Fluorescence correlation spectroscopy and its applications in cell biology

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Diffusion

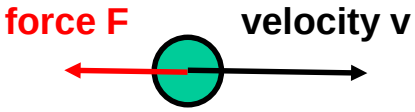
- Diffusion is the random thermal motion (Brownian motion) of molecules
- Time dependence of diffusive motion (Einstein-Schmoluchowski equation):

  - Diffusion in 1D: $\langle \Delta r^2 \rangle = \langle \Delta x^2 \rangle = 2\mathbf{Dt}$
  - Diffusion in 2D: $\langle \Delta r^2 \rangle = \langle \Delta x^2 \rangle + \langle \Delta y^2 \rangle = 4\mathbf{Dt}$
  - Diffusion in 3D: $\langle \Delta r^2 \rangle = \langle \Delta x^2 \rangle + \langle \Delta y^2 \rangle + \langle \Delta z^2 \rangle = \mathbf{6Dt}$

  D (m^2/s) is the diffusion coefficient (~mobility)

Time ∝ square of the distance; thus, to move 10 times as far, we need 100 times longer.
example: 'typical' protein, radius r = 4 nm, D = 60 $\mu m^2/s$
Traversing E. coli cell (r = 1 μm) takes $t = \langle r^2 \rangle / 2D \sim 1$ ms
traversing eukaryotic cell (r = 20 μm) takes about ~400 ms

- Diffusion and friction are related:



$$F = -f \cdot v$$

- *f* is *friction coefficient*
- For a sphere with radius R, $f = 6\pi\eta R$ (Stokes' equation), η: viscosity
- Einstein showed that

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

- (i.e. the diffusion coefficient of a molecule is inversely proportional to its size)

How do we measure diffusion coefficients?

- Forced motion:
  - sedimentation coefficient (analytical ultracentrifugation)
  - electrophoretic mobility
  - gel filtration
- Random motion:
  - classical diffusion measurements
  - dynamic light scattering
  - **fluorescence correlation spectroscopy**

- Diffusion coefficient gives information about
  - Size of a complex
  - Free/bound state
  - Microviscosity

Origins of fluorescence correlation spectroscopy (FCS)

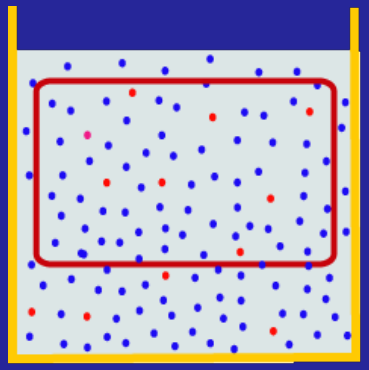
- Belongs to the family of fluctuation spectroscopies (such as elastic light scattering), it is based on fluorescence
- FCS: correlation analysis of fluorescence intensity fluctuations
- 1972: the method was developed for studying kinetic parameters of chemical reactions (e.g. the helix-coil transition of DNA) by restricting observatins to a microscopic volume and detecting fluctuations caused by individual molecules (D. Magde, E.L. Elson, W.W. Webb, R. Rigler)
- Biological application: facilitated by combination with confocal microscopy or two-photon microscopy from the 1990-s

What can cause fluorescence fluctuations?

- Diffusion
- Flow
- Rotational motion
- Photophysical processes (triplet, blinking)
- Conformational changes
- Chemical reactions
- Phase Fluctuations
- …

FCS can study the kinetic pamateres of such processes.

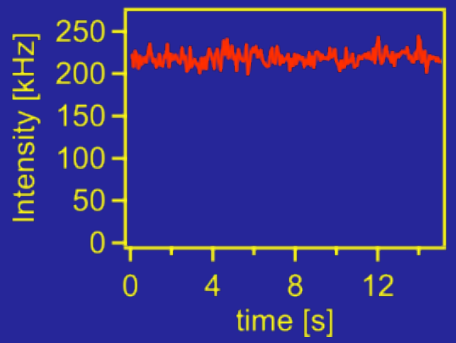
Fluorescence fluctuation analysis is done best around the single molecule limit



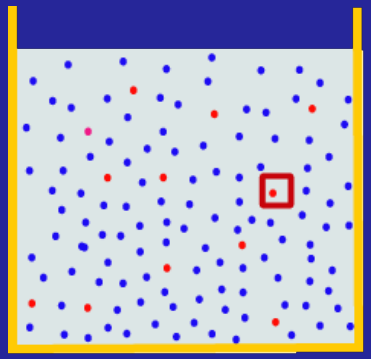
Bulk ensemble

100 nM, 50 µl

~3x10^{12} molecules



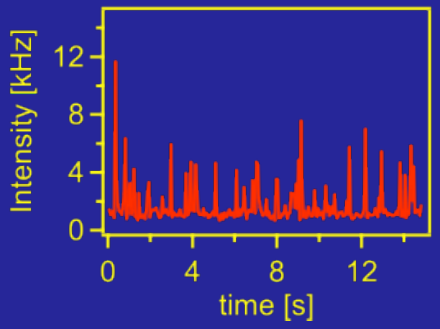
$\frac{\Delta F}{F} = \frac{\sqrt{N}}{N}$ *is small (~10^{-6})*



Single molecule

1 nM, 1 fl

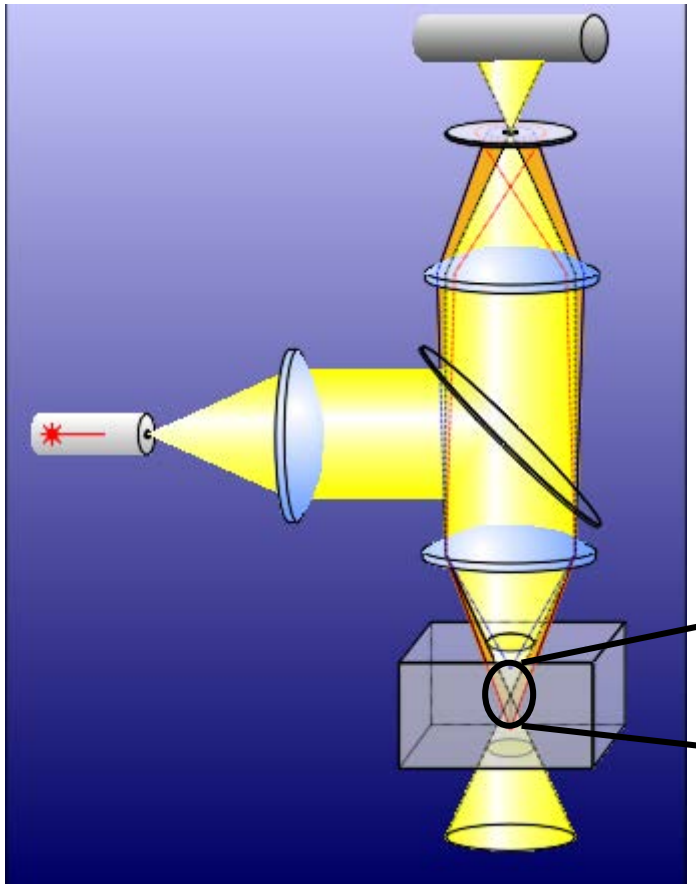
~0.6 molecule

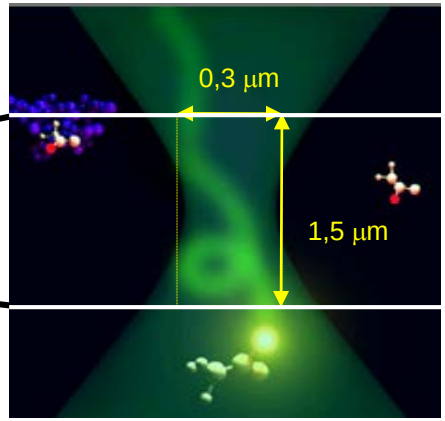


$\frac{\Delta F}{F}$ *is large (~1.3)*

adapted from J. Langowski

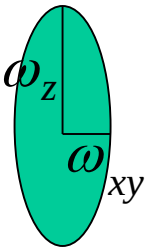
The observation volume in confocal FCS





The signal is proportional to the convolution of the excitation and detection efficiencies.

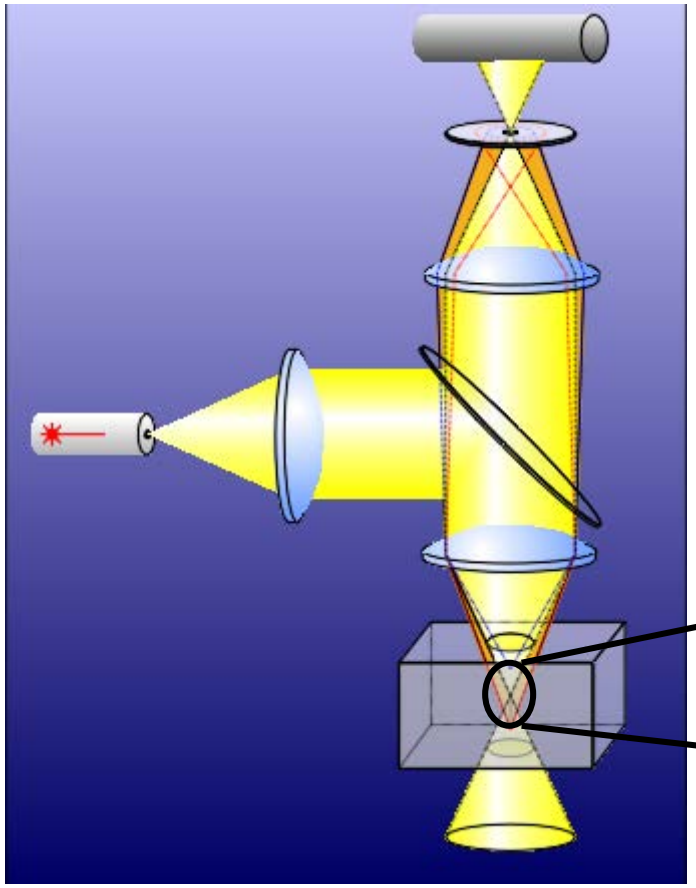
$$I = I_0 \cdot e^{-2\frac{x^2+y^2}{\omega_{xy}^2}} \cdot e^{-2\frac{z^2}{\omega_z^2}}$$

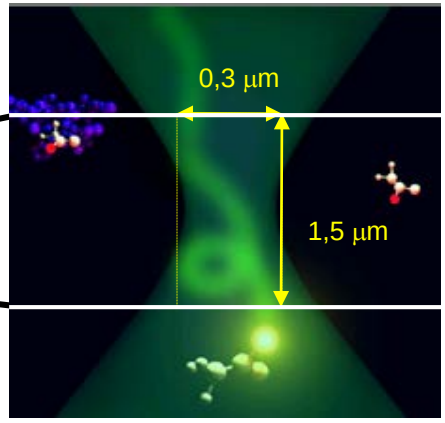


ω_{xy}, ω_z: 1/e^2 radii

$$S = \frac{\omega_z}{\omega_{xy}}$$ Aspect ratio

FCS in a nuthsell

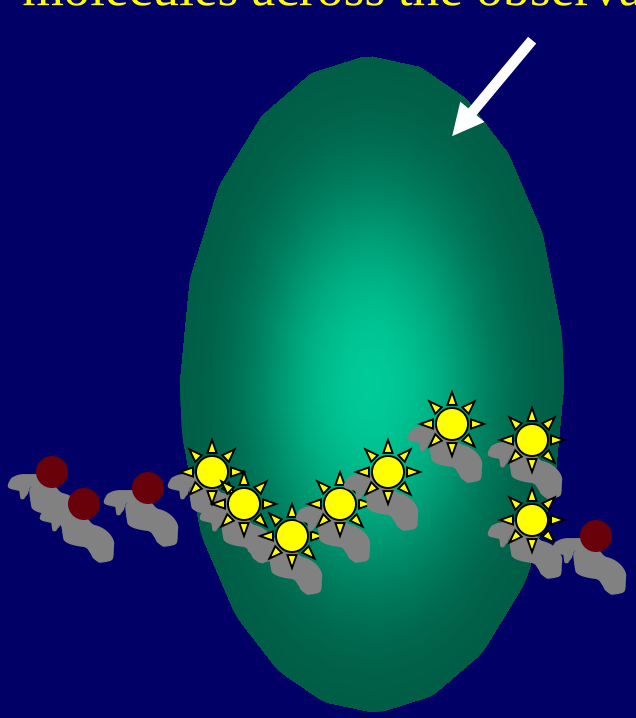


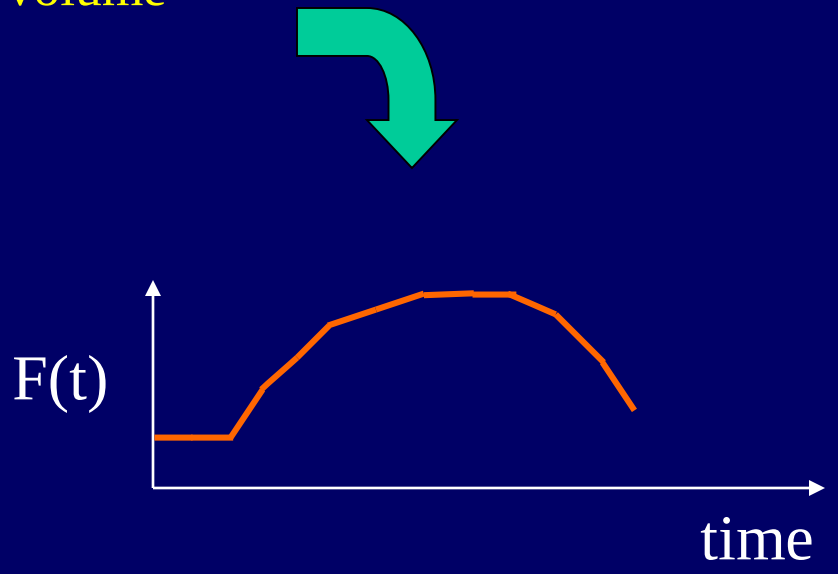


- Fluorescence labeling of molecule of interest (protein, lipid, …)
- Illumination with focused laser beam
- Detection of fluorescence intensity in a confocal configuration with sensitive photodetector (avalanche photodiode) in photon counting mode
- Various processes contribute to the change of fluorescence intensity (motion, photophysical and chemical interactions,…)
- Correlation analysis of fluorescence vs. time

Fluorescence fluctuations may arise from…

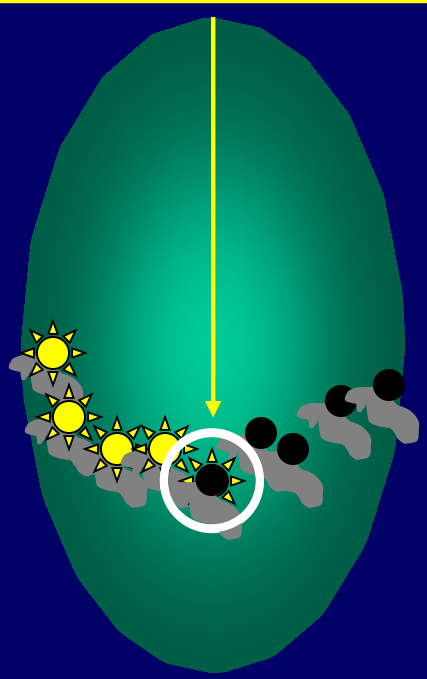
... The motion (diffusion) of fluorescent molecules across the observation volume

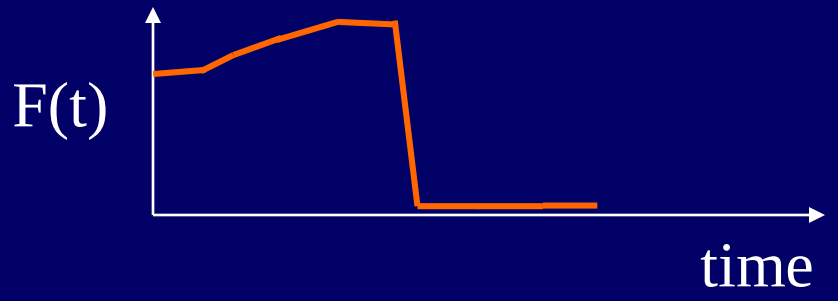




Fluorescence fluctuations may arise from…

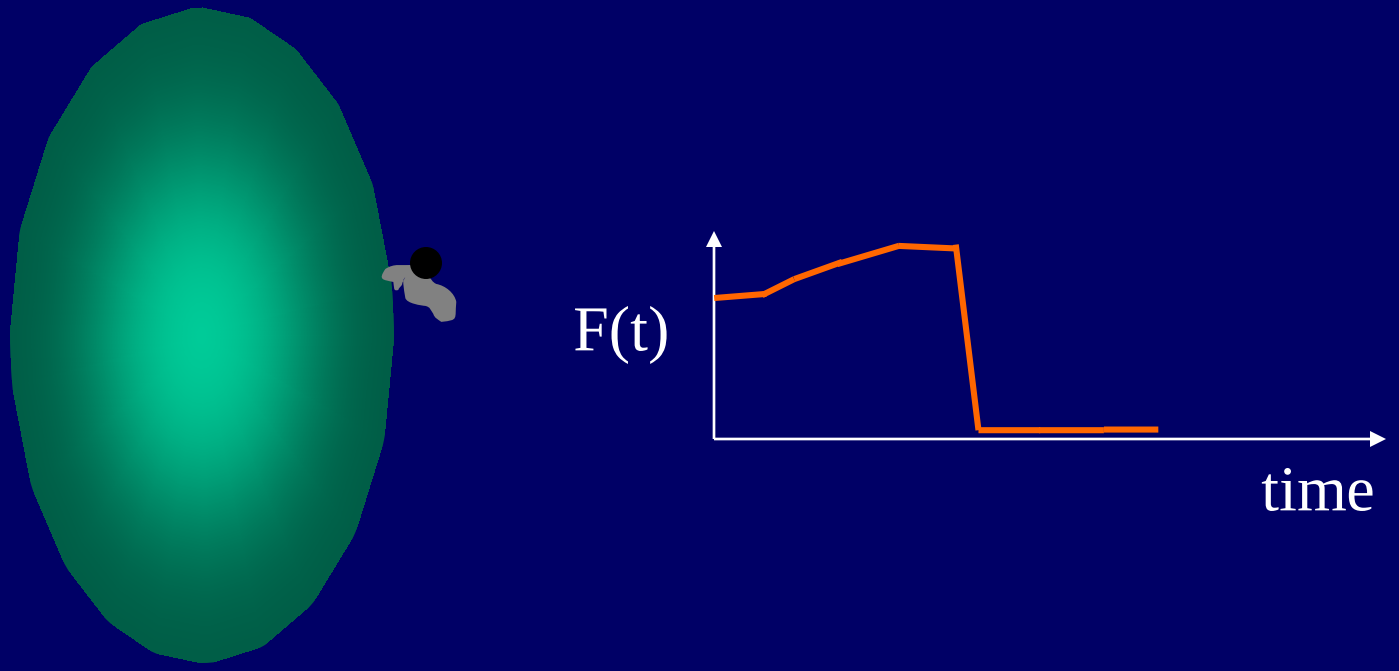
... photophysical processes like photobleaching, triplet state formation, light-induced dark state formation...



