Diffusion and Binding Properties Investigated by Fluorescence Correlation Spectroscopy (FCS)

D. Grünwald[1], M.C. Cardoso[2], H. Leonhardt[3] and V. Buschmann[2,3,*]

[1]*Institute of Medical Physics and Biophysics, Münster, D-48149 Münster Germany,* [2]*Max Delbrück Center for Molecular Medicine, Berlin, D-13125 Berlin, Germany and* [3]*Department of Biology II, Ludwig Maximilians University Munich, D-82152 Planegg-Martinsried, Planegg-Martinsried, Germany*

Abstract: During the last years, Fluorescence Correlation Spectroscopy (FCS) has proven to be a powerful tool for basic research in many applications. The combination of a minimal detection volume in the femtoliter range coupled with very high sensitivity extends the possibilities to design sensitive homogeneous tests. In this article we illustrate the analysis of binding processes with FCS based on the changes in diffusion characteristics of GFP upon binding to an antibody. Problems induced by highly heterogeneous samples are discussed and differences of GFP binding to a monoclonal and a polyclonal antibody are shown and analyzed. We stress data processing, limitations and useful approximations in FCS methodology. Basic ideas of data acquisition and processing as well as new developments and applications are presented.

Key Words: Fluorescence Correlation Spectroscopy, GFP, Kinetics, Binding Assay, Antibody.

INTRODUCTION

With its foundations laid in the early seventies [1-3], Fluorescence Correlation Spectroscopy (FCS) has become a useful tool in biology. Especially its combination with the detection volume of a confocal microscope [4-6] has opened doors for applications in widely different research areas such as ultraanalytics [7], cell biology [8] or photophysics [9, 10]. FCS has been applied for studying the behavior of molecules in solution [11, 12] or microchannels [13], on surfaces [14], membranes [8, 15] or in living cells [16, 17]. Technological progress like correlating two different color channels (Fluorescence Cross Correlation Spectroscopy, FCCS) [18, 19] or combination with different other techniques, as e.g. Fluorescence Resonance Energy Transfer (FRET) [20, 21], or fluorescence fluctuation analysis (photon counting histogram, PCH, Fluorescence intensity distribution analysis, FIDA) [22-24] has contributed to the continuing success of FCS. Furthermore, the number of fluorescent probes available has greatly increased; on the one hand by development of new chemically synthesized fluorophores [25, 26] and on the other hand by the use of genetically encoded fluorescent proteins that can be directly expressed and measured in living cells [27, 28]. Even cross correlation analysis using two the two fluorescent proteins EGFP and mRFP has been performed in live cells [29].

One of the main applications of FCS is to study binding or diffusion of biomolecules free in solution. This review will mainly focus on the way binding processes are studied *in vitro*. We will give an introduction to the processing of FCS data. Furthermore, we will give an overview of problems and possibilities as well as an introduction to helpful, fundamental and recent literature in this field.

*Address correspondence to this author at the Max-Delbrück-Center for Molecular Medicine, Wildtbergstr. 50, 13125 Berlin, Germany; Fax: +49-30 – 94172336; E-mail: volkerbuschmann@web.de

FOUNDATIONS OF FCS

The basic process in FCS measurements is the acquisition of a fluorescence intensity trace. Signal fluctuations in a certain observation volume are followed over time. In the classical case of observing freely diffusing molecules in solution, fluctuations in the fluorescence signal are caused by concentration variation of the fluorophors in the observation volume, which are due to diffusion. If there are only a few molecules in the observation volume, entering or leaving of a single molecule leads to a significant change in the fluorescence intensity, F. These changes are dominated by uncorrelated noise at high concentrations, when many molecules are in the observation volume. Therefore correlation curves cannot be measured at very high concentrations. The principle of processing of a binned intensity trace is shown in Fig. (**1**). The autocorrelation curve is obtained by correlating the fluorescence intensity trace shifting a time interval, as shown in Fig. (**1**). The time shift τ is varied, and the correlation curve is obtained by multiplying the deviation of the average intensity, δF, at the time point t with the deviation at the time point $t + \tau$ and averaging over the whole trace. Finally, the normalized correlation function, $G(\tau)$, is normalized with the squared average signal.

$$G(\tau) = \langle \delta F(t) \delta F(t+\tau) \rangle / \langle F \rangle^2 \quad (1)$$

The autocorrelation function is plotted semi logarithmically. Further practical considerations in the calculation of the FCS curves out of a fluorescence intensity trace are detailed in [30, 31].

