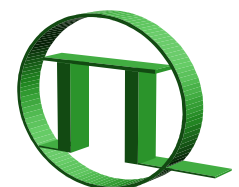


Application Note

FRET analysis of freely diffusing molecules using the MicroTime 200

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Introduction

This application note describes the usage of the MicroTime 200 system for the study of Förster resonance energy transfer (FRET). In order to demonstrate its capabilities, freely diffusing polyproline peptides in water were investigated, which have been established as reference molecules for FRET on biomolecules both in ensemble [1] and single molecule experiments [2]. This special measurement shows results for a peptide containing 20 proline residues, labeled with Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes) as donor and acceptor chromophores (Alexa594-Gly-(Pro)₂₀-Cys-Alexa488), which results in a distance between the chromophores close to the Förster distance of the dye pair (5.4 nm).

Experimental details

A picosecond diode laser running at a repetition rate of 40 MHz with an output wavelength of 468 nm was used to excite the probe. The light beam passes through an excitation filter centered at 450 nm ($\pm 32,5$ nm) to ensure that no parasitic light reaches the probe and was guided to the sample by an dichroic mirror centered at 476 nm through a high numerical objective (100x, N.A. 1.4, oil immersion). The fluorescence is collected through the same objective and dichroic mirror and an additional longpass filter rejected the scattered laser light, while allowing the fluorescence light to pass. Finally, the fluorescence responses from the donor and the acceptor molecules were separated by a dichroic mirror and detected by two SPADs using the method of time-correlated single photon counting and the Time-Tagged Time-Resolved (TTTR) [3] mode of the TimeHarp 200 board. The measurement was performed in a constant detection volume ("point measurement") with a total acquisition time of 900 seconds.

Data Analysis

Using the MicroTime 200 software, the single photon data was binned in each channel in 1 ms bins, resulting in the MCS traces and calculated count rate histograms shown in Figure 1. In order to identify photon bursts, the software uses these histograms to propose a threshold level for each channel with respect to the corresponding background signal level. Every level can also be manually optimised.

