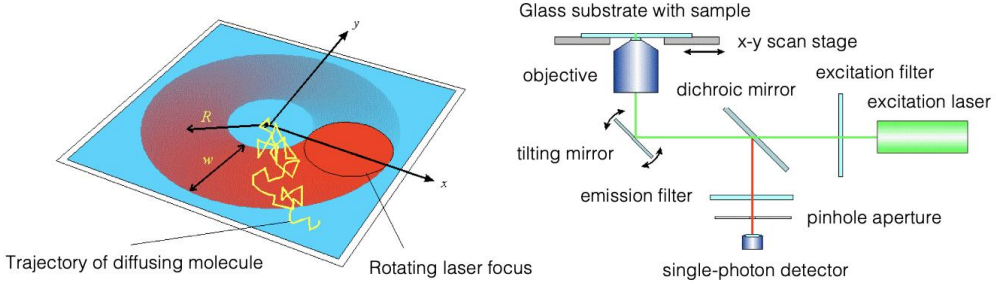
MINFLUX Balzarotti et al, *Science* (2017)



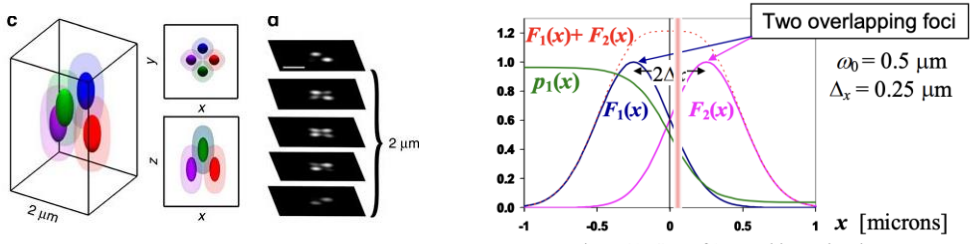
MINSTED Weber et al, *Nature Photonics* (2021)



Orbital tracking Enderlein, ***Appl. Phys. B Lasers Opt.*** **(2000)** Levi et al, ***Biochem. Soc. Trans.*** **(2003)**

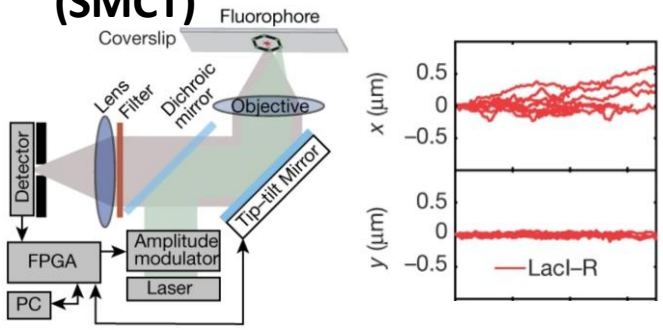


Four-focus particle tracking Perillo et al, ***Nat. Comm.*** **(2015)** Davis et al, ***Opt. Express*** **(2014)**

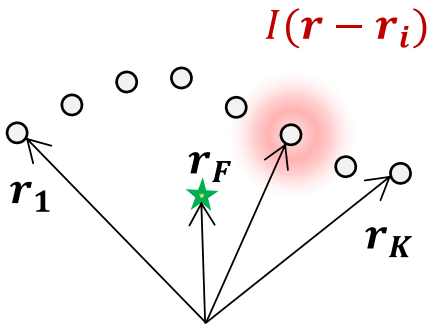


Single-molecule confocal tracking (SMCT)

Marklund et al, ***Nature*** **(2020)**



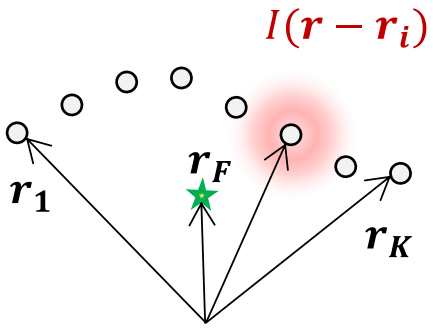
Common framework for SML-SSI



Open-source Python code: https://github.com/Stefani-Lab

Masullo et al, ***Biophysical Reports*** 2, 100036 (2022)

Common framework for SML-SSI



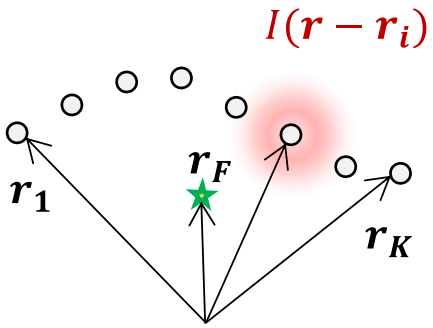
Goal: inferring the position of the emitter $\boldsymbol{r}_F$, given:

$I(\boldsymbol{r})$	The structure of the illumination
$\boldsymbol{r}_i$	The sequence of beam positions
$\boldsymbol{n}_i$	The detected signal in each position

Open-source Python code: https://github.com/Stefani-Lab

Masullo et al, *Biophysical Reports* 2, 100036 (2022)

Common framework for SML-SSI



Goal: inferring the position of the emitter $\boldsymbol{r}_F$, given:

$I(\boldsymbol{r})$	The structure of the illumination
$\boldsymbol{r}_i$	The sequence of beam positions
$\boldsymbol{n}_i$	The detected signal in each position

↓

Maximum likelihood estimation

$$\mathcal{L}(\boldsymbol{r_E}|I(\boldsymbol{r}-\boldsymbol{r}_i),\ \boldsymbol{n}_i)$$

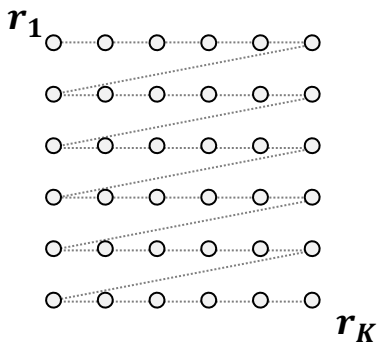
$$\widehat{\boldsymbol{r_E}}^{MLE} = \arg\max\ \mathcal{L}$$

Open-source Python code: https://github.com/Stefani-Lab

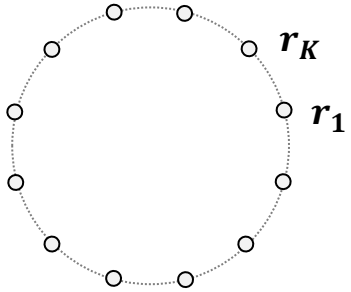
Masullo et al, *Biophysical Reports* 2, 100036 (2022)

Common framework for SML-SSI

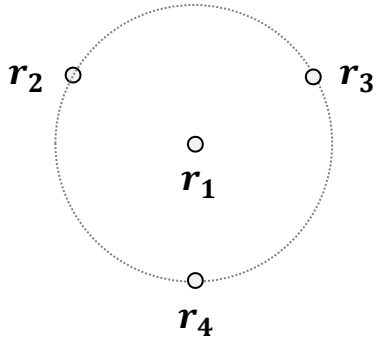
Raster scanning



Orbital scanning



MINFLUX

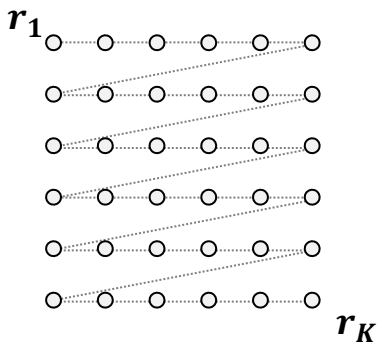


Open-source Python code: https://github.com/Stefani-Lab

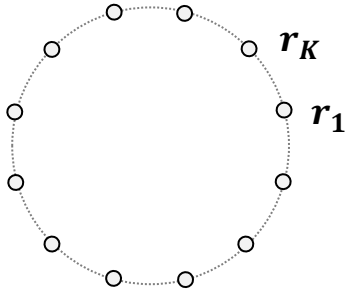
Masullo et al, *Biophysical Reports* 2, 100036 (2022)

Common framework for SML-SSI

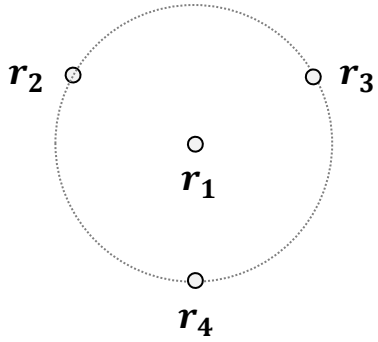
Raster scanning



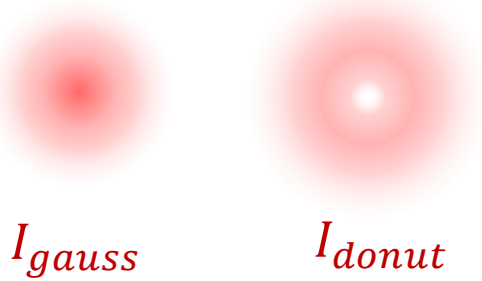
Orbital scanning



MINFLUX



Intensity MAX



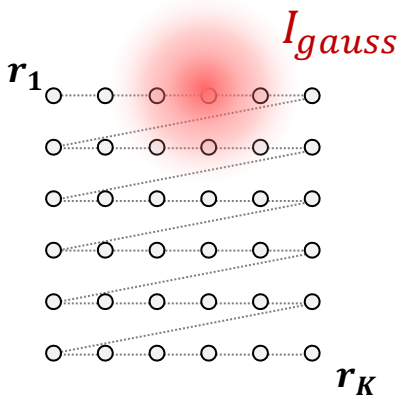
Intensity MIN

Open-source Python code: https://github.com/Stefani-Lab

Masullo et al, *Biophysical Reports* 2, 100036 (2022)

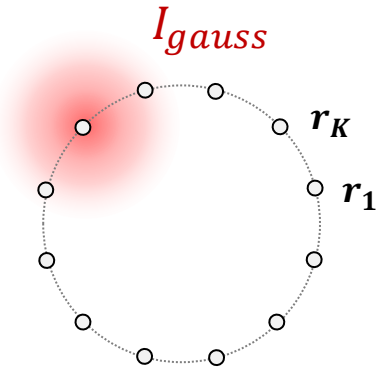
Common description of known methods

Confocal SMLM



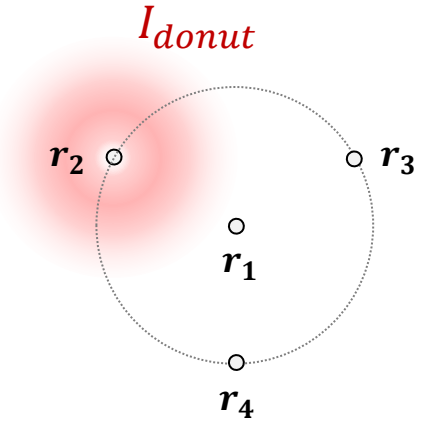
Thiele et al. *ACS Nano* 2020
Zaza et al. *Small Methods* 2023

Orbital tracking / MINSTED



Enderlein *Appl. Phys. B* 2000
Kis-Petikova, et al. Microsc. Res. Tech. 2004
Wehnekamp et al. eLife 2019
Marklund et al. *Nature* 2020
Weber et al. *Nature Phot*. 2021

MINFLUX



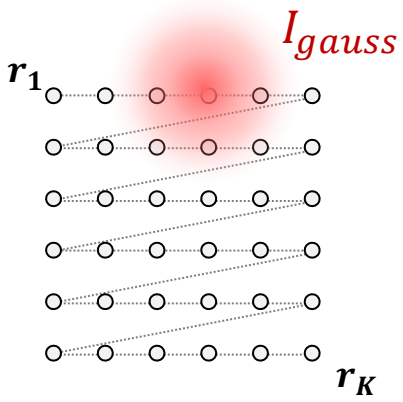
Balzarotti et al. *Science* 2017
Masullo et al. *Nano Lett*. 2021

Open-source Python code: https://github.com/Stefani-Lab

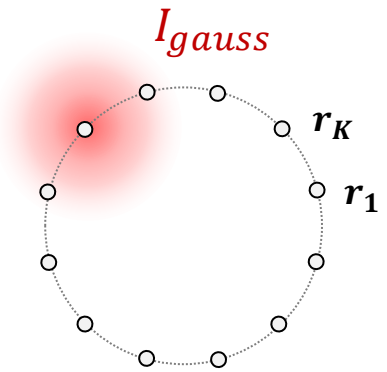
Masullo et al, ***Biophysical Reports*** 2, 100036 (2022)

