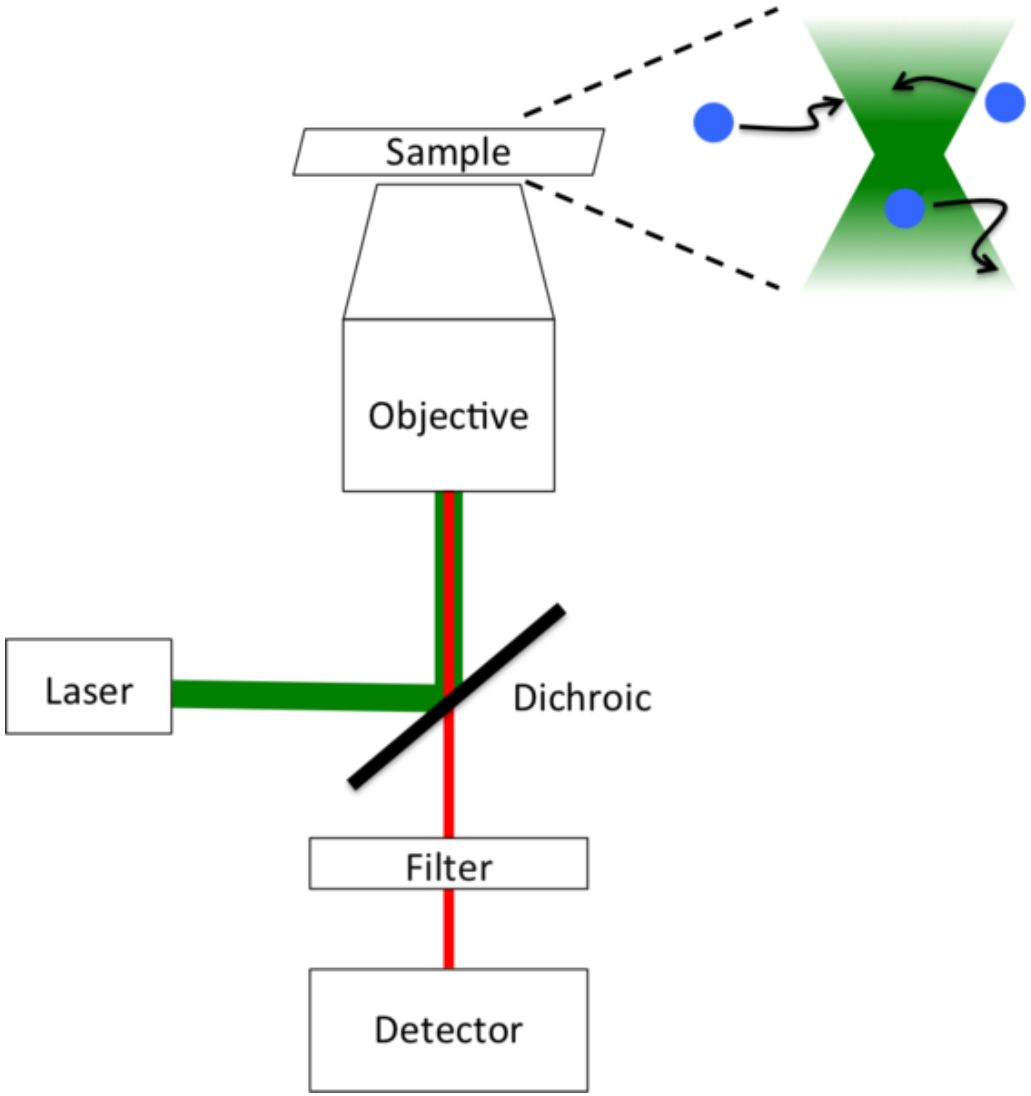
Fluorescence Correlations Spectroscopy

By Andrew Gioe and Kyle Chau

General Principles of FCS

- FCS can be considered a branch of Dynamic Light scattering under non-Guassian Conditions.
- The fluorescent signal may be generated by laser excitation of natively fluorescent molecules or fluorescently tagged molecules.
- It involves the analysis of the intensity of the fluorescent signal originating from a very small volume.

