Lecture 10: STimulated Emission Depletion (STED) microscopy

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Lecture 10: STimulated Emission Depletion (STED) microscopy

- History
- Principles of STED
- Biological examples of STED imaging
- Combining STED and FCS (STED-FCS)
- Biological applications of STED-FCS

History – STED Microscopy

Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy

Stefan W. Hell and Jan Wichmann

Department of Medical Physics, University of Turku, Tykistökatu 6, 20521 Turku, Finland

Received March 7, 1994

We propose a new type of scanning fluorescence microscope capable of resolving 35 nm in the far field. We overcome the diffraction resolution limit by employing stimulated emission to inhibit the fluorescence process in the outer regions of the excitation point-spread function. In contrast to near-field scanning optical microscopy, this method can produce three-dimensional images of translucent specimens.

Hell, S. W., & Wichmann, J. (1994). Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt Lett, 19*(11), 780-782.

Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission

Thomas A. Klar, Stefan Jakobs, Marcus Dyba, Alexander Egner, and Stefan W. Hell†

Max-Planck-Institute for Biophysical Chemistry, High Resolution Optical Microscopy Group, 37070 Göttingen, Germany

Edited by Daniel S. Chemla, E. O. Lawrence Berkeley National Laboratory, Berkeley, CA, and approved May 12, 2000 (received for review March 10, 2000)

The diffraction barrier responsible for a finite focal spot size and limited resolution in far-field fluorescence microscopy has been fundamentally broken. This is accomplished by quenching excited organic molecules at the rim of the focal spot through stimulated emission. Along the optic axis, the spot size was reduced by up to 6 times beyond the diffraction barrier. The simultaneous 2-fold improvement in the radial direction rendered a nearly spherical fluorescence spot with a diameter of 90–110 nm. The spot volume of down to 0.67 attoliters is 18 times smaller than that of confocal microscopy, thus making our results also relevant to three-dimensional photochemistry and single molecule spectroscopy. Images of live cells reveal greater details.

lin) with an intracavity frequency doubler. This system partly converted the Ti:Sapphire pulses into visible ones. The pulse trains were temporally adjusted by an optical delay stage and coupled into the setup by dichroic mirrors (Fig. 1*a*).

The duration of the visible pulses was 0.2 ps to ensure temporally defined excitation of the fluorophore. The near-infrared STED pulses were stretched by a grating to $\tau = 40$ ps. This allowed us to extend STED over a time period much longer than the relaxation time (~0.2 ps) of the vibrational substate of the electronic ground state into which the molecule is quenched. This is important because it allows the quenched molecules to escape re-excitation by the same beam through vibrational relaxation. Because we elected to use dyes with fluorescence

Klar, T., Jakobs, S., Dyba, M., Egner, A., & Hell, S. (2000). Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proc Natl Acad Sci U S A, 97*(15), 8206 – 8821.

History – Hypothesis of "Einstrahlung"

3. Zur Quantentheorie der Strahlung

von **A. Einstein.**

Die formale Ähnlichkeit der Kurve der chromatischen Verteilung der Temperaturstrahlung mit dem Maxwell'schen Geschwindigkeits-Verteilungsgesetz ist zu frappant, als daß sie lange hätte verborgen bleiben können. In der Tat wurde bereits W. Wien in der wichtigen theoretischen Arbeit, in welcher er sein Verschiebungsgesetz

$$\varrho = \nu^3 f\left(\frac{\nu}{T}\right) \qquad (1)$$

ableitete, durch diese Ähnlichkeit auf eine weitergehende Bestimmung der Strahlungsformel geführt. Er fand hiebei bekanntlich die Formel

$$\varrho = \alpha \nu^3 e^{-\frac{h\nu}{kT}} \qquad (2)$$

welche als Grenzgesetz für große Werte von $\frac{\nu}{T}$ auch heute als richtig anerkannt wird (Wien'sche Strahlungsformel). Heute wissen wir, daß keine Betrachtung, welche auf die klassische Mechanik und Elektrodynamik aufgebaut ist, eine brauchbare Strahlungsformel liefern kann, sondern daß die klassische Theorie notwendig auf die Reileigh'sche Formel

$$\varrho = \frac{k\alpha}{h} \nu^2 T \qquad (3)$$

führt. Als dann Planck in seiner grundlegenden Untersuchung seine Strahlungsformel

$$\varrho = \alpha \nu^3 \frac{1}{e^{\frac{h\nu}{kT}} - 1} \qquad (4)$$

auf die Voraussetzung von diskreten Energie-Elementen gegründet hatte, aus welcher sich in rascher Folge die Quantentheorie entwickelte, geriet jene Wien'sche Überlegung, welche zur Gleichung (2) geführt hatte, naturgemäß wieder in Vergessenheit.

Vor kurzem nun fand ich eine der ursprünglichen Wien'schen Betrachtung [1]) verwandte, auf die Grundvoraussetzung der Quanten-

[1]) Verh. d. deutschen physikal. Gesellschaft, Nr. 13/14, 1916, S. 318. In der vorliegenden Untersuchung sind die in der eben zitierten Abhandlung gegebenen Überlegungen wiederholt.

a) *"Ausstrahlung" (Spontaneous Emission).* An oscillating Planck resonator radiates energy in a known manner according to Hertz, independent of whether it is excited by an external field of not. Correspondingly, a molecule shall be able to change from state Z_m into state Z_n under emission of the energy $\varepsilon_m - \varepsilon_n$ with frequency ν without external causes. The probability dW that this actually occurs during the time element dt shall be

$$dW = A_m^n dt, \qquad (A)$$

where A_m^n is a constant characteristic of the index combination under consideration.

The assumed statistical law corresponds to a radioactive reaction, and the assumed elementary process to a reaction where only γ-radiation is emitted. One does not need to assume that this process requires no time; the time need only be negligible compared to the times during which the molecule is in states Z_1, etc.

b) *"Einstrahlung" (Induced Radiation).* In a field of radiation, a Planck resonator changes its energy because the electromagnetic field of the radiation transfers work upon the resonator. Depending upon the phases of the resonator and the oscillating field, this amount of work can be positive or negative. In taking account of this, we introduce the following quantum-theoretic hypotheses. Under the action of the radiation density ρ of frequency ν, the molecule can go from state Z_n to Z_m by absorbing the radiation energy $\varepsilon_m - \varepsilon_n$ according to the probability law

$$dW = B_n^m \rho dt. \qquad (B)$$

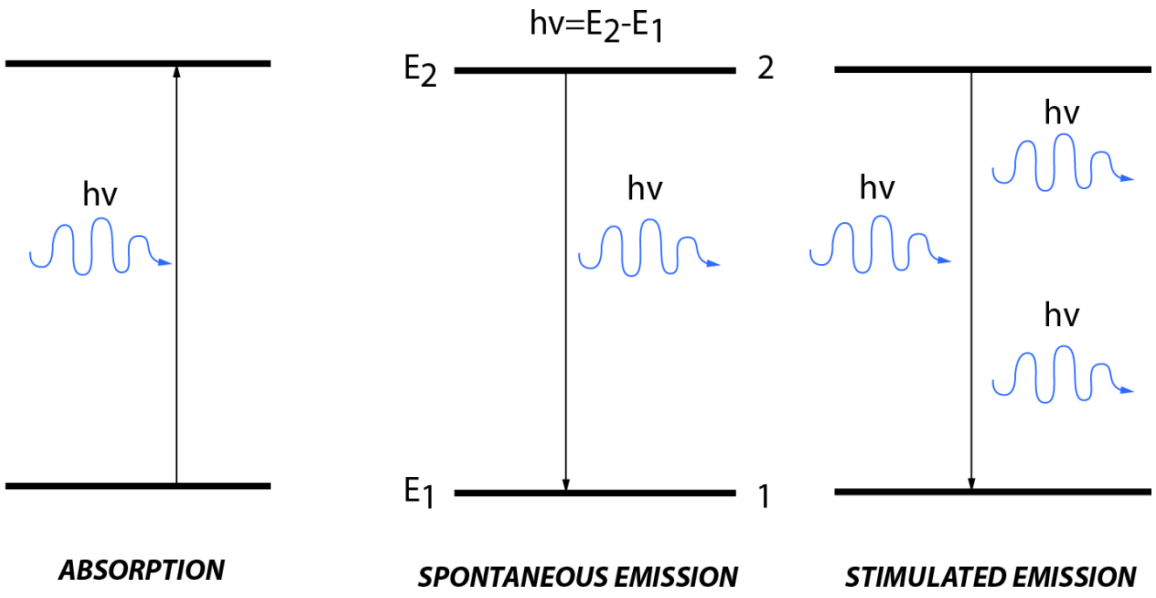
In a similar manner the transition $Z_m \rightarrow Z_n$ is possible under the action of radiation, whereby the radiation energy $\varepsilon_m - \varepsilon_n$ is set free according to the probability law

$$dW = B_m^n \rho dt. \qquad (B')$$

B_n^m and B_m^n are constants. Both processes are called "changes of state by 'Einstrahlung' (induced radiation)."

Einstein, A. (1917). Zur Quantentheorie der Strahlung. *Physikalische Zeitschrift, 18*, 121-128.

Jablonski Diagram Format

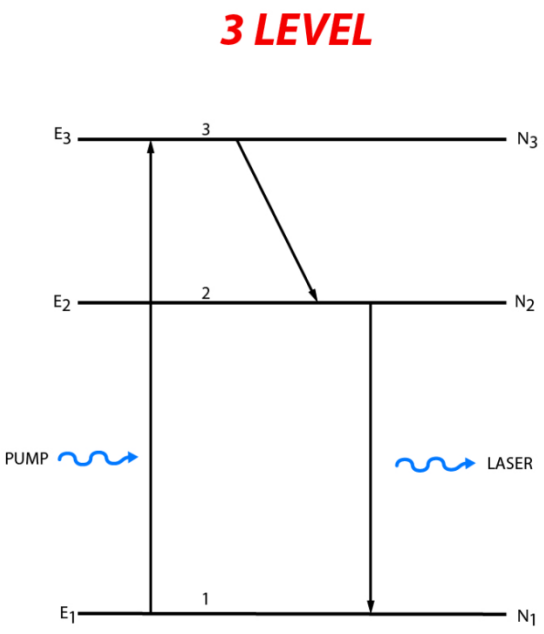


A very brief (but related) sidenote:

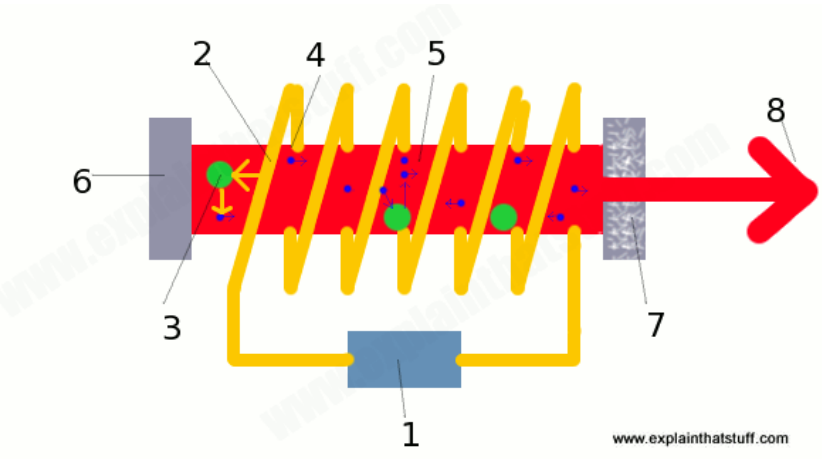
What does acronym LASER stand for?

