Supporting Information for:
Analysis of Brightness of a Single Fluorophore for Quantitative Characterization of Biochemical Reactions

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1 Excitation setup

To record a signal from both ATTO488 and ATTO647N labeled strands we used diode laser system with Pulsed Interleaved Excitation (PIE) mode. Blue and red laser pulses were produced every 50 ns. The blue pulse was delayed by 25 ns with respect to the red one. The signal from labeled oligonucleotides were separated in terms of excitation time gates and fluorescence energy. The same optical pathway was used for both excitation and emission. The donor and acceptor signals were separated after reflection from trichroic mirror (485 / 561 / 635 nm) by Chroma (USA), by a dichroic filter with a dividing edge on 640 nm. Moreover, we applied a band-pass filter (500 to 550 nm) for the blue channel detector and a long-pass 645 nm filter for the red one.

Fig S1: The scheme of data acquisition and analysis using PIE. Using PIE mode we were able to distinguish each of the emitted photon by specific time gate and wavelength range of a channel. For sample with only ATTO488-labeled strand, after blue pulse the photons are observed only in the blue channel, whereas after red pulse only Instrument Response Function (IRF) is recorded. When duplex is formed, energy absorbed by the donor (ATTO488) after a blue laser pulse is transferred to the acceptor (ATTO647N) and fluorescence is observed in the red channel instead of the blue.

2 FRET analysis

We prepared a series of samples, where donor-labeled strand (A) was kept at constant concentration, while the concentration of complementary acceptor-labeled oligonucleotide (B) varied. We performed control FCS measurements with and without the presence of the acceptor. Such checkup allowed us to control the concentration of each strand and relate them to photon counts. The interaction between oligonucleotides strands could be evaluated by means of two approaches: intensity or fluorescence lifetime analysis. We could apply Fluorescence lifetime approach to measure FRET efficiency via induced decrease of donor fluorescence lifetime. However, in case of determining equilibrium constant through titration experiments, fluorescence life time histogram is composed of two overlapping populations of fluorescence lifetimes. Such overlap of two histograms of fluorescence lifetimes requires challenging data analysis for proper separation of residual lifetimes originating from molecules of donor, acceptor and their complex. Thus, we decided to use intensity approach, which is based on the total number of photons transfered from donor to the acceptor channel. The apparent FRET efficiency is given by:

\[
FRET_{eff} = \frac{I_{acc}}{I_{acc} + \eta \cdot I_{don}}
\]

Here \(I_{acc}\) and \(I_{don}\) are background-corrected photon countrate values after blue pulse for the donor and acceptor channel, respectively. In the intensity analysis the \(\eta\) is system-dependent correction factor which depends on two main factors. First, it comprises emission quantum yields of the two dyes as well as collection
yields of the two channels with relation to the emission spectra of the dyes used. Secondly, it includes spectral characteristics of all elements present on the optical path, i.e. of dichroic mirrors, filters and spectral sensitivity of the detectors. For TCSPC system and labeled oligonucleotides we found $\eta$ equal 1.38.\[2\] To calculate binding isotherm for reaction $A+B \rightleftharpoons AB$, we estimated a concentration $C_{eq}^{AB}$ of complexes at equilibrium from FRET efficiency:

$$\frac{FRET_{eff}}{FRET_{MAX}} = \frac{C_{eq}^{AB}}{C_{MAX}^{AB}}$$ (2)

$FRET_{MAX}$ is maximum absolute FRET efficiency measured in the same experimental conditions, where acceptor molecules are in the big excess over the donor ($C_B >> C_A$). Therefore $C_{MAX}^{AB}$ is the initial concentration of the donor labeled strand $C_A$. Measurements carried out in such conditions result in obtaining binding isotherm described by:

$$FRET_{eff} = \frac{C_{eq}^{AB} \cdot FRET_{MAX}}{C_A}$$ (3)

We measured $K$ by FRET using two pairs of double-labeled oligonucleotides. We investigated two oligonucleotide pairs with donor and acceptor dyes either on the same end (3’488/647N) of the formed complex or on the opposite sides (488/647N). Oligonucleotides pairs posses different FRET efficiencies, as a result of longer distance between fluorophores. The $FRET_{MAX}$ was determined, as 0.88 and 0.34 for 3’488/647N and 488/647N pairs, respectively. We performed measurements for the initial concentration of the donor strand $C_A$ for both cases from 10 pM to 30 nM, see Table S1. After fixing the concentration of donor-labeled strand, we varied concentration of acceptor-labeled strand.

