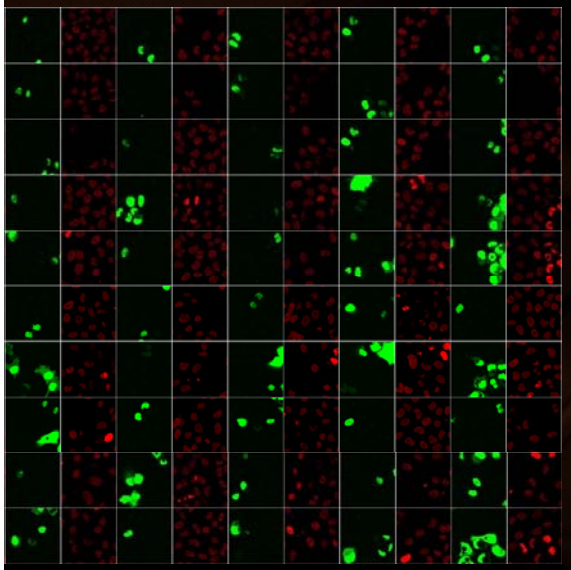
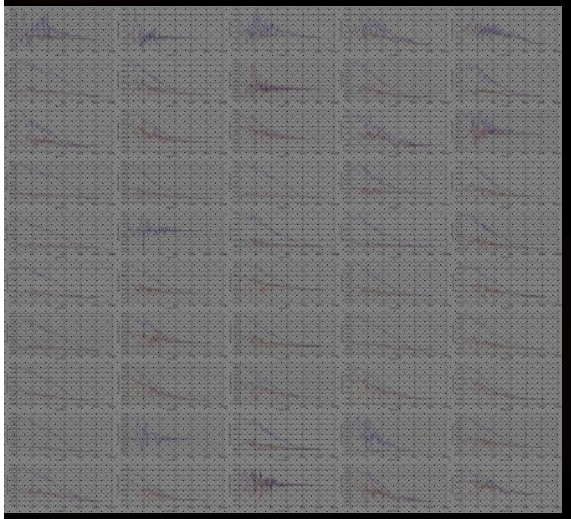


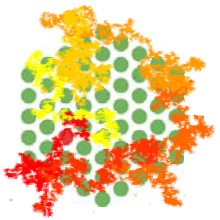
Observing molecular diffusion and binding with light sheet FCS and point FRAP

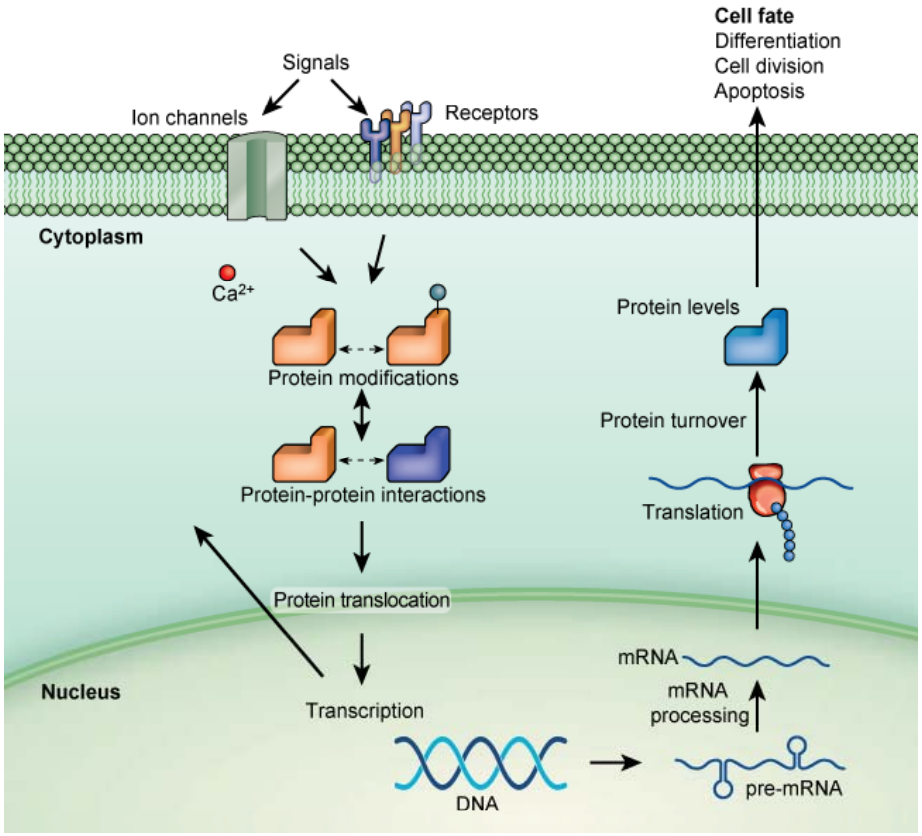


First Irish/UK Light Sheet
Fluorescence Microscopy Workshop
November 21, 2013

Malte Wachsmuth
Cell Biology & Biophysics Unit
European Molecular Biology Laboratory
Heidelberg, Germany

Molecular mobilities and interactions on the cellular level





Based on Spiller et al., Nature 465, 2010

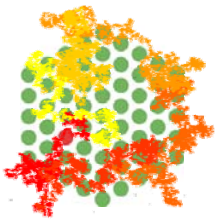
Vital processes on a cellular level rely on

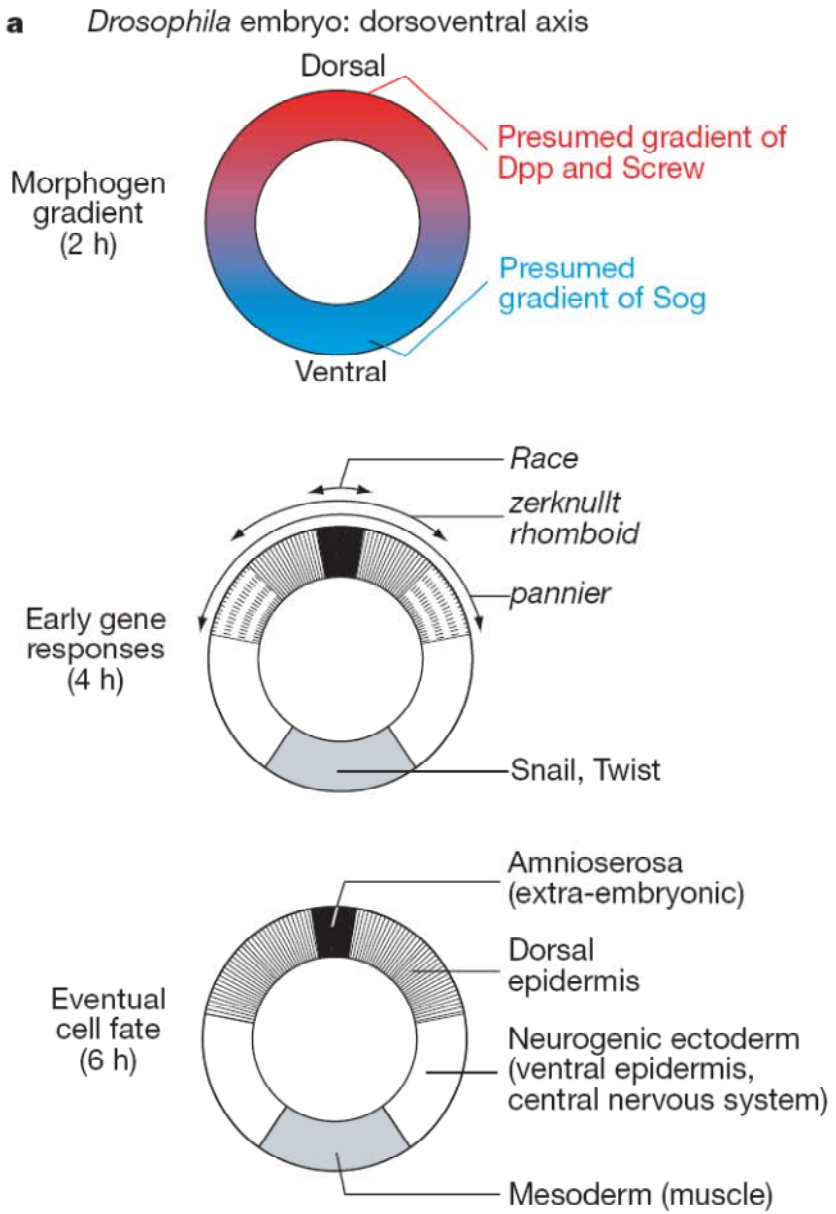
- transport and diffusion
- establishment and maintenance of concentration gradients
- distribution, accessibility, and occupation of specific binding sites
- specific interactions of molecules

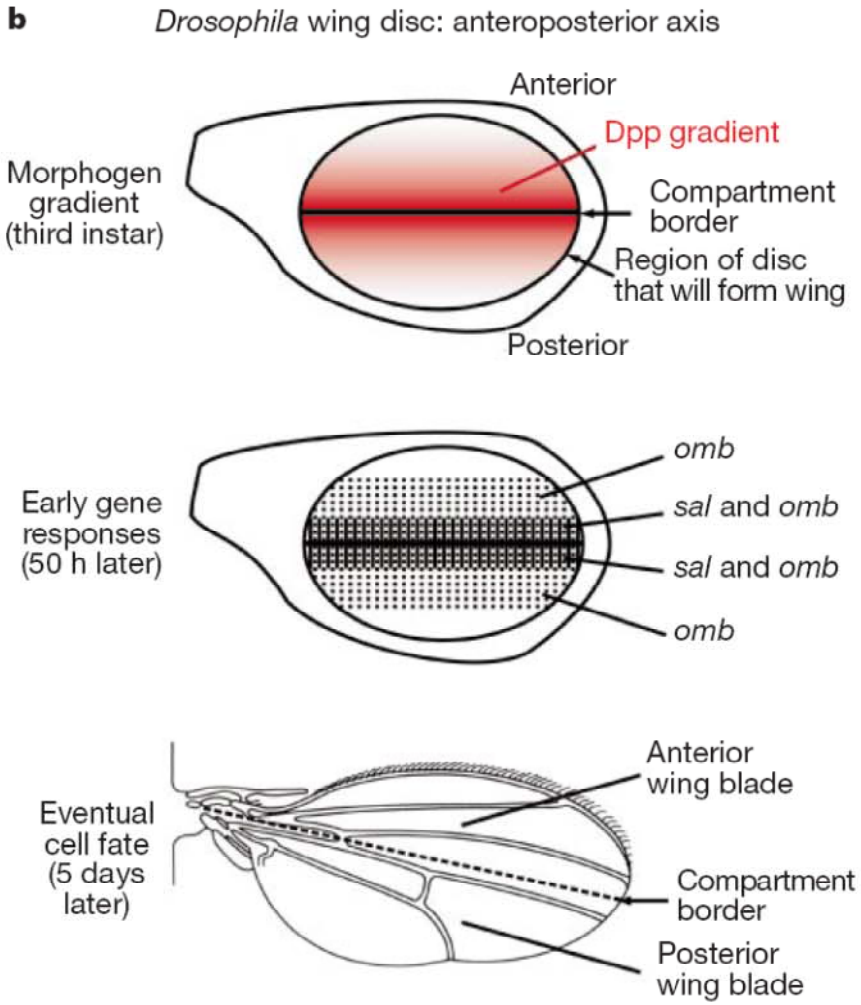
The measurement of molecular mobilities and dynamics yields quantitatively

- biochemical parameters (dissociation constants, degrees of binding/multimerization, ion concentrations, pH)
- biophysical properties (diffusion coefficients, viscosities, connectivities of cellular compartments, elastic parameters)
- in a spatially resolved manner

Morphogen gradients during development

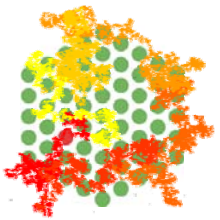


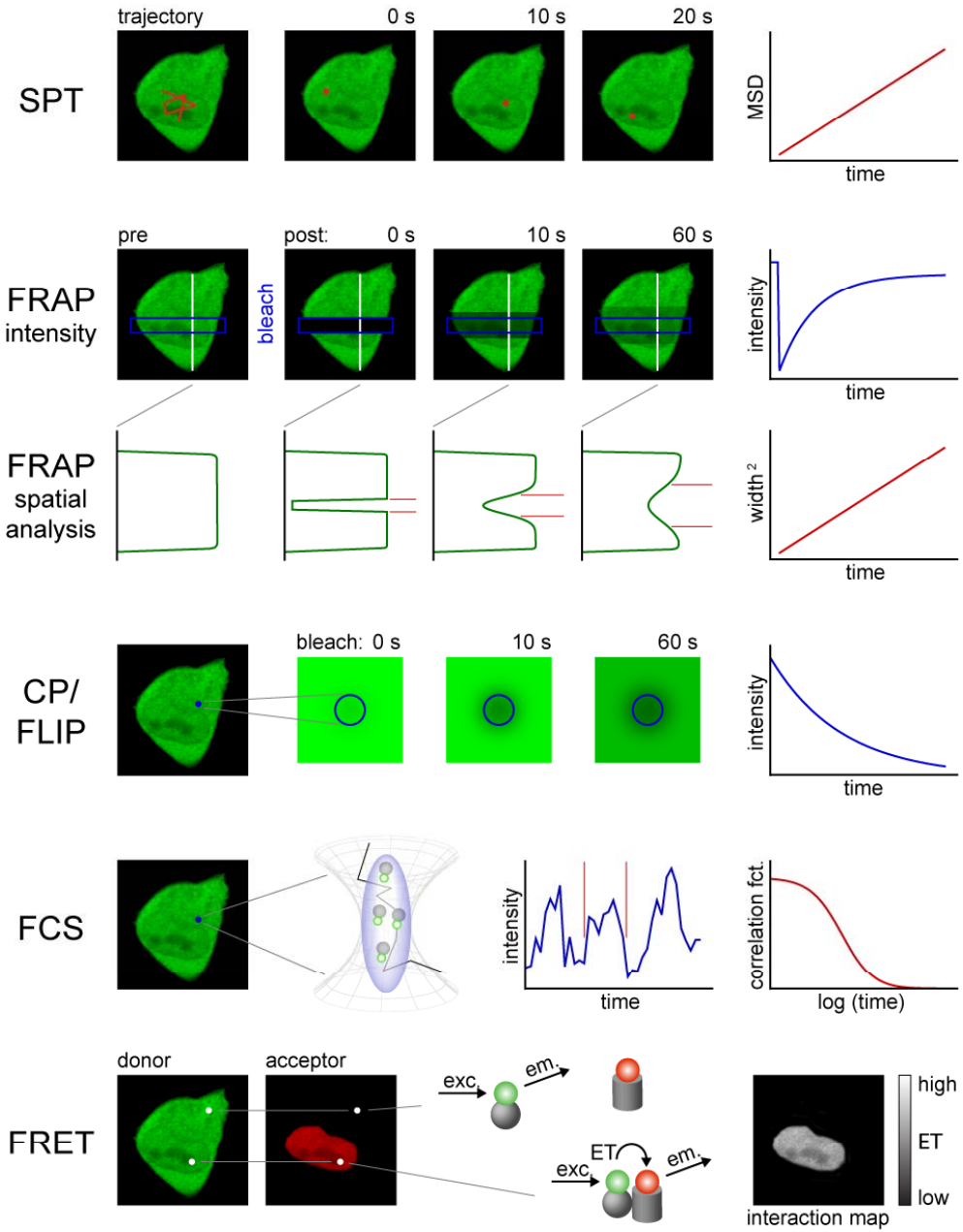


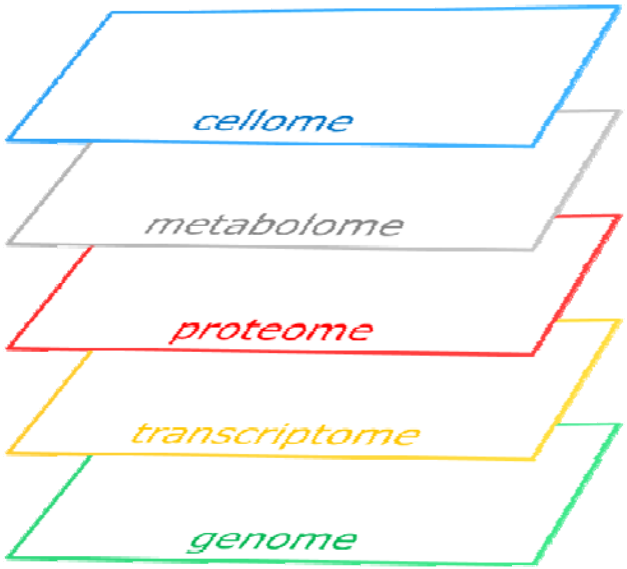


Gurdon & Bourillot, Nature 413 (2001)

Imaging and measuring mobilities and interactions

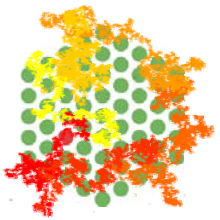




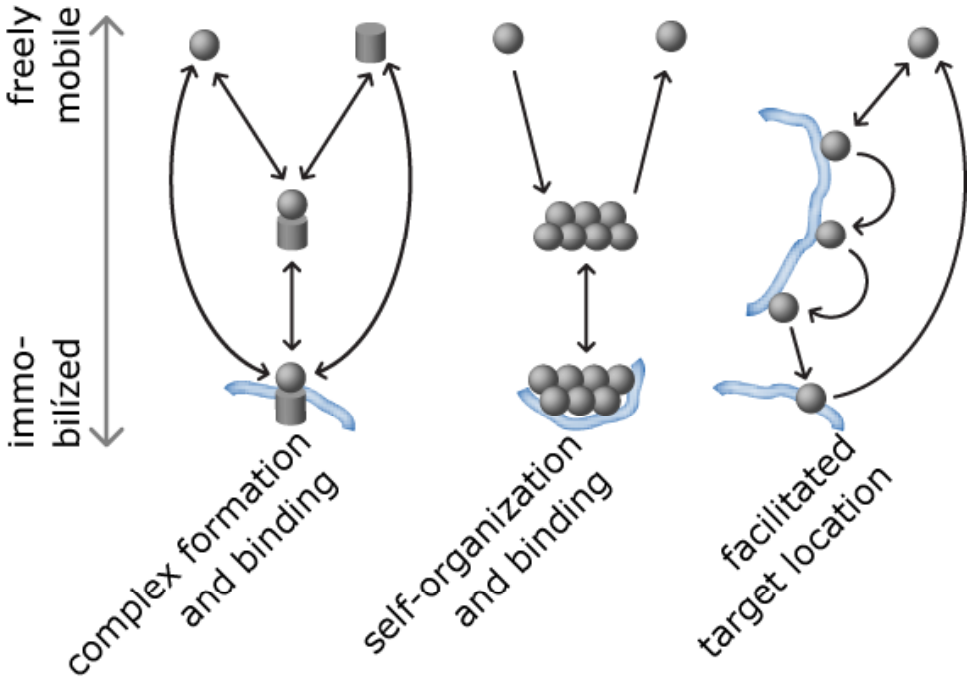


Wachsmuth et al., Biochim. Biophys. Acta 1783, 2008

Mobility = diffusion + binding



impact of biological interactions



complex formation can result in:

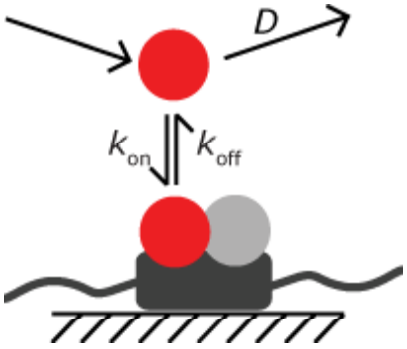
- reduced real diffusion coefficient

$$D \propto \frac{1}{MW^{\beta}}, \quad \beta = \frac{1}{3} \ldots 1$$

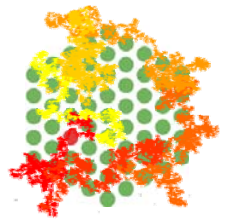
- reduced apparent diffusion coefficient

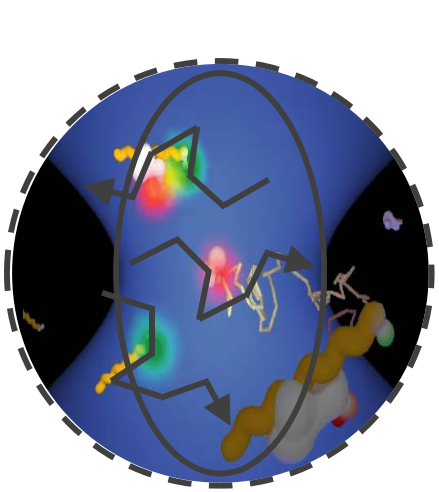
$$D_{\text{app}} = D_{real}\left(1 + \frac{k_{\text{on}}}{k_{\text{off}}}\right)$$

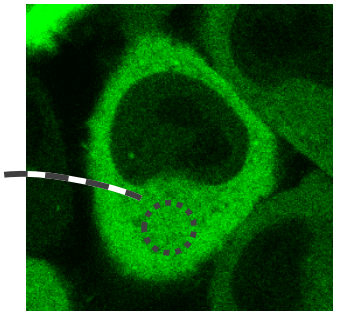
- transient and long-term immobilization to be described as full reaction-diffusion scheme



Confocal fluorescence correlation spectroscopy (FCS)

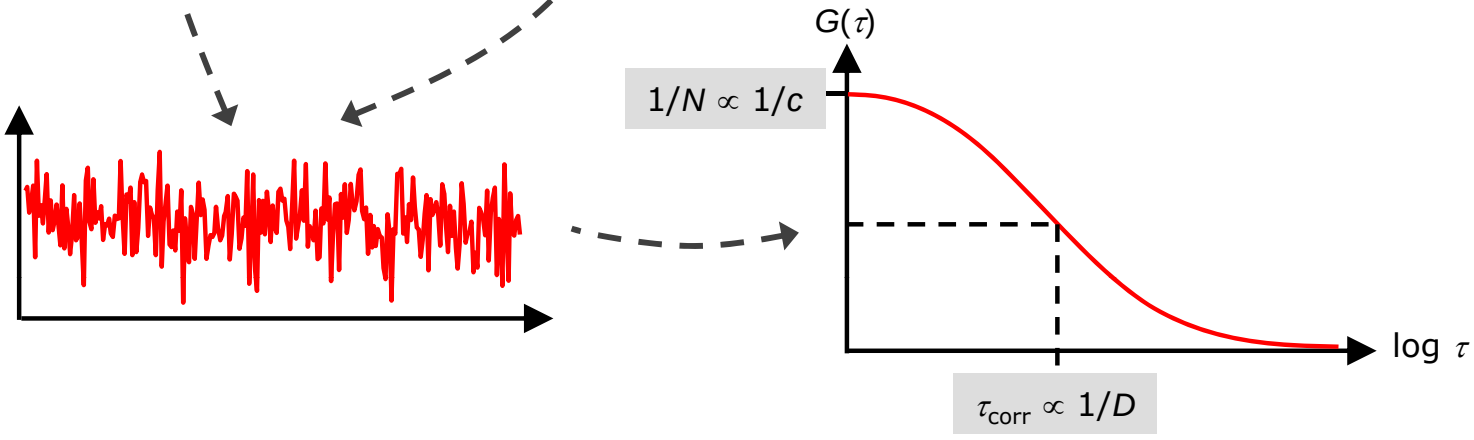




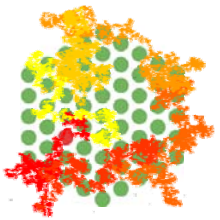


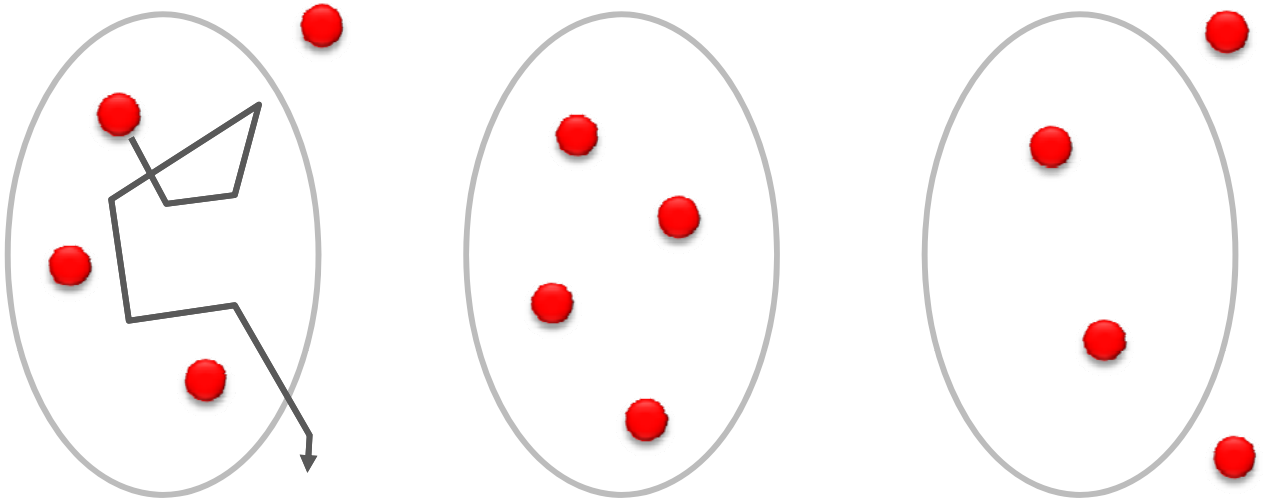
Single color FCS:

- concentrations *c* of fluorescent molecules
- properties of diffusion/ transport processes
- diffusion coefficients *D*



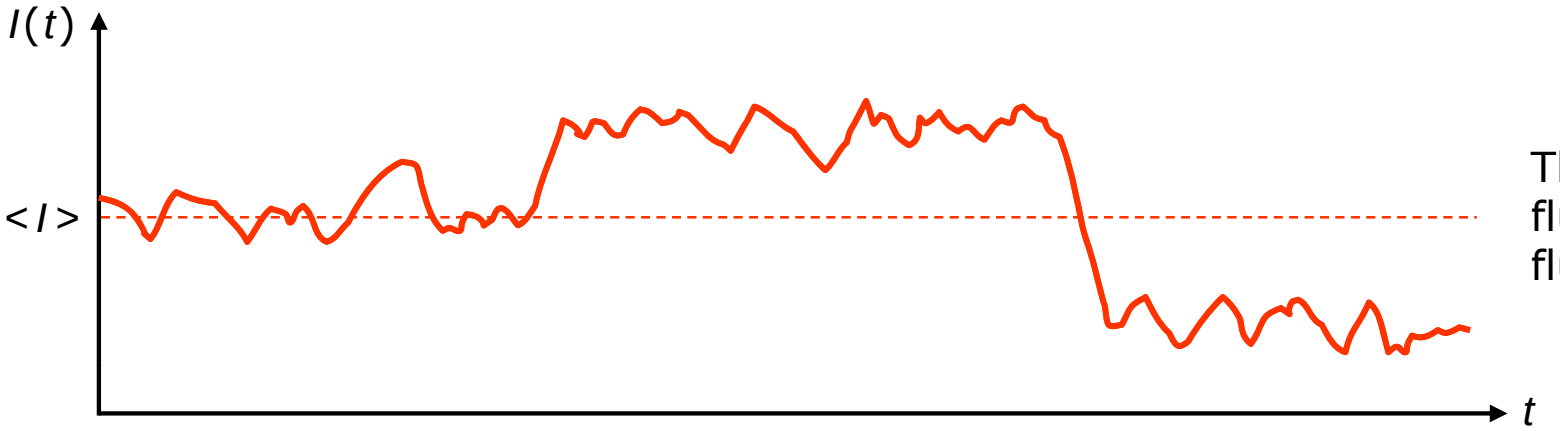
FCS – counting single molecules





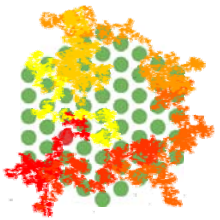
<N> = 3

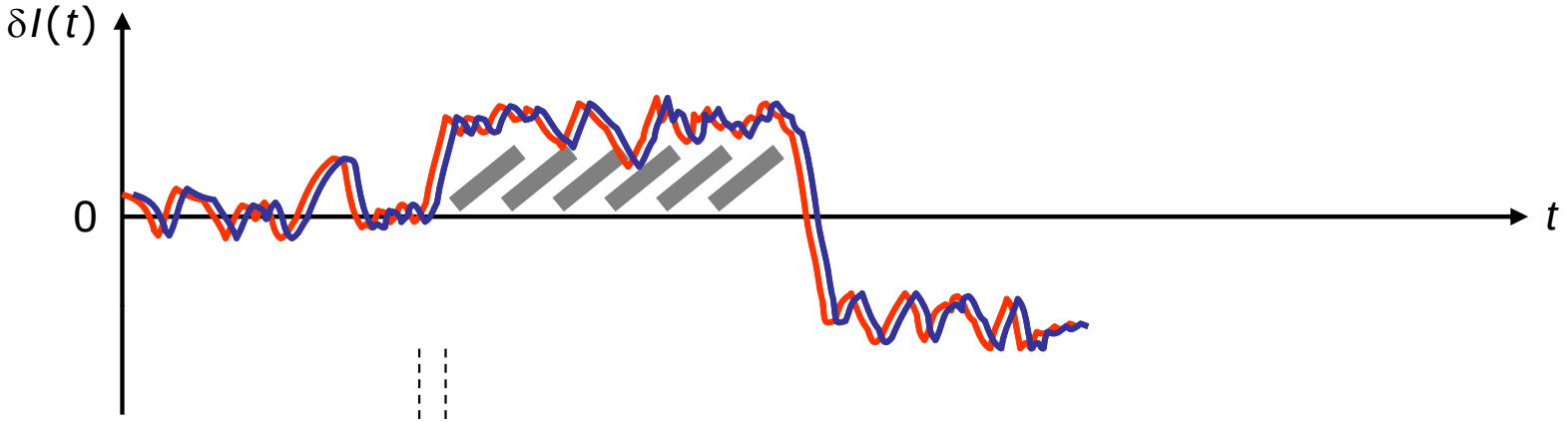
Diffusion induces fluctuations of the number of molecules

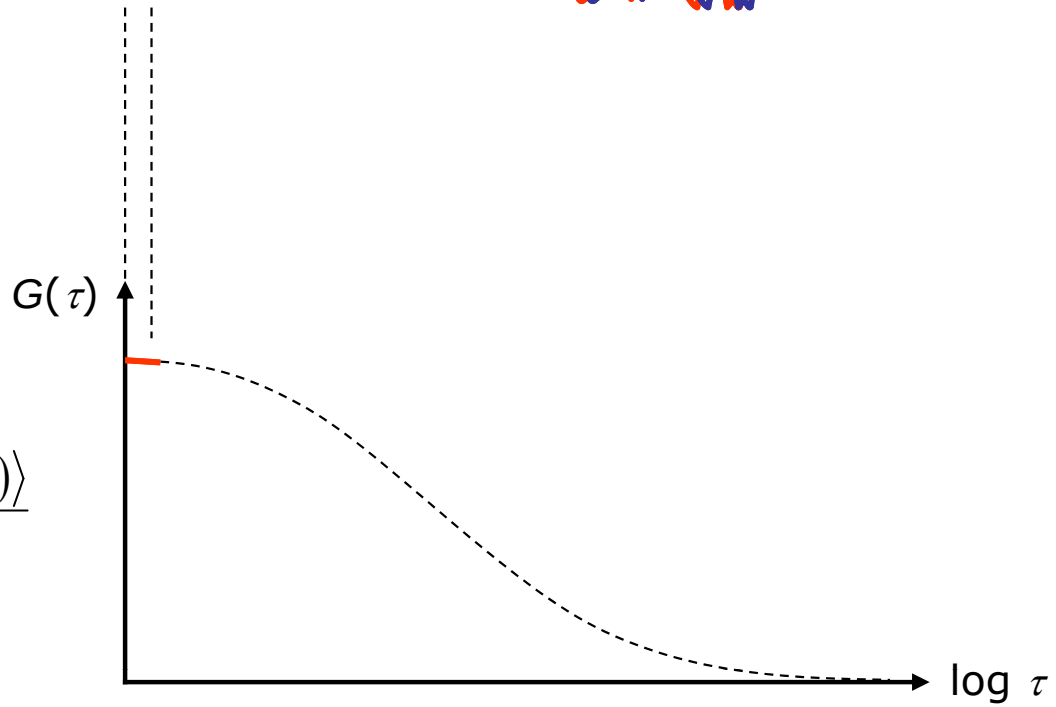


This results in fluctuations of the fluorescence signal

FCS – autocorrelation analysis

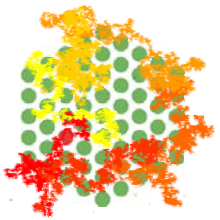


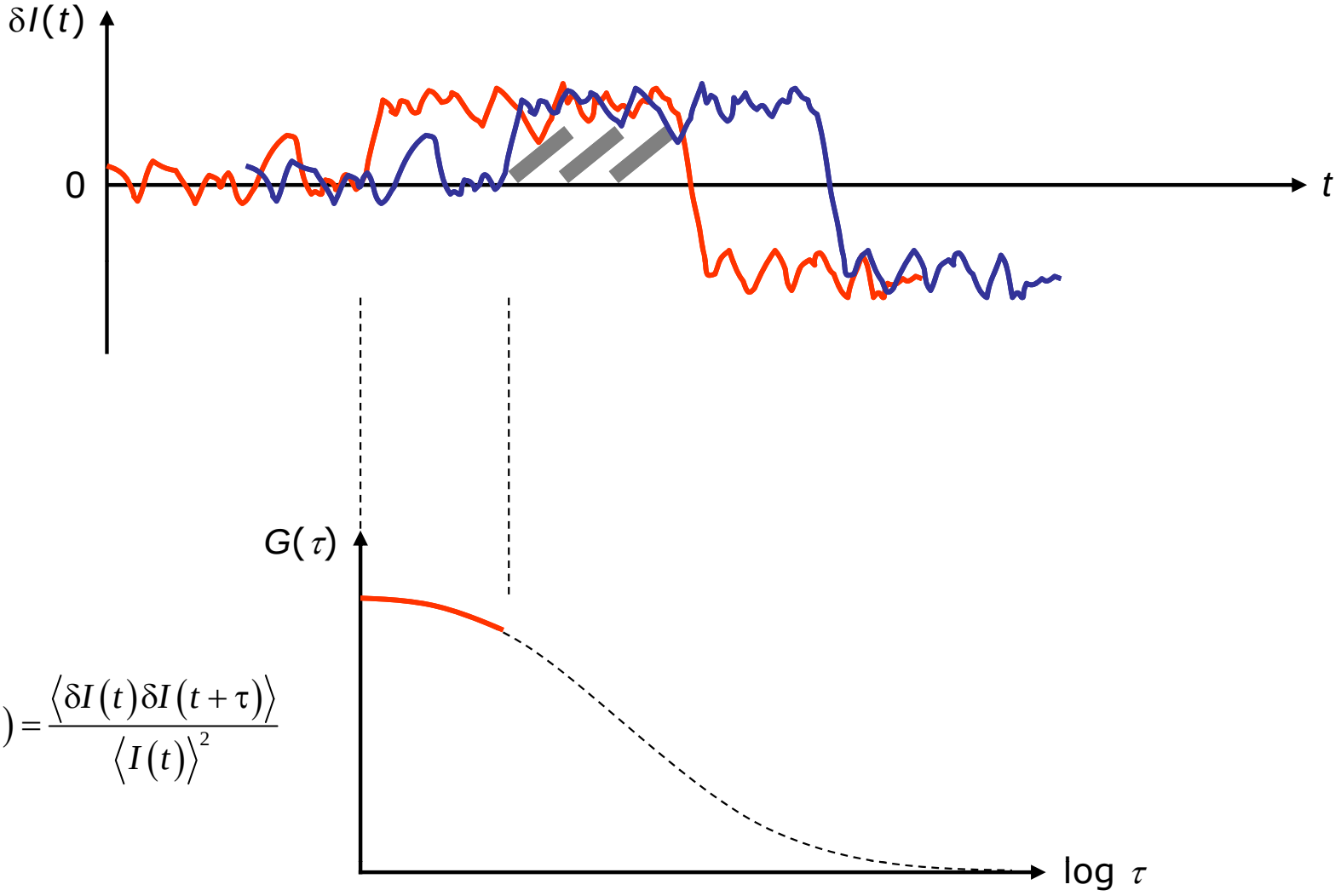




$$G_{\mathrm{O}}(\tau)=\frac{\langle \delta I(t)\,\delta I(t+\tau)\rangle}{\langle I(t)\rangle^{2}}$$

