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8-spot smFRET analysis using two 8-pixel SPAD arrays

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Abstract

Single-molecule Förster resonance energy transfer (smFRET) techniques are now widely used to address outstanding problems in biology and biophysics. In order to study freely diffusing molecules, current approaches consist in exciting a low concentration (<100 pM) sample with a single confocal spot using one or more lasers and detecting the induced single-molecule fluorescence in one or more spectrally- and/or polarization-distinct channels using single-pixel Single-Photon Avalanche Diodes (SPADs). A large enough number of single-molecule bursts must be accumulated in order to compute FRET efficiencies with sufficient statistics. As a result, the minimum timescale of observable phenomena is set by the minimum acquisition time needed for accurate measurements, typically a few minutes or more, limiting this approach mostly to equilibrium studies. Increasing smFRET analysis throughput would allow studying dynamics with shorter timescales. We recently demonstrated a new multi-spot excitation approach, employing a novel multi-pixel SPAD array, using a simplified dual-view setup in which a single 8-pixel SPAD array was used to collect FRET data from 4 independent spots. In this work we extend our results to 8 spots and use two 8-SPAD arrays to collect donor and acceptor photons and demonstrate the capabilities of this system by studying a series of doubly labeled dsDNA samples with different donor-acceptor distances ranging from low to high FRET efficiencies. Our results show that it is possible to enhance the throughput of smFRET measurements in solution by almost one order of magnitude, opening the way for studies of single-molecule dynamics with fast timescale once larger SPAD arrays become available.

Keywords

FRET; single molecule; smFRET; multi-spot; high-throughput; photon-counting; SPAD; SPAD arrays

1. INTRODUCTION

Förster resonance energy transfer (FRET) is a non-radiative dipole-dipole interaction between two fluorescent molecules taking place across a short range of distances (1–10nm). Exploiting this effect makes it possible to measure distances between two fluorescent labels within a biological system, which would nowadays be inaccessible to conventional microscopy. Moreover, when FRET interactions are observed at the single-molecule level, it is possible to distinguish between different subpopulations characterized by different distances, which would be indistinguishable at the ensemble level. Single-molecule FRET (smFRET) has been successfully employed to address outstanding problems in biology and

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biophysics^{1–6}. In smFRET experiments, molecules can be either fixed on a substrate or freely diffusing. The latter approach has the advantages to avoid any interaction between the substrate and the molecules under study.

However, smFRET on freely diffusing molecules suffers from long acquisition times needed to collect sufficient statistics for FRET efficiency estimation. This limitation hinders the ability to observe dynamical phenomena on short timescales (1 s or less) and makes the systematic exploration of vast range of experimental conditions extremely time-consuming⁷.

Recently, 8-spot fluorescence correlation spectroscopy (FCS) at the single-molecule level was been demonstrated using a multiple excitation spot geometry and a single array of 8 SPAD detectors^{8,9}. Shortly after, we presented a proof-of-concept 4-spot freely diffusing smFRET experiment employing the same single 8-pixel SPAD detector, each half of the array being used to detect one of the two FRET spectral channels (donor and acceptor)¹⁰. This experiment was however performed using high FRET efficiency samples only, leaving open the question of its practical extension to other FRET efficiency values.

In this paper we answer this question positively, reporting freely diffusing smFRET experiments using an 8-spot setup and two 8-pixel SPAD arrays. The paper is organized as follows. Section 2 describes our setup, data analysis protocol as well as the 5 FRET samples, spanning the whole range of FRET efficiencies, used to assess the performance of our setup. Section 3 reports our smFRET measurement results. We compare our results with similar ones obtained with a standard single-spot μ s-ALEX setup¹¹. Finally, we discuss the high-throughput performance achieved with the multispot system, showing that measurement times as low as 10 seconds are sufficient for accurate FRET estimation.