### 3 Fluorescence correlation spectroscopy measurements for oligonucleotides in singlex and duplex forms

As a main supporting technique we used fluorescence correlation spectroscopy (FCS). In our model reaction of DNA hybridization $A+B \rightleftharpoons AB$ we can distinguish following components: fluorescent substrate (A), non-fluorescent substrate (B) and fluorescent complex (AB). The fluorescence of the complex AB originates from the fluorophore attached to substrate A. In the initial phase of this project we wanted to present the molecular brightness (MB) per acquisition time per molecule (counts/s/molecule) in a function of non-fluorescent substrate concentration $MB(C_B)$. The auto-correlation function due to translational diffusion (without including triplet states) of this reaction is given by:

$$G(\tau) = \frac{1}{N} \cdot \sum_{i=1}^{2} f_i \cdot \frac{1}{\tau_D} \cdot \sqrt{1 + \frac{\tau_D^2}{\tau_D^2}} \cdot \frac{\omega_0}{z_0}$$ (4)

where $\omega_0$ is axial and $z_0$ the lateral radius of focal volume. The $\tau_D$ is diffusion time across focal volume for a given component and $f_i$ is a mole fraction of each component ($f_1 + f_2 = 1$). In FCS measurements the average number of fluorophores in the focal volume (N) is determined at $\tau = 0$, $N = \frac{1}{G(0)}$. The separation of the fraction $f_1$ and $f_2$ is possible as long as the difference in diffusion coefficients is significant ($D_{AB} > D_A$). The diffusion coefficients must differ by a factor much bigger than 1.6.\[3\] However, in case where diffusion coefficients/hydrodynamic radii are approximately equal ($\Delta D \approx 10\%$), it is not possible to properly separate both components as for short DNA hybridization. As we showed in the Supporting Information hydrodynamic radii of single strand and double strand complex are $r_A = 1.6$ nm and $r_A = 1.7$ nm, respectively. Secondly, Equation 4 assumes that brightness of both components are equal. In reaction where brightness of complex ($\gamma$) is not equal to substrate’s ($\alpha$), the new ratio of components fractions is related by $\frac{f_{MB}^{MB}}{f_2} = \left(\frac{\gamma}{\alpha}\right)^2 \cdot \frac{f_1}{f_2}$. 

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Thus we could only precisely determine MB expressed in number of emitted photon per molecule per acquisition time for: a) solely fluorescent substrate A, and b) complex AB in the experiment when B (non-fluorescent) is in huge excess. Therefore in this work equilibrium constant K determination is based on analysis of emitted photons per second ($\chi$), according to equation $V_0 \cdot (\alpha \cdot C_{eq}^A + \gamma \cdot C_{eq}^{AB}) = \chi$.

To determine brightness of single labelled strand when a complementary strand is not present (ratio $C_B/C_A = 0$), we analyse the auto correlation function (ACF) of the substrate A (donor) in the blue channel (ex. 485 nm). ACF provides crucial information of the number of molecules inside the focal volume. We determine the brightness of observed probe using the ratio $\alpha = \frac{countrate}{N}$, where countrate is averaged number of photons recorded in time and $N$ is the number of the molecules.

![FCS data, $C_A=5$ [nM], 3'Atto488 / Atto647N](image1)

![FCS data, $C_A=5$ [nM], Atto488 / NN](image2)

Fig S2: Series of FCS measurements for oligonucleotide pairs excited by the blue laser (485 nm) and recorded in the blue channel a) FRET pair ATTO488 labelled strand with ATTO647N complementary strand, and b) ATTO488 labelled strand with nonlabelled complementary strand.