Common description of known and new methods

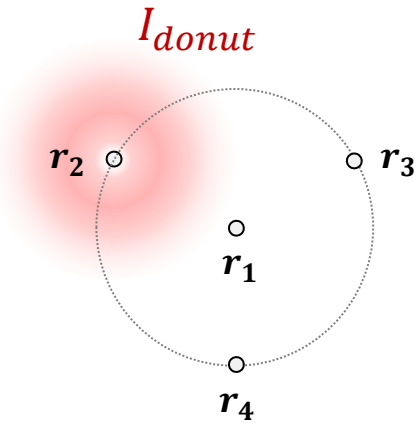
RASTMAX



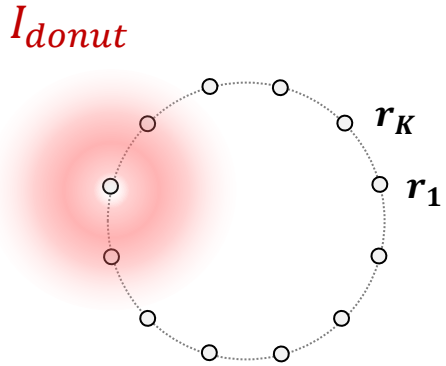
OT



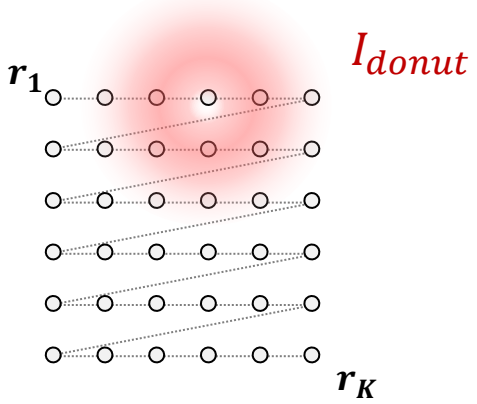
MINFLUX



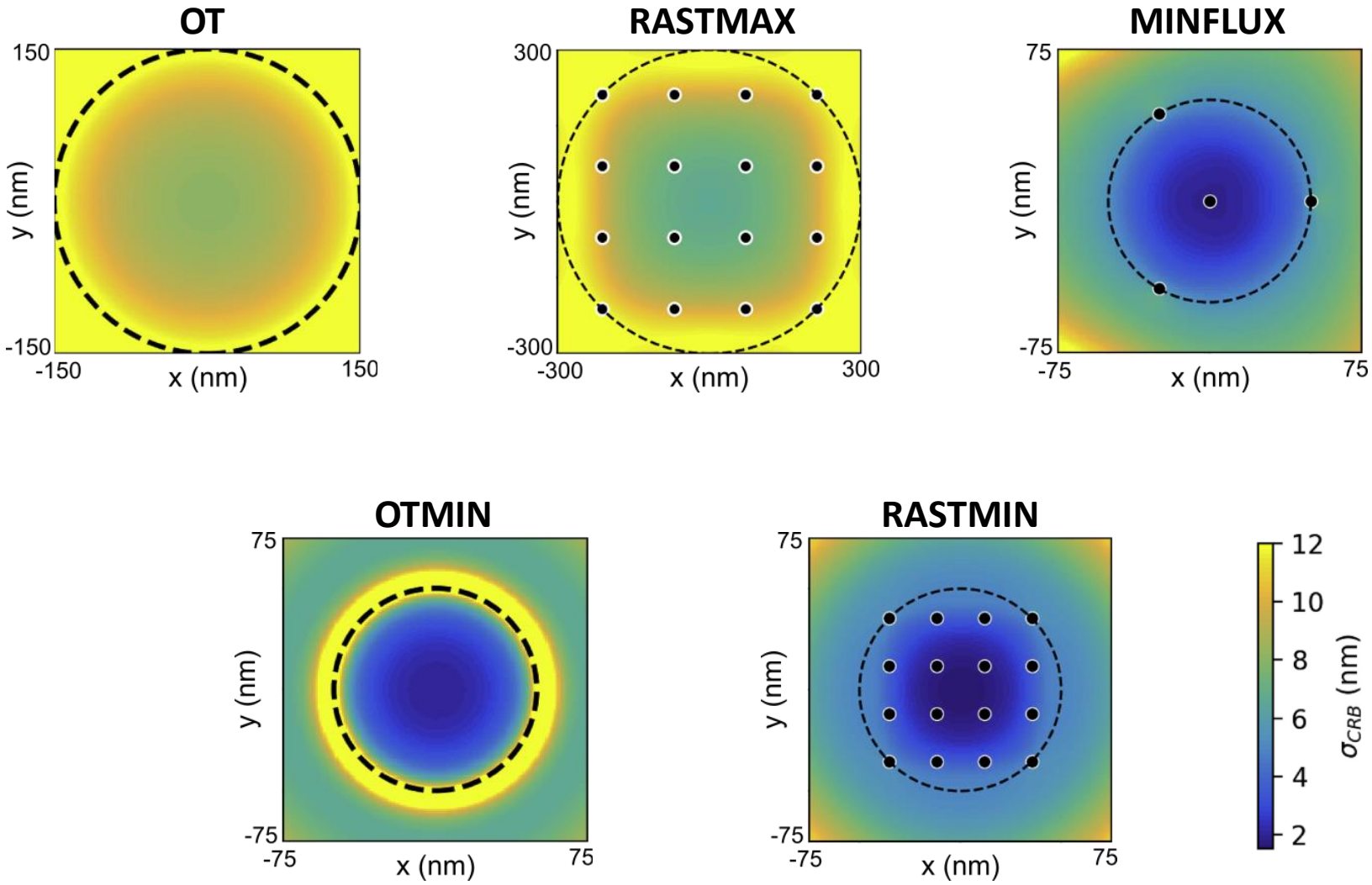
OTMIN



RASTMIN



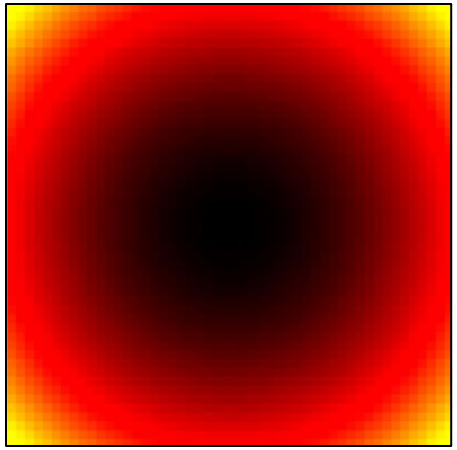
Fair benchmarking of known and new methods



Masullo et al, ***Biophysical Reports*** 2, 100036 (2022)

