Supporting Information

S1: FCS Simulations: test to validate the method.

Simulation of the reaction 1⇔2 with $\boldsymbol{k_{12} = 15 \times 10^{3}\ s^{-1}}$, $\boldsymbol{k_{21} = 5 \times 10^{3}}$ s^{-1} $D_1 = D_2 = 37.5\ \mu m^2.s^{-1}$

The observation volume was modeled by a 3D Gaussian with $\omega_1 = 0.35\ \mu m$ and $\omega_2 = 5\omega_1$. The simulation step time was set to $dt = 1\ \mu s$, and the total simulation time was 0.5s (510^5 cycles)

Input parameters:

N= 1000	//total number of molecules
L1 = 3.2 μm	// semiaxis of the simulation box along dimensions *x* and *y*
L2 = 5**L1*	// semiaxis of the simulation box along dimension *z*
f1 = ¼	// initial fraction of molecules in state 1.
p21 = 0.005	// probability that a molecule in state 2 changes to state 1 (*)
p12 =(1-*f*1)/*f*1**p*21	// probability that a molecule in state 1 changes to state 2 (*)
j1 = 2000	// I_D for a molecule in state 1 (**)
j2 = 200	// I_D for a molecule in state 2 (**)
h1 = 400	// I_A for a molecule in state 1 (**)
h2 = 2000	// I_A for a molecule in state 2 (**)

(*) $p_{21} = k_{21} * dt$, $p_{12} = k_{12} * dt$

(**) Intensities at the center of the observation volume. At any other position, the intensity is

$$I_D = j_{1,2} e^{-2(x^2+y^2)/\omega_1^2} e^{-2z^2/\omega_2^2}$$

$$I_A = h_{1,2} e^{-2(x^2+y^2)/\omega_1^2} e^{-2z^2/\omega_2^2}$$

Results:

Figure 1S shows the donor-autocorrelation (black), acceptor autocorrelation (red) and donor-acceptor crosscorrelation (blue) obtained in this simulation.

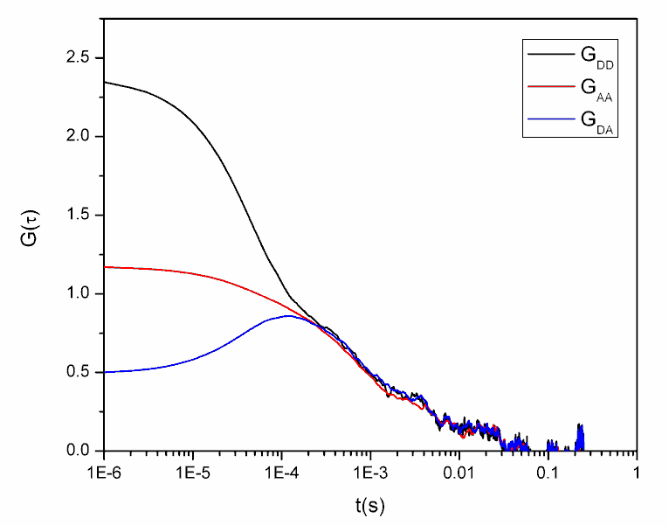

Figure S1

The following analysis shows that these results are consistent with the relaxation time of the process ($\tau_R = (k_{21} + k_{12})^{-1} = (20 \times 10^3\ \mathrm{s}^{-1})^{-1} = 50\ \mu\mathrm{s}$.

1) According to Bonnet et al.[1], the kinetic terms are isolated once the autocorrelation decay of the donor is divided by the diffusion contributions (G_{dif}):

$$G_{Dif}(\tau) = \frac{1}{\langle N \rangle}\left(\frac{1}{1+\tau/\tau_d}\right)\left(\frac{1}{1+\tau\omega_1^2/\tau_d\omega_2^2}\right)^{1/2} \quad ,\tau_d = 4D/\omega_1^2 \qquad \text{eq.S1}$$

---

[1] Bonnet, G.; Krichevsky, O.; Libchaber, A. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 8602.

The mean number of molecules in the observation volume, $\langle N \rangle$, is calculated as N*V_{eff}/V_{box}, where $V_{box} = 2L_2*(2L_1)^2$, and the effective observation volume is $V_{eff} = \pi^{3/2}\omega_1^2\omega_2$. $\langle N \rangle = 0.91$ for the parameters used in this simulation.

