Fluorescence Correlation Spectroscopy

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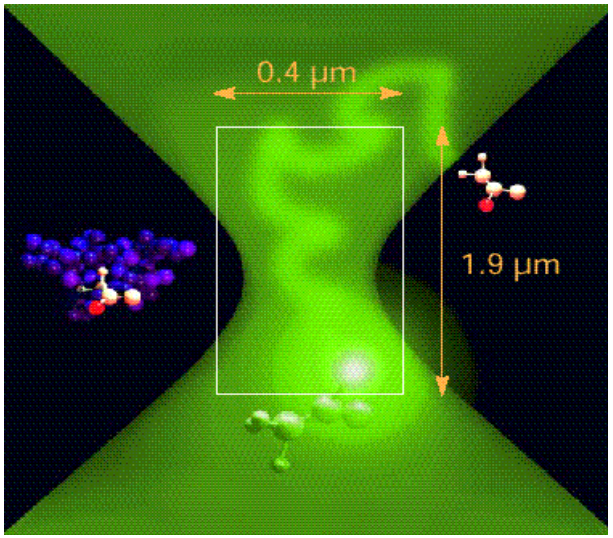
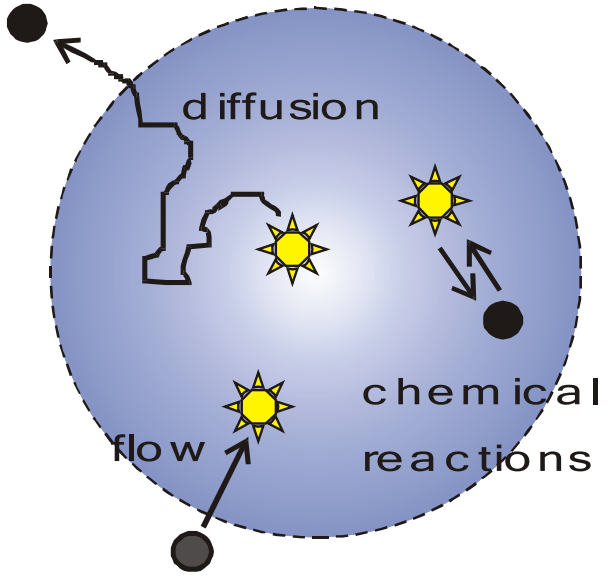
Principles of confocal Fluorescence Correlation Spectroscopy (FCS)

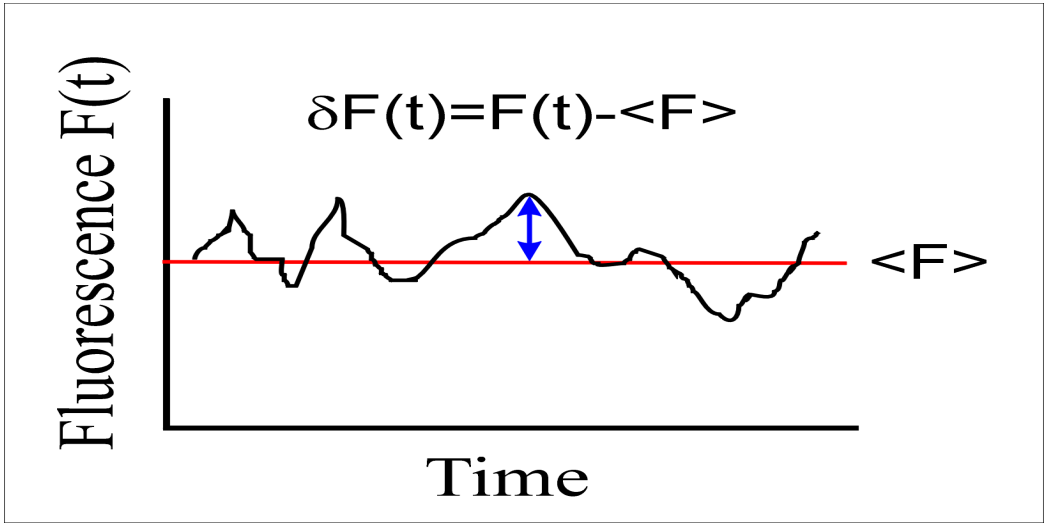


- signal: **fluorescence** (auto or ***labeled***)
- confocal microscope ⇒ volume element in fl range
- dye concentration in nM range → **single molecule**

- Large fluctuations in fluorescence signal by **small volume** and **low concentration**
- Monitored: **time evolution of fluorescence fluctuations (in our case due to diffusion)**



Principles of FCS

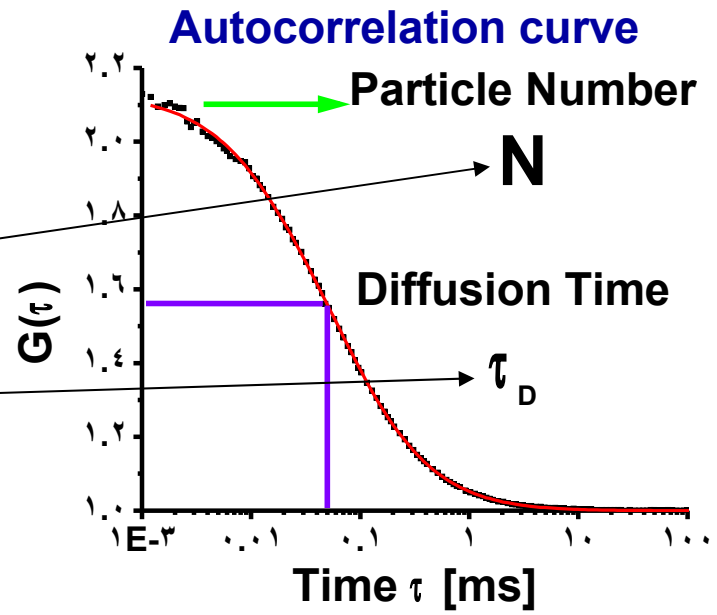


$$G(\tau) = 1 + \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F \rangle^2}$$

For the case 3-D diffusion causes the Fluctuations:

$$G(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{1 + (\tau / \tau_D)} \cdot \frac{1}{\sqrt{1 + S^2 \cdot (\tau / \tau_D)}}$$

S describes the dimension of the volume element



Principles of FCS

If the size of the volume element is known*:

A) τ_D ⟶ Diffusion coefficient D

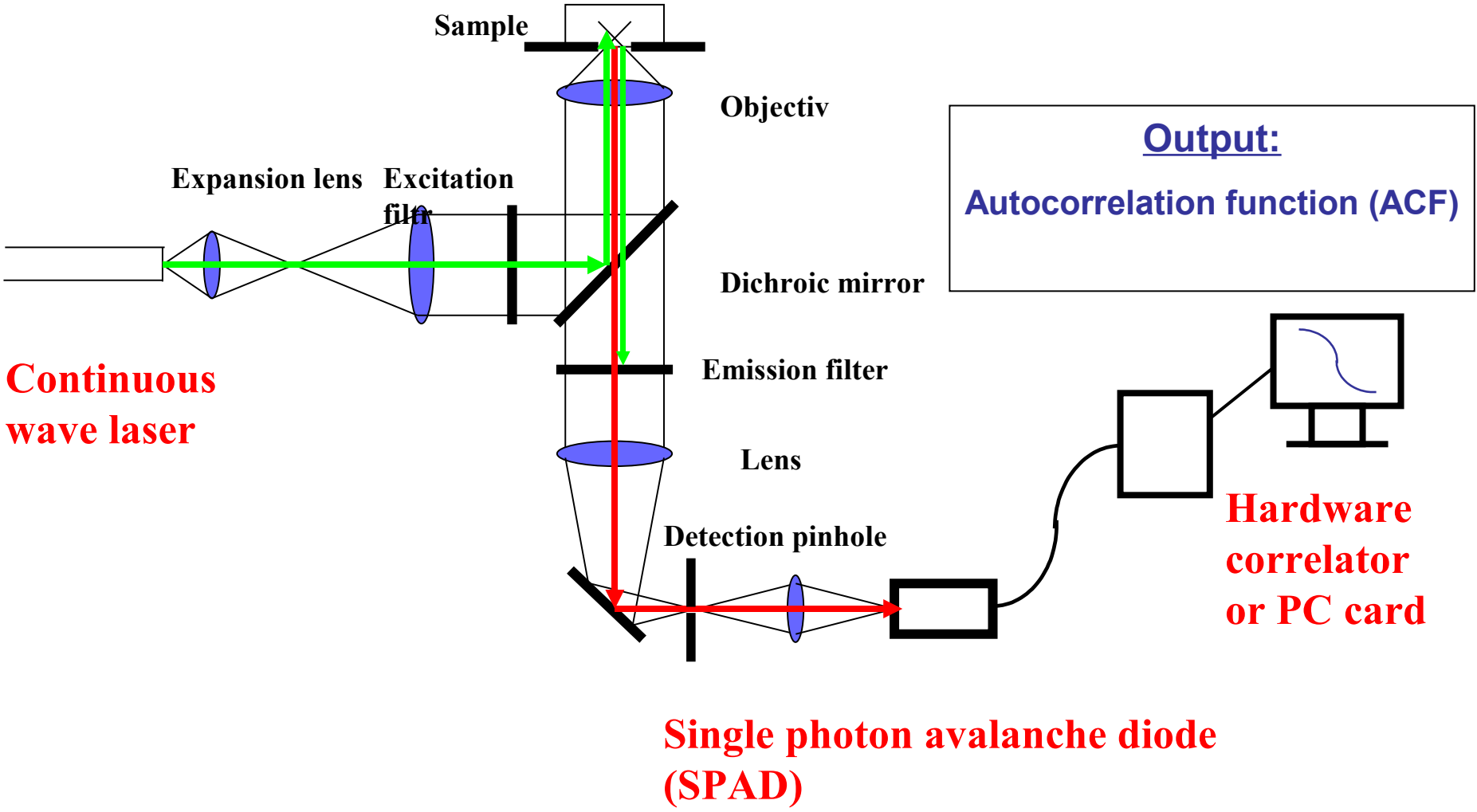
B) N ⟶ Concentration C

Further read out parameter:

C) Count rate ⟶ Fluorescence intensity

***usually determined by calibration experiments using a sample with known D and c**

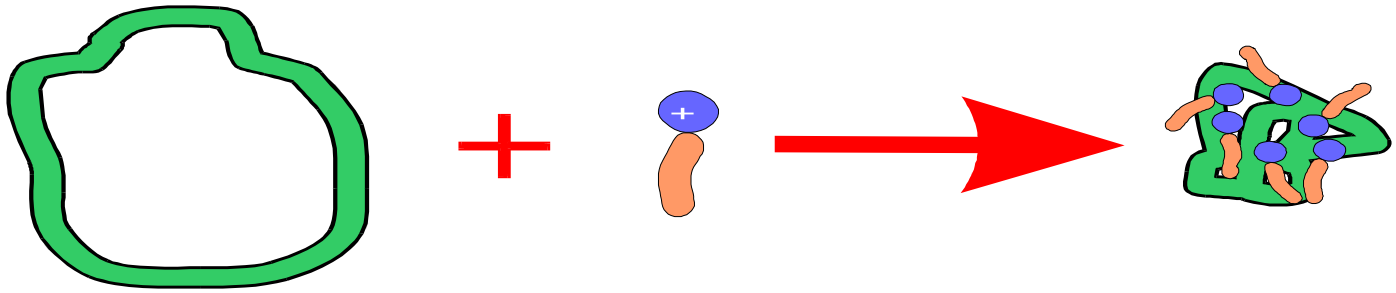
Classic confocal FCS instrument setup



Output:

Autocorrelation function (ACF)

Characterisation of DNA condensation induced by bivalent cations or positively charged amphiphiles (in vitro experiments)

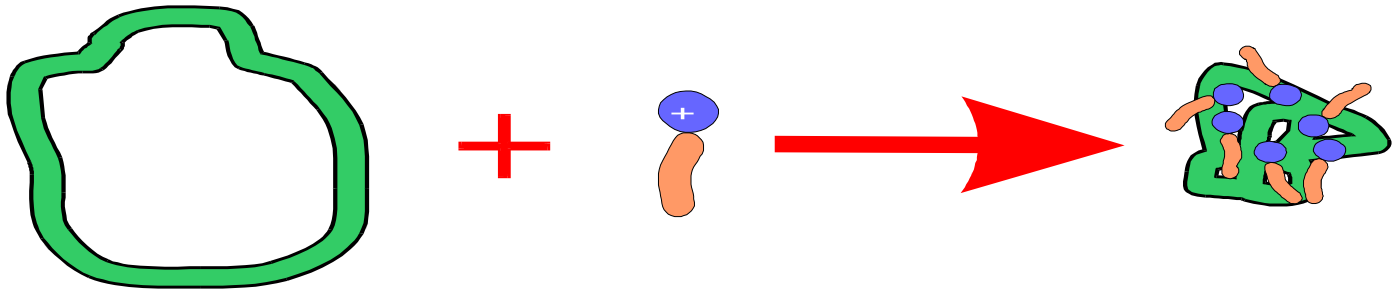


DNA-size: several μm

Condensing agent -DNA
Nanoparticle: down to 50 nm

Background: Condensed plasmids (DNA Nanoparticles) are used in targeted drug delivery systems (Non-Viral Gene Therapy)

Characterisation of DNA condensation induced by bivalent cations or positively charged amphiphiles



Aim: Establishing with FCS a technique which directly monitors the condensation process on a single molecule level

Used plasmids: different sizes: 13 kbp, 10 kbp, 5 kbp, and 3 kbp

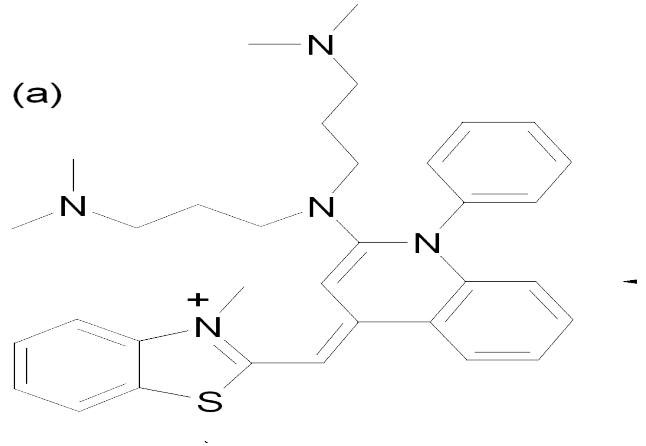
Used dyes: Propidium iodide, ethidium bromide, PicoGreen

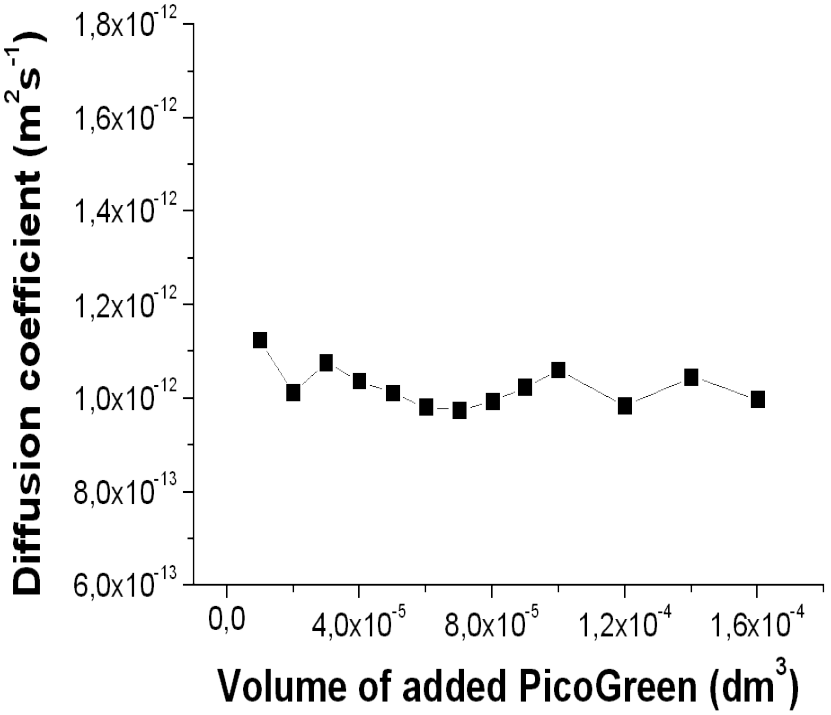
Used condensing agents:

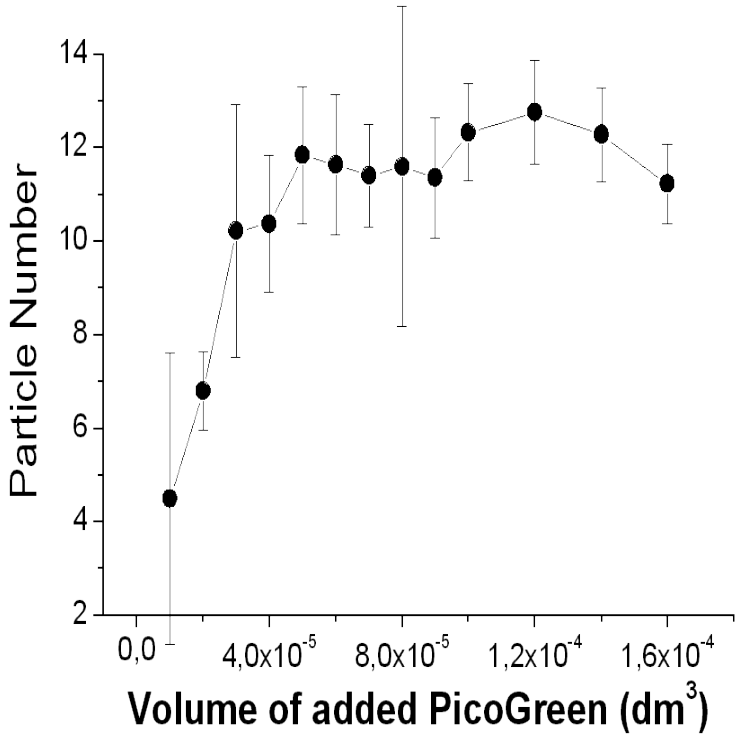
Spermine, hexadecyltrimethyl ammonium bromide (HTAB),…

Newly developed lipopolyamines → **Ideal Nanoparticle Formation**

Titration of DNA (10 kbp) with PicoGreen (buffer TE, pH 8.0)



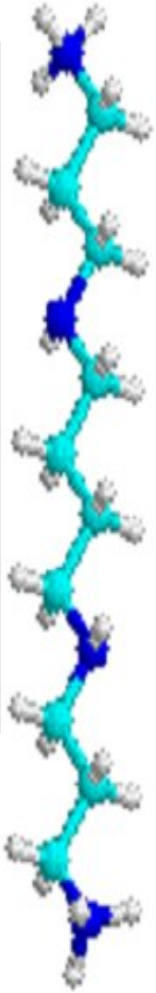




PicoGreen does not influence the DNA conformation
(In opposite to propidium iodide, ethidium bromide)

Typical Condensation Experiment: Labeled plasmid (10 kbp) induced by spermine (water, pH 7.6)

