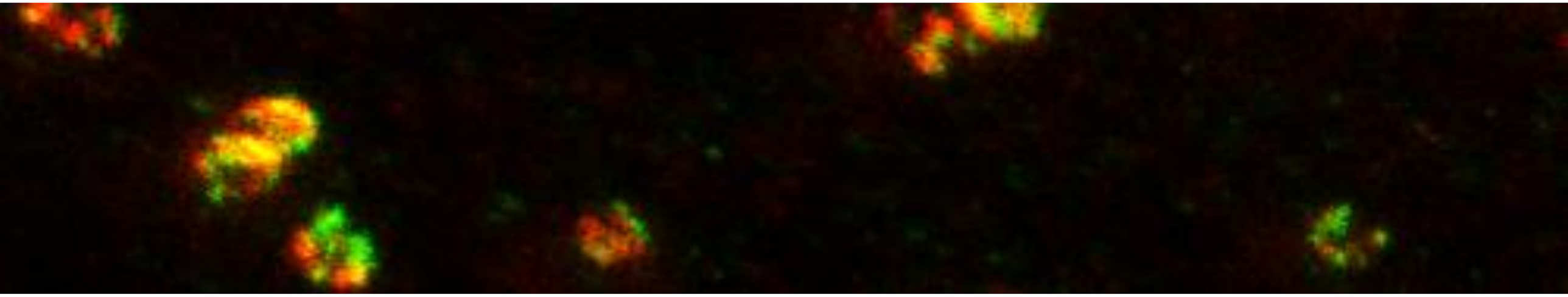
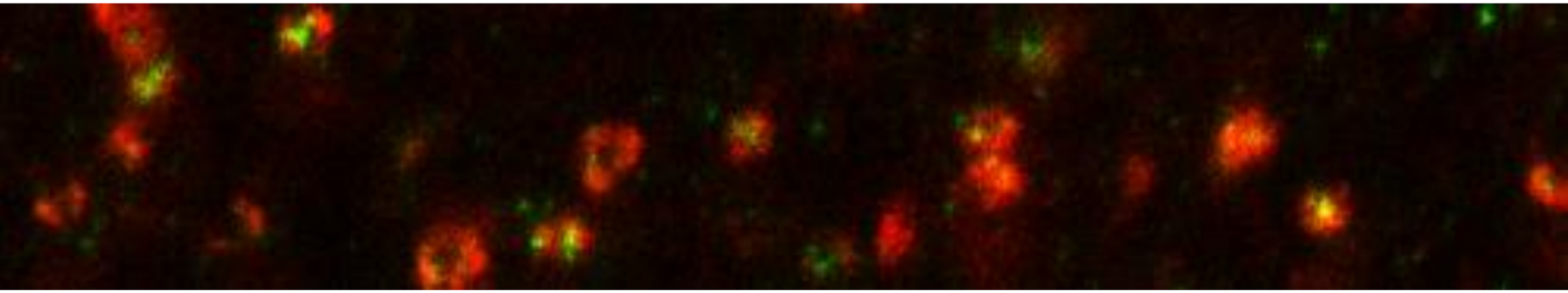


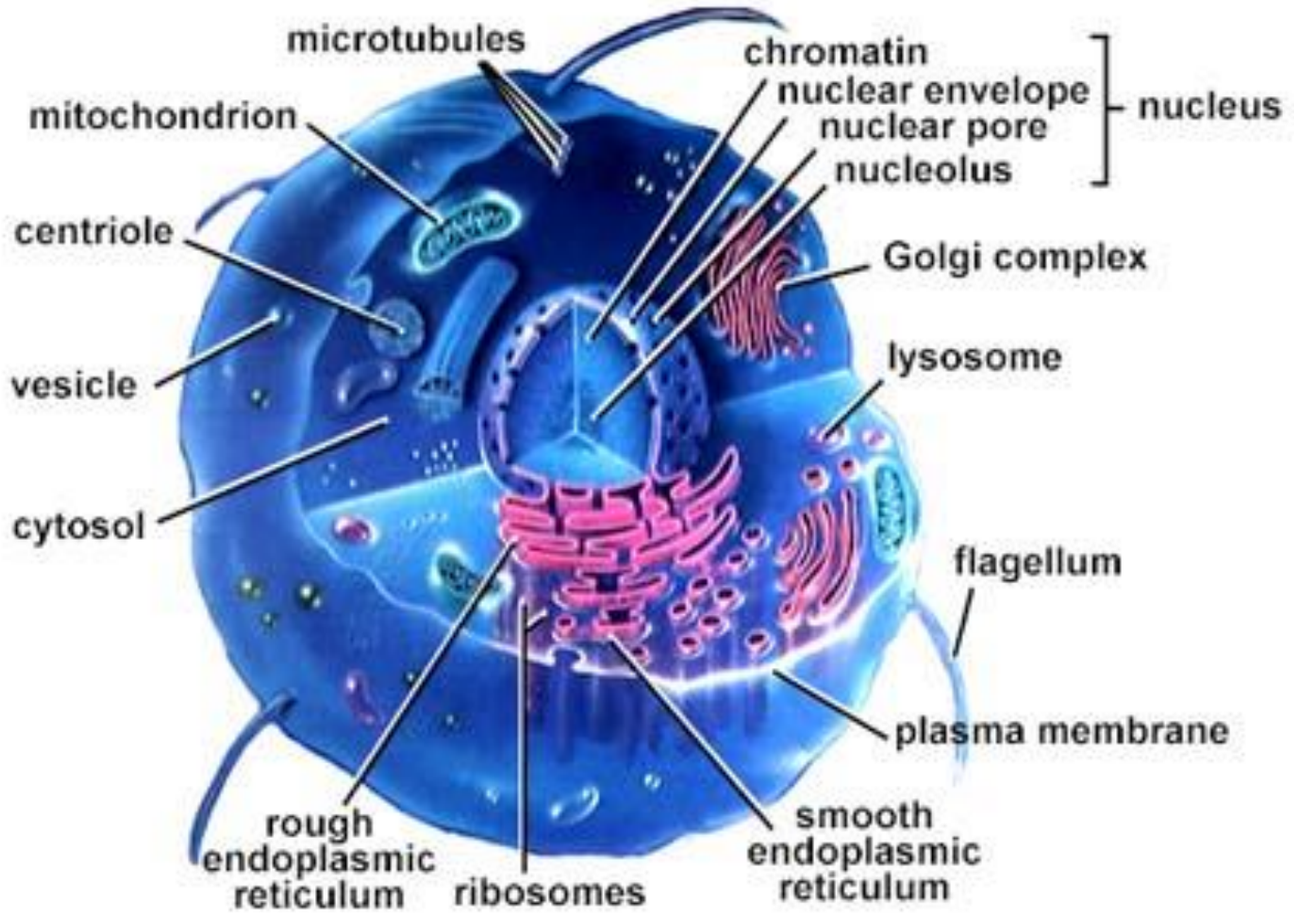
STED microscopy



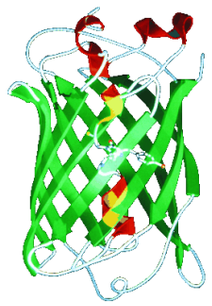
Fluorescence microscopy

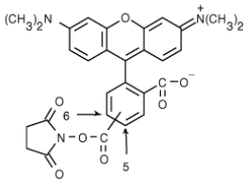
Observation of living cells in far field: Non-Invasive

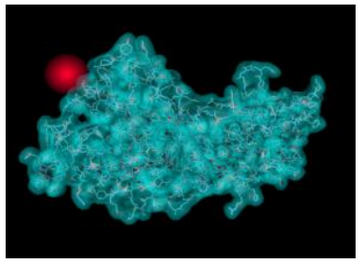
Study specific molecular processes in the living cell:

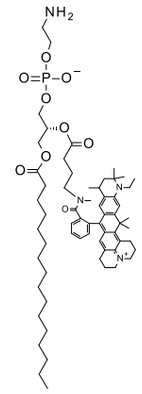


Label specific protein/molecule

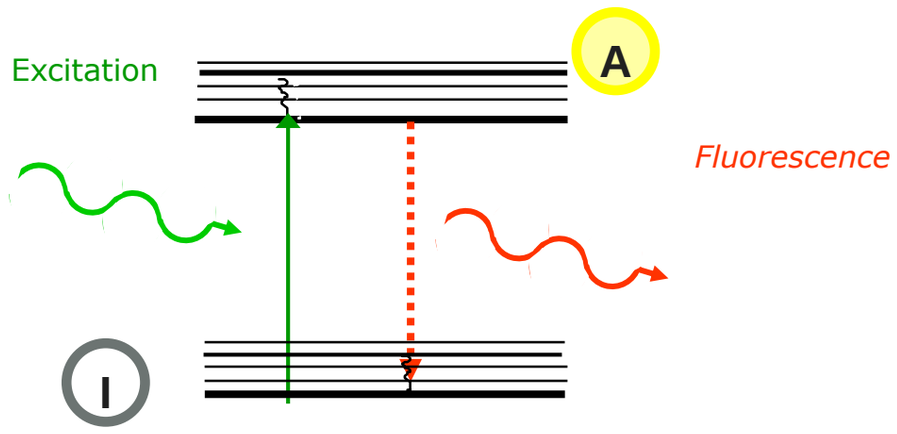




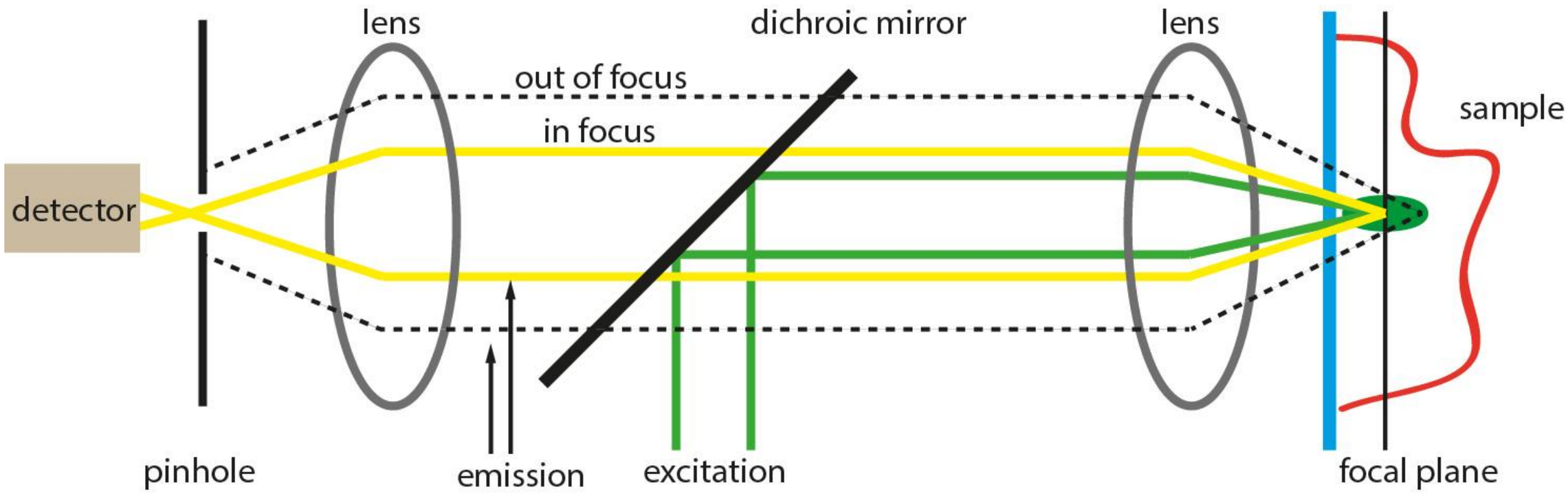


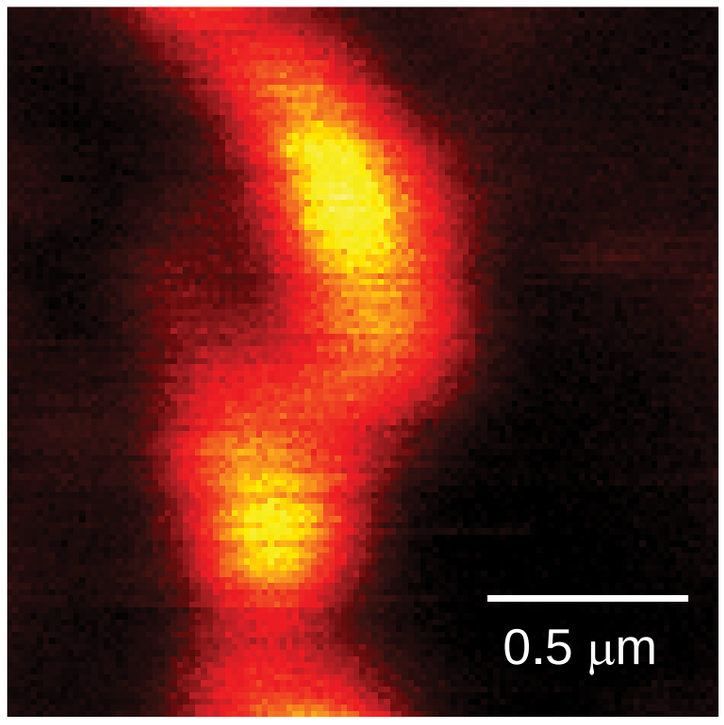


Excite fluorescence by laser light

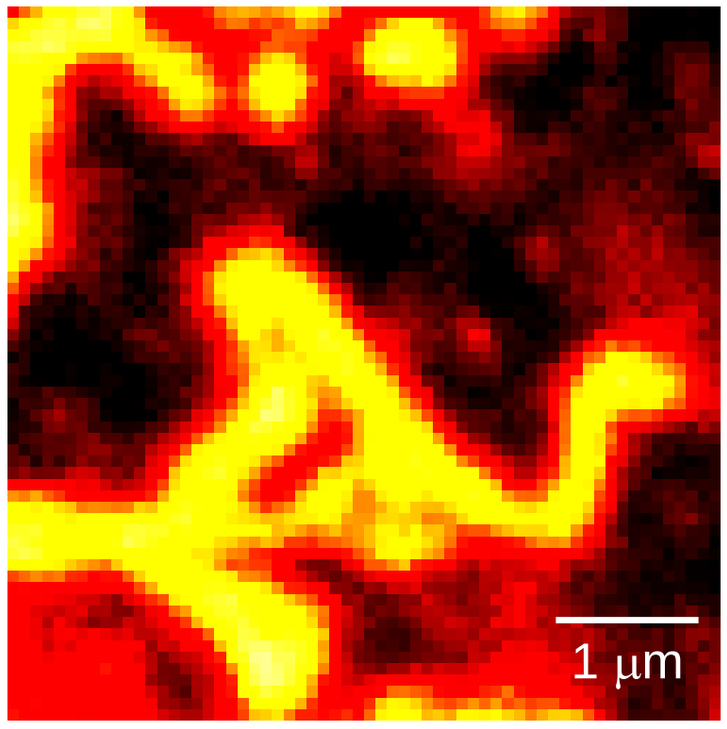


Confocal microscope scheme

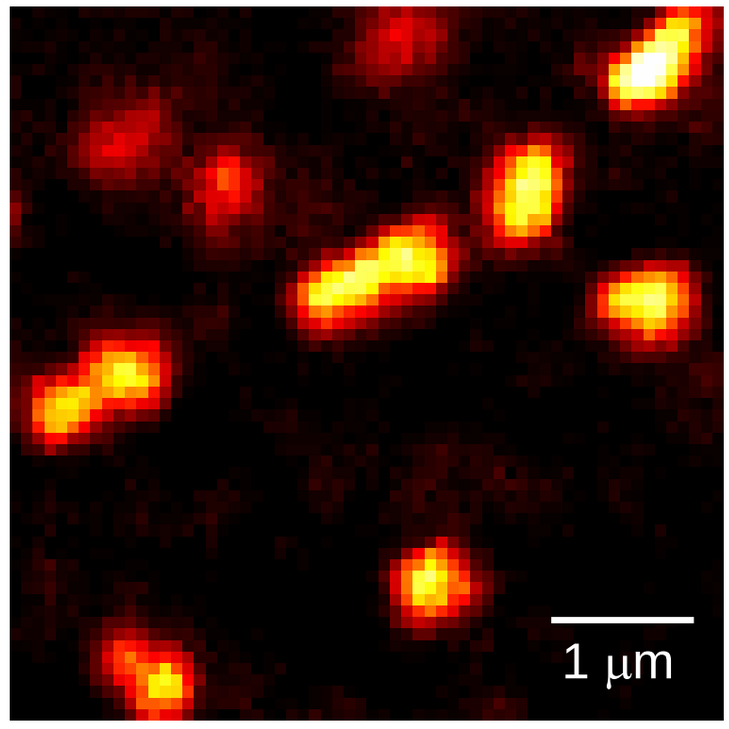




Synaptic vesicles



Mitochondria



Peroxisomes

Confocal microscopy

Biological and biomedical questions have multiple demands

? High spatial resolution

$$d = \frac{\lambda}{2n\sin\alpha}$$

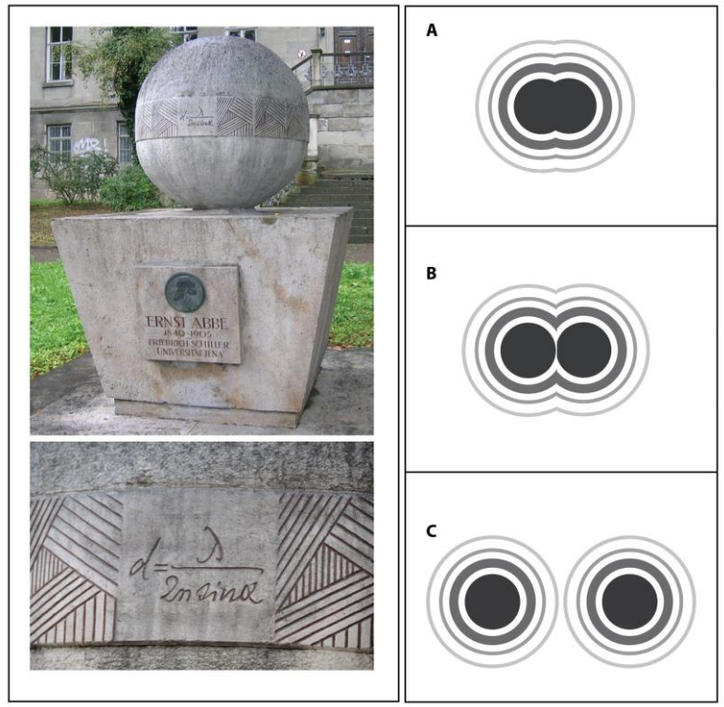
✓ High temporal resolution

✓ Multicolour imaging

✓ 3D imaging

✓ Long acquisition time

✓ Data analysis

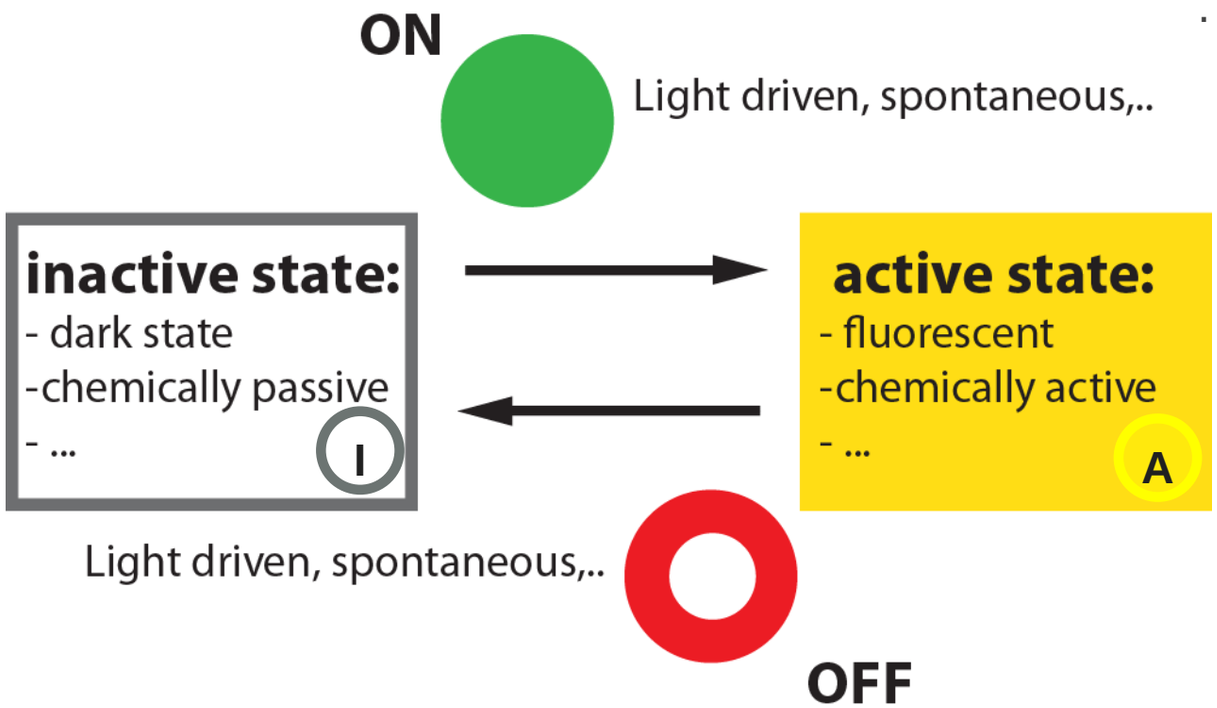


Ernst Abbe's memorial located in front of the physiology building of the University of Jena, Germany

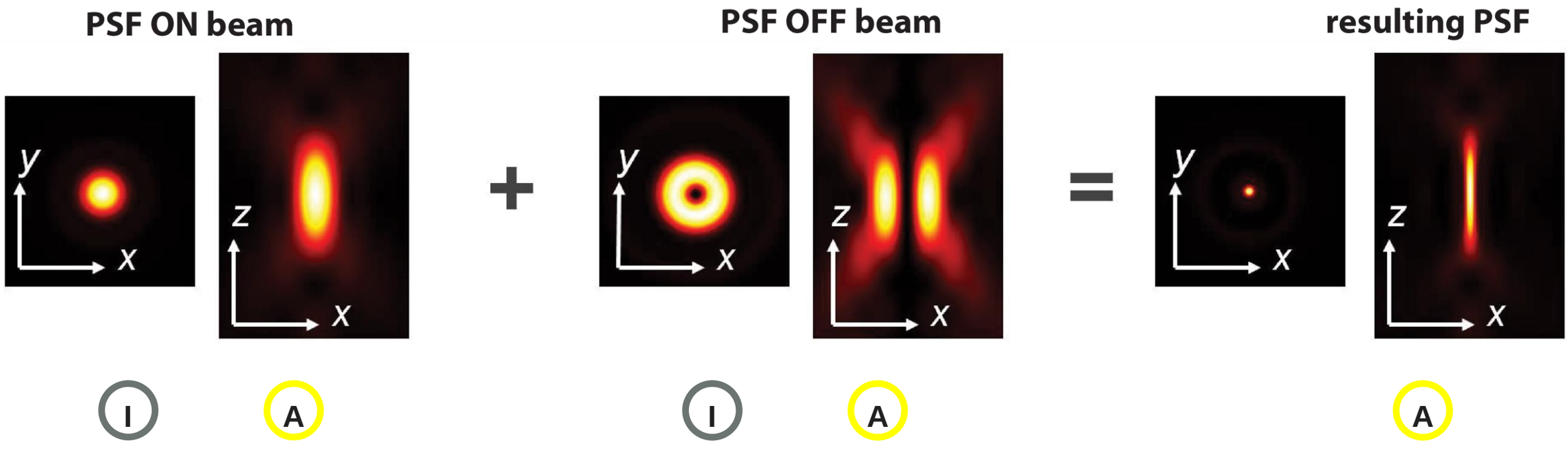
S. W. Hell and J. Wichmann. *Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy.* Opt. Lett., 19:780782, 1994.

Optical nanoscopy

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner "for the development of super-resolved fluorescence microscopy".



$$d_{STED} = \frac{\lambda}{2n\sin\alpha\sqrt{1 + A\frac{I_{max}}{I_s}}}$$



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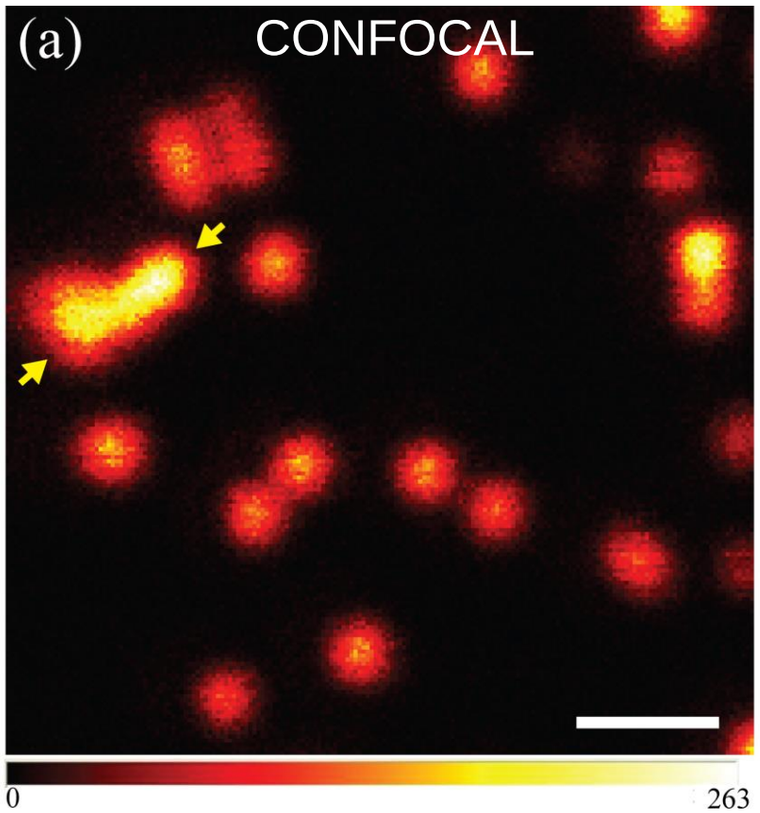

S. W. Hell , Toward fluorescence nanoscopy, Nature Biotechnol. 21,1347–1355 (2003)

Optical nanoscopy

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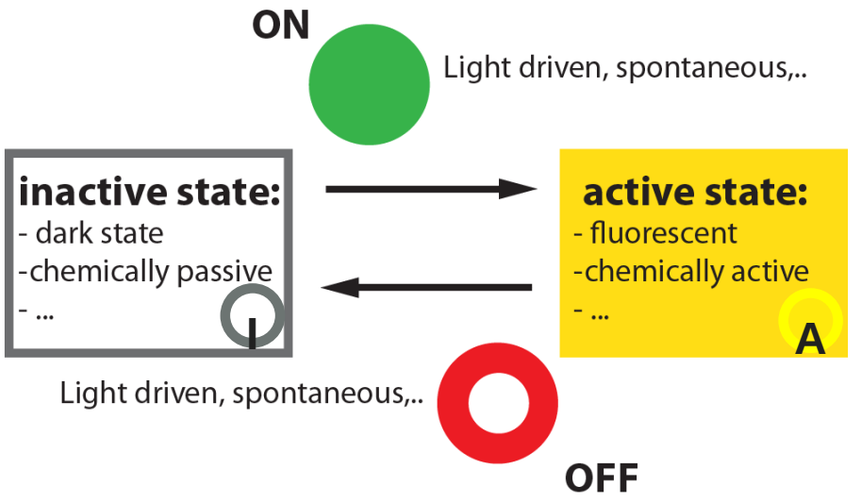
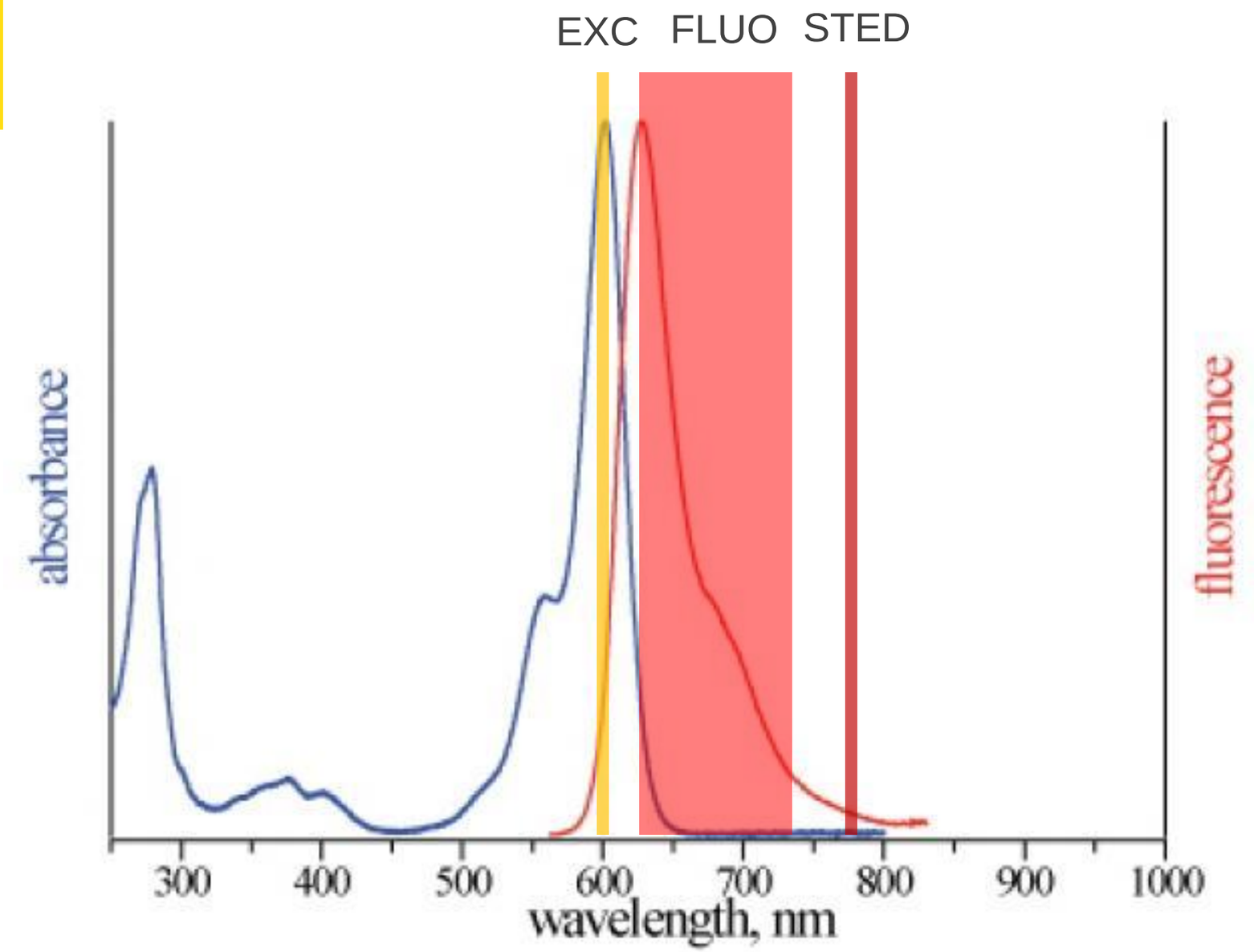
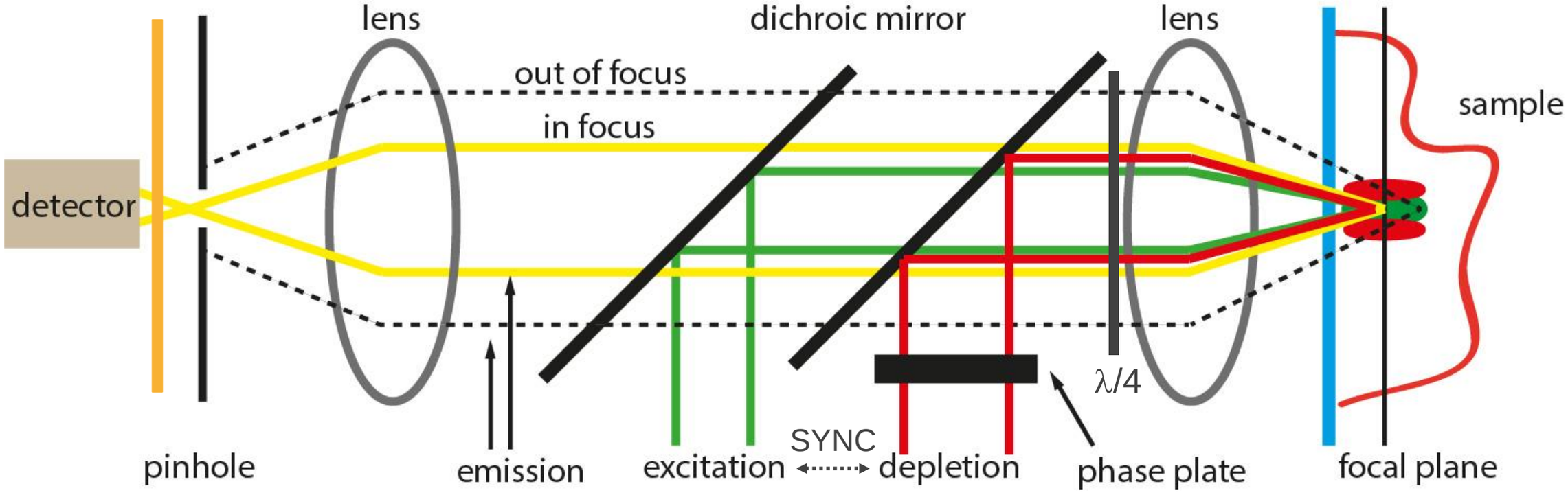
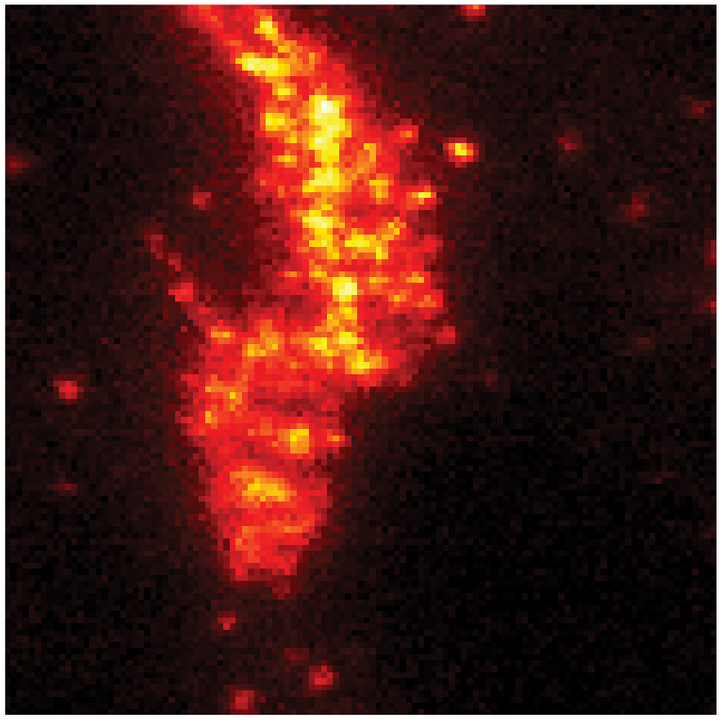




