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

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ABSTRACT

For a spatially restricted excitation volume, the effective modulation of the excitation in time is influenced by the passage times of the molecules through the excitation volume. By applying an additional time-modulated excitation, the build-up of photo-induced reversible dark states in fluorescent molecules can be made to vary significantly with their passage times through the excitation volume. The variations in the dark state populations are reflected by the time-averaged fluorescence intensity, which thus can be used to characterize the mobilities of the molecules.

The concept was experimentally verified by measuring the fluorescence response of freely diffusing Cyanine fluorophores (Cy5), undergoing trans-cis isomerisation when subject to time-modulated excitation in a focused laser beam. From the fluorescence response, and by applying a simple photodynamic model, the transition times of the Cy5 molecules could be well reproduced when applying different laminar flow speeds through the detection volume. The presented approach puts no constraints on sample concentration, no requirements for high time resolution or sensitivity in the detection, nor requires a high fluorescence brightness of the characterized molecules. This can make the concept useful for a broad range of biomolecular mobility studies.

KEYWORDS

Fluorescence correlation spectroscopy, FCS, isomerisation, transient states, diffusion

Introduction

Fluorescence offers several means to measure translational diffusion of biomolecules in cells, cellular membranes, lipid bilayers and in solutions. Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Correlation Spectroscopy (FCS) are together with Single Particle Tracking (SPT) probably the most widely used techniques.¹ The FCS and FRAP techniques date back to the 70's,²⁻⁵ but have experienced an increased interest for diffusion studies in the last decades, in particular for cellular measurements⁶. In FCS, diffusion properties of fluorescent molecules are analyzed via the fluorescence intensity fluctuations as the molecules transit a confocal detection volume. In FRAP, the fluorescent molecules in a defined volume are bleached by a laser pulse, and the subsequent fluorescence intensity recovery as unbleached fluorescent molecules diffuse into the bleached region provides the molecular mobility information. FCS and FRAP are complementary in the sense that they cover different concentration ranges, spatial resolutions and time regions.⁷ As a molecular fluctuation technique FCS is typically limited to concentrations of fluorescent molecules below $500\mu\text{m}^{-2}$ in membranes and 500 nM in solutions. FRAP can not be applied at as low concentrations as FCS (or SPT), but works well at concentrations far higher than the upper limits of the FCS technique. Spatially, FRAP integrates the diffusive properties of the molecules over a much larger region than by FCS, which monitors the diffusive properties of the molecules only when in the detection volume itself. Temporally, FCS can monitor molecular movements down to microseconds, while FRAP is better suited to slower processes in the time range up to seconds. Both FCS and FRAP put restrictions on the fluorophore labels. In FCS, they should be photostable and able to yield high fluorescence emission rates⁸. In FRAP, the fluorophores should be moderately sensitive to photobleaching to minimize the intensity and duration of the bleach beam, but not so sensitive that significant bleaching occurs when measuring the fluorescence recovery by the attenuated probe beam⁹. Moreover, the photobleaching process itself needs to be defined. In particular, reversible photobleaching, i.e. any spontaneous recovery of fluorescence not attributed to diffusion, is a concern and may lead to serious misinterpretations of the diffusion behavior of the studied molecules⁹.

In this work we propose the exploitation of reversible photobleaching for diffusion measurements. By FCS, transitions of dye molecules to and from photo-induced, long-lived, dark or weakly emitting states can be well characterized via the related fluorescence fluctuations¹⁰⁻¹⁴. However, information about these states can also be extracted from the variation in the time-averaged fluorescence of dye molecules under different regimes of time-modulated excitation¹⁴. Unlike FCS and single molecule measurements this, so-called transient state (TRAST) monitoring does not require accurate recording of fluorescence time traces, reflecting the spontaneous blinking behavior of individual or a very low number of fluorescent molecules. Likewise, in contrast to single molecule imaging approaches, where the degree of “blurriness” of the image and its dependence on frame rates or pixel dwell times sometimes can provide information about molecular mobilities^{15, 16} the TRAST concept is not restricted to single molecule concentration samples displaying high molecular fluorescence brightness. It is necessary to modulate of the excitation in a pre-defined manner, but the time information is then entirely kept in the modulation of the excitation. As previously shown [ref 14], the TRAST concept is therefore fully compatible with low time-resolution detection, for instance by a CCD camera, offering the possibility of a massively parallel readout. The TRAST concept has been realized with time-modulated excitation in a confocal arrangement¹⁴, in a total internal reflection fluorescence microscope¹⁷ and by use of wide-field imaging¹⁸. Evidently, the time-modulated excitation experienced by a stationary sample can also be generated by translation of the sample with respect to the excitation, or vice versa. Along this strategy, the TRAST concept was recently established by use of an LSCM¹⁹.

With the TRAST concept, the response in the time-averaged fluorescence to the modulation characteristics of an excitation pulse train (e.g. duration, height and separation of the pulses) is used to characterize the population dynamics of photo-induced, long-lived transient states. Vice versa, it is also possible to get information about the modulation characteristics from the same fluorescence response, if the transient state transition properties are known. For a spatially restricted excitation, the effective excitation modulation of the molecules in time is influenced by their passage times through the excitation volume.