FCS Block Diagram



- Dichroic Mirror: A filter lens that passes light of a single color and reflects light of another color

Effects of Brownian Motion

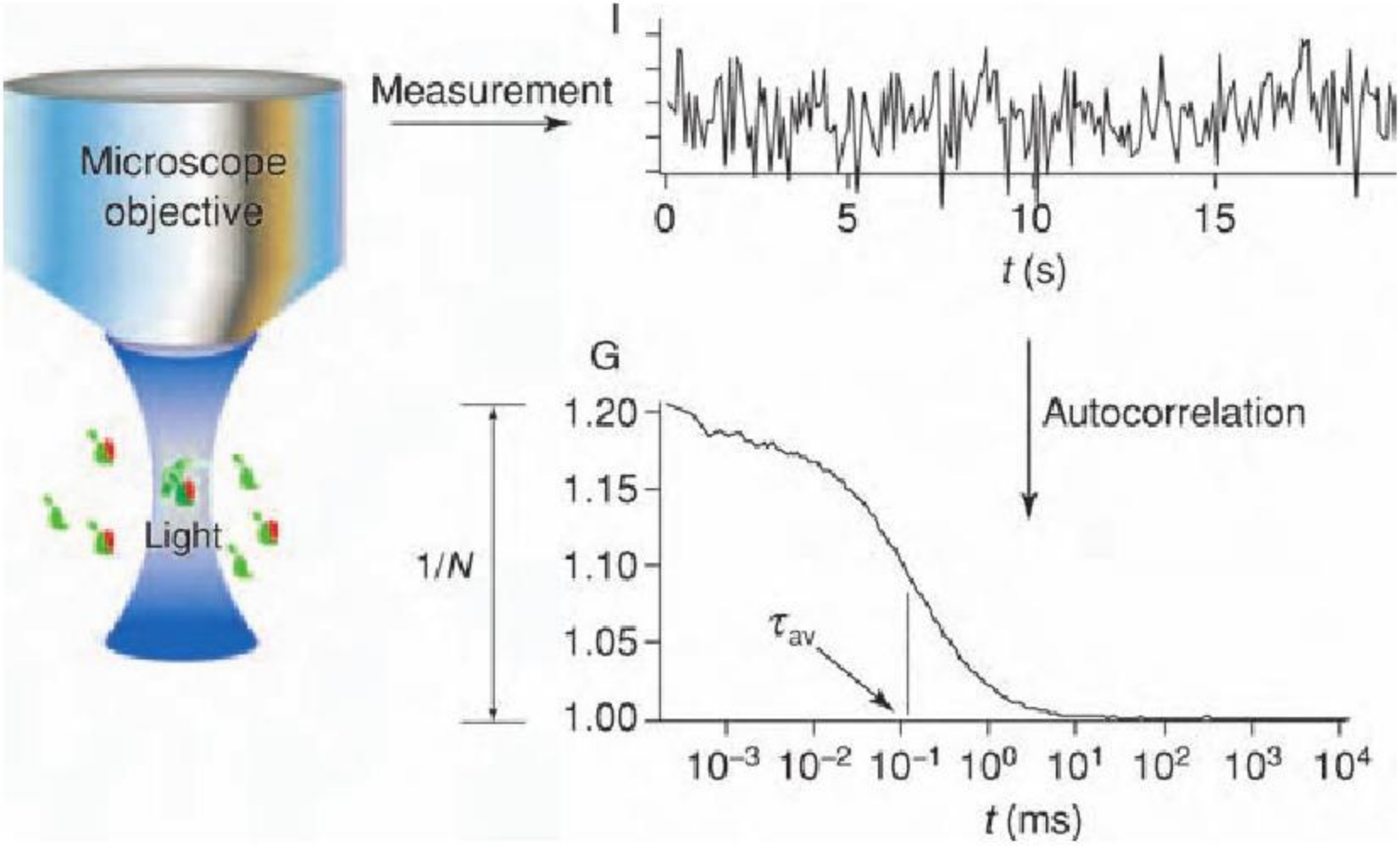
- Diffusion of Fluorescent molecules into and out of the volume illuminated by the laser results in the stochastic fluctuations in the intensity of the florescent signal.
- The relative amplitude of the fluorescent signal intensity is inversely proportional to the square of the number of fluorescent molecules contained in the volume.