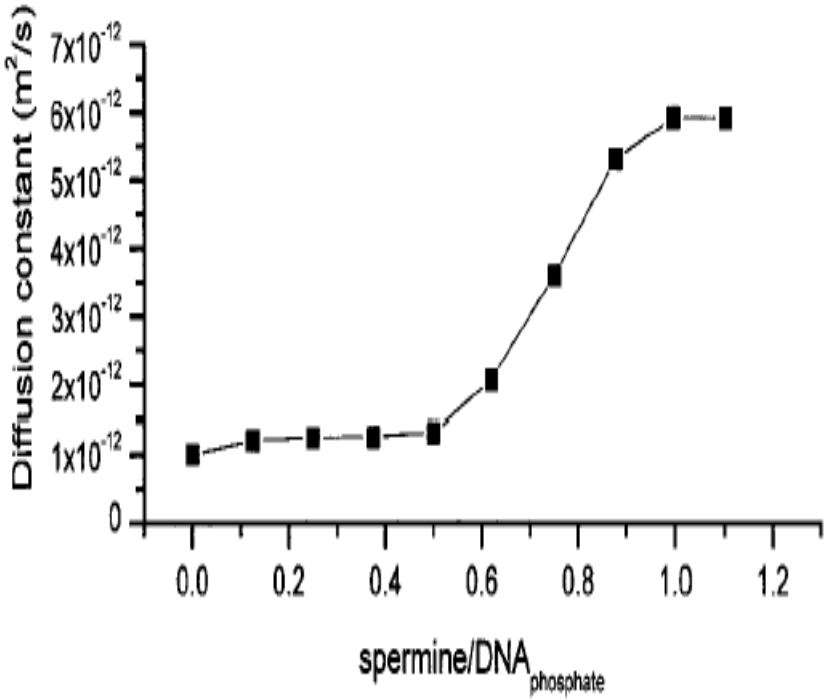
N decreases

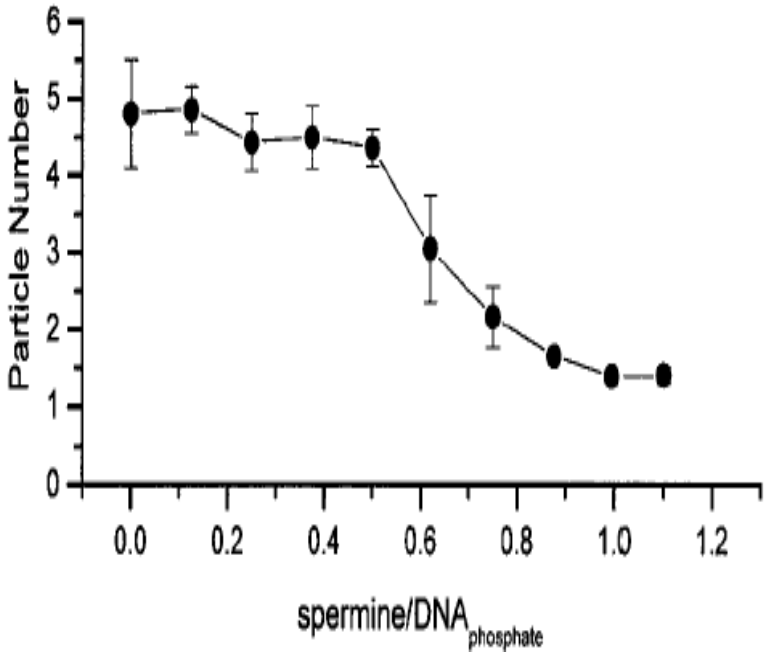


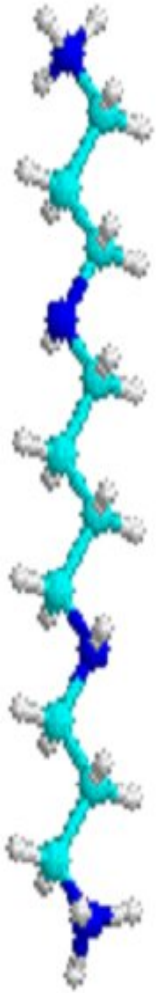
τ_D decreases from 15 to 3 ms!

FCS is extremly sensitive to DNA condensation

Condensation of PicoGreen labeled plasmid induced by spermine (water, pH 7.6)



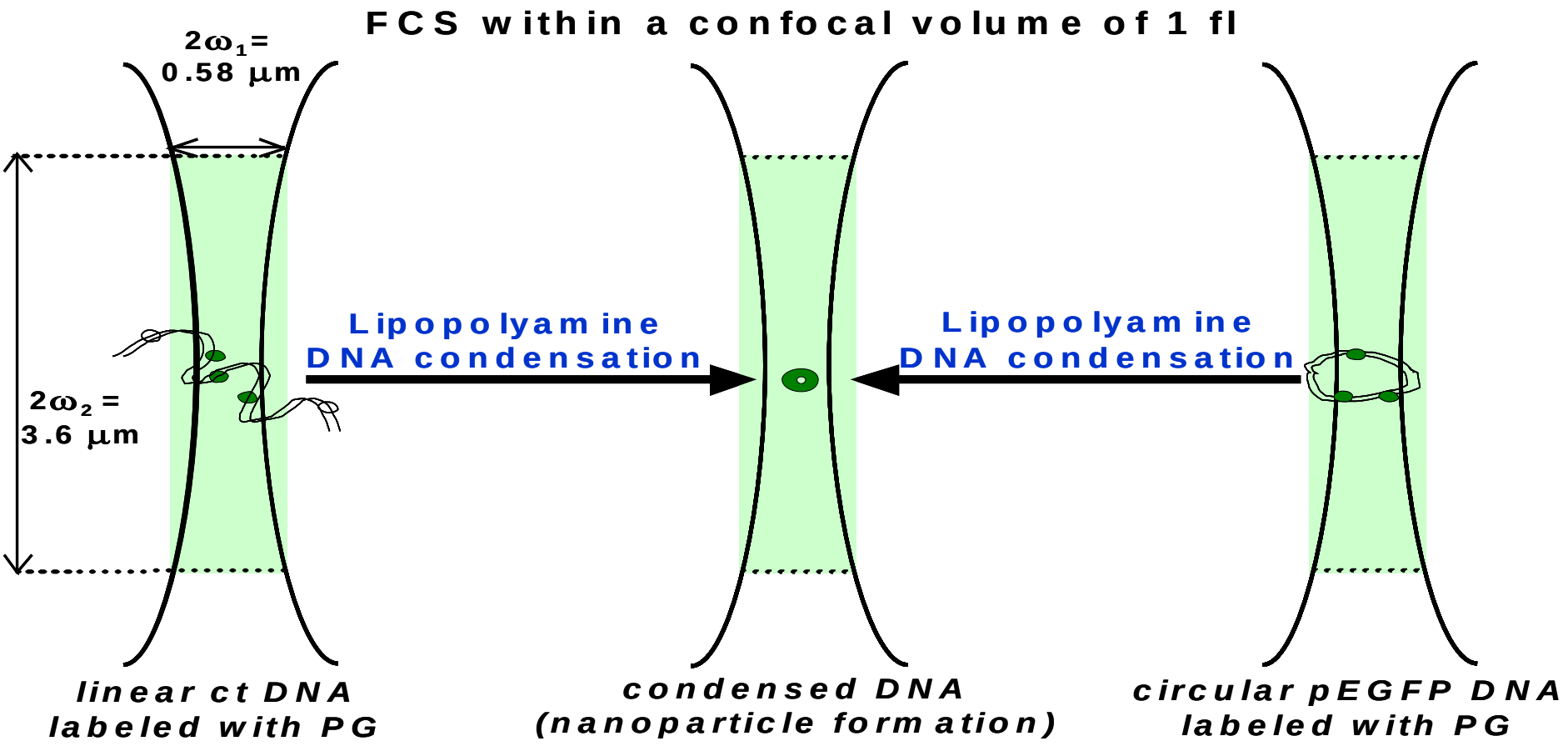




-Spermine-DNA nanoparticle is diffusing much faster than DNA with a length of several μm

-PN before condensation much larger than PN calculated from used DNA Concentration– Why??

FCS is detecting fluctuations: only if particle is much smaller than volume, fluctuation equals concentration



confocal excitation volume (V) ~ 1 femtoliter

intercalated PG within V (excitation)

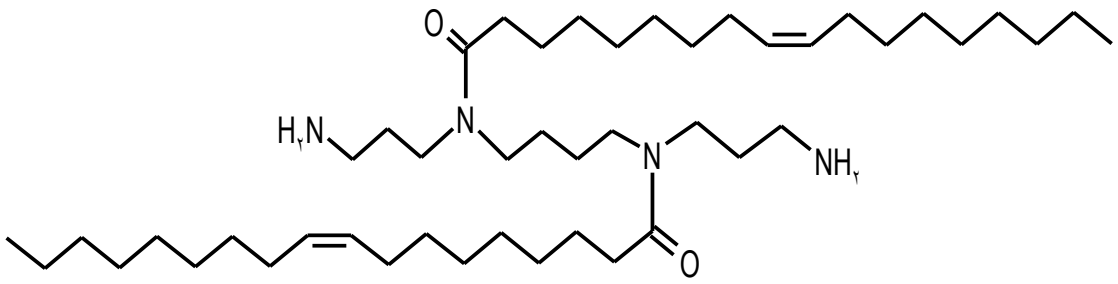
intercalated PG outside V (non-excitation)

Intercalated PG in a DNA nanoparticle within V (excitation)

Summary to “DNA condensation”

- Condensation process: dramatic decrease in τ_D and decrease in N
- If number of fluctuation equals number of particles, ideal DNA-nanoparticle is formed

- Perfect Condenser: *N*4,*N*9-dioleoylspermine (spermine conjugated with two chains of C18 fatty acid, with two positive charges at physiological pH)



Summary to “DNA condensation”

- FCS gives information on conformation and aggregation state of a DNA molecule

Selected Literature:
Kral T, Hof M, Langner M:
Effect of spermine on the plasmid condensation and dye release observed by fluorescence correlation spectroscopy. Biol. Chem 383, 331-335 (2002)

Dual-color FCS

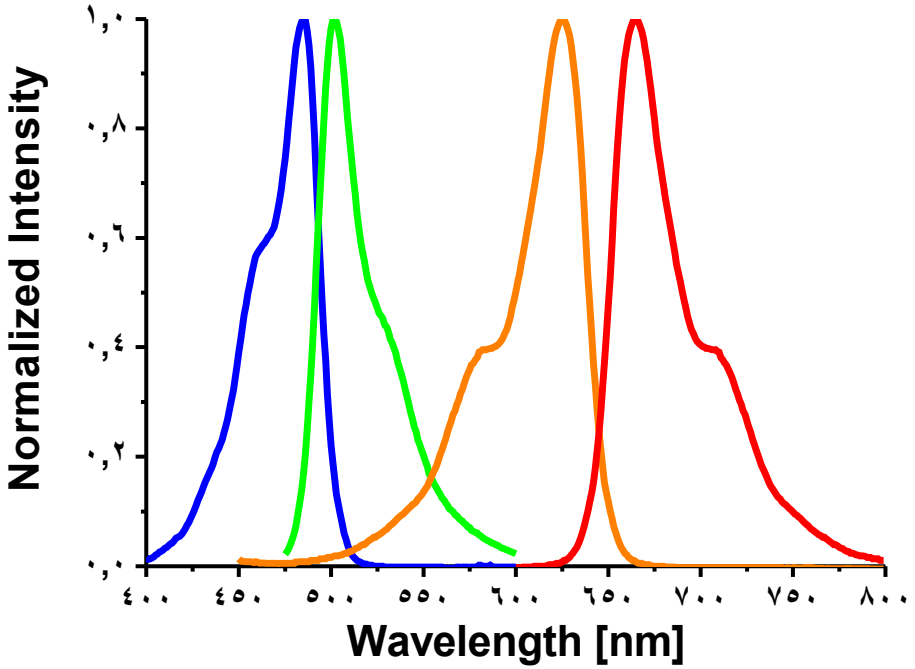
Designed for multicomponent diffusional analysis

Components must have different spectral properties

Autocorrelation functions: Parallel monitoring of concentrations and diffusion characteristics

Cross-correlation function: Interaction of the components

Dual-beam or two-photon excitation setup with several detectors



Schwille, P.; MeyerAlmes, F. J.; Rigler, R., *Biophysical Journal* **1997,** 72, (4), 1878-1886.