Based on this notion, we introduce and experimentally verify an approach where the time-averaged fluorescence intensity response from cyanine dye (Cy5) molecules in an open confocal detection volume is used to determine the mobility of the molecules. The passage times of Cy5 were varied by applying a laminar flow. In FCS measurements, cyanine dyes typically show a prominent build-up of non-fluorescent photo-isomerized states¹². By time-modulated excitation the build-up of these states could be made to strongly correlate to the passage times of the molecules through the confocal detection volume, providing a measure of the molecular mobility. The approach was also tested and verified for the fluorescent protein dsRed, where the extent of build-up of a prominent photo-induced transient state of the protein²⁰ was used to monitor its diffusion speed.

Theory

Electronic state model

Most cyanine dyes in their ground states adopt an all-trans conformation²¹. Upon excitation however, they tend to undergo trans-cis isomerisation, whereby one of the double bonds in their polymethine chains undergoes a π -twist around the bond axis (figure 1A). These formed cis isomers have different absorption spectra and are typically practically non-fluorescent. The isomerisation reactions are predominantly light-induced in both directions, and occur from one of the excited singlet states of the isomers, N_1 and P_1 , to the ground singlet state of the other isomer, P_0 and N_0 , respectively. The rates of isomerisation from N_1 to P_0 and from P_1 to N_0 are denoted k_{ISO} and k_{BISO} , respectively (caption, figure 1A). In the absence of excitation, back-isomerisation can also occur at room temperature in moderately viscous solvents (k_{PN} in figure 1A). k_{PN} is on the order of μ s to ms for most cyanine dyes²²⁻²⁴. For the dye Cyanine 5 (Cy5) in aqueous solution it has been found to be on the order of 100 μ s or slower¹². Given the difference in absorption spectra between the isomers, and that their exchange rates are predominantly light-driven, the steady-state populations of the two isomers relative to each other depend on the excitation wavelength. For Cy-5 under excitation with 594 nm, 633 nm or 647 nm wavelength the fraction of isomers in the trans- and cis-state are both about 50%.¹²

Figure 1.

A. Kinetic scheme modeling the photophysical behavior of carbocyanine dyes. N_0 and N_1 denote the ground singlet and the first excited singlet states of the thermodynamically stable conformation of the dye, normally the all-*trans* form (bottom left). P_0 and P_1 are the corresponding states of the photoisomerised form, usually a mono-*cis* conformation (bottom right). Upon isomerisation the angle, θ , in one of the double bonds of the conjugated hydrocarbon chain connecting the two headgroups of the dye is twisted by 180° . Transitions from N_1 and P_1 take place via the partially twisted intermediate state, $Perp_1$, either to N_0 or to P_0 . $k_{01} = \sigma_{01}I_{EXC}$ denote the excitation rate from N_0 to N_1 (where σ_{01} is the excitation cross section of N_0 , and I_{EXC} is the excitation irradiance). $k_{P01} = \sigma_{P01}I_{EXC}$ is the corresponding excitation rate from P_0 to P_1 . k_{PN} is the rate of thermal deactivation of P_0 to N_0 . The isomerisation rates

from P_1 to N_0 and from N_1 to P_0 can be expressed as $k_{ISO} = k_{Pperp} \frac{k_{perpN}}{k_{perpN} + k_{perpP}}$ and

$k_{BISO} = k_{Nperp} \frac{k_{perpP}}{k_{perpN} + k_{perpP}}$, respectively.

B. Calculated FCS curve (dotted) and the corresponding population of the excited singlet state N_1 for Cy5 versus τ (red), given the initial population probabilities at $\tau=0$ of $N_1=1$, $N_0=0$, $P_1=0$, $P_0=0$.

Fluorescence antibunching time $\tau_{AB} = 10^{-9}$ s, isomerisation relaxation time $\tau_{ISO} = 10^{-6}$ s and characteristic diffusion time $\tau_D = 10^{-3}$ s.

C. Two state model used to characterize the isomerization-induced fluorescence fluctuations observed

in the FCS experiments. Here Trans and Cis correspond to the fractions of dye molecules in the detection volume that are in their trans-singlet ($N_0 + N_1$), and cis-singlet ($P_0 + P_1$) states. k_{ISO}' and k_{BISO}' denote the effective rates of isomerization and back-isomerization (Eqs. 3 and 4).

The quantum yield of intersystem crossing of cyanine dyes is typically quite low, on the order of 10^{-3} .^{21,}

²⁵ Moreover, the lowest triplet state is believed not to be involved in the isomerisation process, which has been reported to occur entirely via the excited singlet state.^{21, 22, 26} Singlet-triplet state transitions are therefore not considered in the model of figure 1A.

In Fluorescence Correlation Spectroscopy (FCS) measurements, as well as in the measurements performed in this study, the fluorescence is collected in a confocal manner. For cyanine dyes, the detected fluorescence intensity can be assumed to be proportional to the population of the first excited state S_1 of the trans- isomer:

$$F(t) = \int \text{CEF}(\vec{r}) c(\vec{r}, t) q k_{10} \Phi_f N_1(\vec{r}, t) dV \quad (1)$$

