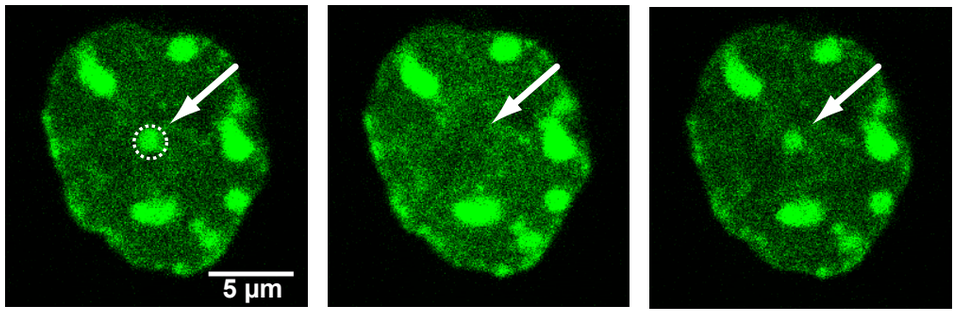
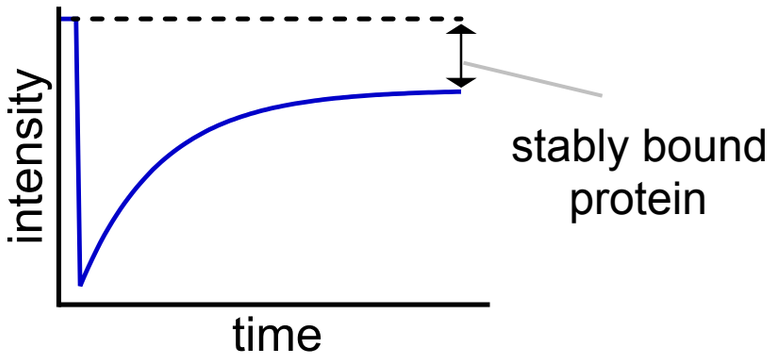
Quantifying molecular mobility and interactions by fluorescence fluctuation microscopy

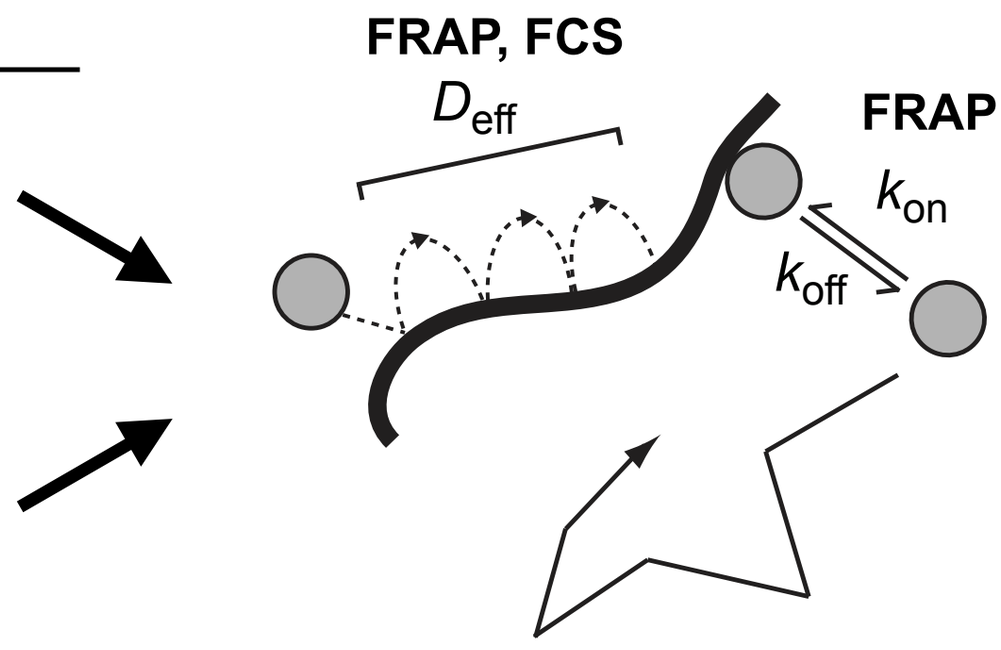
Mobility and interaction analysis of pericentric hetero-chromatin proteins in living cells by FRAP and FCS

Fluorescence recovery after photobleaching (FRAP)

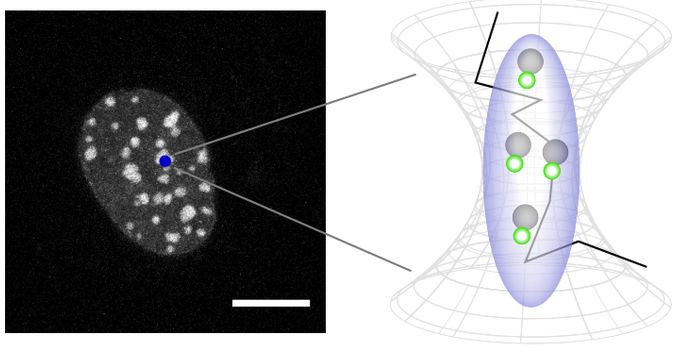


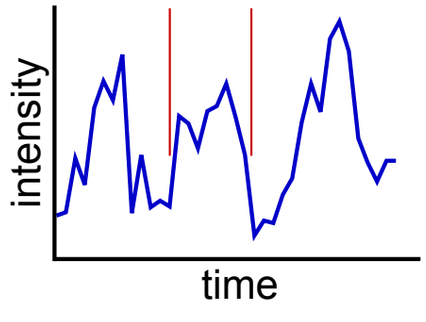


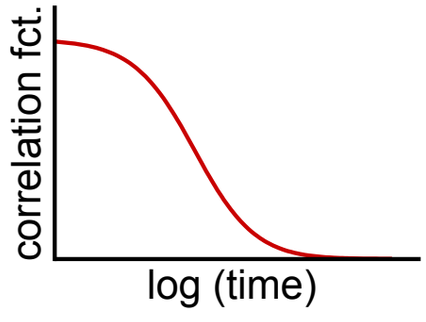
scales: 1 µm, 100 ms - hours



Fluorescence correlation spectroscopy (FCS)



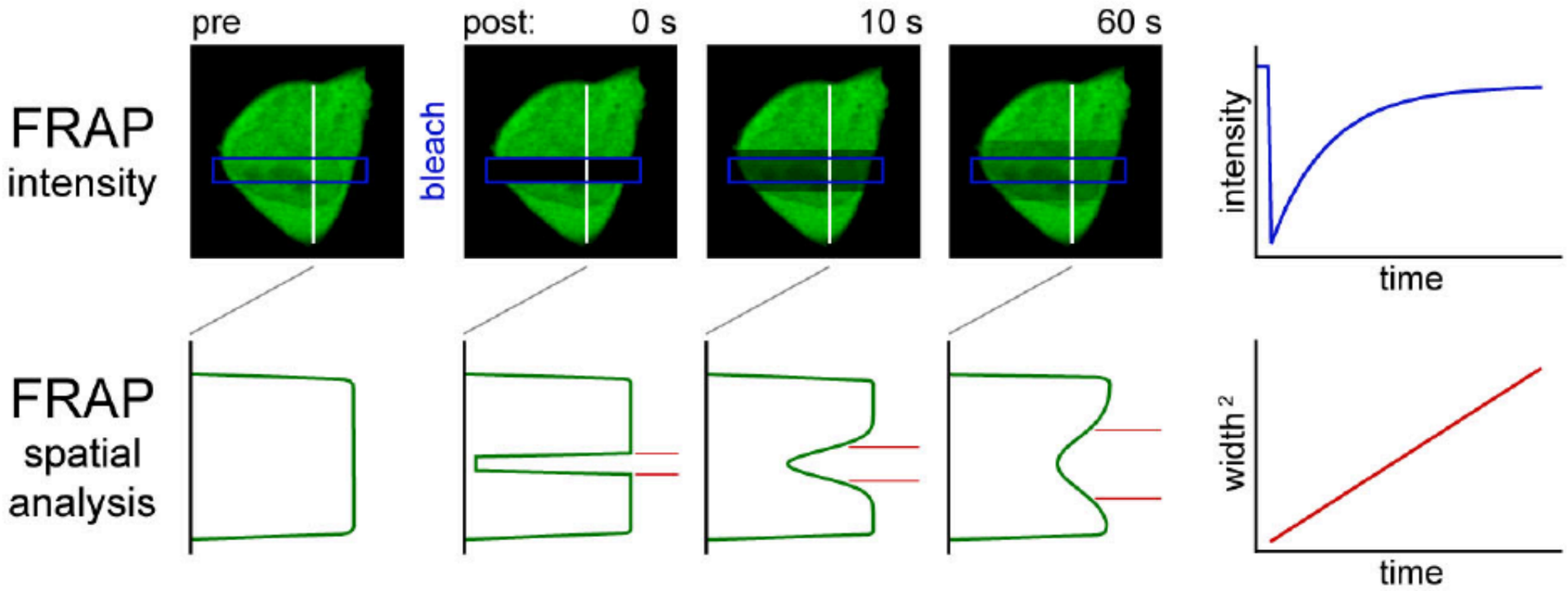




scales: 0.3 µm, 10 µs - 1 s

Müller et al. (2009) *Biophys. J.* **97**, 2876-2885; Erdel, Schubert, Marth, Längst & Rippe (2010) *PNAS* **107**, 19873-19878; Erdel et al. (2011) *Chromosome Res* **19**, 99-115; Erdel & Rippe (2012) *PNAS* **109**, E3221-30.

Fluorescence bleaching/correlation analysis of chromosomal proteins



spatial/
temporal resolution

1 µm
100 ms - hours

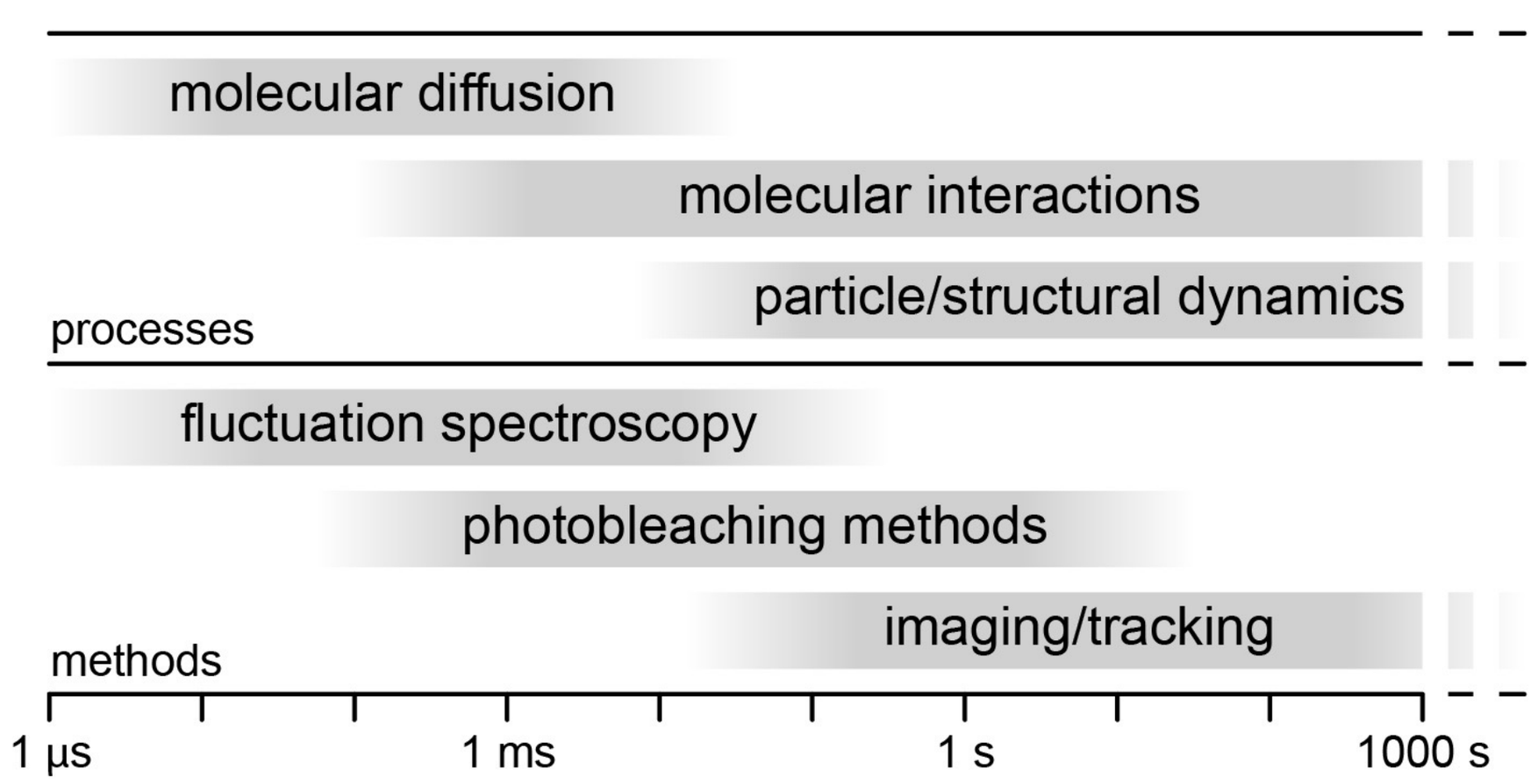
10 µm
100 ms - hours

1 µm
100 ms - hours

0.3 µm
10 µs - 1 s

Wachsmuth, M., Caudron-Herger, M. and Rippe, K. (2008). *Biochim. Biophys. Acta* **1783**, 2061-2079.

Fluorescence bleaching and correlation techniques to dissect protein interactions



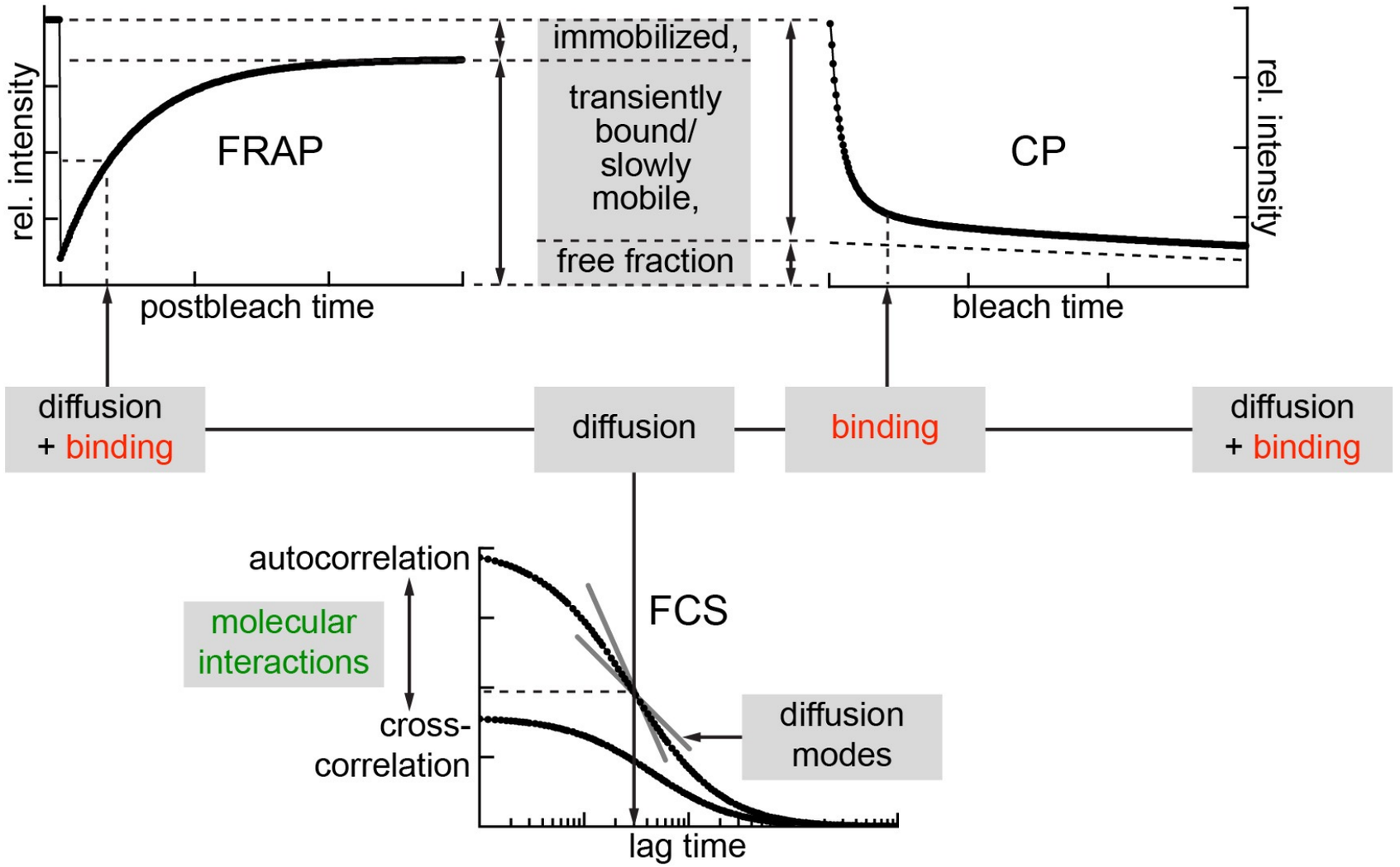
Wachsmuth M et al., *Biochim Biophys Acta* (2008)

