Applications Manual
LSM 510 – ConfoCor 2
Fluorescence Correlation Spectroscopy

Knowledge of this manual is required for the operation of the instrument. Would you therefore please make yourself familiar with the contents of this manual and pay special attention to hints concerning the safe operation of the instrument.

The specifications are subject to change; the manual is not covered by an update service.

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LSM 510 - ConfoCor 2 Applications Manual

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1 INTRODUCTION

1.1 The Concept: Measuring Fluorescence Fluctuations

Applications of fluorescence

Fluorescence is a highly specific property of certain dye molecules. Its principles are used in many aspects of daily life, popular examples of which are neon tubes as well as brilliant fluorescent dyes for traffic signs and signaling. There are also many applications in the life sciences that exploit the properties of fluorescent dyes. They are commonly used as probes for visualization in microscopy. Furthermore, dye lasers exploit fluorescent dyes as the active laser medium.

All applications are based on the property of the dyes to absorb and eventually emit light. The fluorescent dye always causes a shift in light wavelength between the absorbed and the emitted light. Fluorescent tubes convert the ultraviolet light emitted by the gas contained in the tubes into visible light. Similarly, the brilliant „neon“ colors of traffic signs or clothes are generated by the conversion of the UV part of the spectrum of sunlight into visible light. In fluorescence microscopy, the observable spectral shift enables the experimenter to block the omnipresent excitation light by spectral filtering and to detect only the fluorescent light emitted by an object, which might be a ligand labeled with a dye, attached to specific locations of cells or tissues, the so-called binding regions. An image looks as if the cell or the tissue were luminescent on their own.

The fluorescence process

Fluorescence is the result of a three-stage process that is found with certain molecules called fluorochromes, fluorophores or fluorescent dyes. It is best described by a model of electronic energy levels of a molecule as illustrated by the so-called Jablonski diagram, an electronic state diagram (see Fig. 1-1A). In the first stage (excitation), a photon supplied by an external source such as a laser is absorbed by the molecule in the ground state $\mathbf{S_0}$ generating an excited electronic singlet state $\mathbf{S_1}'$ of the molecule with a higher energy level. In the second stage (excited-state lifetime) this level is quickly converted to the so-called relaxed singlet excited state $\mathbf{S_1}$. Depending on the molecule studied and its chemical environment, the excited state is usually populated for an averaged time of 0.1 to 10 ns (the so-called fluorescence life time τ_T). After this delay, in the third step (fluorescent emission), the molecule emits a photon spontaneously from the relaxed singlet excited state $\mathbf{S_1}$ and returns in the ground state.

Due to energy losses (for example, transfer of thermal energy to the surrounding solution during transfer from $\mathbf{S_1}'$ to $\mathbf{S_1}$), the fluorescence photon emitted always carries less energy than the absorbed photon. The corresponding red shift in photon wavelength, the so-called Stokes shift, can be used to separate the emission from the excitation light. For more detailed information, refer to chapter 5.1 and the literature list at the end of this handbook.

Fluorescence spectra

Unless the fluorochrome is irreversibly destroyed in the excitation state, a process called photobleaching, it can be repeatedly excited and will repeatedly emit photons. Thus the process is cyclical. Polyatomic molecules in solution do not show discrete electronic transitions as depicted in Fig. 1-1. Rather they show broad energy spectra, which are referred to as the fluorescence excitation spectrum and the fluorescence emission spectrum, respectively (see Fig. 1-1B). In most of the cases the fluorescent excitation spectrum is identical to the absorption spectrum, and the fluorescent emission spectrum is independent of the excitation wavelength. The emission intensity will be proportional to the amplitude of the fluorescent excitation spectrum at the excitation wavelength.

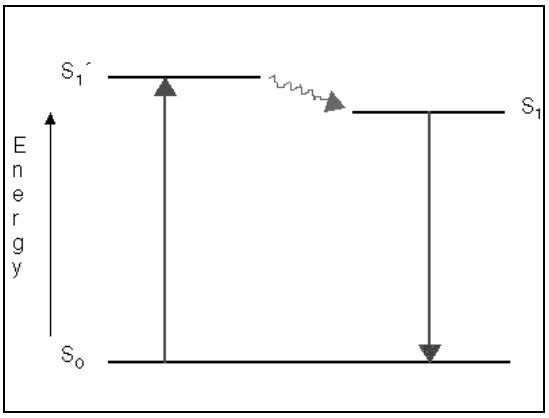


Fig. 1-1A Simple Jablonski diagram

The Jablonski diagram illustrates the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence.

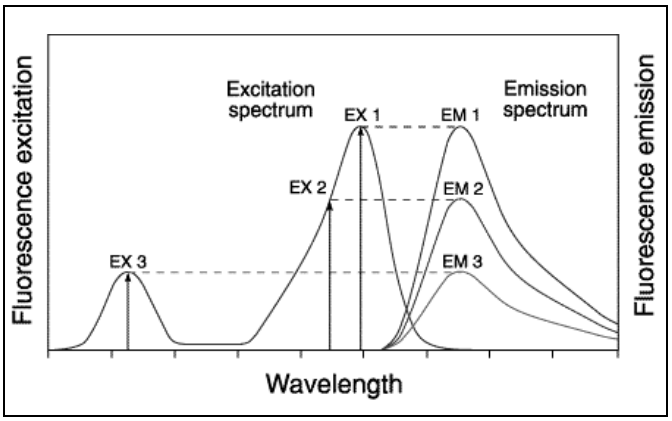


Fig. 1-1B Fluorescence spectra

Excitation of a fluorochrome at three different wavelengths (**EX 1, EX 2, EX 3**) does not change the emission profile but produces variations in fluorescence emission intensity (**EM 1, EM 2, EM 3**) that correspond to the amplitude of the excitation spectrum.

Conventional fluorometry

In conventional fluorometry, a bulk sample in a transparent sample cell is traversed by a ray of excitation light in one direction, and the fluorescence intensity is recorded in a direction orthogonal to the incident beam. In this set-up, a spectrofluorometer, the average properties of the sample are measured. However, using this type of equipment it is difficult to measure fluorescent molecules in dilutions of less than 1 nM. The total signal obtained will be dominated by background signals, such as Raman scattering of the solvent, scattered excitation light which cannot be totally blocked due to the imperfection of the optical filters, and detector noise.

Fluorescence fluctuation analysis

A very elegant way to improve sensitivity is to record fluctuations of the fluorescence intensity instead of just averaging the signal. Fluctuations of a signal are usually associated with noise that is expected to bear no information at all. Under specific circumstances, however, the fluctuations carry a wealth of information, even more than the average intensity. This information can be exploited, the information content being extractable by means of sophisticated signal processing techniques. Such fluctuation analysis was introduced by D. Magde, E. Elson and W. Webb in 1972 [1]. They called it **F**luorescence **C**orrelation **S**pectroscopy (FCS).

The enthusiasm for FCS was somewhat dampened in those early days when it was realized that the full potential of FCS could not be obtained without extremely stable light sources, powerful computers for signal processing, and better optical equipment than was available at that time.

Optimization of the method

In 1992, R. Rigler and co-workers introduced the major prerequisite for the perfect analysis of fluorescence fluctuations - the ultra small detection volume of 1 femtoliter. Together with stable laser light sources, sufficient computer power, and precision optics, a real renaissance was opened up for FCS, demonstrated by a series of papers describing applications of this method [6-10]. Since then, this method has been used successfully for investigating physical properties of certain dyes and for studying interaction kinetics [1-5].

"Light cavity"

Why is the small measuring volume so important? Within 1 ml of a nanomolar solution, about $6x10^{11}$ molecules are present. By contrast, one femtoliter, corresponding to 1 cubic micron (μm^3), contains only 0.6 molecules on average. The diffusion of molecules in solution is subjected to Brownian motion. As a consequence, at any given point of time, the measurement volume may be "empty" or occupied by molecules. Thus, in turn, large fluctuations in fluorescence intensity occur, which are associated with the diffusion process of the molecules, whereas the background signal is relatively constant over time. Thus, fluctuations can be interpreted as diffusion statistics of the fluorescent molecules. It is obvious that a measurement process samples single molecular diffusion events; however, a complete measurement relies on statistics from a multitude of such events.

Autocorrelation

The resulting fluctuations are measured, and the autocorrelation of the signal is calculated in real time. For more details on the mathematical basis of fluctuation analysis, see chapter 5.3 of this handbook.

Confocal optics

As pointed out above, a small measurement volume of about 1 femtoliter is required for precise FCS measurements. Confocal microscope optics can be used to implement such small measurement volumes. Photon counting detectors with a high quantum efficiency in the visible part of the spectrum are another indispensable part of FCS instrumentation.

Avalanche photodiode

Single photon counting avalanche photodiodes (SPAD or APD) are devices ideally suited to this purpose. A modern FCS instrument such as the ConfoCor 2 uses these technologies to register up to 1 million fluorescence photon counts per second from a single dye molecule.

It should be noted, however, that a dye molecule stays in the confocal volume within a microsecond time range only. This is another advantage of the small measurement volume used in FCS. Otherwise, the large number of excitation cycles would result in a rapid photodestruction of the dye molecule and deteriorate the signal considerably.

1.2 Applications: Characterizing Molecular Interactions

Labeling molecules

As described above, fluorescent ligands can be used to visualize other molecules, e.g. within the fluorescence microscope. Therefore, in recent years, fluorescence staining has become a general method for specifically labeling and detecting samples in the life sciences.

Fluorescent dyes

Fluorescent dyes can be attached covalently to, or can intercalate into, several biopolymers and small organic molecules. The covalent linking of dyes to nucleotides, proteins, peptides, enzymes and antibodies enables site-specific labeling.
A large variety of standard labeling procedures has been developed during the past decades. Chapter 3 is dedicated to this important topic, and many protocols can be found in the literature [17-20].

Molecular interactions

Based on the detection of fluorescence fluctuations of individual diffusing molecules, FCS is the method of choice to study molecular interactions. It can be used to characterize the interaction between

- fluorescence-tagged ligands and target molecules such as receptors,
- unidentified, untagged compounds and tagged ligands in competitive binding, and
- protein-protein interactions

FCS may also be used for the study of individual events of signal transduction cascades within cells.

Physical state

Differences in the physical state of the molecule of interest, such as bound vs. free, cleaved vs. intact, can be discerned by FCS provided that the variant forms differ sufficiently in their size-related diffusion properties. For example, FCS can be used to detect

- peptides bound to soluble receptors,
- ligands bound to membrane-anchored receptors,
- viruses bound to cells,
- antibodies bound to prokaryotic or eukaryotic cells,
- primers bound to target nucleic acids,
- regulatory proteins /protein-complexes in interaction with target DNA, or
- enzymatic products.

If the diffusion properties of the reactants are too similar, both reactants have to be labeled with fluorescent dyes with different excitation and emission spectra.

Concentration range

FCS allows molecular interactions to be characterized in homogeneous assays over a wide dynamic range - concentrations of labeled particles between 200 nM to 200 pM can be used. Because of the ultra-low measuring volume of 10^{-15} l required for FCS measurement, nanoliter to microliter sample volumes are sufficient.

1.3 Realization: Instrument Design

Optical design

The optical design of the ConfoCor 2 includes several key elements apart from the microscope core unit (Fig. 1-2). A laser light source, a dichroic mirror to split excitation and fluorescence light, and a photon detection unit are of crucial importance. All this instrumentation serves to limit the measurement volume to a confocal spot about 0.25 fl in size. In the following, we will describe the instrument going by the light path, and thus start with the laser source.

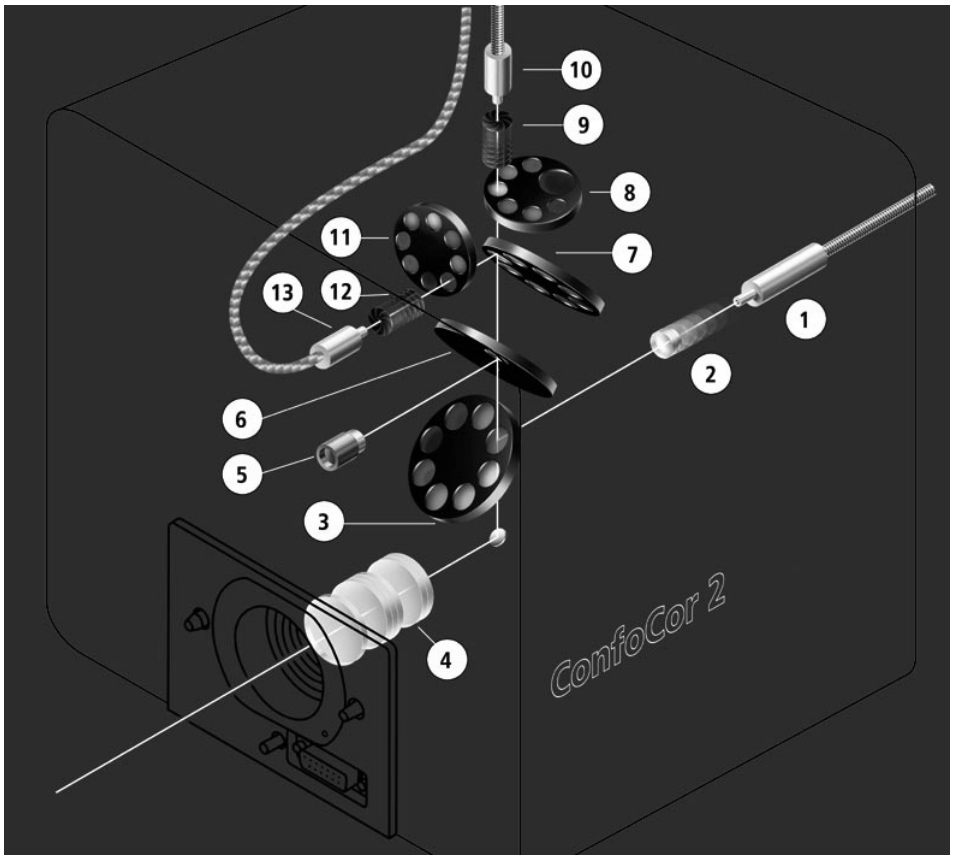


Fig. 1-2 ConfoCor 2 detection head

Up to five laser lines are simultaneously coupled into the detection head via glass fibers (1). Up to two independent detection channels, equipped with fiber-coupled avalanche photodetectors (APD) (10, 13), enable simultaneous measurements of two autocorrelations plus one cross correlation. Each detection channel is equipped with a motorized pinhole.

Laser coupling

Lasers mounted on a laser module are used as light sources. The laser line is selected by an acousto-optical tunable filter (AOTF). Furthermore, the light intensity is modulated by the AOTF for each wavelength independently. The light is delivered to the detection head via optical fibers. The variety of laser lines available (Table 1-1), together with the filter combinations offered, enables a broad spectrum of fluorescent dyes to be used.

Type	Wavelength	Output	Max. power within sample (adjustable by AOTF)
Ar ion	458 nm	> 3 mW	> 0.4 mW
	477 nm	> 10 mW	> 3 mW
	488 nm	> 10 mW	> 1.8 mW
	514 nm	> 15 mW	> 3 mW
HeNe green	543 nm	> 1 mW	> 0.23 mW
HeNe red	633 nm	> 5 mW	> 1.3 mW

Table 1-1 Lasers available for LSM 510 - ConfoCor 2

Focal spot

The laser light is focussed by the objective into a very small, diffraction-limited spot. The focal spot has a diameter of about 0.3 µm. Fluorescent molecules present in this spot are excited by the laser light and emit fluorescent light which is captured partly by the microscope objective. For best results, we recommend a 40x, N.A. 1.2 C-Apochromat, water immersion objective with a working distance of 0.23 mm in combination with a sample carrier that has a glass cover slip thickness of 0.14 – 0.18 mm.

Filters

Behind the objective, a main dichroic mirror (high transmission for emission light, high reflection for excitation light) in combination with a fluorescence-spectra-adapted bandpass or long-pass filter separates laser light (excitation) and fluorescence light (emission).

Detection

The emission light passes through a motor-controlled, variable pinhole, which blocks emission photons produced outside the laser spot, and finally hits a single-photon-counting avalanche photodiode (APD), the hits being analyzed by the Confocor 2 software.

One- and two-channel system

The light path described above applies to the so-called one-channel system. In the two-channel system, a second pathway for emission light is available, allowing the simultaneous registration of two different fluorescent species. For this purpose, additional filters, a second APD and a second pinhole are used.

Correlator

The diffusion of fluorescent particles through the confocal volume causes fluctuations in fluorescence intensity. A software correlator calculates the autocorrelation and, in the two-channel system, the cross-correlation function of the fluorescence signal in real time. The correlation curves depict the intensity fluctuations as a function of particle number and diffusion time.

Assay parameters

The amplitude of the correlation curves corresponds to the sample concentration, while the decay time indicates the mean dwell time of the molecules within the confocal volume element and thus their physical state (e.g. bound or free).

Interpretation

The Confocor 2 software package interprets the autocorrelation function in terms of particle number, diffusion time, concentration ratios (e.g. of bound vs. free molecules) and triplet state. The cross-correlation function yields information about colocalization, i.e. binding of differently fluorescing particles. From these fit results, binding constants as well as rate constants of the system under investigation are accessible.

Sample carrier

Samples can be analyzed using a variety of sample carriers:

- microscope slides,
- microwell plates (and smaller formats),
- capillaries.

Their position is controlled to an accuracy of 1 µm using a high-precision motorized system.

2 REQUIREMENTS FOR FCS MEASUREMENTS

2.1 Assay Components

2.1.1 Dye for Labeling

The first step in performing an FCS measurement is to choose a suitable dye for labeling non-fluorescent objects. A list of FCS-tested dyes in section 3.3 may help you to make an initial choice. The following points must be taken into account:

- The wavelength of the excitation source (laser) has to be compatible with the dye.
- The dye should not show non-specific binding (see section 2.2.2) to assay components or the sample carrier.

For further, more specific criteria refer to section 3.2.

Instrument adjustment

The next step is the adjustment of the instrument, especially the pinhole. In order to adjust the pinhole, perform a measurement on a 100 nM dye solution. Depending on the laser wavelength used, the following dyes are suitable for pinhole adjustment:

- Rhodamine 6G (for 458, 488, and 514 nm)
- Alexa Fluor 488 (for 488 nm)
- Tetramethyl-Rhodamine, (TMR, for 543 nm)
- Cy 5, Alexa Fluor 633 (for 633 nm)

For a precise description please refer to the LSM 510 – ConfoCor 2 or the ConfoCor 2 operating manual.

Characterization of the dye

The next step should be the characterization of the chosen dye in the appropriate buffer under the same conditions as the measurement later on. For this reason, a series of dilutions with different concentrations of the dye in the buffer have to be measured with FCS. Start with a dye concentration of 1 nM and end with a concentration of 100 nM. The concentration range may have to be shifted up or down depending on the characteristics of the dye used.

Ten measurements of 20 seconds each should be carried out with each concentration. These series of dilutions should be measured at a suitable laser intensity. The criteria for a suitable laser intensity are maximal counts per molecule (cpm > 50 kHz) and a resulting triplet fraction of less than 20% (Table 2-1).

Parameter	Typical values of Rh-6G
diffusion time	20 – 30 µs
triplet fraction	less than 20 %
triplet decay time	0.5 ... 3 µs
counts per molecule	80 ... 120 kHz

Table 2-1 Typical parameters of a 10 nM Rh-6G solution, measured at λ = 488nm

2.1.2 Buffer

Biological specimens often require buffers for defined and stable chemical conditions within the assay. In general these buffers should be free of substances that may bind dye molecules and labeled ligands in a non-specific manner or show intrinsic fluorescence.

Detergents

Sometimes it is necessary to use detergents to prevent non-specific binding of the fluorescent components to the surface of the sample carrier or other assay components (see section 2.2.2). Whenever possible, avoid using detergents, because these substances may bind the labeled molecules non-specifically. This results in additional diffusing particles, which will impair the interpretation of the correlation curves due to their fluorescence. If detergents are definitely necessary, please check them separately in FCS measurements for non-specific binding to the dye used for labeling and the labeled substance. This has to be done even when detergents are applied above the cmc (critical micelle concentration).

The interaction of the fluorescent molecule with the detergent is displayed by the following effects on FCS measurements (Table 2-2):

Kind of reaction	Effect on FCS measurement
no interaction	measured diffusion time corresponds to the dye
interaction	two-component fit model is necessary

Table 2-2 Checking detergents for non-specific binding to dye or labeled substance.

Refractive index

Buffers may contain glycerol (up to 10 % per volume). Note that substances such as glycerol, PEG, and saccharides will change the viscosity and the refractive index of the solution. Therefore, adjustments have to be made to compensate for the refractive index using the correction ring of the objective. Prior to pinhole adjustment, the correction ring must be set to a position at which the count rate is maximum.

HPLC-grade chemicals

For best results, use HPLC-grade chemicals for all buffers, and filter the buffer through a filter with a pore diameter of 0.2 µm.

Background count rate

As a general rule, the background count rate of the buffer should be below 10% of the signal derived from the fluorescent molecules.

2.2 Interfering Effects

2.2.1 Intrinsic Fluorescence

Resolution-limiting effect

In measurements made at low concentrations, the intrinsic fluorescence of any substance in the assay has a resolution-limiting effect. Any intrinsic fluorescence increases the fluorescence background which will define the lowest applicable concentration of fluorescent molecules.

To avoid performance losses, check all unlabeled components (buffer, unlabeled interaction partners, etc.) within the assay by doing an FCS measurement on these substances.

For example, the cytoplasm of cells often shows intrinsic fluorescence. In this case, we recommend the use of a HeNe laser (633 nm) and a red excitable dye such as Cy5 in order to minimize the amount of scattered light, as well as self-fluorescence in the red spectral region. Another substance tending to show intrinsic fluorescence is undefined/non-specific DNA, which is often used to suppress non-specific binding.

2.2.2 Non-specific Binding

There are two possibilities of non-specific binding within the assay:

- Binding of the dye to assay components
- Binding of fluorescent molecules to the sample carrier or assay components

Checking the dye

It is very important to check the dye for non-specific binding to buffer components or interaction partners. The following table (Table 2-3) shows possible kinds of reactions and their effects on FCS measurements:

1st reaction partner	2nd reaction partner	Effect on FCS measurement
dye	assay component (e.g. detergent)	additional diffusion component
dye	non-labeled interaction partner	non-specific binding background
dye	labeled ligand	aggregation of the ligand: • lowered free ligand conc. • noise, spikes within the trace
fluorescent molecule	assay component (e.g. detergent)	additional diffusion component
fluorescent molecule	sample carrier	decreasing concentration within the confocal volume

Table 2-3 Effects of non-specific binding on FCS measurement

Fluorescent molecules

Using FCS, the non-specific binding process between fluorescent molecules and assay components can be tested easily through the appearance of an additional diffusing component. This effect is often seen in the presence of BSA, liposomes, membrane vesicles, or intact cells.

The binding of fluorescent substances to the sample carrier is seen as a time-dependent decrease in concentration within the confocal volume element. This effect can often be minimized by the use of detergents. Using NP-40, for example, effectively reduces non-specific binding of several nucleic acid containing samples.

2.2.3 Change of Refractive Index

Optical correction

Some substances change the refractive index of the assay compared to pure water. If using these substances, adapt the optical system of the ConfoCor 2 using the correction ring of the objective. Substances which change the refractive index are, for example, glycerol, PEG, DMSO, and saccharides. These substances also change the viscosity of the solution, which results in longer diffusion times. In FCS measurement, viscosity effects generally have to be taken into account.

2.3 Labeled Ligand

2.3.1 Properties of the Dye

Fluorescence quantum yield

The fluorescence quantum yield (**Q**) describes the ratio of fluorescence intensity to excitation intensity. For a successful FCS experiment the quantum yield for the dye or labeled ligand has to be 0.2 or more.

Triplet state

The triplet excited state is created from the singlet state S1 via an excited-state process called intersystem crossing. This state can last between 0.5 – 10 µs. If in this state, the dye does not contribute to the emission signal. The triplet state should not exceed 20% in the FCS measurements.

Photobleaching

Photobleaching is the irreversible destruction of the fluorochrome. It is caused by high intensities of the exciting light and originates from the triplet state. Therefore the detection sensitivity should be maximized. This is accomplished by using objectives of high numerical aperture and the widest emission longpass or bandpass filters possible. If photobleaching persists, the best remedy is to switch the dye.

2.3.2 Mass Difference

Diffusion time

If only one reactant is labeled, a sufficient difference in diffusion time between the labeled ligand alone and the conjugated forms upon interaction with the unlabeled component is of critical importance for the success of FCS measurements. The diffusion times should differ at least by a factor of 2. If both reactants are differently labeled, no difference in diffusion time is necessary.

Mass ratio

A difference by a factor of 2 in diffusion times corresponds, for ideal spherical particles, to a mass ratio of 1:8. Under certain conditions a ratio of 1:5, e.g. for DNA/protein interactions, may be sufficient. In well-defined systems, differences in diffusion time down to 1:1.6 can be detected. For globular molecules freely diffusing in solution, this corresponds to a mass ratio of labeled ligand to formed complex of 1:4.

Increasing molecular weight

The molecular weight of the unlabeled reactant can often be increased by coupling non-reactive analogs to it, e.g. using common molecular biological methods. In this way a broad spectrum of different substances can be analyzed.

Cross-correlation experiments

If both reactants are differently labeled, no difference in diffusion times is necessary. Thus the reactants do not need to be of different mass.

2.3.3 Purification of the Labeled Ligand

Purification methods

The purification of the labeled ligand has a major influence on the success of an FCS measurement. The method for purification of the labeled ligand depends on the molecular weight MW of the unlabeled ligand.

- If the ligand to be labeled has a molecular weight of less than 10 kD, the method of purification has to be HPLC.
- If the molecular weight of the ligand to be labeled is higher than 10 kD, the product can be separated from excess dye by gel filtration.

An alternative in both cases is extensive dialysis using the correct molecular cut-off size of the dialysis membrane.

For more specific information on purification of the labeled ligand see section 3.6.

2.4 Analysis of Interactions

2.4.1 Solution-Based

Steps in analysis

Steps in performing a solution-based FCS analysis:

1. Choose the dye according to section 2.1.1 and 3.2.
2. Choose buffer/detergents according to section 2.1.2.
3. Adjust the instrument using a suitable dye (Rh-6G, Alexa Fluor 488, TMR, cy 5, Alexa Fluor 633) (2.1.1).
4. Characterize the dye alone using FCS (2.1.1).
5. Characterize the labeled ligand alone (2.3).
6. Check every substance for non-specific binding (2.2.2).
7. Check every substance for intrinsic fluorescence (2.2.1).
8. Analyze the interaction according to section 6.

Vesicles

For measurements on vesicles prepared from cells, we recommend a receptor density of at least 10^5 per cell. Prepare a homogeneous vesicle population from the cells. The diameter of the vesicles should not exceed 100 nm.

2.4.2 Cellular and Intercellular

Intrinsic fluorescence

Measurements of cellular and intercellular samples are performed similarly to the solution-based analysis described above. Cells of interest have to be measured unlabeled first to check for intrinsic fluorescence. Note that only a very low level of auto-fluorescence can be tolerated. The resulting counts per molecule **cpm** should not be higher than 10 kHz.

Observation of positioning

For a precise positioning of the confocal volume element inside a cell (e.g. on the plasma membrane, in the cytosol or nucleus), a direct observation is greatly enhanced by the use of an additional illumination. The focal plane can directly be observed with either the eyepiece or the CCD-camera option within the Confocor 2 software package for instrument control. If the Confocor 2 is used in combination with the LSM 510, the structures can be visualized by confocal scanning.

Reference position

The optional crosshair serves as a reference for the XY position of the sample table on the optical axis. The Z position of the slide can be determined by systematically testing different values in the Z plane (always do this positioning from one side to avoid phase lag of the drives). An illuminated spot must be observable at each surface of the slide (using the eyepiece or the CCD-camera option).

Focusing from above on the plasma membrane of a cell is easily performed by measuring a change in the free ligand concentration.

If the height of the analyzed cell is known, the scale for the area of motion inside a cell is given.

Manual positioning

For cellular measurements, the adjustment of the three axes (XY sample table, objective Z position) is often easier and more precise when carried out by hand. Note that the axes have to be unlocked for manual control.

2.5 Measuring Time

The minimum time required for a correlation curve yielding reliable values for the diffusion time and total number of particles is not constant. It depends on the counts per molecule (**cpm**) and the number of fluorescent particles in solution. This is due to the fact that FCS analyzes fluctuations, which decrease as the number of fluorescent particles in solution increases.

Maximum intensity value

In principle, the upper limit for the total fluorescence intensity is about 1 MHz. Values higher than this are outside the linear range of the APD. This will cause errors in correlation curves, due to the fact that not all fluorescence photons emitted can be registered by the detector. However, especially if dyes with a low quantum efficiency are used, the count rate should be much lower to obtain a good correlation signal. The laser intensity and the dye concentration should be chosen in a way that the number of particles does not exceed 5.

At count rates higher than 1.2 MHz the APD is switched off. In this case the APD can be switched on again by starting a new measurement.

Background signal

Based on our experience, the background signal resulting from buffers and non-fluorescent components used for FCS experiments must be at least 10 times lower than the achievable count rate for the labeled molecule used in the experiment. Otherwise, the autocorrelation curve will be significantly influenced by the background signal. Analysis of such correlation curves yields false values of particle number **N**, **cpm**, etc. Therefore, the background signal of the system under investigation defines the lower limit of concentrations which can be investigated by FCS.

Measuring times

We recommend a measuring time that amounts to 1000 times the diffusion time of the slowest component. Within cells it is often appropriate to use measurement times of 1 s and average up to 100 measurements.

Furthermore, the measuring time has to be adapted to the count rate (Table 2-4).

cpm (kHz)	Measuring time (s)
10	30
50	20
100	10

Table 2-4 Recommended measuring time for solutions containing dye at 10 nM; lower concentrations require larger measuring times.

Precision

These measuring times can only be interpreted as leads. In order to find the optimal measuring time the user should perform repetitive measurements using variable measuring times. To obtain the desired precision of fitting results, the user should choose the measuring conditions from the autocorrelation curve analysis.

3 FLUORESCENT LABELING

3.1 Introduction

Reactive groups

For labeling amino acids, peptides and proteins, different reactive groups of these molecules (thiol, amine, carboxylic acid, ketone) can be used. Protein labeling calls for an optimization of the chemistry used for each individual labeling reaction. This is due to the fact that each protein is a unique molecule, which behaves differently under different conditions. Therefore, the general principles described in the following section can be used as a basis for the optimization of a specific labeling procedure.

3.2 Choosing the Best Dye

Criteria

Refer also to section 2.1.1 for a pre-selection of the dye. In choosing a dye optimally suited for an FCS experiment, the following important physical criteria should be met:

- The highest possible quantum efficiency/yield
- The largest possible cross-section of absorbance (σ)
- A low triplet transition rate (intersystem crossing probability)
- A very short fluorescence lifetime
- A high photo stability

Quantum yield

The quantum yield **Q** has to be higher than 0.2 for a good FCS experiment.

Triplet state

If a triplet state of a molecule is occupied, no fluorescence light can be recorded from this molecule. The higher the laser intensity is, the higher is the occupation number of the triplet state. The triplet lifetime (typically between 0.5 and 10 µs in water) is short compared to the diffusion time of a labeled molecule through the confocal volume element. Therefore, a molecule can be switched on and off several times when reaching its triplet state during its mean dwell time. This process causes its own fluctuation signal resulting in a visible amplitude within the autocorrelation curve.

High triplet rates result in the following effects:

- Reduced fluorescence intensity
- Reduced photostability
- Reduced signal to noise ratio (*S/N*), since a large portion of the fluctuation is produced by the triplet transition.

Laser intensity

Since the presence of triplet occupation will lower the fluorescence intensity measured in an FCS experiment, all dyes and the labeled biomolecules should be measured with a laser excitation that does not result in a triplet fraction higher than 20%.

Intrinsic fluorescence

The choice of the optimal dye is not only dependent on the spectral properties of the dye itself. The intrinsic fluorescence of the biomolecules of interest must be taken into account. The absorption spectra of both the dye and the compound causing intrinsic fluorescence should be separated to the greatest extent possible.