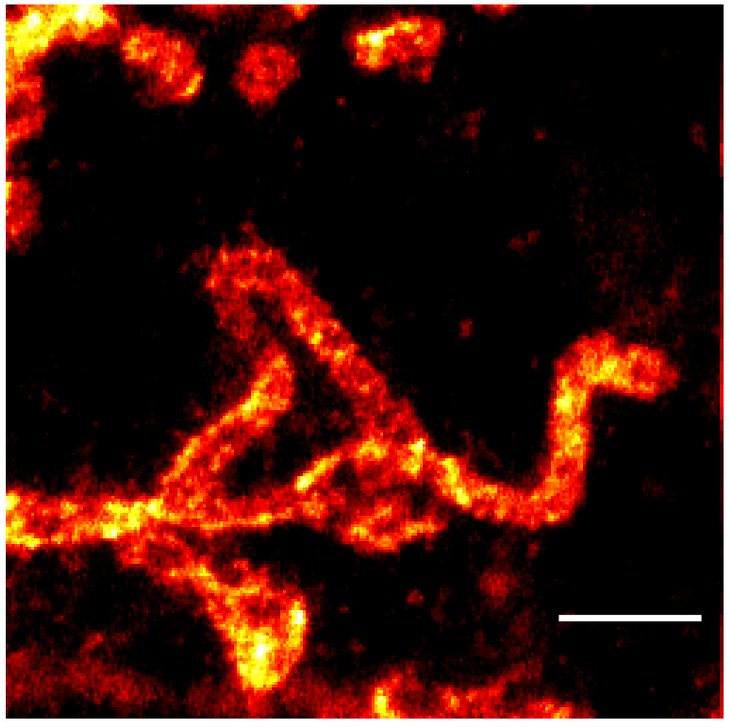
S. W. Hell , Toward fluorescence nanoscopy, Nature Biotechnol. 21,1347–1355 (2003)

STED microscope scheme

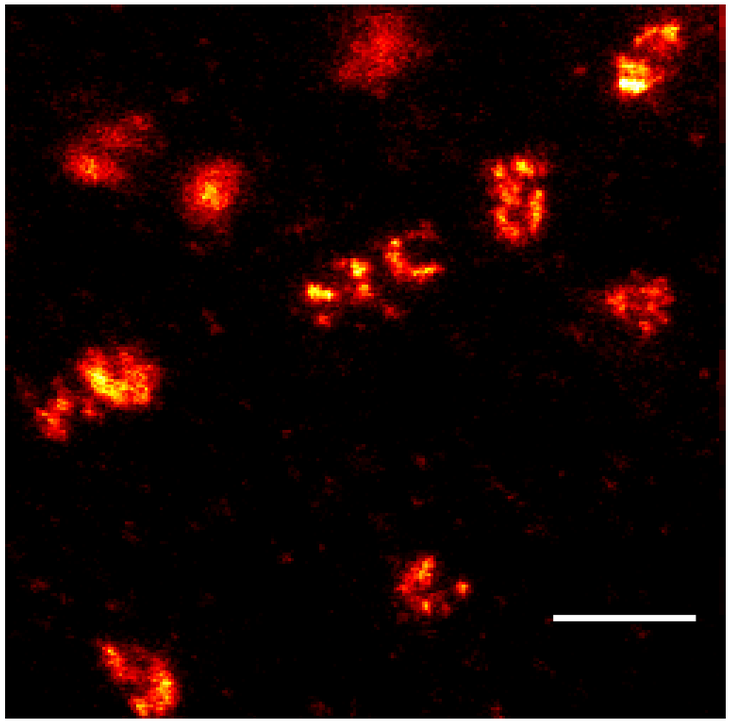




Synaptic vesicles

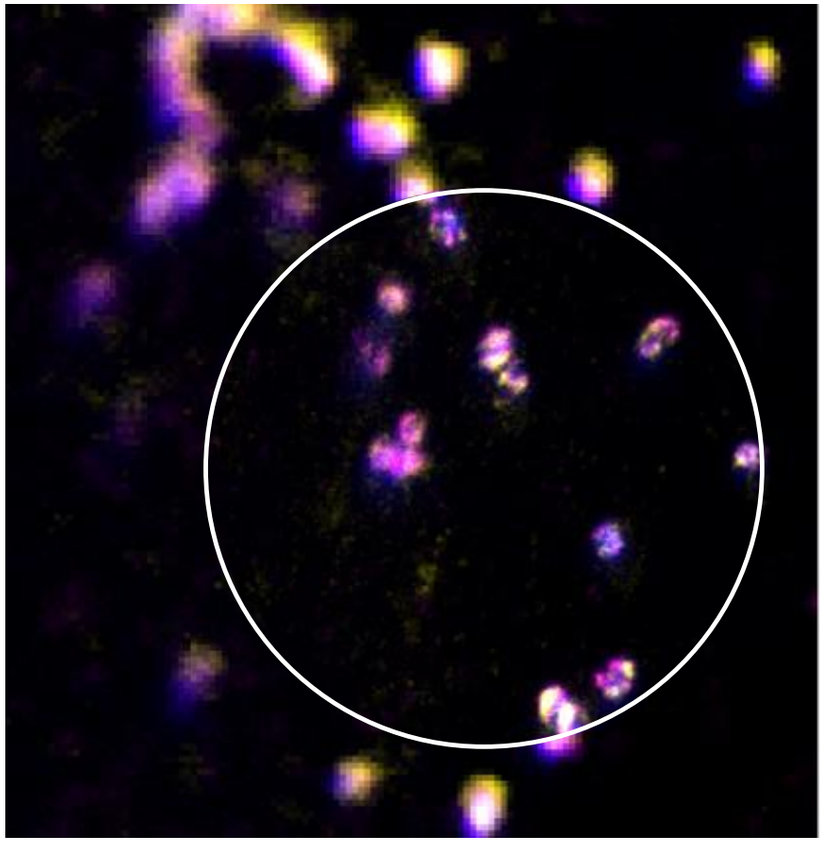


Mitochondria



Peroxisomes

A multicolour STED microscope designed for biological applications



- ✓ High spatial resolution
- ✓ High temporal resolution
- ✓ Multicolour imaging
- ✓ 3D imaging
- ✓ Long acquisition time
- ✓ Data analysis

Göttfert, F., Wurm C. A., Mueller V., Berning S., Cordes V. C., Honigmann A., and Hell S. W., *Coaligned dual-channel STED nanoscopy and molecular diffusion analysis at 20 nm resolution*. Biophys J 105,, L01 - L03 (2013)

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Galiani S / Clausen M / de la Serna Bernardino J, Fritzsche M, Chojnacki J, Lagerholm B C, Eggeling C, Pathways to optical STED microscopy, Nanobioimaging, vol 1, pp. 1–12 (2013)

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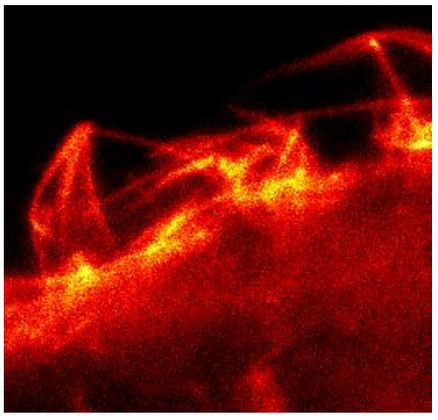
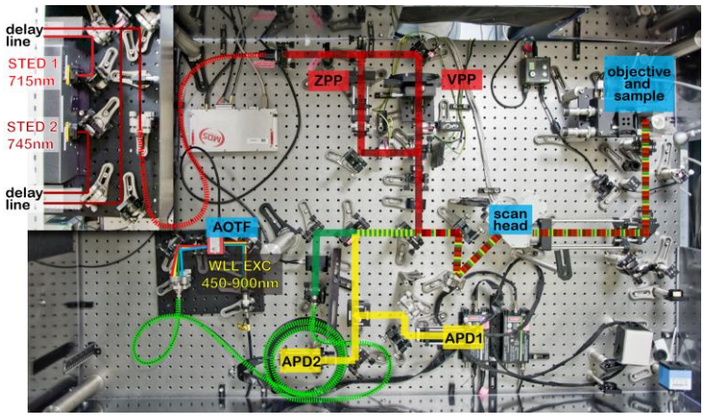
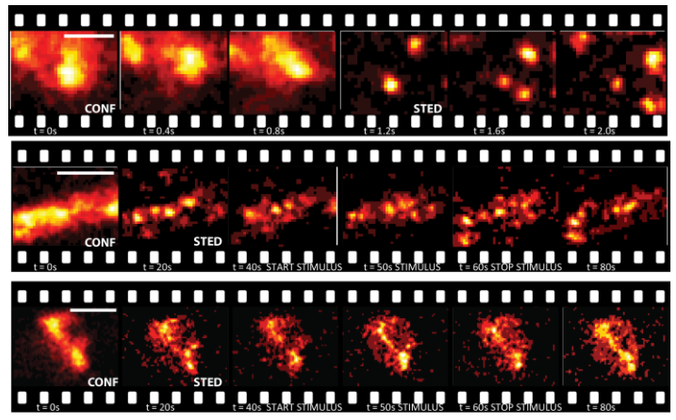
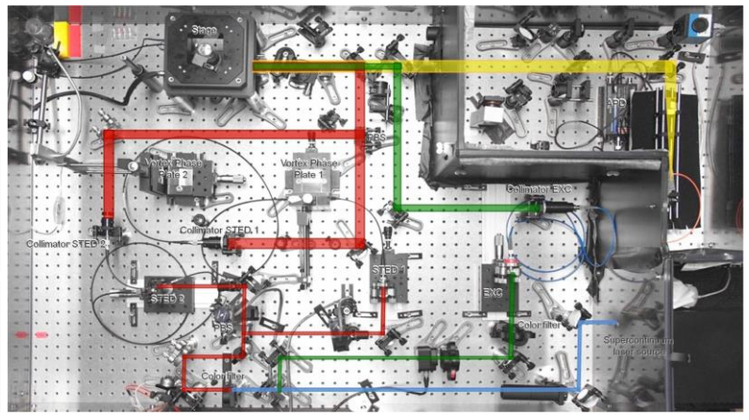
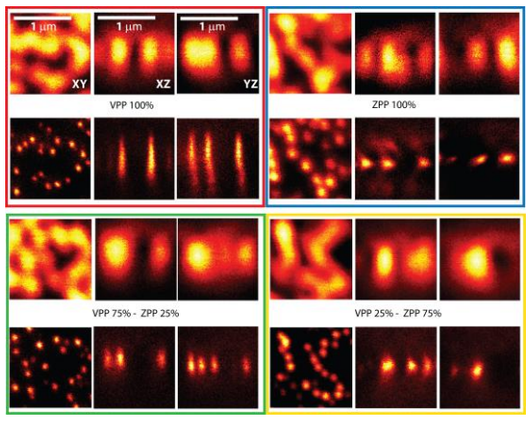


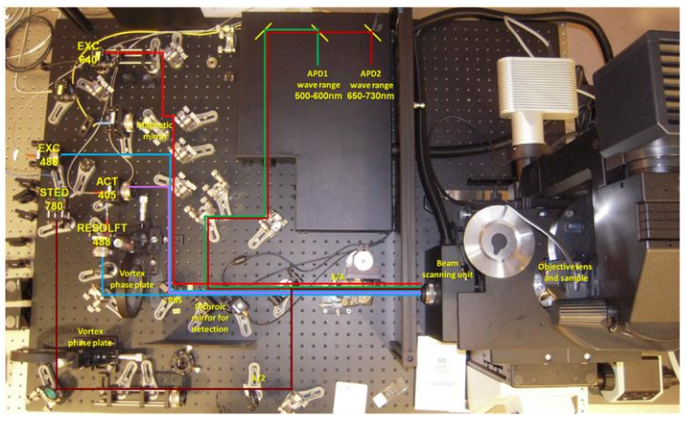


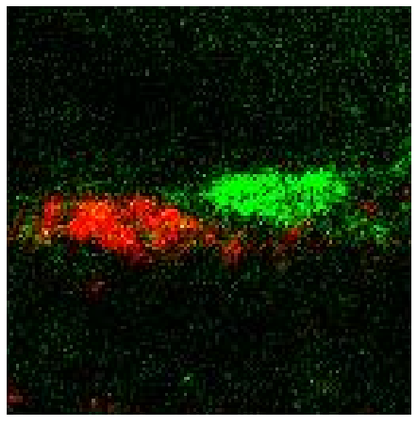


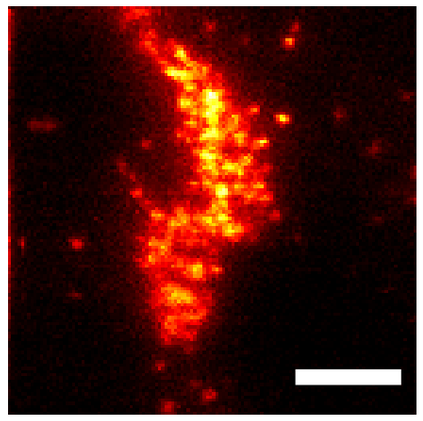


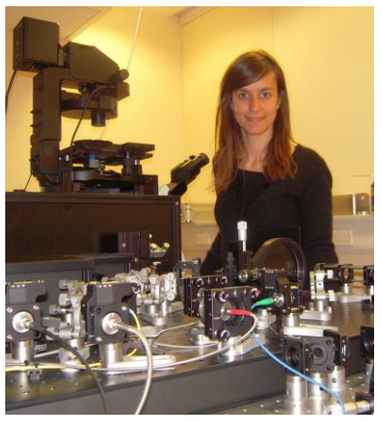
A multicolour STED microscope designed for biological applications





