

# Imaging at the molecular level: F-Techniques (Measuring molecular motion and interactions)

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Wolfson Imaging Centre Oxford

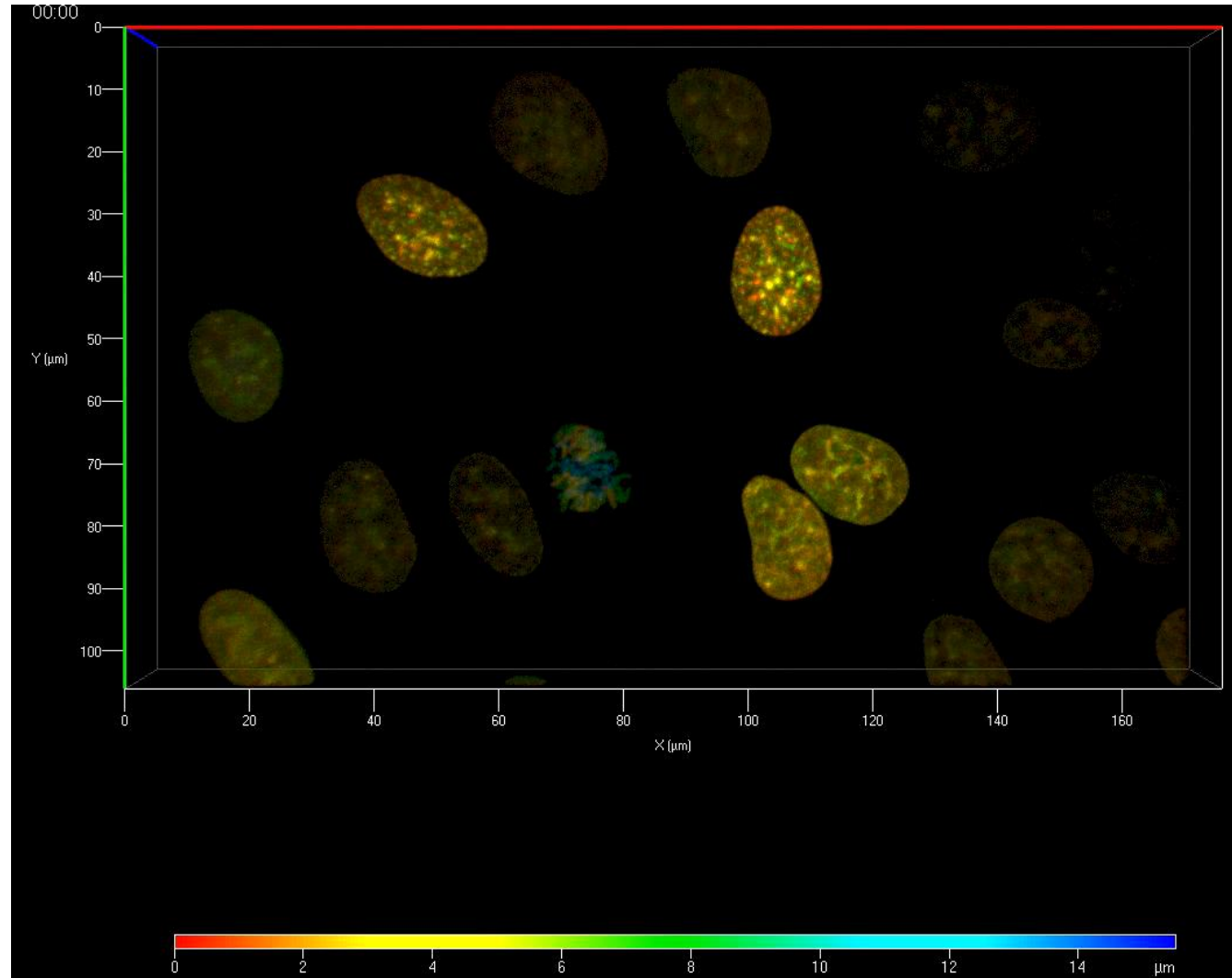
Weatherall Institute of Molecular Medicine

## Imaging at the molecular level: Measuring molecular motion and interactions

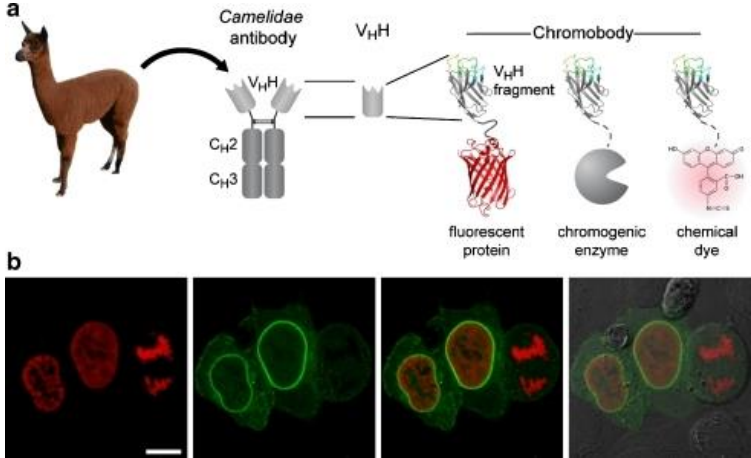
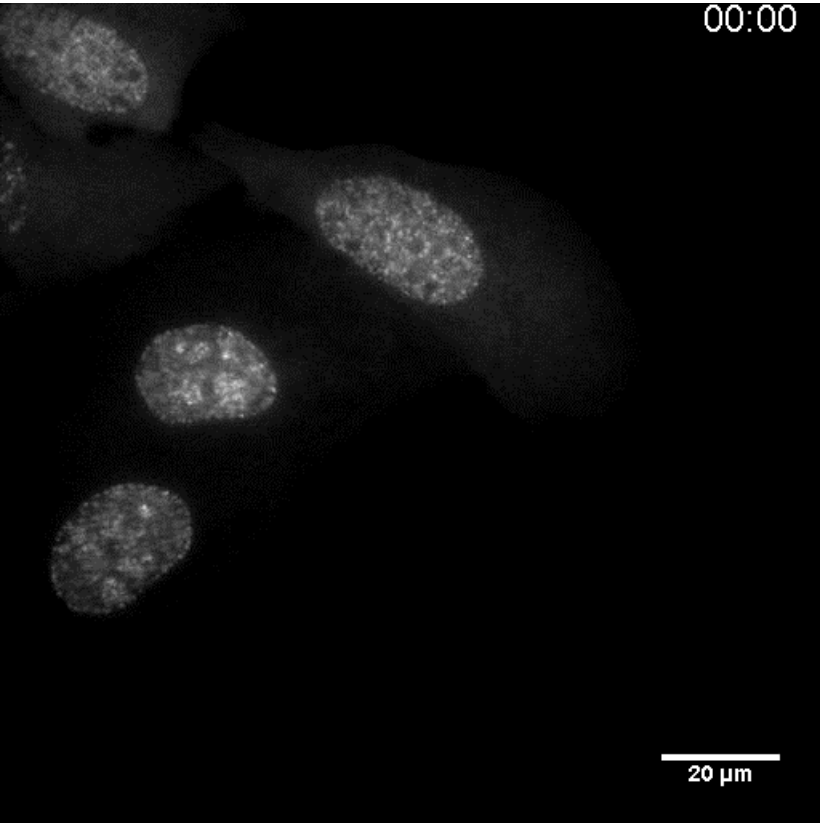
- Brief intro to theory of molecular motion
- Fluorescence techniques for measuring molecular motion
- Single particle tracking (SPT)
- Fluorescence Recovery after Photobleaching (FRAP)
- Fluorescence Correlation Spectroscopy (FCS)
- Fluorescence techniques for measuring molecular interactions
- Förster Resonance Energy Transfer (FRET)
- Fluorescence Cross-Correlation Spectroscopy (FCCS)

Many biological samples are dynamic at a wide range of time-scales

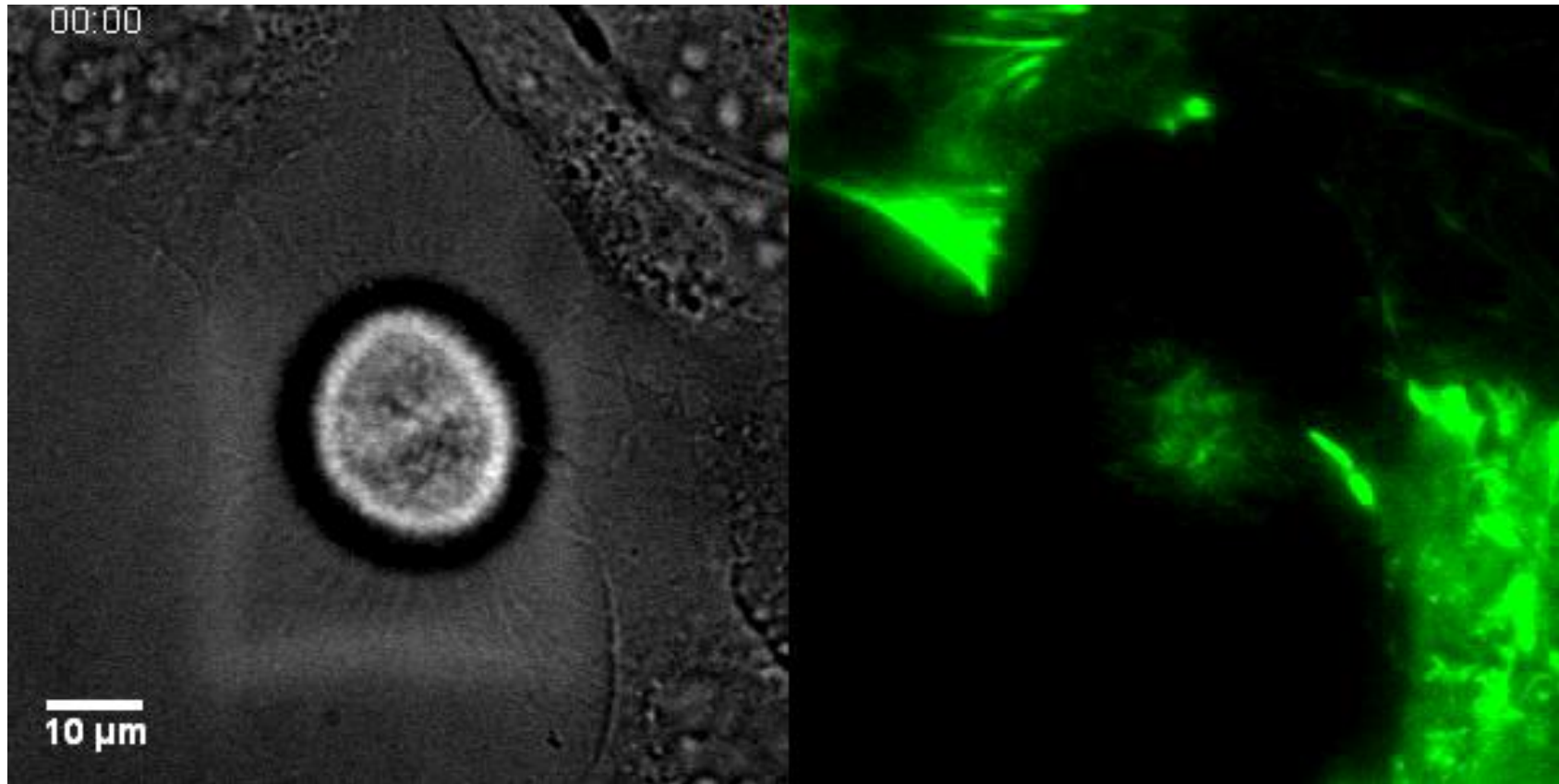
Example 1: Cell cycle; 3D re-construction of HeLa cells expressing H2B-GFP imaged on Spinning-disc Confocal at 6 Z-stacks/h



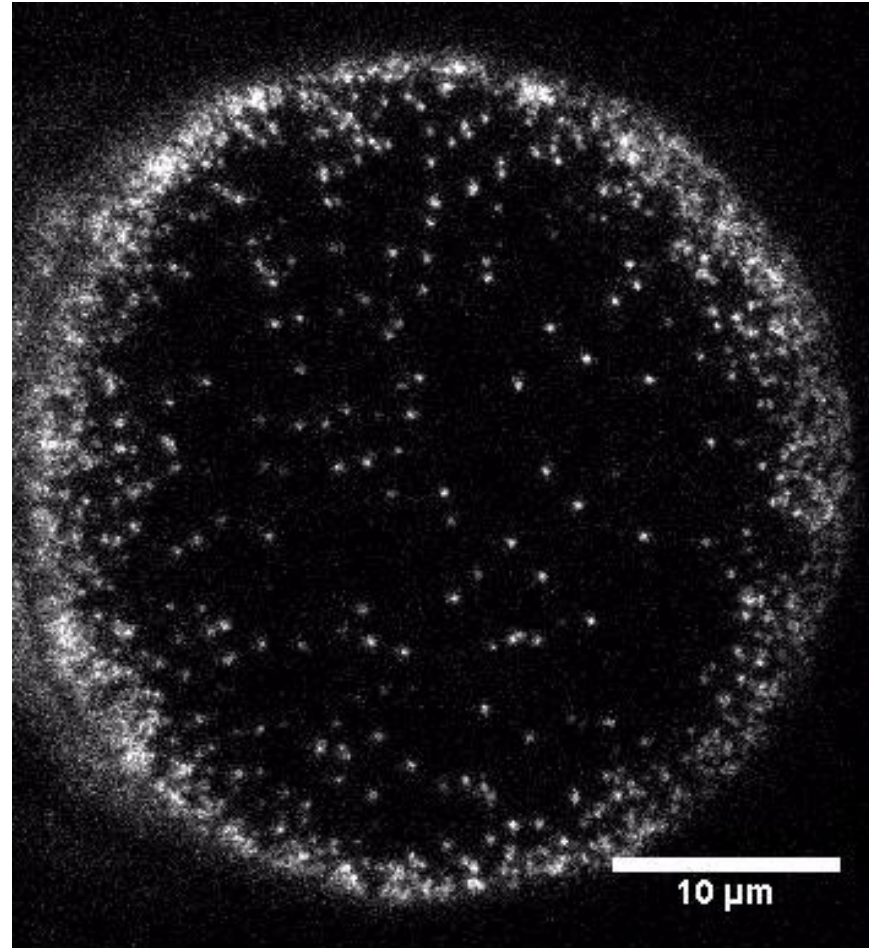
Example 2: Cell cycle; U2OS cells expressing PCNA-Chromobody-RFP imaged on Widefield/TIRF microscope at 24 z-planes/h



Example 3: Mitosis; HeLa cells expressing LifeAct-RFP imaged in TIRF microscope at 24 z-planes/h



Examples 4: Molecular motion of phospholipid analogue (KK114-PE) in substrate supported bilayer (SLB) imaged in TIRF mode at 65 Hz



- ... but time-lapse imaging alone does not reveal quantitative information about movements of cell organelles, proteins, lipids, or DNA
- In particular, time lapse imaging is not sufficient to differentiate between different modes of motion or transient binding of a molecule i.e.
  1. Random (Brownian) diffusion
  2. Directed active (motor driven) transport
  3. Reversible binding and moving by 1, 2, or combination thereof
  4. ...

# Imaging at the molecular level: Measuring molecular motion and interactions

- Brief intro to theory of molecular motion



# Brief intro to theory of molecular motion

## ON THE MOVEMENT OF SMALL PARTICLES SUSPENDED IN A STATIONARY LIQUID DEMANDED BY THE MOLECULAR- KINETIC THEORY OF HEAT

**I**N this paper it will be shown that according to the molecular-kinetic theory of heat, bodies of microscopically-visible size suspended in a liquid will perform movements of such magnitude that they can be easily observed in a microscope, on account of the molecular motions of heat. It is possible that the movements to be discussed here are identical with the so-called "Brownian molecular motion"; however, the information available to me regarding the latter is so lacking in precision, that I can form no judgment in the matter (I).

Mean Squared Displacement:  
(Free (Brownian) diffusion in 1D)  $\langle (x(t) - x_0)^2 \rangle = \int_{-\infty}^{\infty} x^2 N[0, 2Dt] dx = 2Dt$

Stokes-Einstein Relation:  
(3D diffusion in solution)

$$D_S = \frac{k_B T}{6\pi \mu_S R_H}$$

Saffman-Delbrück Relation:  
(2D translational diffusion in membrane)

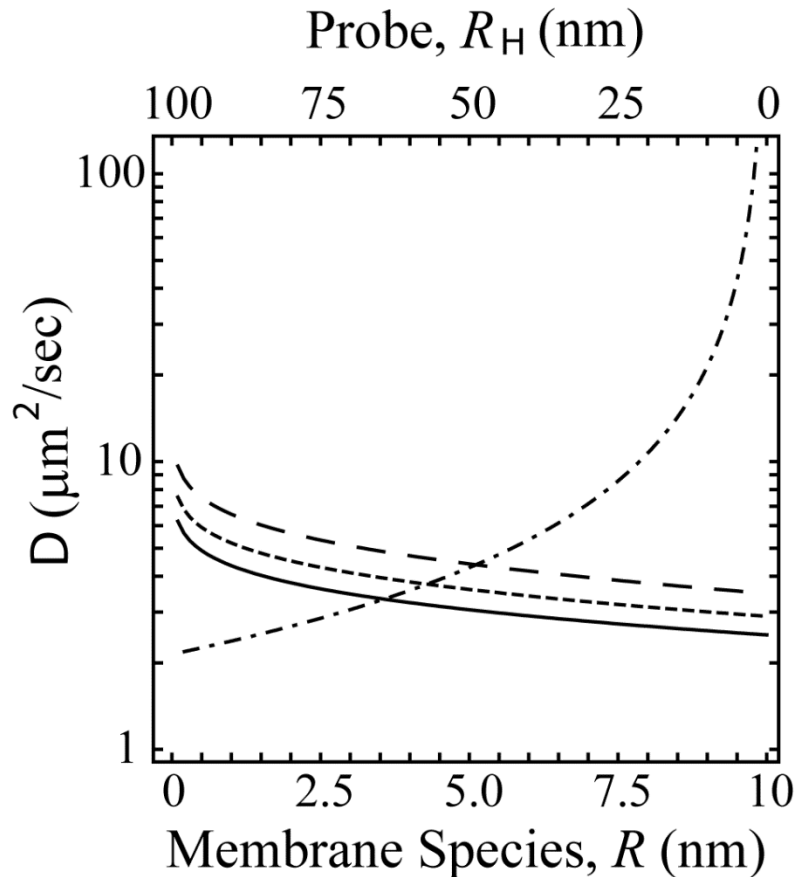
$$D_M = \frac{k_B T}{4\pi \mu_M h} \ln\left[\frac{\mu_M h}{\mu_S R_H} - \gamma\right]$$

Einstein, A. (1905). Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhenden Flüssigkeiten suspendierten Teilchen. *Annalen der Physik*, 17, 549-560.

Einstein, A. (1956). *Investigations on the theory of the Brownian movement* (A. D. Cowper, Trans.). New York, NY: Dover Publications.

Saffman, P. G., & Delbruck, M. (1975). Brownian motion in biological membranes. *Proc Natl Acad Sci USA*, 72(8), 3111-3113.