Fluorescence bleaching and correlation techniques to dissect protein interactions

FRAP:
- slow/immobile particles (~100ms time resolution)
- diffusion coefficients
- rate constants (at immobile substrate)
- immobile fractions

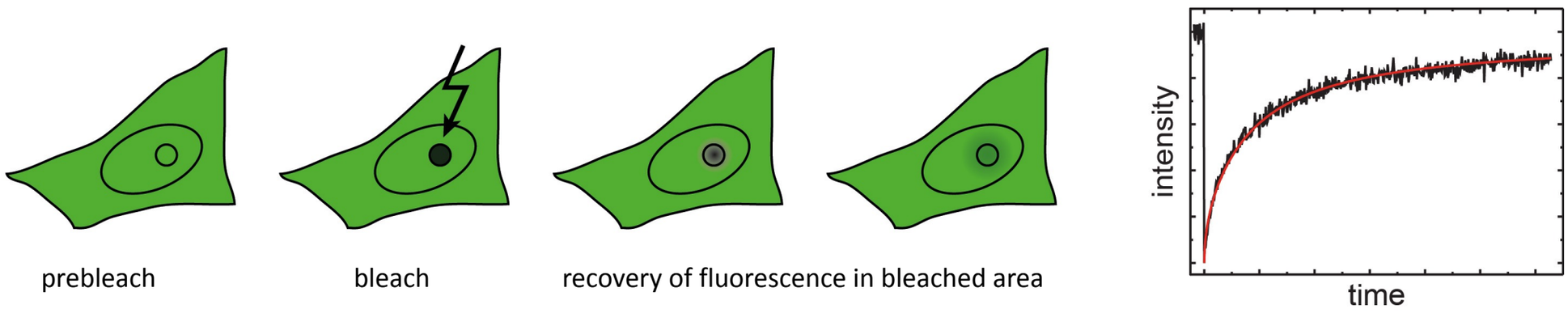
CP:
- faster particles
- dissociation rates (at immobile substrate)

FCS/FCCS:
- fast particles (µs time resolution)
- concentrations
- diffusion coefficients
- anomaly parameters
- molecular interactions



Wachsmuth M et al., *Biochim Biophys Acta* (2008)

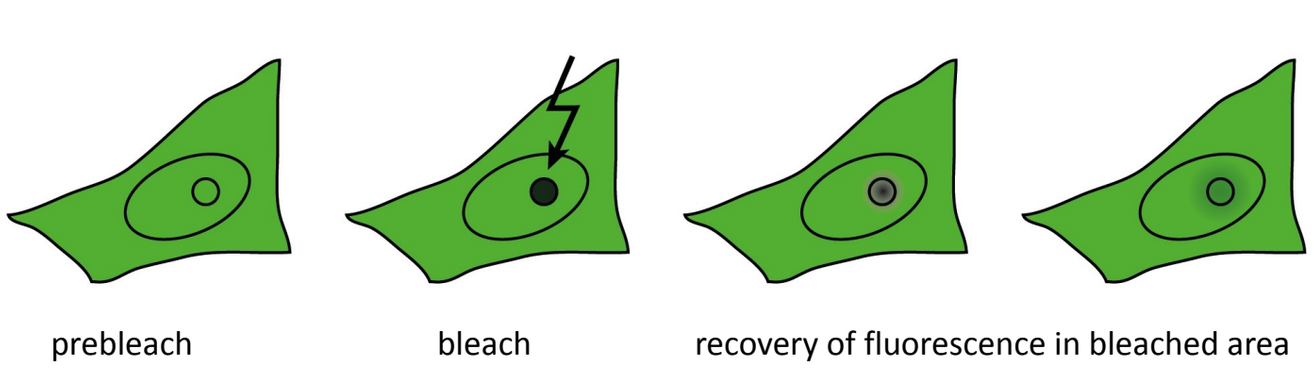
Fluorescence recovery after photobleaching (FRAP)



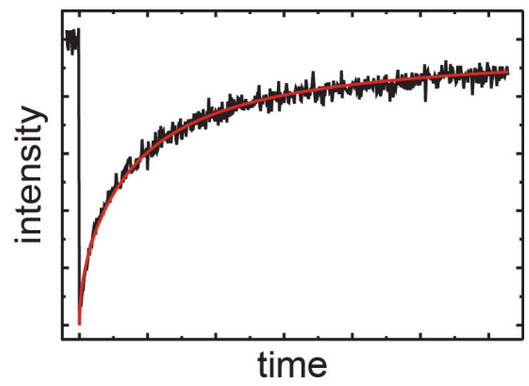
Redistribution of molecules and recovery of the fluorescence signal:

- After the bleach step, bleached molecules leave the bleach region and fluorescent ones enter it owing to diffusion
- Bleached molecules bound to immobile binding sites are released to join the mobile pool
- The binding sites can be re-occupied by mobile fluorescent molecules
- This redistribution due to exchange of both bound and mobile bleached molecules by fluorescent ones results in the recovery of the fluorescence signal

Fluorescence recovery after photobleaching (FRAP)



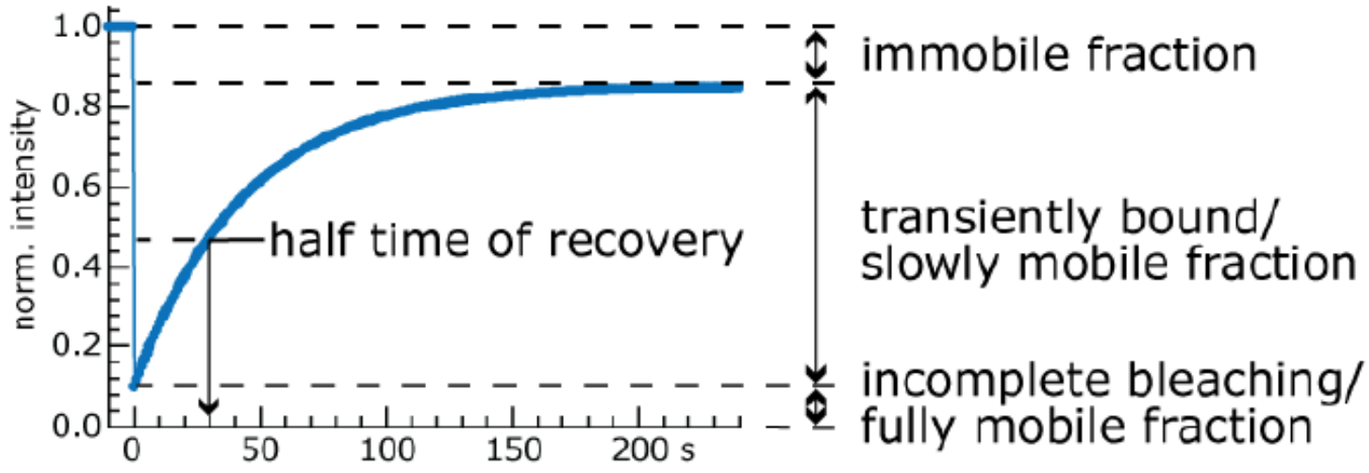
prebleach bleach recovery of fluorescence in bleached area



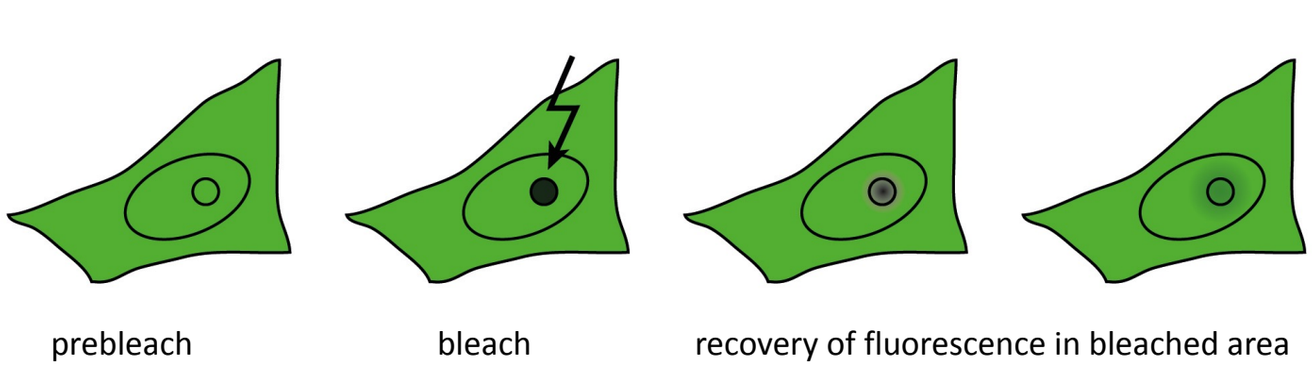
Redistribution of molecules and recovery of the fluorescence signal:

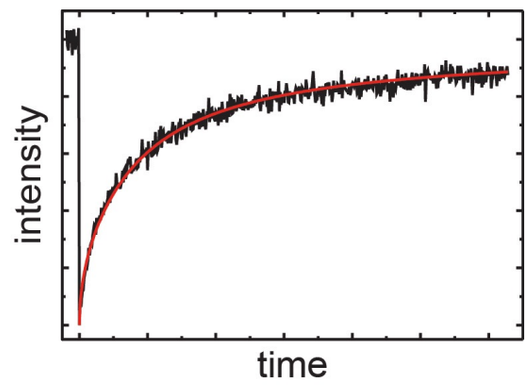
Interpretation of recovery curve:

- **half-time of recovery**
- **immobile fraction**
- **fully mobile fraction**
- **transiently bound fraction**



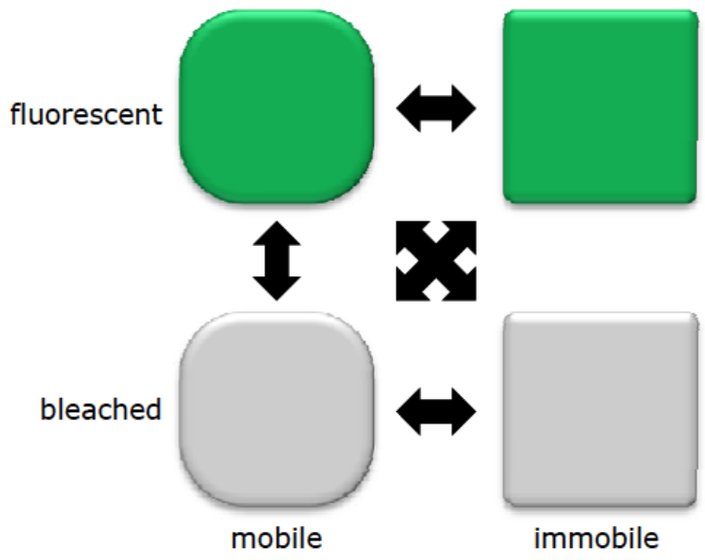
Fluorescence recovery after photobleaching (FRAP)





Redistribution of molecules and recovery of the fluorescence signal:

- A major challenge in FRAP experiments is to dissect the contributions from the **different classes** of molecules…
- … and the **exchange** between these classes
- This requires proper modeling of the data including **diffusion and binding reactions**



The two limiting cases for FRAP data analysis

"free" Diffusion
(no or only transient binding)

$$F_{\mathrm{i}}(t) = e^{-\frac{2\tau_D}{t}}\left[I_0\left(\frac{2\tau_D}{t}\right) + I_1\left(\frac{2\tau_D}{t}\right)\right]$$

diffusion time

$$\tau_D = \omega^2 / 4\, D_{\mathrm{eff}}$$

(ω: beam radius)

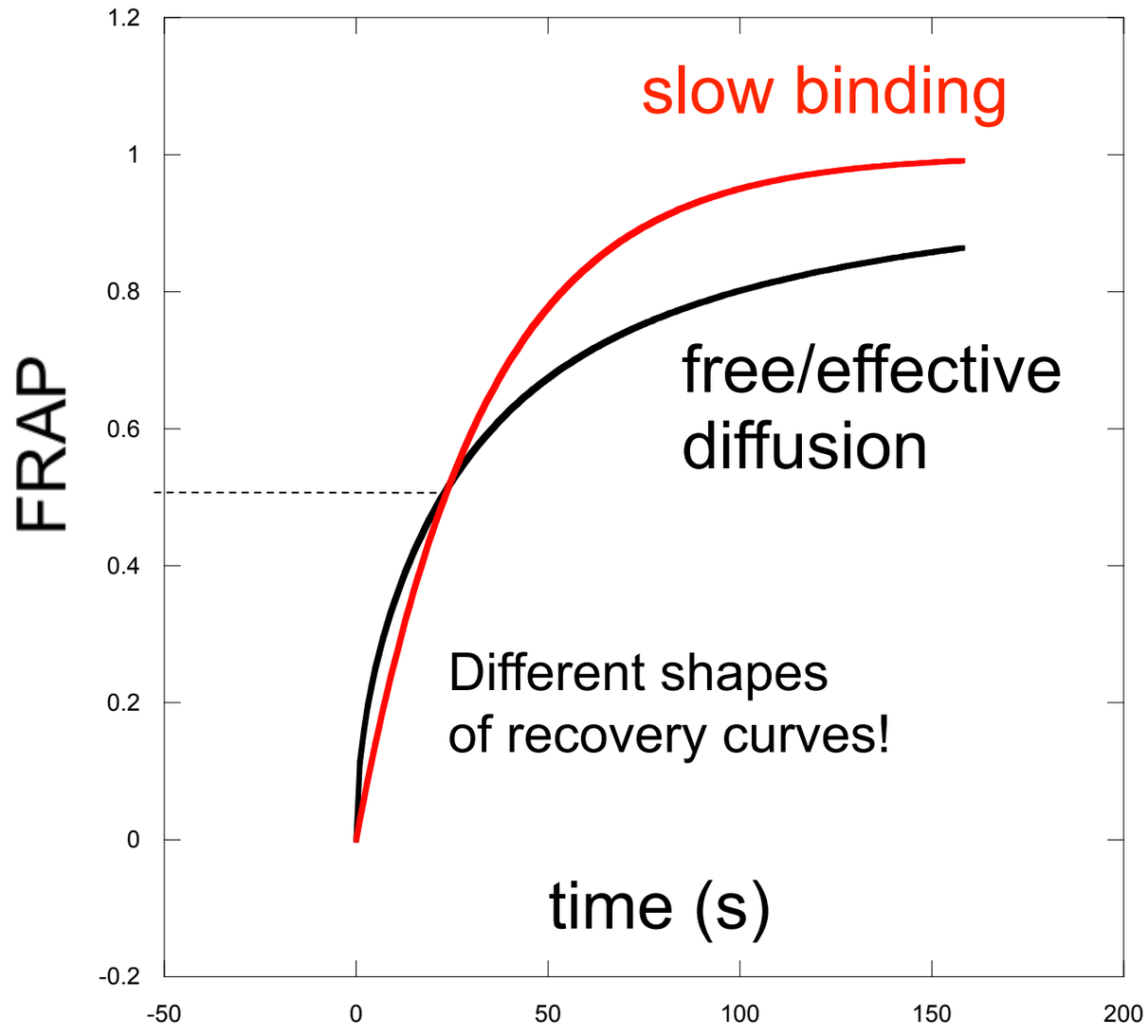
Long-lived binding
(diffusion can be neglected)

$$F_{\mathrm{i}}(t) = 1 - e^{-k_{\mathrm{off}} t}$$

dissociation rate

Diffusion with transient binding
(looks like slow diffusion)

$$D_{\mathrm{eff}} = \frac{D_{\mathrm{free}}}{1 + \frac{k^*_{\mathrm{on}}}{k_{\mathrm{off}}}}$$



Diffusion with transient binding

Transient binding: Reduction of the diffusion coefficient, shape of the FRAP curve remains unchanged from diffusion

„Transient“ means: Many binding events occur during translocations on the length scale of the bleach spot

=> only one fit parameter: the ***effective*** **diffusion coefficient**

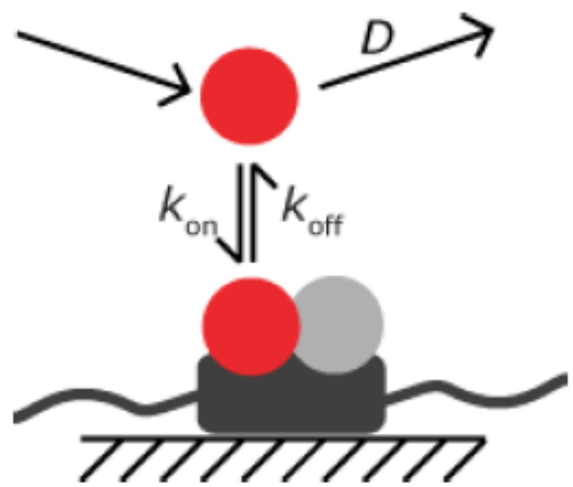
$$D_{\text{eff}} = \frac{D_{\text{free}}}{1 + \boxed{\frac{k^*_{\text{on}}}{k_{\text{off}}}}}$$

If D_{eff} (free diffusion) and D_{free} (free diffusion) are known, the rate constant ratio is obtained