Here, $\text{CEF}(\vec{r})$ is the collection efficiency function of the confocal microscope setup, determined by the dimensions of the pinhole and the optical properties of the objective, $c(\vec{r}, t)$ denotes the concentration of fluorophores, and q accounts for the detector quantum efficiency and the attenuation of the fluorescence in the passage from the sample volume to the detector areas. k_{10} is the deactivation rate from the excited singlet state, Φ_f denotes the quantum yield of fluorescence, and $N_1(\vec{r}, t)$ is the fraction of the fluorophores in the detection volume that are in their excited singlet states of the trans-isomer. In FCS, the correlation function $G(\tau)$ of the detected fluorescence intensity fluctuations can be considered as a probability distribution of detecting a photon from a fluorescent molecule at time τ , given that a photon was detected from the same molecule at time 0. A typical FCS curve for a cyanine dye, measured in aqueous solution and undergoing trans-cis isomerisation upon excitation is shown in figure 1A, and can be expressed as¹²:

$$G_{\text{ISO}}(\tau) = \frac{1}{N} \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \left[1 + \left(\frac{\omega_0}{\omega_z} \right)^2 \frac{\tau}{\tau_D} \right]^{-1/2} \left[1 + \frac{P_{eq}}{1 - P_{eq}} \exp(-\tau / \tau_{ISO}) \right] \quad (2)$$

Here, N is the mean number of fluorescent molecules within the detection volume. ω_0 and ω_z are the distances from the centre of the laser beam focus in the radial and axial direction respectively at which

the collected fluorescence intensity has dropped by a factor of e^2 compared to its peak value. The characteristic diffusion time for the fluorescent molecules is given via the diffusion coefficient, D , by $\tau_D = \omega_0^2 / 4D$. P_{eq} is the time and space averaged fraction of fluorophores within the detection volume being in a non-fluorescent cis photoisomer form and τ_{ISO} is the relaxation time related to the trans-cis isomerisation process.

Considering that equilibration between the ground and excited singlet states within both the isomers ($\sim$ ns) have already occurred at the time scale of the isomerisation ($\sim$ μ s to ms), the electronic state model of figure 1A can be simplified to a two-state model (figure 1C). In this model, it is further assumed that triplet state formation can be neglected. The effective rates of isomerisation and back-isomerisation can then be expressed as:

$$k_{ISO}' = \frac{k_{01}}{k_{10} + k_{01}} k_{ISO} = \frac{\sigma_{01} I_{EXC}}{k_{10} + \sigma_{01} I_{EXC}} k_{ISO} \quad (3)$$

$$k_{BISO}' = \frac{k_{P01}}{k_{P10} + k_{P01}} k_{BISO} + k_{PN} = \frac{\sigma_{P01} I_{EXC}}{k_{P10} + \sigma_{P01} I_{EXC}} k_{BISO} + k_{PN} \quad (4)$$

$$\{k_{P10} \gg \sigma_{P01} I_{EXC}\} = \sigma_{BISO} I_{EXC} + k_{PN}$$

Here σ_{01} and σ_{P01} are the excitation cross sections of the ground singlet states N_0 and P_0 , respectively.

I_{EXC} denotes the mean excitation irradiance within the detection volume. k_{10} and k_{P10} are the deactivation rates of the singlet excited states N_1 and P_1 , respectively. Approximating the excitation irradiance within the detection volume to be uniform,^{12, 14} P_{eq} and τ_{ISO} of Eq. 2 can then be expressed as:

$$P_{eq} = \frac{k_{ISO}'}{k_{ISO}' + k_{BISO}'} \quad (5)$$

$$\tau_{ISO} = \left(k_{ISO}' + k_{BISO}' \right)^{-1} \quad (6)$$

Materials and methods

Instrumental setup

A home-built confocal setup, consisting of an Olympus IX-70 microscope body with a 60x, NA 1.2, UPlanApo Olympus objective was used for the measurements. For measurements with Cy5, excitation was done by a linearly polarized He-Ne laser (594nm, Laser2000 model 30572, Munich, Germany), attenuated by neutral density filters (Schott, Germany) down to 150 μ W. The laser beam was reflected by a dichroic mirror (z488/594rpc, Chroma Technology Corp., USA) and focused down to a $1/e^2$ radius of $\omega_0 = 0.38 \mu\text{m}$ (estimated from the diffusion times of Cy5 acquired from FCS measurements with different pinhole sizes). Emitted fluorescence was collected by the same objective and focused by a 150-mm achromatic lens onto a pinhole of 80- μm diameter in the image plane. Direct Rayleigh-scattered light from the laser beam and Raman-scattered light from the aqueous solution were suppressed by use of band-pass filters (HQ675/135M, Chroma Technology Corp., USA) prior to fluorescence detection by two avalanche photodiodes (APDs) (SPCM AQR-14/16, Perkin-Elmer Optoelectronics, USA) in a 50/50 beam-splitting arrangement (BS010, Thorlabs, USA), to eliminate detector afterpulsing and shot noise. Laser intensity modulation was performed by an acousto-optic modulator (AOM; AA.MT.200/A0,5-VIS, AA Opto-Electronic, Orsay, France).

For measurements with the DsRed fluorescent protein, 514 nm excitation was provided by a linearly polarized multiline argon-ion laser (Lasos LGK 7812 ML, Jena, Germany) and an excitation filter (Z514/10X, Chroma Technology Corp., USA). Laser power was set to 500 μ W. The excitation volume was expanded to a $1/e^2$ radius of $\omega_0 = 1.9 \mu\text{m}$ (estimated from the diffusion times of Rhodamine 6G acquired from FCS measurements with different pinhole sizes). For these measurements a pinhole with diameter 200 μm was used. Dichroic mirror (515/633PC) and fluorescence emission filters (HQ580/80M) were from Chroma Technology Corp., USA.