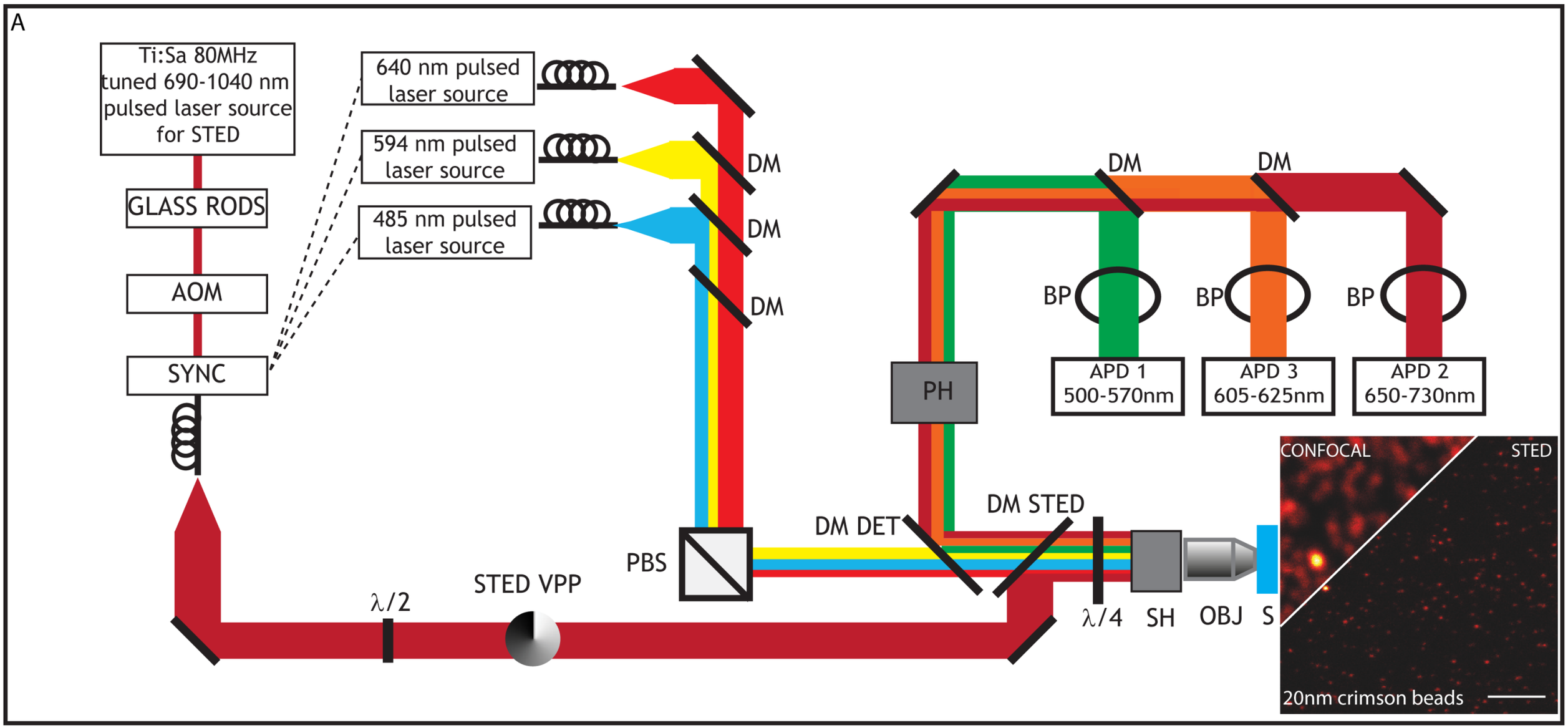




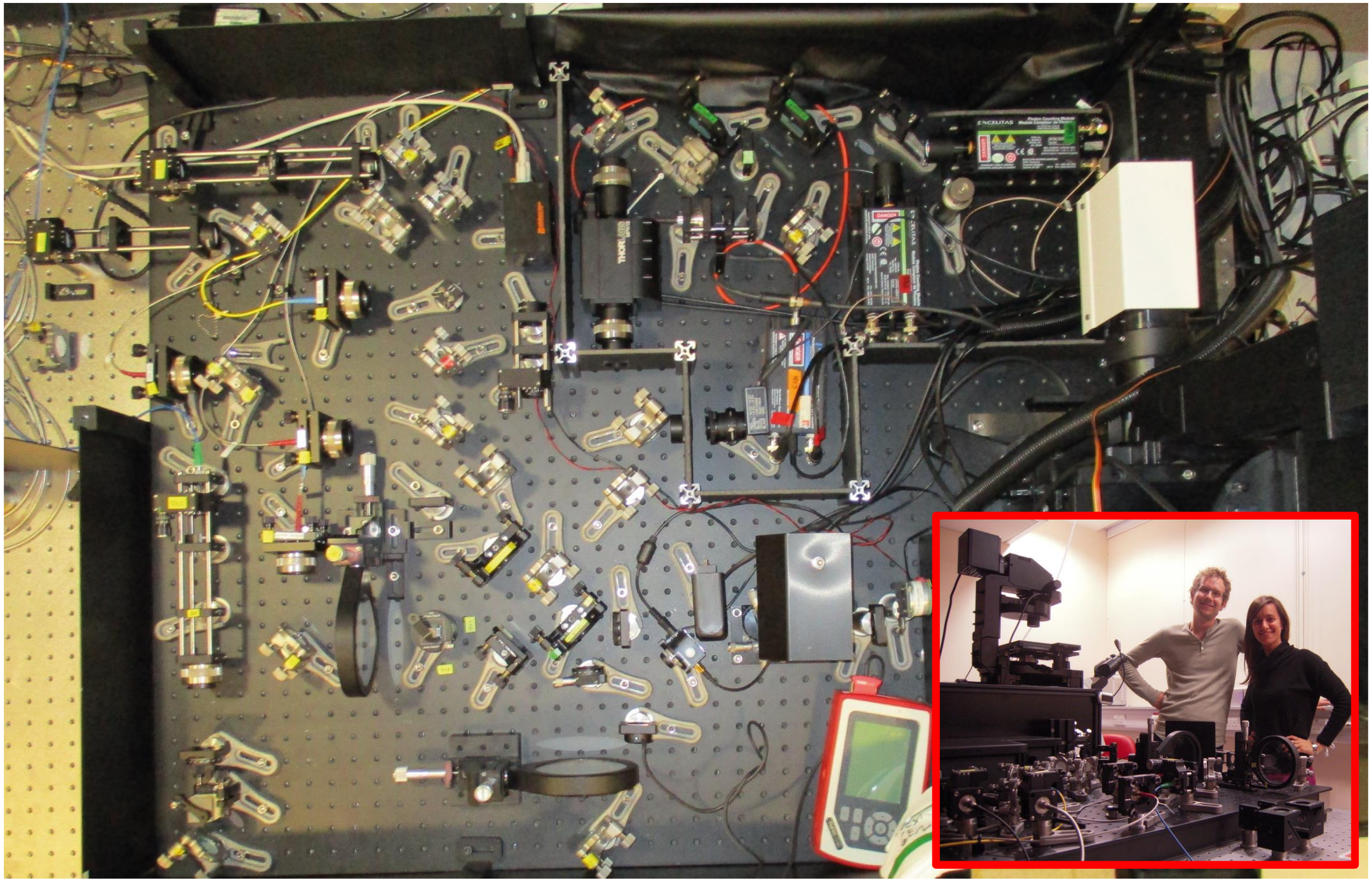


Silvia Galiani Nanoimmunology Group Weatherall Institute of Molecular Medicine University of Oxford

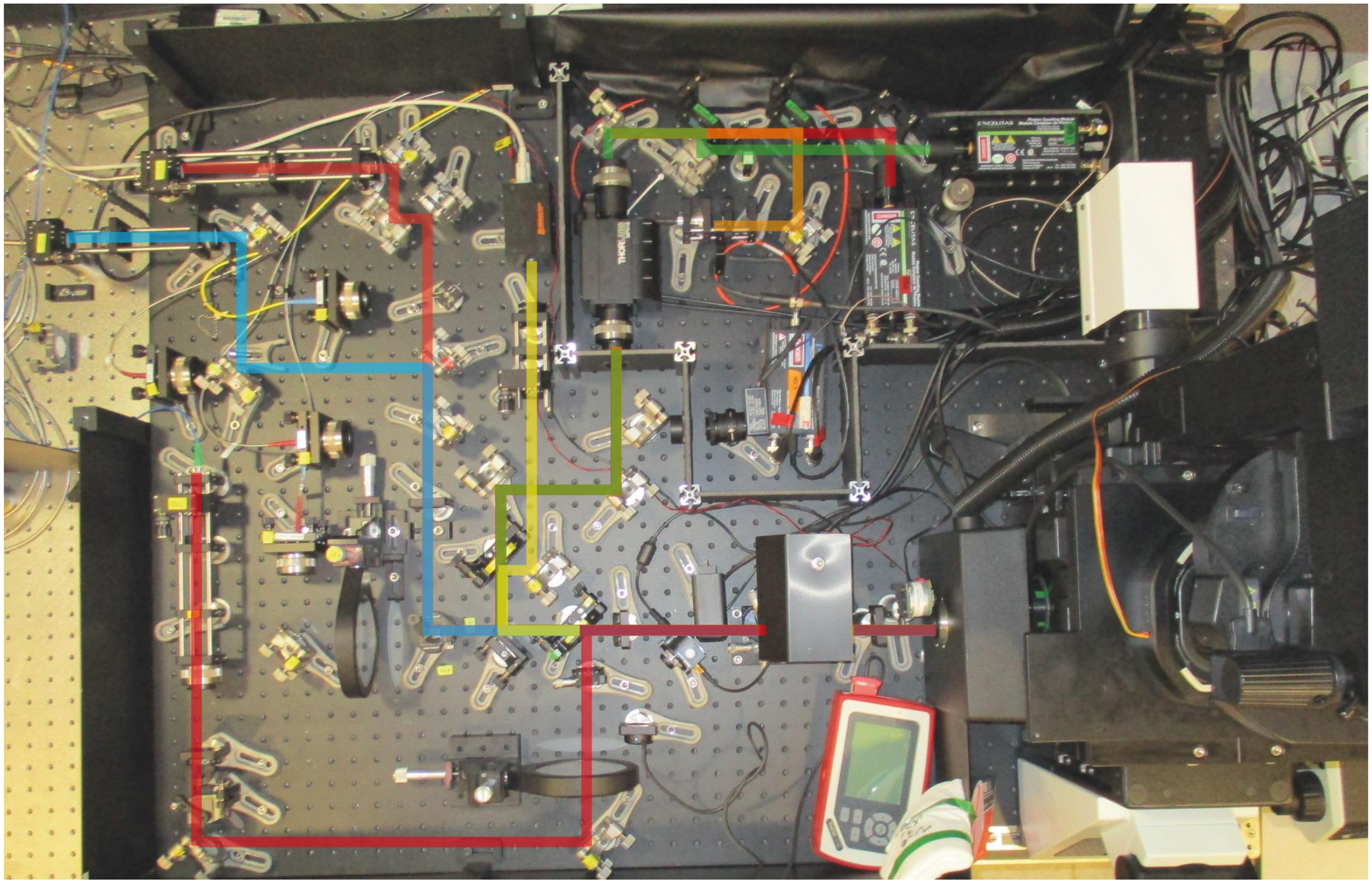
Multicolour STED architecture



Custom made multicolour STED architecture

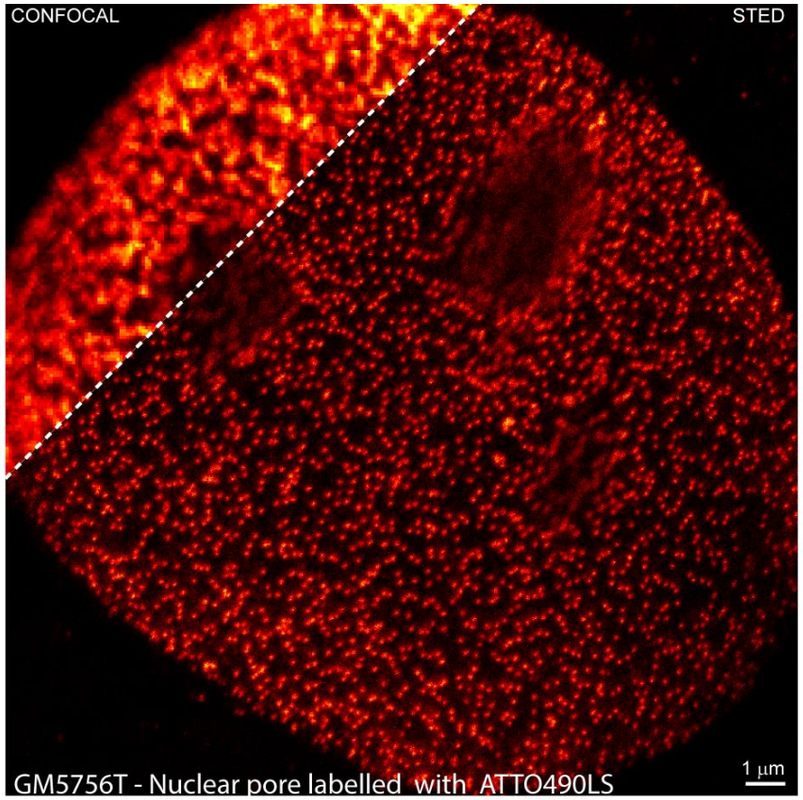


Custom made multicolour STED architecture



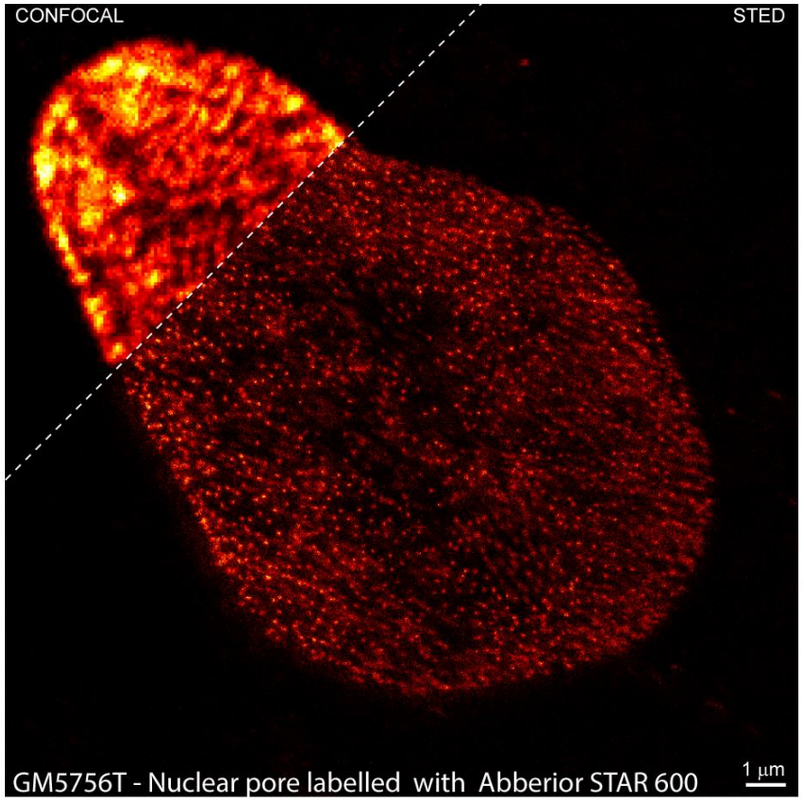
Multicolor STED architecture

ATTO 490LS



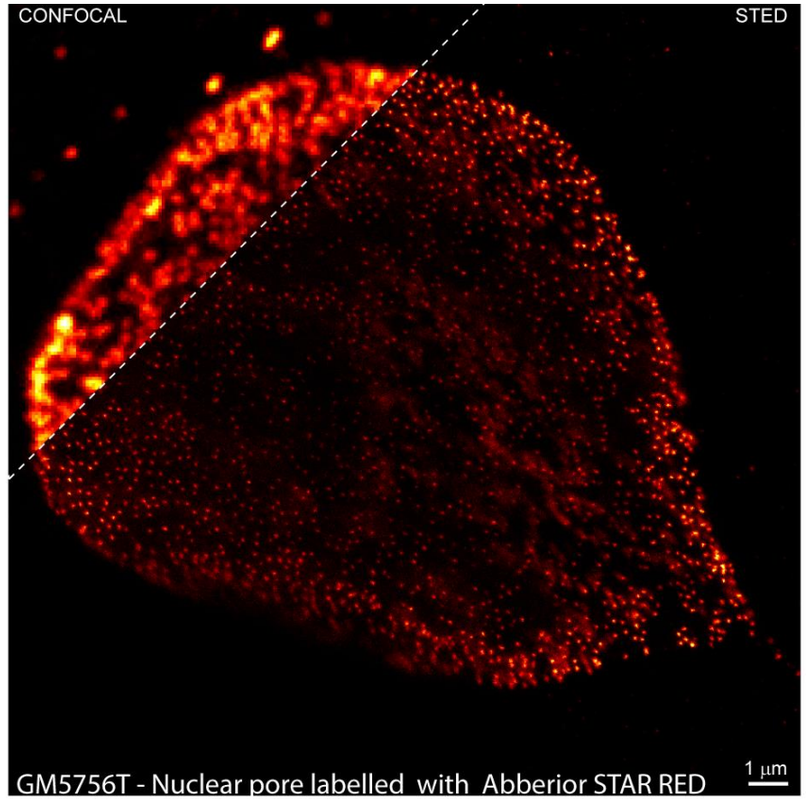
EXC 485nm
STED 755nm
APD2 650-730nm

Abberior STAR 600



EXC 594nm
STED 755nm
APD3 605-625nm

Abberior STAR RED



EXC 640nm
STED 755nm
APD2 650-730nm

Plus one confocal signal EXC 485nm NO STED APD1 500-570nm

Towards an optimized imaging

The microscope needs to be aligned:

1. **Spatial alignment**
2. **Temporal alignment**
3. **Polarization check**

The imaging needs to be controlled

✓ **Chromatic aberration** → when the STED beam is on this system is chromatic aberration free

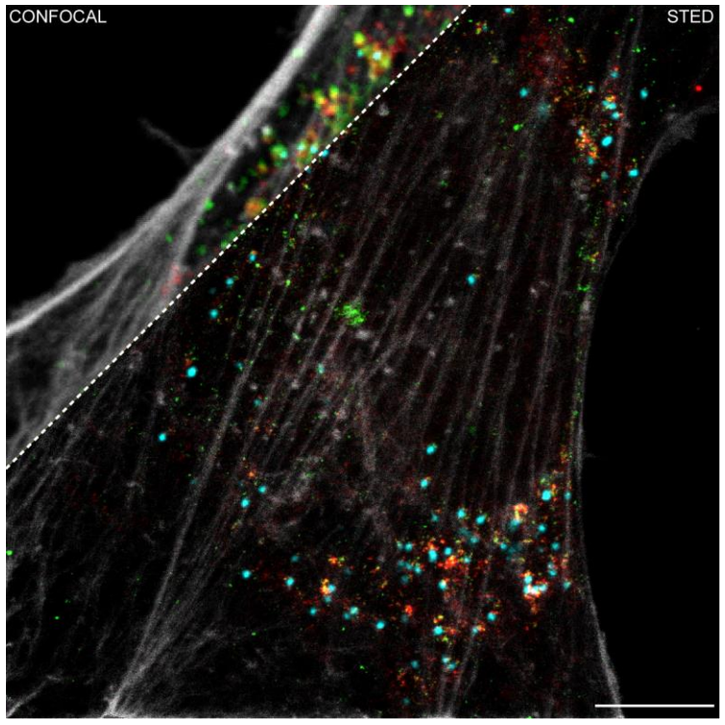
✓ **Crosstalk** → crosstalk negligible between APD1 and APD2 or APD3: acquisition in parallel
→ crosstalk between APD2 and APD3: acquisition in series

✓ **Spatial drift** → due to the acquisition in series a green dye is always introduce to correct for possible spatial drift

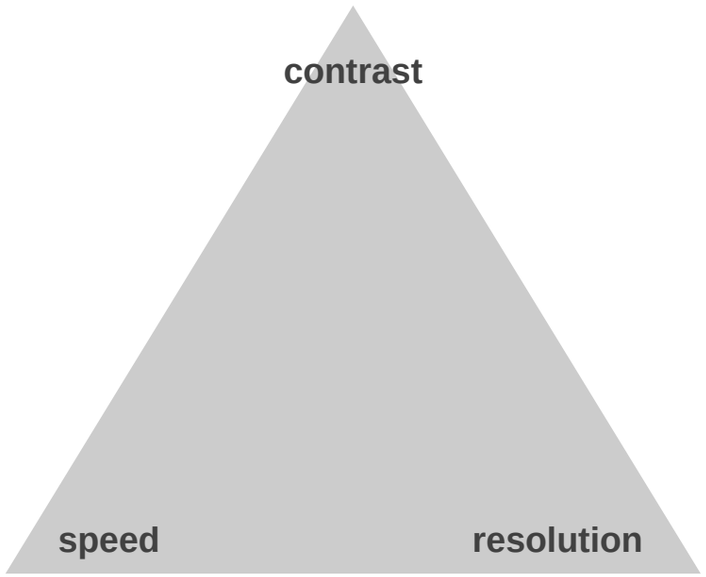
The immunostaining protocol has been optimized

Sample preparation:

- ✓ Fixation: 3% PFA in PBS
- ✓ Permeabilization: pure Methanol
- ✓ Blocking: 2%BSA+5% FCS in PBS
- ✓ Dilution I AB: 1:400
- ✓ Dilution II AB: between 1:125 and 1:250



Challanges of STED microscopy



STED microscopy

High spatial resolution (tens of nm)

High power density

Longer acquisition time

More costly, advanced operation and different sample preparation

When shall I use the technique→ when becomes important for the biological question to disclose details beyond the resolution limit

Imaging the peroxisomal import machinery

Multicolor STED microscopy

The Biologist

The Image Analyst

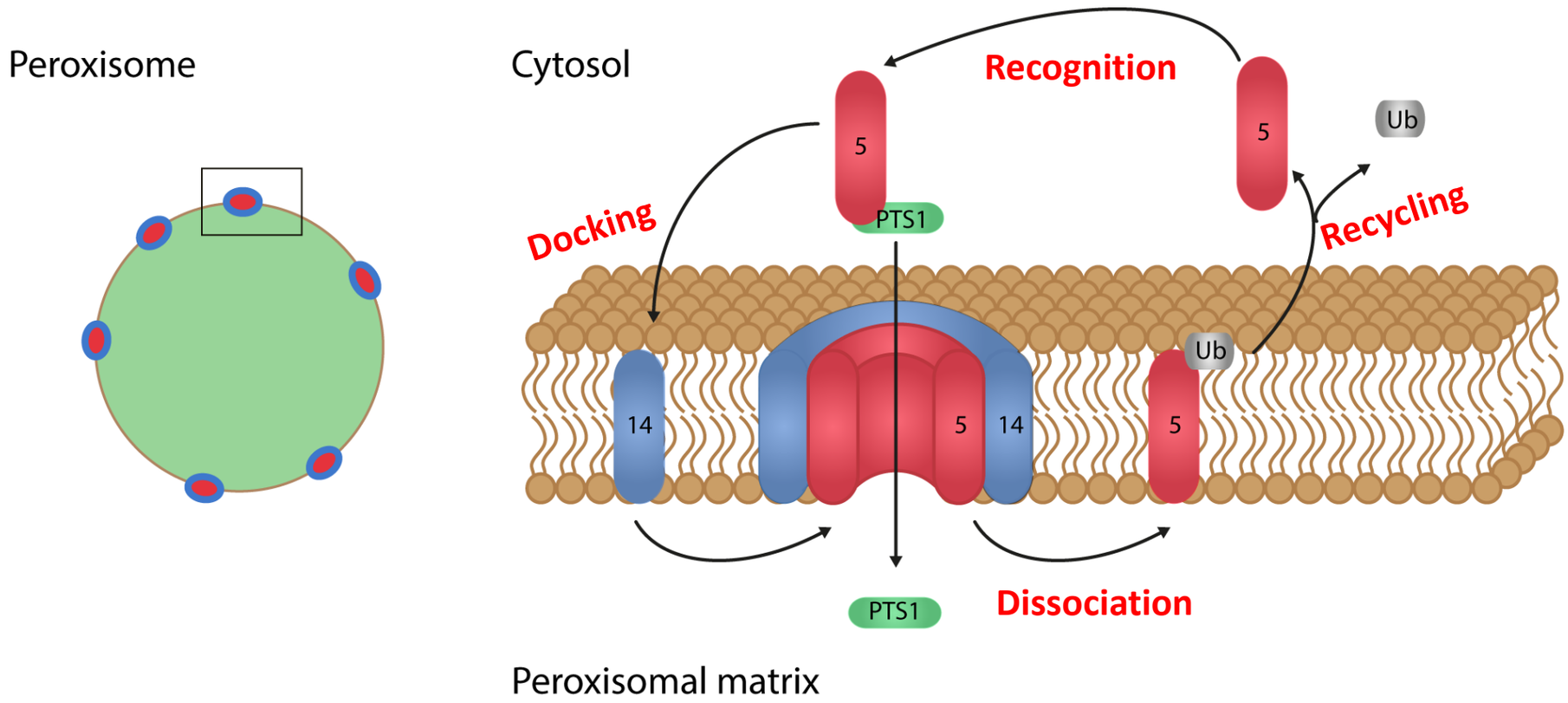
The Microscopist

Peroxisomes function and structure

Peroxisomes have an **indispensable role in the human metabolism:**

- important site to the **breakdown of fatty acid**
- carry out essential steps in **the synthesis of different lipids**

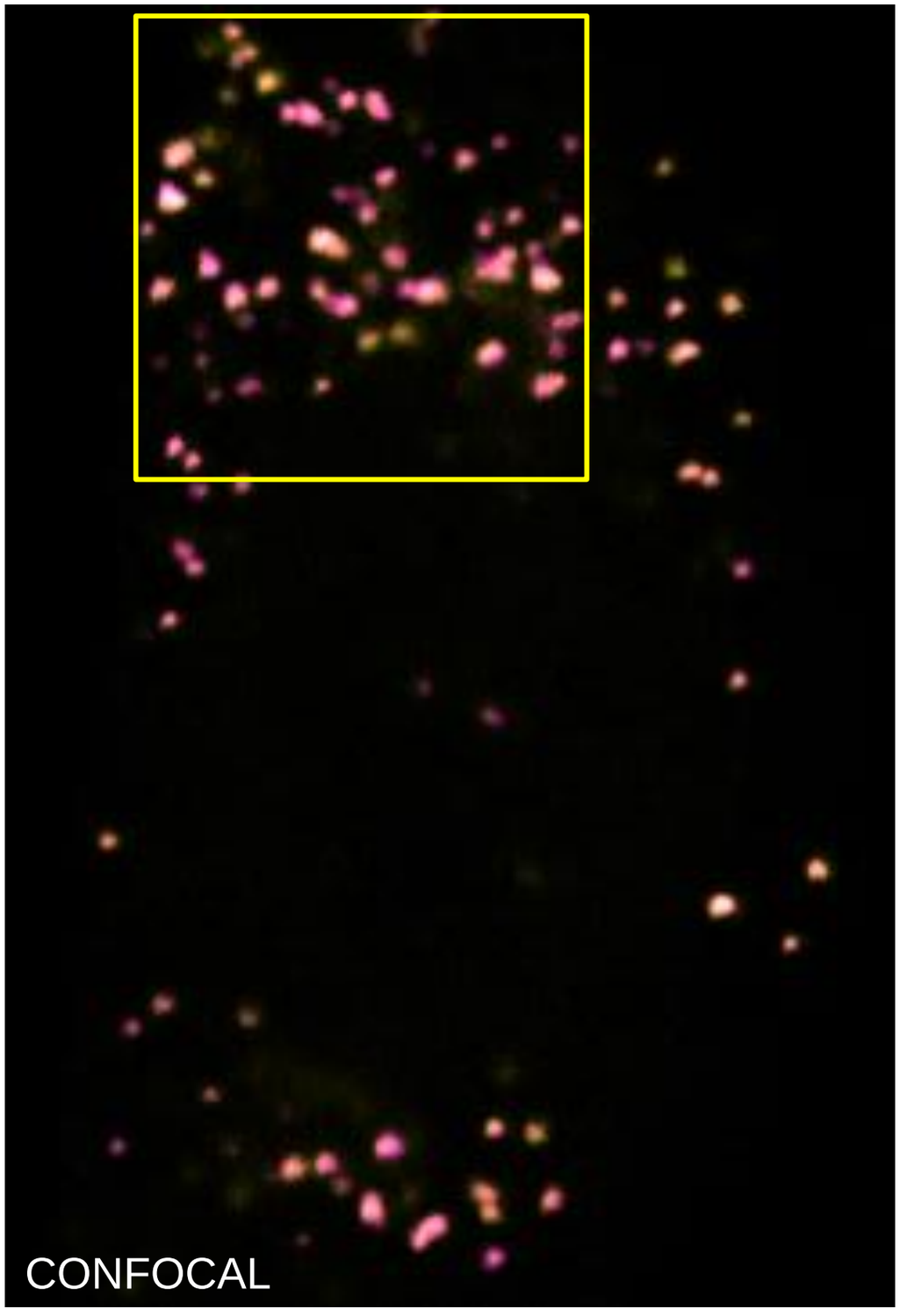
Peroxisomal matrix proteins are nucleus encoded, synthesized on free ribosomes and subsequently folded imported through the peroxisomal membrane.

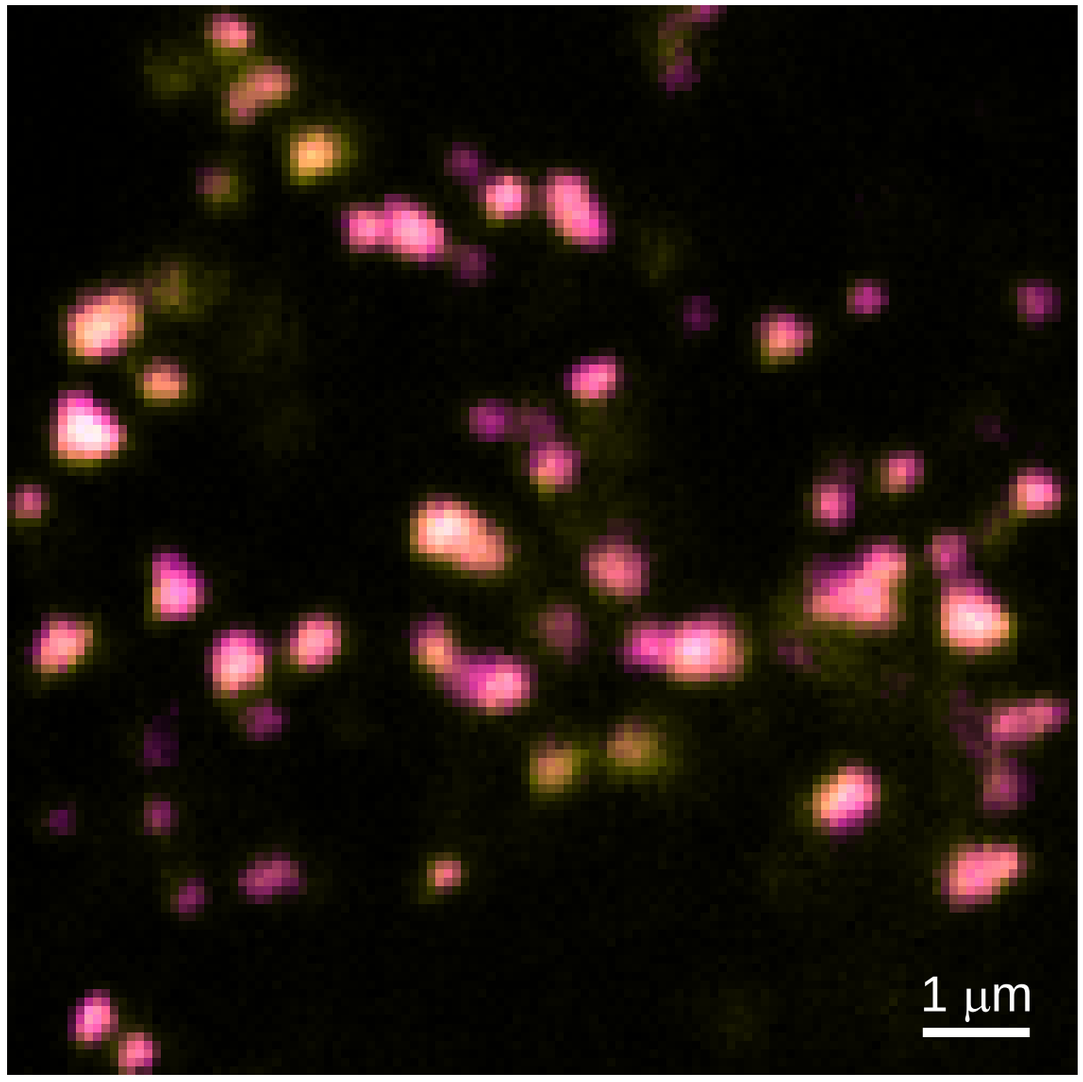


The detailed mechanism of the subcellular dynamics underlying cargo-binding in the cytosol, docking, cargo translocation and receptor release at the peroxisomal membrane is not known.

