FRAP, FCS

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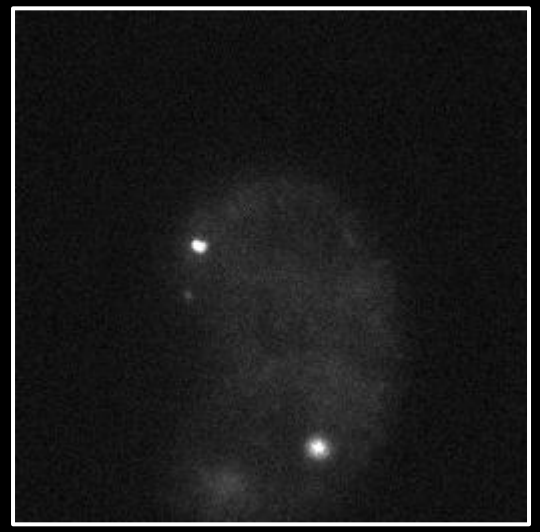
Outline:

- Introduction to kinetic experiments
- FRAP (fluorescence recovery after photobleaching)
  - Analysis of FRAP
  - Example of FRAP
  - Modifications of FRAP
- FCS (fluorescence correlation spectroscopy)
  - Analysis of FCS
  - Example of FCS
  - FCCS (Fluorescence cross-correlation spectroscopy)
- Conclusion

Kinetic experiments

- *Study redistribution of the molecules*
- *Characterize the movement of molecules of interest*
- *Characterize interactions, determine reaction rates*



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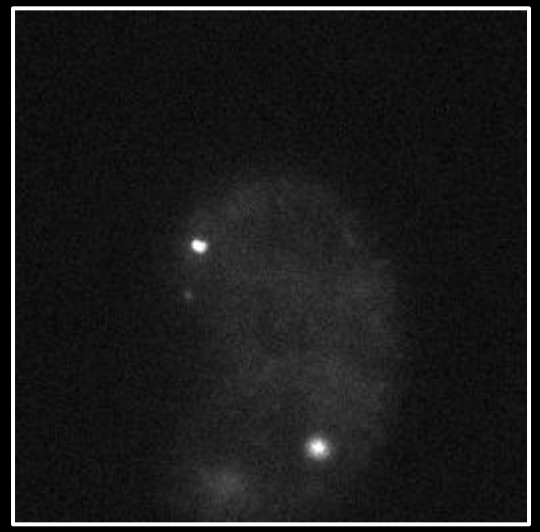
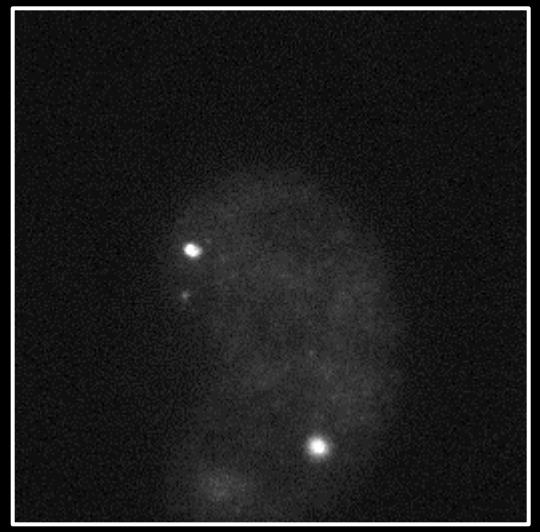




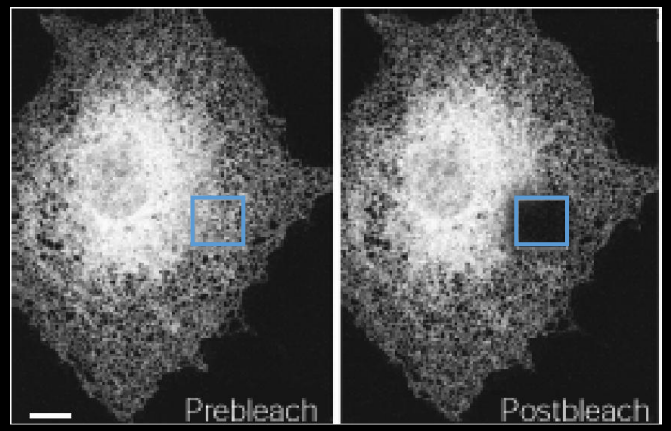
FRAP

What is FRAP

- **“Fluorescence recovery after photobleaching”**
- Selectively *photobleach* a specific area by high intensity laser pulses

Photobleaching

- Photochemical alteration of a dye or fluorophore such that it is permanently unable to fluoresque

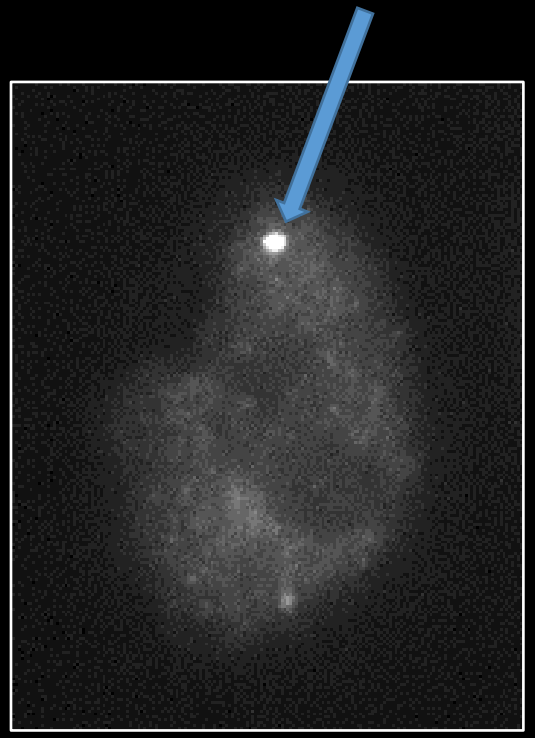


http://cf.gu.se/english/Centre_for_Cellular_Imaging/Techniques/FRAP

FRAP

What is FRAP

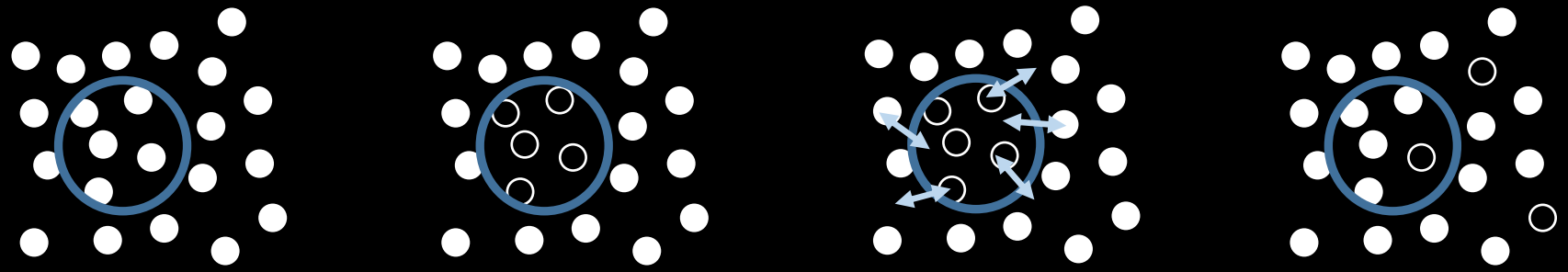
- **“Fluorescence recovery after photobleaching”**
- Selectively *photobleach* a specific area by high intensity laser pulses
- Kinetics of fluorescence recovery is recorded by sampling images at regular time intervals with low intensity illumination **in the same area**
- *Widefield or confocal* fluorescence microscope



- Choose a dye that
  - *Bleaches fast and irreversibly at high illumination power*
  - *Bleaches minimally at low illumination power (to prevent photobleaching)*

- Typically **GFP-tagged proteins**

FRAP



Pre-bleach

- Acquire a few images before photobleaching

Photobleaching

- High illumination power

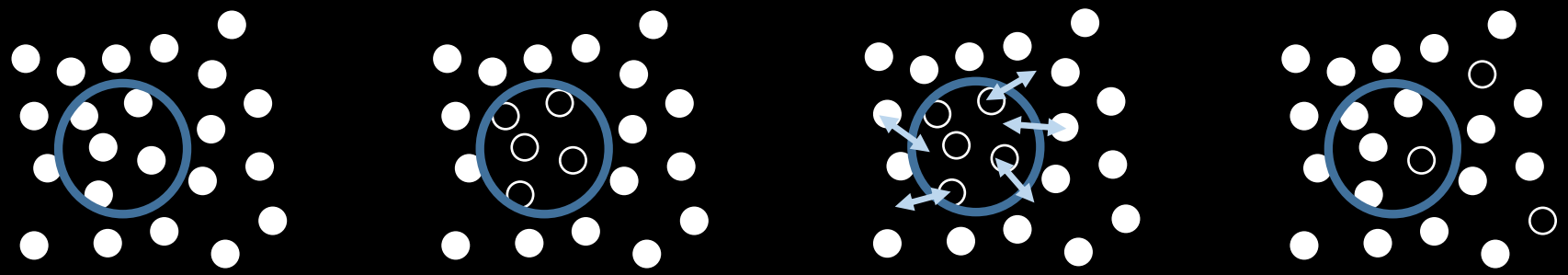
Post-bleach (recovery)

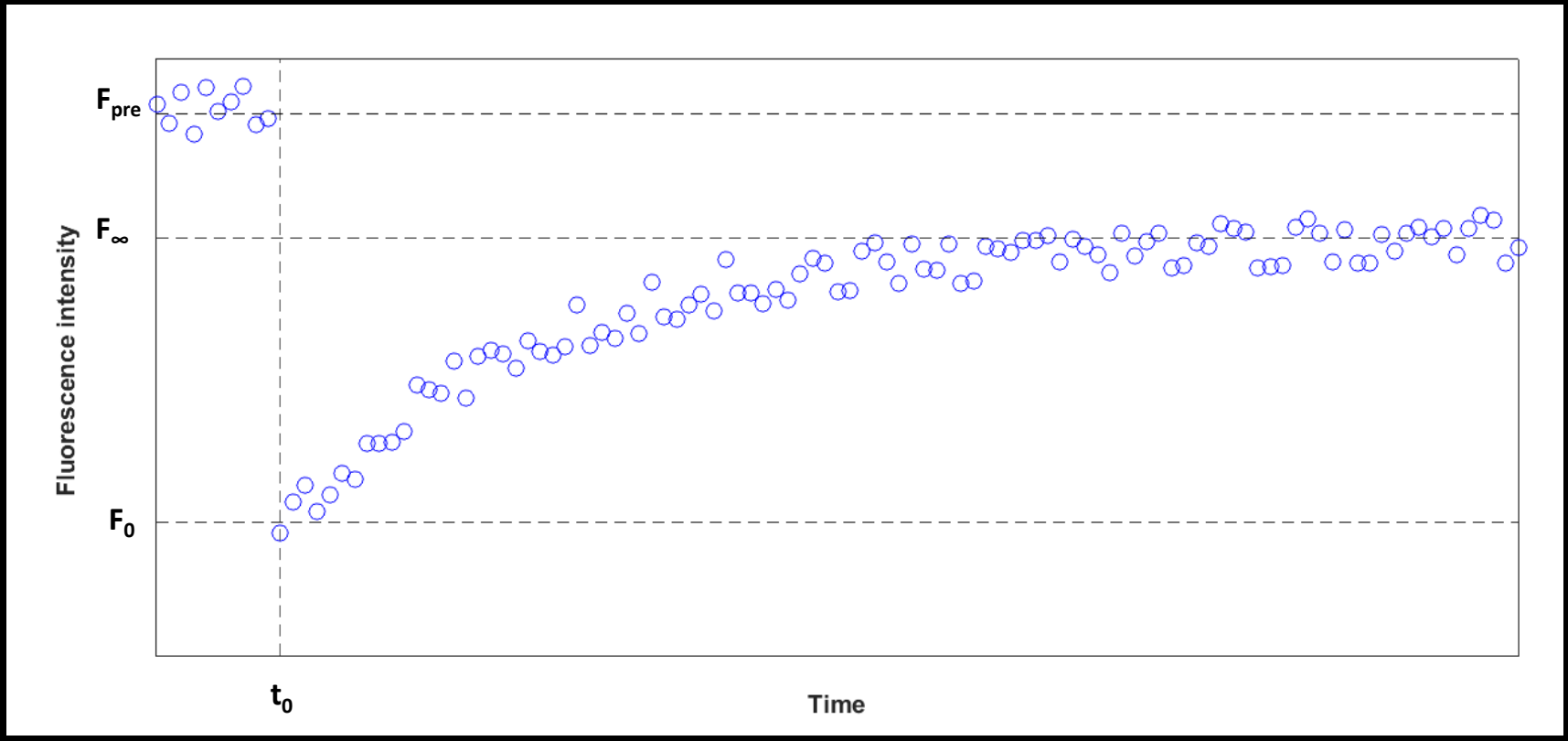
- Acquire images after photobleaching
- Molecules are moving by diffusion or active transport
- Fluorescence in the bleach spot recovers

