Journal of Physics: Conference Series **478** (2013) 012006 doi:10.1088/1742-6596/478/1/012006

Statistics for fluorescence photons of donor and acceptor molecules involved in non-radiative energy transfer (FRET)

A L Shchukina, I S Osad'ko, I Yu Eremchev

Institute for Spectroscopy of Russian Academy of Sciences, Troitsk 142190, Moscow, Russia

E-mail: a.schukina@inbox.ru

Abstract. Fluorescence of single molecular pairs involved in Förster Resonance Energy Transfer (FRET) is considered. Cases when either a donor of the electronic excitation energy or its acceptor has a non-fluorescent "dark" state are described. The influence of such dark states on energy transfer efficiency is analytically studied. Statistics for the number of donor/acceptor photons emitted per bin time is applied for these studies. Experimental distributions for photons of single emitters with "dark" states are presented, as well as their multivariate approximations with simulated distributions. It gives an example of how emitter parameters necessary for FRET efficiency calculations (transitions to and from the dark state) can be determined from the experimental statistics.

1. FRET: general

Single molecule spectroscopy, which emerged in 1990s [1-3], and superresolution techniques, which developed in 2000s [4-6], have naturally discovered new horizons in the research into the matter on the nanoscale level. Despite this, conventional optical techniques such as Förster Resonance Energy Transfer (FRET) are still in some cases the most suitable tool to probe processes undergoing there, especially in fragile biosystems [7].

The modern theory for FRET was presented by Th. Förster in 1948 [8]. In an act of FRET, a chromophore (donor), which has previously been electronically excited, transfers its excitation energy non-radiatively to another chromophore (acceptor) via dipole-dipole interaction. After this the donor is found in the ground state, and the acceptor – in the electronically excited one. In paper [8], the FRET rate was expressed via the spectral overlap between the donor emission and the acceptor absorption, the angle factor (a value depending on the angles between the donor/acceptor dipoles and the line connecting their centers) and the distance between two chromophores. The latter dependence allows using FRET as a "ruler" to measure distances on a nanometer scale.

Experimentally observable values are the donor/acceptor intensities. The quantity characterizing their relation is FRET efficiency E, which is by definition the ratio of the acceptor fluorescence intensity I_A to the summarized intensity of both emitters $I_A + I_D$:

$$E = \frac{I_A}{I_D + I_A}. \quad (1)$$

2. Blinking fluorescence in FRET

According to (1), FRET efficiency E may vary from 0 (no energy transfer) to 1 (full energy transfer). However, expression (1) does not take account of possible intermittences in fluorescence of either of the emitters. Such intermittences, though, are often encountered due to various reasons in fluorescence of organic molecules [9], which are popular FRET donors and acceptors.

Figure 1 illustrates how fluorescence of a single quantum light emitter becomes blinking.

If a chromophore is excited with continuous-wave radiation, it jumps from the ground state *0* to the first excited state *1*. The contribution of higher excited states is negligible, so they are not considered. Level *2* in figure 1a is a long-lived non-fluorescent state. The rate of getting there is much less than the excitation and fluorescence rates, and so is the rate of leaving it. When a chromophore gets to state *2*, its fluorescence stops and resumes when it leaves that state. Thus, so-called off-states (or dark states) alternate with on-states (bright states) – its fluorescence is blinking (figure 1b).

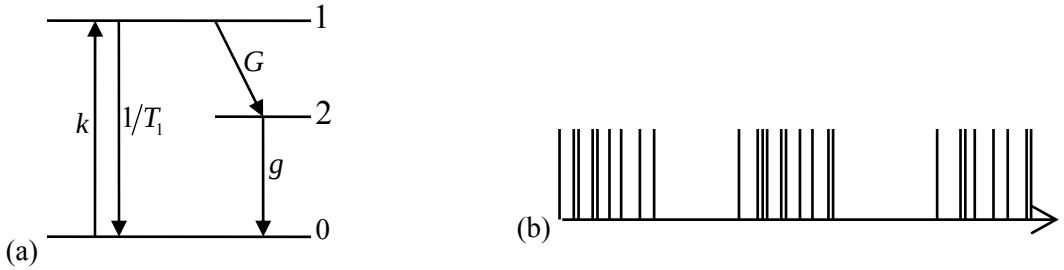


Figure 1. a) Three-level emitter. 0 – ground state, 1 – first excited state, 2 – "dark" state (off-state). k – excitation rate, $1/T_D$ – fluorescence rate, G and g – on-off and off-on transition rates. b) Instants of photon emission: blinking fluorescence of a three-level particle.