For both the Cy5 and the dsRed measurements, the residence times of the molecules inside of the excitation volume were controlled by applying different flow rates through a micro-channel in a silicone film of 200 μm height and 2 mm width. A syringe pump (SP101i, World Precision Instruments Inc., USA) was used to regulate the flow rates.

Data collection and processing

The procedures for the data collection and processing were largely based on those developed in ^{14, 19}.

Data were collected using a PCI-6602 counter/timer card (National Instruments Corp., USA), counting pulses from two APDs connected to separate channels (counters). The acousto-optical modulator driver was externally controlled by a digital signal generated by the card. Communication with the data collecting card PCI-6602 was done via a dynamic-link library (DLL) driver written in C with use of an application programming interface (API) NI-DAQmx. Measurements were automated by Matlab (The MathWorks, Inc., USA) based software developed in-house. With this software, series of excitation pulse trains could be generated, for which the period time, T , and the excitation pulse width, w , could be changed in an automated, pre-programmed manner from one pulse train to the next. The internal 80-MHz clock of the card was used as a time base for the modulation.

For each modulation setting (i.e., for each excitation pulse train with a defined T and w) the fluorescence was recorded and averaged over the full duration of the pulse train (2 s for Cy5 measurements, 15 s for DsRed measurements). After each pulse train, excitation and acquisition were turned off for 0.1 s, to allow for full electronic-state relaxation within the fluorophore molecules before the onset of the next pulse train.

The software generated a matrix with the time-averaged fluorescence intensities, $\bar{F}$, for each pulse train, with period time, T , and pulse width, w . Before measurement on a sample, an intensity matrix was acquired for a blank, i.e., buffer solution in an identical sample container. This background matrix was then directly subtracted from the intensities measured for the samples by the software. $\bar{F}$ were also corrected for possible nonlinear responses of the detectors, appearing at fluorescence inter-photon times approaching those of the dead time of the APDs.

The measured average fluorescence intensities, $\bar{F}$, were divided with the modulation duty cycles, η , of their corresponding excitation pulse trains. Thereby, the average fluorescence intensity during the time the excitation light was actually on, i.e., $\bar{F}_{\text{EXC}}$ of Eq. 16, could be calculated.

For the FCS measurements, the APD signals from each detection channel were processed by an ALV-5000/E correlator (ALV-GmbH, Germany), with an ALV 5000/FAST Tau Extension board giving a 12.5-ns lag time resolution when cross-correlating between channels.

Sample preparation

Cy5 Mono Maleimide was purchased from GE Healthcare (UK), dissolved in DMSO and subsequently diluted to nM concentrations in ultra-filtered, distilled water (Easypure, Wilhelm Werner GmbH, Germany). DsRed fluorescent protein was a kind gift from prof. Masataka Kinjo, Hokkaido University, Japan. The protein was diluted to nM concentrations in Dulbecco's Phosphate Buffered Saline pH 7.4 (Sigma-Aldrich, USA)

Results and discussion

Concept of measurements

As shown recently,^{14, 19} the dependence of the time-averaged fluorescence on the modulation characteristics of the excitation (e.g. duration, height and separation of the pulses within an excitation pulse train) can be used to extract information about the population dynamics of photo-induced, long-lived transient states of dyes. Vice versa, if the transient state transition properties of the dye are known it is also possible to get information about the modulation characteristics from the fluorescence response. For a spatially restricted excitation, the effective excitation modulation of the molecules over time can also be influenced by their passage times through the excitation volume. Based on known transient dark state characteristics of the fluorescent molecules, the laser excitation modulation can be adapted so that the response in the average fluorescence is particularly sensitive to the dwell times of the fluorescent molecules in the excitation volume. This concept to determine molecular dwell times or diffusion properties can in principle be realized, utilizing the kinetic properties of a range of transient photo-induced states. However, trans-cis isomerisation offers an additional advantage, in that it is light driven

in both directions. Apart from a slow thermal deactivation by k_{PN} the two isomers do not interconvert in the absence of excitation. The extent of build-up of cis-isomers thus reflects the amount of excitation irradiation that the molecules have been exposed to. In other words, for a known excitation irradiance, it reflects how much time the molecules on average have spent in the excitation volume. By modulation, the distribution of the excitation irradiation in time can be adapted so that the extent of build-up of the transient state, in our case the cis isomer, is maximally sensitive to changes in the dwell times of the molecules in the excitation volume. For this purpose, we introduced a square-wave modulation of the excitation in time, with the excitation irradiance within each pulse period given by:

$$I_{EXC}(t) = \begin{cases} I_{EXC}, & 0 < t \leq w \\ 0, & w < t \leq T \end{cases} \quad (7)$$

Here, T is the period time, w the duration of the excitation pulse within each period, and I_{EXC} the excitation irradiance. In the following, we use a time variable t , which is reset to $t = 0$ after each pulse period. For the first excitation pulse, assuming uniform excitation irradiance within the detection volume and the molecules to be immobile, the time development of the fluorescence intensity at onset of excitation follows that of the excited singlet state of the trans isomer and is analogous to the relaxation displayed in the FCS curves (Eq. 2):

$$F(t) \propto N_1(t) = 1 - P_{eq} + P_{eq} \exp(-t / \tau_{ISO}) \quad (8)$$

To take diffusion into and out of the detection volume into account, the diffusional exchange is approximated as a chemical reaction inter-converting “in volume” and “bulk” molecules with an effective rate k_D in both directions. Thereby, the dwell times of the fluorophores in the volume are approximated to be exponentially distributed.

