



Förster resonance energy transfer (FRET) measurements from a donor, D, to an acceptor, A, fluorophore are frequently used in vitro and in live cells to reveal information on the structure and dynamics of DA-labeled macromolecules. Accurate descriptions of FRET-measurements by molecular models are complicated because the fluorophores are usually coupled to the macromolecule via flexible long linkers allowing for diffusional exchange between multiple states with different fluorescence properties caused by distinct environmental quenching, dye mobilities, and variable DA-distances. It is often assumed for the analysis of fluorescence intensity decays that DA-distances and D quenching are uncorrelated (homogenous quenching by FRET) and that the exchange between distinct fluorophore states is slow (quasi-static). This allows us to introduce the FRET-induced donor decay, $\epsilon_D(t)$, a function solely depending on the species fraction distribution of the rate constants of energy transfer by FRET, for a convenient joint analysis of fluorescence decays of FRET- and reference samples by integrated graphical and analytical procedures. Additionally, we developed a simulation toolkit to model dye diffusion, fluorescence quenching by the protein surface and FRET. A benchmark study with simulated fluorescence decays of 500 protein structures demonstrates that the quasi-static homogeneous model works very well and recovers for single conformations the average DA-distances with an accuracy of $< 2\%$. For more complex cases, where proteins adopt multiple conformations with significantly

Graphical and Analytical Methods To Analyze Time-Resolved FRET Measurements

different dye environments (heterogeneous case), we introduce a general analysis framework and evaluate its power in resolving heterogeneities in DA-distances. The developed fast simulation methods, relying on Brownian dynamics of a coarse-grained dye in its sterically accessible volume, allow us to incorporate structural information in the decay analysis for heterogeneous cases by relating dye states with protein conformations to pave the way for fluorescence and FRET-based dynamic structural biology.

Finally, we present theories and simulations to assess the accuracy and precision of steady-state and time-resolved FRET measurements in resolving of DA-distances on the single-molecule and ensemble level and provide a rigorous framework for estimating approximation, systematic and statistical errors.