SUPPORTING INFORMATION

RECOVERY OF PHOTO-INDUCED REVERSIBLE DARK STATES UTILIZED FOR MOLECULAR DIFFUSION MEASUREMENTS.

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Calculation of the time-dependence of the fluorescence and transient state populations

The behavior of the box model of figure 2 was checked by calculating the time-dependence of the fluorescence and population levels of the trans- and cis- isomers for square wave excitation pulse trains with different pulse characteristics. The fluorophores were assumed to undergo trans-cis isomerisation and undergo diffusion into and out of the detection volume, as described by eqs 3 and 4 and the model of figure 2. The influence of *F(t)* and the populations of *Trans(t)* and *Cis(t)* on diffusion was tested by comparing the outcomes for immobile molecules ($k_D = 0$) and for molecules undergoing diffusion comparable to that of free Cy5 fluorophores in aqueous solution ($k_D = 0.02$ µs^{-1}). As can be seen in figure S-1, there is a clear distinction between immobile molecules and molecules freely diffusing into and out of the excitation volume. For the immobile fluorophores, some recovery of *Trans(t)* (and *F(t)*) in between the excitation pulses can be seen due to thermal back-isomerisation by k_{PN}. For pulse trains with short excitation pulse durations, and with a limited number of pulses per molecular passage through the excitation volume, also the excitation dose per passage is limited. It then takes a longer time to generate a steady-state population ratio between the trans- and cis- isomers of the fluorescent molecules and a higher $\bar{F}_{EXC}$ can be observed.