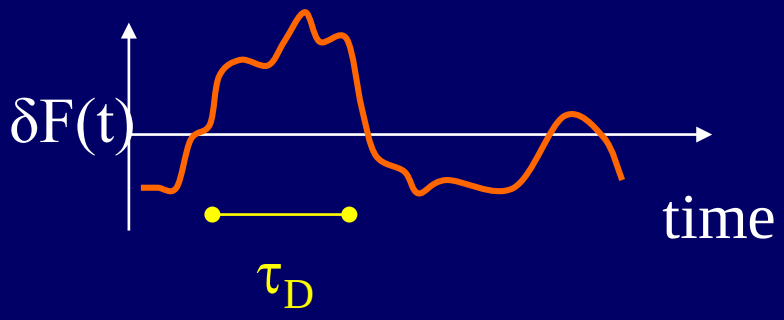


Fluorescence fluctuations may arise from…

... or any other molecular process affecting the fluorescence quantum yield.

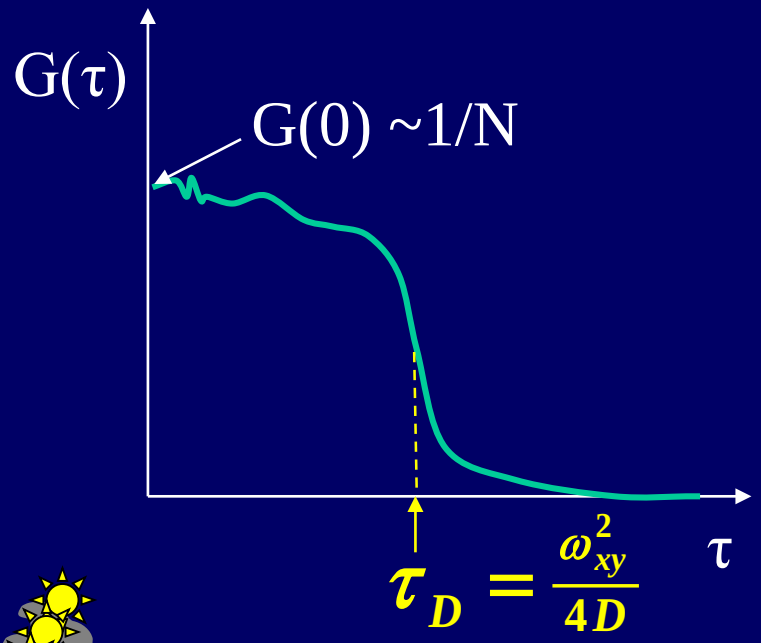


From the time dependence of the fluorescence intensity...



$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F \rangle^2}$$

...the autocorrelation function G(τ) is calculated



... From which the parameters determining the autocorrelation functions can be derived:

- the average number of molecules in the detection volume, *N*
- the diffusion correlation time, τ_D, which is inversely proportional to the diffusion coefficient
- aggregation state (fluorescence /molecule F/N)
- photophysical and chemical kinetic constants, etc.

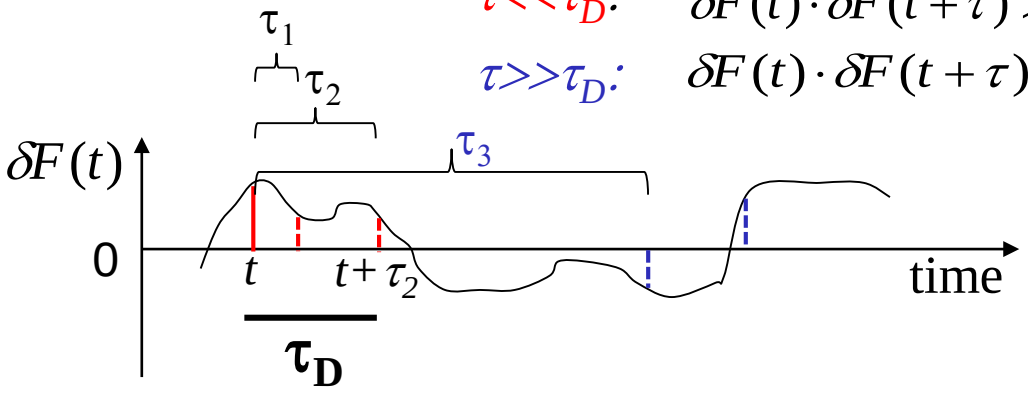
Origin of the autocorrelation curve

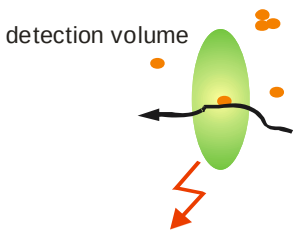
$\delta F(t) = F(t) - \langle F \rangle$ Difference from mean intensity

Time average

$\tau << \tau_D$: $\delta F(t) \cdot \delta F(t+\tau) > 0$

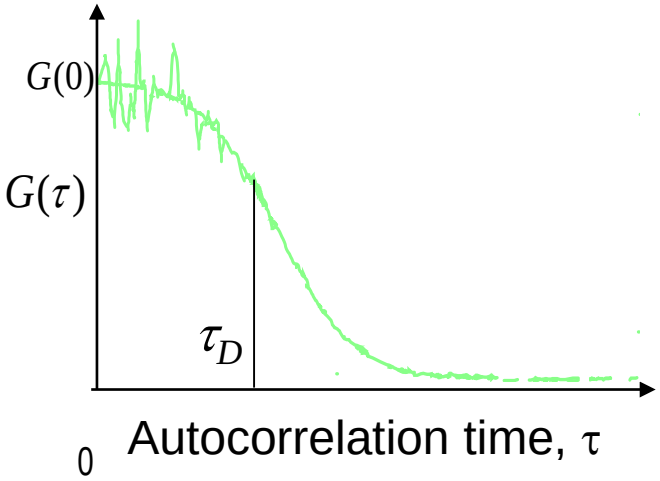
$\tau >> \tau_D$: $\delta F(t) \cdot \delta F(t+\tau) \gtrless 0$





Mean dwell time
(diffusion time)

G(τ) is proportional to the probability of the particle being in the obs. volume at $t=\tau$, provided it was there at t=0.



$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F \rangle^2} = \frac{\frac{1}{T}\int_0^T \delta F(t) \cdot \delta F(t+\tau) dt}{\langle F \rangle^2}$$

Time autocorrelation function of the intensity

The amplitude of the autocorrelation function

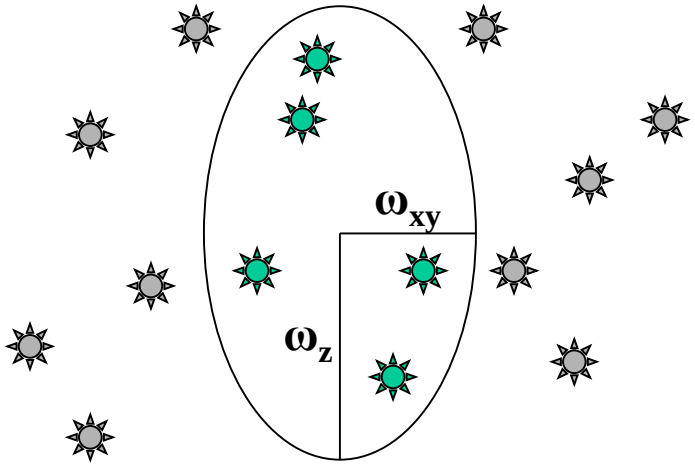
The number of particles *N* in the observation volume follows Poissonian distribution:

Expectation (mean) value of *N*: $\langle N \rangle = \langle c \rangle V_{eff}$

Variance of *N*: $\sigma^2 = \langle N \rangle$

Mean fluorescence of a particle: f

Total fluorescence of *N* particles: $F = Nf$

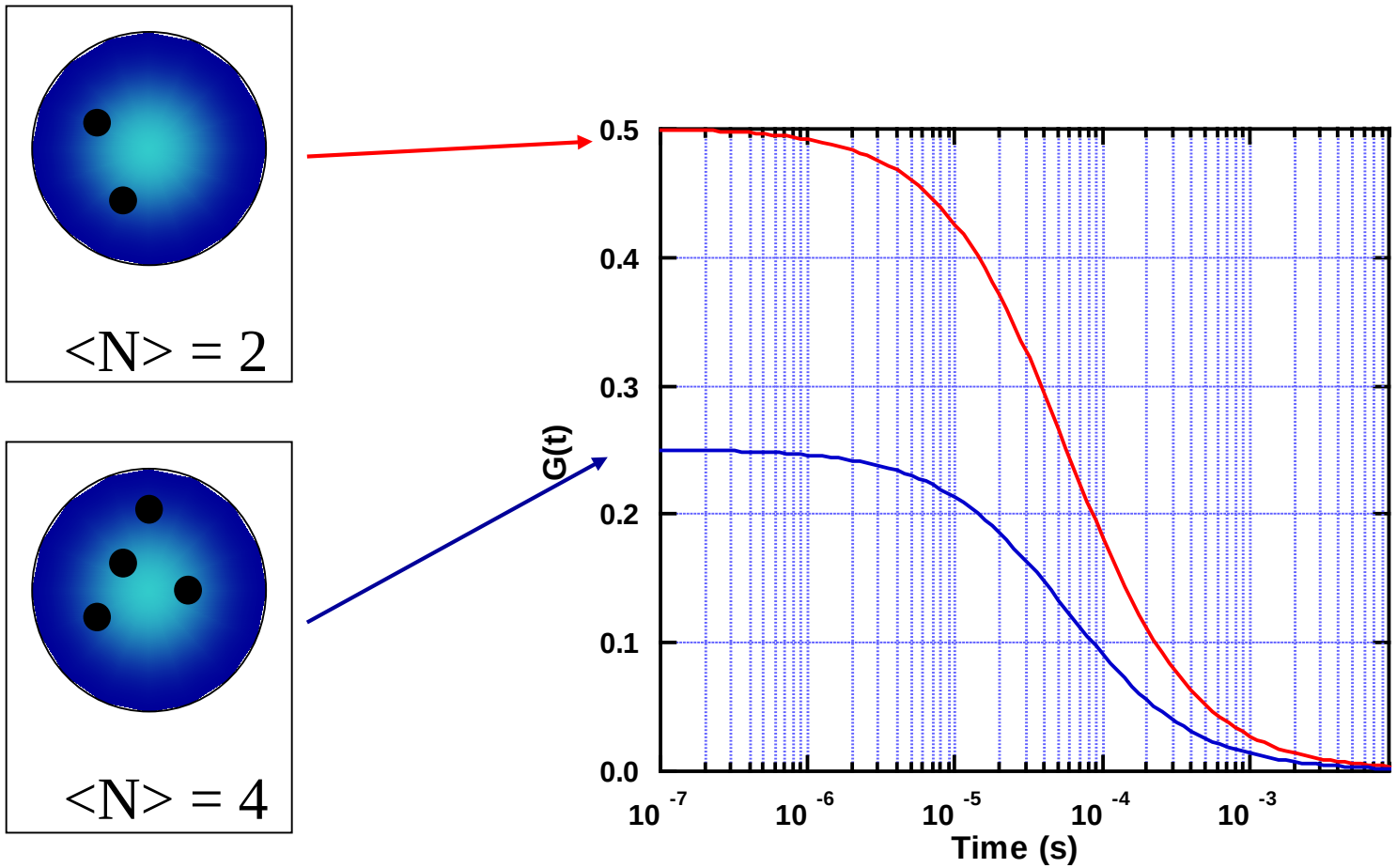


$$G(0) = \frac{\langle \delta F(t) \cdot \delta F(t+0) \rangle}{\langle F^2 \rangle} = \frac{\langle (\delta F(t))^2 \rangle}{\langle F^2 \rangle} = \frac{\langle (\delta N(t) \cdot f)^2 \rangle}{\langle N^2 f^2 \rangle} = \frac{\langle \delta N(t)^2 \rangle}{\langle N^2 \rangle} = \frac{\sigma_N^2}{\langle N^2 \rangle} = \frac{N}{\langle N^2 \rangle} = \frac{1}{\langle N \rangle}$$

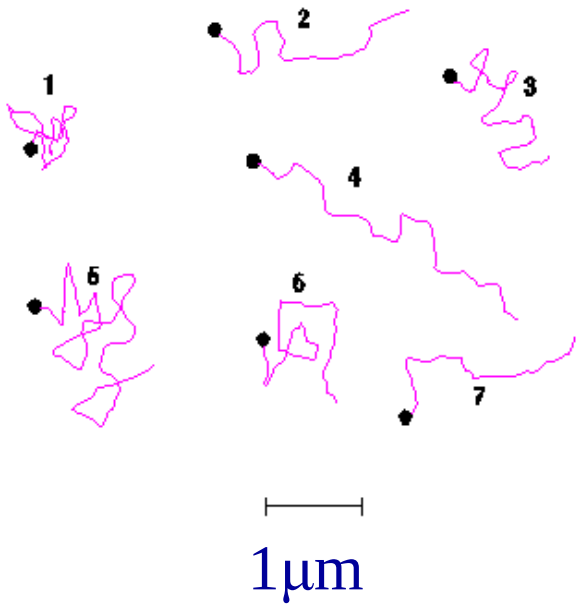
The amplitude of the ACF is the reciprocal of the average number of molecules in the observation volume. The correlation signal is larger if the concentration is lower!

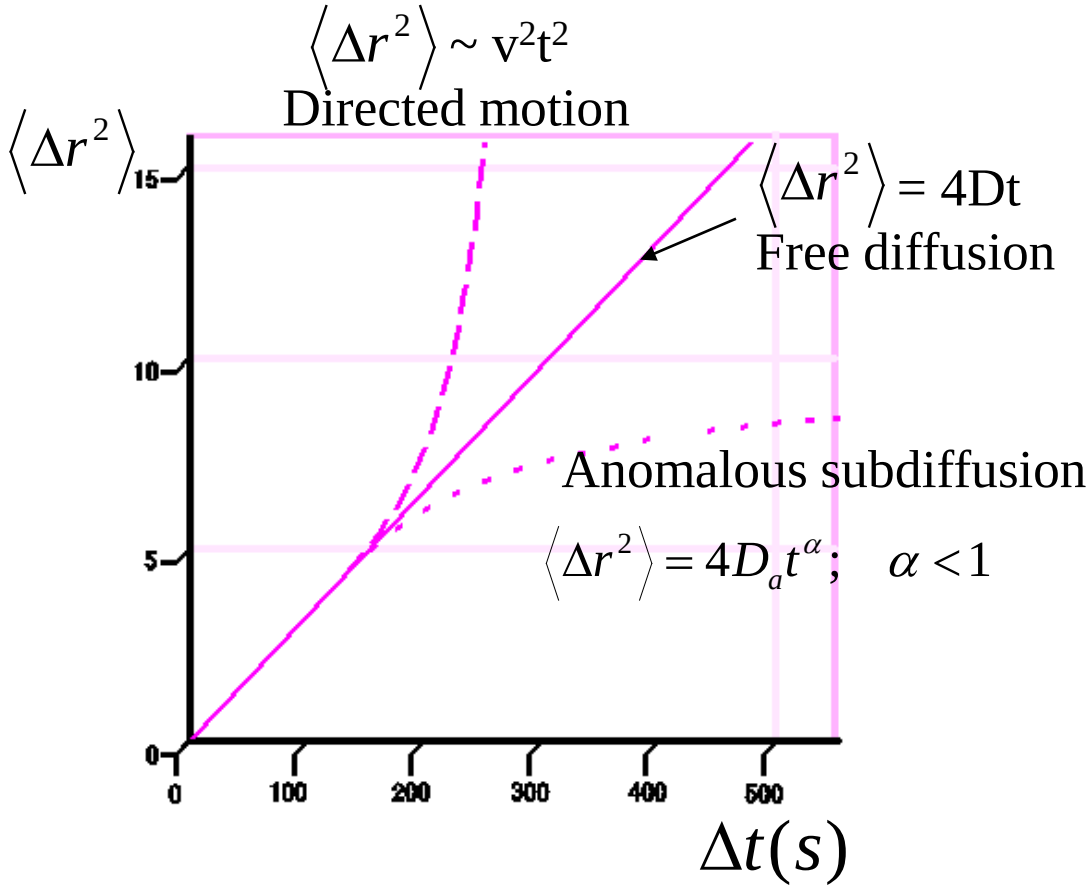
$$G(0) = \frac{1}{\langle N \rangle} = \frac{1}{V_{\text{eff}} \langle C \rangle}, \qquad V_{\text{eff}} = \pi^{3/2} \omega_{xy}^2 \omega_z.$$

Dependence of the ACF on the concentration



Transport mechanisms (in 2D)