# Diffusion in membranes and solution



*Saffman-Delbruck:*

$$D_M = \frac{k_B T}{4\pi \mu_M h} \ln\left[\frac{\mu_M h}{\mu_S R_H} - \gamma\right]$$

$$\mu_S = 1.002 \text{ cP}$$

$$h = 4 \text{ nm}$$

$$T = 293 \text{ K}$$

$$\mu_M/\mu_S = 100 \text{ (solid line)}$$

$$\mu_M/\mu_S = 80 \text{ (short dashed line)}$$

$$\mu_M/\mu_S = 60 \text{ (long dashed line).}$$

*Stokes-Einstein:*

$$D_S = \frac{k_B T}{6\pi \mu_S R_H}$$

$$\mu_S = 1.002 \text{ cP}$$

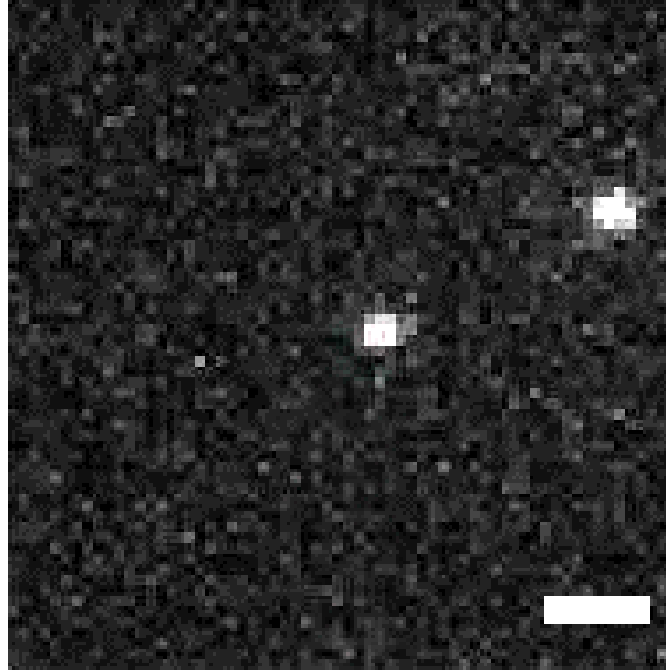
$$T = 293 \text{ K.}$$

# Fluorescence techniques for measuring molecular motion

- 1) Single particle tracking (SPT)
- 2) Fluorescence Recovery after Photobleaching (FRAP)
- 3) Fluorescence Correlation Spectroscopy (FCS)

# 1) Single particle tracking (SPT)

anti-CD73 Fab'-biotin + sAv-605 Qdot



189 Hz ( 5.3 ms integration) with 160X magnification and Andor EMCCD

Playback 100 Hz, 2000 frames  
Scale bar = 1 $\mu$ m

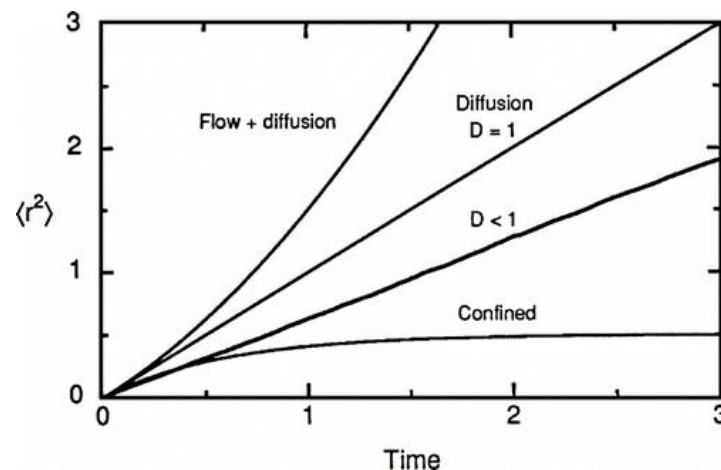
# Single Particle Tracking

- Time lapse imaging of spatially resolved single particles, molecules, or subcellular structures
- Technique results in time trajectories of sub-pixel positions of single objects
- Such trajectories contain information about:
  - diffusion coefficients
  - velocities
  - step sizes
  - spatial and temporal confinement

- Dynamics are analyzed with respect to that predicted by Brownian (random) motion in a 2 D (or 3D) fluid
- For 2D, the mean square displacement (MSD) is

$$\langle (r(t) - r_0)^2 \rangle = 4Dt$$

where D is the diffusion coefficient



*Brownian motion*  $\langle r^2 \rangle = 4Dt$

*Anomalous diffusion*  $\langle r^2 \rangle = 4Dt^\alpha \quad \alpha < 1$

*Diffusion with flow*  $\langle r^2 \rangle = 4Dt + (Vt)^2$

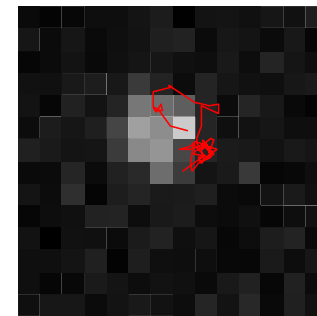
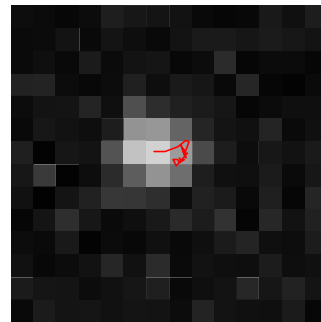
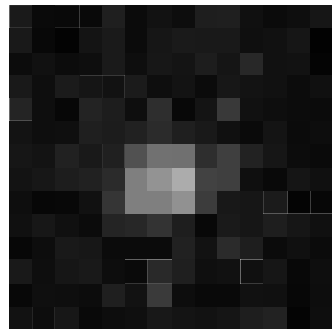
*Confined diffusion*  $\langle r^2 \rangle \approx \langle r_c^2 \rangle [1 - A_1 \exp(-4A_2Dt / \langle r_c^2 \rangle)]$

# Data Analysis

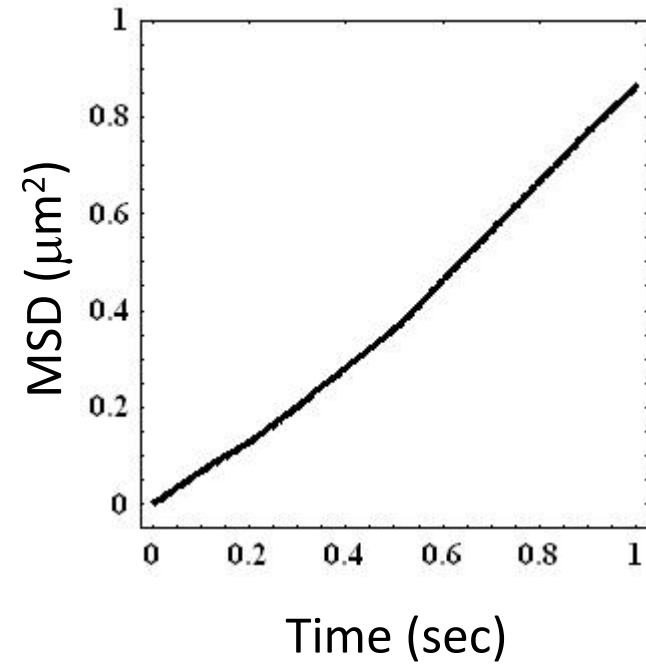
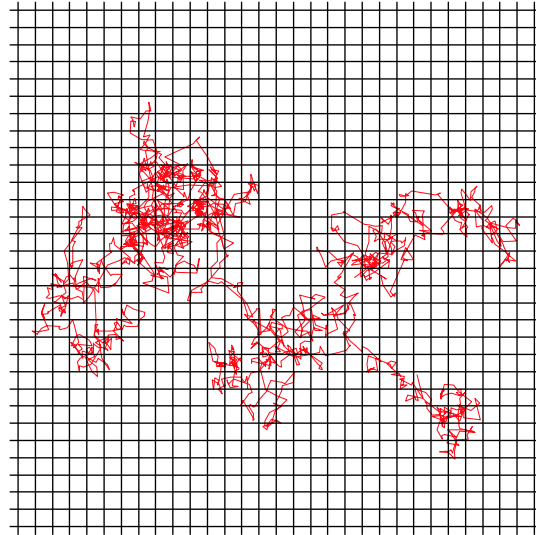
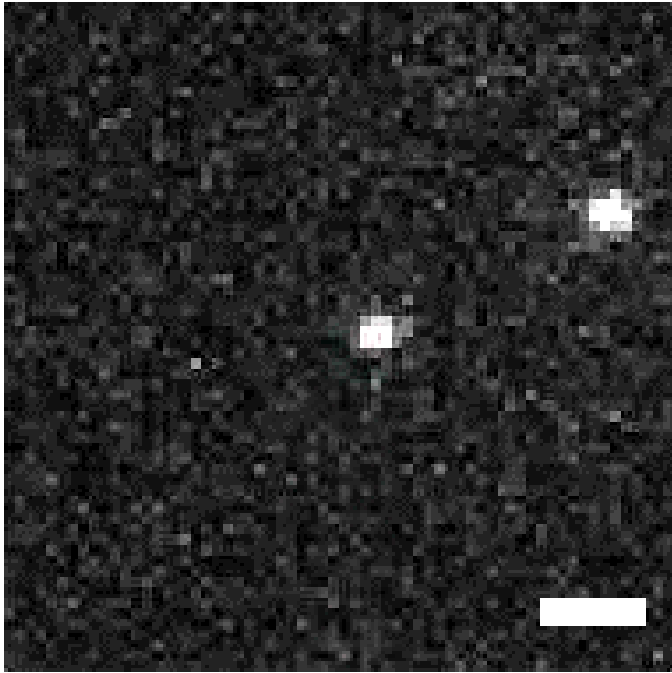
- Obtain sub-pixel resolution by curve fitting to determine centroids of single molecules
- Approximate Airy pattern with a 2D spatial Gaussian and fit each image

$$A + \frac{B}{2\pi w^2} \text{Exp}\left[-\frac{1}{2\pi w^2} ((x - x_0)^2 + (y - y_0)^2)\right]$$

where  $w$  = width of the PSF of the microscope ( $\sim 220$  nm)



# Mode of Motion & Diffusion Coefficient



Brownian Diffusion  
 $D \sim 0.25 \mu\text{m}^2/\text{sec}$

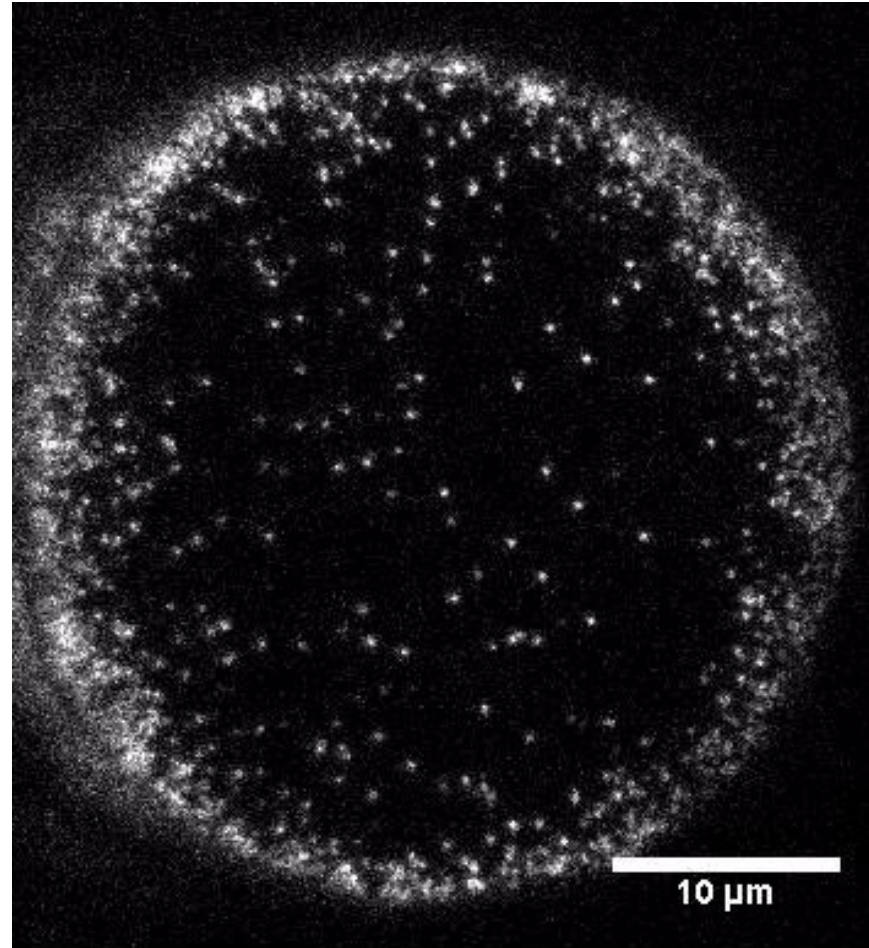


# Probe Considerations for SPT

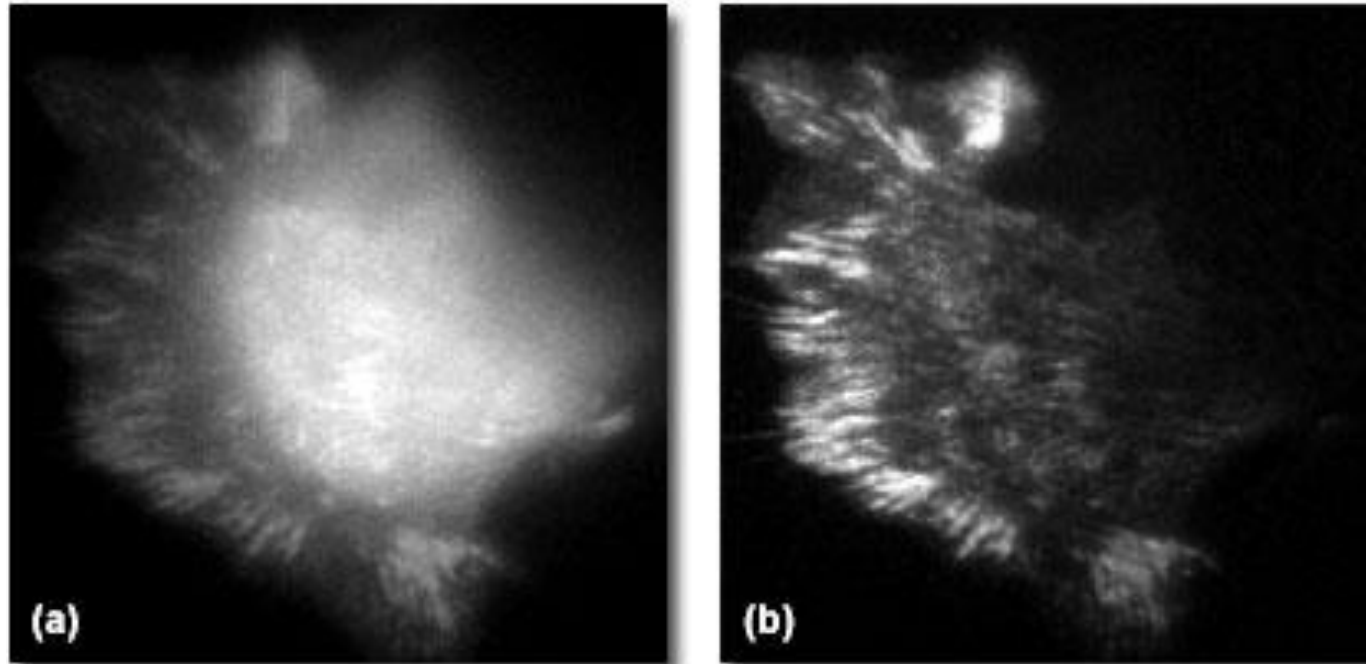
- Very bright and stable
- Small
- Monovalent (i.e. one probe per molecule of interest)
- Low non-specific binding

	<b>Gold particles</b>	<b>Cy3</b>	<b>Quantum dots</b>
<b>Means of detection</b>	<b>Scattering</b>	<b>Fluorescence</b>	<b>Fluorescence</b>
<b>Size (diameter)</b>	<b>40 nm</b>	<b>~1-2 nm</b>	<b>~10-20 nm</b>
<b>Advantages</b>	<b>Photostable</b>	<b>Small Monovalent</b>	<b>Photobleaching resistant</b>
<b>Disadvantages</b>	<b>Immobilization Large Multivalent</b>	<b>Photobleaching (~5 s)</b>	<b>Non-specific binding Blinking</b>

Examples 4: Molecular motion of phospholipid analogue (KK114-PE) in substrate supported bilayer (SLB) imaged in TIRF mode at 65 Hz

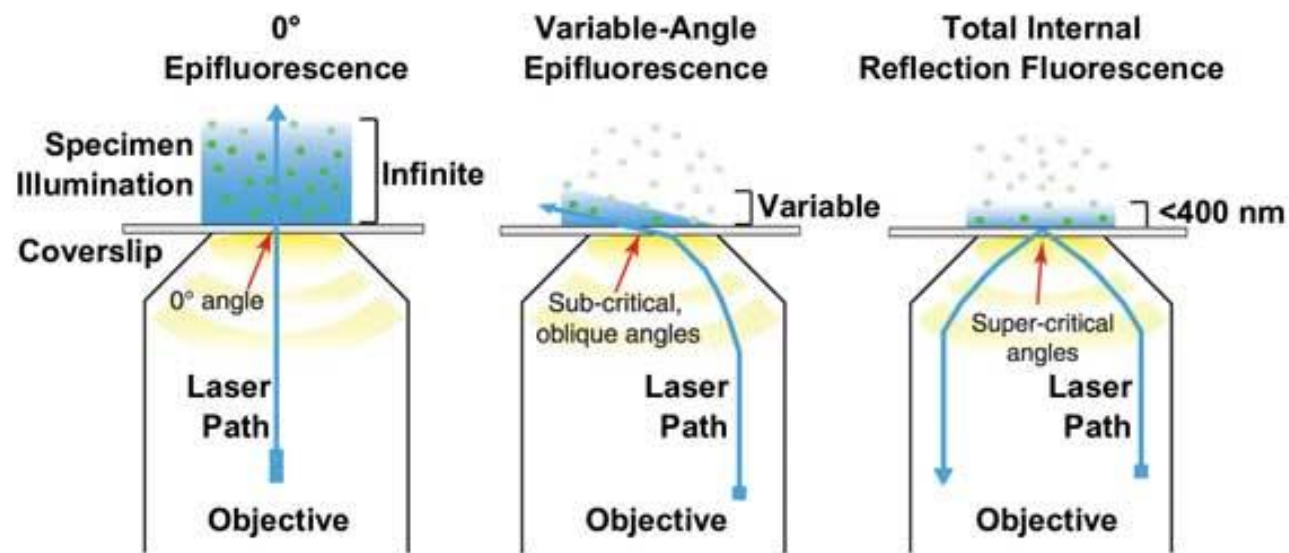


## Cell Focal Adhesions in Widefield and TIR Fluorescence



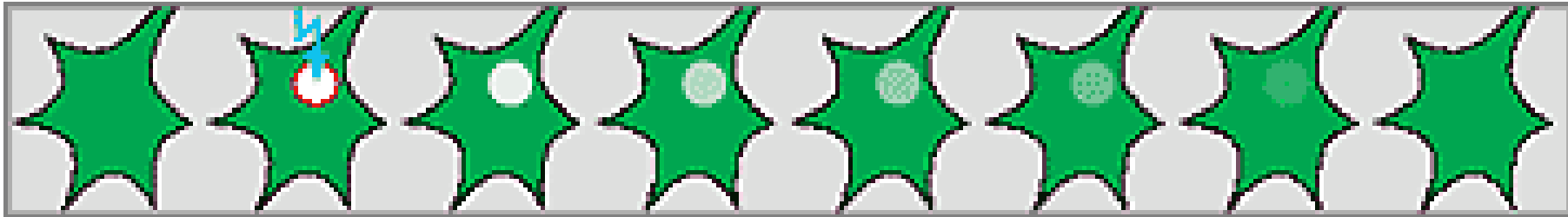
**Figure 5**

<http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html>



## 2) Fluorescence Recovery after Photobleaching (FRAP)

1. Select photobleaching of region of interest (ROI) with very high laser intensity
  2. Monitor the progress of fluorescence recovery in the bleached area with high temporal resolution
- Changes in intensity in the bleached region represent the sum of all movements of fluorescent molecules, whether passive (e.g., diffusion) or active (e.g., transport).
  - The half-recovery time is a measure of the average speed of protein movement.