LASER = Light Amplification by Stimulated Emission of Radiation

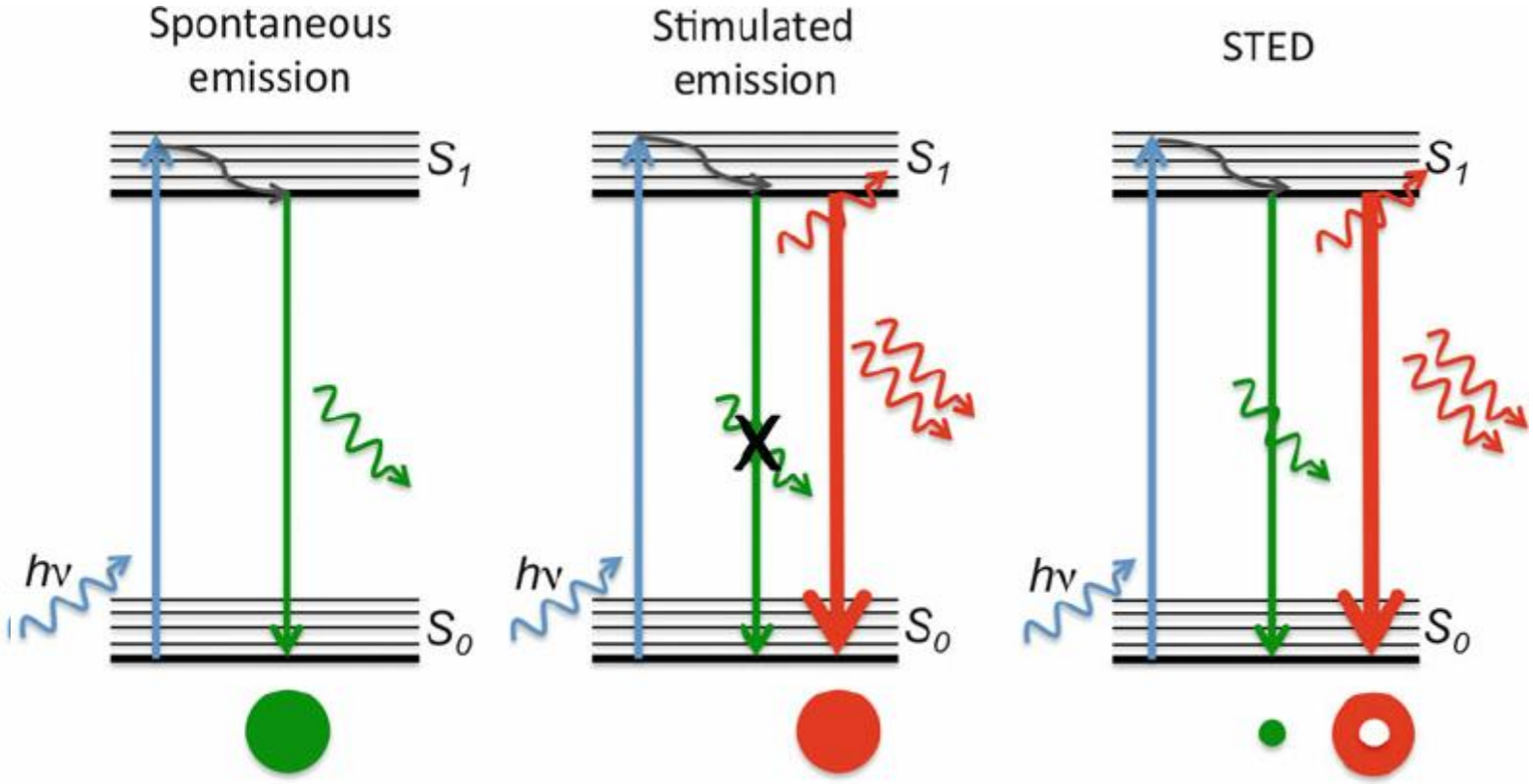
Population inversion



Crystal Laser

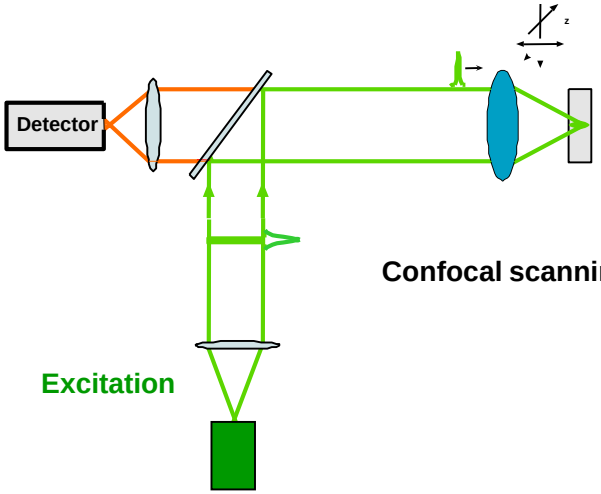


http://www.explainthatstuff.com/lasers.html

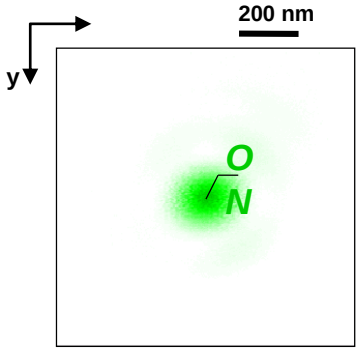

Spontaneous emission
Stimulated emission
STED
S_1
S_0
hν

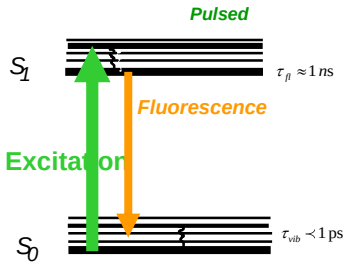
Fluorescence Microscopy

STED Microscopy



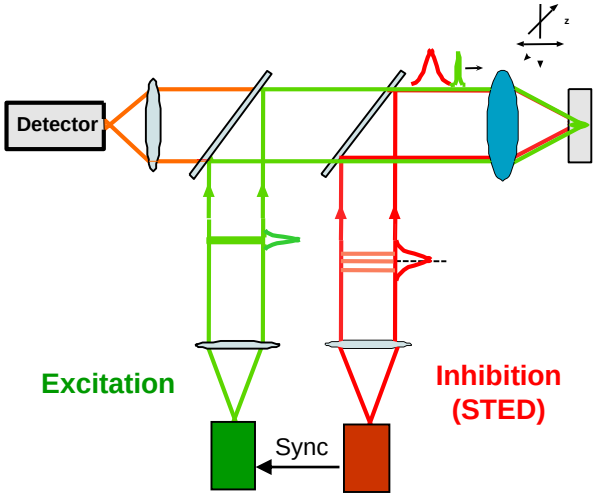
Confocal scanning microscope

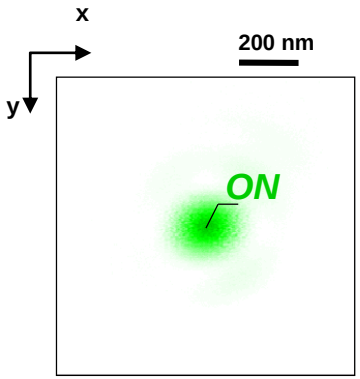


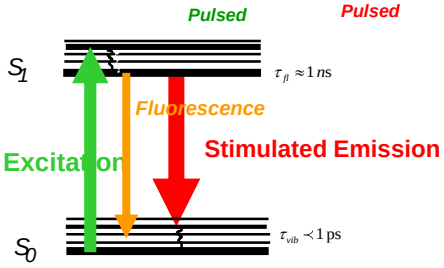


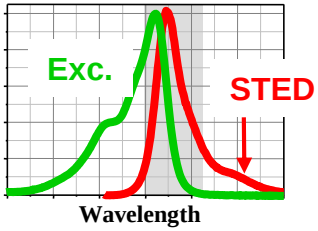
Fluorescence Microscopy

STED Microscopy



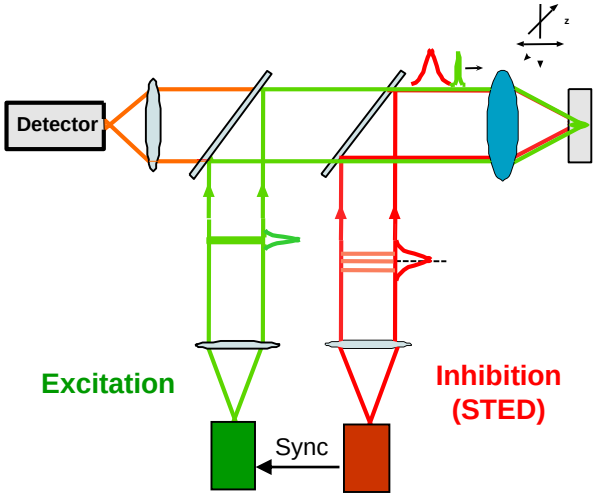


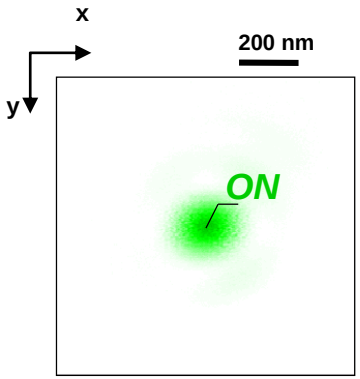


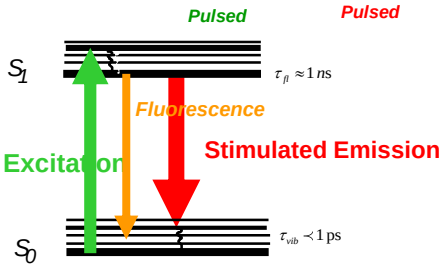


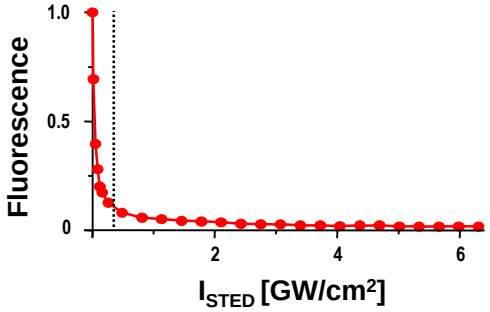
Fluorescence Microscopy

STED Microscopy



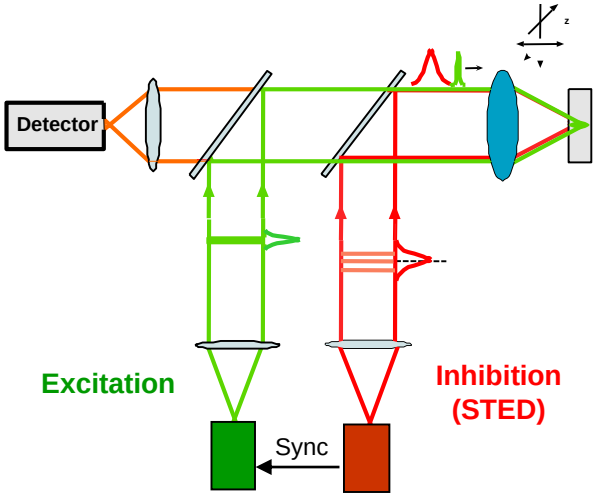


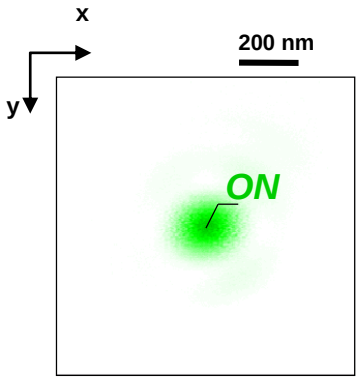


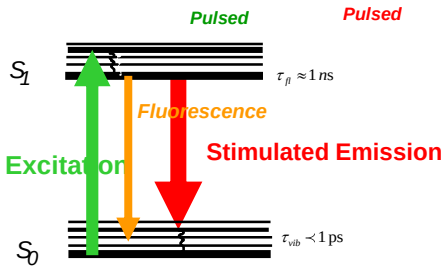


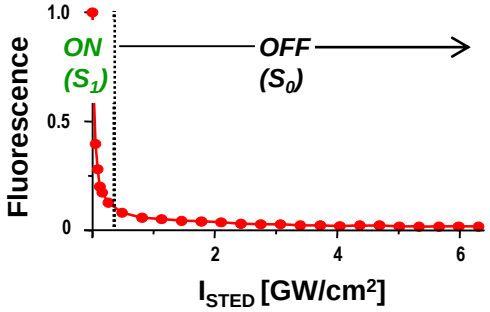
Fluorescence Microscopy

STED Microscopy



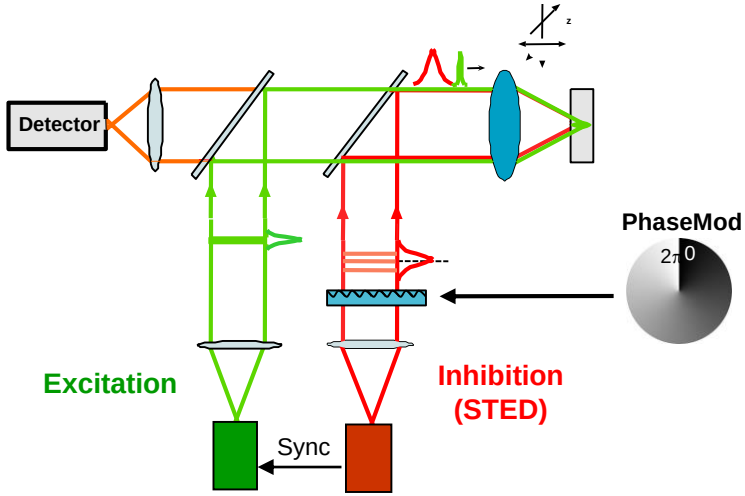


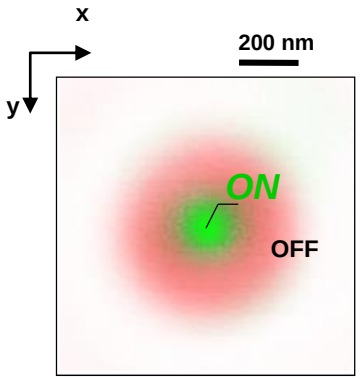


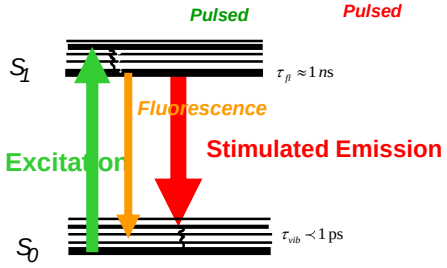


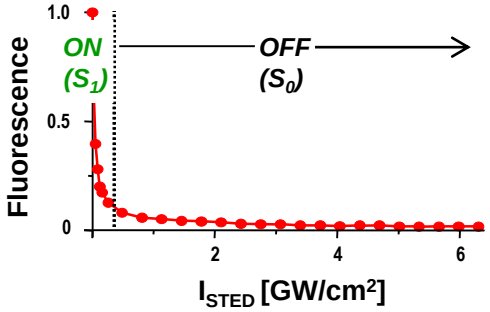
Fluorescence Microscopy

STED Microscopy



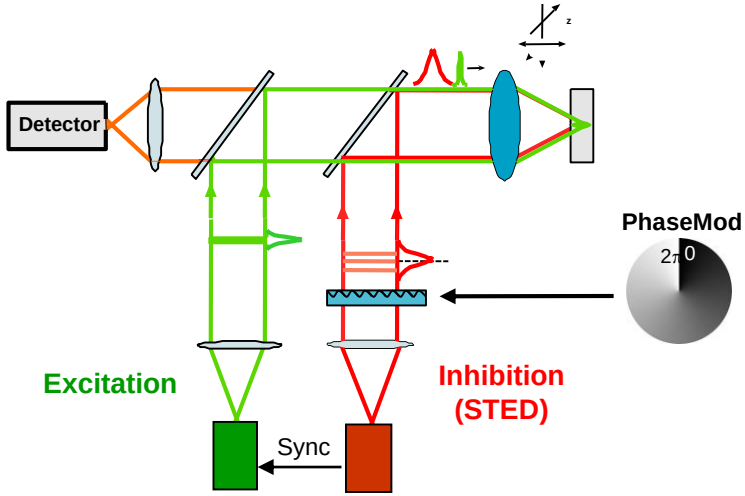


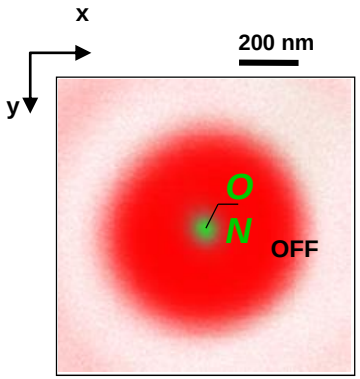


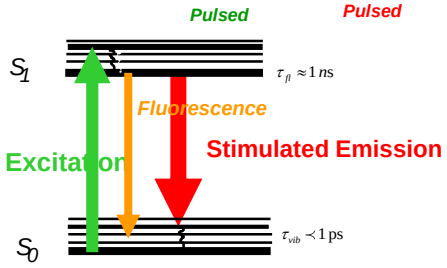


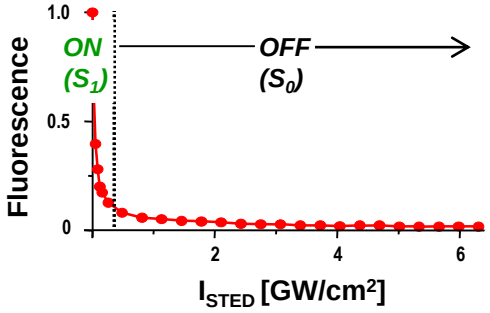
Fluorescence Microscopy

STED Microscopy





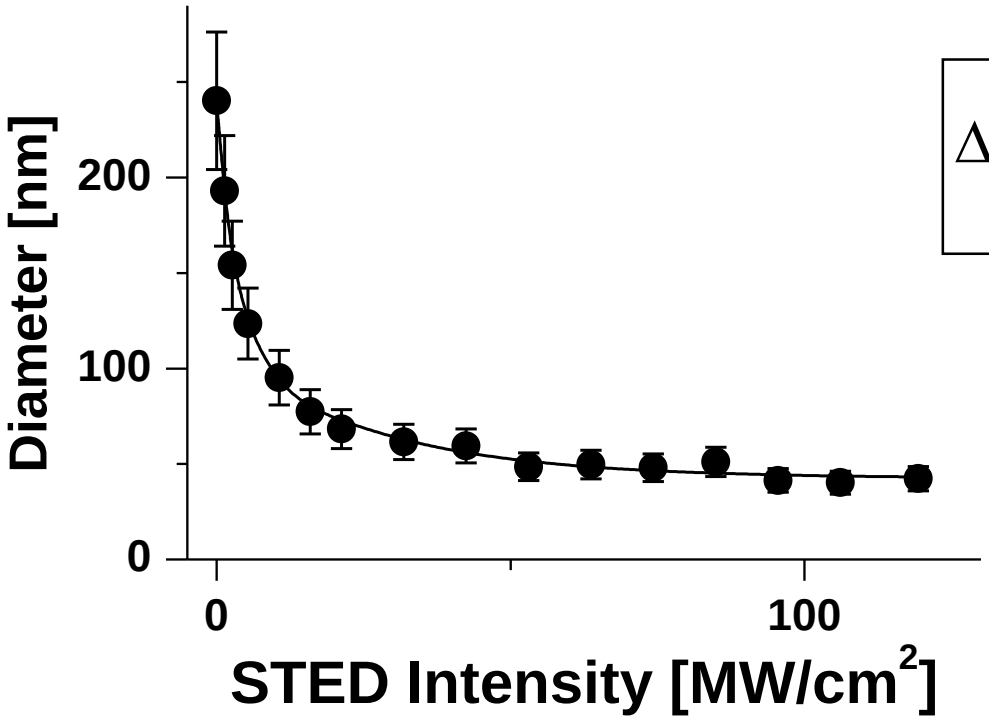




STED Microscopy

Tunable super-resolution imaging

Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!