In FRET studies, blinking is usually neglected. Off-intervals are "cut" from the donor/acceptor fluorescence intensity records (trajectories), and only the remaining parts are used to calculate FRET efficiency. However, the present paper demonstrates a possibility to take into consideration full donor/acceptor trajectories with off-intervals.

3. Specific features of blinking in different types of donor-acceptor pairs

A donor and an acceptor can either be described by a 2-level electronic energy scheme (no off-state *2*, states *0* and *1* in figure 1b are only present, continuous fluorescence) or by a 3-level scheme (blinking fluorescence described above). Let us consider two types of donor-acceptor pairs: 3D-2A (3-level donor and 2-level acceptor) and 2D-3A (2-level donor and 3-level acceptor).

First of all, we have simulated donor and acceptor fluorescence for each type. The results are presented in figure 2.

Instants of donor and acceptor photon emission are shown with blue (upper) and red (lower) segments accordingly. The energy transfer rate F increases from the top to the bottom for each pair. The values of FT_D, where T_D is donor fluorescence characteristic time (fixed), are given on the left.

The present results were obtained with the help of the Monte-Carlo method based on rate equations for the populations of each energy level in a bimolecular system (donor + acceptor) (see Appendix A). The rates of all the transitions between various states of the system are considered constant, except for the energy transfer rate F, which is varied.

As can be seen, the presence of an off-state in one of the emitters affects the fluorescence of the other emitter as well. If a donor has an off-state, it may cause acceptor blinking, and vice versa [10].

In a 3D-2A pair, at medium energy transfer rates F (the third panel from the top of figure 2a) a 3-level donor blinks simultaneously with a 2-level acceptor, which has no dark state of its own. It happens because while a donor is in its dark state, it cannot transfer its energy to the acceptor, so the

acceptor does not fluoresce either. When the transfer rate F becomes much bigger than the donor fluorescence rate $1/T_D$ (the bottom panel of figure 2a), both donor and acceptor fluoresce continuously, as the probability of the energy transfer overwhelms that of the donor on-off transition. Thus, simultaneous blinking is a sign of a donor off-state presence.

In a 2D-3A pair, on the contrary, blinking is not simultaneous, but alternating: either a donor or an acceptor has an off-state at a given moment (the fifth panel of figure 2b). When an acceptor gets into its dark state, the energy transfer stops competing with the donor fluorescence. That is why at large energy transfer rates F the donor fluorescence intensity increases from almost zero (at an acceptor in an on-state) to a considerable maximum (at an acceptor in an off-state). At lower F values (the third and the fourth panels of figure 2b), donor fluorescence is dual: its intensity alternates between two different values (the first is non-zero in this case).

