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# Relaxation Kinetics by Fluorescence Correlation Spectroscopy: Determination of Kinetic Parameters in the Presence of Fluorescent Impurities

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## Abstract

The use of Fluorescence Correlation Spectroscopy (FCS) in combination with Förster Resonance Energy Transfer (FRET) is gaining popularity as a tool to investigate kinetics in equilibrium conditions. The technique is based on the study of fluorescence fluctuations in small numbers of molecules, and is particularly well-suited to investigate conformational dynamics in biopolymers. In practice, its applicability is often hindered by the presence of certain impurities such as partially labeled biomolecules, excess of free fluorophore, or partially dissociated multi-subunit complexes. Here, we show that the simultaneous measurement of the fluctuations in the donor and acceptor intensities allows the determination of the kinetic relaxation time of the reaction in the presence of donor-only particles when cross-talk is negligible, or in cases where all species have the same diffusion coefficient. Theoretical predictions are supported with the results of Monte Carlo simulations, and demonstrate that the applicability of the technique is more general than previously thought.

### Keywords

Fluorescence correlation spectroscopy; Single molecules; Energy-transfer; Kinetics; Fluctuations; Relaxation kinetics

The use of Fluorescence Correlation Spectroscopy (FCS) to investigate conformational dynamics in biopolymers has increased in popularity during the last decade.1⁻6 A common approach takes advantage of the strong distance-dependence of the Förster Resonance Energy Transfer (FRET) phenomenon. The method involves attaching covalently a donor-acceptor FRET pair to the biopolymer so that the timescales of the fluctuations in donor and acceptor fluorescence intensity can be used to determine the relaxation time ($\tau_R$) of the process under equilibrium conditions.[2,7] Briefly, the experiment involves illuminating the sample with a laser that excites selectively the donor, and measuring the fluorescence intensity of the donor and acceptor in an optically-restricted volume containing a low number of molecules in solution (fig. 1). Note that the acceptor is not excited directly by the laser, but only through energy transfer from the donor excited state. Fluorescence fluctuations are then quantified using the auto-and crosscorrelation functions ($G_{DD}$, $G_{AA}$ and $G_{DA}$), which are subsequently fitted to the appropriate physical models to extract the desired kinetic information (i.e. $k_{12}$ and $k_{21}$).[2,8]

Supporting Information Available: Details of Monte Carlo simulations and mathematical derivations, and comparisons between the results of simulations and theoretical equations. This material is available free of charge via the Internet at http://pubs.acs.org.

Contamination with a population of donor-only particles (species 3, fig. 1) creates a potential problem that has been long recognized. These particles contribute to the fluctuations in fluorescence intensity due to translational diffusion within the observation volume, and will in general affect the experimentally measured correlation decays. The problem is significant in biophysical research due to the difficulty of producing fluorescently labeled biopolymers with 100% yield, and because fluorescent acceptors are usually more prone to photochemical degradation (photobleaching). In addition, some biochemically-unstable systems can dissociate at the low concentrations required for the experiment (typically 100 pM–10 nM), leaving donor-only particles diffusing in solution. Although the same is true for acceptor-only particles, they do not contribute to the measured signal because they are not excited during the experiment.

These problems have been addressed recently by applying experimental excitation/emission schemes that allow the filtering of the fluorescence data according to criteria such as color, lifetime and polarization.[5,9–11] Each of these methods has their own strengths and limitations that depend on the characteristics of the system under study. For instance, the use of alternating excitation to distinguish donor-only particles[5] requires strict single-molecule conditions which results in filtered data sets with poor signal-to-noise ratio, especially at timescales close to the diffusion time. Felekyan at al. recently proposed an elegant scheme that introduces fluorescence polarization as a variable for data filtering[10], but its applicability to fluorescently-labeled biopolymers, that often exhibit two or three rotational correlation times[12], has not been demonstrated and does not appear straightforward.

In this letter, we will show that the determination of $\tau_R$ is still possible using conventional FCS in the presence of donor-only particles in many situations of interest in biophysical research. We actually consider the more general situation in which species 3 has visibility in the acceptor channel as well, provided that its relative visibility in the donor and acceptor detectors is constant throughout the experiment. A typical example of this type of situation is the unwanted detection of donor photons in the acceptor detector, known as crosstalk.