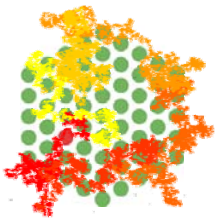
FCS – autocorrelation analysis

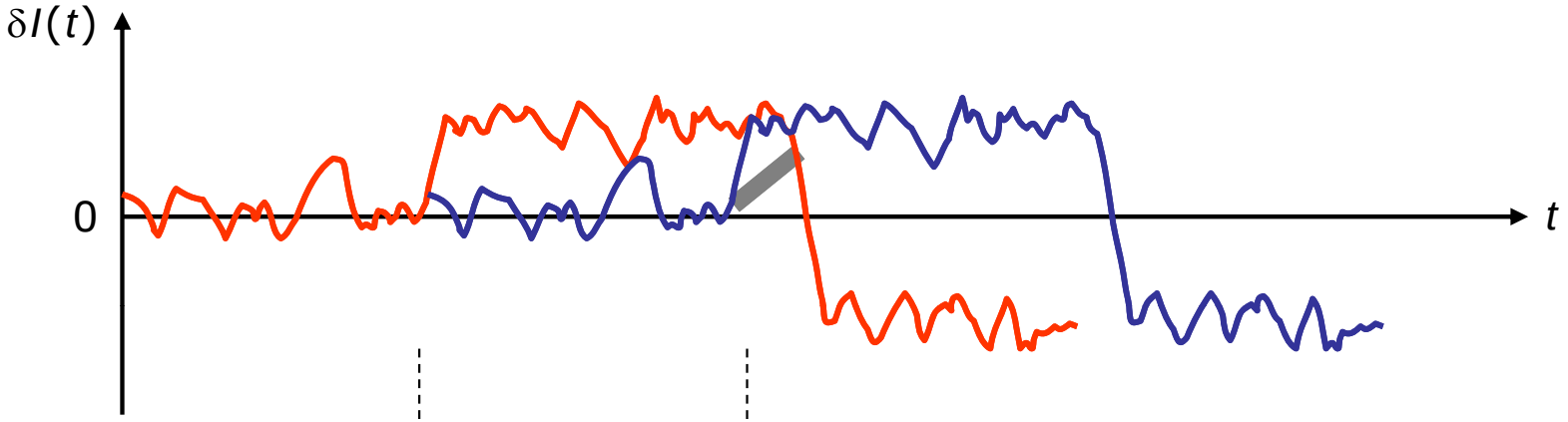


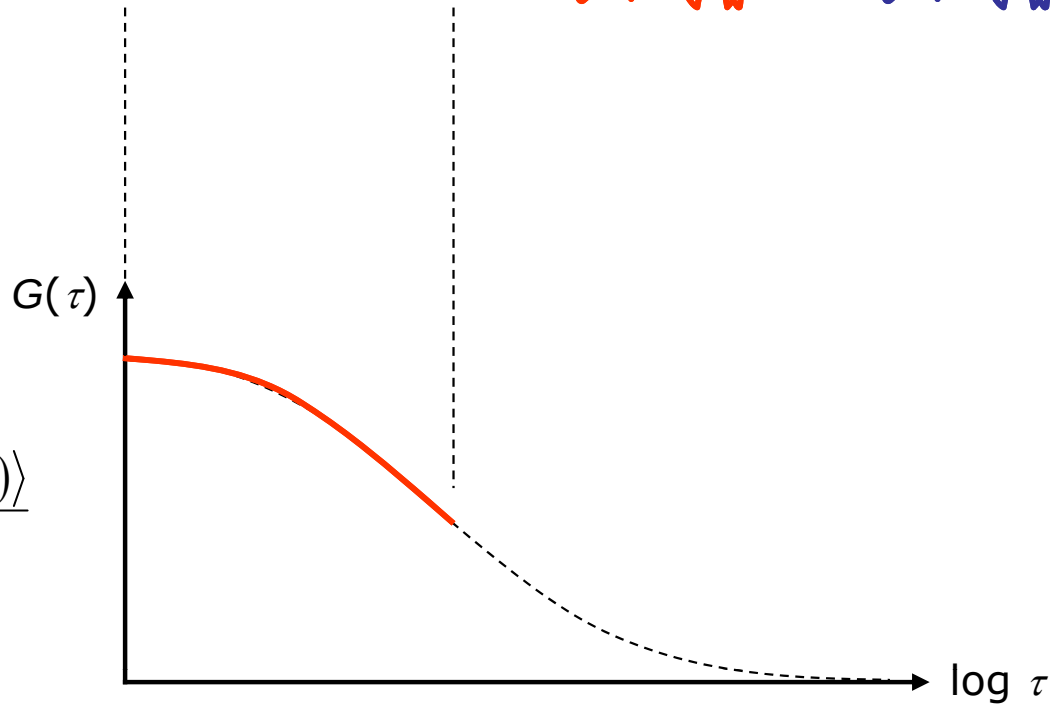


$$G_O(\tau) = \frac{\langle \delta I(t)\, \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

FCS – autocorrelation analysis

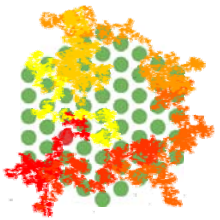


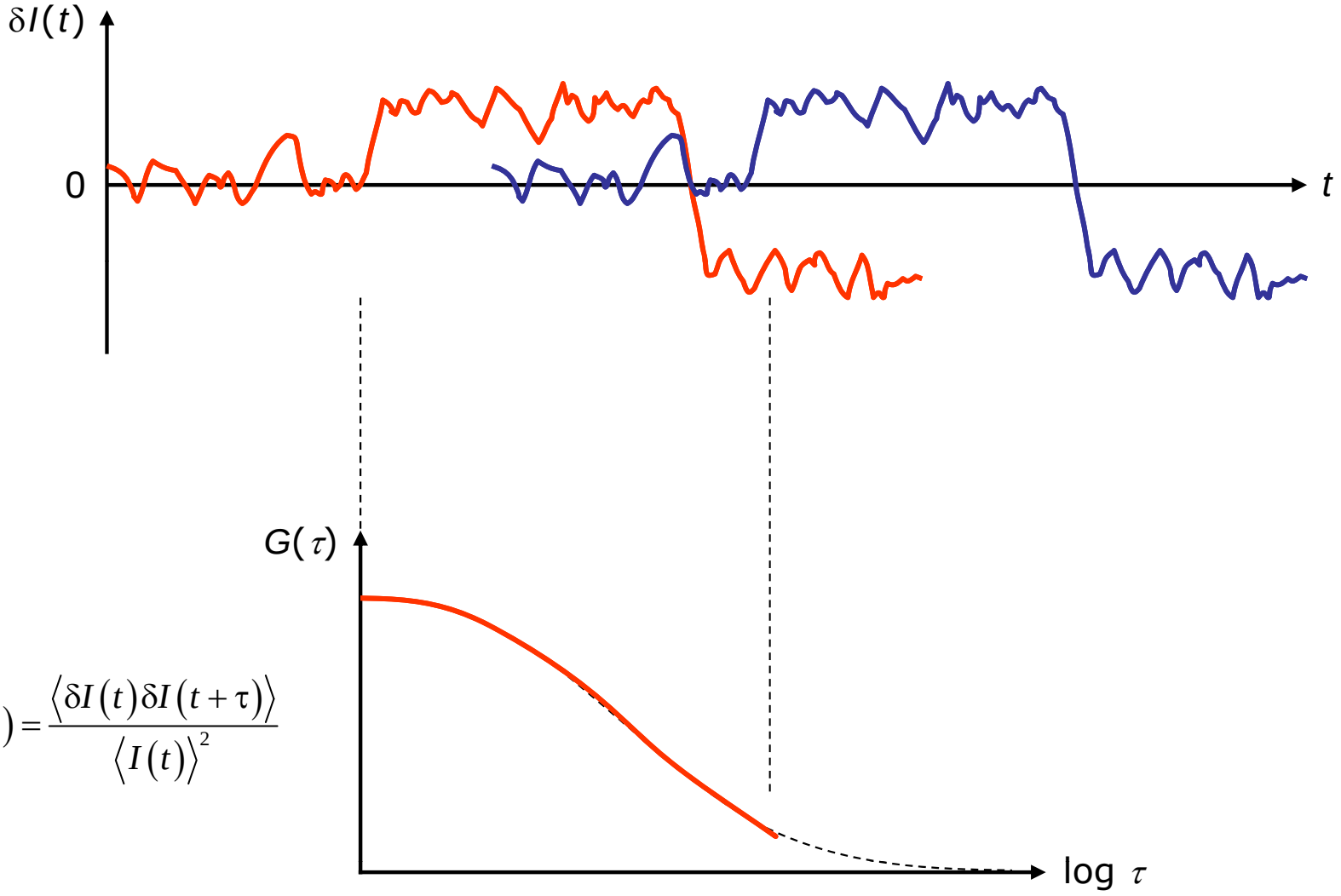




$$G_{\mathrm{O}}(\tau)=\frac{\langle \delta I(t)\,\delta I(t+\tau)\rangle}{\langle I(t)\rangle^{2}}$$

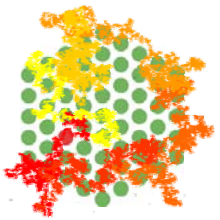
FCS – autocorrelation analysis

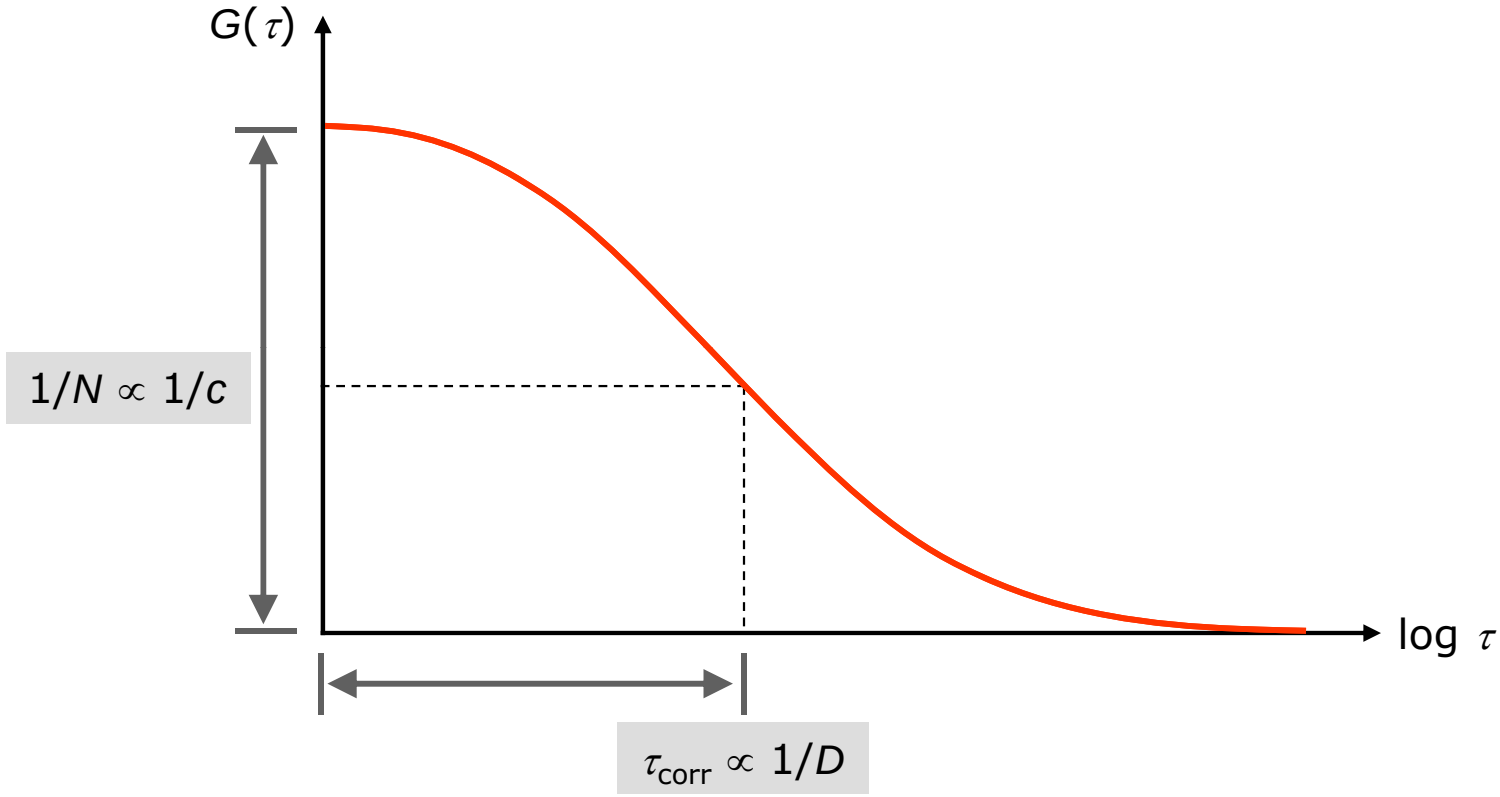




$$G_{\mathrm{O}}(\tau)=\frac{\langle \delta I(t)\,\delta I(t+\tau)\rangle}{\langle I(t)\rangle^{2}}$$

FCS – autocorrelation analysis



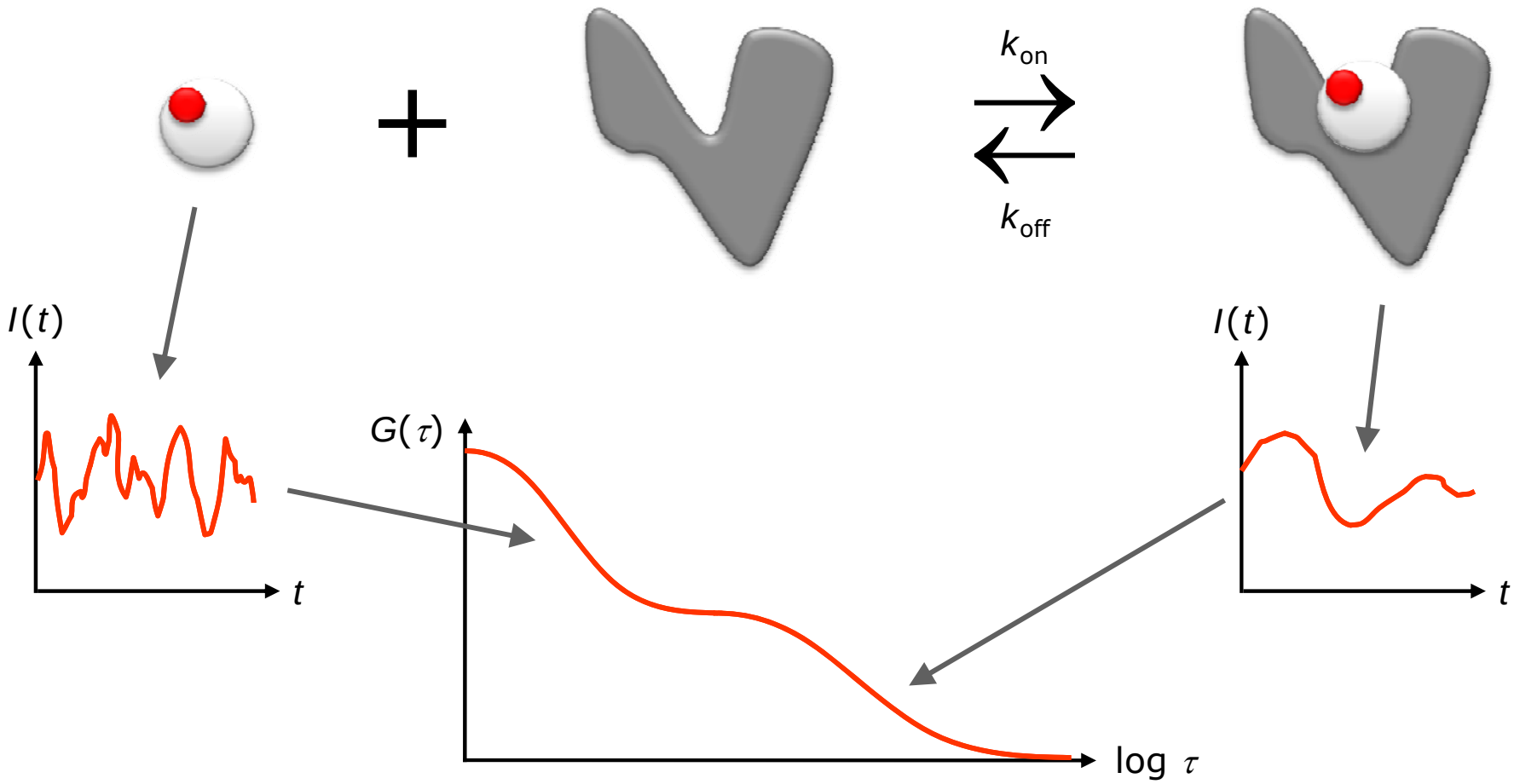


Fitting the autocorrelation function to appropriate model functions results in

- properties of the diffusion process
- the concentration

of several species with different hydrodynamic properties

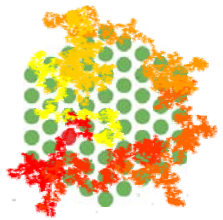
Different species in the autocorrelation function

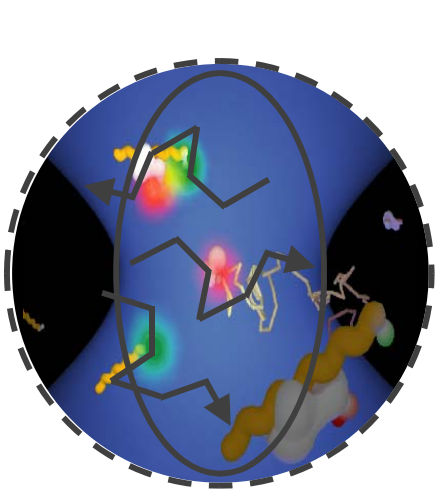


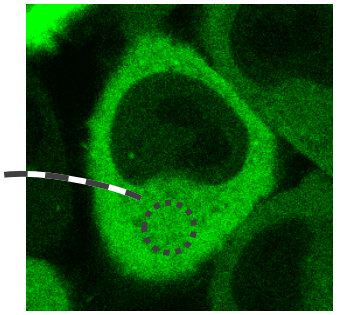
Properties of ligand-receptor interactions:

dissociation constants, reaction rates, concentrations

Confocal fluorescence cross-correlation spectroscopy (FCCS)

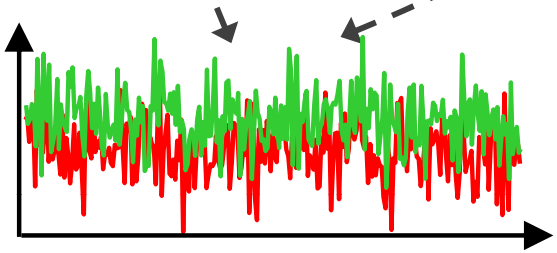


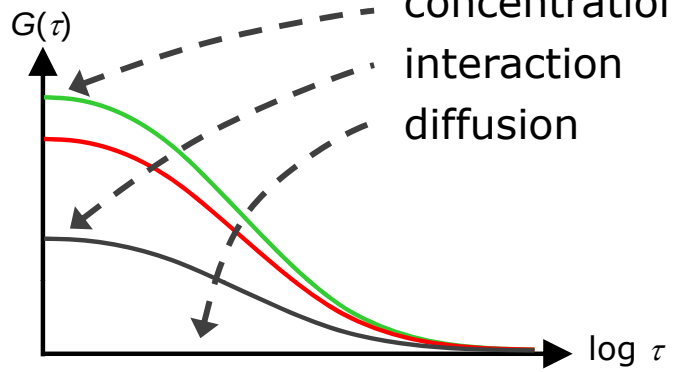




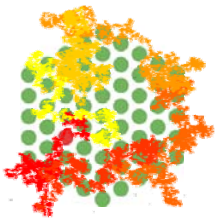
Dual-color FCCS:

- bimolecular interaction properties
- kinetic rates k_{on}, k_{off}
- dissociation constants K_D



