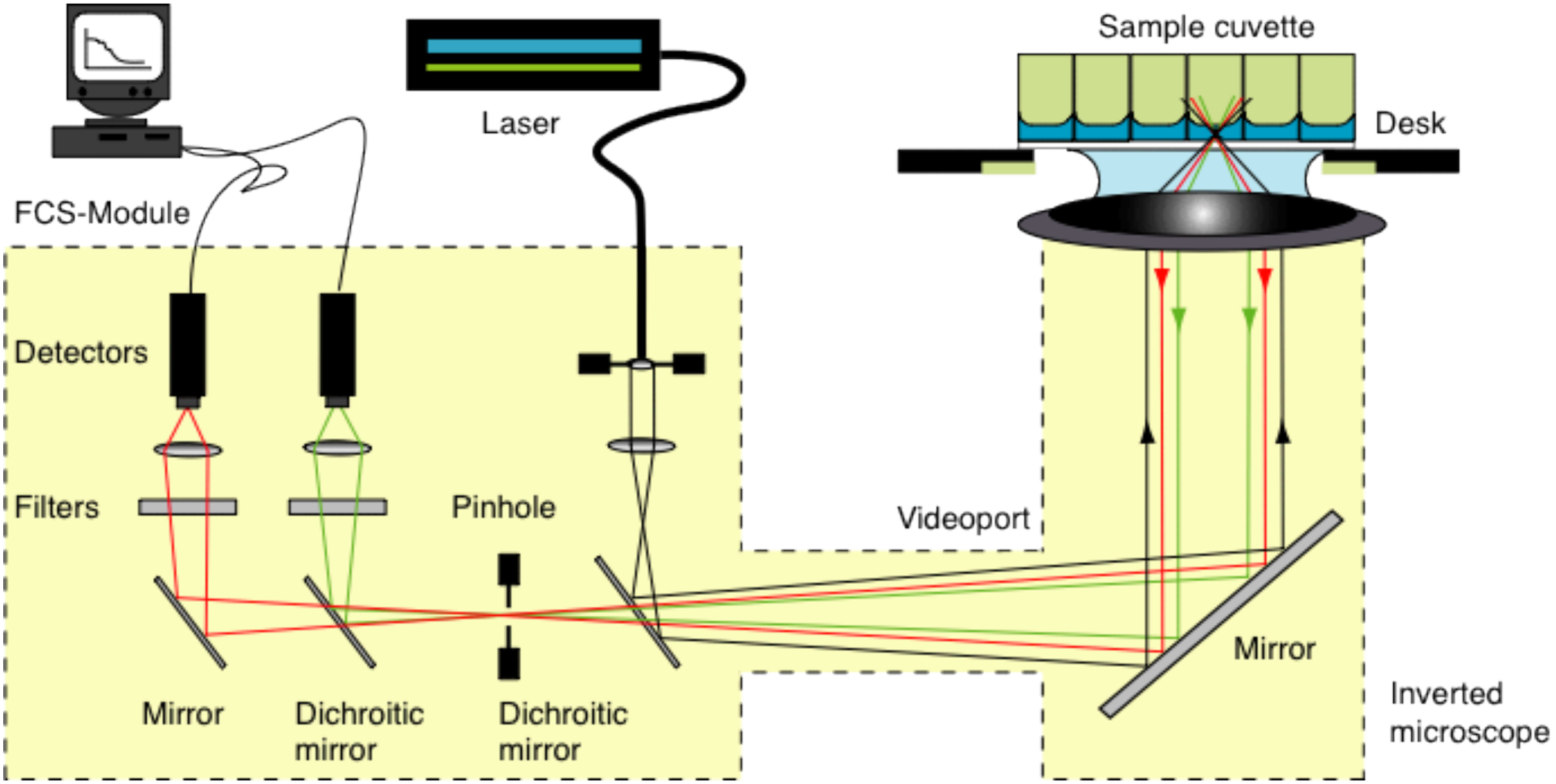
Fluorescence fluctuation microscopy: FRAP and FCS

Confocal laser scanning fluorescence microscope for the mobility analysis in living cells

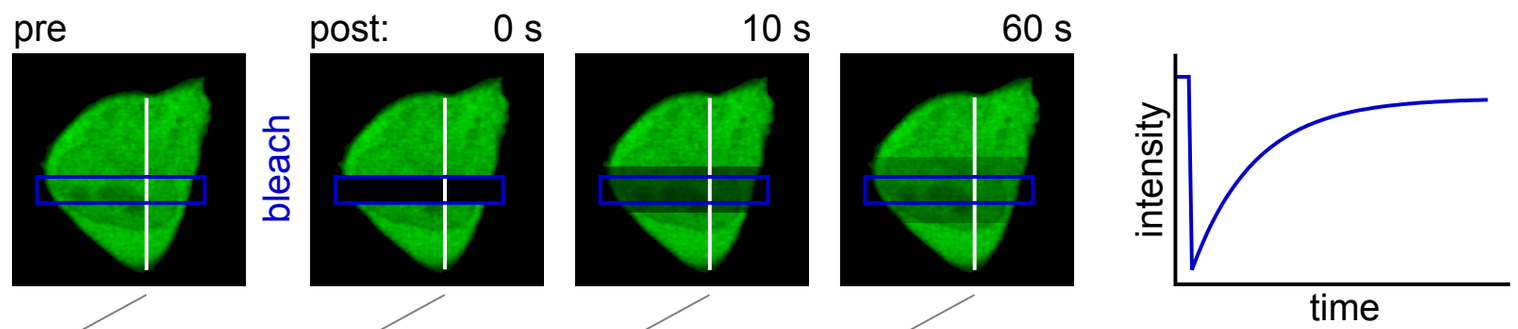


Ensemble measurements of protein mobility and interactions

spatial/temporal resolution

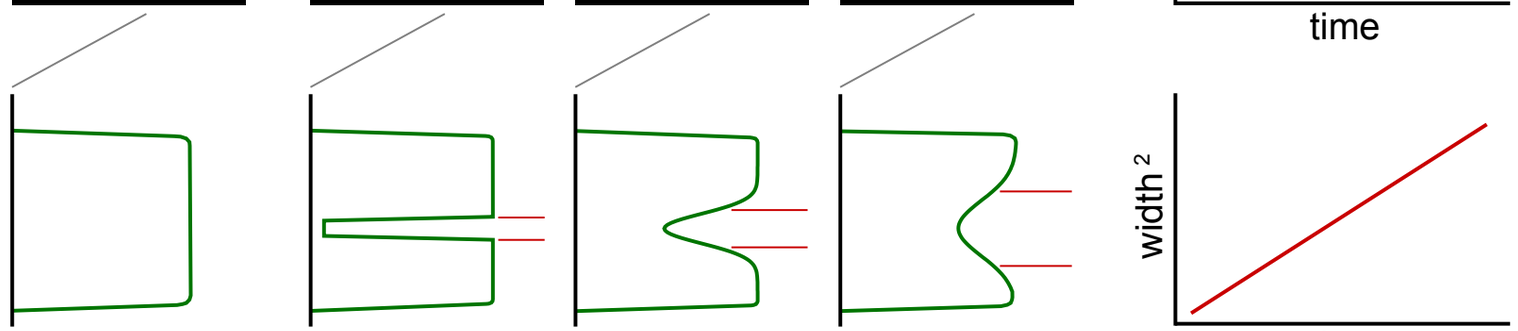
bleach once

FRAP intensity



1 µm
100 ms - hours

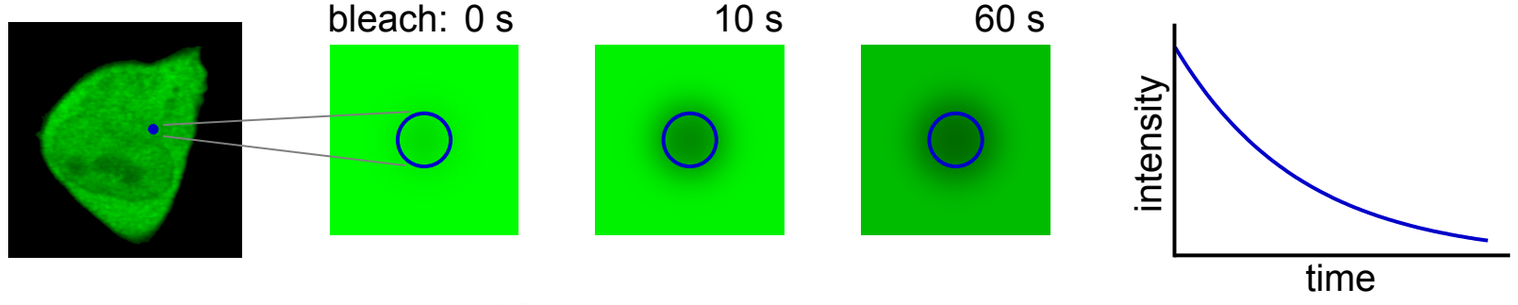
FRAP spatial analysis



10 µm
100 ms - hours

continue bleach

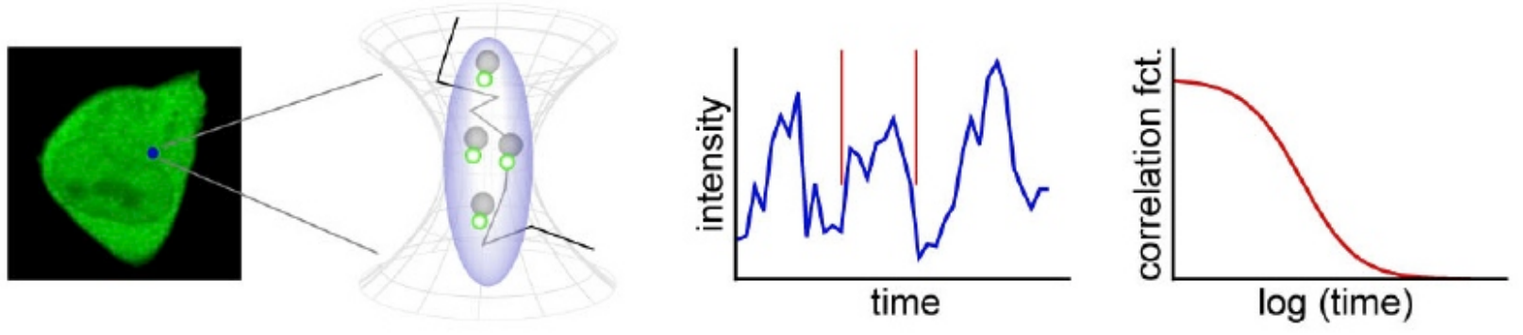
CP/ FLIP



1 µm
100 ms - hours

correlate

FCS



0.3 µm
10 µs - 1 s

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

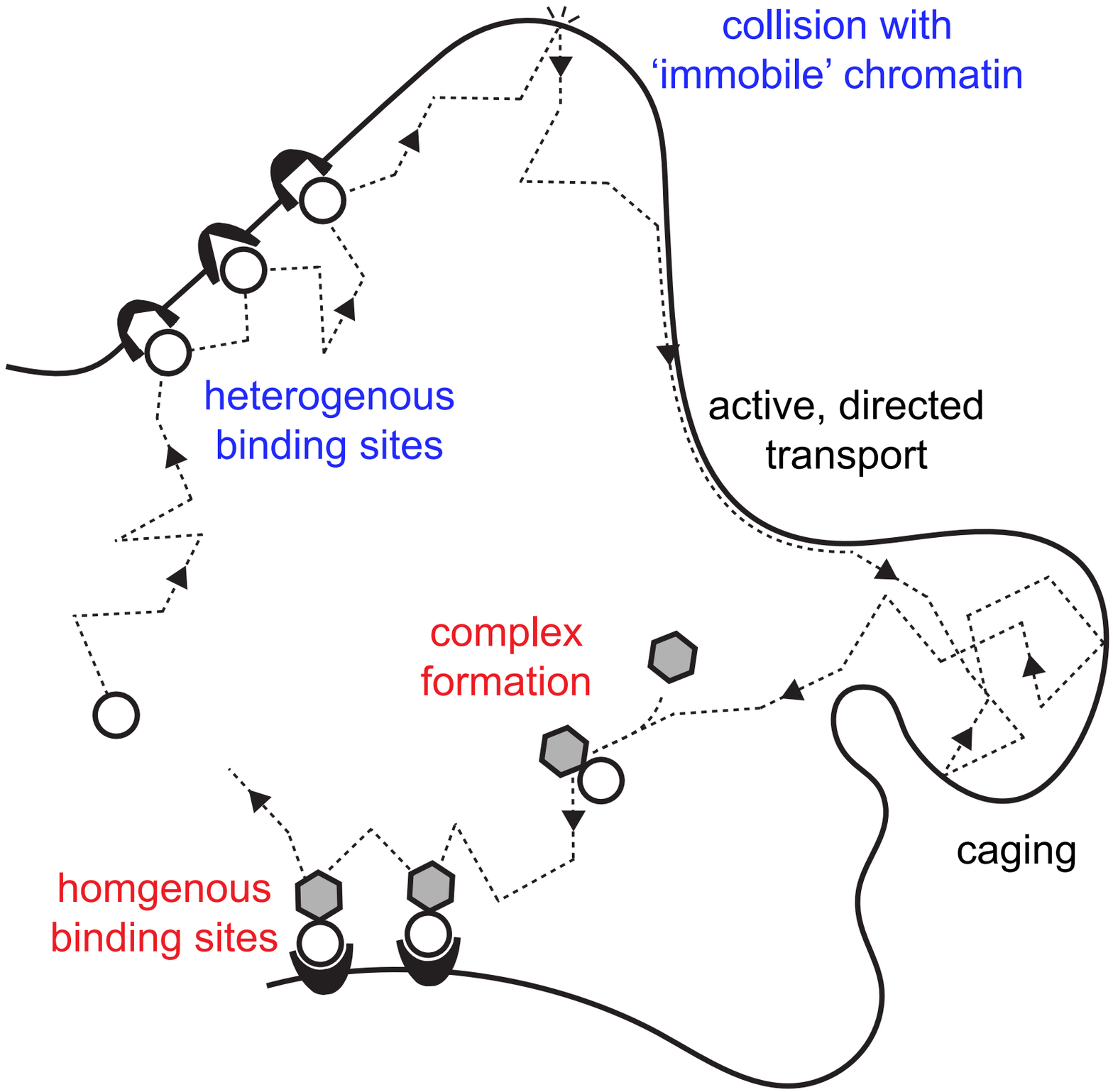
Methods comparison

FRAP (for slow/immobile particles, ~100 ms time resolution)
- diffusion coefficients
- rate constants (at immobile substrates)
- immobile fractions
- photoactivation instead of bleaching (k_{off} measurements)

CP/FLIP (for faster particles)
- dissociation rate (at immobile substrates)

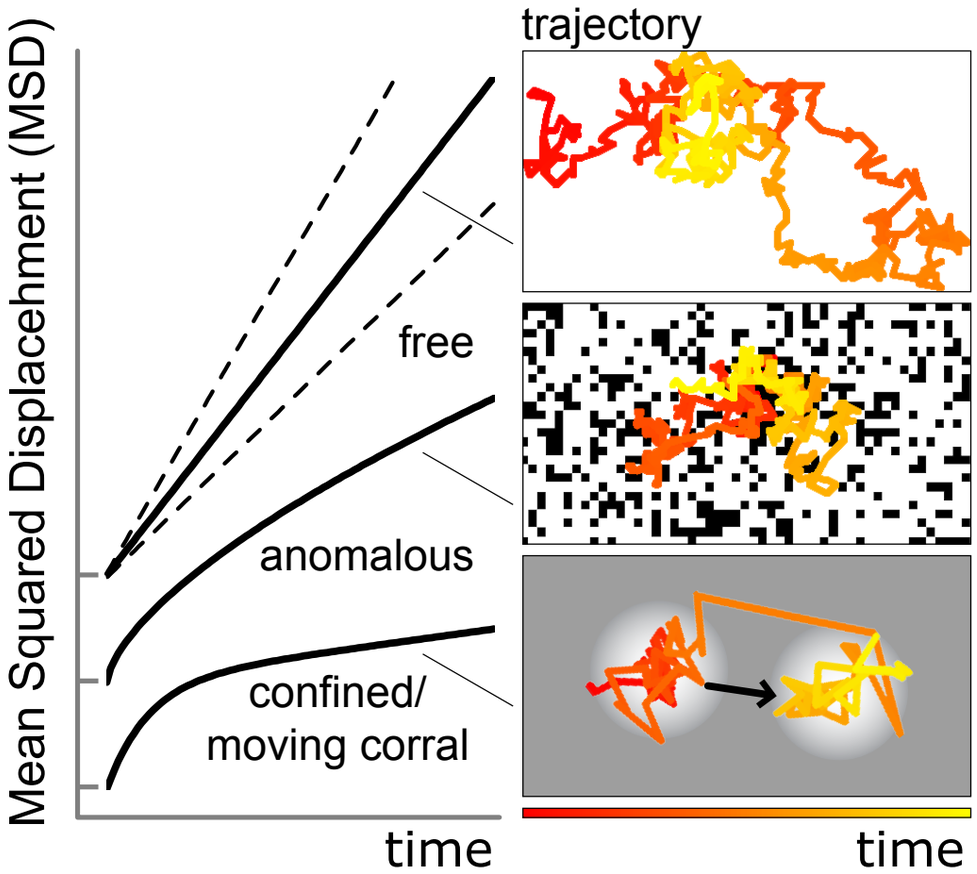
FCS (for fast particles, µs time resolution)
- diffusion coefficients
- rate constants (at mobile substrates)
- anomaly parameters
- concentrations

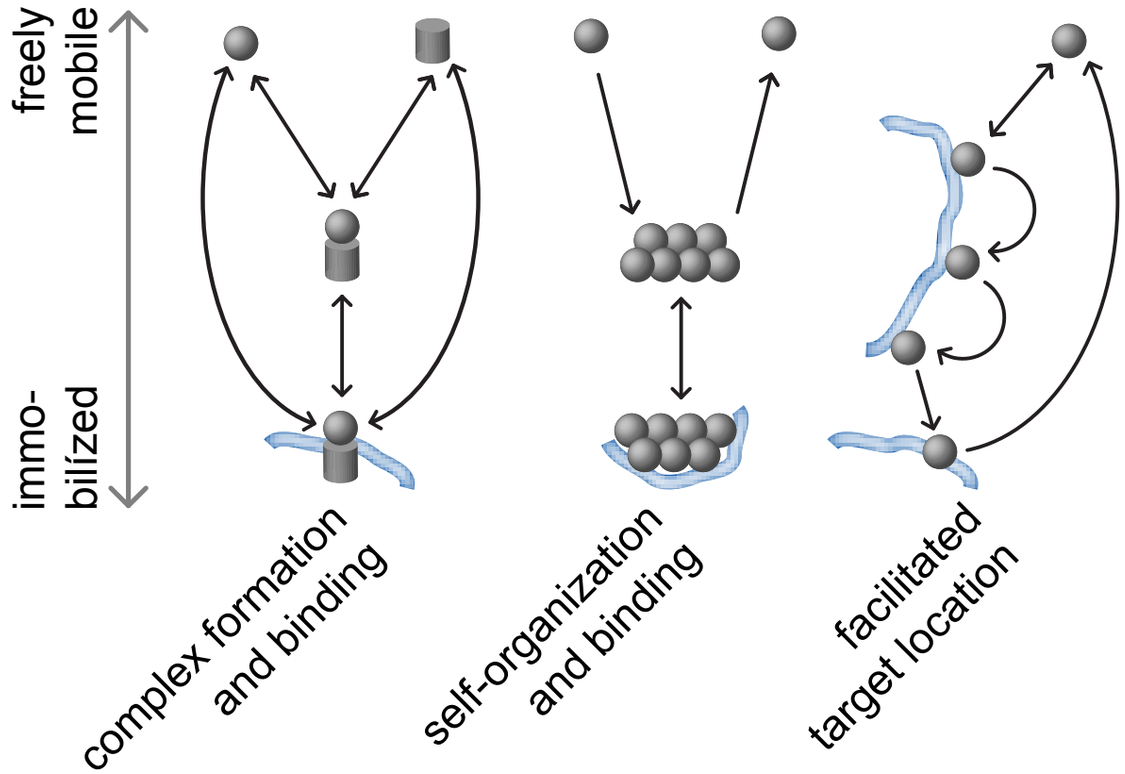
The problem: many things affect the observed mobility



