FCS and STED-FCS

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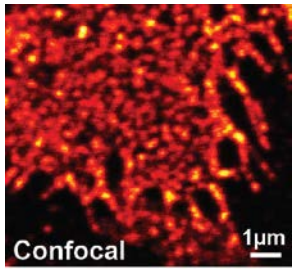
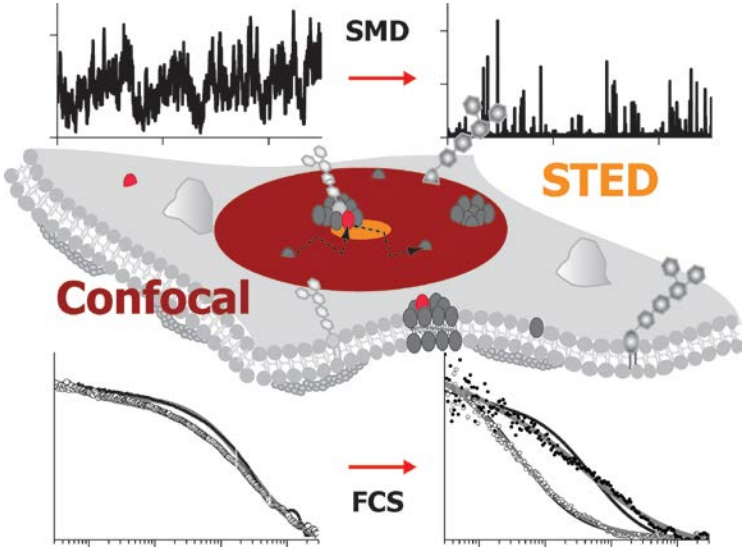
Imaging





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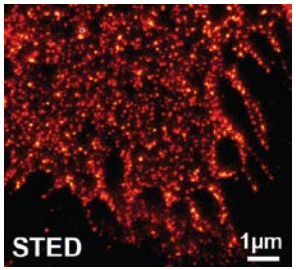


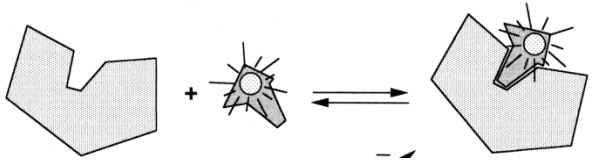
Molecular Diffusion/Mobility

Bioactivity

Example 1

Binding of small peptide to large protein ⇒ increase of mass ⇒ increase of diffusion time τ_D

⇒ Determination of binding affinity via mobility

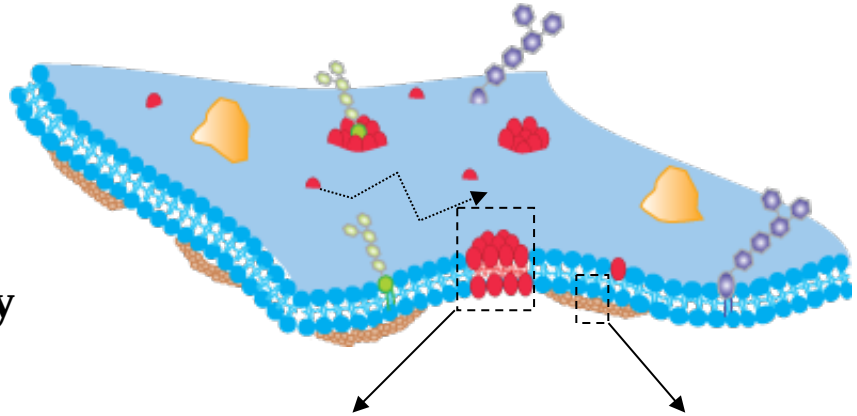


Example 2

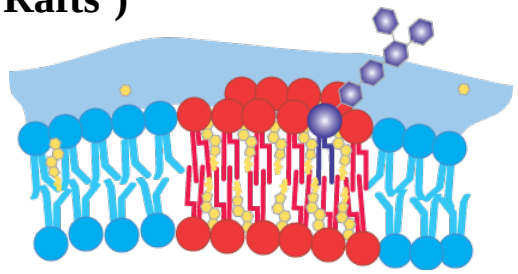
Plasma Membrane:

Constrained diffusion on the nanoscale

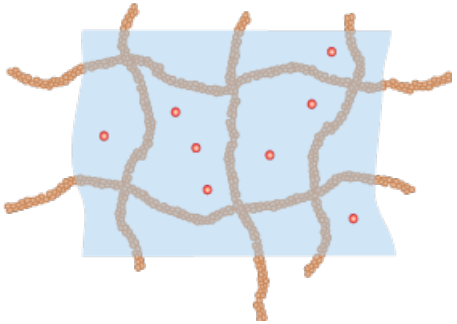
⇒ determines bioactivity



Lipid-dependent Nano-/Microdomains ('Rafts')

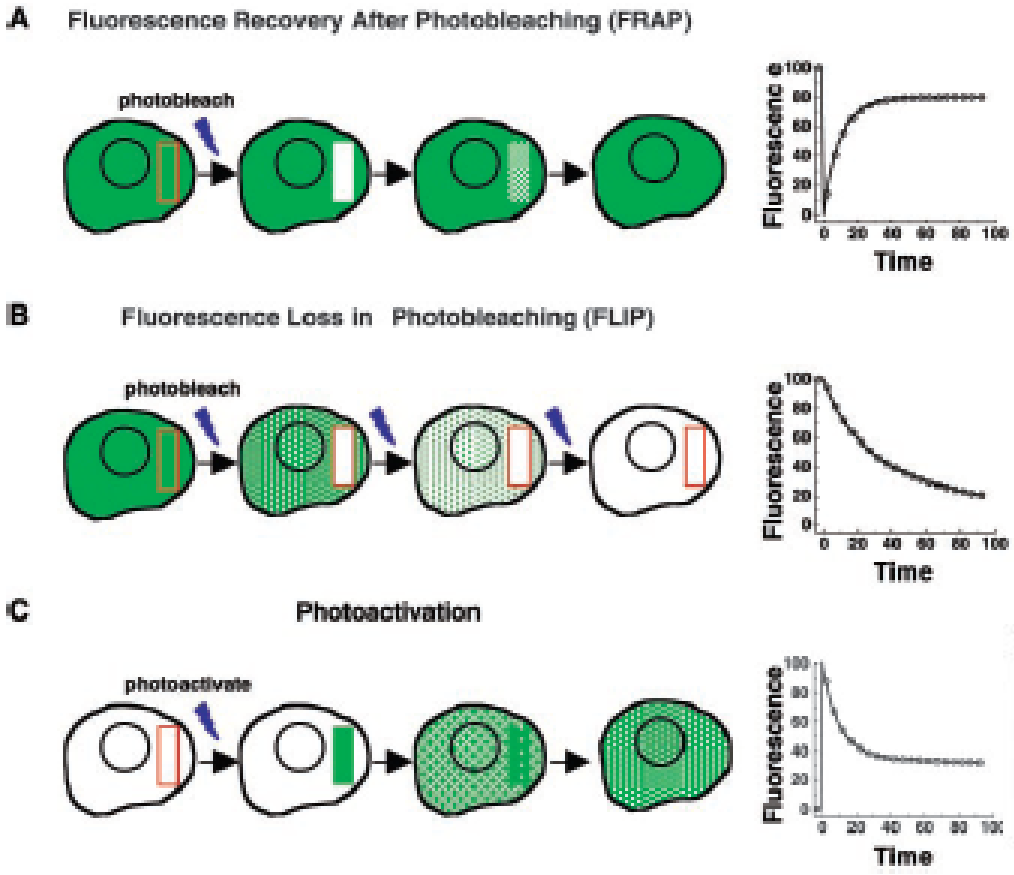


Cytoskeleton- based Meshwork



Molecular Diffusion/Mobility

Bioactivity



Measuring Mobility in Cells

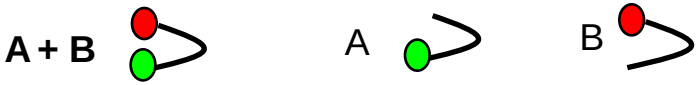
- FRAP

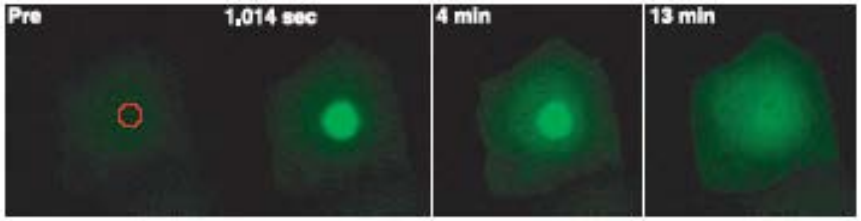
(Fluorescence Recovery After Photobleaching)

- Photoactivation

Problem

- Trigger – disturb system?
- Correlated diffusion





Photoactivatable fluorescent proteins
(PA-GFP. Kaede, Dronpa, asFP595, …)

Lippincott-Schwartz, Science 2003

Molecular Diffusion/Mobility
Single-Molecule Detection

Aim observe biochemical reaction in equilibrium

Why Single-Molecule based experiments?

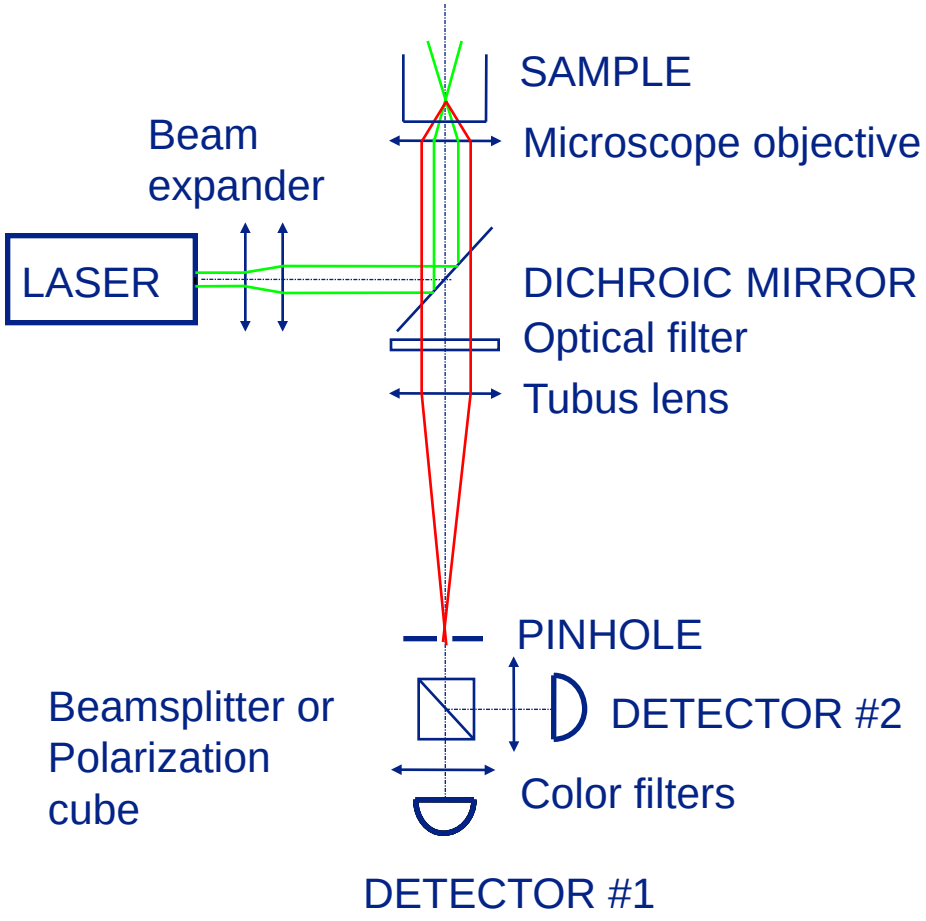
Monitor thermodynamical fluctuation around equilibrium due to kinetics/diffusion
$\Rightarrow$ no trigger of experiment

Analysis on single-molecule level
$\Rightarrow$ distinguish / quantify components with different molecular characteristics
(e.g. weight, brightness)

$\Rightarrow$detect (small) heterogeneities

Far-Field Fluorescence Spectroscopy

Single-Molecule Detection



Background Reduction

⇒ **small detection volume (femto-liter)**
⇒ **confocal detection**

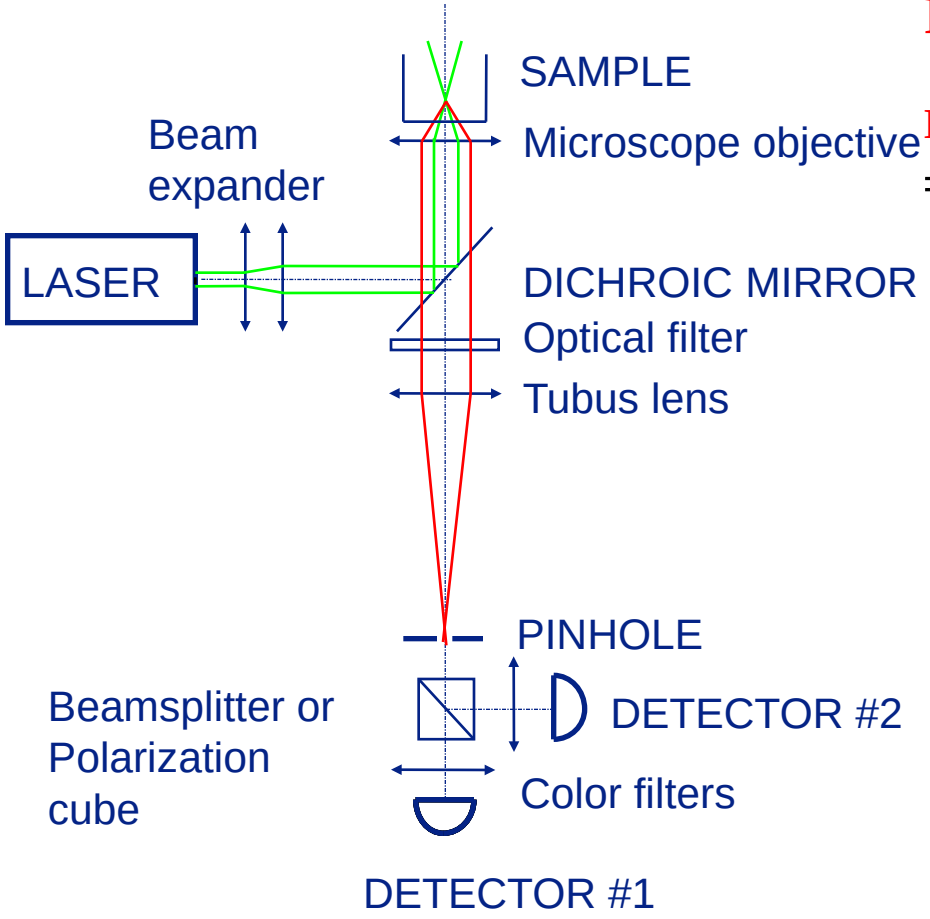
Not a problem to detect signal from single molecule

But: problem to see it in front of background!