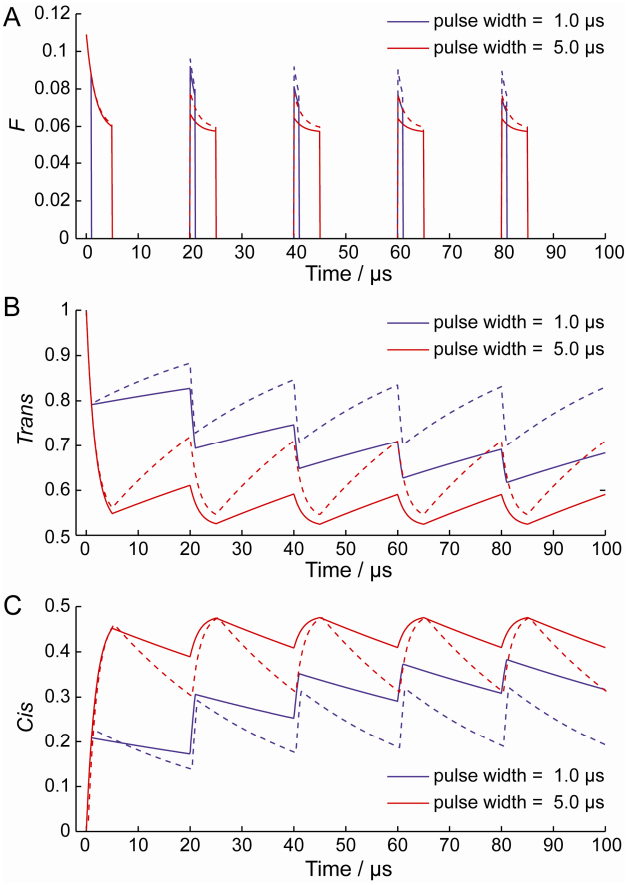

Figure S-1. A realization of the box model of figure 2, and with the solutions given by eq. 15 for the detected fluorescence *F(t)* (subplot A), eqs 12-13 for the populations in *Trans* (subplot B) and *Cis* (subplot C), for five consecutive square excitation pulses ($P = 150$ µW, $w = 1.0$ µs (blue) and 5.0 µs (red), $T = 10$ µs). At the onset of the first excitation pulse all molecules were in a *Trans* isomer conformation. During the pulses, a cis-isomer population is built up, which then decays marginally due to thermal deactivation in-between the pulses. Parameters used in the calculation: $\sigma_{01} = 1.3\times10^{-16}$ cm^2, $k_{10} = 1160$ µs^{-1}, $k_{ISO} = 25$ µs^{-1}, $\sigma_{BISO} = 0.03\times10^{-16}$ cm^2 and $k_{PN} = 0.01$ µs^{-1}. The (uniform) excitation irradiance within the pulses onto the detection volume was set to $I_{EXC} = 24$ kW/cm^2. Solid lines represent the calculated scenario for a stationary fluorophore ($k_D = 0$) and dotted lines that of a diffusion-mediated exchange of the molecules into and out of the excitation/detection volume with $k_D = 1/50$ µs^{-1}.

Calculation of the renormalized average fluorescence intensity within the excitation pulses

In figure S-2 calculations are presented showing the resulting normalized fluorescence intensity within the excitation pulse ($\overline{F}_{\mathrm{EXC}}$) for pulse trains with a duration of 2s, and with the same pulse widths as in figure S-1 (1 µs and 5 µs) and the effect of varying the pulse period for stationary and diffusing fluorophore molecules. As for the calculated *F(t)* trace in figure S-1, it can be seen that for molecules diffusing faster through the excitation volume (shorter diffusion times) $\overline{F}_{\mathrm{EXC}}$ is higher, because the molecule experiences a lower number of excitations per passage.

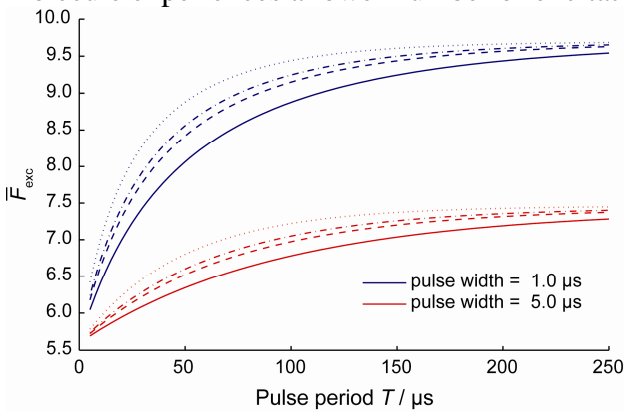

Figure S-2. Calculated plots, based on eqs 12-15, with the time-averaged fluorescence, $\overline{F}_{exc}$, plotted versus the pulse period *T*, with all pulses having the same pulse width *w* of 1.0 µs (blue) and 5.0 µs (red). Solid lines represents calculated $\overline{F}_{exc}$ for diffusion rate k_D=0 μs^{-1}, dashed lines k_D=1/300 μs^{-1}, dashed-dotted lines k_D=1/200 μs^{-1} and dotted lines k_D=1/100 μs^{-1}.

FCS calibration curves for Cy5

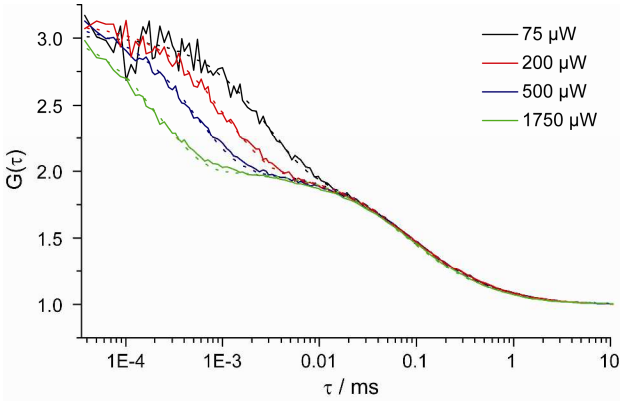

Figure S-3. FCS curves of Cy5 measured in aqueous solution at excitation irradiances of 12, 32, 80 and 280 kW/cm^2 with fits according to equation 2. The amplitude of isomerisation relaxation term P_{eq} does not change significantly, while the relaxation time τ_{ISO} is inversely proportional to the excitation irradiance.