2. MATERIALS AND METHODS

The design of a multispot smFRET system raises several technical challenges. First, a multispot excitation pattern must be generated and projected into the sample. This requires an optical setup able to generate an arrangement of multiple spots from a single laser beam. Second, the emission signal of each excitation spot must be detected individually. Traditionally, to detect the fluorescence signal in a single confocal spot setup¹¹, point-like detectors (single-photon avalanche diodes, SPADs¹²) are employed due to their high photon detection efficiency (PDE) and their ability to emit a pulse for each detected photon with high timing resolution¹³. High PDE SPAD arrays developed in the past few years by the SPADLab at Politecnico di Milano (POLIMI)¹⁴ have allowed parallel single-molecule burst detection from multiple excitation spots¹⁰. Third, suitable multichannel electronic must be used in order to record photon arrival times with nanosecond accuracy and stream the arrival times and channel number to a host computer for further processing. In order to achieve high-throughput performance, a burst search must be performed on each channel, taking into account the different background levels and non-uniformities of each channel. Finally, data from each channel must be calibrated in order to be combined into a single data set.

2.1 Multispot excitation path

The 8-spot excitation pattern was generated using a high power (>1 W) 532 nm pulsed laser (IC-532–1000 ps, High Q Laser Production GmbH, Hohenems, Austria). After laser beam expansion, 8 separate spots were formed by a programmable liquid crystal on silicon spatial light modulator (LCOS-SLM, model X10468-01, Hamamatsu Corp., Bridgewater, NJ) employing a real-space modulation approach described previously⁸. As shown in Fig. 1, the expanded beam was phase-modulated by the LCOS, emulating an array of 8 microlenses. After reflection off the LCOS, 8 spots were focused onto a plane indicated in Fig. 1 as *LCOS focal plane*. The spots were recollimated and directed into an inverted microscope, where a

water-immersion 60 $\times$ objective lens (NA = 1.2, Olympus, Piscataway, NJ) refocused the spots through a microscope coverslip into the sample. The position (x - y), orientation and pitch of the spots can be adjusted by changing the modulation pattern on the software-controlled LCOS device. A linear arrangement of 8 excitation spots was generated in order to match the linear 8-pixel geometry of the detector.

As already described⁸ and shown in Fig. 1, a 300 μ m diameter pindot was inserted after the recollimating lens at a distance equal to the lens focal length in order to block the fraction of unmodulated light reflected off the LCOS. This significantly reduced the out-of-focus background generated in the sample.

2.2 Multispot emission path and detection

Fluorescence emission generated by molecules diffusing through the 8 excitation spots was collected by the objective lens. A dichroic mirror separated the scattered laser light from the dyes emission (Fig. 1). An additional long pass filter was inserted to eliminate any residual fraction of scattered laser light transmitted by the dichroic mirror. The 8-spot emission signal was focused by the microscope tube lens and relayed by a multi-camera port system (Tricam, Cairn Research Ltd., Kent, UK) to two 8-pixel SPAD modules¹⁴, after spectral separation. Each module was individually aligned so that each pixel's active-area received the emission signal from one of the conjugated excitation spots (Fig. 1).

The arrays geometry consisted of eight 50 μ m-diameter pixels linearly distributed with a 250 μ m pitch. The arrays, which have been described previously^{8,9}, use a custom process developed by the SPADLab group at POLIMI¹⁴. This process allows obtaining arrays with low dark-counting rates (DCR) and higher PDE than arrays manufactured using CMOS processes¹⁵⁻¹⁷. However, compared to the thick SPAD process (the detector technology used by the SPCM-AQR detectors of Perkin-Elmer/Excelitas or Count/tau-SPAD detectors of Laser Components/PicoQuant), the PDE of the custom arrays is about two-fold lower in the red part of the visible spectrum (>600 nm). Therefore we expected a low sensitivity of the multispot system for low-FRET populations, characterized by very low photon count in the red part of the spectrum (i.e. acceptor channel). A more detailed comparison of the different SPADs technologies can be found in the literature¹⁸.