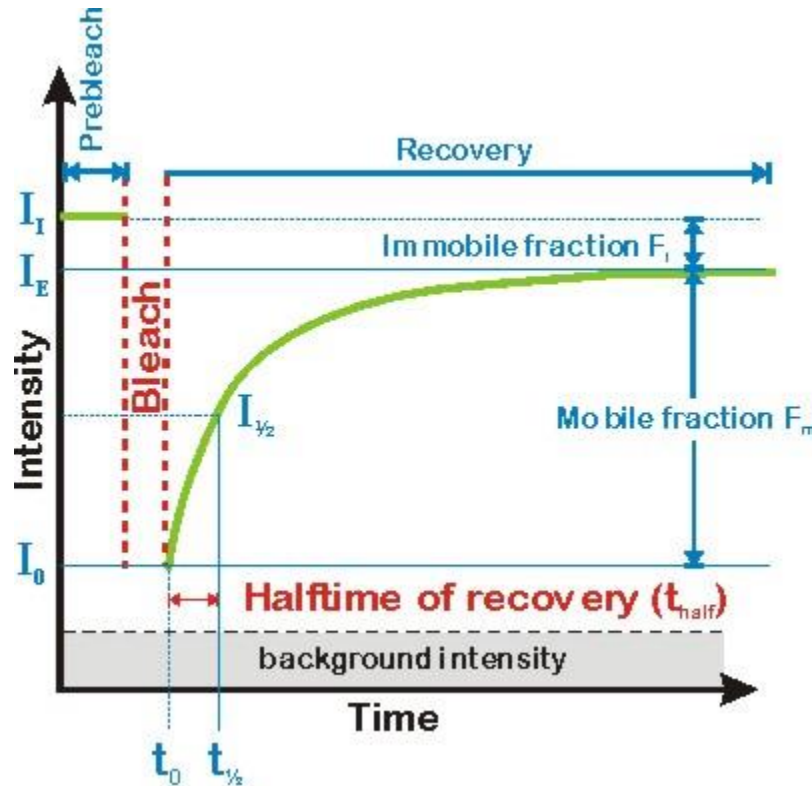


**Bleached spot size ~ 1  $\mu\text{m}$**

**([www.zeiss.com](http://www.zeiss.com))**

# How FRAP works

EAMNET - FRAP module



$I_I$ : initial intensity

$I_0$ : intensity at timepoint  $t_0$

$I_{1/2}$ : half recovered intensity

$(I_{1/2} = (I_E - I_0) / 2)$

$I_E$ : endvalue of the recovered intensity

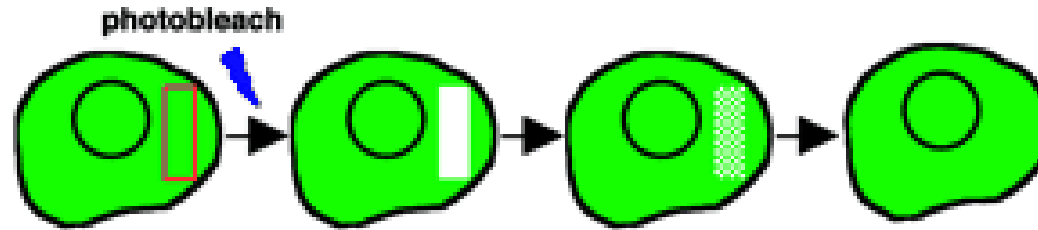
$$\text{Halftime for recovery } t_{1/2}(I_{1/2}) = \frac{t(I_E) - t(I_0)}{t(I_I) - t(I_0)}$$

$$\text{Mobile fraction } F_m = \frac{I_E - I_0}{I_I - I_0}$$

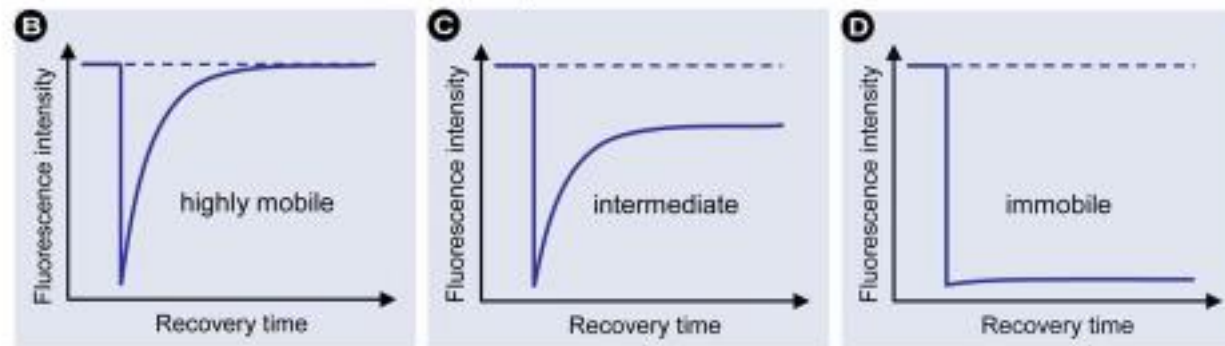
For 2D diffusion:

$$D = \frac{w^2}{4 t_{1/2}} \quad w: \text{laser spot radius}$$

- Can also do FRAP with most confocal microscopes



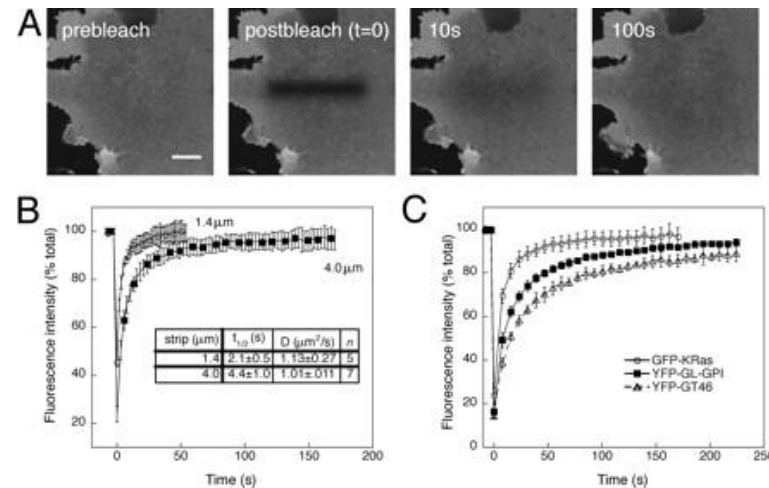
- In this geometry,  $D$  is typically calculated numerically by comparing fluorescence recovery curves with simulated curves  
(See i.e. [http://www.embl.de/eamnet/html/frap\\_analysis.html](http://www.embl.de/eamnet/html/frap_analysis.html))



# Dynamics of putative raft-associated proteins at the cell surface

Anne K. Kenworthy,<sup>1,2,3</sup> Benjamin J. Nichols,<sup>1,4</sup> Catha L. Remmert,<sup>2</sup> Glenn M. Hendrix,<sup>2</sup> Mukesh Kumar,<sup>5</sup> Joshua Zimmerberg,<sup>5</sup> and Jennifer Lippincott-Schwartz<sup>1</sup>

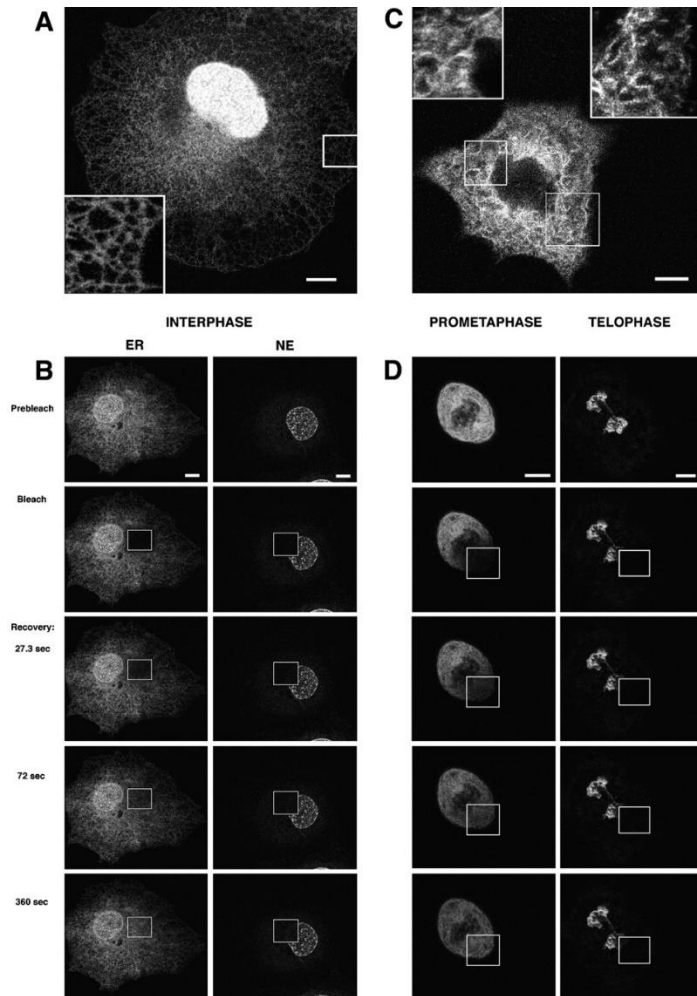
The Journal of Cell Biology, Volume 165, Number 5, June 7, 2004 735–746



Large-scale lateral diffusion measurements by confocal microscopy. (A) Selected images from a confocal FRAP experiment at 37°C of GFP-KRas expressed in COS-7 cells. Bleach box, 4 μm wide. Bar, 10 μm. (B) Kinetics of recovery for 1.4- (circles) versus 4-μm-wide (squares) bleach box. Calculated D and t<sub>1/2</sub> values are indicated. Data shown are for GFP-KRas expressed in COS-7 cells at 37°C. (C) Kinetics of recovery for YFP-GT46 (triangles), YFP-GL-GPI (squares), and GFP-KRas (circles) in COS-7 cells at 37°C using a 4-μm-wide bleach box. Each curve shows the mean ± SD from seven to nine cells from a single experiment. The calculated Ds were as follows: GFP-KRas, 1.01 ± 0.11 μm<sup>2</sup>/s; YFP-GL-GPI, 0.47 ± 0.07 μm<sup>2</sup>/s; YFP-GT46, 0.23 ± 0.02 μm<sup>2</sup>/s.

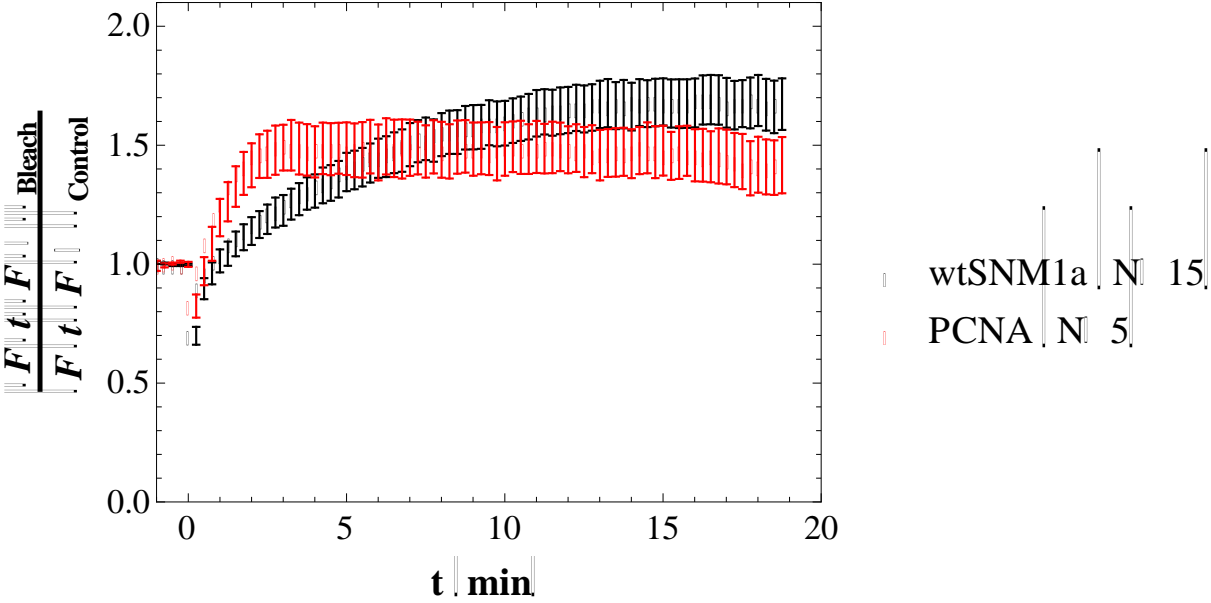
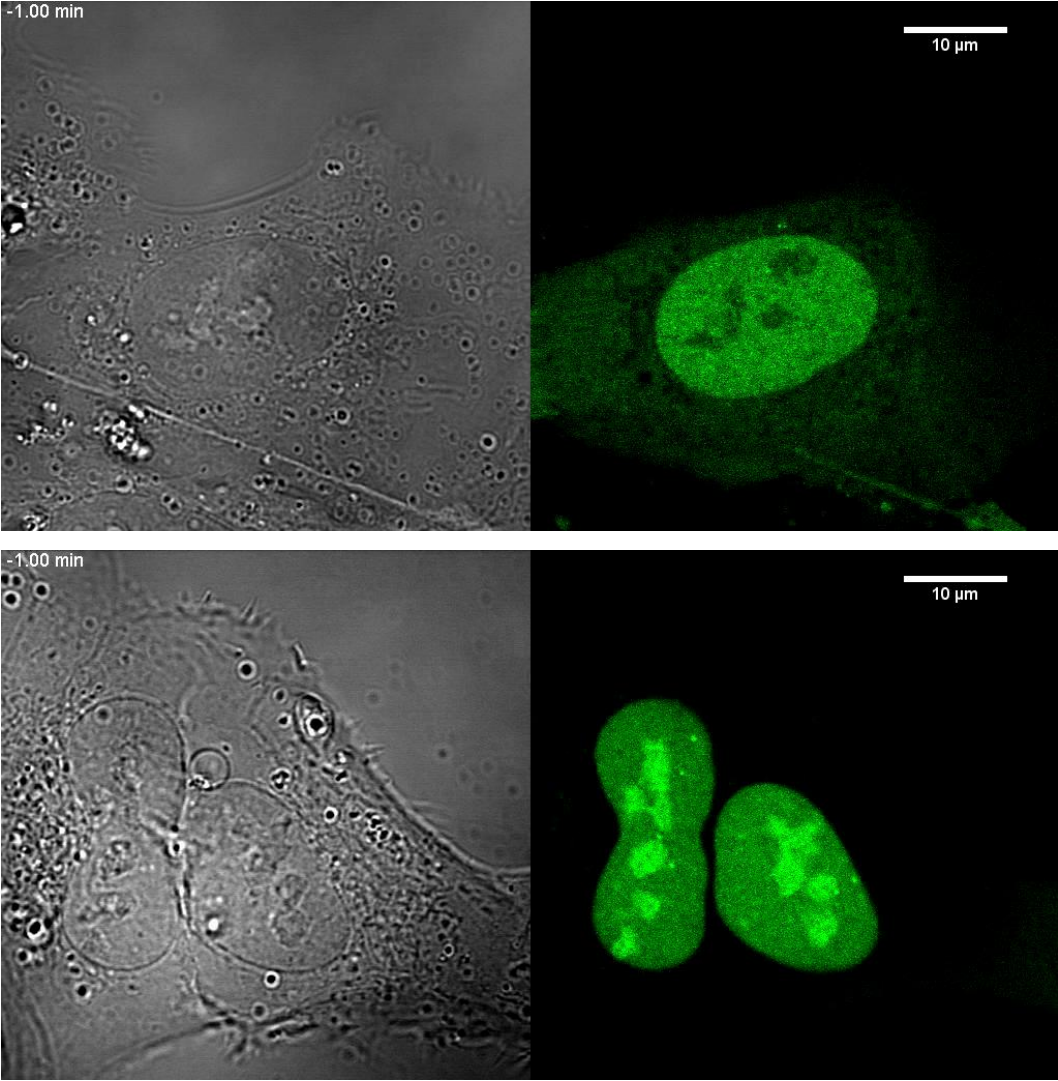


J. Ellenberg, E. D. Siggia, J. E. Moreira, C. L. Smith, J. F. Presley, H. J. Worman, and J. Lippincott-Schwartz (1997)  
 Nuclear Membrane Dynamics and Reassembly in Living Cells: Targeting of an Inner Nuclear Membrane Protein  
 in Interphase and Mitosis. *J. Cell Biol.* 138: 1193-1206



Distribution and mobilities of LBR-GFP in interphase and mitotic membranes. (A) Confocal section close to the lower cell surface showing steady-state expression of LBR-GFP in an interphase cell. *(Inset)* Boxed region at higher magnification showing LBR-GFP distribution within the ER network. (B) Qualitative FRAP experiments in ER and NE membranes in interphase cells expressing LBR-GFP. *(Left)* Photobleach recovery in ER membranes. *(Right)* Photobleach recovery in NE membranes. Note the complete recovery of fluorescence in the ER and the lack of recovery in the NE. (C) Thin confocal section through the mitotic apparatus showing the steady-state expression pattern of LBR-GFP in metaphase cells. *(Insets)* Boxed regions at higher magnification showing the tubular membrane network within which LBR-GFP redistributed. Note its resemblance to the interphase ER shown in A. (D) Qualitative FRAP experiments in mitotic membranes of cells expressing LBR-GFP. *(Left)* Photobleach recovery in prometaphase membranes. *(Right)* Photobleach recovery in telophase membranes.