Dual-color FCS x FLCS

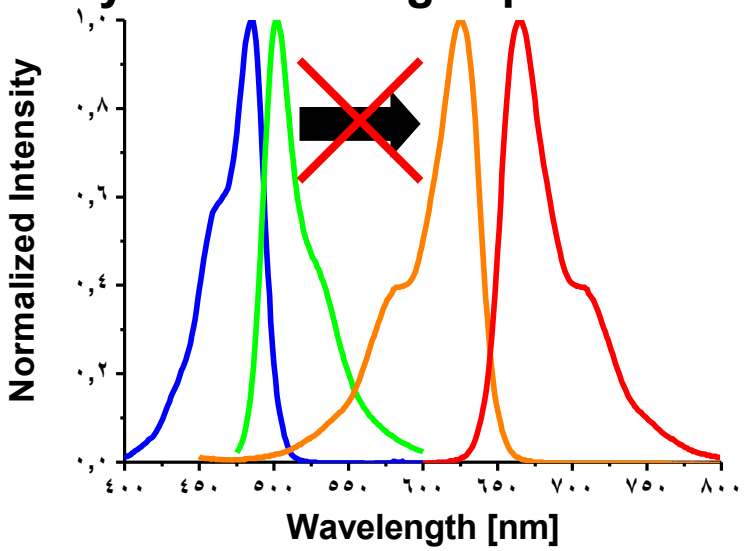
Designed for similar purpose
Different principle and realization

Immense advantage of FLCS

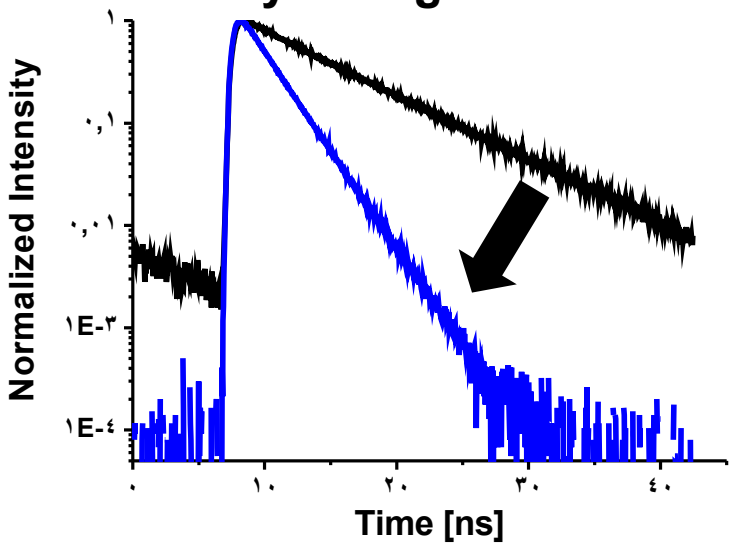
SINGLE DYE LABELING

Chemical, conformational or microenvironmental change can

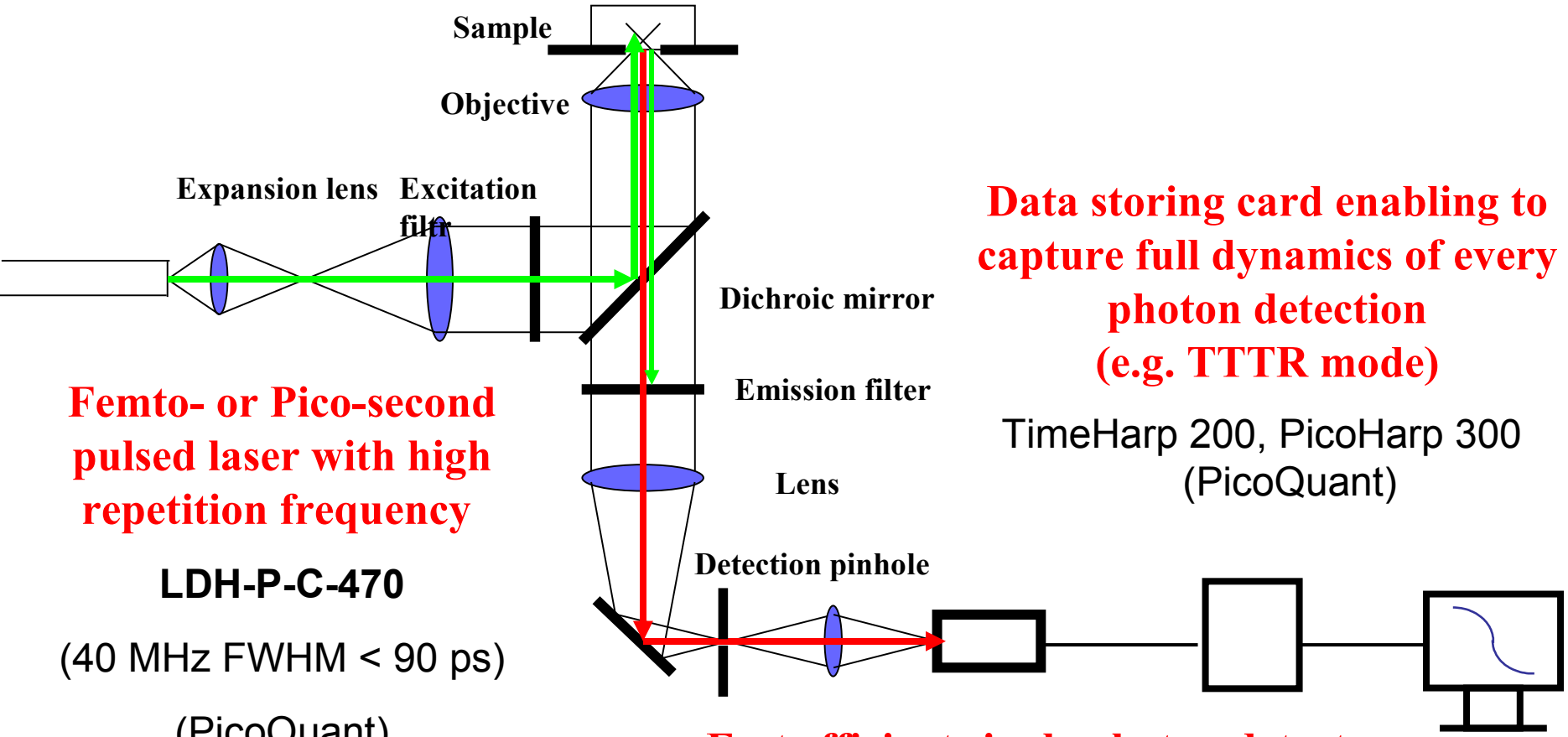
hardly induce a large spectral shift



easily change a lifetime



Schematic setup for FLCS measurements



Femto- or Pico-second pulsed laser with high repetition frequency

LDH-P-C-470

(40 MHz FWHM < 90 ps)

(PicoQuant)

Data storing card enabling to capture full dynamics of every photon detection (e.g. TTTR mode)

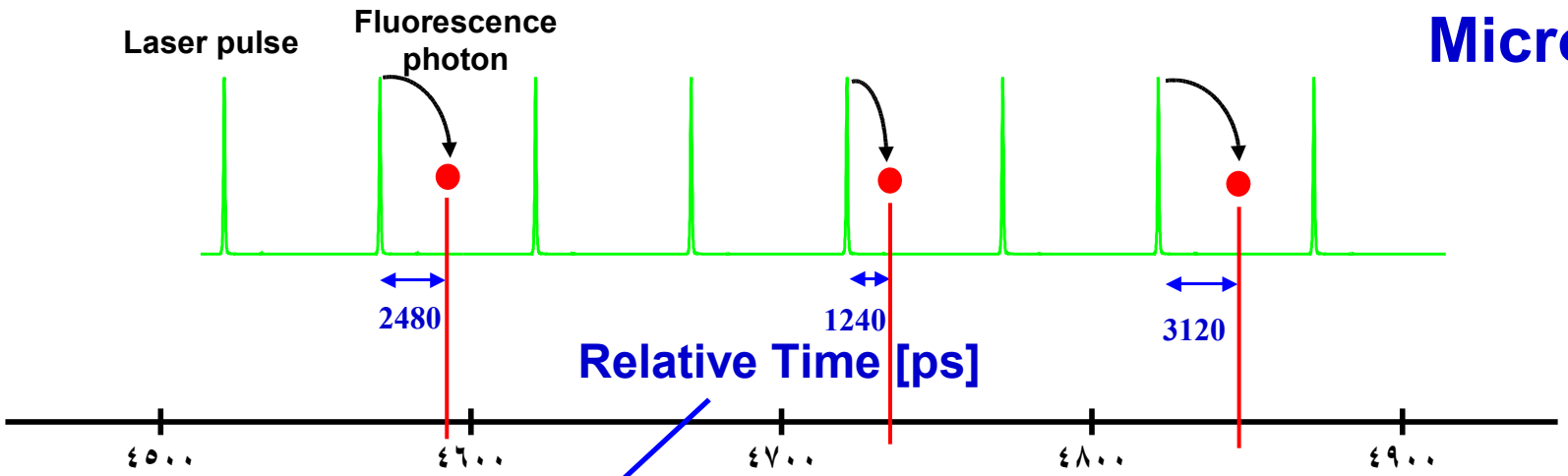
TimeHarp 200, PicoHarp 300 (PicoQuant)

Fast efficient single photon detector

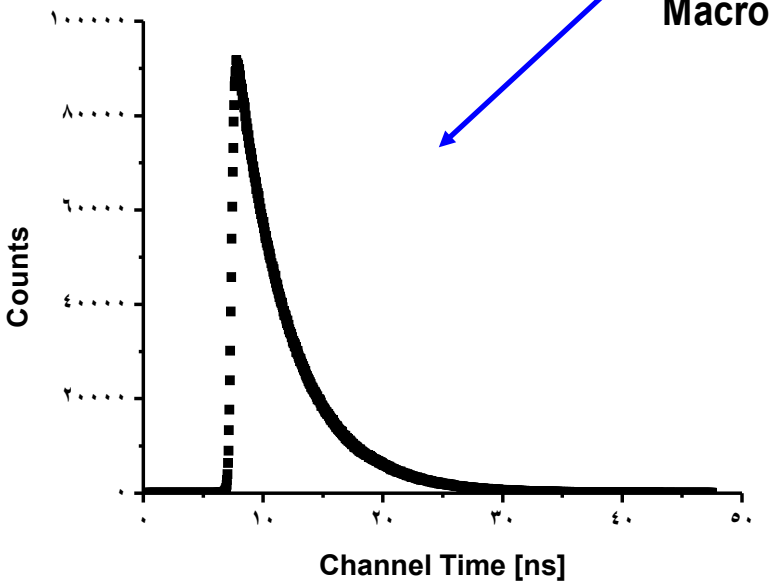
Actively quenched SPCM-AQR-13-FC
(Perkin-Elmer)