Upon mixing of substrates, the observed system is containing three species: observed initial substrate, second substrate and the complex. Analysis of such multi-component ACF is complicated. In Figure S2 we presented one of the experiments performed on same-ends labelled strand (3’ATTO488 / 647N). We changed ratio between donor and acceptor labelled strand while keeping the initial concentration of donor labelled strand constant; same as in experiments described in main article. The increase of the auto correlation function with the increase of $C_A/C_B$ ratio means that less and less molecules of donor are observed within focal volume, which is in line with our expectations. However, from the FCS analysis we obtain incorrect donor
If we assumed that for this duplex reaction $K = 3.3 \cdot 10^{-9} M^{-1}$, the concentration of the donor should decrease 65 times between the ratio $\frac{C_A}{C_B} = 0$ and $\frac{C_A}{C_B} = 5$, but from FCS we got only 5 times decrement. This result is observed due to two main factors. Firstly, the brightness of the donor decreases significantly upon complex formation. As a result, two "blue" components (from a donor-strand and a complex) are observed in the system. The effect of the complementary labelled strand with ATTO647N is negligible (up to hundreds of nM) due to selection of spectral filters. Taking this into account, ACF is not separating each component properly. There are methods proposed for a correction of such issues in the FCS experiments analysis, and that could be apply for a determination of the equilibrium constants. However, to quantitatively study complex formation by FCS, the size (and a diffusion coefficient) between substrate probe and the complex must vary significantly. In our model case, it is impossible to separate each fraction due to similar hydrodynamic radii of 1.6 and 1.7 nm for single strand and duplex, respectively. Similar observation was made for single labelled pair (ATTO488 / NN), please see Figure S2b. In addition, in case of ATTO488 / NN the molecular brightness is increased by 20% while for 3’ATTO488 / 647N we observed a decrease. This also affects analysis of ACF as an artefact showing the increase in concentration.

4 Workflowchart of brightness analysis method for equilibrium constant determination

For ease of understanding of brightness analysis method for model reaction of $A + B \rightleftharpoons AB$, we present it in a form of workflowchart.

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Fig S3: Flow chart for brightness analysis
5 Concentration working regime

The molecular brightness technique is sensitive for any differences in photon countrate changes. This effect is enable due to the application of Single Photon Avalanche Detectors (SPAD). In presented article even the fluctuations of 5% were enough to quantitatively analyse the interactions between non labelled strand and strand labelled with ATTO647N (NN / ATTO67N). Such sensitivity level has it own limitations. The first one is limitations due to low signal to noise ratio (SN). Each detector has it own spectral sensitivity and its always provided by manufacturer. Hence a photon with different energy has a different detection level. Therefore photons of higher energy induce more avalanche events on first dynode of detector, which results in lower SN ratio.

Fig S4: Equilibrium constants calculated by molecular brightness method for different initial concentrations of the donor.

On the other hand with the increasing number of molecules inside of focal volume, more photons are getting emitted. The limit of the linear response of SPAD is usually at the level of around 100k counts per second. After this point, photons start to getting lost between electronic feedback loop which falsifies the further analysis. In Figure S4 we provide schematic representation of $K$ obtained for a number of experimental series for 3′488/647N pair. The initial concentration of the donor strand labelled with ATTO488 was prepared in the range from 10 pM to 50 nM and we varied the concentration of acceptor strand; from 0.1 x concentration of the donor to 10 times excess. Therefore for the highest concentration of the donor in our experiments (i.e. 50 nM) we need to use 500 nM of the acceptor. At such high concentration the detectors are saturated by the emitted photons. In this case, $K$ cannot be validated (data point not shown on plot). For example, we were not able to characterize $K$ of 11 nM donor sample in the red channel while it was possible in the blue channel - the red channel was saturated already at lower concentration of the fluorophores.