Any process that leads to fluctuations in the fluorescence intensity can be analyzed by FCS. Apart from diffusion processes, also photophysical processes, molecular rotations or molecular interactions can be studied [32-34]. Artifacts may be introduced by insufficient isolation of the set up from environmental vibrations as well as unstable illumination sources. In principle, the timescale that can be analyzed

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spreads from nanoseconds to several seconds. While the upper limit of time is mainly governed by the measurement time, most processes of interest are fast, and the lower limit of time is set by the fluorescent lifetime. The fluorescence lifetime of a fluorophor is typically in the order of nanoseconds and is a limit for the rate a fluorophor can emit.

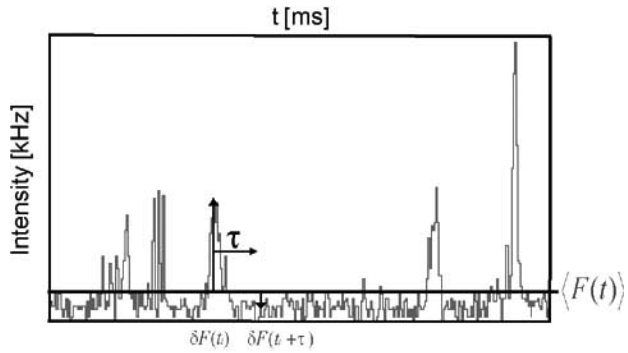

Fig. (1). Relevant data for FCS calculation out of a fluorescence intensity trace. Diffusion of molecules in and out of the detection volume result in intensity changes over time. For calculation of the FCS curve, the deviations of the intensity from the average signal <F(t)> at a time t, $\delta F(t_i)$, is multiplied with the deviation at a time $t_i + \tau$. The result is averaged for all t_i and normalized with the squared average signal.

In order to keep the number of fluorescent probe molecules in the observation volume small, which is a prerequisite to detect intensity fluctuations due to diffusion of the probe molecules, the detection volume needs to be very small. While a combination of FCS with total internal reflection as method for restriction of the detection volume was invented already in the eighties [35], generally a confocal microscope for measurements of fluorescence is used. [6]. Here, the observation volume is limited to less than a femtoliter. In a diluted sample of nanomolar concentration, the number of molecules in the observation volume is relatively small, so that the change upon entering or leaving the observation volume of one fluorescent molecule or a change of its fluorescent characteristics leads to a detectable signal change. With a confocal observation volume, even fluorescent traces of single surface - attached molecules can be recorded, and a fluorescence correlation function of a single fluorescent molecule can be calculated [36].

PARAMETERS AFFECTING FCS RESULTS – POTENTIAL PROBLEMS AND SOLUTIONS

Information Obtainable by FCS

While in the first papers Elson, Magde and Webb studied diffusion of molecules in and out of a laser beam [1-3], fitting correlation data from diffusion measurements using a confocal detection volume must take into account not only the illumination intensity distribution, but also the spatial filtering by the pinhole of the confocal microscope. The resulting ideal probe volume is approximated by a Gaussian profile with the extention ω_0 in x and y and z_0 in the z direction [37]. The diffusion of one single component is usually fitted with the standard model [6]:

$$G(\tau) - 1 = \frac{1}{N}\left(1 + \frac{4D\tau}{\omega_0^2}\right)^{-1}\left(1 + \frac{4D\tau}{z_0^2}\right)^{-1/2} \qquad (2)$$