Confocal microscopy on peroxisomal proteins

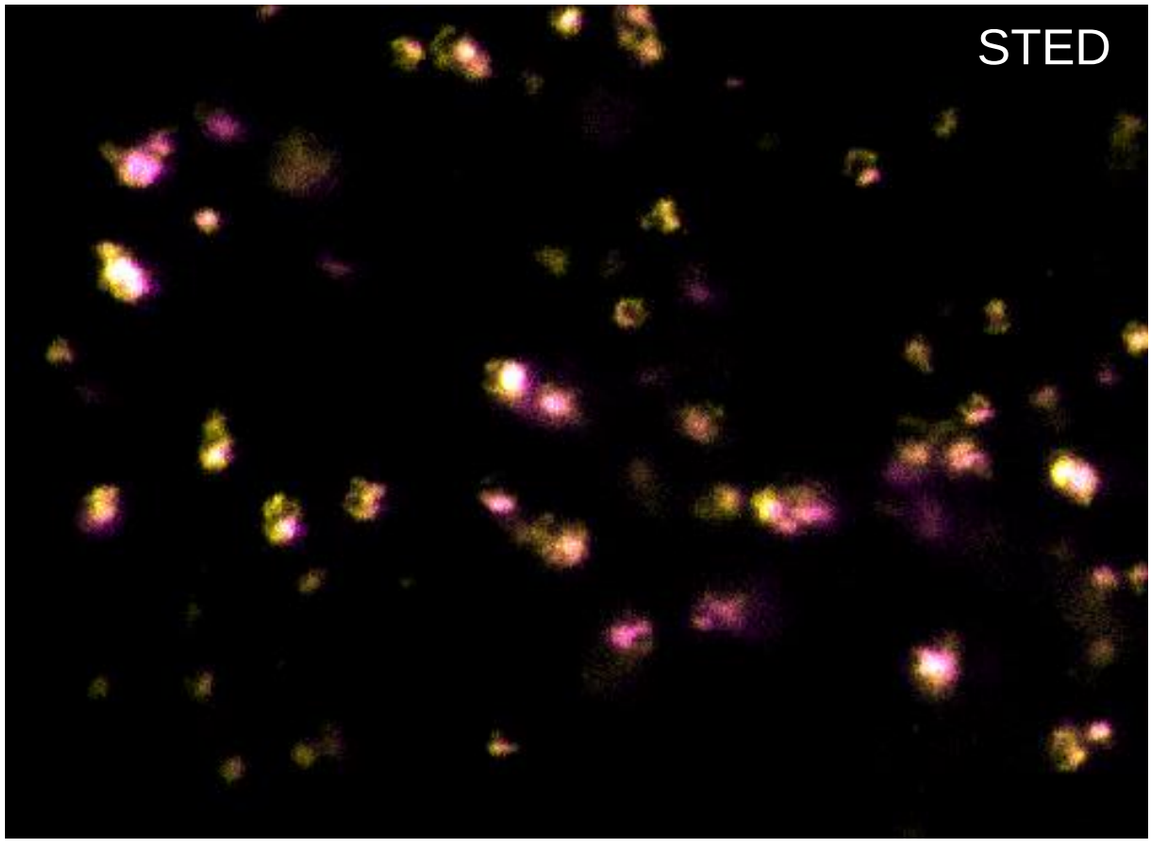
GM5756T human fibrobast
PEX14-Ab*RED and PTS1-Ab*600



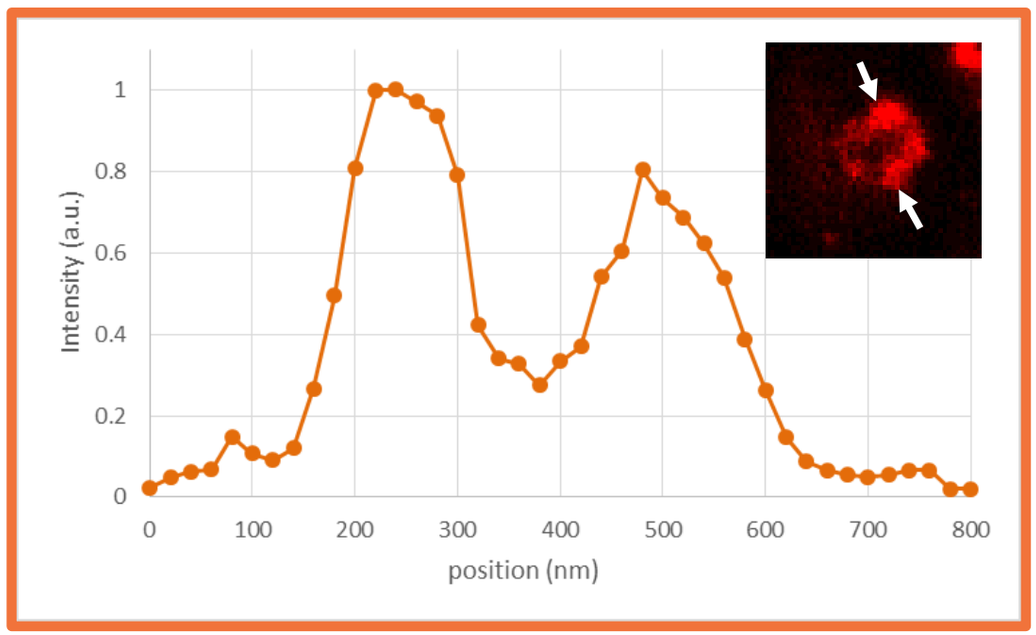


STED microscopy on peroxisomal proteins

PEX14-Ab*RED and PTS1-Ab*600

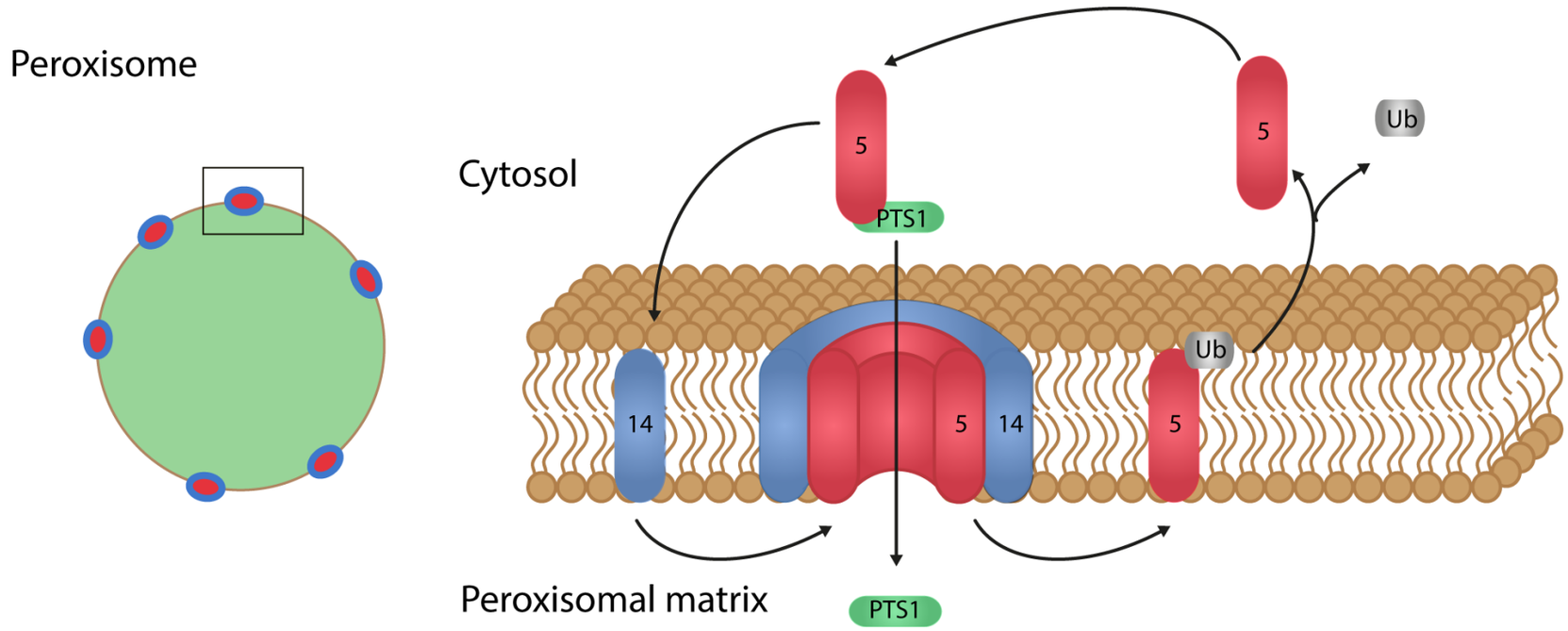


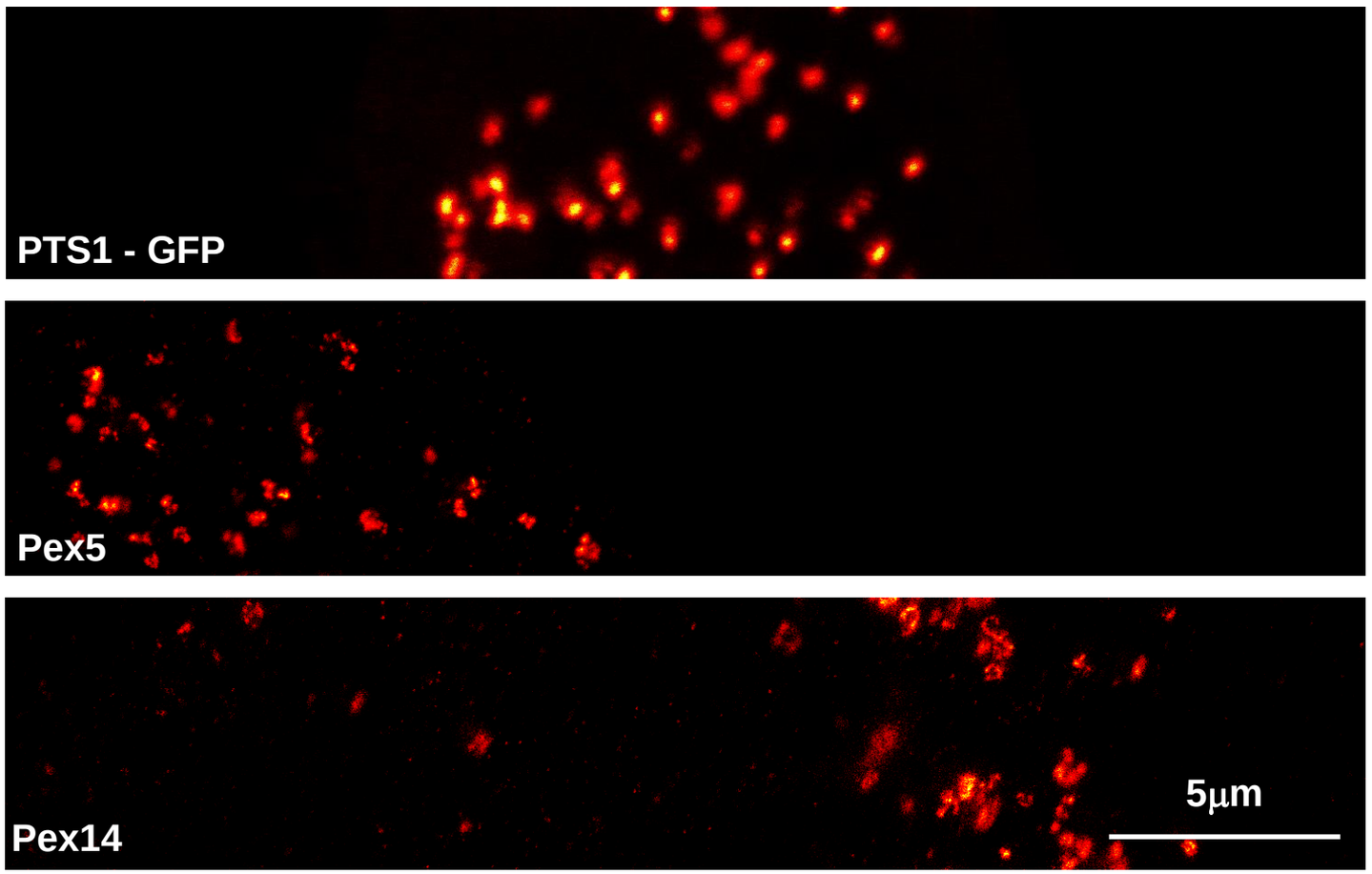
Heterogeneous size of peroxisomes



FWHM PEX14 staining 330±125nm (n=100)

1 colour images





Cell line: GM5756T human fibroblast
Imaged protein:

- PTS1 - cargo protein - GFP
- Pex 5 - import receptors
- Pex14 – traslocon

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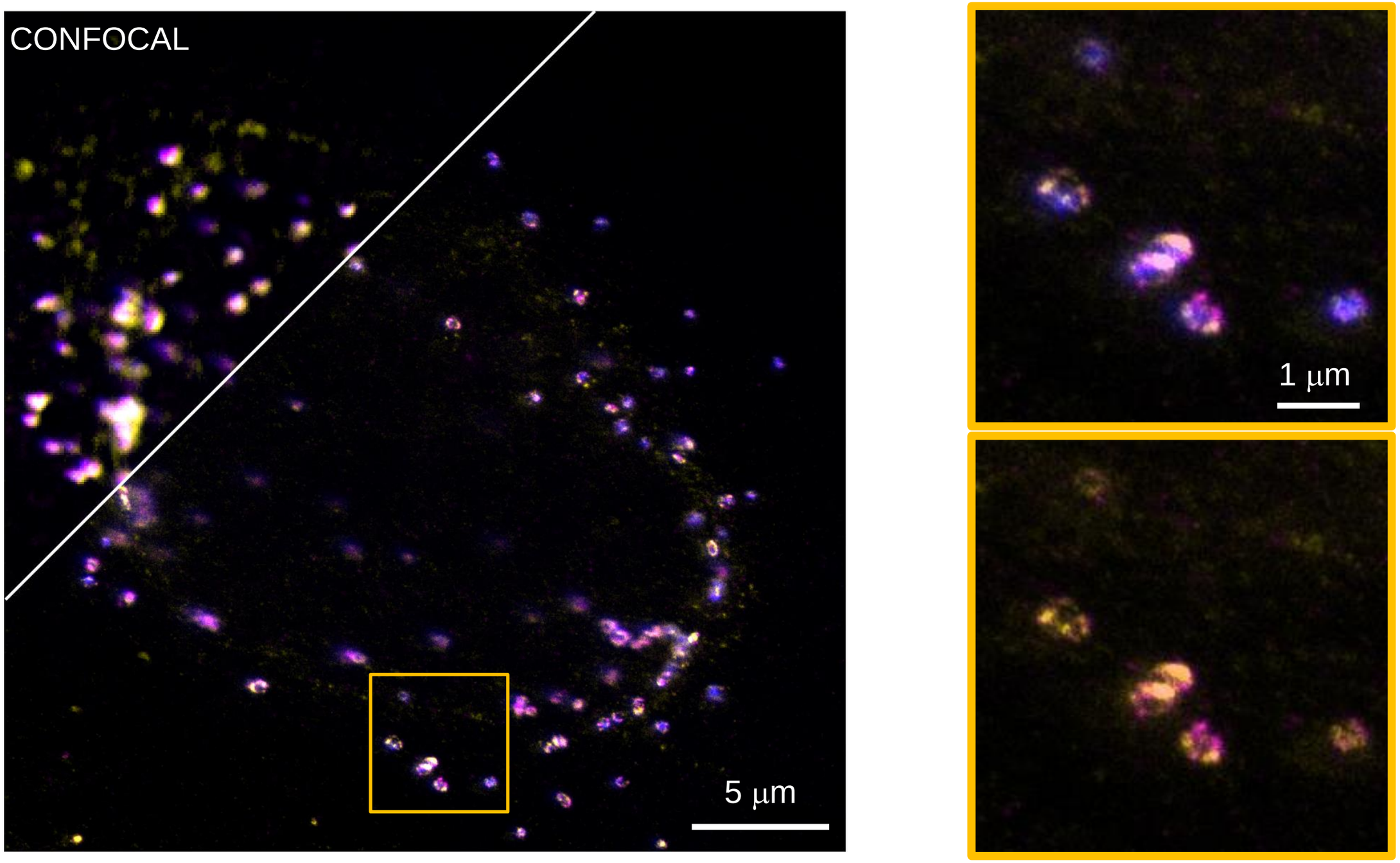
3 colour images