Protein mobility and interactions in the cell

$$MSD = 6\,D\,t^{\alpha}$$





Dependence of diffusion coefficient D and molecular mass *M*

protein: $D \propto M^{-\frac{1}{3}}$ DNA: $D \propto M^{-\frac{1}{2}}$

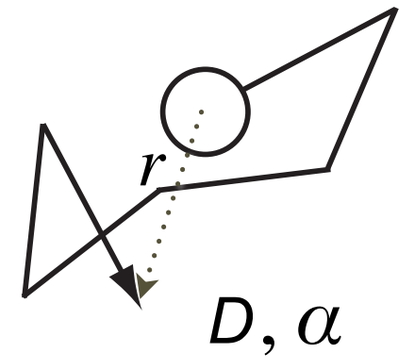
double mass *M* => 0.8 fold lower *D* double mass *M* => 0.7 fold lower *D*

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

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

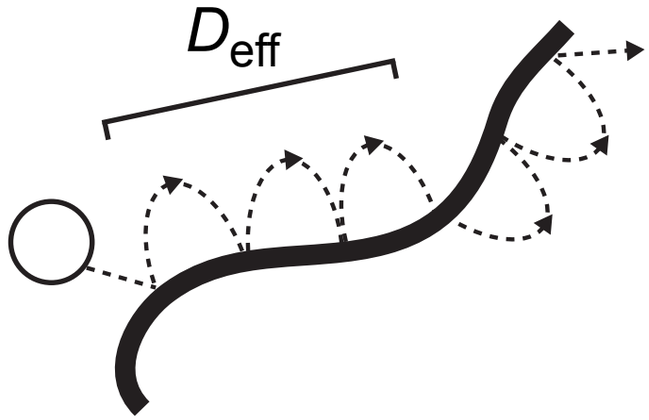
diffusion without binding,

α = 1 for free diffusion



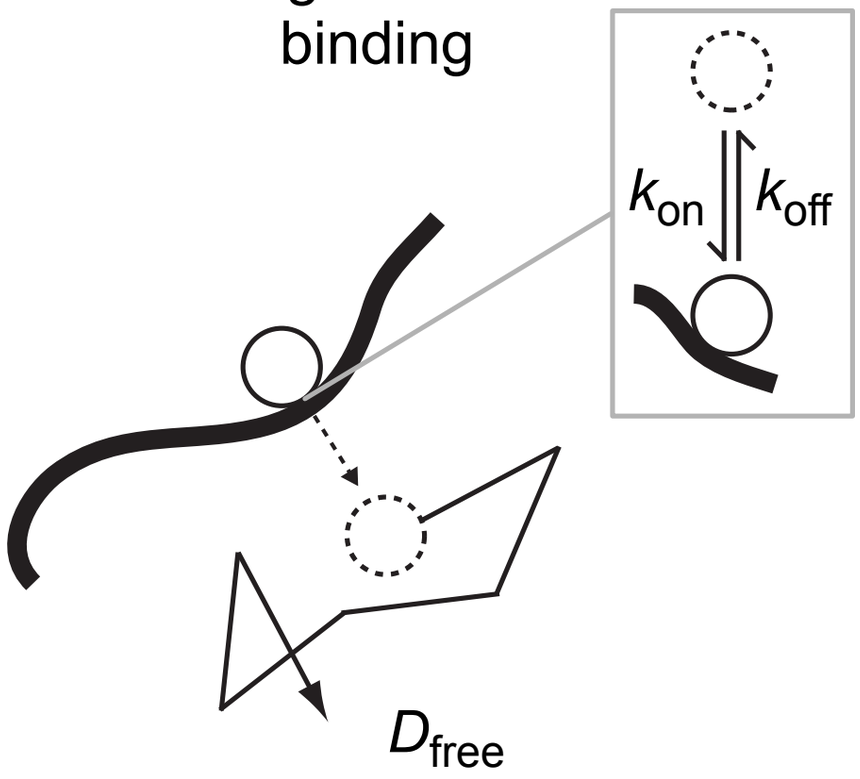
$$\left\langle r^2 \right\rangle = 6 \cdot D \cdot t^{\alpha}$$

transient chromatin binding



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

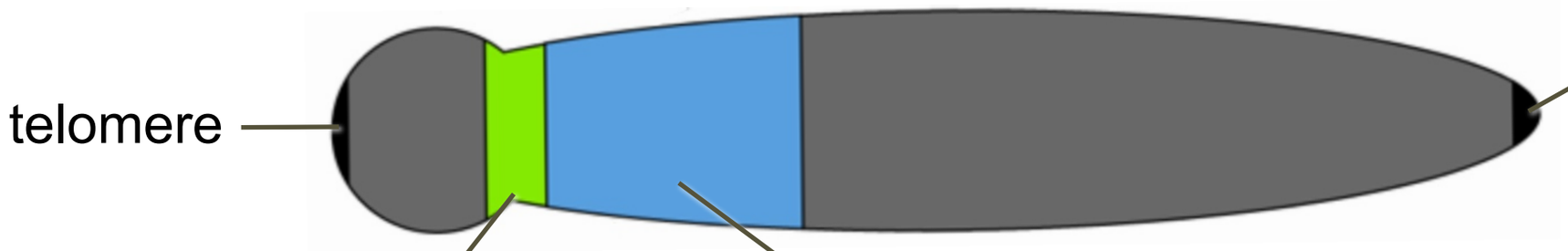
strong chromatin binding



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

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

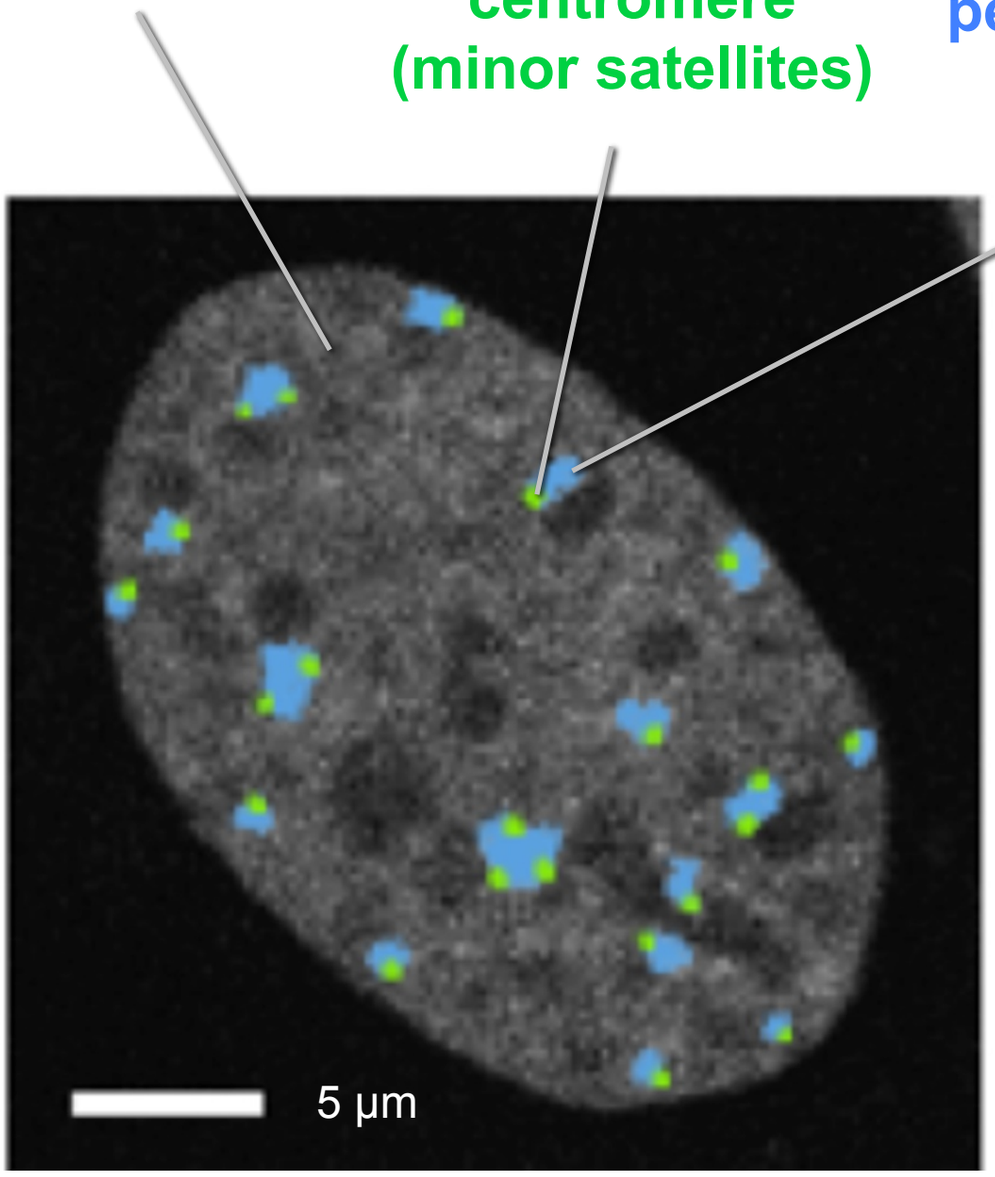
Pericentric heterochromatin (PCH) in mouse cells



“euchromatin”

centromere (minor satellites)

pericentric heterochromatin (PCH) (major satellites repeats)

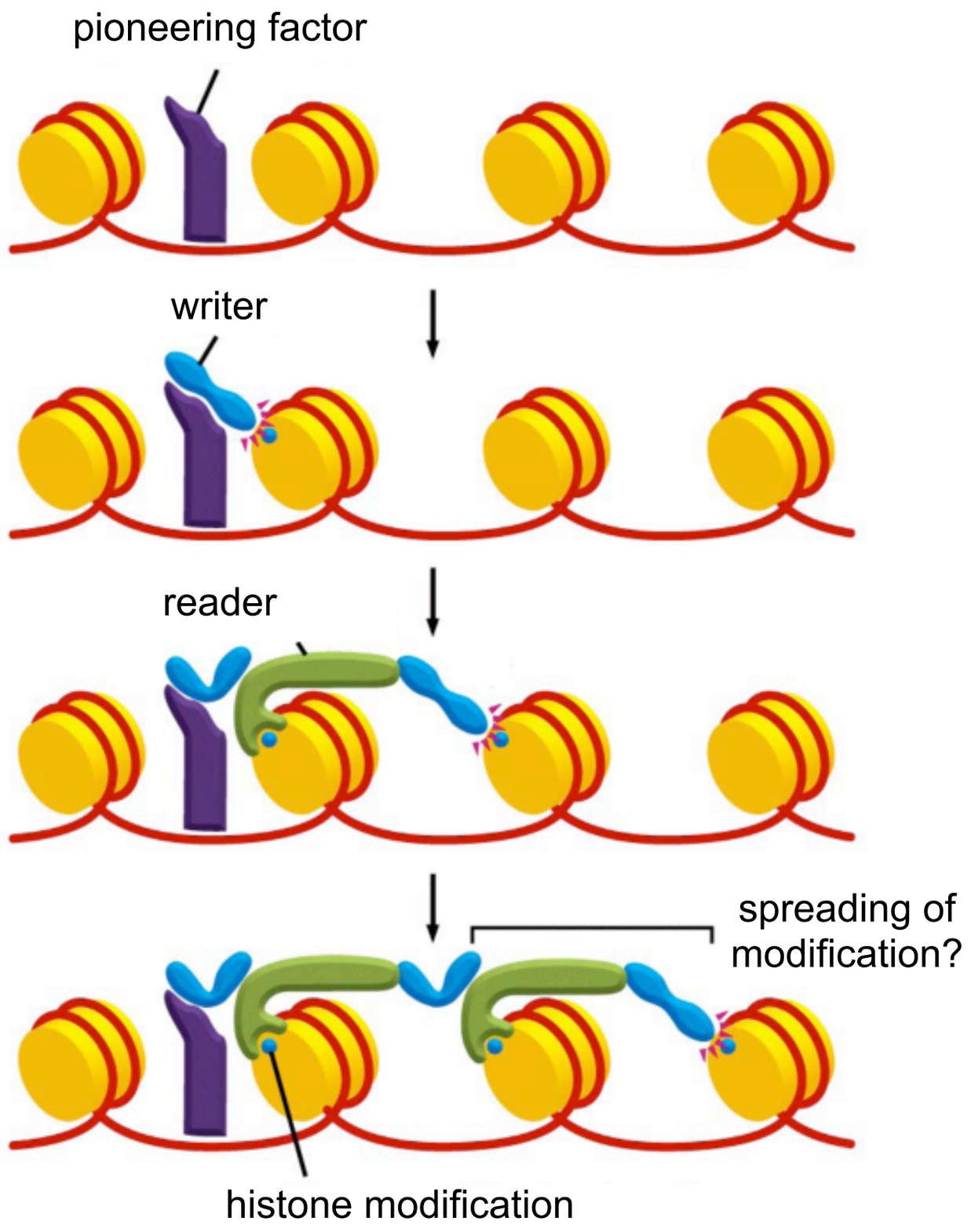


Mouse pericentric heterochromatin

- “chromocenters” visualized by DAPI staining
- 5meC, H3K9me3, H4K20me3
- HP1, Suv39h, Suv4-20h
- silences transcription of repeats (requires Suv39h)
- important for chromosome segregation

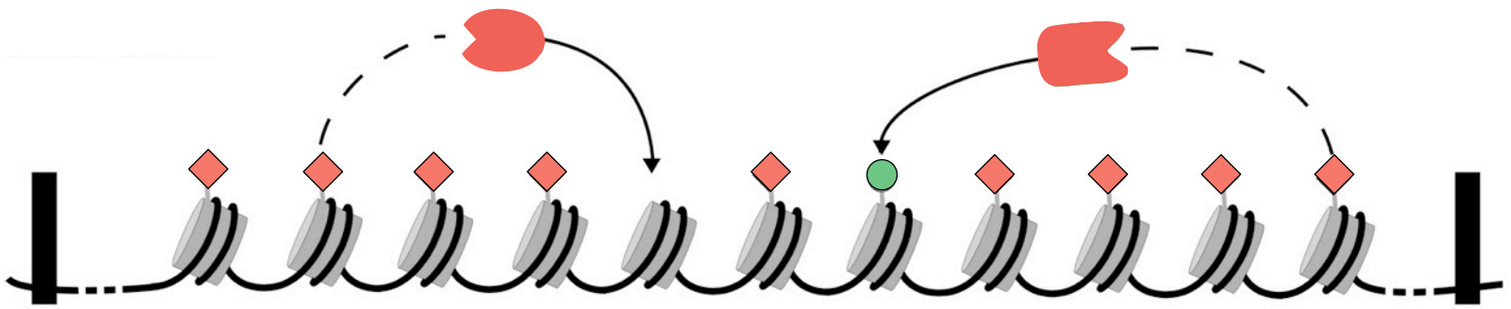
Specificity, propagation and epigenetic memory?

Writing, reading and transmitting epigenetic signals



from Molecular Biology
of the Cell

Distinct chromatin states can be established and maintained via interlinked feedback loops



silenced H3K9me3
heterochromatin

H3K9me3

histone H3
lysine 9
methylation

DNA methylase

DNMT

HP1

SUV39h

histone
methylase

SUV39h, DNMT

Jmjd2

histone
demethylase

5meC

MeCP1

HDAC

histone
deacetylase

histone
acetylase

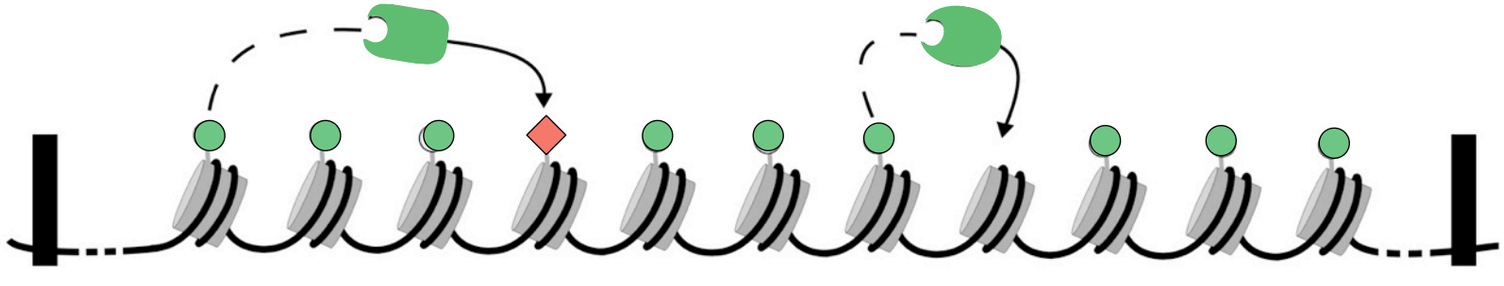
HAT

HAT

HDAC

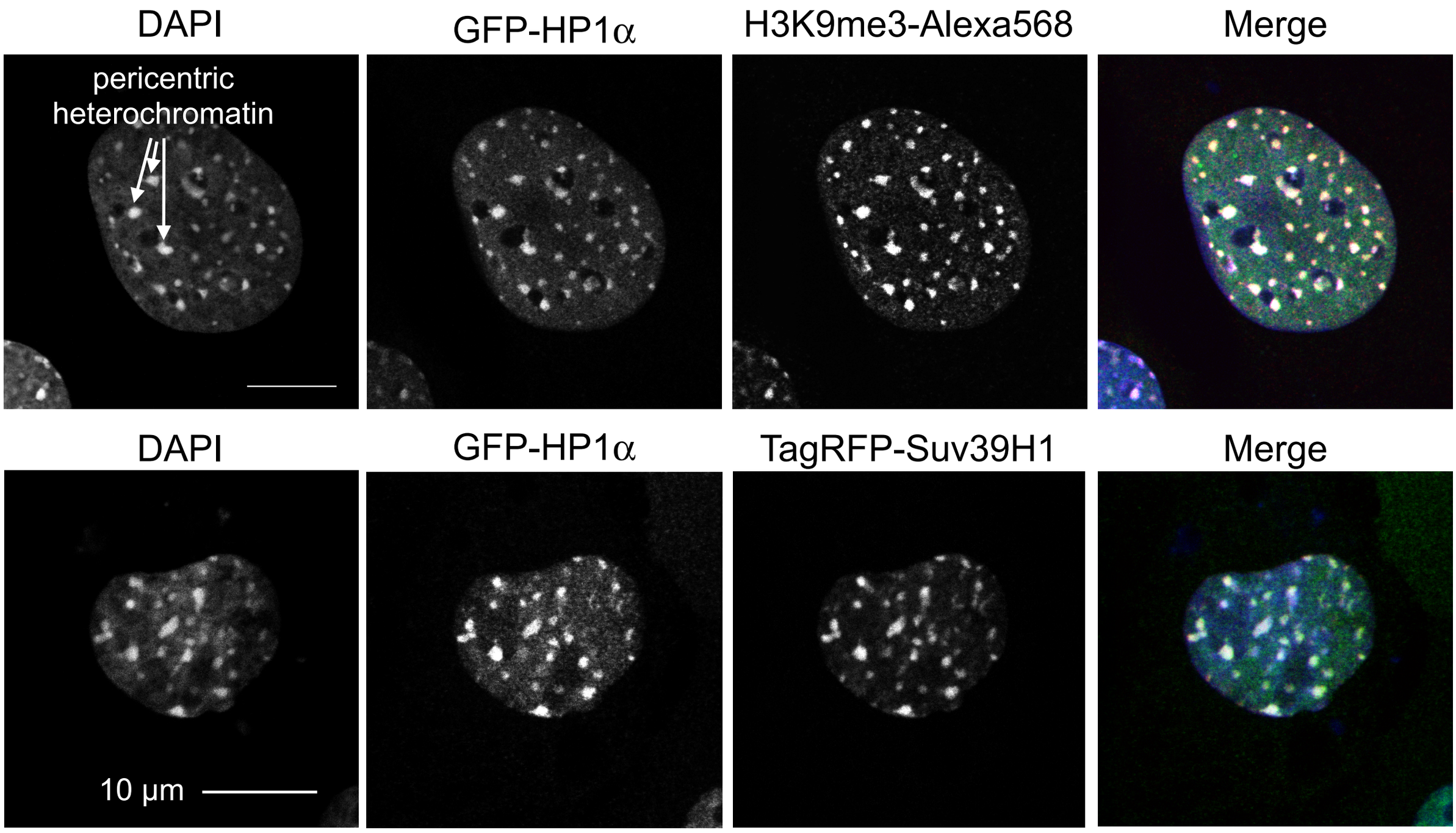
histone H3
lysine 9
acetylation

H3K9ac



active H3K9ac
euchromatin

Colocalization of heterochromatin protein 1 (HP1), Suv39H1 histone methylase and H3K9 methylation