Fluorescence recovery with both **diffusion** and **binding**

Coupled diffusion and reaction:

Consider a diffusive fraction A and an immobile, bound fraction B that exchange according to



$$\mathrm{A} + \mathrm{B} \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftarrows}} \mathrm{AB}$$

This can be described with the diffusion-reaction equations

$$\frac{\partial c_{\mathrm{diff}}(\mathbf{r},t)}{\partial t} = D\nabla^2 c_{\mathrm{diff}}(\mathbf{r},t) - c_B k_{\mathrm{on}} c_{\mathrm{diff}}(\mathbf{r},t) + k_{\mathrm{off}} c_{\mathrm{bound}}(\mathbf{r},t),$$

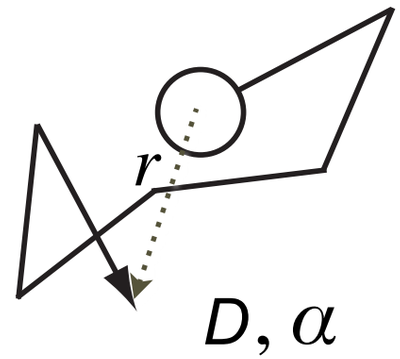
$$\frac{\partial c_{\mathrm{bound}}(\mathbf{r},t)}{\partial t} = -k_{\mathrm{off}} c_{\mathrm{bound}}(\mathbf{r},t) + c_B k_{\mathrm{on}} c_{\mathrm{diff}}(\mathbf{r},t)$$

This system of coupled differential equations is difficult or impossible to solve, especially for complex boundary conditions (bleach geometry, molecular kinetics)

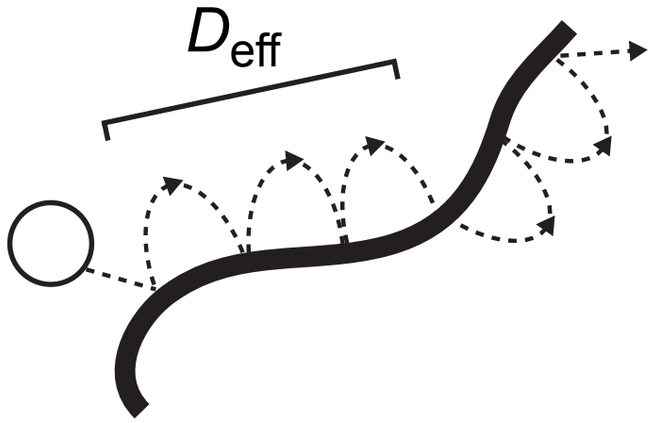
Determining diffusion coefficient D, kinetic binding rates k_{on} and k_{off}, and the apparent equilibrium constant K_{eq}^*

diffusion without binding,

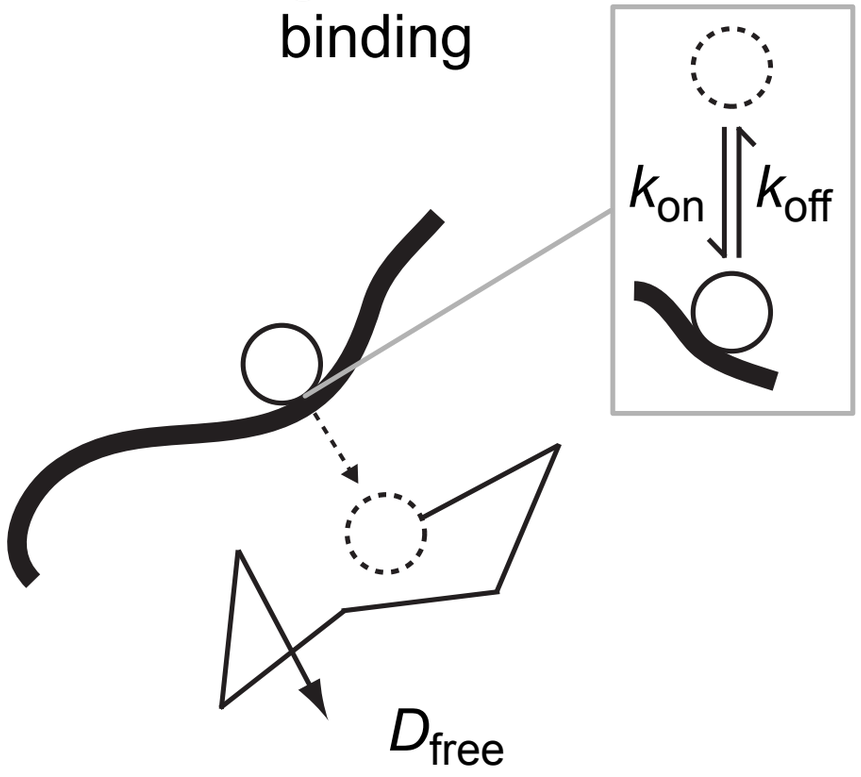
α = 1 for free diffusion



transient chromatin binding



strong chromatin binding



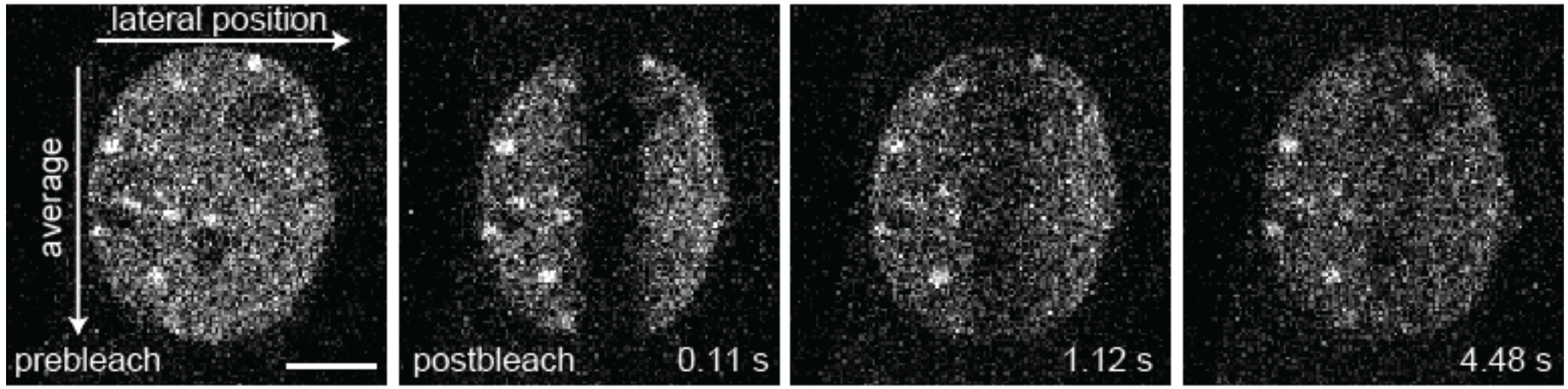
$$\left\langle r^2 \right\rangle = 6 \cdot D \cdot t^{\alpha}$$

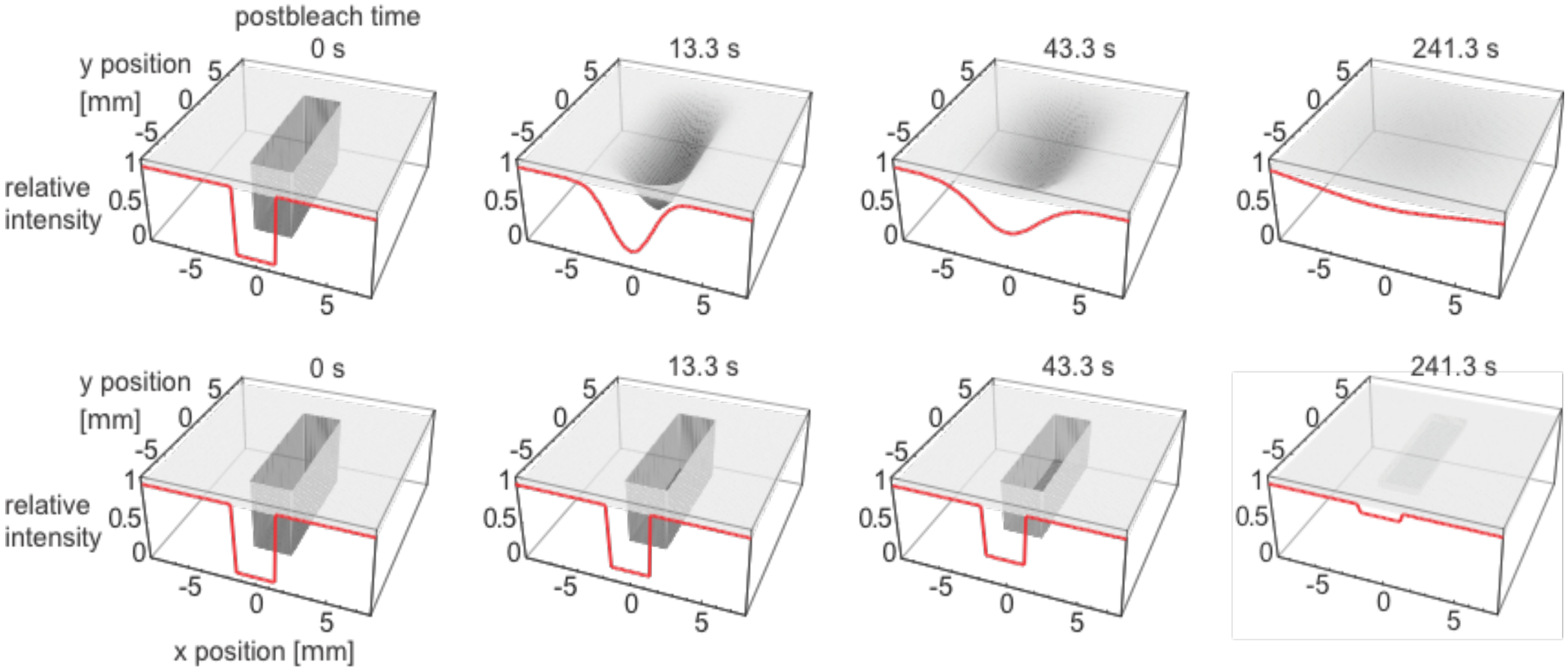
$$D_{\text{eff}} = \frac{D}{1 + K_{\text{eq}}^*}$$

$$K_{\text{eq}}^* = \frac{k_{\text{on}}^*}{k_{\text{off}}} = \frac{k_{\text{on}} \cdot [S]_{\text{eq}}}{k_{\text{off}}}$$

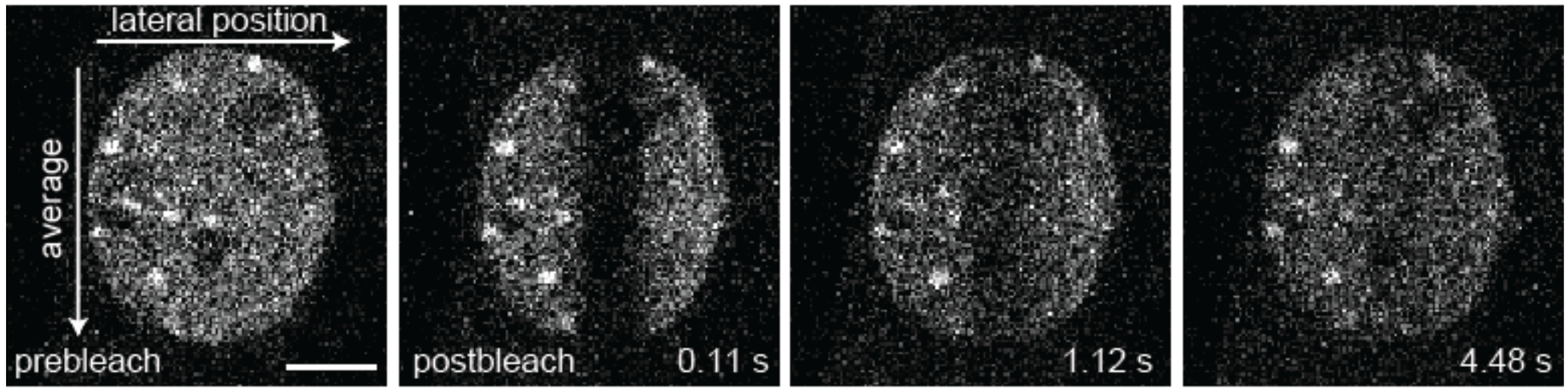
Erdel, Müller-Ott, Baum, Wachsmuth & Rippe (2011) *Chromosome Res* **19**, 99–115.

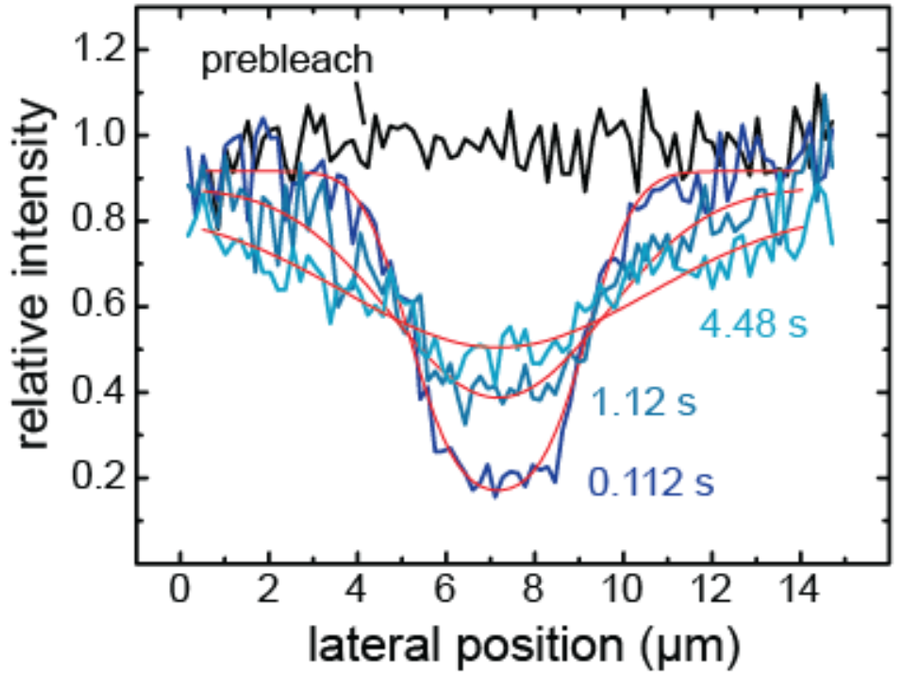
Profile FRAP – hints on diffusion and reaction dependencies

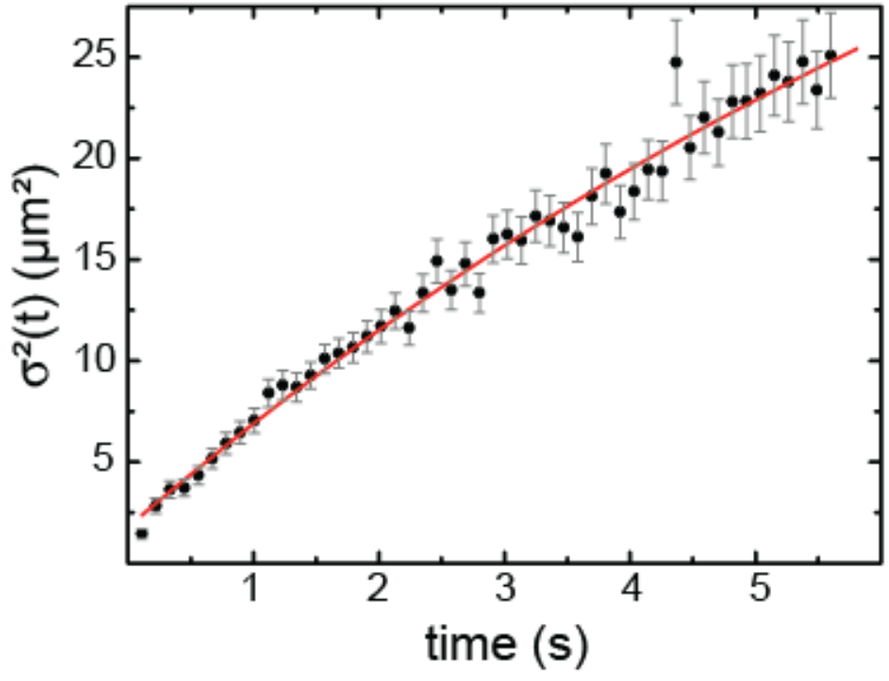




Profile FRAP – hints on diffusion and reaction dependencies







- fit postbleach profiles to obtain the squared width $\sigma^2(t)$ of the bleach strip over time
- plot $\sigma^2(t)$ reveals diffusive behavior, determine *D*

