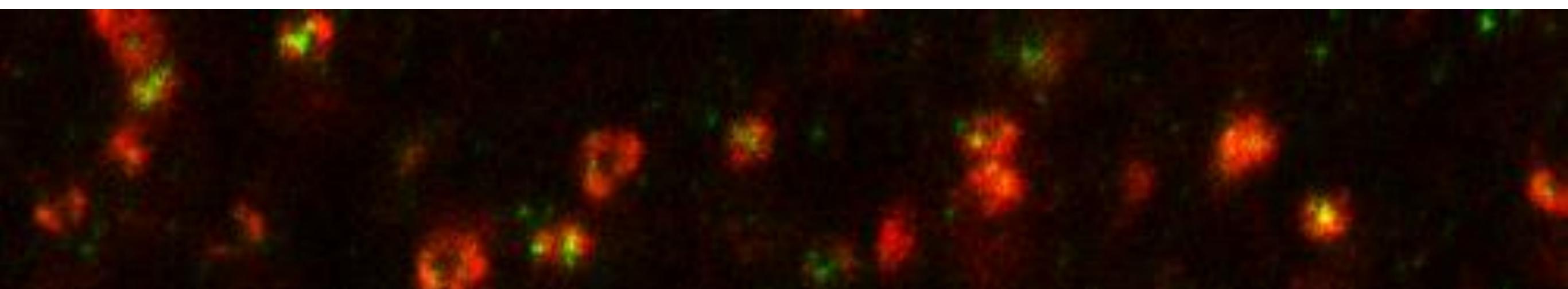


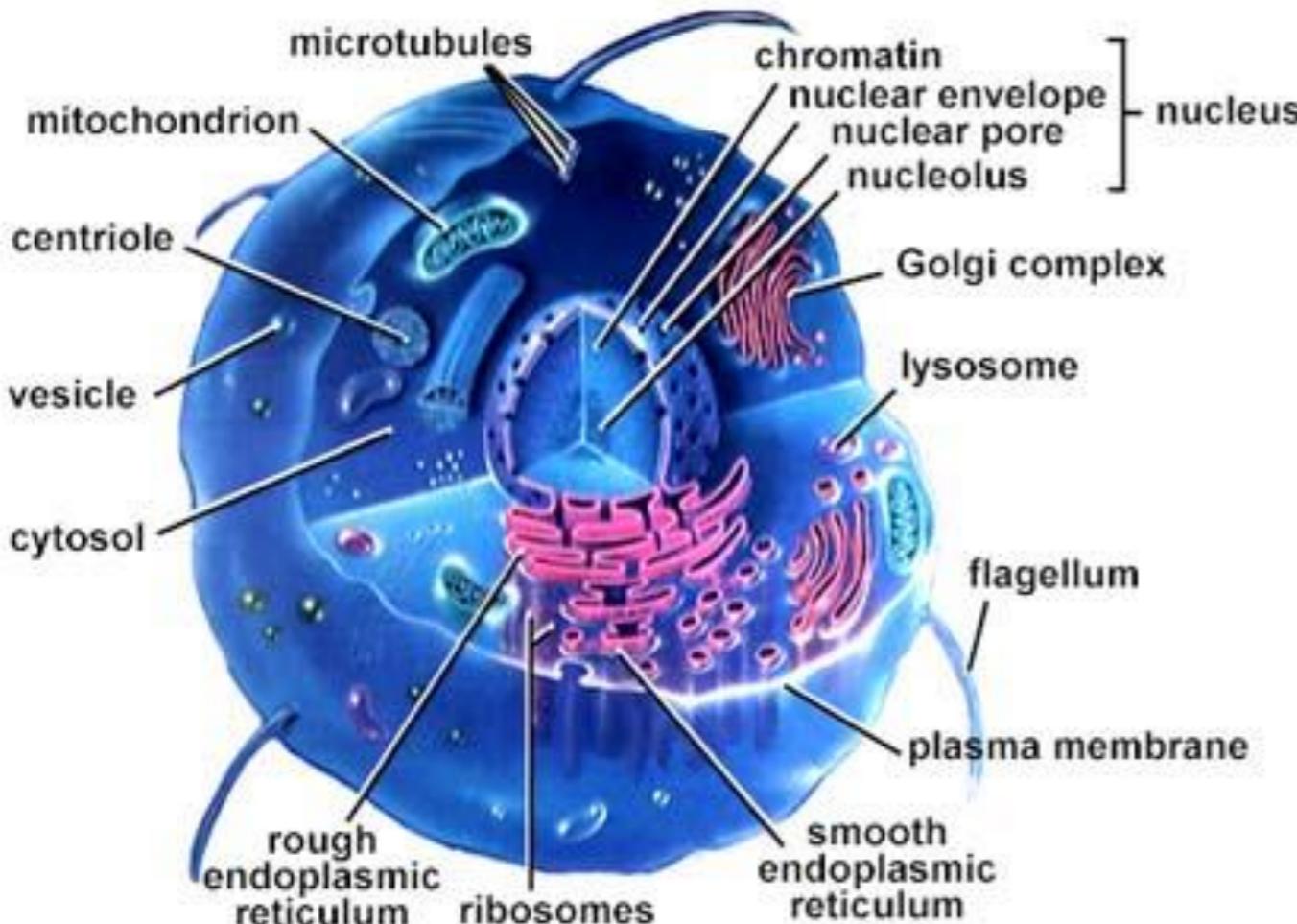
# STED microscopy



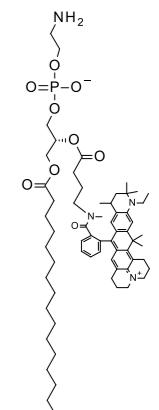
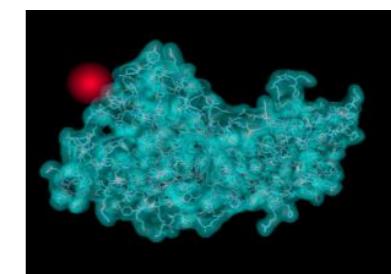
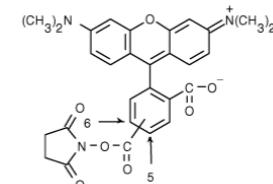
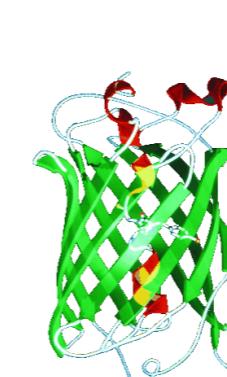
# From microscopy to nanoscopy

*Observation of living cells in far field: Non-Invasive*

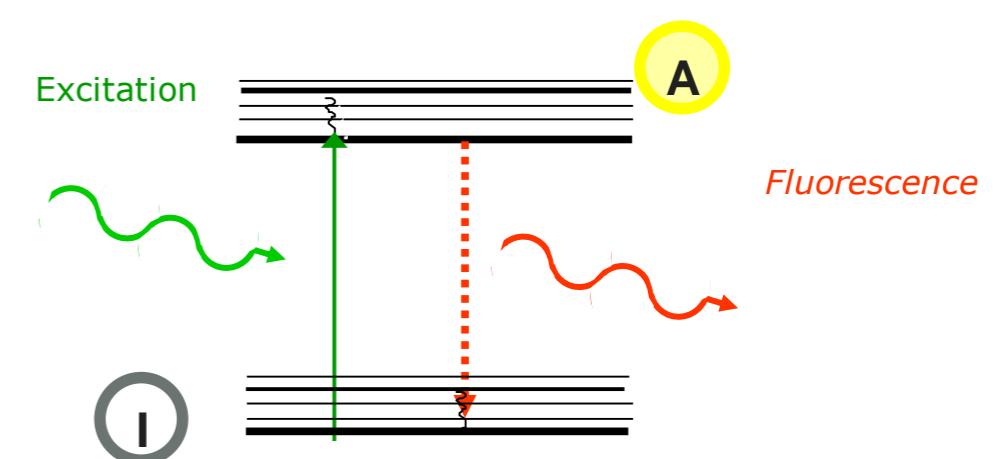
Study specific molecular processes in the living cell:



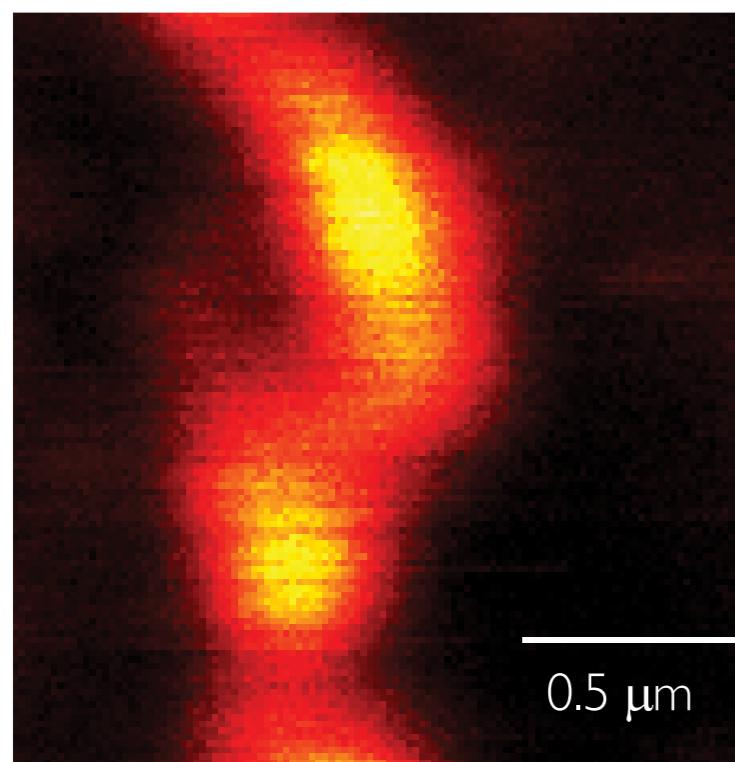
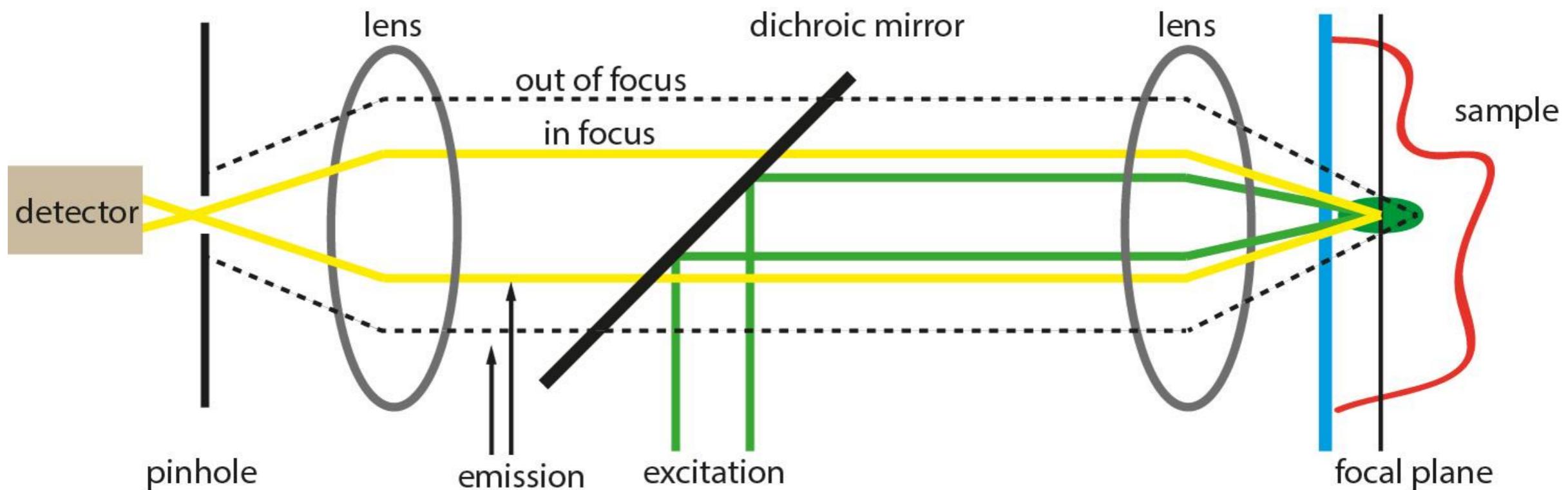
Label specific protein/molecule