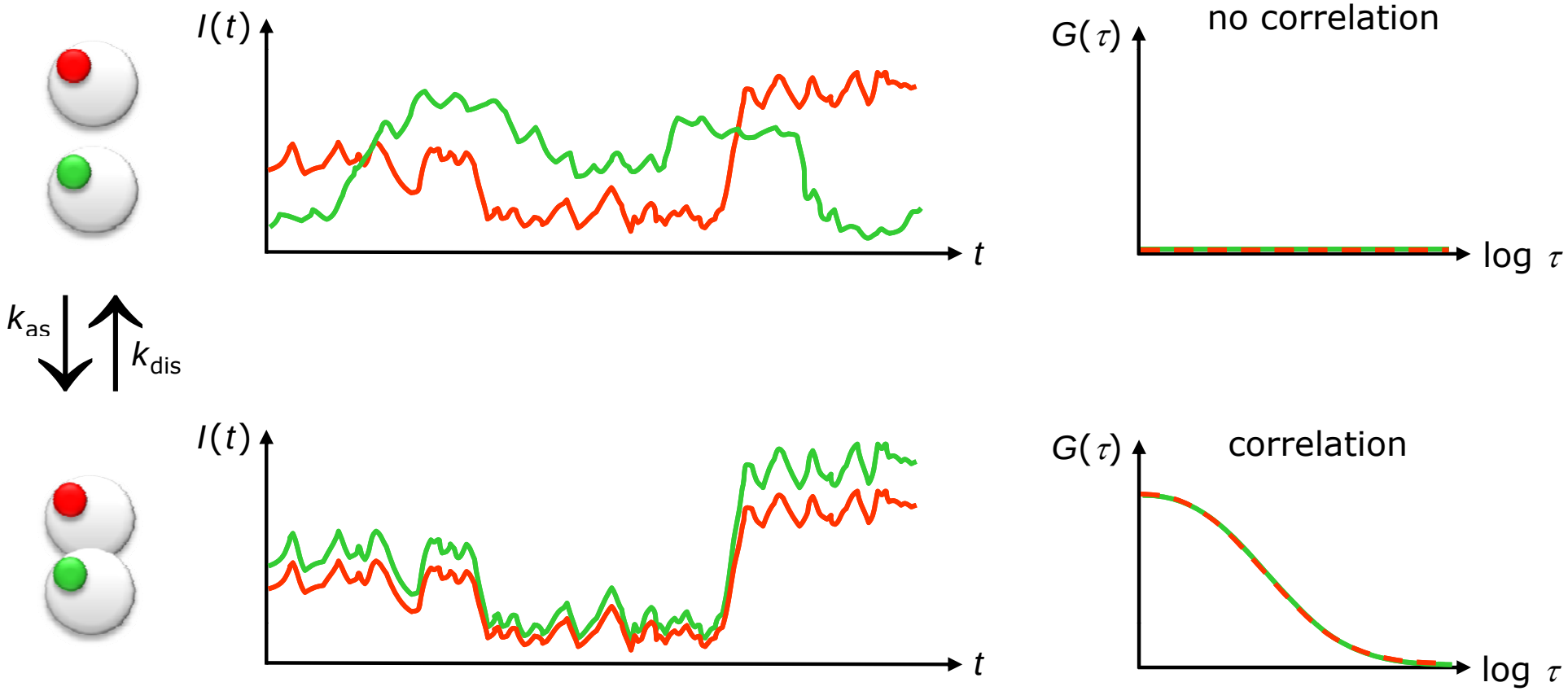


FCCS – fluorescence cross correlation spectroscopy

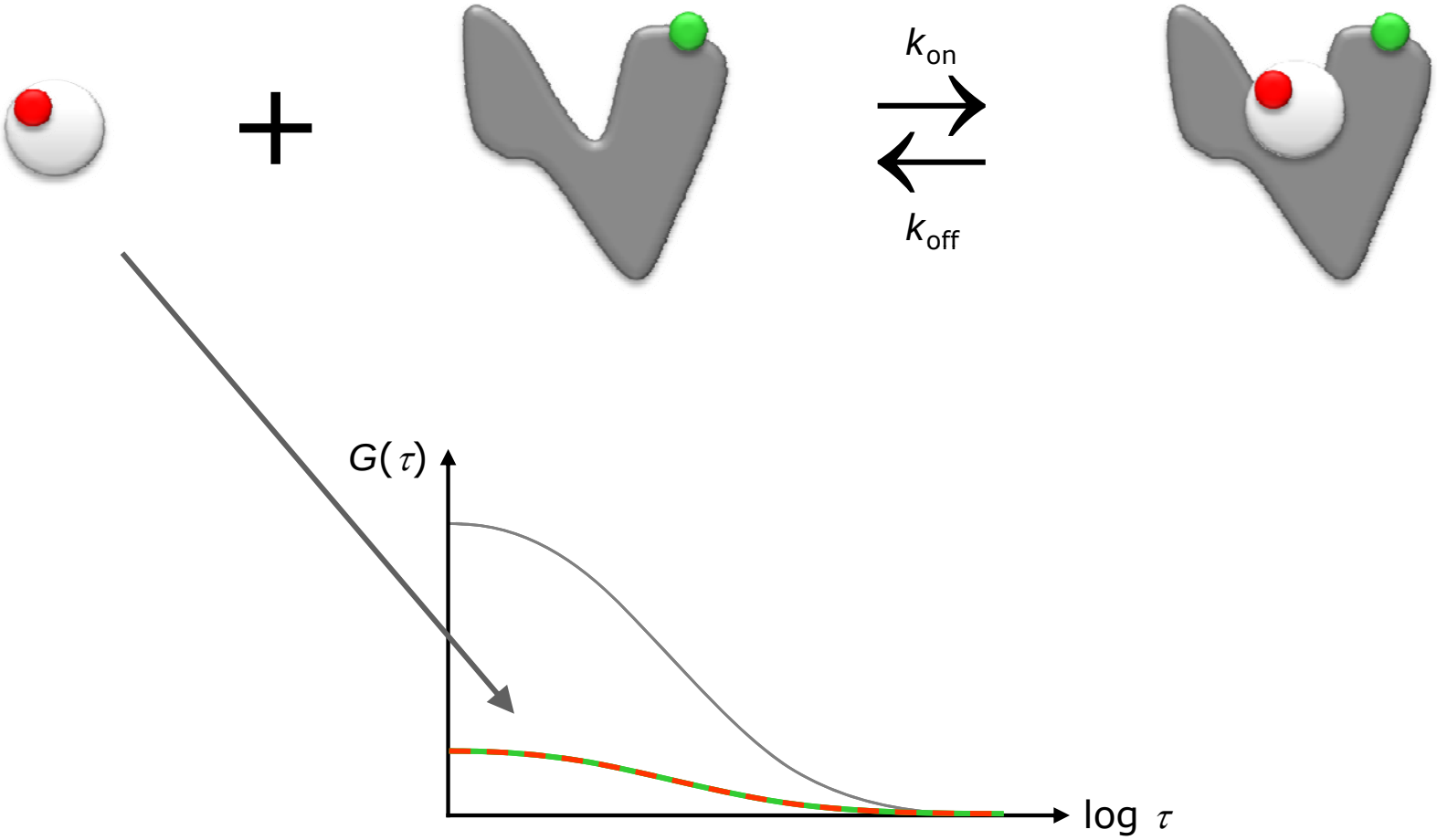


Extended concept:

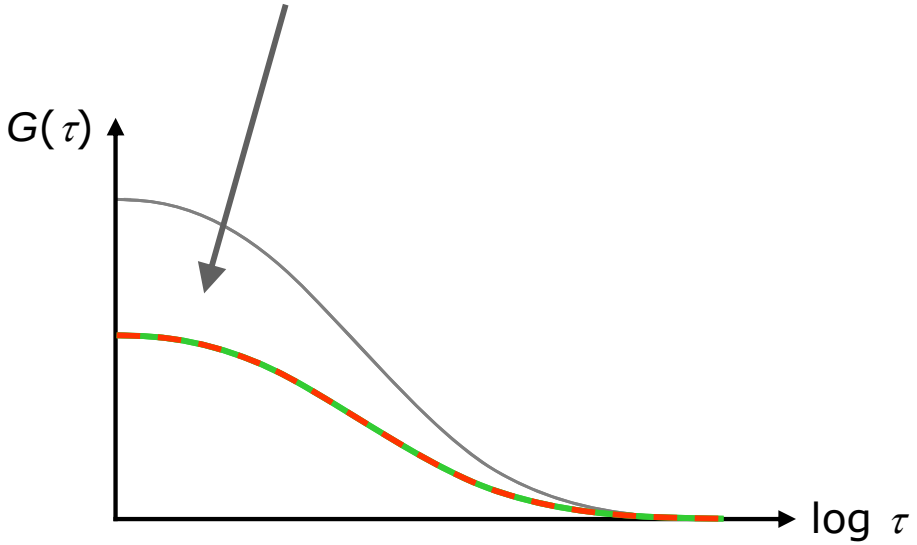
- labeling of potential binding partners with spectrally different fluorophores
- looking for correlations between the corresponding signals



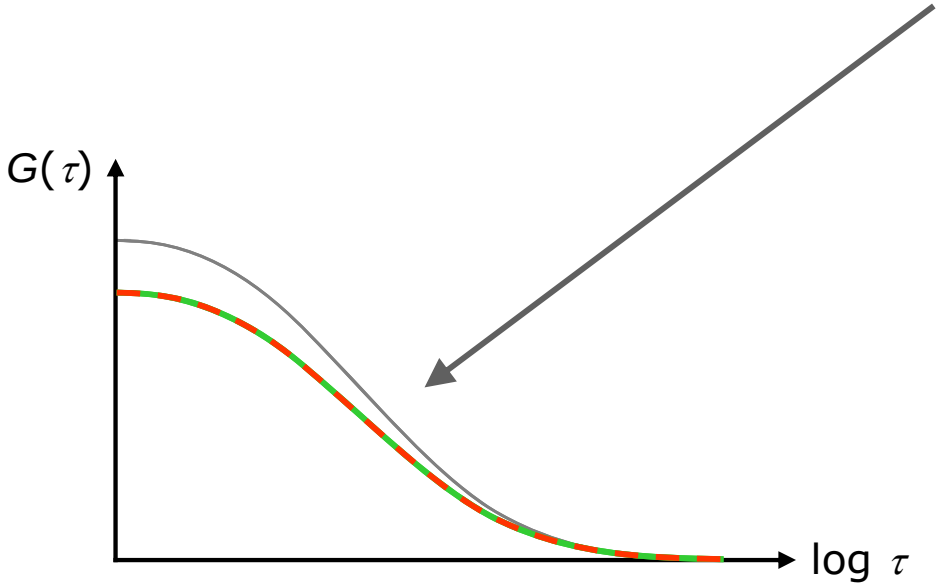
FCCS – model application

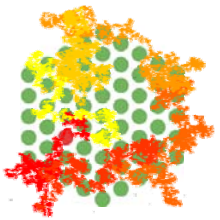


FCCS – model application



FCCS – model application





EMBL

Systems Biology of Epigenetic Networks

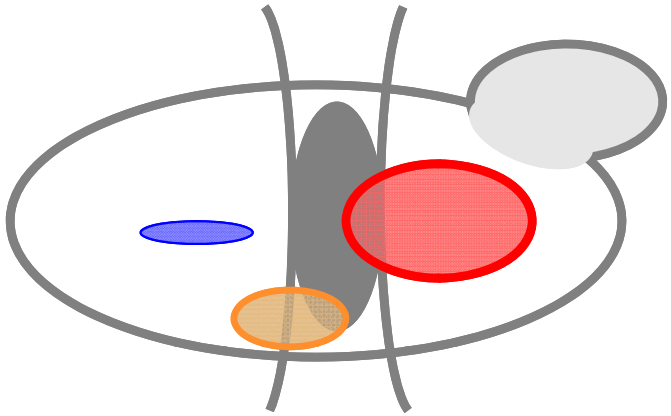
GEFÖRDERT VOM

Bundesministerium
für Bildung
und Forschung

Light-sheet FCCS

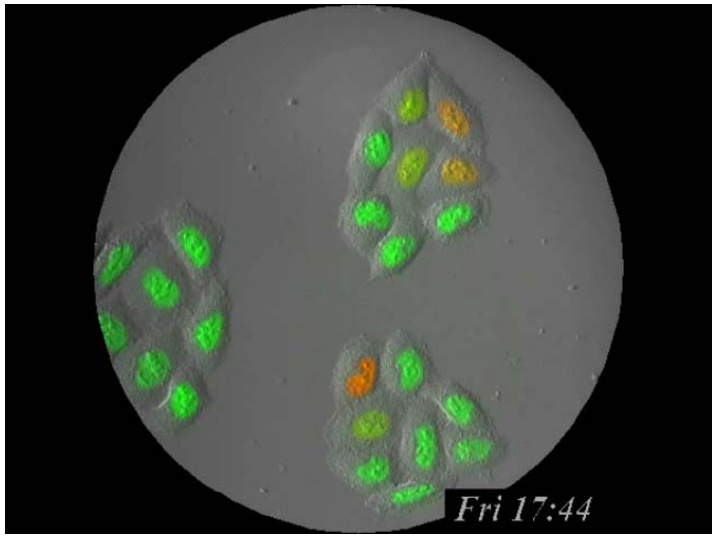
Jérémie Capoulade
Friedhelm Serwane
Michael Knop
Tim Saunders
Lars Hufnagel

Limitations of conventional and sequential point FCS



- Limited pool of (fluorescently labelled) molecules
- Heterogeneous cellular interior
- Slow cellular movements and changes
- Long integration time for 2D maps of FCS data

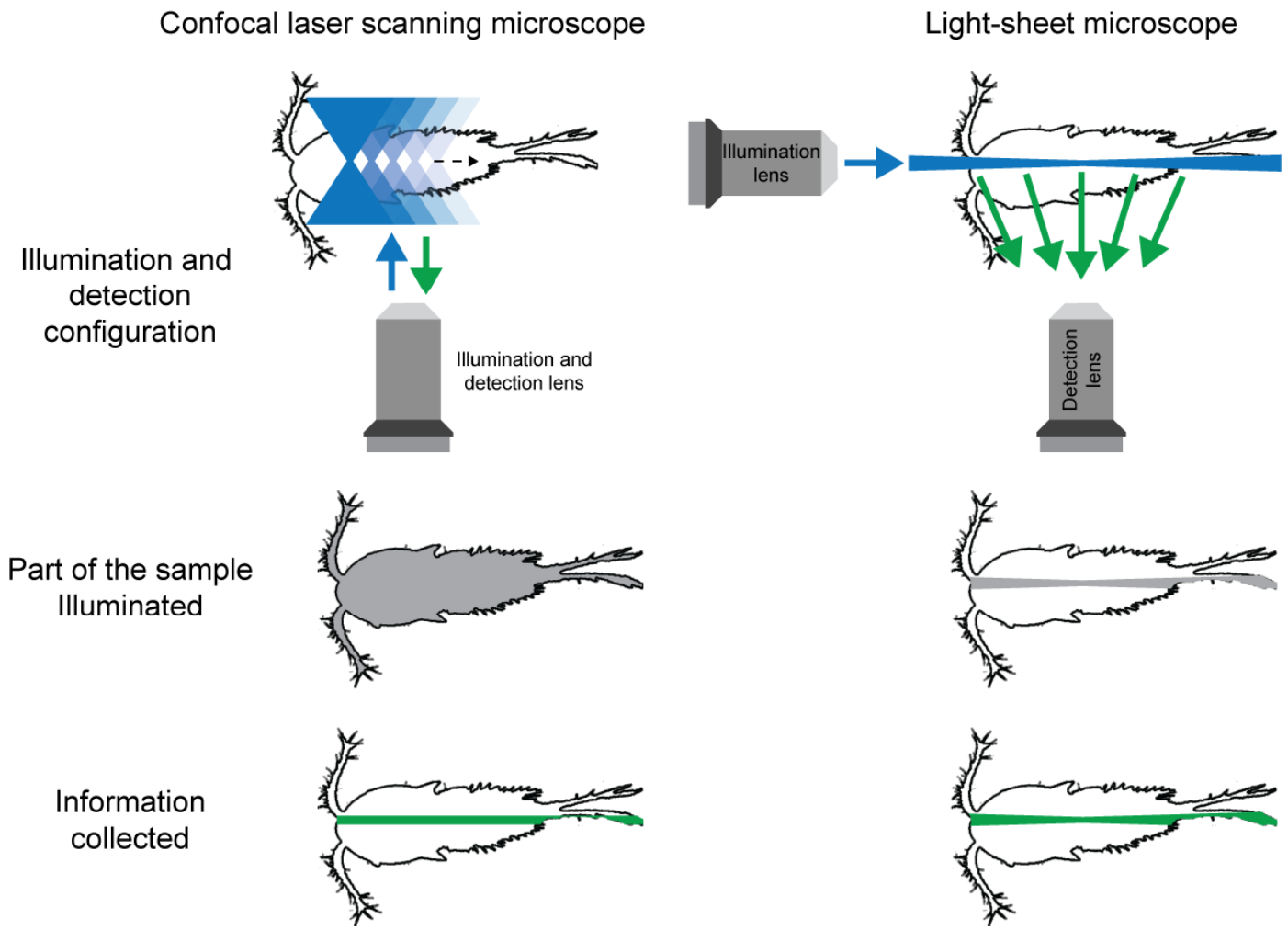
Single and sequential FCS measurements from single cells often suffer from large errors



Biological requirements:

- good statistics in single cells
- spatially resolved measurements

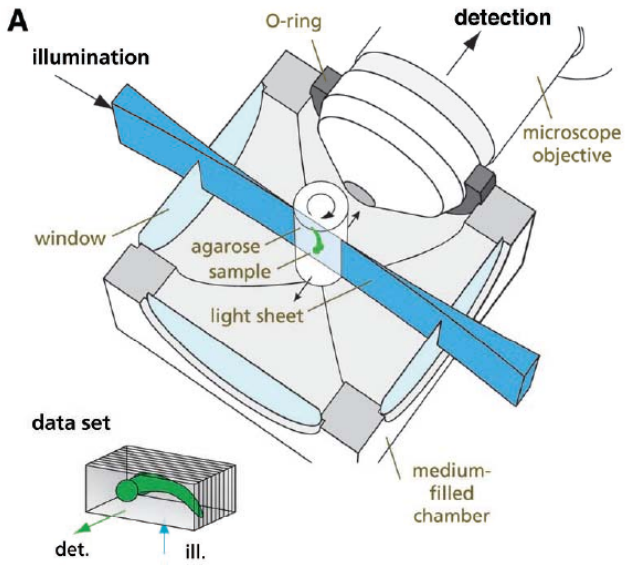
Epi- vs. light-sheet fluorescence microscopy



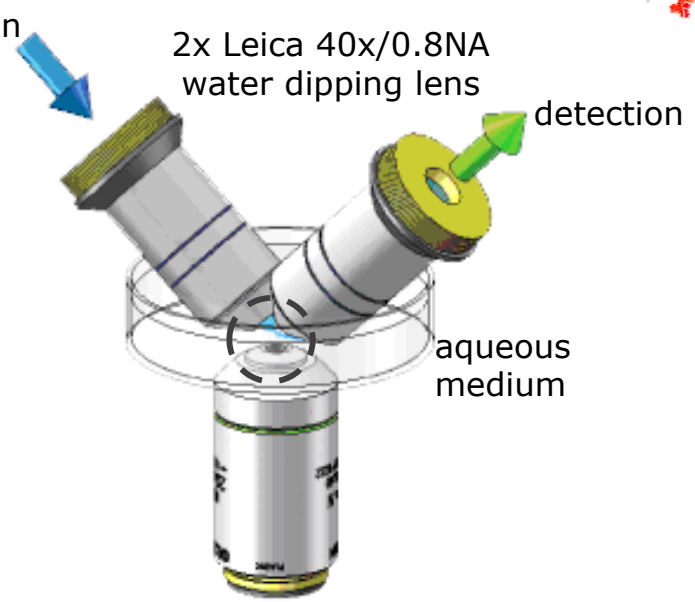
Fluorescence excitation only in the detection focal plane

- no out of focus fluorescence generated
- optical sectioning deep inside 3D specimens
- good S/N ratio