Enhanced fluorescence detection
Multi-color (excitation + detection)
Polarized excitation + detection
Fluorescence lifetime (pulsed exc. + TCSPC)
Raw data detection (photon-by-photon)
Online analysis (correlation, distribution)

Far-Field Fluorescence Spectroscopy

Single-Molecule Detection

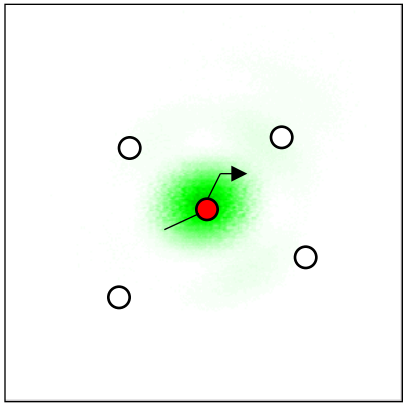


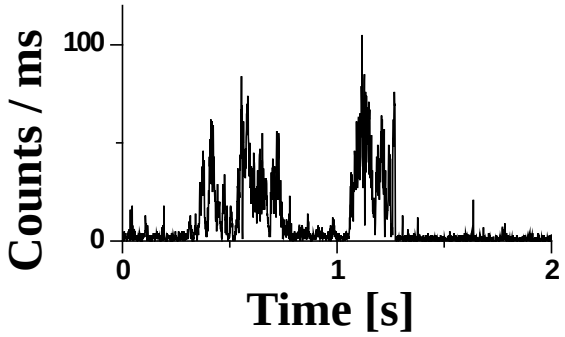
Fluorescence intensity over time

Low fluorescent concentration

⇒ **diffusion of single-molecules**

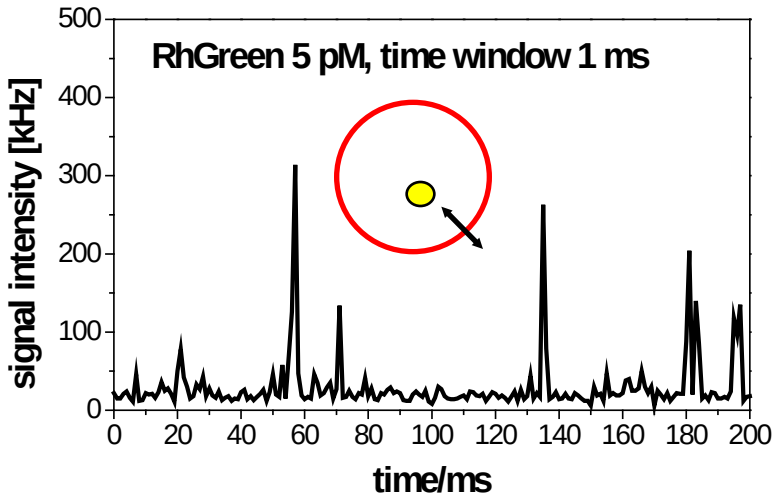
= fluorescence bursts





Single-Molecule Detection

Signal Fluctuations



Single molecule

-diffusion in/out detection volume

-fluorescence changes due to reaction kinetics

⇒ **Fluctuations**

Only detectable if mean number of observed molecules small (near one)

⇒ **small detection volume** (< µm)

⇒ **low concentration** (nM)

Information

Length – diffusion time (or reaction kinetic)

Height – brightness

Density – concentration

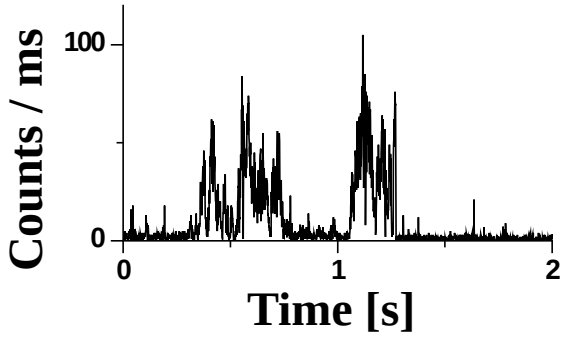
Analysis

Statistics in **time** – correlation

Statistics in **amplitude** – distribution analysis

Fluorescence Fluctuation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS)

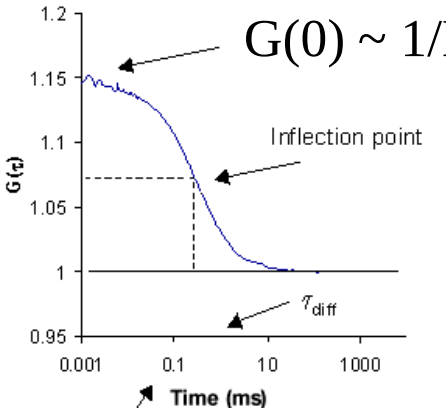


data acquisition

calculation of correlation function

$$G(\tau_c) = \frac{\sum_t A(t) \cdot A(t+\tau_c)}{normalize}$$

Statistics on Time Axis



logarithmic x-axis

data analysis

$$G(\tau_c) = 1 + (1/N)[1 - T + T\exp(-\tau_c / t_T)] \times (1 - \tau_c / \tau_{diff})^{-1} \times (1 + (\omega / z)^2 \tau_c / \tau_{diff})^{-1/2}$$

⇒ **<u>mean transit time</u>** τ_d (~ mass) of each fluorescent species – diffusion coefficient

⇒ **concentration N** (mean number of particles) N = conc * V_{Det}

⇒ **kinetic rate constants**

Small = fast

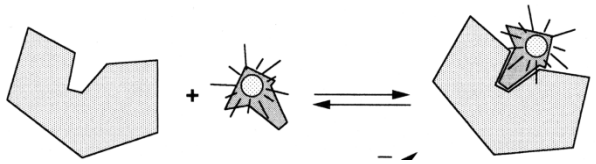
Big = slow

FCS

Application: Mobility Measurement

FCS-Example

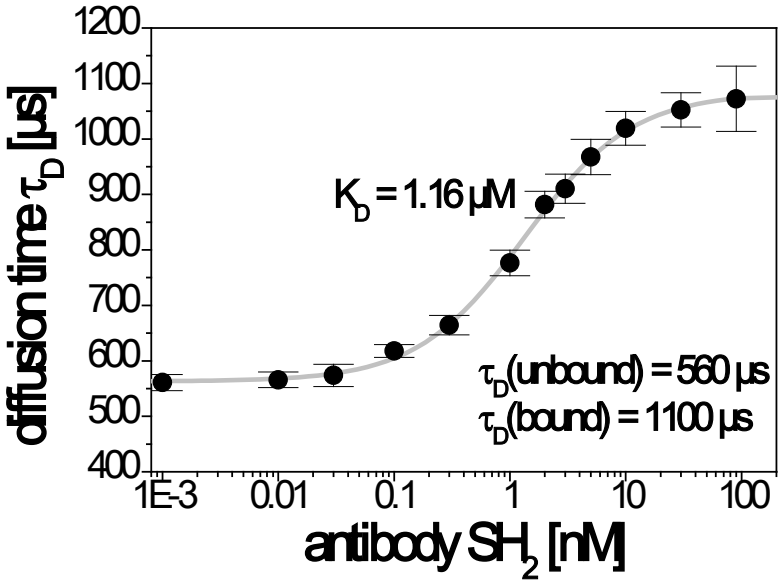
Binding of small peptide to large protein ⇒ increase of mass ⇒ increase of diffusion time τ_D



Measurement

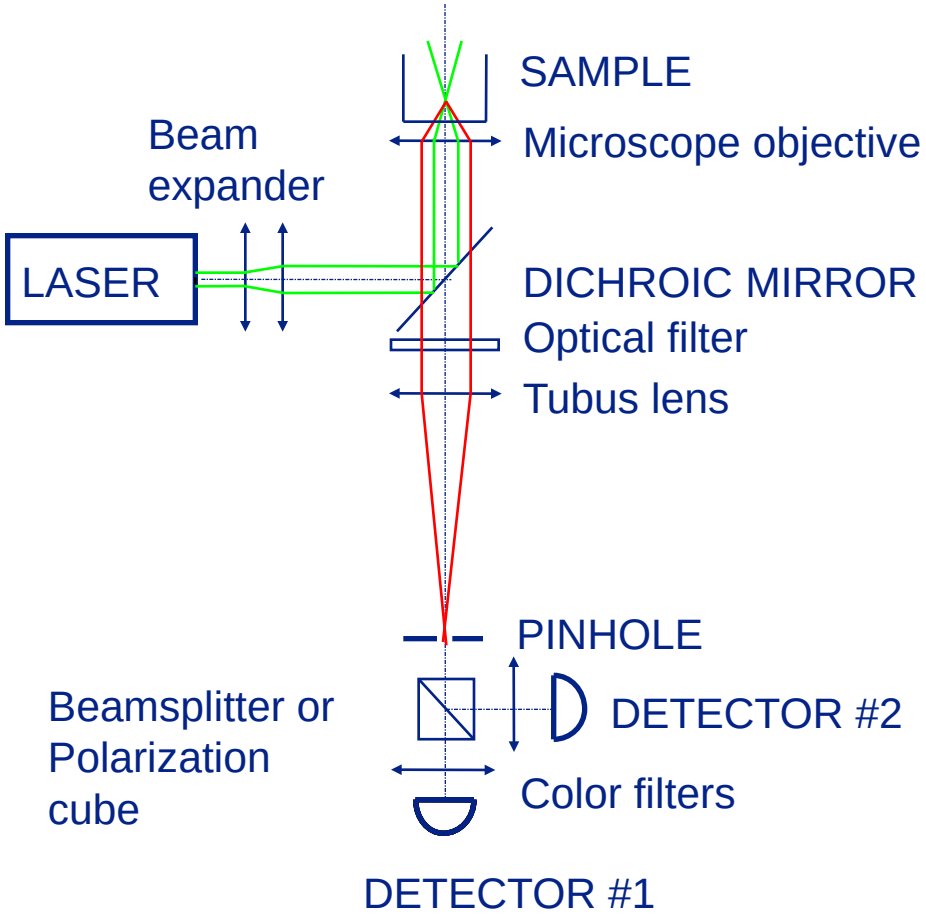
Tyrosine Kinase Growth Factor Receptor Bound
Protein [Grb2 SH2]
+ **labelled ligand**
(phosphotyrosine Ac-Y*VNVK(Cy5)-CO-NH2)

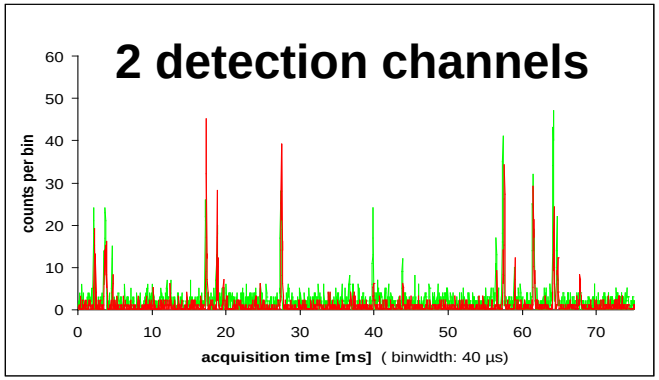
Titration of Grb2 SH2 (10 s measurement time)



Fluorescence Fluctuation Spectroscopy

Two-Color FFS –FCCS





Coincidence/cross correlation

A + B

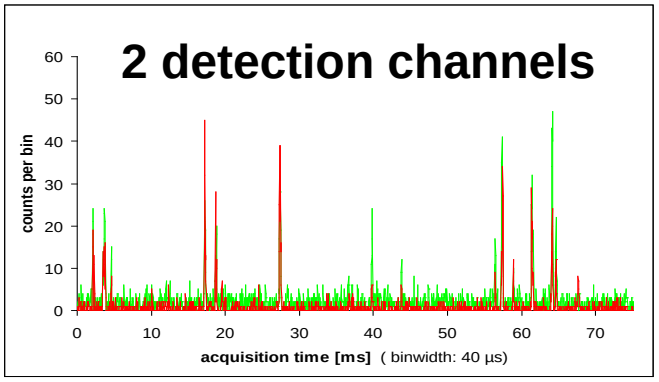
A

B

no coincidence/cross correlation

Fluorescence Fluctuation Spectroscopy

Two-Color FFS – 2D-FIDA and FCCS



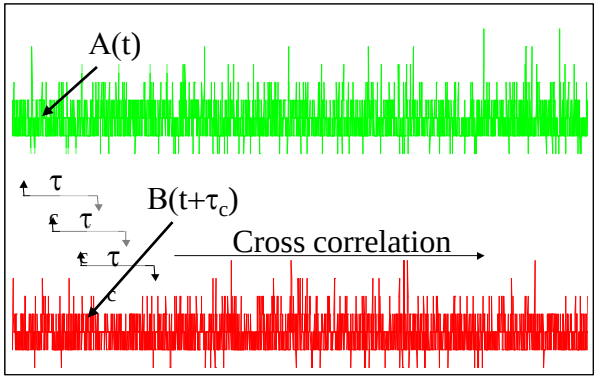
Coincidence/cross correlation

$\mathbf{A + B \Rightarrow N_{AB}}$

A

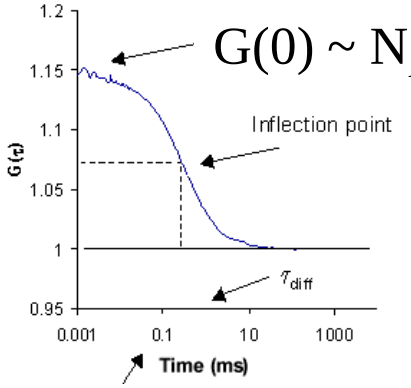
B

no coincidence/cross correlation



$$G(\tau_c) = \frac{\sum_t A(t) \cdot B(t+\tau_c)}{normalize}$$

FCCS (cross correlation)

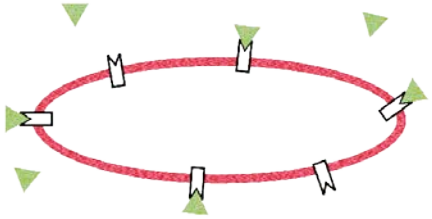


Fluorescence Fluctuation Spectroscopy

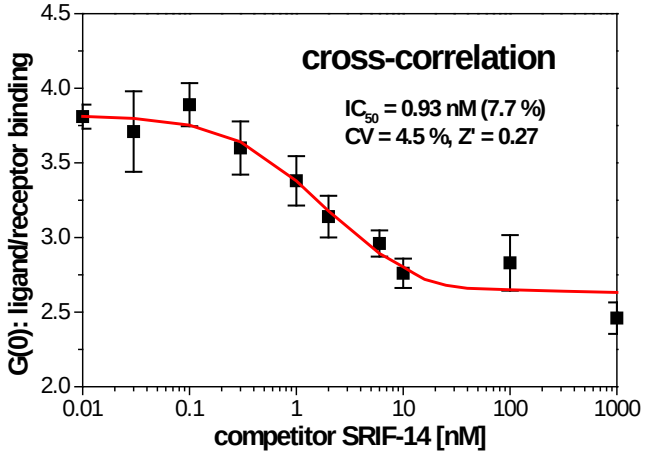
Two-Color FFS – 2D-FIDA and FCCS

SOMATOSTATIN Receptor (SST2)

• Human Type-2 High Affinity SOMATOSTATIN **Receptor** (SSTR2) + small fluorescently TAMRA-l**abelled peptide ligand** (Somatostatin-14 , SMS)
• Small Membrane **Vesicles** from overexpressing CCL39 cells (stained with red dye DiD) carrying SSTR-2
Somatostatin-14 (SRIF-14) as **competitor** (unlabelled)
•**controls: q(unbound) = 12 / 0 kHz + 0 / 300 kHz**
q(bound) ≈ 800 / 300 kHz
•**analysis: 3-components (coincidence) ⇒ fraction bound/coincidence**



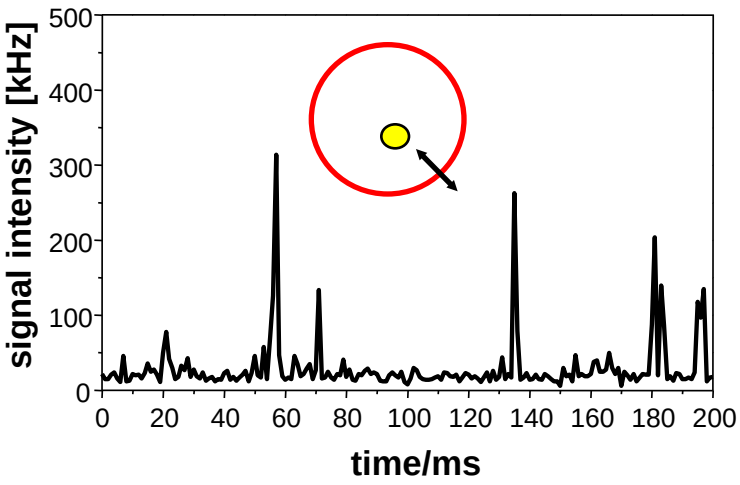
Competition with SRIF-14

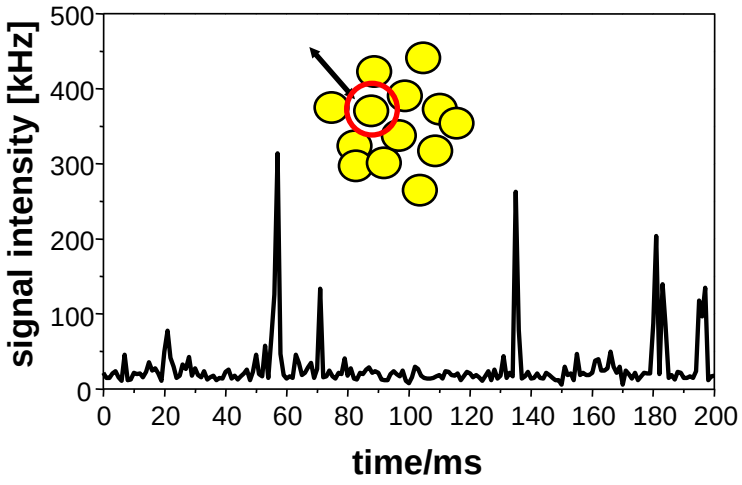


Fluorescence Correlation Spectroscopy

Use of STED Nanoscopy?

FCS – need for low concentration (< 20 nM)





Concentration Problem

endogenous protein / molecule concentration higher

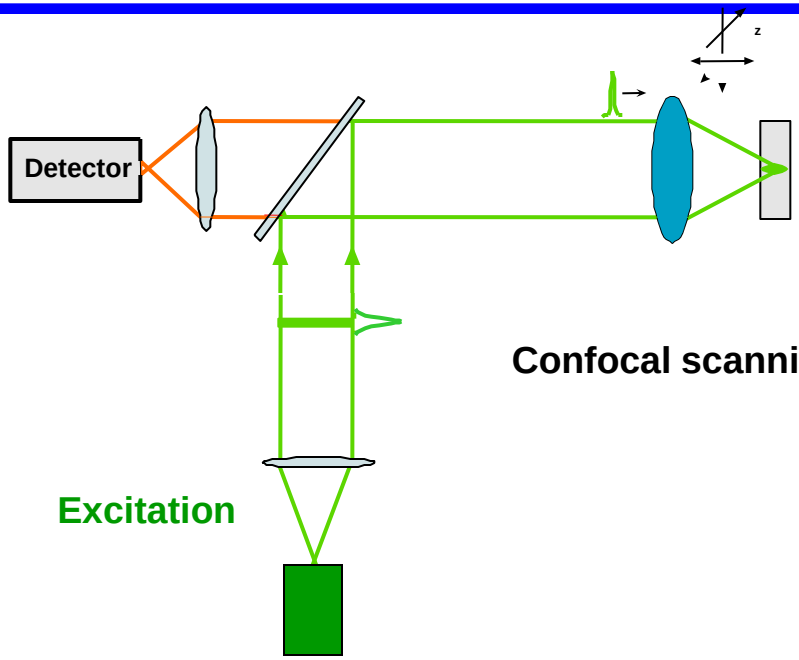
low affinity binding or enzymatic activity reactions - high concentration (~ 1µM)

⇒ smaller/more confined detection volumes:

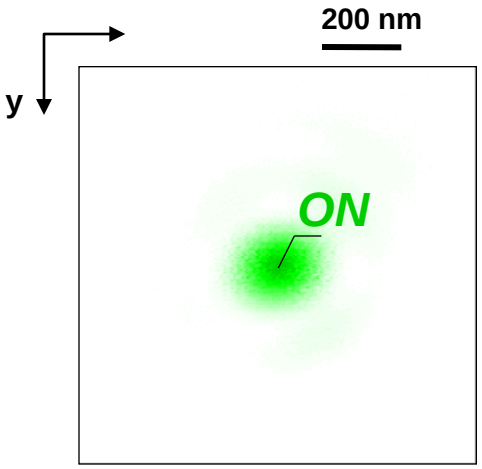
measure at higher concentration!