It has been well-established that the one-color autocorrelation function of a mixture of species is given by a linear combination of the individual correlation functions, $g_i(\tau)$, in which each species contributes proportionally to its number of molecules ($N_i$) and the square of its brightness ($B_i$):[8,13]

$$G(\tau)=\sum_i N_i B_i^2 g_i(\tau)/\left(\sum_i N_i B_i\right)^2 \quad (1)$$

In the previous equation, the individual components $g_i(\tau)$ represent just the shapes of the decays, or in other words, they are normalized so that $g_i(0) = 1$.

Analogously, we can express the two-color correlation functions as:

$$G_{xy}(\tau)=\frac{\sum_i N_i B_{i,x} B_{i,y} g_{i,xy}(\tau)}{\sum_i N_i B_{i,x} \sum_i N_i B_{i,y}}=\sum_i \frac{f_{i,x} f_{i,y}}{N_i} g_{i,xy}(\tau) \quad (2)$$

where $B_{i,x}$ and $B_{i,y}$ represent the brightness of species $i$ in detectors $x$ and $y$ respectively and $G_{xy}$ represents the total auto- ($x = y$) or cross- ($x \neq y$) correlation function of the system. The

right-hand side of the equation is expressed in terms of the fractional intensities in detectors *x* and *y*, defined as $f_{ix}=N_iB_{ix}/\sum_i N_iB_{ix}$ and $f_{iy}=N_iB_{iy}/\sum_i N_iB_{iy}$.

For species 3, fluctuations arise from translational diffusion only, and therefore the correlation functions ($g_{3,DD} = g_{3,AA} = g_{3,DA}$) can be written in terms of its diffusion coefficient ($D_3$) and the radial and axial semiaxis of the Gaussian observation volume ($\omega_1$ and $\omega_2$) as[13–14]

$$T_3(\tau)=\left[\left(1+4D_3\tau/\omega_1^2\right)\left(1+4D_3\tau/\omega_2^2\right)^{1/2}\right]^{-1} \quad (3)$$

For species 1 and 2, fluctuations in fluorescence intensity arise from both diffusion and conformational dynamics. The corresponding correlation functions can be expressed as the product of a diffusion ($T(\tau)$) and a kinetic term ($Q_{x,y}(\tau)$) provided that the diffusion coefficients of the two states are identical ($D_1 = D_2$).[1,7] Equation 3 can be used to describe the contributions of diffusion by using the diffusion coefficients of species 1 and 2. The kinetic term $Q_{x,y}(\tau)$ has been derived in previous work[7], and can be expressed in terms of the equilibrium constant of the process ($N_2/N_1$) and the fractional intensities of states 1 and 2 as:

$$Q_{x,y}=\frac{(f_{1x}+f_{2x})\left(f_{1y}+f_{2y}\right)-\left(f_{1y}-f_{2y}N_1/N_2\right)(f_{2x}-f_{1x}N_2/N_1)\,e^{-\tau/\tau_R}}{(N_1+N_2)\left(f_{1x}f_{1y}/N_1+f_{2x}f_{2y}/N_2\right)} \quad (4)$$

From eq. 2, and assuming that the diffusion coefficients of states 1 and 2 are identical, the auto- and cross-correlation decays of the system described in fig. 1 can be expressed as:

$$G_{xy}(\tau)=\frac{f_{3x}f_{3y}}{N_3}T_3(\tau)+\left(\frac{f_{1x}f_{1y}}{N_1}+\frac{f_{2x}f_{2y}}{N_2}\right)T_1(\tau)Q_{x,y}(\tau) \quad (5)$$

Although the presence of species 3 affects in principle all measured correlation decays, we will show that the determination of $\tau_R$ is still possible in many cases of interest even if $N_3$, $D_3$ and $B_{3x,y}$ are unknown.

The assumption that the diffusion coefficients of states 1 and 2 are identical is necessary to obtain closed-form expressions for the contributions to the correlation function.[14] Although this may seem too restrictive, the differences in diffusion coefficient between two conformational states of a biomolecule are often small and hard to measure by FCS.[15] An increase in hydrodynamic radius of about 50% has been measured, however, in studies of denaturation of globular proteins[16–17], indicating that deviations can be expected when such dramatic changes in molecular shape are involved. Although the auto- and cross- correlation functions of a two-state system cannot be expressed analytically when $D_1 \neq D_2$, they can still be calculated numerically, providing a means by which the effects of neglecting variations in frictional coefficient can be evaluated.