2.3 Alignment

The alignment procedure was performed using a concentrated (100 nM) Cy3B solution. The Cy3B dye has a wide enough emission band to be detectable by both the donor and acceptor channels. During alignment the laser power was adjusted such as to achieve a high signal-to-noise ratio (SNR) on the SPADs, making the dark counting rates (DCR) of each pixel negligible.

Alignment comprised two steps, one for each detector. For the first detector (either donor or acceptor) the LCOS pattern was programmed in LabVIEW (National Instruments - NI, Austin, TX) to emulate a single lens, thus generating a single spot. While recording the output of the detector to be aligned (see below), the pattern was moved across the LCOS in the x and y directions. By maximizing the signal on each pixel, the coordinates of the conjugated positions of the 8 pixels on the LCOS plane can be determined.

The same procedure was repeated for the second detector. Knowing the coordinates of the 8 pixels on the LCOS plane for the second detector, the second SPAD position (x , y) and orientation (ϕ) were adjusted in order to match the position and orientation of the first SPAD. The procedure was iterated multiple times until no further alignment was needed.

2.4 Data acquisition and analysis

The two 8-pixel detectors provided 16 independent TTL outputs. The 16 signals were routed to a FPGA-based processing board (PXI-7813R, NI), which was programmed in LabVIEW to provide a time-stamp with 12.5 ns resolution for each pulse. Timestamp and channel number were asynchronously streamed to a host PC using a communication bridge (PXI PCI 8330, NI). The host PC ran a LabVIEW software displaying a real-time time-trace of the 16 channels for monitoring purposes and stored the data on disk.

Further processing was performed offline using Python scripts. Burst search was performed using all photons (merging donor and acceptor channels)¹¹ for each excitation spot. This burst search algorithm was similar to the sliding window algorithm proposed by Eggeling *et al.*¹⁹ and consisted in selecting a minimum rate as threshold for burst identification and in searching through the photon stream for regions with local rate above this threshold value¹⁰. The local rate was computed using m consecutive photons. The minimum rate for burst detection was computed as a function of the total background rate in the channel. An empirical analysis of the background counts distribution led to the choice of a minimum rate for burst detection equal to 4-times the background rate. After burst detection, corrections for leakage (donor emission in the acceptor spectral band) and background subtraction were performed^{10,11} and bursts with more than 50 photons were selected in order to reduce shot-noise broadening of the FRET histograms²⁰. These parameters were used in all the results reported below.

Finally, FRET efficiency for each burst was computed ratiometrically²¹ according to eq. (1):

$$E = \frac{F_A}{F_A + \gamma F_D} \quad (1)$$

where F_D and F_A are the number of burst photons in the donor and acceptor channel (after correction) and γ is the detection correction factor between donor and acceptor channels¹¹. Section 3.2 will describe how the γ -factor was computed for the multispot system.

2.5 Samples

In order to characterize the multispot system and compare its performance with a standard single confocal spot μ s-ALEX setup, we prepared a series of 5 doubly-labeled DNA (dsDNA) samples. All samples were 40 base-pair (bp) long, with a common “upper” strand labeled with the acceptor dye (ATTO647N) attached to the 5' end. The donor dye (ATTO550) was attached on the complementary strand at 5 different positions in order to achieve a donor-acceptor separation of 7, 12, 17, 22 and 27 bp. This set of base-pair distances was chosen in order to cover a wide range of FRET efficiencies¹¹. Furthermore, for comparison with the low FRET samples, a donor-only sample using an unlabeled upper strand was prepared.

The sequence for the upper strand was 5'-TAAATCTAAAGTAACATAAGGTAACATAACGGTAAGTCCA-3'. For both donor and acceptor strand, NHS-ester chemistry and C6 linker were used. Dual HPLC purified singly-labeled ssDNA samples were purchased from IDT (Coralville, IO) and used without further purification.

In the following, each sample will be designated by the donor-acceptor separation in base pairs, using for example 7 bp for the sample with a 7 base-pair separation between donor and acceptor and a similar notation for the other samples.