- Directed motion: flow, active transport
- Anomalous subdiffusion (long-range diffusion gets slower than short-range diffusion): ”Molecular crowding”, transient binding, corralling.
- α: anomaly parameter

ACF of diffusion: model functions

Free diffusion in 3D

$$G(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}\cdot\left(1+\frac{\tau}{S^2\tau_D}\right)^{-\frac{1}{2}}$$

Free lateral diffusion in 2D (membrane)

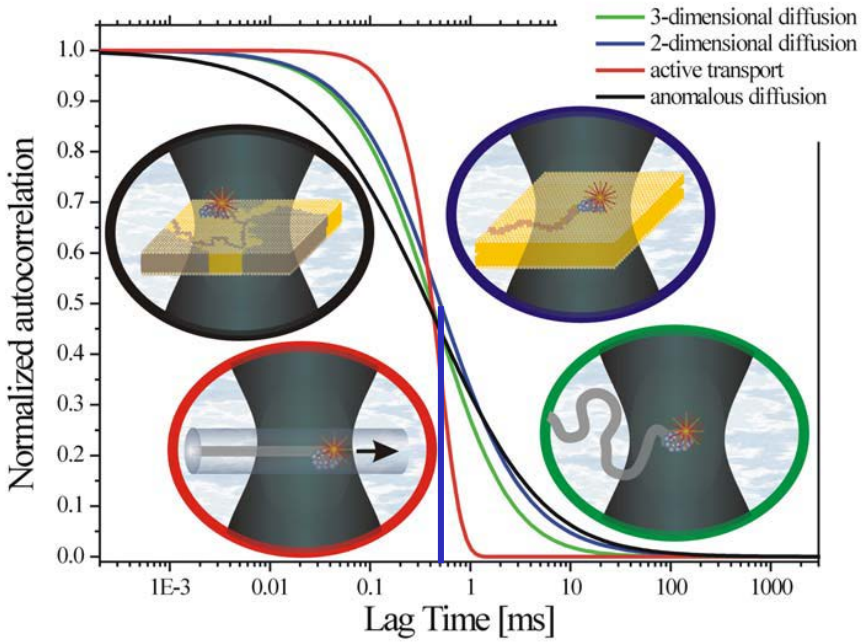
$$G(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_D}\right)^{-1}$$

Anomalous subdiffusion in 3D (α<1):

$$G(\tau) = \frac{1}{N}\left(1+\left(\frac{\tau}{\tau_D}\right)^{\alpha}\right)^{-1}\cdot\left(1+S^{-2}\left(\frac{\tau}{\tau_D}\right)^{\alpha}\right)^{-\frac{1}{2}}$$

Active transport with velocity v

$$G(\tau) = \frac{1}{N}\exp\left(-\left(\tau v/\omega_{xy}\right)^2\right)$$



P. Schwille, E. Hausstein
http://www.biophysics.org/portals/1/pdfs/education/schwille.pdf

The mechanism of transport affects the shape of the ACF
Parameters can be determined by nonlinear fitting

$D = \omega_{xy}^2/4\tau_D$ (single-photon exc.),

$D=\omega_{xy}^2/8\tau_D$ (2-photon exc.)

N: mean number of particles in the observation volume

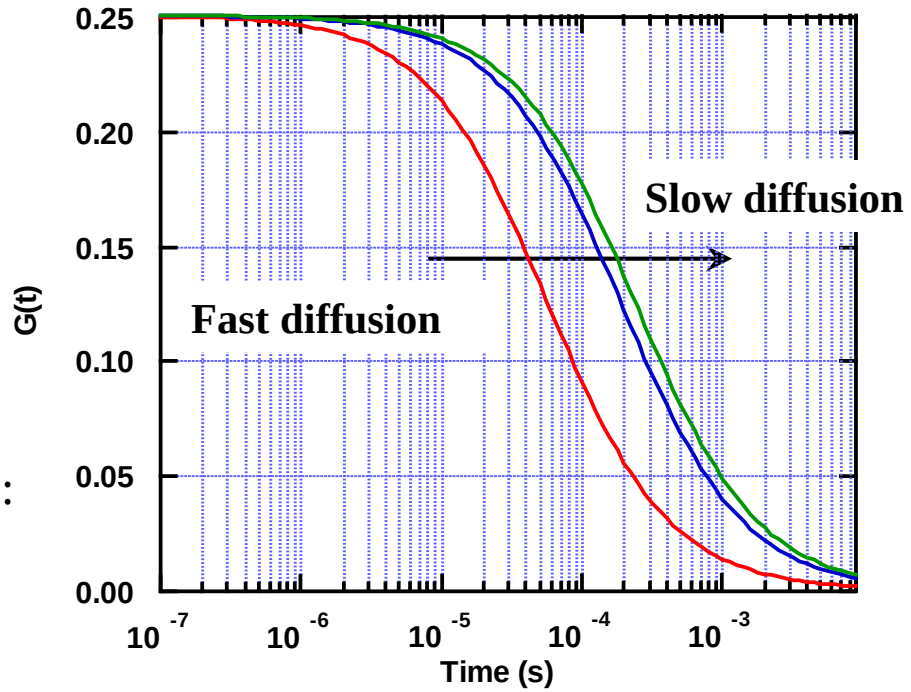
τ_D: diffusion time (mean dwell time in the observation volume)

$S = \omega_z/\omega_{xy}$: aspect ratio of observation volume

Dependence of the ACF on the molecular weight

Diffusion coefficient

300 µm²/s
90 µm²/s
71 µm²/s



Stokes-Einstein equation for a sphere:

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

radius

$$r \propto \sqrt[3]{V} \propto \sqrt[3]{M}$$

$$D \propto \frac{k \cdot T}{\eta} \sqrt[3]{\frac{1}{M}}$$

Monomer → Dimer:

D decreases by a factor of $2^{1/3} = 1.26$ only

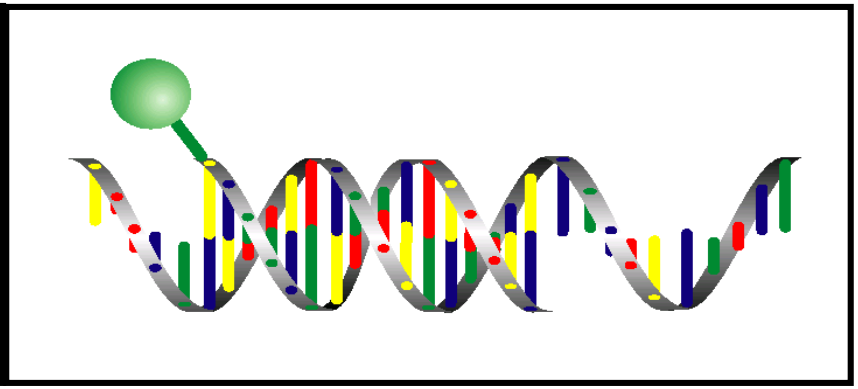
It can indicate binding of small molecule (ligand) to macromolecule

adapted from E. Gratton

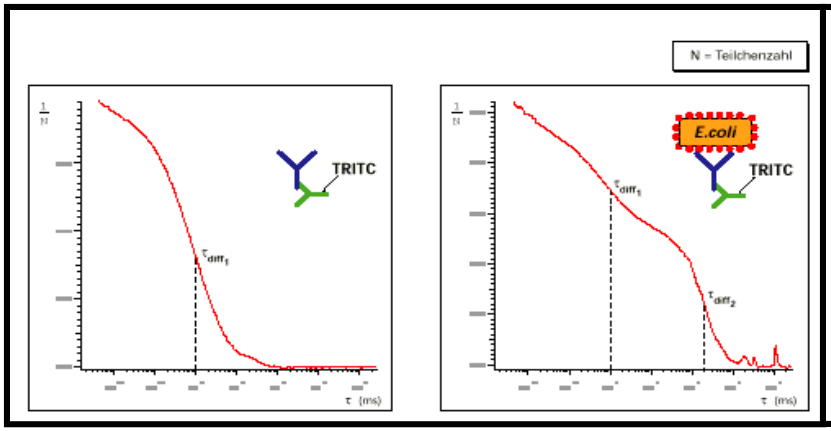
Examples of biomolecular interactions studied with FCS based on change in diffusion time

Protein - DNA

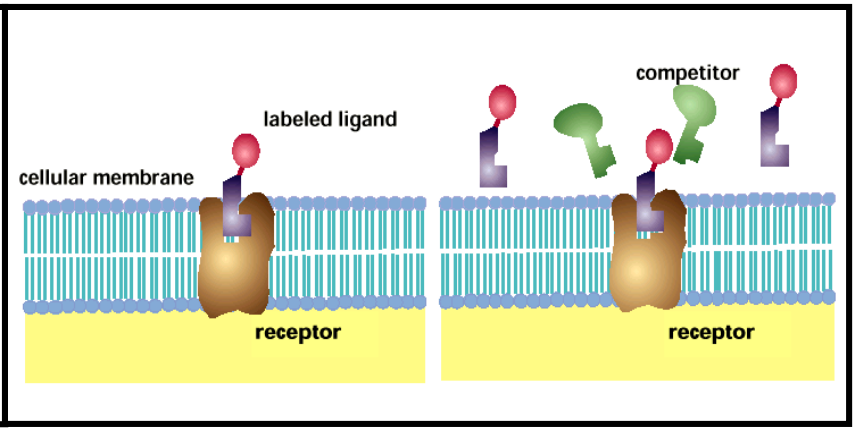
Nucleic acid hybridisation



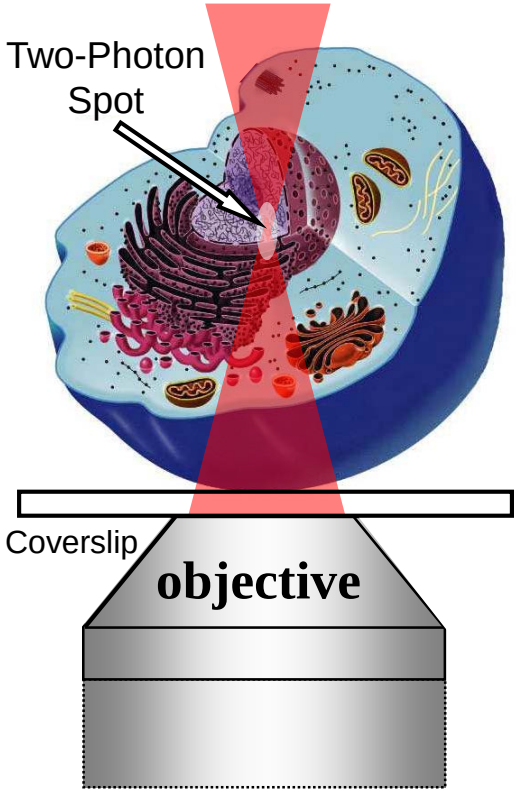
Antigen - Antibody

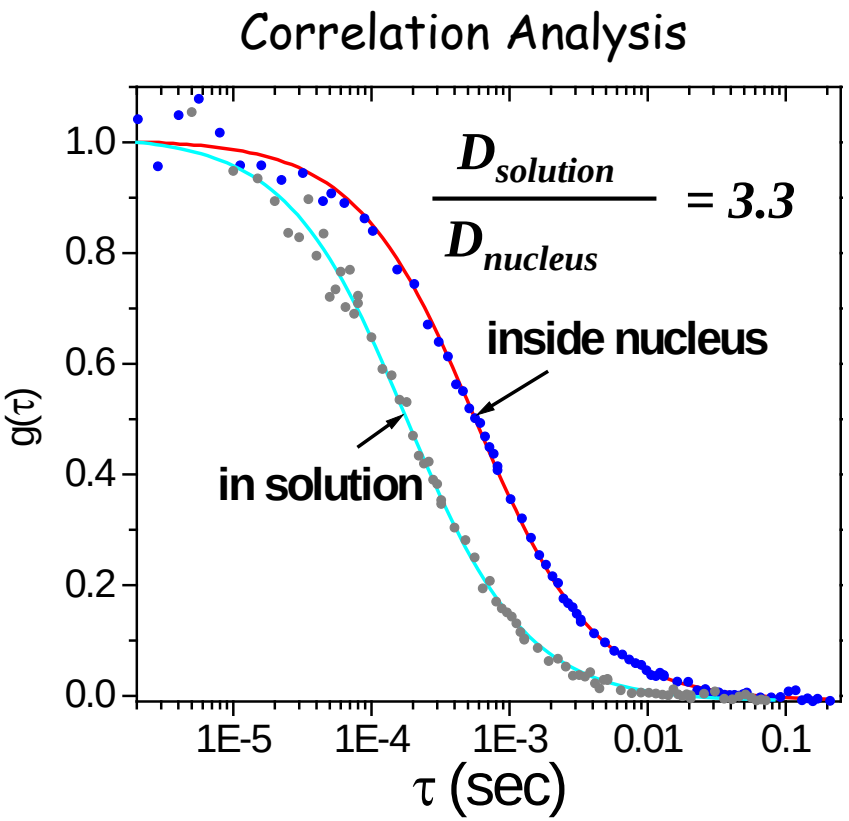


Receptor – Ligand



Dependence of the ACF on the viscosity: diffusion of GFP in the nucleus and vs. water





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

The nucleus is a more viscous ”solution” than water

adapted from E. Gratton

Multicomponent systems

The contribution of a component to the amplitude of the ACF is proportional to the product of the molecular fraction Y_i and the square of the specific brightness F_i

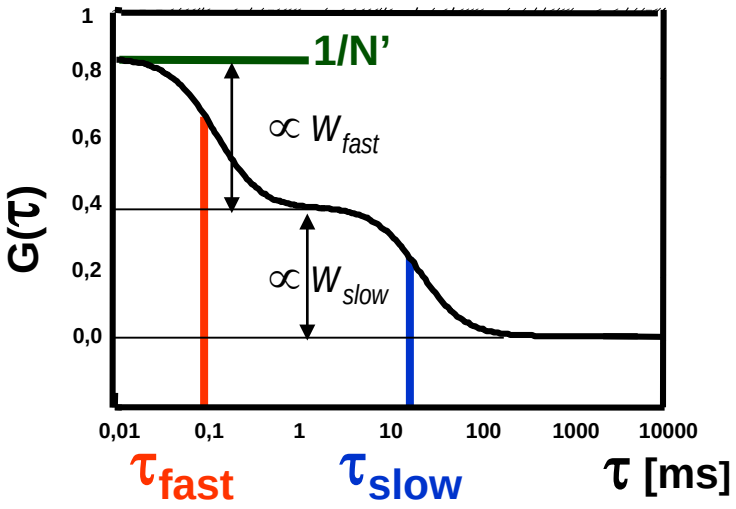
$$G(\tau) = \frac{1}{N\left(\sum_i Y_i F_i\right)^2} \cdot \sum_i Y_i F_i^2 \cdot \frac{1}{1+\frac{\tau}{\tau_i}} \cdot \frac{1}{\sqrt{1+\frac{\tau}{S^2 \cdot \tau_i}}} = \frac{1}{N'} \cdot \sum_i w_i \cdot \frac{1}{1+\frac{\tau}{\tau_i}} \cdot \frac{1}{\sqrt{1+\frac{\tau}{S^2 \cdot \tau_i}}}$$

Y_i - molecular fraction of the i^{th} component

F_i - brightness of the i^{th} component

$w_i = Y_i F_i^2 \Big/ \sum_i Y_i F_i^2$ - weight of the i^{th} component

$N' = N \cdot \sum_i Y_i F_i^2 \Big/ \left(\sum_i Y_i F_i\right)^2$ - apparent number of molecules

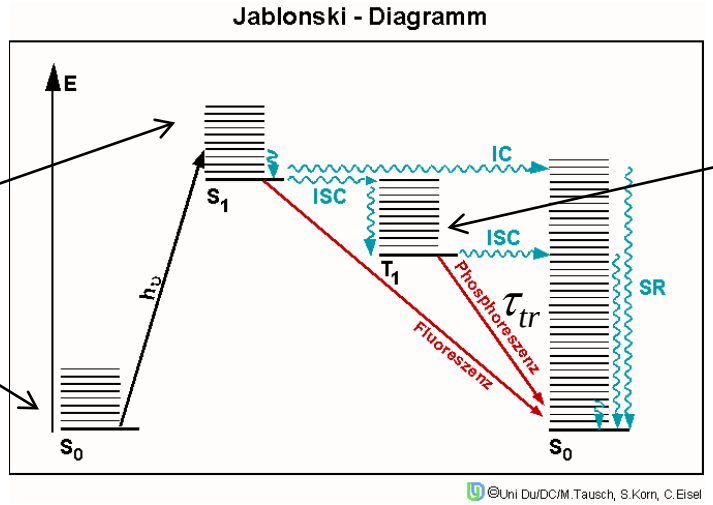


• **weight is proportional to the square of brightness** → dim species are ”underrepresented” in the ACF

• ***N’* = 1/*G*(0) < *N*** if F_i-s are different → **the total number of particles is underestimated**

• average ***F*/*N* is overestimated** if F_i-s are different → state of aggregation can only be assessed if we have a single species