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

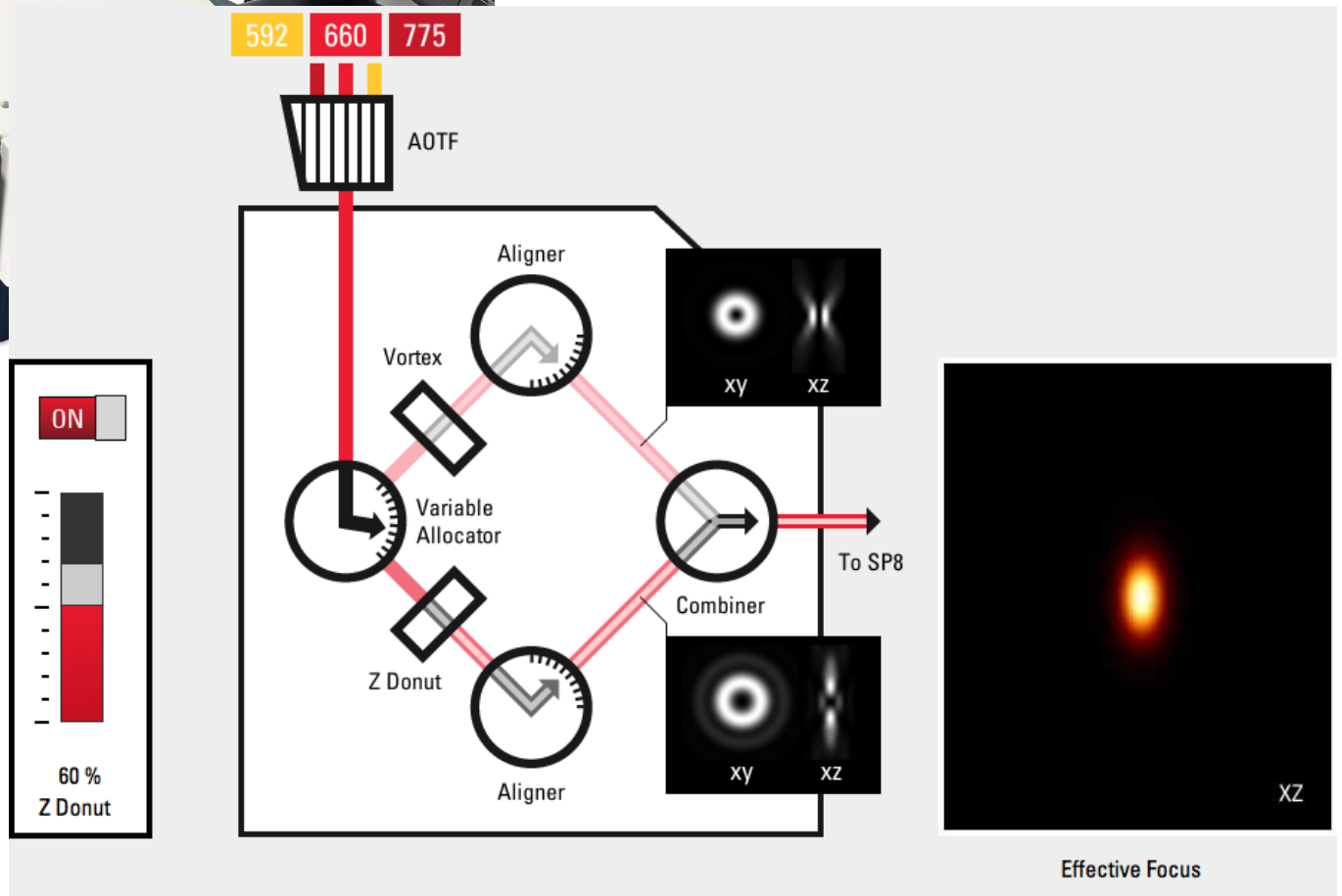
Leica SP8 gSTED 3X with PicoQuant SMD upgrade (FLIM, FCS)

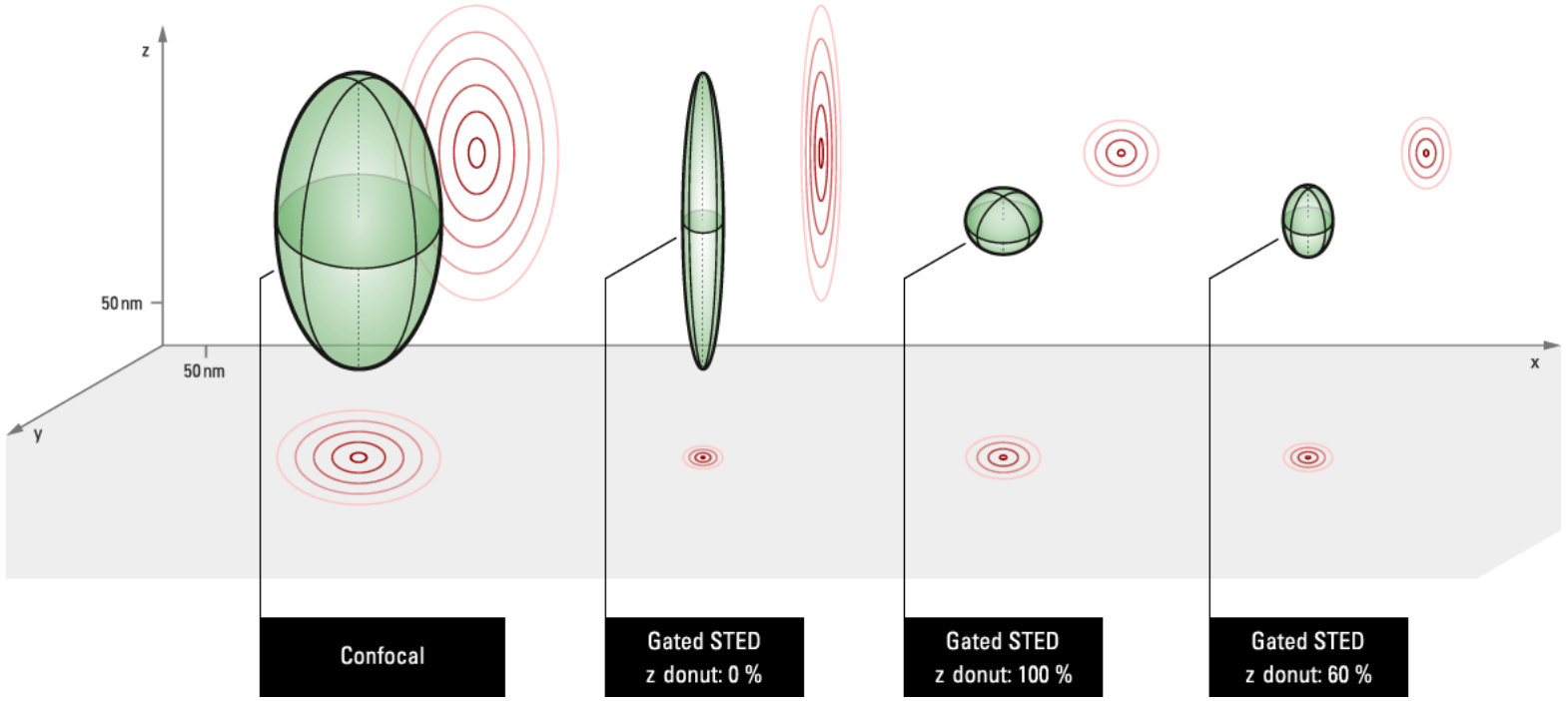
- The LEICA SP8 is a conventional inverted laser-scanning confocal microscope that is additionally equipped for 3D super-resolution gated STED imaging (Specified resolution$_{x,y}$ ~ 50 nm)

- This system is equipped with continuous wave (CW) lasers (@ 405, 458, 488, and 514 nm), a tunable pulsed white light laser (WLL; 470-670 nm), a pulsed laser at 440 nm, high power CW lasers at 592 nm, 660 nm, and a pulsed 775 nm for STED imaging and STED-FCS.

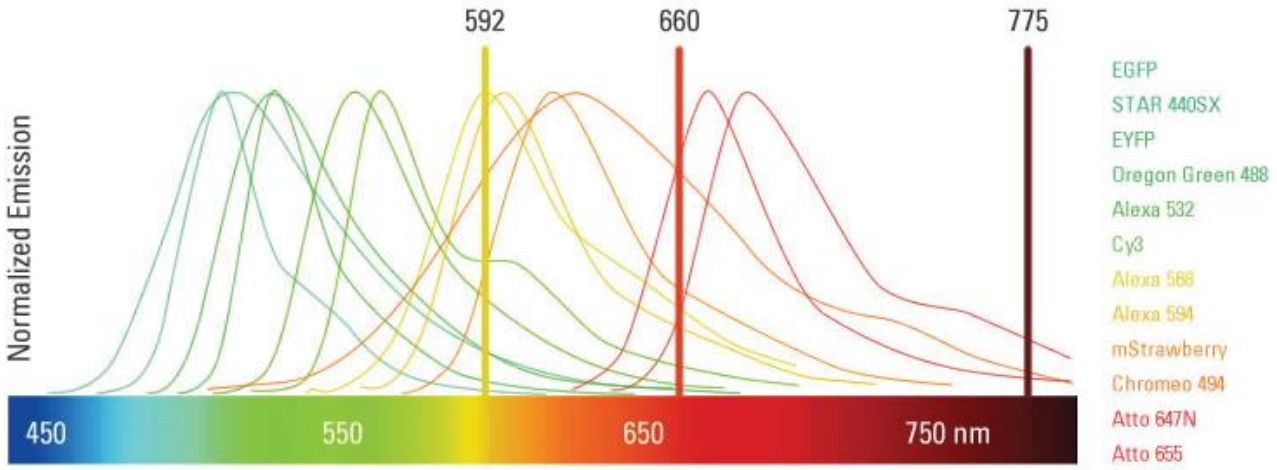
- Designed to operate as “closed” turn-key system with auto-alignment procedure of 592 nm STED laser and WLL and with Engineer co-alignment of 660 nm and 775 nm STED lasers

Leica STED Implementation



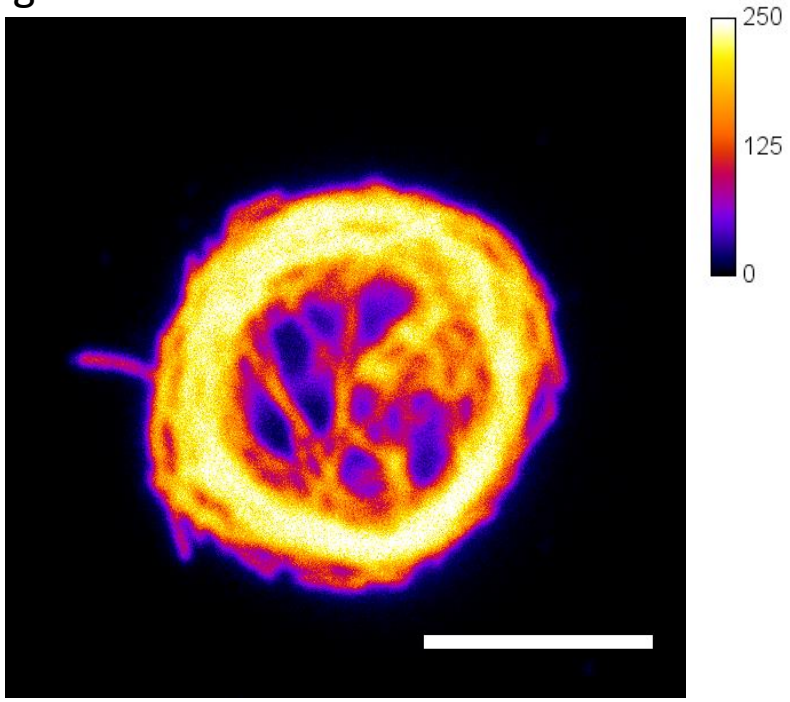


Multicolor STED

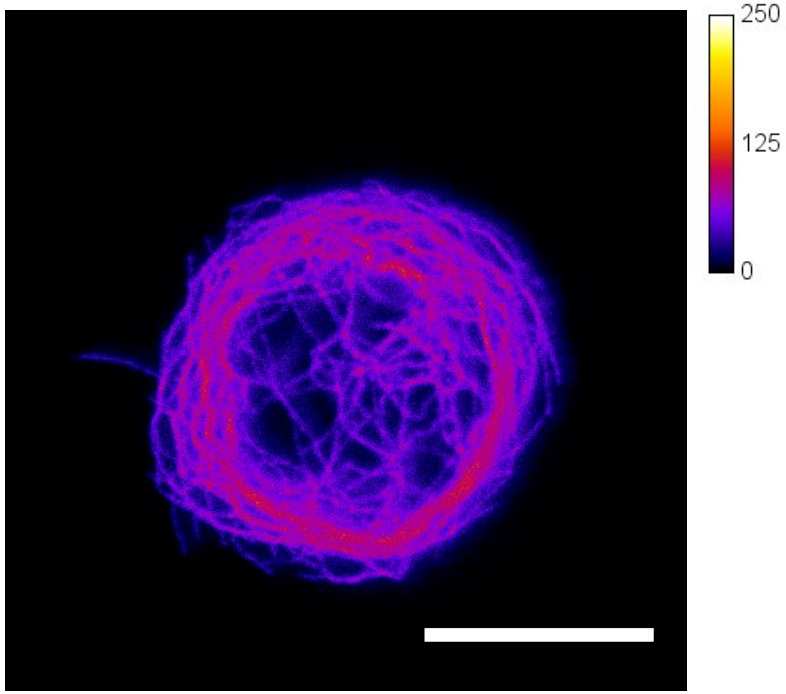


Some Image Examples

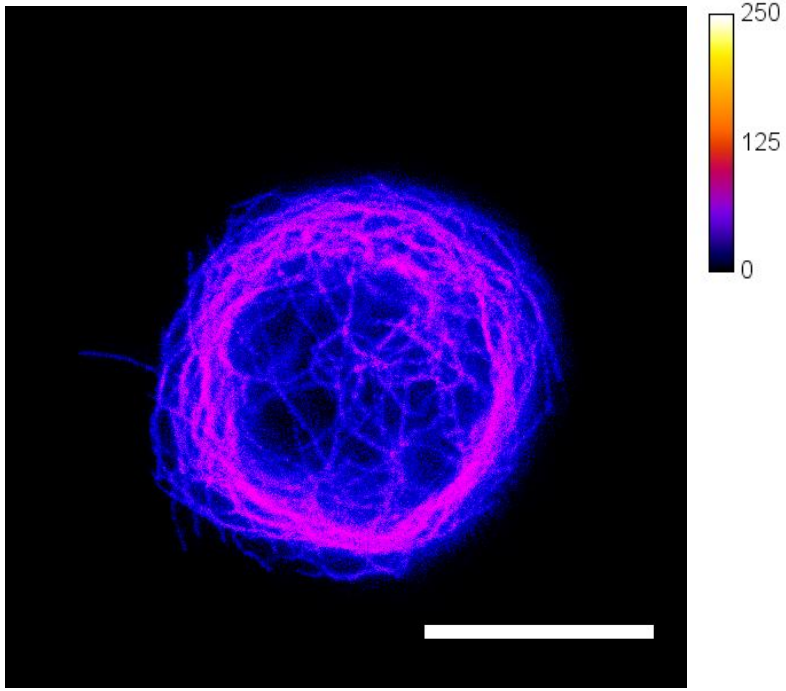
Demonstration of gSTED in images



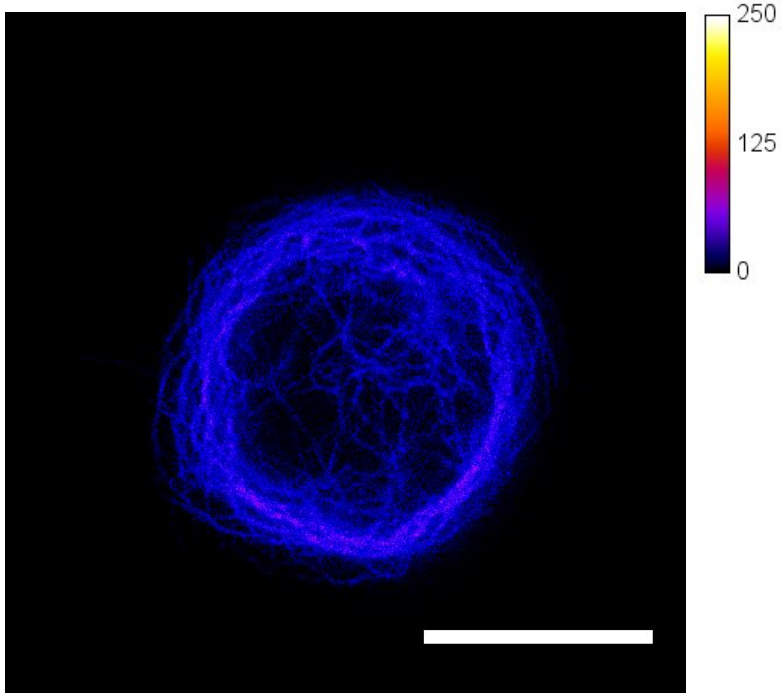
Confocal image
Microtubule Network in Drosphila macrophage
mouse anti-α-tubulin (clone DM1A) / goat ant-mouse IgG Alexa 488
Exc: WLL @488 nm at 80 MHz



gSTED image (25% of max STED power (75 mW) at 592 nm)
Microtubule Network in Drosphila macrophage
mouse anti-α-tubulin (clone DM1A) / goat ant-mouse IgG Alexa 488
Exc: WLL @488 nm at 80 MHz; gSTED592 ($1.5<\tau_g<6.5$ ns)



gSTED image (50% of max STED power (150 mW) aty 592 nm)
Microtubule Network in Drosphila macrophage
mouse anti-α-tubulin (clone DM1A) / goat ant-mouse IgG Alexa 488
Exc: WLL @488 nm at 80 MHz; gSTED592 ($1.5<\tau_g<6.5$ ns)



gSTED image (100% of max STED power (300 mW) at 592 nm)
Microtubule Network in Drosphila macrophage
mouse anti-α-tubulin (clone DM1A) / goat ant-mouse IgG Alexa 488
Exc: WLL @488 nm at 80 MHz; gSTED592 ($1.5<\tau_g<6.5$ ns)