A modified kinetic scheme based on this approximation is presented in figure 2. From this scheme, a set of coupled first-order differential equations can be established:

Figure 2. Three-state model used to characterize the fluorescence intensities observed in the modulated excitation experiments. Here, the states *Trans* and *Cis* and the rates k_{ISO}' and k_{BISO}' are defined as in figure 1C. The "Bulk" state represents the molecules outside of the excitation volume, and k_{D} is the rate of exchange of molecules into and out of this volume.

$$\frac{d}{dt} \begin{pmatrix} Trans(t) \\ Cis(t) \\ Bulk(t) \end{pmatrix} = \begin{bmatrix} -k_{ISO}' - k_D & k_{BISO}' & k_D \\ k_{ISO}' & -k_{BISO}' - k_D & 0 \\ k_D & k_D & -k_D \end{bmatrix} \begin{pmatrix} Trans(t) \\ Cis(t) \\ Bulk(t) \end{pmatrix} \quad (9)$$

where k_{ISO}' and k_{BISO}' are the effective rates of isomerisation, and back-isomerisation, as defined in Eqs. 3 and 4, and k_D denotes the rate of exchange of molecules into and out of the excitation volume (Figure 2).

Trans and *Cis* denote the normalized populations of the trans- (in either N_0 , or N_1) and cis- (in either P_0 or P_1) isomers respectively. *Bulk* is the population of the fluorophores in the solution outside of the detection volume. The concentration of fluorescent molecules is assumed to be constant, and normalized to unity both inside and outside of the detection volume:

$$\begin{aligned} Trans(t) + Cis(t) &= 1 \\ Bulk(t) &= 1 \end{aligned} \quad (10)$$

At onset of the first excitation pulse of the pulse train, the following initial condition is valid

$$\begin{pmatrix} Trans_0^{\text{off}}(T) \\ Cis_0^{\text{off}}(T) \\ Bulk_0^{\text{off}}(T) \end{pmatrix} = \begin{pmatrix} Trans_1^{\text{on}}(0) \\ Cis_1^{\text{on}}(0) \\ Bulk_1^{\text{on}}(0) \end{pmatrix} = \begin{pmatrix} 1 \\ 0 \\ 1 \end{pmatrix} \quad (11)$$

Here, X_i^p signify the population probability of state X ($= Trans, Cis$ or $Bulk$), with the superscript p ($=$ on or off) denoting whether the excitation is active or idle, and the subscript i the number of the pulse within the pulse train.

From the initial condition of Eq. 11 and the set of differential equations of Eq. 9, the population dynamics of the trans- and cis- state within the excitation pulse ($0 < t \leq w$) can be described by:

$$\begin{aligned} Trans_i^{\text{on}}(t) &= 1 - \frac{k_{ISO}'}{k_{\Sigma}} + \exp(-t \cdot k_{\Sigma}) \left[-Cis_{i-1}^{\text{off}}(T) + \frac{k_{ISO}'}{k_{\Sigma}} \right] \\ Cis_i^{\text{on}}(t) &= \frac{k_{ISO}'}{k_{\Sigma}} + \exp(-t \cdot k_{\Sigma}) \left[Cis_{i-1}^{\text{off}}(T) - \frac{k_{ISO}'}{k_{\Sigma}} \right] \end{aligned} \quad (12)$$

Here $k_{\Sigma} = k_{ISO}' + k_{BISO}' + k_D$.

When the excitation is turned off, k_{ISO}' is 0, and the population time development within the time period between two consecutive pulses ($w < t \leq T$) is given by

$$\begin{aligned} Trans_i^{\text{off}}(t) &= 1 - Cis_i^{\text{on}}(w) \cdot \exp(-[t-w] \cdot [k_D + k_{PN}]) \\ Cis_i^{\text{off}}(t) &= Cis_i^{\text{on}}(w) \cdot \exp(-[t-w] \cdot [k_D + k_{PN}]) \end{aligned} \quad (13)$$

Applying the initial condition of Eq. 11, at the start of the excitation pulse train, and then using Eqs. 12 and 13 recursively for $i = 1, 2, 3, \dots, n$, the population dynamics can be calculated for a square-wave pulse train of n pulse periods, as described by Eq. 7.

Equilibration between the ground and first excited singlet states occur on the ns timescale, which is much shorter than the typical pulse widths applied (several μs). Consequently, within the i th excitation pulse ($0 < t \leq w$):

$$N_{1,i}(\vec{r}, t) = \frac{\sigma_{10} I_{\text{EXC}}}{k_{10} + \sigma_{10} I_{\text{EXC}}} Trans_i^{\text{on}}(\vec{r}, t) \quad (14)$$

When the excitation pulse is ended, N_1 , decay within nanoseconds to N_0 . In between the excitation pulses ($w < t \leq T$), the population of N_1 can thus be set to zero.

For a measurement with n excitation pulses, the average fluorescence signal during the measurement is then given by:

$$\bar{F} = \frac{1}{n \cdot T} \int_0^{n \cdot T} F(t) dt = Q \frac{1}{n \cdot T} \sum_{i=1}^n \left[\int_0^w N_1(t) dt \right] \quad (15)$$

Here $n \cdot T$ is the total duration of the excitation pulse train and Q is the product of the constants q , k_{10} and Φ_f from Eq. 1, and the integral $\int CEF(\vec{r})c(\vec{r}, t) dV$, which is assumed to remain constant (no photobleaching).