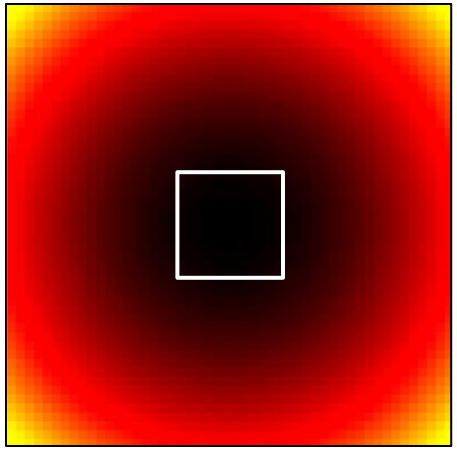
Indefinite zooming-in with zeroes

Parabolic minimum

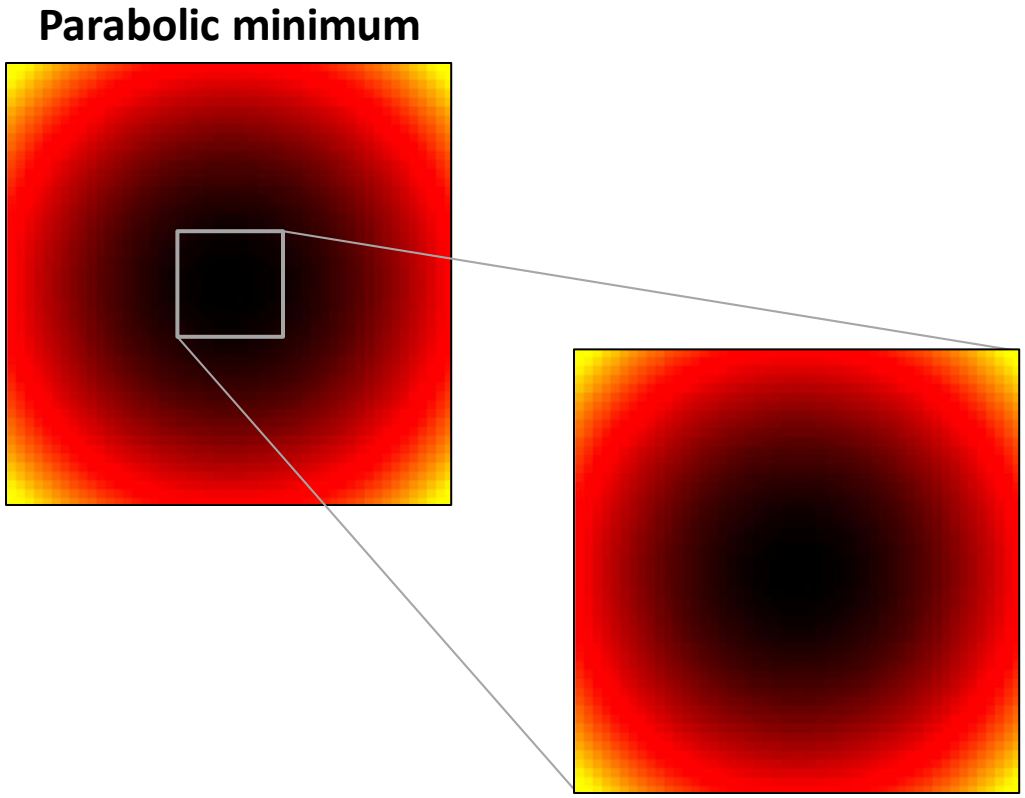


Indefinite zooming-in with zeroes

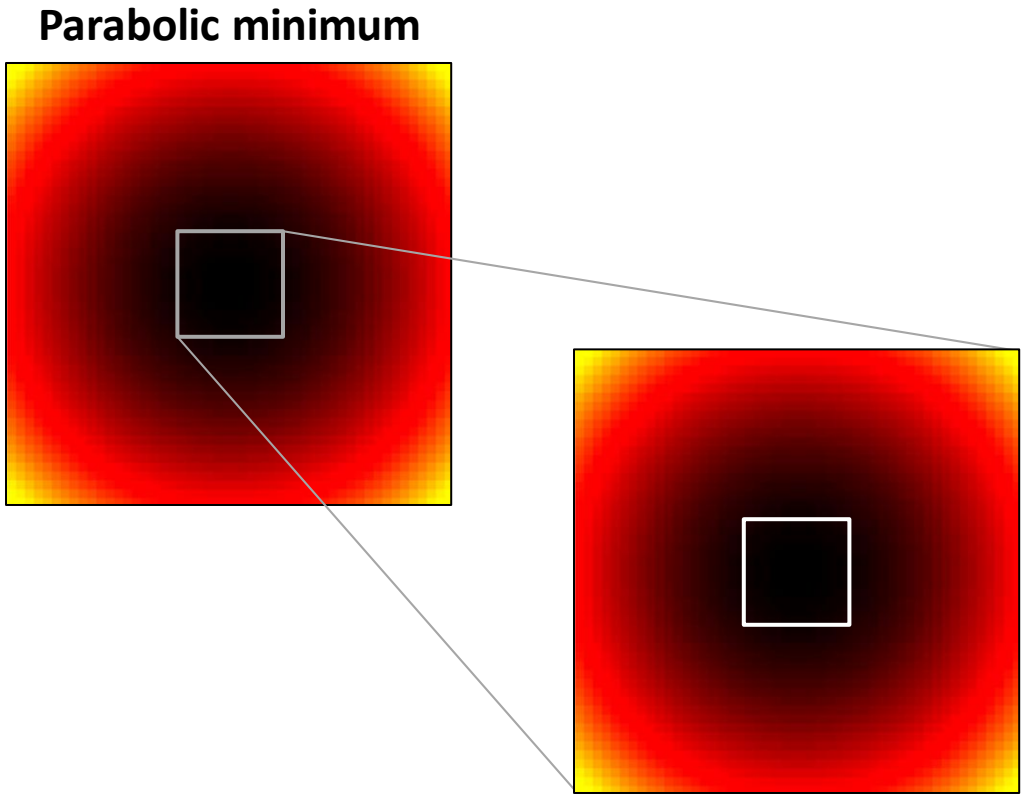
Parabolic minimum



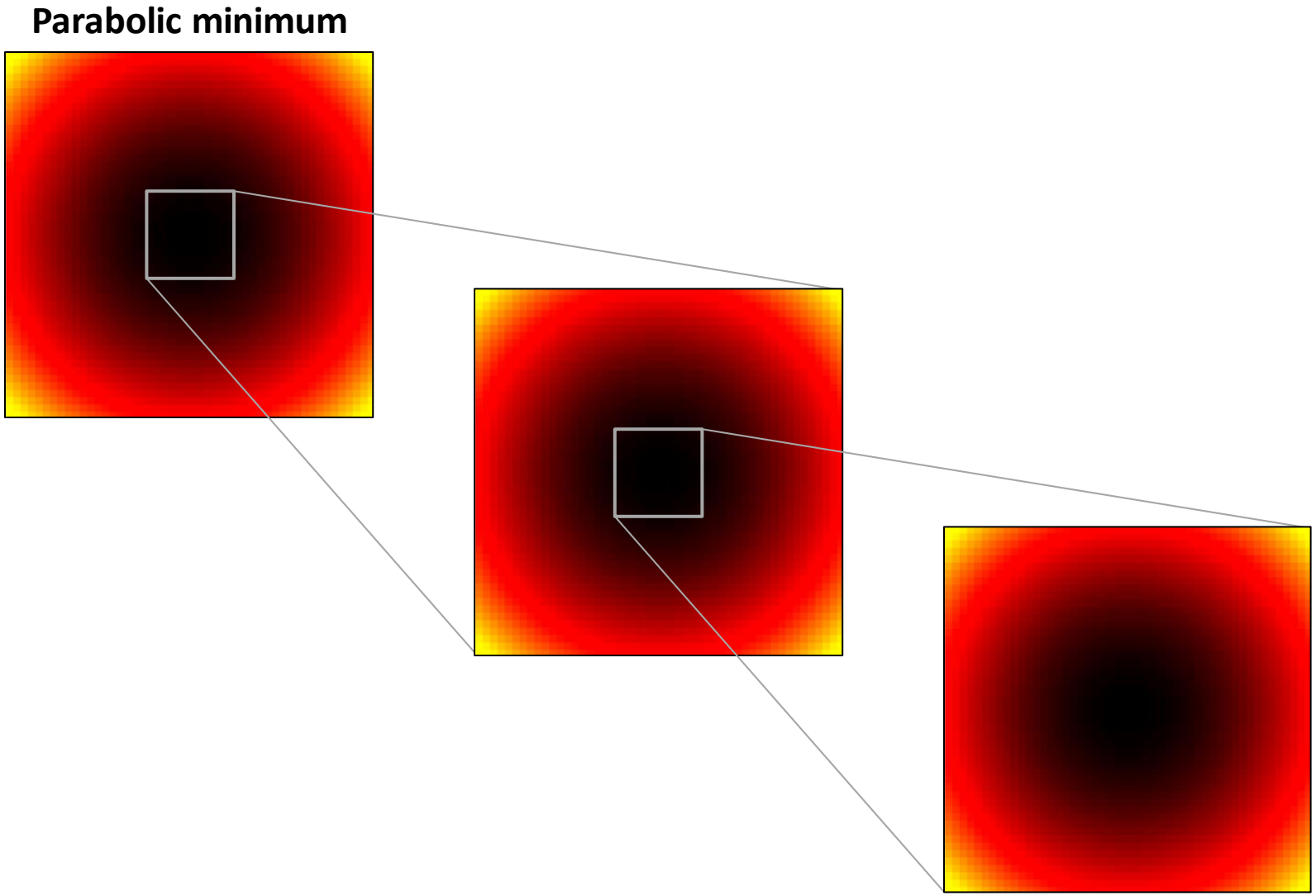
Indefinite zooming-in with zeroes



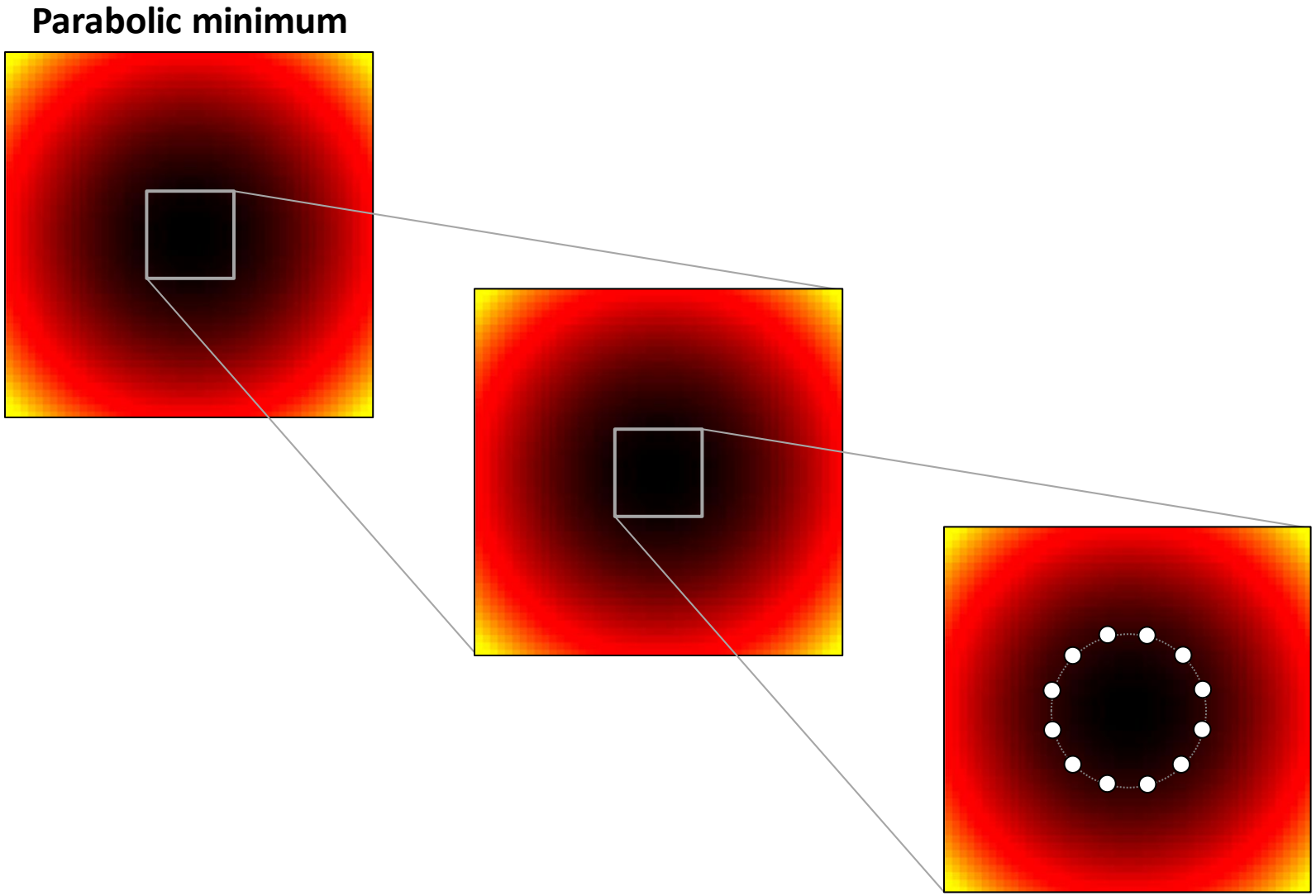
Indefinite zooming-in with zeroes



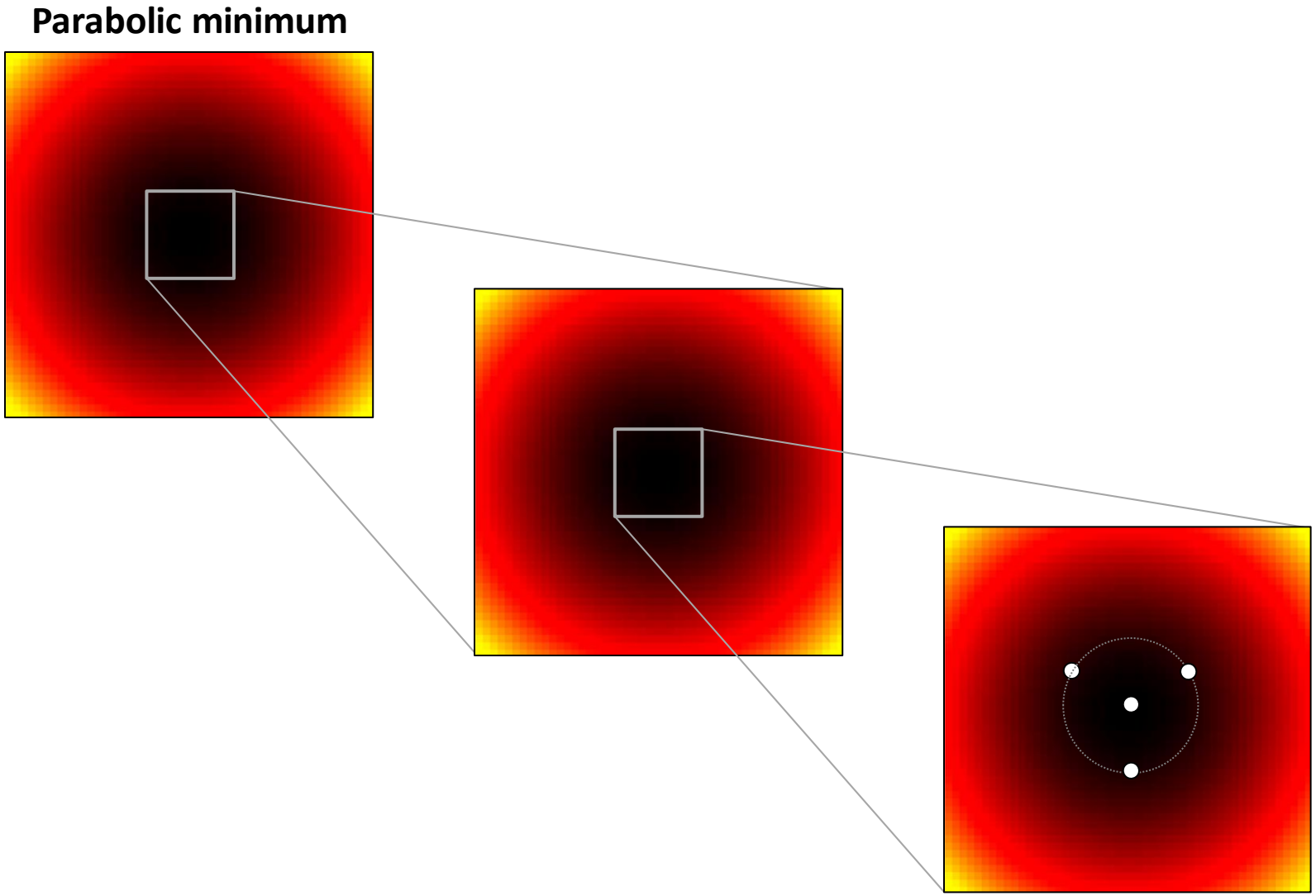
Indefinite zooming-in with zeroes



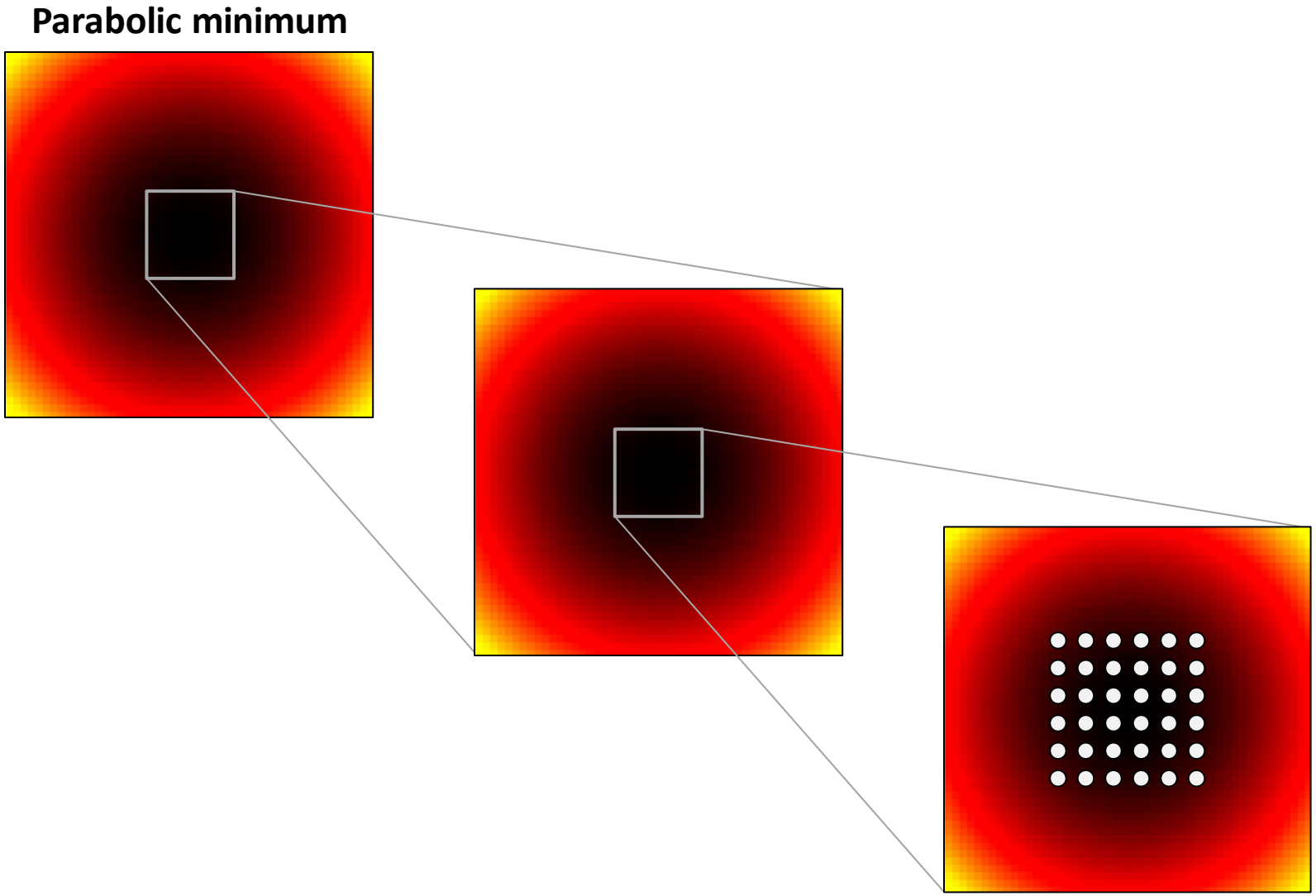
Indefinite zooming-in with zeroes



Indefinite zooming-in with zeroes

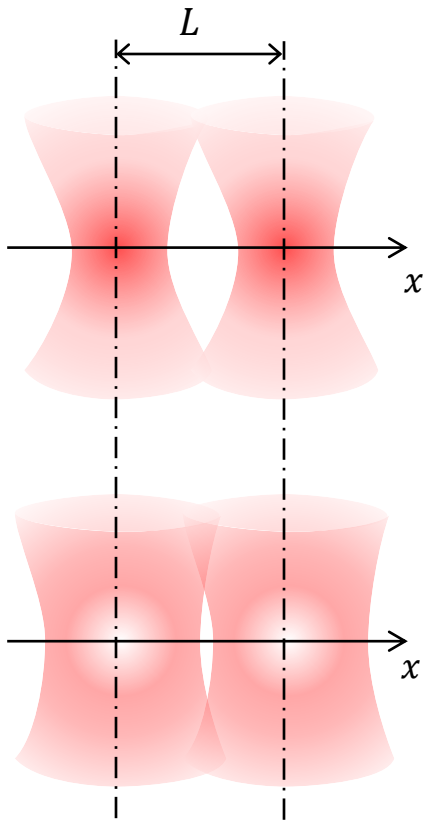


Indefinite zooming-in with zeroes

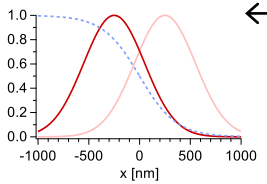


Indefinite zooming-in with zeroes

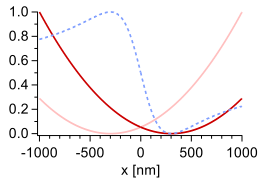
$I_1(x) = I(x + L/2)$ $I_2(x) = I(x - L/2)$ $p(x) = \frac{I_2(x)}{I_1(x) + I_2(x)}$



$L = 600$ nm



x [nm]

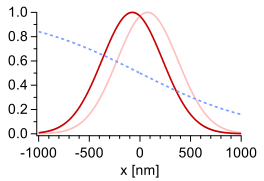


x [nm]

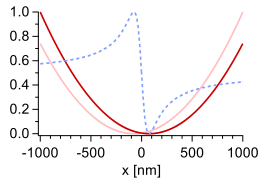
Zooming-in with maxima

Zooming-in with minima

$L = 150$ nm



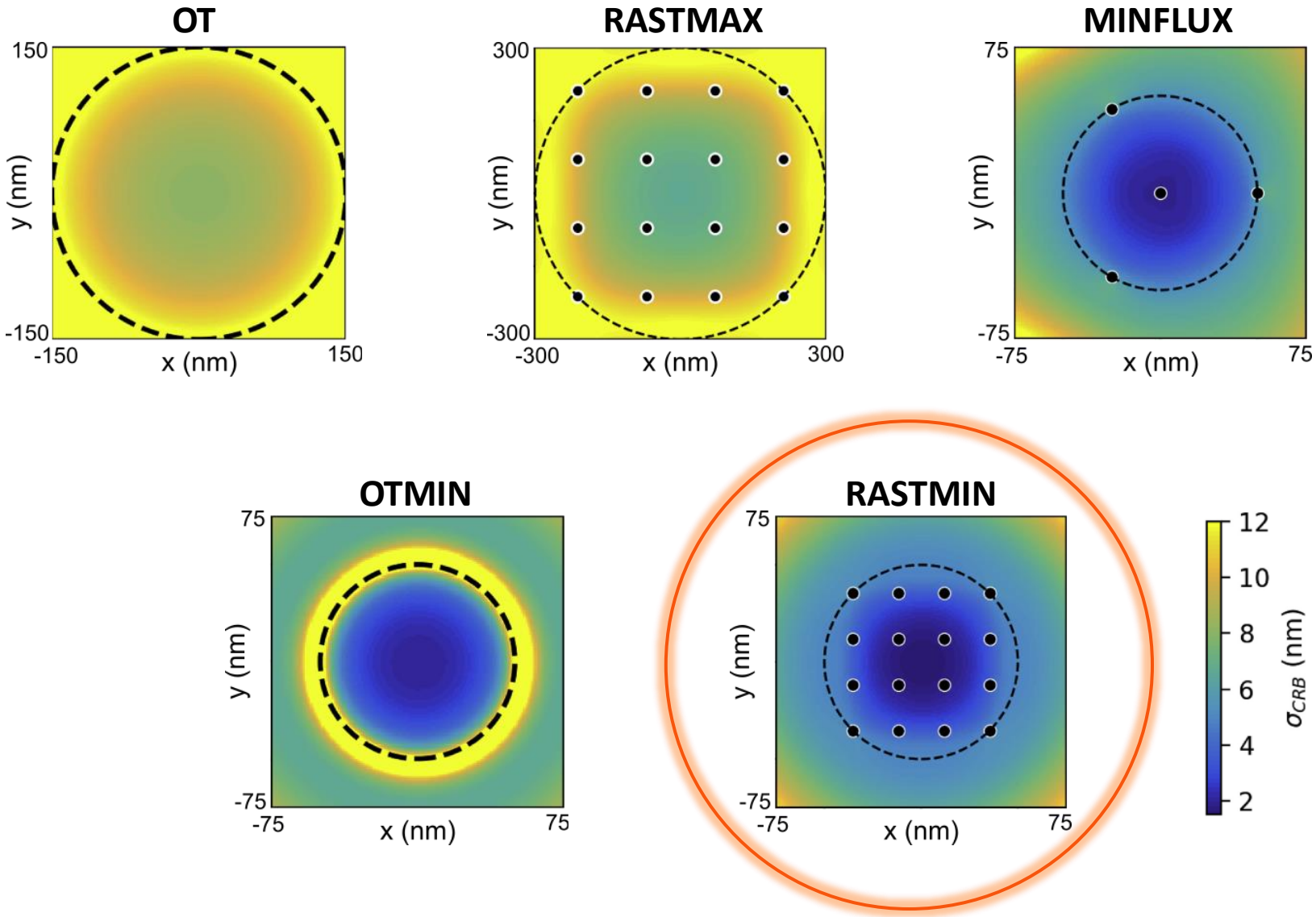
x [nm]



x [nm]

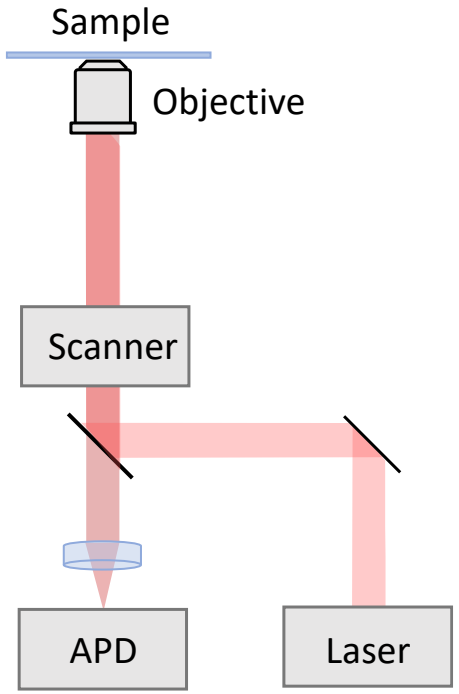
Fernando D. Stefani, "Tracking nanoscopic motion with minima of light" ***Nature Photonics*** 17 (2023) 552–553

Fair benchmarking of known and new methods



Masullo et al, ***Biophysical Reports*** 2, 100036 (2022)

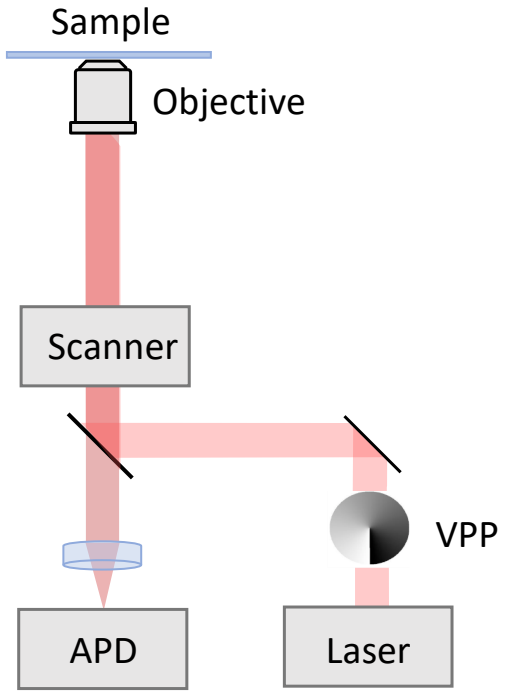
RASTMIN instrumentation



Standard scanning microscope
(confocal, two-photon)

Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

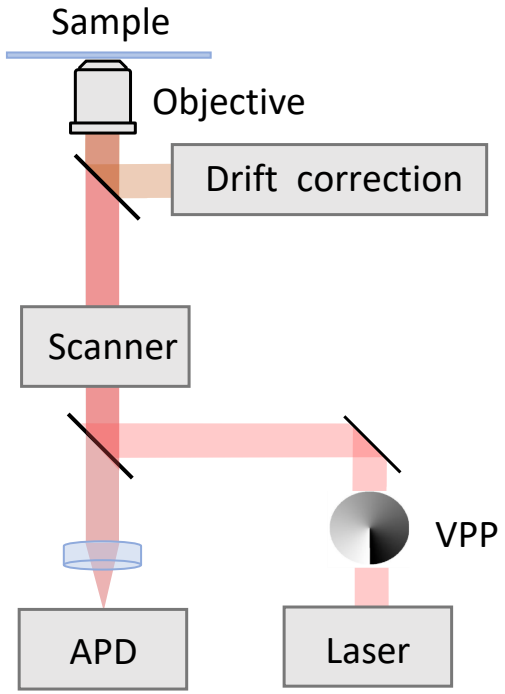
RASTMIN instrumentation



Standard scanning microscope
(confocal, two-photon)
+
Vortex Phase Plate (or SLM)

Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

RASTMIN instrumentation

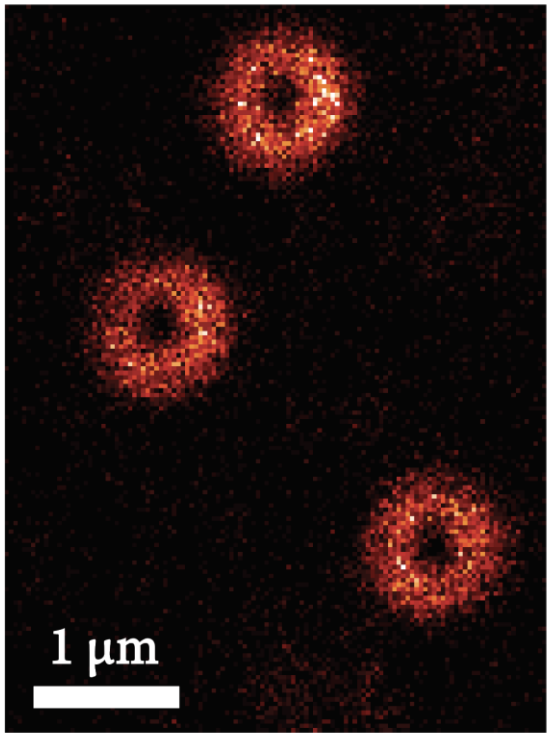


Standard scanning microscope
(confocal, two-photon)
+
Vortex Phase Plate (or SLM)
+
Active drift correction

Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

Single-molecule localization with RASTMIN

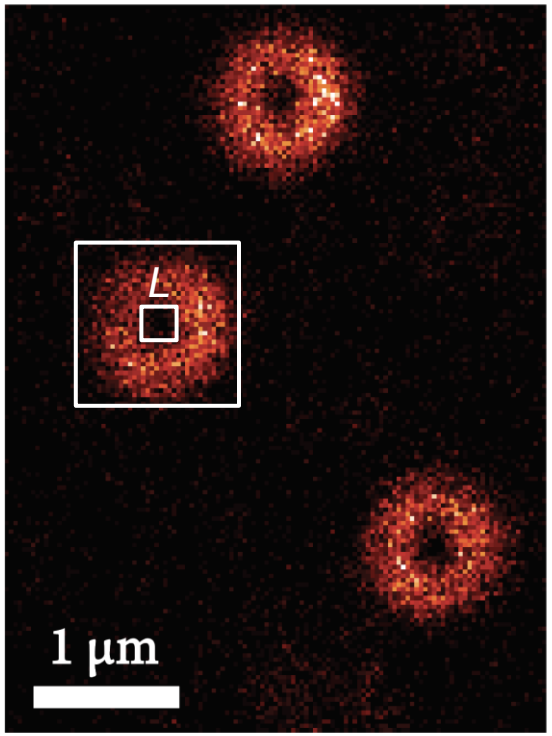
Single molecules

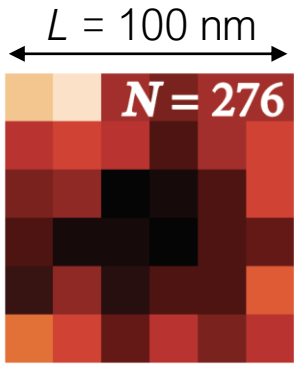


Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

Single-molecule localization with RASTMIN

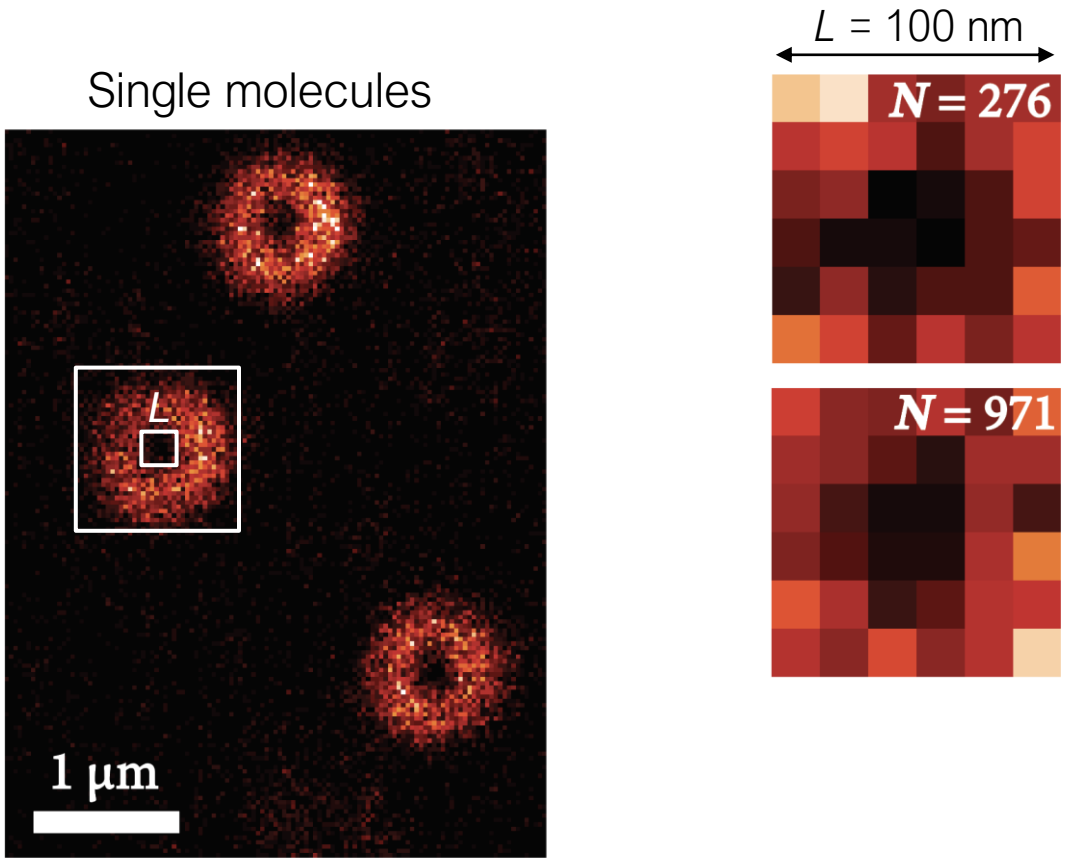
Single molecules





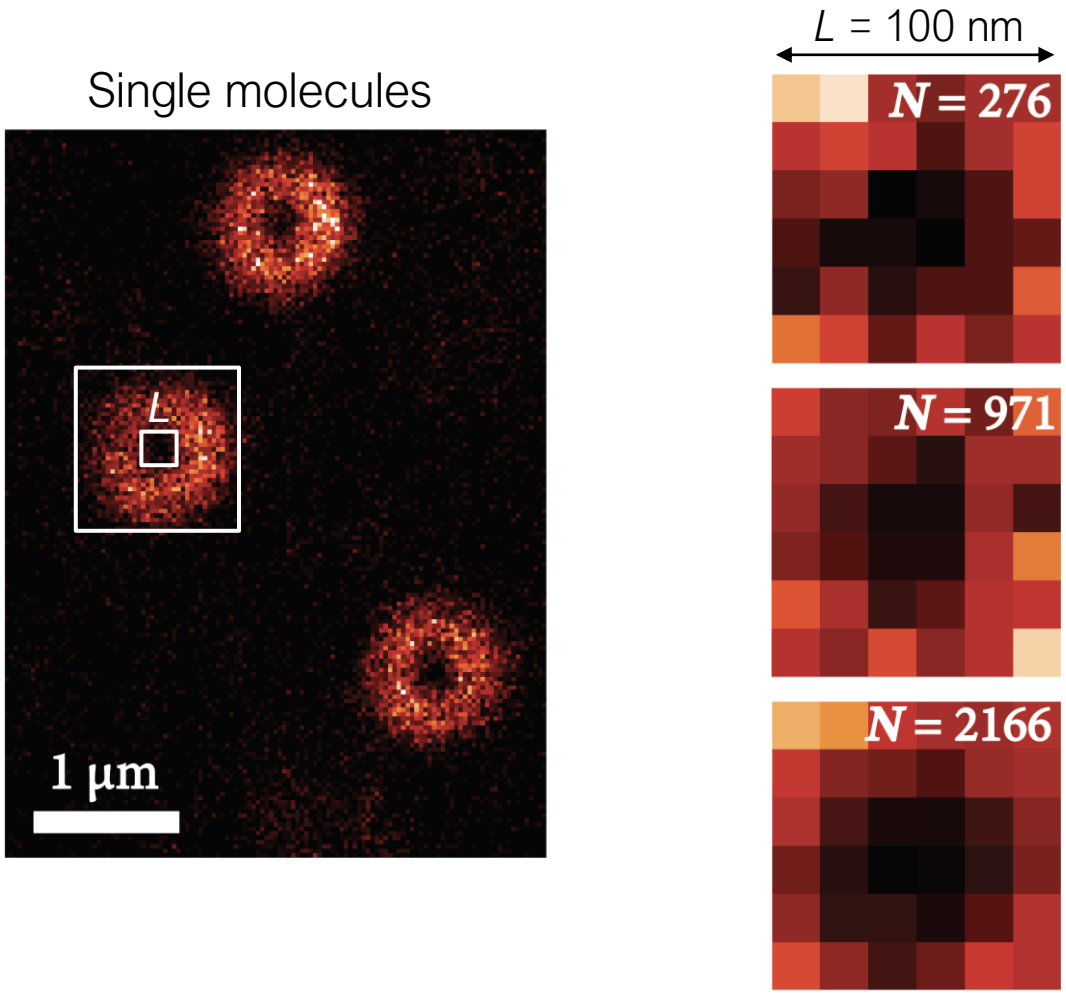
Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

Single-molecule localization with RASTMIN



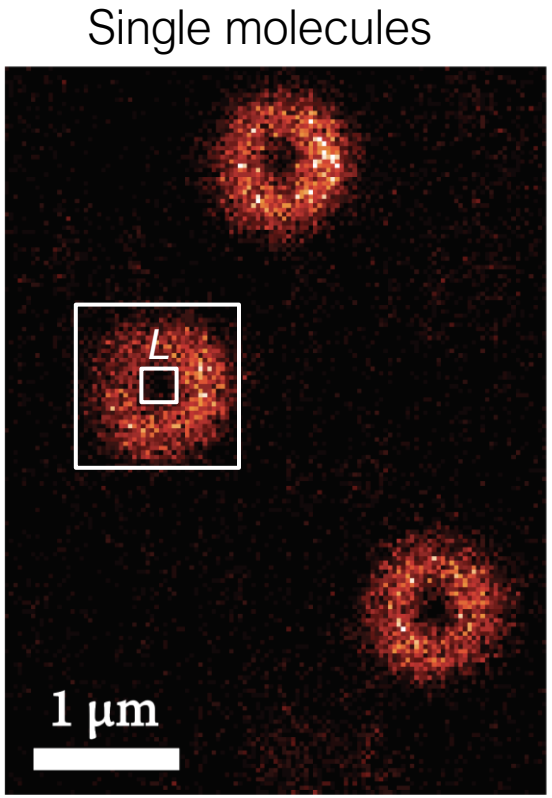
Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

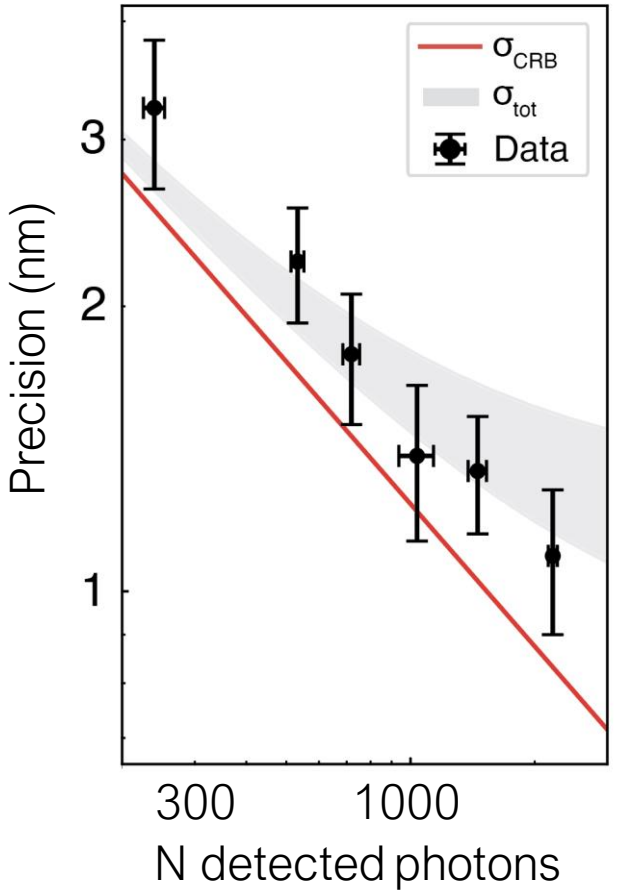
Single-molecule localization with RASTMIN



Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

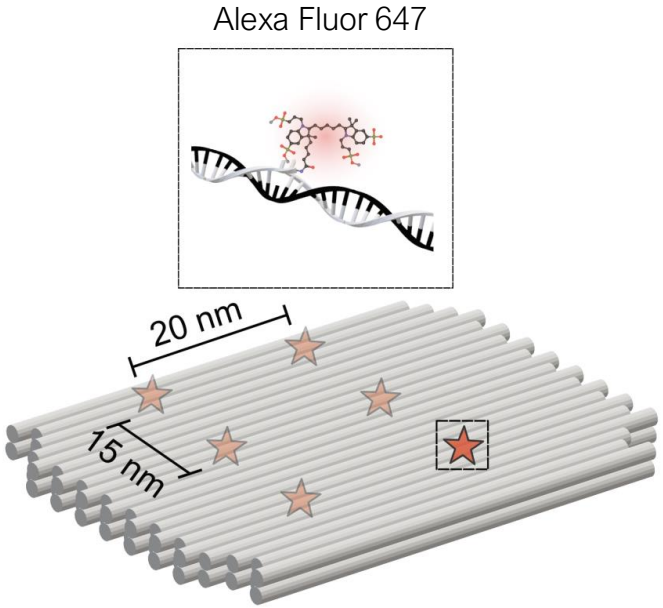
Single-molecule localization with RASTMIN