DNA damage; U2OS cells expressing PCNA-GFP or wtSNM1A-GFP imaged on Confocal microscope at 240 z-planes/h



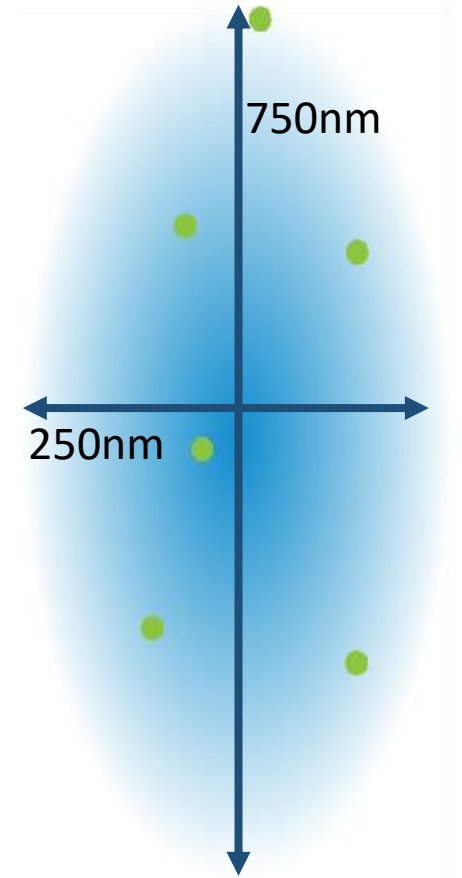
Characterization of role of *SNM1A*, a 5′–3′ exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuahini, Christoffer Lagerholm, Peter McHugh)

### 3) FCS – Fluorescence Correlation Spectroscopy

- FCS is used to get time-resolved information about an ensemble with single molecule sensitivity in a small confocal volume
- Widely used technique to obtain quantitative information such as
  - diffusion coefficients
  - hydrodynamic radii
  - average concentrations
  - kinetic chemical reaction rates
  - singlet-triplet dynamics

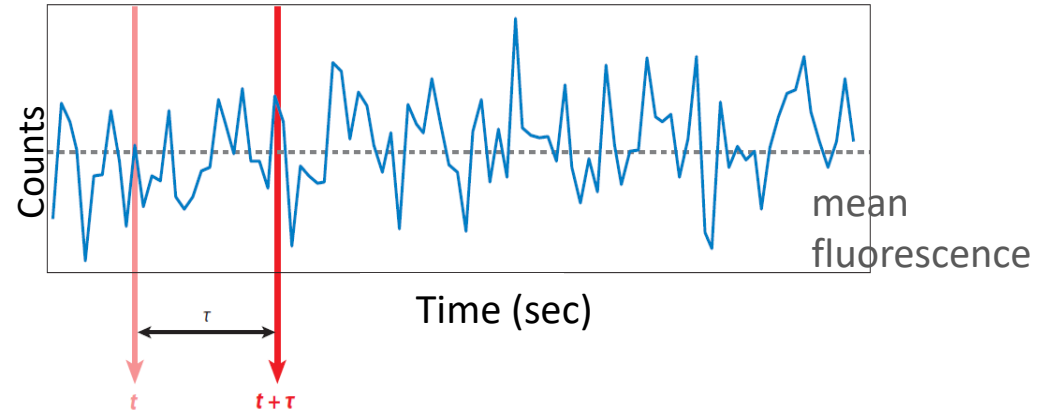
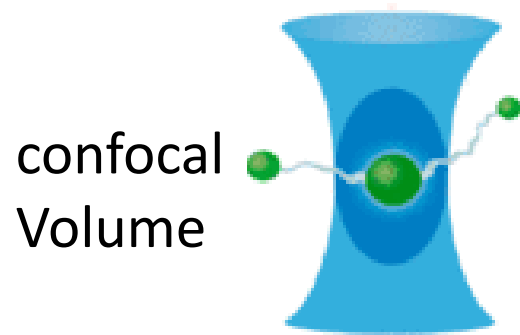
$$D = \frac{k_B T}{6\pi \eta r}$$

Diffusion in solution (3D) – Stokes-Einstein relation



Volume  $\approx 10^{-15}$

# FCS – Autocorrelation of Fluorescence Fluctuations

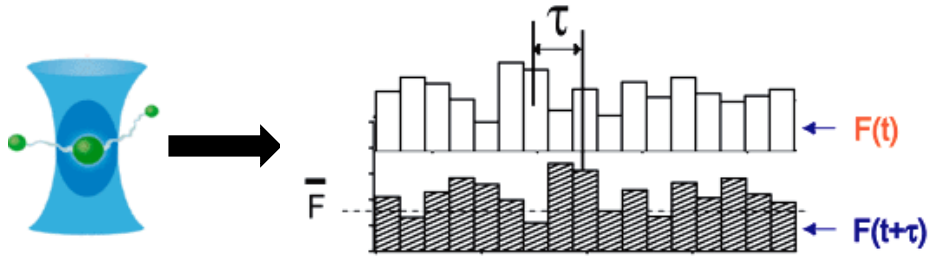


What causes fluctuations in intensity? a) Poisson noise,  
b) diffusion of molecules



Bursts of photons are seen as single  
fluorophores diffuse in and out of the  
laser beam

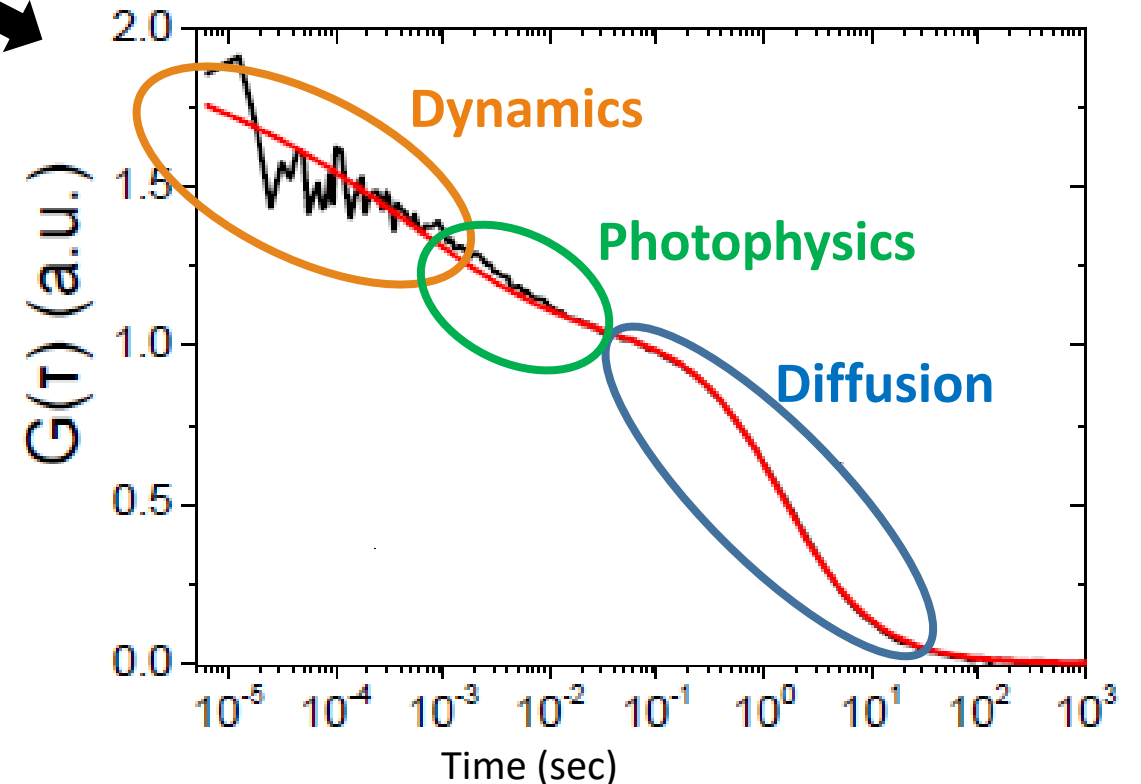
# FCS – Autocorrelation of Fluorescence Fluctuations



$$G(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} (1 + A \exp(-k\tau))^\beta$$

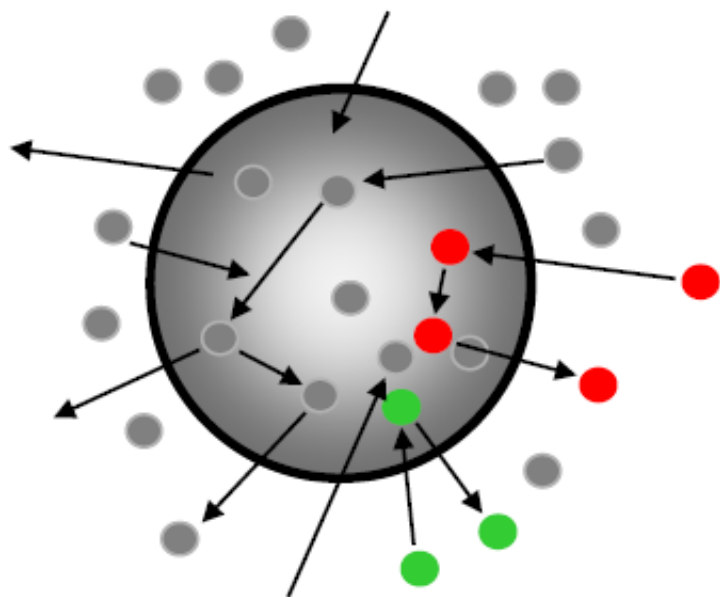
2D Gaussian Model

- What does FCS tell us?
  - diffusion rates -  $D \propto m^{1/3}$ .
  - the number of fluorescent objects in volume and hence concentration.
  - Relaxation rates and folding times