Hydrophobicity

For measurements on hydrophobic samples, such as cells or vesicles, we recommend the use of dyes such as TMR, Rhodamine Green™ or Alexa Fluor dyes. Dyes such as Rhodamine-6-G or Rhodamine B are not useful for these applications due to their strong hydrophobic interaction with the surface or membrane of cells. Due to their high hydrophobicity, these dyes are not optimal for the labeling of small organic molecules or peptides either. Additionally, the fluorescence of these dyes is often quenched when they are covalently bound to a biomolecule.

Proteins

Based on our experience, proteins labeled with Texas Red®, Cy5-NHS-esters, tetramethylrhodamine-isothiocyanates and Alexa Fluor succinimidyl esters or maleimides work best and result in excellent conjugates well suited for FCS measurements.

Nucleic acids

For the labeling of nucleic acids, we recommend the use of Alexa dyes, TAMRA, ROX or Rhodamine Green™. Intercalating dyes, such as ethidium bromide, propidium iodide and Pico Green®, may also be used.

Fluorescein

Based on our experience, fluorescein, which is one of the most commonly used dyes in confocal microscopy, is not useful for FCS measurements because of its pH dependence, rapid protonation reaction and its strong photobleaching effect, which obscures the analysis of the measured correlation curves.

3.3 Dyes for FCS-grade Labeling

Table 3-1 lists dyes that are suitable for FCS measurements.

Dye	Supplier	Excitation [nm]	Emission [nm]	Laser line [nm]
Alexa Fluor 488	Molecular Probes	495	519	488
Alexa Fluor 546	Molecular Probes	556	573	543
Alexa Fluor 633	Molecular Probes	632	647	633
BODIPY® 581/591	Molecular Probes	582	590	543
Cy2™	Amersham	489	505	488
Cy3™	Amersham	550	570	543
Cy5™	Amersham	649	670	633
Ethidium bromide	Molecular Probes	518	605	514
Lissamin rhodamine B	Molecular Probes	574	602	543
PicoGreen	Molecular Probes	498	520	543, (514)
Propidium iodide	Molecular Probes	536	617	543
Resorufin (RESOS)	Sigma	571	585	543,(514)
Rhodamine-6-G	Radiant Dye Laser, Aldrich	525	555	514, (543)
Rhodamine B	Aldrich, Lambda Physik	555	580	543
Rhodamine Green™	Molecular Probes	502	527	488
Rhodol Green™	Molecular Probes	500	525	488
5-ROX	Molecular Probes	574	602	543
TAMRA	Molecular Probes	555	580	543, (514)
Texas Red	Molecular Probes	595	620	543
TMR	Molecular Probes	555	576	543, (514)
DY-630	Dyomics	621	652	633
DY-635	Dyomics	634	664	633
EvoBlue™ 30	Dyomics	635	670	633

Table 3-1 List of FCS suitable dyes. The table lists the supplier, the excitation and emission wavelength as well as the laser lines of the ConfoCor 2 that should be used for excitation. Laser lines in parenthesis can also be used, however, they are less sufficient.

3.4 Labeling Strategies

Reactive groups

A variety of functional groups can be used for labeling proteins and oligonucleotides:

- Thiols (cystine, cysteine, methionine)
- Amines (lysines, α-amino groups)
- Carboxylic acids
- Ketones and aldehydes (for glycoproteins through oxidation of vicinal diols)

Primary amines, thiols and phenols are especially useful for the labeling of proteins and peptides.

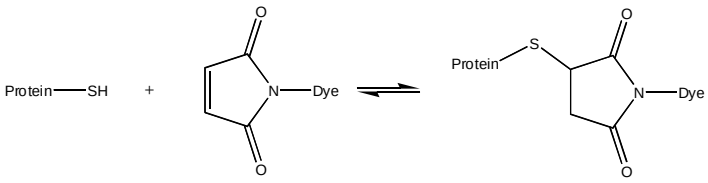
On the following pages, general reactions for labeling different functional groups on proteins with suitable reactive forms of dyes are shown.

3.4.1 Thiols

Thiols can be labeled using the following reactive forms of dyes.

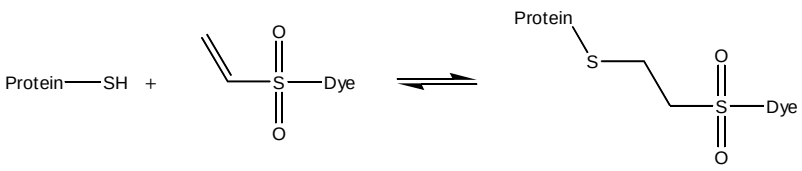
Haloacetyl

Maleimide



Disulphide

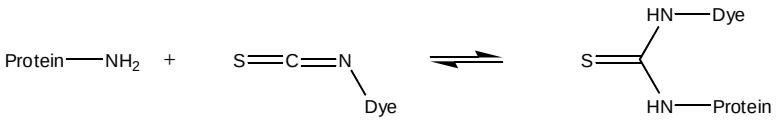
Vinyl Sulfone



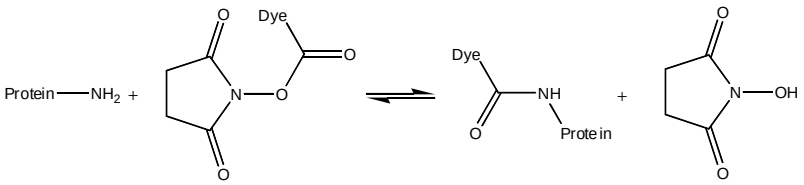
3.4.2 Amines (Lysines, α-Amino Groups)

Amines can be labeled using the following reactive forms of dyes.

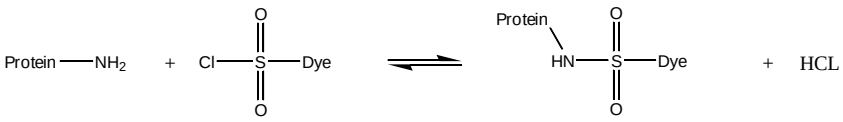
Isothiocyanates



NHS ester



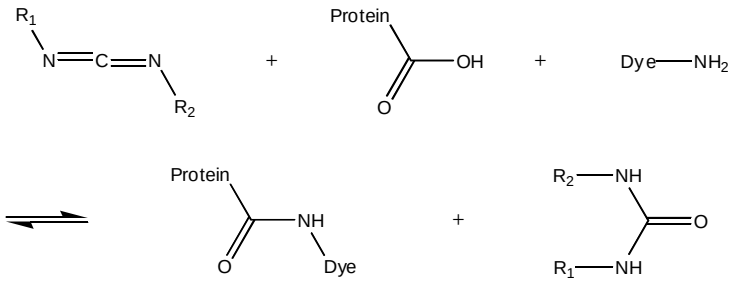
Sulfonyl halides



3.4.3 Carboxylic Acids

Carboxylic acids can be labeled using the following reactive forms of dyes.

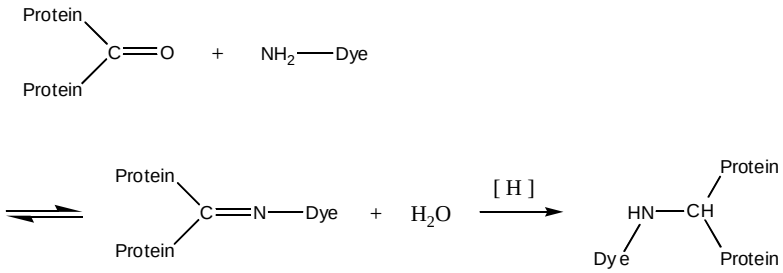
Carbodiimide



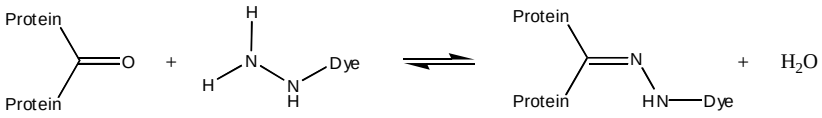
3.4.4 Ketone Groups

Ketones can be labeled using the following reactive forms of dyes.

Primary amines



Hydrazines



3.5 Protocols for Labeling

In this section, a general procedure is described for the labeling of proteins containing amino groups. Additionally, specific protocols are given for labeling FCS-suitable reference substances which can be used for testing the performance of the instrument. Companies like Molecular Probes often offer kits with a detailed description of the labeling procedure. The section closes with examples of correlation curves for those reference substances measured under standard conditions on the ConfoCor 2. Refer also to the literature [21-25] given in the appendix.

3.5.1 General Labeling Protocol

Step 1 Dissolve the protein to be labeled in 0.1 M bicarbonate buffer, pH 9.5. The protein concentration in the reaction should be between 5 and 20 mg/ml for optimal results. Often such high concentrations cannot be obtained. Efficient labeling can also be done with concentrations as low as 0.1 mg. The reaction volume is of no major concern, albeit large volumes make gel filtration less efficient to remove the unreacted dye. A more crucial point is that the ratio between protein and dye should be maintained.

Step 2 Dissolve the amine-reactive dye in dehydrated, freshly distilled N,N-dimethylformamide (DMF) or dehydrated dimethyl sulfoxide (DMSO) immediately before starting the reaction. (Concentration of the dye: 5 mg in 500 µl). Reduce the amount of dye if less protein is used.

Step 3 While stirring or vortexing the protein solution, slowly add the reactive dye solution. Incubate the reaction at room temperature for 1 hour, stirring or rotating continuously.

Step 4 Halt the reaction by adding 0.1 ml of freshly prepared 1.5 M hydroxylamine, pH 8.5 to obtain a final concentration of 0.15 M. Incubate at room temperature for another hour, stirring moderately.

Step 5 Separate the conjugate from unreacted labeling reagent by twice passing the solution through a gel filtration column (e.g. NAP 10™) equilibrated with PBS or buffer of choice. Often, especially if the dye has nearly completely reacted, only one gel filtration is enough.

Degree of substitution

Determine the degree of substitution fluorometrically using the following formula:

$$\frac{A_x}{d \cdot \varepsilon} \cdot \frac{MW_{protein}}{mg_{protein}/ml} = \frac{mol_{dye}}{mol_{protein}}$$

A_X: absorbance value of the dye at the absorption wavelength.

ε: molar extinction coefficient of the dye or reagent at absorption wavelength. For example:

Cy5:	ε = 250.000 l/(mol · cm)	at λ = 647 nm
Texas Red:	ε = 116.000 l/(mol · cm)	at λ = 592 nm

d: optical pathlength

Primary amino groups

Using the general labeling protocol given above, primary amino groups of biomolecules can be labeled with the following reactive forms of the dyes:

- NHS-esters
- Sulfonyl chlorides
- Isothiocyanates

Optimization

The protocol given above is intended only to serve as a general guideline. For a specific labeling strategy, optimization of the above procedure may be needed for the specific biomolecule and dye used.

Concentration

The final biomolecule concentration in the reaction mixture depends on the mass of the molecule to be labeled. For a protein with a molecular mass of 140 kD, we recommend to use a concentration of 3.5 nmol in a total reaction volume of 100 µl. Smaller molecules will require a higher concentration, larger molecules a lower concentration in the labeling reaction mixture.

Temperature

For sufficient labeling of a protein, four free accessible lysines or two free thiols are necessary. The best suited reaction temperature depends on the type of biomolecule. Typically, room temperature (20 °C) is used. For very sensitive molecules lower temperatures down to 4 °C may be required.

3.5.2 Alkaline Phosphatases with Cy-5

Materials

Labeling buffer:

- 0.1 M sodium carbonate/sodium bicarbonate buffer, pH 9.5
- 1 mM $MgCl_2$
- 0.1 mM $ZnCl_2$

Chemicals

- Alkaline phosphatase (e.g. Merck), 5 mg/ml in labeling buffer
- 1 vial Cy-5 monoreactive, Amersham (Biological Detection System)

- 1.5 M freshly prepared hydroxylamine solution

Storage buffer

- 3 M NaCl
- 1 mM $MgCl_2$
- 0.1 mM $ZnCl_2$
- 30 mM triethylamine, pH 7.5
- NAP 5 columns (Pharmacia) equilibrated in storage buffer

Reaction

Reaction

- Add 100 µl (3.55 nmol) of the alkaline phosphatase solution to a vial Cy-5. Mix the solution carefully.
- Incubate the reaction at room temperature (20 °C) for 3 hours, stirring continuously.
- Halt the reaction by adding 10 µl of the freshly prepared hydroxylamine solution. Incubate at room temperature for another hour, stirring moderately.

Purification

Purification

- Apply the total reaction mixture to an NAP 5 column. Allow the entire reaction mixture to enter the gel. Next add 400 µl of storage buffer to the column and discard the flow-through. Then add 500 µl of the storage buffer and recover the flow-through.
- Apply the sample eluate from purification step 1 to a fresh NAP column (equilibrated with 500 µl storage buffer). Allow the entire sample volume to enter the gel. Add 1000 µl of storage buffer to elute the labeled sample. Recover the flow-through.

Analysis

Determine the degree of substitution (labeling) spectroscopically, using the molar extinction coefficients attached.

3.5.3 Alkaline Phosphatases with Texas Red

Materials

Labeling buffer:

- 0.1 M sodium carbonate/sodium bicarbonate buffer, pH 9.5
- 1 mM $MgCl_2$
- 0.1 mM $ZnCl_2$
- Alkaline phosphatase, 5 mg/ml in labeling buffer (e.g. Merck)
- 1 ampule (1 mg) Texas Red® (Molecular Probes), dissolved in 100 µl dehydrated DMF.
- 1.5 M freshly prepared hydroxylamine solution NAP 5 column equilibrated in storage buffer

Reaction

- Add 4.4 µl (71 nmol) Texas Red® to 100 µl (3.55 nmol) of the alkaline phosphatase solution, mixing carefully.
- Incubate the reaction at room temperature (20 °C) for 3 hours, stirring continuously.
- Halt the reaction by adding 10 µl of the freshly prepared hydoxylamine solution. Incubate at room temperature for another hour stirring moderately.

Purification

- Apply the total reaction mixture to an NAP 5 column. Allow the entire reaction mixture to enter the gel. Next add 400 µl of storage buffer to the column and discard the flow-through. Then add 500 µl of the storage buffer and recover the flow-through.
- Apply the sample eluate from purification step 1 to a fresh NAP column (equilibrated with 500 ml storage buffer). Allow the entire sample volume to enter the gel. Add 1000 µl of storage buffer to elute the labeled sample. Recover the flow-through.

3.6 Purification of the Ligand

Purity

The highest degree of purity of the labeled ligand is required. If possible, no free dye should be present in the solution. The labeled ligand must be completely soluble in the buffer used for FCS measurement. Please check the ligand for its tendency to form aggregates in the buffer used. Such aggregation phenomena alone may obscure the analysis of the measured correlation curves of the interaction under investigation.

Purification methods

For an efficient purification of protein conjugates, we recommend HPLC or gel filtration depending on the molecular weight of the unlabeled ligand:

MW < 10kD

- If the ligand to be labeled has a molecular weight of less than 10 kD, the method of purification has to be HPLC. The required purity of the labeled ligand is 95 %. Due to the similar hydro-dynamic dimensions of the dye in its free and coupled form, the fraction of both components, the free dye, and the labeled ligand, cannot be determined by FCS with the precision needed. For this reason a mass spectrum is strongly recommended to confirm purity.

MW > 10kD

- If the molecular weight of the ligand to be labeled is more than 10 kD, the product can be separated from excess dye by gel filtration. We recommend filtration with an NAP™-10 column (Pharmacia Biotec AB) carried out two or three times. The number of gel filtrations needed for purification depends on the amount of non-bound dye.
- If small volumes (<200 µl) have been used, removal of the unreacted dye could also be performed by spin columns.

The quality of the product may be checked by HPLC or FCS. The result of HPLC analysis has to be 90 % of labeled ligand or better. For the checking of the quality of the HPLC purified ligand with FCS, an amount of approx. 70 % labeled ligand or more must be observable. The labeled ligand is represented by the slow-diffusing particles.

(The explanation for the different results of HPLC and FCS analysis is given at the end of this section.)

Analysis

For best results, analyze a dilution series of the ligand in the buffer of choice (1..100 nM) at a laser intensity which does not result in a triplet fraction higher than 20 %.

The following parameters have to be measured:

- Diffusion time of the labeled ligand
- Triplet percentage of the labeled ligand
- Triplet lifetime of the labeled ligand
- Concentration of the labeled ligand
- Amount of free dye at rest (using two-copponent model)

Different results

The differences in the fraction of free dye determined by HPLC and FCS arise due to changes of the fluorescence intensity of the dye in the free as well as in the coupled state. Such changes are measurable in FCS by a change of the counts per molecule **cpm**. A **cpm** difference of more than 15 % has to be corrected after FCS analysis (see section 6.6 for correction procedure).

3.7 Storage of the Labeled Ligand

The storage of the labeled ligand should be identical to the storage requirements for the unlabeled molecule. If possible, add NaN_3 or Thimerosal at 0.05% to stabilize the product. For higher stability, it may be of advantage to store labeled enzymes in glycerol at -20 °C. However, it should be taken into account that glycerol may disturb the FCS measurement. Labeled ligands may be stored at +4 °C or -20 °C in aqueous buffer, or lyophilized.

4 USING THE LSM 510 - CONFOCOR 2 SOFTWARE

4.1 Introduction

The LSM 510 - ConfoCor 2 instrument is controlled completely by a special software package. The software uses the 32-bit Windows NT 4.0 operating system. It permits control of the LSM 510 - ConfoCor 2 hardware and the convenient analysis of the measured data. Both single measurements and software-controlled multiple measurements are possible.

The software permits the calculation of the auto- and cross-correlation functions from the raw data to be performed quasi-simultaneously with the current measurement. Furthermore, the primary data are optionally provided to permit individual analysis by the user. For the analysis of correlation curves, a software module is available to match the measuring data to model functions by means of a non-linear fitting technique. The fit is based on a Marquardt algorithm (a non-linear least-square algorithm using $\chi 2$ criterion), and it can be used for the precise determination of diffusion times and particle number in real time.

For a detailed description of software functions, refer to the operating manual. The aim of this chapter is to show the reader how to analyze correlation curves. It will train users in deciding which of the fit models available is best suited to analyze a given correlation curve.

4.2 Fitting Algorithm

4.2.1 Mathematical Procedure

Mathematical model for autocorrelation

The fluorescence signal is measured in real time and the autocorrelation function calculated by a software correlator. For a two-component model this function can be described by he following equation:

$$G'(\tau) = 1 + \frac{1}{N} \cdot \left[(1-Y) \cdot \left(\frac{1}{1+\tau/\tau_{D1}} \right) \cdot \left(\frac{1}{1+\tau/S^2\tau_{D1}} \right)^{\frac{1}{2}} + Y \cdot \left(\frac{1}{1+\tau/\tau_{D2}} \right) \cdot \left(\frac{1}{1+\tau/S^2\tau_{D2}} \right)^{\frac{1}{2}} + f(T) \right]$$

N: number of fluorescent particles
S: structural parameter - synonym for ω_2/ω_1, axis ratio **AR** (defining the dimension of the confocal volume)
τ_{D1}: diffusion time of component 1 in the assay, e.g. the faster one
1-Y: fraction of particles with diffusion time τ_{D1} in the total number of fluorescent particles
τ_{D2}: diffusion time of component 2 in the assay, e.g. the slower one.
Y: fraction of particles with diffusion time τ_{D2} in the total number of fluorescent particles
f(T): function used for fitting the triplet characteristics τ_T and % τ_T of the fluorescent label within the assay

By means of an iterative least-square method (specifically developed for the analysis of FCS data), the values calculated by the algorithm are compared repeatedly to the experimentally generated autocorrelation curve and approximated until the difference between the two curves is minimized.

4.2.2 Preliminary Studies

Illustration

The reader may compare the search for the best mathematical approximation with the smallest degree of error to a walk through a landscape in which valleys and mountains (representing the value of deviation) are close to one another. The deepest valley then corresponds to the minimum deviation between the fit and the autocorrelation curve.

Optimal fit

The fitting algorithm used by the Confocor 2 software is designed to generate a mathematical representation of the actual correlation curve measured without the need for guessing the parameters to be fitted. An optimal fit, therefore, can be defined as a mathematical representation of the data which shows a minimum deviation from the correlation curve measured.

A special algorithm had to be developed for this purpose, due to the fact that some of the parameters to be fitted are not independent of one another. This applies to the one-component model as well as to more complex models.

Complex systems

For more complex experimental systems the number of fitting parameters necessary to describe the autocorrelation curve increases mathematically. In general, the probability of unique mathematical solution decreases with increasing numbers of free fitting parameters. This is due to the fact that some of the fitting parameters are no longer independent.

Returning to the landscape analogy, a straightforward solution would correspond to a single deep valley representing the best fit. More complex systems, those with two or three diffusing particles, can be compared to a landscape in which many valleys, or local minima, exist. In this case, the search for the best mathematical solution is not straightforward. If the diffusion times of the components do not vary greatly, several local minima exist within the solution landscape. Without additional information, the algorithm will find a possible solution, but due to the interdependency of some of the parameters, other solutions exist which may be equally valid.

Noise

Noise within the correlation curves prevents the algorithm from finding the best solution, too, because the fit landscape corresponding to a noisy correlation curve shows no sharp minima representing the unequivocal fitting result.

Preliminary information

Previously determined information about one or more parameters of the system under investigation can be valuable in assisting the algorithm in finding the best fit, by decreasing the number of compatible good fitting results. Information which can be of use for increasing the significance of the fitting results consists in the characteristic diffusion times of one or more of the components, the triplet lifetime, and the axis ratio, which defines the size of the confocal volume element. With this information, the portion that each type of diffusing particles contributes to the total number of observed particles can be determined with higher precision.

4.3 Models for Data Analysis

4.3.1 Choosing an Evaluation Model

Depending on the autocorrelation function measured on the assay under investigation, the user has to decide for the one-, two- or three-component model (7) for evaluation. The suitability of the model depends on the amount of fluorescent particles within the assay. The choice may be based on knowledge of the assay or made by following a stepwise procedure as described in this section. The fitting process should proceed from simple to more complex models, because a more complex model with additional parameters will generally result in a better fit quality, but may not always reflect the actual situation.

One important criterion for determining the quality of a fit is the shape of the residuals curve (17), which describes the differences between the measured and the simulated correlation curve. A wavy shape is a strong indicator for systematic deviations, as shown in Fig. 4-1 below.

Unsatisfactory fit

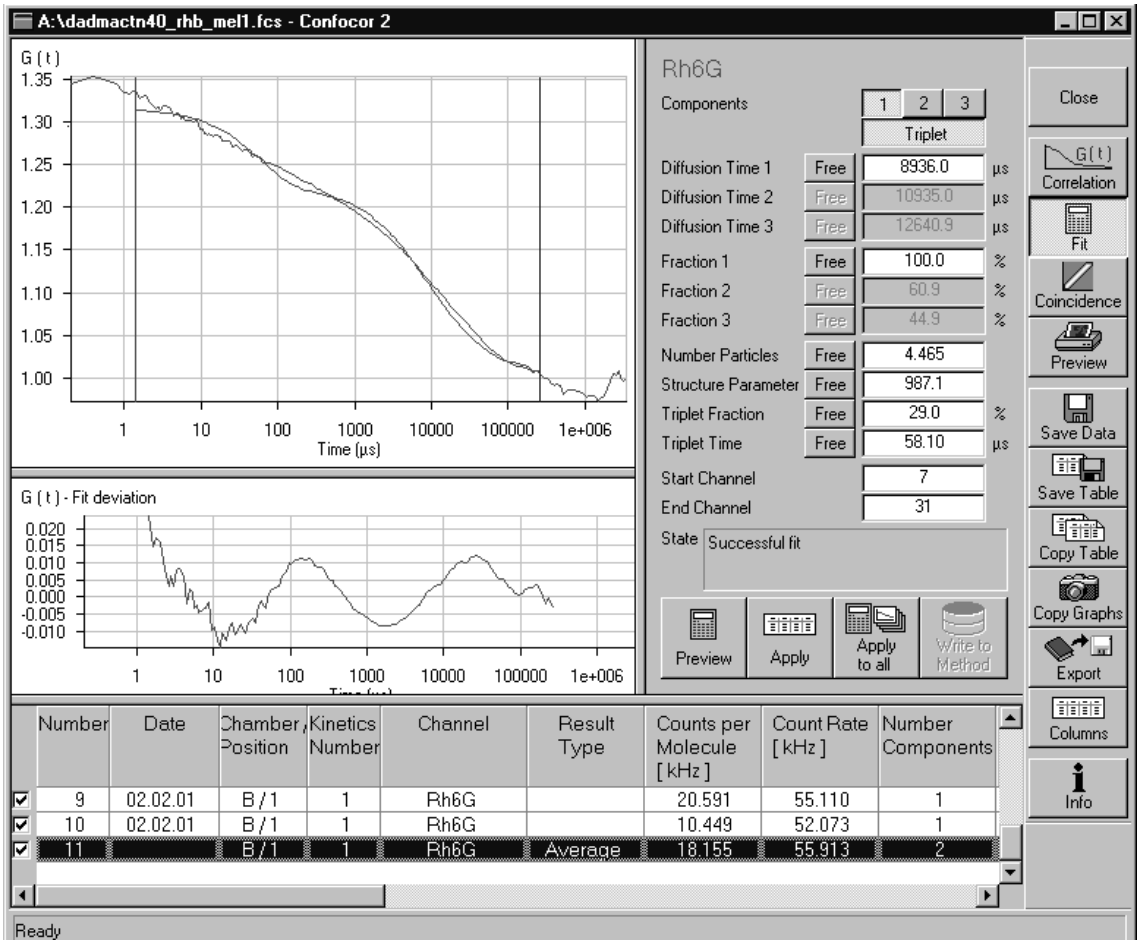


Fig. 4-1 Fit of a correlation curve using the one-component model. The result obtained is unsatisfactory, indicated by the wavy shape of the residuals curve.

The curves given in Fig. 4-1 represent a typical correlation curve measured for a solution containing a minimum of two different populations of fluorescent molecules, faster and slower ones. For this reason the correlation curve can not be approximated in a satisfactory way using the one-component model.

More complex model

By choosing a more complex model, i.e. the two- or three- instead of the one-component model, the wavy shape of the residuals curve is not observed, and the relative least square value is minimized. Figure 4-2 shows an excellent fit of the same correlation curve using the two-component model.

Satisfactory fit

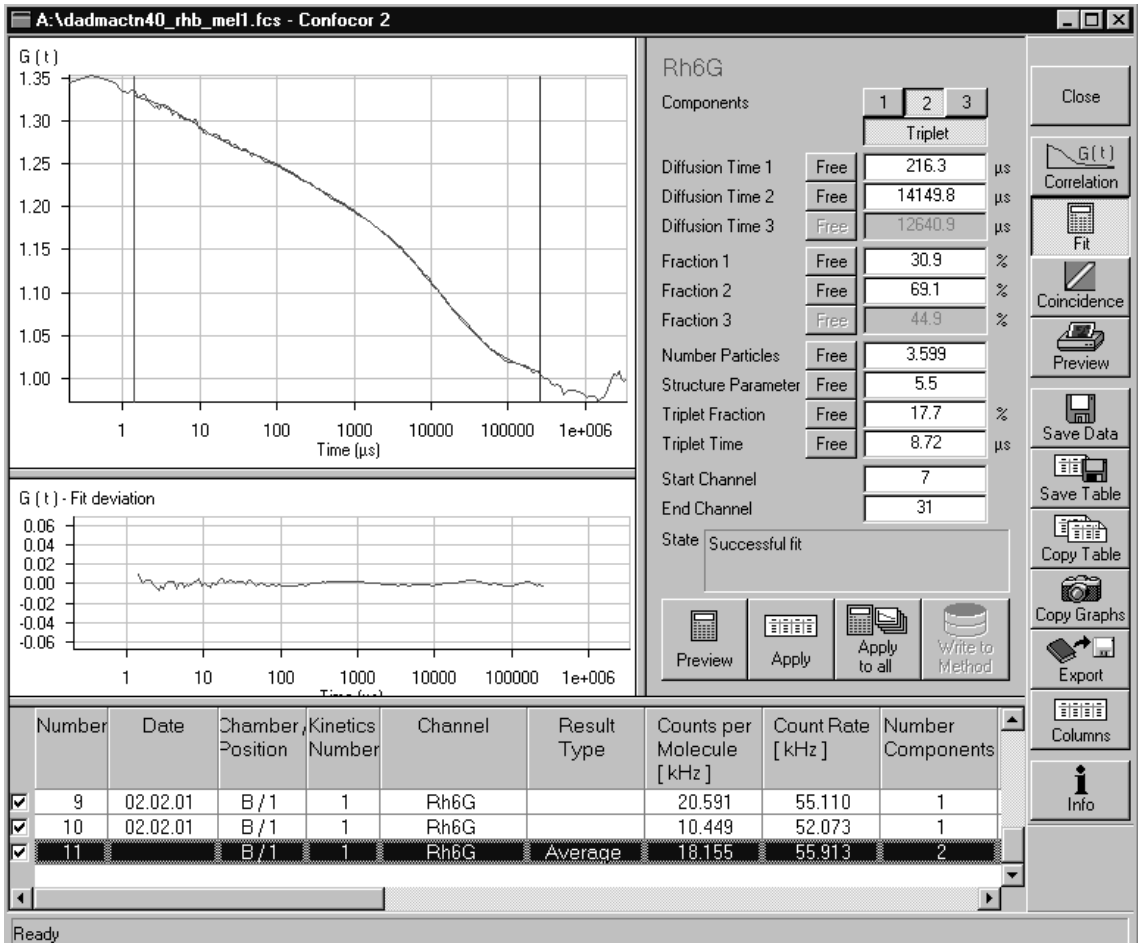


Fig. 4-2 Fit of the same datafile as in Fig. 4-1. The two-component model was chosen and led to an excellent fit.

4.3.2 One-Component Model

One fluorescent component

The one-component model is generally applied to fit correlation curves resulting from the measurements of solutions containing only dye molecules or a labeled ligand of high purity. Such data can be fitted with the ConfoCor 2 software without the need for guesswork regarding any of the fitting parameters. The fit of each individual datafile yields reproducible values for all parameters (Fig. 4-3).

Excellent fit

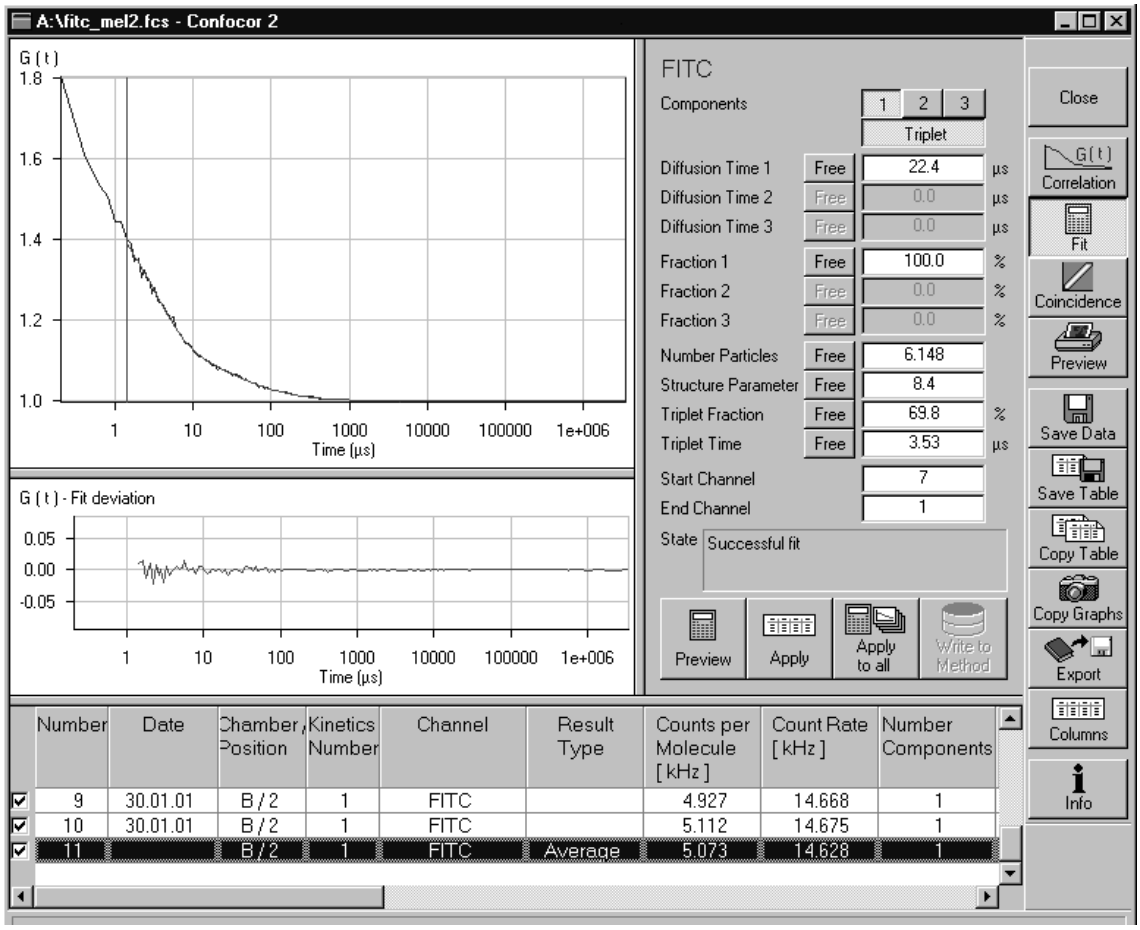


Fig. 4-3 Fit obtained with the one-component model of a correlation curve measured for a dye. No start values or fixation of a parameter were needed to obtain an excellent fit with stable values.

