Imaging at the molecular level: Measuring molecular motion and interactions

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Imaging at the molecular level: Measuring molecular motion and interactions

- <u>Brief intro to theory of molecular motion</u>

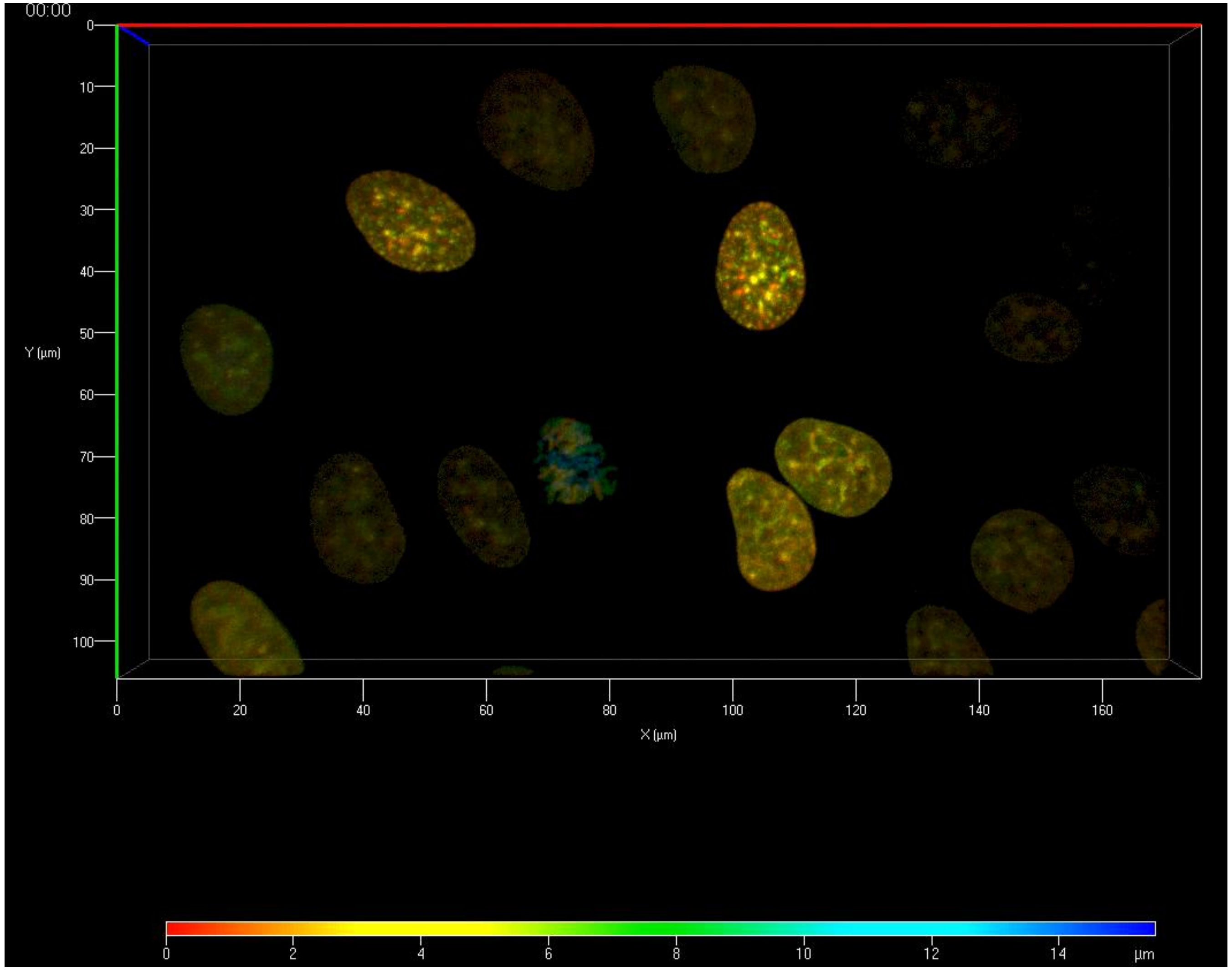
- <u>Fluorescence techniques for measuring molecular motion</u>

- Single particle tracking (SPT)
- Fluorescence Recovery after Photobleaching (FRAP)
- Fluorescence Loss in Photobleaching (FLIP)
- Fluorescence Localization after Photobleaching (FLAP)
- Fluorescence Correlation Spectroscopy (FCS)
- Raster Image Correlation Spectroscopy (RICS)

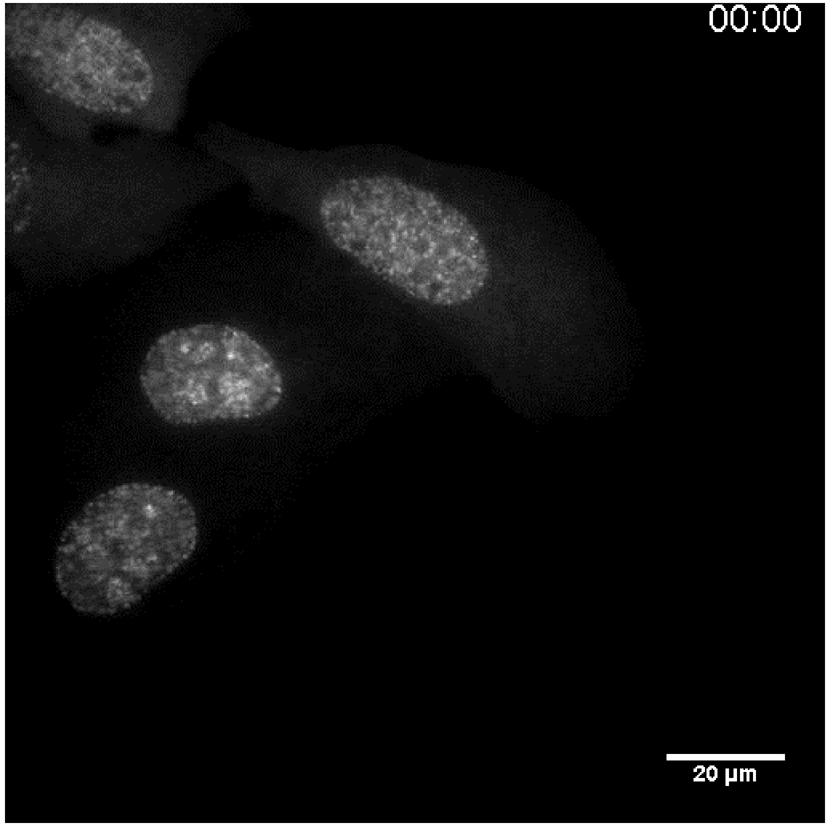
- <u>Fluorescence techniques for measuring molecular interactions</u>
- Förster Resonance Energy Transfer (FRET)
- Fluorescence Cross-Correlation Spectroscopy (FCCS)

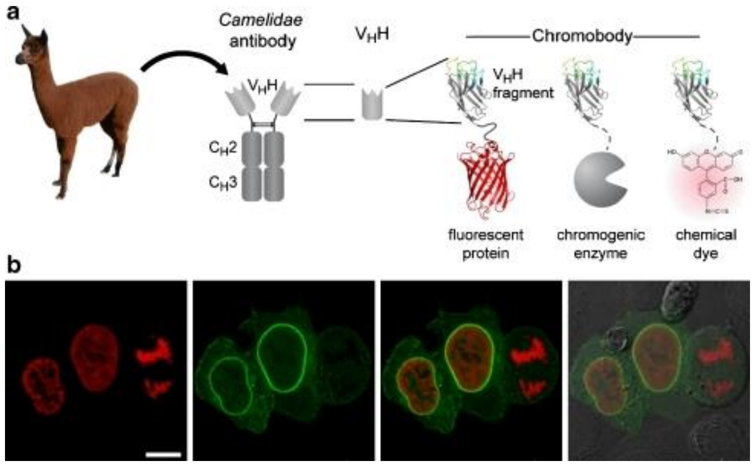
Many biological samples are dynamic at a wide range of time-scales

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

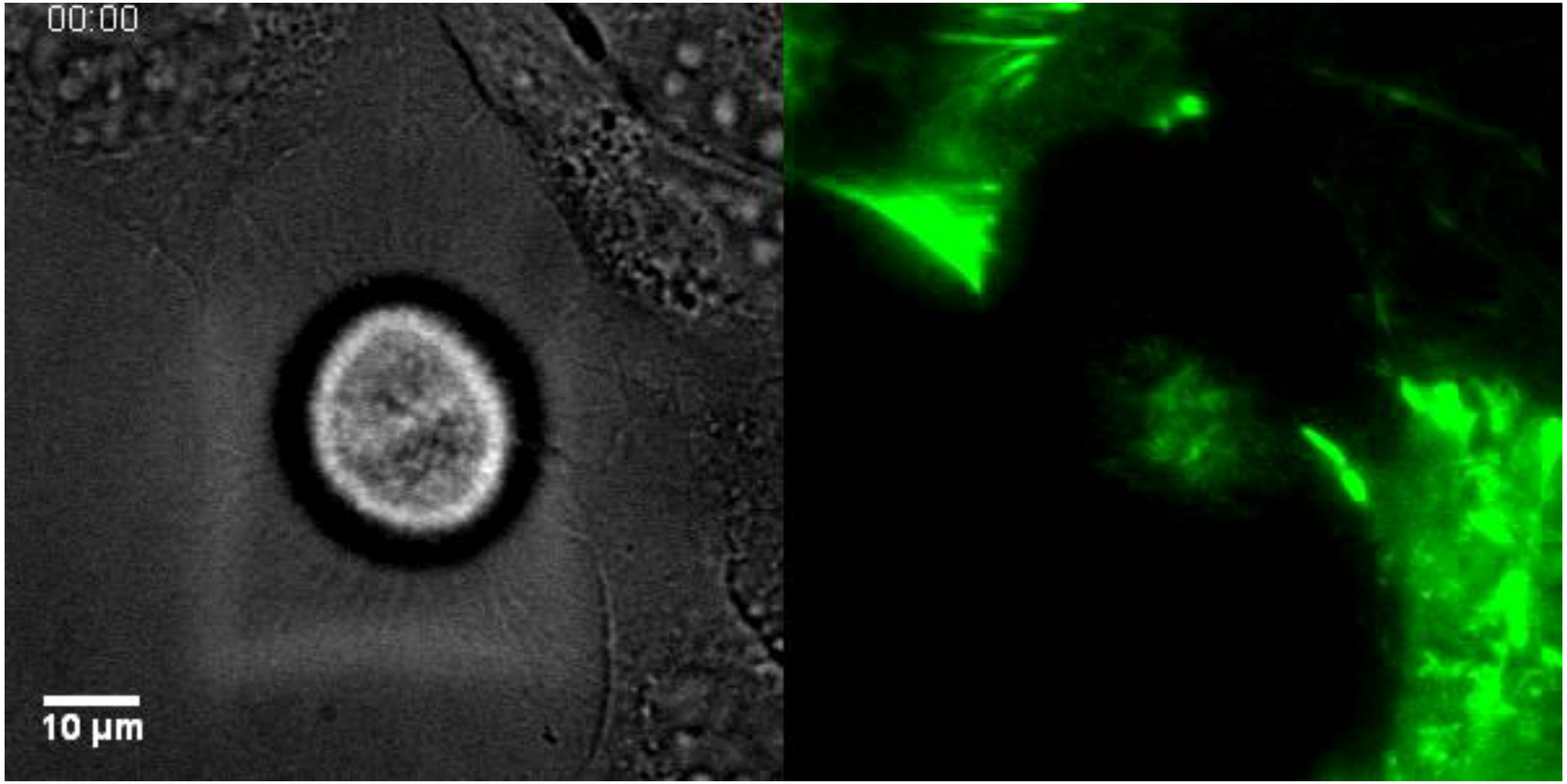


Example 2: Cell cycle; U2OS cells expressing PCNA-Chromobody-RFP imaged on Widefield/TIRF microscope at 24 z-planes/h

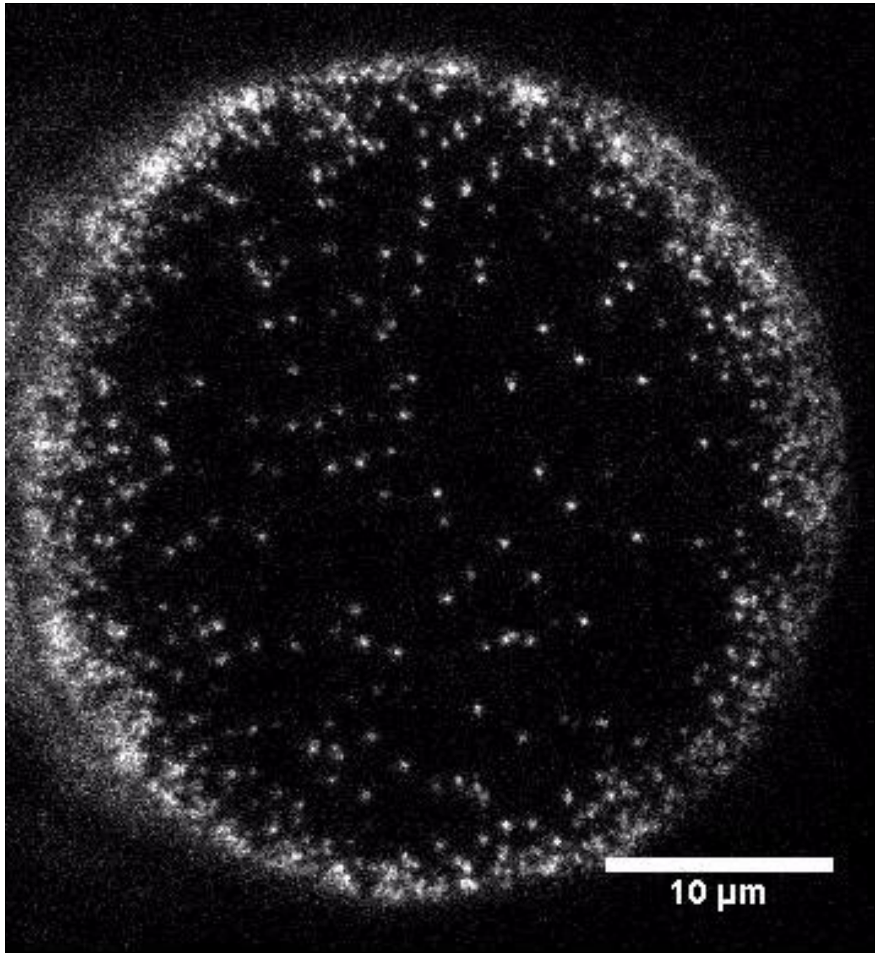




Example 3: Mitosis; HeLa cells expressing LifeAct-RFP imaged in TIRF microscope at 24 z-planes/h



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

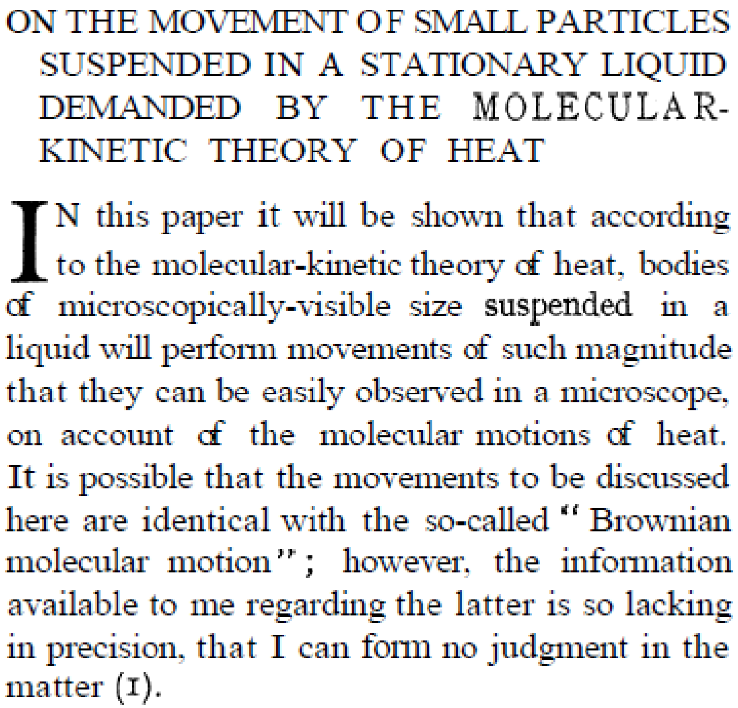


- … but time-lapse imaging alone does not reveal quantitative information about movements of cell organelles, proteins, lipids, or DNA
- In particular, time lapse imaging is not sufficient to differentiate between different modes of motion or transient binding of a molecule i.e.
    1. Random (Brownian) 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

- <u>Brief intro to theory of molecular motion</u>

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

IN 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 (1).

Mean Squared Displacement:
(Free (Brownian) diffusion in 1D)

$$\left\langle (x(t)-x_0)^2 \right\rangle = \int_{-\infty}^{\infty} x^2 \; N[0,2Dt]\, dx = 2Dt$$

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)

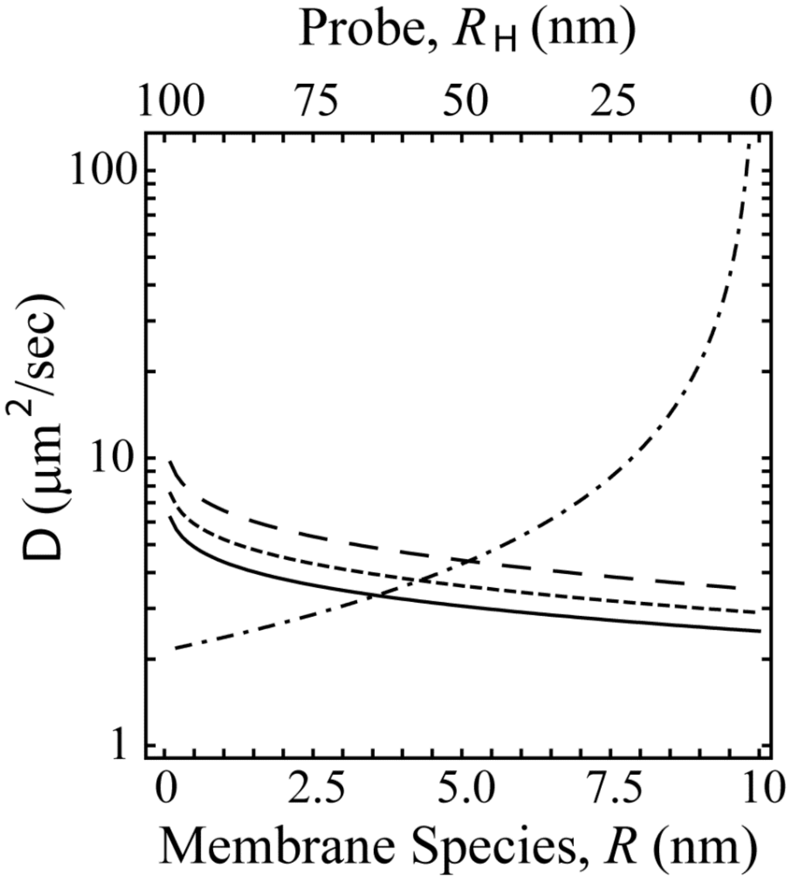
$$D_M = \frac{k_B\, T}{4\pi\, \mu_M\, h} \ln\left[\frac{\mu_M\, h}{\mu_S\, R_H} - \gamma\right]$$

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:

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

μ_S = 1.002 cP
h = 4 nm
T = 293 K
μ_M/μ_S = 100 (solid line)
μ_M/μ_S = 80 (short dashed line)
μ_M/μ_S = 60 (long dashed line).