Light-sheet-based imaging and multifocal FCS



Huisken et al., Science 305, 2004

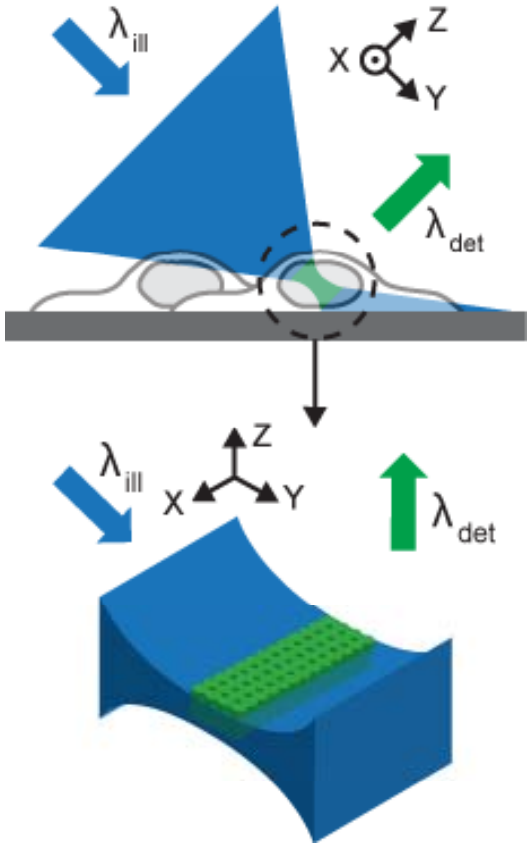


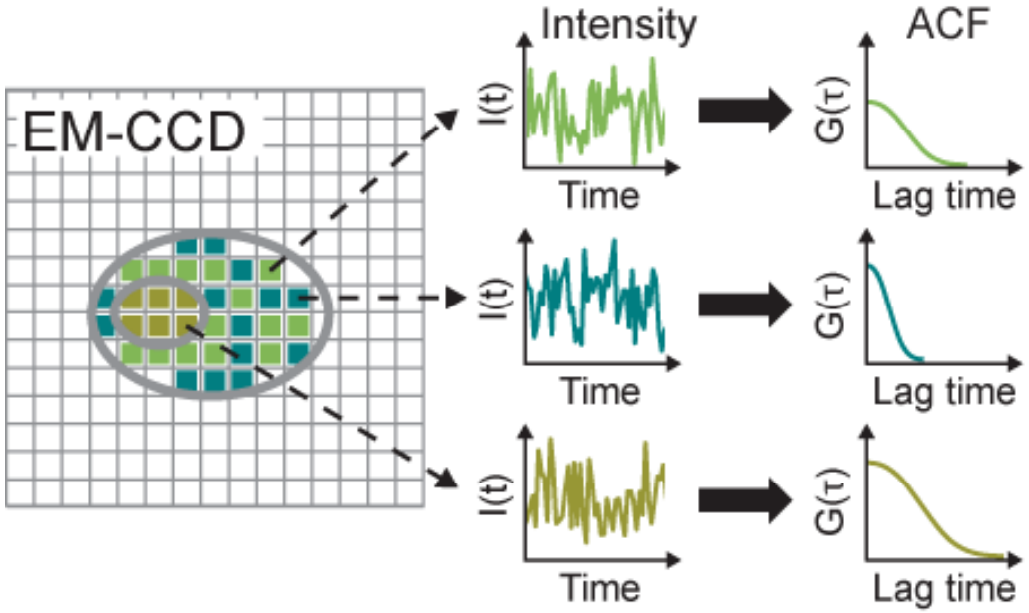
Light-sheet configuration:

- Illumination & detection with high NA water dipping lenses
  ⇨ optimized optical performance
- No agarose-based sample mounting
  ⇨ suitable for living cells
- Combination with an inverted microscope
  ⇨ simplified sample positioning

Capoulade et al., Nat. Biotechnol. 29, 2011

2D-FCS: concept





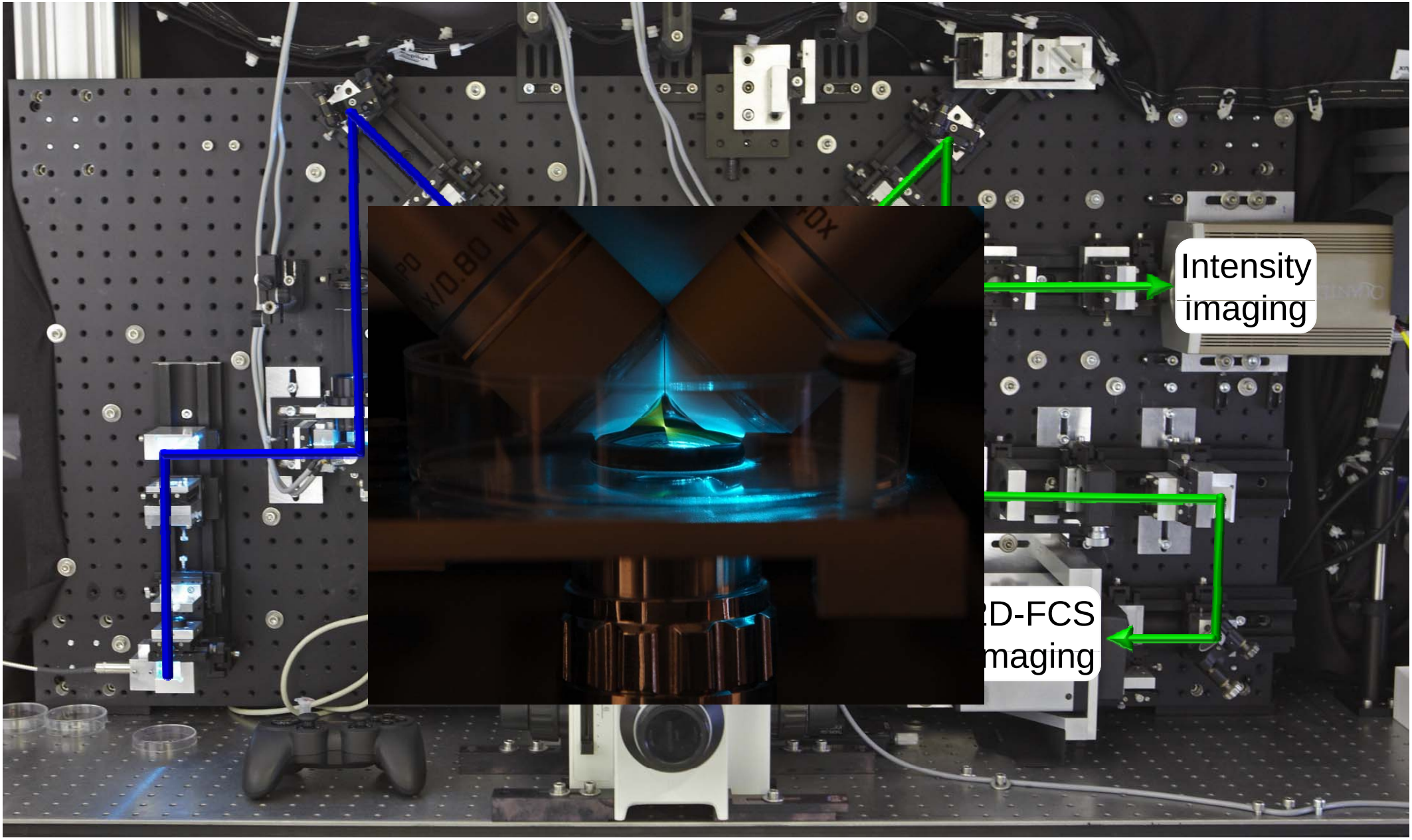
Optical sectioning under 45°:

- Array of close-to-confocal volume elements
- Every volume element corresponds to a camera pixel

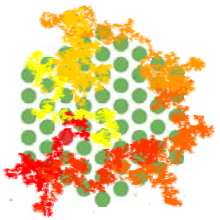
EM-CCD-based detection:

- Single photon counting
- Every pixel serves as FCS point detector

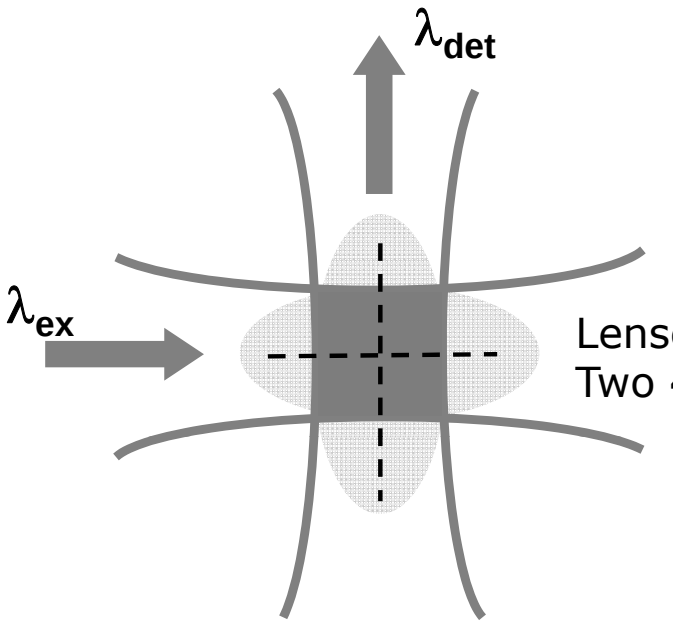
2D-FCS: the setup



PSF measurements



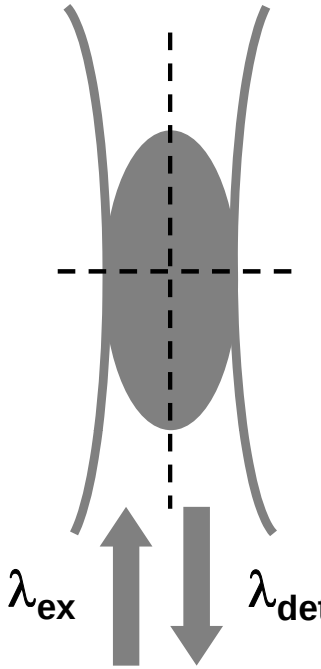
2D-FCS microscope



Lenses:
Two 40x, 0.8 NA

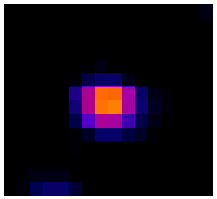
Sample: 20nm beads
(Invitrogen) in agarose

Confocal microscope Leica SP5

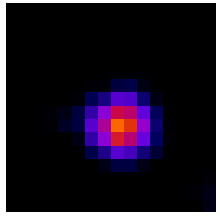


Lens:
63x, 1.2 NA

(XY)



(XZ)

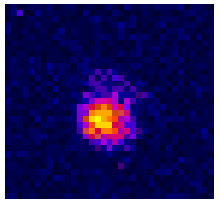


w_{XY} = 370 nm

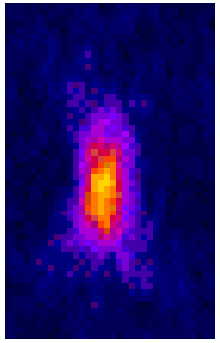
w_Z = 410 nm

V = 0.31 fL

(XY)



(XZ)



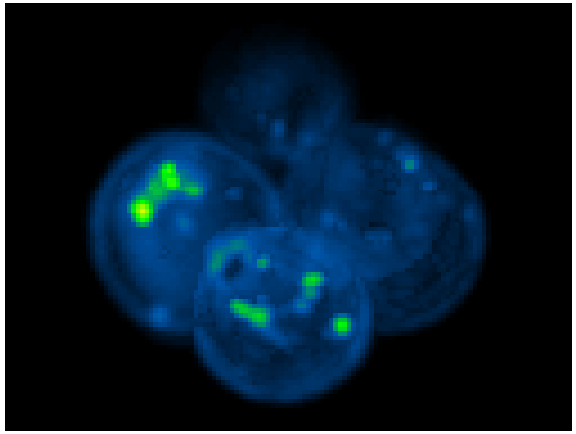
w_{XY} = 240 nm

w_Z = 600 nm

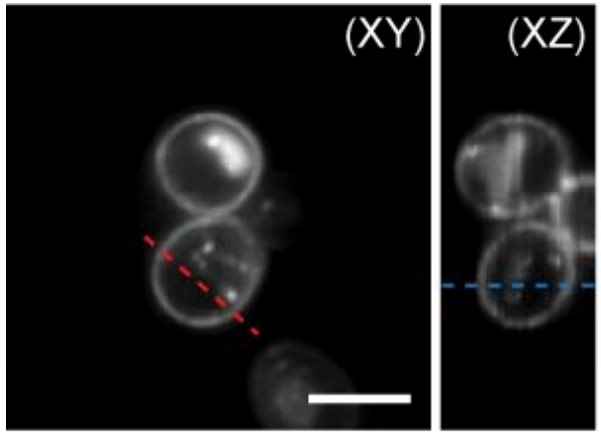
V = 0.19 fL

Light-sheet microscopy of yeast with isotropic resolution

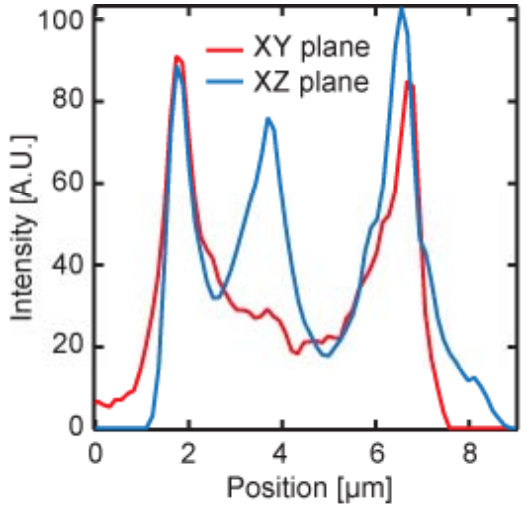
2D-FCS microscope



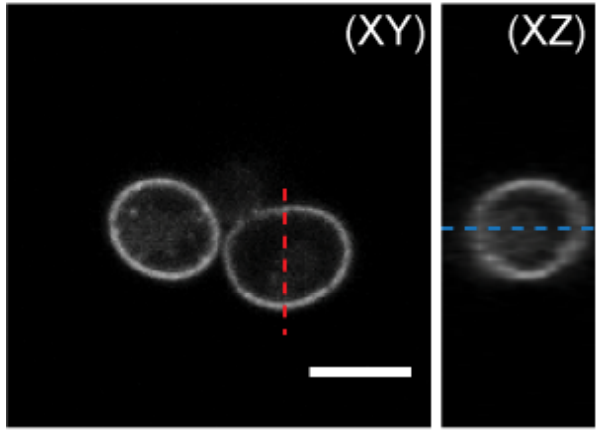
3D reconstruction
image stack, z step 280 nm
scale bar 2.5 µm



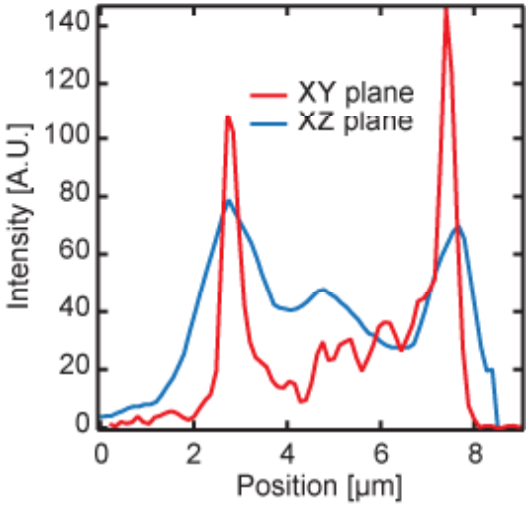
scale bar 5 µm



confocal microscope



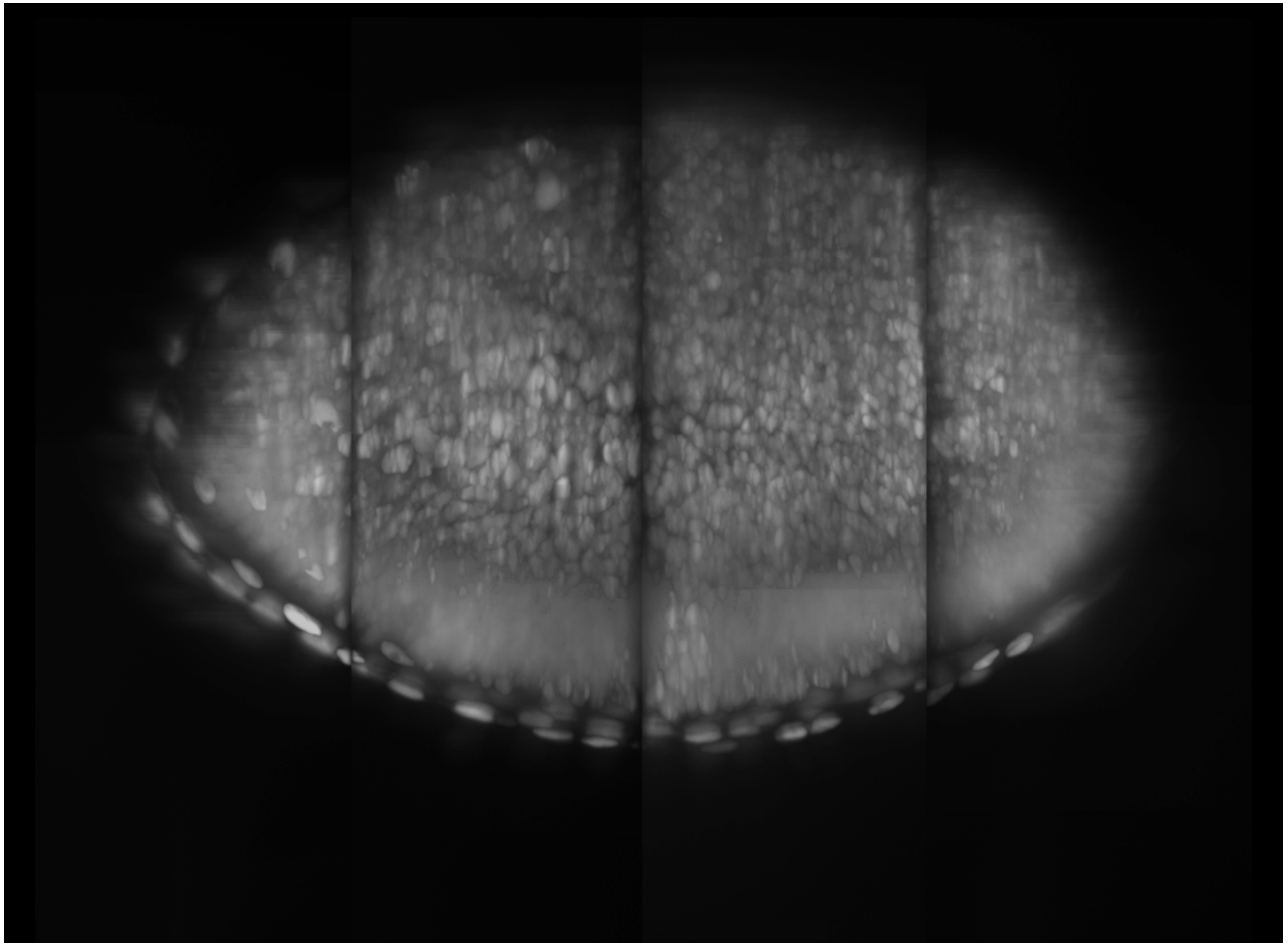
scale bar 5 µm



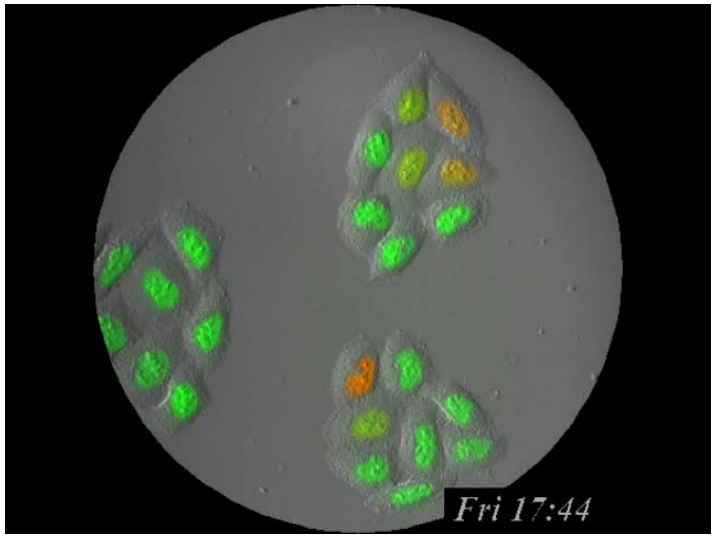
S. cerevisiae expressing
plasma membrane
protein Pma1-GFP