Dividing the average detected fluorescence with the pulse train duty cycle ($\eta = w/T$) yields the average fluorescence intensity within the excitation pulses:

$$\bar{F}_{\text{EXC}} = \bar{F} / \eta \quad (16)$$

Experimental verification

The concept was experimentally tested by measurements of freely diffusing Cy5 in aqueous solution. FCS measurements (figure S-3) were performed at different excitation irradiancies (from 10 to 300 kW/cm²) to determine the rates of photo-induced isomerisation and back-isomerisation and to check the diffusion properties of the dye. By fitting the FCS curves to Eq. 2, the diffusion coefficient of Cy5 was determined to $D=3.9 \cdot 10^{10} \text{ m}^2/\text{s}$ (at 22°C). From the excitation dependence of τ_{ISO} and P_{eq} , obtained by fitting the FCS curves to Eq. 2 and then using Eqs. 3-6, yielded $k_{\text{ISO}}=23 \text{ } \mu\text{s}^{-1}$ and $\sigma_{\text{BISO}}=0.03 \times 10^{-16} \text{ cm}^2$. Here, σ_{01} was set to $1.3 \times 10^{-16} \text{ cm}^2$, and k_{10} to $1160 \text{ } \mu\text{s}^{-1}$.¹² The values of k_{ISO} and σ_{BISO} , as well as the diffusion coefficient, D , of Cy5 are well in agreement with previously determined parameter values using FCS¹² and dual-foci FCS [REF enderlein 2008]. Based on the determined isomerization parameters, the behavior of the box model of figure 2 was tested 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. Further, pulse characteristics were identified yielding a clear distinction in the measured $\bar{F}_{\text{EXC}}$ for immobile molecules and molecules freely diffusing into and out of the excitation volume (see Supporting Information).

Based on these pulse characteristics, excitation-modulated measurements on fluorescent molecules were performed with different excitation volume residence times. Different residence times can either be achieved by coupling the Cy5 fluorophore molecule to molecules of different sizes. However, a more convenient and tunable approach is to vary the residence times of the fluorophores by applying different flow speeds in a microchannel. The flow speeds were analyzed with FCS (figure S-4) using the following expression:^{27, 28}

$$G(\tau) = G_{\text{ISO}}(\tau) \cdot \exp \left(- \left[\frac{\tau}{\tau_{\text{flow}}} \right]^2 \cdot \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \right) \quad (17)$$

where $G_{\text{ISO}}(\tau)$ is defined by Eq 2. τ_{flow} is the average (laminar) flow time of the fluorescent molecules through the detection volume, from which the flow velocity is given by $V = \tau_{\text{flow}} / \omega_0$. V was found to

depend linearly on the applied flow rates of aqueous solution through the microchannel (inset of figure S-4).

At low flow rates (few $\mu\text{l}/\text{min}$), the characteristic flow times τ_{flow} , as obtained from the FCS measurements are lower than the diffusion time τ_D . τ_{flow} is then not representative for the molecular residence times. As a more general estimate of the residence times at different flow rates, we therefore used the integral of the area of the correlation curve representing diffusion and/or flow:

$$\tau_{\text{residence}} = \int_0^{\infty} G_D(\tau) G_{\text{flow}}(\tau) d\tau \quad (18)$$

$$\text{where } G_D(\tau) = \left(1 + \frac{\tau}{t_D}\right)^{-1} \left(1 + \frac{\tau}{(z_0/\omega_0)^2 \cdot \tau_D}\right)^{-\frac{1}{2}} \text{ and } G_{\text{flow}}(\tau) = \exp\left(-\left[\frac{\tau}{\tau_{\text{flow}}}\right]^2 \cdot \left[1 + \frac{\tau}{\tau_D}\right]^{-1}\right)$$

By applying different flow rates in the microchannel, $\tau_{\text{residence}}$ was varied from 130 μs to $\sim 40 \mu\text{s}$, as measured by FCS in the same setup, but without excitation modulation, before and after each series of excitation-modulated measurements. For each adjusted flow rate, $\bar{F}_{\text{exc}}$ was measured under excitation-modulation, varying the pulse widths, w (1-5 μs), and the pulse periods, T (5-300 μs) of the different pulse trains. A significant increase in $\bar{F}_{\text{exc}}$ could be observed with increasing T , for pulse widths shorter than the residence times of the fluorophores (figure 3A). The duration of each pulse train was 2 seconds. The power of the pulses was kept constant for all pulse trains, and corresponded to an average excitation irradiance of 34 kW/cm^2 in the detection volume.

The measured $\bar{F}_{\text{exc}}$ and its variation with T and w were analyzed by the model of figure 2.