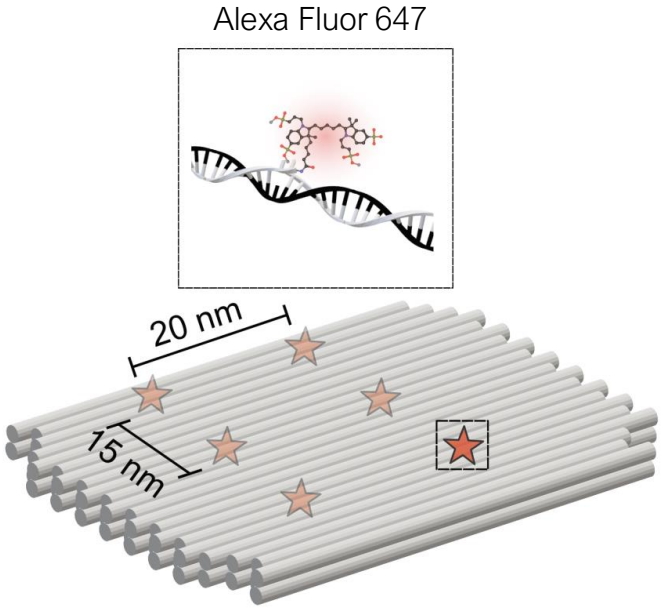


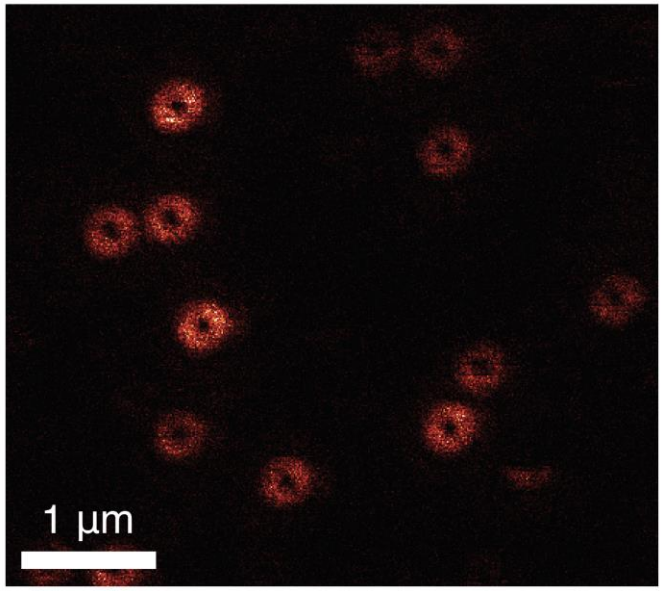
Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

RASTMIN nanoscopy

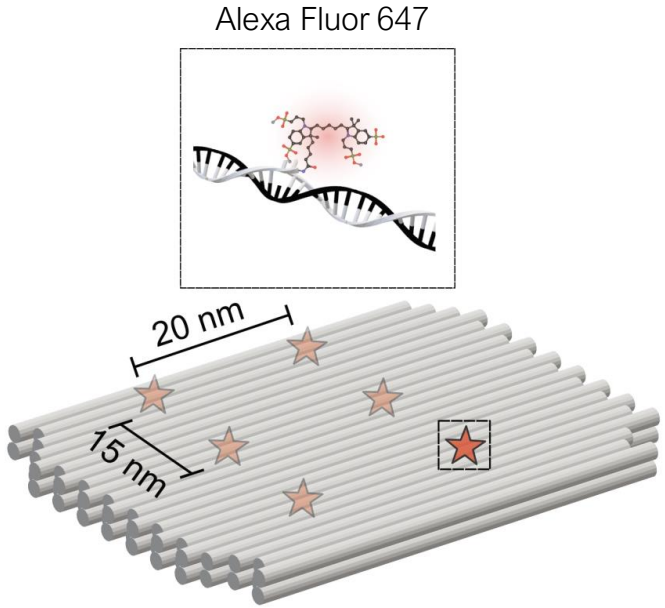


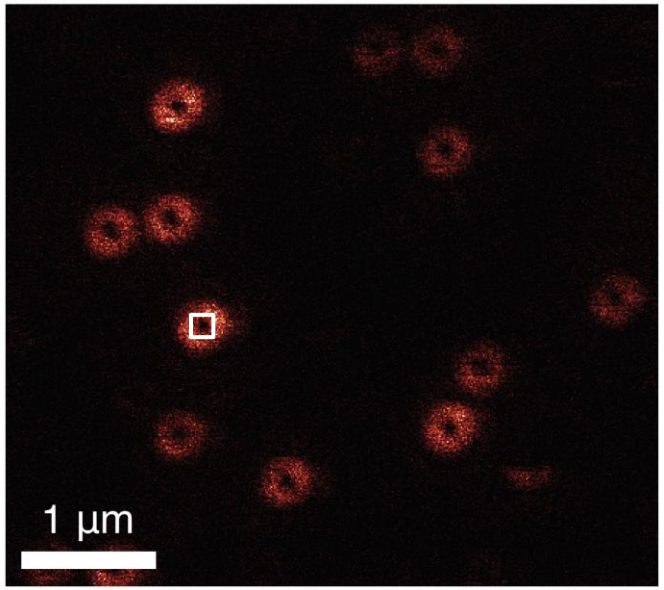
RASTMIN nanoscopy

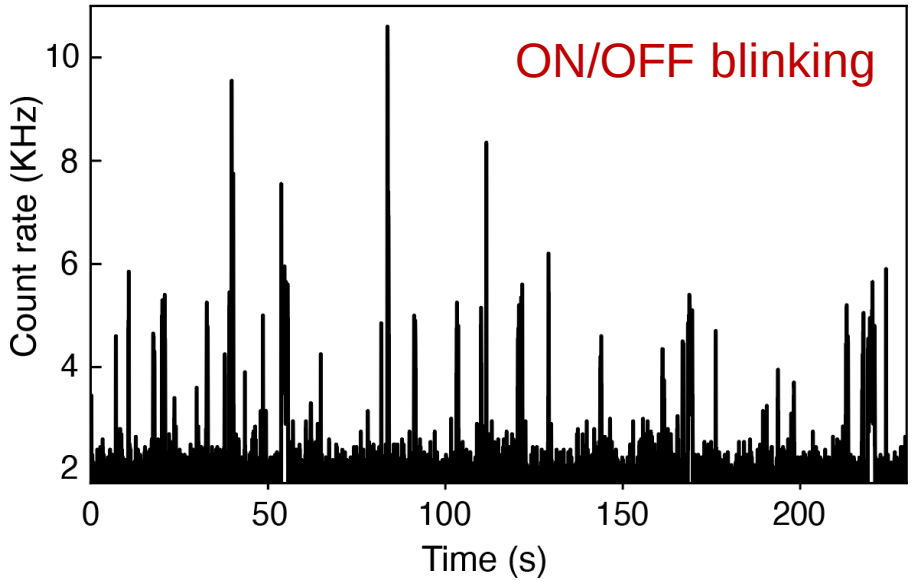




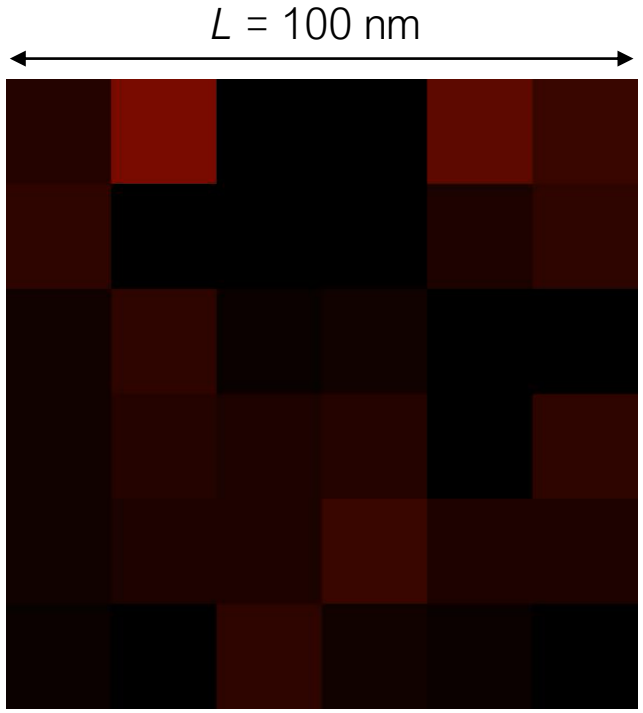
RASTMIN nanoscopy



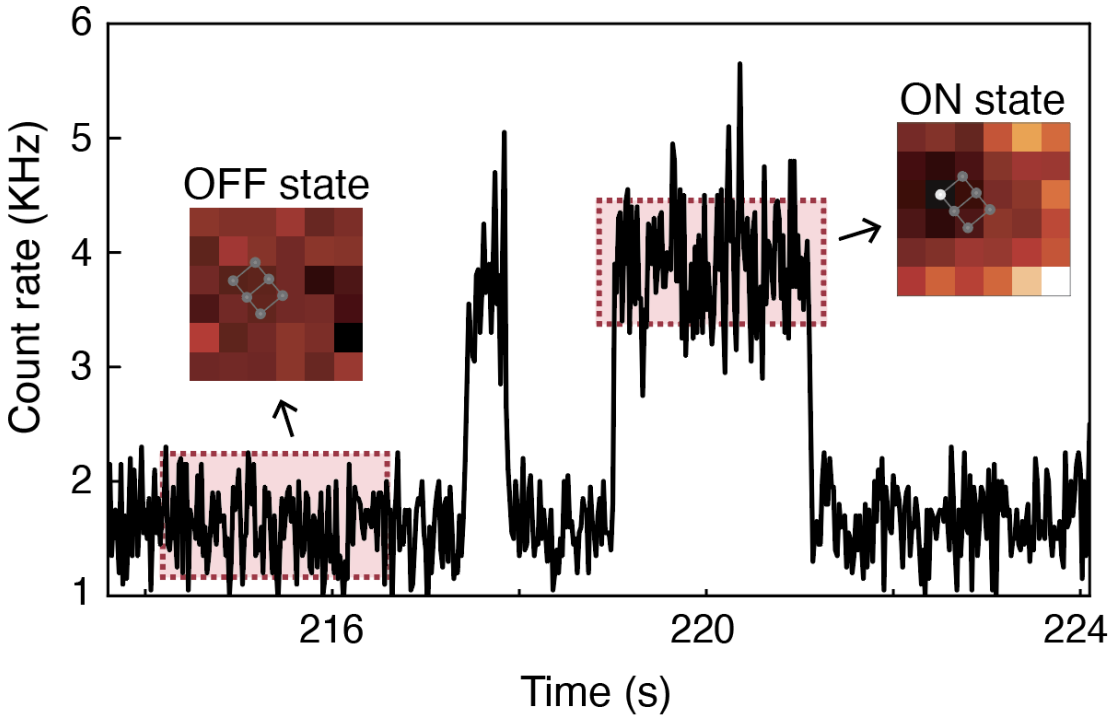




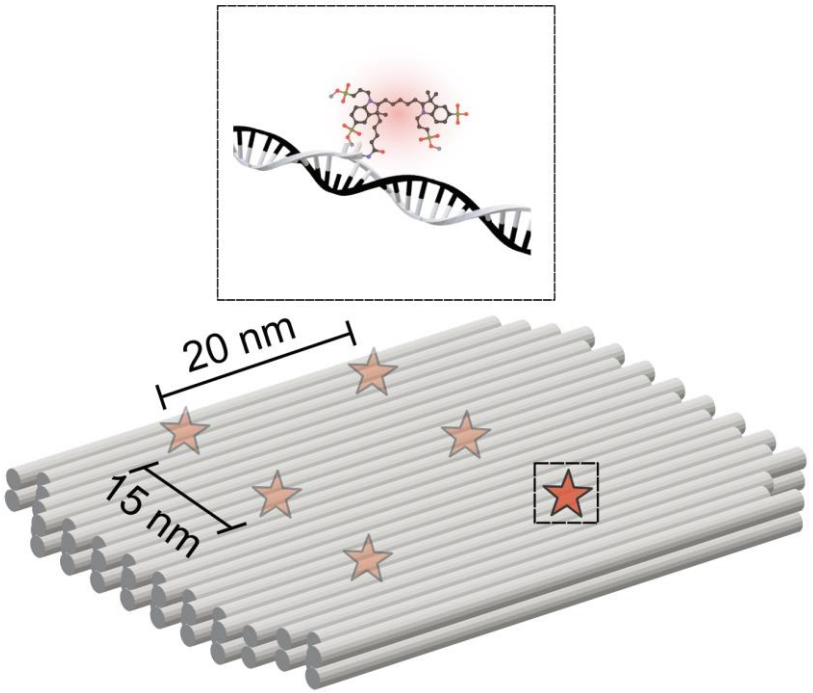
RASTMIN nanoscopy



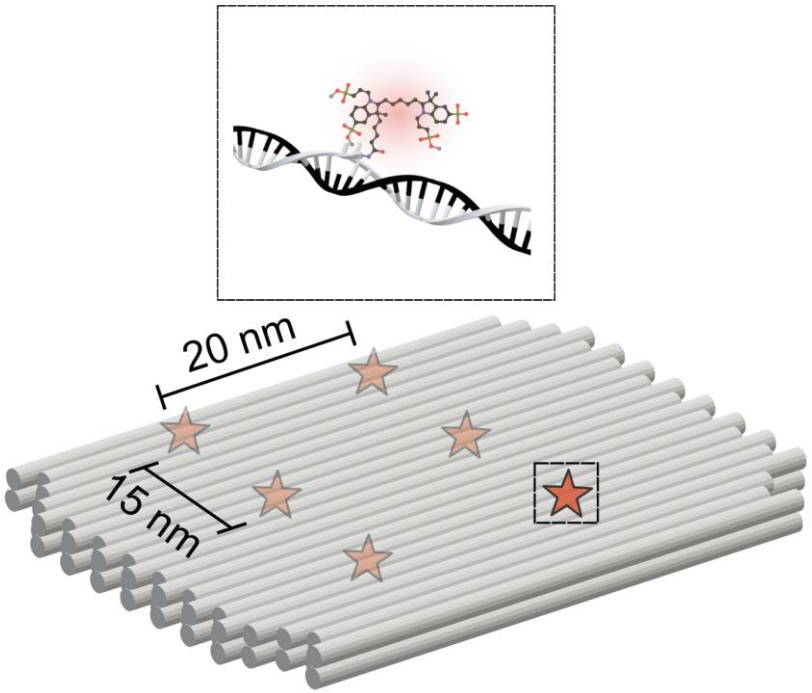
Confocal time-lapse (raw data)