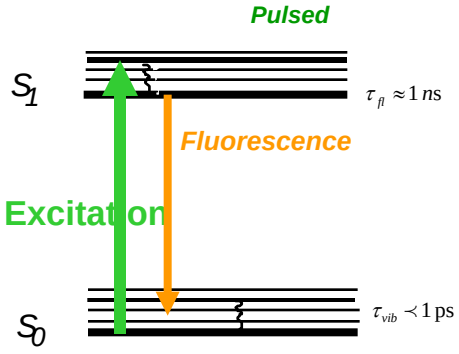
Fluorescence Microscopy

STED Microscopy



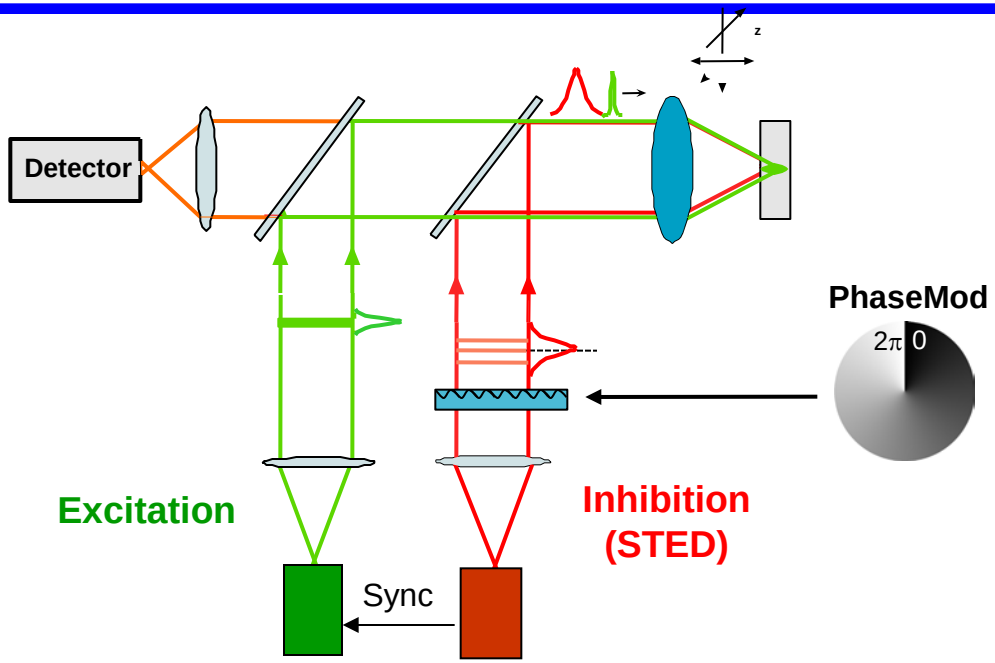
Confocal scanning microscope

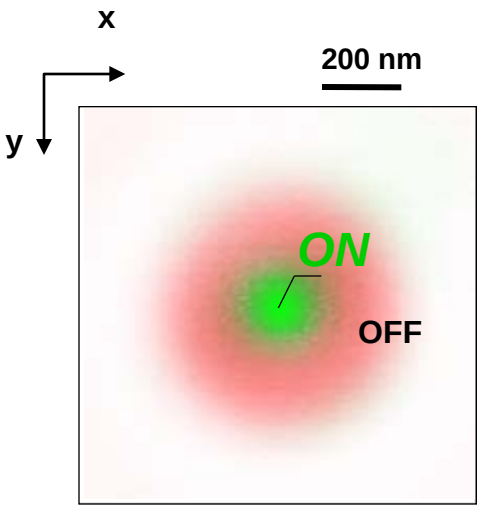


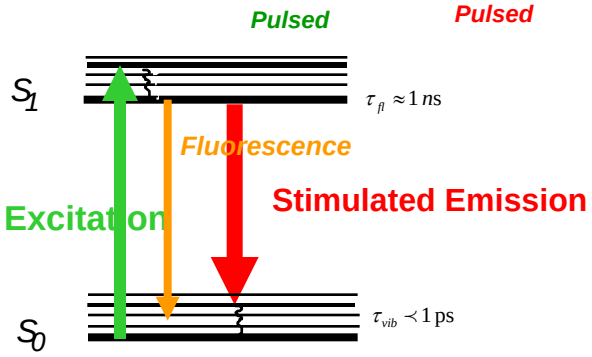


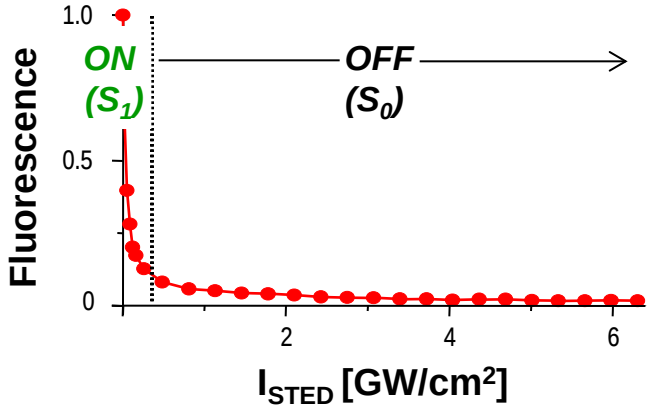
Fluorescence Microscopy

STED Microscopy



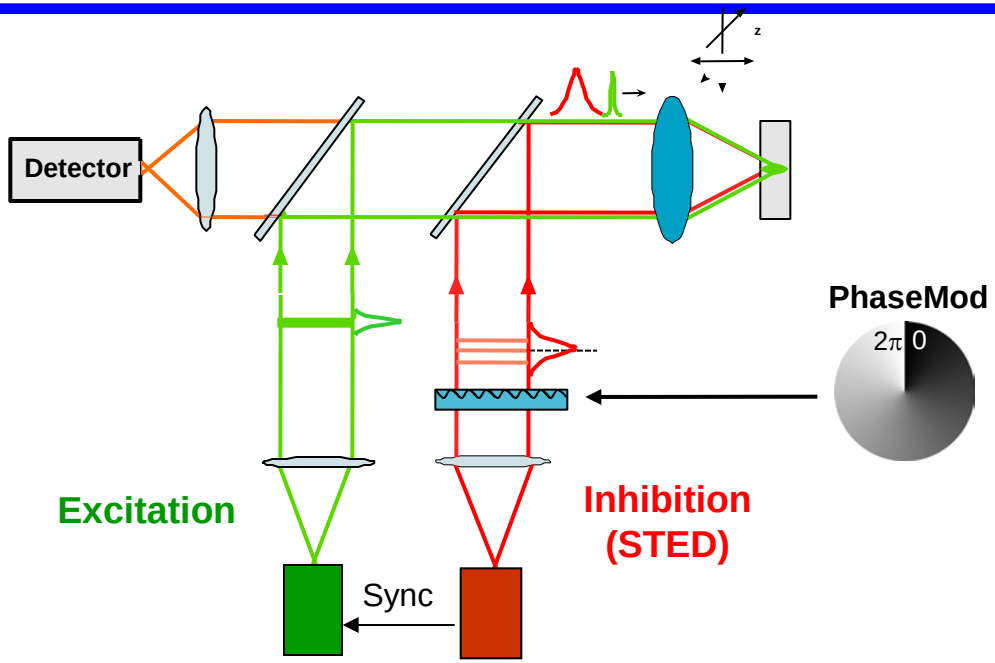


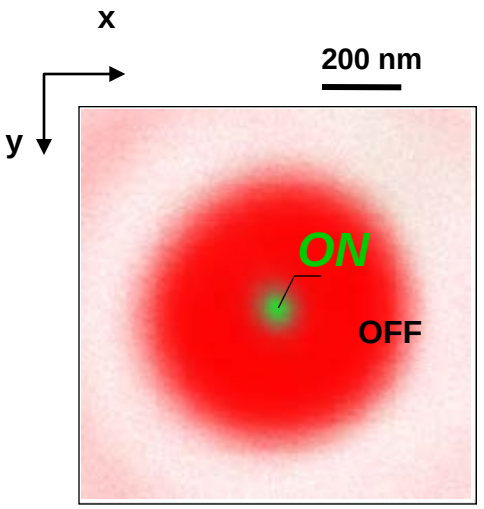


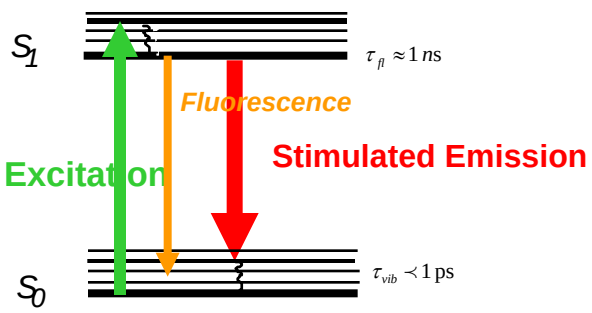


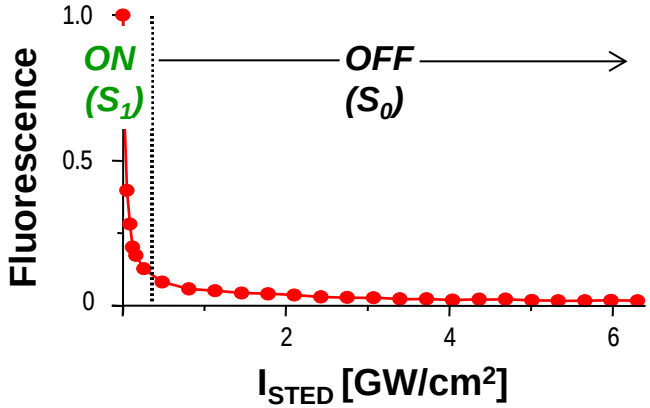
Fluorescence Microscopy

STED Microscopy





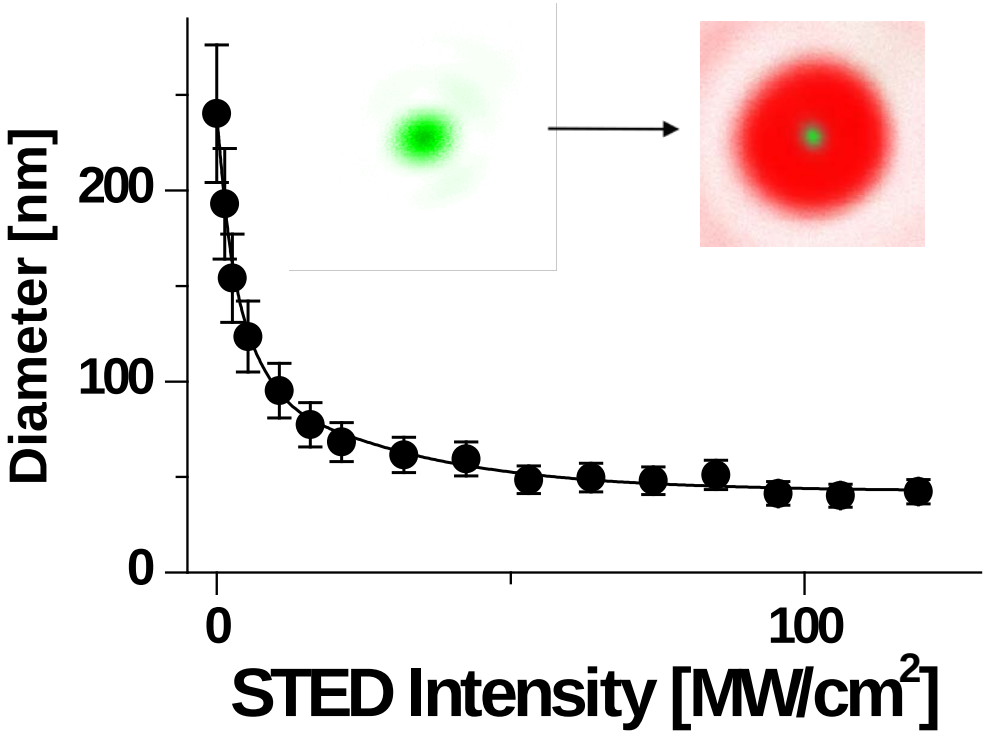




STED Microscopy

Dynamical confinement of resolution

Nanoscale observation areas: CONTINUOUS TUNING of spatial resolution!



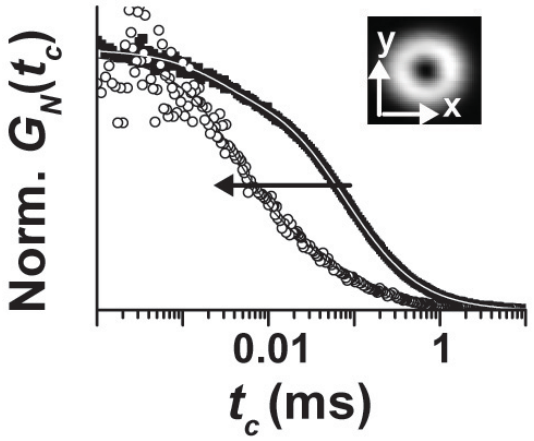
$$\Delta r \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+I/I_{sat}}}$$

Fluorescence Correlation Spectroscopy

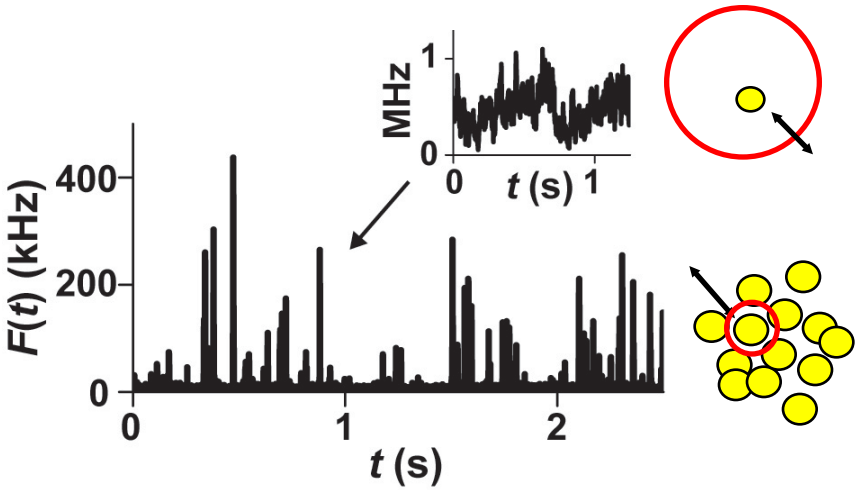
STED Nanoscopy: Concentration Issue

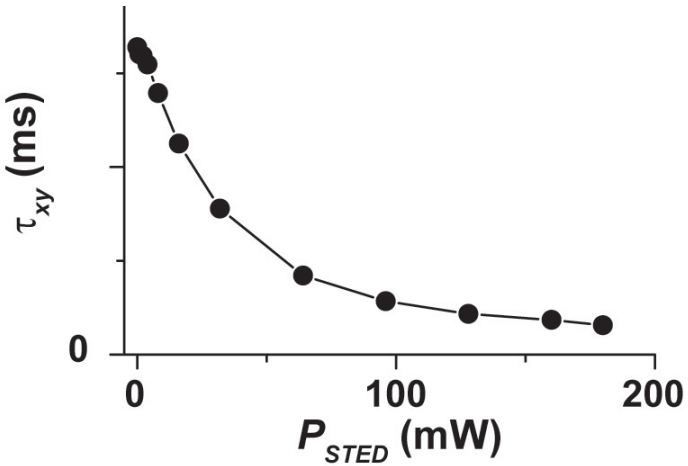
STED-FCS in open volumes: Atto647N in aqueous solution

FCS data with increasing STED power, i.e., increasing focal confinement



Diffusion time decreases as expected (10-fold)