Effect of photophysical processes on the ACF: singlet-triplet transition



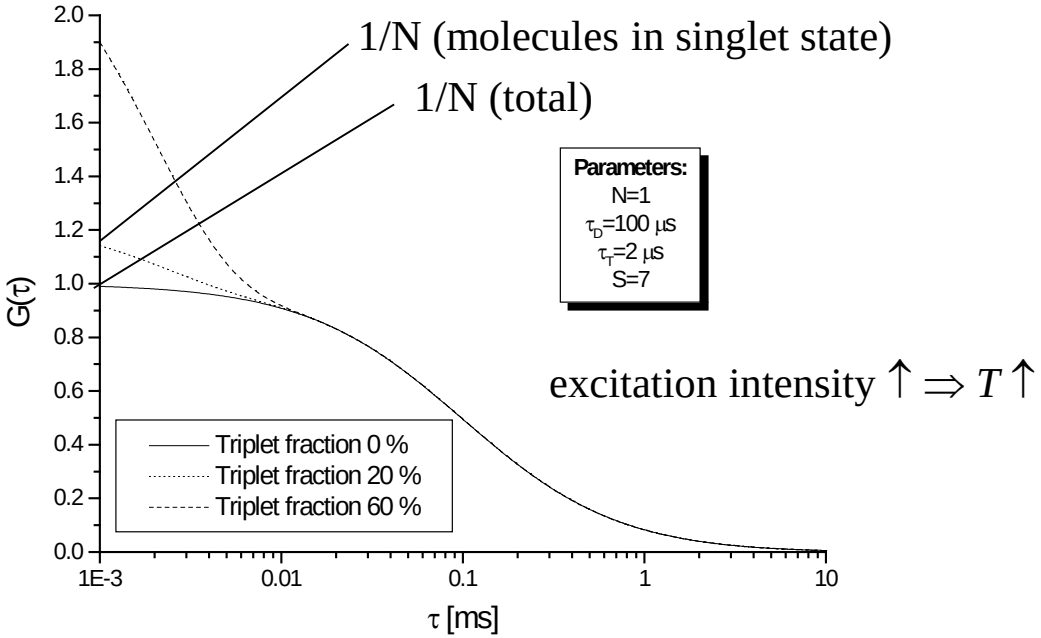
singlet states

Triplet state

$$G(\tau) = \frac{1}{N} G_{diff} \frac{1 - T + Te^{-\tau/\tau_{tr}}}{1 - T}$$

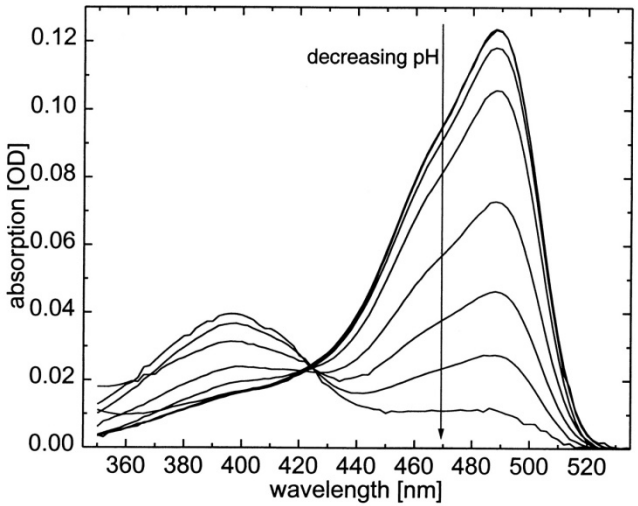
T: triplet fraction

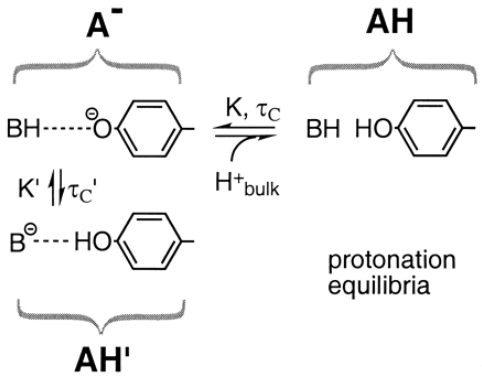
τ_{tr}: triplet lifetime



Effect of GFP blinking on the ACF

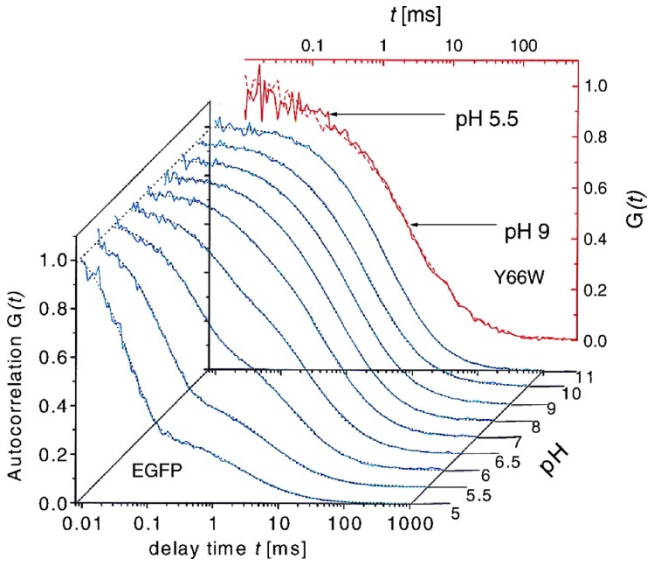
Absorption spectra of EGFP at varying pH (pH 10 … pH 5)





The protonated form of EGFP does not absorb or emit, it is dark

Normalized ACFs of EGFP at different pH values



Low pH: protonation dominates, high pH: diffusion dominates the ACF

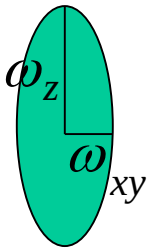
$$G(\tau) = \frac{1}{N} G_{diff}(\tau)\left(1 + Pe^{-\tau/\tau_C} + P'e^{-\tau/\tau_C'}\right)$$

P, P': (pH-dependent) amplitudes

τ_C, τ'_C: time constants of external (pH-dep.) and internal (pH-indep.) protonation

Haupts U et al. PNAS 1998;95:13573-13578

Calibration of the observation volume



• For calculating D from τ_D, $D = \omega_{xy}^2/4\tau_D$, knowledge of the lateral radius, ω_{xy}, is required

• Method 1 (from known D of standard): Measurement of the diffusion time of a dye with known diffusion coefficient:

$$\omega_{xy} = \sqrt{(4D\tau_D)}$$

Frequently used standards: Alexa dyes, Atto dyes, Rhodamine 6G

Usually this is the method of choice.

• Method 2. (from known concentration of standard)

- a): measurement of particle number N in a solution of known concentration:

$$N, c \rightarrow V$$

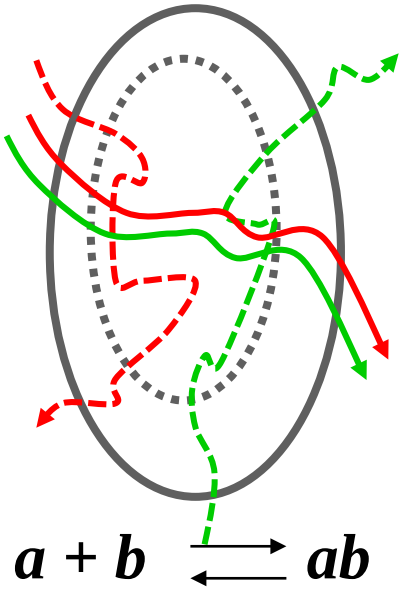
- b) measurement of the axial radius of the obervation volume (ω_z) by taking a z-scan from a monolayer
- c) calculation of ω_{xy} based on V and ω_z

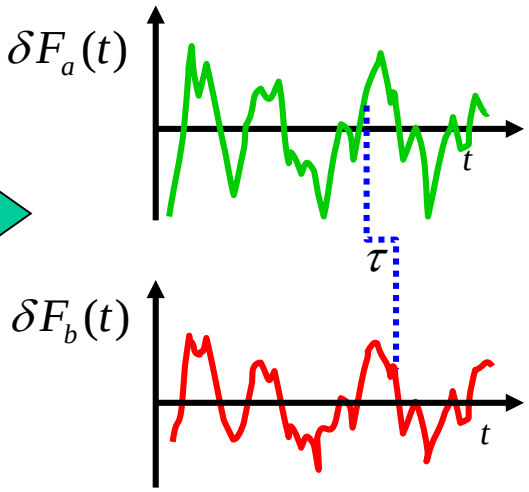
Fluorescence crosscorrelation spectroscopy (FCCS)

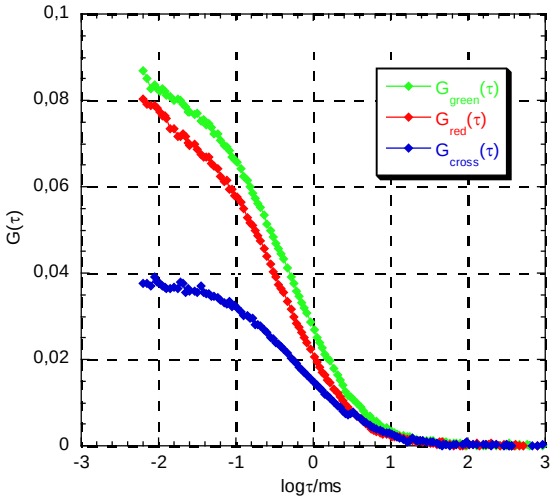
Co-mobility of associated molecules

Parallel fluctuations of green and red signals

Positive crosscorrelation amplitude







$$G_{ab}(\tau) = \frac{\langle \delta F_a(t) \cdot \delta F_b(t+\tau) \rangle}{\langle F_a \rangle \langle F_b \rangle}$$

$$G_{ab}(0) \propto \frac{c_{ab}}{V_{eff}\left(c_{a,tot}\right)\left(c_{b,tot}\right)}$$

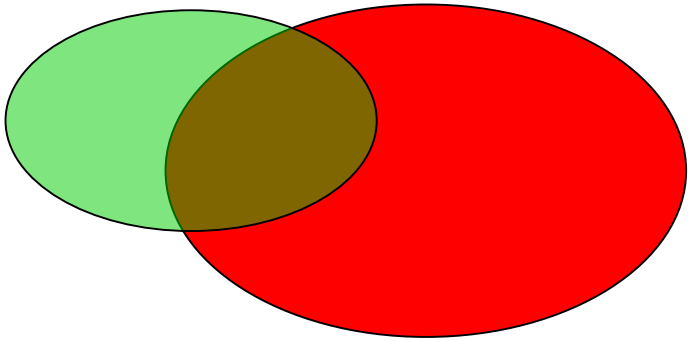
FCCS can indicate the stable association of molecules (in the τ_D time window)

Ries J et al, New Journal of Physics 12 (2010) 113009

Experimental Concerns

Observation volumes don't overlap perfectly because of

Laser alignment
Chromatic aberration
Spherical aberrations



These result in
- lengthening of the diffusion time of the CCF
- decrease of the crosscorrelation amplitude

Baseline of CCF is usually not zero because of spectral overspill between the detection channels (e.g. GFP signal correlates with itself in the green and red channels).

Define dynamic range:
measurement of a positive control (100% doubly labeled molecule) and a negative control (mixture of independently moving dyes) is measured

Simplified formula of the cross-correlation function

Definition $G^{\times}(\tau) = \dfrac{\langle \delta F_a(t) \cdot \delta F_b(t+\tau) \rangle}{\langle F_a \rangle \langle F_b \rangle}$

Intensity fluctuations of dyes *a* and *b* at time t and t+τ

τ: „lag time"

Mean intensity of *a* and *b*

CCF of diffusion $G^{\times}(\tau) = \underbrace{\dfrac{c_{ab}}{V_{eff}\left(c_{a,tot}\right)\left(c_{b,tot}\right)}}_{\text{Amplitude}} \cdot \underbrace{\dfrac{1}{\gamma_{xy}\sqrt{\gamma_z}}}_{\text{Focus geometry}} \cdot \underbrace{\dfrac{1}{1+\frac{\tau}{\gamma_{xy}\cdot\tau_{ab}}} \cdot \dfrac{1}{\sqrt{1+\frac{\tau}{\gamma_z \cdot S_a^2 \cdot \tau_{ab}}}}}_{\text{diffusion}}$

Diffusion coefficient ← $D \propto \dfrac{\omega_{xy}^2}{4\tau_{ab}}$

ω_{xy}^2 → Lateral radius of observation volume

$4\tau_{ab}$ → Diffusion time of the *ab* complex

Relation of the amplitudes of the CCF and the ACFs $G^{\times}(0) \leq G_a(0),\ G_b(0)$

Usually the same model functions are used as for ACFs, but without triplet term.

T. Weidemann, PhD thesis Heidelberg

Bacia K, Nat Protoc. 2007;2:2842-56.

Some variants of FCS

• FCS with 2-photon excitation: less photobleaching, no pinhole needed, better overlap for FCCS

• 2-focus FCS: measures crosscorrelation between signals from two separate foci. ACF does not depend on the exact shape of the observation volume, more precise measurement of D

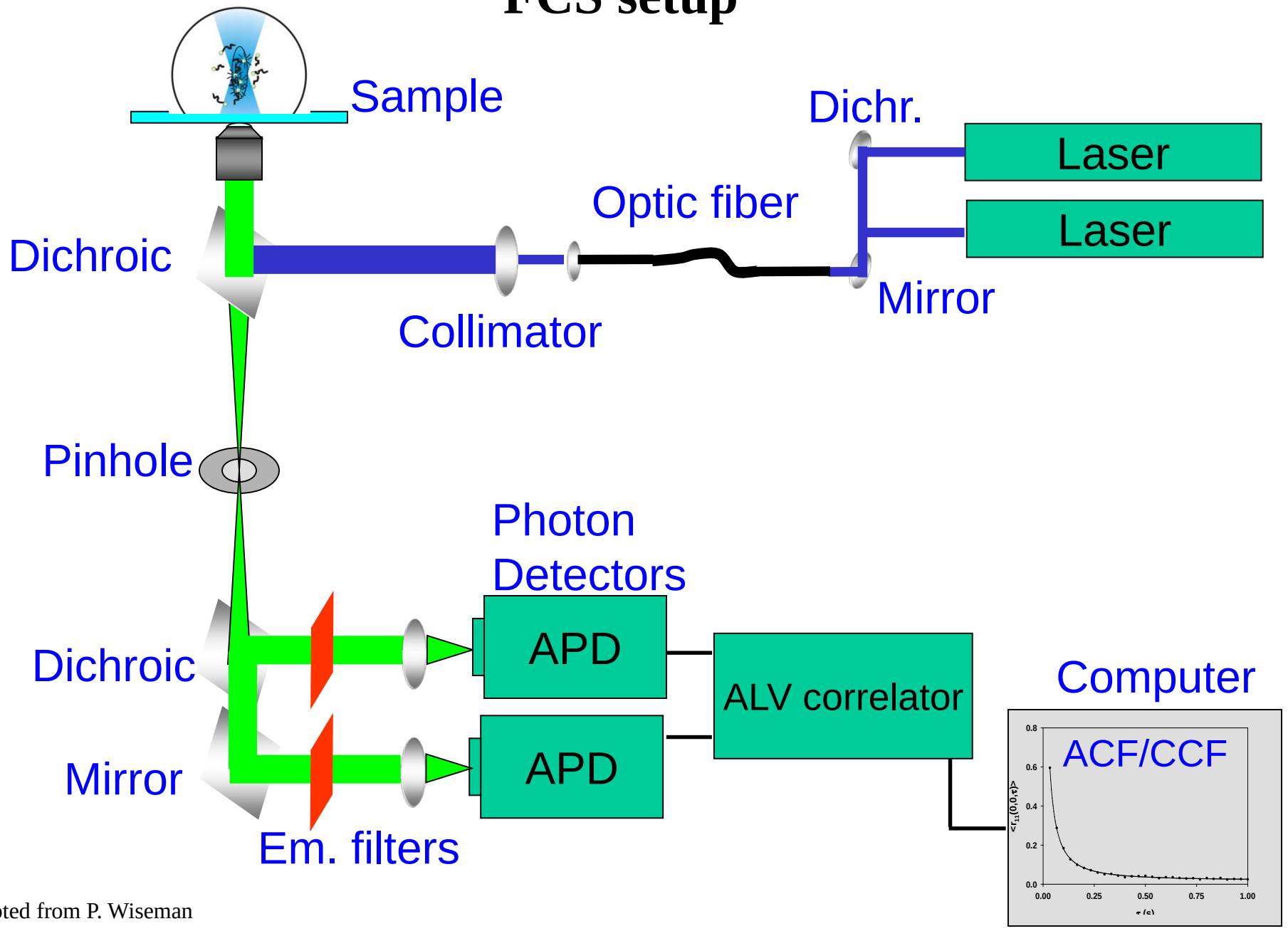
• Scanning FCS: simultaneous measurement at multiple foci, less photobleaching, spatial correlation, detection of vector of directed motion

• Image correlation spectroscopy (ICS): FCS measured in a whole image using CCD camera or APD array

• SPIM-FCS: ICS using selected plane illumination

• Photon counting histogram (PCH): analysis of photon count distribution per unit time, measurement of molecular brightness of multiple species. Yields aggregation state, not based on measurement of diffusion

FCS setup



Adapted from P. Wiseman

FCS instruments at our institute

Olympus FluoView 1000 confocal microscope with 2-channel FCS/FCCS ALV 5000E hardware correlator (prototype, designed by J. Langowski)