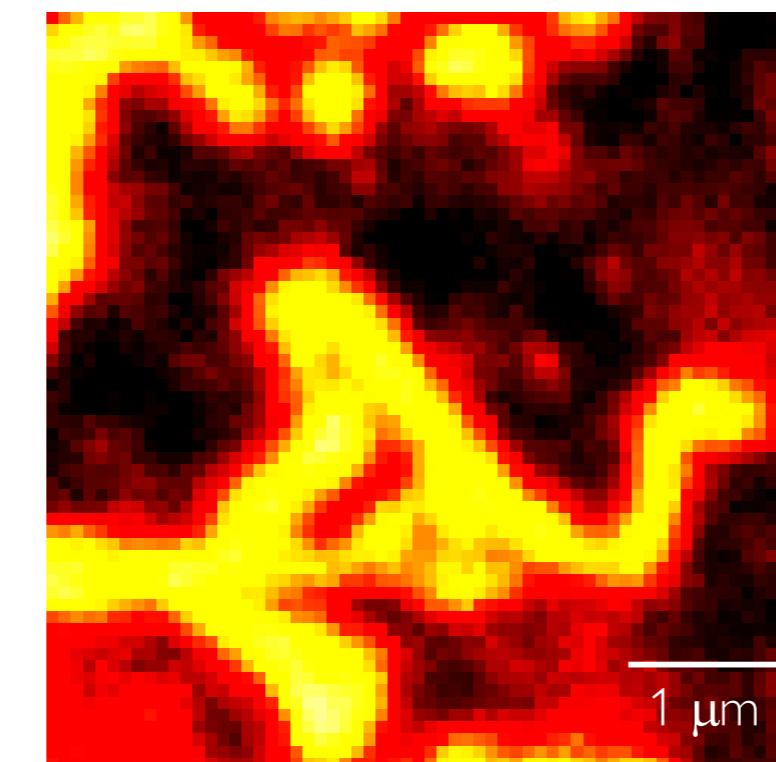
Excite fluorescence by laser light



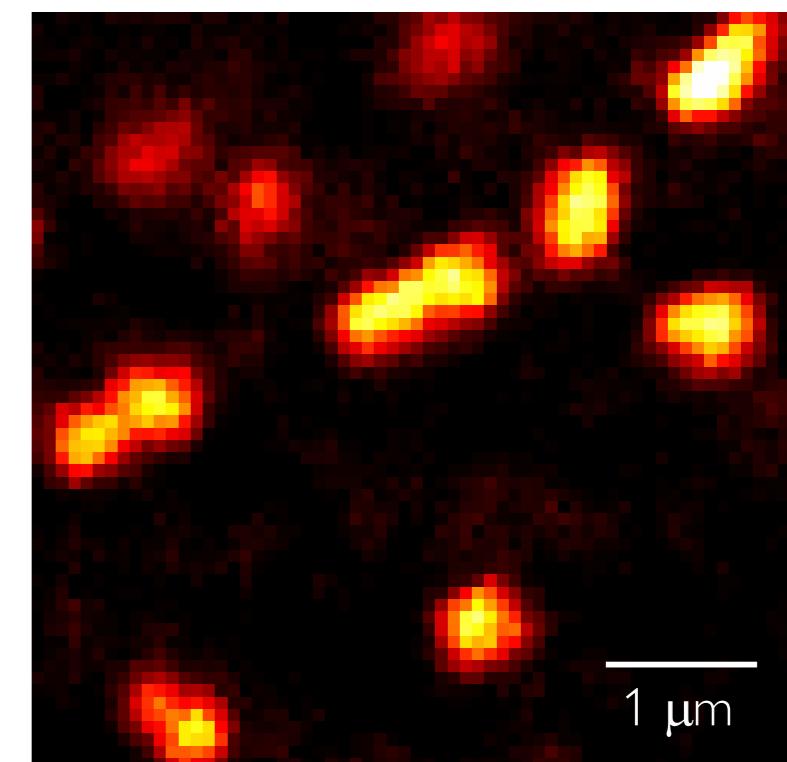
# Confocal microscope scheme



Synaptic vesicles



Mitochondria



Peroxisomes

## Biological and biomedical questions have multiple demands

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

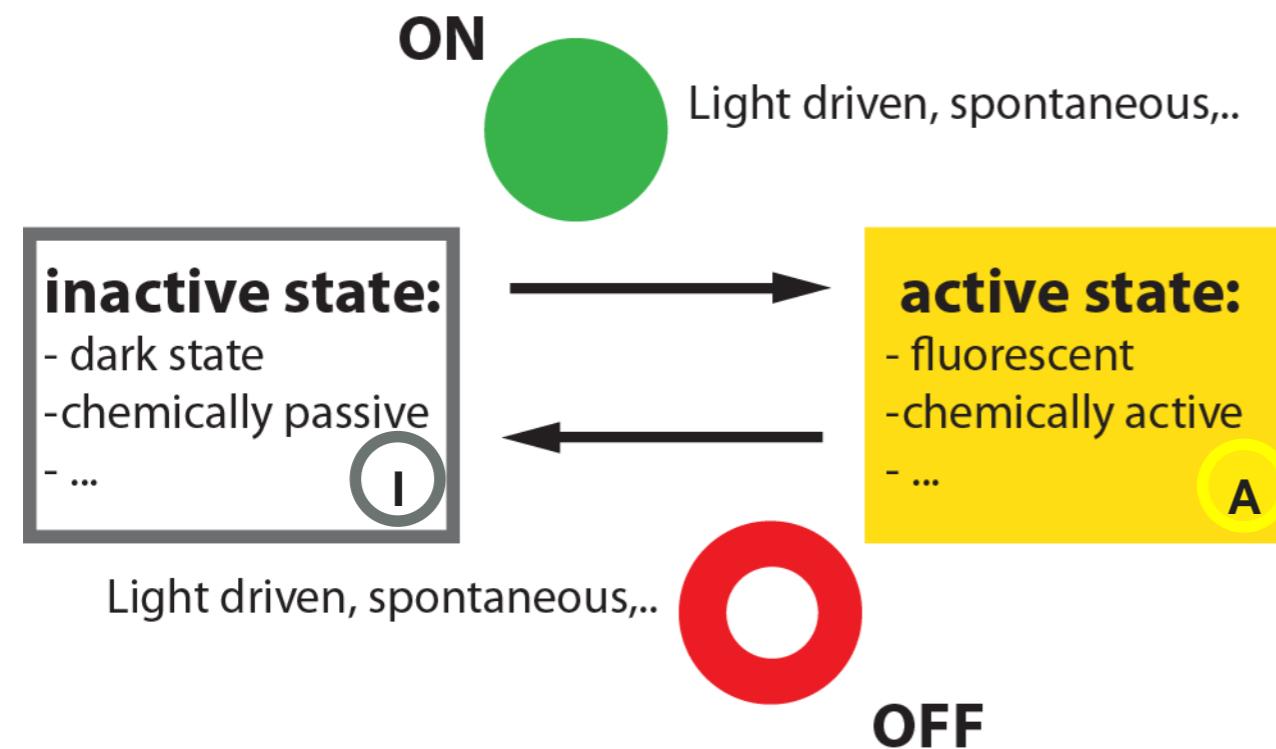
$$d = \frac{\lambda}{2n \sin \alpha}$$



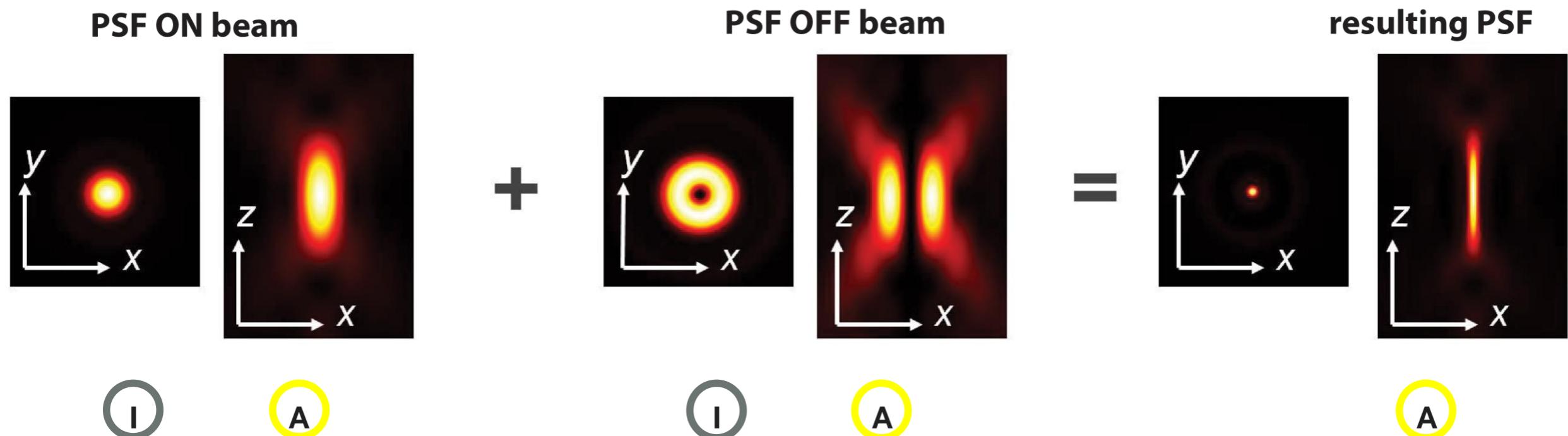
Ernst Abbe's memorial located in front of the physiology building of the University of Jena, Germany

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

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".

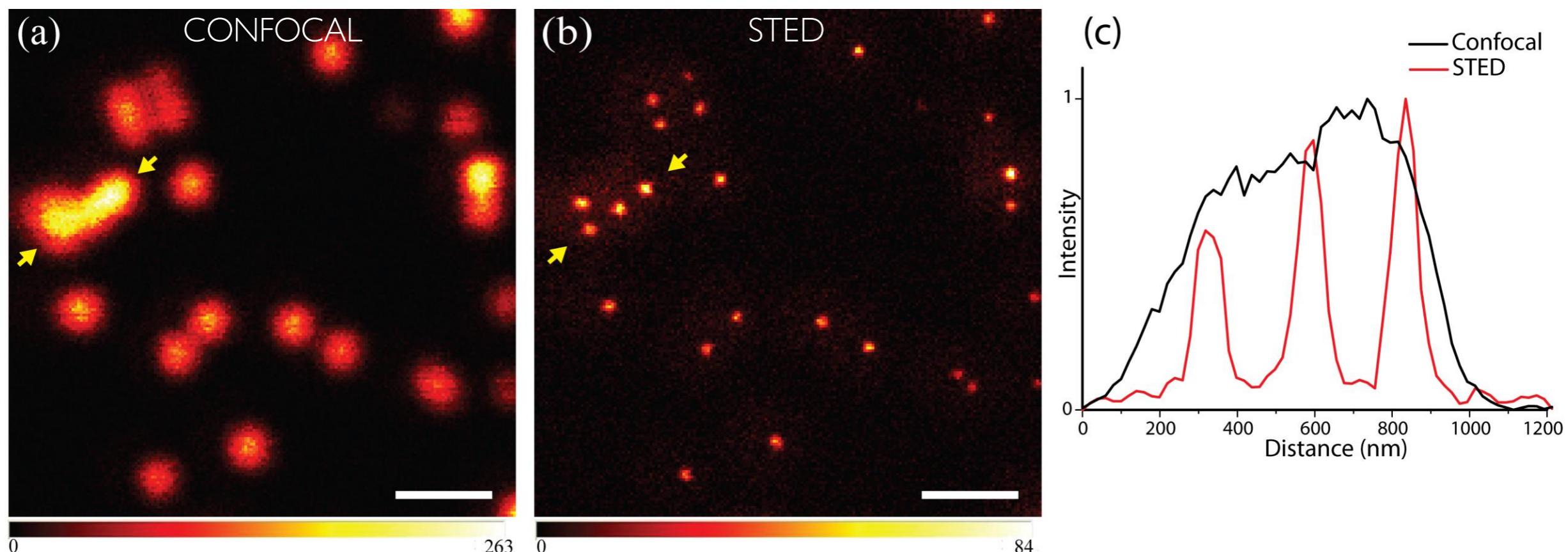


$$d_{STED} = \frac{\lambda}{2n \sin \alpha \sqrt{1 + A \frac{I_{\max}}{I_s}}}$$



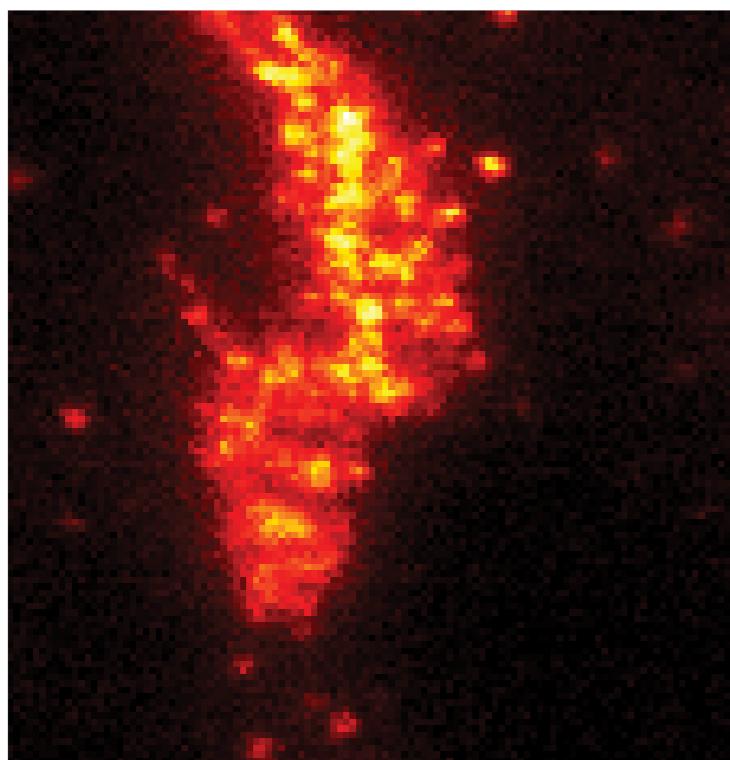
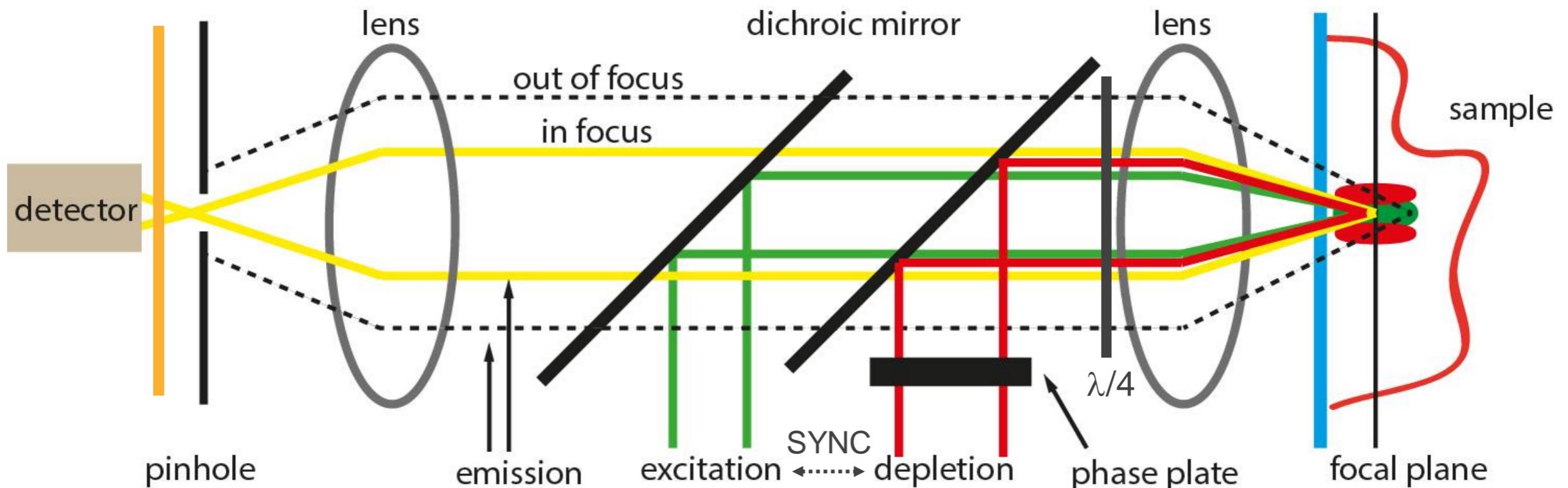
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner "for the development of super-resolved fluorescence microscopy".