All 5 samples in the series were measured at single-molecule concentrations (~ 100 pM). Measurement times ranged from 100 to 300 seconds.

3. DISCUSSION AND RESULTS

3.1 FRET histograms

Fig. 2 & 3 report FRET efficiency histograms (one for each channel) for the 7 bp and 27 bp samples, respectively the highest and the lowest FRET samples of the series. A γ -correction factor of 0.5 was used, as justified in the next section.

In the 7 bp sample, we observed both a FRET population peak and a donor-only peak (centered on $E = 0$). The donor-only population was not present for the 27 bp sample and the FRET histograms only showed a low-FRET peak. To demonstrate this point, we also reported in Fig. 3 the measured histograms for the donor-only sample (light gray), which showed peaks exactly centered on the $E = 0$ value. The low-FRET peaks of the 27 bp sample were thus clearly separated from the donor-only peaks. Therefore, although we expected a lower sensitivity on the multi-spot system for low-FRET samples (see section 2.2), these results demonstrated that FRET efficiencies as low as 0.1 could clearly be identified, thanks to careful alignment and accurate background and leakage corrections, as detailed in section 2.4.

3.2 FRET *versus* distance

Fig. 4 reports the position of the FRET population peak for all 5 samples and all 8 channels (each channel is represented by a “+” symbol) as a function of the donor-acceptor separation reported in bp. As mentioned previously for Fig. 2 & 3, γ -correction factor of 0.5 was used. This value was calculated by comparison with μ s-ALEX results as described in the following paragraphs.

Fig. 4 also reports the FRET peak position of the 5 samples as obtained with a standard single-spot μ s-ALEX setup (*red line*)^{11,22}. Since the μ s-ALEX system use a second (red) laser for direct acceptor excitation, an additional S parameter can be computed for each burst. As previously reported¹¹, using this additional information allows extracting the γ -factor of the μ s-ALEX setup directly from a set of measurements with different FRET samples. This method was used with the 5 FRET samples described here reported and resulted in a γ -correction factor of 0.96 for the μ s-ALEX setup.

In the multispot setup, the lack of an acceptor-excitation laser prevented from extracting the γ -correction factor in this manner. However, by measuring each same sample with the multispot and the μ s-ALEX setup and using the μ s-ALEX results as a reference, it is possible to compute the γ -correction factor of the multispot setup. Here we used the 7 bp sample for this calibration and we computed a γ -correction factor of 0.5 for the multispot setup. Once calculated, the same value ($\gamma = 0.5$) was used for all the remaining 4 samples.

Fig. 4 first shows that, with proper γ -correction, the FRET peak positions obtained with the multispot setup is in very good agreement with the values obtained with the state-of-the-art single-spot μ s-ALEX setup. Secondly, the spread of FRET values amongst the 8 channels is rather modest and in any case, allowing unambiguously discrimination of the different samples.

Comparison of the measured values with a simple geometrical model of the labeled dsDNA strand is indicated in Fig. 4 by grey curves. The donor-acceptor separation was computed using a simple cylindrical model for the dsDNA²³ and a fixed Förster radius (two extreme values, 6.5 nm and 5 nm, were used). We observed however, that regardless of the chosen

Förster radius, the model was not able to provide a satisfactory explanation of the experimental results, as has been noted before^{11,24}. Clearly, a more comprehensive analysis will be needed to account for the experimental data.

3.3 FRET *versus* measurement time

Although the variance or maximum spread of FRET values amongst channels reported in Fig. 4 were modest (the maximum spread was 6.1% for the 17 bp sample), they would affect the precision of the FRET value obtained by pooling all bursts detected by different channels, as would be done ideally in order to achieve a higher throughput measurement.