Axis ratio

Please note that the structural parameter which relates to the dimensions of the confocal volume element should result in a mean value of about 5 (calculated from approximately 10 individual measurements). This indicates good adjustment of the instrument.

4.3.3 Two-Component Model

Two fluorescent components

The two-component model is of use for the analysis of correlation curves resulting from the measurement of a labeled ligand. Such data can be initially fitted by means of the one-component model and a fixed value of the structural parameter as determined in preliminary measurements of the dye used for labeling the ligand (Fig. 4-4).

Unsatisfactory fit

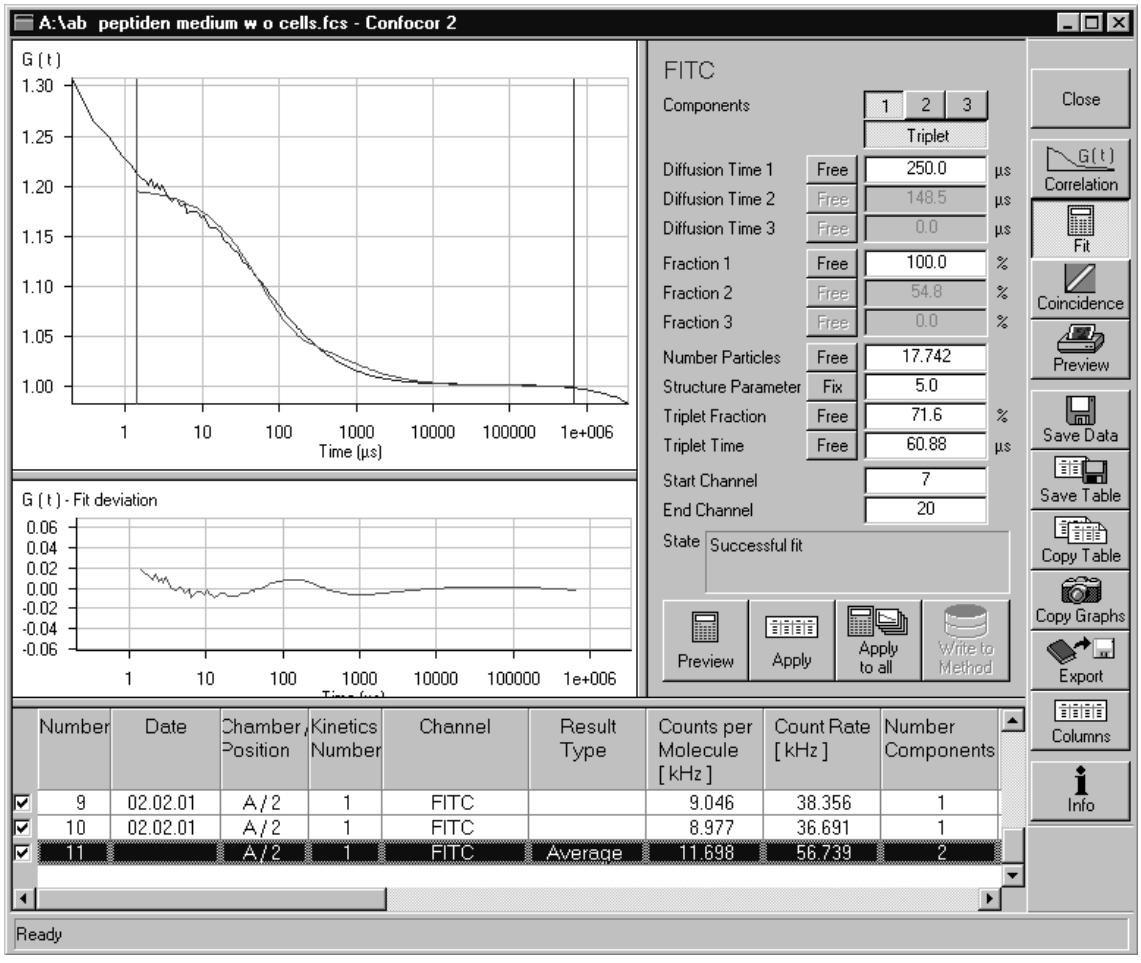


Fig. 4-4 Fit obtained with the one-component model of a correlation curve measured for a labeled ligand. Only the value of the axis ratio S has been fixed. The wavy shape indicates the unsatisfactory quality of the fitting result.

Residual curve

As shown in the example above, the wavy nature of the residuals curve indicates that the one-component model is not sufficient for a high-precision analysis of the data. Highly precise analysis is obtained only by using the two-component model as shown in the next figure.

By fixing diffusion time 1 and the structural parameter at values obtained from measurements of the dye used for labeling, a labeled ligand of lower purity can be analyzed using the two-component model (Fig. 4-5).

Satisfactory fit

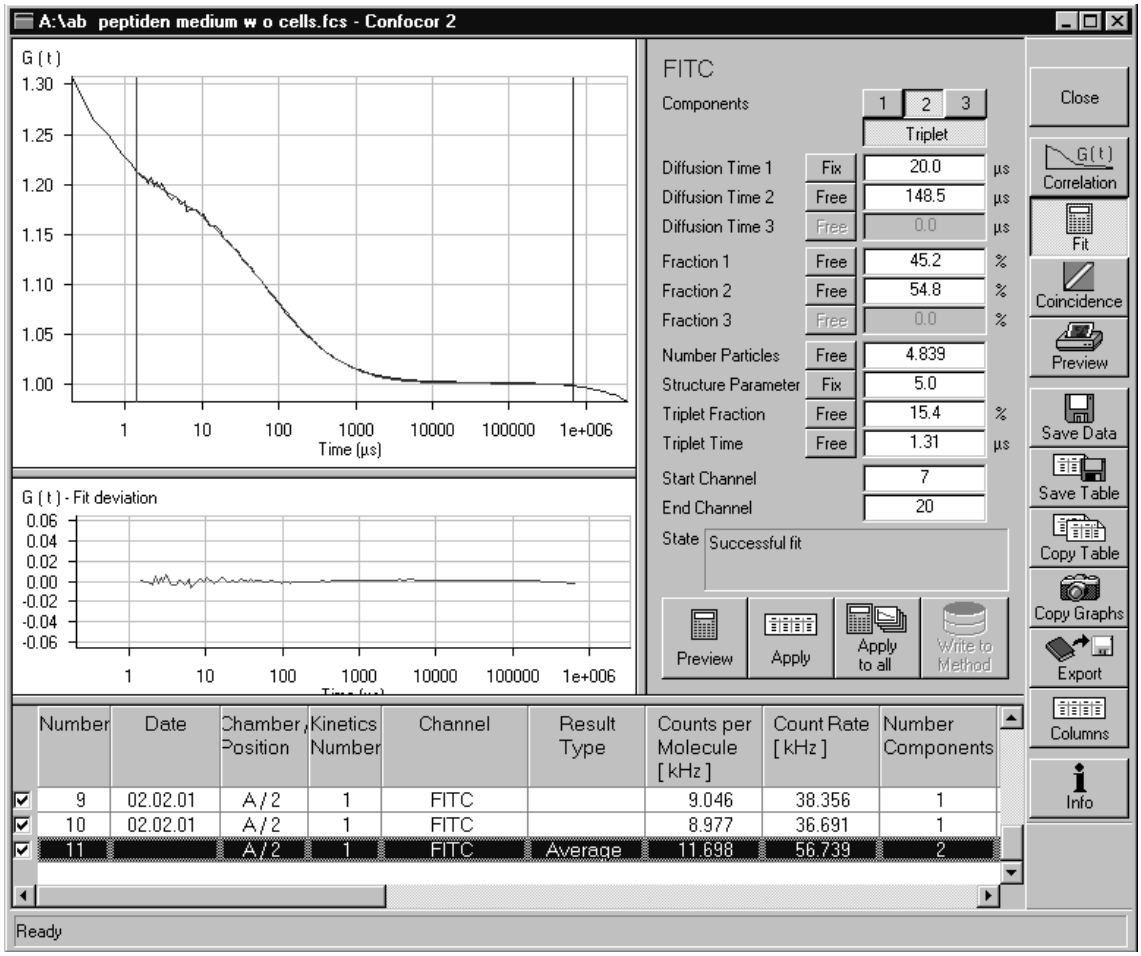


Fig. 4-5 **Fit of the correlation curve shown in Fig. 4-4 using the two-component model. Fixation of S and τ_{D1} leads to a good fitting result. In this example the axis ratio had to be fixed at 5.0, τ_{D1} at a value of 20 µs. These values were obtained from a series of correlation curves resulting from measurements with solutions containing the dye alone.**

Relative least square

The quality of the fit obtained with this method is excellent. Note that the value of the relative least square obtained from the fit deviation is much smaller than the one obtained with a one-component fit of the same data.

4.3.4 Three-Component Model

Titration

The three-component model is often applied for the analysis of a typical titration experiment in which one labeled reactant (the smaller substance) interacts with one unlabeled reactant (the larger substance).

Dye parameters

To evaluate this kind of assay, first the dye used for labeling must be evaluated in a series of correlation curves, and the mean value of the diffusion time τ_{D1} and the structural parameter **S** have to be obtained from the fit of the correlation curves with the one-component model.

Labeled reactant

As previously shown, this information can be used to analyze correlation curves resulting from the measurement of the labeled reactant using the two-component model with fixed values of **S** and τ_{D1}. From a series of correlation curves of the labeled interaction partner, the mean diffusion time τ_{D2} of this molecule can then be determined.

Using this additional information, the interaction of an unlabeled molecule and the labeled ligand can be investigated in a series of correlation curves which are analyzed using the three-component model with fixed values for **S**, τ_{D1} and τ_{D2}.

Unsatisfactory fit

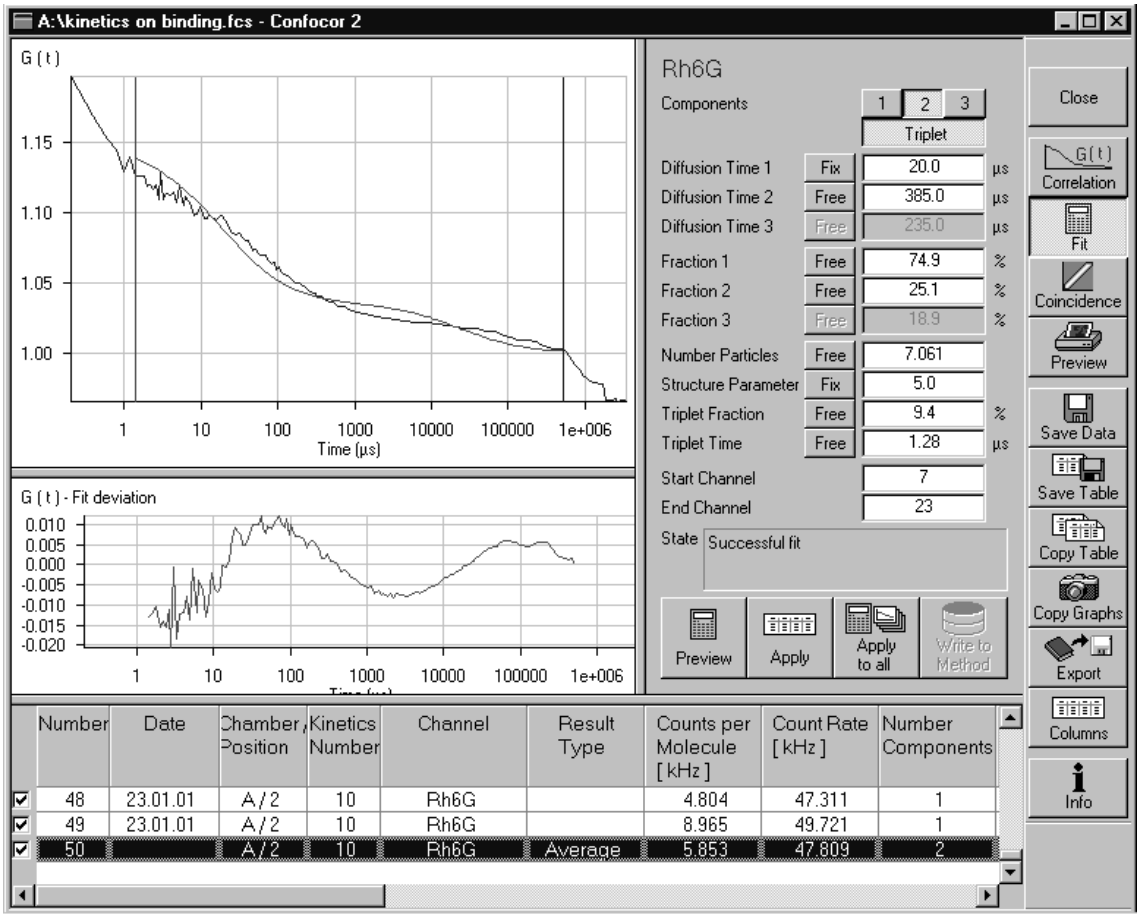


Fig. 4-6 **Fit obtained with the two-component model of a correlation curve measured for an interaction of the labeled molecule characterized in Fig. 4-5 with another biomolecule. The wavy shape of the residuals curve indicates the poor fitting result.**

Residuals curve

Note that a fit obtained with the two-component model does not result in a fit of sufficient quality. This can be concluded by the wavy shape of the residuals curve, which indicates systematic deviations (Fig. 4-6).

With the three-component model and fixed values of τ_{D1}, τ_{D2} and **S,** a much better fit is obtained. Note that the value of the **relative least square** is much smaller than that obtained using the two-component fit of the same data (Fig. 4-7).

Satisfactory fit

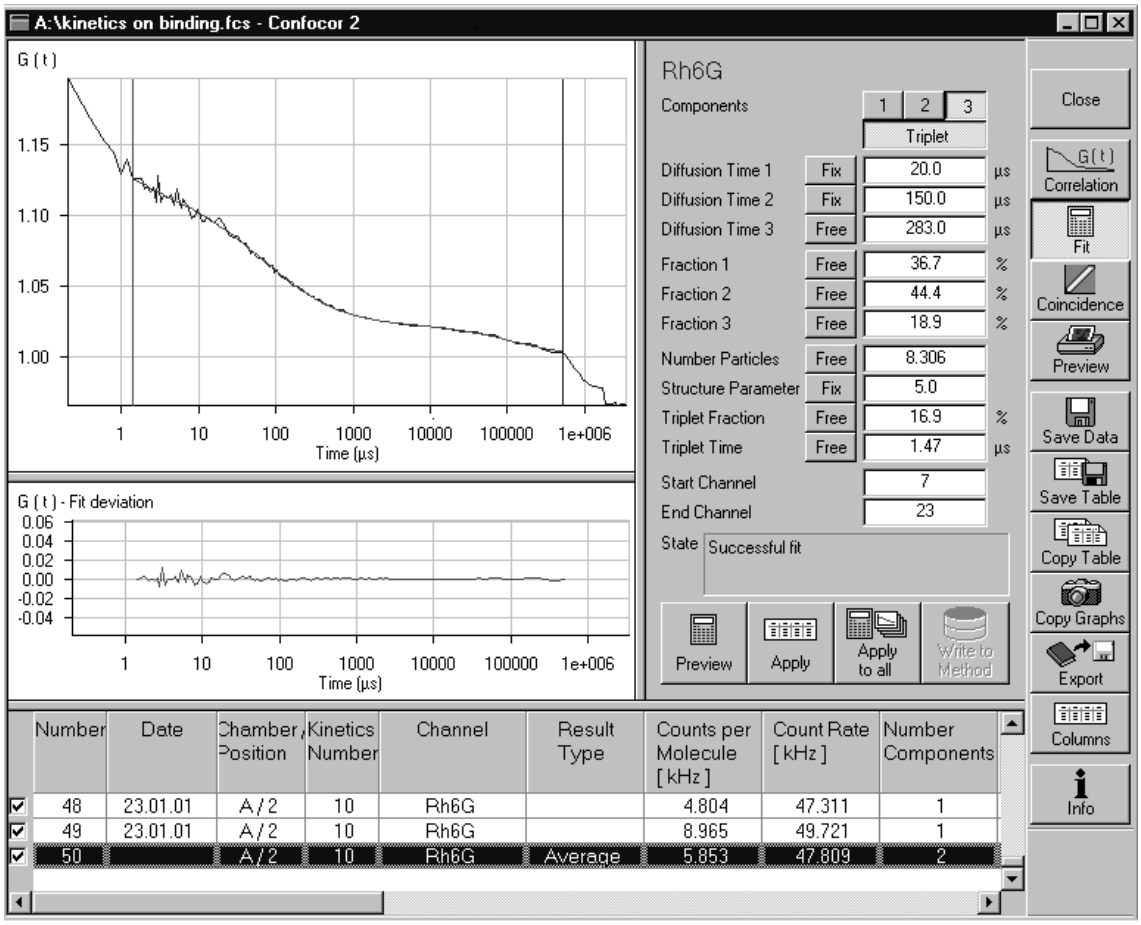


Fig. 4-7 Fit obtained with the three-component model of the correlation curve shown in Fig. 4-6. Fixation of S, τ_{D1} and τ_{D2} leads to an excellent fitting result.

4.4 Data Acquisition and Export

The following sections will provide a brief survey on which data sets can be acquired, how they can be analyzed and how the raw data are exported for use in other software programs. For more details, the reader should consult the Operating Manual.

4.4.1 Data Analysis

All data are acquired simultaneously.

Name	Description	Acquisition	Calculation	Display	Analysis
Autocorrelation	Analysis of interactions 1 reactant labeled Differentiation by diffusion properties	1 or 2 channels	Multiple tau algorithm	Autocorrelation curve	Number of molecules, 2 diffusion times, 2 fractions, triplet time / fraction
Cross-correlation	Analysis of interactions 2 reactants labeled Differentiation by diffusion properties	2 channels	Multiple tau algorithm	Cross-correlation curve	Number of molecules, diffusion times
Count rate histogram FIDA = PCH	Analysis of interactions 1 reactant labeled Differentiation by brightness	1 or 2 channels	Binning and counting	Count rate histogram	Separate software needed (use data export)
Coincidence histogram	Rare event detection 2 reactants labeled	2 channels	Binning and counting	2D count rate histogram	Separate software needed (use count rate export)

All fit results can be fixed.

4.4.2 Additional Information

All data are acquired simultaneously.

Name	Description	Acquisition	Calculation	Display
Raw data	Time between photons = intensity Stored as file using patented data compression	1 or 2 channels	Data compression	File name
Count rate	Photons / sec = intensity	1 or 2 channels	Binning	Intensity curve
Pulse distance histogram	Histogram of time between photons	1 or 2 channels	Counting	Density curve

4.4.3 Data Import and Export

Name	Description	Format	Procedure
Raw data	Intensity represented by all detected photons	Time interval between two detected photons (time resolution 50 ns) in compressed, patented and documented format	Before measurement, select files to which data is to be written.
Save data	All measured and calculated data (except raw data), instrumental settings	Binary AINSI-Text	Standard Save dialog
Export data	Count rate, correlation curve, restricted set of instrumental settings. Compatible to ConfoCor 1	Binary AINSI-Text	Standard Save dialog
Save / Copy table	Fit results	Text file	Context menu: save or copy
Copy graphs	Bitmap of all displayed graphs	Bitmap	Switch on diagrams Press button
Save / Copy count rate	Intensity data represented by count rate (resolution depending on measurement time)	Text file	Context menu: save or copy
Save / Copy correlation curve	Correlated data	Text file	Context menu: save or copy
Save / Copy count rate histogram	Data of count rate histogram	Text file	Context menu: save or copy
Save / Copy pulse density histogram	Data of pulse density histogram	Text file	Context menu: save or copy
Save / Copy count rate (pinhole adjustment) for XYZ	Count rate versus pinhole positions	Text file	Context menu: save or copy
Save / Copy count rate (Z scan)	Count rate versus focus position	Text file	Context menu: save or copy

4.5 Evaluating a Binding/Kinetic Study

Reaction system

The following procedure is suitable for the determination of rate constants or for the evaluation of a binding study. It is based on the following reaction system:

$$A + B \Leftrightarrow C$$

Here A represents the labeled ligand, which interacts with B forming the fluorescent complex C. By determining the portion of the fluorescent components A and C it is possible to calculate the binding or kinetic constant of this reaction.

4.5.1 Analysis of the Dye

Step 1 Analyzing the dye

Ten FCS measurements of the diffusion characteristics of the dye used for labeling the ligand should be carried out.

These measurements have to be performed under the same experimental conditions which will be used for the binding or kinetic study. The correlation curves are then analyzed with the one-component fit model (Fig. 4-8).

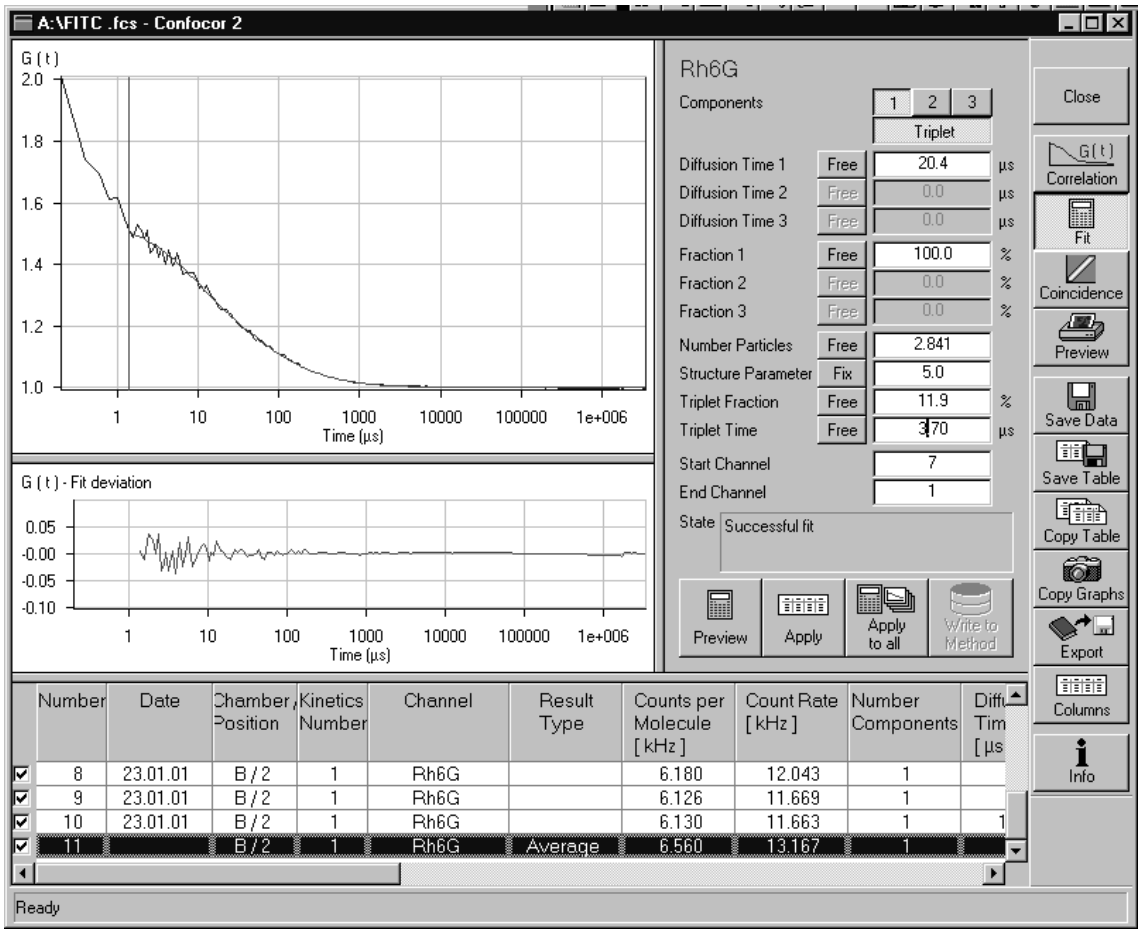


Fig. 4-8 **The fit window for a one-component fit of a correlation curve measured for a dye.**

Fixing parameters

Refer to the criteria listed in section 4.3.1 to determine the quality of the fit. Get the mean **diffusion time** and the mean structural parameter **S** for the dye from the fit of the averaged autocorrelation of the ten correlation curves. These values will then be fixed in the analysis of the correlation curves generated by the measurement of the interaction under investigation.

4.5.2 Analysis of the Labeled Ligand

Step 2 Analyzing the labeled ligand

Analyze the correlation curves generated by measurements of the labeled ligand A under experimental conditions identical to those for the measurement of the dye. Use the one-component model (7), thereby fixing the value of the structural parameter **S** at the value determined in analysis of the dye measurements, as shown in Fig. 4-9:

Unsatisfactory fit

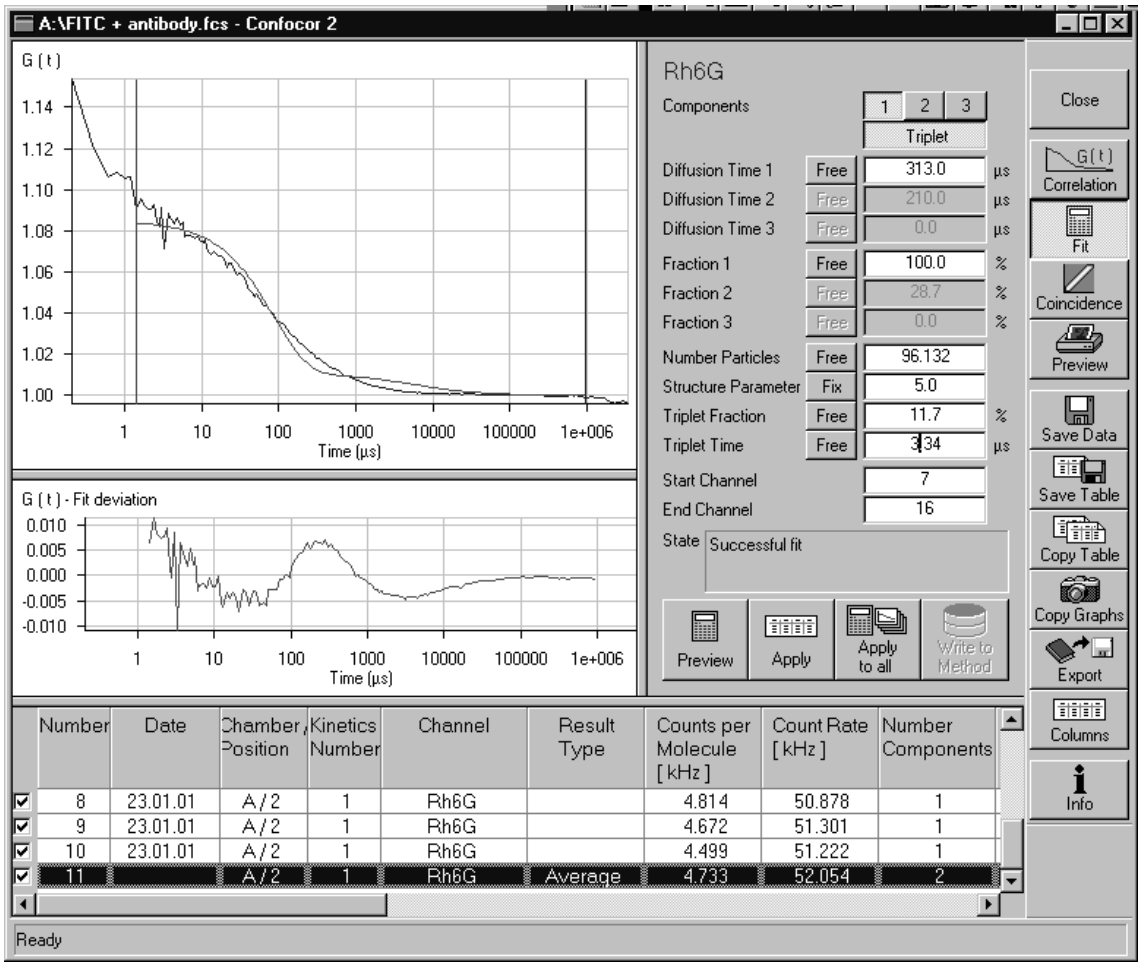


Fig. 4-9 Fit obtained with the one-component model of the correlation curve measured for a labeled molecule. Fixation of S yields an unsatisfactory result in the one-component fit.

If the **residuals curve** shows systematic deviations from the zero baseline, as shown in the figure, use the two-component model, and fix the values of the structural parameter **S** and τ_{D1}, the diffusion time of the dye used for labeling (Fig. 4-10).

Satisfactory fit

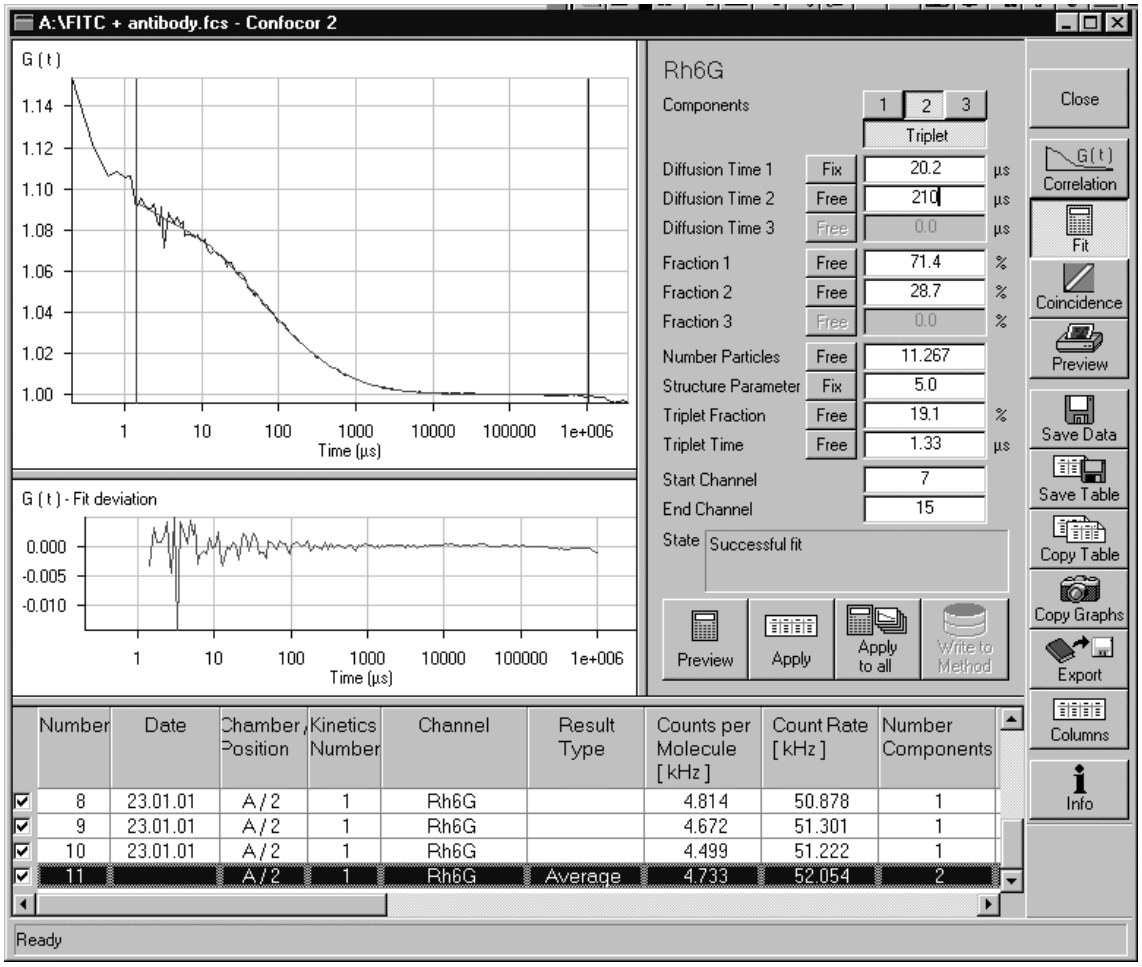


Fig. 4-10 Fit obtained with the two-component model of the correlation curve shown in Fig. 4-9. Fixation of S and τ_{D1} of the dye used for labeling results in a good fit obtained with the two-component model.