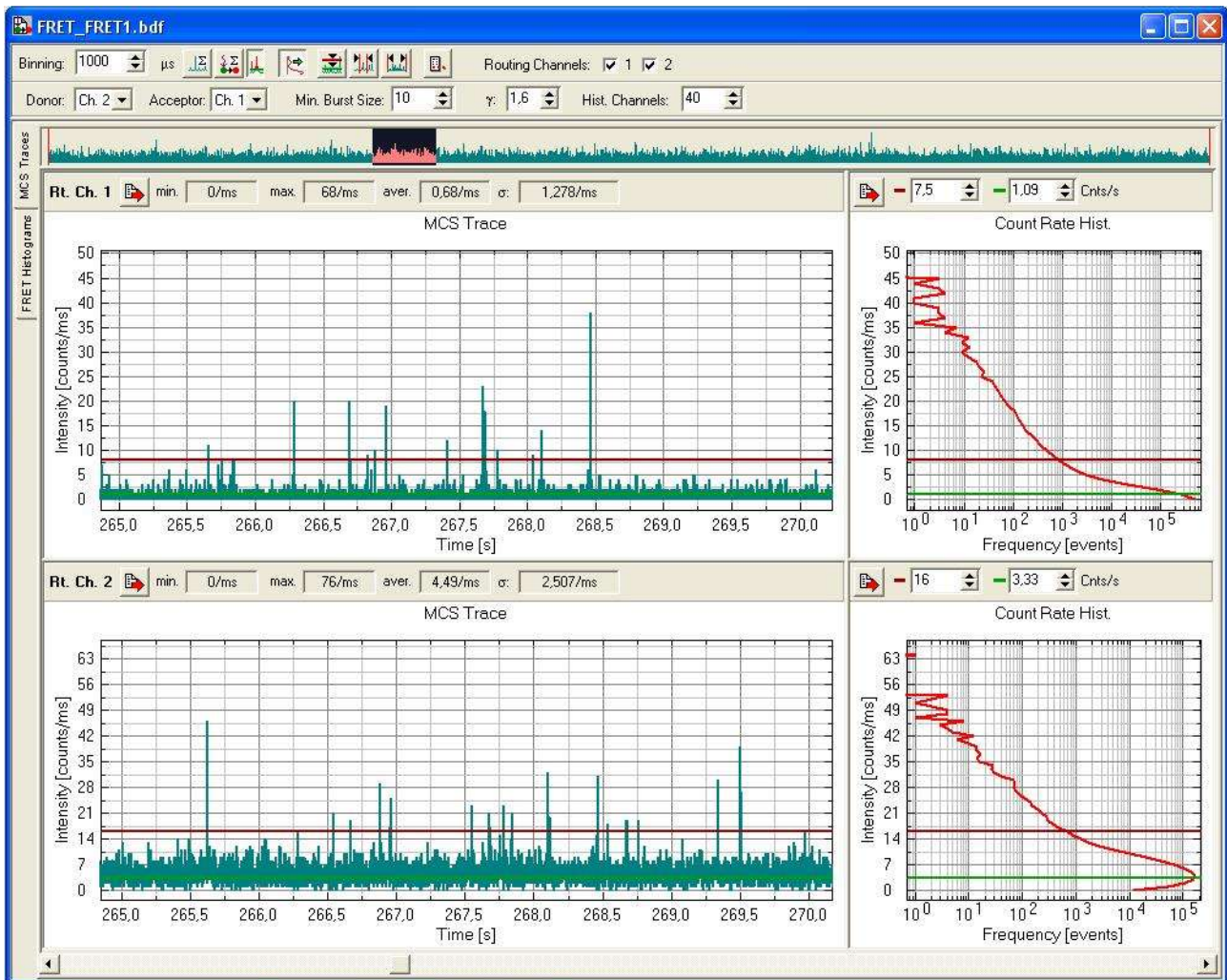


Figure 1: MCS traces and count rate histograms of the measurement

Single molecule bursts are then identified, if the count rate in both channels exceeds the corresponding threshold value. The information of adjacent bins can optionally be binned together to get a sum photon number for each burst which is assumed to be caused by a FRET pair. Finally the FRET efficiency and Donor-Acceptor distance is calculated for corresponding events in Channel 2 (Donor) and Channel 1 (Acceptor) from extracted sum photons n_D and n_A taking into account the possible difference in the detection efficiency (γ), provided by the user (see Figure 2).

As expected for this special sample, the FRET efficiency histogram is dominated by a maximum close to an efficiency of 0.5 and the maximum of the distribution in the calculated distance between the two chromophores is close to the Förster radius R_0 . The peak at a FRET efficiency close to zero is due to molecules lacking the acceptor dye because of photo bleaching or residual impurities. The large width of the distribution is mostly due to shot noise.