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

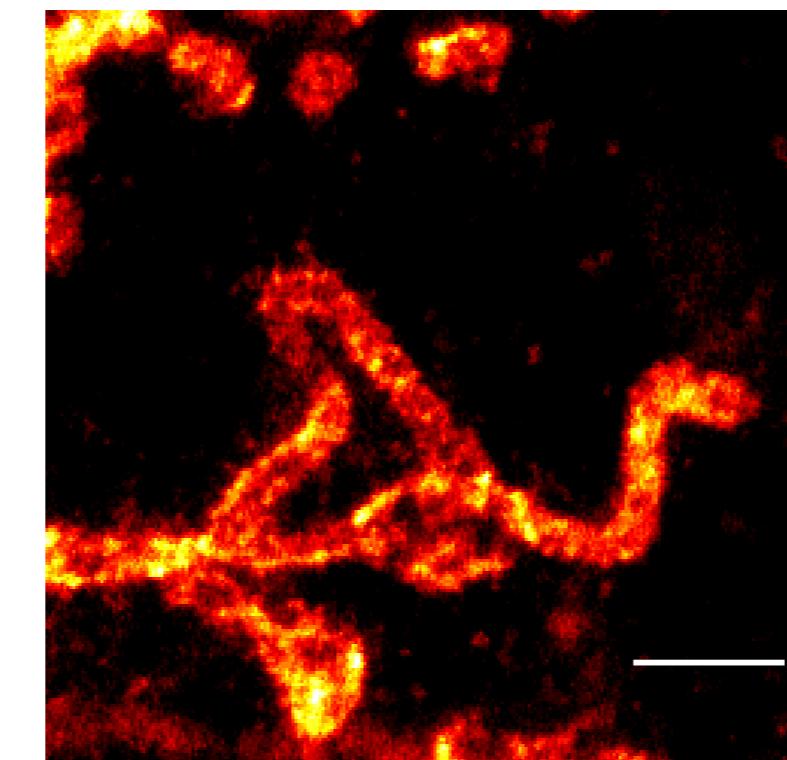


S. W. Hell , Toward fluorescence nanoscopy, Nature Biotechnol. 21,1347–1355 (2003)

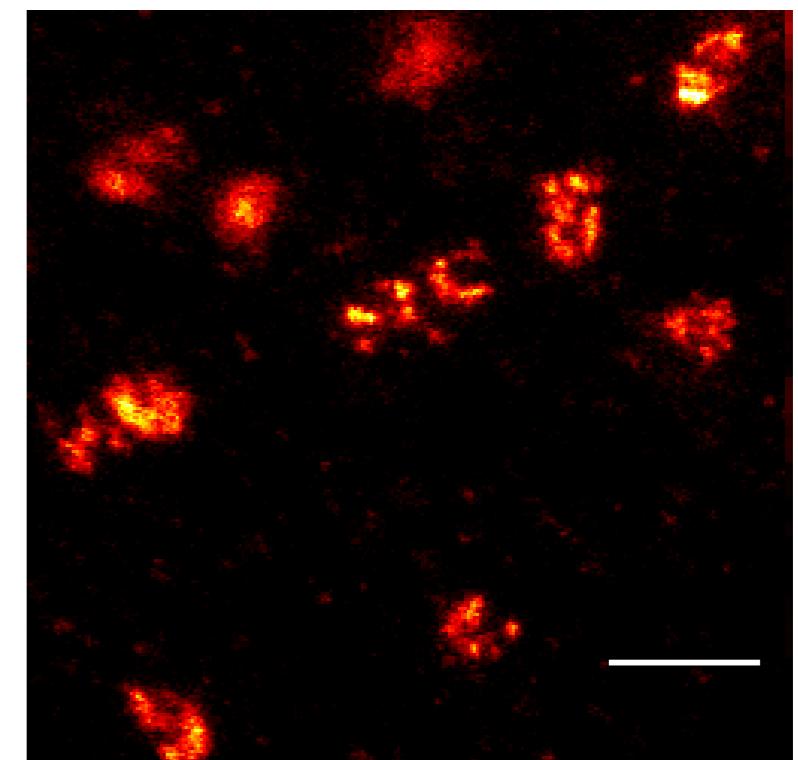
# STED microscope scheme



Synaptic vesicles

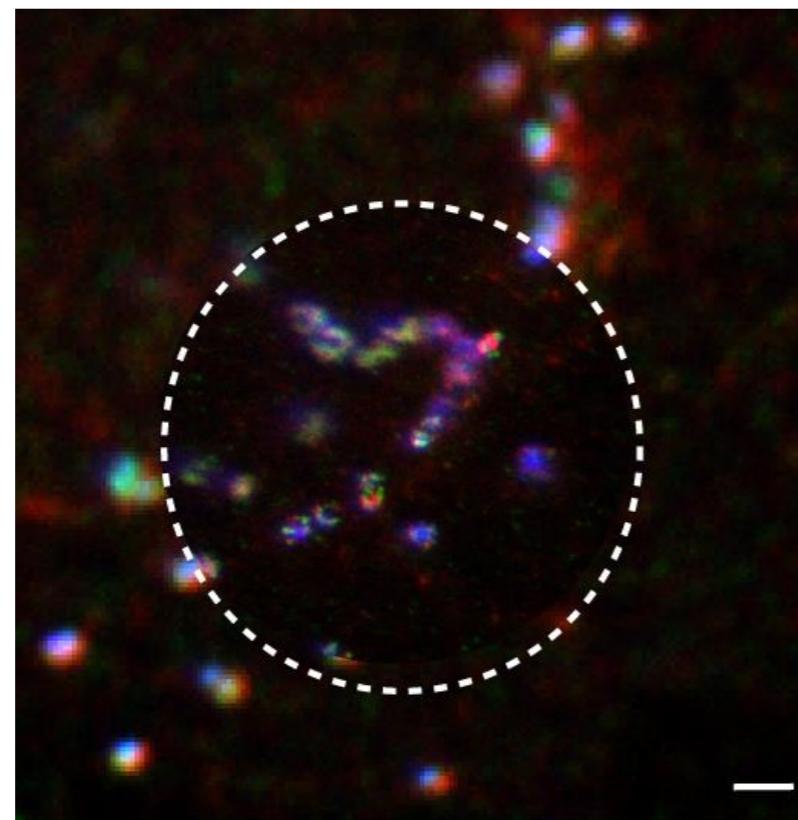


Mitochondria



Peroxisomes

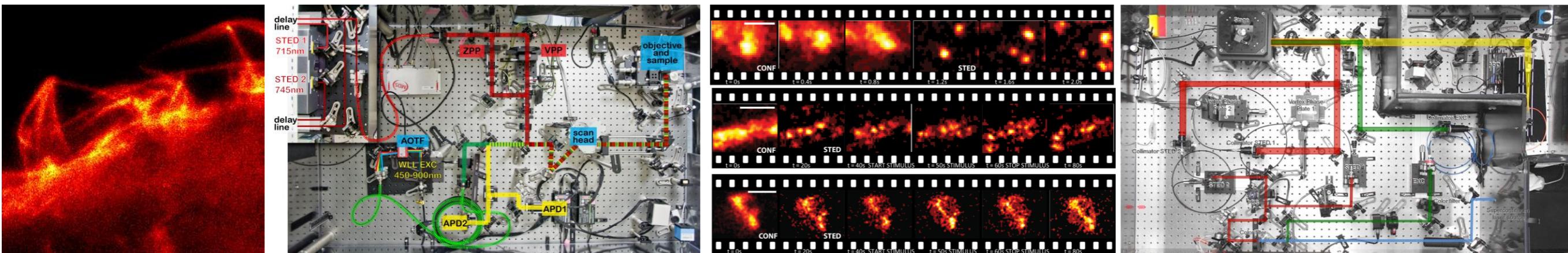
# A multicolour STED microscope designed for biological applications



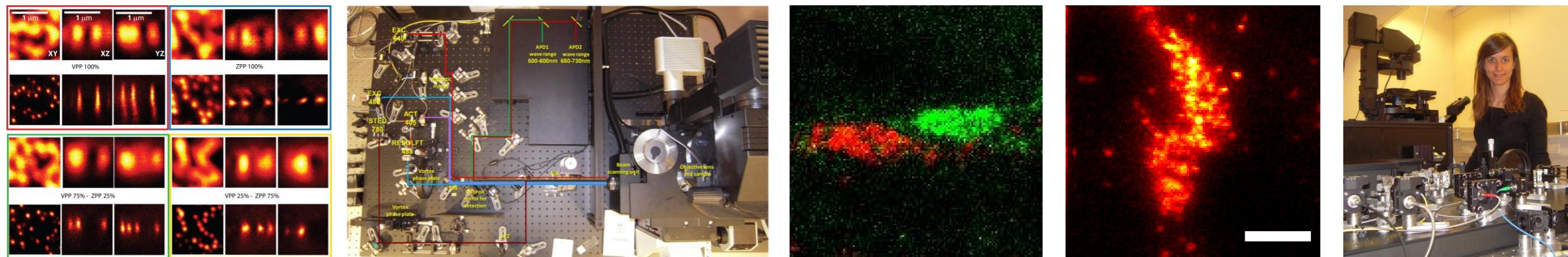
- ✓ High spatial resolution
- ✓ High temporal resolution
- ✓ Multicolour imaging
- ✓ 3D imaging
- ✓ Long acquisition time
- ✓ Data analysis

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)

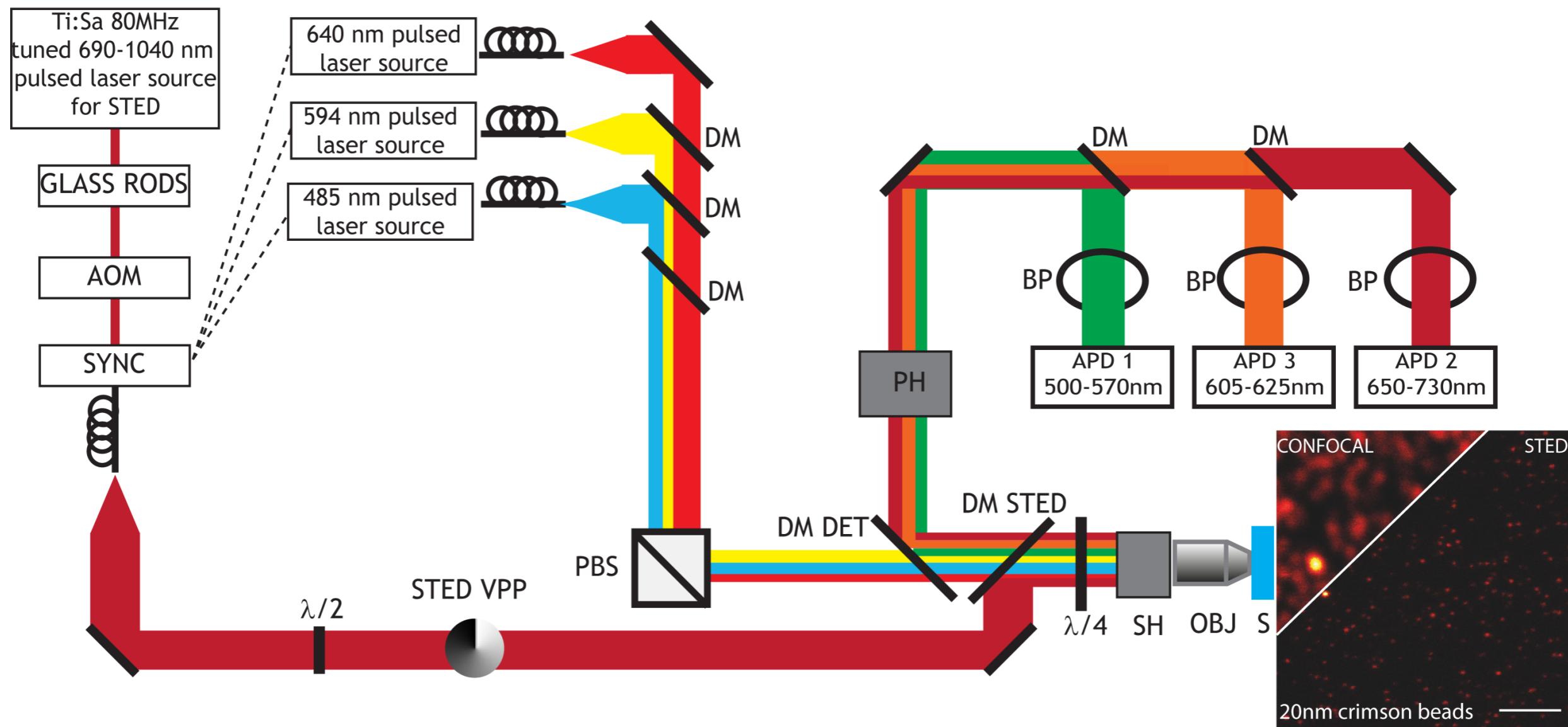
Galiani S / Clausen M / de la Serna Bernardino J, Fritzsch M, Chojnacki J, Lagerholm B C, Eggeling C, Pathways to optical STED microscopy, Nanobioimaging, vol 1, pp. 1–12 (2013)



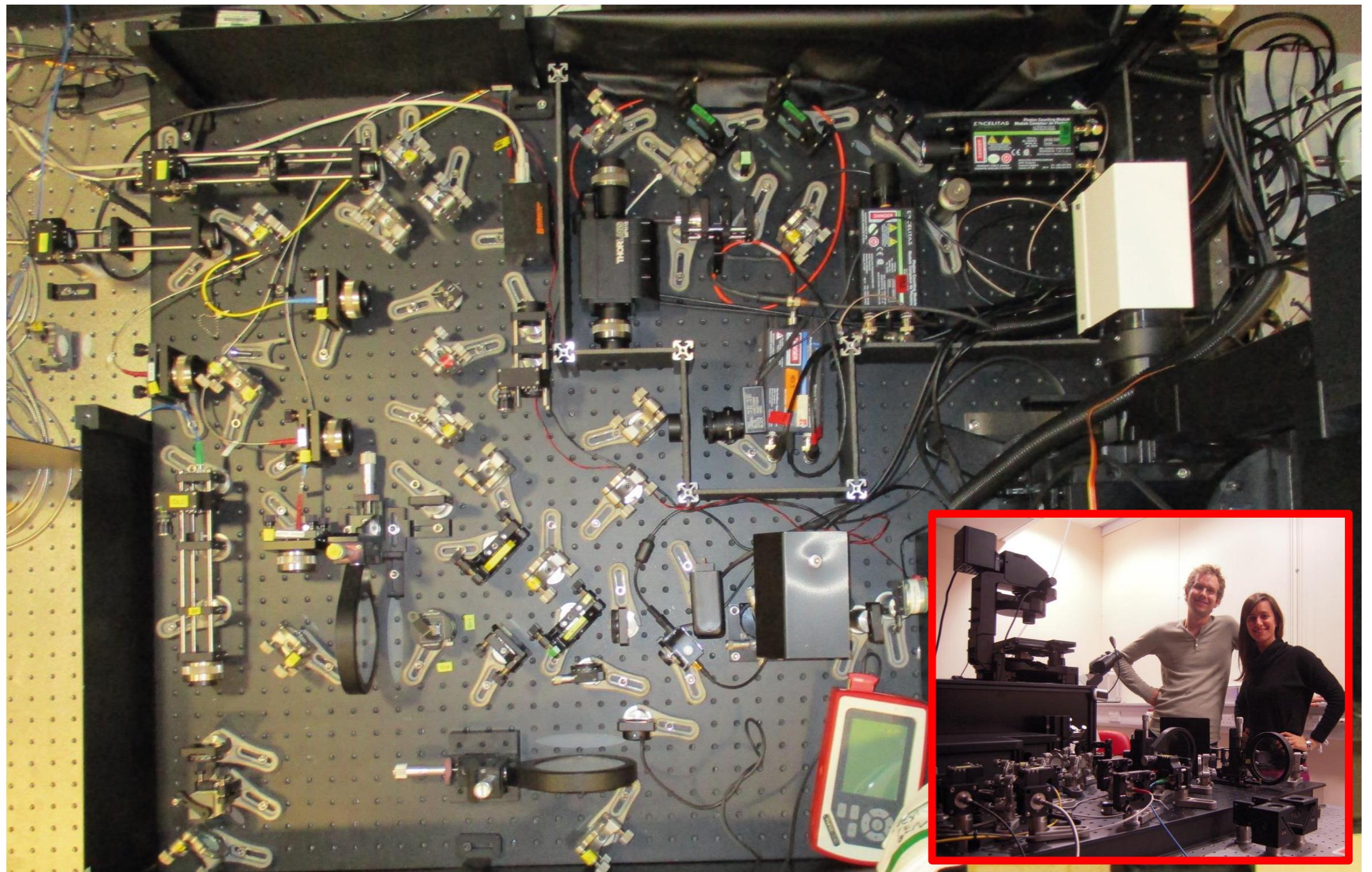
## A multicolour STED microscope designed for biological applications