Post-bleach (recovery)

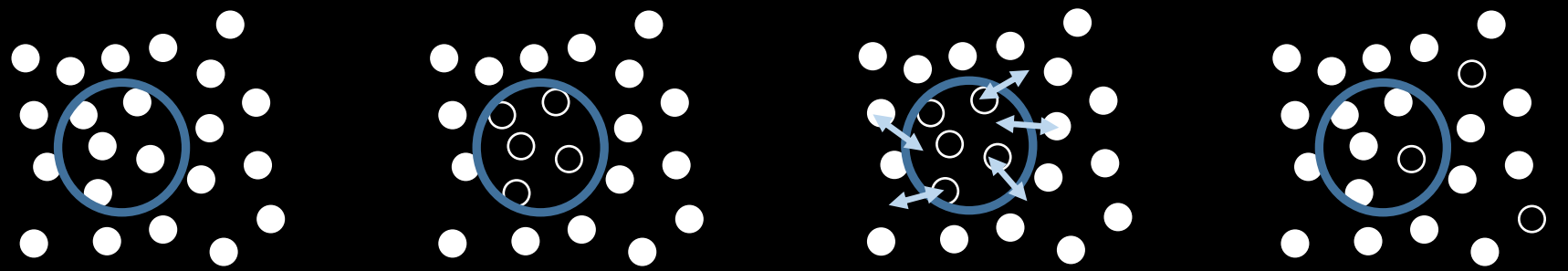
- Incomplete recovery due to some fraction of molecules that are immobilized at the bleached spot

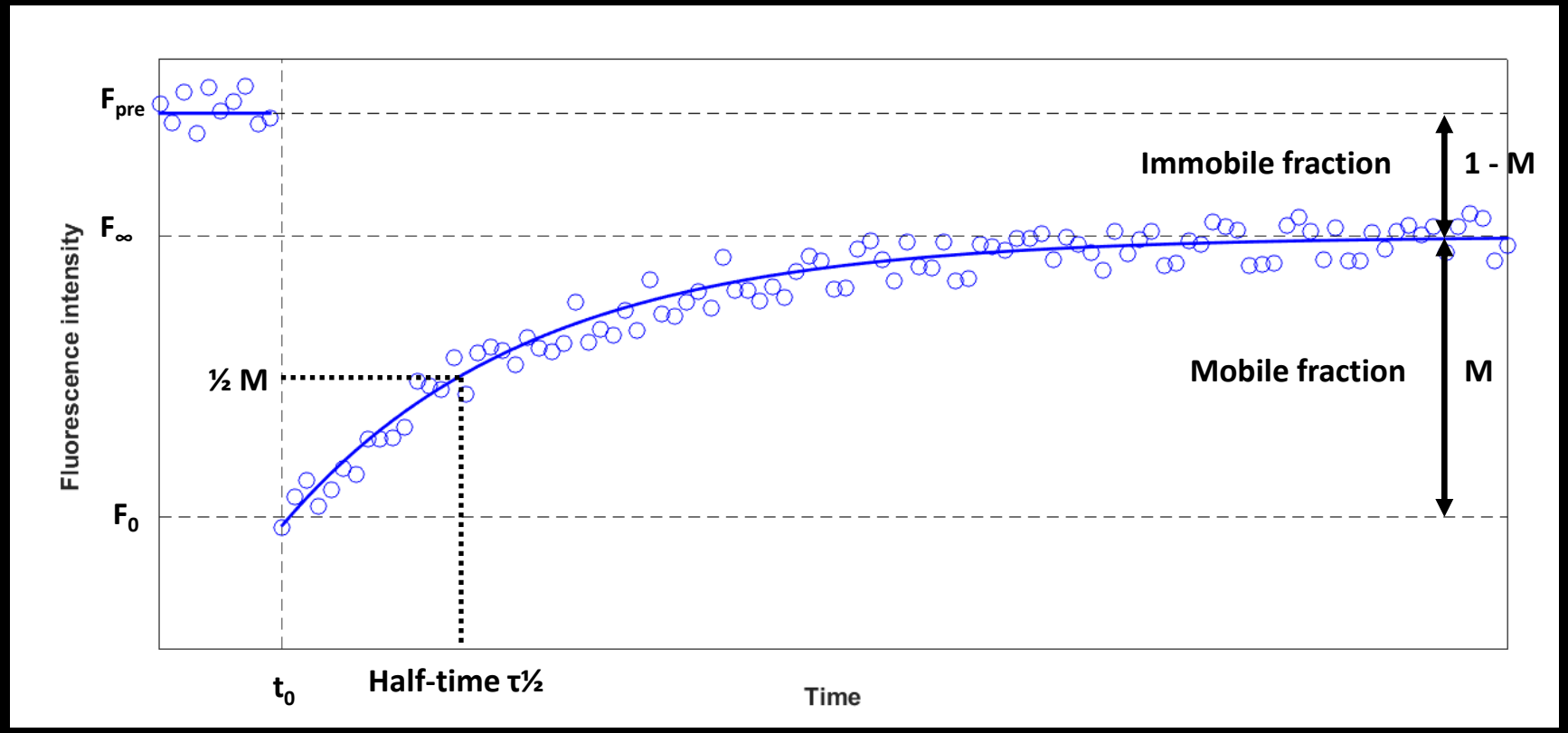
FRAP



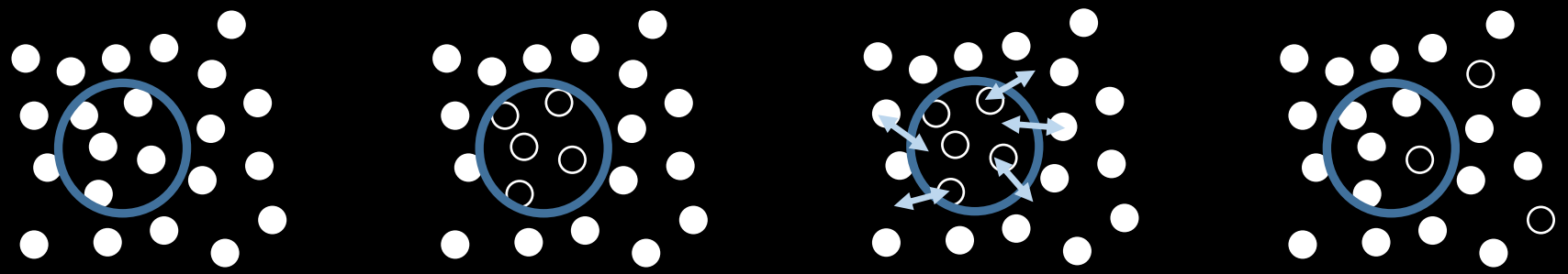


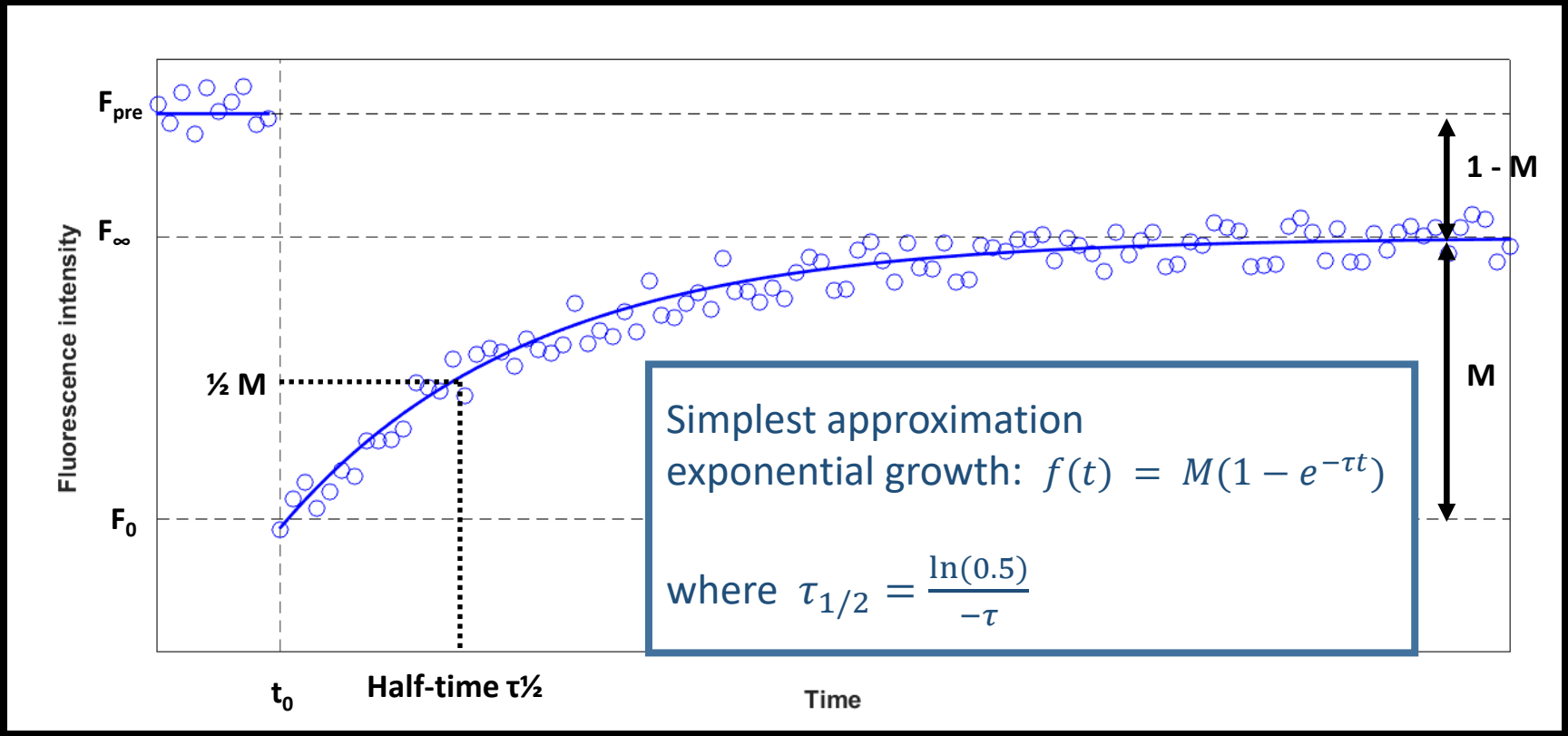
FRAP




F_pre
F∞
½ M
F0
Fluorescence intensity
Immobile fraction
1 - M
Mobile fraction
M
t0
Half-time τ½
Time