By fitting with this equation, two different pieces of information are obtained. On the one hand, the amplitude at $\tau = 0$, which corresponds to the variance of the intensity trace, equals the inverse number of molecules present in the observation volume. Thus it provides information about the sample concentration. If there is a higher contribution of non-correlated background, as for example in a cellular environment, the number of molecules present is actually lower than assumed from the amplitude of the correlation function. Several aspects of the influence of noise have been discussed in detail by several authors [38-40]. While one kind of noise is due to the statistical limitations of the measurements themselves, other sources of noise have more practical causes, like detector afterpulsing [41], highly scattering environment or fluorescent impurities, especially in living cells [42]. While the amplitude at $\tau = 0$ provides a concentration information, the inflection point of the correlation function corresponds approximately to the average time, a probe molecule remains in the observation volume. As this time corresponds to $\tau_D = \omega_0^2/4D$, the diffusion coefficient D is obtained, and so the hydrodynamic radius can be calculated and the molecular weight estimated.

Factors Influencing the Focal Volume

The standard model as shown in equation (2) assumes a three dimensional Gaussian profile, being radial in xy-direction [6]. In order to achieve such a profile, a water objective must to be used and correctly adjusted for the right coverglass thickness for measurements in aqueous solution. As shown in Fig. (**2**), changes in the adjustment of the coverglass correction at the objective result not only in a change of the overall signal intensity, but also in changes of the diffusion times and the number of fluorescent molecules in the observation volume [43]. These settings are optimized, when the number of fluorescent molecules in the focus and their diffusion time are minimal, while the fluorescence intensity and especially the count rate per molecule are maximal, so in the example (Fig. **2**) at the coverglass correction for a thickness around 150 µm. The exact size of the probe volume is measured with a fluorescent compound with known diffusion coefficient D, and the obtained values for characterizing the observation volume, ω_0 and z_0, are then used in measurements of the sample (eq. 2). A non Gaussian beam profile may result in false fitting of a second component [44]. For oil objectives, the exact beam profile has also been calculated and used for FCS [45]. In this case the beam profile for measurements in an aqueous environment depends on the distance to the surface of the coverglass.

Saturation Effects

For the fit with the standard model, a linear relationship between the intensity of the excitation light at any given point within the excitation profile and the fluorescence intensity of a molecule at this point is assumed. If saturation effects come into play, either by saturating the fluorescent dye, that cannot cycle between fluorescent and non-fluorescent

states infinitely fast, or by saturation of the detector upon passing of a highly labeled fluorescent sample, this linear relationship may be disturbed and leads to an apparent increase of the measured diffusion times [43, 46]. In practice, saturation due to pumping of the dye into the triplet state can easily occur at high excitation intensities.

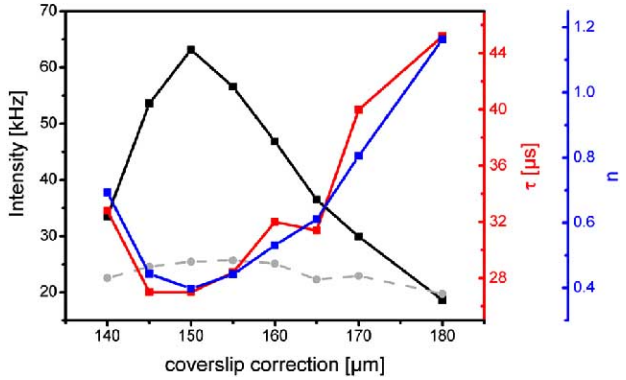

Fig. (2). Effect of the cover glass correction of the water immersion objective on the FCS data. As objective, a 40x water immersion objective with a numerical aperture of 1.2 was used. As can be easily seen, small adjustments of ≈10 µm can have a large influence on the different parameters. While the influence on the overall intensity (grey) is relatively small, non-optimal sample positioning leads to an increase of the measured diffusion time (red) and number of molecules present in the detection volume (blue) (corresponding to a bigger detection volume). At the same time, the count rate per molecule (black), which is calculated from the overall count rate divided by the number of fluorescent molecules in the detection volume, N, is reduced.