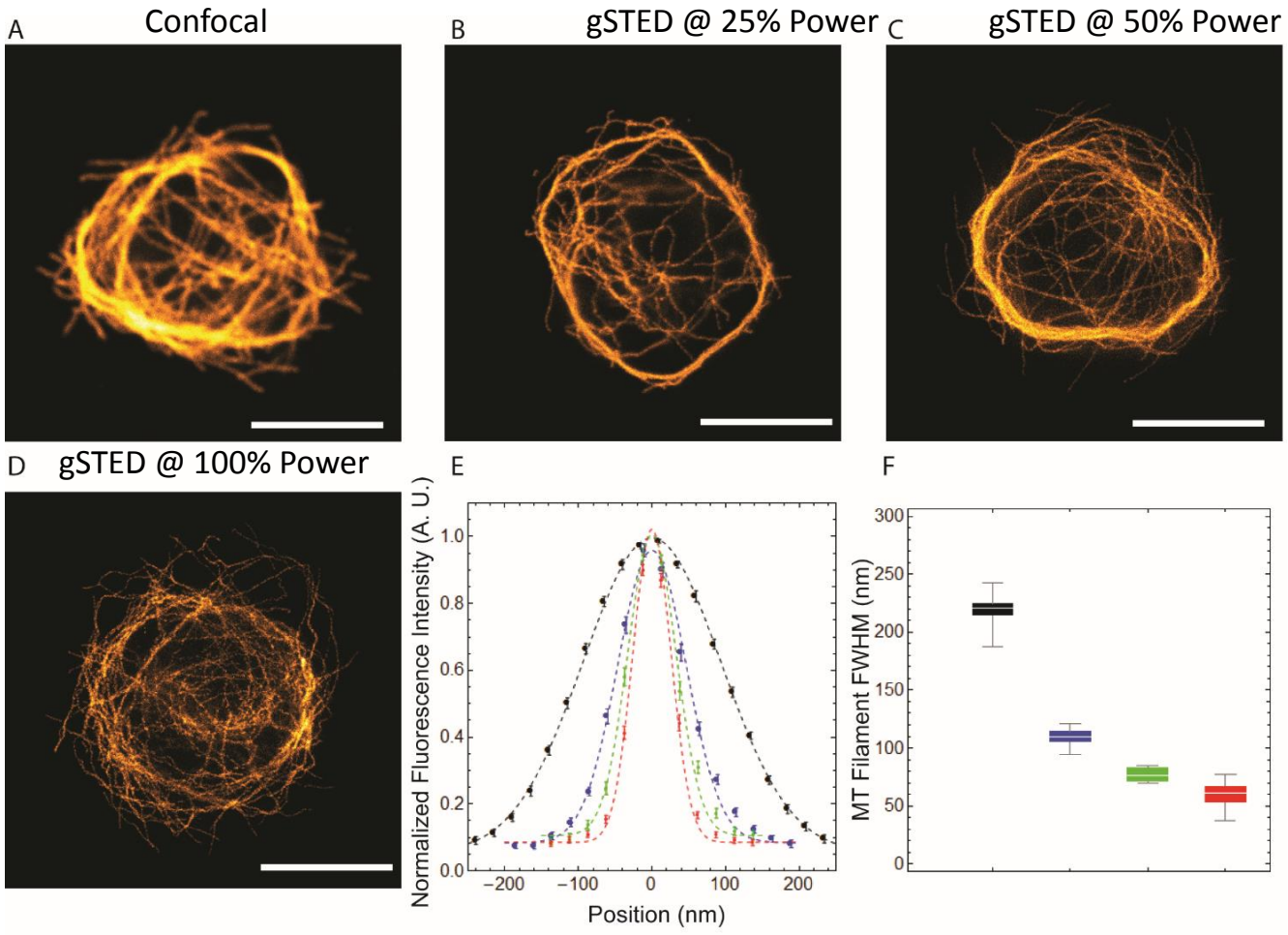
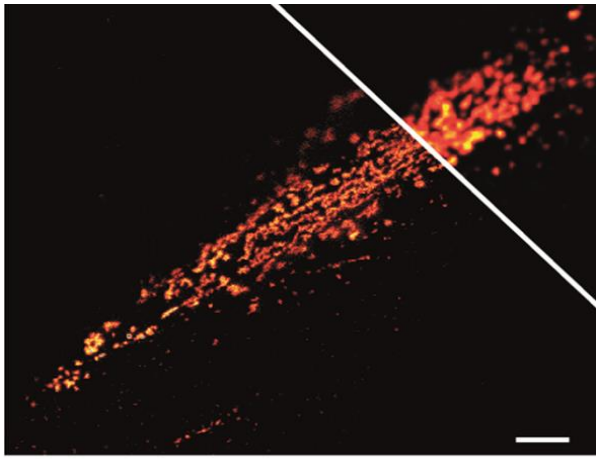
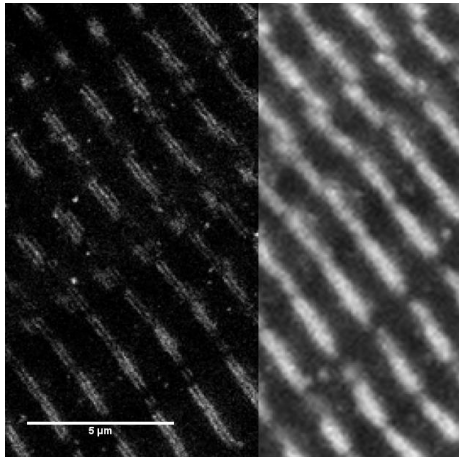

A
Confocal
B
gSTED @ 25% Power
C
gSTED @ 50% Power
D
gSTED @ 100% Power
E
Normalized Fluorescence Intensity (A. U.)
1.0
0.8
0.6
0.4
0.2
0.0
−200
−100
0
100
200
Position (nm)
F
MT Filament FWHM (nm)
300
250
200
150
100
50
0

Image Examples – 2D (xy) STED Imaging



STED/Confocal image of Acetylcholine Receptor Clusters in Myotubes.
Dr. Judith Cossins / Prof. David Beeson
Molecular Neurosciences
University of Oxford

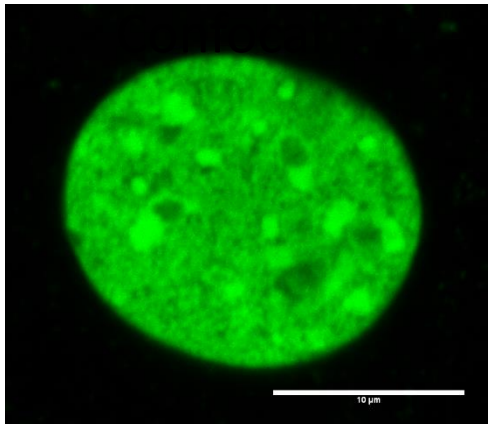


STED/Confocal image of Titin Z-disk in cardiomyocytes.
Dr. Katja Gemlich
Department of Cardiovascular Medicine
University of Oxford

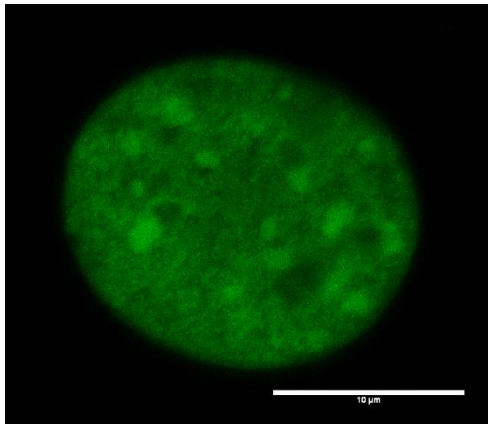
Application Example: 3D (xyz) STED of Sytox Green stained cell nuclei in HeLa cells

Dr. Lothar Schermelleh, Biochemistry & Micron

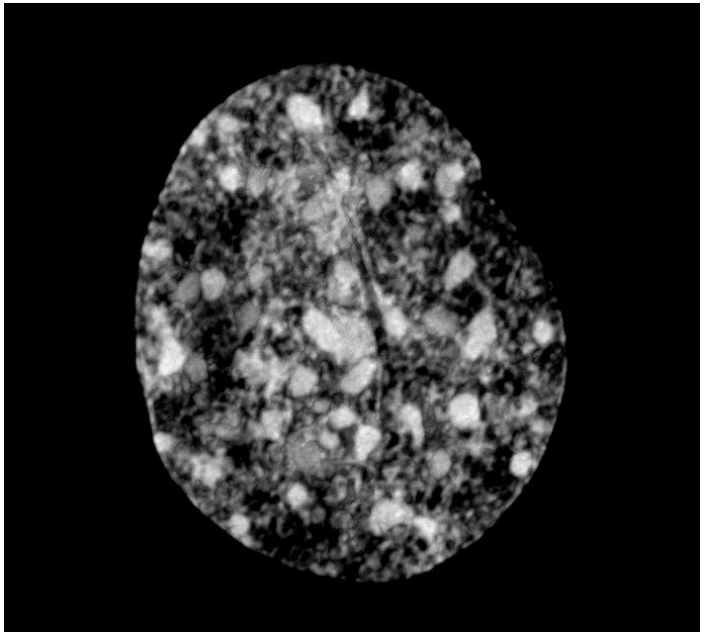
Confocal



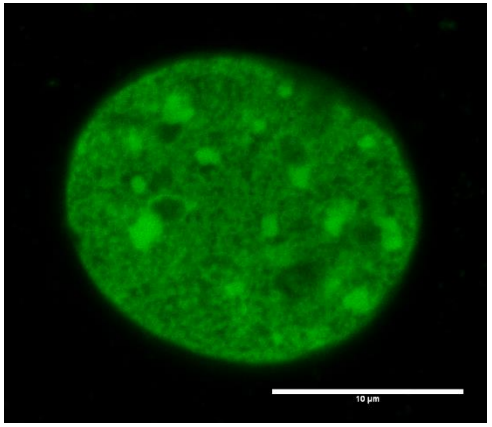
gSTED at 25% Power (xy:z=100:0)



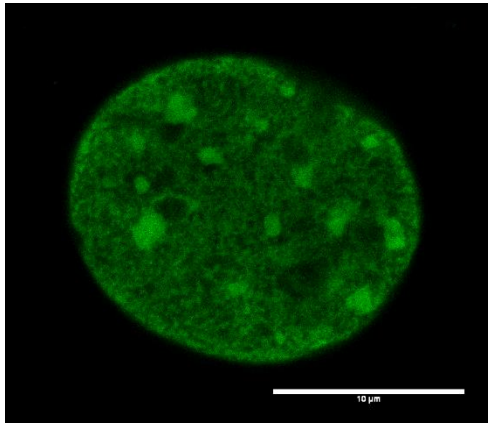
gSTED at 100% Power (xy:z=50:50)



gSTED at 25% Power (xy:z=0:100)



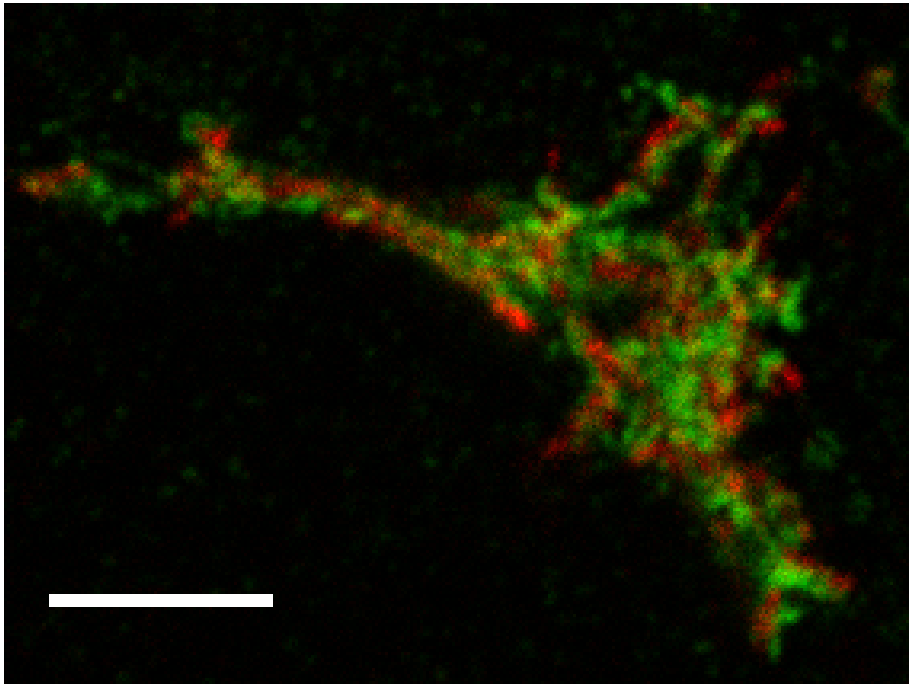
gSTED at 100% Power (xy:z=50:50)



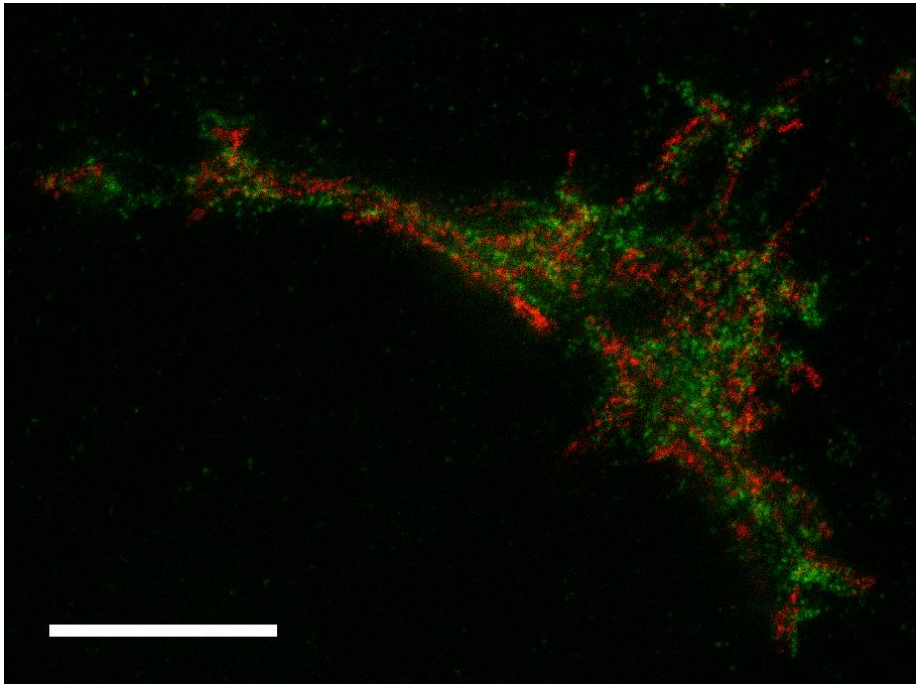
Application Example: Two color 2D STED of cis- and trans-golgi in COS7

TGN46 (trans-golgi) / Alexa488 GM130 (cis-golgi) / TMR

Dr. Eva Wegel, Micron Advanced Bioimaging Unit, Dept of Biochemistry, University of Oxford



Confocal



gSTED

1) 561 Exc & 660 nm STED ($1.5<\tau_g<6.5$ ns)