Stokes-Einstein:

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

μ_S = 1.002 cP
T = 293 K.

Fluorescence techniques for measuring molecular motion

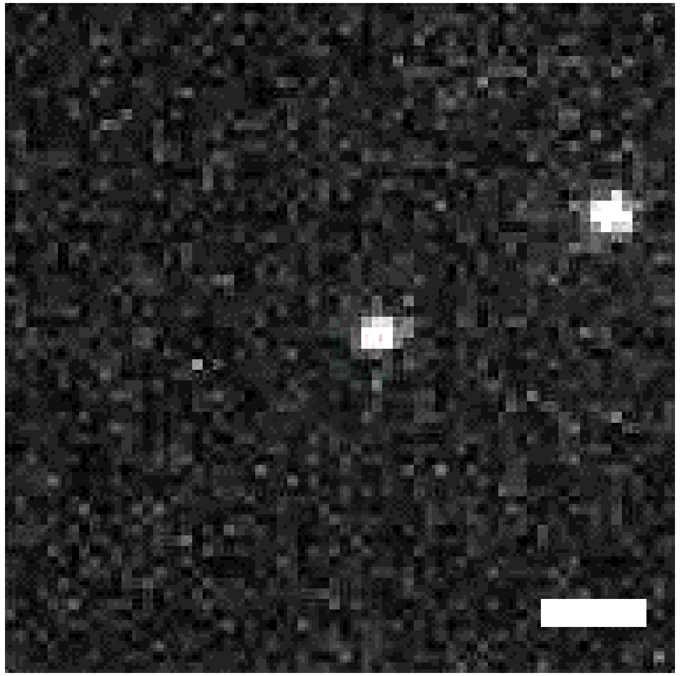
- 1) Single particle tracking (SPT)

- 2) Fluorescence Recovery after Photobleaching (FRAP)
- 3) Fluorescence Loss in Photobleaching (FLIP)
- 4) Fluorescence localization after Photobleaching (FLAP)

- 5) 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 = 1µm

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

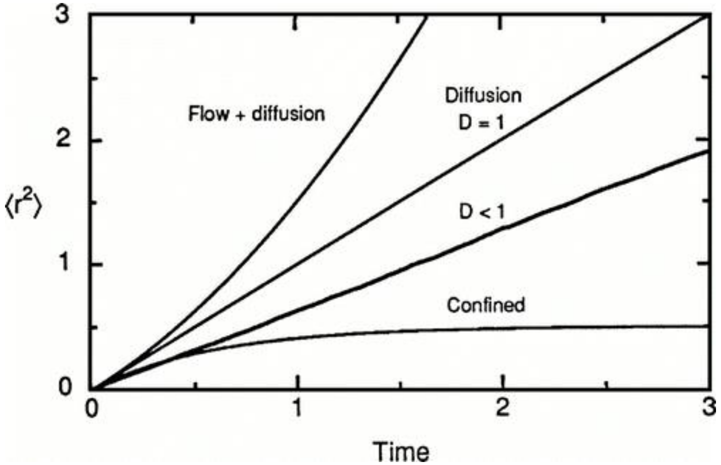
- Such trajectories contain information about:
  - diffusion coefficients
  - velocities
  - step sizes
  - spatial and temporal confinement

- 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

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

where D is the diffusion coefficient



$$Brownian\ motion <r^2>=4Dt$$

$$Anomalous\ diffusion <r^2>=4Dt^{\alpha} \quad \alpha<1$$

$$Diffusion\ with\ flow <r^2>=4Dt+(Vt)^2$$

$$Confined\ diffusion <r^2>\approx<r_C^2>[1-A_1\exp(-4A_2Dt/<r_C^2>)]$$

Saxton and Jacobson (1997) Annu. Rev. Biophys. Biomol. Struct. 26: 373-99

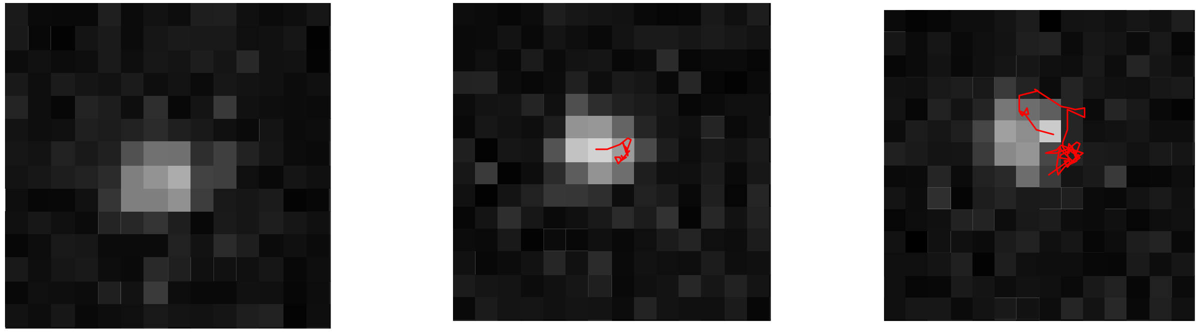
Data Analysis

- Obtain sub-pixel resolution by curve fitting to determine centroids of single molecules

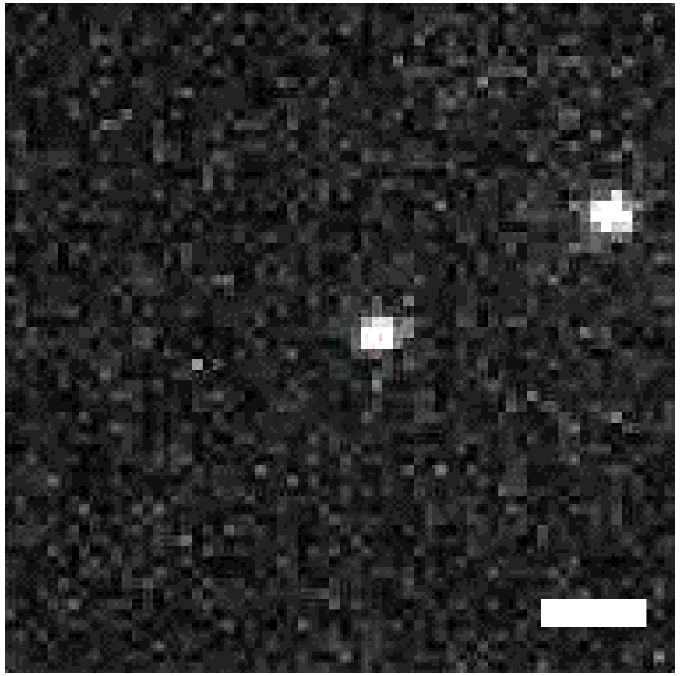
- Approximate Airy pattern with a 2D spatial Gaussian and fit each image

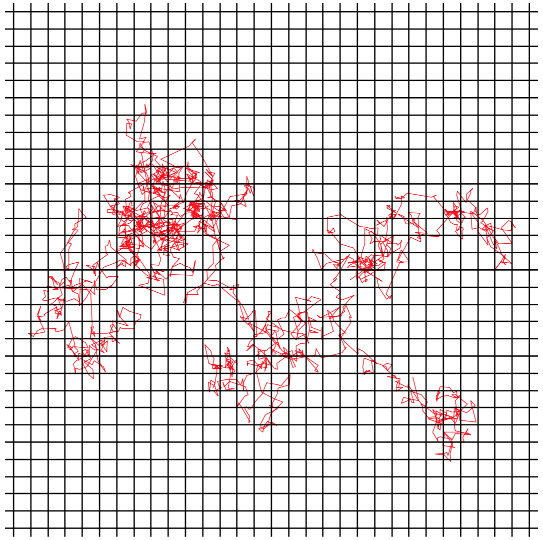
$$\boldsymbol{A}+\frac{\boldsymbol{B}}{2\pi \boldsymbol{w}^2}\boldsymbol{Exp}[-\frac{1}{2\pi \boldsymbol{w}^2}((\boldsymbol{x}-\boldsymbol{x_o})^2+(\boldsymbol{y}-\boldsymbol{y_o})^2)]$$

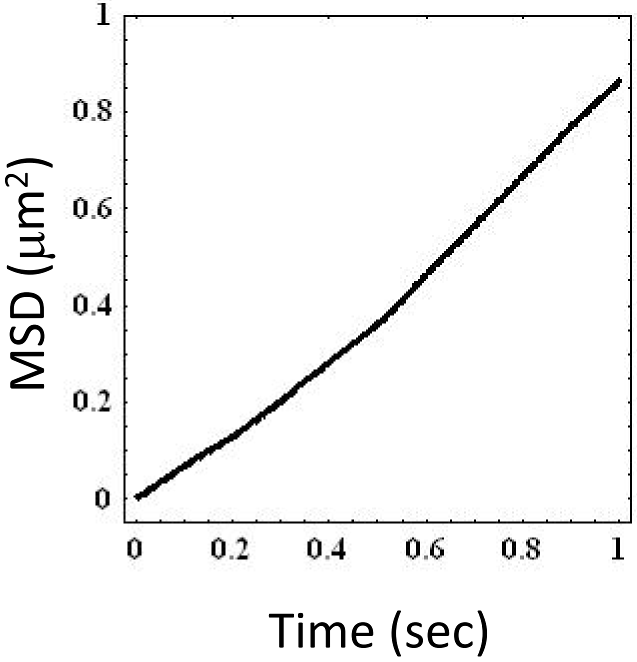
where w = width of the PSF of the microscope (~220 nm)



Mode of Motion & Diffusion Coefficient







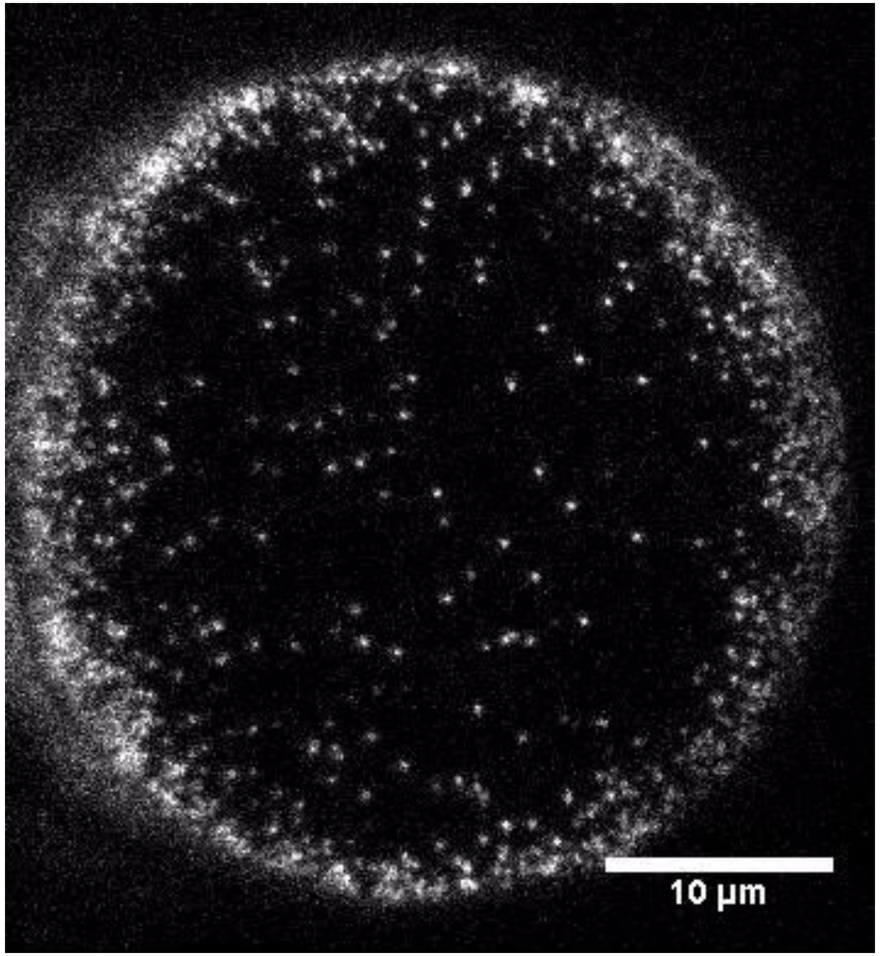
Brownian Diffusion
D ~ 0.25 μm^2/sec

Probe Considerationsfor SPT

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

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

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

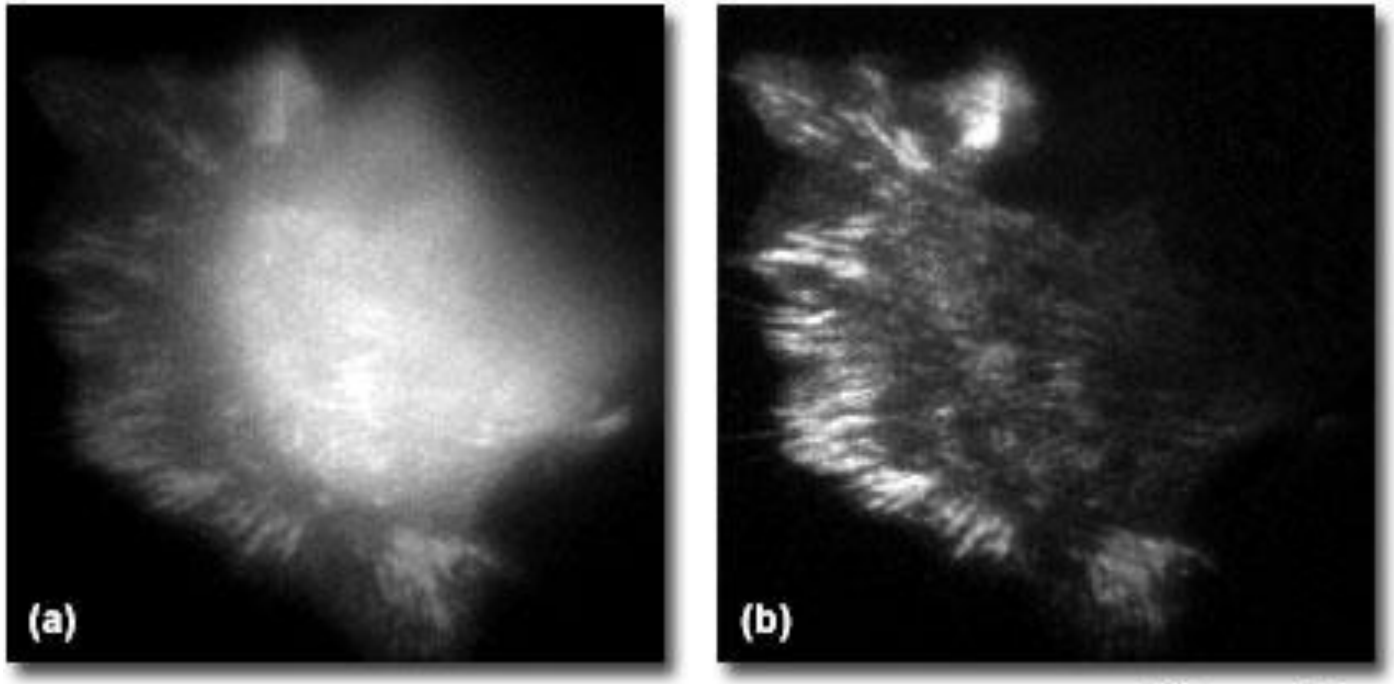
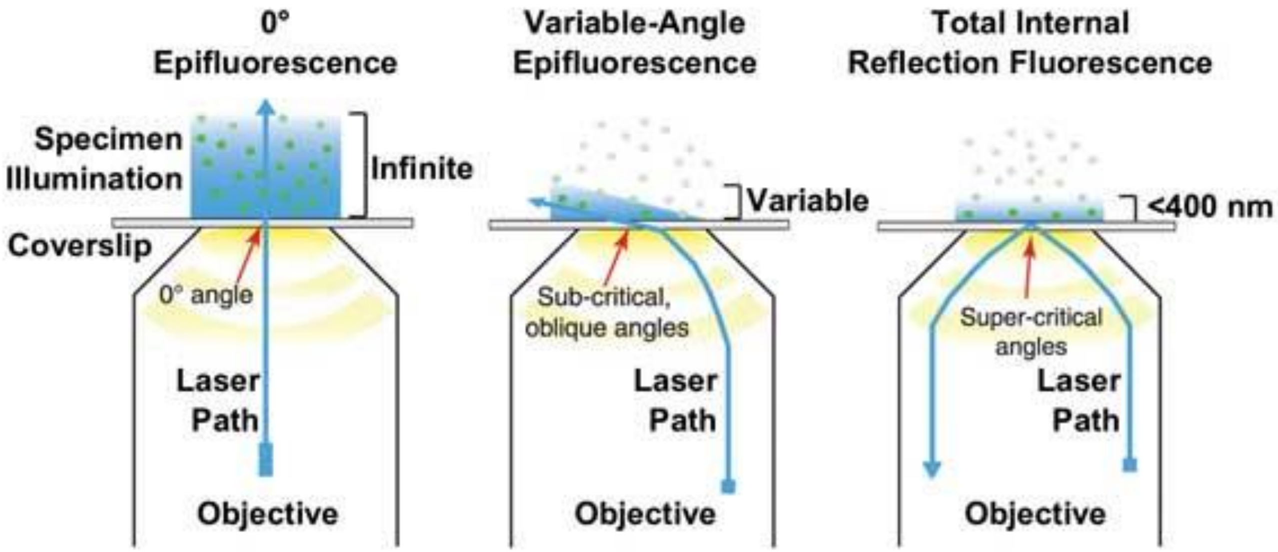


Figure 5

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

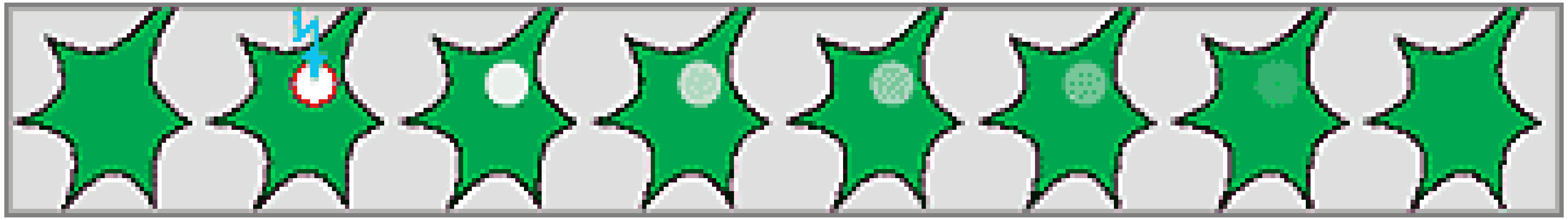

0° Epifluorescence
Variable-Angle Epifluorescence
Total Internal Reflection Fluorescence
Specimen Illumination
Infinite
Variable
<400 nm
Coverslip
0° angle
Sub-critical, oblique angles
Super-critical angles
Laser Path
Laser Path
Laser Path
Objective
Objective
Objective


OLYMPUS
Apo N
60x/1.49 Oil
∞/0.13–0.19/FN22
OLYMPUS
UApo N
100x/1.49 Oil
∞/0.13–0.19/FN22
OLYMPUS
UApo N
150x/1.45 Oil
∞/0.13–0.19/FN22

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.