The red line in fig. S2 shows the ratio G_{DD}/G_{dif}, where G_{DD} is the simulated donor autocorrelation and G_{dif} is the diffusion contribution calculated from eq.S1 using the input simulation parameters. The black line is the exponential function $1+A*\exp(t/50\times10^{-6}\text{s})$

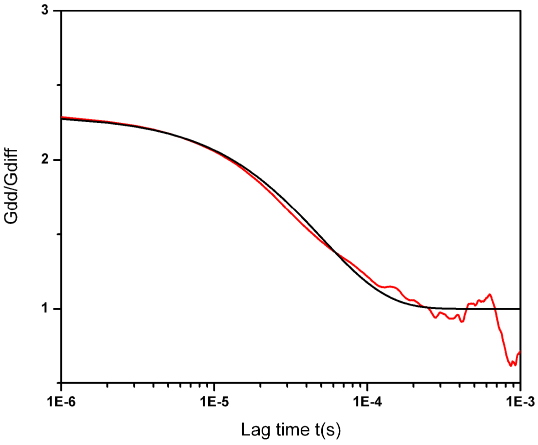

Figure S2

2) According to Torres and Levitus[2], the kinetic terms are isolated once the autocorrelation decay of the donor is divided by the donor-acceptor cross-correlation calculated from the same experimental intensity fluctuations:

S2

$$G_{DD}(\tau) = G_{Dif}(\tau)X_{DD}(\tau)$$
$$G_{DA}(\tau) = G_{Dif}(\tau)X_{DA}(\tau)$$

S3

---

[2] Torres, T.; Levitus, M. *J. Phys. Chem. B* **2007**, *111*, 7392.

$$X_{DD}(\tau) = 1 + \frac{k_{12}k_{21}(E_1 - E_2)^2}{\left(k_{21}(1-E_1) + k_{12}(1-E_2)\right)^2} e^{-(k_{12}+k_{21})\tau}$$

$$X_{DA}(\tau) = 1 - \frac{k_{12}k_{21}(E_1 - E_2)^2}{\left(k_{21}(1-E_1) + k_{12}(1-E_2)\right)\left(k_{21}E_1 + k_{12}E_2\right)} e^{-(k_{12}+k_{21})\tau}$$

where E_1 and E_2 represent the FRET efficiencies in states 1 and 2 (identical to the proximity factors in an ideal case where the two dyes have the same fluorescence quantum yield and are detected with equal efficiency).

The black line in fig. S3 shows the ratio G_{DD}/G_{DA}, where G_{DD} is the simulated donor autocorrelation and G_{DA} is the simulated donor-acceptor cross-correlation. The red line represents the ratio of the theoretical correlation decays (X_{DD}/X_{DA}, eq. S3) using the input parameters used in the simulation.

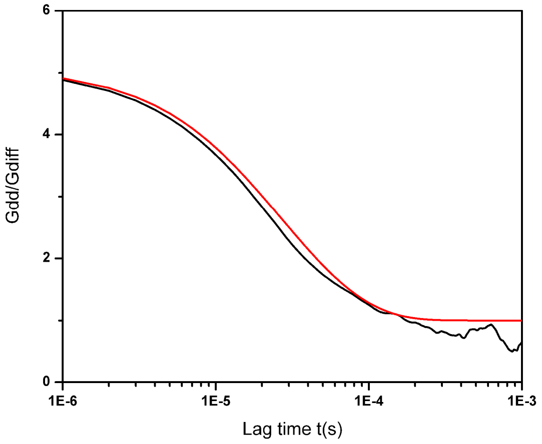

Figure S3

S2: parameters used in all FCS Simulations.

Table S1: Parameters used in simulations presented in figures 3-5 of the manuscript. See section S1 for more details regarding the meaning of these variables.