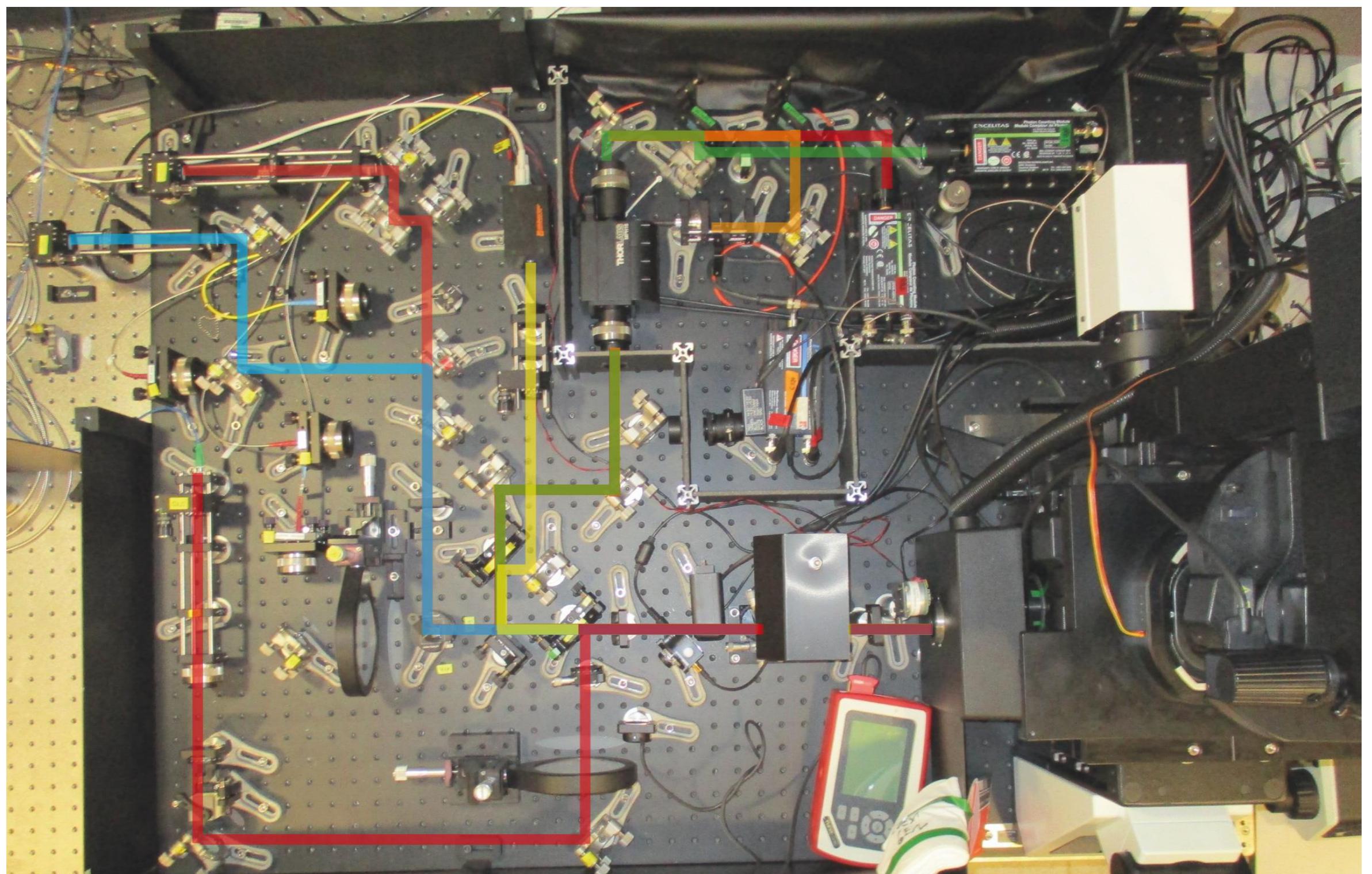


A

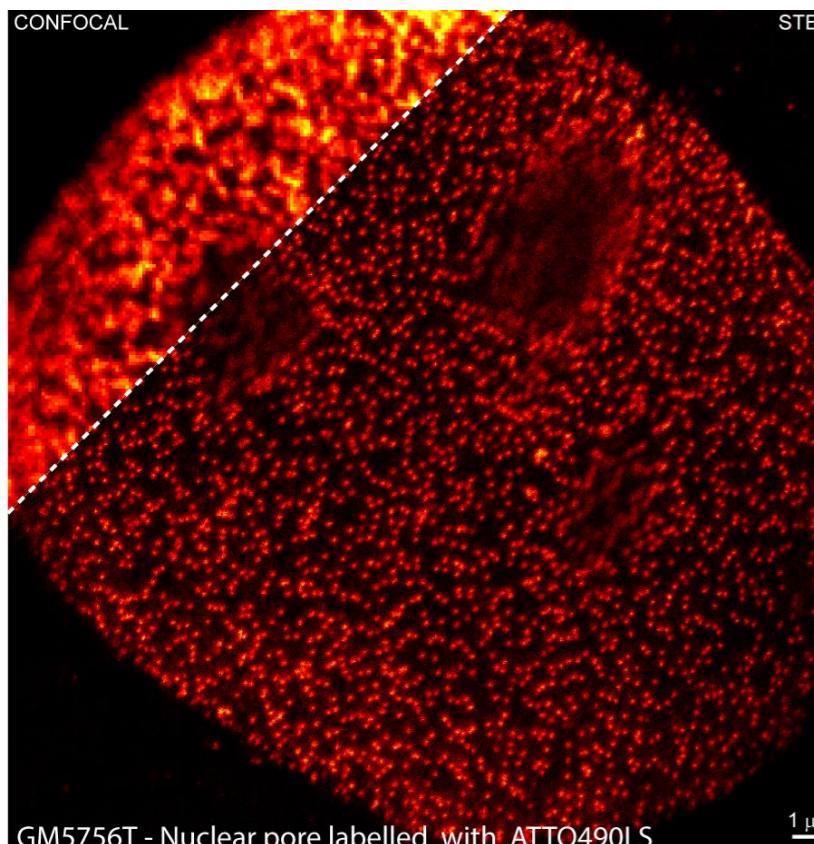


# Custom made multicolour STED architecture





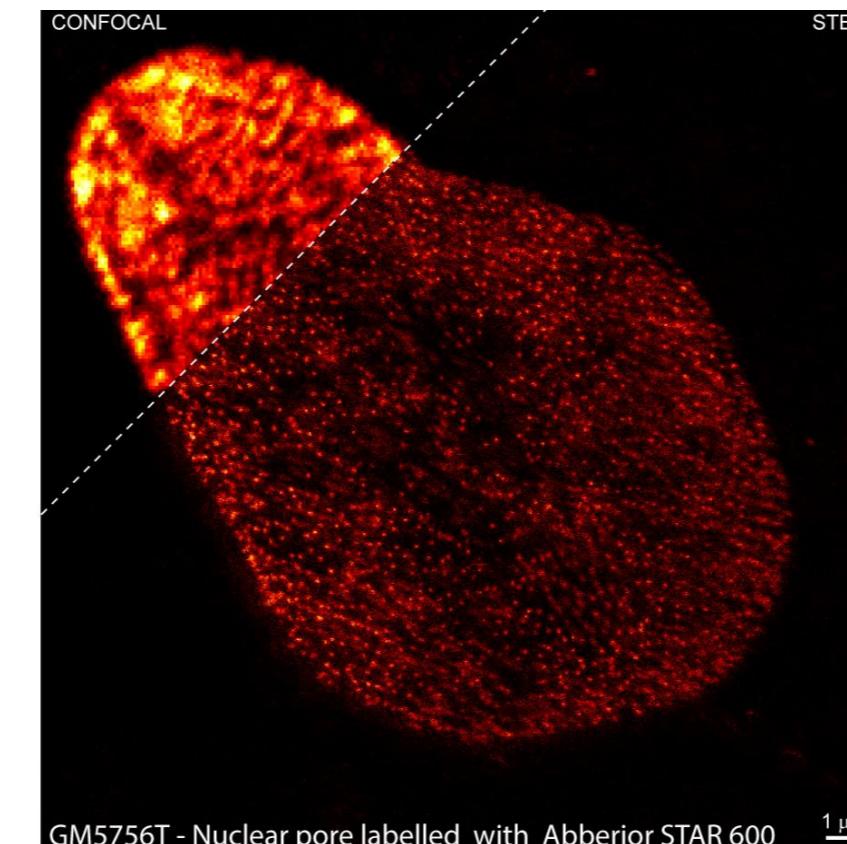
ATTO 490LS



GM5756T - Nuclear pore labelled with ATTO490L

**EXC 485nm  
STED 755nm  
APD2 650-730nm**

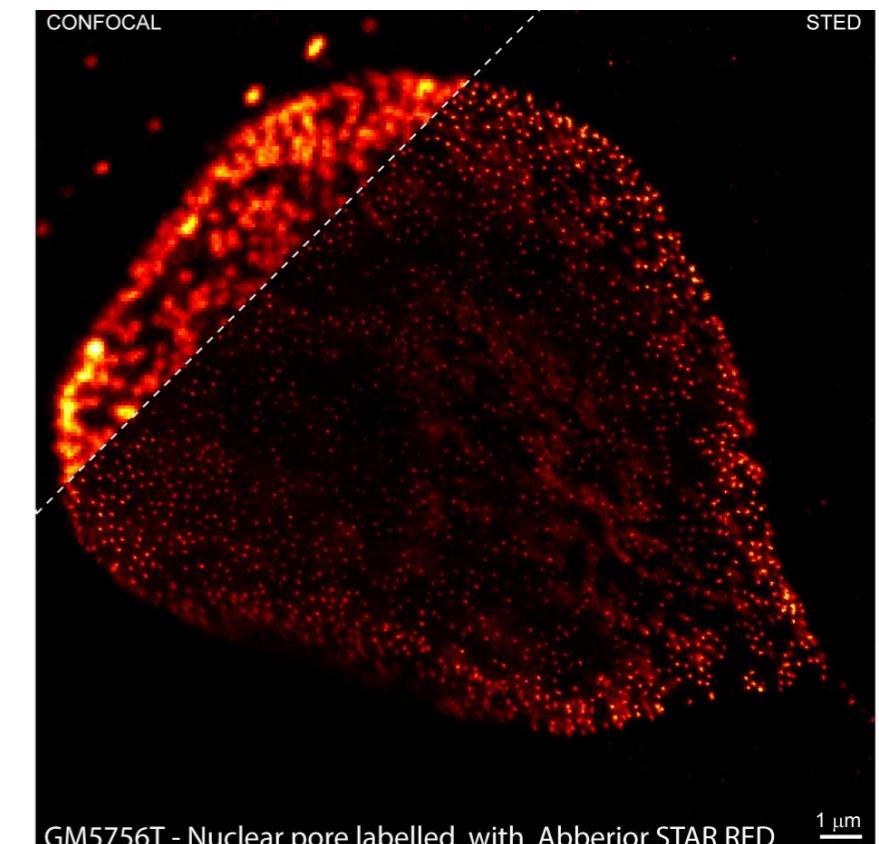
Abberior STAR 600



GM5756T - Nuclear pore labelled with Abberior STAR 600

EXC 594nm  
STED 755nm  
APD3 605-625nm

Abberior STAR RED



GM5756T - Nuclear pore labelled with Abberior STAR RED 1 μm

**EXC 640nm  
STED 755nm  
APD2 650-730nm**

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

**The microscope needs to be aligned:**

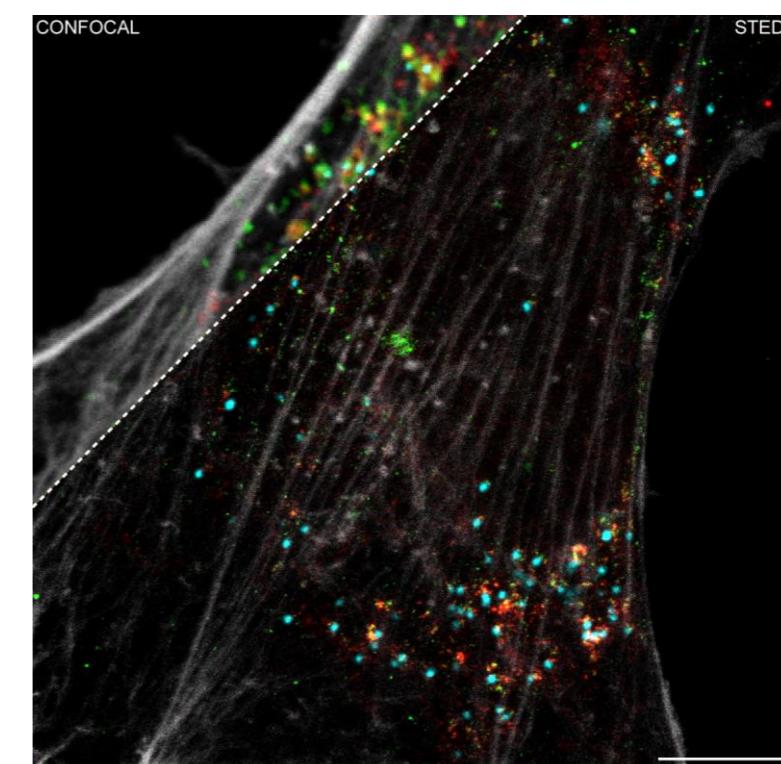
1. Spatial alignment
2. Temporal alignment
3. Polarization check

**The imaging needs to be controlled**

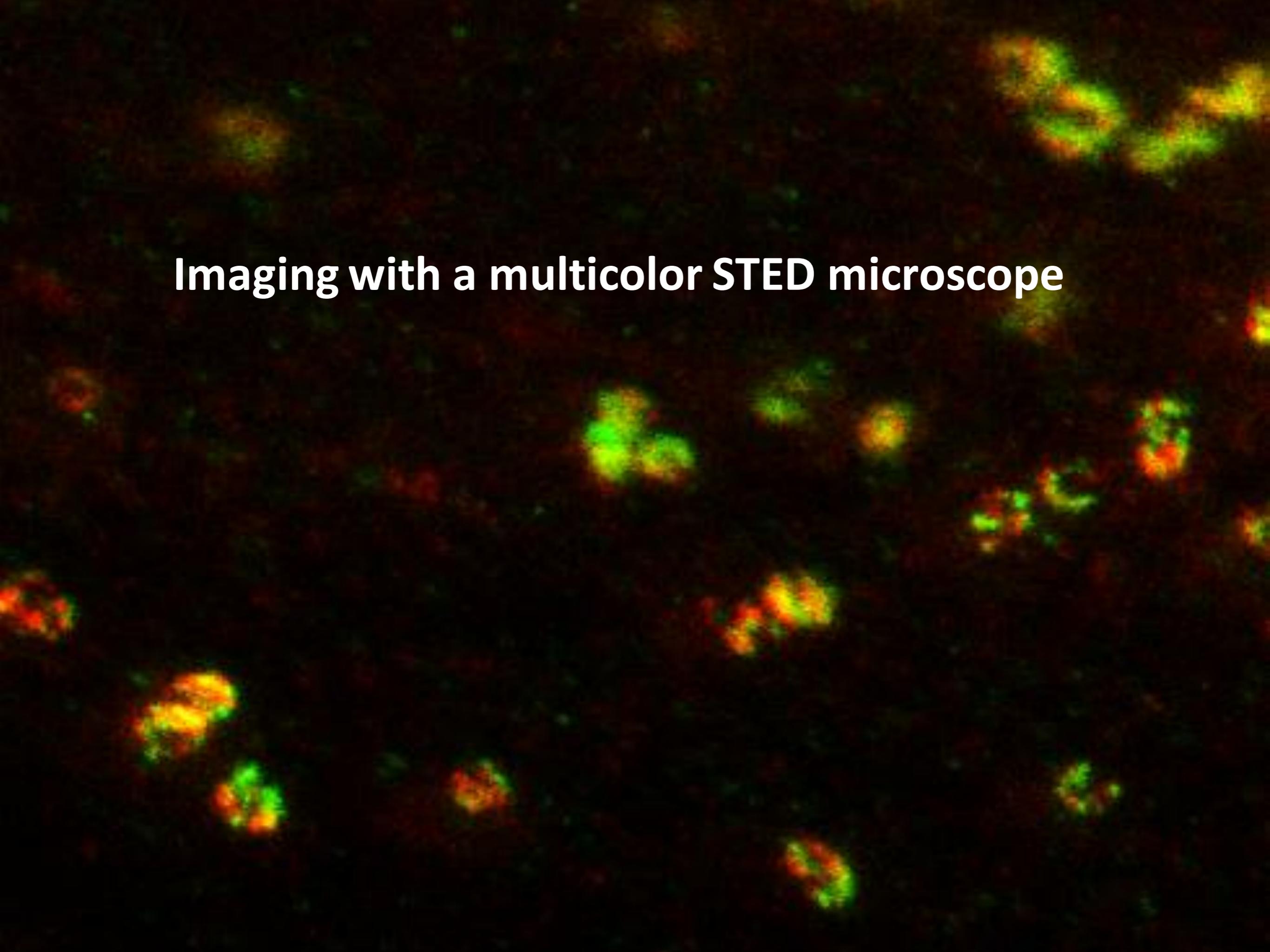
- ✓ Chromatic aberration → when the STED beam is on this system is chromatic aberration free
- ✓ Crosstalk → crosstalk negligible between APD1 and APD2 or APD3: acquisition in parallel  
→ crosstalk between APD2 and APD3: acquisition in series
- ✓ Spatial drift → due to the acquisition in series a green dye is always introduced to correct for possible spatial drift