Size Determinations by FCS

For determination of the detection volume at 543 nm excitation, Rhodamin6G with a diffusion coefficient of 2.8 x $10^{-10}m^2s^{-1}$ at 20°C is generally used [3]. For 488 nm excitation, fluorescein (D=2.7 x $10^{-10}m^2s^{-1}$ [47]) can be used for calibration. We measured the characteristic diffusion time of fluorescein on a ConfoCor II with a pinhole size of 70 µm – corresponding to one Airy unit - to be 34 µs. As fluorescein is small compared to proteins the diffusion time of proteins will be longer. In general, the range of proteins is in the order of several hundreds of µs, linearly depending on the hydrodynamic diameter r of the protein as well as on the viscosity of the medium η stated in Stokes law [48].

$$D = \frac{k_B T}{6\pi \eta r} \qquad (3)$$

In this equation, T is the temperature and k_B the Boltzmann constant.

Fluorescence fluctuation due to photophysical events can be distinguished by varying the pinhole diameter and thereby the observation volume, which results also in changed characteristic correlation times, while any fluctuation due to photopysical events [49] remain unaltered. In case of very large and therefore slow complexes, scanning of the sample beam can be performed [50, 51]. Alternatively, a movement of the sample solution or the solution as a whole may be induced, as in applications in microchannels [52, 53] or even for detection of bacteria in water for water quality control purposes [54].

FCS APPLICATION FOR STUDYING BINDING OF FREELY DIFFUSING MOLECULES

The analysis of translational diffusion is a popular application for FCS, as also changes in the diffusion properties caused by binding processes can be easily analyzed [3, 55]. Upon binding of a small fluorescent molecule to a larger and unlabeled molecule like an antibody, the complex moves slower than the unbound fluorescent probe due to its larger size. The fluorescent probe and the complex with the bound fluorescent probe contribute to the overall correlation function with amplitude corresponding to their fractional presence, if both components are assumed to have the same molecular brightness [42]. If the diffusion times are sufficiently separated, a clear "step" appears in the correlation function and the inverted first derivative shows two maxima approximately at the respective correlation times of the two components. As shown in Fig. (**3**), the two maxima are not longer distinguishable, when the correlation times are too similar. Several papers have dealt with the error in determining the correct diffusion times, the resolution capacity of FCS [56] and the influence of noise [39, 40]. With 50% unbound ligand and a count rate of 15 kHz per particle, correlation times differing by a factor of 1.6 can be distinguished. This corresponds for spherical molecules to a 4 -

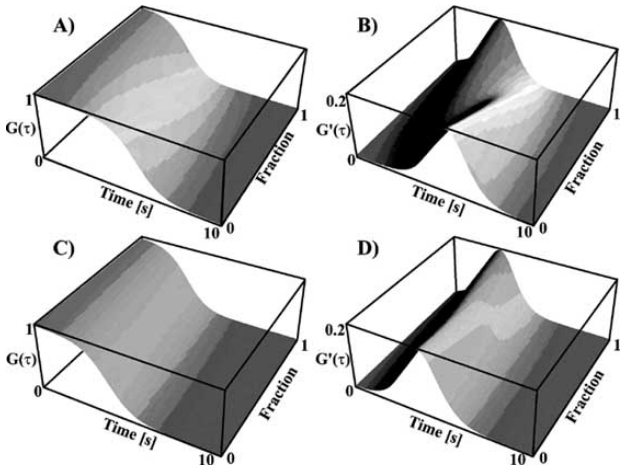

Fig. (3). Simulated diffusion curves demonstrating the resolution of two components with different diffusion times in FCS (A and C) and their first deviations (B and D). In A), relative fractions of two components with diffusion times of 93 µs and 4000 µs are varied from a fraction of 0 to 1 of the component with 93 µs. The log of the time is plotted on the x – axis (log s). As both diffusion times mark a inflection point in the curve, in the inverted first derivative of the correlation functions (B), a maximum becomes visible for both diffusion times. On the other hand, if the diffusion times of the two components are too close together, as in C), where the two components have diffusion times of 93 µs and 170 µs, only one maximum gradually shifts with increasing amounts of the second component. For the two dimensional case (as shown) the inflection point is exactly the diffusion time. In case of a three dimensional process the relation between z and x, y extension of the focal element should be taken into account.

fold molecular weight difference, as the diffusion coefficient scales with the radius, while the weight scales with the volume. If one component is present in much higher concentration than the other, the molecular weight difference needs to be higher in order to be resolved [56].