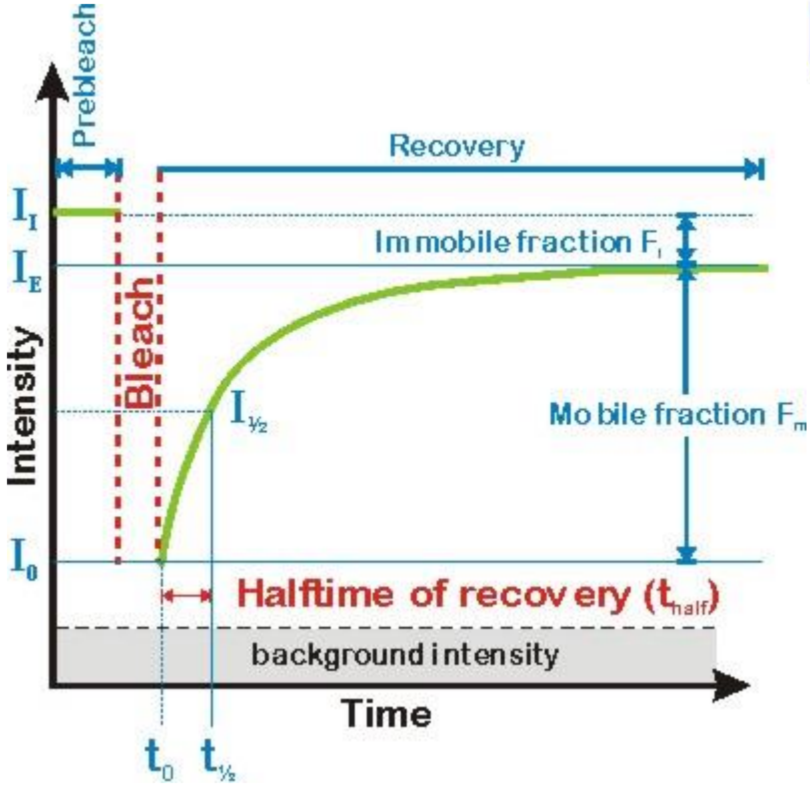


Bleached spot size ~ 1 µm

(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

For 2D diffusion:

$$D = \frac{w^2}{4\, t_{1/2}}$$

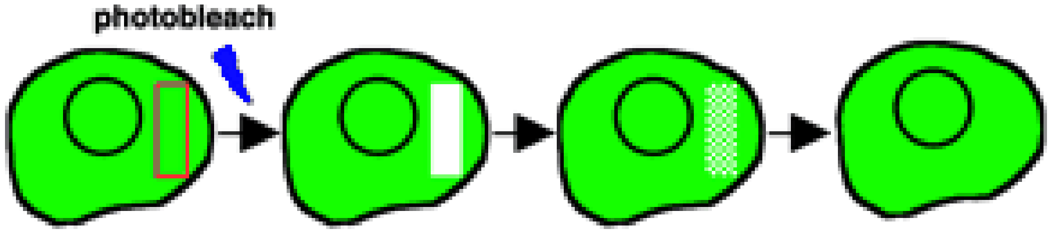
w: laser spot radius

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

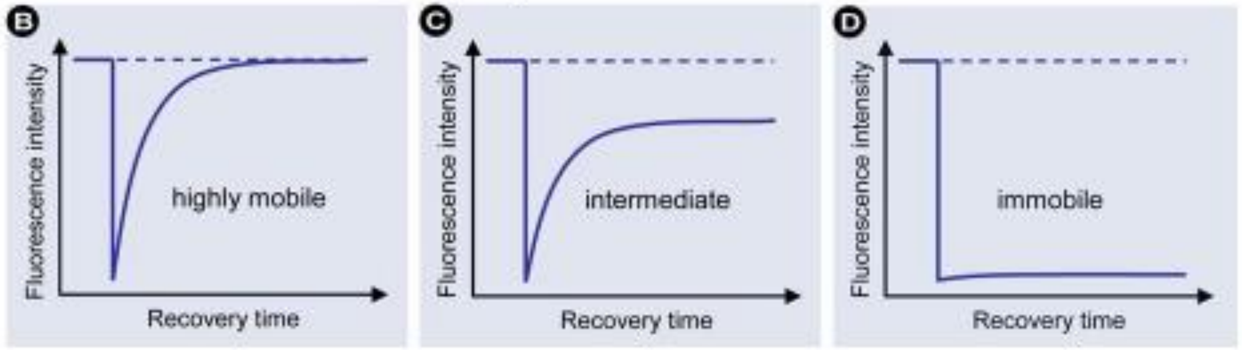
Mobile fraction $F_m = \dfrac{I_E - I_0}{I_I - I_0}$

http://www.embl-heidelberg.de/eamnet/frap/html/how_frap_works.html

- Can also do FRAP with most confocal microscopes



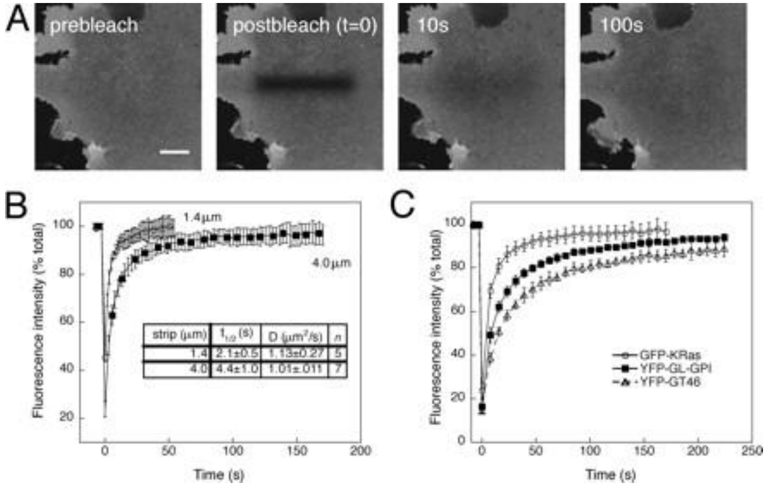
- In this geometry, D is typically calculated numerically by comparing fluorescence recovery curves with simulated curves
(See i.e. http://www.embl.de/eamnet/html/frap_analysis.html)



Dynamics of putative raft-associated proteins at the cell surface

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

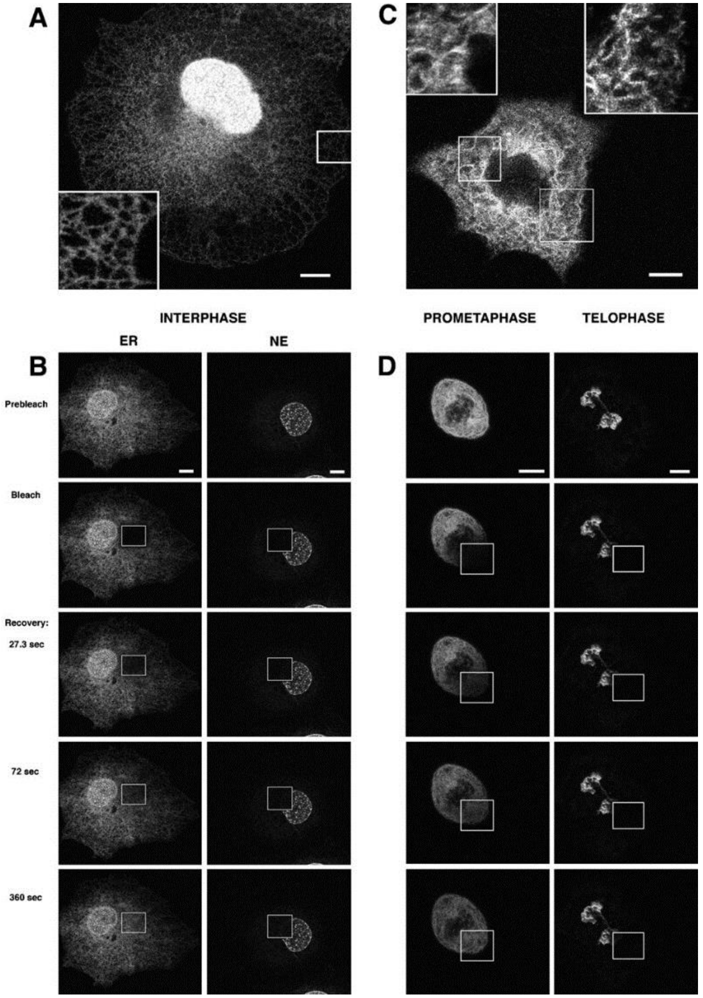
The Journal of Cell Biology, Volume 165, Number 5, June 7, 2004 735–746



strip (µm)	$t_{1/2}$ (s)	D (µm²/s)	*n*
1.4	2.1±0.5	1.13±0.27	5
4.0	4.4±1.0	1.01±.011	7

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 t1/2 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 µm2/s; YFP-GL-GPI, 0.47 ± 0.07 µm2/s; YFP-GT46, 0.23 ± 0.02 µm2/s.

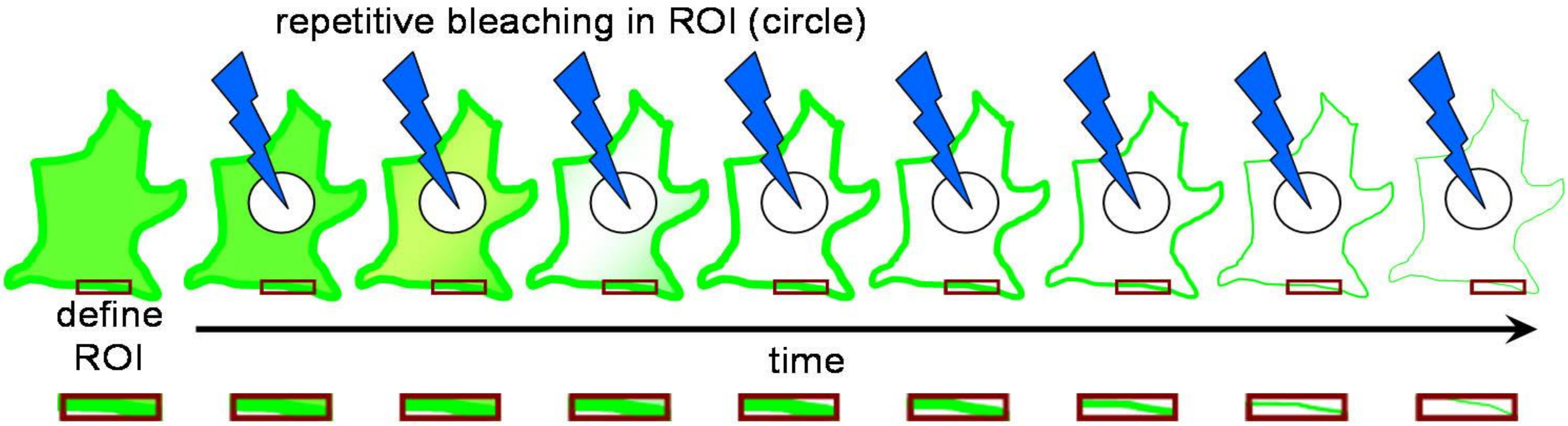
J. Ellenberg, E. D. Siggia, J. E. Moreira, C. L. Smith, J. F. Presley, H. J. Worman, and J. Lippincott-Schwartz (1997)
Nuclear Membrane Dynamics and Reassembly in Living Cells: Targeting of an Inner Nuclear Membrane Protein
in Interphase and Mitosis. J. Cell Biol. 138: 1193-1206



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.

3) FLIP – Fluorescence Loss in Photobleaching

- a small region is repeatedly bleached and the loss of fluorescence in another region is measured.
- useful to show connectivity of compartments or for measuring turnover of molecules between different compartments



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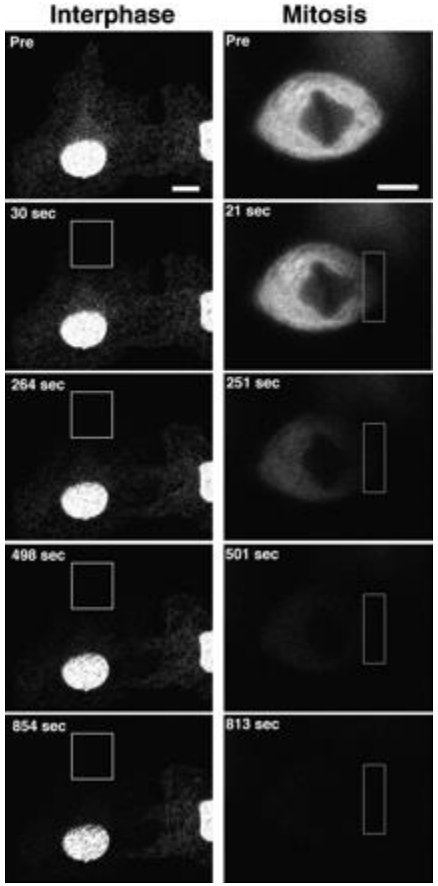
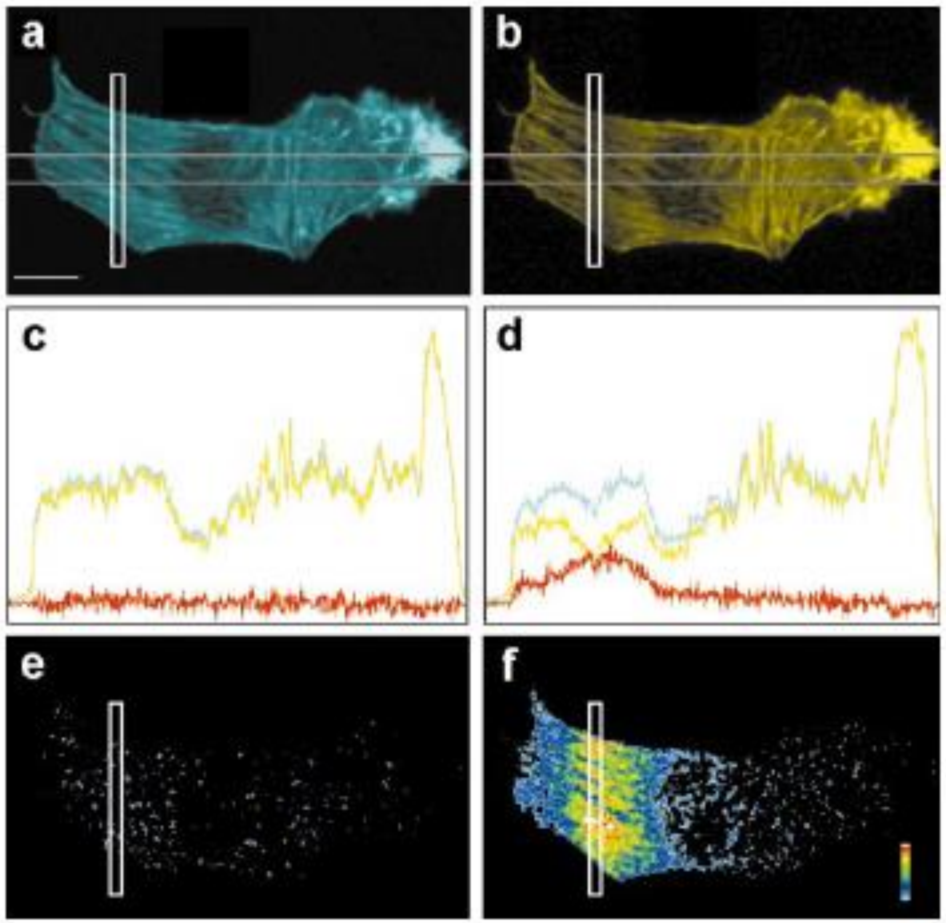


Fig. 6. FLIP to probe the continuity of interphase and mitotic membranes containing LBR-GFP. FLIP experiments were performed on interphase membranes (*left*) and metaphase membranes (*right*). Note the complete loss of fluorescence from both interphase ER membranes and mitotic membranes over a similar time course, but not from NE membranes in interphase. ER fluorescence that remained in interphase is from an adjacent cell whose membranes were not connected to those within the photobleached box. Bars, 10 µm.

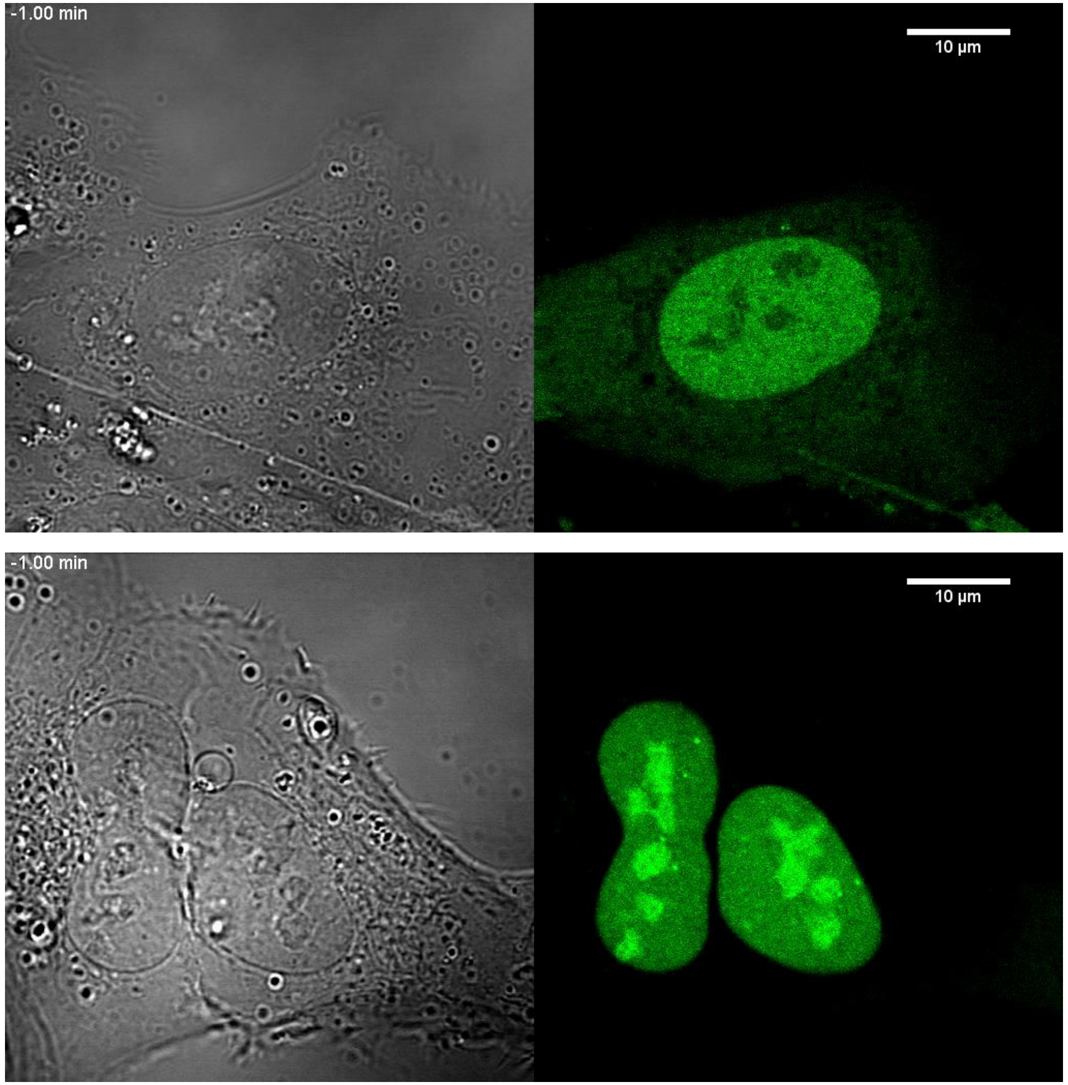
4) FLAP – Fluorescence Localization after Photobleaching