Figure 3	Figure 4	Figure 5	Description
ω_1 = 0.35 μm	ω_1 = 0.4 μm	ω_1 = 0.35 μm	radial semiaxis of Gaussian obs. volume
$\omega_2 = 5\omega_1$	$\omega_2 = \omega_1$	$\omega_2 = \omega_1$	axial semiaxis of Gaussian obs. volume
dt = 10 μs	dt = 1 μs	dt = 1 μs	simulation step time
T = 5s	T = 0.5s	T = 0.5s	total simulation time
N= 1000	*N*= 1000	*N*= 2000	total number of molecules
dr =0.04 μm	*dr* = 0.015 μm	*dr* = 0.035 μm	Simulation step length ($dr^2 = 6D\,dt$)
L1 = 3.2 μm	*L1* = 3.2 μm	*L1* = 3.2 μm	semiaxis of the simulation box along dimensions *x* and *y*
L2 = 5**L1*	*L2* = *L1*	*L2* = *L1*	semiaxis of the simulation box along dimension *z*
f1 =1/2	*f1* =3/4	*f1* =1/2	initial fraction of molecules in state 1.
p21 = 1×10^{-4}	*p21* =0.02	*p21* =0.002	probability that a molecule in state 2 changes to state 1
p12 =1×10^{-4}	*p12* =6.7×10^{-3}	*p12* =0.002	probability that a molecule in state 1 changes to state 2
j1 = 2000	*j1* = 2000	*j1* = 2000	I_D for a molecule in state 1 located at the center
j2 = 100	*j2* = 500	*j2* = 500	I_D for a molecule in state 2 located at the center
h1 = 200	*h1* = 500	*h1* = 500	I_A for a molecule in state 1 located at the center
h2 = 2000	*h2* = 2000	*h2* = 2000	I_A for a molecule in state 2 located at the center

S3: Diffusion analyzed as a stretched exponential

Figure S4 shows the fluorescence autocorrelation decay of the dye Tetramethylrhodamine diffusing freely in an aqueous buffer at room temperature (red dots). The black line represents the results of a non-linear fit to a stretched exponential kinetic model:

$$G_p(\tau) = G_p(0)\exp\left[-(\tau/\tau_R)^{\beta}\right] \quad \text{S4}$$

The parameters obtained in the fitting procedure are τ_R = 99.4 μs and β = 0.37.

The purpose of this comparison is to demonstrate that FCS decays that are dominated by diffusion can be successfully fitted with a stretched exponential model.

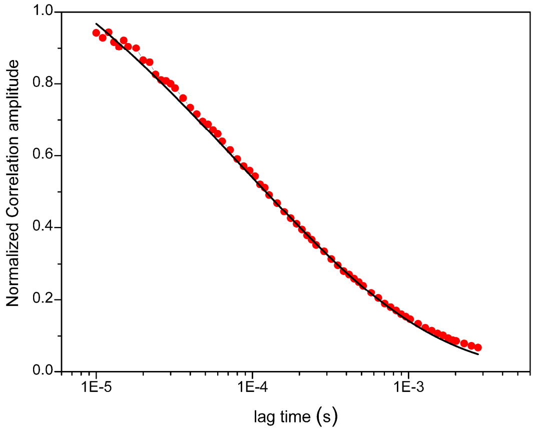

Figure S4.

S4: The amplitude of the Gp decay: Simulations

We derived an expression of the amplitude of the Gp decay in situation where the kinetic contributions dominate the fluorescence fluctuations ($\tau_D >> \tau_R$)

$$G_p(0) = \frac{1}{\langle N \rangle + 1}\frac{f_1 p_1^2 + f_2 p_2^2}{(f_1 p_1 + f_2 p_2)^2} - 1 = \frac{1}{\langle N \rangle + 1}\frac{K(1-Q)^2}{(1+QK)^2} \quad \text{S5}$$

With Q = p_2 / p_1 and K = f_2 / f_1,

$\langle N \rangle$ is calculated as N^*V_{eff}/V_{box}, where N is the number of molecules in the simulation box of volume V_{box}, and V_{eff} is the effective observation volume defined as $\pi^{3/2}\omega_1^2\omega_2$.

We have performed 12 simulations spanning a variety of parameters to test this equation.