The acquired $\bar{F}_{\text{exc}}$ data were fitted to Eqs. 15 and 16. In the fitting, σ_{exc} and σ_{BISO} were fixed to $1.3 \times 10^{-16} \text{ cm}^2$ and $0.03 \times 10^{-16} \text{ cm}^2$, respectively¹², k_{10} to $1.16 \times 10^9 \text{ s}^{-1}$, k_{ISO} to $25 \times 10^6 \text{ s}^{-1}$ and k_{pN} to $1 \times 10^4 \text{ s}^{-1}$. Only the diffusion rate (k_D) was entered as a free parameter. In figure 3B, the resulting inverse diffusion rates, $1/k_D$, are plotted versus $\tau_{\text{residence}}$, as determined from the corresponding FCS

Figure 3. A. Measured average fluorescence intensity within the excitation pulses, $\bar{F}_{exc}$, for Cy5 with different flow rates 0-300 $\mu l/min$. For all measurements, the pulse width $w=2.0 \mu s$ and the pulse period T was varied from 5.1 to 300 μs . $\bar{F}_{exc}$ is normalized to 1 for the longest $T=300 \mu s$. Excitation irradiance was 34 kW/cm^2 . The measurement time was 2 s for each data point. Dotted lines: fit according to Eq. 15

B. Inverse of the diffusion rate k_D obtained by fitting the data in plot A to Eq. 15. On the abscissa: the normalized residence time ($\tau_{residence}/\tau_D$) calculated according to Eq. 18. A linear dependence of $1/k_d$ versus $\tau_{residence}$ could be observed.

C: The inverse of the single exponential decay time t_l obtained as a result of fitting the data in A to Eq. 19. The dependence of t_l versus the normalized residence time follows an inverse single exponential decay, given by $t_l = t_{PN} [1 - \exp[(\tau_{residence} / \tau_D) / \tau_{PN}]]$, yielding the parameter values $t_{PN}=68 \mu s$ and $\tau_{PN}=0.09$.

measurements. This plot yields a close to linear relationship between $1/k_D$ and $\tau_{\text{residence}}$, as predicted from the model of figure 2. For high flow rates, the flow speed $V=2\omega_0 k_D$. In absolute values, the flow speeds derived from the data in Figure 3B were also found to agree well with the flow speeds determined from the FCS measurements (Figure S-4).

To test how a wrong estimation of the different photophysical parameters in the model may affect the determined k_D above, fits to the data in figure 3A were performed where the parameter values of σ_{exc} , σ_{BISO} , k_{10} and k_{PN} were fixed as stated above, except that one of these parameters was set to different values, from -50% and up to +50% off the correctly fixed value. Variation of the fixed parameter values of σ_{exc} , σ_{BISO} , k_{10} one by one then resulted in comparable relative errors in the determined k_D values. By variation of the fixed k_{PN} value however, several-fold larger deviations in the determined k_D values were found. This strong dependence of k_D on the fixed k_{PN} value reflects that the analysis relies on a strong correlation between the excitation irradiation dose and the dark state build-up, which in turn depends on the extent to which the transitions to and from the dark state are light-induced, i.e. on the value of k_D . Apart from the analysis based on this model, we noted that the normalized fluorescence $\bar{F}_{\text{exc}}$, plotted versus the pulse period T (figure 3A) could also be well fitted to a negative single exponential function:

$$y(t) = y_0 - A \exp(-t/t_1) \quad (19)$$

where the parameters y_0 and A were found to be relatively constant for the different flow rates ($y_0 \sim 2050$ kHz and $A \sim 800$), and where t_1 initially increases with increasing $\tau_{\text{residence}}$, and then reaches an asymptotic value (figure 3C). We assume that this asymptotic value of t_1 reflects the isomerization relaxation time, given by the thermal back-isomerisation of the dyes, $1/k_{\text{PN}}$, which then also defines a lower limit for how slow molecular transits that can be monitored via this particular photo-physical process.

A range of fluorescent proteins display long-lived non- or weakly emitting states with photodynamic features very similar to trans-cis isomerisation of cyanine dyes.²⁹⁻³² For this reason, excitation-modulated measurements were also performed in the same manner on the fluorescent protein DsRed. In

agreement with previous observations²⁰, FCS measurements of DsRed in aqueous solution revealed a prominent dark state relaxation component, with relative amplitude of about 30% and with a relaxation time in the sub-ms time range. As for trans-cis isomerisation of Cy5, the amplitude of the relaxation component remained constant (except for a minor drop for lower I_{EXC} , possibly attributed to a larger relative influence of thermal back-isomerization), while the relaxation time decreased with higher excitation intensities (figure S-5). Given the relatively slow isomerisation dynamics of dsRed, the excitation volume was enlarged, yielding diffusion times of DsRed of ~15 ms, as measured by FCS. The isomerisation dynamics in DsRed is significantly more complex than in Cy5. As for many fluorescent proteins a multitude of photo-induced states can be expected, with relaxation times found over a broad time range. We therefore did not apply the model described by Eqs 15 and 16 to evaluate the DsRed measurements. However, as in the Cy5 measurements the normalized fluorescence $\bar{F}_{\text{EXC}}$ versus the pulse train period was found to fit well to Eq. 19 (figure 4). Moreover, as for Cy5, the t_1 parameter values obtained from these fits displayed a negative exponential dependence to $\tau_{\text{residence}}$, (inset of figure 4). Consequently, although the complex photophysics of DsRed at least at a first attempt excludes an evaluation using the model of figure 2, the recovery curves of figure 4 support the applicability of the proposed method for protein mobility studies using also fluorescent proteins.