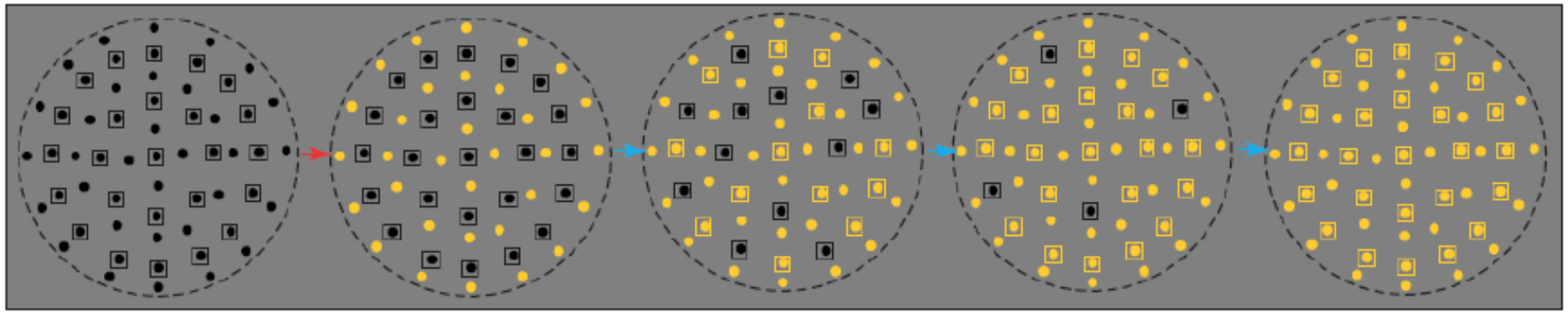
Fluorescence recovery due to diffusion and reaction processes

Diffusion and binding on different time scales:

Then, the diffusion and the reaction contribution can be treated independently

$$\frac{\partial c_{\text{diff}}(\mathbf{r},t)}{\partial t} = D\nabla^2 c_{\text{diff}}(\mathbf{r},t) - c_B k_{\text{on}} c_{\text{diff}}(\mathbf{r},t) + k_{\text{off}} c_{\text{bound}}(\mathbf{r},t),$$

$$\frac{\partial c_{\text{bound}}(\mathbf{r},t)}{\partial t} = -k_{\text{off}} c_{\text{bound}}(\mathbf{r},t) + c_B k_{\text{on}} c_{\text{diff}}(\mathbf{r},t)$$



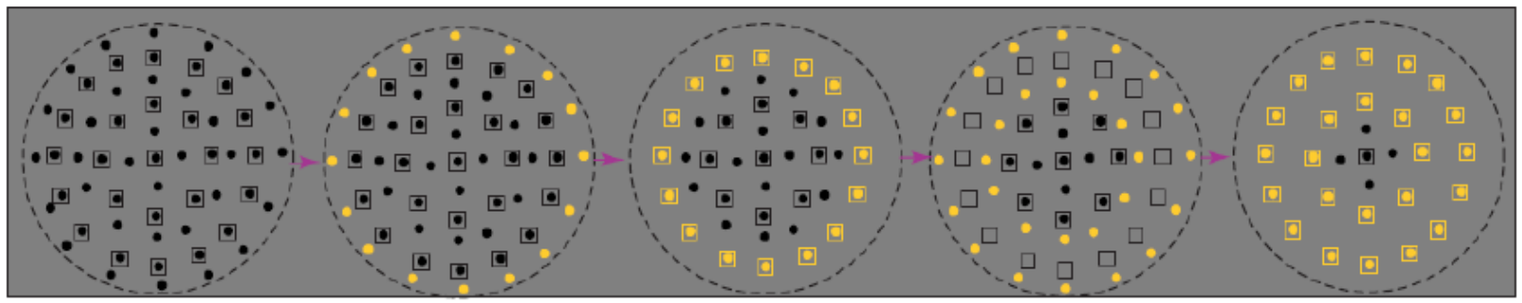
Fluorescence recovery due to diffusion and reaction processes

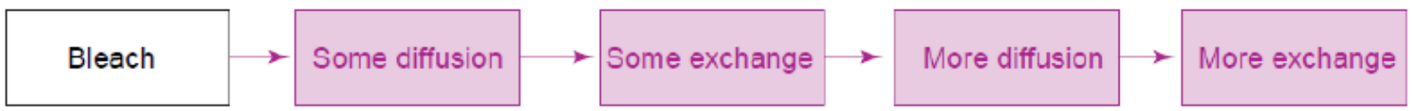
Diffusion and binding on the same time scales:

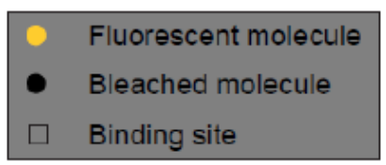
The full coupled reaction-diffusion scheme must be solved.

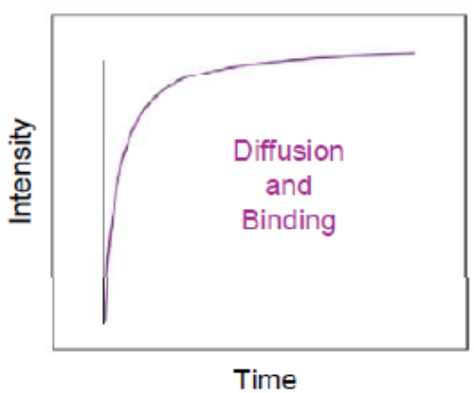
$$\frac{\partial c_{\text{diff}}(\mathbf{r},t)}{\partial t} = D\nabla^2 c_{\text{diff}}(\mathbf{r},t) - c_B k_{\text{on}} c_{\text{diff}}(\mathbf{r},t) + k_{\text{off}} c_{\text{bound}}(\mathbf{r},t),$$

$$\frac{\partial c_{\text{bound}}(\mathbf{r},t)}{\partial t} = -k_{\text{off}} c_{\text{bound}}(\mathbf{r},t) + c_B k_{\text{on}} c_{\text{diff}}(\mathbf{r},t)$$





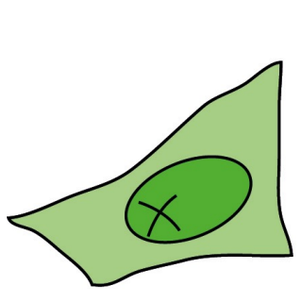


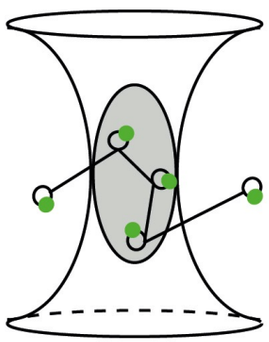


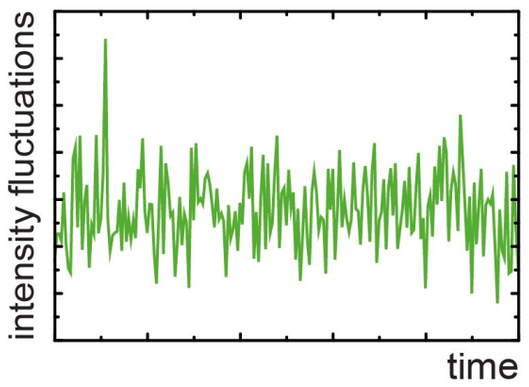
full model: reaction-diffusion model

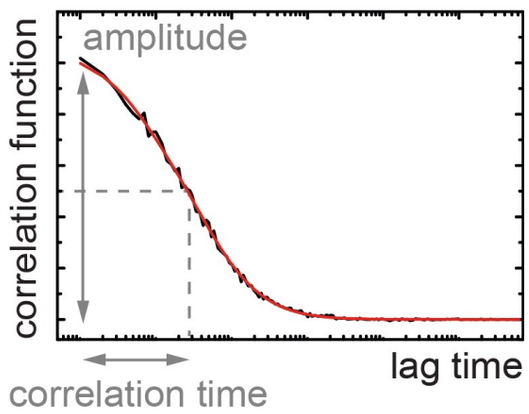
$$\overline{frap}(p) = \frac{1}{p} - \frac{F_{\text{eq}}}{p}\left(1 - 2K_1(q\omega)I_1(q\omega)\right)\left(1 + \frac{k^*_{\text{on}}}{p + k_{\text{off}}}\right) - \frac{C_{\text{eq}}}{p + k_{\text{off}}} \quad \xrightarrow{\text{Laplace}} \quad frap(t)$$

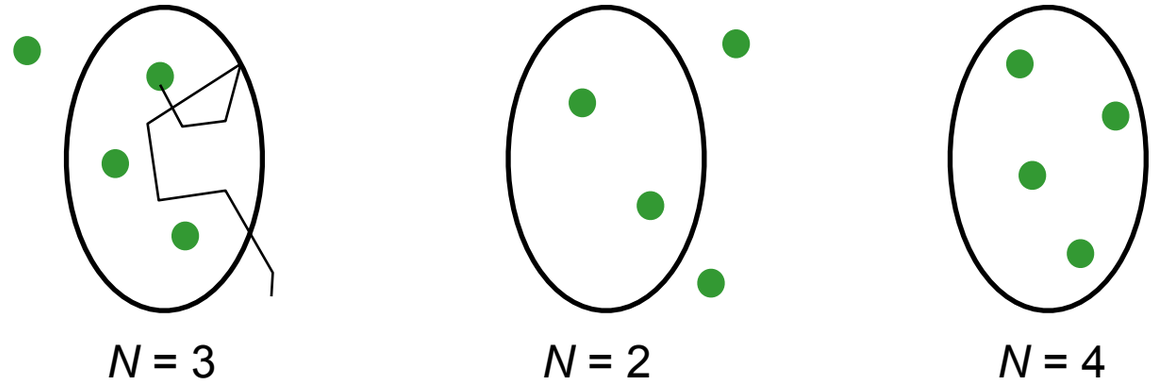
Fluorescence correlation spectroscopy (FCS)





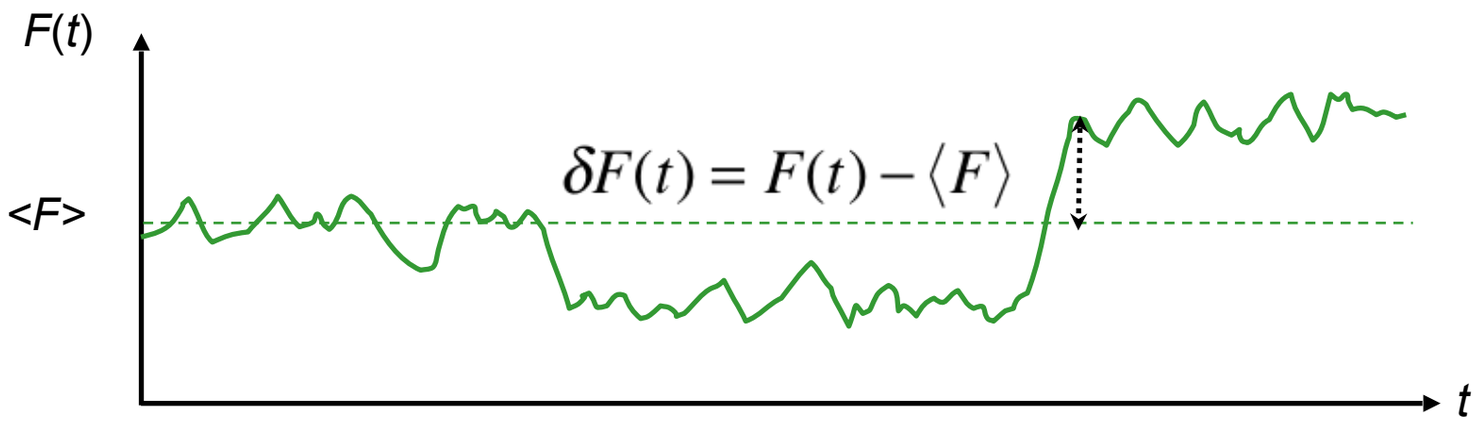






Diffusion induces fluctuations of the number of molecules

<*N*> = 3

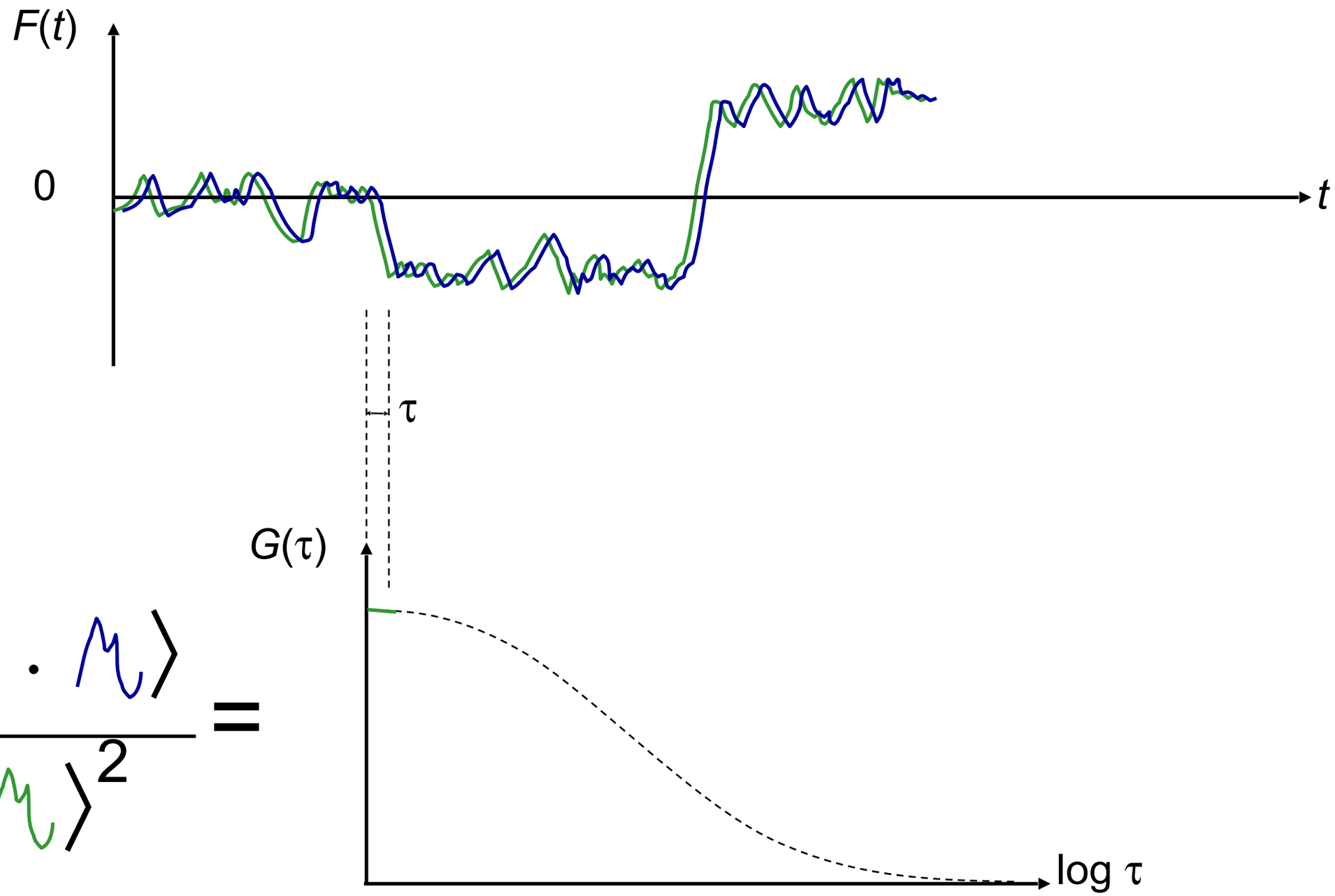


This results in fluctuations of the fluorescence signal

Fluctuations of the particle number of a 1 nM rhodamine solution in dependence of the observation volume