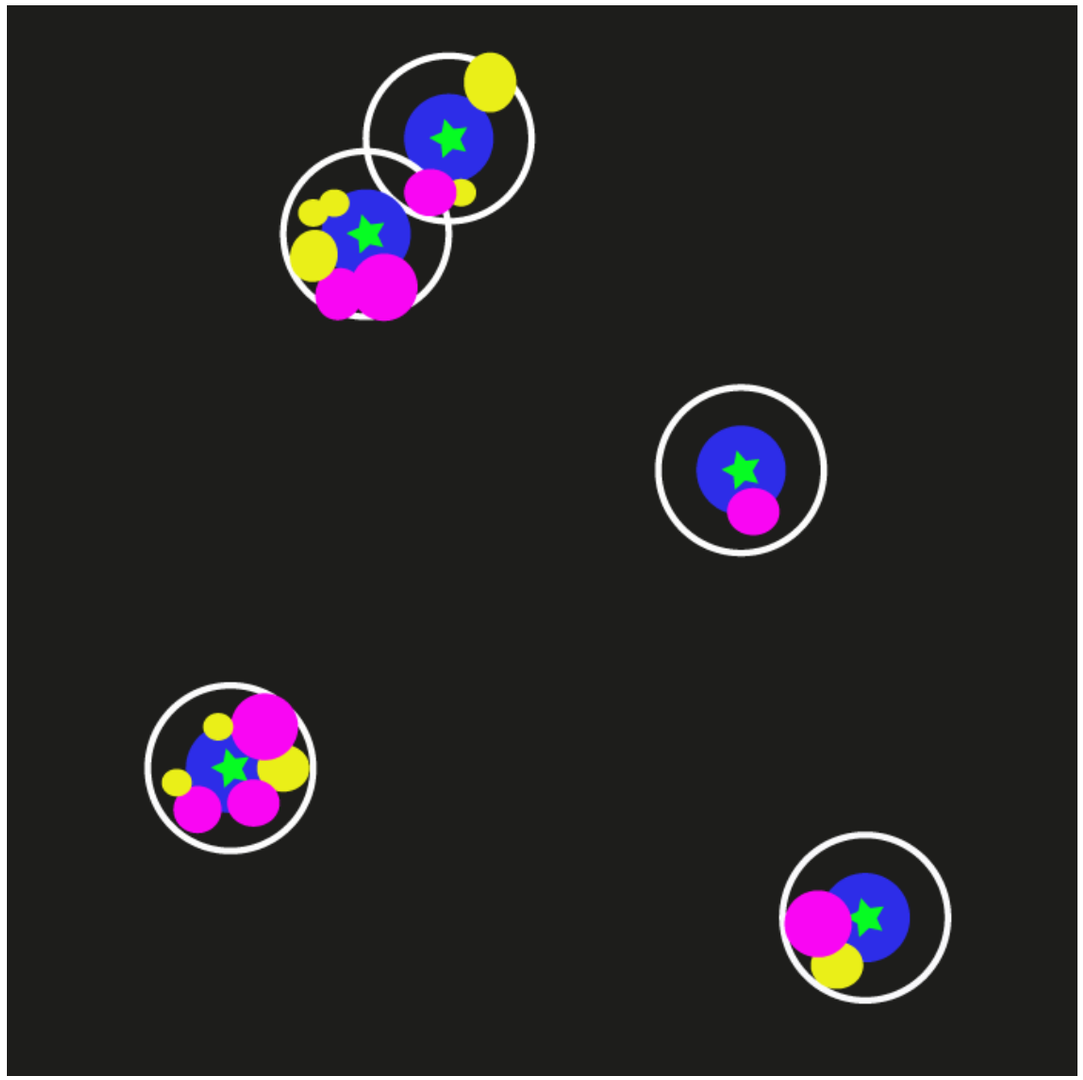
MRC Human Immunology Unit

Protein co-localization of import receptors (Pex5) and translocon (Pex14) respect to the peroxisomal matrix marker



GM5756T PTS1 labelled with GFP
PEX14-Ab*RED
PEX5-Ab*600

Colocalization Analysis

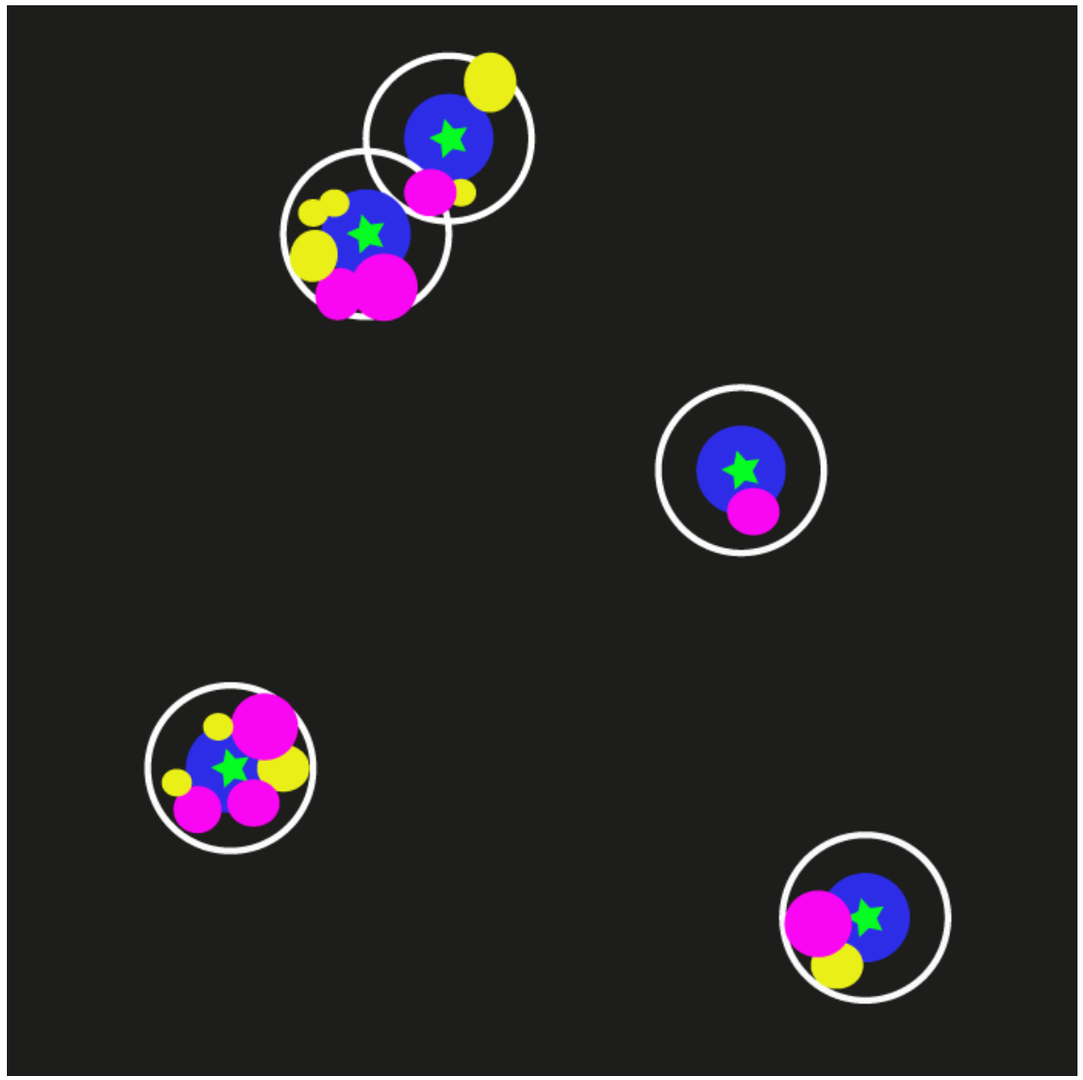


1 - Find coordinates of the peroxisomes using the PTS1-GFP signal

2 - Measure intensity in each circular patch (radius 190 nm) for both super-resolved signals (594nm and 640nm excitation lines)

Images statistic: at least 10 images on at least 3 different samples have been collected per each condition

Colocalization Analysis



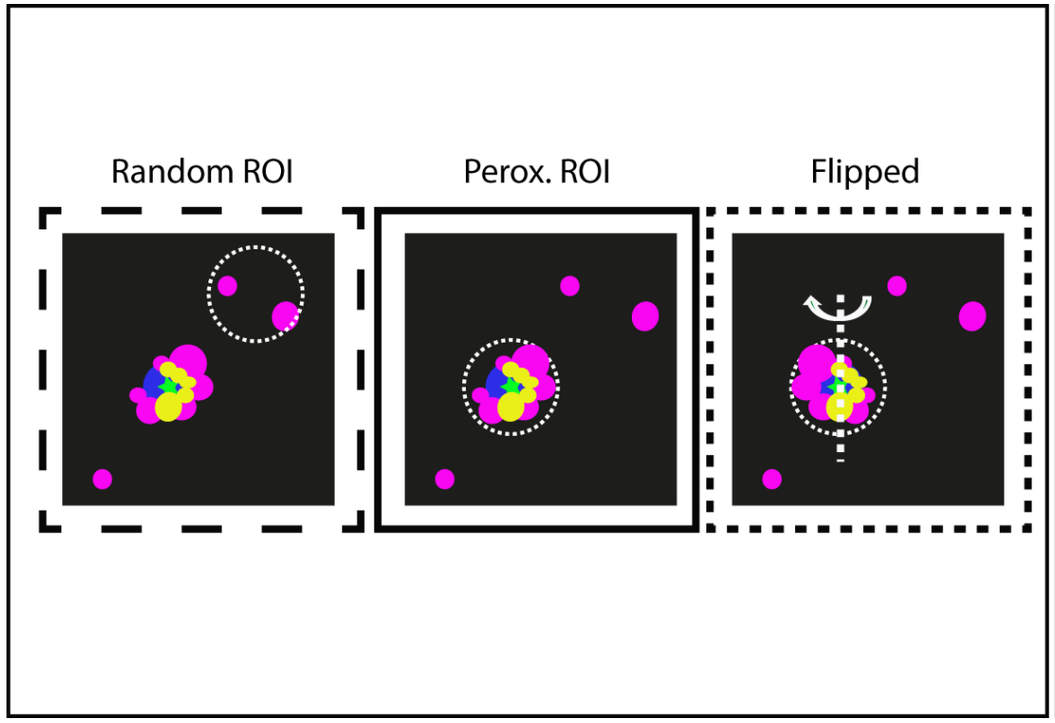
1 - Find coordinates of the peroxisomes using the PTS1-GFP signal

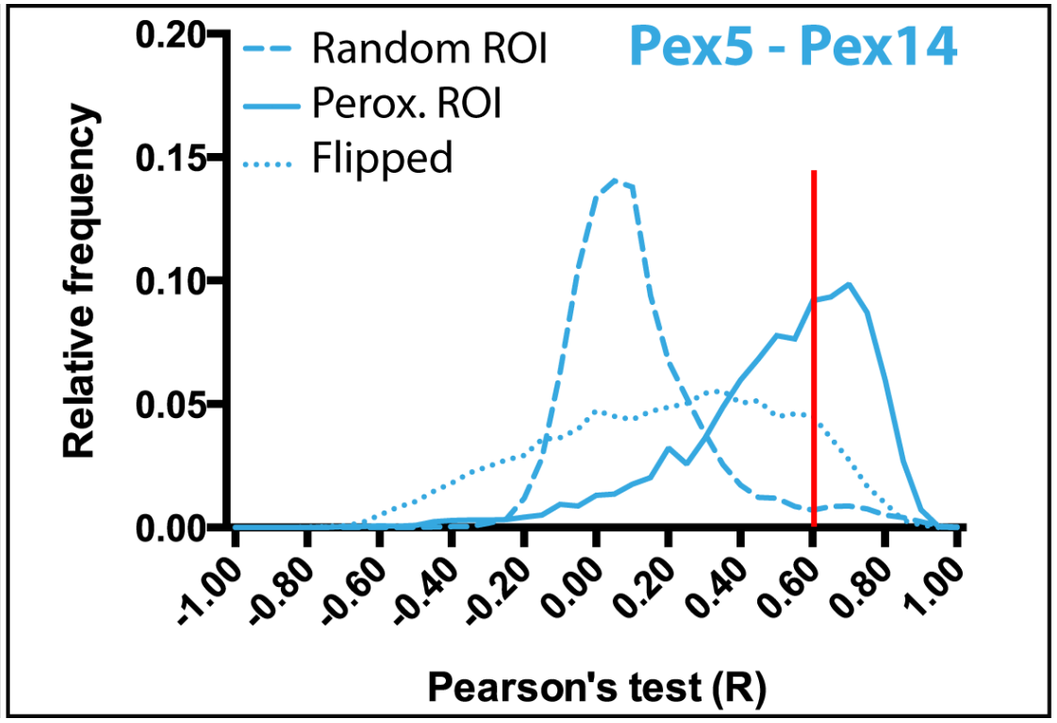
2 - Measure intensity in each circular patch (radius 190 nm) for both super-resolved signals (594nm and 640nm excitation lines)

3 – Correlation of pixel intensities in the patches with Pearson's Test results in colocalisation value for each peroxisome

Images statistic: at least 10 images on at least 3 different samples have been collected per each condition

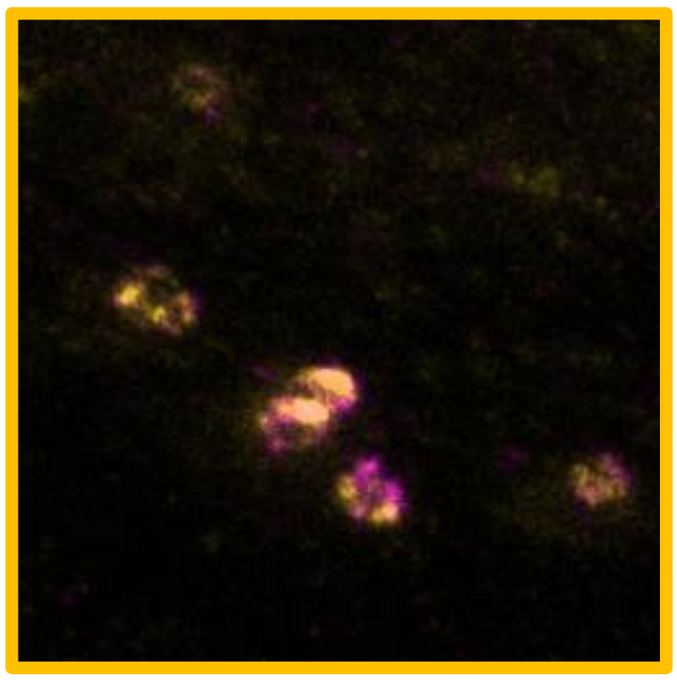
PEX5-PEX14 Colocalization Analysis





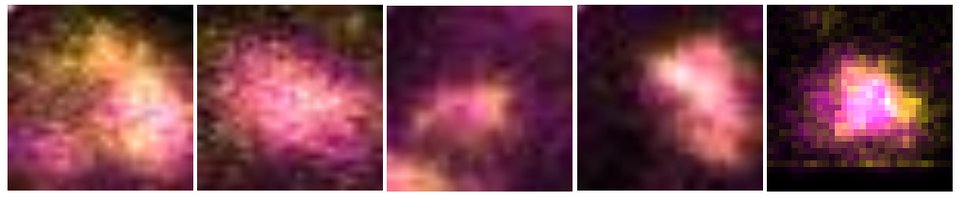
Images statistic: at least 10 images of at least 3 different samples have been collected for each condition

- PEX5-PEX14 show high colocalization
- Co-localisation does not occur through random.
- Flipped control: Specific correlation at peroxisomes