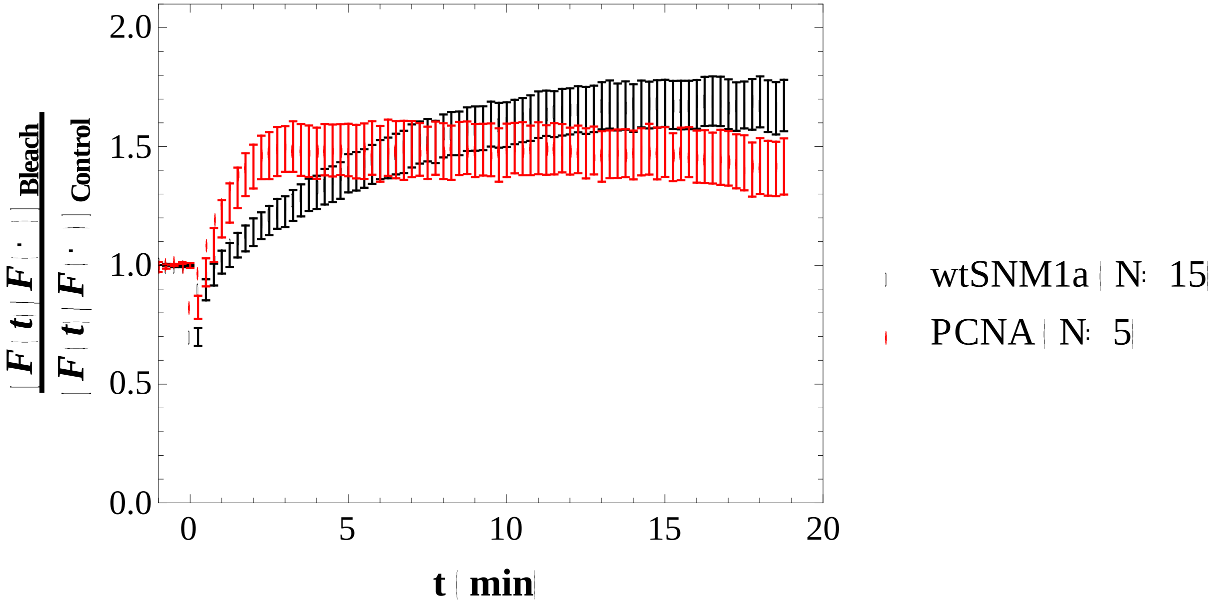
- In FLAP a protein is tagged with two fluorescent labels: one is photobleached and the other acts as a reference
- The use of a reference fluorochrome allows the tracking of the distribution of the labelled molecules by simple image differencing (**I**) and thus enables measurement of fast relocation dynamics.



Dunn, G. A., Dobbie, I. M., Monypenny, J., Holt, M. R., & Zicha, D. (2002). Fluorescence localization after photobleaching (FLAP): a new method for studying protein dynamics in living cells. *J Microsc, 205*(Pt 1), 109-112.

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



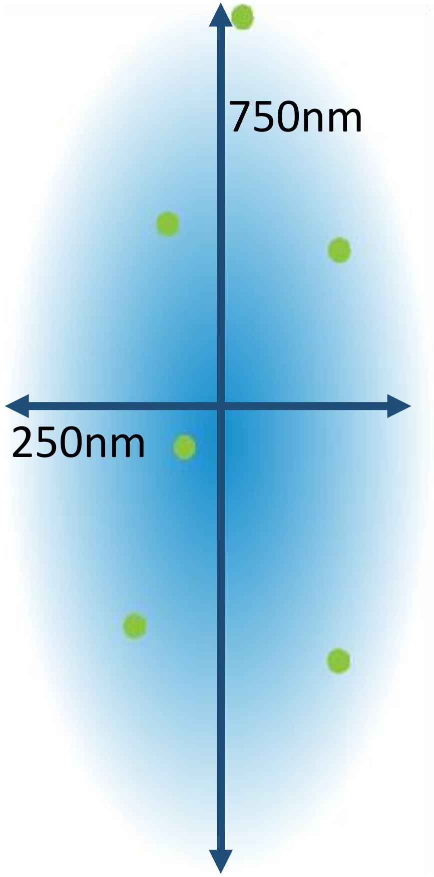


Characterization of role of *SNM1A*, a 5′–3′ exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)

5) 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



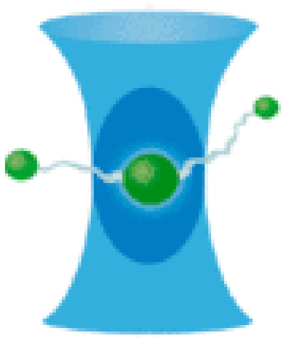
$$D = \frac{k_B T}{6\pi \, \eta \, r}$$

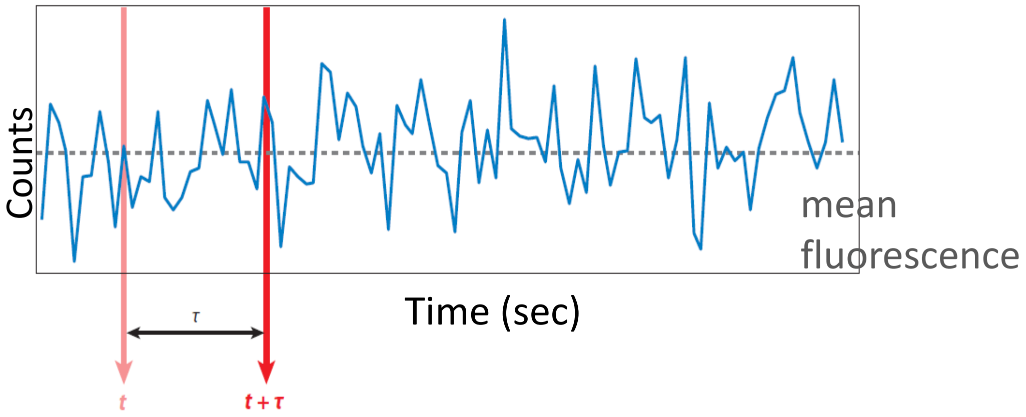
Diffusion in solution (3D) – Stokes-Einstein relation

Volume ≈ 10^{-15}l

FCS – Autocorrelation of Fluorescence Fluctuations

confocal
Volume

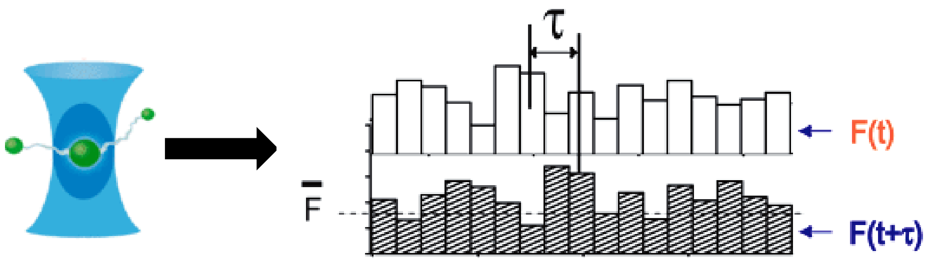




What causes fluctuations in intensity? a) Poison noise, b) diffusion of molecules

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

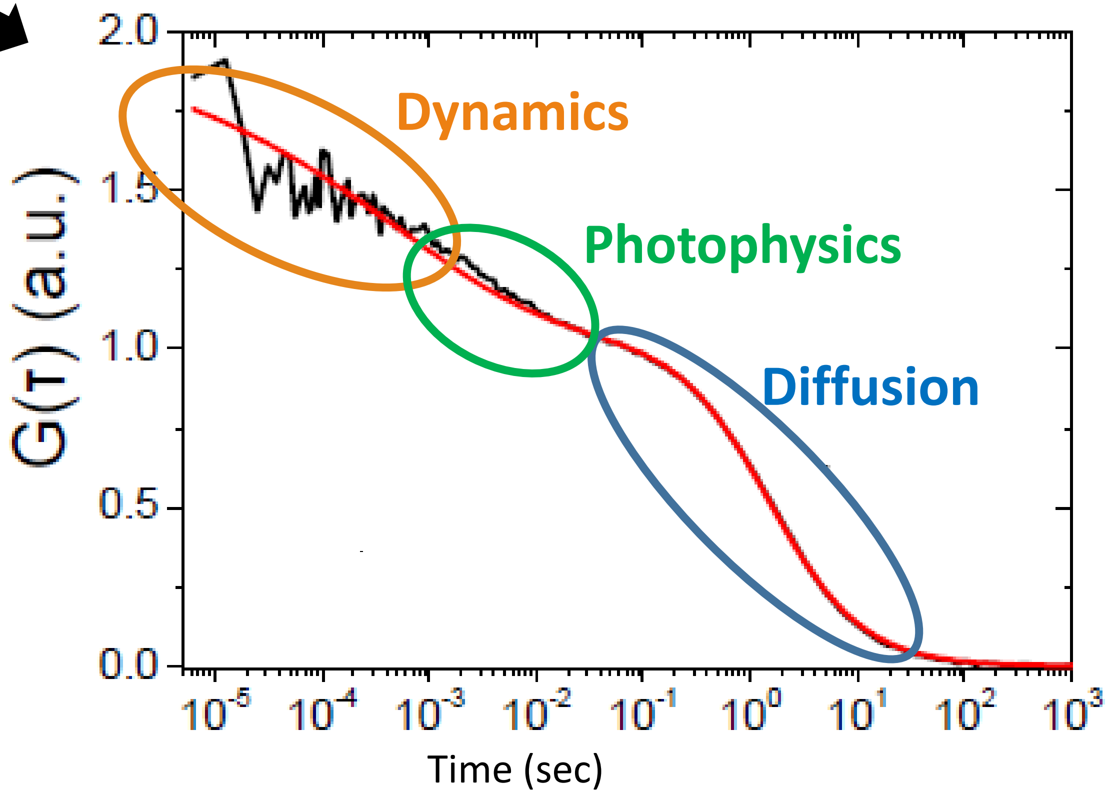
FCS – Autocorrelation of Fluorescence Fluctuations



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

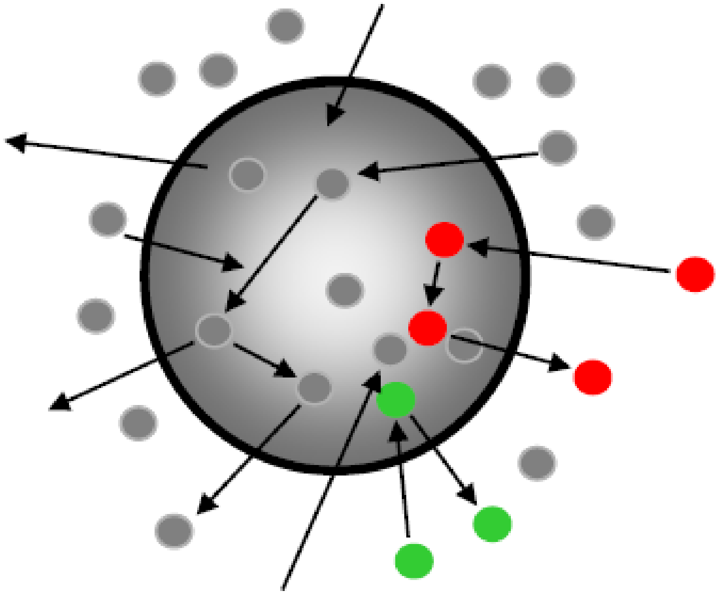
2D Gaussian Model

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

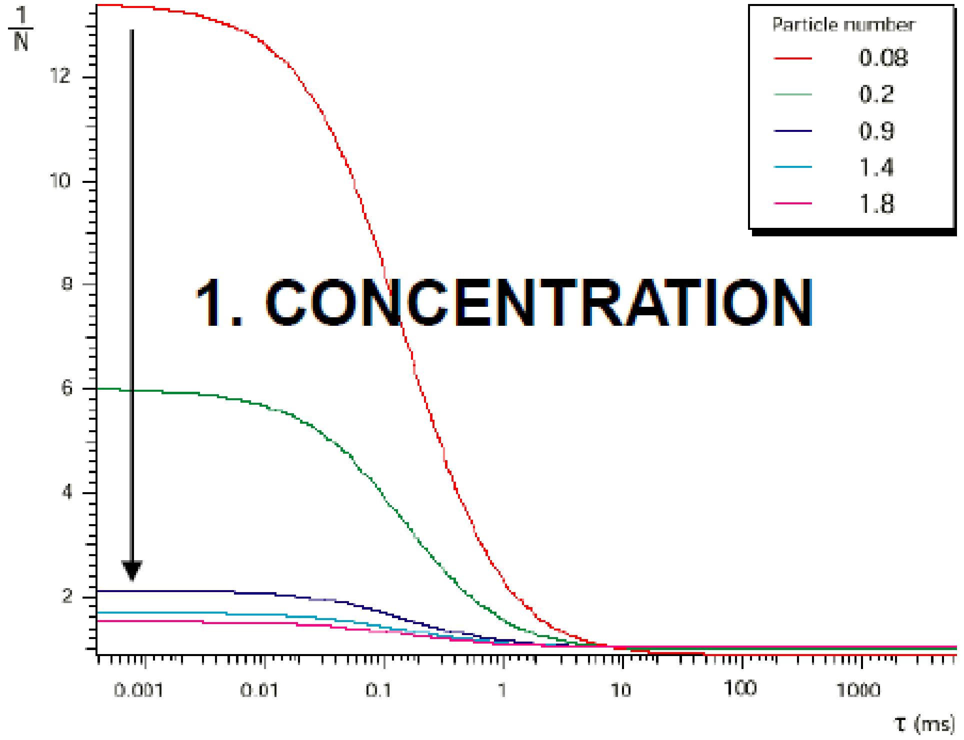


Fluorescence Correlation Spectroscopy

Wrapping it up: How many



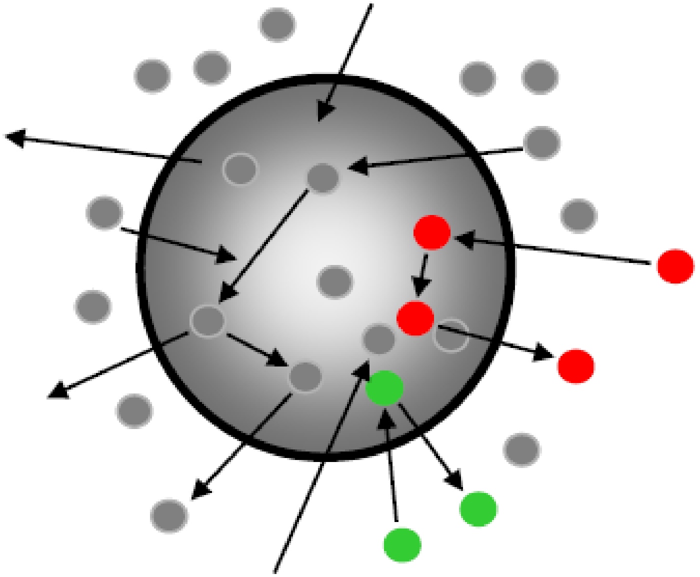
$$g^{(2)}(0) \sim 1 / N$$



1. Concentration dynamic range: pM - μM

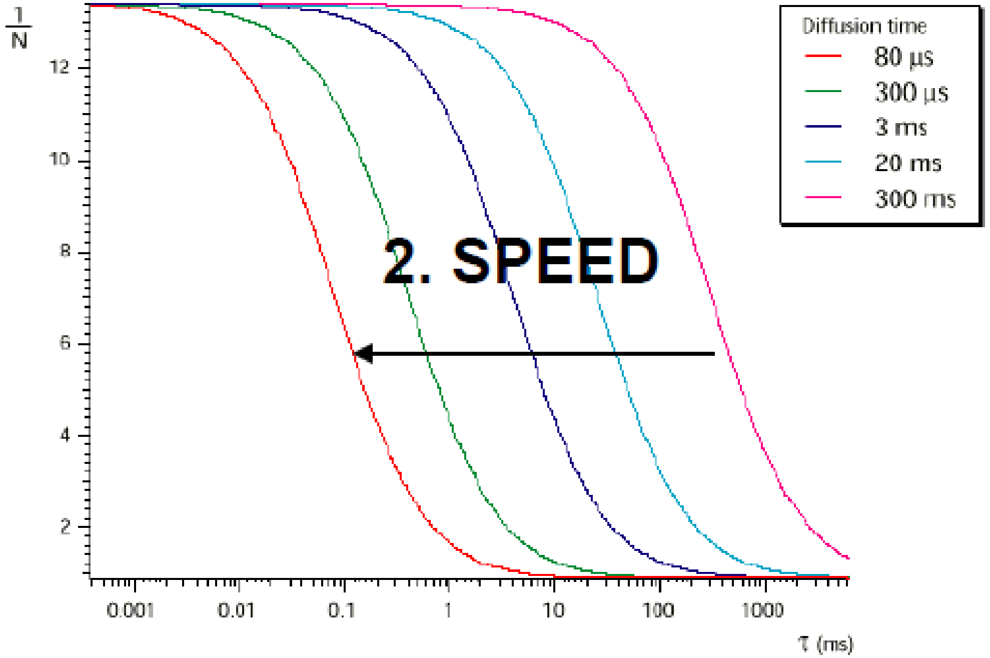
Fluorescence Correlation Spectroscopy

Wrapping it up: How many, how fast



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

$$\tau_D \sim 1 / D$$

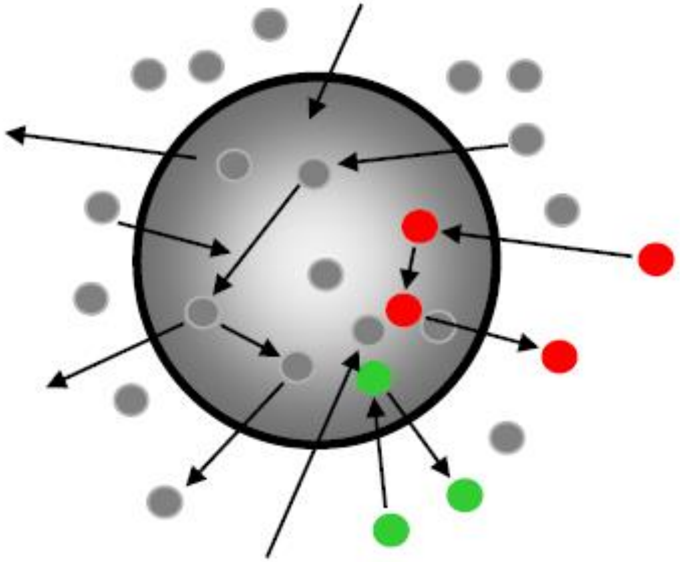


1. Concentration dynamic range: pM – µM

2. Time scale range: µs - s

Fluorescence Correlation Spectroscopy

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



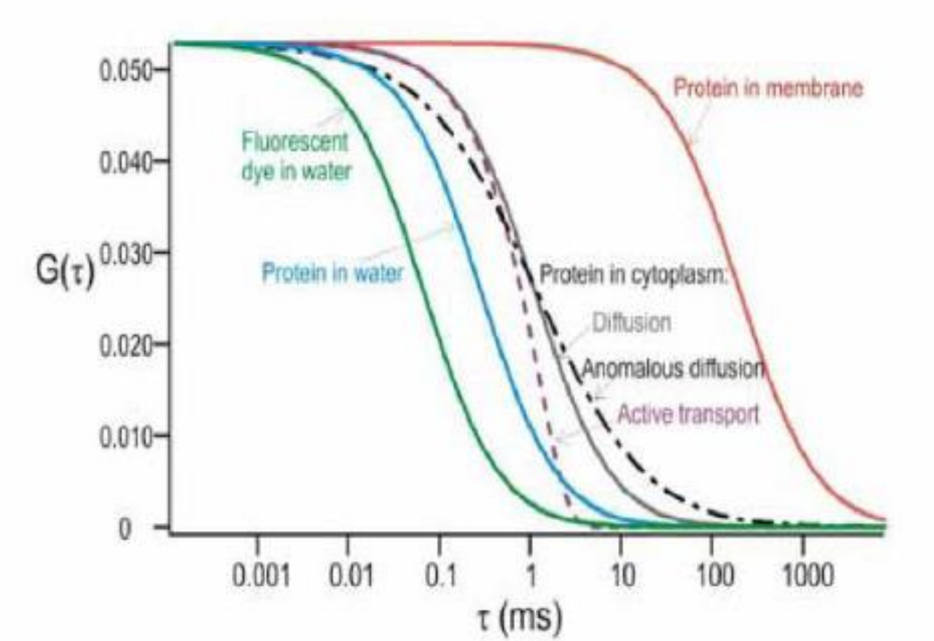
$g^{(2)}(0) \sim 1 / N$

$\tau_D \sim 1 / D$

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

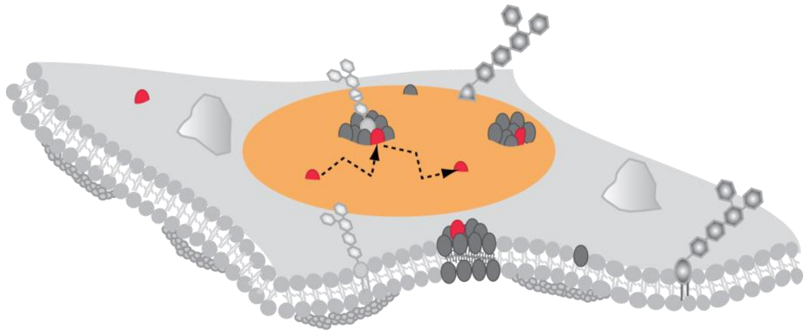
$$g^{(2)}(\tau) = 1 + \frac{A}{1 + \frac{\tau \cdot 4D}{w^2}}$$