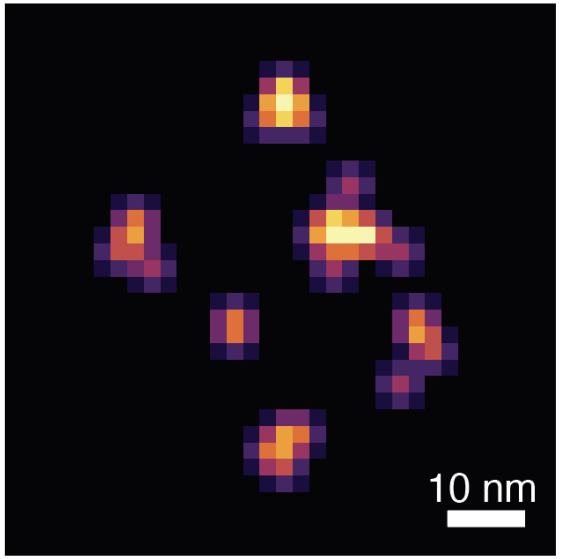


RASTMIN nanoscopy

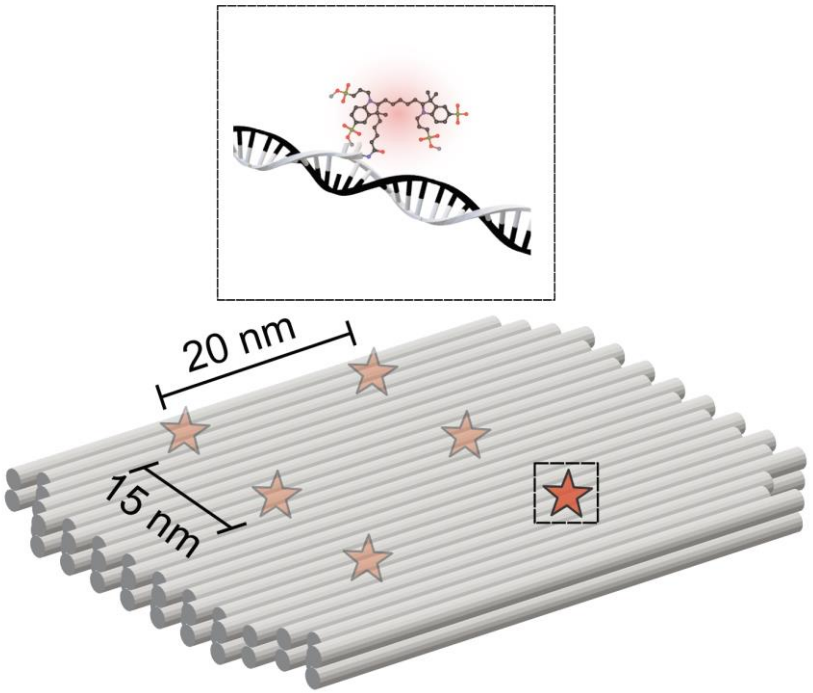


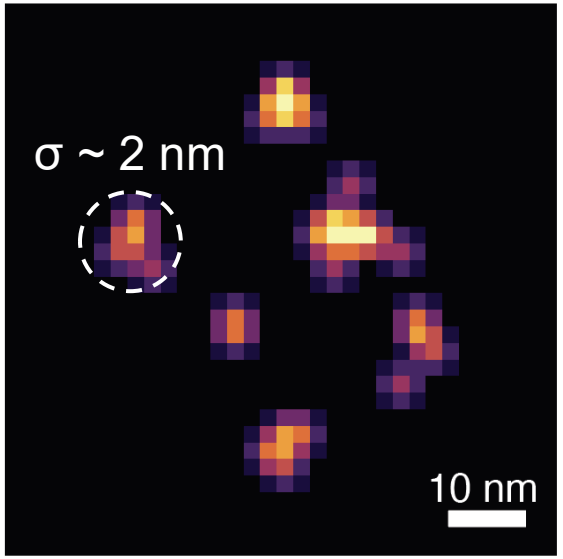
RASTMIN nanoscopy





RASTMIN nanoscopy

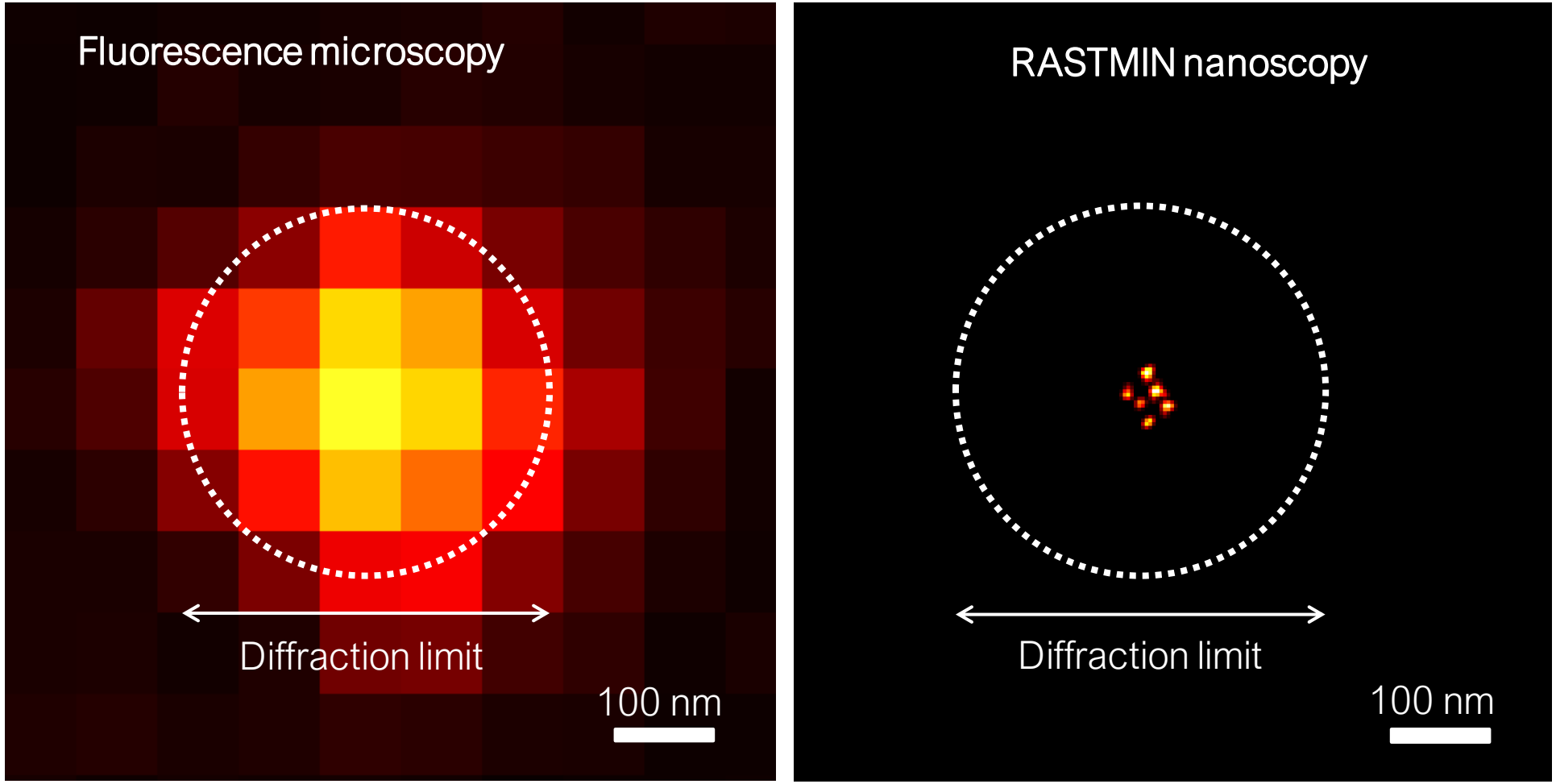




Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

Masullo et al, ***Light: Science and Applications*** 11, 70 (2022

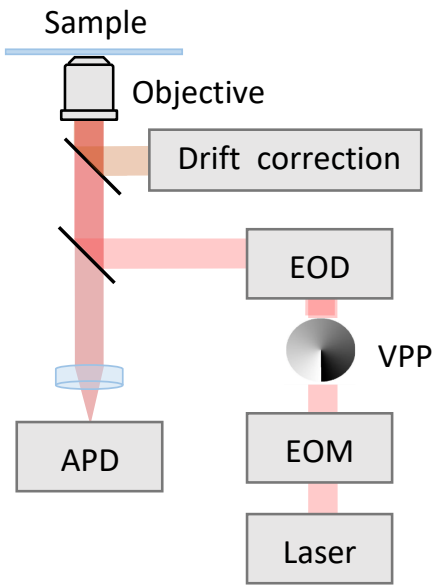
RASTMIN nanoscopy



Masullo et al, ***Light: Science and Applications*** 11, 199 (2022)

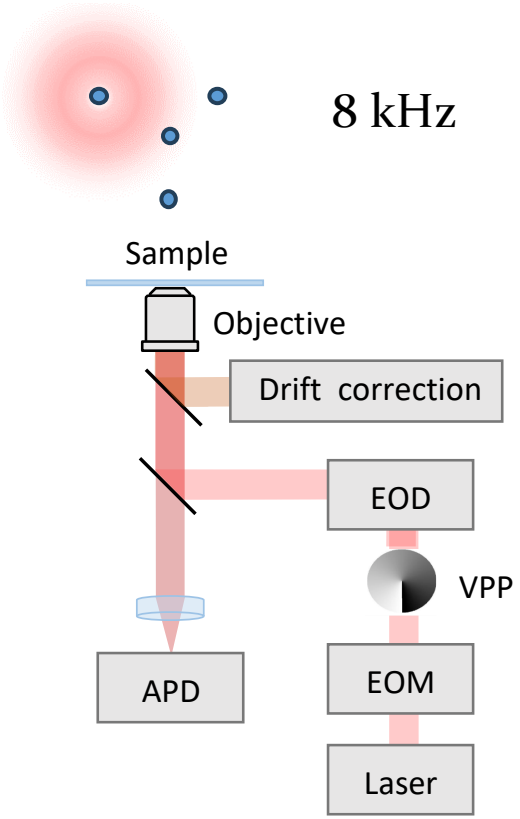
Masullo et al, ***Light: Science and Applications*** 11, 70 (2022

MINFLUX instrumentation



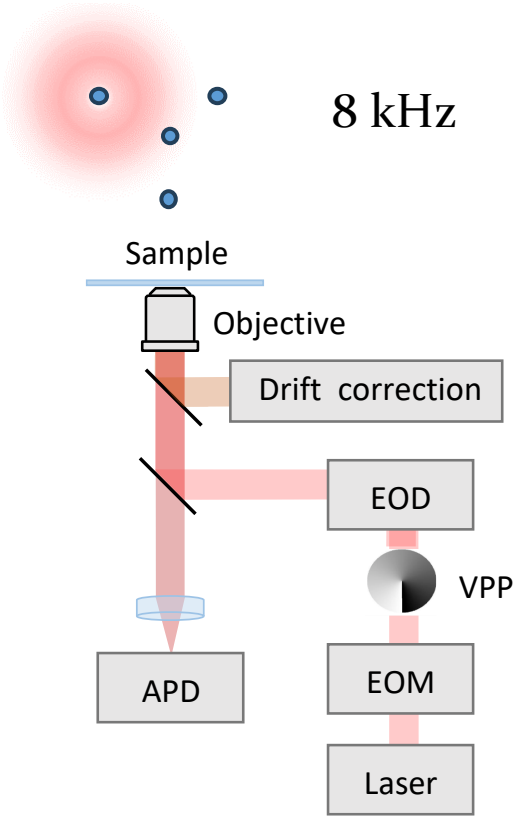
Balzarotti et al. ***Science*** 355 (2017) 606-612