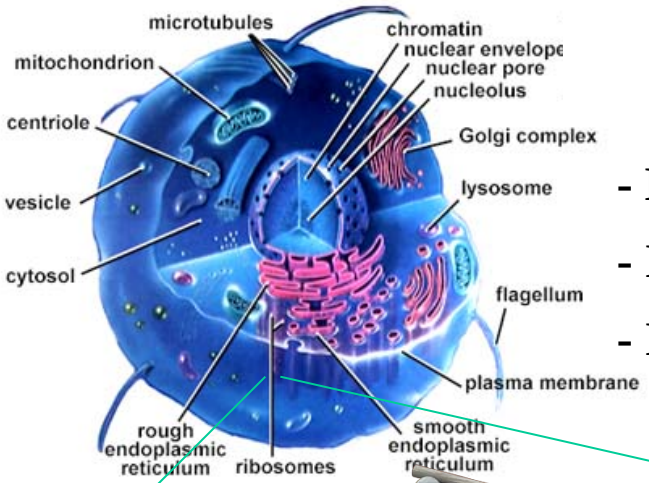




Ringemann et al.
New J. Physics 2009

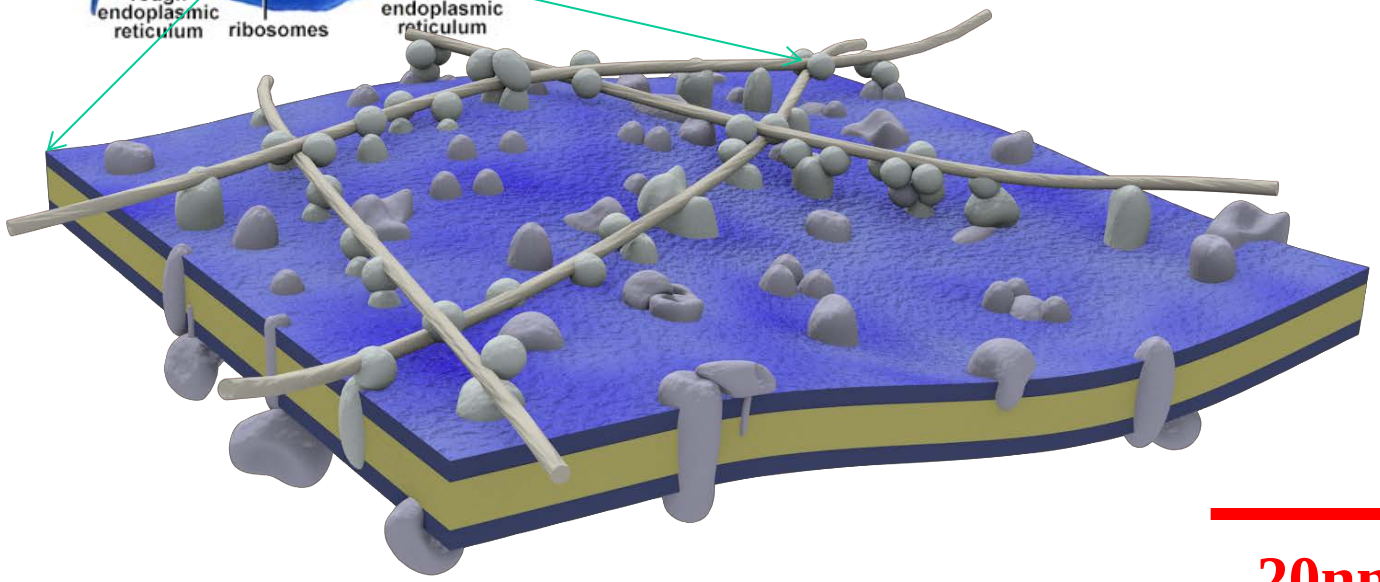
Lipid Plasma Membrane Organization

Nanoscale



Lipid Plasma Membrane Organization:

- **Heterogeneous distribution (viscosity, curvature, …)**
- **Interaction with proteins**
- **Interaction with cortical cytoskeleton**



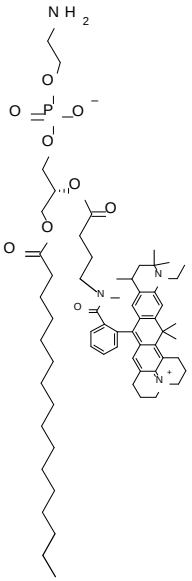
20nm

Small spatial scales!!!!

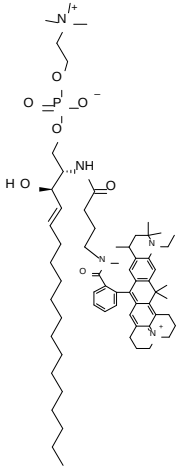
Lipid Plasma Membrane Organization

Fluorescence Recordings: Lipids

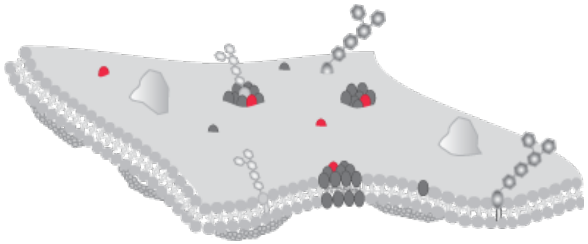
Phosphoglycerolipid:
Atto647N-phosphoethanolamine (PE)

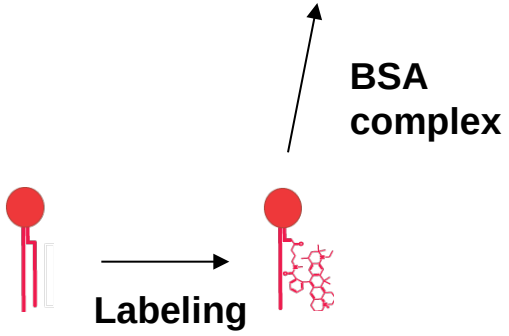


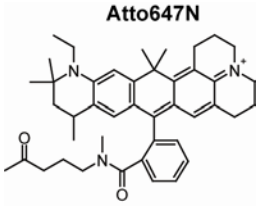
Sphingolipid:
Atto647N-sphingomyelin (SM)



Live PtK2 cells:
physiological conditions
incorporation in plasma membrane

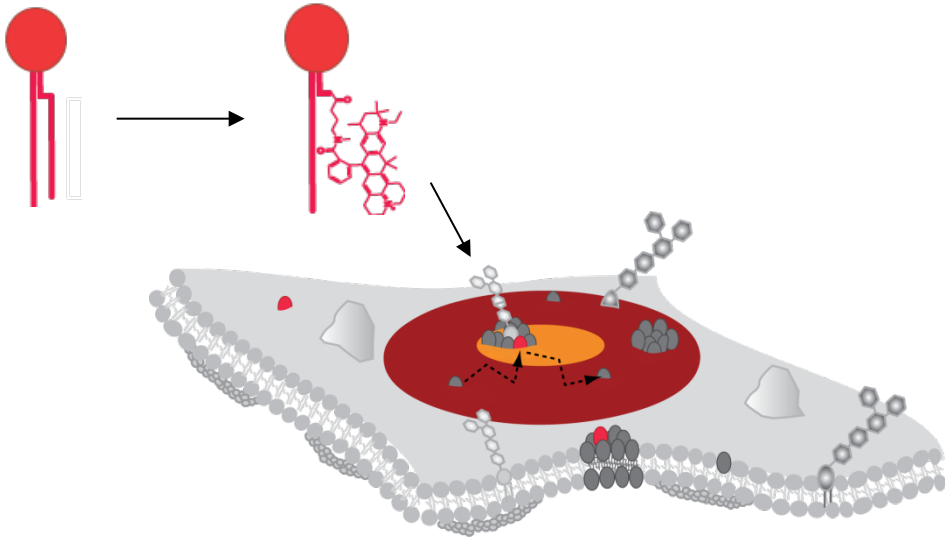


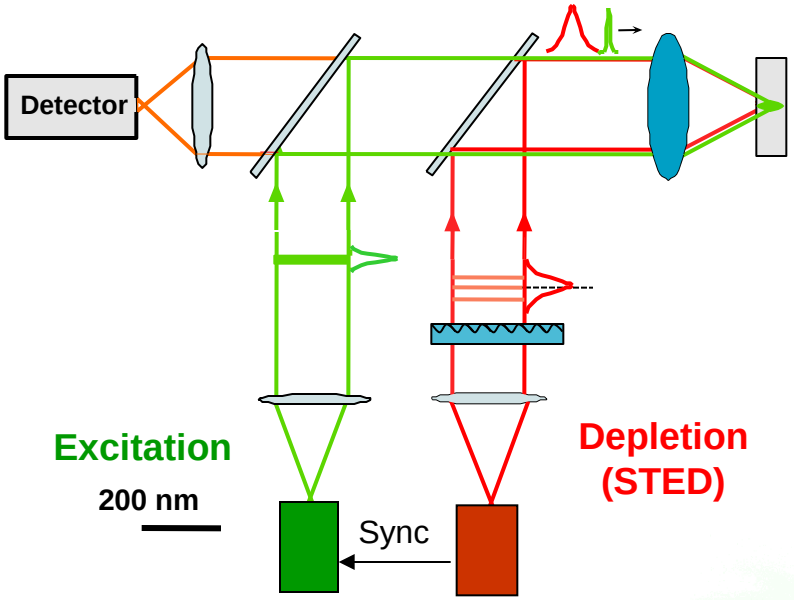


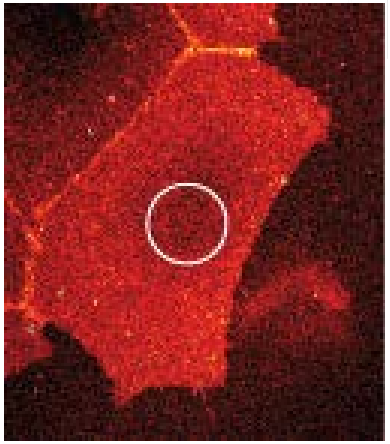


Lipid Plasma Membrane Organization

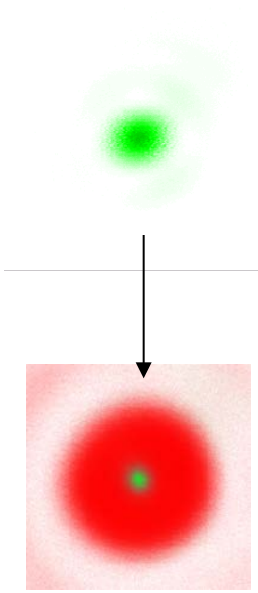
STED Nanoscopy Measurement





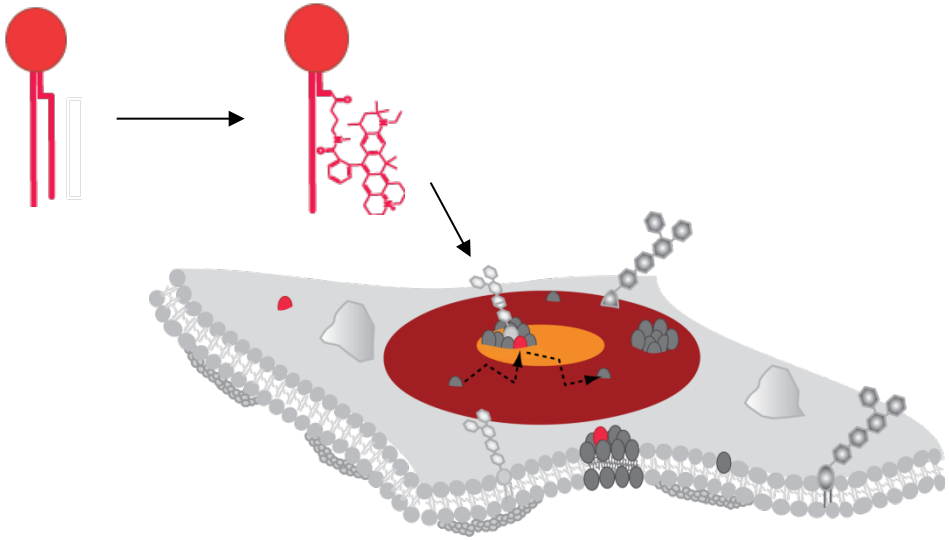


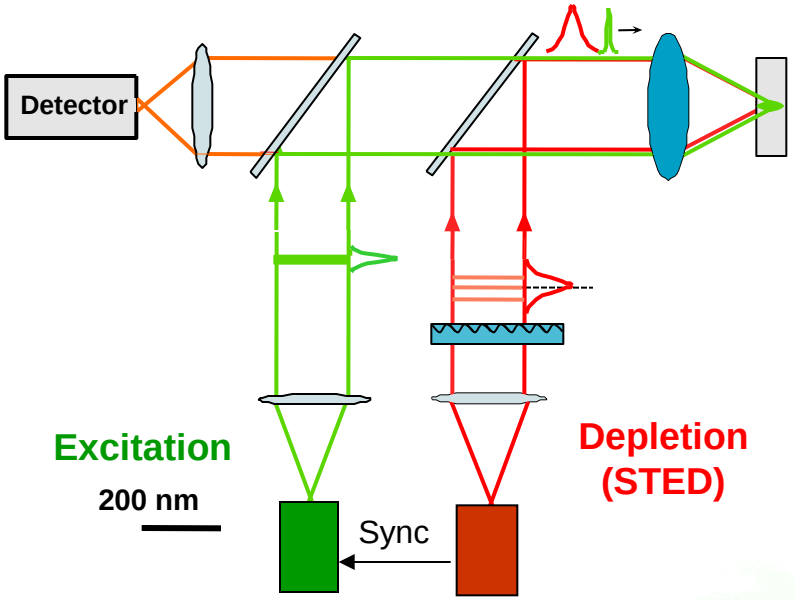
Homogeneous distribution

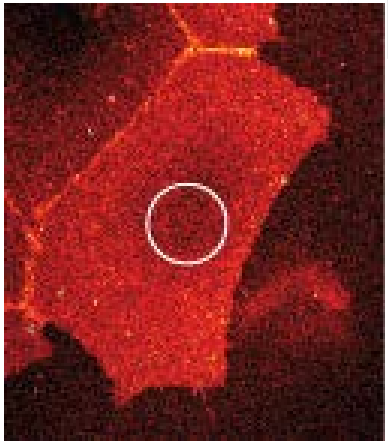


Lipid Plasma Membrane Organization

STED Nanoscopy Measurement

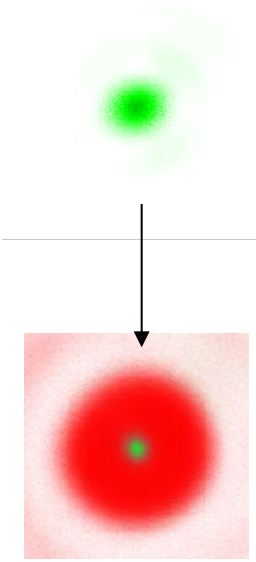






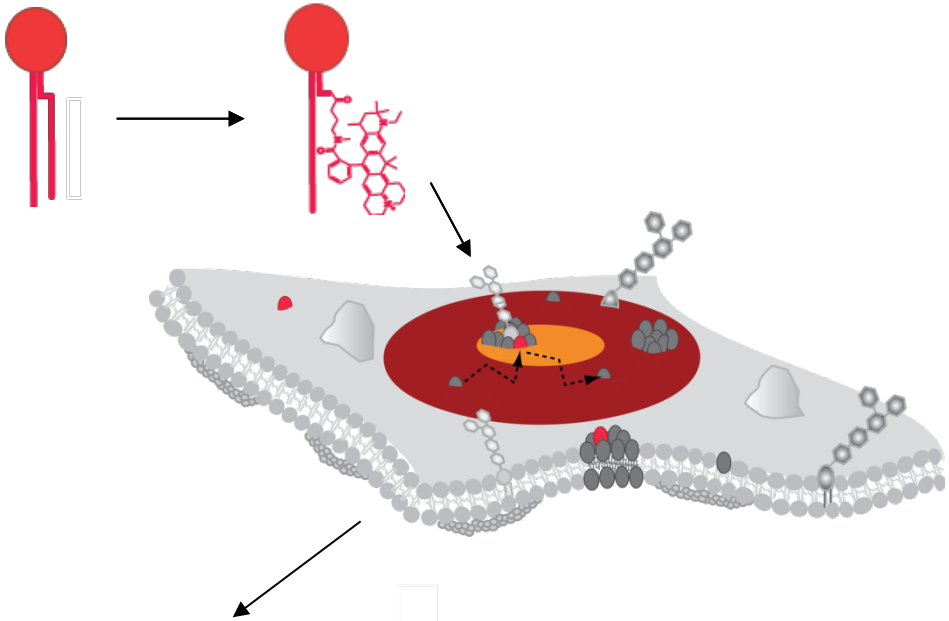
Homogeneous distribution

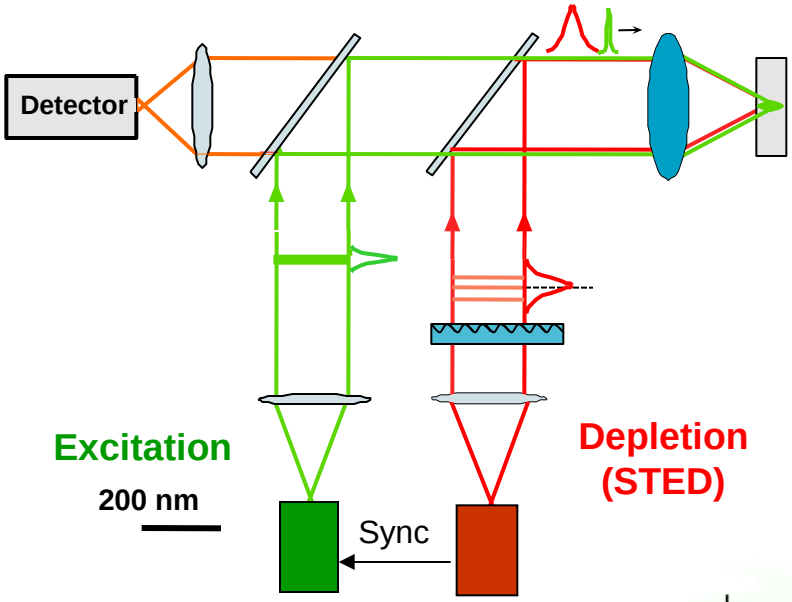
Fast diffusion → Limited temporal resolution!