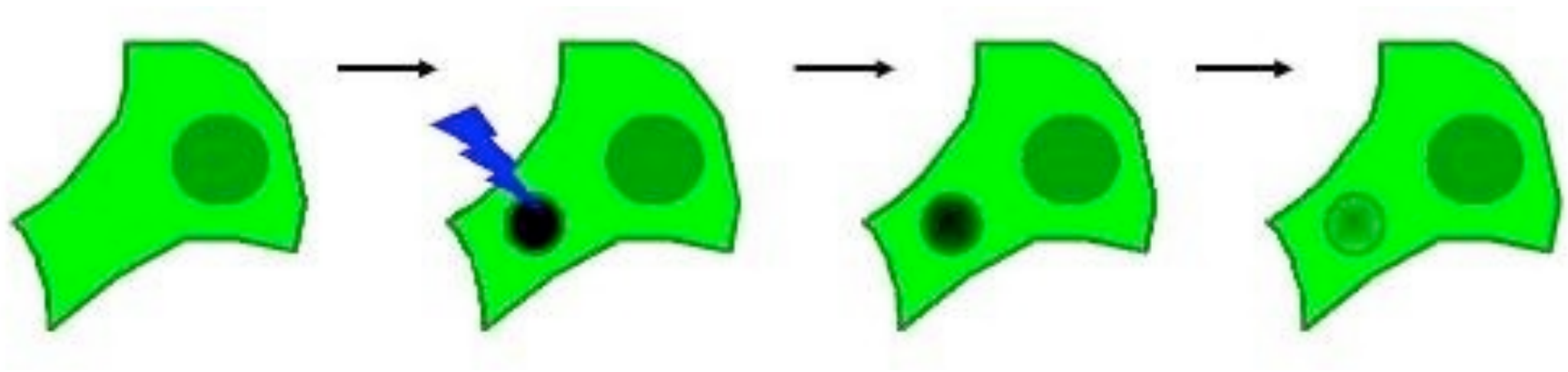


Fluorescence Recovery After Photobleaching (FRAP)

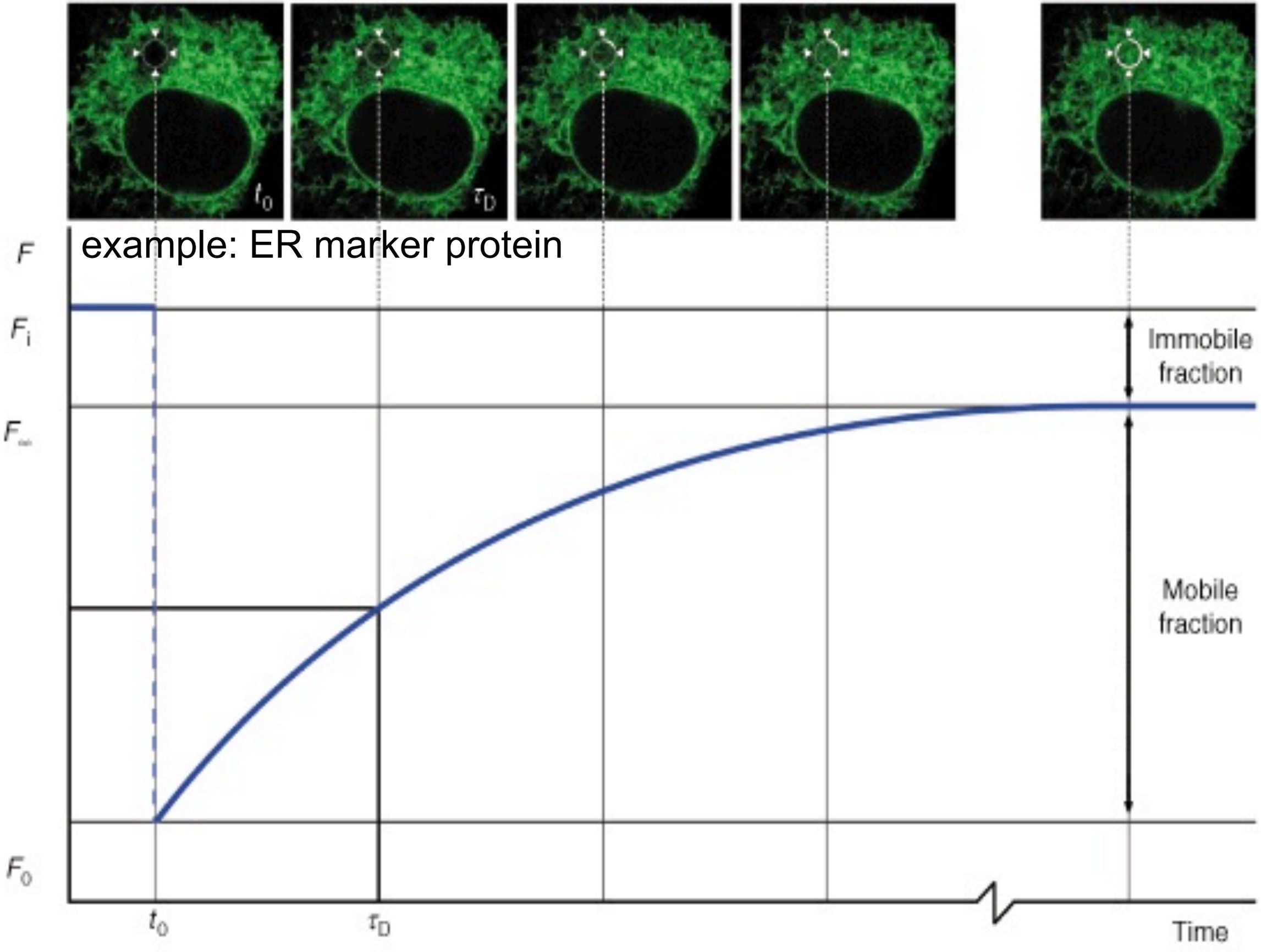
Fluorescence Recovery After Photobleaching (FRAP)

Protocol:

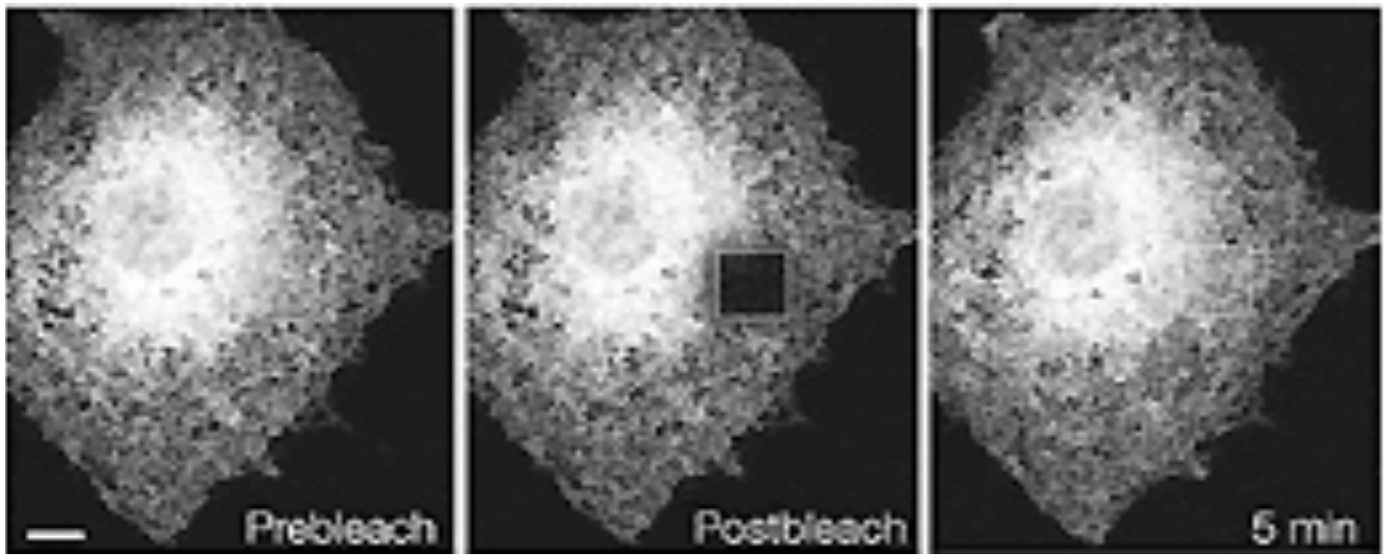
1. Bleach particles (with a laser)
2. Wait and watch during they diffuse away
3. Fit the fluorescence-over-time curve or profile

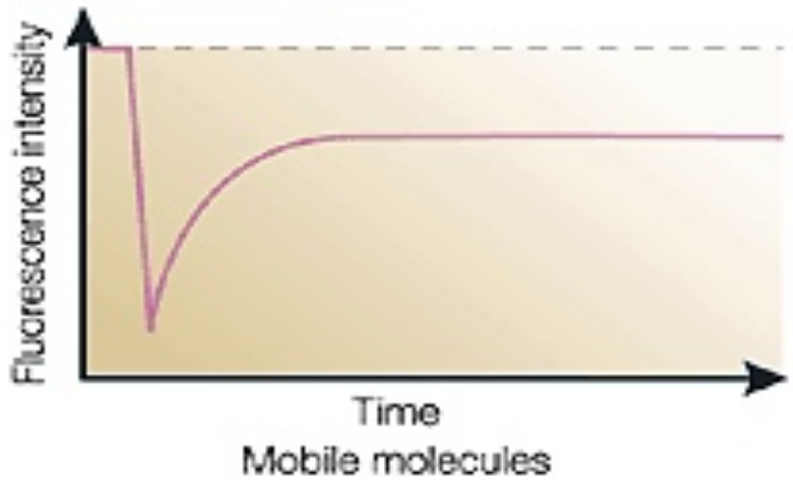


Source: Wikipedia

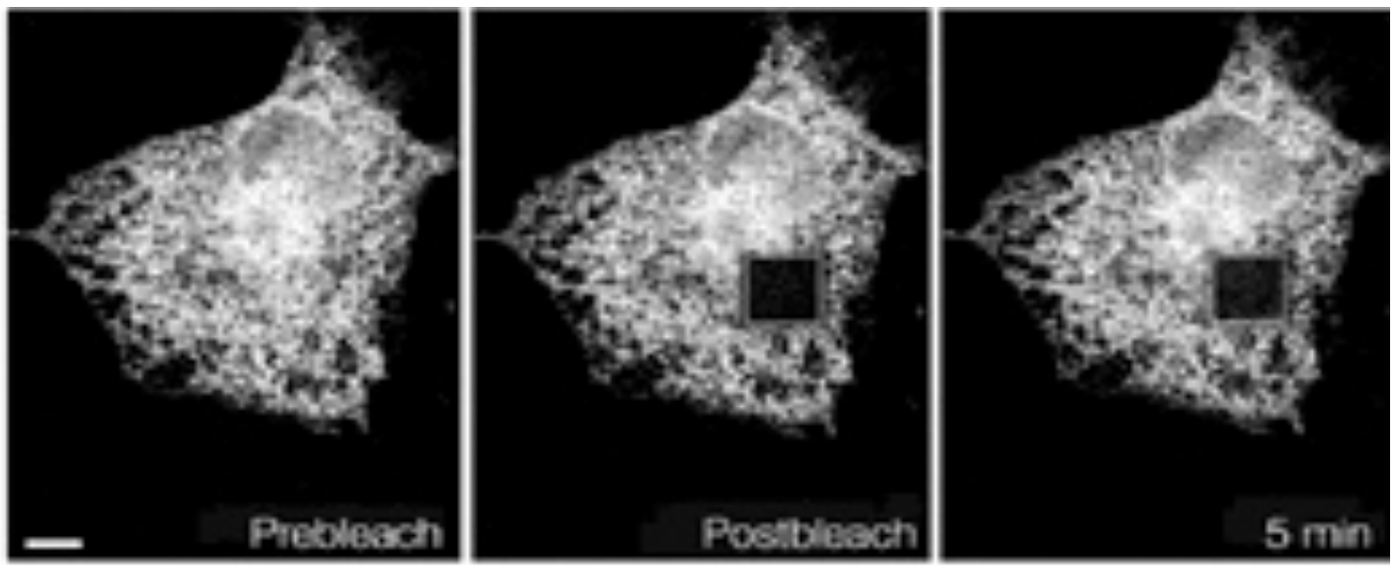


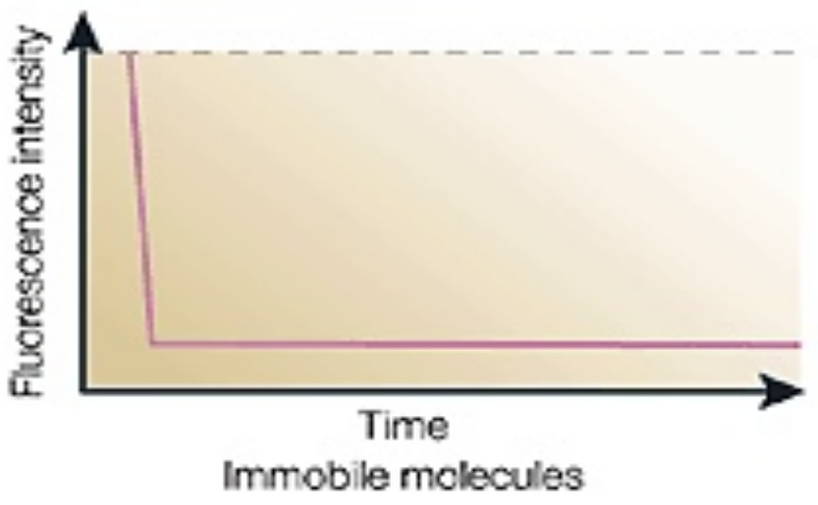
Reits et al. 2001 (From fixed to FRAP: measuring protein mobility and activity in living cells)





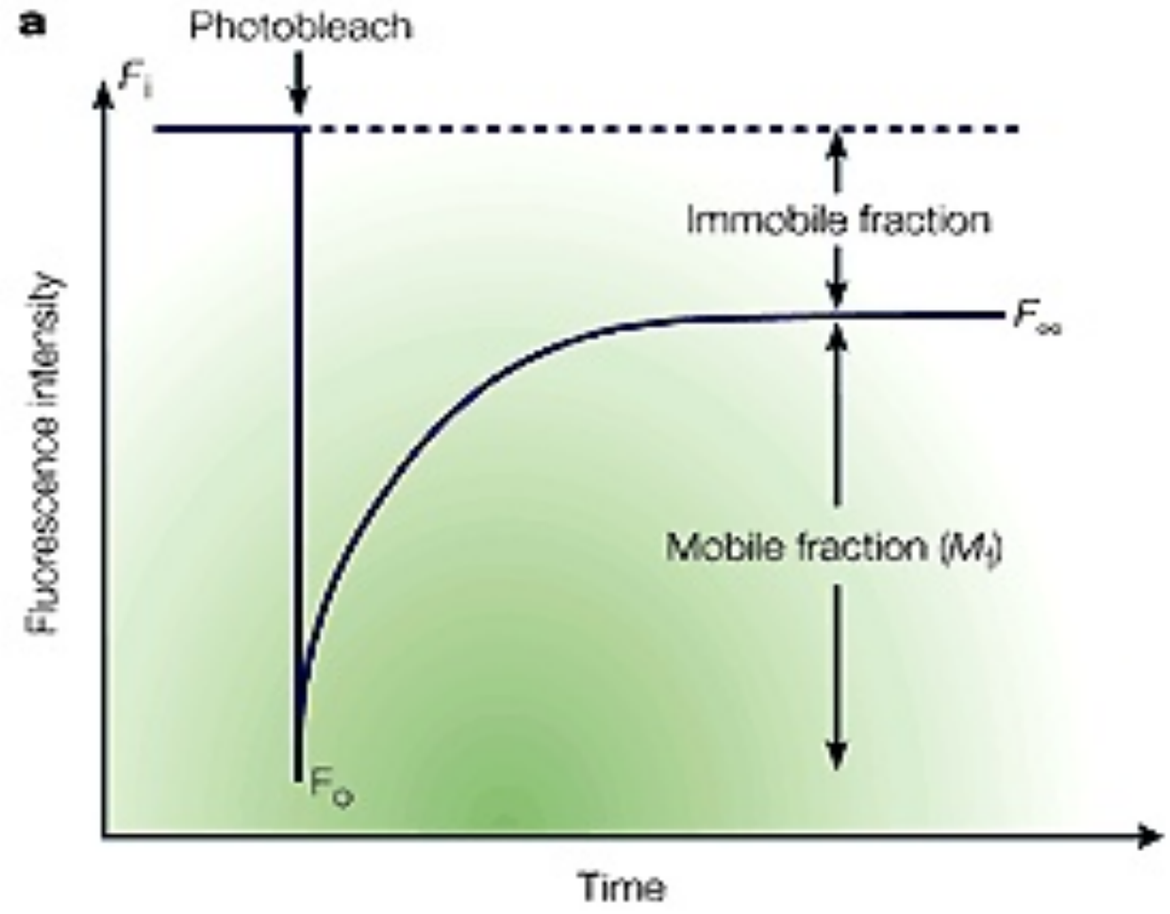
mobile protein





immobile protein

Typical FRAP curve



F_i: f.i. before bleaching
F_0: f.i. just after bleaching
F_∞: f.i. in recovery region

mobile fraction $M_f = \frac{F_\infty - F_0}{F_i - F_0}$

characteristic diffusion time $\tau_D = \frac{w^2}{4D}$

bleach radius w
diffusion coefficient D

Equation for the recovery curve in the absence of binding:

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

I_0, I_1: Modified Bessel functions

FRAP with fast binding

Fast binding: Reduction of the diffusion coefficient, **shape** of the curve remains **unchanged**