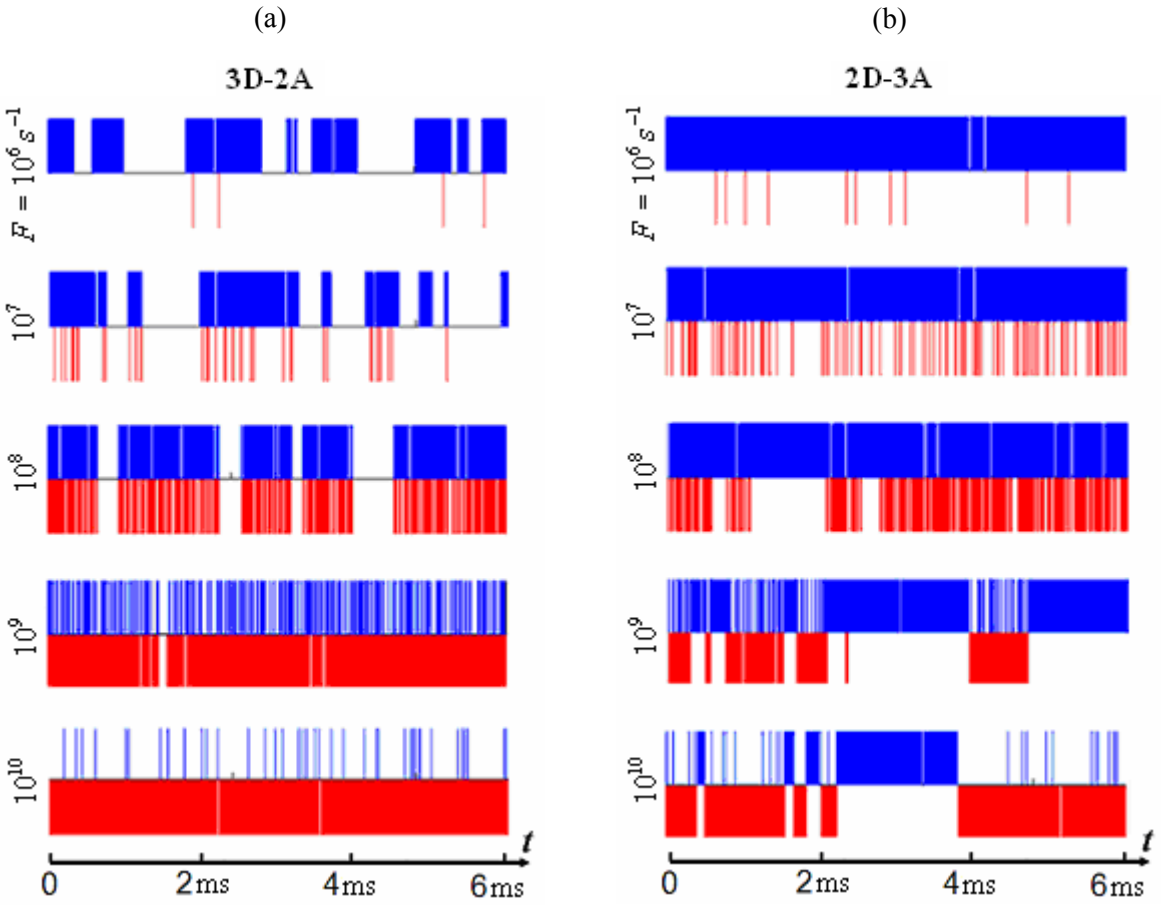


Figure 2. Instants of donor (upper blue segments) and acceptor (lower red segments) photon emission for pairs a) 3D-2A, b) 2D-3A. Parameters: $k = 10^6\, s^{-1}$ (excitation rate), $1/T_D = 2 \cdot 10^8\, s^{-1}, 1/T_A = 10^8\, s^{-1}$ (donor/acceptor fluorescence rates), $G_D = 10^6\, s^{-1}$, $g_D = 10^3\, s^{-1}$, $G_A = 5 \cdot 10^5\, s^{-1}$, $g_A = 500 s^{-1}$ (donor/acceptor on-off and off-on transitions).

This is how we gain the possibility to identify the case (3D-2A or 2D-3A pair) judging by the character of the donor/acceptor blinking [10].

4. Probability distribution functions for fluorescence photon numbers

An advantageous way to analyze the intensity trajectories like those presented in section 3 is to calculate the probability distribution functions for photon numbers emitted by a donor/acceptor at a given bin time. The method was developed in work [11]. According to this method, the time axis is divided into equal bin times (corresponding to those allowed by the time resolution in an experiment), and the number of photons emitted is counted at each bin time. Then the probability to observe a given photon number is calculated.

Figure 3 presents such photon number distributions for continuous (3a) and blinking (3b) fluorescence of a single emitter.

The distribution for continuous fluorescence (figure 3a) is close to a Poisson one. As it was shown in reference [12], they become indistinguishable when there is a big (about two orders of magnitude or more) difference between the excitation rate and the fluorescence rate, which is usually the case.

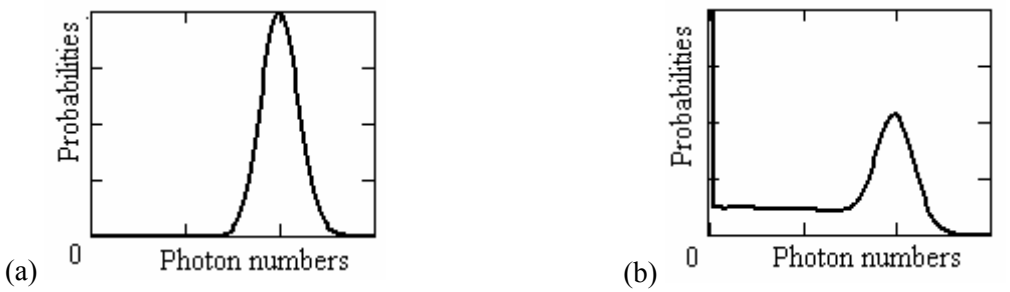


Figure 3. Photon number distributions (probabilities to register a given photon number at a bin time) for continuous (3a) and blinking (3b) fluorescence.