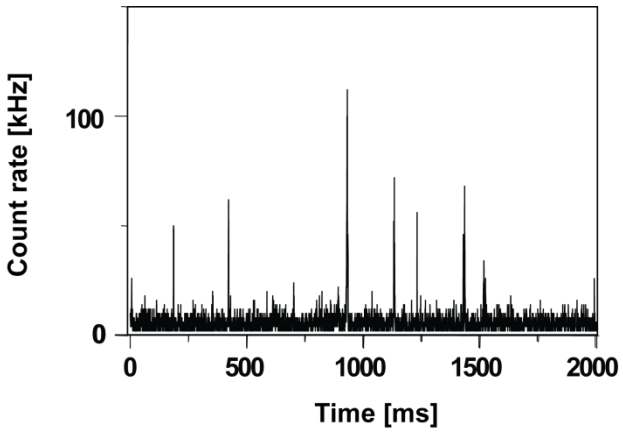


Lipid Plasma Membrane Dynamics

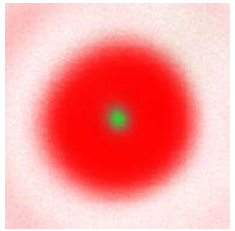
STED Nanoscopy Measurement







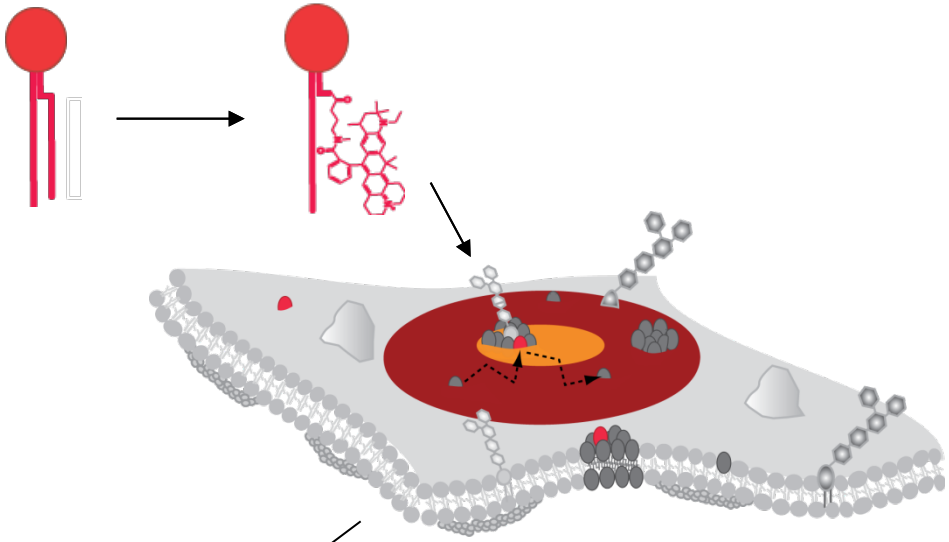
Discover diffusion dynamics!!!

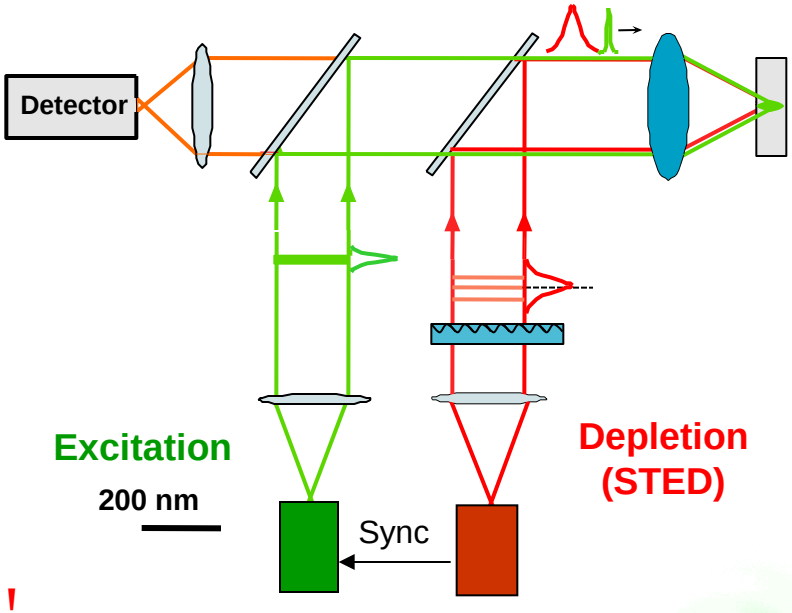


Eggeling et al Nature 2009

Lipid Plasma Membrane Dynamics

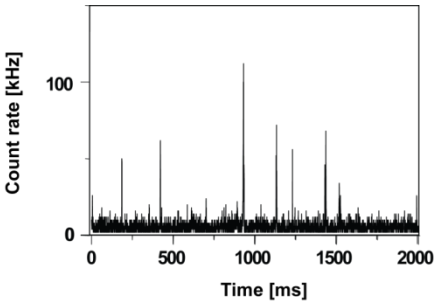
STED Nanoscopy Measurement

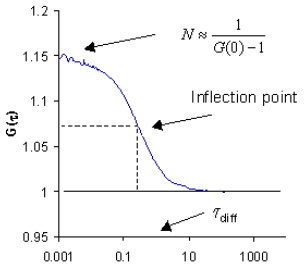




Discover diffusion dynamics!!!

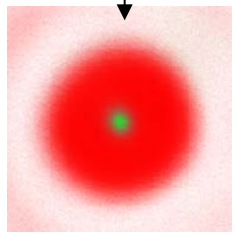
Fluorescence Correlation Spectroscopy (FCS)





Log. x-axis: t_c

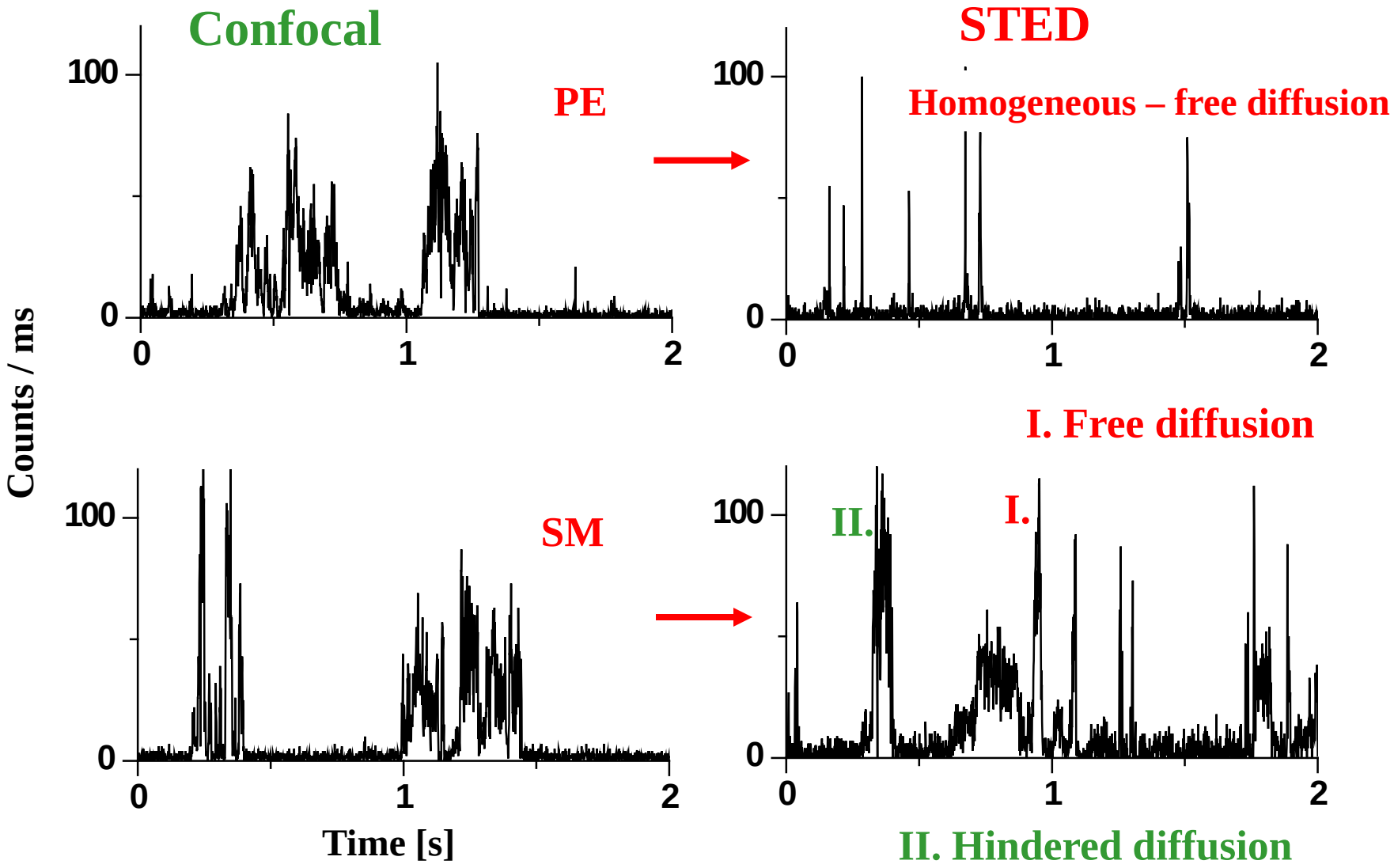
molecular diffusion coefficient = molecular mobility



Eggeling et al Nature 2009

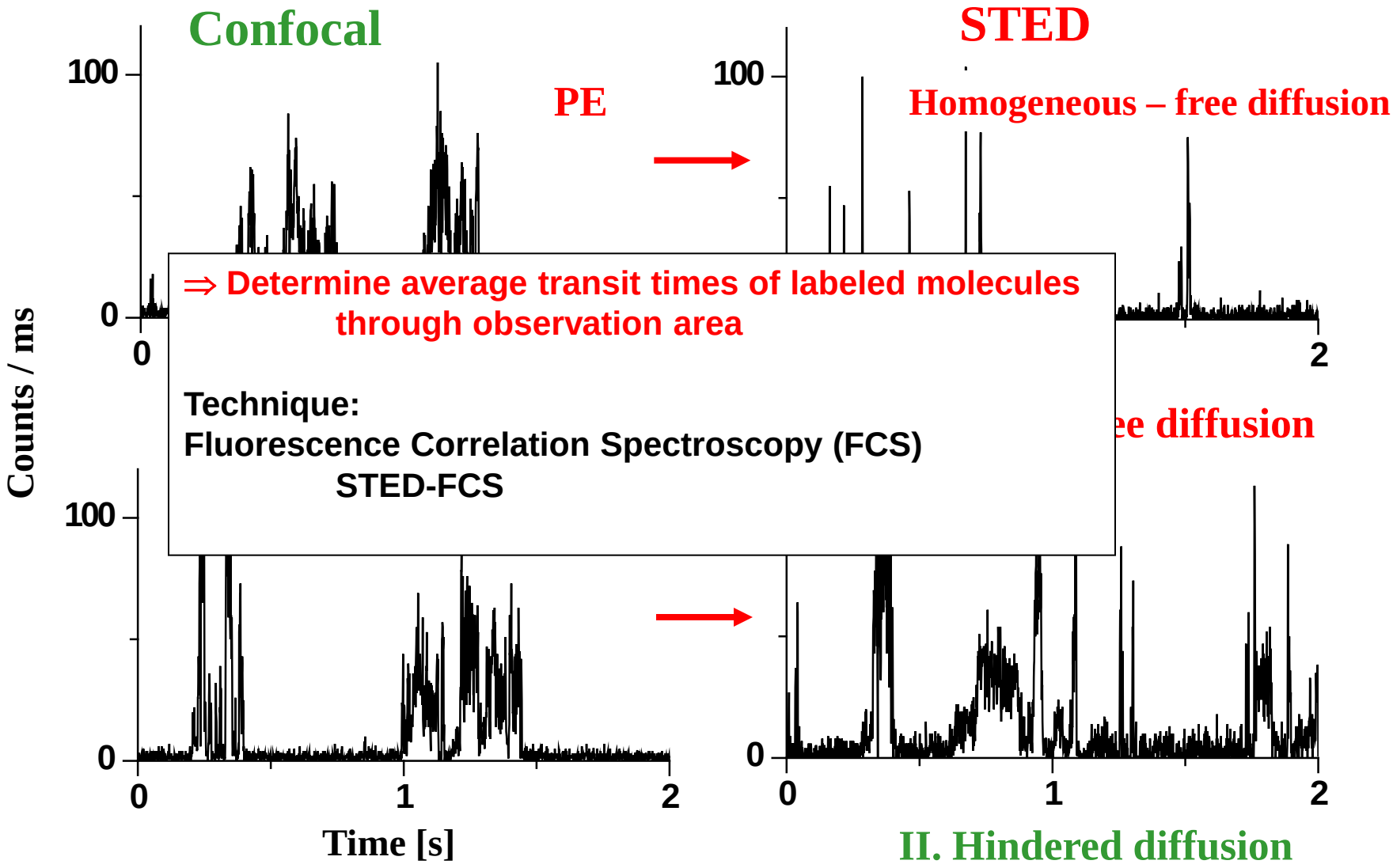
STED Live Cell Spectroscopy

Single Lipid Dynamics



STED Live Cell Spectroscopy

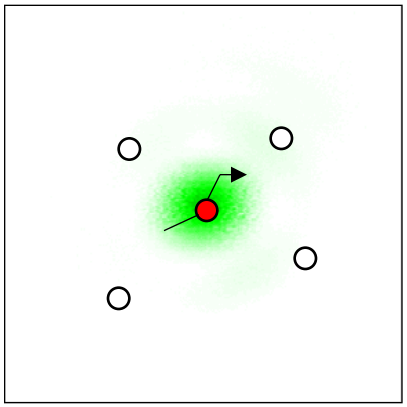
Single Lipid Dynamics

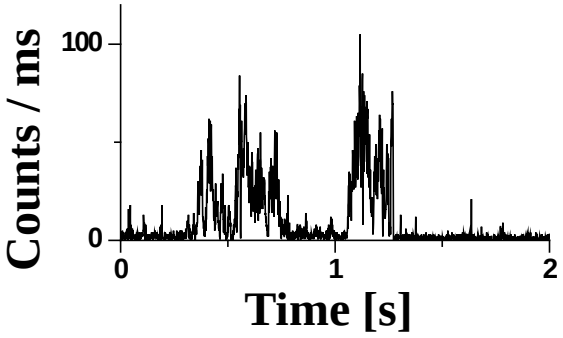


Fluorescence Correlation Spectroscopy
FCS

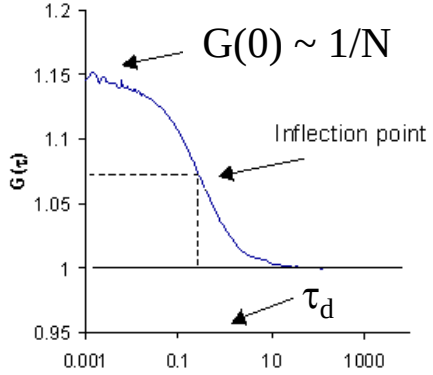
Fluorescence intensity over time

Low fluorescent concentration
⇒ **<u>diffusion of single-molecules</u>**
= fluorescence bursts





Statistics in Time



Log. x-axis: $\mathbf{t_c}$

Fluorescence Correlation Spectroscopy (FCS)
data acquisition - calculation of correlation function
data analysis – length and density of fluctuations

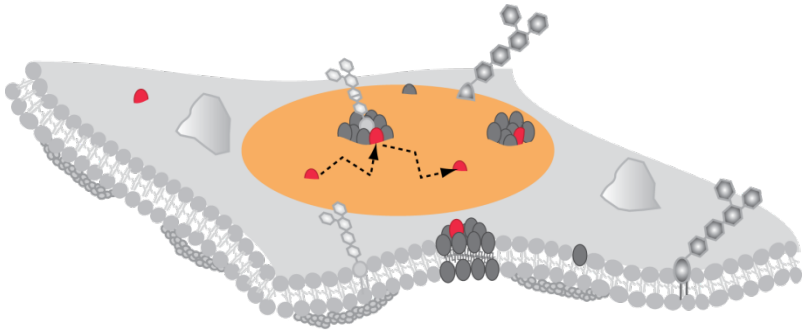
Fitting: anomalous sub-diffusion: $G(t_c) \sim 1/(1 + (t_c/\tau_d)^{\alpha})$
⇒ **<u>transit time</u>** τ_d **(~ mass, obs. area) = decay time**
~ d^2 / D

⇒ **<u>anomaly 1/α:</u>**
(1/α) = 1: normal free diffusion
(1/α) > 1: anomalous diffusion (e.g. trapping)

Lipid Plasma Membrane Dynamics

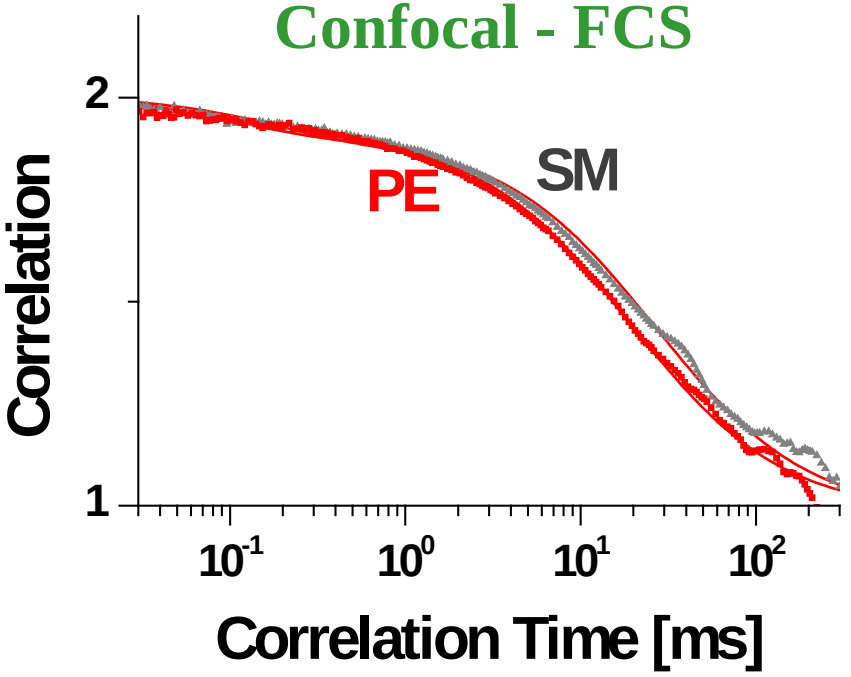
Confocal Recordings

Confocal: Limited spatial resolution !!!