6 Setting the laser power for experiments

One of the main factors which affects molecular brightness is excitation laser power (LP). The most comparable method of laser power measurement is to set the photodiode sensor at the end of optical path-way. The measured value is considered as a total amount of photons excited by the sample. Setting the LP is crucial not only for our measurements but also for the biological samples. For biological samples it is preferable to use the lowest possible power where samples can still obtain highest SN ratio. This reduces issues like a
photodamage and a phototoxicity. Therefore to choose optimal laser power we acquired a series of countrate of photons for single strand oligonucleotides labelled both with ATTO488 and ATTO647N at different laser power levels. The timetrace of photons was stable at each point which lies on linear region of function presented in Figure S5. However, where the linearity falls apart the time trace became decreasing during acquiring time of measurement. Therefore to obtain highest possible SN ratio we chose the laser power before the inflection point.

Fig S5: Series of countrate measurements for every component in systems for different laser powers.
7 Summarized fitted values estimated by FRET and brightness analysis method

Table S1: Summarized results for FRET analysis, where $C_A$ is initial donor concentration, $\sigma(C_A)$ is an error of $C_A$, and $K$, $\sigma(K)$ are equilibrium constant with its error, respectively.

|     | $C_A$ [nM] | $\sigma(C_A)$ [nM] | $K \cdot 10^9$ M$^{-1}$ | $\sigma(K) \cdot 10^9$ M$^{-1}$ |
|-----|------------|---------------------|--------------------------|--------------------------------|
| 488 / 647N |
| 0.251 | 0.138 | 1.723 | 0.179 |
| 1.05  | 0.29  | 1.194 | 0.187 |
| 2.34  | 0.55  | 0.851 | 0.166 |
| 5.00  | 1.10  | 0.718 | 0.223 |
| 9.20  | 4.45  | 1.401 | 2.805 |
| 10.76 | 0.85  | 0.705 | 0.139 |
| 15.70 | 1.96  | 0.392 | 0.101 |
| 29.71 | 4.59  | 0.239 | 0.076 |

| 3'488 / 647N |
| 0.05  | 0.02  | 3.40  | 3.61  |
| 0.09  | 0.02  | 2.38  | 0.21  |
| 0.18  | 0.18  | 1.58  | 0.23  |
| 1.73  | 0.14  | 2.04  | 0.23  |
| 2.98  | 0.47  | 5.78  | 4.59  |
| 5.45  | 0.22  | 5.96  | 1.63  |
Table S2: Summary of molecular brightness Analysis for both used FRET pairs with the opposite-ends and same-ends labelling. The table represents equilibrium constants fitted by analysis countrate recorded by both blue and red channel separately.