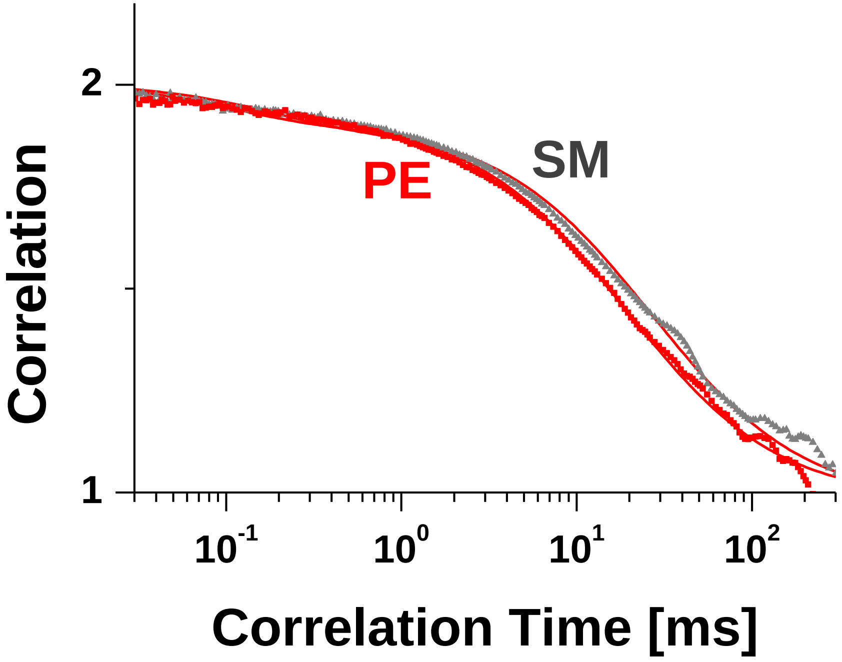
3. TYPE OF MOVEMENT



1. Concentration dynamic range: pM – μM
2. Time scale range: μs – 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 ms (PE) / 30ms (SM)
$(1/\alpha) \approx 1$ (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_{Airy}

- FRAP, FLIP, FLAP, FCS, and RICS 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)

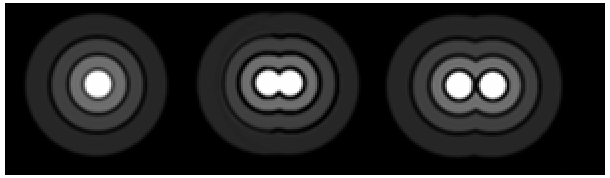
- 2) Fluorescence Cross-Correlation Spectroscopy (FCCS)

Why can’t we image molecular interactions directly?

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



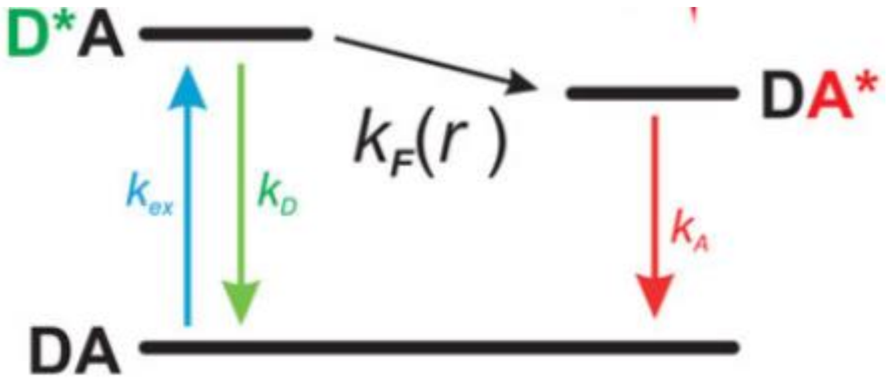
Airy patterns of two point sources

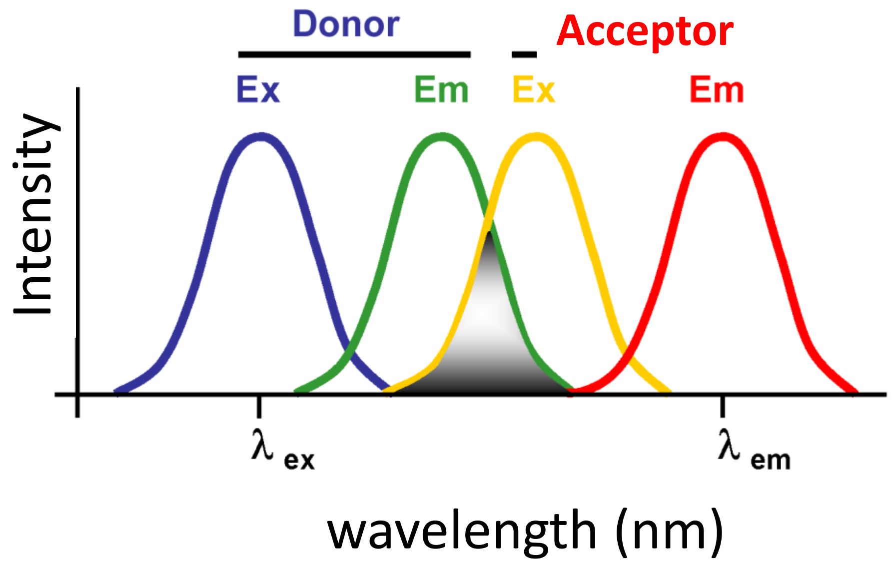
- For 100X magnification, 1.4 NA oil immersion objective and illumination with green light (500 nm = 0.5 µm)

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

1) FRET – Förster Resonance Energy Transfer

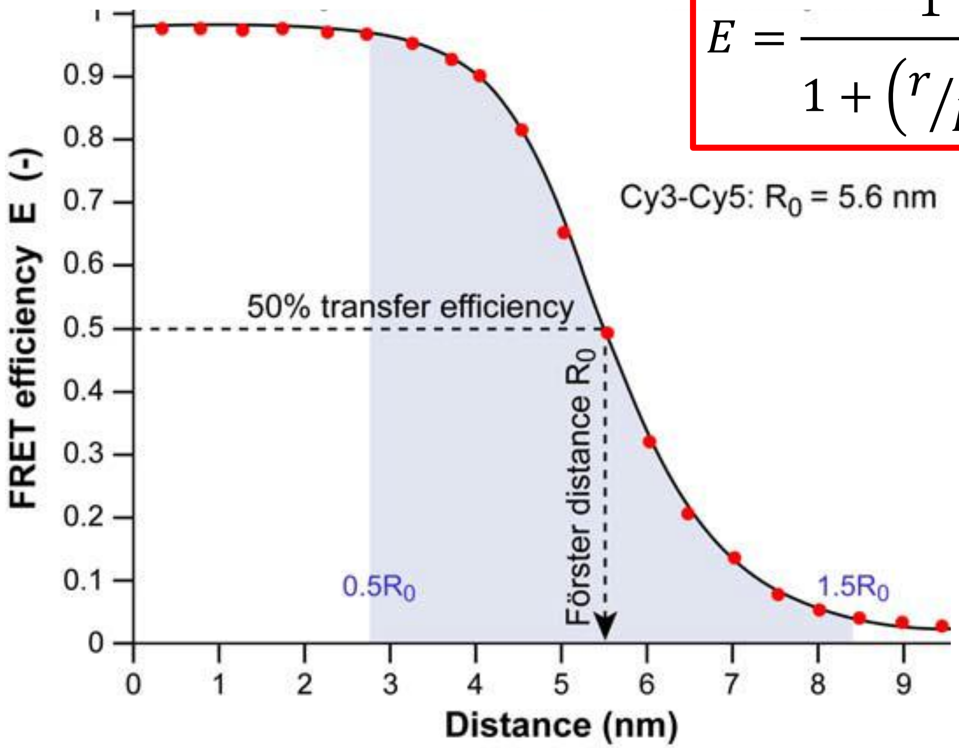
- energy is transferred non-radiatively (via long-range dipole-dipole coupling)
- relies on the close physical interaction of the two fluorophores (0.5 – 10nm)
- used to determine molecular interactions beyond the resolution limits

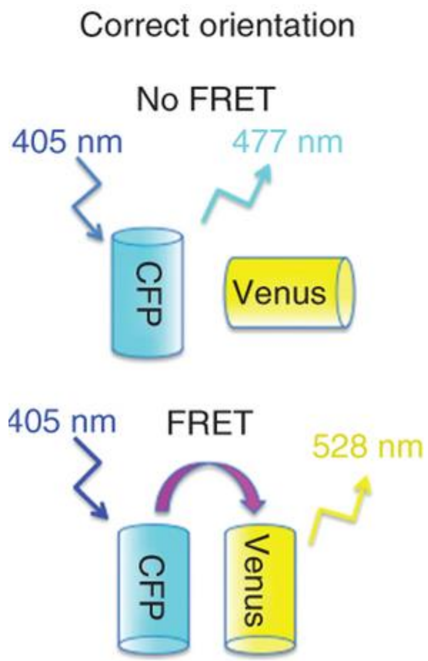


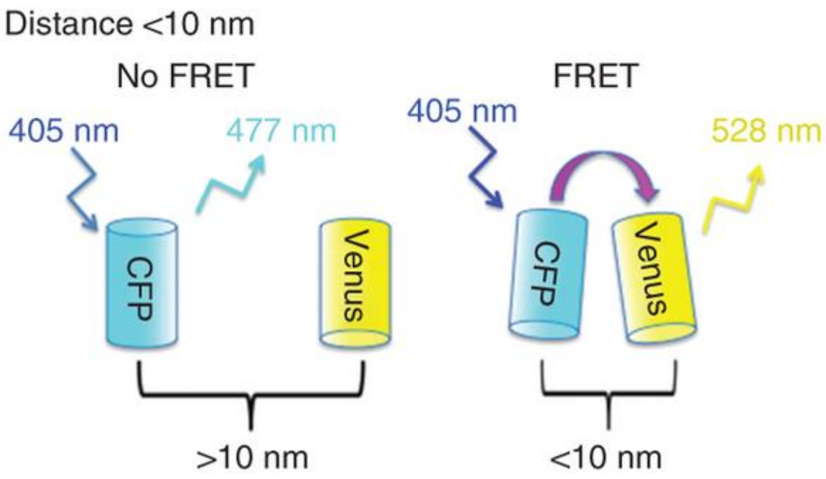


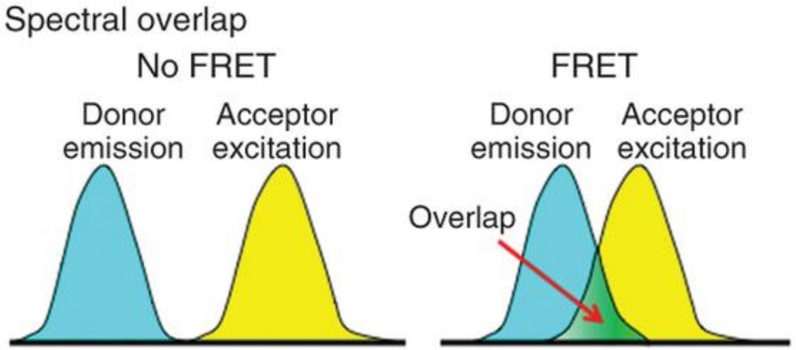
- distance between two molecules
- spectral overlap
- the quantum yield of the donor
- the relative orientation of the transition dipoles

$$E = \frac{1}{1+\left(r/R_0\right)^6}$$



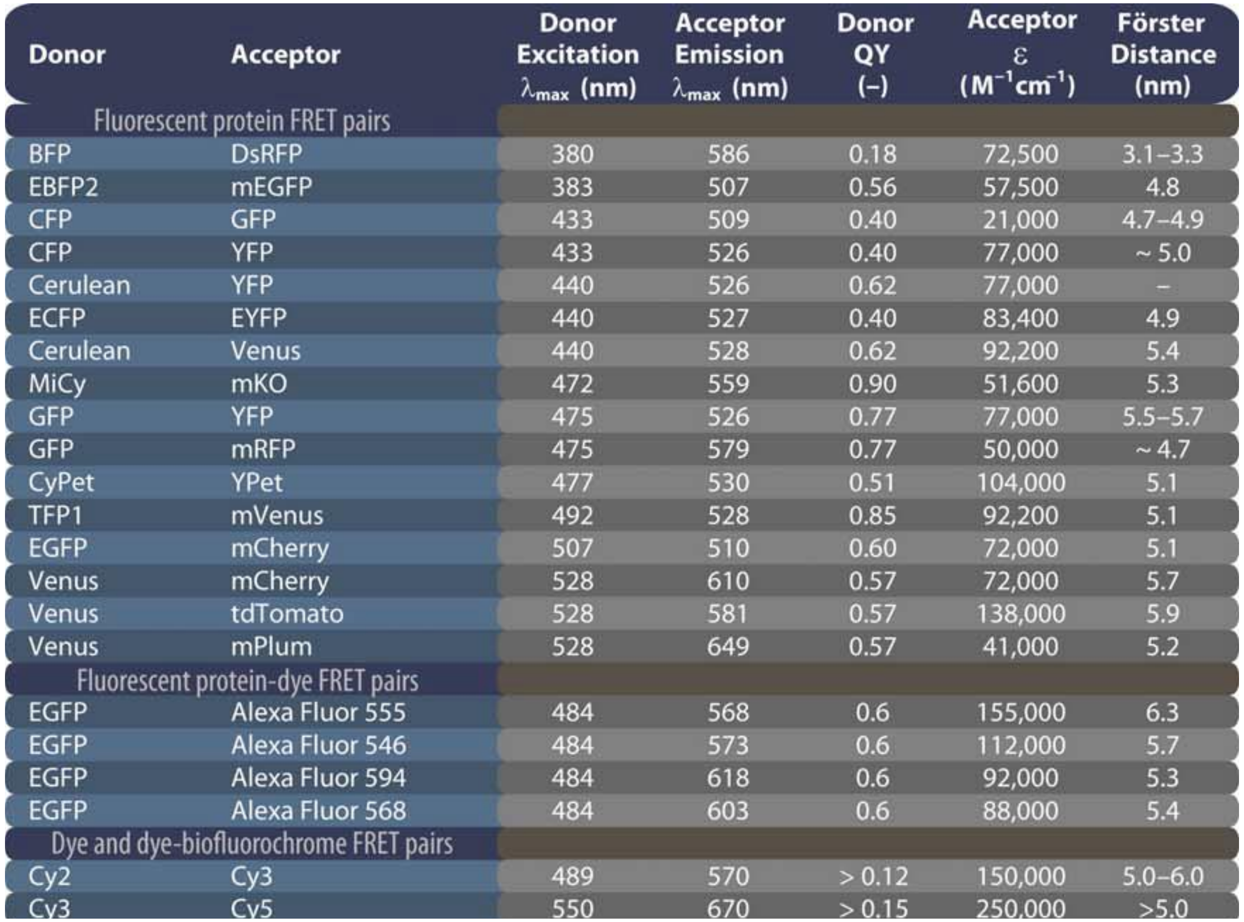
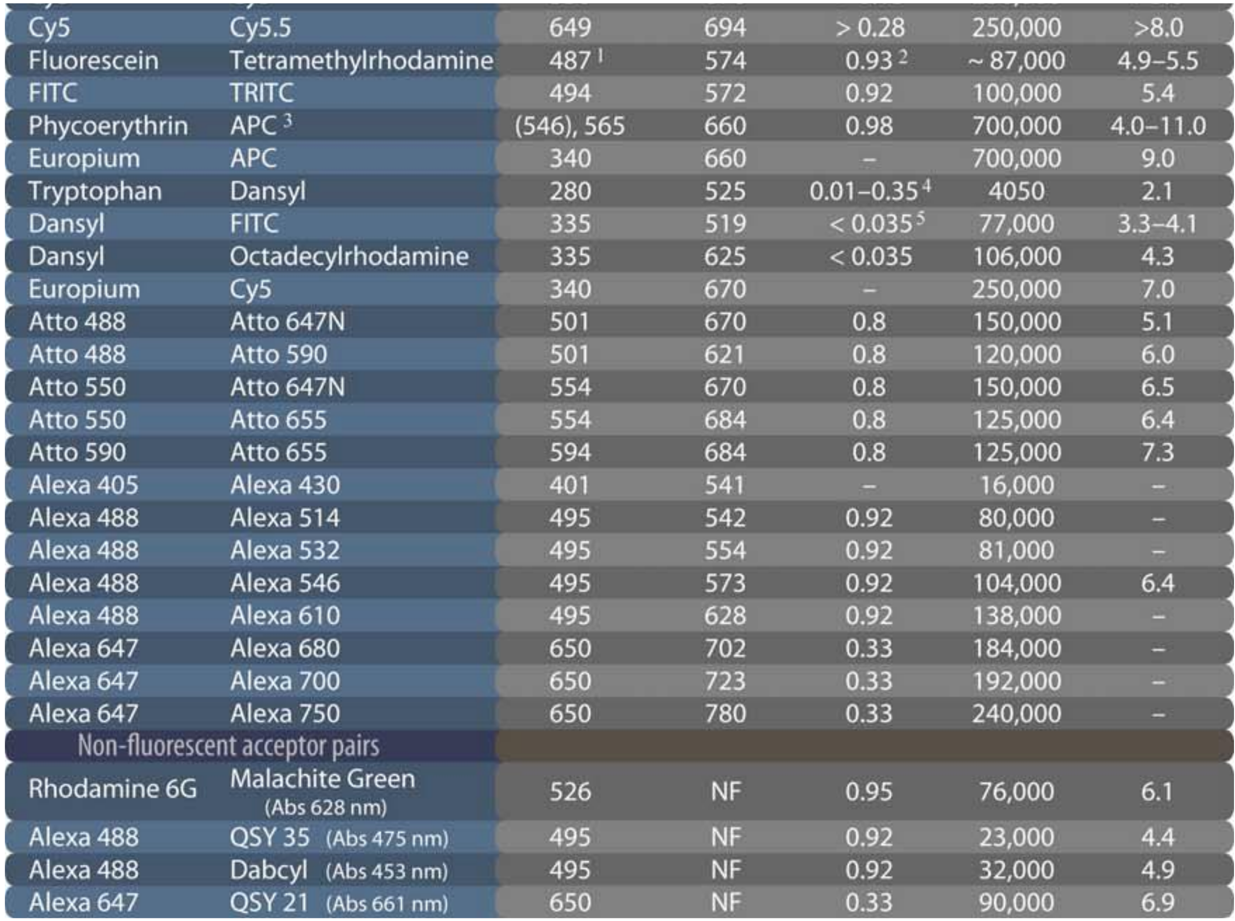