Table S2 Comparison between amplitudes obtained in simulations and expected amplitudes

$\langle N \rangle$	p_1	p_2	f_1	f_2	**A1(*)**	**A2 (#)**
0.68	0	1.00	0.50	0.50	0.64	0.60
2.72	0	1.00	0.50	0.50	0.33	0.27
0.91	0	1.00	0.50	0.50	0.53	0.52
1.36	0	1.00	0.25	0.75	0.17	0.14
1.36	0	1.00	0.75	0.25	1.31	1.27
0.91	0.11	0.89	0.33	0.667	0.20	0.18
1.36	0.20	0.80	0.50	0.50	0.16	0.15
1.36	0.20	0.80	0.75	0.25	0.24	0.23
0.91	0.33	0.67	0.50	0.50	0.07	0.06
1.36	1.00	0	0.70	0.30	0.22	0.18
0.91	1.00	0	0.40	0.60	0.83	0.79
0.91	1.00	0	0.20	0.80	2.00	2.09

* A1 is the amplitude obtained in the simulation

A2 is the expected amplitude calculated from eq. S5.

Results show an excellent correlation between the value of the amplitude obtained from the equation derived in this work and the amplitude obtained from the simulations. The black line represents the result of a linear fit, which gives a slope of 1.003.

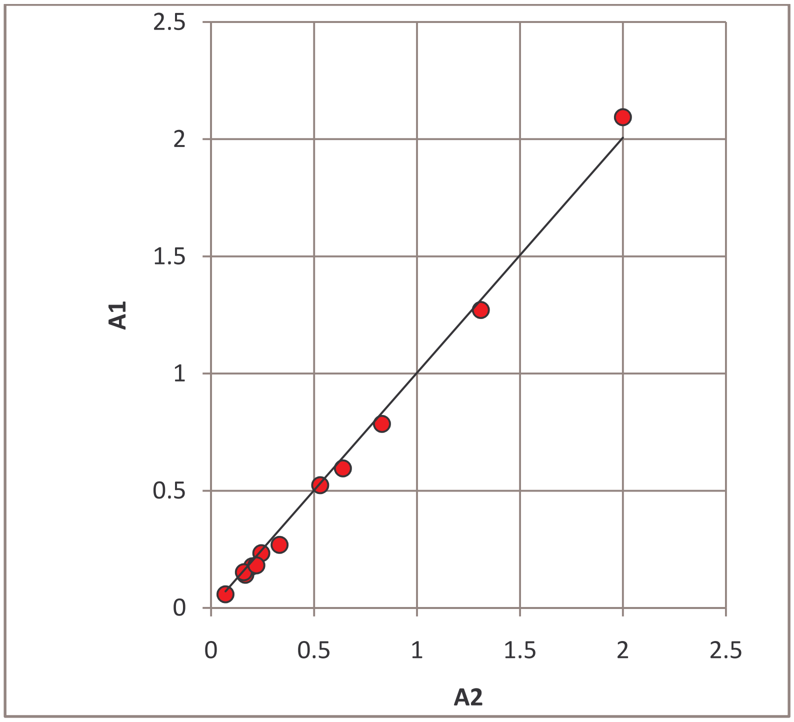

Figure S5.

S5: Influence of Background

It has been well-established that the presence of non-correlated background does not distort the shape of the correlation decays of the fluorescence intensities, but just affects their amplitudes.[3] In this section, we investigate the effects of background on the G_P decay through Monte Carlo simulations. Simulations were run with no background using the parameters listed below. The obtained data was then "contaminated" with different levels of non-correlated background by adding a random number to

---

[3] Thompson, N. L. Fluorescence Correlation Spectroscopy. In *Topics in Fluorescence Spectroscopy: Techniques*; Lakowicz, J. R., Ed.; Plenum Press: New York, 1991; Vol. 1.

the fluorescence intensity. Random numbers were chosen from a Poisson distribution with a mean equal to a given fraction of the mean intensity signal measured in the absence of background:

$D(t) = d(t)$ + poisssrnd(mean(d)*f_d)

$A(t) = a(t)$ + poisssrnd(mean(a)*f_a)

Here, the lower-case variables a and d refer to the acceptor and donor intensities obtained from the simulation (no background), and the upper-case variables refer to the intensities contaminated with background. The function poissrnd(μ) (Matlab) chooses a random number from a Poisson distribution with a mean μ. In this case, the mean was chosen as a fraction f of the average intensities detected in each detector.