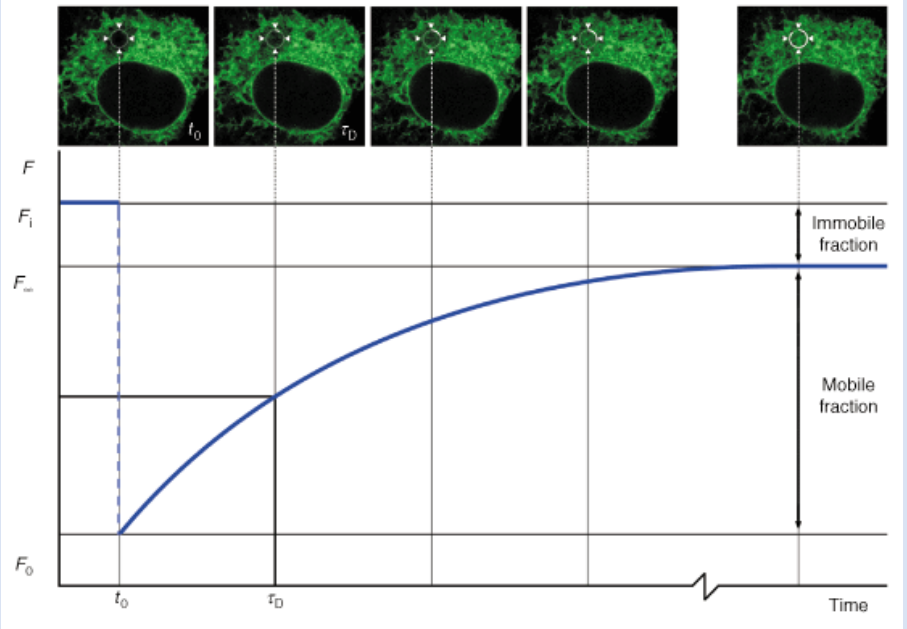
FRAP




F_pre
F∞
½ M
F0
Fluorescence intensity
1 - M
M
t0
Half-time τ½
Time
Simplest approximation
exponential growth: $f(t) = M(1 - e^{-\tau t})$
where $\tau_{1/2} = \frac{\ln(0.5)}{-\tau}$

FRAP in cell biology

Parameters of FRAP measurement



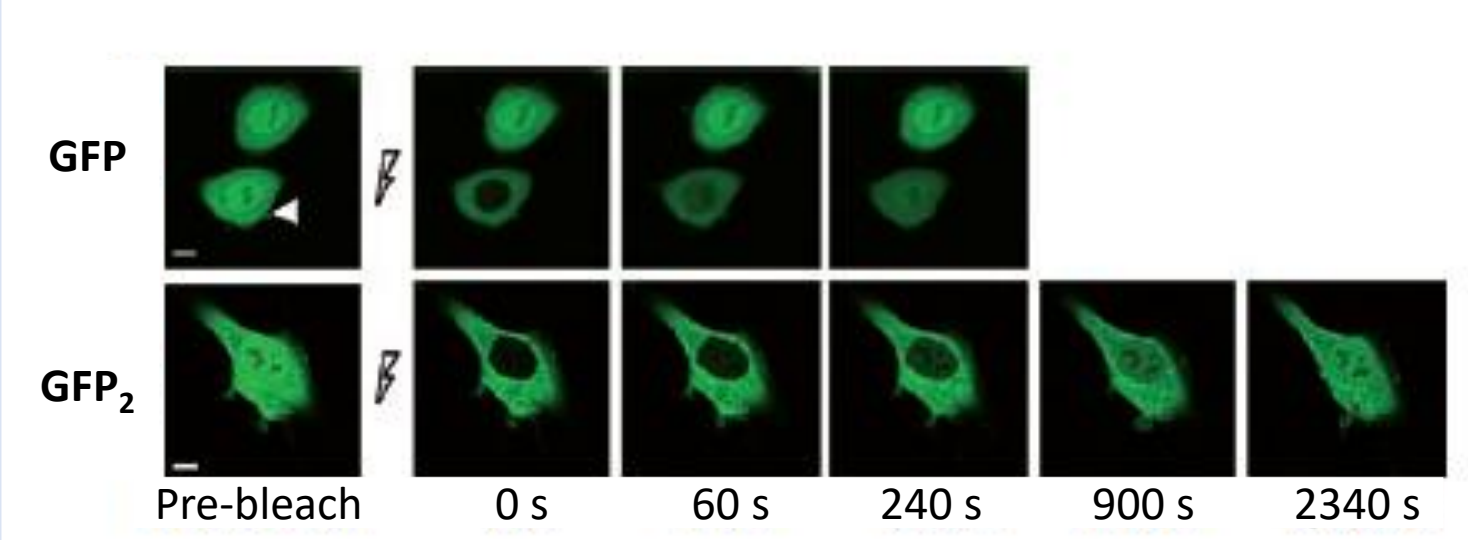
- Number of images before photobleaching
- Shape of the photobleached area
- Size of the photobleached area
- Intensity of the laser for photobleaching
- Time needed for photobleaching
- Intensity of the laser for image acquisition after photobleaching
- Time of the first image after photobleaching
- Time intervals between images acquired after photobleaching
- Total time of image acquisition after photobleaching

E.A. Reits & J.J. Neefjes, Nature Cell Biology 2001, 3 (6), E145-E147

FRAP in cell biology

What can be photobleached – Region Of Interest (ROI)

- Part of the cell (spot, multiple spots, area)
- Cell compartment



What can be determined by FRAP

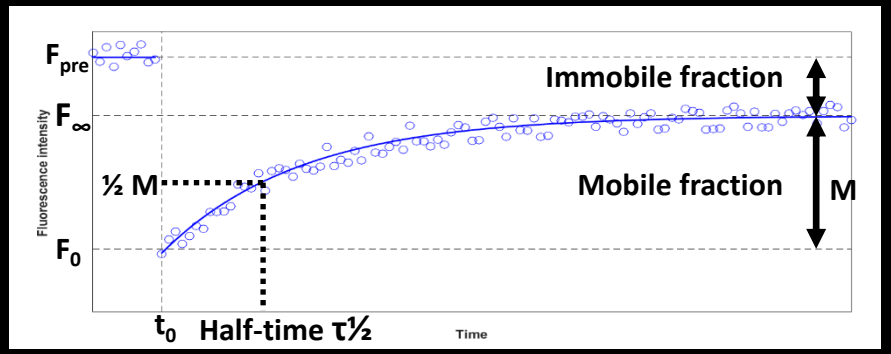
- **Diffusion coefficients** , type of motion
- **If it is bound** to any surrounding structure
- **If it is freely diffusing or the movement is restricted** by surrounding structures
- **Chemical reaction rates** using a specific kinetic model

Francesco Cardarelli, Molecular Therapy 2007, 15 (7), 1313-1322

Analysis of FRAP

Curve fitting – if one exponential cannot fit the curve, use different function instead (e.g. biexponential)

- *Mobile fraction*
- *Half-time*



- **<u>Comparison between different cell compartments, proteins, etc. and/or conditions</u>**
- **<u>No information about specific processes (e.g. interactions)</u>**

Analysis of FRAP

Curve fitting – if one exponential cannot fit the curve, use different function instead (e.g. biexponential)

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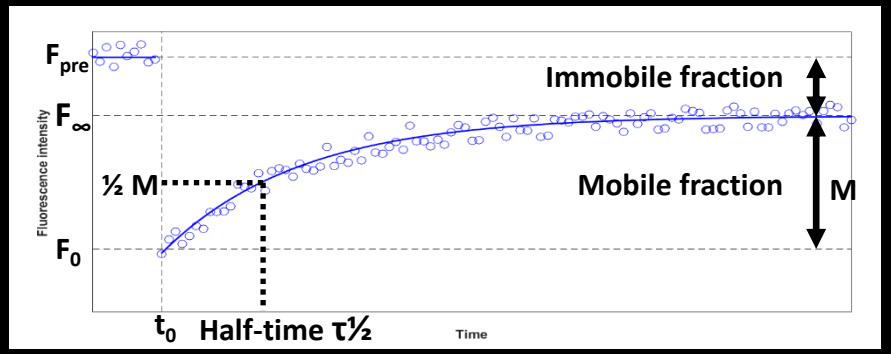

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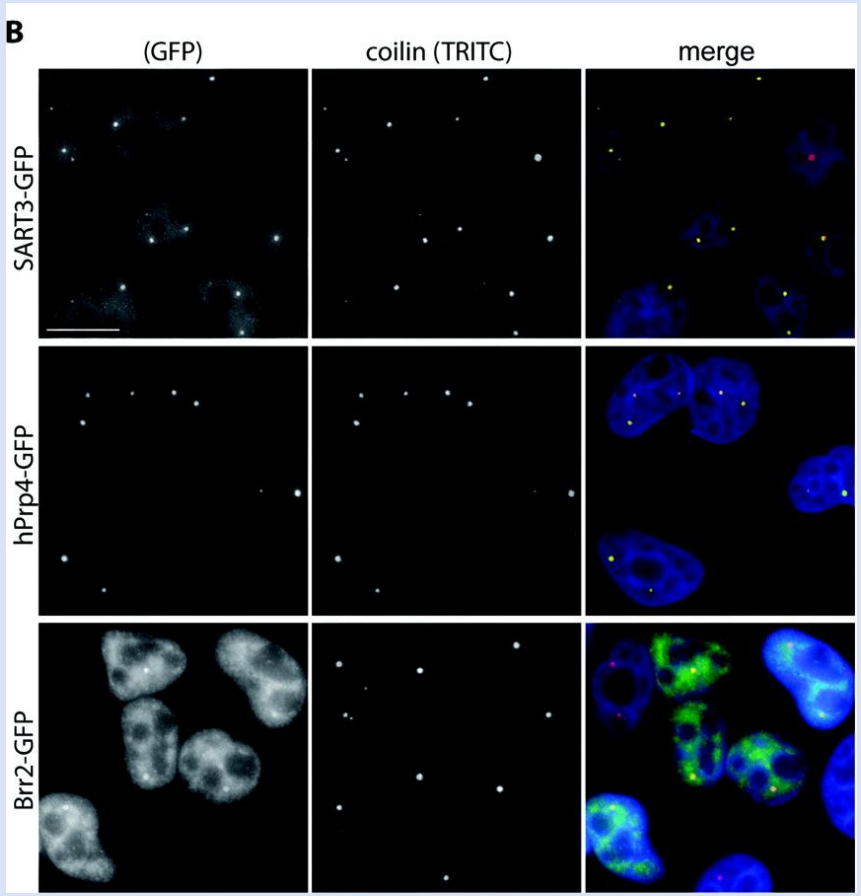
Mathematical modeling