In order to compensate for channel variability, a channel-dependent γ -correction factor was introduced. First, the FRET efficiencies were computed with a common γ -correction factor, as explained in section 3.2. Once the FRET peak position were determined for each channel ($\hat{E}_{ch}$), the average $\bar{E}$ of these 8 values was computed. Finally, the γ -factor of each channel was adjusted in order to obtain a FRET peak position equal to $\bar{E}$. In other words, the channel-dependent γ -correction factor (γ_{CH}) was computed according to Eq. (2):

$$\gamma_{ch} = \frac{\left(\frac{1}{\bar{E}} - 1\right)}{\left(\frac{1}{\hat{E}_{ch}} - 1\right)} \bar{\gamma} \quad (2)$$

where $\bar{\gamma}$ is the channel independent γ -correction factor (0.5 in this case) computed by comparison with the μ s-ALEX results. The expression in Eq. (2) follows straightforwardly from the ratiometric FRET efficiency formula, Eq. (1).

In order to assess the high-throughput capabilities of the system, we used the same FRET data reported in Fig. 2–4, but only considered the first few seconds of measurement, applying the channel-dependent γ -correction factor calibration approach described above and pooling bursts data from all 8 channels into a single FRET histogram per sample. The resulting FRET histograms for the 5 samples were reported in Fig. 5 after 10 seconds of measurement. It is clear that after this short period of time, the number of bursts collected from all channels is large enough to obtain clearly defined FRET histograms and accurate FRET efficiencies for all samples.

Fig. 6 next shows the fitted FRET peak position as a function of measurement time for all 5 samples. The light blue region represents the $\pm 3\sigma$ confidence interval around the estimated fit position, where σ is the standard deviation of the peak position estimator assuming a gaussian distribution. For reference, we reported the gaussian fit of the FRET peaks after 60 seconds of measurement (Fig. 6, left). Note that if the experimental distribution of FRET efficiencies exhibited asymmetries or other departure from a Gaussian distribution, the confidence limit of Fig. 6 would not be correct anymore but would likely be a lower bound for the actual uncertainty, showing a rapid $1/T$ decay as a function of measurement time T . This representation, which extends to all times the conclusion drawn from Fig. 5 for $T = 10$ s, is an indication of the significant throughput increase provided by the 8-channel set up. Note that peak position uncertainty would need to be complemented by information on the peak width to address the question of how rapidly two different samples with similar but different FRET efficiencies within a mixture could be identified.

4. CONCLUSIONS AND PERSPECTIVES

We have demonstrated and thoroughly characterized an 8-spot smFRET setup able to detect a broad range of FRET efficiencies and whose results are in very good agreement with the

results obtained with a state-of-the-art single-spot μ s-ALEX setup. Our results showed that, even for the lowest FRET efficiency sample (27 bp, $E \sim 0.1$), we obtained a good estimation of the FRET peak position, which was clearly distinguishable from a donor-only sample. The setup showed limited channel-to-channel variations, which could be easily corrected after a channel-specific γ -correction factor calibration. We also illustrated the high-throughput performance of the setup by accurate FRET peak position identification after only 10 seconds of measurements for all 5 samples.

This setup opens the way to high-throughput applications such as high-throughput screening or rapid exploration of a large parameter space in conjunction with a microfluidic formulator⁷. Another important application of this ability will be the observation of fast evolving non-equilibrium processes such as for instance conformational changes of macromolecules or enzymatic activity.

Future improvements of the setup will include increasing the number of excitation spots as new SPAD arrays with more pixels become available as well as implementing an ALEX excitation scheme^{11,22}.