MINFLUX instrumentation

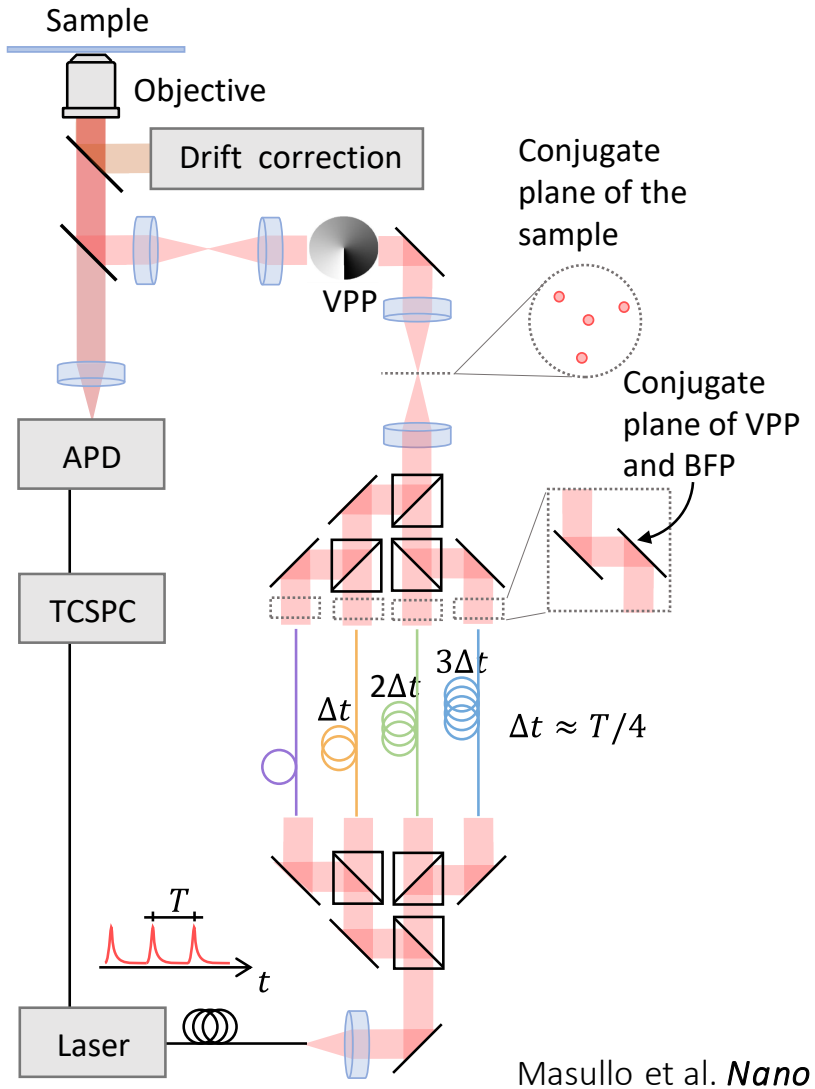


Balzarotti et al. ***Science*** 355 (2017) 606-612

MINFLUX instrumentation

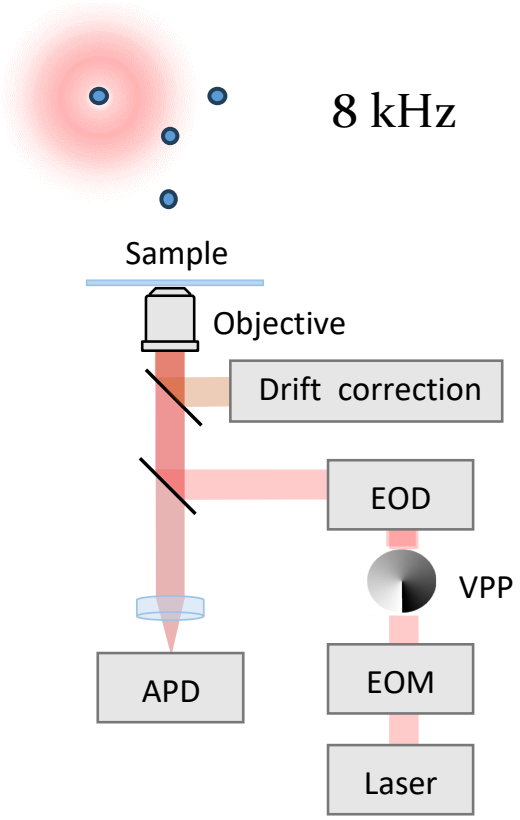


Balzarotti et al. ***Science*** 355 (2017) 606-612

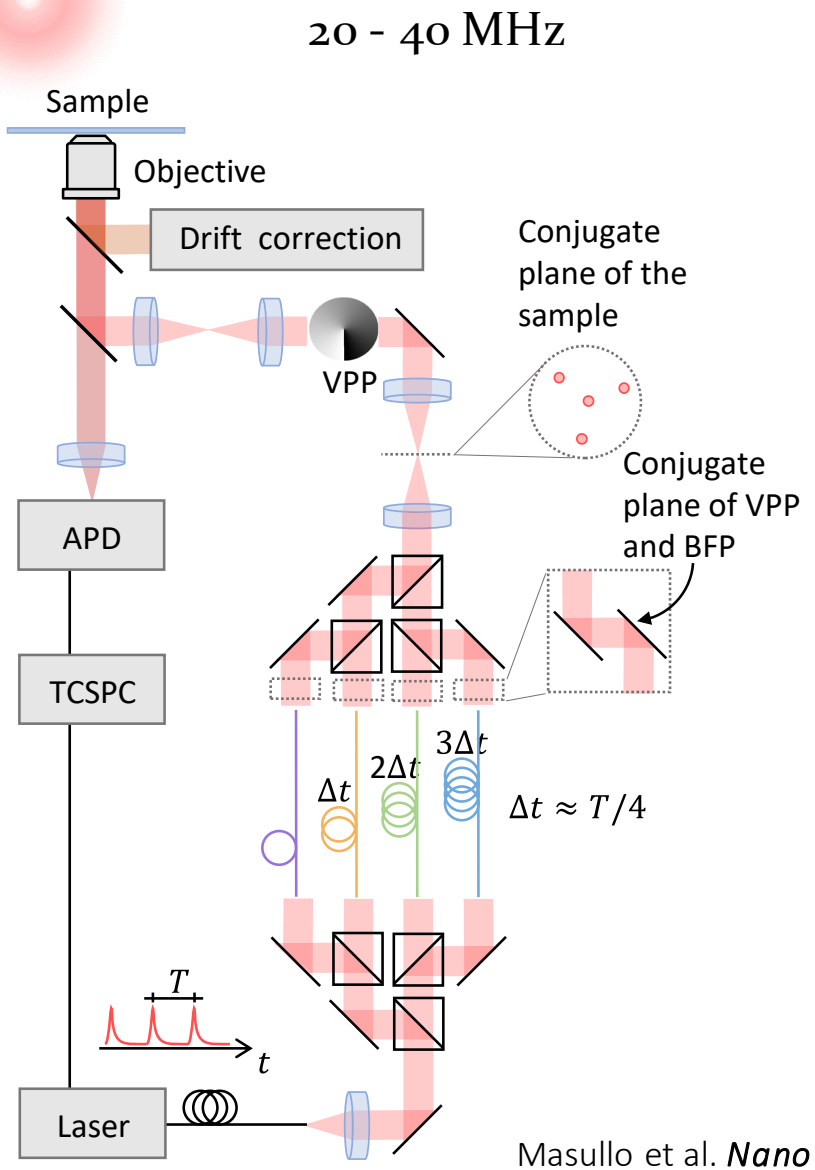


Masullo et al. ***Nano Letters*** 21 (2021) 840-846

MINFLUX instrumentation

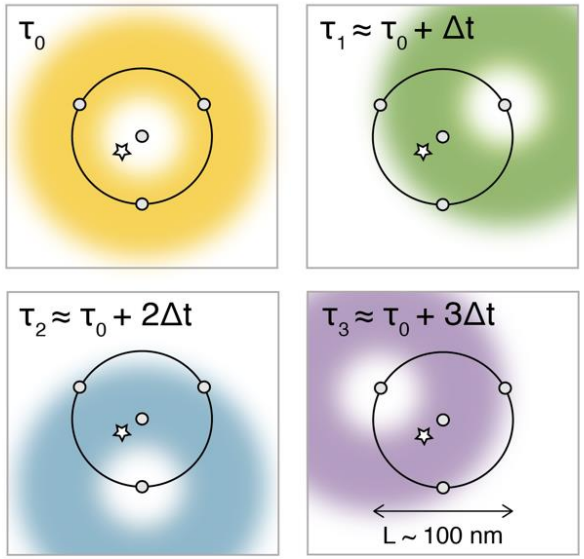


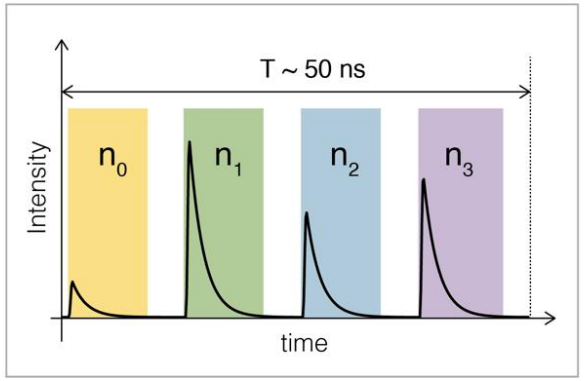
Balzarotti et al. ***Science*** 355 (2017) 606-612

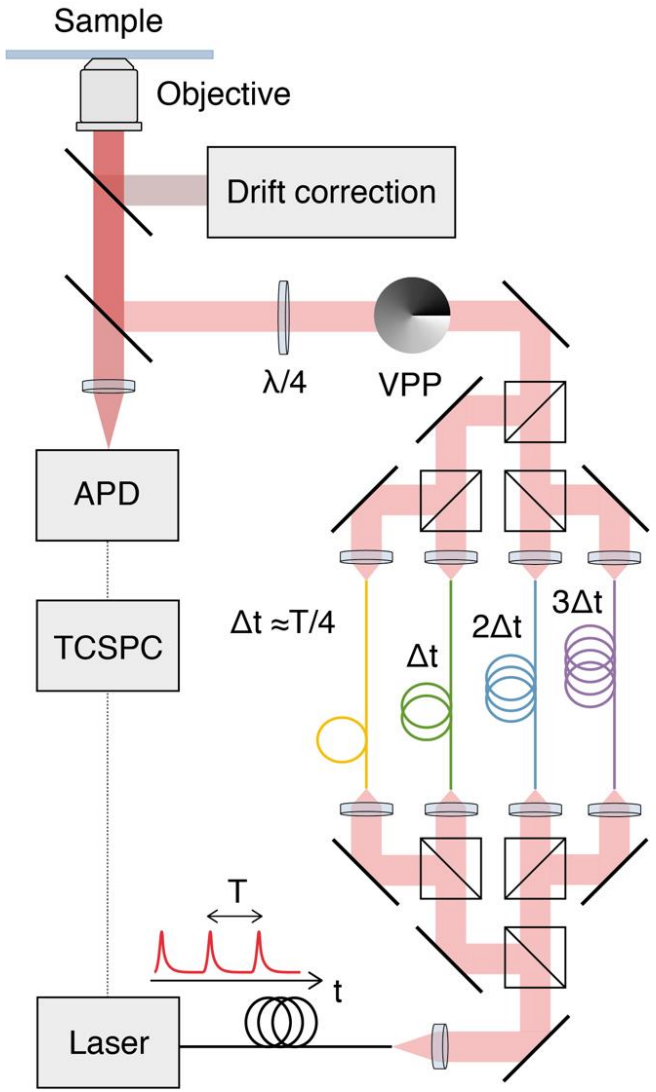


Masullo et al. ***Nano Letters*** 21 (2021) 840-846

Pulsed-interleaved MINFLUX (p-MINFLUX)

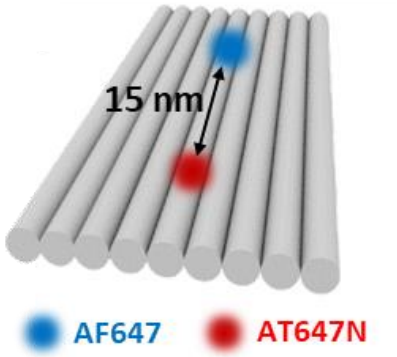


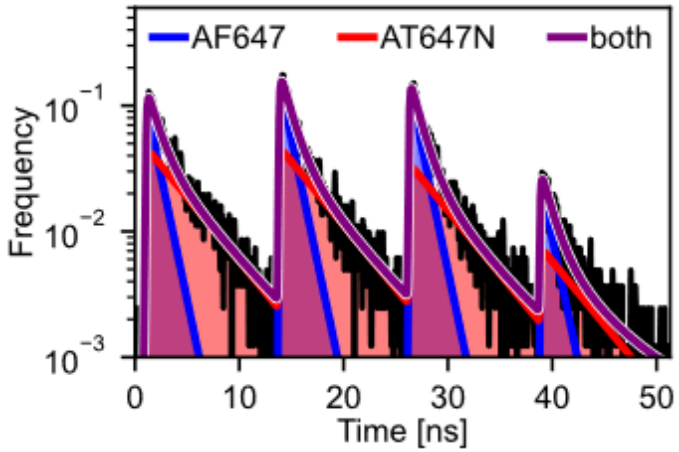


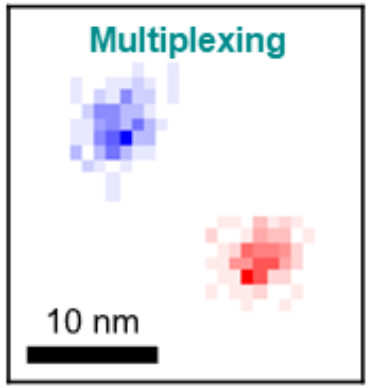


Masullo et al. ***Nano Letters*** 21 (2021) 840-846

Lifetime multiplexed localization with p-MINFLUX

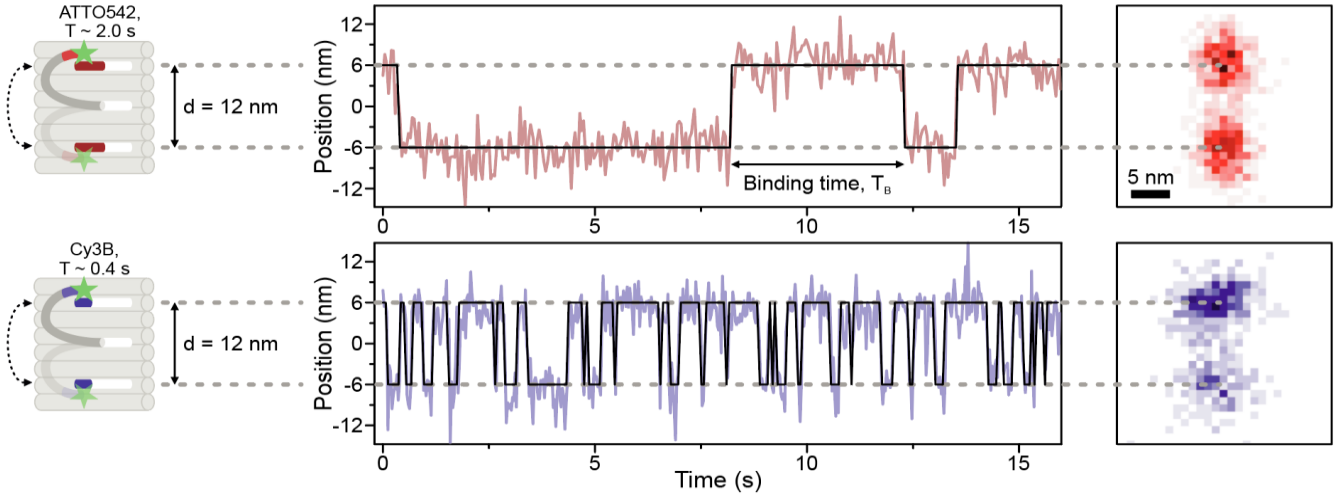


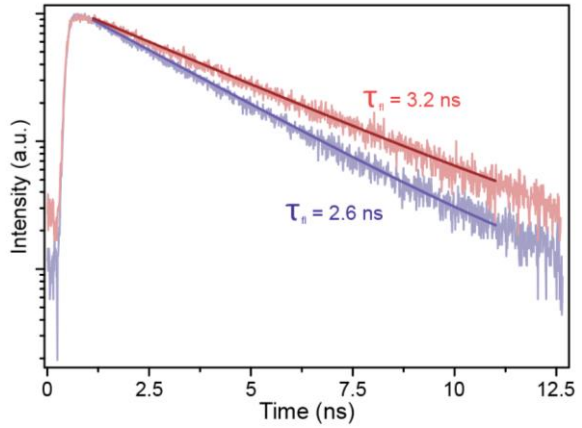


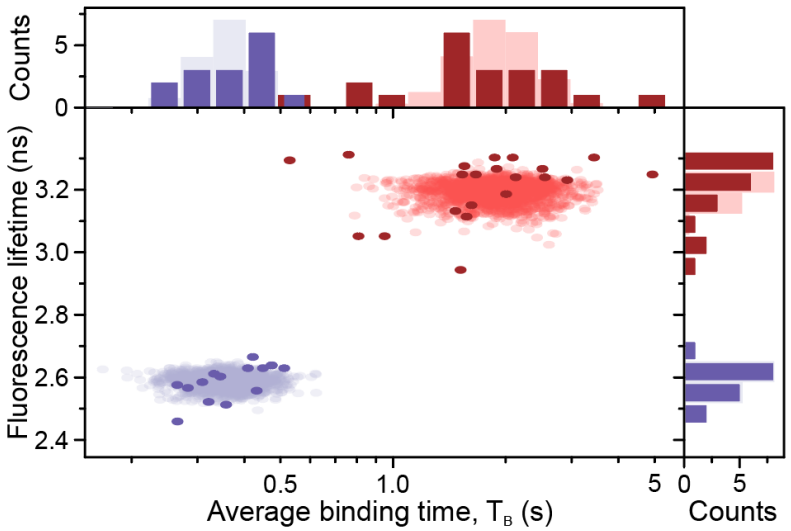


Phillip Tinnefeld & Fernando Stefani Labs - Cole et al. ***Nature Photonics*** *(2024). https://doi.org/10.1038/s41566-024-01384-4*

Lifetime multiplexed single-molecule tracking with p-MINFLUX

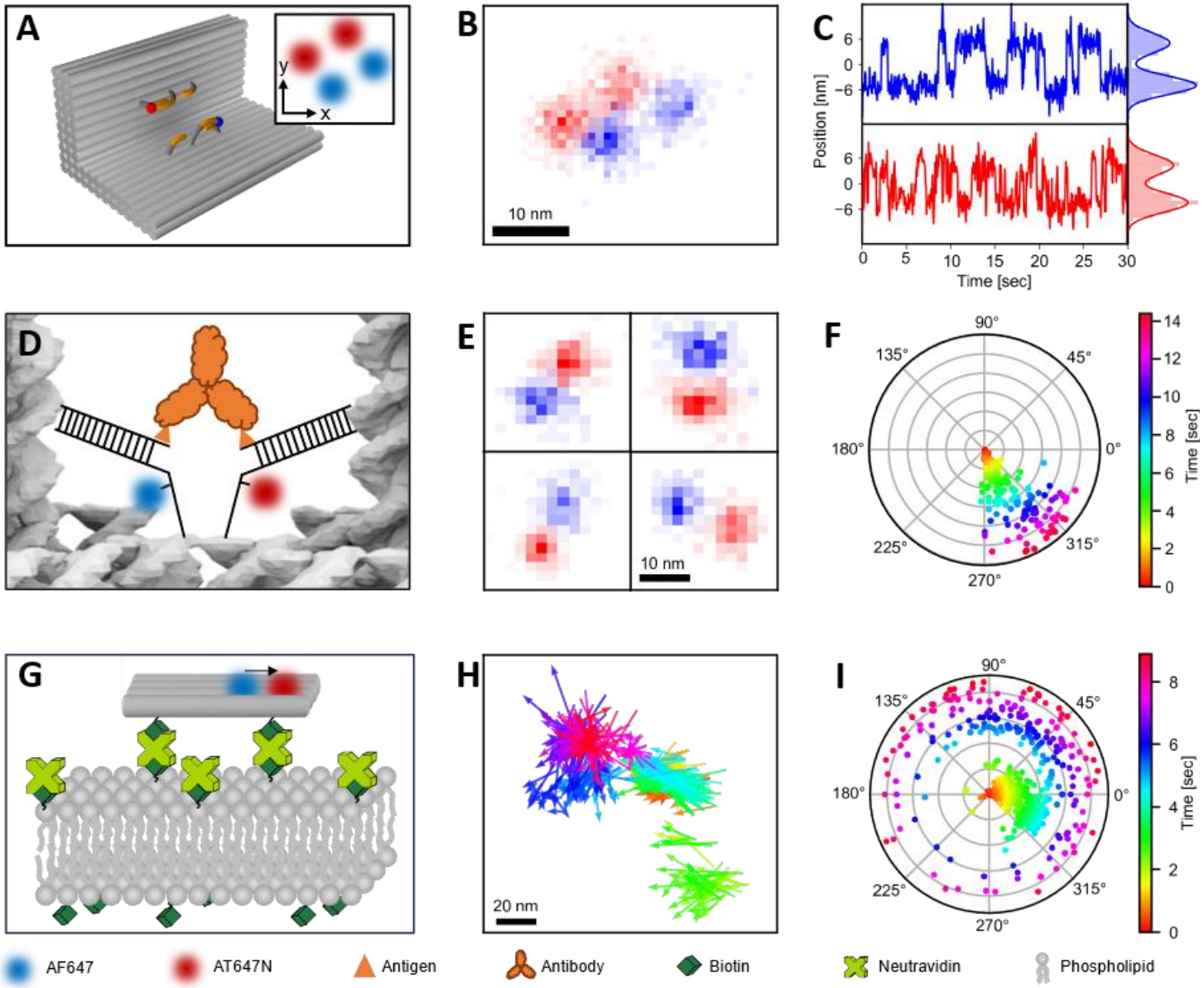






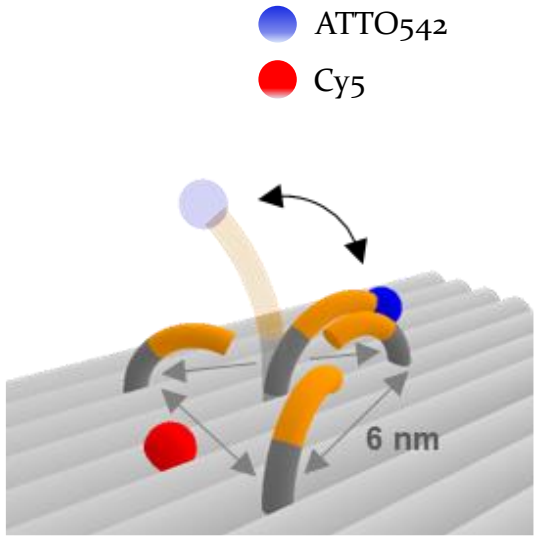
Fernando Stefani & Phillip Tinnefeld Labs - Masullo et al. *Nano Letters* 21 (2021) 840-846