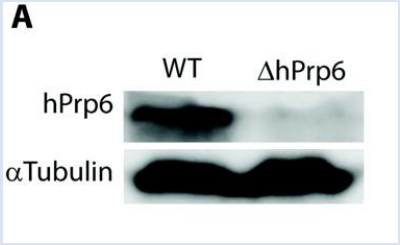
- Diffusion
  - *Effective diffusion coefficient* D_{eff} [m^2s^{-1}] reflects the mean squared displacement explored by the proteins through a random walk over time
- Specific interactions
- Combination of both

Example of FRAP

In vivo kinetics of U4/U6·U5 tri-snRNP formation in Cajal bodies



hPrp6-KD

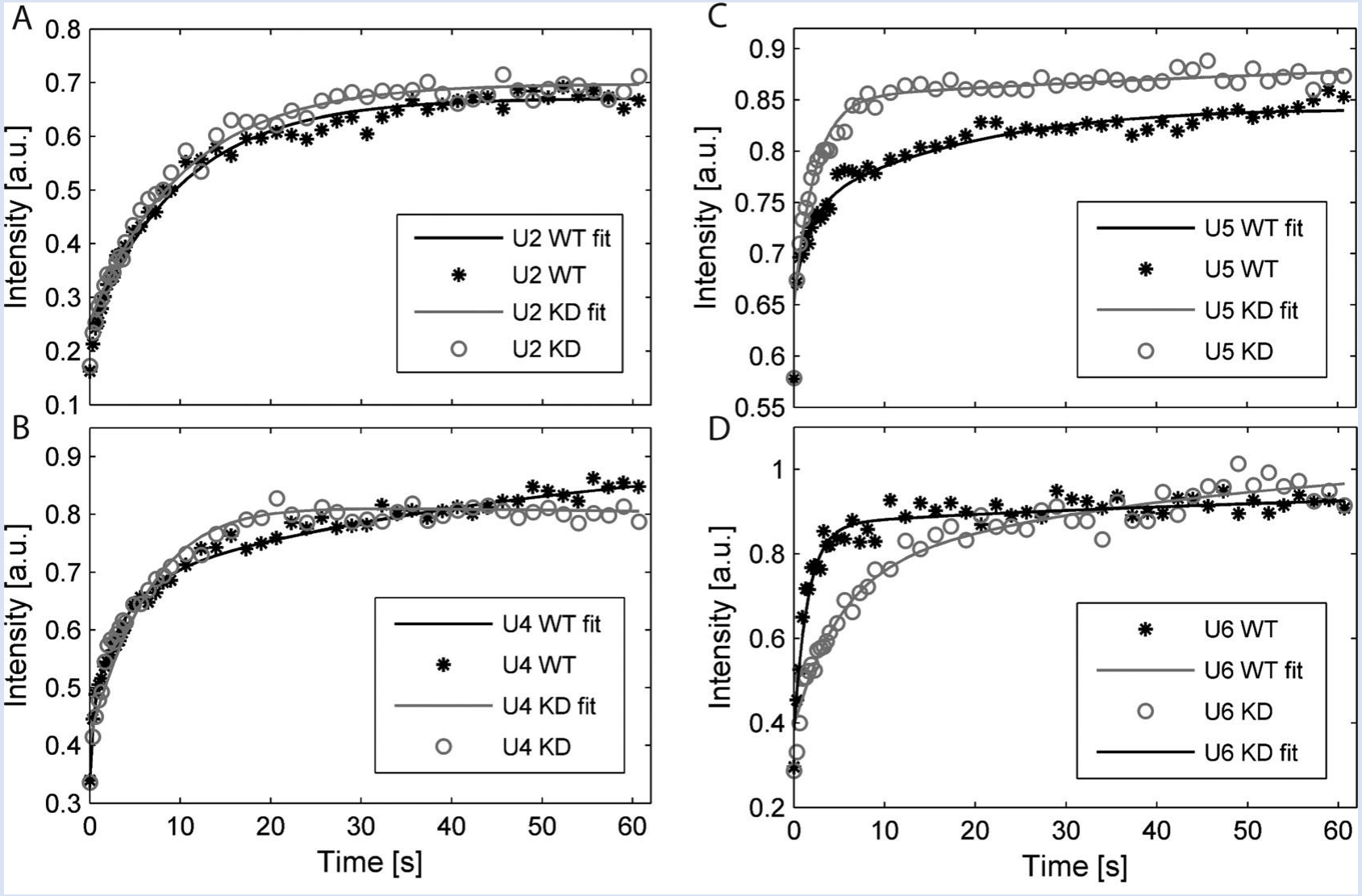


Comparison between WT and hPrp6-KD

- Higher accumulation of U6 and U4 in CBs (depicted by coilin immunostaining)
- Localization of U2 (Brr2-GFP) was not significantly altered - control

Novotny et al., Molecular Biology of the Cell 22(4):513-23 (2011)

Example of FRAP



Novotny et al., Molecular Biology of the Cell 22(4):513-23 (2011)

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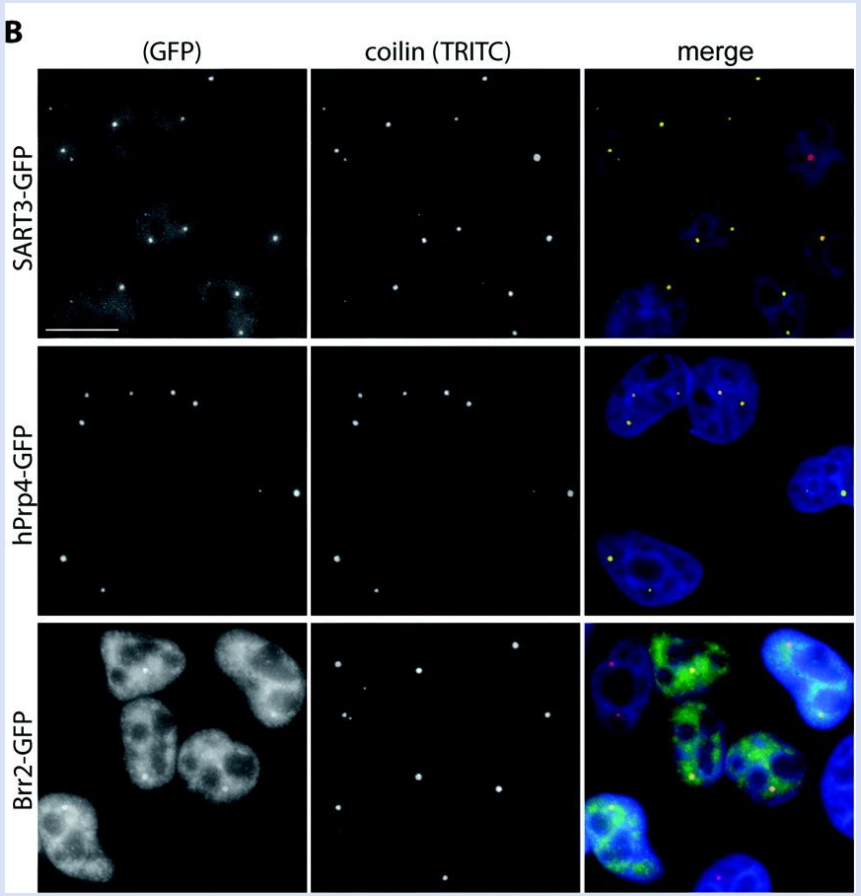
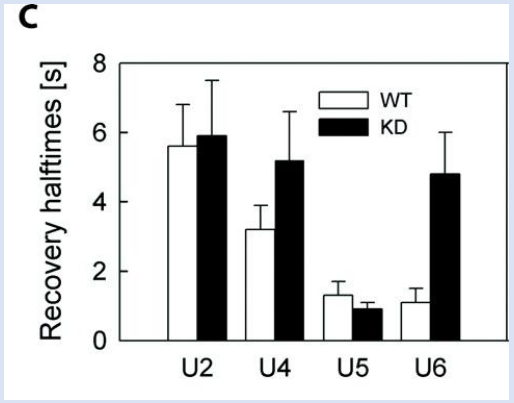


hPrp6-KD



Comparison between WT and hPrp6-KD

- The halftime values $\tau_{1/2}$ were obtained from bi-exponential fits of the measured fluorescence intensities
- Slower recovery of U4 and U6

Novotny et al., Molecular Biology of the Cell 22(4):513-23 (2011)

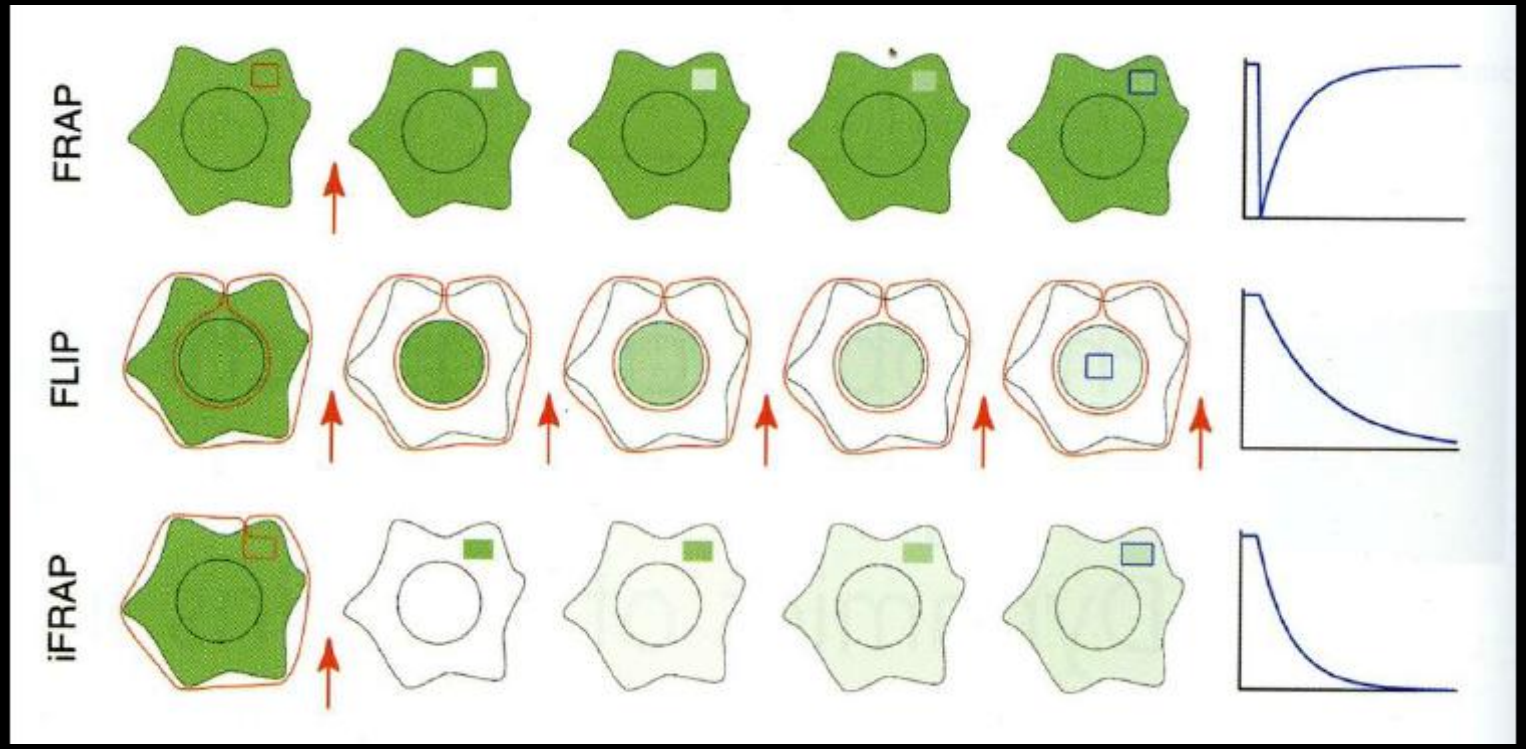
Modifications of FRAP

FLIP (fluorescence loss in photobleaching)