FCS curves for Cy5 in the presence of laminar flow

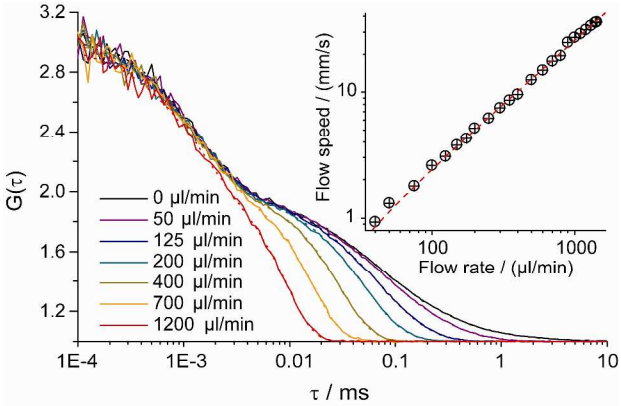

Figure S-4. FCS curves for Cy5 in the presence of laminar flow in a microchannel with fits according to eq. 17. Excitation irradiance is 16 kW/cm^2. Diffusion time τ_D = 70 µs, flow time τ_{flow} varies from 230 µs at a microchannel flow rate of 50 µl/min to 10 µs at a flow rate of 1200 µl/min. At fast flow rates (>1.5 ml/min) a decrease in the amplitude P_{eq} and the decay time τ_{ISO} of the isomerisation relaxation term could be observed, reflecting that the flow time (and the residence time in the excitation volume) approaches the time range of the isomerisation relaxation time at this excitation irradiance, τ_{ISO} = 1.4 µs. The inset shows the observed linear dependence of the flow speed $V = \tau_{flow} / \omega_0$ versus the applied flow rate generated by the syringe pump.

The determined flow speeds, V, in the inset can be compared to the V obtained from the measurements presented in figure 3, where the speed is given by $V=k_D 2\omega_0$, at least for flow rates in the higher range, where the diffusion influence on k_D is less pronounced. From figure 3B we obtain for the fasted flow rate (300µl/min) a $1/k_D$ of 100µs. This corresponds to a flow speed of $V= k_D 2\omega_0 = 10^4 s^{-1} \times 2 \times 0{,}38 µm = 7{,}6 mm/s$. From the FCS measurements and the inset get a flow speed of 6,5mm/s for an applied flow rate of 300 µl/min, which is reasonably in agreement with the V determined from the data in figure 3.

FCS curves for dsRed protein

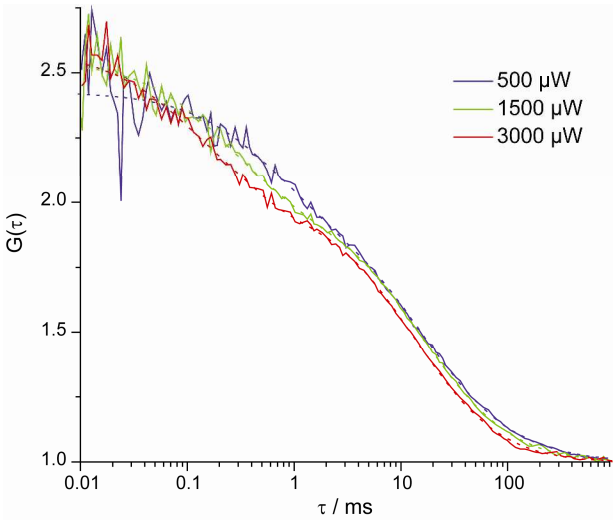

Figure S-5. Measured FCS curves of DsRed in aqueous solution at excitation irradiances of 4.4, 13 and 26 kW/cm^2 with fits according to eq.(2). The amplitudes of the isomerisation relaxation term P_{eq} and the corresponding relaxation times τ_{ISO} are: 0.30 / 780 µs, 0.35 / 370 µs and 0.36 / 212 µs, respectively.