Molecular Weight by FCS

$$\left\langle \left(\frac{\Delta N}{\langle N \rangle} \right)^2 \right\rangle = \left\langle \left(\frac{\Delta C}{\langle C \rangle} \right)^2 \right\rangle = \frac{1}{N} \qquad \text{(A)}$$

where C is the (wt/vol) concentration of the molecules. The fewer the number of molecules, the larger are the fractional fluctuations. Therefore, for given concentration $\langle C \rangle$, the size of the fluctuations increases with molecular weight. By measuring these fluctuations (via any parameter that is sensitive to concentration), one can determine from Eq. (A) the number of molecules N in a given volume V within the measured fluctuations. Then knowing the average concentration $\langle C \rangle$, the molecular weight is determined from

$$M = \langle C \rangle \left(\frac{\Delta C}{\langle C \rangle} \right)^2 V N_A \qquad \text{(B)}$$


Microscope objective
Light
Measurement
I
0
5
10
15
t (s)
G
1.20
1.15
1.10
1.05
1.00
1/N
Autocorrelation
τav
10−3 10−2 10−1 100 101 102 103 104
t (ms)

Autocorrelation function definitions

Comment D11.2 Autocorrelation functions in FCS

In most applications of FCS one of the following definitions of the autocorrelation function is used:

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

or

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

where $\langle\rangle$ denotes the time average

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

denotes the fluctuation around the mean intensity and for a long time average of F (no bleaching),

$$G^{\delta F} = G^{F} - 1$$

Diffusion Time

- The relation between the auto correlation function G(τ) and the diffusion time τ_{av} is:

$$G(\tau) = \frac{1}{N}\left(\frac{1}{1+\frac{\tau}{\tau_{av}}}\right)\left(\frac{1}{1+\left(\frac{r}{l}\right)^2\frac{\tau}{\tau_{av}}}\right)^{1/2} \qquad \text{(D11.1)}$$

- The diffusion time τ_{av} characterizes the average time it takes for a molecule to diffuse though the radial part of the observation volume of the microscope.

For Variable Volumes

- Diffusion time is not constant when changes are made to the observation volume illuminated by the laser. The relationship between diffusion time and diffusion coefficient is

$$\tau_{av} = \frac{r^2}{4D} \qquad \text{(D11.2)}$$

Accounting for Diffusion in 3 Dimensions

$$G(\tau) = \frac{1}{N}\left(\frac{1}{1+\tau/\tau_{\text{av}}}\right)^{1/2} \qquad \text{(D11.3)}$$

In Vivo FCS

- FCS may be carried out in vitro as well as in vivo.
- In a complex biological environment no conventional diffusion constant can be specified because there are many different modes of mobility.
- That is, molecules may diffuse through multiple dimensions and mediums or be aided by cellular events which speed their movement.

The figure shows that the autocorrelation curve for active transport displays the sharpest decay, whereas the one for anomalous diffusion decreases rather slowly. If one treats the autocorrelation curve as a kind of distribution function of residence times within the focal volume, this behaviour becomes clearer. Particles

