Analysis of Photobleaching in Single-Molecule Multicolor Excitation and Förster Resonance Energy Transfer Measurements†

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Received: August 15, 2005; In Final Form: January 12, 2006

Dye photobleaching is a major constraint of fluorescence readout within a range of applications. In this study, we investigated the influence of photobleaching in fluorescence experiments applying multicolor laser as well as Förster resonance energy transfer (FRET) mediated excitation using several red-emitting dyes frequently used in multicolor experiments or as FRET acceptors. The chosen dyes (cyanine 5 (Cy5), MR121, Alexa660, Alexa680, Atto647N, Atto655) have chemically distinct chromophore systems and can be excited at 650 nm. Several fluorescence analysis techniques have been applied to detect photobleaching and to disclose the underlying photophysics, all of which are based on single-molecule detection: (1) fluorescence correlation spectroscopy (FCS) of bulk solutions, (2) fluorescence cross-correlation of single-molecule trajectories, and (3) multiparameter fluorescence detection (MFD) of single-molecule events. The maximum achievable fluorescence signals as well as the survival times of the red dyes were markedly reduced under additional laser irradiation in the range of 500 nm. Particularly at excitation levels at or close to saturation, the 500 nm irradiation effectively induced transitions to higher excited electronic states on already excited dye molecules, leading to a pronounced bleaching reactivity. A theoretical model for the observed laser irradiance dependence of the fluorescence brightness of a Cy5 FRET acceptor dye has been developed introducing the full description of the underlying photophysics. The model takes into account acceptor as well as donor photobleaching from higher excited electronic states, population of triplet states, and energy transfer to both the ground and excited states of the acceptor dye. Also, photoinduced reverse intersystem crossing via higher excited triplet states is included, which was found to be very efficient for Cy5 attached to DNA. Comparing continuous wave (cw) and pulsed donor excitation, a strong enhancement of acceptor photobleaching by a factor of 5 was observed for the latter. Thus, in the case of fluorescence experiments utilizing multicolor pulsed laser excitation, the application of the appropriate timing of synchronized green and red laser pulses in an alternating excitation mode can circumvent excessive photobleaching. Moreover, important new single-molecule analysis diagnosis tools are presented: (1) For the case of excessive acceptor photobleaching, cross-correlation analysis of single-molecule trajectories of the fluorescence signal detected in the donor and acceptor detection channels and vice versa shows an anticorrelated exponential decay and growth, respectively. (2) The time difference, $T_g - T_r$, of the mean observation times of all photons detected for the donor and acceptor detection channels within a single-molecule fluorescence burst allows one to identify and exclude molecules with an event of acceptor photobleaching. The presented single-molecule analysis methods can be constrained to, for example, FRET-active subpopulations, reducing bias from FRET-inactive molecules. The observations made are of strong relevance for and demand a careful choice of laser action in multicolor and FRET experiments, in particular when performed at or close to saturation.