To analyze the binding process of a fluorescently labeled ligand to a nonlabeled receptor, a titration of the receptor into a solution of the ligand can be performed. In this way the diffusion times of the free ligand (before adding the receptor) and of the pure complex (after complete binding of the ligand with an excess of receptor) can be determined. At non - saturating conditions, the correlation curves can be fitted with a two component model with fixed diffusion times for both ligand and complex. If a single-component model is used, an intermediate time is obtained. From this titration the dissociation constant can be determined [3, 57]. If present, photophysical processes like triplet transitions have to be taken into account. In this case, the correlation function with several diffusing components is given by:

$$G(\tau)-1=\left(1+\frac{T}{1-T}e^{-\tau/\tau_{tr}}\right)\frac{1}{N}\sum_i \varphi_i\left(1+\frac{4D\tau_i}{\omega_0^2}\right)^{-1}\left(1+\frac{4D\tau_i}{z_0^2}\right)^{-1/2} \quad (4)$$

The contribution of the additional fluctuation is found in the first factor, with T being the fraction of molecules residing in the non-fluorescent state and τ_{tr} the characteristic time for the photophysical process, in our example molecules entering and leaving the triplet state [49]. In this equation, it is assumed that all components i have the same photophysical properties. In case that all components have the same molecular brightness, they contribute to the total correlation curve with their molecular fraction φ_i [42].

As an example, we added a monoclonal mouse antibody directed against GFP (α - GFP) to a GFP solution at unequal molar ratios. With increasing amounts of antibody, the correlation curve shifted to longer times as shown in Fig. (**4**). The concentration of bound GFP is given by the law of mass action. As each antibody contains two equal affinity binding sites, [α-GFP] represents the concentration of the binding sites [58].

$$\frac{[GFP]\,[\alpha-GFP]}{[GFP\circ\alpha-GFP]}=K_d \quad (5)$$

In Fig. (**4**) can be seen that the two different diffusion times for the bound and unbound fraction are not resolved. In general, the diffusion times of the free component and the bound component – determined under excess of antibody - are then kept fixed and only the fraction of the two components is varied. Alternatively, data can be fitted with a one-component model and the amount of bound and unbound GFP can be calculated from the average diffusion time. By this procedure, only a small error is introduced. If saturation is not reached during the titration, the diffusion coefficient of the bound ligand can be obtained by applying a two component fit and keeping the diffusion coefficient of the free ligand fixed. Thus, even when mixtures of complexes have a difference in molecular weight too small to be resolved by the two component model with two free diffusion times, the binding constant can be determined. Errors may be introduced by unspecific wall adsorption of the compounds.

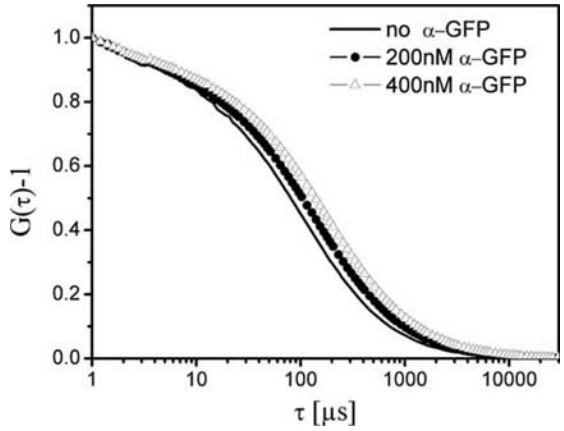

Fig. (4). FCS curves of freely diffusing GFP molecules in aqueous solution and changes of the diffusion time upon addition of a GFP – specific monoclonal antibody (α - GFP). The correlation times of the normalized curves shift to longer times, as more and more GFP is bound to the ≈ 5 fold heavier antibody.