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REFERENCES

- [1]. Weiss S. Fluorescence Spectroscopy of Single Biomolecules. *Science*. 1999; 283(5408):1676–1683. [PubMed: 10073925]
- [2]. Weiss S. Measuring conformational dynamics of biomolecules by single molecule fluorescence spectroscopy. *Nat. Struct. Mol. Biol.* 2000; 7(9):724–9.
- [3]. Michalet X, Weiss S, Jäger M. Single-molecule fluorescence studies of protein folding and conformational dynamics. *Chem. Rev.* 2006; 106(5):1785–813. [PubMed: 16683755]
- [4]. Kapanidis AN, Margeat E, Ho SO, Kortkhonja E, Weiss S, Ebright RH. Initial transcription by RNA polymerase proceeds through a DNA-scrunching mechanism. *Science*. 2006; 314(5802):1144–7. [PubMed: 17110578]
- [5]. Kapanidis AN, Margeat E, Laurence TA, Doose S, Ho SO, Mukhopadhyay J, Kortkhonja E, Mekler V, Ebright RH, et al. Retention of transcription initiation factor sigma70 in transcription elongation: single-molecule analysis. *Mol. Cell*. 2005; 20(3):347–56. [PubMed: 16285917]
- [6]. Chakraborty A, Wang D, Ebright YW, Korlann Y, Kortkhonja E, Kim T, Chowdhury S, Wigneshweraraj S, Irschik H, et al. Opening and closing of the bacterial RNA polymerase clamp. *Science*. 2012; 337(6094):591–5. [PubMed: 22859489]
- [7]. Kim S, Streets AM, Majumdar DS, Quake SR, Weiss S. High-throughput single-molecule optofluidic analysis. *Nat. Methods*. 2011; 8(3):242–5. [PubMed: 21297618]
- [8]. Colyer RA, Scalia G, Rech I, Gulinatti A, Ghioni M, Cova S, Weiss S, Michalet X. High-throughput FCS using an LCOS spatial light modulator and an 8×1 SPAD array. *Biomed. Opt. Express*. 2010; 1(5):1408–1431. [PubMed: 21258559]
- [9]. Colyer RA, Scalia G, Kim T, Rech I, Resnati D, Marangoni S, Ghioni M, Cova S, Weiss S, et al. High-throughput multispot single-molecule spectroscopy. *Proc. of SPIE*. 2010; 7571:75710G–75710G11.
- [10]. Ingargiola A, Colyer RA, Kim D, Panzeri F, Lin R, Gulinatti A, Rech I, Ghioni M, Weiss S, et al. Parallel multispot smFRET analysis using an 8-pixel SPAD array. in *Proc. of SPIE*. 2012; 8228:82280B–82280B-8.