One interesting case of importance in biophysical research is the situation where species 3 has visibility in the donor detector only. This could be due to incomplete chemical labeling, photochemical degradation of the acceptor, or due to partial dissociation of a subunit containing the donor, and assumes that crosstalk is negligible. Partial dissociation is particularly relevant in research involving biochemical systems comprised of many subunits that might dissociate at the low concentrations used in these experiments. In this case, the donor-only particle does

not necessarily have the same diffusion coefficient as the donor-acceptor particles, but interestingly, we will show that the measurement of the relaxation time of the reaction is still possible.

In this case, $f_{3A} = 0$, and therefore $G_{DA}$ and $G_{AA}$ are proportional to $T_1(\tau)Q_{DA}(\tau)$ and $T_1(\tau)Q_{AA}(\tau)$ respectively (eq. 5) as it is the case when $N_3 = 0$. In other words, the presence of species 3 does not affect the shapes of these two correlation decays, but just their amplitudes. This is the basis for the successful determination of $\tau_R$ even when $N_3 \neq 0$. It should be noted that the amplitude of $G_{DA}$ does in fact depend on $N_3$ ($f_{1D}$ and $f_{2D}$ are functions of $N_3$), so it is not true that the presence of donor-only particles does not affect the cross-correlation decay as it has been assumed elsewhere[18]. Yet, although the amplitude of the decay is affected, the relaxation time can be determined from the temporal decay of the ratio $F_A(\tau) = G_{AA}(\tau)/G_{DA}(\tau)$ as follows. This ratio is independent of the diffusion contributions, and can be written as $[K_1 + K_2\, exp(-\tau/\tau_R)]/[K_3 + K_4\, exp(-\tau/\tau_R)]$, where the constants $K_{1-4}$ are functions of the fractional intensities, $N_1$ and $N_2$ (eq. 4). It can be shown by simple algebraic manipulation that the function

$$Y(\tau)=\frac{F_A(\tau=0)-F_A(\tau\to\infty)}{F_A(\tau)-F_A(\tau\to\infty)}=\frac{K_4}{K_3+K_4}+\frac{K_3}{K_3+K_4}e^{\tau/\tau_R}\qquad f_{3A}=0 \tag{6}$$

does not depend on $N_3$ (see supplemental material for more details). In addition, a plot of $ln\ Y(\tau)$ vs $\tau$ is linear with slope $1/\tau_R$ at long lag times ($K_4 \ll K_3 exp\ (\tau/\tau_R)$), allowing the determination of the relaxation time from measurements of $G_{AA}(\tau)/G_{DA}(\tau)$ even in the presence of donor-only species. This procedure was tested using Monte Carlo simulations as discussed below.

Another case of interest is the situation where $D_3 = D_{1,2}$, with any value of $f_{3A}$. This represents the case where a fraction of the particles is not dynamic (i.e. the donor-acceptor distance remains constant during the experiment), or a situation of donor-only labeled particles with significant cross-talk. In this case, $T_3(\tau) = T_{1,2}(\tau)$, and an analysis analogous to the one presented in the previous paragraph shows that the ratios $F_A(\tau) = G_{AA}(\tau)/G_{DA}(\tau)$ and $F_D(\tau) = G_{DD}(\tau)/G_{DA}(\tau)$ can both be used to construct functions that yield $\tau_R$ even in the presence of species 3 (see supplemental information for more details):

$$Y(\tau)=\frac{F_A(\tau=0)-F_A(\tau\to\infty)}{F_A(\tau)-F_A(\tau\to\infty)}=\frac{F_D(\tau=0)-F_D(\tau\to\infty)}{F_D(\tau)-F_D(\tau\to\infty)}=\frac{K_4}{K_3+K_4}+\frac{K_3}{K_3+K_4}e^{\tau/\tau_R}\qquad D_3=D_{1,2} \tag{7}$$