Lifetime multiplexed single-molecule tracking with p-MINFLUX

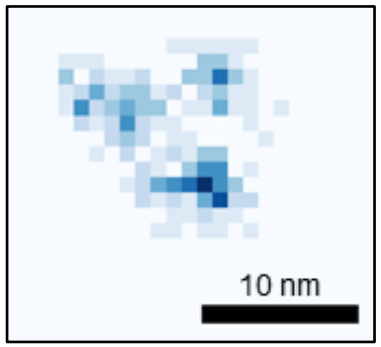


Phillip Tinnefeld & Fernando Stefani Labs - Cole et al. ***Nature Photonics*** *(2024). https://doi.org/10.1038/s41566-024-01384-4*

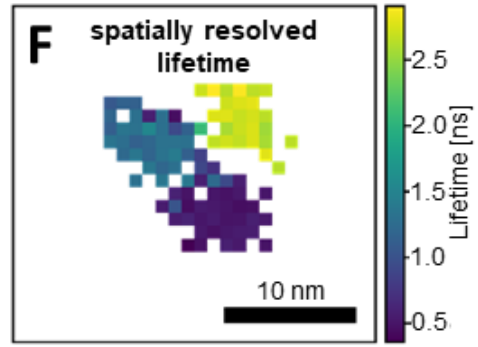
Localizing a dark absorber with p-MINFLUX



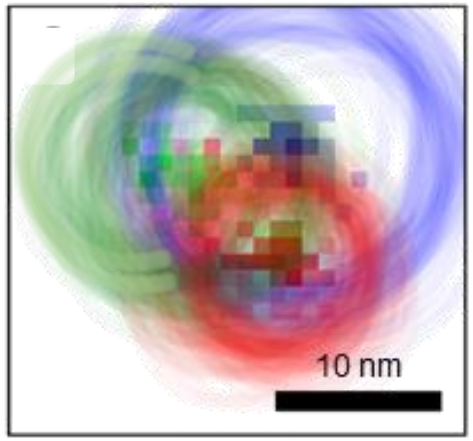
Localizations



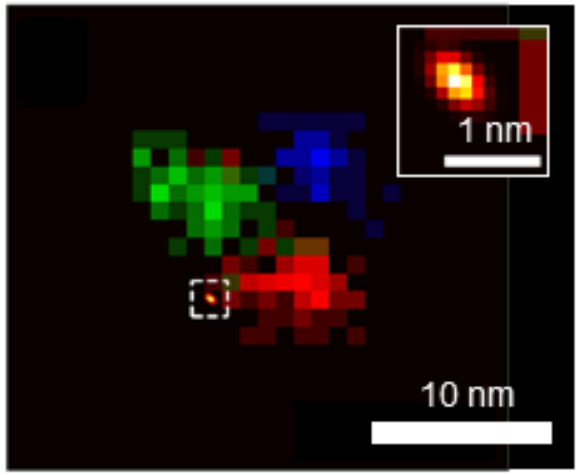
Lifetime



Acceptor triangulation

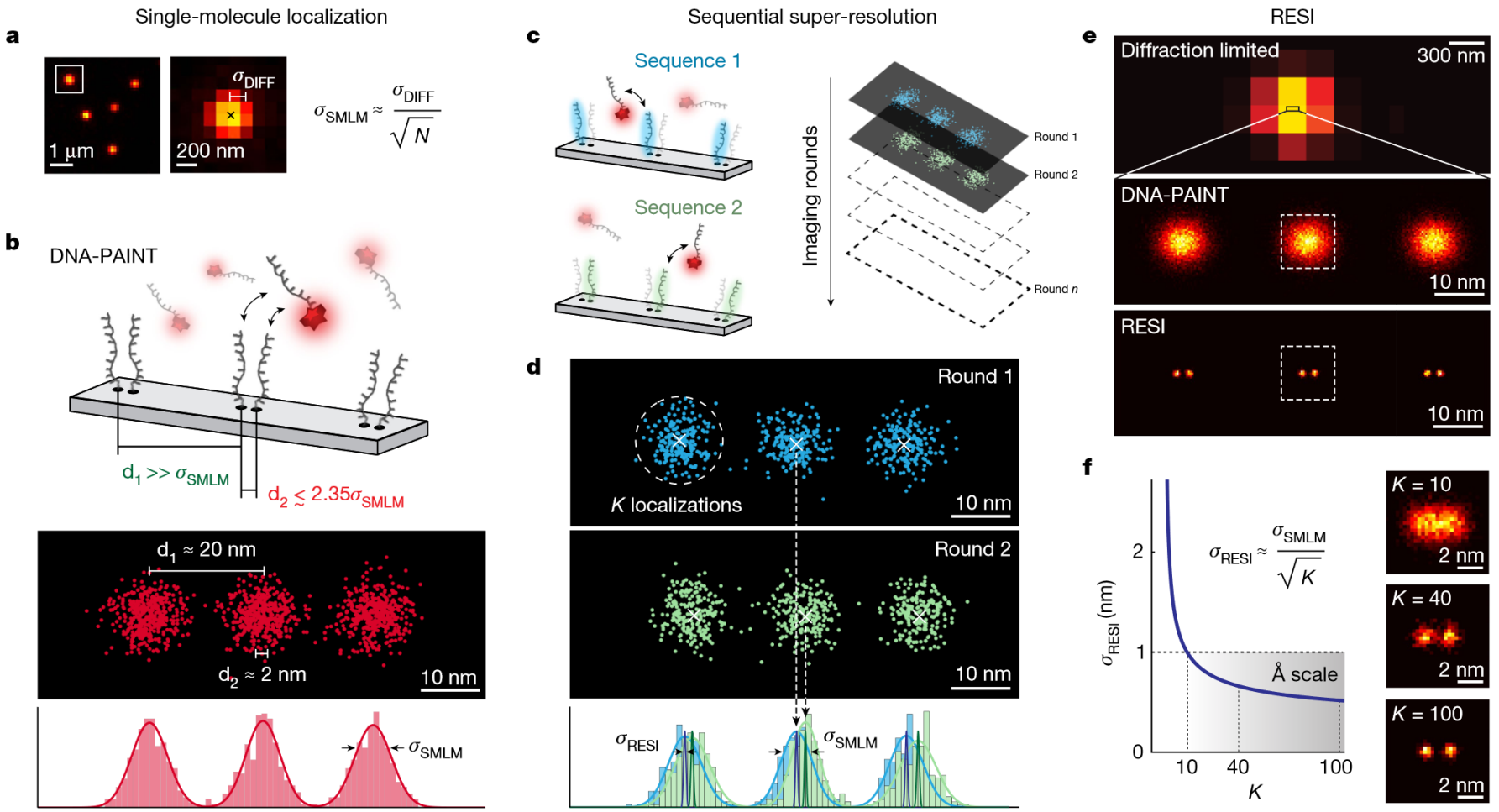


Acceptor localization



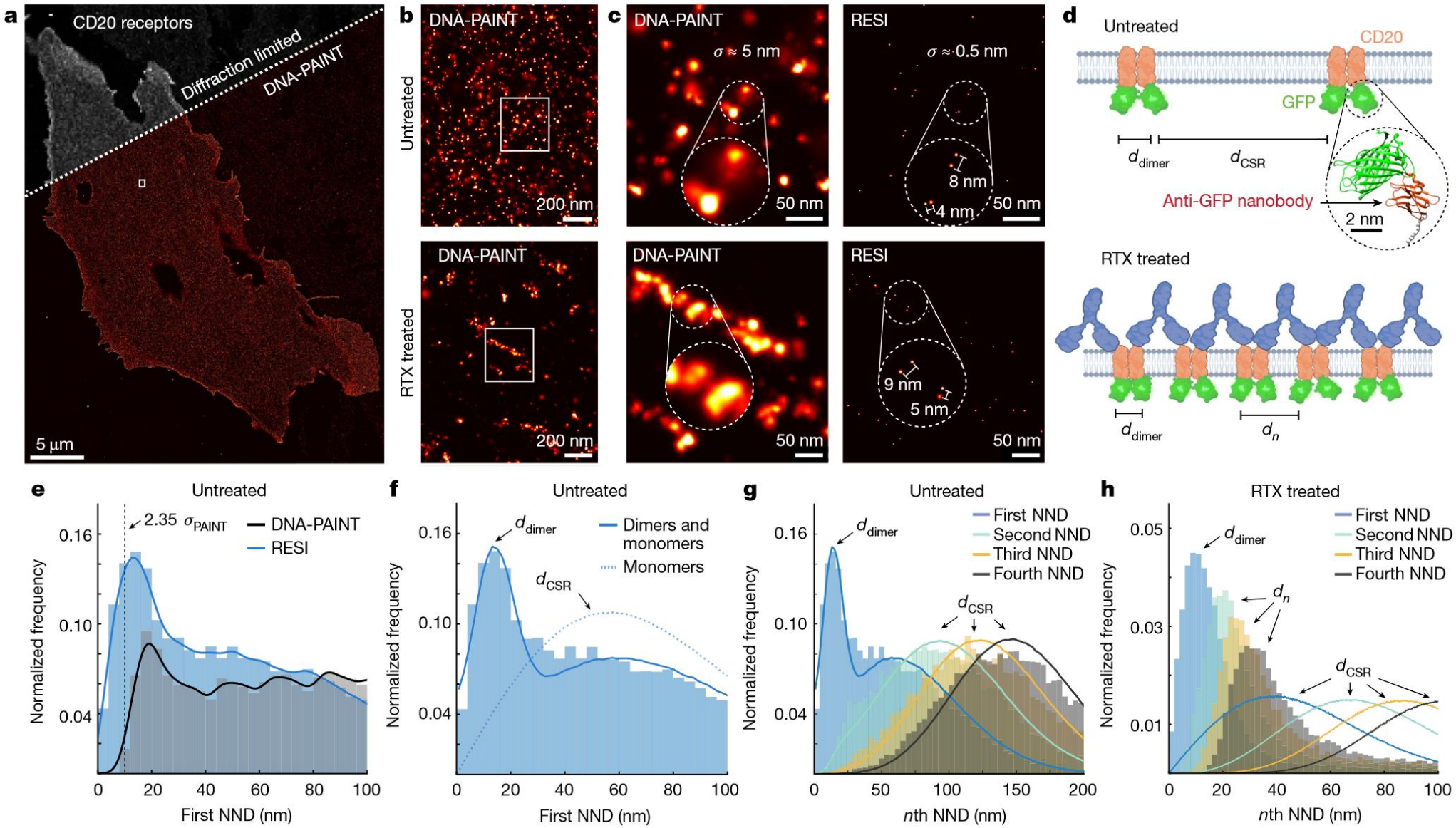
Phillip Tinnefeld & Fernando Stefani Labs - Cole et al. ***Nature Photonics*** *(2024). https://doi.org/10.1038/s41566-024-01384-4*

Resolution Enhancement by Sequential Imaging (RESI)



Ralf Jungmann Lab - Reinhardt et al. ***Nature*** 617 (2023) 711–716

Resolution Enhancement by Sequential Imaging (RESI)



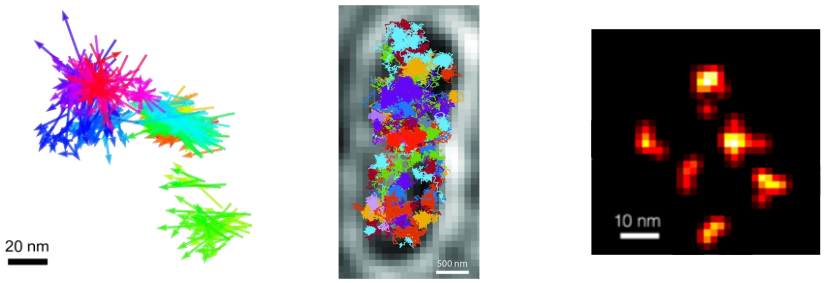
Ralf Jungmann Lab - Reinhardt et al. ***Nature*** 617 (2023) 711–716

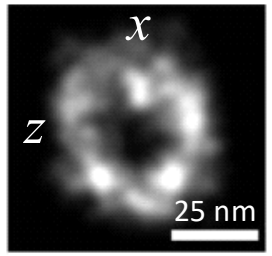
Sub-10 nm resolution fluorescence nanoscopy

https://stefani-lab.ar/

fernando.stefani@df.uba.ar

SML-SSI





SIMPLER

Sub-10 nm 3D TIRF nanoscopy

p-MINFLUX: fast / accurate tracking

RASTMIN / MINFLUX:
the high photon-efficiency is losing significance for highest resolution: tricks to get more photons, label size limit,…

Another edge: 10-20 nm resolution with “bad” fluorophores

Combination with camera-based localization for photophysical studies (absorption-emission)

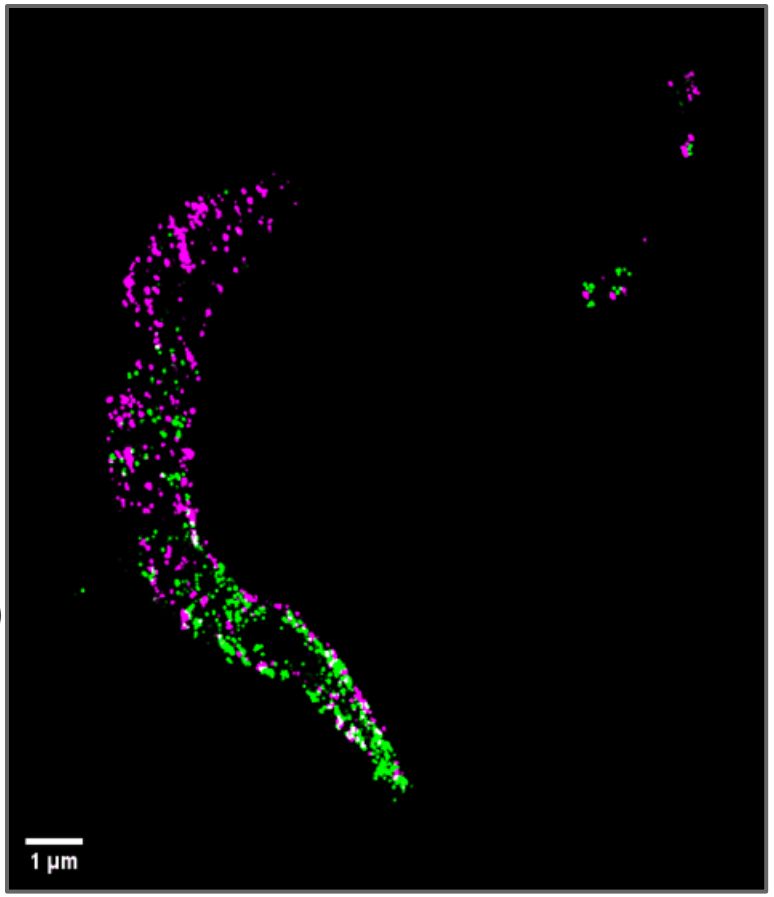
STED-FRET

Super-resolved energy transfer / biomlecular interaction

https://stefani-lab.ar/

fernando.stefani@df.uba.ar

Current projecs: applications to biomedicine



Luciano Masullo (now at MPI biochem, Munich)

Cecilia Zaza (now at UC London)

Romina Landa (now at Collective.ai, Buenos Aires)

Fernando Caprile (now at Iquall Networks, Buenos Aires)

German Chiarelli (now at Fribourg University))

Julián Gargiulo (now at UNSAM, Buenos Aires)

Ianina Violi (now at UNSAM, Buenos Aires)

Valeria País (now at ICFO, Barcelona)

Emiliano Cortés (now Prof. at LMU, Munich)

Martín Bordenave (now at Satellogic, Buenos Aires)

Federico Barabas (now at Spotify, Stockholm)

CONICET AGENCIA UNIVERSIDAD DE BUENOS AIRES MAX-PLANCK-GESELLSCHAFT Alexander von Humboldt Stiftung/Foundation exemys