Mean diffusion time

Compile at least 10 correlation curves for this compound. Get the diffusion time τ_{D2} for the labeled ligand A from the fit of the averaged autocorrelation function. This value will later be used to analyze the correlation curves resulting from the investigation being conducted with the unlabeled interaction partner *B*.

4.5.3 Analysis of the Interaction

Labeled ligand of high purity

Correlation curves generated by measuring the interaction of interest can be analyzed using the two-component model if the labeled ligand A is free of unbound dye (Fig. 4-11). For this analysis, it is necessary to fix the diffusion time of the smaller, labeled interaction partner A (determined in the second step of this procedure, section 4.4.2), and the structural parameter **S** determined under the same experimental settings and conditions (a standard value determined by measurement of the dye alone).

Unsatisfactory fit

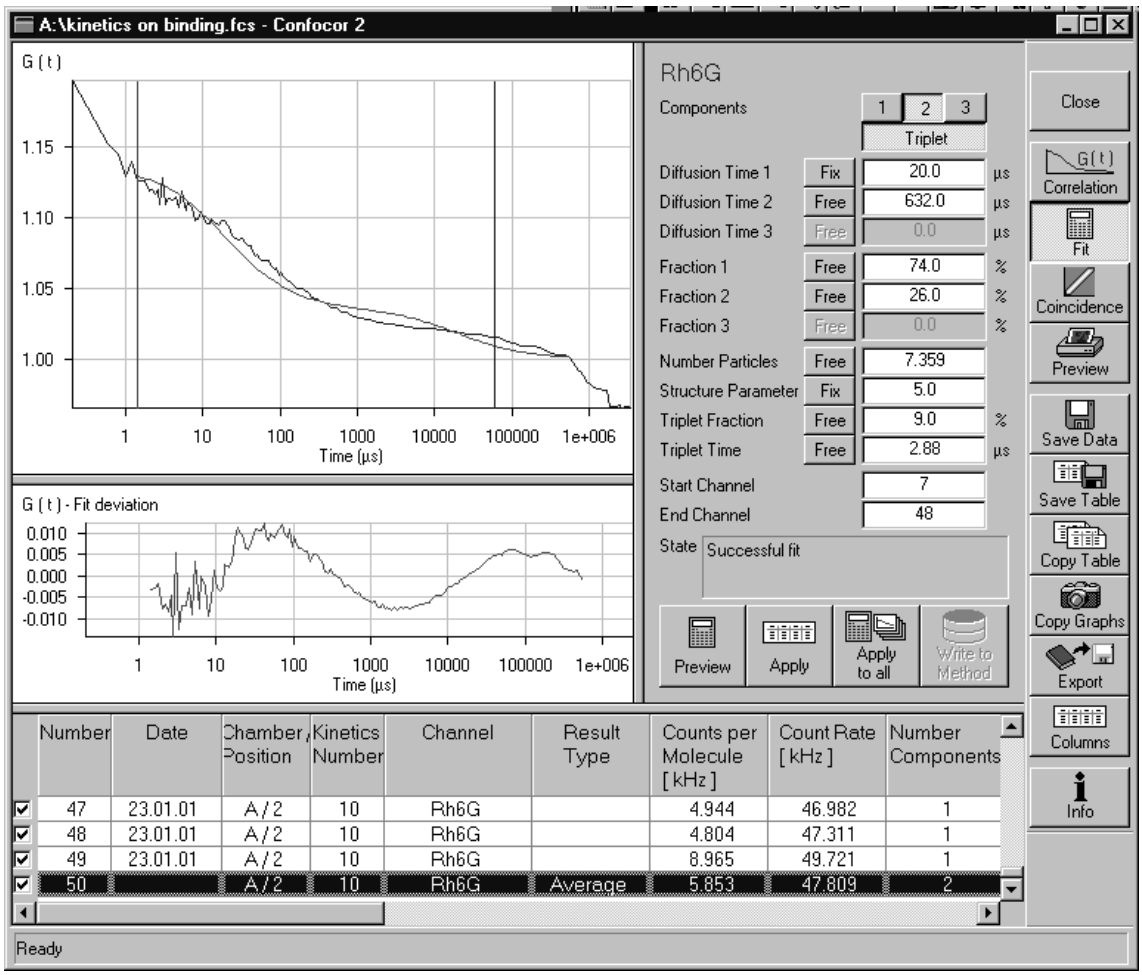


Fig. 4-11 Fit obtained with the two-component model of a correlation curve measured for the interaction of the labeled molecule characterized in Fig. 4-10. Fixation of S and τ_{D1} does not result in a good fit obtained with the two-component model.

Labeled ligand of inferior purity

Note: If the labeled ligand is not of sufficient purity (a large proportion of unbound dye present) requiring the application of a two-component model for the labeled ligand alone, the three-component model may be applied. In this case, it is necessary to fix τ_{D1}, the diffusion time of the dye, τ_{D2}, the diffusion time of the labeled ligand, and **S**, as shown in Fig. 4-12.

Excellent fit

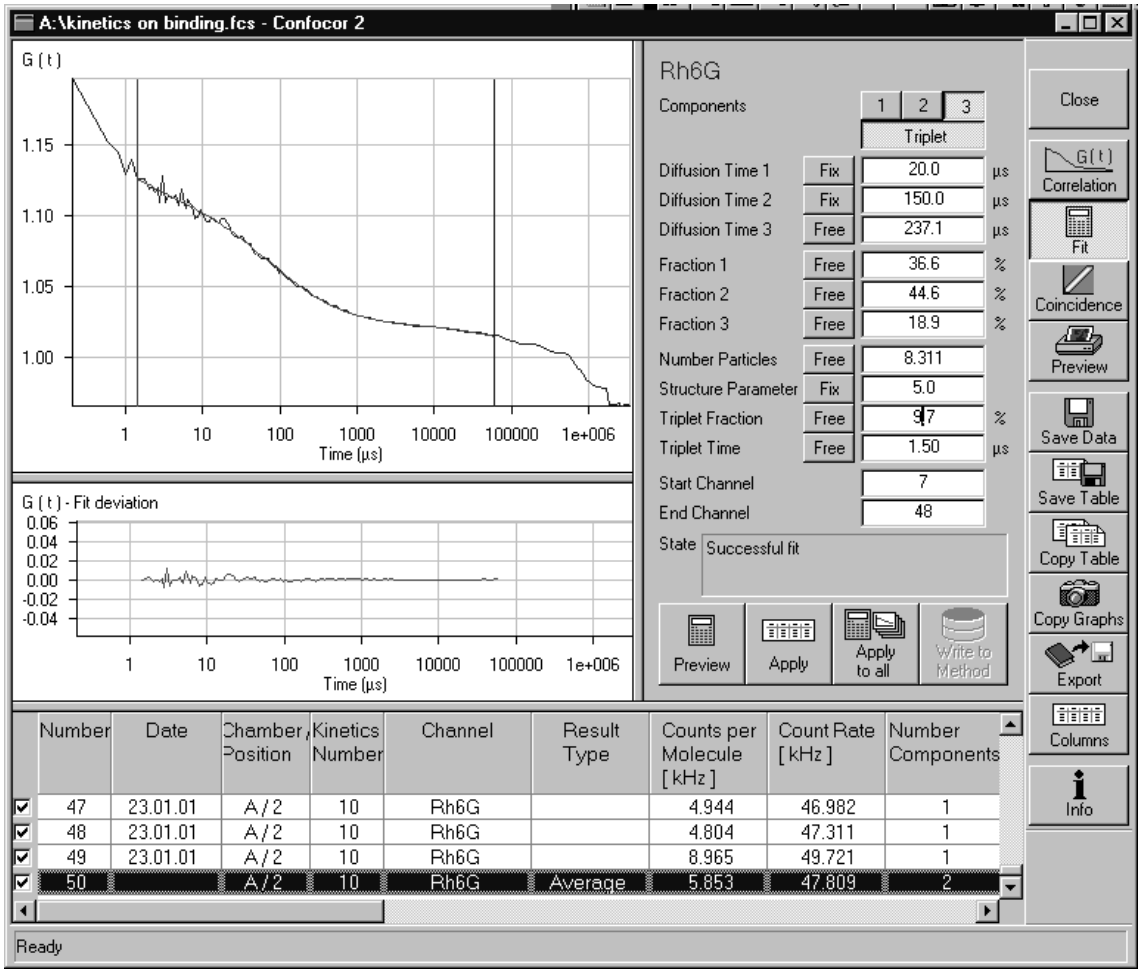


Fig. 4-12 Fit obtained with the three-component model of the correlation curve shown in Figure 4-11. Fixation of S, τ_{D1}, and τ_{D2} yields an excellent result.

Similar diffusion times

If the difference in diffusion time between the labeled ligand A and the resulting complex C is small, e.g. a factor of two or less, the determination of the diffusion time of the complex C and the number of molecules complexed is of low precision. Under such conditions, it is not only necessary to measure the labeled ligand A alone (as done in step two of this protocol, section 4.4.2), but a measurement of the complex C alone must also be done. To determine the diffusion time of the complex, a measurement can be carried out in the presence of a large excess of the non-labeled ligand B relative to the amount of labeled interaction partner.

If a two-channel system is available one should use cross-correlation for investigating reactants of similar diffusion times. For this purpose, both reactants have to be labeled with dyes possessing different excitation (488 nm, 633 nm) and emission spectra. The cross-correlation function describes the binding of both reactants.

Binding of the ligand

In principle, analyzing the correlation curves measured during the interaction of all components, with fixed values for the diffusion time of the labeled ligand A and the resulting complex C, leads to a precise quantification of the portion of bound versus non-bound ligand. This information can be used for the determination of binding or kinetic constants.

4.5.4 Autocorrelation versus Cross-Correlation

Autocorrelation is only suitable if the diffusion times between the labeled ligand alone and its bound form can be distinguished. This is normally the case if there is a difference by a factor of 2, which, for perfect spherical proteins, corresponds to a mass difference of eight. However, it depends on the forms of the molecules how large the mass difference really has to be. Hence it might be worth testing your special system.

If the difference in diffusion is not sufficiently great, cross-correlation has to be used. In this case both binding partners have to be labeled separately. In order to avoid crosstalk of the emission light of shorter wavelength into the channel set for detection of the longer emission wavelength, the excitation wavelengths of the dyes should be well separated. We recommend using dyes that will be excited at 488 nm and 633 nm, respectively. Cross-correlation can be generally used, independent of the size of the partner molecules. The readout of a cross-correlation experiment will be three correlation curves, two autocorrelation curves corresponding to each of the labeled molecules, which will give you the total number of these species, and one cross-correlation curve, from which the numbers of bound molecules can be determined (see Fig. 4-13).

You can either determine the free ligand concentration in each case by a two component fit of the autocorrelation functions or by simply subtracting the value of the bound molecules, as specified by the cross-correlation function, from the total number of labeled species, as seen with the autocorrelation function. The results will be the number of free and bound molecules for each labeled reactant. The evaluation of the binding parameters can then be done as outlined in the previous sections.

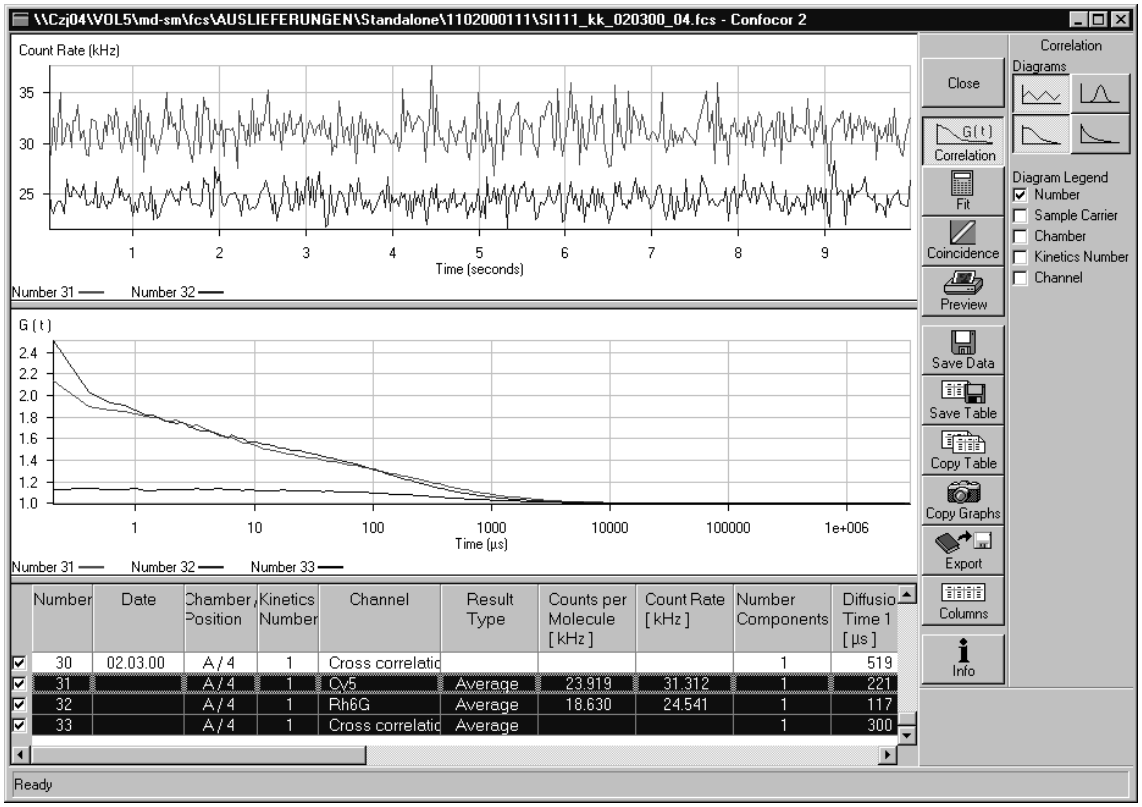


Fig. 4-13 **A typical cross-correlation experiment where two differently labeled molecules form a complex. There is an autocorrelation function for each molecule (blue and red) and a cross-correlation function for the complex (black).**

4.6 Measurements Taken on the Surface or within a Cell

4.6.1 Introduction

In combination with the LSM 510, the ConfoCor 2 allows you to take measurements within, or on the membrane of, a living cell. The only prerequisite is that you must observe diffusion, i.e. your labeled molecule of interest should not be bound by a rigid structure in order to be recognized in FCS measurements. Data evaluation is in principle the same as for solution, however, the model of free translational diffusion might not be applicable on the cell membrane or inside a cell. In this case it is advisable to export the raw and correlated data and obtain fitting routines from the literature or commercially.

Before doing cell work, the beam paths between the LSM 510 and the ConfoCor 2 are precisely matched. The accuracy of this alignment is within 1 µm. The structure of interest is then pinpointed with the LSM 510 and, after mode switching, FCS measurements can be taken at the very spot.

4.6.2 Potential Problems Associated with Cell Work

Phenol red in the medium cannot be tolerated due to its high intrinsic fluorescence. But even without it, media may show high background rates. In this case, check if fetal calf serum is present. Look for particulate matter and remove it by filtration, or check other charges. If the problem persists, check out different media or sera. Remember that some background can be tolerated as long as your specific signal is at least ten times stronger.

Some structures within cells may be autofluorescent. In this case you could check out different cell lines or try to improve on your sample by choosing different dyes or reducing the laser power. Using laser lines with higher wavelength can also be helpful.

Many dyes bind unspecifically to cell membranes. This can easily be checked in the LSM. If membrane fluorescence occurs with the dye alone, then the dye is not suitable, and you should switch your fluorescence label. If the count rate in the medium decreases in the presence of cells versus their absence, this also is indicative of an unspecific binding.

4.6.3 How to Bring Your Protein of Interest into the Cell

There are several possibilities to target a protein to the cell. One is by microinjection, where you can take any label that is best for your experiment. When you set up patch clamp experiments, the labeled protein can be introduced by the electrode. Another possibility is the expression of the protein after transfection of the cell. In this case you have to fuse your sequence of interest to a fluorescent living color like GFP. Yet another possibility is to fuse your protein to a sequence that allows the protein to penetrate the membrane. Such a sequence is, for example, represented by parts of the tat protein from HIV. You also might transiently perforate the membrane of the cell using detergents or treatment with a UV-Laser.

4.6.4 Matching up the Beam Paths between the LSM 510 and ConfoCor 2

In a chamber with a glass slide at the bottom, fill in a 10^{-6}M dilution of a dye, e. g. Rhodamine 6 Green, in ethanol. The bottom should be well covered. Dry the dye with the lid uncovered overnight. This leaves you with a thin layer of dye at the bottom. With the LSM locate the dye layer, define positions, and with the help of the FCS burn holes at these sites. Match up the positions of the holes with the sites specified by the LSM. Correct for any deviations in the FCS settings. For more details consult the operation manual. The instrument is then ready to do the cell experiments. Only choose your site of interest with the help of the LSM510 and start recording with the ConfoCor 2.

Alternatively, in the optimization method, burn a hole in the middle of your scan, mark that position and place your structure of interest right beneath it when doing your cell experiments.

4.6.5 Locating Structures of Interest

If there is enough fluorescence provided by the labeled molecule, the structure of interest, such as a membrane or a cytosolic compartment, can be localized by epi-fluorescence or scanning. Once determined, the position is marked in the LSM mode and after a switch to the FCS mode, records are taken. In the Measurement mode you can define multiple points of interest, and FCS measurements will be taken at all of them one by one (fig. 4-14).

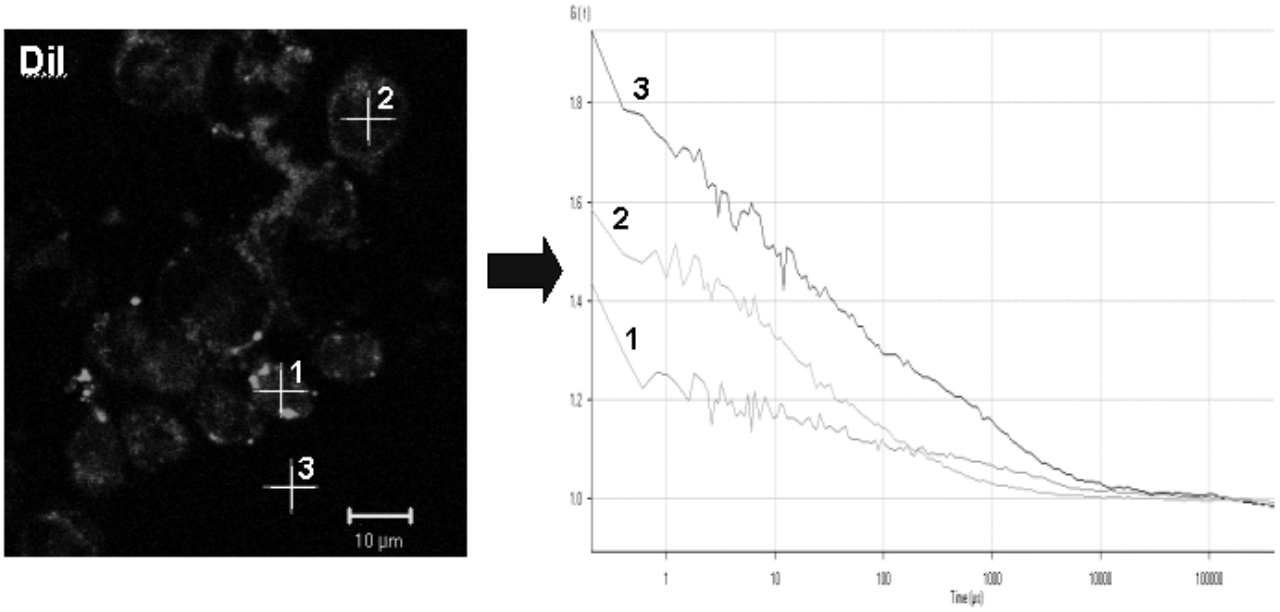


Fig. 4-14 The distribution of DiI (Molecular Probes), a lipophilic dye that incorporates into the lipid bilayers of cells, was analyzed by FCS. Spots were selected within the membrane (1), cytosol (2) and medium (3). Note that the correlation is least with the highest dye concentration in the membrane.

In the optimization method, measurements can be taken at only one position, i.e. in the middle of the scan. To take measurements in this mode, you need to place your structure of interest right under this position, which you need to determine as outlined in paragraph 4.6.3.

The optimization method will also help you to home in on a membrane. In this case, record a Z-stack in the LSM mode containing the membrane. Then make a measurement in FCS in the Z-stack mode, taking the boundary values determined with the LSM. If a labeled ligand binds to the membrane, the maximum value will show the position of the membrane. Select this position and take your FCS measurements.

An example of results from measurements on a membrane is shown in fig. 4-15.

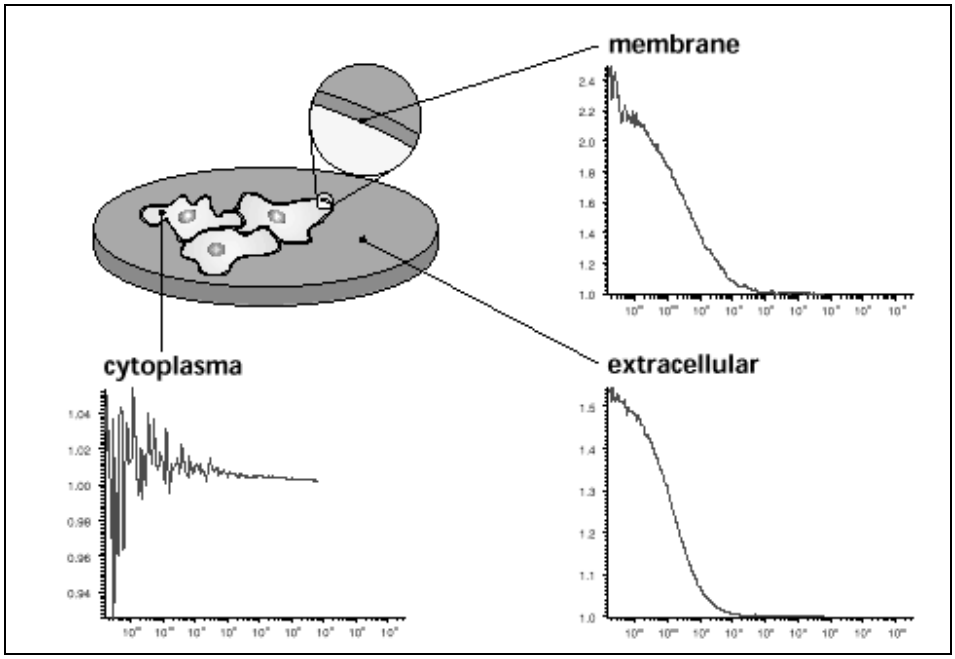


Fig. 4-15 Binding of the EGF receptor to the membrane. Note that within the membrane there is a diffusion time in the ms range, whereas in the extracellular medium the diffusion is within the µs range. There is no EGF within the cytosol of the cell, hence no correlation is observed.

4.6.6 Evaluation of the Data

A crucial point in the evaluation is to use the right fitting procedure. Within a cell or on a cell membrane, the algorithm provided for by the ConfoCor 2 software, i.e. a fitting procedure for free translational diffusion, is not applicable in general. Consult the literature or Zeiss to obtain the correct fitting algorithms. You can export the raw and correlated data in various formats allowing you to import the data into Excel or other commonly used software to do your evaluations.

5 BIOPHYSICAL FUNDAMENTALS OF FCS

5.1 Theoretical Principles of Fluorescence

5.1.1 Fluorescence Correlation Spectroscopy

Principle

When a population of fluorochromic molecules is excited by light of an appropriate wavelength, fluorescent light is emitted. Using a sensitive detection unit, such as the avalanche photodiode in the ConfoCor 2, the light intensity can be measured in a time-resolved manner.

The diameter of the observed volume created by the ConfoCor 2 is diffraction limited and thus amounts to about 0.3 µm. The confocal optics project the fluorescent light radiating out of the confocal volume onto a single photon detector. When a fluorescent molecule moves through the laser-illuminated volume, data are collected corresponding to the fluorescent signal. Using a software correlator, the autocorrelation function derived from these data is calculated.

In contrast to standard fluorescence techniques where whole fluorescence spectra or time-averaged fluorescence intensities are recorded, FCS analysis measures fluctuations of fluorescent light in a tiny volume element and analyzes them by auto- and cross-correlation of these fluctuations.

For an optimal assay design, some knowledge about the fluorescence properties of the dyes used is required. Therefore, the aim of the following sections is to introduce the user to some basic principles of fluorescence.

5.1.2 Excitation and Emission

Specific wavelength

Fluorochromes have characteristic light absorption and emission spectra. The absorption of a light quantum of a certain wavelength boosts an electron from the ground state energy level (S_0) to a higher energy level (an unstable, excited state S_n), which means that excitation occurs. When the excited electron falls back to the ground state, energy is released from the fluorochrome in the form of an emitted light quantum possessing a specific wavelength.

Excitation spectrum

The probability that incident light of a given wavelength will excite the fluorochrome can be determined from the excitation spectrum of the fluorochrome. The excitation spectrum is a plot of emitted fluorescence at a certain emission wavelength versus the excitation wavelength.

Emission spectrum

The probability that the emitted photon will have a particular wavelength is described by the emission spectrum, which is a plot of the relative intensity of emitted light as a function of the emission wavelength when excited at a fixed excitation wavelength.

Stokes shift

The emission spectrum of a fluorochrome is always shifted towards longer wavelengths (lower energy) relative to the excitation spectrum, as shown in Fig. 5-1. The difference in wavelength between the apex of the emission peak and the apex of the excitation peak is known as the Stokes shift. This shift in wavelength (energy) represents the energy dissipated during the lifetime of the excited state before fluorescent light is emitted.

The Stokes shift makes it possible to separate excitation light from emission light with the use of optical filters. The intensity of the emitted light can then be measured without the detector being affected by excitation light.

Fluorescence spectra

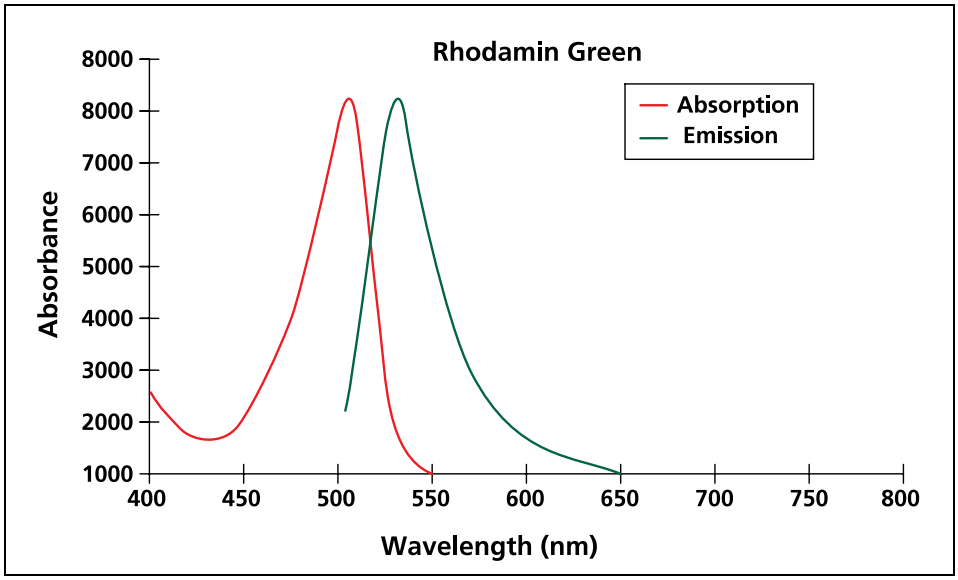


Fig. 5-1 Fluorescence spectra of Rhodamine Green.

Energy difference

The wavelength λ_{ex} of photons that can be absorbed by the fluorochrome is correlated to the energy difference E_{ex} between the ground and excited energy levels.

$$\lambda_{ex} = hc/E_{ex}$$

The excitation spectrum in Fig. 5-1 shows that light within a certain wavelength range is absorbed. The width of the excitation spectrum reflects the fact that an electron starting from any one of several vibrational and rotational energy levels within the ground state can end up in any of several vibrational and rotational energy levels within the excited state. The apex of the excitation peak is at a photon energy equal to the energy difference between the ground state of the fluorochrome and a favored vibrational level of the first excited state (S_1) of the molecule.

Likewise, the energy of an emitted photon equals the difference between the energy of the electron in the exited state and the energy level of the ground state to which it drops. This energy difference determines the **wavelength** λ_{em} of the emitted light.

$$\lambda_{em} = hc/E_{em}$$

As it can be seen from the emission spectrum in Figure 5-1, the light is not emitted at a singular wavelength but rather within a certain wavelength range. The wavelength of the emission spectrum's maximum represents the energy equal to the energy difference between the base level of the excited state and a favored vibrational level in the ground state.

The photophysical processes are summarized in Fig. 5-2.

Jablonski Diagram

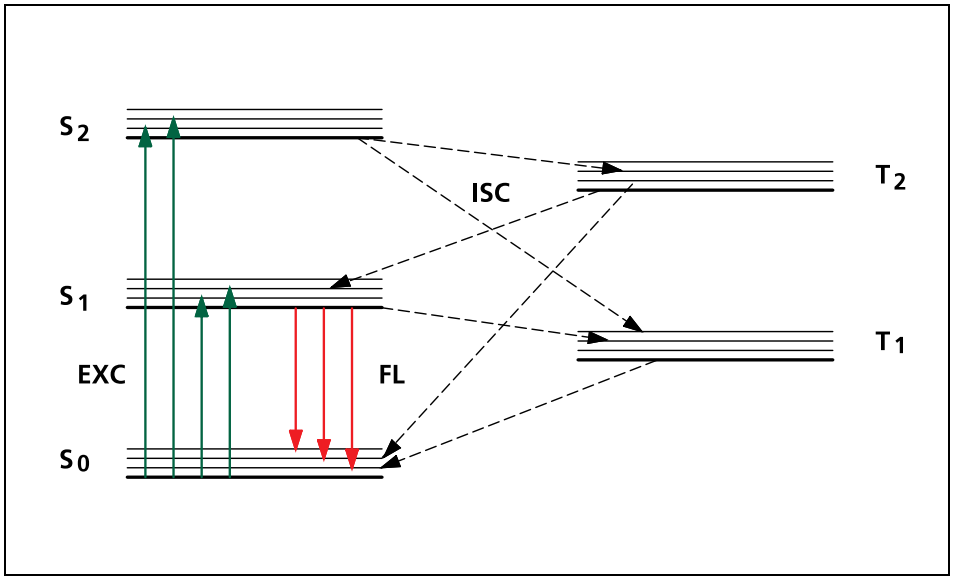


Fig. 5-2 **Jablonski diagram summarizing the typical photophysical processes. The broken lines represent intersystem crossings (ISC), solid lines either excitation (EXC) or fluorescence emission (FL). S_0 represents the singlet ground state, whereas S_1 and S_2 represent the first and the second excited singlet state. T_1 and T_2 are the triplet states, which have slightly less energy than their corresponding singlet states. Transitions between singlet and triplet states are spin-forbidden and therefore comparatively slow.**

Excited state lifetime

During the brief lifetime of the excited state, the excited electron generally decays toward the lowest vibrational energy level within the electronic excited state. The energy lost in this decay is dissipated as heat. When the electron drops from the excited state to the ground state, light is often emitted. For the fluorochromes commonly used in FCS, the half-life of the excited state $\mathbf{S_1}$ (and therefore of the emissions) is usually a few nanoseconds.