Zeiss LSM 510 – Confocor 2 confocal with 2-channel FCS/FCCS

What is required for an FCS measurement

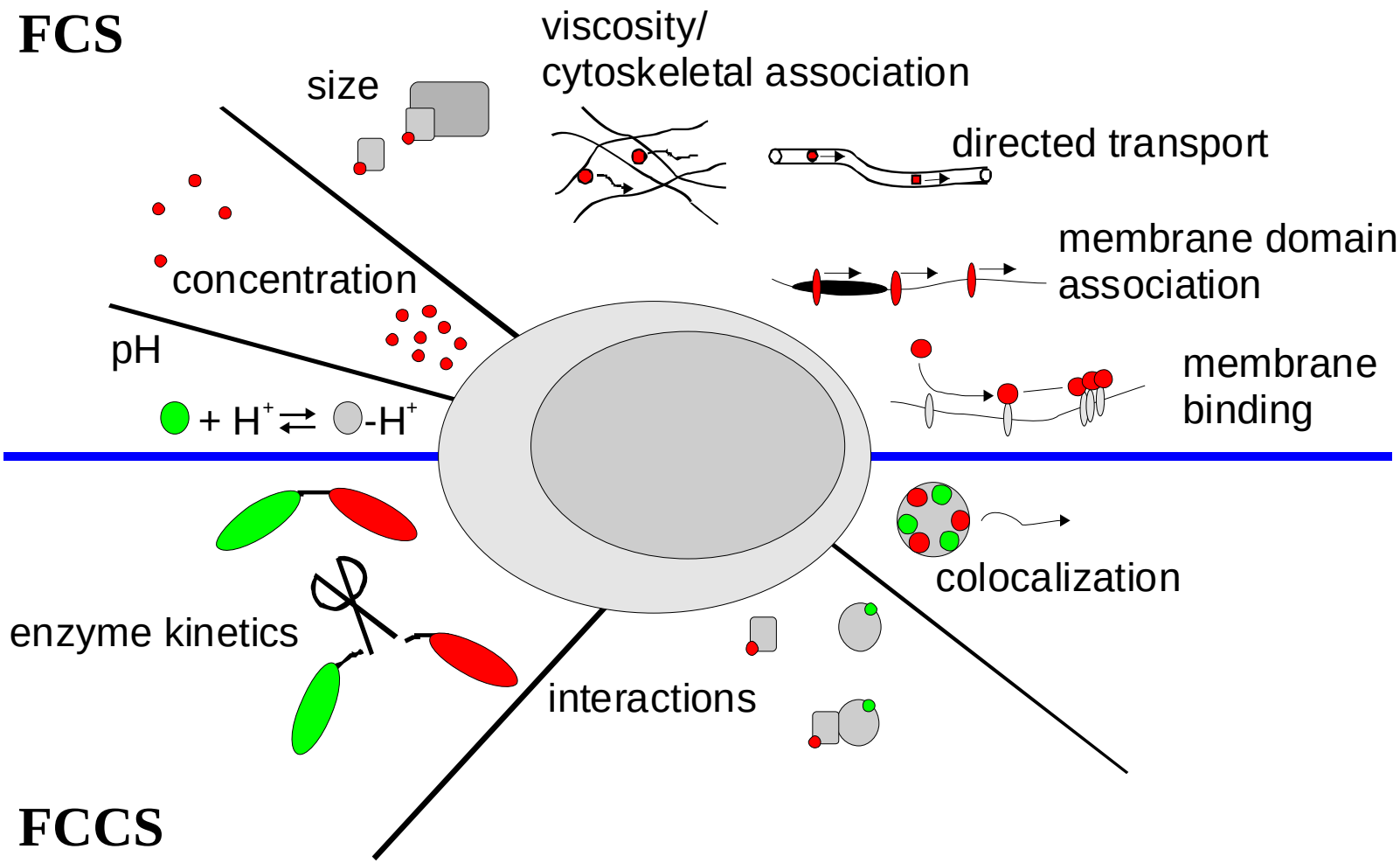
FCS (mobility)

- **Specific fluorescent labeling:**
  - **Fab fragment (monovalent but weaker), mAb (bivalent but stronger)**
  - **ligand**
  - **FP-fusion protein (GFP, YFP, mRFP, mCherry…)**
  - **The dye should be highly photostable**
- **Appropriate concentration of the labeled molecules (ca. 10^{-11} – 10^{-7} M, for membrane proteins 10^{3}-10^{6} molecules / cell)**
- **Not crucial to label all molecules**
- **The labeled membrane protein should not internalize instantly (fixation is not possible)**

FCCS (co-mobility)

- **Labeling with two spectrally distinct dyes**
- **Fraction of unlabeled molecules should be as small as possible (<50%)**

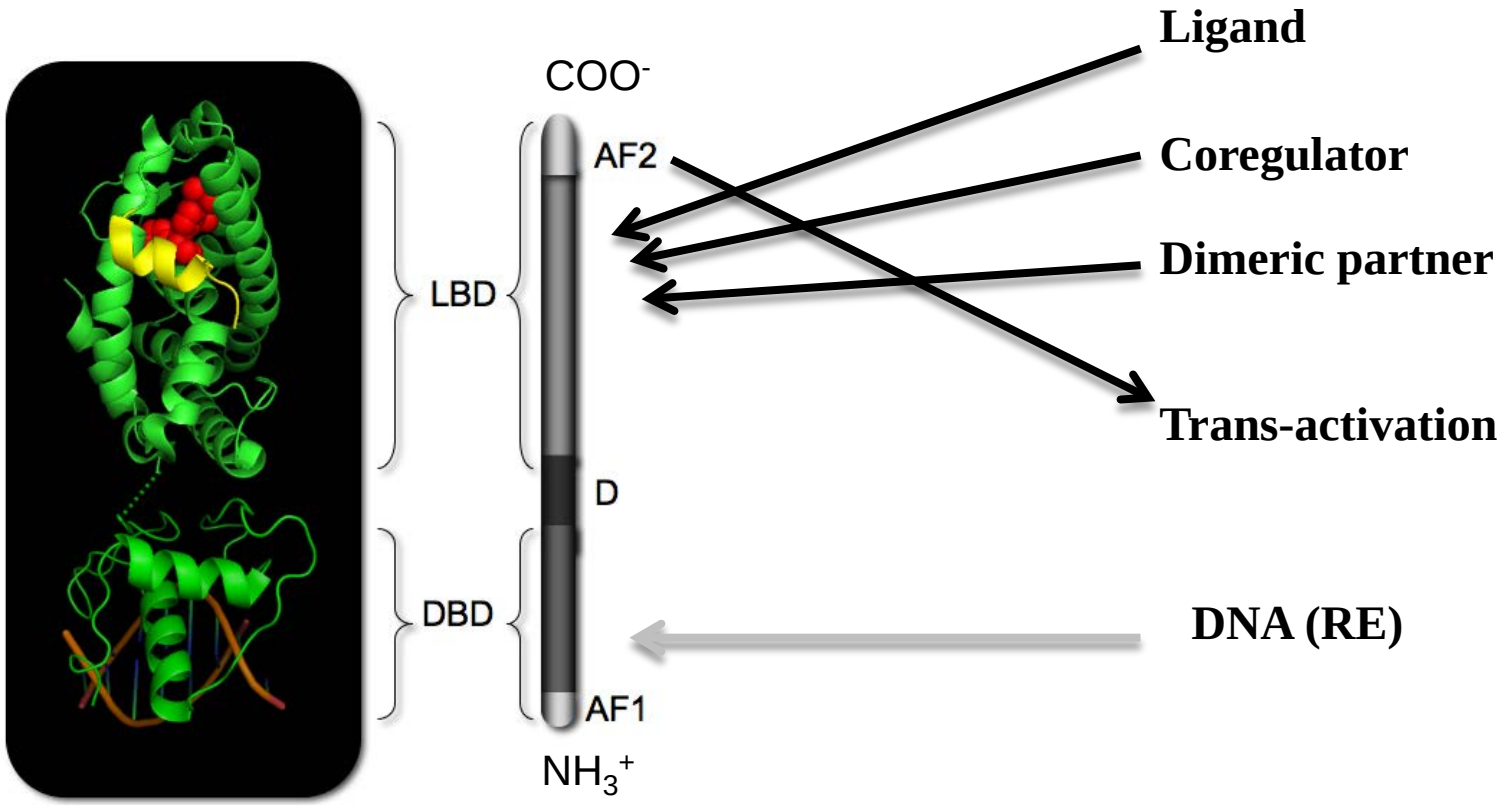
Intracellular F(C)CS - applications



Adapted from Bacia et al., Nature Methods, 3 (2006) 83-89

Following nuclear receptor (NR) activation by FCS

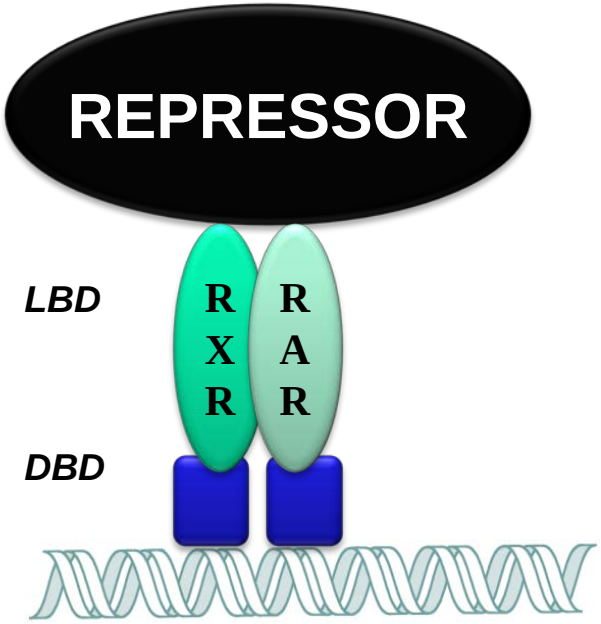
(P. Brázda et al. J Cell Sci 124:3631)



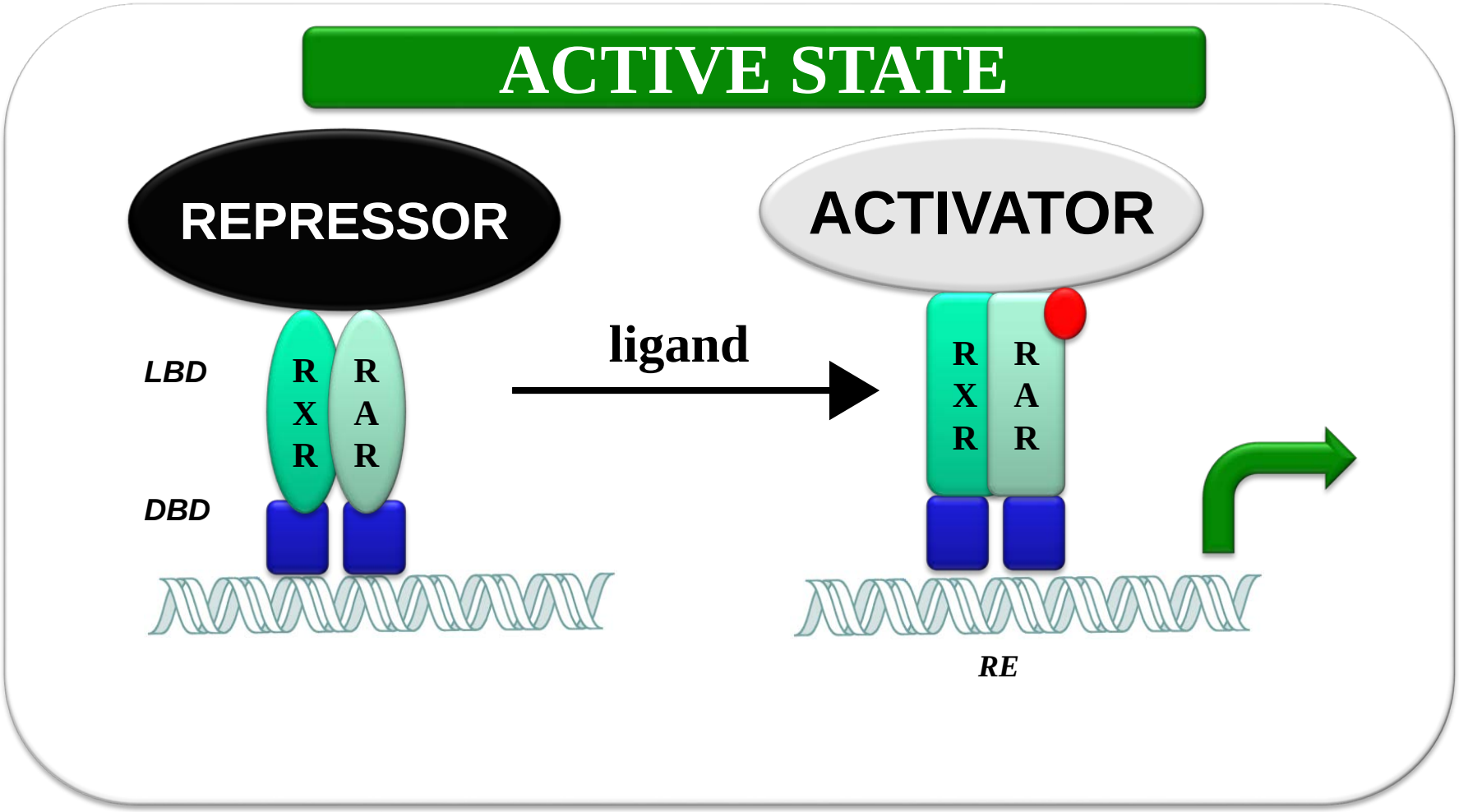
Ligand dependent transcription factors
Have lipophilic ligands (e.g. 9-cis retinoic acid, all-trans retinoic acid)
Retinoic acid receptor (RAR) and retinoid X receptor (RXR) act as a heterodimer

NRs in action I.
the *molecular switch model*

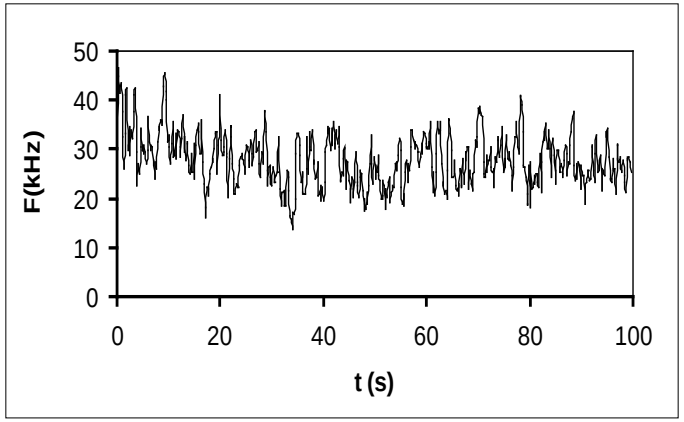
REPRESSED STATE



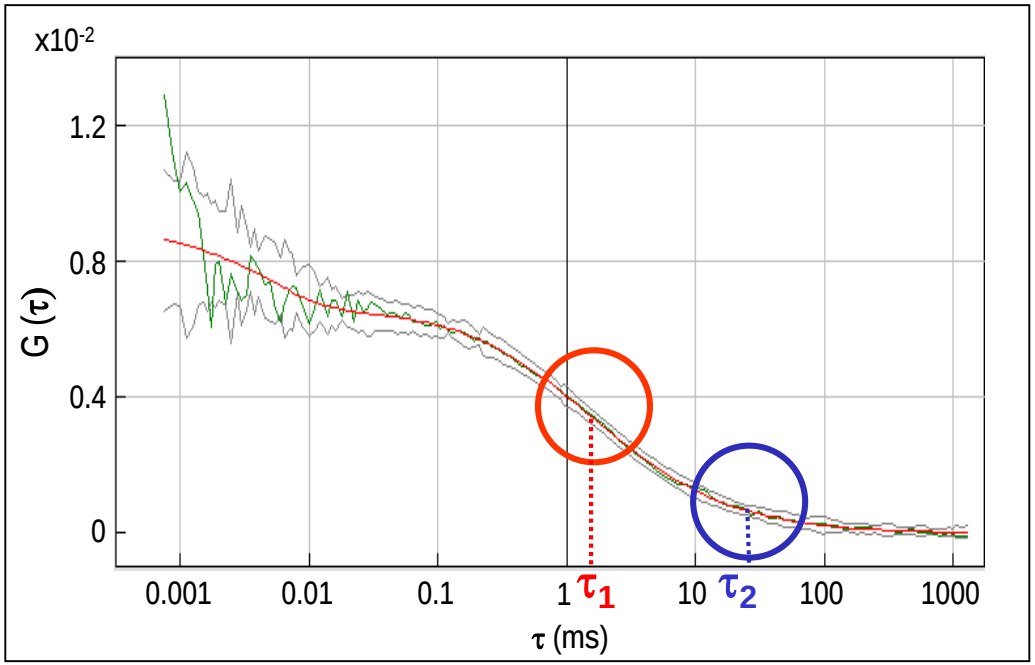
NRs in action I.
the *molecular switch model*



Processes affecting the ACF



Fluorescence intensity fluctuations



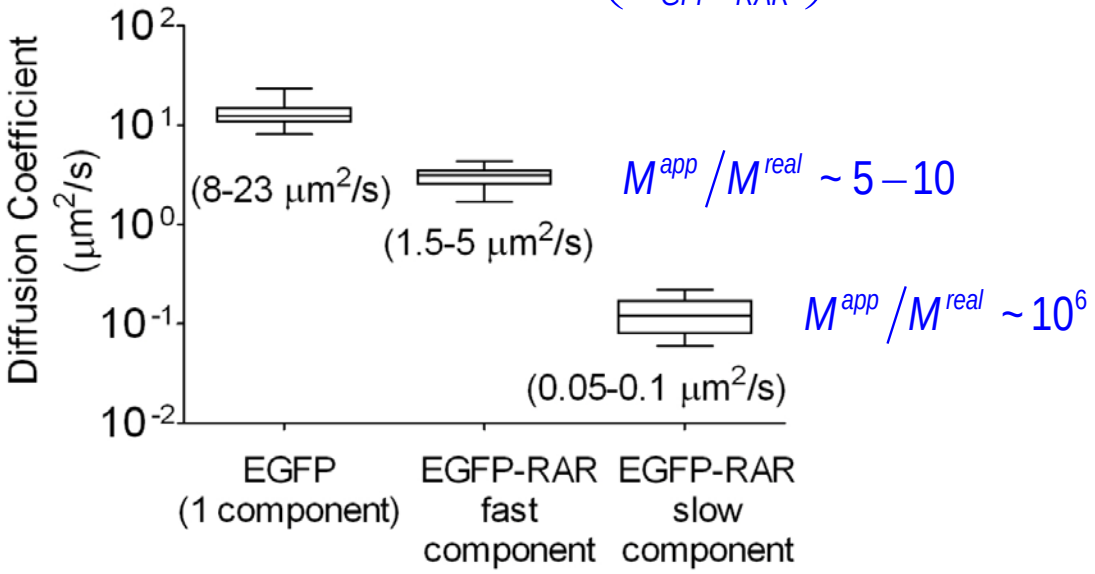
Hela cell expressing GFP-RAR

- Singlet-triplet transition
- GFP blinking (dark state formation)
- Fast diffusion component
- Slow diffusion component

$$G(\tau)=\frac{1}{N}\times\frac{\left(1-T+Te^{-\tau/\tau_{tr}}-\Theta_c+\Theta_c e^{-\tau/\tau_c}\right)}{1-T-\Theta_c}\times\left[r_1\left(1+\frac{\tau}{\tau_{d,1}}\right)^{-1}\left(1+\frac{\tau}{S^2\tau_{d,1}}\right)^{-1/2}+r_2\left(1+\frac{\tau}{\tau_{d,2}}\right)^{-1}\left(1+\frac{\tau}{S^2\tau_{d,2}}\right)^{-1/2}\right]$$