Relative large confocal observation area:
averages over details on nanoscale
cannot distinguish normal diffusion
from nanoscale hindered diffusion

Confocal - FCS



SM diffusion slightly prolonged but still normal

$\tau_d \approx$ 20 ms (PE) / 30ms (SM)

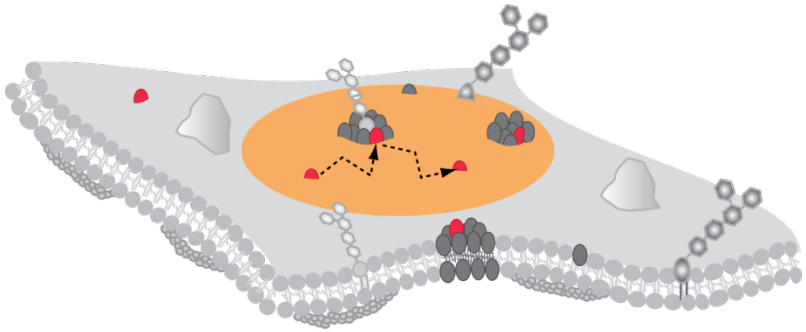
$(1/\alpha) \approx 1$ (PE / SM)

Slower normal diffusion
but no anomalous diffusion???

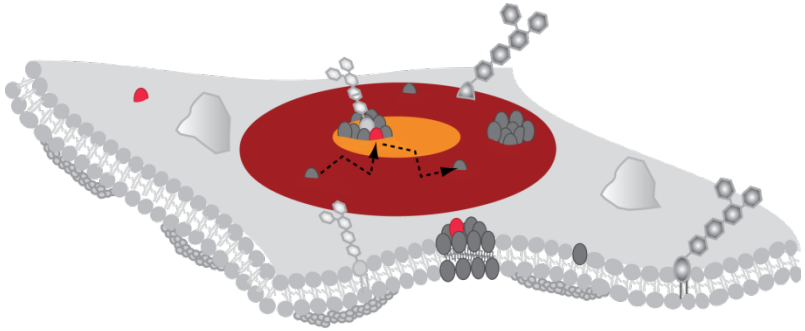
Lipid Plasma Membrane Dynamics

Move to STED

Confocal: Limited spatial resolution !!!



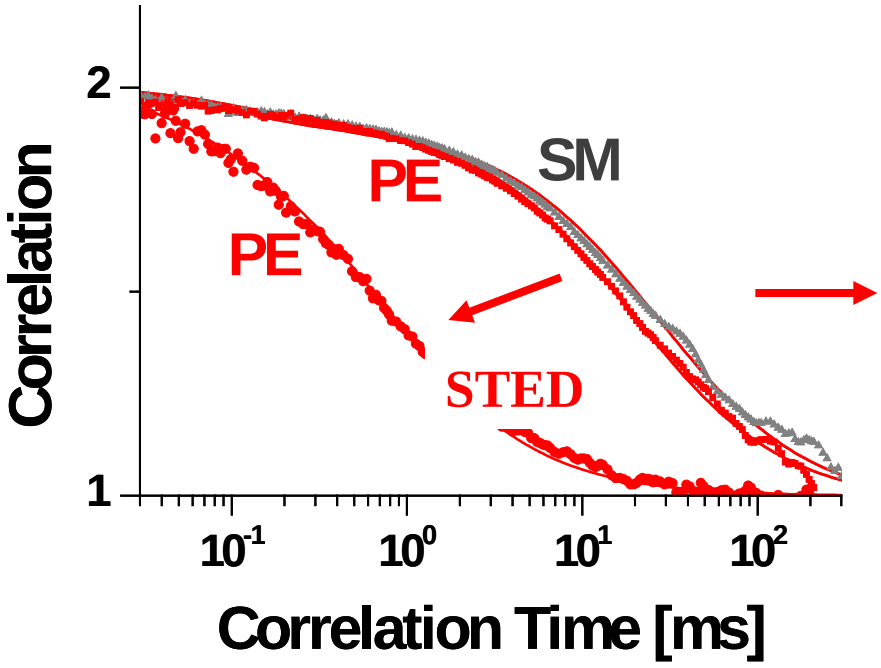
STED!!!!



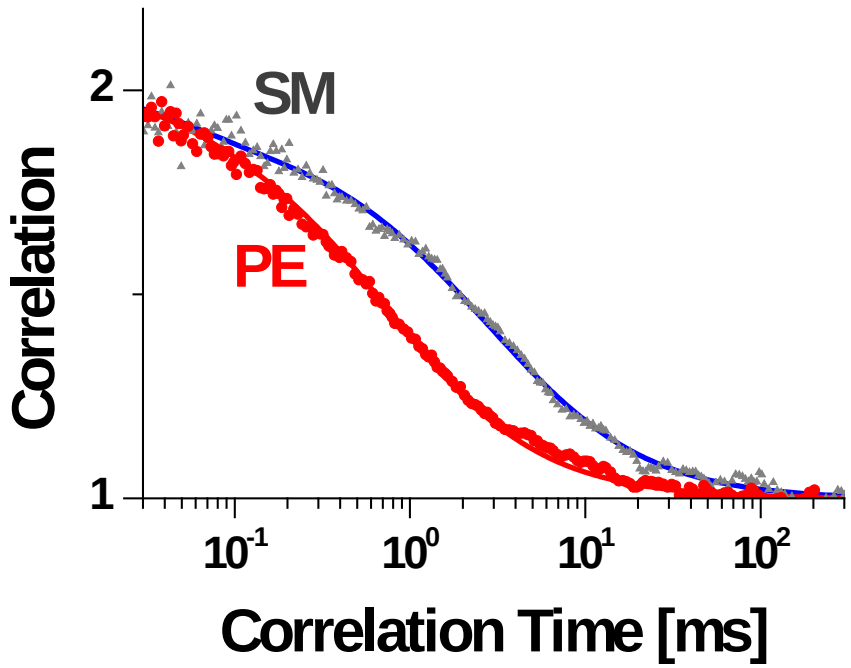
STED Live Cell Spectroscopy

Single Lipid Dynamics - FCS

Confocal



STED – 40nm



STED (240 -> 40nm):

PE diffusion scales with area reduction

τ_d: 20 -> 0.6ms (35-fold)

and still normal

$(1/\alpha) \approx 1$

STED:

SM diffusion much longer than PE

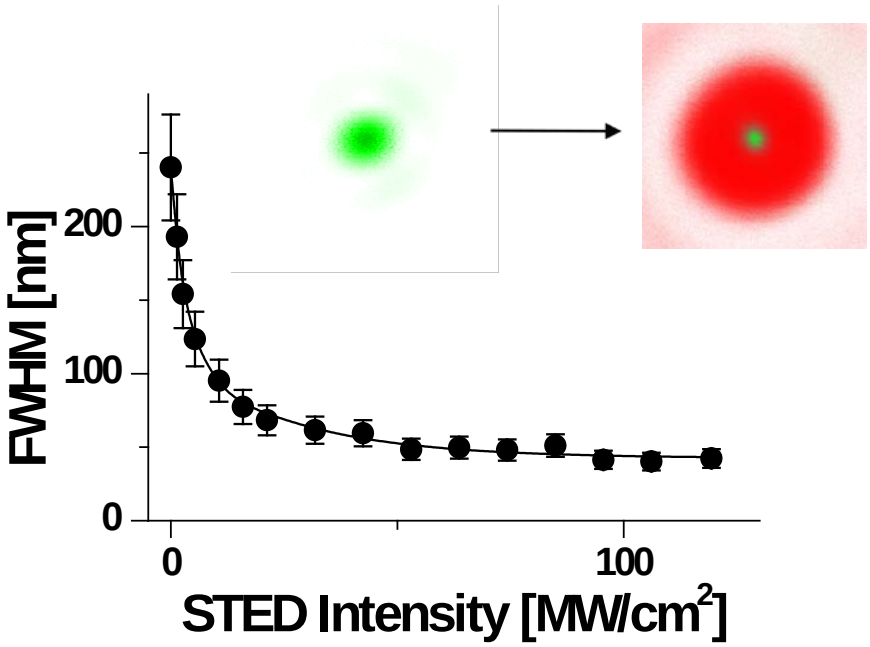
τ_d: 30 -> 3ms (10-fold)

and anomalous

$(1/\alpha) \approx 1.5$

Live Cell Nanoscopy
STED-FCS

STED-Microscopy: Tuning of observation area



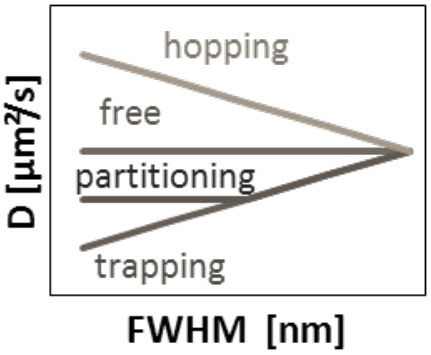
STED-FCS
Determine transit time
for different sizes of observation areas
(different STED intensities)

Calculate
apparent diffusion coefficient:

D ~ area / transit time

Dependencies: D(diameter)
240nm → 30/40nm

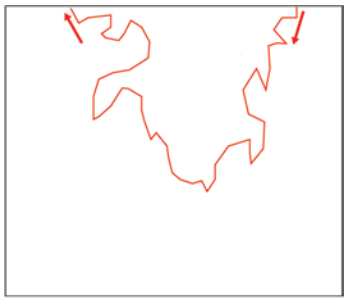
Varies for different diffusion modes



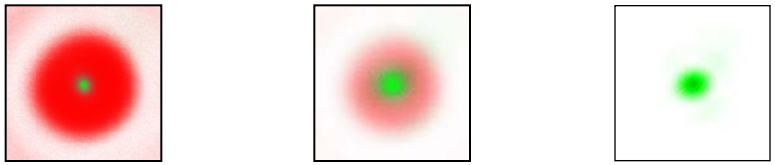
Live Cell Nanoscopy

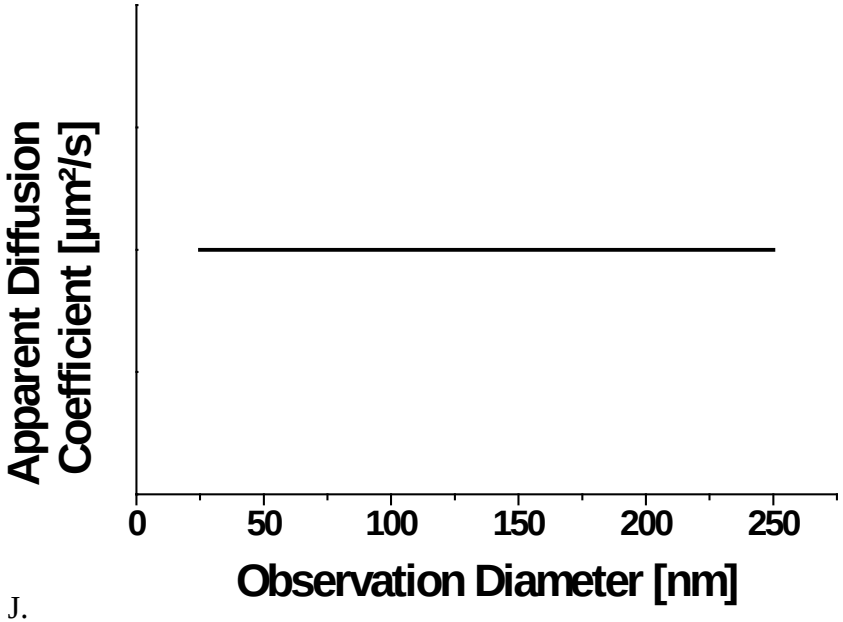
STED-FCS - Diffusion Models

Free diffusion



← STED Intensity





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

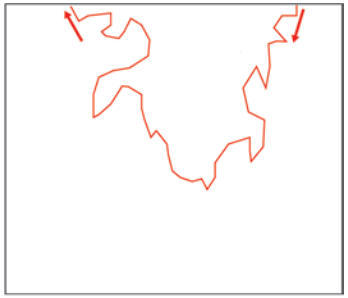
Apparent diffusion coefficient:

D ~ area / transit time

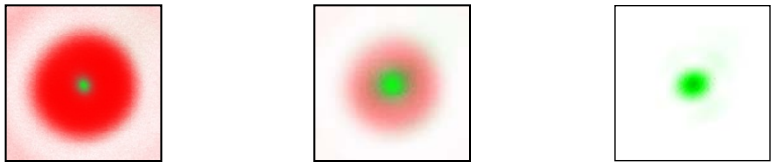
Live Cell Nanoscopy

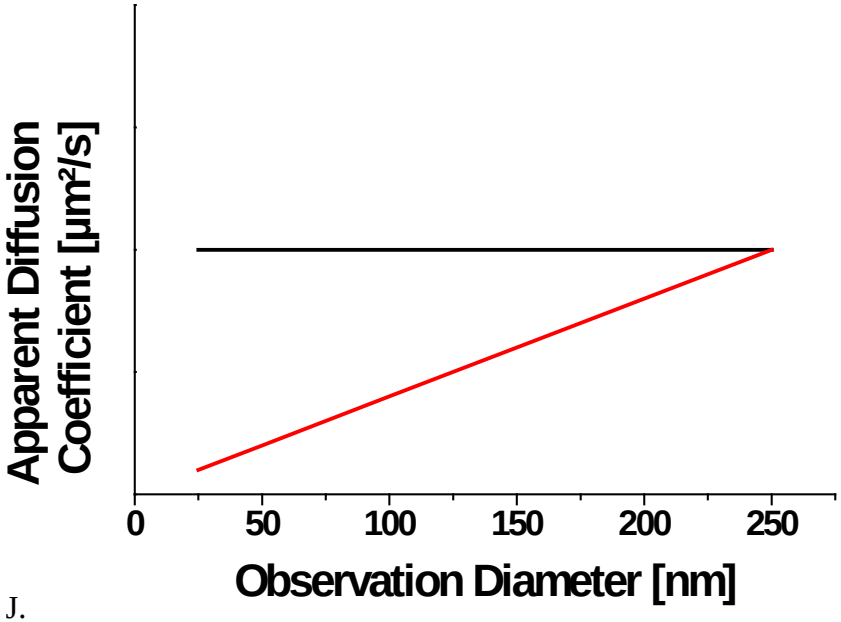
STED-FCS - Diffusion Models

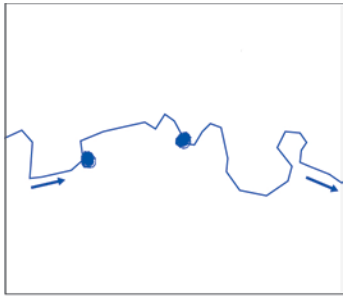
Free diffusion



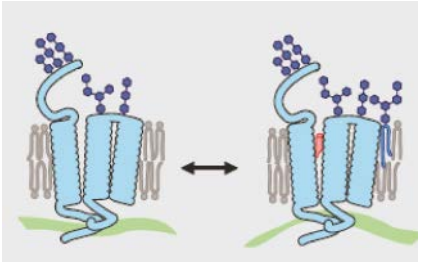
← **STED Intensity**







Trapping



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Mueller et al. *Biophys J* 2011

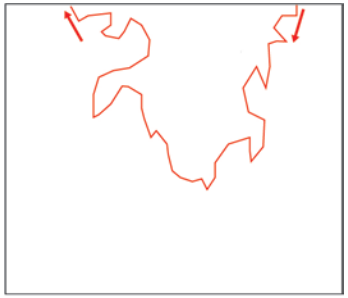
Apparent diffusion coefficient:

D ~ area / transit time

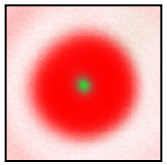
Live Cell Nanoscopy

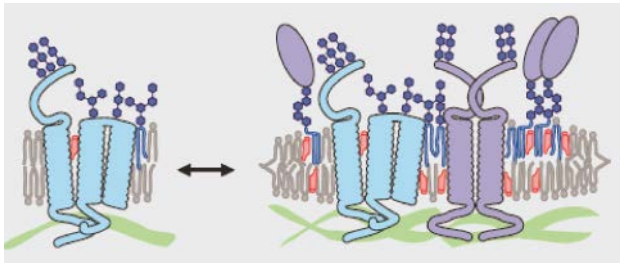
STED-FCS - Diffusion Models

Free diffusion

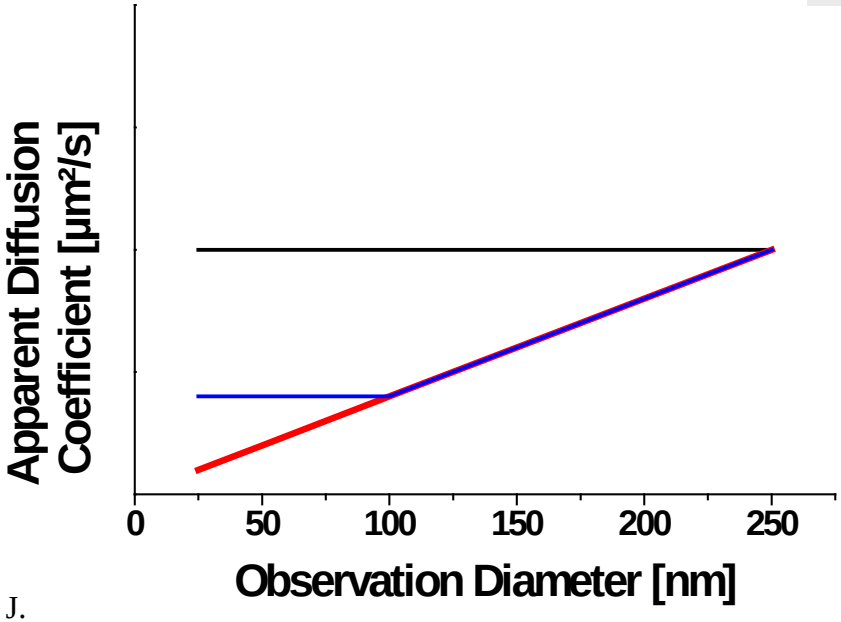


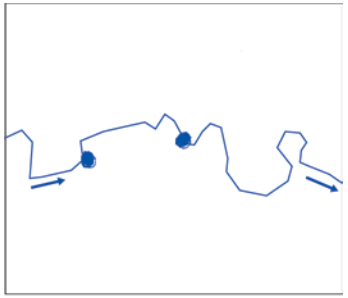
← STED Intensity



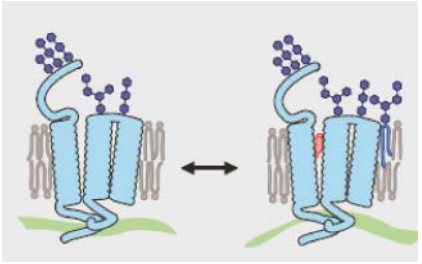


Domain incorporation





Trapping



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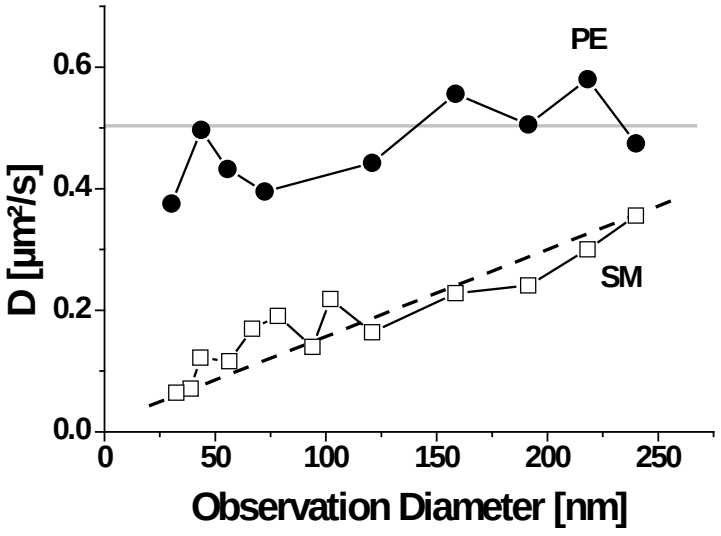
Mueller et al. *Biophys J* 2011

Apparent diffusion coefficient:

D ~ area / transit time

STED-FCS

Lipid Membrane Diffusion + Interactions: PE + SM



→ **Complex on molecular scale**

(proteins, lipid-shells, …)

~10 ms, no movement during trapping

Cholesterol-assisted

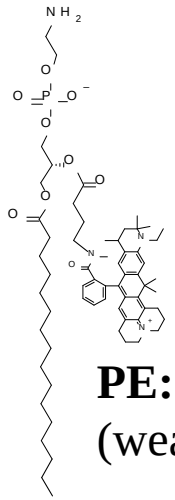
(COase/ß-Cyclo-Dextrin/Zaragozic acid…)

Binding partner bound to cytoskeleton

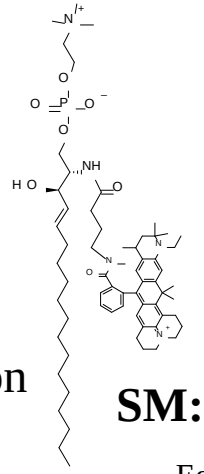
(Latrunculin/Jasplakinolide/Nocodazole…)

Dependence on lipid structure – proteins as well

(not label)

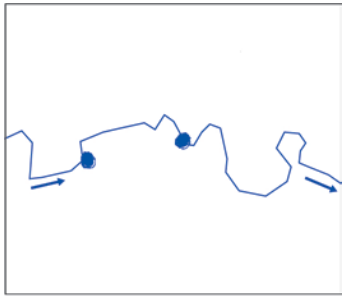


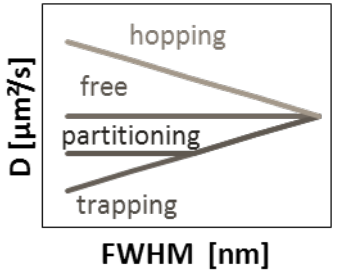
PE: free diffusion
(weak trapping)



SM: trapping

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



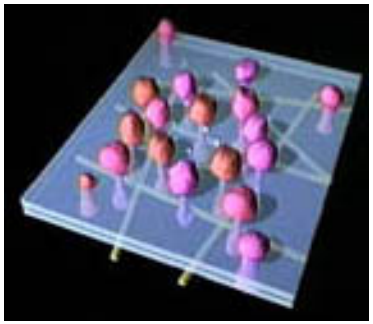


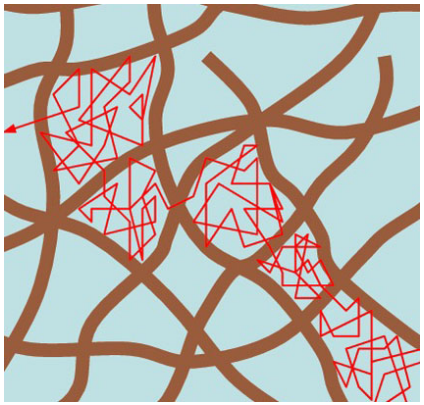
Lipid Plasma Membrane Organization

Interactions on the Nanoscale: Cytoskeleton

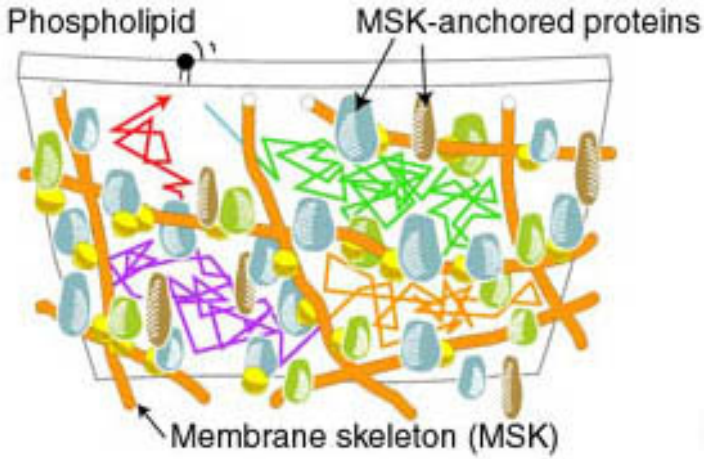
Cytoskeleton

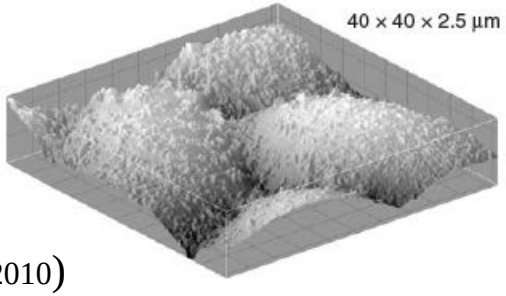
- **Membrane divided in compartments**
- **Proteins: fence/hindrance in diffusion path**
- **Hopping diffusion**





Kusumi



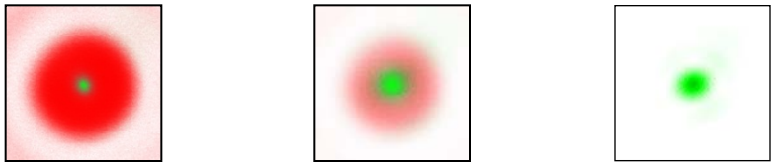


Curvature (Parmryd, NM2010)

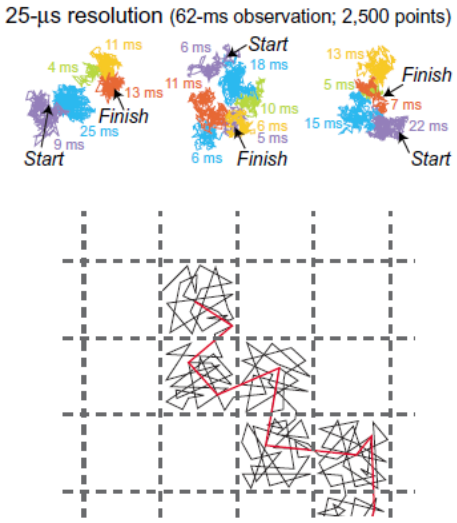
Live Cell Nanoscopy

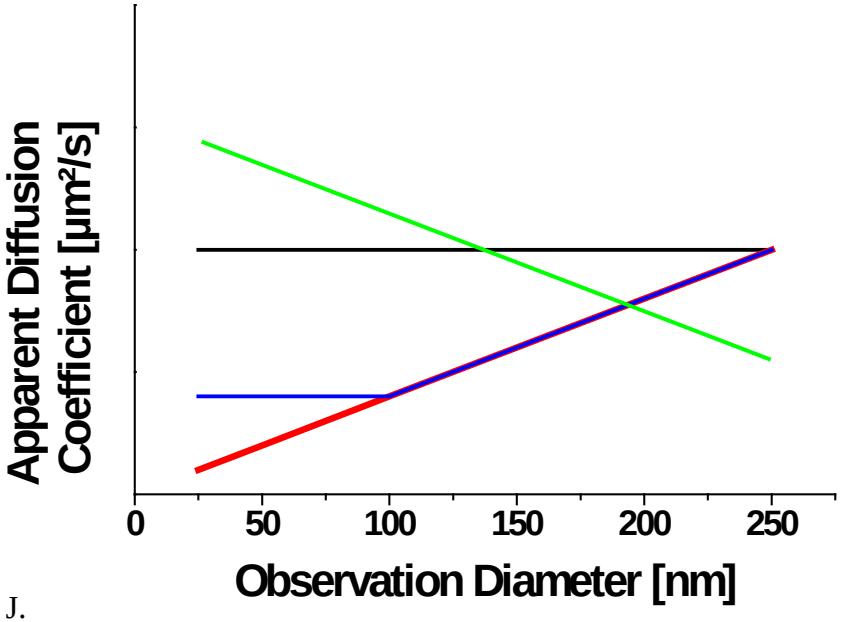
STED-FCS - Diffusion Models

← STED Intensity



Hopping (Kusumi)
meshwork, curvature,…





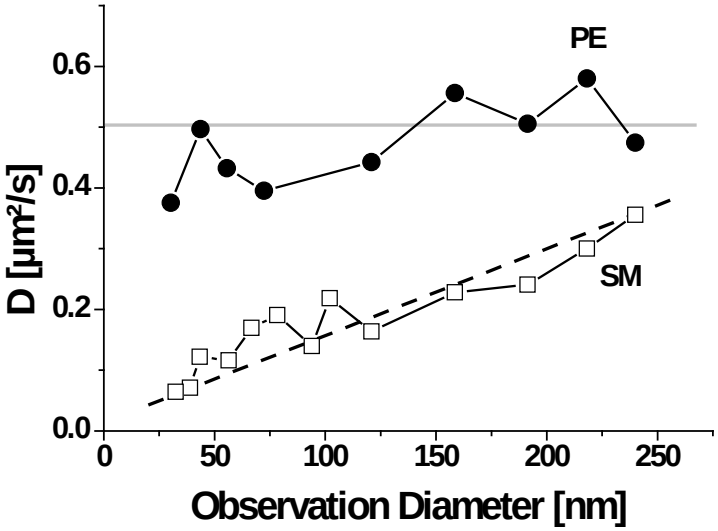
Wawrezinieck et al. Biophys J. 2005 December; 89(6)
Eggeling et al. *Nature* 457, 1159-1162 ,2009
Mueller et al. *Biophys J* 2011

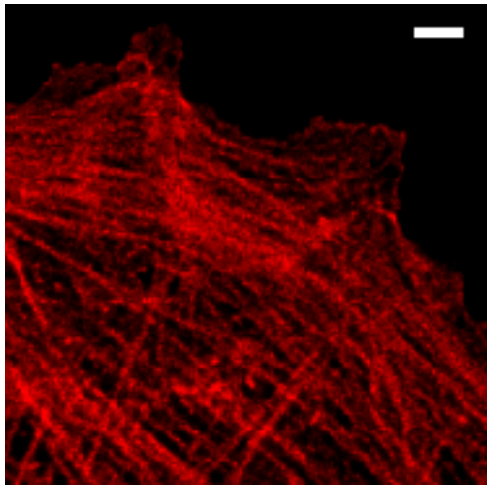
Apparent diffusion coefficient:

D ~ area / transit time

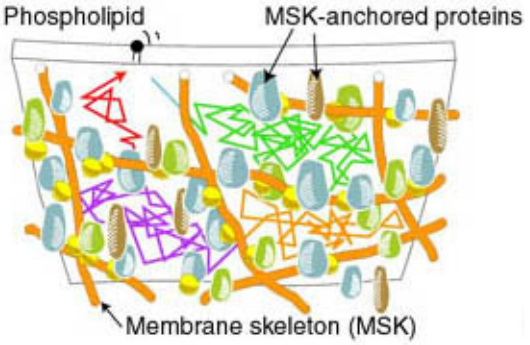
Lipid Plasma Membrane Dynamics

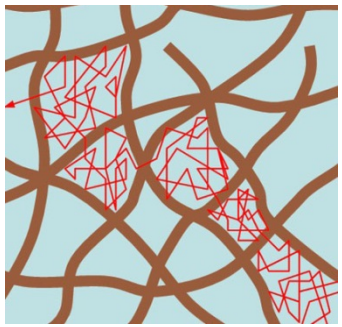
Hopping of Lipids?