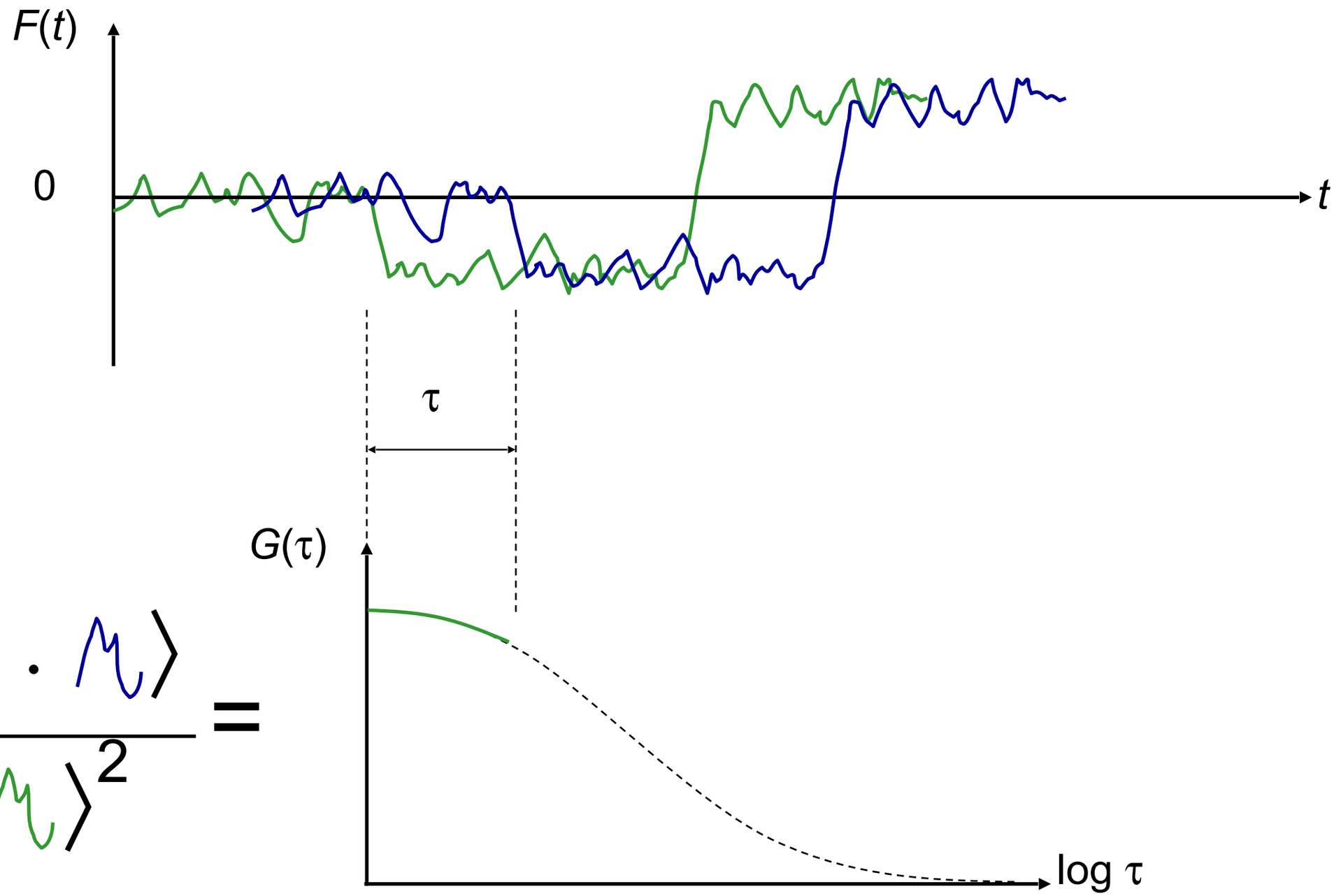
Size [mm]	Volume [l]	particles	ΔN	ΔN/N [%]
10	10^{-3}	$6.023 \cdot 10^{11}$	776080	0.00013
1	10^{-6}	$6.023 \cdot 10^{8}$	24541	0.0041
0.1	10^{-9}	$6.023 \cdot 10^{5}$	776	0.129
0.01	10^{-12}	602.3	24.5	4.075
0.001	10^{-15}	0.6023	0.776	128.9

The concept of autocorrelation analysis



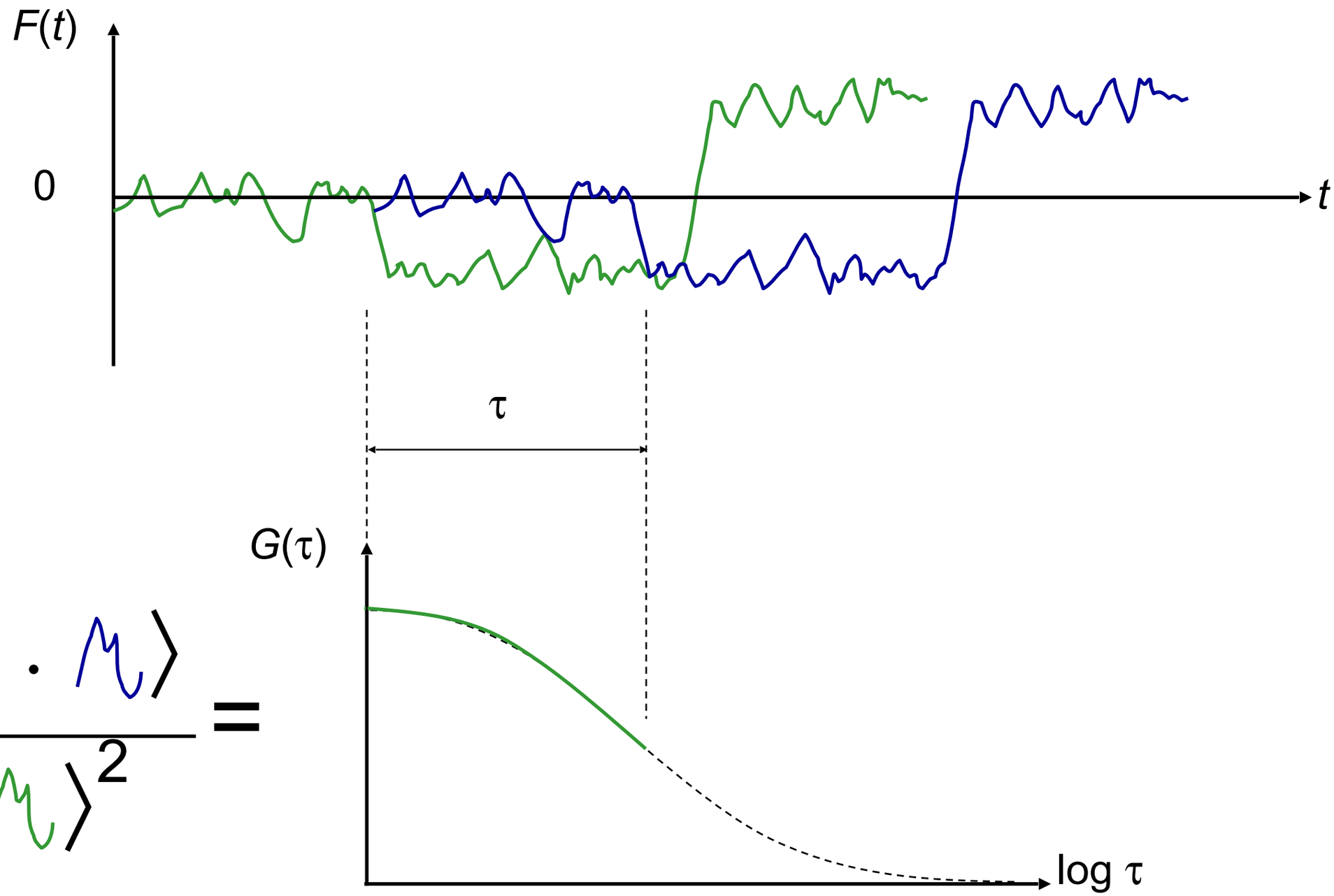
$$\frac{\langle \sim \cdot \sim \rangle}{\langle \sim \rangle^2} =$$

The concept of autocorrelation analysis



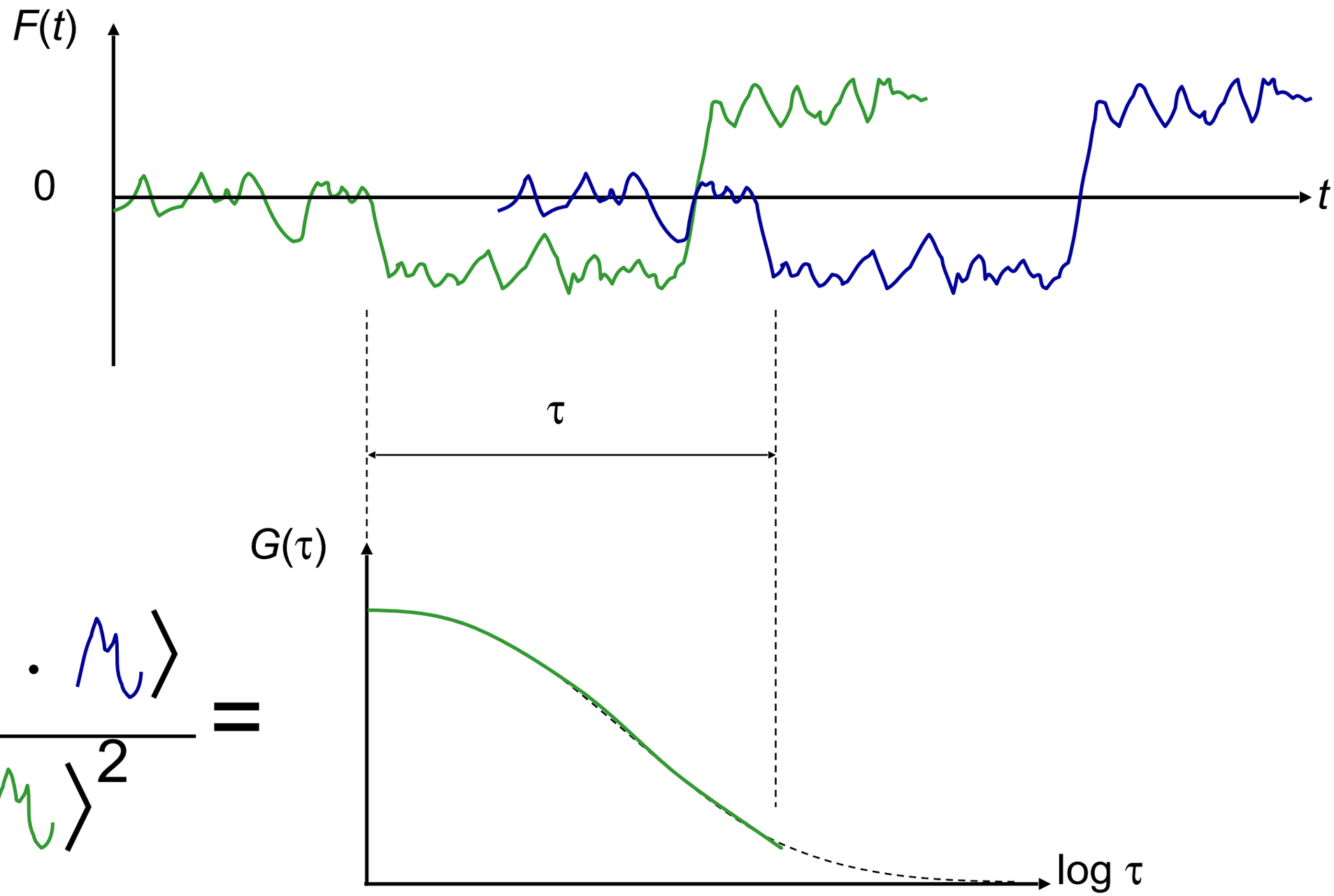
$$\frac{\langle \sim \cdot \sim \rangle}{\langle \sim \rangle^2} =$$

The concept of autocorrelation analysis



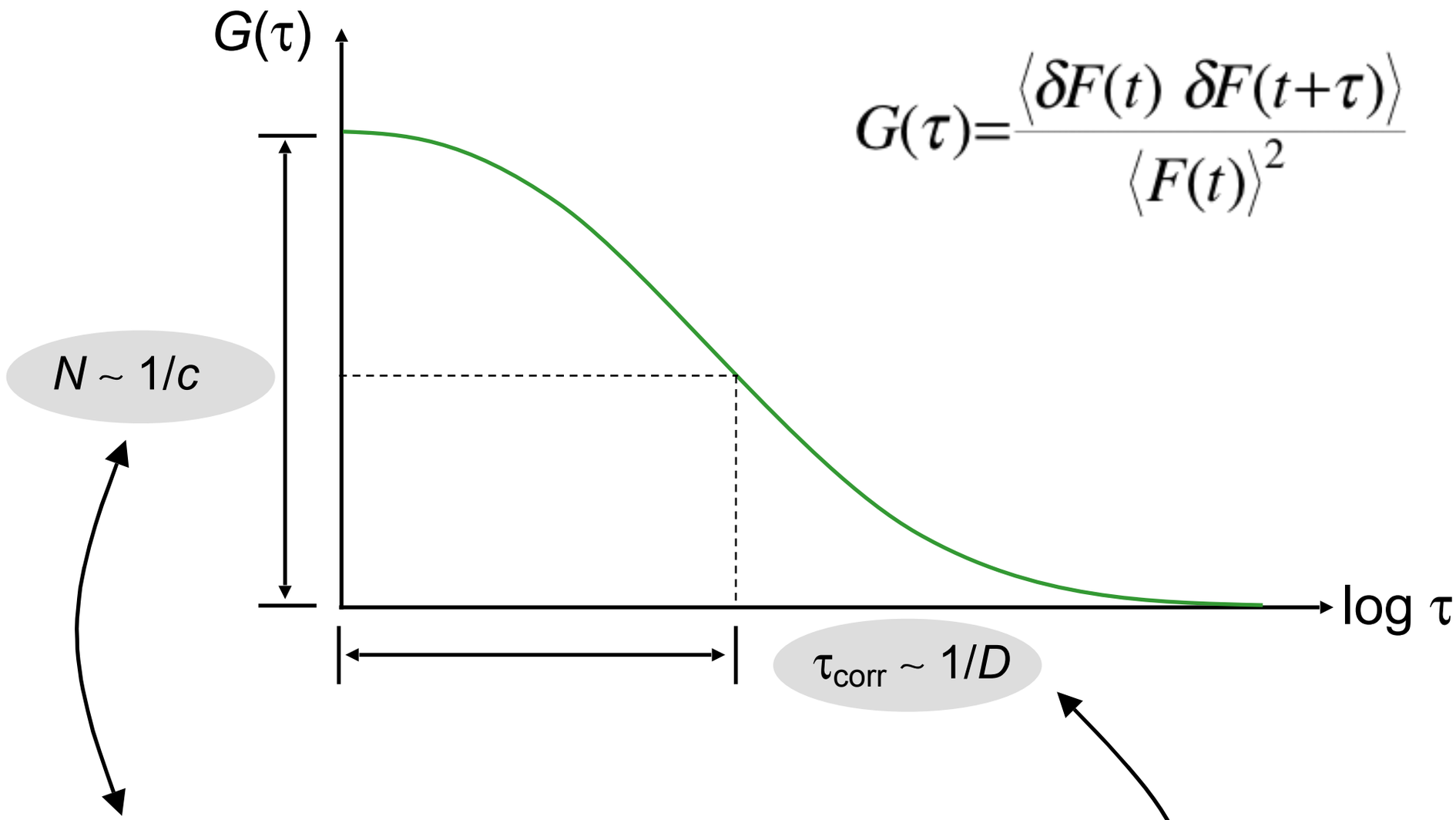
$$\frac{\langle \wedge \cdot \wedge \rangle}{\langle \wedge \rangle^2} =$$

The concept of autocorrelation analysis



$$\frac{\langle \sim \cdot \sim \rangle}{\langle \sim \rangle^2} =$$

The autocorrelation function (ACF) encodes molecule concentration and dynamics

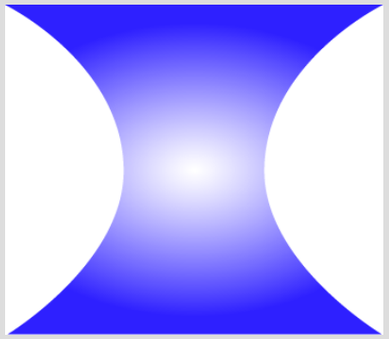


$$G(\tau)=\frac{\langle \delta F(t)\ \delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}$$

- variance of fluctuations ~ concentration
- length of fluctuations ~ diffusion coefficient

Extracting mobility information by fitting a correlation function for free diffusion in 3D

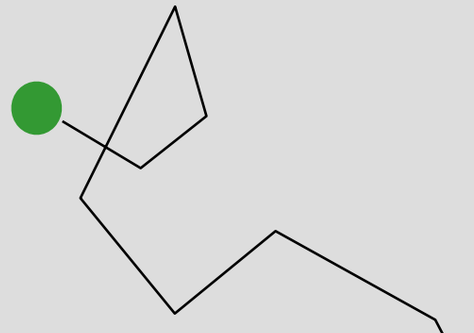
Properties of the
optical system



$F(\mathbf{r}) = \ldots$

•

Properties of the
diffusion process



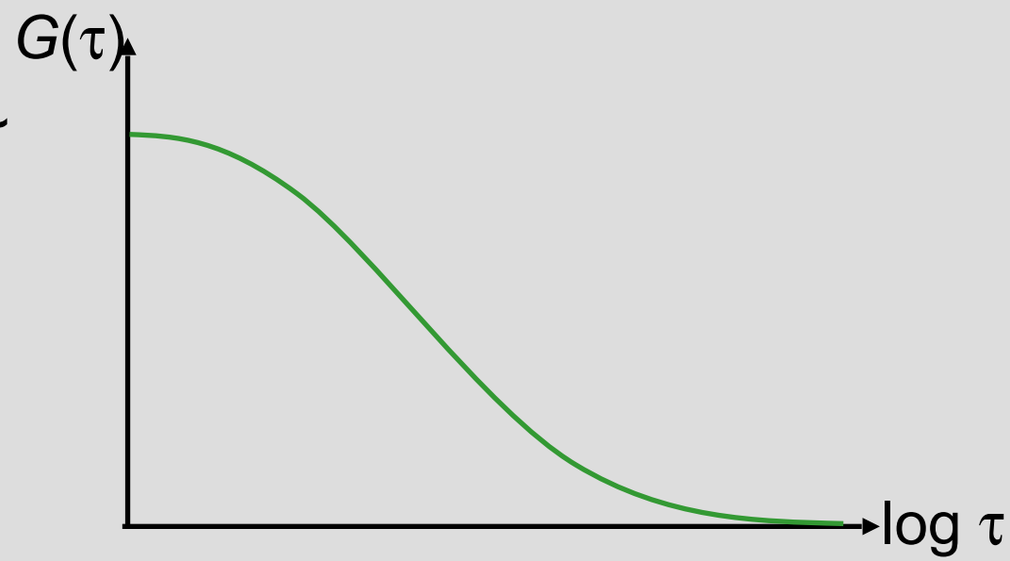
$c(\mathbf{r},t) = \ldots$

=

Analytical autocorrelation function ~

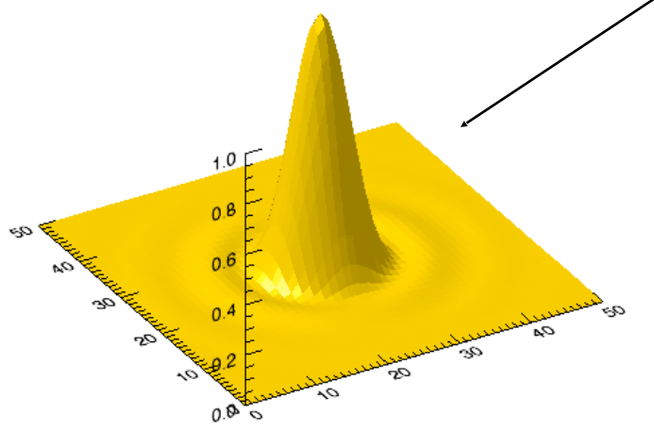
concentration

diffusion properties of different species



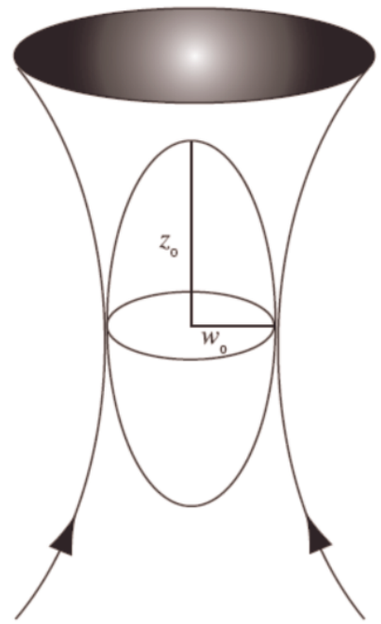
Extracting the mobility information by fitting a correlation function for free diffusion in 3D