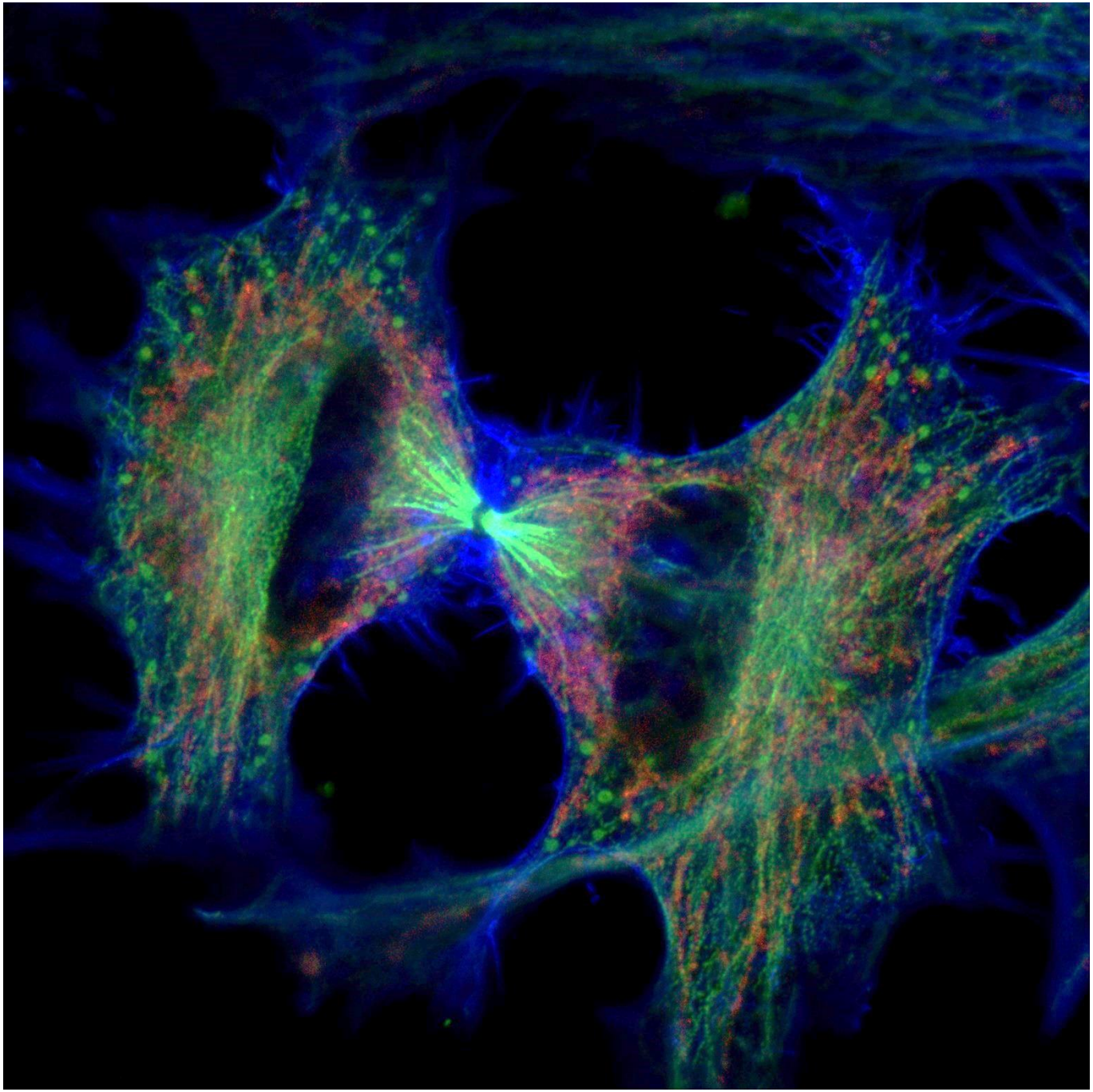
2) 488 nm Exc & 592 nm STED ($1.5<\tau_g<6.5$ ns)

3 Color STED Example - HeLa

TOM20/Abberior Star 635P
Exc633; STED775-25;

Tubulin/Alexa555
Exc: 561 nm; STED660-25;

F-actin/Phalloidin-OG488
Exc: 488 nm; STED592-25;

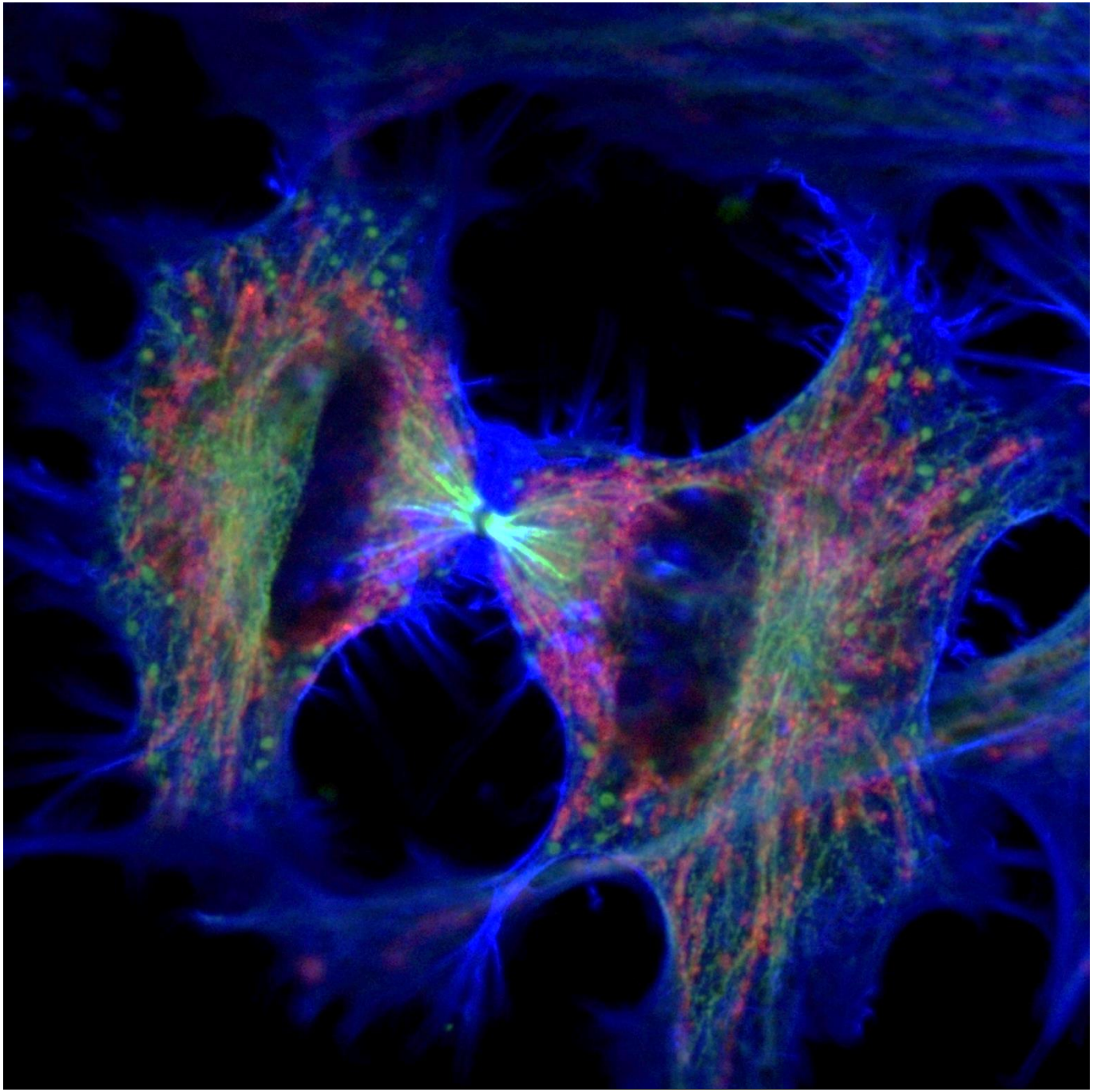


3 Color Confocal Example - HeLa

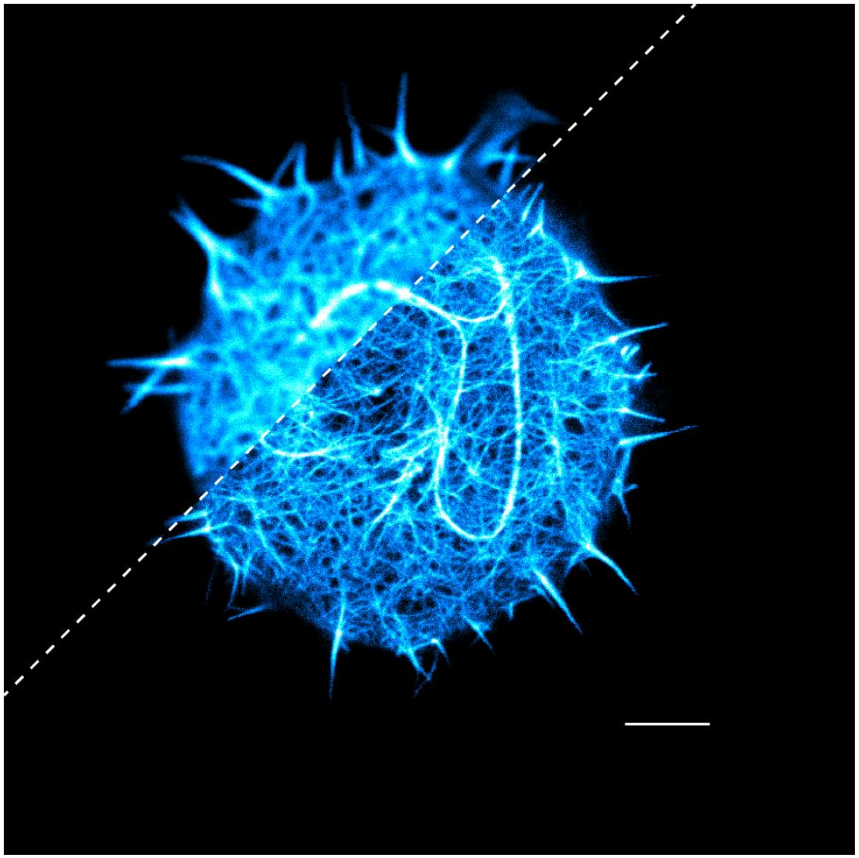
TOM20/Abberior Star 635P
Exc633;

Tubulin/Alexa555
Exc: 561 nm;

F-actin/Phalloidin-OG488
Exc: 488 nm;

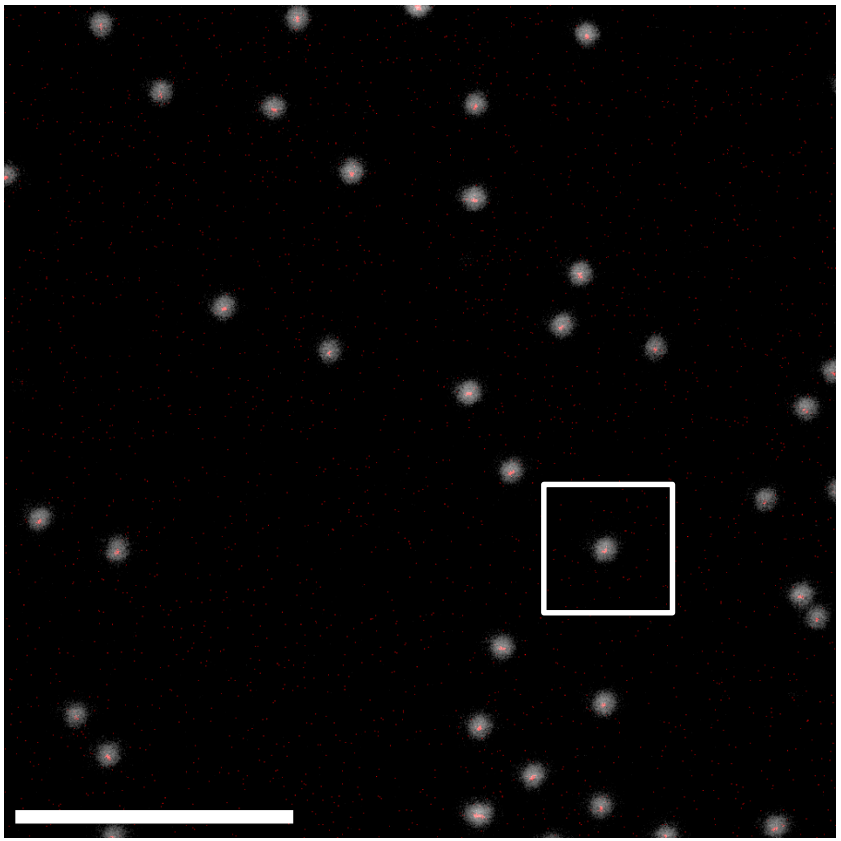


LIVE3D STED of Citrin-Actin in live T-cell (gSTED592 @10% Laser Power)
Dr. Marco Fritsche, Dr. Mathias Clausen, Prof. Christian Eggeling, MRC-HIU, WIMM

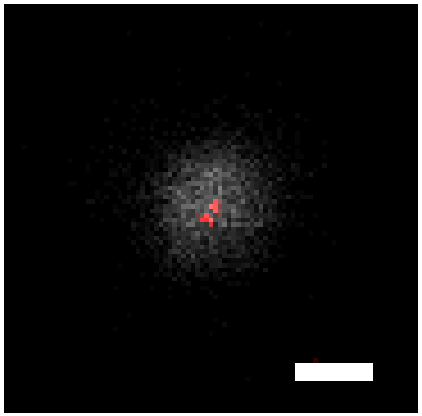


Validation of resolution with GATTAQUANT Rulers

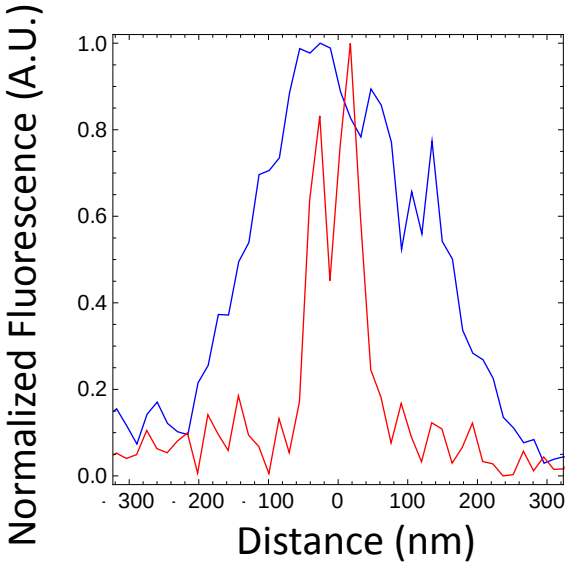
GATTAQUANT 50R (Atto647N)



Scale bar = 5 µm



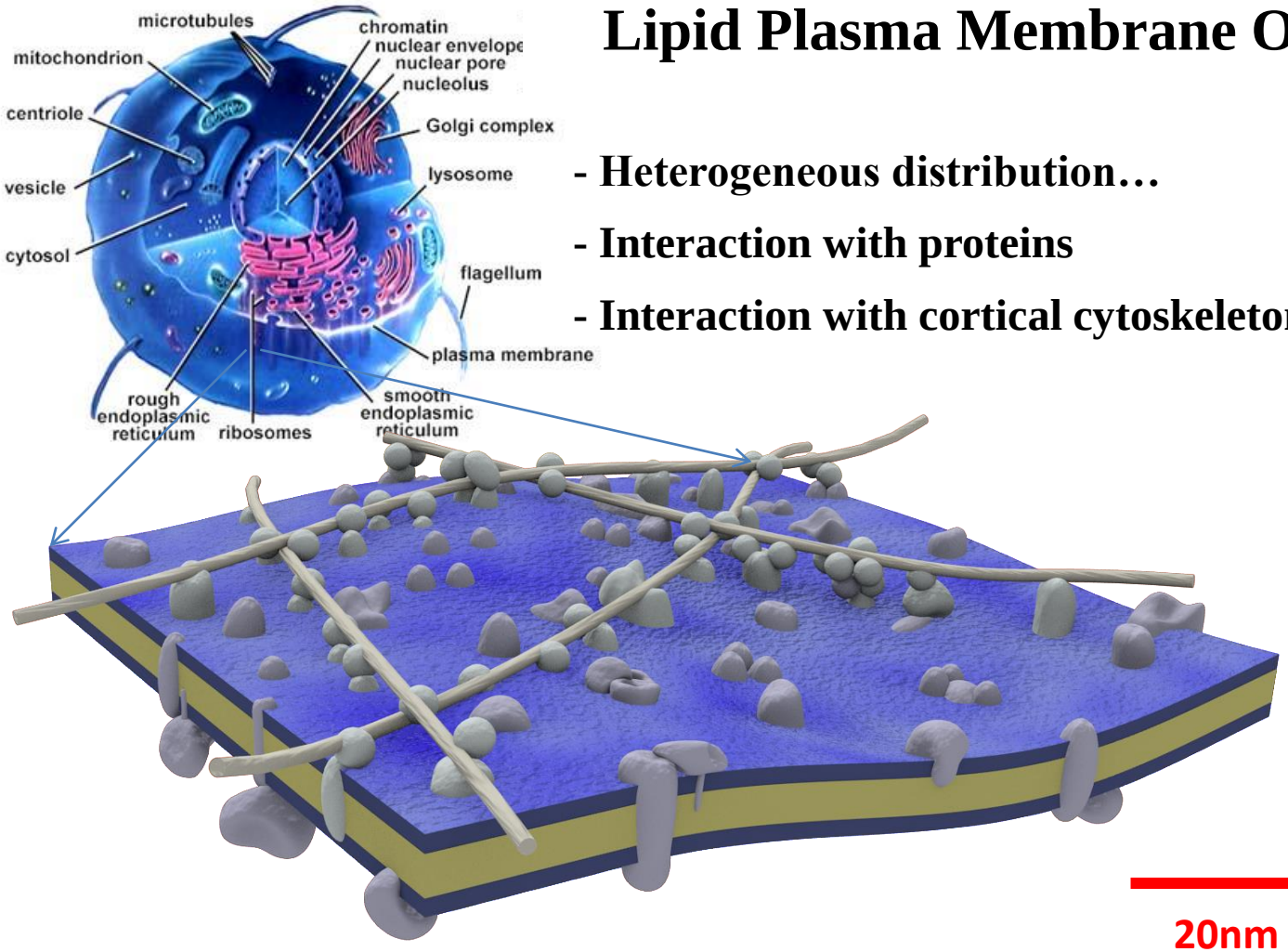
Scale bar = 250 nm



	FWHM (nm)	Peak Separation (nm)
Confocal	300 +/- 20 (n=9)	N.A.
STED775-80 (P=280 mW)	40 +/- 4 (n=9x2)	54 +/- 6 (n=9)