The distribution for blinking fluorescence (figure 3b) drastically diverges from the Poisson one. Figure 3b corresponds to a case when the bin time is comparable to the average on- and off-interval durations in blinking fluorescence. The presence of a plateau in the area of small photon numbers N, as well as a large probability to encounter zero photons (due to the possibility to cover a part of an off-interval with the observation interval) is a sign of blinking fluorescence.

Figure 4 shows some examples of photon number distributions for the intensity trajectories given in figure 2. Distributions for 3D-2A pairs were calculated both analytically and treating the computer simulated experiments (the two ways gave the same result). Distributions for 2D-3A pair were obtained with the help of computer simulation only. Dotted lines show Poisson distributions for comparison.

Having at our disposal distribution functions like those presented in figure 4, we can make a conclusion about the blinking or continuous character of donor/acceptor fluorescence – judging by the presence or absence of a plateau on the probability plot.

5. The contribution of background fluorescence

The model described above can be brought closer to real experiments by taking into account background fluorescence and bin time averaging. Figure 5 demonstrates simulated fluorescence of a 3-level donor shown previously in figure 2d (the second panel from the top) observed through bin times without background fluorescence (figure 5a) and with it (figure 5b).

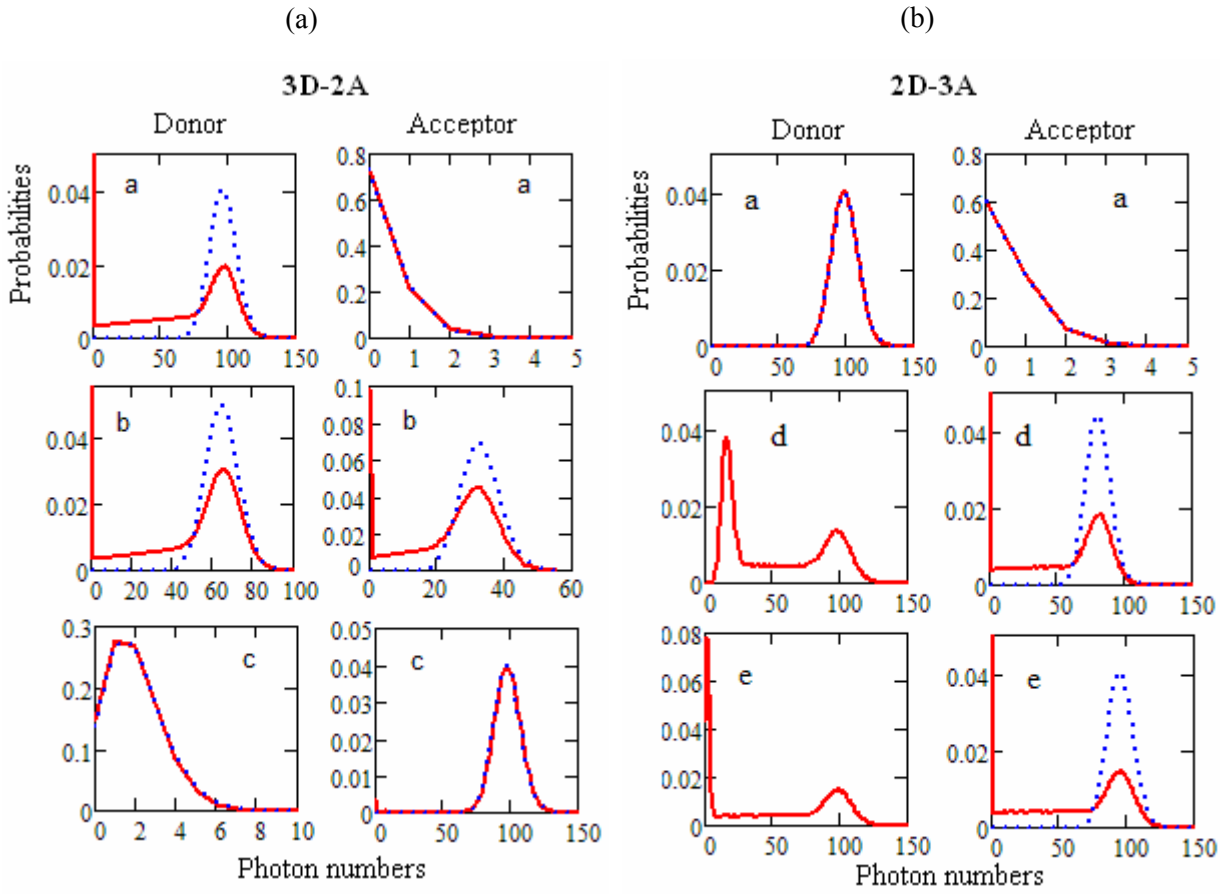


Figure 4. Photon number distributions for donor/acceptor fluorescence in 3D-2A and 2D-3A pairs. For parameters see figure 2. Bin time – 10ms. Letters a-e correspond to panels with different energy transfer rate F in figure 2.