<u>detection efficiency</u>

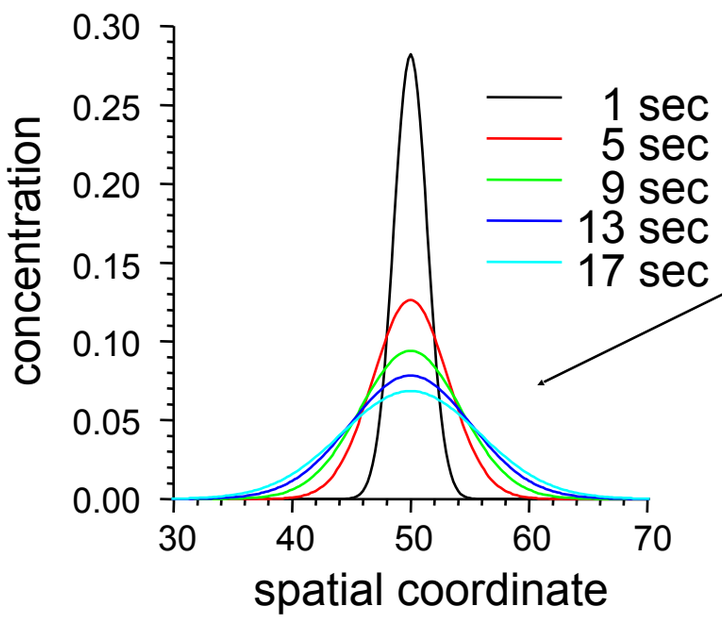


That's how a 'point' in the confocal microscope looks like

$$\Psi_k(\mathbf{r}) = \exp\left(-2\frac{x^2+y^2}{w_0^2} - 2\frac{z^2}{z_0^2}\right)$$



<u>Green's function for free diffusion</u>



That's how a diffusing molecule spreads out over time (in 1D)

$$P(\mathbf{r}_1,\mathbf{r}_2,\tau) = (4\pi D\tau)^{-3/2}\exp\left(-\frac{(\mathbf{r}_2-\mathbf{r}_1)^2}{4D\tau}\right)$$ for 3D

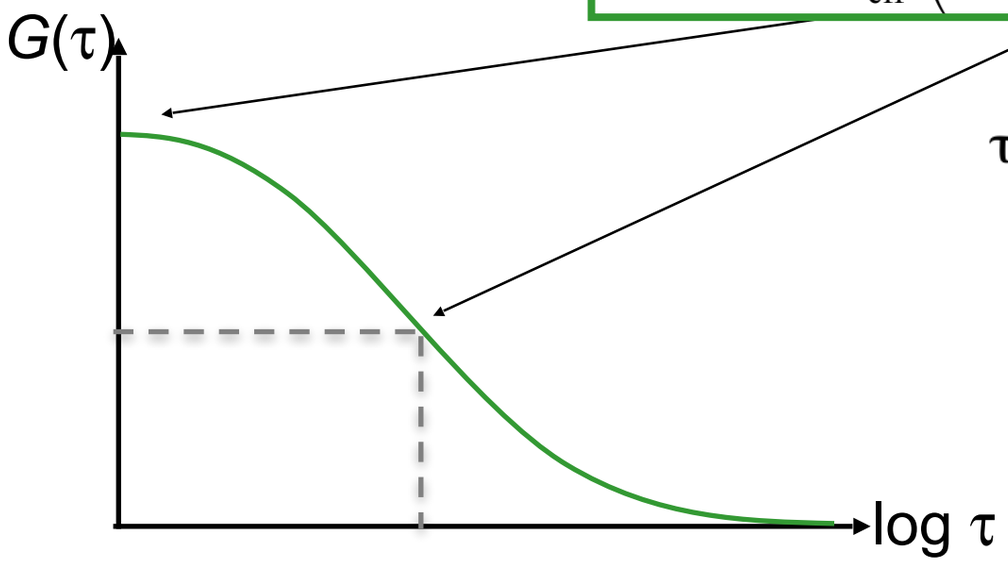
Extracting the mobility information by fitting a correlation function for free diffusion in 3D

definition of the correlation function $G(\tau)$:

probability to detect a particle before and after it has diffused a distance $|\mathbf{r}_2-\mathbf{r}_1|$ for time τ

$$G_{kl}(\tau)=\frac{\int d^3r_1\int d^3r_2\,\Psi_k(\mathbf{r}_1)P(\mathbf{r}_1,\mathbf{r}_2,\tau)\Psi_l(\mathbf{r}_2)}{\int d^3r\,\Psi_k(\mathbf{r})\int d^3r\,\Psi_l(\mathbf{r})}$$

$$G_{kl}(\tau)=\frac{1}{cV_{\text{eff}}}\left(1+\frac{\tau}{\tau_{\text{diff}}}\right)^{-1}\left(1+\frac{1}{\kappa^2}\frac{\tau}{\tau_{\text{diff}}}\right)^{-1/2}$$

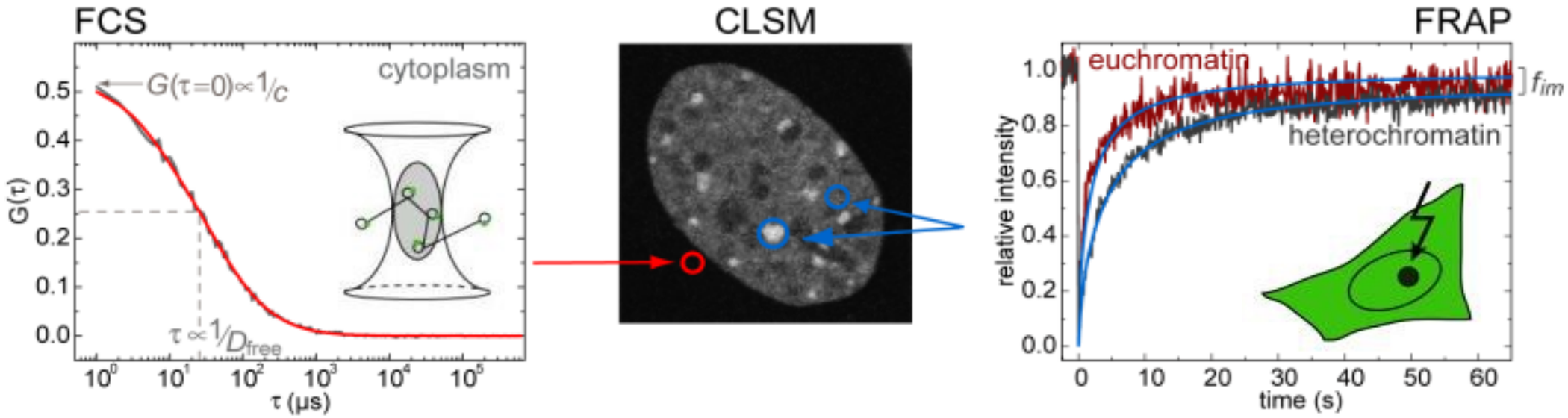


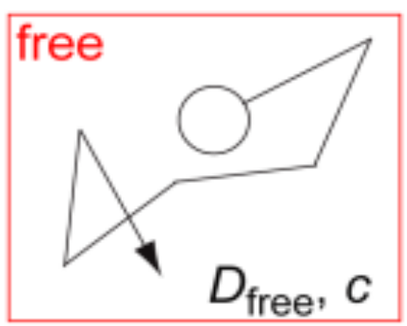
$$\tau_{\text{diff}}=\frac{w_0^2}{4D},\quad V_{\text{eff}}=\pi^{3/2}w_0^2z_0,\quad \kappa=\frac{z_0}{w_0},\quad w_0^2=\frac{w_k^2+w_l^2}{2}$$

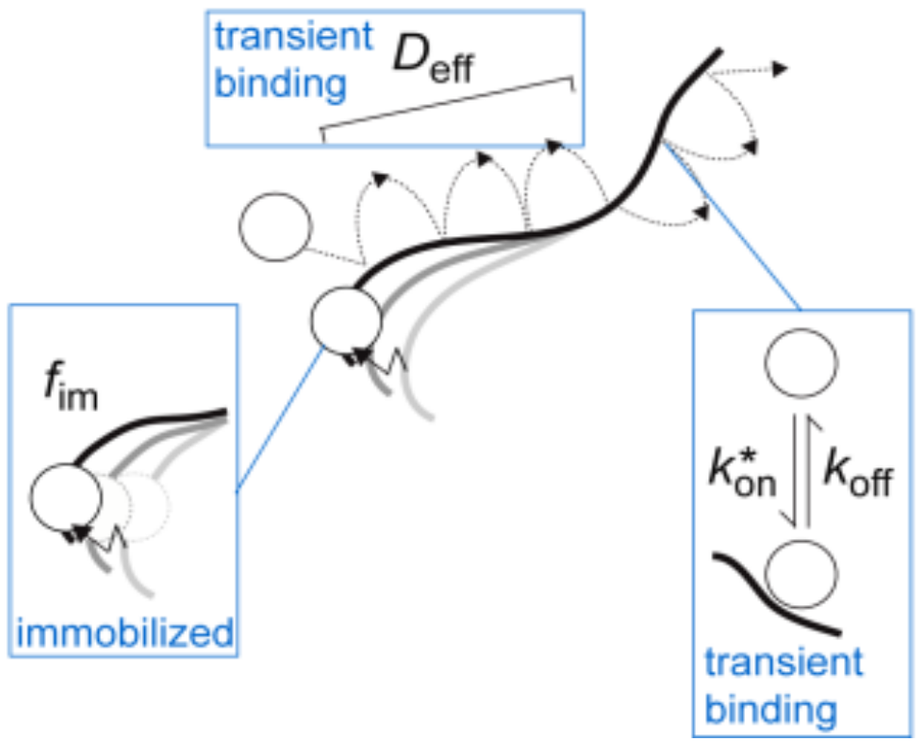
focal volume, structure parameter, focus radius

diffusion coefficient D, concentration c (different species)

Combine fluorescence bleaching and correlation techniques to dissect diffusion and chromatin interactions of heterochromatin protein 1 (HP1)

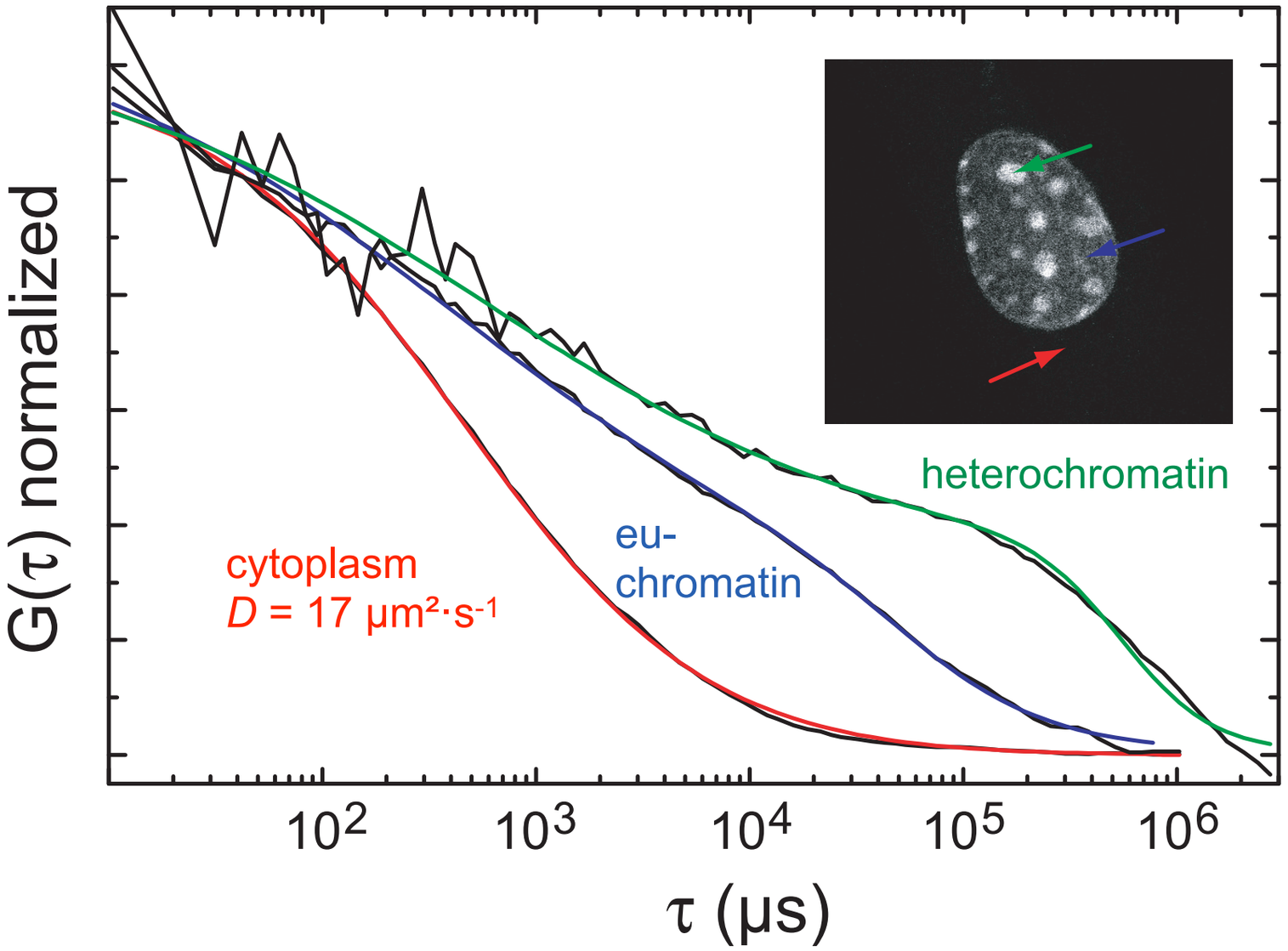


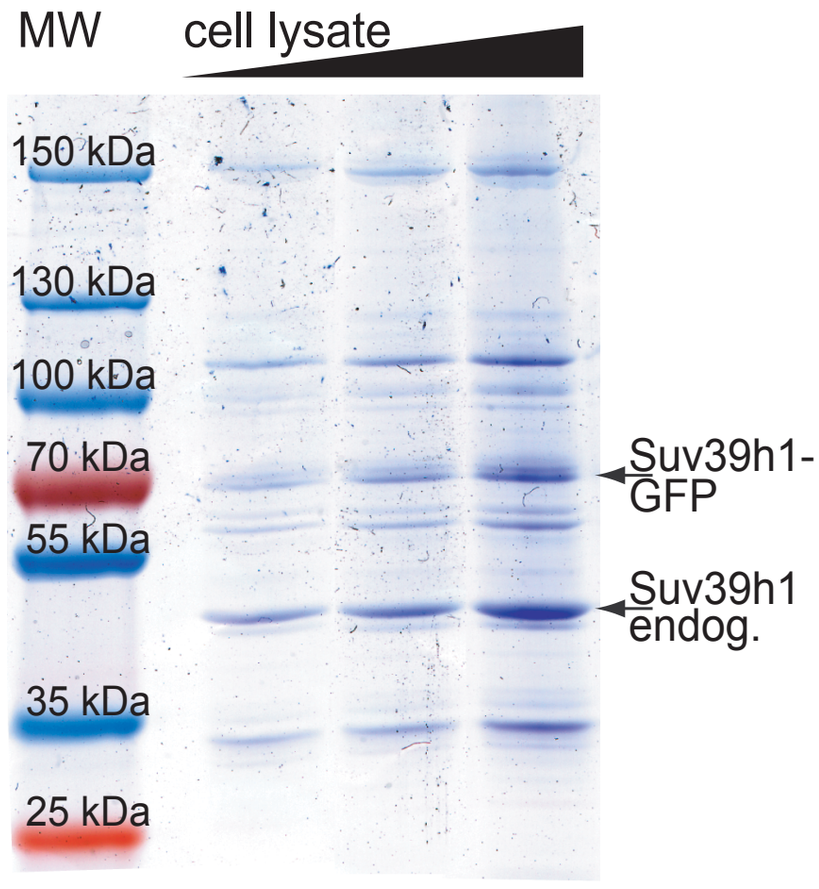




Erdel and Müller-Ott et al., *Chrom Res* (2010)