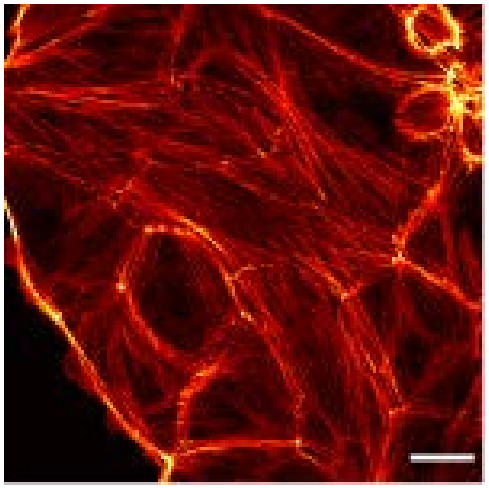




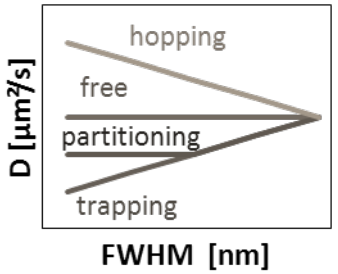
NRK cells





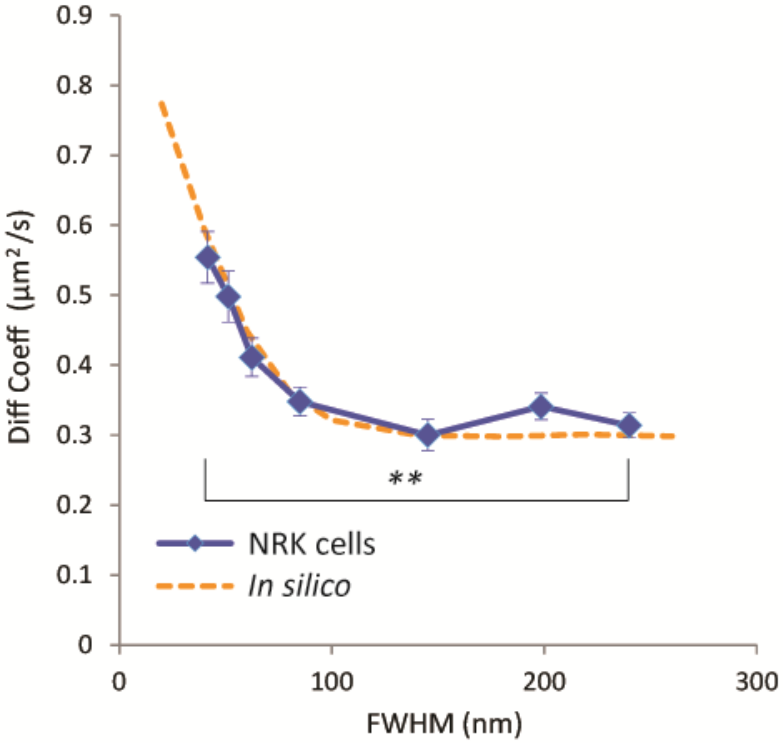


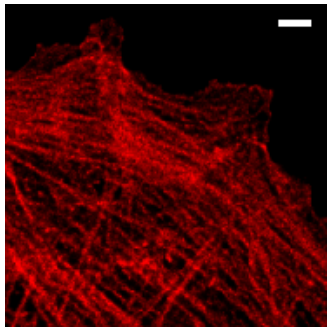
PtK2



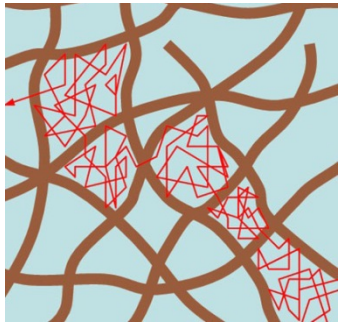
Lipid Plasma Membrane Dynamics

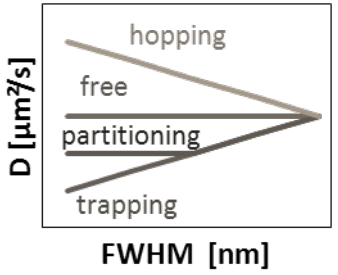
Hopping of Lipids?





NRK cells



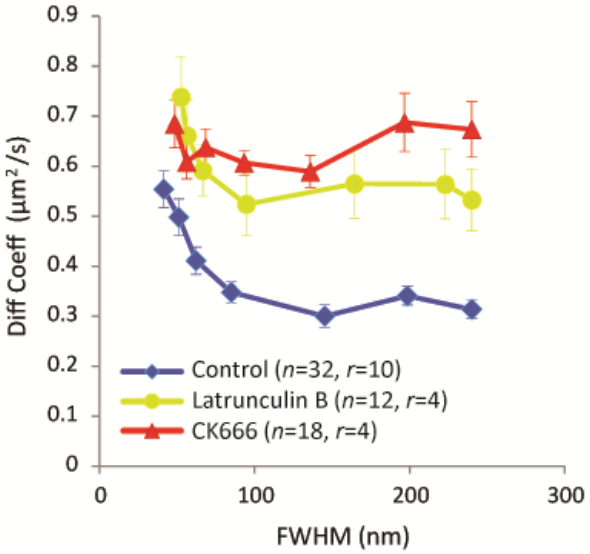


D = 0.8 μm²/s
P_{hop} = 0.1
L = 80nm

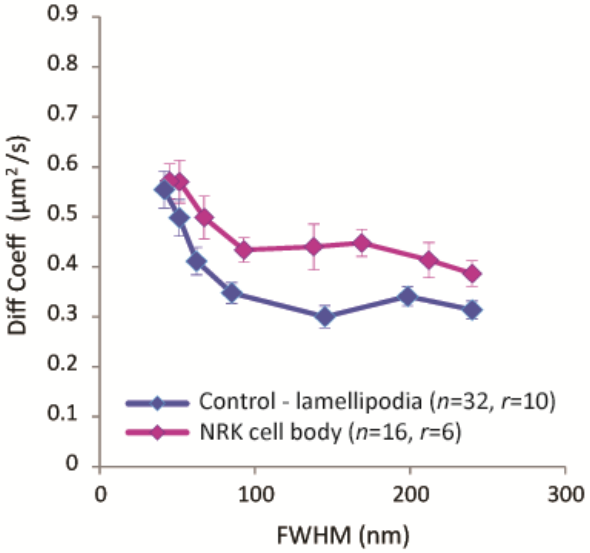
Machado Andrade 2012/2013

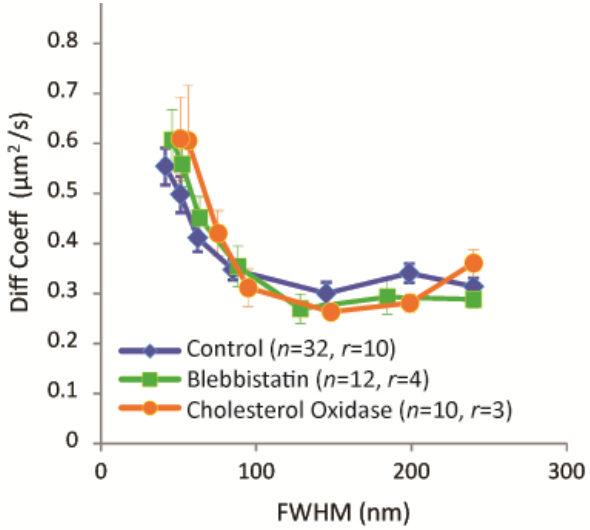
Lipid Plasma Membrane Dynamics

Hopping of Lipids?

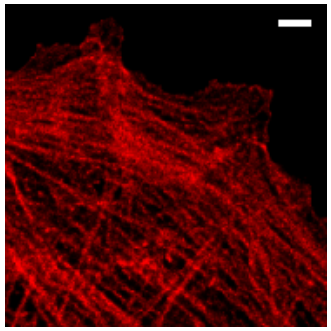


Latrunculin B: actin depolymerization
CK666: Arp2/3 inhibitor
(nucleation core/new branching)

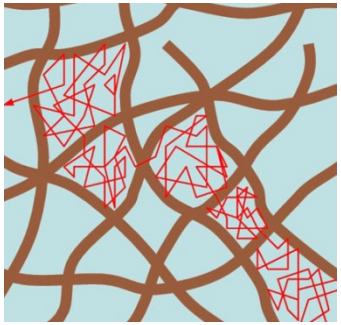


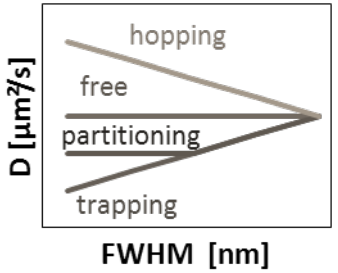


COase: cholesterol oxidation
Blebbistatin: Inhibitor myosin II



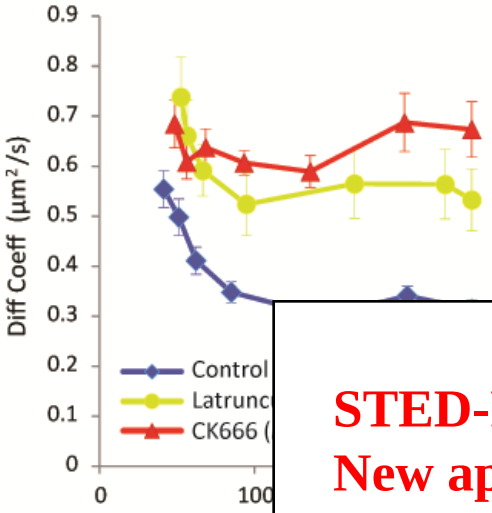
NRK cells

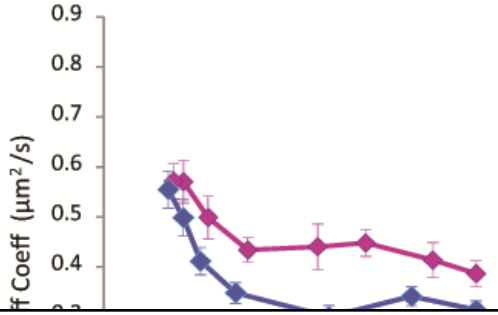




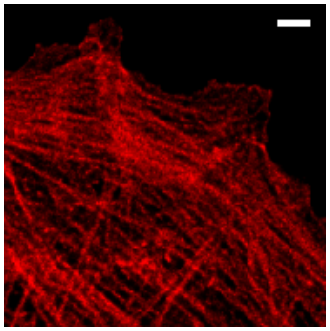
Lipid Plasma Membrane Dynamics

Hopping of Lipids?

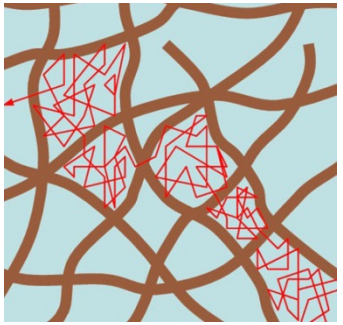




STED-FCS:
New approach to study molecular interactions!



NRK cells



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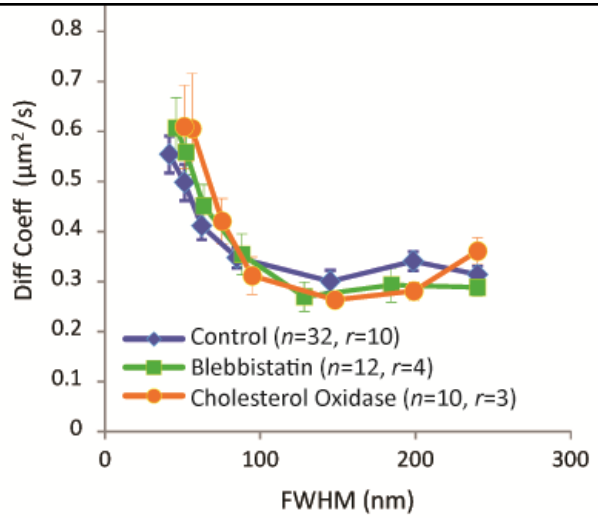
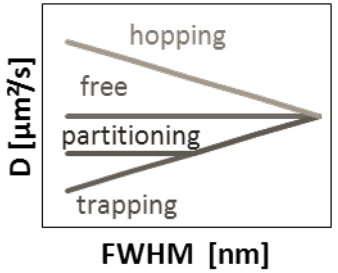
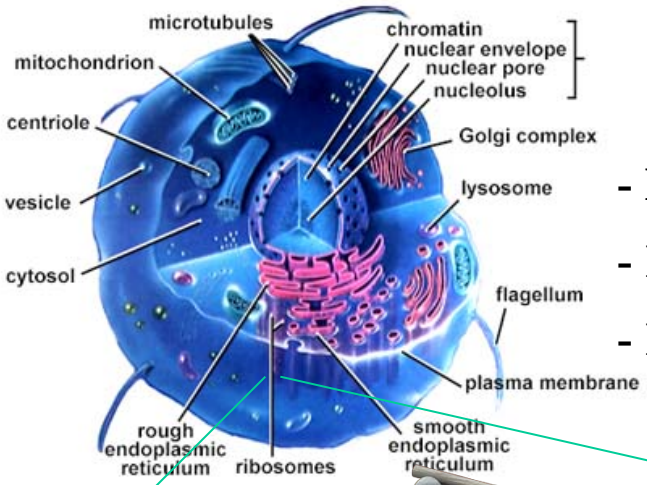
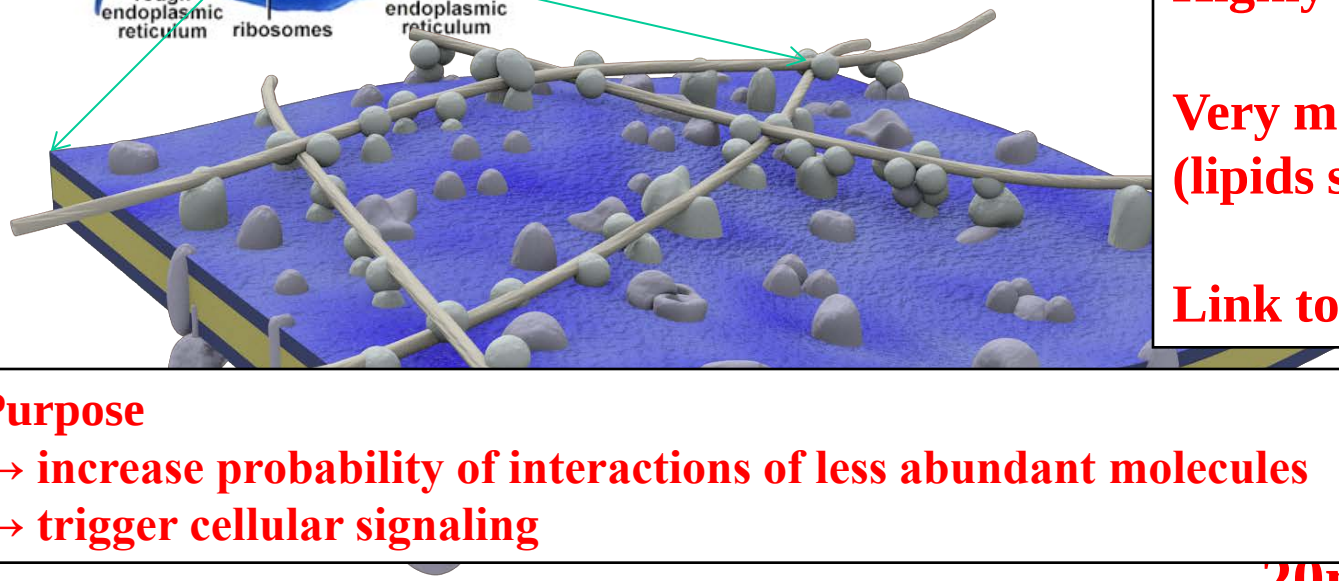




Lipid Plasma Membrane Dynamics

Nanoscale Diffusion





Molecular Membrane Dynamics:

- **Heterogeneous diffusion (viscosity, curvature…)**
- **Interaction with proteins / lipids**
- **Interaction with cortical cytoskeleton**

Highly dynamic!

Very molecule-specific!!!! (lipids specific function)

Link to functionality!?

Purpose
→ increase probability of interactions of less abundant molecules
→ trigger cellular signaling

20nm

Small spatial scales!!!!

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Lipid Experiments

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Birka Lalkens

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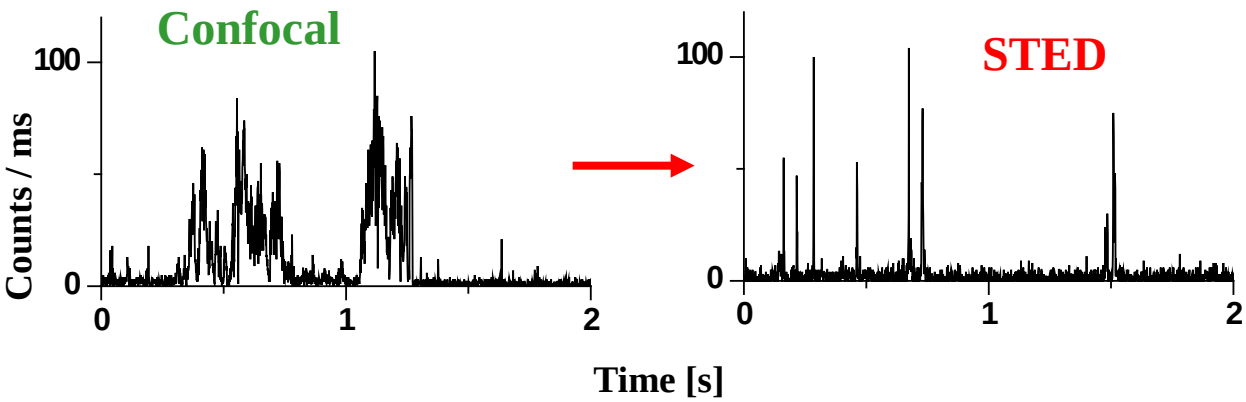
Haisen Ta

Andreas Schönle

Lipid labeling

Dr. V. Belov

S. Polyakova



Stefan Hell

+ whole group

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Mathias Clausen (Biophysics - membrane)
Silvia Galiani (Physics – nanoscope setup/organelles)
Marco Fritzsche (Physics - cytoskeleton)
Erdinc Sezgin (Biophysics – membrane)
Jakub Chojnacki (Biochemistry - virus)
Huw Colin York (Physics – microscopy/force)
Tess Sanley (Biology – receptor)
Antonio Gregorio Dias (Biology – virus)
Sumita Ganglui (Biology)

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Christoffer Lagerholm (manager)
Veronica Buckle

WIMM

Vincenzo Cerundolo

Doug Higgs

Simon Davis

David Jackson , Graham Ogg ...

Labeling

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Del Besra (Birmingham)

Micron/Oxford

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Philipp Kukura…
Mike Dustin...