Quantum efficiency

The probability that light will be emitted is characterized by the fluorescence quantum yield or quantum efficiency Q of the fluorochrome which is defined as:

$$\mathbf{Q} = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

Values for **Q** range from 0 for non-fluorescent compounds to 1 for 100 % efficiency. Three fluorochromes that demonstrate the range of values obtained are fluorescein (**Q**≈0.9) , Cy5 ™ (**Q**≈0.3) and Rhodamine-6-G (**Q**≈0.9). In practice, **Q** is usually listed as the fluorescence quantum efficiency at the wavelength of maximum absorption.

5.1.3 Quantification of Fluorescence

The wavelength (energy) of the emission maximum is a function of the available energy levels in the fluorochrome and is independent of the intensity of the incident light. In contrast, the intensity of the emitted fluorescent light is a function of the intensity and wavelength of incident light, the quantum yield **Q** of the fluorochrome, and the concentration of fluorochrome present.

Fluorochrome concentration

When the excitation wavelength and intensity are held constant (for example, by using a controlled laser light source), the number of photons emitted is a linear function of the fluorochrome concentration.

Self-absorption

At very high concentrations, the signal becomes non-linear because the density of the fluorochrome molecules is so high that excitation occurs only at or near the surface of the volume and because some of the emitted light is reabsorbed by other fluorochrome molecules (self-absorption).

Illumination intensity

Furthermore, the number of photons emitted (the number of electrons returning to the ground state) increases with increasing illumination intensity over a certain range of excitation. If the illumination is very intense, a majority of the fluorochrome molecules will be in the excited state for a majority of the time, resulting in strong photobleaching effects.

5.1.4 Environmental Effects

The environment of a fluorochrome can affect its quantum efficiency, its excitation and emission spectra. Environmental effects on the fluorescence properties of a certain fluorochrome may be caused by changes in factors such as temperature, pH, ionic strength, solvents etc.

In addition, covalent binding to a molecule as well as non-covalent interaction can give rise to changes of the micro-environment, which may also affect the fluorescence properties of the dye molecule.

Photobleaching

An additional photophysical effect, photodestruction of the fluorochrome (photobleaching), affects the resulting fluorescence intensity of the dye, because the excited state is generally much more chemically reactive than the ground state. A small fraction of the excited fluorochromic molecules participates in chemical reactions that alter the molecular structure of the fluorochrome and create a molecule with reduced fluorescence. The rate of these reactions depends on the sensitivity of the particular fluorochrome to bleaching, the chemical environment, the excitation light intensity, and the dwell time of the excitation beam.

Antifading agent

Photobleaching often results from the reaction of molecular oxygen with the triplet excited state of dyes, producing highly-reactive singlet oxygen. To reduce photobleaching, antifading agents, such as the antioxidants phenylalanine or azide, anoxic conditions, or low-light conditions may be of help.

5.2 The Principle of FCS

Limitations of conventional fluorometry

In conventional fluorescence spectroscopy, unlike FCS, a relatively large volume element is illuminated. Therefore, the average fluorescence intensity is recorded against a high background noise of scattered light and other sources of luminescence.

This explains the limitations in resolution and sensitivity experienced in conventional fluorescence spectroscopy.

Small volume element

In FCS, a sharply focused laser beam illuminates a volume element of about 0.25 fl, a quarter of an *E. coli* cell. This volume is so small that, at a given point in time, it can host only one particle out of the many present in the sample solution.

Time resolved detection

As single molecules diffuse through the illuminated volume over time, they give rise to bursts of fluorescent light quanta. Each individual burst, resulting from a single molecule, can be registered. Hereby, confocal imaging eliminates any interference from back-ground signals. The photons are recorded in a time-resolved manner by a highly sensitive single-photon detection unit. The measured diffusion time is related to the size and shape of the particle.

Intensity fluctuations

Diffusion events of more than one particle at concentrations of >1 nM are registered as fluctuations of the emitted fluorescence intensity. All signals resulting from the diffusion of a series of particles through the confocal volume during the measurement period are recorded. The quanta belonging to a particular fluorescing molecule are identified by the auto-correlation software. The number of molecules in the illuminated volume element, as well as their characteristic translational diffusion times, can be computed from the autocorrelation curve.

Diffusion times

The autocorrelation of the time-dependent fluorescence signal allows faster and slower diffusing particles to be differentiated. Properties such as binding or catalytic activity can be calculated directly from the diffusion times as well as the ratio of smaller to larger molecules (Fig. 5-3).

Schematic correlation curve

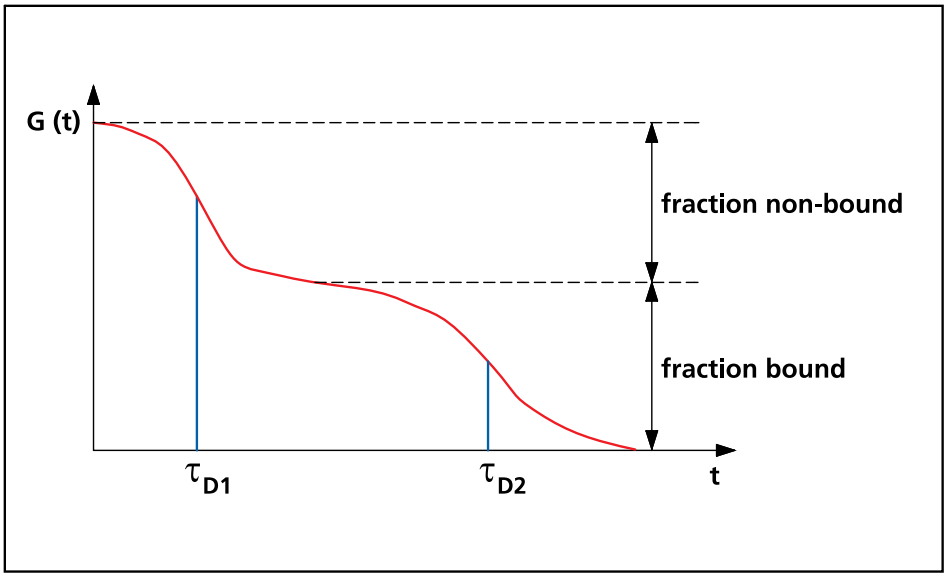


Fig. 5-3 **Schematic correlation curve describing the interaction between a small labeled ligand and a larger molecule, e.g., a receptor. The difference in the diffusion time τ_D is used to distinguish between the bound (index 2) and non-bound ligand (index 1).**

5.3 Autocorrelation

The autocorrelation of the measured fluorescence fluctuation is the key operation in elucidating the behavior of a reaction studied by FCS. Therefore, we will focus this chapter on the principle behind the term of correlation.

Dynamic process

As mentioned previously, FCS is a method for studying dynamic processes of fluorescence-marked molecules diffusing in an open volume element illuminated by a focused laser beam. Fluctuations in fluorescence are caused by molecules entering and leaving the volume element of detection and measured by an APD in a time-resolved manner. See Fig. 5-4 for an example of such a measured signal.

Intensity fluctuations

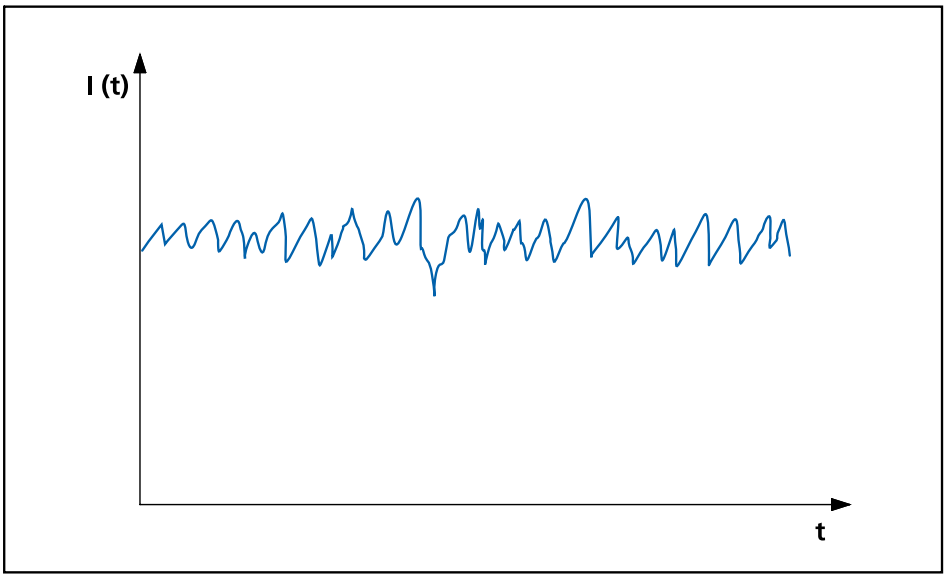


Fig. 5-4 Intensity fluctuations due to diffusion of fluorescent molecules into and out of the detection volume. I(t) represents the time-dependent fluorescence intensity measured.

Fluctuation statistics

This fluctuation can be analyzed using the autocorrelation function, which analyzes time-dependent „noise“ in order to find similarities within the measured signal that reveal its statistical properties. Fig. 5-4 shows a sketch of the measured fluctuation. It looks like noise, which is exactly what it is. The detailed behavior is of no interest, since we care only about its statistical properties, which are its mean value ⟨I⟩ (the photon flux) and the fluctuating part of the emitted light δI(t) = I(t) - ⟨I⟩.

Autocorrelation function

In case of constant intensity of excitation, the autocorrelation function G(t) is given as:

$$G(\tau) = \langle I(t) \cdot I(t+\tau) \rangle = \lim_{T \to \infty} \frac{1}{2T} \int_{-T}^{T} \langle I(t) \cdot I(t+\tau) \rangle dt$$

The autocorrelation function compares the value of the signal at any arbitrary time t with the value of a short time interval τ later. The definition of the autocorrelation is written for one particular interval τ; the complete autocorrelation function specifies G(τ) for every τ.

Since

$$I(t) = I + \langle \delta I(t) \rangle$$

the autocorrelation function has a constant term ⟨I⟩² and a time-dependent part, containing information about the kinetics in the process under investigation.

$$G(\tau) = \langle I \rangle^2 + \langle \delta I(t) \delta I(t+\tau) \rangle$$

If we divide by the squared mean ⟨I⟩² we obtain the normalized autocorrelation function

$$G'(\tau) = \frac{G(\tau)}{\langle I \rangle^2} = 1 + \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I \rangle^2} \quad .$$

Poisson distribution

Considering the Poisson-distributed statistics for small numbers of independently moving molecules and normalizing G(τ) by ⟨I⟩² shows that the limit of the amplitude of the time dependent term at G'(τ=0) is given by the inverse number of molecules **N** within the volume element.

$$\lim_{\tau \to 0} \langle \delta I(t) \delta I(t+\tau) \rangle \,/\, \langle I \rangle^2 = \frac{1}{N}$$

I(t): time-dependent fluorescence intensity
⟨I⟩: mean value of fluorescence intensity (photon flux)
N: number of molecules, N = c·V
c: particle concentration
V: size of detection (confocal) volume element

Number of molecules

FCS enables the direct measurement of average numbers of fluorescent molecules diffusing in solution, based on the interpretation of the autocorrelation curve.

What does the correlation curve of a noisy signal as shown in Fig. 5-4 look like? In order to explain the form and the information contained within G'(τ) we will focus again on the confocal volume element wherein our fluctuating fluorescence signal is measured.

Consider a single molecule diffusing through the sample. The time-dependent fluorescence I(t) is high as long as this molecule stays inside the sample volume element. When the molecule leaves this element, the intensity drops. The average length of time the molecule is present in the volume element depends on its translational diffusion coefficient.

Repetition of diffusion times

In order to extract information about the time required for a molecule to diffuse through the volume element, the signal at a given (but arbitrary) time t, I(t), is compared to the intensity I(t+τ) some time τ later, by multiplying both values. If t is sufficiently small, the product will be high since the molecule has not yet left the detection volume. For large values of t, when the molecule has left the volume, I(t+τ) is zero and so is the product. The product is calculated for various time intervals t and averaged over the measuring time. The result is the autocorrelation function $G'(\tau) = 1 + \langle \delta I(t) \cdot \delta I(0) \rangle / \langle I \rangle^2$, here in its normalized form.

A general solution of the three-dimensional autocorrelation function regarding translational diffusion and excited state - ground state transitions yields the following formula for the situation that the fluorescence decay ($\boldsymbol{\tau}_L$) and translational diffusion $\boldsymbol{\tau}_D$ are well separated in time ($\boldsymbol{\tau}_L << \boldsymbol{\tau}_D$):

$$G'(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{1 + 4D\tau / \omega_1^2} \left\{ \frac{1}{1 + 4D\tau / \omega_2^2} \right\}^{1/2}$$

D: diffusion coefficient, $D = \omega_1^2/4\tau_D$

$\boldsymbol{\omega}_1$: radius of the volume element in XY direction
(at the e^{-2} point of the Gaussian laser beam intensity)

$\boldsymbol{\omega}_2$: half length of the volume element in Z direction.

Fit results

The number of molecules, **N**, as well as their characteristic translational diffusion times, $\boldsymbol{\tau}_D$, can now be computed from the autocorrelation curve as shown in Fig. 5-5.

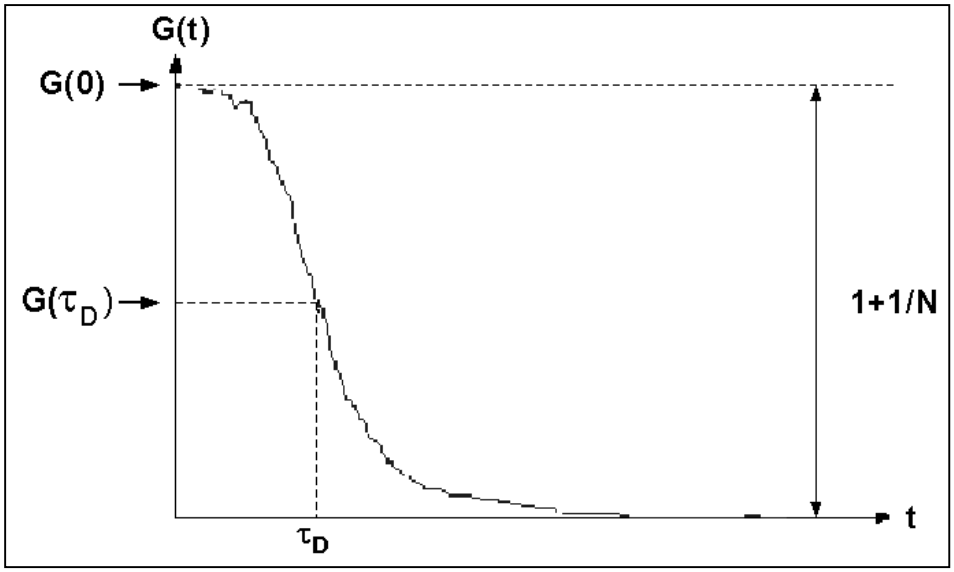


Fig. 5-5 **Sketch of an autocorrelation curve for a single diffusing particle species. The amplitude for G(0) is proportional to 1/N (G(0)=1+1/N). Therefore, an average of the number of fluorescent particles in solution is possible. τ_D corresponds to the particle's characteristic diffusion time. It is the correlation time obtained at the correlation G($\tau=\tau_D$).**

Summation of correlation functions

If there are several species present in the sample, each represented by its characteristic mean diffusion times τ_{D1}, τ_{D2}, ... , the resulting autocorrelation function G(t) is the sum of the correlation function of each species.

As shown in Fig. 5-3, two amplitudes and two mean diffusion times are represented in the correlation function, providing information about the concentration and diffusion coefficients of two distinct species.

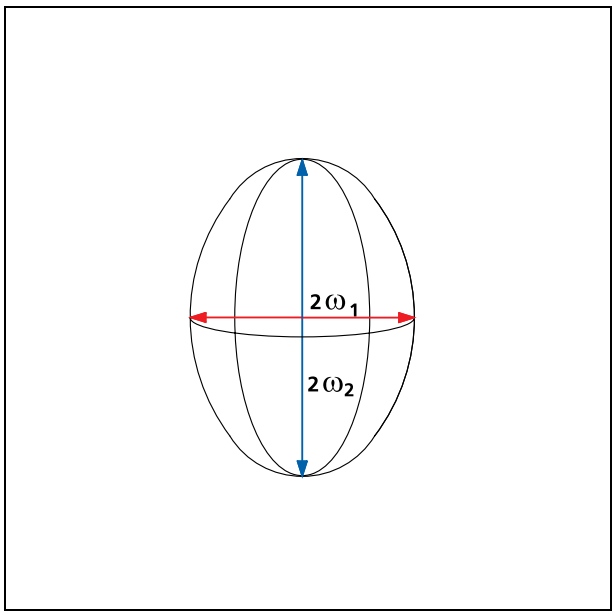


Fig. 5-6 Shape of the confocal volume element.

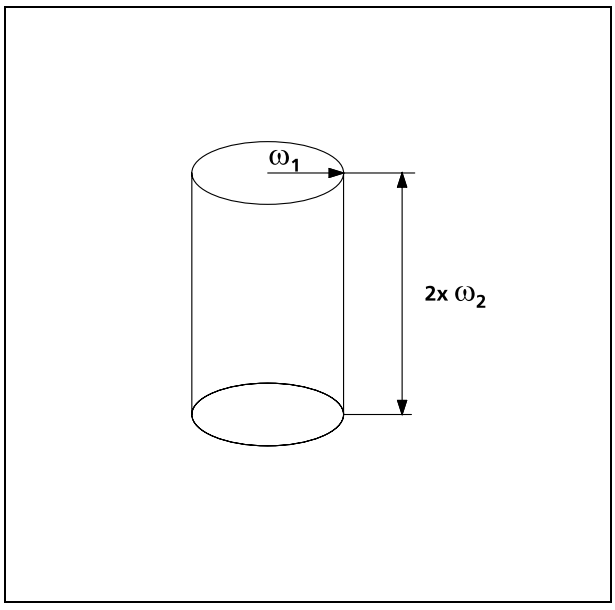


Fig. 5-7 The confocal volume as a cylinder

5.4 Confocal Volume and Concentration Determination

Using ConfoCor 2 software, the number of fluorescent particles per volume element of detection (i.e. the confocal volume) can be calculated from the correlation curve of an FCS measurement. If the size of the confocal volume element is known, the molar concentration of molecules under FCS analysis can be calculated directly from the observed number of fluorescent particles.

In the following section, some equations are shown which will be of use for the determination of concentration on the basis of the size of the confocal volume. The size of the confocal volume can easily be calculated from several parameters obtained from the fit of the correlation curve.

Confocal volume element

ω_1 represents half of the short axis of the „American football"-like volume element, ω_2 corresponds to half of the long axis of the volume (Fig. 5-6).

Simplification

The picture shown in Fig. 5-7 is an assumption for a special optical situation. The form of the volume is altered with changes in the optical settings, e.g. a change in the pinhole diameter or of the objective used. Therefore, for simplification we look at the confocal volume element as a cylinder (Fig. 5-7).

Laser beam radius

Using the following formula, normally used for calculation of the particle diffusion time through the volume element, the radius ω_1 of the laser beam can be calculated from the measured diffusion time τ_D, if the diffusion coefficient **D** of the substance under analysis is known.

$$\tau_D = \frac{\omega_1^2}{4D} \qquad \Rightarrow \qquad \omega_1 = \sqrt{4D * \tau_D}$$

(To confirm the dimensions of the confocal volume we recommend the use of a Rhodamine-6-G solution (30 nM in 150 mM NaCl), with a known **D** of 2.8 x 10^{-10} m^2/s) for the blue channel and Cy5 (30 mM in 150 mM NaCl), with a known **D** of 3.16 x 10^{-10} m^2/s for the red channel.

Length of cylinder

The length of the cylinder is given as 2x ω_2. This value can be calculated easily from the known value of ω_1, and the axial structural parameter **S** (also called the axial ratio **AR**) obtained from the fit of the correlation curve.

$$\omega_2 = S \cdot \omega_1$$

With this information, the volume of the confocal detection area can be calculated as follows:

$$V = \omega_1^2 \cdot \pi \cdot 2 \cdot \omega_2 = 2 \cdot \pi \cdot S \cdot \omega_1^3$$

Molar concentrations

If the size of the confocal volume is known, the molar concentration of the fluorescent particles can be calculated from the particle number N using the formula below:

$$c = \frac{N}{N_A \cdot V}$$

N: number of fluorescent particles per confocal volume
$\mathbf{N_A}$: Avogadro's number 6.023 x 10^{23} mol^{-1}

The following table (Table 5-1) shows the molar concentrations for different values of **N** in volume elements of different sizes corresponding to the laser lines 488 nm and 633 nm (S = 5).

N	Concentrations in different volumes, in [nmol/l]	
	V_{488nm}=0.38 fl	V_{633nm}=0.93 fl
0.1	0.44	0.18
0.2	0.87	0.36
0.5	2.2	0.89
1.0	4.4	1.8
2.0	8.7	3.6
5.0	22.4	8.9
10	43.6	17.8
20	87.4	35.7
50	224	89.2
100	436	178

Table 5-1 Molar concentrations dependent on particle number N and the size of the confocal volume element. The size of the volume elements corresponds to the laser lines 488 and 633 nm, assuming S = 5.

5.5 Theoretical Calculation of Diffusion Times

Diffusion coefficient

The diffusion of a molecule is characterized by its specific diffusion coefficient **D**. The diffusion times of different molecules have been calculated for

- spherical, globular molecules, such as proteins or antibodies;
- rod-like molecules such as DNA-helices.

5.5.1 Spherical, Globular Molecules

The diffusion coefficient can be calculated using the following formula:

Globular molecules

$$D = \frac{k \cdot T}{6\pi \cdot \eta \cdot r}$$

k: Boltzmann constant, k = 1.38 x10 $^{-23}$ J/K
T: absolute temperature (e.g. 293 K)
η: viscosity (e.g. 0.01 g/cm·s)
r: hydrodynamic radius of the molecule

The radius r can be calculated from the molecular mass m of the molecule under analysis using the following formula:

Hydrodynamic radius

$$r = \sqrt[3]{\frac{3 \cdot m / N_A}{4\pi \cdot \rho}}$$

$\mathbf{N_A}$: Avogadro's number 6.023 x 10 ^{23} mol $^{-1}$
ρ: mean density of the molecule, e.g. 1 g/cm^3

Attention: Different classes of molecules such as proteins, nucleic acids etc. exhibit deviating values for **ρ** (Table 5-2)

Mean density

Molecule class	Mean density ρ
Proteins	1.2 g/cm3
Nucleic acids	1.8 g/cm3
Lipids, etc.	0.9 - 1.1 g/cm3

Table 5-2 Mean densities ρ for different classes of molecules.

With the following formula, the diffusion coefficient can be used to calculate the diffusion time of the molecule through the focus of the laser, i.e. the confocal volume element:

Diffusion time

$$\tau_D = \frac{\omega_1^2}{4D}$$

Here ω_1^2 is equal to the square of the radius of the laser focus

Examples

Using the values of the radii corresponding to the laser lines 488 and 633 nm, i.e. 0.23 and 0.31 nm, respectively, the diffusion times τ_D can be calculated for molecules of different size. **D** represents the characteristic diffusion coefficient of the species under analysis (Table 5-3, Fig. 5-8).

Substance	MW [g/mol]	Radius [cm]	D [m^2/s]	τ_D in [µs]	
				$\omega_{1,488nm}$=230 nm	$\omega_{1,633nm}$=310nm
Dye	550	6.02 x10^{-8}	3.60 x 10^{-10}	37	67
EGF-ligand	6800	1.4 x 10^{-7}	1.54 x 10^{-10}	86	156
Antibody	140000	3.8 x 10^{-7}	5.62 x 10^{-11}	235	427
Prim./sec. antibody	280000	4.8 x 10^{-7}	4.46 x 10^{-11}	297	538
E.coli	-	1.5 x 10^{-4}	1.43 x 10^{-13}	92500	168000
Latex 100nm	-	5.0 x 10^{-6}	4.29 x 10^{-12}	3080	5600
Latex 10nm	-	5.0 x 10^{-7}	4.29 x 10^{-11}	308	560
unspecified	6000	1.34 x 10^{-7}	1.61 x 10^{-10}	82	149
unspecified	8000	1.47 x 10^{-7}	1.46 x 10^{-10}	91	164
unspecified	10000	1.58 x 10^{-7}	1.36 x 10^{-10}	97	176
unspecified	15000	1.81 x 10^{-7}	1.18 x 10^{-10}	112	203
unspecified	20000	1.99 x 10^{-7}	1.08 x 10^{-10}	122	222

Substance	MW [g/mol]	Radius [cm]	D [m^2/s]	**τ_D in [µs]**	
				$\omega_{1,488nm}$=230 nm	**$\omega_{1,633nm}$=310nm**
unspecified	25000	2.15×10^{-7}	9.99×10^{-11}	132	240
unspecified	30000	2.28×10^{-7}	9.40×10^{-11}	141	256
unspecified	35000	2.40×10^{-7}	8.93×10^{-11}	148	269
unspecified	40000	2.51×10^{-7}	8.54×10^{-11}	155	281
unspecified	45000	2.61×10^{-7}	8.21×10^{-11}	161	293
unspecified	50000	2.71×10^{-7}	7.93×10^{-11}	167	303
unspecified	60000	2.88×10^{-7}	7.46×10^{-11}	177	322
unspecified	70000	3.03×10^{-7}	7.09×10^{-11}	186	339
unspecified	80000	3.17×10^{-7}	6.78×10^{-11}	195	354
unspecified	90000	3.29×10^{-7}	6.52×10^{-11}	203	368
unspecified	100000	3.41×10^{-7}	6.29×10^{-11}	210	382
unspecified	200000	4.30×10^{-7}	4.99×10^{-11}	265	481
unspecified	250000	4.63×10^{-7}	4.64×10^{-11}	285	518
unspecified	500000	5.83×10^{-7}	3.68×10^{-11}	359	653
unspecified	1000000	7.35×10^{-7}	2.92×10^{-11}	453	823

Table 5-3 Diffusion coefficient D and the corresponding diffusion times τ_D for molecules of different size. τ_D is calculated for the radii of the confocal laser spot 0.23 and 0.32 µm, corresponing to the laser lines 488 and 633 nm.

Diffusion times of globular molecules

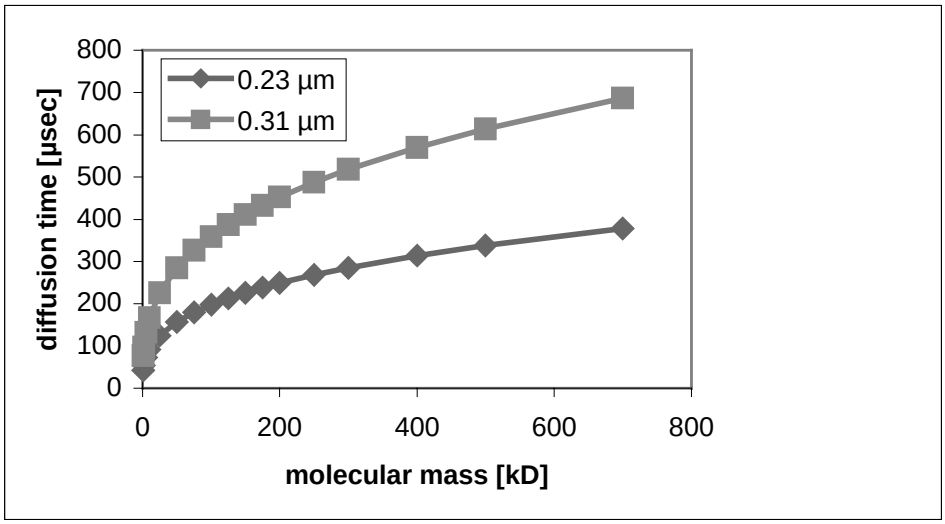


Fig. 5-8 **Diffusion time τ_D for different spherical molecules as a function of the molecular mass calculated for different confocal laser spot radii.**

5.5.2 Rod-like Molecules

The translational diffusion coefficient of a rod-like molecule, such as a short DNA molecule, is defined by the following formula [30]:

Diffusion coefficient

$$D = \frac{A \cdot k \cdot T}{3\pi \cdot \eta \cdot L}$$

A represents a correction factor

Correction factor A

$$A = \ln(L/d) + 0.312 + 0.565/(L/d) - 0.1/(L/d)^2$$

L: length of a double stranded DNA, e.g. 3.4 Å (rise per base pair) multiplied by the number of base pairs
d: diameter of the rod, e.g. d = 23.8 Å

In the following table the diffusion coefficients and corresponding diffusion times for DNA strands of various lengths are summarized. τ_D is calculated for different radii of the confocal laser spot (ω_1: 0.24, 0.30, 0.34 and 0.38 µm; Table 5-4, Fig. 5-9):

Subst.	MW [g/mol]	A	L/d	D [$10^{-12}m^2/s$]	τ [µsec]	
					ω_{488nm}=230 nm	ω_{633nm}=310nm
Dye	550	-	-	360	37	67
10 bp	6600	1.0151	1.4	128	103	188
20 bp	13200	1.5473	2.9	97.6	135	246
23 bp	15180	1.6643	3.1	91.3	145	263
25 bp	16500	1.7353	3.3	87.6	151	274
30 bp	19800	1.8937	4.3	79.7	166	301
40 bp	26400	2.1508	5.7	67.8	195	354
50 bp	33000	2.3553	7.1	59.4	223	404
100 bp	66000	3.0103	14.3	38.0	348	632
200 bp	132000	3.6841	28.6	23.2	570	1040
250 bp	165000	3.9033	35.7	19.7	671	1220
500 bp	330000	4.5886	71.4	11.6	1140	2070
1000 bp	660000	5.2778	143	6.66	1990	3610

Table 5-4 Diffusion coefficient D and the corresponding diffusion times τ_{D1}, τ_{D2}, τ_{D3}, and τ_{D4} for DNA strands of various lengths. τ_D is calculated for different radii of the confocal laser spot (ω_1: 0.24, 0.30, 0.34 and 0.38 µm).

With the calculated diffusion coefficient **D**, the diffusion time of the molecule through the focus of the laser beam can be calculated using the following formula:

$$\tau_D = \frac{\omega_1^2}{4D}$$

Diffusion times of rod-like molecules

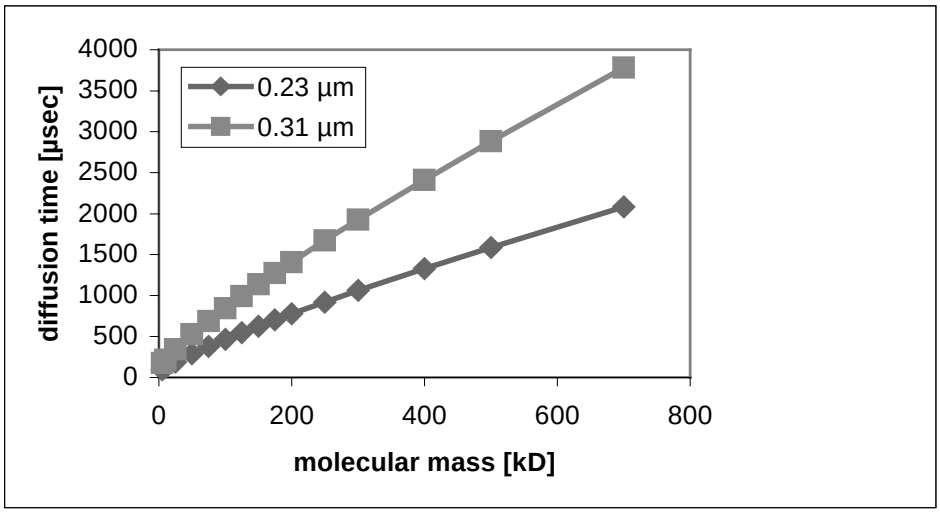


Fig. 5-9 **Diffusion time τ_D of rod-like molecules as a function of molecular mass for different confocal laser spot radii.**

5.6 Cross-correlation

5.6.1 Correlation functions

If I_g and I_r are the intensities emitted in two colors, e.g. blue and red, we obtain the following autocorrelation functions

(1a) $G_g(\tau) = \langle I_g(t) \cdot I_g(t+\tau) \rangle$ and

(1b) $G_r(\tau) = \langle I_r(t) \cdot I_r(t+\tau) \rangle$

as well as the cross-correlation function

(1c) $G_{gr}(\tau) = G_{rg}(\tau) = \langle I_g(t) \cdot I_r(t+\tau) \rangle$

Using the intensity I(t) written as a mean intensity <I> plus fluctuations δI(t)

(2) $I(t) = \langle I \rangle + \delta I(t)$

we obtain the following alternative spellings for the autocorrelation functions:

(3a) $G_g(\tau) = \langle \delta I_g(t) \cdot \delta I_g(t+\tau) \rangle + \langle I_g \rangle^2$

(3b) $G_r(\tau) = \langle \delta I_r(t) \cdot \delta I_r(t+\tau) \rangle + \langle I_r \rangle^2$

and the crosscorrelation function

(3c) $G_{gr}(\tau) = \langle \delta I_g(t) \cdot \delta I_r(t+\tau) \rangle + \langle I_g \rangle \cdot \langle I_r \rangle$.