Fluorescence correlation spectroscopy (FCS) of the histone methylase SUV39H1





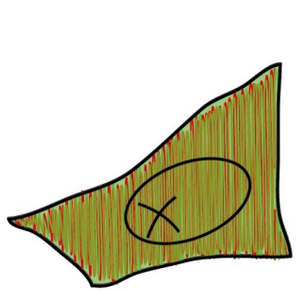
cytoplasm: 0.015 ± 0.006 µM
euchromatin: 0.11 ± 0.07 µM
heterochromatin: 0.8 ± 0.5 µM

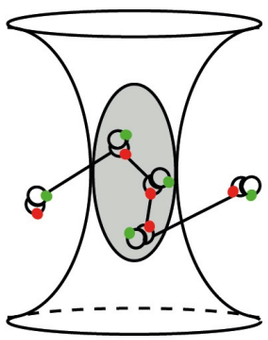
FCS experiments

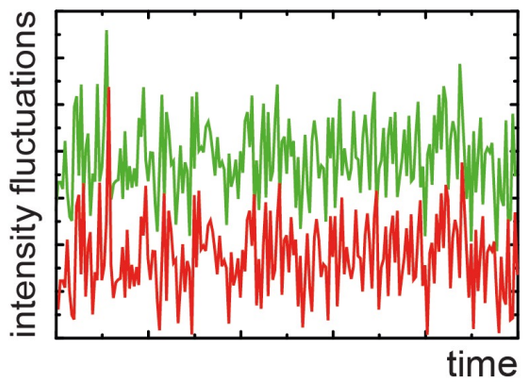
Heterochromatin: D_1 = 0.02 ± 0.01 µm² s^{-1}; D_2 = 18 ± 2 µm² s^{-1}

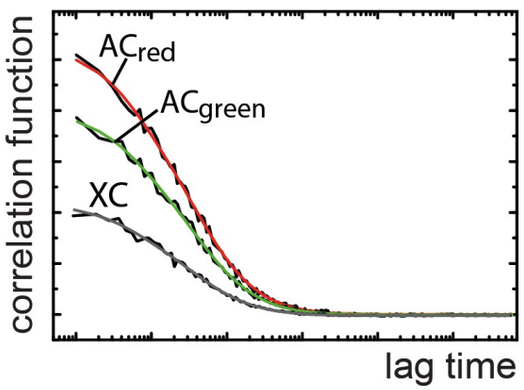
Cytoplasm: D = 26 ± 3 µm² s^{-1}

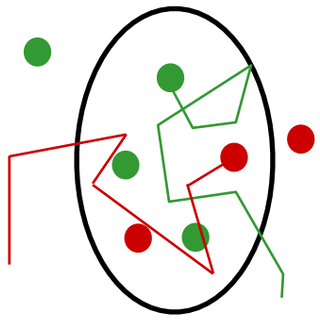
Fluorescence cross-correlation spectroscopy (FCCS)

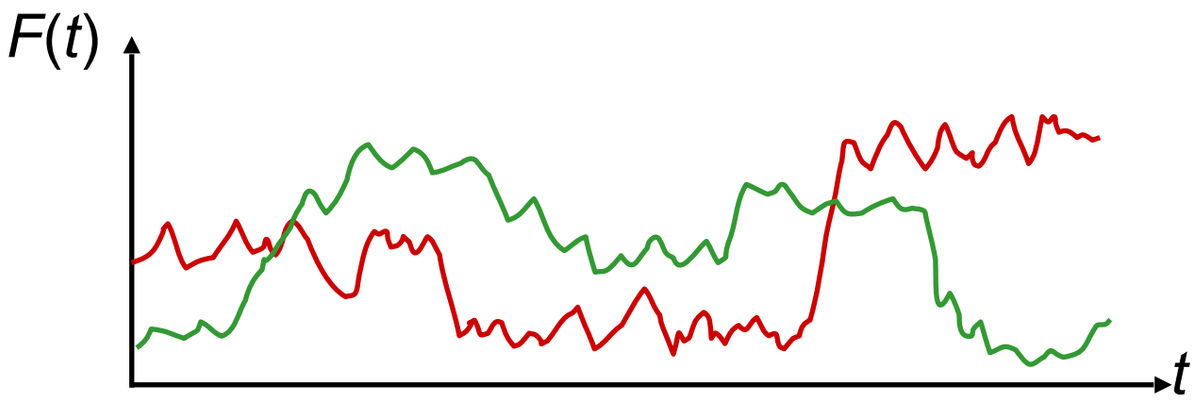




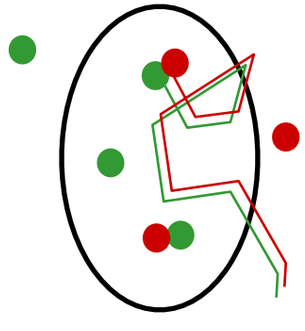


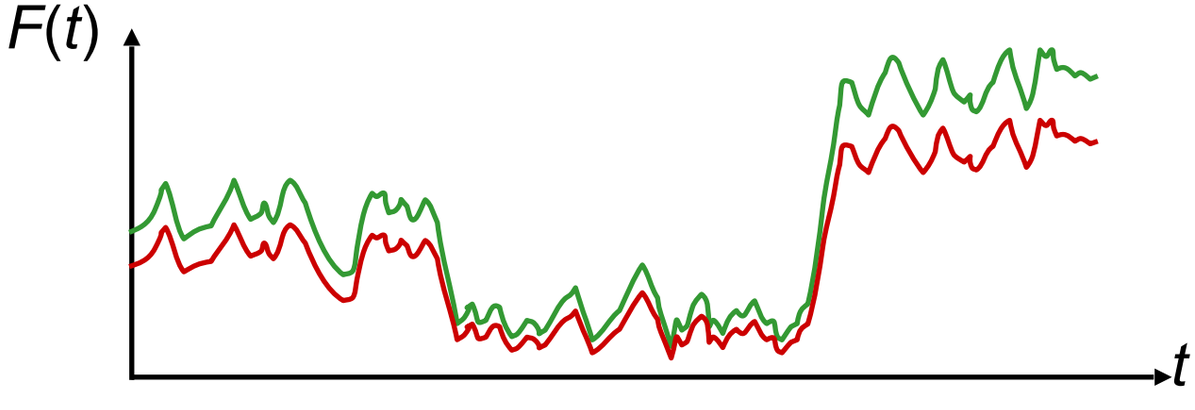






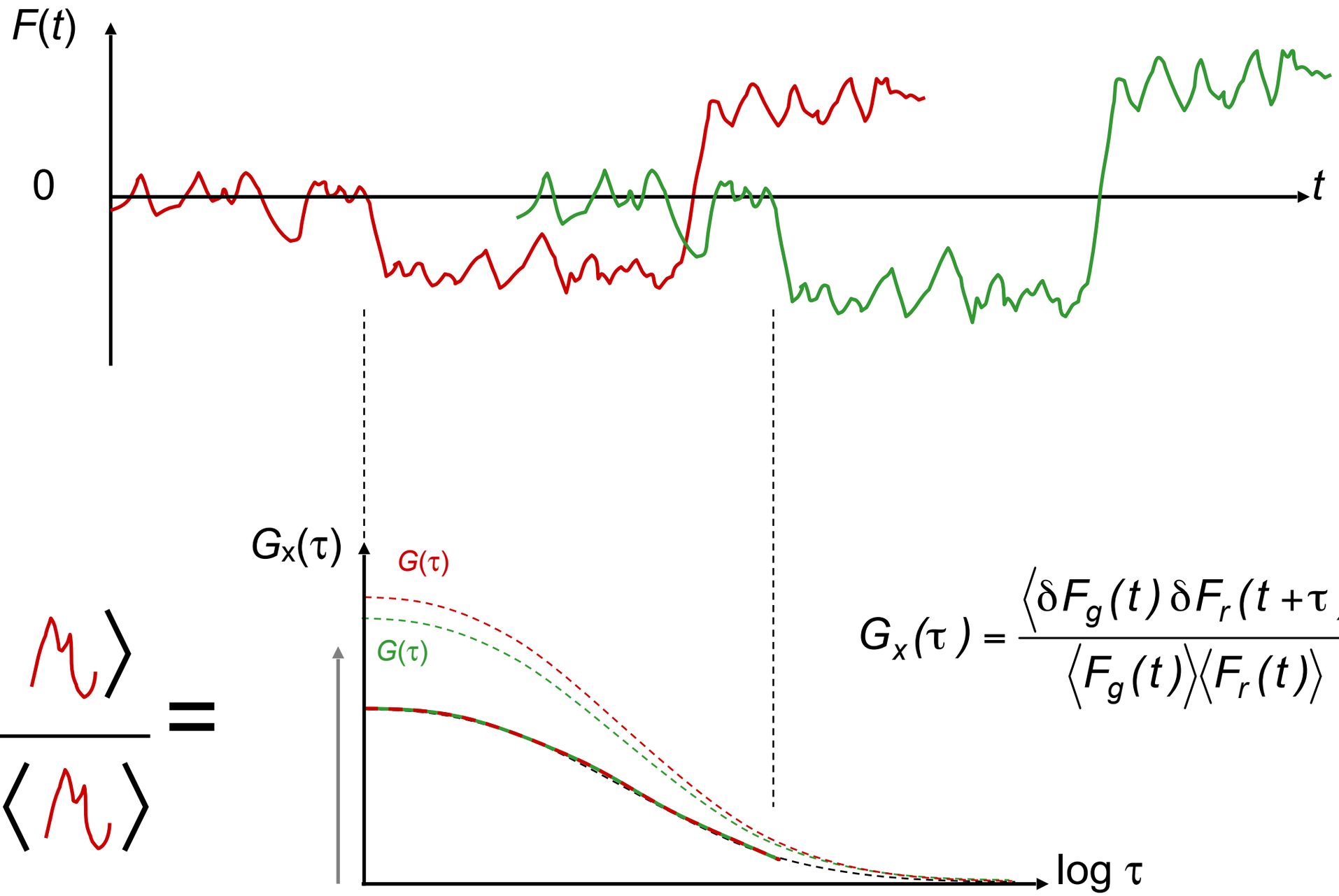
no correlation





correlation!

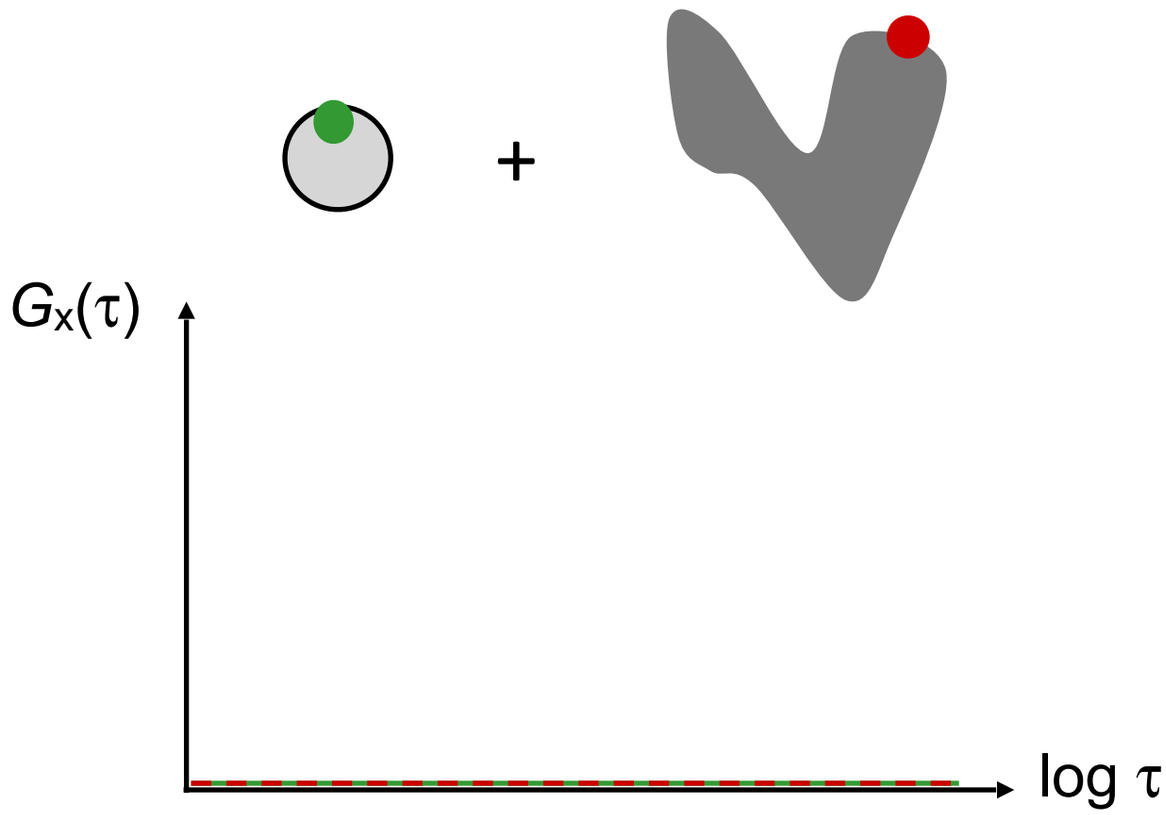
A cross-correlation analysis reveals the degree of molecular interactions

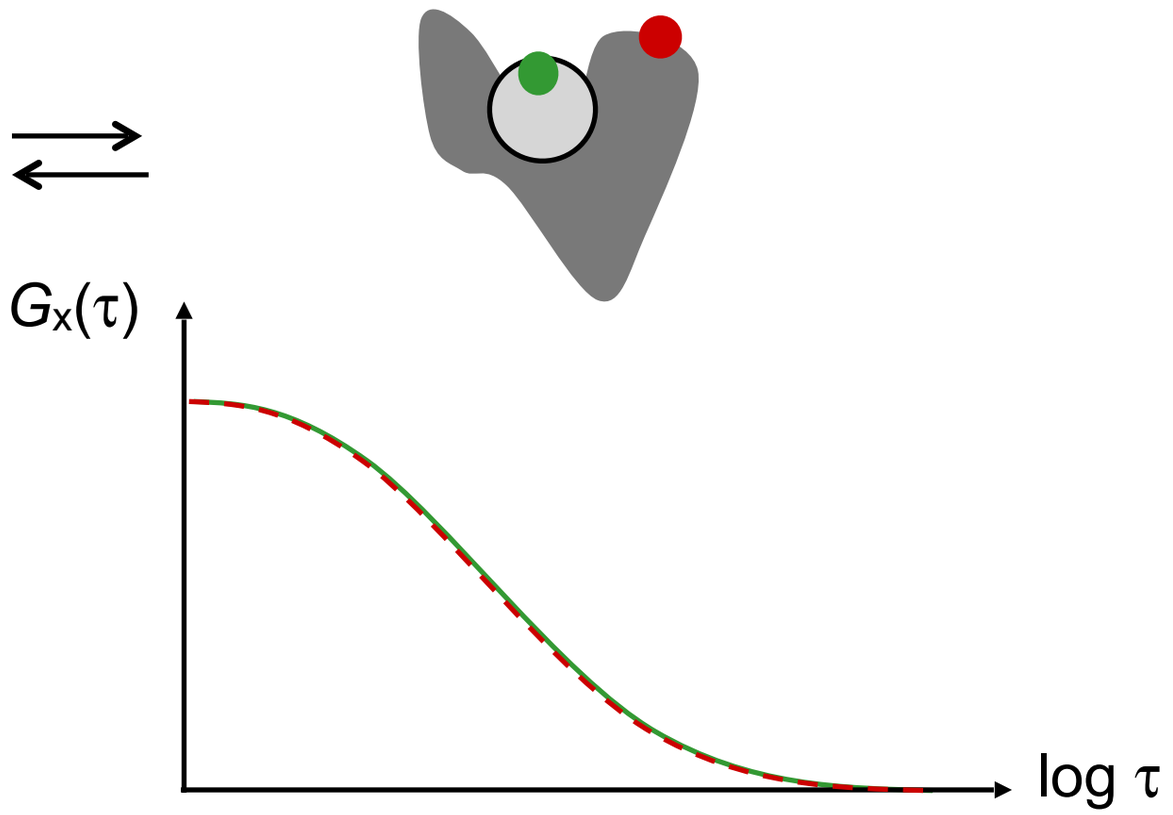


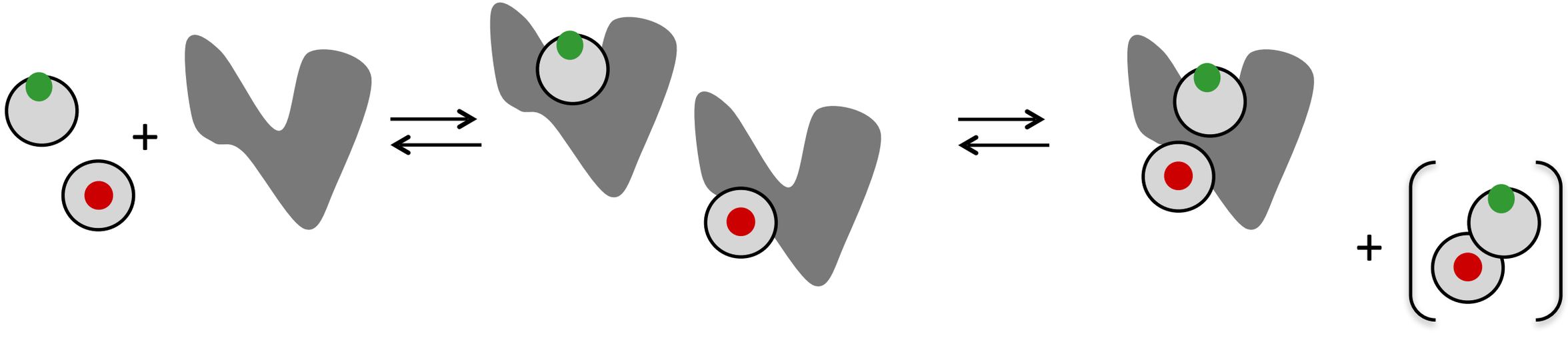
$$\frac{\langle \cdot \rangle}{\langle \rangle\langle \rangle} =$$

$$G_x(\tau) = \frac{\langle \delta F_g(t)\, \delta F_r(t+\tau) \rangle}{\langle F_g(t) \rangle \langle F_r(t) \rangle}$$