Upgraded Confocor 1 (Carl Zeiss, PicoQuant), MicroTime 200 (Olympus, PicoQuant)

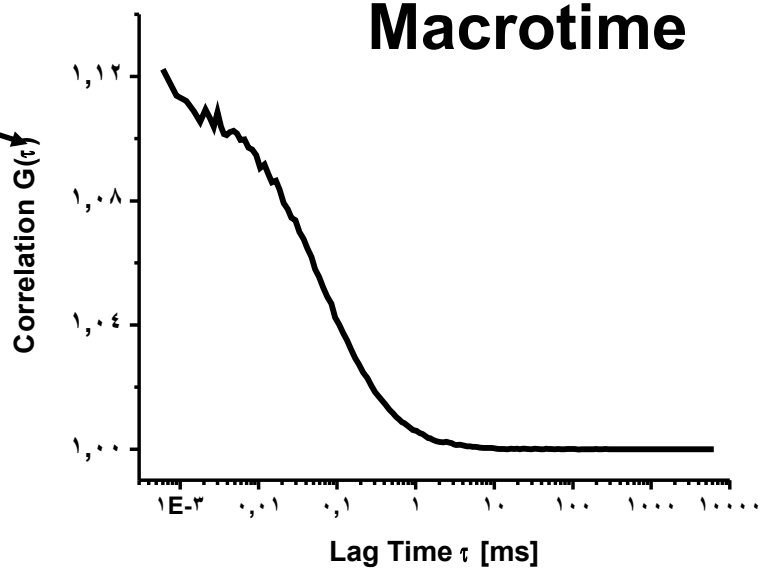
Data storage: microtime vs macrotime



Macro Time [ns]



+



= FLCS

FLCS mathematics

- **Provided that** $I_j(t) = \sum_{i=1}^{N} w^{(i)}(t) p_j^{(i)}$

2) We can find a filter function f_j^i, which gives the most likely values of $w^{(i)}$ for every particular macrotime.

$$\sum_{j=1}^{L} f_j^{(i)} I_j(t) = w^{(i)}(t)$$

6) Calculated by

$$f_j^{(i)} = \left(\left[M \cdot diag\left\langle I_j(t) \right\rangle_t^{-1} \cdot M^T \right]^{-1} \cdot M \cdot diag\left\langle I_j \right\rangle^{-1} \right)_{ij}$$

$$\hat{M}_{ij} = p_j^{(i)}$$

4) Orthonormal with fluorescence patterns

$$\sum_{j=1}^{L} f_j^{(i)} p_j^{(k)} = \begin{cases} 1, & i = k, \\ 0, & i \neq k. \end{cases}$$

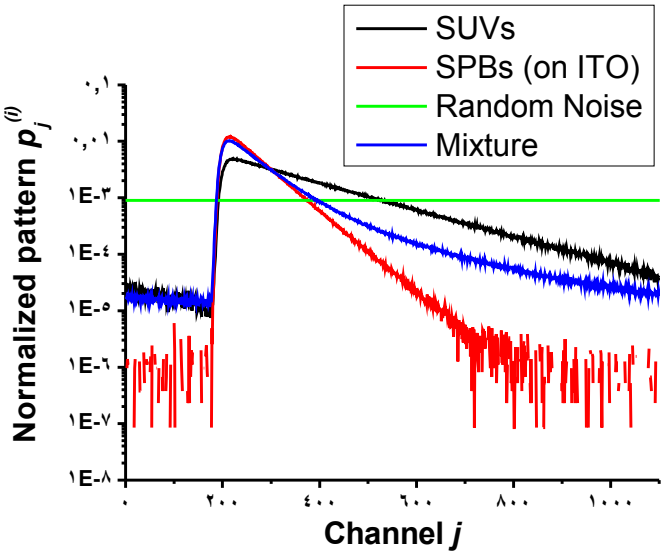
5) Auto- and crosscorelation functions can be calculated

FLCS correlation functions

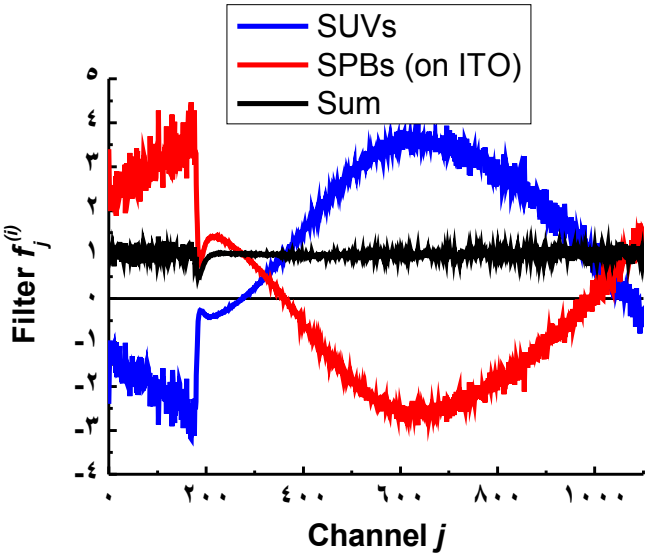
$$g^{(ij)}(\tau) = \left\langle w^{(i)}(t) w^{(j)}(t+\tau) \right\rangle_t$$

$$= \left\langle \left(\sum_{k=1}^{L} f_k^{(i)} I_k(t) \right) \left(\sum_{l=1}^{L} f_l^{(k)} I_l(t+\tau) \right) \right\rangle_t$$

$$= \sum_{k=1}^{L} \sum_{l=1}^{L} f_k^{(i)} f_l^{(k)} \left\langle I_k(t) I_l(t+\tau) \right\rangle_t$$



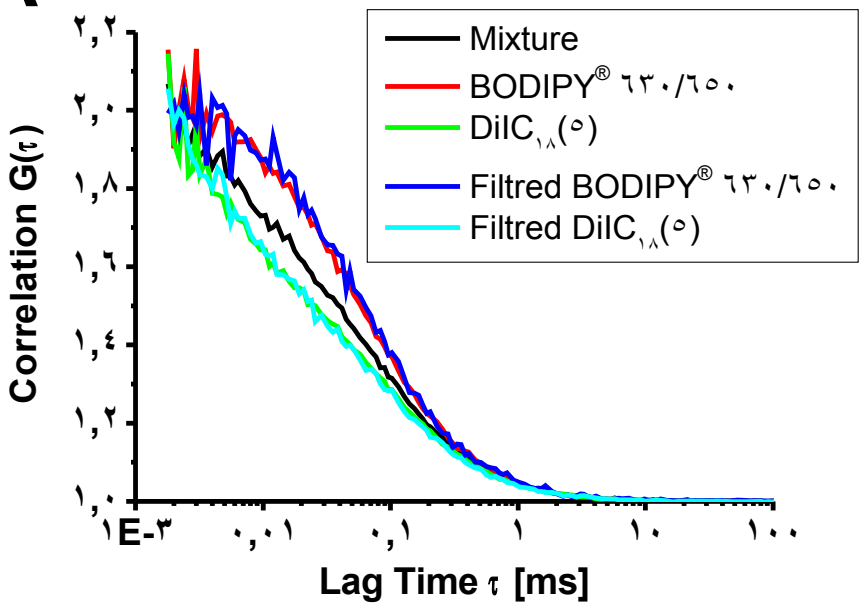
No exact intensity separation in advance, the required averaging happens during the correlation!!!



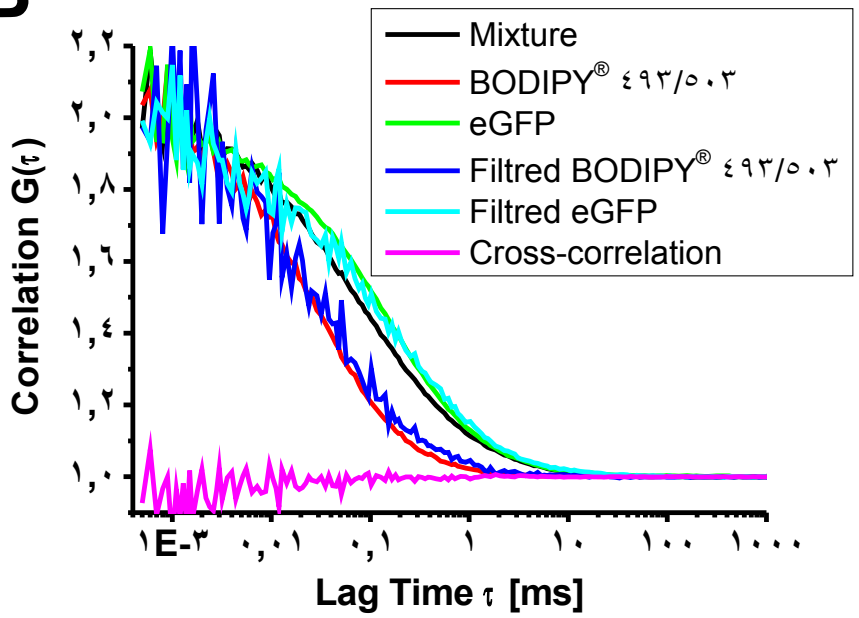
Examples

- Mixture of BODIPY 630/650 and $DiIC_{18}(5)$ in methanol – 639 nm laser
- Mixture of BODIPY 493/503 and eGFP in water – 470 nm laser
- Small and large unilamellar vesicles in water labeled with BODIPY 630/650 and $DiIC_{18}(5)$, respectively – 639 nm laser