| Brightness Analysis | $C_A$ [nM] | $\sigma(C_A)$ [nM] | $K \cdot 10^9$ M$^{-1}$ | $\sigma(K) \cdot 10^9$ M$^{-1}$ |
|---------------------|-------------|---------------------|--------------------------|---------------------------------|
|                     | Blue Channel |                     |                          |                                 |
| 3'488 / 647N        | 0.02        | 0.00                | 7.88                     | 2.99                            |
|                     | 0.09        | 0.00                | 2.38                     | 0.21                            |
|                     | 0.19        | 0.00                | 1.83                     | 0.16                            |
|                     | 1.62        | 0.03                | 3.27                     | 0.79                            |
|                     | 1.72        | 0.03                | 2.15                     | 0.23                            |
|                     | 6.74        | 0.07                | 3.98                     | 0.80                            |
|                     | 15.96       | 0.13                | 3.80                     | 1.07                            |
|                     | 36.68       | 0.82                | 1.38                     | 0.83                            |
|                     | Red Channel |                     |                          |                                 |
|                     | 0.08        | 0.08                | 4.68                     | 2.17                            |
|                     | 0.19        | 0.16                | 3.35                     | 1.13                            |
|                     | 1.17        | 0.72                | 2.60                     | 1.06                            |
|                     | 1.57        | 0.31                | 5.22                     | 1.24                            |
|                     | 4.12        | 1.52                | 3.47                     | 2.51                            |
|                     | 5.35        | 1.38                | 7.08                     | 5.65                            |
|                     | 0.31        | 0.00                | 0.42                     | 0.12                            |
|                     | 0.56        | 0.01                | 0.20                     | 0.07                            |
|                     | 1.19        | 0.02                | 1.16                     | 0.89                            |
|                     | 3.11        | 0.02                | 0.39                     | 0.40                            |
|                     | 5.60        | 0.07                | 2.24                     | 3.43                            |
|                     | 11.40       | 0.17                | 1.57                     | 3.65                            |
|                     | 17.60       | 0.15                | 0.70                     | 0.89                            |
|                     | 27.06       | 0.21                | 0.52                     | 0.60                            |
|                     | 37.80       | 1.12                | 0.41                     | 1.51                            |
|                     | 488 / 647N  |                     |                          |                                 |
|                     | 0.27        | 0.01                | 2.83                     | 0.26                            |
|                     | 0.61        | 0.01                | 1.87                     | 0.08                            |
|                     | 1.79        | 0.02                | 1.05                     | 0.07                            |
|                     | 2.69        | 0.09                | 0.79                     | 0.17                            |
|                     | 5.41        | 0.19                | 0.33                     | 0.07                            |
|                     | 10.47       | 0.14                | 0.43                     | 0.05                            |
|                     | 13.84       | 0.34                | 2.21                     | 1.36                            |
|                     | 27.38       | 0.70                | 2.11                     | 1.43                            |
Table S3: Summary of molecular brightness Analysis for single labelled pairs where labelling dye was either ATTO488 or ATTO647N.

| Brightness Analysis | $C_A$ [nM] | $\sigma(C_A)$ [nM] | $K \cdot 10^9$ M$^{-1}$ | $\sigma(K) \cdot 10^9$ M$^{-1}$ |
|---------------------|------------|---------------------|--------------------------|--------------------------------|
|                     | 0.02       | 0.00                | 20.87                    | 154.98                         |
|                     | 0.08       | 0.01                | 6.12                     | 15.68                          |
|                     | 0.24       | 0.01                | 0.60                     | 1.03                           |
|                     | 0.68       | 0.01                | 3.15                     | 1.85                           |
|                     | 1.20       | 0.03                | 0.48                     | 0.51                           |
|                     | 1.61       | 0.02                | 0.43                     | 0.26                           |
|                     | 1.78       | 0.02                | 4.83                     | 4.01                           |
|                     | 2.94       | 0.10                | 2.36                     | 0.93                           |
|                     | 6.57       | 0.23                | 4.46                     | 2.98                           |
|                     | 6.78       | 0.06                | 1.87                     | 1.36                           |
|                     | 10.02      | 0.12                | 0.85                     | 0.66                           |
|                     | 25.56      | 0.12                | 0.93                     | 0.65                           |
|                     | 26.07      | 0.22                | 0.53                     | 0.44                           |
|                     | 31.34      | 0.65                | 0.52                     | 1.31                           |
|                     | 38.91      | 0.32                | 1.52                     | 2.98                           |
|                     | 4.26       | 0.13                | 1.04                     | 3.85                           |
|                     | 7.01       | 0.30                | 0.87                     | 4.32                           |
|                     | 13.88      | 0.23                | 0.70                     | 1.86                           |
|                     | 14.73      | 0.28                | 0.13                     | 0.22                           |
|                     | 16.40      | 0.16                | 1.28                     | 2.81                           |
|                     | 26.45      | 0.25                | 1.32                     | 3.87                           |
8 Emission spectra

We recorded emission spectra of the oligonucleotides using Agilent, model: Cary Eclipse, excitation wavelength: 480 and 630 nm at 25°C. The single strand sample was 50 nM whereas double strand, was mix of initial strand with 10 times excess of complementary thread. We did not observe any change in shape or shift of the maximum emission peak.

![Emission spectra graphs](image)

Fig S6: Emission intensity ratio of single strand to duplex form.

References

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