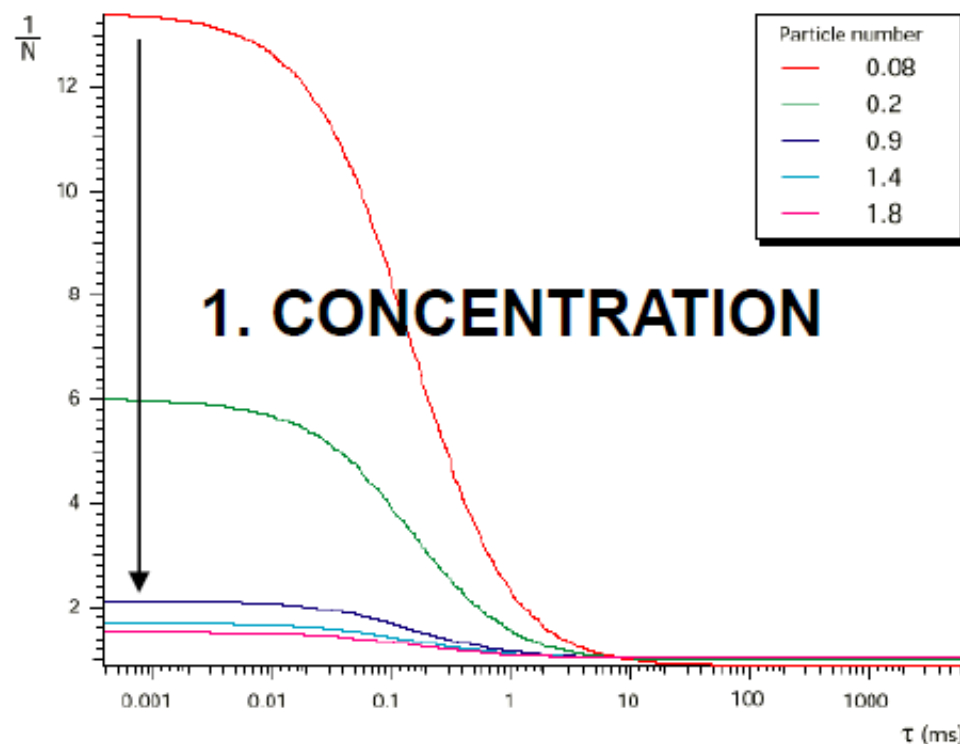


# Fluorescence Correlation Spectroscopy

*Wrapping it up: How many*



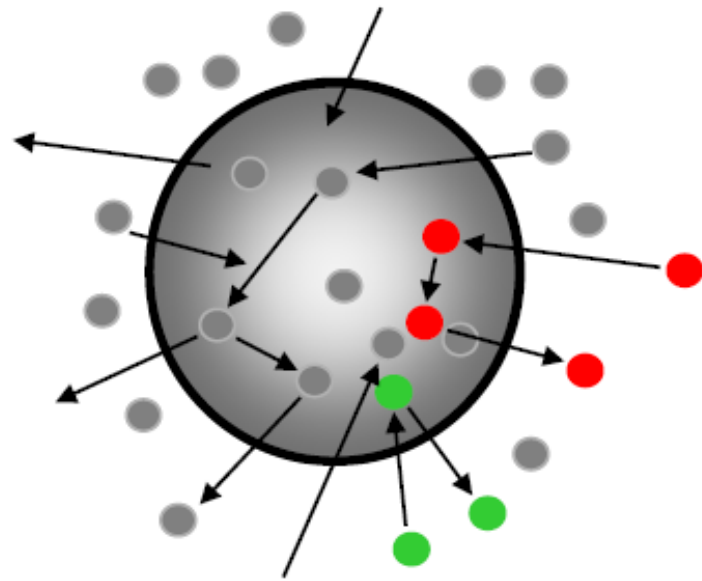
$$g^{(2)}(0) \sim 1 / N$$



**1. Concentration dynamic range: pM -  $\mu$ M**

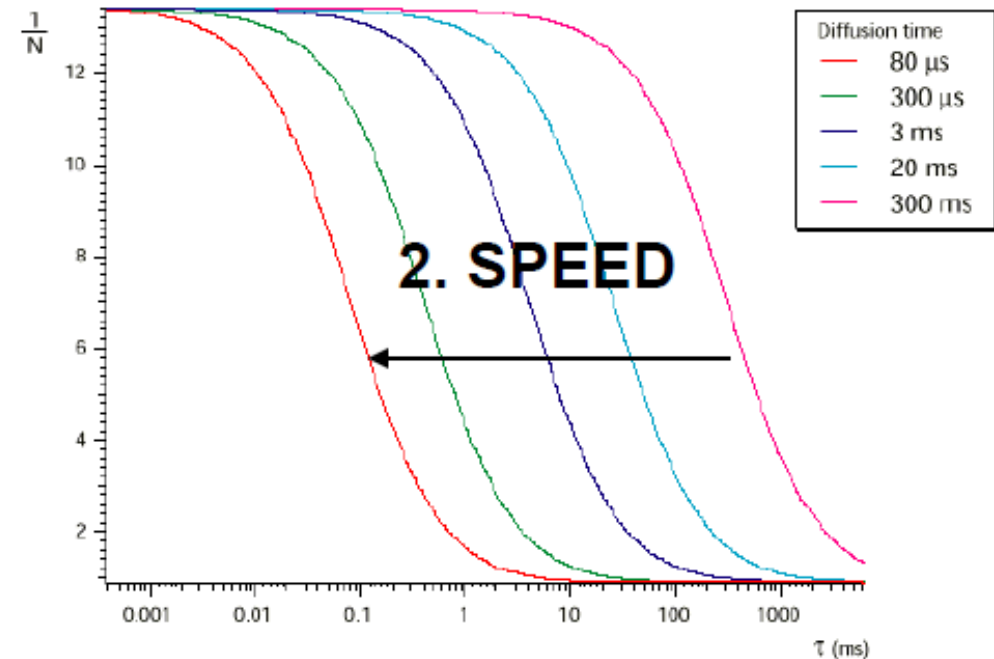
# Fluorescence Correlation Spectroscopy

*Wrapping it up: How many, how fast*



$$g^{(2)}(0) \sim 1 / N$$

$$\tau_D \sim 1 / D$$

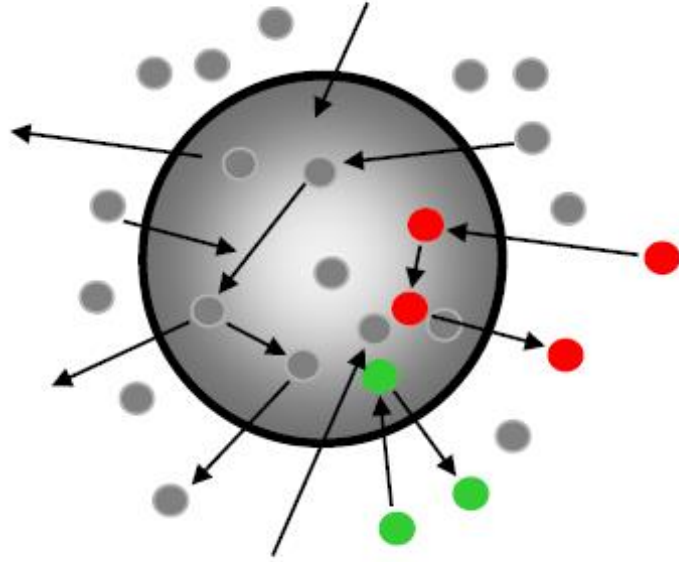


1. Concentration dynamic range: pM –  $\mu$ M
2. Time scale range:  $\mu$ s - s



# Fluorescence Correlation Spectroscopy

*Wrapping it up: How many, how fast, what mechanism*



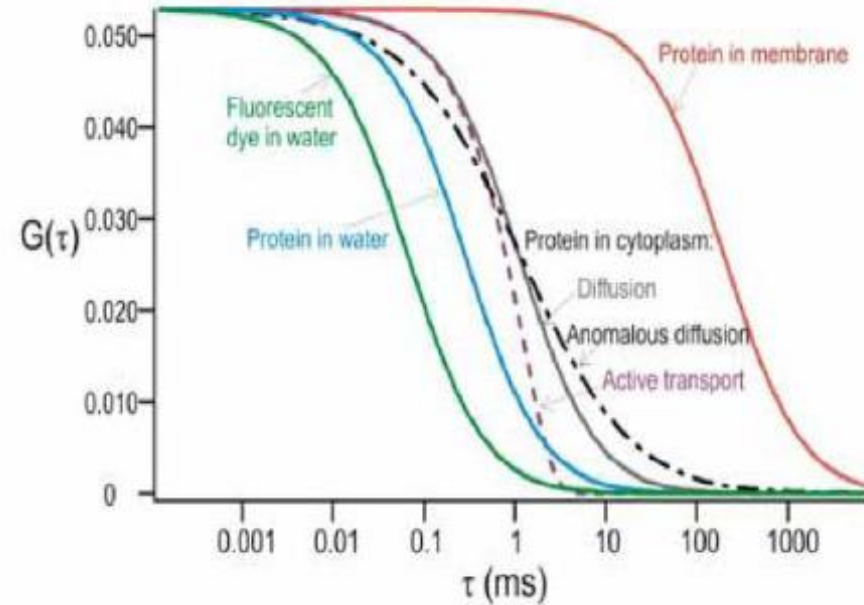
$$g^{(2)}(0) \sim 1 / N$$

$$\tau_D \sim 1 / D$$

$$g^{(2)}(\tau) \sim \text{shape}$$

$$g^{(2)}(\tau) = 1 + \frac{A}{1 + \frac{\tau \cdot 4D}{w^2}}$$

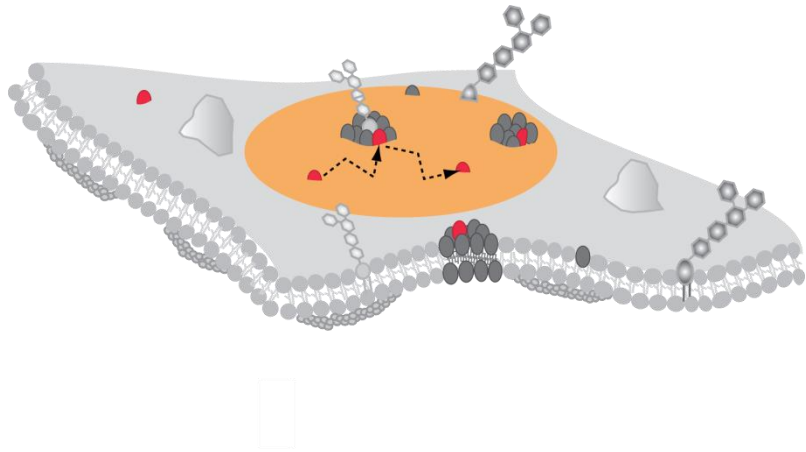
## 3. TYPE OF MOVEMENT



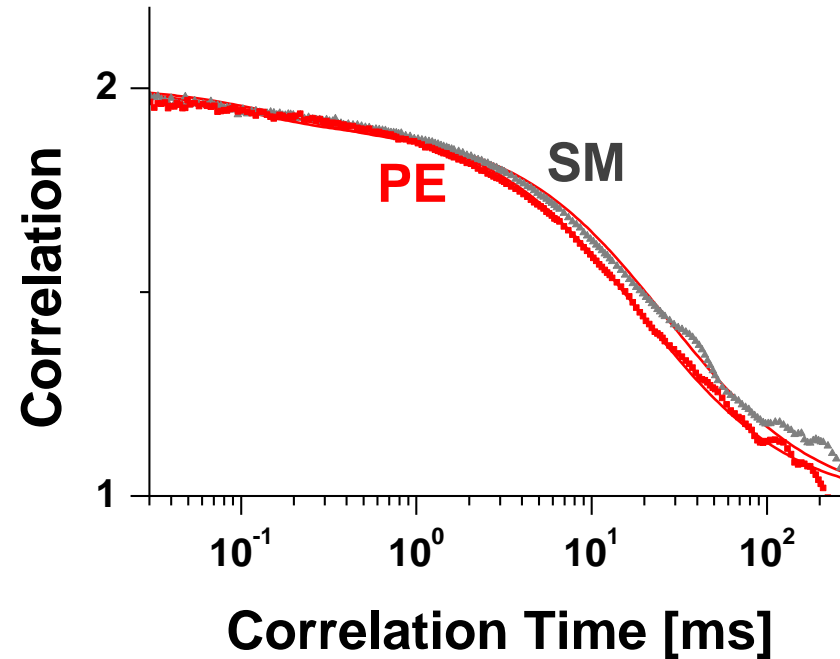
1. Concentration dynamic range: pM –  $\mu$ M
2. Time scale range:  $\mu$ s – s
3. Type of dynamics: rafts, flow, free, ...



# Example: Translational diffusion of lipids in plasma membrane of live cells



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



SM diffusion slightly prolonged but still normal

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

$(1/\alpha) \approx 1 \text{ (PE / SM)}$

# Summary - Fluorescence techniques for measuring molecular motion

- All methods are dependent on the availability of theoretical models such that experimental data is compared to theoretical models by curve fitting
- SPT can yield information about “heterogeneties” of single molecule behavior that is not apparent in ensemble average techniques and at a spatial resolution of ~ tens of nanometers  
...but only for single molecules that are separated by a distance greater than  $r_{\text{Airy}}$
- FRAP and FCS are ensemble average techniques that are often easy to implement on a standard confocal microscope
- FCS is best choice for very fast dynamics including 3D diffusion in solution of small molecules
- Techniques can also be used to investigate binding constants and equilibria (with suitable models)

## Fluorescence techniques for measuring molecular interactions

- 1) Förster Resonance Energy Transfer (FRET)
- 2) Fluorescence Cross-Correlation Spectroscopy (FCCS)

Why can't we image molecular interactions directly?

Co-localization?

# Microscope Resolution

- The diffraction of light causing the Airy disk is also the limiting factor of the resolution of a microscope
- The resolution of a microscope is defined as the minimum distance two objects have to be separated by to be resolved as two separate objects



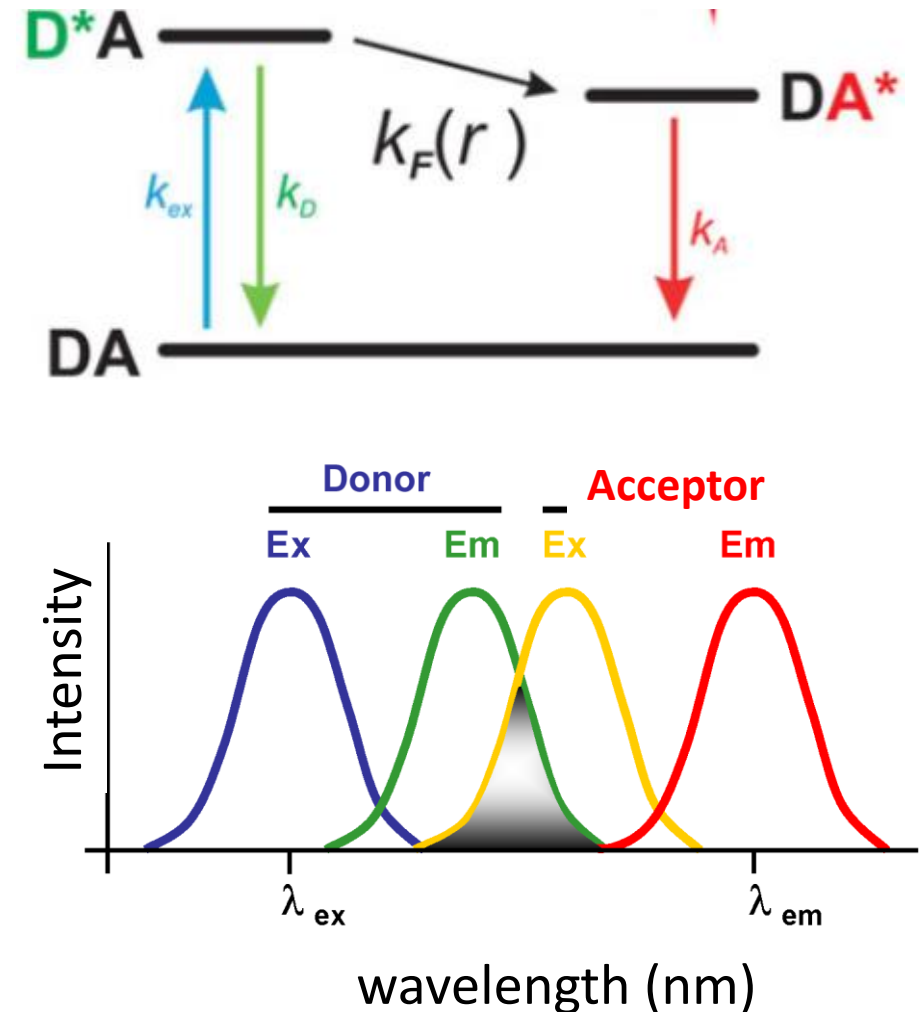
Airy patterns of two point sources

- For 100X magnification, 1.4 NA oil immersion objective and illumination with green light (500 nm = 0.5  $\mu\text{m}$ )

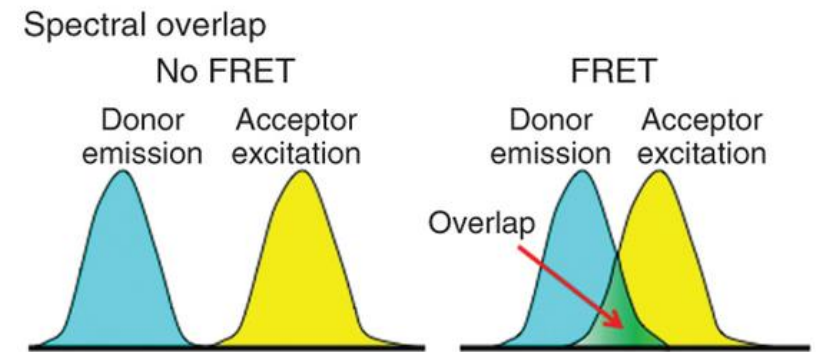
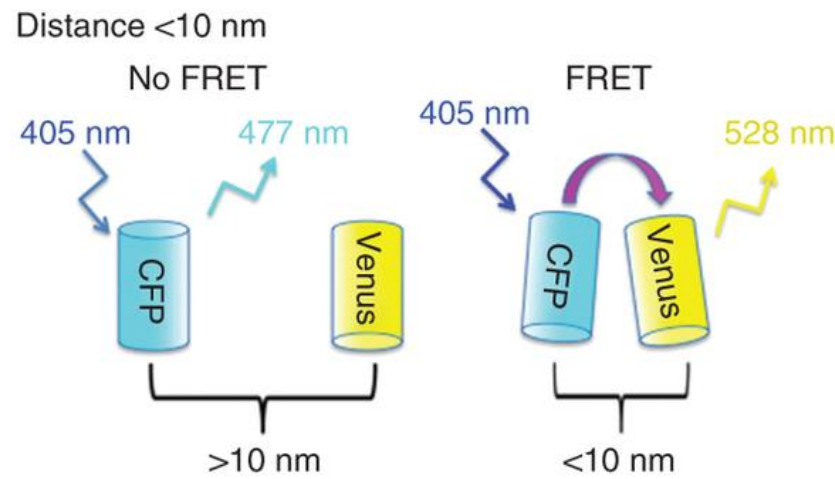
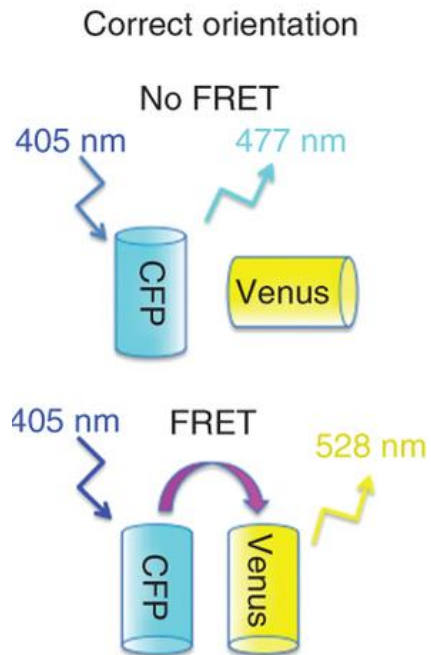
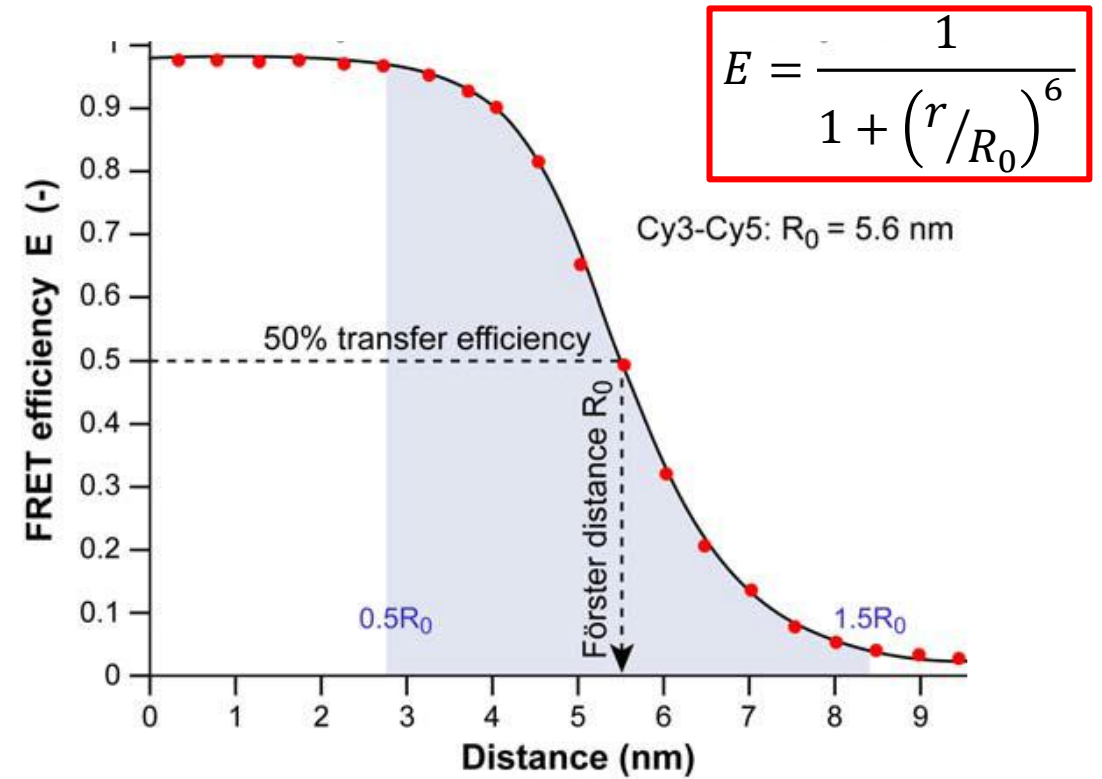
$$\text{Resolution} = \frac{(0.61) (0.5 \mu\text{m})}{1.4} \approx 220 \text{ nm}$$

# 1) FRET – Förster Resonance Energy Transfer

- energy is transferred non-radiatively (via long-range dipole-dipole coupling)
- relies on the close physical interaction of the two fluorophores (0.5 – 10nm)
- used to determine molecular interactions beyond the resolution limits



- distance between two molecules
- spectral overlap
- the quantum yield of the donor
- the relative orientation of the transition dipoles



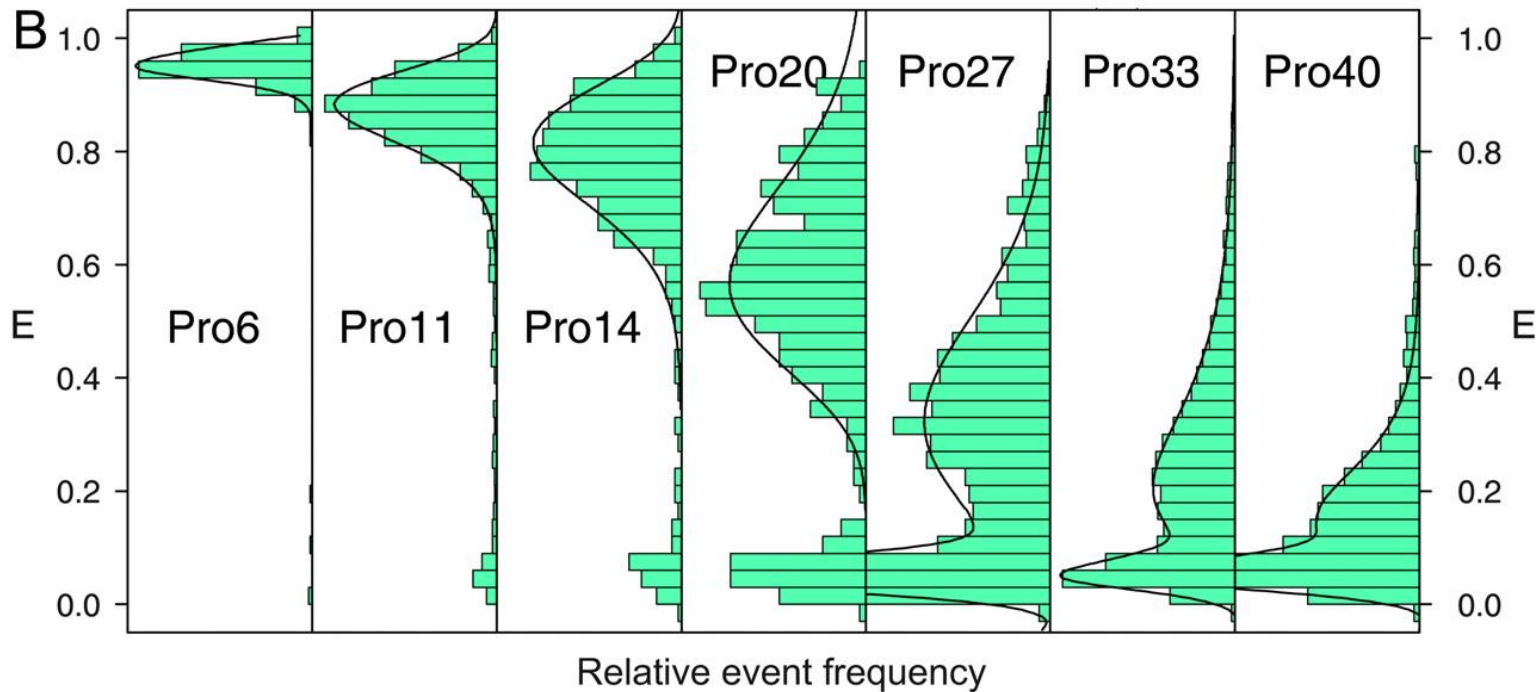
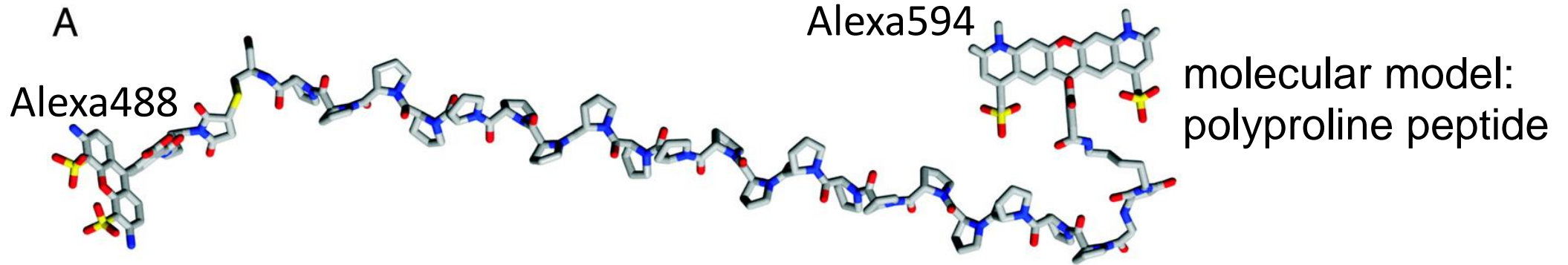
# FRET Pairs