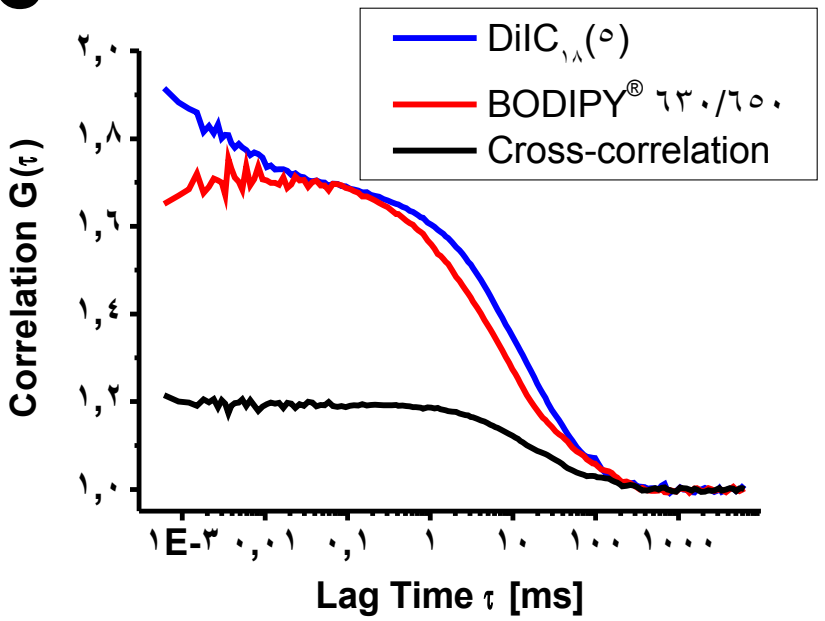
A



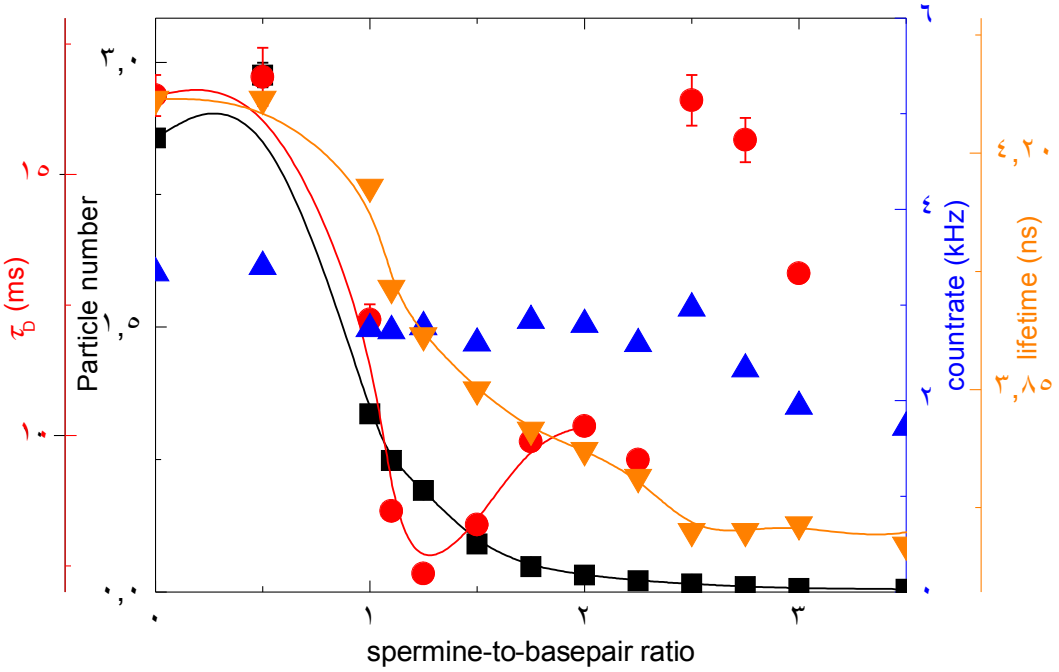
B



C

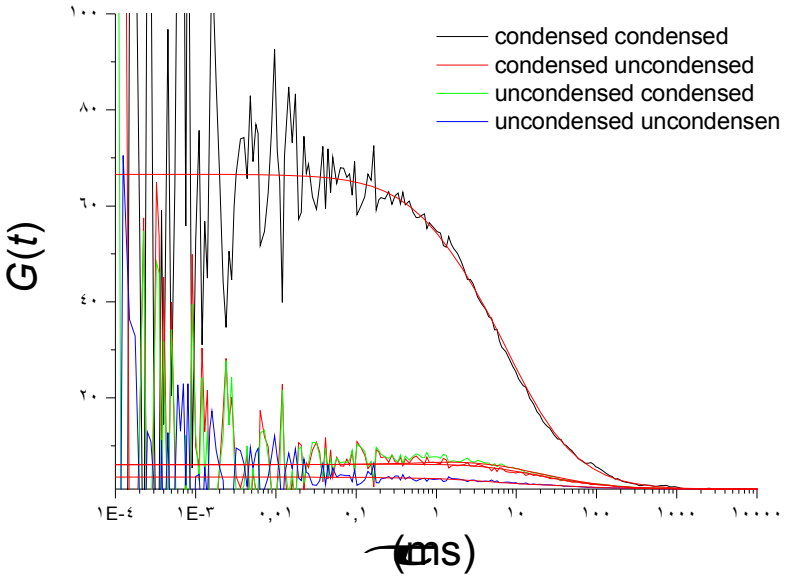


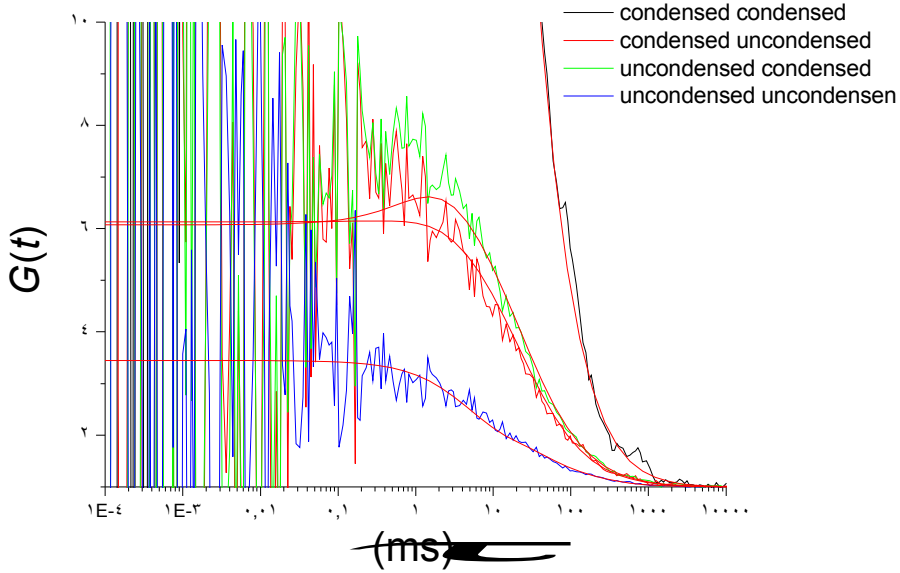
Simultaneous Measurement of Lifetime and FCS for DNA Condensation



Lifetime change during the condensation
Middle point – coexistence of condensed and uncondensed DNA
Can **FLCS** separate the signal for condensed and uncondensed DNA?

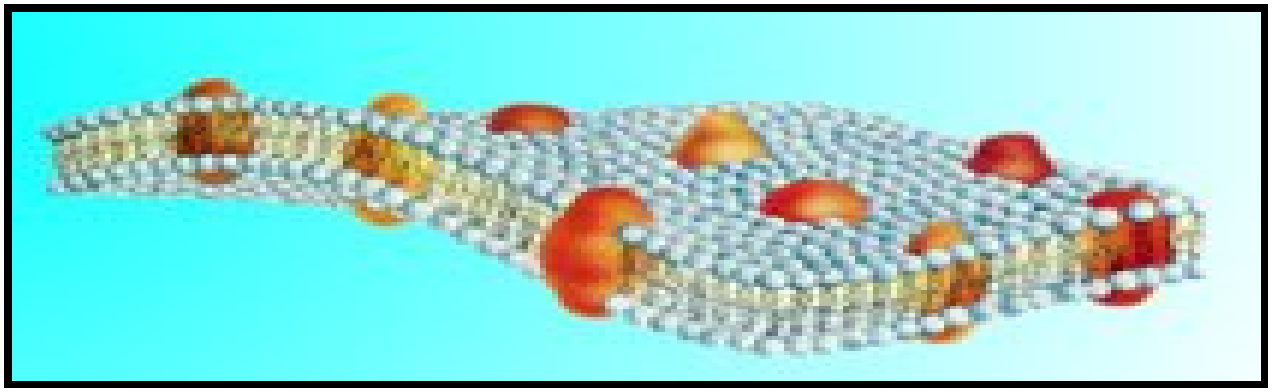
FLCS at the “point of coexistence”





Autocorrelation curves correspond to the ACFs for condensed and uncondensed DNA measured separately
Crosscorelation curves correspond to the intramoleculardynamics on the milisecond scale

How to determine lateral diffusion coefficients in membranes by confocal FCS



Background: Recent Studies (e.g. Webb PNAS 99) show large standard deviations of 40 % to 100 % in diffusion coefficient

↓

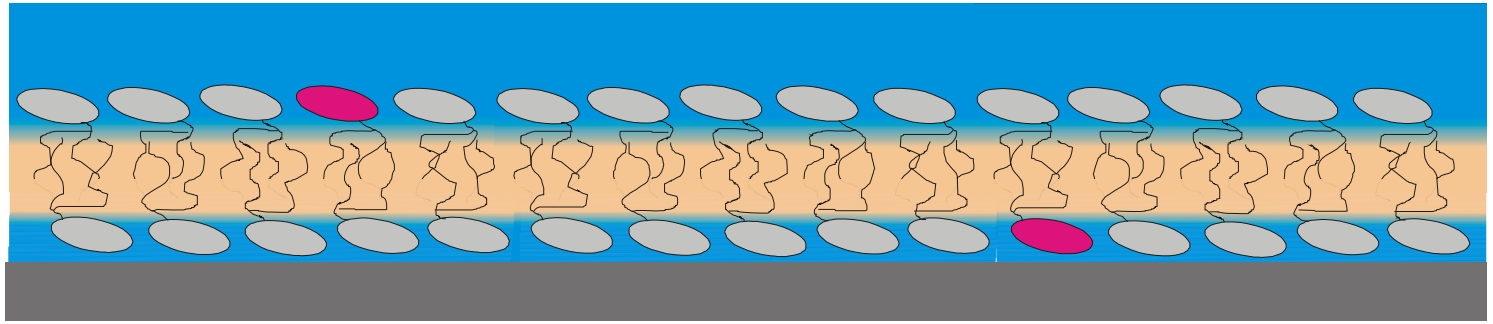
Only 3 publications on this topic till 2002

↓

Confocal FCS not established for lipid diffusion within bilayers

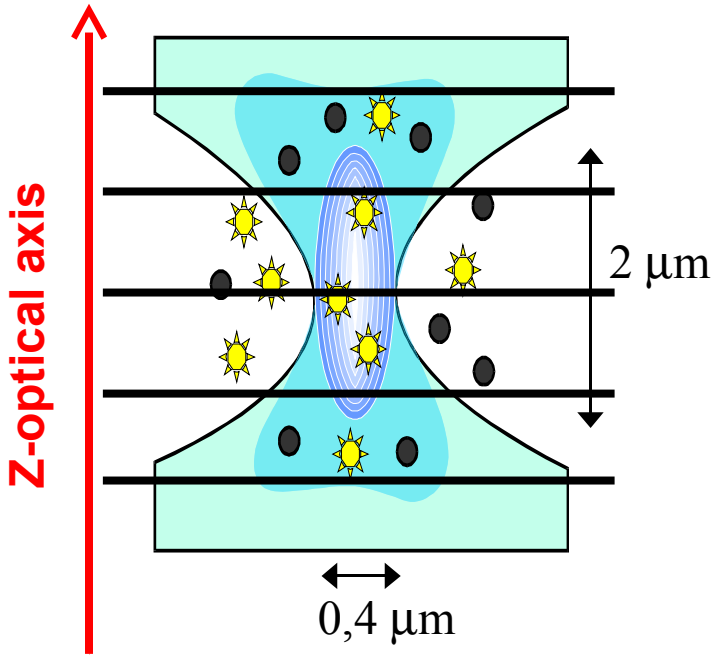
How to determine lateral diffusion coefficients in membranes by confocal FCS