Compartmentalization of membrane proteins

PEX5-PEX14 High Colocalization (> 0,6)

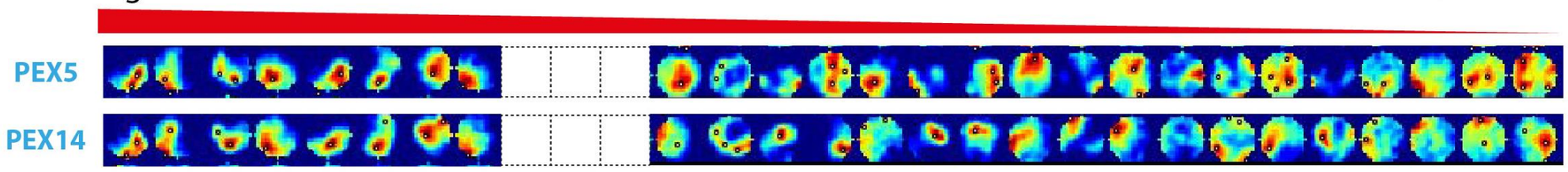


Low Compartmentalization

Low Colocalization (< 0,4)

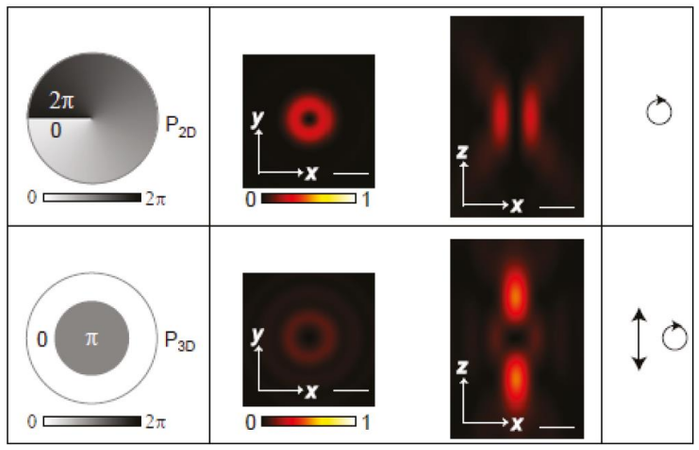
High Compartmentalization

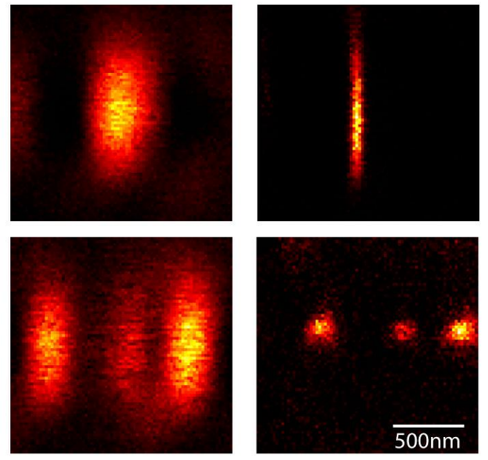
High Colocalization Low Colocalization

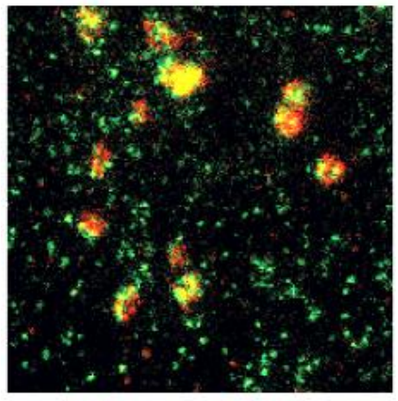


Work in progress:

- 3D STED implementation







- Use CRISPR/Cas9 deletion cell lines KO PEX5 and KO PEX14: complementation of PEX5/PEX14

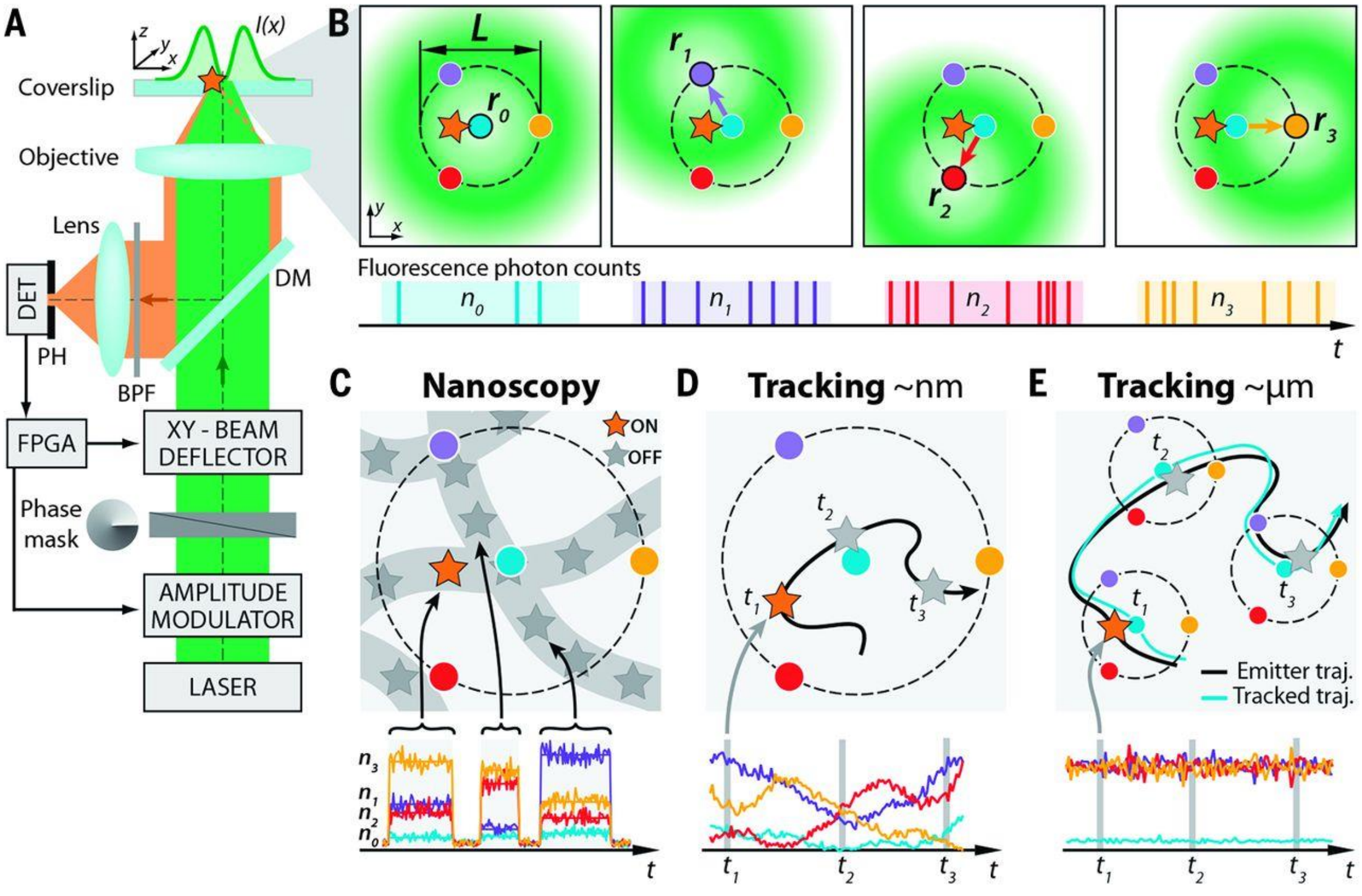
Super resolution-STED microscopy reveals compartmentalization of peroxisomal membrane proteins. Galiani S, Waithe D, Reglinski K, Zaragoza L D C, Clausen M P, Schliebs W, Erdmann R, Eggeling C, The Journal of Biological Chemistry, 291, 16948-16962 (2016)

Minflux

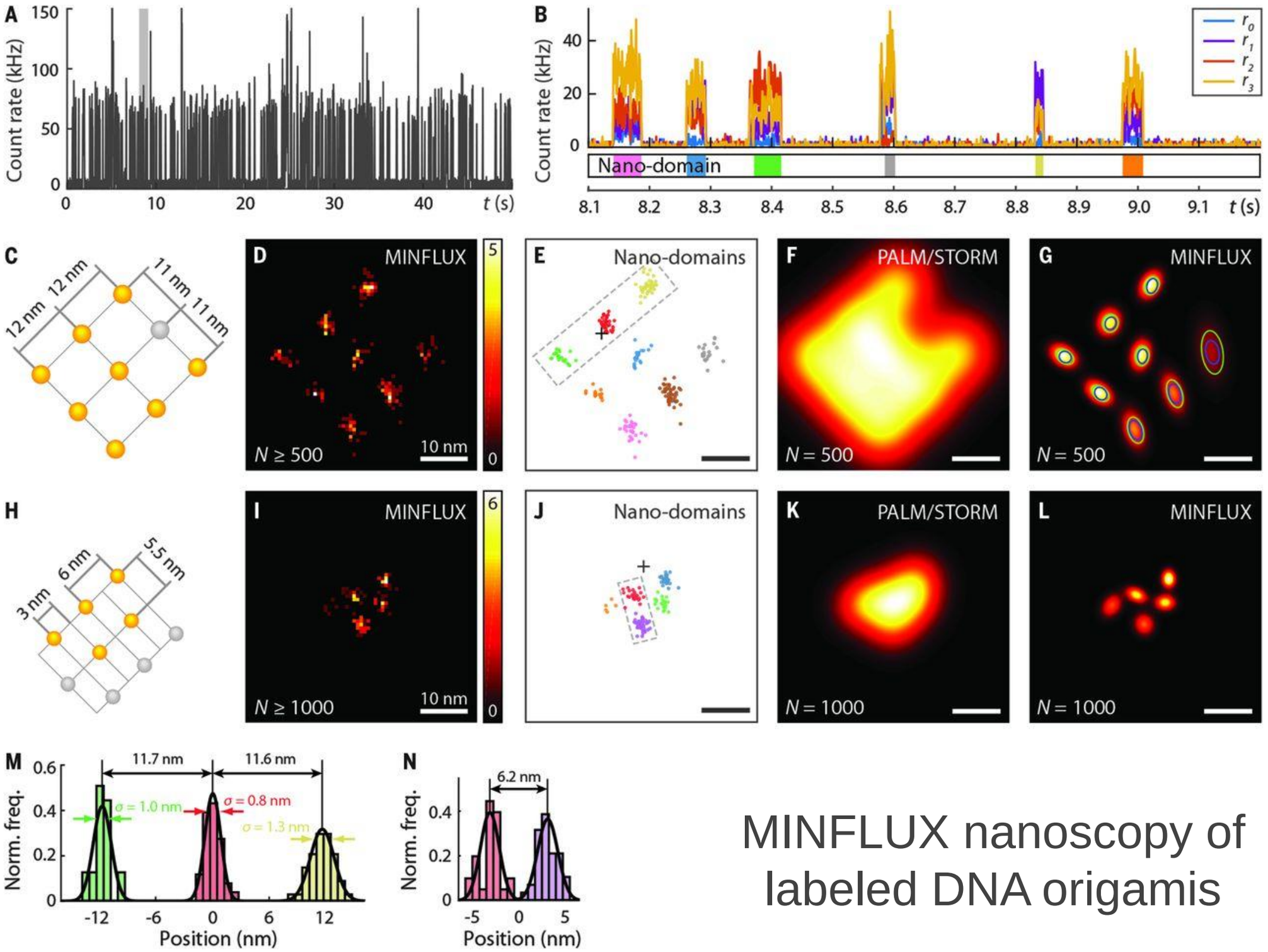
combining super-resolution techniques

Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes
Balzarotti F, Eilers Y, Gwosch K C, Gynnå A H, westphal V, Stefani F D, Elf J, Hell S W. Science 2017

2D Minflux

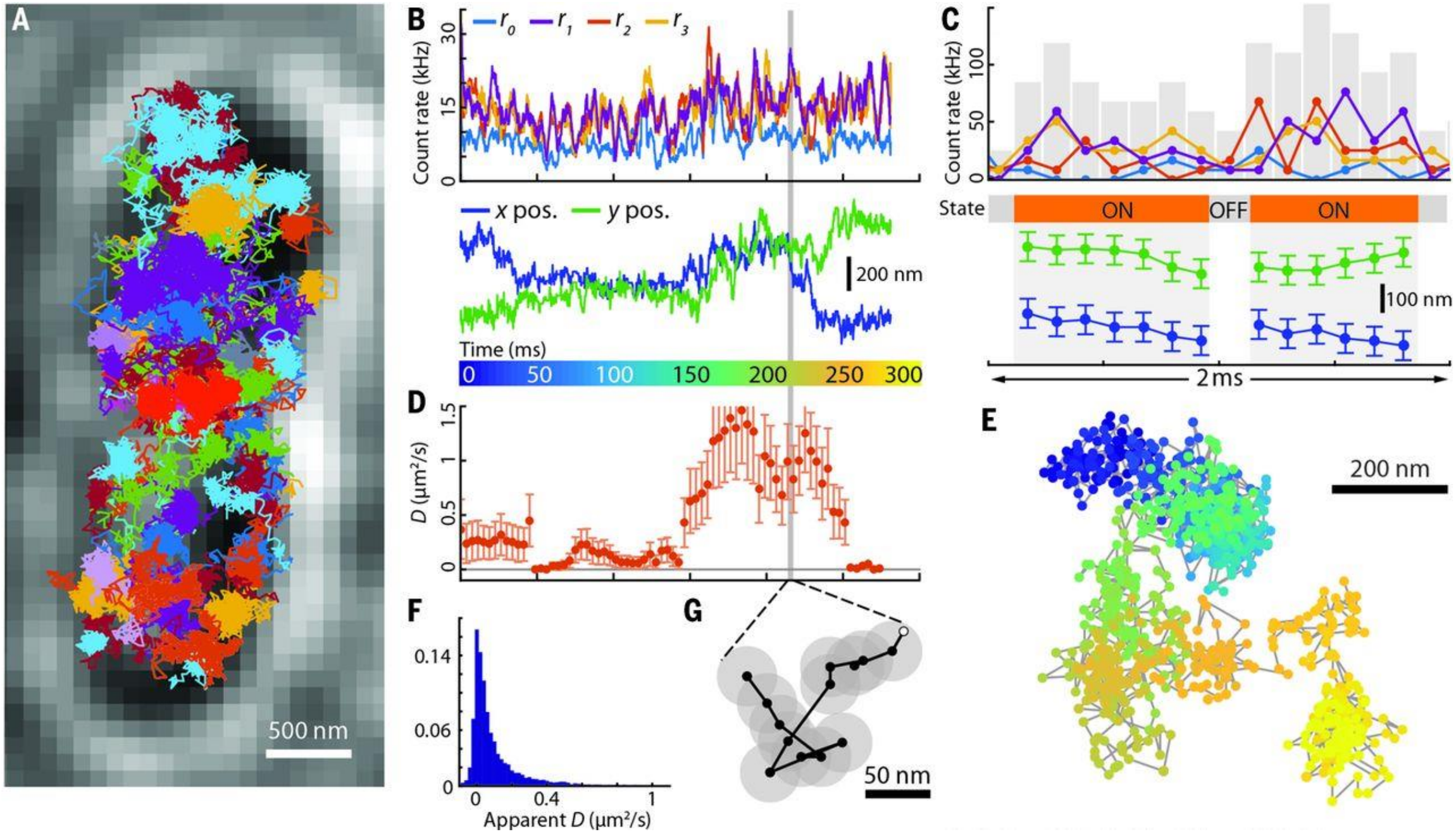

A
I(x)
Coverslip
Objective
Lens
DM
DET
PH
BPF
FPGA
XY - BEAM DEFLECTOR
Phase mask
AMPLITUDE MODULATOR
LASER
B
L
r_0
r_1
r_2
r_3
Fluorescence photon counts
n_0
n_1
n_2
n_3
t
C
Nanoscopy
ON
OFF
D
Tracking ~nm
E
Tracking ~µm
t_1
t_2
t_3
Emitter traj.
Tracked traj.

Imaging with Minflux - Nanoscopy



MINFLUX nanoscopy of labeled DNA origamis

Particle tracking with Minflux



Single-molecule MINFLUX tracking in living E. coli bacteria. Single 30S ribosomal protein subunits fused to the switchable fluorescent protein mEos2 are tracked.

… Thank you for your attention!