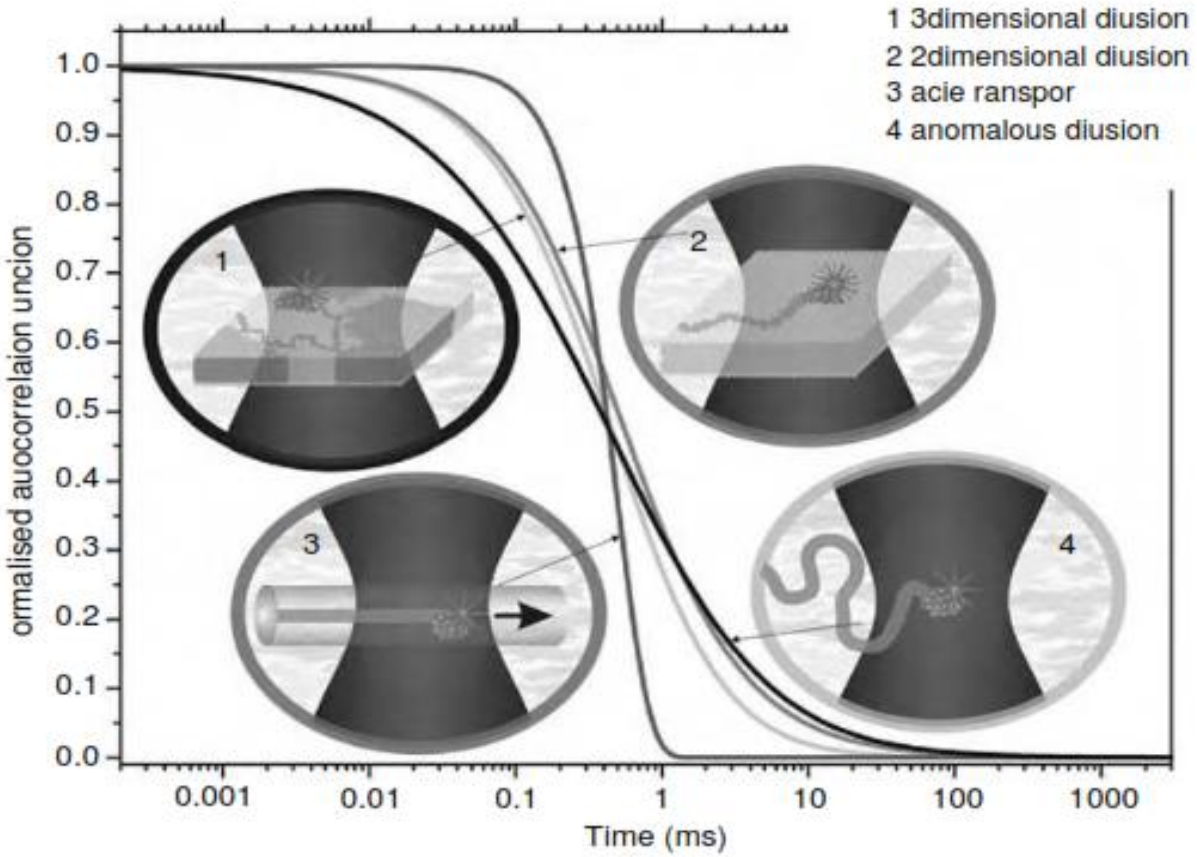
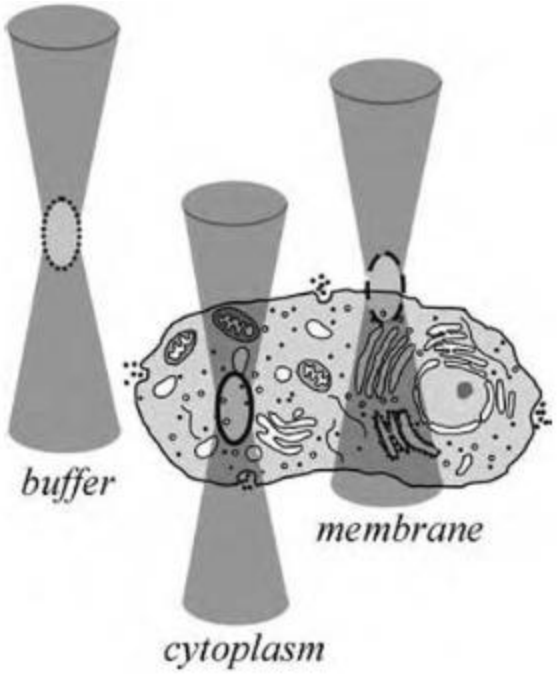


Fig. D11.2 Model autocorrelation curves for different particle mobilities: (1) three- and (2) two-dimensional diffusion, (3) active transport and (4) anomalous diffusion (see text). (Adapted from Hausten and Schwille, 2003.)

Anomalous diffusion: diffusion with a non-linear relationship to time

Tackling Multi-Component Systems

- However, if the environment is broken up into components and multiple kinds of fluorescent probes are used, diffusion coefficients for each different environment may be obtained.
- In a multicomponent system, the correlation curve is evaluated by standard Marquardt non-linear least-squares fitting routine.