The procedures described above are illustrated in fig. 2, which represents the results of three Monte Carlo simulations of the behavior of the system of fig. 1. A three dimensional random walk was used to simulate translational diffusion, and the back and forth chemical reactions between species 1 and 2 were simulated as Poisson processes as described elsewhere[19] (see supporting information for details). The parameters used in the simulations were $N_2/N_1 = 3$, $N_2 + N_1 = 0.91$, $\tau_R = 125$ μs, $B_{1D} = 2{,}000$, $B_{2D} = 200$, $B_{3D} = 3{,}000$, $B_{1A} = 400$, $B_{2A} = 2{,}000$, $\omega_1 = \omega_2 = 0.35$ μm, and $D_1 = 50$ μm$^2$ s$^{-1}$. The remaining parameters varied as follows: case A: $N_3 = 0$, case B: $N_3 = N_1$, $D_3 = 400$ μm$^2$ s$^{-1}$, $B_{3A} = 0$ (species 3 is smaller than species 1 and 2 and has no visibility in the acceptor detector), and case C: $N_3 = N_1$, $D_3 = D_1$ and $B_{3A} = 300$ (species 3 has visibility in the acceptor detector and the same diffusion coefficient as species 1 and 2). The diffusion coefficients represent typical values for a protein (D ~ 50 μm$^2$ s$^{-1}$)[20] and a free fluorescent dye (D ~ 400 μm$^2$ s$^{-1}$)[21], the latter being the extreme example of donor-only contamination.

The correlation decays obtained in all three simulations are in excellent agreement with the curves predicted by equations 3–5 (see supporting information). Figures 2A–C show the simulated $G_{DD}$ (black), $G_{DA}$ (red) and $G_{AA}$ (blue) decays for the three cases described above. As expected, $G_{DD}$ for case B is greatly influenced by the presence of large quantities of fast-diffusing donor-only particles. However, because in this case species 3 has no visibility in the acceptor channel, $G_{AA}$ overlaps with the decay obtained for case A (supporting information fig. S5). The cross-correlation decay overlaps only in shape, but the amplitude of $G_{DA}$ is lower because the large excess of donor-only particles affects the average donor intensity measured in the experiment (supporting information, fig. S6).

Importantly, despite the significant differences in the individual correlation decays, the three cases yield the same $ln\ Y(\tau)$ vs $\tau$ plot (fig. 2D), which is linear with a slope $1/\tau_R$ at long times. In figure 2D, the red, blue and green lines represent the natural logarithm of $Y(\tau)=[F_A(\tau = 0) - F_A(\tau \rightarrow \infty)]/[F_A(\tau) - F_A(\tau \rightarrow \infty)]$ for cases $\tau$ A, B $\tau$ and C respectively, while the orange line represents the same function calculated from $F_D(\tau) = G_{DD}(\tau)/G_{DA}(\tau)$ for case C ($f_{3A} \neq 0$, $D_3 = D_{1,2}$). The black straight line represents the term $ln[K_3 e^{\tau/\tau_R}/(K_3 + K_4)]$ (see eq. 6) calculated from the parameters used in the simulation, and therefore has a slope of $1/\tau_R$. The dotted vertical line represents the relaxation time for the simulated reaction, indicating that linearity is poor at $\tau \ll \tau_R$. The inset in fig. 2D shows $Y(\tau)$ calculated from the donor autocorrelation function ($F_D(\tau) = G_{DD}(\tau)/G_{DA}(\tau)$) for case B, showing a clear deviation from linearity. This is expected because the fast-diffusing contaminating particles greatly affect the donor-autocorrelation decay, but leave the shape of the cross-correlation decay unaltered because their visibility in the acceptor detector is negligible.