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

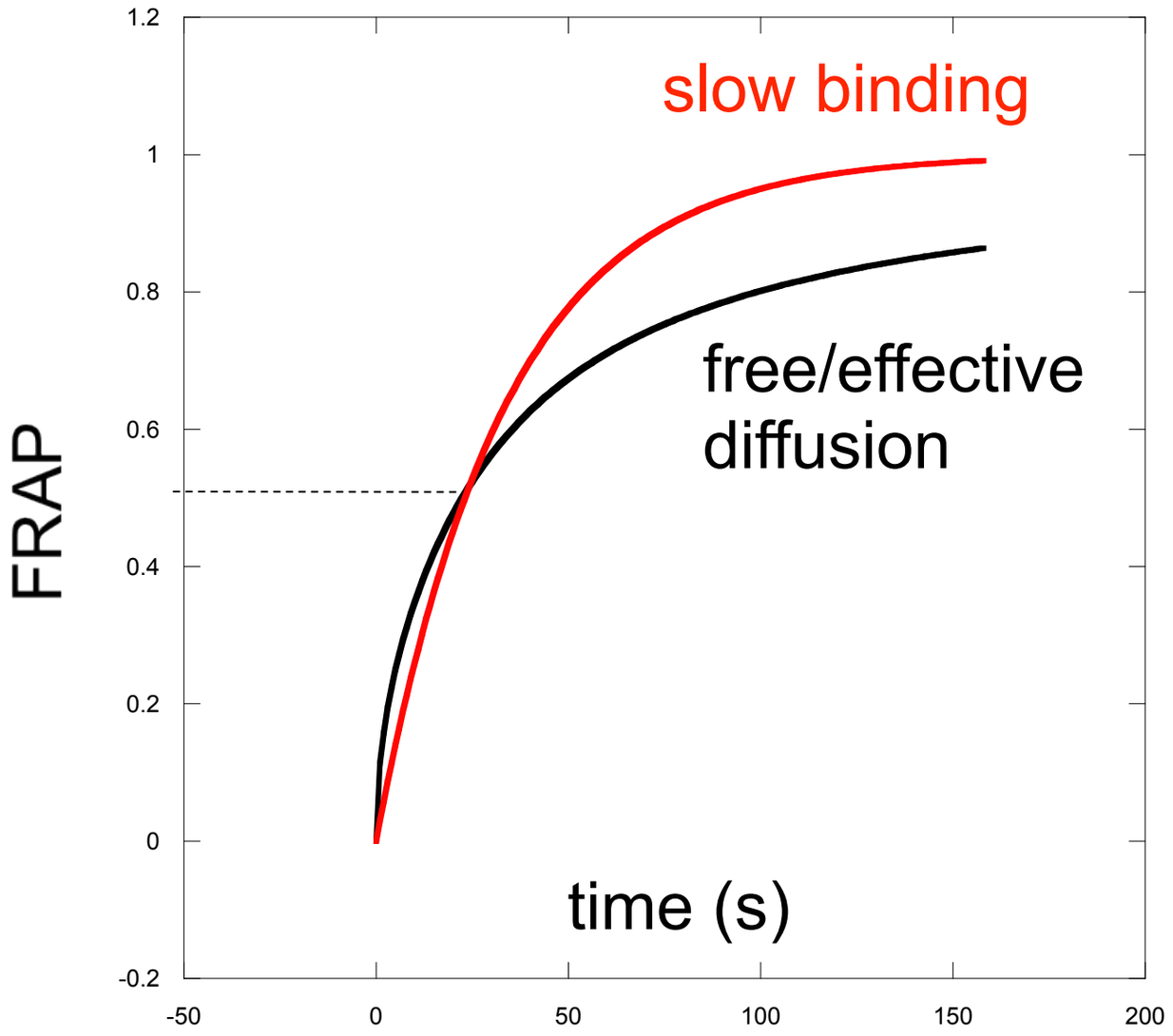
Only one fit parameter: Effective diffusion coefficient

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

If *D* is known, the ratio of the rate constants is obtained

FRAP with slow binding

Long-lived binding events lead to different shape of recovery curve



Effective diffusion (no or fast binding):

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

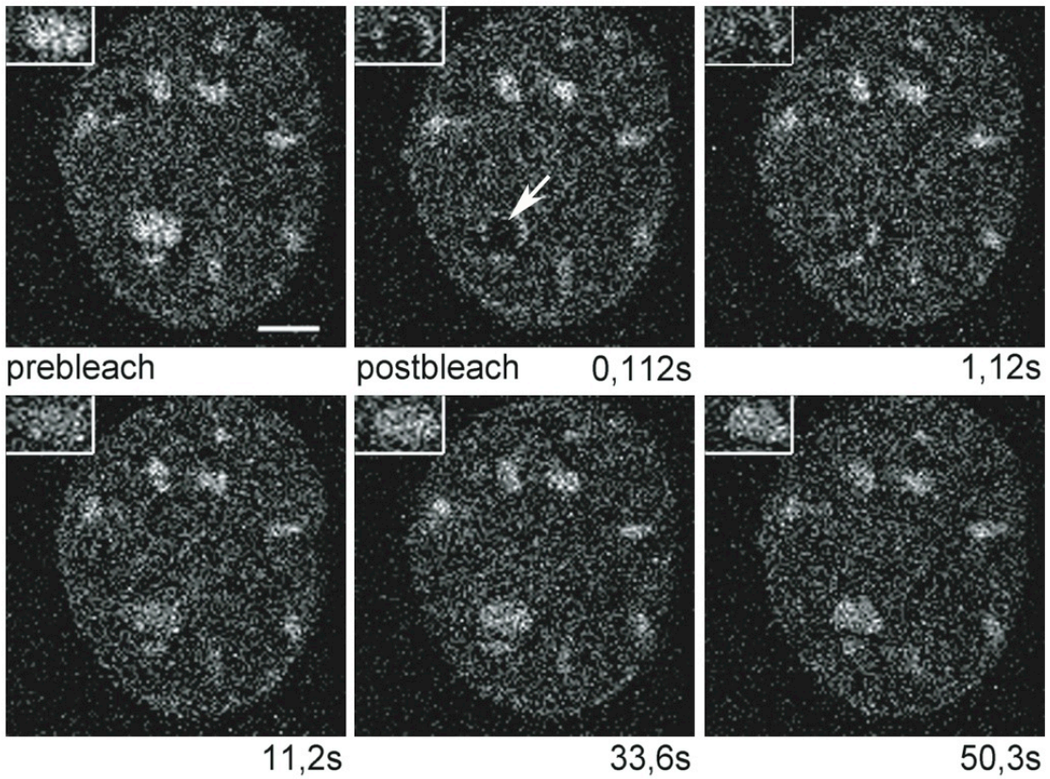
Slow binding:

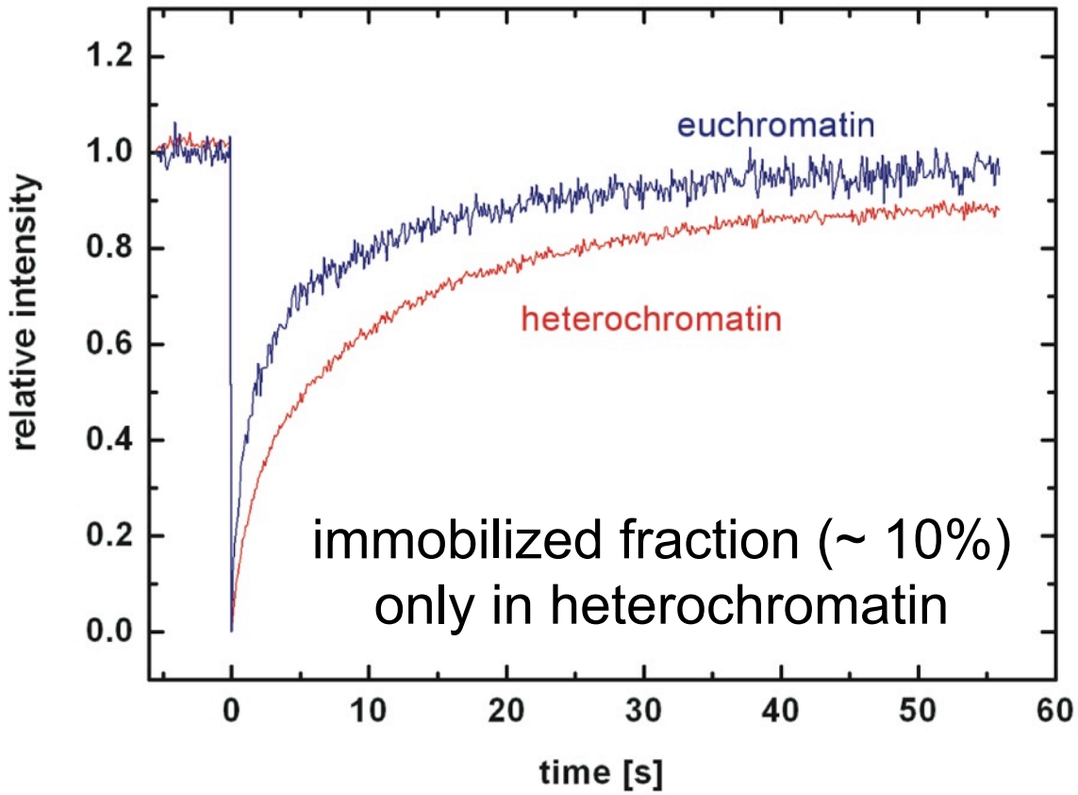
$$F_{\mathrm{i}}(t) = 1 - e^{-k_{\mathrm{off}} t}$$

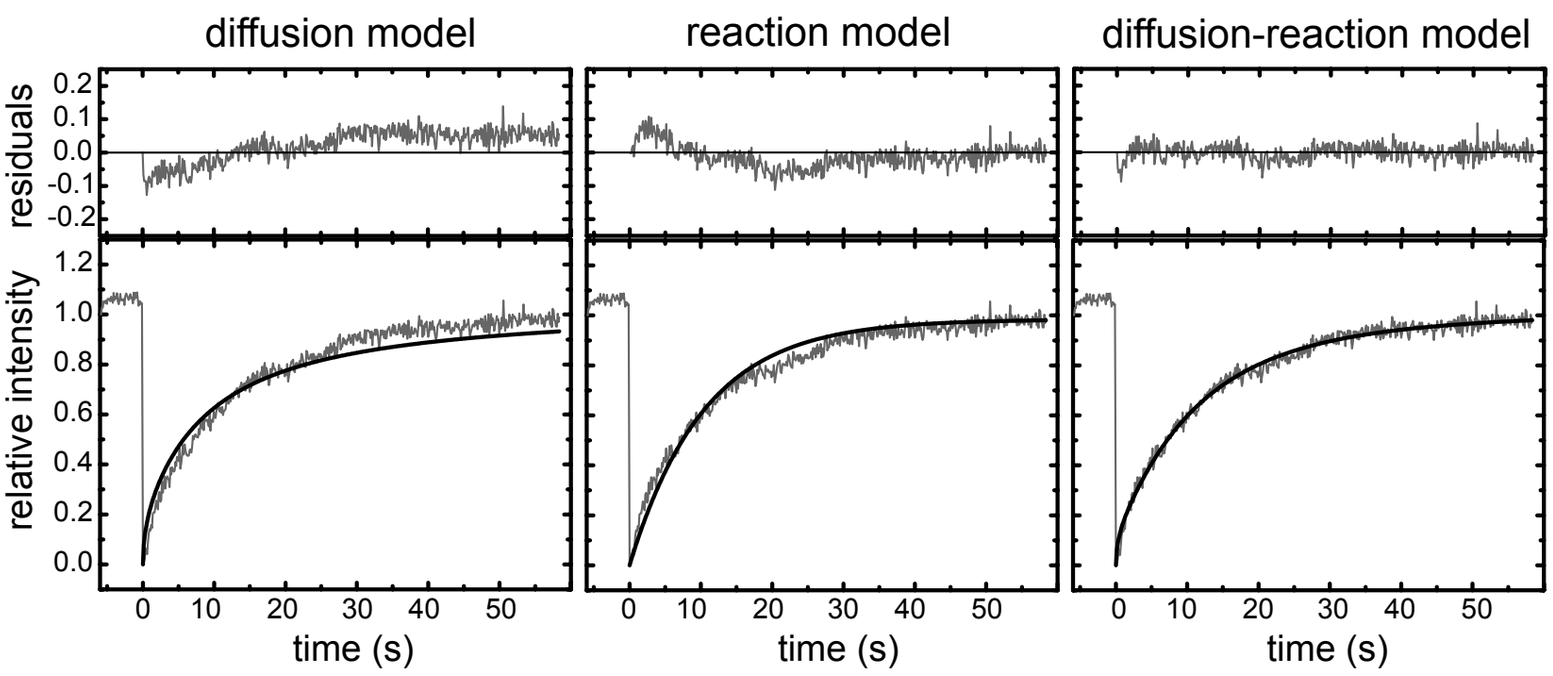
k_{off}: dissociation rate

Moderately fast binding: no analytical solution

Intensity analysis FRAP resolves HP1 diffusion and interactions on the 1 µm and second time scale

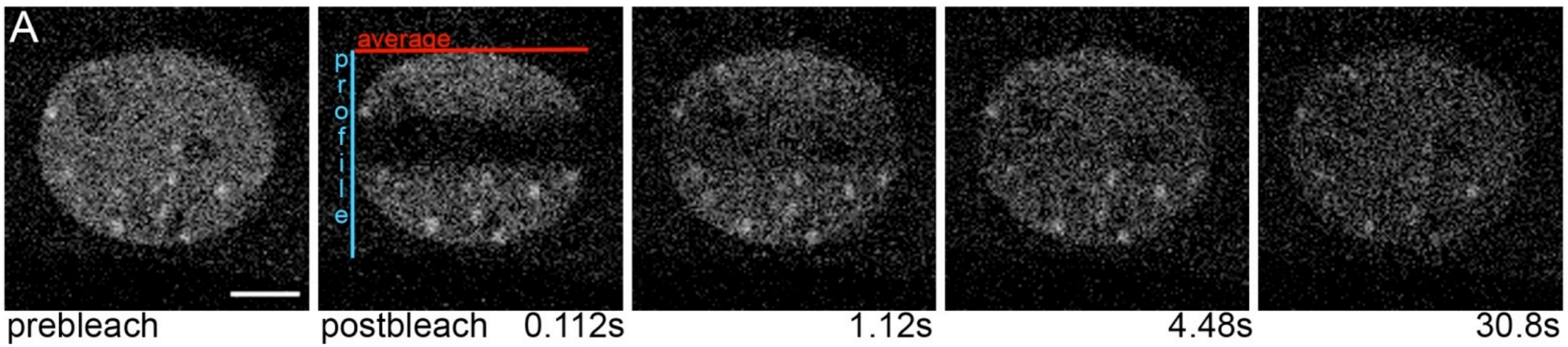


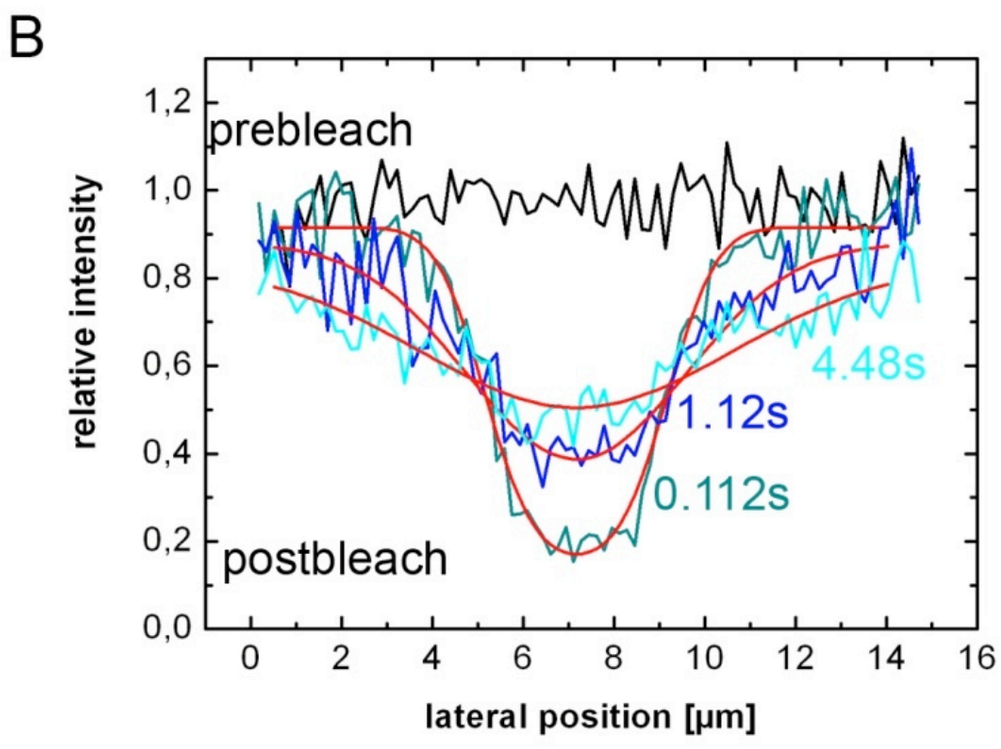


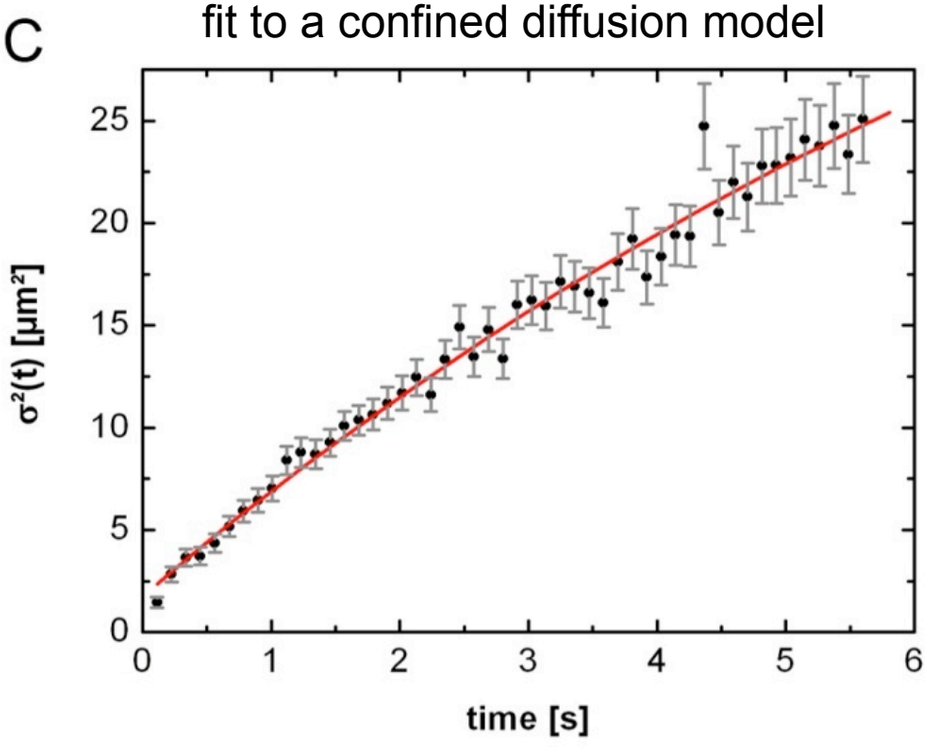


diffusion-reaction analysis (Sprague & McNally 2004, *Biophys. J.* **86**, 3473) yields k_{off} = 0.15 ± 0.07 s^{-1} in heterochromatin

FRAP profile analysis yields an effective nuclear diffusion coefficient of D_{eff} = 1.4 µm²·s^{-1} of HP1 (10 µm and second scale)