Donor	Acceptor	Donor Excitation $\lambda_{\max}$ (nm)	Acceptor Emission $\lambda_{\max}$ (nm)	Donor QY (-)	Acceptor $\epsilon$ ( $M^{-1}cm^{-1}$ )	Förster Distance (nm)
Fluorescent protein FRET pairs						
BFP	DsRFP	380	586	0.18	72,500	3.1–3.3
EBFP2	mEGFP	383	507	0.56	57,500	4.8
CFP	GFP	433	509	0.40	21,000	4.7–4.9
CFP	YFP	433	526	0.40	77,000	~ 5.0
Cerulean	YFP	440	526	0.62	77,000	–
ECFP	EYFP	440	527	0.40	83,400	4.9
Cerulean	Venus	440	528	0.62	92,200	5.4
MiCy	mKO	472	559	0.90	51,600	5.3
GFP	YFP	475	526	0.77	77,000	5.5–5.7
GFP	mRFP	475	579	0.77	50,000	~ 4.7
CyPet	YPet	477	530	0.51	104,000	5.1
TFP1	mVenus	492	528	0.85	92,200	5.1
EGFP	mCherry	507	510	0.60	72,000	5.1
Venus	mCherry	528	610	0.57	72,000	5.7
Venus	tdTomato	528	581	0.57	138,000	5.9
Venus	mPlum	528	649	0.57	41,000	5.2
Fluorescent protein-dye FRET pairs						
EGFP	Alexa Fluor 555	484	568	0.6	155,000	6.3
EGFP	Alexa Fluor 546	484	573	0.6	112,000	5.7
EGFP	Alexa Fluor 594	484	618	0.6	92,000	5.3
EGFP	Alexa Fluor 568	484	603	0.6	88,000	5.4
Dye and dye-biofluorochrome FRET pairs						
Cy2	Cy3	489	570	> 0.12	150,000	5.0–6.0
Cy3	Cy5	550	670	> 0.15	250,000	>5.0

Cy5	Cy5.5	649	694	> 0.28	250,000	>8.0
Fluorescein	Tetramethylrhodamine	487 <sup>1</sup>	574	0.93 <sup>2</sup>	~ 87,000	4.9–5.5
FITC	TRITC	494	572	0.92	100,000	5.4
Phycoerythrin	APC <sup>3</sup>	(546), 565	660	0.98	700,000	4.0–11.0
Europium	APC	340	660	–	700,000	9.0
Tryptophan	Dansyl	280	525	0.01–0.35 <sup>4</sup>	4050	2.1
Dansyl	FITC	335	519	< 0.035 <sup>5</sup>	77,000	3.3–4.1
Dansyl	Octadecylrhodamine	335	625	< 0.035	106,000	4.3
Europium	Cy5	340	670	–	250,000	7.0
Atto 488	Atto 647N	501	670	0.8	150,000	5.1
Atto 488	Atto 590	501	621	0.8	120,000	6.0
Atto 550	Atto 647N	554	670	0.8	150,000	6.5
Atto 550	Atto 655	554	684	0.8	125,000	6.4
Atto 590	Atto 655	594	684	0.8	125,000	7.3
Alexa 405	Alexa 430	401	541	–	16,000	–
Alexa 488	Alexa 514	495	542	0.92	80,000	–
Alexa 488	Alexa 532	495	554	0.92	81,000	–
Alexa 488	Alexa 546	495	573	0.92	104,000	6.4
Alexa 488	Alexa 610	495	628	0.92	138,000	–
Alexa 647	Alexa 680	650	702	0.33	184,000	–
Alexa 647	Alexa 700	650	723	0.33	192,000	–
Alexa 647	Alexa 750	650	780	0.33	240,000	–
Non-fluorescent acceptor pairs						
Rhodamine 6G	Malachite Green (Abs 628 nm)	526	NF	0.95	76,000	6.1
Alexa 488	QSY 35 (Abs 475 nm)	495	NF	0.92	23,000	4.4
Alexa 488	Dabcyl (Abs 453 nm)	495	NF	0.92	32,000	4.9
Alexa 647	QSY 21 (Abs 661 nm)	650	NF	0.33	90,000	6.9

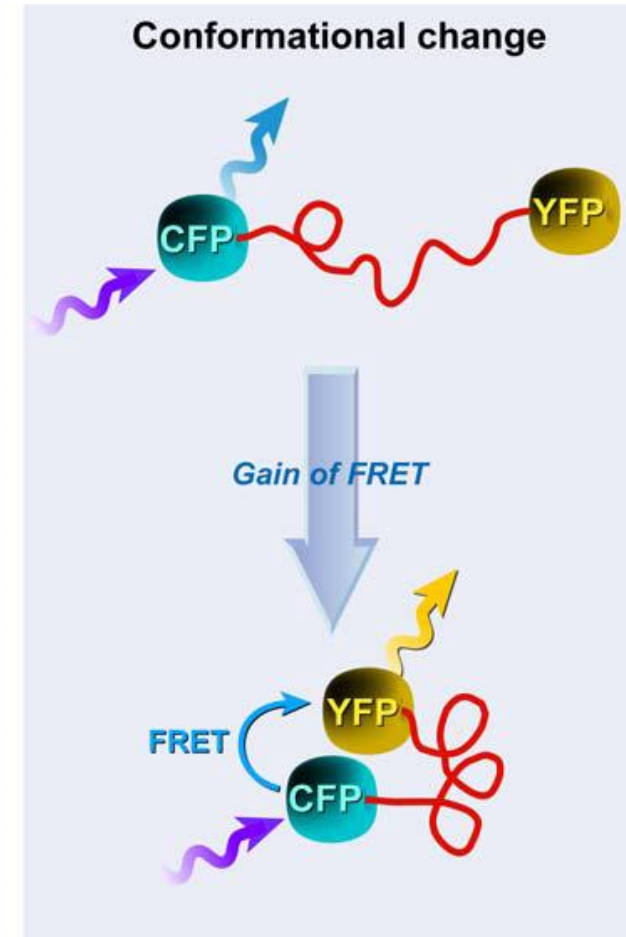
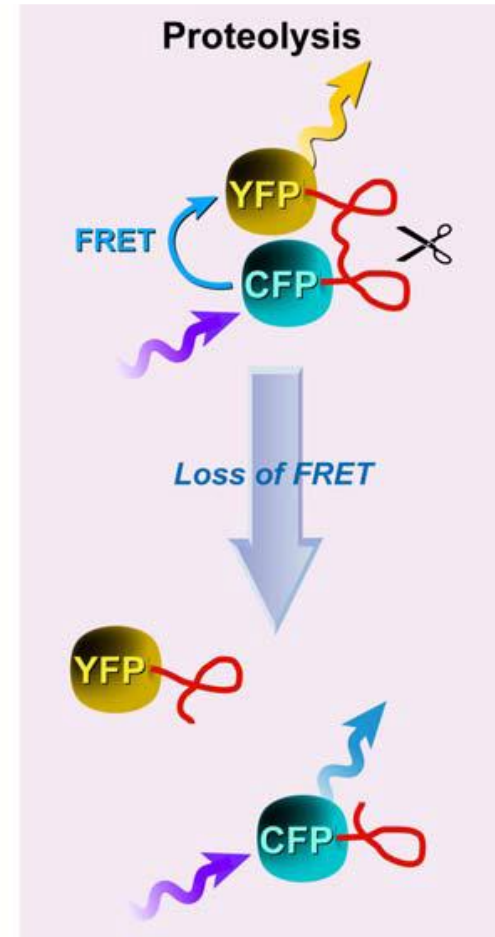
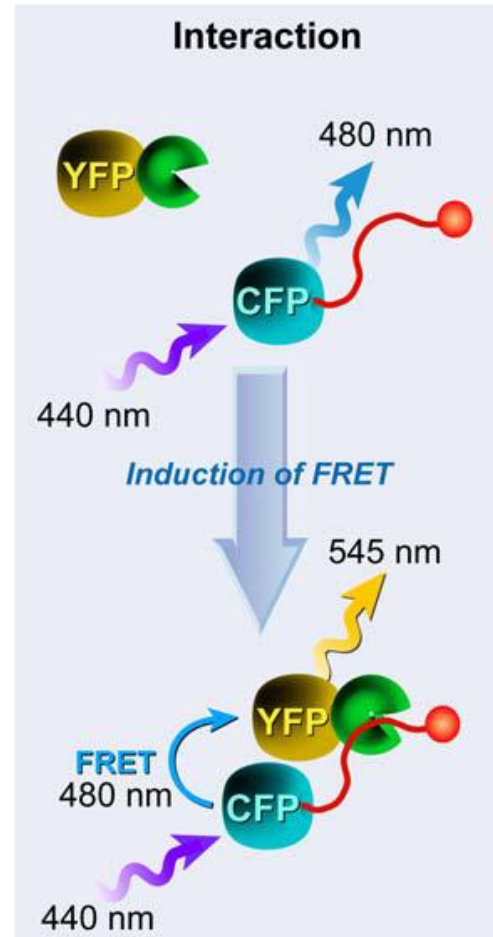


# FRET – traditionally used as Molecular Ruler



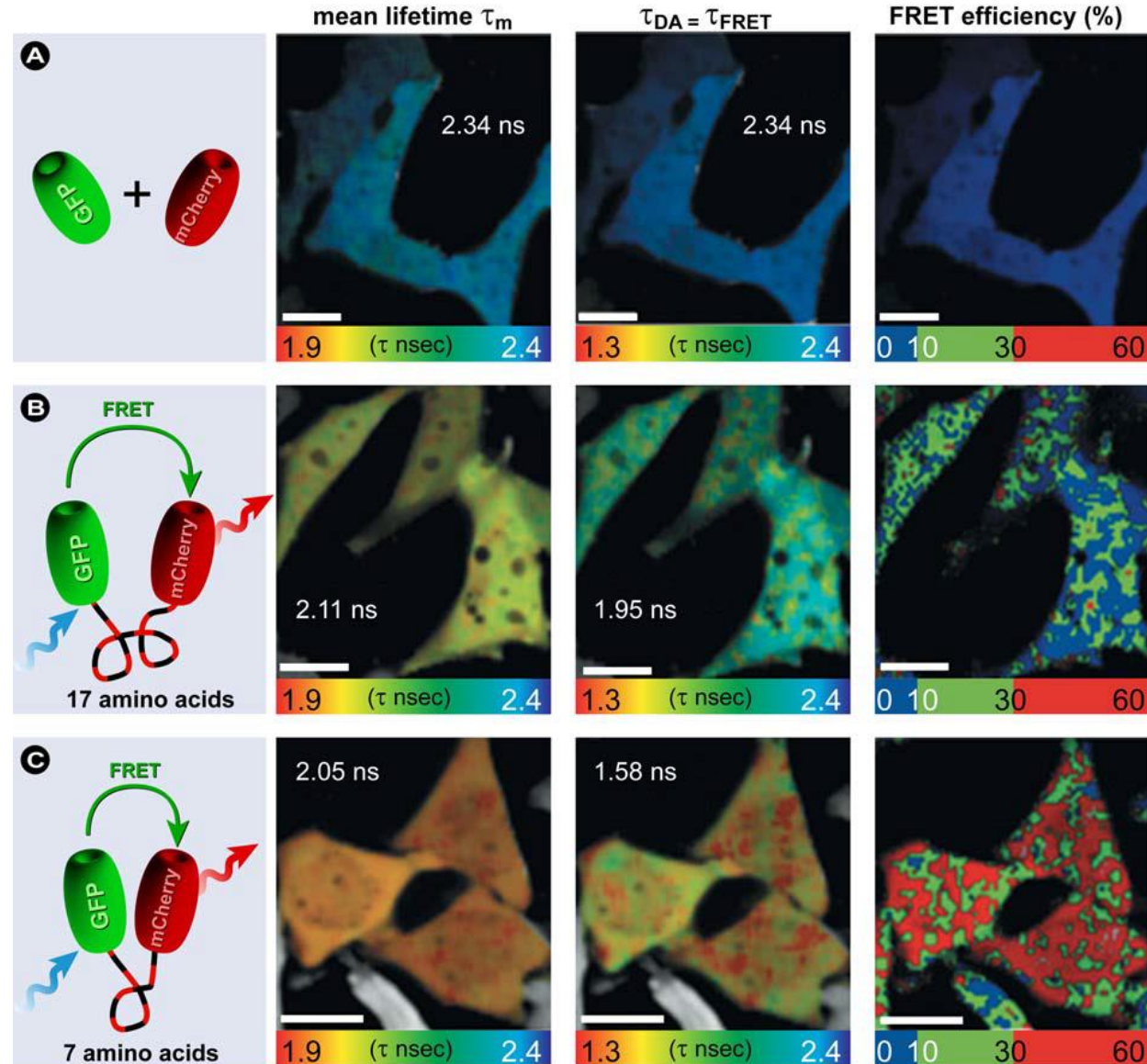
# Fundamental cellular FRET-Approaches

- FRET-biosensors: fusion proteins or other appropriate pairs linked by a sensory domain
- monitoring of donor/acceptor channels and detection of changes in the FRET signal as a result of biological activity



# FRET-FLIM

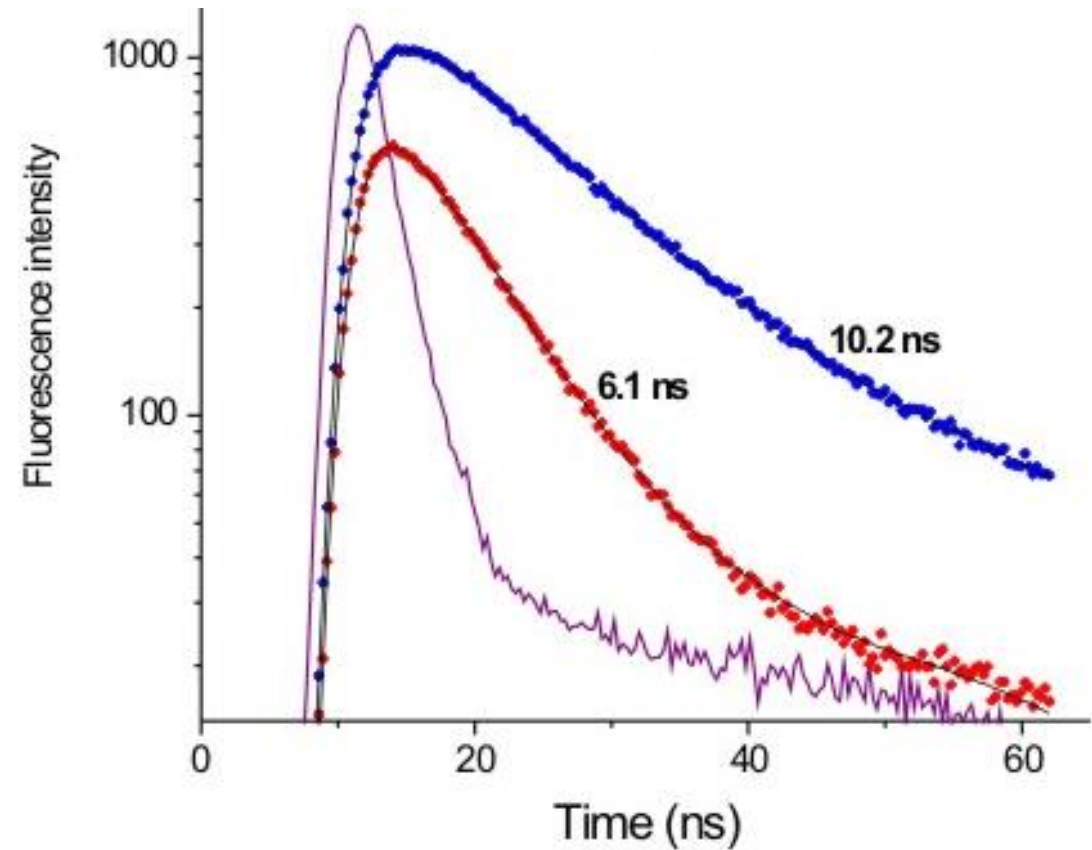
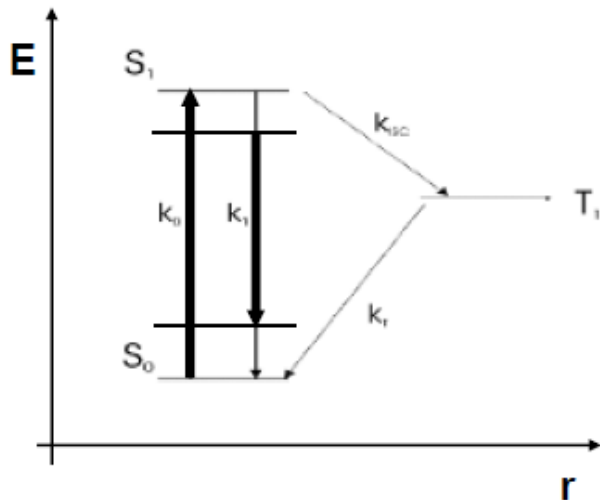
- measurement of lifetime dynamics pixel-by-pixel
- mapping of spatial distributions to measure biomolecule concentrations, interactions between biomolecules, and conformational changes with a much higher accuracy than conventional FRET methods



# A variation of FRET: FRET-FLIM

## FLIM – Fluorescence Lifetime Imaging

- ...is an imaging technique for producing an image based on the differences in the exponential decay rate of fluorophores.
- ...is independent of probe concentration
- the lifetime of the fluorophore signal, not the intensity, is used to create the image in FLIM.



# FLIM with TCSPC in practice

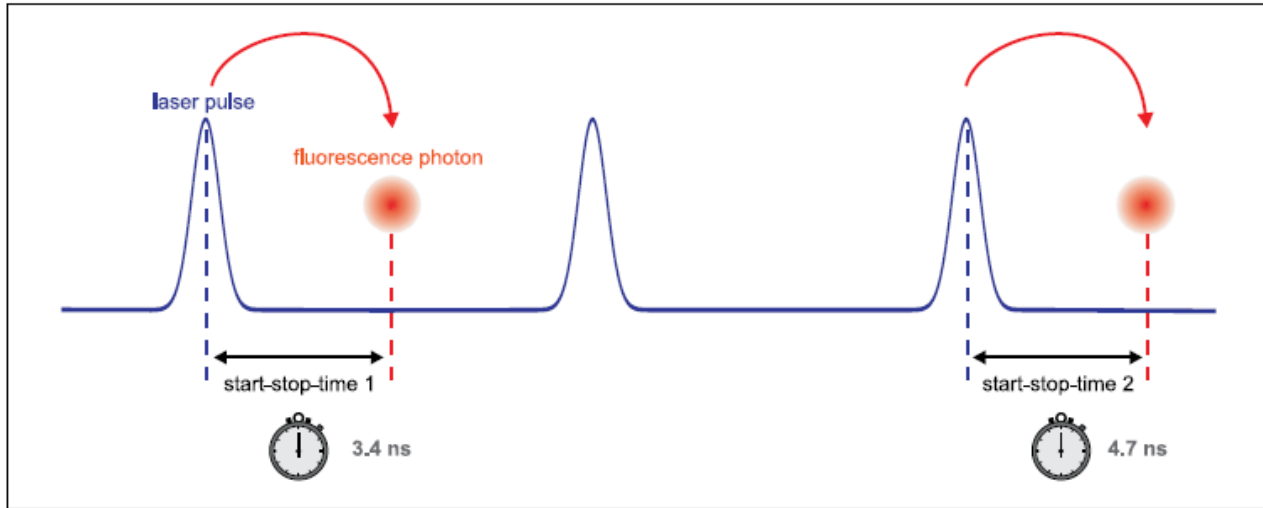


Figure 1: Measurement of start-stop times in time-resolved fluorescence measurement with TCSPC.