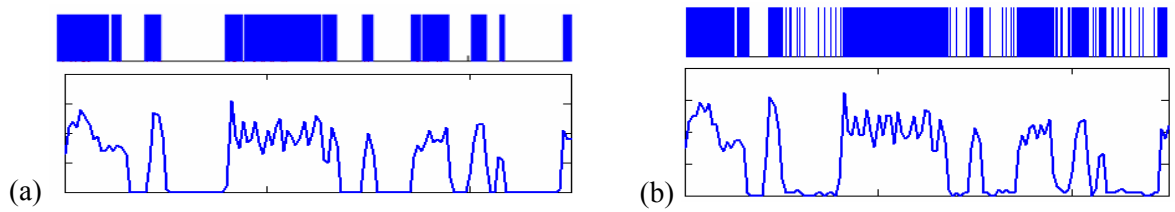


Figure 5. Simulated blinking fluorescence of a 3-level donor: separate photon emission instants (at the top) and photons counted within bin times (at the bottom): a) without background fluorescence; b) with background fluorescence of average intensity 20 times less than the on-interval donor intensity.

Though background fluorescence will blur the simultaneity/alternation of donor/acceptor blinking, it will still evidently allow making the necessary conclusions for choosing the right model – 3D-2A or 2D-3A. Off-intervals can be easily identified qualitatively from data like those in figure 5b. As for quantitative results, they can be obtained with the help of probability distribution functions for photon numbers (see section 4). Figure 6 presents such distributions for “pure” blinking fluorescence and that convolved with background fluorescence.

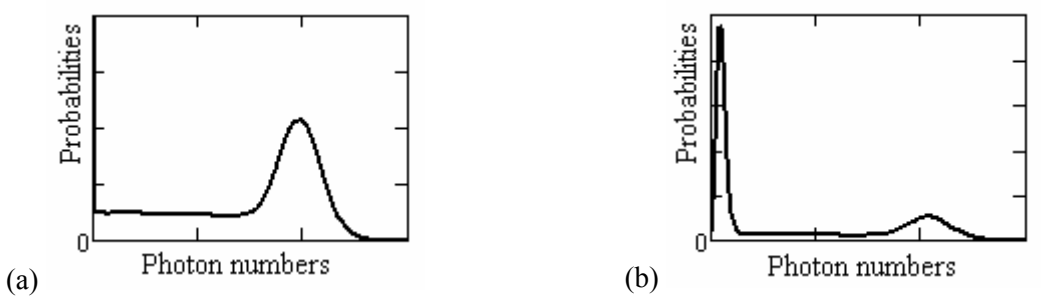


Figure 6. Photon number distributions a) for fluorescence of single 3-level emitter b) for fluorescence of 3-level emitter plus background fluorescence with Poisson statistics (intensity 20 times less than of the emitter during on-interval).

It was shown in reference [13] that when there are two sources of photons (say one is a fluorescing particle, and the other is background fluorescence), their joint signal should be treated with the help of the following formula:

$$w_{(2)}(N,T) = \sum_{i=0}^{N} \left(w_1(i,T) \cdot w_2(N-i,T) \right), \qquad (2)$$

that is, the probability $w_{(2)}(N, T)$ to register N photons from both sources at a bin time T is a convolution of the probabilities for each of the sources, w_1 and w_2. Thus, when background fluorescence is just "cut out", the distributions are distorted. So it is important to consider the convolution instead.