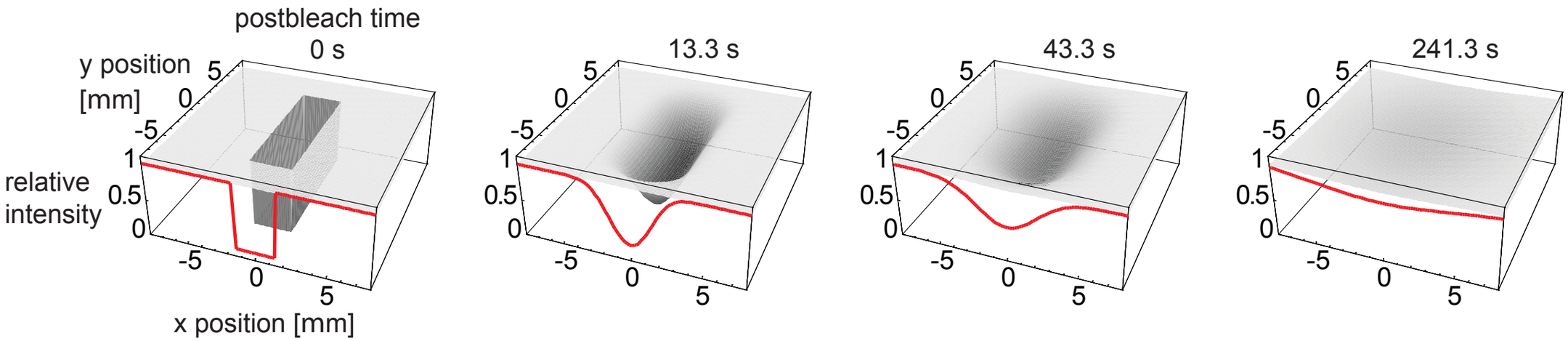




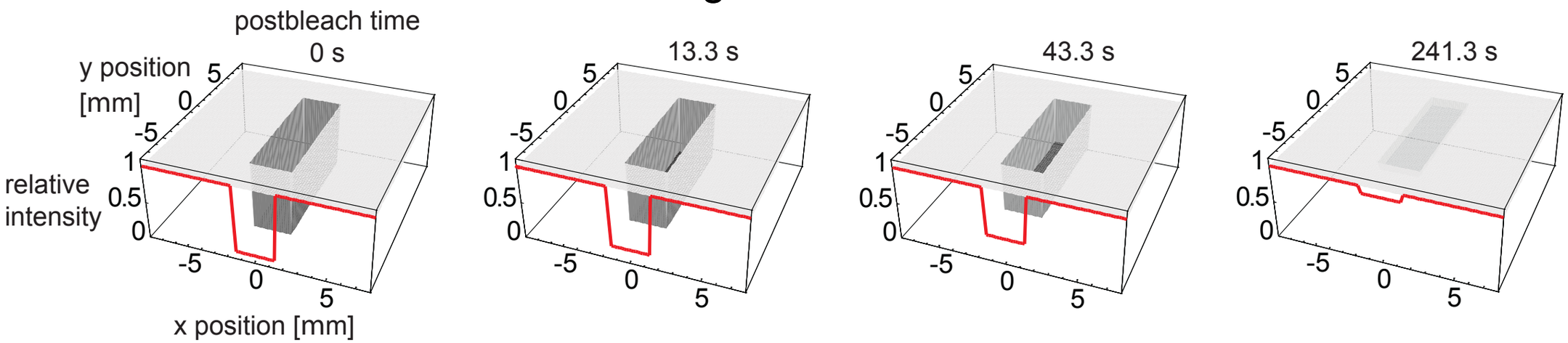


Diffusion versus binding in FRAP profile analysis

diffusion dominant case



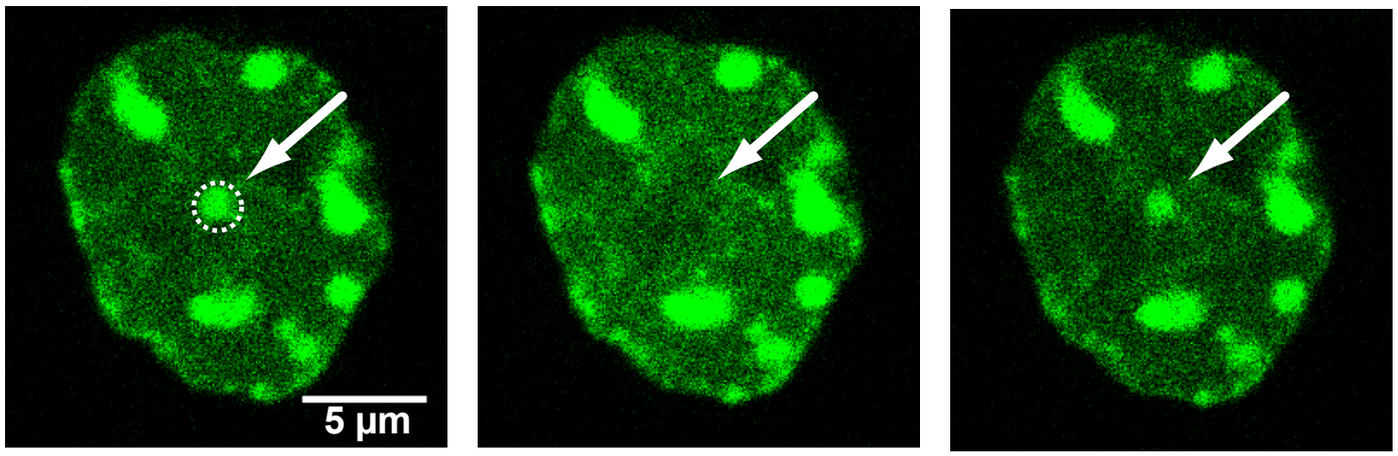
binding dominant case



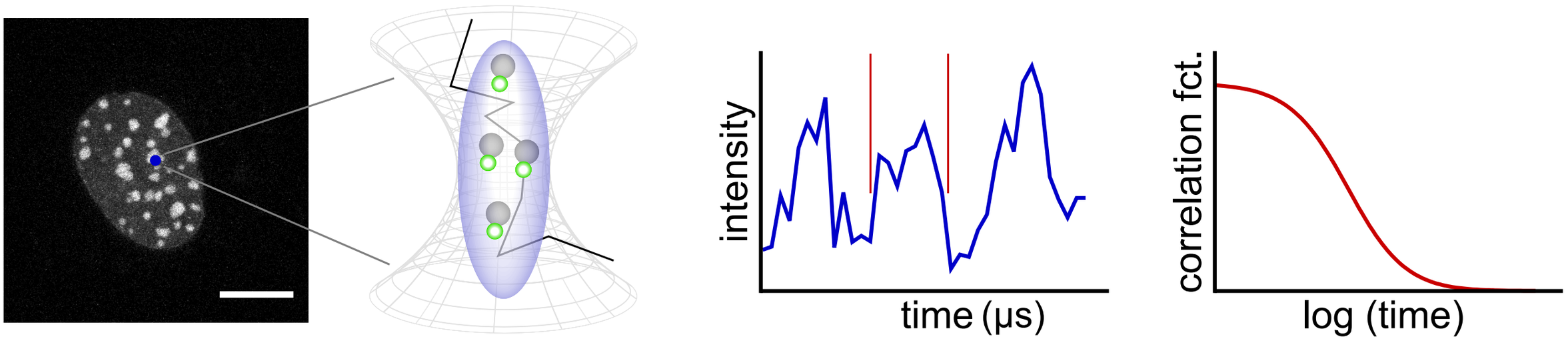
Calculations Malte Wachsmuth

Mobility and interaction analysis in living cells

Fluorescence recovery after photobleaching (FRAP)



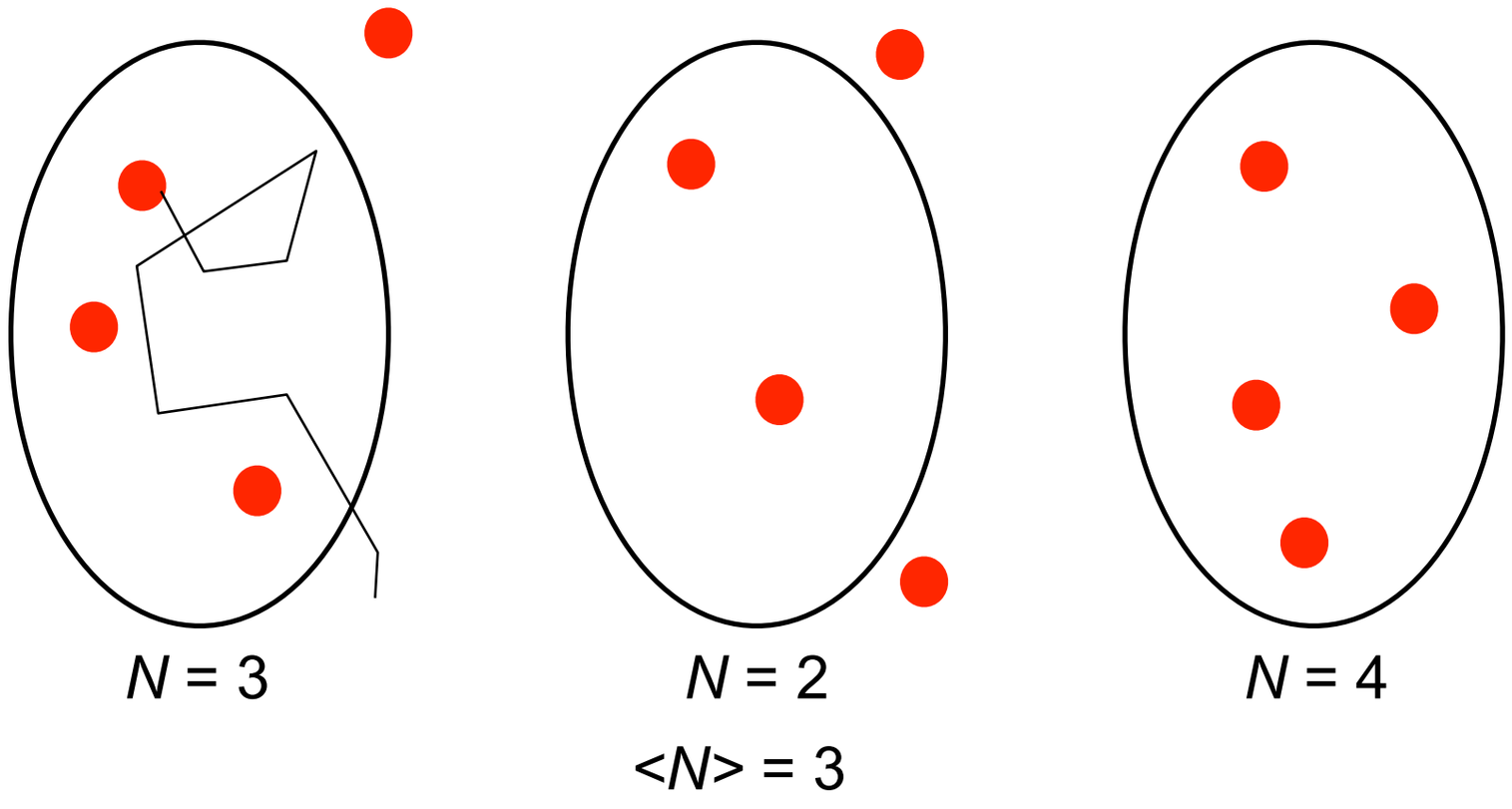
Fluorescence correlation spectroscopy (FCS)



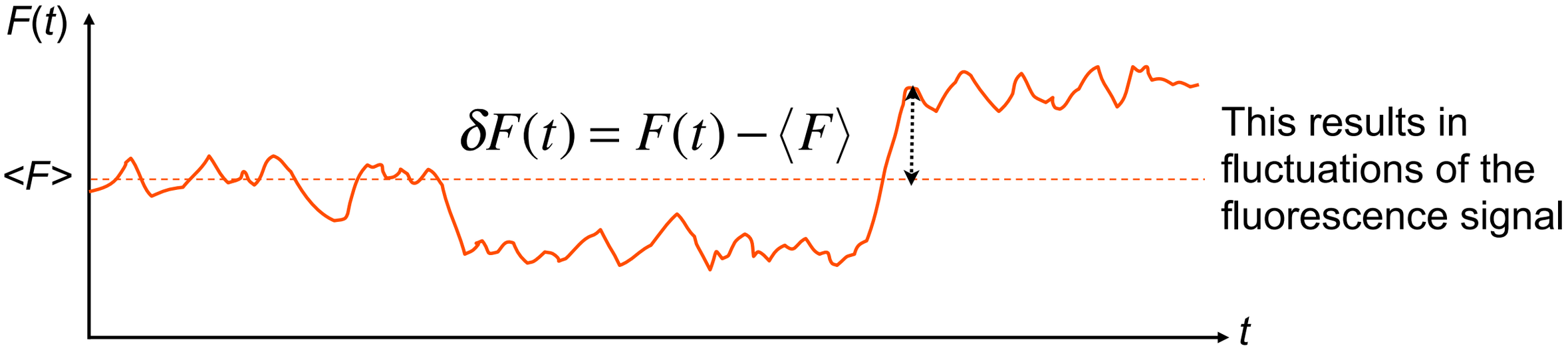
Müller et al. (2009). *Biophys. J.* **97**, 2876-2885; Erdel et al. (2011) *Chromosome Res* **19**, 99-115.

Fluorescence correlation spectroscopy

The concept: measuring number fluctuations of fluorescent particles in the focus



Diffusion induces fluctuations of the number of molecules

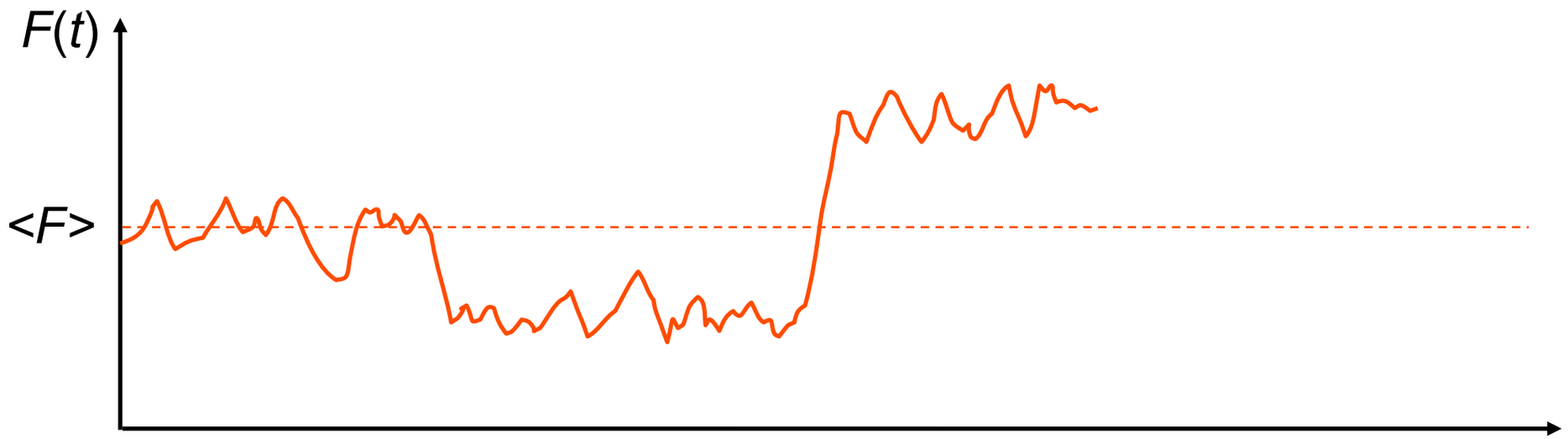


This results in fluctuations of the fluorescence signal

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

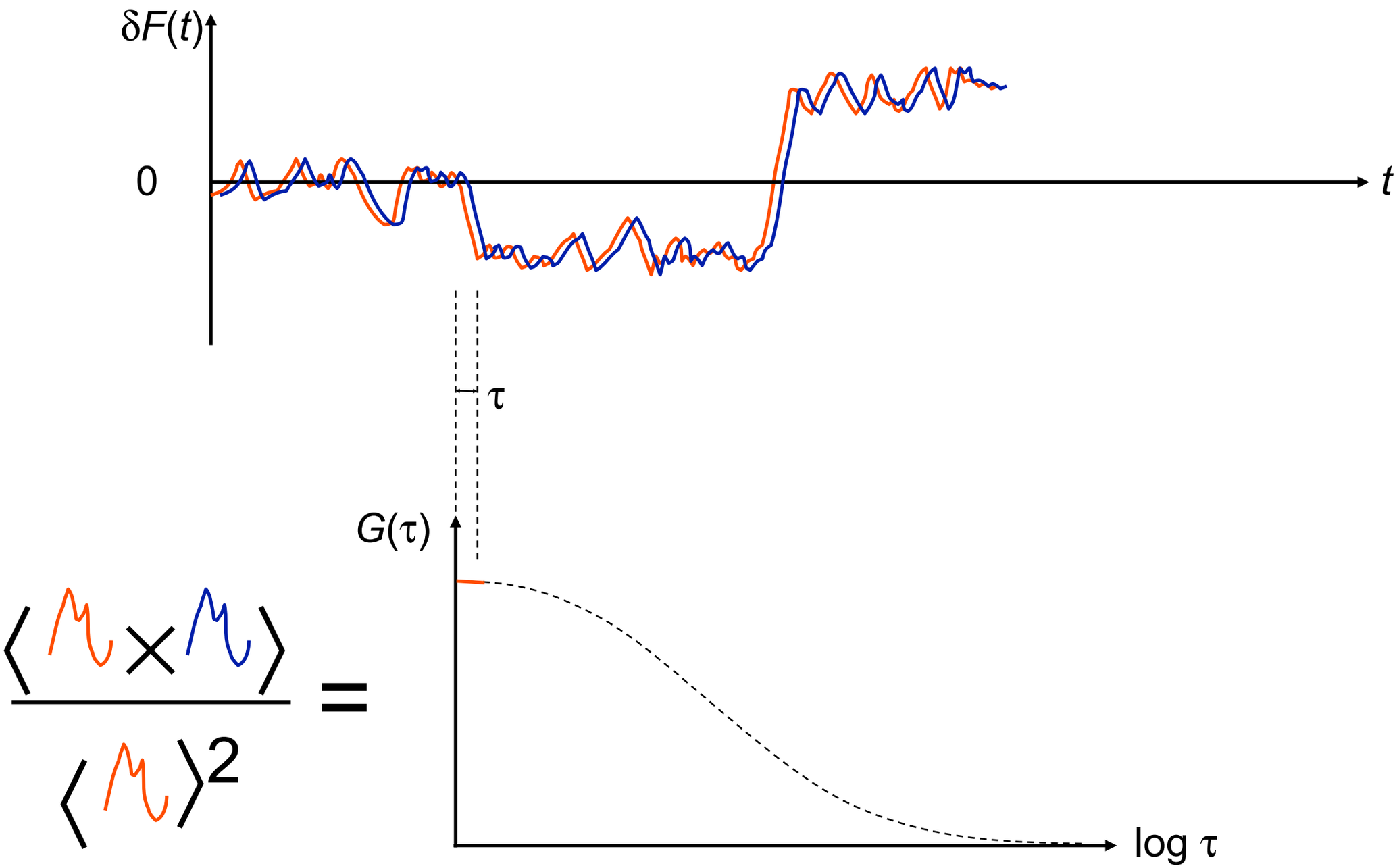
FCS data analysis



We want to know:

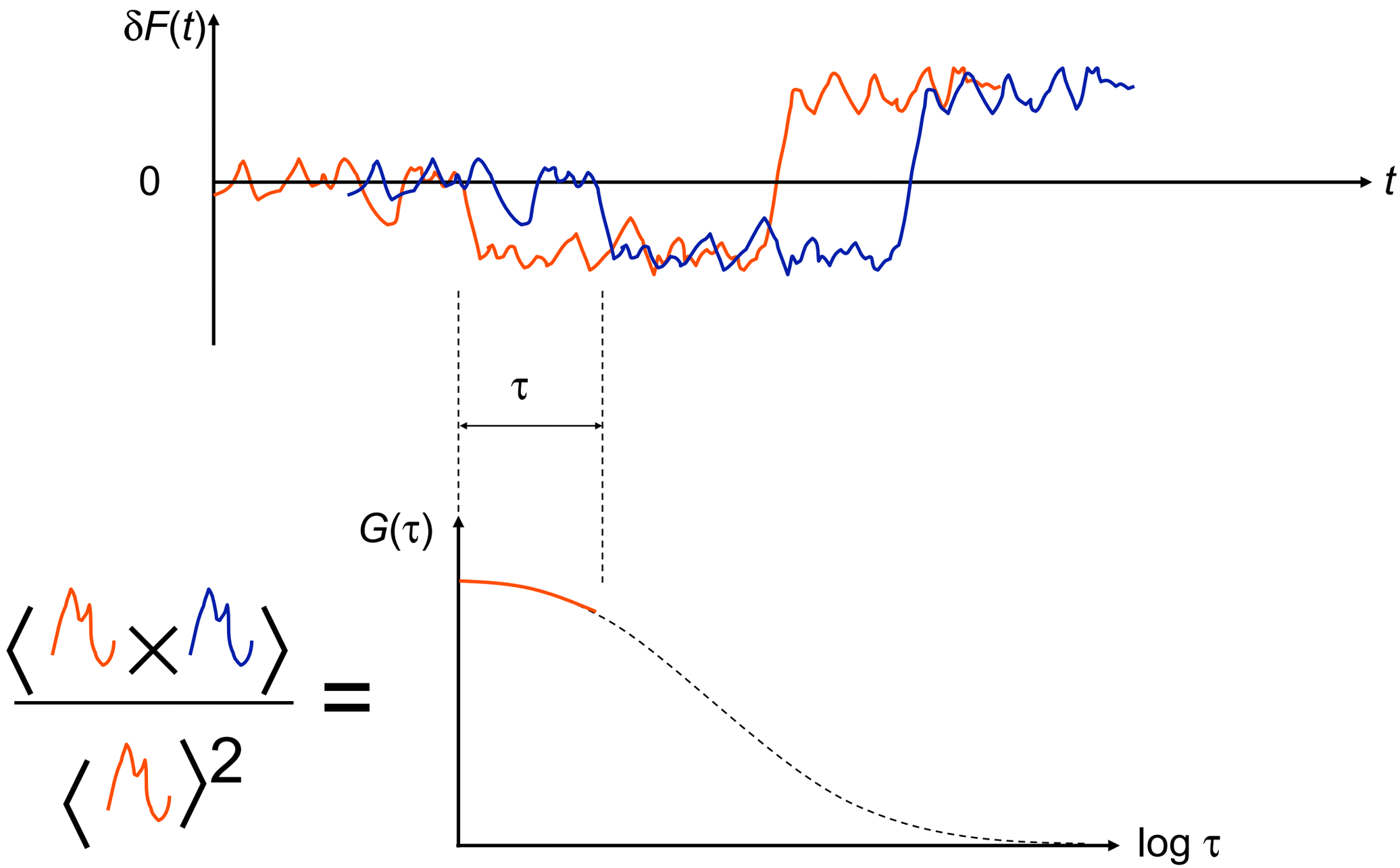
- the average number of molecules in the focus ⇒ concentration
- the dwell time in the focus ⇒ diffusion coefficient

The concept: autocorrelation analysis



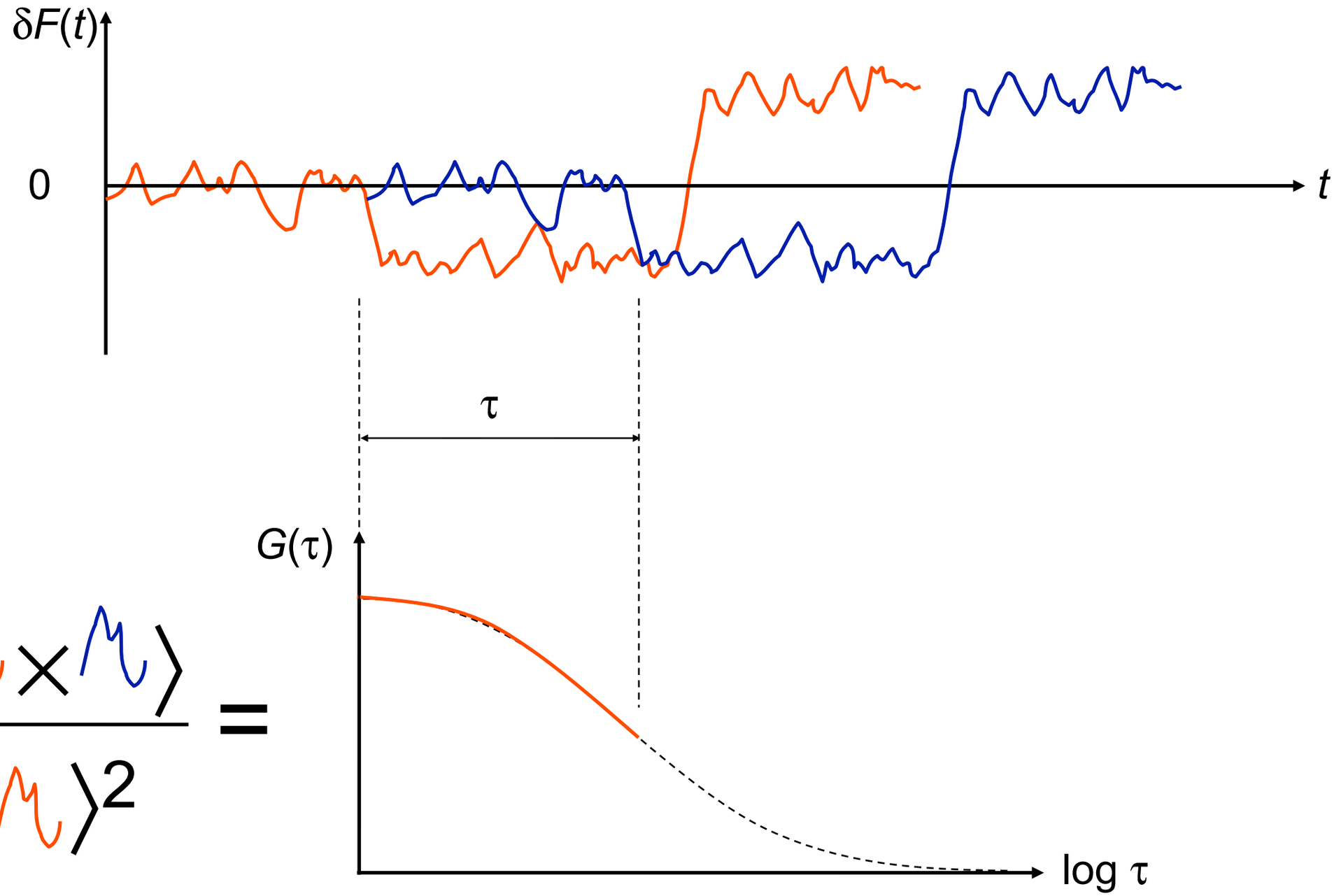
$$\frac{\langle \sim \times \sim \rangle}{\langle \sim \rangle^2} =$$

The concept: autocorrelation analysis



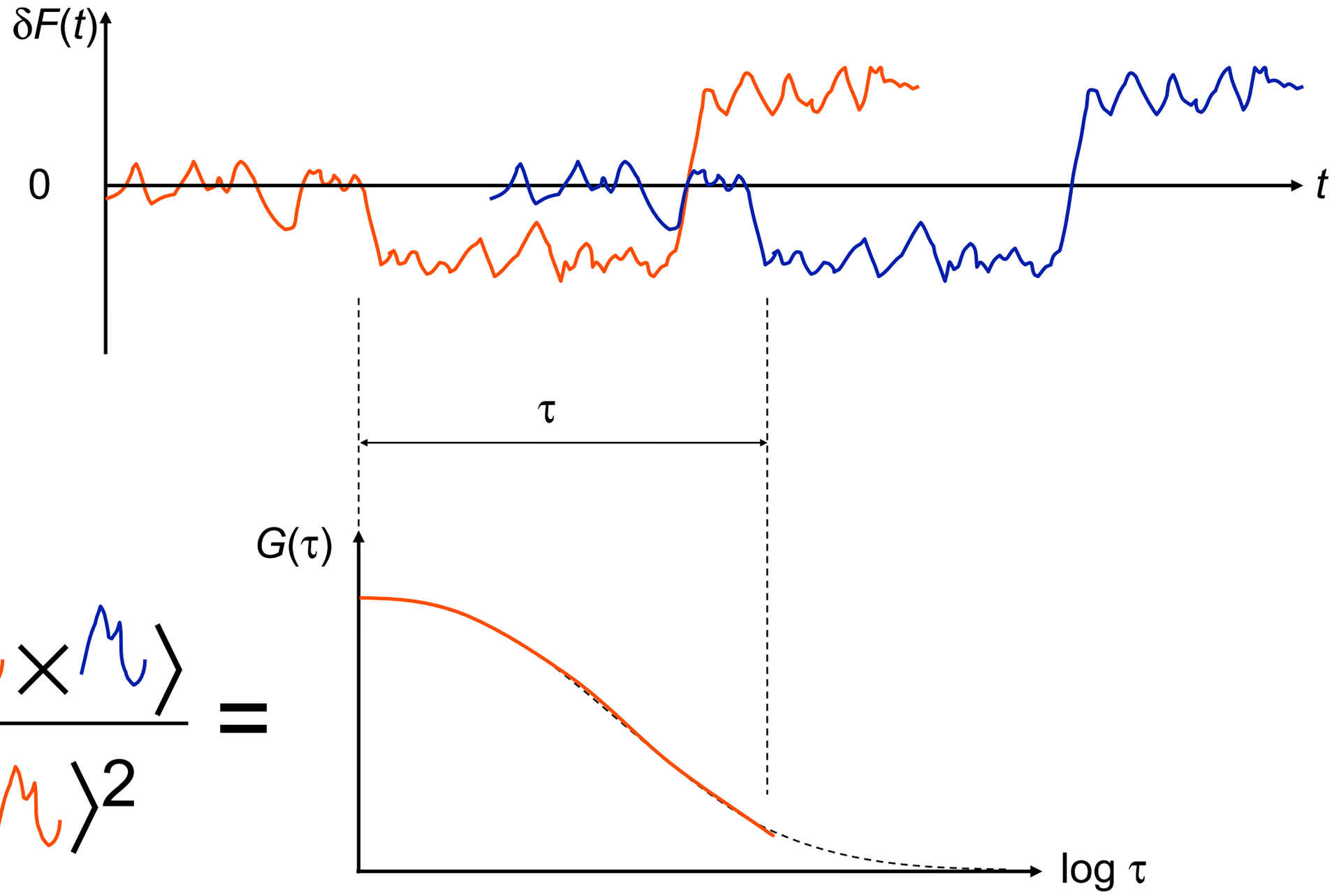
$$\frac{\langle \sim \times \sim \rangle}{\langle \sim \rangle^2} =$$

The concept: autocorrelation analysis



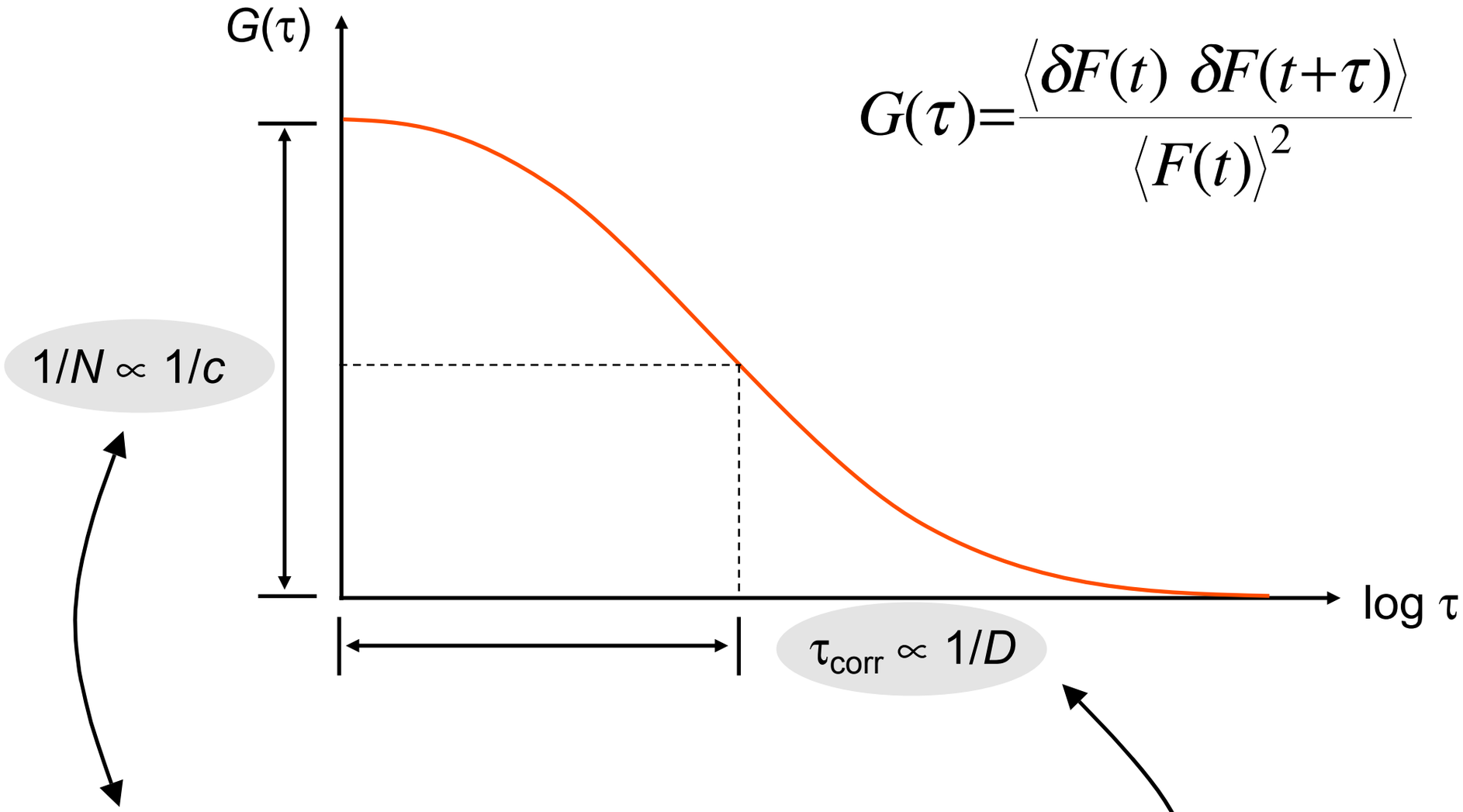
$$\frac{\langle \sim \times \sim \rangle}{\langle \sim \rangle^2} =$$

The concept: autocorrelation analysis



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

Results from FCS experiments

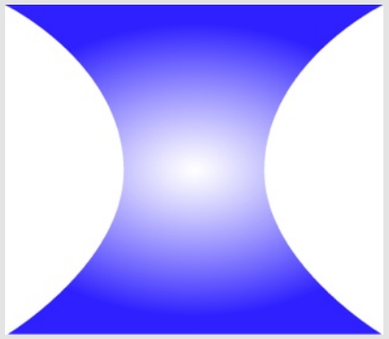


$$G(\tau)=\frac{\langle \delta F(t)\ \delta F(t+\tau)\rangle}{\langle F(t)\rangle^2}$$

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

Theoretical approach - formulas

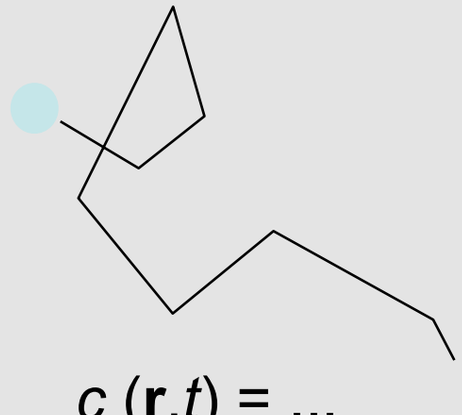
Properties of the optical system



$I(\mathbf{r}) = ...$

×

Properties of the diffusion process

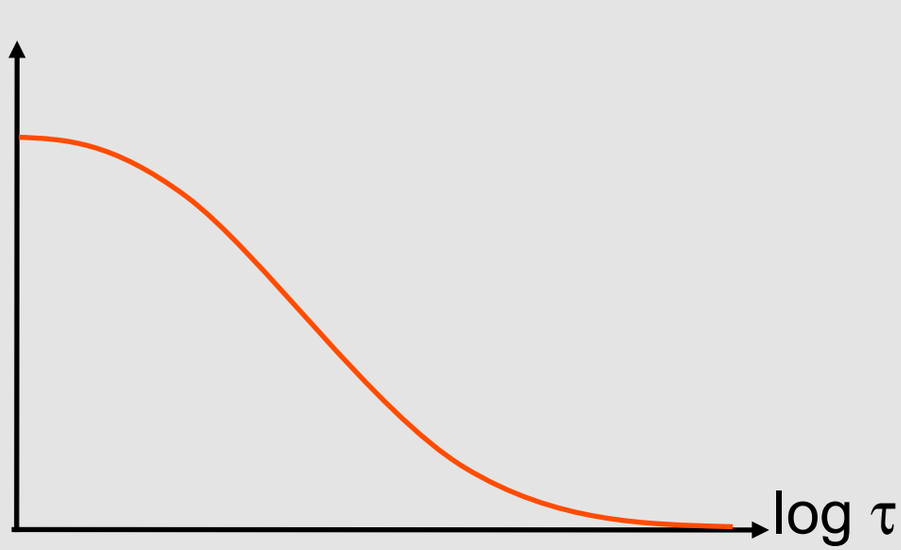


$c(\mathbf{r},t) = ...$

=

Analytical autocorrelation function ⇒

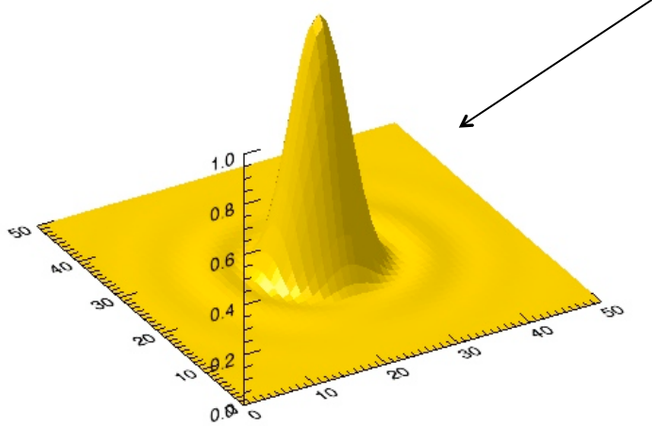
concentration, brightness,
diffusion properties of particles,
interactions, cellular environment...