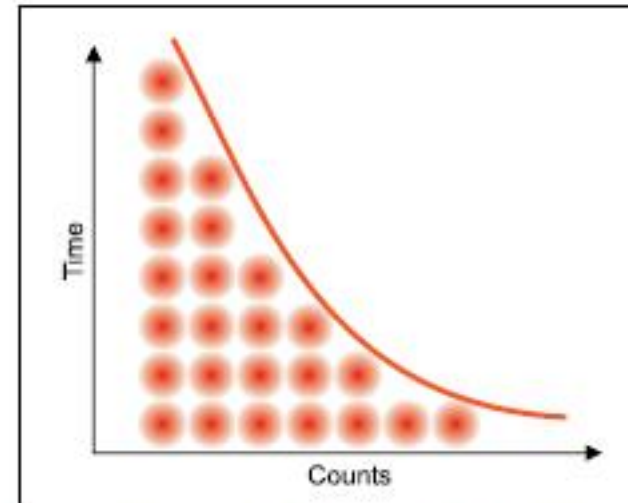


Figure 2: Histogram of start-stop times in time-resolved fluorescence measurement with TCSPC.

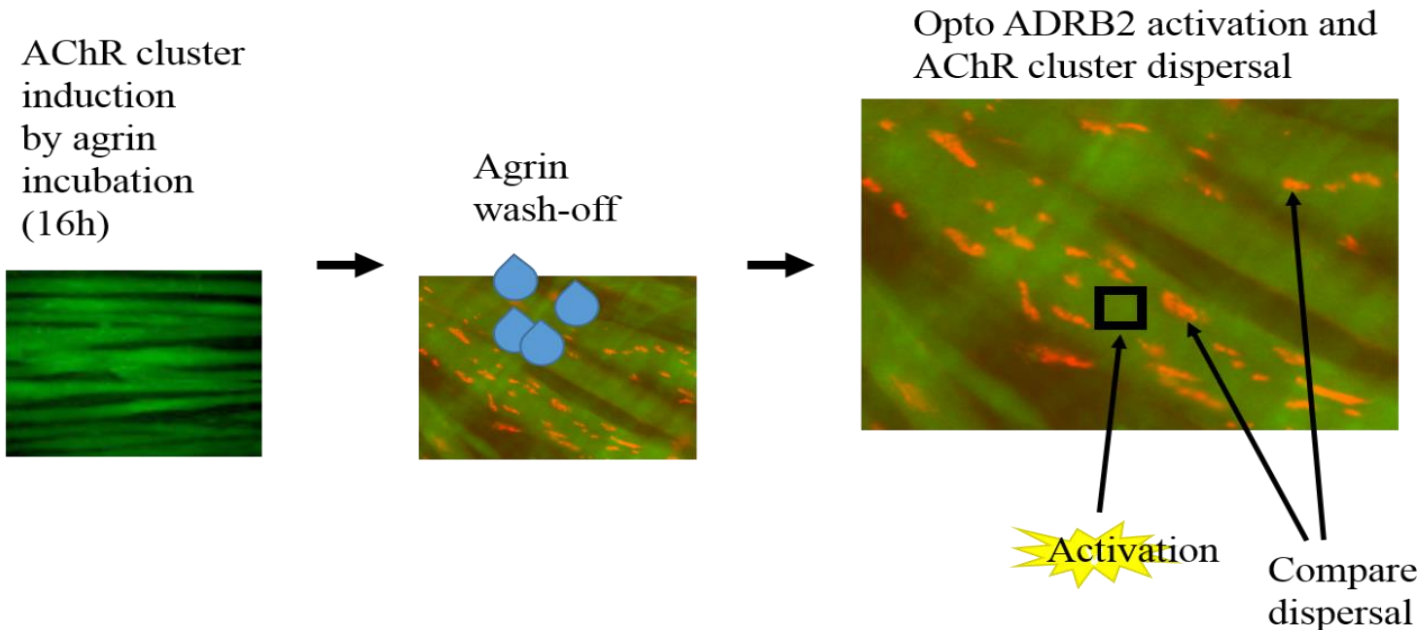
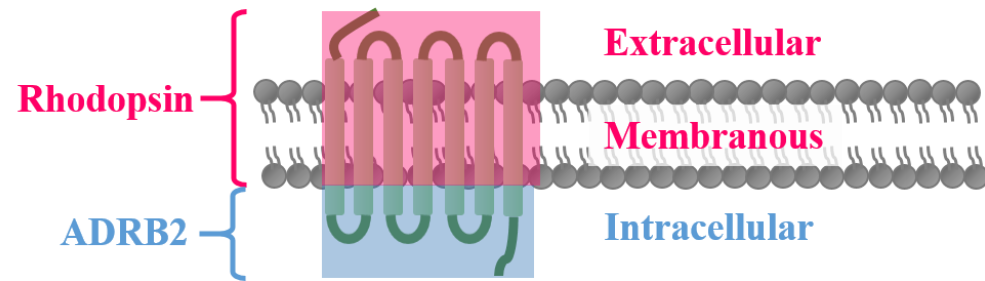


## A recent FLIM-FRET Example

An investigation in the role of cAMP in a signal transduction pathway that appears to link beta-2 adrenergic receptor activation to the stability of acetylcholine receptor (AChR) clusters at the neuromuscular junction (NMJ) by use of the cAMP fluorescent biosensor mTurquoise2 $\Delta$ -Epac(CD, $\Delta$ DEP, Q270E)-tdDark<sup>cp173</sup>Venus.

(a collaboration with Lisa Clausen, Judith Cossins, David Beeson)

Our approach: Combine optogenetics using light activated Rhodopsin-ADRB2 fusion protein with cAMP fluorescent biosensor

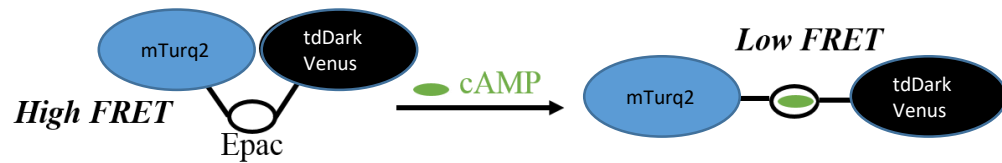


## A recent FLIM-FRET Example

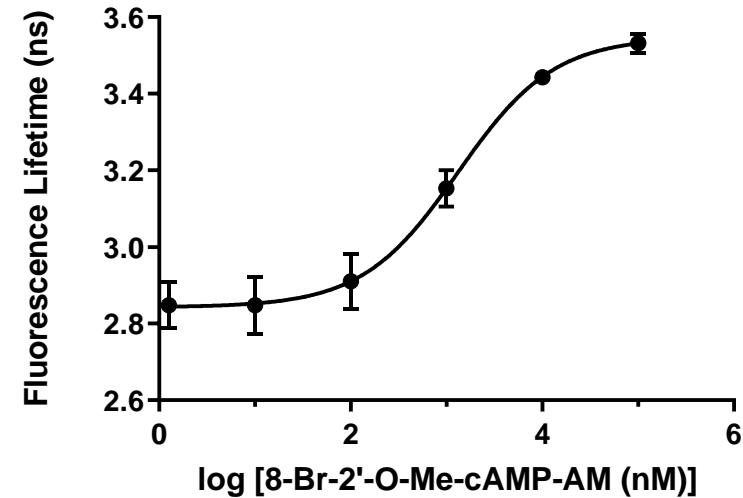
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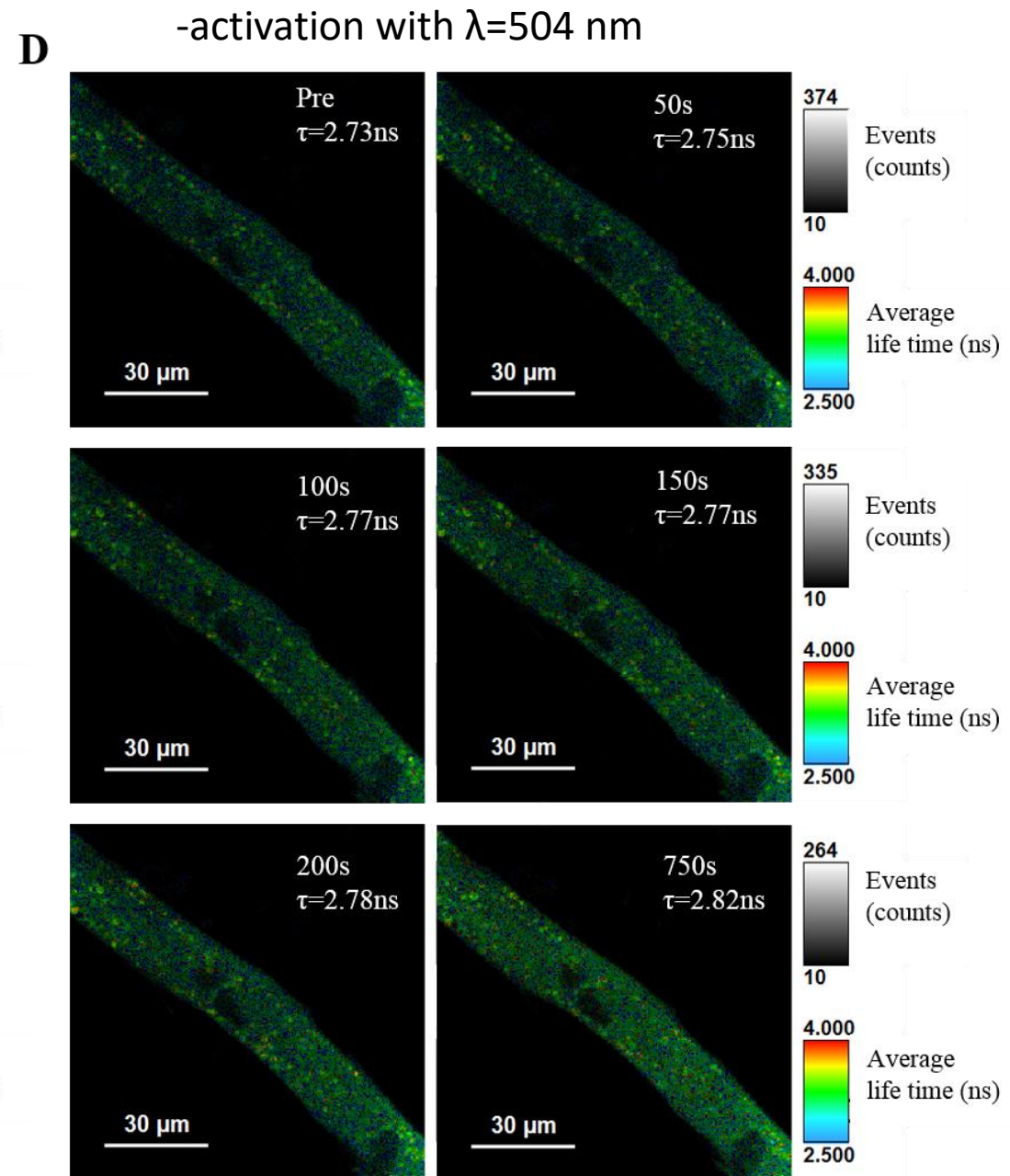
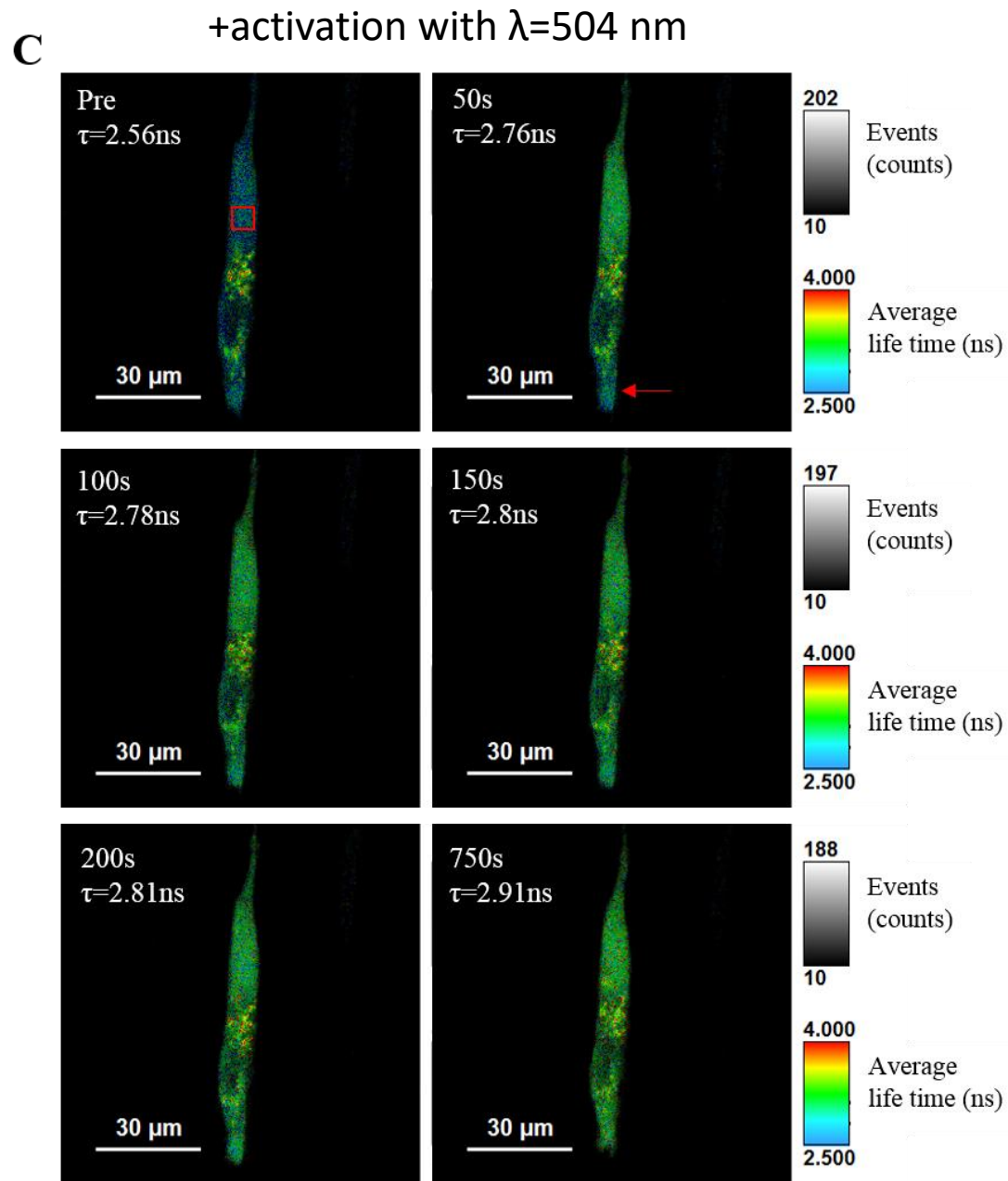
mTurquoise2 $\Delta$ -Epac(CD, $\Delta$ DEP, Q270E)-tdDark<sup>cp173</sup>Venus



**Titration of [cAMP] with  
mTurq2 $\Delta$ -Epac(CD, $\Delta$ DEP, Q270E)-tdDark<sup>cp173</sup>Ven  
in C2C12 myotubes**



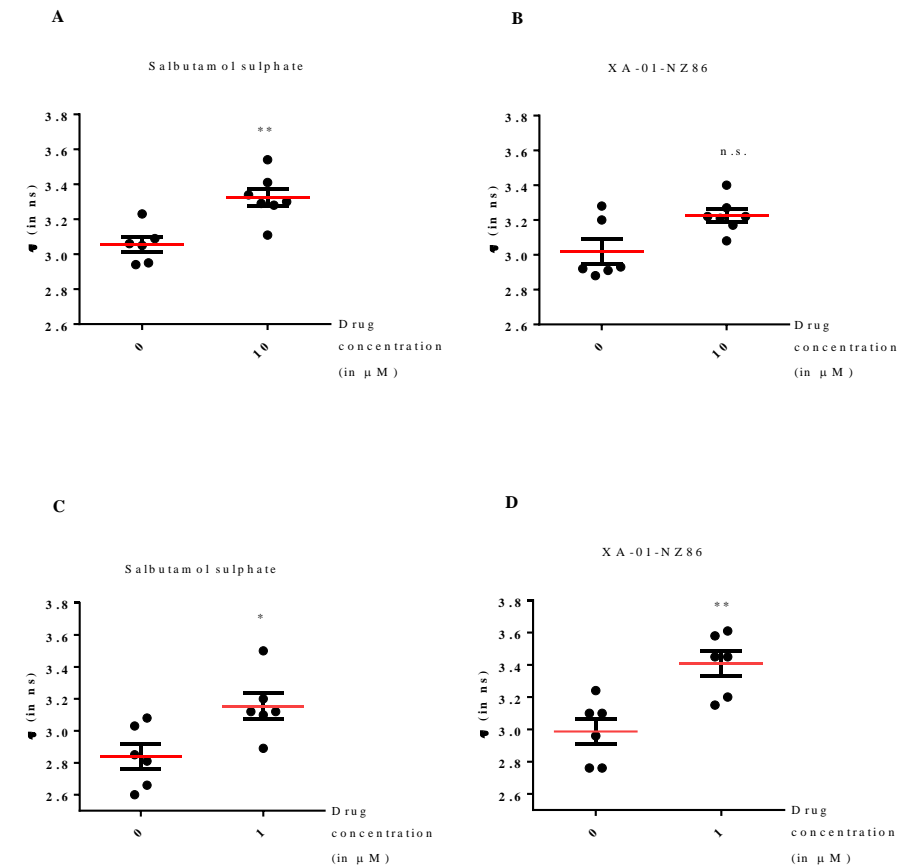
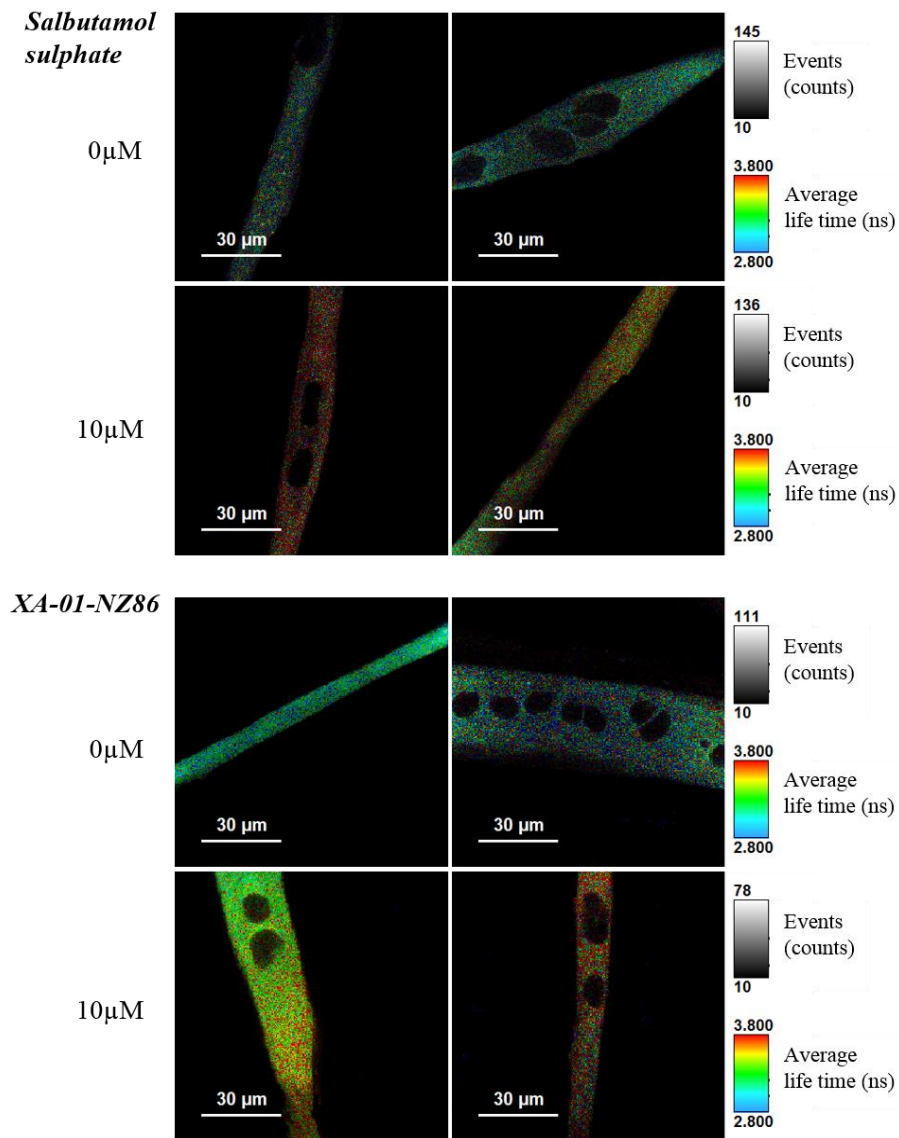
Day 8 of differentiation: Myotube state of the cells



