Suppression of the Zero Emission Peak through Pulsed Interleaved Excitation (PIE) Single Pair FRET Using a Confocal Microscope Based on Time-Correlated Single Photon Counting (TCSPC)

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In common laser scanning fluorescence microscopes a large amount of information is lost because only averaged information of a number of photons, e.g. the intensity, is recorded. In contrast, Time-Correlated Single Photon Counting offers the possibility to overcome these limitations, because it allows to store spatial, timing and spectral information for every detected photon individually. Traditional acquisition techniques like imaging (including FRET and FLIM) and sophisticated point measurements (e.g. FCS) can now be implemented based on this ultimately flexible data collection scheme called Time-Tagged Time-Resolved (TTTR) mode. All possible analysis methods can thereby be applied to the same raw data set.

The optical design of the presented system (MicroTime 200) is based on a standard inverted microscope equipped with a three dimensional piezo scanning device which allows either for sample or probe (objective) scanning, which also permits to investigate heavy and/or bulky samples. Compact pulsed diode lasers are used for excitation to get discrete timing information. The fluorescence is detected on a single photon base with highly efficient avalanche diodes in a freely configurable detection scheme consisting of up to four detectors. The presentation will illustrate this powerful approach with different examples like pulsed-interleaved FRET experiments (PIE-FRET) on freely diffusing dual labeled polypeptides and lifetime assisted FCS (FLCS) measurements. In PIE measurements the donor and the acceptor molecule of a FRET pair are alternatingly excited and the measurement result can be used to identify and rule out partial inactive FRET molecules, which cause interfering artefacts in standard FRET efficiency analysis.[1]

This system also allows a more sophisticated FCS analysis. Time-gating can be applied to discriminate the fluorescence signal from scattered light contributions (Rayleigh, Raman). In addition fluorescence lifetime can be exploited for sorting and weighting of the detected photons to improve the significance in common FCS analysis. This allows even to separate FCS curves for species which differ in their fluorescence lifetime but cannot be distinguished spectrally [2].