**The immunostaining protocol has been optimized**Sample preparation:

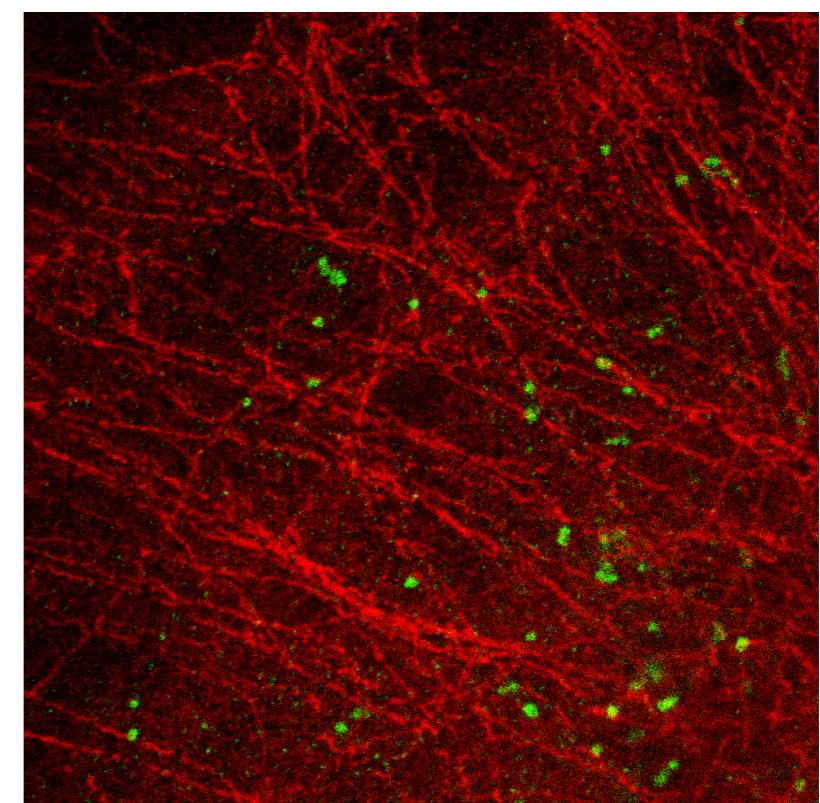
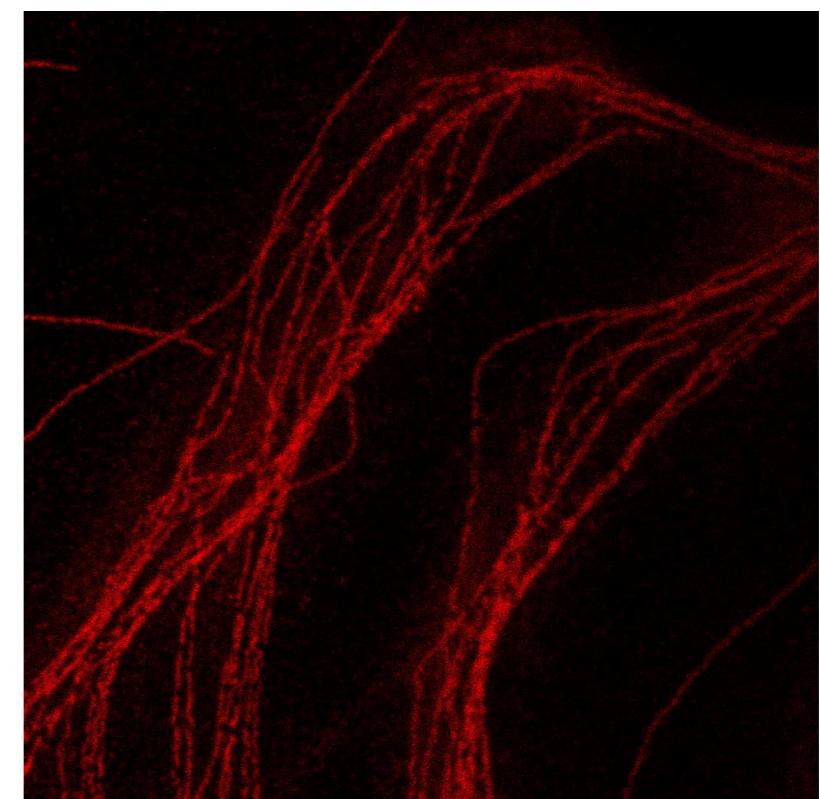
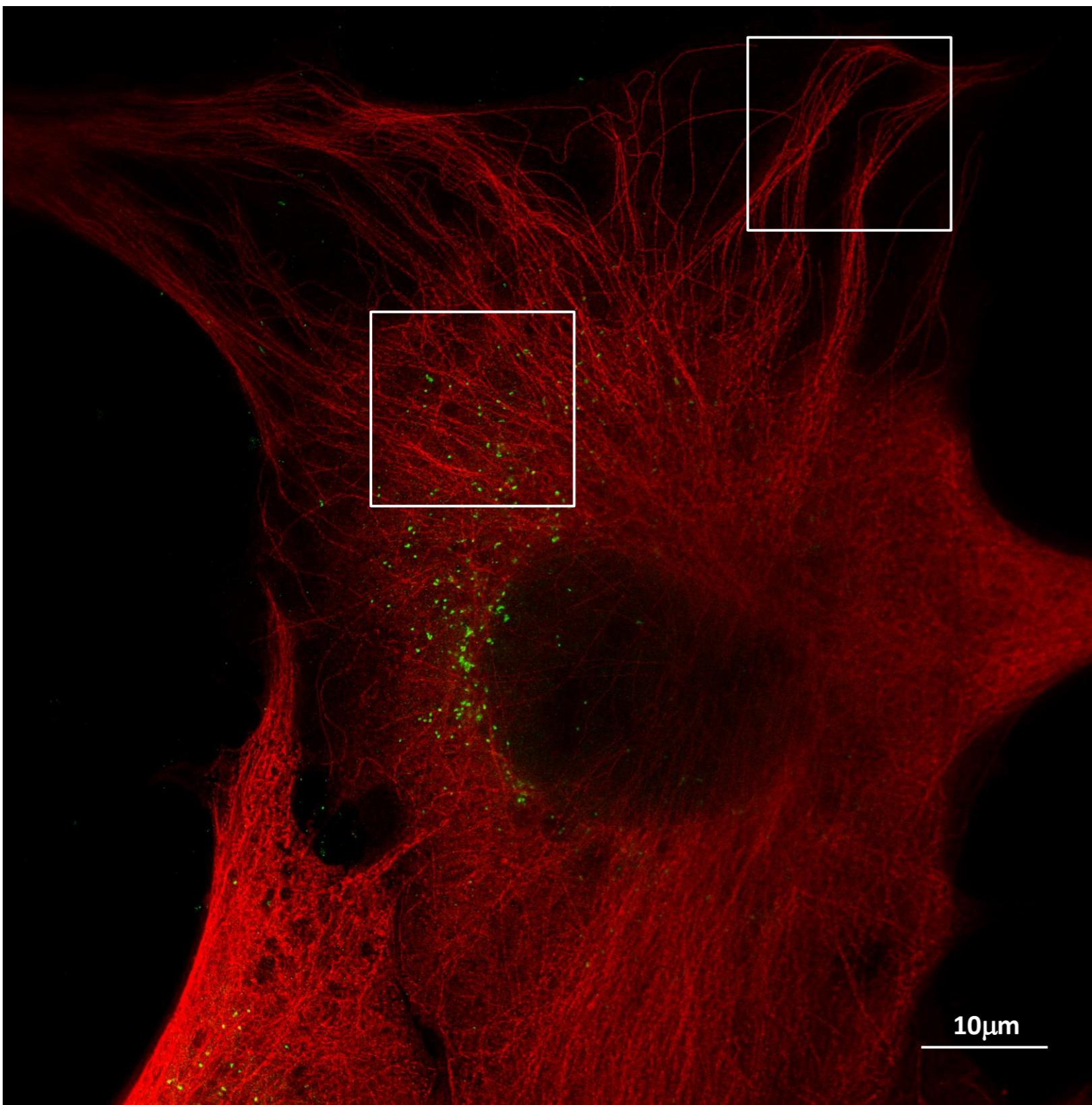
- ✓ Fixation: 3% PFA in PBS
- ✓ Permeabilization: pure Methanol
- ✓ Blocking: 2%BSA+5% FCS in PBS
- ✓ Dilution I AB: 1:400
- ✓ Dilution II AB: between 1:125 and 1:250



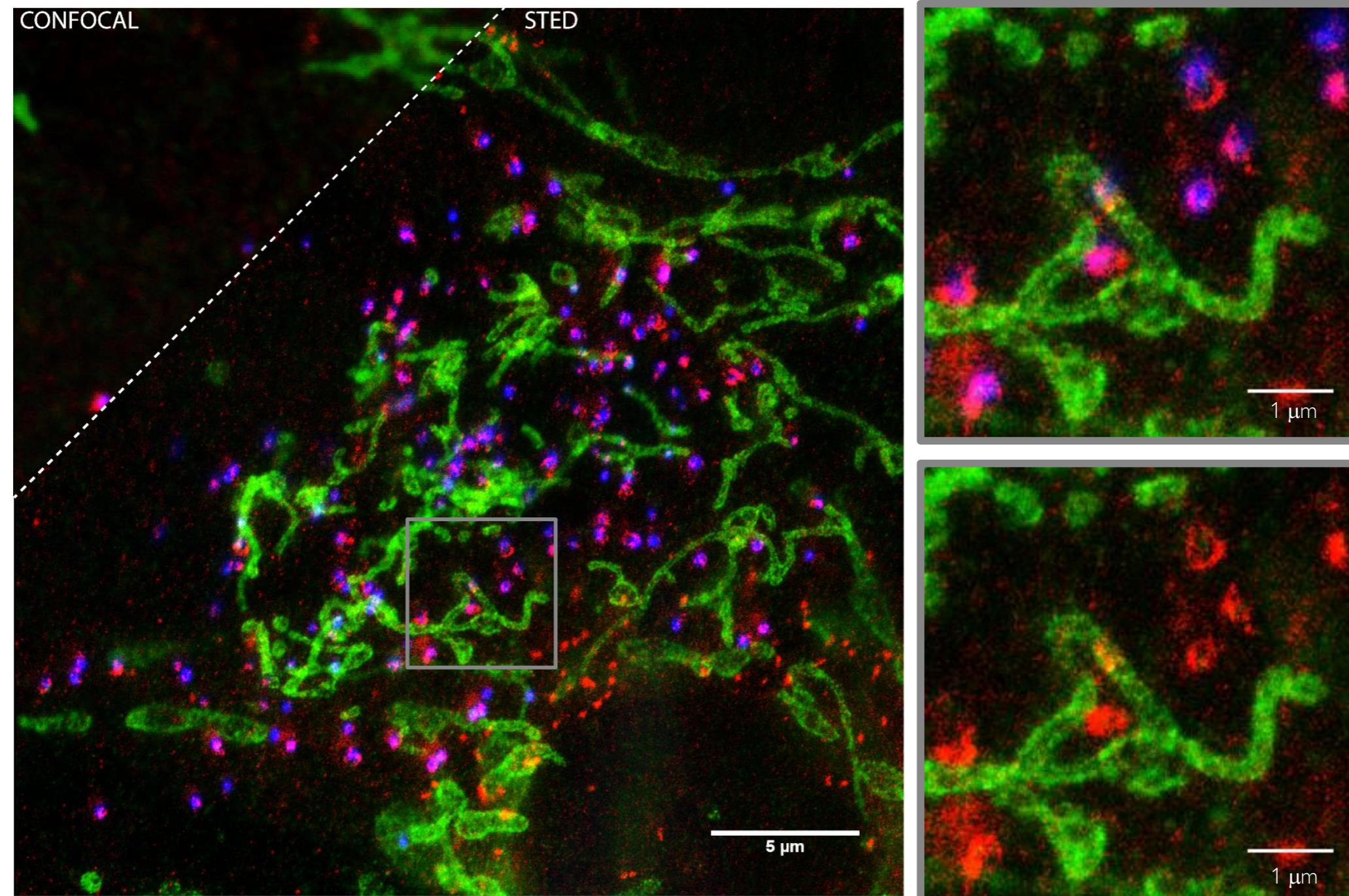
# Imaging with a multicolor STED microscope



# Some examples

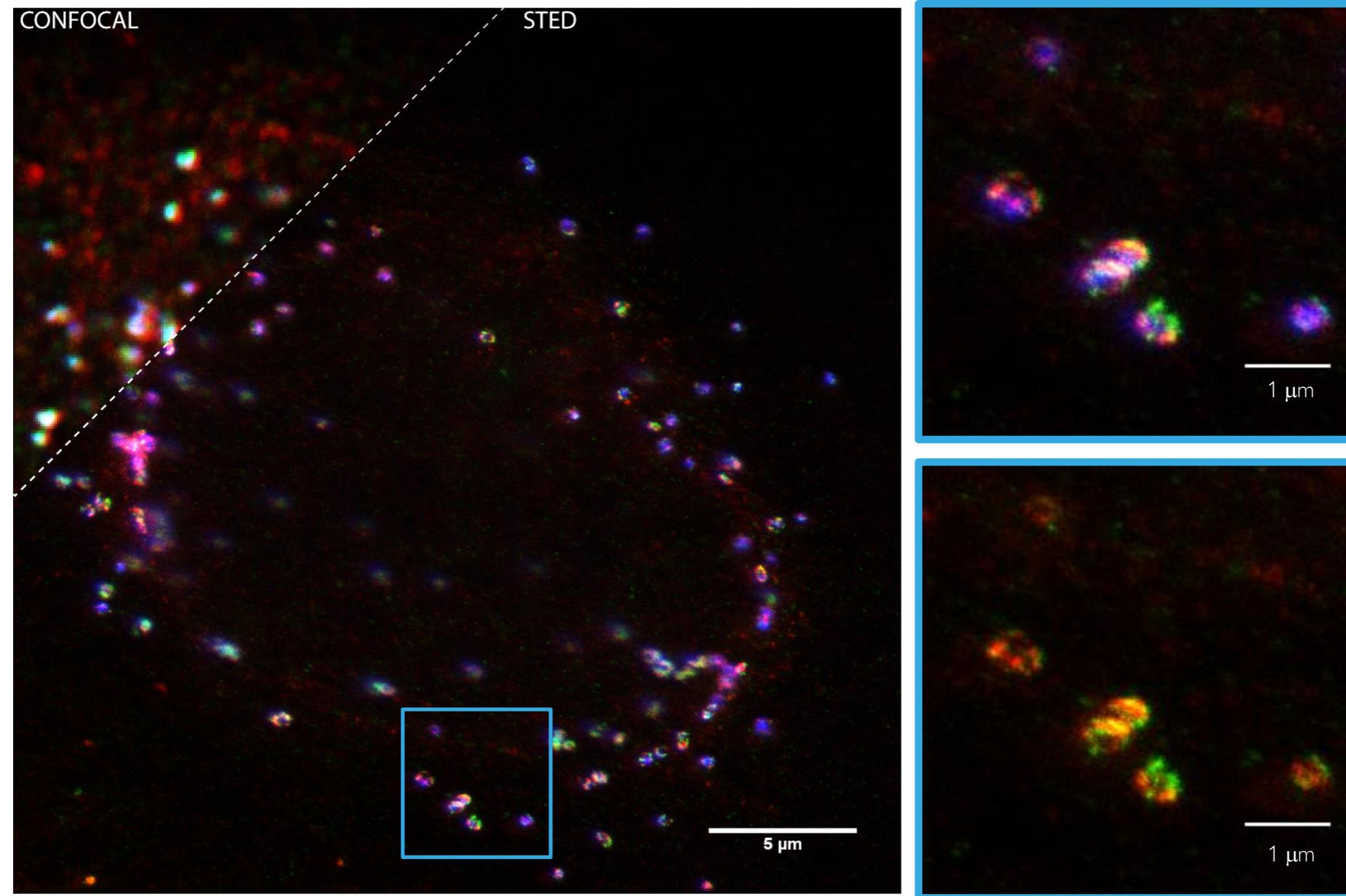


## Protein co-localization of mitochondrial import receptor subunit (Tom20) and import receptors (Pex5) respect to the peroxisomal matrix marker