Diffusion coefficients and apparent molecular masses

$$M^{app}_{GFP-RAR} = M_{GFP} \times \left(\frac{D_{GFP}}{D_{GFP-RAR}} \right)^3$$



$M^{app} / M^{real} \sim 5 - 10$

$M^{app} / M^{real} \sim 10^6$

- Fast fraction: 30%
- Receptor in a small complex

- Slow fraction: 70%
- Cannot be diffusion of a free receptor
  - Diffusion of chromatin-bound receptor
  - Time of transient binding to chromatin

(P. Brázda et al. J Cell Sci 124:3631)

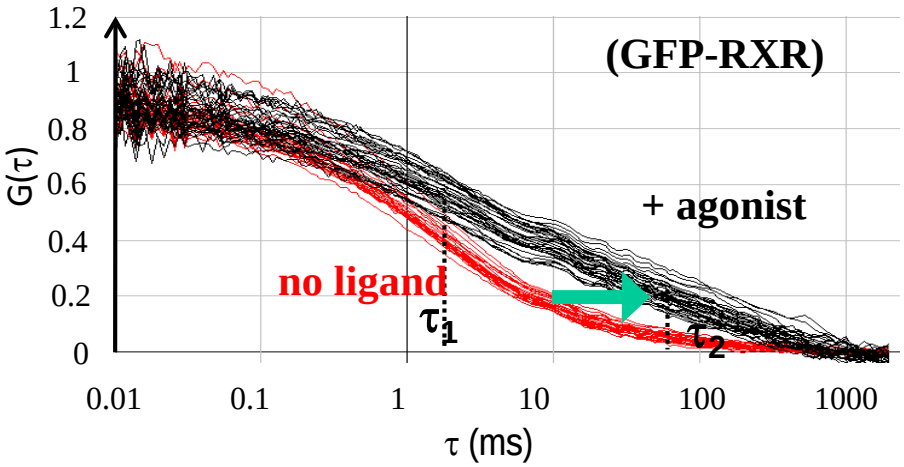
Ligand binding increases the amount of the slow component of GFP-RAR

Prior to activation

τ_1 = 2.6 ms (70%)

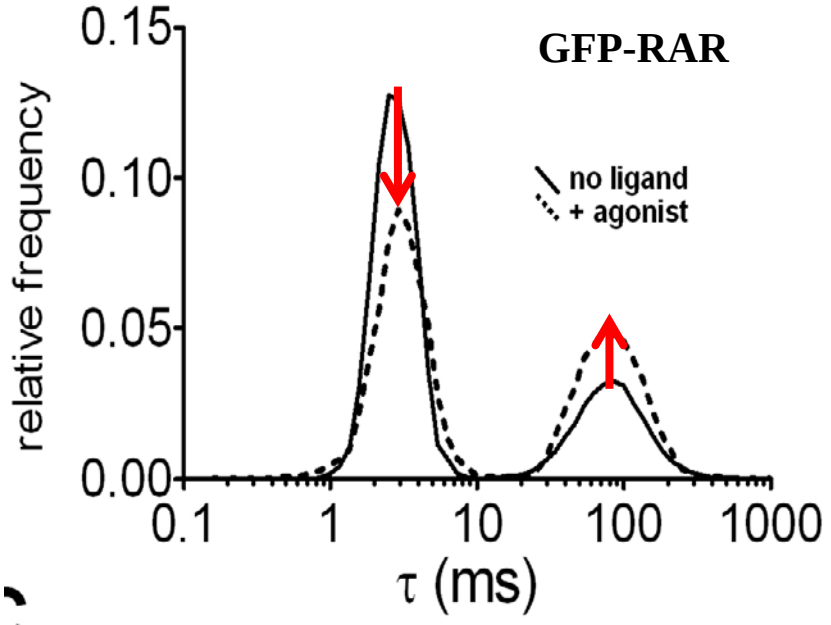
τ_2 = 96 ms (30%)

• Small ‘slow’ population (30%)



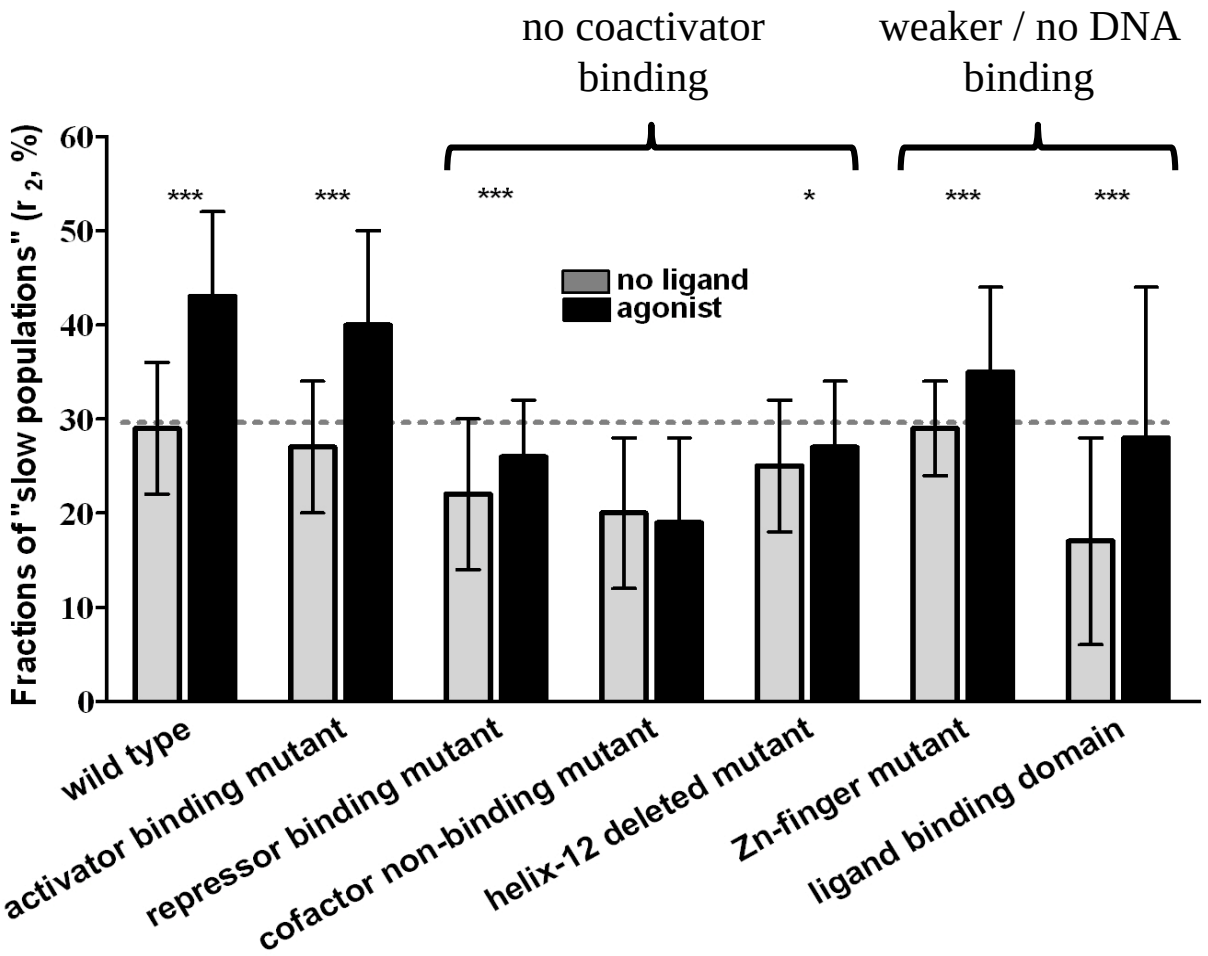
After activation

• No change in diffusion times
• Increased ratio of ‘slower’ population (+15%)



(P. Brázda et al. J Cell Sci 124:3631)

The binding of coactivator is needed for the transition to the slow state

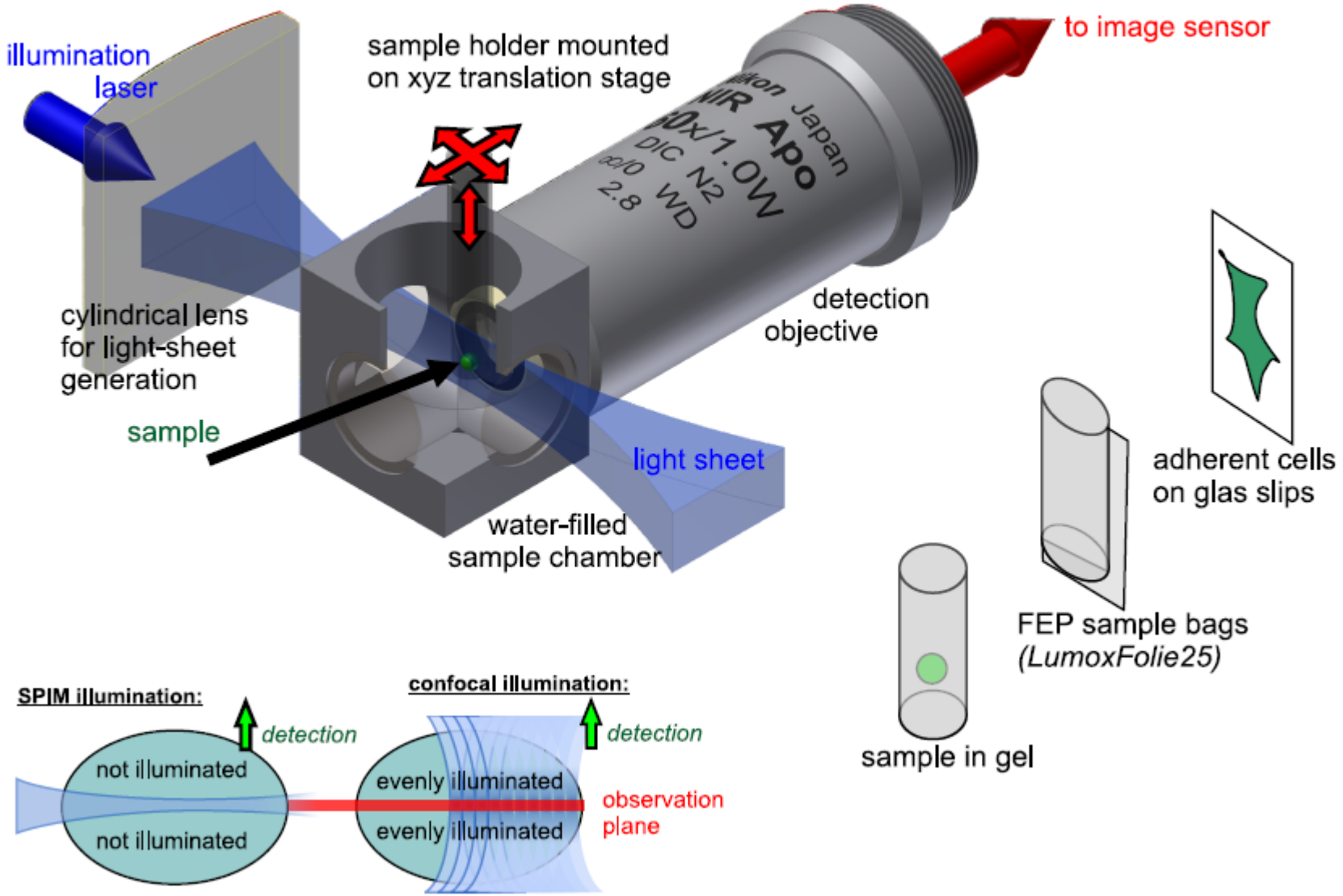


(P. Brázda et al. J Cell Sci 124:3631)

- In contrast with the original assumption of the molecular switch model, in non-activated state the **majority of receptors diffuses freely**. Receptor activation induces the **fast increase of the slow population**, for RAR by ~10%.

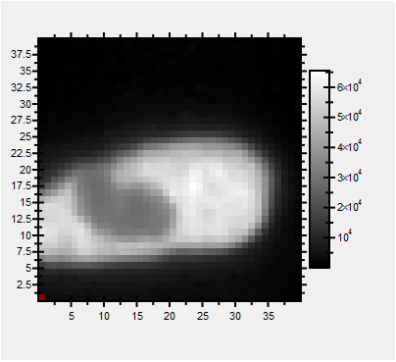
- The diffusion of the fast component corresponds to MW of a receptor oligomer or a small complex. The slow component is probably related to **coregulator binding** and stable or transient **binding to DNA**.

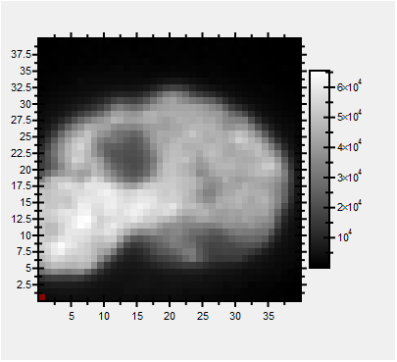
Single Plane Illumination for FCS



J. Buchholz, J. Krieger et al. Optics Express, 20:17767 (2012)

First results on mapping GFP-RXR diffusion by imaging SPIM-FCS

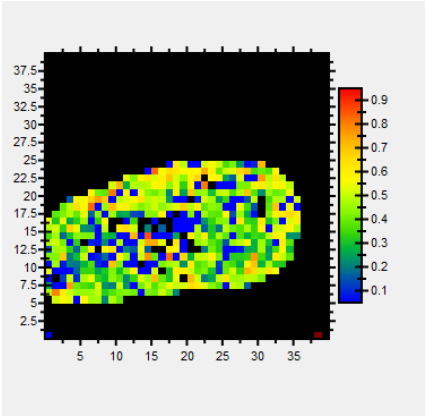


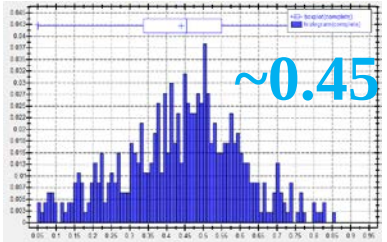


P. Brázda (U. Debrecen)
J. Krieger (DKFZ, Heidelberg)

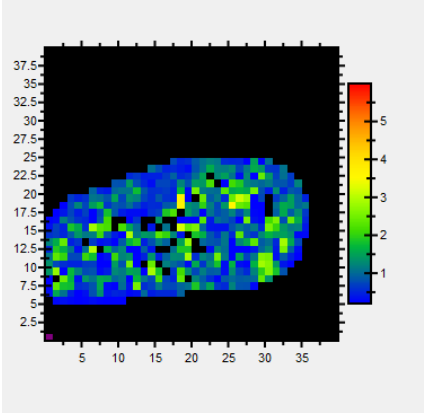
No ligand

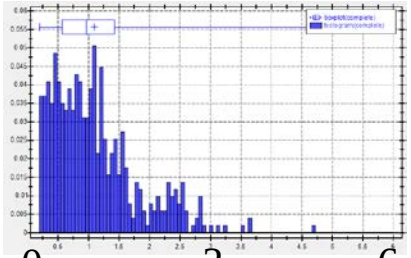
r_2 (slow fraction)





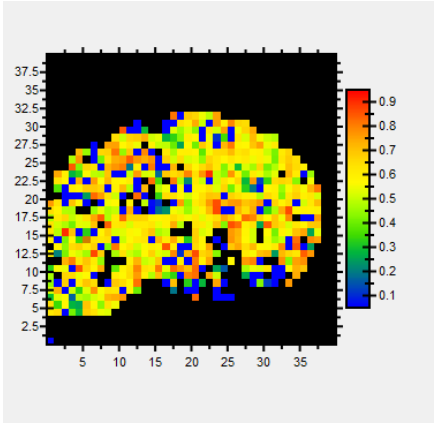
D_2 ($\mu m^2/s$)

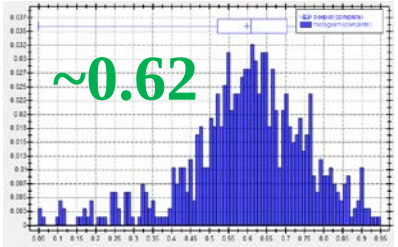




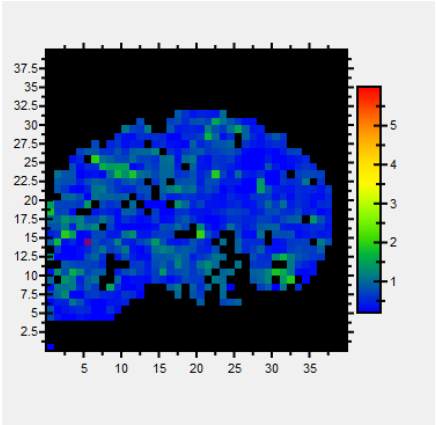
+ Agonist (LG 268)

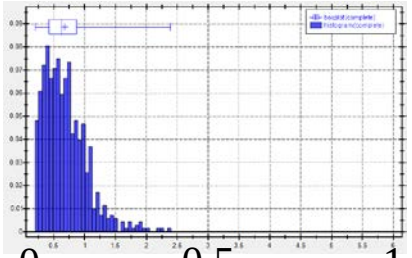
r_2 (slow fraction)