After normalization by $\langle I_g \rangle^2$ and $\langle I_r \rangle^2$, respectively, for the autocorrelation functions and by $\langle I_g \rangle \cdot \langle I_r \rangle$ for the cross-correlation function we obtain

(4a) $$G_g'(\tau) = 1 + \frac{\langle \delta I_g(t) \cdot \delta I_g(t+\tau) \rangle}{\langle I_g \rangle^2}$$

(4b) $$G_r'(\tau) = 1 + \frac{\langle \delta I_r(t) \cdot \delta I_r(t+\tau) \rangle}{\langle I_r \rangle^2}$$

(4c) $$G_{gr}'(\tau) = 1 + \frac{\langle \delta I_g(t) \cdot \delta I_r(t+\tau) \rangle}{\langle I_g \rangle \cdot \langle I_r \rangle}.$$

Remark

In the literature the normalized correlation function G´(τ) is also referred to as G(τ), g(τ) and very often as G(t). In this manual we use the expression G´(τ) in the text sections. However, in the figures we have used G(t). Please note that the ConfoCor 2 software also uses G(t) for the normalized correlation function in its displays.

5.6.2 Biophysical model

The correlation function for translational diffusion in a 3D Gaussian volume element after normalization with the squared mean intensity $\langle I \rangle^2$ is given by

(5) $$G'(\tau) = 1 + \frac{1}{N} \cdot \left(\frac{1}{1 + \frac{\tau}{\tau_D}} \right) \cdot \left(\frac{1}{1 + \left(\frac{\omega_1}{\omega_2} \right)^2 \cdot \frac{\tau}{\tau_D}} \right)^{1/2}$$ with

(6) $$\tau_D = \frac{\omega_1^2}{4D};$$

(7) $$S = AR = \frac{\omega_2}{\omega_1};$$

(8) $$V = \pi^{3/2} \cdot \omega_1^2 \cdot \omega_2$$ and

(9) $$c = \frac{N}{V \cdot A}$$

D: Diffusion coefficient

τ_D: Characteristic diffusion time

ω_1: Radius of the volume element in xy-direction

ω_2: Half length of the volume element in z-direction

N: Number of excitable molecules in the volume element

V: Volume element

S=AR: Structural parameter or axis ratio

c: Concentration of excitable molecules

A: Avogadro number ($6.023 \cdot 10^{23}$ mol^{-1})

5.6.3 Determination of particle numbers

At a certain concentration c the number of molecules N is dependent on the size of the volume element V. Therefore N varies with the wavelength λ of the exciting laser:

(10) $$\frac{\omega_{1,g}}{\omega_{1,r}} = \frac{\omega_{2,g}}{\omega_{2,r}} = \frac{\lambda_g}{\lambda_r}$$ and hence

(11a) $$\frac{V_r}{V_g} = \left(\frac{\lambda_r}{\lambda_g}\right)^3$$ and

(11b) $$\frac{\tau_{D,r}}{\tau_{D,g}} = \left(\frac{\lambda_r}{\lambda_g}\right)^2 .$$

The corresponding functions for the crosscorrelation function $G_{gr}(t)$ are:

(12) $$V_{gr} = \left(\frac{\pi}{2}\right)^{3/2} \cdot \left(\omega_{1,g}^2 + \omega_{1,r}^2\right) \cdot \left(\omega_{2,g}^2 + \omega_{2,r}^2\right)^{1/2}$$ and

(13) $$\tau_{D,gr} = \frac{\left(\tau_{D,g} + \tau_{D,r}\right)}{2} = \frac{\left(\omega_{1,g}^2 + \omega_{1,r}^2\right)}{8D}$$

In the limit $\tau = 0$ we obtain from equation (4c)

(14) $$G_{gr}^{'}(0) - 1 = \frac{\left\langle \delta I_g(t) \cdot \delta I_r(t) \right\rangle}{\left\langle I_g \right\rangle \cdot \left\langle I_r \right\rangle} = \frac{1}{N} = \frac{N_{gr}}{\left(N_g + N_{gr}\right) \cdot \left(N_r + N_{gr}\right)}$$

For extreme cases the equation simplifies to

(15) $\frac{1}{N} = \frac{1}{N_{gr}}$ if $N_{gr} >> N_g, N_r$ and to

(16) $\frac{1}{N} = \frac{N_{gr}}{N_g \cdot N_r}$ if $N_{gr} << N_g, N_r$,

where N_{gr} is the number of crosscorrelated molecules and N_g and N_r are the numbers of molecules exhibiting no correlation.

5.6.4 Crosstalk

Equation (14) is exact only if there is no cross-talk from one channel to the other, i.e. no green emission seen in the red channel and no red emission seen in the green channel. If this is, however, case we have to correct for the crosstalk Q:

(17) $Q_{r(g)} = \frac{C_g}{C_{gr}}$,

(18) $Q_{g(r)} = \frac{C_r}{C_{rg}}$

C_g: Count rate in the green channel if only the laser light for the green dye is switched on

C_r: Count rate in the red channel if only the laser light for the red dye is switched on

C_{rg}: Count rate in the red channel if both laser lights for the green and red dyes are switched on

C_{gr}: Count rate in the green channel if both laser lights for the green and red dyes are switched on

$Q_{r(g)}$: Crosstalk of the red emission into the green channel

$Q_{g(r)}$: Crosstalk of the green emission into the red channel

Equation 14 than takes the form

(19) $$\frac{1}{N} = \frac{N_{gr}}{\left[\left(N_g + N_{gr}\right) + Q_{r(g)} \cdot \left(N_r + N_{gr}\right)\right] \cdot \left[\left(N_r + N_{gr}\right) + Q_{g(r)} \cdot \left(N_g \cdot N_{gr}\right)\right] + N_{gr}\left(Q_{r(g)} + Q_{g(r)}\right)}$$

5.6.5 Procedure for obtaining particle concentrations

1. Step: Determination of the volume elements

Make measurement with suitable dyes in both channels. For wavelengths of 488 nm and 633 nm, which is best for crosscorrelation, we recommend using Rhodamine 6 Green and Cy5, respectively. Leave all parameters free.

Adjust dye concentration (10nM – 200nM) and laser intensities to obtain the following parameters:

- Number of particles N: 0.1 – 2
- Counts per molecule: maximum (typically 10 – 200 kHz)
- Triplet fraction: <25%.

From the diffusion time τ_D and the known diffusion coefficient D of the dye you obtain the radius ω_1 of your volume element according to equation (6):

$$\omega_{1,g} = \sqrt{\tau_{D,g} \cdot 4D_g} ,$$

$$\omega_{1,r} = \sqrt{\tau_{D,r} \cdot 4D_r} .$$

From the structural parameter S (which is identical to the axis ratio AR=ω_1/ω_2) you obtain the half-height ω_2 of your volume element according to equation (7):

$$\omega_{2,g} = \omega_{1,g} \cdot S ,$$

$$\omega_{2,r} = \omega_{1,r} \cdot S .$$

From ω_1 and ω_2 you obtain your volume elements according to equations (8) and (11):

$$V_g = \pi^{3/2} \cdot \omega_{1,g}^2 \cdot \omega_{2,g} ,$$

$$V_r = \pi^{3/2} \cdot \omega_{1,r}^2 \cdot \omega_{2,r} ,$$

$$V_{gr} = \left(\frac{\pi}{2}\right)^{3/2} \cdot \left(\omega_{1,g}^2 + \omega_{1,r}^2\right) \cdot \left(\omega_{2,g}^2 + \omega_{2,r}^2\right)^{1/2} .$$

2. Step: Determination of particle numbers

Set up your binding experiment. You will obtain two autocorrelation and one cross-correlation function. From the amplitude G´(0) of the autocorrelation and cross-correlation functions you obtain according to equation (5) at $\tau = 0$:

- the number of free and complexed molecules labeled with the green dye $N_{AC,g}$ according to $N_{AC,g} = N_g + N_{gr} = \frac{1}{G_g^{'}(0) - 1}$;
- the number of free and complexed molecules labeled with the red dye $N_{AC,r}$ according to $N_{AC,r} = N_r + N_{gr} = \frac{1}{G_r^{'}(0) - 1}$;
- the number N_{CC} according to $N_{CC} = \frac{1}{G_{gr}^{'}(0) - 1}$

Hence you can calculate the number of molecules labeled with both the red and green dyes according to equation (14):

$$N_{gr} = \frac{N_{AC,g} \cdot N_{AC,r}}{N_{CC}} = \frac{\left(N_g + N_{gr}\right) \cdot \left(N_r + N_{gr}\right)}{N_{CC}}.$$

From this value the number of single labeled molecules can be calculated:

$N_g = N_{AC,g} - N_{gr}$ and

$N_r = N_{AC,r} - N_{gr}$.

If there is crosstalk Q the numbers of double labeled molecules are obtained according to equation (19):

$$N_{gr} = \frac{\left(N_{AC,g} + Q_{r(g)} \cdot N_{AC,r}\right) \cdot \left(N_{AC,r} + Q_{g(r)} \cdot N_{AC,g}\right)}{N_{CC} - \left(Q_{g(r)} + Q_{r(g)}\right)}.$$

From this value the numbers of single labeled molecules can be calculated:

$N_g = N_{AC,g} - \left(Q_{r(g)} \cdot N_{AC,r} + N_{gr}\right)$ and

$N_r = N_{AC,r} - \left(Q_{g(r)} \cdot N_{AC,g} + N_{gr}\right)$.

Often the crosstalk between the red emission into the green channel can be neglected, i.e. $Q_{r(g)}=0$. The molecule numbers can be derived from the following equations:

- $N_{gr} = \dfrac{N_{AC,g} \cdot \left(N_{AC,r} + Q_{g(r)} \cdot N_{AC,g}\right)}{N_{CC} - Q_{g(r)}}$,
- $N_g = N_{AC,g} - N_{gr}$ and
- $N_r = N_{AC,r} - \left(Q_{g(r)} \cdot N_{AC,g} + N_{gr}\right)$.

3. Step: Calculations of concentrations

Concentrations are calculated according to equation (9):

- $c_g = \dfrac{N_g}{V_g \cdot A}$,
- $c_r = \dfrac{N_r}{V_r \cdot A}$,
- $c_{gr} = \dfrac{N_{gr}}{V_{gr} \cdot A}$.

6 DETERMINING BIOPHYSICAL PARAMETERS

6.1 Monitoring Affinities

6.1.1 Introduction

Physico-chemical investigations

Biological functions result from a complex network of interactions between biological molecules and macromolecules. For a comprehensive understanding of the biological function it is necessary to characterize these interactions by physico-chemical investigations in terms of binding constants and kinetic parameters.

FCS is the method of choice for the analysis of biological systems because of its great sensitivity, the small amount of material required and its capability to deliver relevant thermodynamic and kinetic parameters in a fast and efficient way. The FCS method is based on homogeneous assays, hence the results are not influenced by surface artifacts. Therefore FCS yields precise information on physico-chemical parameters.

6.1.2 Chemical Equilibrium and Equilibrium Constant

A + B <=> C

In order to introduce some of the mathematical foundations and to provide a basis for the discussion of the FCS method, let us begin with the particularly simple case in which a macromolecule *B* binds a labeled molecule *A* forming a complex *C* .

$$A + B \Leftrightarrow C$$

In the following figure representing such a simple reaction, the change in concentration is shown as a function of the reaction time (Fig. 6-1).

Component concentration

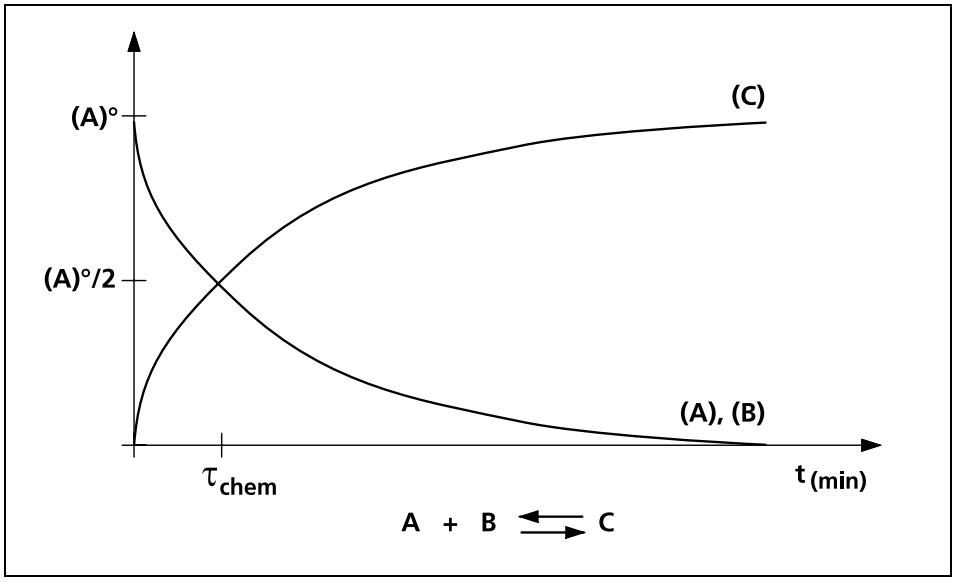


Fig. 6-1 Change in the concentration of the reactants with time

Chemical equilibrium

By definition, when considering molecules which interact with each other, chemical equilibrium is reached if no further change in concentration can be measured.

The equilibrium state is reached after a specific length of time, which is dependent upon the concentration of the reactants used and the rate constants. Therefore, it is essential to wait a sufficient length of time to allow chemical equilibrium to be attained.

“Pseudo first order” condition

In order to estimate this length of time, we assume that the concentration of *B* is at least ten times higher than that of *A*. In FCS measurements A would represent the labeled ligand. This condition is routinely employed in kinetic experiments and is known as the „pseudo first order“ condition. Now, using the following equation, the chemical relaxation time τ_{chem} can be evaluated:

Chemical relaxation time

$$\tau_{chem} = 1/\left(k_{ass} \cdot [A]_0 + k_{diss}\right)$$

$\mathbf{k_{ass}}$: association rate constant

$\mathbf{k_{diss}}$: dissociation rate constant

$[A]_0$: concentration of *A* at t=0

Example:

$k_{ass} = 10^6\ M^{-1}s^{-1}$;

$k_{diss} = 10^{-3}\ s^{-1}$;

$[A]_O = 10^{-9}$ M

=> $\boldsymbol{\tau_{chem}}$ = 8.3 minutes

General rule

As a general rule, equilibrium is attained after 6 x $\boldsymbol{\tau_{chem}}$. In the example given, equilibrium is reached after t ≈ 50 min.

Equilibrium constant

The binding constant **K** in the case of chemical equilibrium is defined for the reaction

$$A + B \underset{k_{diss}}{\overset{k_{ass}}{\Leftrightarrow}} C$$

as

$$K = \frac{[C]}{[A]\cdot[B]} = \frac{k_{ass}}{k_{diss}}$$

[*A*],[*B*] and [*C*] correspond to the equilibrium concentrations. The equilibrium binding constant **K** can be evaluated by measuring the concentration of *A* and *C*, or the concentration of free and bound ligand as in an FCS measurement. $\mathbf{k_{ass}}$ and $\mathbf{k_{diss}}$ are the rate constants for association and dissociation (section 6.3).

If we use conventional techniques which require a separation step such as filter binding, HPLC, GC, or absorption techniques for the evaluation of affinities, the binding constant **K** must be very high. This means the complex *C* must have a long half-life time $1/\mathbf{k}_{\mathbf{diss}}$, longer than the time required for separation.

Separation-free determination

If the half-life of the complex ($1/k_{diss}$) is shorter than, or similar to, the time required for separation, it will be necessary to measure the concentration in solution. With FCS and the ConfoCor 2, such concentration values can be measured in a fast and sensitive way directly in solution without the need for the separation procedures mentioned above.

6.1.3 Determination of Binding Constants

The binding constant K can easily be calculated using the results of an FCS experiment obtained after fitting a measured correlation curve of a reaction system. The procedure is described in this chapter.

Two-component fit

In the case of the simple interaction between a fluorescent ligand *A* and a larger molecule *B* resulting in the complex *C*, it is possible to determine the total number of fluorescent particles **N**, consisting of bound (*C*) and unbound ligand (*A*) as well as the portion of the two forms from the results of a two-component fit of the correlation curves resulting from an FCS measurement of this interaction process.

In the following, we will use the parameter Y defined as the ratio of the concentration of the complex *C* versus the total concentration of fluorescent particles, consisting of the complex *C* plus the free labeled molecule *A*. This value is calculated by the FCS software as a portion of Component 2.

$Y = [C] / ([A] + [C])$

With the value of Y given by the evaluation and the known size of the confocal volume V as an instrument parameter, the concentration of *A* and *C* can be calculated using the relation ([*A*]+[*C*]=N/V).

Concentrations

$$[C] = Y \cdot N/V \quad \text{and} \quad [A] = \frac{[C]}{Y} - [C]$$

When all concentrations of labeled species in solution are known, it is possible to calculate the binding constant of the interaction. For the concentration of the unlabeled *B* we can write [*B*] = $[B]_0$ - [*C*] with $[B]_0$ as the concentration of *B* at the time t = 0.

Affinity constant

$$K = [C] / \left[\left([B]_o - [C] \right) \cdot [A] \right]$$

In principle, for the simplest case of a one-to-one binding process, only one FCS experiment is needed to calculate the affinity constant **K**.

Restrictions

For practical considerations the following restrictions on the determination of binding constants using FCS have to be taken into account:

- Background fluorescence, e.g. from the buffer used, must be no more than one tenth of the signal, otherwise the determination of particle number will be inaccurate.
- A change in quantum yield as a result of the binding process will influence the calculation of the particle fractions based on the total number of fluorescent particles. It is, however, possible to apply a correction for this effect (section 6.6).
- Non-specific binding to buffer components such as BSA can obscure the results and should be avoided (see section 2.1.2 for buffer quality requirements).
- Non-specific adsorption to the walls of the sample carrier can lower the effective concentration of compounds in solution. Therefore, these effects must be minimized using detergents (section 2.2.2).

6.1.4 Procedure for the Determination of Binding Constants

Dissociation process

Sometimes it is more useful to regard the reaction under investigation as a dissociation process rather than a binding process,

$$C \Leftrightarrow A + B$$

in which we can write the dissociation constant

Dissociation constant

$$K_D = \frac{[A] \cdot [B]}{[C]} = \frac{k_{diss}}{k_{ass}} \;,\; K_D = \frac{1}{K}$$

Using $[A]_0$ and $[B]_0$ as the concentration of *A* and *B* at the time t = 0, we get:

$$K_D = \frac{\left([A]_o - [C] \right) \cdot \left([B]_o - [C] \right)}{[C]}$$

To express the results in terms which are experimentally accessible, we need a parameter which represents the average number of labeled molecules bound to each unlabeled ligand. From the fit result of the correlation curve such a parameter can be derived in form of the ratio Y.

In order to demonstrate the way from the correlation curve fitting results to the binding data, the following explanation will be focused on the simple case of a one-to-one binding process. If the number of moles of the complex *C* per unit volume (e.g. the confocal volume) is equal to [C], and the total number of moles of fluorescent particles per unit volume is given by the sum ([*A*] + [*C*]), we define the ratio Y as:

Proportion Y

$$Y = \frac{[C]}{[A] + [C]}$$

The following form, with [C] = K [A] [B], is obtained after rearrangement.

$$Y = \frac{K \cdot [B] \cdot [A]}{[A] + K \cdot [B] \cdot [A]}$$

$$Y = \frac{K \cdot [B]}{1 + K \cdot [B]}$$

From a plot of Y as a function of the free unlabeled ligand concentration $[B]_0$ at the beginning of each experiment, as shown in the next graph, the value can be determined easily (Fig. 6-2).

The value of 1/**K** can be obtained as the value of $[B]_0$ at Y = 0.5 (50 % saturation point).

Titration curve

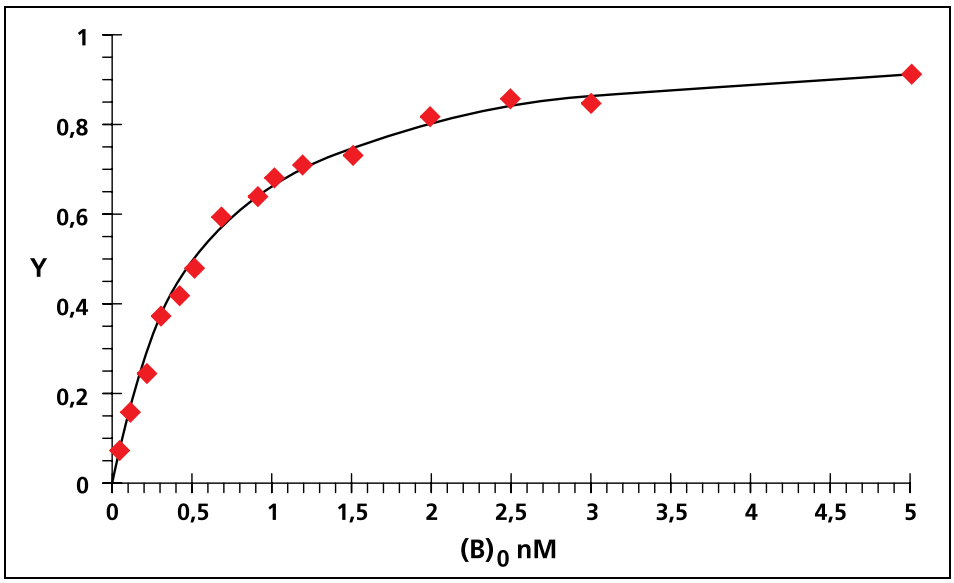


Fig. 6-2 **Titration curve leading to a value of K = 2x10⁹ M⁻¹ ($[B]_0$ = 0,5 nM) from the half saturation point. Each dot represents a measurement with a different start concentration $[B]_0$ of *B*.**

If $\mathbf{K_D}$ is used in the equation given above, the value of $\mathbf{K_D}$ instead of **K** can be read directly from the titration curve as the value of $[B]_0$ at half saturation point.

Binding mechanism

Normally, based solely on the graphical representation of binding data, it is not easy to determine which type of binding mechanism applies in any particular case. To help determine the type of binding mechanism, it is better to rearrange the equation for Y to produce a function that yields a linear graph.

One formulation for doing this is the so called Scatchard plot, which is widely used:

$$\frac{Y}{[B]} = K(n - Y)$$

for n=1, single binding site:

$$\frac{Y}{[B]} = K - K \cdot Y$$

From a Scatchard plot, as shown in the figure below, **K** can be obtained from the slope of the graph (Fig. 6-3).

Scatchard plot

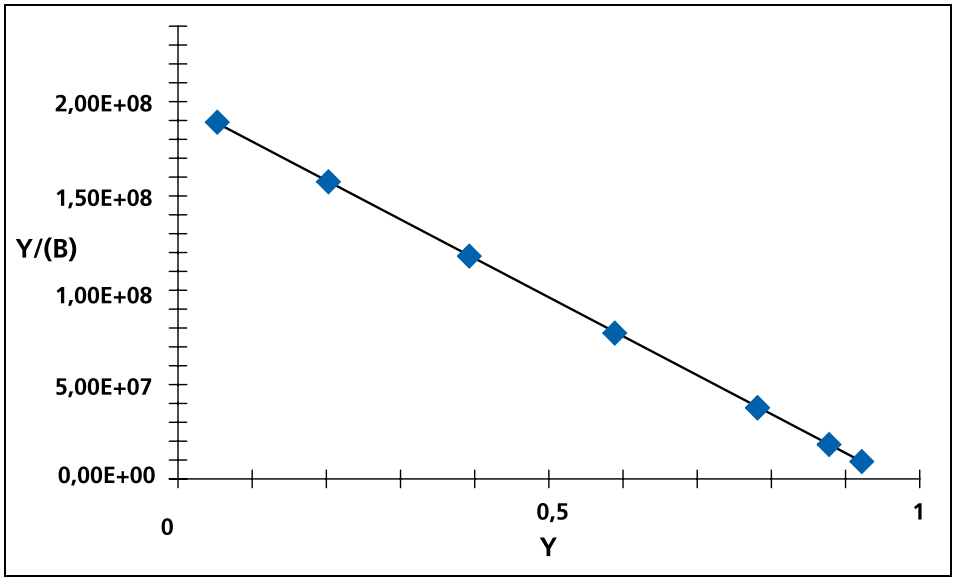


Fig. 6-3 From the Scatchard plot a binding constant of 2 x 10^8 M^{-1} can be obtained.

Avoiding artifacts

Scatchard analysis as well as other indirect analysis types for the interpretation of binding data are sensitive to small artifacts. Therefore, a direct analysis of the measured data by fitting $\mathbf{K_D}$ directly to the measured data yields stable solutions.

For this purpose, use the following equation applicable to most of the scientific data analysis and technical graphics software packages available, such as MircoCAL Origin™ :

Standard equation

$$Y = \frac{K_D + [B]_o + [A]_o - \sqrt{\left(K_D + [B]_o + [A]_o\right)^2 - 4[B]_o \cdot [A]_o}}{2[A]_o}$$

$[A]_o$ and $[B]_o$ are the concentrations of A and B at t=0; Y is determined for different $[B]_o$.

6.2 Competition Studies

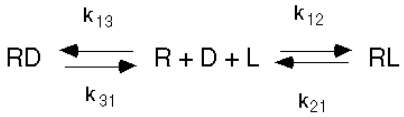
Applications

Competition studies are performed for different purposes:

- to distinguish specific binding of a ligand from non-specific binding,
- to search for new ligands with identical biological activity and possibly the same affinity or specificity,
- to find inhibitors for a specific ligand (e.g. binders without so-called intrinsic activity).

Competitor

A competitor **D** is defined as a substance which is able to bind to the same position on a receptor **R** as the specific ligand **L**.



Competition mechanism

Typically, competitors must either be preincubated with the receptor population under investigation or mixed with the labeled ligand before addition to a solution containing the receptor. Therefore, the competitor can act as shown in the equation above via the competitive pathway with an association rate of $\mathbf{k_{13}}$ and a dissociation rate of $\mathbf{k_{31}}$. The receptor, on the other hand, can also interact with the labeled ligand forming **RL**. This reaction pathway is characterized by the association rate of $\mathbf{k_{12}}$ and the dissociation rate of $\mathbf{k_{21}}$.

The time course of the association process is given in the following diagram. In general, a competitor retards the reaching of the equilibrium (Fig. 6-4).

Time course of association

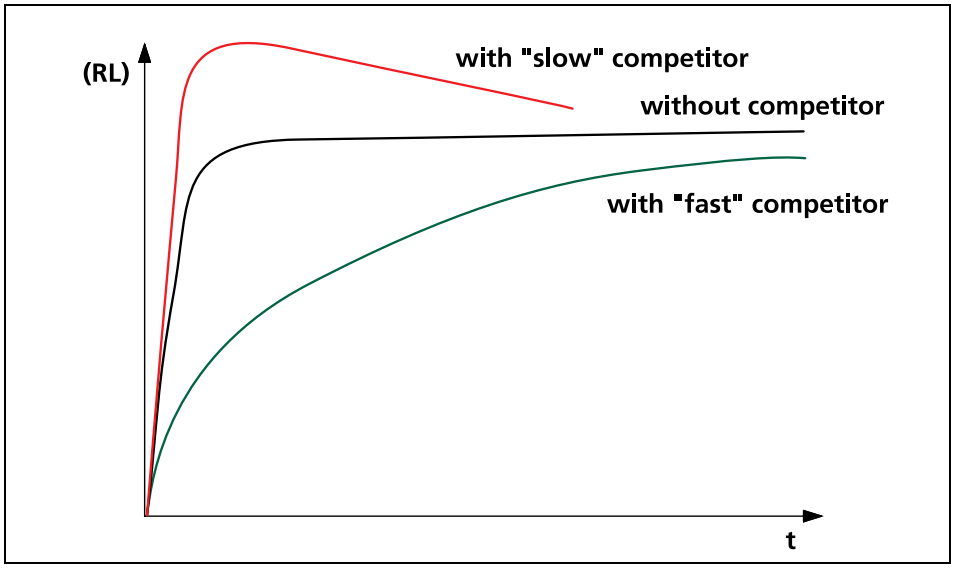


Fig. 6-4 Effect of a competitor on the time course of the association

At equilibrium the following equation can be used to evaluate the concentration of a receptor ligand complex of interest:

$$[RL]_{eq} = \frac{[R]_0 \cdot K_L \cdot [L]_{eq}}{\left(1 + K_L \cdot [L]_{eq} + K_C \cdot [D]_{eq}\right)}$$

$[\mathbf{R}]_0$: total receptor concentration

$[\mathbf{L}]_{eq}$: labeled ligand concentration at equilibrium

$[\mathbf{D}]_{eq}$: competitor concentration at equilibrium

$\mathbf{K}_L$: affinity constant of the labeled ligand

$\mathbf{K}_c$: affinity constant of the competitor

In the following graph, three examples are given for titration curves with a competitor (Fig. 6-5).

Titration with competitor

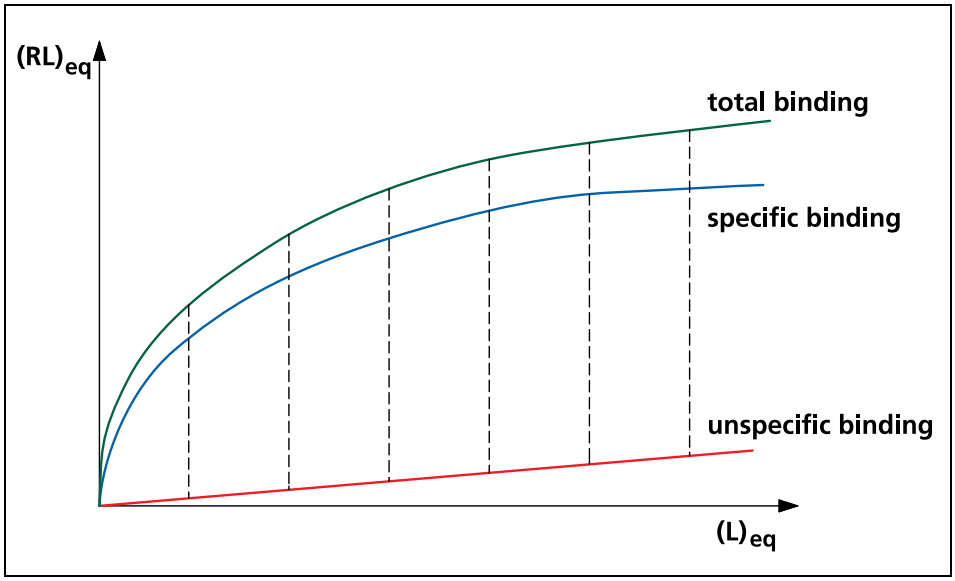


Fig. 6-5 **Effect of a competitor on the form of a titration curve. Subtraction of the amount of unspecific binding (lower curve) from the total binding curve yields the titration curve for the specific binding**

Analyzing with a Scatchard plot

Measuring binding curves, as shown above, in the presence or absence of a large excess (100-fold) of the competitor leads to corrected binding curves, which can be analyzed in the form of a Scatchard plot. An estimate of the binding constant and the number of receptors can be obtained from such a graph.