6. FRET efficiency for different types of donor-acceptor pairs

Formula (1) leads to a conventional expression of FRET efficiency E via donor fluorescence rate $1/T_D$ (T_D is the average lifetime of the first excited donor state in absence of an acceptor) and energy transfer rate F for a 2D-2A pair:

$$E = \frac{FT_D}{1 + FT_D}. \qquad (3)$$

This expression can be generalized for each type of a donor-acceptor pair by replacing rate F in (3) with a function which contains the constants of all possible transitions in the system of the two emitters [10]. The corresponding formulas for 3D-2A and 2D-3A cases are given in Appendix B. They are derived with the help of the rate equations for the energy level populations (see Appendix A) in a stationary regime, i.e., setting the derivatives equal to zero, so that the donor/acceptor intensities become proportional to the populations of levels with an excited emitter.

The dependence of the FRET efficiency E on the energy transfer rate F for various types of donor-acceptor pairs is plotted in figure 6. 2D-2A (trivial case not) and 3D-2A pairs obey formula (2). Thus, the dark donor state does not influence the FRET efficiency. However, pairs with a 3-level acceptor do not exhibit full energy transfer even at large energy transfer rates ($E(F)$ considerably lower than unity). So it can be concluded that an acceptor dark state hampers FRET efficiency [10].

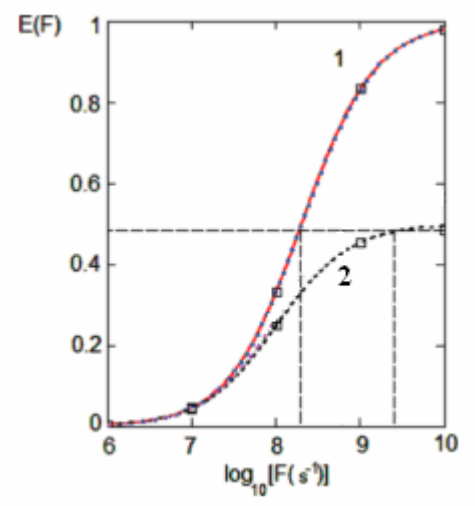


Figure 6. FRET efficiency *E(F)* depending on energy transfer rate *F* (logarithmic scale) for 3D-2A (1) and 2D-3A (2) pairs. Parameters for calculations are the same as in figure 2.

An experimentally established value of FRET efficiency, say, about 0.5 can thus correspond to two considerably different values of energy transfer rate (see dashed lines in figure 6). The difference can be of more than an order of magnitude, as is illustrated (the horizontal axis of figure 6 is in the logarithmic scale). The criterion, according to which the "real" value of the transfer rate should be chosen, is the simultaneity/alternation of donor/acceptor blinking. If they have simultaneous off-intervals, it is a 3D-2A pair, and we should follow line 1. If their off-intervals are alternating, it is a 2D-3A pair, and line 2 is the necessary one.

7. Experimental determination of transition rates in blinking fluorescence

To determine energy transfer rate *F* out of measurable FRET efficiency *E*, as section 6 and Appendix B show, we need to know the rates of excitation and on-off/off-on transitions of each molecule. The present section will show an example of how these parameters can be found from experimental photon number distributions of single organic molecules.

Single molecules fluorescence time tracks were obtained with a home-build luminescence microscope with epi-excitation scheme and multichannel registration system: cooled CCD with electronic multiplication (PCO Sensicam EM). Experiments were performed with Nikon high-numerical aperture microobjective. Exposition time was 30 ms. The sample (ATTO 610 dye molecules on a surface) was prepared from a water solution drop by spin-coating on a thin glassy plate (0.15 mm thickness). Single molecules were excited near their absorption peak by dye laser CR-599.

Single molecules images in each CCD frame (at a definite time point) were recognized and put in correspondence to different single molecules automatically by a custom computer program. It allows obtaining fluorescence time tracks for all single molecules in a microscope field of view. These tracks were used to calculate the experimental distributions of photon numbers per bin time (20 ms).