- Monitor loss of fluorescence intensity in the unbleached area, the photobleaching is repeated

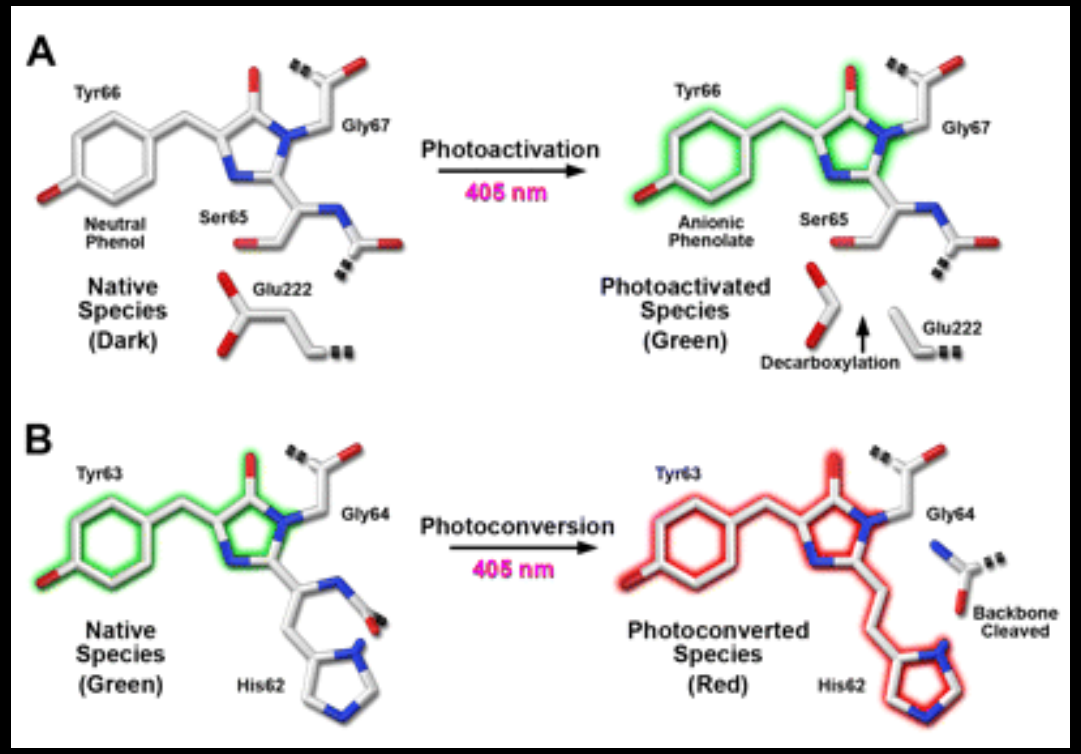
iFRAP (inverse FRAP)

- Photobleaching of the whole cell except a small area where we measure the fluorescence recovery



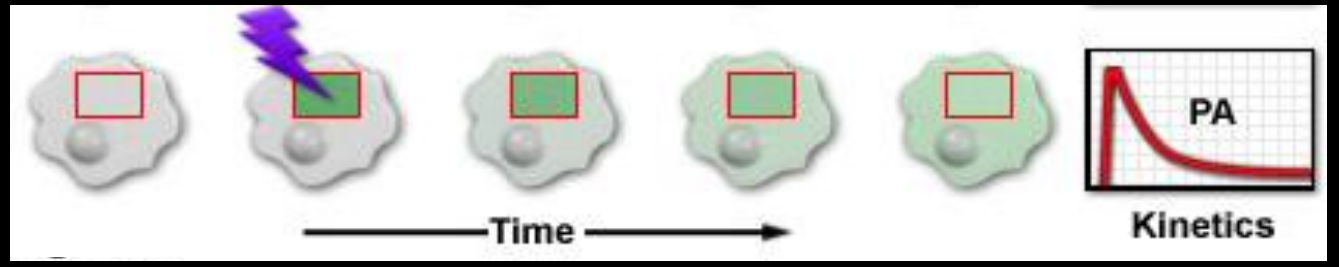
Raut and Ellenberg, Live Cell imaging, Lab Manual, p.101

Modifications of FRAP



Photoactivation of PA-GFP and PS-CFP2 from dark state to green fluorescence

Photoconversion of Kaede, KikGR, Dendra2 and Eos from green to red fluorescence

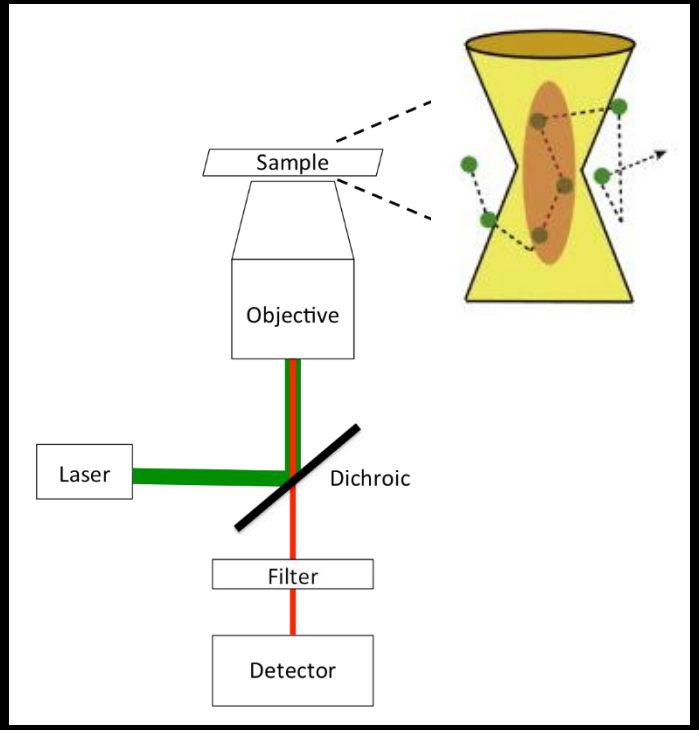


http://www.olympusfluoview.com, N.C. Shaner et al., Journal of Cell Science 2007, 120, 4247-4260

FCS

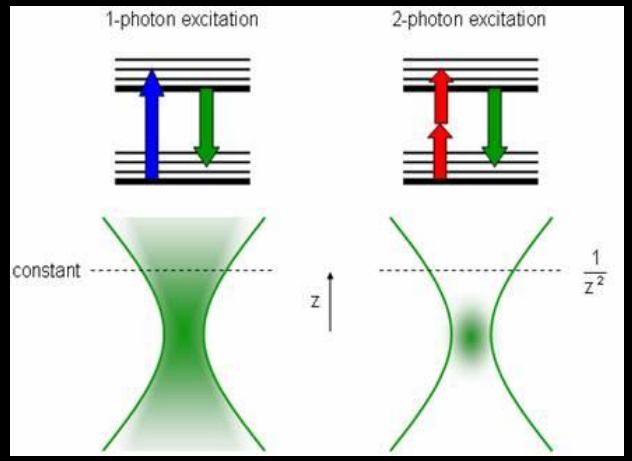
What is FCS

- **“Fluorescence Correlation Spectroscopy”**
- The fluorescence emitted from a very tiny space in solution containing a small number of fluorescent molecules is observed
- Fluctuation of fluorescence intensity due to Brownian motion of particles



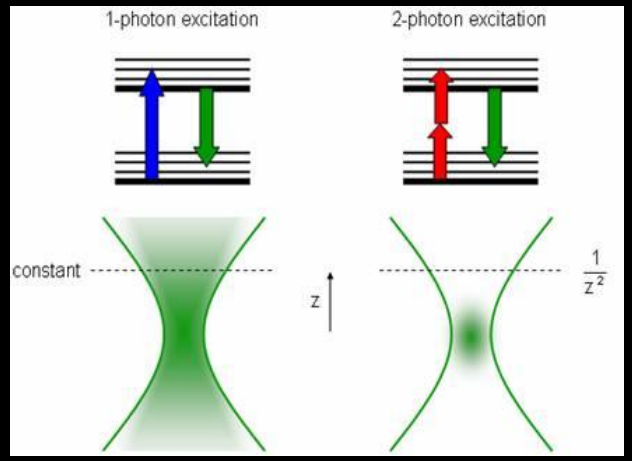
Sub-femtoliter volume (~μm^3)

FCS



- **One-photon excitation - confocal**
- **Two-photon excitation**
  - ***Only few molecules to be simultaneously detected***
  - ***Concentrations in nanomolar range***

FCS



- **One-photon excitation - confocal**
- **Two-photon excitation**
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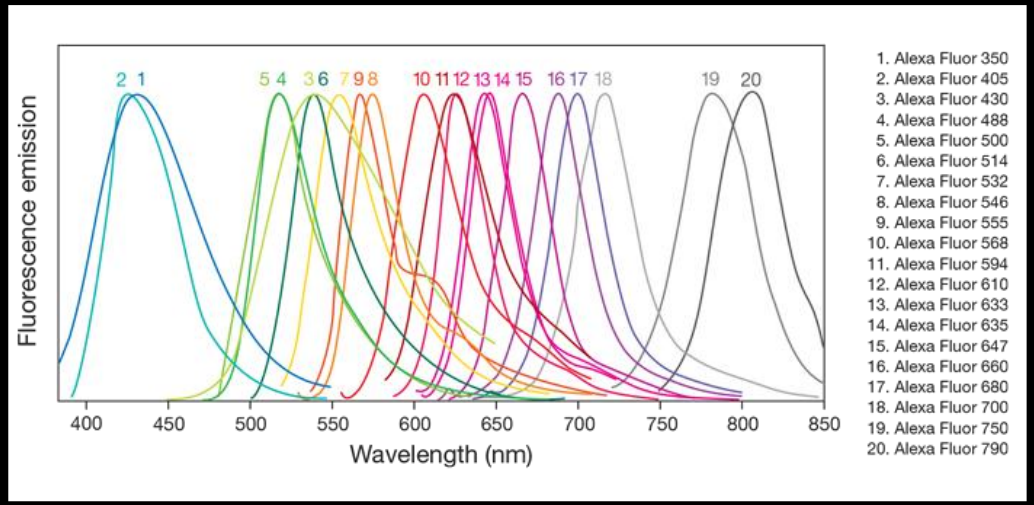
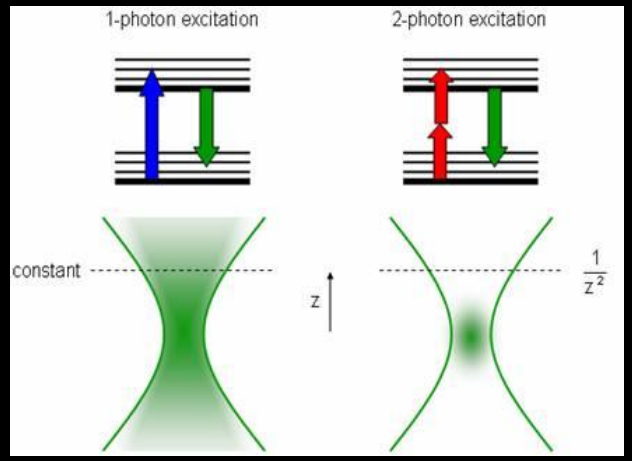
Fluorescent dyes