In our example we determined the diffusion time of bound GFP fixing the diffusion time of free GFP in the measurement with the highest antibody concentration. Data are then fitted with a two component model with fixed diffusion times at different $[GFP]_0$ / $[\alpha - GFP]_0$ – ratios. The relative fraction of bound GFP, F_b = [GFP _ α - GFP] / $[GFP]_0$ is plotted as shown in Fig. (**5**). In all fits, a contribution of triplet transitions was taken into account. The index 0 stands for the overall concentration of bound and unbound substrate. Data are analyzed with the simple binding model describing binding of one GFP molecule to one antibody site as shown in equation (6); *m* and *c* are scaling factors [59]:

$$F_b=\frac{[GFP\circ\alpha-GFP]}{[GFP]_0}=\frac{m\,[\alpha-GFP]}{[\alpha-GFP]+K_d}+c \quad (6)$$

Using this method, the binding affinity of a digoxigenin specific antibody yielded the same results as using fluorescence polarization techniques [59].

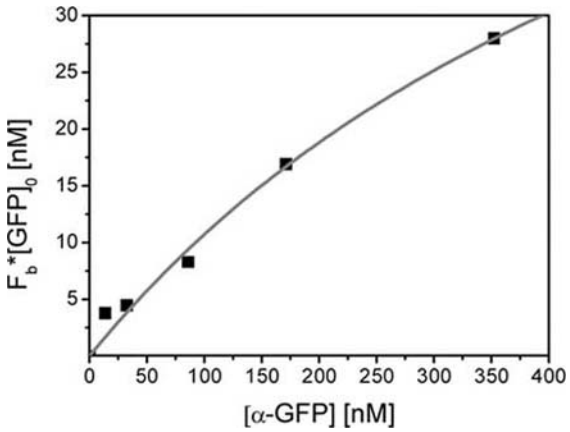

Fig. (5). Titration of α - GFP into a GFP solution. The concentration of bound GFP, given by the total GFP concentration $[GFP]_0$ times the fraction F_b = [GFP ∘ α - GFP] / $[GFP]_0$, is plotted vs. concentration of the free antibody binding sites [α-GFP]. The dissociation constant was determined by a nonlinear fit to be 620 ± 260 nM.

In many cases, binding does not lead to one or two defined products, but to aggregates of different sizes, as may be illustrated by the comparison of the correlation curves obtained after binding of an anti - GFP monoclonal antibody and a polyclonal antibody to GFP (Fig. **6a**). In Fig. **6c** the distribution of the average diffusion times – fitted with a single component model - upon addition of the monoclonal antibody is shown. An increasing antibody concentration just causes an increase in the fitted average diffusion times, while for polyclonal antibodies a broad range of diffusion times is obtained (Fig. **6b**). The FCS curves vary strongly from measurement to measurement, as the number of larger complexes originating from multiple binding sites and passing through the detection volume during measurement time is relatively small. The occurrence of large undefined complexes obviously is a problem in FCS analysis. Nevertheless, it can also be exploited for analytic purposes, as shown for binding of the coumarin derivative COU2 to the serotonin receptor expressed in Escherichia coli bacteria [60], where bright spikes with anomalous high intensities were detected.

The problem of multi - component samples with a variety of different diffusion times has recently been addressed by introducing Maximal Entropy Method (MEM) analysis into treatment of FCS diffusion data [61, 62]. In MEM – analysis, a distribution of diffusion times is assumed and the amplitudes are fitted, minimizing χ^2 while maximizing the entropy.

In common drug testing, binding of an unlabeled small drug to an unlabeled receptor is measured. In this case, displacement of a fluorescently labeled ligand from a ligand receptor complex by an unlabeled drug may be monitored [63].