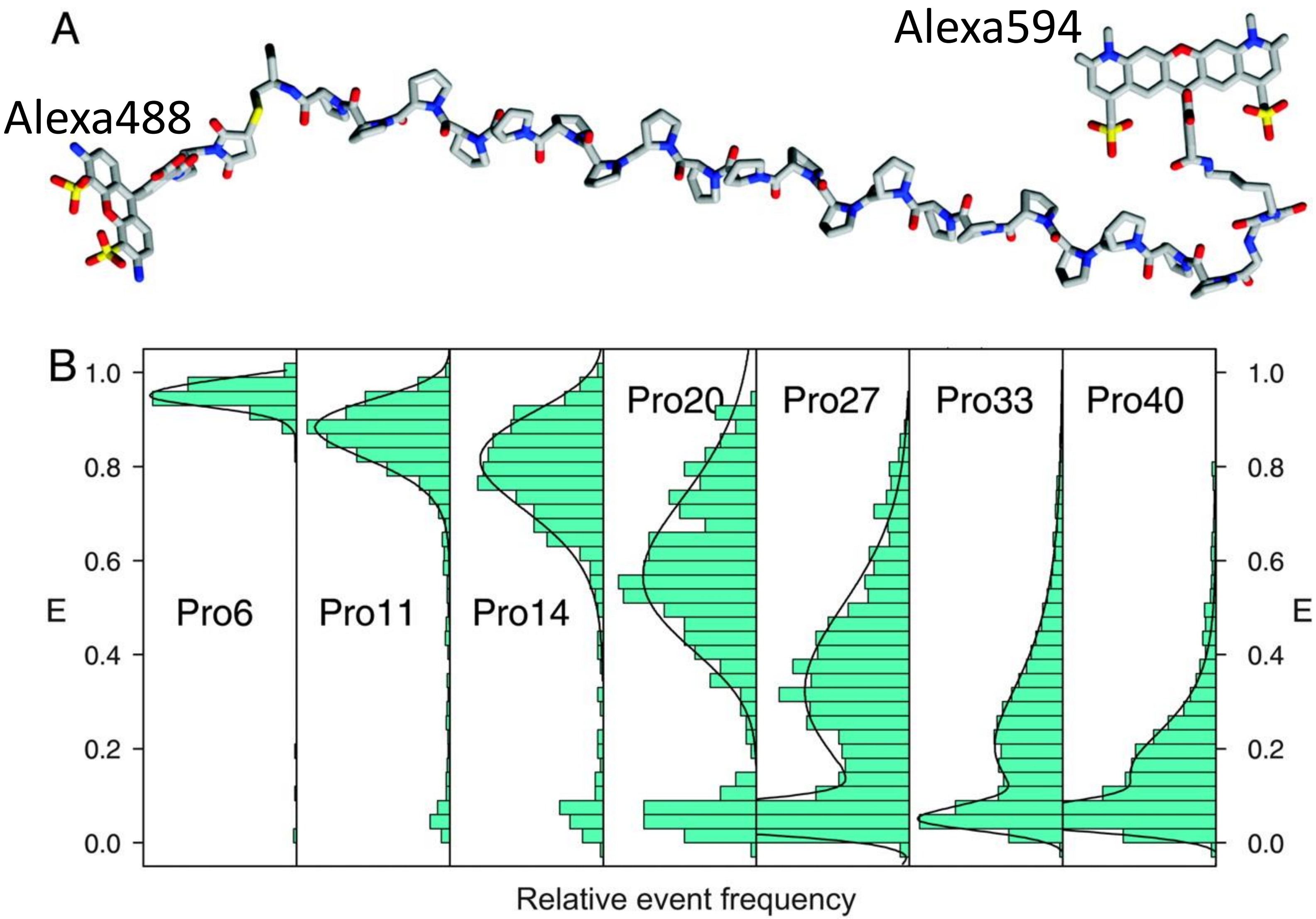
NatProt, 8, 265-281, 2013

FRET Pairs

Donor	Acceptor	Donor Excitation λ_{max} (nm)	Acceptor Emission λ_{max} (nm)	Donor QY (–)	Acceptor ε ($M^{-1}cm^{-1}$)	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[1]	574	0.93[2]	~ 87,000	4.9–5.5
FITC	TRITC	494	572	0.92	100,000	5.4
Phycoerythrin	APC[3]	(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[4]	4050	2.1
Dansyl	FITC	335	519	< 0.035[5]	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

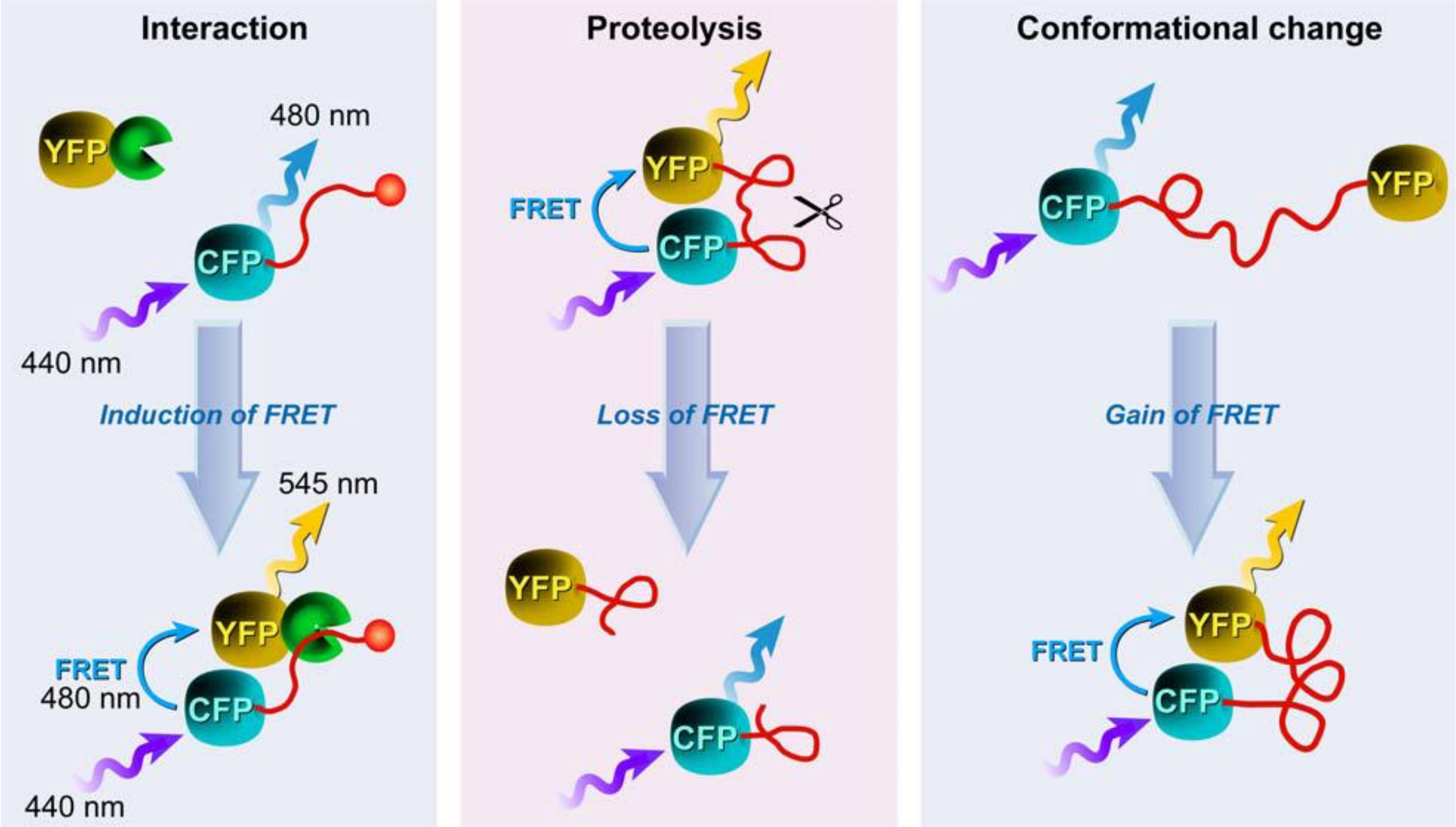


molecular model:
polyproline peptide

PNAS,102,2754-2759, 2005

Fundamental cellular FRET-Approaches

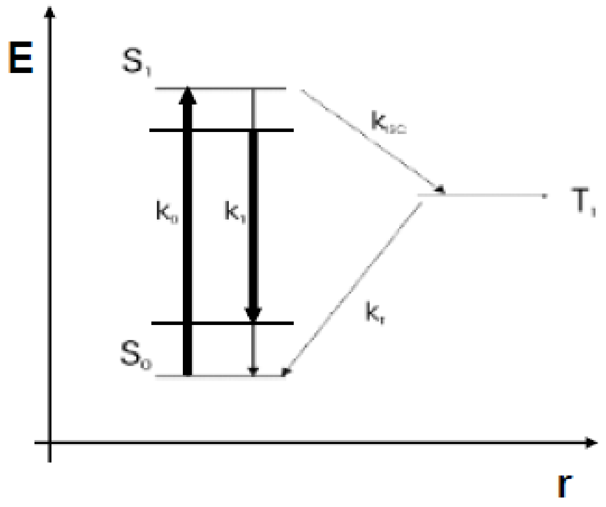
- FRET-biosensors: fusion proteins or other appropriate pairs linked by a sensory domain
- monitoring of donor/ acceptor channels and detection of changes in the FRET signal as a result of biological activity

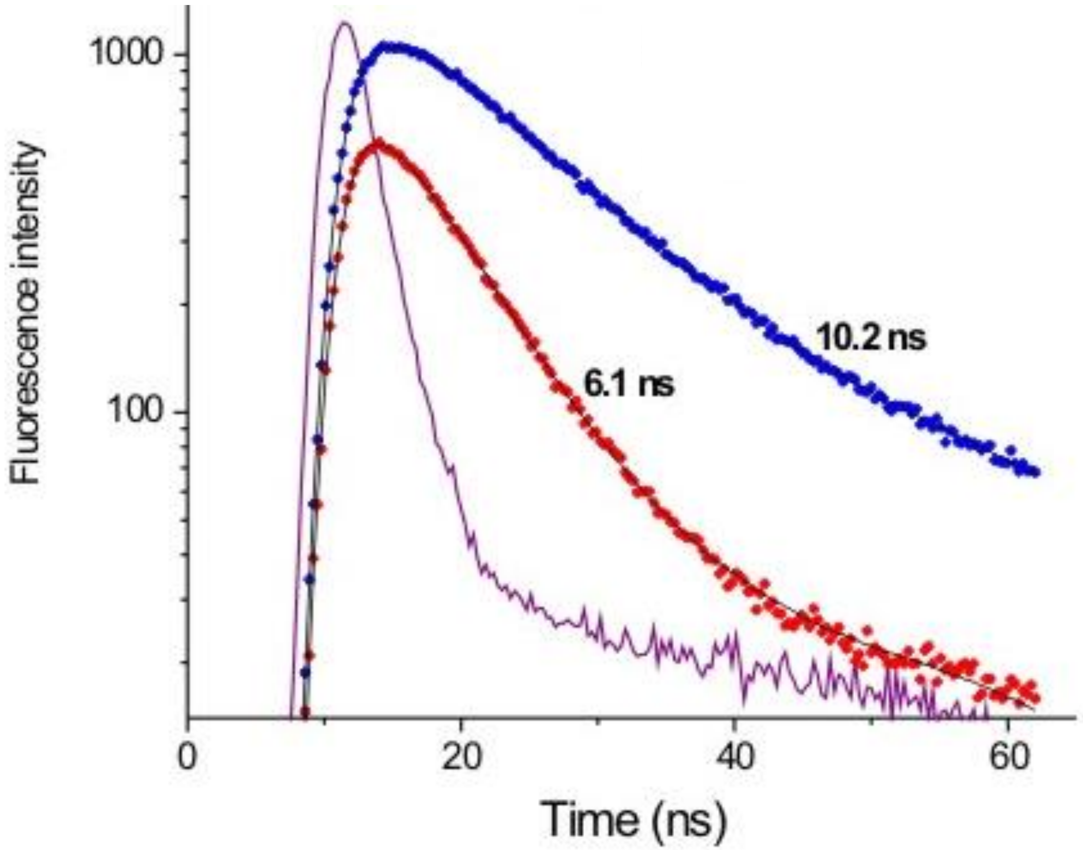


A variation of FRET: FRET-FLIM

FLIM – Fluorescence Lifetime Imaging

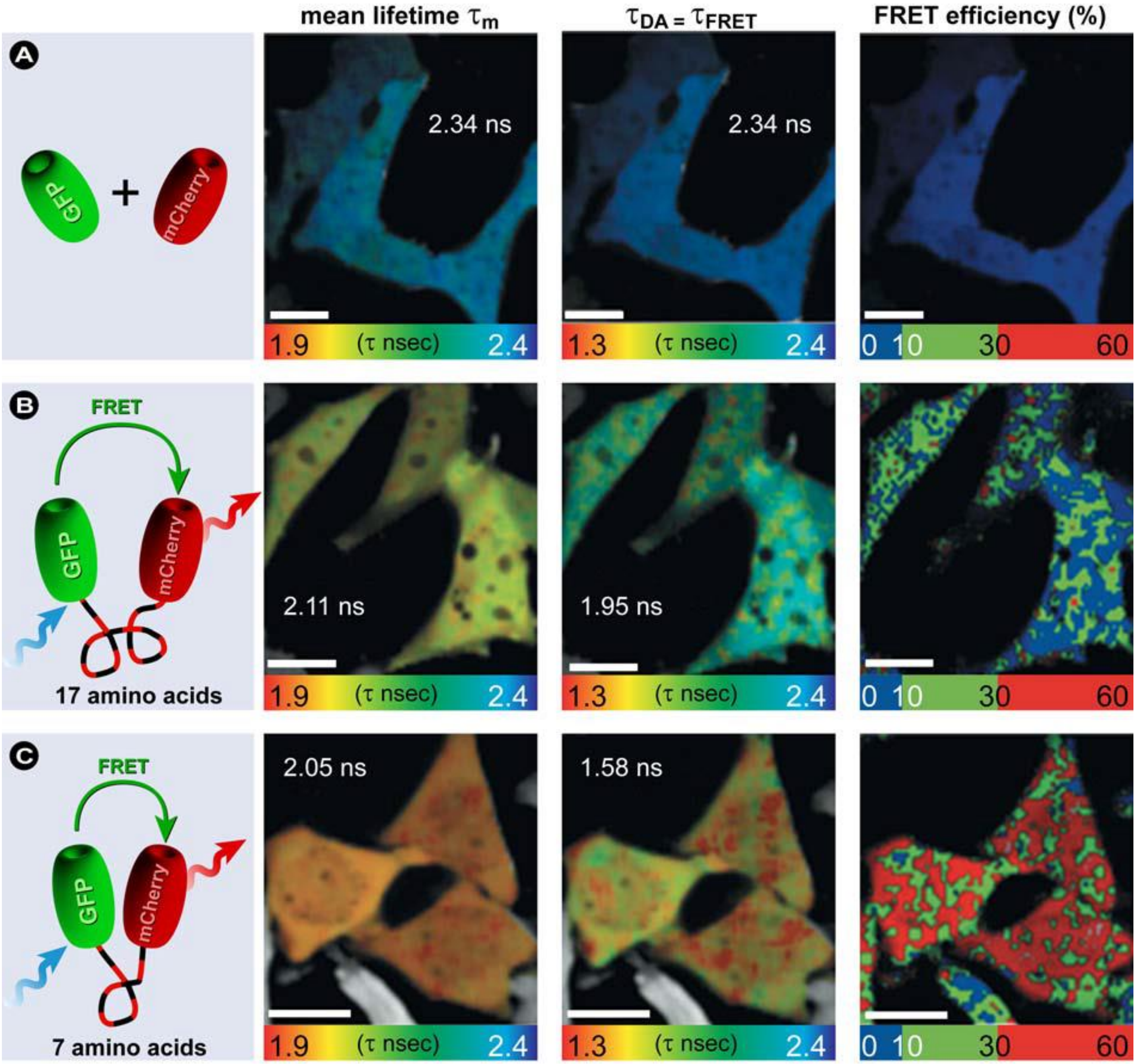
- **…**is an imaging technique for producing an image based on the differences in the exponential decay rate of fluorophores.
- ...is independent of probe concentration
- the lifetime of the fluorophore signal, not the intensity, is used to create the image in FLIM.