- *High quantum efficiency*
- *Large absorption cross section*
- ***Photostability*** *(high laser power)*

- Typically **Alexa Fluor dyes**

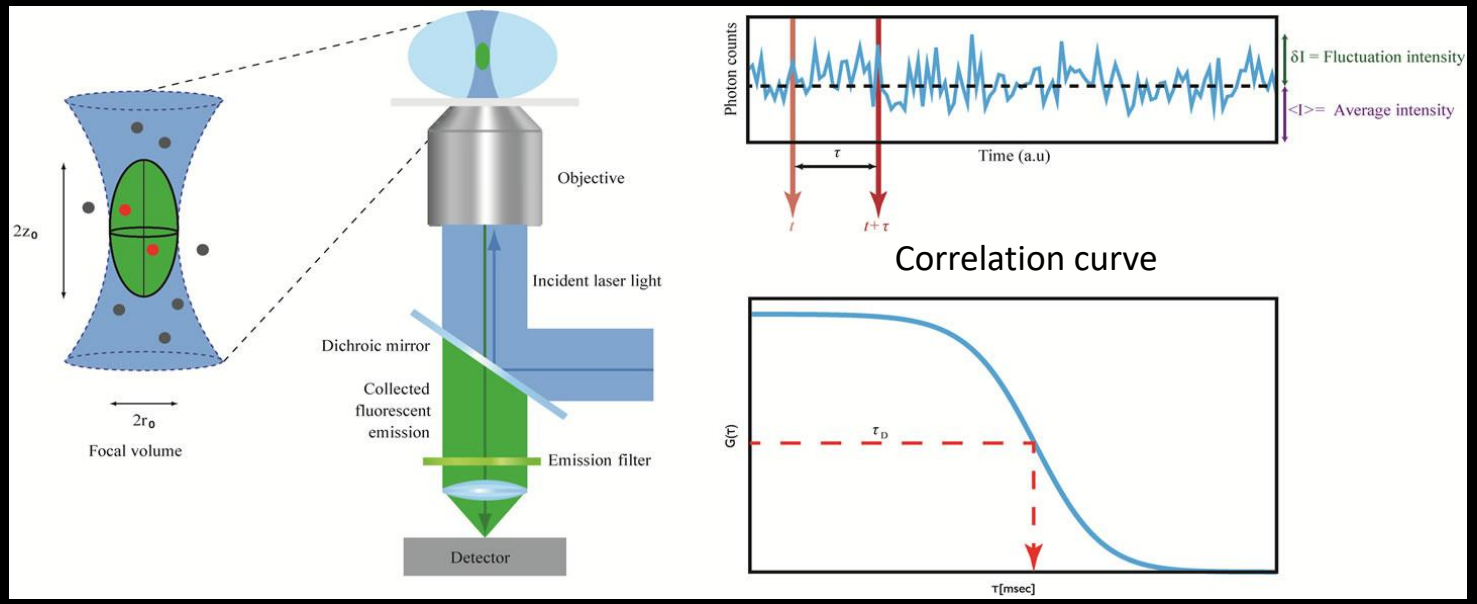


FCS



- **One-photon excitation - confocal**
- **Two-photon excitation**
  - ***Only few molecules to be simultaneously detected***
  - ***Concentrations in nanomolar range***

Fluorescence signal



Rasmussen et al., Journal of Modern Physics 4(11B), ID:40289 (2013)

FCS – autocorrelation curve

Autocorrelation curve

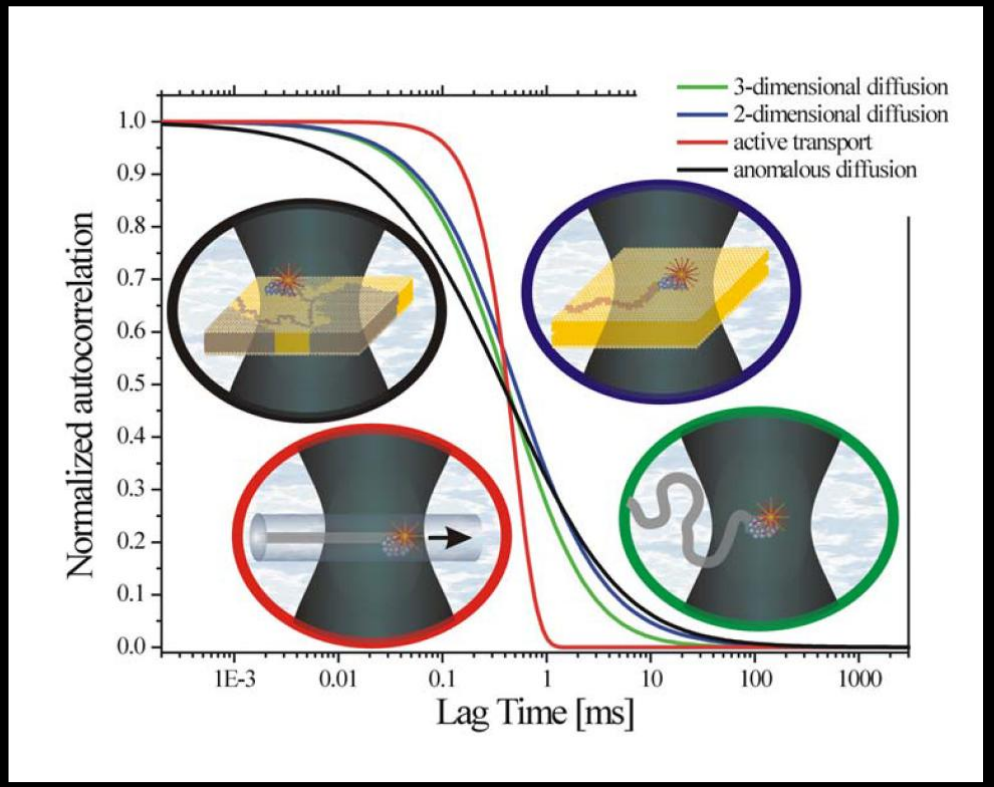
- One particular species of fluorescent particles
- Temporal autocorrelation of the recorded intensity signal (a measure of self-similarity of a time series signal)

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

Fluctuations of the fluorescence signal – defined as deviations from the temporal average of the signal

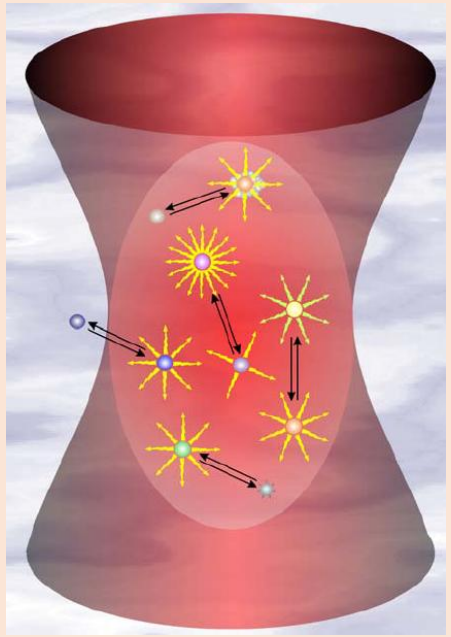
$$G(\tau) = \frac{\langle \delta F(t) . \delta F(t+\tau) \rangle}{\langle F(t) \rangle}$$

Normalized autocorrelation function



https://www.researchgate.net/publication/235410358

FCS in cell biology



Molecular mechanisms that might give rise to florescence fluctuations comprise

- particle movements
- conformational changes
- chemical or photophysical reactions

What can be determined by FCS

- **Diffusion coefficients** , type of motion of the particles
- **Hydrodynamic radii** of the particles
- **Average concentrations** in the focal volume
- **Chemical reaction rates** using dual-color cross-correlation analysis