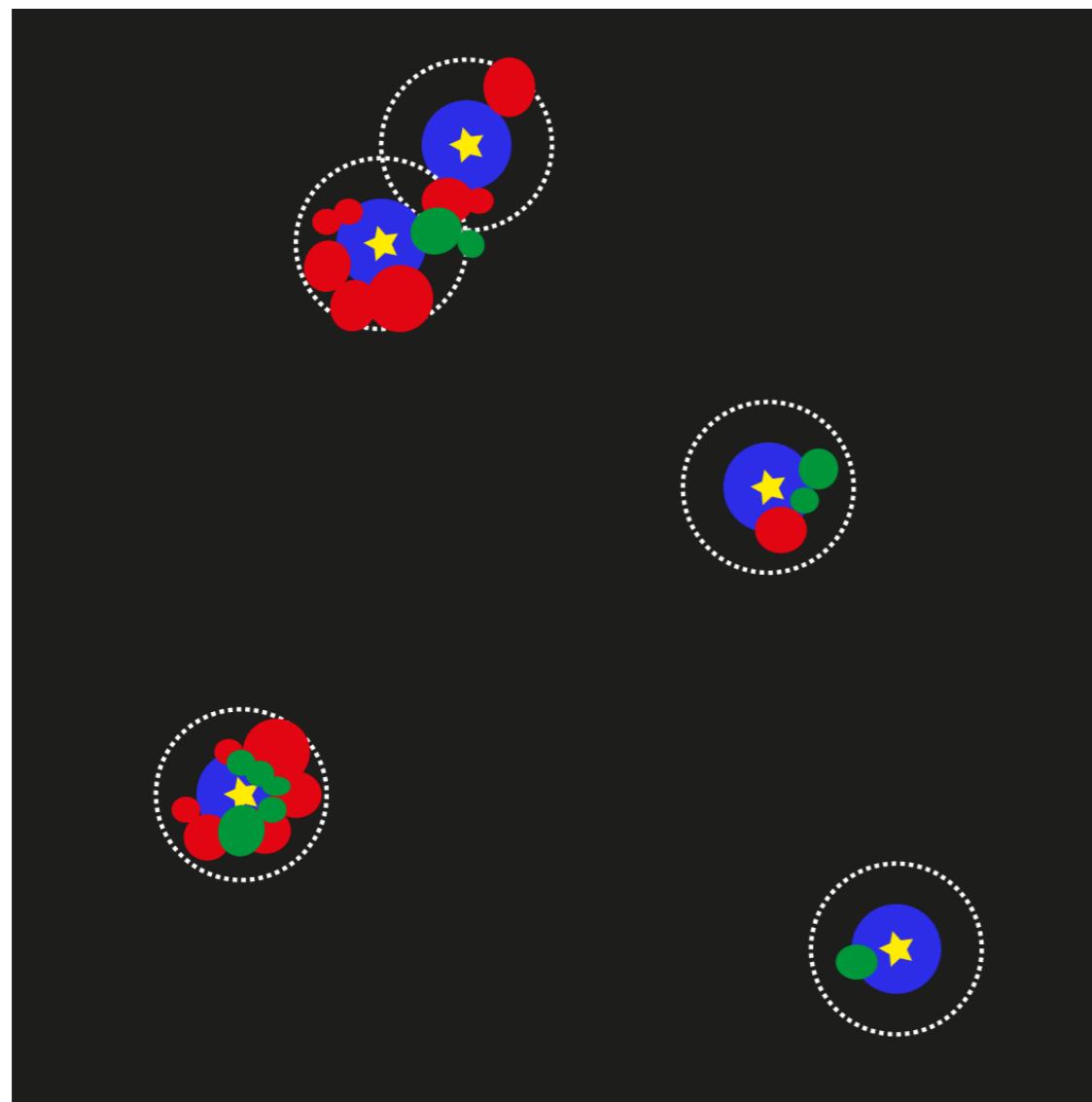


GM5756T   PTS1 labelled with GFP  
Pex5 labelled with Abberior\*600  
Tom20 with KK114

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



GM575T   PTS1 labelled with GFP  
Pex14 labelled with Abberior\*600  
Pex5 with KK114

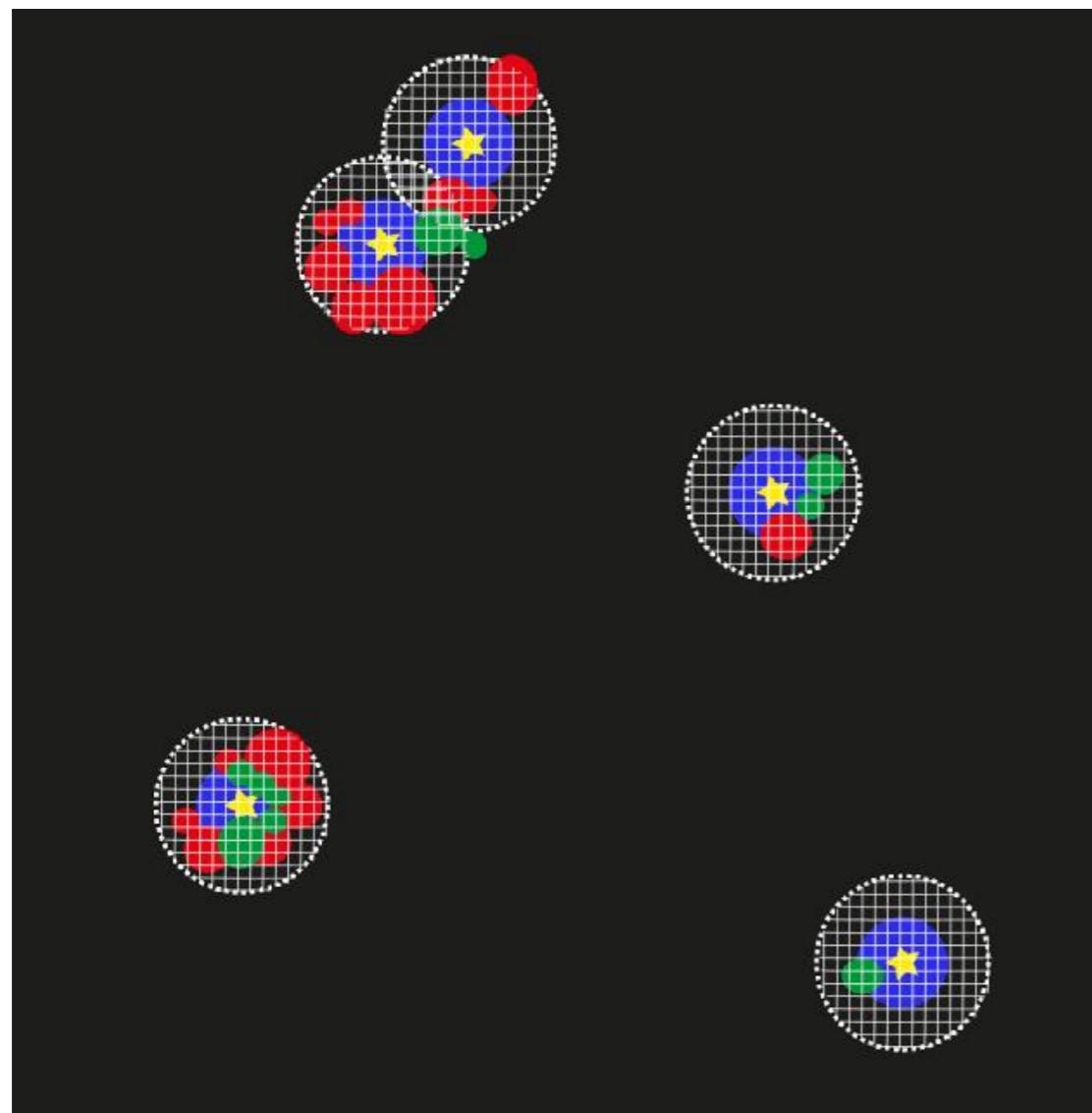


1 - Find coordinates of the peroxisomes using the PTS1-GFP signal

2 - Measure intensity in each circular patch (radius 200 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



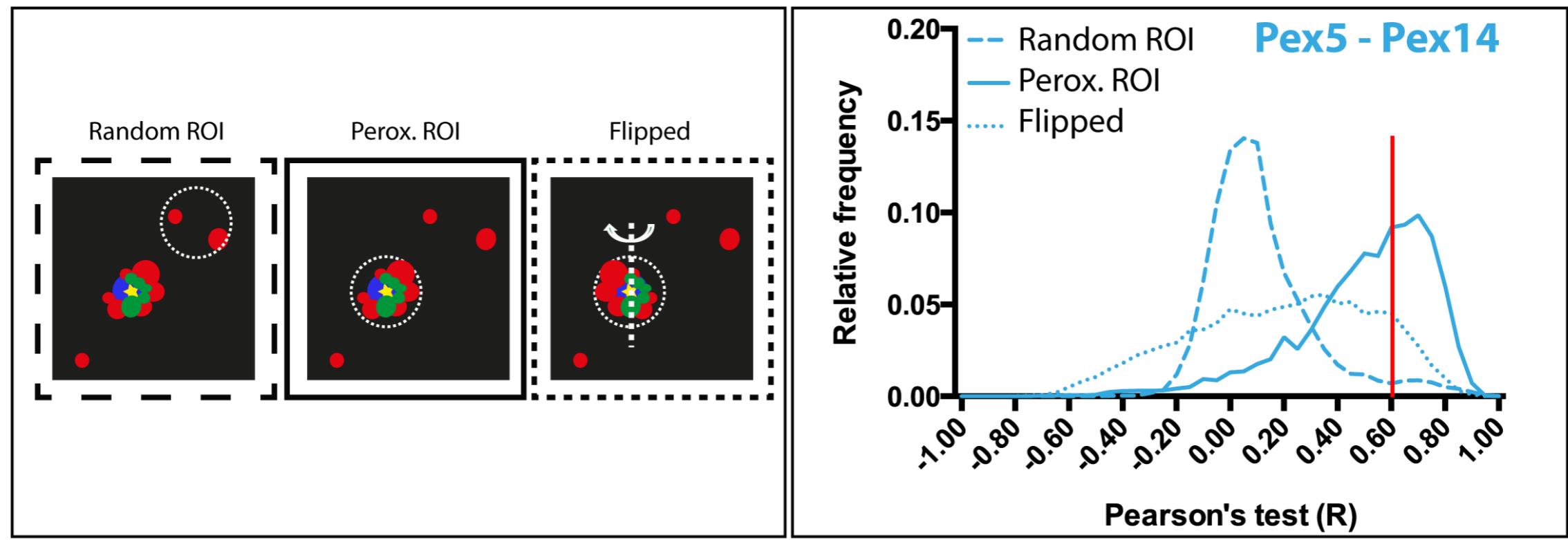
1 - Find coordinates of the peroxisomes using the PTS1-GFP signal

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

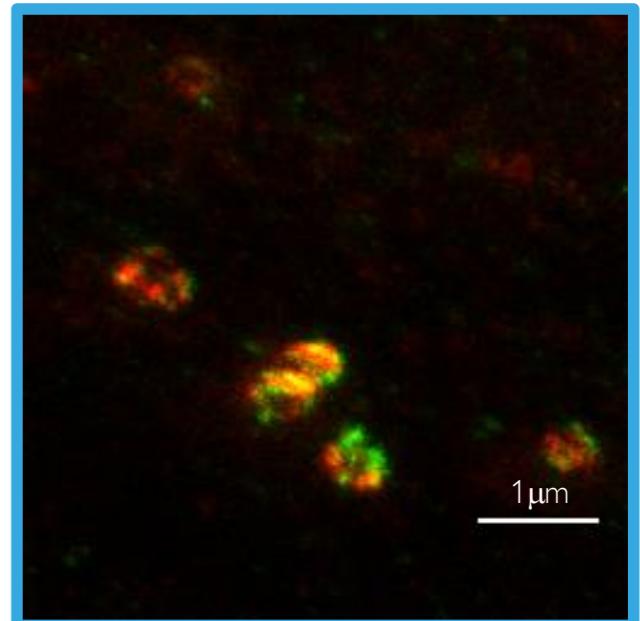
Images statistic: at least 10 images on at least 3 different samples have been collected per each condition

# Colocalization Analysis



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

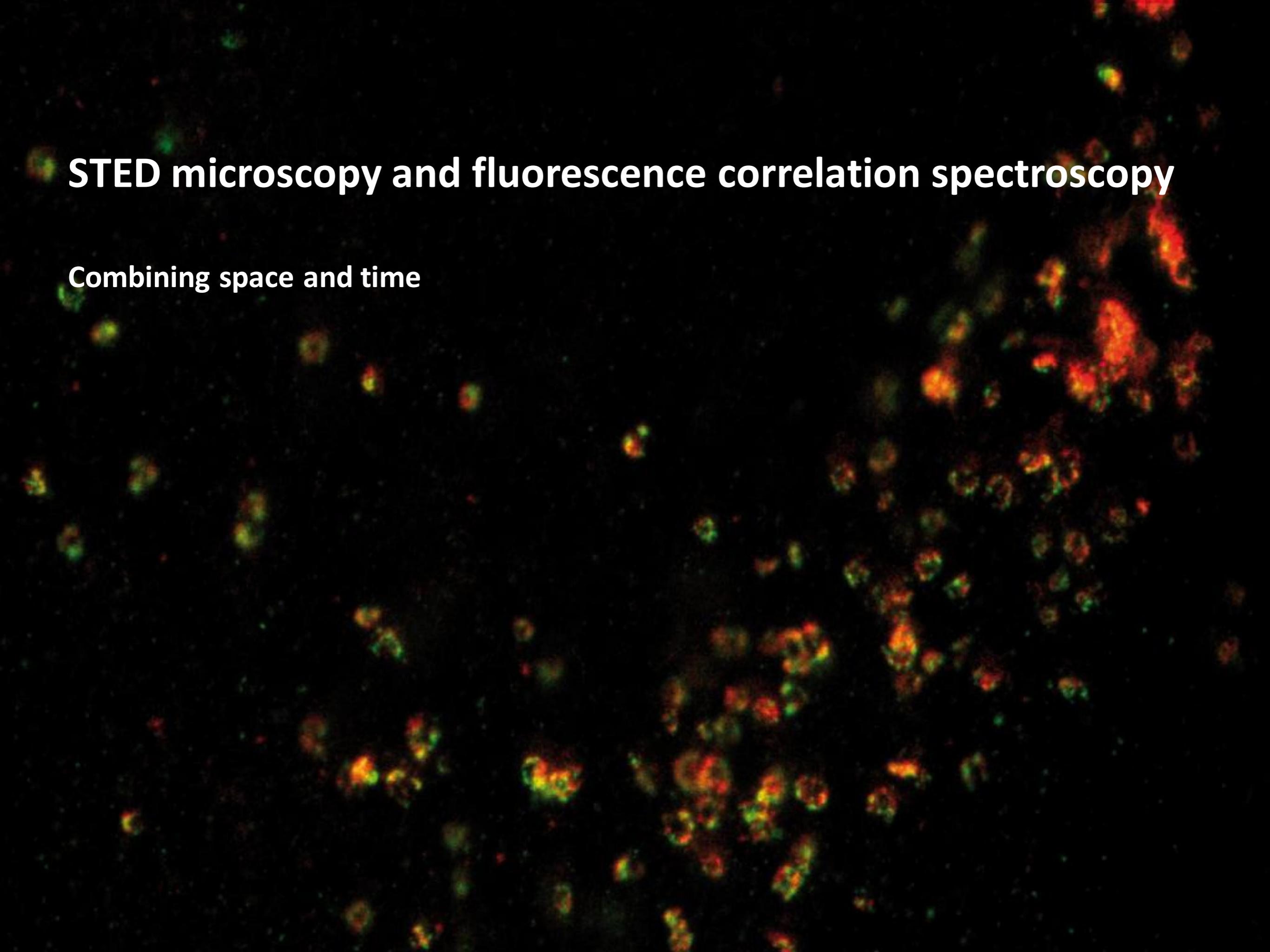
- PEX5-PEX14 show high colocalization
- Co-localisation does not occur through random.
- Flipped control: Specific correlation at peroxisomes