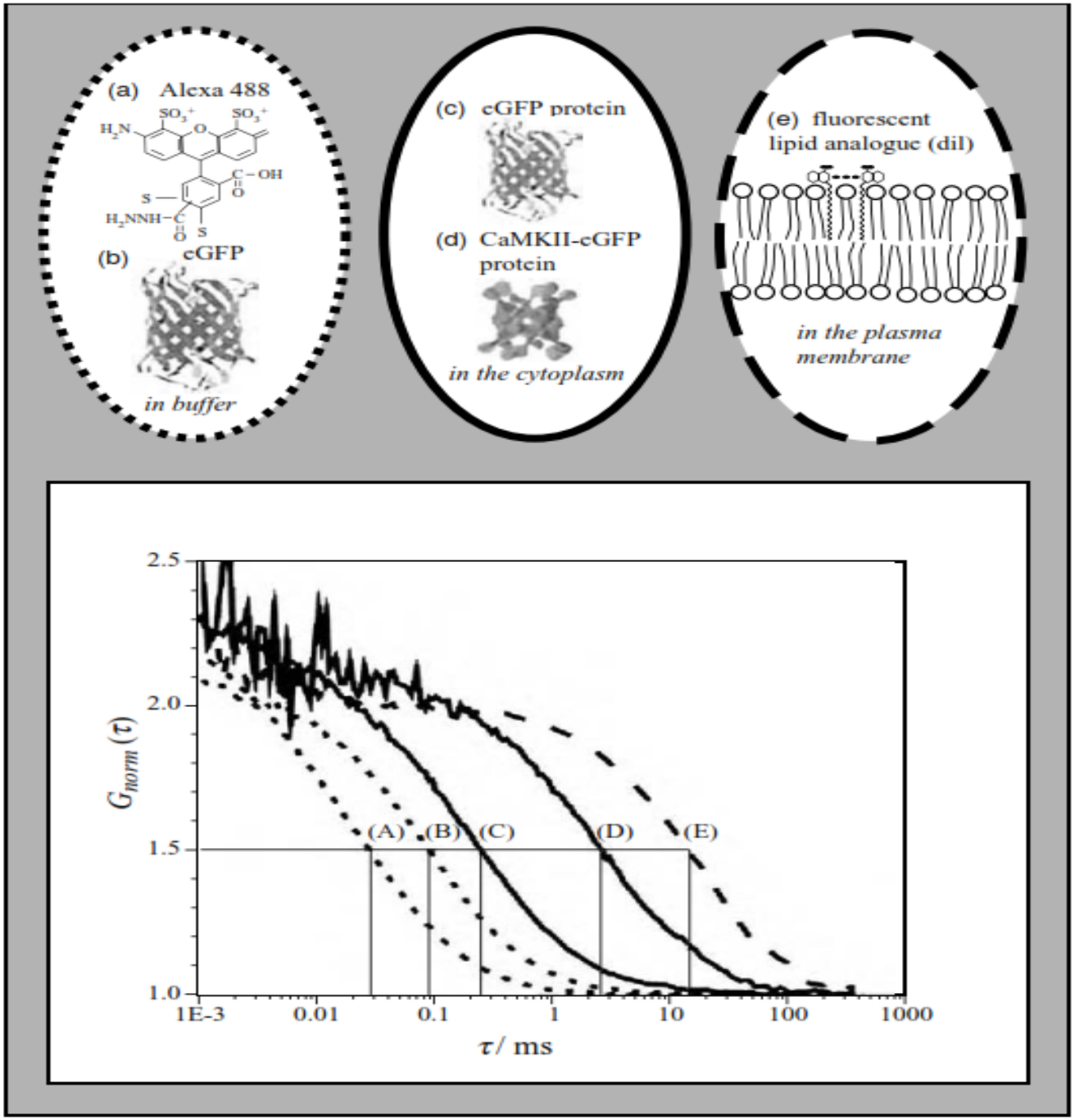
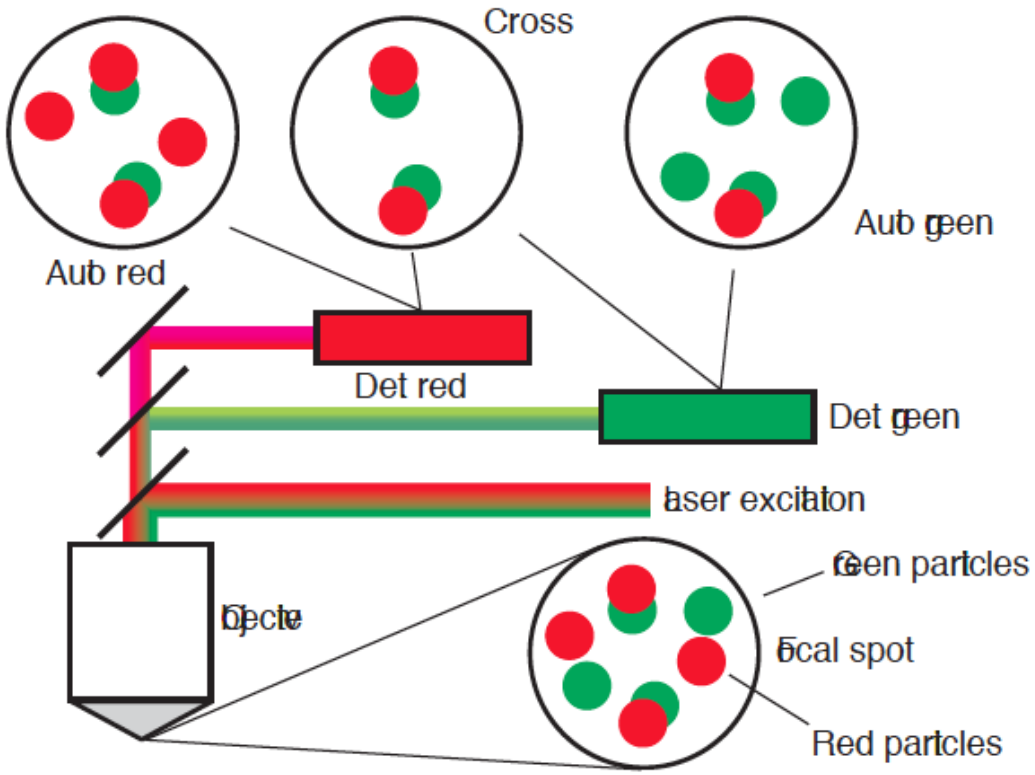