The parameters used in the simulations discussed below are:

$\omega_1 = \omega_2$ 0.4 μm

$L1 = L2 = 3.2$ μm

dt = 1 μs

dr =0.04 μm

$f1$ =3/4

$p21 = 0.02$

$p12 = 6.7 \times 10^{-3}$

$j1 = 2000$

$j2 = 200$

$h1 = 200$

$h2 = 2000$

The remaining parameters varied in different simulations as described below:

Case A) $N = 500$, $f_a = f_d = 0$ (No background).

Case B) $N = 500$, $f_a = 0.3$ (30% background), $f_d = 0.05$ (5% background).

Case C) $N = 500$, $f_a = 0.3$ (30% background), $f_d = 0.3$ (30% background).

Case D) $N = 5000$, $f_a = f_d = 0$.

Case E) $N = 5000$, $f_a = 0.3$ (30% background), $f_d = 0.05$ (5% background).

In all cases, the intensity autocorrelation functions (G_{DD}, G_{DA} and G_{AA}) calculated from these simulations are identical in shape to the correlation functions calculated in the absence of background, and their amplitudes decrease exactly as predicted by theory. [3] However, simulation shows that the decay of the proximity factor correlation can become contaminated with diffusion contributions even if $\tau_D >> \tau_R$.

Figures S6 and S7 show the results of simulations A and B. Figure S6 shows a representative time window (the total simulation time was 0.5 s) for the case with no background (left) and with 30% background in the acceptor detector and 5% background in the donor channel (right). The acceptor intensity is plotted in blue (bottom), while the calculated time-dependent proximity factor is plotted in green (top). The donor trace is not shown for clarity. In the absence of background, the proximity factor clearly fluctuates between a high and low value as the system exchanges conformation. The intensity trace shows that the volume is empty a significant fraction of the time, as expected for a system with $\langle N \rangle = 0.68$ (red arrows). However, because there is no background, the small intensities detected in this case still give rise to the same proximity factors. In the presence of background, the values of p in these regions are determined by the background levels, and not from the small contributions from the fluorescent molecules. This apparent p value depends exclusively on the background levels (compare with figure S8 below). As a consequence, the proximity factor fluctuates between a high and low value due to kinetics only in the time periods in which the volume is occupied with fluorescent molecules. However, when the volume is empty, the measured p is determined by the relative background levels.

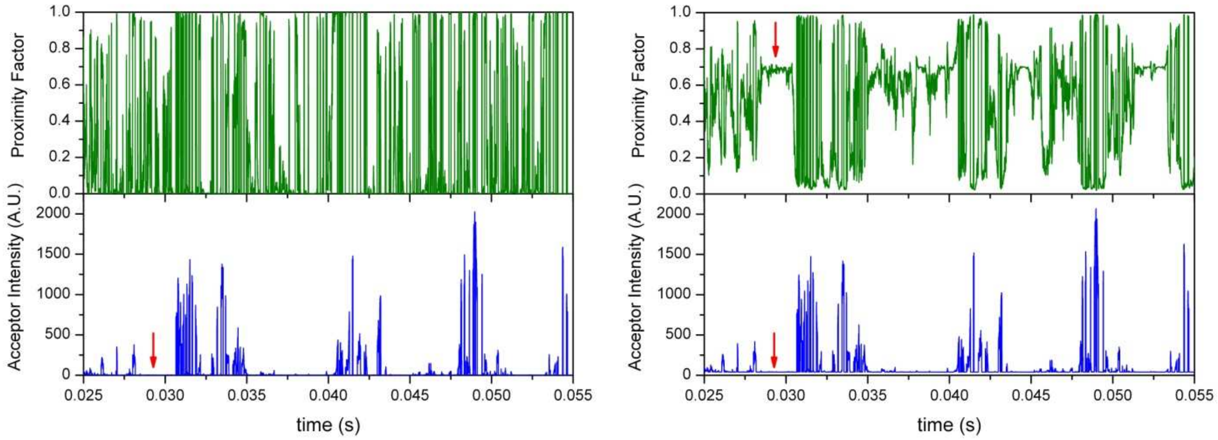

Figure. S6: Simulations with $\langle N \rangle$ =0.68 and f_d = f_d =0 (left) and f_d = 0.05 and f_a = 0.3 (right). The bottom plots represent the acceptor intensities (donor intensities not shown for clarity) and the bottom plots represent the calculated proximity factors. The red arrows point to regions in time where no fluorescent molecules are inside the confocal region.