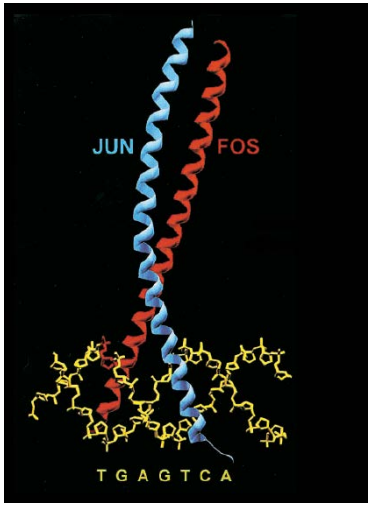
D_2

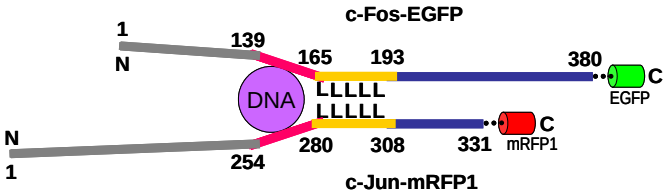


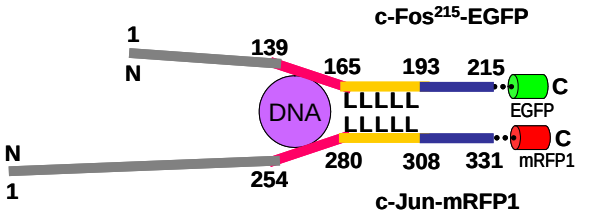


Interactions and function of transcription factors I.
the Fos-Jun complex

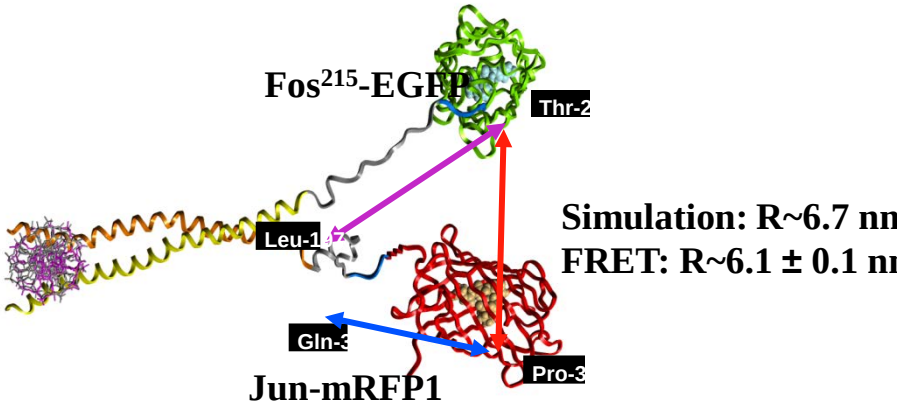
- AP-1 (Fos-Jun complex)
  - Regulates proliferation, differentiation, apoptosis and oncogenesis.
  - Part of the IL-2 signaling pathway
  - Basic leucine zipper TF
  - Function as dimers
    - Fos-Jun heterodimer
    - Jun homodimer
    - Fos homodimer is instable in vitro
  - cFos overexp. correlates with bad tumor prognosis (breast, cervix, osteosarcoma)
  - Earlier we characterized the conformation of the Fos-Jun complex







- Question:
  - Are there stable Fos homodimers in live cells?
- Collaboration
  - Tóth Katalin, Jörg Langowski (DKFZ, Heidelberg)
  - István Komáromi, MD simulation (UD Clinical Research Center

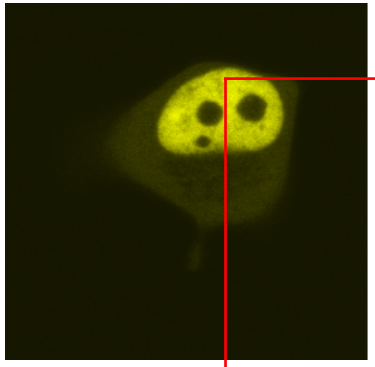


Aims

- **Does c-Fos form stable homodimers in live cells?**
- **How can we measure the dissociation constant of from FRET data in live cells? What is the K_d of Fos-Fos and Fos-Jun dimers?**
- **Does the Fos homodimer and the Fos-Jun heterodimer bind to the chromatin?**

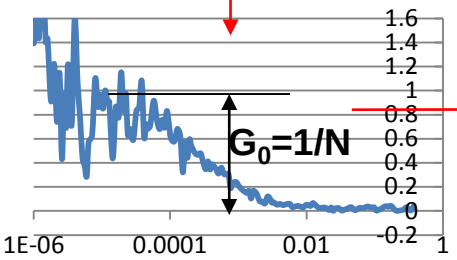
Calibration of fluorescence intensity as a measure of concentration is required for FRET titrations

Confocal microscopy:
Fluorescence intensity

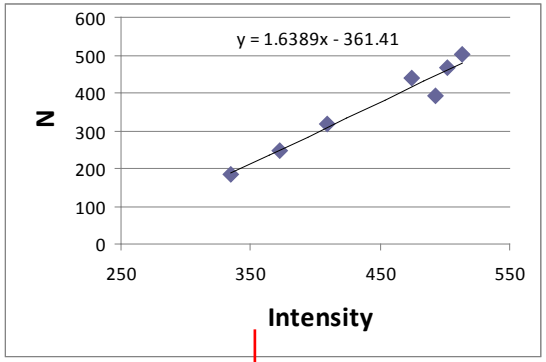


Fluorescence intensity

FCS:



Number of particles N in the detection volume



N (F)

Calibration of FCS detection volume
with dye of known concentration
(or known diffusion coefficient)

$$N = N_A c \pi^{3/2} \omega_{xy}^2 \omega_z$$

c (F):
Concentration as a function of fluorescence intensity

Weidemann T, et al. J Mol Biol. 2003, 334:229-40.

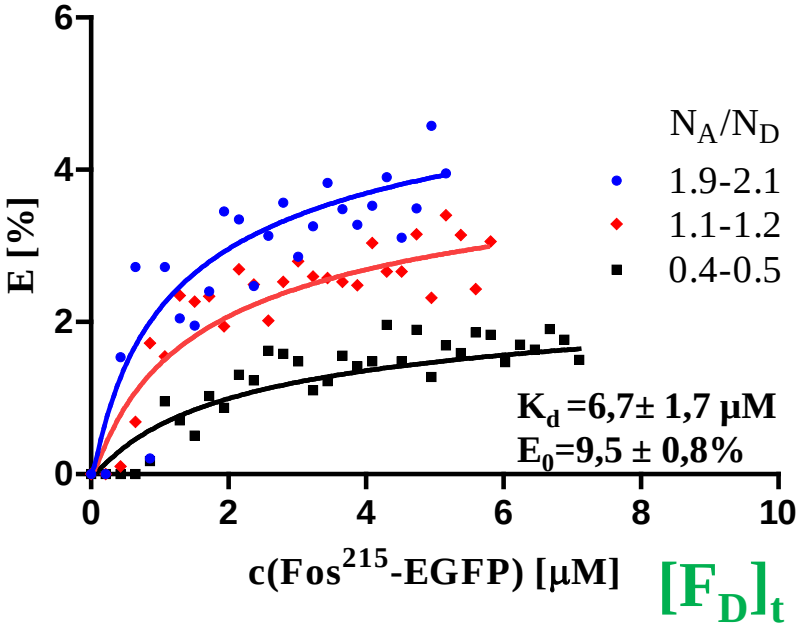
Determining the dissociation constant of c-Fos homodimers in live cells from FRET data

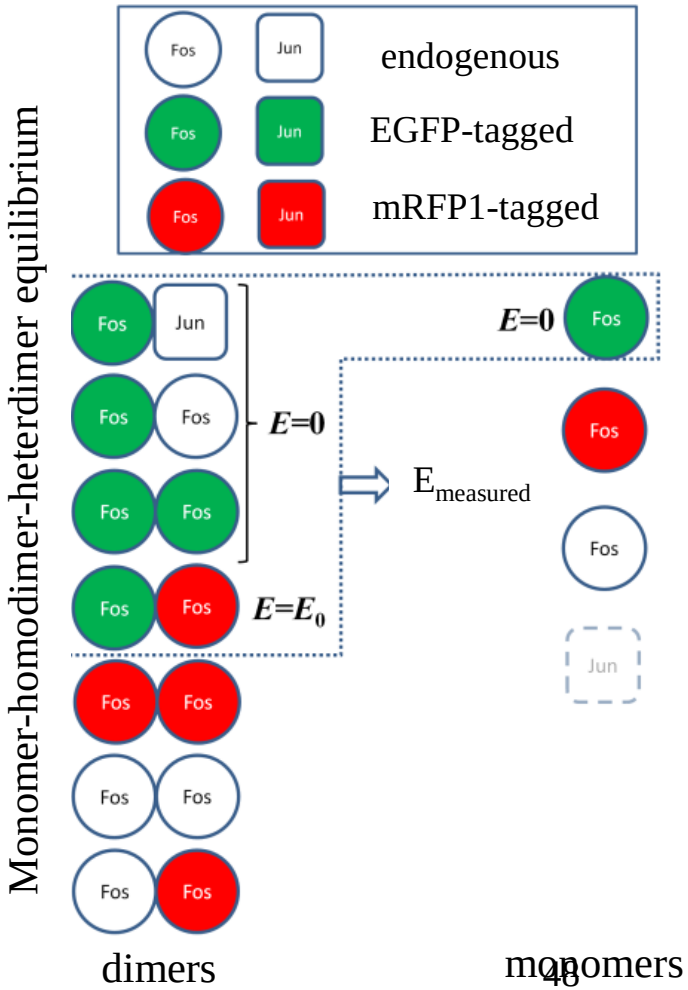
$$[F][F]/[FF] = K_d^{FF},$$
$$[J]_t = [FJ], \quad [F]_t = [F] + [FJ] + 2[FF]$$

$$[FF] = \frac{1}{8}\left(4[F]_t - 4[J]_t + K_d^{FF} - \sqrt{8K_d^{FF}[F]_t - 8K_d^{FF}[J]_t + \left(K_d^{FF}\right)^2}\right)$$

$$E_{measured} = \frac{1}{4}\left(4[F]_t - 4[J_e]_t + K_d^{FF} - \sqrt{K_d^{FF}}\sqrt{8[F]_t - 8[J_e]_t + K_d^{FF}}\right)\frac{[F_D]_t \frac{N_A}{N_D} E_0}{[F]_t^2}$$

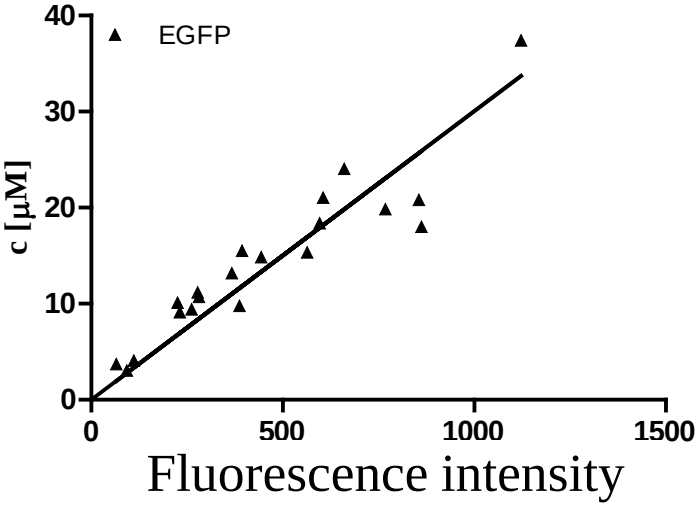
$$[F]_t = [F_D]_t + [F_A]_t + [F_e]_t = [F_D]_t\left(1 + \frac{N_A}{N_D}\right) + [F_e]_t$$

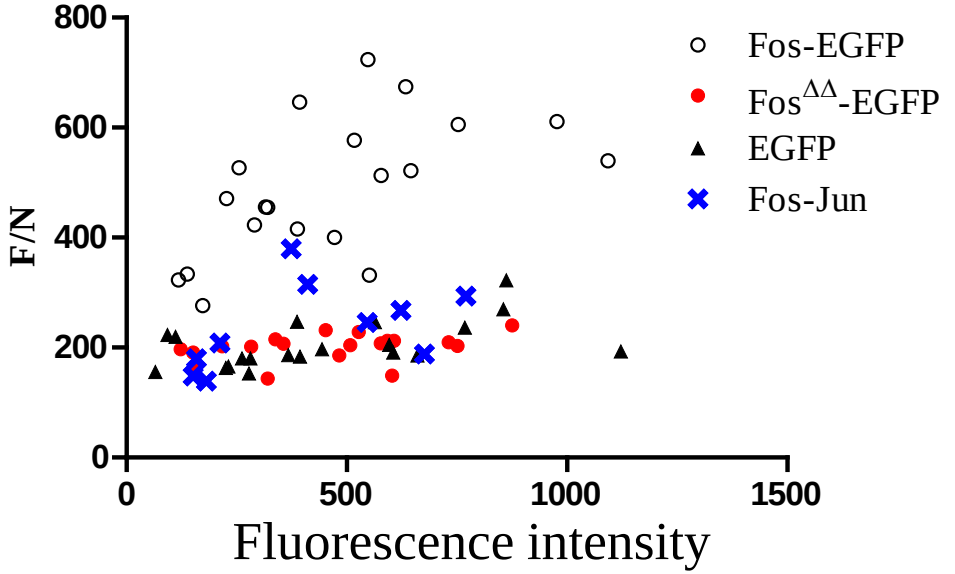


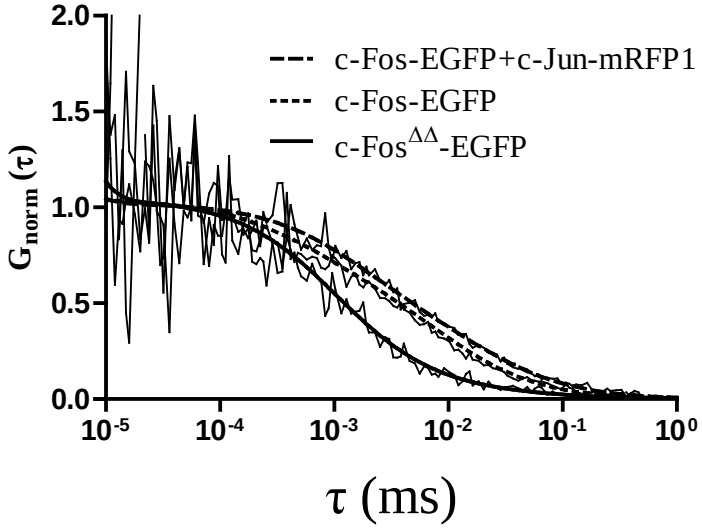


Szalóki et al Mol Cell Biol 2015

Fluorescence brightness (F/N) proves the formation of c-Fos homodimers, slow diffusion shows their DNA-binding







	D_1 ± SEM (μm²/s)	D_2 ± SEM (μm²/s)	Fraction of slow component ± SD	n
EGFP	19.8 ± 1.5	0.11 ± 0.02	0.07 ± 0.05	11
c-Fos$^{\Delta\Delta}$-EGFP	11.8 ± 1.9	0.38 ± 0.2	0.19 ± 0.12	16
c-Fos-EGFP+ c-Jun-mRFP1	5.3 ± 0.4	0.19 ± 0.03	0.38 ± 0.10	19
c-Fos-EGFP	5 ± 0.6	0.26 ± 0.06	0.35 ± 0.14	17

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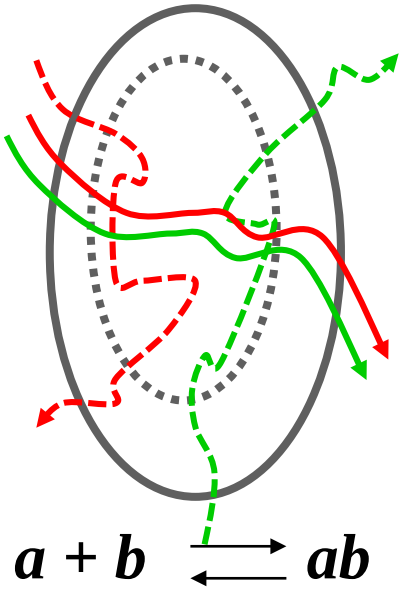
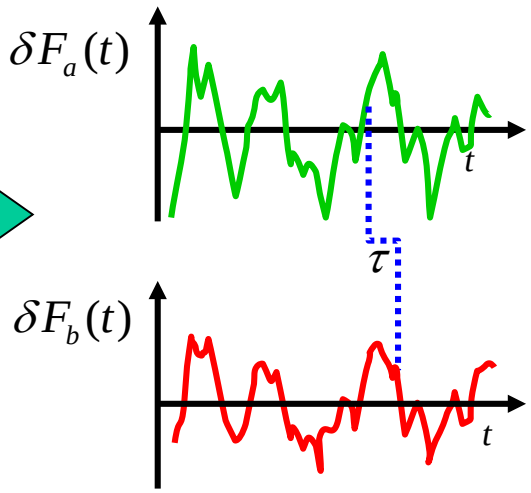
Fluorescence crosscorrelation spectroscopy (FCCS)

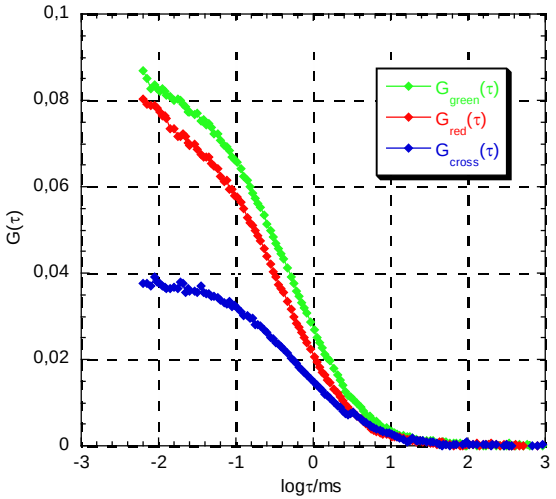
Co-mobility of associated molecules

Parallel fluctuations of green and red signals

Positive crosscorrelation amplitude







$$G_{ab}(\tau) = \frac{\langle \delta F_a(t) \cdot \delta F_b(t+\tau) \rangle}{\langle F_a \rangle \langle F_b \rangle}$$

$$G_{ab}(0) \propto \frac{c_{ab}}{V_{eff}(c_{a,tot})(c_{b,tot})}$$

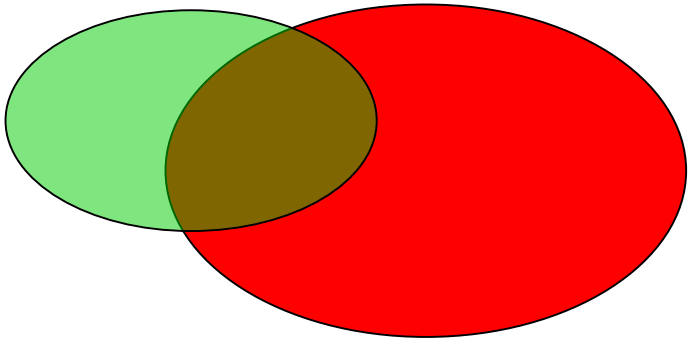
FCCS can indicate the stable association of molecules (in the τ_D time window)

Ries J et al, New Journal of Physics 12 (2010) 113009

Experimental Concerns

Observation volumes don't overlap perfectly because of

Laser alignment
Chromatic aberration
Spherical aberrations



These result in
- lengthening of the diffusion time of the CCF
- decrease of the crosscorrelation amplitude

Baseline of CCF is usually not zero because of spectral overspill between the detection channels (e.g. GFP signal correlates with itself in the green and red channels).