Fig. D11.4 Depending on the probe and the environment, diffusion coefficients of different orders of magnitude are obtained: (a) small dye molecule (Alexa 488, M ~ 0.6 kDa) in water, $D = 3 \times 10^{-6}$ cm^2 s^{-1}; (b) green fluorescent protein (eGFP) in aqueous solution (M ~ 27 kDa); (c) the same protein in the cytoplasm of a HEL cell, $D = 3 \times 10^{-7}$ cm^2 s^{-1}; (d) large protein complex (multimeric complex of calmodulin-dependent kinase II-eGFP fusion protein) in the cytoplasm of a HEK cell, $D = 3 \times 10^{-8}$ cm^{2}s^{-1}; (e) two-dimensional diffusion of a fluorescent lipid analogue (long-chain carbocyanine dye 'dif C_{18}') in the plasma membrane of a HEK cell, $D = 6 \times 10^{-9}$ cm s^{-1}. (Adapted from Bacia and Schwille, 2003.)

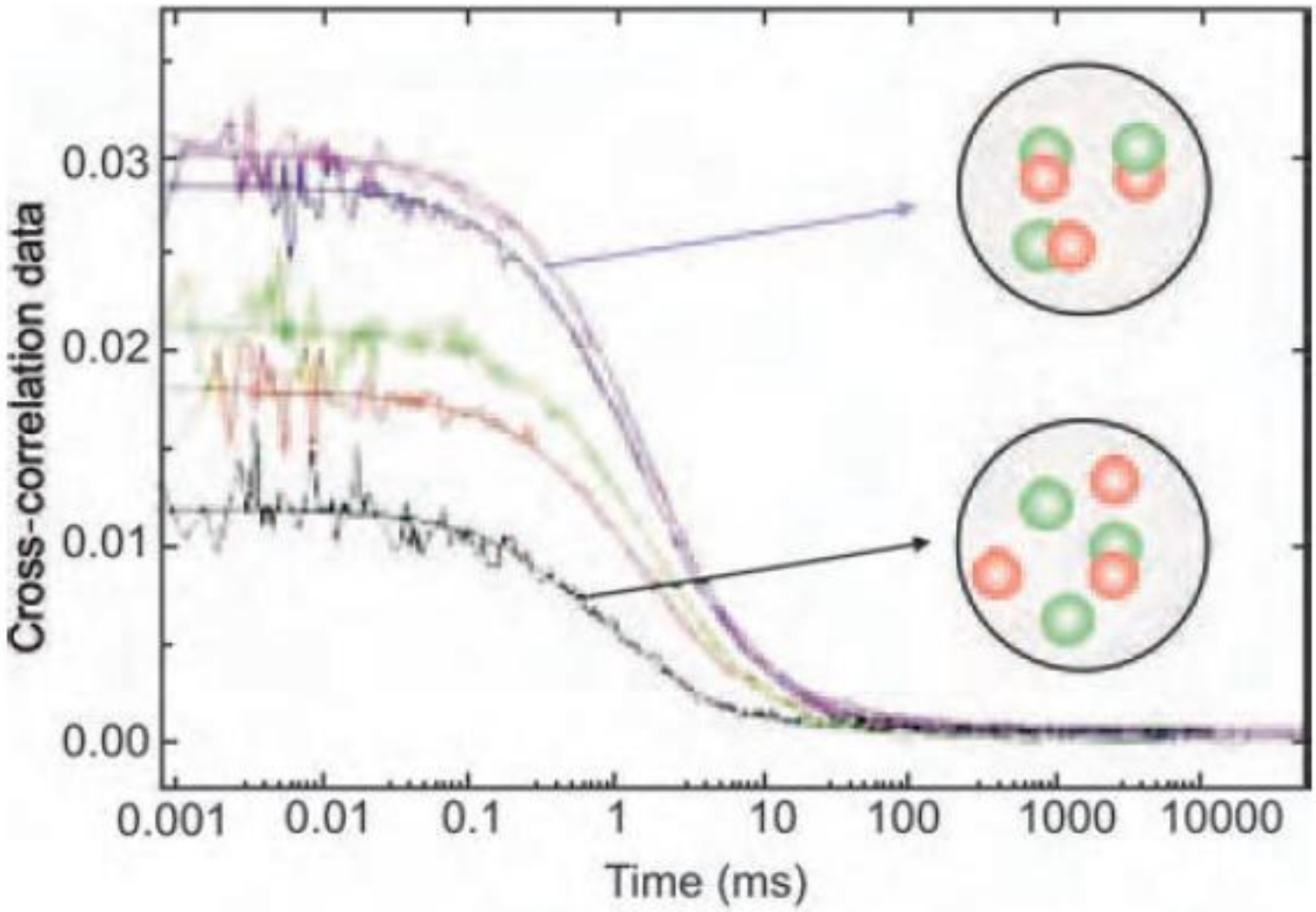
Dual-Color Fluorescence Cross-Correlation Spectroscopy (FCCS)