In order to obtain the necessary values of the parameters, the experimental distributions have been approximated with those simulated by the Monte-Carlo method. Firstly, "pure" distributions of a single blinking emitter like those presented in figure 3b were simulated. Then they were convolved with normal distributions (entailed by background fluorescence) in accordance with formula (2). Five parameters determine the shape of the convolved distribution in this case: for the blinking emitter – the excitation rate, the on-state lifetime and the off-state lifetime; for the background fluorescence – the average intensity (average number of photons per bin) and the width of its distribution. So, multivariate approximation of the experimental distribution was carried out to determine these five parameters. The method was a combination of Nelder-Mead algorithm and brute force (as there are many local minima of the difference between the experimental distribution and the simulated one). Figure 7 shows the results.

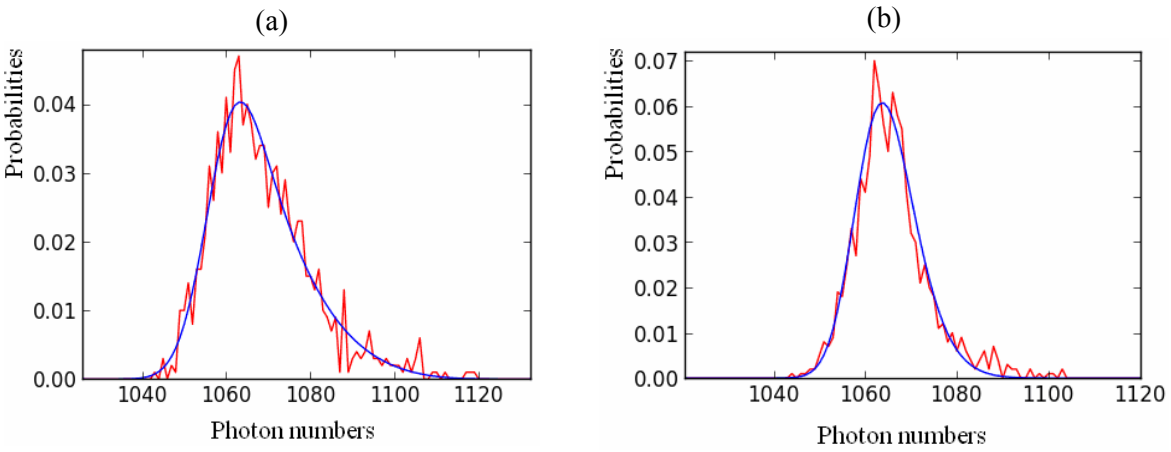


Figure 7. Two examples of experimental photon number distributions approximated by simulated ones. Parameters determined: a) $k = 1.3 \cdot 10^3$ s^{-1} (excitation rate), t_{on}=7.2ms (on-state lifetime), t_{off}=28.2ms (off-state lifetime), m=1060 (average background photons per bin), s=6.4 (distribution width for background fluorescence); b) $k = 7.8 \cdot 10^2$ s^{-1}, t_{on}=4.0ms, t_{off}=14.3ms, m=1060, s=4.9. Bin time 30ms.

8. Conclusion

It has been shown that the type of one emitter involved in FRET (3-level or 2-level, i.e. producing blinking or continuous fluorescence) influences the fluorescence character of the other one. That is, a 3-level donor can make a 2-level acceptor blinking and vice versa. The difference is that in the first case (3D-2A) pair off-intervals occur simultaneously, while in the second one (2D-3A) they alternate.

Besides, it has been shown that a 3-level acceptor hampers FRET efficiency considerably and does not allow it to reach the value of 1 (full transfer) even at fast energy transfer. In this case, FRET efficiency depends not only on the energy transfer rate, but also on the rates of the transitions to and from the third “dark” acceptor state.

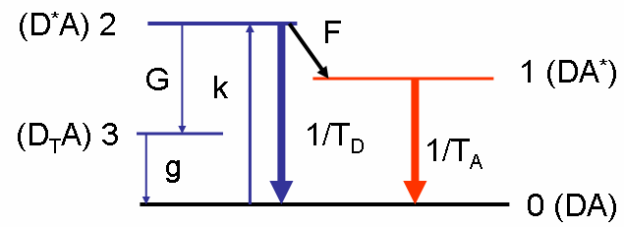
Finally, a way to determine these rates (included to the updated FRET efficiency dependence for a 2D-3A case) from experimental statistics has been described, and examples have been provided.