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



FLIM with TCSPC in practice

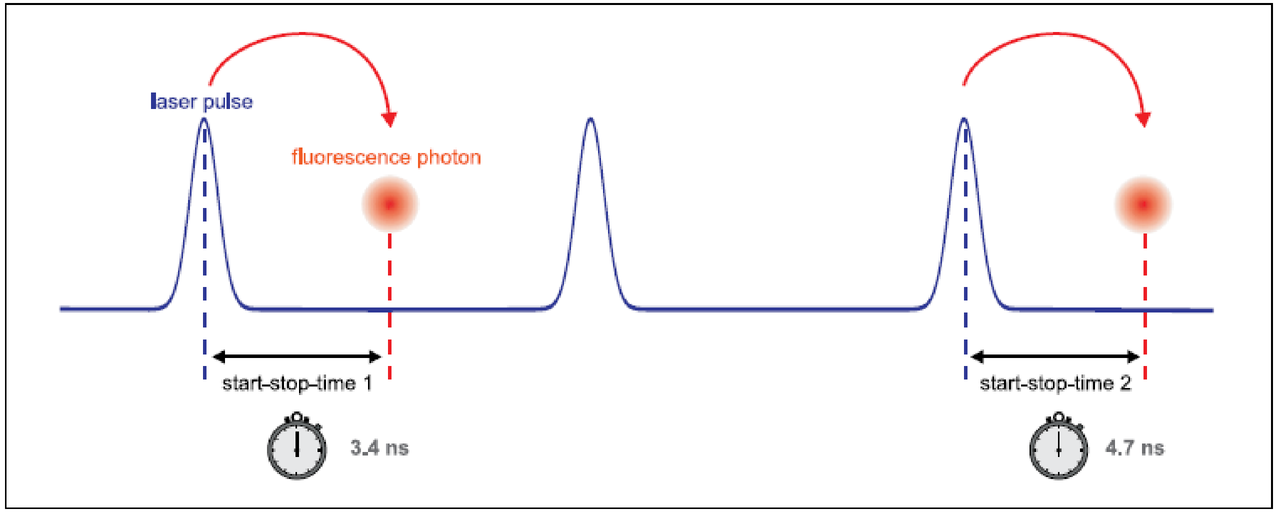


Figure 1: Measurement of start-stop times in time-resolved fluorescence measurement with TCSPC.

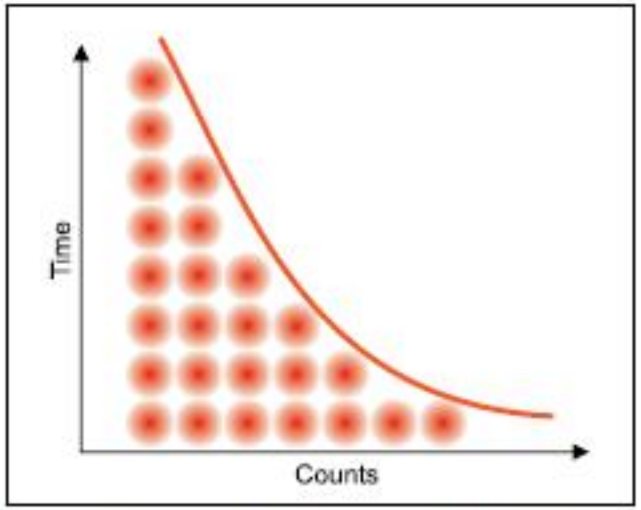
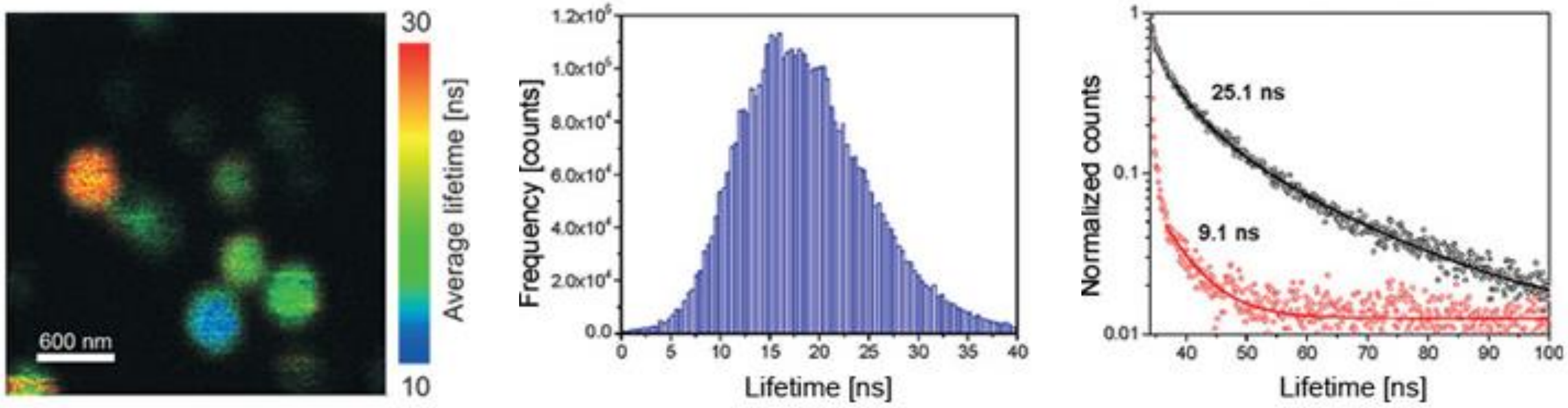


Figure 2: Histogram of start-stop times in time-resolved fluorescence measurement with TCSPC.

FLIM Measurements

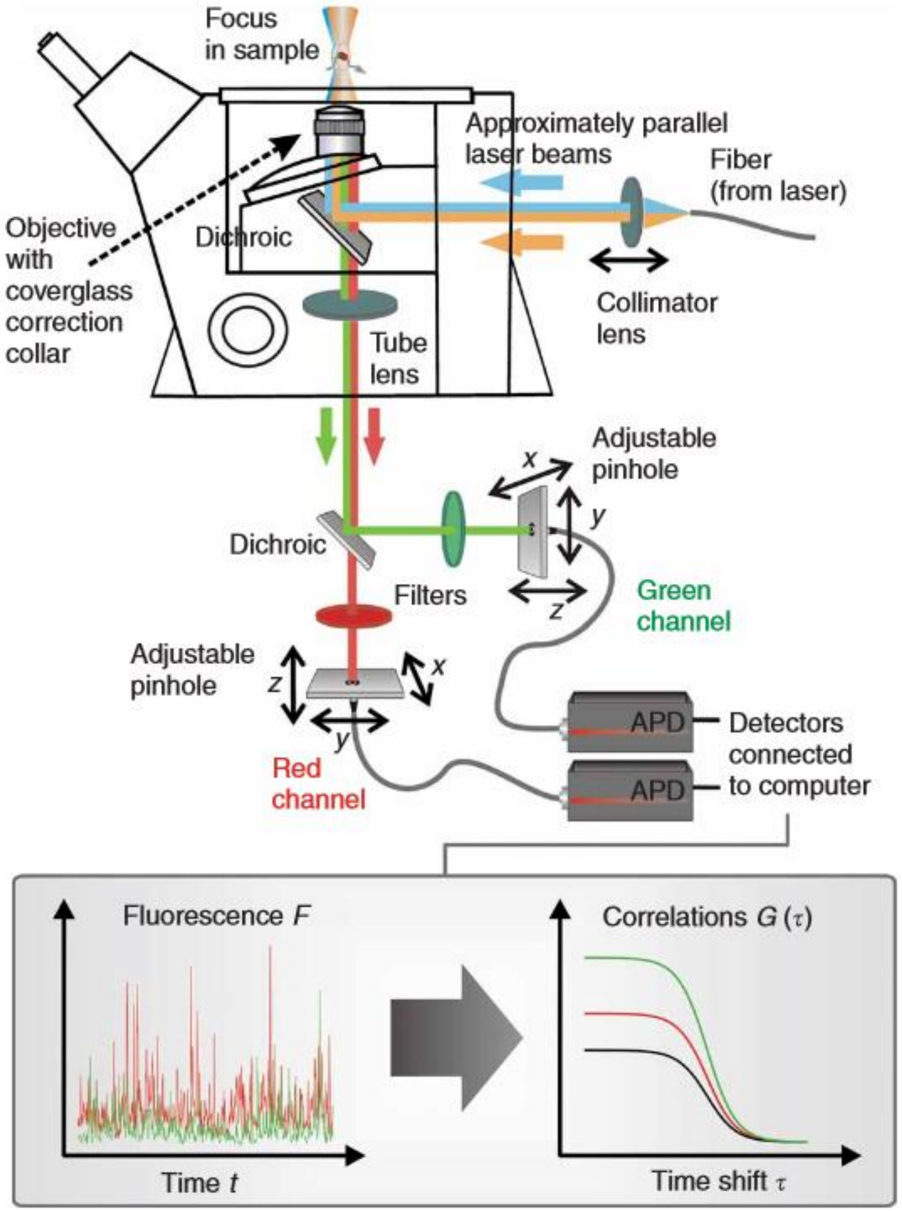
- essential components of a FLIM set-up:
  - pulsed laser source
  - detector (APD or PMT)
  - TCSPC unit to measure the time between excitation and fluorescence emission (time-domain FLIM)

- the delay times are sorted into a histogram

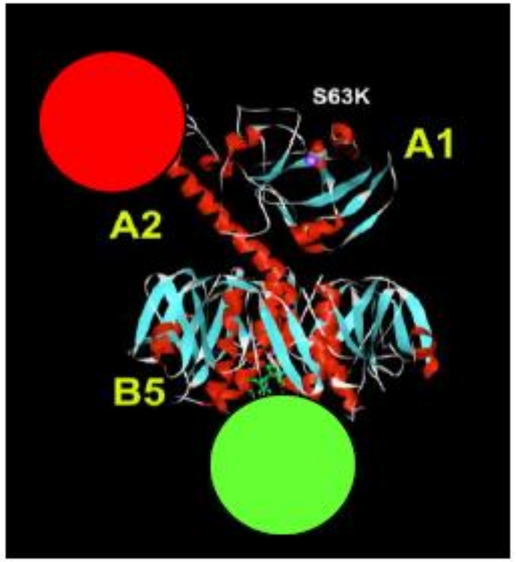


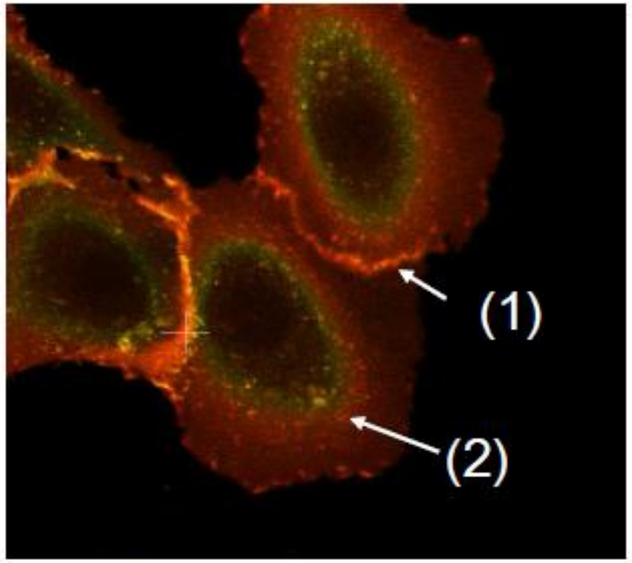
2) FCCS – Dual-Colour Extension of FCS

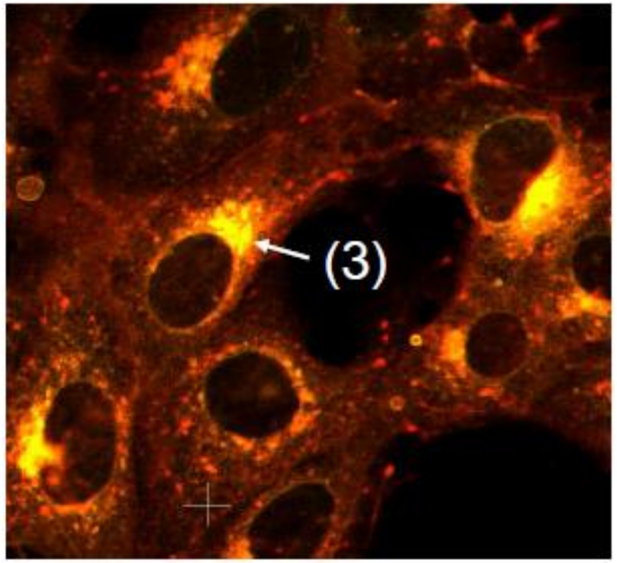
- interaction of two differently labeled molecular species with higher precision than single-color FCS
- high specificity: FCCS curve is only formed if the differently labeled molecules are bound and moving together (co-diffuse).
- From amplitude and decay time: binding constants, mobility of the bound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

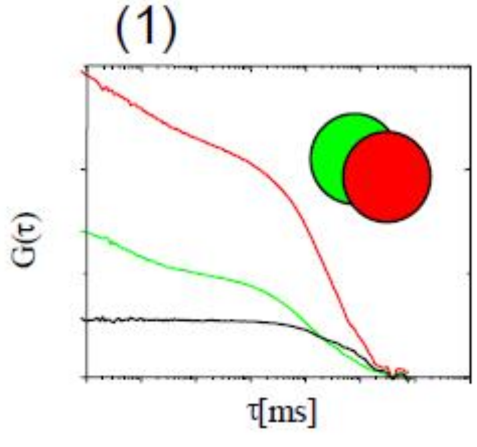


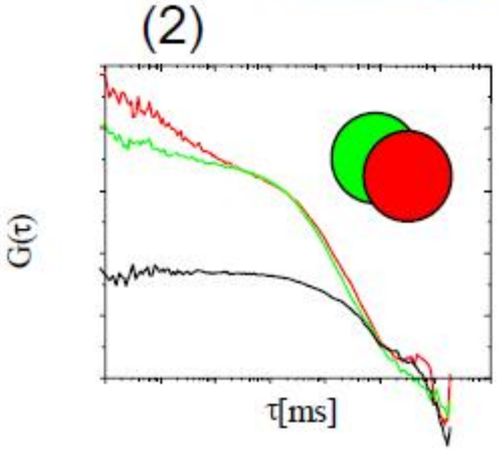
Toxicity of Choleratoxin unfolds as the active A-units separated from B_5 in Golgi

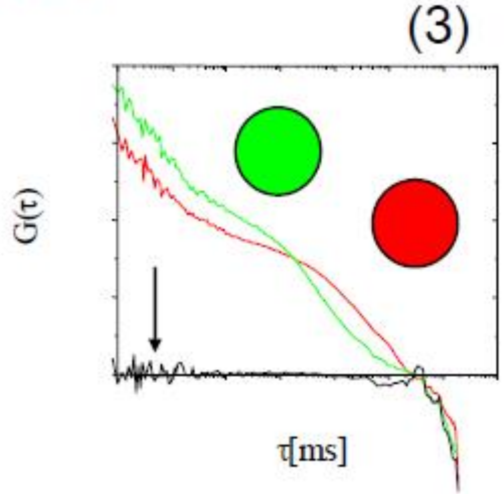












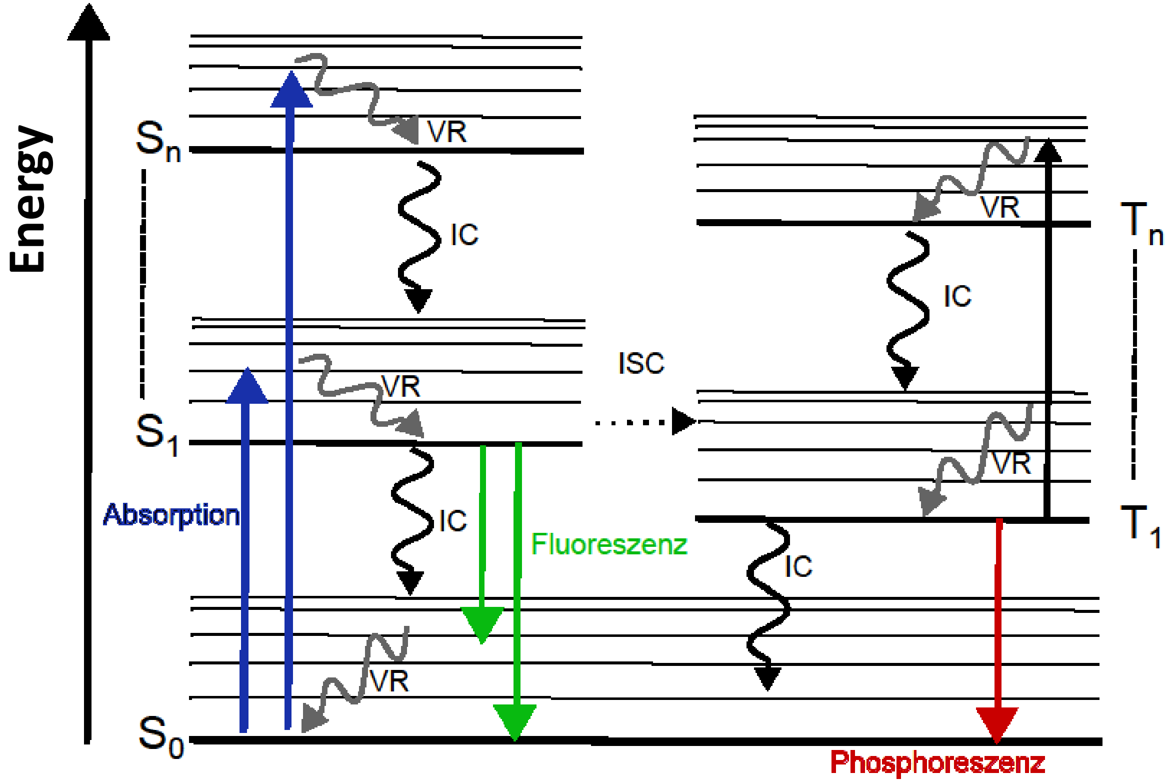
Bacia, Majoul, Schwille, Biophys. J. 2002

Summary - Fluorescence techniques for measuring molecular interactions

- FRET is a direct method for measuring molecular interactions beyond the resolution limits (~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-Diagram