- FCCS is an extension of FCS
- Correlation of intensity from one channel, with that from the other channel, shifted by a time interval
- Applications
  - Molecular Interaction
  - Characterize enzymatic cleavage
  - Analysis of complex systems
  - Analysis of intracellular transport

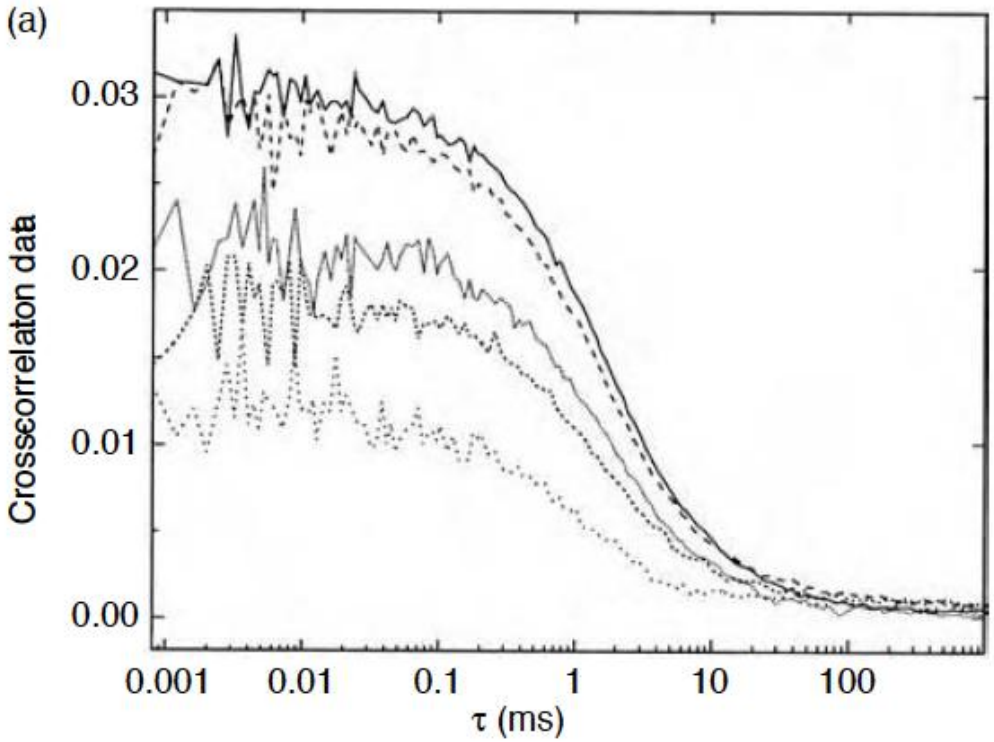
FCCS Schematic



- Light from 2 lasers into a single-mode fiber.
- Illuminates the back of the objective and is focused in the sample.
- Fluorophores in the sample are excited
- The fluorescence emission is collected back through the objective and separated from the excitation by the first mirror.


Cross-correlation data
0.03
0.02
0.01
0.00
0.001
0.01
0.1
1
10
100
1000
10000
Time (ms)