It is of great importance that the binding of the competitor to the receptor is measured at equilibrium, as in the example above. A constant amount of competitor is incubated with increasing amounts of labeled ligand (Fig. 6-6). Take into account that the competitor must be in excess.

IC_{50}

In pharmacology, a parameter called $\mathbf{IC_{50}}$ is often used, which is defined as the inhibitor concentration at which 50 % of the ligand bound to the receptor is displaced.

Different affinities

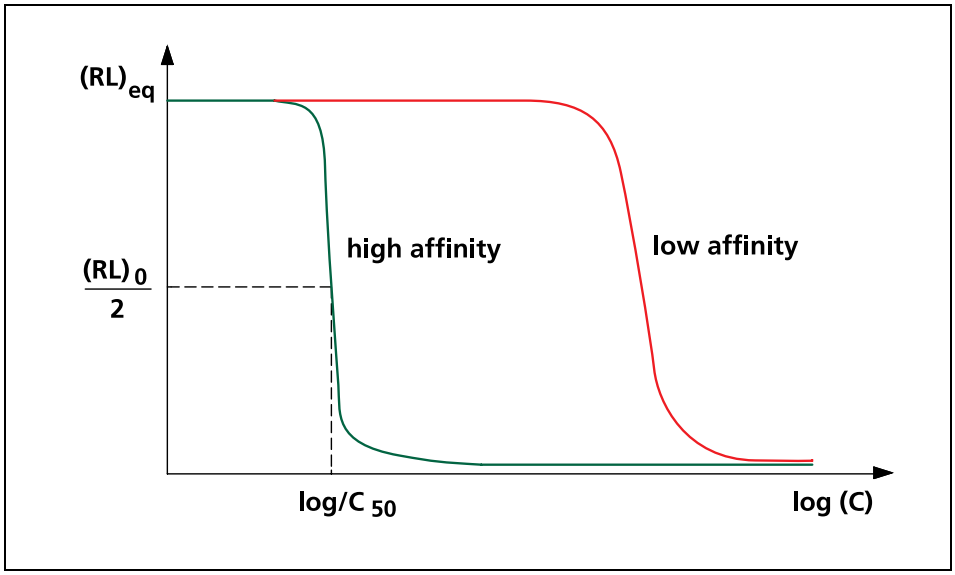


Fig. 6-6 **Form of competition curves for competitors with different affinities.**

Binding constant

Using the IC_{50} value, the affinity/binding constant **K** can be calculated from the equation given above. $[RL]_{eq}$ is equal to ½·$[R]_0$ if, at the beginning, nearly all receptors are occupied by the labeled ligand.

$$\frac{1}{K} = \frac{IC_{50}}{1 + K_L \cdot [L]_{eq}}$$

K_L: affinity constant of the labeled ligand
$[L]_{eq}$: free ligand concentration

It is important to note that a competitor will be unable to displace a labeled ligand from a non-specific binding site, for example on a receptor or a cell. This is due to the fact that non-specific binding is not mediated by structural recognition of the ligand at a defined binding site, and it is exactly the structural recognition which is shared by the competitor.

Specific binding site

In order to obtain accurate data from competition studies, it is important to be sure that only competition between the unlabeled competitor and the labeled ligand for the specific binding site is measured. Any non-specific binding of either the labeled ligand or unlabeled competitor will obscure the results.

In general, displacement will occur only up to the level of non-specific binding. This level will be identical for all competitors of a given system under investigation, as shown in Fig. 6-7.

Competition curves

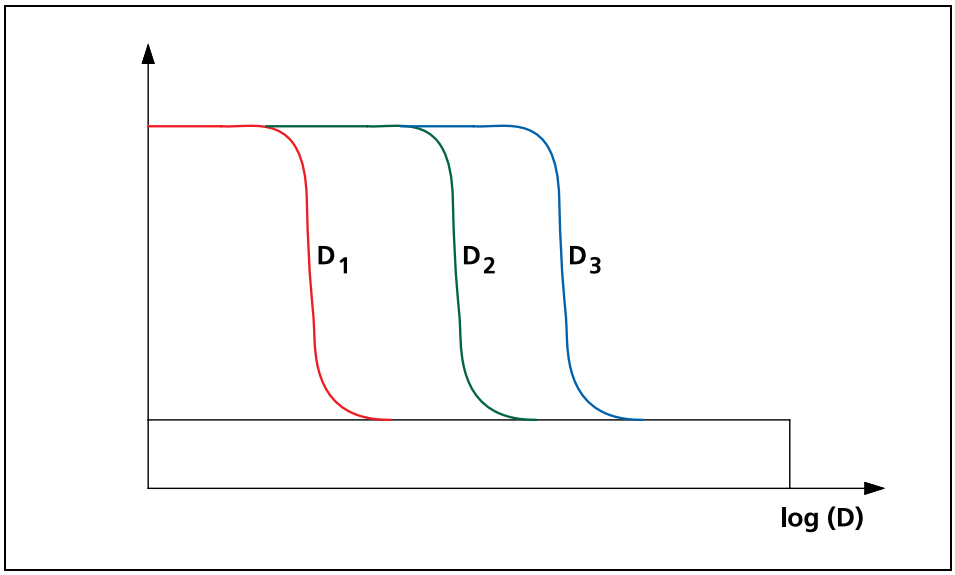


Fig. 6-7 **Competition curves for different competitors D_1, D_2 and D_3 . Displacement occurs only up to the level of non-specific binding**

6.3 Monitoring Chemical Kinetics

Undisturbed equilibrium

With FCS it is possible to measure chemical kinetic constants of molecules at low concentrations in very small sample volumes. Unlike relaxation methods, where a macroscopic perturbation is applied in the form of a temperature, electrical or pressure change, the reaction rates may be determined from spontaneous fluctuations without disturbing the equilibrium of the reacting system. In order to gain information about a reaction process by FCS, the reaction must manifest itself as a change in fluorescence or in diffusion properties.

Diffusion property changes

We will initially look only at changes in diffusion properties as a result of the reaction. Later, examples will be given of changes in fluorescence intensity and a change in the concentrations of the reactants.

For an explanation of the process of interest, let us first look at a reaction of the following type:

Reaction system

$$A + B \underset{k_{diss}}{\overset{k_{ass}}{\Leftrightarrow}} C$$

The general form of the autocorrelation function for this case contains an array of terms reflecting different combinations of diffusion and reaction mechanisms.

Limiting cases

For coupling translational diffusion and chemical relaxation, two limiting cases are of interest.

Short relaxation times

- The chemical relaxation times, given as $1/\boldsymbol{\tau}_{\mathbf{chem}} = \mathbf{k}_{\mathbf{diss}} + \mathbf{k}_{\mathbf{ass}} \cdot [B]_o$ are much shorter than the characteristic diffusion times, i.e. the chemical exchange is equilibrated during the course of diffusion through the volume element.

$$\tau_{chem} << \tau_D = \omega^2 / 4D$$

Long relaxation times

- The chemical relaxation times are much longer than the characteristic diffusion times, and the chemical exchange does not take place on the same time scale as diffusion through the confocal volume:

$$\tau_{chem} >> \tau_{D}$$

Autocorrelation function

In the second case, the autocorrelation function is given as:

$$G'(\tau) = 1 + \frac{1}{N}\left\{\frac{1-Y}{1+4D_{free}\cdot\tau\,/\,\omega^2} + \frac{Y}{1+4D_{bound}\cdot\tau\,/\,\omega^2}\right\}$$

Y: portion of ligand bound, Y = [C]/([A]+[C])

D_{free}: diffusion coefficient of free labeled ligand

D_{bound}: diffusion coefficient of bound labeled ligand

Change in quantum yield

Often a change in the quantum yield of the labeled ligand occurs if it is bound to the unlabeled interaction partner. This quantum yield change is taken into consideration in the following formula.

$$G'(\tau) = 1 + \frac{1}{N[(1-Y)+YQ]^2}\left\{\frac{1-Y}{1+4D_{free}\cdot\tau\,/\,\omega^2} + \frac{\mathbf{Q}^2\cdot Y}{1+4D_{bound}\cdot\tau\,/\,\omega^2}\right\}$$

where

$$\mathbf{Q} = \frac{\left(\Phi_{\mathbf{f}}\varepsilon_{abs}\right)_{bound}}{\left(\Phi_{\mathbf{f}}\varepsilon_{abs}\right)_{free}}$$

Φ_f: fluorescence intensity

ε_{abs}: extinction coefficient

Ratio of bound to total ligand

The association or dissociation reaction can be analyzed from a plot of the ratio of bound to total ligand (e.g. percentage of complex formed) against reaction time.

Relaxation time

If the ratio increases exponentially, the following formula can be used to calculate the relaxation time τ_{chem} for the reaction given before.

$$\frac{[C]}{[A]_0} = 1 - \exp^{\left(-\frac{t}{\tau_{chem}}\right)}$$

In the next figure, a typical time course of the association process is shown. Using the formula given, a theoretical curve was calculated for a relaxation time τ_{chem} = 7.1 min (Fig 6-8) .

Typical time course

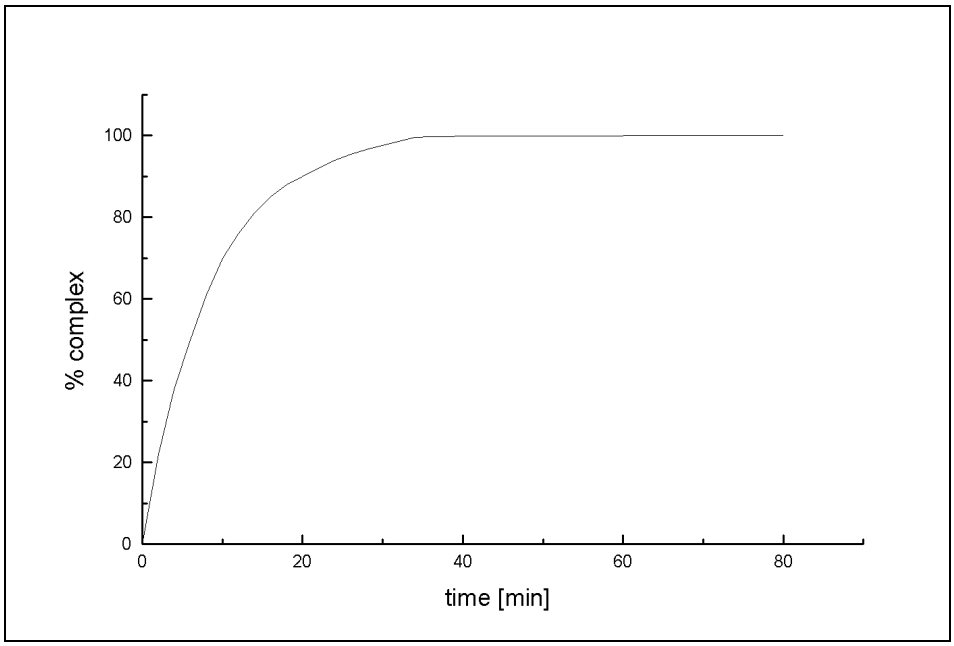


Fig. 6-8 Theoretical time course of an association process, τ_{chem} = 7.1 min.

The relaxation time τ_{chem} for the reaction given above is calculated by use of the following formula:

$$\frac{1}{\tau_{chem}} = k_{ass} \cdot [B]_0 + k_{diss}$$

If association experiments are carried out at different start concentrations of B ($[B]_0$) yielding the corresponding τ_{chem}, the association rate constant k_{ass} can be obtained from the slope of a plot of the reciprocal relaxation time as a function of $[B]_0$. The dissociation rate constant k_{diss} can be extracted from the intercept of the linear regression (Fig. 6-9).

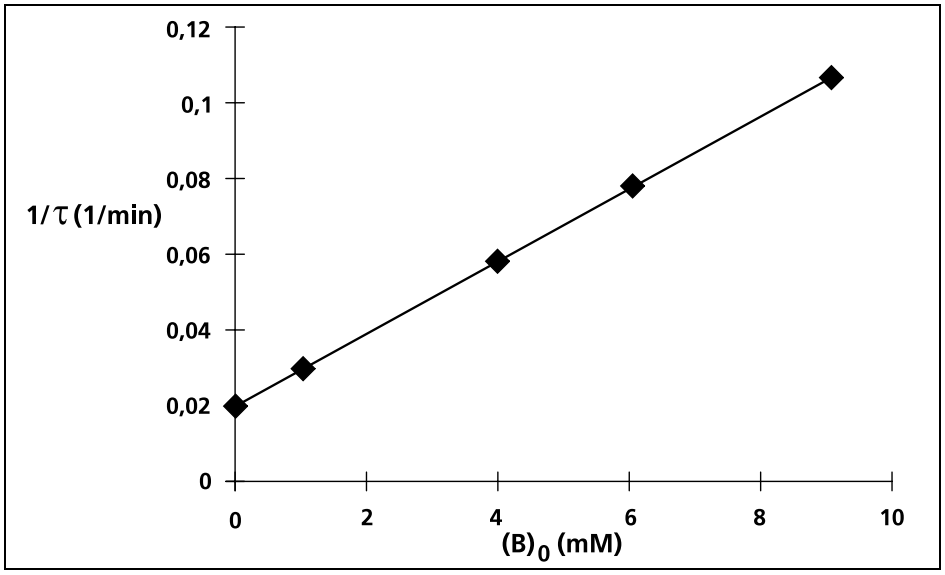


Fig. 6-9 Dependence of the reciprocal relaxation time on the concentration of one reactant.

In most cases k_{diss} is too small to be determined accurately from such plots. Therefore, the dissociation constant must be determined in a separate experiment, such as the experiment described in section 6.3.2.

An excess (100-fold) of unlabeled ligand must be added to the solution. Under these conditions, re-association is prohibited and the dissociation process can then be described as a simple one-step mechanism following a single exponential decay:

$$y(t) = \exp^{\left(\frac{-t}{\tau_{chem}}\right)} \cdot [C]_0$$

The time constant $\boldsymbol{\tau_{chem}}$ can be determined from the slope of a logarithmic plot:

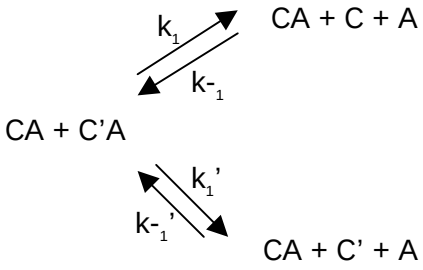
$$\ln\left([C]_t - [C]_0 \right) = \frac{-t}{\tau_{chem}}$$

For the case of dissociation, $\boldsymbol{\tau_{chem}}$ is the direct inverse of $\mathbf{k_{diss}}$.

$$\frac{1}{\tau_{chem}} = k_{diss}$$

Dissociation steps

For a dissociation mechanism such as



consisting of two dissociation steps, two relaxation times will be observed, leading to two dissociation rate constants:

$$y(t) = \exp^{\left(\frac{-t}{\tau_{chem}}\right)} \cdot [C'A]_o + \exp^{\left(\frac{-t}{\tau_{chem}}\right)} \cdot [CA]_o$$

The dissociation process following this equation is biphasic.

6.3.1 Procedure for On-Rate Determination

The following procedure for the determination of on-rates is based on the following reaction system:

"On-rate" reaction system

$$A + B \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} C$$

For purposes of simplification, the rate determination procedure is carried out under pseudo first order conditions whereby the unlabeled reactant is present in at least 10-fold excess. The formulas given here must be corrected for conditions other than first order.

Step 1: Determine τ_{D1} of labeled ligand

- First, pipette the labeled interaction partner (here A) into the Nunc chamber, which is positioned above the objective. Ten FCS measurements are then taken and the correlation curves are analyzed using the one-component fit model. Consult the criteria listed in section 4.5 to determine the quality of the fit. If the fit is of insufficient quality, the correlation curves must be analyzed using the two-component fit model by fixing the values of **S** and τ_{D1} (the diffusion time of the dye used for labeling). Calculate the mean diffusion time for the labeled ligand from the results of these fits. This value will later be used to analyze the correlation curves resulting from the kinetic investigation conducted with the unlabeled interaction partner.

Step 2: Measure association

- Second, mix the unlabeled interaction partner (B) with the existing solution containing the first interaction partner (A). Immediately start repeated correlation measurements. It is often advantageous to include a break between individual measurements to allow for regeneration of the solution and protection against photo damage, though this is not necessary for all types of solutions.

Step 3: Analyze association

- After this series of correlation curves have been measured, analyze the curves using the two-component model. For this analysis, it is necessary to fix the diffusion time of the smaller, labeled interaction partner (determined in the first step of this procedure), and axis ratio **S**, a standard value determined by measurement of the dye alone under the same experimental settings and conditions.

Note: If the labeled ligand is not of sufficient quality (a large proportion of unbound dye present), requiring the application of a two-component model for the labeled ligand alone, the three-component model may be applied, necessitating the fixation of τ_{D2} along with τ_{D1} and **S**.

Complex forming

From the results of the fits, the percentage of complex C formed at a specific time during the course of the reaction is of interest. These percentages are plotted against reaction time, and the resulting curve is compared to various kinetic models to describe the reaction mechanism (Fig. 6-10).

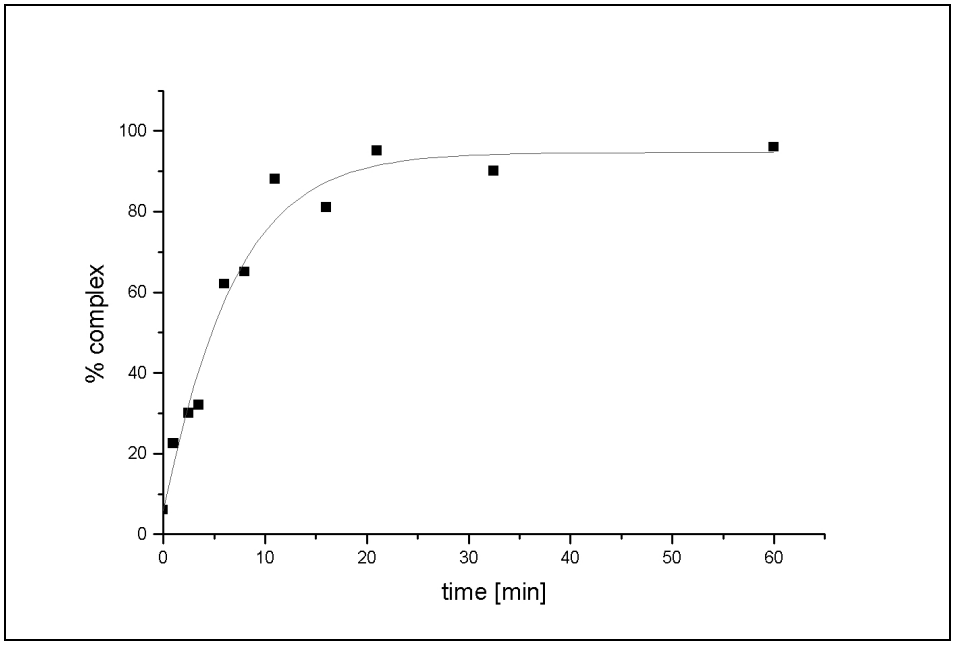


Fig. 6-10 **On-rate determination. The dots represent the measured amounts of formed complex as a function of time. Measurement time per dot was 30 seconds; fits were performed using the two-component model with fixed values of τ_{D1} , τ_{D2} and S.**

6.3.2 Procedure for Off-Rate Determination

The following procedure is used for the determination of off-rates based on the following reaction system:

"Off-rate" reaction system

$$C \underset{k_1}{\overset{k_{-1}}{\Leftrightarrow}} B + A$$

where C and A are fluorescent. A represents the smaller one of the two fluorescent particles, e.g. the labeled ligand.

Step 1: Diffusion time of the labeled ligand

- First, measure the labeled ligand A alone in the buffer used for the kinetic study. Compile at least 10 correlation curves for this component. Analyze the resulting autocorrelation curves using the one-component model. If the quality of the fit is insufficient (consult the criteria in section 4.5), analyze the correlation curves using the two-component model by fixing the values of the structural parameter and diffusion time 1, i.e. the diffusion time of the dye used for labeling. Calculate the mean diffusion time for the labeled ligand from the results of the ten fits. This value is then used for analyzing the correlation curves resulting from the kinetic study carried out.

Step 2: Measurement of complex

- Second, pipette an equilibrated mixture of *A* and *B* (the complex *C* is also included in this solution) into the Nunc chamber and measure the correlation curve of the solution 10 times under the same settings and conditions as used for the measurements of the labeled ligand.

Step 3: Analysis of complex

- Analyze the resulting correlation curves using the two-component model included in the ConfoCor 2 fitting software. Evaluate the mean value of the diffusion time and the amount of complex formed, e.g. slow-diffusing particles.

Step 4: Measurement of dissociation

- Now add a 10- to 100-fold excess of unlabeled A to the solution, mix well and start a series of repetitive correlation measurements (identical to the on-rate measurements). The unlabeled A is now displacing the labeled A in the complex. The proportion of fast-diffusing molecules in the solution increases as the amount of slow (complexed A and B) molecules decreases.

Step 5: Analysis of dissociation

- From a fit of the correlation curves, identical to the fits for on-rate determination, the amount of complex formed is obtained and is plotted against reaction time. From this plot, the off-rate can be determined.

Displacement kinetics

The following figure shows the results of a displacement kinetic (Fig. 6-11).

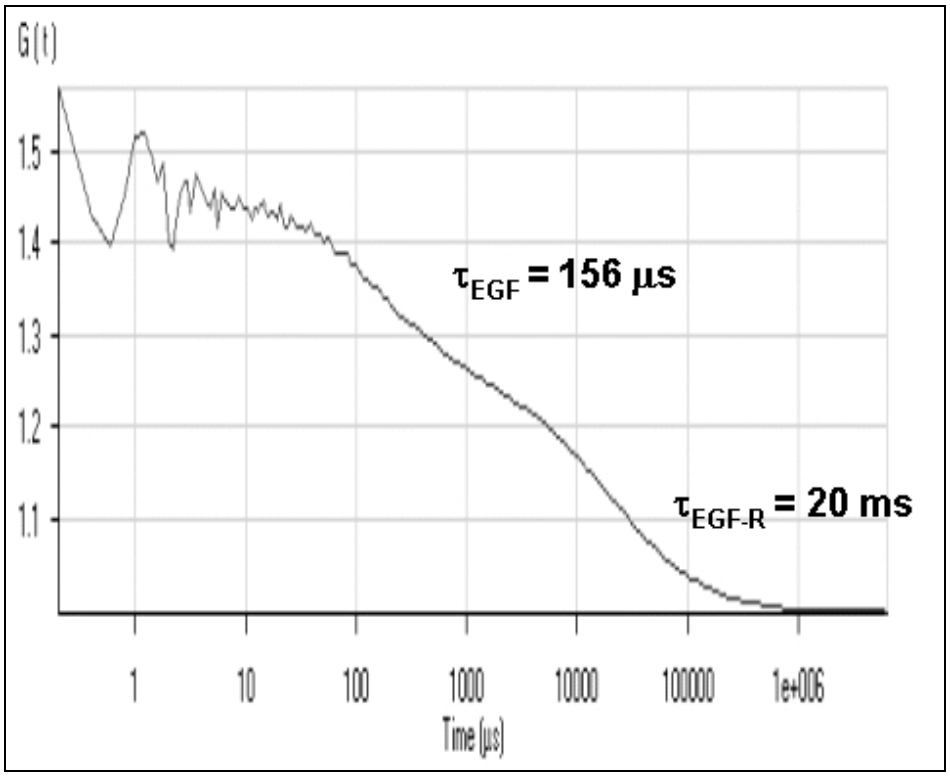


Fig. 6-11 Correlation curve of a receptor binding study. The interaction was measured with EGF-receptors on membrane vesicles with labeled EGF

Reaction system

Vesicles (membrane preparation 259 µg/ml) formed from A431-cells bearing 10^6 EGF receptors per cell were premixed with labeled EGF (5 nM), resulting in 55% complex. A 100-fold excess of unlabelled EGF was then added and the correlation curves were measured and evaluated as described above.

Two-step mechanism

In the following graph the amount of slow-diffusing particles (% complex) is plotted against time. The plot yields a best description of the results as a two-step (biphasic) mechanism in which the fast step corresponds to the dissociation of the ligand from the low affinity binding site on the receptor, and the slow dissociation process corresponds to dissociation from the high affinity binding site (Fig. 6-12).

Dissociation kinetics

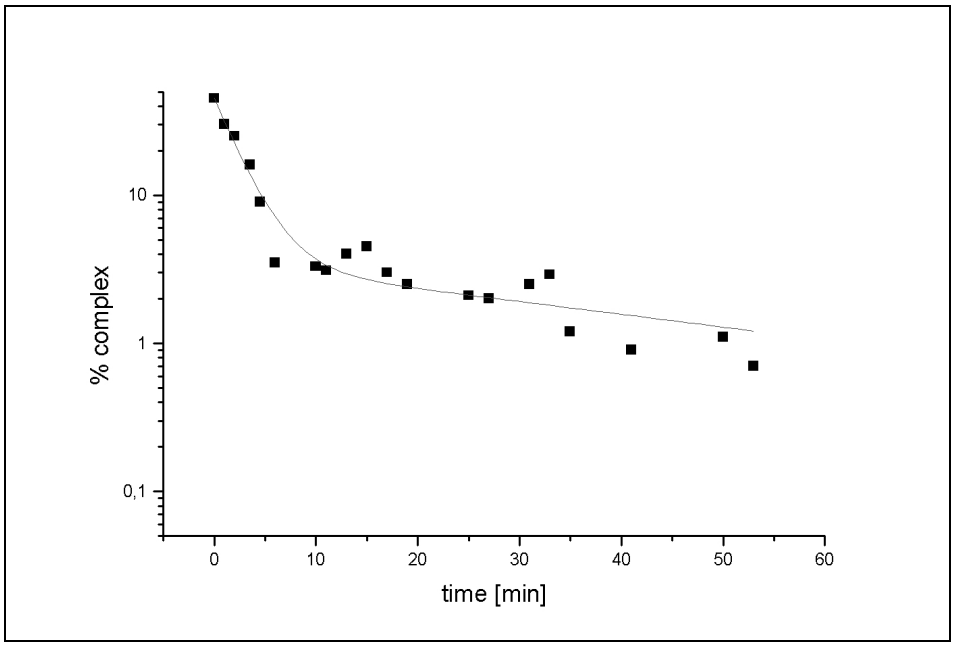


Fig. 6-12 Dissociation kinetics of EGF from the EGF-receptor, measured on membrane vesicles.

In general, any information about a reaction of interest can be used to elucidate a reaction mechanism, as long as this information changes over time. In FCS, we can use the relative quantity of different diffusing particles, as described above, or the absolute number of particles and the measured fluorescence intensity for analyzing reaction kinetics; the only condition is that a change over time must be observable.

6.4 Examples for Analyzing Kinetics

6.4.1 Protease Assay

Digestion of casein-resorufin

In the following example the potential of FCS to analyze enzyme reactions is shown for a protease assay. Casein, which is multilabeled with resorufin, is digested by chymotrypsin. The assay is easily performable; a standard protocol and some correlation curves will be shown at the end of this chapter in order to visualize the procedure.

As the reaction proceeds, the total count rate of fluorescence photons increases as the self-quenching of the resorufin fluorescence is minimized. This can be seen in the following figure (Fig. 6-13).

Total count rate

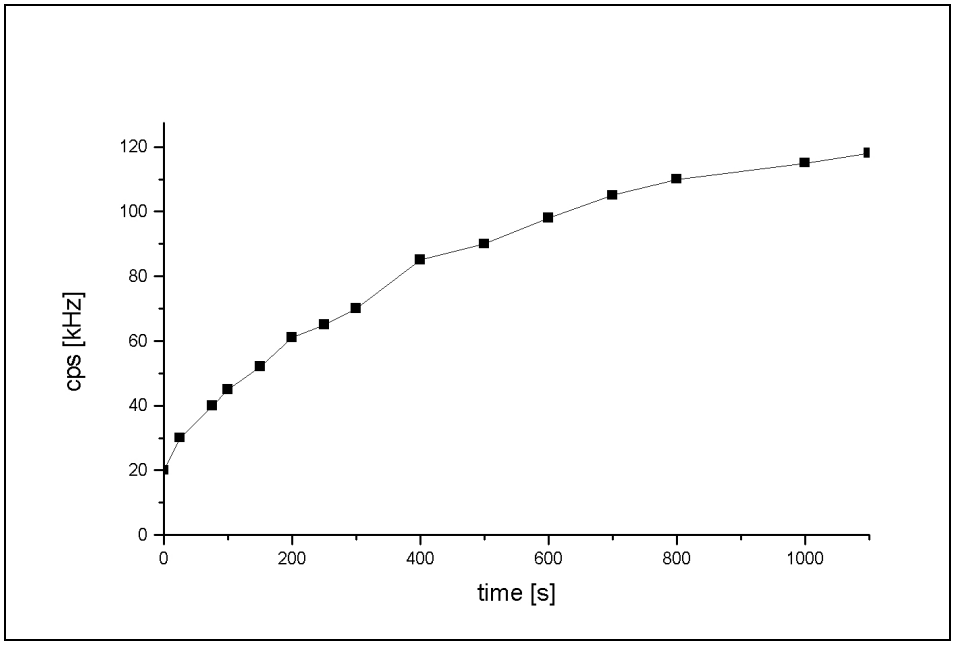


Fig. 6-13 Increase in the total count rate (cps) as a function of time for the digestion of chymotrypsin

Also, a change in the number of fluorescent particles can be observed and used for the characterization of the reaction kinetics (Fig. 6-14).

Number of particles

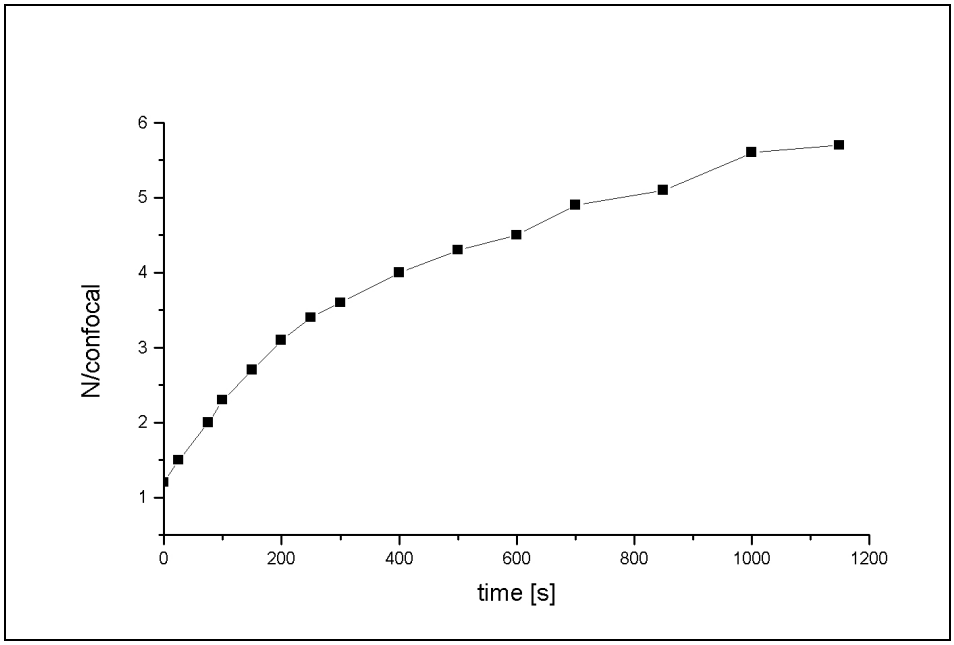


Fig. 6-14 Increase in the number of particles (N) per confocal volume as a function of time.