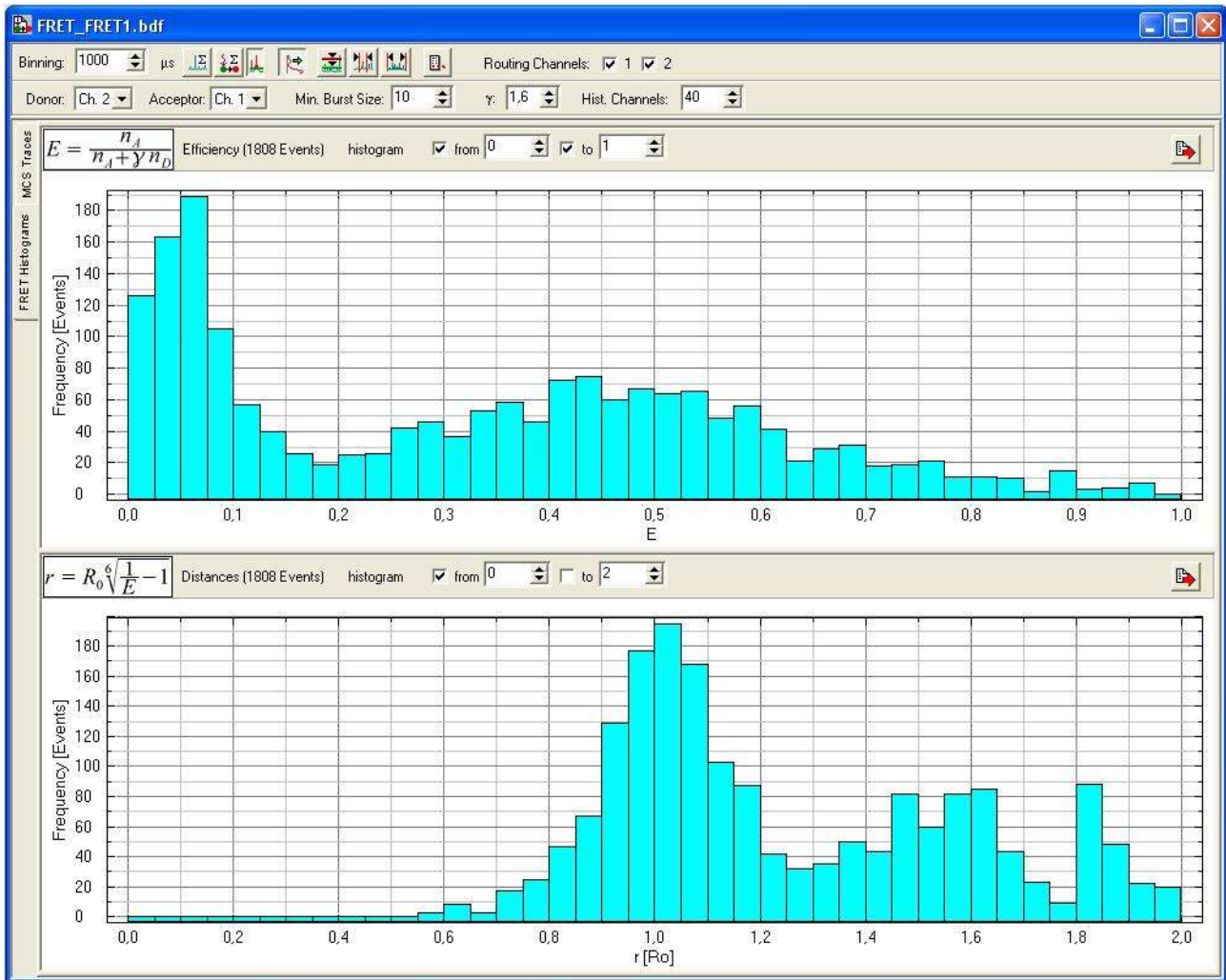


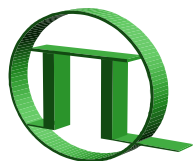
Figure 2: FRET-Analysis

The same kind of data analysis can also be applied to the data of a single immobilised FRET pair to investigate its FRET fluctuations. In this case the photons of adjacent bins are typically not summed, but the analysis is carried out just bin by bin along the MCS trace. The used bin width can be freely set at the start of the analysis.

Data courtesy of Prof. Ben Schuler, Universität Potsdam, Germany.

Further Reading

- [1] Stryer, L., Haugland, R.P., Proc. Natl. Acad. Sci. USA 58, Vol. 2, p.719-726 (1967)
- [2] Schuler B., Lipman E. A., Eaton W. A., Nature, Vol.419, p.743-747 (2002)
- [3] Wahl M., Erdmann R., Lauritsen K., Rahn H.-J., Proceedings of SPIE, Vol.3259, p.173-178 (1998)



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