Correlation



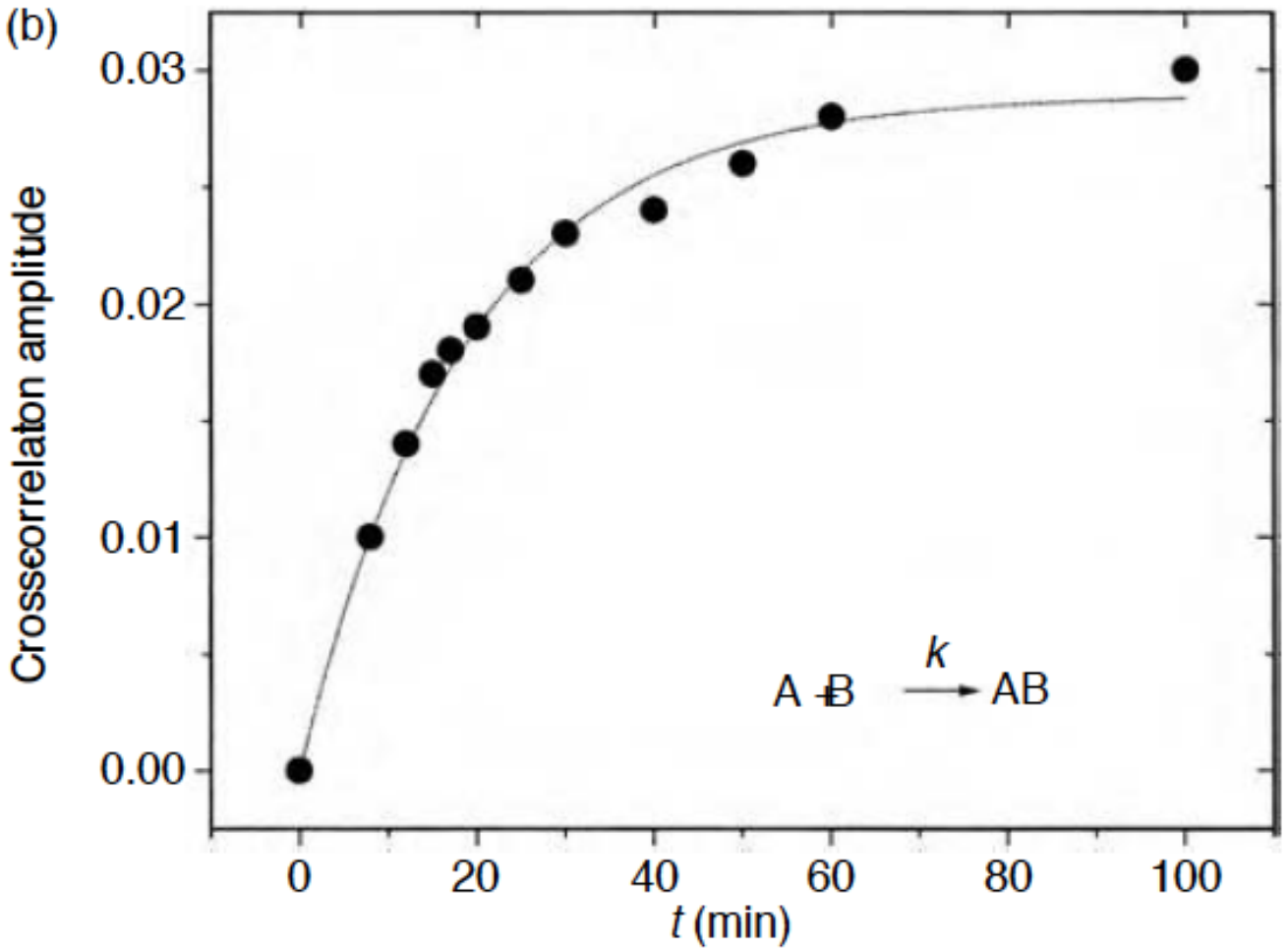
$$g_\times(\tau) = \frac{\langle (F_1(t) - \langle F_1 \rangle)(F_2(t+\tau) - \langle F_2 \rangle) \rangle}{\langle F_1 \rangle \langle F_2 \rangle}$$

$$g_1(0) = \frac{1}{n_1 + n_{12}}, \quad g_2(0) = \frac{1}{n_2 + n_{12}}, \quad g_\times(0) = \frac{n_{12}}{(n_1 + n_{12})(n_2 + n_{12})}$$

$\frac{g_\times(0)}{g_2(0)} = \frac{n_{12}}{n_1 + n_{12}}$ → fraction of green in complex with red

$\frac{g_\times(0)}{g_1(0)} = \frac{n_{12}}{n_2 + n_{12}}$ → fraction of red in complex in complex with green

Hybridisation Kinetic



Advantages and Disadvantages of FCCS

Advantages:

- Measures single and multiple component diffusion
- Measures physiological concentrations
- Measures also fast dynamics

Disadvantages:

- Measurements depend on concentration fluctuations
- Does not measure immobile fraction
- Both binding partners need to be labeled completely
- Quantitative measurements sometimes problematic