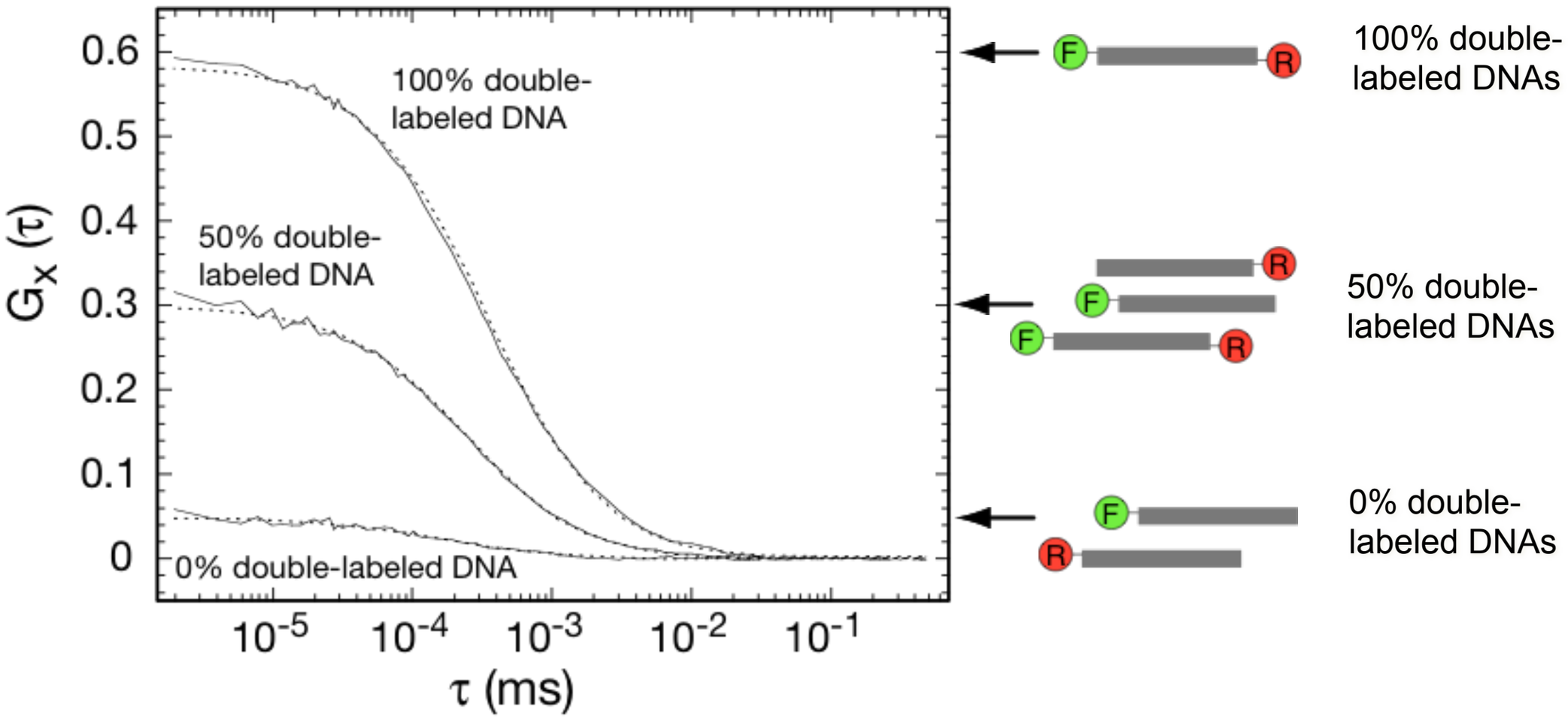
Studying ligand binding by FCCS



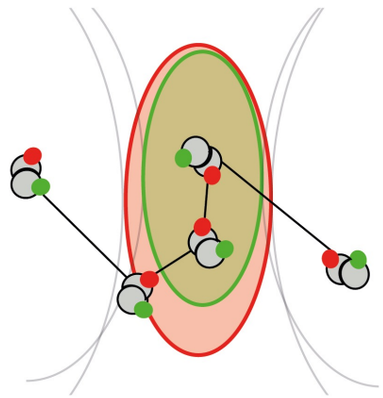




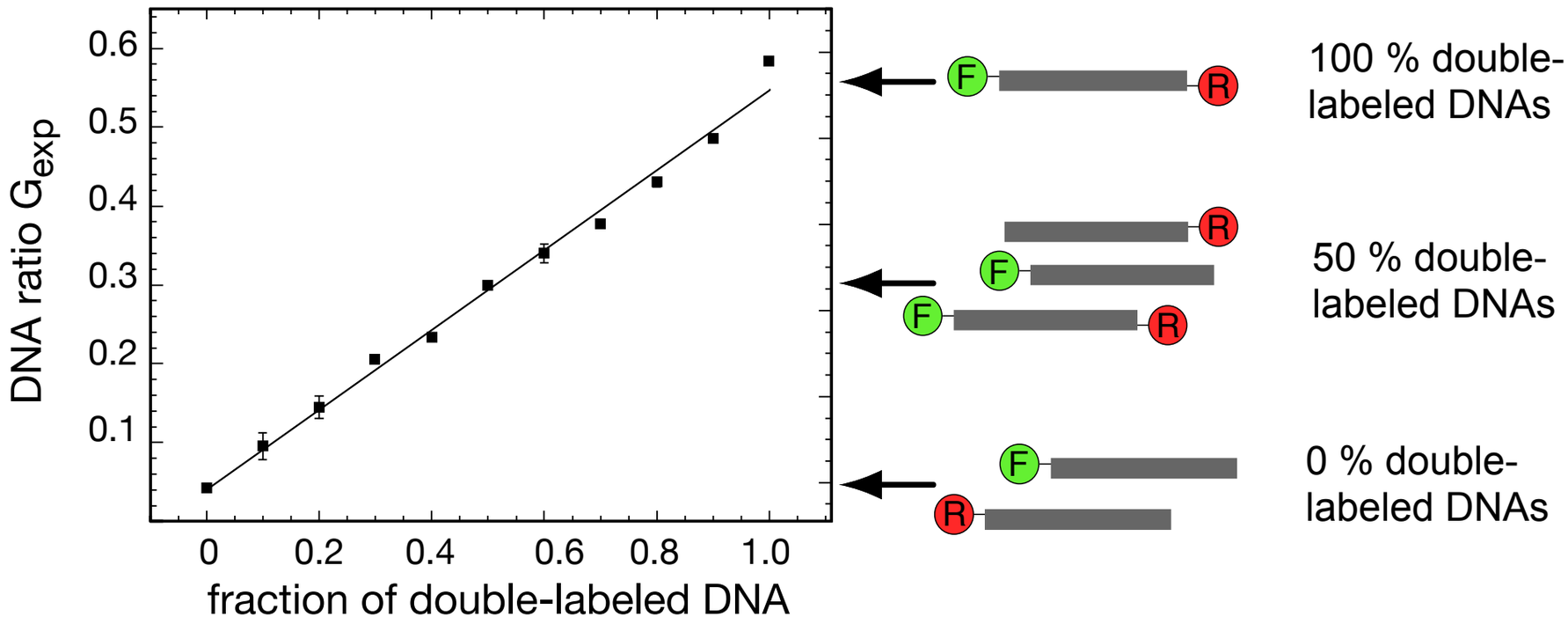
FCCS measurements of single and double-labeled DNAs



- $G_x(0)$ increases with the fraction of double-labeled DNAs
- cross talk between detection channels causes $G_x(0) > 0$ also for 0% double-labeled DNA
- $G_{x,max}(0) < 1$ due to incomplete overlap of excitation focus volumes



Quantitating the amount of double-labeled molecules



$$ratio\ G = \frac{G'_{x}(0)}{\sqrt{G'_{0,f}(0)G'_{0,r}(0)}}$$

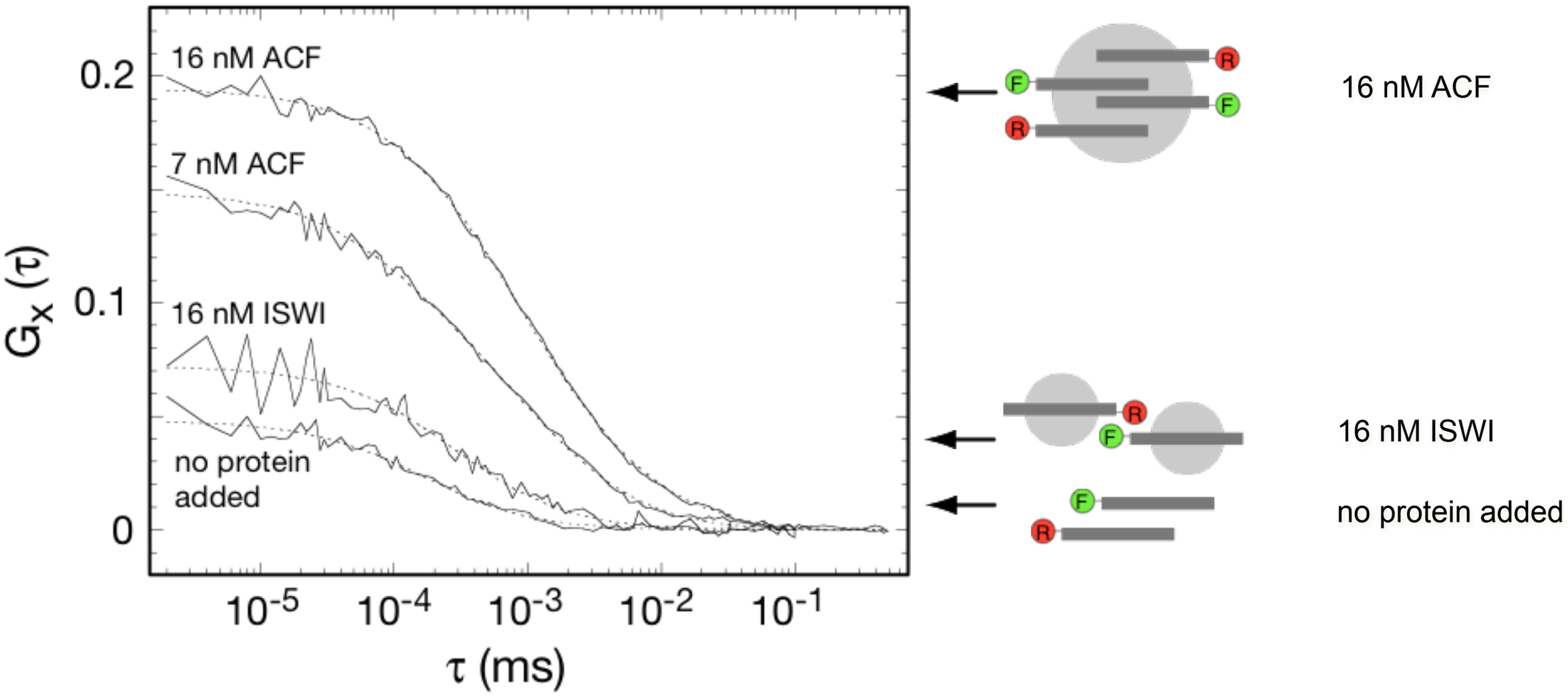
theoretical value of 1 for 100% double-labeled sample

instrument correction for cross-talk, chromatic abberrations etc.

$$ratio\ G_{cor} = \frac{ratio\ G_{exp} - ratio\ G_{min}}{ratio\ G_{max} - ratio\ G_{min}}$$

Rippe, K. (2000) *Biochemistry* **39**, 2131-2139.
Weidemann, T., Wachsmuth, M., Tewes, M., Rippe, K. & Langowski, J. (2002) *Single Molecules* **3**, 49-61.

Analysis of the DNA binding capacity of ACF and ISWI by FCCS



ISWI alone shows little cross-correlation above background

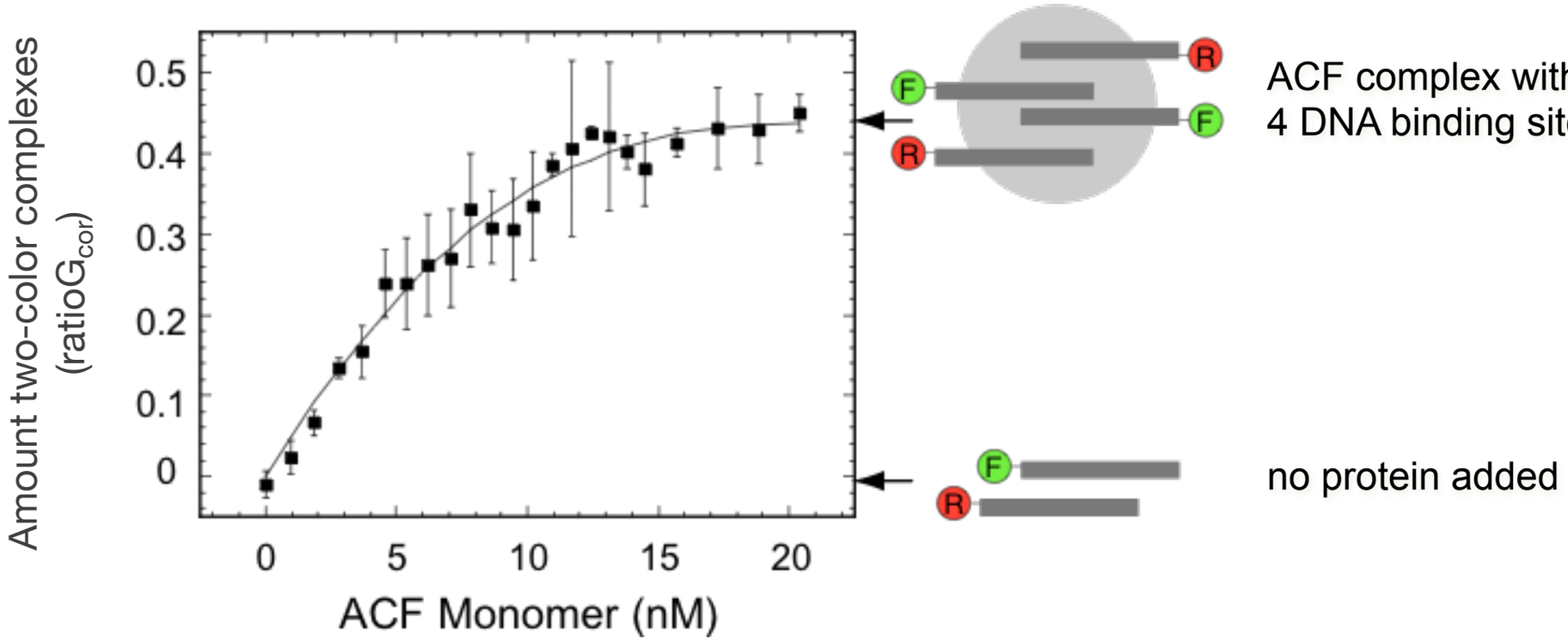
⇒ binds only a single DNA duplex (maybe some ISWI dimers present)

ACF shows strong cross-correlation signal

⇒ binds *at least two* DNA duplexes simultaneously

Strohner R et al., *Nat Struct Mol Biol* (2005)

Analysis of the DNA binding capacity of ACF and ISWI by FCCS



$ratioG_{cor} = f($binding sites n, subunits k, diss. const. K_d $)$

n = 4 DNA binding sites per ACF complex

k = 2.1 ± 0.8 ISWI-Acf1 subunits

K_d ~ 10 nM

Strohner R et al., *Nat Struct Mol Biol* (2005)