In conclusion, these results support the idea that it is possible to measure the relaxation time of certain reactions in equilibrium using FRET and FCS even in the presence of excess donor-only particles provided that cross-talk is not significant. It should be noted that the effects of crosstalk can be eliminated by performing alternating laser excitation as described in reference [9]. Yet, if species 3 has significant visibility in the acceptor detector, the determination is possible if its diffusion coefficient is identical to that of species 1–2. In this case, the ratios $F_A(\tau) = G_{AA}(\tau)/G_{DA}(\tau)$ or $F_D(\tau) = G_{DD}(\tau)/G_{DA}(\tau)$ can be used to construct functions that yield $\tau_R$ independently of $N_3$ and $f_{3A}$. The results of further simulations that support these conclusions are included as supporting material.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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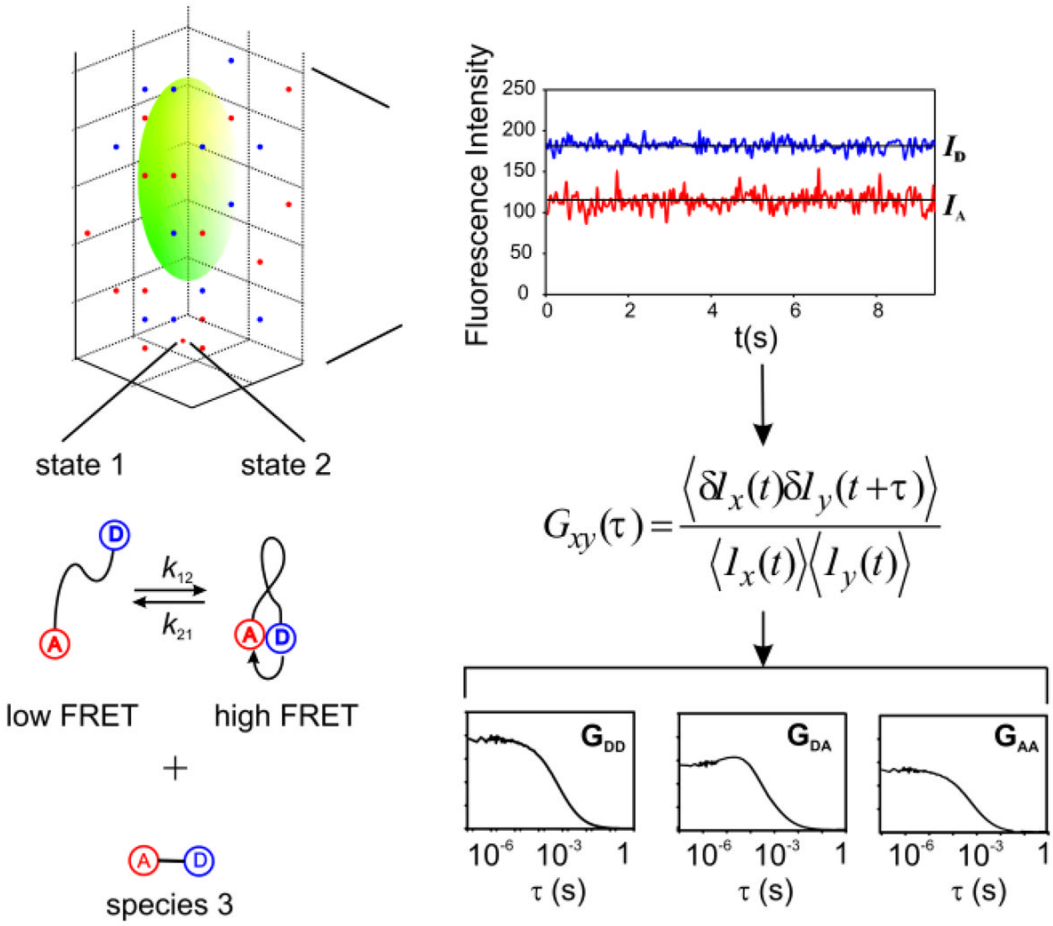


**Figure 1.**

Schematics of the FCS experimental measurement and chemical system investigated in this work. Point molecules diffuse freely throughout a Gaussian observation volume (green surface). Species 1 and 2 exist in equilibrium, and interconvert with rates $k_{12}$ and $k_{21}$ for the forward and backward reaction respectively. The two states are distinguishable by their FRET efficiency, which determines the visibility of each state in the donor and acceptor detectors. Species 3 has constant brightness in both detectors throughout the experiment. The fluorescence intensity, which is the sum of the contributions of all molecules, is measured in two independent detectors (blue: donor, red: acceptor). Fluctuations arise due to translational diffusion (all species), and due to chemical kinetics (species 1 and 2). Fluctuations are analyzed in terms of their auto- or cross-correlation functions ($G_{DD}$, $G_{DA}$, and $G_{AA}$), defined as: $G_{xy}(\tau) = \langle \delta I_x(t)\, \delta I_y(t+\delta t) \rangle / [\langle I_x(t) \rangle \langle I_y(t) \rangle]$ where $\delta I(t) = I(t) - \langle I(t) \rangle$ represents the deviation from the mean.