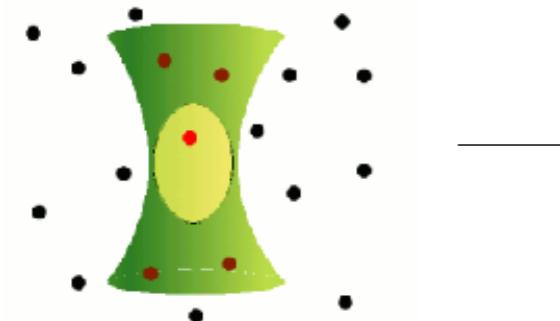


PEX5 – PEX14

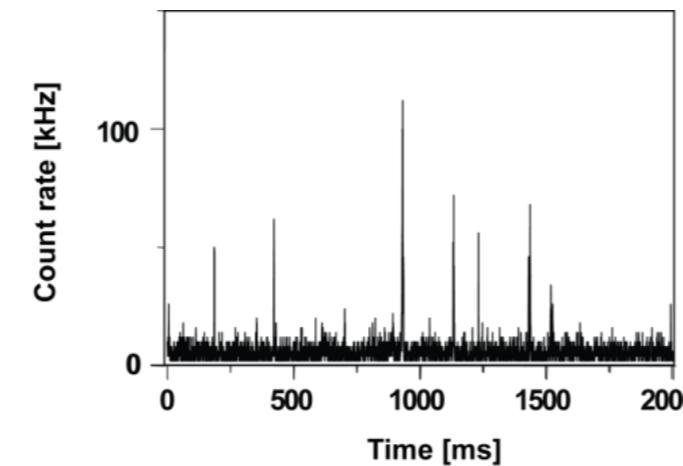
# STED microscopy and fluorescence correlation spectroscopy

Combining space and time





Point FCS  
Time scale  $\approx 0.1\mu\text{s} - 1\text{s}$



FCS has the temporal resolution to investigate fast molecular diffusion and highlight transient interactions in molecular assemblies

Determine average transit times of labeled molecules through observation area

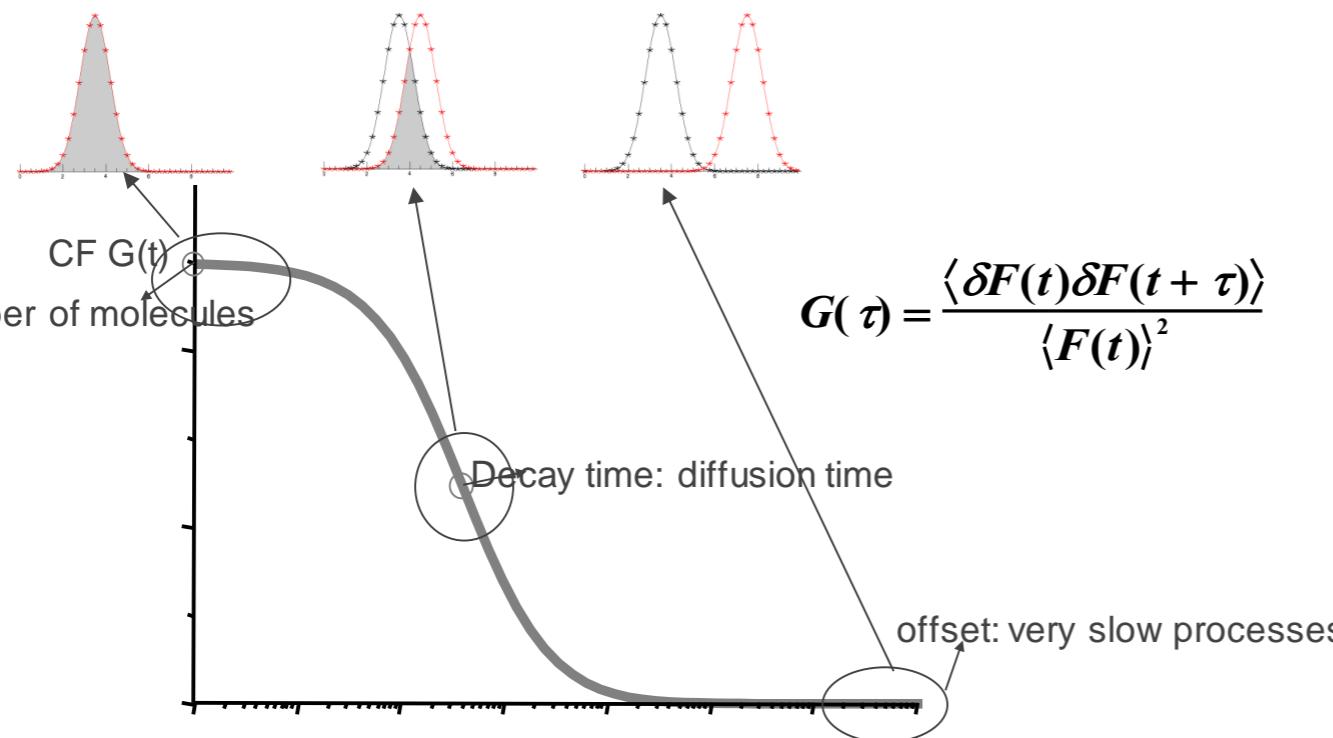
Molecular mobility

- The correlation function

$t=0$

$t=t_D$

$t=\infty$



Diffusion coefficient:

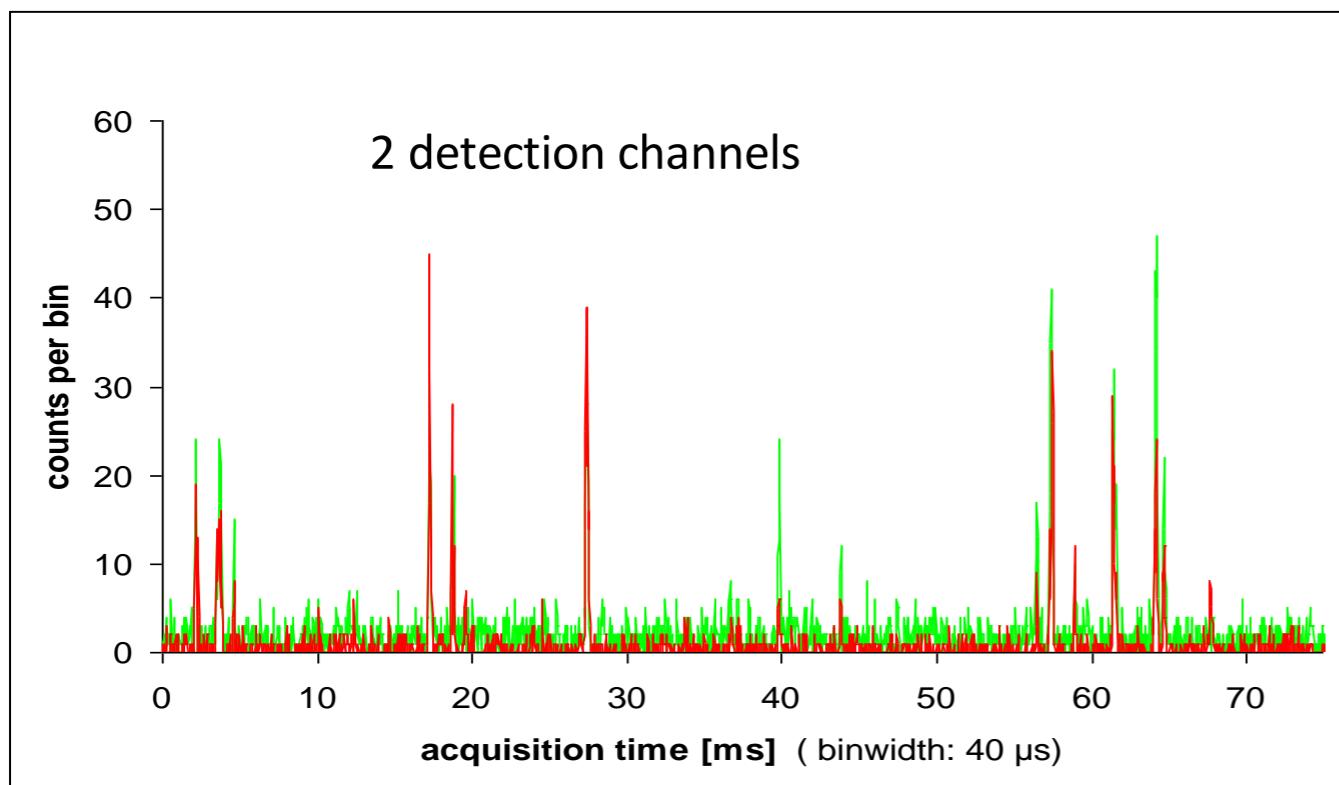
$$D = \frac{w^2}{2}$$

$$4t_d \\ ,_i$$

molecular diffusion coefficient (D)

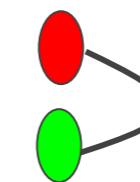
=

Molecular mobility



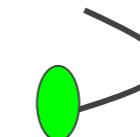
Coincidence/cross correlation

A + B



no coincidence/cross correlation

A



B

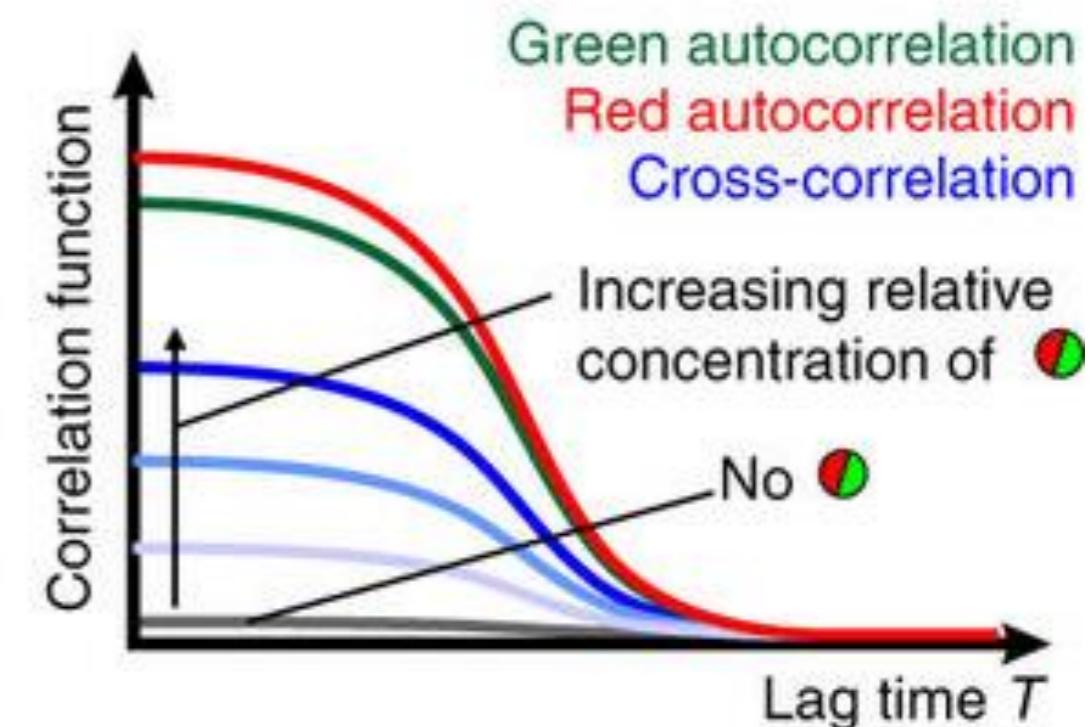


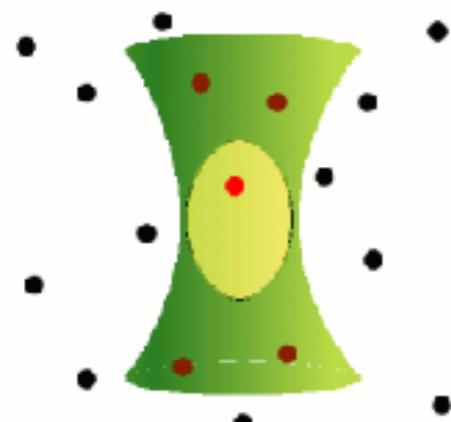
Cross-Correlation uses spectrally separable fluorophores to probe for interaction

$$G_{cc}(\tau) = \frac{\langle \delta I_{Ch1}(t) \delta I_{Ch2}(t + \tau) \rangle}{\langle I_{Ch1}(t) I_{Ch2}(t) \rangle} + 1$$

Discover interaction/diffusion dynamics!!!