Define dynamic range:
measurement of a positive control (100% doubly labeled molecule) and a negative control (mixture of independently moving dyes) is measured

Simplified formula of the cross-correlation function

Definition $G^{\times}(\tau) = \dfrac{\langle \delta F_a(t) \cdot \delta F_b(t+\tau) \rangle}{\langle F_a \rangle \langle F_b \rangle}$

Intensity fluctuations of dyes *a* and *b* at time t and t+τ

τ: „lag time"

Mean intensity of *a* and *b*

CCF of diffusion

$$G^{\times}(\tau) = \underbrace{\frac{c_{ab}}{V_{eff}\left(c_{a,tot}\right)\left(c_{b,tot}\right)}}_{\text{Amplitude}} \cdot \underbrace{\frac{1}{\gamma_{xy}\sqrt{\gamma_z}}}_{\text{Focus geometry}} \cdot \underbrace{\frac{1}{1+\frac{\tau}{\gamma_{xy}\cdot\tau_{ab}}} \cdot \frac{1}{\sqrt{1+\frac{\tau}{\gamma_z \cdot S_a^2 \cdot \tau_{ab}}}}}_{\text{diffusion}}$$

Diffusion coefficient ← $D \propto \dfrac{\omega_{xy}^2}{4\tau}$

ω_{xy}^2 → Lateral radius of observation volume

τ → Diffusion time of the *ab* complex

Relation of the amplitudes of the CCF and the ACFs

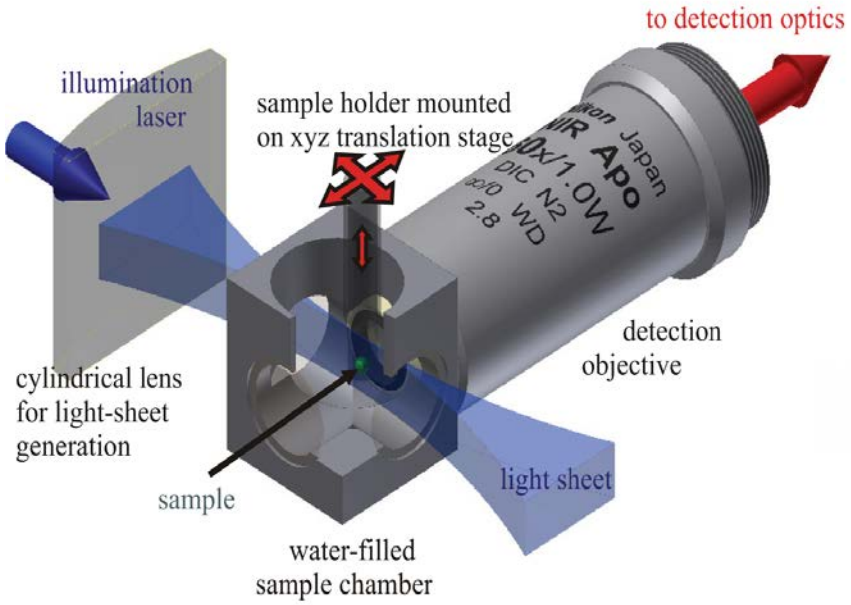
$$G^{\times}(0) \leq G_a(0),\ G_b(0)$$

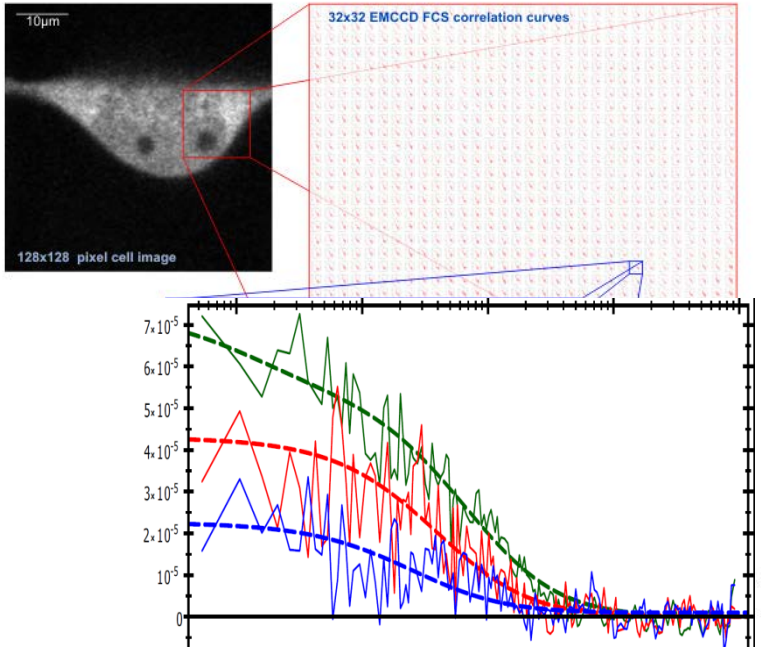
Usually the same model functions are used as for ACFs, but without triplet term.

T. Weidemann, PhD thesis Heidelberg

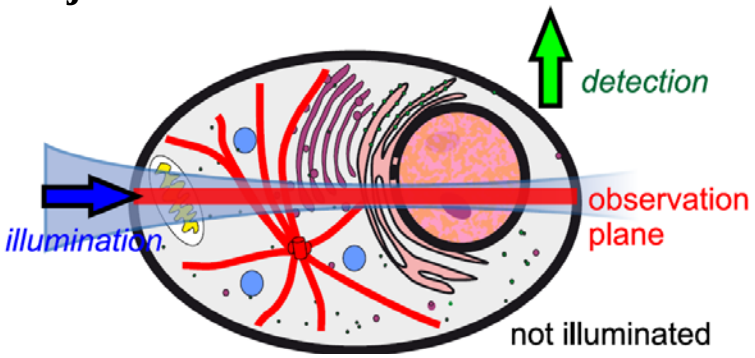
Bacia K, Nat Protoc. 2007;2:2842-56.

Selected plane illumination microscopy based fluorescence cross-correlation spectroscopy (SPIM-FCCS)





Illumination and detection objectives

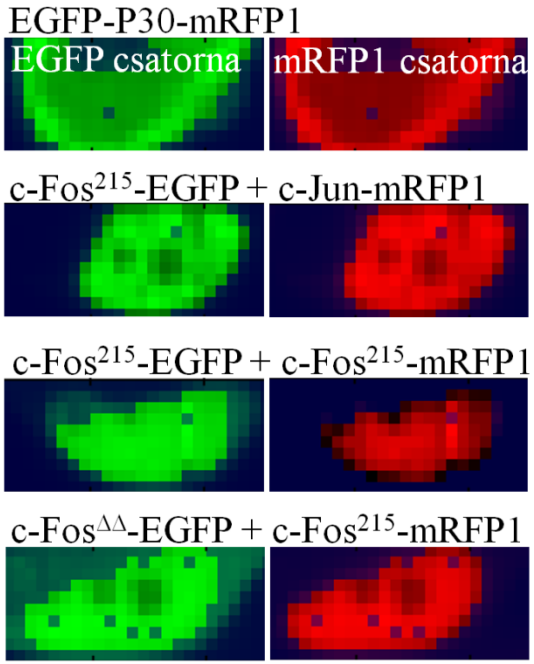


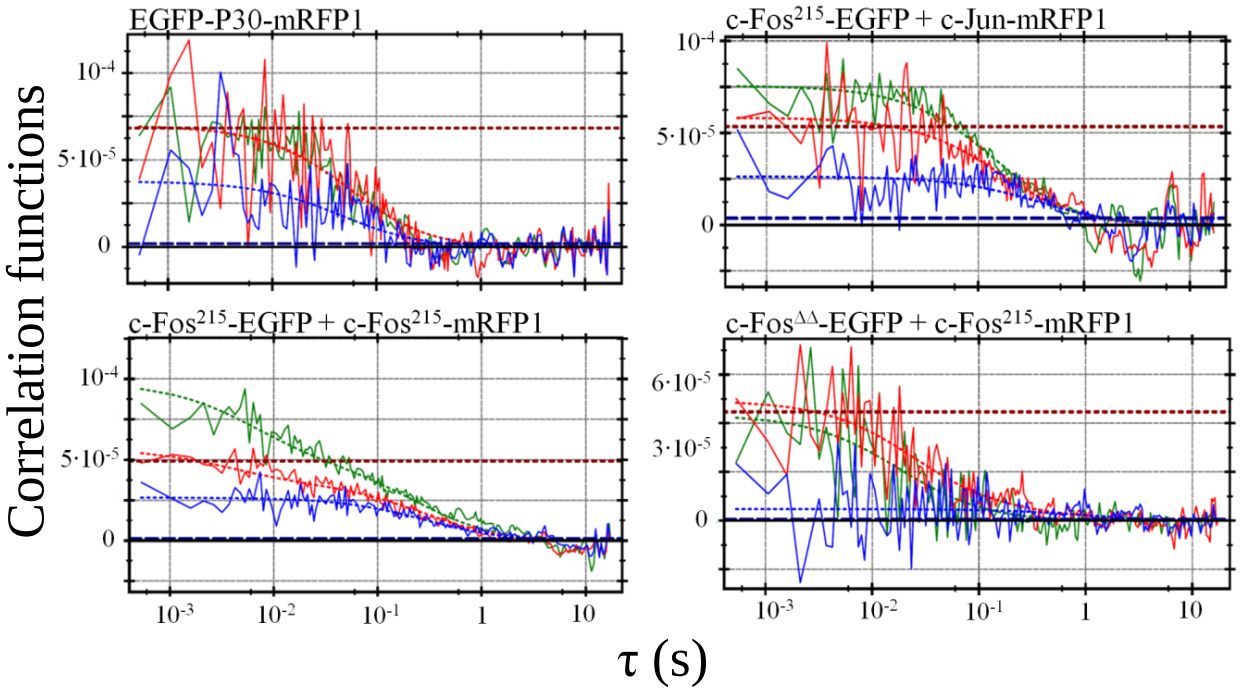
Light sheet: 2 µm

https://www.dkfz.de/Macromol/research/spim.html

Mobility and DNA-binding of dimers

Fluorescence intensity

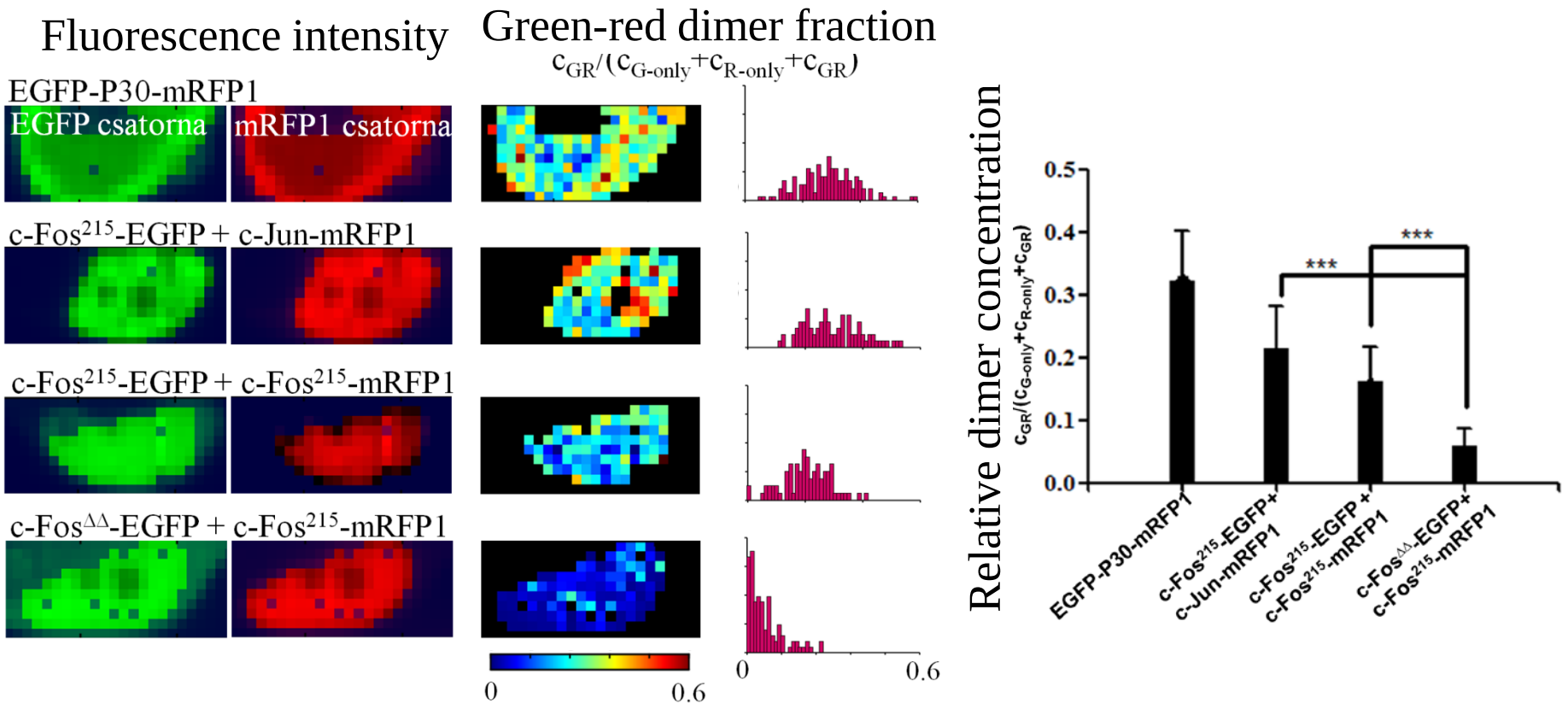




Positive cross-correlation amplitude proves the presence of Fos-Fos and Fos-Jun dimers

Szalóki et al Mol Cell Biol 2015

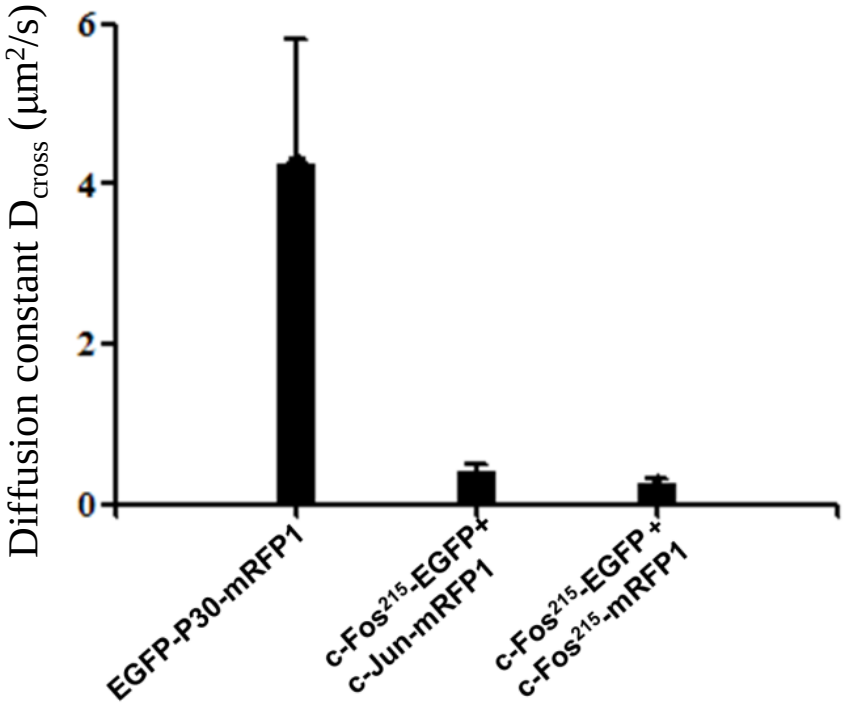
Mobility and DNA-binding of dimers



Green-red dimer fraction of Fos-Fos and Fos-Jun sample lies between that of the negative and positive controls

Szalóki et al Mol Cell Biol 2015

Mobility and DNA-binding of dimers



Slow diffusion of Fos-Fos and Fos-Jun dimers suggests their binding to chromatin

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