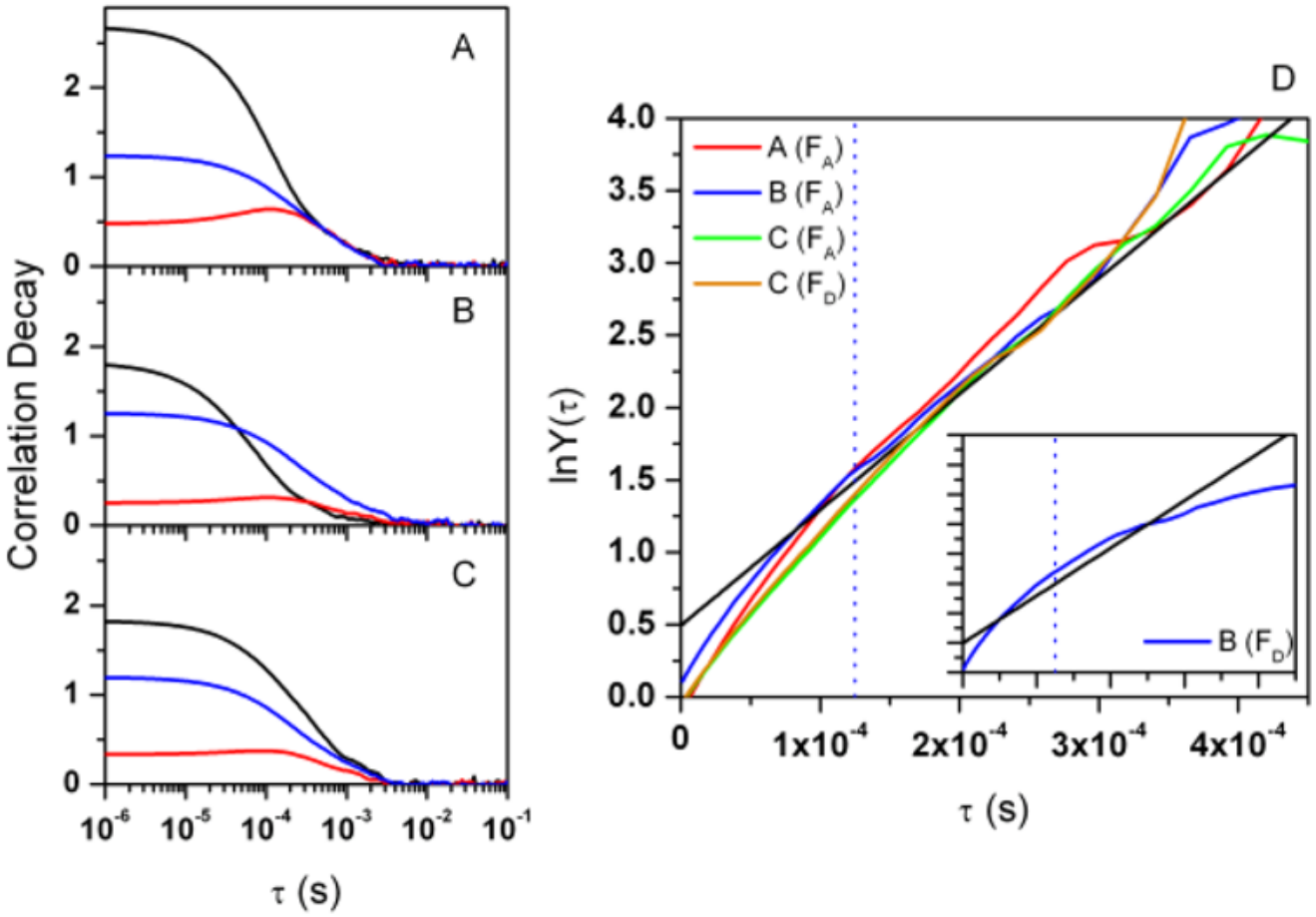


**Figure 2.**

Left: Correlation decays obtained from Monte Carlo simulations for cases A–C. The black, blue and red lines represent $G_{DD}$, $G_{AA}$, and $G_{DA}$, respectively. Right: Results of Monte Carlo simulations analyzed as described in the text (eq. 7). The red, blue and green curves are constructed from the $G_{AA}/G_{DA}$ ratio ($F_A$) for cases A, B and C respectively. The orange trace was constructed from the $G_{DD}/G_{DA}$ ratio ($F_D$) for case C. The black straight line represents $ln[K_3e^{\tau/\tau_R}/(K_3 + K_4)]$ calculated from the parameters used in the simulation. The blue dotted vertical line denotes the relaxation time of the reaction (125 μs). Inset: $lnY(\tau)$ calculated from $G_{DD}/G_{DA}$ for case B (blue) showing poor linearity at all times. The scales of both axes are the same as in the parent plot.