Acknowledgements

Authors acknowledge A.V. Naumov (Institute for Spectroscopy of Russian Academy of Sciences) for the custom program for fluorescence data retrieval. A.Shchukina acknowledges B.Elkin (Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany) for stimulating discussions and ideas. Financial support from the Grants of the President of Russia (MK-6382.2012.2, MD-465.2012.2) is gratefully acknowledged.

Appendix A
Rate equations for two types of donor-acceptor pairs.

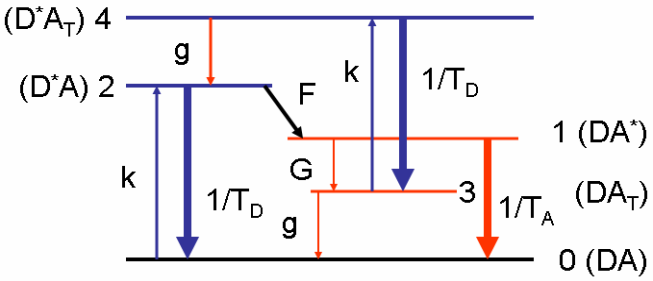
3D-2A:



$$\dot{\rho}_0 = -k\rho_0 + \rho_1 / T_A + \rho_2 / T_D + g\rho_3,$$
$$\dot{\rho}_1 = -\rho_1 / T_A + F\rho_2,$$
$$\dot{\rho}_2 = k\rho_0 - (F + G + 1/T_D)\rho_2,$$
$$\dot{\rho}_3 = G\rho_2 - g\rho_3.$$

D*, A* - excited donor/acceptor. Here and in what follows we neglect the level D*A* (both donor and acceptor are excited), as its population is diminishable. D_T – donor in a dark state.
k – excitation rate, $1/T_D$ – fluorescence rate, *G* and *g* – on-off and off-on transition rates,

2D-3A:



$$\dot{\rho}_0 = -k\rho_0 + \rho_1 / T_A + \rho_2 / T_D + g\rho_3,$$
$$\dot{\rho}_1 = -(G + 1/T_A)\rho_1 + F\rho_2,$$
$$\dot{\rho}_2 = k\rho_0 - (F + 1/T_D)\rho_2 + g\rho_4,$$
$$\dot{\rho}_3 = G\rho_1 - (k + g)\rho_3 + \rho_4 / T_D,$$
$$\dot{\rho}_4 = k\rho_3 - (g + 1/T_D)\rho_4.$$

A_T – acceptor in a dark state

Appendix B
Formulas for FRET efficiency for various types of donor/acceptor pairs:

$$E(F) = \frac{\bar{F}(F)T_D}{1 + \bar{F}(F)T_D}$$

for 2D-2A and 3D-2A:

$$\bar{F}(F) = F$$

for 2D-3A:

$$\bar{F}(F) = \frac{F}{1 + GT_A + \frac{G}{g}\frac{FT_D kT_A}{(k+g)T_D + 1}}$$

References

[1] Moerner W E, Kador L 1989 Phys. *Rev. Lett.* **62** 21 2535
[2] Orrit M, Bernard J 1990 *Phys. Rev. Lett.* **65** 2716
[3] Ambrose W P, Basche Th, Moerner W E 1991 *J. Chem. Phys.* **95** 7150
[4] Dyba M, Jakobs S, Hell S W 2003 *Nat. Biotechnol.* **21** 1303
[5] Gustafsson M G L 2005 *Proc. Natl. Acad. Sci. USA* **102** 1308
[6] Betzig E, Patterson G H, Sougrat R, Lindwasser O E, Olenych S, Bonifacino J S, Davidson M W, Lippincott-Schwartz J, Hess H F 2006 *Science* **313** 1642
[7] Grecco H E, Verveer P J 2011 *Chemphyschem.* **12** 3 484
[8] Förster Th 1948 *Annalen der Physik* **6**(2) 55
[9] Orlov S V, Naumov A V, Vainer Yu G, Kador L 2012 *J. Chem. Phys.* **137** 194903
[10] Osad'ko I S, Shchukina A L 2012 *Phys. Rev. E* **85** 061907
[11] Osad'ko I S, Fedyanin V V 2009 *J. Chem. Phys.* **130** 064904
[12] Osad'ko I S 2005 *ZhETF* **128**(1) 77
[13] Osad'ko I S, Shchukina A L 2010 *J. Phys. Chem. C* **114**(23) 10349