Figure S7 shows the G_P decays of the two cases described above. The black curve corresponds to case A (no background), and it’s described accurately in terms of a purely kinetic model because $\tau_D >> \tau_R$. The red curve corresponds to case B, and shows a small contribution due to diffusion in the ~200 μs timescale. This contribution is caused by the added fluctuations between the *p* values given by background (red arrow in figure S6) and the *p* values determined by the kinetic system. Because these fluctuations are due to fluctuations in the number of molecules in the observation volume, their contribution occurs in the timescales of molecular diffusion. Therefore, the autocorrelation decay in the presence of background presents a diffusion contribution even when $\tau_D >> \tau_R$.

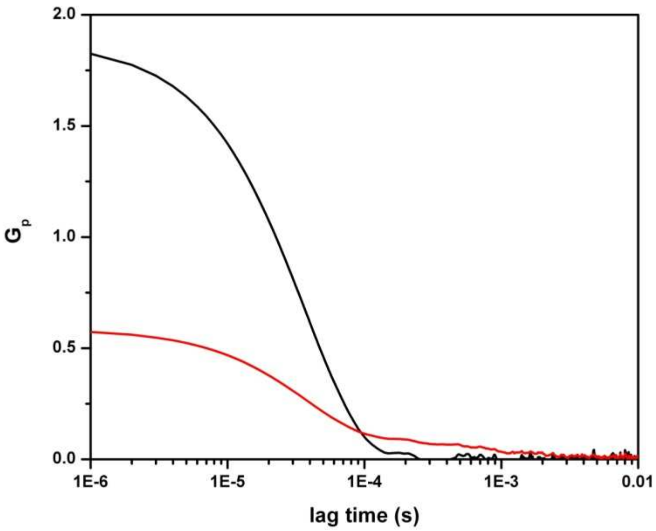

Figure S7: G_p decays for the simulations with $\langle N \rangle$ =0.68 and f_d = f_d =0 (black) and f_d = 0.05 and f_a = 0.3 (red).

It is interesting to note that these diffusion contributions dissapear when the apparent proximity factor measured from background equals the average proximity factor of the kinetic system (the value one would measure in the absence of background). This situation is illustrated in figure S8, which compares the same simulation with different levels of added backgound. The red curves represent the results for case B described above. The blue curves repesent the results for case C, in which the donor background was increased from 5% to 30%. Because the background levels in both channels are now the same fraction of the average intensities in each channel (f_a = f_d), the apparent proximity factor measured when the observation volume is empty is the average proximity factor that would be measured in the absence of background. In this case, the proximity factor fluctuates between a high and low value in the timescale of the kinetic process. These fluctuations are averaged out at longer timescales, and because the *p* value measured for the background is identical to this average, no new fluctuations due to diffusion are observed in this case.