https://www.researchgate.net/publication/235410358

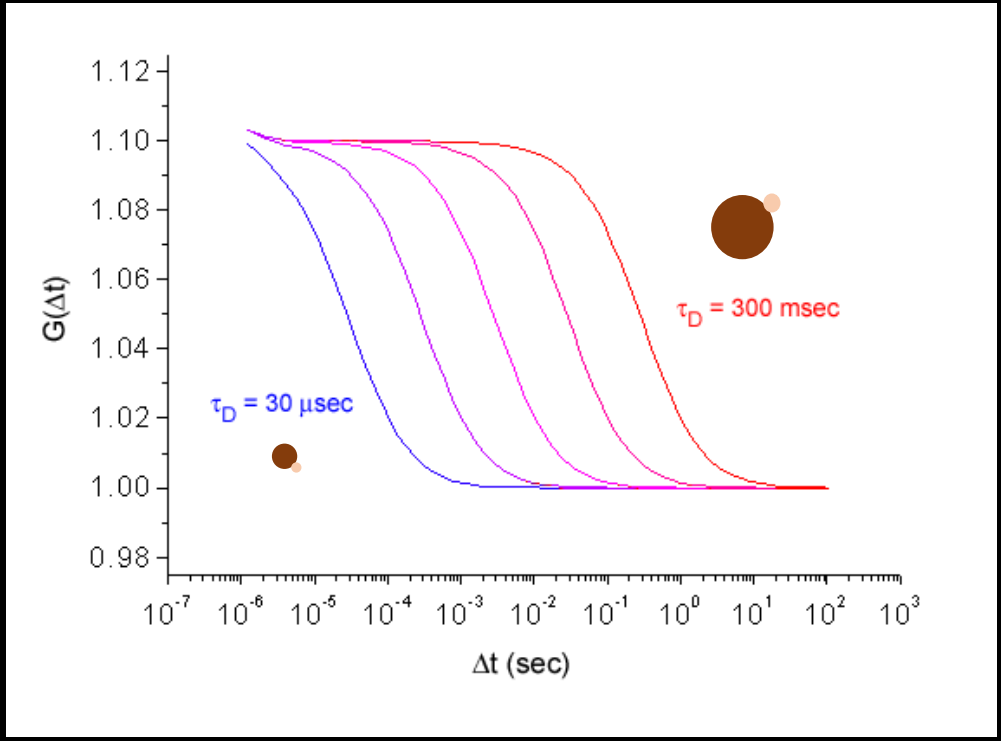
Analysis of FCS

Diffusion analysis

$$\tau_D = \frac{r_0^2}{4D}$$

lateral diffusion time that a molecule stays in the focal volume

diffusion coefficient



- Heavy molecule moves slower than light molecule

Analysis of FCS

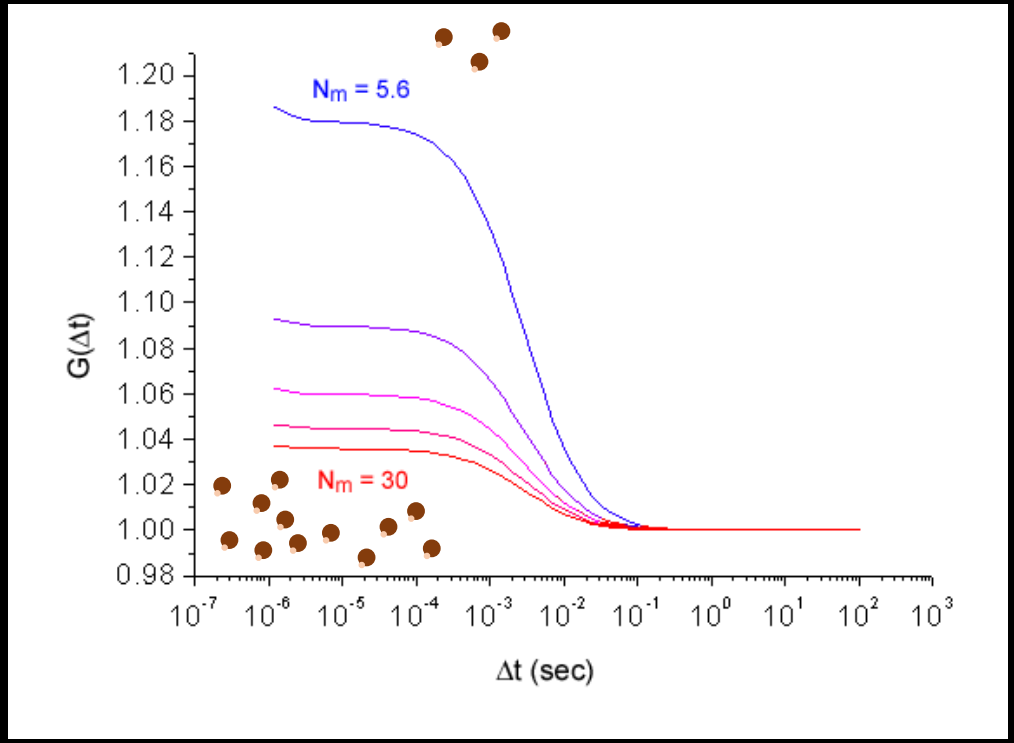
Concentration of fluorescent molecules

$$G_0 = \frac{1}{\langle N \rangle} = \frac{1}{V_{eff}.\langle C \rangle}$$

amplitude of the autocorrelation curve

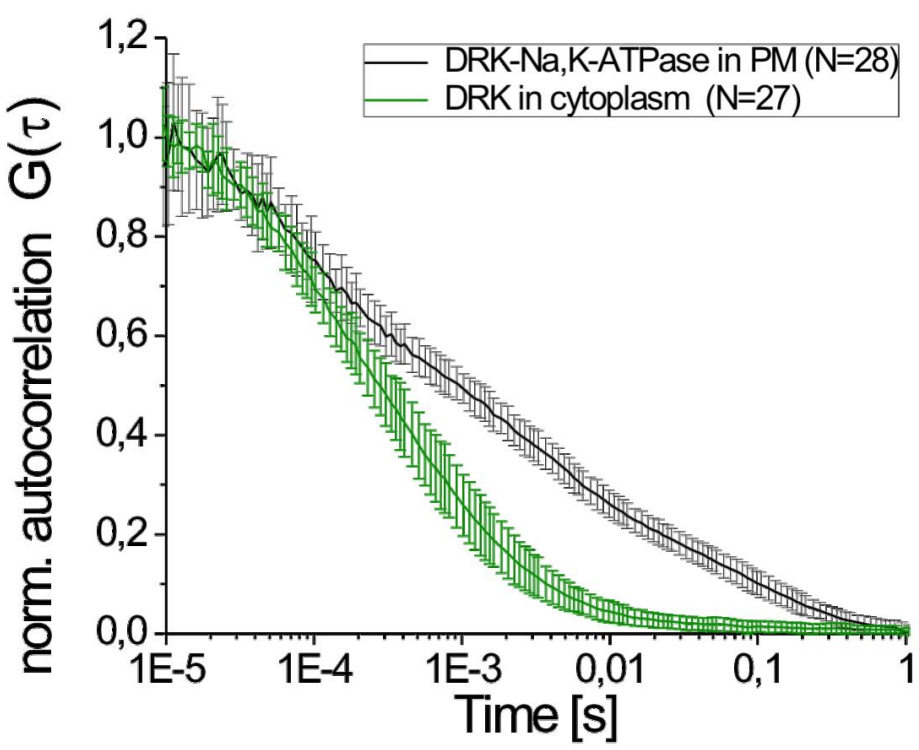
number of particles in the focal volume

local concentration of molecules



- Correlation amplitude is inversely proportional to concentration of fluorophores

Example of FCS



Normalized autocorrelation curves with standard deviation from the DRK protein expressed in the cytoplasm and DRK-labelled Na,K-ATPase in the plasma membrane (PM)

- DRK-labeled Na,K-ATPase exhibited *much slower diffusion*
- DRK-Na,K-ATPase fusion protein has a much larger molecular weight (about 184 kDa) than the isolated DRK protein (27 kDa)
- Dynamic viscosity of lipid membranes is high compared to the aqueous media

https://www.researchgate.net/publication/296633109

Example of FCS

	diffusion time τ	diffusion constant D [$m^2 \cdot s^{-1}$]
R6G	(27 ± 3) µs (eGFP settings, PH70) (29 ± 2) µs (DRK settings, PH70) (33 ± 3) µs (DRK settings, PH 80)	$2.8 \cdot 10^{-11}$ (refs. [47, 49])
DRK in PBS	(106 ± 22) µs	$(8.5 \pm 1.8) \cdot 10^{-11}$
eGFP in PBS	(95 ± 2) µs	$(7.6 \pm 0.2) \cdot 10^{-11}$
DRK in cytosol	(479 ± 222) µs	$(1.9 \pm 0.9) \cdot 10^{-11}$
eGFP in cytosol	(376 ± 69) µs	$(1.9 \pm 0.3) \cdot 10^{-11}$
DRK-Na,K-ATPase in PM	τ_1 = (318 ± 212) µs ((50 ± 7)%)	$D_1 = (2.8 \pm 1.9) \cdot 10^{-11}$
	τ_2 = (44 ± 14) ms ((50 ± 7)%)	$D_2 = (1.8 \pm 0.6) \cdot 10^{-13}$
eGFP-Na,K-ATPase in PM	τ_1 = (974 ± 331) µs ((31 ± 4)%)	$D_1 = (0.7 \pm 0.3) \cdot 10^{-11}$
	τ_2 = (67 ± 17) ms ((69 ± 4)%)	$D_2 = (1.1 \pm 0.3) \cdot 10^{-13}$

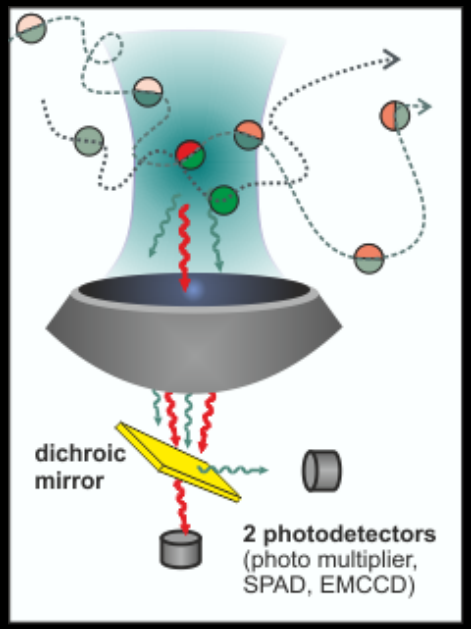
Comparison of the diffusion behavior of DRK- and eGFP-labeled Na,K-ATPase constructs in the plasma membrane of HEK293T cells

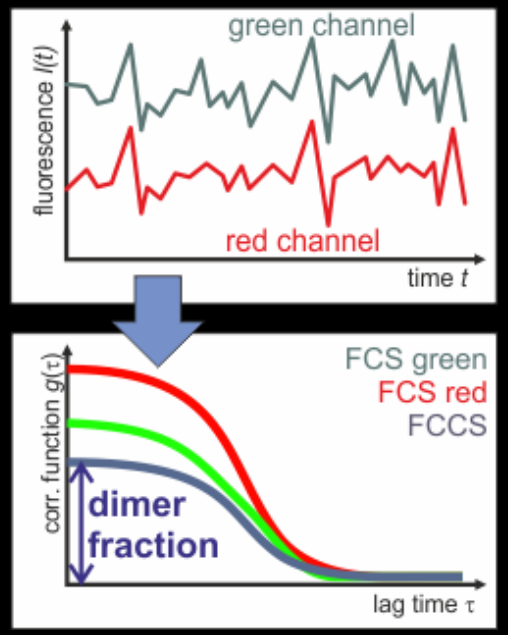
https://www.researchgate.net/publication/296633109

FCCS

Cross-correlation curve

- Dual color mode
- Interactions between different molecular species
  - *Stoichiometry*
  - *Binding constants*





http://www.dkfz.de/Macromol/research/spim.html, http://www.dbs.nus.edu.sg/lab/BFL/lscm_fcs.html