FCS – correlation function for free diffusion in 3D

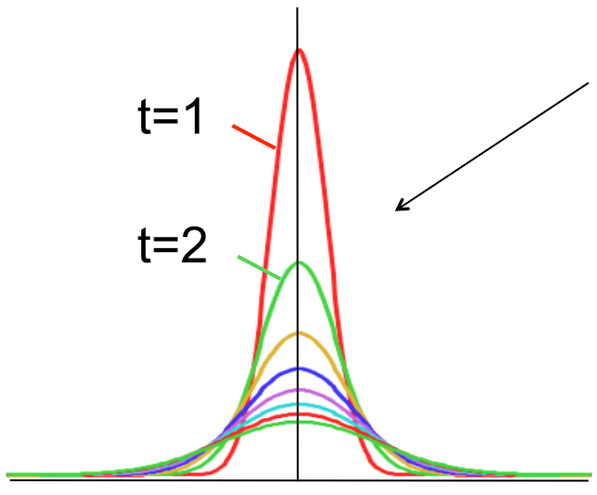
The correlation function $G(\tau)$ gives the probability to detect a particle at time t **and** at time $t + \tau$

detection efficiency



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

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



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

Green's function for free 3D diffusion

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

FCS – correlation function for free diffusion in 3D

Probability to detect a particle **before** and **after** it has diffused for **time** τ

definition of the correlation function

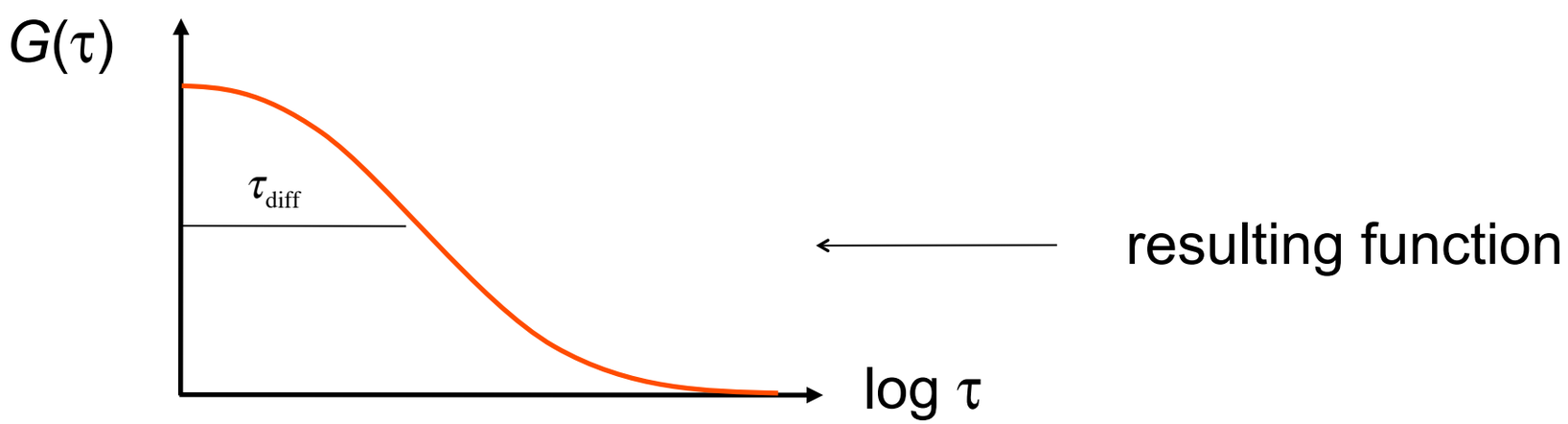
$$G_{kl}(\tau) = \frac{\int d^3 r_1 \int d^3 r_2 \Psi_k(\mathbf{r}_1) P(\mathbf{r}_1, \mathbf{r}_2, \tau) \Psi_l(\mathbf{r}_2)}{\int d^3 r \Psi_k(\mathbf{r}) \int d^3 r \Psi_l(\mathbf{r})}$$

correlation function

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

diffusion time, concentration, focal volume, structure parameter, focus radius

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

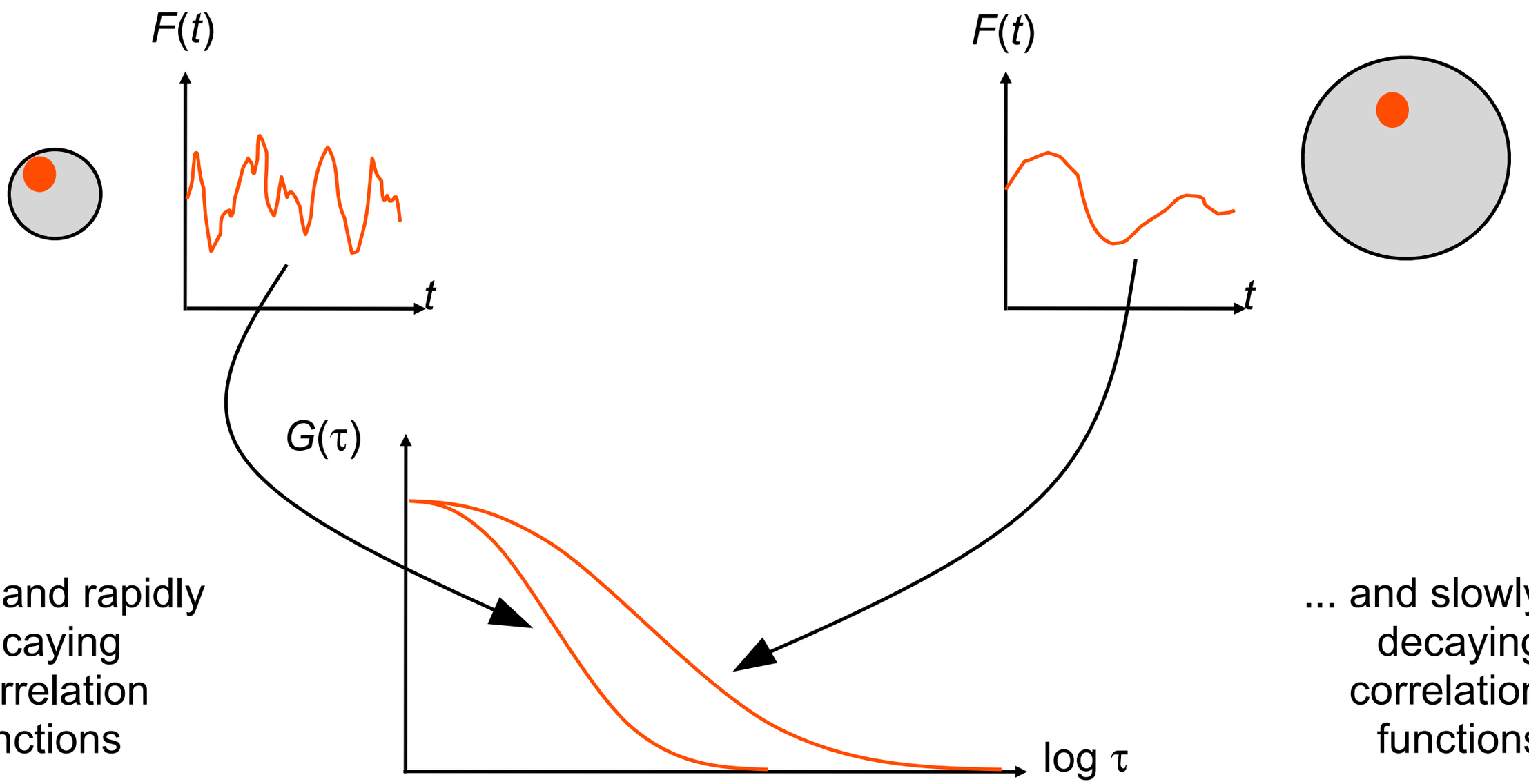


resulting function

Determining diffusion coefficients

small molecules generate short fluctuations...

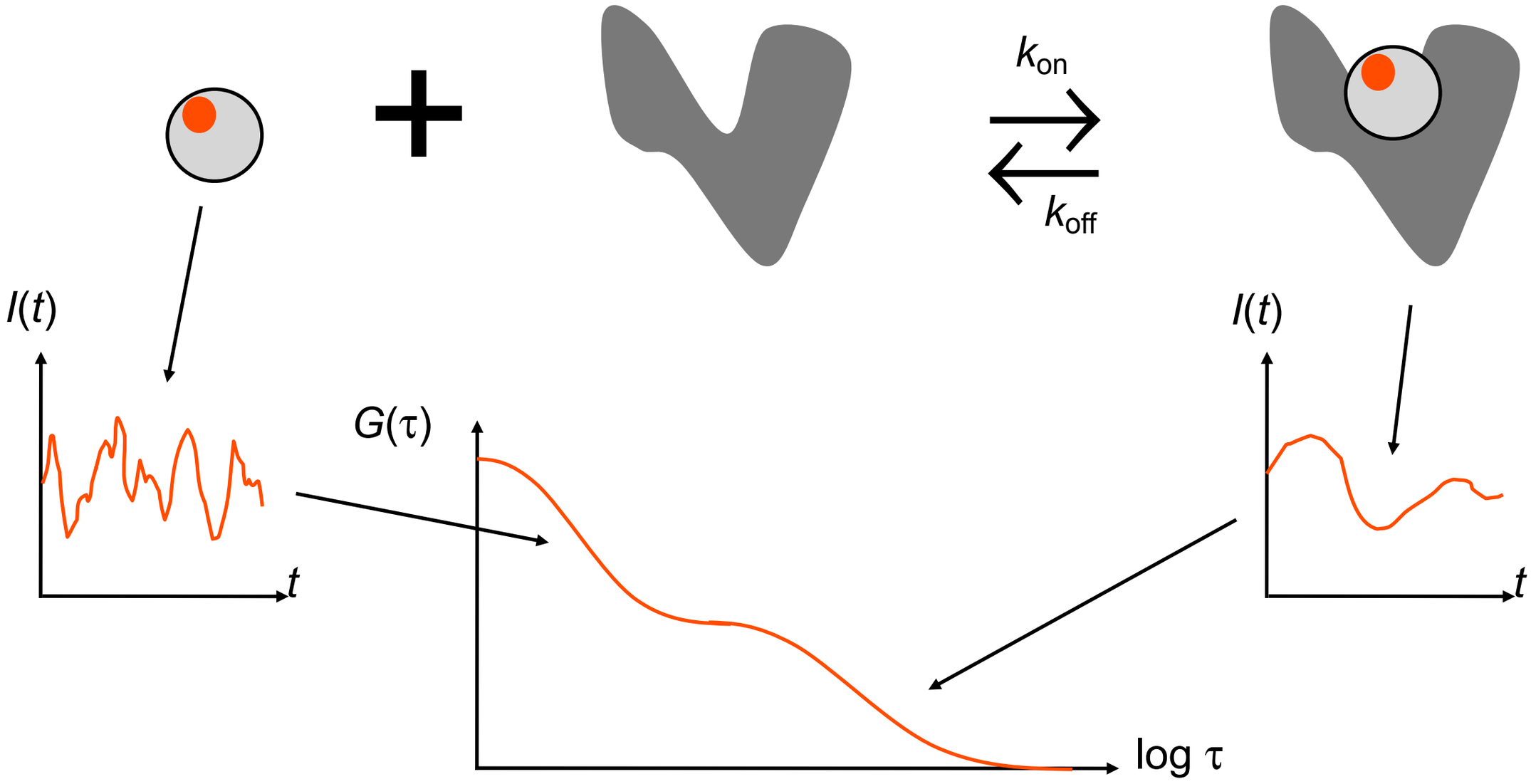
larger complexes generate longer fluctuations...



... and rapidly decaying correlation functions

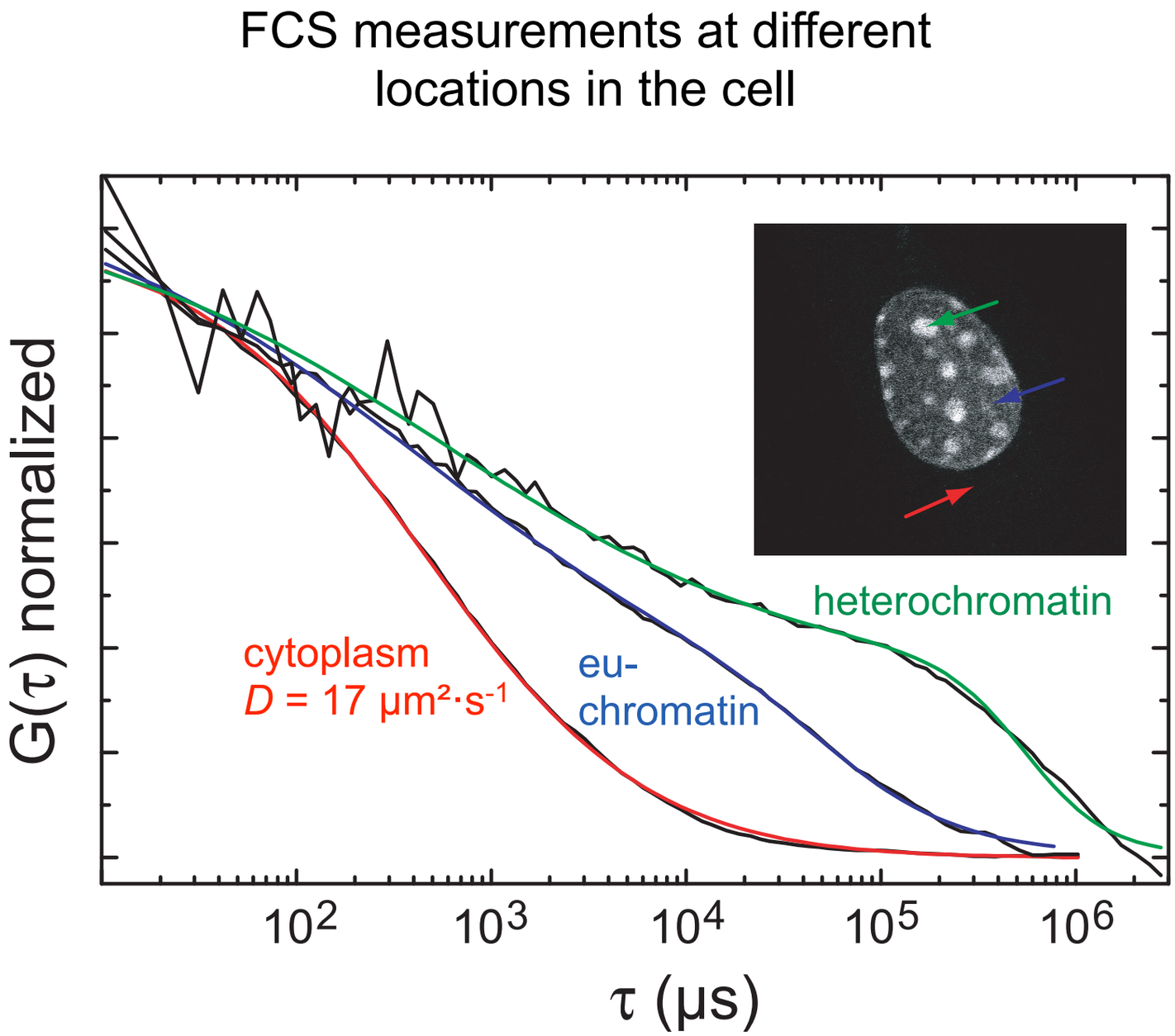
... and slowly decaying correlation functions

Measuring ligand binding affinity

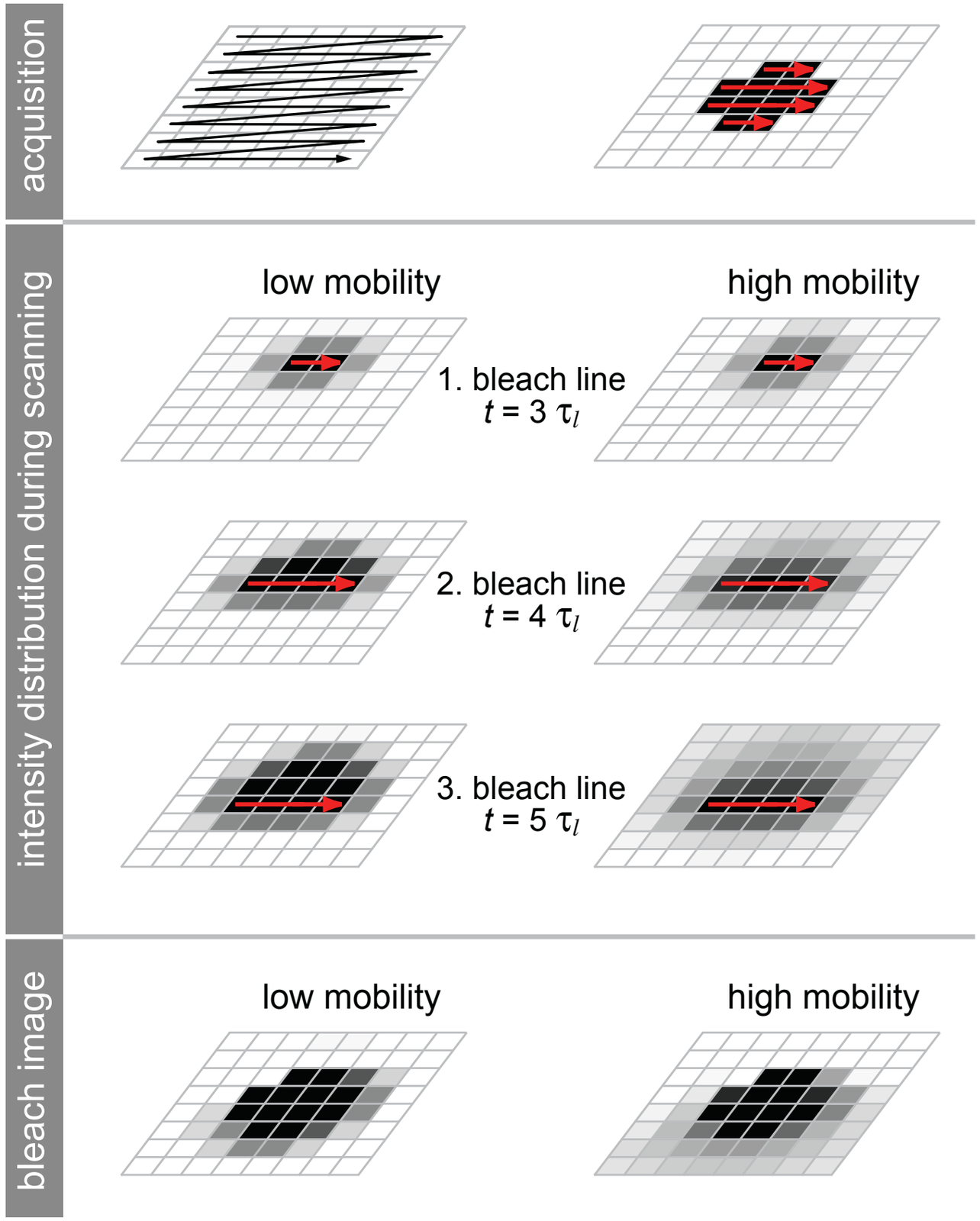


Properties of ligand-receptor interactions: dissociation constant, reaction rates, concentrations

Fluorescence correlation spectroscopy (FCS) of the H3K9me3 histone methylase Suv39h1