Cross Correlation Curve amplitude directly relates to interaction



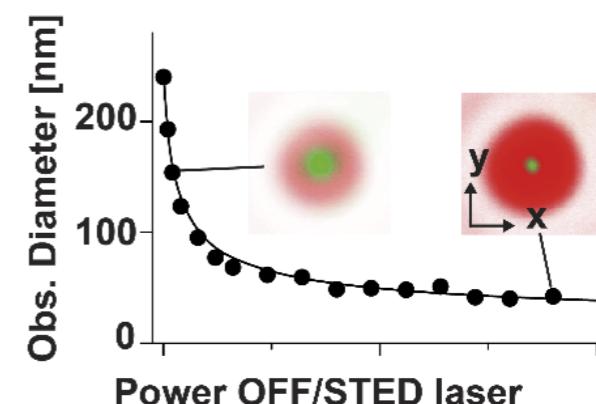


**Fluorescence Correlation Spectroscopy (FCS)**  
**Average transit time through observation spot**

**STED-FCS**

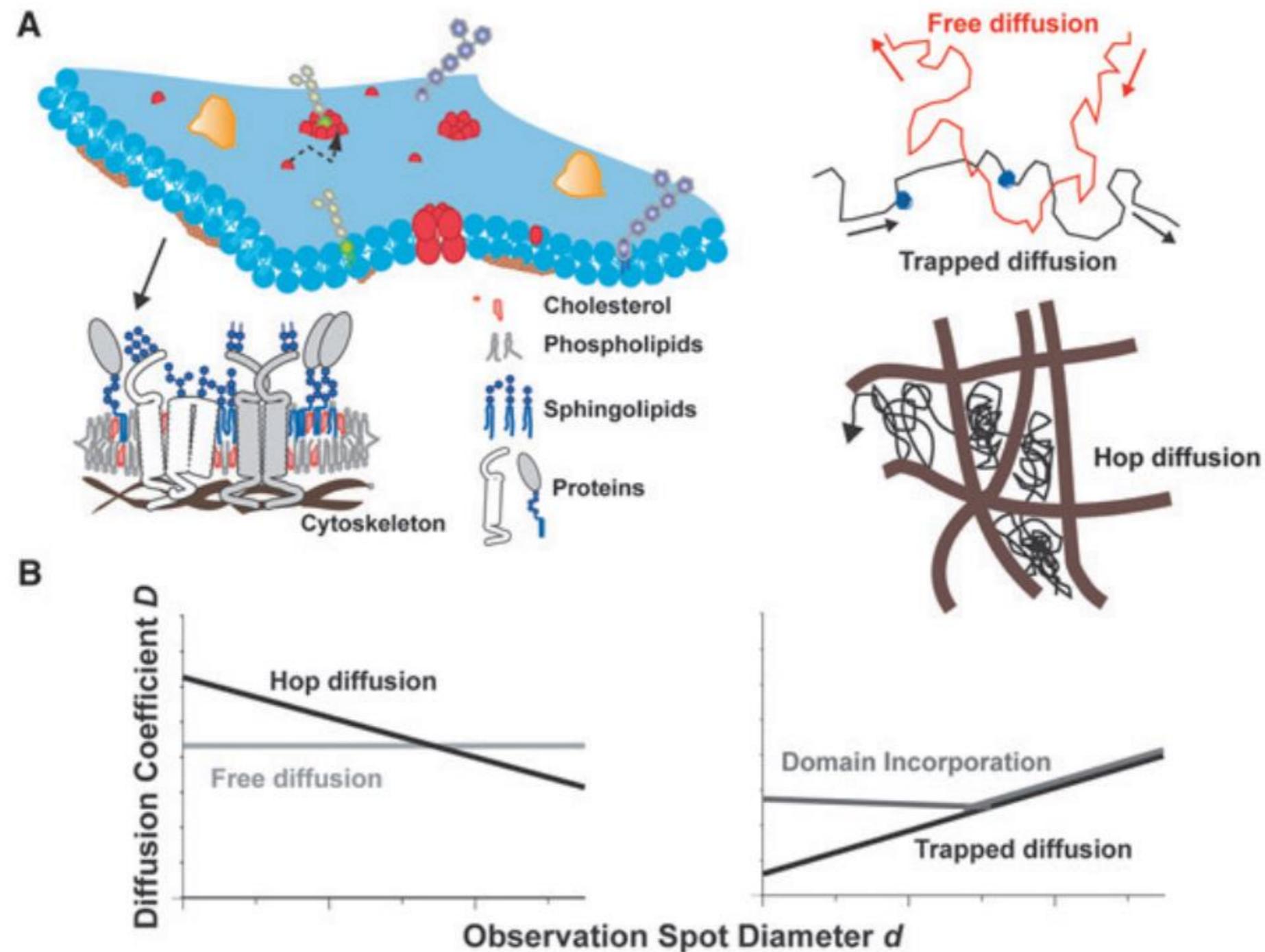
**Transit Time vs. Spot Diameter**

Diffusion modes/interactions

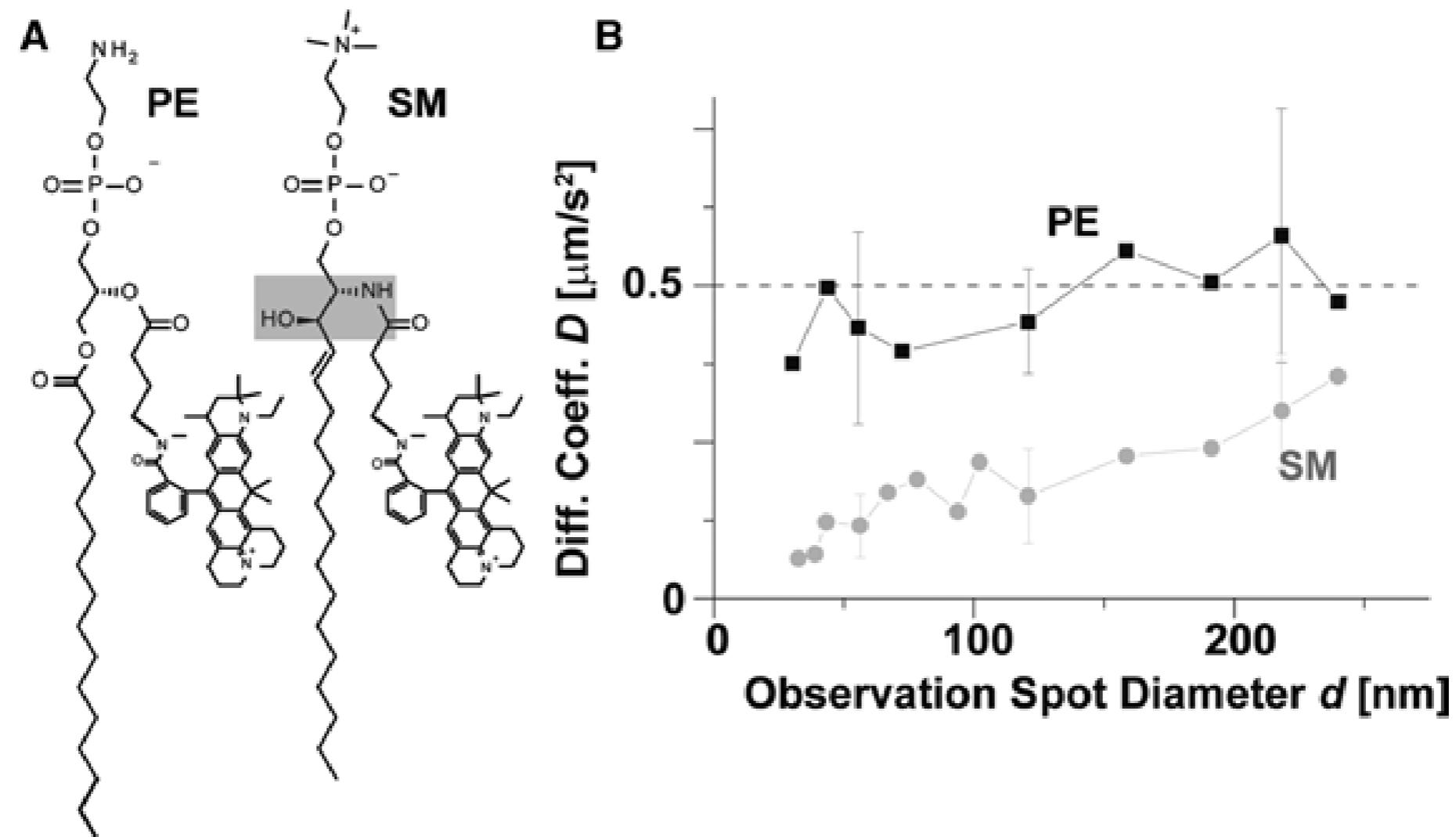


STED microscopy allows recording FCS data and thus investigating molecular diffusion modes for different sizes of the observation spot, as tuned the power of the STED laser

Eggeling C. "Super-resolution optical microscopy of lipid plasma membrane dynamics"; Essays Biochem. volume 57 2015



Eggeling C. "Super-resolution optical microscopy of lipid plasma membrane dynamics"; Essays Biochem. volume 57 2015



(B) Dependency of the apparent diffusion coefficient [ $D(d)$ ] on the diameter ( $d$ ) of the observation spot for the diffusion of PE, phosphatidylethanolamine, (black squares) and SM, sphingomyelin, (grey circles) in the plasma membrane of live mammalian cells, indicating close to free diffusion for PE and trapped diffusion of SM.

Eggeling C. "Super-resolution optical microscopy of lipid plasma membrane dynamics"; Essays Biochem. volume 57 2015

# Many thanks ...

## Nanoimmunology Group

### Group Leader

Christian Eggeling

### Postdocs

Dilip Shrestha

Erdinc Sezgin

Iztok Urbancic

Katharina Reglinski

### PhD Students

Francesco Reina

Falk Scheneider

Maximilian Buttner

### Alumni

Mathias P. Clausen

Jorge Bernardino de la Serna

Jakub Chojnacki

Antonio Gregorio Dias Jr

Tess Archana Stanly

## Collaborators

Dr. Philip Hublitz

Dr. Debora Andrade

Dr. Jacopo Antonello

PhD student Aurelien Barbotin

Prof. Dr. Martin Booth

## DFG research unit PerTrans

Dr. Luis Daniel Cruz Zaragoza

PhD student Jessica Kluemper

Prof. Dr. Wolfgang Schliebs

Dr. Wolfgang Girzalsky

Prof. Dr. Ralf Erdmann



### Associated Members

Christopher Lagerholm - Facility Manager

Jana Koth - Facility co-manager

Dominic Waithe - Image Analyst

Ulrike Schulze - Image Analyst

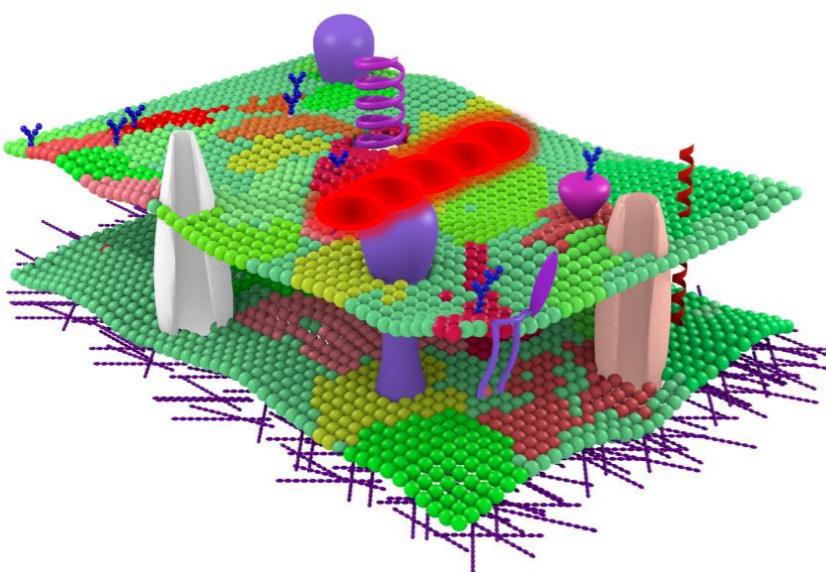
... Thank you for your attention!

# Scanning FCS measurements

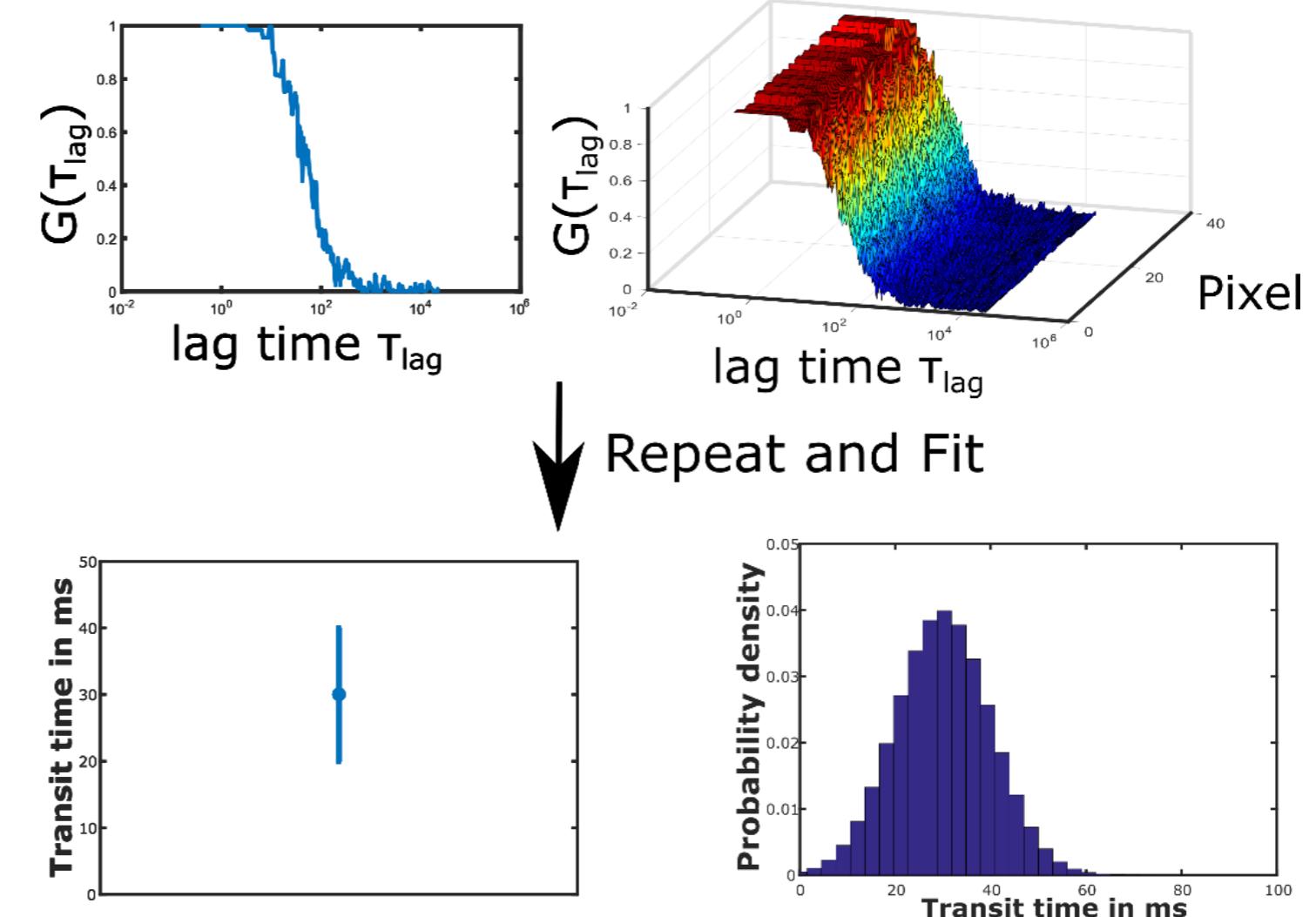


# Scanning FCS measurements

Highly heterogeneous  
Highly dynamic  
Organised on nano-scales

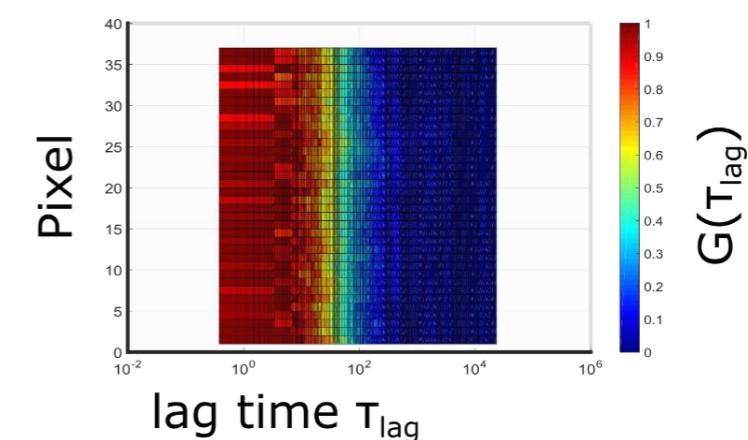


Sezgin et al. (2017), Nature Reviews

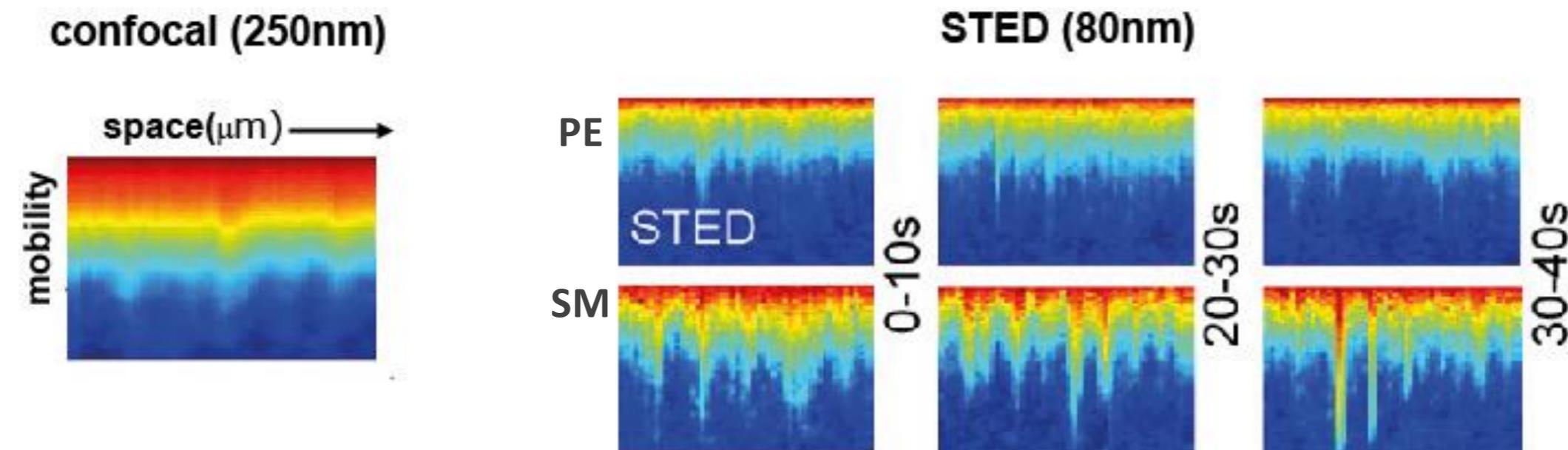


→ Adds spatial information and large statistics

→ Time scale ≈ 1ms - 1s



Honigmann et al. (2014), Nature Communications



SM shows sites of strong transient interactions with slow-moving or immobilized molecules (such as proteins), while these are less prominent for PE.

Marty, it's perfect,  
you're just not thinking  
fourth-dimensionally!!!