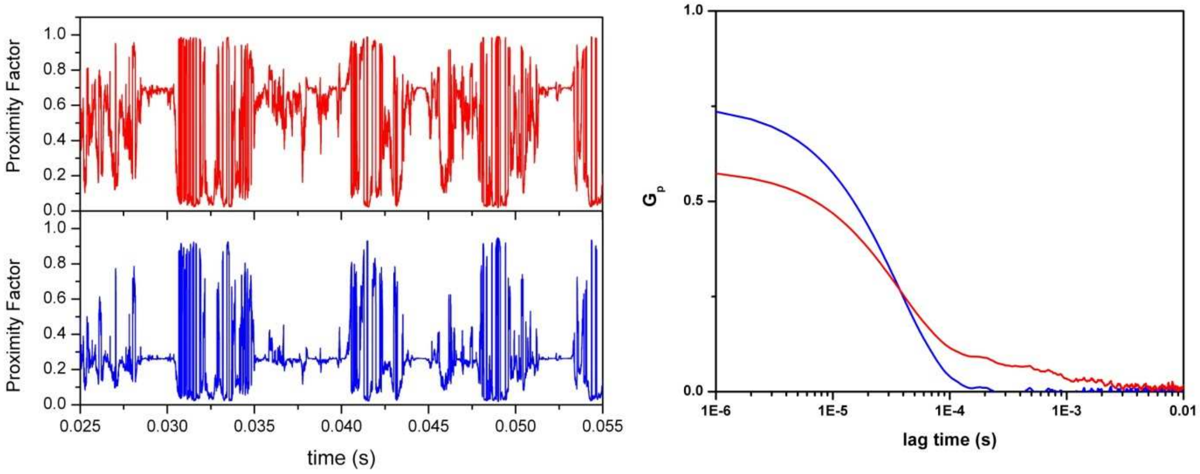

Figure S8: Simulations with $\langle N \rangle$ =0.68 and f_d = 0.05 and f_a = 0.3 (red) and f_d = 0.3 and f_a = 0.3 (blue). The left plots contain the simulated time-dependent proximity factors, while the decays on the right represent the corresponding G_p decays.

The above discussion suggests that diffusion contributions will not be significant at concentrations of sample high enough so that the proximity factor is never dominated by background alone. This is in fact observed in simulations D and E, in which $\langle N \rangle$ = 6.8. Figure S9 shows the result of these simulations. The blue trace on the left represents the acceptor intensity for case E (f_a = 0.3, f_d = 0.05), and the green trace represents the corresponding proximity factor. Because in this case the observation volume is likely to be occupied with several fluorescent molecules at all times, the fluctuations between the two proximity factor regimes observed before disappear. The plots on the right represent the G_p decays of the simulations with conditions D (no background, blue trace) and E (f_a = 0.3, f_d = 0.05, red trace). The two traces are overlapped in amplitude in the inset, where it is clear that the diffusion contributions observed at low concentrations are not significant in this case.

To conclude, non-correlated background has the potential of causing fluctuations in the proximity factor in the timescales of diffusion, even when kinetics is much faster than diffusion ($\tau_D >> \tau_R$). However, it is expected that these contributions are significant only at low concentrations, where the observation volume is likely to be found empty at times.

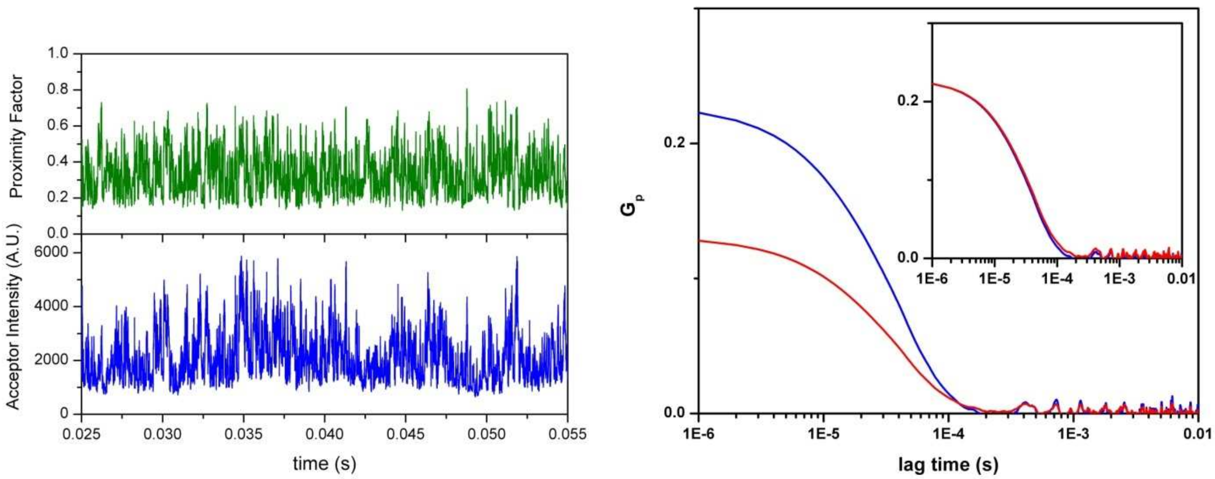

Figure S9: Simulation with $\langle N \rangle$ =6.8. Left: Acceptor intensity (blue) and proximity factor (green) for the simulation using f_a = 0.3 and f_d = 0.05 (case E). Right: G_p decays for cases E (red) and D ($f_a = f_d = 0$, blue). The inset shows the two curves arbitrarily overlapped in amplitude.