Investigated model system: Supported Phospholipid Bilayers (SPB's)

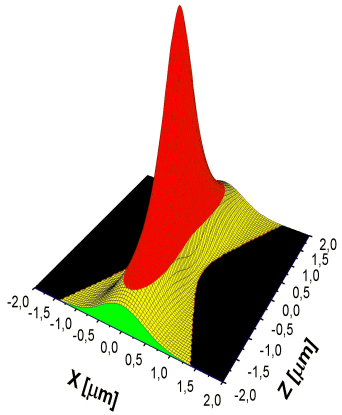


Aim: Reproducible and accurate confocal FCS results on planar lipid systems

Reason for "bad" results: surface localization problem



The intensity distribution of the excitation beam can be described as a z-symmetric Lorentz-Gauss profil



Radius ***w*** of sample illuminated area strongly depends on a mutual position of sample and focus planes

=>

Direct influence on PN (c) and

τ_D (D)

- how to place the sample in a defined z-position relative to the focus ?

-how to measure *w* ?

Solution: "Z-scan"

PN and τ_D are functions of the relative position Δz of sample and focus planes

Z-scan = to measure at different well-defined Z-positions around the focus plane

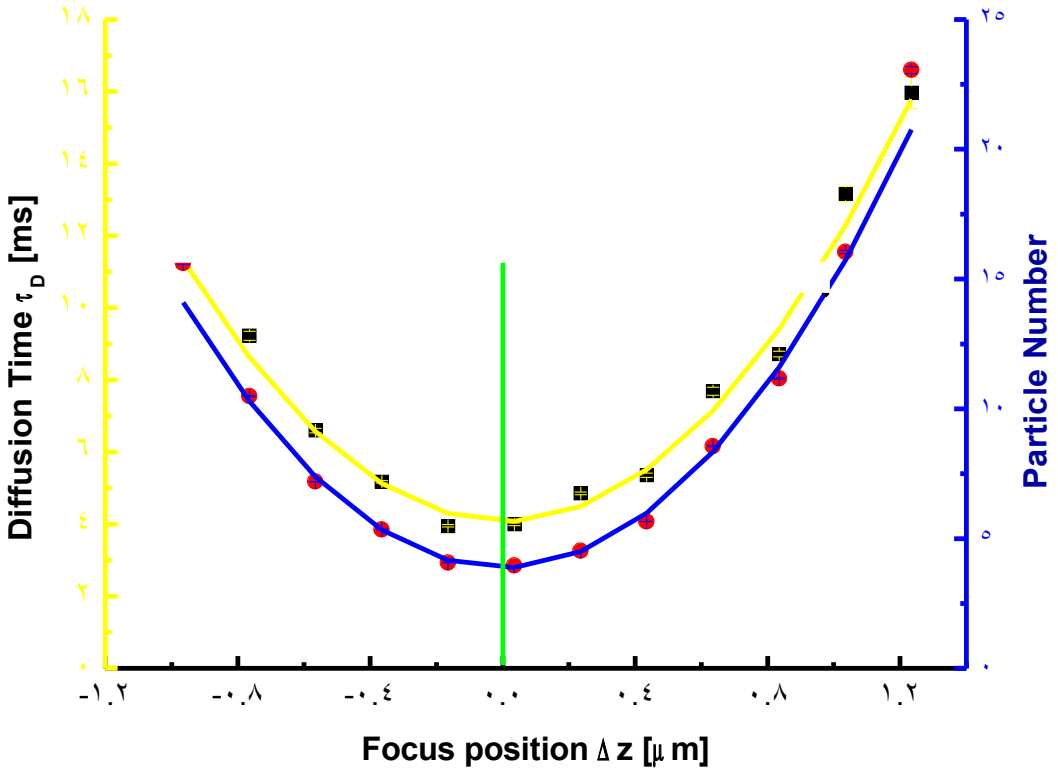
Diffusion time

$$\tau_D = \frac{w_0^2}{4D}\left(1 + \frac{\lambda^2 \Delta z^2}{\pi^2 n^2 w_0^4}\right)$$

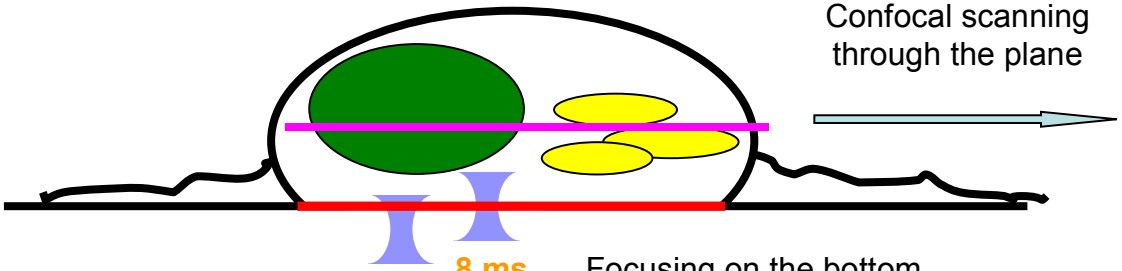
Particle number

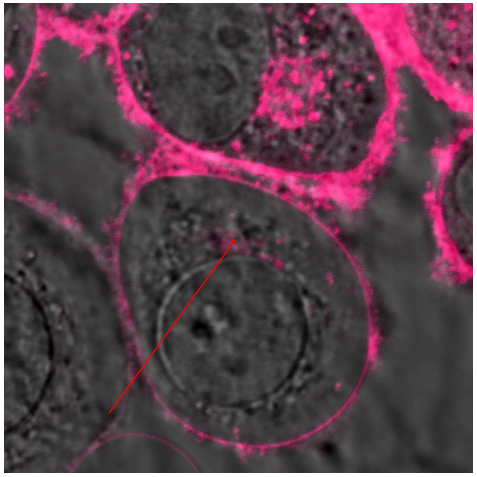
$$PN = \pi c w_0^2 \left(1 + \frac{\lambda^2 \Delta z^2}{\pi^2 n^2 w_0^4}\right)$$

⇒D and C without external calibration



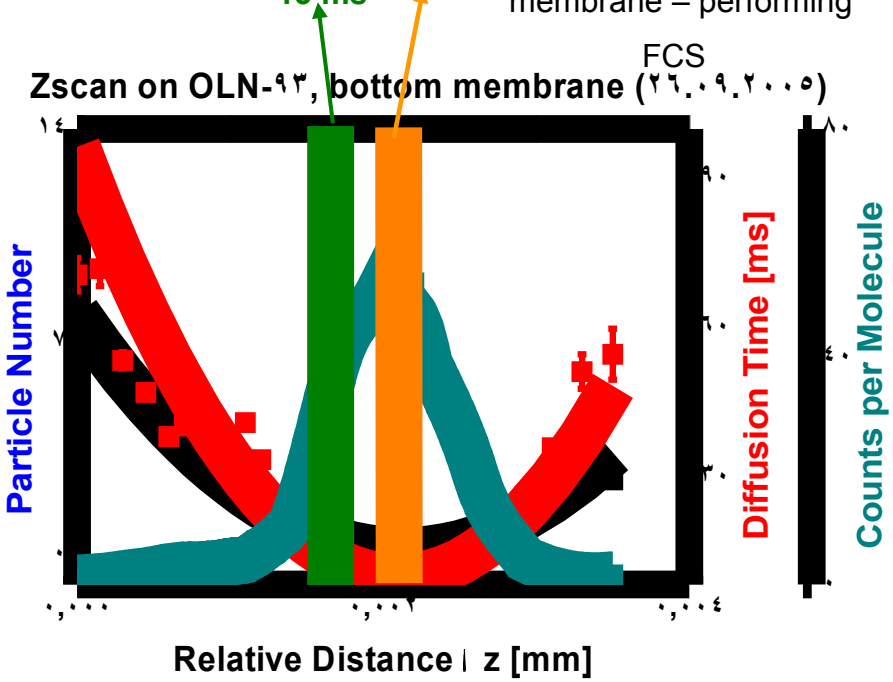
Application of Z-scan in living cells





OLN-93 labeled with dye DiD (lipid-like molecule present in liquid disordered phase) – cross-section

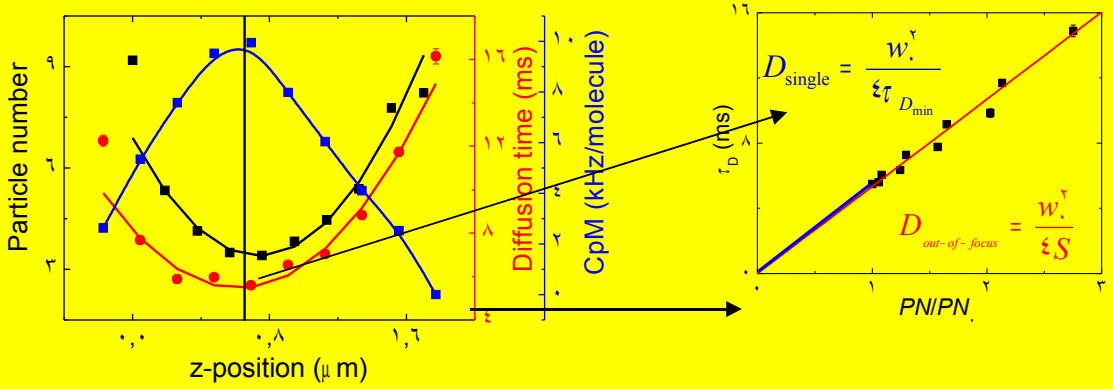
Focusing on the bottom membrane – performing FCS



Advantages of Z-scan

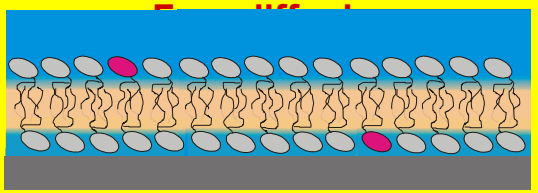
- **D** in cell membranes with z-scan **10-times lower error than by standard way!**
- unspecific signals (e.g. by immobile dye molecules) are easy to identify and to separate from the lipid diffusion