Lipid Plasma Membrane Dynamics

Nanoscale



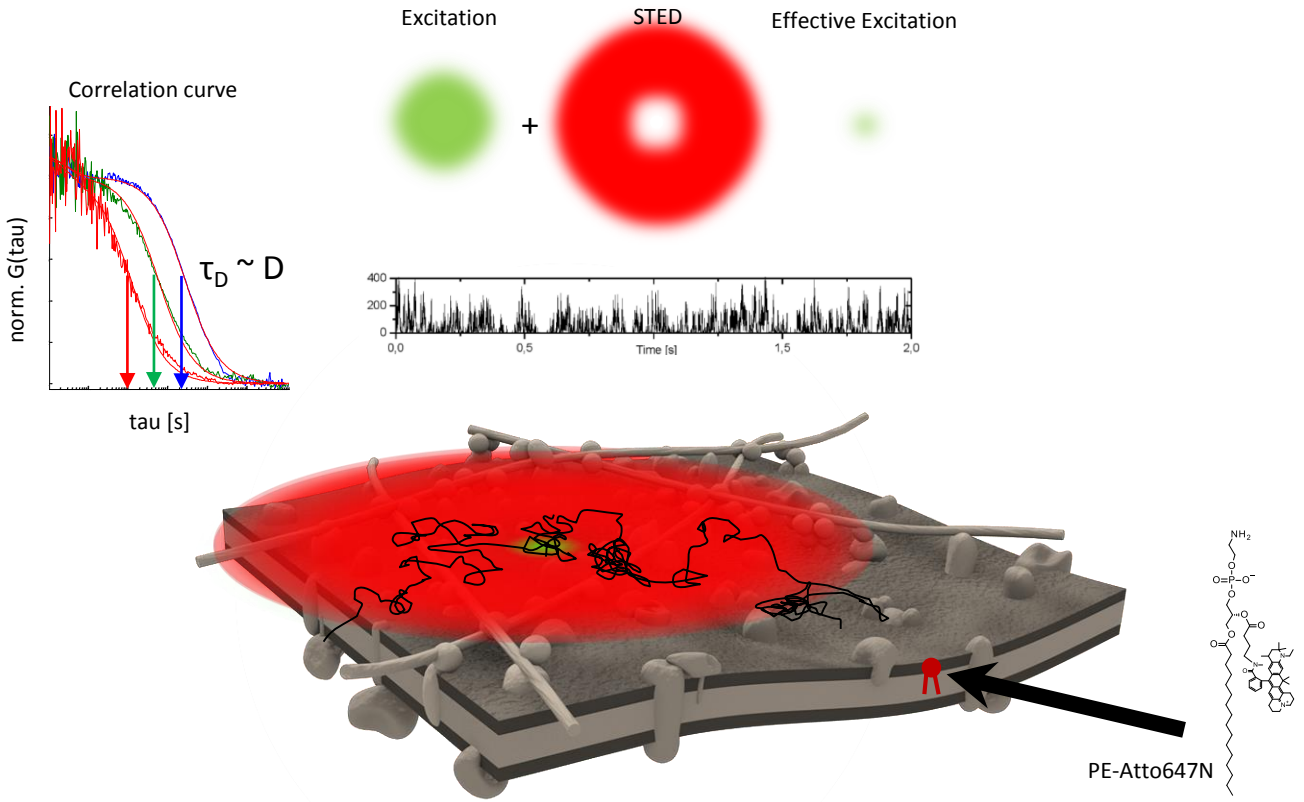
Lipid Plasma Membrane Organization:

- Heterogeneous distribution…
- Interaction with proteins
- Interaction with cortical cytoskeleton

20nm

Small spatial scales!!!!

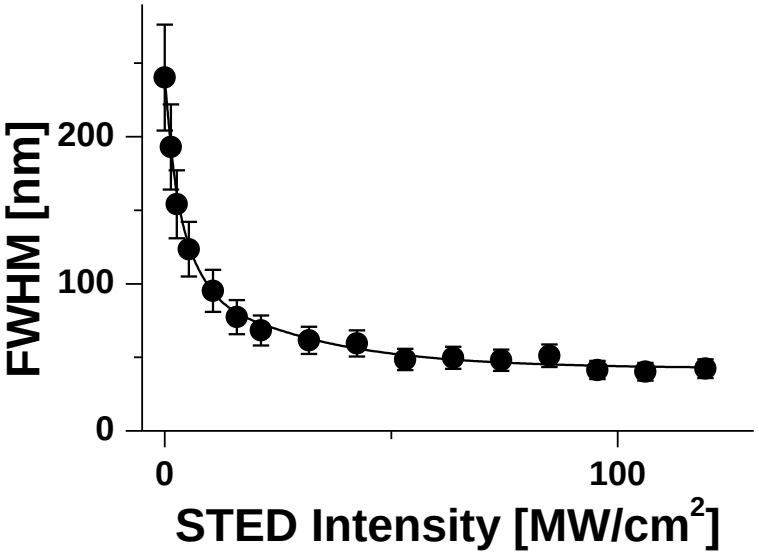
STED-**FCS**



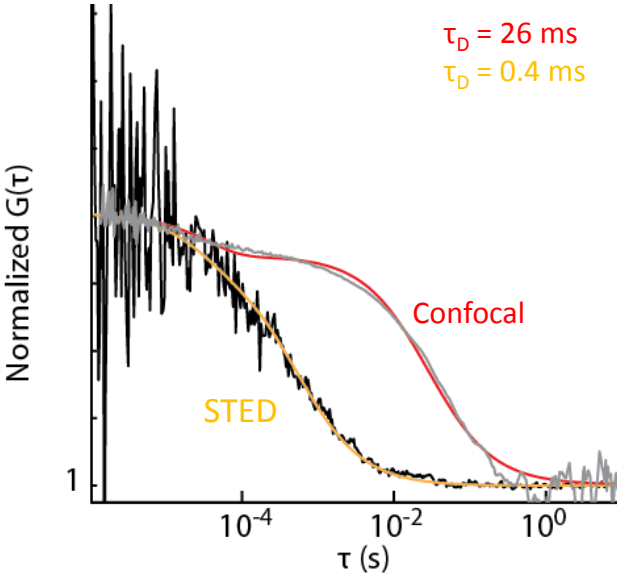
Animation by Dr. Alf Honigmann

STED-FCS

STED-Microscopy: Tuning of observation area

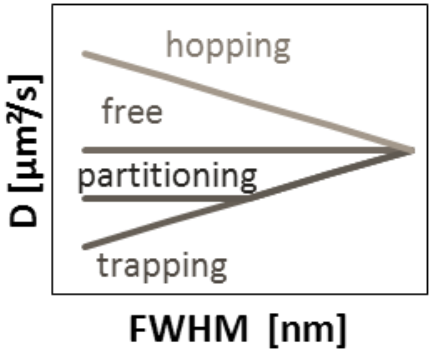


STED-FCS
Determine transit time
for different sizes of observation areas
(different STED intensities)



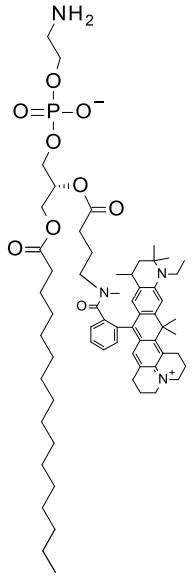
Calculate apparent diffusion coefficient and plot spatial dependence:

D ~ area / transit time

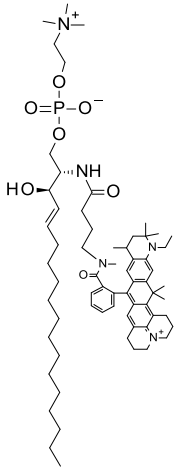


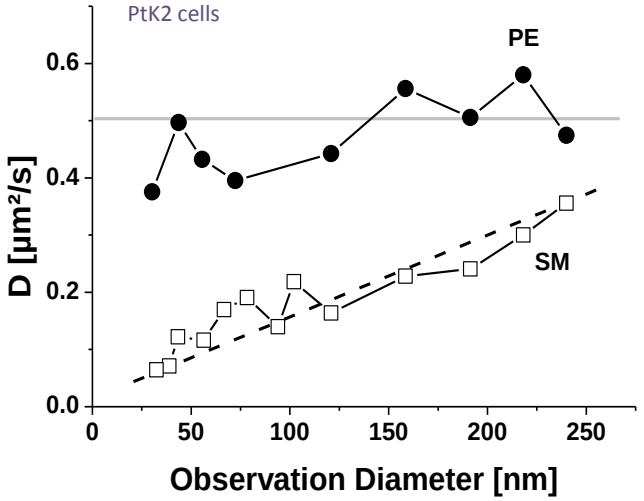
STED-FCS Example: Direct observation of the nanoscale dynamics of membrane lipids in a living Ptk2 cell

Phosphoglycerolipid:
Atto647N-phosphoethanolamine (PE)



Sphingolipid:
Atto647N-sphingomyelin (SM)





Eggeling et al., Nature, 2009

Eggeling, C., Ringemann, C., Medda, R., Schwarzmann, G., Sandhoff, K., Polyakova, S., . . . Hell, S. W. (2009). Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature, 457*(7233), 1159-1162.

SCIENTIFIC REPORTS

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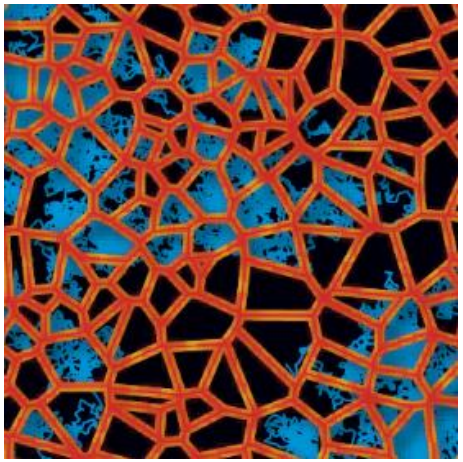
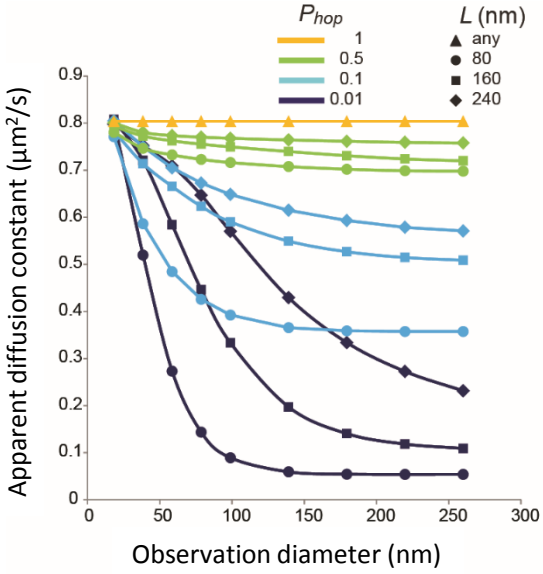
Cortical actin networks induce spatio-temporal confinement of phospholipids in the plasma membrane – a minimally invasive investigation by STED-FCS

Débora M. Andrade[1,3,*], Mathias P. Clausen[1,2,4,*], Jan Keller[1], Veronika Mueller[1], Congying Wu[5], James E. Bear[5,6], Stefan W. Hell[1], B. Christoffer Lagerholm[3,4] & Christian Eggeling[1,2]



Simulation of compartmentalised diffusion



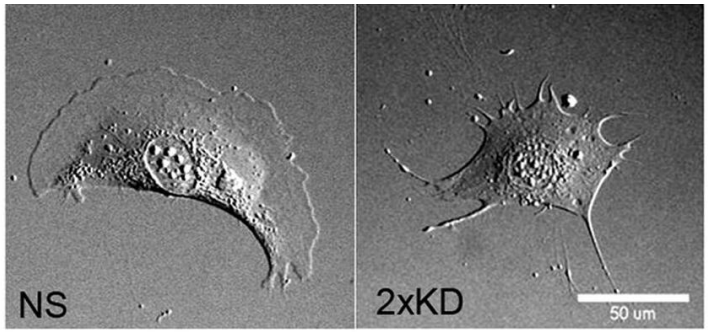
Molecules diffuse free with diffusion constant D_{free} within compartments of size L

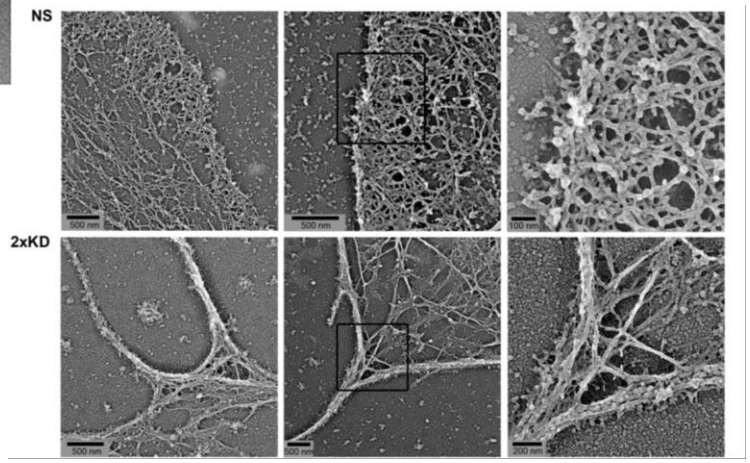
Molecules cross the barrier with probability P_{hop}

Andrade, D. M., Clausen, M. P., Keller, J., Mueller, V., Wu, C., Bear, J. E., Lagerholm, B. C., Eggeling, C. (2015). Cortical actin networks induce spatio-temporal confinement of phospholipids in the plasma membrane – a minimally invasive investigation by STED-FCS. *Scientific Reports, 5*(May), 11454-11454.