- [11]. Lee NK, Kapanidis AN, Wang Y, Michalet X, Mukhopadhyay J, Ebricht RH, Weiss S. Accurate FRET measurements within single diffusing biomolecules using alternating-laser excitation. *Biophys. J.* 2005; 88(4):2939–53. [PubMed: 15653725]
- [12]. Cova S, Ghioni M, Lotito A, Rech I, Zappa F. Evolution and prospects for single-photon avalanche diodes and quenching circuits. *J. Mod. Opt.* 2004; 51(9-10):1267–1288.
- [13]. Michalet X, Siegmund OHW, Vallergera JV, Jelinsky P, Millaud JE, Weiss S. Detectors for single-molecule fluorescence imaging and spectroscopy. *J. Mod. Opt.* 2007; 54(2-3):239. [PubMed: 20157633]
- [14]. Rech I, Marangoni S, Resnati D, Ghioni M, Cova S. Multipixel single-photon avalanche diode array for parallel photon counting applications. *J. Mod. Opt.* 2009; 56(2-3):326–333.
- [15]. Colyer, R. a.; Scalia, G.; Villa, F. a.; Guerrieri, F.; Tisa, S.; Zappa, F.; Cova, S.; Weiss, S.; Michalet, X. Ultra high-throughput single molecule spectroscopy with a 1024 pixel SPAD. in *Proc. of SPIE*. 2011; 7905:790503–790503-8.
- [16]. Michalet X, Colyer RA, Scalia G, Weiss S, Siegmund OHW, Tremsin AS, Vallergera JV, Villa F, Guerrieri F, et al. New photon-counting detectors for single-molecule fluorescence spectroscopy and imaging. in *Proc. of SPIE*. 2011:803316–803316-12.
- [17]. Guerrieri F, Tisa S, Tosi A, Zappa F. Two-Dimensional SPAD Imaging Camera for Photon Counting. *IEEE Photonics J.* 2010; 2(5):759–774.
- [18]. Michalet X, Colyer RA, Scalia G, Ingargiola A, Lin R, Millaud JE, Weiss S, Siegmund OHW, Tremsin AS, et al. Development of new photon-counting detectors for single-molecule fluorescence microscopy. *Phil. Trans. R. Soc. B.* 2013; 368(1611):20120035. [PubMed: 23267185]
- [19]. Eggeling C, Berger S, Brand L, Fries JR, Schaffer J, Volkmer A, Seidel CAM. Data registration and selective single-molecule analysis using multi-parameter fluorescence detection. *J. Biotechnol.* 2001; 86(3):163–80. [PubMed: 11257530]
- [20]. Nir E, Michalet X, Hamadani KM, Laurence TA, Neuhauser D, Kovchegov Y, Weiss S. Shot-noise limited single-molecule FRET histograms: comparison between theory and experiments. *J. Phys. Chem. B.* 2006; 110(44):22103–24. [PubMed: 17078646]
- [21]. Dahan M, Deniz AA, Ha T, Chemla DS, Schultz PG, Weiss S. Ratiometric measurement and identification of single diffusing molecules. *Chem. Phys.* 1999; 247(1):85–106.
- [22]. Kapanidis AN, Laurence TA, Lee NK, Margeat E, Kong X, Weiss S. Alternating-laser excitation of single molecules. *Acc. Chem. Res.* 2005; 38(7):523–533. [PubMed: 16028886]
- [23]. Clegg RM, Murchie AI, Zechel A, Carlberg C, Diekmann S, Lilley DM. Fluorescence resonance energy transfer analysis of the structure of the four-way DNA junction. *Biochemistry.* 1992; 31(20):4846–56. [PubMed: 1591245]
- [24]. Sindbert S, Kalinin S, Nguyen H, Kienzler A, Clima L, Bannwarth W, Appel B, Müller S, Seidel CAM. Accurate distance determination of nucleic acids via Förster resonance energy transfer: implications of dye linker length and rigidity. *J. Am. Chem. Soc.* 2011; 133(8):2463–80. [PubMed: 21291253]

Figure1. Schematic representation of the multispot setup. See Section 2 for a detailed description of the system.

Figure2.

FRET histograms for the 7 bp sample. Each plot corresponds to one channel of the multispot system. A gaussian fit (*grey line*) of the FRET population and the fitted peak position (*red dashed lines*) are shown with the estimated FRET efficiency for each channel (*text box*).

Figure 3.

FRET histograms for the 27 bp sample. Each plot corresponds to one channel of the multispot system. A gaussian fit (*grey line*) of the FRET population and the fitted peak position (*red dashed lines*) are shown with the estimated FRET efficiency for each channel (*text box*). Histograms for the donor-only sample are shown in light gray for comparison.

Figure 4.

FRET peak position as a function of the base-pair separation between donor and acceptor dyes for the 5 dsDNA samples. Each "+" symbol represents the estimated FRET population peak position for each of the 8 channels of the multispot setup. The maximum difference between channels is reported as " Δ " for each sample. The red line reports the FRET population peak position as measured with the reference single-spot μ s-ALEX setup. The theoretical dependence of FRET versus distance (*grey lines*) assuming a constant Förster radius R_0 and a donor-acceptor distance computed according to a cylindrical dsDNA model. Two values for R_0 (6.5 nm and 5 nm) were used. See text for details (section 3.2).

Figure 5. FRET histograms computed from 10 seconds of data acquired by and pooled from 8-channels, reported for all 5 dsDNA samples, illustrating the high-throughput performance of the multispot setup.

Figure 6. (right) FRET peak position estimation for the 5 samples as a function of measurement time (black lines). The light-blue regions represent $\pm 3\sigma$ confidence limits of the peak position. (left) Gaussian fit and peak position (red dashed lines) of the FRET peaks after 60 seconds of measurements.