The potential for using FCS related techniques in immunoassays and diagnostics [64] has been recently reviewed [65]. Thus, Lagerkvist *et al*. were able to bind Cy5 labeled cognate antigen to the envelope protein E2 of the hepatitis C virus and monitor the displacement with free antigen [66]. These techniques have also the potential to be extended for measuring surface binding kinetics by combining FCS with total internal reflection (TIR) [63, 67].

ALTERNATIVE FLUORESCENCE – BASED METHODS TO STUDY MOLECULAR INTERACTIONS

FCS measurements have become popular in studying ligand/receptor interactions [68], because they are fast, easy to perform and require minute amounts of reagents. Still one drawback is the limitation, that a substantial molecular weight difference of ligand and receptor is necessary in order to observe an effect on the diffusion time. Several groups have shown examples of binding processes that work with inter- or intramolecular quenching effects, for example caused by tryptophan that is known to quench several dyes upon binding [26, 32, 69]. Apart from fluorescence quenching, FRET-based probes may be used for monitoring binding events. Binding of a ligand labeled with a donor dye to a receptor labeled with an acceptor dye results in a measurable fluorescence energy transfer from donor to acceptor [70]. If several fluorescent ligands bind to one receptor, the complex has a higher molecular brightness, which can be analyzed by fluorescence intensity distribution methods like FIDA [71] or PCH [22]. If both molecules can be fluorescently labeled with dyes that exhibit a different spectrum, cross-correlation can be applied [18]. With two spectrally different emitting dyes, this has become a standard tool (for a comprehensive review see [72]). The concept has recently been extended to three different dyes [21] or two dyes that do not differ in the fluorescence spectra, but have a different fluorescence lifetime [73].

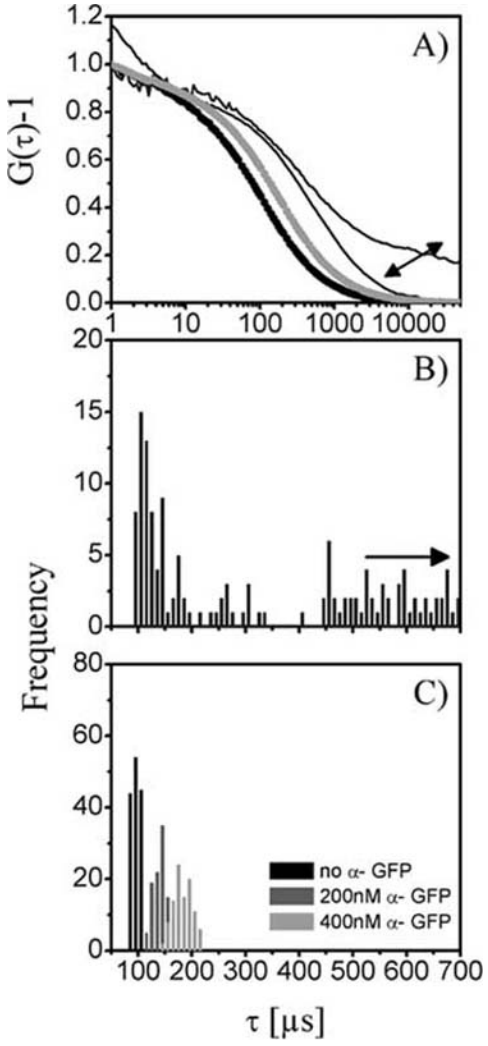

Fig. (6). Comparison of the binding of GFP to a monoclonal and a polyclonal antibody. While binding to a monoclonal antibody just result in a slightly shifted correlation curve, whose maximum remains constant after saturation, binding of a polyclonal antibody results in very heterogeneous correlation curves (A) resulting from the detection of a few large complexes. Consequently, a broad range of diffusion times is fitted in various measurements (B). In contrast, binding of a monoclonal antibody (α - GFP) results in a narrow distribution of well defined single – component fits (C). As two components contribute to the fitted average time, free GFP and GFP bound to antibody, the maxima of the distributions in (C) shift to longer values upon increase of the bound GFP fraction.

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