Arp2/3 Is Critical for Lamellipodia and Response to Extracellular Matrix Cues but Is Dispensable for Chemotaxis

Congying Wu,[1,4,7] Sreeja B. Asokan,[1,4,7] Matthew E. Berginski,[2] Elizabeth M. Haynes,[1,4] Norman E. Sharpless,[3,4] Jack D. Griffith,[4,5] Shawn M. Gomez,[2] and James E. Bear[1,4,6,*]
[1]Department of Cell and Developmental Biology
[2]Departments of Biomedical Engineering, Computer Science, and Pharmacology
[3]Department of Genetics
[4]Lineberger Comprehensive Cancer Center
[5]Department of Biochemistry
[6]Howard Hughes Medical Institute
University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA
[7]These authors contributed equally to this work
*Correspondence: jbear@email.unc.edu
DOI 10.1016/j.cell.2011.12.034

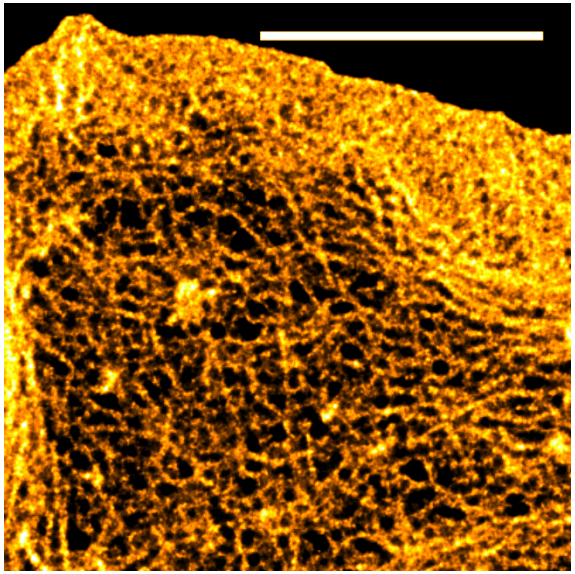




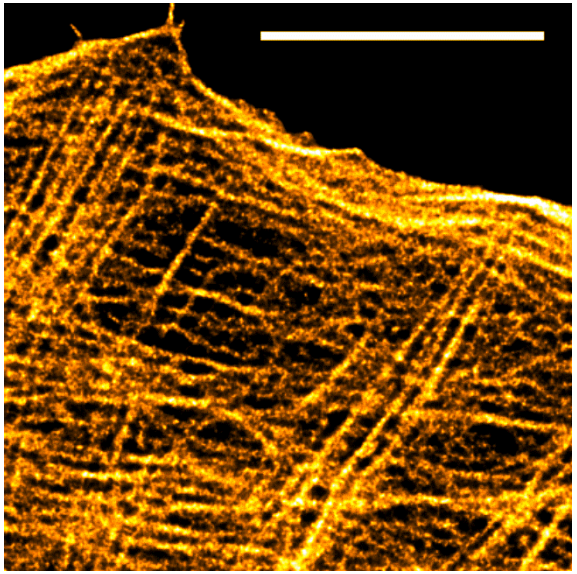
Wu, C., Asokan, S. B., Berginski, M. E., Haynes, E. M., Sharpless, N. E., Griffith, J. D., . . . Bear, J. E. (2012). Arp2/3 is critical for lamellipodia and response to extracellular matrix cues but is dispensable for chemotaxis. *Cell, 148*(5), 973-987.

STED images – F-actin

IA32 MEFs

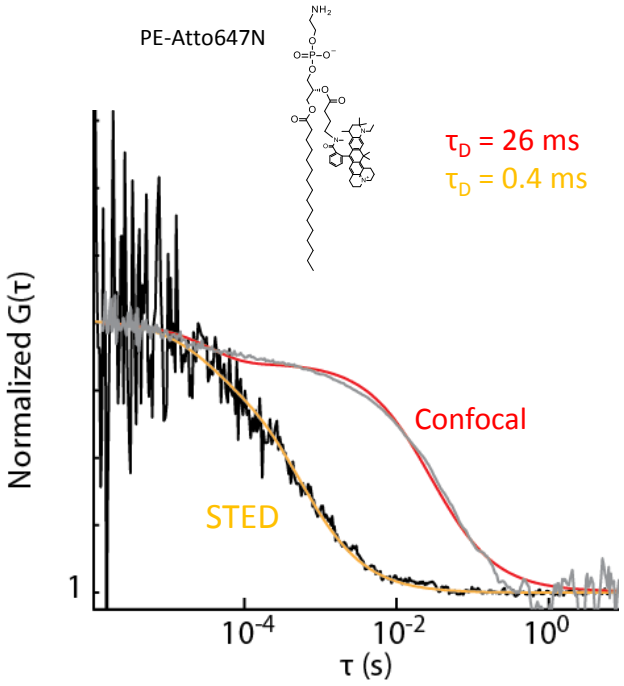


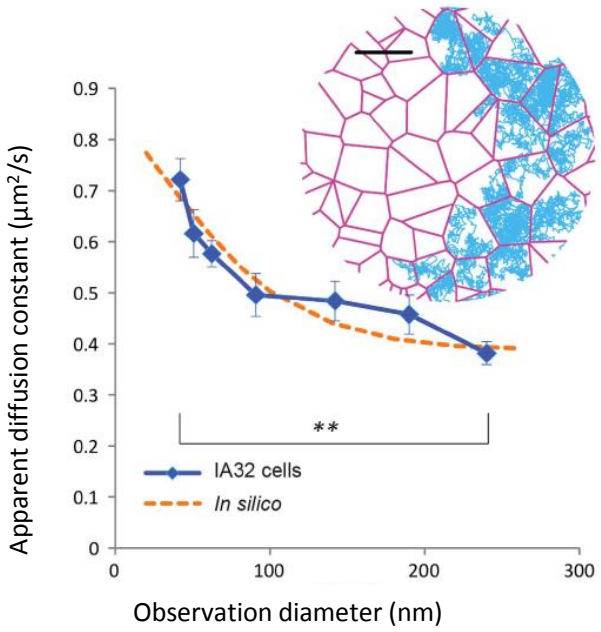
IA32 2xKD MEFs



Scale bar = 5 μm

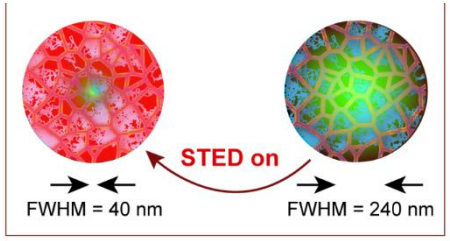
Compartmentalised diffusion in cells



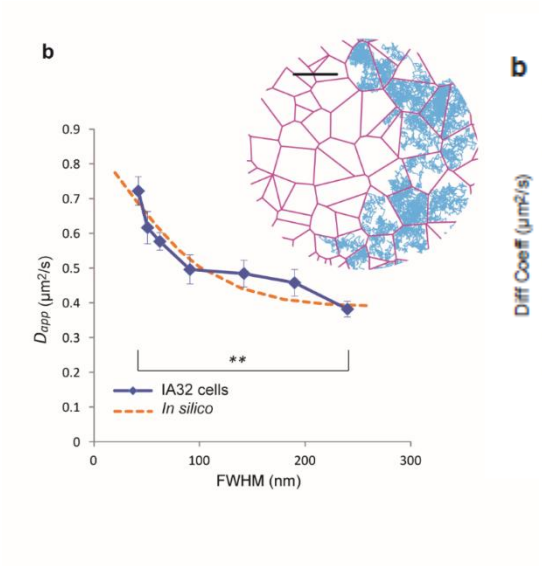


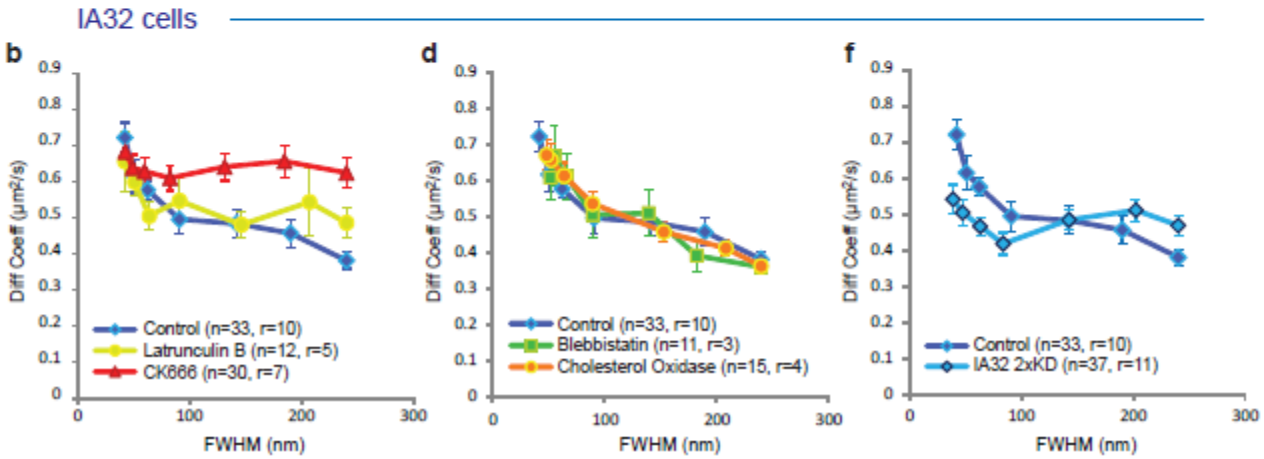
L = 150 nm

STED-FCS with Atto647N-DPPE and Simulation data

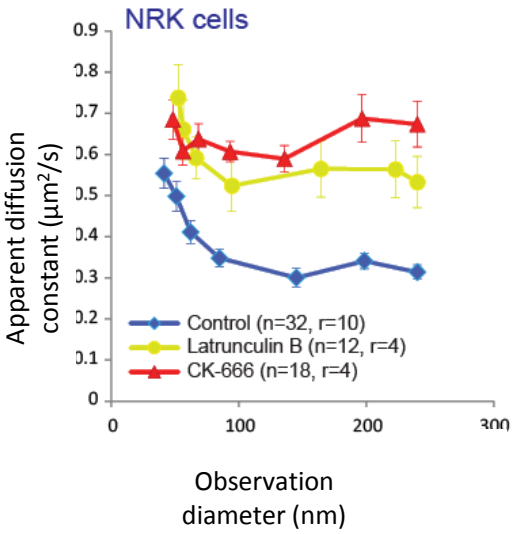


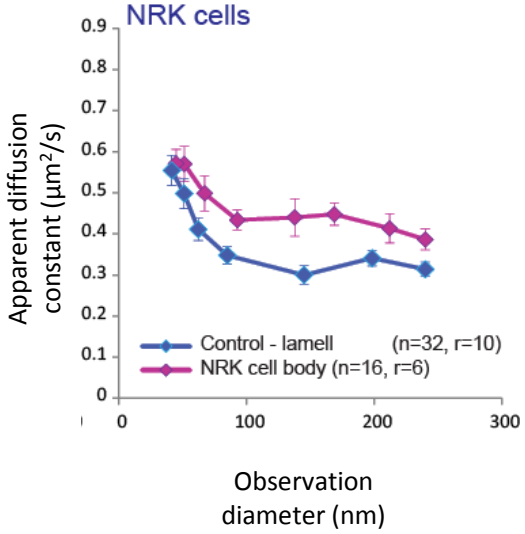
Mechanism of Hop-Diffusion in IA32 MEFs

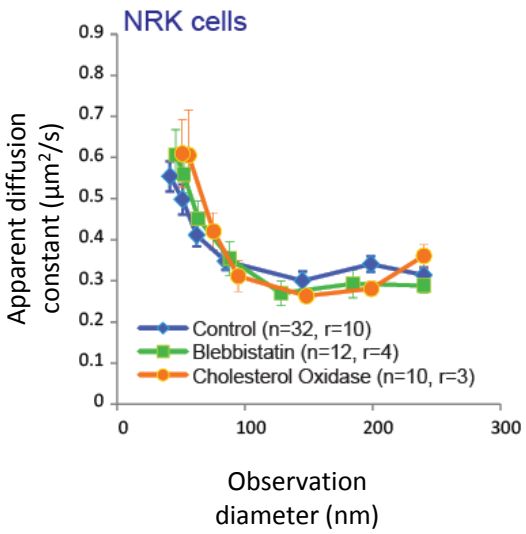




Molecular mechanism behind compartmentalised diffusion







ACKNOWLEDGEMENTS

Funding: Wolfson Foundation, MRC, Wellcome Trust Multi-User Equipment Grant, EPA Fund, ISS, User Access Fees

Quality Control of Leica STED performance

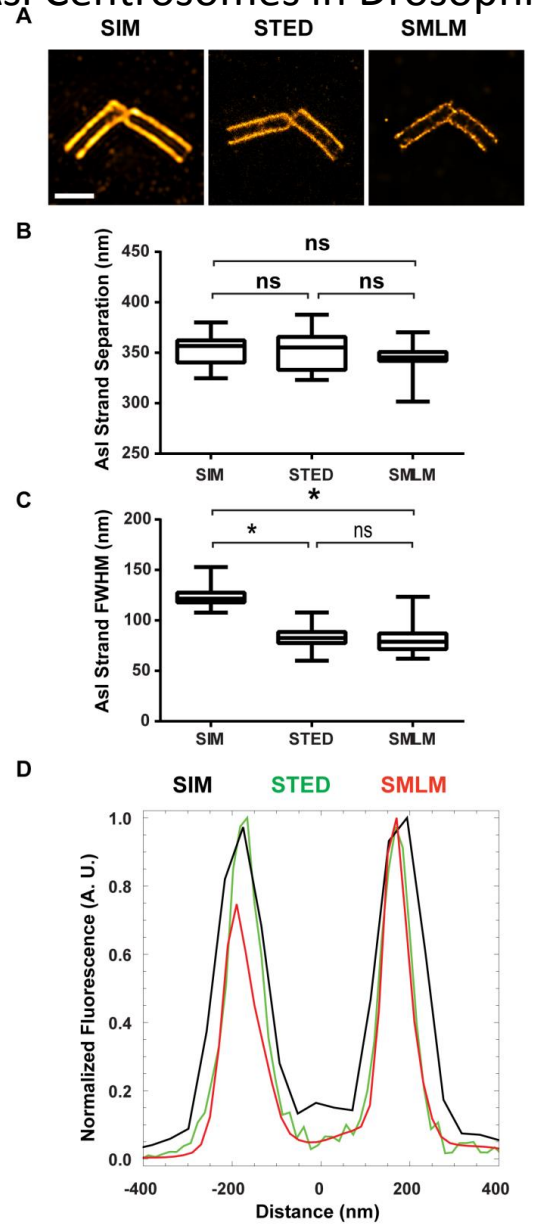
- Dr. Esther Garcia, Wolfson Imaging Centre, U. of Oxford
- Prof. Christian Eggeling; U. of Oxford
- Dr. Dominic Waithe; Dr Silvia Galiani, , U. of Oxford

Image Comparison of SIM, STED, and dSTORM

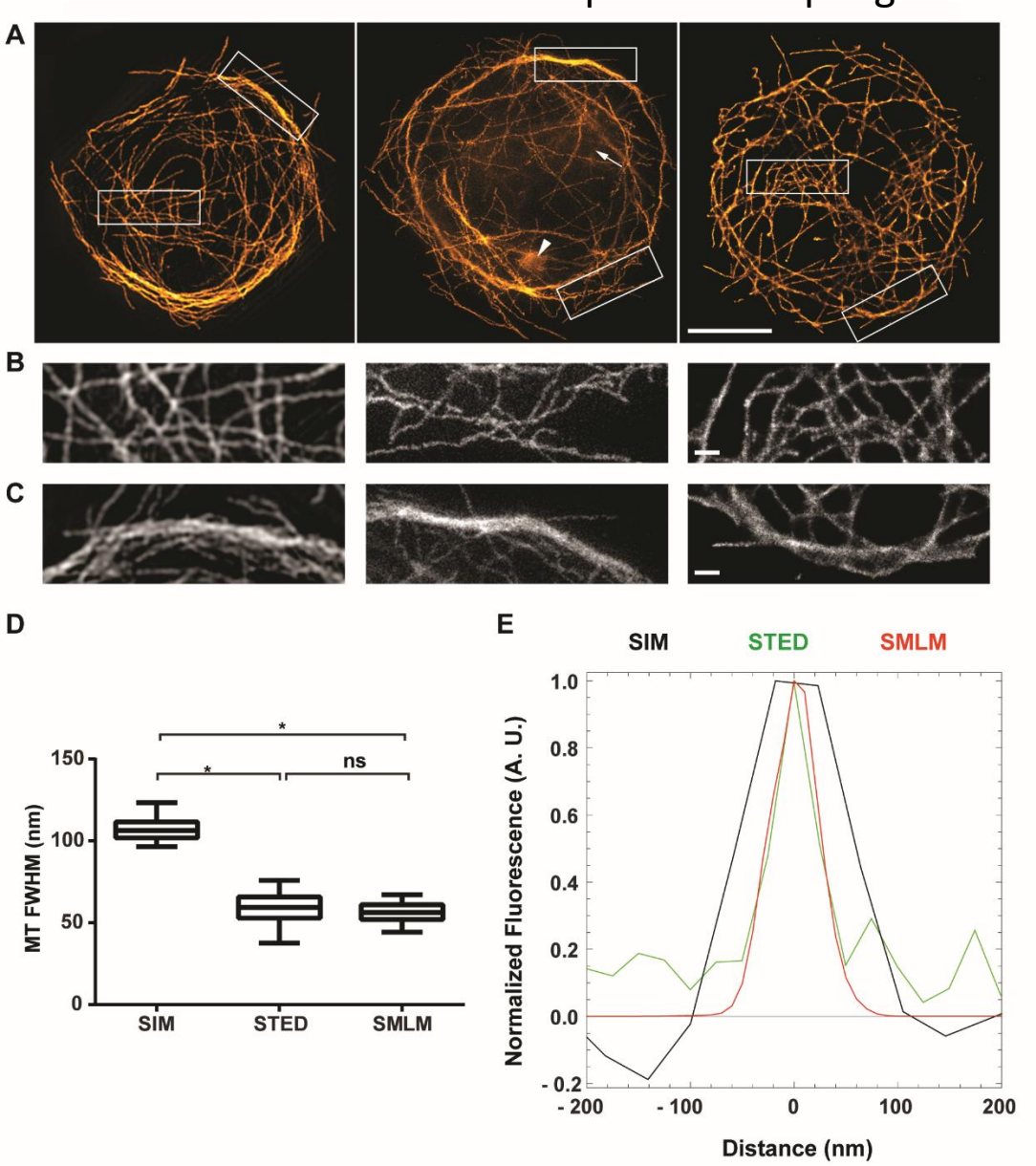
- Dr. Ian Dobbie, Antonia Goehler, Rainer Kauffman, Micron, Biochemistry, U. of Oxford
- Dr. Eva Wegel, John Innes Centre (previously Micron, Biochemistry, U. of Oxford)