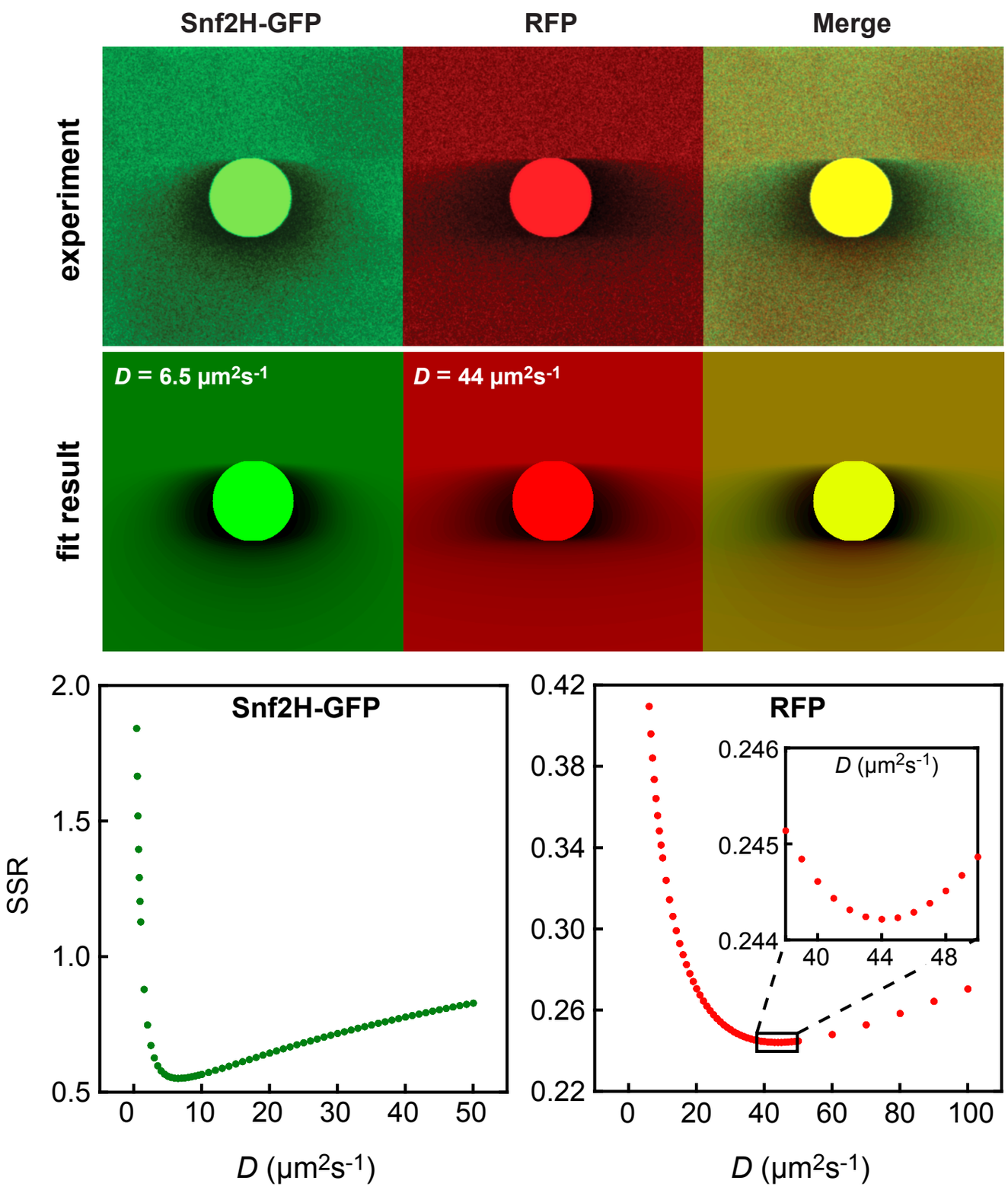


Pixel-wise **P**hotobleaching **P**rofile **E**volution **A**nalysis - **3PEA**

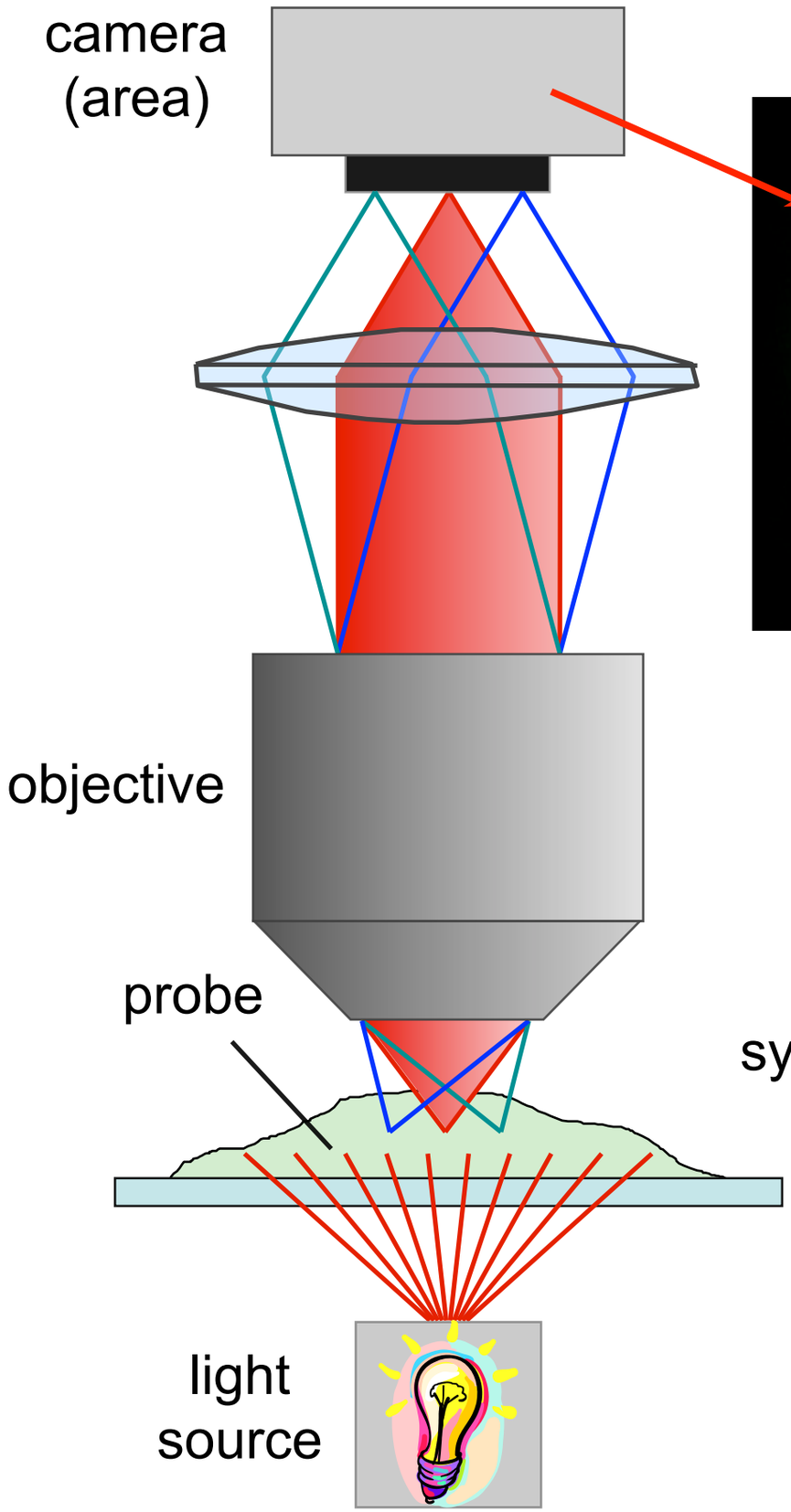


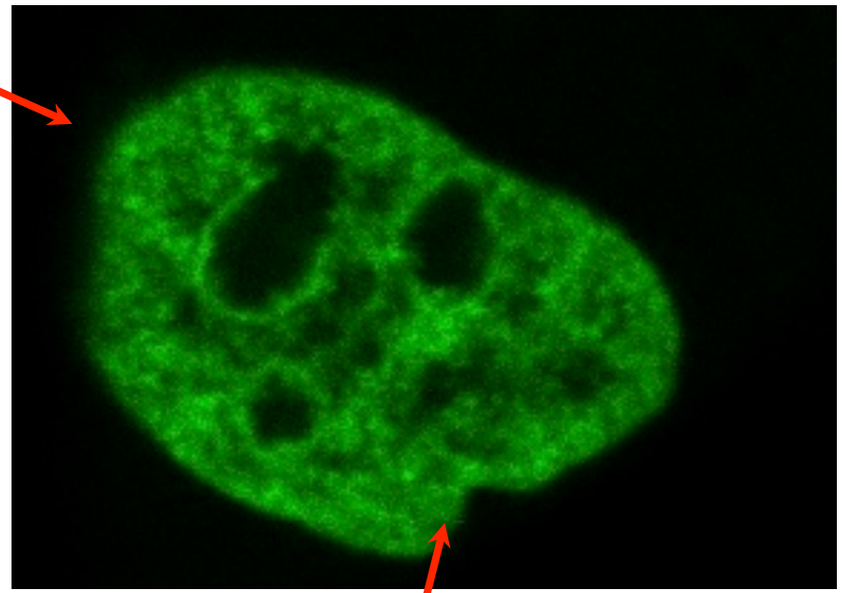
Erdel & Rippe (2012).
PNAS 109, E3221-30.

3PEA analysis of Snf2H chromatin remodeler:
D_{eff} = 6.5 µm^2 s^{-1} (FRAP: 0.7 µm^2 s^{-1})



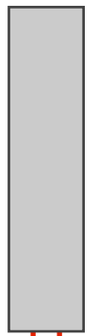
Wide-field verus confocal microscopy setup

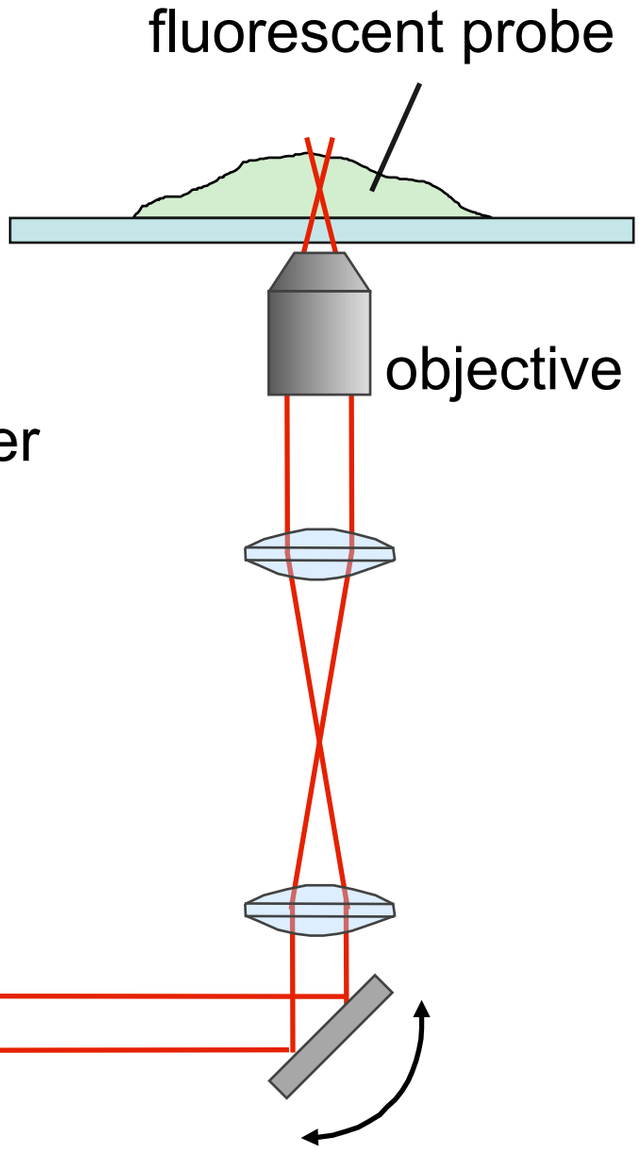


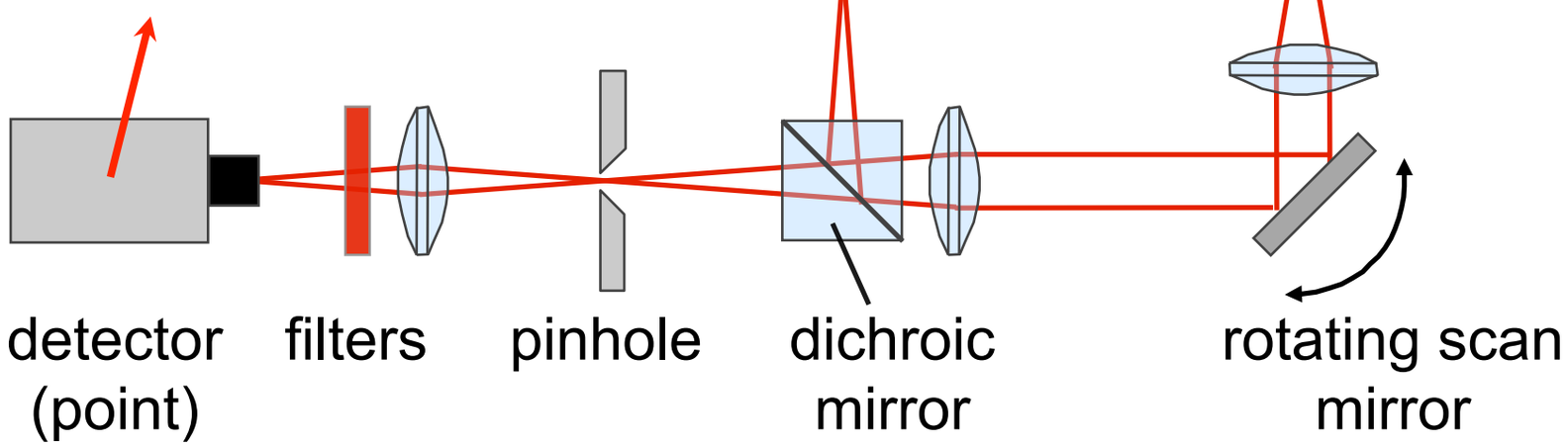


reconstruction of the spatial
fluorescence distribution

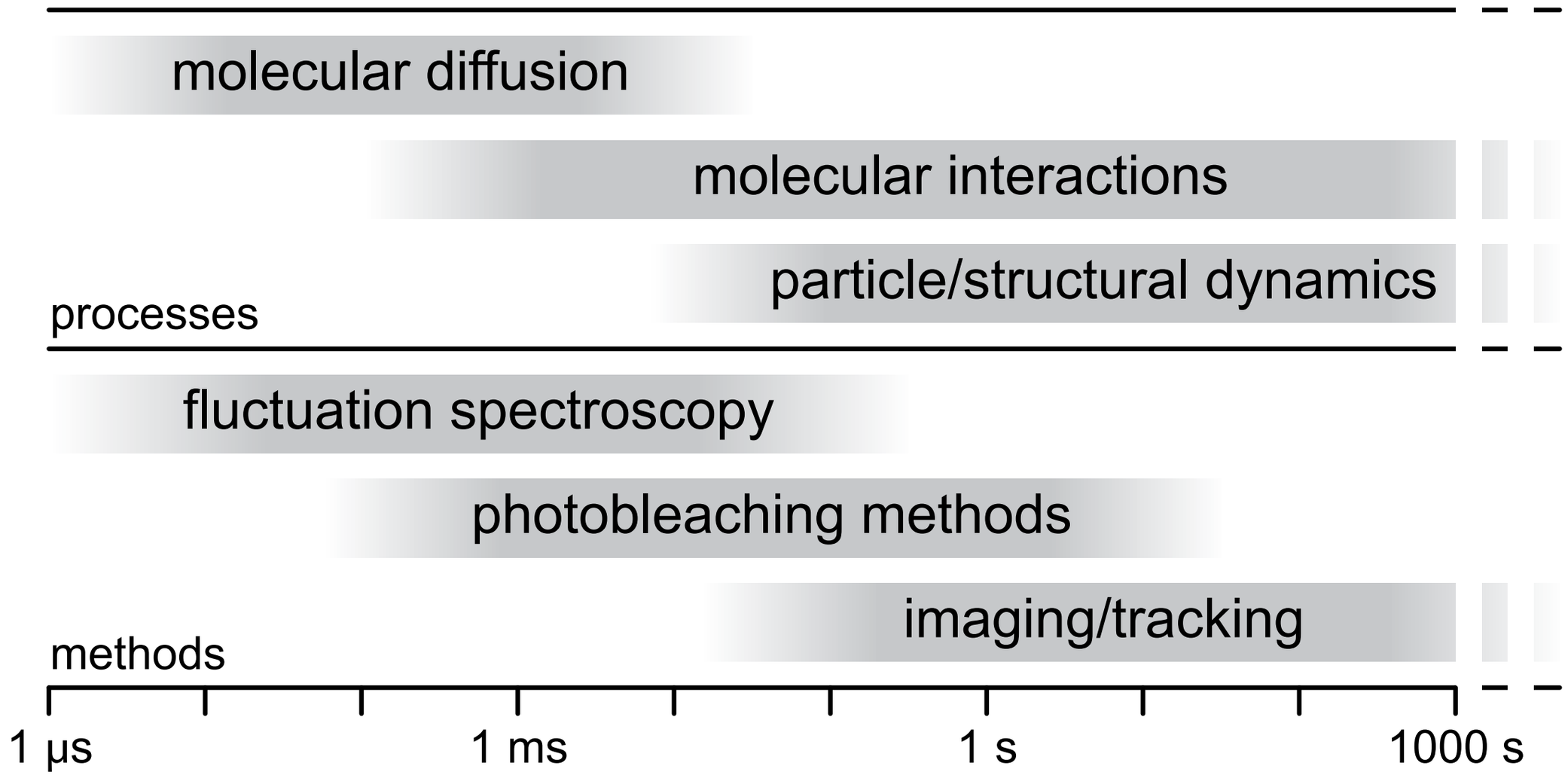
synchronisation of scan mirror
and signal acquisition







Dynamic processes in the cell nucleus take place on the microsecond to hour time scale



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

	typical resolution	acquisition rate (frames/sec)	light exposure
wide field	250 nm (x,y) > 2 µm (z)	200	low
confocal	250 nm (x,y) 600 nm (z)	1-10	high
line scanner confocal	250 nm (x) 380 nm (y) 700 nm (z)	30	low

Typical values for diffusion coefficients in the nucleus

	D_{min} ($\mu m^2 s^{-1}$)	accesible corral radius	methods
chromatin/ telomeres	$2 \cdot 10^{-3}$ $4 \cdot 10^{-4}$ $2 \cdot 10^{-5}$	0.08 µm 0.2 µm 0.3-0.8 µm	CLSM, single particle tracking
transcription factor	10-15 (free) 0 - 0.01	up to 10 µm (nucleus)	FRAP (bound) FCS (free, transiently bound)
membrane proteins	2-20 (2-D)	10 µm (nuclear membrane)	FRAP FCS

Accessible range of diffusion coefficients for CLSM, FRAP and FCS measurments

	t_{min} / t_{max}	typical analysis volume	D_{min} / D_{max}
confocal (single particle tracking)	0.4-2 sec / infinite	10 x 10 µm (x,y) 600 nm (z)	0 $\mu m^2 s^{-1}$ / 10 $\mu m^2 s^{-1}$
FRAP	0.4-2 sec / infinite	2 x 2 µm (x,y) 0.6 - 5 µm (z)	0 $\mu m^2 s^{-1}$ / 10 $\mu m^2 s^{-1}$
FCS	1 µsec / 1 sec	250 nm (x,y) 600 nm (z)	0.05 $\mu m^2 s^{-1}$ / 200 $\mu m^2 s^{-1}$