A fast check of the enzyme reaction may be performed as described below:

Materials

Buffer:

- 0.1 M Tris/HCl, pH 8.0
- 0.02 M $CaCl_2$
- 0.1 % PEG 6000

Substrate:

- Casein-resorufin (λ_{max} = 571 nm, λ_{em} = 585 nm)
- Stock solution (to be freshly prepared): 2 mg/ml in twice-distilled H_2O corresponding to a concentration of 80 µM.
- Assay concentration: 5 nM (optimal conditions are met if 0.5 to 1 particles on average are present within the confocal volume element before the enzyme is added)

Enzyme:

- Stock solution of trypsin (freshly prepared): 1 mg/ml in buffer corresponding to 40 µM
- Assay concentration: 400 nM

Measurement

Preparation:

- Pipette 160 µl buffer and 20 µl substrate (50 nM); final concentration: 5nM

Repetitive measurements on the ConfoCor 2:

- Measuring time: 15 sec
- Breaks: about 10 sec
- Number of measurements: about 40
- Excitation at 543 nm

Start of reaction

- After three measurements, start the reaction within the break by adding 20 µl enzyme (4 µM); final concentration: 400 nM (mix the solution well with a pipette).

Analysis

Analysis:

- Take measurements of the substrate alone and at various points of time after adding the enzyme.
- Use the one-component model with a fixed value of **S**, determined within the adjustment measurement.

6.4.2 Characterizing Hybridization Kinetics

Another example, which demonstrates the potential of FCS for analyzing reaction kinetics in a homogeneous assay, is the hybridization reaction of a fluorescence-labeled primer to its specific DNA binding site.

DNA binding assay

Prof. Rudolf Rigler's group at the Karolinska Institute in Stockholm was the first to use FCS for studying the reaction. In this assay, a fluorescence-labeled DNA primer (18 nucleotides) anneals to its homologous ssDNA binding site (viral M13-DNA, 7250 nucleotides). This assay has been further analyzed, and a standard protocol has been developed (see end of section). Additionally, the assay is well suited for learning how to use the ConfoCor 2 and for gaining a hands-on feeling of the potential of the FCS method.

Labeled primer

In the FCS assay, the binding process may be characterized by the diffusion time of the labeled primer, which has

- a long diffusion time in its bound form, and
- a short diffusion time in its unbound form.

The reaction will follow the simple equation:

Reaction system

$$P + M13 \underset{k_{diss}}{\overset{k_{ass}}{\Longleftrightarrow}} M13 - P$$

where P represents the labeled primer and M13 corresponds to the viral DNA.

Two different diffusion times, τ_{free} and τ_{bound}, can be observed, corresponding to the two differently diffusing fluorescent species resulting from the hybridization process.

The correlation function describing the hybridization at any point in time during the interaction is given by the following equation:

Correlation function for hybridization

$$G'(\tau) = 1 + \frac{1}{N}\left[\frac{1-Y}{1+\tau \,/\, \tau_{free}} + \frac{Y}{1+\tau \,/\, \tau_{bound}}\right]$$

Y corresponds to the bound primer portion.

The binding process of the primer to its ssDNA binding site is described by the following rate equation:

Rate equation

$$-\frac{d[P]}{dt} = k \cdot [P] \cdot [M13]$$

With $[P]_o = [M13|_o$ => [P] = [M13], the equation is simplified as

$$-\frac{d[P]}{dt} = k \cdot [P]^2$$

Integration of the differential equation yields

Integrated rate equation

$$[P]\,(t) = \frac{[P]_o}{1 + k[P]_o \cdot t}$$

The measured change in the amount of primer bound can be described by the equation given above. The following diagram (Fig. 6-15) summarizes the change in concentration of the free and bound primer over time.

Concentration change

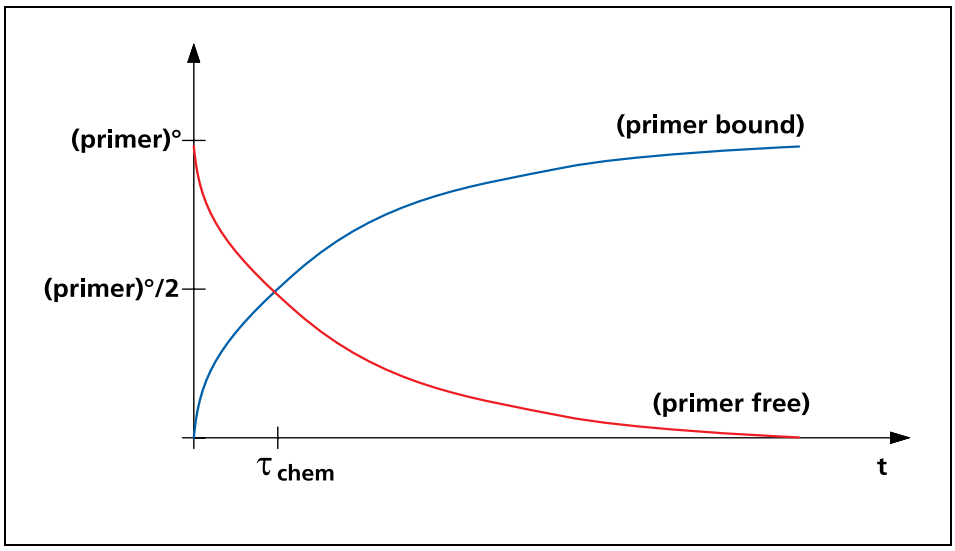


Fig. 6-15 Change of concentration during the reaction time.

The stoichiometry of the reaction can be characterized using a plot such as that shown in Fig. 6-16:

Hybridization stoichiometry

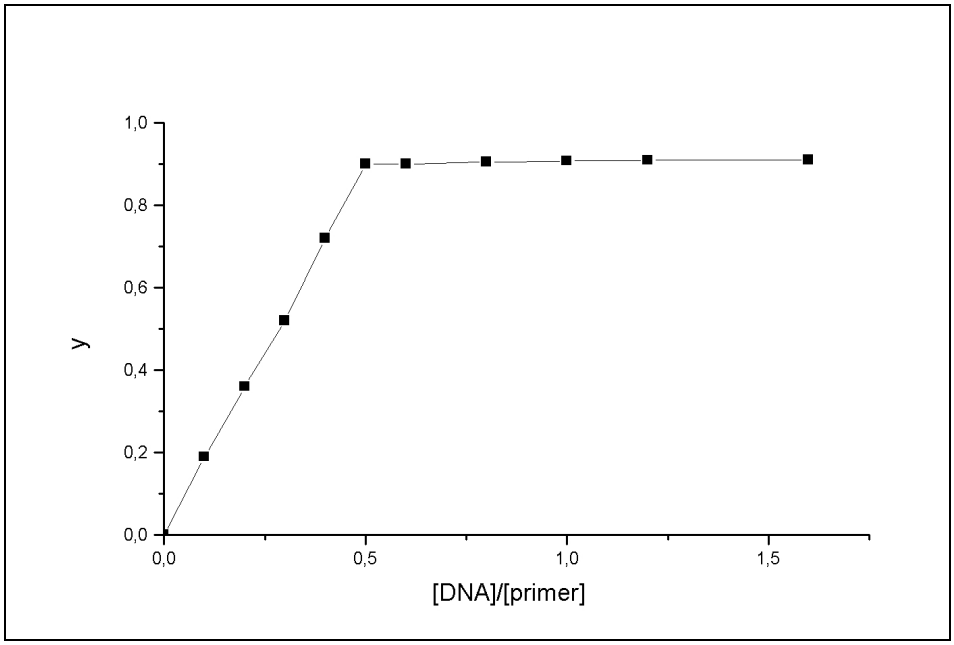


Fig. 6-16 The binding degree y = [complex]/[primer]$_o$, obtained from a series of experiments at a given point in time (e.g. after 120 min), is plotted against the DNA/primer ratio.

The change in the number of particles in a given reaction is only a reflection of the behavior of the system under particular experimental conditions and should only be used to draw general conclusions if unspecific binding reactions can be fully neglected.

The M13 Hybridization Assay is described on this and the following page:

Materials

Buffer:

- 200 mM Tris, pH 7.5
- 100 mM $MgCl_2$
- 250 mM NaCl

ssDNA:

- M13mp18+ (Pharmacia Biotech product no. 27-1546-01, Lot: 4091546012, 0.02 µg/µl)
- Assay concentration: 50 nM

Primer:

- G-Tamra-21 primer (Applied Biosystems product no. 408-025 40pmol/100 µl)
- Assay concentration: 50 nM

Basis solution

Prepare 12 reaction tubes with the following basis solution:

- 4 µl buffer
- 13 µl H_2O (twice distilled)

Assay / control solution

Incubate the ssDNA (18 µl) and the primer (6.5 µl) separately at 65°C for 3 min. Wait until the substances have slowly cooled down to room temperature before pipetting the following solutions:

Hybridization assay:	Control solution:
• 3.7 µl primer	• 2.5 µl primer
• 18.0 µl ssDNA	• 4.0 µl buffer
• 6.0 µl buffer	• 13.5 µl H_2O (twice distilled)
• 2.3 µl H_2O (twice distilled)	

Incubate the mixtures at 40° C and simultaneously start time series (t = 0 min.)

Samples

After 0, 3, 6, 10, 15, 20, 30, 45 , 60, 90 minutes prepare the samples using the following procedure:

1. Take 3 µl of the hybridization assay and dilute it in one of the prepared reaction tubes (basis solution) described above.
2. Put the diluted sample on ice before starting FCS measurement in order to prevent further primer association/hybridization.
3. Measure the sample as quickly as possible on the ConfoCor 2 as described below.
4. Finally, on the ConfoCor 2, measure 3 µl of the control solution diluted within one of the reaction tubes (basis solution).

Measurement

Perform an FCS-measurement using the ConfoCor 2 (excitation at λ=543 nm, measuring time is 3 x 60 sec. for each sample).

Data analysis

First analyze the FCS data using the one-component model with fixed values of AR for the determination of

- diffusion time of the primer,
- diffusion time of the formed complex (analysis of only the FCS correlation curves measured after 90 min hybridization).

Then analyze each sample using the two-component model with fixed values of

- S,
- τ_{D1} (labeled primer),
- τ_{D2} (complexed primer)

for the determination of the degree of complexed oligonucleotide (Fig. 6-17).

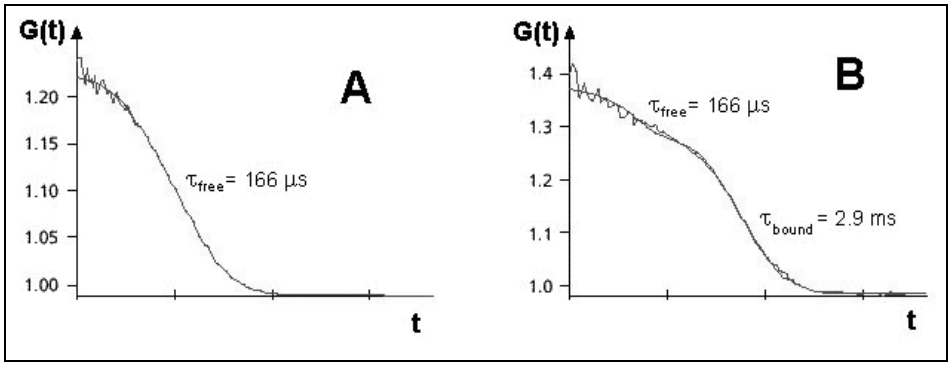


Fig. 6-17 **Correlation curves of the primer measured alone (A) and at the end of the hybridization process. $\tau_{D1} = \tau_{free}$ = 166 µs of the free primer and $\tau_{D2} = \tau_{bound}$ = 2.9 ms of the complexed primer have been determined from these correlation functions. (Note that the x-axis is not a time scale but indicates channels).**

The following plot represents the results of such a kinetic study (Fig. 6-18).

Kinetics

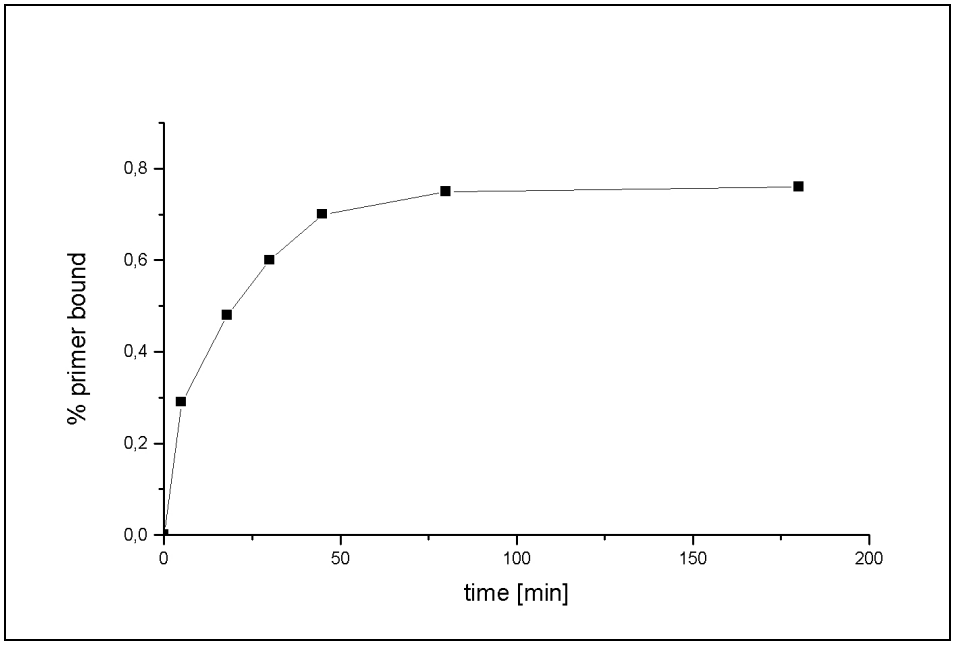


Fig. 6-18 Amount of primer bound as a function of the reaction time.

A value of 1.7 x 10^4 mol^{-1} s^{-1} was obtained for the reassociation constant.

6.5 Statistical Accuracy of FCS Experiments

Possible errors

The accuracy of FCS data is influenced by several factors, which can be divided into three classes of error:

- Optical and hardware misalignment, such as distorted confocal volume, scattered laser light, and dark counts of the photon detector
- Statistical errors, the noise of the autocorrelation function calculated from the initial data
- Errors introduced as a result of the fitting procedure

Avoidance of errors

By performing frequent quality controls for instrument adjustment, thereby detecting and avoiding optical and hardware misalignment, and choosing the correct model for fitting the measured correlation curves, sources of error resulting from the first and third classes can largely be avoided.

Under these conditions, only the second class of errors remains to be of interest and will be described in this section.

6.5.1 Statistical Errors and Noise

Noise of fluctuation

Although autocorrelation is a common technique for signal processing in science and technology, a method for the calculation of the associated statistical error has only recently become available. The complexity of the calculations required to obtain a good approximation may explain this lack of a method for error evaluation to some extent. FCS measurements are based on the fluctuation of the fluorescence intensity. Calculating the statistical error of these measurements means calculating the fluctuations of the fluctuations!

Literature

In the fluorescence fluctuation field, the first paper by Koppel [14] dates from 1976 and gives an estimate of the upper concentration limits applicable for such measurements. In 1990, Qian published a more detailed calculation [15], also valid for lower concentrations, and more realistic profiles of the confocal volume. A new calculation, including approximations for several critical cases and a set of profiles of the confocal volume, was described by Kask et al. in 1996 [16].

For specific details of error calculation, the reader is referred to one of the above-mentioned articles. The following section is a discussion on how to perform FCS experiments with a high signal-to-noise ratio S/N.

6.5.2 FCS Detection Process and Sources of Noise

During an FCS experiment, fluorescence-labeled particles diffuse in and out of the confocal volume created by a focused laser beam. Fluorescence photons are emitted in bursts. When such a particle passes through the focus, it is collected by the microscope objective, and guided to the detector. The photon counts are summed over a specific time interval t giving a series of counts for consecutive time intervals. Finally, the autocorrelation curve for all these intervals is calculated.

Fluctuation sources

Fluctuations occur on several levels in an FCS experiment:

- The fluctuation of the number of fluorescent particles due to diffusion
- The quantum nature of light resulting in a Poissonian distribution of photon numbers detected by the photon-counting avalanche photo diode
- The fluctuation in diffusion time of the individual particles

Whereas the first kind of fluctuation is the measurement principle underlying FCS, and thus desired, the Poissonian distribution of counts imposes a limit on the accuracy of the photon number measured during each time interval. Another undesired fluctuation is the fluctuation in diffusion time and related molecular fluctuations: though detecting diffusion events of single molecules, a minimum number of diffusion events must be averaged to get an accurate estimate of the diffusion time of the molecule. Moreover, a sampling time that is shorter than the average diffusion time measures the same molecule several times and does not lead to improved diffusion statistics.

Today, existing calculation methods still do not include every experimental FCS parameter. Dark counts and scattered light are neglected, as well as saturation effects of the photon counting device. In a strict sense, the calculations only represent the case for the correlation time $\tau = 0$, but any differences are expected to be small for reasonable, positive values of τ.

6.5.3 Mathematical Expressions for FCS Noise

The most exact approximation to the signal-to-noise ratio *S/N* in FCS takes the following form:

$$S/N = \frac{Nq\sqrt{m}}{\sqrt{N\left(\left(q^2\gamma_4^{-1}\right)\left(1+2K_1\right)+4q\gamma_3^{-1}+2\right)+N^2\left(2q^2\left(1+K_2\right)+4q+2\right)}}$$

with the average number of particles **N** in the confocal volume, the average count rate per particle and sampling interval (dwell time) **q**, and the number of samplings ***m***, equivalent to the measurement time. g_3 and g_4 are constants related to the shape of the confocal volume. For the ideal case of a 3-D Gaussian volume element, $g_3 = 0.5$ and $g_4 = 0.2$.

To the first approximation, the expressions K_1 and K_2 can be replaced by $K_i = 2\,\boldsymbol{\tau}_D$, where $\boldsymbol{\tau}_D$ represents the average diffusion time. The expression for the signal-to-noise ratio thus takes the form

Signal-to-noise ratio

$$S/N = \frac{Nq\sqrt{m}}{\sqrt{N\left(\left(q^2/5\right)\left(1+4\tau_D\right)+2q+2\right)+N^2\left(2q^2\left(1+2\tau_D\right)+4q+2\right)}}$$

which is still rather complicated. If we simplify for high count rates and thus neglect the quantum nature of light, we obtain

$$S/N = \frac{N\sqrt{m}}{\sqrt{\left(m/5\right)\left(1+4\tau_D\right)+2N^2\left(1+2\tau_D\right)}}$$

On the other hand, if we account for photon noise, but not for diffusion time effects, we obtain

$$S/N = \frac{N\sqrt{m}}{\sqrt{N\left(\left(q^2/5\right)+2q+2\right)+2N^2\left(q^2+2q+1\right)}}$$

In the simplest assumption, photon noise and molecular noise are both neglected. In this case, the expression takes the rather simple form

$$S/N = \frac{N\sqrt{m}}{\sqrt{N/5+2N^2}}$$

Brightness effect

It is not surprising that, if photon noise is not accounted for, the signal-to-noise ratio becomes independent of the molecular fluorescence intensity **cpm**. However, if background signals such as scattered laser light and dark counts from the detector were accounted for, the signal-to-noise ratio S/N would still depend on the molecular brightness (however, no formula incorporating the background signal has been derived so far). At a low molecular fluorescence intensity, the dependence of the S/N on **q** is almost linear. In almost every practical application, the molecular brightness is still the most important factor influencing the S/N.

Measurement time

With respect to the total measurement time, FCS measurement is comparable to most other measurement principles:

A two-fold increase in signal-to-noise ratio requires a four-fold increase in measurement time.

Concentration effect

At low concentrations, the *S/N* is linearly dependent on the square root of the concentration of the labeled molecule. It becomes independent of the concentration at a number of 10 - 100 molecules in the confocal volume.

For a size of 1 femtoliter ($1x10^{-15}$ l), which is typical with the ConfoCor 2 optics, this corresponds to a molar concentration of 17 - 170 nM.

Diffusion time effect

Finally, the diffusion time plays an important role in the evaluation of statistical error. It takes more time to accumulate diffusion statistics if the molecule of interest is diffusing rather slowly. This effect is reflected by the appearance of the average diffusion time in the denominator of the S/N. For long diffusion times, in the range of milliseconds, the S/N drops significantly.

6.5.4 Guidelines for a Good Signal-to-Noise Ratio

A high signal-to-noise ratio can be obtained if the relevant FCS parameters are in line with the following criteria:

High CPM

- The counts per molecule **cpm** should always be as high as possible (but without increasing the triplet state of the molecule to more than 10 - 20 % of the autocorrelation amplitude).

Optimal concentration

- The concentration of the labeled molecule should be chosen to be in the nanomolar to micromolar range. Lower concentrations decrease the signal-to-noise ratio or require longer measurement times. Moreover, in the picomolar range and lower, the background signal contributes significantly to the total signal, distorting the autocorrelation data. In this case, the autocorrelation function overestimates the particle concentration. This concentration range is sometimes called the „non-linear“ range of the ConfoCor 2.

Diffusion time

- The diffusion time should be as short as possible. However, the difference in diffusion time between the particles being measured should at least amount to a factor of 2 if relative concentrations are to be measured accurately in a system containing two fluorescence-labeled species.
- The confocal volume should show no distortion.

Distortion-free volume element

The maximum detectable count rate of the detector sets a maximum for the product of the concentration and the count rate per particle. Generally, the count rate should be less than 750,000 counts per second for the EG&G SPCM-AQ.

6.6 Correction for Differences in Quantum Yields

The correlation function for translational diffusion

$$G'(\tau) = 1 + \frac{1}{N}\left\{\frac{1}{1 + 4D\tau \,/\, \omega^2}\right\}$$

relates to a volume element with a Gaussian intensity distribution in the XY plane and an infinite dimension in the Z plane. A limiting case can be visualized as a volume element with Gaussian intensity distributions in three dimensions:

Intensity distribution

$$G'(\tau) = 1 + \frac{1}{N} \cdot \frac{1}{1 + 4D\tau \,/\, \omega_{xy}^2}\left\{\frac{1}{1 + 4D\tau \,/\, \omega_z^2}\right\}^{1/2}$$

The given correlation function describes the situation for a single type of diffusing particle. More than one type of diffusing particle can be found in solution when, for example, a labeled ligand binds to a macromolecule.

Single component

In this case the correlation function has the following form (for simplification, only the two-dimensional form is given):

$$G'(\tau) = 1 + \frac{1}{N}\left\{\frac{1-Y}{1 + 4D_{free}\tau \,/\, \omega^2} + \frac{Y}{1 + 4D_{bound}\tau \,/\, \omega^2}\right\}$$

Two components

When two types of fluorescent molecules (e.g. labeled macromolecules and free dye molecules) are present in solution, they may be present in different concentrations, i.e.. average number of molecules per sample volume n_1 and n_2 , and exhibit different fluorescence detection intensities per molecule, I_1 and I_2. In other words, the two species with different diffusion behaviors are characterized by different quantum yields. Therefore, the measured autocorrelation function for the mixture of these molecules must be corrected for this effect. For our correction procedure we assume that the solution of the particles is ideal and the molecules diffuse independently of each other.

The time-dependent portion of the correlation function can generally be expressed by the equation below for time t = 0:

$$G'(0)-1=\frac{1}{N}=\frac{1}{n_1+n_2}$$

n_1: average number of component 1 molecules per sample volume

n_2: average number of component 2 molecules per sample volume

Amplitude correction

If we consider that both species have different quantum yields, then the amplitude of the time-dependent portion of the correlation function is given as

$$G'(0)-1=\frac{\overline{n}_1 I_1^2+\overline{n}_2 I_2^2}{(\overline{n}_1 I_1+\overline{n}_2 I_2)^2}$$

where

$\overline{n}_1 \cdot I_1^2$: probability to detect a series of photons of component 1

$\overline{n}_2 \cdot I_2^2$: probability to detect a series of photons of component 2

$(\overline{n}_1 \cdot I_1+\overline{n}_2 \cdot I_2)$: average fluorescence intensity

By substitution and introduction of the real concentration ratio c, we get

$$N\cdot(G'(0)-1)=\frac{c\cdot\alpha^2+(1-c)}{(c\cdot\alpha+(1-c))^2}$$

c: real concentration ratio, [0.....1], $c=n_2/\left(\overline{n}_1+\overline{n}_2\right)$

V: confocal volume

α: Q_c/Q_{1-c}, quantum yield ratio for the two species

N: number of fluorescent particles

Using

$$c = \frac{Y}{Y + \alpha^2 - Y\alpha^2}$$

with Y = measured concentration ratio (apparent ratio),we obtain the following equation for quantum correction

$$\frac{1}{N} = (G'(0) - 1)\frac{(Y + \alpha - Y\alpha)^2}{Y + \alpha^2 - Y\alpha^2}$$

Quantum correction

In the following figure the dependence of the real concentration on the correction term is plotted for the apparent concentration ratio (Fig. 6-19).

Correction graphs

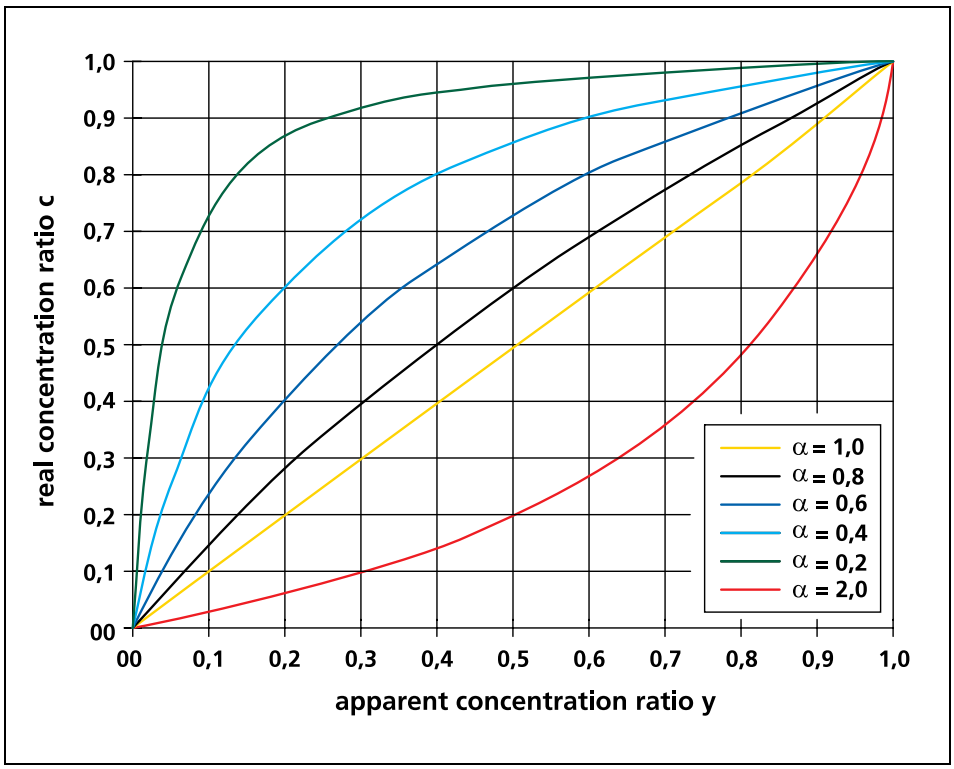


Fig. 6-19 **The real concentration ratio c is plotted as a function of the measured apparent concentration y for different values of α.**

6.6.1 Example of a Correction Process

In the following section an example of a correction is shown, which was done using the equations given in the previous section.

Purity check

A mixture of Texas Red™ (**TR**) and a protein (**TR-prot**) labeled with this dye has to be analyzed, a typical situation when the purity of a labeling product is to be checked.

Well defined mixtures of both substances, as well as the purified substances alone, were measured on the ConfoCor 2 in order to obtain **α**. With this value, the real concentration ratio can be determined from the calibration graph (Fig. 6-20).

The following graph demonstrates the differences between the concentration ratio Y result obtained from the ConfoCor 2 software and the corrected values **c**. Both parameters are plotted against the theoretical concentration ratio Y_{theo}.

$$Y_{theo} = \frac{N_{TR-Prot}}{N_{TR-Prot} + N_{TR}}$$

N_{TR}: number of free Texas Red reactants

$N_{TR\text{-}Prot}$: number of protein particles labeled with Texas Red

Concentration ratio

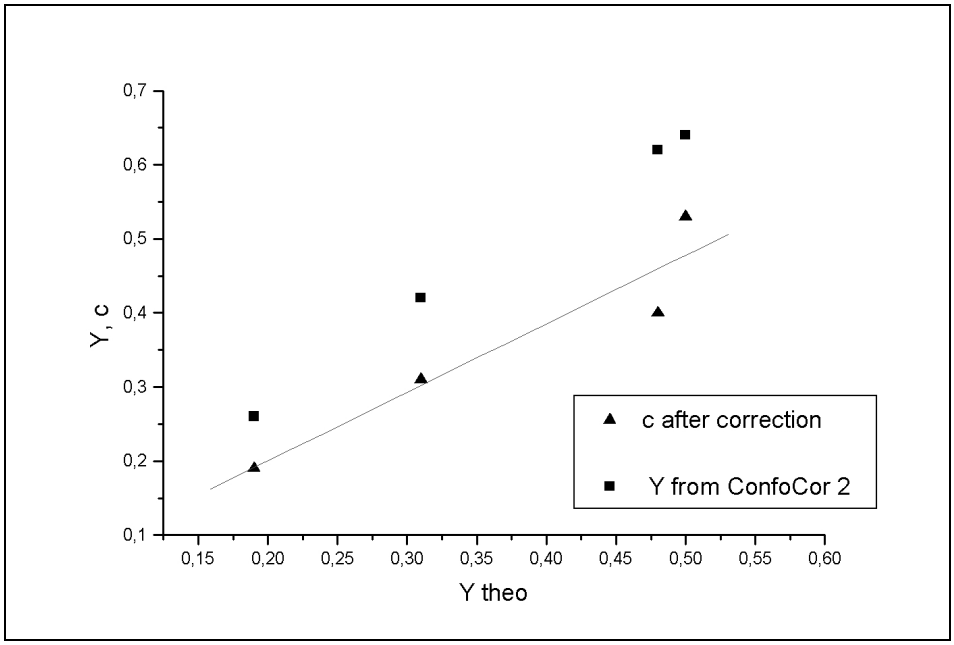


Fig. 6-20 Plot of the fit result Y (concentration ratio) and the corrected values c against the theoretical concentration ratio Y_{theo}.

APPENDIX

A. Abbreviations

AR :	axis ratio, **AR** = ω_2/ω_1; synonym for the structural parameter S
BSA :	bovine serum albumin
cmc :	critical micelle concentration
cpm :	counts per molecule [s^{-1}]
D :	diffusion coefficient [m^2/s]
DMSO :	dimethylsulphoxide
HPLC :	high-performance liquid chromatography
FCS :	fluorescence correlation spectroscopy
fl :	femtoliter
K :	equilibrium binding constant, affinity constant [M^{-1}]
$\mathbf{K_D}$:	binding dissociation constant [M]
k :	Boltzmann constant, 1.38 x10^{-23} J/K
$\mathbf{k_{ass}}$:	association rate constant [$mol^{-1} \cdot s^{-1}$]
$\mathbf{k_{diss}}$:	dissociation rate constant [s^{-1}]
kD :	kilodalton
MW :	molecular weight [g/mol]
m :	molecular mass [g/mol]
ms :	milliseconds
$\mathbf{N_A}$:	Avogadro's number, 6.023 x 10^{23} mol^{-1}
N :	number of fluorescent particles
PEG :	polyethylene glycol
Q :	quantum yield
r :	hydrodynamic radius [m]
Rh-6G :	Rhodamine-6G
S :	structural parameter; synonym for the axis ratio AR
$\mathbf{S_0}$:	ground state energy level

S_1 :	first excited state
S_n :	excited-state energy level
S/N :	signal-to-noise ratio
ssDNA :	single stranded DNA
T :	absolute temperature [K]
T_n :	triplet state
TMR :	tetramethyl Rhodamine
t :	time [s]
τ_{chem} :	chemical relaxation time constant [s]
τ_D :	diffusion time [s]
τ_{D1} :	diffusion time of the faster component [s]
τ_{D2} :	diffusion time of the slower component [s]
τ_T :	triplet lifetime [s]
ω_1 :	radius of the laser beam [m]
ω_2 :	half length of the confocal volume in Z-direction [m]
η :	viscosity [cp]
µs :	microseconds
ρ :	mean density [g/cm^3]
σ :	cross-section of absorbance [m^2]

B. Bibliography

(a) FCS theory

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