Light-sheet microscopy of Drosophila wing imaginal disc

Wing imaginal disc of Drosophila larva expressing GFP-NLS



FCS of MDCK cells expressing the Fucci system

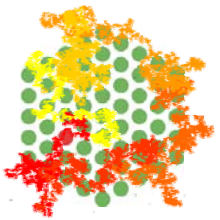


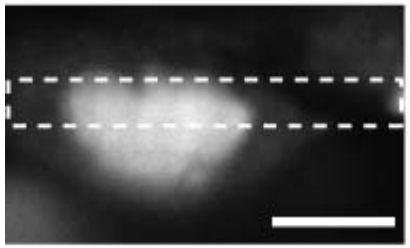
The fluorescent ubiquitination-based cell cycle indicator system Fucci:

Sakaue-Sawano et al., Cell (2008)

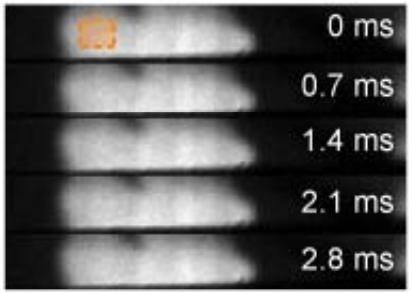
- green in S to M phase
- red in G_1
- in S to M, point FCS shows free diffusion of green construct with D ~ 24 $\mu m^2.s^{-1}$

2D-FCS of MDCK cells expressing the Fucci system

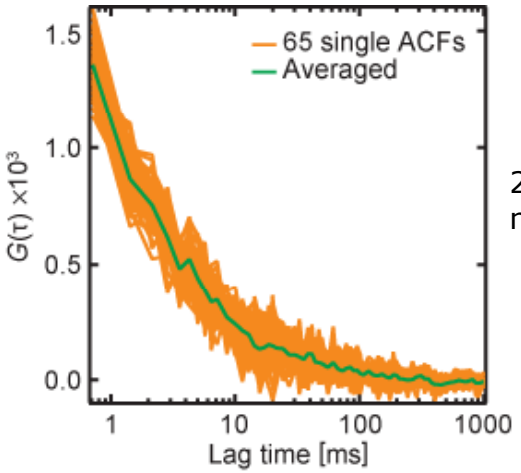




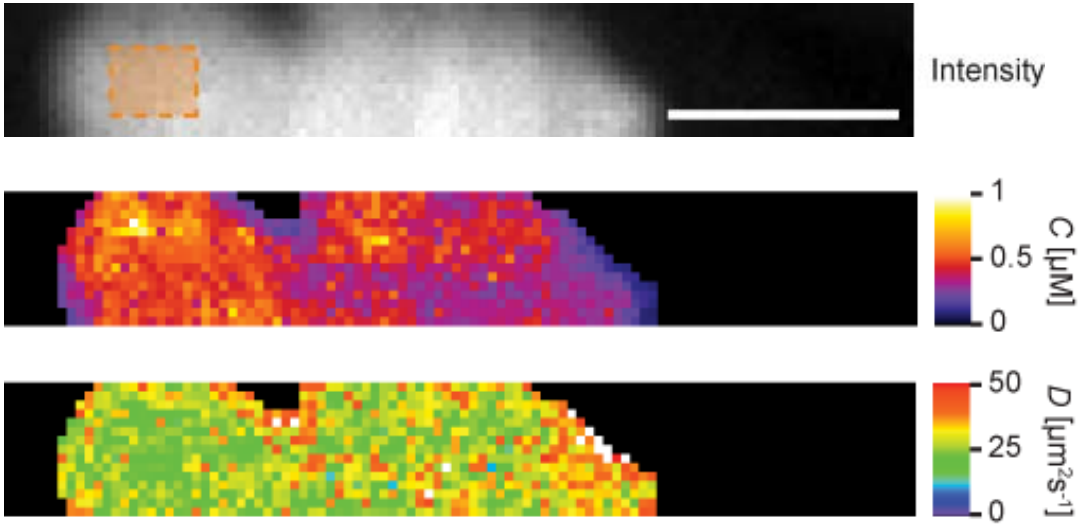
QuantEM
EM-CCD



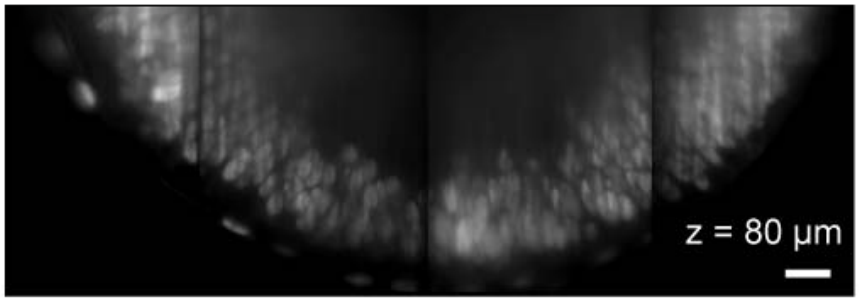
Sensovation
EM-CCD

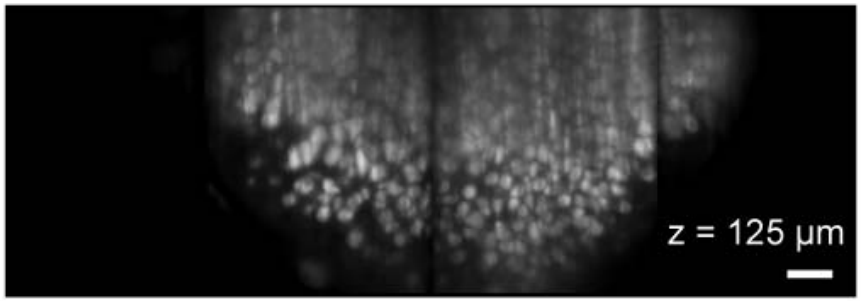


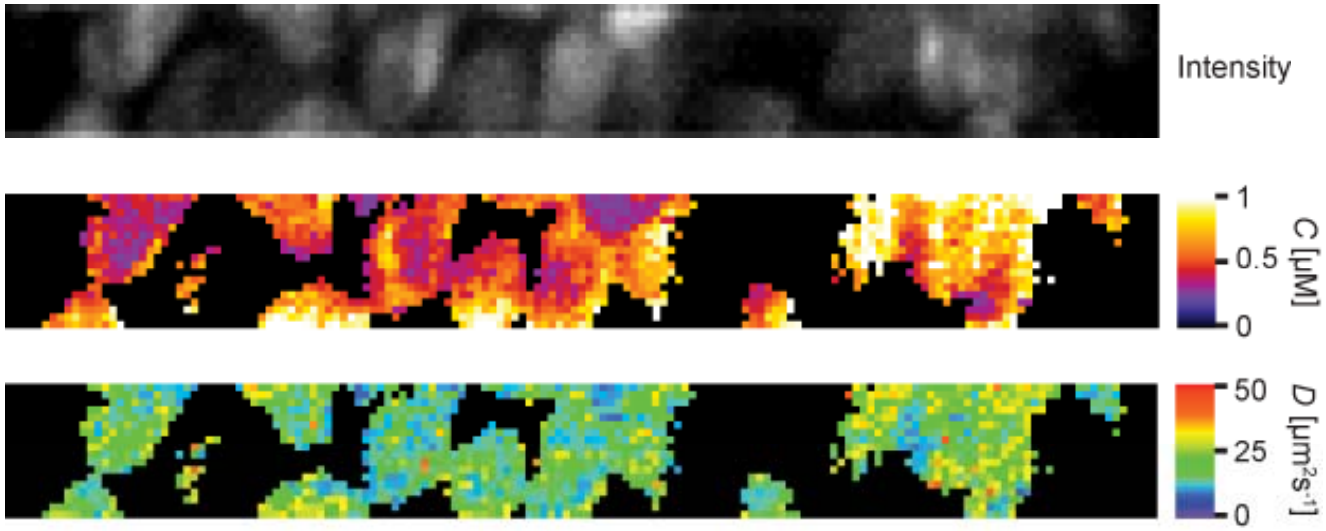
2D-FCS
measurements



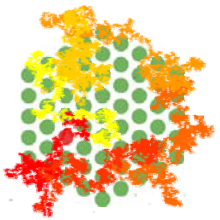
2D-FCS of GFP-NLS in Drosophila wing imaginal discs

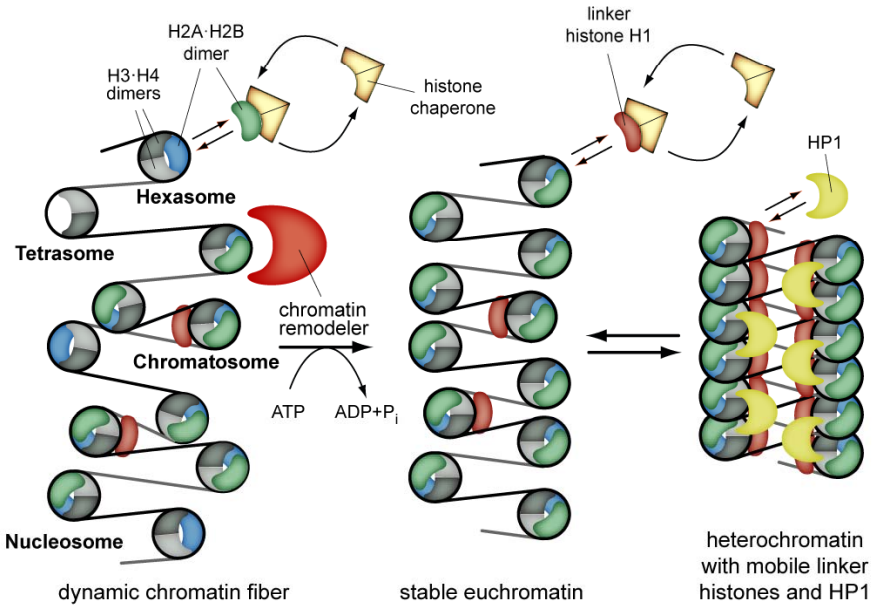






Chromatin organization in interphase

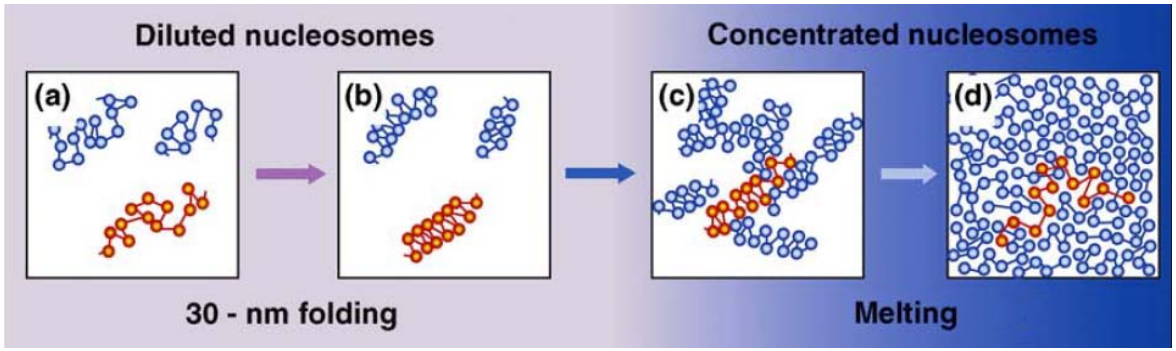




From histones to the chromatin fiber:

- compaction
- conservation and protection
- dynamic organisation
- regulation of transcription, replication, repair
- adoption of different epigenetic states

Wachsmuth et al., Biochim. Biophys. Acta 1783, 2008

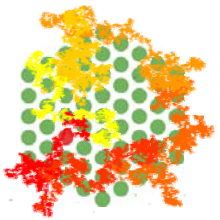


Maeshima et al., Curr. Op. Cell. Biol. 22, 2010

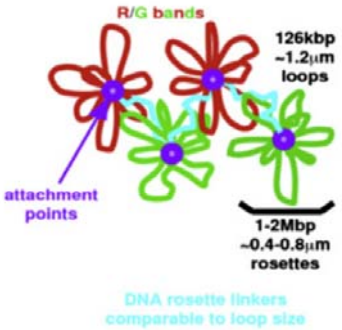
Controversial:

- 30 nm chromatin fiber – as observed under (semi-)dilute conditions
- poorly structured – sea of nucleosomes, polymer melt

Chromatin organization in interphase

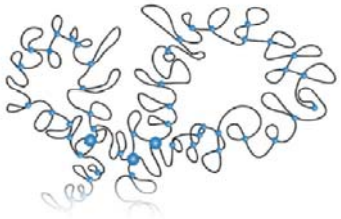


Models for higher-order organisation:



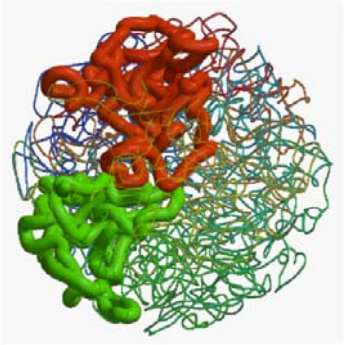
Jhunjhunwala et al.,
Cell 133, 2008

Multi-loop sub-compartment model



Mateos-Langerak et al.,
PNAS 106, 2009

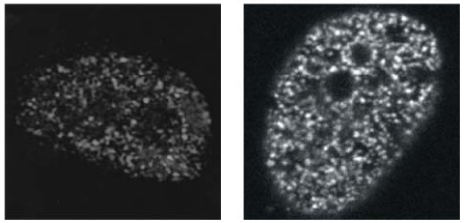
Random-loop model



Lieberman-Aiden et al.,
Science 326, 2009

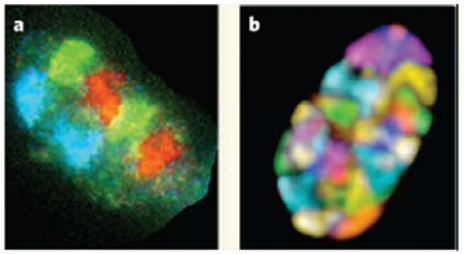
Fractal globule model

?



Verschure et al., J. Cell. Biol. 147, 1999
Cook, Science 284, 1999

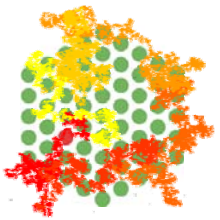
Submicrometer-sized subchromosomal replication and transcription foci



Meaburn & Misteli, Nature 445, 2007

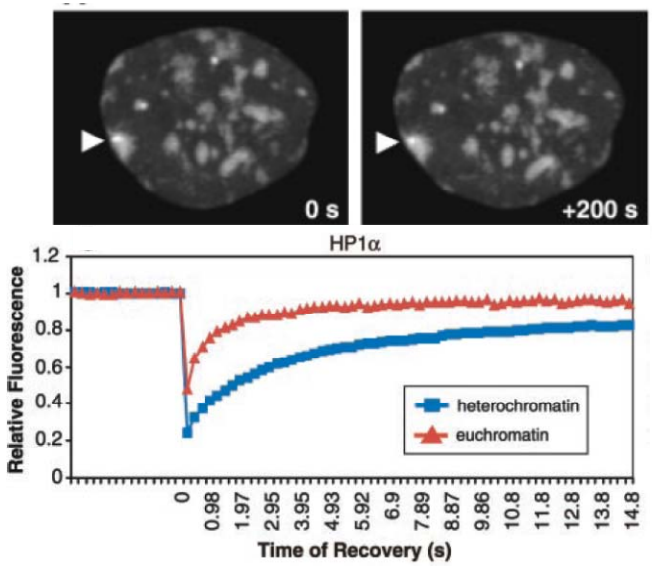
Occupation of distinct chromosome territories in the nucleus

3T3 cells expressing HP1α-EGFP: diffusion and binding

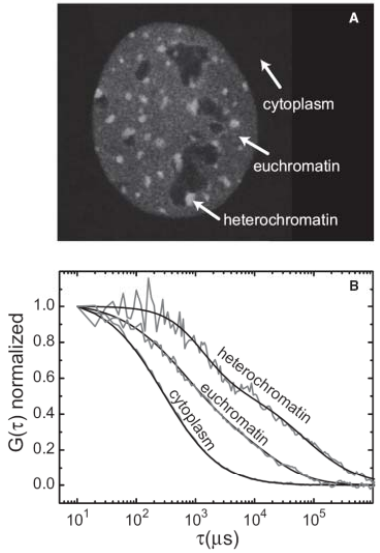


Heterochromatin protein 1 isoform α (HP1α):

- involved in heterochromatin formation
- binds globally to chromatin
- and with higher affinity to heterochromatin

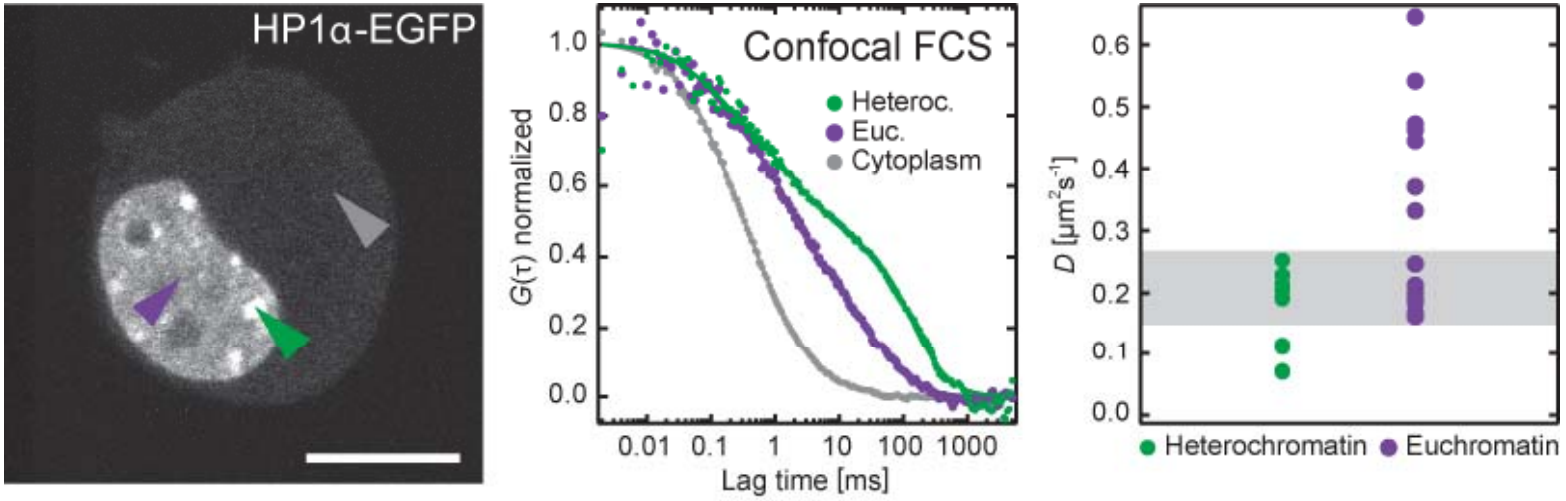


Cheutin *et al.* (2003) *Science* **299**



Müller *et al.* (2009) *Biophys. J.* **97**

Confocal FCS of 3T3 cells expressing HP1α-EGFP



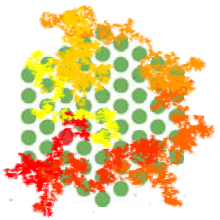
2-component fit:

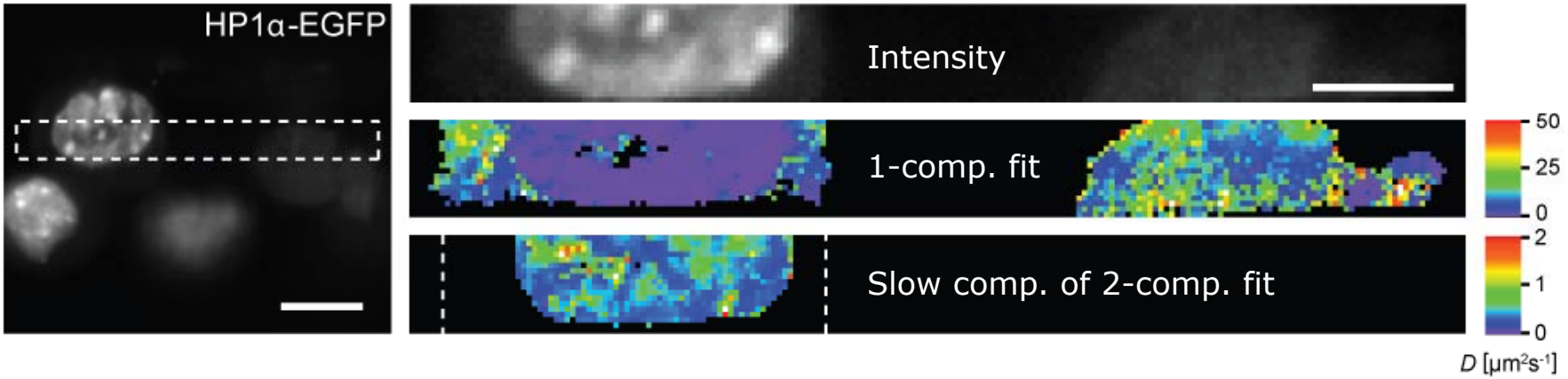
- 1st component is free fraction (fast diffusion)
- 2nd compoment is bound fraction (slow reaction-diffusion) with different properties in eu- and heterochromatin

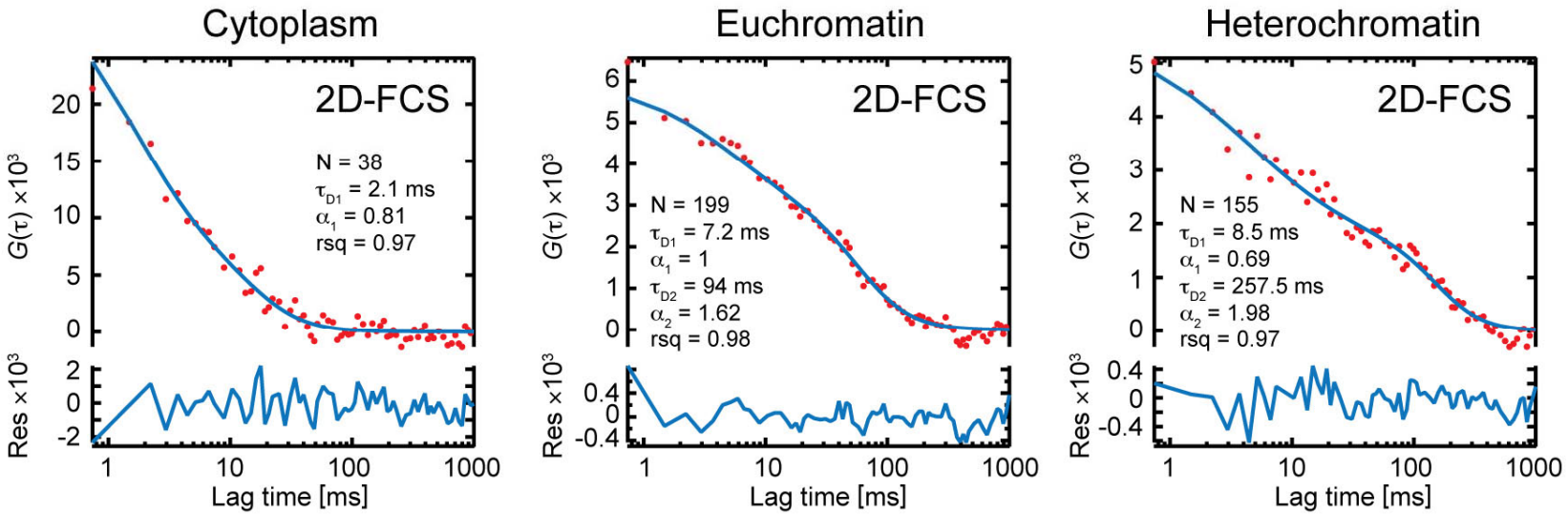
However:

- overlap of eu- and heterochromatin distribution
- noise? significant? spatial distribution?

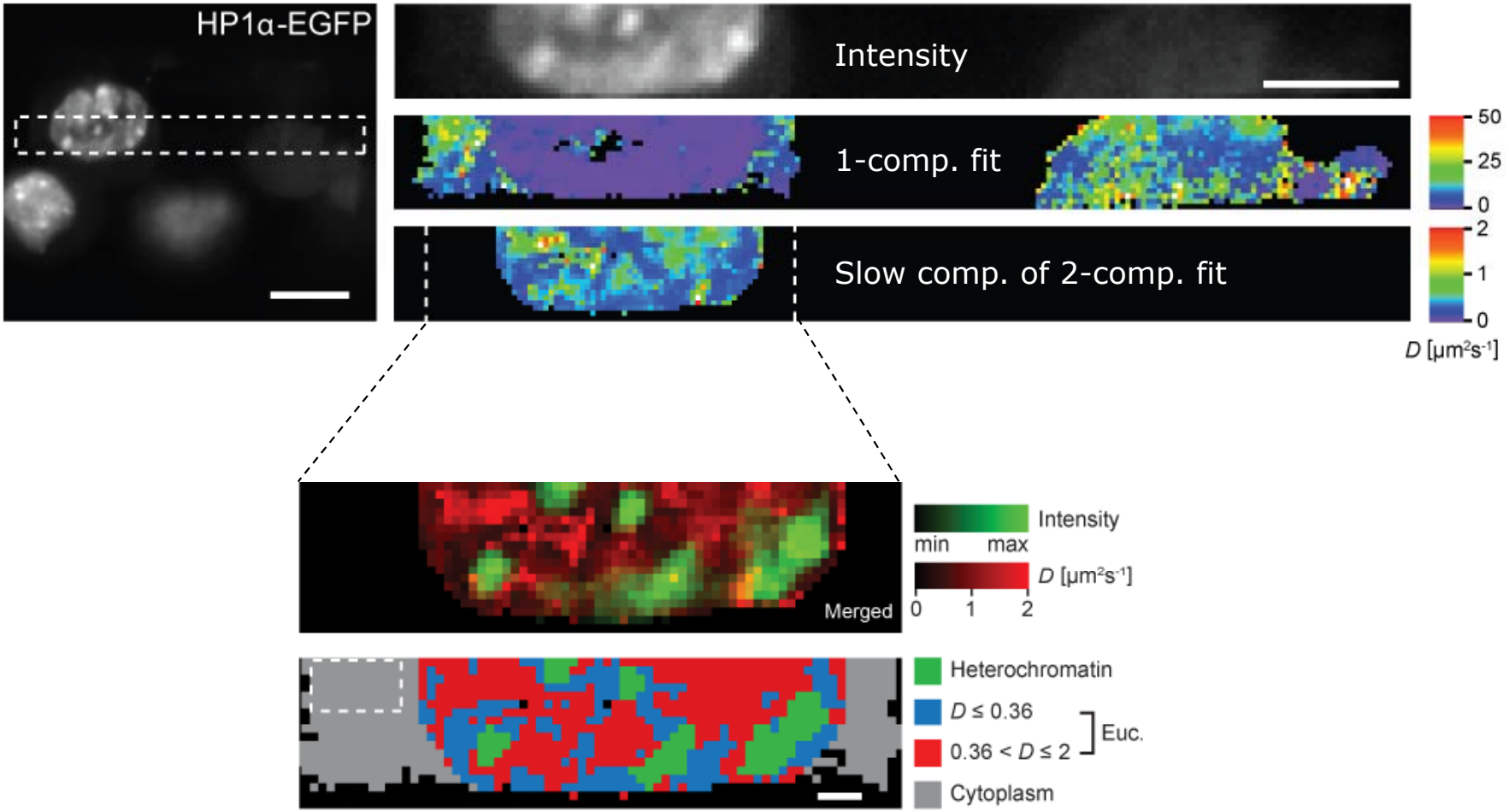
2D-FCS of 3T3 cells expressing HP1α-EGFP



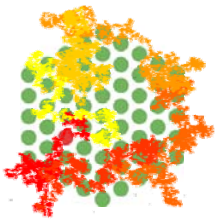


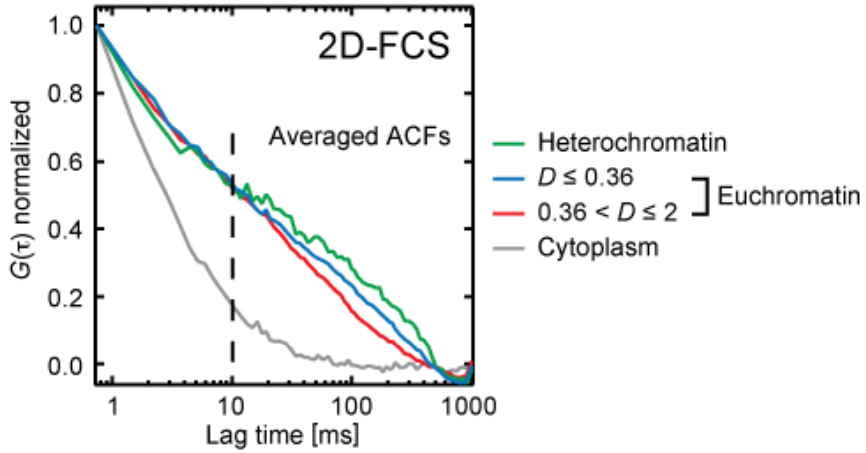


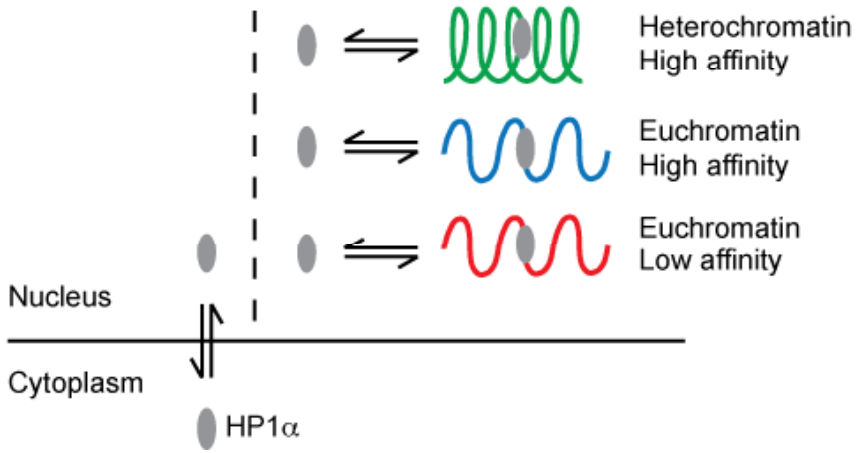
2D-FCS of 3T3 cells expressing HP1α-EGFP

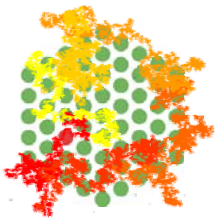


2D-FCS of 3T3 cells expressing HP1α-EGFP









EMBL

Systems Biology of Epigenetic Networks

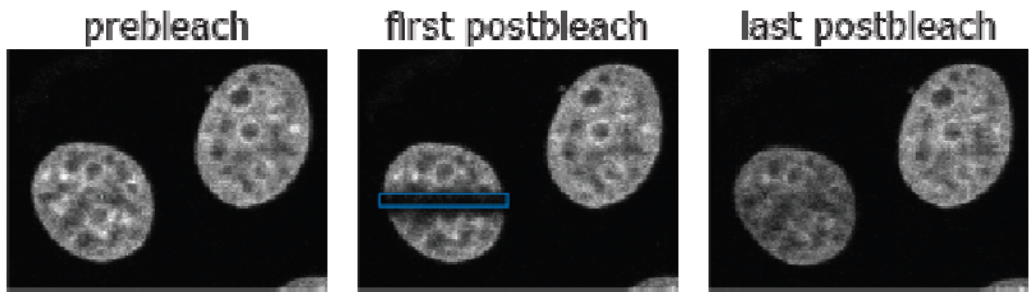
GEFÖRDERT VOM

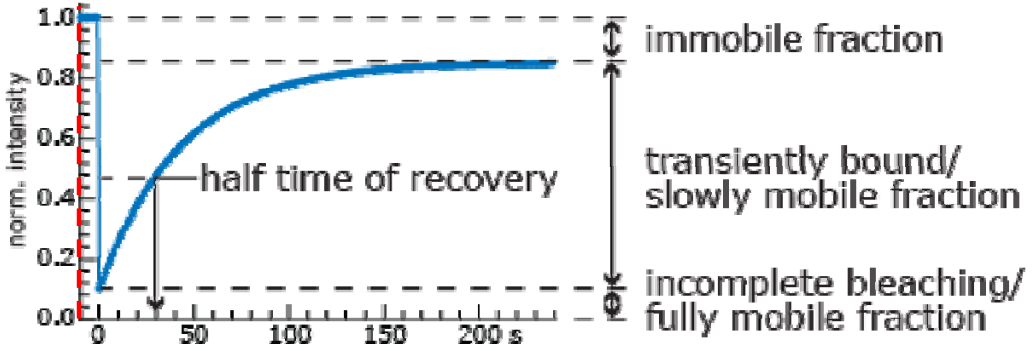
Bundesministerium
für Bildung
und Forschung

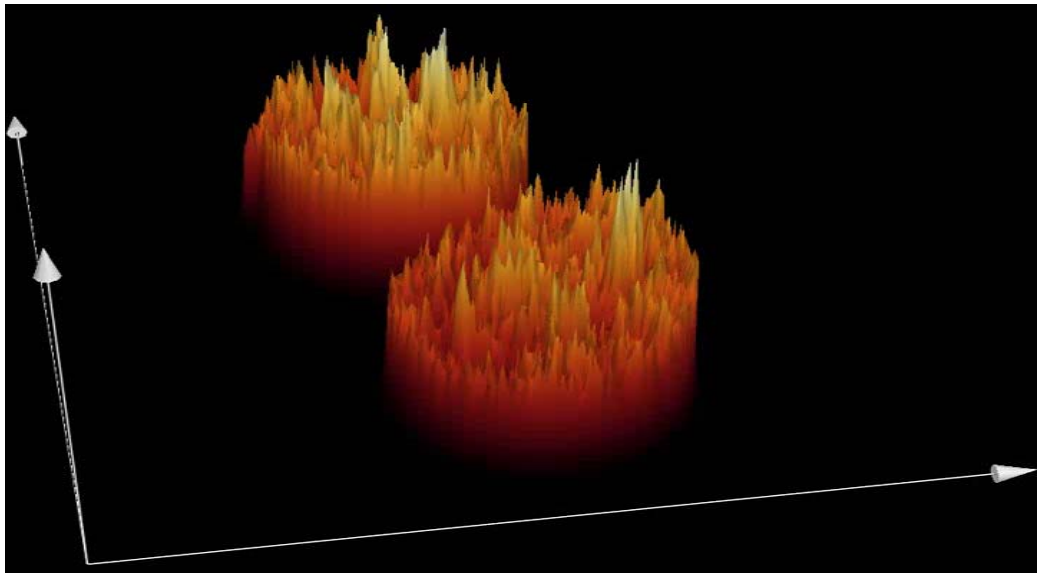
Combined point FRAP and FCS

Kang-Bin Im
Ute Schmidt
Moonsik Kang
Ji Young Lee
Felix Bestvater

Fluorescence recovery after photobleaching (FRAP)



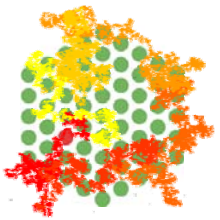




Results of FRAP experiments:

- properties of diffusion/transport processes
- diffusion coefficients *D*
- properties of interactions
- association, dissociation rates k_{on}, k_{off}
- relative concentrations of different fractions

Combining FRAP and FCS



Comparing FRAP and FCS experiments:

- FCS and FRAP address different time, space and concentration regimes
- FCS allows precise determination of diffusion properties
- FRAP assesses properties of binding processes that involve immobilization

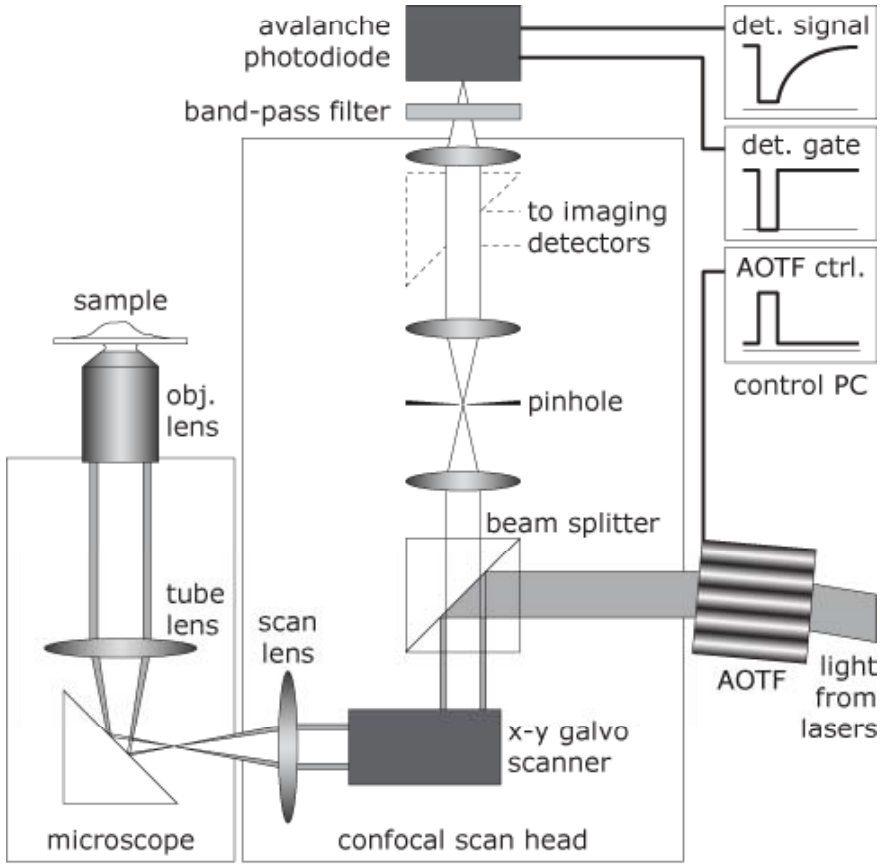
Limitations and drawbacks:

- unwanted diffusion during bleach step of FRAP sequence
- imaging FRAP too slow to sample diffusion processes
- FCS is "conceptually blind" for immobilized molecules
- application of generalized diffusion-reaction models difficult due to the heterogeneous cellular geometry and topology
- results for diffusion coefficients and transport parameters are often inconsistent

-> Combined point FRAP and FCS using a confocal microscope

-> Comprehensive theoretical description of FRAP and FCS

Confocal point FRAP and FCS

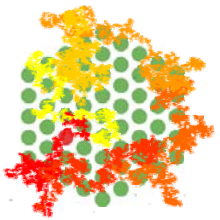


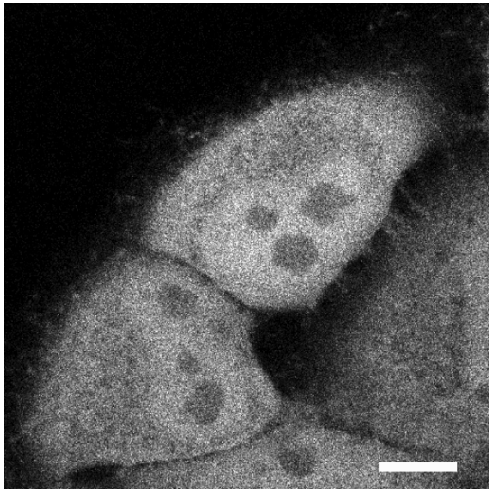
Modified confocal FCS microscope:

- flexible AOTF control with 10 µs time resolution
- flexible APD readout with 1 µs time resolution
- flexible raw data processing

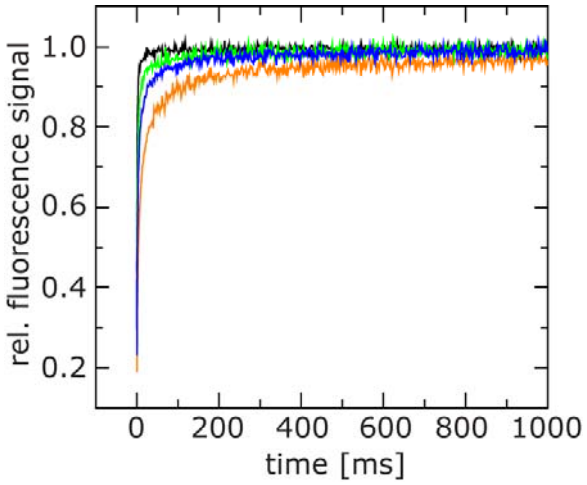
-> Combined point FRAP and FCS measurements

Point FRAP and FCS of EYFP in HeLa cells

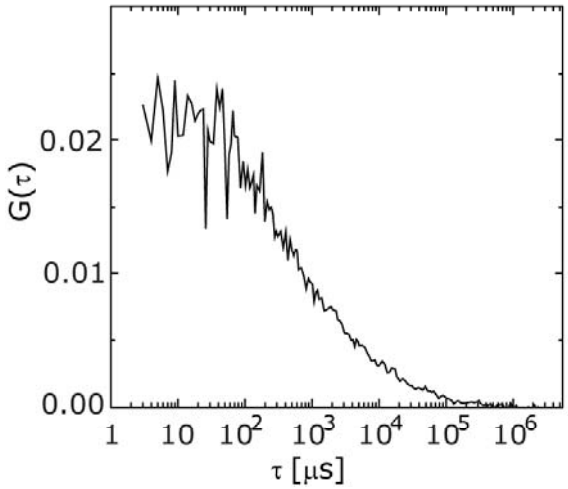




HeLa cells expressing EYFP
(scale bar 10 µm)

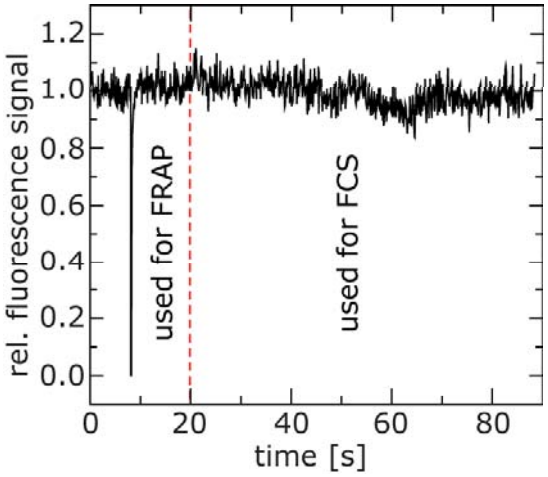


point FRAP with bleach times
0.1, 0.5, 1, 5 ms



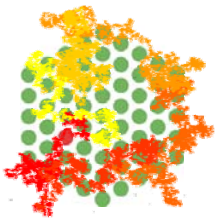
FCS

bleach time T [ms]	half-time of recovery $\tau_{1/2}$ [ms]	immobilized fraction f_{immo} [%]	diffusion time τ_{diff} [ms]	
			3D FRAP (Axelrod 1976)	3D FRAP with diffusion
5.0	6.23 ± 0.42	< 1	9.76 ± 0.24	1.58 ± 0.16
1.0	2.23 ± 0.20	< 1	2.84 ± 0.08	0.72 ± 0.11
0.5	1.25 ± 0.17	< 1	1.22 ± 0.04	0.63 ± 0.12
0.3	1.03 ± 0.13	< 1	0.96 ± 0.04	0.63 ± 0.13
0.1	0.78 ± 0.09	< 1	0.56 ± 0.02	0.45 ± 0.09
extrapolated to 0.0	0.78 ± 0.13	< 1	0.49 ± 0.18	0.50 ± 0.05
global fit	—	—	1.61 ± 1.61	0.45 ± 0.05
FCS	—	—	—	0.46 ± 0.07



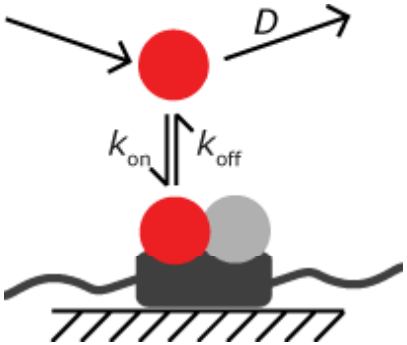
D = 24 ± 3 µm²s⁻¹

Closed-form coupled reaction-diffusion model for FRAP



Ansatz:

- solution of differential equations including diffusion, association and dissociation
- approach similar to FCS theory established in Elson & Madge (1974)
- closed-form expression for FRAP intensity parametrized with reaction rates and diffusion coeff.
- validation with simulated data



parameter ranges:

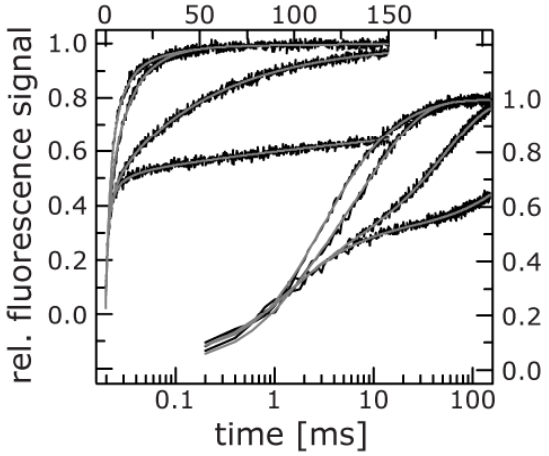
k_{on} = 2...2000 s^{-1}

k_{off} = 2...2000 s^{-1}

D = 20 $\mu m^2 s^{-1}$

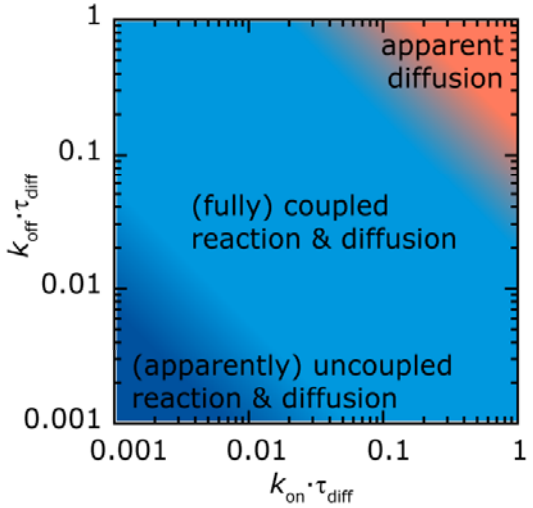
w_0 = 0.2 µm

τ_{diff} = 0.5 ms

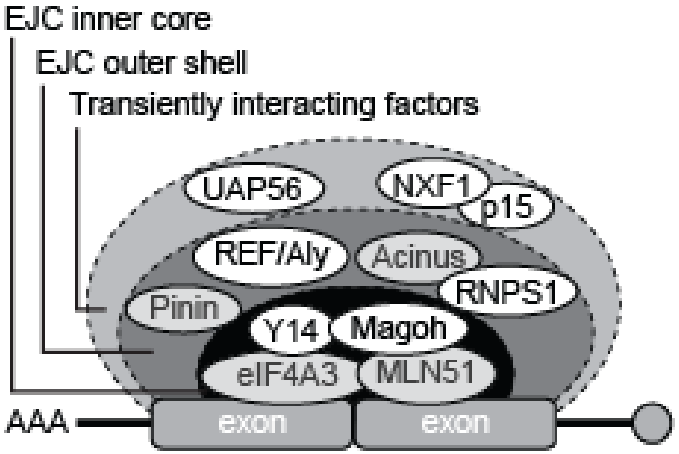


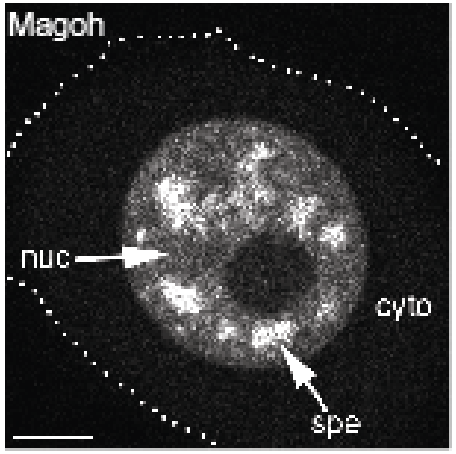
Result:

- model is applicable to a wide range of reaction vs. diffusion rates
- coverage of (fully) coupled and (apparently) uncoupled reaction-diffusion schemes



Dynamics of the exon-exon junction complex





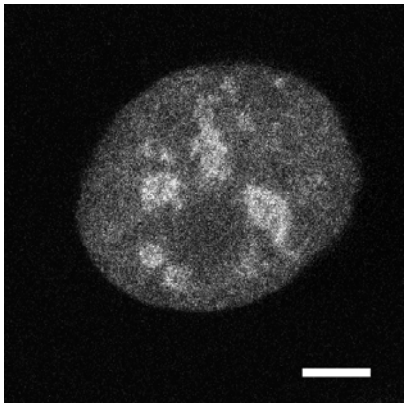
(Schmidt et al., RNA, 2009)

Exon-exon junction complex (EJC):

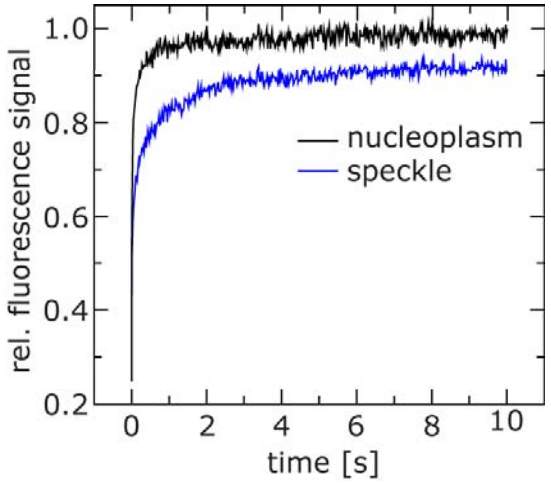
- forms upon intron excision on maturating mRNA molecules in the nucleus
- serves as adaptor for nuclear-cytoplasmic export and for mRNP quality control
- core-shell model of the EJC: core formed from proteins binding after, shell formed from proteins binding prior to intron excision
- strong nuclear localization with accumulation in splicing speckles
- highly dynamic and mobile

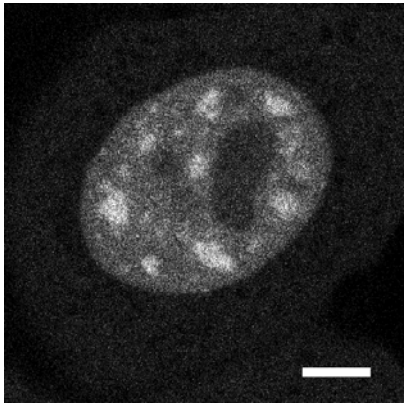
What contributes to the mobilities of the EJC components Magoh and REF2-I?

Point FRAP and FCS of EJC factors REF2-II and Magoh

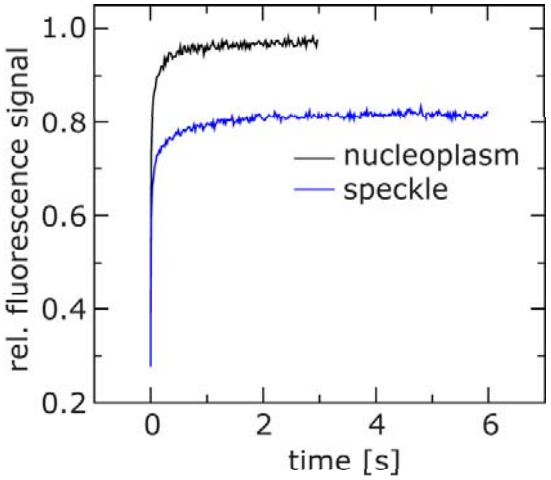


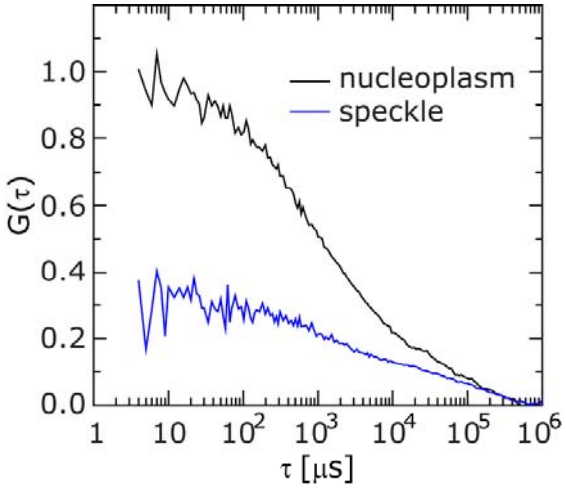
REF2-II-EGFP
scale bar 5 µm



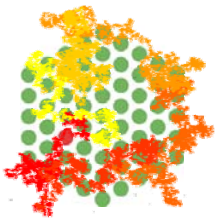


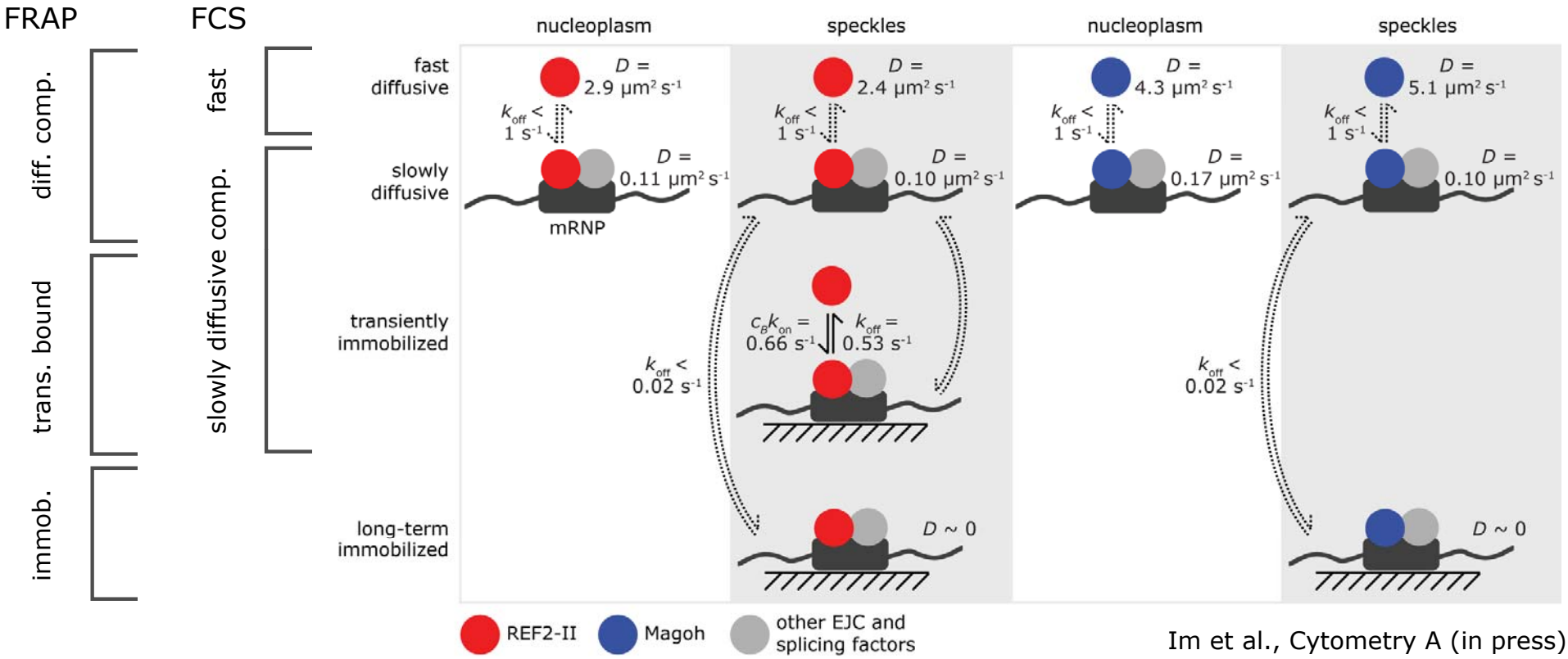
Magoh-EGFP
scale bar 5 µm





Binding and diffusion properties of REF2-II and Magoh





Im et al., Cytometry A (in press)