- 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



FCS – Conformational Dynamics

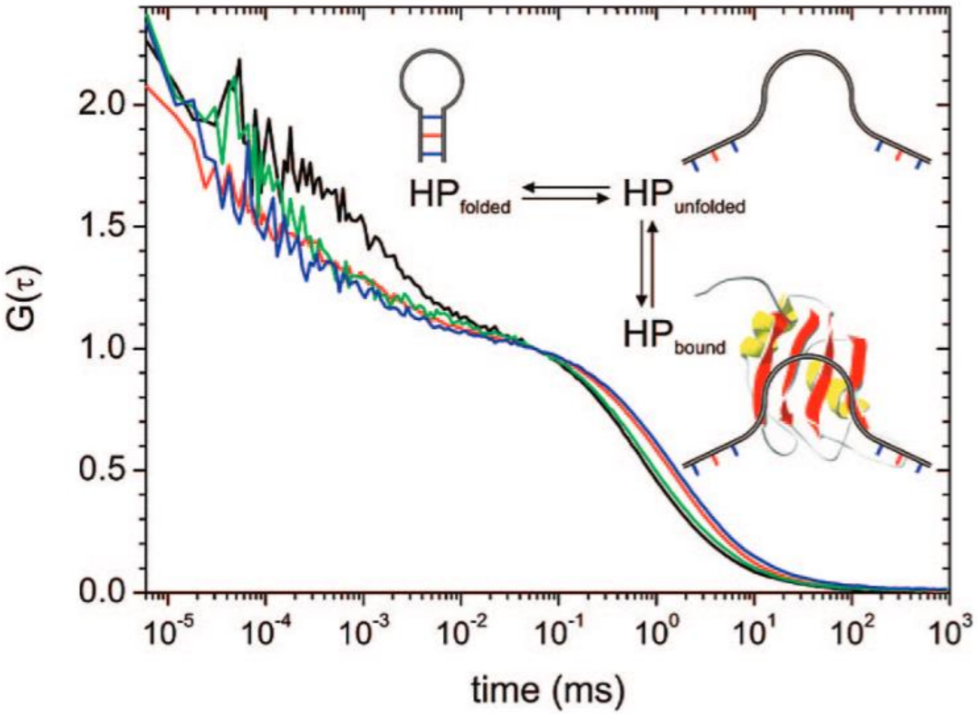
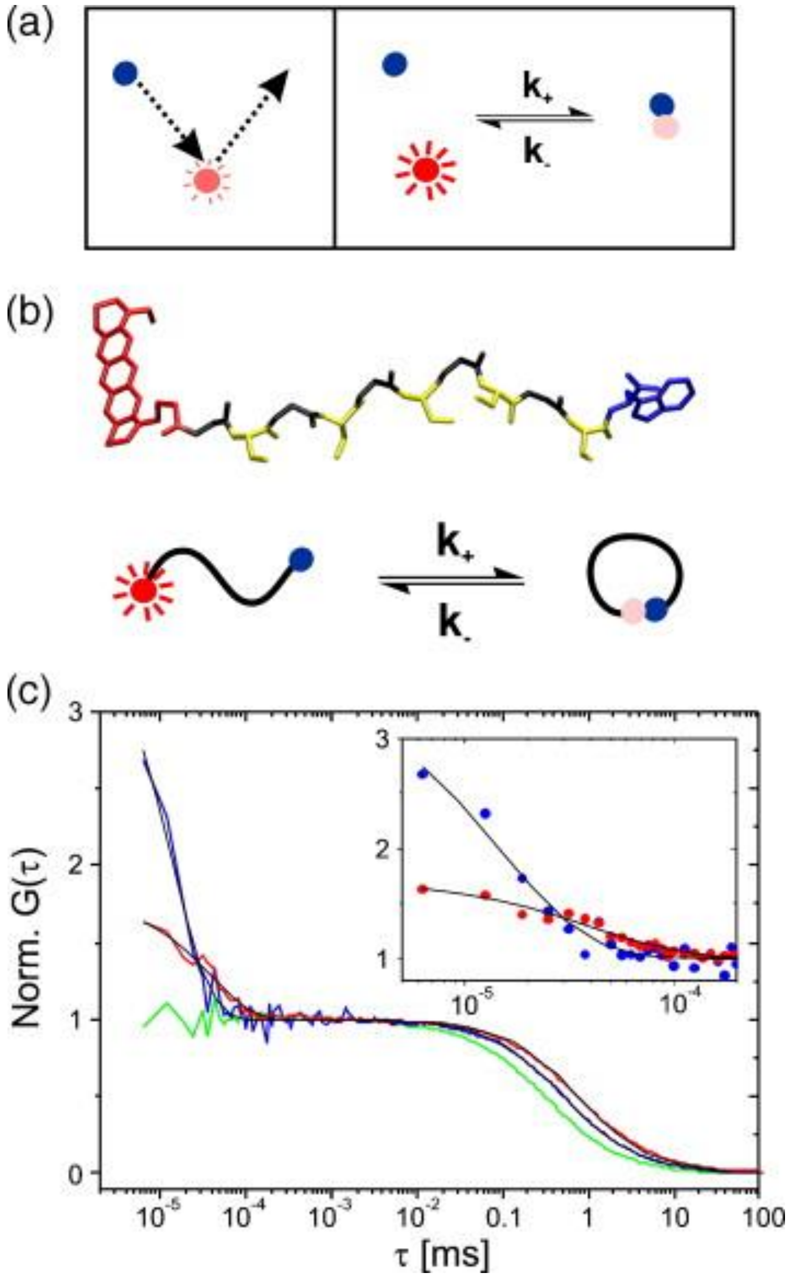
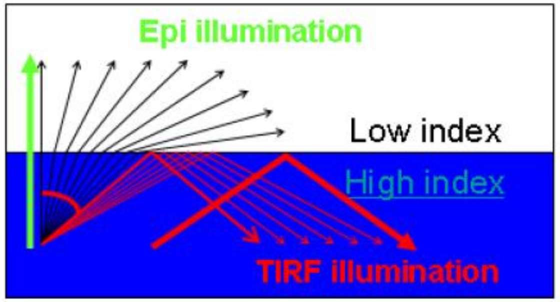


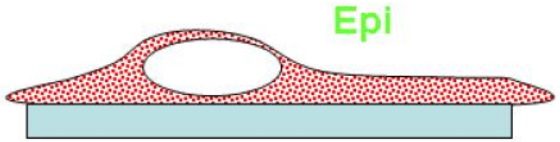
Figure 3. Normalized FCS curves measured for 10^{-9} M solutions of the RNA hairpin HP and the native ORN NT in the absence and presence of *At*GRP7 protein. The fluorescence correlation functions of free HP and NT are shown in black and green, respectively. The curve for HP bound to *At*GRP7 is shown in red and that for NT bound to *At*GRP7 in blue. The proposed model of a two-state hairpin-folding equilibrium whose unfolded state can be bound by the protein[33] is illustrated in the inset.

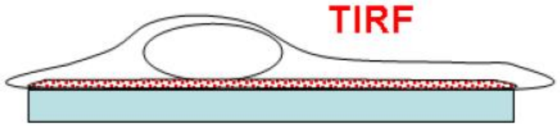
JACS., 2008, 130 (29)



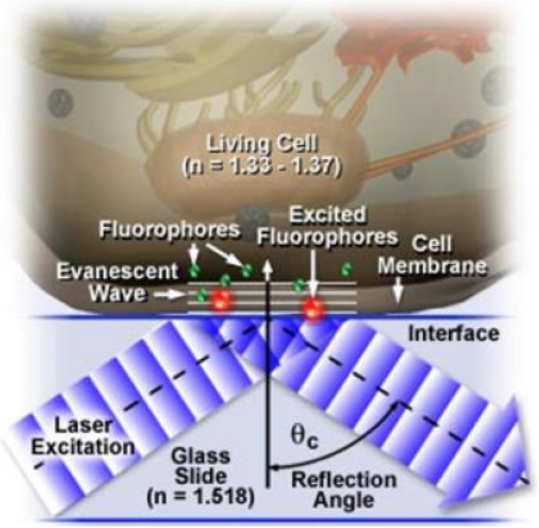
How does TIRF microscopy work?







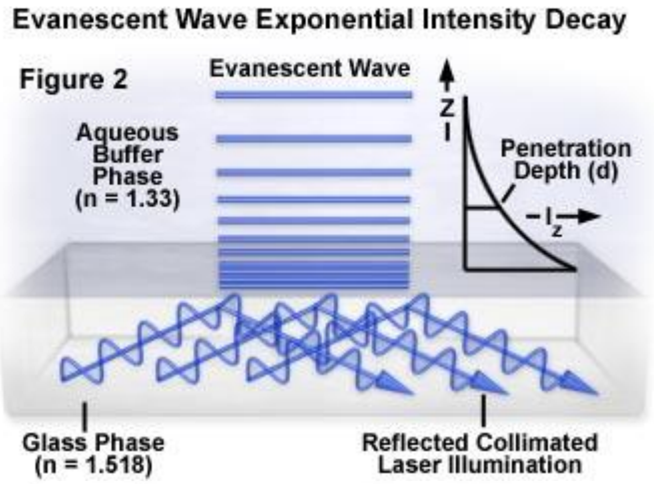
In TIRF, only those fluorophore molecules that are near the cover glass surface are excited



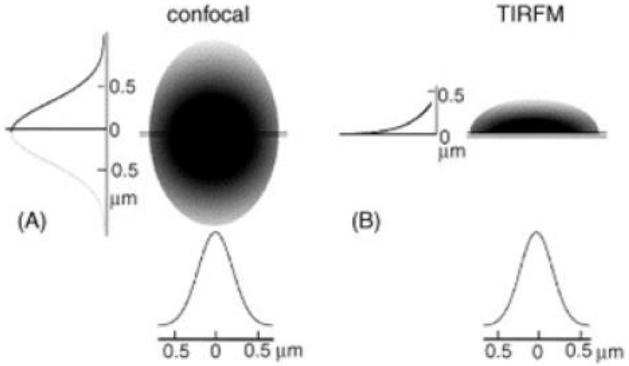
When the incident angle is greater than the critical value, the laser light undergoes **total internal reflection** from the glass-water interface.

http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfhome.html

Advantages of the evanescent field



Point-spread function for confocal *versus* TIRF



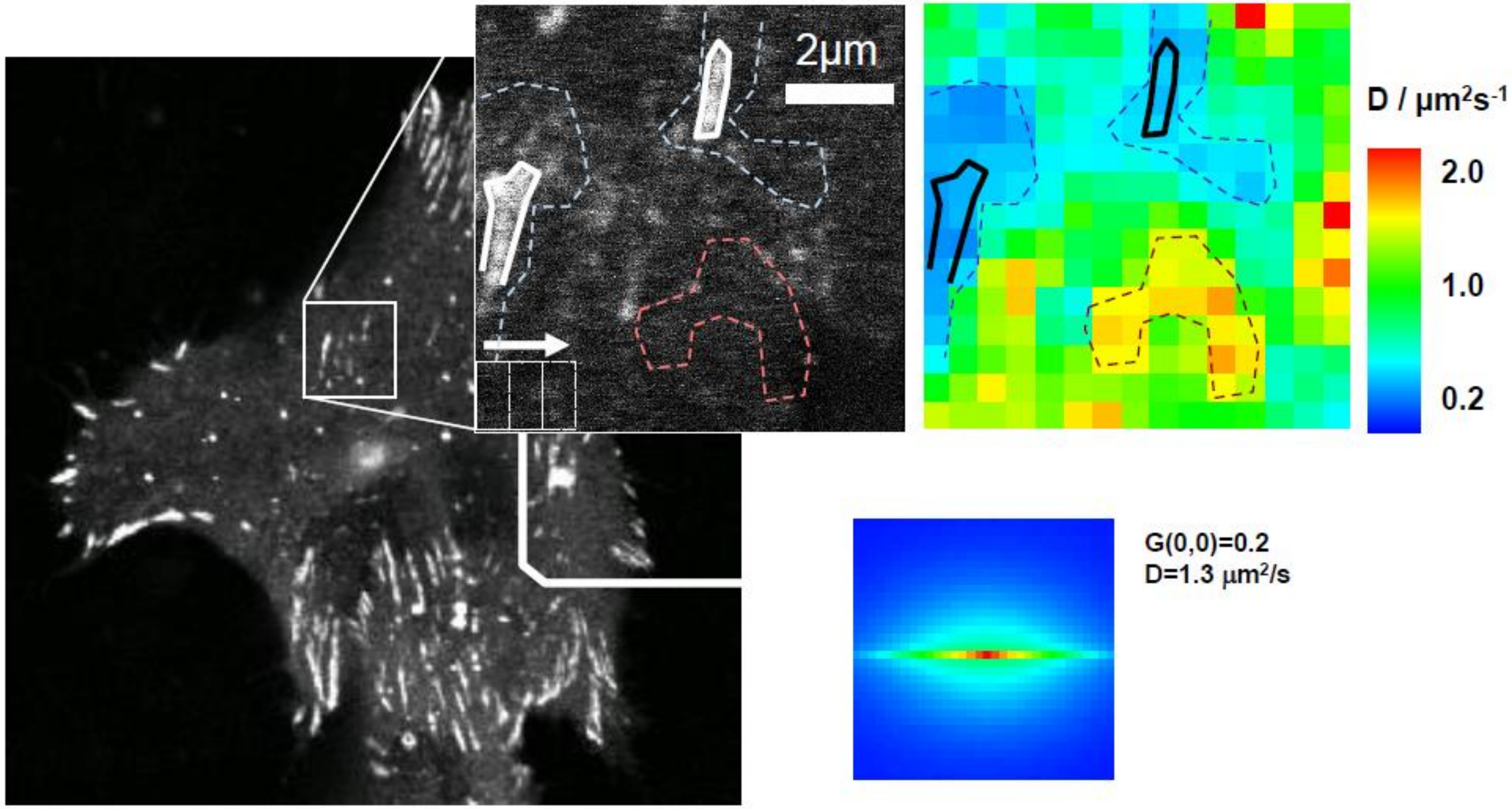
FWHM of point-spread function:

Confocal	300 nm X 800 nm
TIRF	250 nm X ~100 nm (length constant)

6) RICS – Raster Image Correlation Spectroscopy

- RICS is a point confocal imaging form of FCS
- Technique is used to obtain quantitative diffusion maps of heterogeneous samples

Mobility of Eos-Pax between cell adhesion structures – like a weather map.

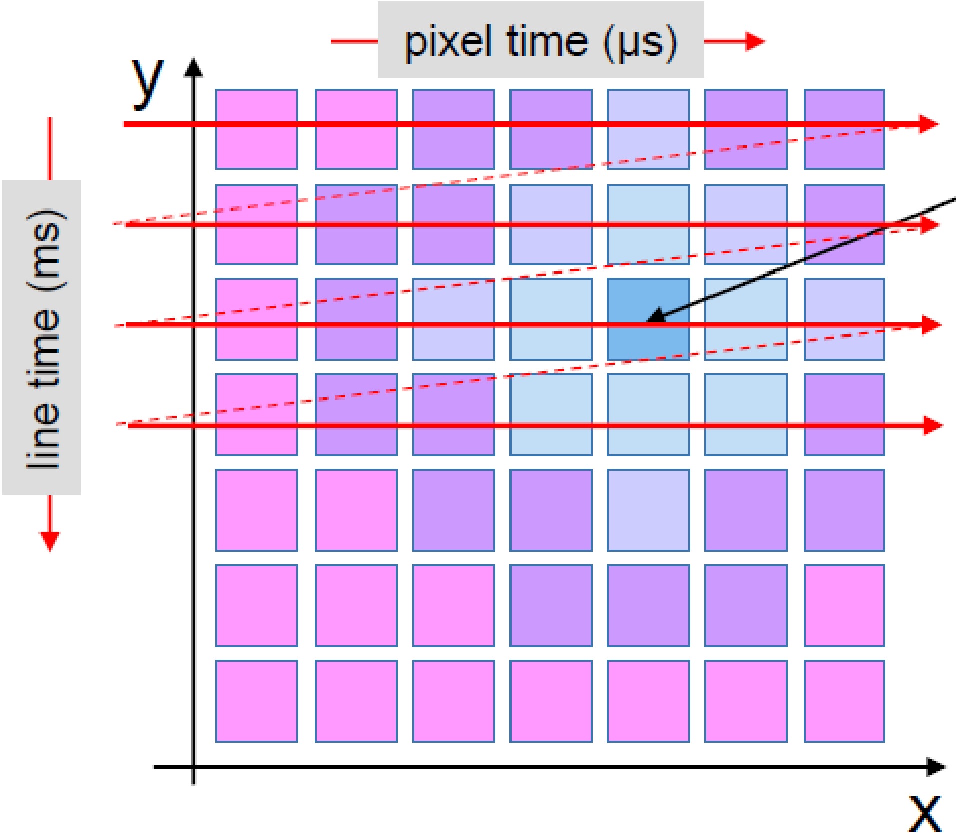


Sample: Hari Shroff and Eric Betzig, Janelia Farm Research Campus, Ashburn, VA, USA

RICS = Raster Image Correlation Spectroscopy

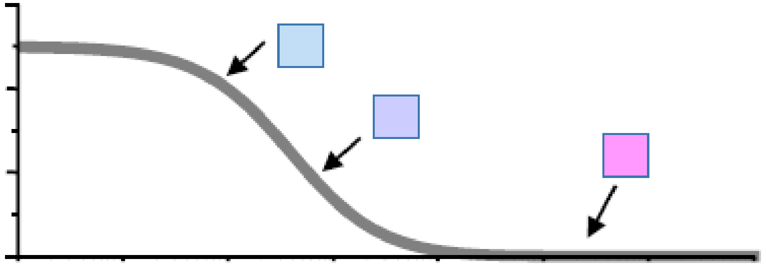
In an image pixels are small **time bins** separated in **space** (**I**CS)

In a raster scanned image pixels are **time bins** separated in **space and time** (**R**ICS)



If something is happening in pixel A then, what is the probability of it happening in pixel B?

The answer is: It depends…



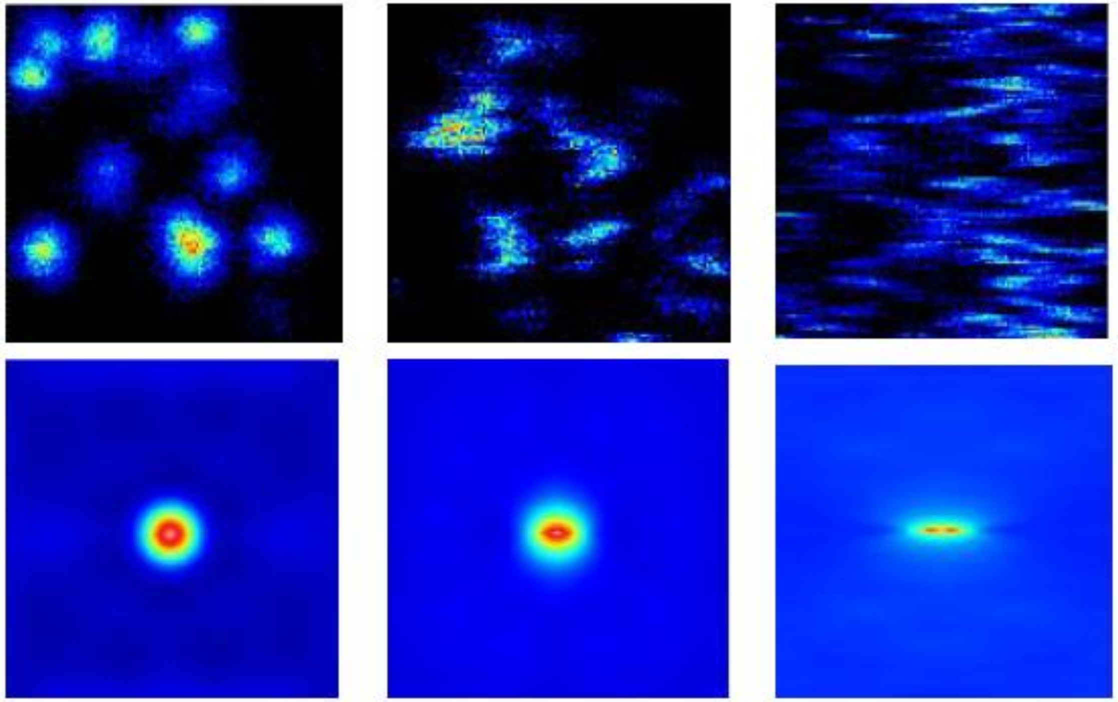
distance from pixel A
and time delay from pixel A

What is shown here for one individual pixel is now repeated for all pixels…

The recipe is exactly the same!

$$g^{(2)}(\xi,\psi)=\frac{\langle I_r(x,y)\cdot I_r(x+\xi,y+\psi)\rangle}{\langle I_r\rangle^2}$$

What **can** change is the pattern, but **only** if the objects move (if **not:** see ICS)



D = 0.1 μm²/s D = 5 μm²/s D = 90 μm²/s

Raster scanned images of moving (sub-resolution) beads

This is the ICS applied to a raster scanned image of moving (sub-resolution) beads.

RICS analysis

$$g_{tot}^{(2)}(\xi,\psi)=\frac{\langle I_r(x,y)\cdot I_r(x+\xi,y+\psi)\rangle}{\langle I_r\rangle^2}=\boxed{g_{diff}^{(2)}(\xi,\psi)}\times\boxed{g_{scan}^{(2)}(\xi,\psi)}$$

Diffusional term:
Finding the same
particle in time

multiplied by

Scan term:
Finding the same
particle in space

Both are connected via the diffusion coefficient…