Single waist versus “out-of-focus” FCS

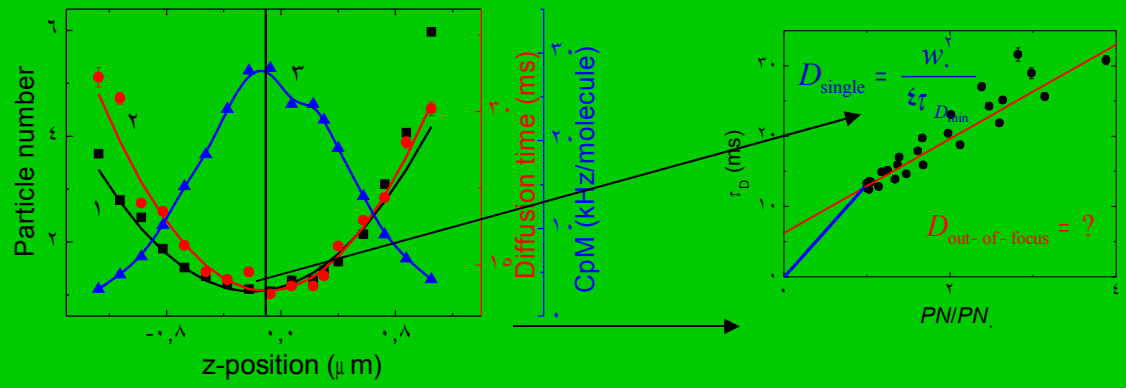


For diffusion of DiD in SPBs on Mica:

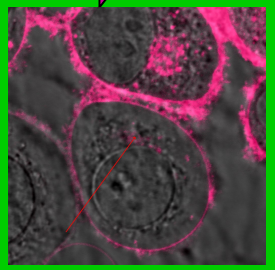
$D_{single} = D_{out\text{-}of\text{-}focus}$



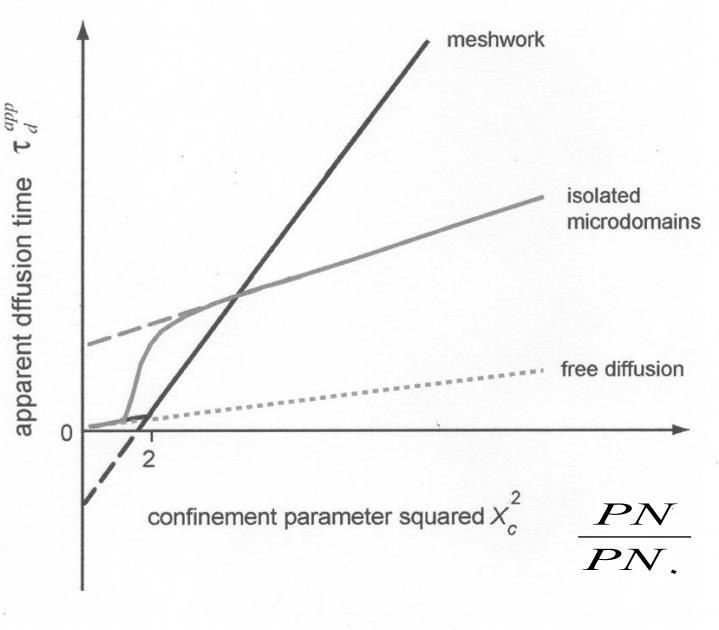
$$\frac{PN}{PN_0} \propto w^2$$

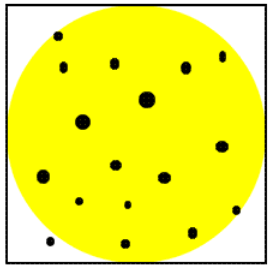


Diffusion of DiD in the bottom cellular membrane is **no more free**

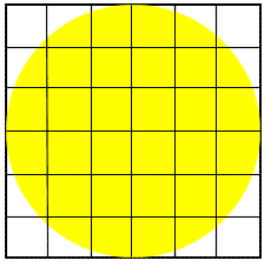


What`s going on in the cellular membranes: “FCS diffusion laws”





Domains



Meshwork

FCS diffusion law in general:

$$\tau_D^{app} = t_. + \frac{1}{4 D_{eff}} \omega^2$$

$t_0 = 0$ free diffusion

$t_0 > 0$ presence of domains – rafts

$t_0 < 0$ interaction with meshwork

- increasing size of the illuminated spot reveals restrictions on the diffusion behavior → need of a beam expander
- performing z-scan enables to increase the illuminated area
- number of particles in the spot is directly proportional to the spot`s size

Wawrezinieck, L., H. Rigneault, D. Marguet and P. F. Lenne. 2005. Fluorescence correlation spectroscopy diffusion laws to probe the submicron cell membrane organization. *Biophys. J.* 89: 4029-4042.

Achievements of Z-scan

- Z-scan allows the accurate determination of D in model membranes

- Recent studies on living cells show that z-scan is a must when determining D in membranes

Selected Literature:

Benda A, Beneš M, Mareček V, Lhotský A, Hermens W, Hof M:

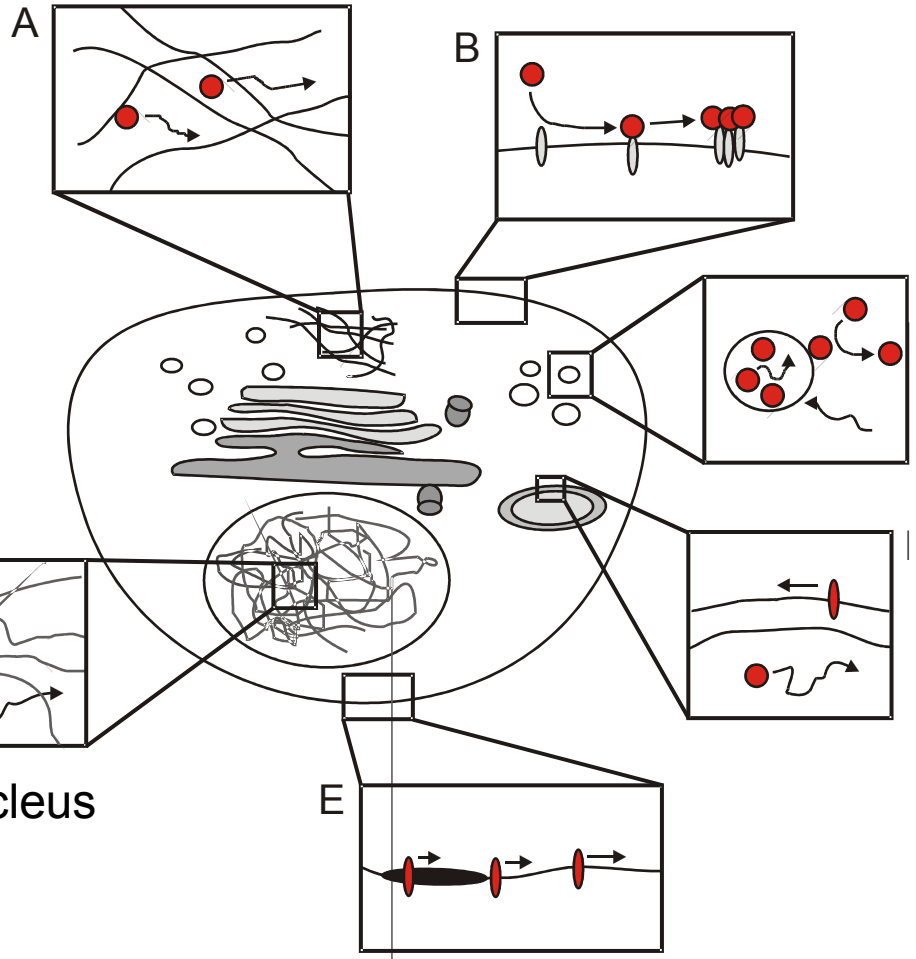
How to Determine Diffusion Coefficients in Planar Phospholipid Systems by Confocal Fluorescence Correlation Spectroscopy.

Langmuir 19, 4120-4126 (2003).

Potential accesibility of cellular processes to FCS

B: binding of ligands to cell surface receptors

A: diffusion of molecules inside cytoplasma



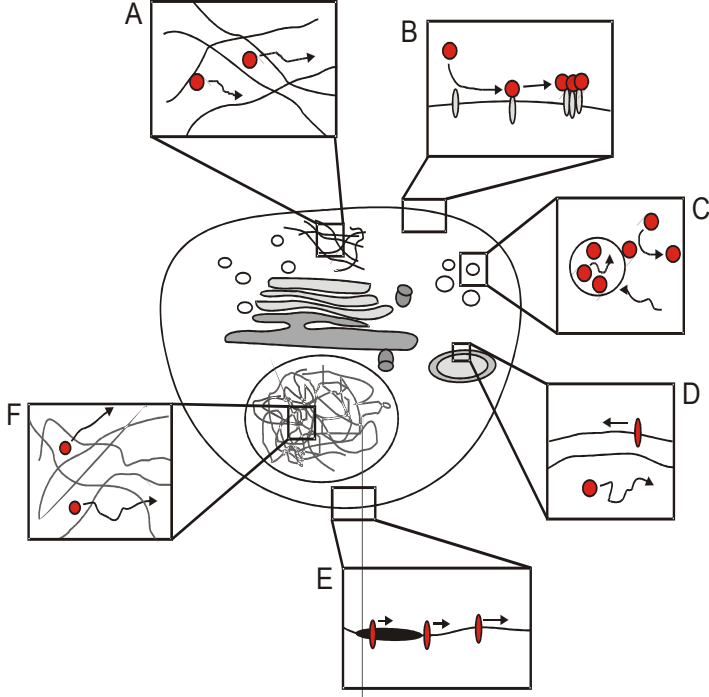
C: Vesicle transport and association of molecules with vesicles

D: Molecular mobility in compartments, like e.g. mitochondria

F: diffusion inside of nucleus

E: 2-D diffusion in cell membranes gives information on membrane organization (microdomain formation)

Problems of cellular FCS



- Self- or Unspecific aggregation of labeled species leading to large highly fluorescent aggregates ➔distortion of FCS curves
- Association of labeled species with immobile structures ➔photobleaching
- Measurement time (several seconds up to minutes) too long for cellular process of interest
- Photobleaching

➔FCS alone is strongly limited in cellular application

➔Solution: Combination of detection modalities: intensity and lifetime imaging, time-resolved fluorescence, FRET (energy transfer), photobleaching recovery