Image Comparison of SIM, STED, and dSTORM

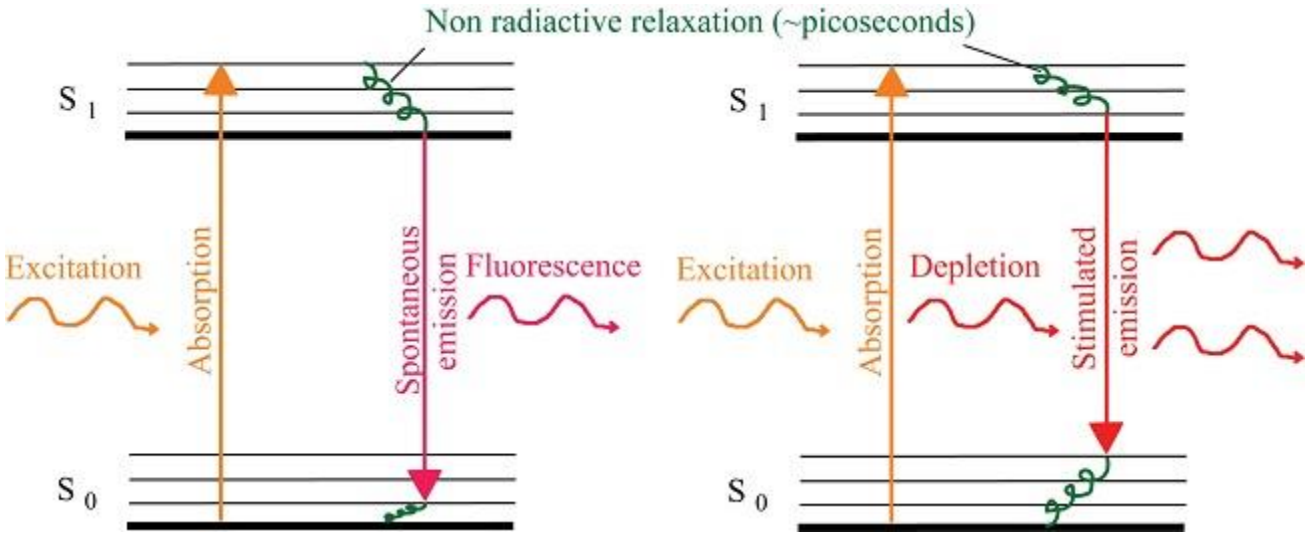
Asl Centrosomes in Drosophila testes



Microtubules in Drosophila macrophages

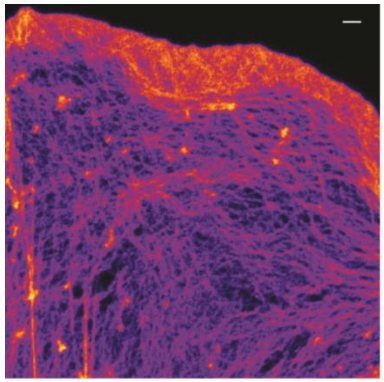


Dr. Ian Dobbie, Antonia Goehler, Alan Wainman, Rainer Kauffman, Micron, Biochemistry, U. of Oxford
Dr. Eva Wegel, John Innes Centre (previously Micron, Biochemistry, U. of Oxford)

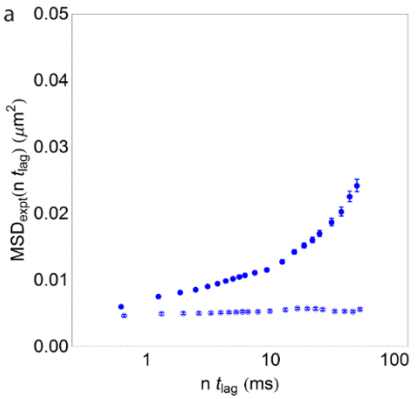

Non radiactive relaxation (~picoseconds)
S 1
S 0
Excitation
Absorption
Spontaneous emission
Fluorescence
S 1
S 0
Excitation
Absorption
Depletion
Stimulated emission

Graphical Overview of Results – SPT & STED-FCS data for lateral diffusion of DPPE in IA32 MEF

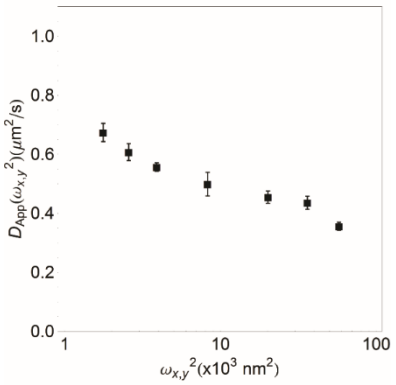
F-actin in IA32 MEF

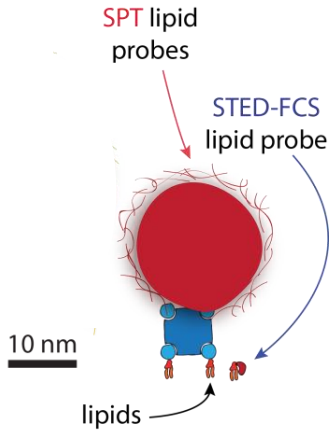


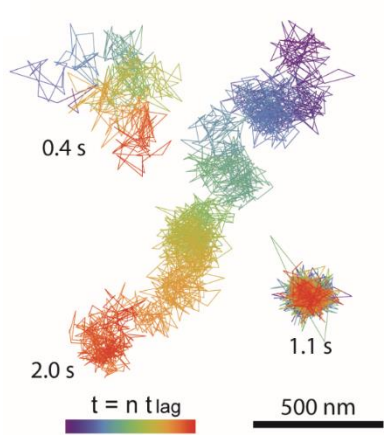
SPT Data

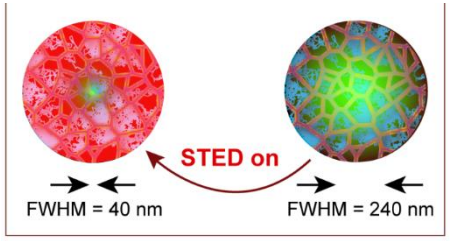


STED-FCS Data

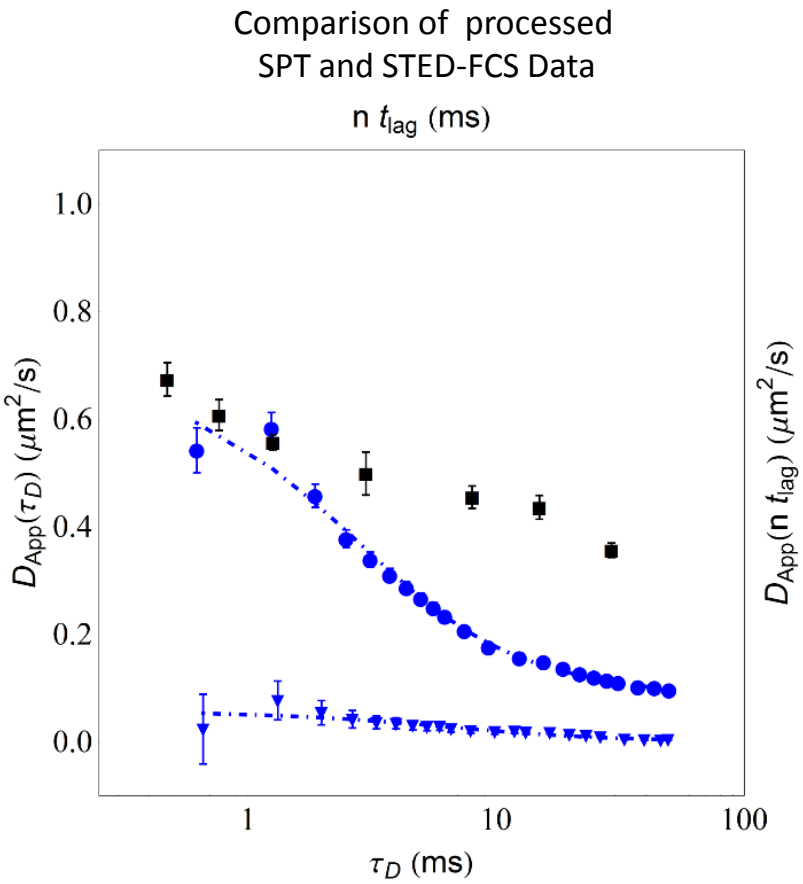


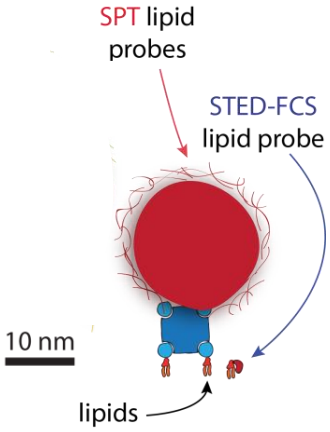




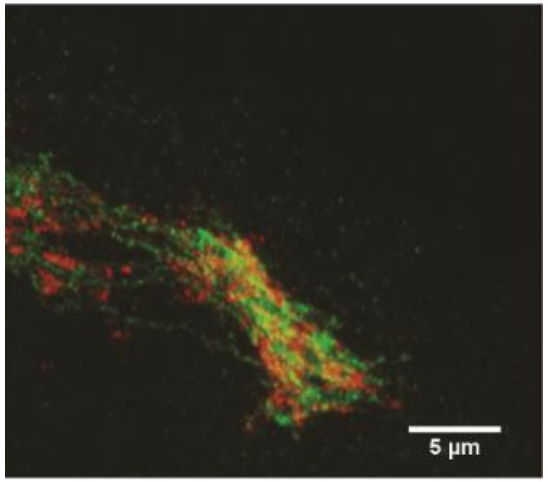


Graphical Overview of Results – SPT & STED-FCS data for lateral diffusion of DPPE in IA32 MEF

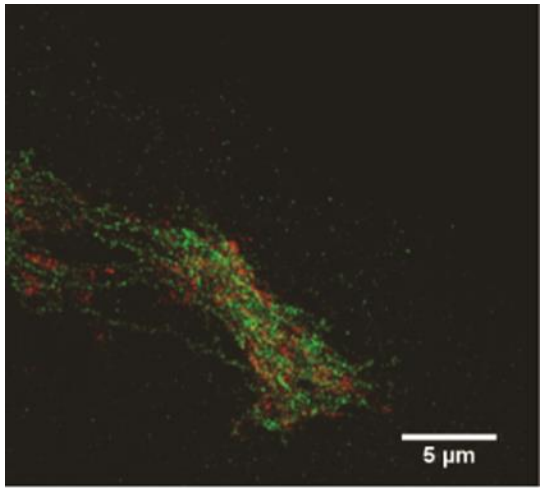




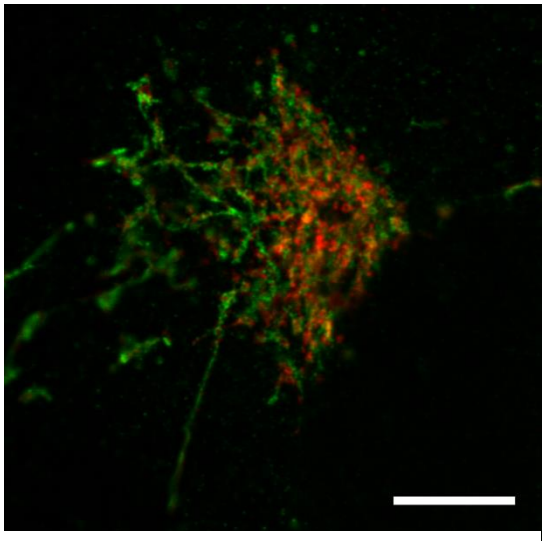
Confocal



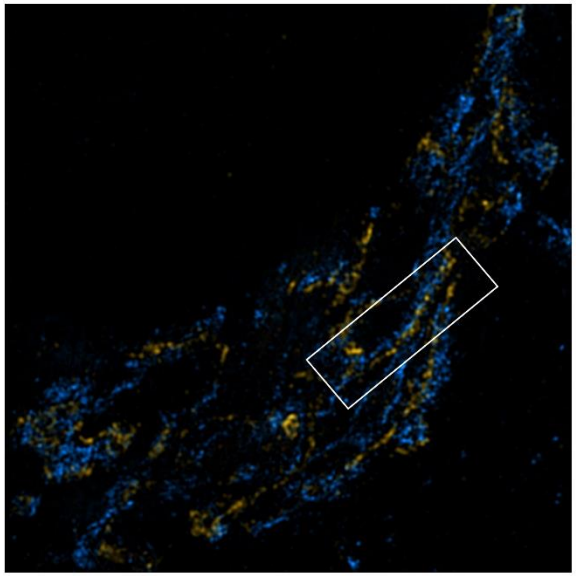
Zeiss Airyscan



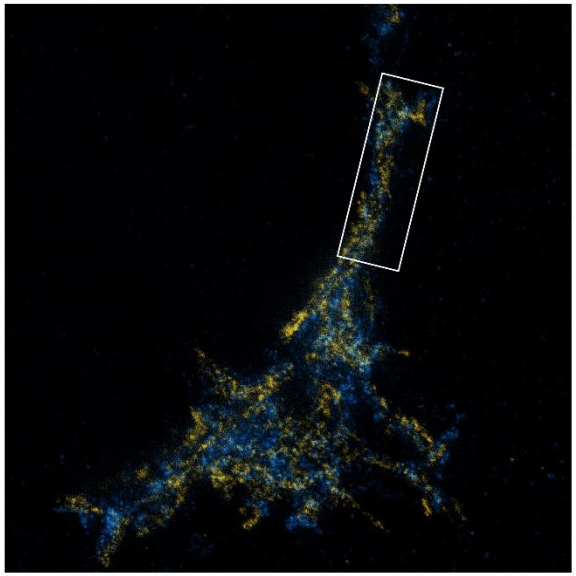
Olympus FV1200 Super Resolution Software Module



SIM



STED



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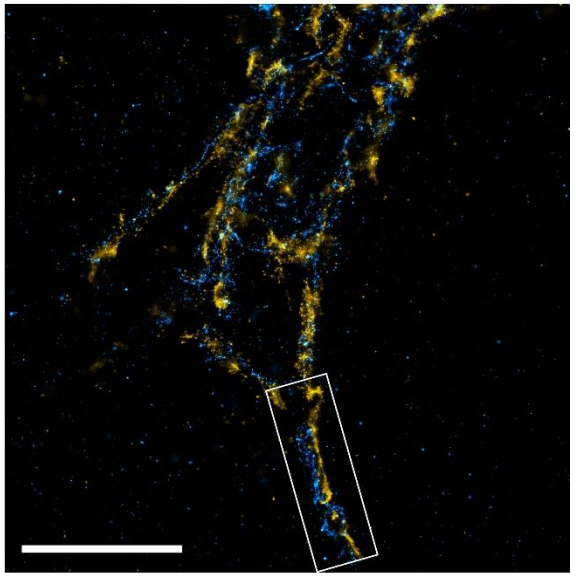

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