$\sim \Delta\tau = +0.2-0.4$  ns  $\rightarrow$  0.2-1  $\mu$ m cAMP



# Equivalent cAMP response with ADRB2 agonists salbutamol and XA-01-NZ86

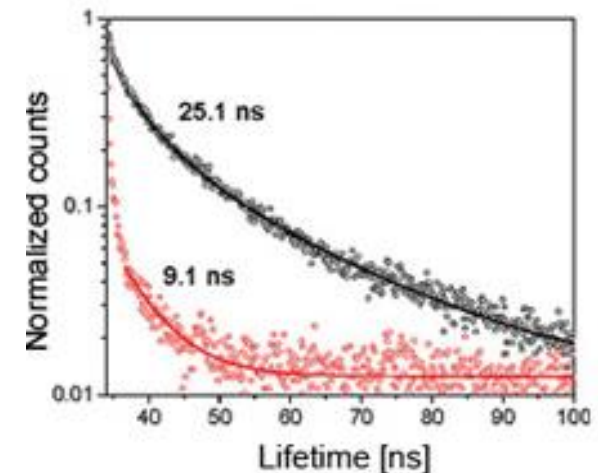
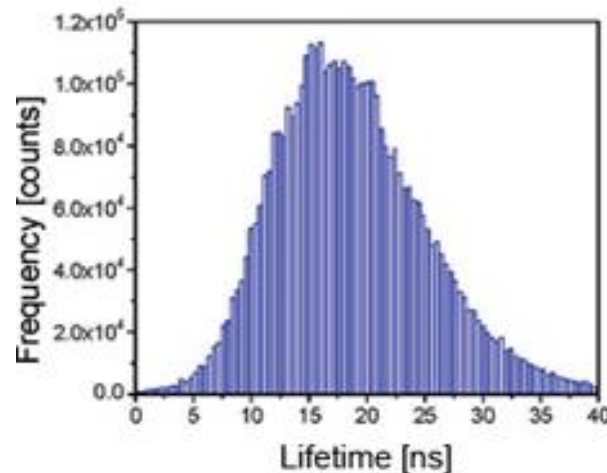
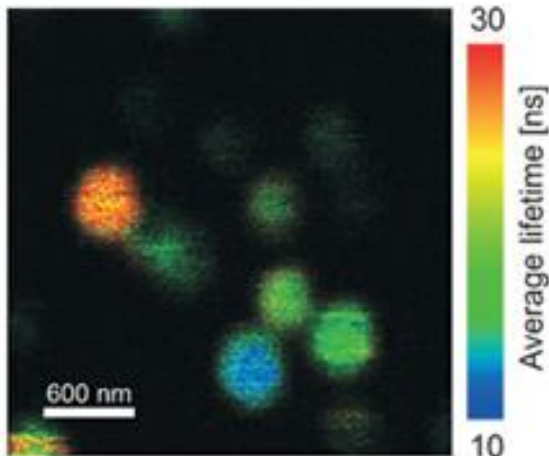


**Figure 5.10 (continued)** (E) Representative microscopic colour-coded images of C2C12<sup>WT-ADRB2+Epac</sup> myotubes obtained through TCSPC before and after 5min of incubation with 10μM salbutamol sulphate or XA-01-NZ86 on day 8 after electroporation with the FRET sensor. The shift in colour towards bright green/red illustrates the rise in cAMP levels.

**Figure 5.10 Effects of agonist-mediated activation of endogenous ADRB2 on cAMP levels in C2C12<sup>WT-ADRB2+Epac</sup> myotubes.** Measurements were taken before and after 5min of incubation with salbutamol sulphate or XA-01-NZ86 on day 8 after electroporation with the FRET sensor. (A) At 10μM, salbutamol sulphate significantly increased cAMP levels (difference=0.27ns, Mann-Whitney test p=0.002). N=6 (‘0μM’), N=7 (‘10μM’). (B) No significant difference occurred with 10μM XA-01-NZ86 (difference=0.2ns, Mann-Whitney test p=0.07). N=6 (‘0μM’), N=7 (‘10μM’). (C) Lowering the dose of the drugs to 1μM caused a larger increase in cAMP levels. Salbutamol sulphate raised the fluorescence lifetime by 0.32ns (Mann-Whitney test p=0.009). N=6 (‘0μM’), N=7 (‘10μM’). (D) Incubation with 1μM of XA-01-NZ86 raised the lifetime by 0.42ns (Mann-Whitney test p=0.007). Both N=6. Error bars indicate the standard error of the mean. n.s.>0.05, \*p<0.05, \*\* p<0.01.

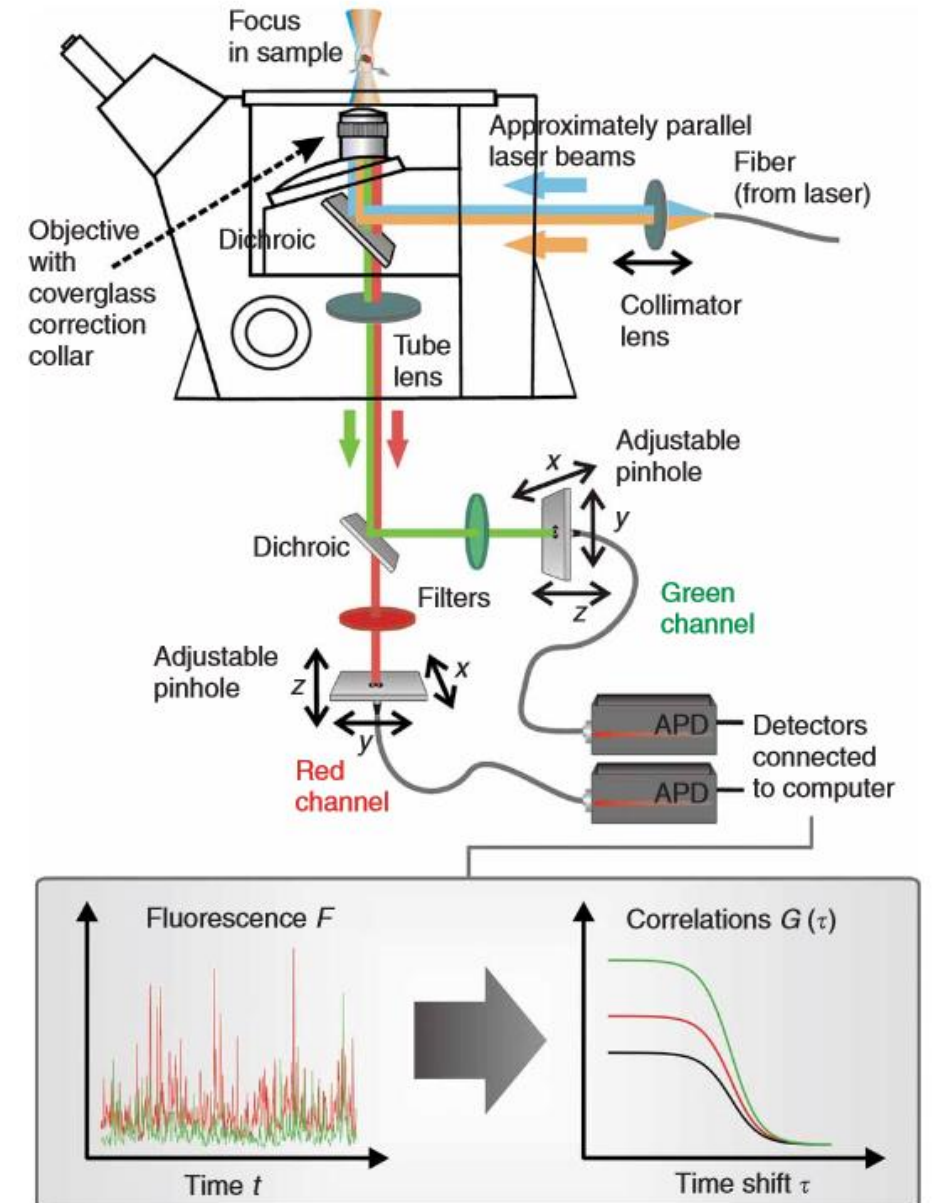
# FLIM Measurements

- essential components of a FLIM set-up:
  - pulsed laser source
  - detector (APD or PMT)
  - TCSPC unit to measure the time between excitation and fluorescence emission (time-domain FLIM)
- the delay times are sorted into a histogram



## 2) FCCS – Dual-Colour Extension of FCS

- interaction of two differently labeled molecular species with higher precision than single-color FCS
- high specificity: FCCS curve is only formed if the differently labeled molecules are bound and moving together (co-diffuse).
- From amplitude and decay time: binding constants, mobility of the bound complex, concentrations of all of the species

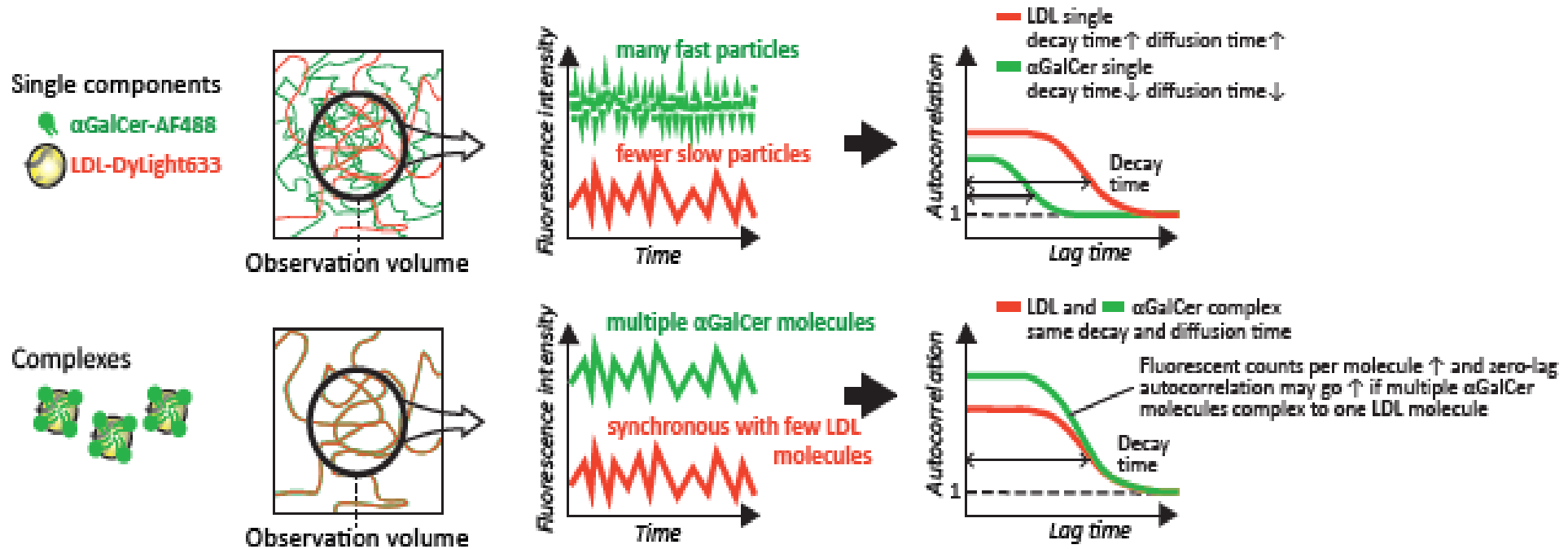


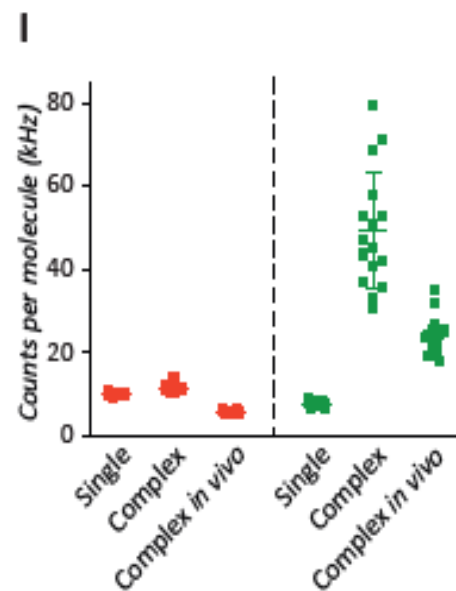
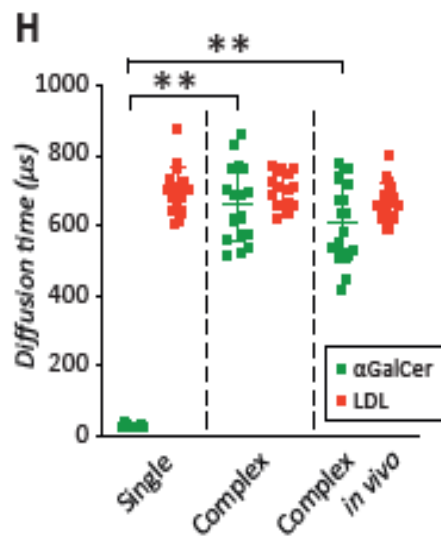
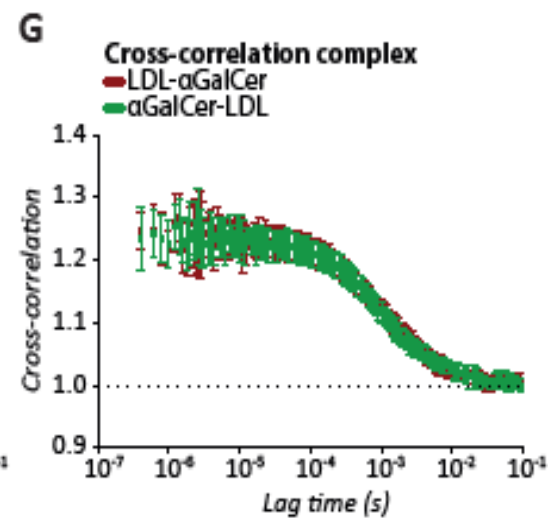
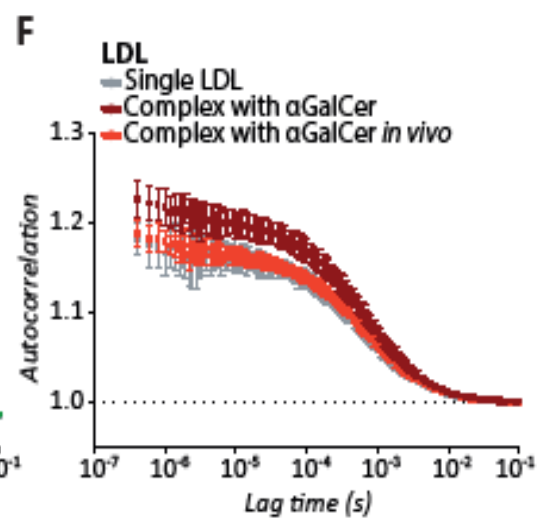
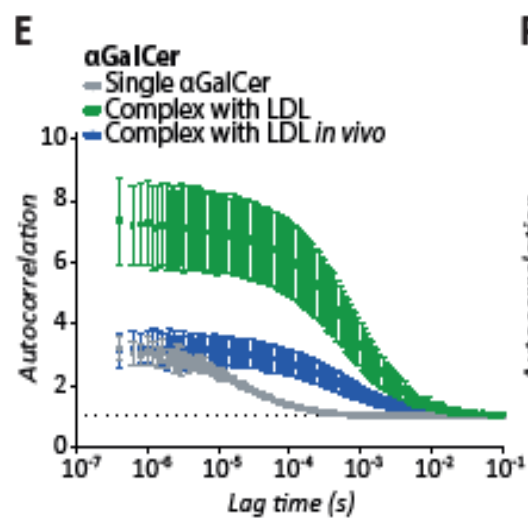
## A recent FCCS Example

An investigation of the role of lipoproteins in the mechanism by which foreign lipid antigens are delivered to antigen presenting cells (APCs) for downstream presentation and activation of invariant Natural Killer T-cells (iNKT cells).

(Suzanne E. Engelen, ..., Henk S. Schipper. Manuscript submitted)

### D 2-color Fluorescence cross-correlation spectroscopy





**J**

Lipoprotein	Apo	Stable complex	Molar ratio	Aggregates
hLDL	B	Yes	10:1	No
hLDL <i>in vivo</i>	B	Yes	5:1	No
hLDL	B	Yes	50:1	Yes
hVLDL	B,C,E	Yes	10:1	Few
hHDL	A,C,E	No	-	-
mVLDL	B,C,E	Yes	10:1	Few
mVLDL ApoE <sup>-/-</sup>	B,C	Yes	10:1	Few

## Summary - Fluorescence techniques for measuring molecular interactions

- FRET is a direct method for measuring molecular interactions beyond the resolution limits ( $\sim 0.5-10$  nm)
- FRET-FLIM is particularly powerful method because it minimizes spectral cross-talk problems and concentration dependent artefacts
- FCCS is extension of FCS that enables the direct measurement of molecular interactions provided that the interacting molecules are mobile

# Jablonski-Diagramm

- molecules have electronic, vibrational and rotational energy levels
- absorption = electron moves to a higher excited state
- vibrational relaxation (Stokes-Shift), internal conversion, intersystem crossing
- return to the ground state: fluorescence, phosphorescence