FCCS

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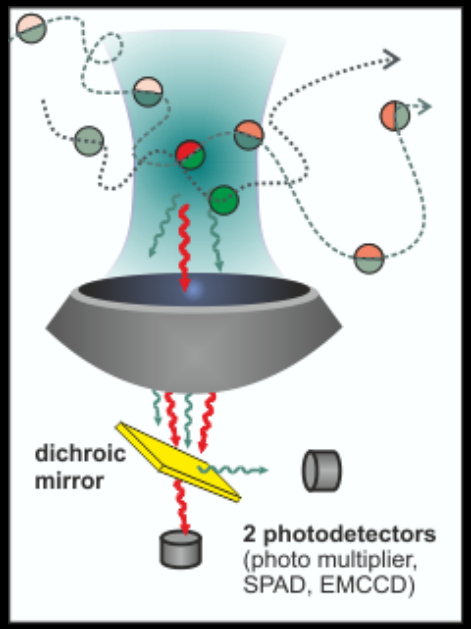
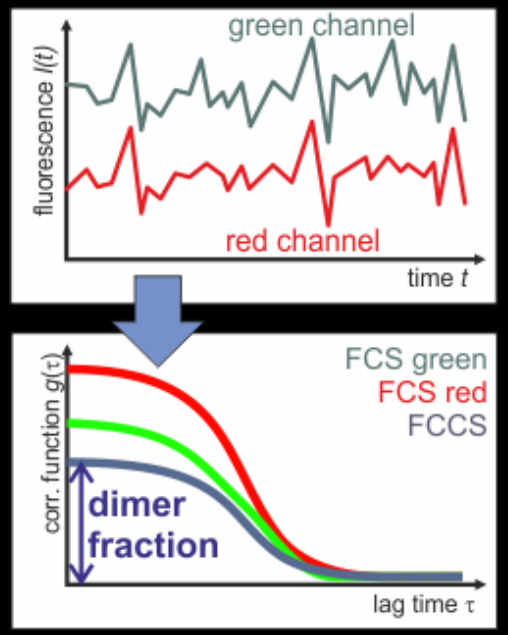
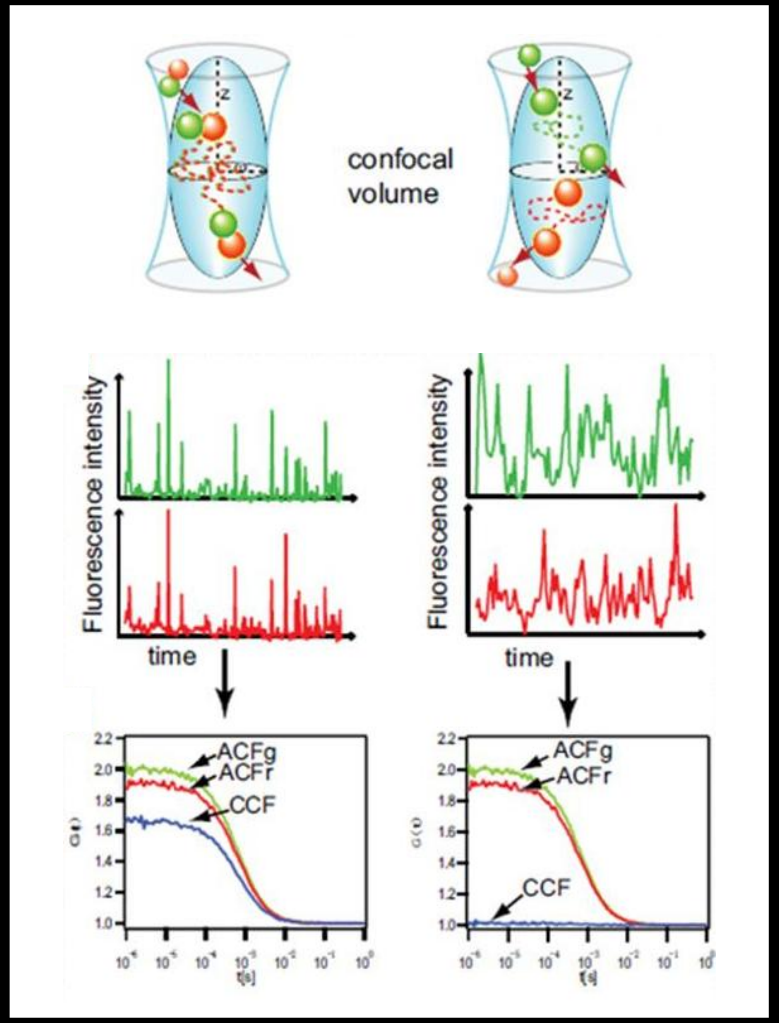
- Dual color mode
- Interactions between different molecular species
  - *Stoichiometry*
  - *Binding constants*



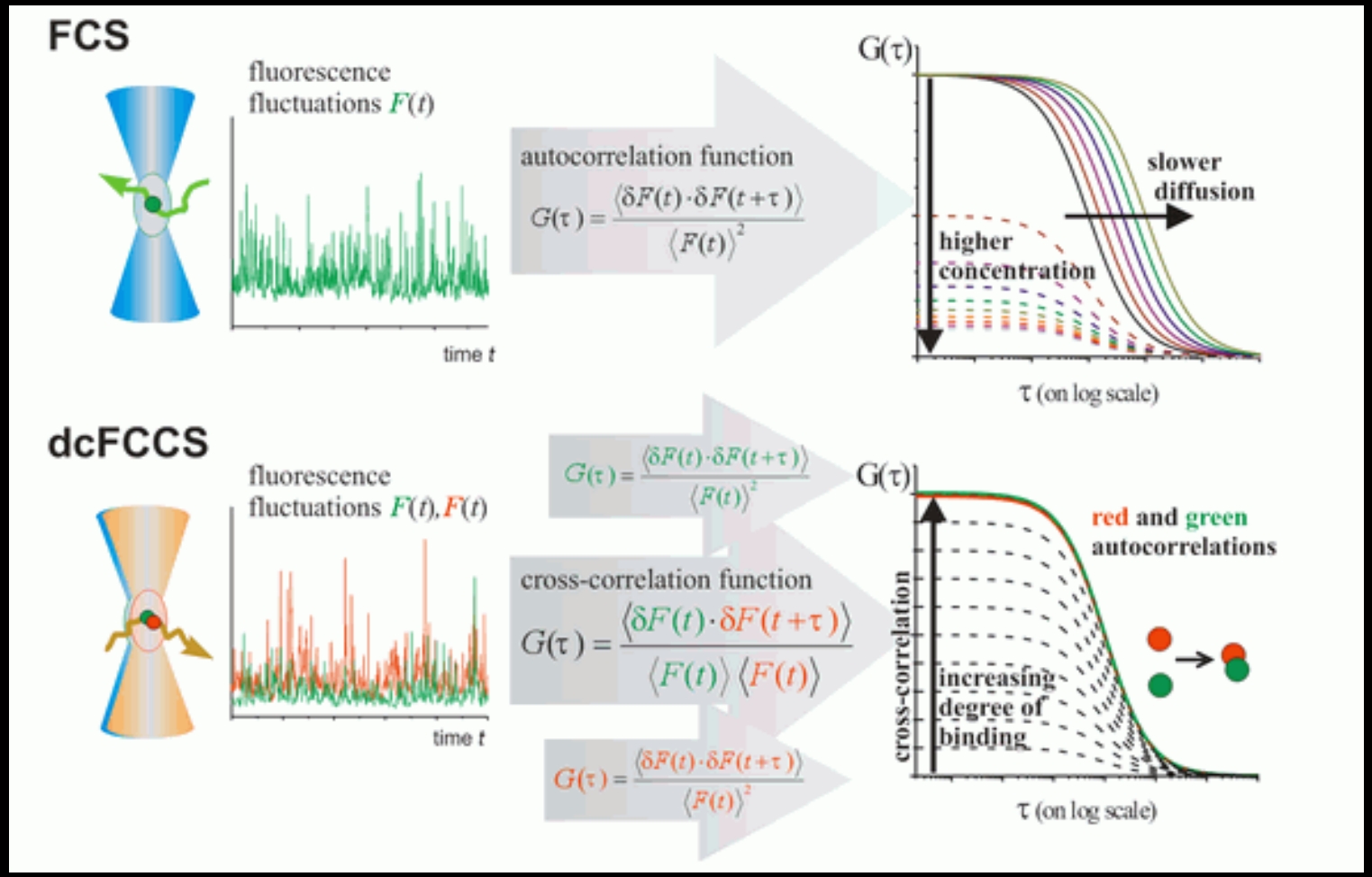




Interaction **No interaction**

http://www.dkfz.de/Macromol/research/spim.html, http://www.dbs.nus.edu.sg/lab/BFL/lscm_fcs.html

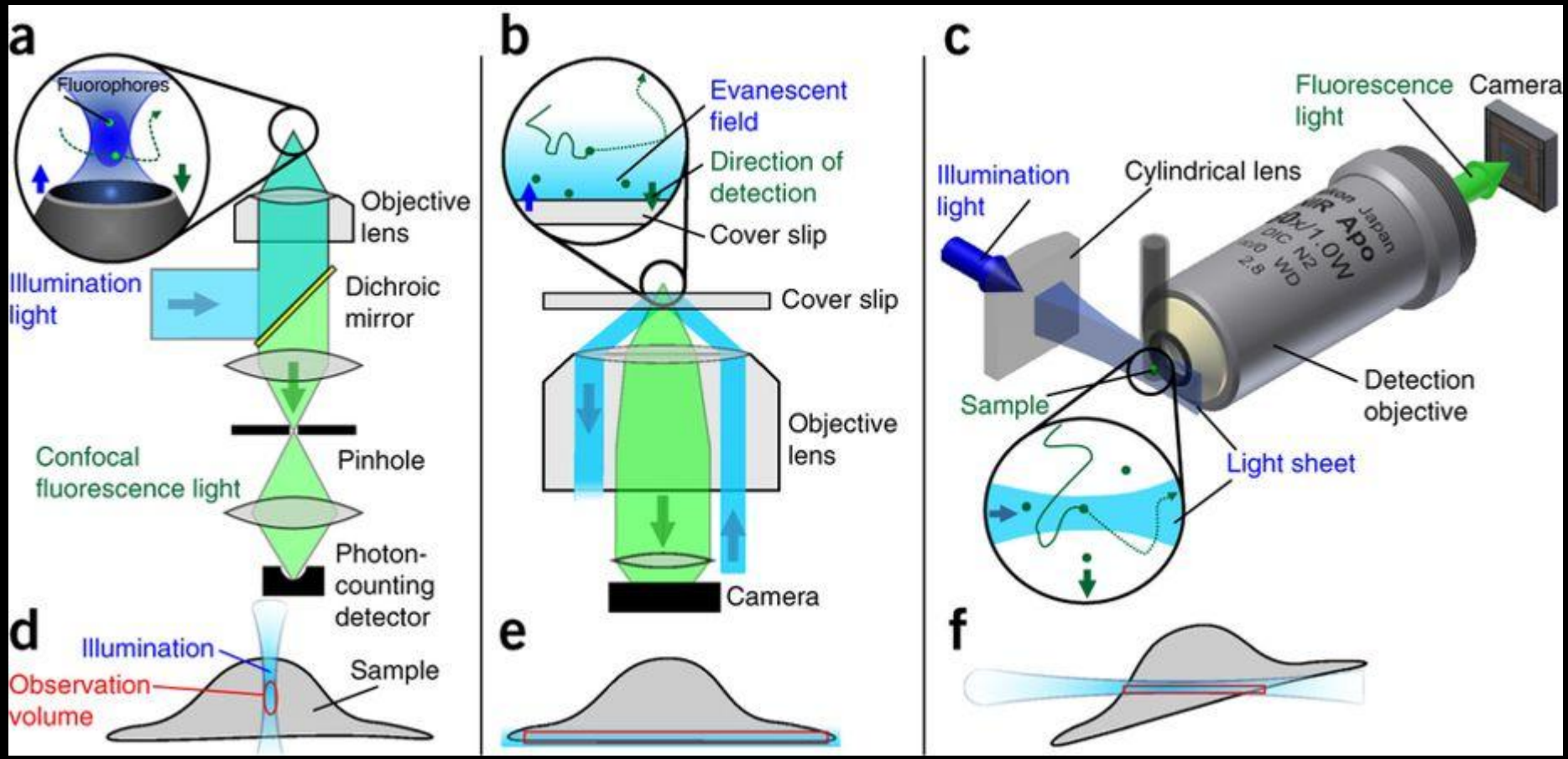
FCCS



Nature Methods 3, 83 - 89 (2006)

Imaging FCS

- Camera-based approach



Confocal microscope | Camera-based TIRF | SPIM microscope

Krieger J.W. et al., Nature Protocols 2015, Vol.10, No.12, 1948-1974

	Fluorescence Recovery After Photobeaching **FRAP**	Fluorescence Correlation Spectroscopy **FCS**
Diffusion Rates	Yes	Yes
Multicomponent Diffusion	No	Yes
Mobile Fraction	Yes	No
Concentration	No	Yes
Complexing	No	Yes
Complex Stoichiometry	No	Yes
Binding Kinetics	Yes	Yes

	Fluorescence Recovery After Photobeaching **FRAP**	Fluorescence Correlation Spectroscopy **FCS**	Förster Resonance Energy Transfer **FRET**
Diffusion Rates	Yes	Yes	No
Multicomponent Diffusion	No	Yes	No
Mobile Fraction	Yes	No	No
Concentration	No	Yes	No
Complexing	No	Yes	Yes
Complex Stoichiometry	No	Yes	No
Binding Kinetics	Yes	Yes	Yes

Thank you for your attention!