Figure 4. $\bar{F}_{exc}$ obtained from time-modulated excitation measurements on DsRed applying different flow rates (5-30 $\mu\text{l/min}$). For all measurements, $w = 300 \mu\text{s}$ and T was varied from 0.4 ms to 20 ms. Curves are normalized to 1 for the longest $T=20 \text{ ms}$. The excitation irradiance 4.4 kW/cm^2 . Measurement times: 15 s for each data point. Dotted lines: single exponential fit according to Eq. 19. Inset: The t_l parameter values obtained from fitting $\bar{F}_{exc}$ versus T to Eq. 19, plotted versus the residence time, calculated according to Eq. 18. Parameter values obtained from an inverse exponential fit, as in figure 3C: $t_{PN}=1.9 \text{ ms}$ and $\tau_{PN}=0.06$.

Concluding remarks

In this work, we demonstrate a concept where the mobilities of fluorescent molecules into and out of a spatially restricted excitation volume, and subject to properly adapted time-modulated excitation in this volume, can strongly influence the build-up of non-fluorescent transient states in the fluorophores. This transient state build-up, is in turn reflected in the detected time-averaged fluorescence intensity, which can be used to characterize the mobilities of the molecules.

By the concept, diffusion measurements can be performed in the same spatial and temporal range as that of FCS and in contrast to FRAP it exploits a mechanism that does not irreversibly deplete the sample fluorescence. In contrast to FCS measurements, it can be performed with very small constraints in the concentration range, and with practically no need for a high fluorescence brightness of the molecules studied. Here, molecular mobility measurements were demonstrated for fluorophores traversing a single focused laser beam. However, since the concept can be performed with very minor requirements in terms of time resolution and sensitivity in the detection, also a more parallelized approach should be possible. Such readout can be realized by introducing a patterned or wide-field excitation, modulated in time and/or space, and by using a standard CCD camera for fluorescence detection. For the Cy5 measurements in this study, the relevant photophysical parameters were known and the fluorescence data was fitted with the k_D rate as the only free parameter. However, in general the photophysical parameters can be rather environment sensitive and for more complex environments not always easily determined. Our parameter stability tests show that in particular the thermal deactivation rate of the dark cis state (k_{PN}) is critical, and also sets a lower limit for how slow transits that can be monitored.

Nonetheless, when measuring under similar environmental conditions the proposed approach should be able to provide relative mobility information, as illustrated by the dsRed measurements. Moreover, if the k_{PN} rate of Cy5 would not be small enough to monitor slowly moving molecules, dsRed or other fluorophores or fluorescent proteins can be used having considerably smaller thermal deactivation rates [REF t.ex. J Fuchs et al Nat Meth 7(8), aug 2010]. With these aspects in mind, we expect the proposed method to be useful for biomolecular mobility imaging, in cells or in-vitro, where the mobility of the

molecules is too fast to be recorded within the frame rates of commonly used microscopic techniques, such as laser-scanning confocal microscopy and wide-field or total-internal reflection microscopy using a CCD camera for fluorescence detection. It may also provide a complement to FCS and FRAP for localized molecular mobility studies, where instrumental constraints or the concentration range and/or the molecular fluorescence brightness of the sample may pose limitations.

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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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 ($k_D = 0.02 \mu s^{-1}$). 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 ($P = 150 \mu W$, $w = 1.0 \mu s$ (blue) and $5.0 \mu s$ (red), $T = 10 \mu s$). 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: $\sigma_{01} = 1.3 \times 10^{-16} \text{ cm}^2$, $k_{10} = 1160 \mu s^{-1}$, $k_{ISO} = 25 \mu s^{-1}$, $\sigma_{BISO} = 0.03 \times 10^{-16} \text{ cm}^2$ and $k_{PN} = 0.01 \mu s^{-1}$. The (uniform) excitation irradiance within the pulses onto the detection volume was set to $I_{EXC} = 24 \text{ kW/cm}^2$. 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 $k_D = 1/50 \mu s^{-1}$.

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 ($\bar{F}_{\text{exc}}$) 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) $\bar{F}_{\text{exc}}$ 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, $\bar{F}_{\text{exc}}$, 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 $\bar{F}_{\text{exc}}$ for diffusion rate $k_D=0 \mu\text{s}^{-1}$, dashed lines $k_D=1/300 \mu\text{s}^{-1}$, dashed-dotted lines $k_D=1/200 \mu\text{s}^{-1}$ and dotted lines $k_D=1/100 \mu\text{s}^{-1}$.

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/cm² 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 $\tau_D = 70 \text{ }\mu\text{s}$, flow time τ_{flow} varies from $230 \text{ }\mu\text{s}$ at a microchannel flow rate of $50 \text{ }\mu\text{l/min}$ to $10 \text{ }\mu\text{s}$ at a flow rate of $1200 \text{ }\mu\text{l/min}$. At fast flow rates ($>1.5 \text{ ml/min}$) 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, $\tau_{\text{ISO}} = 1.4 \text{ }\mu\text{s}$. The inset shows the observed linear dependence of the flow speed $V = \tau_{\text{flow}} / \omega_0$ 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 fastest flow rate ($300 \mu\text{l/min}$) a $1/k_D$ of $100 \mu\text{s}$. This corresponds to a flow speed of $V = k_D 2\omega_0 = 10^4 \text{ s}^{-1} \times 2 \times 0.38 \mu\text{m} = 7.6 \text{ mm/s}$. From the FCS measurements and the inset get a flow speed of 6.5 mm/s for an applied flow rate of $300 \mu\text{l/min}$, 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.